Isolation and Characterization of ISA Degrading Alkaliphilic Bacteria

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Dedication

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Abstract

Radioactive waste disposal in the UK is expected to be managed via the construction of a deep geological disposal facility (GDF) backfilled with cementitious grouts. Post closure the repository is expected to have a hyper alkaline, anoxic environment. The UK's intermediate-level radioactive waste (ILW) inventory contains significant quantities of cellulosic materials that expected to undergo alkaline hydrolysis under repository conditions to form Iso-saccharinic acids (ISA). The isomers of ISA (α- and β-) are able to form water-soluble complexes with radioelements, increasing their solubility and enhancing their migration. The biological removal of ISA through microbial activity would therefore have a positive impact on repository performance by reducing complex formation.

This research aimed to isolate pure cultures capable of degrade ISAs under anaerobic and alkaline conditions analogous to GDF. The microcosms of mineral media and CDPs-fed cycle as a sole carbon source inoculated by alkaline soil samples from the Buxton site. The degradation process monitored under a fermentation and an anaerobic respiration by adding terminal electron acceptors. All processes carried out under alkaline and strict anaerobic conditions. Different types of agar plate media used to obtain pure culture. The results of the current study indicate that alkaliphilic bacterial communities were capable of fermenting ISA to acetate up to pH 11.0. In addition, the ISA (α- and β- isomers) degradation through terminal electron acceptors at pHs (7.0, 8.0, 9.0 and 10.0) were tested, resulted to a significant amount of ISA was degraded in Nitrate-reducing culture, and small amount in Iron (III)-reducing culture, whilst there was no sign of degradation in Sulphate-reducing culture.

A 16SrRNA gene sequencing showed a significant reduction of the bacterial community in the microcosms compared with the crude soil sample. The phyla that detected in all microcosms at different pH values dominated by Firmicutes, followed by Proteobacteria, Bacteroidetes, Actinobacteria and Archaea Euryarchaeota. However, in pure culture many of the bacteria isolated were unable to degrade ISA eventhough they had been isolated and enriched under ISA degrading conditions. Only two bacterial strains purified which were capable of degrading ISA. These strains *Macellibacteroides fermentans* HH-ZS a Gram negative, strictly anaerobic bacterium and a strain of *Aeromonas* sp.

The biochemical analysis, metal and NaCl tolerance, pH profile, biofilm and extracellular polymeric substances detection, fatty acid methyl ester profile, and whole genome sequencing analysis used for characterization of the some isolated Alkaliphiles. The phylogenetic and biomarker results led to identifying a novel strain of *Macellibacteroides fermentans* HH-ZS strain is the first Gram negative, strictly anaerobic bacteria able to degrade ISA.

Future work; the genome of the *M. fermentans* HH-ZS strain harboured a number of carbohydrate degrading enzymes, which merit further investigation to determine the metabolic pathways associated with ISA degradation. In addition, some of isolated Alkaliphiles considered as rare strains and some of them identified as new strains; further investigation to find the possibility to introduce these isolates in the bioremediation and an industrial application.

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List of Abbreviations

BF	Bacterial biofilm
BLAST	Basic Local Alignment Search Tool
CDP	Cellulose Degradation Products
COGs	Clusters of Orthologous Groups
DNA	Deoxyribonucleic Acid
eDNA	Extracellular DNA
EPS	Exopoly Saccharide
FAA	Fastidious Anaerobe Agar
FAME	Fatty Acid Methyl Ester
FFC	First Fed Cycle
GC-FID	Gas chromatography with flame ionisation detection
GC-TCD	Gas Chromatography with Thermal Conductivity Detection
GDF	Geological Disposal Facility
HLW	High Level Waste
HM	Heavy Metals
HPAEC-PAD	High Performance Anion Exchange Chromatography with Pulsed Amperometric Detection
IC	Ion Chromatography
ILW	Intermediate Level Waste
IRB	Iron Reducing Bacteria
ISA	Isosaccharinic Acid
LLW	Low level waste
MEGA	Molecular Evolutionary Genetics Analysis
MIDI	Microbial Identification Inc.
MIS	Microbial Identification System
MM	Mineral Media
MUSLE	MUltiple Sequence Comparison by Log-Expectation
NCBI	National Centre for Biotechnology Information
NDA	Nuclear Decommissioning Authority
OTUs	Operational taxonomic units
OrthoANI	Orthologous Average Nucleotide Identity
PCR	Polymerase Chain Reaction
PLFA	Phospholipid Fatty Acid
R	Microcosm Reactor
RAST	Rapid Annotation using Subsystem Technology
SEM	Scanning Electron Microscopy
SRB	Sulphate Reducing Bacteria
TEA	Terminal electron acceptor
UPGMA	Unweighted Pair Group Method with Arithmetic Mean
VFAs	Volatile Fatty Acids

Chapter 1
1. Introduction

The United Kingdom's radioactive waste stock began to accumulate in the 1940s. To date the total volume (m^3) of accumulated radioactive waste from all sources is in the region of $4.72x10^6$ m^3 with an estimated volume of future production of $4.55x10^6$ m^3. This waste is classified into Low Level Waste (LLW) which accounts for 93.9% of the total volume, followed by Intermediate Level waste (ILW) (6.1%) and High Level waste (HLW) (0.02%) (Anon, 2011). Although HLW is the smallest component on a volume basis, it accounts for the majority of the radioactivity.

The UK's preferred solution for the disposal of its ILW and HLW is via a geological disposal facility (GDF) (Anon, 2011). In the case of ILW, one of the design options is an engineered facility backfilled with a cement based grout which will create an alkaline environment. A major component of ILW is cellulosic materials originating from contaminated clothing, packaging and paper. Under the alkaline, anoxic environment expected within a ILW GDF cellulosic materials are expected to be subject to alkaline hydrolysis which generated cellulose degradation products (CDP) including the α and β stereoisomers of isosaccharinic acid (ISA) (Glaus, Van Loon, Achatz, Chodura, & Fischer, 1999; Humphreys, Laws, & Dawson, 2010). These organic acids are able to form stable complexes with certain radionuclides increasing their solubility and promoting their transport via groundwater (Greenfield, Hurdus, Spindler, & Thomason, 1997; Randall, Rigby, Thomson, & Trivedi, 2012).

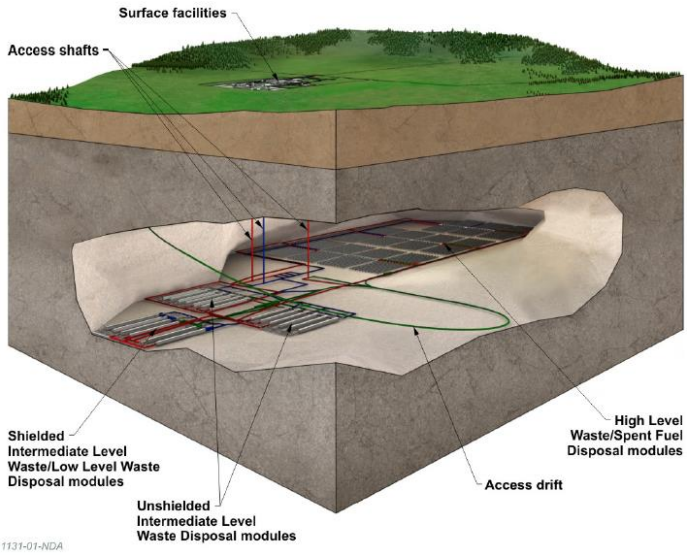
In view of the importance of ISA in promoting the transport of radionuclides the microbial degradation of these compounds has received considerable attention (Bassil, Bryan, & Lloyd, 2015; Humphreys, West, & Metcalfe, 2010; Rout, Charles, Doulgeris, et al., 2015; Rout et al., 2014; S. L. Smith, Rizoulis, West, & Lloyd, 2016). Given the cementitious nature of the proposed ILW GDF alkaliphilic bacteria are expected to play a crucial role in ISA degradation. The research described in this thesis has focussed on the isolation and characterisation of alkaliphilic organisms from anthropogenic alkaline soils such as those found at the Harpur Hill lime kiln waste site, Buxton, Derbyshire, UK (Rout, Charles, et al., 2015a).

Although ISA degrading bacterial communities have been detected in sediment samples from neutral pH sites, alkaline sediments e.g. Buxton, are expected to provide a better chance of isolating novel alkaliphiles which may also be able to degrade ISA. Previous investigations have shown that the bacterial community in the hyper-alkaline sediments at

Harpur Hill are able to degrade ISAs under aerobic, nitrate and iron (III) reducing conditions. Consequently, this site is likely to be a source of novel alkaliphilic or alkali tolerant species that may be capable of degrading ISAs.

1.2. Geological disposal of radioactive waste

The UK Nuclear Decommissioning Authority (NDA) has the responsibility for establishing a geological disposal facility (GDF) for the long term disposal of radioactive wastes (Figure 1.1 A), this facility is expected to protect people and the environment from the impacts of radioactive wastes (Defra, Food, & Affairs, 2008). Radioactive wastes are classified into three types depending on the physical properties of the radioactive materials; low level waste (LLW), intermediate level (ILW) and high level radioactive wastes (HLW) (Anon, 2011; Humphreys, Laws, et al., 2010; Wild & Mathieson, 2003). ILW contains a range of heterogeneous materials including a significant amount of the cellulosic materials (Anon, 2010b).



A) Deep geological disposal facility

B) Drum of 500 litre capacity, contains a compressed wastes

Figure 1. 1: Radioactive wastes repository and deep geological disposal facility concept

Taken from the 2010 NDA (Anon, 2010b), the schematic illustrates the organisation of radioactive waste under the GDF system, access is provided by shafts as a pathway between surface facilities and the different levels of the disposal modules underground (A), Drums of 500 litre capacity, contains compressed wastes for storage facility until transport to the GDF (B).

1.2.1. Intermediate–level wastes under GDF

One of the potential designs for the United Kingdom's ILW GDF is a multi-barrier repository where various packages of waste will be placed in engineered vaults backfilled with a cementitious grout (Figure 1.2) (Anon, 2010b). The cementitious grouts is composed of ordinary Portland cement and limestone flour and is designed to limit the transport of radionuclides from the radioactive waste to the environment (Chapman & Hooper, 2012). ILW contains significant amounts of cellulosic material that are derived from contaminated, wood, paper and cloth (Anon, 2014; Chapman & Hooper, 2012), it also contains steel, demolition debris and contaminated soil. Post closure, the GDF is expected to re-saturate with ground water which will react with the $Ca(OH)_2$ and other alkaline compounds such as KOH and NaOH present in the grout to generate an alkaline pH (>pH 12.0). In addition the corrosion of metallic waste and construction materials will generate anaerobic conditions through the utilization of the oxygen during corrosion (Anon, 2010a).

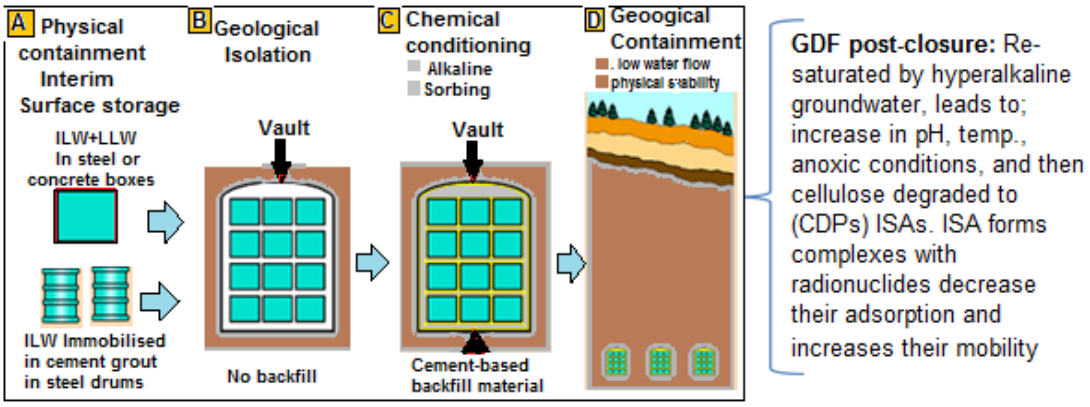


Figure 1. 2: Depicts a GDF for safely dispose of radionuclides. ***(Nirex Report N/075, 2003)***

Surface storage in steel drums or concrete boxes (A) and then in vault for geological segregation (B) of cement-based backfill (C) in deep sites for optimum isolation (D).

1.2.1.1. The Fate of Cellulosic Materials

Cellulosic materials account for 2,000 tonnes of the UK's ILW inventory (Anon, 2010b). Cellulose is a polysaccharide composed of ß-D-glucose units that are linked together by ß-1,4-glycosidic linkages (Figure 1.3) and with neighbouring celluloses by hydrogen bonds to form a linear macromolecule (Cordeiro, 2016)**.** This structure under alkaline and anaerobic conditions undergoes chemical degradation to produce a range of soluble organic compounds with α and β isosaccharinic acids (ISA) (Figure 1.4) being the most abundant and small amount of other short-chain hydrocarbons include volatile fatty acids such as

acetic acid, butyric acid and propionic acid (Greenfield et al., 1997; Heath & Williams, 2005).

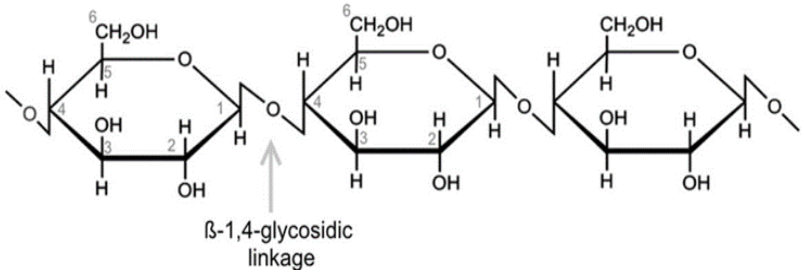


Figure 1. 3: Chemical structure of cellulose, taken from (Cordeiro, 2016).

The degradation reaction of cellulose under alkaline condition are carried out through three main pathways of chemical reactions. The peeling reaction where the terminal anhydro-glucose unit at the reducing end of the cellulose is removed one by one to form ISA isomers. However, the peeling reaction is blocked due to a formation of alkali-stable terminal groups compounds of glucometasaccharinic acids (MSA), this process is called stopping reaction. The third chemical reaction is the mid-chain scission that generate a new reducing terminal groups by a random cleavage of the glycosidic links along the molecular chain.

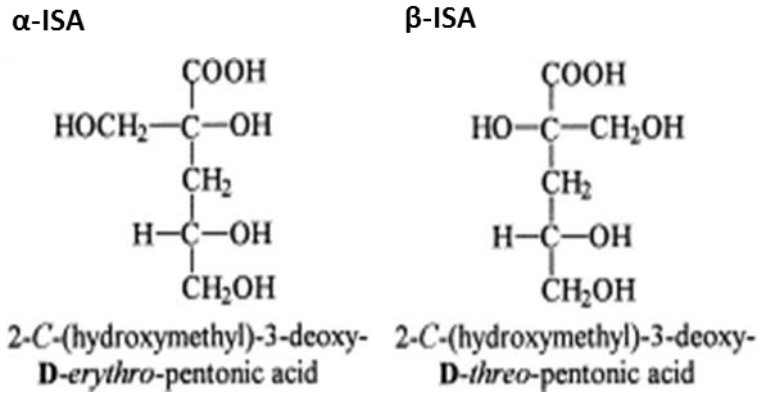


Figure 1. 4: **ISA stereoisomers**

ISA has been shown to form complexes with radioelements, enhancing their migration and potentially increasing the associated radiological risks (Greenfield et al., 1997; Heath & Williams, 2005; Humphreys, West, & Metcalfe, 2009; Pedersen, 2000). For instance it is able to form complexes with Th (IV) (K Vercammen, Glaus, & Van Loon, 2001), U(IV) (Warwick, Evans, Hall, & Vines, 2004) and Np(IV) (Rai et al., 2003). Research by Lawson et al (1994) showed that the leachates produced from cellulose degradation had the ability to changes the plutonium behaviour, by increase its solubility and reducing sorption (A. Lawson et al., 1994)

1.3. Microbial Processes Relevant to Radioactive Waste Disposal

Microbial activity will have both direct and indirect effects on the performance of a GDF. The direct effects include gas production and biofilm formation, which may lead to pressurisation and the blocking of pores. The indirect effects include the alteration of pH and redox resulting in changes to radionuclide mobility, solubility and sorption (Brainard, 1992; McCabe, 1990; Pederson, 1992). However, the extreme environmental conditions associated with a GDF such as hyper-alkalinity, radiation and radionuclide toxicity are expected to suppress microbial activities. As the GDF evolves with time it is possible that microorganism may be tolerant to such extreme conditions (Chicote et al., 2004; Rizoulis, Steele, Morris, & Lloyd, 2012) and be able to utilise the organic carbon present either by fermentation or terminal electron accepting processes (anaerobic respiration). Under anaerobic conditions, instead of oxygen, microorganism can respire using a broad range of terminal electron acceptors (TEA) (Lloyd, 2003) including nitrate (NO_3^-), Iron (III), sulfate (SO_4^{2-}) and carbon dioxide (CO_2) (Achtnich, Bak, & Conrad, 1995).

The most significant degradable organics within an ILW GDF are cellulose and CDP. However, the quantities of terminal electron acceptors such as nitrate and sulphate are likely to be limited and consequently the fermentation of ISAs to volatile fatty acids, carbon dioxide and hydrogen gases is expected to dominate (Askarieh et al., 2000, Greenfield et al., 1990). With the exception of carbon dioxide, the only other significant TEA is likely to be Fe (III), which will be generated from the corrosion of steel drums, construction steel and iron waste components. This corrosion will initially remove oxygen from the system and lead to the production of Iron (III) minerals, once the oxygen is depleted anaerobic corrosion will take over with the associated generation of hydrogen (Humphreys, West, et al., 2010).

In terms of CDP Rout *et al*. (2014), demonstrated that microbial communities in near-surface neutral pH sediments were able to degrade both forms of ISAs at pH 7.0 through iron (III) and sulphate reduction as well as through fermentation and methanogenesis with no significant difference in α and β ISA degradation rates (Rout et al., 2014). Similarly Kuippers *et al*. (2015), found that the bacterial community in the alkaline sediments at Harpur Hill were capable of utilizing α-ISA with oxygen, nitrate, sulphate and Fe (III) as TEAs when incubated at pH 7.0 (Kuippers, Bassil, Boothman, Bryan, & Lloyd, 2015).

Using the same sediments at alkaline pH values Williamson *et al*. (2013), documented the reduction of Iron (III) in the form of ferrihydrite using lactate as an electron donor

(Williamson et al., 2013). As did Rizoulis *et al*. (2012) who demonstrated that this bacterial community was capable of reducing Iron (III) (ferrihydrite and ferric citrate) between pH 10.0 and pH 12.0 using lactate and yeast extract as electron donors (Rizoulis et al., 2012). Bassil *et al*. (2015), expanded this work to include ISA and demonstrate the fermentation of ISA to acetate at pH 10.0 in the presence of insoluble ferrihydrite. The resulting populations were dominated by the genus *Anaerobacillus* (~99%) of the phylum Firmicutes (Bassil et al., 2015). Bassil *et al*. (2014), found that the rate of ISA biodegradation under alkaline conditions was higher in the presence of oxygen as a TEA, similar via nitrate reduction, reduced during Fe(III) reduction, and very low during sulphate reduction (Bassil et al., 2015), with no sulphate reduction detected ≥ pH 10.0 (Bassil et al., 2015). This observation agrees with Rizoulis *et al*. (2012), who were unable to detect sulphate reduction at pH 10.0, 11.0 or 12.0 when yeast extract, acetate or lactate were provided as electron donors (Rizoulis et al., 2012). From a review of the literature it is clear that SRB can grow in alkaline environments, however the majority of these studies suggest that sulphate reduction is uncommon above pH 10.0.

In terms of fermentation, Rout *et al*. (2015) found that the bacterial community in a neutral pH sediment was able to ferment ISAs under alkaline condition up to pH 10.0, with the degradation rate decreasing as the pH increased. A shift to alkaline conditions led to a significant reduction in bacterial community diversity and a shift in the dominant bacteria. Methanogenesis was active up to pH 10.0 via the hydrogenotrophic pathway and the accumulation of acetate. The metabolic activity was suppressed when the pH of the microcosm was raised to pH 11.0 when ISA accumulated (Rout, Charles, Doulgeris, et al., 2015). When working on Harpur Hill sediments Rout *et al*. (2015), were able to confirm that ISAs were generated in situ due to the alkaline pH of pH 12.5. In addition, they found that the microbial communities in the sediments were active and capable of degrading both forms of ISA with the associated production of acetic acid, hydrogen and methane at pH 11.0. The associated bacterial community was found to be dominated by alkaliphilic *Clostridia* and hydrogenotrophic Methanobacteriaceae (Rout, Charles, et al., 2015a).

Work by Charles *et al*. (2015), on cotton samples immersed for several weeks in the hyper-alkaline soils at Harpur Hill observed a biofilm dominated by *Clostridia* and hydrogenotrophic methanogens. However, that community was significantly reduced and the methanogens became undetectable when the cotton sample was used to seed a CDP fed

microcosm operated at pH 11.0, in this case the bacterial community was dominated by *Alishewanella* and was able to completely degrade ISA (Charles et al., 2015).

1.3.1. Pure Culture Degradation of ISA

Biodegradation of ISA by bacterial communities under aerobic and anoxic conditions at alkaline pH has been extensively reported. However, there are a limited number of reports on single isolates able to degrade ISA. Work carried out by Stand *et al*. (1984), used media containing 1g/l Gluco-Isosaccharinic acid (ISA) to test the capability of 22 aerobic and facultative anaerobic soil bacteria for their ability to utilize ISA as a sole carbon source. The results showed that none of the bacterial strains were able to degrade ISA, except some strains of *Ancylobacter aquaticus*, which were capable of growing aerobically on ISA at pH 7.2 and 9.5 in media supplemented with vitamins, peptone and phosphate (Strand, Dykes, & Chiang, 1984). Bailey (1986) screened 50 bacterial strains from soil contaminated with black water (the black liquor is a waste of the sulphate cellulose (Kraft pulping) process where the wood under alkaline and high temperature converted into pure cellulose fibre and the Glucoisosaccharinic acid is the major component of the waste) at pH 6.0- 9.7 for their ability to grown on ISA. Only two of these bacteria were able degrade ISA, at pH 6.2 (Bailey, 1986). Work by Francis *et al*. (2009), found that aerobic, Gram positive rods were capable of ISA biodegradation (Francis & Dodge, 2009).

The degradation of the organic compounds by bacteria depends on many factors include; the nature of that organic compounds, biochemical composition, molecular size, concentration and physical factors in its environment including pH, temperature, oxygen level (Reference 1). During the degradation process there are two types of transport mechanisms that used by bacterial cells for the nutrition and metabolism process for cell survival and growth; the passive transport mechanism by which the molecules across the cell membrane either by passive diffusion or facilitated diffusion of the solutes, this mechanism does not require energy. The second mechanism is active transport, it always requires energy in the form of ATP, proton motive force, or by phosphoenylpyruvate (PEP), several types of transport proteins are involved in this mechanism, these proteins are embedded in the cytoplasmic membrane that include; the group translocation transporters, ATP-Binding Cassette (ABC) transports, antiporters, and symporters. By using ATP-dependent ABC-transports, permeases, and even by passive diffusion using specific facilitator proteins; the nutrient is taken up by carrier protein of phosphoenolpyruvate-dependent phosphotransferase system

(PTS). The PTS is a first glucose transporter (GLT) was identified in *E. coli* and described in 1966, about 30 of GLT systems are characterized in more than 20 bacterial species (Amon & Benner, 1996; Castro et al., 2009; Erni, 1989; Jahreis, Pimentel-Schmitt, Brückner, & Titgemeyer, 2008).

1.4. Microbial Extremophiles

1.4.1. Alkaliphilic Microbiology

Successful microbial metabolism requires a cytoplasmic pH of between pH 7.4 and 7.8 for neutrophilic organisms and between pH 7.5 to 8.3 for some alkaliphiles (Slonczewski, Fujisawa, Dopson, & Krulwich, 2009). A review by Booth *et al*. (1985), reported that fermentative micro-organism are able to grow at a wider pH range than those utilizing respiration pathways (Booth, 1985). Alkaliphiles have a growth optimum between pH 8.5 and 11.5; neutrophiles between 5.5 and 8.0; and acidophiles 0.0 to 5.5 (Figure 1.5 A). A relationship between the occurrence of alkaliphilic microorganisms and the pH of the sample origin is illustrated in (Figure 1.5 B) (Koki Horikoshi, 1999). Many natural high pH habitats exist, for example, geothermal springs and soda lakes. There are also a range of manmade alkaline environments that includes paper pulping industrial sites, steel slag disposal sites (Rizoulis et al., 2012) and lime working sites such as Harpur Hill (Milodowski, Shaw, & Stewart, 2013).

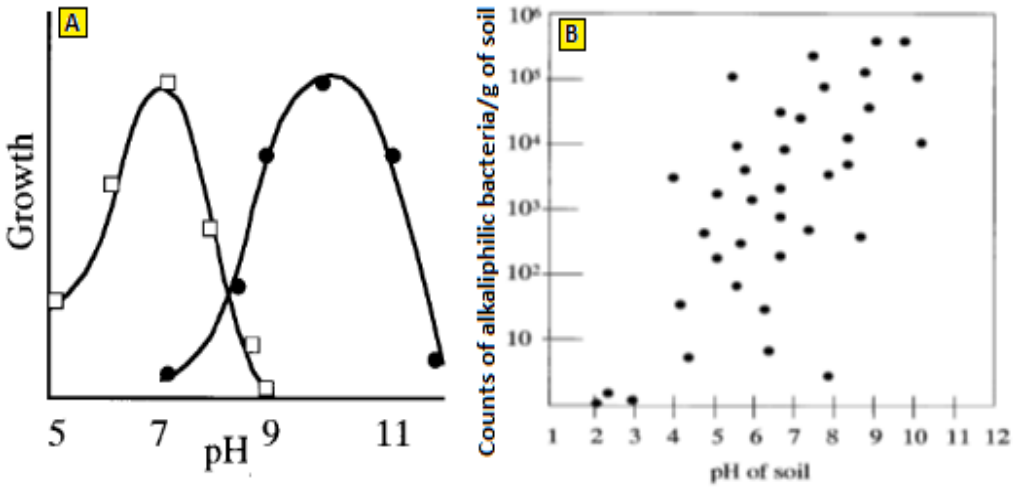


Figure 1. 5: pH profile of abacterial community (Koki Horikoshi, 1999)

A) The typical pH dependency of neutrophilic and alkaliphilic bacteria is shown by open squares and solid circles, respectively. B) Distribution of alkaliphilic microorganisms in environments at various pHs (Koki Horikoshi, 1999).

In general, different microorganisms have characteristic pH growth ranges and a distinct pH growth optimum. For instance, a facultative alkaliphilic *Bacillus halodurans* C-125 lost its pH homeostatic ability and did not grow at pH above 10.8 (K. Horikoshi, 2006). There are numerous adaptation strategies used by bacteria in order to maintain a cytoplasmic pH between pH 7.2-8.0 (Koki Horikoshi, 1999) and these strategies were discussed in a review by Padan *et al*. (Padan, Bibi, Ito, & Krulwich, 2005) and include:

(i) metabolic changes that lead to increased acid production through amino acid deaminases and sugar fermentation**;**

(ii) increased ATP synthase that couples H^+ entry to ATP generation;

(iii) changes in cell surface properties, for example a quantities of poly-γ-L-glutamic acid (plg) in the cell walls increase with increasing culture pH (K. Horikoshi, 2006); and

(iv) elevated expression levels and activity of monovalent cation/proton antiporters (Figure 1.6).

The later play an essential and dominant role in alkaline pH homeostasis in many bacteria (Padan et al., 2005). For instance, *Bacillus firmus* and *Exiguobacterium auranticum* use Na^+/H^+ antiporter systems in the region of pH 7.0 to 9.0, (Krulwich, Ito, Hicks, Gilmour, & Guffanti, 1998)**.**

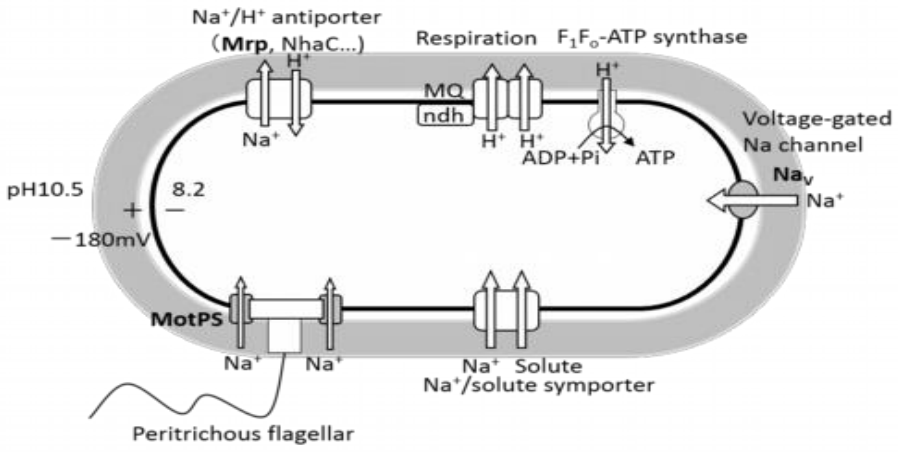


Figure 1. 6: Homeostasis in alkaliphilic bacteria

The diagram represented the strategies of bacterial cell to tolerate high pH at surrounding environment. Taken from Ito Laboratory in Toyo University website http://www2.toyo.ac.jp/~ito1107/researchen.html, [cited 13.03.2017].

The degradation processes catalysed by alkaliphilic bacteria depends on alkaline adapted enzyme systems. These extracellular alkaline-adapted enzymes often show activities in a

broad pH range, with good thermostability and a tolerance to oxidants when compared with neutral enzymes (Fujinami & Fujisawa, 2010). *Bacillus* species for example are able to produce a variety of alkaline extracellular enzymes, such as cellulases, proteases and pectinases that have been exploited in various industrial processes (Fujinami & Fujisawa, 2010; Ito, 1997; J. Singh, Batra, & Sobti, 2004). In general, cytoplasmic pools of polyamines and a low membrane permeability are two modes of passive regulation in bacterial cells, whereas sodium ion channels drive the active regulation. For example, the low molecular weight, aliphatic polycations and their positively charges the polyamine can bind to the DNA, RNA and proteins of the cell and play crucial roles in bacterial growth, modulation of cell signalling, in stress responses, biofilm formation, regulation of gene expression and stabilization of the cell membrane (Shah & Swiatlo, 2008). (Shah & Swiatlo, 2008)

1.4.2. Heavy metal tolerance.

Heavy metals (HM) are defined as metallic elements with density above 5 g/cm^3 (Nies, 1999). Although, some heavy metal ions are essential for microbial growth in trace amounts e.g. iron, nickel, zinc and copper, most heavy metals are toxic at high concentrations. Metals such as silver, cadmium and mercury have no biological role and are considered toxic to bacteria even in trace amounts (Lima e Silva et al., 2012). Toxic heavy metals can form complex compounds with vital cellular components, such as nucleic acids, enzymes, and structural proteins interfering with their function (Lemire, Harrison, & Turner, 2013). Bacteria overcome heavy metal toxicity by many mechanisms. Barkay *et al*. (2003), stated that bacteria are able to be become resistant to inorganic and organic mercury compounds (HgR), through the expression of a mercuric reductase enzyme (MerA) (Barkay, Miller, & Summers, 2003). Work by De *et al*. (2008), found that many marine bacteria are able to volatilize mercury through the putative *merA* gene and other catalyses by the reduction of Hg(II) to the elemental form, Hg(0), these bacteria also able to remove the toxicity of Cd and Pb (De, Ramaiah, & Vardanyan, 2008).

Nies (1999) pointed out that bacteria have different strategies for survival and growth under extreme conditions and that the resistance against toxic heavy metals depends on two systems (Nies, 1999). The first type is a high substrate specific ATP efflux system, such as metal transport ATPases. The second resistance system depends on a chemiosmotic gradient and cation/proton antiporters, which is a faster, none specific system (Nies, 1999; Silver & Phung, 1996). Bacteria can develop a resistance against high levels of heavy metals because

of exposure to those metals and this resistance may not be specific. For instance in soils polluted with Cu, in addition to increased tolerance to Cu, the bacterial community also has increased tolerance to Zn, Cd, and Ni (Díaz-Raviña, Bååth, & Frostegård, 1994). Furthermore, bacterial communities that acquired a tolerance to Zn and Cd from a polluted environment, demonstrated an increased tolerance to all five metals (Díaz-Raviña et al., 1994).

1.5. Biofilm formation

Bacterial biofilms (BF) are a structural mass of bacterial cells coated with a polymer matrix attached to either biotic or abiotic surfaces (Davey & O'toole, 2000). The formation of a biofilm occurs via an established pattern, for instance (Figure 1.7) that illustrating biofilm formation in (Bacillus subtilis) (Vlamakis, Chai, Beauregard, Losick, & Kolter, 2013). Biofilms isolated from various environments share common characteristics (Stewart & Franklin, 2008): (i) the bacterial cells are held together by a polymeric matrix composed of exopolysaccharides, proteins and nucleic acids; (ii) the development of the biofilm occurs in response to extracellular signals, either in the environment or produced by the bacterial cells (Vlamakis et al., 2013).

Biofilm formation protects bacteria and allows them to survive in hostile environmental conditions (Kolenbrander, Palmer, Periasamy, & Jakubovics, 2010). Biofilms can withstand harsh environmental conditions for instance high pH, temperature and are much more resistant to heavy metals than planktonic bacterial cells. Teitzel and Parsek (2003), found that free-swimming cells of *Pseudomonas aeruginosa* were less resistant (from 2 to 600 times) to heavy metal stress than the same bacteria in a biofilm (Teitzel & Parsek, 2003). Biofilms protect microbes from extreme conditions whilst allowing cells to utilize the surrounding nutrients (Costerton, Lewandowski, Caldwell, Korber, & Lappin-Scott, 1995; Sutherland, 2001b), through the highly permeable water channels present in the biofilm (Davey & O'toole, 2000). A gradient of nutrients is observed from the top of the biofilm to its base. This observation reinforces the idea that the metabolic state of bacteria within a biofilm is dependent on its location within the structure. Moreover, biofilms can provide environmental conditions that support nutritional cooperation between multispecies consortia embedded in the biofilms matrix. Work by Charles *et al*. (2015) found that biofilms play an essential role in the protection of bacterial communities from hyper-alkaline conditions (pH 11.0) by creating a pH gradient within the film (Charles et al., 2015).

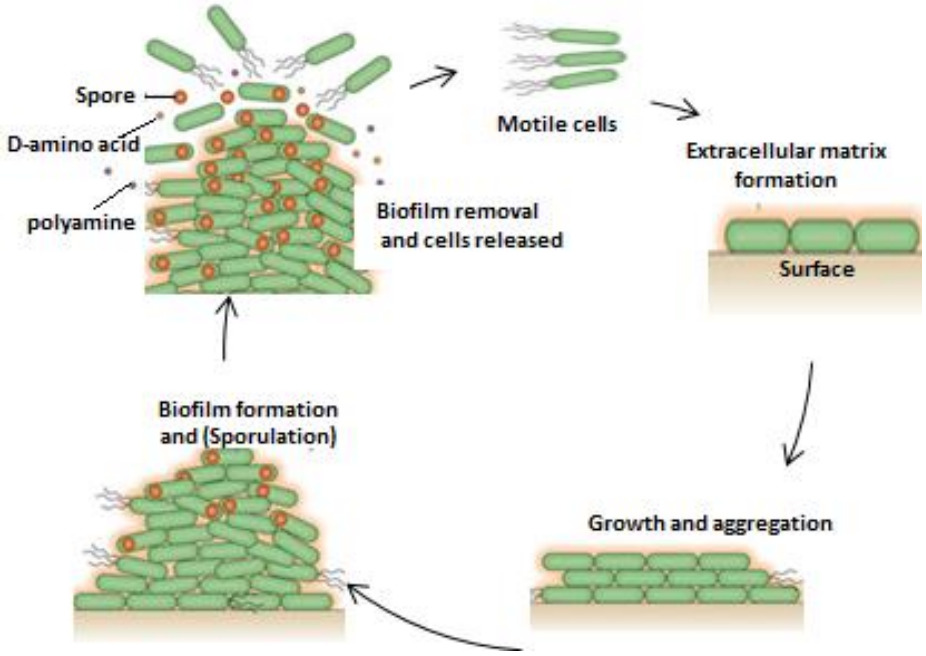


Figure 1. 7: Diagram describe a bacterial biofilm formation in *Bacillus subtilis*

Biofilm formation in (*Bacillus subtilis*) (Vlamakis et al., 2013), Bacteria start sticking together to build a biofilm, the EPS production is the first step to form a matrix, then bacteria start growing and aggregation to build up a sticky matrix called a biofilm.

1.5.1. Extracellular polymeric substances

Extracellular polymeric substances (EPS) are high molecular weight polymers, composed of sugar residues, proteins, DNA, lipids, and humic substances (Sutherland, 2001a). These EPS are secreted by microorganisms into the surrounding environment and the physiochemical properties of bacterial biofilms depend on the EPSs components (Sutherland, 2001a). EPSs provide protection against anti-microbial substances, starvation conditions and also extremes of pH and temperature (Nichols, Guezennec, & Bowman, 2005; Sutherland, 2001a). The biofilm matrix is highly hydrated and can contain up to 97% water (Sutherland, 2001b).

In alkaliphilic multispecies biofilms a wide range of EPS have been observed. Charles *et al*. (2017), observed complex mixtures of EPS including polysaccharides, lipids and proteins with an extracellular deoxyribonucleic acid (eDNA) matrix that formed due to autolysis of the bacterial cells under a control of quorum sensing system which allowed the associated community to maintain a lower pH within the biofilm system than that of the wider environment(C. Charles et al., 2017). Related work by the same author, demonstrated that alkaliphilic biofilms formed on different surfaces, formed with different structures. This was

most pronounced on steel where the biofilms had an eDNA basal layer upon which the rest of the biofilm formed(C. J. Charles et al., 2017).

1.6. Identification and characterization of Microorganisms

Although, bacterial identification and characterization still depend on the isolation of bacteria in a pure culture, not all environmental bacteria have been or are culturable. This is particularly the case since some bacteria are syntrophic, depending on growth factors released by other bacteria (Jiao et al., 2012; Morris, Henneberger, Huber, & Moissl-Eichinger, 2013). Current methodologies for the determination and identification of bacteria are dependent on molecular-based methods that are used for both pure isolates and communities.

1.6.1. Identification of bacteria by 16SrRNA gene sequencing

Molecular analysis is often used for the study of bacterial and archaeal communities. This is commonly based on 16S rRNA gene sequencing since this gene is a highly conserved component of the transcriptional machinery of all DNA-based life forms (Patel, 2001). Consequently, it is highly specific as a target gene for microbial identification in samples that potentially contain thousands of different species. Universal PCR primers specific to the conserved regions of the 16S rRNA gene are available, making it possible to amplify the gene in a wide range of different microorganisms from a single sample. Conveniently, the 16S rRNA gene consists of both conserved and variable regions. While the conserved region makes universal amplification possible, sequencing the variable regions allows discrimination between specific different prokaryotic microorganisms such as bacteria and archaea (Wintzingerode, Göbel, & Stackebrandt, 1997).

1.6.2. Biochemical test-based identification

Biochemical test-based identification systems are based on the ability of bacteria to utilize substrates. Bacterial growth and substrate utilisation is detected through pH shifts, redox reactions or product generation. These biochemical systems usually involve plates or strips of wells coated with substrates such as the Biolog MicroPlate^{TM} (Hayward, USA), and the API (BioMe´rieux) system. Although these systems are considered simple to use compared with DNA sequencing and FAME analysis (Cook, Turenne, Wolfe, Pauls, & Kabani, 2003; O'Hara, 2005), these systems are dependent on the data-base that has been collated for that

specific system. Therefore, their usage is limited to the number of bacterial species (Kunitsky, Osterhout, & Sasser, 2006).

1.6.3. Cellular fatty acids test-based identification

Fatty acids (FAs) with between 2 and 24 carbons are present across a wide range of microorganisms and have been used to provide an insight into bacterial community characterization (Banowetz et al., 2006). The cellular fatty acids content is stable, and is not affected by mutations or death (Fakruddin & Mannan, 2013). This analysis is also called the fatty acid methyl ester (FAME) method, by transesterification of fats with methanol in the presence of an acid catalyst. Another approach is to us the phospholipid fatty acid (PLFA) compositions, these differ between microbial communities and provide a method that can provide an insight into bacterial community structure (Tunlid, 1992). Chintalapati *et. al*. (2004) found that bacteria are able to adapt the low temperature through modulate the fluidity of the membrane by various strategies such as altering their FAs composition that include the FAs chain length and the proportion of cis to trans FAs (Chintalapati, Kiran, & Shivaji, 2004).

1.6.4. Whole genome sequencing based characterization

Whole Genome Sequencing (WGS) provides important information about the characteristics of novel bacterial isolates. For example, it has been used to identify non-pathogenic *Pseudomonas* strains for bioremediation, industry and agriculture applications by determining the absence of known virulence factors (Nelson et al., 2002). WGS for bacterial isolates are constructed using next generation sequencing (NGS) techniques. The NGS is a method of DNA sequencing which provides a rapid, high-throughput approach for the characterisation of genomes, transcriptome profile analysis of mRNAs, small RNAs and transcription factor regions tRNA (Ansorge, 2009). It has also been used to describe microbial community structure in different ecosystems (Indugu, Bittinger, Kumar, Vecchiarelli, & Pitta, 2016). NGS methods include Pyrosequencing (454) (Roche, Basel, Switzerland), Ion semiconductor (Ion Torrent sequencing), Sequencing by ligation (SOLiD sequencing) (Applied Biosystems, California, USA) and Sequencing by synthesis (Illumina (Solexa) sequencing) (Illumina, San Diego, USA) (Indugu et al., 2016; Quail et al., 2012).

The Roche 454 sequencer uses pyrosequencing technology, based on the detection of pyrophosphate (PPi) that released during amplification of a single strand DNA and nucleotide incorporation. In this process, the following enzymes DNA polymerase, ATP sulfurylase, luciferase and apyrase are used. The PPi converted to ATP, the ATP drives the luciferin into oxyluciferin and generates visible light. The light is detected by a camera and analysed in a pyrogram (Liu et al., 2012)**.** Ion semiconductor (Ion Torrent sequencing), the genomic DNA are divided into fragments, each fragment attached to the adaptor at the both sides for the amplification process. After that each single DNA strand attached to the beads, the coated beads then added to the semiconductor chips in the presence of the ion sensitive layer. In this process the DNA sequencing measurements based on hydrogen ions that released after aneling of each nucleotide. The four nucleotides are added one nucleotide sequence at a time. This method takes a shorter time, but it produces a short read length compared with other methods (Liu et al., 2012)**.** Sequencing by ligation (SOLiD sequencing), two types of sample preparation; fragment library single DNA fragment or mate-paired library two DNA fragments can be used. In both cases the adaptor ligated to the end of the targeted DNA fragment, the fragment amplified on beads in emulsion PCR, then the beads covalently attached to glass slide, with barcoding many samples can be run at the same time. The empty beads can be removed by centrifugation. In this process ligation based sequencing with Di-base probes that fluorescently labelled with 4 dyes. During sequencing, laser excites the fluorescent dye then the detector measures the energy photon that corresponding to dye colour (Salipante et al., 2014)**.**

Sequencing by synthesis (Illumina (Solexa) sequencing), it has a high-throughput community sequencing and low cost per sequence of amplicon compared with others NGS methods. It also called a bridge method where both sides of the DNA fragments are attached on the oligo-contains flowcell slide by adapters to form a bridge shape before amplification started. In this method the single strand sequencing started by adding four types of nucleotides (ddATP, ddTTP, ddGTP, ddCTP) which labelled by different cleavable fluorescent dye, the fluorescent signal that released during the sequencing process for each nucleotide could be detected through the images taken by camera (Caporaso et al., 2012; Liu et al., 2012; Salipante et al., 2014; Whiteford et al., 2009)**.**

1.7. Aims and objectives

The UK's geological disposal facility was proposed for the long term fate of radioactive materials supported by multi-barrier of the cementitious materials. the presence of a heterogeneous wastes include cellulosic materials in the ILW is an issue of particular concern due to a formation of CDPs include abundant amount of ISAs. The ISAs are capable to form complex with certain radionuclides increase their solubility in water. The ability of microbial consortia from the anthropogenic sites to degrade ISAs was carried out by previous studies.

There is currently an absence in the literature of characterised pure cultures able to utilise ISAs under alkaline and strictly anaerobic conditions. The recent detection of ISAs in the alkaline soils of the Harpur Hill site (Rout, Charles, et al., 2015a) suggests that ISA degradation may be more common than previously thought. The identification and characterization of novel alkaliphiles and ISA degrading bacteria in particular could provide a platform for the metabolic pathways underpinning ISA degradation to be determined and the associated genes identified. In addition, the isolation and identification of alkaliphilic bacteria may provide species that may be used for further applications, e.g. bioremediation processes.

The objectives of this study are:

- The establishment and characterisation of CDP fed microcosms established under GDF relevant conditions that include anaerobic, high pH and the CDPs as a sole carbon source, this to be as a selective environmental condition for ISA degraded alkaliphiles.
- The isolation of novel alkaliphilic bacteria from alkaline sediments of the CDPs fed microcosms after a period of an incubation time under an anaerobic condition and fed-cycles protocol. The isolation process through a series of an inoculation processes using agar plates to obtain pure culture.
- Test the ability of the bacterial isolates (single and pure bacterium) for ISA degradation under an anaerobic and alkaline conditions.
- The biochemical and genomic characterisation of these novel isolates, through comparing the results with the available information of the previous studies.

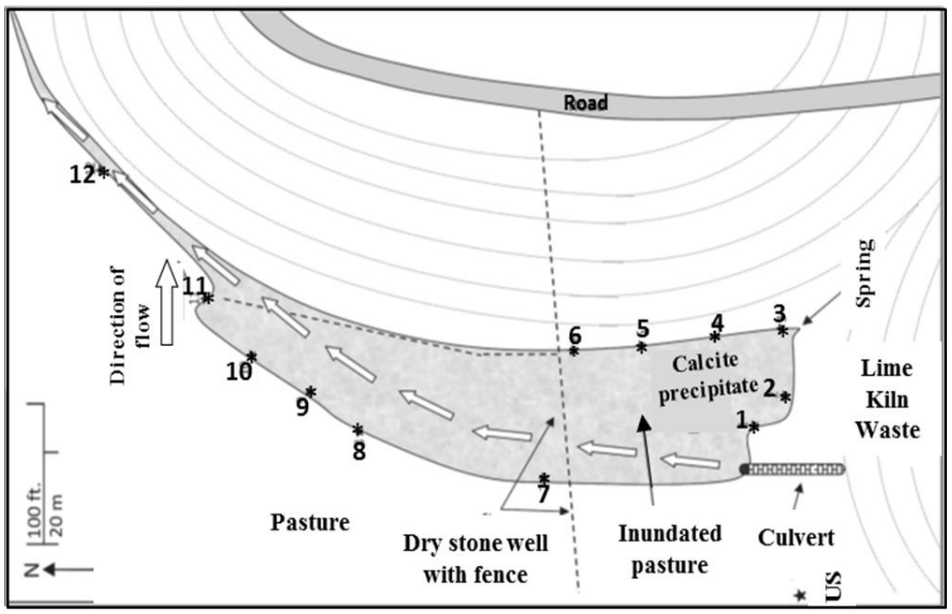
- The determination of the environmental capabilities (pH, salinity, heavy metal tolerance profiles) of these novel isolates that may use in bioremediation and novel industrial applications.

Chapter 2
2. Materials and methods

2.1. Samples

2.1.1. Alkaline soils samples

Samples from hyper-alkaline sediments were used in order to isolate alkaliphilic bacteria. The main source of alkaline soils was the Harpur Hill site, Buxton, Derbyshire, UK (Figure 2.1 and 2.2). The site has been contaminated for decades by high pH, lime kiln wastes (Milodowski et al., 2013). The soil samples were taken at depth (~10cm) from the points indicated on Figure 2.1 (Rout, Charles, et al., 2015a). These sediments are hyperalkaline, where the pH values of soil *in-situ* > pH 11.0 (Milodowski et al., 2013; Rout, Charles, et al., 2015a; Salah, Rout, & Humphreys, 2016) and contain high calcium and silicate concentrations, analogous to a cementitious radioactive waste repository (Rizoulis et al., 2012). The second source of samples were cotton samples that had been immersed in the alkaline environment of the Buxton site for three months prior to retrieval, further details are provide by Charles (Charles et al., 2015).



Site*	pH
1	11.7
2	7.7
3	11.2
4	11.07
5	11.35
6	10.80
7	10.17
8	10.30
9	8.00
10	9.00
11	7.85
12	10.30
US	7.40

Figure 2. 1: Overview of lime kiln waste site that was targeted for alkaline samples collection

This figure was taken from Rout *et al*. (2015), showing the site at Brookbottom, Harpur Hill, near Buxton in Derbyshire, UK. Sampling points are indicated by black stars and numbers with the pH value of each soil sample for each site. The US= Uncontaminated Site where the pH was near neutral (Rout, Charles, et al., 2015a). This site developed in the 1830's for the large scale production of lime for the alkali-carbonate industry which continued until the early 1950's, the area has a highly alkaline (pH >11) (Milodowski et al., 2013)

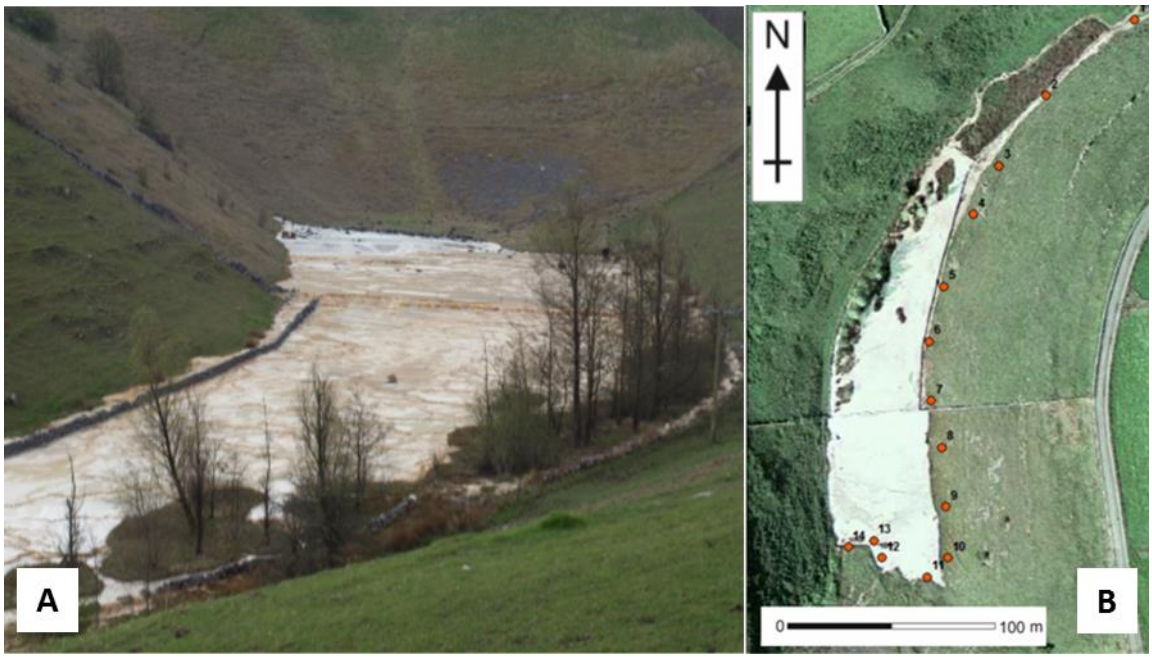


Figure 2. 2: View across the Harpur Hill site, showing the lime waste, white deposits of calcareous and tufa.

With a numerous of small "islands" with trees that have grown since 1971 (British Geological Survey © NERC 2014) (A). Aerial photograph of the site, showing the extent of the high alkaline leachate and tufa deposits of the calcium carbonate (Aerial photography ©UKP/Getmapping Licence No. UKP2006/01) (B) (Milodowski et al., 2013).

2.2. Media

2.2.1. Cellulose degradation products (CDP)

CDP was prepared by the method described by Rout et al. (2014) Briefly laboratory tissues (200 g, Pristine Paper Hygiene, London, UK) were hydrolysed in ultrapure water (1800 ml) in the presence of $Ca(OH)_2$ 10 g/l (18 g) and 0.1M of NaOH (7.2 g) in an anaerobic jar under anaerobic conditions. In order to remove oxygen from the vessel, the contents of the vessel were flushed with nitrogen gas for 30 min. The vessel was sealed and placed in the oven at 80°C for 30 d. Finally, the product was filtered via a 0.22 µm Millipore filter under anaerobic conditions and then stored in the dark. Following preparation the CDP was analysed for its ISA and volatile fatty acids content (Rout et al., 2014).

2.2.2. Isosaccharinic acid in the form of Ca $(ISA)_2$

α-ISA in the form of $Ca(ISA)_2$ was prepared as outlined by Vercammen *et al*. (1999) (Karlien Vercammen, Glaus, & Van Loon, 1999).

2.2.3. Mineral medium

Mineral medium (MM) was used as a basal broth for bacterial cultivation. The MM was prepared in deionised water (g/l): KH_2PO_4 (0.27g), $Na_2HPO_4.12H_2O$ (1.12g), NH_4Cl (0.53g), $CaCl_2.2H_2O$ (0.075g), $MgCl_2.6H_2O$ (0.1g), $FeCl_2.4H_2O$ (0.02g), $NaS.9H_2O$ (0.1g) and 10 ml of trace element solution (B.S.I, 2005).The components of the Trace elements (g/l) were: $MnCl_2.4H_2O$ (0.05g), H_3BO_3 (0.005g), $ZnCl_2$ (0.003g), $Na_2MoO_4.H_2O$ (0.001g), $CoCl_2.6H_2O$ (0.01g), $NiCl_2.6H_2O_2$ (0.01g), $NaScO_3$ (0.005) and $NaWO_4.2H_2O$ (0.002g). Resazurin as an oxygen indicator 0.001g, sodium thioglycolate 0.05% and L-Cystine 0.05% as reducing agents were added to 1L of mineral medium. In order to remove oxygen, the MM was purged with nitrogen gas for 30 minutes. The MM pH levels were adjusted with 4 M of NaOH to increase the pH and create an alkaline condition. MM was used as the basic solution for experiments, supplemented by either CDP or ISA, depending on the experiment requirements.

2.2.4. Minimal medium

Minimal medium (Min) was prepared in serum bottle 1000ml capacity, containing g/l of $NaHCO_3$ (2.52g), NH_4Cl (0.251g), $NaH_2PO_4.H_2O$ (0.6g), KCl (0.1g) supplemented with vitamins solution 1% (2.2.6.) (Lovley, Greening, & Ferry, 1984), and mineral solution of trace elements 1% that containing g/L: $MgSO_4.7H_2O$ (3.0), $MnSO_4.2H_2O$ (0.5), NaCl (1), $FeSO_4.7H_2O$ (0.1), $ZnSO_4$ (0.1), $Na_2MoO_4.2H_2O$ (0.001), $CoCl_2.6H_2O$ (0.1), $CuSO_4.5H_2O$ (0.01), $Na_2MnO_4.2H_20$ (0.01) (Bassil et al., 2015).

2.2.5. Modified ‘Horikoshi’ Broth medium (HBM)

Horikoshi broth medium was supplemented with yeast extract 0.1% (w/v) and peptone 0.25% (w/v) added to mineral components in (g/l); K_2HPO_4 (1.0g), $Mg_2SO_4.7H_2O$ (0.2g) and Na_2CO_3 (10g) (Koki Horikoshi, 1999). ISA was added to this medium as a sole carbon source instead of glucose. The medium was prepared under a stream of nitrogen gas to avoid any contact with oxygen.

2.2.6. Vitamins solution (supplement)

The formulation is based on Wolfe’s vitamin solution and contains folic acid 2.0 mg/l, pyridoxine hydrochloride 10.0 mg/l riboflavin 5.0 mg/l, biotin 2.0 mg/l, thiamine 5.0 mg/l,

nicotinic acid 5.0 mg/ml, calcium pantothenate 5.0 mg/l, vitamin B12 0.1 mg/l, p-aminobenzoic acid 5.0 mg/l, thioctic acid 5.0 mg/l, monopotassium phosphate 900.0 mg/l.

2.2.7. Agar plate media

A range of different agar media for the purpose of bacterial isolation include commercial and prepared media.

2.2.7.1. Commercial media

Commercial media including tryptic soy agar (TSA), fastidious anaerobic agar (FAA) and R2A agar from Oxoid (Thermo Scientific) were used. In all cases the pH of these media were adjusted to pH 9.5 and 10.0 before autoclaving in order to isolate alkaliphilic bacteria. A reducing agent 0.05% of L-Cysteine HCl was added to TSA and R2A before autoclaving to remove oxygen from the media.

2.2.7.2. Prepared medium

The ingredients of these media are MM or Min supplemented with a source of ISA as a sole carbon source either from CDPs 30% to 50% (V/V) or by using $Ca(ISA)_2$ or Na-ISA at 5 to 10 mM L^{-1} (W/V). In some cases 1% (V/V) multivitamins solution was added to the media, the media were solidified by 1.5% of agar No.1 (Oxoid^{TM}).

2.3. Microbiological Methods

2.3.1. The Gram-stain method

Gram staining was carried out as outlined by Harrigan *et al.* (2014), it divides bacterial cells into two groups, Gram-negative and Gram-positive, depending on the bacterial cell wall structure (Harrigan & McCance, 2014). The slides were examined under oil immersion using an Olympus microscope (Olympus BX 40F, Japan). Controls employing known Gram positive and Gram negative bacteria were used.

2.3.2. Electron microscopy

Scanning electron microscopy (SEM) was undertaken using a JEOL JSM-6060LV microscope (JEOL, USA). Samples were dehydrated using serial ethanol dilution of 25, 50, 75 and 100% for 2 min per step and then sputter coated via a gold palladium plasma (CA7625 Polaron, Quorum Technologies Ltd, UK).

2.3.3. Identification of pure culture and bacterial community by 16SrRNA gene sequences

2.3.3.1. Ultra Clean® Microbial DNA Isolation Kit

The Ultra Clean® Microbial DNA Isolation Kit (Mo Bio, lab USA) was used to isolate genomic DNA of pure bacterial isolates according to the manufacturer's instructions.

2.3.3.2. Isolation genomic DNA from soil sample

The PowerSoil® DNA Isolation Kit (MO-BIO, Carlsbad, CA, US) includes a humic substance removal technique for isolating genomic DNA from environmental samples, and it was used according to the manufacturers' instructions.

2.3.3.3. Griffiths method for DNA extraction

DNA was extracted from some environmental samples by following a modified version of the method provided by (Griffiths, Whiteley, O'Donnell, & Bailey, 2000).

2.3.3.4. Quantification of DNA sample

Quantification of genomic DNA was carried out using the Jenway genova nano spectrophotometer (Jenway, UK) at 260 nm, by adding 1 µl of the DNA at the top of the sensor, 1µl of ultrapure water was used as a blank.

2.3.3.5. DNA Electrophoresis

One percent (1% w/v) agarose gel (Bioline, London) was prepared incorporating 1µl of gel dye (SYBR® safe stain, USA). Loading dye was added to each DNA sample (5µl sample and 1µl loading dye (Bioline, London, UK)). 5µl of a 1Kb ladder (Invitrogen, USA) and 6 µl DNA product was loaded into the wells. The gels were run for 60 minutes at 90 volts. The bacterial DNA bands were detected and compared with the 1Kb HyperLadder™ on a gel documentation system (BioDoc-It® Imaging System, Cambridge, UK).

2.3.3.6. Amplification of 16S rRNA gene by PCR

The PCR of 16S rRNA gene was carried out using a universal primer (10% primer mix of the primer pA and pH). PCR was run on a thermal cycler (Techne, Staffordshire, UK) under the following programme: initial temperature 95ºC for 5min, followed by 35 cycles, each

cycle pass through 3 steps: denaturation of DNA at 94°C for 1 minute, annealing of primer at 60°C for 45 second, extension at 72°C for 1 minutes and final extension of 72°C. The PCR product was annealed in the thermocycler at 4°C. Agarose gel electrophoresis was performed to confirm the PCR products before purification.

2.3.3.7. PCR product purification

The PCR products were purified using the QIAquick purification (QUAGEN, UK) kits. The purified DNA concentration was measured by spectrophotometer (Genway, Genova Nano, Stone, UK) at 260nm and confirmed by agarose gel electrophoresis. Purified DNA was prepared as per the instructions provided by the Eurofins Genomics DNA sequencing services (UK).

2.3.3.8. Analysis of bacteria sequencing

The 16S rRNA gene sequencing results obtained from Eurofins MWG Operon (London, UK) were used to identify bacterial isolates, by comparing the sequence with the Genebank database (K. S. Park et al., 2012). Isolates were identified to the species or genus level using Basic Local Alignment Search Tool (BLAST) searches (Jisun Kim, Jung, Sung, Chun, & Park, 2012). In addition a number of online databases were used for gene sequence analysis such as the Ribosomal Database Project (RDP) (Cole et al., 2014) which is provided by the National Centre for Biotechnology Information (Acland et al., 2014). RDP (Release 11, Update, September 30, 2016) was used to identify and classify bacterial strains using the hierarchy browser http://rdp.cme.msu.edu/index.jsp. The obtained sequences together with the bacterial isolate sequences were submitted to Genbank through MEGA5 (Tamura et al., 2011). For Eubacterial sequences the 16S rRNA sequence for *Escherichia coli* (Genbank accession number J01695), was used as a reference for chimera checking in the Genbank at the following URL http://www.ncbi.nlm.nih.gov/genbank (Wheeler et al., 2007).

2.3.3.8.1. Construction of phylogenetic trees

Bacteria gene sequences were aligned with reference sequences obtained either from Genbank or the hierarchy browser component of RDP were used to build phylogenetic trees using the MEGA 5 software programme (Tamura et al., 2011). Multiple Sequence Comparison by Log-Expectation (MUSCLE) and Neighbour-Joining was used to estimate

phylogeny using a 1000 replicate bootstrap analysis of Maximum Composite Likelihood methods (Kumar, Nei, Dudley, & Tamura, 2008).

Unweighted Pair Group Method with Arithmetic Mean (UPGMA), this programme used to build a rooted tree (dendrogram) that shows the pairwise similarity matrix structure between two clusters by phylogenetic distances, it also reflect the similarities between Operational Taxonomic Units (OTUs) http://www.icp.ucl.ac.be/~opperd/private/upgma.html

2.3.4. Community analysis via 16SrRNA gene sequence analysis

Community analysis (Eubacteria & Archaea) was carried out based on 16S rRNA gene sequences generated via next-generation sequencing (NGS) technologies, and the CLcommunity™ software provided by ChunLab (Korea).

2.3.5. Storage of pure bacterial cells

Pure cultures were stored on Microbank beads (ProLab Diagnostics Ltd) at -80 °C. In the case of spore forming bacteria, spore suspensions were made and stored in 50% ethanol at -20°C.

2.3.6. Identification of pH profile for bacterial isolates

The pH profile of the bacterial isolates was determined via the Bioscreen C Automated Growth Curve System (Finland) taking measurements at 450-600nm in either; fastidious anaerobic broth, TSB or ISO-sensitest^{TW} broth CM0473 (OXOID). The pH was adjusted to a pH range of pH 4-12 in 1.0 pH increments using HCl 2M or NaOH 4M accordingly. Replicates were prepared in the honeycomb 100 well microplates designed for this system. In the case of strict anaerobic bacteria, the plates were sealed using gas proof tape. The Bioscreen C System was set for automatically OD measurement each hour, under continual shaking for between 24 hours to two days (depends on the generation time). In order to find the optimum pH, the area under growth curve was calculated, and the optimum pH value was determined for each bacterium. In order to determine the impact of pH and temperature, pH profile experiments were repeated at a range of temperatures either through adjustment of the instrument or by placing the Bioscreen in a cooled incubator.

2.3.7. Heavy metals tolerance

In order to determine heavy metal tolerance the minimal inhibitory concentrations (MIC) of nickel, copper, lead, cadmium, cobalt and zinc were determined using the Bioscreen C instrument. Stock solutions were prepared from the metal chlorides and serially diluted to give a final concentration range from 0.5 to 10 mM at 0.5 mM intervals. In order to maintain the correct concentration of the metal in the instrument the stock solution for each metal was prepared at double strength and sterilized at 121ºC for 15min. The ISO-sensitest broth CM 0473 (OXOID^{TM}) employed was also prepared in double strength 4.68% (W/V) and sterilized at 121ºC for 15 min. Bacterial test suspensions were prepared from fresh bacterial colonies and adjusted to an optical density at 620 nm that were equivalent to 10^7 to 10^8 CFU/ml. The test plates were set up with duplicates for each metal concentration, and triplicate positive controls and duplicate negative controls at each metal concentration. The Bioscreen System was set to run from 24 to 36 hours at 30ºC with low shaking and hourly readings with a wide band wavelength (420-600nm).

The MIC was calculated as per the method outlined by Lambert and Pearson (2000), where the fractional are is calculated as per below (Lambert & Pearson, 2000):

$$fa = (X1 - NC) / (PC - NC) \qquad \text{eq. 1}$$

Where X is the average O.D. at each concentration, NC is an average of the O.D. of the negative control; PC is an average of the O.D. of the positive control. The MIC is then calculated via the fitting of a modified Gomperts function. The fractional area (y) to the log of heavy metal concentration (x) was analysed using a Gompertz equation eq. 2 in order to get calculated values for y (ycal.):

$$ycal = A + C * EXP(-EXP(B * (X - M))) \qquad \text{eq. 2}$$

Where A, C, B and D were calculated by Data-Solver program (Microsoft Excel to determine the maximum or minimum value), by solving the total of the error ^{2} value that was calculated by subtracting (fa) value from y calculated value for each HM concentration values, and the random values of the A, C, B and M. In order to get the lower asymptote of y that is A, slope parameter B, the distance between the upper and lower asymptote C, and M is the log concentration of the inflexion point (Lambert & Pearson, 2000).

2.3.8. Extracellular polymeric substances detection

The production of ESP was detected fluorescence microscopy, using 0.1% fluorescent brightener 28 (FB28) in deionised water. A smear was prepared for each isolate, flooded with FB28 for 10 minutes, and then the slide was examined by fluorescence microscopy.

2.3.9. Detection of bacterial biofilms

After incubation for 24 to 48 hours at 25°C on an agar plate, a bacterial suspension was prepared in TSB broth at an optical density equivalent to 10^8 CFU/ml. The bacteria suspension (200 µl) was transferred to a 96 well plate, four wells for each isolate, the plate was incubated at 25°C for 24 to 48 hours. Following incubation the plate was rinsed and washed four times using sterile 0.9% sodium chloride. The formed biofilm was fixed by adding methanol (200 µl) for 15 minutes. The methanol was then rinsed and a crystal violet stain 2% (v/v) (200 µl) added for 5 minutes before the plate was rinsed again (x4) with 0.9% buffer saline. Finally glacial acetic acid (33% v/v) was added as a decolorizing (160 ml) agent before the optical density was measured by spectrophotometer (FLUO star OPTIMA (BMG Lab tech)) at 570 nm. Biofilm formation was evaluated by calculating the average OD of four replicates for each strain and comparison with the OD of the negative control (medium without inoculation). The extent of biofilm formation was evaluated as outlined below (Stepanović, Vuković, Dakić, Savić, & Švabić-Vlahović, 2000).

$OD \leq ODc$	No biofilm formed
$ODc < OD \leq 2x\ ODc$	Weak biofilm formed
$2x\ ODc < OD \leq 4x\ ODc$	Moderate
$4x\ ODc < OD$	Strong

Optical density cut-off value (ODc) = average OD of negative control + (3x standard deviation (SD) of negative control).

2.3.10. Quinones and polar lipids

Menaquinones are abbreviated MK-n, where (M) stands for menaquinone that represent the functional group of "quinone" ring that are found in all (K) vitamins and the (n) represents the number of isoprenoid units in the side chain. Where the name of MK refer to the length and degree of saturation of the carbon tail (Figure 2.3).

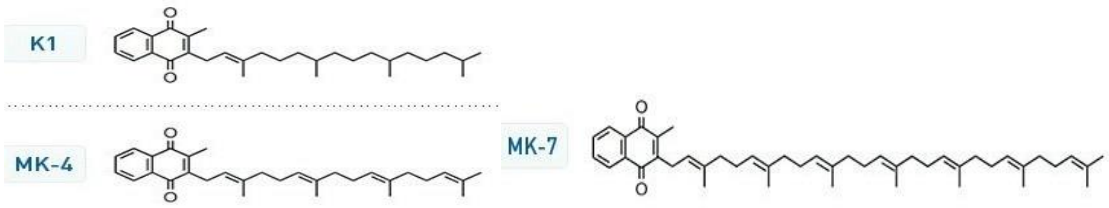


Figure 2.3: An example of the menaquinone structure

Analysis of the respiratory quinones and polar lipids (PLs) were carried out by DSMZ (Braunschweig, Germany). Two dimensional Thin Layer Chromatography (TLC) was used to separate the polar lipids, the separated PLs were identified using Molybdatophosphoric acid and heating at 200ºC for 10 min.

2.3.11. Cellular fatty acids contents of bacterial isolates

In order to identify and analyse the fatty acid content of the isolates, one isolate was sent to DSMZ (Braunschweig, Germany) whereby freeze dried cells of the pure culture were prepared as specified by DSMZ. For some isolates, the fatty acid methyl esters (FAME) were extracted from 40 mg of bacterial cells using the method Sherlock^{TM} Microbial Identification Incorporation (MIDI Inc., Newark, USA) (Sasser, 1990). In this study, the analysis was performed by using Gas chromatography-mass spectrometry (GC-MS) system (Algilent technologies, Edinburgh, UK) (2.4.4) and the library of the National Institute of Science and Technology (NIST).

2.3.12. Biolog® EcoPlate for R1 pH10, R2 pH11 and R4 pH10.5

Biolog® EcoPlates enable the metabolic profiling of microbial communities. The Biolog plates contain 96 wells containing 31 different carbon sources (substrate) plus a blank well (water) with each treatment present in triplicate. Utilization of the carbon sources is indicated by the reduction of Tetrazolium Violet Redox Dye (TVRD), which changed from colourless to purple (Gryta, Frąc, & Oszust, 2014). In addition, substrates were subdivided into five group substrates, carbohydrates, carboxylic and ketonic acids, amines and amides, amino acids, and polymers, according to Weber and Legge (Zak, Willig, Moorhead, & Wildman, 1994). EcoPlates were prepared by spinning down (10,000 rpm for 5min) 5ml of test material and resuspending the pellet in 15ml sterile (0.9%) NaCl prior to inoculating each well with 150 µl of this suspension, followed by incubation at 25ºC. Microbial activity in each well was expressed as colour development after 24 hours to 5 days incubation.

2.3.13. Whole genome sequencing

The whole genome sequencing for some isolates were performed by microbesNG https://microbesng.uk/ Birmingham, UK, and by BaseClear DNA sequencing services, Netherlands. Genomic DNA was extracted using an Ultraclean microbial isolation kit (Mo-Bio, USA) and associated protocol. The genome was then sequenced using a whole genome shotgun sequence (WGS) strategy. Illumina HiSeq 2500 were used to generate paired-end 125 cycle sequence read (BaseClear), Illumina CASAVA and CLC Genomics Workbench version 7.0 were used to generate FASTQ sequence file and assembly, respectively. Scaffolds or supercontigs were generated by linking the contigs (Boetzer et al., 2011). Finally, Rapid Annotation in the Subsystem Technology (RAST) (Aziz et al., 2008) and the National Centre for Biotechnology Information (NCBI) Prokaryotic Genome Automatic Annotation were used to construct the final de novo annotation of the genome (Benson et al., 2013).

2.4. Methods for chemical analysis

2.4.1. ISA concentration measurements

ISA concentration was measured by High-Performance Anion-Exchange Chromatography on either a Dionex 3000 or 5000 system (Dionex, Camberley, UK) with a Pulsed Amperometric Detection (HPAEC-PAD) and a CarboPac PA20 Column, at a flow rate of 0.5 mL min^{-1}. The mobile phase was 50 mM NaOH and the column was regenerated by eluting buffer 200 mM NaOH for 20 minutes. The internal control was 40 mg/l D-ribonic acid which was added to each sample prior to analysis. A volume of 10 µL from each sample was automatically injected onto the column. The integration process was performed using the 'Chromeleon 7.0' Software package. The results were calculated using a set of ISA standards (100, 75, 50 and 25 mg/l) (Appendix-1) and associated peak areas for each sample (Shaw, Robinson, Rice, Humphreys, & Laws, 2012).

2.4.2. VFAs concentration measurements

VFAs were measured using a Gas Chromatograph (GC), Model GC6890 (Hewlett Packard, UK) and FID, a HP-FFAP Agilent Technology (30 m x 0.535 m x 1.00 µm) column, and a helium mobile phase. The operating conditions were: an initial temperature of 95°C for 2 minutes, then 140°C no hold, followed by 200°C with a hold of 10 minutes, after that to a post run temperature of 50°C was employed. Samples were acidified using 85% phosphoric

acid prior to the injection of 1µl of sample. Concentrations were determined by comparison to a range of standard stock solutions of volatile fatty acids (Supelco analytical, Pennsylvania, US). The calibration curve (Appendix-1) was performed for each acid at a linearity >0.99 in each case.

2.4.3. Head space gas analysis

Head space gas analysis (CH_4, H_2, CO_2) was performed via gas chromatography using a Model 6850 Agilent GC with a thermal conductivity detector fitted with a GS-Q column (30m x 0.53mm ID, Agilent technologies, Berkshire, UK) and a helium carrier gas, the column temperature was 30°C and the detector temperature 200°C. Sample from the headspace of cultures or microcosm reactors were collected by a lockable gas syringe prior to injection. The retention time for each gas was determined using known volumes of each gas.

2.5. Microcosm studies

A wide range of microcosm studies were performed in this investigation. These studies are described below and summarised in Table 2.1.

2.5.1. CDPs-fed microcosm reactors at pH 10.0, pH 11.0 and pH 12.0

An alkaline soil sample (30g) was divided equalise into three 250 ml Schott bottles (Figure 2.4) containing 150 ml MM (2.2.3) and 25 ml CDP. The three microcosms were adjusted to pH 10, 11, and 12 respectively. The microcosms were purged for 20 minutes with nitrogen gas before being sealed. Twenty-five (25) ml of CDPs was added monthly to each microcosm and the pH readjusted to the target pH, until a final volume of 250 ml. Once the microcosm volume had reached 250 ml, 25ml of waste was removed prior to feeding, the microcosms were fed monthly for 12 months. After this period, the protocol was switched from 30 to 10 days waste feed cycles. In order to keep the pH levels for each reactor at the particular values for isolation of the bacteria that can tolerance alkaline environment.

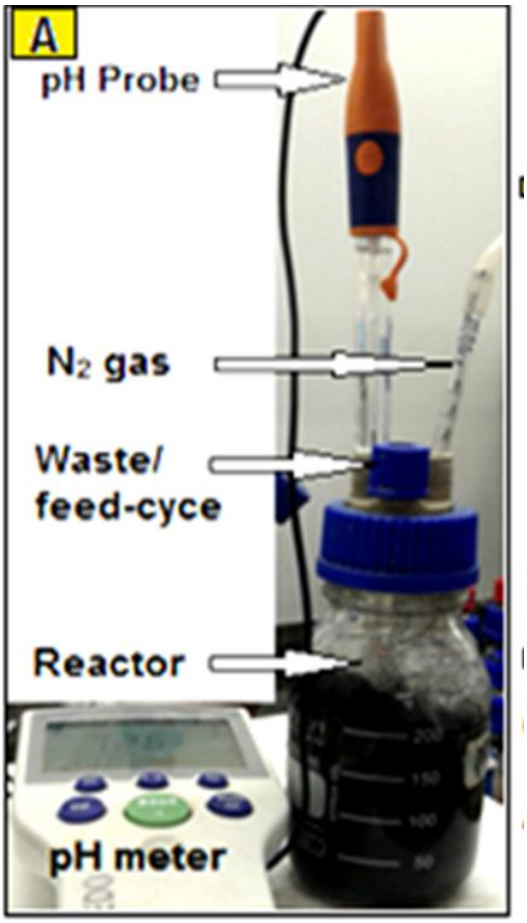


Figure 2. 4: Scheme of the experiments procedures in this project.

Setting up microcosm reactors subjected to a particular pH under anaerobic conditions, nitrogen gas free oxygen used in order to prevent any contact with oxygen during CDP feed-cycle and pH adjustment, that followed by sampling for chemical and biological analysis under anaerobic condition. The sediment samples were used to isolate bacteria in pure culture on agar plates under anaerobic condition, the bacterial isolates were identified by molecular methods, DNA extraction, DNA amplification by PCR, then the purified PCR products were confirmed on agarose gel electrophoresis, after that the purified DNA was used for bacterial identification through 16SrRNA gene sequencing. The filtered broth was used to measure the ISA concentration by using ion exchange chromatography and VFAs concentration was measured by gas chromatography.

2.5.2. Additional microcosms at pH 8.0, 8.5 and 9.5

Harpur Hill soil samples were incubated in MM supplemented with CDP (50:50 v/v mix). The media was dispensed into six 50 ml bottles, which were then inoculated with 3.0g of soil. The pH of the microcosms reactors was then adjusted to either pH 8, pH 8.5 or pH 9.5 respectively. Control reactors were autoclaved and all reactors incubated at 25ºC in the dark. All reactors were sampled every 5 days for 30 days for the usual suite of analyses. The microcosms at pH 9.5 was adjusted to a pH 9.0 (microcosm R5) and incubated for further incubation under a protocol of CDPs feed-cycle each 30 days to give three microcosms reactors operating at three pH values one unit apart with the previous microcosms R1-pH 10 and R2-pH 11 (2.5.1.).

2.5.3. Establishment of a batch fed reactor with an evolving pH (pH 8.5 to 9.5 to 10.5)

Microcosm reactor 4 of mineral media and CDP 4 was set up with a CDP waste-feed cycle and a pH that was adjusted from 8.5 to 9.5 and then 10.5 every 30 day intervals. Initially two 250 ml reactors were set up at pH 8.5 one of them as a control, each bottle containing 50 ml of CDPs and 130 ml MM. For the control bottle, 10 grams of soil was autoclaved and added into the bottle under a stream of nitrogen gas. Another 10 grams of soil was added directly into the test bottle. Following incubation condition, 2ml from each reactor was collected every 7 days throughout the study. All samples were collected in sterile Eppendorf tubes under anaerobic condition by purging with nitrogen gas and stored for chemical and biological analysis.

2.5.4. Bacterial isolation

In order to isolate alkaliphilic bacteria in pure culture, microcosm sediments were subcultured onto a range of media under anaerobic conditions. Before subculturing the agar plates were kept inside an anaerobic workstation (CO_2 10%, H_2 10% and N_2 80%) for two days in order to remove oxygen from the media. This step was confirmed by adding 0.001% resazurin as an oxygen indicator. The samples were inoculated both directly and after a serial dilution onto the following media: FAA, CDP/MM, $Ca(ISA)_2$ 5mM/MM and (R2A+0.5% glucose). All plates were incubated anaerobically at 25°C. Once growth was observed, individual colonies were removed and plated out on identical media. The isolation steps were repeated several times, followed by Gram-staining in order to ensure a pure culture. Initially

isolates were tested to determine if they were strict or facultative anaerobes, by incubation under both aerobic and anaerobic conditions. Isolates were identified by 16SrRNA gene amplification, purification, sequencing and reference to sequence libraries.

2.5.5. The Influence of pH and the Availability of Terminal Electron Acceptors on the Degradation of CDP

In order to determine the influence of pH and TEA on population diversity, a range of microcosms (100 ml for each bottle) (Figure 2.5), 3 bottles for each TEA were set up at pH 7.0, pH 8.0, pH 9.0 and pH 10.0 using CDP as an electron donor and Nitrate, Sulphate and Iron (III) as potential electron acceptors, where the total of the bottles were 12. The relative concentration of Nitrate and Sulphate was established by the addition of 33.6mM $NaNO_3$, Na_2SO_4 (21mM) and 30mM Ferrihydrite. The ferrihydrite (Fe (III) oxyhydroxide) was prepared as outlined by Wu *et al.* (Y. Wu et al., 2015). Additional fermentation microcosms were also set up. The microcosms were set up with MM broth supplemented with CDPs (30% v/v), 30ml CDP was added to 70ml broth of MM, the redox indicator resazurin (Fisher, UK) was also added. The media was prepared under nitrogen and autoclaved prior to the addition of 5 grams of Harpur Hill soil and then incubated in the dark at 25ºC for 1 week.

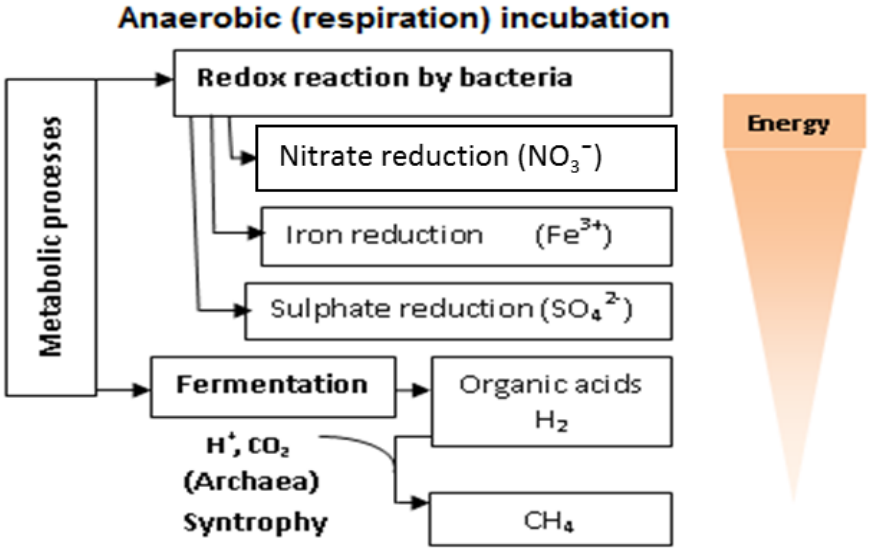


Figure 2. 5: Redox reaction compared with a fermentation process for ISAs

After 1 week's incubation the microcosms were sub-cultured into identical microcosms (MM, pH, TEA, CDP) and incubated for a further week and then subcultured into duplicate microcosms and incubated for a further 30 days with regular sub sampling. All samples were filtered (0.45 µm syringe filters) and preserved in -20ºC prior to analysis for ISA, VFA,

Nitrate, Sulphate and Iron (III). Furthermore, at the end the incubation period, the contents remaining in the microcosm was centrifuged and the DNA associated with the resulting pellet extracted for community analysis.

2.5.5.1. Quantification of Nitrate, Sulphate and Iron concentrations

Sulphate and nitrate concentrations were measure via ion chromatography with a Metrosep AS7 column (Metrohm IC 850 professional, Metrohm, Cheshire, UK). Sample concentration was determined by reference to a standard curve. Sodium carbonate (3.2 mmol L^{-1}) and sodium hydrogen carbonate (1.0 mmol L^{-1}) were used as an eluting buffer. Iron-(III) and Iron-(II) concentrations were determine using the ferrozine method described by (Viollier, Inglett, Hunter, Roychoudhury, & Van Cappellen, 2000).

2.6. Pure culture studies

In order to find alkaliphilic bacteria with the ability to degrade ISA a wide range of broth based pure culture studies were performed. The protocols for bacterial isolation from the microcosm reactors has already been outlined (section 2.5). These investigations employed two types of basal media, mineral media (2.2.3) and minimal medium (2.2.4) supplemented by one of the different forms of ISAs with and without other supporting growth factors such as vitamins solution, yeast extract, peptone and, under alkaline and in anaerobic conditions. Microbial metabolism was determined via substrate and product profiles alongside headspace analysis and microbial growth data.

2.7. Statistical analysis

The arithmetic mean of the values that obtained after a chemical analysis of the samples that collected from the microcosms during an incubation time include ISA and VFAs concentration. These values were used to calculate the standard deviation to measure the amount of variation values. The variation value for each reading was represented by error bars on the graphs.

Table 2. 1: Summary of the ISAs driven microcosms experiments

The table provide a summary of the microcosm experiments, including experiment number, type of each microcosm (R), the sources of sample for each experiment, pH levels, incubation time, the aim of each experiment and the result number for each experiment.

Experiment number	Rs	Sample source	Broth media	Supplements	pH and incubation time	Results numbers	Aims of each experiment
2.5.1.	R1, R2, and R3	Alkaline Soils from Buxton site	MM and CDPs waste-feed cycle each 30 days	No	Three of Rs, pH 10.0, 11.0 and 12.0. Incubated for 12 months at room temp.	**3.1.1.**	The CDPs waste-feed cycle was carried out after 4 months of an incubation time, under a control of the pH each 30 days for 12 month, in order to isolate ISA degrading alkaliphilic bacteria
2.5.1.	Sub-microcosms	Sediments 0.2ml from the R1	MM/ $Ca(ISA)_2$ 5mM	No	pH 9.0, at 25°C, for 10 days of an incubation time	**3.3.1.**	In this experiment ISA in the form of $Ca(ISA)_2$, as a sole carbon source, without supplements
2.5.2.	R5 pH 9.0	2nd batch of soils	MM/CDPs 50%	No	Three bottles at pH 8, 8.5 and 9.5, for 30 days. Followed by waste-feed cycle each 30 days at pH 9.0.	**3.1.1.**	Using soil samples from a different place of the Buxton site, it may provide a different microbial community
2.5.1.	Sub-microcosm	Sediments 0.2ml from the R1	MM/Na-ISA 10mM, and MM/$Ca(ISA)_2$ 5mM	No	Adjusted to pH 9.0, and incubated at 25°C, for 30 days	**3.3.1.**	The experiment was carried out to compare a biodegradation of ISA in the form of Na-ISA with $Ca(ISA)_2$ by bacterial community
2.5.3.	R4 pH 10.5	3RD batch of soils	MM/CDPs 30%,	No	Adjusted to pH 8.5, then after 30days, CDPs waste-feed cycle and adjusted to pH 9.5, then after 30 days, CDPs waste-feed cycle and adjusted to pH 10.5 for 30 days incubation at 25°C	**3.1.2.**	In order to adapt bacterial community for high pH using new alkaline soils samples of the Buxton site, the CDPs 30ml waste-feed cycle protocol was carried out each 30 days, associated with a protocol of bacterial isolation process.
2.5.1.	R1, R2, and R5	Rs (The same reactors)	MM and CDPs after 12 month of waste-feed cycle each 30 days, the CDPs waste-feed cycle was switched to each 10 days.	No	pH 10.0, pH 11.0 and pH 9.0, after 12 month incubation the CDPs waste-feed cycle was switched to each 10 days, for 40 days of an incubation time	**3.1.3.**	This process was carried out in order to keep the pH levels at particular values, each 10 days for 40 days, for isolation of Alkaliphiles

Experiment number	Rs	Sample source	Broth media	Supplements	pH and incubation time	Results numbers	Notes and comments
2.5.1. and 2.5.3.	Sub-microcosms	Sediment 0.2ml from R2, and 0.2ml from R4	Minimal medium (2.2.4.)/ $Ca(ISA)_2$ 2mM	vitamins solution 1%, for supporting the growth	The Rs were adjusted to pH 9.0 and incubated at 25°C	**3.3.1.**	In this experiment a broth of minimal medium was used instead of MM to provide a different nutritional components for bacterial community, and in order to isolate alkaliphilic bacteria.
2.5.5.	Microcosms	Alkaline Soils from Buxton site	MM/CDPs 30%,	Nitrate, Sulfate and Iron(III), to be used as TEAs and CDPs contents to be used as an electron donor	Each metabolic condition of each R were adjusted to four pH levels; pH 7.0, pH 8.0, pH 9.0 and pH 10.0 and incubated for 7 days. Subcultured for a further 7days, and then subcultured again and monitored 30 days.	**3.5.**	TEA experiment at pH 7, 8, 9 and 10, to investigate community evolution in response to energy pathways and pH levels.

Chapter 3
3. Results and discussion

3.1. Alkaline Microcosm Studies

A wide range of alkaline microcosms were operated throughout this study (Table 2.1) with a view to:

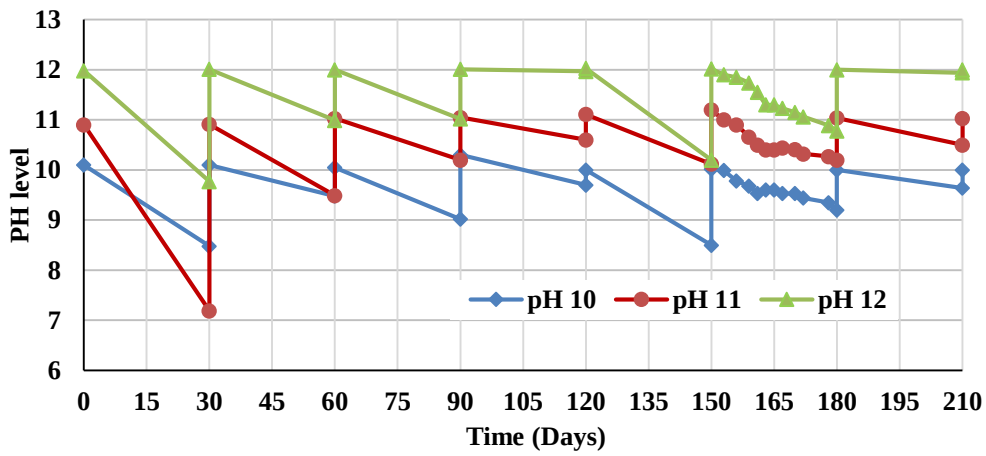
- Gaining an insight into the microbiology of CDP and ISA degradation and the associated microbial communities;
- Isolating and characterising alkaliphilic, ISA degrading bacteria.

The data presented here are from those microcosms that contributed significantly to the overall progress of the project. Those microcosms not described in detail are outlined in Table 2.1, and are not reported in detail either because the duration of operation was short or the results generated replicated those already reported for other microcosms.

3.1.1. Initial alkaline microcosms

The first batch of soil microcosm reactors (2.5.1) were established in order to isolate alkaliphiles and to determine their ability to degrade ISAs under alkaline, anaerobic conditions. Three microcosms were run on a 10%, 30 day waste/feed cycle and maintained at pH 10.0, pH 11.0 and pH 12.0. The 30 day feed cycle was chosen to impose a longer minimum growth rate than previously employed by Rout (Rout, Charles, Doulgeris, et al., 2015) and Charles (Charles et al., 2015). The pH of the microcosms was measured regularly with associated volatile fatty acid (VFA) concentrations recorded from day 120 onward.

A)



B)

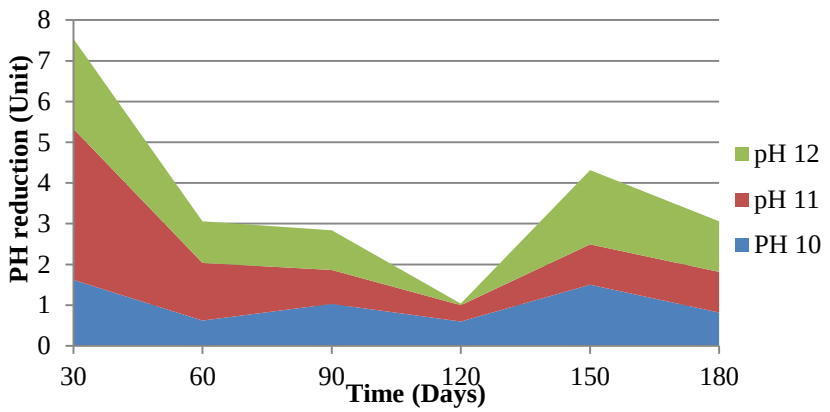


Figure 3. 1: pH decrease in the microcosm at pH 10, 11 and 12

The reduction of the pH at the end of each 30 day cycle (A), is an indicator of bacterial activity. The magnitude of reduction was variable between 30 day feed cycles (B).

Between feed cycles a reduction of pH (Figure 3.1 A) of between 0.8 and 3.0 units (Figure 3.1 B) was observed in all microcosms due to acetic acid generation via fermentation. It should be noted that a small amount (≈0.5 mM) of acetate is a component of the CDPs stock solution. The amount of acetate generated within the microcosms varied at all pH levels and peaked around 20 mM after 150 days in all cases (Figure 3.2). Towards the end of operation (Days 160 to 180) the acetate levels levelled off with the pH 10.0 system having higher level of acetate than the higher pH values suggesting that the fermentation activity at pH 10.0 was greater than that seen at pH 11.0 and pH 12.0.

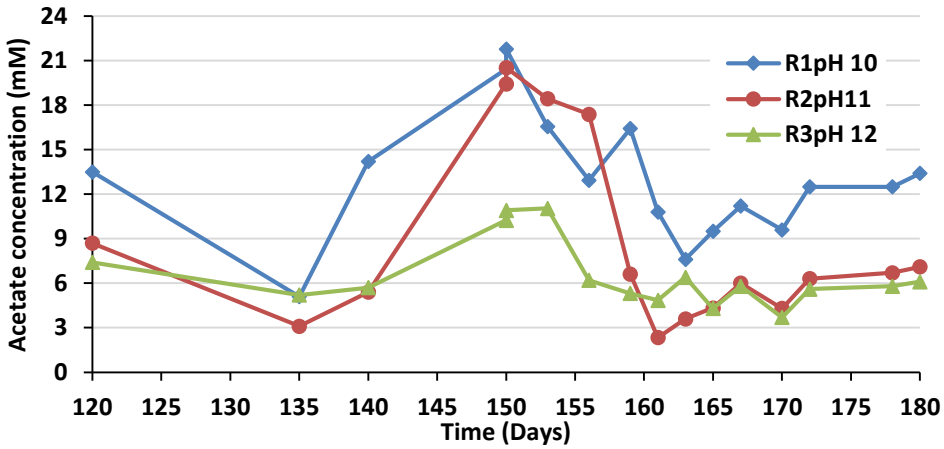


Figure 3. 2: Acetate concentrations at different pH levels pH 10, pH 11 and pH 12.

Acetate concentrations varied across the experimental period indicating that the levels of fermentation also varied. The amounts of acetate levelled off towards the end of the operational period with the pH 10.0 reactor having a higher level of acetate suggesting that this reactor was sustaining a higher level of fermentation.

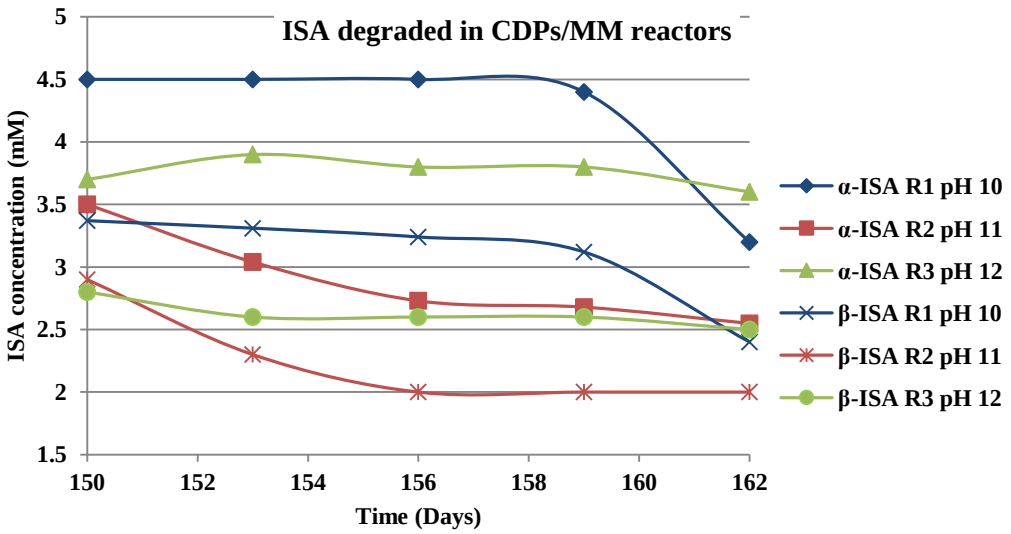


Figure 3. 3: ISA concentrations at different pH levels pH 10, pH 11 and pH 12.

There was a small amount of α-ISA degradation at pH 10.0 and pH 11.0, but no signs of any reduction at pH 12.0. While the β-ISA concentration decreased at all pH levels include pH 12.0 (Figure 3.5 B), suggesting that β-ISA may be more degradable at high pH than α-ISA.

Analysis of the head space gases indicated that methane was accumulating in the headspace of the pH 10.0 microcosm (Figure 3.4 A), whilst hydrogen was accumulating at pH 11.0 (Figure 3.4 B) with no changes in the headspaces gases detected at pH 12.0. The gases were identified by using a known sample of both gases (Figure 3.4 C and D). This data indicates that the pH 10.0 microcosm had an active fermentative and methanogenic community whilst the pH 11.0 microcosm was primarily fermentative and that pH 12.0 microcosm had limited microbial activity, when the pH level increased, the microorganism activity decreased and it was inhibited at pH 12.

The pH of these microcosms meant that any CO_2 generated would remain in solution and would not be detected in the headspace. Although no biogenic gases were detected at pH 12.0 the reduction in pH observed suggests that some microbial activity was occurring. This data contradicts that published by Rout *et al.* (2015) who established methanogenic communities from these sediments at pH 11.0 (Rout, Charles, et al., 2015a), however, this fermenter was run on shorter waste/feed cycle than those described here. When operated at pH 9.5 the microcosm gave similar results to those obtained at pH 10.0 with a reduction of pH associated with the fermentation of both forms of ISA generating acetic acid (Figure 3.5 A-D). The concentration of acetic acid (40 mM) being greater than that observed at the higher pH values.

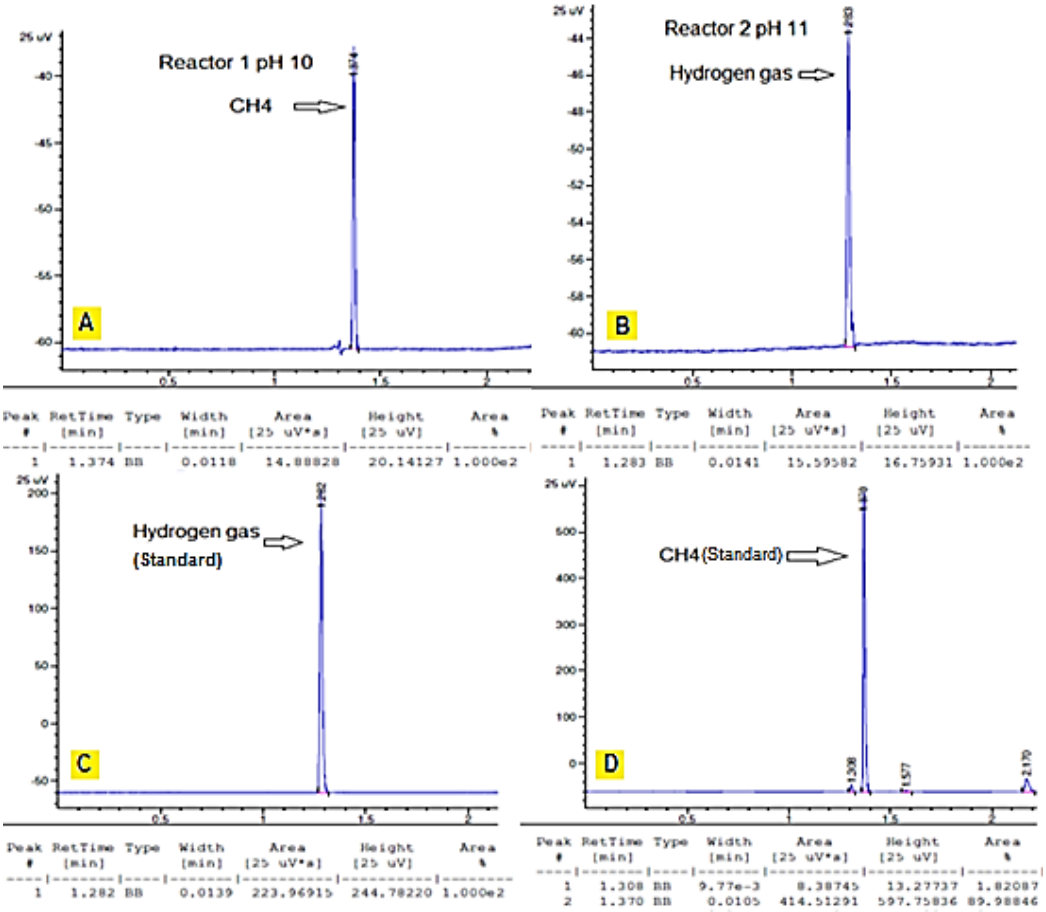


Figure 3. 4: Biogas detected from the head space of the microcosm at pH 10 and pH 11.

CH_4 was detected after 24 hours of each CDPs feed-cycle at pH10.0 (A), Hydrogen was detected after 2 days of incubation after each CDPs feed-cycle at pH 11.0 (B). These gases were identified by comparing the retention time for each peak with standards of hydrogen and methane (H) and (D) respectively.

Considerable amount of ISAs were degraded during 15 days of incubation (Figure 3.5 A) generating acetate, CH_4, CO_2 and H_2. Initially H_2 gas was detected (after 4 days) which was accompanied by CH_4 and CO_2 after 20 days (Figure 3.5 F). The CO_2 was detected once the microcosm had acidified (Figure 3.5 D). The results indicated of the presence of hydrogenotrophic methanogens as a part of the bacterial community in the microcosm. This result is in agreement with Rout *et al*. (2015) who found that CDP fed microcosm operated at pH 10.0 were dominated by fermentative populations that included hydrogenotrophic methanogens (Rout, Charles, Doulgeris, et al., 2015). A significant amount of acetate was detected (Figure 3.5 E) which decreased once the pH had decreased suggesting aceticlastic methanogenesis progressed once the pH had been reduced.

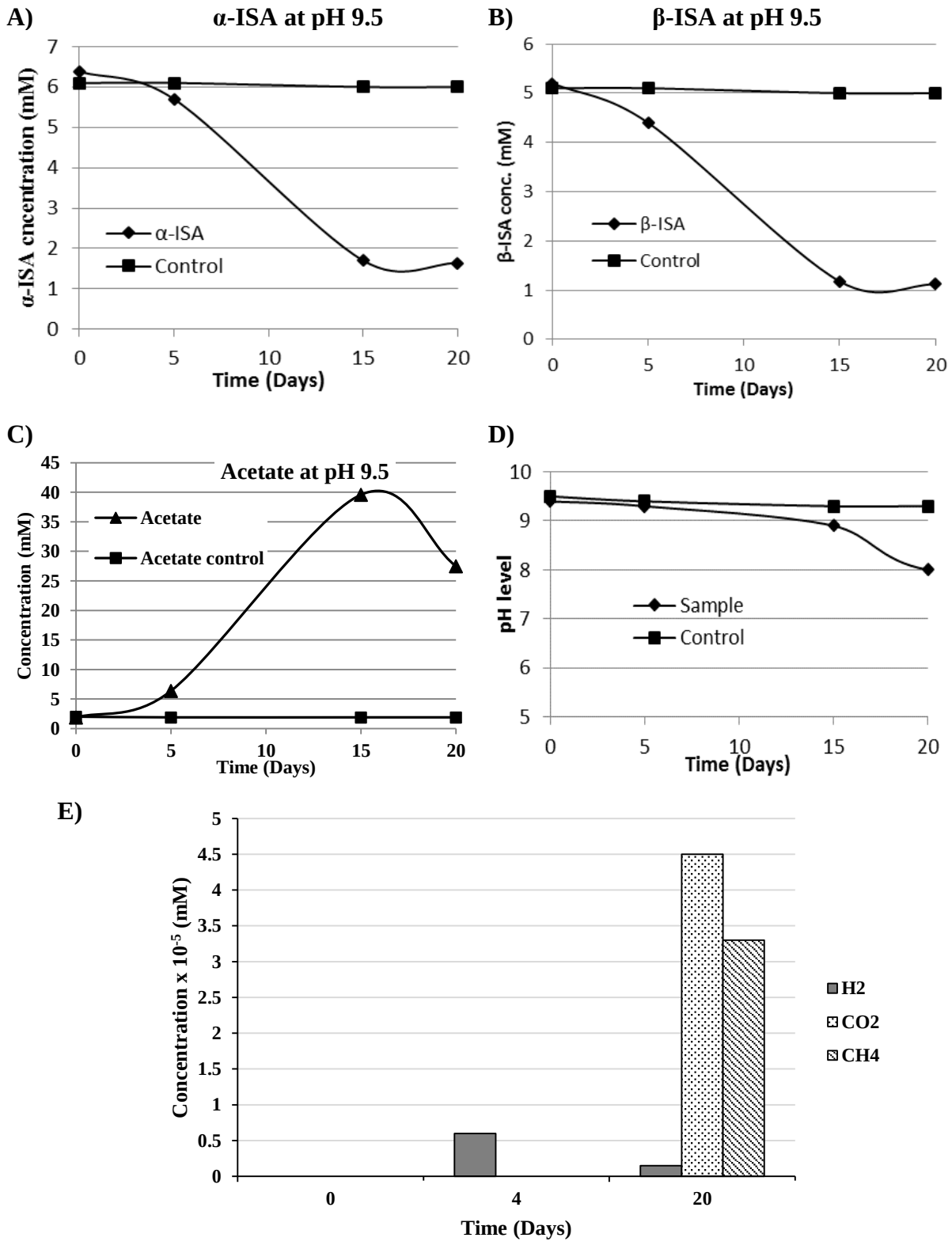


Figure 3. 5: Bacterial utilization of CDPs at pH 9.5

About 5.5mM of α-ISA was degraded (A), and about 4.5mM of β-ISA was degraded (B) during 15 day incubation period, a significant amount of acetate (40mM) was detected at the end of the incubation time (C), that causes a reduction of pH (D). H_2 was detected after 4 days which was replaced by CH_4 after 20 day (E).

3.1.2. Evolving pH microcosms

Employing the same protocol as the initial microcosms, a microcosm was established on a 30 day CDP feed-cycle with a gradual increase in pH from pH 8.5, to pH 9.5 and finally pH 10.5 (2.5.4). The pH was increased at the end of each 30day waste/feed cycle. The aim being to see if a more gradual increase in pH would select for alkaliphilic microbes in the inoculum.

At pH 8.5 complete degradation of α-ISA (4.5mM) and β-ISA (4.0mM) (Figure 3.6 A and B) was observed within 25 days of incubation time with H_2, CO_2 and CH_4 detected in the headspace (Figure 3.6 D). When the pH was adjusted to pH 9.5 similar results were observed, with significant degradation of both forms of ISA associated with VFA production and a pH decrease to pH 8.0 after 30 day incubation (data not shown). At pH 10.5 the degradation of α-ISA was incomplete with 1.5 mM remaining after 30 days, whilst β-ISA was completely degraded by 20 days. As anticipated, this drop was associated with a drop in pH and an increase in acetic acid and methane generation. The data demonstrated a greater reduction in ISA concentration at pH 10.5 (Figure 3.7 A and B) than seen in the pH 10.0 reactor described in the previous section, suggesting that a more gradual increase in pH was more successful in establishing an alkaliphilic microbial population. However, the greater ISA degradation is associated with a greater acetate generation and pH decrease (Figure 3.7 C). Consequently, it is hard to separate the impact of the gradual increase in initial pH with the impact of the microcosm spending more time at a lower pH that is more favourable for microbial activity.

The results of this microcosm are broadly similar to those obtained from the fixed pH microcosms with the fermentation of ISA being associated with the accumulation of acetate and the generation of methane (Figure 3.7 D) with the extent of ISA degradation decreasing as the operational pH increases. The results are in broad agreement with those published by Rout *et al*. (2015) who found that CDP fed microcosm operated at pH 10.0 were dominated by fermentative populations that included hydrogenotrophic methanogens (Rout, Charles, Doulgeris, et al., 2015).

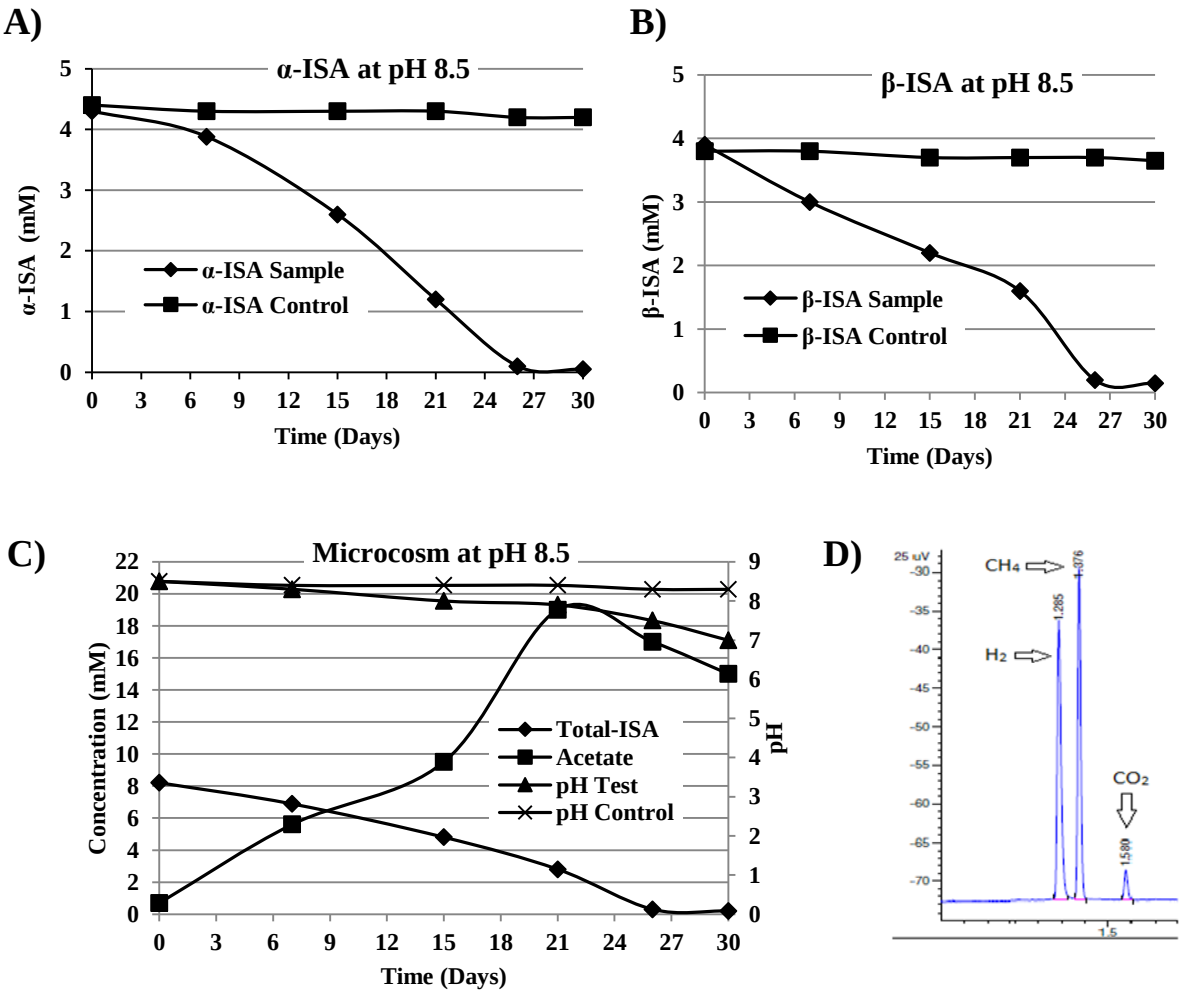


Figure 3. 6: Degradation of ISAs at pH 8.5

Complete degradation of α-ISA and β-ISA (A and B) led to an increase of acetate concentration (C), causing a reduction in pH from 8.5 to 7.0 at the end of 30 days (C). A gradual reduction of ISAs was associated with a gradual increase of acetate (C), the fermentation process was associated with gas production (H_2, CH_4 and CO_2) (D).

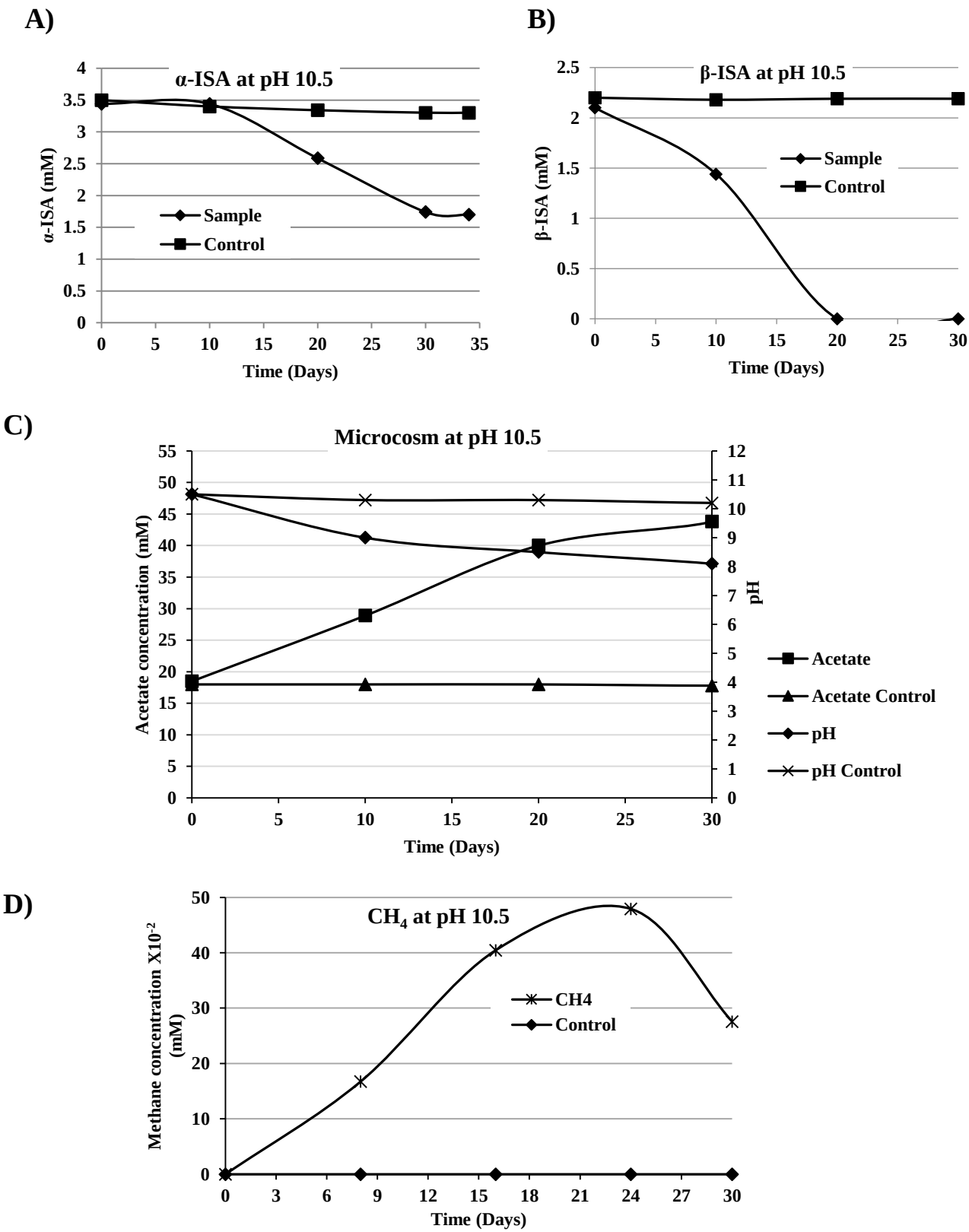


Figure 3. 7: Complete degradation of β-ISA at pH 10.5

Significant amounts of α-ISA (~2.0 mM) at pH 10.5 (A), and all β-ISA (~2.2 mM) (B) were degraded at pH 10.5 leading to an increases in acetate from ≈18 mM to ≈43mM at the end of 30 days (C) causing a reduction in pH from 10.5 to 8.1 (C). CH_4 gradually increased peaking after 21 days and then decreasing (D).

3.1.3. Operation of alkaline microcosms at shorted waste feed cycles

The pH 9.0, 10.0, and 11.0 30 day waste/feed cycle microcosms were operated for 12 months in total. These microcosms were prone to significant reduction in pH which moved the microcosms towards a more neutral pH environment. In order to maintain the microcosms at alkaline pH values the microcosms were modified to a 10 day waste feed cycle. The pH values of each microcosm being readjusted each 10 days for 40 days (2.5.5.).

Complete degradation of ISA was achieved at pH 9.0 however this was associated with a drop in pH to near neutral conditions (Figure 3.8 A-C). Adaption to the shorter feed cycle resulted in an initial accumulation of acetate and other VFA, these subsided as the microbial population adapted to the revised feed cycle. These two VFAs could be used as a substrate by butyrate-, and propionate-degrading syntrophic acetogens, which are able to grow syntrophically with hydrogenotrophic methanogens (Schink, 1997; W.-M. Wu, Jain, De Macario, Thiele, & Zeikus, 1992). The reduction in VFA reflects the presence of methanogens as a part of the bacterial community utilising acetic acid to produce methane (McInerney et al., 2008; Schink, 1997; W.-M. Wu et al., 1992).

Although the two forms of ISA were completely degraded at pH 9.0 (Figure 3.8 A) within the 10 days of each feed cycle. At pH 10.0 β-ISA was completely removed whilst the α-ISA accumulated (Figure 3.9 A). This result disagrees with the previous results by Rout *et al*. (2015) who found a complete removal of α-ISA (~ 0.4 mM) in alkaline microcosm up to pH 9.5 and accumulation of β-ISA in microcosm at pH 9.5 and pH 10.0 (Rout, Charles, Doulgeris, et al., 2015). This suggests that the population has diverged from that established by Rout *et al*. (2015) due to the differing feed cycles. At pH 10.0 the microcosm pH reduced with the generation of acetic acid (Figure 3.9 B), however there was not the accumulation of other VFA seen in the pH 9.0 microcosm (Figure 3.8 B). At pH 10.0, the acetic acid accumulated and remained in the system. This results agrees with the previous data presented by Rout *et al*. (2015) who reported a gradual removal of acetate at pH 7.5 but an accumulation at pH 9.5 and pH 10.0 (Rout, Charles, Doulgeris, et al., 2015).

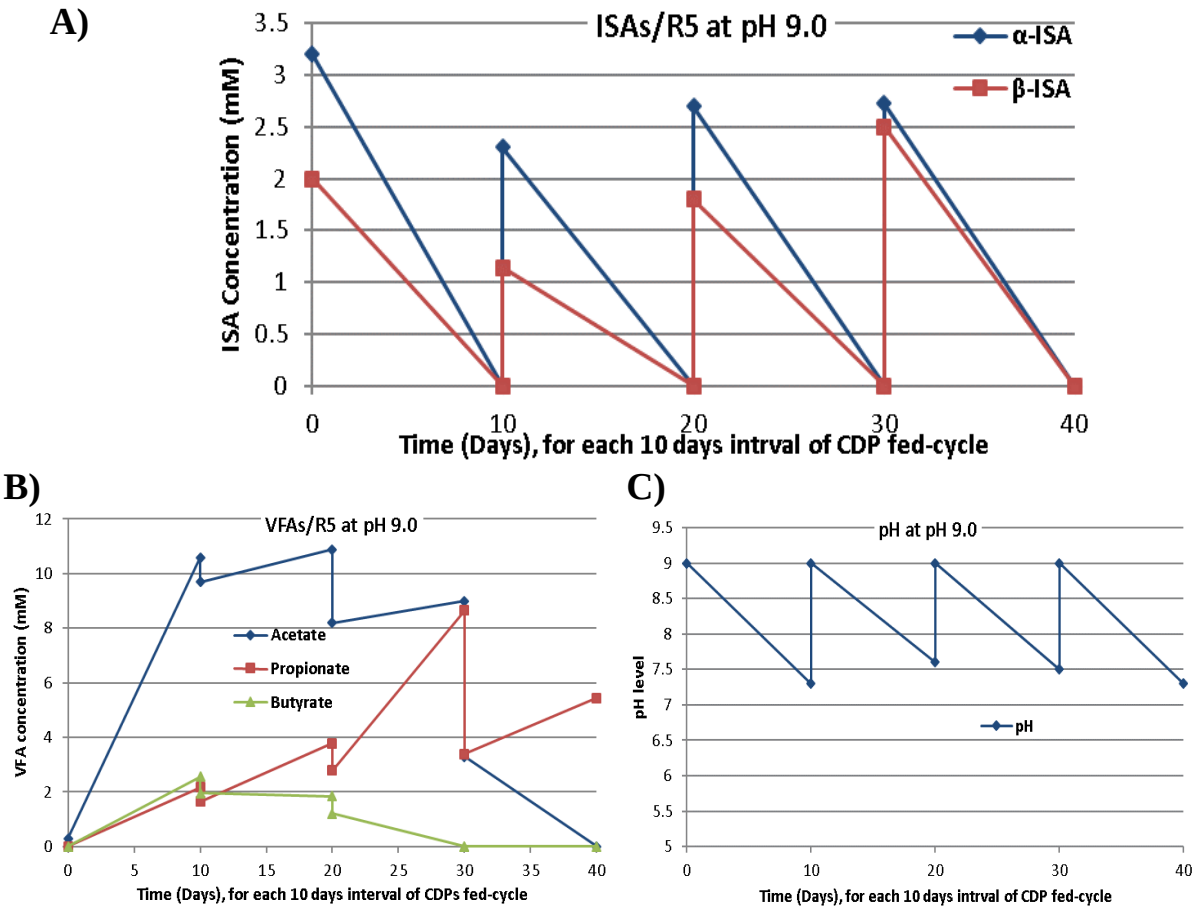


Figure 3. 8: ISAs degradation at pH 9.0 under CDPs feed-cycle each 10 days.

Complete degradation of α-ISA (~3.3 mM) and β-ISA (~2.0 mM) (A), at the end of each 10 days feed cycle followed by an increase of variable amount of VFAs dominated by acetate (B). The pH value decreased to ~ pH 7.3 (C) due to VFAs and carbonates formation.

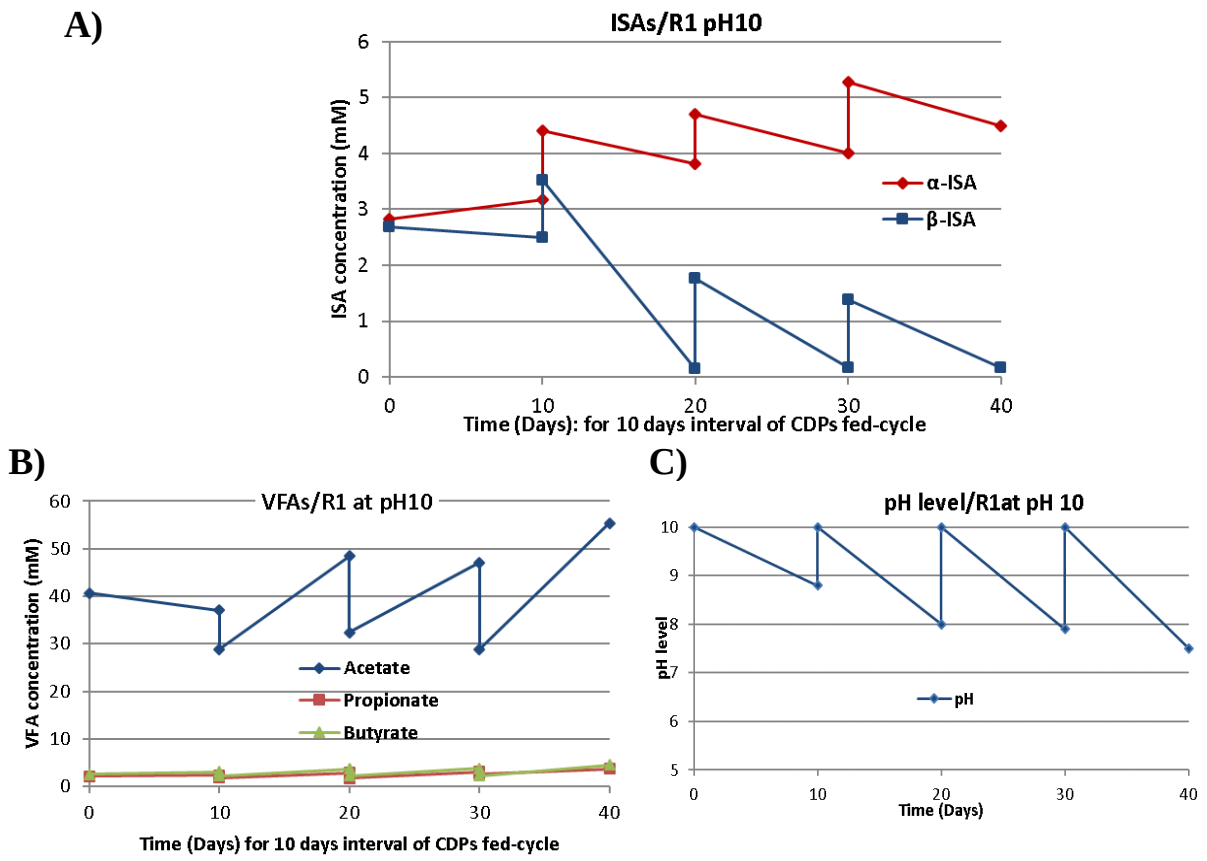


Figure 3. 9: ISAs degradation at pH 10.0 under CDPs fed-cycle each 10 days.

Small amounts of α-ISA (≤ 1.0 mM) were degraded alongside complete degradation of β-ISA (~2.0 mM) (A), at the end of 10 days. This was accompanied by a variable amount of VFAs dominated by acetate (B), that led to decrease in the pH value to ≈pH 8.0 (C). No reduction in acetate concentration was observed, suggesting that the methanogens were not active at pH 10.0.

At pH 11.0 microbial activity was significantly reduced with limited degradation of both ISA isomers detected through the accumulation of acetate, whist the overall ISA profile was one of accumulation (Figure 3.10 A and B). The accumulation of acetate produced a reduction in the pH, which remained above pH 10.0 for much of the experiment (Figure 3.10 C). Work by Yuan *et al*. (2006), found that the rates of fermentation of protein and carbohydrate are decreased as pH increased up to pH values of 10.0 and 11.0 (Yuan et al., 2006). Rout *et al*. (2015) found that an increase in pH of the CDPs driven microcosm to pH 11.0 resulted to an accumulation of ISA and the loss of acetoclastic methanogens from the microcosm (Rout, Charles, Doulgeris, et al., 2015).

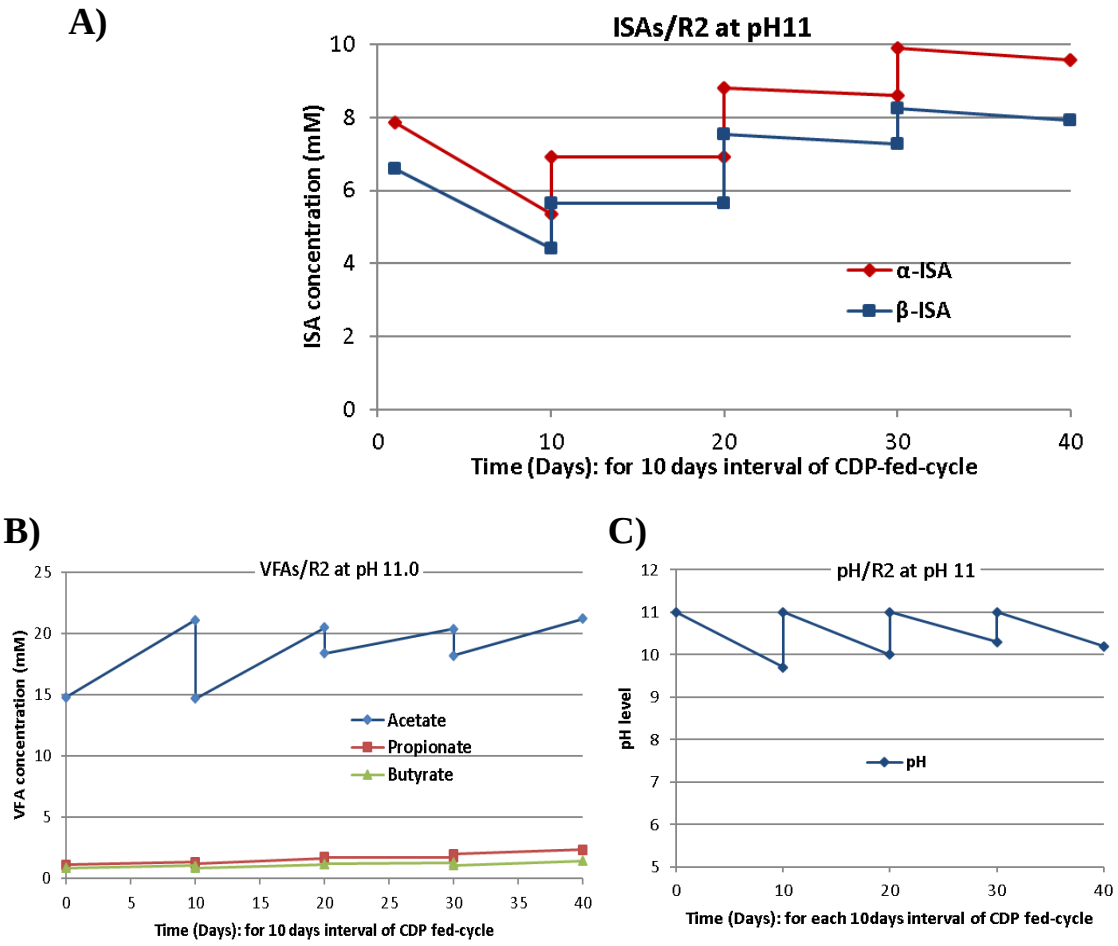


Figure 3. 10: ISAs degradation at pH 11.0 under CDPs feed-cycle each 10 days.

Insignificant amount of both α-ISA and β-ISA (≤ 1.0 mM) was gradually degraded (A), at the end of the first 10 days followed by increase of variable amount of VFAs concentration dominated by acetate (B), that led to decrease in the pH value to about pH 10 (C) that indicated to the metabolic activity of the bacterial community at pH 11.0

The shift from a 30 days feed cycle to a 10 days feed cycle had the impact of reducing the biodegradation potential of the microcosms. Although the 30 days fed-cycle showed significant degradation of ISA at all pH levels including pH 11.0. The 10 days feed cycle resulted in a complete degradation of both forms of ISA only at pH 9.0 and significant β-ISA degradation at pH 10.0 and negligible degradation of both forms of ISA at pH 11.0.

The results suggest that the microbial consortia in the microcosms were able to degrade both form of ISA at an optimum pH for alkaliphilic bacteria between pH 9.0 and pH 10.0. In addition, these microorganisms have an ability to tolerate and survival at up to pH 11.0. The metabolic activity of these microorganisms caused a slow reduction in pH during that shifted the pH into a region more favourable for microbial growth.

3.1.4. Degradation of ISA over extended incubation times.

The pH 10.0 and pH 11.0 microcosms operated on a 10 day waste/feed cycle were run for 4 cycles (40 days) in total. At the end of this period, they were left operational and their performance monitored. The data presented below covers a 60 day period after the final waste/feed event.

In the pH 10.0 microcosm a significant amount of α-ISA had accumulated during the 10 day feed cycles. When left to run for 60 days fermentation of both forms of ISA generated acetate resulting in a drop in overall pH. As the pH fell the extent of ISA degradation increased. Significant amounts of α-ISA (~5.8mM) and all the β-ISA (3mM) were degraded (Figure 3.11 A and B), when the pH of the microcosm had fallen to pH 8.8 followed by complete degradation of α-ISA (~9mM) (Figure 3.11 B) and a reduction in pH to pH 7.7 by the end of the incubation time (Figure 3.11 D) due to an accumulation of acetic acid (Figure 3.11 C).

