Characterisation of Novel Isosaccharinic Acid Degrading Bacteria and Communities

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Dedication

I would like to dedicate this work to two people.

Firstly, to my dad of blessed memory, Nana Appiah Kubi for all the investments he made towards my education but sadly is not here to see its success.

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Abstract

The current plan for the permanent disposal of Low Level and Intermediate Level nuclear wastes is the cement-based geological disposal where the wastes will be disposed off in geological disposal facility (GDF). The chemical evolution of the GDF is expected to cause cellulosic materials in the waste to degrade into cellulose degradation products (CDP) of which isosaccharinic acids (ISA) are the major components. ISAs are reported to form complexation reactions with radionuclides, potentially enhancing their migration out of the GDF.

Recent studies have shown that microbial consortia indigenous to anthropogenic sites can potentially degrade these ISAs but have focused on the use of different nitrogen sources $Ca(ISA)_2$ as analogue for CDP. This study therefore aimed at characterizing novel ISA degrading bacteria and investigate the metabolic potentials of microorganism within soil sediments from Harpur Hill site to biodegrade ISAs and assess the impact of using $Ca(ISA)_2$ or CDP with NH_4^+ against NH_4^+ free systems on the chemical and microbial community evolution under conditions representative of the GDF.

In a batch/fed microcosms, microorganisms within the soil sediment were able to biodegrade ISAs in pH ranging from 9-11.5 irrespective of the source. The microbial community evolution in these systems were however different suggesting that the type of carbon source and the presence of a nitrogen source impacted on the selection of these communities. The evolution of the microcosms gave rise to a complex methanogenic, polymicrobial communities where the degradation of ISAs led to the formation of acetic acid and gases including CH_4, CO_2 and H_2. The formation of these gaseous products are likely to contribute to the pressurization of the GDF as a result of which the porosity and permeability factors should be taken into account in the formulation of the cement backfill materials. Molecular characterization of the ISA degrading communities and pure isolates (*Exiguobacterium* sp. strain Hud and *Oceanobacillu* sp. strain Hud) will allow for studies into genes associated with ISA degradation which is currently lacking in the literature. The Harpur Hill site presents a diverse pool of microorganisms with the metabolic potentials to degrade ISA hence it could be a good candidate site for the GDF.

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List of Abbreviations

AGR	Advanced Gas-Cooled Reactor
ATP	Adenosine Triphosphate
BLAST	Basic Local Alignment Search Tool
cDNA	Complementary Deoxyribonucleic Acid
CDP	Cellulose Degradation Products
CSH	Calcium-Silica-Hydrate
DNA	Deoxyribonucleic Acid
DNase	Deoxyribonuclease
EPS	Exopolysaccharide
FAME	Fatty Acid Methyl Ester
GC-FID	Gas Chromatography With Flame Ionisation Detection
GC-MS	Gas Chromatography Mass Spectrometer
GC-TCD	Gas Chromatography With Thermal Conductivity Detection
GDF	Geological Disposal Facility
HLW	High Level Waste
HPAEC-PAD	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
LLWR	Low Level Waste Repository
MEGA	Molecular Evolutionary Genetics Analysis
MIC	Minimum Inhibitory Concentration
MIDI	Microbial Identification Inc.
MPN	Most Probable Number
mRNA	Messenger Ribonucleic Acid
MSA	Metasaccharinic Acid
NCBI	National Centre For Biotechnology Information
NDA	Nuclear Decommissioning Authority
NGS	Next Generation Sequencing
NIC	Non Inhibitory Concentration
NMR	Nuclear Magnetic Resonance
NRVB	Nirex Reference Vault Backfill
OTUs	Operational Taxonomic Units
PCR	Polymerase Chain Reaction
PFLA	Phospholipid Fatty Acid
qPCR	Quantitative Polymerase Chain Reaction
RAST	Rapid Annotation Using Subsystem Technology
RNA	Ribonucleic Acid
RNase	Ribonuclease
rRNA/rDNA	Ribosomal Ribonucleic Acid/Deoxyribonucleic Acid
SE	Standard Error

SEM	Scanning Electron Microscopy
SRB	Sulphate Reducing Bacteria
TC	Total Carbon
TIC	Total Inorganic Carbon
TOC	Total Organic Carbon
U.S.A	United States of America
UK	United Kingdom
UPGMA	Unweighted Pair Group Method With Arithmetic Mean
VFA	Volatile Fatty Acid
WGS	Whole Genome Sequencing
WIPP	Waste Isolation Pilot Plant
X-ISA	Xyloisosaccharinic Acid