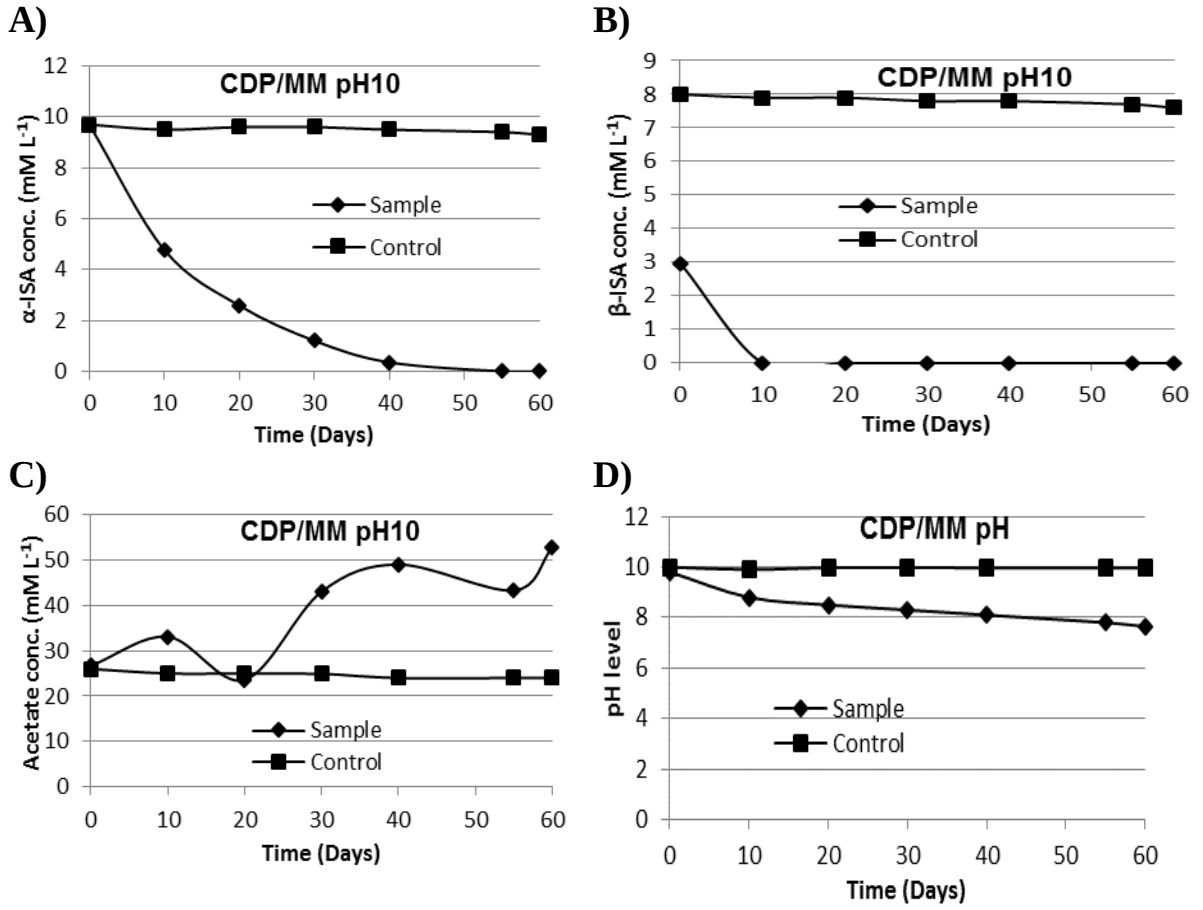


Figure 3. 11: Degradation of ISA in the R1 at pH 10 after 60 days incubation

Complete degradation of β-ISA (3.0mM) occurred after 10 days (B), at the same time as a significant degradation of α-ISA (5.8mM) (A), the pH fell during the incubation period to a final pH of pH 7.7 (D), a significant amount of acetate was detected due to fermentation (C).

The extended incubation pH 11.0 system gave similar results to those seen at pH 10.0 with the degradation of α-ISA only proceeding once the pH had decreased. The fermentation of β-ISA proceeded quickly generating the acetate that reduced the pH and facilitated the onset of α-ISA fermentation (Figure 3.12 A, B and C). The fermentation of β-ISA initiated at pH 11.0 although the low levels present meant that a detailed profile was not characterised (Figure 3.12 B). While the α-ISA was only significantly degraded when the pH was reduced to below pH 9.5 (Figure 3.12 A and D).

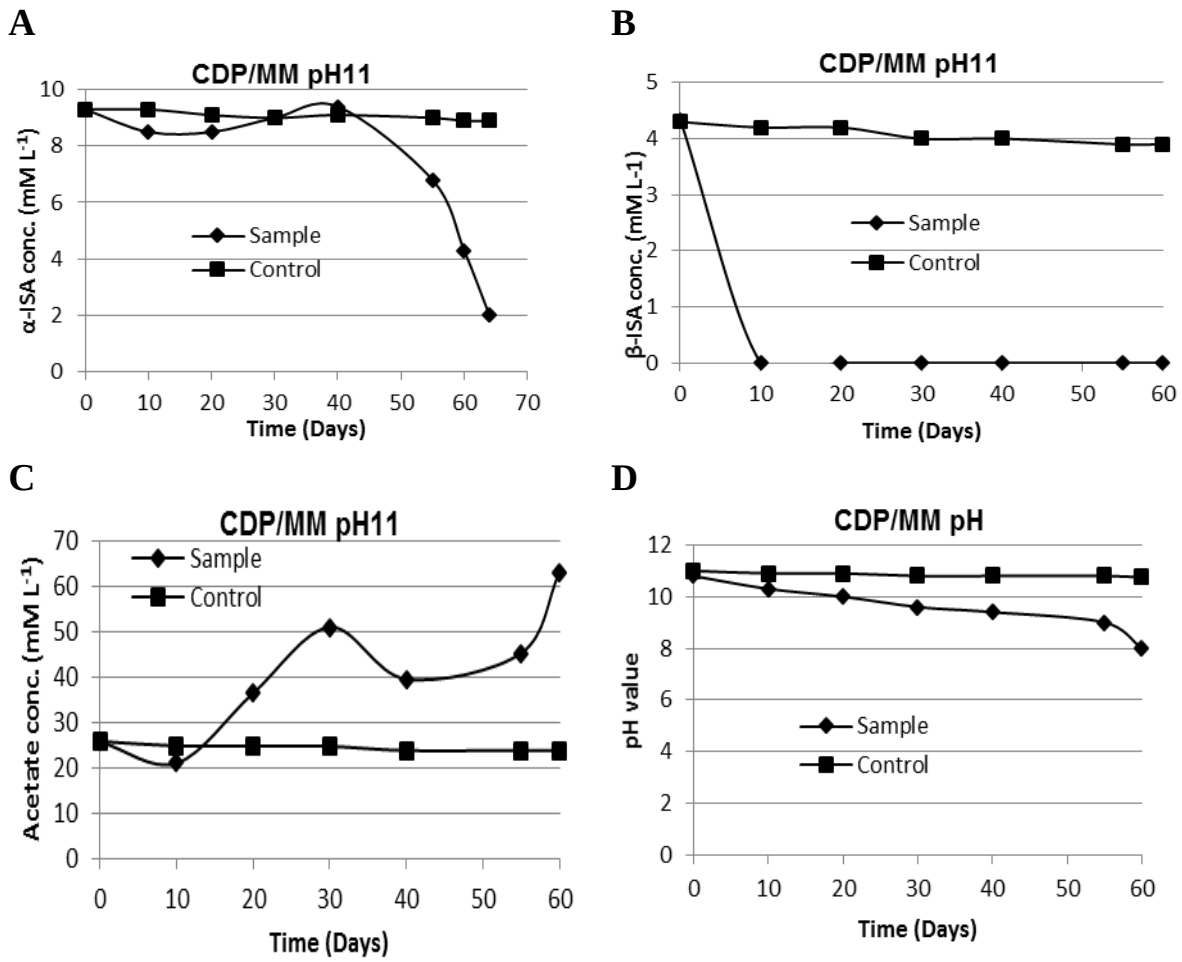


Figure 3. 12: Degradation of ISA at pH 11 during 60 days incubation time

Fermentation resulted in a decreasing the concentration of; α-ISA mM (A) and β-ISA mM (B), followed by increase of acetate concentration mM L (C), and a decrease in pH (D).

3.1.5. Discussion

The review of Humphreys *et al*. (2010) suggested that the degradation of CDPs at alkaline pH would dominate the post-closure period of a repository and suggested that fermentative bacteria are likely to be confined to low pH niches generated by the production of gases and organic acids (Humphreys, Laws, et al., 2010). In the previous work reported by Grant *et al*., it was

suggested that the optimum pH values for ISA degradation were between 9.0 and 10.0 with the highest pH value for its degradation being pH 11.5 (Grant, 2002). More recently, a number of studies have confirmed that the microbial consortia present in the hyper-alkaline environments of Harpur Hill are capable of degrading ISAs at pH 10.0 (Bassil et al., 2015; Rout, Charles, Doulgeris, et al., 2015) and able to generate methane up to pH 11.0 and that the community was suppressed above pH 11.0 (Rout, Charles, et al., 2015a). However, other work has found that the same microbial consortia is capable of survival up to pH 12.0 in the form of polymicrobial flocs (C. Charles et al., 2017).

The data presented here broadly agree with previously published work with the consortia established in the microcosms being able to initiate ISA degradation up to pH 11.0 with degradation accelerating as the pH is reduced. In line with other published work (Bassil et al., 2015; Rout, Charles, Doulgeris, et al., 2015) the fermentation of ISA results in the accumulation of acetate with only low levels of longer chain VFA. The generation of methane was less consistent, again an observation which aligns with the literature where Bassil *et al*. (2015) did not observe methane generation whilst Rout *et al*. (2015) did. This suggests that the methanogen population in the original inoculum is low resulting in an inconsistent colonisation of the microcosms. The accumulation of acetate suggests that the methanogenic pathway is primarily via the hydrogenotrophic pathway, although acetate degradation appeared to occur at lower pH levels.

3.1.6. Key findings

- The bacterial community present in the alkaline soil inoculum is able to adapt to an alkaline medium and degrade CDP containing both forms of ISA.
- The bacterial communities established in these microcosms were able to degrade CDP to methane at pH 10.0. At pH 11.0 methanogenesis was lost and the community became purely fermentative in nature. At pH 12.0 no clear evidence of microbial activity was evident.
- The bacterial community has shown an affinity for the utilization of β-ISA, rather than α-ISA, even at pH10.0.
- Significant ISA degradation was initiated when the pH fell below pH 9.5.
- Acetate accumulation was an indicator of ISA fermentation and facilitated the reduction in pH.

3.2. Bacterial community analysis of the CDP fed alkaline microcosms

3.2.1. Microbial community analysis of the pH 9.0, pH 10.0 and pH 11.0 microcosms that were operated on a 30 day waste feed cycle

The microbial communities in the pH 9.0, pH 10.0 and pH 11.0 microcosms operated on a 30 day waste feed cycle for 12 months were further investigated via 16s rRNA gene based community analysis and compared to the community present in the initial sample. The pH 12.0 microcosm not investigated further due to the lack of degradation observed in this reactor. Community analysis was performed using ChunLab's pipeline analysis software (Appendix-2), EzBioCloud 16S database (Jisun Kim et al., 2012; S.-H. Yoon et al., 2017). This was used alongside CD-hit (Fu, Niu, Zhu, Wu, & Li, 2012) and UCLUST (Edgar, 2010) software tools to obtain operational taxonomic unit (OTUs) (M. Kim et al., 2013) and alpha diversity indices (Cardoso, Rigal, & Carvalho, 2015). The initial soil inoculum (crude soil) showed a complex bacterial community (~1030 OTUs) that decreased in the microcosms; pH 9 (549 OTUs), pH 10 (~607 OTUs) and pH 11 (~242 OTUs) (Figure 3.13).

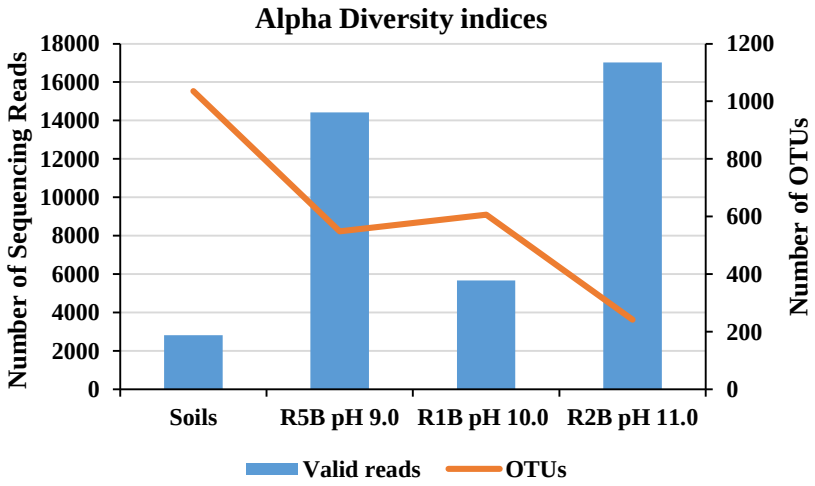
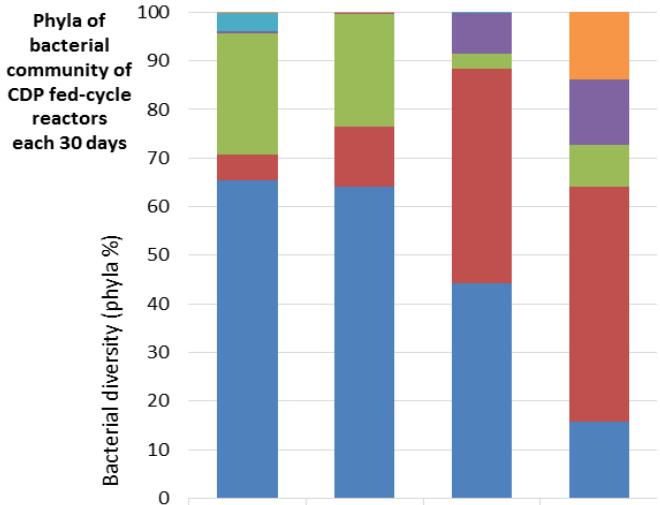


Figure 3. 13: Microbial diversity analysis of the starting inoculum, and CDPs driven microcosms each 30 days.

The results indicate that the microcosms exerted a significant selective pressure on the original community. Given that these original soils are highly alkaline (pH 11.0 to pH 13.0) it seems likely that this pressure was provided by the combination of pH, anaerobic conditions and the CDP provided as the primary carbon source. Generally speaking, the pH 11.0 microcosm most closely matched that seen in the crude soil, perhaps reflecting that this microcosm was closest to the crude soil in terms of pH. The bacterial communities showed a sharp reduction of Proteobacteria phylum, from 48% in crude soil to about 5.4% and 12% at pH 9.0 and pH 10.0

respectively, the proportion of Proteobacteria then recovered at pH 11.0 (Figure 3.14). On the other hand, the Firmicutes increased from 15.8% in the crude soil to about 64% at pH 9.0 and pH 10.0 and to 44.2% at pH 11.0. The Bacteroidetes had a similar profile to the Firmicutes with similar proportions at pH 9.0 (24.8%) and pH 10.0 (23%) and much lower proportions at pH 11.0 and the crude soil. Although, the Archaea that includes methanogenic bacteria were undetected in the crude soil, the phylum Euryarchaeota was recovered in the microcosm at pH 9.0 (3.7%) and pH 11.0 (0.2%), but were undetectable at pH 10.0.



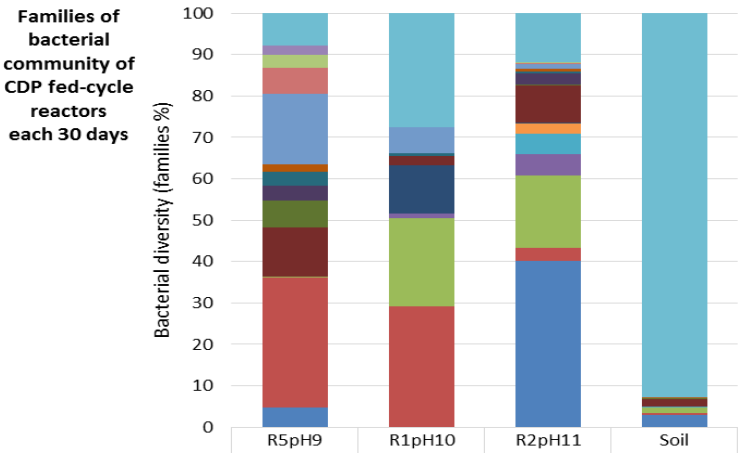
	R5 pH9.0	R1 pH10	R2 pH11	Crud soil
ETC < 2%	0.2	0.09	0.01	13.93
Euryarchaeota	3.74	0	0.21	0
Actinobacteria	0.43	0.21	8.35	13.36
Bacteroidetes	24.8	23.22	3.14	8.72
Proteobacteria	5.4	12.36	44.02	48.19
Firmicutes	65.43	64.12	44.27	15.8

Figure 3. 14: Phyla level description of the microbial communities of 30 days waste/feed cycle

Firmicutes was a dominant phylum in all microcosms followed by Proteobacteria 44% in microcosm pH 11, Bacteroidetes 24.8%, 23.2% in microcosms pH 9 and pH 10 respectively. *Actinobacteria* 8.4% in pH 11, while the dominant phylum in crude soil sample was Proteobacteria 48% followed by Firmicutes 15.8%, Actinobacteria 13% and Bacteroidetes 8.7%.

The Firmicutes phylum was represented by families including the Ruminococcaceae, Tissierellaceae and Clostridiaceae as previously observed by Rout *et al*. and Kuippers *et al*. (Kuippers et al., 2015; Rout, Charles, Doulgeris, et al., 2015). Although, as observed by Bassil *et al*. (2015), who found that the Proteobacteria dominated the crude soil, this phylum was found in variable levels in the microcosms (pH 9.0 (5.4%), pH 10.0 (12.4%) and pH 11.0 (44%)) (Bassil et al., 2015). At pH 11.0 this phylum was represented by the Rhodocyclaceae family which was dominated in turn by member of the *Azonexus* genus (Figure 3.16), whilst at pH 10.0 the Alcaligenaceae (11.6%) were dominant. The phylum Bacteroidetes was identified in all

microcosm and the crude soil and was represented by the Porphyromonadaceae which includes the genus of Macellibacteroides that was found in all microcosms with the highest percentage recorded at pH 9.0. The Actinobacteria phylum was most prevalent at pH 11.0 and represented by the Coriobacteriaceae and Dietziaceae families (Figure 3.15). The presence of the Dietziaceae is in agreement with observations made by Charles et al. (2017) who associated *Dietzia* sp. with biofilm formation at alkaline pH (C. Charles et al., 2017).



	R5pH9	R1pH10	R2pH11	Soil
ETC < 2%	7.74	27.49	11.84	92.8
Clostridium	2.27	0.12	0	0
Metanobacteriaceae	3.11	0	0.21	0
Lintimicrobiaceae	6.29	0	0.17	0
Porphyromonadaceae	17	6.14	1.18	0
Clostridiaceae	2	0.05	0.74	0.18
Sedimentibacter	3.36	0.6	0.37	0
Eubacteriaceae	3.55	0	2.74	0
Christensenellaceae	6.36	0	0.11	0.14
Tissierellaceae	11.85	2.42	9.14	1.95
Alcaligenaceae	0	11.59	0.05	0
Dietziaceae	0.04	0	2.61	0.04
Coriobacteriaceae	0	0.07	4.94	0.18
Anaerobranca	0.08	1.09	5.03	0
Anaerovirgula	0.22	21.16	17.5	1.38
Ruminococcaceae	31.28	29.25	3.25	0.32
Rhodocyclaceae	4.85	0.02	40.12	3.01

Figure 3. 15: Family level in the microbial communities of 30 days waste/feed cycle

Families that represented less than 2% of the microbial community of the crude soil sample made up >90% of the soil population. The microcosm at pH 9.0 was dominated by Ruminococcaceae 31.2%, porphyromonadaceae 17% and Tissierellaceae 11.9%. The microcosm at pH 10 was dominated by Ruminococcaceae 29%, Anaerovirgula 21.2% and Alcaligenaceae 11.6%. The microcosm at pH 11 was dominated by Rhodocyclaceae 40% and Anaerovirgula 17%.

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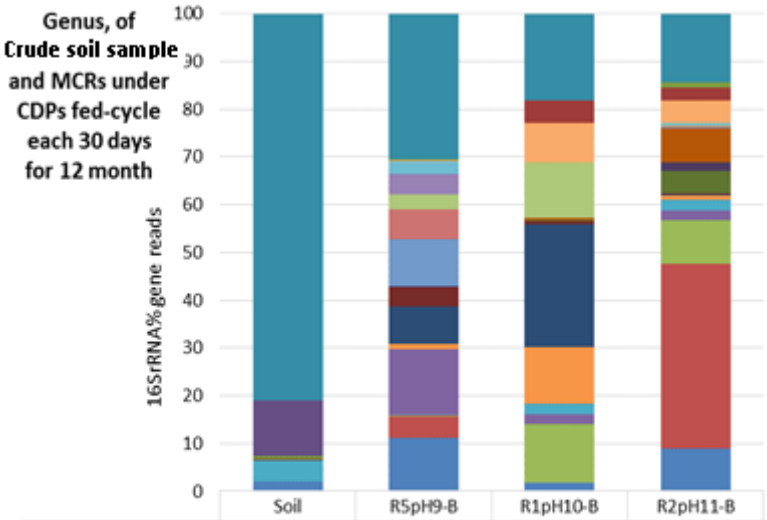


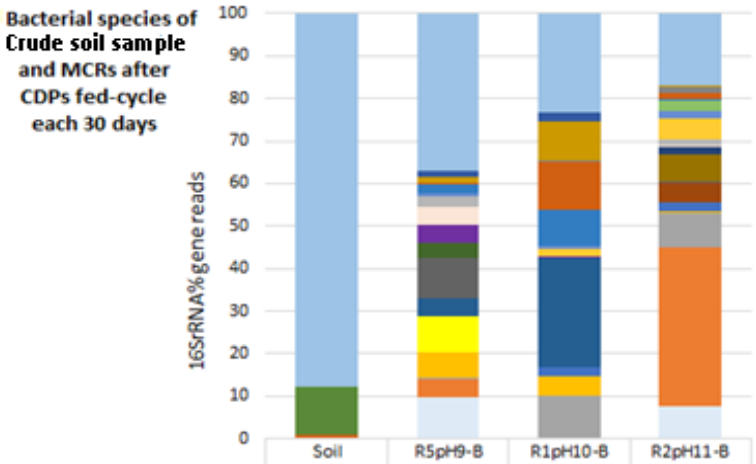
	Soil	R5pH9-B	R1pH10-B	R2pH11-B
ETC < 1.0%	81.01	30.49	18.20	14.29
Propionibacterium	11.55	0.00	0.00	0.00
Rhodocyclaceae_uc	0.78	0.22	0.00	1.28
Dietzia	0.04	0.04	4.64	2.59
Alcaligenes	0.00	0.00	0.00	0.00
Coriobacteriaceae_uc	0.00	0.13	8.39	4.81
Sedimentibacter	0.00	2.77	0.00	0.33
Parabacteroides	0.00	4.19	0.04	0.00
Methanobacterium	0.00	3.06	11.53	0.21
Lentimicrobium	0.00	6.25	0.04	0.17
Fermentimonas	0.00	9.97	0.04	0.25
Natronincola	0.00	0.00	0.39	7.14
DF968182_g	0.00	0.00	0.00	0.00
Comamonas	0.00	0.07	0.00	1.79
Anaerobranca	0.00	0.00	0.14	4.83
Ruminococcaceae_uc	0.14	4.13	0.74	0.18
AB630534_g	0.00	7.78	25.69	0.16
DQ677001_g	0.00	1.14	11.80	0.88
AB237727_g	4.54	0.04	2.22	2.19
Ercella	0.04	13.86	2.03	2.00
Alkaliphilus	0.00	0.19	12.38	9.32
Azonexus	0.00	4.53	0.02	38.61
Tissierella	1.91	11.14	1.76	8.95

Figure 3. 16: Genera level of the microbial communities of 30 days waste/feed cycle

The microcosm at pH 9.0 was dominated by; *Ercella* 13.8%, *Tissierella* 11%, and *Fermentimonas* 10%; the microcosm at pH 10 was dominated by *AB630534* genus 26%, *DQ677001* genus 11.8%, and *Alkaliphilus* 12.4%; while, microcosm at pH 11 dominated by *Azonexus* genus 38.6%, and *Tissierella* genus 9.0%.

At the species levels, all three microcosms were dominated by unknown species identified via accession numbers reflecting the uncharacterised nature of the environment the microcosms were established from. The dominant known species in the microcosms were at pH 9.0 *Fermentimonas caanicola* 9.7% and *Macellibacteroides fermentans* 4%. In the pH 10.0 microcosm they were Ruminococcaceae species 25.7% and Rhodocyclaceae species 11.27%.

Whilst at pH 11.0 were *Anaerovirgula* species 6.5%, Coribacteriaceae species 4.8%, *Dietzia* species 2.5% and *Azonexus* species 1.43% were present.



	Soil	R5pH9-B	R1pH10-B	R2pH11-B
ETC < 2.0%	87.81	36.966	23.359	16.894
Propionibacterium acnes	11.23	0	0	0
Methanobacterium*	0.00	1.47	2.17	0
Proteiniphilum_uc	0.00	1.27	9.011	0.44
Azonexus_uc	0.00	0.22	0.37	1.43
Rhodocyclaceae_uc_s	0.78	0.22	11.27	1.28
Acetobacterium woodii*	0.00	2.13	8.4	0.52
Dietzia psychralcaliphila*	0.00	0.04	0	2.5
Tissierella_uc	0.00	0.6	0.9	1.7
Coriobacteriaceae_uc_s	0.04	0.13	1.71	4.8
Ercella succinigenes	0.00	2.48	0.02	1.62
AB630534_g_uc	0.00	4.19	0	0.05
Macellibacteroides fermentans*	0.00	4.07	0.24	0
JX391172_s	0.00	3.58	0.03	0.12
Comamonas kerstersii*	0.00	0.06	0	1.7
Anaerovirgula multivorans*	0.00	0	0	6.53
Fermentimonas caenicola	0.00	9.68	0	0.24
JF159990_s	0	0.00	0	4.5
Ruminococcaceae_uc_s	0.14	4.13	25.7	0.18
JQ245572_s	0.00	8.36	0	0.006
DQ266900_s	0.00	0.014	2	2.1
AY570585_s	0.00	6.11	4.6	0.16
JN178047_s	0.00	0.15	10.22	8.36
DQ088747_s	0.00	4.29	0	37.16
GQ132209_s	0.00	9.84	0	7.71

Figure 3. 17: Species level of the microbial communities of 30 days waste/feed cycle

All of three microcosms were dominated by unknown species that identified by accession numbers. The dominant known species in the microcosms were at pH 9.0: *Fermentimonas caanicola* 9.7% and *Macellibacteroides fermentans* 4%, at pH 10: Ruminococcaceae species 25.7% and *Rhodocyclaceae* species 11.27%, at pH 11: *Anaerovirgula* species 6.5%, Coriobacteriaceae species 4.8%, *Dietzia* species 2.5% and *Azonexus* species 1.43%.

Methane was generated in a number of the microcosms indicating the presence of methanogenic archaea. The microbial community analysis confirmed the presence of Archaeal groups that occupied 3.7% of the total prokaryotic diversity. The Archaeal population was dominated (39%) by hydrogenotrophic methanogens of *Methanobacterium bryantii* (39%), followed by

unknown strain AY454550 of *Methanobacterium* genus with 97.1% similarity to *Methanobacterium uliginosum strain P2St.* A third Archaeal strain had high similarity (99.74%) to the acetotrophic and hydrogenotrophic methanogens *Methanosarcina horonobensis* (9%) (accession number CP009516). The fourth strain (5.5%) had 97.9% similarity to *Methanoculleus hydrogenitrophicus* (accession number FJ977567). These findings are in agreement with the community analysis of microcosms based on the same sediments carried out via cloning and sequencing by Rout *et al*. 2015 (Rout, Charles, et al., 2015a). The dominance of hydrogenotrophic methanogens in these alkaline microcosms is supported by the accumulation of acetic acid in these microcosms. A position in agreement with Rout *et al*. (2015) who found that the acetoclastic methanogenesis was suppressed in microcosm at pH 11.0 (Rout, Charles, Doulgeris, et al., 2015).

3.2.2. Bacterial community analysis of microcosms operated on a 10 day waste/feed cycle.

When the waste feed cycle was reduced from 30 days to 10 days the impact on the microbial communities was a reduction in diversity as indicated by the number of OTUs (Figure 3.18). This change represented a reduction of 44 and 45% at pH 9.0 and pH 10.0, but only 20% at pH 10.0. This reduction is despite the fact that the shorter waste feed cycle resulted in greater substrate availability but required the microbial community to be growing at a higher rate to ensure they can maintain a population in the microcosm.

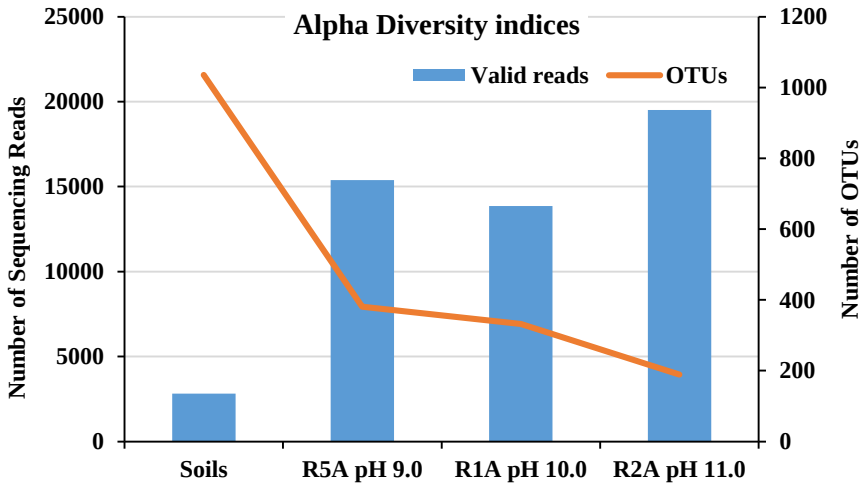
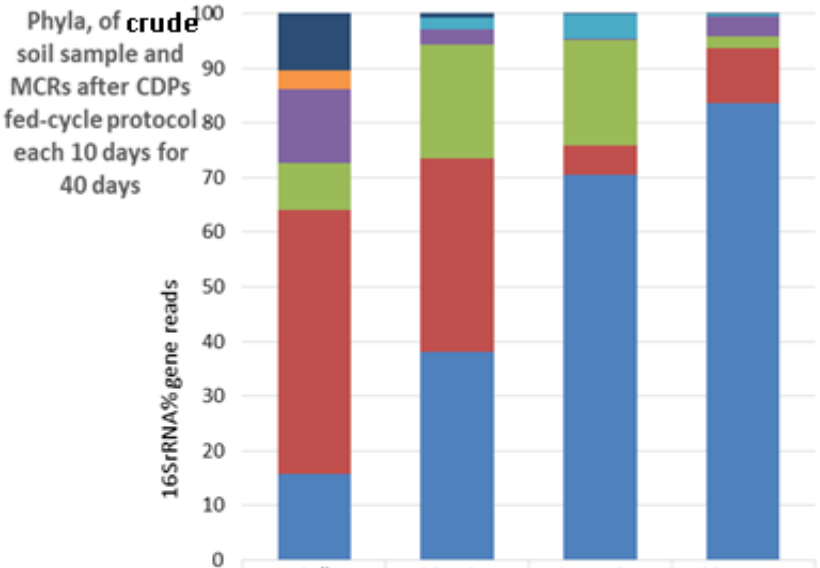


Figure 3. 18: Bacterial community of the starting inoculum, and CDPs driven microcosms.

The α-diversity plot shows the number of OTUs in the microcosms at different pH levels.

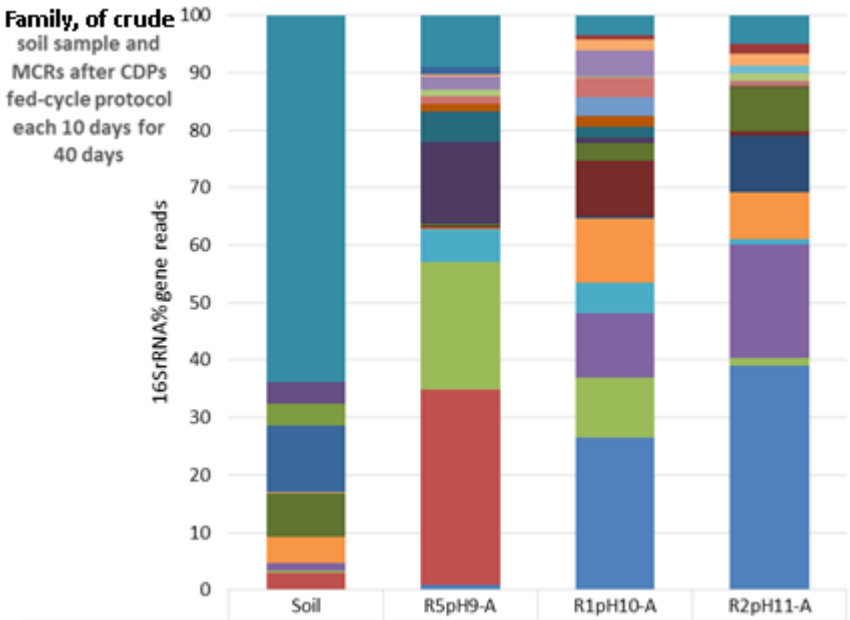
The reduction in microbial diversity was associated with an increased dominance by the Firmicutes which increased as the pH increased (38%, 70.5%, and 83.5% at pH 9.0, pH 10.0 and pH 11.0 respectively) an increase accompanied by a decrease in the Proteobacteria and the Bacteroidetes (Figure 3.19). Gram positive bacteria from the Ruminococcaceae family dominated microcosms under both of CDP feed-cycles at pH 9.0 and pH 10.0, while, Anaerovirgula and Tissierellaceae families dominated both sets of microcosms at pH 11.0 (Figure 3.20). Although the overall diversity had reduced the microcosms still retained bacteria from the *Tissierella* genus, the *Azonexus* genus and the *Alkaliphilus* genus (Figure 3.21). At the species level the microcosms were dominated by unknown species with *Macellibacteroides fermentans* and *Dietzia* species being notable exceptions (Figure 3.22).



	Soil	R5pH9-A	R1pH10-A	R2pH11-A
ETC < 2.0%	10.39	0.75	0.08	0.04
Acidobacteria	3.54	0.00	0.00	0.00
Euryarchaeota	0.00	2.17	4.45	0.59
Actinobacteria	13.36	2.76	0.35	3.48
Bacteroidetes	8.71	20.73	19.36	2.32
Proteobacteria	48.20	35.44	5.22	10.07
Firmicutes	15.80	38.14	70.55	83.49

Figure 3. 19: Phyla level of the microbial communities of 10 days waste/feed cycle.

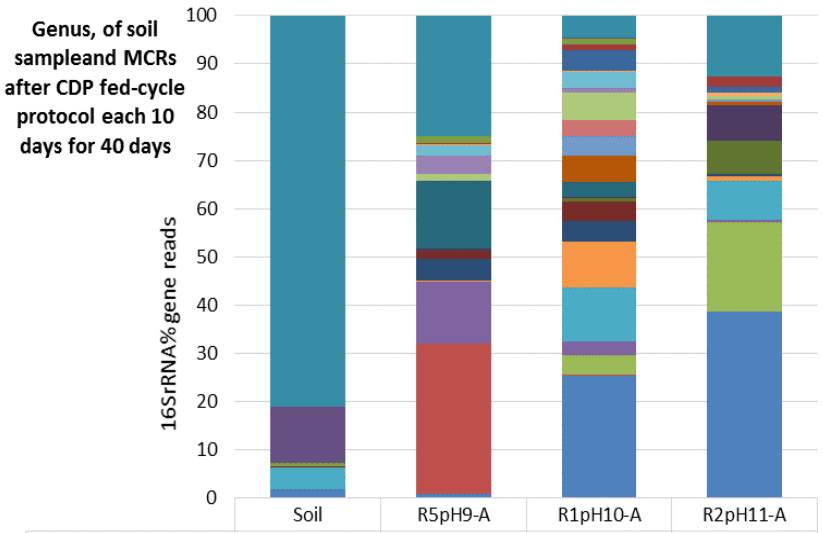
Bacterial diversity of the inoculum compared with microcosms at different pH levels. bacteria in the crude soil were dominated by Proteobacteria 48% that was substituted by the Firmicutes phylum in microcosms at pH 10.0 and pH 11.0, while the microcosm at pH 9.0 was dominated by three phyla; Firmicutes 38%, Proteobacteria 35% and Bacteroidetes 20.7%.



	Soil	R5pH9-A	R1pH10-A	R2pH11-A
ETC < 1.0%	63.86	8.88	3.37	4.97
Acidaminobacter_f	3.69	0.01	0.00	0.00
Pseudomonadaceae	3.79	0.06	0.00	0.03
Bacillaceae	0.14	0.04	0.94	1.65
Propionibacteriaceae	11.59	1.39	0.00	0.08
Dietziaceae	0.04	0.24	1.72	2.05
Alcaligenaceae	0.00	0.10	0.17	1.29
Sedimentibacter_f	0.00	2.24	4.43	0.10
Coriobacteriaceae	0.00	1.08	0.17	1.21
Methanobacteriaceae	0.00	1.42	3.39	0.59
Lentimicrobiaceae	0.00	0.00	3.39	0.15
Eubacteriaceae	0.00	1.20	1.86	0.41
Christensenellaceae	0.14	5.49	1.86	0.14
DF968182_f	0.00	14.18	0.95	0.00
Comamonadaceae	7.51	0.36	3.01	7.45
EU845084_f	0.00	0.37	9.75	0.87
Anaerobranca_f	0.00	0.09	0.27	9.85
Erysipelotrichaceae	4.54	0.03	11.21	8.13
Porphyromonadaceae	0.00	5.68	5.27	1.02
Anaerovirgula_f	1.38	0.05	11.21	19.60

Figure 3. 20: Family level of the microbial communities of 10 days waste/feed cycle

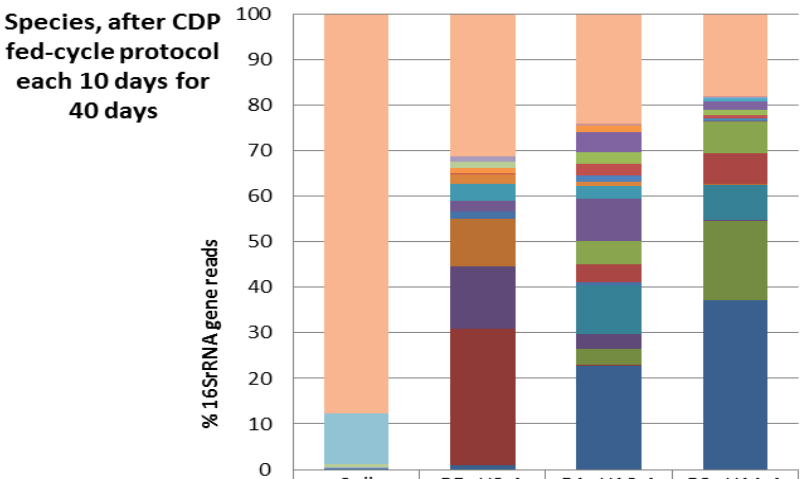
Family level diversity of the inoculum and at pH 9.0, pH 10.0 and pH 11.0. There was a significant reduction in families (50%) when compared to the crude soil. The microcosm at pH 9.0 was dominated by Rhodocyclaceae 33.9%, this family was represented by very low percentages (<0.2%) in the other two microcosms, whereas, the Tissierellaceae family dominated at pH 10.0 (26.4%) and pH 11.0 (39%).



	Soil	R5pH9-A	R1pH10-A	R2pH11-A
ETC < 1.0%	81.01	24.94	4.57	12.62
Propionibacterium	11.55	0.00	0.22	0.00
Rhodocyclaceae_uc	0.78	1.44	1.31	0.00
Dietzia	0.04	0.24	0.98	2.04
Alcaligenes	0.00	0.07	4.43	1.27
Coriobacteriaceae_uc	0.00	0.23	0.09	0.64
Sedimentibacter	0.00	2.04	3.37	0.10
Parabacteroides	0.00	3.85	1.00	0.03
Methanobacterium	0.00	1.41	5.57	0.59
Lentimicrobium	0.00	0.00	3.37	0.15
Fermentimonas	0.00	0.00	4.03	0.50
Natronincola	0.00	0.00	5.57	0.68
DF968182_g	0.00	14.13	2.94	0.00
Comamonas	0.00	0.36	0.25	7.25
Anaerobranca	0.00	0.00	0.82	7.01
Ruminococcaceae_uc	0.14	1.72	4.14	0.04
AB630534_g	0.00	4.36	4.14	0.50
DQ677001_g	0.00	0.37	9.64	0.85
AB237727_g	4.54	0.01	11.05	8.02
Ercella	0.04	12.70	2.92	0.49
Alkaliphilus	0.00	0.03	4.01	18.49
Azonexus	0.00	31.13	0.09	0.04
Tissierella	1.91	0.96	25.48	38.71

Figure 3. 21: Genera level of the microbial communities of 10 days waste/feed cycle

Bacterial diversity of the inoculum compared with pH 9.0, pH 10.0 and pH 11.0 communities. High prevalence was recorded for the *Tissierella* genus 38% and 25.5% at pH 11.0 and pH 10.0 respectively followed by the *Azonexus* genus, 31% from pH 9.0 and the *Alkaliphilus* genus 18.5% from the pH 11.0.



	Soil	R5pH9-A	R1pH10-A	R2pH11-A
ETC < 1.0%	87.60	31.10	23.91	18.03
Propionibacterium acnes	11.23	0.00	0.12	0.00
HQ716403_g_uc	0.00	1.16	0.00	0.00
Rhodocyclaceae_uc_s	0.78	1.44	0.01	0.00
Acetobacterium woodii*	0.00	0.00	0.43	0.15
Anaerovirgula_f_uc_s	0.18	0.00	0.02	0.26
Ercella_uc	0.04	1.11	1.29	0.03
Methanobacterium flexile	0.00	0.00	0.00	0.59
Dietzia psychralcaliphila*	0.00	0.00	4.38	1.98
Tissierella_uc	0.00	0.05	2.53	1.06
Coriobacteriaceae_uc_s	0.04	0.23	2.71	0.64
Ercella succinigenes	0.00	0.01	1.28	0.31
AB630534_g_uc	0.00	2.17	0.97	0.12
Macellibacteroides fermentans*	0.00	3.73	2.87	0.03
JX391172_s	0.00	2.20	9.34	0.38
Comamonas kerstersii*	0.00	0.00	4.93	6.97
JF159990_s	0.00	0.00	3.92	6.69
Ruminococcaceae_uc_s	0.14	1.72	0.82	0.04
JQ245572_s	0.00	10.37	0.05	0.10
DQ266900_s	0.00	0.00	10.69	7.79
AY570585_s	0.00	13.82	3.22	0.14
JN178047_s	0.00	0.01	3.52	17.54
DQ088747_s	0.00	30.05	0.09	0.04
GQ132209_s	0.00	0.83	22.85	37.12

Figure 3. 22: Species level of the microbial communities of 10 days waste/feed cycle

The microcosms were dominated by unknown bacterial species. *Macellibacteroides fermentans* was detected in all microcosms pH 9.0 (3.7%), pH 10.0 (2.8%) and pH 11.0 (0.03%) and *Dietzia* species represented 4.4% of the population at pH 10.0 and ~2% at pH 11.0.

The genus *Tissierella* was the dominant group in the microcosms at pH 10.0 and pH 11.0. *Tisserella* species are strictly anaerobic, Gram-positive, and non-spore-forming bacteria. Although it has recently been determined that some species can form a terminal oval spore and can be Gram variable (Alauzet et al., 2014). Representatives of this genus have been isolated from various environments including, a creatinine degrading *Tissierella creatinine* BN1lT strain isolated from the sediment of a wastewater pool at a sugar refinery, the optimum pH for

this strain was pH 8.3 and the pH range was from pH 6.7 to 9.1 (Farrow, Lawson, Hippe, Gauglitz, & Collins, 1995). *T. creatinophila* was isolated from sewage sludge and showed an optimum growth on creatinine in the presence of formate up to pH 8.5 (Harms, Schleicher, Collins, & Andreesen, 1998). *T. praeacuta* on the other hand is a Gram-negative rod isolated from clinical and environmental samples (Caméléna et al., 2016).

The results presented here suggest that the strain (GQ132209) which has 99% similarity to the *Tissierella* genus and represent 44.4% of the total Firmicutes in the pH 11.0 microcosm may play a role in ISA degradation at high pH level. The phylogenetic tree below was constructed to compare the sequence of GQ132209 with the closest sequences (95% match) obtained via BlastN (Figure 3.23).

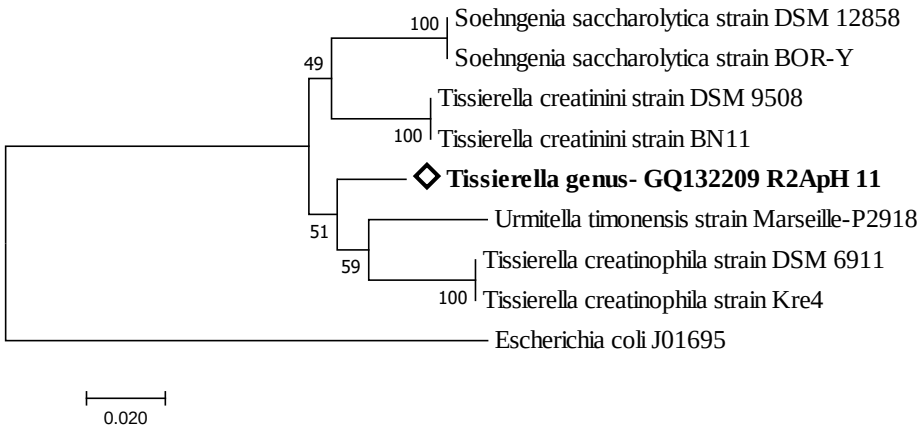


Figure 3. 23: phylogenetic tree based on 16SrRNA gene sequences.

Maximum-likelihood phylogenetic trees based on 16S rRNA gene sequences indicating the relationship between GQ132209 and the closest phylogenetic relatives of these strain. Bootstrap values (expressed as percentages of 1000 replicates) are shown at each node where the bar represents 0.02 changes per nucleotide position. Evolutionary analyses was conducted in MEGA7

3.2.3. Discussion

Whilst the initial alkaline soil demonstrated a high microbial diversity, much of this was lost when it was used to establish anaerobic CDP fed microcosms at alkaline pH. The observed shift from a Proteobacteria dominated soil (Bassil et al., 2015) to a microbial communities dominated by the Firmicutes is in line with previous observations by (Kuippers et al., 2015; Rout, Charles, Doulgeris, et al., 2015). The shift towards the Firmicutes was most obvious when the microcosms were shifted to a 10 day waste/feed cycle.

Comparison of the microbial populations found in each microcosm at both the 30 and 10 day waste/feed cycle was carried via the UniFrac (uniquIn order to determine whether the

communities are significantly different (Appendix-2; Table 1 and 2). UniFrac measures the fraction of the branch length of the phylogenetic distance between sets of taxa in a phylogenetic tree (Lozupone & Knight, 2005), and Fast Unifrac allowing integration of larger numbers of sequences (Hamady, Lozupone, & Knight, 2010). The microbial community at pH 9.0 was similar to the community in pH 10.0 when run on a 30 day waste feed cycle. Whilst under the 10 day waste feed cycle the pH 10.0 and the pH 11.0 microcosms clustered together. The results suggest that neither pH nor feed cycle had an overriding control over the communities in these microcosms (Figure 3.24).

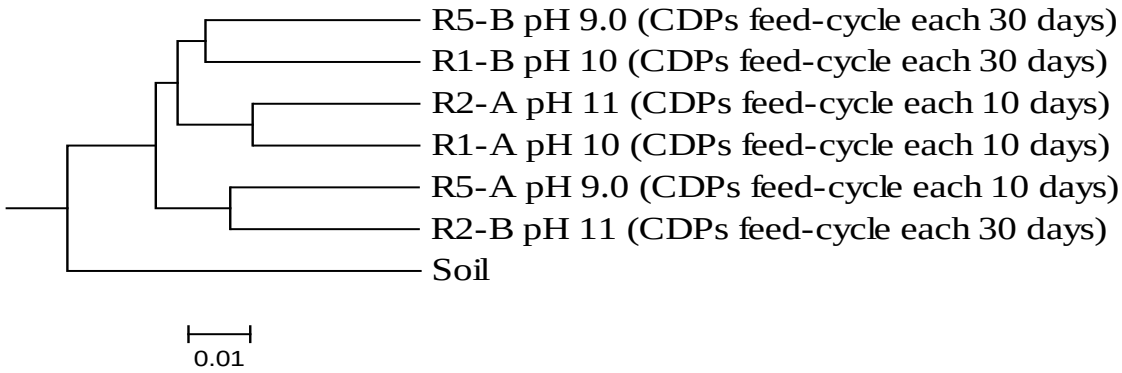


Figure 3. 24: Dendrogram of β-diversity; comparison between microbial communities of the inoculum soils sample and CDPs driven microcosms based on UPGMA.

The community at pH 10.0 was similar to pH 9.0 at a 30 day CDP feed-cycle (A), when the protocol was switched to a 10 day CDPs feed-cycle the pH 10.0 microcosm became similar to that at pH 11.0 (B).

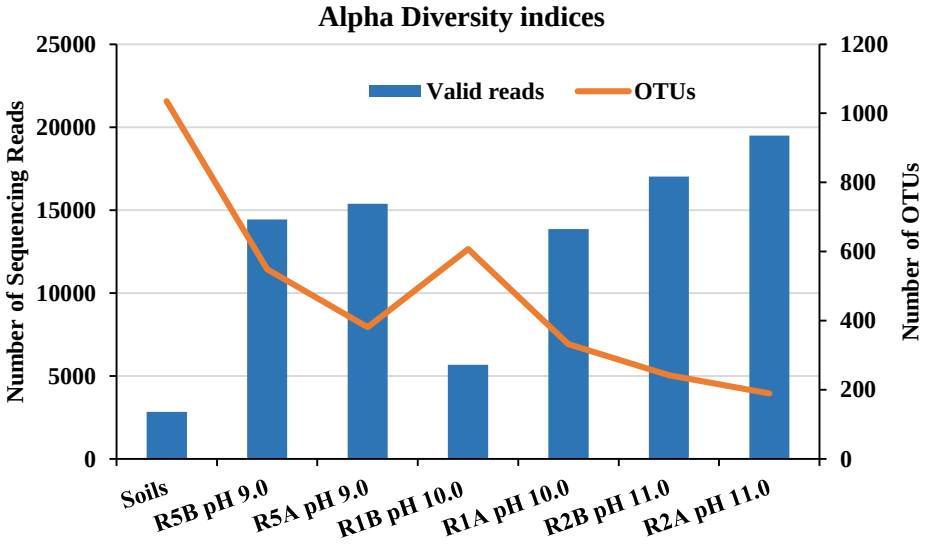


Figure 3. 25: Alpha diversity indices of the starting inoculum compared with CDPs driven microcosms.

The α-diversity plot shows the number of OTUs in the microcosms at different pH levels under both the 30 day and 10 day feed cycles compared with the OTUs of the inoculum.

As the communities evolved the populations became dominated by a more limited group of organisms. Investigation of this is complicated by the dominance of undefined species. However, as the populations became more limited some species became prominent. This is most obvious in the case of the Firmicutes where *Tissierella* sp. became prominent as the conditions became challenging. Unfortunately, it was not possible to isolate a representative from this Genus.

3.2.4. Key Findings

- A significant reduction in bacterial diversity occurred in all microcosms when compared to the bacterial community of the background sediment.
- Eubacteria of the following phyla; Firmicutes, Proteobacteria, Bacteroidetes, Actinobacteria and Archaea of the Euryarchaeota were detected in all microcosms at different pH values.
- The Firmicutes were the dominant phylum in all the CDPs driven microcosms represented by the following families Ruminococaceae, Anaerovirgula, Anaerobranca, Tissierellaceae, Eubacteriaceae, Clostridiaceae, Sedimentibacter and Clostridium.
- The most dominant genus at microcosm pH 11.0 was the *Tissierella* genus.
- A wide range of pure bacterial cultures have been isolated from these microcosms.

3.3. Sub-microcosm studies

A range of sub-microcosms were operated in order to facilitate the isolation of ISA degrading bacteria and to provide a more detailed insight into both CDP and $Ca(ISA)_2$ degradation. These microcosms were generally run as batch systems with multiple subcultures. The calcium salt of ISA ($Ca(ISA)_2$) has been used in both geochemical and microbiological studies as an alternative to CDP (Bassil et al., 2015), presumably due to its ease of manufacture and availability on the open market. However, the calcium salt of α-ISA is sparingly soluble in water (Rai, Rao, & Xia, 1998) when compared to the sodium salt. Its use in microbiological studies has resulted in low levels of degradation and an absence of fermentative degradation at alkaline pH (Kuippers et al., 2015). This contrasts with higher levels of degradation and the establishment of methanogenic communities when CDP has been used (Rout, Charles, Doulgeris, et al., 2015). In addition comparisons between CDP and $Ca(\alpha\text{-}ISA)_2$ as carbon sources for alkaline anaerobic metabolism have indicated that these two carbon sources generate significantly different populations when the same inoculum is employed. Consequently, experiments were run to investigate the degradation of $Ca(ISA)_2$ by CDP degrading communities, to provide an enrichment step prior to the isolation of ISA degrading organisms.

3.3.1. Sub-microcosm of the pH 10.0, pH 11.0 and pH 10.5 microcosms

In order to isolate ISA degrading alkaliphiles a range of alternative culture conditions were investigated. Sediments from the pH 11.0 and pH 10.5 microcosms were incubated in a minimal medium (2.2.4) supplemented with $Ca(ISA)_2$ at a pH of pH 9.0 (2.5.7.). In both cases direct degradation of ISA was evident (Figure 3.26). An increase in optical density (OD) indicated bacterial growth which was accompanied by a decrease in ISA concentration, an increase in acetate concentration, gas generation and a decrease of pH value.

A significant amount of acetate (Figure 3.26 C and F) was produced from ISA during the fermentation process. The concentration of acetate was proportional with ISA degradation. About half the amount of ISA 2.5mM in the form of $Ca(ISA)_2$ (Figure 3.27) was degraded by the pH 11.0 microcosm community which resulted in the generation of ≈5.0mM of acetate in <10 days at 25°C. In contrast all the ISA (4.5mM) was degraded by bacterial from the pH 10.5 microcosm, generating ≈10 mM of acetate over 15 days under the same incubation conditions. The microbial community from the microcosm at pH 10.5 was more active than microbial community of microcosm at pH 11.0.

Culturing of the sediments in these sub microcosms on FAA agar at pH 9.5 recovered a range of Gram negative bacteria including; *Aeromonas salmonicida* ZS-66, *Alcaligenes aquatilis* ZS-22, *Citrobacter* sp., and *Macellibacteroides fermentans* ZS-69 strain.

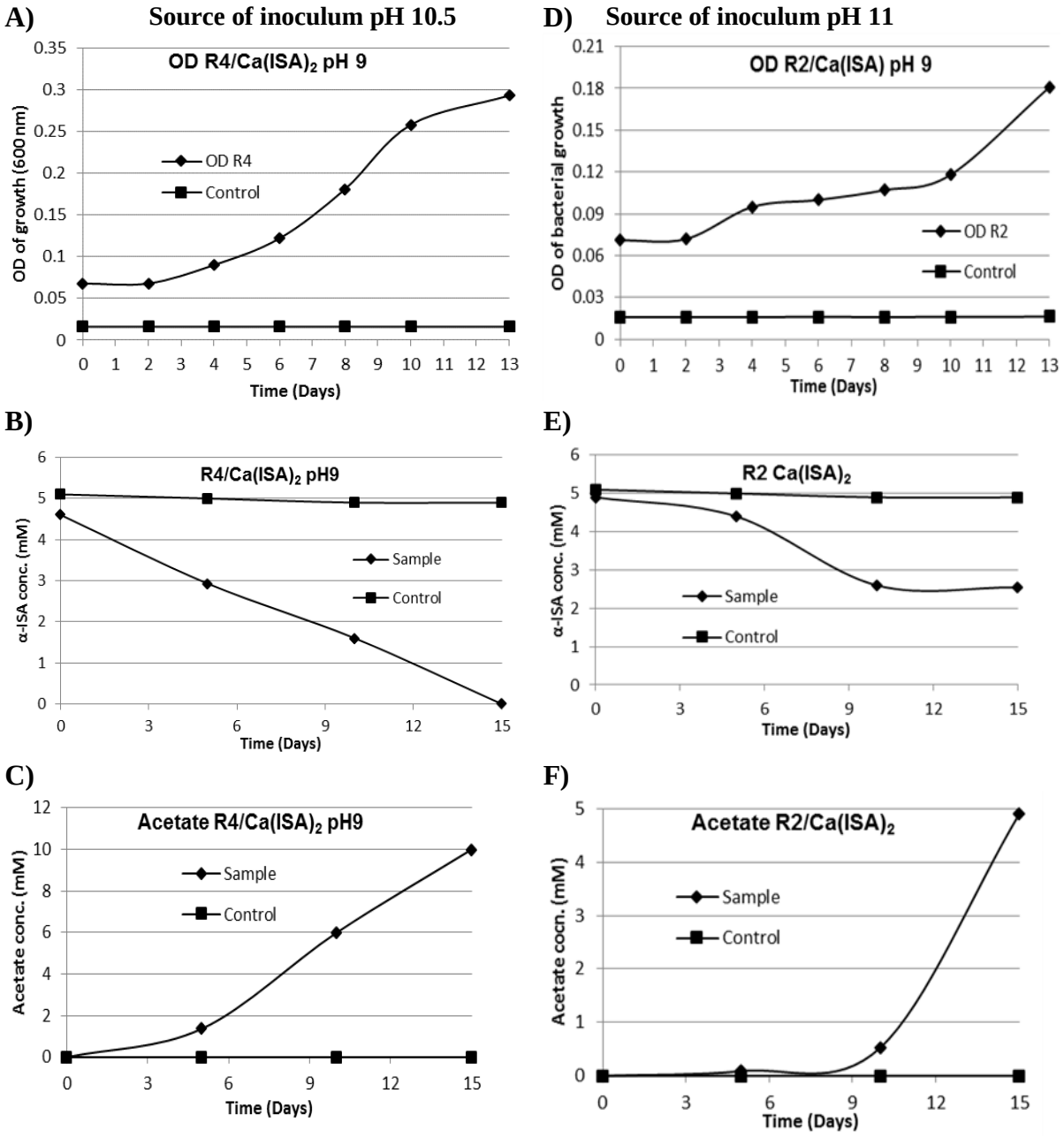


Figure 3. 26: ISA degradation in the form of $Ca(ISA)_2$ by a sediments samples from (R2 pH 11) and (R4 pH 10.5)

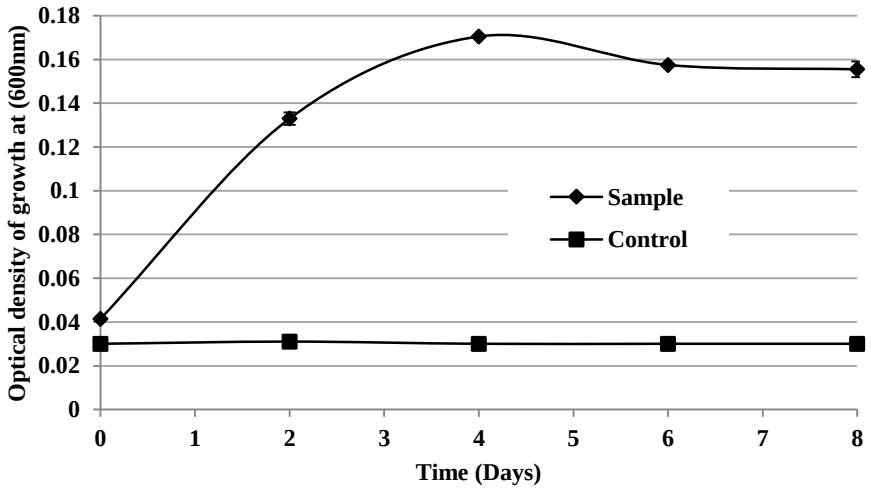
The bacterial community from the pH 10.5 microcosm was more active than bacterial community from pH 11.0; the optical density of growth in $Ca(ISA)_2$ broth medium (A) was higher than the OD in the broth of medium (D), a complete degradation of ISA (B) while half the amount of ISA was degraded in the broth medium (E), associated with an increase of acetate in a broth medium to about 10 mM L^{-1} (C), but in the second broth medium about 5mM of acetate was produced (F).

When sediments were taken from the pH 10.0 microcosm (2.5.1.2) and inoculated into minimal medium supplemented with 2.0 mM of $Ca(ISA)_2$, at pH 9.0 and 25°C there was a complete

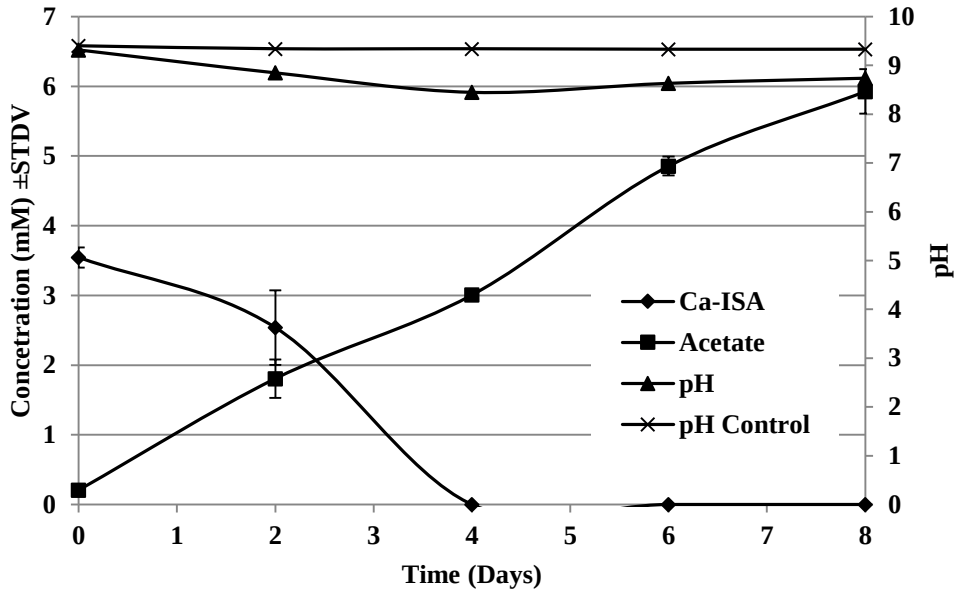
degradation of $Ca(ISA)_2$ after 4 days (Figure 3.27 B). Degradation was confirmed by an increase of optical density (Figure 3.27 A) production of acetate and a reduction in pH (Figure 3.27 B). In addition, a significant amount of gas (H_2, CH_4 and CO_2) was detected in the headspace (Figure 3.27 C).

Microscopic investigation of the microcosm community indicated that it was dominated by Gram negative rods. This was confirmed by community analysis based on 16S ribosomal RNA gene sequencing (Figure 3.28) which indicated that the community was dominated (>90%) by *Azonexus hydrophilus* which was subsequently isolated from the microcosm and is discussed in greater detail in section (3.6.5.4.). These experiments demonstrated that prolonged alkaline incubation (12 months) with CDP can lead to the establishment of communities with limited species diversity which retain their ability to degrade ISA. Previous studies have demonstrated $Ca(ISA)_2$ degradation at pH 10.0 via respiratory processes (aerobic and denitrification) (Bassil et al., 2015). However, the data presented confirms the fermentation and subsequent methanogenesis of $Ca(ISA)_2$ at alkaline pH (Kyeremeh, Charles, Rout, Laws, & Humphreys, 2016), which aligns with previous studies employing CDP as a source of ISA (Charles et al., 2015; Rout, Charles, et al., 2015a).

A)



B)



C)

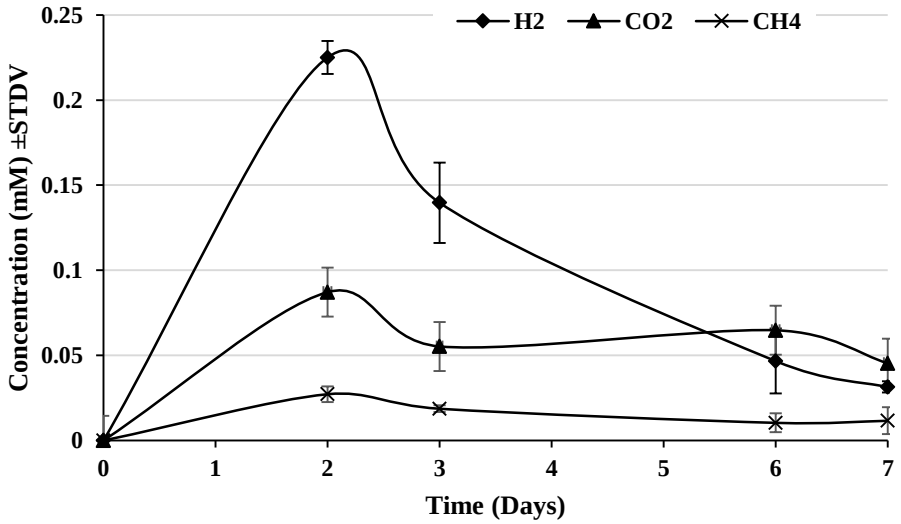


Figure 3. 27: Biodegradation of ISA by a bacterial community pH 9.0.

The gradual increase in optical density indicated bacterial growth at the expense of $Ca(ISA)_2$ degradation over a 4day incubation period. This was associated with an increase in acetate production and a reduction in the pH 8.7 at the end of 8 days (B). Fermentation gases accumulated in the headspace between first day and 7 days starting with significant amounts of hydrogen gas, followed by carbon dioxide and methane (C).

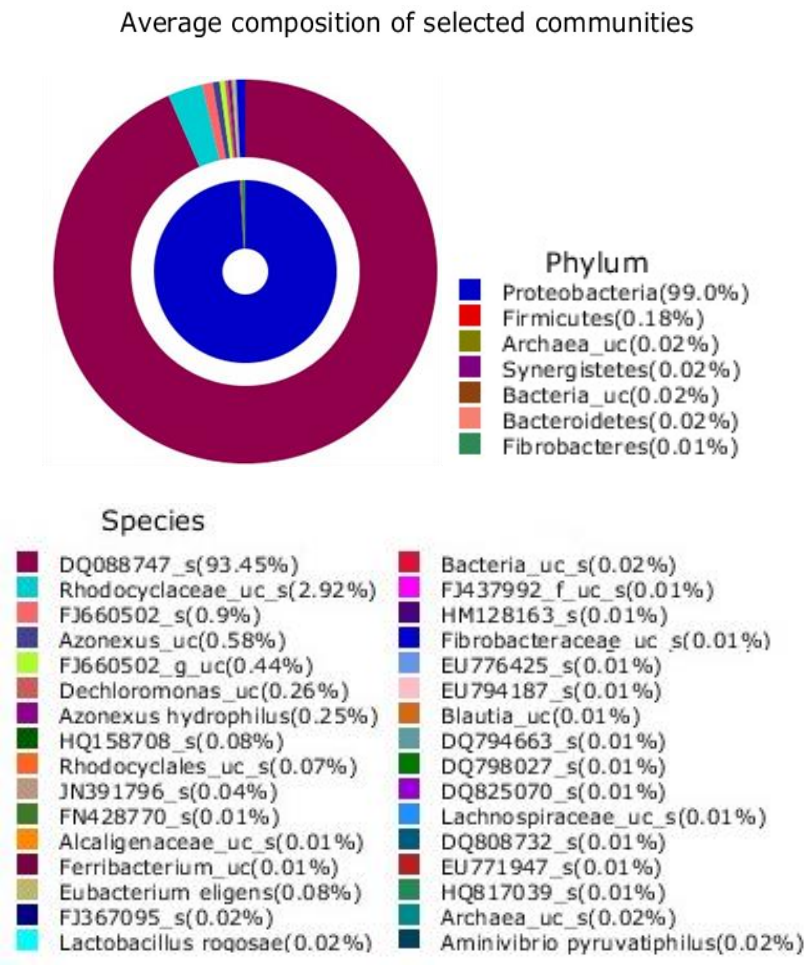


Figure 3. 28: Bacterial diversity of the microcosm supplemented by $Ca(ISA)_2$ at pH 9.0

The bacterial community analysis of the sediment demonstrated that the microcosm was dominated by unclassified species (accession number DQ088747) *93.45%* (outer circle) of the Proteobacteria phylum 99% (central circle).

Based on the 16SrRNA gene sequence the dominant strain in the community had an accession number DQ088747 which showed a 99% match to *Azonexus hydrophilus* ZS02 that was isolated in pure culture from the microcosm (Figure 3.29), this strain is described in greater detail in later sections.

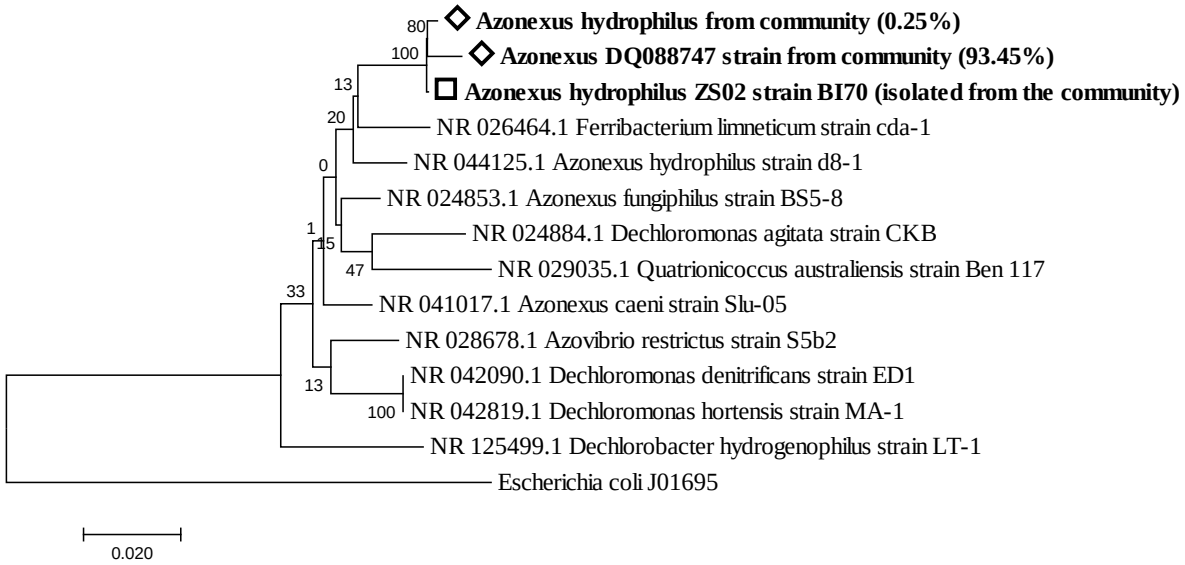


Figure 3. 29: Phylogenetic tree for the *Azonexus* genus and isolated strain related to this genus based on 16SrRNA gene sequences.

Maximum-likelihood phylogenetic trees based on 16S rRNA gene sequences demonstrating the relationships between the dominated strain DQ088747 and other strains from the community and related representatives of their genus and the closest phylogenetic relatives of these two strains with an isolated *Azonexus hydrophilus* ZS02 strain. Bootstrap values (expressed as percentages of 1000 replicates) are shown at each node where the bar represents 0.02 changes per nucleotide position. Evolutionary analyses were conducted in MEGA7.

Although $Ca(ISA)_2$ is the most commonly used salt of ISA, the sodium salt can be obtained (Shaw et al., 2012). The advantage of using Na-ISA is the fact that it is far more soluble than the calcium salt. In the case of other complexants e.g. citric acid there is evidence that the counter ion does influence the biodegradability of the associated organic compound. In view of this, experiments (2.5.3.) were carried out to compare the degradation rates of both forms of ISA by the microbial consortia present in R1 pH 10.0.

The community present in the pH 10.0 microcosm was able to degrade both forms of ISA, with an associated accumulation of acetate and a reduction in a pH (Figure 3.30 A to F). However, the extent of degradation was more extensive with the sodium salt (~8.5 mM), rather than the calcium salt with the $Ca(ISA)_2$ (4.0 mM) (Figure 3.30 A and B). There was a complete degradation of Na-ISA while 2mM of $Ca(ISA)_2$ remained recalcitrant within the microcosm. This observation in similar to the data presented in other studies employing the calcium salt,

where 100% degradation is not observed (Bassil et al., 2015), however it contradicts data presented earlier in this section where 100% degradation was observed.

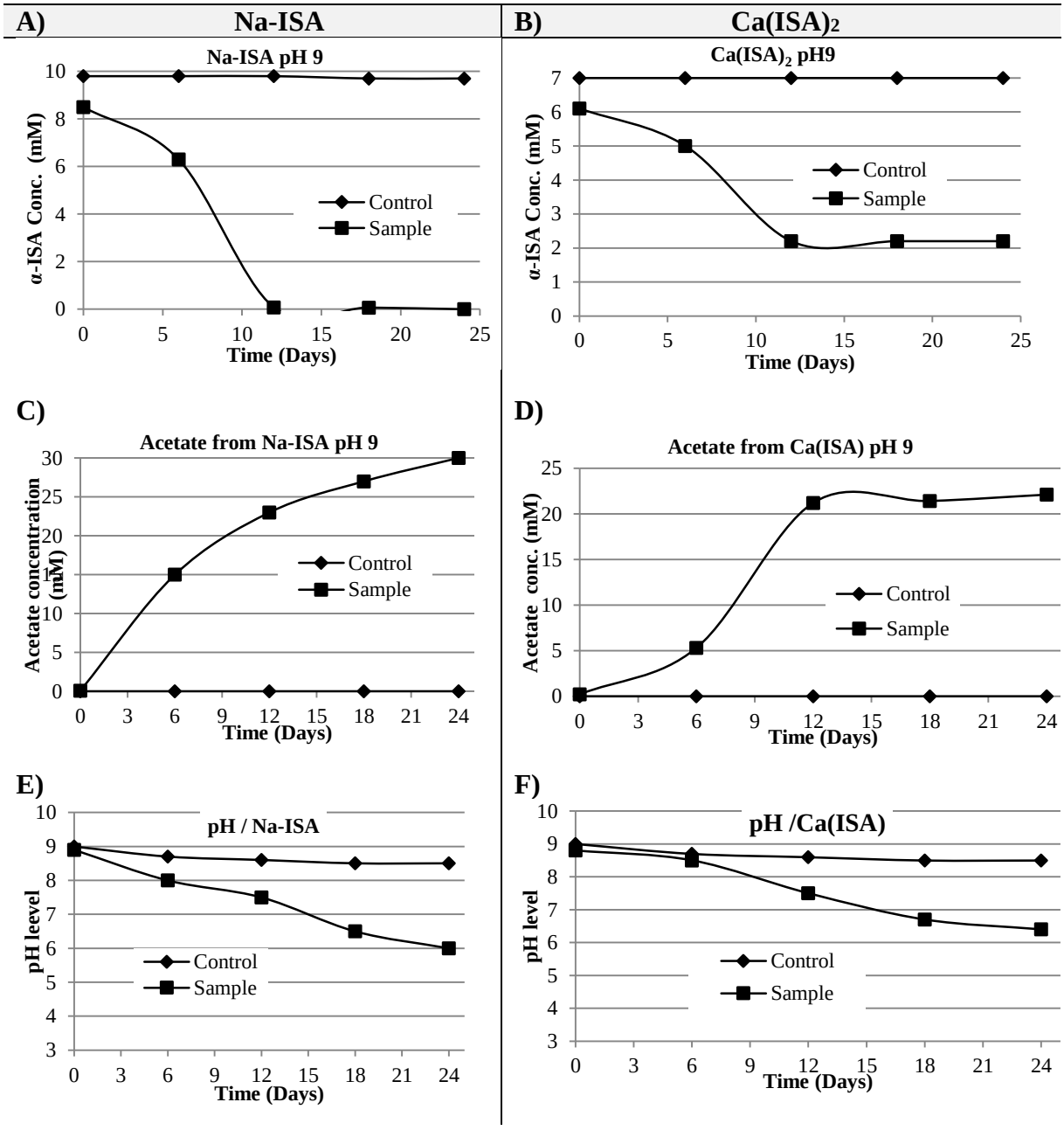


Figure 3. 30: Comparing Na-ISA and $Ca(ISA)_2$ degradation by bacterial community.

Complete degradation of Na-ISA (~9.0mM) during 12 days (A), at the same period of an incubation time only (4.0 mM) of ISA in the form of $Ca(ISA)_2$ was degraded by bacterial community (B), significant amount of acetate was produced (C and D), that causes a reduction in pH values for both microcosms (E and F).

3.4. ISA degradation in defined cultures

In order to find alkaliphilic isolates with the ability to degrade ISA, pure cultures and mixtures of pure cultures were tested in microcosms using mineral media with and without the ATCC

vitamins (1% v/v) solution supplemented with ISA in the form of either $Ca(ISA)_2$, Na-ISA and CDPs (α- and β-ISA). These were carried out using a mixture of these isolates; *Aeromonas salmonicida* (BI 14), *Citrobacter gillenii* (BI 30), *Alcaligenes aquatilis* (BI 22), *Brevundimonas diminuta sp*. (BI 36) and *Ensifer adhaerens* (BI 35). Although all these strains were isolated from microcosms fed on CDPs/MM and isolated on CDP/MM plates, there was no evidence of ISA degradation in anaerobic broth culture at pH 9.0 and 25°C. This suggests that these isolates were either secondary members of the microcosm population, perhaps feeding on cellular breakdown products (e.g. proteins) from the death of other bacteria in the system. Or that ISA degradation by these bacteria required growth factors provided by uncultivable microorganisms present the microcosms.

This work was continued by investigating a mixture of *Aeromonas salmonicida* (BI55), *Citrobacter gillenii* and *Macellibacteroides fermentans* (BI40) (Table 3.4). These isolates had been isolated from CDP driven microcosms at pH 9.0, pH 10.0 and pH 11.0. In addition, these isolates were isolated in pure culture on FAA at pH 9.5 as well as CDPs/MM plates at pH 9.5. These three isolates were inoculated in minimal medium supplemented by $Ca(ISA)_2$, under anaerobic condition, at pH 9.0 and at 25ºC.

This combination showed an ability to degrade α-ISA (about 1.7mM) which levelled off after 18 days of incubation (Figure 3.31 A). The fermentation of ISA generated acetate that gradually increased to about 6.0mM by the end of 25 days (Figure 3.31 B), leading to a reduction in pH value to about pH 8.1 (Figure 3.31 C). Given the ability of this combination of bacteria to degrade ISA, the isolates were investigated in greater detail. These investigations focussed on the two Gram-negative bacteria (*Macellibacteroides fermentans* HH-ZS strain (BI40) and *Aeromonas salmonicida* strain (BI55)) in mineral medium containing either $Ca(ISA)_2$ or CDP at either pH 8.0 or pH 9.0 under anaerobic condition at 25ºC.

When combined at pH 9.0 these two strains completely degraded Ca-ISA within 24 hours with a 5 day lag period (Figure 3.32 A). This lag was similar to that seen when the three bacteria were combined (Figure 3.31 A). As expected ISA degradation was associated with acetate accumulation in a molar ratio of 1:2, acetate accumulation reduced the pH to pH 7.8 (Figure 3.32 A). Microbial growth was associated with ISA degradation as indicated by the increase in optical density (Figure 3.32 B). When fed on CDP rather than $Ca(ISA)_2$ at pH 9.0 degradation started at 5 days with approximately 2.1mM of both α-ISA and β-ISA was degraded up to day 8 beyond which no further degradation was observed (Figure 3.33 A). Unlike the pure ISA experiments this ISA degradation was associated with a range of VFAs being produced dominated by acetate 18mM, butyrate 4.0mM and propionate 2.5Mm (Figure 3.33 B) which

reduced the pH to ≈pH 7.9 (Figure 3.33 D). The results were similar at pH 8.0 however at this pH there was no lag in the onset of α-ISA degradation (Figure 3.34 and 3.35), although there was a lag in β-ISA degradation (Figure 3.35 A).

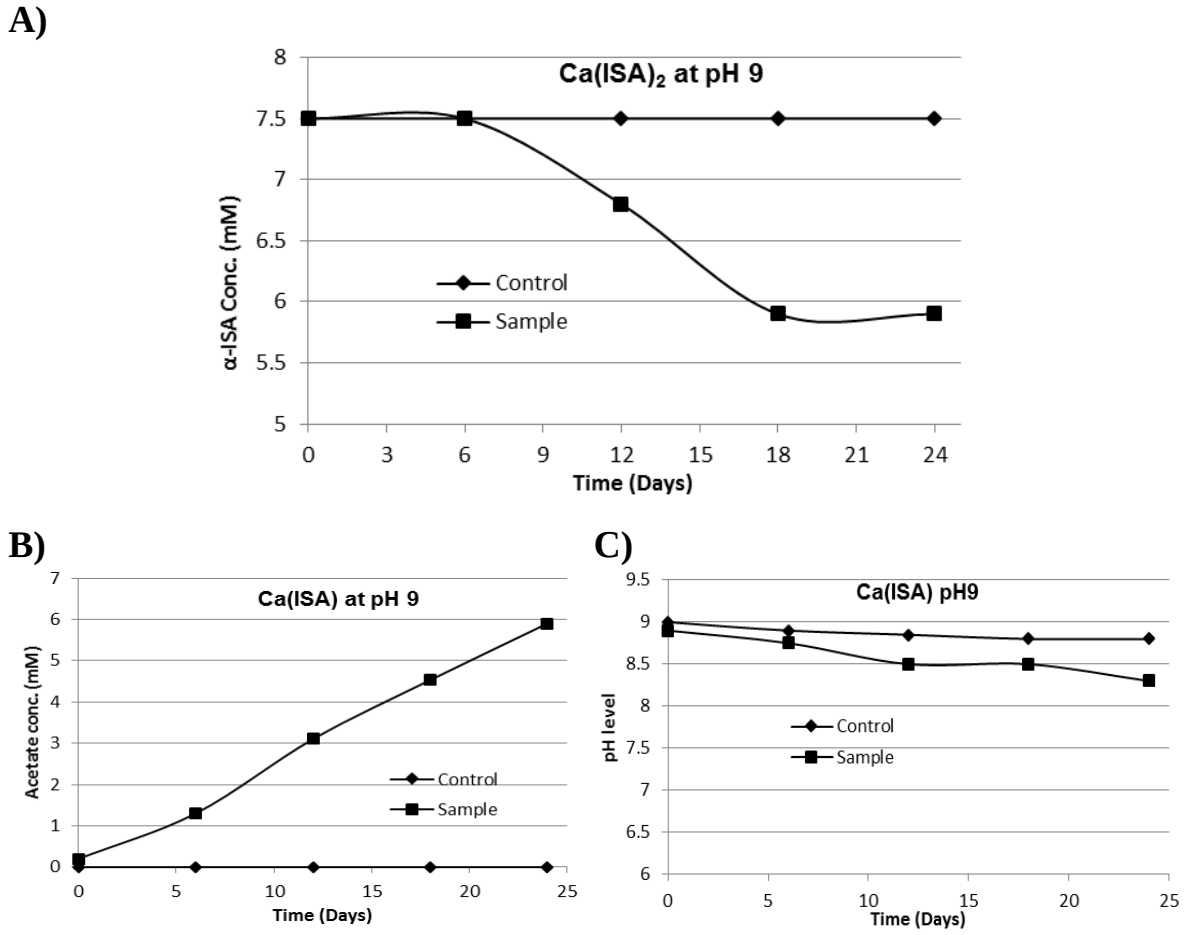


Figure 3.31: Degradation of $Ca(ISA)_2$ by a combination of three bacterial isolates at pH 9.0

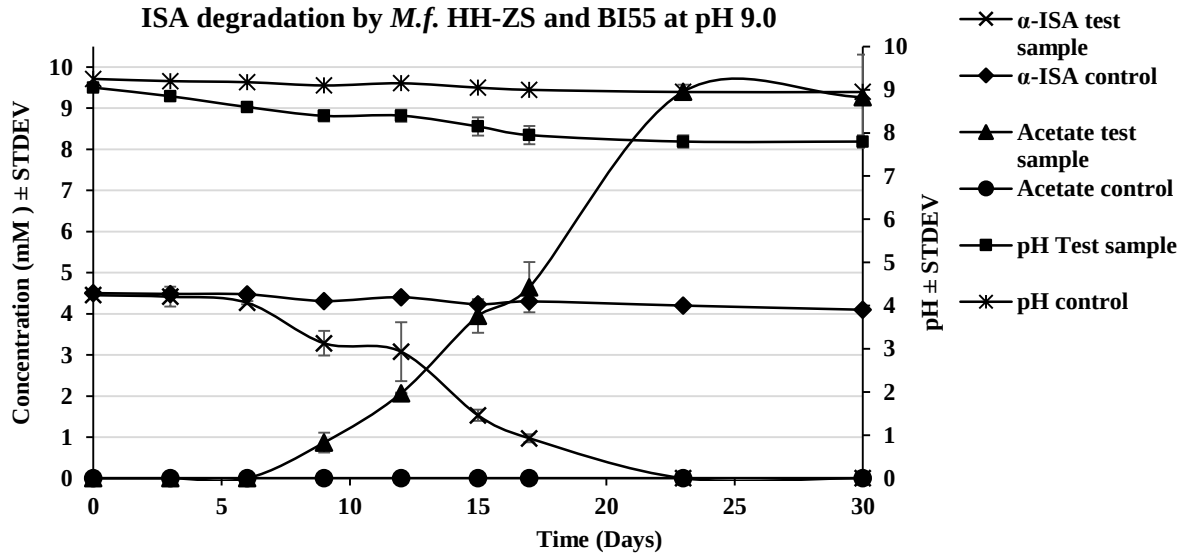
About 1.7mM of ISA was degraded after 18 days of incubation (A), the degradation of ISA was associated with acetate production (B), leading to a reduction in pH value to about pH 8.1 (C).

When investigated on its own *Macellibacteroides fermentans (M.f.* HH-ZS*)* was able to degrade α-ISA as a sole carbon source at both pH 9.0 (Figure 3.36) and pH 8.0 (Figure 3.37) generating acetic acid. However, in this case the molar stoichiometry was closer to 1:1 rather than the 1:2 observed in the mixed culture. When cultured in the presence of CDP both forms of ISA were degraded without a lag at pH 9.0 (Figure 3.38), but there was a lag in β-ISA degradation at pH 8.0 (Figure 3.39). In both cases a greater amount of acetate was generated than when fed on Ca-ISA.

At both pH 9.0 and pH 8.0 *Aeromonas salmonicida* (BI55) was also able to degrade $Ca(ISA)_2$ with a lag period observed at both pH values (Figure 3.40 and 3.41). Degradation was associated with acetate generation which was approached a molar stoichiometry closer to 1:2 that the 1:1

Macellibacteroides fermentans. This pattern was repeated with the CDP fed cultures where both forms of ISA were degraded with an acetate degradation profile approaching a 1:2 stoichiometry. As observed with *Macellibacteroides fermentans* there was a significant lag period observed with β-ISA degradation (Figure 3.42 and 3.43). Microbial growth was associated with ISA degradation as indicated by the increase in optical density in all cases.

A)



B)

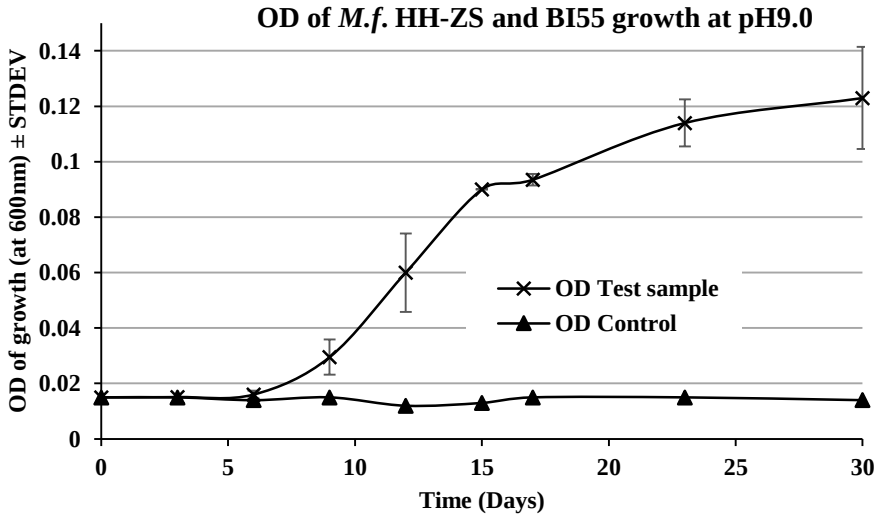
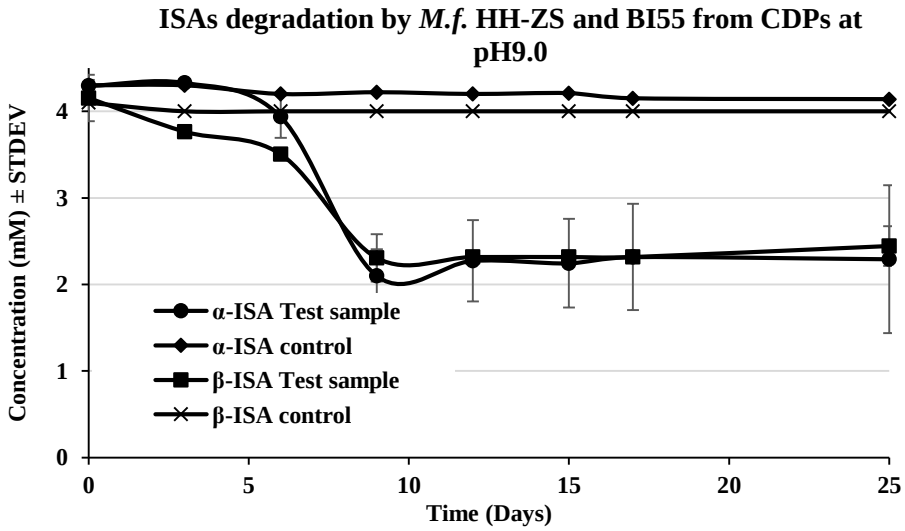
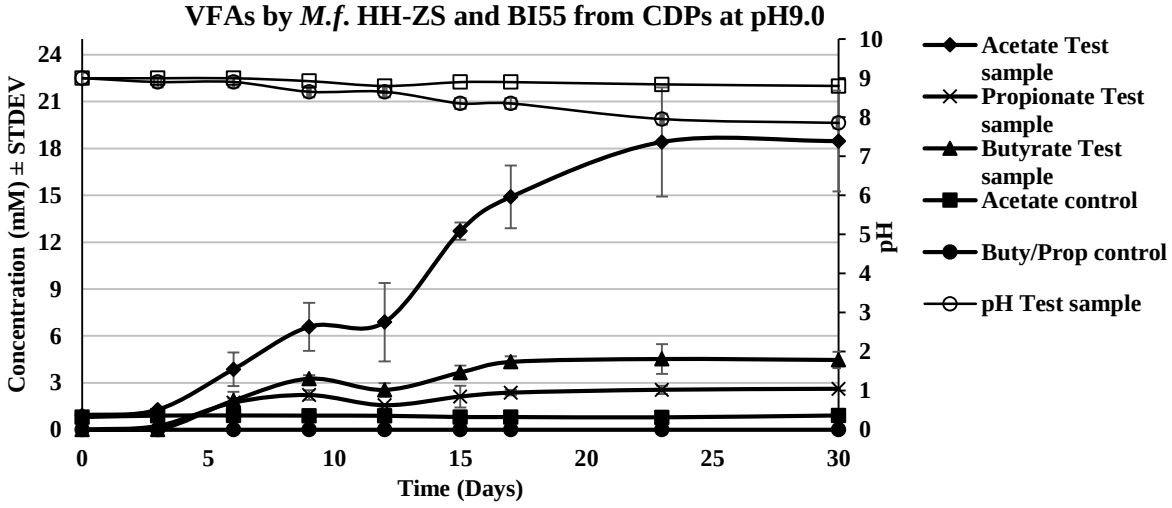


Figure 3. 32: Degradation of $Ca(ISA)_2$ by *M. fermentans* HH-ZS and *Aeromonas* sp. (BI 55) at pH 9.0.

A)



B)



C)

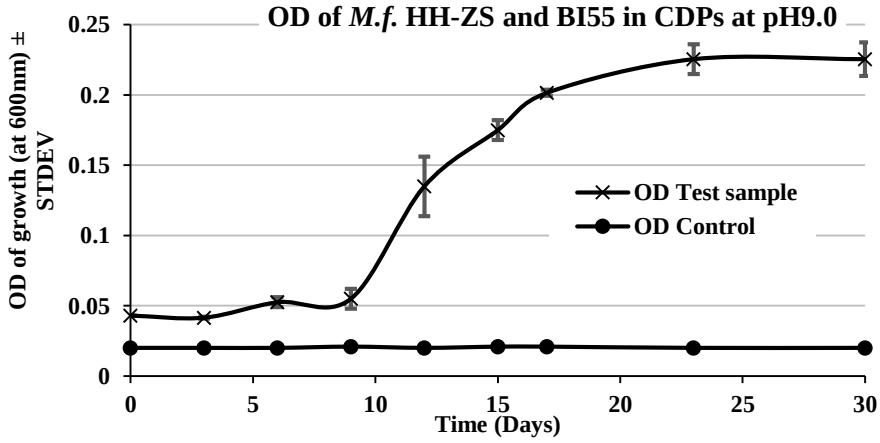
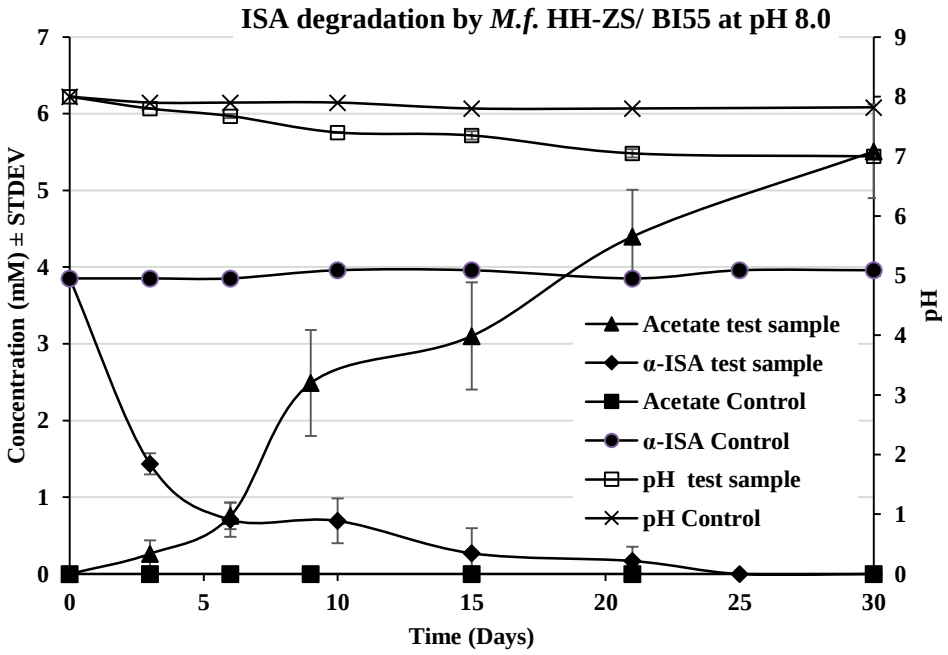


Figure 3. 33: Degradation of ISAs from CDPs by *M. fermentans* HH-ZS and *Aeromonas* sp. at pH 9.0

A)



B)

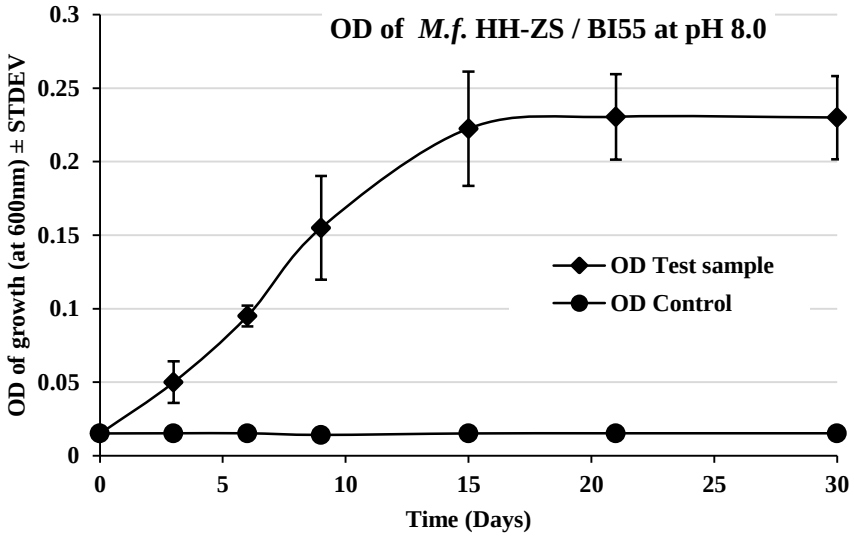
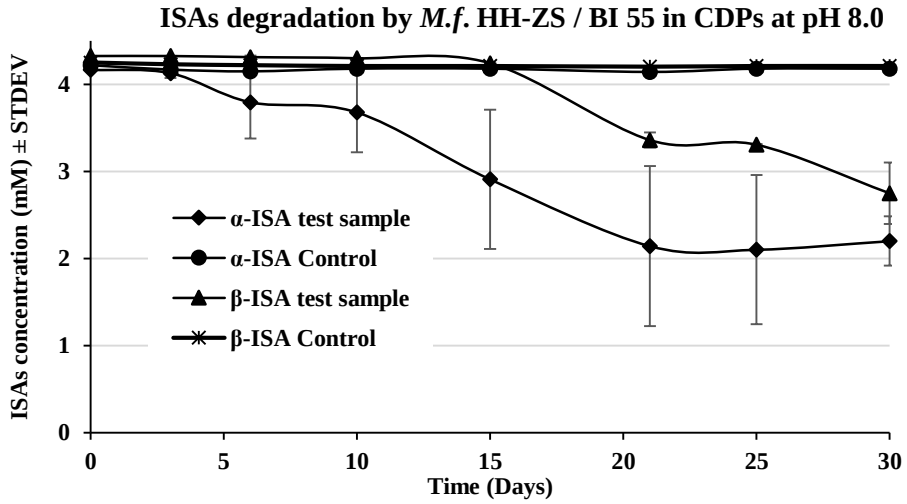
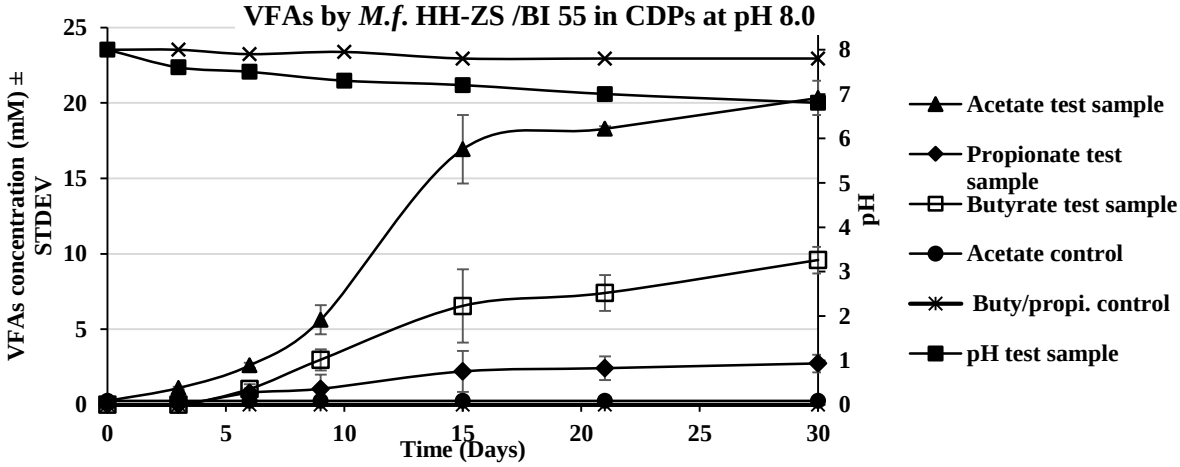


Figure 3. 34: Degradation of $Ca(ISA)_2$ by *M. fermentans* HH-ZS and *Aeromonas* sp. (BI 55) pH 8.0

A)



B)



C)

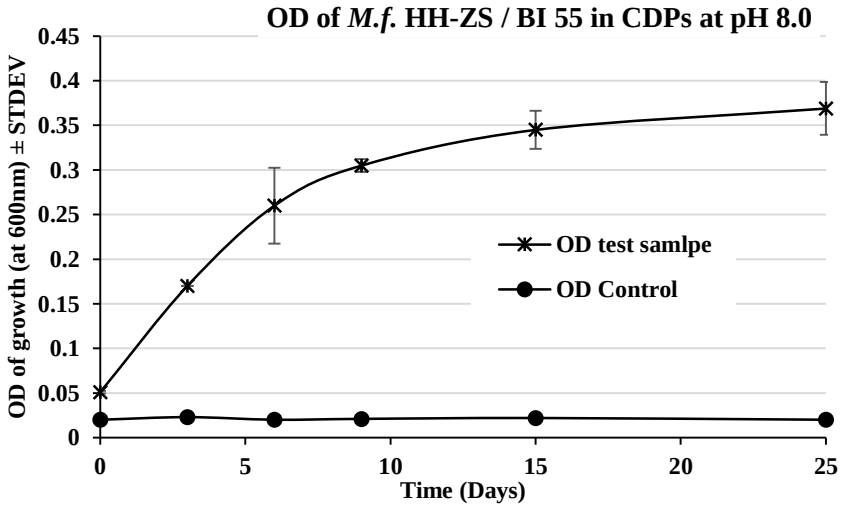


Figure 3. 35: Degradation of ISA from CDPs by *M. fermentans* HH-ZS and *Aeromonas* sp. (BI55) pH 8.0

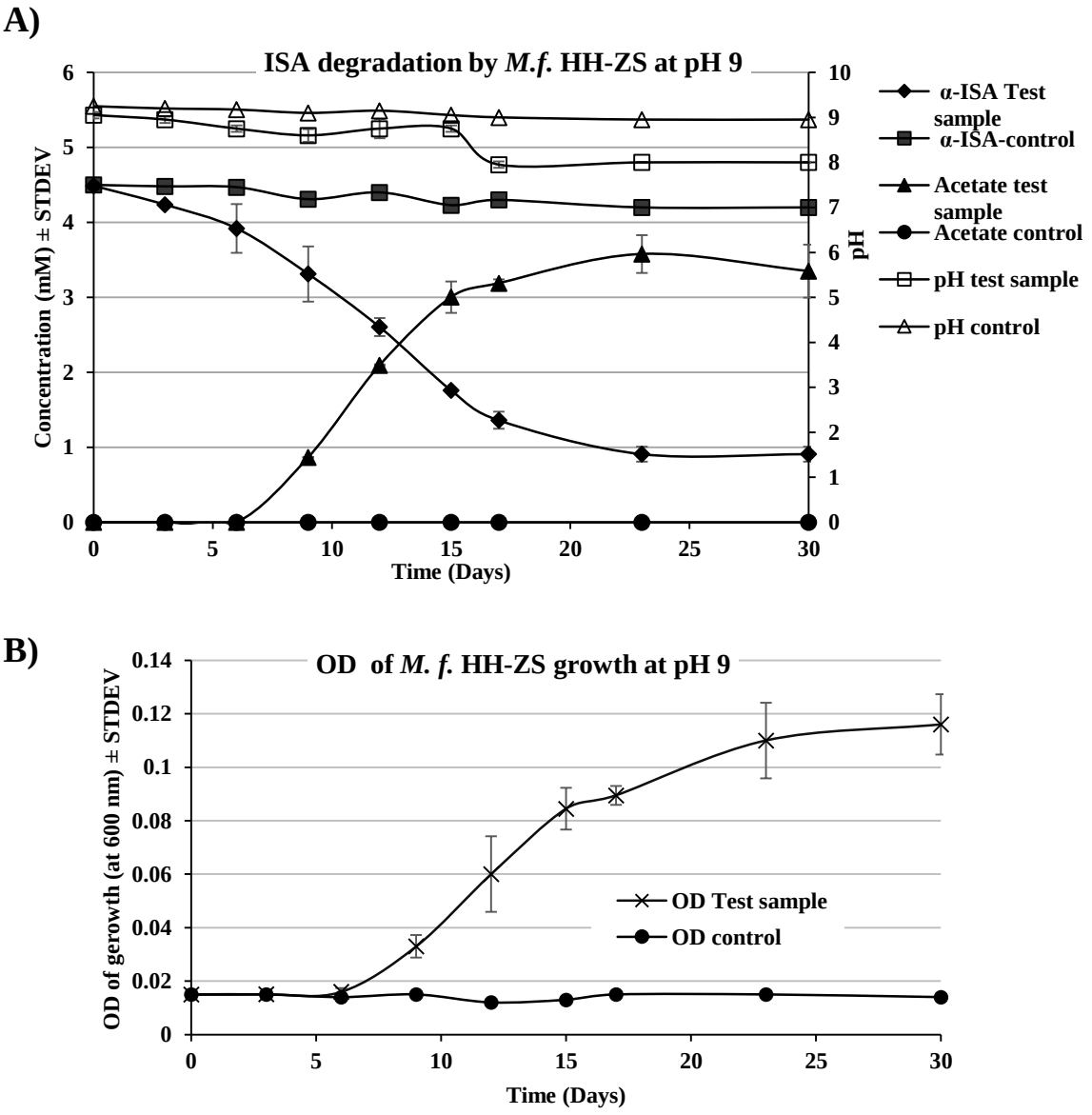
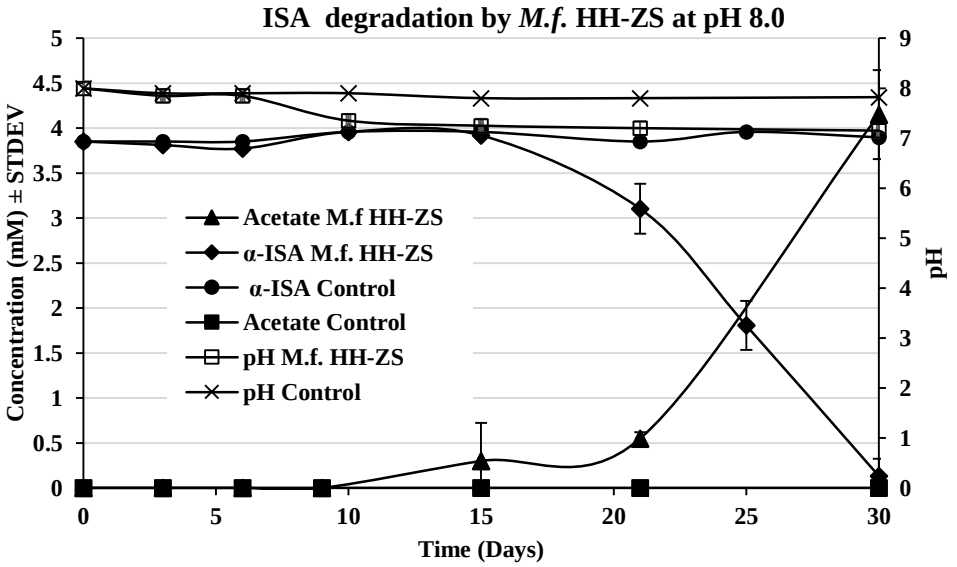


Figure 3. 36: Degradation of $Ca(ISA)_2$ by *M. fermentans* HH-ZS at pH 9.0

A)



B)

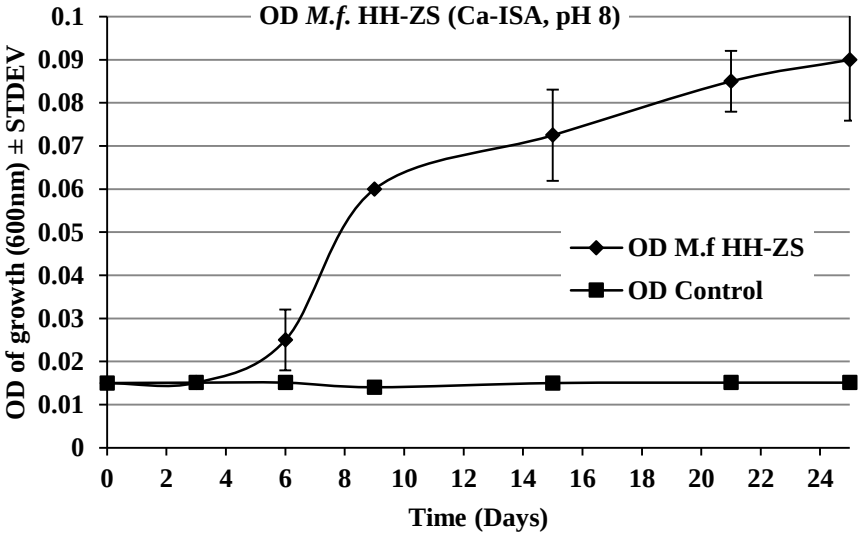


Figure 3. 37: Degradation of Ca(ISA)2 by *M. fermentans* HH-ZS at pH 8.0

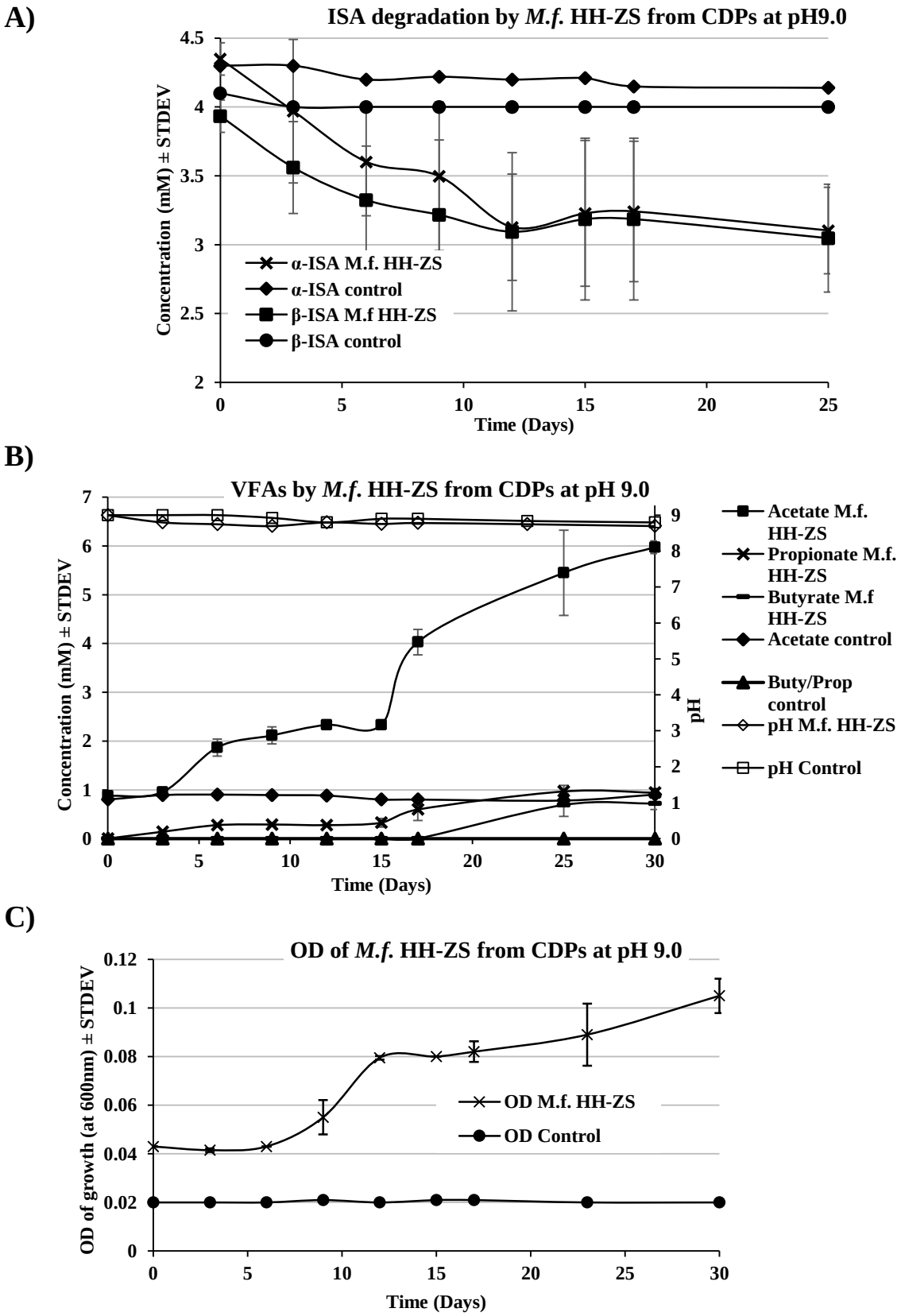


Figure 3. 38: Degraded of ISAs from CDPs by *M. fermentans* HH-ZS strain at pH 9.0

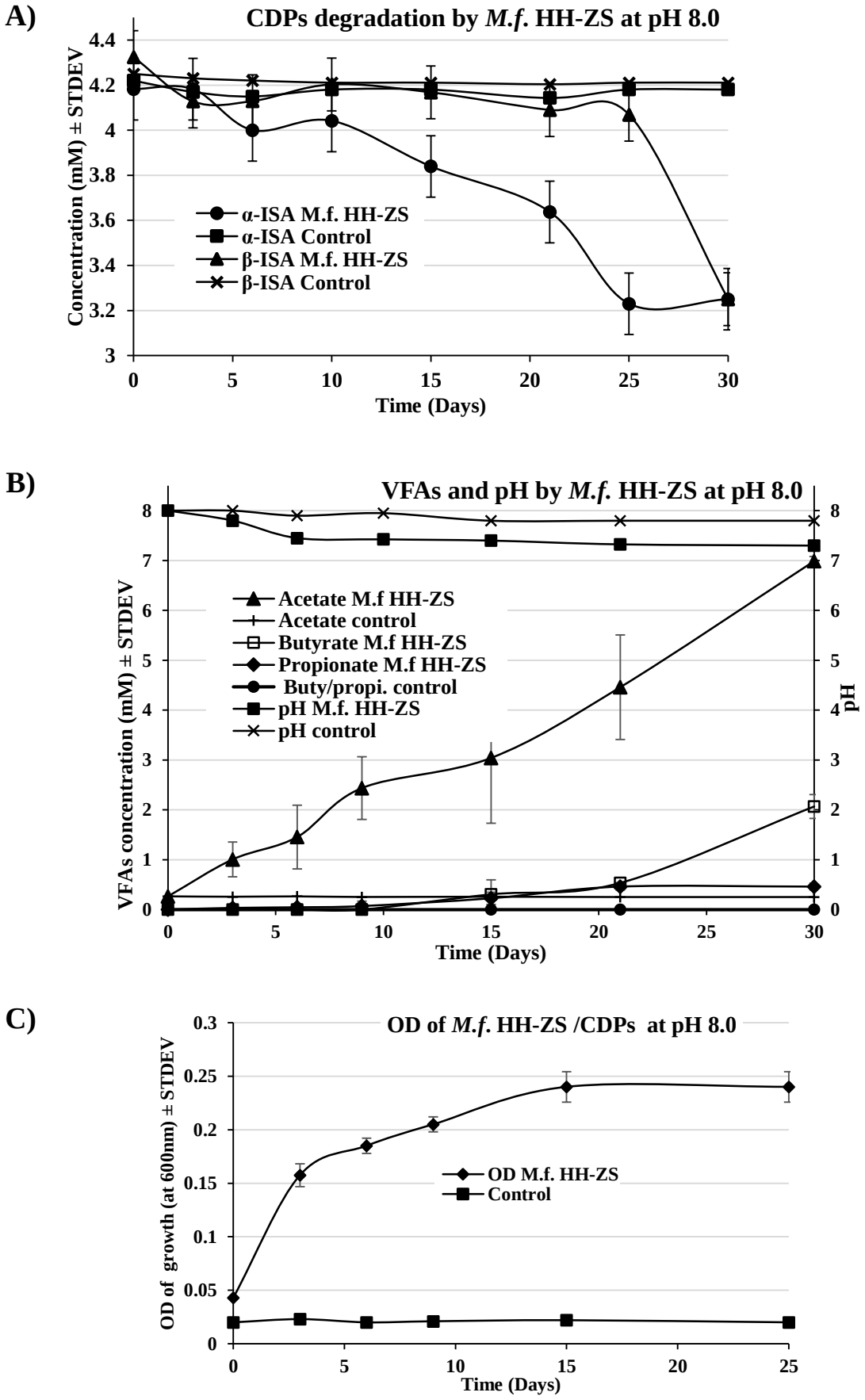


Figure 3. 39: Degradation of ISAs from CDPs by *M. fermentans* HH-ZS *strain* at pH 8.0

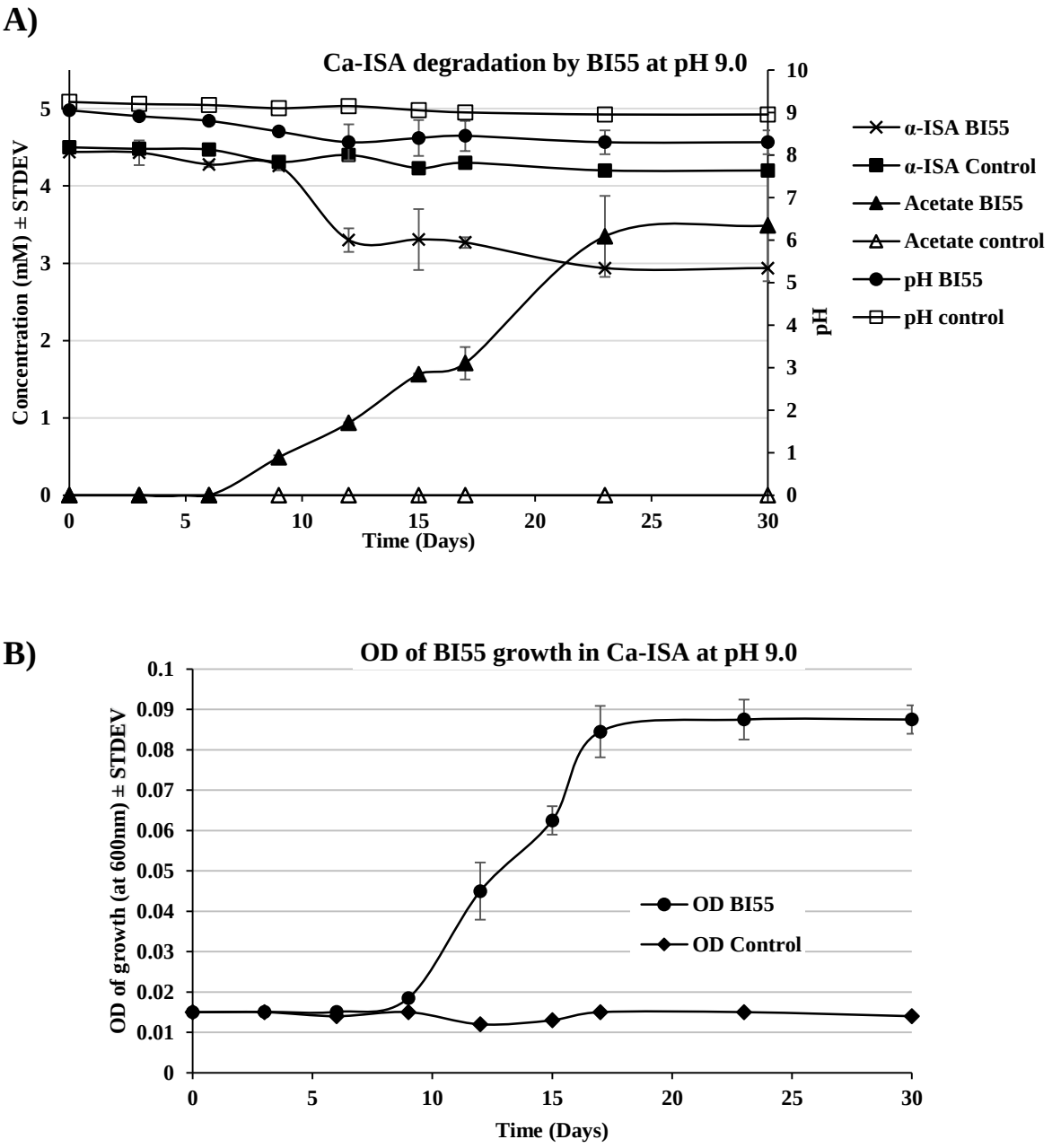


Figure 3. 40: Degradation of $Ca(ISA)_2$ by *Aeromonas* sp. (BI 55) strain at pH 9.0

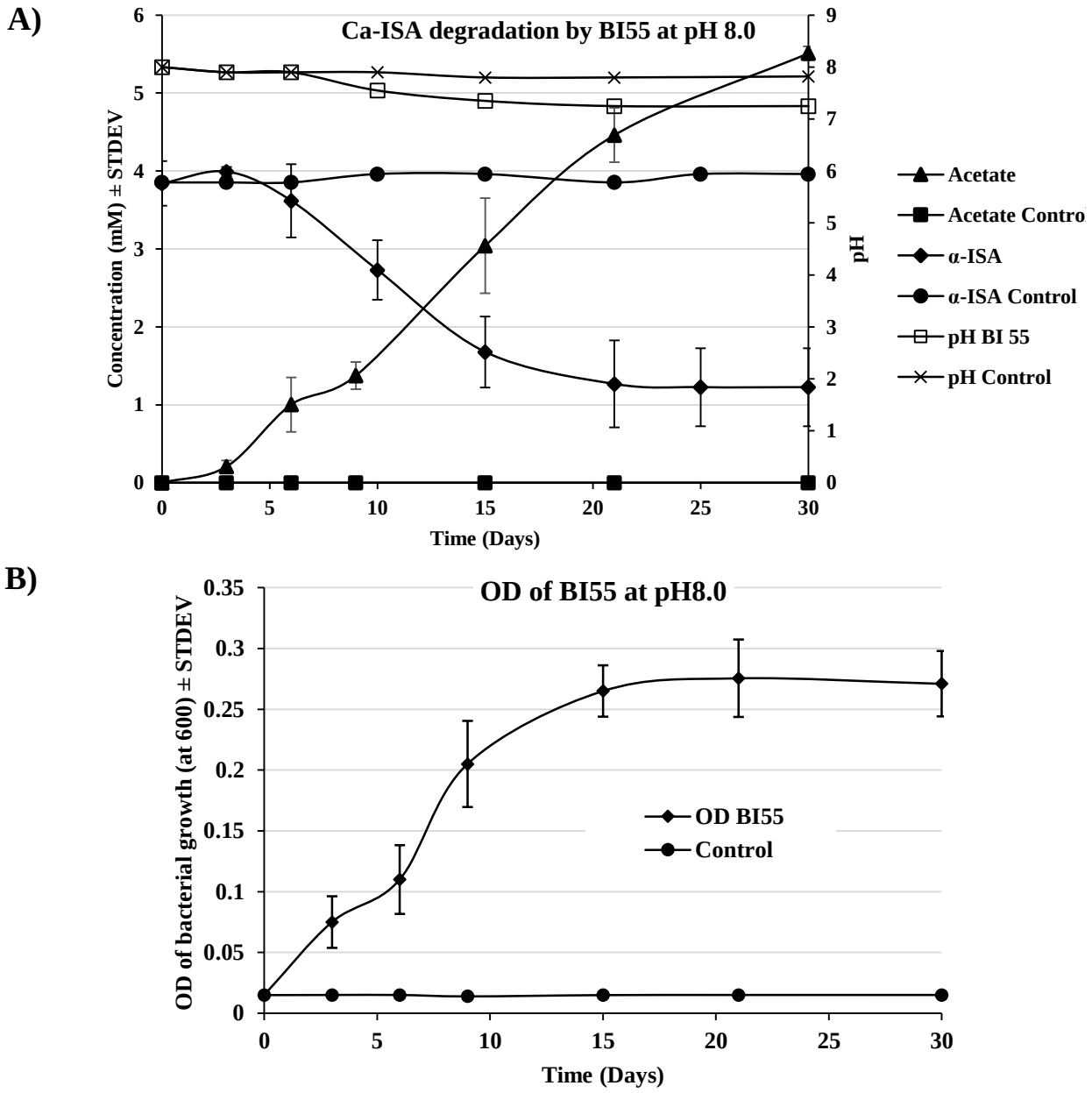


Figure 3. 41: ISA degradation by *Aeromonas* sp. (BI 55)

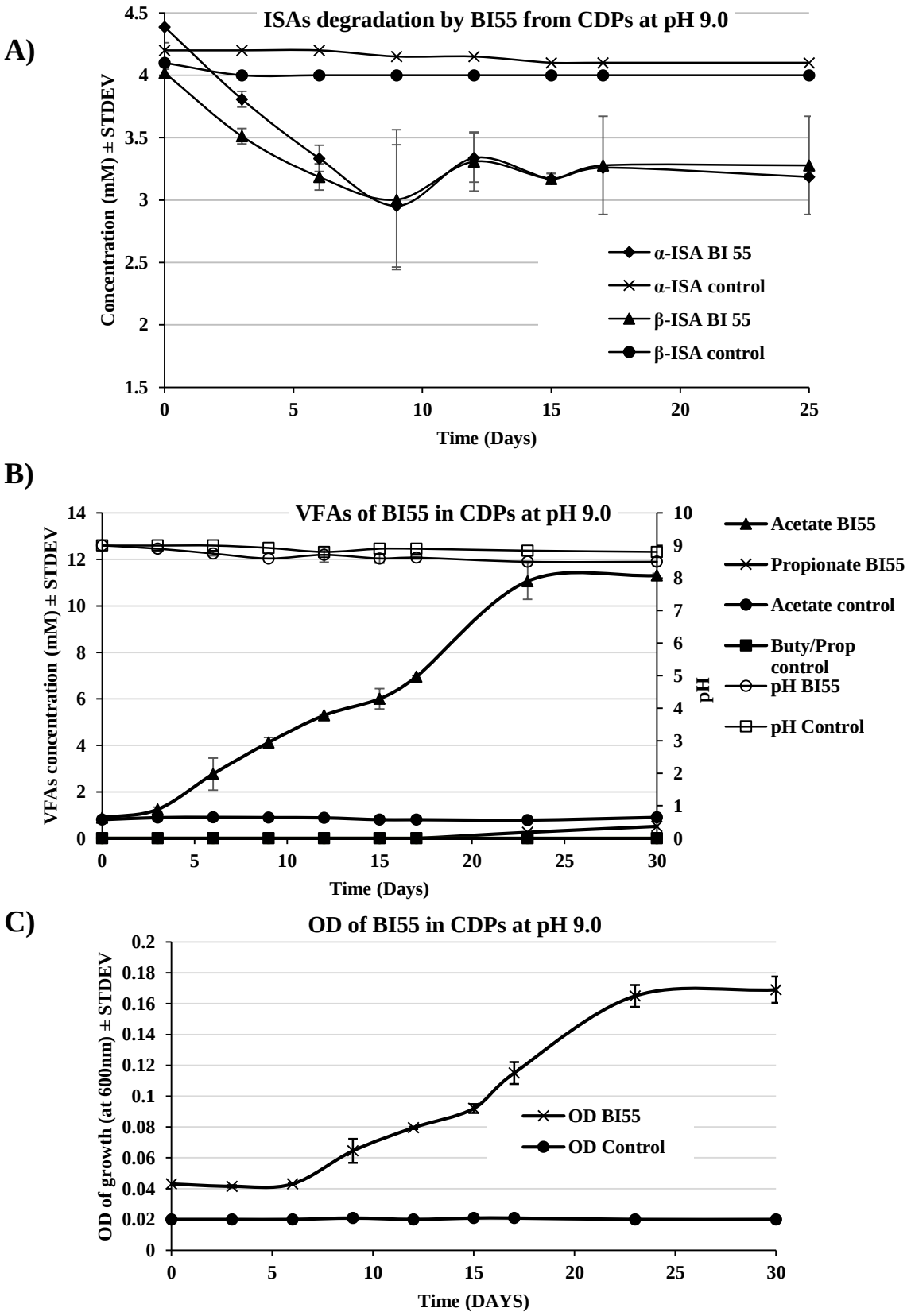
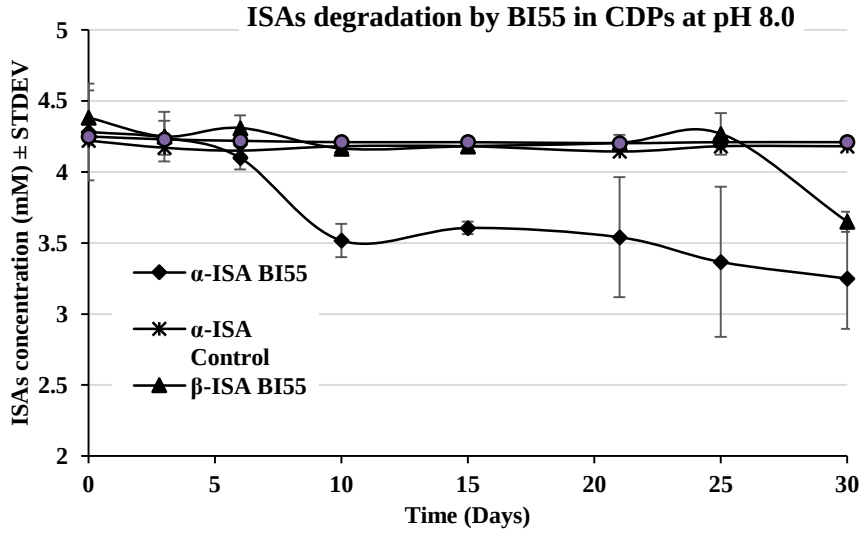
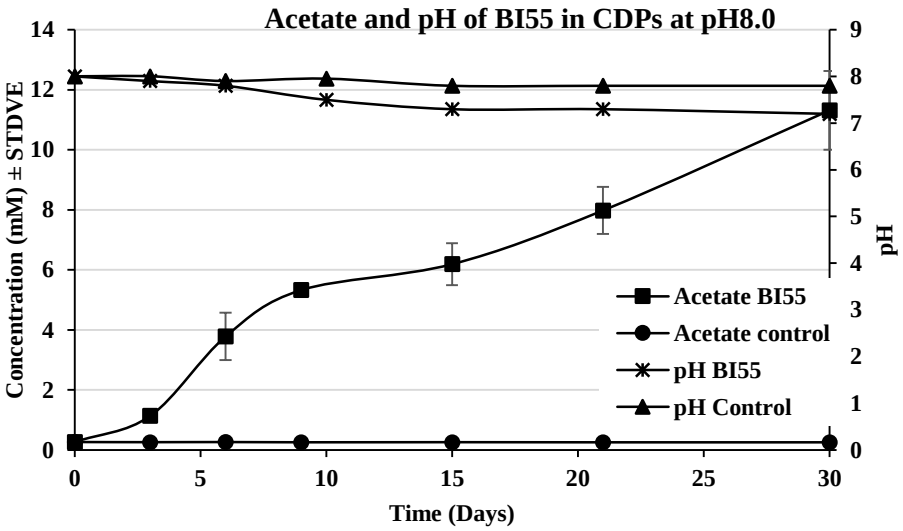


Figure 3. 42: Degradation of ISAs from CDPs by *Aeromonas* sp. (BI55) at pH 9.0

A)



B)



C)

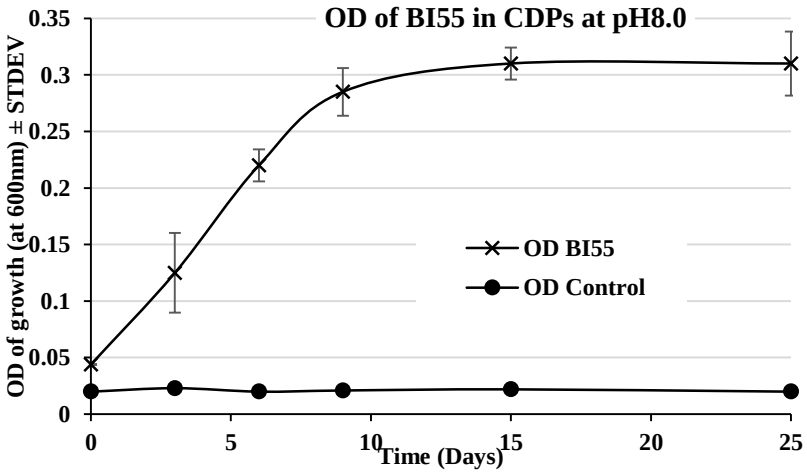


Figure 3. 43: Degradation of ISAs isomers from CDPs by *Aeromonas* sp. (BI 55) at pH 8.0

ISA has the same general formula as glucose and is generated from cellulose which is a polymer of glucose, however it has a different structure. Given these similarities it is interesting to contrast the degradation profiles when metabolised by the same organism. In this case the degradation of glucose by *Macellibacteroides fermentans* HH-ZS was compared with α-ISA degradation at pH 8.5 under the same incubation conditions of minimal medium and anaerobic condition (Figure 3.44). In this case glucose was degraded in <10 days (A) whilst α-ISA was gradually degraded, and complete degradation required around 30 days (B). This indicates that whilst the structure is similar, ISA degradation is more difficult that glucose degradation. This may be due to the transport of the relative compounds into the cells, since glucose transport systems are common in bacteria and ISA is a rare compound for bacteria to experience.

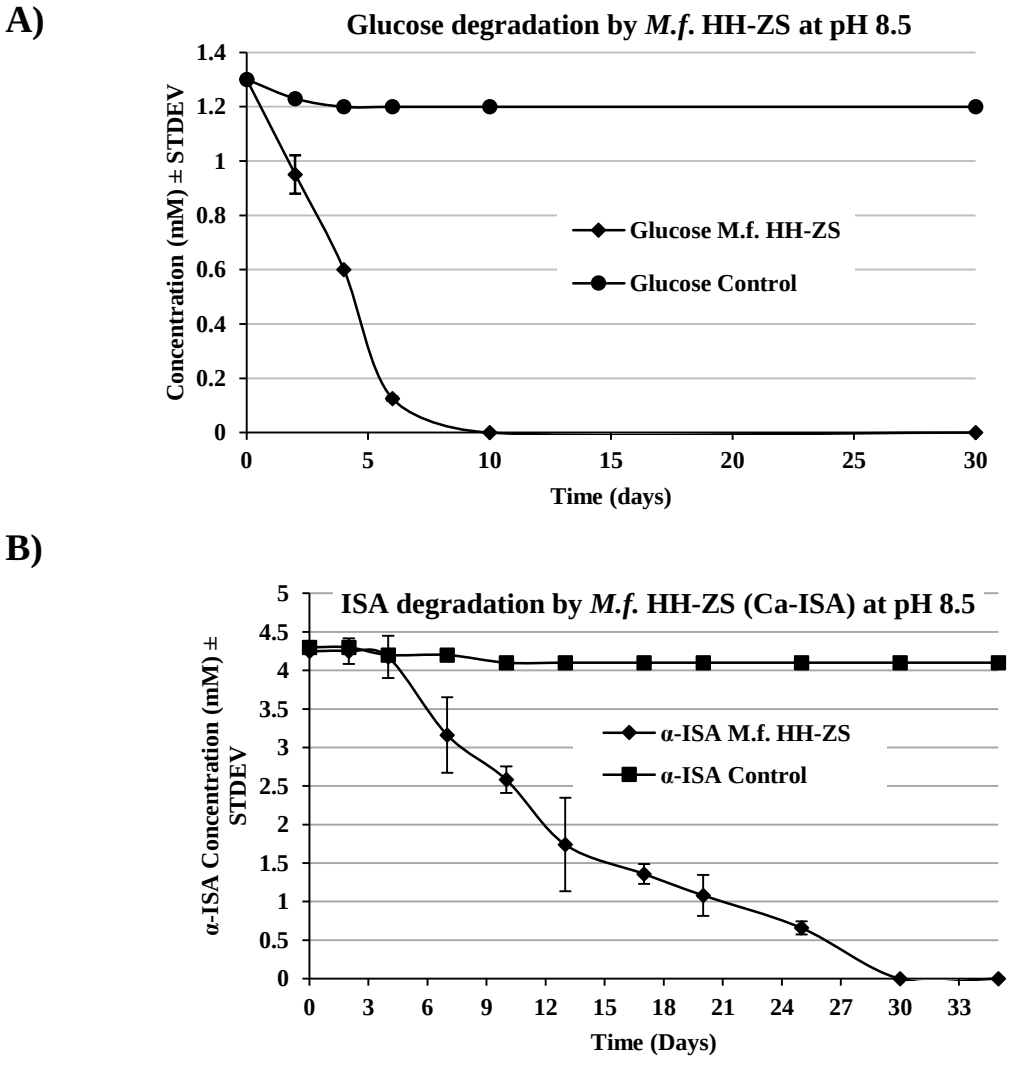


Figure 3. 44: *M. fermentans* HH-ZS strain able to degrade both glucose and α-ISA.

3.4.1. Discussion

A wide range of isolates were obtained from a range of alkaline microcosms fed on CDP. The analysis generated two strains, i.e. *A. salmonicida* (BI55) and *M. fermentans* HH-ZS (BI40) able to degrade α- ISA in the form of $Ca(ISA)_2$ and in the form of CDPs, both α- and β-ISA. The degradation of ISA by these two strains was detected in broth media at pH 8.0 and pH 9.0. The degradation of ISA by these two strains was carried out through a fermentation pathway that primarily generated acetic acid. In addition, the degradation rate of $Ca(ISA)_2$ by *M. fermentans* HH-ZS strain (BI40) was faster than that demonstrated by *A. salmonicida* (BI55). The stoichiometry of ISA degradation to acetate production was closer to 1:1 for *M. fermentans* and 1:2 for *A. salmonicida.*

Macellibacteroides fermentans of the Bacteroidetes phylum was detected within the microbial communities of the CDP fed microcosms operated between pH 9.0 and pH 11.0 (section 2) indicating that it was a significant component of these communities. In these microcosms the Bacteroidetes phylum was found to be the second most dominant phylum in the microcosms at pH 9.0 (24.8%) and pH 10.0 (23.22%) and at low concentration (3.14%) in the microcosm at pH 11.0. Work by Edward *et al*. (2010) found that the cellulose-degrading microbial communities in the marine environment was also dominated by members of the *Bacteroidetes* (Edwards et al., 2010). Although, *M. fermentans* was a minor member of the microbial community (4%, 2.8% and 0.03% at pH 9.0, pH 10.0 and pH 11.0 respectively) (Table 3.4), previous authors have outlined the fact that the culture dependent methods do not always represent the dominant species of the inoculum sources (Hugenholtz, 2002). The general characterisation of the members of this family are Gram negative rods, heterotrophic, non-motile, strictly anaerobic, non-spore-forming, that ferment a broad spectrum of sugars (Class & KRieg, 2011). The species *M. fermentans* was identified for the first time by Jabari *et al*. (2012) through their work that carried on the anaerobic treatment of abattoir wastewaters in Tunisia (Jabari et al., 2012). *M. fermentans* HH-ZS (BI40) is the first strictly anaerobic bacterium able to degrade ISA, more details are provided in section (3.6.5.).

3.4.1.1. Key findings

- The sediments from the microcosms at pH 10.0, pH 10.5 and pH 11.0 contained microbial communities able to degrade $Ca(ISA)_2$ in MM at pH 9.0.
- The microbial community present in the pH 10.0 microcosm contains a bacterial community able to degrade both $Ca(ISA)_2$ and Na-ISA.

- Complete degradation of both forms of ISA was observed, with Na-ISA being more rapidly utilised.
- *Alcaligenes* sp., *Citrobacter* sp., and *M. fermentans* strains were the most dominant culturable isolates isolated on FAA at pH 9.5.
- Extended incubation (>12 months) of sediments under alkaline conditions can lead to the selection of communities dominated by a small number of species.
- A microbial community dominated by the Gram negative bacillus, *Azonexus hydrophilus* was established allowing this organism to be isolated in pure culture.
- *M. fermentans* (BI40) and *A. salmonicida* (BI55) were both able to degrade ISA.

3.5. Degradation of CDPs using the TEAs Nitrate, Iron (III) and Sulphate

Previous experiments showed that the bacterial community present in the alkaline soils from the Harpur Hill site was capable of utilizing the ISA present in CDP via fermentative and methanogenic pathways. The objectives of this experiment was to determine the ability of this community to metabolise CDP via a wider range of both TEA (nitrate sulphate, and/or Fe (III)) and environmental pH values (pH 7.0, pH 8.0, pH 9.0, and pH 10.0) (2.5.9.). There were no investigations carried out under aerobic conditions since CDP are only generated in the absence of oxygen and the aerobic phase of a GDF is expected to have a limited duration.

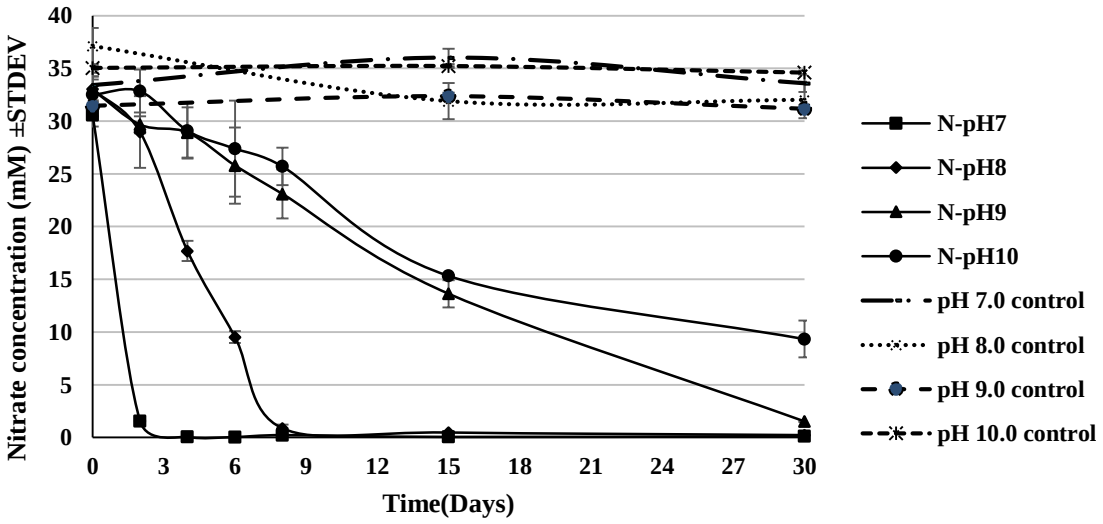
3.5.1. Chemical analysis of the CDPs driven microcosms under TEAs

As might be expected the rate of nitrate removal decreased as the pH increased (Figure 3.45 a) with the rate reducing exponentially as the pH increased (r^2 =>0.9, Figure 3.45 b). The metabolism appeared to be primarily respiratory as indicated by the stable pH (Figure 3.46) seen in all the experiments, this is supported by the lack of significant volatile acid generation (Figure 3.47). The only pH where there was any suggestion of fermentation is at pH 10.0 where there was a slight increase in acetic acid towards the end of the incubation period. At pH 7.0, 8.0 and 9.0 the small amounts of acetic acid present in the CDP was removed within the first 12 days irrespective of the pH. At pH 10.0 the acetic acid concentration was relatively stable with a slight increase toward the end of the experiment after a decrease midway through the incubation period. Whilst acetic acid removal was consistent between pH 7.0 and 9.0 the removal of β-ISA was significantly greater at pH 7.0 where complete removal was achieved after 8 days (Figure 3.47). At pH 8.0 β-ISA removal ceased after 12 days, 3 days after complete nitrate removal. This gap between the end of nitrate removal and the end of β-ISA removal was seen at both pH 7.0 and 8.0 and may reflect the fact that denitrification is a multistep process

(Oehmen, Lopez-Vazquez, Carvalho, Reis, & Van Loosdrecht, 2010; R. L. Smith, Böhlke, Garabedian, Revesz, & Yoshinari, 2004) and the monitoring of nitrate only measures the first stage. At pH 9.0 and 10.0 the β-ISA removal profiles are generally the same. The biodegradation of α-ISA was more limited than the degradation of β-ISA with the degradation at all pH values being generally similar (Figure 3.48 and 3.49). Overall nitrate reduction systems appeared to preferentially removal acetic acid followed by β-ISA and finally α-ISA with both β-ISA and α-ISA persisting in the presence of nitrate throughout the 30 day experimental period.

There was no significant evidence of fermentation even though the fermentation of both forms of ISA has been extensively documented in the absence of electron acceptors (Charles et al., 2015; Rout, Charles, Doulgeris, et al., 2015; Rout et al., 2014) where strictly anaerobes of the Clostridia phylum appeared to drive the fermentation of the both forms of ISA at high pH. It may be that the oxidising environment generated by the presence of nitrate inhibited the anaerobic bacteria required for the fermentation of ISA. This in turn suggests that the fermentation of ISA in these systems is driven by strictly anaerobic organisms rather than facultative anaerobes. The degradation of α-ISA through nitrate reduction at pH 10.0 was performed by Bassil *et al*. (2014), they found that the complete degradation of α-ISA followed the production of acetate via fermentation. Acetate generation was associated with a reduction of both ISA and nitrate. The bacterial community in these systems was dominated by facultative anaerobic Proteobacteria (65%) followed by strictly anaerobic *Firmicutes* (24%) and Bacteroidetes (21%) (Bassil et al., 2015).

A)



B)

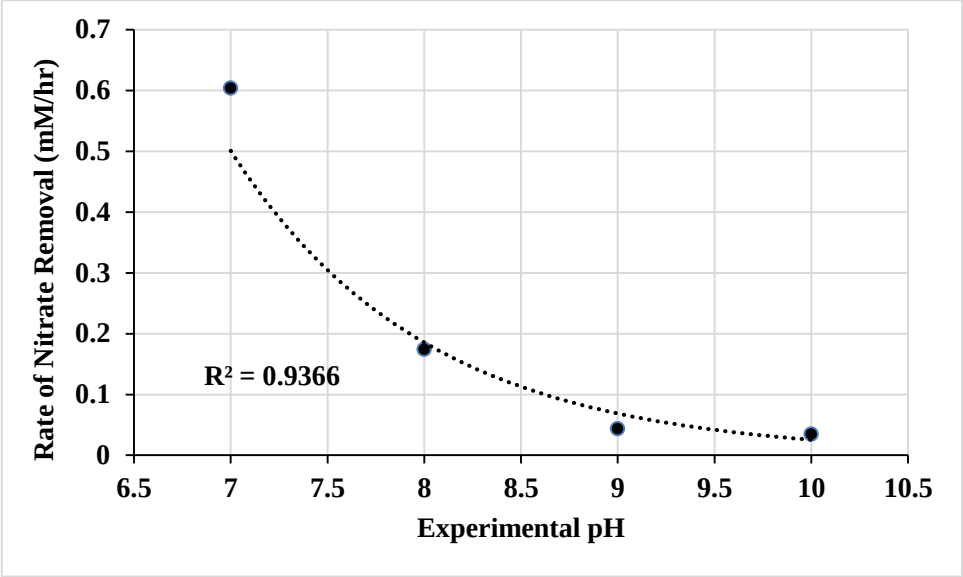


Figure 3. 45: Nitrate Removal.

Nitrate reduction, complete removal of nitrate at pH 7.0 during < 3 days and at pH 8.0 during < 9 days, whilst at pH 9.0 it takes about 30 days and significant reduction at pH 10.0 during the same period of time (A). The rate of nitrate removal reduced as the pH increased in an exponential manner (B).

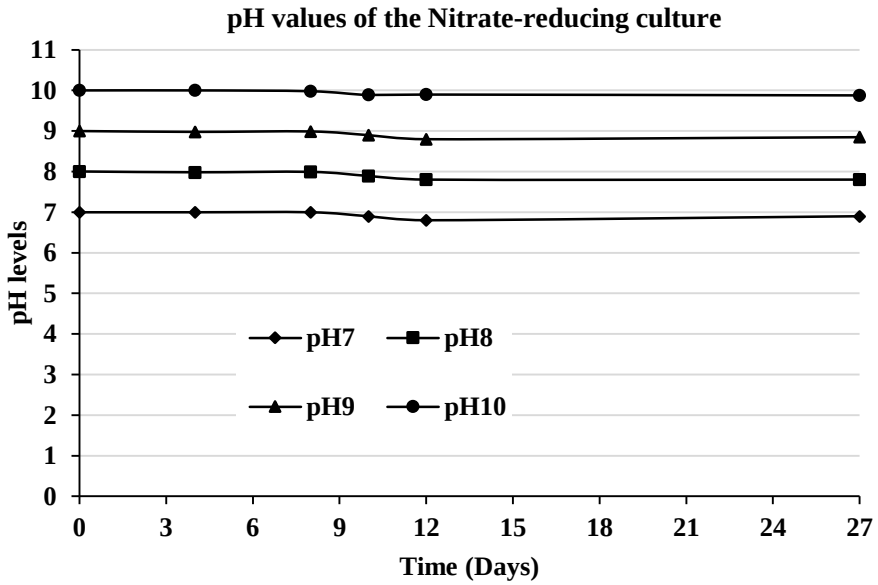


Figure 3. 46: pH profiles in the nitrate reduction experiments.

There was no evidence of pH decrease in the nitrate reduction experiments suggesting a primarily oxidative microbiology. If significant amounts of fermentation was occurring then the pH would be expected to decrease as volatile acids were generated.

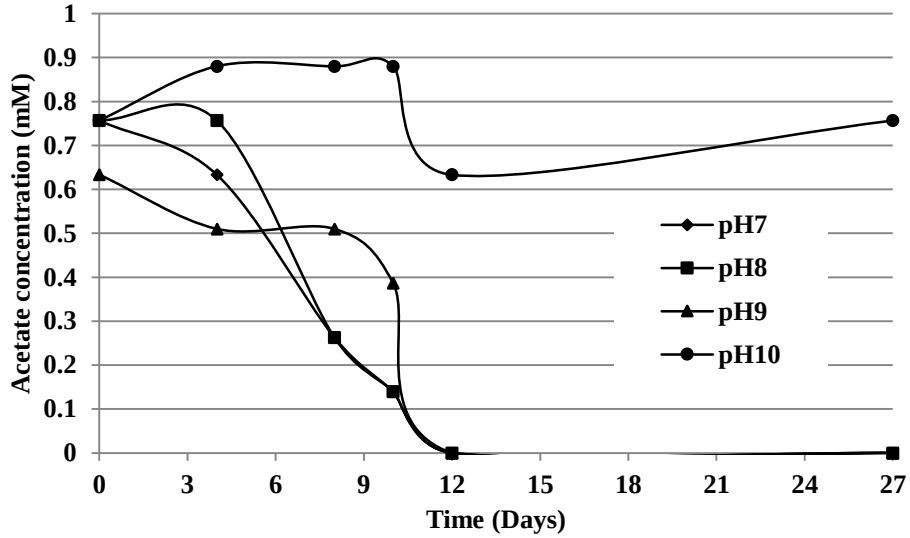


Figure 3. 47: Acetic acid profiles of the nitrate reduction experiments.

The only suggestion of fermentation occurs at pH 10.0 where the acetic acid concentration increases towards the end of the incubation period.

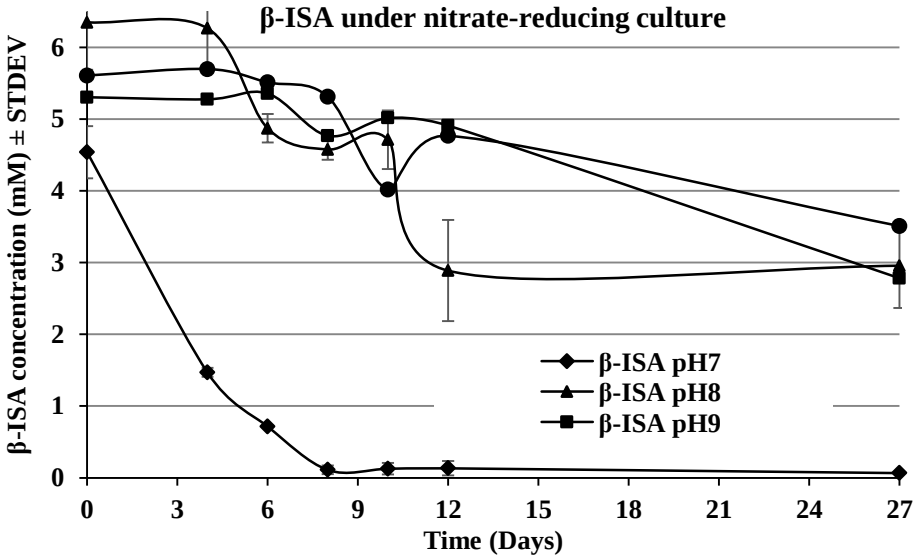


Figure 3. 48: Removal of β-ISA under nitrate reducing conditions.

The degradation of β-ISA was significantly greater at pH 7.0 than the other pH values investigated.

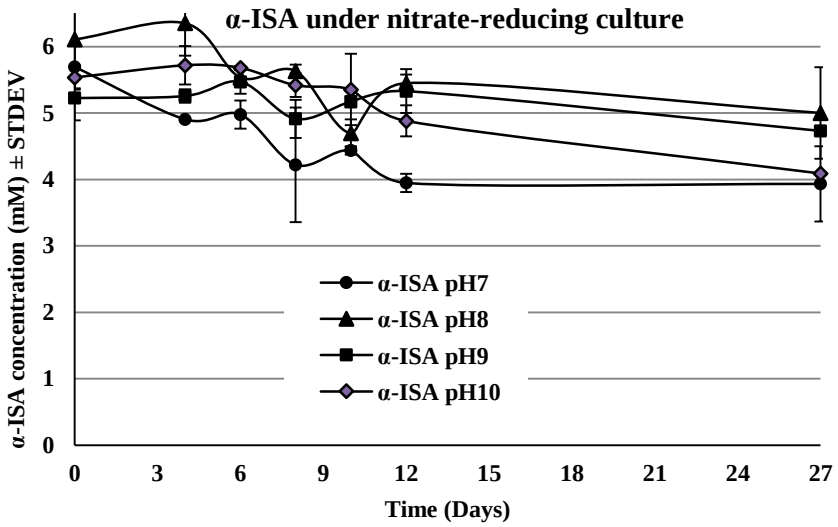


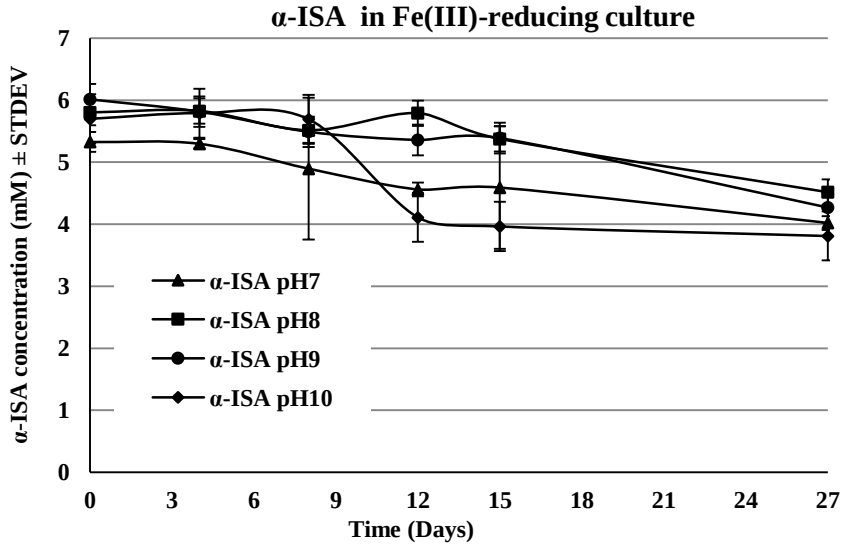
Figure 3. 49: Removal of α-ISA under nitrate reducing conditions.

The degradation of α-ISA was limited across all pH values investigated.

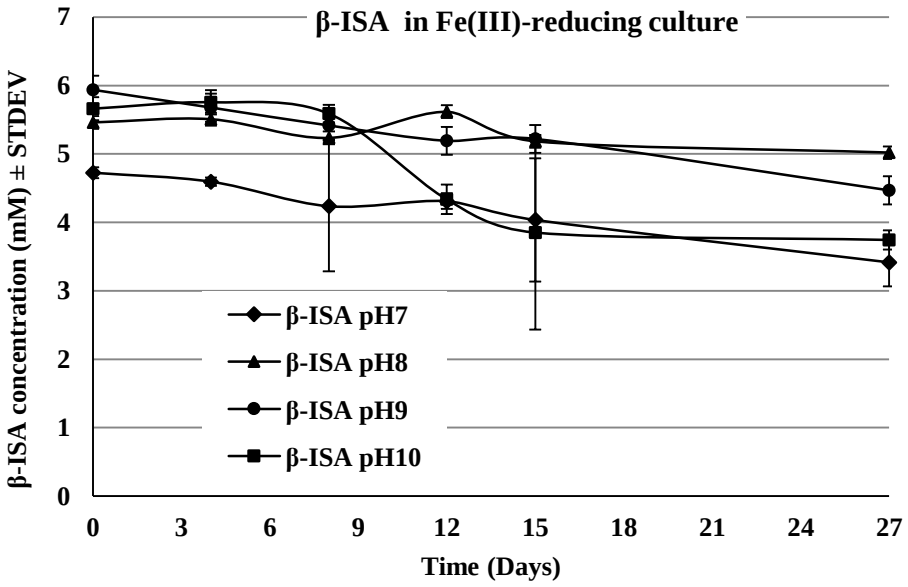
Under iron reducing conditions the degradation of both forms of ISA was limited with 1-2 mM being removed over the 30 day incubation period (Figure 3.50 a and b). As expected, the pH levels of the microcosms were slightly decreased (Figure 3.50 c). The degradation was accompanied by an increase is ferrous iron concentration which is indicative of iron reduction. After an initial increase, the ferrous iron concentration either levelled off or continued to increase slowly (Figure 3.50 d). This profile suggests that later in the incubation period the concentration of ferrous iron was solubility controlled most likely due to the presence of siderite an iron (II) carbonate or magnetite a mixed Fe(II)/Fe(III) oxide.

Similar results were obtained by Bassil *et al*. (2014) who found that only about 36% of the total α-ISA was degraded under Iron (III)-reducing culture at pH 10.0 after 90 days of incubation. This ISA removal was associated with a ≈21% reduction of Iron (III) and a drop in pH to pH 9.5 (Bassil et al., 2015). Rout *et al* (2014), found that the microbial community in anoxic sediments was able to degrade both forms of ISA by utilising a range of terminal electron acceptors at neutral pH. In this study similar amounts ISA were degraded under iron reducing, sulphate reducing and methanogenic conditions (Rout et al., 2014).

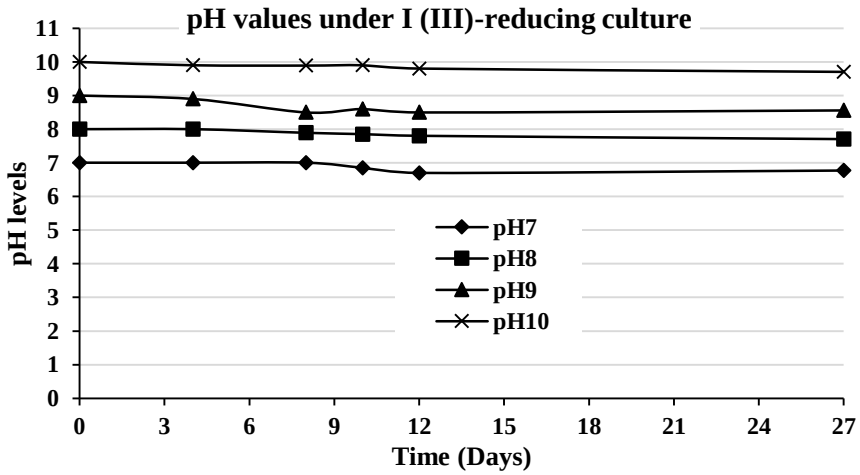
a)


α-ISA in Fe(III)-reducing culture
α-ISA concentration (mM) ± STDEV
Time (Days)
α-ISA pH7
α-ISA pH8
α-ISA pH9
α-ISA pH10

b)


β-ISA in Fe(III)-reducing culture
β-ISA concentration (mM) ± STDEV
Time (Days)
β-ISA pH7
β-ISA pH8
β-ISA pH9
β-ISA pH10

c)


pH values under I (III)-reducing culture
pH levels
Time (Days)
pH7
pH8
pH9
pH10

d)

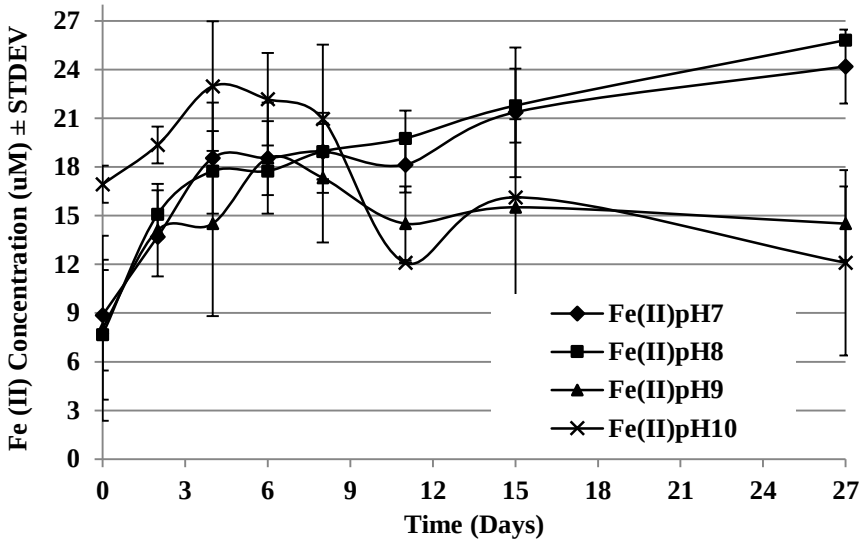
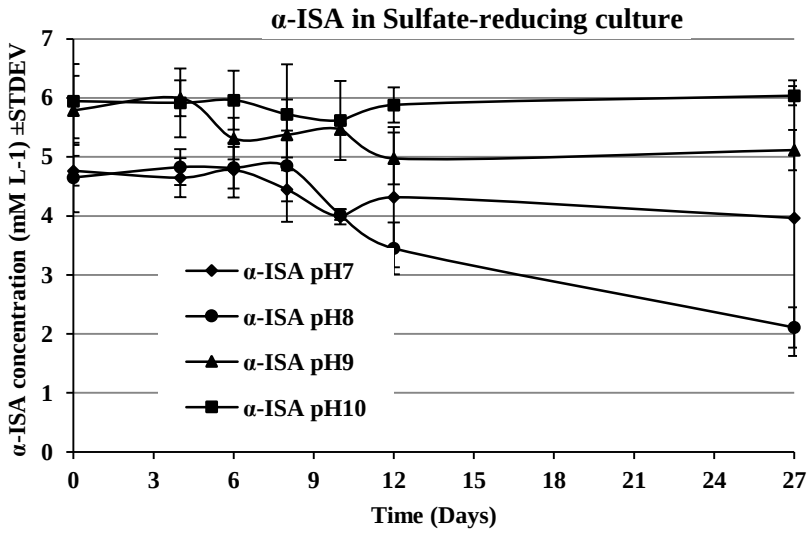


Figure 3. 50: ISA degradation under Iron (III) reducing conditions.

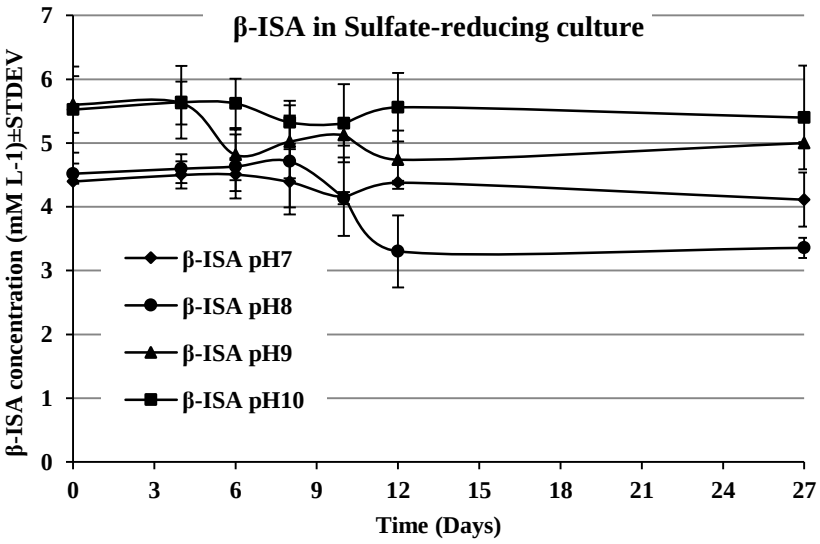
The degradation of both forms of ISA was limited with 1-2 mM being removed over the 30 day incubation period (a and b). The pH levels of the microcosms were slightly decreased (c). The ISA degradation was accompanied by an initial increase of ferrous iron indicating active iron reduction, the ferrous iron concentration either levelled off or continued to increase slowly (d).

Unlike the nitrate and iron reducing systems the sulphate reducing showed clear evidence of fermentation as indicated by the accumulation of a range of VFA (Figure 3.51 d to f) at all pH values with the exception of pH 10.0. There was no evidence of microbial activity under sulphate reducing conditions at pH 10.0, a trend consistent across the sulphate and ISA removal data (Figure 3.51 a, b and c). The sulphate removal curves at pH 7.0 and 8.0 were broadly similar, whilst at pH 9.0, sulphate removal began after 6 days once the ambient pH had been reduced due to the VFA generation (Figure 3.51 c). Although the sulphate curve at pH 9.0 demonstrated a lag until day 6, the VFA data indicated an immediate accumulation. This demonstrated a fermentation driven system with the sulphate reducing bacteria metabolising the VFA's rather than the ISA. The degradation of both forms of ISA was modest between pH 7.0 to 9.0 and in the case of the α-ISA the degradation at pH 8.0 was more extensive than at pH 7.0 which may reflect the alkaline nature of the environment sampled and the presence of alkaliphilic organisms. This data is similar to that of Rout *et al*. (2014) who demonstrated a complete removal of both forms of ISA (α- and β-) in the sulphate-reducing microcosm at neutral pH (Rout et al., 2014). The results of the microcosm at pH 10.0 (Figure 3.51 h) are consistent with previous results, obtained using similar sediments incubated under sulphate reducing conditions with $Ca(ISA)_2$ (Bassil et al., 2015). In addition, other experiments operated under sulphate reducing conditions with acetate and lactate as electron donors failed to demonstrate sulphate reduction even after 140 days (Rizoulis et al., 2012). These observations underpin the observation that sulphate reduction is limited at alkaline pH value >pH 9.0.

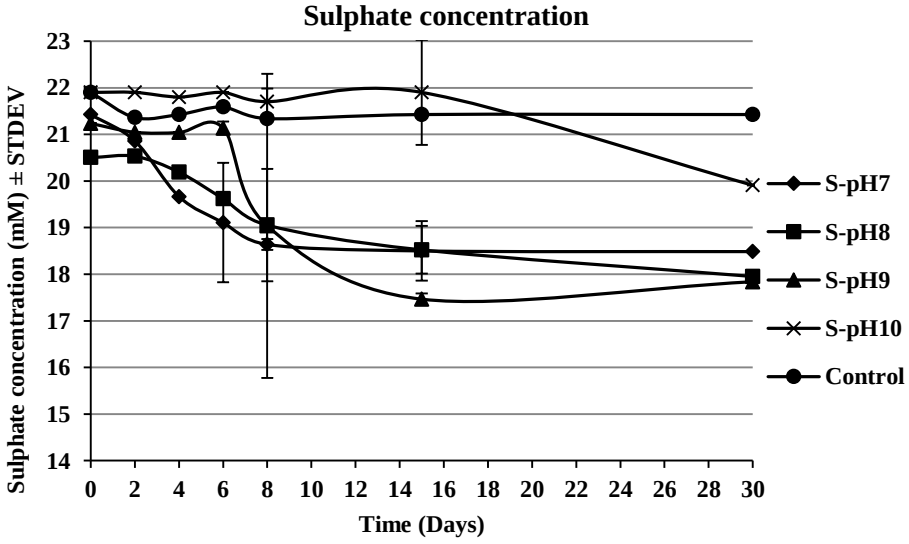
a)


α-ISA in Sulfate-reducing culture
α-ISA concentration (mM L-1) ±STDEV
Time (Days)
α-ISA pH7
α-ISA pH8
α-ISA pH9
α-ISA pH10

b)


β-ISA in Sulfate-reducing culture
β-ISA concentration (mM L-1)±STDEV
Time (Days)
β-ISA pH7
β-ISA pH8
β-ISA pH9
β-ISA pH10

c)


Sulphate concentration
Sulphate concentration (mM) ± STDEV
Time (Days)
S-pH7
S-pH8
S-pH9
S-pH10
Control

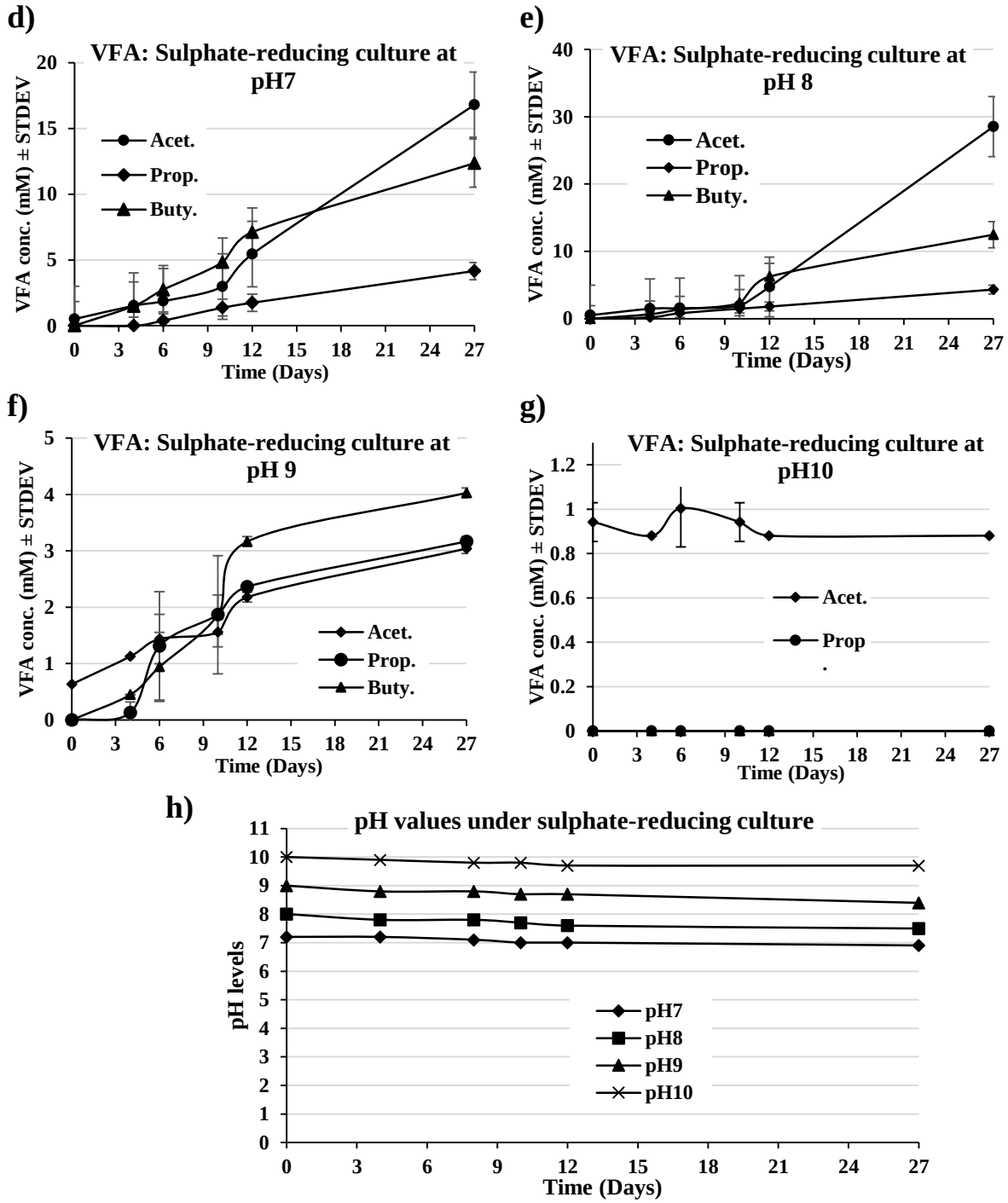


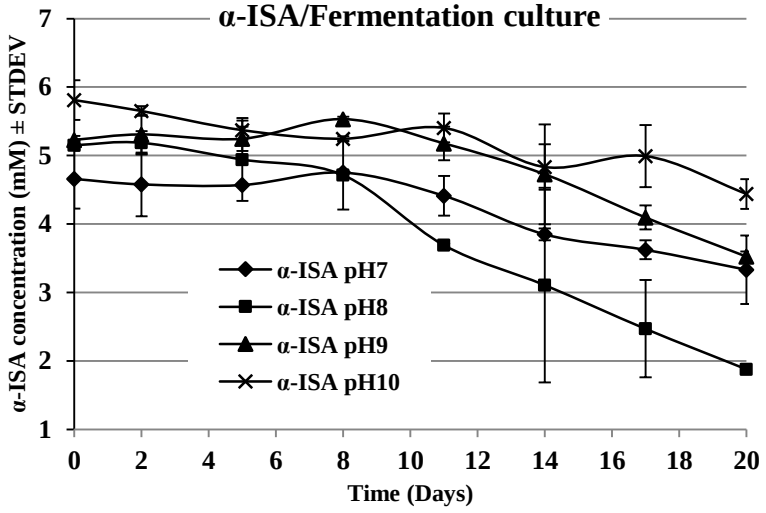
Figure 3. 51: ISA degradation under sulphate reducing conditions.

Clear evidence of fermentation in the sulphate reducing culture was indicated by the accumulation of a range of VFA (d to f) at all pH values with the exception of pH 10.0. There was also a reduction of pH (h). The sulphate removal curves at pH 7.0 and 8.0 were broadly similar, whilst at pH 9.0 sulphate removal began after 6 days once the ambient pH had been reduced due to the VFA generation (c).

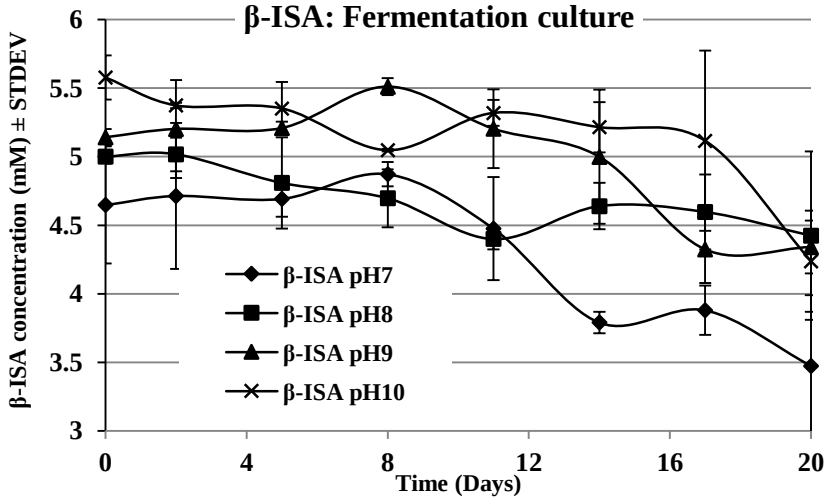
In the absence of terminal electron acceptors, a purely fermentative system was established. These microcosms demonstrated greater ISA degradation than observed under iron and sulphate reducing conditions with the removal of both forms of ISA up to pH 10.0 (Figure 3.52 a and b). The ISA degradation rate was pH dependent with a general trend of decreased ISA removal as the pH increased. However, as with the sulphate reducing systems the degradation of α-ISA

was greater at pH 8.0 than at pH 7.0. VFAs accumulated at all pH values with acetic and butyric acids being the most abundant (Figure 3.52 e to g), this VFA generation was accompanied with a slight decrease in pH. Degradation of both forms of ISA via fermentation processes at different pH values has been confirmed by previous studies. Rout *et al*. (2014) found that a complete degradation of both forms of ISA during 3 days of an incubation time at neutral pH accompanied by the generation of VFAs and methane (Rout et al., 2014). The same authors was demonstrated that the bacteria in Buxton sediments were capable of fermenting both forms of ISA at pH 11.0 with the production of acetic acid, H_2 and CH_4 (Rout, Charles, et al., 2015a).

a)



b)



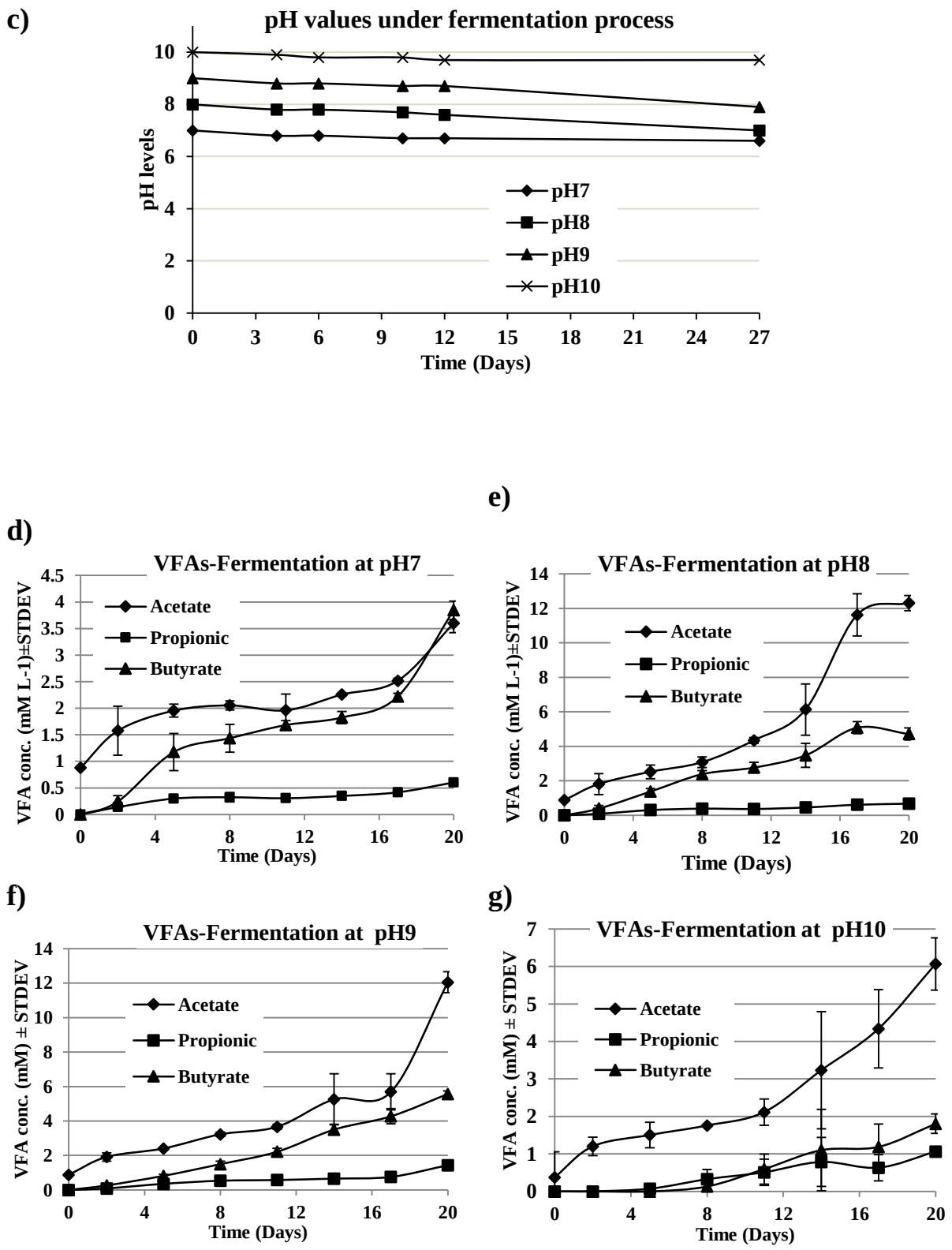


Figure 3. 52: Biodegradation of ISA by a purely fermentative system.

The ISA degradation rate was pH dependent with a general trend of decreased ISA removal and the pH increased (a and b), VFAs accumulated at all pH values with acetic and butyric acids being the most abundant (e to g), this VFA generation was accompanied with a slight decrease in pH (c).

3.5.2. Bacterial community analysis of the CDPs driven microcosms under Nitrate, Sulphate and Iron (III)-reducing cultures compared with fermentation process at different pH levels

The number of OTUs (1035 OTUs) in the background alkaline sediment was represented by approximately 30 phyla dominated by Gram-negative bacteria (Proteobacteria 48% and Bacteroidetes 8.7%) and Gram-positive bacteria (Firmicutes 15% and Actinobacteria 13.3%) (Table 3.5). This profile is consistent with previous data presented by Bassil *et al.* (2015) where the background sediment was dominated by Gram-negative bacteria (Proteobacteria 44%, Bacteroidetes 24%) followed by low percentages of Gram-positive bacteria (Firmicutes 6%) (Bassil et al., 2015). The microbial communities in the microcosms demonstrated a reduction in diversity as indicated by the number of OTUs when compared to the original soil. Given the diversity of terminal electron acceptors and pH values investigated, the only common factor is the use of CDP, it is therefore likely that this reduction in OTU is due to the use of CDP as the common energy source. The diversity was lower under the more oxidising nitrate and iron reducing systems than when active fermentation was occurring in the presence of sulphate or the absence of a terminal electron acceptor (Figure 3.53). The communities are described below on a system by system basis.

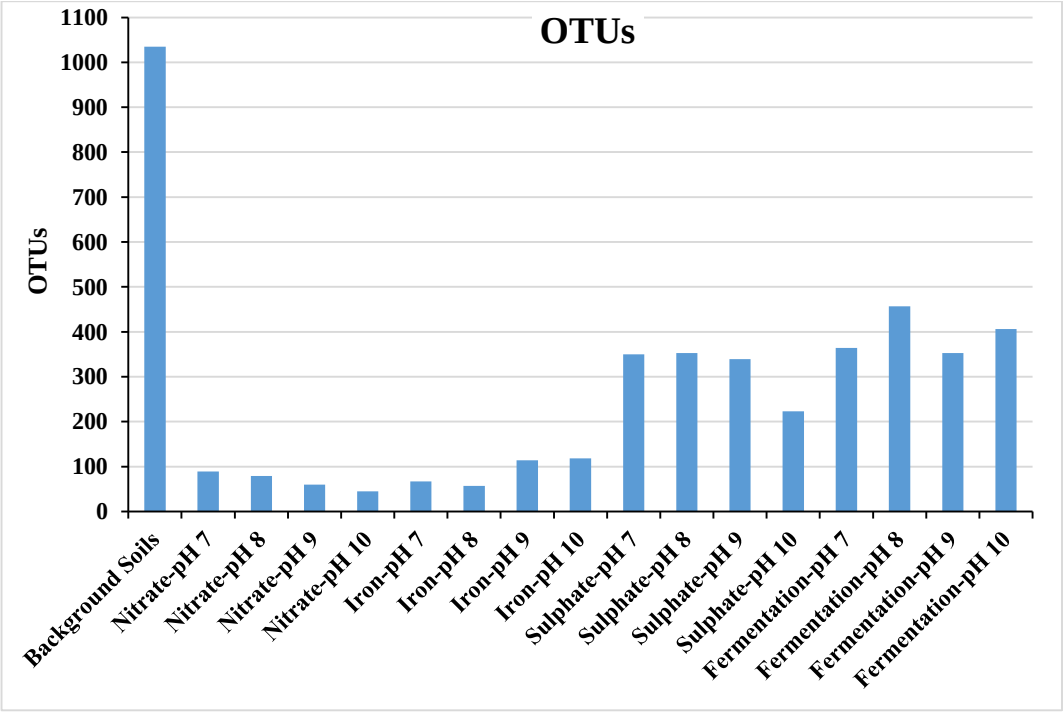
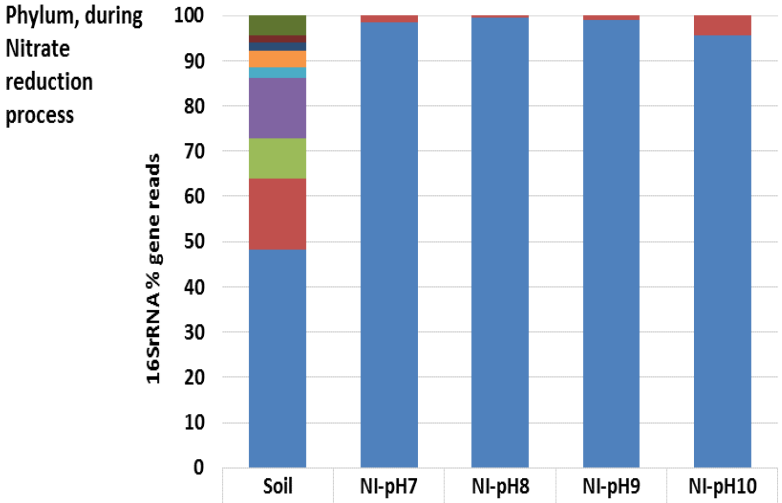


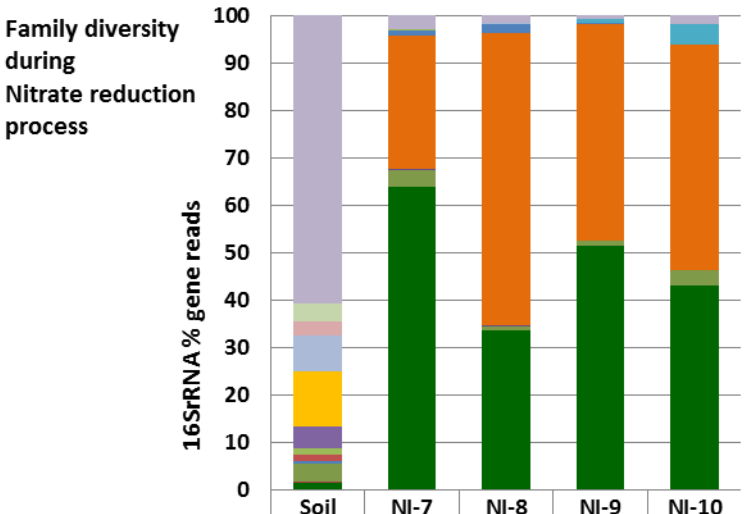
Figure 3. 53: Alpha diversity indices of the microbial diversity calculated via ChunLab's CLcommunity analysis pipeline.

The establishment of nitrate reducing conditions promoted the expansion of bacteria from the phylum Proteobacteria from the 48% seen in the soil to around 99% at all pH levels (Figure 3.54 A). At the phylum level, the presence of nitrate had a greater impact than the increase in pH from pH 7.0 to 10.0. Although as the pH increased the proportion of Firmicutes in the population increased. At the family level the Proteobacteria were represented by the Brucellaceae (approximately 50%) followed by Alcaligenaceae (approximately 45%) (Figure 3.54 B). These families were in turn dominated by the following species: *Brucella melitensis* 61.7% at pH7, 50.1% at pH 9.0 and 41.5% at pH 10.0; and *Achromobacter* sp. dominated by *Achromobacter marplatensis* 36.3% at pH 8.0, 32.5% at both pH 9.0 and pH 10.0 (Figure 3.54 (C)). Other Proteobacteria present in the systems were represented by the Pseudomonadales (<5%) represented by *Pseudomonas tuomuerensis*. The small proportion (<0.8%) of Firmicutes present were represented by the following families Ruminococaceae, Sedimentibacter, Clostridiaceae, Lachnospiraceae and Bacillaceae, except the last family that represented 4.2% in microcosm at pH 10 (Table 3.5) and was dominated by *Anaerobacillus macyae* (3.6%).



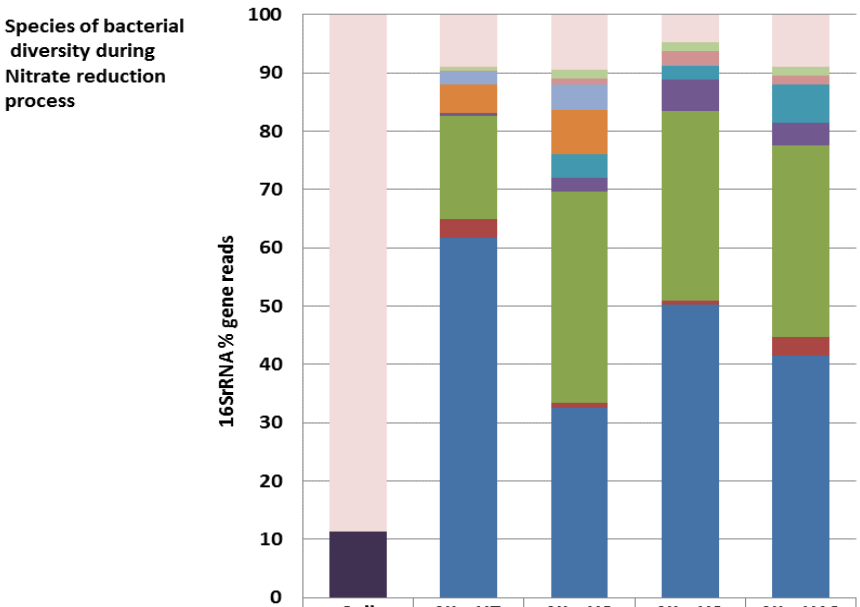
	Soil	NI-pH7	NI-pH8	NI-pH9	NI-pH10
ETC < 1.0%	4.29	0.00	0.00	0.01	0.01
Gemmatimonadetes	1.59	0.00	0.00	0.00	0.00
Chloroflexi	1.98	0.00	0.00	0.01	0.00
Acidobacteria	3.54	0.00	0.00	0.00	0.00
Planctomycetes	2.52	0.00	0.00	0.00	0.00
Actinobacteria	13.36	0.06	0.00	0.03	0.00
Bacteroidetes	8.72	0.02	0.03	0.02	0.02
Firmicutes	15.80	1.57	0.51	0.93	4.49
Proteobacteria	48.19	98.35	99.46	99.01	95.48

A) The Proteobacteria dominated bacterial diversity at the phylum level it increased from 48.1% in crude soil to high percentages 98.3, 99.4, 99.0 and 95.4% in all microcosms at all pHs (7.0, 8.0, 9.0 and 10.0 respectively).



	Soil	NI-7	NI-8	NI-9	NI-10
ETC < 1.0%	60.81	2.80	1.55	0.81	1.82
Acidaminobacter_f	3.69	0.00	0.00	0.00	0.00
Rhodocyclaceae	3.01	0.00	0.00	0.00	0.00
Comamonadaceae	7.51	0.11	0.11	0.00	0.00
Propionibacteriaceae	11.59	0.00	0.00	0.00	0.00
Bacillaceae	0.14	0.04	0.03	0.78	4.19
Erysipelotrichaceae	4.54	0.02	0.01	0.01	0.00
Anaerovirgula_f	1.38	0.27	0.00	0.02	0.01
Paludibacter_f	1.13	0.01	0.00	0.01	0.01
Xanthomonadaceae	0.57	0.86	1.92	0.18	0.01
Porphyromonadaceae	0.00	0.01	0.02	0.00	0.01
Alcaligenaceae	0.00	28.30	61.69	45.73	47.68
Sedimentibacter_f	0.00	0.03	0.10	0.00	0.02
Pseudomonadaceae	3.79	3.61	1.04	0.85	3.21
Ruminococcaceae	0.32	0.02	0.01	0.01	0.04
Brucellaceae	1.52	63.91	33.52	51.60	43.01

B) The dominant families in the all nitrate-reducing microcosms were; Brucellaceae followed by the Alcalligenaceae. The Brucellaceae family was increased from being undetectable in the crude soil sample to 63.9, 33.55, 51.6 and 43.0% in the microcosms at pHs (7.0, 8.0, 9.0 and 10.0 respectively), followed by Alcaligenaceae family 28, 61.6, 45.7 and 47.68% in microcosms at pHs (7.0, 8.0, 9.0 and 10.0 respectively).



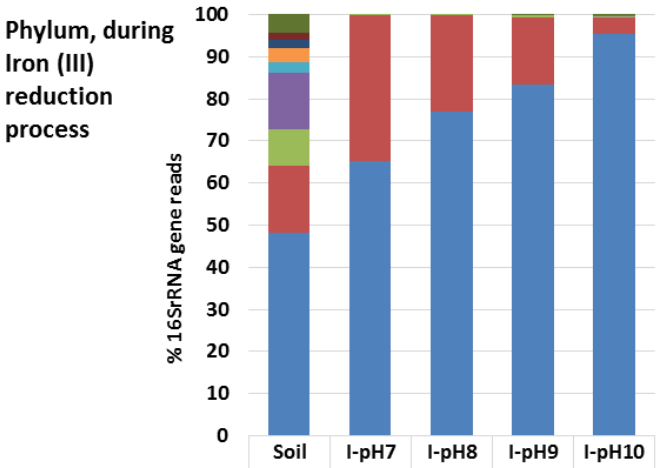
	Soil	NI-pH7	NI-pH8	NI-pH9	NI-pH10
ETC < 1.)%	88.77	8.90	9.45	4.80	8.94
Propionibacterium acnes	11.23	0.00	0.00	0.00	0.00
Achromobacter_uc	0.00	0.74	1.48	1.46	1.48
AKMR_s	0.00	0.20	1.06	2.40	1.64
Bordetella trematum	0.00	2.21	4.40	0.11	0.00
Eiseniicola composti	0.00	4.78	7.59	0.08	0.01
Achromobacter piechaudii	0.00	0.06	4.02	2.36	6.45
Alcaligenes aquatilis	0.00	0.57	2.32	5.42	3.95
Achromobacter marplatensis	0.00	17.71	36.32	32.45	32.91
Pseudomonas tuomuerensis	0.00	3.07	0.82	0.74	3.09
Brucella melitensis	0.00	61.77	32.53	50.18	41.53

C) The dominant species in the nitrate-reducing cultures were *Brucella melitensis* 61.7% at pH7, it was 50.1% at pH 9.0, 41.5% at pH 10; *Achromobacter marplatensis* 36.3% at pH 8.0, about 32.5% at pH 9 and pH 10.

Figure 3. 54: Bacterial diversity of the microcosm under a Nitrate reduction

As was the case in the nitrate reducing systems, the iron reducing systems were dominated at Phylum level by the Proteobacteria at all pH levels, which grew to 95% at the highest pH level (Figure 3.55 (A)). The only other phyla represented in any significant manner were the Firmicutes which increased steadily as the pH increased. However, at the family level whilst the Brucellaceae were still highly represented, it was the Pseudomonadaceae rather than the Alcaligenaceae that made up the rest of this family. Unlike the nitrate reducing systems the *Sedimentibacter* were present at the lower pH values in proportions >30% which reduced to <1% at pH10.0. The proportion of the Sedimentibacter and Pseudomonadaceae decreased as the pH increased, whilst the Brucellaceae increased up to >40% at pH 9.0 and pH 10.0. There was also an increase in the proportion of Aeromonadaceae in the mid pH ranges (19% at pH 9.0) but was unable to maintain this presence at the highest pH (Figure 3.55 (B)).

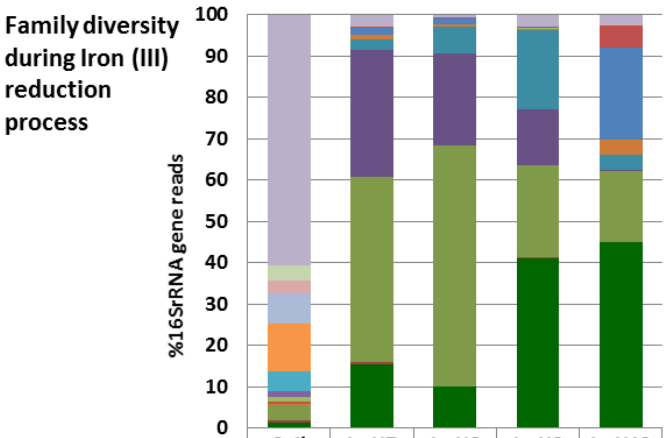
The dominant species under iron reducing conditions were similar to those seen under nitrate reducing conditions with *Pseudomonas tuomuerensis* having a greater presence at the lower pH values (42.4% at pH 7.0, 55.9% at pH 8.0, 20.8% at pH 9.0 and 11% at pH 10.0). The second species in common with the nitrate microcosms was the *Brucella melitensis* group which was more prevalent at the higher pH values (43.3% at pH 10.0, 40% at pH 9.0, 9.89% at pH 8.0 and 14.9% at pH 7.0). Two other species with a significant presence were the unclassified EF059533 which decreased as the pH increased and *Rhanella bruchi* which became more dominant at pH 10.0 (≈20%) (Figure 3.55 (C)). These results are inconsistent with previous data provided by Basil *et al*, where α-ISA was used as an analogue to CDP and the pH values of the microcosms were reduced by fermentation and an accumulation of VFA. In that study the population showed an increase of the Firmicutes phylum from 6% in the background sediment to about 24% in the nitrate-reducing culture that was dominated by Gram-negative bacteria of the Proteobacteria phylum, whilst the Firmicutes was dominated of the Fe-(III)-reducing culture represented almost 100% of the bacteria community (Bassil et al., 2015).



	Soil	I-pH7	I-pH8	I-pH9	I-pH10
ETC < 1.0%	4.29	0.00	0.00	0.10	0.23
Gemmatimonadetes	1.59	0.00	0.00	0.00	0.00
Chloroflexi	1.98	0.00	0.01	0.05	0.00
Acidobacteria	3.54	0.00	0.00	0.00	0.01
Planctomycetes	2.52	0.00	0.00	0.02	0.05
Actinobacteria	13.36	0.01	0.01	0.07	0.05
Bacteroidetes	8.72	0.05	0.01	0.35	0.34
Firmicutes	15.80	34.74	23.11	15.95	4.08
Proteobacteria	48.19	65.20	76.86	83.46	95.24

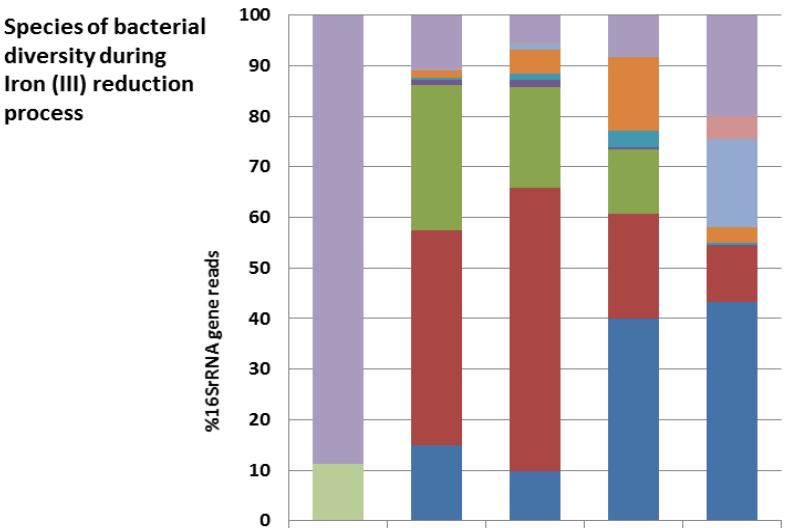
A): Bacterial community analysis on phyla level under Iron-(III) reducing culture

The dominant phylum was Proteobacteria in all microcosms 65.2, 76.86, 83.46 and 95.2% at pHs (7.0, 8.0, 9.0 and 10.0 respectively) followed by Firmicutes 34.7, 23.1, 15.95 and 4.0% at pHs (7.0, 8.0, 9.0 and 10.0 respectively).



	Soil	I-pH7	I-pH8	I-pH9	I-pH10
ETC < 1.0%	60.56	2.63	0.78	2.87	2.45
Acidaminobacter_f	3.69	0.00	0.00	0.00	0.00
Rhodocyclaceae	3.01	0.32	0.00	0.00	0.03
Comamonadaceae	7.51	0.01	0.00	0.01	0.02
Propionibacteriaceae	11.59	0.00	0.00	0.00	0.00
Erysipelotrichaceae	4.54	0.00	0.00	0.03	0.09
Anaerovirgula_f	1.38	0.01	0.01	0.15	0.01
Paludibacter_f	1.13	0.00	0.00	0.27	0.07
Xanthomonadaceae	0.57	0.12	0.00	0.03	5.39
Enterobacteriaceae	0.07	1.74	1.59	0.10	22.30
Lachnospiraceae	0.28	1.21	0.56	0.16	3.40
Aeromonadaceae	0.04	2.45	6.51	19.25	3.91
Sedimentibacter_f	0.00	30.78	22.30	13.50	0.15
Pseudomonadaceae	3.79	44.73	58.01	22.31	17.20
Ruminococcaceae	0.32	0.61	0.05	0.11	0.09
Brucellaceae	1.52	15.40	10.20	41.22	44.92

B) Bacterial community analysis on the family level under Iron-(III) reducing conditions. The most common families were the Pseudomonadaceae, 44.7, 58, 22.3 and 17.2%, followed by Brucellaceae 15.5, 10.2, 41.2 and 44.9% and Sedimentibacter 30.7, 22.3, 13.5 and only 0.15% at pHs (7.0, 8.0, 9.0 and 10.0 respectively).

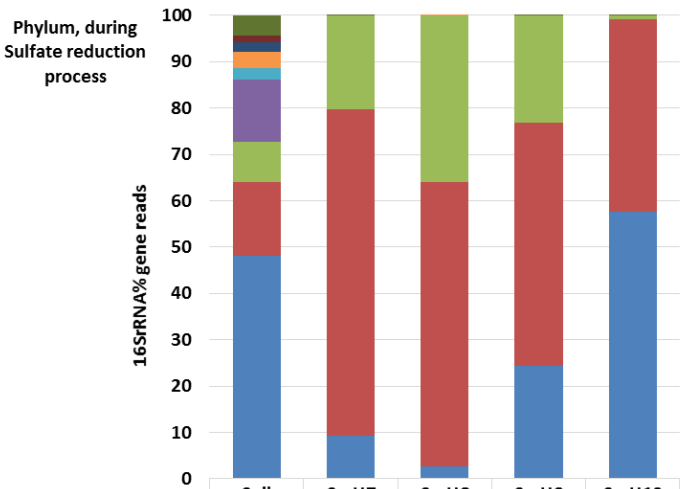


	Soil	I-pH7	I-pH8	I-pH9	I-pH10
ETC < 1.0%	88.77	10.54	5.47	8.07	20.05
Propionibacterium acnes	11.23	0.00	0.00	0.00	0.00
Pseudomonas putida group	0.00	0.10	0.04	0.06	4.45
Rahnella bruchi group	0.00	0.34	1.22	0.05	17.38
Aeromonas veronii group	0.00	1.41	4.76	14.69	3.03
Aeromonas hydrophila group	0.00	0.35	1.22	3.25	0.60
Sedimentibacter_uc	0.00	1.12	1.49	0.47	0.01
EF059533_s	0.00	28.75	19.95	12.57	0.11
Pseudomonas tuomuerensis group	0.00	42.43	55.98	20.83	11.03
Brucella melitensis group	0.00	14.96	9.89	40.00	43.34

C) The phylogenetic analysis on the species level under Iron (III)-reducing cultures. *Brucella melitensis* was dominated the community 40% and 43.3% in the microcosms at pH 9.0 and pH 10.0 respectively, it was presented by 14.9% and 9.89% in microcosms at pH 7.0 and pH 8.0 respectively, followed by *Pseudomonas* group that dominated the microcosms at pH 8.0 and pH 7.0 by 55.98% and 42.4% respectively it also found in the microcosms at 9.0 and pH 10.0 by 20.8% and 11.0% respectively

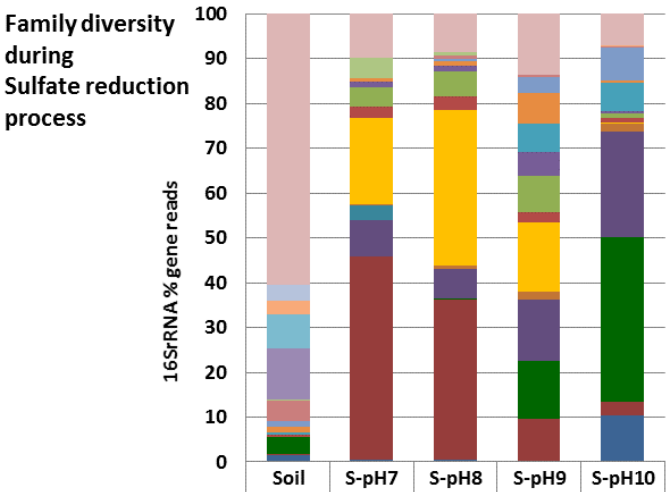
Figure 3. 55: Bacterial community analysis under Iron-(III) reducing culture

Under sulphate reducing conditions the most obvious change when compared to the more oxidising nitrate and iron reducing systems is the more prominent role take by the Firmicutes and the Bacteroidetes alongside the Proteobacteria. This is most obvious between pH 7 and pH 9, since at pH 10 the Bacteroidetes fail to maintain their position within the population (Figure 3.56 (A)). At the family level the Ruminococcaceae (45%, at pH 7.0 and 35% at pH 8.0) and the Prophyromonadaceae (19% at pH7.0 and 35% at pH 8.0) dominate at the lower pH values. Whilst >pH 8.0 the Pseudomonadaceae (36% at pH 10.0 and 13% at pH 9.0) and Sedimentibacter (23% at pH 10.0 and 14% at pH 9.0) take over (Figure 3.56 (B)). At the species level the populations were not dominated by small number of species as observed in the more oxidising microcosms. In these systems, a number of unclassified species had prominent roles, whilst *Pseudomonas turmuerensis* and *Brucella melitensis* were still present in significant proportions (Figure 3.57 (C)).



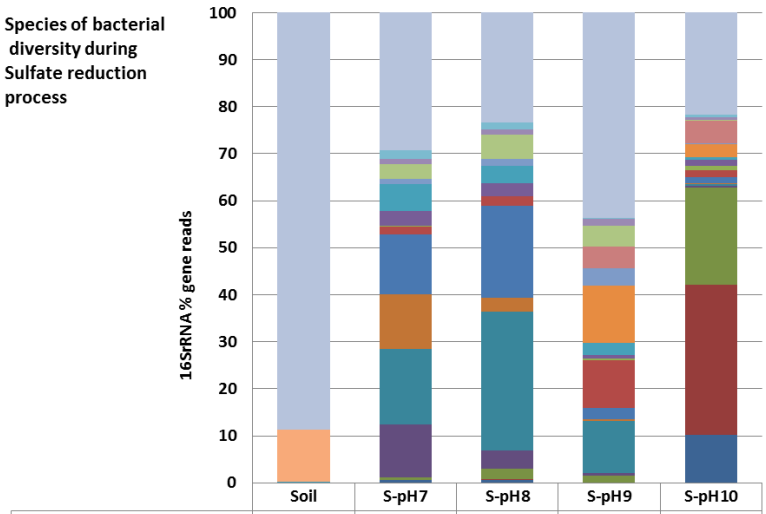
	Soil	S-pH7	S-pH8	S-pH9	S-pH10
ETC < 1.0%	4.29	0.01	0.00	0.03	0.03
Gemmatimonadetes	1.59	0.00	0.00	0.00	0.00
Chloroflexi	1.98	0.00	0.00	0.01	0.00
Acidobacteria	3.54	0.00	0.01	0.01	0.01
Planctomycetes	2.52	0.00	0.00	0.01	0.01
Actinobacteria	13.36	0.01	0.00	0.01	0.09
Bacteroidetes	8.72	20.23	35.91	23.12	0.81
Firmicutes	15.80	70.37	61.35	52.52	41.44
Proteobacteria	48.19	9.37	2.73	24.30	57.63

A) Bacterial phyla under a sulphate-reducing culture were dominated by the Firmicutes 70.3, 61.3, 52.55 and 41.4% in microcosms, followed by Bacteroidetes 20.2, 35.9, 23.1 and 0.81% and Proteobacteria 9.3, 2.7, 24.3 and 57.6% in microcosms at pHs (7.0, 8.8, 9.0 and 10.0 respectively).



	Soil	S-pH7	S-pH8	S-pH9	S-pH10
ETC < 1.0%	60.38	9.89	8.45	13.47	7.01
Acidaminobacter_f	3.69	0.00	0.00	0.01	0.00
Rhodocyclaceae	3.01	0.00	0.00	0.00	0.18
Comamonadaceae	7.51	0.00	0.00	0.00	0.02
Propionibacteriaceae	11.59	0.00	0.00	0.00	0.01
Desulfovibrionaceae	0.07	4.37	0.84	0.00	0.02
Erysipelotrichaceae	4.54	0.01	0.78	0.49	0.37
Anaerovirgula_f	1.38	0.00	0.56	3.77	7.31
Paludibacter_f	1.13	0.84	0.98	6.62	0.31
Xanthomonadaceae	0.57	0.01	0.01	6.38	6.55
Clostridiaceae	0.18	1.29	1.24	5.45	0.44
Clostridium_g7_f	0.00	4.16	5.64	7.96	0.96
Lachnospiraceae	0.28	2.74	2.85	2.49	0.91
Porphyromonadaceae	0.00	19.28	34.79	15.42	0.45
Aeromonadaceae	0.04	0.24	0.79	1.71	1.76
Alcaligenaceae	0.00	3.17	0.07	0.01	0.03
Sedimentibacter_f	0.00	8.14	6.40	13.76	23.58
Pseudomonadaceae	3.79	0.00	0.27	12.76	36.64
Ruminococcaceae	0.32	45.33	35.76	9.68	2.93
Brucellaceae	1.52	0.55	0.59	0.03	10.51

B) On the family level the microcosms communities were dominated by the following families; Ruminococcaceae 45.3, 35.7, 9.6 and 2.9%, followed by Pseudomonadaceae alkaliphilic species 0.0, 0.27, 12.7 and 36.6%; Sedimentibacter 8.1, 6.4, 13.7 and 23.5% and porophyromonadaceae 19.2, 34.7, 15.4 and 0.45% in the microcosms at pHs (7.0, 8.0, 9.0 and 10.0 respectively).

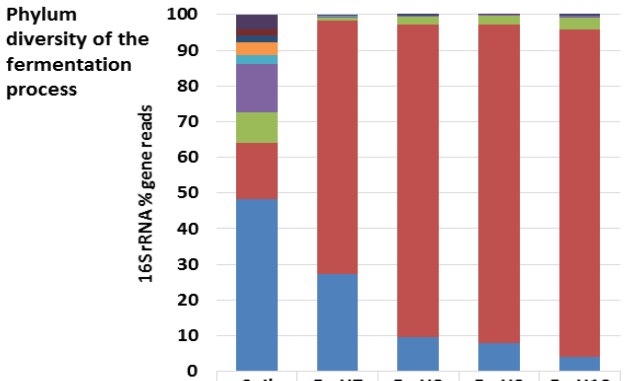


	Soil	S-pH7	S-pH8	S-pH9	S-pH10
ETC < 1.0%	88.63	29.32	23.39	43.60	21.64
Propionibacterium acnes	11.23	0.00	0.00	0.00	0.00
Clostridium sphenoides	0.00	1.75	1.54	0.25	0.57
Sedimentibacter_f_uc_s	0.00	1.12	1.01	1.53	0.57
Porphyromonadaceae_uc_s	0.00	3.16	5.25	4.46	0.19
CP001111_s	0.00	0.00	0.00	4.48	4.76
AB298756_s	0.00	1.08	1.35	3.75	0.23
Pseudomonas putida	0.00	0.00	0.00	12.11	2.85
Ruminococcaceae_uc_s	0.14	5.84	3.74	2.59	0.41
Alkalibaculum bacchi	0.00	3.09	2.81	0.77	1.31
Aeromonas veronii	0.00	0.15	0.00	0.39	1.01
Sedimentibacter_uc	0.00	1.72	2.03	10.07	1.48
AJ488078_s	0.00	12.73	19.49	2.50	1.26
JX391172_s	0.00	11.52	2.98	0.43	0.25
Proteiniphilum_uc	0.00	16.12	29.53	10.96	0.25
AB630534_g_uc	0.00	11.21	3.95	0.56	0.51
EF059533_s	0.00	0.69	2.10	1.52	20.57
Pseudomonas tuomuerensis	0.00	0.00	0.26	0.00	31.93
Brucella melitensis	0.00	0.50	0.57	0.03	10.21

C) Bacterial diversity on the species level under a sulphate-reducing culture; three species were dominated the microcosm at pH 10; *Brucella melitensis* 10.2%, *Pseudomonas tuomuerensis* 31.9% and unclassified sp (EF059533) 20.57%.

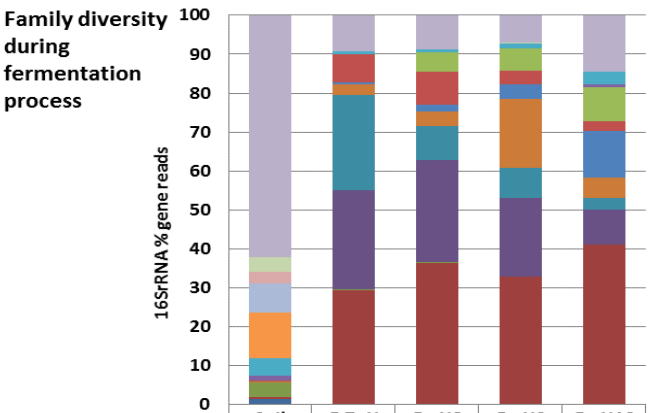
Figure 3. 56: Analysis of the sulphate-reducing bacterial communities

In the absence of a terminal electron acceptor, the Firmicutes dominated the communities with the Proteobacteria and the Bacteroidetes reducing as the pH increased. (Figure 3.57 A). The dominant family was the Ruminococcaceae at all pH values, followed by the Sedimentibacter and the Aeromonadaceae families at the lower pH values (Figure 3.57 B). At the species level, no one identified species had a dominant position with the only possible exception being *Aeromonas sp* (19.6% at pH 7.0).



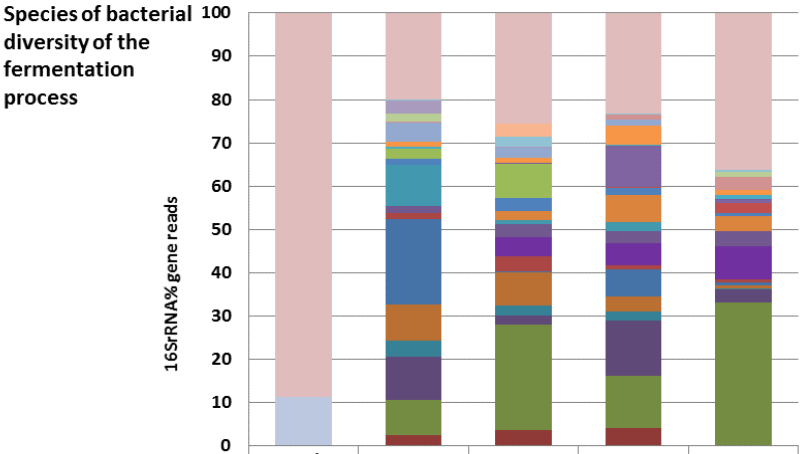
	Soil	F-pH7	F-pH8	F-pH9	F-pH10
ETC < 1.0%	4.18	0.19	0.13	0.05	0.09
Streptophyta	0.11	0.03	0.00	0.00	0.00
Gemmatimonadetes	1.59	0.03	0.02	0.01	0.01
Chloroflexi	1.98	0.07	0.15	0.00	0.11
Acidobacteria	3.54	0.09	0.04	0.02	0.05
Planctomycetes	2.52	0.20	0.11	0.01	0.10
Actinobacteria	13.36	0.26	0.21	0.10	0.39
Bacteroidetes	8.72	0.92	2.01	2.67	3.54
Firmicutes	15.80	71.00	87.87	89.20	91.74
Proteobacteria	48.19	27.22	9.47	7.94	3.97

A) Fermentative phylum was dominated the microcosms at all pHs (71% - 91%). Although the Proteobacteria were dominant in the crude soil, they decreased to < 9.5% at pHs (8.0, 9.0 and 10).



	Soil	F-7pH	F-pH8	F-pH9	F-pH10
ETC < 1.0%	62.15	9.28	8.71	7.08	14.46
Acidaminobacter_f	3.68	0	0.01	0.04	0.06
Rhodocyclaceae	3.01	0.01	0	0	0
Comamonadaceae	7.51	0.02	0.01	0	0.01
Propionibacteriaceae	11.58	0	0	0	0
Erysipelotrichaceae	4.54	0.64	0.82	1.25	3.05
Anaerovirgula_f	1.38	0	0	0.01	0.91
Eubacteriaceae	0	0.01	5.05	5.76	8.62
Clostridiaceae	0.18	7.3	8.39	3.47	2.563
Clostridium_g7_f	0	0.47	1.89	3.83	11.96
Lachnospiraceae	0.28	2.66	3.68	17.81	5.36
Aeromonadaceae	0.03	24.56	8.69	7.58	2.94
Sedimentibacter_f	0	25.36	26.3	20.37	8.87
Pseudomonadaceae	3.79	0.29	0.07	0	0.01
Ruminococcaceae	0.31	29.37	36.41	32.75	41.13
Brucellaceae	1.52	0	0.012	0.02	0.01

B) The families present under fermentative conditions Ruminococcaceae 29.3, 36.4, 32.7 and 42%; Sedimentibacter 25.5, 26, 20 and 8.8%; and Aeromonadaceae 24, 8.7, 7.5 and 2.9% in microcosms at pHs (7.0, 8.0, 9.0 and 10.0 respectively).



	Soil	F-pH7	F-pH8	F-pH9	F-pH10
ETC < 1.0%	88.59	19.98	25.57	23.26	36.30
Propionibacterium acnes	11.23	0.00	0.00	0.00	0.00
Aeromonas sobria	0.00	0.01	3.09	0.01	0.01
Clostridium intestinale	0.00	0.21	2.31	0.18	0.28
HQ697815_s	0.00	3.15	0.06	0.00	0.00
Aeromonas salmonicida	0.00	1.69	0.01	0.00	1.33
Sedimentibacter saalensis	0.00	0.23	0.03	1.16	3.03
FN436134_s	0.00	4.53	2.36	1.50	0.00
AJ229234_s	0.04	1.16	1.13	4.25	1.14
Clostridium propionicum	0.00	0.32	0.16	0.41	0.95
FN436095_s	0.00	0.01	0.23	9.40	0.80
HQ896300_s	0.00	2.39	7.71	0.06	0.00
Clostridium sphenoides	0.00	0.00	0.04	0.09	2.31
Sedimentibacter_f_uc_s	0.00	1.32	2.97	1.70	0.82
EU266824_s	0.00	0.08	2.15	6.26	3.35
DQ168650_s	0.00	9.55	0.86	2.00	0.16
Ruminococcaceae_uc_s	0.14	1.64	3.06	2.92	3.41
Alkalibaculum bacchi	0.00	0.00	4.42	5.12	7.56
Aeromonas veronii	0.00	1.39	3.58	0.81	0.83
Aeromonas hydrophila	0.00	19.62	0.12	6.38	0.60
Sedimentibacter_uc	0.00	8.37	7.61	3.42	0.71
AJ488078_s	0.00	3.80	2.49	1.97	0.35
JX391172_s	0.00	9.80	1.96	12.96	2.89
AB630534_g_uc	0.00	8.21	24.47	11.96	33.16
EF059533_s	0.00	2.52	3.60	4.15	0.00
Brucella melitensis	0.00	0.00	0.01	0.01	0.01

C) On species level the highest percentages was represented by *Aeromonas* (19.6%) at pH 7; *AB630534* (24.4%) at pH 8; *JX391172* (12.9%), *AB630534* (11.96%) at pH 9 and *AB630534* (33%) at pH 10.

Figure 3. 57: Bacterial diversity at different pH level under fermentative conditions

Comparison of the communities using the unique fraction metric (UniFrac) and Fast Unifrac provided by CLcommunity ChunLab demonstrated the clustering of the different populations (Figure 3.58). In this analysis the nitrate reducing and fermentative systems all clustered together. The iron reduction and sulphate reduction communities were more diverse, with the communities from ≤ pH 9.0 clustering together whilst the iron and pH 10.0 sulphate communities were clustered loosely. The initial soil used as the inoculum did not cluster with any of the other communities.

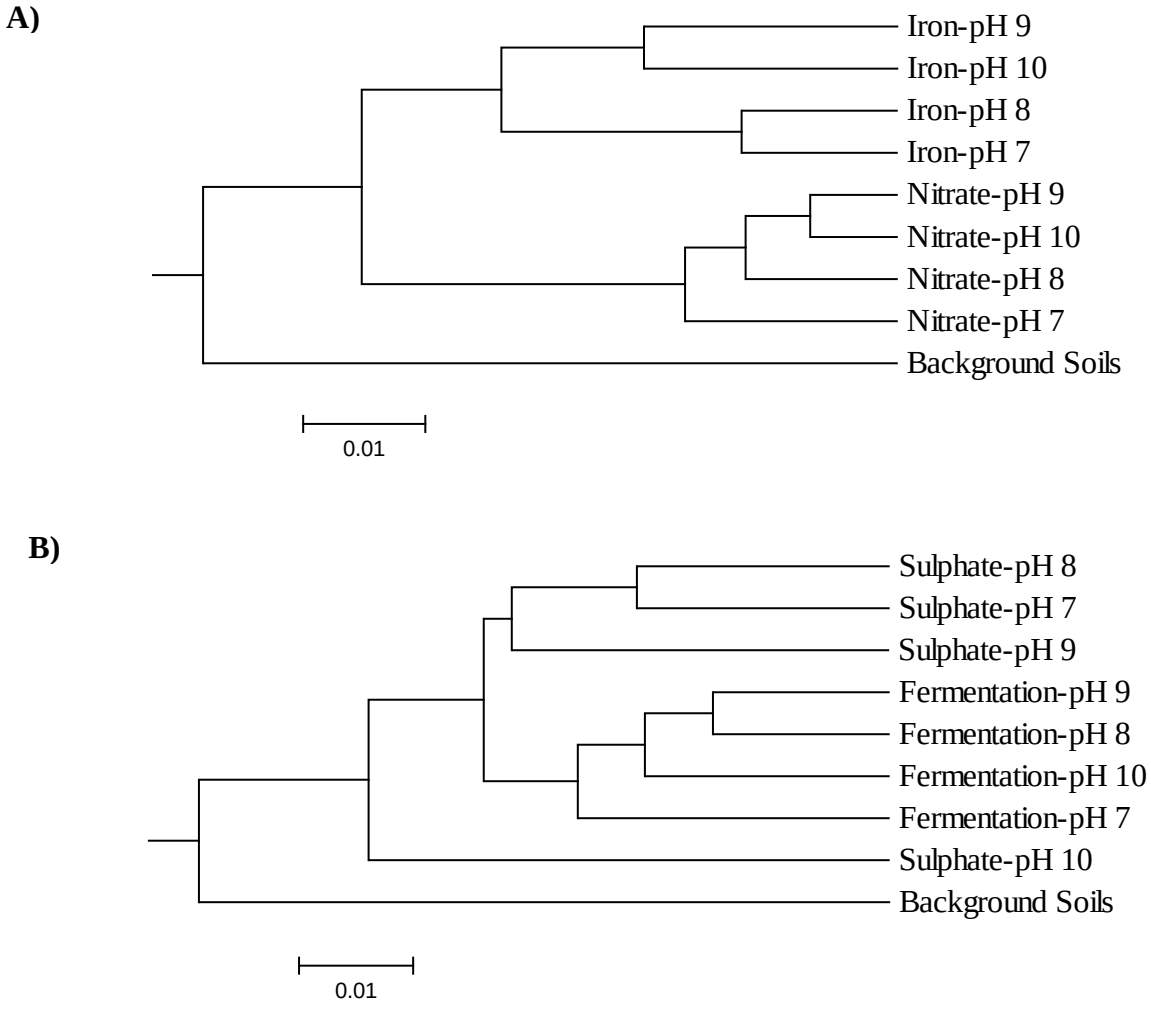


Figure 3. 58: Beta-diversity, dendrogram, comparison of the microbial communities present in all the microcosms compared to the initial soil based on UPGMA.

3.5.3. Discussion

This experiment was carried out to determine the impact of terminal electron acceptors and environmental pH on the CDP degrading communities present in alkaline contaminated soils collected from the Harper Hill site. Under nitrate-reducing conditions the rate of the ISA degradation decreased in an exponential manner as the pH increased. The degradation of β-ISA was more significant than that of α-ISA and a complete reduction of nitrate at pH 7.0, pH 8.0, pH 9.0 and significant amount at pH 10.0. Basil *et al*. investigated the same sediments and reported a complete degradation of α-ISA at pH 10 over a 6 day under nitrate reducing conditions. In Basil *et al*. experiments, the reduction of nitrate was associated with fermentation indicated by acetate production, the acetate was then removed via the reduction of nitrate (Bassil et al., 2015). This significant role of fermentation resulted in a drop in pH which in turn promoted ISA degradation. In the study reported here the alkaline pHs was maintained, and as such this was a more valid comparison of the impact of pH. The experiments reported here were also able to maintain the oxidising conditions promoted by the presence of nitrate, which is indicated by the absence of an accumulation of volatile fatty acids. At neutral pH Kuippers *et al*. (2015), observed the biodegradation of α-ISA under nitrate-reducing conditions (about 88% of ISA was degraded during 28 days) with no evidence of fermentation. These observations are more closely aligned with the data generated during this study.

Bacterial species that were prominent in the nitrate fed systems included *Brucella melitensis,* **(**with 99.75% similarity) which had a significant presence at pH 7.0 (61.7%) at pH 8 (32.5%), at pH 9.0 (50.1%) and at pH 10.0 (41.5). It was also present in the Iron (III)-reducing microcosm at pH 7.0 (15%), pH 8.0 (10%), pH 9.0 (40%) and pH 10.0 (43%). *Brucella* species are facultative anaerobic bacteria and *Brucella melitensis* is a nitrate reducing species with two regulatory 16M: NarR genes (Haine, Dozot, Dornand, Letesson, & De Bolle, 2006) which is able to respire nitrate to dinitrogen. It also has a hydrogen-transporting ATP synthase that used by bacterial cells for regulate high pH value (DelVecchio et al., 2002). Furthermore *Brucella melitensis* has also been identified as an Iron (III) reducing strain (Danese et al., 2004) which explains its presence in the iron reducing microcosms.

In addition to *Brucella melitensis, Achromobacter marplatensis* (with 99.5% similarity) was also detected in the nitrate-reducing microcosms at pH 7.0 (17.7%), pH 8.0 (36.3%), pH 9.0 (32.4%) and at pH 10.0 (32.9%). Bacteria of this genus have been isolated from a range of environmental sources (water, soil) and from a number of clinical samples (Vandamme et al., 2013). *Achromobacter marplatensis* was isolated from soil contaminated by pentachlorophenol

(Murialdo, Fenoglio, Haure, & Gonzalez, 2003) and is able to assimilate citrate, malate and phenylacetate. It is also capable of nitrate and nitrite reduction, although this species was unable to utilize a wide range of carbohydrates. However, other closely related species are able to utilize numerous carbohydrate carbon sources (Gomila et al., 2011) and have been associated with cellulose-decomposing communities (Dumova & Kruglov, 2009) where it contributes to the community by producing β-glucosidase (X. Chen, Wang, Yang, Qu, & Li, 2015) whilst a number of *Achromobacter* species have been identified as highly active nitrate reducing strains (Eltarahony, Zaki, hassan Khairalla, & Abd-El-Haleem, 2015) this study is the first to report alkaliphilic *Achromobacter* species.

Under Fe(III)-reducing condition (Figure 3.50), there was limited ISA degradation with Fe(III) reduction indicated by the generation of Fe (II). The microbial populations were broadly similar to those observed in the nitrate reducing microcosms that were dominated by Gram-negative *Proteobacteria* e.g. *Brucella melitensis* and *Pseudomonas tuomuerensis,* followed by *Firmicutes* represented by the *Sedimentibacter* which occupied a proportion of the population in the mid pH ranges. This member of the Firmicutes phyla was the only significant Gram positive organism present within these microcosms. This contrasts with Bassil *et al*. (2015) who observed populations dominated by the Gram-positive *Anaerobacillus* genus (≈95.5%) in Fe-(III)-reducing alkaline cultures fed on α-ISA at pH 10.0 (Bassil et al., 2015). This once again emphasis the fact that ISA is not a valid analogue of CDP as demonstrated by Kyeremeh *et al*. (2016).

The Pseudomonadaceae were present within these populations at the lower pH values represented by *Pseudomonas tuomuerensis*. Some pseudomonads are able to reduce many metals, such as Fe (III) (Lonergan et al., 1996; Tao, Zhou, He, Hu, & Li, 2014) and have been associated with cellulose-decomposing bacterial community (Dumova & Kruglov, 2009). At neutral pH Kuippers *et al*. (2015), found that Iron-reducing communities were initially dominated by Firmicutes which were replaced by Gram-negative Betaproteobacterium (40% sequences) later in the fermentation. They suggested that this is likely to the formation of insoluble iron (III) and the presence of co-factors generated by other metabolic processes including fermentation (Kuippers et al., 2015).

Rhanella bruchi accounted for 17.4% of the iron-reducing communities at pH 10.0 (Table 3.5). The *Rahnella genus* was proposed in 1979, and between 2009-2012 this genus was isolated from the tissues of the following; Oak Decline, alder and walnut log tissue, and buprestid

beetles resulting in the classification of five *Rhanella* species including *Rahnella bruchi* sp. nov. (Type strain FRB 226T). *Rahnella bruchi* is a Gram-negative facultative anaerobic rod which is oxidase negative and positive for catalase, α-galactosidase and gelatinase. This strain able to reduce nitrate to nitrite and uses a wide range of carbohydrates during fermentation (Brady, Hunter, Kirk, Arnold, & Denman, 2014).

As with iron reduction, there was only modest amounts of ISA degradation (~1.5mM of each ISAs) and associated sulphate reduction (Figure 3.51). The ISA reduction was combined with VFAs production that suggest the ISA reduction occurred via fermentation rather than sulphate reduction. These results are broadly consistent with previous data provided by Bassil *et al*. (2015), who did not find any evidence of ISA degradation under sulphate-reducing conditions at pH 10.0 (Bassil et al., 2015) and with the data of Rizoulis, *et al*. (2012) who fed similar sediments from Harpur Hill the Buxton site with sulphate and lactate or acetate at alkaline pH (Rizoulis et al., 2012). At neutral pH Kuippers *et al*. (2015) also found that sulphate reduction was associated with the fermentation of ISA (Kuippers et al., 2015). The lack of sulphate reduction at alkaline pH is underinned by the lack of any of the classic sulphate reducing bacteria. Rather the population had a similar profile to that seen in the iron reducing systems with the exception that a significant number of unclassified organisms began to appear in the population.

In the lower pH sulphate microcosms, the Porphyromonadaceae were the dominant bacterial family and these strict anaerobes were only detected under sulphate-reducing conditions (19%, 34.8%, 15.4% and 0.5% at pH 7.0, pH 8.0, pH 9.0 and pH 10.0 respectively). This family was represented by the *Proteiniphilum*, a genus of Gram-negative, non-spore-forming rods. Isolates of this genus isolated from brewery wastewater, were proteolytic and able to ferment peptone, yeast extract, and L-arginine to acetic acid. The optimum pH (7.5-8.0) and range (pH 6.0–9.7) (S. Chen & Dong, 2005) supports the dominace of these bacteria in the lower pH microcosms. This genus has been found in other sulphate-reducing systems for example a toluene-degrading microbial community of a Contaminated Aquifer (Kuppardt et al., 2014). Even though these bacteria have been seen in other sulphate reducing conditions there is no explanation to date as to why they dominate in these sulphate reducing conditions.

Ruminococcaceae and Lachnospiraceae are two strictly anaerobic families found in the sulphate microcosms, these families include cellulose-degrading strains (Chassard, Delmas, Robert, Lawson, & Bernalier-Donadille, 2012) and carbohydrate fermenters commonly found in the

mammalian gut (Flint, Scott, Duncan, Louis, & Forano, 2012). For instance, *Ruminococcus champanellensis* is able to metabolize cellulose and cellobiose to acetate and succinate (Chassard et al., 2012). The *Sedimentibacter* are strictly anaerobic, polymorphous, rods with wide pH ranges 5.8-8 (Breitenstein et al., 2002). These observations suggest that the cellulosic origins of CDP had a greater impact on the population structure than the presence of sulphate.

In the absence of a terminal electron acceptor, the communities (Appendix-3; Table 1, 2 and 3). were dominated by Gram-positive bacteria, which aligns with the observation of Bassil *et al*. (2015). Which in turn supports the observation that theirs was a fermentation driven rather than a respiratory system established due to the use of ISA rather than CDP.

3.5.4. Key findings

- The bacterial community in the Harpur Hill sediments was able to degrade α- and β-ISAs under all provided metabolic pathways except sulphate reduction.
- The amount and the rate of the ISA degradation differed depending on the terminal electron acceptor employed and the pH level.
- Phylogenetic analysis of the CDPs driven microcosms showed a significant reduction in bacterial community diversity when compared with the crude soil.
- A significant change of the bacterial diversity structure was under the different metabolic pathways of fermentation, Nitrate, Sulphate and Iron (III) reduction at different pH level.
- The Nitrate reducing-microcosms at different pH levels were dominated by two genera of Proteobacteria represented by *Brucella melitensis* and *Achromobacter marplatensis.*
- Increases in pH led to significant changes in bacterial community structure under all provided metabolic pathways, it also has a direct effect on the rate of the ISA degradation.
- Generally, the bacterial diversity of the microcosms under fermentation metabolic pathway was similar to the bacterial diversity of the microcosms at sulphate reduction (Figure 3.58 B).
- The microcosms under nitrate and iron (III) reduction (Figure 3.58 A) were dominated by Gram-negative bacteria of phylum Proteobacteria at all pH levels.

3.6. Isolation and identification of alkaliphilic bacteria

3.6.1. Isolation and identification of alkaliphiles from CDP fed sulphate reducing and fermentative microcosms

Alkaliphiles were isolated from CDP fed sulphate reducing and fermentative microcosms operated by Charles (2017) which had been in operation for 22 weeks at pH 11.0 and pH 12.0 (Charles 2017). After 7 days of anaerobic incubation on FAA (pH 10.0 and pH 11.0) at 25°C, a mixed growth was observed (Figure 3.59 A) with the fermentative microcosm at pH 11.0 providing more diverse and extensive growth than the fermentative microcosm at pH 12.0 and sulphate reducing microcosms at pH 11.0 and pH 12.0.

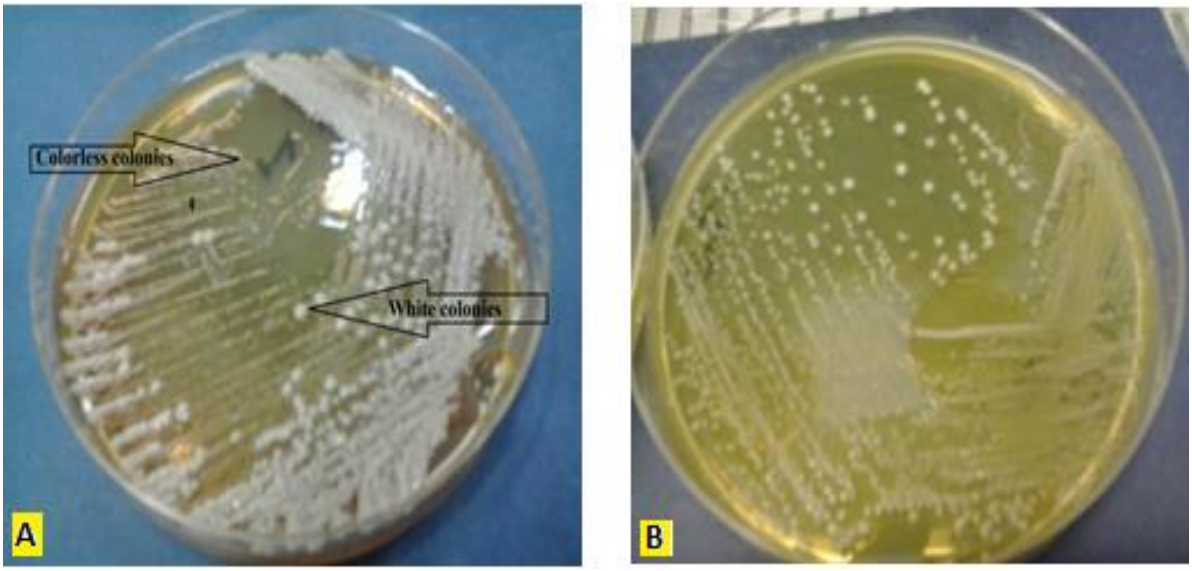


Figure 3. 59: streak plate technique for bacterial isolation

Mixed bacterial growth on FAA plate at pH 10 (A), isolated pure colonies (B).

The dominant colonies from these plates were isolated and purified (Figure 3.59 A and B). The resulting isolates were also tested for their ability to grow in aerobic condition (Table 3.1) to differentiate between facultative and obligate anaerobes.

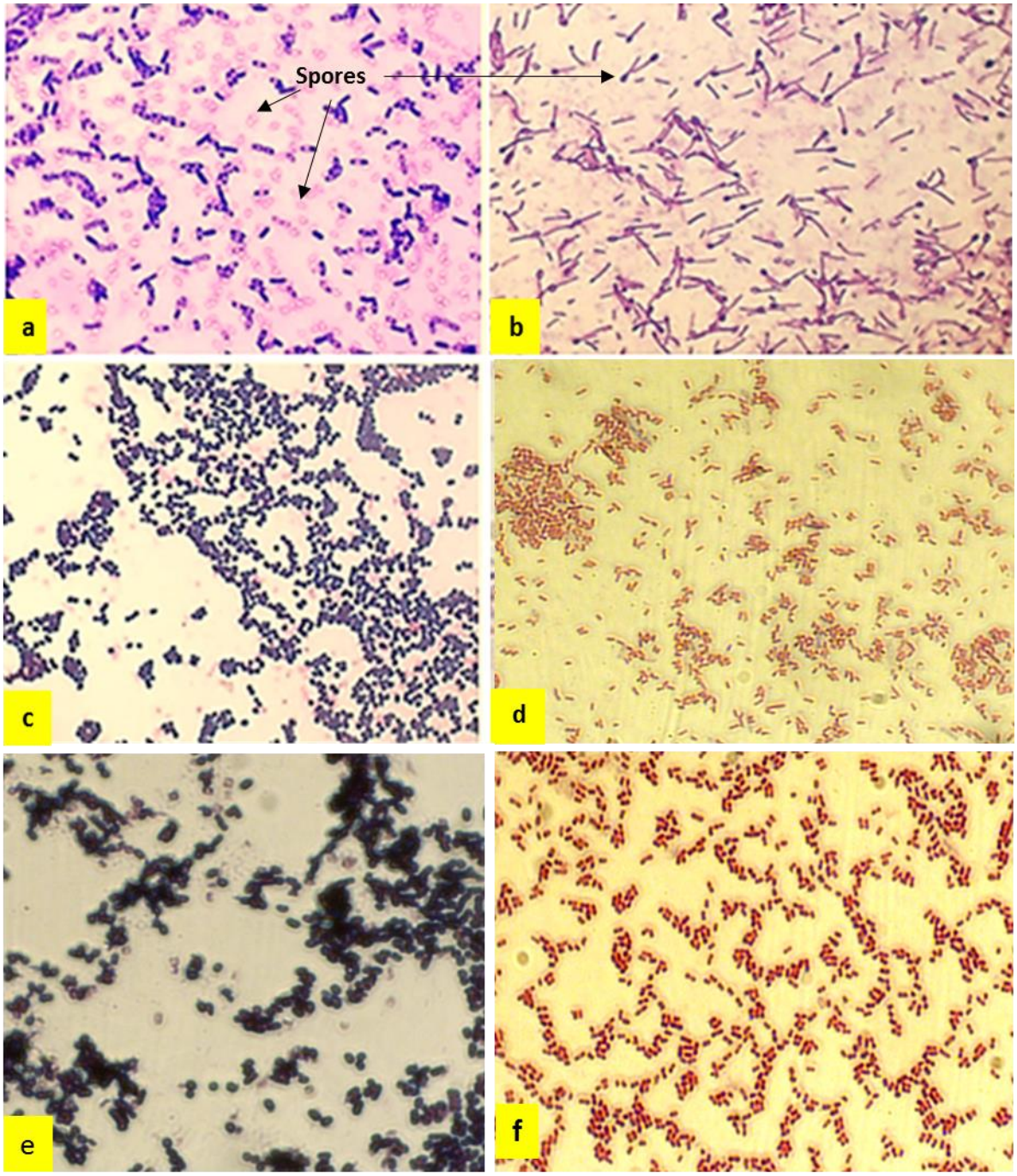


Figure 3. 60: Gram stains of the pure cultures derived from the pH 11 and 12 microcosms.

Gram positive bacilli with a central spore (a), Gram positive bacilli with a terminal spore (b), Gram positive cocci (c), Gram positive polymorphic coccobacilli (e) and Gram negative polymorphic coccobacilli (d and f)

Nine isolates were recovered, but it was not possible to maintain one isolate. Consequently, this strictly anaerobic Gram positive bacillus with terminal spores was lost from the investigation (Figure 3.60 b). Of the remaining eight isolates, four were strictly anaerobic, Gram-positive, spore forming bacilli that generated white colonies with a regular edge and smooth texture on

FAA. The other four isolates were facultative anaerobic, non-spore forming bacteria. Two of which were Gram-positive (Figure 3.60 c and e) and formed colourless colonies with a regular edge and a smooth texture on FAA at pH 10.0. The remaining two isolates were Gram-negative rods (Figure 3.60 d and f) that formed colourless colonies with a regular edge and smooth texture on FAA at pH 10.0.

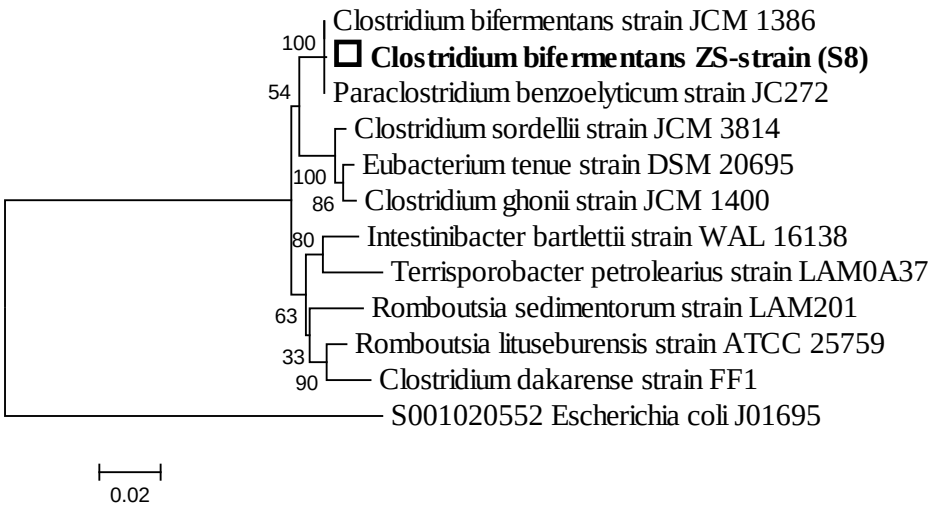
The eight pure cultures isolated were subject to phylogenetic analysis via 16s rRNA gene sequence analysis (See section 2.3.3.). Identification by BLASTN (Zhang, Schwartz, Wagner, & Miller, 2000) (Table 3.2) indicated that the FAA were dominated by *Enterococcus gallinarum* (Figure 3.60 c). This was followed by moderate growth by a strain of *Aeromonas salmonicida* (Figure 3.60 d) and *Clostridium bifermentans* (Figure 3.60 a). The strains associated with more limited growth were identified as an *Acinetobacter guillouiae* (Figure 3.60 f), *Exiguobacterium mexicanum* (Figure 3.60 e) and three *Clostridium sp.* (Table 3.1). Generally, FAA was dominated by Gram-positive bacteria classified under the Firmicutes phylum, an observation consistent with the dominant phylum identified via 16s rRNA community analysis of the original microcosms (Charles et al., 2015).

Table 3. 1: Identification of bacterial isolates from CDP feed sulphate reducing and fermentative microcosms at pH 11 and pH 12

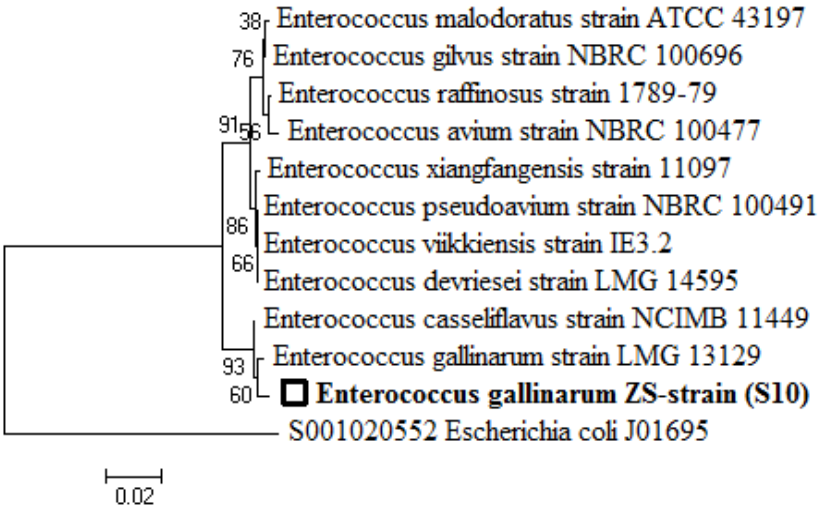
Isolate Identification (Strain number)	Bacteria isolated from the Microcosms on FAA plates at pH 10.0 and pH 11.0						
	Fragment length	% match	FAA pH	Aerobic growth	Gram stain	Morphology	Spore forming
***Clostridium bifermentans* (S8)**	980	99	10, 11	-	+	Bacilli	+
***Clostridium sordelli* (S17)**	956	99	10	-	+	Bacilli	+
***Clostridium mangenotii* (S20)**	947	99	10	-	+	Bacilli	+
***Clostridium malenominatum* (S16)**	906	98	10	-	+	Bacilli	+
***Enterococcus gallinarum* (S10)**	935	99	10,11	+	+	Cocci	-
***Exiguobacterium mexicanum* (S15)**	956	99	10,11	+	+	Bacilli	-
***Aeromonas salmonicida* (S9)**	848	100	10,11	+	-	Bacilli	-
***Acinetobacter guillouiae* (S011)**	1019	99	10	+	-	Bacilli	-
Terminal spore forming strain	-	-	10	-	+	Bacilli	+

F= fermentative microcosm, S= sulphate reducing microcosm, UD= undetected

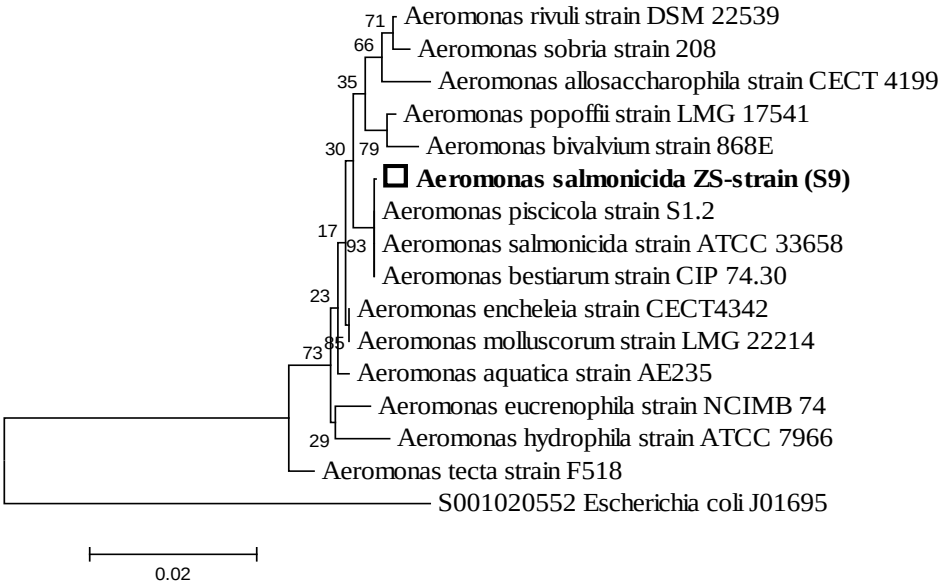
A)


Clostridium bifermentans strain JCM 1386
Clostridium bifermentans ZS-strain (S8)
Paraclostridium benzoelyticum strain JC272
Clostridium sordellii strain JCM 3814
Eubacterium tenue strain DSM 20695
Clostridium ghonii strain JCM 1400
Intestinibacter bartlettii strain WAL 16138
Terrisporobacter petrolearius strain LAM0A37
Romboutsia sedimentorum strain LAM201
Romboutsia lituseburensis strain ATCC 25759
Clostridium dakarense strain FF1
S001020552 Escherichia coli J01695
100
54
100
86
80
63
33
90
0.02

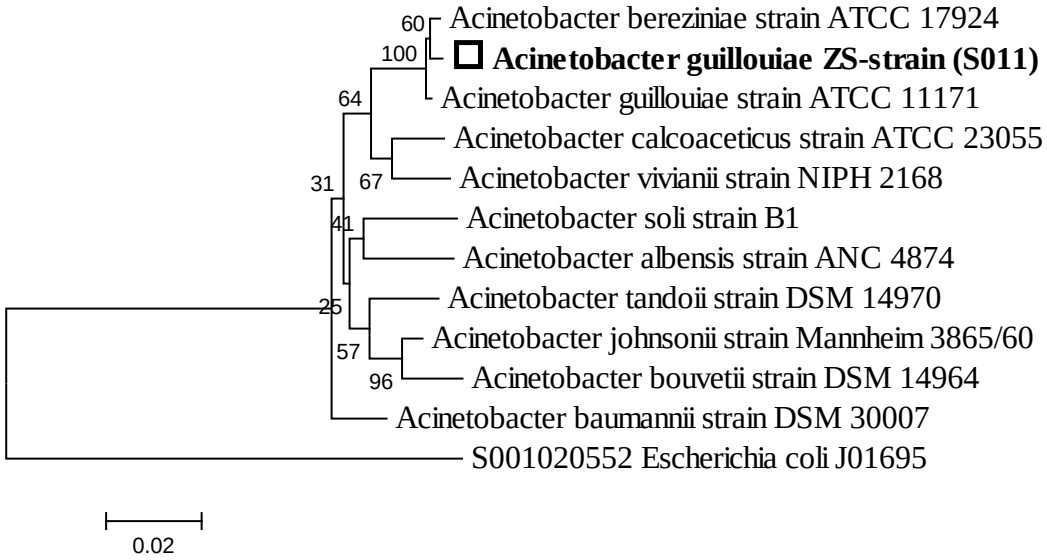
B)


Enterococcus malodoratus strain ATCC 43197
Enterococcus gilvus strain NBRC 100696
Enterococcus raffinosus strain 1789-79
Enterococcus avium strain NBRC 100477
Enterococcus xiangfangensis strain 11097
Enterococcus pseudoavium strain NBRC 100491
Enterococcus viikkiensis strain IE3.2
Enterococcus devriesei strain LMG 14595
Enterococcus casseliflavus strain NCIMB 11449
Enterococcus gallinarum strain LMG 13129
Enterococcus gallinarum ZS-strain (S10)
S001020552 Escherichia coli J01695
38
76
91
86
66
93
60
0.02

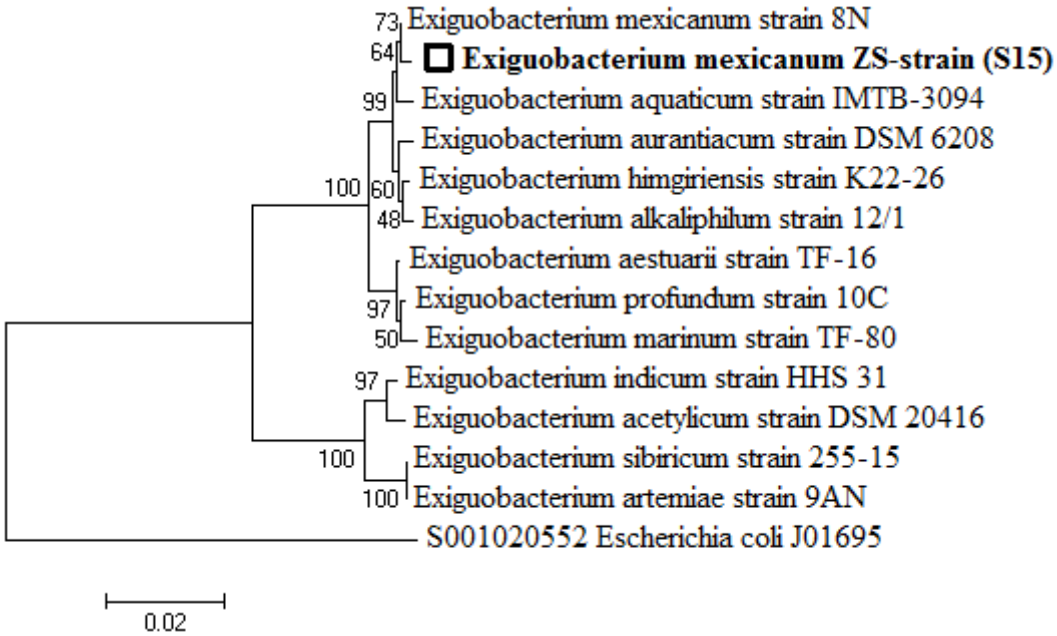
C)


Aeromonas rivuli strain DSM 22539
Aeromonas sobria strain 208
Aeromonas allosaccharophila strain CECT 4199
Aeromonas popoffii strain LMG 17541
Aeromonas bivalvium strain 868E
Aeromonas salmonicida ZS-strain (S9)
Aeromonas piscicola strain S1.2
Aeromonas salmonicida strain ATCC 33658
Aeromonas bestiarum strain CIP 74.30
Aeromonas encheleia strain CECT4342
Aeromonas molluscorum strain LMG 22214
Aeromonas aquatica strain AE235
Aeromonas eucrenophila strain NCIMB 74
Aeromonas hydrophila strain ATCC 7966
Aeromonas tecta strain F518
S001020552 Escherichia coli J01695
71
66
35
30
79
17
93
23
73
85
29
0.02

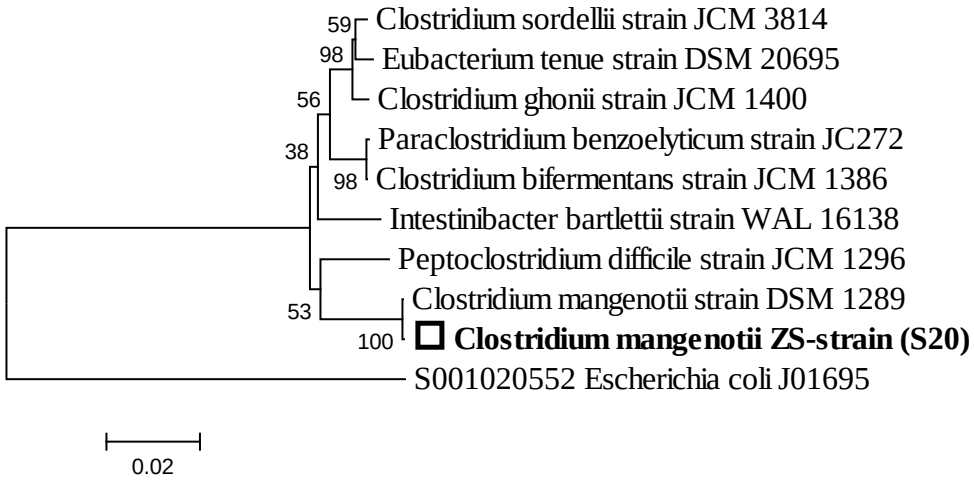
D)


60
100
64
31
67
41
25
57
96
Acinetobacter bereziniae strain ATCC 17924
Acinetobacter guillouiae ZS-strain (S011)
Acinetobacter guillouiae strain ATCC 11171
Acinetobacter calcoaceticus strain ATCC 23055
Acinetobacter vivianii strain NIPH 2168
Acinetobacter soli strain B1
Acinetobacter albensis strain ANC 4874
Acinetobacter tandoii strain DSM 14970
Acinetobacter johnsonii strain Mannheim 3865/60
Acinetobacter bouvetii strain DSM 14964
Acinetobacter baumannii strain DSM 30007
S001020552 Escherichia coli J01695
0.02

E)


73
64
99
100
60
48
97
50
97
100
100
Exiguobacterium mexicanum strain 8N
Exiguobacterium mexicanum ZS-strain (S15)
Exiguobacterium aquaticum strain IMTB-3094
Exiguobacterium aurantiacum strain DSM 6208
Exiguobacterium himgiriensis strain K22-26
Exiguobacterium alkaliphilum strain 12/1
Exiguobacterium aestuarii strain TF-16
Exiguobacterium profundum strain 10C
Exiguobacterium marinum strain TF-80
Exiguobacterium indicum strain HHS 31
Exiguobacterium acetylicum strain DSM 20416
Exiguobacterium sibiricum strain 255-15
Exiguobacterium artemiae strain 9AN
S001020552 Escherichia coli J01695
0.02

F)


59
98
56
38
98
53
100
Clostridium sordellii strain JCM 3814
Eubacterium tenue strain DSM 20695
Clostridium ghonii strain JCM 1400
Paraclostridium benzoelyticum strain JC272
Clostridium bifermentans strain JCM 1386
Intestinibacter bartlettii strain WAL 16138
Peptoclostridium difficile strain JCM 1296
Clostridium mangenotii strain DSM 1289
Clostridium mangenotii ZS-strain (S20)
S001020552 Escherichia coli J01695
0.02

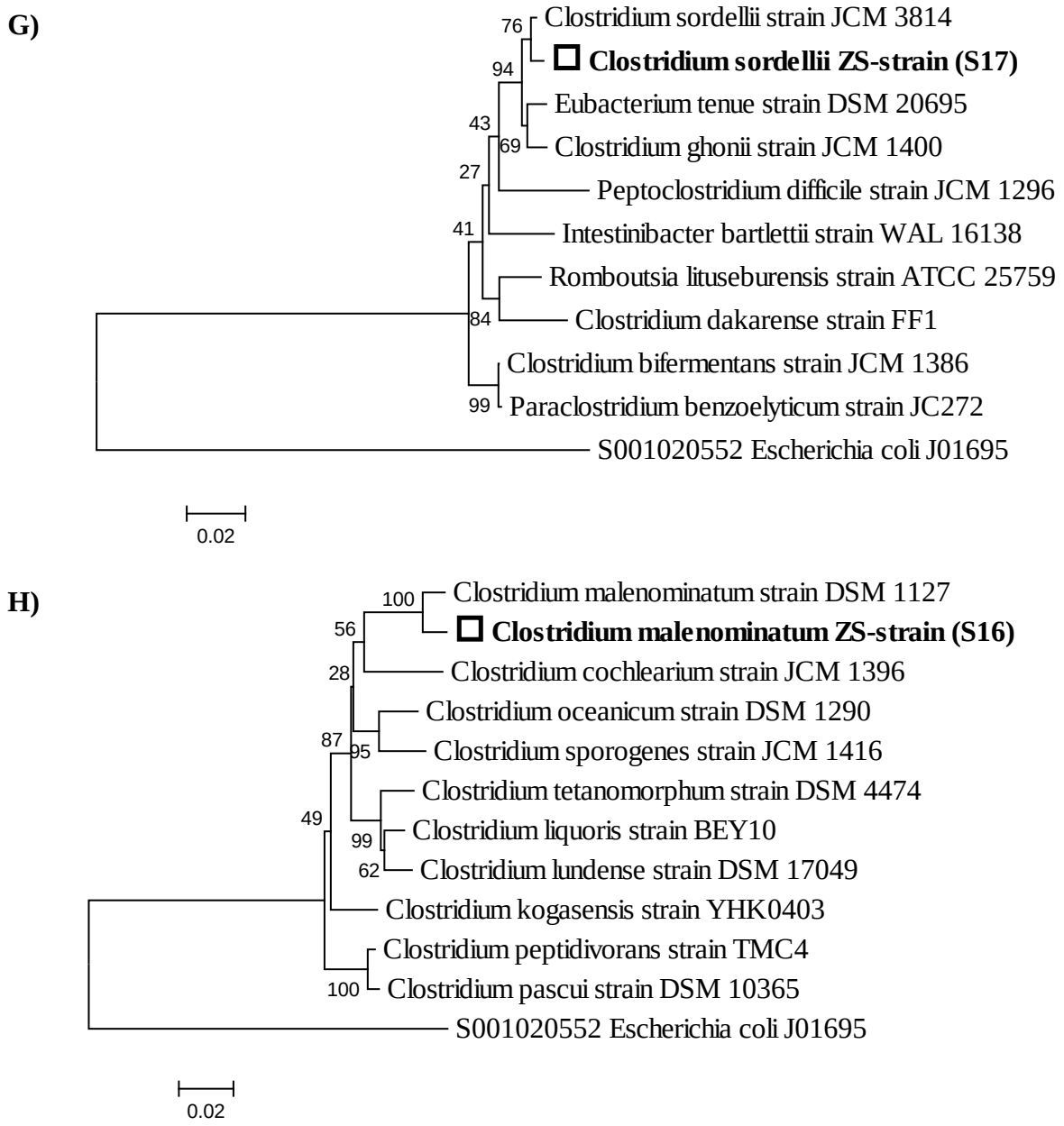


Figure 3. 61: phylogenetic trees for each of the isolates based on 16SrRNA gene sequences. A) *C. bifermentans,* B) *E. galinarum*, C) *A. salmonicida,* D) *A. guillouiae,* E) *E. mexicanum*, F) *C. mangenotii,* G) *C. sordelliim,* H) *C. malenominatum*.

Maximum-likelihood phylogenetic trees based on 16S rRNA gene sequences provide the relationships between these strains and related representatives of their families. Bootstrap values (expressed as percentages of 1000 replicates) are shown at each node where the bar represents 0.02 changes per nucleotide position. Evolutionary analyses were conducted in MEGA7.

Table 3. 2: Closest neighbours of the Alkaliphiles isolated from the pH 11.0 and 12.0 microcosms

The following results represent the evolutionary relationships of the bacterial isolates that analysed in the phylogenetic trees (Figures 3.62 with serial letters A-H).

Figure (4.2)	Bacterial isolates (strain number)	Closest relative strain from the Gene-bank database	
		Strain name	Identification number in gene bank
A	*C. bifermentans* ZS-8	*C. bifermentans*	ATCC 638
B	*E. gallinarum ZS*-10	*E. gallinarium*	AF039900
C	*A. salmonicida* ZS-9	*A. salmonicida* *A.piscicola* *A. bestiarum*	ATCC 33658 S1.2 CIP 74.30
D	*A. guillouiae* ZS-011	*A. guillouiae*	DSM 590X81659
E	*E. mexicanum* ZS-15	*E. mexicanum*	8N AM072764
F	*C. mangenotii* ZS-20	*C. mangenotii*	M5 9098
G	*C. sordillii* ZS-17	*C. sordillii*	ATCC 9714
H	*C. malenominatum* ZS-16	*C. malenominatum*	DSM 1127

Investigation of a number of CDPs driven microcosms operated under fermentation and sulphate reducing conditions at pH 11.0 and pH 12.0 resulted in the isolate a wide range of *Alkaliphiles*. The *Clostridium bifermentans* ZS-8 (Figure 3.61 A, Table 3.1) isolated showed a 99% match to the *C. bifermentans* Type strain ATCC 638. *Clostridium bifermentans* is a known alkaliphilic bacterium as demonstrated by the work of Joe *et al*. (2008) who found that the *C. bifermentans* strains are alkaline tolerant up to pH 12.0. In that case strains isolated from soil were able to decolorize textile azo dyes at a wide range of pH values (pH 6.0-12.0) with the optimum decolourization activity observed at pH 10.0 in the presence of glucose (Joe, Lim, Kim, & Lee, 2008). In general *C. bifermentans* strains are known to utilize a wide range of polysaccharides and proteins (C. Wang et al., 2003), which is consistent with the carbohydrate based alkaline environment *C. bifermentans* ZS-8 was isolated from CDP driven microcosm (Charles 2017, PhD thesis).

The other strictly anaerobic Gram-positive rods isolated were *Clostridium mangenotii* ZS-20 and *Clostridium sordelli* ZS-17 (Figure 3.61 F and G respectively) both of which showed 99% matches to their respective Type strains (Table 3.2). The remaining anaerobic Gram-positive rod (ZS-16) showed a 98% similarity to the *Clostridium malenominatum* Type strain DSM 1127 (Table 3.2, Figure 3.61 H). In previous studies, *Clostridium mangenotii* has been associated with African soils and a draft genome is available for an isolate originating from the faecal material of a timber rattlesnake (McLaughlin et al., 2014). Type strain descriptions

indicate that optimum growth is between 30-37^{o}C and up to pH 8.5 (P. A. Lawson, Citron, Tyrrell, & Finegold, 2016), the latter being consistent with the alkaline conditions employed to isolate ZS-16. It has been recently proposed that *Clostridium mangenotii* be reclassified as *Clostridioides mangenotii* (P. A. Lawson et al., 2016).

The literature on *C. sordellii* is dominated by the fact that it is associated with serious and often fatal infections (Aldape, Bryant, & Stevens, 2006); however, pathogenicity in *C. sordellii* is due to the presence of plasmid borne virulence genes (Couchman et al., 2015). In addition to medical sources, *C. sordellii* is often isolated from soils with diverse characteristics e.g. pH up to pH 8.2 (del Mar Gamboa, Rodríguez, & Vargas, 2005) and low temperatures (Nakamura, Shimamura, & Nishida, 1976) with some authors classifying these soil associated isolates as none pathogenic (Watanabe, Miwa, Imamura, Kohata, & Mochizuki, 1975). *C. malenominatum* has also been isolated from soils but at much lower abundance (del Mar Gamboa et al., 2005) and is considered to be a classic glutamate fermenting Clostridium (Wilde, Collins, & Hippe, 1997).

The most numerous bacteria isolated from the microcosms was *E. gallinarum* ZS-10 suggesting that this strain is able to utilize CDPs at high pH levels. The closest match via 16s rRNA gene sequencing was to a strain originating from a clinical study (Joulian, Ollivier, Patel, & Roger, 1998) and *E. gallinarum* strains have been associated with bacteraemia in particular (Reid, 2001). However, *E. gallinarum* also has a significant none pathogenic profile. Mesophilic strains of *E. gallinarum* have been isolated from rumen fluid which have significant anaerobic cellulose hydrolysis capabilities (A. Wang, Gao, Ren, Xu, & Liu, 2009). The ability *E. gallinarum* to utilise a wide range of carbohydrates has also been reported by other authors (G. Kim et al., 2005). Other strains isolated from tannery waste-contaminated soil (Sayel et al., 2012) were able to reduce hexavalent chromium Cr(VI) at a wide range of pH values (7.0–11.0) with an optimum pH of pH 10.0 and temperature range of 25-45°C. In this case, the presence of glucose assisted in the Cr (VI) reduction process. The strain was also able to tolerate heavy metals including Cu^{2+}, Ni^{2+}, Pb^{2+}, Co^{2+} and Zn^{2+}. In addition, textile wastewaters were the source of *E. gallinarum* strain able to decolourise azo dyes (Bafana, Krishnamurthi, Devi, & Chakrabarti, 2008). The metabolic diversity and broad carbohydrate degradation capabilities of *E. gallinarum* means that it is ideally suited to the CDP fed environments of the microcosms investigated.

Gram-negative bacillus, *Acinetobacter guillouiae* ZS-11, was also isolated from the microcosms on the FAA agar plate at pH 10. The type strain of *A. guillouiae* originated from sewage contaminated gas works effluent (Nemec et al., 2010). Other strains of *A. guillouiae* (SFC 500-1A) have been isolated from tannery sediment contaminated soil, in this case the isolate had the ability to simultaneously remove high Cr(VI) and phenol concentrations (Ontañon, González, & Agostini, 2015). *Acinetobacter* species are commonly isolated from a range of different environmental sources include soil and water (Krizova, Maixnerova, Sedo, & Nemec, 2014). For example, a group of *Acinetobacter* strains were isolated from soil contaminated with atrazine (atrazine is a pesticide used for the nonselective weed control with 3 ppb is a permissible level in water and soil). These *Acinetobacter* strains had the ability to grow up to 250 ppm of atrazine in alkaline media (pH 8) (P. Singh, Suri, & Cameotra, 2004). Despite the fact that *Acinetobacter* sp. are classically aerobic bacteria, some studies have demonstrated that the *Acinetobacter guillouiae* shows an optimum growth in facultative anaerobic condition through redox reaction in the presence of copper (Majumder, Gangadhar, Raghuvanshi, & Gupta, 2015). Other studies have demonstrated that facultative anaerobic metabolism is common in metal-respiring bacteria (Csotonyi, Stackebrandt, & Yurkov, 2006; Shen & Wang, 1994). However, in the reactors that this isolate was recovered from it is not clear what terminal electron acceptor this isolate would be employing to facilitate its metabolism.

A facultative anaerobic strain of *Aeromonas salmonicida* ZS-9 was also isolated from the alkaline microcosms. In a previous studies, *Aeromonas* sp. were isolated from a variety of aquatic environments worldwide include water and sewage with a wide temperature range of 0°C to 45°C and an optimum of 22-32°C (Didugu et al., 2015). *Aeromonas salmonicida* is known as an etiological agent of bacterial disease in fish (Daly, Kew, Moore, & Olivier, 1996; Joseph & Carnahan, 1994; Reith et al., 2008; Wiklund & Dalsgaard, 1998). This species can utilize a wide range of carbohydrates (Abbott, Cheung, & Janda, 2003), under anaerobic condition this genus can also utilise either a fermentative or anaerobic respiration energy generation strategy. *Aeromonas* are also able to survive in extreme conditions such as high pH through biofilm formation (Kirov, Castrisios, & Shaw, 2004). The high interspecies sequence similarity found in the 16S rRNA gene sequences (96.7% to 100%) of the *Aeromonas* genus (A. J. Martínez-Murcia, Figueras, Saavedra, & Stackebrandt, 2007; Vega-Sánchez et al., 2014) makes it difficult to distinguish between closely related *Aeromonas* species. Additional to the similarity of the genes, a lack of definitive biochemical markers causes complexity in the

taxonomy of this genus (A. Martínez-Murcia et al., 2013; Soler et al., 2003) in both environmental and clinical isolates (Beaz-Hidalgo, Alperi, Figueras, & Romalde, 2009; Ørmen, Granum, Lassen, & Figueras, 2005). These issues explain the clustering of closely related *Aeromonas* sp. found in the phylogenetic tree presented above (Figure 3.61 C).

Distinctive orange coloured colonies were detected on FAA at pH 10.0 and 11.0. Phylogenetic analysis showed that the isolated bacterium belonged to the genus *Exiguobacterium* and was related to *Exiguobacterium mexicanum* Type strain 8N AM072764 (99.0 %). This strain *Exiguobacterium mexicanum* ZS-15 is Gram-positive, non-spore-forming (Figure 3.60 e), facultatively anaerobic coccobacilli. *Exiguobacterium* was proposed as a new genus, belonging to the family Bacillaceae of the Firmicutes phylum in 1983 by (Collins, Lund, Farrow, & Schleifer, 1983) and includes 16 species (Vishnivetskaya et al., 2014). In previous studies, *Exiguobacterium* sp. have been isolated from markedly diverse sources including, ancient Siberian permafrost, Greenland glacial ice, hot springs, with temperature range from -12 to 55°C (Vishnivetskaya, Kathariou, & Tiedje, 2009). This diversity has resulted in the classification of *Exiguobacterium* sp. into two groups depending on temperature ranges for growth, with few exceptions, such as Greenland ice isolate *GIC31*-strain that was classified to belong to both groups (Vishnivetskaya et al., 2014; Vishnivetskaya et al., 2009). Alkaliphilic strains of *Exiguobacterium* able to tolerate up to pH 12.0, have been isolated from the alkaline drain sludge of a beverage facility in New Delhi, India (Kulshreshtha, Kumar, Begum, Shivaji, & Kumar, 2013), This genus is know to have a range of stress response genes that allow survival in extreme environments. For examples one *Exiguobacterium* strain isolated from a salt flat in the Atacama Desert, South America carries a number of stress-related genes including heavy metals/metalloid tolerance genes for cadmium, chromium, mercury, copper, tellurium, arsenic and UV stress response genes (Castro-Severyn et al., 2017). Therefore, this genus is worthy of attention as its members have the ability to adapt to extreme conditions including psychrophilic, thermophilic, alkaliphilic and heavy metal contaminated environments.

3.6.2. Isolation and identification of alkaliphiles from CDP feed fermentative microcosms at pH 10, pH 11 and pH 12

A range of isolates were recovered from the initial pH 10.0, 11.0 and 12.0 microcosms on FAA pH 9.5. These isolates represented a wide range of alkaliphiles (Table 3.3). These broke down to a Gram-positive group that included *Enterococcus gallinarum*, *Bacillus mycoides*, *Dietzia*

natronolimnaea and *Tessaracoccus lubricantis*, and a Gram-negative group composed of *Aeromonas salmonicida*, *Macellibacteroides fermentans* (a strictly anaerobic bacterium), *Alcaligenes aquatilis*, and *Citrobacter gillenii*. There were also two isolates recovered from the microcosm which operated at pH 12.0, these were *Alishewanella aestuarii* and *Bacillus cohnii*.

Table 3. 3: Identification of bacterial isolates from CDP feed fermentative microcosms at pH 10.0, pH 11.0 and pH 12.0.

N	Bacterial isolates (strain number)	bp (%match)	Closest related strain from the Gene-bank database (RDP)	
			Strain name	Identification number in gene bank
1	*Bacillus mycoides ZS-4*	924 (100%)	*B.mycoides* *B. weihenstephanensis*	NBRC 101228 DSM 11821
2	*Bacillus cohnii ZS-27*	807 (99%)	*Bacillus cohnii*	NBRC 15565
3	*Alcaligenes aquatilis ZS-13*	976 (99%)	*Alcaligenes aquatilis*	LMG 22996
4	*Citrobacter gillenii ZS-30*	1147 (98%)	*Citrobacter gillenii*	CDC 4693-86
5	*E. gallinarum ZS-46*	968 (99%)	*E. gallinarum*	AF039900
6	*Macellibacteroides fermentans ZS-40*	888 (99%)	*Macellibacteroides fermentans*	*LIND7H*
7	*Dietzia natronolimnaea ZS-51*	850 (99%)	*Dietzia natronolimnaea*	DSM 44860
8	*Tessaracoccus lubricantis ZS-41*	928 (98%)	*Tessaracoccus lubricantis*	*KSS-17Se*
9	*Aeromonas salmonicida ZS-55*	839 (99%)	*A. salmonicida* *A.piscicola* *A. bestiarum*	ATCC 33658 S1.2 CIP 74.30
10	*Alishewanella aestuarii ZS-28*	936 (99%)	*Alishewanella aestuarii*	B11

The Gram-positive cocci *Enterococcus gallinarum* ZS-46 formed colourless colonies with a regular edge and a smooth texture on FAA at pH 9.5. Based on the 16SrRNA gene sequence this isolated strain showed a 99% match to the *E.gallinarum* type strain AF039900, this strain is also similar (with 99% match) to the *E. gallinarum* ZS-10 strain described in a previous section. The *Aeromonas salmonicida* ZS-55 strain isolated showed 99% similarity to the *A. salmonicida* type strain ATCC 33658 and a 99% match to the *A. salmonicida* ZS-9 discussed in the previous section.

The following bacterial isolates (serial numbers from 6-10) are discussed in more detail in future section 3.6. *Macellibacteroides fermentans* ZS-40 a strictly anaerobic, Gram negative rod, formed white colonies with a regular edge, these were soft in texture and convex in shape. The isolate showed a 99% match to the closest phylogenetic relatives of *Macellibacteroides fermentans* type strain LIND7H (Table 3.3). *Dietzia natronolimnaea* ZS-51 is a Gram-positive, non-spore forming cocci that formed pink colonies with regular edges, raised or convex and

soft in texture which showed a 99% match to the *Dietzia natronolimnaea* type strain *DSM 44860* (Table 3.2). *Tessaracoccus lubricantis* ZS-41 strain is Gram-positive cocci, non-spore-forming bacteria, which formed yellow, translucent and shiny colonies with entire edges within two days on FAA plates at pH 9.5. This isolate showed a 98% match to the *Tessaracoccus lubricantis* type strain KSS-17Se (Table 3.3). *Alishewanella aestuarii* ZS-28 is a Gram-negative rod, isolated from the microcosm at pH 12. This isolate formed colonies with a regular edge, smooth, slightly raised, beige in colour and darker at the colony centre and showed a 99% match to the *Alishewanella aestuarii* type strain B11 (Table 3.3).

Bacillus mycoides ZS-4 strain is a spore-forming, Gram-positive bacilli, this bacterial species was often isolated from the pH 10.0 microcosm. *Bacillus mycoides* ZS-4 showed a 100% match to the *Bacillus mycoides* type strain NBRC 101228 and *Bacillus weihenstephanensis* strain DSM 11821 (Figure 3.62-1). The strain formed rhizoid colonies on the FAA at pH 9.5 that were able to overgrow a full plate in 5-7 days of incubation. *Bacillus mycoides* is a common soil organism worldwide (Stratford, Woodley, & Park, 2013) which is able to utilize a wide range of carbon and nitrogen sources for growth (Borah, Thakur, & Nigam, 2002). Through 16S rRNA gene sequencing *B. mycoides* is (99.5%) closely related to the human pathogenic strains of *B. anthracis, B. thuringiensis* and *B. cereus*. However, *B. myocoides* is non-motile with a distinct rhizoidal colony morphology (von Wintzingerode, Rainey, Kroppenstedt, & Stackebrandt, 1997).

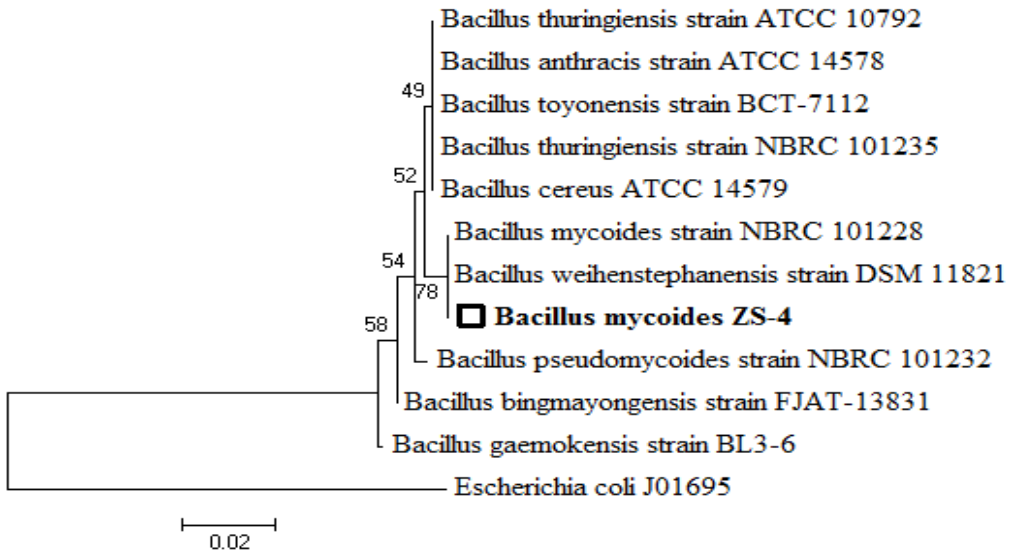
Bacillus cohnii ZS-27 was isolated from the pH 12.0 microcosm and showed a 99% match to the *Bacillus cohnii* type strain NBRC 15565 (Figure 3.62-2), when grown on FAA at pH 9.5 this strain showed small, cream white colonies. The *Bacillus* genus was described by Ferdinand Cohn in 1872 and the name *Bacillus cohnii* was proposed in 1993 for a novel, obligate alkaliphilic, oval spore-Forming *Bacillus* (Spanka & Fritze, 1993). A number of *Bacillus cohnii* strains have been investigated for their ability to generate industrially important enzymes. For example *Bacillus cohnii* US147 isolated from soil in Tunisia, generated an amylase activity in both acid and alkaline pH up to pH 9.0 at 70°C (Ghorbel, Maktouf, Massoud, Bejar, & Chaabouni, 2009), whereas *Bacillus cohnii* APT5 strain produced an extracellular alkaline protease with an optimum activity at pH 11.0 and 50°C (Tekin et al., 2012).

Another commonly isolated strain was *Alcaligenes aquatilis ZS-13,* which was isolated from the microcosm at both pH 10.0 and pH 11.0 showed a 99% match to the *Alcaligenes aquatilis* type strain LMG 22996 (Figure 3.62-3). This Gram negative rod, formed white circular colonies

with a regular edge, a low-convex profile and a smooth texture, older colonies formed irregular margins on FAA at pH 9.5. This genus was proposed in 1919 and classified under *Alcaligenaceae* family of the *Proteobacteria* phylum in 1986 (De Ley, Segers, Kersters, Mannheim, & Lievens, 1986). The two isolated strains NCCP-650^{T} and NCCP-667 of *Alcaligenes pakistanensis* showed tolerance to toxic concentrations of heavy metals, including Cr^{+2}, As^{+2}, Pb^{+2} and Cu^{+2} and pH range of 5.5–10.0 (Abbas et al., 2015). Strains of *A. aquatilis* are motile and positive for catalase and oxidase. Anaerobic growth of some strains in the presence of nitrate and nitrite was detected, some strains were also able to reduce nitrite but not nitrate. They were also able to utilize sodium acetate and produces acid from, xylose, mannitol, L-arabinose and maltose (Van Trappen, Tan, Samyn, & Vandamme, 2005).

Citrobacter gillenii ZS-30 showed a 98% match to the *Citrobacter gillenii* type strain CDC 4693-86 (Figure 3.62-4), this strain is a Gram-negative, facultative anaerobic, non-spore-forming short rod that formed translucent colonies with a regular edge. The genus *Citrobacter* belongs to the Enterobacteriaceae family of the Proteobacteria phylum. Species of the genus *Citrobacter* can be found in varied environments and are considered to be inhabitants of human and other animal guts. In humans, some species are regarded as opportunistic pathogens (Samonis et al., 2009). *Citrobacter* species isolated from soil were able to decolorize several recalcitrant dyes that used in a textile and dyeing industry under an optimal pH 7.0–9.0 and 35–40°C (An et al., 2002). They are motile and negative for oxidase and catalase, some are also able to utilize a wide range of carbohydrates and produce acid from a range of sugars including D-glucose (Clermont et al., 2015).

1)



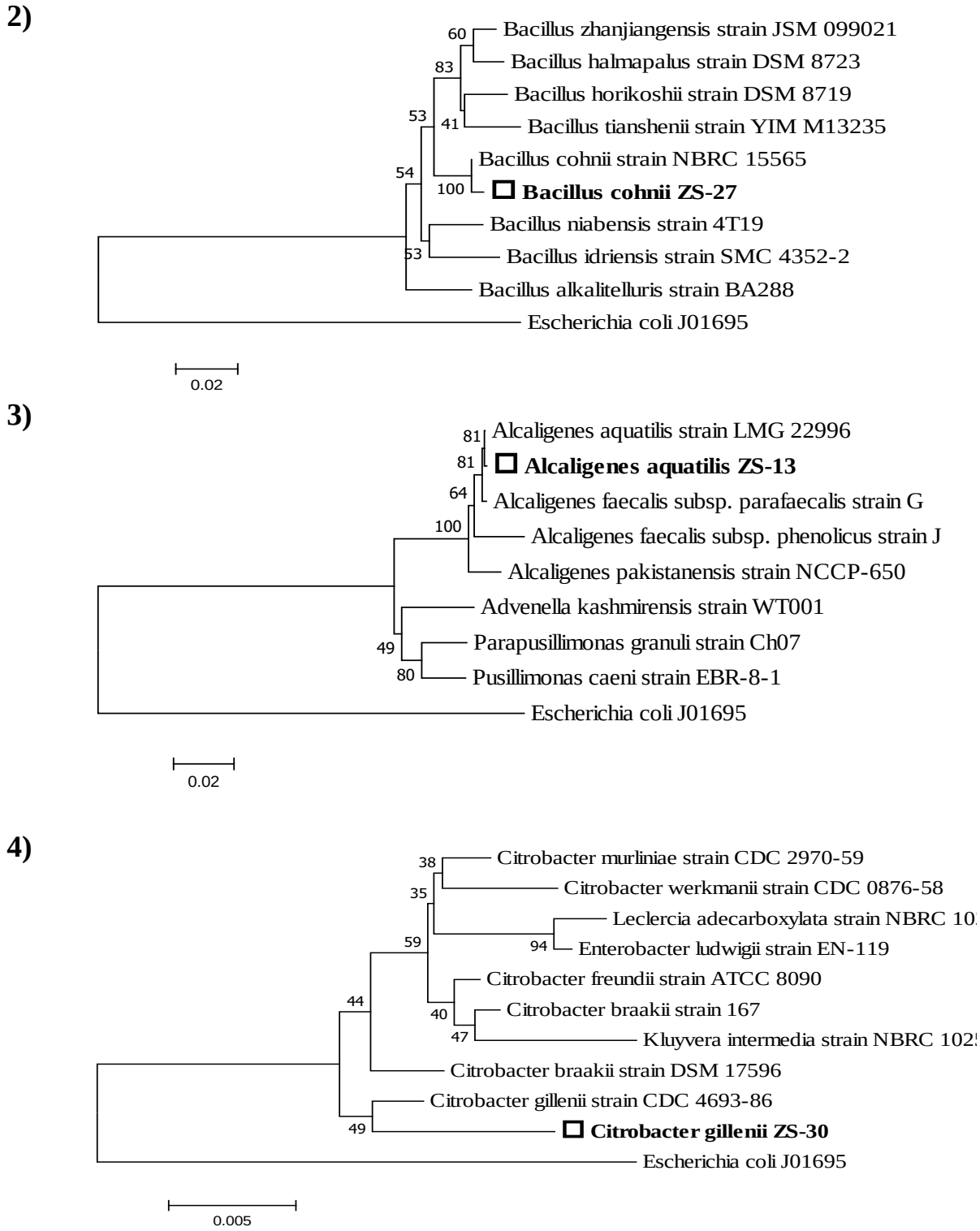


Figure 3. 62: phylogenetic trees for each of the isolates based on 16SrRNA gene sequences.

1) Bacillus mycoides ZS-4, **2)** ***Bacillus cohnii ZS-27,*** **3)** ***Alcaligenes aquatilis ZS-13,*** **4)** ***Citrobacter gillenii ZS-30.*** Maximum-likelihood phylogenetic trees based on 16S rRNA gene sequences. Bootstrap values (expressed as percentages of 1000 replicates) are shown at each node where the bar represents between 0.005 and 0.02 changes per nucleotide position. Evolutionary analyses were conducted in MEGA7.

3.6.3. Isolation of bacteria from the pH adaption microcosms

A number of alkaliphiles were isolated from the CDP fed microcosm that was started at pH 8.5 and the increased to pH 9.5 and finally pH 10.5 (Figure 3.63) and identified via 16s rRNA gene sequencing (Figure 3.64). These alkaliphiles included; *Shewanella putrefaciens* ZS-53, *Brevundimonas diminuta* ZS-36, *Rhodococcus erythropois* ZS-49, *Alcaligenes aquatilis ZS-34*, *Bacillus mycoides ZS-06* and ZS-09, *Clostridium tertium* ZS-83 and *Aeromonas salmonicida* ZS-24 (Table 3.4). The following two bacteria isolates; *Brevundimonas diminuta* ZS-36, *Rhodococcus erythropois* ZS-49 were selected for further investigation and are discussed in detail in later section (3.6.).

Shewanella putrefaciens ZS-53 is a facultative anaerobic Gram-negative rod (Figure 3.63 A), which when grown on FAA at pH 9.5 formed bright pink colonies, with a slightly raised regular edge and smooth texture. The *S.putrefaciens* ZS-53 isolated strain showed a 99% match to the *Shewanella putrefaciens* Type strain NBRC 3908 (Figure 3.64 A). The genus *Shewanella* are aquatic microorganisms inhabiting a wide range of environments with a worldwide distribution (Nealson & Scott, 2006). They are also associated with food spoilage due to exopolysaccharide production and biofilm formation during food processing and may cause bacterial induced corrosion on steel surface (Bagge, Hjelm, Johansen, Huber, & Gram, 2001). Some species of *Shewanella* has been isolated from deep sea sediments (Kato & Nogi, 2001; Martín-Gil, Ramos-Sánchez, & Martin-Gil, 2004) and they can use insoluble Fe(III) as terminal electron acceptors during anaerobic growth (Blakeney, Moulaei, & DiChristina, 2000).

Clostridium tertium ZS-83 Gram-positve spore -forming rods (Figure 3.63 B) demonstrated a 98% match to the Type strain JCM 6289 (Figure 3.64 B). This is the first time *Clostridium tertium* has been isolated from an alkaline environment. This strain was found in the microcosms at pH 10.5 and pH 9.0 prior to isolation on FAA at pH 9.5. *Clostridium tertium* is an aero tolerant clostridium species (Lew, Wiedermann, Sneed, Campos, & McCullough, 1990) found in both the soil and in gastrointestinal tracts of the human and animal (Miller, Brazer, Murdoch, Reller, & Corey, 2001). It is rarely associated with disease in the human and it is considered to be a non-toxin-producing strain with low pathogenic potential (Tappe et al., 2005; Vanderhofstadt et al., 2010). *Clostridium tertium* strains are a ble to utilise a wide range of carbohydrates (Kataoka & Tokiwa, 1998). *Clostridium tertium* ZS-83 was selected for further investigation and is described in greater detail in later section (3.6).

Bacillus mycoides ZS-06 and ZS-09 were isolated from the microcosm at pH 9.5 and pH 10.5 respectively. These Gram positive rods are found in chains (Figure 3.63 E and F), however these two strain are 100% match to *Bacillus mycoides* Type strain NBRC101228 (Figure 3.64 C). The Gram negative bacilli *Aeromonas* salmonicida ZS-24 is a 99% match to the *Aeromonas salmonicida* Type strain ATCC 33658 (Figure 3.64 D).

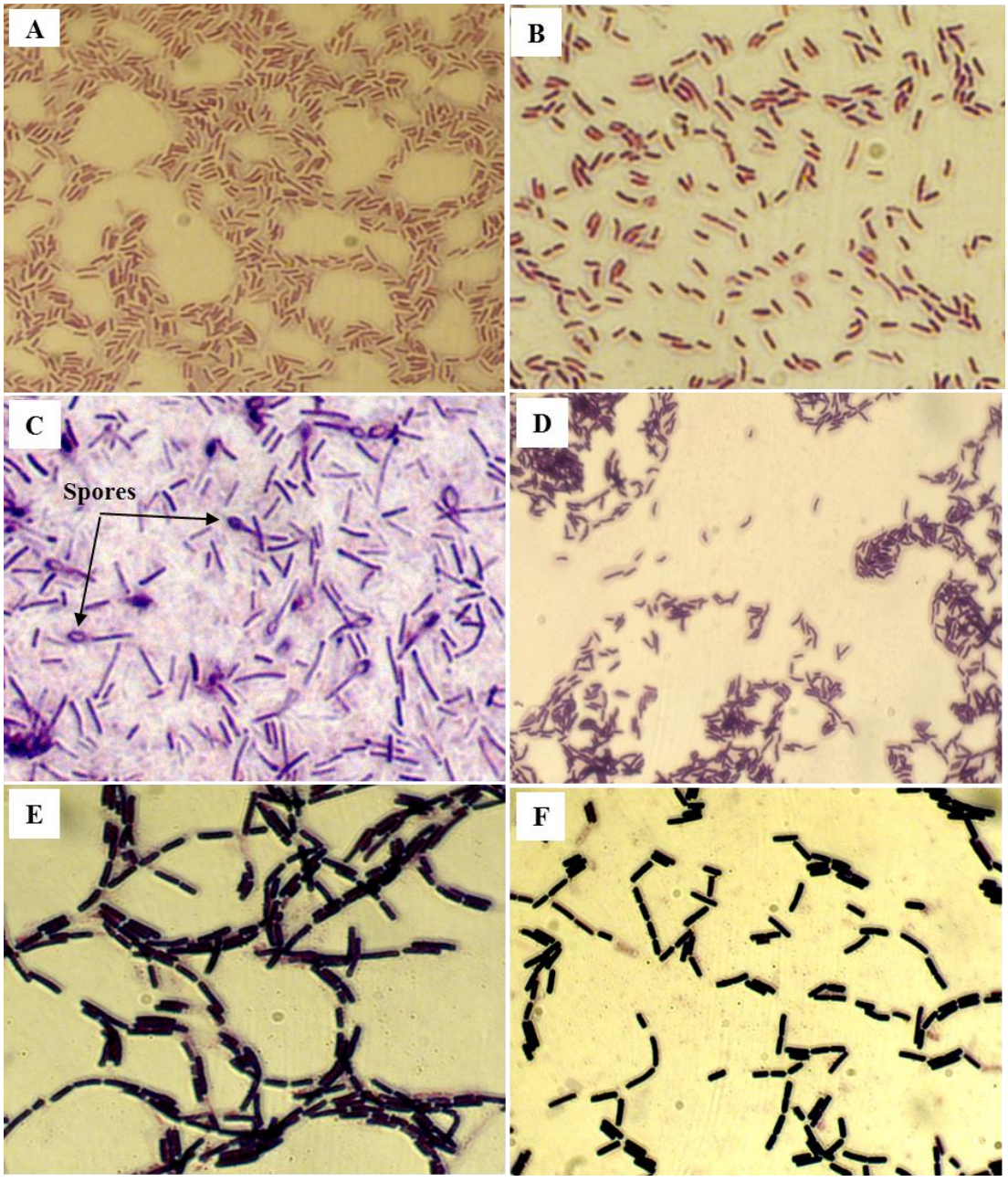
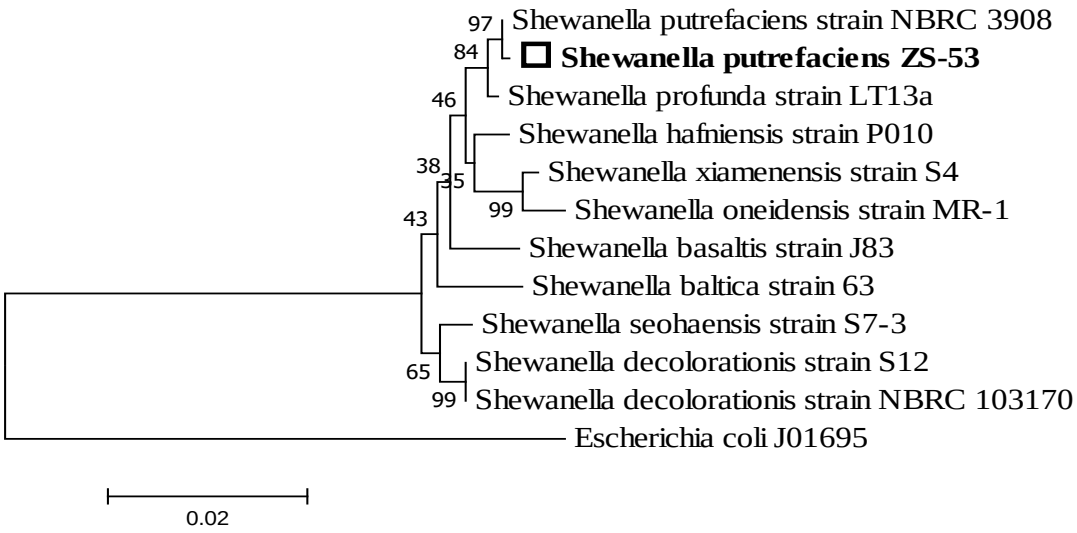


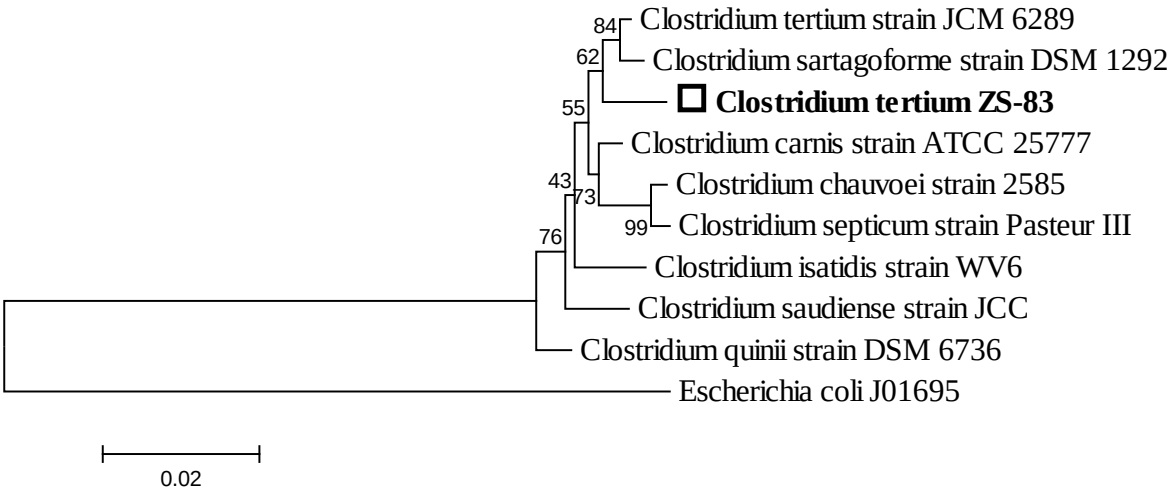
Figure 3. 63: Gram stains of the pure cultures derived from the pH 8.5-10.5 microcosms.

Gram negative bacilli of *S.putrefaceiens* (A) and *A. aquatillis* (B), Gram positive rods of *terminal spore forming C. tertium* (C), Gram positive-rods of *R.erythropois* ZS-49 (D) and Gram positive rods of *Bacillus mycoides* (E and F)

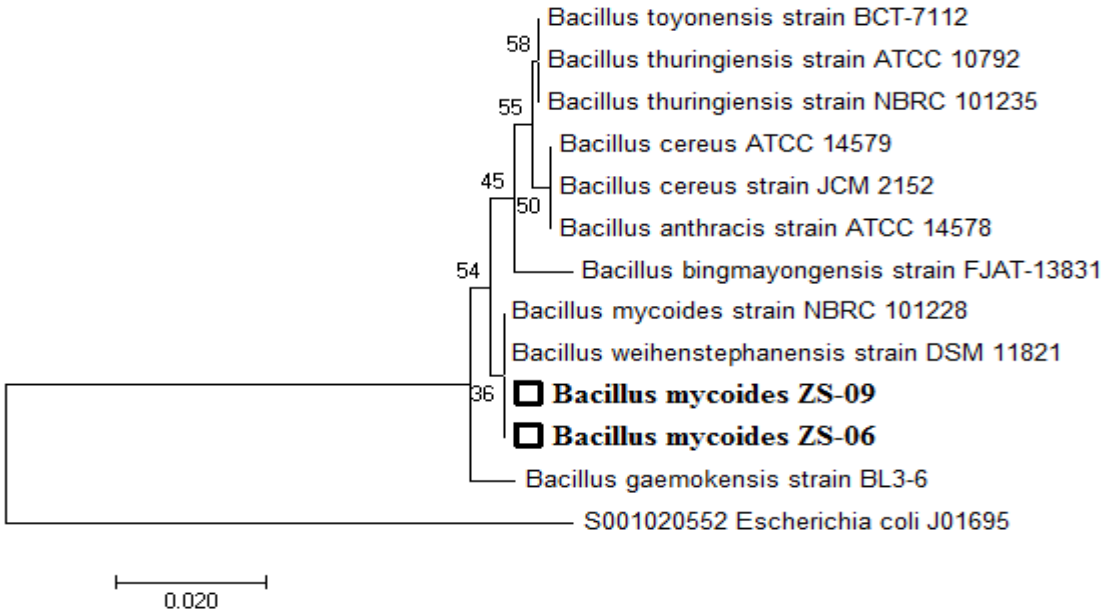
A)


97
Shewanella putrefaciens strain NBRC 3908
84
Shewanella putrefaciens ZS-53
46
Shewanella profunda strain LT13a
Shewanella hafniensis strain P010
38
35
Shewanella xiamenensis strain S4
99
Shewanella oneidensis strain MR-1
43
Shewanella basaltis strain J83
Shewanella baltica strain 63
Shewanella seohaensis strain S7-3
65
Shewanella decolorationis strain S12
99
Shewanella decolorationis strain NBRC 103170
Escherichia coli J01695
0.02

B)


84
Clostridium tertium strain JCM 6289
62
Clostridium sartagoforme strain DSM 1292
Clostridium tertium ZS-83
55
Clostridium carnis strain ATCC 25777
43
73
Clostridium chauvoei strain 2585
99
Clostridium septicum strain Pasteur III
76
Clostridium isatidis strain WV6
Clostridium saudiense strain JCC
Clostridium quinii strain DSM 6736
Escherichia coli J01695
0.02

C)


Bacillus toyonensis strain BCT-7112
58
Bacillus thuringiensis strain ATCC 10792
55
Bacillus thuringiensis strain NBRC 101235
Bacillus cereus ATCC 14579
45
Bacillus cereus strain JCM 2152
50
Bacillus anthracis strain ATCC 14578
54
Bacillus bingmayongensis strain FJAT-13831
Bacillus mycoides strain NBRC 101228
Bacillus weihenstephanensis strain DSM 11821
36
Bacillus mycoides ZS-09
Bacillus mycoides ZS-06
Bacillus gaemokensis strain BL3-6
S001020552 Escherichia coli J01695
0.020

D)

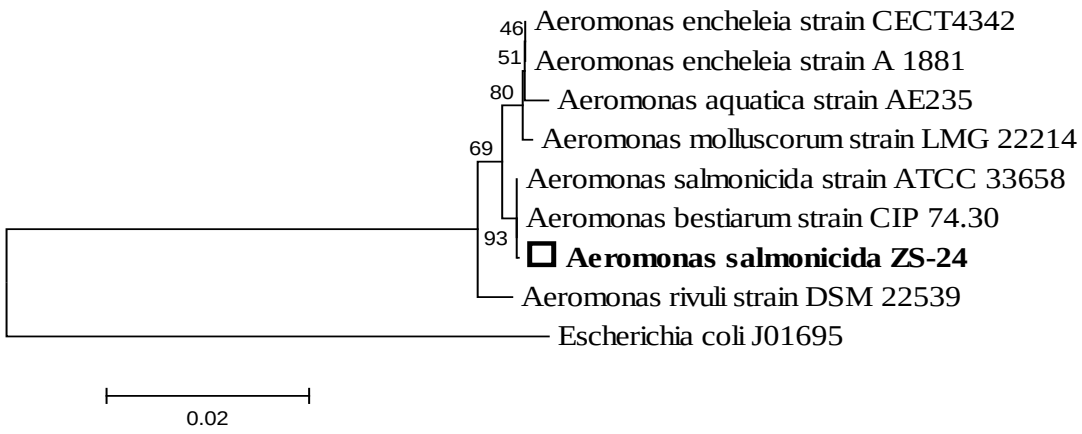


Figure 3. 64: phylogenetic trees for A) *S. putrefaciens* ZS-53, B) *Clostridium tertium* ZS-83, C) *Bacillus mycoides* ZS-09 and ZS-06 and D) *Aeromonas* salmonicida ZS-24

Maximum-likelihood phylogenetic trees based on 16S rRNA gene sequences provide the relationships between these strains and related representatives of their species and the closest phylogenetic relatives of these strains. Bootstrap values (expressed as percentages of 1000 replicates) are shown at each node where the bar represents 0.02 changes per nucleotide position. Evolutionary analyses were conducted in MEGA7.

3.6.4. Discussion

Phylogenetic analysis of all the isolates recovered employing the CLcommunity™ software (ChunLab (Korea)) indicated that the majority of the isolates were Firmicutes, followed by lower numbers of Proteobacteria, Bacteroidetes and Actinobacteria (Table 3.4). The isolates represented the following genus; *Aeromonas, Bacillus, Acinetobacter, Alcaligenes, Citrobacter, Enterococcus, Rhodococcus, Microbacterium, Dietzia, Tessarococcus, Ensifer, Brevundimonas, Azonexus, Shewanella, Clostridium XIVb, Clostridium XIVa and Clostridium XI*. In addition member of the newly proposed genera *Macellibacteroides* (Jabari et al., 2012), *Terrisporobacter* and *Romboutsia* (Gerritsen et al., 2014) were also recovered (Appendix-4, Table 1). The majority of these isolates were obtained from the lower pH microcosms (pH 8.5 to pH 10.0), (Table 3.4) however, some isolates were obtained from microcosm operating at pH 12.0 including *Alishewanella aestuarii* and *Bacillus cohnii*. Facultative anaerobic isolates were more prevalent than the obligate anaerobic bacteria, with the latter being dominated by the *Clostridium* sp. which are easier to isolate and manipulate due to their ability to generate endospores. The notable exception to this was the single *Macellibacteroides* sp. that was isolated since this was a non-spore forming obligate anaerobe.

3.6.5. Key findings

- Isolation from the fermentative microcosm at pH 11.0 provided a more diverse and extensive growth than the fermentative microcosm at pH 12.0 and the sulphate reducing microcosms at pH 11.0 and pH 12.0.
- FAA at pH 10.0 and pH 11.0 were dominated by Gram positive bacteria of the Firmicutes Phylum.
- The Gram positive cocci *Enterococcus gallinarum* ZS-10 was the most common Gram positive isolate followed by *Clostridium bifermentans* ZS-8.
- In the case of the Gram negative bacteria the most common isolate was *Aeromonas salmonicida* ZS-9 isolated from the Charles (2017) microcosms.
- Isolates present at lower levels of abundance included the Gram positive *Exiguobacterium mexicanum* ZS-15, Gram negative *Acinetobacter* sp. ZS-11 and a range of *Clostridium* species ZS strains (16, 17 and 20).

3.7. Characterization of isolated Alkaliphiles

3.7.1. Overview of the isolated alkaliphiles

A wide range of bacterial isolates were recovered from the microcosms operated in this study. Since the overall aim was to isolate ISA degrading bacteria, bacterial isolates were screened on MM agar plates supplemented with either CDP or $Ca(ISA)_2$ (10mM) at pH 9.5 (2.5.8). Bacterial growth on CDP/ISA was compared with growth on general media plates (FAA) and growth under aerobic conditions. The incubation period for these isolates ranged from 3 to 10 days on ISA/MM agar plates. Generally speaking, CDP was more successful than the $Ca(ISA)_2$ plates in growing bacteria which reflects the fact that CDP contains a wider range of substrates. After growth on solid media, isolates were screened for their ability to degrade ISA in liquid culture. The purity of the isolates was confirmed by Gram staining with the majority of isolates being Gram-positive. Once purity was confirmed the isolates were identified by 16SrRNA gene sequencing prior to more detailed characterisation. The majority of these isolates were obtained from the lower pH microcosms (pH 8.5 to pH 10.0), (Table 3.4) however, some isolates were obtained from microcosms operating at pH 12.0 including *Alishewanella aestuarii* and *Bacillus cohnii*. Facultative anaerobic isolates were more prevalence than the obligate anaerobic bacteria.

Many of the isolates were unable to degrade ISA as a sole carbon source in liquid culture however some them were still worthy of further investigation primarily due to their rarity and potential applications in the field of bioremediation and biotechnology. These strains were characterised through both molecular (WGS) and biochemical approaches that included metal tolerance, pH profile, NaCl tolerance, biofilm production, EPS production and FAME analysis.

These bacterial isolates (Table 3.4) include; *Dietzia natronolimnaea* (BI51), *Tessaracoccus lubricantis* (BI41), *Alishewanella aestuarii* (BI28), *Brevundimonas diminuta* (BI36), *Rhodococcus erythropois* (BI49), *Azonexus hydrophilus* (BI70), *Clostridium tertium* (BI85), *Shewanella putrefaciens* (BI53). The resulting genome sequences provided information regarding the metabolic capabilities and survival strategies of these organisms; with the metabolic characterisation providing experimental underpinning of this potential.

Table 3. 4: Summary of alkaliphilic isolates obtained during this research

Alkaliphiles isolated from soil samples of Buxton site after incubated at 25ºC in the microcosms of MM supplemented by CDPs, under anaerobic condition at pH between 8.5 and 12.

SN	Micro-Banking	Phylogeny (PG)	PG %	MM/CDPs Broth pH	ISA Plates pH 9.5		Growth in		Gram reaction
					CDPs	Ca-ISA	AnO_2	O_2	
1	BI 1	*Aeromonas salmonicida*	100	10	+	+	+	+	- B
2	BI 2	*Aeromonas salmonicida*	99	10	++	+	+	+	- B
3	BI 3	*Aeromonas popoffii*	99	10	+	+	+	+	- B
4	BI 4	*Bacillus mycoides*	100	11	+	+	+	+	+ B
5	BI 5	*Bacilli toyonensis*	98	9.5	+	+/-	+	+	+ B
6	BI 6	*Bacillus mycoides*	100	10.5	+	+	+	+	+ B
7	BI 7	*Bacillus mycoides*	100	8.5	+	+	+	+	+ B
8	BI 8	*Acinetobacter guillouiae*	99	9.5	++	+	+	+	+ CB
9	BI 9	*Bacillus mycoides*	99	9	+	+	+	+	+B
10	BI 10	*Alcaligenes aquatilis*	100	11	++	+	+	+	-B
11	BI 11	*Acinetobacter guillouiae*	99	9.0	++	+	+	+	+B
12	BI 13	*Alcaligenes aquatilis*	99	10	++	+	+	+	-B
13	BI 14	*Aeromonas salmonicida*	91	9.0	++	+	+	+	+B
14	BI 15	*Alcaligenes aquatilis*	100	10.4	++	+	+	+	-B
15	BI 16	*Alcaligenes aquatilis*	99	11.0	++	+	+	+	-B
16	BI 17	*Acinetobacter Johnsonii*	99	9.0	++	+	+	+	-B
17	BI 18	*Enterococcus gallinarum*	99	10	+	+/-	+	+	+C
18	BI 19	*Alcaligenes aquatilis*	99	9.0	++	+	+	+	-B
19	BI 21	*Exiguobacterium mexicanum*	99	10	+	+	+	+	+CB
20	BI 22	*Alcaligenes aquatilis*	98	9.0	++	++	+	+	-B
21	BI 23	*Acinetobacter johnsonii*	99	9.0	++	+	+	+	-B
22	BI 24	*Aeromonas salmonicida*	94	9.0	++	+	+	+	-B
23	BI 26	*Aeromonas salmonicida*	94	9.0	++	+	+	+	-B
24	BI 27	*Bacillus cohnii*	99c	12.0	+/-	-	+	+	+B
25	**BI 28**	***Alishewanella aestuarii***	94	12.0	+/-	-	+	+	-B
26	BI 30	*Citrobacter gillenii*	98	8.5	+	+	+	+	-B

SN	Micro-Banking	Phylogeny (PG)	PG %	MM/CDPs Broth pH	ISA Plates pH 9.5		Growth in		Gram reaction
					CDPs	Ca-ISA	AnO_2	O_2	
27	BI 34	*Alcaligenes aquatilis*	98	10.5	+	-	+	+	-B
28	BI 35	*Ensifer adhaerens*	99	8.5	+	+	+	+	+C
29	**BI 36**	***Brevundimonas diminuta***	99	10	+	+	+	+	-CB
30	BI 38	*Microbacterium kitamience*	99	9.0	+	+	+	+	
31	**BI 40**	***Macellibacteroides fermentans***	100	10	+	+	+	-	+B
32	**BI 41**	***Tessaracoccus lubricantis***	98	9.5	+	+	+	+	+ C
33	BI 42	*Dietzia natronolimnaea*	99	10.5	+	+	+	+	+C
34	BI 45	*Dietzia natronolimnaea*	89	10	+/-	+/-	+	+	+C
35	BI 46	*Enterococcus gallinarum*	99	9.0	+	+/-	+	+	+C
36	**BI 49**	***Rhodococcus erythropois***	99	9.0	+	-	+	+	+B
37	**BI 51**	***Dietzia natronolimnaea***	99	10	+	+	+	+	+C
38	BI 53	*Shewanella putrefaciens*	100	9.0	+	+/-	+	+	-B
39	BI 54	*Citrobacter gillenii*	100	9.0	+	+	+	+	-B
40	BI 55	*Aeromonas salmonicida*	100	10	+	+	+	+	-B
41	BI 59	*Dietzia natronolimnaea*	99	9.5	+	+	+	+	+C
42	BI 60	*Shewanella putrefaciens*	99	9.0	+	-	+	+	-B
43	BI 66	*Aeromonas salmonicida*	99	9.0	+	+	+	+	-B
44	BI 69	*Macellibacteroides fermentans*	100	9.0	+	+	+	-	+B
45	**BI 70**	***Azonexus hydrophilus***	98	9.0	+	+/-	+	+	-B
46	BI 74	*Shewanella putrefaciens*	99	9.0	+	+	+	+	-B
47	BI 80	*Clostridium propionicum*	99	9	+	+	+	-	+B
48	BI 82	*Clostridium celerecrescens*	99	9.0-10	+	+	+	-	+B
49	**BI 83, 85**	***Clostridium tertium***	99	9.0-10	+	+	+	-	+B
50	BI 89	*Clostridium sartagaforme*	99	9.0	+	+	+	-	+B
51	BI 86, 91	*Terrisporobacter petrolearius*	99	9.0-10	+	+	+	-	+B
52	BI 87, 92	*Aeromonas taiwanensis*	99	9.5-10	+	+	+	+	-B
53	BI 93, 97	*Romboutsia sedimentorium*	99	9.5-10.5	+	+	+	-	+B
54	BI 99	*Alcaligenes aquatilis*	99	10	+	+	+	+	-B

BI= bacterial isolate, -B= Gram negative bacilli, +C= Gram positive cocci, Bold= Whole genome sequencing, yellow shading= ISA degrading bacteria

3.7.2. Overall characterisation of the Alkaliphiles isolates

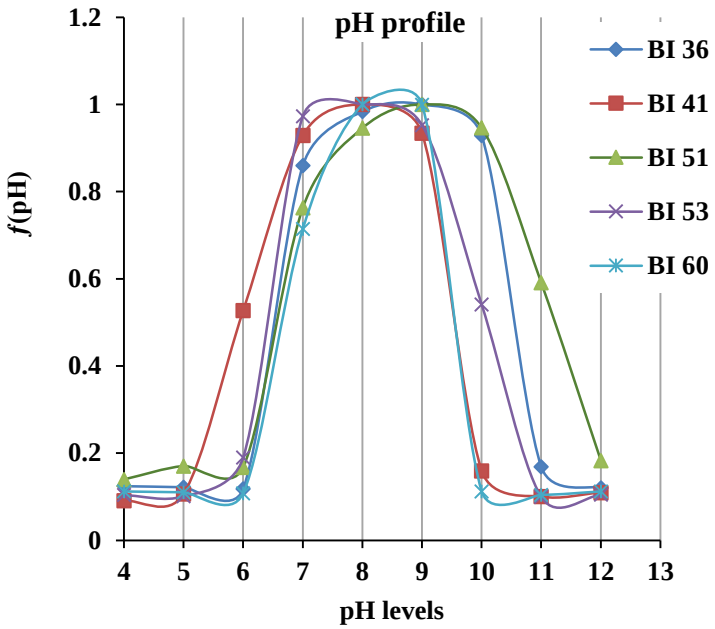
The aim of this part of the research was to characterise the isolates based on their metabolic capabilities. The overall aim being to identify strains that are unique when compared to those already described in the scientific literature.

3.7.2.1. pH profiles

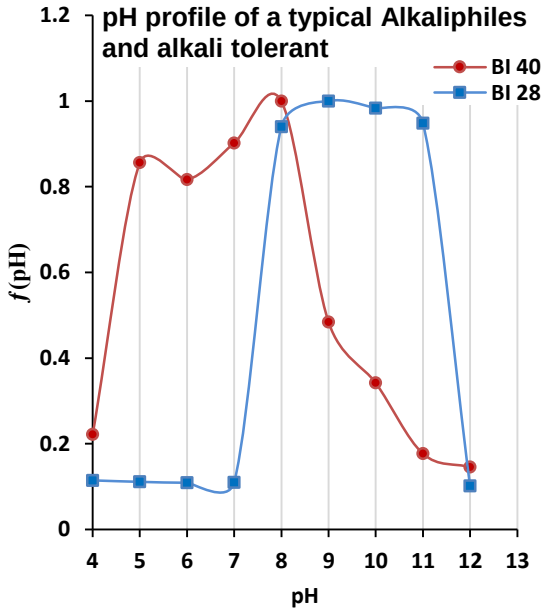
The majority of these isolates were able to grow on CDPs/MM and/or ISA/MM media at pH 9.5, under anaerobic condition at 25°C. The pH profiles of these isolates indicated that the majority were moderately alkaliphilic with optima between pH 7.0 and pH 9.0, whilst a smaller number had much wider pH profiles with optima ranging up to pH 10.0 (Figure 3.65).

When all the isolates are considered, the isolates *Brevundimonas diminuta* (BI36), *Tessaracoccus lubricantis* (BI41), *Dietzia natronolimnaea* (BI51), *Rhodococcus erythropois* (BI49) and *Azonexus hydrophilus* (BI70) all had pH ranges between pH 6.0 and pH 10 with optimum growth of pH between pH 7.0 and pH 9.0 (Figure 3.65 A). Some isolates were able to survive at pH values up to pH 12.0. These strains include *Alishewanella aestuarii* HH-ZS strain (BI 28) which had a growth range between pH 7.0 and pH 11 with an optimum of pH 9.0 and *Macellibacteroides fermentans* HH-ZS strain (BI 40) which had a growth range between pH 5.0 and 10 and an optimum pH between pH 7-8 (Figure 3.65 B). The other group of alkaliphiles presented below (Figure 3.65 C), include *Bacillus cohnii* (BI 27) with an optimum pH 9 and a growth range of pH 7-11, *Dietzia natronolimnaea* (BI 45) with a wide pH range for growth of pH 7.0-11 and an optimum of pH 9.0, the other strains (*Bacillus toyonensis* (BI5), *Alcaligenes aquatilis* (BI34), *Shewanella putrefaciensis* (BI60) and *Shewanella putrefaciensis* (BI53) and *Citrobacter gillenii* (BI54)) all had pH ranges between pH 6.0-9.5 and optimum pH 8.0.

A)


pH profile
BI 36
BI 41
BI 51
BI 53
BI 60
f(pH)
pH levels
1.2
1
0.8
0.6
0.4
0.2
0
4
5
6
7
8
9
10
11
12
13

B)


pH profile of a typical Alkaliphiles and alkali tolerant
BI 40
BI 28
f(pH)
pH
1.2
1
0.8
0.6
0.4
0.2
0
4
5
6
7
8
9
10
11
12
13

C)

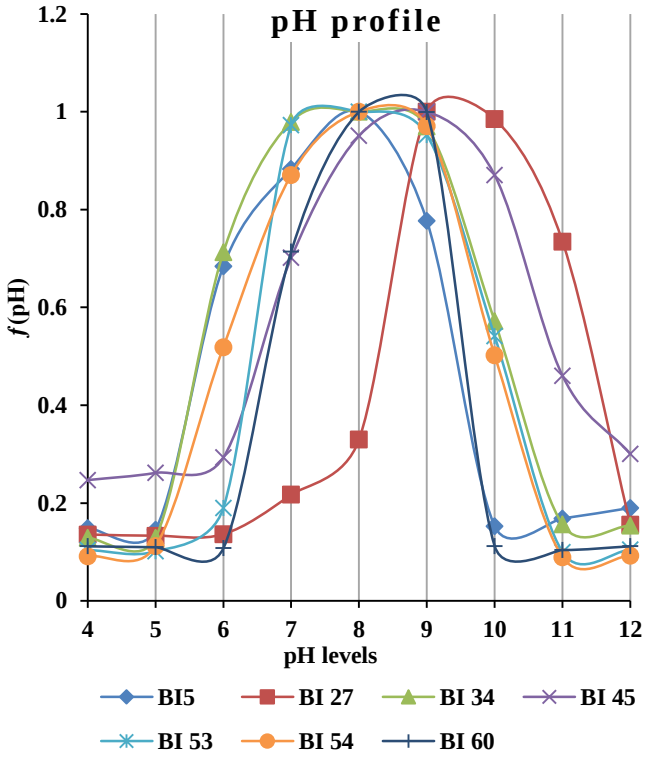


Figure 3. 65: pH profiles curves of Alkaliphiles and alkali tolerant bacterial isolates

The sum of growth curves illustrating the pH ranges for bacterial growth. *Brevundimonas diminuta* (BI 36), *Tessaracoccus lubricantis* (BI 41), *Dietzia natronolimnaea* (BI 51), *Rhodococcus erythropois* (BI 49), *Azonexus hydrophilus* (BI 70) *Alishewanella aestuarii* HH-ZS strain (BI 28) *Macellibacteroides fermentans HH-ZS* (BI 40) *Bacillus cohnii* (BI 27) *Dietzia natronolimnaea* (BI 45) *Bacillus toyonensis* (BI 5), *Alcaligenes aquatilis* (BI 34), *Shewanella putrefaciensis* (BI 60) and *Shewanella putrefaciensis* (BI 53) and *Citrobacter gillenii* (BI 54)

3.7.2.1.1. Discussion

The pH profiles of these isolates are in agreement with related strains provided by previous studies. For instance, bacterial isolate *Brevundimonas diminuta* (BI36), had pH range between pH 6.0 and pH 10 (Figure 3.65 A and Appendix-5), the related strain of this isolated strain was able to release an extracellular metalloproteases that had an optimum pH for proteolytic activity ranging from 7.0 to 11.0 (Chaia et al., 2000). *Tessaracoccus lubricantis* (BI41), had a pH range between pH 6.0 and pH 9.5, this result is similar with data provided in the previous studies for *T. bendigoensis* Ben 106^{T} (Maszenan, Seviour, Patel, Schumann, & Rees, 1999) and for *T. lubricantis* KSS-17Se^{T} (Kämpfer, Lodders, Warfolomeow, & Busse, 2009), however a higher pH range was demonstrated by *T. flavescens* SST-39^{T} from pH 6.1 to pH 12.1 (D. W. Lee & Lee, 2008).

Dietzia natronolimnaea (BI 51), had a pH range from pH 7.0 to pH 11.0, optimum at pH 9.0, which is a wider pH range than that reported for most other *Dietzia* sp. For instance, *Dietzia natronolimnaea* JQ-AN is able to degrade aniline in an alkaline environment at optimum pH 8.0 (Jin et al., 2012).

Rhodococcus erythropois (BI49) had a pH range from pH 6.0 to pH 10 and an optimum ranging from pH 7.0 to pH 8.0. In a previous study, *Rhodococcus erythropolis* strain Y2 isolated from soil contaminated with haloalkanes compounds had a pH optima ranging from pH 9.2-9.5 (Sallis, Armfield, Bull, & Hardman, 1990). *Bacillus cohnii* (BI 27), had a pH range from pH 7.0 to pH 11.5 and an optimum from 9.0 to pH 10.0.

3.7.2.2. Biochemical characterization of alkaliphilic isolates

There were significant differences in substrate utilisation when the isolates were compared using the API20A (Table 3.5) and Biolog systems (Tables 3.6 and 3.7). When compared on their carbohydrate utilisation *Macellibacteroides fermentans* (BI40), *Tessaracoccus lubricantis* (BI41), *Bacilli toyonensis* (BI5), *Citrobacter gillenii* (BI54), *Clostridium celerecrescens* (BI82) and *Clostridium sartagaforme* (BI89) were capable of utilizing almost all substrates, while the following strains; *Alishewanella aestuarii* (BI28), *Ensifer adhaerens* (BI35), *Brevundimonas diminuta* (BI36), *Dietzia natronolimnaea* (BI45), *Rhodococcus erythropois* (BI49), *Dietzia natronolimnaea* (BI51), *Azonexus hydrophilus* (BI70) were unable to utilize any of the carbohydrates provided in the system.

Although all these organisms were isolated on media fed with carbohydrate derived carbon sources (ISA and CDP), there were numerous strains with a limited ability to utilize carbohydrates. This suggests that ISA degradation is not a marker for carbohydrate utilisation, presumably due to the structural differences between ISA and sugars such as glucose.

Table 3. 5: Biochemical identification of bacterial isolates in anaerobic condition

The biochemical tests indicated that not all isolates were capable of utilizing all the substrates provided by the API 20A system, however, a smaller number of strains were capable of utilizing almost all the substrates. *Macellibacteroides fermentans* (BI40), *Tessaracoccus lubricantis* (BI41), *Bacilli toyonensis* (BI5), *Citrobacter gillenii* (BI54), *Clostridium sartagaforme* (BI89), *Aeromonas samonicida* (BI55). *Alishewanella aestuarii* (BI28), *Ensifer adhaerens* (BI35), *Brevundimonas diminuta* (BI36), *Dietzia* sp. (BI 45, 51, 59), *Rhodococcus erythropois* (BI49), *Shewanella putrefaciensis* (BI53), *Azonexus hydrophilus* (BI70), *Clostridium tertium* (BI 85), *Terrisporobacter mayombei* (BI 91), *Romboutsia sedimentorum* (BI 93) and *Clostridium celerecrescens* (BI82).

TESTS	BI28	BI35	BI36	BI40	BI41	BI45,51, 59	BI5	BI49	BI 54	BI 55	BI 53	BI70	BI85	BI91	BI93	BI82	BI89
Oxidase	+	+	+	-	-	-	+	-	-	+	+	+	-	-	-	-	-
Catalase	+	-	+	+	+	+	-	+	+	+	-	-	-	-	-	-	-
Indol	-	-	-	-	-	-	-	-	-	-	-		-	-	-	+	-
Urease	-	-	+	-	-	-	-	-	-	+	-	+	-	-	-	-	-
Protease (gelatine)	-	-	+	-	-	-	+	-	-	-	-	-	-	-	+	-	-
B-glucosidase (Esculin)	+	-	-	+	+	-	+	+	+	-	-	-	+	+	+	+	+
H_2S	+	-	-	+	+	-	-	+	+	+	-	-	+	+	+	+	-
Acidification of Carbohydrates																	
D-Glucose	-	-	-	+	+	-	+	-	+	-	-	-	+	+	+	+	+
D-Mannitol	-	-	-	+	+	-	+	-	-	-	-	-	+	-	-	+	+
D-Lactose	-	-	-	+	+	-	+	-	+	-	-	-	-	-	-	+	+
D-Sucrose	-	-	-	+	+	-	+	-	-	+	-	-	-	-	-	-	+
D-Maltose	-	-	-	+	+	-	+	-	+	-	-	-	+	+	+	+	+
Salicin	-	-	-	+	+	-	+	-	+	+	-	-	+	-	-	+	+
D-xylose	-	-	-	+	+	-	+	-	-	+	+	-	-	+	-	+	+
L-arabinose	-	-	-	+	+	-	+	-	+	+	+	-	-	-	-	+	+
Glycerol	-	-	-	+	+	-	+	-	+	+	-	-	-	+	+	-	+
D-cellobiose	-	-	-	+	-	-	+	-	+	+	-	-	+	-	-	+	+
D-mannose	-	-	-	+	+	-	+	-	+	+	-	-	+	-	+	+	+
D-melezitose	-	-	-	+	-	-	+	-	-	-	-	-	-	-	-	-	+
D-raffinose	-	-	-	+	+	-	+	-	+	-	-	-	-	+	-	+	+
D-sorbitol	-	-	-	+	+	-	+	-	+	-	-	-	-	+	+	-	+
L-rhamnose	-	-	-	+	+	-	+	-	+	-	-	-	-	-	-	+	+
D-trehalose	-	-	-	+	+	-	+	-	+	-	-	-	-	-	+	+	+

Table 3. 6: Biolog of PM1 microplate of carbon sources for Alkaliphiles isolates

Substrates PM1	BI 28	BI 41	BI 51	BI 55	BI 70	Substrates PM1	BI 28	BI 41	BI 51	BI 55	BI 70
Negative Control	-	-	-	-	-	L-Asparagine	-	+	+	+	+
L-Arabinose	-	+	-	+	-	D-Aspartic Acid	-	+	-	+	-
N-Acetyl-D-Glucosamine	+	+	-	+	-	D-Glucosaminic	+	+	-	+	-
D-Saccharic Acid	+	-	-	-	-	1,2-Propanediol	-	+	-	+	-
Succinic Acid	-	-	-	+	+	Tween 40	+	+	+	+	-
D-Galactose	+	+	-	+	-	α -Keto-Glutaric acid	+	+	-	+	+
L-Aspartic Acid	+	-	-	+	+	α -Keto-Butyric acid	+	+	-	+	-
L-Proline	+	-	-	+	-	α -Methyl-D-Galactoside	-	+	-	+	-
D-Alanine	-	-	-	+	-	α-D-Lactose	-	+	-	+	-
D-Trehalose	-	+	+	+	-	Lactulose	-	+	-	+	-
D-Mannose	-	+	+	+	-	Sucrose	-	+	+	+	-
Dulcitol	-	-	-	+	-	Uridine	-	+	-	+	-
D-Serine	+	+	-	+	-	L-Glutamine	+	+	+	+	+
D-Sorbitol	-	-	-	+	-	m-Tartaric Acid	-	-	-	-	-
Glycerol	-	+	-	+	-	D-Glucose-1-phosphate	+	+	-	+	-
L-Fucose	-	+	-	+	-	D-Fructose-6-phosphate	+	+	-	+	-
D-Glucuronic Acid	-	+	-	+	-	Tween 80	+	+	+	+	-
D-Gluconic Acid	+	+	-	+	-	α –Hydroxy Glutaric acid	+	-	-	+	-
D,L-α-Glycerol Phosphate	-	+	-	+	-	α –Hydroxy Butyric acid	+	+	-	+	-
D-Xylose	-	+	-	+	-	β-Methyl-DGlucoside	+	+	-	+	-
L-Lactic Acid	-	+	-	+	+	Adonitol	-	+	-	+	-
Formic Acid	-	-	-	+	-	Maltotriose	-	+	+	+	-
D-Mannitol	-	+	-	+	-	2-Deoxy Adenosine	-	+	-	+	-
L-Glutamic Acid	-	+	+	+	+	Adenosine	-	+	-	+	-
D-Glucose-6-phosphate	-	+	-	+	+	Glycyl-L-Aspartic acid	-	+	-	+	-
D-Galactonic Acid	-	-	-	+	-	Citric Acid	-	-	+	+	-
D,L-Malic Acid	-	-	-	+	+	m-Inositol	-	+	-	+	-
D-Ribose	+	+	-	+	+	D-Threonine	+	+	-	-	-
Tween 20	+	+	+	+	-	Fumaric Acid	-	-	-	+	+
L-Rhamnose	-	+	-	+	-	Bromo Succinic acid	+	-	-	+	+
D-Fructose	+	+	+	+	-	Propionic Acid	+	+	+	+	-
Acetic Acid	-	+	+	+	+	Mucic Acid	+	-	-	+	-
Glycyl-LGlutamic acid	-	-	-	+	-	D-Psicose	-	+	-	+	-
Tricarballylic acid	-	-	-	-	-	L-Lyxose	-	+	+	+	+
L-Serine	+	-	-	+	-	Glucuronamide	+	+	-	+	-
L-Threonine	+	+	-	+	-	Pyruvic Acid	+	+	+	+	+
L-Alanine	+	-	-	+	-	L-Galactonic acid	-	+	-	+	-
L-Alanyl-Glycine	+	-	-	+	-	D-Galacturonic acid	-	+	-	+	-
Acetoacetic Acid	+	+	+	+	-	Phenylethylamine	-	-	-	-	-
N-Acetyl-β-D-Mannosamine	-	+	-	+	-	2-Aminoethanol	-	-	-	-	-
Mono Methyl succinate	-	+	+	+	-	α -D-Glucose	-	+	+	+	-
Methyl Pyruvate	-	+	+	+	+	Maltose	-	+	-	+	-
D-Malic Acid	-	-	-	-	-	D-Melibiose	-	+	-	+	-
L-Malic Acid	-	-	-	+	+	Thymidine	-	+	-	+	-
Glycyl-L-Proline	+	+	-	+	-	Glycolic Acid	-	-	-	+	-
p-Hydroxy phenyl acetic	-	-	-	-	+	Glyoxylic Acid	-	-	-	-	-
m-Hydroxy phenyl acetic	+	-	-	-	-	D-Cellobiose	-	+	-	+	-
Tyramine	+	+	-	+	-	Inosine	-	+	-	+	-

Table 3. 7: Biolog of PM2 microplate of carbon sources for Alkaliphiles isolates

Substrates MP2	BI 28	BI 41	BI 51	BI 55	BI 70	Substrates PM2	BI 28	BI 41	BI 51	BI 55	BI 70
Negative Control	-	-	-	-	-	D-Raffinose	-	+	-	+	-
Chondroitin Sulfate C	-	+	-	+	-	Salicin	+	+	-	+	-
α -Cyclodextrin	+	-	-	+	-	Sedoheptulosan	-	+	-	+	-
β -Cyclodextrin	+	-	-	+	-	L-Sorbose	-	+	-	+	-
γ-Cyclodextrin	+	-	-	+	-	Stachyose	-	+	-	+	-
Dextrin	+	+	+	+	-	D-Tagatose	+	+	-	+	-
Gelatin	+	+	_	+	_	Turanose	-	+	-	+	-
Glycogen	-	+	_	+	_	Xylitol	-	+	-	-	-
Inulin	-	+	_	+	_	N-Acetyl-D-Glucosaminitol	+	+	-	+	-
Laminarin	+	-	_	+	_	γ-Amino Butyric Acid	-	-	-	+	-
Mannan	-	-	_	+	_	σ-Amino Valeric Acid	-	-	-	+	-
Pectin	-	+	_	+	_	Butyric Acid	+	-	+	+	+
N-Acetyl-D-Galactosamine	-	+	_	+	_	Capric Acid	+	+	-	-	-
N-Acetyl-Neuraminic Acid	-	+	_	+	_	Caproic Acid	+	-	+	+	+
β -D-Allose	-	+	_	+	_	Citraconic Acid	-	-	_	+	_
Amygdalin	+	-	_	+	_	Citramalic Acid	-	-	_	-	_
D-Arabinose	+	+	_	+	_	D-Glucosamine	+	+	_	+	+
D-Arabitol	-	+	_	-	_	2-Hydroxy Benzoic Acid	-	-	_	-	_
L-Arabitol	-	+	-	+	-	4-Hydroxy Benzoic Acid	-	-	_	-	_
Arbutin	-	+	-	+	-	β-Hydroxy Butyric Acid	+	+	+	+	+
2-Deoxy-D-Ribose	+	+	-	+	+	γ-Hydroxy Butyric Acid	-	-	_	+	_
i-Erythritol	-	-	-	-	-	a-Keto-Valeric Acid	+	-	_	+	_
D-Fucose	-	+	-	+	-	Itaconic Acid	-	-	_	-	_
3-0-β-D-Galacto-pyranosyl-D-Arabinose	-	+	-	+	-	5-Keto-D-Gluconic Acid	+	+	_	+	+
Gentiobiose	+	+	-	+	-	D-Lactic Acid Methyl Ester	-	-	_	-	+
L-Glucose	-	-	-	+	-	Malonic Acid	-	-	+	+	+
Lactitol	-	+	-	+	-	Melibionic Acid	+	+	+	+	_
D-Melezitose	-	+	-	+	-	Oxalic Acid	+	+	+	+	_
Maltitol	-	+	-	+	-	Oxalomalic Acid	+	+	-	+	-
a-Methyl-D-Glucoside	-	+	-	+	-	Quinic Acid	-	-	-	+	-
β-Methyl-D-Galactoside	-	+	-	+	-	D-Ribono-1,4-Lactone	-	-	_	+	_
3-Methyl Glucose	-	+	-	+	-	Sebacic Acid	-	-	_	-	_
Acetamide	-	-	_	+	_	D,L-Carnitine	-	-	-	+	_
L-Alaninamide	+	-	_	+	_	Sec-Butylamine	-	-	+	-	_
N-Acetyl-L-Glutamic Acid	+	-	_	+	_	D.L-Octopamine	-	-	-	+	_
L-Arginine	-	-	_	+	_	Putrescine	-	-	+	+	_
Glycine	+	-	_	+	_	Dihydroxy Acetone	-	+	-	+	+
L-Histidine	+	-	_	+	_	2,3-Butanediol	-	-	-	+	_
L-Homoserine	-	-	_	+	_	2,3-Butanone	-	-	-	+	_
Hydroxy-L-Proline	-	-	_	+	_	3-Hydroxy 2-Butanone	-	-	-	+	_
L-Isoleucine	+	-	_	+	_	β-Methyl-D-Glucuronic Acid	-	-	+	+	_
L-Leucine	+	-	-	+	-	α-Methyl-D-Mannoside	-	+	-	-	-
L-Lysine	-	-	-	+	-	β-Methyl-D-Xyloside	-	+	-	+	-
L-Methionine	-	-	-	-	-	Palatinose	-	+	-	+	-
L-Ornithine	-	-	-	+	-	Sorbic Acid	-	-	-	-	-
L-Phenylalanine	-	-	-	+	-	Succinamic Acid	+	-	-	+	+
L-Pyroglutamic Acid	-	-	-	+	-	D-Tartaric Acid	-	-	-	-	-
L-Valine	+	-	-	+	-	L-Tartaric Acid	-	-	-	-	-

3.7.2.3. Heavy metal and sodium chloride tolerance

The isolates demonstrated a varied ability to tolerate heavy metals and sodium chloride (Table 3.8). An example data set for heavy metal tolerance is provided in Figure 3.66 and the other graphs in appendix-5. Some bacteria can tolerant high concentration of NaCl up to 20%. For example, *Halothemothrix orenii*, is a Gram-negative, halophilic, thermophilic, strictly anaerobic bacterium isolated from a saline lake sediment in Tunisia. This strain showed growth at up to 20% NaCl, with an optimum between 5-10% at a pH up to pH 8.2 (Cayol et al., 1994). Highly halotolerant *Haloincola saccharolytica* showed growth at a NaCl concentration up to 30%, with an optimum at 10% at pH 8.0 (Zhilina et al., 1992). The highest tolerance for NaCl (%) in this study was recorded by *Clostridium tertium* (BI 85), at MIC 13.7% and *Macellibacteroides fermentans* (BI40) at MIC 13%. In a previous study, strain LIND7H^T of the *Macellibacteroides fermentans* showed a growth in the presence of up to 2% NaCl (Jabari et al., 2012).

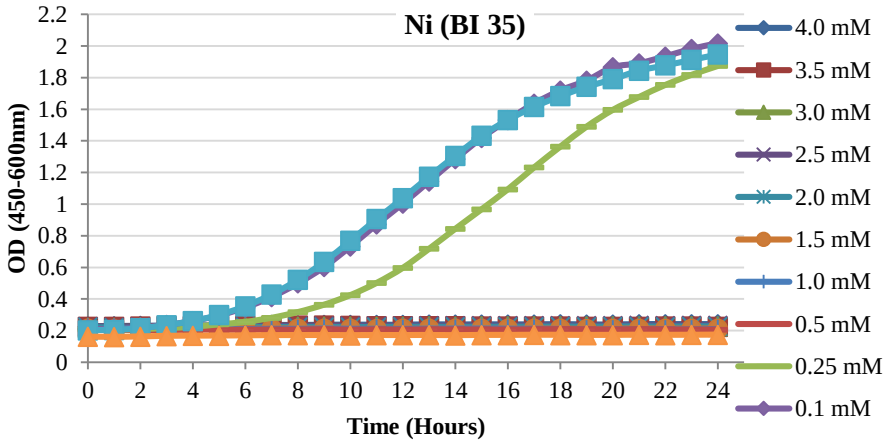
In respect of heavy metals, the highest tolerance was showed by *Brevundimonas diminuta* (BI36) with lead (MIC 30 mM) and Zinc (MIC 12 mM) and by *Aeromonas samonicida (*BI55), *Rhodococcus erythropois* (BI49) and *Clostridium tertium* (BI 85) with Lead (MIC >5.0 mM), followed by Zinc, (MIC >5.0 mM). In addition, *Clostridium tertium* (BI 85) showed a tolerance to all heavy metals (Table 3.15).

Table 3. 8: Alkaliphiles heavy metals and NaCl tolerance for the selected isolates

Alishewanella aestuarii (BI28), *Brevundimonas diminuta* (BI36), *Macellibacteroides fermentans* (BI40), *T. lubricantis* (BI41), *R. erythropois* (BI49), *D. natronolimnaea* (BI51), *Aeromonas samonicida* (BI55), *Azonexus hydrophilus* (BI70), *CL. tertium* (BI 85).

Heavy metals tolerance MIC (mM) and NaCl tolerance (%)									
Heavy metal	**16SrRNA identified bacteria isolates**								
	BI 28	**BI36**	**BI40**	**BI41**	**BI49**	**BI51**	**BI 55**	**BI70**	**BI85**
$NiCl_2$	0.86	0.44	2.10	0.01	2.0	0.42	2.4	1.0	2.5
$CdCl_2$	NG	0.95	1.85	5.1	NG*	0.2	0.4	0.25	(>5.0)*
$CoCl_2$	0.92	1.93	0.57	2.3	2.0	0.47	2.8	1.5	5.0
$PbCl_2$	3.8	30	1.06	2.4	(>5.0)*	2.5	(>5.0)*	0.25	(>5.0)*
$ZnCl_2$	1.2	12.0	3.39	8.2	(>5.0)*	1.09	3.0	0.5	(>5.0)*
$CuCl_2$	2.8	3.63	2.25	1.87	6.0	4.9	4.0	NG*	5.0
Sodium chloride tolerant %									
NaCl %	4.7	5.6	13	5.4	4.6	11	3.7	8	13.5

A) An example of a growth curve under a different heavy metal concentration



B) Inhibition profile

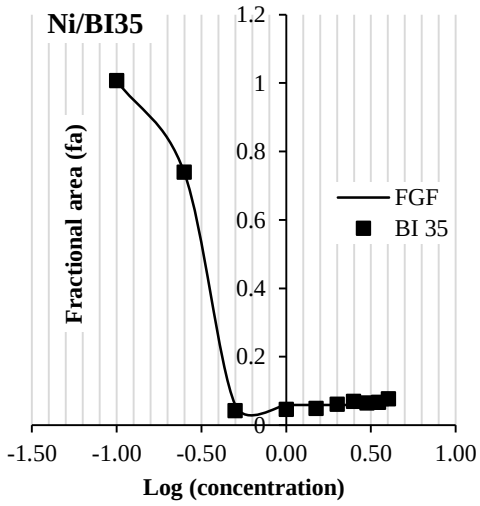


Figure 3. 66: An example of the heavy metal tolerant growth curve and inhibition profile

The inhibition profile of Nickel chloride against *Ensifer adhaerens* (BI35) (A): (■), observed FA; (▬), fitted Gompertz function (FGF) (B).

3.7.2.3.1. Discussion

The response of microorganisms to heavy metals depends on many factors such as the natural habitat of the organism, the type of metal, the concentration and bioavailability of metals and the detoxification mechanisms available to the organism (Coblenz & Wolf, 1994; De Rore, Top, Houwen, Mergeay, & Verstraete, 1994; Farrow et al., 1995; Hashemi, Leppard, & Kushnert, 1994). However, the strains isolated in this study showed higher resistance to heavy metals when compared to previous published studies performed on 25 isolates for Cu, Cr (MIC from 0.02 to 1.5 mM), Co (MIC from 0.05 to 0.8 mM) and Cd, Zn (from 0.2 to 1.5) (Hassen, Saidi, Cherif, &

Boudabous, 1998). In previous study, *Alishewanella* sp. WH16-1 strain showed the MICs for Pb, Cd, and Cu were 10, 0.08 and 1.0 mM, respectively (Xia et al., 2016).

3.7.2.4. Production of extracellular polymeric substances and biofilm formation

Eleven of the bacterial isolates were clear EPS producers: *Brevundimonas diminuta* (BI36), *Tessaracoccus lubricantis* (BI41), *Dietzia natronolimnaea* (BI45, 51), *Enterococcus gallinarum* (BI46), *Rhodococcus erythropois* (BI49), *Aeromonas samonicida* (BI55), *Shewanella putrefaciensis* (BI53, 60), *Citrobacter gillenii* (BI54 and BI62), this finding are consistent with the fact that a biofilm and EPS are formed by bacteria as a protective barrier against extreme conditions such as high pH. The EPS was detected by a fluorescence microscope, using 0.1% fluorescent brighter 28, it is a blue haze surrounded bacterial cells (Figure 3.67). Further studies would be needed to identify and characterise the EPS produced.

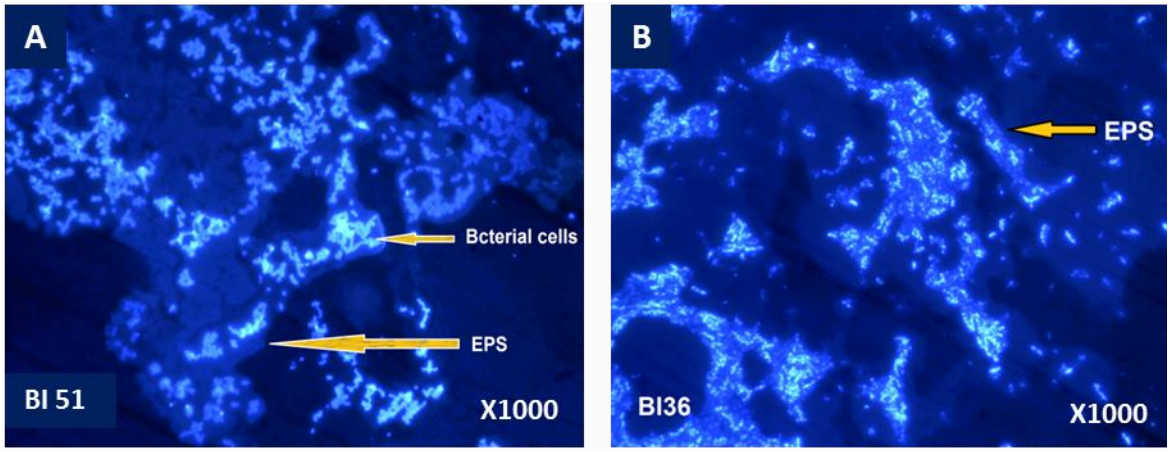


Figure 3. 67: Detection of EPS of bacterial isolates

Examples of EPS producer bacteria; *Dietzia natronolimnaea* (BI 51) (A) and *Brevundimonas diminuta* (BI36) (B); the EPS (blue haze) and bacterial cell (white).

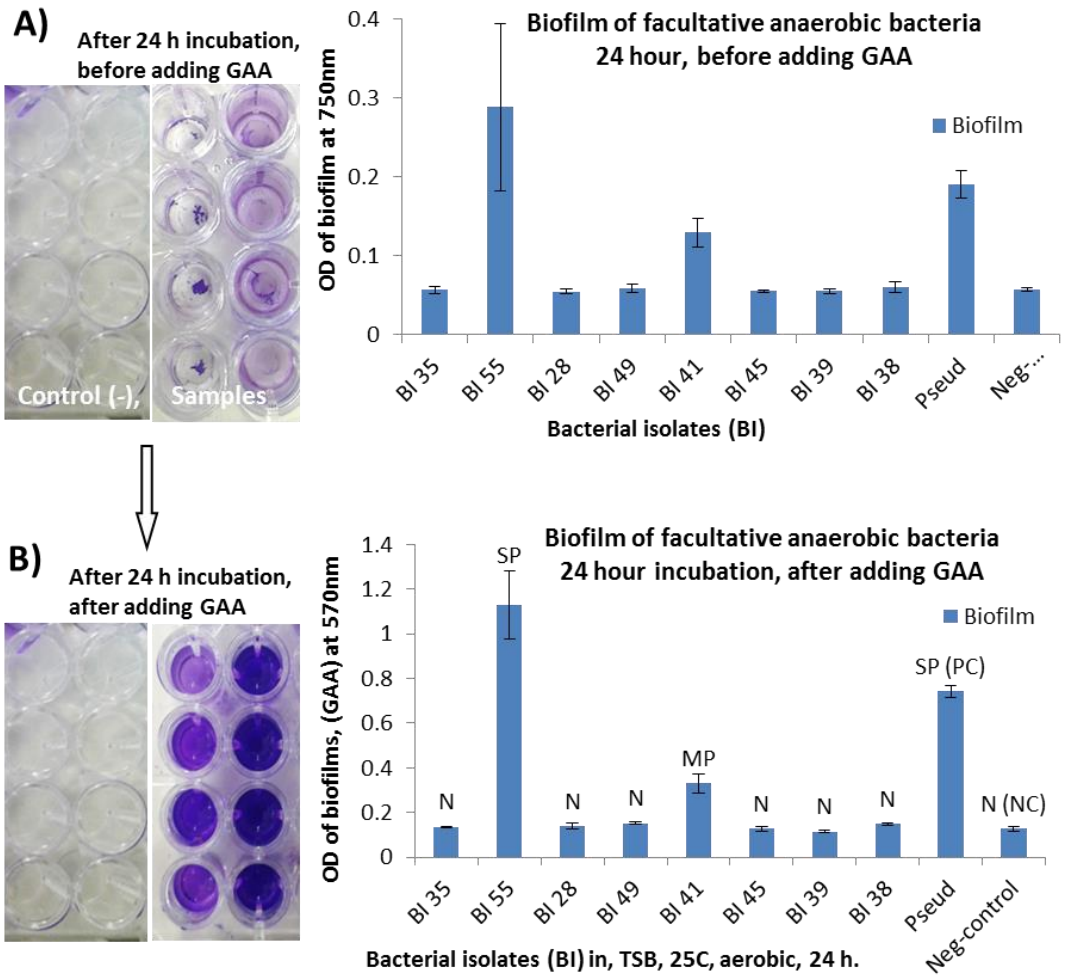


Figure 3. 68: Biofilm formation of some Alkaliphiles sp.

The graphs indicate that a biofilm was formed by *Aeromonas* sp. (BI55) strong positive (SP), and moderate positive (MP) by *Tessaracoccus* sp. (BI41), while negative (N) results were detected by other strains; *Ensifer adhaerens* (BI35), *Rhodococcus erythropois* (BI49), *Dietzia natronolimnaea* (BI45), *Microbacterium kitamience* (BI38 and BI39), the results were compared with *Pseudomonas sp* as a positive control (PC).

The results of the current study show the ability of most of these isolates to form of EPS and biofilm formation. After 24 hr incubation clear biofilms were formed by *Aeromonas* sp. (BI55) (SP) and *Tessaracoccus* sp. and moderate biofilm (MP) was formed by *Tessaracoccus* sp. (BI41), whereas more time (48 hours) was taken by some strains to form the moderate biofilms by *Alishewanella aestuarii* (BI28) and *Brevundimonas diminuta* (BI36), the same time (48 hours) was taken by *Dietzia natronolimnaea* (BI59) to form a strong biofilm (SP) (Figures 3.68 and 3.69). No biofilm detected by the *Ensifer adhaerens* (BI35), *Rhodococcus erythropois* (BI49), *Dietzia natronolimnaea* (BI45), *Microbacterium kitamience* (BI38 and BI39).

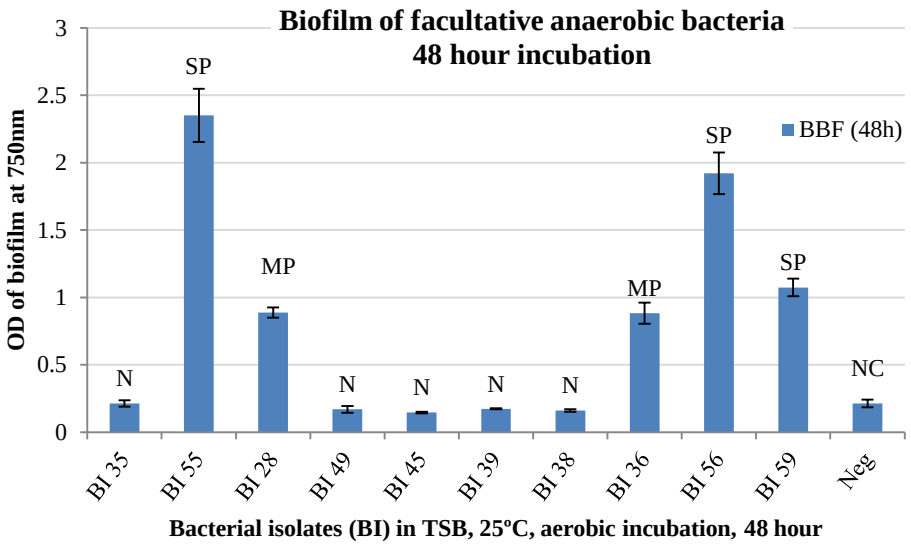


Figure 3. 69: Biofilm detection after 48 hours incubation

The biofilm was detected after 48 hours incubation for some *Alkaliphiles* strains, moderate positive; *Alishewanella aestuarii* (BI28), *Brevundimonas diminuta* (BI36); strong positive, *Dietzia natronolimnaea* (BI59), the density of the biofilm increased when the incubation period increased for *Aeromonas sp* (BI55 and BI56). No biofilm was formed by *Ensifer adhaerens* (BI35), *Rhodococcus erythropois* (BI49), *Dietzia natronolimnaea* (BI45), *Microbacterium kitamience* (BI38 and BI39). GAA=Glacial acetic acid, N= Negative, W= Weak positive, MP= Moderate positive, SP=Strong positive, NC= Negative control, PC= Positive control.

3.7.2.4.1. Discussion

The presence of biofilm producing bacteria in a community may play a crucial role in metabolic cooperation between species by offering and facilitating interspecies substrate exchange (cross-feeding) and the removal of metabolic wastes (syntrophic relationships) (Davey & O'toole, 2000). Some of these isolates have been previously detected in biofilms associated with this research, where these bacteria have been shown to grow in flocs composed of bacteria embedded in EPS. These floc based communities were dominated by *Alishewanella* and *Dietzia* species, in addition this community was capable to metabolize all form of ISA, with > 60% of the carbon was used for EPS formation (Charles et al., 2015). These results suggested that EPS and biofilm production have a main role in the protection of microbial communities from hyperalkaline environment (C. Charles et al., 2017) and provide suitable condition that support bacterial growth. In another study, the same author concluded that pH 13.0 suppressed biofilm formation on a range surfaces (graphite, stainless steel, and Nirex reference vault backfill (NRVB)) with biofilm formation only possible when the ambient pH was below pH 13.0 (C. J. Charles et al., 2017).

3.7.3. Key findings

- The bacterial isolates recovered from the CDP fed microcosms had a varied ability to utilize different carbohydrates.
- A few strains were able to metabolise a wide range of carbohydrates including: *Macellibacteroides fermentans* (BI40), *Tessaracoccus lubricantis* (BI41), *Bacillus toyonensis* (BI5), *Citrobacter gillenii* (BI54), *Aeromonas salmonicida* (BI55); spore-forming bacteria, *Clostridium sartagaforme* (BI89) and *Clostridium celerecrescens* (BI82).
- MIC values for heavy metals and NaCl varied considerable with *Clostridium tertium* (BI 85) demonstrating a tolerance to all heavy metals and NaCl.
- EPS was produced by most of isolates and strong biofilm formation was detected by *Aeromonas salmonicida* (BI55 and BI56) and *Dietzia natronolimnaea* (BI59).

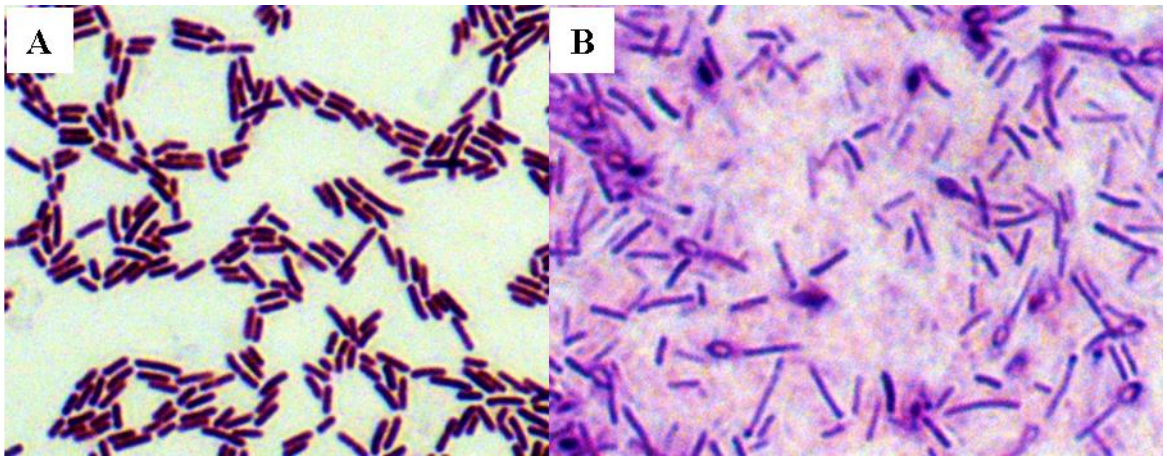
3.7.4. Characterization of Spore forming bacterial strains isolated from alkaline microcosms

Bacterial spores allow bacteria to survive times of extreme stress by preserving the cell's genetic material. In a GDF these stresses are represented by high temperature, irradiation, desiccation, and high pH. Therefore, it is interesting to investigate alkaliphilic spore forming bacteria with the ability to degrade ISA. A number of strictly anaerobic spore forming bacteria were isolated from (soil/CDP/MM) microcosm reactors operated at pH 9.0 and pH 10.0 (Table 3.9).

These bacteria were recovered on FAA at pH 9.5 and identified via 16s rRNA gene sequencing as: *Cl. propionicum* (BI 80), *Cl. tertium* (BI 85) (Figure 3.70 A) with oval terminal and bulging spores (Figure 3.70 B), *Cl. celerecrescens* (BI82) with spherical terminal and bulging spores (Figure 3.70 G and H), *Cl. sartagoforme* (BI 89), *Terrisporobacter petrolearius* (BI 86) with oval sub terminal and non-bulging spores (Figure 3.70 C and D) and *Romboutsia sedimentorum* ZS strain (BI93). These bacterial strains showed an ability to grow on CDPs/ISA/MM agar plates at pH 9.0, where the ISA and CDPs acted as the sole carbon source.

3.7.4.1. Microscopic characterisation of spore-forming alkaliphilic isolates

Clostridia species have differentiated by their spore morphology; i.e. size, shape and their location inside the vegetative cells (endospore). Although *Clostridia* species are typically Gram positive and strictly anaerobic, some *Clostridia* species are found to be aero tolerant and appear Gram-negative when stained (Wells & Wilkins, 1996). The Gram stains for these bacteria are illustrated below (Figure 3.70).



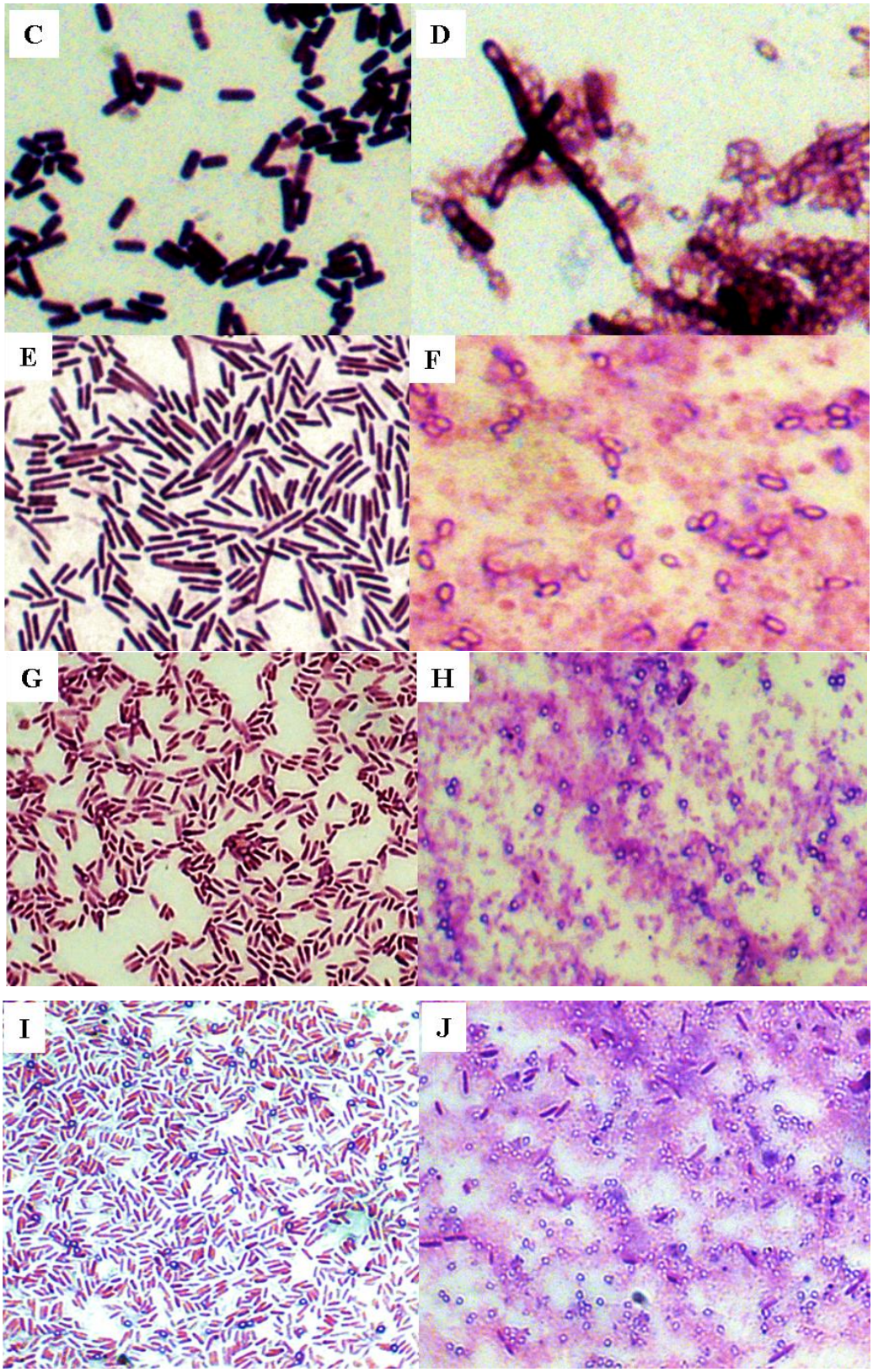
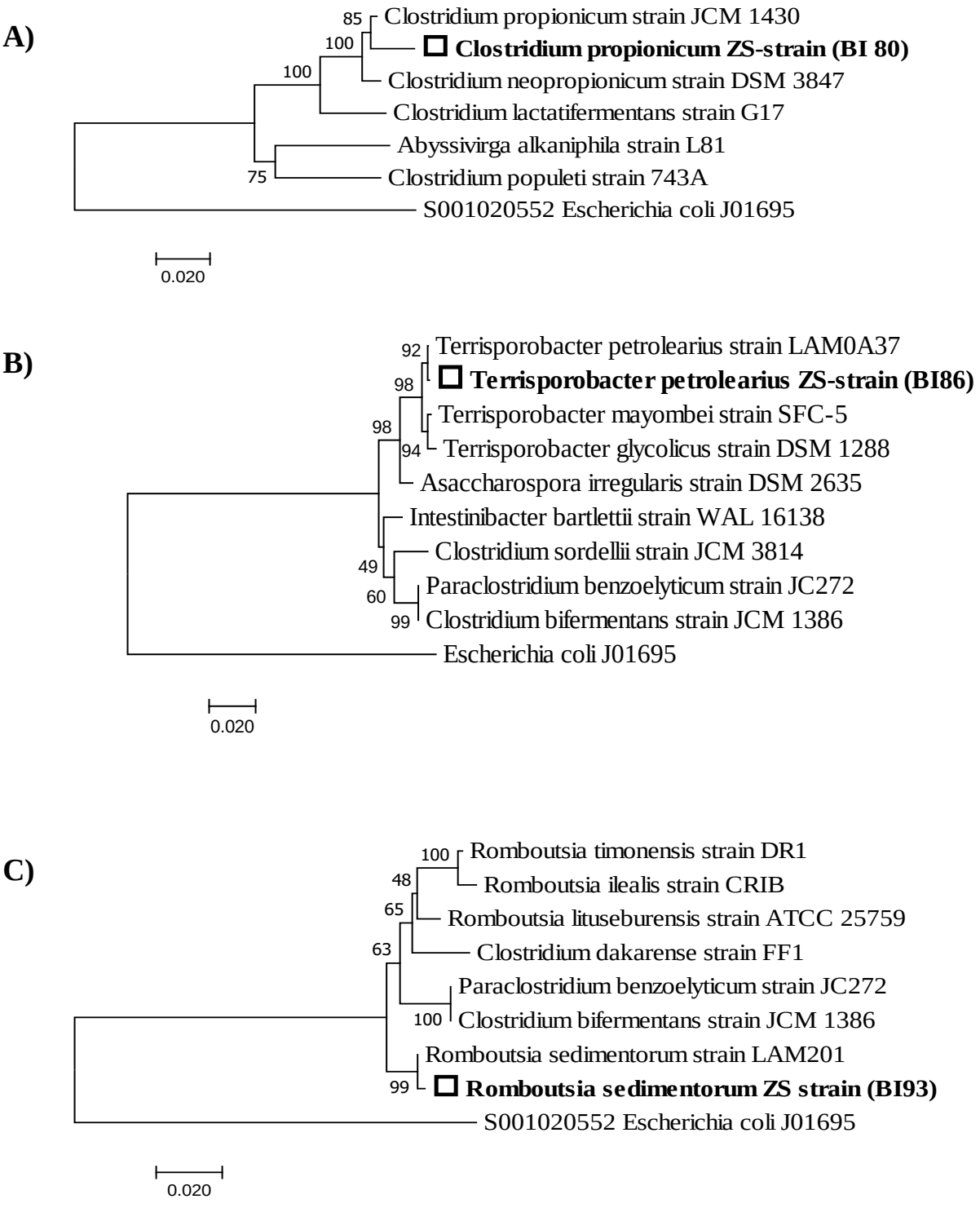


Figure 3. 70: Gram-stain for Clostridia sp. (endospore forming bacteria)

Oval terminal and bulging (BI 85) (B), Oval sub terminal and non bulging (BI 86) (D), spherical terminal and bulging (BI 80 and BI 82) (G and I) and include free spores.

3.7.4.2. Phylogenetic analysis of the spore forming bacterial isolates

The 16s rRNA identification and phylogenetic analysis of the spore forming strains are outlined below (Figure 3.71). The closest related strains are identified in Table 3.9.



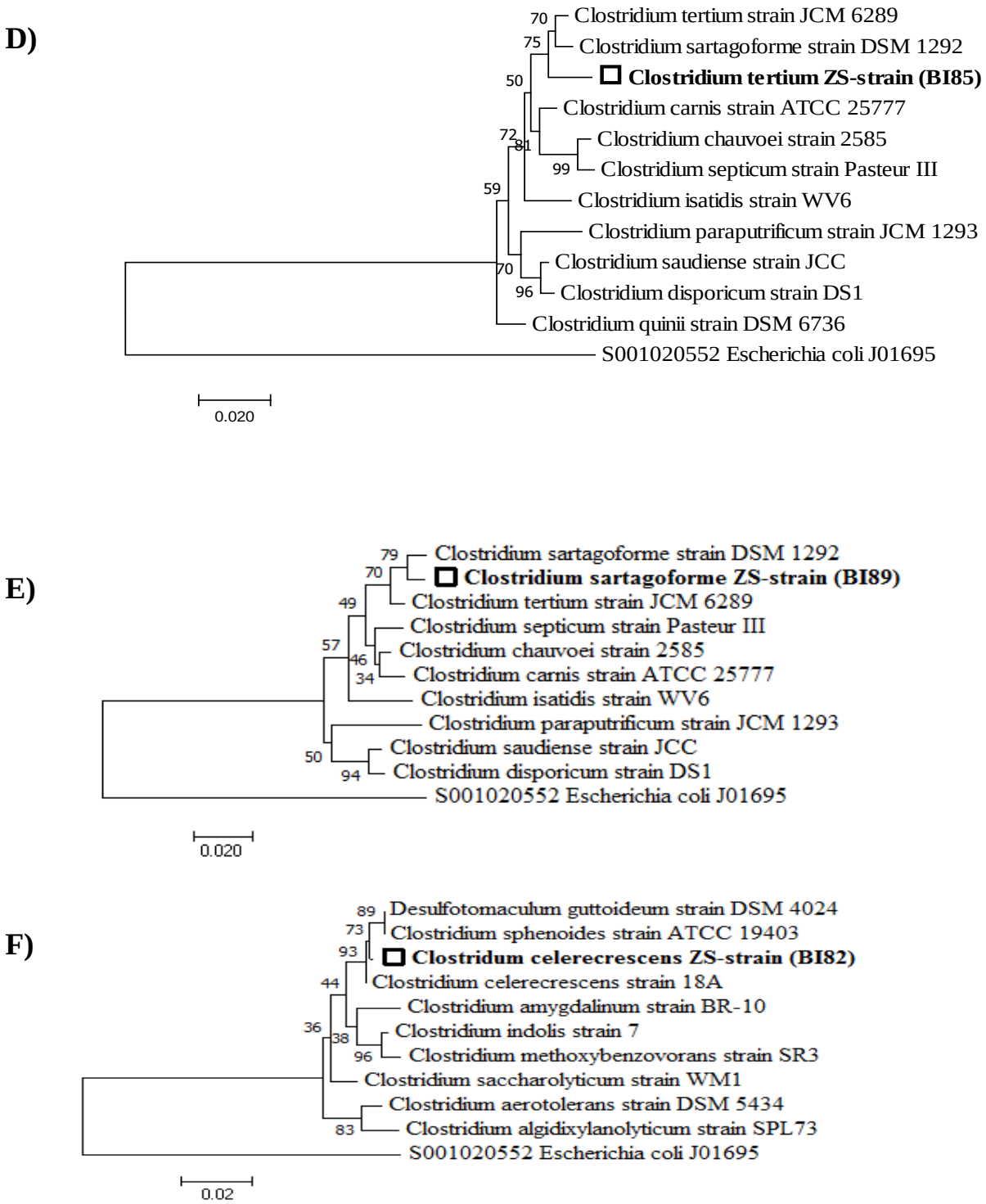


Figure 3. 71: Phylogenetic consensus tree for spore forming bacterial isolates

The evolutionary history was inferred using the Neighbour-Joining method (Saitou & Nei, 1987). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches (Felsenstein, 1985). The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura, Nei, & Kumar, 2004) and are in the units of the number of base substitutions per site.

Table 3. 9: Relationships of taxa from the gene bank to the Alkaliphiles of spore forming bacteria isolated from alkaline microcosms

Figure 3.71	Bacterial isolates, this study (strain number)	bp	% match	Closest relatives strain from the Gene-bank database (RDP)	
				Strain name	Identification number in gene bank
A	*Cl.propionicum* (BI 80)	981	99	*Cl. propionicum*	JCM 1430
B	*Terrisporobacter petrolearius* (BI86)	858	99	*Terrisporobacter petrolearius*	LAM0A37
C	*Romboutsia sedimentorum* (BI93)	915	99	*R. sedimentorum*	LAM201
D	*Cl. tertium* (BI85)	903	98	*Cl. tertium*	JCM 6289
E	*Cl. sartagoforme* (BI89)	688	99	*Cl. sartagoforme*	DSM1292
F	*Cl. celerecrescens* (BI82)	828	99	*Cl. celerecrescens*	18A

3.7.4.3. Biochemical characteristics and pH profiles of the bacterial isolates

Clostridum celerecrescens (BI82), *Clostridium tertium* (BI 85), *Cl. sartagoforme* (BI 89), and *Terrisporobacter petrolearius* (BI 86) were all negative for catalase, oxidase, and urease (Table 3.10). All strains were positive for D-Glucose but had variable performance against the other carbohydrates with *Cl. sartagoforme* (BI 89) being able to utilize all the substrates provided. Whereas *Terrisporobacter petrolearius* showed moderate positives (+/-) for almost all the substrates but it was negative for D-melezitose, D-raffinose, L-rhamnose. *Clostridium tertium* was positive for D-mannitol, D-maltose, D-cellobiose, D-mannose and salicin and moderate positive for D-lactose and D-raffinose.

Although, some of these spore-forming bacteria were able to tolerate and showed growth at pH values up to pH 12 including *Cl. propionicum* (BI 80), *Terrisporobacter petrolearius* (BI 86) and *Cl. sartagoforme* (BI 89). The pH profile of these strains prove of these bacterial isolates are moderate alkaliphiles. The pH range was variable for all isolates ranging from pH 5.0 and pH 6.0 to pH 10.0 and up to pH 12.0 for some strains. In general, the optimum pHs are from pH 7.0 to pH 9.0 (Figure 3.72). Some strains were able to adapt to high pH values as indicated by the lag phases that increased as the pH increased, these strains were *Cl. celerecrescens* (BI82), *Cl. tertium* (BI 85), and *Cl. sartagoforme* (BI 89). The strain *Terrisporobacter petrolearius* (BI 86), showed fluctuating growth that may be due to bacterial cells adapting to high pH before it begins to grow.

Table 3. 10: Biochemical characteristics of the bacterial isolates by API20A

	BI 82	BI 85	BI 89	BI 86
Oxidase	-	-	-	-
Catalase	-	-	-	-
Indole	+	-	-	-
Urease	-	-	-	-
Protease (gelatine)	-	-	-	-
B-glucosidase (Esculin)	+/-	+	+	+/-
H_2S	+/-	-	-	+/-
Acidification of Carbohydrates				
D-Glucose	+	+	+	+
D-Mannitol	+	+	+	+/-
D-Lactose	+	+/-	+	+/-
D-Sucrose	+	-	+	+/-
D-Maltose	+	+	+	+/-
Salicin	+	+	+	+/-
D-xylose	+	-	+	+/-
L-arabinose	+	-	+	+/-
Glycerol	+/-	-	+	+/-
D-cellobiose	+	+	+	+/-
D-mannose	+	+	+	+/-
D-melezitose	+/-	-	+	-
D-raffinose	+	+/-	+	-
D-sorbitol	+/-	-	+	+
L-rhamnose	+	-	+	-
D-trehalose	+	-	+	+/-

Cl. celerecrescens (BI82)

Cl. tertium (BI 85)

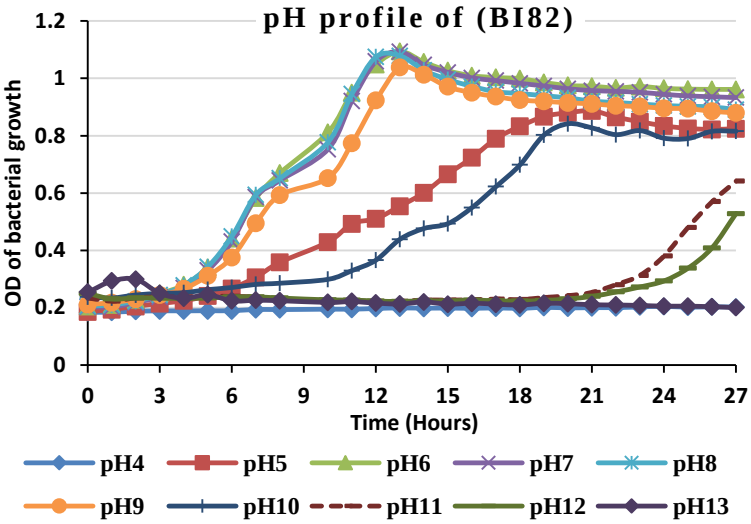
Cl. sartagoforme (BI 89)

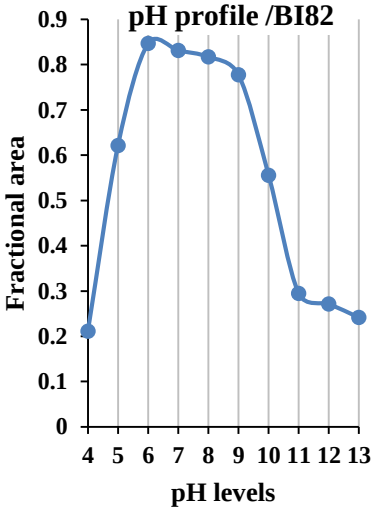
Terrisporobacter petrolearius (BI 86),

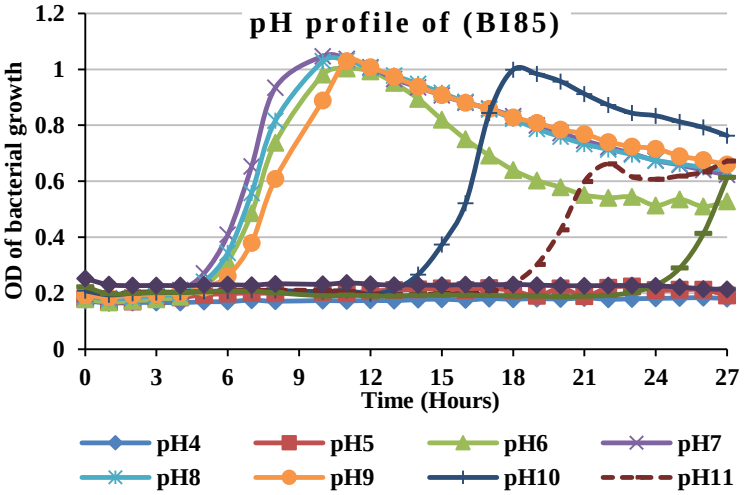
Positive +

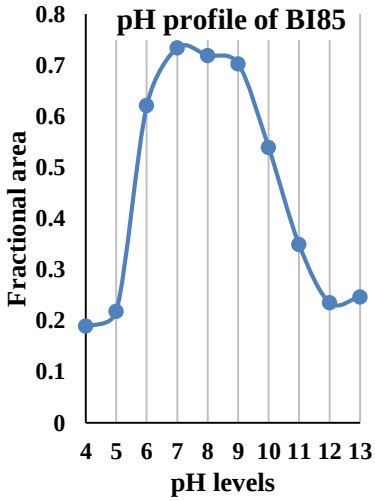
Negative -

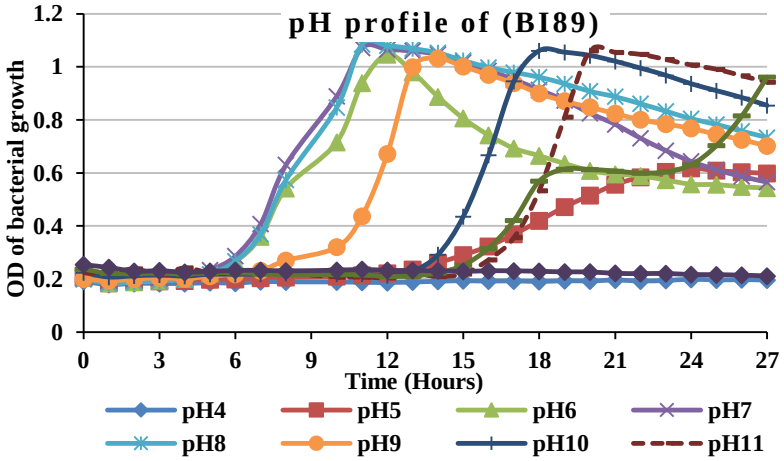
Moderate positive +/-

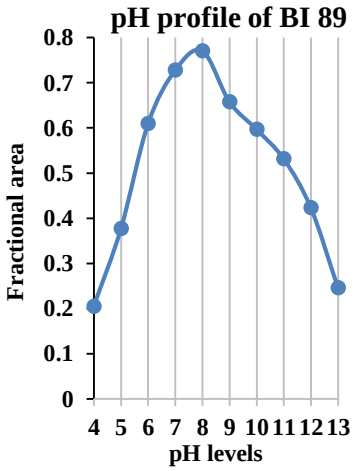

pH profile of (BI82)
OD of bacterial growth
Time (Hours)
pH4
pH5
pH6
pH7
pH8
pH9
pH10
pH11
pH12
pH13


pH profile /BI82
Fractional area
pH levels


pH profile of (BI85)
OD of bacterial growth
Time (Hours)
pH4
pH5
pH6
pH7
pH8
pH9
pH10
pH11


pH profile of BI85
Fractional area
pH levels


pH profile of (BI89)
OD of bacterial growth
Time (Hours)
pH4
pH5
pH6
pH7
pH8
pH9
pH10
pH11


pH profile of BI 89
Fractional area
pH levels

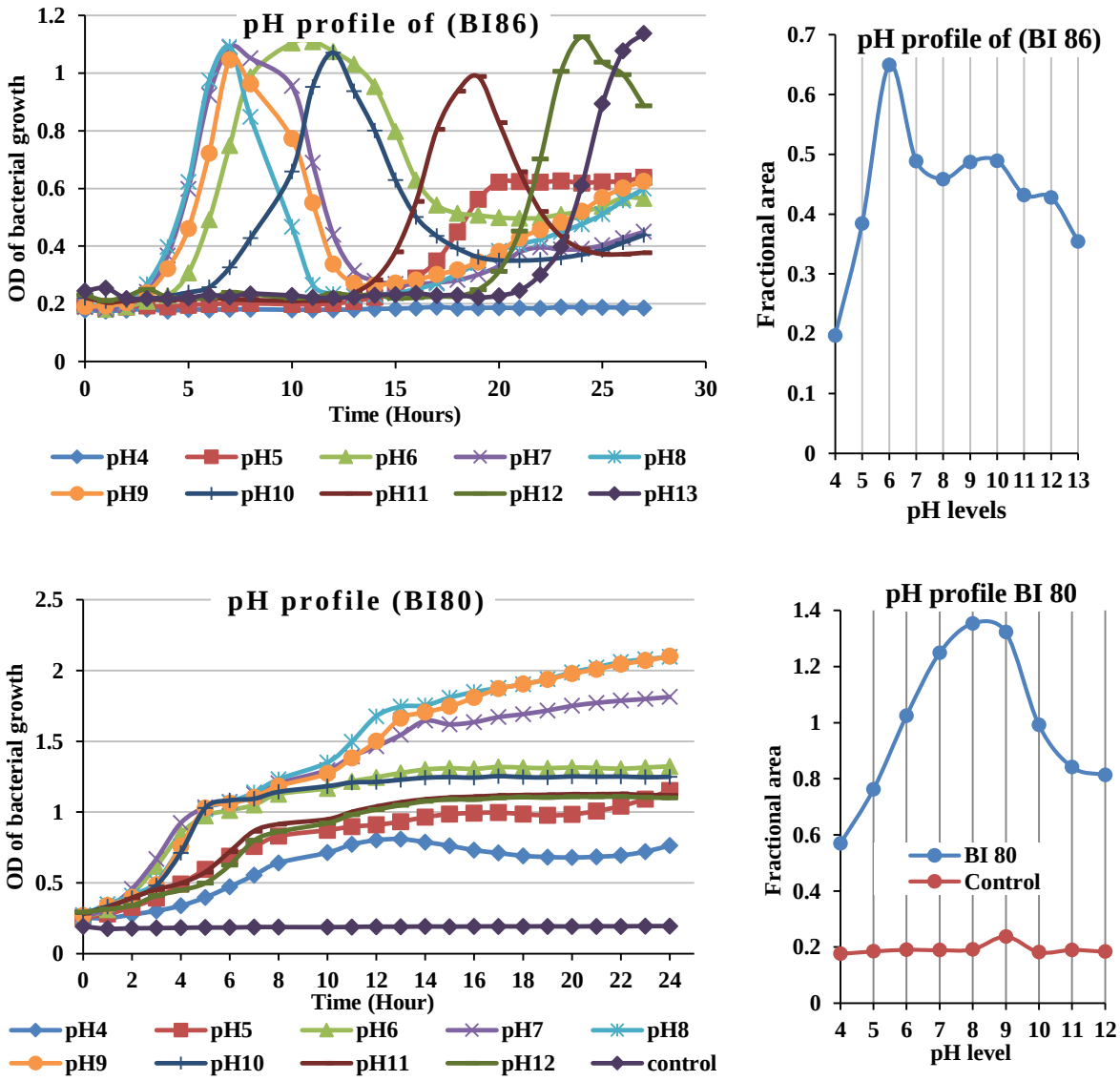


Figure 3. 72: pH profiles of the spore forming bacterial isolates

In this study *Cl. sartagoforme* (BI89) was able to utilize all available carbohydrates (Table 3.5) including glucose, xylose and cellobiose. Recently Zhang *et al*. (2015), reported that bio-hydrogen was produced from the direct degradation of cellulosic biomass by *Cl. sartagoforme FZ11*, which was able to degrade glucose, xylose, hemicellulose, xylan and cellobiose (Y. Wang et al., 2015). The results indicated that this strain was able to multiply in CDP fed microcosms it was unable to degrade both forms of ISA when it inoculated as a pure culture in a broth of mineral media either in the presence of CDPs or by adding $Ca(ISA)_2$ at pH 8.0 and pH 9.0. This strain had an optimum pH for growth between pH 7.0 and pH 9.0 (Figure 3.72 (BI89)), with a pH range from pH 5.0 to pH 12.0. In this case, the lag phase took more than 12 hours at pH 5.0 as well as at pH 10.0, 11.0 and 12.0). The results suggest that this strain has an ability to adapt to both low and high pH conditions in the environment.

Clostridium tertium ZS (BI85) was isolated from microcosms at both pH 9.0 and pH 10.0. This strain was able to grow on agar supplemented with CDPs or ISA as a sole of carbon source at pH 9.0. This strain was positive for D-mannitol, D-maltose, D-cellobiose, D-mannose and salicin and moderate positive for D-lactose and D-raffinose (Table 3.10). The optimum pH was between pH 7.0 and pH 9.0, with a pH range from pH 5.5-11 (Figure 3.72 (BI85)). However, this strain was unable to degrade ISA in both forms (α- and β-) in mineral media at pH 8.0 and pH 9.0. In previous studies, this species is an aerotolerant strain able to ferment a wide range of carbohydrates (Kataoka & Tokiwa, 1998). *Cl. tertium KT-5A* strain, which was isolated from lotus soil, paddy soil and pond sediment, their pH range for growth 5-8, it was reported as an active mannanase-producing anaerobic bacterium (Kataoka & Tokiwa, 1998). *Cl. tertium* (BI85) showed a 98% match to the *Cl. tertium* Type strain JCM 6289, together with their ability for growth at high pH, this strain was selected for whole genome sequencing analysis for a first time.

Clostridum celerecrescens (BI82) was isolated from CDP microcosms at pH 9.0 and pH 10 on FAA at pH 9.5 and was positive for a wide range of sugars (Table 3.10). The optimum pH for growth was between pH 6.0 and pH 9.0 (Figure 3.72 (BI82)), with a pH range between pH 5.0 and pH 10, however it showed a growth at pH 11 and pH 12 after a long lag phase. In a previous study, *Cl. celerecrescens* K-2 was isolated from waste waters had a similar sugar utilissaiton profile and had a high efficiency for the production of caproate and butyrate (Hu, Du, & Xu, 2015).

Terrisporobacter petrolearius (BI 86) was isolated from microcosms at pH 9.0 and pH 10. This strain was positive for D-glucose, and D-sorbitol and showed a moderate positive for a range of other substrates (Table 3.10). The genus *Terrisporobacter* was proposed by Gerritsen *et al*. (2014), this genus belongs to the family Peptostreptococcaceae of the phylum Firmicutes (Gerritsen et al., 2014). The closest match to BI 86 was the type strain *T. petrolearius* LAM0A37^T which was isolated from a petroleum reservoir in China. The optimum pH for this strain was (7.0-7.5) (Figure 3.73 (BI86)), with a pH range for growth 5.5–9.5, their optimum growth occurred without NaCl, but it tolerated NaCl up to 3.0%, it was able to utilize D-glucose and a range of sugars. The main products of glucose fermentation was acetate and CO_2 (Deng et al., 2015).

Romboutsia sedimentorum ZS (BI93) was isolated from CDPs driven microcosms at pH 9.5 and pH 10.5. In a previous study, *Romboutsia* gen. was proposed by Gerritsen *et al*. (2014), where the first isolate was *Romboutsia ilealis* CRIB^T which originated from the gastrointestinal tract of a rat. The CRIB^T strain was able to utilize a very limited number of organic compounds, it was

positive for L-fucose, D-glucose, raffinose, and sucrose, it showed a moderate positive for D-arabinose and D-galactose (Gerritsen et al., 2014). *Romboutsia sedimentorum* LAM201^{T} was isolated from sediment samples collected from an alkaline-saline lake of Daqing oilfield, China, the strain was able to utilize glucose, maltose, fructose, sorbitol and trehalose as the sole carbon source. The main products of glucose fermentation were ethanol, acetic acid, iso-valeric acid and iso-butanoic acid. The pH range for growth was pH 6.0–9.0 (optimum: pH 7.0) (Y. Wang et al., 2015).

Clostridium propionicum (BI 80) was isolated from a microcosm at pH 9.0. This strain had a pH range for growth between pH 4.0 and pH 12.0, the optimum pH for growth was between pH 7.0 and pH 9.0 (Figure 3.72 (BI80)). A previous study found that *Cl. propionicum* was able to utilize a narrow pH range (pH 6.6 to pH 8.35). The strain was able to utilized a limited number of organic compounds for growth including acrylate and lactate by fermentation producing acetate and propionate. The strain was able to grow in the presence of 4% NaCl (Hetzel et al., 2003; Janssen, 1991). Due to the strains wide pH range (pH 4.0 to pH 12.0) its ability to form biofilms was investigated to determine if biofilm formation was the reason the strain's ability to resit such a wide pH range.

Biofilm formation is known to be used by spore-forming bacteria to protect against extremes of pH. Work by Dumitrache *et al*. (2013) found that the obligately anaerobic cellulolytic strain *Clostridium thermocellum* 27405 was capable of the complete metabolism of cellulose through biofilm formation. In addition, terminal endospores appeared throughout all stages of biofilm growth. The results suggest that the biofilm formation depends on the substrate availability for cell attachment that effects the efficiency of cellulose utilization (Dumitrache, Wolfaardt, Allen, Liss, & Lynd, 2013). In this study, a strong biofilm was detected by *Clostridium propionicum* ZS-strain (BI 80) at an optimum pH 8.0 followed by pH 7.0. Moderate biofilm production was detected at pH 4.0, 5.0, 6.0 and at pH 9.0 (Figure 3.73). A weak biofilm was formed between pH 10.0 and pH 12.0. This gives an indicator of the ability of this strain to tolerate acidic condition and its ability to grow at a high pH through a biofilm formation.

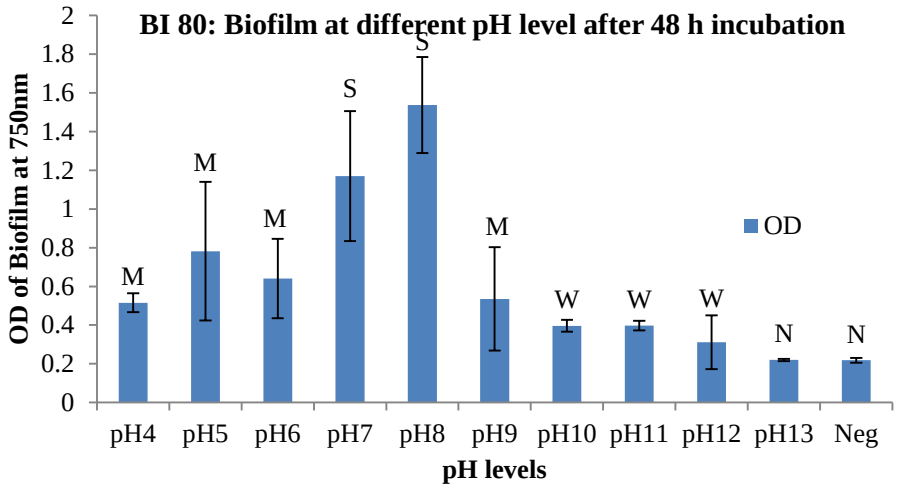


Figure 3. 73: Biofilm formation by *Clostridium propionicum* (BI 80)

N= Negative, W= Weak positive, MP= Moderate positive, SP=Strong positive, NC= Negative control

3.7.4.4. Key findings

- Strictly anaerobic spore forming bacteria were isolated from a wide range of microcosms operated at pH 9.0 and pH 10.0. These strains showed an ability to grow on CDPs/ISA/MM agar plates at pH 9.0.
- The pH range for these isolates was from pH 5.0 and 6.0 to pH 10.0 and up to pH 12.0 for some strains. In general, the optimum pHs were from pH 7.0 to pH 9.0.
- *Cl. celerecrescens* (BI82), *Cl. tertium* (BI 85), *Cl. sartagoforme* (BI 89), and *Terrisporobacter petrolearius* (BI 86) were able to utilize D-Glucose, D-mannitol, D-maltose, D-cellobiose, D-mannose, salicin, D-lactose and D-raffinose.
- However, these bacterial strains; *Cl. sartagoforme* ZS strain (BI89), *Cl. tertium* ZS (BI85), *Cl. celerecrescens* (BI82), *Terrisporobacter petrolearius* (BI 86), *Romboutsia sedimentorum* ZS strain (BI93) and *Cl. propionicum* (BI 80) were unable to degrade ISA either from the CDPs or in the form of $Ca(ISA)_2$ as a sole carbon source in liquid culture at either pH 8.0 and pH 9.0.
- In the case of *Cl. propionicum* (BI 80) spore formation (sporulation) was detected after 72-hour incubation while biofilms were detected after 48-hour incubation. The results suggest that the formation of a biofilm is the first defence used by spore-forming bacteria againt extreme pH levels.

3.7.5. Characterisation and whole genome sequencing (WGS) of the selected Alkaliphilic strains

A number of strains were selected for further investigation either based on the rarity of the isolate or the presence of specific properties such as ISA degradation. In some cases, the WGS was carried out and annotated via RAST (Table 3.11). Key features and associated functions are summarised below.

Table 3. 11: Subsystem features counts of isolated Alkaliphiles strains

Macellibacteroides fermentans (BI40), *Alishewanella aestuarii* (BI28), *Azonexus hydrophilus* (BI70), *Brevundimonas diminuta* (BI36); *Dietzia natronolimnaea* (BI51), *Rhodococcus erythropois* (BI49), *Tessaracoccus lubricantis* (BI41) and *Clostridium tertium* (BI 85). The highlighted numbers in the table showing the highest numbers of a group of genes

Subsystem Statistics								
Subsystem Feature Counts	**number of genes in the bacterial isolates BI**							
	BI 40	**BI 28**	**BI 70**	**BI 36**	**BI 51**	**BI 49**	**BI 41**	**BI 85**
Cofactors, Vitamins, Prosthetic, Pigments	178	231	262	162	294	**421**	207	166
Cell Wall and Capsule	98	144	147	71	74	104	88	**207**
Virulence, Disease and Defense	86	100	82	109	98	113	55	74
Potassium metabolism	18	15	24	9	19	14	12	13
Photosynthesis	0	0	0	0	0	0	0	0
Miscellaneous	24	29	25	43	42	45	30	11
Phages, Transposable elements, Plasmids	34	1	7	16	13	23	0	7
Membrane Transport	73	107	154	115	72	102	69	85
Iron acquisition and metabolism	2	11	11	9	13	21	7	25
RNA Metabolism	107	**216**	165	144	76	93	97	163
Nucleosides and Nucleotides	65	81	72	70	96	124	104	141
Protein Metabolism	229	269	271	240	237	266	206	252
Cell Division and Cell Cycle	38	30	31	26	24	29	26	47
Motility and Chemotaxis	0	122	114	97	3	11	4	87
Regulation and Cell signaling	14	61	78	62	32	69	24	48
Secondary Metabolism	0	4	4	5	0	8	0	0
DNA Metabolism	99	129	82	111	75	96	77	121
Fatty Acids, Lipids, and Isoprenoids	85	91	128	106	251	**328**	73	92
Nitrogen Metabolism	29	30	**108**	8	32	30	18	13
Dormancy and Sporulation	4	3	1	3	2	4	2	**68**
Respiration	54	76	**193**	105	121	116	90	67
Stress Response	53	124	**157**	91	93	136	63	88
Metabolism of Aromatic Compounds	3	18	13	27	13	**89**	7	1
Amino Acids and Derivatives	236	350	304	313	374	**696**	238	197
Sulfur Metabolism	20	30	48	7	28	89	33	18
Phosphorus Metabolism	34	41	37	39	42	42	31	43
Carbohydrates	297	220	235	207	344	**537**	398	**524**
Total	1880	2533	2753	2195	2468	3606	1959	2558

3.7.5.1. Characterization of *Aeromonas* sp. ZS strain (BI 55)

Aeromonas sp. have been isolated from a variety of aquatic environments worldwide including water and sewage with a wide temperature ranging from 0°C to 45°C and optimum of 22-32°C (Didugu et al., 2015). Under anaerobic conditions this genus can utilise either fermentative or anaerobic respiration for the generation of energy. *Aeromonas* sp. are able to survive extreme conditions such as high pH through a biofilm formation (Kirov et al., 2004).

3.7.5.1.1. Phylogenetic analysis of *Aeromonas* sp. ZS strain (BI 55)

This strain was identified through 16S rRNA gene sequencing and BLASTN analysis as *A. salmonicida* subsp. *Salmonicida* (Figure 3.74, Table 3.12) with 99% similarity to *A. salmonicida* type strain ATCC 33658. The high interspecies sequence similarity found in the 16S rRNA gene sequences (96.7% to 100%) of the *Aeromonas genus* (A. J. Martínez-Murcia et al., 2007; Vega-Sánchez et al., 2014) makes it difficult to distinguish between closely related *Aeromonas* sp.

The Aeromonadaceae were the third dominant family of the bacterial community present in the CDPs fed fermentative microcosm comprising 24.5% at pH 7 which dropped to less than 9% at pH 8.0 and pH 9.0 (Figure 3.57 B and C). *A. salmonicida* species; *A. salmonicida* subspecies

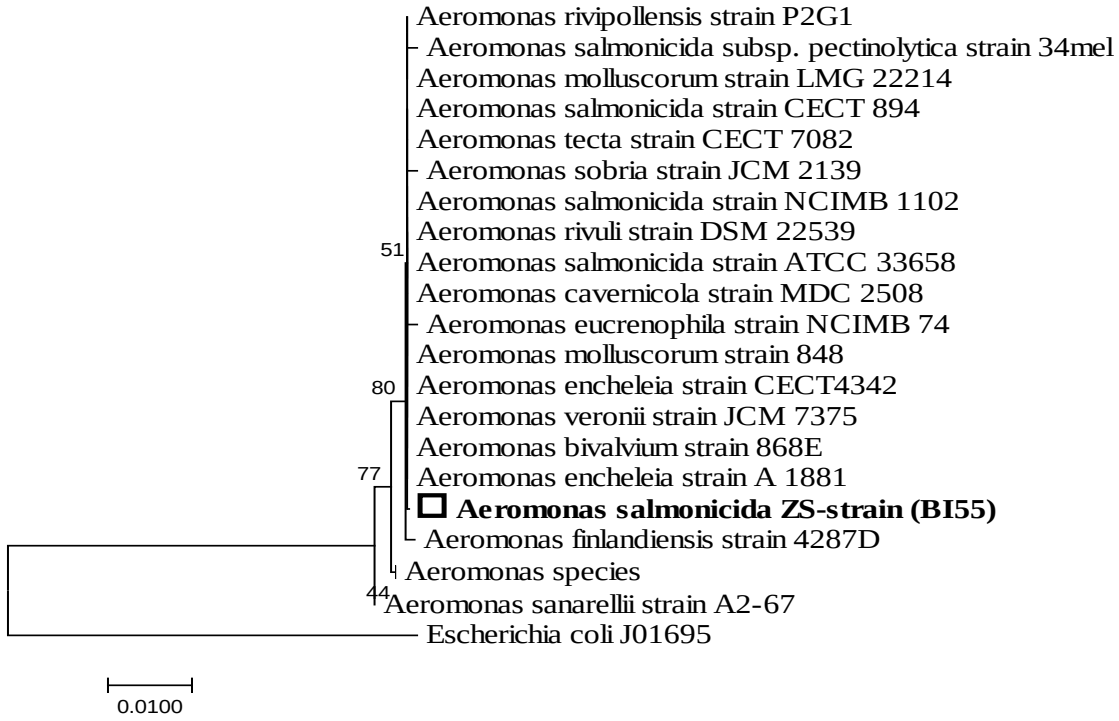


Figure 3. 74: *Aeromonas* sp. ZS strain (BI 55), evolutionary relationships of taxa

Table 3. 12: The closest strains to the *A. salamonicida* ZS-strain (BI55).

Rank	Name	Strain	Authors	Accession	Pairwise Similarity (%)
1	*A. salmonicida subsp. Salmonicida*	ATCC 33658	(Lehmann and Neumann 1896) Schubert 1967	LSGW01000109	100
2	*A. rivuli*	DSM 22539	Figueras et al. 2011	CDBJ01000088	100
3	*A. molluscorum*	848	Miñana-Galbis et al. 2004	AY532690	100
4	*A. salmonicida subsp. achromogenes*	NCIMB 1110	(Smith 1963) Schubert 1967	X60407	99.76
5	*Haemophilus piscium*	CIP 106116	Snieszko et al. 1950	JN175340	99.76
6	*A. rivipollensis*	P2G1	Martí and Balcázar 2016	FR775967	99.76
7	*A. veronii*	CECT 4257	Hickman-Brenner et al. 1988	CDDK01000015	99.76
8	*A. eucrenophila*	CECT 4224	Schubert and Hegazi 1988	CDDF01000007	99.76
9	*A. bestiarum*	CECT 4227	Ali et al. 1996	CDDA01000036	99.76
10	*A. tecta*	CECT 7082	Demarta et al. 2010	CDCA01000001	99.76
11	*Aeromonas jandaei*	CECT 4228	Carnahan et al. 1992	CDBV01000055	99.76
12	*A. bivalvium*	CECT 7113	Miñana-Galbis et al. 2007	CDBT01000022	99.76
13	*A. sanarellii*	LMG 24682	Alperi et al. 2010	CDBN01000061	99.76
14	*A. piscicola*	LMG 24783	Beaz-Hidalgo et al. 2010	CDBL01000074	99.76
15	*A. salmonicida subsp. masoucida*	NBRC 13784	Kimura 1969	BAWQ01000150	99.76
16	*A. cavernicola*	CCM7641	Martínez-Murcia et al. 2012	HQ436040	99.76
17	*A. ichthiosmia*	DSM6393	Schubert et al. 1991	X71120	99.76
18	*A. salmonicida subsp. pectinolytica*	34mel	Pavan et al. 2000	ARYZ01000167	99.76
19	*A. encheleia*	LMG 16331	Esteve et al. 1995	AJ458409	99.76
20	*A. sobria*	ACC 43979	Popoff and Veron 1981	X74683	99.64
21	*A. finlandiensis*	4287D	Beaz-Hidalgo et al. 2015	LM654283	99.64
22	*A. aquatica*	AE235	Beaz-Hidalgo et al. 2015	JRGL01000106	99.52
23	*A. hydrophila subsp. hydrophila*	ATCC 7966	(Chester 1901) Schubert 1964	CP000462	99.52
24	*A. media*	CECT 4232	Allen et al. 1983	CDBZ01000012	99.52
25	*A. popoffii*	CIP 105493	Huys et al. 1997	CDBI01000096	99.52
26	*A. hydrophila subsp. ranae*	LMG 19707	Huys et al. 2003	AJ508766	99.52
27	*A. salmonicida subsp. smithia*	CCM4103	Austin et al. 1989	AJ009859	99.52
28	*A. taiwanensis*	LMG 24683	Alperi et al. 2010	CDDD01000060	99.28
29	*A. caviae*	CECT 838	(ex Eddy 1962) Popoff 1984	CDBK01000019	99.28
30	*A. dhakensis*	CIP 107500	(Huys et al. 2002) Beaz-Hidalgo et al. 2015	CDBH01000037	99.28
31	*A. lacus*	AE122	Beaz-Hidalgo et al. 2015	JRGM01000126	99.16

3.7.5.1.2. Morphological and metabolic characterisation of *Aeromonas* sp. ZS (BI 55)

Aeromonas sp. ZS (BI 55) was isolated from CDP fed alkaline microcosms operated at pH 8.5-11.0. When grown on FAA at 25ºC the strain produced colonies that were smooth in texture with a regular edge and a convex profile. Microscopic observation revealed a short, non-spore forming Gram-negative rod with rounded edges (coccobacilli) (Figure 3.75). This strain was capable of degrading $Ca(ISA)_2$ and both α- and β-ISA (CDP) in a broth of minimal medium at pH 8.0 and pH 9.0 section (3.3.).

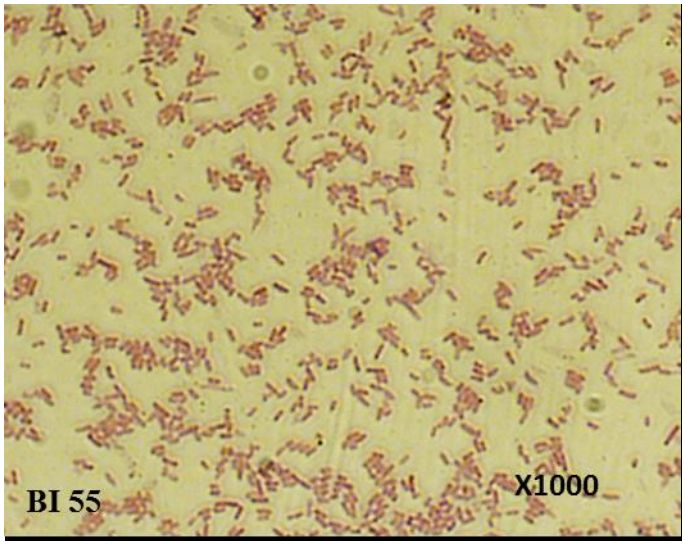


Figure 3. 75: *Aeromonas* sp. ZS strain (BI 55), Gram negative coccobacilli.

Aeromonas salmonicida ZS (BI 55) is a mesophilic strain, with an optimum temperature of 30ºC and no growth at 40ºC. The pH range was from pH 6.0-10.0 with optimum growth at pH 8.0 (Figure 3.76). When pH and temperature were combined temperature was the dominant factor between pH 6.0 and pH 9.0 (Figure 3.77). However, growth at the optimum temperature allowed the strain to sustain significant growth at pH 10.0, which was not seen at any other temperature (Figure 3.77). This strain was positive for; oxidase, catalase, urease, H_2S and negative for; protease (gelatine), β-glucosidase (esculin) and indole metabolism. The strain was also able to utilize a wide range of carbohydrates provided by the API system: (Table 3.5). In addition, the strain was able to utilising 90% of the substrates provided by the Biolog PM1 plate (Table 3.6) and 70% of the substrates in the Biolog PM2 (Table 3.7) microplate.

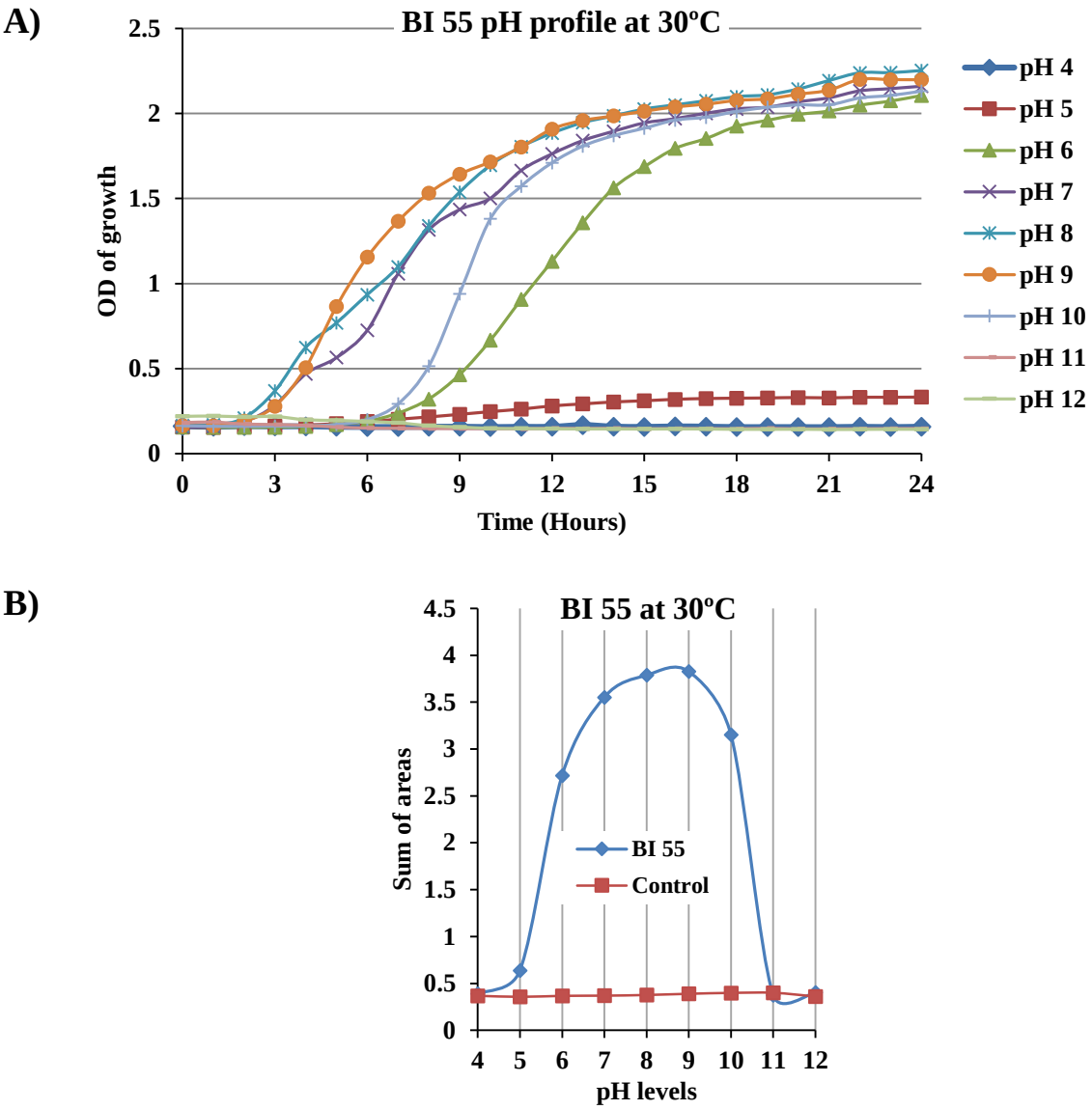


Figure 3. 76: *Aeromonas* sp. ZS strain (BI 55), pH profile curve

The graphs showing a growth curves of this strain at pH 6, 7, 8, 9 and 10 (A); with an optimum pH is pH 8 (B) at an optimum temperature is 30ºC.

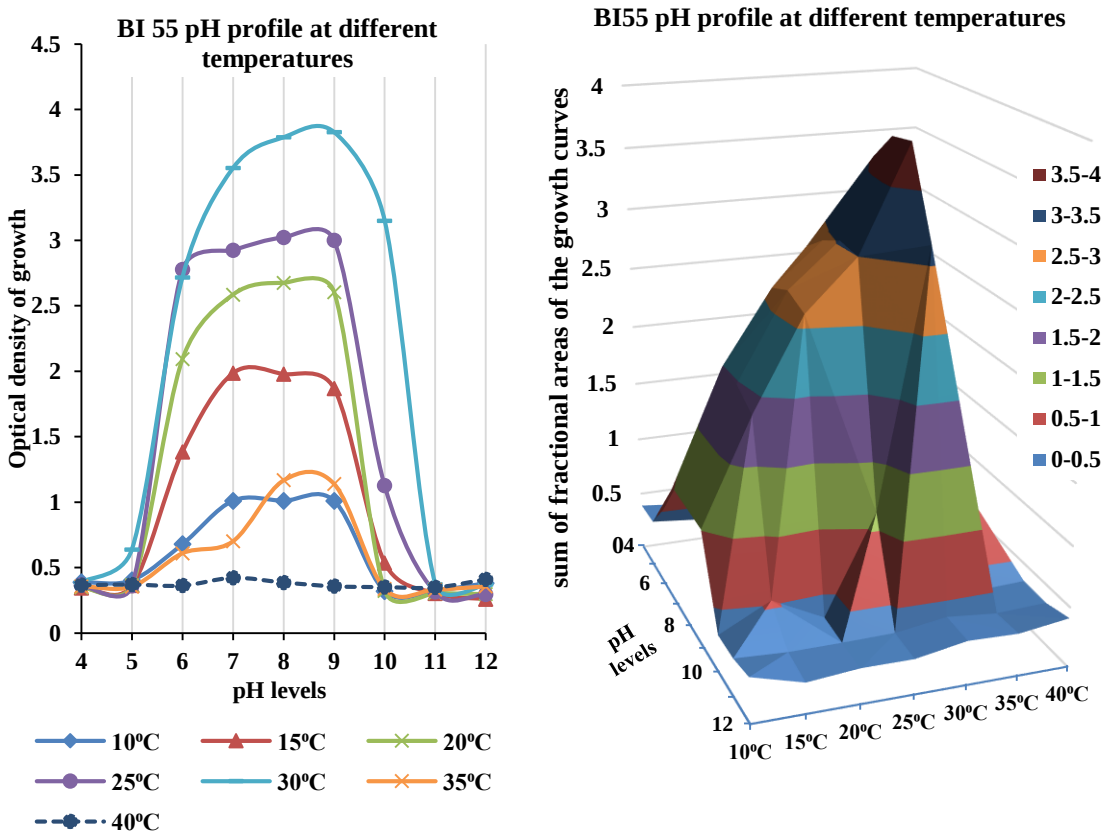


Figure 3. 77: *Aeromonas* sp. (BI 55), pH profile at different temperatures

The strain also showed an ability to tolerate heavy metals as demonstrated through the minimal inhibitory concentration (MIC) for: Nickle (2.4 mM), Cadmium (0.4 mM), Cobalt (2.8 mM), Zinc (3.0 mM), Copper (4.0 mM) and Lead (> 5.0 mM) (Table 3.8). When investigated for its response to NaCl the MIC was 0.633 M (Figure 3.78). In addition, a strong capacity for biofilm formation was demonstrated by *Aeromonas* sp. ZS strain (BI 55) (Figure 3.68 and 3.69). Biofilm formation is an indicator of the strain's ability to tolerate extreme conditions such as those experienced in an alkaline environment (Kirov et al., 2004).

In view of the extensive research that has been carried out on *Aeromonas* sp. a WGS for *Aeromonas* sp. ZS strain (BI 55) was not performed.

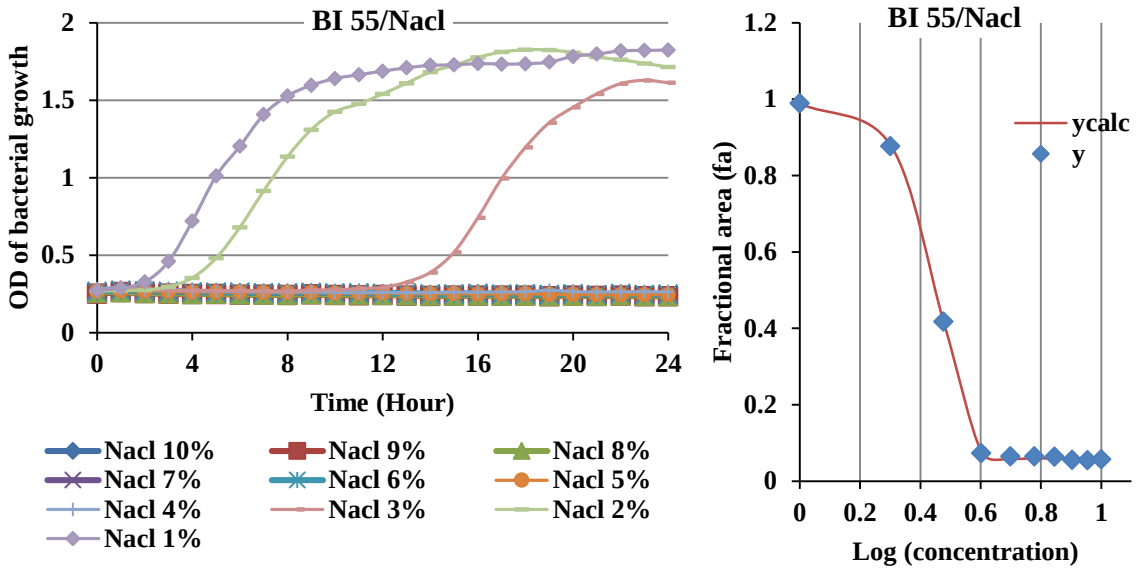


Figure 3. 78: *Aeromonas* sp. (BI 55) NaCl tolerance

The graphs showing curves of a bacterial growth in the presence of NaCl up to 3% (A), the MIC of NaCl was calculated through fractional area at different concentration and plotted on the graph against a log (concentration) using Gompertz function to find the MIC that equal (0.633 M) 3.7% of NaCl. The MIC was detected through calculation of; (■), observed FA; (—), fitted Gompertz function illustrated on a graph (B).

3.7.5.1.3. Key findings

- *Aeromonas salmonicida* ZS-strain (BI55) was able to degrade ISA in the form of $Ca(ISA)_2$.
- The characteristics of *Aeromonas salmonicida* ZS-strain (BI55) were similar to previous studies which found that *Aeromonas* species can utilize a wide range of carbohydrates (Abbott et al., 2003), with more than 90% of *Aeromonas* species characterized as fermentative bacteria (Janda, 1985).
- In addition, the isolated *Aeromonas salmonicida* ZS-strain (BI55) was able to form a strong biofilm which will provide some protection from extreme environmental conditions (Kirov et al., 2004).

3.7.5.2. Draft Whole Genome Sequence of the *Cl. tertium* ZS strain (BI85)

The biochemical and physiological characterisation of *Cl. tertium* (BI85) has been outline in the sections above. *Cl. tertium* ZS strain (BI85) has a high percentage (20.5%) of genes for carbohydrate metabolism compared with the subsystem groups of the other isolates (Table 3.11). However, this strain was unable to degrade ISA either from CDPs or in the form of $Ca(ISA)_2$ in liquid culture at pH 8.0 and at pH 9.0. This strain showed only 98% similarity to the closest strain

of a *Cl. tertium* Type strain JCM 6289. This observation along with the ability for growth at high pH resulted in this strain being selected for whole genome sequencing. In this case it is the first time a whole-genome sequence has produced for this species.

RAST annotation (Figure 3.79) indicated the presence of a numerous of functional genes particularly for stress responses, metal resistance and carbohydrate degradation. The number of genes for dormancy and stress response (heat shock, cold shock, oxidase stress and detoxification) indicate the adaption of this bacterium to harsh environments.

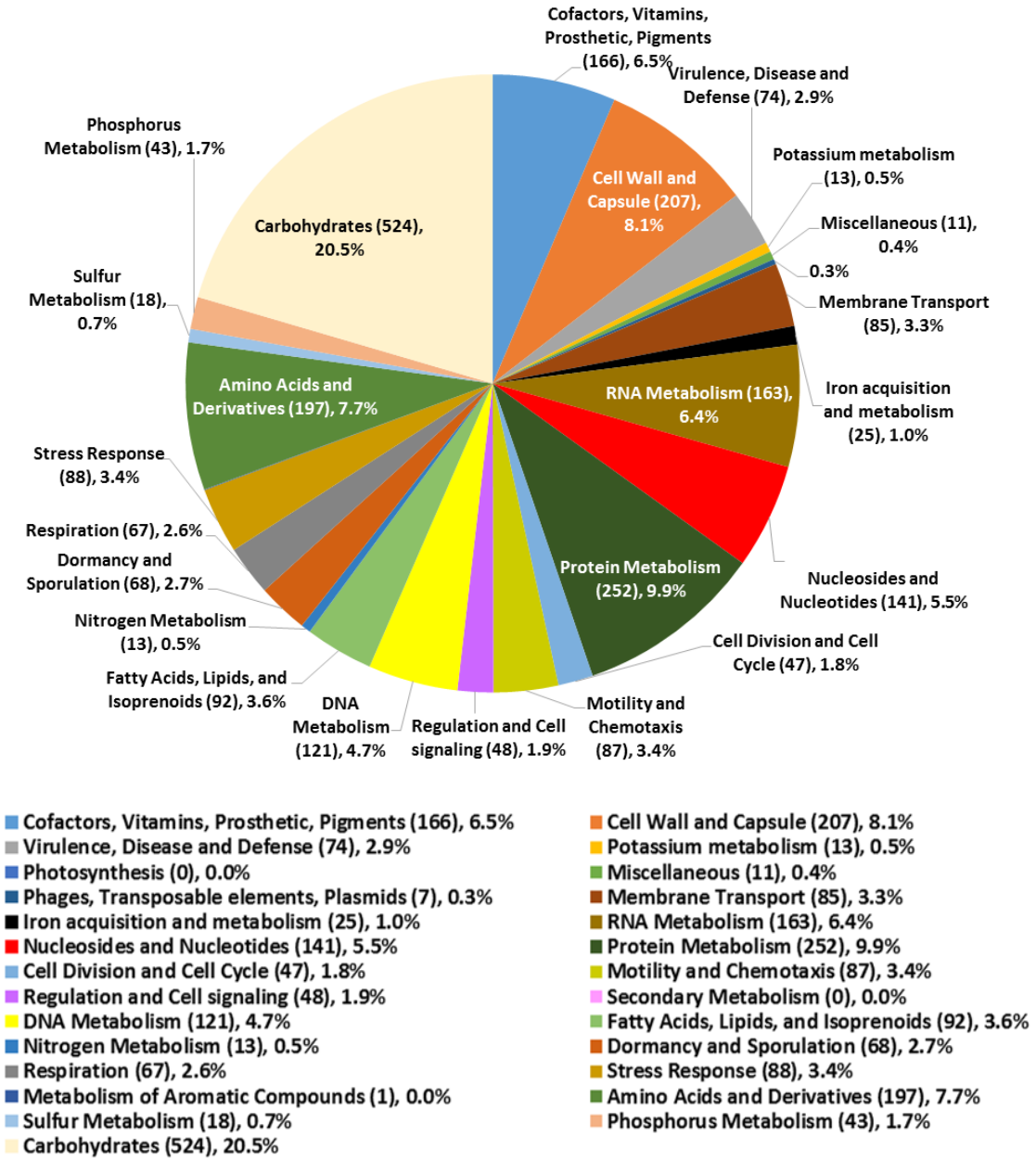


Figure 3. 79: Whole genome sequencing and Subsystems group distribution of *Clostridium tertium* ZS strain (BI85).

3.7.5.3. Characterisation and WGS of *Alishewanella* sp. HH-ZS strain

The genus *Alishewanella* assigned to the class Gammaproteobacteria by Fonnesbech Vogel *et al*. (2000). At present, several species belonging to the genus *Alishewanella* have been identified such as: *Alishewanella fetalis* strain CCUG 30811T, isolated from an autopsy of a human foetus in Sweden (Vogel et al., 2000); *Alishewanella aestuarii* B11T, isolated from tidal flat sediments in Korea (Roh et al., 2009); *Alishewanella tabrizica* RCRI4T isolated from Qurugöl Lake in north-west Iran (Tarhriz, Nematzadeh, Vahed, Hejazi, & Hejazi, 2012); *Alishewanella solinquinati* KMK6(T), isolated from soil contaminated with textile dyes located in India (Kolekar, Pawar, et al., 2013); *Alishewanella jeotgali* MS1T, isolated from gajami sikhae, a traditional fermented food in Korea made from flatfish (M.-S. Kim et al., 2009); *Alishewanella agri* BL06T isolated from landfill soil in Pohang, Korea (M.-S. Kim, Jo, Roh, & Bae, 2010). Recently *Alishewanella* sp. have been found to be dominant members of a floc based bacterial communities of CDP fed microcosm inoculated with colonised cotton that recovered from Harpur Hill (Charles et al., 2015).

3.7.5.3.1. Phylogenetic analysis of *Alishewanella aestuarii* HH-ZS strain (BI28)

Analysis of the partial 16SrRNA sequence placed *Alishewanella aestuarii* HH-ZS strain (BI28) (*A.* HH-ZS) is homology to *Alishewanella aestuarii* B11^{T} (Roh et al., 2009) with a 99% similarity (Table 3.13 and Figure 3.80).

Table 3. 13: *Alishewanella* sp. and related strains with the closest strains to the *Alishewanella* sp. HH-ZS strain (BI28).

List of hits from EzBioCloud 16S database

No	**Name**	**Strain**	**Accession**	**Pairwise Similarity (%)**	**Authors**
1	*Alishewanella aestuarii*	B11	ALAB01000035	100.0	Roh *et al*. 2009
2	*Alishewanella jeotgali*	KCTC 22429	AHTH01000032	100.0	Kim *et al*. 2009
3	*Alishewanella agri*	BL06	AKKU01000023	98.3	Kim *et al*. 2010
4	*Alishewanella fetalis*	CCUG 30811	AF144407	98.1	Fonnesbech Vogel *et al*. 2000
5	*Rheinheimera aquatica*	GR5	GQ168584	97.2	Chen *et al*. 2011
6	*Alishewanella tabrizica*	RCRI4	GQ505294	97.1	Tarhriz *et al*. 2012
7	*Alishewanella solinquinati*	KMK6	EU574916	96.7	Kolekar *et al*. 2014
8	*Rheinheimera longhuensis*	LH2-2	EU183319	96.7	Liu *et al*. 2012

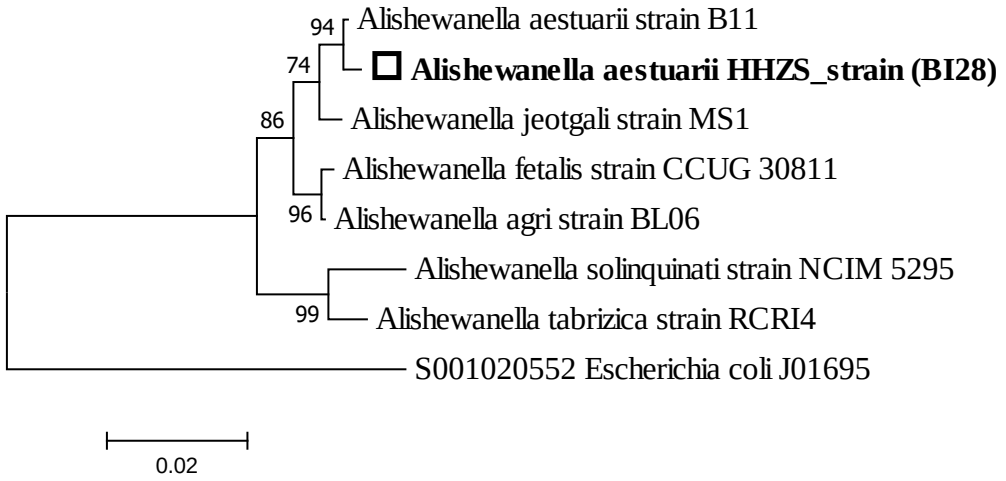


Figure 3. 80: Phylogenetic consensus tree based on 16S rRNA gene sequences.

The tree showing the position of strain *Alishewanella* sp. HH-ZS belongs in the genus *Alishewanella* sp. Phylogenetic analysis was performed by the neighbour-joining method with 1000 random replicates of the 16SrRNA gene sequencing (Fasta). The tree was configured using the neighbour-joining in MEGA 7.0.

Comparison of *A.*HH-ZS (BI28) with the *Alishewanella* sp. that dominated the floc based community formed in the CDP fed microcosm reported by Charles *et al*. (C. Charles et al., 2017) indicated that *Alishewanella* sp. from the community showed a 99% identity to the *A.HH-ZS* (BI28). A phylogenetic tree generated using the neighbour-joining methods in MEGA 7.0 showing that the *Alishewanella* sp. (community) was closely related to *A.*HH-ZS (BI28) and three other strains BL06, B11 and MS1 with 99% sequence identity (Figure 3.81).

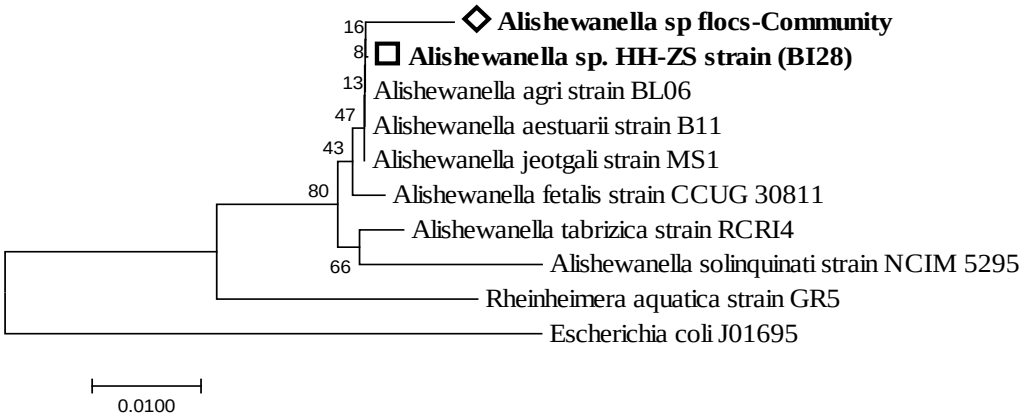


Figure 3. 81: Phylogenetic consensus tree of the *Alishewanella* sp. community strain and *Alishewanella* sp. HH-ZS strain (BI28).

3.7.5.3.2. Morphological and metabolic characterisation of *Alishewanella* sp. HH-ZS strain

When grown on FAA, *Alishewanella* sp. HH-ZS formed colonies that had a regular edge, smooth surface, slightly raised profile, beige colouration which was darker at the centre of the colony (Figure 3.82 A).

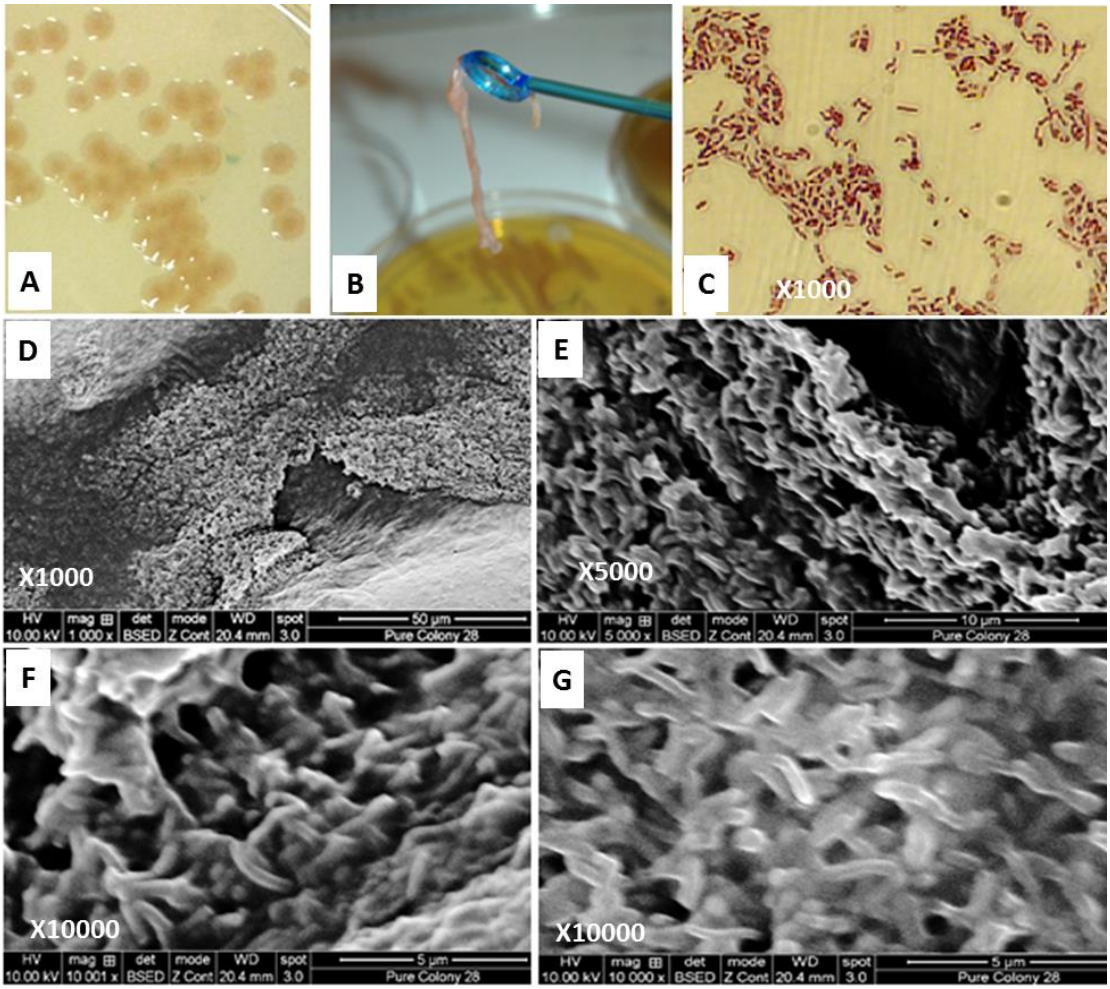


Figure 3. 82: **morphological characterisation of the *Alishewanell*a sp. HH-ZS**

Colonies were pale and darker at the centre on FAA (A), sticky texture after 7 days incubation in an anaerobic condition on FAA agar at pH 9.5 (B), Gram-negative rods (C), images by SEM, bacilli cells attached to each other (D, E, F and G).

The colonies were 'sticky' (Figure 3.82 B) after 5 days incubation in an anaerobic workstation at 25^{o}C. Microscopic investigation revealed *A.* HH-ZS to be a Gram-negative, motile rod, arranged as single cells (Figure 3.82 C). The cells under SEM were bacilli attached to each other (Figure 3.82 D, E, F and G), suggesting EPS production and biofilm formation. This was confirmed when the strain was evaluated for its ability to form biofilms and it was shown to form a moderate biofilm in a 96 well plate test (Figure 3.83).

Compared with its closest relatives that have been characterised (Table 3.14), *A.*HH-ZS was broadly similar. The only exception being a higher maximum pH and the absence of protease as indicated by its inability to hydrolyse gelatine.

Table 3. 14: Comparison of the *Alishewanella* sp. HH-ZS strain and its closest related strains

The strains are: 1, *A. jeotgali* sp. MS1^T (M.-S. Kim et al., 2009); 2, *A. fetalis* CCUG 30811^T (Vogel et al., 2000); 3, *A. aestuarii* B11^T (Roh et al., 2009). The physiological and biochemical characteristics results of *A.* HH-ZS strain is distinct from other strains. ND, No data available.

Test	*A.HH-ZS*	1	2	3
Source	Buxton soil	Gajami sikhae (jeotgal)	Human fetus	Tidal flat sediment
Motility	+	+	-	+
Temp. range (optim. ^OC)	10-44(30)	4-40(37)	25-42(37)	18-44(37)
NaCl range (optimum %)	0-4(1-2)	1-2 (1)	1-8	0-5(3)
pH range (optimum)	7-11(8-10)	6.5-9.5(6.5-9.0)	ND	ND
Oxidase	+	+	+	+
Catalase	+	+	+	+
Indol	-	-	-	-
Urease	-	-	-	-
Protease (gelatine)	-	+	+	+
B-glucosidase (Esculin)	+	+	+	-
D-Glucose	-	-	-	-
D-Mannitol	-	-	+	-
D-Lactose	-	-	ND	ND
D-Sucrose	-	+	+	+
D-Maltose	-	+	+	-
Salicin	-	-	ND	ND
D-xylose	-	-	ND	ND
L-arabinose	-	-	ND	-
Glycerol	-	-	+	-
D-cellobiose	-	-	ND	ND
D-mannose	-	-	ND	-
D-melezitose	-	-	ND	ND
D-raffinose	-	-	-	+
D-sorbitol	-	-	ND	ND
L-rhamnose	-	-	ND	ND
D-trehalose	-	+	-	-
G+C content (mol%)	51	53.6	51	49.5

Although, the *Alishewanella aestuarii* HH-ZS strain showed inability to metabolize almost all substrates provided by the API20A system (Table 3.13), the strain was able to utilize about 35.7% of the substrates of carbon sources provided by PM 1 Biolog microplate, and 30% of the substrates provided by PM2 Biolog microplate, the names of the substrates is shown in Tables, 3.6 and 3.7, and they are included in the discussion section.

The cellular fatty acid profile of *A.* HH-ZS strain (BI28) (Table 3.15) were compared to two if its closest relatives *A.aestuarii* B11^T and *A. fetalis* CCUG 30811^T strain, the dominant fatty acid in these two strains was C18:1 ω7C (22.6% and 17.7% respectively) (Roh et al., 2009) which was also present in significant amounts in *Alishewanella* sp. HH-ZS (BI28). However, in *A.* HH-ZS

(BI28) significant amounts of C17:1 ω7C was present and not detected in the comparative strains (Table 3.15). Generally, all the three strains had similar FA profiles. The major fatty acids of *Alishewanella* sp. HH-ZS strain were C17:1 ω7C (26.71%), C18:1 ω7C (17.95%), C17:0 (17.7%) and C18:0 (8.1%).

Table 3. 15: Cellular fatty acids contents (%) of *Alishewanella* sp. HH-ZS strain

Fatty acid amounting to more than 1.5% of the cellular *A.* sp. HH-ZS strain (this study) are illustrated. Tr, Trace (<1.5%); -, not detected. Results from the others two strains ($B11^T$ and $CCUG\ 30811^T$) were obtained from Roh *et al*. (Roh et al., 2009).

Fatty acid	HH-ZS (BI28)	$B11^T$	$CCUG\ 30811^T$
C13:0	1.4	0	0
C13:0 3OH	0	3.6	3.6
C14:0	0	Tr	2.1
C15:0	2.5	1.2	1.9
C16:0	3.8	11	14.3
C17:0	17.7	8.8	8.6
C17:1 ω7C	26.71	0	0
C17:1 ω8C	0	18	16
C18:0	8.1	1.6	1.3
C18:1 ω7C	17.95	22.6	17.7
C18:1 ω7C	0	1.3	2.0
C18:0 Cyclo ω7C	2.21	0	0

Alishewanella sp. were detected as the dominant species in the flocs of the microbial community in the CDPs fed microcosms of alkaline broth media up to pH 12.0. The same author concluded that floc formation in hyperalkaline conditions protects bacterial communities from alkaline pH value up to pH 13.0 (Charles et al., 2015; C. Charles et al., 2017). Evaluation of the biofilm forming potential of *A.* HH-ZS strain (BI28) indicated that under these conditions it was only a moderate biofilm former. This may indicate that it was other members of the floc communities reported by Charles *et al*. (2017) e.g. *Dietzia* sp. that were responsible to the biofilm formation reported.

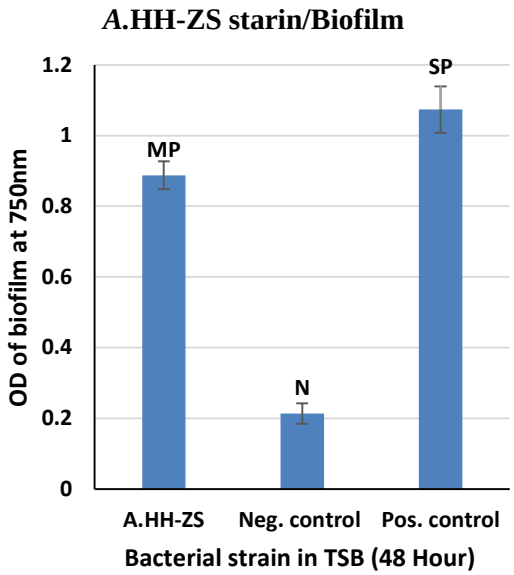


Figure 3. 83: *Alishewanella* sp. HH-ZS (BI 28) strain and biofilm formation

Moderate positive (MP) of a biofilm was formed by *A*.HH-ZS strain, compared with negative control (N) and strong positive (SP) control that detected at the same incubation time using 96- wells plate method.

The pH response of *A*.HH-ZS (BI28) was evaluated at a range of temperatures. When grown at the optimal growth temperature (30°C) the strain maintained optimal growth across a wider pH range (Figure 3.84) up to pH 11.0. At lower temperature, the optimal pH range narrowed with pH 9.0 being the maximum pH supporting significant growth at 20, 25 and 40^{o}C. *A*.HH-ZS was isolated from CDPs driven microcosm at pH 12.0, a pH greater than the upper pH limit of the strain in isolation. This suggests that in the microcosms the strain is protected from the external pH, potentially through interactions with other species as observed by Charles *et al*. (2017). In a previous study, a pH profile for *A jeotgali* sp. MS1^{T}, isolated from a traditional fermented food had a pH range from pH 6.5-9.5 and optimum pH was at pH 6.5-9.0 (M.-S. Kim et al., 2009) which is similar to that reported here.

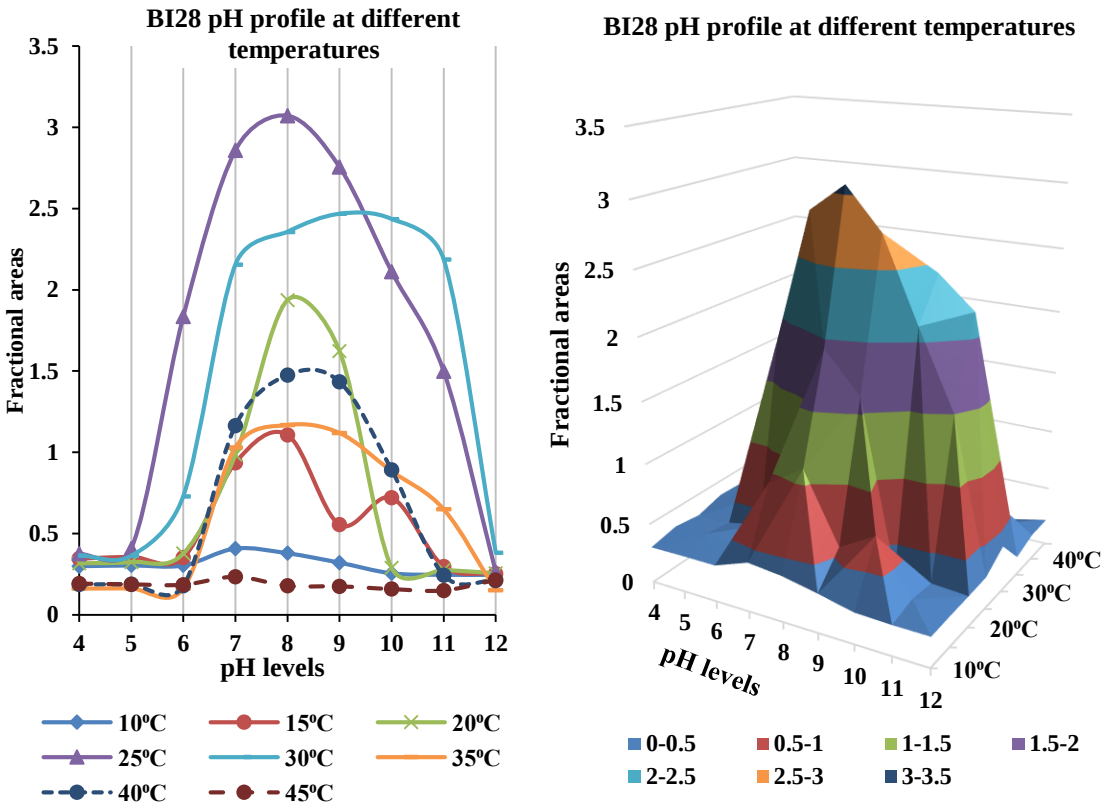


Figure 3. 84: *Alishewanella* sp. HH-ZS strain pH versus temperature for bacterial growth

The optimum temperature for this strain is 30ºC where this strain showed capability of growth at high pH at pH 8.0 to pH 11.0, the lag phase was increased at high pH as the temperature decreased but the optimum pH was still at pH 8.0, while the growth was suppressed at a temperature 10ºC and 45ºC, (A). On the other hand, the lag phase was decreased as the temperature was increased above 25ºC to 40ºC. 3D surface plot showing the effects of pH and temperature on the growth.

The minimal inhibitory concentration of sodium chloride was 4.7% (w/v), and the optimum concentration of NaCl for *A.*HH-ZS growth was 1-2% (w/v) (Figure 3.85). The results of the tolerance to heavy metals have been illustrated in Table 3.8. The strain demonstrated greatest tolerance to lead and copper with MICs 3.8, 2.8 mM respectively, and least tolerance to cadmium where it was inhibited by 0.1 mM. In a previous study, *Alishewanella* sp. WH16-1 strain was isolated from a heavy metal-rich soil of an iron and copper mine, was able to reduce chromate (Cr^{6+}) to Cr^{3+} (less toxic) and sulfate ($SO4^{2-}$) to S^{2-}. The latter being able to react with Cd^{2+} resulting in the precipitated of Cadmium sulfide (CdS), the strain was also able to tolerant other heavy metals with MICs for Cd, Pb, Cu, Chromate (Cr) and Arsenite (As) were 0.08, 10, 1.0, 45, and 1.0 mM, respectively (Xia et al., 2016).

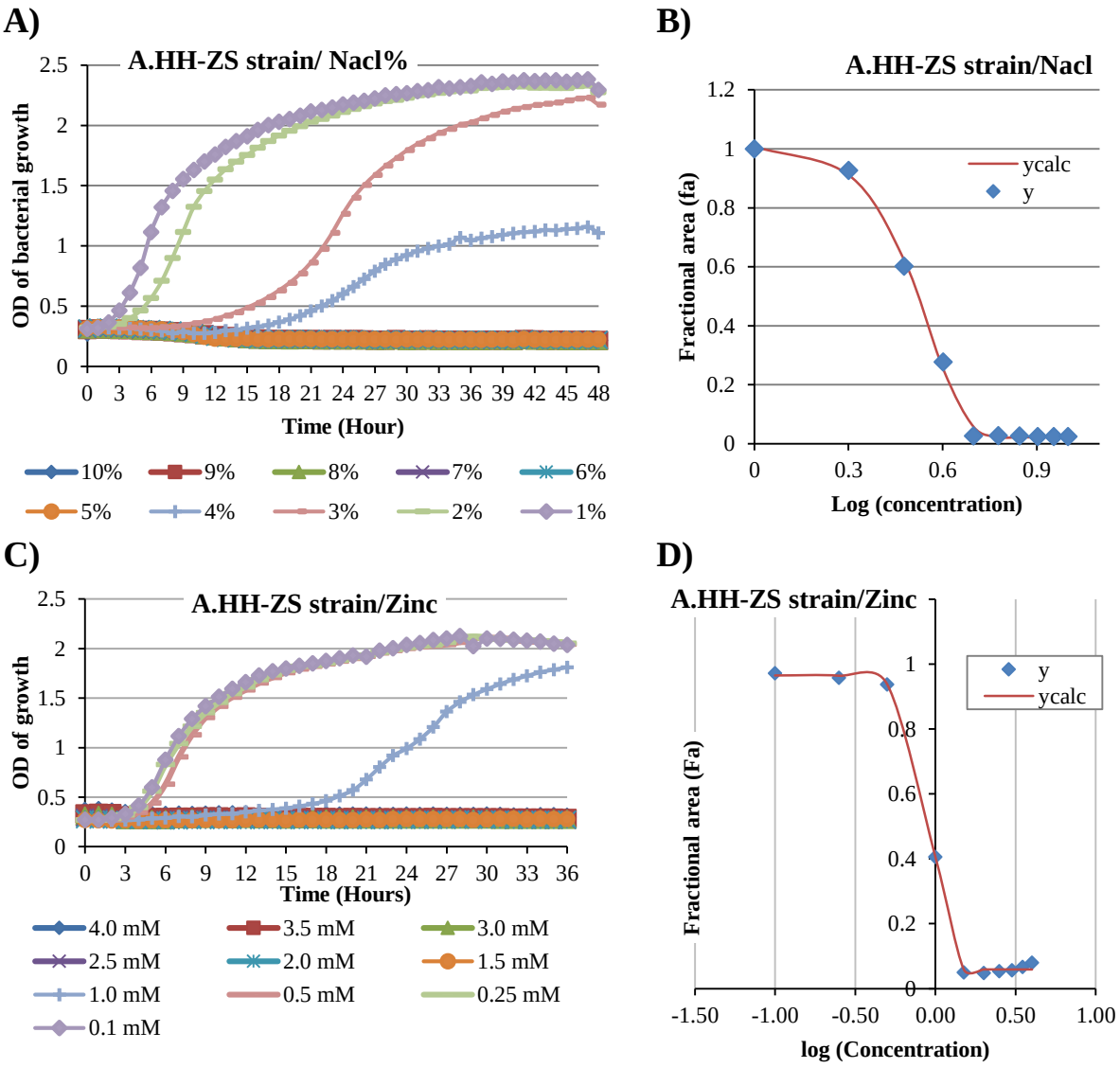


Figure 3. 85: Sodium chloride and heavy metals (Zinc) tolerance of *Alishewanella* sp .HH-ZS strain

Growth curves of bacteria at different NaCl concentration (A), the inhibition profile of NaCl against *A.HH-ZS* (BI28) MIC was detected through calculation of; (■), observed FA; (▬), fitted Gompertz function illustrated on a graph (B). An example growth curves for one of the total 6 heavy metals that already added in the appendix, this strain capable to tolerate Zinc up to 1.0 mM (C).

3.7.5.3.3. Draft Whole Genome Sequence

The WGS of *Alishewanella aestuarii* HH-ZS strain contained 3,531,586 bp encoding for 3,304 putative coding sequences, of which 71 have been classified as pseudogenes, 3,236 as hypothetical proteins, and 3,165 predicted to form known functional proteins. The genome has a GC content of 51.0% and contains 68 genes RNA; rRNAs (5S, 16S, 23S), 60 tRNA, and 5 noncoding RNA (ncRNA). The WGS from this project has been deposited at DDBJ/EMBL/GenBank under the accession numbers LZEJ00000000 (*Alishewanella aestuarii* HH-ZS).

Furthermore, RAST annotation (Figure 3.86) indicated the presence of 220 genes involved in carbohydrate metabolism (Table 3.16). This number is low when compared with the ISA fermentative *M.f.*HH-ZS strain that has 297 genes. For example, it has less than 50% of genes associated with monosaccharides and di- and oligosaccharides metabolism (Table 3.16).

Table 3. 16: Comparison of carbohydrate metabolism genes between *Alishewanella* HH-ZS and *M.fermentans* HH-ZS.

Alishewanella aestuarii HH-ZS strain = (A) and *M.f.*HH-ZS strain = (B)

No	Comparison the Subsystem features of the carbohydrate metabolism between *Alishewanella aestuarii* HH-ZS = (A) (220 genes) and *M.f.HH-ZS* strain = (B) (297 genes)
1.	**A) Central carbohydrate metabolism (101);** Glycolysis and Gluconeogenesis (14), Methylglyoxal Metabolism (7), Pyruvate metabolism II: acetyl-CoA, acetogenesis from pyruvate (8), Pyruvate Alanine Serine Interconversions (6), Glyoxylate bypass (6), Entner-Doudoroff Pathway (13), Dehydrogenase complexes (11), TCA Cycle (17), Pentose phosphate pathway (9), Pyruvate metabolism I: anaplerotic reactions, PEP (10) **B) Central carbohydrate metabolism (78);** Glycolysis and Gluconeogenesis (14), Methylglyoxal Metabolism (2), Pyruvate metabolism II: acetyl-CoA, acetogenesis from pyruvate (4), Pyruvate Alanine Serine Interconversions (5), Glycolate and glyoxylate interconversions (6), Pentose phosphate pathway (8), TCA Cycle (10), Entner-Doudoroff Pathway (12), Pyruvate metabolism I: anaplerotic reactions and PEP (10), Dehydrogenase complexes (4), Dihydroxyacetone kinases (2), Pyruvate:ferredoxin oxidoreductase (1).
2.	**A) Fermentation (24);** Butanol Biosynthesis (6), Acetolactate synthase subunits (4), Fermentations: Lactate (1) and Acetyl-CoA fermentation to Butyrate (13) **B) Fermentation (19)** include; Mixed acid (8), Acetolactate synthase subunits (2), Fermentations: Lactate (3), Acetoin, butanediol metabolism (6)
3.	**A) Monosaccharides (21);** Mannose Metabolism (2), D-ribose utilization (1), Deoxyribose and Deoxynucleoside Catabolism (6), D-Galacturonate and D-Glucuronate Utilization (12) **B) Monosaccharides (56)** include; Mannose Metabolism (20), D-ribose utilization (4), Xylose utilization (10), Deoxyribose and Deoxynucleoside Catabolism (7), D-Galacturonate and D-Glucuronate Utilization (15)
4.	**A) Polysaccharides (7);** Glycogen metabolism (7) **B) Polysaccharides (19)** include; Glycogen metabolism (4), Cellulosome (15)
5.	**A) Aminosugars (6);** Chitin and N-acetylglucosamine utilization (6) **B) Aminosugars (18)** include; Chitin and N-acetylglucosamine utilization (18)
6.	**A) Di- and oligosaccharides (16);** Sucrose (5), Maltose and Maltodextrin Utilization (11) **B) Di- and oligosaccharides (49)** include; Maltose and Maltodextrin Utilization (22), Lactose and Galactose Uptake and Utilization (18), Lactose utilization (9)
7.	**A) One-carbon Metabolism (35);** Serine-glyoxylate cycle (30), by tetrahydropterines (5) **B) One-carbon Metabolism (26)**; Serine-glyoxylate cycle (20), by tetrahydropterines (6)
8.	**A) Organic acids (3);** Glycerate metabolism (3) **B) Organic acids (6)** include; Propionyl-CoA to Succinyl-CoA Module (6)
9.	**A) Sugar alcohols (6);** Glycerol and Glycerol-3-phosphate Uptake and Utilization (6) **B) Sugar alcohols (26)**; Glycerol and Glycerol-3-phosphate Uptake and Utilization (16), Inositol catabolism (10)

The presence of these genes enable *Alishewanella aestuarii* HH-ZS strain to utilize 35.7% of the substrates provided by the PM 1 Biolog microplate (Table 3.6).

A comparison between *Alishewanella* sp. HH-ZS strain (BI28) and its closest relatives (Table 3.14) indicated that there was a similarity in most parameters. The biochemical tests indicated that all strains were incapable of utilizing the substrates provided by the API 20A system, except D-Sucrose and D-Maltose which were utilized by *A. jeotgali* sp. nov. MS1^T and *A. fetalis* CCUG 30811^T (M.-S. Kim et al., 2009; Vogel et al., 2000), whilst *Alishewanella aestuarii* B11^T capable to utilizing D-Maltose and D-raffinose (Roh et al., 2009). In addition, a new strain WH16-1 of *Alishewanella* sp. showed similar results in the case of oxidase, catalase, and aesculinase and negative for indole. It was also able to use maltose and D-sucrose and was unable to use L-arabinose, D-glucose, D-mannitol, D-mannose (Xia et al., 2016).

This strain is well equipped with a range of resistance genes associated with beta lactamase, cobalt, zinc, arsenic, copper, chromium and several genes of efflux pumps. It also had an array of genes covering dormancy and stress responses (heat shock, cold shock, oxidase stress and detoxification) indicated the adaption of this bacterium to harsh environmental conditions. The *Alishewanella* sp. was used for Pectin degradation (Kolekar, Konde, et al., 2013; Wei et al., 2016), biological treatment of wastewater that was contaminated with a textile dye and converted to non-toxic compounds (Kolekar & Kodam, 2012), it has also described as be able to reduce the bioavailability of pH in soil (Zhou et al., 2016).

This WGS of this strain was published as follow:

Salah, Z. B., Rout, S. P., & Humphreys, P. N. (2016). Draft whole-genome sequence of the alkaliphilic *Alishewanella aestuarii* strain HH-ZS, isolated from historical lime kiln waste-contaminated soil. *Genome announcements*, 4(6), e01447-16.

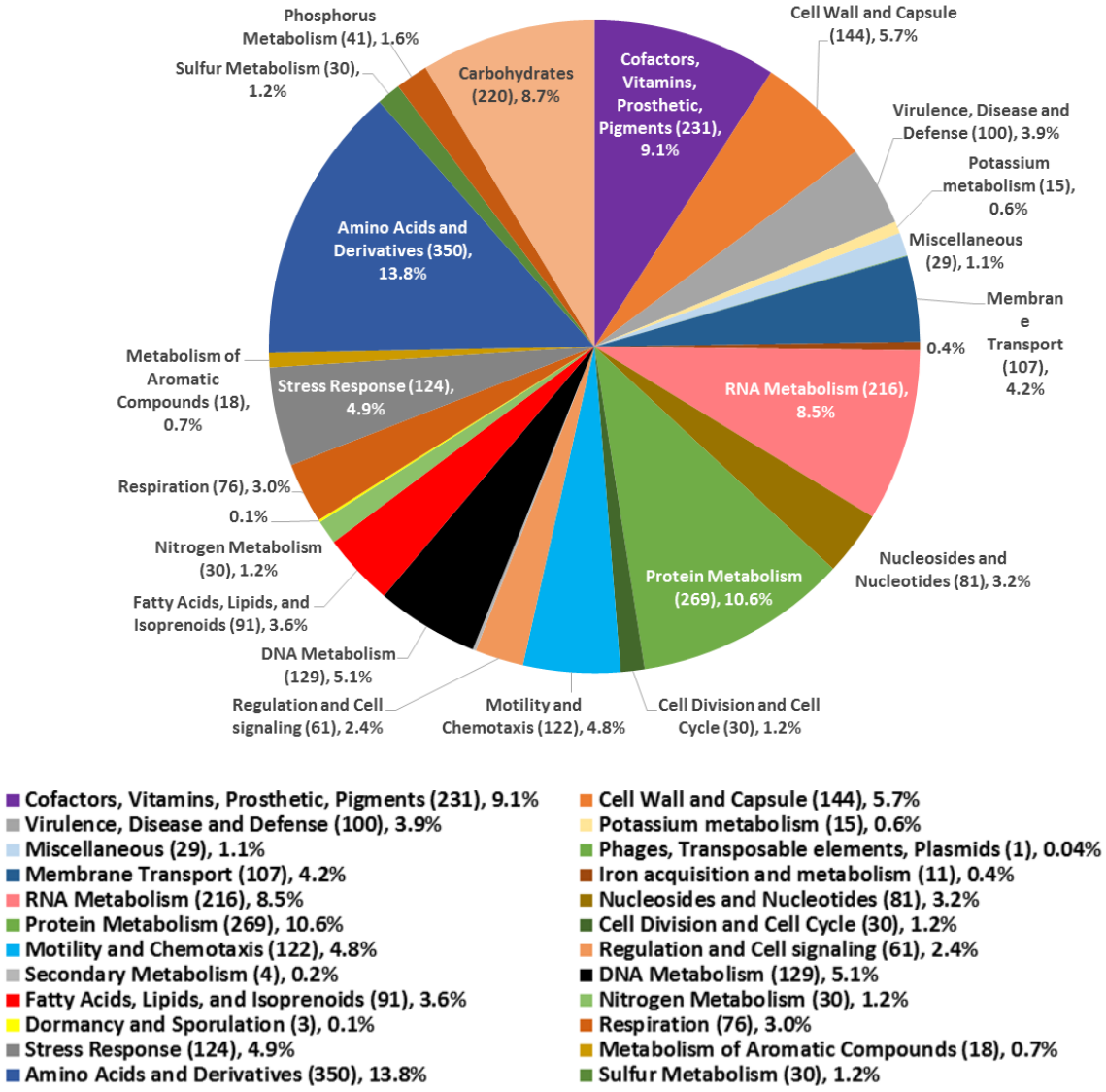


Figure 3. 86: Subsystems groups distribution statistic of *Alishewanella* HH-ZS strain WGS

Based on genome annotations via RAST. The pie chart presents the abundance of each subsystem group and the number of each subsystem featured (in brackets). The highest percentages were: 13.8% represented a wide range of genes involved in utilization of amino acids and derivatives, protein metabolism 10.6% and carbohydrates 8.7%.

3.7.5.3.4. Key findings

- This strain was unable to degrade ISA in the form of either CDPs or $Ca(ISA)_2$.
- The strain was able to tolerate extreme environmental conditions, such as pH up to pH 12, temperatures up to 44ºC and exposure to heavy metals.
- The strain possessed a wide range of genes covering capabilities such as stress response proteins, membrane transport proteins and proteins involved in defence system.

3.7.5.4. Characterisation and WGS of *Azonexus hydrophilus* ZS02 (BI 70)

The location of this genus in the *Betaproteobacteria*, under the *Rhodocyclaceae* family was proposed by Reinhold-Hurek & Hurek (2000). Currently this genus includes two species *Azonexus fungiphilus* and *Azonexus caeni*. More recently Chou (2008), proposed a third species *Azonexus hydrophilus* sp. nov., which was isolated from freshwater (Chou et al., 2008). *Aznexus* strains have been identified as members of bacterial communities in an Indian coal bed at 600-700m depth. In these communities the *Azonexus* sp. enhanced the biotransformation of coal into methane through the nitrogen fixation abilities (D. N. Singh, Kumar, Sarbhai, & Tripathi, 2012). *Azonexus hydrophilus ZS02* (BI70) (this study) was isolated from a minimal medium/$Ca(ISA)_2$ microcosm operating at pH 9.0 where the population had become dominated by *Azonexus* species following a period of starvation. In addition, the Gram-stain result of the (sediment) sample was dominated by Gram-negative rods that showed similar morphological characteristics compared with the Gram-stain of the pure culture of *Azonexus hydrophilus* ZS02 strain (BI 70) (this study) (Figure 3.87).

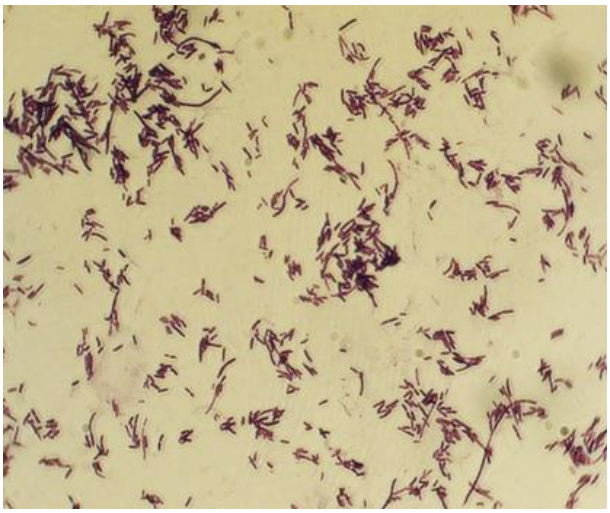


Figure 3. 87: Gram-negative rods dominated the $Ca(ISA)_2$ microcosm sediment

3.7.5.4.1. Phylogenetic analysis of *Azonexus hydrophilus* ZS02 strain

Analysis of the 16S rRNA sequence (Figure 3.88) identified the strain as *Azonexus hydrophilus* which aligned most closely with the existing strain (d8-1) identified by (Chou et al., 2008) (Figure 3.88). The isolated strain showed a 98% match to the *Azonexus hydrophilus* Type strain d8-1 and it showed a 95% match to an uncharacterised *Azonexus* sp. (Table 3.17). (DQ088747) (that dominated the community parent 94.6%). However, the latter showed 97% match to the *Azonexus hydrophilus* Type strain d8-1.

Table 3. 17: *Azonexus* sp. and related strains with the closest strains to the *Azonexus* sp. ZS02 strain (BI70). List of hits from EzBioCloud 16S database

No	Name	Strain	Accession	Similarity (%)	Authors
1	*Azonexus hydrophilus*	DSM 23864 (d8-1)	AUCE01000006	98.0	Chou *et al*. 2008
2	AY124797_s	LT-1	AY124797	96.5	
3	*Azonexus fungiphilus*	BS5-8	AF011350	95.9	Reinhold-Hurek and Hurek 2000
4	*Azonexus caeni*	Slu-05	AB166882	95.6	Quan *et al*. 2006
5	AJ009452_s	SJA-10	AJ009452	95.4	
6	JF775627_s	S33	JF775627	95.2	
7	FN436157_s	HAW-R60-B-924d-I	FN436157	95.2	
8	*Dechloromonas denitrificans*	ATCC BAA-841	LODL01000012	95.1	Horn *et al*. 2005
9	DQ088747_s	BE23FW031301A4RD-1	DQ088747	95.0	

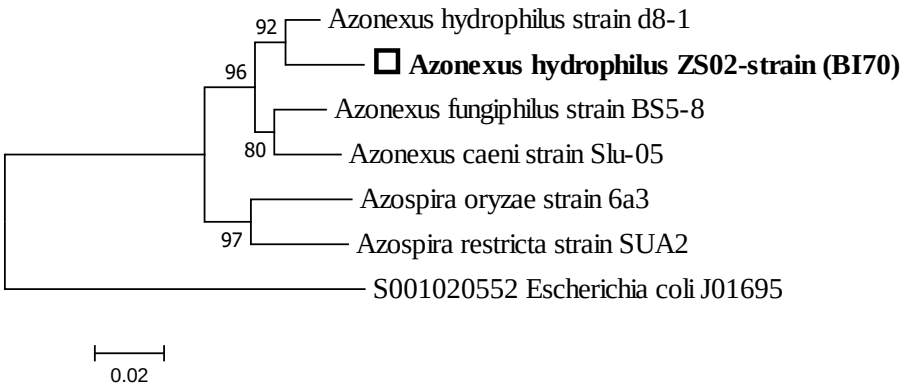


Figure 3. 88: Evolutionary relationships of taxa

The evolutionary history was inferred using the Neighbour-Joining method (Saitou & Nei, 1987). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches (Felsenstein, 1985). The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura et al., 2004) and are in the units of the number of base substitutions per site. Evolutionary analyses were conducted in MEGA7 (Indugu et al., 2016).

A number of *Azonexus* sp. were detected in the CDP fed microcosms allowing the isolated strain to be placed alongside these strains based on their 16S rRNA gene sequences (Figure 3.89). *Azonexus* sp. were detected a number of times in the CDP fed microcosms, these *Azonexus* sp. showed 97.5% similarity to an unclassified *Azonexus* strain (accession number DQ088747). In these communities *Azonexus* sp. occupied between 0.1% (pH 10.0 CDP microcosm), 30.7% (pH 9.0 CDP microcosm) and 94.6% (extended incubation $Ca(ISA)_2$ pH 9.0 microcosm). *A.*

hydrophilus ZS02 (BI70) was closely related to both the community associated *Azonexus* sp. and the unclassified *Azonexus* strain (DQ088747) (Figure 3.89).

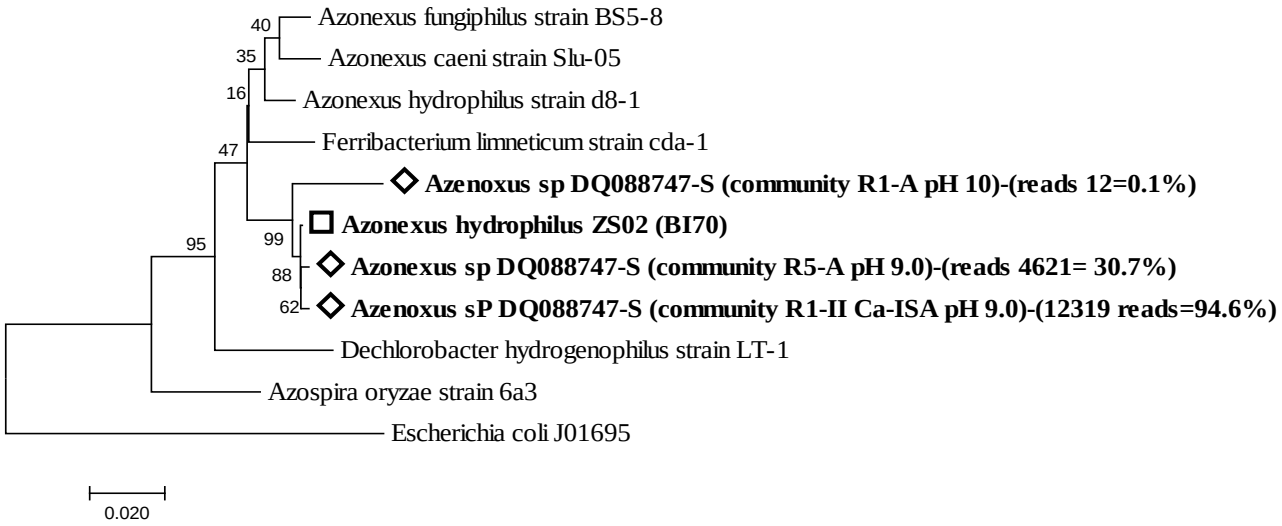


Figure 3. 89: Evolutionary relationships of taxa from the community of the microcosms

The evolutionary history was inferred using the Neighbour-Joining method (Saitou & Nei, 1987). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches (Felsenstein, 1985). The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura et al., 2004) and are in the units of the number of base substitutions per site. Evolutionary analyses were conducted in MEGA7 (Indugu et al., 2016).

3.7.5.4.2. Morphological and metabolic characterisation *A. hydrophilus* ZS02

The isolate generated small colonies on FAA at pH 9.0 (Figure 3.90 A and B). *Azonexus hydrophilus* ZS02, is a Gram-negative, non-spore-forming, polymorphic cells (Figure 3.90 C and D), forming chains of slightly curved pointed rods (Figure 3.90 E, F and G). This strain was unable to utilize all sugars provided by API 20A (Table 3.5), while only 18.7% of the organic substrates of the Biolog PM1(Table 3.6). In addition, this strain was capable of utilizing 10% of the substrate provided by PM2 microplate (Table 3.7).

The strain had a narrow temperature range with an optimum at 25^{o}C (Figure 3.91). The pH profile of the *Azonexus* sp. ZS02 showed a relatively level response between pH 6.0 to pH 9.0 suggesting that it is an alkali tolerant strain. As the temperature increased the optimum pH shifted toward the alkaline end of the growth range. The lag phase of the bacterial growth curves decreased as the temperature increased towards the optimum. This strain was able to tolerate NaCl up to 8.98% (Figure 3.92) but had relatively low metal tolerance, with greatest tolerance being seen with cobalt (Nickel (1.0), Cadmium (0.25), Cobalt (1.5), Lead (0.25) and Zinc (0.5)) (Table 3.8).

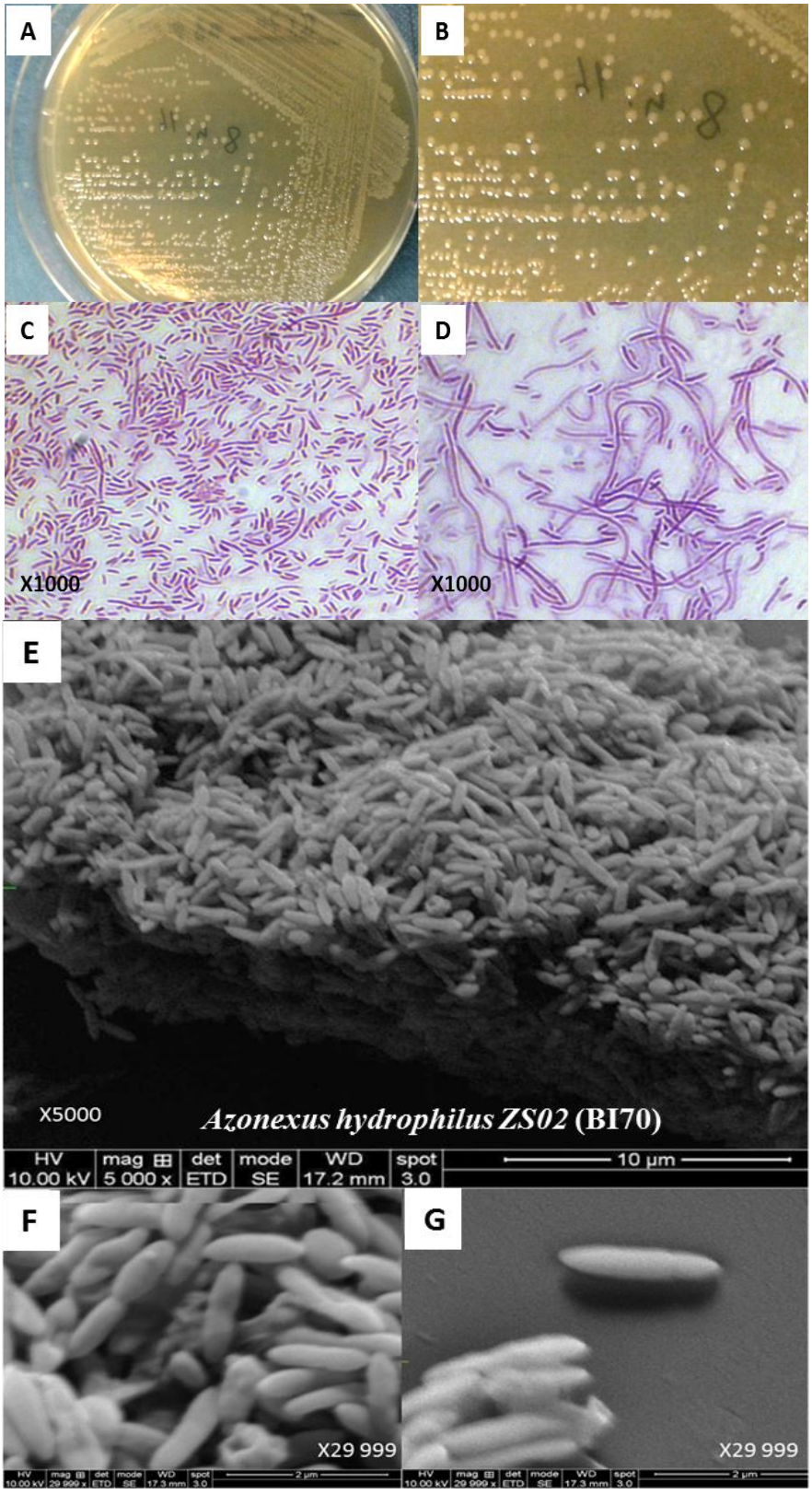


Figure 3. 90: Morphological charecterization of *Azonexus hydrophilus* ZS02

*Pure colonies on the FAA agar at pH 9.5 after 5 days incubation at 25^0C (A); small colonies, semi-soft in texture, regular edge and slightly raised (B); Gram-negative rods, short and long rods (C) and filament-like in shape (D); SEM, the bacterial cells are arranged in cluster(A); diplobacilli and streptobacilli (F), with rounded ends (G).

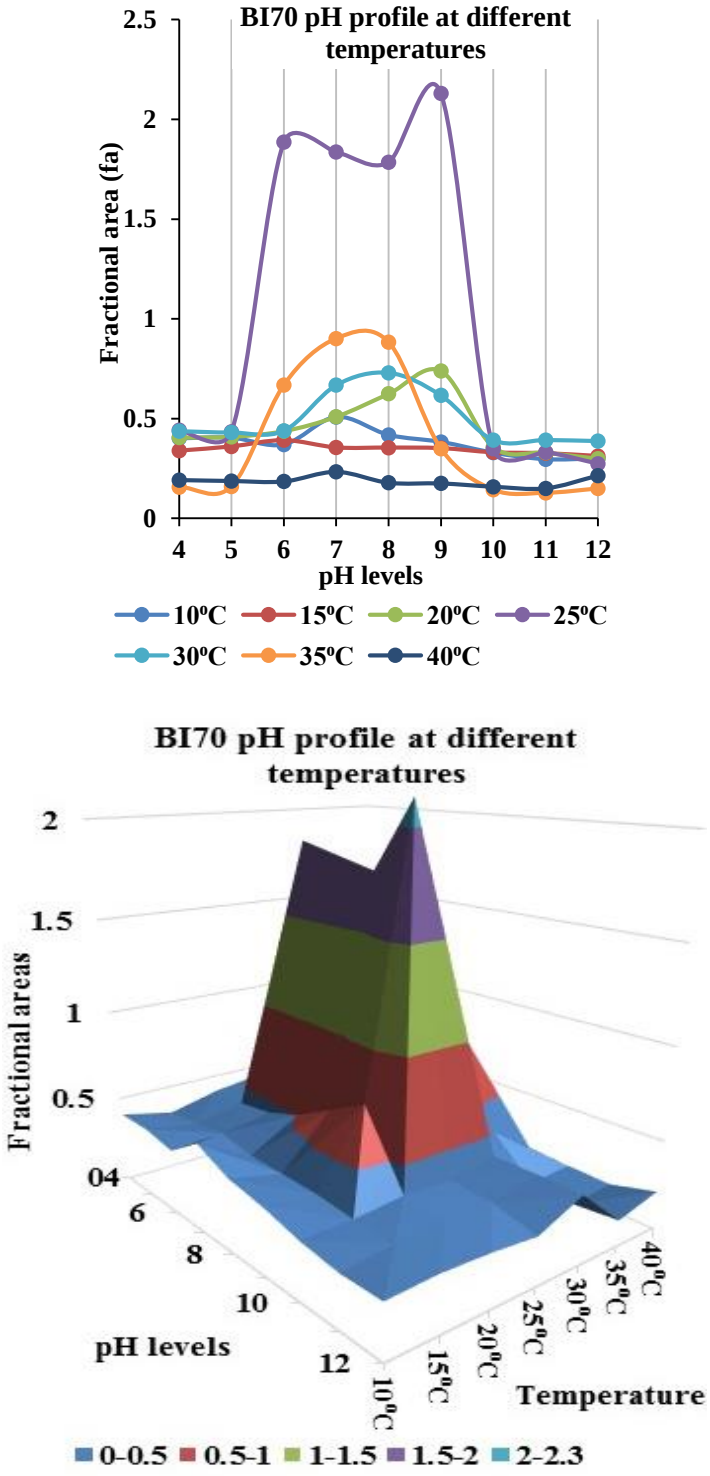


Figure 3. 91: pH profile of BI 70 at different temperature degrees

Poor growth at 10°C in neutral pH of pH 7.0, the bacterial activity was increased at a temperature above 10°C, the lag phase of the bacterial growth curves decreased as the temperature increased, the optimum temperature for this strain is 25°C at this temperature the strain showed good growth at pH from pH 6.0 to pH 9.0, the optimum pH was pH 8.0, this strain was incapable of growing at 40°C from pH 4.0 to pH 12.0.

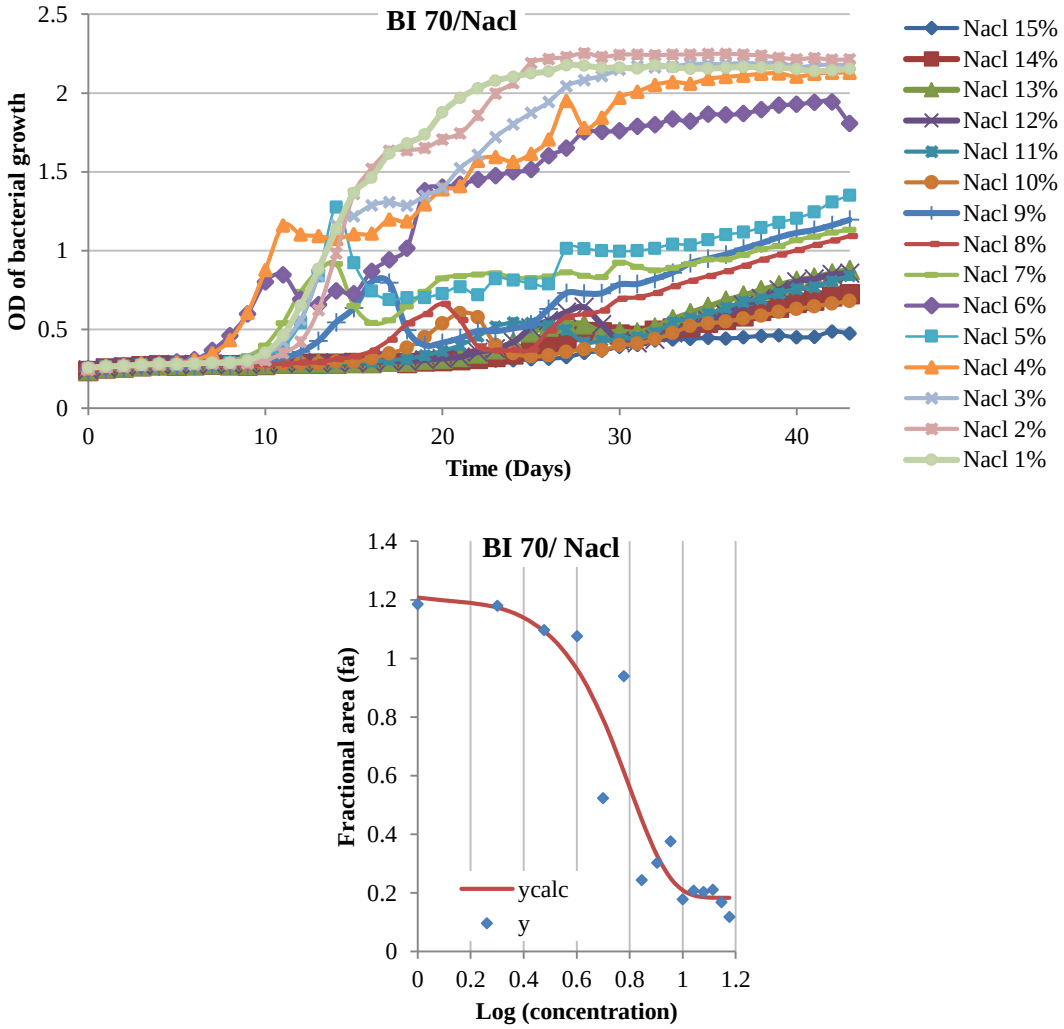


Figure 3. 92: sodium chloride tolerance of *Azonexus* sp. ZS02 (BI70)

3.7.5.4.3. Draft Whole Genome Sequence of *Azonexus* sp. ZS02 (BI70)

In this study the WGS of *Azonexus* sp. ZS02 (BI70) contained 3,596,517 bp encoding for 3,493 putative coding sequences, of which 35 have been classified as pseudogenes, 3,430 as hypothetical proteins, and 3,395 predicted to form known functional proteins. The genome has a high GC content of 61.8% and contains 68 genes RNA; rRNAs 1, 1, 1 of (5S, 16S, and 23S), 56 tRNAs, and 5 noncoding RNA (ncRNA). The *Azonexus hydrophilus* whole genome shotgun (WGS) project has project accession MTHD00000000. This version of the project (01) has the accession number MTHD01000000, and consists of sequences MTHD01000001-MTHD01000017.

Annotation of the genome (Figure 3.94) indicated the presence of 57 resistance genes associated with beta lactamase, cobalt, zinc, arsenic, copper, cobalt, chromium and several genes for efflux pumps. Capsular and extracellular polysacchrides were also associated with 40 genes. Furthermore, 25 genes for nitrogen fixation were present. In addition, a wide range of genes (235 genes) were involved in different type of carbohydrate fermentation (monosaccharide,

disaccharide, oligosaccharide and polysaccharide), dormancy and stress response (heat shock, cold shock, oxidase stress and detoxification) indicated the adaption of this bacterium to harsh environments.

RAST annotation indicated that the *Azonexus* sp. ZS02 genome includes more than 50% of genes present in the fermentative strains of *M.fermentans* HH-ZS (Table 3.19). For instance, mixed acid fermentation genes are absent in *Azonexus* sp. ZS02 strain, these genes enable bacteria to ferment six-carbon sugar (B. Xu, Jahic, Blomsten, & Enfors, 1999). This suggests that although this strain was isolated from the same microcosm dominated by *Azonexus* sp. (94.6%) it is likely to be different. This is because that microcosm was operated at pH 9.0 and fed on the six carbon compound $Ca(ISA)_2$. *Azonexus* sp. ZS02 strain has nitrogen-fixing genes (nif) that codes for the nitrogen fixing nitrogenase enzyme. Nitrogen-fixing microorganisms are enzymatically able to transform dinitrogen gas into ammonium that is utilized for the biosynthesis of essential macromolecules (Gaby & Buckley, 2012). There are many PCR primers available have been used to target the nifH gene (Zehr, Mellon, & Zani, 1998). The agarose-gel figure 3.93 showing the band of the nifH-gene present in this strain.

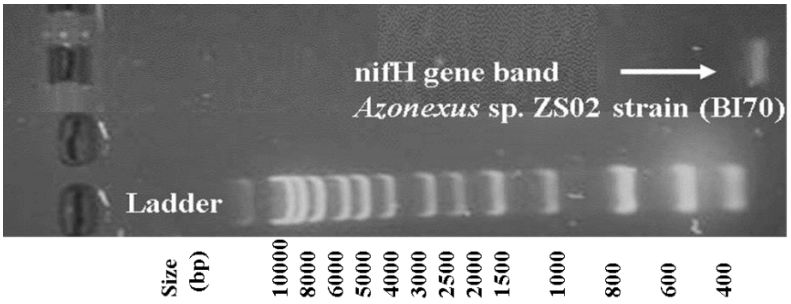


Figure 3. 93: Agarose gel of PCR product of nifH gene in *Azonexus* sp. ZS02 (BI70)

Nitrogen metabolism in this new strain was represented by 108 genes present within subsystems of the WGS, these include; dissimilatory nitrite reductase (11 genes), nitrogen fixation (25 genes), nitrate and nitrite ammonification (17 genes), ammonia assimilation (14 genes), denitrification (38 genes), denitrifying reductase gene clusters (16 genes).

Nitrogen fixation genes are associated with a conversion of molecular nitrogen to ammonia through catalyzed process by nitrogenase enzyme, this complex process provides the nitrogen in soil that is used by plants (Franche, Lindström, & Elmerich, 2009). This process is associated with the eubacteria and methanogenic Archaea (Young & Stacey, 1992). Nitrogen fixation processes are presented by cyanobacteria and Azotobacter (Thiel, 1993). However, a nif gene that produces nitrogenase was detected a first time in *Klebsiella oxytoca* strain (Arnold, Rump, Klipp, Priefer,

& Pühler, 1988) and new class of this gene was detected in *Rhodobacter capsulatus* (Schmehl et al., 1993). In this study, the *nif* genes (25 genes) was detected in the *Azonexus sp*. ZS02 strain (BI70), for instance, *NifA, NifS, NifU, NifB, NifX, NifE, NifN, NifQ, NifV, NifW, NifM, NifH, NifD, NifK, NifZ, NifT, NifO, NifY*, these genes code for nitrogenase and nitrogenase-associated proteins that are involved in the complex process of nitrogen fixation. In addition, this strain has 22 genes for denitrifaction, such as, NirS gene for cytochrome cd1 nitrite reductase; cNor-B, cNor-C, NorE, NorD, NorQ encoded for Nitric-oxide reductase functional group and Nitrous reductase group that encoded by NosZ, NosD, NosF, NosY, NosL. These genes were not detected in the genome of the other strains isolated in this study (Table 3.18). Denitrification genes have been detected in numerous groups of bacteria such as, *Rhodobacter sphaeroides* (Kwiatkowski & Shapleigh, 1996), *Pseudomonas aeruginosa* (Toyofuku et al., 2008).

Table 3. 18: Comparison of nitrogen metabolism genes between *Azonexus hydrophilus* ZS02 genome and other genomes of the isolated strains (this study).

Azonexus hydrophilus ZS02 (BI70) has a greater number of genes (108 genes) for nitrogen metabolism compared with the genomes of the other isolated strains: *Macellibacteroides fermentans* (BI40), *Alishewanella aestuarii* (BI28), *Azonexus hydrophilus* (BI70), *Brevundimonas diminuta* (BI36); *Dietzia natronolimnaea* (BI51), *Rhodococcus erythropois* (BI49), *Tessaracoccus lubricantis* (BI41) and *Clostridium tertium* (BI 85).

Genome Subsystem Feature Counts of genes for Nitrogen metabolism		**Bacterial isolates (BI)**							
		BI70	**BI40**	**BI28**	**BI51**	**BI41**	**BI49**	**BI36**	**BI85**
Nitrogen Metabolism total of genes (A and B)		**108**	**29**	**30**	**32**	**18**	**30**	**8**	**7**
A	**Nitrogen Metabolism - no subcategory**	(70)	(25)	(26)	(32)	(14)	(30)	(8)	(7)
1	Dissimilatory nitrite reductase	**11**	0	0	0	0	0	0	0
2	Nitrogen fixation	**25**	0	0	0	0	0	0	0
3	Nitrosative stress	3	8	2	1	1	1	1	6
4	Nitrate and nitrite ammonification	17	7	12	7	6	5	0	0
5	Ammonia assimilation	14	10	12	17	7	16	7	7
6	Cyanate hydrolysis	0	0	0	4	0	0	0	0
7	Allantoin Utilization	0	0	0	3	0	0	0	0
8	Nitric oxide synthase	0	0	0	0	0	7	0	0
9	Amidase clustered with urea and nitrile hydratase functions	0	0	0	0	0	1	0	0
B	**Denitrification**	(38)	(4)	(4)	(0)	(4)	(0)	(0)	(0)
1	Denitrifying reductase gene clusters	**16**	4	4	0	4	0	0	0
2	Denitrification	**22**	0	0	0	0	0	0	0

Table 3. 19: Comparison of carbohydrate metabolism genes between *Azonexus hydrophilus* ZS02= (A) and *M. fermentans* HH-ZS strain = (B)

No	Comparison the Subsystem features of the carbohydrate metabolism between *Azonexus hydrophilus ZS02*= (A) (235 genes) and *M.f.HH-ZS* strain = (B) (297 genes)
1.	**A) Central carbohydrate metabolism (108)** Methylglyoxal Metabolism (10), Pyruvate metabolism II: acetyl-CoA, acetogenesis from pyruvate (21), Pyruvate Alanine Serine Interconversions (6), Glycolysis and Gluconeogenesis (14), Glycolate, glyoxylate interconversions (7), Dehydrogenase complexes (10), TCA Cycle (17), Pentose phosphate pathway (11), Pyruvate metabolism I: anaplerotic reactions, PEP (6) and Glyoxylate bypass (6). **B)Central carbohydrate metabolism (78);** Methylglyoxal Metabolism (2), Pyruvate metabolism II: acetyl-CoA, acetogenesis from pyruvate (4), Pyruvate Alanine Serine Interconversions (5), Glycolysis and Gluconeogenesis (14), Glycolate and glyoxylate interconversions (6), Dehydrogenase complexes (4), TCA Cycle (10), Pentose phosphate pathway (8), Pyruvate metabolism I: anaplerotic reactions and PEP (10), Entner-Doudoroff Pathway (12), Dihydroxyacetone kinases (2), and Pyruvate:ferredoxin oxidoreductase (1).
2.	**A) Fermentation (48),** Acetolactate synthase subunits (5), Fermentations: Lactate (5), Butanol Biosynthesis (14), Acetyl-CoA fermentation to Butyrate (24) **B) Fermentation (19)** include; Acetolactate synthase subunits (2), Fermentations: Lactate (3), Acetoin, butanediol metabolism (6) and Mixed acid (8).
3.	**A) Monosaccharides (5),** Mannose Metabolism (3), D-ribose utilization (2) **B) Monosaccharides (56)** include; Mannose Metabolism (20), D-ribose utilization (4), Xylose utilization (10), Deoxyribose and Deoxynucleoside Catabolism (7), D-Galacturonate and D-Glucuronate Utilization (15)
4.	**A) Polysaccharides (0)** **B) Polysaccharides (19)** include; Glycogen metabolism (4), Cellulosome (15)
5.	**A) Aminosugars (0)** **B) Aminosugars (18)** include; Chitin and N-acetylglucosamine utilization (18)
6.	**A) Di- and oligosaccharides (1),** Lactose utilization (1) **B) Di- and oligosaccharides (49)** include; Maltose and Maltodextrin Utilization (22), Lactose and Galactose Uptake and Utilization (18), Lactose utilization (9)
7.	**A) One-carbon Metabolism (5),** One-carbon metabolism by tetrahydropterines (5) **B) One-carbon Metabolism (26)**; Serine-glyoxylate cycle (20), by tetrahydropterines (6)
8.	**A) Organic acids (27),** Propionyl-CoA to Succinyl-CoA Module (5), Glycerate metabolism (5), Lactate utilization (10), Malonate decarboxylase (7) **B) Organic acids (6)** include; Propionyl-CoA to Succinyl-CoA Module (6)
9.	**A) Sugar alcohols (0)** **B) Sugar alcohols (26)**; Glycerol and Glycerol-3-phosphate Uptake and Utilization (16), Inositol catabolism (10)
10.	**A) CO_2 fixation (41),** CO_2 uptake, carboxysome (8), Photorespiration (oxidative C2 cycle) (16), Calvin-Benson cycle (17) **B) CO2 fixation (0)**

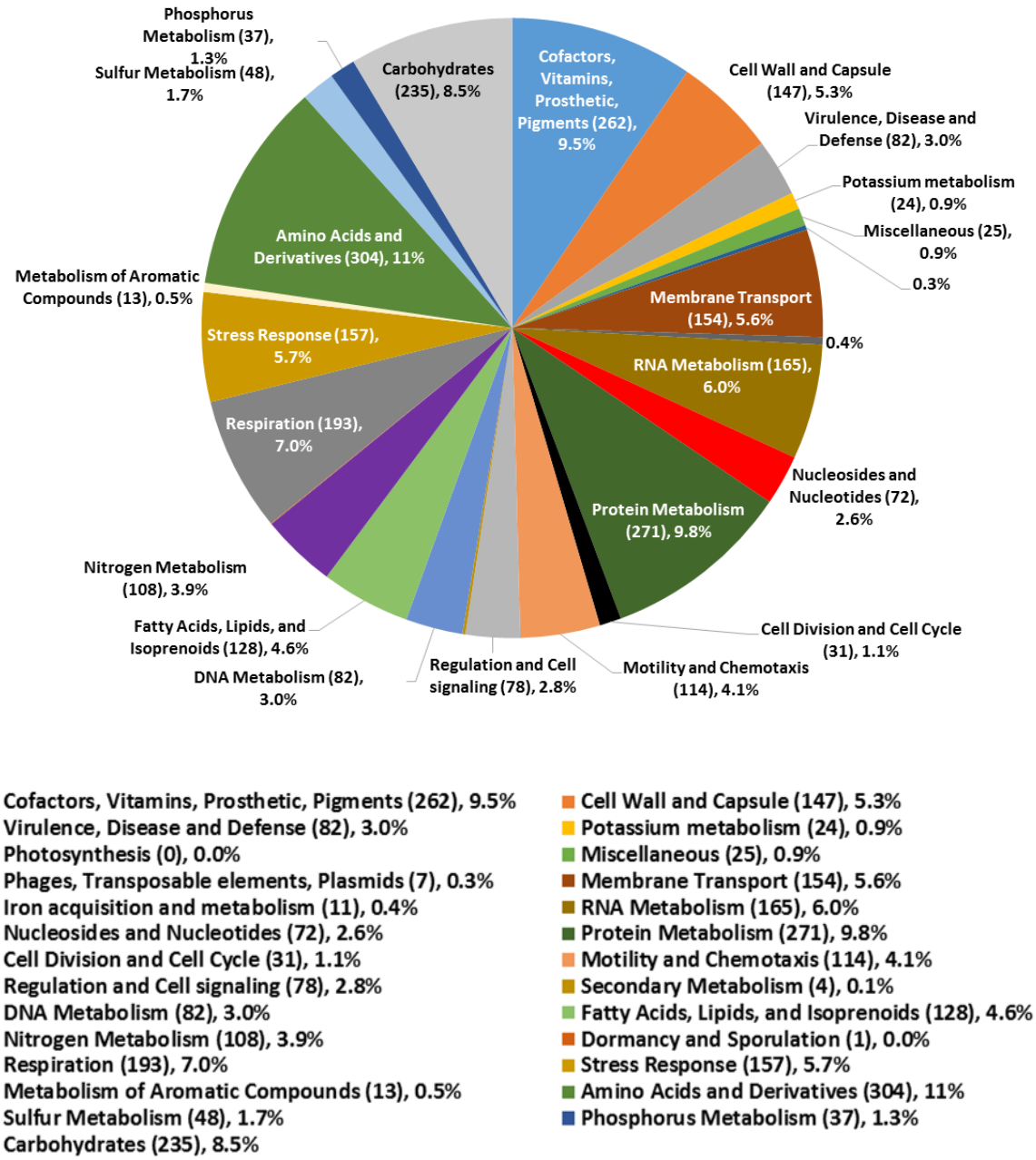


Figure 3. 94: Subsystems groups distribution statistic of *Azonexus* sp. ZS02 (70) WGS

The pie chart presents the abundance of each subsystem group and the count of each subsystem feature that shown between curved brackets at the chart legend, the highest percentages 11% represented a wide range of genes involved in utilization of amino acids and derivatives, protein metabolism 9.8% and carbohydrates 8.5%.

3.7.5.4.4. Key findings

- *Azonexus hydrophilus* ZS02 (BI70) is an alkali tolerant strain that shows a 98% match to the *Azonexus hydrophilus* Type strain d8-1
- The isolated strain shows a 95% match to the uncultured *Azonexus* sp. that has accession number DQ088747 that dominated (94.6%) the $Ca(ISA)_2$ fed microcosm at pH 9.0.
- However, this strain was unable to utilize ISA from CDPs or in the form of $Ca(ISA)_2$.
- This strain is distinct from the other strains isolated in this study since it has the nitrogen fixation genes.

3.7.5.5. Characterization and WGS of *Dietzia* sp. ZS03 (BI51).

Dietzia sp. have been isolated from oil field samples (Borzenkov, Milekhina, Gotoeva, Rozanova, & Belyaev, 2006), deep sea samples and soda lakes (Duckworth, Grant, Grant, Jones, & Meijer, 1998) (Takami, Inoue, Fuji, & Horikoshi, 1997), and from several of petroleum-contaminated sediments (Brito et al., 2006; von der Weid et al., 2007). *Dietzia* sp. are capable of utilizing water-immiscible hydrocarbons (Bødtker, Hvidsten, Barth, & Torsvik, 2009) and aliphatic hydrocarbons as a sole carbon source (Yumoto et al., 2002). In this study, *Dietzia* ZS03 strain (BI51) was isolated on FAA at pH 9.5 from an alkaline microcosm supplemented by CDPs.

3.7.5.5.1. Phylogenetic analysis of the *Dietzia* sp. ZS03-strain

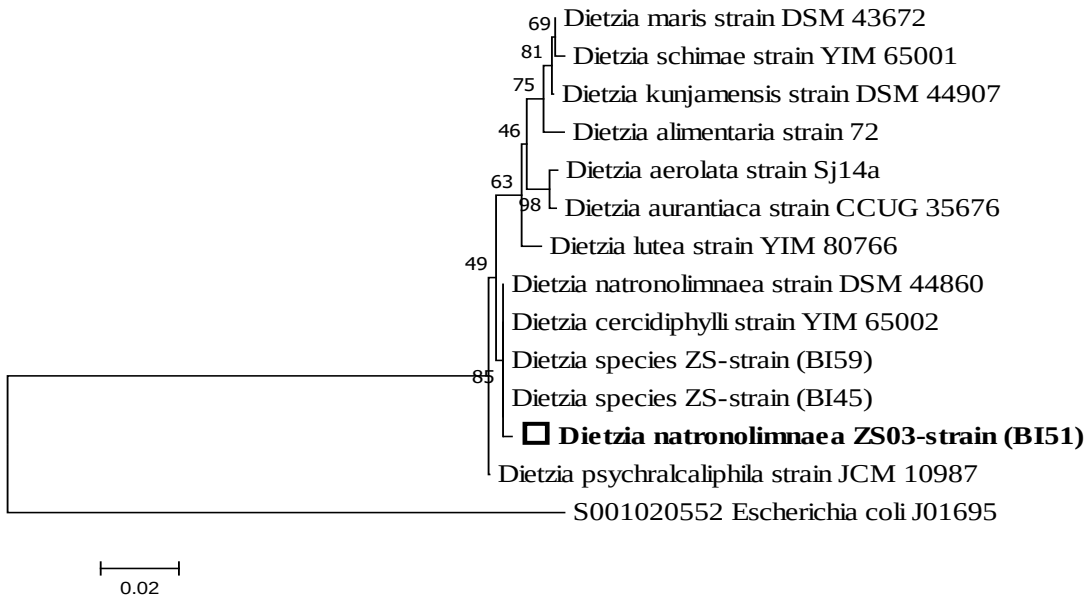
16SrRNA gene sequencing analysis (Figure 3.95 A) indicates that ZS03 strain (BI51) belongs to the genus *Dietzia*. The other two *Dietzia* strains (BI45 and BI59) all cluster with *D. natronolimnaea* and *D. cercidiphylli* these are the closest species match to strain BI51 (Figure 3.95 B, Table 3.25). When compared with the *Deitza* sp. that dominated the bacterial floc forming community reported by Charles *et al.* (C. Charles et al., 2017), *Deitzia* sp. ZS03 (BI51) had a 99% similarity. A phylogenetic tree generated on the basis of the 16S rRNA gene sequence (850 bp, Figure 3.95 D) showed that *Deitza* sp. ZS03 (BI51), was closely related to *Dietzia natronolimnaea* DSM 44860, *D. cercidiphylli* YIM 65002 and the two other isolated strains (BI59 and BI45) (Figure 3.95 B) with 99% sequence identity. Whilst the *Dietzia* sp. obtained from the phylogenetic community analysis of Charles *et al.* (2017), formed new branch on the tree (Figure 3.95 B) that may be due to a short of the DNA sequence (407 bp) that used to build the tree.

Table 3. 20: *Dietzia* sp.and related strains with the closest strains to the *Dietzia* sp. ZS03 (BI51).

List of hits from EzBioCloud 16S database

No	Name	Strain	Accession	Pairwise Similarity (%)	Authors
1	***Dietzia cercidiphylli***	YIM 65002	EU375846	99.6	(Li et al., 2008)
2	***Dietzia psychralcaliphila***	JCM 10987	AB159036	99.4	(Yumoto et al., 2002)
3	***Dietzia natronolimnaea***	CBS 107.95	X92157	99.4	(Duckworth et al., 1998)
4	***Dietzia lutea***	YIM 80766	EU821598	98.3	(Li et al., 2009)
5	***Dietzia aerolata***	Sj14a	FM995533	97.9	(Kämpfer et al., 2010)
6	***Dietzia aurantiaca***	CCUG 35676	FR821260	97.9	(Kämpfer et al., 2012)
7	***Dietzia maris***	DSM 43672	X79290	97.8	(Rainey et al., 1995, Nesterenko et al., 1982)
8	***Dietzia schimae***	YIM 65001	EU375845	97.6	(Li et al., 2008)
9	***Dietzia kunjamensis***	K30-10	AY972480	97.4	(Mayilraj et al., 2006)
10	***Dietzia alimentaria***	72	AGFF01000033	97.3	(Kim et al., 2011)
11	***Dietzia cinnamea***	IMMIB RIV-399	AJ920289	96.9	(Yassin et al., 2006)

A)



B)

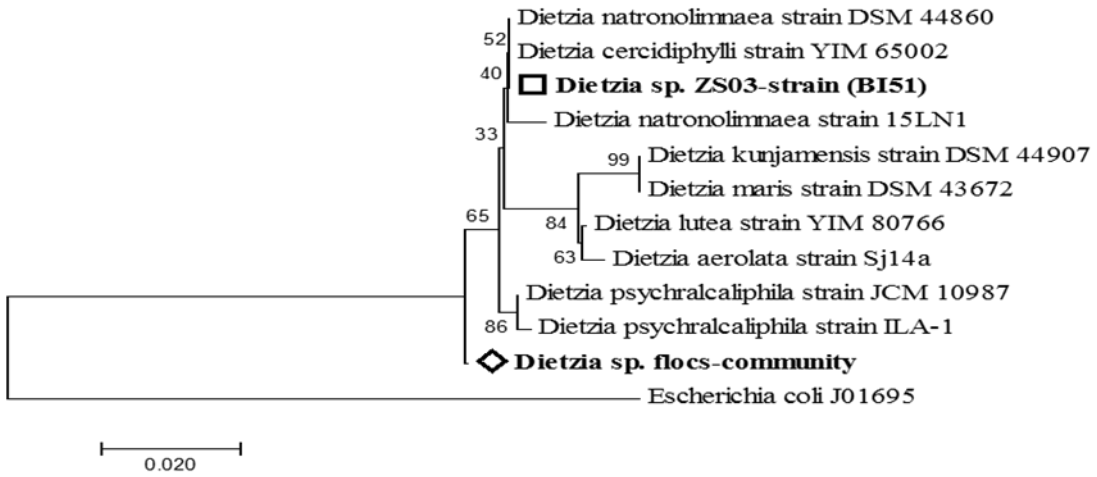


Figure 3. 95: Phylogenetic consensus tree based on 16S rRNA gene sequences.

A phylogenetic tree was generated using neighbour-joining methods in MEGA 7.0 on the basis of the sequencing of 16S rDNA for *Deitza* sp. ZS03 (BI51), showed closely related to the other two strains *Dietza* sp. (BI59 and BI45) and to DSM 44860 and YIM 65002 (A). Whilst, the *Deitzia* strain that dominated the community in the flocs was related to the same taxa that showed 99% similarity to the *Deitza sp* ZS03 (BI51) by BlastN program (B).

3.7.5.5.2. Morphological and metabolic characterisation of *Dietzia* sp. ZS03 (BI51).

Based on colony morphology, three *Dietzia* sp. isolates were obtained (Figure 3.96 A). *Dietzia* sp. ZS03 strain (BI 51) (Figure 3.96 B) had a bright pink pigment, a regular edge, a raised convex profile and was soft in texture. Microscopic examination indicated Gram-positive, non-spore forming cocci that associate into what appear to be short rods (Figure 3.97 C).

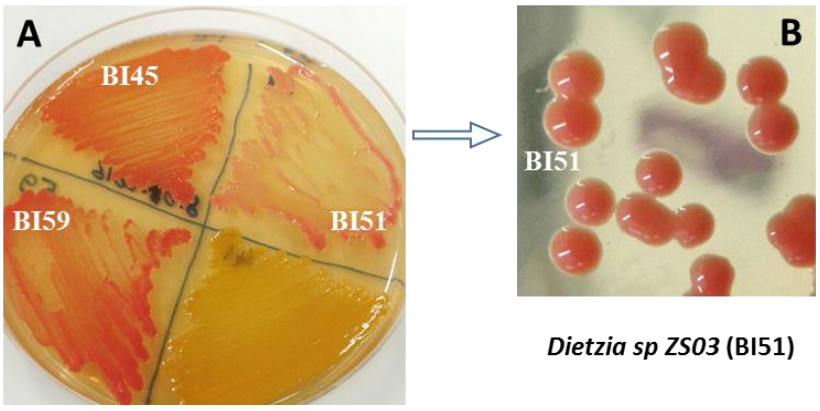


Figure 3. 96: Morphological characteristics of the *Dietzia* sp. ZS03 (BI51) on FAA plate

Three bacterial isolates of *Dietzia* species differ in the morphology of the colonies on the FAA plate at pH 9.5, BI 59 colonies are rough in texture and dark pink in colour, BI 45 colonies are softer and pale pink in colour compared with BI 59 (A), while *Dietzia* sp. ZS03 (BI51) colonies were soft colonies and brighter (B) orange.

Dietzia sp. ZS03 strain (BI 51) is catalase positive, oxidase negative and indole positive. It is able to grow between pH 7.0 and pH 11.0 with an optimum pH of pH 9.0 (Figure 3.98 A). It has a high salt tolerance with a minimum inhibitory concentration of 11% (Figure 3.98 B). In previous studies some *Dietzia* strains had a pH range of 7.0-10 for *Dietzia kunjamensis* K30-10^T and MIC for NaCl 15% (Mayilraj, Suresh, Kroppenstedt, & Saini, 2006); *Dietzia. psychralcaliphila* ILA-1^T had a MIC for NaCl of 10% (Yumoto et al., 2002) as does *Dietzia alimentaria* 72^T (Jandi Kim et al., 2011). *Dietzia aurantiaca* CCUG 35676^T had a pH range of 5.5–12.5 with optimum pH 7.0–8.0 and MIC for NaCl 12% (Kämpfer, Falsen, Frischmann, & Busse, 2012). In the addition *Dietzia* sp. ZS03 was mildly tolerant to heavy metals with MIC (mM) of: Nickel (0.42), Cadmium (0.2), Cobalt (0.47), Lead (2.5), Zinc (1.09) Copper (4.9), the results are compared with other bacterial isolates in Table 3.8.

Fluorescent staining indicated that this strain was able to produce EPS (Figure 3.99). *Dietzia* species were one of the two bacterial genera that were detected in bacterial flocs formed in hyperalkaline microcosms fed on CDP. The authors Charles *et al.* (2107) demonstrated that this floc formation and EPS generation was associated with the protection of the bacteria from hyperalkaline pH (C. Charles et al., 2017)**.**

Metabolic profiling indicated that the strain was unable to metabolise a number of substrates provided by the API20A (anaerobically) (Table 3.5) including D-Glucose. In addition, this strain only utilized 20% of the substrates provided by Biolog system PM1 (Table 3.6) (aerobically) and only 7.3% substrates of PM2 Biolog system (Table 3.7). In a recent, review by Gharibzahedi *et al.* (2014), the authors stated that among 12 *Dietzia* strains only *Dietzia timorensis* ID05-A0528^T (Yamamura et al., 2010) was able to utilize a wide range of carbon sources including sugars, whilst the remaining strains were able to utilize <50% of the carbohydrates available (Gharibzahedi, Razavi, & Mousavi, 2014).

Comparison of the biochemical characteristics of *Dietzia* sp. ZS03 strain (BI51) (this study) and other closely related strains (*Dietzia natronolimnaea* DSM 44860^T and *Dietzia psychralcaliphila* strains) showed that all three showed similar results based on the utilization of acetate, D-glucose and urea. In addition DSM 44860 utilized sucrose, trehalose and citrate (aerobically)(Yassin, Hupfer, & Schaal, 2006), and *D. cercidiphylli* YIM 65002^T strain was able to utilizing a few additional carbon sources including L-arabinose, D-glucose, D-lactose, D-mannose, maltose, arbutin, D-lyxose, potassium 5-ketogluconate and D-tagatose (Li et al., 2008).

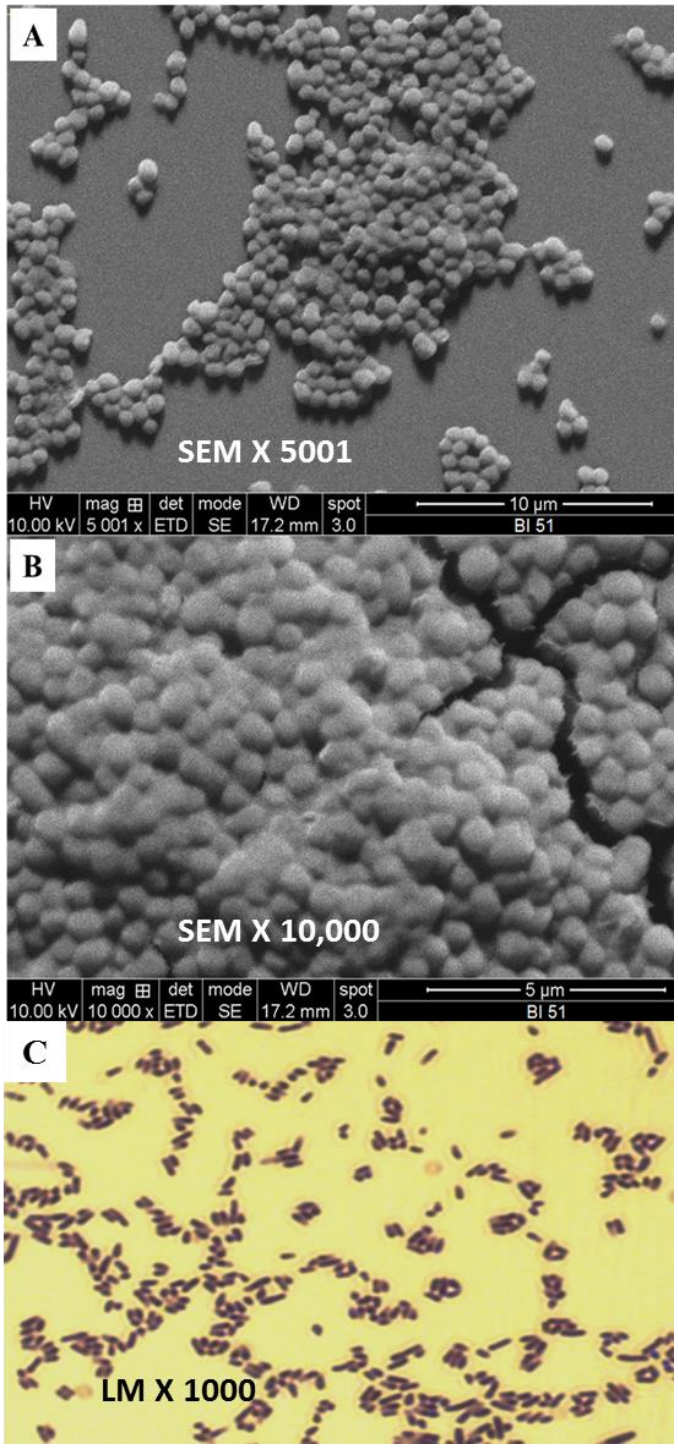


Figure 3. 97: *Dietzia* sp. (BI 51) strain cells by SEM and Gram-stain by light microscope

Cocci attached to each other (A). These cells formed in a cluster as an indicator of EPS production by this strain (B). Gram-positive cocci and short rods arranged in pairs and single cells (C).

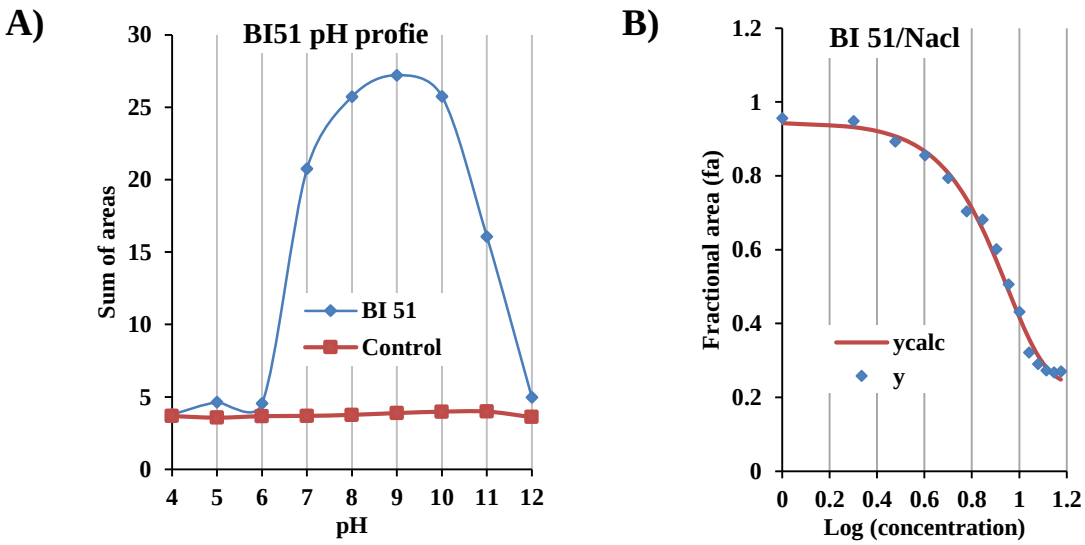


Figure 3. 98: pH profile and MIC of NaCl for *Dietzia* sp. ZS03 strain (BI 51)

Bacteria *Dietzia* sp. ZS03-strain (BI51) has a pH range 7.0-11.0 and optimum pH 9.0 (A), this strain can tolerant NaCL up to 11% (anti-log of 1.04 on the x-axis) the MIC of NaCl was calculated through fractional area at different concentration and plotted on the graph against a log (concentration) using Gompertz function to find the MIC that equal 11% of NaCl. The MIC was detected through calculation of; (■), observed FA; (▬), fitted Gompertz function illustrated on a graph (B).

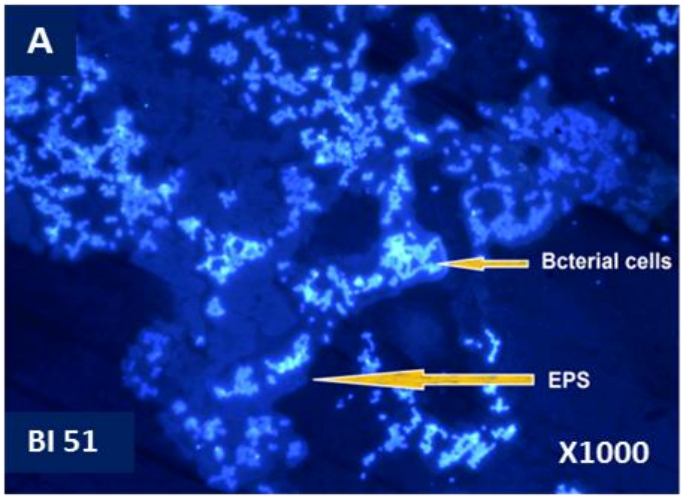


Figure 3. 99: Detection of EPS of *Dietzia* sp. ZS03 (BI51) strain

The EPS (blue haze) and bacterial cell (white).

3.7.5.5.3. Draft Whole Genome Sequence of the *Dietzia* sp. ZS03 (BI51).

Dietzia sp. ZS03 (BI51) has a genome of 4,003,539 bp with a high GC content (70.2%). RAST annotation (Figure 3.100) indicated the presence of numerous of functional genes.

A recent review (2014) described a range of industrial application for this genus such as, therapeutic biotreatments for paratuberculosis animals, food ingredients as animal feed additives,

carotenoid pigments production, biosurfactant and extracellular polymeric substances production, biodegradation of hydrocarbons and industrial fermentation (Gharibzahedi et al., 2014). These functions are reflected in the genome of the isolated strain that contains 13 genes involved in the metabolism of aromatic compounds; for salicylate ester degradation (1), Quinate degradation (1), benzoate degradation (6), salicylate and gentisate catabolism (3) and gentisate degradation (2). In addition, there are a several genes encoded for invasion and intracellular resistance (36) that include; mycobacterium virulence operon involved in protein synthesis (SSU ribosomal proteins) (4).

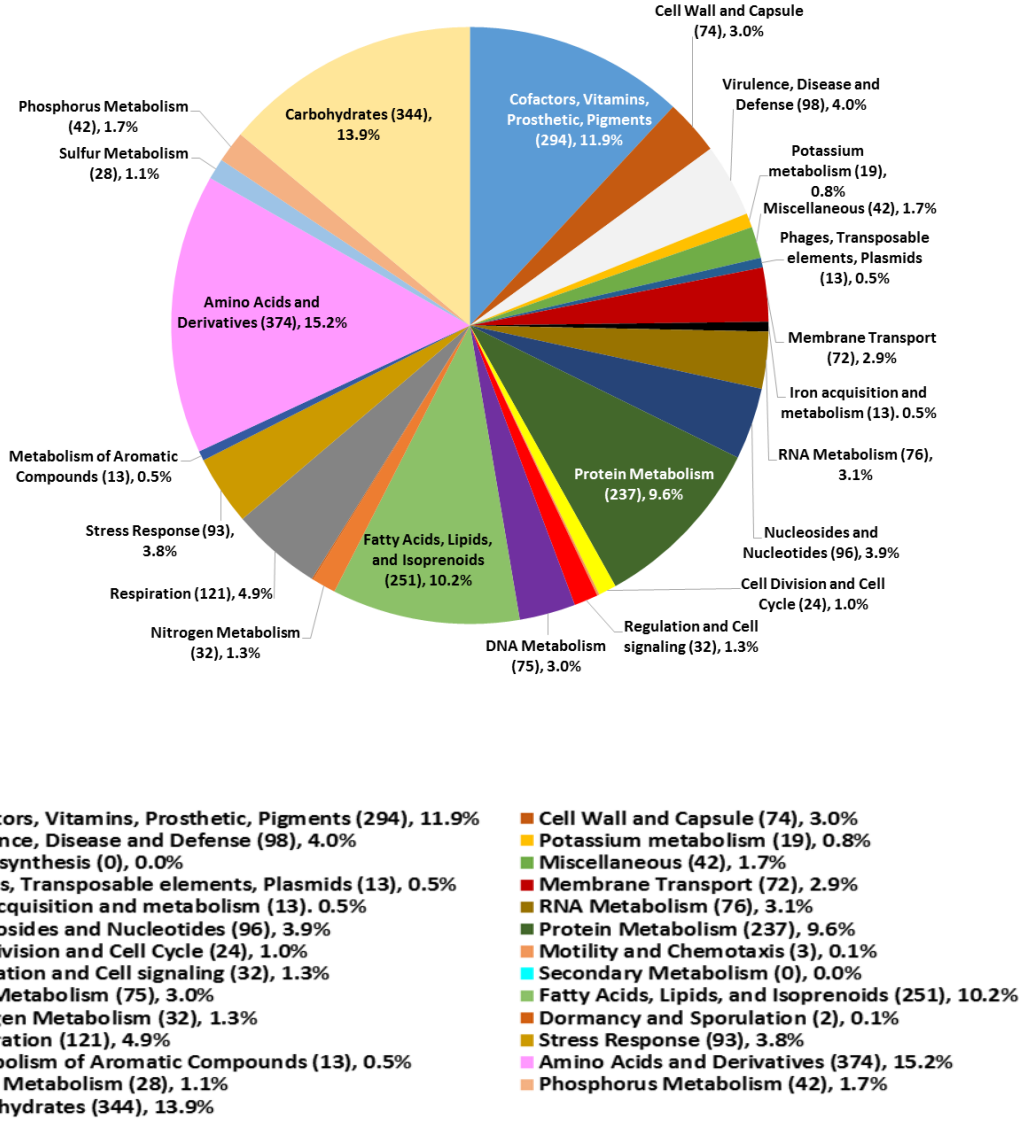


Figure 3. 100: Subsystems group distribution statistics for of *Dietzia* sp. ZS03 (BI51) WGS

The pie chart presents the abundance of each subsystem group and the count of each subsystem feature that shown between curved brackets at the chart legend, the highest percentages 15.2% represented a wide range of genes involved in utilization of amino acids and derivatives, carbohydrate 13.9%, fatty acids, lipids, isoprenoids 10.2% and protein metabolism 9.6%.

3.7.5.5.4. Key findings

- This strain *D.* sp. ZS03 (BI51) and the other two isolated strains of *Detzia* sp. (BI 45 and 59) were unable to utilise ISA either from CDPs or in the form of $Ca(ISA)_2$ as a sole carbon source in pure culture.
- The presence of this strain and closely related strain of *Dietzia* in anaerobic floc forming communities fed on CDP indicate that these *Dietzia* sp are able to utilise CDP and related compounds only in cooperation with other member of the community.
- The WGS of these *Dietzia* strains indicate that they have a broad metabolic capability that may provide useful biotechnological applications.

3.7.5.6. Characterization and WGS of *Brevundimonas diminuta* ZS04 (BI36).

The genus *Brevundimonas* was proposed by Segers *et al.* (1994), who reclassified two species of *Pseudomonas* (*p. diminuta* and *p.vesicularis*) through their genetic characteristics into the new genus *Brevundimonas* (Segers et al., 1994).

3.7.5.6.1. Phylogenetic analysis of the *Brevundimonas* sp. ZS04 (BI 36)

16SrRNA gene sequencing analysis indicates that ZS04 strain (BI36) belongs to the genus *Brevundimonas* showing closest alignment with *Brevundimonas diminuta* (Figure 3.101andTable 3.21).

Table 3. 21: *Brevundimonas* sp. and related strains with the closest strains to the *Brevundimonas* sp. ZS04 (BI 36). List of hits from EzBioCloud 16S database

No	Name	Strain	Accession	Pairwise Similarity (%)	Authors
1	***Brevundimonas diminuta***	ATCC 11568	GL883089	99.9	(Leifson and Hugh 1954) Segers et al. 1994
2	***Brevundimonas vancanneytii***	LMG 2337	AJ227779	99.9	Estrela and Abraham 2010
3	***Brevundimonas naejangsanensis***	DSM 23858	ATXN01000003	99.7	Kang et al. 2009
4	***CP015614_s***	B1	CP015614	99.7	unavailable
5	***Brevundimonas faecalis***	CS20.3	FR775448	99.3	Scotta et al. 2012
6	***Brevundimonas olei***	MJ15	GQ250440	99.0	Lee et al. 2010
7	***Brevundimonas terrae***	KSL-145	DQ335215	98.7	Yoon et al. 2006
8	***Brevundimonas halotolerans***	MCS 24	M83810	98.0	Abraham et al. 2010
9	***Brevundimonas bullata***	IAM 13153	D12785	97.7	(Gray and Thornton 1928) Kang et al. 2009

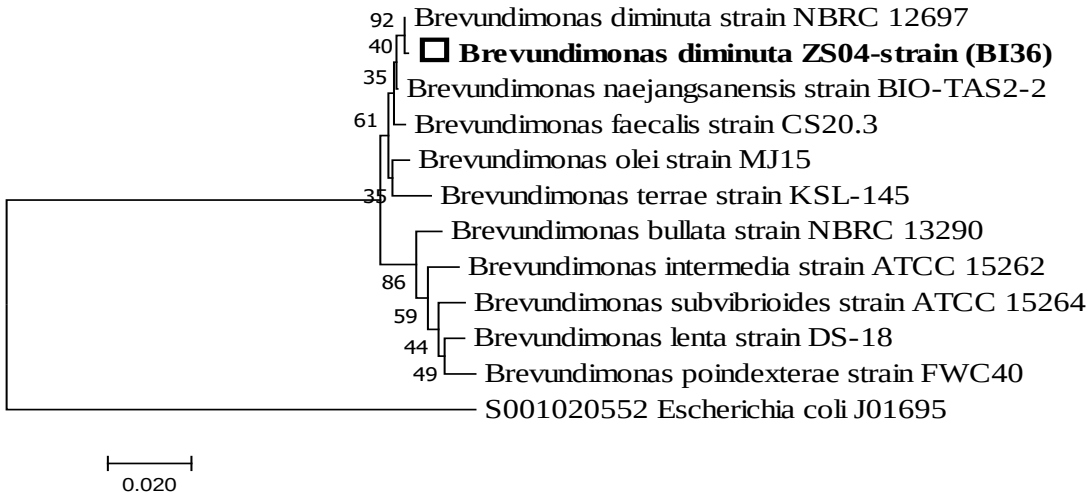


Figure 3. 101: *Brevundimonas* sp. ZS04 (BI 36) Phylogenetic consensus tree

3.7.5.6.2. Morphological and metabolic characterisation of *Brevundimonas* sp. ZS04 (BI 36)

A Gram-negative, rod-shaped, *Brevundimonas* ZS04 (BI 36) (Figure 3.102) was isolated from microcosms inoculated with alkaline soil. The closest related strain was KSL-145^T was isolated from alkaline soil in Korea (J.-H. Yoon, Kang, Lee, & Oh, 2006) which has similar morphological, biochemical and physiological characteristics. After 3 days incubation at 25ºC on FAA, the colonies are circular, smooth, slightly convex and grey in colour. This strain is facultative anaerobic and an EPS producer (Figure 3.67 B).

The optimal temperature for this strain was 30ºC and growth occurs between pH 5.0 and 10.0 with an optimal pH between 7.5 and 9.0 (Figure 3.103 A). The optimum temperature for growth was 30ºC and at this temperature, the strain demonstrated growth between pH 5.0 and pH 10. The lag phase increased at higher pH as the temperature decreased but the optimum pH was still pH 8.0, whilst growth was suppressed at 10ºC and 40ºC (Figure 3.103 A). The 3D diagram shows the optimum growth at the middle of the pyramid (Fa 3.0-3.5) at 30ºC and pH 8.0 (Figure 3.103 B).

In addition, the strain has a variable tolerance to heavy metals, with a wide range of MIC values (mM); Nickel (0.44), Cadmium (0.95), Cobalt (1.93), Lead (30), Zinc (12) and copper (3.63) (Table 3.8). With high tolerance to lead and zinc. Metal (Zn^{+2}, Mn^{+2}, and Pb^{+2}) tolerance has been documented in *Brevundimonas diminuta* strains isolated from water and a sediment of Mariout Lake, Egypt (Abou-Shanab, Angle, & Van Berkum, 2007). The strain did not require NaCl for growth, however it was tolerant up to 5.6% (wt/v) (Figure 3.104).

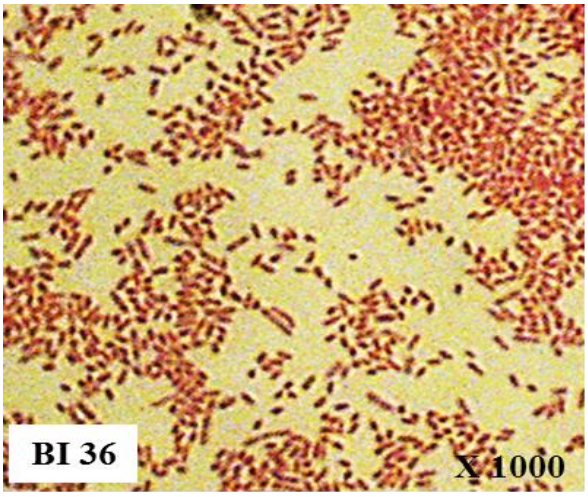
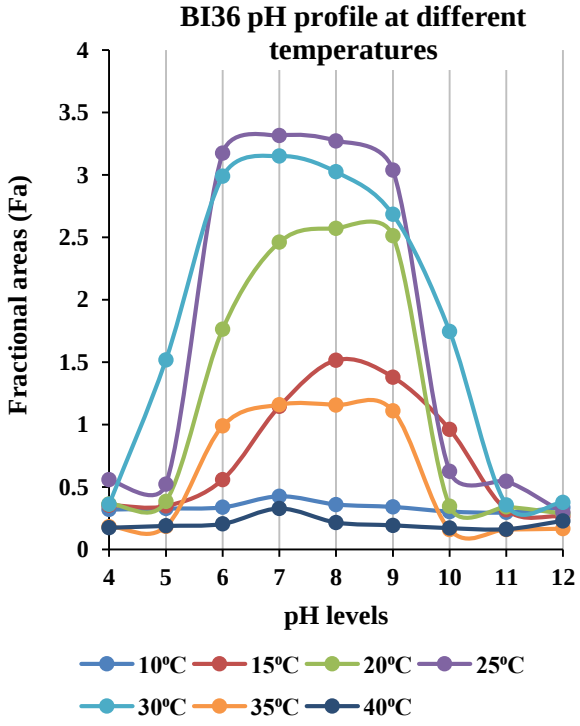


Figure 3. 102: Gram-negative short rods of *Brevundimonas* sp. ZS04 (BI 36) strain

A)



B)

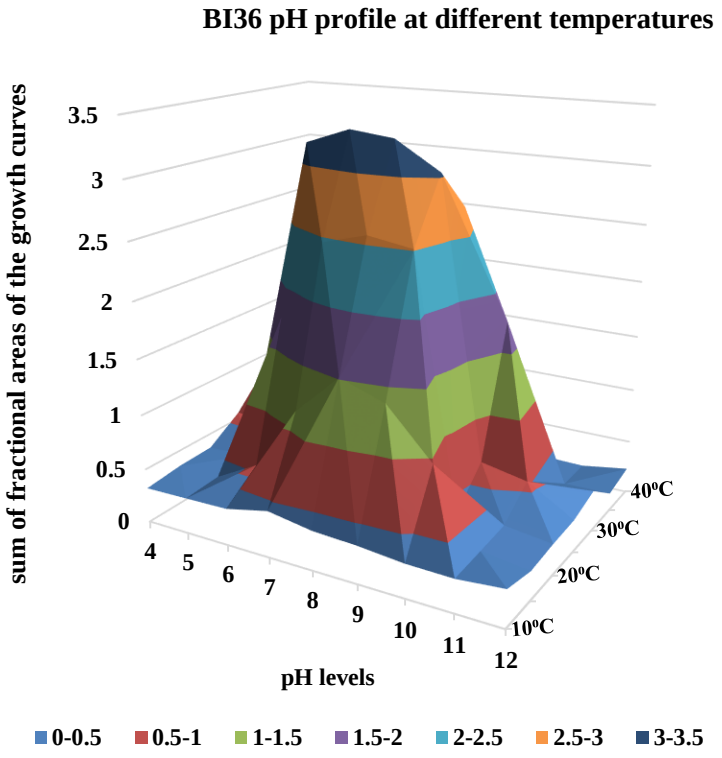
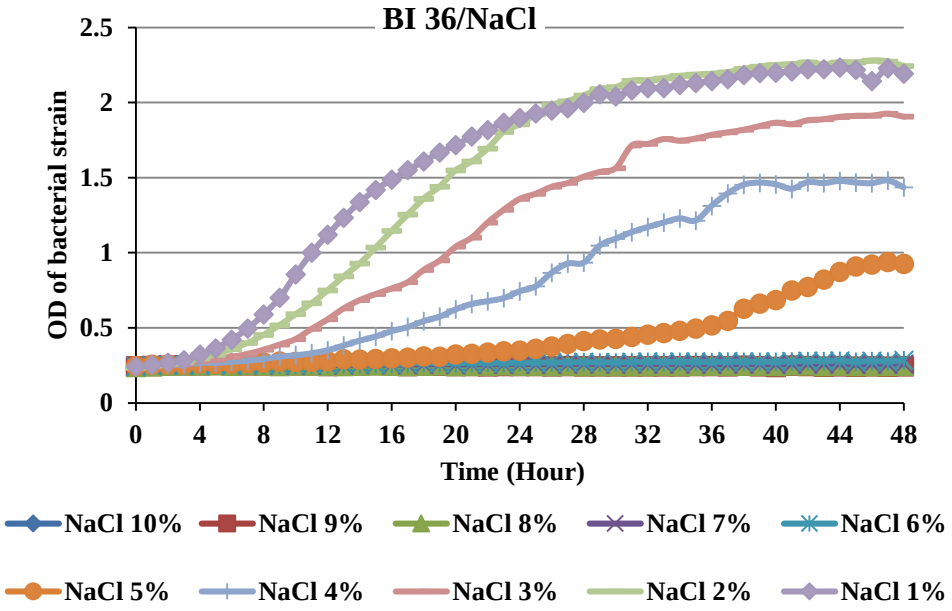


Figure 3. 103: pH profile of *Brevundimonas* sp. ZS04 (BI 36) strain at different temperatures

The effect of the pH levels and temperature degrees on the bacterial growth are illustrated on the graph (A) and on the 3D diagram (B). The optimum pH 8.0 at a temperature 30ºC.

A)



B)

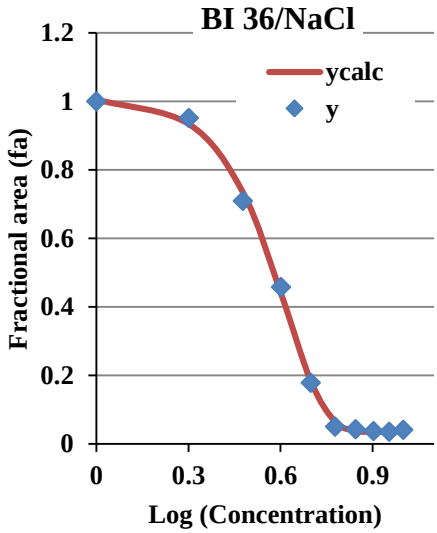


Figure 3. 104: NaCl tolerance for *Brevundimonas* sp. ZS04 (BI 36)

Brevundimonas ZS04 (BI 36) has a similar G to C content (67.4%) to other species of the *Brevundimonas* Genus (J.-H. Yoon et al., 2006). The strain has broad degradation capabilities but is distinct from the other related strains, since it is positive for urease and able to hydrolysis gelatin (J.-H. Yoon et al., 2006).

3.7.5.6.3. Draft Whole Genome Sequence of the *Brevundimonas* sp. ZS04 (BI36)

The *Brevundimonas* sp. ZS04 (BI 36) genome contained 3,421,534 bp encoding for 3,367 putative coding sequences, of which 39 have been classified as pseudogenes, 3,310 as hypothetical proteins, and 3,271 are predicted to form known functional proteins. The genome contains 57 genes for RNA; rRNAs 1, 1, 1 of (5S, 16S, and 23S), 50 tRNAs, and 4 noncoding RNA (ncRNA). The *Brevundimonas* sp. ZS04 whole genome shotgun (WGS) project has the project accession number MTHE00000000.

This version of the project (01) has the accession number MTHE01000000, and consists of sequences MTHE01000001-MTHE01000079. Annotation was added by the NCBI Prokaryotic Genome Annotation Pipeline (released 2013).

RAST annotation (Figure 3.105) indicated the presence of numerous functional genes. Dormancy and stress response (heat shock, cold shock, oxidase stress and detoxification) indicated the adaption of this bacterium to harsh environments. This strain was compared with an available data in RAST for *Brevundimonas* sp. BAL3 that was isolated from the Baltic Sea at a depth of 4 meters (2008). The BAL3 strain whole genome has accession number: PRJNA19287 in NBCI BioProject.

The isolated strain had a greater number of genes compared with *Brevundimonas diminuta* BAL3 strain, particularly in genes that encode for defence and membrane transport system (Table 3.22). These additional membrane transport and cation transport systems may be associated with the survival of *B.* sp. ZS04 strain (BI36) in alkaline environments.

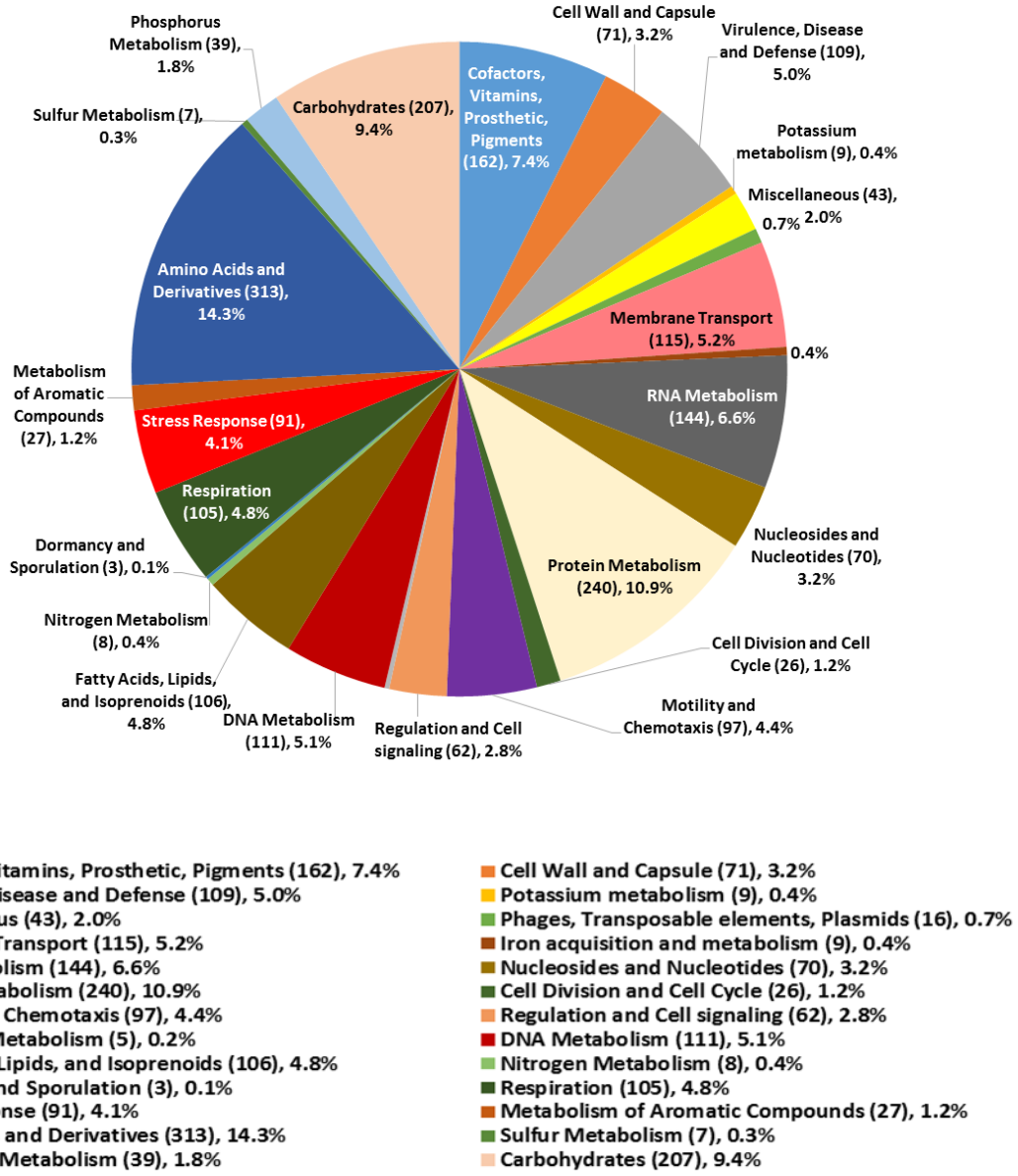


Figure 3. 105: Subsystems group distribution of *Brevundimonas* sp. ZS04 (BI36)

Table 3. 22: Comparison of subsystem features for *Brevundimonas* sp. (BI36) this study and *Brevundimonas* sp. BAL3 strain

Subsystem statistics, comparison between *B. sp.* BI36 and *B.sp. BAL3* strain in the number of genes					
Subsystem Feature Counts	**BI 36**	**BAL3**	**Subsystem Feature Counts**	**BI36**	**BAL3**
Cofactors, Vitamins, Prosthetic, Pigments	162	173	**Regulation and Cell signaling**	**62**	**33**
Cell Wall and Capsule	71	76	Secondary Metabolism	5	5
Virulence, Disease and Defense	**109**	**48**	DNA Metabolism	111	98
Potassium metabolism	9	14	Fatty Acids, Lipids, and Isoprenoids	106	72
Photosynthesis	0	0	**Nitrogen Metabolism**	**8**	**29**
Miscellaneous	43	36	Dormancy and Sporulation	3	1
Phages, Transposable elements, Plasmids	**16**	**1**	Respiration	105	116
Membrane Transport	115	89	Stress Response	91	96
Iron acquisition and metabolism	**9**	**0**	Metabolism of Aromatic Compounds	27	20
RNA Metabolism	144	123	Amino Acids and Derivatives	313	313
Nucleosides and Nucleotides	70	61	**Sulfur Metabolism**	**7**	**22**
Protein Metabolism	240	211	Phosphorus Metabolism	39	31
Cell Division and Cell Cycle	26	24	**Carbohydrates**	207	181
Motility and Chemotaxis	**97**	**58**	Total	2195	1931

3.7.5.6.4. Key findings

- *B. sp.* ZS04 strain (BI36) had a high resistance to Lead and Zinc at MIC 30 mM and 12 mM respectively which aligns with the number of genes that encoded for metals resistance.
- Although this strain was isolated from CDPs fed microcosms, it showed an inability to metabolise ISA from CDPs and in the form of $Ca(ISA)_2$.
- The broad metabolic capabilities of this strain of *Brevundimonas* suggests that it may have industrial and bioremediation applications.

3.7.5.7. Characterisation and WGS of *Rhodococcus erythropolis* ZS (BI49)

The species of *Rhodococcus erythropolis* is of interest in bioremediation studies due to its bioconversion and degradation capabilties. This species possesses enzymes for metabolic processes such as the desulphurisation of hydrocarbons (Borole et al., 2002) and the conversion of petroleum compounds such as benzene to more valuable compounds such as phenol by hydroxylation (Kulikova & Bezborodov, 2000). Some species are also capable of alcohol dehydrogenation (Gröger et al., 2004) epoxidations, dehalogenations and hydrolysis (De Carvalho & Da Fonseca, 2005). Recently, a group of isolates including *Rhodococcus* sp. were isolated from concrete samples to be utilised for their ability to biomineralization and generate calcium carbonate or calcite precipitation (Montaño-Salazar, Lizarazo-Marriaga, & Brandão, 2017).

3.7.5.7.1. Phylogenetic analysis of the *Rhodococcus erythropolis* ZS-strain (BI49)

A phylogenetic analysis showed that *R. erythropolis* ZS-49 was closely related to *Rhodococcus baikonurensis* GTC1041 AB07951, *Nocardia coeliaca* DSM44595 FR733721 and three other strains (Figure 3.106) with 99% sequence identity (Table 3.23).

Table 3. 23: *Rhodococcus* sp. and related strains with the closest strains to the *Rhodococcus erythropolis* ZS-strain (BI49).

No	Name	Strain	Accession	Pairwise Similarity (%)	Authors
1	***Rhodococcus qingshengii***	JCM 15477	LRRJ01000016	100.0	Xu et al. 2007
2	***Rhodococcus degradans***	CCM 4446	JQ776649	100.0	Svec et al. 2015
3	***Rhodococcus erythropolis***	NBRC 15567	BCRM01000055	100.0	(Gray and Thornton 1928) Goodfellow and Alderson 1979
4	***Rhodococcus baikonurensis***	GTC 1041	AB071951	99.7	Li et al. 2004
5	***Rhodococcus globerulus***	NBRC 14531	BCWX01000023	98.7	Goodfellow et al. 1985
6	***Rhodococcus marinonascens***	NBRC 14363	BCXB01000074	98.4	Helmke and Weyland 1984
7	***Rhodococcus jostii***	DSM 44719	FNTL01000001	98.1	Takeuchi et al. 2002
8	***Rhodococcus koreensis***	DSM 44498	FNSV01000005	98.1	Yoon et al. 2000
9	***Rhodococcus nanhaiensis***	SCSIO 10187	JN582175	98.0	Li et al. 2012

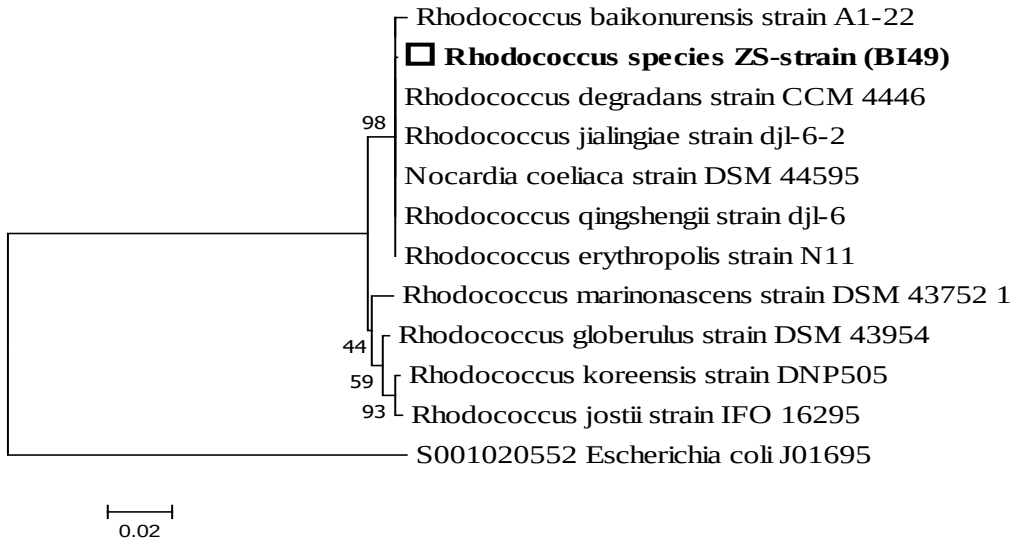


Figure 3. 106: *Rhodococcus erythropolis* ZS-strain (BI49), Phylogenetic consensus tree

The tree showing the position of *R. erythropolis* ZS-strain (BI49) within the genus *Rhodococcus*. Phylogenetic analysis was performed by the neighbour-joining method with 1000 random replicates of the 16SrRNA gene sequencing (Fasta), the tree was configured using the neighbour-joining. This tree showing the position of this strain with 99% matching with other closest strains.

3.7.5.7.2. Morphological and metabolic characterisation of *R. erythropolis* (BI49)

Rhodococcus erythropolis (BI49) was isolated from a CDPs driven microcosm reactors at pH 9.0. *Rhodococcus erythropolis* (BI49) was a facultative anaerobic, Gram-positive bacillus (Figure 3.107 B), of mycelial morphology which fragments into rod-shaped or coccoid elements, an observation also reported by (Chung, Maeda, Song, Horikoshij, & Kudo, 1994; Lechevalier, 1989). In addition, the cells of older colonies contained dark intracellular granules (Figure 3.107 C). When grown on FAA at pH 9.5, the colonies were circular, convex, opaque, glistening, creamish white in colour with a smooth texture (Figure 3.107 A). This strain is also an EPS producer which is visible as a pale staining region on the Gram stain, and a more visible region under fluorescent microscopy (Figure 3.107 D).

The strain is catalase-positive, oxidase-negative, urease-negative and indole-negative and is unable to degrade a range of sugars and other substrates (Table 3.5).

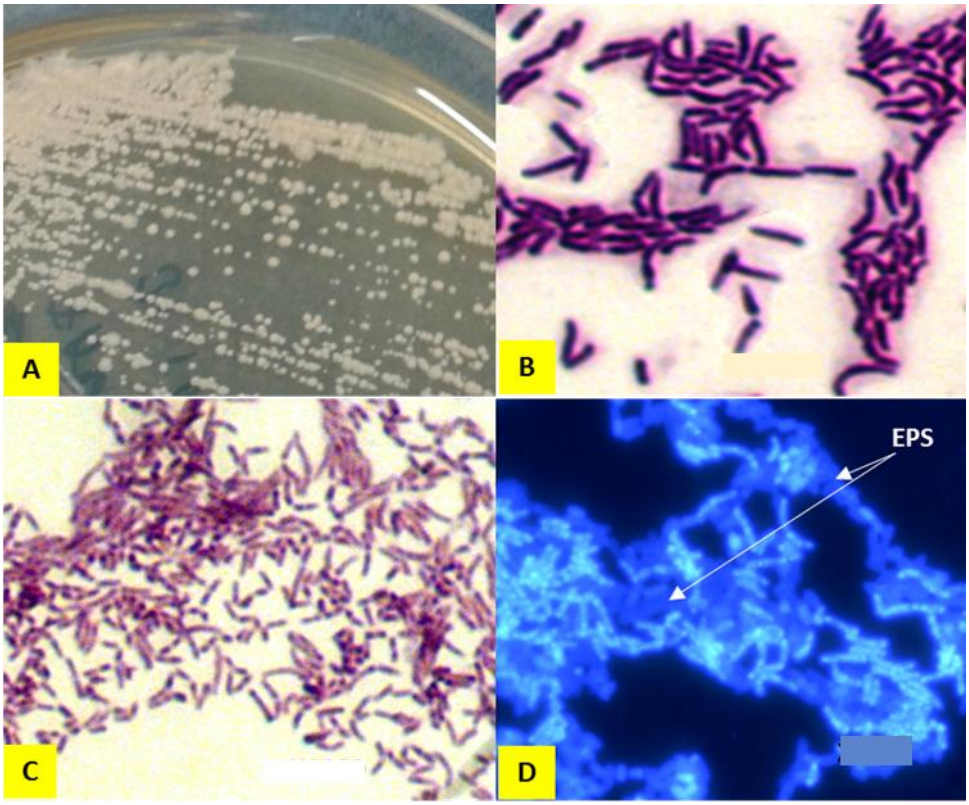


Figure 3. 107: *Rhodococcus erythropolis* ZS-strain (BI49), colonies and cell morphology

Colonies on FAA at pH 9.5 (A), Gram-positive rods surrounded by EPS (B), Gram-stain after 3 days from dried colonies showed a Gram-negative rods contains a dark granules (C) and EPS (blue haze) and bacterial cells (white) (D)

This strain was able to grow between pH 5.0 and pH 10.0 with an optimum between pH 6.0-8.0 at 25ºC (Figure 3.108 B), the MIC of NaCl was 0.79M (4.6%) (Figure 3.108 A). In a previous study, the optimum growth of the closest strain *Rhodococcus qingshengii* was observed at pH 7.5–8.0 (J.-L. Xu et al., 2007) and there was three strains *R. degradans* CCM 4446^{T}, *R. baikonurensis* CCM 8450^{T} and *R. erythropolis* CCM 277^{T} which were able to grown in the presence of up to 6.5 % NaCl (Švec et al., 2015). The *R. sp.* ZS-strain (BI49) was able to tolerant some heavy metals particularly lead, zinc and copper. The MIC results in (mM) were; Nickel (2.0), Cadmium (it was inhibited by 0.1 mM), Cobalt (2.0), Lead (>5.0), Zinc (>5.0) and copper (6.0) (Table 3.8).

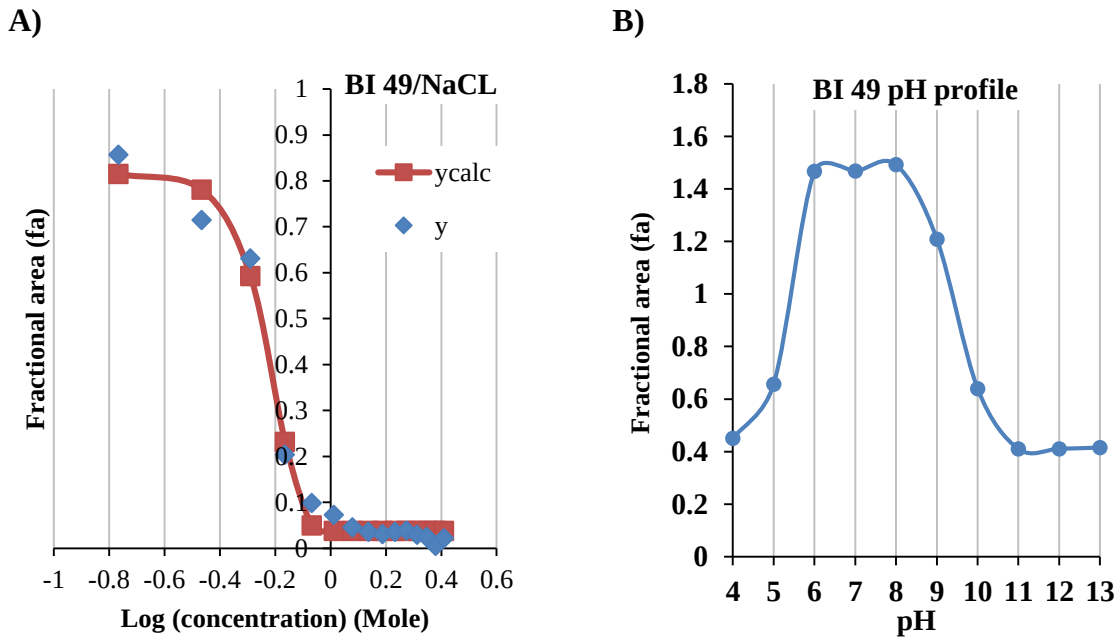


Figure 3. 108: *Rhodococcus erythropolis* ZS-strain (BI49), pH profile and MIC for NaCl

From graph MIC was calculated (anti log of -0.1 concentration) that equal 0.79 M (4.6%) of NaCl (A), the pH range is from pH 5.5-10 with optimum pH is from 6.0-8.0 (B)

The cellular FA profile of *R. sp.* ZS-strain (BI49) was compared to two if its closest relatives. Data for *R. degradans* CCM 4446^{T} was obtained from (Švec et al., 2015) and data for *R. qingshengii* was taken from (J.-L. Xu et al., 2007). The contents of the FA profiles of these two strains were different to that of *R. erythropolis* (B149). Two FAs C16:0 and C18:0 were detected in all three strains (Table 3.24). Whilst, the FA C17:0, C19:1 ω9c, C15:0 were only detected and dominated the FAs profiles of the *R. erythropolis* (BI49).

Table 3. 24: Cellular fatty acids contents (%) of *R. erythropolis* ZS-strain (BI49)

Fatty acid amounting to more than 1.5% of the cellular *R. erythropolis* ZS-strain (BI49) are illustrated. 1- *R. sp.* ZS-strain (BI49), 2- *R. degradans*, 3- *R. qingshengii*. -, not detected.

Fatty acid	1	2	3
C14:0	-	3.8	8.12
C15:0	12	-	-
C16:0	3.5	31.7	25.97
C17:1 ω7c	8	-	-
C17:0	36	-	-
C18:1 ω7c	3	-	-
C18:1 ω9c	-	31.2	7.32
C18:0	4	11.6	7.0
C19:1 ω9c	15	-	-
C19:0	5	-	4.3
C19:1 ω8c	4	-	-

3.7.5.7.3. Draft Whole Genome Sequence of the *R. erythropolis* ZS (BI49)

The genome of *Rhodococcus erythropolis* ZS-strain (BI49) contained 6,741,898 bp encoding for 6,430 putative coding sequences and was analysed via RAST analysis (Figure 3.109). The genome has a 62.4% G to C content and contains 68 RNA genes. In respect to carbohydrate utilization, this strain has a greater number of genes compared with other isolated strains in this study (537). Despite this factor it was not able to degrade ISA. The contents of ZS-strain genome (this study) was compared (Table 3.25) with the complete genome of *Rhodococcus* sp. *RHA1*, which contain 67% of GC (McLeod et al., 2006). The *R. jostii* RHA1 WGS has accession number PRJNA314082 in NBCI BioProject. The presence of groups of genes responsible for the metabolism of aromatic compounds (89) means that this strain may be able to contribute to biotechnological and bioremediation processes. In a recent study (2017) *Rhodococcus qingshengii* YL-1 (which is the closest species to *R.* sp. *ZS*-strain (BI49)) was able to utilize buprofezin (insecticide) as a sole source of carbon and energy for growth (X. Chen et al., 2017).

The isolated strain has a similar total number of genes when compared with *R. jostii* RHA1. However, there are significant differences in some groups of gene. In general *R.* sp. ZS (BI49) has a greater number of genes than RHA1 strain in key areas such as cell wall and capsules, membrane transport system, virulence, disease and defence. *R.* sp. (BI49) has also a number of genes associated with phage, transposable elements and plasmids that were not detected in the RHA1 strain. The results suggest that the *Rhodococcus* sp. ZS-strain (BI49) has more adaptions to survival in harsh environments than the *R. jostii* RHA1strain.

Table 3. 25: Comparason of the subsystem features of *Rhodococcus* sp. ZS (BI49) and *R. jostii* RHA1

Subsystem statistics, comparison between *R. sp.* (BI49) and *R.sp.* RHA1 strain in the number of genes					
Subsystem Feature Counts	**BI49**	**RHA1**	**Subsystem Feature Counts**	**BI49**	**RHA1**
Cofactors, Vitamins, Prosthetic, Pigments	421	394	Regulation and Cell signaling	69	56
Cell Wall and Capsule	**104**	**71**	Secondary Metabolism	8	8
Virulence, Disease and Defense	**113**	**75**	DNA Metabolism	96	81
Potassium metabolism	14	24	Fatty Acids, Lipids, and Isoprenoids	328	249
Photosynthesis	0	0	Nitrogen Metabolism	30	49
Miscellaneous	45	79	Dormancy and Sporulation	4	3
Phages, Transposable elements, Plasmids	**23**	**0**	Respiration	116	230
Membrane Transport	**102**	**42**	Stress Response	136	181
Iron acquisition and metabolism	21	17	Metabolism of Aromatic Compounds	89	167
RNA Metabolism	93	134	Amino Acids and Derivatives	696	706
Nucleosides and Nucleotides	124	137	Sulfur Metabolism	89	85
Protein Metabolism	266	236	Phosphorus Metabolism	42	47
Cell Division and Cell Cycle	29	25	**Carbohydrates**	537	639
Motility and Chemotaxis	**11**	**2**	**Total**	**3606**	**3737**

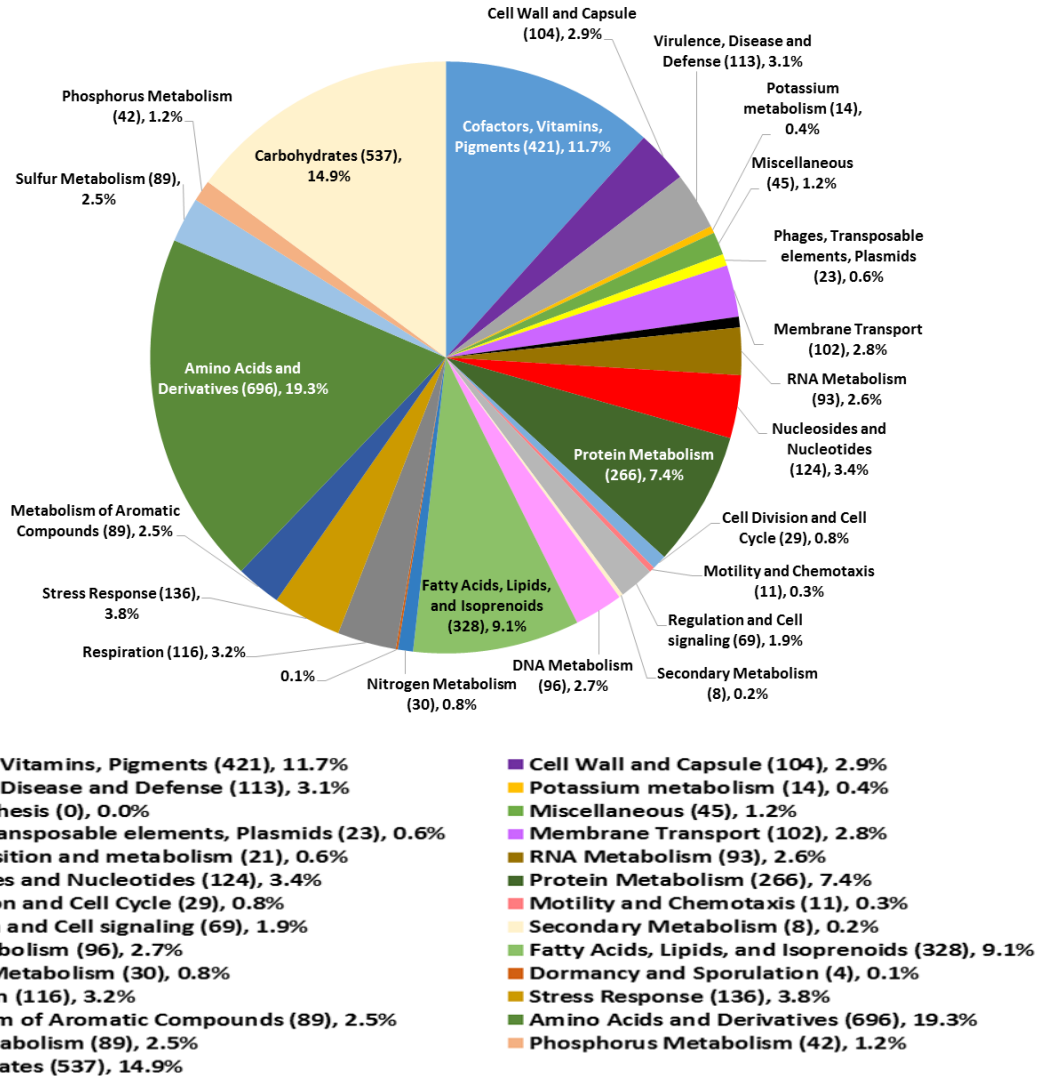


Figure 3. 109: Subsystems group distribution of *R. erythropolis* ZS03 strain (BI49)

3.7.5.7.4. Key findings

- *Rhodococcus erythropolis* ZS (BI49) has a broad range of genes that may contribute to the bioremediation or biotransformation of hydrocarbons (hydrophobic compounds) and in biomineralization processed.
- This strain has plasmids that may help the cells to utilize unusual substances of organic compounds and also provide resistance against toxic materials.
- However, this strain showed an inability to utilize ISA from the CDPs and in the form of $Ca(ISA)_2$.

3.7.5.8. Characterisation and WGS of *Tessarococcus* sp. ZS01 strain (BI 41)

Tessaracoccus sp. ZS01 strain (BI41), is a member of the Propionibacteriaceae family, of Acinetobacteria phylum (Table 3.26). *Tessaraccus* species have been isolated from a range of different environments; *T.* KSS-17Se^T strain was isolated from metalworking fluid in Germany (Kämpfer et al., 2009); *T. flavescens* SST-39^T was isolated from a marine sediment sample in Korea (D. W. Lee & Lee, 2008) and *T. bendigoensis Ben* 106^T was isolated from a sewage-treatment plant, Australia (Maszenan et al., 1999). *Tessarococcus lapidicaptus* is capable of precipitating carbonate and Fe-rich phosphate minerals and was isolated from the subsurface of Rio Tinto basin, Spain (Sánchez-Román, Puente-Sánchez, Parro, & Amils, 2015).

3.7.5.8.1. Phylogenetic analysis of the *Tessaracoccus* sp. ZS01 (BI41)

Tessaracoccus strain BI41 showed a 98% sequence similarities with *T. lubricantis* strain KSS-17Se, 97% with *T. massiliensis* SIT-7 strain and less than 97% with strains of all species of the genus *Tessaracoccus*. A phylogenetic tree resulting from neighbour-joining reconstruction (Saitou & Nei, 1987) is shown in (Figure 3.110, Table 3.31).

Table 3. 26: *Tessaracoccus* sp. and related strains with the closest strains to the *Tessaracoccus* ZS01 (BI41).

No	Name	Strain	Accession	Pairwise Similarity (%)	Authors
1	***T. lubricantis***	KSS-17Se	FM178840	98.4	Kämpfer et al. 2009
2	**FJ675335_s**	LL141-7O10	FJ675335	97.1	Unavailable
3	**CP019606_s**	NSG39	CP019606	96.8	Unavailable
4	**CU918461_s**	QEEA2BA09	CU918461	96.7	Unavailable
5	***T. flavus***	RP1	FNPU01000023	96.2	Kumari et al. 2016
6	***T. defluvii***	LNB-140	KC295452	96.1	Srinivasan et al. 2017
7	***T. flavescens***	SST-39	AM393882	96.1	Lee and Lee 2008
8	**FJ672019_s**	Ll142-2N1	FJ672019	96.0	Unavailable
9	***T. oleiagri***	CGMCC 1.9159	jgi.1058080	95.4	Cai et al. 2011

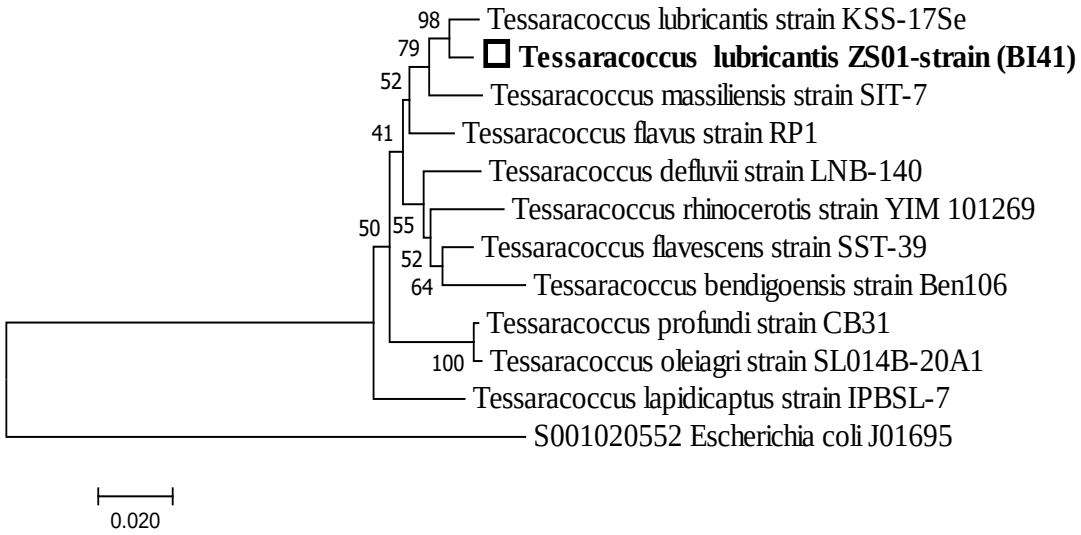


Figure 3. 110: *Tessaracoccus* sp. ZS01 strain (BI41), Phylogenetic consensus tree

The tree shows the position of *T.* ZS01 strain (BI41) in the genus *Tessaracoccus*. Phylogenetic analysis was performed by the neighbour-joining method with 1000 random replicates of the 16SrRNA gene sequencing (Fasta), the tree was configured using the neighbour-joining. This tree shows the position of this strain with 97% matches with the other closest strains.

3.7.5.8.2. Morphological and metabolic characterisation of *Tessarococcus* sp. ZS01 strain (BI 41)

Tessarococcus sp. ZS01 strain BI41 produces yellow pigmented, translucent and shiny colonies with an entire edge on FAA at pH 9.5 (Figure 3.111 A), it is a Gram-positive, non-spore-forming cocci (Figure 3.111 B), EPS producer and biofilm-forming bacteria.

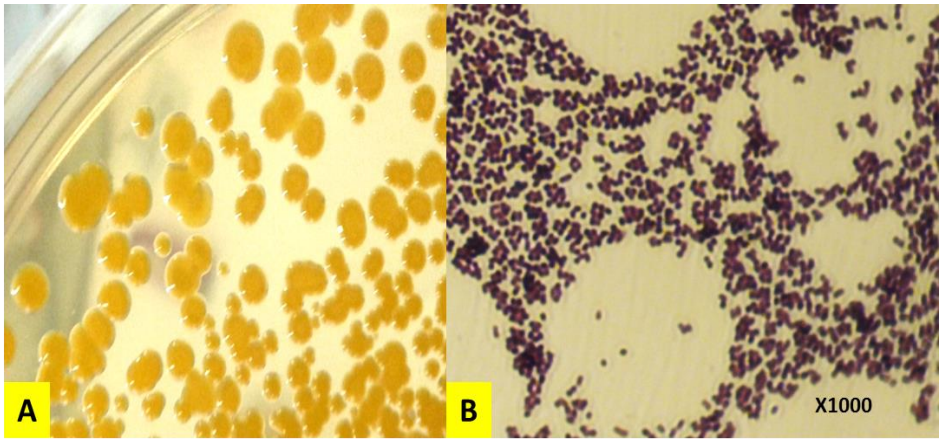


Figure 3. 111: *Tessaracoccus* sp. ZS01 strain BI41 morphoogh

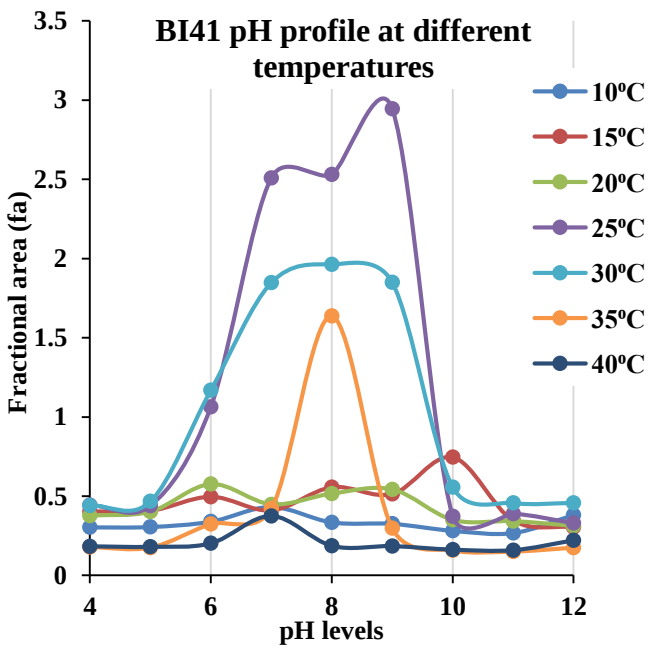
Yellow colonies on FAA (A), Gram-positive cocci that appear as single cells and in pairs (diplococci) (B).

The strain is catalase positive, oxidase negative, urease negative and indole negative. It utilized a wide range of the carbohydrates and utilize 69% and 45% of the substrates provided by the Biolog PM1 and PM2 plates (Table 3.6 to 3.7). In respect to the carbohydrate metabolism (Table 3.28),

the finding are consent with the biochemical characteristics results of *Tessaracoccus lubricantis* KSS-17Se^T (Kämpfer et al., 2009). In addition, *Tessarococcus* sp. ZS01 (BI41) was able to tolerant NaCl up to 0.92 M (5.38%) (Figure 3.113) and heavy metals (Table 3.8). The strain had a pH range of pH 6.0 to 9.5 with an optimum pH of pH 8.0 (Figure 3.112) at 25ºC.

The optimum temperature for growth was 25ºC where this strain demonstrated the ability to grow between pH 6.0 and pH 9.5. The lag phase increased at higher pH as the temperature decreased but the optimum pH was still pH 8.0, while growth was suppressed at a temperature less than 20ºC and above 40ºC, (Figure 3.112 A). A similar pH range and optimum temperature were observed in *Tessaracoccus lubricantis* KSS-17Se^T that had a pH range of pH 6.5-9.5 and an optimum temperature of 25ºC (Kämpfer et al., 2009) and by *T. bendigoensis* Ben 106^T strain that had pH range pH 5.5-9.3 and the same optimum temperature (Maszenan et al., 1999). A higher pH range 6.1-12.1 was observed in *T. flavescens* SST-39^T isolated from marine sediment samples that had an optimum temperature range 20ºC -30ºC (D. W. Lee & Lee, 2008).

A)



B)

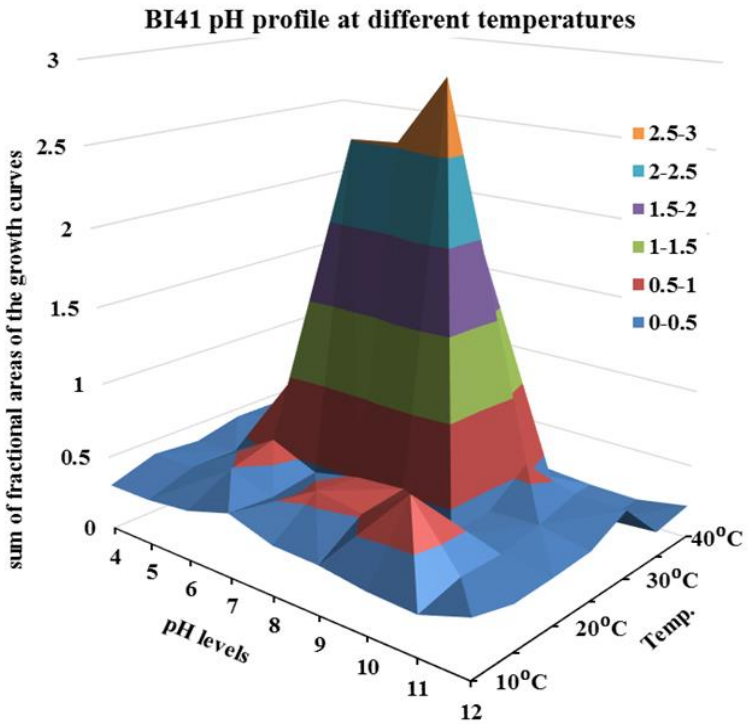


Figure 3. 112: pH profile of *Tessarococcus* sp. ZS01 strain (BI41) at different temperatures

The effect of pH and temperature on bacterial growth are illustrated in graph (A) and on the 3D diagram (B), showing optimum growth at 25ºC and pH 8.0.

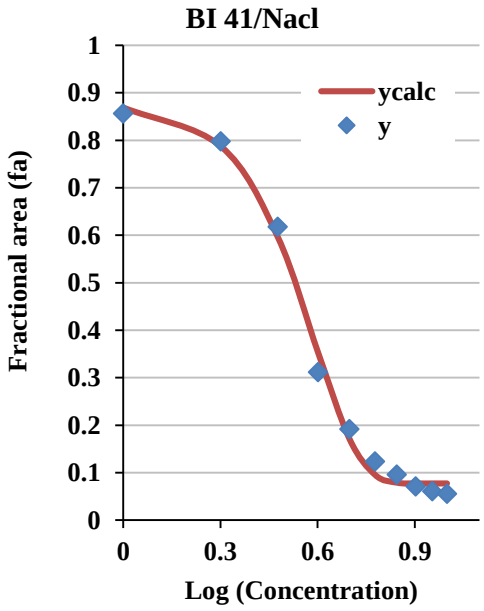


Figure 3. 113: *Tessarococcus* sp. ZS01 (BI41) tolerated NaCl up to 5.0% (MIC 5.38%)

Table 3. 27: Comparative phenotypic characters of *Tessaracoccus* sp. ZS01 strain (BI41) and *Tessaracoccus* type strains

All four strains are Gram-positive and non-spore-forming, oxidase-negative, catalase-positive, urease-negative and indole negative. All four strains utilize D-Maltose, D-xylose, L-arabinose. Data for *Tessaracoccus lubricantis* KSS-17Se^T strain were obtained from (Kämpfer et al., 2009). Data for *T. flavescens* SST-39^T were taken from (D. W. Lee & Lee, 2008) and data for *T. bendigoensis* Ben 106^T were obtained from (Maszenan et al., 1999). ND, No data available.

Phenotypic character	ZS01 (BI41)	KSS-17Se^T	Ben 106^T	SST-39^T
Cell morphology	Cocci	Short rods	Cocci	Short rods
Isolation source	Alkaline soils	Metalworking fluid	Sewage-treatment plant	Marine sediment
Temperature range (optimum °C)	>20-35 (25)	15-36 (25)	20-37 (25)	20-30 (ND)
pH range (optimum)	6.0-9.5 (8.0)	6.5-9.5 (ND)	5.5-9.3 (7.5)	6.1-12.1 (ND)
DNA G+C %	68.4	ND	74	68.4
Oxidase	-	-	-	-
Catalase	+	+	+	+
Indole	-	-	-	-
Urease	-	-	-	-
Protease (gelatine)	-	ND	ND	ND
B-glucosidase	+	ND	ND	-
H_2S	+	ND	ND	ND
D-Glucose	+	+	+	-
D-Mannitol	+	-	+	-
D-Lactose	+	+	+	ND
D-Sucrose	+	-	+	+
D-Maltose	+	+	+	+
Salicin	+	ND	ND	ND
D-xylose	+	+	+	+
L-arabinose	+	+	+	+
Glycerol	+	ND	+	ND
D-cellobiose	-	ND	ND	+
D-mannose	+	+	+	-
D-melezitose	-	ND	ND	ND
D-raffinose	+	ND	ND	-
D-sorbitol	+	ND	ND	ND
L-rhamnose	+	+	-	+
D-trehalose	+	+	-	+

The cellular fatty acid profile of *T.* sp. ZS01 strain (BI41) was compared to two if its closest relatives. Data for *T. lubricantis* KSS-17Se^T strain was obtained from (Kämpfer et al., 2009). There were two sources of FAs results for *T. flavescens* SST-39^T, data for SST-39^T (I) was taken from (Kämpfer et al., 2009), and for the same strain SST-39^T (II) data was obtained from (D. W. Lee & Lee, 2008), the dominant FA in these two strains was C15:0 AISO (83.2.6%. 55.8% and 49.6 respectively). Whilst, the FAs of the *T.* sp. ZS01 strain (BI41) (this study) was dominated by

C16:0 (68%). A similar quantity for C14:0 ISO and C15:0 ISO was found in all four results (Table 3.27).

Table 3. 28: Cellular fatty acids contents (%) of *Tessaracoccus* sp. ZS01 strain (BI41)

Fatty acids found in *Tessaracoccus* ZS01 strain (BI41) (this study) are illustrated below. Tr, Trace (<1.5%); -, not detected. The FA profile of *T.* sp. ZS01 strain (BI41) is composed mainly of C16: 0 (68%). It is clearly different from the FA profiles of the other two strains KSS-17Se^T and two results of *T. flavescens* SST-39^T (I and II).

Fatty acid	ZS01 (BI41)	KSS-17Se^T	SST-39^T (I)	SST-39^T (II)
C14:0 ISO	4	2.0	5.2	2.4
C15:0 ISO	4.5	3.4	8.8	3.2
C15:0 AISO	-	83.2	55.8	49.6
C16:0	68	2.6	3.0	11.5
C18:0	-	-	2.7	17.5

3.7.5.8.3. Draft Whole Genome Sequence of the *Tessarococcus* sp. ZS01 (BI41)

The genome of *Tessaracoccus* sp. ZS01 strain (BI41), contained 3,184,301 bp encoding for 2,870 putative coding sequences, of which 47 have been classified as pseudogenes, 2,773 as hypothetical proteins, and 2,773 predicted to form known functional proteins. The genome has a high GC content of 68.4% and contains 50 genes RNA; rRNAs 1, 1, 1 of (5S, 16S, and 23S), 44 tRNAs, and 4 noncoding RNA (ncRNA). The *Tessaracoccus* sp. ZS01 strain (BI41) whole genome shotgun (WGS) project has the project accession MTHC00000000.

This version of the project (01) has the accession number MTHC01000000, and consists of sequences MTHC01000001-MTHC01000041. Annotation was added by the NCBI Prokaryotic Genome Annotation Pipeline (released 2013). RAST annotation (Figure 3.114) indicated the presence of numerous of functional genes. Dormancy and stress response (heat shock, cold shock, oxidase stress and detoxification) genes indicated the adaption of this bacterium to harsh environments.

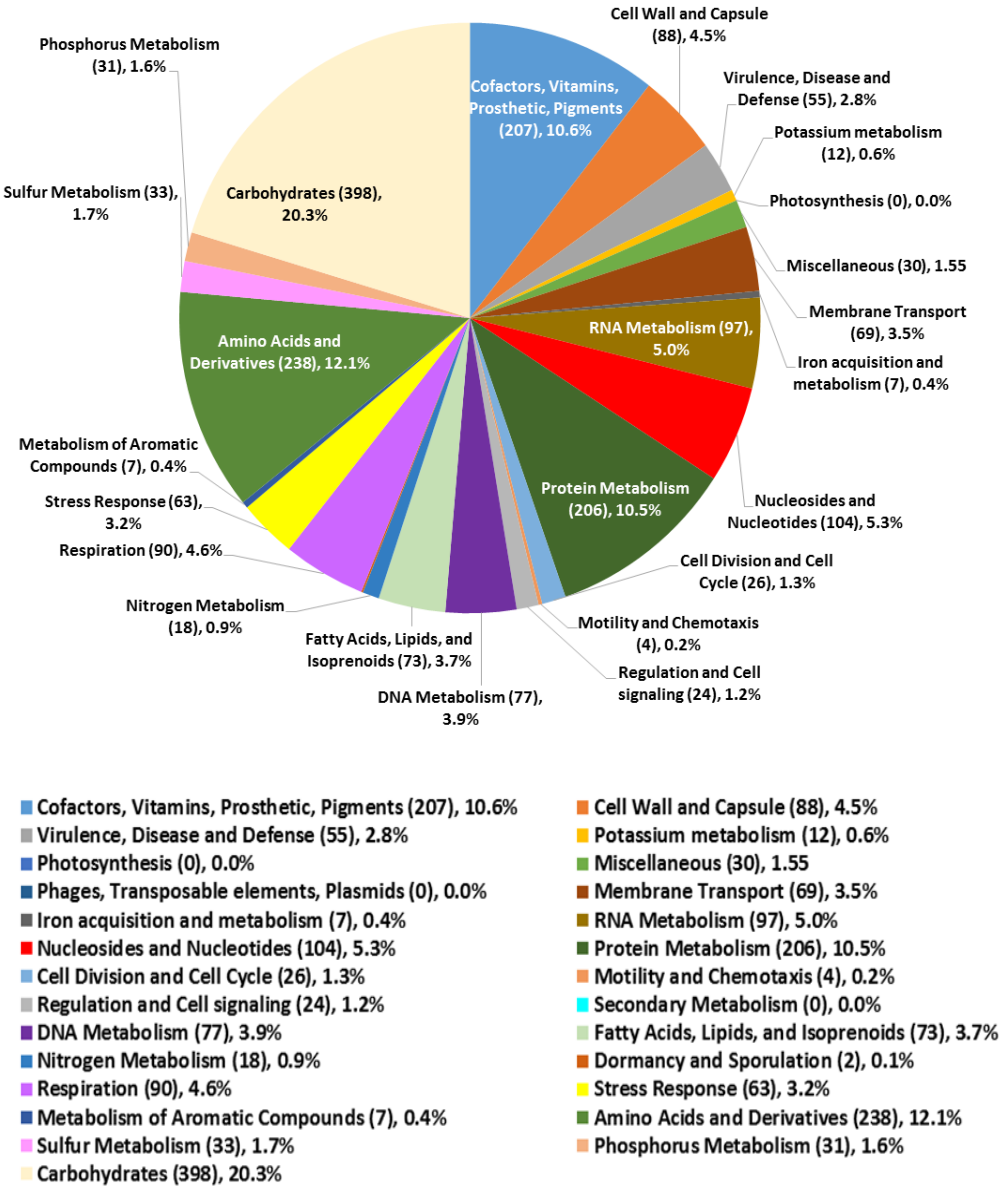


Figure 3. 114: Subsystems group distribution of *Tessaracoccus* sp. ZS01 strain (BI41).

3.7.5.8.4. Key findings

- *Tessarococcus* sp. ZS01 strain (BI41) was able to utilize a wide range of carbohydrates.
- It has a numerous of genes for carbohydrates utilization, with an ability to produce EPS and biofilm formation.
- This strain showed an inability to utilize ISA from CDPs and in the form of $Ca(ISA)_2$.

3.7.5.9. Morphological and WGS of *Macellibacteroides fermentans* HH-ZS strain (BI40).

More than 100 bacterial isolates were obtained from the CDP fed microcosm established over a range of alkaline pH values with the overall aim of identifying pure culture ISA degrading isolates. One such isolate was *Macellibacteroides fermentans* HH-ZS (BI 40) which is a member of the Bacteroidetes phylum. This section of the thesis provides a detailed description of that isolate. The genus of *Macellibacteroides* falls within the family of Porphyromonadaceae within the phylum of Bacteroidetes (Jabari et al., 2012). This genus was recently identified as being distinct from the Genus *Parabacteroides* by Jabari *et al.* (2012) through the identification of the LIND7H strain that had been isolated from the anaerobic treatment of abattoir wastewaters in Tunisia.

3.7.5.9.1. Phylogenetic analysis of *M. fermentans* HH-ZS strain

Macellibacteroides fermentans HH-ZS was identified via BLASTn (Zhang et al., 2000) from its partial 16SrRNA sequence of (888 bp) (Figure 3.115, Table 3.29).

>*Macellibacteroides fermentans* HH-ZS strain (BI40)
GGCTTACACATGCAAGTCGAGGGAGCAGCATAAAAGTAGCAATACTTTGGTGGCGACCGG
CGCACGGGTGAGTAACGCGTATGCAACCTACCTATCAGAGGGGAATAACCCGGCGAAAGT
CGGACTAATACCGCATAAAACAGGGGCACCGCATGGTGATATTTGTTAAAGAAATTCGCT
GATAGATGGGCATGCGTTCCATTAGGTAGTTGGTGAGGTAACGGCTCACCAAGCCGACGA
TGGATAGGGGAACTGAGAGGTTGGTCCCCCACACTGGTACTGAGACACGGACCAGACTCC
TACGGGAGGCAGCAGTGAGGAATATTGGTCAATGGGCGAGAGCCTGAACCAGCCAAGTCG
CGTGAAGGAAGAAGGATCTATGGTTCGTAAACTTCTTTTGCAGGGGAATAAAGTGCAGGA
CGTGTCCTGTTTTGTATGTACCCTGAGAATAAGGATCGGCTAACTCCGTGCCAGCAGCCG
CGGTAATACGGAGGATCCGAGCGTTATCCGGATTTATTGGGTTTAAAGGGTGCGTAGGTG
GTTTGATAAGTCAGCGGTGAAAGTTTGCAGCTTAACTGTAAAAATGCCGTTGAAACTGTC
GGACTTGAGTGTAAATGAGGTAGGCGGAATGCGTGGTGTAGCGGTGAAATGCATAGATAT
CACGCAGAACTCCGATTGCGAAGGCAGCTTACTAAGCTACAACTGACACTGAAGCACGAA
AGCGTGGGGATCAAACAGGATTAGATACCCTGGTAGTCCACGCAGTAAACGATGATTACT
AGCTGTTTGCGATACACAGTAAGCGGCACAGCGAAAGCGTTAAGTAATCCACCTGGGGAG
TACGCCGGCAACGGTGAAACTCAAAGGAATTGACGGGGGCCCGCACAA

Figure 3. 115: 16SrRNA gene sequencing result of *M. fermentans* HH-ZS strain

Table 3. 29: Porphyromonadaceae family and related genus and species with the closest strains to the *M. fermentans* HH-ZS strain (BI40).

Rank	Name	Strain	Authors	Accession	Pairwise Similarity (%)
1	Macellibacteroides fermentans	LIND7H	Jabari et al. 2012	HQ020488	99.89
2	Parabacteroides chartae	NS31-3	Tan et al. 2012	JN029805	99.66
3	HQ767315_s	ELU0061-T404-S-NIPCRAMgANa_000364		HQ767315	95.15
4	Parabacteroides goldsteinii	DSM 19448	(Song et al. 2006) Sakamoto and Benno 2006	AQHV01000028	94.36
5	HQ784009_s	ELU0098-T213-S-NI_000223		HQ784009	94.25
6	HQ761618_s	ELU0048-T276-S-NIPCRAMgANa_000181		HQ761618	94.14
7	Parabacteroides gordonii	MS-1	Sakamoto et al. 2009	KQ033919	94.13
8	Parabacteroides faecis	157	Sakamoto et al. 2015	AB739697	94.00
9	AY571434_s	RsaP101		AY571434	93.69
10	JQ608169_s	NLAE-zl-C468		JQ608169	93.00
11	Parabacteroides merdae	ATCC 43184	(Johnson et al. 1986) Sakamoto and Benno 2006	AAXE02000112	92.45
12	Parabacteroides johnsonii	DSM 18315	Sakamoto et al. 2007	ABYH01000014	92.00
13	EU771909_s	arma_aaj53e03		EU771909	92.00
14	HQ767595_s	ELU0061-T404-S-NIPCRAMgANa_000644		HQ767595	91.87
15	FJ374176_s	PeHg86		FJ374176	91.75
16	JN680579_s	SL34		JN680579	91.62
17	FJ879254_s	R-6301		FJ879254	91.52
18	HM450028_s	WM06x_B4F		HM450028	91.09
19	AMCI01005881_s			AMCI01005881	91.09
20	Parabacteroides chinchillae	DSM 29073	Kitahara et al. 2013	jgi.1108062	91.08
21	HM557221_s	BICP1211		HM557221	90.98
22	AJ576384_s	PeM14		AJ576384	90.95
23	AB255914_s	Nt2-152		AB255914	90.91
24	JN680584_s	SL38		JN680584	90.88
25	HM124306_s	M121		HM124306	90.84
26	FJ374177_s	PeHg27		FJ374177	90.66
27	DQ263706_s	GA62		DQ263706	90.61
28	JN680583_s	SL41		JN680583	90.53
29	Parabacteroides distasonis	ATCC 8503	(Eggerth and Gagnon 1933) Sakamoto and Benno 2006	CP000140	90.52
30	JN680585_s	SL42	Jabari et al. 2012	JN680585	90.52

A neighbour-joining phylogenetic tree shows the relationships between *M. fermentans* HH-ZS strain and related representatives of the family Porphyromonadaceae (Figure 3.116). Although the closest phylogenetic relatives of strain *M. fermentans* HH-ZS is the LIND7H strain, phylogenetic and phenotypic properties indicate that *M. fermentans* HH-ZS is a distinct strain within the genus *Parabacteroides.*

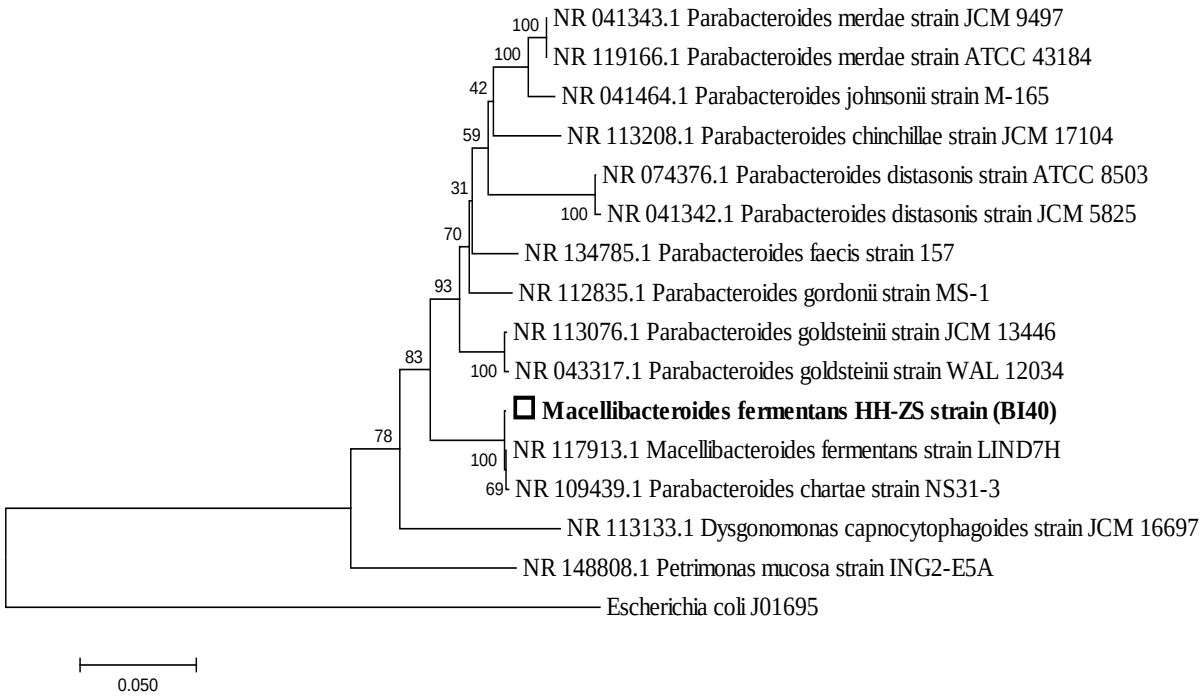


Figure 3. 116: Neighbour-joining phylogenetic tree for *M. fermentans* HH-ZS strain

Maximum-likelihood algorithm phylogenetic tree based on 16S rRNA gene sequences provide the relationships between *M. fermentans* HHZS strain and related representatives of the family Porphyromonadaceae.

3.7.5.9.2. Morphological and metabolic characterisation of *M. fermentans* HH-ZS

When grown on FAA colonies of *M. fermentans* HH-ZS are concave, with a regular edge, soft texture and white colour (Figure 3.117 A) which became viscous when exposed to air (Figure 3.117 B). Direct examination of the cells via light microscopy indicate that *M. fermentans* HH-ZS is a Gram-negative non-spore forming bacillus, with rods being ~1µm in length, and arranged in pairs or chains (Figure 3.118 A). The cells appear to be surrounded by a thin capsule like layer. Cell morphology was confirmed by SEM that showed the aggregation of bacterial cells that is suggestive of biofilm formation (Figure 3.118 B).

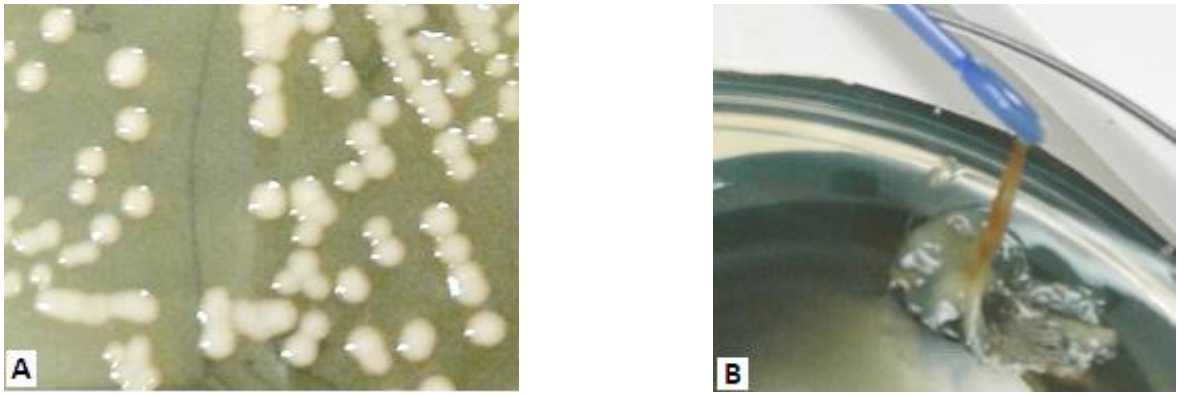


Figure 3. 117: *M. fermentans* HH-ZS strain colonies on FAA plates

Pure colonies after 4days incubation (A), sticky texture of the moist colonies, after 4.0 hours incubation on FAA plate of hard air dried colonies (B).

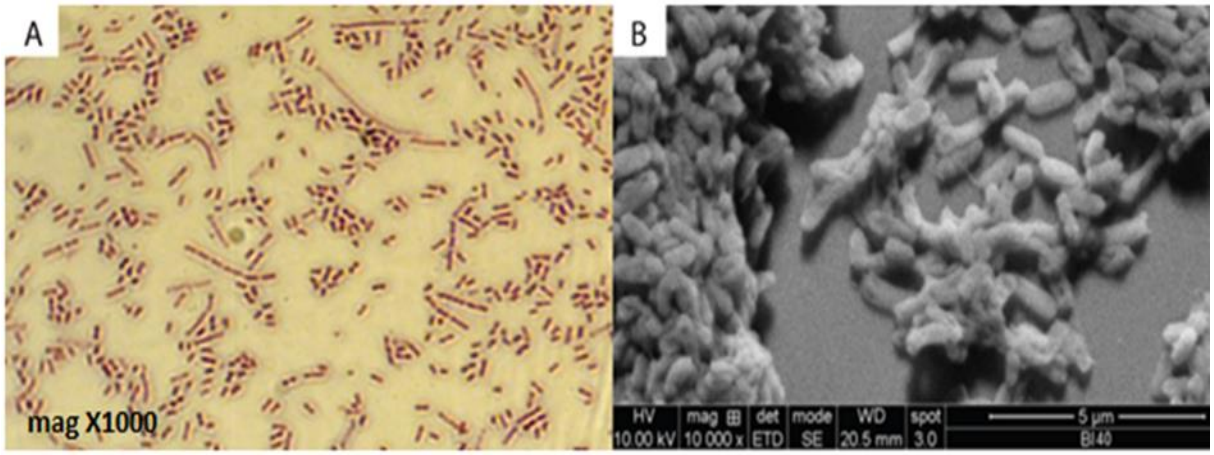


Figure 3. 118: Gram stain and SEM for *M. fermentans* HH-ZS strain

Gram negative bacilli as single cells, arranged in chains and surrounded by a thin layer like a capsule (A), the SEM (x10,000) shows the bacterial cells that attached together to gives an indicator of biofilm formation (B).

Although *M. fermentans* HH-ZS was non-spore forming, it has the ability to survive when exposed to air. When exposed to air on a dry sterile surface at room temperature for 3 days the soft colonies of *M. fermentans* HH-ZS became viscous and developed a fragile crust (Figure 3.119 A). However, these dried colonies were revived once transferred to FAA and incubated under anaerobic conditions (Figure 3.119 C). This ability may be related to biofilm formation and the capability to deal with Reactive Oxygen Species (ROS) as indicated by the presence of catalase.

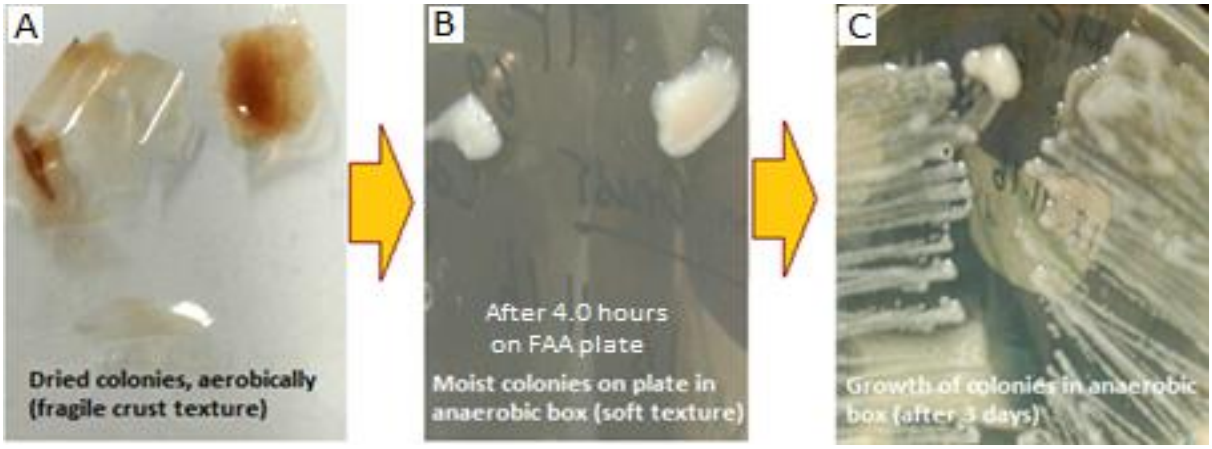


Figure 3. 119: *M. fermentans* HH-ZS strain is an aerotolerant bacterium

The moist colonies were air dried when they acquire a fragile crust like texture after more than three days at room atmosphere (A). The colonies become moist and sticky after 4.0 hours on FAA (B), and at this point are able to grow again in an anaerobic workstation (C).

The cellular fatty acid profile of *M.f.*HH-ZS was compared to two if its closest relatives LIND7H^T and *P.distasonis* CCUG 4941^T (Jabari et al., 2012) (Table 3.30). The major fatty acids of *M.f.*HH-ZS strain were anteiso-C15:0 (31.2%), C17:0 2OH (18.35%), anteiso w9c C17:0 (7.32%). The biggest difference in fatty acid profile was the greater percentage of C17:0 2OH and the presence of a range of other acids (1-5%) not present in the other bacteria.

The menaquinone profile of *M.f.*HH-ZS was compared with the menaquinones of the LIND7H strain (Jabari et al., 2012) and the results provided by Sakamoto & Benno, (2006) for the species of the genus *Parabacteroides;* include the following: *P. merdae*, *P. goldsteinii*, *P. gordonii*, *P.johnsonii*, *P. distasonis* JCM 5825T (Sakamoto & Benno, 2006; Sakamoto, Kitahara, & Benno, 2007; Sakamoto et al., 2009), (Table 3.31). When compared to LIND7H the major differences were the presence of MK8 and the absence of MK9(H2), in terms of the Parabacteroides the greater abundance of MK8 and lower levels of MK10 were the most obvious differences. There were slight differences in the polar lipids contents (Figure 3.120) of *M.f.*HH-ZS and LIND7H^T (Jabari et al., 2012). The images of the TLC plate indicate that lipid (L) and Aminolipid (AL) were only detected and clearly identified in *M. fermentans* HH-ZS whilst the glycolipid (GL), and Phosphatidylglycerol (PG) were only detected in LIND7H^T strain. Both of the two strains have unidentified phospholipids (PL).

Table 3. 30: Cellular fatty acids contents (%) of *M. fermentans* HH-ZS strain

Fatty acid amounting to more than 1% of the cellular *M.f.* HH-ZS strain (this study) are illustrated. Tr, Trace (<1%); -, not detected. Results from the others two strains (LIND7H and CCUG 4941) were obtained from (Jabari et al., 2012), all results were obtained from the identification service of the DSMZ (Braunschweing. Germany).

Fatty acid	*M.f.HH-ZS* strain	LIND7H	CCUG 4941
anteiso C13:0	1.56	2.7	Tr
iso C15:0	5.11	5.4	8.5
anteiso C15:0	31.20	30.1	43.5
w8c C15:1	1.11	-	-
w6c C15:1	1.44	-	-
Sum in feature3*	2.73	-	-
C16:0	4.31	1.8	4.2
Sum in feature9**	6.92	-	-
anteiso w9c C17:0	7.32	-	-
anteiso C17:0	1.08	-	-
w8c C17:1	1.98	-	-
C17:0	1.15	-	-
iso C16:0 3OH	1.02	-	-
C16:0 3OH	1.43	1.1	5.6
iso C17:0 3OH	6.97	6.3	13.6
C17:0 2OH	18.35	8.0	1.3
C17:0 3OH	1.19	-	-

*the summed in feature3 comprises C16:1 w7c and/or C16:1 w6c
**the summed in feature9 comprises C17:1 iso w9c or C16:0 10-methyl

Table 3. 31: Menaquinone content of *M. fermentans* HH-ZS strain

The Menaquinone contents of *M.f.* HH-ZS strain compared to the two closest strains; LIND7H strain and *Parabacteroides* species (Jabari et al., 2012).

Menaquinone%	*M.f.*HH-ZS strain	LIND7H	*Parabacteroides sp.*
MK-8	12	0	1-5 (4)
MK-9	49	52	10-54 (24)
MK-9(H_2)	0	33	0
MK-10	16	15	37-72 (67)
MK-11	0	0	Tr*-13 (4)

*Tr, Trace amount

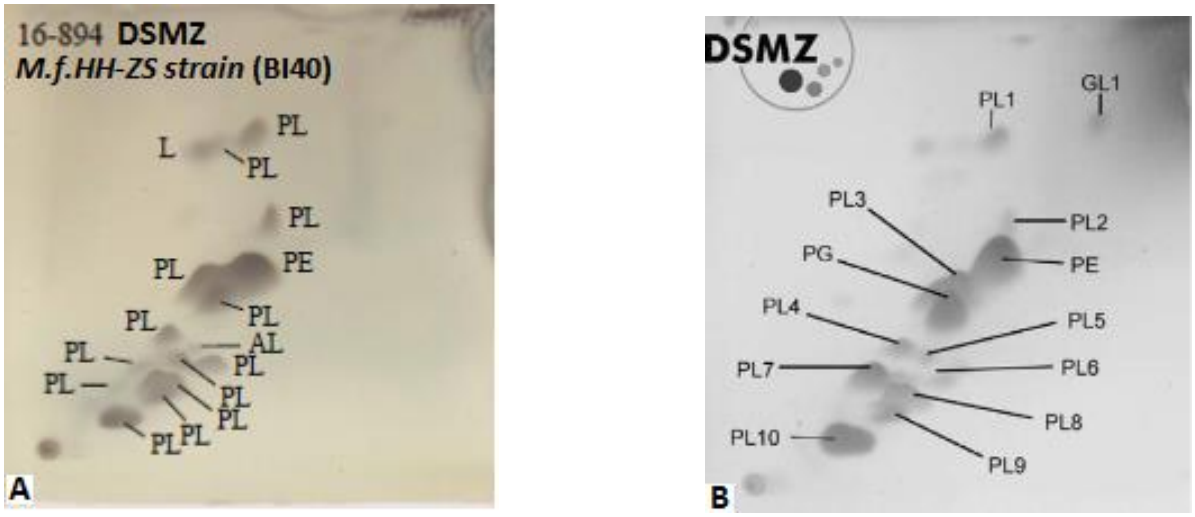


Figure 3. 120: Total polar lipids spots of *M. fermentans* HH-ZS strain by thin Layer Chromatography image

TLC of *M.f.* HH-ZS strain (this study) polar lipids represented by; L, Lipid; AL, Aminolipid; PL, Phospholipid (unidentified phospholipids); PE, Phosphatidylethanolamine (A), compared with *LIND7HT* Strain; Polar Lipid 1–10, unidentified phospholipids; PE, phosphatidylethanolamine; GL1, unidentified glycolipid; PG, Phosphatidylglycerol (B) (Jabari et al., 2012).

Biomarkers such as polar lipids, fatty acids and menaquinones have been routinely used in the characterization of microbial community structure and the differentiation of microbial isolates (Rossel et al., 2008). For example work by Rutters (2002), found that both polar lipids and fatty acid composition allowed the differentiation of three marine sulfate-reducing bacteria (Rütters, Sass, Cypionka, & Rullkötter, 2002). Differences in polar lipid, fatty acid and menaquinone compositions provide further evidence that *M.f.* HH-ZS is a distinct strain when compared with its closest relative LIND7H^{T}.

The biochemical characterisation of *M.f.* HH-ZS focussed on the strains enzymic and catabolic capabilities. Biolog and API analysis confirmed that *M.f.* HH-ZS has broad catabolic capabilities primarily focussed on carbohydrate metabolism (Table 3.32 and 3.33). The API analysis indicated that the enzymic activity *M.f.* HH-ZS was broadly similar to LIND7H^{T} (Jabari et al., 2012) with the exception of *M. fermentans* HH-ZS being catalase and salicin positive and negative for protease (gelatin). The biochemical profile of *M.f.* HH-ZS particularly the presence of catalase provides further evidence that *M. fermentans* HH-ZS is a novel strain of *M. fermentans.*

Table 3. 32: Biolog 96 wells Microplate for *M. fermentans* HH-ZS strain

This strain was utilized more than 50% of the substrates that are listed under substrate (+), while none metabolised substrates are listed under the substrate (-).

***M.f.HH-ZS* strain biochemical tests**			
Substrate positive (+)		**Substrate negative (-)**	
N-Acetyl-DGlucosamine	α-D-Glucose	Adonitol	D-Galacturonic acid,
α-D-Glucose-1-Phosphate	α-Cyclodextrin	i-Erythritol	D-Glucosaminic acid
D-Fructose	D-Galactose	Glycerol	α-Hydroxybutyric acid
N-Acetyl-β-DMannosamine	D-Cellobiose	Acetic acid	2'-Deoxy adenosine
α-Methyl-DGalactoside	Maltose	Fumaric acid	N-Acetyl-DGalactosamine
D-Melezitose,	Lactulose	L-Lactic acid	D,L-α-Glycerol Phosphate
β-Methyl-DGalactoside	Palatinose	D-Saccharic acid	D-Lactic acid methyl ester
α-Methyl-DGlucoside	D-Raffinose	L-Asparagine	β- Hydroxybutyric acid
Stachyose	Sucrose	L-Glutamic acid	Thymidine-5'-Mono-phosphate
α-Ketobutyric acid	α-Ketovaleric acid	L-Glutamine	L-Valine plus L-Aspartic acid
Pyruvic acid methyl ester	Succinic acid	Formic acid	L-Fucose
L-Alanyl-LThreonine	Thymidine,	Dulcitol	D-Mannitol
Glycyl-LAspartic acid	Glycyl-LGlutamine	m-Inositol	Glyoxylic acid
L-Alanyl-LGlutamine	Uridine	L-Serine	Itaconic acid
Amygdalin	Arbutin	Inosine	L-Malic acid
β-Cyclodextrin	Dextrin	Propionic acid	Urocanic acid
Gentiobiose	D-Gluconic Acid	m-Tartaric acid	Succinamic acid
D-Glucose-6-Phosphate	α-D-Lactose,	L-Valine	L-Phenylalanine
Maltotriose	D-Mannose	D-Arabitol	L-Methionine
3-Methyl-DGlucose	D-Melibiose		
β-Methyl-DGlucoside	Salicin		
L-Rhamnose	D-Sorbitol		
D-Trehalose	Turanose		
D,L-Lactic acid	Pyruvic acid		
Succinic acid mono-methyl ester	Alaninamide		
L-Alanyl-LHistidine	L-Alanine		
Glycyl-LMethionine	Glycyl-LProline		
Uridine-5'- Monophosphate	L-Threonine		

Table 3. 33: Metabolic activity of the *M. fermentans* HH-ZS (this study) and LIND7H^T strain studied by Jabari *et al.* (2012).

The enzyme profile of *M. fermentans* HH-ZS showed similarities with LIND7H^T (Jabari et al., 2012), with the exception of catalase, salicin and gelatine degradation.

Activity	*M.f.HH-ZS* strain	LIND7H^T
Oxidase	-	ND
Catalase	+	-
Indol	-	ND
Urease	-	-
Protease (gelatine)	-	+
B-glucosidase (Esculin)	+	+
H_2S	+	+
Acidification of Carbohydrates		
D-Glucose	+	+
D-Mannitol	+	+
D-Lactose	+	+
D-Sucrose	+	+
D-Maltose	+	+
Salicin	+	-
D-xylose	+	+
L-arabinose	+	+
Glycerol	+	+
D-cellobiose	+	+
D-mannose	+	+
D-melezitose	+	+
D-raffinose	+	+
D-sorbitol	+	+
L-rhamnose	+	+
D-trehalose	+	+

M. fermentans HH-ZS has the ability to survive and grow between pH 5.0 and pH 11.0 (Figure 3.121 A), with a relatively flat response to pH between pH 5.0 and pH 8.0 (Figure 3.121 C). In term of an adaptation strategy, this bacterium exhibits a longer lag time at extreme acidic and alkaline conditions (Figure 3.121 A). Acid generation was evident between pH 7.0 and 11.0 (Figure 3.121 B) which clearly assists the bacteria to modify its environment towards a more amenable pH value (Figure 3.121 B). Whilst, the lower pH for growth is similar to other strains such as LIND7H strain which had an optimum pH (6.5-7.5) and a range of growth between pH (5.0-8.5) (Jabari et al., 2012), and *Parabacteroides chartae* sp. nov. NS31-3^T, where the optimium pH was (7.0–7.5) and the range for growth pH (5.5-8.5) (Tan et al., 2012). The upper pH limit for *M. fermentans* HH-ZS was considerably greater (pH 11.0).

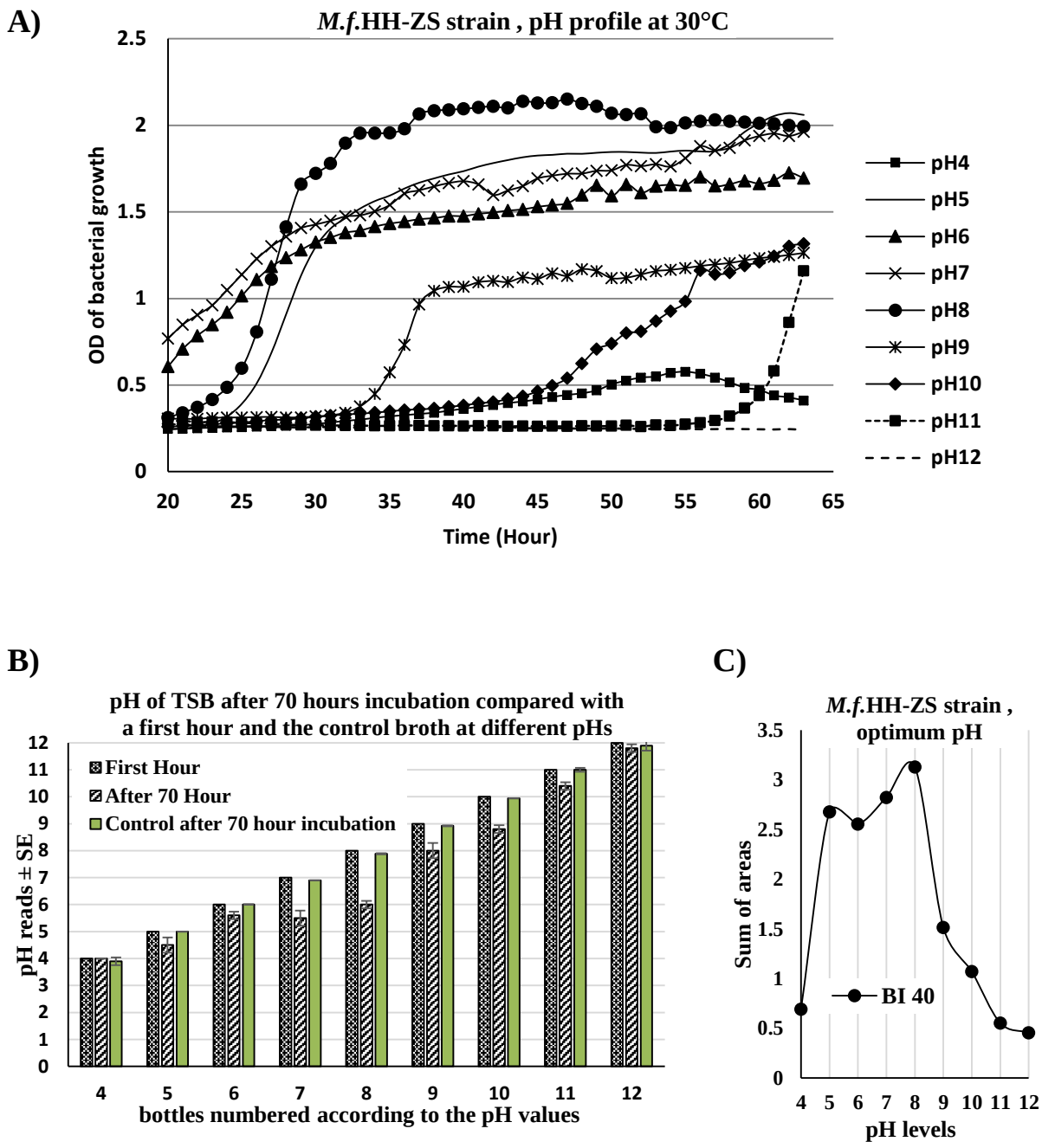


Figure 3. 121: *M.fermentans* HH-ZS strain pH profile

The *M.f.* HH-ZS strain has an ability to survive and grow at a wide range of the pH levels between pH 5.0 and pH 11.0

3.7.5.9.3. Biofilm formation by *M. fermentans* HH-ZS strain

Biofilm formation is one approach that bacteria may us to provide protection against extremes of pH. When evaluated for its ability to form a biofilm, *M. fermentans* HH-ZS generated a moderate biofilm at its optimum pH value (pH 8.0) (Figure 3.122) and weak or no biofilm at either side of its optimum. This suggests that this strain was not a strong biofilm former.

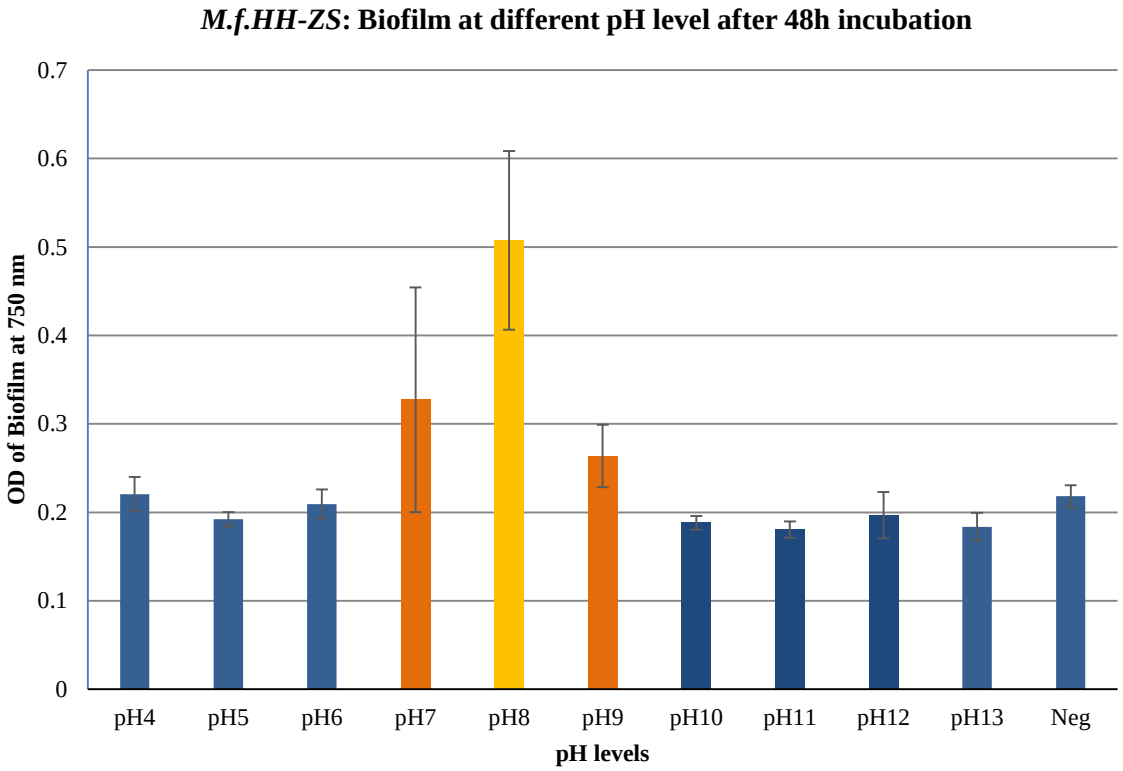


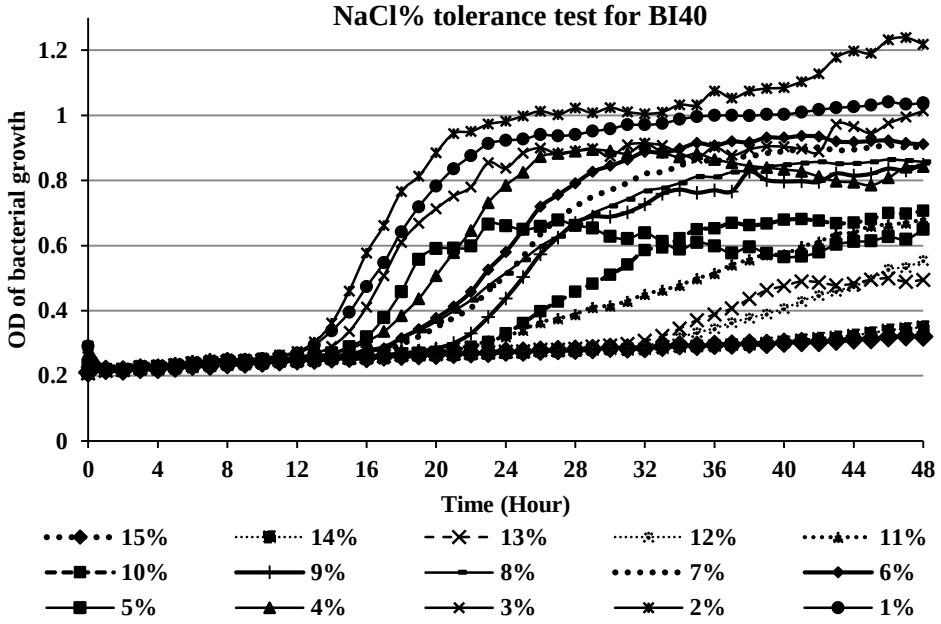
Figure 3. 122: Biofilm formation by *M.fermentans* HH-ZS strain (BI40) at different pH levels

The blue colour indicates no biofilm formation, yellow colour indicates to moderate and orange indicate to weak biofilm formation.

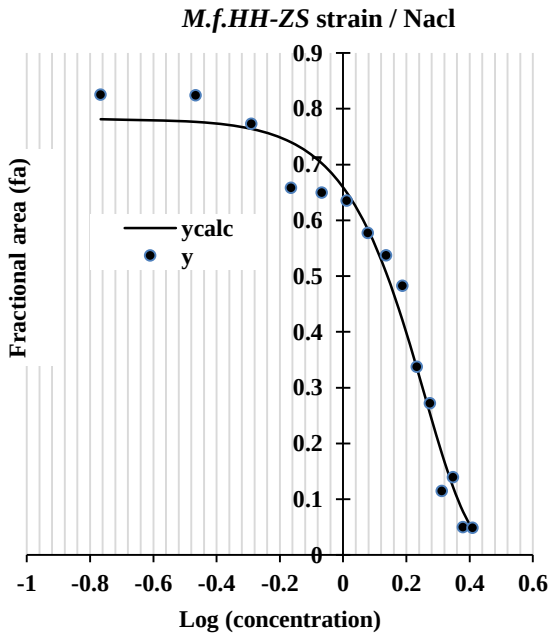
M. fermentans HH-ZS demonstrated a tolerance to NaCl up to a MIC of just above 13% (w/v) (Figure 3.123 A and B) even though the optimum NaCl concentration was between 1-2% (w/v). At extreme NaCl levels, the strain exhibited an extended lag phase, a response similar to that seen with pH (Figure 3.123 C), and a reduced overall growth (Figure 3.123 D). The strain was able to tolerate much greater NaCl levels than its closest relative strains which only tolerated NaCl up to 2% for *M. fermentans* LIND7H^{T} (Jabari et al., 2012)and *Parabacteroides chartae* NS31-3^{T} (Tan et al., 2012). This tolerance to NaCl may reflect the fact that the strain originates from an environment high is alkaline earth metals including calcium (>30%), and some sodium and potassium (<0.54%) (Burke et al., 2012). When exposed to heavy metals (Cd, Co, Cu, Zn, Pb and Ni) the strain was most sensitive to Co and least sensitive to Zn (Table 3.34). The same pattern of an extended lag phase seen with pH and NaCl exposure was also seen at heavy metal concentration that approached the MIC value. Work by Riley *et al*. (1982), carried out the sensitivity test of 105 strains of *Bacteroides* to Pb, Co, Cd, Ni, Ce, As Ag and Hg. They found that all *Bacteroides* strains were multiply resistant when exposed to a concentration of 0.1 mM. When the

concentration was increased to 1.0 mM, the strains was 100% resistant to Ni, Co, Pb, Cd and Cr (Riley & Mee, 1982).

A)



B)



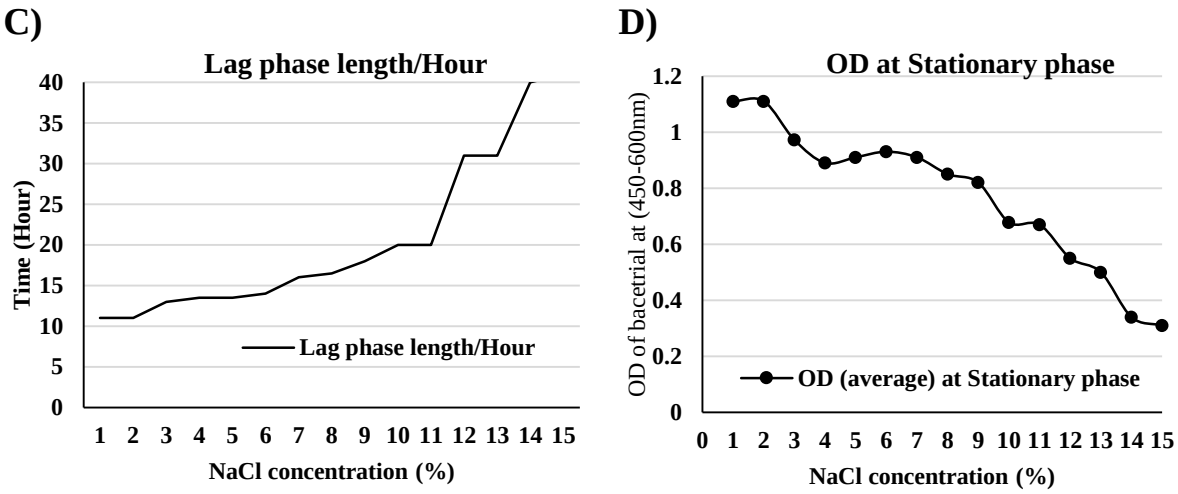


Figure 3. 123: Growth curve of *M.fermentans* HH-ZS at a different NaCl concentration

M.f. HH-ZS *strain* can tolerant to about 13% of NaCl. The area undergrowth curves were calculated and the MIC of NaCl was detected about (2.52 M). The inhibition profile of NaCl against *M.f.* HH-ZS *strain* (BI40) (A): (■), observed fa; (▬), fitted Gompertz function (B), although. The optical density of the inoculum-broth at the lag phase remained at the same values at a different NaCl concentration, the length time of the lag phase was increased (C) and the optical density (average) of the bacterial growth at stationary phase was decreased (D) when the NaCl concentration was increased.

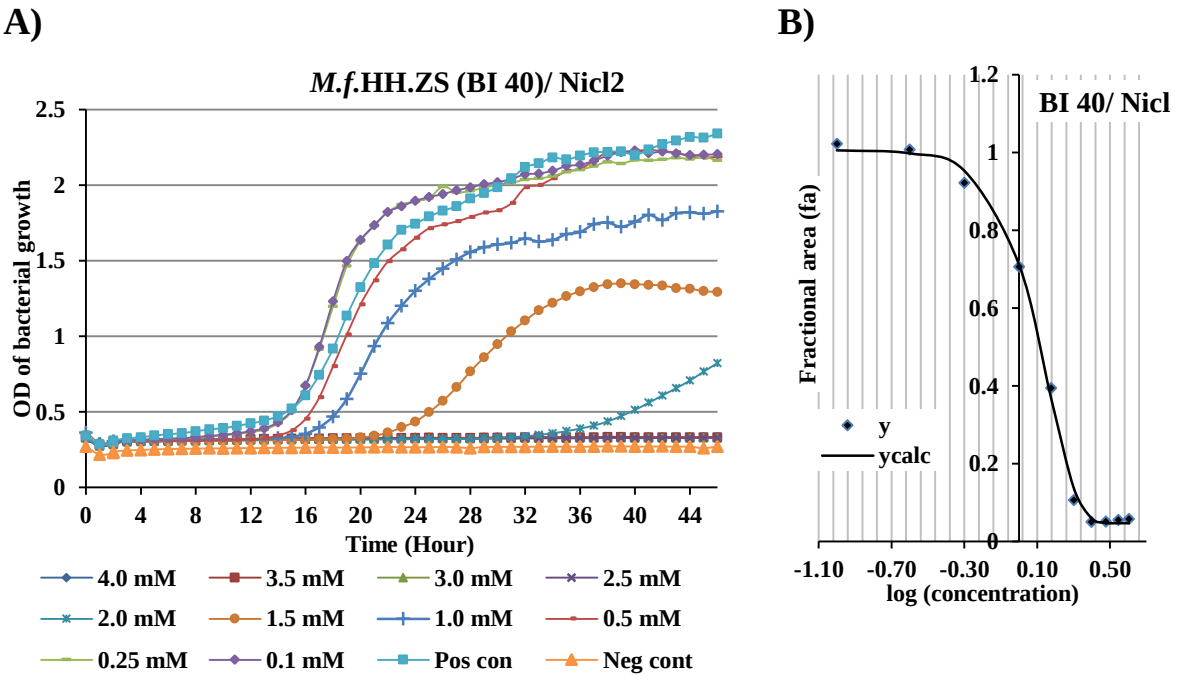


Figure 3. 124: *M.fermentans* HH-ZS growth curves in a different concentration of heavy metals.

An example growth curves (A) for one of the 6 heavy metals that can be found in the appendix. The inhibition profile of $NiCl_2$ against *M.f.* HH-ZS (BI40) was detected by calculate the fractional area at different concentration and plotted on the graph against a log (concentration) using Gompertz function to find the MIC (D) that equal 2.10 mM. The MIC was detected through calculation of; (■), observed FA; (▬), fitted Gompertz function illustrated on a graph (B).

Table 3. 34: The MIC of the heavy metals and NaCl of *M. fermentans* HH-ZS

***Macellibacteroids fermentans* HH-ZS strain**						
Heavy metals tolerance, MIC (mM)						**NaCl%**
Ni	**Cd**	**Co**	**Pb**	**Zn**	**Cu**	
2.10	1.85	0.57	1.06	3.39	2.25	13

3.7.5.9.4. Whole genome sequencing of *M. fermentans* HH-ZS.

The WGS annotation of *Macellibacteroides fermentans* HH-ZS was carried out by RAST (Aziz et al., 2008; Overbeek et al., 2014). The genome assembly consisted of a total 4,081.835 bp within 67 scaffolds, representing a coverage of 127.36x encoding for 3,345 putative coding sequences, of which 3,241 were protein coding, 69 were RNA coding and 35 were pseudogenes. The genome has a GC content of 41.71% and contains rRNAs 1,1,1 for 5S, 16S, 23S respectively, 64 tRNAs, and 2 ncRNAs. RAST annotation (Figure 3.125) displayed the presence of 65 genes responsible for resistance to toxic compounds such as in arsenic, copper and cobalt, 12 genes for efflux pumps, 2 genes for iron acquisition and 11 genes for regulation and cell signalling. The highest percentages of the subsystem features were represented by groups of genes involved in a wide range of carbohydrate degradation processes (Figure 3.125, Table 3.35).

RAST analysis indicated 267 genes involved in the utilization of a wide range of carbohydrates including an extracellular multi-enzyme complex (Table 3.35) known as cellulosome that was detected only in *M. fermentans* HH-ZS strain (BI40) (Table 3.36). This enzyme acts as a mediating factor attaching bacterial cells to complex substrates and along with a multi-functional integrating subunit (scaffolding), work synergistically to degrade a complex substrate (Schwarz, 2001) such as cellulose.

In addition, a wide range of genes (229) involved in protein metabolism were present along with dormancy and stress response (53) genes. The latter covering; osmotic stress (10), oxidative stress (21), heat shock (14), detoxification (7), periplasmic stress (5), these features indicated that this bacterium capable of surviving in a strict environment and reflect the physiological evidence for pH, NaCl and heavy metal tolerance.

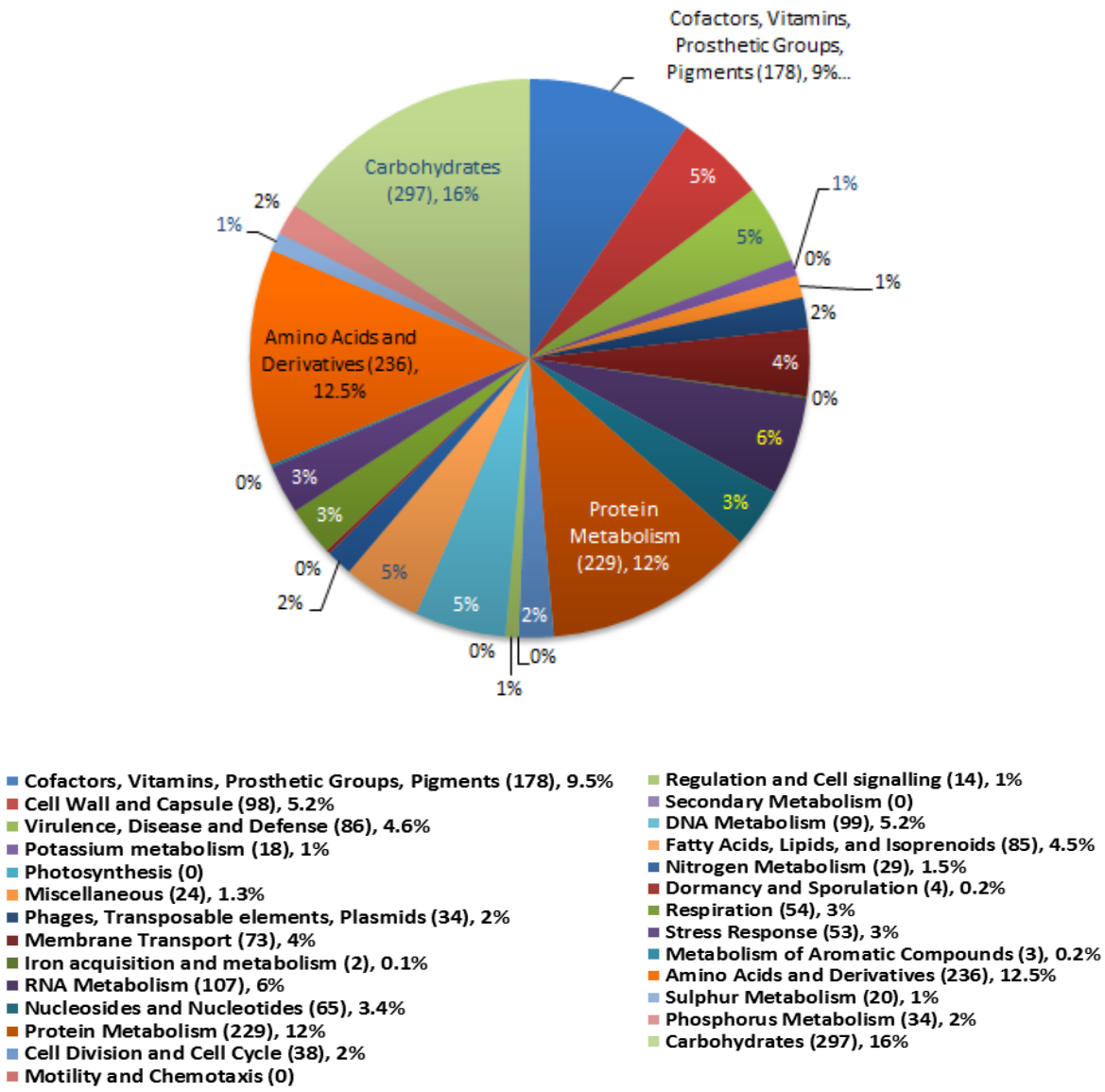


Figure 3. 125: Subsystems groups distribution statistic of *M.fermentans* HH-ZS strain

RAST genome annotations presenting the abundance of each subsystem and the number of each subsystem feature (shown in brackets), the highest percentage (16%) represented a wide range of genes involved in fermentation of different carbohydrate potentially including ISA.

The whole genome sequencing of this new strain has been deposited at BioProject under PRJNA324192, GenBank ID accession number LZEK00000000.

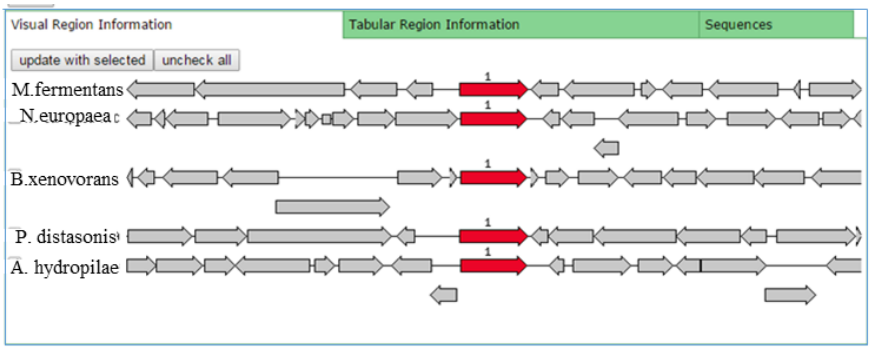
Table 3. 35: Carbohydrates Subsystem Feature in the *M. fermentans* HH-ZS srain

No	Subsystem features of the carbohydrate metabolism by *M.f.* HH-ZS strain (297 genes)
1.	**Central carbohydrate metabolism (78)** involved the following number of (genes); Glycolysis and Gluconeogenesis (14), Glycolate and glyoxylate interconversions (6), Pentose phosphate pathway (8), TCA Cycle (10), Entner-Doudoroff Pathway (12), Pyruvate metabolism I: anaplerotic reactions and PEP (10), Pyruvate Alanine Serine Interconversions (5), Pyruvate metabolism II: acetyl-CoA, acetogenesis from pyruvate (4), Dehydrogenase complexes (4), Methylglyoxal Metabolism (2), Dihydroxyacetone kinases (2), Pyruvate:ferredoxin oxidoreductase (1).
2.	**Fermentation (19)** include the following; Mixed acid (8), Acetolactate synthase subunits (2), Fermentations: Lactate (3), Acetoin, butanediol metabolism (6)
3.	**Monosaccharides (56)** include; Mannose Metabolism (20), D-ribose utilization (4), Xylose utilization (10), Deoxyribose and Deoxynucleoside Catabolism (7), D-Galacturonate and D-Glucuronate Utilization (15)
4.	**Polysaccharides (19)** include; Glycogen metabolism (4), Cellulosome (15)
5.	**Aminosugars (18)** include; Chitin and N-acetylglucosamine utilization (18)
6.	**Di- and oligosaccharides (49)** include; Maltose and Maltodextrin Utilization (22), Lactose and Galactose Uptake and Utilization (18), Lactose utilization (9)
7.	**One-carbon Metabolism (26)** include; Serine-glyoxylate cycle (20), One-carbon metabolism by tetrahydropterines (6)
8.	**Organic acids (6)** include; Propionyl-CoA to Succinyl-CoA Module (6)
9.	**Sugar alcohols (26)** include; Glycerol and Glycerol-3-phosphate Uptake and Utilization (16), Inositol catabolism (10)

Although, this strain was an obligate anaerobe, it was catalase positive an enzyme involved in protection against oxidative stress and ROS (Figure 3.126). This enzyme is very rare in anaerobic bacteria, however, recent research suggests that obligate anaerobic archaea and bacteria contain antioxidant enzymes (Jennings, Schaff, Horne, Lessner, & Lessner, 2014). Brioukhanov (2004) reviewed the presence of oxidative defence enzymes such as catalase and superoxide dismutase (SOD) in anaerobes and concluded that they are important oxidative tolerance function in some groups of strictly anaerobic archaea, sulphate-reducing bacteria, *Bacteroides* sp. and *Clostridium* sp. (Brioukhanov & Netrusov, 2004).

The presence of the catalase gene in *M. fermentans* HH-ZS was determined by comparing the location of this gene in four known catalase positive bacteria (Figure 3.126). The sets of genes (catalase) with the same sequence are indicated by number 1 and the colour red relates to the function of the gene catalase, whereas, the other arrows coloured grey represent the other genes (for other functions), the diagram was downloaded from RAST (Aziz et al., 2008).

A)



B)

>fig|6666666.197520.peg.1881 Catalase (EC 1.11.1.6) [Macellibacteroides fermentans]

MEGKKKLTTESGAPVGDNQNIQTAGPHGPALLQNAWMIEKLAHFNRERIPERVVHAKGSGAFGTLTITHDITMYTKAA
IFSKIGKKTDLFLRFSTVAGERGAADTERDVRGFAIKFYTEEGNWDLVGNNTPVFFLRDPLKFPDFIHTQKRDPKTNLRS
STAAWDFWSLSPESLHQVMILMSDRGIPQNLRQMHGFGSHTFSFINDVNKRFWVKFHFKSMQGIANFTNEEAARIVAD
DREYSQRDLYNNIAGGNFPKWRMCIQIMPEAEAHTCGFNPFDLTKVWSQKNYPLIEVGIMELNKNPENYFATVEQAAF
NPANVVPGIGYSPDKMLQGRLFAYGDAARYRLGINHGSLPVNAPRCPFHNYHHDGTMRTGDNGKGSVNYEPNSFDGP
VENSQYNEPALALEGAAFNYNHREDKDYYTQPGNLYRLVPADEKERIHSNVAAAMEGVPDFIKIRAIARFYQADENC
GKGIAAKAGIQLKDVLTEVERQKDE

Figure 3. 126: Catalase gene location for Oxidative stress and the protein sequence

The graphic is centred on the focus gene, which is red and numbered 1; the identification that include, function of the gene catalase (EC1.11.1.6), coting-00012, the gene starts from 14792 bp stop 13311 bp, size 1482 bp, 494 aa, set 1. This feature is part of a subsystem; in Oxidative stress and protection from Reactive Oxygen Species (RAST server) (A). Using BlastP program, the protein sequences showed 100% identity to catalase (B).

Clusters of Orthologous Groups (COGs) analysis allows the proteins associated with the genome to be identified (Tatusov, Galperin, Natale, & Koonin, 2000). The functional categories are classified in 26 groups and each functional group is labelled from A-Z (Tatusov et al., 2003). Functional classification of the *M. fermentans* HH-ZS genome was performed using the BIOiPLUG function of the ChunLab "genome service". The analysis resulted in the annotation of 3068 genes (Figure 3.127). Similar results were also obtained using WebMGA server (S. Wu, Zhu, Fu, Niu, & Li, 2011). Leaving aside the genes with unknown functions, the largest groups were those associated with cell wall and membrane synthesis (M), carbohydrate transport and metabolism (G) and inorganic ion transport (P), all functions that might be expected to be dominant in an ISA degrading alkaliphilic isolate. However, COG includes the function of unknown proteins under the category (S; 1177 genes (38.36%)) which may include genes that coded for ISA degradation. It is not unusual for proteins with unknown function to dominate protein identified via COG (Tatusov et al., 2000). For example a similar result was found in the genome analysis of *Bifidobacterium bifidum* S17, where the category for unclassified proteins estimated 37.4% (Wei et al., 2016).

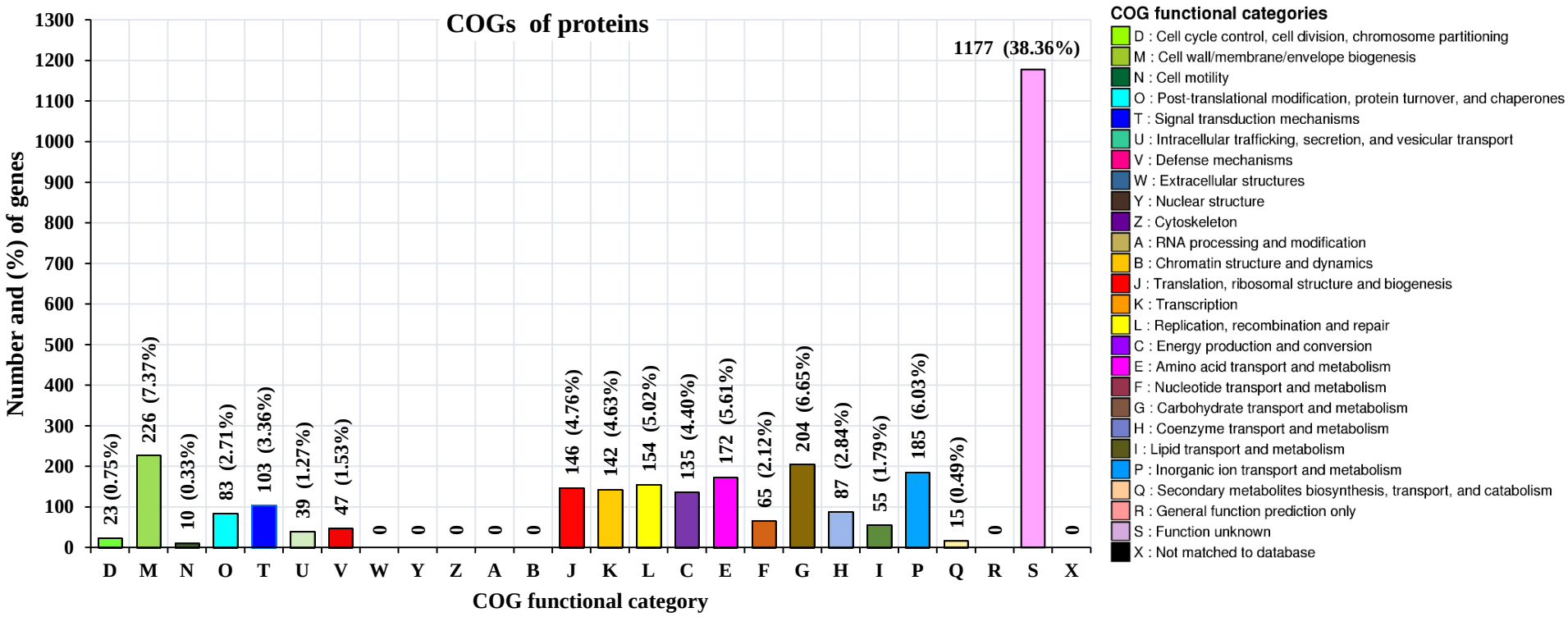


Figure 3. 127: The number of genes for protein-coding sequences (CDS) frequency per COG category for *M.fermentans* HH-ZS

A bar graph of the number of genes for protein-coding sequences (CDS) frequency per COG category after comprehensive annotation, each bar showing the number of CDS in that category https://www.bioiplug.com/genome/explore?puid=77474

Table 3. 36: Carbohydrate subsystem features of *M.fermentans* HH-ZS and other strains

Subsystem Feature Counts	MfHH-ZS	BI85	BI 49	BI 70	BI 41	BI 28	BI 36	BI 51
Carbohydrates	297	524	537	235	398	220	207	344
Central carbohydrate metabolism	78	128	148	108	117	101	105	124
Methylglyoxal Metabolism	2	5	29	10	4	7	8	27
Pyruvate:ferredoxin oxidoreductase	1	3	0	0	2	0	0	0
Pyruvate metabolism II: acetyl-CoA, acetogenesis from pyruvate	4	9	32	21	12	8	9	27
Pyruvate Alanine Serine Interconversions	5	5	9	6	5	6	9	4
Glycolysis and Gluconeogenesis, including Archaeal enzymes	0	16	0	0	0	0	0	0
Dihydroxyacetone kinases	2	8	1	0	6	0	0	6
Glycolysis and Gluconeogenesis	14	21	11	14	16	14	13	16
Dehydrogenase complexes	4	0	20	10	14	11	14	0
TCA Cycle	10	0	22	17	17	17	16	19
Entner-Doudoroff Pathway	12	27	0	0	14	13	11	0
Pyruvate metabolism I: anaplerotic reactions, PEP	10	12	4	6	5	10	6	5
Pentose phosphate pathway	8	20	11	11	16	9	10	8
Glycolate, glyoxylate interconversions	6	2	0	7	0	0	4	4
Glyoxylate bypass	0	0	6	6	6	6	5	7
Ethylmalonyl-CoA pathway of C2 assimilation	0	0	3	0	0	0	0	1
Aminosugars	18	64	11	0	35	6	0	0
Chitin and N-acetylglucosamine utilization	18	21	11	0	35	6	0	0
N-Acetyl-Galactosamine and Galactosamine Utilization	0	16	0	0	0	0	0	0
Hyaluronate utilization	0	27	0	0	0	0	0	0
Di- and oligosaccharides	49	157	32	1	58	16	5	25
Fructooligosaccharides(FOS) and Raffinose Utilization	0	13	0	0	0	0	0	0
Maltose and Maltodextrin Utilization	22	32	9	0	25	11	0	9
Trehalose Uptake and Utilization	0	17	11	0	13	0	5	14
Beta-Glucoside Metabolism	0	61	0	0	0	0	0	0
Lactose and Galactose Uptake and Utilization	18	24	10	0	11	0	0	0
Lactose utilization	9	10	2	1	6	0	0	2
Sucrose utilization	0	0	0	0	0	5	0	0
One-carbon Metabolism	26	6	80	5	36	35	37	62
One-carbon metabolism by tetrahydropterines	6	5	7	5	5	5	4	5
Serine-glyoxylate cycle	20	0	73	0	31	30	33	57
Organic acids	6	5	23	27	12	3	3	4
Glycerate metabolism	0	5	5	5	6	3	3	2
Propionyl-CoA to Succinyl-CoA Module	6	0	6	5	0	0	0	0
Lactate utilization	0	0	7	10	6	0	0	2
Malonate decarboxylase	0	0	0	7	0	0	0	0
Fermentation	19	37	141	48	39	24	35	89
Butanol Biosynthesis	0	11	64	14	10	6	12	43
Fermentations: Mixed acid	8	18	0	0	13	0	0	0
Fermentations: Lactate	3	8	5	5	5	1	4	3
Acetolactate synthase subunits	2	0	4	5	2	4	0	3
Acetoin, butanediol metabolism	6	0	9	0	5	0	3	0
Acetyl-CoA fermentation to Butyrate	0	0	59	24	4	13	16	40
CO2 fixation	0	2	0	41	0	0	11	0
CO2 uptake, carboxysome	0	2	0	8	0	0	0	0
Photorespiration (oxidative C2 cycle)	0	0	0	16	0	0	11	0
Calvin-Benson cycle	0	0	0	17	0	0	0	0
Sugar alcohols	26	33	35	0	16	6	4	7
Glycerol and Glycerol-3-phosphate Uptake and Utilization	16	9	15	0	7	6	4	7
Mannitol Utilization	0	8	0	0	0	0	0	0
Ethanolamine utilization	0	16	0	0	0	0	0	0
Inositol catabolism	10	0	20	0	9	0	0	0
Carbohydrates - no subcategory	0	7	0	0	7	1	1	0
Carbon storage regulator	0	2	0	0	0	1	0	0
Lacto-N-Biose I and Galacto-N-Biose Metabolic Pathway	0	5	0	0	7	0	0	0
Polysaccharides	19	8	27	0	10	7	0	14
Glycogen metabolism	4	7	7	0	7	7	0	5
Alpha-Amylase locus in Streptocococcus	0	1	20	0	3	0	0	9
Cellulosome	**15**	0	0	0	0	0	0	0
Monosaccharides	56	77	40	5	68	21	6	19
Mannose Metabolism	20	20	6	3	6	2	3	4
D-ribose utilization	4	8	7	2	11	1	3	5
Deoxyribose and Deoxynucleoside Catabolism	7	18	6	0	5	6	0	4
D-gluconate and ketogluconates metabolism	0	6	7	0	0	0	0	2
D-galactarate, D-glucarate and D-glycerate catabolism	0	4	0	0	0	0	0	0
Fructose utilization	0	17	12	0	10	0	0	4
D-galactarate, D-glucarate and D-glycerate catabolism - gjo	0	4	2	0	0	0	0	0
Xylose utilization	10	0	0	0	12	0	0	0
D-Galacturonate and D-Glucuronate Utilization	15	0	0	0	9	12	0	0
L-Arabinose utilization (15)	0	0	0	0	15	0	0	0

Further annotation using the Carbohydrate-Active enzymes (CAZyme) analysis tool kit (B. H. Park, Karpinets, Syed, Leuze, & Uberbacher, 2010) identified a further 69 genes and placed all of these into CAZY domains (Figure 3.128). Through the WGS analysis, the number of glycoside hydrolase proteins family was greater in *M. fermentans* HH-ZS compared with other available *Parabacteroides* species.

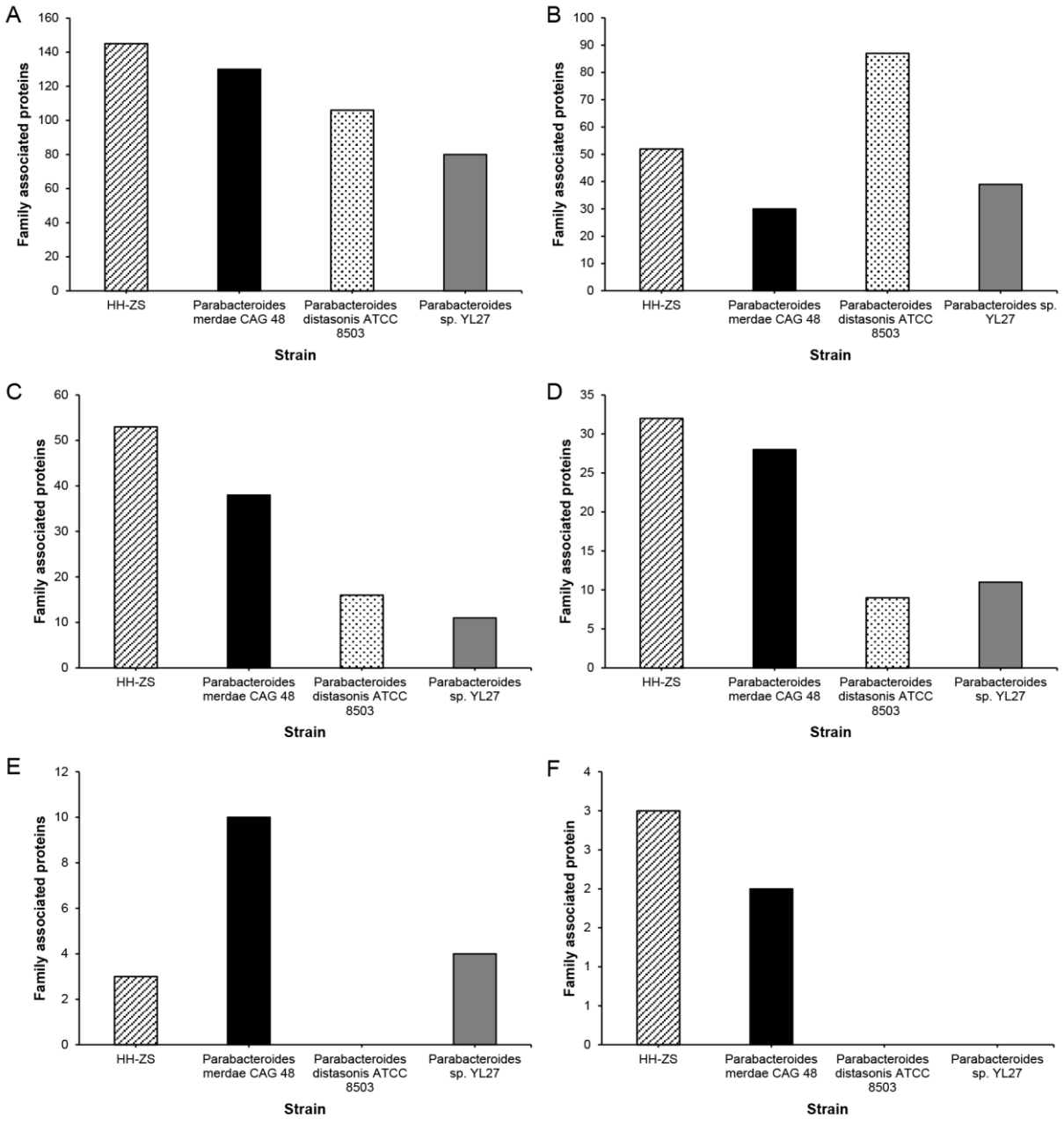


Figure 3. 128: CAZy assignment of carbohydrate active enzymes of *M. fermentans* HH-ZS

Comparison of the CAZy assignment with available whole genome sequences of *Parabacteroides* sp. Glycoside Hydrolase (A), Glycosyl Transferase (B), Carbohydrate Binding Module (C), Carbohydrate Esterase (D), Polysaccharide Lyase (E) and Auxiliary Activities (F) associated families are shown.

The presence of a number of carbohydrate binding module families (CBM) in the genome, (in particular calcium dependent CBM4), are involved in the metabolism of a number of the organic materials present in soils include cellulosic materials, with the exception of crystalline cellulose (Kataeva, Seidel, Li, & Ljungdahl, 2001). Interestingly alkaline conditions will result in the swelling of cellulose and potentially its partial hydrolysis, making the cellulose fibres less crystalline.

The availability of the whole genome of some related strains (Table 3.37) provides an alternative approach to identifying the bacteria concerned. BLASTn analysis using the partial sequence of the 16S rRNA gene (1528 bp) from the full sequence was employed generated with a partial sequences of a closest match with *Macellibacteroides fermentans* type strain LIND7H by the EzBioCloud database (S.-H. Yoon et al., 2017). A phylogenetic tree (Figure 3.129 A) using an Unweighted Pair Group Method with Arithmetic Mean (UPGMA) clustered *M.f* HH-ZS (BI40) with *Macellibacteroides fermentans* type strain LIND7H, accession number HQ020488.

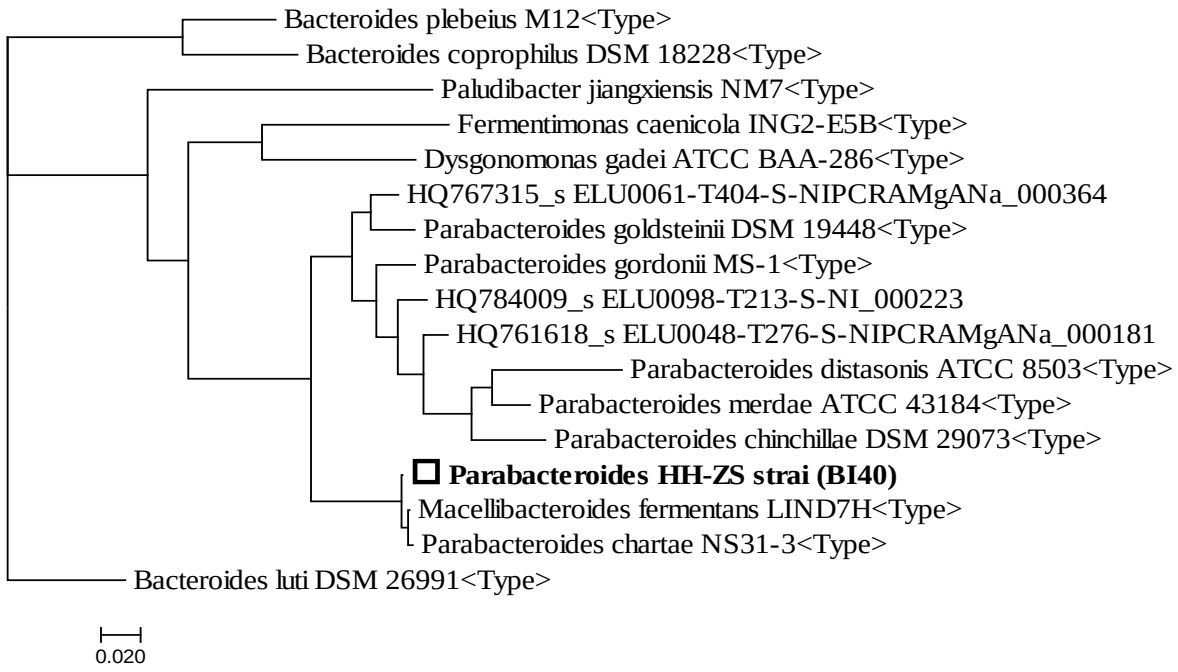
Whilst, the Orthologous Average Nucleotide Identity (OrthoANI) analysis provides an alternative approach which has similarities to the traditional DNA/DNA hybridisation approach employed to identify relatedness between bacterial isolates (I. Lee, Kim, Park, & Chun, 2016; S.-H. Yoon et al., 2017). When this approach was employed *M.f* HH-ZS (BI40) was aligned with *Parabacteroides chartae* Type strain DSM 24967 (Table 3.37, Figure 3.129 B) instead of the type strain LIND7H, that was due to the unavailability of the whole genome sequence of the type strain LIND7H. It should be noted that, when the HH-ZS strain was analysed via the updated EzBioCloud programme (S.-H. Yoon et al., 2017) the data base attributed it to the *Parabacteroides genus* rather than the *Macellibacteroides genus.* This result combined with the previous results provide further evidence that *M.f.* HH-ZS is a distinct strain when compared with its closest relative strains of *Macellibacteroides fermentans* LIND7H^{T} and *Parabacteroides chartae* Type strain DSM 24967.

Table 3. 37: Whole Genome based identification of *M. fermentans* HH-ZS strain

Similarity search results sorted on 16S rRNA and identity by OrthoANI values or one of them if the information from one of them is not available (NA). NA=not available

	Taxon name	OrthoANIu (figure 3.131 B)		16S rRNA (phylogenetic tree, figure 3.131 A)		
		Hit	Identity (%)	Hit	Similarity (%)	Accession
1	Macellibacteroides fermentans	NA	NA	LIND7H(T)	99.6	HQ020488
2	Parabacteroides chartae	DSM 24967(T)	95.7	NS31-3(T)	99.4	JN029805
3	HQ767315_s	NA	NA	ELU0061-T404-S-NIPCRAMgANa_000364	93.9	HQ767315
4	HQ784009_s	NA	NA	ELU0098-T213-S-NI_000223	93.9	HQ784009
5	HQ761618_s	NA	NA	ELU0048-T276-S-NIPCRAMgANa_000181	93.7	HQ761618
6	Parabacteroides goldsteinii	DSM 19448(T)	71.3	DSM 19448(T)	93.2	AQHV01000028
7	Parabacteroides gordonii	MS-1(T)	71.2	MS-1(T)	93.2	KQ033919
8	Parabacteroides chinchillae	DSM 29073(T)	71.0	DSM 29073(T)	91.8	jgi.1108062
9	Parabacteroides merdae	ATCC 43184(T)	70.5	ATCC 43184(T)	93.2	AAXE02000112
10	Parabacteroides distasonis	ATCC 8503(T)	70.2	ATCC 8503(T)	90.7	CP000140
11	Bacteroides luti	DSM 26991(T)	68.5	DSM 26991(T)	86.8	jgi.1107761
12	Paludibacter jiangxiensis	NM7(T)	68.1	NM7(T)	86.1	BDCR01000002
13	Dysgonomonas gadei	ATCC BAA-286(T)	68.1	ATCC BAA-286(T)	88.9	GL891979
14	Bacteroides plebeius	DSM 17135(T)	67.9	M12(T)	84.3	AB200217
15	Bacteroides coprophilus	DSM 18228(T)	67.8	DSM 18228(T)	85.0	ACBW01000012
16	Fermentimonas caenicola	ING2-E5B(T)	67.0	ING2-E5B(T)	88.0	LN515532

A)



B)

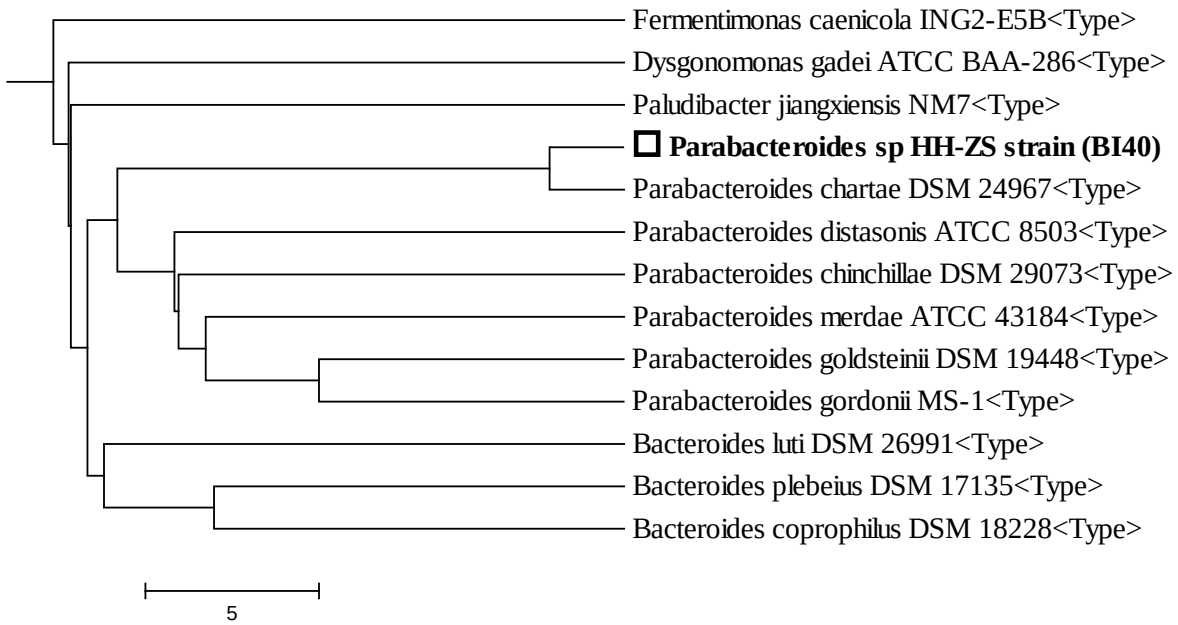


Figure 3. 129: A phylogenetic tree based on 16S rRNA sequence and UPGMA clustering of OrthoANI similarity. https://www.bioiplug.com/genome/explore?puid=77474

The similarity identification results from the EzBioCloud genome DB through BIOiPLUG, ChunLab's "genome service" DB. Maximum-likelihood algorithm phylogenetic tree based on 16S rRNA gene sequences showed closest strains to the *M.f* HH-ZS (BI40), with 99.6% similarity to *Macellibacteroides fermentans* type strain LIND7H^T, accession number HQ020488 (A). The Dedrogram based on UPGMA of OrthoANI showed a similarity between *M.f* HH-ZS (BI40) and *Parabacteroides chartae* type strain DSM 24967 (B).

3.7.5.9.5. Discussion

This study identified a single strictly anaerobic bacterial strain capable of ISA degradation using either CDPs or $Ca(ISA)_2$ at pH 8.0 and pH 9.0. The final products of ISA fermentation by *Macellibacteroides fermentans* HH-ZS was acetic acid when $Ca(ISA)_2$ was provided as a carbon source. In addition, this strain also has the ability to ferment a wide range of sugars (Table 3.32 and 3.33). Whole genome annotation (Figure 3.125) demonstrated the presence of a range of genes (297 (16%)) encoding proteins associated with carbohydrate metabolism (Table 3.35). This included a group of 15 genes that encoding for the cellulosome not present in the genome of any of the other strains (Table 36). This multi-enzyme systems was first discovered in the strictly anaerobic of cellulolytic thermophile, *Clostridium thermocellum* (E. Bayer, Setter, & Lamed, 1985; E. A. Bayer, Kenig, & Lamed, 1983), and has also been detected in other anaerobic bacteria include; *C. cellulovorans*, *C. cellulolyticum*, *Bacteroides ceflulosolvens*, *Ruminococcus flavefaciens* and *Acetivibrio cellulolyticus*. The cellulosome in most of these anaerobic bacteria are similar and composed of Dockerin domine protein that bind to type I cohesins and CBD (Complete scaffoldin), except in *Bacteroides* species, where the cellulosome is composed of type II cohesins and CBD without Dockerin (Incomplete scaffoldin) or Cell-surface anchoring proteins (E. A. Bayer, Chanzy, Lamed, & Shoham, 1998). CAZy analysis revealed that the strain harbored a number of carbohydrate degrading enzymes, which merit further investigation to determine the metabolic pathways associated with ISA degradation.

M.f. HH-ZS grew between pH 5.0–10.0 with an optimum between pH 5.0–8.0 (Figure 3.121), it did not require NaCl for growth, but was capable of growth in the presence of NaCl up to 2.52 M (Figure 3.123). It was also able to tolerant heavy metals with MIC values indicating that it was more sensitive to Co and least sensitive to Zn (Table 3.34). In previous research carried out on copper contaminated sediments from contaminated lakes in Finland. It was found that there was a significant shift in the community structure, with the Bacteroidetes phyla becoming the dominated phyla in the contaminated lake due to their high resistance to heavy metals (X.-P. Chen et al., 2017).

Identification of phylogeny using partial 16S rRNA sequencing have classified *M.f.* HH-ZS as a new strain within the newly established species *Macellibacteroides fermentans*. This strain shows homology to the *Macellibacteroides fermentans* LINND7H strain (Figure 3.116). However, the physical and biochemical characteristics including biomarker analysis and alkali and NaCl tolerance separated this isolate from the type strain of *Macellibacteorides fermentans*.

Macellibacteroides fermentans HH-ZS strain was also aerotolerant by virtue of the possession of catalase. The biomarker studies supported the position that this is a new strain based on the fact that there were significant differences in fatty acid, polar lipid and menaquinone profiles when compared with the closest related strains LIND7H^T strain and *Parabacteroides* species (Jabari et al., 2012).

The data collected regarding *Macellibacteroides fermentans* HH-ZS strain contributed to the publication:

Simon P. Rout, Zohier B. Salah, Christopher J. Charles, Paul N. Humphreys: *Whole-Genome Sequence of the Anaerobic Isosaccharinic Acid Degrading Isolate, Macellibacteroides fermentans Strain HH-ZS*. Genome Biology and Evolution 08/2017; 9(8-8):2140-2144., DOI:10.1093/gbe/evx151

3.7.5.9.6. Key findings

- *Macellibacteroides fermentans* HH-ZS was a novel strain with a number of characteristics, which separate it from its closest relatives including catalase activity, the presence of a cellulosome, fatty acid and polar lipid contents.
- The strain was a strictly anaerobic bacterium with specific defence mechanisms against oxidative stress e.g. catalase.
- *Macellibacteroides fermentans* HH-ZS was the first Gram negative, strictly anaerobic bacteria able to degrade ISA to be identified.

4. General Discussion and Conclusions

4.1. Discussion

The deep cementitious geological disposal facility (GDF), about 500 m underground, is a current strategy for a long term storage of the United Kingdom's nuclear waste legacy (Anon, 2010a). depending on the heat and radioactive outputs, the GDF are classified into three levels, low- intermediate- and high level waste (Anon, 2011). The intermediate waste (ILW) of GDF will be expected to contain a number of heterogeneous of the radionuclide-contaminated waste that include a significant amount of the cellulosic materials. Post closure of the facility, it is expected to re-saturate with the groundwater which causes the dissolution of the cementitious backfill that leading to create high pH (Evans, 2008). Under the alkaline and anaerobic environmental conditions that generated within the GDF, the cellulosic materials are subjected to alkaline hydrolysis and produce cellulose degradation products including the α and β stereoisomers of isosaccharinic acid (ISA) (Glaus et al., 1999; Humphreys, Laws, et al., 2010). These organic acids are able to form stable complexes with certain radionuclides increasing their solubility and promoting their transport via groundwater (Greenfield et al., 1997; Randall et al., 2012).

The generation of ISA in the proposed GDF poses both safety and microbiological challenges (Glaus et al., 1999; Humphreys, Laws, et al., 2010). The research described in this thesis has focussed on the isolation and characterisation of alkaliphilic bacteria from anthropogenic alkaline soils and test their ability to degrade ISA isomers (α- and β-) in the CDP and in the form of $Ca(ISA)_2$, under alkaline and anaerobic incubation conditions through the fermentation and anaerobic respiration by using TEA. The microcosms of CDPs/MM broth were inoculated with soil samples that collected from Haripur Hill lime kiln waste site, Buxton site, Derbyshire, UK. This site is likely to be a source of novel alkaliphilic or alkali tolerant species which may be capable of the degradation of ISAs (Bassil et al., 2015; Humphreys, West, et al., 2010; Rout, Charles, Doulgeris, et al., 2015; Rout, Charles, et al., 2015a; Rout et al., 2014; S. L. Smith et al., 2016). In addition, these novel alkaliphiles may have alternative applications in biotechnology and bioremediation.

When used to establish CDP fed microcosms, these alkaline sediments generated communities able to degrade both forms of ISA (α- and β-) via fermentation up to pH 11.0. At pH 12.0 there was no clear evidence of microbial activity, although isolation studies demonstrated that viable organisms remained viable at the pH. These microcosms were operated for extended incubation period (12 months) in order to establish stable communities. Despite the fact that the Harpur

Hill sediments are highly alkaline (pH 12-13) and that ISA is generated in the sediments at the site (Rout, Charles, et al., 2015b)**,** ISA degradation in these microcosms was clearly pH dependent with increased degradation below pH 9.0. This is in agreement with previous observations by a number of authors (Bassil et al., 2015; Kuippers et al., 2015; Rout, Charles, Doulgeris, et al., 2015). The fermentation of ISA consistently generated acetic acid as the primary fermentation end product with other VFA rarely observed.

Adaption to CDP fed microcosms significantly impact the associated microbial diversity. These observation were also consistent with previous observations by Rout *et al.* and Kuippers *et al*. where the microbial community shifted from a Proteobacteria dominated community to a Firmicutes dominated community in the microcosms (Kuippers et al., 2015; Rout, Charles, Doulgeris, et al., 2015). This loss in diversity from ~ 1030 OTUs in the soil to ≤600 OTUs in the microcosms is not surprising considering the fact that the soil will contain large numbers of aerobes, plant associated organisms, transient organisms and spores that will not be able to maintain populations in the microcosms.

Previous studies have looked at ISA degradation at the expense of TEA at high pH (pH 10.0) (Bassil et al., 2015) and neutral pH (Kuippers et al., 2015) there was no systematic investigation looking at a range of pH environments employing CDP. These experiments demonstrated two trends in common with the microcosms firstly that the rate of ISA degradation decreased at high pH and secondly the community diversity seen in soil inoculum decreased significantly when incubated anaerobically with CDP and the TEA. The use of CDP in these experiments rather than Ca-ISA generated different outcomes when compared to similar studies (Bassil et al., 2015) and neutral pH (Kuippers et al., 2015). This difference is that in the previously published Ca-ISA studies nitrate reduction were two stages process with nitrate reduction following fermentation. In the experiments reported here, nitrate reduction progressed directly with no evidence of fermentation. Under Fe(III)-reducing condition there was limited ISA degradation with Fe(III) reduction indicated by the generation of Fe (II). An observation similar to that recorded by Kuippers *et al*. (2015). The microbial populations were broadly similar to those observed in the nitrate reducing microcosms dominated by Gram-negative Proteobacteria such as *B. melitensis* and *P. tuomuerensis* followed by the Firmicutes. This results are in contrast with the observations of Bassil *et al*. (2015), who observed populations dominated by Gram-positive Firmicutes represented by the *Anaerobacillus* genus at pH 10 (Bassil et al., 2015).

Under sulphate-reducing cultures, the degradation of ISA was associated with a fermentation process at pH 7.0 and pH 8.0 but not above pH 9.0. These results are consistent with previous data provided by Bassil *et al.* (2015), who did not find any evidence of ISA degradation under sulphate-reducing conditions at pH 10 (Bassil et al., 2015) and Rizoulis *et al.* (2012), who failed to find evidence for sulphate reduction at the expense of lactate or acetate at alkaline pH (Rizoulis et al., 2012). They are also in agreement with Kuippers *et al*. (2015), who demonstrated sulphate reduction at neutral pH was associated with fermentation. The absence of sulphate reduction under alkaline conditions is further illustrated by the absence of any classical SRB in the community analysis of these experiments. Rather, large numbers of unclassified organisms dominated these experiments.

The calcium salt of ISA ($Ca(ISA)_2$) has been used in both geochemical and microbiological studies as an alternative to CDP (Bassil et al., 2015). It was used in the studies presented here as a step towards isolating pure cultures of ISA degrading bacteria. A large number of microcosms were run with Ca-ISA, one important example became dominated by Gram-negative rods primarily composed of *Azonexus* species. This then led to the isolation of a novel alkaliphilic nitrogen fixing strain of *Azonexus.*

This *Azonexus* sp. was one of many bacteria isolated during the course of this investigation. Many of these isolates were novel and isolated from CDP fed microcosms and isolated on CDP and Ca-ISA plates. However, with the exception of two isolates, none was able to degrade ISA when cultured in anaerobic broths with ISA as a soul carbon source. This suggests that ISA degradation in the microcosms reported here is a multispecies process that could not be reproduced in pure culture.

Two Gram-negative strains were isolated, which were able to degrade ISA in pure culture. These were the obligate anaerobic *Macellibacteroides fermentans* HH-ZS strain (BI40) and the facultative anaerobic *Aeromonas salmonicida* strain (BI55). These are the first examples of pure cultures able to degrade ISA under alkaline, anaerobic conditions via fermentation. In view of its novelty, *M. fermentans* HH-ZS received more detailed attention than *Aeromonas salmonicida* (BI55).

Macellibacteroides fermentans is a member of the Bacteroidetes phylum and was detected in the microbial communities of the CDP microcosms, but only at a low level (4%, 2.8% and 0.03% at pH 9.0, pH 10.0 and pH 11.0 respectively). The strain is able to ferment ISA to acetic

acid under strictly anaerobic conditions. The species *M. fermentans* was identified for a first time by Jabari *et al*. (2012) through their work on isolate LIND7H which was obtained from anaerobic abattoir wastewaters in Tunisia (Jabari et al., 2012). *M. fermentans* HH-ZS strain (BI40) (this study) was significantly different to *M.fermentans* LIND7HT in terms of its pH profile, salt tolerance and the presence of catalase. Biochemical and molecular (WGS) of *M. fermentans* HH-ZS indicated that it has broad carbohydrate degradation capabilities including the presence of a cellulosome.

4.2. Conclusions

4.2.1. Conclusion 1

After a number of CDP fed-cycle protocol in the microcosms under the pH values between 8.0 and 11.0, the results concluded to that, the bacterial community present in the alkaline soil was able to adapt to an alkaline broth medium and degrade CDP containing both forms of ISA. The bacterial communities established in these microcosms were able to degrade CDP to methane at pH 10.0. The bacterial community has shown an affinity for the utilization of β-ISA, rather than α-ISA, even at pH 10.0. Significant ISA degradation was initiated when the pH fell below pH 9.5. Acetate accumulation was an indicator of ISA fermentation and facilitated the reduction in pH. A significant reduction of bacterial diversity occurred during the adaption process.

4.2.1. Conclusion 2

When subcultured in liquid culture and provided with ISA as a sole carbon source, microbial populations may become dominated by small numbers of bacteria. The clearest example being the establishment of a microbial community dominated by the Gram-negative bacillus, *Azonexus hydrophilus*. These microcosms proved to be an effective “half way house” in the process of isolating novel isolates.

4.2.2. Conclusion 3

Many of the bacteria present in the microcosms were not recovered in pure culture, supporting the well known observation that large proportions on environmental bacteria are uncultivable via conventional techniques. Of those bacteria that were isolated, many were unable to degrade ISA when provided as a sole carbon source in liquid culture. This suggests that the degradation of ISA in the CDP fed microcosms was either associated with the uncultivable bacteria or required the cooperation of more than one species.

4.2.3. Conclusion 4

This study identified two bacterial strains capable of ISA degradation under anaerobic conditions using either CDPs or $Ca(ISA)_2$ at pH 8.0 and pH 9.0. These two strains were the Gram-negative *Aeromonas* species ZS-strain (BI55) and *Macellibacteroides fermentans* HH-ZS. *Macellibacteroides fermentans* HH-ZS is the first Gram negative, strictly anaerobic bacteria able to degrade ISA to be identified.

4.3. Future work

The large difference between the numbers of bacteria present within the microcosms and those recoverable via conventional culturing techniques means that there is a gap in the currently available techniques. The molecular analysis of these populations provides an insight into the organisms present. Future studies will use this analysis to identify target organisms for isolation, this isolation will be guided by a molecular understanding of what these organisms are capable of and which culture supplements they may require.

The genome of *Macellibacteroides fermentans* HH-ZS harboured a number of carbohydrate degrading enzymes, which merit further investigation to determine the metabolic pathways associated with ISA degradation. In addition, some of the isolated alkaliphiles are poorly characterised. The characterization of these isolates would provide a platform for further investigation regarding their biotechnological and bioremediation potential.

This study isolated two strain of *Aeromonas salmonicida,* only one of which was able to degrade ISA. These strains provide an opportunity to identify the mechanisms by which ISA is degraded by Gram-negative bacteria. This analysis could be performed via a comparison of the whole genomes of these two strains.

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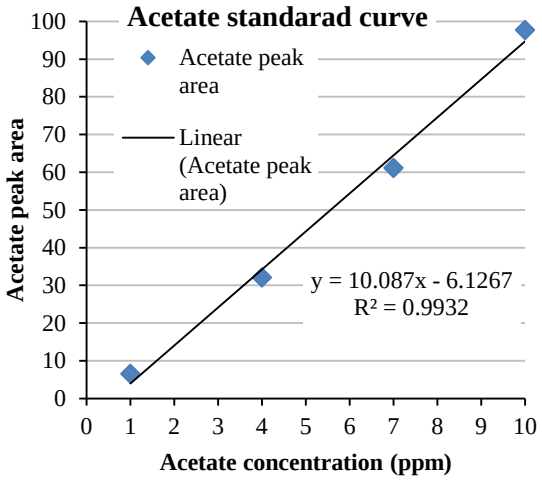
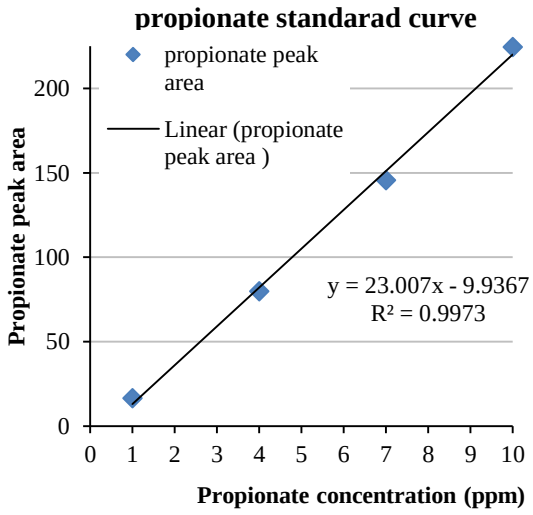
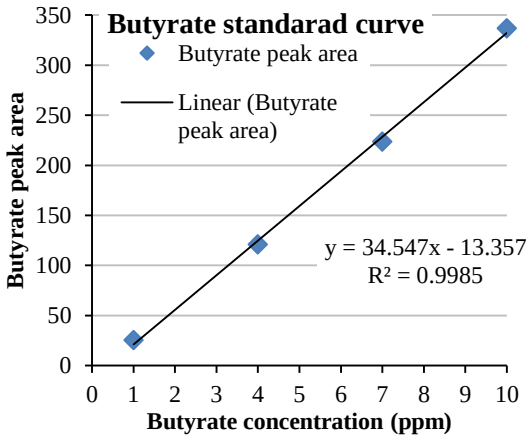
Appendix
Chapter 3

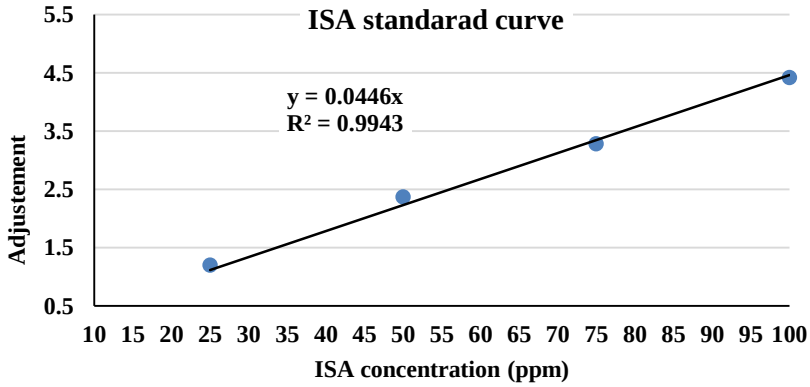
Appendix-1

Standard curves to calculate the concentration of the ISA isomers and volatile fatty acids of the samples.





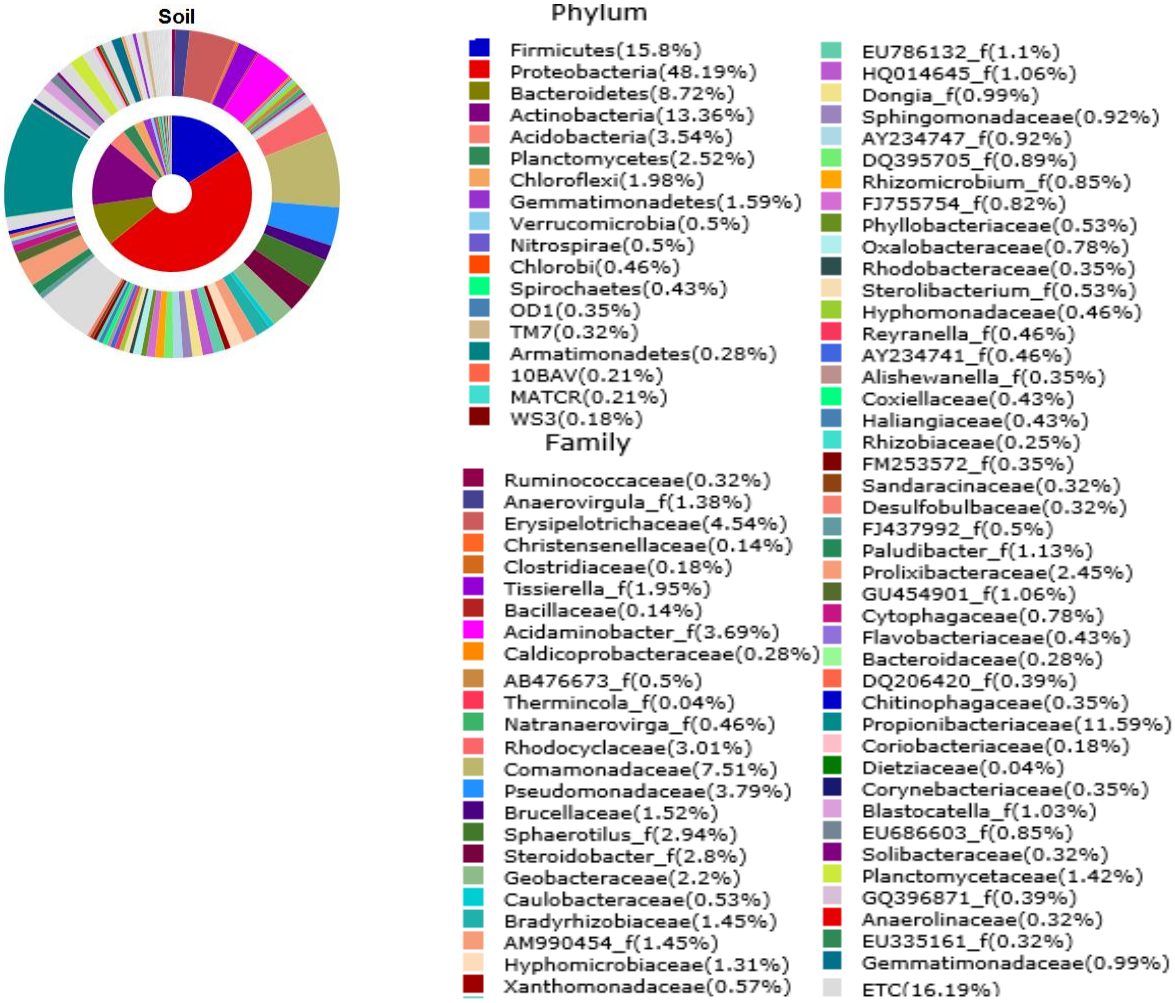


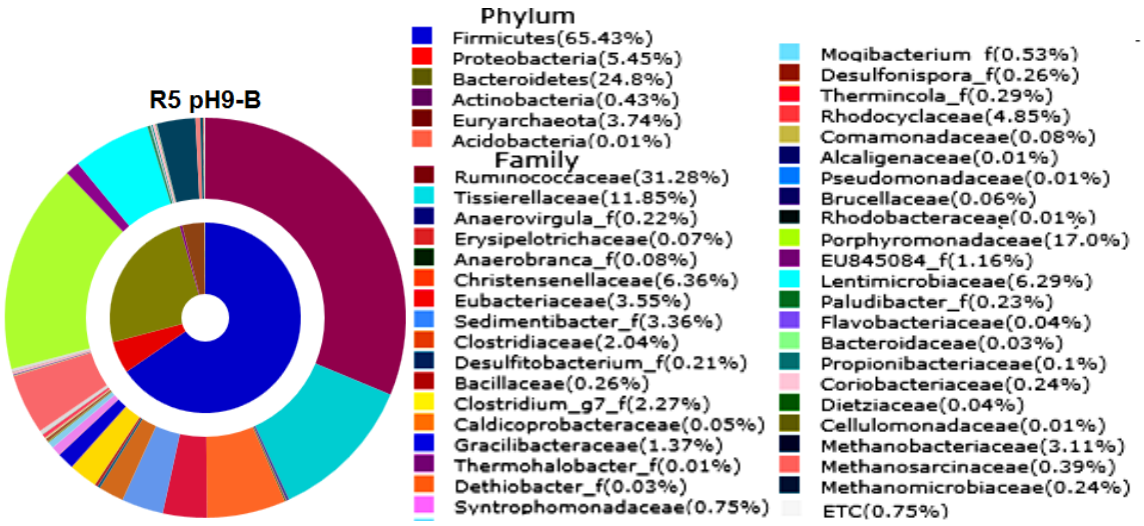


Appendix-2; Bacterial community analyses under a fermentation process

Bacterial diversity of the microcosms; R1 at pH 10, R2 at pH 11 and R5 at pH 9.0.

After CDPs feed-cycle each 30 days for 12 month incubation




R5 pH9-B
Phylum
Firmicutes(65.43%)
Proteobacteria(5.45%)
Bacteroidetes(24.8%)
Actinobacteria(0.43%)
Euryarchaeota(3.74%)
Acidobacteria(0.01%)
Family
Ruminococcaceae(31.28%)
Tissierellaceae(11.85%)
Anaerovirgula_f(0.22%)
Erysipelotrichaceae(0.07%)
Anaerobranca_f(0.08%)
Christensenellaceae(6.36%)
Eubacteriaceae(3.55%)
Sedimentibacter_f(3.36%)
Clostridiaceae(2.04%)
Desulfitobacterium_f(0.21%)
Bacillaceae(0.26%)
Clostridium_g7_f(2.27%)
Caldicoprobacteraceae(0.05%)
Gracilibacteraceae(1.37%)
Thermohalobacter_f(0.01%)
Dethiobacter_f(0.03%)
Syntrophomonadaceae(0.75%)
Mogibacterium_f(0.53%)
Desulfonispora_f(0.26%)
Thermincola_f(0.29%)
Rhodocyclaceae(4.85%)
Comamonadaceae(0.08%)
Alcaligenaceae(0.01%)
Pseudomonadaceae(0.01%)
Brucellaceae(0.06%)
Rhodobacteraceae(0.01%)
Porphyromonadaceae(17.0%)
EU845084_f(1.16%)
Lentimicrobiaceae(6.29%)
Paludibacter_f(0.23%)
Flavobacteriaceae(0.04%)
Bacteroidaceae(0.03%)
Propionibacteriaceae(0.1%)
Coriobacteriaceae(0.24%)
Dietziaceae(0.04%)
Cellulomonadaceae(0.01%)
Methanobacteriaceae(3.11%)
Methanosarcinaceae(0.39%)
Methanomicrobiaceae(0.24%)
ETC(0.75%)
R1 pH10-B
Phylum
Firmicutes(64.12%)
Proteobacteria(12.36%)
Bacteroidetes(23.22%)
Actinobacteria(0.21%)
Verrucomicrobia(0.04%)
Synergistetes(0.05%)
Family
Ruminococcaceae(29.25%)
Anaerovirgula_f(21.16%)
Erysipelotrichaceae(2.31%)
Anaerobranca_f(1.09%)
Christensenellaceae(1.15%)
Eubacteriaceae(0.23%)
Sedimentibacter_f(0.6%)
Clostridiaceae(0.05%)
Tissierella_f(2.42%)
Desulfitobacterium_f(2.19%)
Bacillaceae(0.69%)
Clostridium_g7_f(0.12%)
Caldicoprobacteraceae(0.69%)
Gracilibacteraceae(0.02%)
Dethiobacter_f(0.18%)
Syntrophomonadaceae(0.23%
Mogibacterium_f(0.18%)
Clostridiales_uc(1.15%)
Lachnospiraceae(0.09%)
Desulfonispora_f(0.05%)
Thermincola_f(0.05%)
Rhodocyclaceae(0.02%)
Comamonadaceae(0.16%)
Alcaligenaceae(11.59%)
Brucellaceae(0.42%)
Caulobacteraceae(0.05%)
Rhodobacteraceae(0.02%)
Porphyromonadaceae(6.14%)
EU845084_f(12.29%)
FJ437992_f(4.64%)
Paludibacter_f(0.11%)
Propionibacteriaceae(0.12%)
Coriobacteriaceae(0.07%)
Corynebacteriaceae(0.02%)
ETC(0.48%)
R2 pH11-B
Phylum
Firmicutes(44.27%)
Proteobacteria(44.02%)
Bacteroidetes(3.14%)
Actinobacteria(8.35%)
Euryarchaeota(0.21%)
Family
Ruminococcaceae(3.52%)
Tissierellaceae(9.14%)
Anaerovirgula_f(17.5%)
Erysipelotrichaceae(2.23%)
Anaerobranca_f(5.03%)
Christensenellaceae(0.11%)
Eubacteriaceae(2.74%)
Sedimentibacter_f(0.37%)
Clostridiaceae(0.74%)
Bacillaceae(0.55%)
Caldicoprobacteraceae(1.04%)
Lachnospiraceae(0.23%)
Desulfonispora_f(0.17%)
Rhodocyclaceae(40.12%)
Comamonadaceae(1.86%)
Alcaligenaceae(0.05%)
Pseudomonadaceae(0.02%)
Brucellaceae(0.11%)
Caulobacteraceae(0.76%)
Desulfomicrobiaceae(0.72%)
Rhodobacteraceae(0.18%)
Alishewanella_f(0.05%)
Rhizobiaceae(0.05%)
Porphyromonadaceae(1.18%)
EU845084_f(0.89%)
Lentimicrobiaceae(0.17%)
Paludibacter_f(0.79%)
Bacteroidaceae(0.05%)
Propionibacteriaceae(0.1%)
Coriobacteriaceae(4.94%)
Dietziaceae(2.61%)
Corynebacteriaceae(0.59%)
Cellulomonadaceae(0.06%)
Methanobacteriaceae(0.21%)
ETC(0.38%)

After CDPs feed cycle each 10 days for further 40 day incubation.

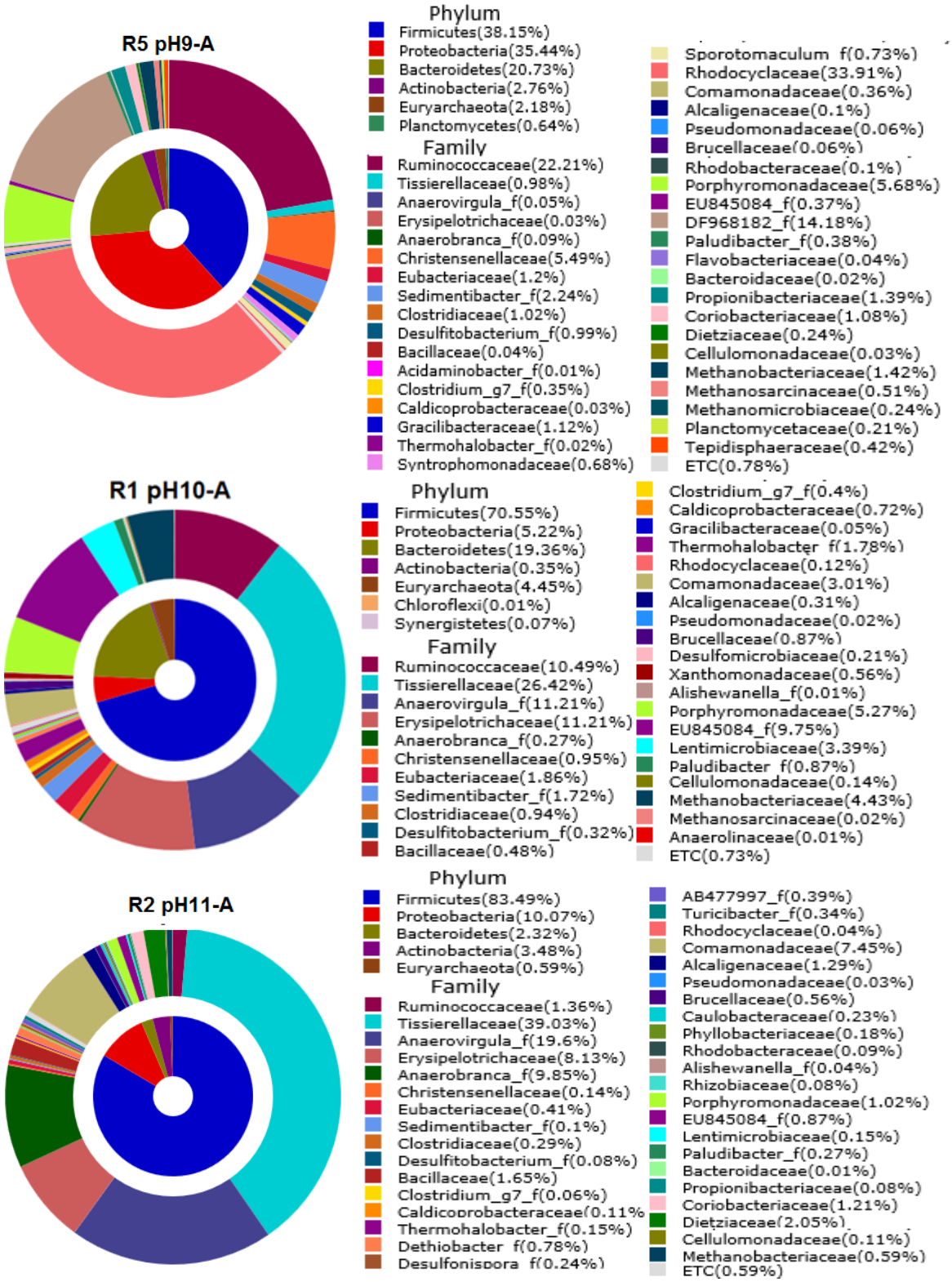


Table 1: Bacterial diversity of crude soil sample compared with CDPs driven microcosms at pH 9.0, pH 10.0 and pH 11.0, under two CDPs feed-cycle protocols; each 30 days for 12 month and followed by each 10 days for 40 days.

Alkaliphiles community of CDPs driven microcosms at pH 9.0, pH 10.0 and pH 11.0, were dominated by Firmicutes phylum under both of incubation conditions protocols, followed by Proteobacteria 44% at pH 11, 35.4% at pH 9.0 that was dominated phylum in a crud soil sample 48%. The phylum Bacteroidetes was presented by high percentages at pH 9.0 and pH 10.0 under both conditions with an average 23% and 21.5% respectively. Actinobacteria phylum was recorded a low percentage at all pH values under both of incubation conditions. UD= undetected.

Name of phyla%		30 days of CDPs feed-cycle for 12 months			10 days of CDPs feed-cycle for 40 days			Crude soil
		R5pH 9.0-B	R1pH 10-B	R2pH 11-B	R5pH 9.0-A	R1pH 10-A	R2pH 11-A	
Firmicutes		65.43	64.12	44.27	38.14	70.55	83.5	15.8
Proteobacteria		5.4	12.36	44.02	35.4	5.22	10.0	48.19
Bacteroidetes		24.8	23.22	3.14	20.7	19.36	2.3	8.72
Actinobacteria		0.43	0.21	8.35	2.76	0.35	3.48	13.36
Euryarchaeota		3.74	UD	0.21	2.17	4.45	0.6	UD
ETC < 2.0%		0.2	0.09	0.01	0.83	0.07	0.12	13.93
	Families%							
Firmicutes	Ruminococcaceae	31.28	29.25	3.25	22.21	10.5	1.36	0.32
	Anaerovirgula	0.22	21.16	17.5	0.05	11.21	19.6	1.38
	Anaerobranca	0.08	1.09	5.03	0.1	0.27	9.85	UD
	Tissierellaceae	11.85	2.42	9.14	0.1	26.42	39	1.95
	Eubacteriaceae	3.55	UD	2.74	1.2	1.86	0.41	UD
	Clostridiaceae	2.0	0.05	0.74	1.0	0.94	0.3	0.18
	Sedimentibacter	3.36	0.6	0.37	2.24	1.72	0.1	UD
	Clostridium	2.27	0.12	UD	0.35	0.4	0.06	UD
Proteobacteria	Rhodocyclaceae	4.85	0.02	40.12	34	0.12	0.04	3.01
	Alcaligenaceae	UD	11.59	0.05	0.1	0.31	1.3	UD
Actinobacteria	Coriobacteriaceae	UD	0.07	4.94	1.0	UD	1.2	0.18
	Dietziaceae	0.04	UD	2.61	0.24	UD	2.0	0.04
Bacteroidetes	Porphyromonadaceae	17.0	6.14	1.18	5.68	5.27	1.0	UD
Unknown	Total of unknown families	UD	UD	UD	14.9	11	1.0	0.4
Euryarchaeota	Metanobacteriaceae	3.11	UD	0.21	1.42	4.43	UD	UD
ETC < 2.0%	ETC < 2.0%	20.39	27.49	12.12	15.41	25.55	22.78	92.54

Table 2: Bacterial diversity on a species level of crud soil sample compared with CDPs driven microcosm at pH 9.0, pH 10.0 and pH 11.0, under two CDPs feed-cycle protocols; each 30 days for 12 month and followed by each 10 days for 40 days.

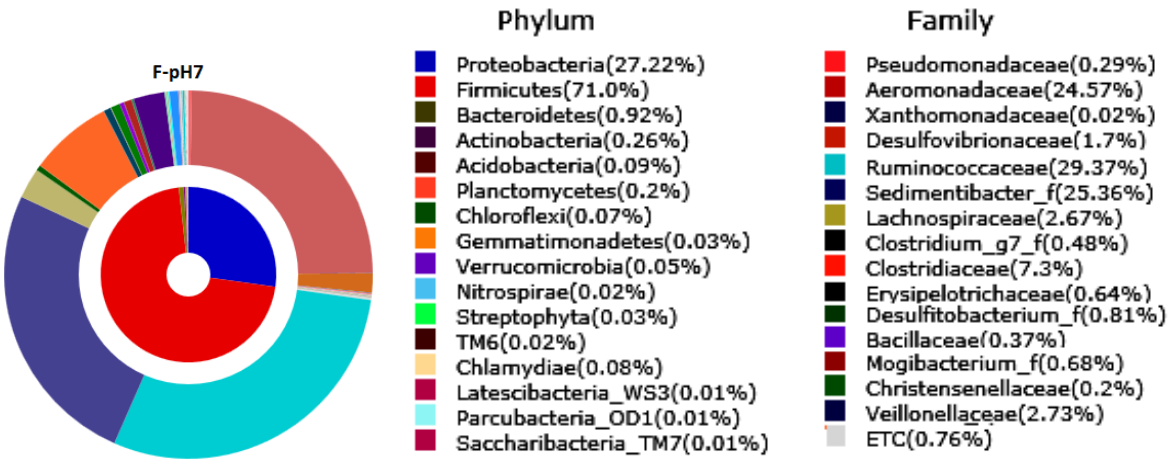
On a species level, the highest percentage was recorded by DQ088747 species of *Azonexus* genus at pH11 37% under FFCP and pH 9.0 30% under S-FCP, followed by *Ruminococcus* sp. 25.7% under F-FCP at pH 10.0. The species *GQ132209* of *Tissierella* genus *(*37%, 23%, at pH 11 and pH 10 respectively, under S-FCP). *JN178047* sp. of *Alkaliphilus* genus was presented by 17.5% under S-FCP at pH 11 and by 10% under F-FCR at pH 10.
UD= undetected, Gram positive (G+), Gram negative (G-), *Archaea, Bold species = whole genome seq was performed from pure culture of these strains

16SrRNA gene reads			30 days CDPs feed-cycle for 12 months			10 days of CDPs feed-cycle for 40 days			Crude soil
Name of families	**Genus**	**Species%**	**R5pH 9.0-B**	**R1pH 10-B**	**R2pH 11-B**	**R5pH 9.0-A**	**R1pH 10-A**	**R2pH 11-A**	
Ruminococcaceae (G+)	*Ruminococcus,*	*Ruminococcus*	4.13	25.7	0.18	1.72	0.82	0.04	0.14
	Ercella	*Er. succinigenes*	2.48	0.02	1.62	0.01	1.28	0.31	UD
		JQ245572	8.36	UD	0.006	10.37	0.05	0.10	UD
	AB630534_g	*JX391172*	3.58	0.03	0.12	2.20	9.34	0.38	UD
		AB630534 g	4.19	UD	0.05	2.17	0.97	0.12	UD
Anaerovirgula (G+)	*Anaerovirgula*	*An. multivorans*	UD	UD	6.53	UD	5.00	0.60	UD
	Alkaliphilus	*JN178047*	0.15	10.22	8.36	0.01	3.52	17.54	UD
Tissierellaceae (G+)	*Tissierella*	*Tissierella*	0.6	0.9	1.7	0.05	2.53	1.06	UD
		GQ132209	9.84	UD	7.71	0.83	22.85	37.12	UD
Eubacteriaceae (G+)	*Acetobacterium*	*Acetobacterium woodii*	2.13	8.4	0.52	UD	0.43	0.15	UD
Rhodocyclaceae (G-)	***Azonexus,***	***Azonexus***	**0.22**	**0.37**	**1.43**	**0.90**	**0.00**	**0.00**	UD
		DQ088747	**4.29**	**UD**	**37.16**	**30.05**	**0.09**	**0.04**	UD
	Rhodocyclus	*Rhodocyclus*	0.22	11.27	1.28	1.44	0.01	UD	0.78
Coriobacteriaceae (G+)	*Coriobacterium*	*Coriobacteria*	0.13	1.71	4.8	0.23	2.71	0.64	0.04
Dietziaceae (G+)	***Dietzia***	***Diet.psychralcaliphila***	**0.04**	**UD**	**2.5**	**UD**	**4.38**	**1.98**	**UD**
Porphyromonadaceae (G-)	*Fermentimonas;,*	*Ferm. caenicola*	9.68	UD	0.24	UD	4.00	0.50	UD
	Parabacteroides	***Macellibacteroides fermentans***	**4.07**	**0.24**	**UD**	**3.73**	**2.87**	**0.03**	UD
	Proteiniphilum	*Proteiniphilum*	1.27	9.011	0.44	UD	0.10	0.20	UD
Lentimicrobiaceae (G-)	*Lentimicrobium*	*AY570585*	6.11	4.6	0.16	13.82	3.22	0.14	UD
Erysipelotrichaceae (G+)	*AB237727_g*	*DQ266900*	0.014	2	2.1	UD	10.69	7.79	UD
Syntrophomonadaceae (G+)	*Anaerobranca*	*JF159990*	UD	UD	4.5	UD	3.92	6.69	UD
Metanobacteriaceae	*Methanobacterium*	*Methanobacterium**	1.47	2.17	UD	UD	4.40	0.60	UD
Comamonadaceae (G-)	*Comamonas*	*Com. kerstersii*	0.06	UD	1.7	UD	4.93	6.97	UD
Propionibacteriaceae(G+)	*Propionibacterium*	*Propionibacterium*	UD	UD	UD	UD	0.12	UD	11.23
ETC < 2.0%			36.966	23.359	16.894	32.47	11.78	17.01	87.81

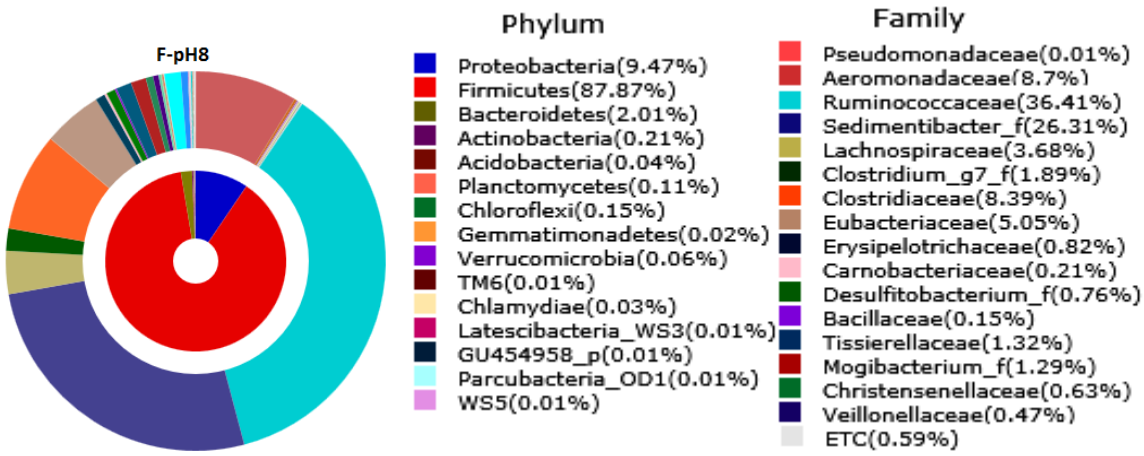
UD= undetected, Gram positive (G+), Gram negative (G-), *Archaea, Bold species = whole genome seq was performed from pure culture of these strains

Appendix-3; Bacterial diversity analysis under a redox reaction processes

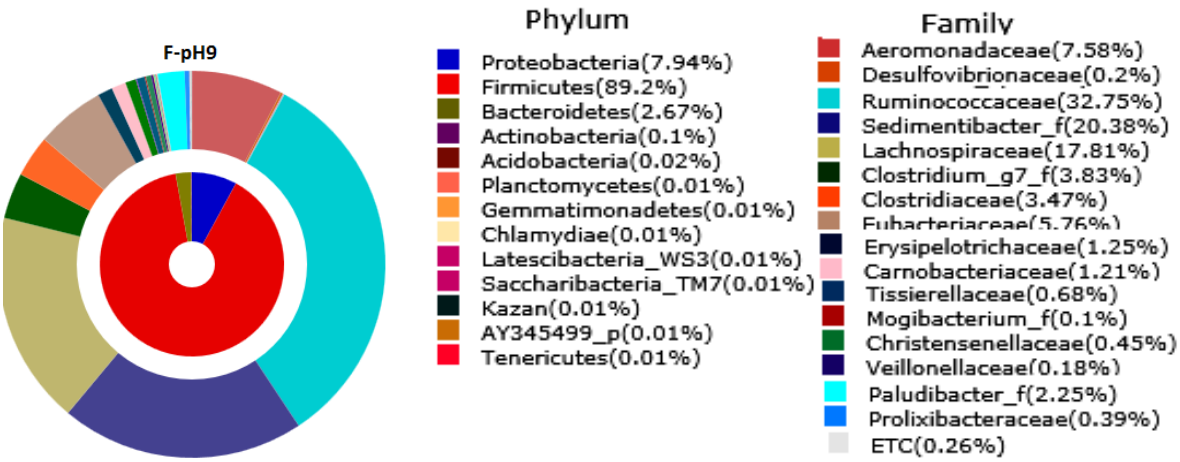
I - Phylogenetic diversity under a fermentation process, at pH7.0, 8.0, 9.0 and 10.0



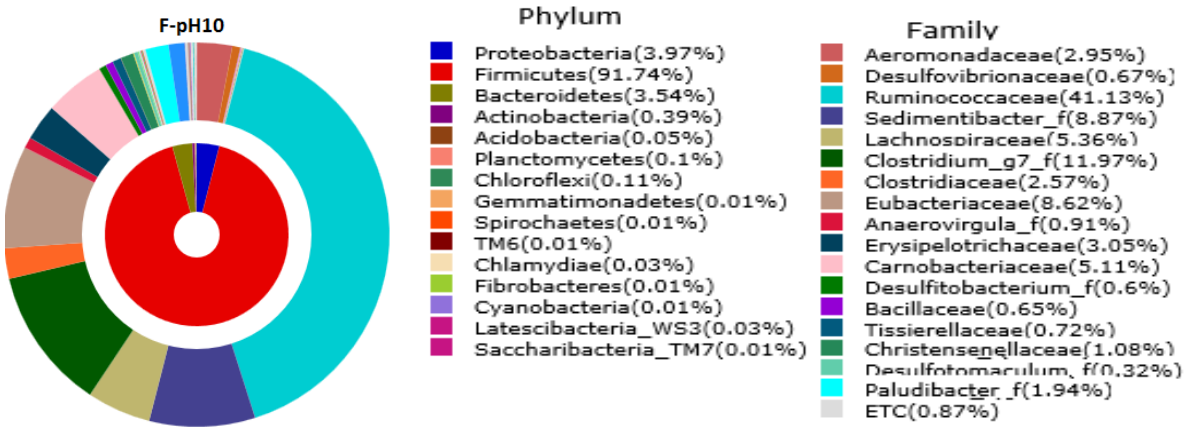
A) Bacterial community through fermentation (F) process at pH 7.0



B) Bacterial community through fermentation (F) process at pH 8.0

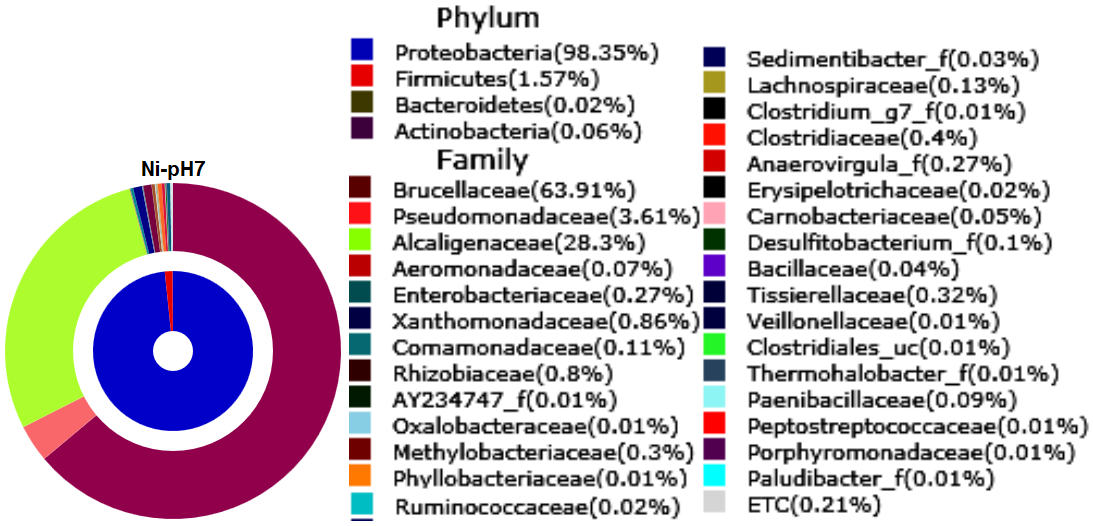


C) Bacterial community through fermentation (F) process at pH 9.0

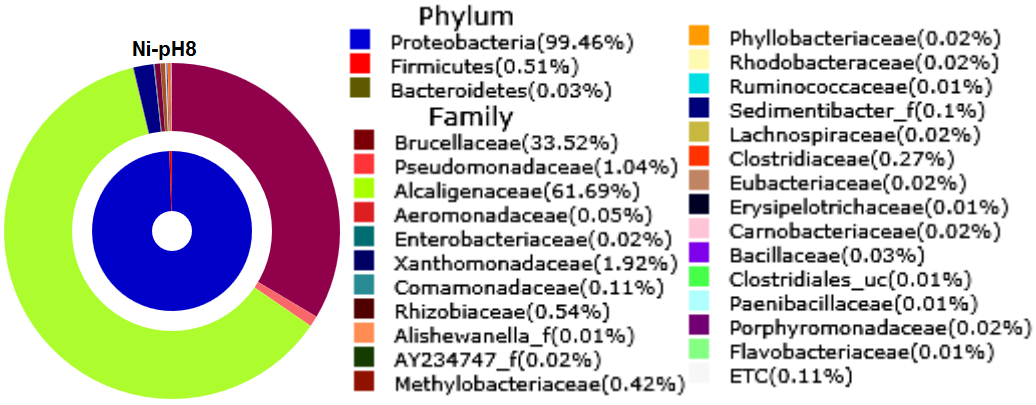


D) Bacterial community of fermentation (F) process at pH 10.0

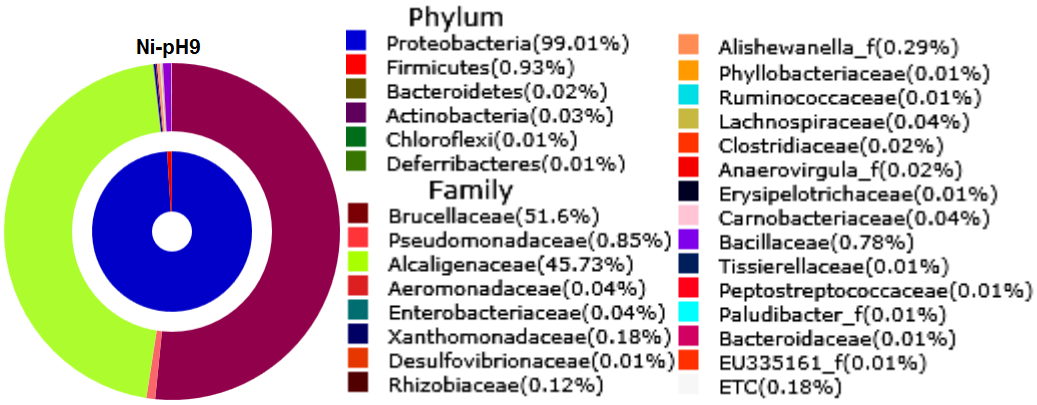
II - Phylogenetic analysis under Nitrate reduction process (RX-NI)



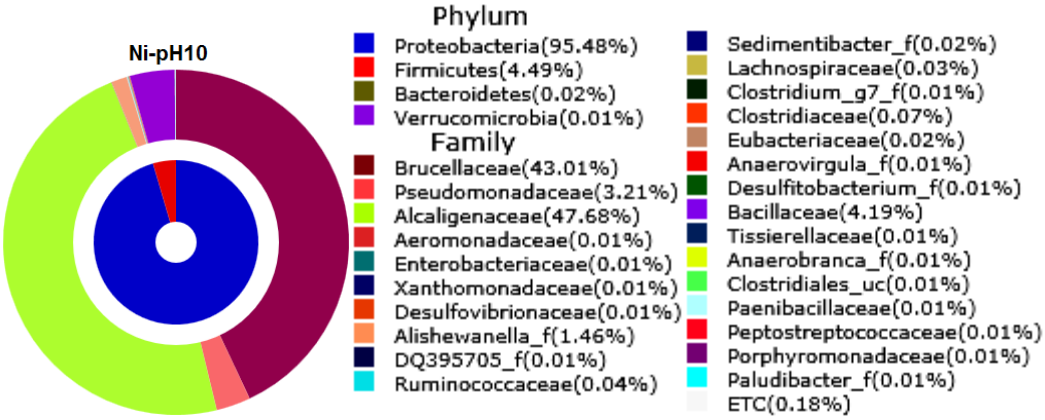
A) Bacterial diversity under nitrate reduction at pH 7.0



B) Bacterial diversity under nitrate reduction at pH 8.0

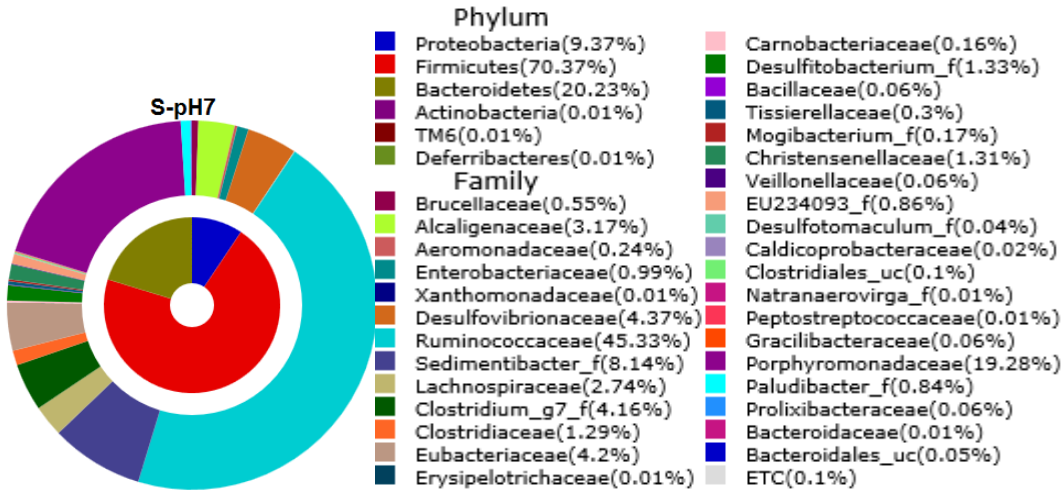


C) Bacterial diversity under nitrate reduction at pH 9.0

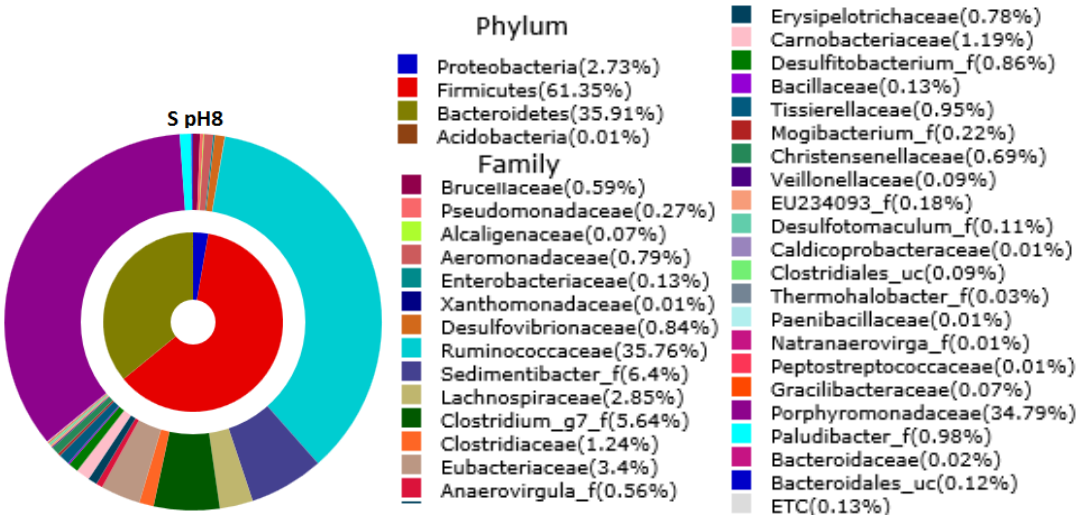


D) Bacterial diversity under nitrate reduction at pH 10.0

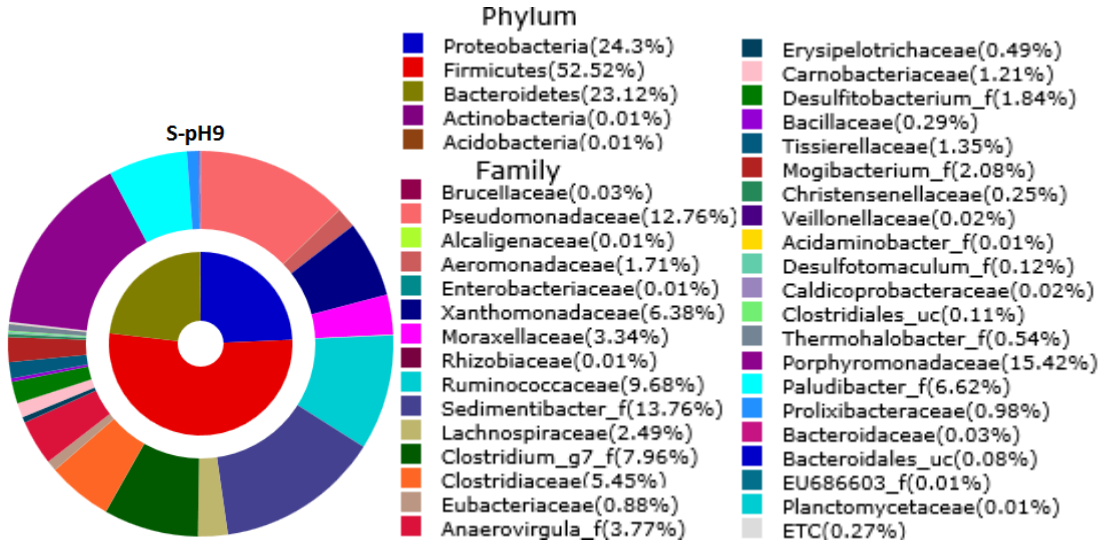
III- Sulphate reduction process (RX-S):



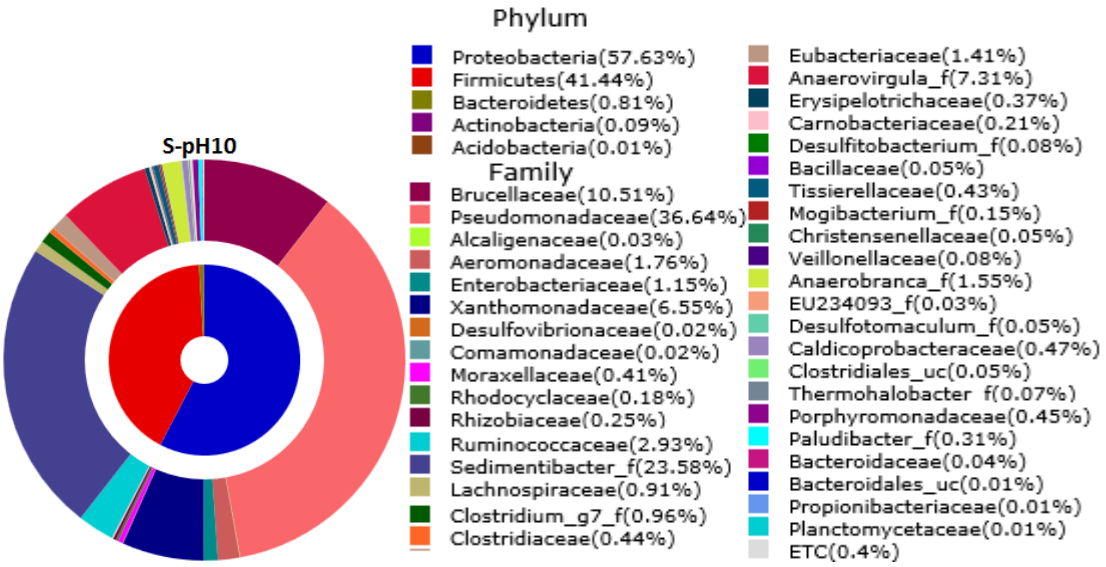
A) Bacterial diversity under Sulphate reduction condition at pH 7.0



B) Bacterial diversity under Sulphate reduction condition at pH 8.0

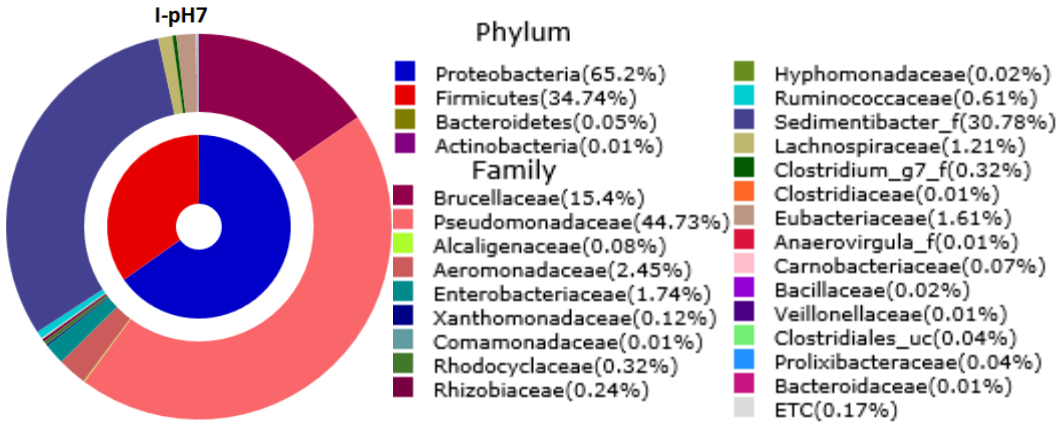


C) Bacterial diversity under Sulphate reduction condition at pH 9.0

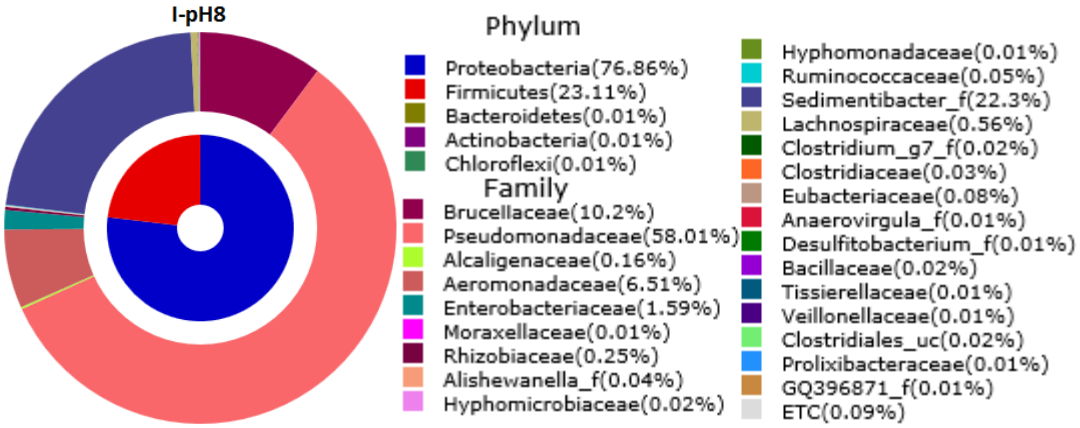


D) Bacterial diversity under Sulphate reduction condition at pH 10.0

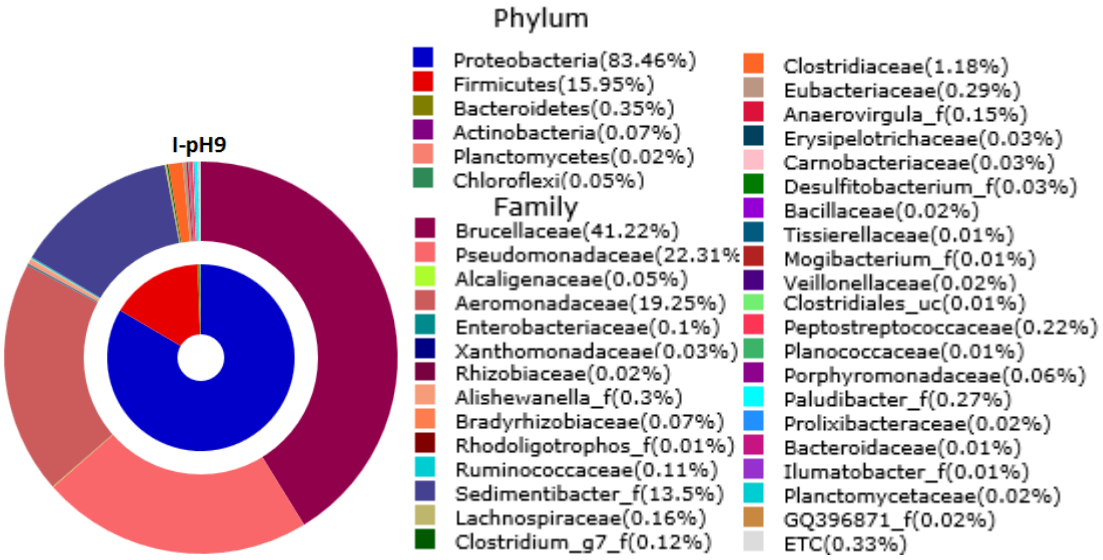
IV- Phylogenetic analysis under Iron (III) reduction process (RX-I (III))



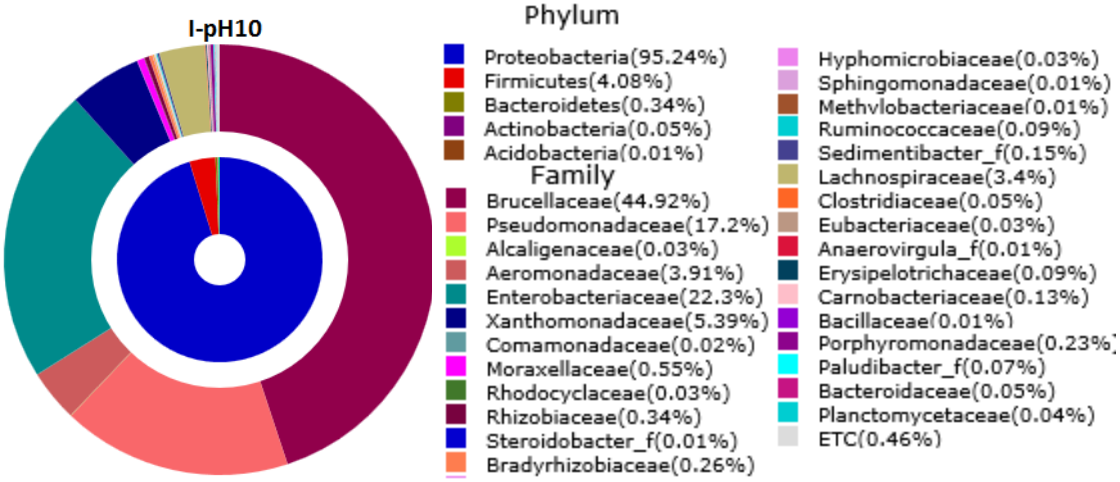
A) Bacterial diversity under Iron (III) reduction condition at pH 7.0



B) Bacterial diversity under Iron (III) reduction condition at pH 8.0



C) Bacterial diversity under Iron (III) reduction condition at pH 9.0



D) Bacterial diversity under Iron (III) reduction condition at pH 10

Table 1: Bacterial diversity of the CDPs driven microcosms under a fermentation and a redox reaction at different pH levels

Microbial Community (%) using 16SrRNA gene sequencing analysis																	
Phylum%	Metabolic processes under anaerobic condition at different pH levels																Direct Crude Soil
	Fermentation				Nitrate reduction				Sulphate reduction				Iron (III) reduction				
	pH7	pH8	pH9	pH10	pH7	pH8	pH9	pH10	pH7	pH8	pH9	pH10	pH7	pH8	pH9	pH10	
Firmicutes	71	87.8	89.2	91.7	1.57	0.51	0.93	4.47	70.4	61.3	52.5	41.4	34.7	23.1	15.9	4.08	15.8
Proteobacteria	27.2	9.47	7.94	3.97	98.3	99.4	99.0	95.5	9.37	2.73	24.3	57.6	65.2	76.8	83.4	95.2	48.2
Bacteroidetes	0.92	2.0	2.67	3.54	0.02	0.03	0.02	0.02	20.2	35.9	23.1	0.81	0.05	0.01	0.34	0.34	8.72
Acetinobacteria	0.29	0.21	0.1	0.39	0.06	0.0	0.03	0.0	0.01	0.01	0.01	0.09	0.01	0.01	0.07	0.05	13.36
Others	0.59	0.52	0.09	0.4	0.04	0.06	0.02	0.01	0.02	0.06	0.09	0.1	0.04	0.08	0.29	0.33	13.92
Family%																	
Ruminococaceae	29.3	36.4	32.7	41.1	0.02	0.01	0.01	0.04	45.3	35.7	9.7	3.0	0.6	0.05	0.11	0.09	0.32
Sedimentibacter	25.3	26.3	20.3	8.3	0.03	0.1	UD	0.02	8.1	6.4	13.7	23.5	30.8	22.3	13.5	0.1	UD
Clostridiaceae	7.3	8.4	3.47	2.6	0.4	0.27	0.02	0.07	1.3	1.24	5.4	0.44	0.01	0.03	1.1	0.05	0.18
Bacillaceae	0.37	0.15	UD	0.65	0.04	0.03	0.78	4.2	0.06	0.13	0.3	0.05	0.02	0.02	0.02	0.01	0.14
Lachnospiraceae	2.6	3.6	17.8	5.3	0.13	0.02	0.04	0.03	2.74	2.85	2.5	0.9	1.2	0.66	0.16	3.4	0.28
Aeromondaceae	24.5	8.7	7.6	3.0	0.07	0.05	0.04	0.01	0.24	0.79	1.7	1.7	2.4	6.5	19.2	4	UD
Brucellaceae	UD	UD	UD	UD	64	33.5	52	43	0.55	0.59	0.03	10.5	15.4	10.2	41.2	45	1.52
Alcaligenaceae	UD	UD	UD	UD	28.3	61.7	45.7	47.6	3.1	0.07	0.01	0.03	0.08	0.16	0.05	0.03	UD
Pseudomonadaceae	0.29	0.01	UD	UD	3.6	1.0	0.85	3.2	0.0	0.27	12.7	36.6	44.7	58	22.3	17.2	3.79
Porphyromonadaceae	UD	UD	UD	UD	0.01	0.02	UD	0.01	19.0	34.8	15.4	0.5	UD	UD	0.06	UD	UD

UD= Undetected

Table 2: The genera and related classified species of the dominate bacterial diversity that identified by name through 16 SrRNA gene sequencing of the CDPs driven microcosms under a fermentation and a redox reaction at different pH levels

Classified genera and related species (%) of the microbial community under the following metabolic activities																	Soil
Genera and related species%	Fermentation				Nitrate reduction				Sulphate reduction				Iron (III) reduction				
	pH7	pH8	pH9	pH10	pH7	pH8	pH9	pH10	pH7	pH8	pH9	pH10	pH7	pH8	pH9	pH10	
Aeromonas sobria	0.01	3.09	0.01	0.01	UD	UD	UD	UD	UD	UD	UD	UD	UD	UD	UD	UD	UD
Clostridium intestinale	0.21	2.31	0.18	0.23	UD	UD	UD	UD	UD	UD	UD	UD	UD	UD	UD	UD	UD
Aeromonas salmonicida	1.69	0.01	0	1.33	UD	UD	UD	UD	UD	UD	UD	UD	UD	UD	UD	UD	UD
Sedimentibacter saalensis	0.23	0.03	1.16	3.03	UD	UD	UD	UD	UD	UD	UD	UD	UD	UD	UD	UD	UD
Clostridium propionicum	0.32	0.15	0.41	0.95	UD	UD	UD	UD	UD	UD	UD	UD	UD	UD	UD	UD	UD
Clostridium sphenoides	0	0.04	0.09	2.31	UD	UD	UD	UD	1.75	1.54	0.25	0.57	UD	UD	UD	UD	UD
Sedimentibacter sp.	1.32	2.97	1.7	0.82	UD	UD	UD	UD	1.12	1.01	1.53	0.57	UD	UD	UD	UD	UD
Porphyromonadaceae sp.	UD	UD	UD	UD	UD	UD	UD	UD	3.16	5.25	4.46	0.19	UD	UD	UD	UD	UD
Ruminococcaceae sp.	1.64	3.06	2.92	3.41	UD	UD	UD	UD	5.84	3.74	2.59	0.41	UD	UD	UD	UD	0.14
Alkalibaculum bacchi	0	4.42	5.12	7.56	UD	UD	UD	UD	3.09	2.81	0.77	1.31	UD	UD	UD	UD	UD
Aeromonas veronii	1.39	3.58	0.81	0.83	UD	UD	UD	UD	0.15	0	0.39	1.01	1.41	4.76	14.7	3.03	UD
Aeromonas hydrophila	19.62	0.12	6.38	0.6	UD	UD	UD	UD	UD	UD	UD	UD	0.35	1.22	3.25	0.60	UD
Sedimentibacter sp.	8.37	7.61	3.42	0.71	UD	UD	UD	UD	1.72	2.03	10.1	1.48	1.12	1.49	0.47	0.01	UD
Propionbacterium acnes	UD	UD	UD	UD	UD	UD	UD	UD	UD	UD	UD	UD	UD	UD	UD	UD	11.23
Achromobacter sp.	UD	UD	UD	UD	0.74	1.48	1.46	1.48	UD	UD	UD	UD	UD	UD	UD	UD	UD
Brodetella termatum	UD	UD	UD	UD	2.21	4.4	0.11	0	UD	UD	UD	UD	UD	UD	UD	UD	UD
Eisenicola composri	UD	UD	UD	UD	4.78	7.59	0.08	0.01	UD	UD	UD	UD	UD	UD	UD	UD	UD
Achromobacter piechaudii	UD	UD	UD	UD	0.06	4.02	2.36	6.45	UD	UD	UD	UD	UD	UD	UD	UD	UD
Alcaligenes aquatilis	UD	UD	UD	UD	0.57	2.32	5.42	3.95	UD	UD	UD	UD	UD	UD	UD	UD	UD
Achromobacter marplatensis	UD	UD	UD	UD	17.71	36.32	32.45	32.91	UD	UD	UD	UD	UD	UD	UD	UD	UD
Pseudomonas tuomuerensis	UD	UD	UD	UD	3.07	0.82	0.74	3.09	0	0.26	0	31.93	42.4	56	20.8	11.03	UD
Brucella melitensis	UD	UD	UD	UD	61.77	32.53	50.18	41.53	0.5	0.57	0.03	10.21	15	10	40	43.34	UD
Proteiniphilum sp.	UD	UD	UD	UD	UD	UD	UD	UD	16.1	29.5	10.9	0.25	UD	UD	UD	UD	UD
Pseudomonas putida	UD	UD	UD	UD	UD	UD	UD	UD	UD	UD	UD	UD	0	0	12.1	2.85	UD
Rahnella bruchi	UD	UD	UD	UD	UD	UD	UD	UD	UD	UD	UD	UD	0.34	1.22	0.05	17.38	UD

UD= undetected bacterial species or less than 2%

Table 3: unclassified species of the dominate bacterial diversity that identified by numbers through 16 SrRNA gene sequencing of the CDPs driven microcosms under a fermentation and a redox reaction at different pH levels

UD= undetected bacterial species or less than 2% between all compared microcosms. F=Family and G=Genus with 93-99.5% similarity

The most prevalent unclassified species (%) that dominated of the microbial community under the following metabolic activities																	
Unclassified species % (93-99.5%similarity)	Fermentation				Nitrate reduction				Sulphate reduction				Iron (III) reduction				Soil
	pH7	pH8	pH9	pH10	pH7	pH8	pH9	pH10	pH7	pH8	pH9	pH10	pH7	pH8	pH9	pH10	
HQ697815 (Ruminococcaceae-F)	3.15	0.06	0	0	UD	UD	UD	UD	UD	UD	UD	UD	UD	UD	UD	UD	UD
FN436134 (Clostridiaceae-F)	4.53	2.36	1.5	0	UD	UD	UD	UD	UD	UD	UD	UD	UD	UD	UD	UD	UD
AJ229234 (Lachnospiraceae-F)	1.16	1.13	4.25	1.14	UD	UD	UD	UD	UD	UD	UD	UD	UD	UD	UD	UD	UD
FN436095 (Lachnospiraceae-F)	0.01	0.23	9.4	0.8	UD	UD	UD	UD	UD	UD	UD	UD	UD	UD	UD	UD	UD
HQ896300 (Sedimentibacter-G)	2.39	7.71	0.06	0	UD	UD	UD	UD	UD	UD	UD	UD	UD	UD	UD	UD	UD
EU266824 (Sedimentibacter-G)	0.08	2.15	6.26	3.35	UD	UD	UD	UD	UD	UD	UD	UD	UD	UD	UD	UD	UD
DQ168650 (Sedimentibacter-G)	9.55	0.86	2.0	0.16	UD	UD	UD	UD	UD	UD	UD	UD	UD	UD	UD	UD	UD
AJ488078 (Ruminococcaceae-F)	3.8	2.49	1.97	0.35	UD	UD	UD	UD	12.7	19.5	2.5	1.26	UD	UD	UD	UD	UD
JX391172 (Ruminococcaceae-F)	9.8	1.96	12.7	2.89	UD	UD	UD	UD	11.5	3.0	0.43	0.25	UD	UD	UD	UD	UD
AB630534 (Ruminococcaceae-F)	8.21	24.47	12	33.16	UD	UD	UD	UD	11.2	3.95	0.56	0.51	UD	UD	UD	UD	UD
EF059533 (Sedimentibacter-G)	2.52	3.6	4.15	0	UD	UD	UD	UD	0.69	2.1	1.52	20.57	28.7	20	12.6	0.11	UD
CP001111 (Stenotrophomonas-G)	UD	UD	UD	UD	UD	UD	UD	UD	0	0	4.48	4.76	UD	UD	UD	UD	UD
AB298756 (Clostridium-G)	UD	UD	UD	UD	UD	UD	UD	UD	1.08	1.35	3.75	0.23	UD	UD	UD	UD	UD

Appendix-4; 16SrRNA gene sequencing for bacterial identification

Table 6: Isolated strains, phylogenetic identification by percentage of compatibility using BLASTN

BI	domain	phylum	class	order	Family	genus	Species	ID %
BI49	Bacteria	Actinobacteria	Actinobacteria	Actinomycetales	Nocardiaceae	Rhodococcus	***Rho. erythropolis***	**99%**
BI38					Microbacteriaceae	Microbacterium	*Mic. Kitamience**	99%
BI39							*Mic. Kitamience**	99%
BI59					Dietziaceae	Dietzia	*Di.natronolimnaea**	99%
BI51							***Di.natronolimnaea****	**99%**
BI45							*Di.natronolimnaea**	89%
BI41					Propionibacteriaceae	Tessaracoccus	***Tes. lubricantis***	**98%**
BI40		Bacteroidetes	Bacteroidia	Bacteroidales	Porphyromonadaceae	Parabacteroides	***Macellibacteroides fermentans****	**99%**
BI69							*Macel. Fermentans**	99%
BI27		Firmicutes	Bacilli	Bacillales	Bacillaceae	Bacillus	*B. cohnii*	99%
BI5							*B. weihenstephanensis*	99%
BI4							*B. mycoides**	99%
BI88							*B. mycoides**	99%
BI21						Exiguobacterium	*E. mexicanum*	99%
BI80			Clostridia	Clostridiales	Lachnospiraceae	Clostridium XIVb	*Cl. propionicum*	99%
BI82						Clostridium XIVa	*Cl. celerecrescens*	99%
BI85					Clostridiaceae	Clostridium sensu stricto	***Cl. Tertium***	**98%**
BI89							*Cl. sartagoforme*	99%
BI86					Peptostreptococcaceae	Clostridium XI	*Terrisporobacter mayombei*	99%
BI35		Proteobacteria	Alphaproteobacteria	Rhizobiales	Rhizobiaceae	Ensifer	*Ensifer adhaerens*	99%
BI36				Caulobacterales	Caulobacteraceae	Brevundimonas	***Bre. diminuta***	**99%**
BI70			Betaproteobacteria	Rhodocyclales	Rhodocyclaceae	Azonexus	***Azonexus hydrophilus***	**98%**
BI15				Burkholderiales	Alcaligenaceae	Alcaligenes	*Alc. aquatilis*	100%
BI18							*Alc. aquatilis*	97%
BI55			Gammaproteobacteria	Aeromonadales	Aeromonadaceae	Aeromonas	*Are. salmonicida*	99%
BI87							*Are.taiwanensis*	99%
BI90							*Are. media*	99%
BI28				Alteromonadales	Alteromonadaceae	Alishewanella	***Alishewanella aestuarii***	**99%**
BI53					Shewanellaceae	Shewanella	*Sh. Putrefaciens**	99%
BI60							*Sh. Putrefaciens**	99%
BI54				Enterobacteriales	Enterobacteriaceae	Citrobacter	*C. gillenii*	100%
BI23				Pseudomonadales	Moraxellaceae	Acinetobacter	*Acin. johnsonii*	99%
BI8							*Acin. guillouiae*	99%
BI58				Xanthomonadales	Xanthomonadaceae	Stenotrophomonas	*Sten. maltophilia*	99%

BI= Bacterial Isolates vial number. * distinguished through slight difference in pigment and texture. Yellow shading, bacteria selected for WGS

Phylogenetic trees

> *Aeromonas salmonicida* ZS-strain (BI 1) 946 100%

AGCGGGAAAGTAGCTTGCTACTTTTGCCGGCGAGCGGCGGACGGGTGAGTAATGCCTGGGGATCTGCCCAGTCGAGGGGGAT
AACAGTTGGAAACGACTGCTAATACCGCATACGCCCTACGGGGGAAAGGAGGGGACCTTCGGGCCTTTCGCGATTGGATGAA
CCCAGGTGGGATTAGCTAGTTGGTGGGGTAATGGCTCACCAAGGCGACGATCCCTAGCTGGTCTGAGAGGATGATCAGCCAC
ACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGGGAAACCCTGATGCAGC
CATGCCGCGTGTGTGAAGAAGGCCTTCGGGTTGTAAAGCACTTTCAGCGAGGAGGAAAGGTTGGCGCCTAATACGTGTCAAC
TGTGACGTTACTCGCAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGTGCAAGCGTTAATCGGA
ATTACTGGGCGTAAAGCGCACGCAGGCGGTTGGATAAGTTAGATGTGAAAGCCCCGGGCTCAACCTGGGAATTGCATTTAAA
ACTGTCCAGCTAGAGTCTTGTAGAGGGGGGTAGAATTCCAGGTGTAGCGGTGAAATGCGTAGAGATCTGGAGGAATACCGGT
GGCGAAGGCGGCCCCCTGGACAAAGACTGACGCTCAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGT
CCACGCCGTAAACGATGTCGATTTGGAGGCTGTGTCCTTGAGACGTGGCTTCCGGAGCTAACGCGTTAAATCGACCGCCTGGG
GAGTACGGCCGCAAGGTTAAAACTCAAATGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGATGCAA
CGCGAAGAACCTTACCTGGCCTTGACATGTCTGGAATCCTGTAAA

> *Aeromonas salmonicida* ZS-strain (BI 3) 785 99%

AAAGTAGCTTGCTACTTTTGCCGGCGAGCGGCGGACGGGTGAGTAATGCCTGGGGATCTGCCCAGTCGAGGGGGATAACAGT
TGGAAACGACTGCTAATACCGCATACGCCCTACGGGGGAAAGGAGGGGACCTTCGGGCCTTTCGCGATTGGATGAACCCAGG
TGGGATTAGCTAGTTGGTGGGGTAATGGCTCACCAAGGCGACGATCCCTAGCTGGTCTGAGAGGATGATCAGCCACACTGGA
ACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGGGAAACCCTGATGCAGCCATGCC
GCGTGTGTGAAGAAGGCCTTCGGGTTGTAAAGCACTTTCAGCGAGGAGGAAAGGTTGGCGCCTAATACGTGTCAACTGTGAC
GTTACTCGCAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGTGCAAGCGTTAATCGGAATTACT
GGGCGTAAAGCGCACGCAGGCGGTTGGATAAGTTAGATGTGAAAGCCCCGGGCTCAACCTGGGAATTGCATTTAAAACTGTC
CAGCTAGAGTCTTGTAGAGGGGGGTAGAATTCCAGGTGTAGCGGTGAAATGCGTAGAGATCTGGAGGAATACCGGTGGCGA
AGGCGGCCCCCTGGACAAAGACTGACGCTCACGTGCGAAAGCGTGGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCAC
GCCGTAAACGATGTCGATTTGGAGGCTGTGTCCTTGAGACGTGGCTTCCG

>*Bacillus mycoide* ZS-strain (BI 4) 924 100%

TCGAGCGAATGGATTAAGAGCTTGCTCTTATGAAGTTAGCGGCGGACGGGTGAGTAACACGTGGGTAACCTACCCATAAGAC
TGGGATAACTCCGGGAAACCGGGGCTAATACCGGATAATATTTTGAACTGCATAGTTCGAAATTGAAAGGCGGCTTCGGCTG
TCACTTATGGATGGACCCGCGTCGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCGACGATGCGTAGCCGACCTGAG
AGGGTGATCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACG
AAAGTCTGACGGAGCAACGCCGCGTGAGTGATGAAGGCTTTCGGGTCGTAAAACTCTGTTGTTAGGGAAGAACAAGTGCTAG
TTGAATAAGCTGGCACCTTGACGGTACCTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTG
GCAAGCGTTATCCGGAATTATTGGGCGTAAAGCGCGCGCAGGTGGTTTCTTAAGTCTGATGTGAAAGCCCACGGCTCAACCGT
GGAGGGTCATTGGAAACTGGGAGACTTGAGTGCAGAAGAGGAAAGTGGAATTCCATGTGTAGCGGTGAAATGCGTAGAGAT
ATGGAGGAACACCAGTGGCGAAGGCGACTTTCTGGTCTGTAACTGACACTGAGGCGCGAAAGCGTGGGGAGCAAACAGGAT
TAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAAGTGTTAGAGGGTTTCCGCCCTTTAGTGCTGAAGTTAACGCAT
TAAGCACTCCGCCTGGGGAGTACGGCCGCAAGGCTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATG
TGGTTTAATTCGAAGCAACGCGAA

>*Clostridium bifermentans* ZS-strain (BI S8) 981 99%

GCTACATCATGCAGTCGAGCGATCTCTTCGGAGAGAGCGGCGGACGGGTGAGTAACGCGTGGGTAACCTGCCCTGTACACAC
GGATAACATACCGAAAGGTATACTAATACGGGATAACATACGAAAGTCGCATGGCTTTTGTATCAAAGCTCCGGCGGTACAG
GATGGACCCGCGTCTGATTAGCTAGTTGGTAAGGTAATGGCTTACCAAGGCAACGATCAGTAGCCGACCTGAGAGGGTGATC
GGCCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGAAAGCCTGA
TGCAGCAACGCCGCGTGAGCGATGAAGGCCTTCGGGTCGTAAAGCTCTGTCCTCAAGGAAGATAATGACGGTACTTGAGGAG
GAAGCCCCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGGGCTAGCGTTATCCGGAATTACTGGGCGTAAAGGGT
GCGTAGGTGGTTTTTTAAGTCAGAAGTGAAAGGCTACGGCTCAACCGTAGTAAGCTTTTGAAACTAGAGAACTTGAGTGCAG
GAGAGGAGAGTAGAATTCCTAGTGTAGCGGTGAAATGCGTAGATATTAGGAGGAATACCAGTAGCGAAGGCGGCTCTCTGG
ACTGTAACTGACACTGAGGCACGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGT
ACTAGGTGTCGGGGGTTACCCCCCTCGGTGCCGCAGCTAACGCATTAAGTACTCCGCCTGGGAAGTACGCTCGCAAGAGTGA
AACTCAAAGGAATTGACGGGGACCCGCACAAGTAGCGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCTAAG
CTTGACATCCCACTGACCTCTCCCTAATCGGAGATTCCCTTCGGGGACAGTGGTGACAGGTGGTGCATGGTTGTCGTCAGC

>*Acinetobacter guillouiae* ZS-strain (BI 8) 916 99%

CAGTCGAGCGGGGGAGATTGCTTCGGTAATTGACCTAGCGGCGGACGGGTGAGTAATACTTAGGAATCTGCCTATTAATGGG
GGACAACATCTCGAAAGGGATGCTAATACCGCATACGCCCTACGGGGGAAAGCAGGGGATCACTTGTGACCTTGCGTTAATA
GATGAGCCTAAGTCGGATTAGCTAGTTGGTGGGGTAAAGGCCTACCAAGGCGACGATCTGTAGCGGGTCTGAGAGGATGATC
CGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGGGGAACCCTGA
TCCAGCCATGCCGCGTGTGTGAAGAAGGCCTTATGGTTGTAAAGCACTTTAAGCGAGGAGGAGGCTCTCTTGGTTAATACCCA
AGATGAGTGGACGTTACTCGCAGAATAAGCACCGGCTAACTCTGTGCCAGCAGCCGCGGTAATACAGAGGGTGCGAGCGTTA
ATCGGATTTACTGGGCGTAAAGCGTGCGTAGGCGGCTTTTTAAGTCGGATGTGAAATCCCCGAGCTTAACTTGGGAATTGCAT
TCGATACTGGGAAGCTAGAGTATGGGAGAGGATGGTAGAATTCCAGGTGTAGCGGTGAAATGCGTAGAGATCTGGAGGAAT
ACCGATGGCGAAGGCAGCCATCTGGCCTAATACTGACGCTGAGGTACGAAAGCATGGGGAGCAAACAGGATTAGATACCCT
GGTAGTCCATGCCGTAAACGATGTCTACTAGCCGTTGGGGCCTTTGAGGCTTTAGTGGCGCAGCTAACGCGATAAGTAGACCG
CCTGGGGAGTACGGTCGCAAGACTAAAACTCAAATGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCG
ATGCAACGCGAAAA

>*Bacillus mycoides* ZS-strain (BI 9) 970 99%

CTGCAGTCGAGCGAATGGATTAAGAGCTTGCTCTTATGAAGTTAGCGGCGGACGGGTGAGTAACACGTGGGTAACCTACCCA
TAAGACTGGGATAACTCCGGGAAACCGGGGCTAATACCGGATAATATTTTGAACTGCATAGTTCGAAATTGAAAGGCGGCTT
CGGCTGTCACTTATGGATGGACCCGCGTCGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCGACGATGCGTAGCCGA
CCTGAGAGGGTGATCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAAT
GGACGAAAGTCTGACGGAGCAACGCCGCGTGAGTGATGAAGGCTTTCGGGTCGTAAAACTCTGTTGTTAGGGAAGAACAAGT
GCTAGTTGAATAAGCTGGCACCTTGACGGTACCTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGT
AGGTGGCAAGCGTTATCCGGAATTATTGGGCGTAAAGCGCGCGCAGGTGGTTTCTTAAGTCTGATGTGAAAGCCCACGGCTC
AACCGTGGAGGGTCATTGGAAACTGGGAGACTTGAGTGCAGAAGAGGAAAGTGGAATTCCATGTGTAGCGGTGAAATGCGT
AGAGATATGGAGGAACACCAGTGGCGAAGGCGACTTTCTGGTCTGTAACTGACACTGAGGCGCGAAAGCGTGGGGAGCAAA
CAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAAGTGTTAGAGGGTTTCCGCCCTTTAGTGCTGAAGTTA
ACGCATTAAGCACTCCGCCTGGGGAGTACGGCCGCAAGGCTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGG
AGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGTCTTGACATCCTCTGAAAACTCTAGAG

> *Alcaligenes aquatilis* ZS-strain (BI 10) 872 100%

AACGGCAGCACGAGAGAGCTTGCTCTCTTGGTGGCGAGTGGCGGACGGGTGAGTAATATATCGGAACGTGCCCAGTAGCGGG
GGATAACTACTCGAAAGAGTGGCTAATACCGCATACGCCCTACGGGGGAAAGGGGGGGATTCTTCGGAACCTCTCACTATTG
GAGCGGCCGATATCGGATTAGCTAGTTGGTGGGGTAAAGGCTCACCAAGGCAACGATCCGTAGCTGGTTTGAGAGGACGACC
AGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATTTTGGACAATGGGGGAAACCCTGA
TCCAGCCATCCCGCGTGTATGATGAAGGCCTTCGGGTTGTAAAGTACTTTTGGCAGAGAAGAAAAGGTATCTCCTAATACGAG
ATACTGCTGACGGTATCTGCAGAATAAGCACCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGTGCAAGCGTTAA
TCGGAATTACTGGGCGTAAAGCGTGTGTAGGCGGTTCGGAAAGAAAGATGTGAAATCCCAGGGCTCAACCTTGGAACTGCAT
TTTTAACTGCCGAGCTAGAGTATGTCAGAGGGGGGTAGAATTCCACGTGTAGCAGTGAAATGCGTAGATATGTGGAGGAATA
CCGATGGCGAAGGCAGCCCCCTGGGATAATACTGACGCTCAGACACGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTG
GTAGTCCACGCCCTAAACGATGTCAACTAGCTGTTGGGGCCGTTAGGCCTTAGTAGCGCAGCTAACGCGTGAAGTTGACCGCC
TGGGGAGTACGGTCGCAAGATTAAAACTCAAAGGAATTGACGGGGACCCGCC

>*Acinetobacter guillouiae* ZS-strain (BI011) 1019 99%

ACCTGCAGTCGAGCGGGGGAGATTGCTTCGGTAATTGACCTAGCGGCGGACGGGTGAGTAATACTTAGGAATCTGCCTATTA
ATGGGGGACAACATCTCGAAAGGGATGCTAATACCGCATACGCCCTACGGGGGAAAGCAGGGGATCACTTGTGACCTTGCGT
TAATAGATGAGCCTAAGTCGGATTAGCTAGTTGGTGGGGTAAAGGCCTACCAAGGCGACGATCTGTAGCGGGTCTGAGAGGA
TGATCCGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGGGGAAC
CCTGATCCAGCCATGCCGCGTGTGTGAAGAAGGCCTTATGGTTGTAAAGCACTTTAAGCGAGGAGGAGGCTCTCTTGGTTAAT
ACCCAAGATGAGTGGACGTTACTCGCAGAATAAGCACCGGCTAACTCTGTGCCAGCAGCCGCGGTAATACAGAGGGTGCGAG
CGTTAATCGGATTTACTGGGCGTAAAGCGTGCGTAGGCGGCTTTTTAAGTCGGATGTGAAATCCCCGAGCTTAACTTGGGAAT
TGCATTCGATACTGGGAAGCTAGAGTATGGGAGAGGATGGTAGAATTCCAGGTGTAGCGGTGAAATGCGTAGAGATCTGGAG
GAATACCGATGGCGAAGGCAGCCATCTGGCCTAATACTGACGCTGAGGTACGAAAGCATGGGGAGCAAACAGGATTAGATA
CCCTGGTAGTCCATGCCGTAAACGATGTCTACTAGCCGTTGGGGCCTTTGAGGCTTTAGTGGCGCAGCTAACGCGATAAGTAG
ACCGCCTGGGGAGTACGGTCGCAAGACTAAAACTCAAATGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAA
TTCGATGCAACGCGAAGAACCTTACCTGGTCTTGACATAGTAAGAACTTTCCAGAGATGGATTGGTGCCTTCGGGAACTTACA
TACAGGTGCTGCATGGCTGTCGTCAGCTCGTGT

>*Aeromonas salmonicida* ZS-strain (BI 12) 576 99%

GACCGCATACGCCCTACGGGGGAAAGGAGGGGACCTTCGGGCCTTTCGCGATTGGATGAACCCAGGTGGGATTAGCTAGTTG
GTGGGGTAATGGCTCACCAAGGCGACGATCCCTAGCTGGTCTGAGAGGATGATCAGCCACACTGGAACTGAGACACGGTCCA
GACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGGGAAACCCTGATGCAGCCATGCCGCGTGTGTGAAGAAGG
CCTTCGGGTTGTAAAGCACTTTCAGCGAGGAGGAAAGGTTGGCGCCTAATACGTGTCAACTGTGACGTTACTCGCAGAAGAA
GCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCACG
CAGGCGGTTGGATAAGTTAGATGTGAAAGCCCCGGGCTCAACCTGGGAATTGCATTTAAAACTGTCCAGCTAGAGTCTTGTA
GAGGGGGGTAGAATTCCAGGTGTAGCGGTGAAATGCGTAGAGATCTGGAGGAATACCGGTGGCGAAGGCGGCCCCCCTGGA
CAAA

>*Clostridium malenominatum* ZS-strain (BI S16) 906 98%

AGAATCCCTTCGGGGACGATTCTAGCGGCGGACGGGTGAGTAACACGTGGGTAACCTGCCTCAAAGAGGGGGATAGCCCTCC
GAAAGGAGGATTAATACCGCATAAAGTTAAGAATTCGCATGAATTCATAACCAAAGGAGAAATCCGCTTTGAGATGGACCCG
CGTCCCATTAGCTAGTTGGTGAGGTAATAGCTCACCAAGGCGACGATGGGTAGCCGACCTGAGAGGGTGATCGGCCACATTG
GAACTGAGATACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCGCAATGGGGGAAACCCTGACGCAGCAACG
CCGCGTGGGTGACGAAGGTCTTCGGATTGTAAAACCCTGTCTTCTGGGACGATAATGACGGTACCAGAGGAGGAAGCCACGG
CTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCGAGCGTTGTCCGGAATTACTGGGCGTAAAGGGTGCGTAGGCGG
ATGTTTAAGTGGGATGTGAAATACCTGAGCTCAACTCGGGTGCTGCATTCCAAACTGGATATCTAGAGTGCAGGAGAGGAGA
ATGGAATTCCTAGTGTAGCGGTGAAATGCGTAGAGATTAGGAAGAACACCAGTGGCGAAGGCGATTCTCTGGACTGTAACTG
ACGCTGAGGCACGAAAGCGTGGGTAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAATACTAGGTGTA
GGGGGGAACCTCCCTCTGTGCCGCAGTTAACACAATAAGTATTCCGCCTGGGGAGTACGATCGCAAGATTAAAACTCAAAGG
AATTGACGGGGGCCCGCACAAGCAGCGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCTGAACTTGACATCC
CCTGC

>*Alcaligenes aquatilis* ZS-strain (BI 16) 608 99%

TTGTGGCTAATACCGCATACGCCCTACGGGGGAAAGGGGGGGATTCTTCGGAACCTCTCACTATTGGAGCGGCCGATATCGG
ATTAGCTAGTTGGTGGGGTAAAGGCTCACCAAGGCAACGATCCGTAGCTGGTTTGAGAGGACGACCAGCCACACTGGGACTG

AGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATTTTGGACAATGGGGGAAACCCTGATCCAGCCATCCCGCGTG
TATGATGAAGGCCTTCGGGTTGTAAAGTACTTTTGGCAGAGAAGAAAAGGTATCTCCTAATACGAGATACTGCTGACGGTATC
TGCAGAATAAGCACCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGTGCAAGCGTTAATCGGAATTACTGGGCGT
AAAGCGTGTGTAGGCGGTTCGGAAAGAAAGATGTGAAATCCCAGGGCTCAACCTTGGAACTGCATTTTTAACTGCCGAGCTA
GAGTATGTCATAGGGGGGTAGAATTCCACGTGTAGCAGTGAAATGCGTAGATATGTGGAGGAATACCGATGGCGAAGGCAG
CCCCCTGGGATAATACTGACGCTCACACACGAAG

>*Clostridium sordillii* ZS-strain (BI S17) 956 99%

CATTTGCAGTCGAGCGACCCTTCGGGGTGAGCGGCGGACGGGTGAGTAACGCGTGGGTAACCTGCCCTGTACACACGGATAA
CATACCGAAAGGTATGCTAATACGGGATAACATATGAGAGTCGCATGGCTTTTGTATCAAAGCTCCGGCGGTACAGGATGGA
CCCGCGTCTGATTAGCTAGTTGGTAAGGTAACGGCTTACCAAGGCAACGATCAGTAGCCGACCTGAGAGGGTGATCGGCCAC
ATTGGAACTGAGACACGGTCCAAACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGAAAGCCTGATGCAGC
AACGCCGCGTGAGCGATGAAGGCCTTCGGGTCGTAAAGCTCTGTCCTCAAGGAAGATAATGACGGTACTTGAGGAGGAAGCC
CCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGGGCTAGCGTTATCCGGAATTACTGGGCGTAAAGGGTGCGTAG
GCGGTCTTTCAAGCCAGAAGTGAAAGGCTACGGCTCAACCGTAGTAAGCTTTTGGAACTGTAGGACTTGAGTGCAGGAGAGG
AGAGTGGAATTCCTAGTGTAGCGGTGAAATGCGTAGATATTAGGAGGAACACCAGTAGCGAAGGCGGCTCTCTGGACTGTAA
CTGACGCTGAGGCACGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTACTAGGT
GTCGGGGGTTACCCCCCTCGGTGCCGCAGCTAACGCATTAAGTACTCCGCCTGGGAAGTACGCTCGCAAGAGTGAAACTCAA
AGGAATTGACGGGGACCCGCACAAGTAGCGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCTAAGCTTGACA
TCCCATTGACCTCTCCCTAATCGGAGATTTCCCTTCGGGGACAGTGGTGACAGGT

>*Acinetobacter johnsonii* ZS-strain (BI 17) 1014 99%

TGCAGTCGAGCGGGGAAGGGTAGCTTGCTACCTGACCTAGCGGCGGACGGGTGAGTAATGCTTAGGAATCTGCCTATTAGTG
GGGGACAACATTCCGAAAGGAATGCTAATACCGCATACGCCCTACGGGGGAAAGCAGGGGATCTTCGGACCTTGCGCTAATA
GATGAGCCTAAGTCAGATTAGCTAGTTGGTGGGGTAAAGGCCTACCAAGGCGACGATCTGTAGCGGGTCTGAGAGGATGATC
CGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGCGAAAGCCTGA
TCCAGCCATGCCGCGTGTGTGAAGAAGGCCTTTTGGTTGTAAAGCACTTTAAGCGAGGAGGAGGCTACCGAGATTAATACTCT
TGGATAGTGGACGTTACTCGCAGAATAAGCACCGGCTAACTCTGTGCCAGCAGCCGCGGTAATACAGAGGGTGCGAGCGTTA
ATCGGATTTACTGGGCGTAAAGCGTGCGTAGGCGGCTTTTTAAGTCGGATGTGAAATCCCTGAGCTTAACTTAGGAATTGCAT
TCGATACTGGGAAGCTAGAGTATGGGAGAGGATGGTAGAATTCCAGGTGTAGCGGTGAAATGCGTAGAGATCTGGAGGAAT
ACCGATGGCGAAGGCAGCCATCTGGCCTAATACTGACGCTGAGGTACGAAAGCATGGGGAGCAAACAGGATTAGATACCCT
GGTAGTCCATGCCGTAAACGATGTCTACTAGCCGTTGGGGCCTTTGAGGCTTTAGTGGCGCAGCTAACGCGATAAGTAGACCG
CCTGGGGAGTACGGTCGCAAGACTAAAACTCAAATGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCG
ATGCAACGCGAAGAACCTTACCTGGTCTTGACATAGTAAGAACTTTCCAGAGATGGATTGGTGCCTTCGGGAACTTACATACA
GGTGCTGCATGGCTGTCGTCAGCTCGTGT

>*Clostridium mangenotii* ZS-strain (BI S20) 947 99%

ATGCAGTCGAGCGACCCCTTCGGGGGAGAGCGGCGGACGGGTGAGTAACGCGTGGGTAACCTACCCTGTACACACGGATAAC
ATACCGAAAGGTTTACTAATACGTGATGACATATCAGACAGGCATCTGTTTGATATCAAAGGTCAGCTGGTACAGGATGGAC
CCGCGTCTGATTAGCTAGTTGGTGAGATAAAAGCTCACCAAGGCGACGATCAGTAGCCGACCTGAGAGGGTGATCGGCCACA
TTGGAACTGAGACACGGTCCAAACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGAAAGCCTGATGCAGCA
ACGCCGCGTGAGTGATGAAGGTTTTCGGATCGTAAAACTCTGTCCTCAAGGAAGATAATGACGGTACTTGAGGAGGAAGCCC
CGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGGGCTAGCGTTATCCGGATTTACTGGGCGTAAAGGGTGCGTAGG
CGGTCTTTCAAGTCAGAAGTTAAATTCTACGGCTCAACCGTAGCCAGCTTTTGAAACTGGAAGACTTGAGTGCAGGAGAGGA
GAGTAGAATTCCTAGTGTAGCGGTGAAATGCGTAGATATTAGGAGGAATACCAGTAGCGAAGGCGGCTCTCTGGACTGTAAC
TGACGCTGAGGCACGAAAGCGTGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTACTAGGT
GTCGGGGGTTACCCCCCTCGGTGCCGCAGCTAACGCATTAAGTACTCCGCCTGGGGAGTACGCTCGCAAGAGTGAAACTCAA
AGGAATTGACGGGGACCCGCACAAGTAGCGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCTAAGCTTGACA
TCCTGGTGACCTCTCCCTAATCGGAAATTTCCCTTCGGGGACACCAG

>*Exiguobacterium mexicanum* ZS-strain (BI 21) 936 99%

GCAGTCGAGCGCAGGAATCGACGGACCCTTCGGGGGGAAGTCGACGGAATGAGCGGCGGACGGGTGAGTAACACGTAAAGA
ACCTGCCCTCAGGTCTGGGATAACCACGAGAAATCGGGGCTAATACCGGATGGGTCATCGGACCGCATGGTCCGAGGATGAA
AGGCGCTTCGGCGTCGCCTGGGGATGGCTTTGCGGTGCATTAGCTAGTTGGTGGGGTAATGGCCCACCAAGGCGACGATGCA
TAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTT
CCACAATGGACGAAAGTCTGATGGAGCAACGCCGCGTGAACGATGAAGGCCTTCGGGTCGTAAAGTTCTGTTGTAAGGGAAG
AACAAGTGCCGCAGGCAATGGCGGCACCTTGACGGTACCTTGCGAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTA
ATACGTAGGTGGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGCGCGCGCAGGCGGCCTCTTAAGTCTGATGTGAAAGCCCC
CGGCTCAACCGGGGAGGGCCATTGGAAACTGGGAGGCTTGAGTATAGGAGAGAAGAGTGGAATTCCACGTGTAGCGGTGAA
ATGCGTAGAGATGTGGAGGAACACCAGTGGCGAAGGCGACTCTTTGGCCTATAACTGACGCTGAGGCGCGAAAGCGTGGGG
AGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAGGTGTTGGAGGGTTTCCGCCCTTCAGTGCTG
AAGCTAACGCATTAAGCACTCCGCCTGGGGAGTACGGTCGCAAGGCTGAAACTCAAAGGAATTGACGGGGACCCGCACAAG
CGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAG

>*Alcaligenes aquatilis ZS-strain* (BI 19) 880 99%

AACGGCAGCACGAGAGAGCTTGCTCTCTTGGTGGCGAGTGGCGGACGGGTGAGTAATATATCGGAACGTGCCCAGTAGCGGG
GGATAACTACTCGAAAGAGTGGCTAATACCGCATACGCCCTACGGGGGAAAGGGGGGGATTCTTCGGAACCTCTCACTATTG
GAGCGGCCGATATCGGATTAGCTAGTTGGTGGGGTAAAGGCTCACCAAGGCAACGATCCGTAGCTGGTTTGAGAGGACGACC
AGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATTTTGGACAATGGGGGAAACCCTGA
TCCAGCCATCCCGCGTGTATGATGAAGGCCTTCGGGTTGTAAAGTACTTTTGGCAGAGAAGAAAAGGTATCTCCTAATACGAG
ATACTGCTGACGGTATCTGCAGAATAAGCACCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGTGCAAGCGTTAA

TCGGAATTACTGGGCGTAAAGCGTGTGTAGGCGGTTCGGAAAGAAAGATGTGAAATCCCAGGGCTCAACCTTGGAACTGCAT
TTTTAACTGCCGAGCTAGAGTATGTCAGAGGGGGGTAGAATTCCACGTGTAGCAGTGAAATGCGTAGATATGTGGAGGAATA
CCGATGGCGAAGGCAGCCCCCTGGGATAATACTGACGCTCAGACACGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTG
GGTAGTCCACGCCCTAAACGATGTCAACTAGCTGTTGGGGGCCGTTAGGCCTTAGTAGCGCAGCTAACGCGTGAAGTTGACC
GCCCTGGGGGAGTACGGTCGCAAGATTAAAACTCAAAGGGAATTGACGGGGGACCCGCACA

>*Aeromonas media* ZS-strain (BI 29) 665 99%

TTAATACCGCATACGCCCTACGGGGGAAAGCAGGGGACCTTCGGGCCTTGCGCGATTGGATATGCCCAGGTGGGATTAGCTT
GTTGGTGAGGTAATGGCTCACCAAGGCGACGATCCCTAGCTGGTCTGAGAGGATGATCAGCCACACTGGAACTGAGACACGG
TCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGGGAAACCCTGATGCAGCCATGCCGCGTGTGTGAAGA
AGGCCTTCGGGTTGTAAAGCACTTTCAGCGAGGAGGAAAGGTTGATACCTAATACGTATCAGCTGTGACGTTACTCGCAGAA
GAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGC
ACGCAGGCGGTTGGATAAGTTAGATGTGAAAGCCCCGGGCTCAACCTGGGAATTGCATTTAAAACTGTCCAGCTAGAGTCTT
GTAGAGGGGGGTAGAATTCCAGGTGTAGCGGTGAAATGCGTAGAGATCTGGAGGAATACCGGTGGCGAAGGCGGCCCCCTG
GACAAAGACTGACGCTCAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGT
CGATTTGGAGG

> *Citrobacter gillenii* ZS-strain (BI 30) 1147 98%

AGGGCCTATTTCTTCTACTGGTGCGTCGAACGGTAGCACAGAGGAGCTTGCTCCTTGGGTGACGAGTGGCGGACGGGTGAGT
AATGTCTGGGAAACTGCCCGATGGAGGGGGATAACTACTGGAAACGGTAGCTAATACCGCATAACGTCGCAAGACCAAAGA
GGGGGACCTTCGGGCCTCTTGCCATCGGATGTGCCCAGATGGGATTAGCTAGTAGGTGGGGTAACGGCTCACCTAGGCGACG
ATCCCTAGCTGGTCTGAGAGGATGACCAGCCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGA
ATATTGCACAATGGGCGCAAGCCTGATGCAGCCATGCCGCGTGTATGAAGAAGGCCTTCGGGTTGTAAAGTACTTTCAGCGA
GGAGGAAGGGGATGTGGTTAATAACCGCATTCATTGACGTTACTCGCAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCG
CGGTAATACGGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCACGCAGGCGGTCTGTCAAGTCGGATGTGAAA
TCCCCGGGCTCAACCTGGGAACTGCATCCGAAACTGGCAGGCTAGAGTCTTGTAGAGGGGGGTAGAATTCCAGGTGTAGCGG
TGAAATGCGTAGAGATCTGGAGGAATACCGGTGGCGAAGGCGGCCCCCTGGACAAAGACTGACGCTCAGGTGCGAAAGCGT
GGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGTCGACTTGGAGGTTGTTCCCTTGAGGAGTGGCT
TCCGGAGCTAACGCGTTAAGTCGACCGCCTGGGGAGTACGGCCGCAAGGTTAAAACTCAAATGAATTGACGGGGGCCCGCAC
AAGCGGTGGAGCATGTGGTTTAATTCGATGCAACGCGAAGAACCTTACCTACTCTTGACATCCACAGAACTTAGCAGAGATG
CTTTAGTGCCTTCGGGACTCTGAGACAGGTGCTGCATGGCTGTCGTCAGCCTCGTGTGTGAAATGTGGGTTAAGTCCCGCCAA
CGAGCGCAACCCATTATCATTTGTGCAGCGGATCGGCCGGACTCAAAGGAAACTGCCAGTGATTAACCTGGAGGAAAGTGGA

>*Aeromonas salmonicida* ZS strain (BI029) 583 99%

TTTTGGATGAACCCAGGTGGGATTAGCTAGTTGGTGGGGTAATGGCTCACCAAGGCGACGATCCCTAGCTGGTCTGAGAGGA
TGATCAGCCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGGGAAAC
CCTGATGCAGCCATGCCGCGTGTGTGAAGAAGGCCTTCGGGTTGTAAAGCACTTTCAGCGAGGAGGAAAGGTTGGCGCCTAA
TACGTGTCAACTGTGACGTTACTCGCAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGTGCAAG
CGTTAATCGGAATTACTGGGCGTAAAGCGCACGCAGGCGGTTGGATAAGTTAGATGTGAAAGCCCCGGGCTCAACCTGGGAA
TTGCATTTAAAACTGTCCAGCTAGAGTCTTGTAGAGGGGGGTAGAATTCCAGGTGTAGCGGTGAAATGCGTAGAGATCTGGA
GGAATACCGGTGGCGAAGGCGGCCCCCCTGGACAAAGACTGACGCTCAGGTGCGAAAGCGTGGGGAGCAACAGGATTAGAT
ACCCTGGTAGT

>*Aeromonas salmonicid* ZS-strain (BI 31) 591 99%

GGACCTTCGGGCCTTTCGCGATTGGATGAACCCAGGTGGGATTAGCTAGTTGGTGGGGTAATGGCTCACCAAGGCGACGATC
CCTAGCTGGTCTGAGAGGATGATCAGCCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATA
TTGCACAATGGGGGAAACCCTGATGCAGCCATGCCGCGTGTGTGAAGAAGGCCTTCGGGTTGTAAAGCACTTTCAGCGAGGA
GGAAAGGTTGGCGCCTAATACGTGTCAACTGTGACGTTACTCGCAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGG
TAATACGGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCACGCAGGCGGTTGGATAAGTTAGATGTGAAAGCC
CCGGGCTCAACCTGGGAATTGCATTTAAAACTGTCCAGCTAGAGTCTTGTAGAGGGGGGTAGAATTCCAGGTGTAGCGGTGA
AAATGCGTAGAGATCTGGAGGAATACCGGTGGCGAAGGCGGCCCCCTGGACAAAGACTGACGCTCAGGTGCGAAAGCGTGG
GGGAGCAAACAGGATAAG

>*Ensifer adhaerens* ZS-strain (BI 35) 855 99%

CGCTGTGCGGTTAGCGCACTACCTTCGGGTAGAACCAACTCCCATGGTGTGACGGGCGGTGTGTACAAGGCCCGGGAACGTA
TTCACCGCAGCATGCTGATCTGCGATTACTAGCGATTCCAACTTCATGCACTCGAGTTGCAGAGTGCAATCCGAACTGAGATG
GCTTTTGGAGATTAGCTCGACCTCGCGGTCTCGCTGCCCACTGTCACCACCATTGTAGCACGTGTGTAGCCCAGCCCGTAAGG
GCCATGAGGACTTGACGTCATCCCCACCTTCCTCTCTCGGCTTATCACCGGCAGTCCCCTTAGAGTGCCCAACTGAATGCTGGCA
ACTAAGGGCGAGGGTTGCGCTCGTTGCGGGACTTAACCCAACATCTCACGACACGAGCTGACGACAGCCATGCAGCACCTGT
CTCCGATCCAGCCGAACTGAAGGATCACATCTCTGTAATCCGCGATCGGGATGTCAAGGGCTGGTAAGGTTCTGCGCGTTGCT
TCGAATTAAACCACATGCTCCACCGCTTGTGCGGGCCCCCGTCAATTCCTTTGAGTTTTAATCTTGCGACCGTACTCCCCAGGC
GGAATGTTTAATGCGTTAGCTGCGCCACCGAACAGTAAACTGCCCGACGGCTAACATTCATCGTTTACGGCGTGGACTACCAG
GGTATCTAATCCTGTTTGCTCCCCACGCTTTCGCACCTCAGCGTCAGTAATGGACCAGTGAGCCGCCTTCGCCACTGGTGTTCC
TCCGAATATCTACGAATTTCACCTCTACACTCGGAATTCCACTCACCTCTTCCATACTCTAGACACCCAGTATCAAAGGCAGTT
CCAGAGTTGAGCTCTGGGATTTCA

>*Staphylococcus saprophyticus* ZS-strain (BI 37) 969 99%

GCAGTCGAGCGAACAGATAAGGAGCTTGCTCCTTTGACGTTAGCGGCGGACGGGTGAGTAACACGTGGGTAACCTACCTATA
AGACTGGGATAACTTCGGGAAACCGGAGCTAATACCGGATAACATTTGGAACCGCATGGTTCTAAAGTGAAAGATGGTTTTG
CTATCACTTATAGATGGACCCGCGCCGTATTAGCTAGTTGGTAAGGTAACGGCTTACCAAGGCAACGATACGTAGCCGACCTG
AGAGGGTGATCGGCCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGG
CGAAAGCCTGACGGAGCAACGCCGCGTGAGTGATGAAGGGTTTCGGCTCGTAAAACTCTGTTATTAGGGAAGAACAAATGTG
TAAGTAACTGTGCACATCTTGACGGTACCTAATCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGT
GGCAAGCGTTATCCGGAATTATTGGGCGTAAAGCGCGCGTAGGCGGTTTCTTAAGTCTGATGTGAAAGCCCACGGCTCAACC
GTGGAGGGTCATTGGAAACTGGGAAACTTGAGTGCAGAAGAGGAAAGTGGAATTCCATGTGTAGCGGTGAAATGCGCAGAG
ATATGGAGGAACACCAGTGGCGAAGGCGACTTTCTGGTCTGTAACTGACGCTGATGTGCGAAAGCGTGGGGATCAAACAGGA
TTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAAGTGTTAGGGGGTTTCCGCCCCTTAGTGCTGCAGCTAACGCA
TTAAGCACTCCGCCTGGGGAGTACGACCGCAAGGTTGAAACTCAAAGGAATTGACGGGGACCCGCACAAGCGGTGGAGCAT
GTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAAATCTTGACATCCTTTGAAAACTCTAGAGATA

>*Microbacterium kitamiense* ZS-strain (BI 38) 891 99%

CTCCACGAAGGGTTGGGCCACCGGCTTCAGGTGTTACCGACTTTCATGACTTGACGGGCGGTGTGTACAAGACCCGGGAACG
TATTCACCGCAGCGTTGCTGATCTGCGATTACTAGCGACTCCGACTTCATGAGGTCGAGTTGCAGACCTCAATCCGAACTGGG
ACCGGCTTTTTGGGATTCGCTCCACCTCGCGGTATTGCAGCCCTTTGTACCGGCCATTGTAGCATGCGTGAAGCCCAAGACAT
AAGGGGCATGATGATTTGACGTCATCCCCACCTTCCTCCGAGTTGACCCCGGCAGTATCCCATGAGTTCCCACCATTACGTGC
TGGCAACATAGAACGAGGGTTGCGCTCGTTGCGGGACTTAACCCAACATCTCACGACACGAGCTGACGACAACCATGCACCA
CCTGTTCACGAGTGTCCAAAGAGTTCTACATTTCTGCAGCGTTCTCGTGTATGTCAAGCCTTGGTAAGGTTCTTCGCGTTGCAT
CGAATTAATCCGCATGCTCCGCCGCTTGTGCGGGTCCCCGTCAATTCCTTTGAGTTTTAGCCTTGCGGCCGTACTCCCCAGGCG
GGGAACTTAATGCGTTAGCTGCGTCACGGAATCCGTGGAATGGACCCCACAACTAGTTCCCAACGTTTACGGGGTGGACTAC
CAGGGTATCTAAGCCTGTTTGCTCCCCACCCTTTCGCTCCTCAGCGTCAGTTACGGCCCAGAGATCTGCCTTCGCCATCGGTGT
TCCTCCTGATATCTGCGCATTCCACCGCTACACCAGGAATTCCAATCTCCCCTACCGCACTCTAGTCTGCCCGTACCCACTGCA
GGCTGAGGGTTGAGCCCCCAGATTTCACAGCAGACGCGACAAACCGCCTACGAGCTCTTT

>*Dietzia natronolimnaea* ZS-strain (BI 45) 991 89%

ACCCCCTCCTCCTCCCTAGTTCTGGTTCTCATATAGTTTGGGAAGGGCCTAACCATGGTTTTTGGGGGTGCTTCCCCGTTGGGT
GGGCCGCCCCCCCTCTCGTGATGGTGTTCGGATTTTTGCTCTAGGCTGTTGTTGTTCTTTTGTTTGTTGGCTTTTTGCTGGATTG
CGAGGGCGATTTTTGTGGTAGTTTTGCCAGATGTGAACCCGGTGGGGTAATGGCCTACCAAGGTGACGACGGGTAACGGCCT
GAGAGGGTGATCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGG
GCGAAAGCCTGATGCAGCGACGCCGCGTGGGGGATGACGGTCTTCGATTGTAACCCTTTCATAGGGACGAAGCGAAATGACG
GTACCTGCAGAAGAACACCGGCCAACTACGTGCCGCAGCCGCGGTAATACGTAGGGTGCAGCGTTGTCCGGAATTACTGGGC
GTAAAGAGCTCGTAGGCGGTTTGTCACGTCTCTGTGAATCCTCCAGCTCACTGGGGCGTGCAGCGATACGGGCAACTGATACT
ACAGGGAGACTGGATTCCTGGGAGCGTGAATGCGCAGATATCAGGAGAACCCGGTGCGAAGCGGTCTCTGGTAGTACTGAC
CTGAGAGCGAAGCATGGGAGCAACAGGATAGAACCTGGAGTCATGCCGTAACGTGGCGCAGTGTGGGTCCTCCCGGATCCTG
CGTACTACGCTTAACGCCCGCTGGGATACGGCGCAGGCAACTCAAGAATGACGGGGCCCACAACGCTGACATGTGATAATCG
AGCACCGAAACCTACTAGCTGACTAACAGACACGCAAATGCGTTCCTTCGCTGAACAGGGGGAGGTGCTCACTCGGCTGAAA
GTGGTAACCCCACACCCACCTGTTAGTGCAAATTATGTGGACTGAAACGCGGTCATCGAGAGGGGAACTCACCATGCCTAGT
AGG

>*Enterococcus gallinarum* ZS-strain (BI 46) 968 99%

ACATGCAGTCGAACGCTTTTTCTTTCACCGGAGCTTGCTCCACCGAAAGAAAAAGAGTGGCGAACGGGTGAGTAACACGTGG
GTAACCTGCCCATCAGAAGGGGATAACACTTGGAAACAGGTGCTAATACCGTATAACACTATTTTCCGCATGGAAGAAAGTT
GAAAGGCGCTTTTGCGTCACTGATGGATGGACCCGCGGTGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCAACGAT
GCATAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAA
TCTTCGGCAATGGACGAAAGTCTGACCGAGCAACGCCGCGTGAGTGAAGAAGGTTTTCGGATCGTAAAACTCTGTTGTTAGA
GAAGAACAAGGATGAGAGTAGAACGTTCATCCCTTGACGGTATCTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCG
CGGTAATACGTAGGTGGCAAGCGTTGTCCGGATTTATTGGGCGTAAAGCGAGCGCAGGCGGTTTCTTAAGTCTGATGTGAAA
GCCCCCGGCTCAACCGGGGAGGGTCATTGGAAACTGGGAGACTTGAGTGCAGAAGAGGAGAGTGGAATTCCATGTGTAGCG
GTGAAATGCGTAGATATATGGAGGAACACCAGTGGCGAAGGCGGCTCTCTGGTCTGTAACTGACGCTGAGGCTCGAAAGCGT
GGGGAGCGAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAAGTGTTGGAGGGTTTCCGCCCTTCAG
TGCTGCAGCAAACGCATTAAGCACTCCGCCTGGGGAGTACGACCGCAAGGTTGAAACTCAAAGGAATTGACGGGGGCCCGCA
CAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGTCTTGACTTCCTTTG

>*Pseudomonas hibiscicola* ZS-strain (BI 58) 881 99%

AACGAGCAGCACAGAGGAGCTTGCTCCTTGGGTGGCGAGTGGCGGACGGGTGAGGAATACATCGGAATCTACTTTTTCGTGG
GGGATAACGTAGGGAAACTTACGCTAATACCGCATACGACCTACGGGTGAAAGCAGGGGATCTTCGGACCTTGCGCGATTGA
ATGAGCCGATGTCGGATTAGCTAGTTGGCGGGGTAAAGGCCCACCAAGGCGACGATCCGTAGCTGGTCTGAGAGGATGATCA
GCCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGCGCAAGCCTGAT
CCAGCCATACCGCGTGGGTGAAGAAGGCCTTCGGGTTGTAAAGCCCTTTTGTTGGGAAAGAAATCCAGCCGGCTAATACCTG
GTTGGGATGACGGTACCCAAAGAATAAGCACCGGCTAACTTCGTGCCAGCAGCCGCGGTAATACGAAGGGTGCAAGCGTTAC
TCGGAATTACTGGGCGTAAAGCGTGCGTAGGTGGTCGTTTAAGTCCGTTGTGAAAGCCCTGGGCTCAACCTGGGAACTGCAGT
GGATACTGGACGACTAGAGTGTGGTAGAGGGTAGCGGAATTCCTGGTGTAGCAGTGAAATGCGTAGAGATCAGGAGGAACA
TCCATGGCGAAGGCAGCTACCTGGACCAACACTGACACTGAGGCACGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTG
GTAGTCCACGCCCTAAACGATGCGAACTGGATGTTGGGTGCAATTTGGCACGCAGTATCGAAGCTAACGCGTTAAGTTCGCC
GCCTGGGGAGTACGGTCGCAAGACTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGT

> *Dietzia natronolimnaea* ZS-strain (BI 59) 931 99%

CTTACACATGCAAGTCGAACGGTAAGGCCCTTTCGGGGGTACACGAGTGGCGAACGGGTGAGTAACACGTGGGTAATCTGCC
CTGCACTTCGGGATAAGCCTGGGAAACCGGGTCTAATACCGGATATGAGCTCCTGCCGCATGGTGGGGGTTGGAAAGTTTTTC
GGTGCAGGATGAGTCCGCGGCCTATCAGCTTGTTGGTGGGGTAATGGCCTACCAAGGCGACGACGGGTAGCCGGCCTGAGAG

GGTGATCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGAA
AGCCTGATGCAGCGACGCCGCGTGGGGGATGACGGTCTTCGGATTGTAAACTCCTTTCAGTAGGGACGAAGCGAAAGTGACG
GTACCTGCAGAAGAAGCACCGGCCAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGTGCAAGCGTTGTCCGGAATTACTG
GGCGTAAAGAGCTCGTAGGCGGTTTGTCACGTCGTCTGTGAAATCCTCCAGCTCAACTGGGGGCGTGCAGGCGATACGGGCA
GACTTGAGTACTACAGGGGAGACTGGAATTCCTGGTGTAGCGGTGAAATGCGCAGATATCAGGAGGAACACCGGTGGCGAA
GGCGGGTCTCTGGGTAGTAACTGACGCTGAGGAGCGAAAGCATGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCATGCC
GTAAACGGTGGGCGCTAGGTGTGGGGTCCTTCCACGGATTCCGTGCCGTAGCTAACGCATTAAGCGCCCCGCCTGGGGAGTA
CGGCCGCAAGGCTAAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGCGGAGCATGTGGATTAATTCGATGCAACGCG
AAGAACCTTACCTAGGCTTGACATATACAGG

>*Aeromonas salmonicida* ZS-strain (BI 66) 888 99%

ACCTACTTTTGCCGGCGAGCGGCGCAACGGGTGAGTAATGCCTGGGGATCTGCCCAGTCGAGGGGGATAACAGTTGGAAACG
ACTGCTAATACCGCATACGCCCTACGGGGGAAAGGAGGGGACCTTCGGGCCTTTCGCGATTGGATGAACCCAGGTGGGATTA
GCTAGTTGGTGGGGTAATGGCTCACCAAGGCGACGATCCCTAGCTGGTCTGAGAGGATGATCAGCCACACTGGAACTGAGAC
ACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGGGAAACCCTGATGCAGCCATGCCGCGTGTGTG
AAGAAGGCCTTCGGGTTGTAAAGCACTTTCAGCGAGGAGGAAAGGTTGGCGCCTAATACGTGTCAACTGTGACGTTACTCGC
AGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAA
AGCGCACGCAGGCGGTTGGATAAGTTAGATGTGAAAGCCCCGGGCTCAACCTGGGAATTGCATTTAAAACTGTCCAGCTAGA
GTCTTGTAGAGGGGGGTAGAATTCCAGGTGTAGCGGTGAAATGCGTAGAGATCTGGAGGAATACCGGTGGCGAAGGCGGCC
CCCTGGACAAAGACTGACGCTCAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAAC
GATGTCGATTTGGAGGCTGTGTCCTTGAGACGTGGCTTCCGGAGCTAACGCGTTAAATCGACCGCCTGGGGAGTACGGCCGC
AAGGTTAAAACTCAAATGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGATGCAAC

>*Clostridium propionicum* ZS-strain (BI 80) 1904 99% (8f & 1510r)

AGGGGGGAAAAAAATGACGGTACCTGAATAAGAAGCCCCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGGGCA
AGCGTTATCCGGAATTACTGGGTGTAAAGGGAGAGTAGGCGGCATGGTAAGTTAGATGTGAAAGCCCGAGGCTTAACCTCGG
GATTGCATTTAAAACTATCAAGCTAGAGTACAGGAGAGGTAAGTGGAATTCCTAGTGTAGCGGTGAAATGCGTAGATATTAG
GAAGAACACCAGTGGCGAAGGCGACTTACTGGACTGAAACTGACGCTGAGGCTCGAAAGCGTGGGGAGCGAACAGGATTAG
ATACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAGGTGTCGGGGGGGAACCCTCGGTGCCGCAGCTAACGCAATAAGCA
CTCCACCTGGGGAGTACGATCGCAAGATTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTA
ATTCGAAGCAACGCGAAGAACCTTACCAAGGCTTGACATCCCTCTGACCGGTGTAGAGATACACCTTCTCTTCGGAGCAGAG
GTGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCCTATTCTTA
GTAGCCATCATTCAGTTGGGCACTCTAGGGAGACTGCCGTGGATAACACGGAGGAAGGTGGGGATGACGTCAAATCATCATG
CCCCTTATGTCTTGGGCTACACACGTGCTACAATGGCTGGTAACAAAGTGACGCAAAACGGCGACGTCGAGCAAATCACAAA
AACCCAGTCCCAGTTCGGATTGTAGTCTGCAACTCGACTACATGAAGCTGGAATCGCTAGTAATCGCGAATCAGAATGTCGCG
GTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCATGGGAGTTGGAAGCACCCGAAGTCGGTGACCTGACCTTAC
ACCGGTCAGAGGGATGTCAAGCCTTGGTAAGGTTTTTCGCGTTGCTTCGAATTAAACCACATGCTCCACCGCTTGTGCGGGCC
CCCGTCAATTCCTTTGAGTTTCAATCTTGCGATCGTACTCCCCAGGTGGAGTGCTTATTGCGTTAGCTGCGGCACCGAGGGTTC
CCCCCCGACACCTAGCACTCATCGTTTACGGCGTGGACTACCAGGGTATCTAATCCTGTTCGCTCCCCACGCTTTCGAGCCTCA
GCGTCAGTTTCAGTCCAGTAAGTCGCCTTCGCCACTGGTGTTCTTCCTAATATCTACGCATTTCACCGCTACACTAGGAATTCC
ACTTACCTCTCCTGTACTCTAGCTTGATAGTTTTAAATGCAATCCCGAGGTTAAGCCTCGGGCTTTCACATCTAACTTACCATG
CCGCCTACTCTCCCTTTACACCCAGTAATTCCGGATAACGCTTGCCCCCTACGTATTACCGCGGCTGCTGGCACGTAGTTAGCC
GGGGCTTCTTATTCAGGTACCGTCATTTTTTTCGTCCCTGTTGATAGAAGTTTACGAGCCGAAACCCTTCTTCCTTCACGCGGC
GTTGCTGCATCAGGCTTTCGCCCATTGTGCAATATTCCCCACTGCTGCCTCCCGTAGGAGTTTGGGCCGTGTCTCAGTCCCAAT
GTGGCCGATCACCCTCTCAGGTCGGCTACTGATCGTTGCCTTGGTGGGCTGTTATCTCACCAACTAGCTAATCAGATGCGGGC
CCATCCTGTACCGAATAAATCCTTTTCTTGCAAGAGGATGCCCTCTCGCAATGACATGCGGTATTAGTCACCGTTTCCAGTGAT
TATTCCACAGTACAGGGCAGGTTGCCCACACGTTACTCACCCGTCCGCCGCTAAAATATGTAATCTTCCTGCCGAATCTGC

>*Clostridium tertium* ZS strain (BI83) 897 98%

ACGGGTGAGTAACACGTGGGCAACCTGCCTTGTAGAGGGGAATAGCCTTCCGAAAGGAAGATTAATACCGCATAACATTGCT
TTATCGCATGATAAAGTAATCAAAGGAGCAATCCGCTACAAGATGGGCCCGCGGCGCATTAGCTAGTTGGTGAGGTAACGGC
TCACCAAGGCGACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCACATTGGGACTGAGACACGGCCCAAACTCCTACGGGA
GGCAGCAGTGGGGAATATTGCACAATGGGGGAAACCCTGATGCAGCAACGCCGCGTGAATGATGAAGGTCTTCGGATCGTAA
AGTTCTGTCTTCAGGGACGATAATGACGGTACCTGAGGAGGAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGT
AGGTGGCAAGCGTTGTCCGGATTTACTGGGCGTAAAGGGAGCGTAGGCGGATTTTTAAGTGAGATGTGAAATACTCAGGCTC
AACCTGGGGGCTGCATTTCAAACTGGAAGTCTAGAGTGCAGGAGAGGAGAGTGGAATTCCTAGTGTAGCGGTGAAATGCGTA
GAGATTAGGAAGAACACCAGTGGCGAAGGCGACTCTCTGGACTGTAACTGACGCTGAGGCTCGAAAGCGTGGGGAGCAAAC
AGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAATACTAGGTGTAGGGGTTGTCATGACCTCTGTGCCGCCGCTAAC
GCATTAAGTATTCCGCCTGGGGAGTACGGTCGCAAGATTAAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCAGCGGAG
CATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCTAGACTTGACATCTCCTGCATTACTCTTAATCGAGGAAGT

>*Aeromonas sp* ZS-strain (BI 87)

TTGATACTTTTGCCGGCGAGCGGCGCAACGGGTGAGTAATGCCTGGGAAATTGCCCAGTCGAGGGGGATAACAGTTGGAAAC
GACTGCTAATACCGCATACGCCCTACGGGGGAAAGCAGGGGACCTTCGGGCCTTGCGCGATTGGATATGCCCAGGTGGGATT
AGCTTGTTGGTGAGGTAATGGCTCACCAAGGCGACGATCCCTAGCTGGTCTGAGAGGATGATCAGCCACACTGGAACTGAGA
CACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGGGAAACCCTGATGCAGCCATGCCGCGTGTGT
GAAGAAGGCCTTCGGGTTGTAAAGCACTTTCAGCGAGGAGGAAAGGTTGGTAGCTAATAACTGCCAACTGTGACGTTACTCG
CAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAA
AGCGCACGCAGGCGGTTGGATAAGTTAGATGTGAAAGCCCCGGGCTCAACCTGGGAATTGCATTTAAAACTGTCCAGCTAGA
GTCTTGTAGAGGGGGGTAGAATTCCAGGTGTAGCGGTGAAATGCGTAGAGATCTGGAGGAATACCGGTGGCGAAGGCGGCC
CCCTGGACAAAGACTGACGCTCAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAAC
GATGTCGATTTGGAGGCTGTGTCCTTGAGACGTGGCTTCCGGAGCTAACGCGTTAAATCGACCGCCTGGGGAGTACGGCCGC
AAGGTTAAAACTCAAATGAATTGACGGGGGCCCGCACAAGCGGTGGAACAT

>*Bacillus mycoide* ZS-strain (BI 88) 874 99%

TCTTATGAAGTTAGCGGCGGACGGAGTGAGTAACACGTGGGTAACCTACCCATAAGACTGGGATAACTCCGGGAAACCGGGG
CTAATACCGGATAATATTTTGAACTGCATAGTTCGAAATTGAAAGGCGGCTTCGGCTGTCACTTATGGATGGACCCGCGTCGC
ATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCAACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTG
AGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCAACGCCGCGT
GAGTGATGAAGGCTTTCGGGTCGTAAAACTCTGTTGTTAGGGAAGAACAAGTGCTAGTTGAATAAGCTGGCACCTTGACGGT
ACCTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTATCCGGAATTATTGGG
CGTAAAGCGCGCGCAGGTGGTTTCTTAAGTCTGATGTGAAAGCCCACGGCTCAACCGTGGAGGGTCATTGGAAACTGGGAGA
CTTGAGTGCAGAAGAGGAAAGTGGAATTCCATGTGTAGCGGTGAAATGCGTAGAGATATGGAGGAACACCAGTGGCGAAGG
CGACTTTCTGGTCTGTAACTGACACTGAGGCGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGT
AAACGATGAGTGCTAAGTGTTAGAGGGTTTCCGCCCTTTAGTGCTGAAGTTAACGCATTAAGCACTCCGCCTGGGGAGTACGG
CCGCAAGGCTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAACA

>*Aeromonas media* ZS-strain (BI 90) 855 99%ZS

TTTGATACTTTTGCCGGCGAGCGGCGCAACGGGTGAGTAATGCCTGGGAAATTGCCCAGTCGAGGGGGATAACAGTTGGAAA
CGACTGCTAATACCGCATACGCCCTACGGGGGAAAGCAGGGGACCTTCGGGCCTTGCGCGATTGGATATGCCCAGGTGGGAT
TAGCTTGTTGGTGAGGTAATGGCTCACCAAGGCGACGATCCCTAGCTGGTCTGAGAGGATGATCAGCCACACTGGAACTGAG
ACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGGGAAACCCTGATGCAGCCATGCCGCGTGTG
TGAAGAAGGCCTTCGGGTTGTAAAGCACTTTCAGCGAGGAGGAAAGGTTGATACCTAATACGTATCAGCTGTGACGTTACTC
GCAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTA
AAGCGCACGCAGGCGGTTGGATAAGTTAGATGTGAAAGCCCCGGGCTCAACCTGGGAATTGCATTTAAAACTGTCCAGCTAG
AGTCTTGTAGAGGGGGGTAGAATTCCAGGTGTAGCGGTGAAATGCGTAGAGATCTGGAGGAATACCGGTGGCGAAGGCGGC
CCCCTGGACAAAGACTGACGCTCAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAAC
GATGTCGATTTGGAGGCTGTGTCCTTGAGACGTGGCTTCCGGAGCTAACGCGTTAAATCGACCGCCTGGGGAGTACGGCCGC
AAGGTTAAAACTCAAATGAATTGACGGGGGGCCGCA

>*Alcaligenes aquatilis* ZS-strain (BI 99) 929 99%

CACTCTTGGTGGCGAGTGGTGGACAGGGTGAGTAATATATCGGAACGTGCCCAGTAGCGGGGGATAACTACTCGAAAGAGTG
GCTAATACCGCATACGCCCTACGGGGGAAAGGGGGGGATTCTTCGGAACCTCTCACTATTGGAGCGGCCGATATCGGATTAG
CTAGTTGGTGGGGTAAAGGCTCACCAAGGCAACGATCCGTAGCTGGTTTGAGAGGACGACCAGCCACACTGGGACTGAGACA
CGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATTTTGGACAATGGGGGAAACCCTGATCCAGCCATCCCGCGTGTATGA
TGAAGGCCTTCGGGTTGTAAAGTACTTTTGGCAGAGAAGAAAAGGTATCTCCTAATACGAGATACTGCTGACGGTATCTGCA
GAATAAGCACCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAG
CGTGTGTAGGCGGTTCGGAAAGAAAGATGTGAAATCCCAGGGCTCAACCTTGGAACTGCATTTTTAACTGCCGAGCTAGAGT
ATGTCAGAGGGGGGTAGAATTCCACGTGTAGCAGTGAAATGCGTAGATATGTGGAGGAATACCGATGGCGAAGGCAGCCCC
CTGGGATAATACTGACGCTCAGACACGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCCTAAACGAT
GTCAACTAGCTGTTGGGGCCGTTAGGCCTTAGTAGCGCAGCTAACGCGTGAAGTTGACCGCCTGGGGAGTACGGTCGCAAGA
TTAAAACTCAAAGGAATTGACGGGGACCCGCACAAGCGGTGGATGATGTGGATTAATTCGATGCAACGCGAAAAACCTTACC
TACCCTTGACATGTCTGGAATGCCAAAG

>*Clostridium propionicum* ZS strain (BI 80)

AGGGGGGAAAAAAATGACGGTACCTGAATAAGAAGCCCCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGGGCA
AGCGTTATCCGGAATTACTGGGTGTAAAGGGAGAGTAGGCGGCATGGTAAGTTAGATGTGAAAGCCCGAGGCTTAACCTCGG
GATTGCATTTAAAACTATCAAGCTAGAGTACAGGAGAGGTAAGTGGAATTCCTAGTGTAGCGGTGAAATGCGTAGATATTAG
GAAGAACACCAGTGGCGAAGGCGACTTACTGGACTGAAACTGACGCTGAGGCTCGAAAGCGTGGGGAGCGAACAGGATTAG
ATACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAGGTGTCGGGGGGGAACCCTCGGTGCCGCAGCTAACGCAATAAGCA
CTCCACCTGGGGAGTACGATCGCAAGATTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTA
ATTCGAAGCAACGCGAAGAACCTTACCAAGGCTTGACATCCCTCTGACCGGTGTAGAGATACACCTTCTCTTCGGAGCAGAG
GTGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCCTATTCTTA
GTAGCCATCATTCAGTTGGGCACTCTAGGGAGACTGCCGTGGATAACACGGAGGAAGGTGGGGATGACGTCAAATCATCATG
CCCCTTATGTCTTGGGCTACACACGTGCTACAATGGCTGGTAACAAAGTGACGCAAAACGGCGACGTCGAGCAAATCACAAA
AACCCAGTCCCAGTTCGGATTGTAGTCTGCAACTCGACTACATGAAGCTGGAATCGCTAGTAATCGCGAATCAGAATGTCGCG
GTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCATGGGAGTTGGAAGCACCCGAAGTCGGTGACCTGACC

>*Terrisporobacter petrolearius* ZS-strain (BI86)

AAAGAGCGAGCGGACGGGTGAGTAACGCGTGAGGTAACCTGCCTCATACACATGGATAACATACCGAAAGGTATGCTAATA
CAGGATAATATAAGAGATTCACATGGATTTTTTATCAAAGCTCCGGCGGTATGAGATGGACCCGCGTCTGATTAGCTAGTTGG
TAAGGTAATGGCTTACCAAGGCGACGATCAGTAGCCGACCTGAGAGGGTGATCGGCCACATTGGAACTGAGACACGGTCCAA
ACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGAAAGCCTGATGCAGCAACGCCGCGTGAGTGATGAAGGC
CTTCGGGTCGTAAAACTCTGTCCTCAAGGAAGATAATGACGGTACTTGAGGAGGAAGCCCCGGCTAACTACGTGCCAGCAGC
CGCGGTAATACGTAGGGGGCTAGCGTTATCCGGATTTACTGGGCGTAAAGGGTGCGTAGGTGGTTTTTTAAGTCAGGAGTGA
AAGGCTACGGCTCAACCGTAGTAAGCTCTTGAAACTGGAAAACTTGAGTGCAGGAGAGGAAAGTGGAATTCCTAGTGTAGCG
GTGAAATGCGTAGATATTAGGAGGAACACCAGTAGCGAAGGCGGCTTTCTGGACTGTAACTGACACTGAGGCACGAAAGCGT
GGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTACTAGGTGTCGGGGGTTACCCCCCTCGGTG
CCGCAGCTAACGCATTAAGTACTCCGCCTGGGGAGTACGCTCGCAAGAGTGAAACTCAAAGGAATTGACGGGGACCCGCACA
AGTAGCGGAGCATGTGGTTTAATTCGAAGCAACGCGAAG

>*Romboutsia sedimentorum* ZS strain (BI93)

GCGGAGGGGTGAGTAACGCGTGAGGTAACCTGCCCTGTACACACGGATAACATACCGAAAGGTATGCTAATACGGGATAATG
TACTTTTGTCGCATGGCAAAAGTATCAAAGCTCCGGCGGTACAGGATGGACCCGCGTCTGATTAGCTAGTTGGAGAGGTAAT
GGCTCACCAAGGCGACGATCAGTAGCCGACCTGAGAGGGTGATCGGCCACATTGGAACTGAGACACGGTCCAAACTCCTACG
GGAGGCAGCAGTGGGGAATATTGCACAATGGGCGAAAGCCTGATGCAGCAACGCCGCGTGAGCGATGAAGGCCTTCGGGTC
GTAAAGCTCTGTCCTCAAGGAAGATAATGACGGTACTTGAGGAGGAAGCCCCGGCTAACTACGTGCCAGCAGCCGCGGTAAT
ACGTAGGGGGCTAGCGTTATCCGGAATTACTGGGCGTAAAGGGTGCGTAGGTGGTTTCTTAAGTCAGAGGTGAAAGGCTACG
GCTCAACCGTAGTAAGCCTTTGAAACTGAGAAACTTGAGTGCAGGAGAGGAGAGTAGAATTCCTAGTGTAGCGGTGAAATGC
GTAGATATTAGGAGGAATACCAGTTGCGAAGGCGGCTCTCTGGACTGTAACTGACACTGAGGCACGAAAGCGTGGGGAGCA

AACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTACTAGGTGTCGGGGGTTACCCCCCTCGGTGCCGCAGCT
AACGCATTAAGTACTCCGCCTGGGAAGTACGCTCGCAAGAGTGAAACTCAAAGGAATTGACGGGGACCCGCACAAGTAGCG
GAACATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCTAAGCTTGACATACTTATGACCGATACCTAATAGTATTTTTC
CCTTCGGGGACATGA

>*Clostridium tertium* ZS strain (BI85)

GGGGAAGAACCTAGCGGCGGACGGGTGAGTAACACGTGGGCAACCTGCCTTGTAGAGGGGAATAGCCTTCCGAAAGGAAGA
TTAATACCGCATAACATTACTTTATCGCATGATGAAGTAATCAAAGGAGCAATCCGCTACAAGATGGGCCCGCGGCGCATTA
GCTAGTTGGTGAGGTAACGGCTCACCAAGGCGACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCACATTGGGACTGAGAC
ACGGCCCAAACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGGGAAACCCTGATGCAGCAACGCCGCGTGAAT
GATGAAGGTCTTCGGATCGTAAAGTTCTGTCTTCAGGGACGATAATGACGGTACCTGAGGAGGAAGCCACGGCTAACTACGT
GCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGATTTACTGGGCGTAAAGGGAGCGTAGGCGGATTTTTAAGT
GAGATGTGAAATACTCAGGCTCAACCTGGGGGCTGCATTTCAAACTGGAAGTCTAGAGTGCAGGAGAGGAGAGTGGAATTCC
TAGTGTAGCGGTGAAATGCGTAGAGATTAGGAAGAACACCAGTGGCGAAGGCGACTCTCTGGACTGTAACTGACGCTGAGGC
TCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAATACTAGGTGTAGGGGTTGTCA
TGACCTCTGTGCCGCCGCTAACGCATTAAGTATTCCGCCTGGGGAGTACGGTCGCAAGATTAAAACTCAAAGGAATTGACGG
GGGCCCGCACAAGCAGCGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCTAGACTTGACATCTCCTGCATTA
CTC

>*Clostridium sartagoforme* ZS-strain (BI89)

GGGAAGAACCTAGCGGCGGACGGGTGAGTAACACGTGGGCAACCTGCCTTATAGAGGGGAATAGCCTTCCGAAAGGAAGAT
AATACCGCATAAGATTACATCTTCGCATGAAGAAGTAATTAAAGGAGCAATCCGCTATAAGATGGGCCCGCGGCGCATTAGC
TAGTTGGTGAGGTAACGGCTCACCAAGGCGACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCACATTGGGACTGAGACAC
GGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGGGAAACCCTGATGCAGCAACGCCGCGTGAGTGA
TGAAGGTCTTCGGATCGTAAAGCTCTGTCTTCAGGGACGATAATGACGGTACCTGAGGAGGAAGCCACGGCTAACTACGTGC
CAGCAGCCGCGGTAATACGTAGGTGGCGAGCGTTGTCCGGATTTACTGGGCGTAAAGGGAGCGTAGGCGGATTTTTAAGTGA
GATGTGAAATACCCGGGCTCAACTTGGGTGCTGCATTTCAAAACTGGAAGTCTAGAGTGCAGGAGAGGAGAGTGGAATTCCT
AGTGTAGCGGTGAAATGCGTAGAGATTAGGAAGAACACCAGTGGCGAAGGCGACTCTCTGGACTGTAACTGACGCTGAGGCT
CGAAAGCGTGGGGAGCAACAGGATTAGATACCT

>*Clostridum celerecrescens* ZS strain (BI82)

ATTGACTAAGCGGCGGACGGGTGAGTAACGCGTGGGTAACCTGCCTCATACAGGGGGATAACAGTTGGAAACGACTGCTAAT
ACCGCATAAGCACACAGTGCCGCATGGTACGGTGTGAAAAACTCCGGTGGTATGAGATGGACCCGCGTCTGATTAGGTAGTT
GGTGAGGTAACGGCCCACCAAGCCGACGATCAGTAGCCGACCTGAGAGGGTGACCGGCCACATTGGGACTGAGACACGGCC
AAACTCCTACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGGGAAACCCTGATCCAGCGACGCCGCGTGAGTGAAGAAG
TATTTCGGTATGTAAAGCTCTATCAGCAGGGAAGAAAATGACGGTACCTGACTAAGAAGCCCCGGCTAACTACGTGCCAGCA
GCCGCGGTAATACGTAGGGGGCAAGCGTTATCCGGATTTACTGGGTGTAAAGGGAGCGTAGACGGCACTGCAAGTCTGGAGT
GAAAGCCCGGGGCTCAACCCCGGGACTGCTTTGGAAACTGTGGTGCTAGAGTGCAGGAGAGGTAAGTGGAATTCCTAGTGTA
GCGGTGAAATGCGTAGATATTAGGAGGAACACCAGTGGCGAAGGCGGCTTACTGGACTGTAACTGACGTTGAGGCTCGAAAG
CGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAATACTAGGTGTTGGGGAGCAAAGCTCTTC
GGTGCCGCCGCTAACGCAATAAGTATTCCACCTGGGGAGTACGTTCGCAAGAATGAAACTCAAAGGAATTGACGGGGACCCG
CACAAGCGG

>*Alishewanella aestuarii* HH-ZS strain (BI28)

AGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCACGCAGGCGGCTTTTTAAGTCGGATGTGAAAGCCCCGGGCTC
AACCTGGGAATTGCATCTGATACTGGGAAGCTAGAGTATGTGAGAGGGGGGTAGAATTCCAAGTGTAGCGGTGAAATGCGTA
GAGATTTGGAGGAATACCAGTGGCGAAGGCGGCCCCCTGGCACAATACTGACGCTCAGGTGCGAAAGCGTGGGGAGCAAAC
AGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGTCTACTAGCTGTTCGCGGCCTTGTGTTGTGAGTAGCGCAGCTAAC
GCATTAAGTAGACCGCCTGGGGAGTACGGTCGCAAGATTAAAACTCAAATGAATTGACGGGGGCCCGCACAAGCGGTGGAG
CATGTGGTTTAATTCGACGCAACGCGAAGAACCTTACCTACTCTTGACATCTACAGAAGAACGCAGAGATGTGTTTGTGCCTT
CGGGAACTGTAAGACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTTGTGAAATGTTGGGTTAAGTCCCGCAACGAGCGCAAC
CCTTATCCTTAGTTGCCAGCGATTCGGTCGGGAACTCTAGGGAGACTGCCGGTGATAAACCGGAGGAAGGTGGGGACGACGT
CAAGTCATCATGGCCCTTACGAGTAGGGCTACACACGTGCTACAATGGTATGTACAGAGGGAGGCAAGCTGGCGACAGTGAG
CGGATCTCTTAAAGCATATCGTAGTCCGGATTGGAGTCTGCAACTCGACTCCATGAAGTCGGAATCGCTAGTAATCGCAAATC
AGAATGTTGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCATGGGAGTGGGTTGCAAAAGAAGTAGGTA
GCTTAACCTTCGGGAGGGCGCTTACCACTTTGT

>*Azonexus hydrophilus* ZS02 strain (BI70)

GAACGAGCAGCACGGGCTTCGGTCTGGTGGCGAGTGGCGAACGGGTGAGTAATGCATCGGAACGTACCCGGGAGTGGGGGA
TAACTATCCGAAAGGATAGCTAATACCGCATATTCTGTGCGCAGGAAAGCAGGGGATCTTCGGACCTTGTGCTCCCGGAGCG
GCCGATGTCAGATTAGCTAGTTGGTGGGGTAAAGGCCTACCAAGGCGACGATCTGTAGCGGGTCTGAGAGGATGATCCGCCA
CACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATTTTGGACAATGGGCGCAAGCCTGATCCAGC
CATGCCGCGTGAGTGAAGAAGGCCTTCGGGTTGTAAAGCTCTTTCGGCCGGGAAGAAATCGTACGGGTTAATACCCTGTGCG
GATGACGGTACCGGCATAAGAAGCACCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGTGCGAGCGTTAATCGGA
ATTACTGGGCGTAAAGCGTGCGCAGGCGGTTTTTTAAGATAGGCGTGAAATCCCCGGGCTCAACCTGGGAACTGCGCTTATG
ACTGGAAGACTAGAGTATGGCAGAGGGGGGTGGAATTCCACGTGTAGCAGTGAAATGCGTAGAGATGTGGAGGAACACCGA
TGGCGAAGGCAGCCCCCTGGGCCAATACTGACGCTCATGCACGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGT
CCACGCCCTAAACGATGTCAACTAGGTGTTGGGTGGGTAAAACCATTTAGTACCGGAGCTAACGCGTGAAGTTGACCGCCTG
GGGAGTACGGCCGCAAGGTTAAAACTCAAAGGAATTGACGGGGACCCGCACAAGCGGTGGATGATGTGGATTAATTCGATG
CAACGCGAAAAACCTTACCTACCCTTGACTTGCCA

>*Dietzia* sp. ZS03-strain BI51

CGAACGGTAAGGCCCTTTCGGGGGTACACGAGTGGCGAACGGGTGAGTAACACGTGGGTAATCTGCCCTGCACTTCGGGATA
AGCCTGGGAAACCGGGTCTAATACCGGATATGAGCTCCTGCCGCATGGTGGGGGTTGGAAAGTTTTTCGGTGCAGGATGAGT
CCGCGGCCTATCAGCTTGTTGGTGGGGTAATGGCCTACCAAGGCGACGACGGGTAGCCGGCCTGAGAGGGTGATCGGCCACA
CTGGGACTGAGACACAGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGAAAGCCTGATGCAGCG
ACGCCGCGTGGGGGATGACGGTCTTCGGATTGTAAACTCCTTTCAGTAGGGACGAAGCGAAAGTGACGGTACCTGCAGAAGA
AGCACCGGCCAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGTGCAAGCGTTGTCCGGAATTACTGGGCGTAAAGAGCTC
GTAGGCGGTTTGTCACGTCGTCTGTGAAATCCTCCAGCTCAACTGGGGGCGTGCAGGCGATACGGGCAGACTTGAGTACTAC
AGGGGAGACTGGAATTCCTGGTGTAGCGGTGAAATGCGCAGATATCAGGAGGAACACCGGTGGCGAAGGCGGGTCTCTGGG
TAGTAACTGACGCTGAGGAGCGAAAGCATGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCATGCCGTAAACGGTGGGC
GCTAGGTGTGGGGTCCTTCCACGGATTCCGTGCCGTAGCTAACGCATTAAGCGCCCCGCCTGGGGAGTACGGCCGCAAGGCT
AACACTCAAAGGAATTGACGGGGGCCCGCACAA

>*Dietzia* sp. from the flocs –community

TGGGGAATATTGCACAATGGGCGAAAGCCTGATGCAGCGACGCCGCGTGGGGGATGACGGTCTTCGGATTGTAAACTCCTTT
CAGTAGGGACGAAGCGAAAGTGACGGTACCTGCAGAAGAAGCACCGGCCAACTACGTGCCAGCAGCCGCGGTAATACGTAG
GGTGCAAGCGTTGTCCGGAATTACTGGGCGTAAAGAGCTCGTAGGCGGTTTGTCACGTCGTCTGTGAAATCCTCCAGCTCAAC
TGGGGGCGTGCAGGCGATACGGGCAGACTTGAGTACTACAGGGGAGACTGGAATTCCTGGTGTAGCGGTGAAATGCGCAGAT
ATCAGGAGGAACACCGGTGGCGAAGGCGGGTCTCTGGGTAGTAACTGACGCTCAGGTGCGAAAGCGTGGGGAGCAAACA

>*Brevundimonas* ZS04 (BI 36)

CGTGCCAGCAGCCGCGGTAATTCGAAGGGGGCTAGCGTTGCTCGGAATTACTGGGCGTAAAGGGCGCGTAGGCGGATCGTTA
AGTCAGAGGTGAAATCCCAGGGCTCAACCCTGGAACTGCCTTTGATACTGGCGATCTTGAGTATGAGAGAGGTATGTGGAAC
TCCGAGTGTAGAGGTGAAATTCGTAGATATTCGGAAGAACACCAGTGGCGAAGGCGACATACTGGCTCATTACTGACGCTGA
GGCGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGATTGCTAGTTGTCGGGCTGC
ATGCAGTTCGGTGACGCAGCTAACGCATTAAGCAATCCGCCTGGGGAGTACGGTCGCAAGATTAAAACTCAAAGGAATTGAC
GGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGCAGAACCTTACCACCTTTTGACATGCCTGGACC
GCCACGGAGACGTGGCTTTCCCTTCGGGGACTAGGACACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGG
TTAAGTCCCGCAACGAGCGCAACCCTCGCCATTAGTTGCCATCATTTAGTTGGGAACTCTAATGGGACTGCCGGTGCTAAGCC
GGAGGAAGGTGGGGATGACGTCAAGTCCTCATGGCCCTTACAGGGTGGGCTACACACGTGCTACAATGGCAACTACAGAGGG
TTAATCCTTAAAAGTTGTCTCAGTTCGGATTGTCCTCTGCAACTCGAGGGCATGAAGTTGGAATCGCTAGTAATCGCGGATCA
GCATGCCGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCATGGGAGTTGGTTCTACCCGAAGGCGGTGCG
CTAACCAGCAACTGGAGGCAGCCGACCACGGTAGTCAG

>*Rhodococcus* sp. ZS-strain (BI49)

CGTTGTCCGGAATTACTGGGCGTAAAGAGTTCGTAGGCGGTTTGTCGCGTCGTTTGTGAAAACCAGCAGCTCAACTGCTGGCT
TGCAGGCGATACGGGCAGACTTGAGTACTGCAGGGGAGACTGGAATTCCTGGTGTAGCGGTGAAATGCGCAGATATCAGGAG
GAACACCGGTGGCGAAGGCGGGTCTCTGGGCAGTAACTGACGCTGAGGAACGAAAGCGTGGGTAGCGAACAGGATTAGATA
CCCTGGTAGTCCACGCCGTAAACGGTGGGCGCTAGGTGTGGGTTCCTTCCACGGAATCCGTGCCGTAGCTAACGCATTAAGCG
CCCCGCCTGGGGAGTACGGCCGCAAGGCTAAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGCGGAGCATGTGGATT
AATTCGATGCAACGCGAAGAACCTTACCTGGGTTTGACATATACCGGAAAGCTGCAGAGATGTGGCCCCCCTTGTGGTCGGT
ATACAGGTGGTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCCTATCTTATG
TTGCCAGCACGTTATGGTGGGGACTCGTAAGAGACTGCCGGGGTCAACTCGGAGGAAGGTGGGGACGACGTCAAGTCATCAT
GCCCCTTATGTCCAGGGCTTCACACATGCTACAATGGCCAGTACAGAGGGCTGCGAGACCGTGAGGTGGAGCGAATCCCTTA
AAGCTGGTCTCAGTTCGGATCGGGGTCTGCAACTCGACCCCGTGAAGTCGGAGTCGCTAGTAATCGCAGATCAGCAACGCTG
CGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACGTCATGAAAGTCGGTAACACCCGAACGCCGGT

>*Tessaracoccus* sp. ZS01 strain BI41

AACGGTAAGGCCCTTTCGGGGGTACACGAGTGGCGAACGGGTGAGTAACACGTGAGTAACCTGCCCTTGACTTTGGGATAAC
TCCTGGAAACAGGTGCTAATACCGGATACCAGCCTTCACGGCATCGTGTTGGTTTGAAAGCTCCGGCGGTCAAGGATGGACTC
GCGGCCTATCAGCTTGTTGGTGAGGTAGTGGCTCACCAAGGCTTCGACGGGTAGCCGGCCTGAGAGGGTGACCGGCCACATT
GGGACTGAGATACGGCCCAAACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGACGCAAGTCTGATGCAGCAAC
GCCGCGTGCGGGATGACGGCCTTCGGGTTGTAAACCGCTTTCAGCAGGGACGAAGCGAGAGTGACGGTACCTGCAGAAGAA
GCACCGGCTAACTACGTGCCAGCAGCCGCGGTGATACGTAGGGTGCGAGCGTTGTCCGGATTTATTGGGCGTAAAGAGCTTG
TAGGCGGTTTGTTGCGTCGGTAGTGAAAACTCAGGGCTTAACCCTGAGCCTGCTTCCGATACGGGCAGACTTGAGGAAGGTA
GGGGAGAATGGAATTCCTGGTGAAGCGGTGGAATGCGTAGATATCAGGAGGAACACCAGTGGCGAAGGCGGTTCTCTGGAC
CTTTCCTGACGCTGAGAAGCGAAAGCGTGGGGAGCAAACAGGCTTAGATACCCTGGTAGTCCACGCCGTAAACGGTGGGTAC
TAGGTGTGGGGTTCATTCCACGAACTCCGTGCCGCAGCTAACGCATTAAGTACCCCGCCTGGGGAGTACGGCCGCAAGGCTA
AAACTCAAAGGAATTGACGGGGCCCCGCACAAGCGGCGGAGCATGCGGATTAATTCGATGCAACGCGAAGAACCTTACCTG
GGTTTGACATATGCCGGAAACATCTAGAG

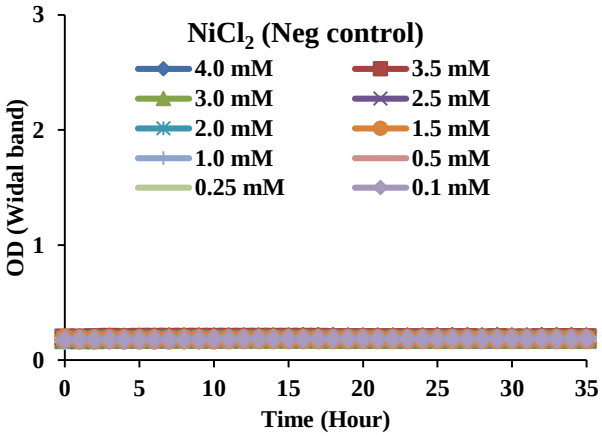
>*Aeromonas* SP. ZS strain (BI55)

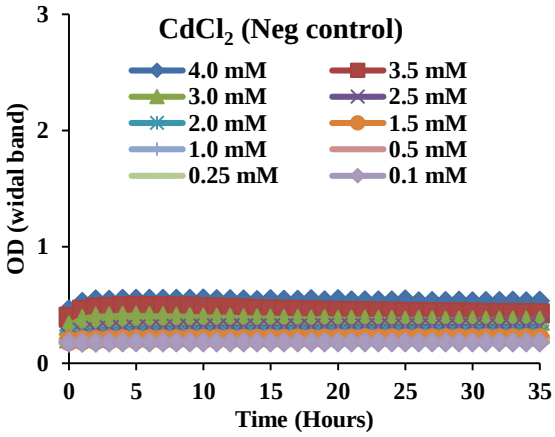
CACGCAGGCGGTTGGATAAGTTAGATGTGAAAGCCCCGGGCTCAACCTGGGAATTGCATTTAAAACTGTCCAGCTAGAGTCT
TGTAGAGGGGGGTAGAATTCCAGGTGTAGCGGTGAAATGCGTAGAGATCTGGAGGAATACCGGTGGCGAAGGCGGCCCCCT
GGACAAAGACTGACGCTCAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATG
TCGATTTGGAGGCTGTGTCCTTGAGACGTGGCTTCCGGAGCTAACGCGTTAAATCGACCGCCTGGGGAGTACGGCCGCAAGG
TTAAAACTCAAATGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGATGCAACGCGAAGAACCTTACC
TGGCCTTGACATGTCTGGAATCCTGTAGAGATACGGGAGTGCCTTCGGGAATCAGAACACAGGTGCTGCATGGCTGTCGTCA
GCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCCTGTCCTTTGTTGCCAGCACGTAATGGTGGGAACTC
AAGGGAGACTGCCGGTGATAAACCGGAGGAAGGTGGGGATGACGTCAAGTCATCATGGCCCTTACGGCCAGGGCTACACAC
GTGCTACAATGGCGCGTACAGAGGGCTGCAAGCTAGCGATAGTGAGCGAATCCCAAAAAGCGCGTCGTAGTCCGGATCGGA
GTCTGCAACTCGACTCCGTGAAGTCGGAATCGCTAGTAATCGCGAATCAGAATGTCGCGGTGAATACGTTCCCGGGCCTTGTA
CACACCGCCCGTCACCACCAT

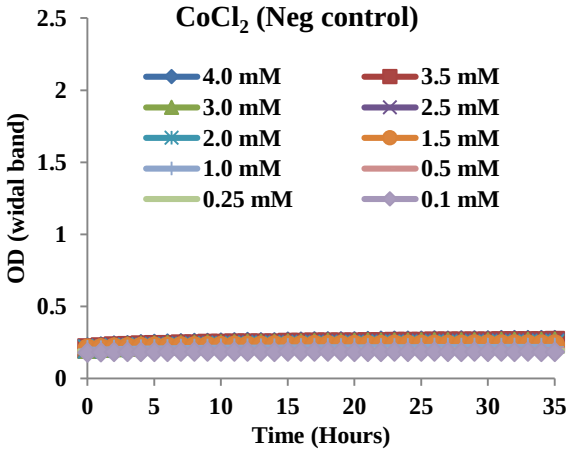
Appendix-5; Overall characterization of isolates

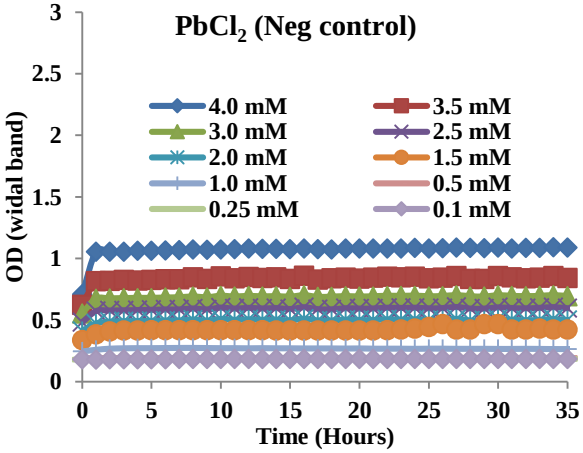
1. Heavy metals tolerant

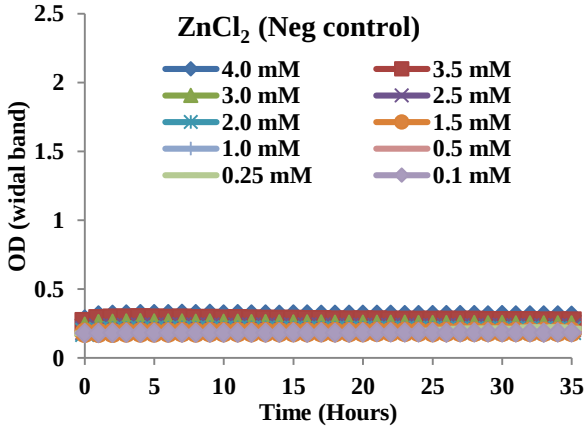
1.1 Negative control for HMs

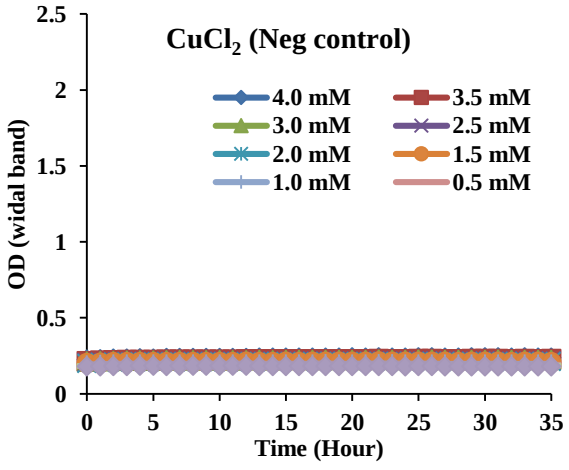




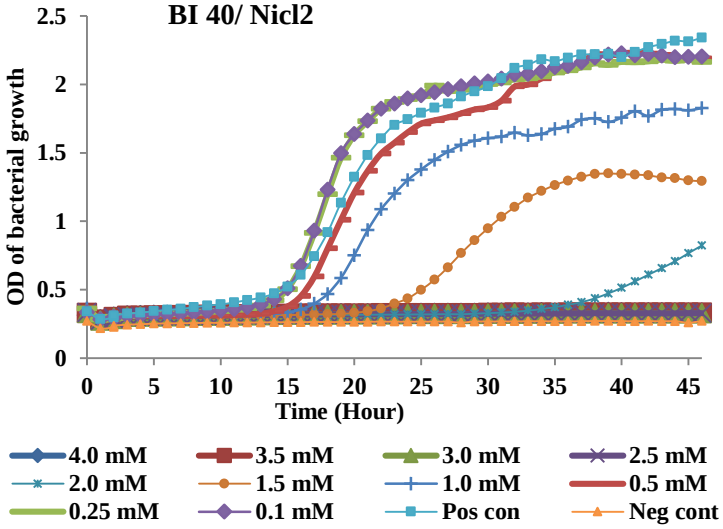


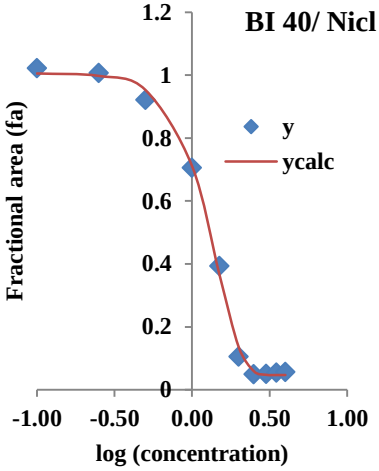


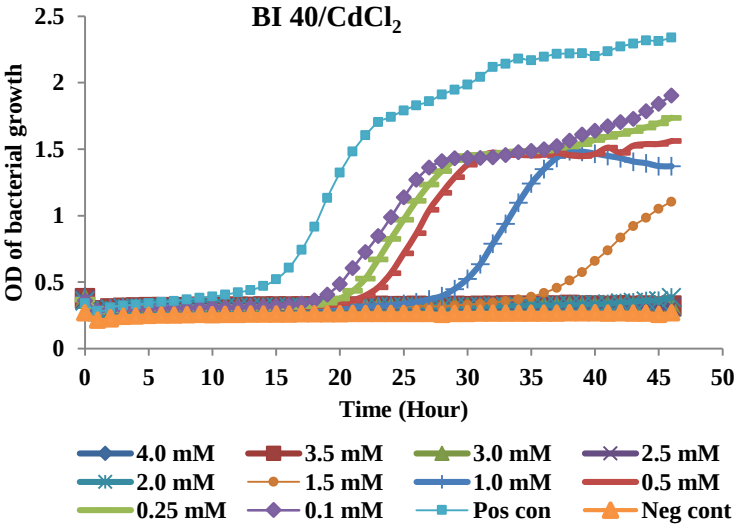


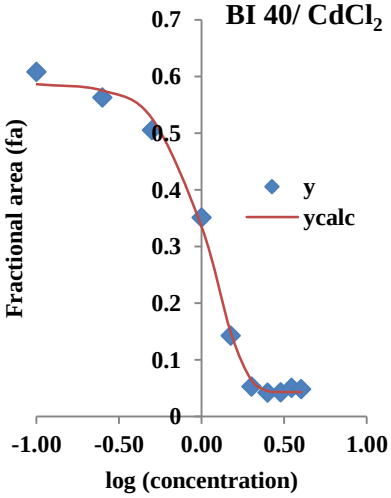


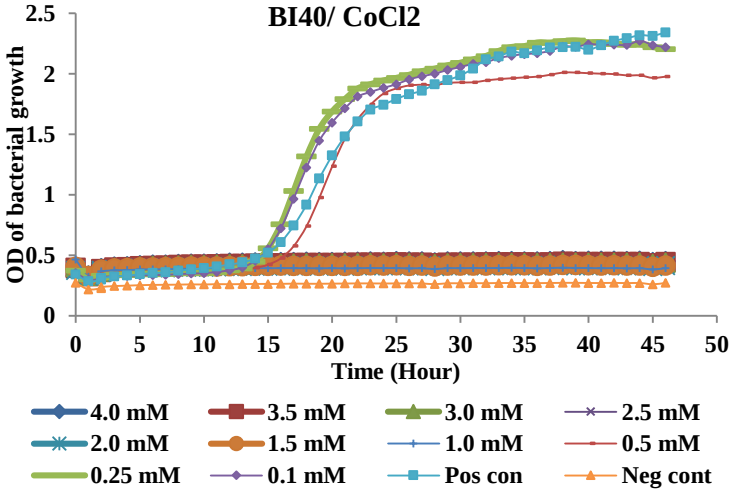
Macellibacteroides fermentans HH-ZS strain (BI 40)

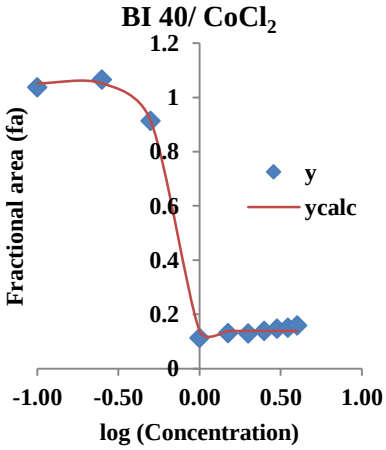


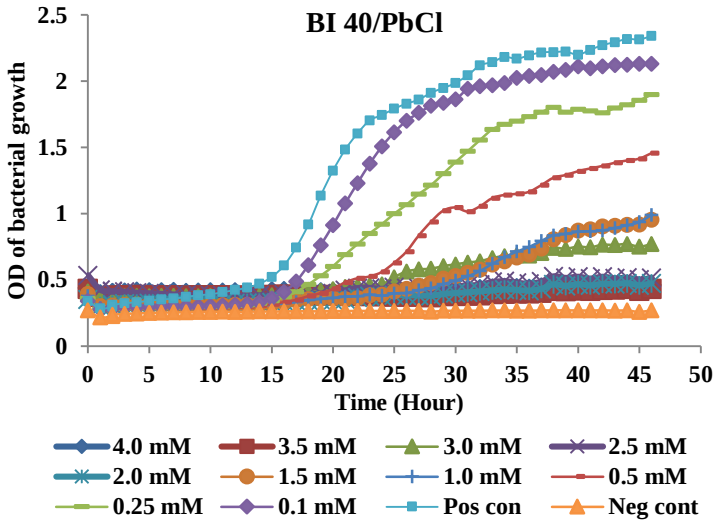


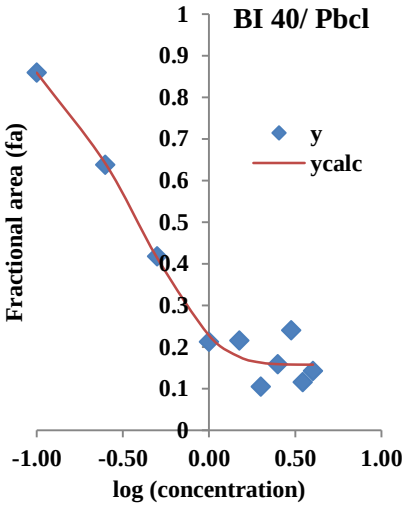


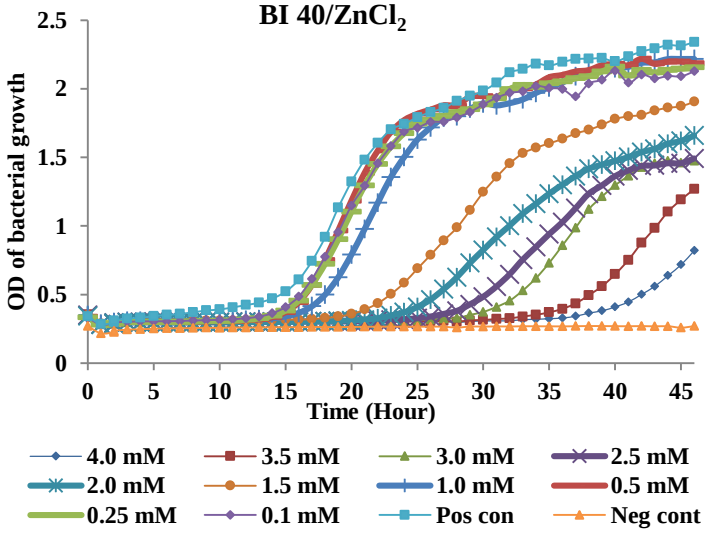


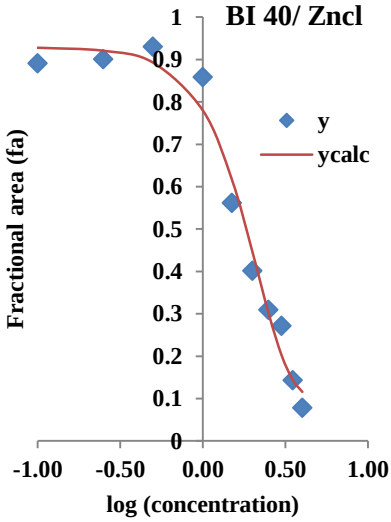


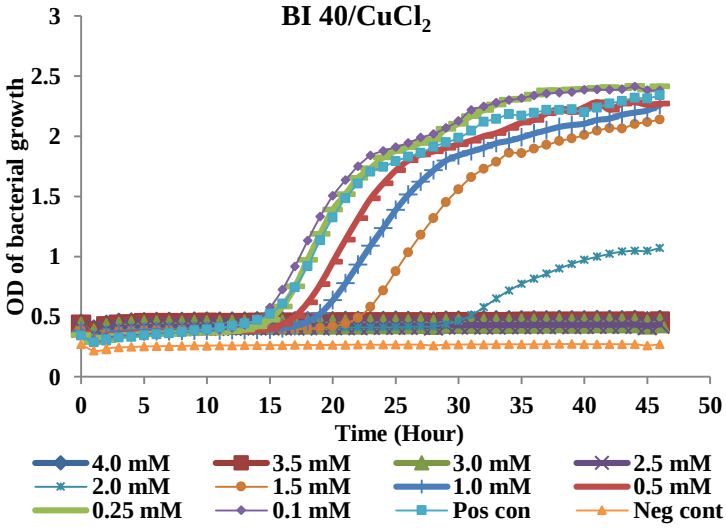


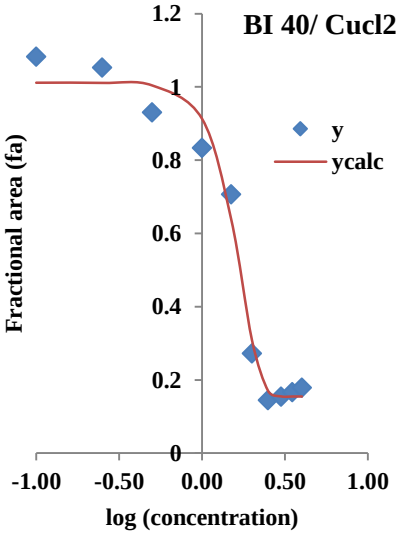

BI 40/PbCl
OD of bacterial growth
Time (Hour)
4.0 mM
3.5 mM
3.0 mM
2.5 mM
2.0 mM
1.5 mM
1.0 mM
0.5 mM
0.25 mM
0.1 mM
Pos con
Neg cont


BI 40/ Pbcl
Fractional area (fa)
log (concentration)
y
ycalc

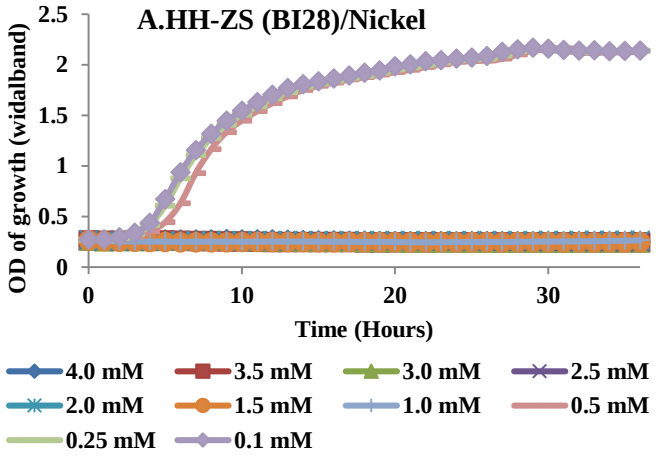

BI 40/ZnCl2
OD of bacterial growth
Time (Hour)
4.0 mM
3.5 mM
3.0 mM
2.5 mM
2.0 mM
1.5 mM
1.0 mM
0.5 mM
0.25 mM
0.1 mM
Pos con
Neg cont

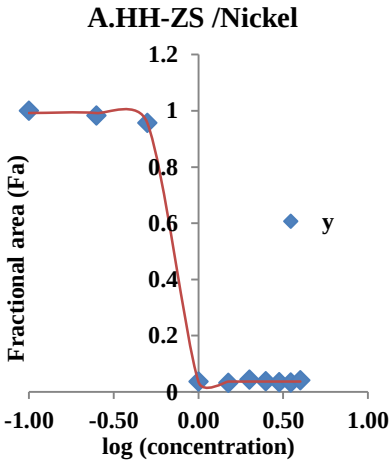

BI 40/ Zncl
Fractional area (fa)
log (concentration)
y
ycalc

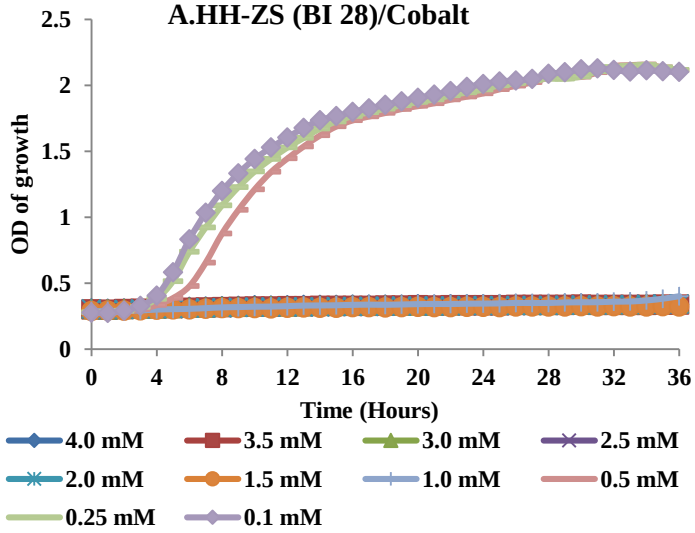

BI 40/CuCl2
OD of bacterial growth
Time (Hour)
4.0 mM
3.5 mM
3.0 mM
2.5 mM
2.0 mM
1.5 mM
1.0 mM
0.5 mM
0.25 mM
0.1 mM
Pos con
Neg cont

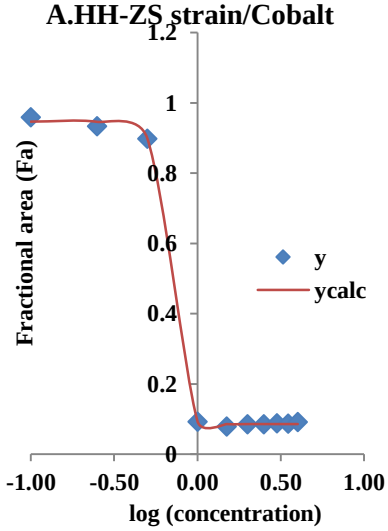

BI 40/ Cucl2
Fractional area (fa)
log (concentration)
y
ycalc

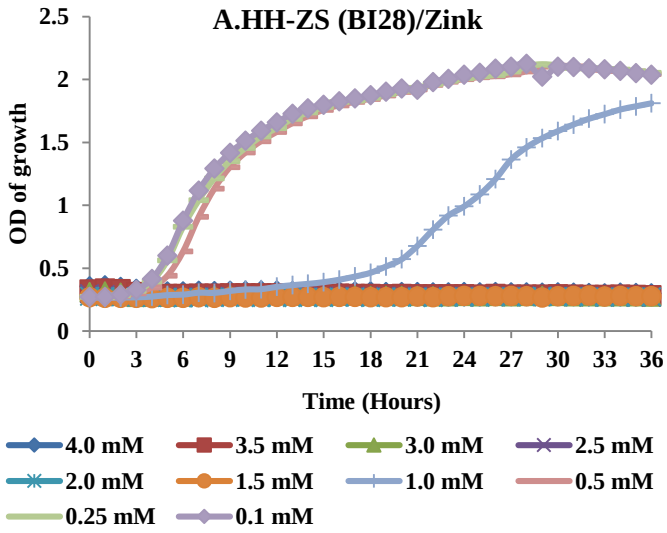
Alishewanella sp. HH-ZS strain (BI 28)

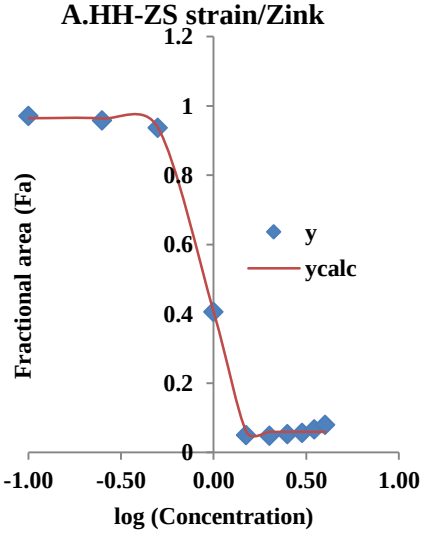


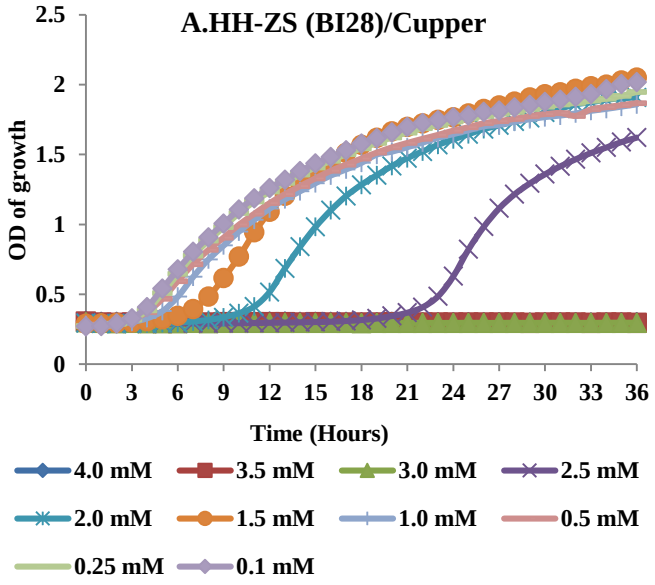


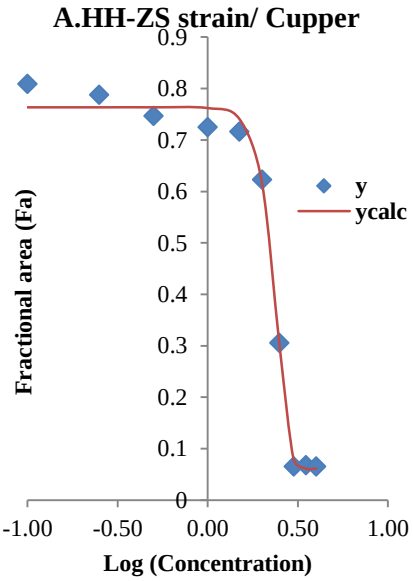


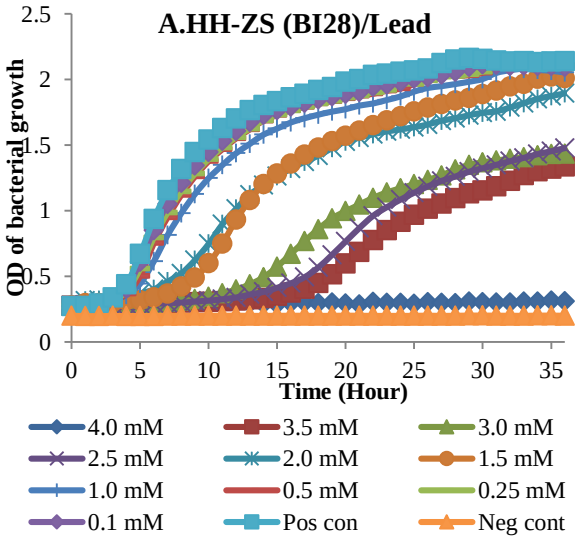


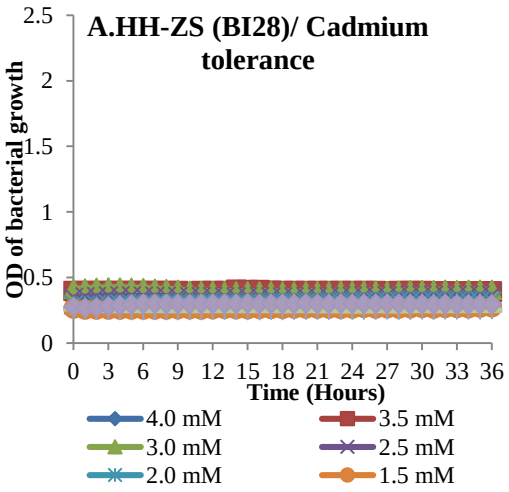


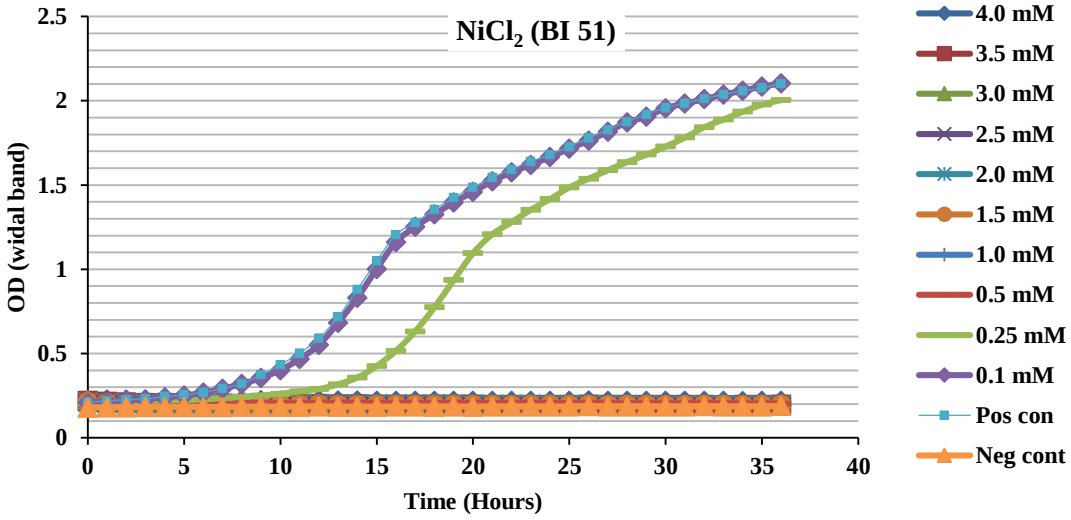


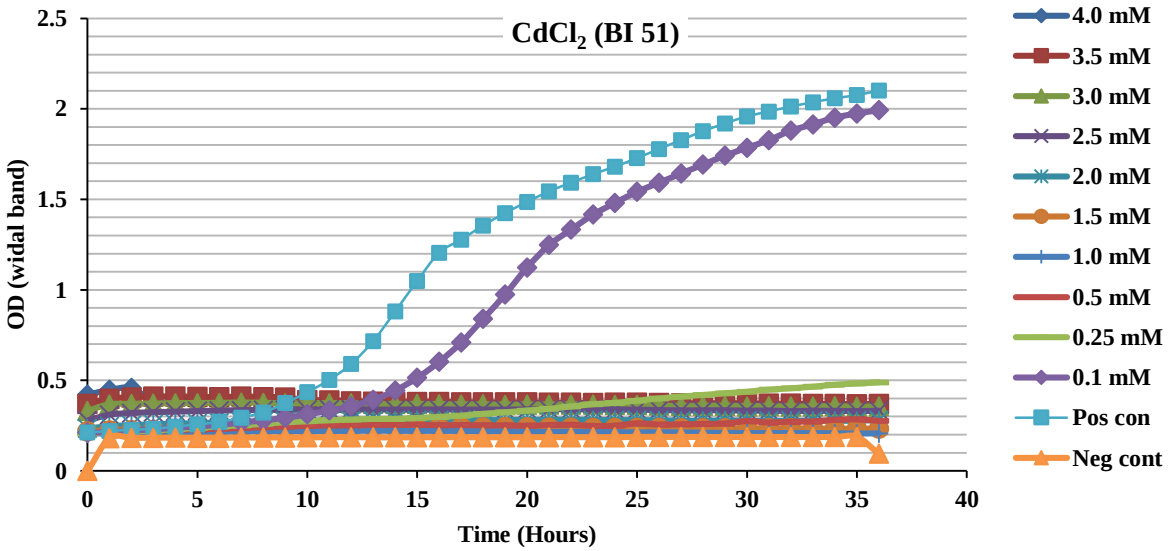

A.HH-ZS (BI28)/Cupper
OD of growth
Time (Hours)
4.0 mM
3.5 mM
3.0 mM
2.5 mM
2.0 mM
1.5 mM
1.0 mM
0.5 mM
0.25 mM
0.1 mM

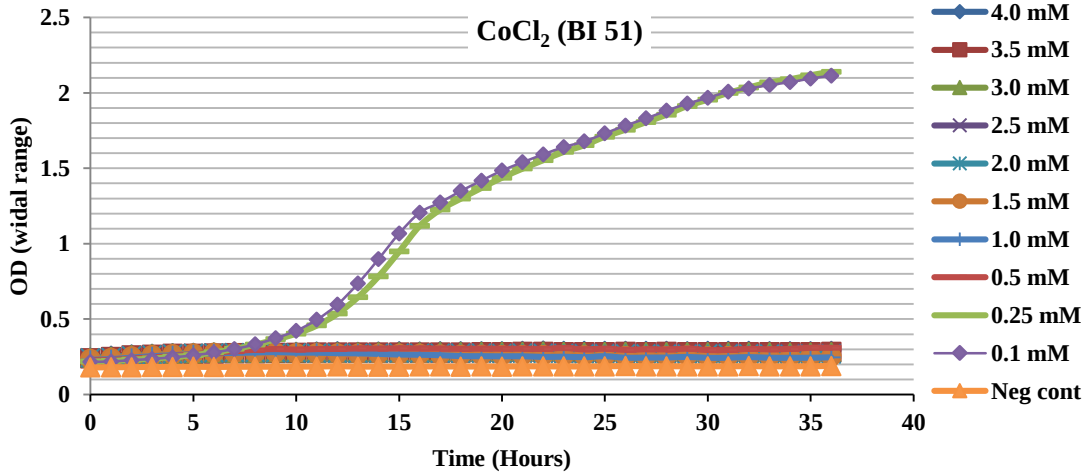

A.HH-ZS strain/ Cupper
Fractional area (Fa)
Log (Concentration)
y
ycalc

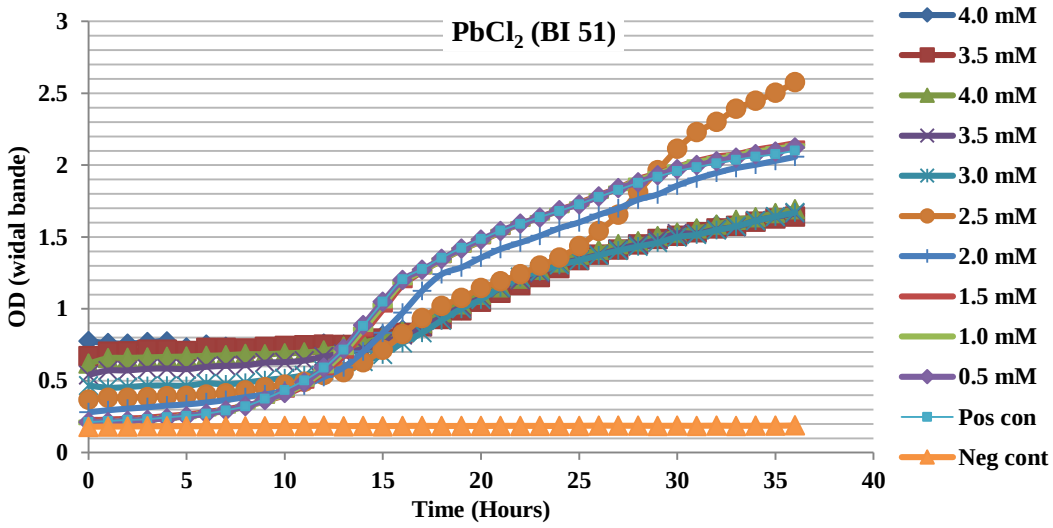

A.HH-ZS (BI28)/Lead
OD of bacterial growth
Time (Hour)
4.0 mM
3.5 mM
3.0 mM
2.5 mM
2.0 mM
1.5 mM
1.0 mM
0.5 mM
0.25 mM
0.1 mM
Pos con
Neg cont

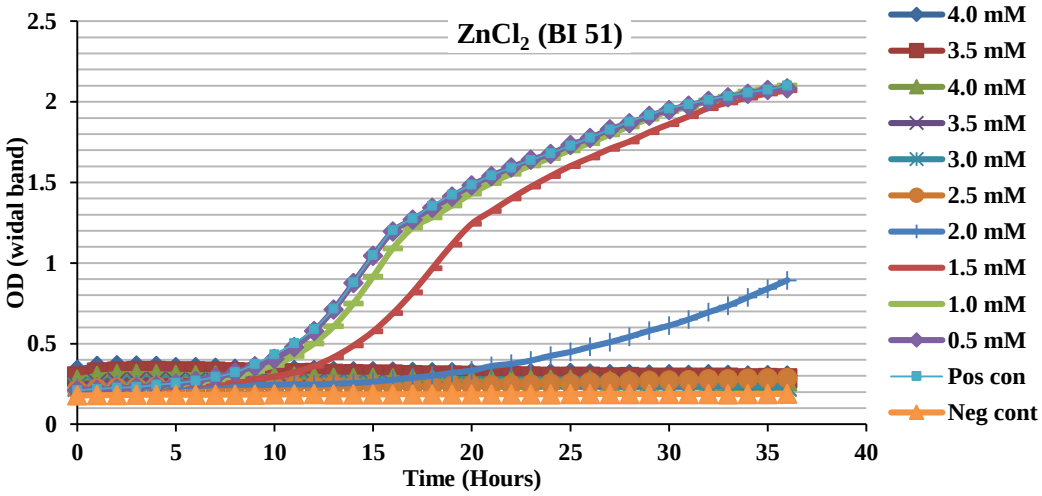

A.HH-ZS (BI28)/ Cadmium tolerance
OD of bacterial growth
Time (Hours)
4.0 mM
3.5 mM
3.0 mM
2.5 mM
2.0 mM
1.5 mM

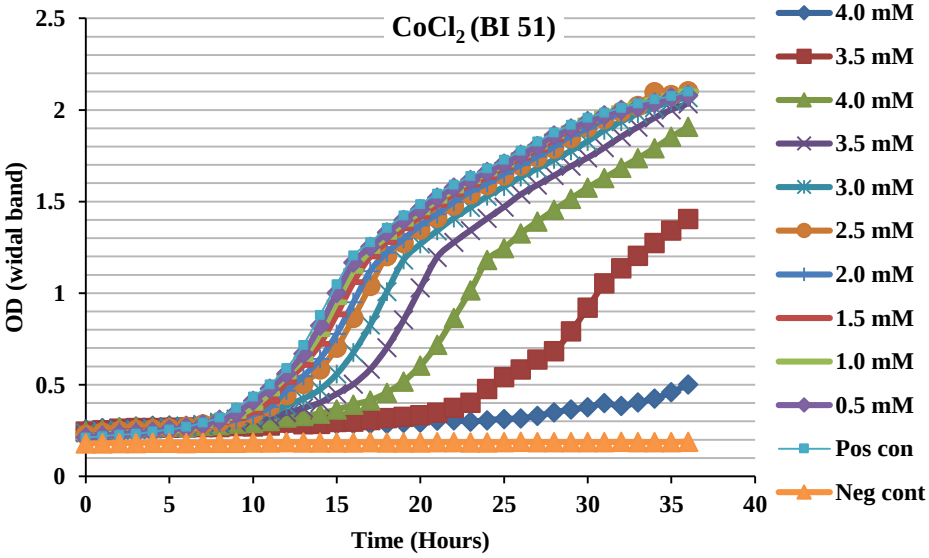

NiCl2 (BI 51)
OD (widal band)
Time (Hours)
4.0 mM
3.5 mM
3.0 mM
2.5 mM
2.0 mM
1.5 mM
1.0 mM
0.5 mM
0.25 mM
0.1 mM
Pos con
Neg cont

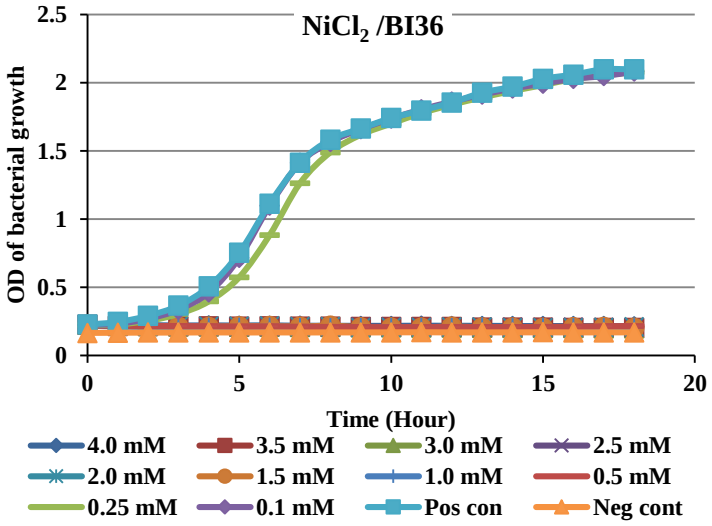

CdCl2 (BI 51)
OD (widal band)
Time (Hours)
4.0 mM
3.5 mM
3.0 mM
2.5 mM
2.0 mM
1.5 mM
1.0 mM
0.5 mM
0.25 mM
0.1 mM
Pos con
Neg cont

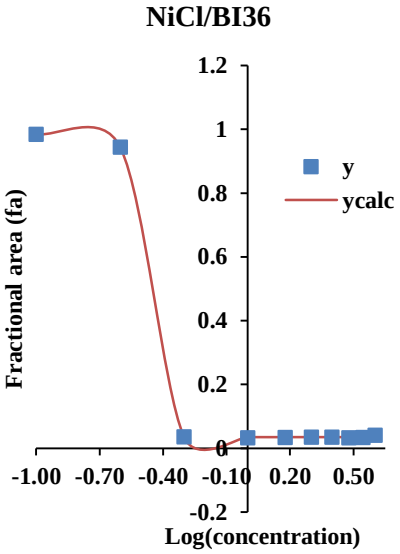

CoCl2 (BI 51)
OD (widal range)
Time (Hours)
4.0 mM
3.5 mM
3.0 mM
2.5 mM
2.0 mM
1.5 mM
1.0 mM
0.5 mM
0.25 mM
0.1 mM
Neg cont

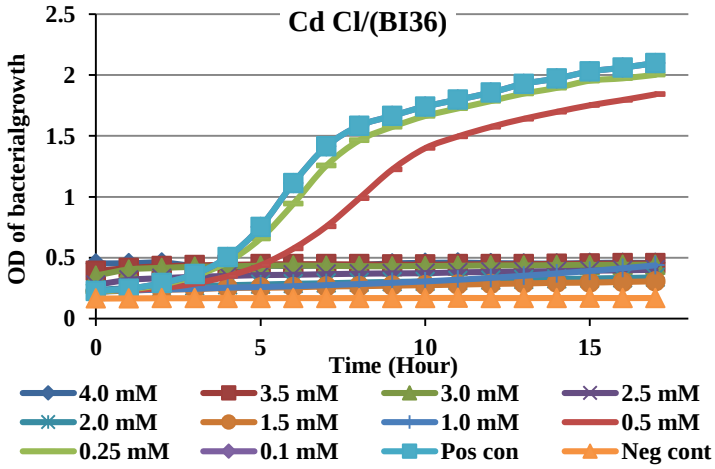

PbCl2 (BI 51)
OD (widal bande)
Time (Hours)
4.0 mM
3.5 mM
4.0 mM
3.5 mM
3.0 mM
2.5 mM
2.0 mM
1.5 mM
1.0 mM
0.5 mM
Pos con
Neg cont

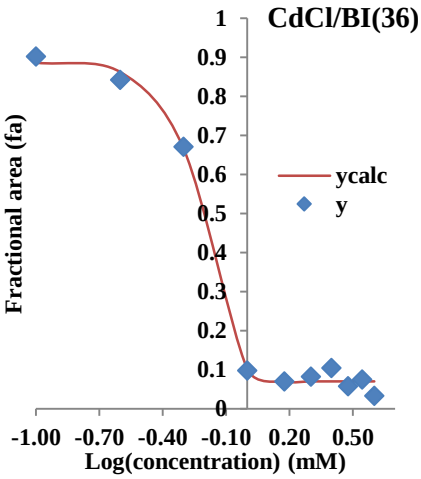

ZnCl2 (BI 51)
OD (widal band)
Time (Hours)
4.0 mM
3.5 mM
4.0 mM
3.5 mM
3.0 mM
2.5 mM
2.0 mM
1.5 mM
1.0 mM
0.5 mM
Pos con
Neg cont


CoCl2 (BI 51)
OD (widal band)
Time (Hours)
4.0 mM
3.5 mM
4.0 mM
3.5 mM
3.0 mM
2.5 mM
2.0 mM
1.5 mM
1.0 mM
0.5 mM
Pos con
Neg cont



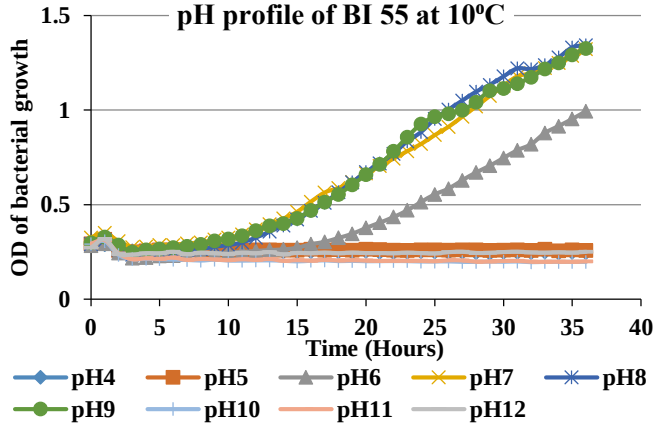


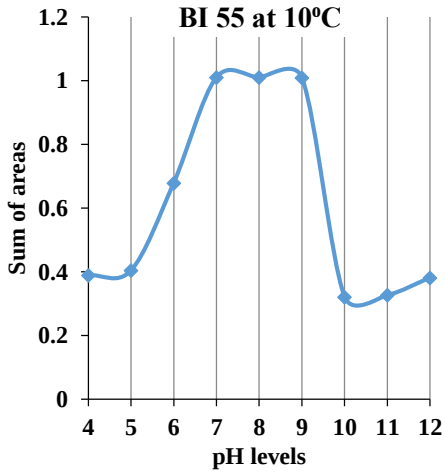


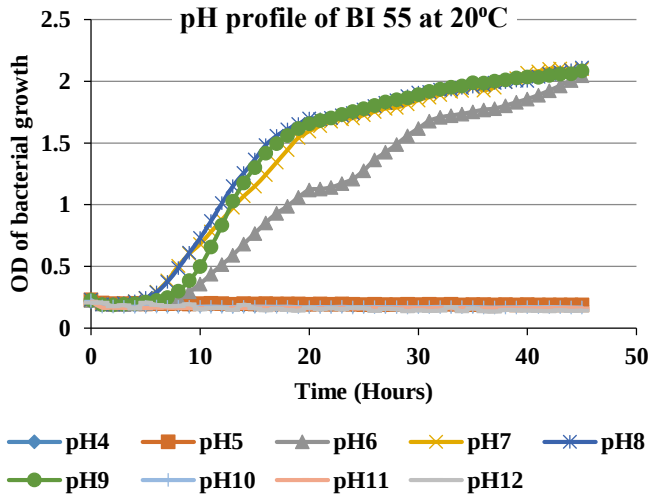


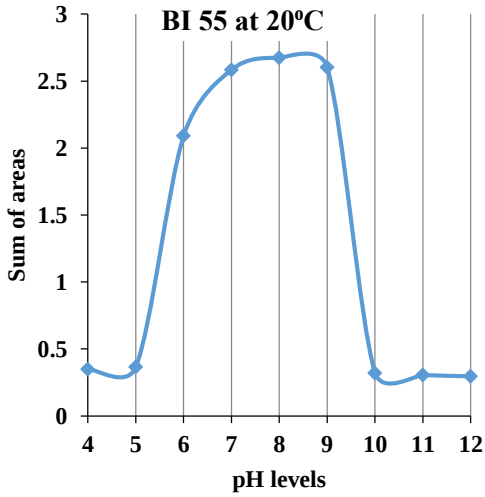
2. pH profile of isolates

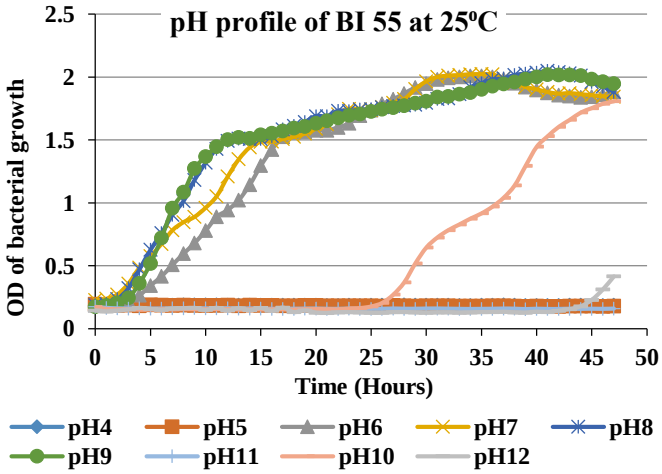
Aeromonas sp. ZS-strain (BI 55) pH profile at different temperatures

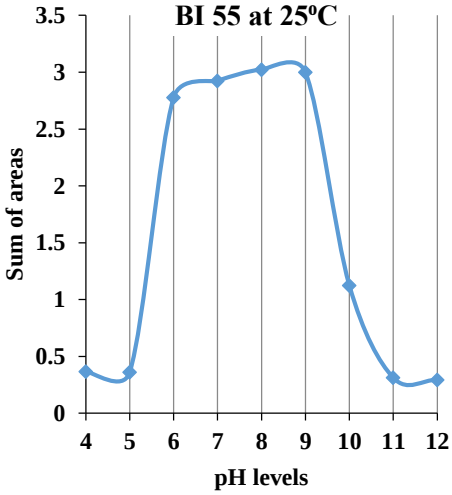


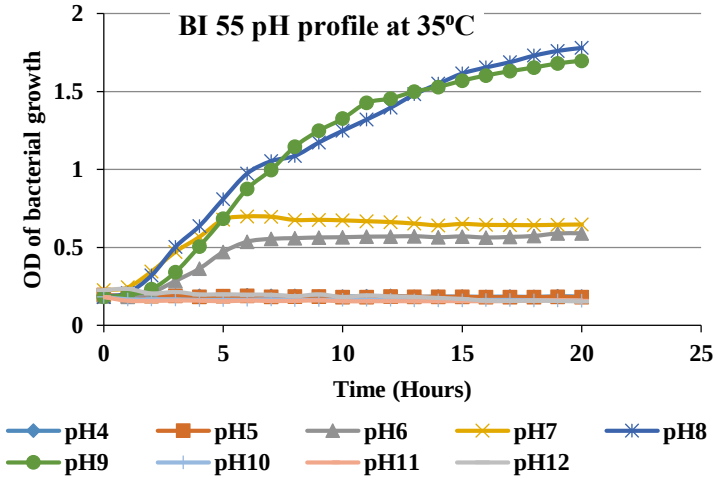


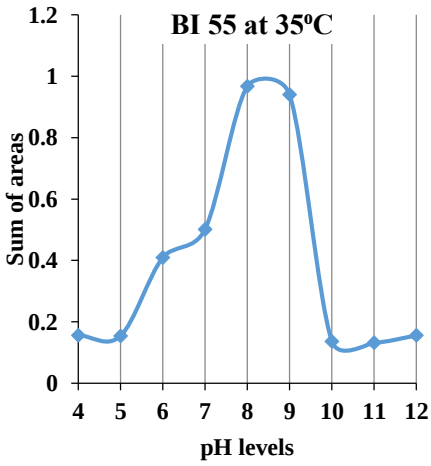

pH profile of BI 55 at 20ºC
OD of bacterial growth
Time (Hours)
pH4
pH5
pH6
pH7
pH8
pH9
pH10
pH11
pH12

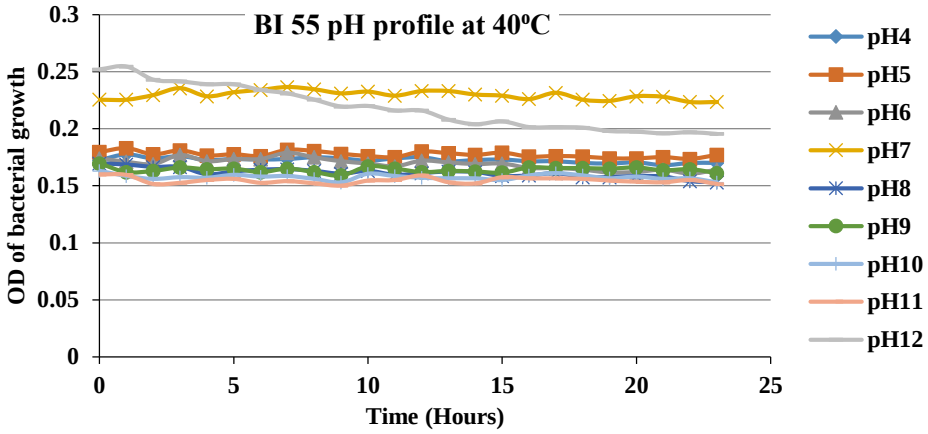

BI 55 at 20ºC
Sum of areas
pH levels


pH profile of BI 55 at 25ºC
OD of bacterial growth
Time (Hours)
pH4
pH5
pH6
pH7
pH8
pH9
pH11
pH10
pH12

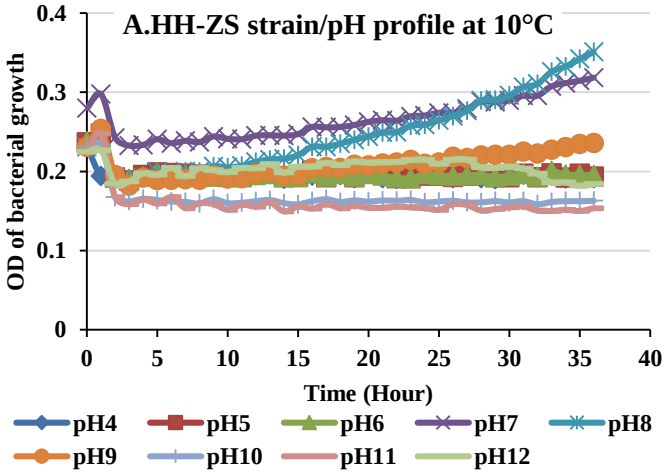

BI 55 at 25ºC
Sum of areas
pH levels

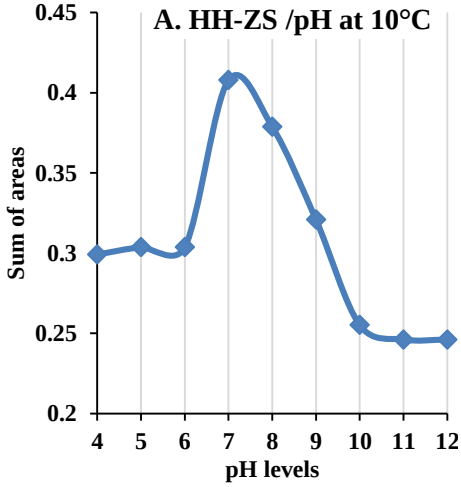

BI 55 pH profile at 35ºC
OD of bacterial growth
Time (Hours)
pH4
pH5
pH6
pH7
pH8
pH9
pH10
pH11
pH12

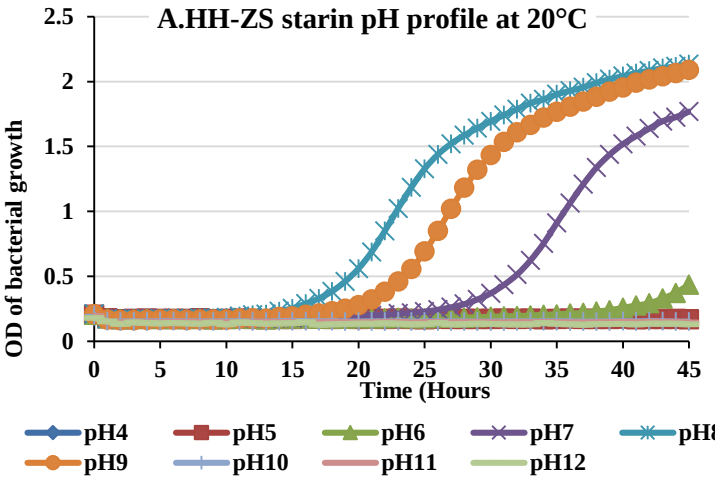

BI 55 at 35ºC
Sum of areas
pH levels

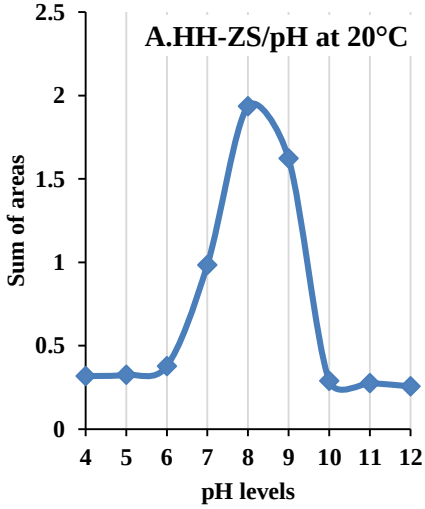


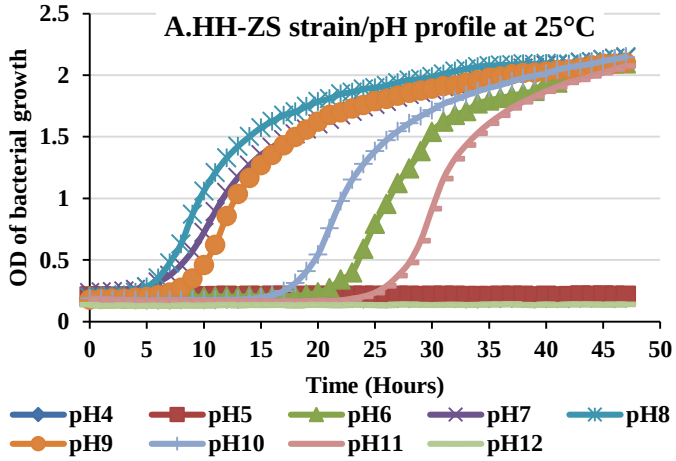
***Alishewanella* sp. HH-ZS strain (BI28), pH profile at different temperature degrees**

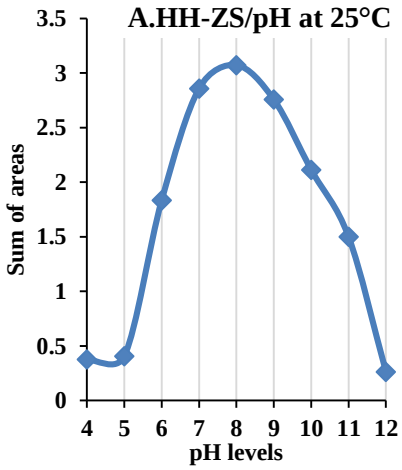


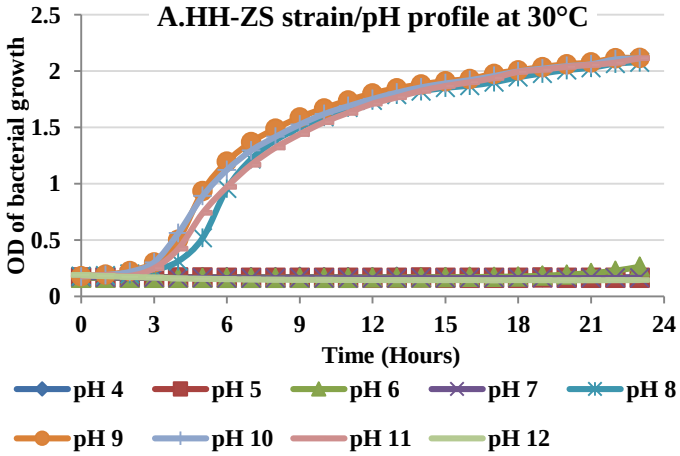


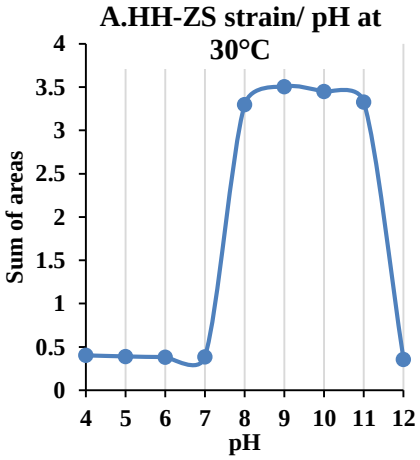


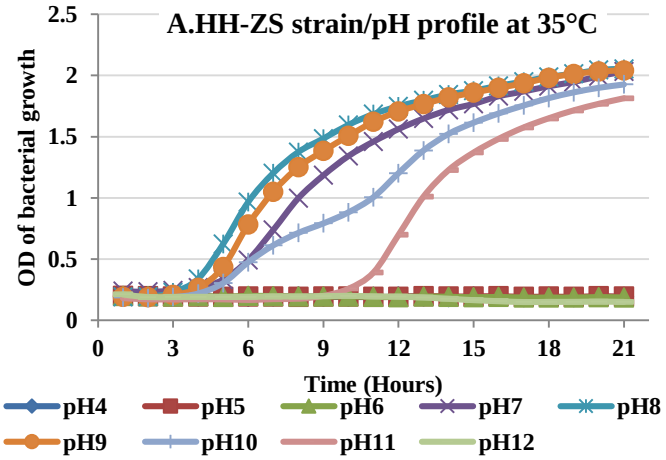


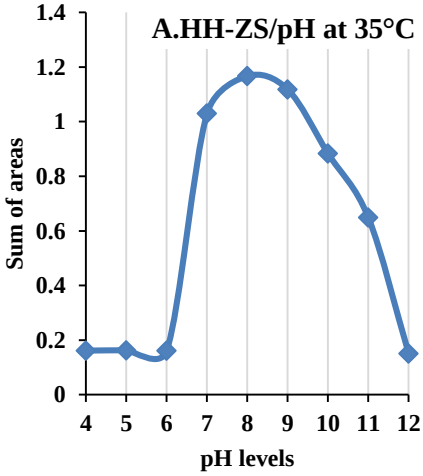

A.HH-ZS strain/pH profile at 25°C
OD of bacterial growth
Time (Hours)
pH4
pH5
pH6
pH7
pH8
pH9
pH10
pH11
pH12


A.HH-ZS/pH at 25°C
Sum of areas
pH levels

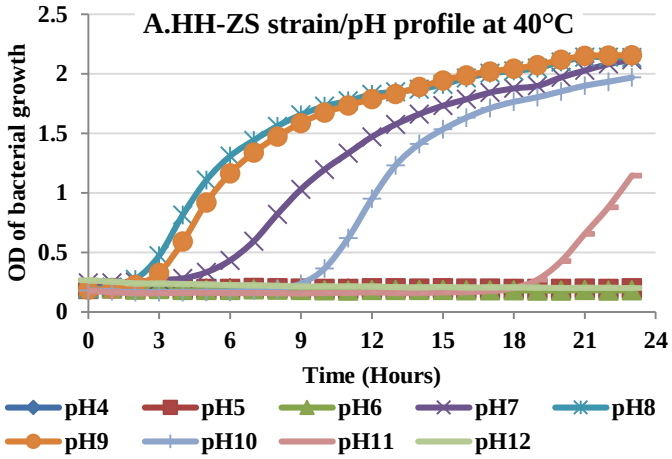

A.HH-ZS strain/pH profile at 30°C
OD of bacterial growth
Time (Hours)
pH 4
pH 5
pH 6
pH 7
pH 8
pH 9
pH 10
pH 11
pH 12

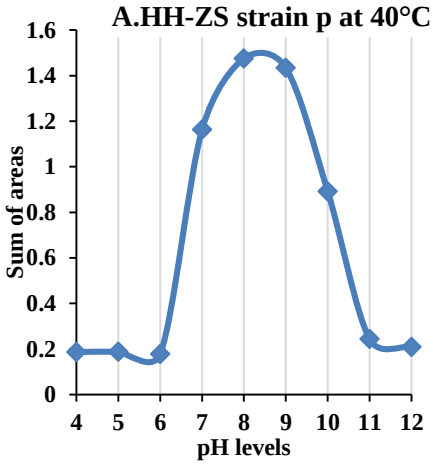

A.HH-ZS strain/ pH at 30°C
Sum of areas
pH


A.HH-ZS strain/pH profile at 35°C
OD of bacterial growth
Time (Hours)
pH4
pH5
pH6
pH7
pH8
pH9
pH10
pH11
pH12

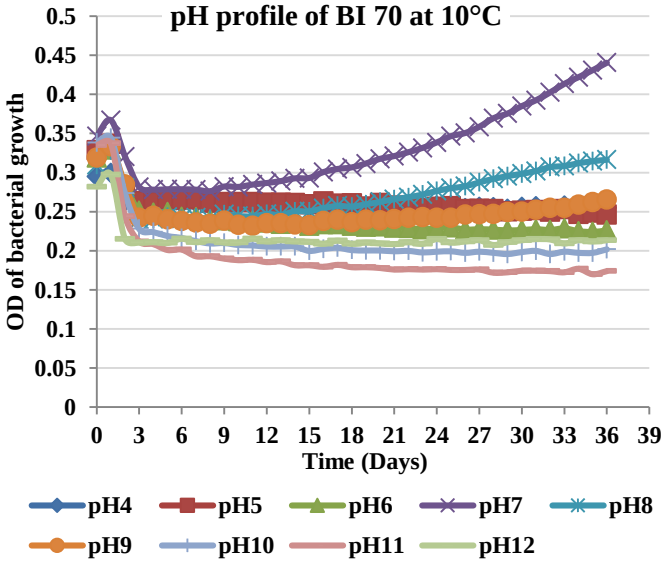

A.HH-ZS/pH at 35°C
Sum of areas
pH levels

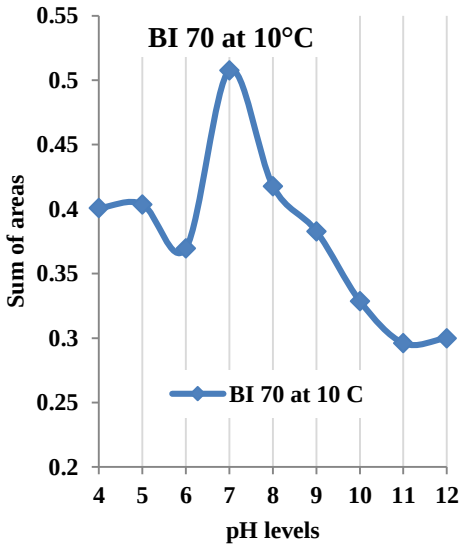
F)

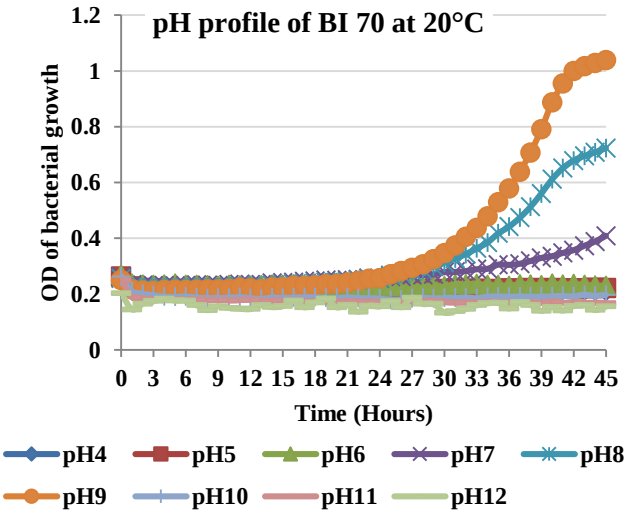


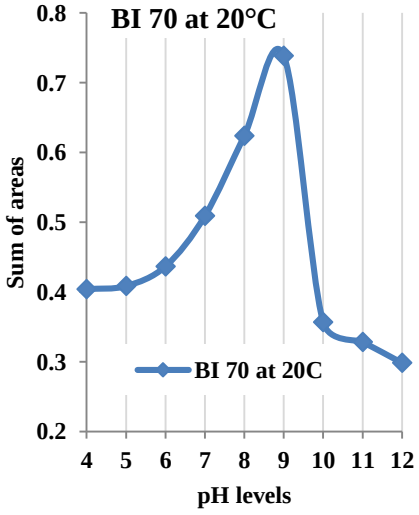


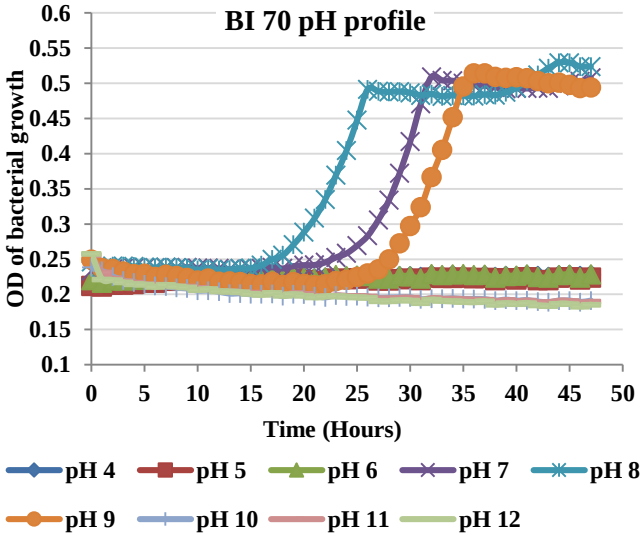
pH profile of *Azonexus* sp. *ZS02* strain (BI70) at different temperatures

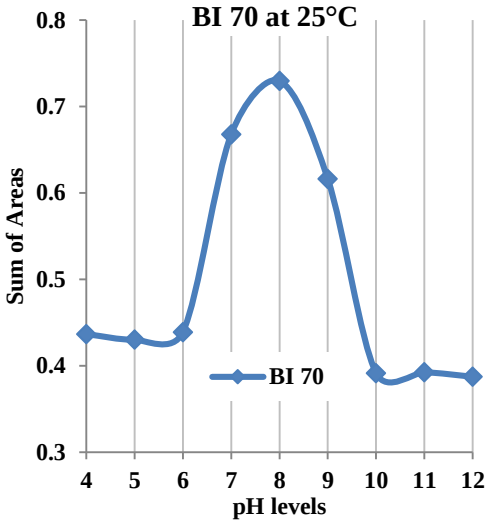


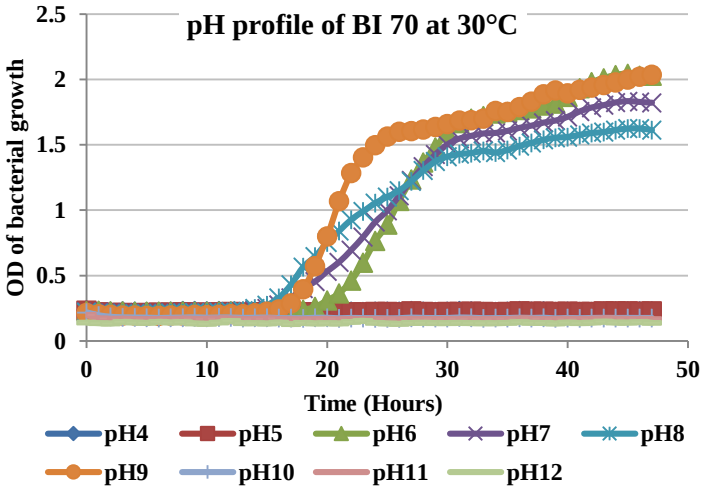


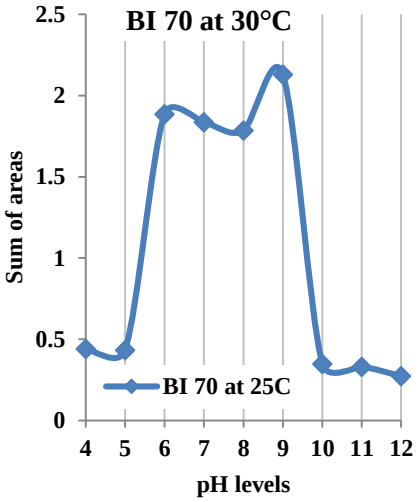


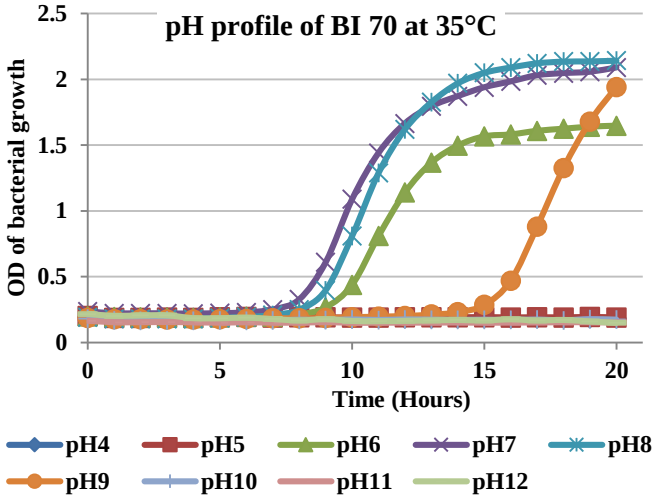


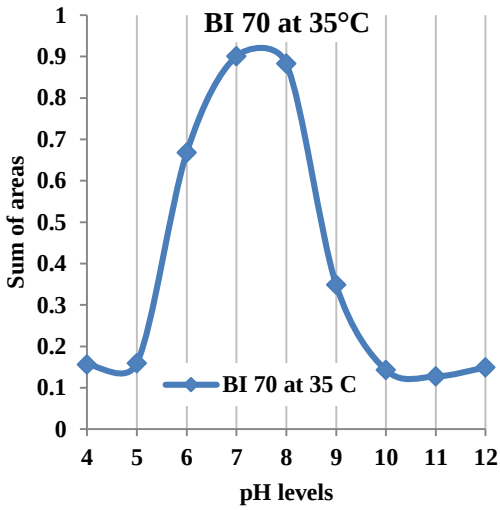

BI 70 pH profile
OD of bacterial growth
Time (Hours)
pH 4
pH 5
pH 6
pH 7
pH 8
pH 9
pH 10
pH 11
pH 12

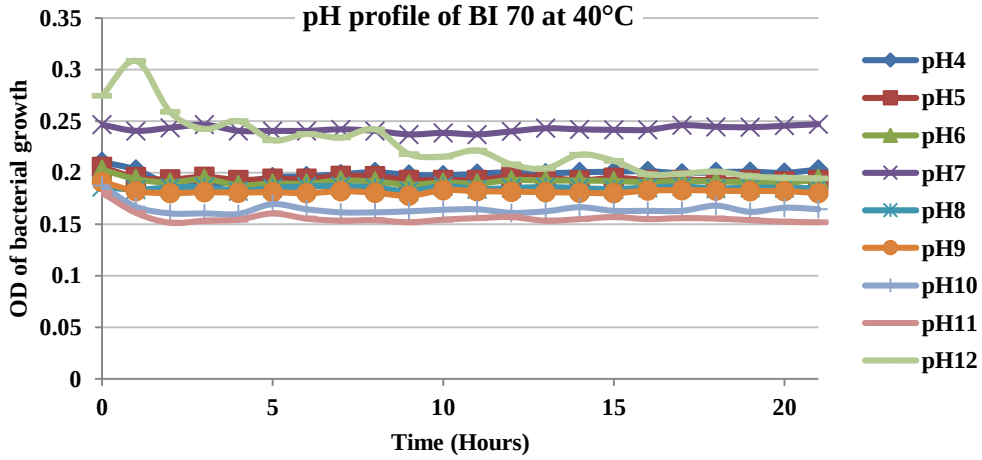

BI 70 at 25°C
Sum of Areas
BI 70
pH levels


pH profile of BI 70 at 30°C
OD of bacterial growth
Time (Hours)
pH4
pH5
pH6
pH7
pH8
pH9
pH10
pH11
pH12

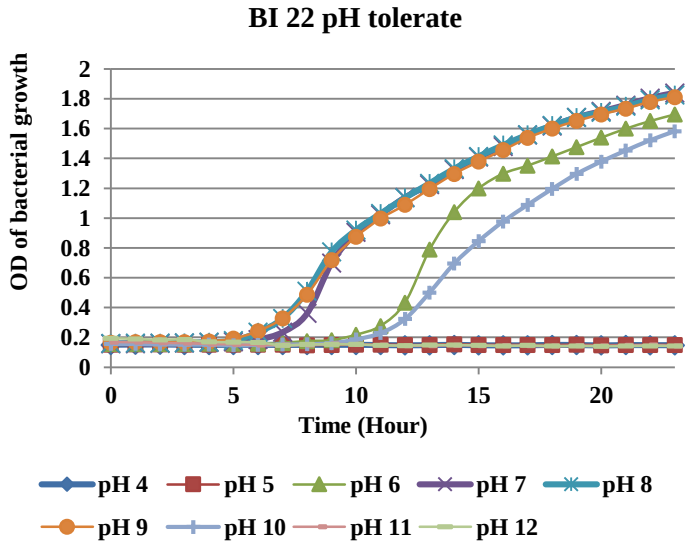

BI 70 at 30°C
Sum of areas
BI 70 at 25C
pH levels

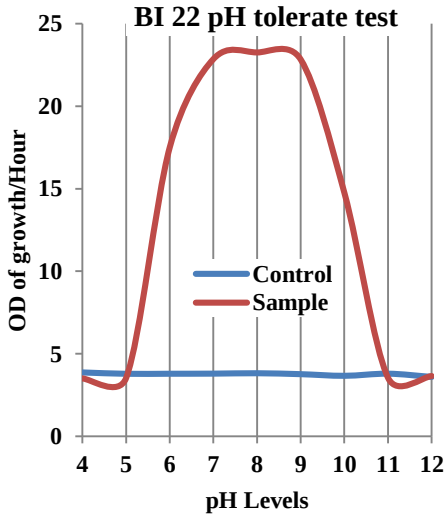




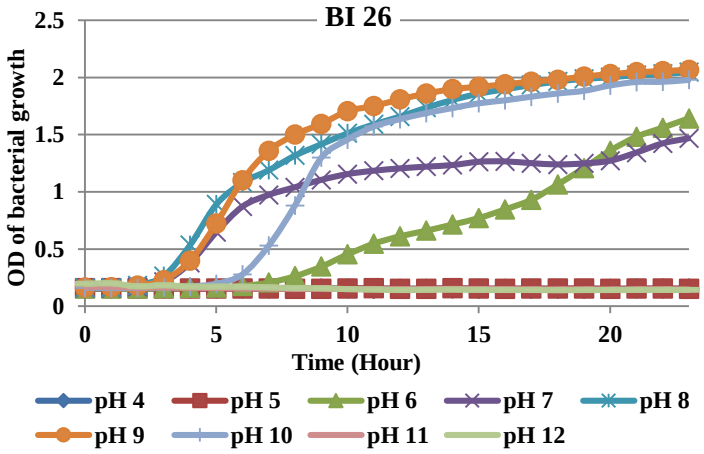


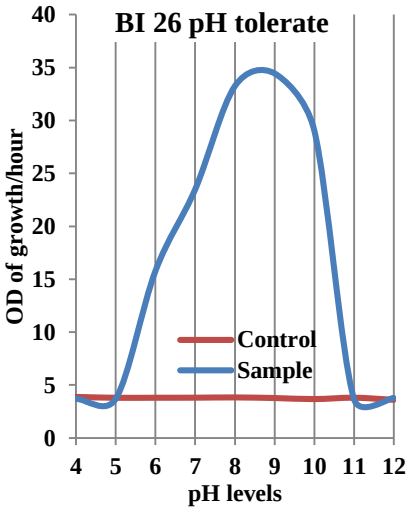
***Alcaligenes aquatilis* (BI 22) pH profile include range and optimal pH**



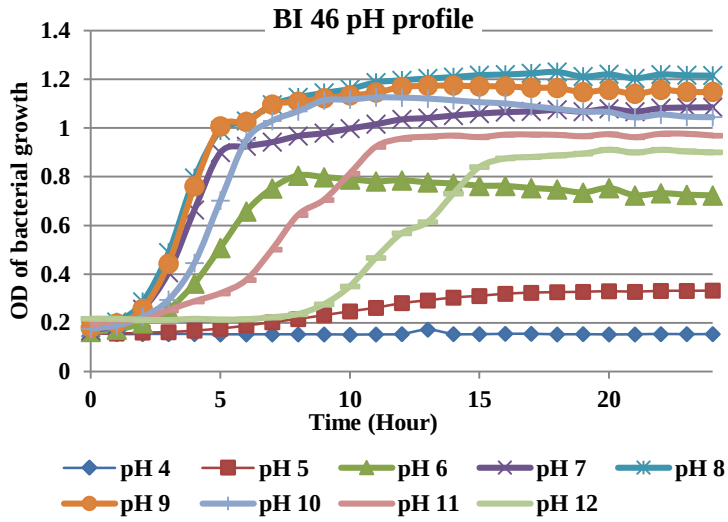


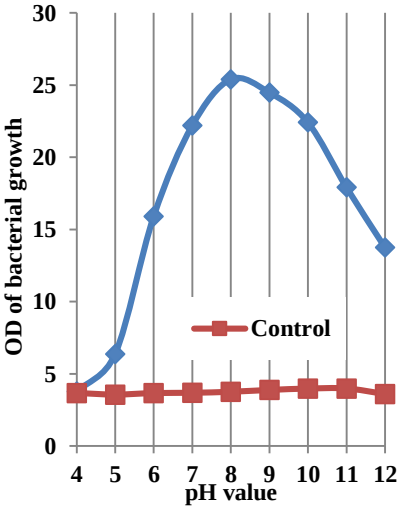
***Aeromonas salmonicida* (BI 26) pH profile include range and optimal pH**



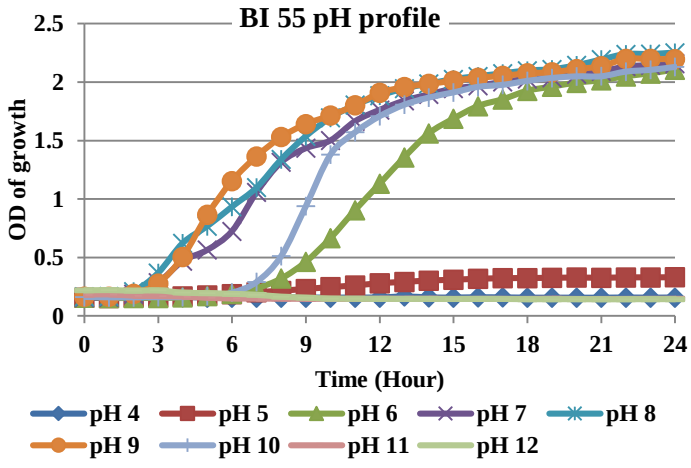


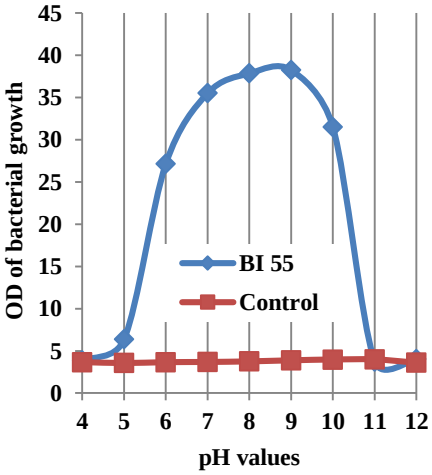
Enterococcus gallinarum **(BI46) pH profile, range and optimal pH**



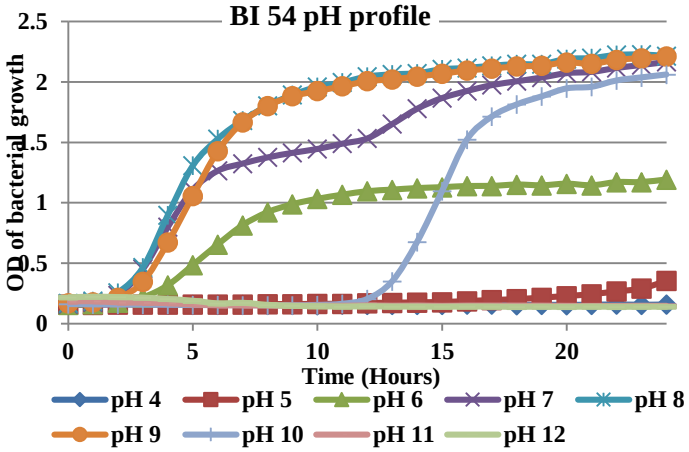


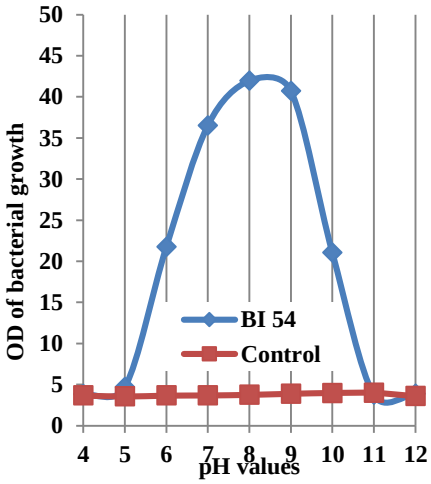
Aeromonas salmonicida **(BI55) pH profile, range and optimal pH**



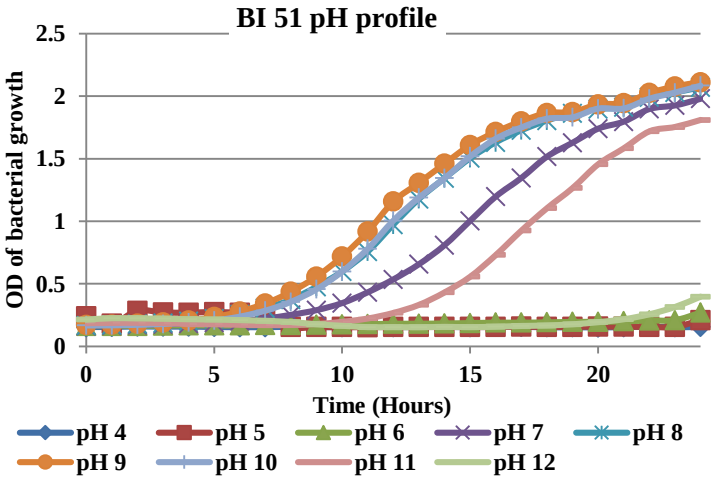


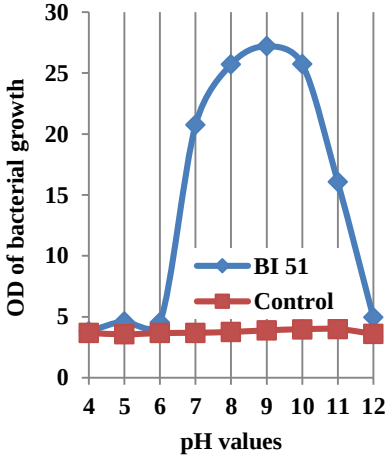
Citrobacter gillenii **(BI54) pH profile, range and optimal pH**



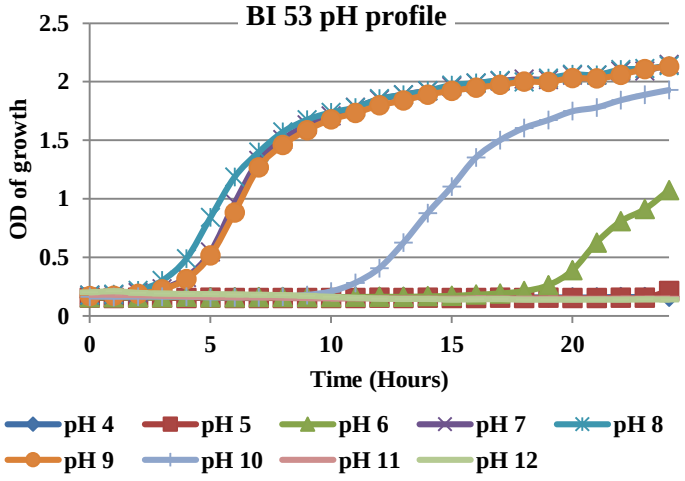


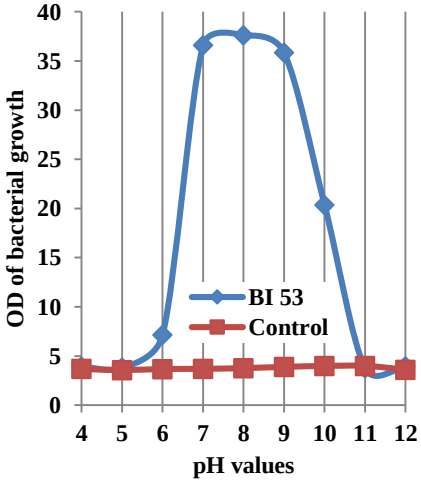
Dietzia natronolimnaea **(BI51) pH profile, range and optimal pH**



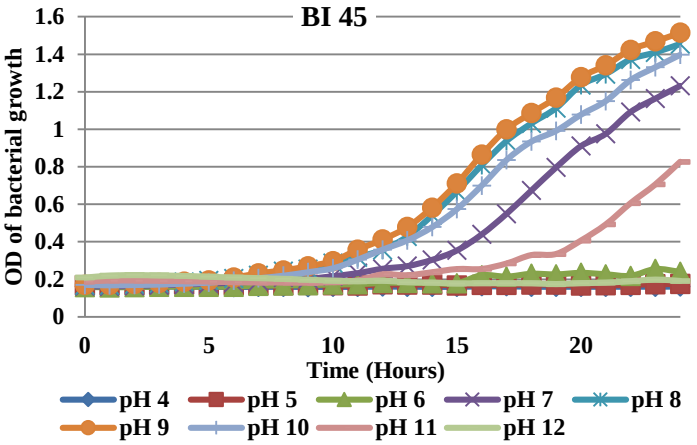


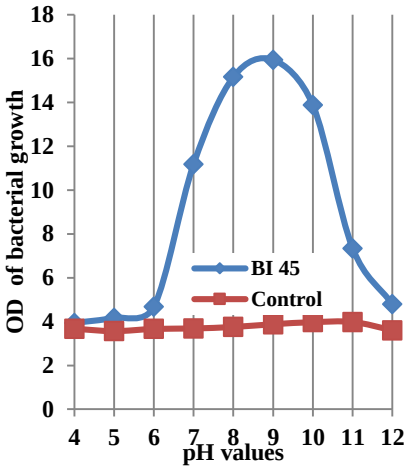
Shewanella putrefaciens **(BI 53) pH profile, range and optimal pH**



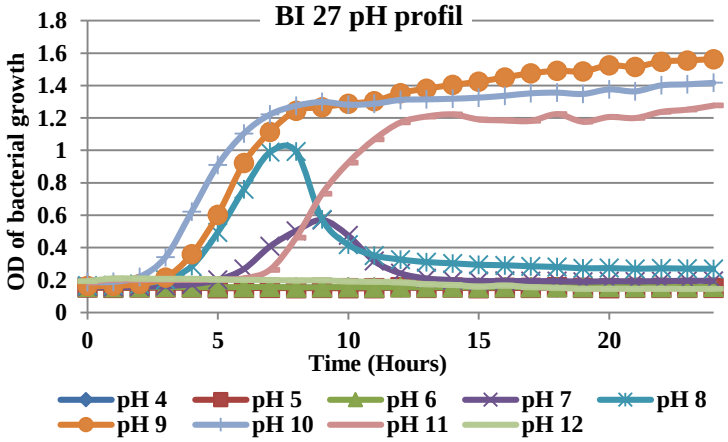


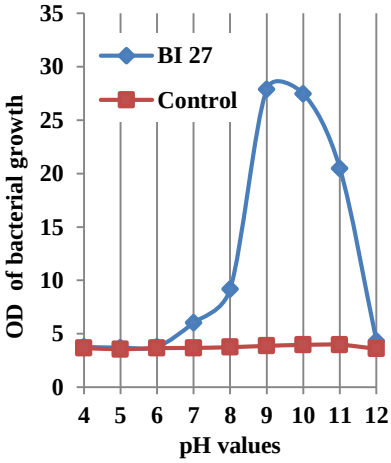
Dietzia natronolimnaea **(BI 45), pH profile, range and optimal pH**



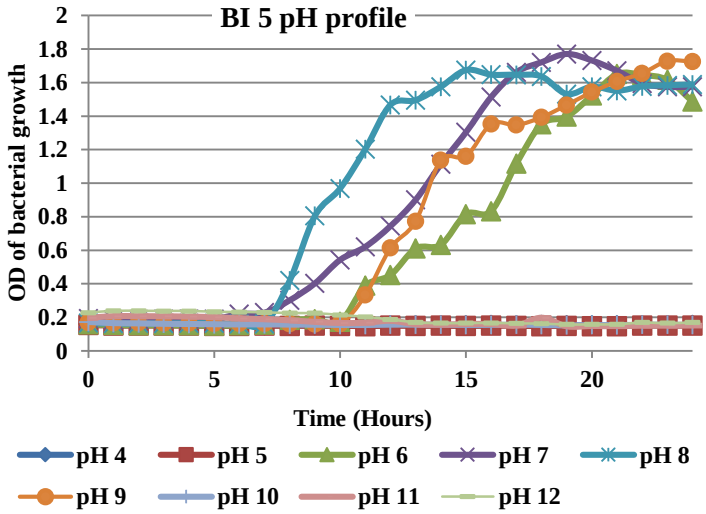


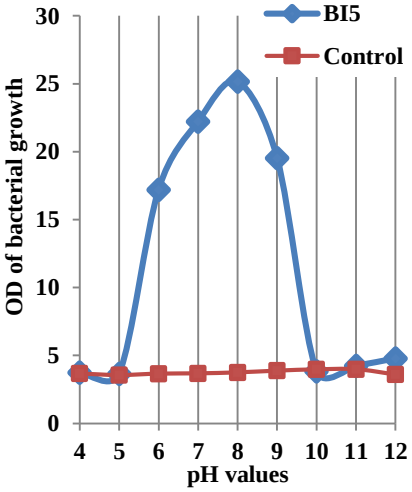
***Bacillus cohnii* (BI 27), pH profile, range and optimal pH**





***Bacilli toyonensis* (BI 5), pH profile, range and optimal pH**





***Alcaligenes aquatilis* (BI 34), pH profile, range and optimal pH**