Chapter 1

1. Introduction

1.1. The United Kingdom's Nuclear Waste Legacy

The UK's nuclear waste generation started in the 1940s when sites were established for the development of the nuclear industry. Also during the cold war, facilities were built for the provision of weapons materials which accounted for a substantial component of the nuclear waste legacy. These include the two Windscale Pile reactors, a storage pond for nuclear fuel and a waste storage silo [1]. Currently, many industrial, medical, research and defence activities involving radioactivity and radiations contribute to the nuclear waste inventory in the UK where most of the waste is from the nuclear electricity generating industry [2]. As a result, the UK has a substantial accumulation of nuclear wastes, some of which are already in storage whereas others still form part of existing facilities still in operation and will eventually become waste as these plants are shut down and decommissioned [1].

1.1.1. Categories of nuclear wastes

A material that has no further use is considered nuclear waste if it is contaminated by radioactivity at levels above the threshold defined in the UK legislation [3]. Nuclear waste includes those with only low-levels of contamination such as those from industrial and medical sources to those with high concentrations of radioactivity such as those from defence and nuclear power stations. The classification of nuclear wastes in the UK is carried out according to the nature and quantity of their radioactivity and the amount of heat that this radioactivity can generate [4]. The main categories of nuclear waste are High Level Wastes (HLW), Intermediate Level Waste (ILW) and Low Level Wastes (LLW).

HLW includes all nuclear wastes which generate significantly higher temperature due to their radioactivity such that the heat-generating factor has to be considered in designing storage or disposal facilities for them [3]. HLW is generated in a

liquid form from spent nuclear fuel reprocessing which is subsequently treated to form solid glass blocks [4].

ILW includes wastes in which the radioactivity levels exceed the upper limits of LLW but does not generate enough heat to be considered in the design of storage or disposal facilities [3]. The major components (Figure 1.1) which make up the ILW are metals which include reactor components, graphite from the cores of nuclear reactor and sludges arising from treating radioactive liquid effluents [4].

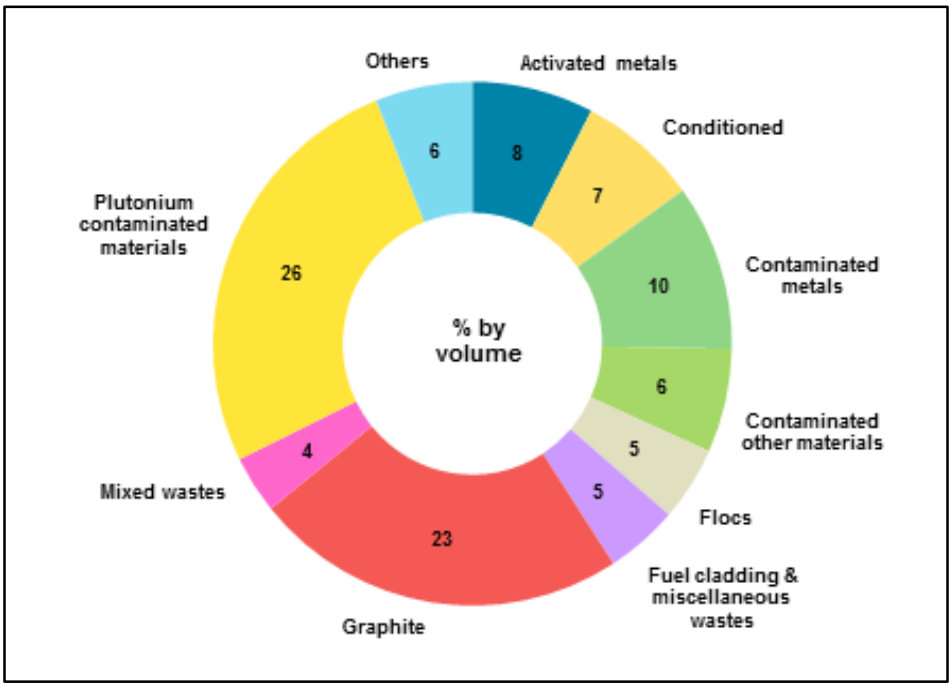


Figure 1.1 Different kinds of wastes that make up ILW taken from [2].

The LLW category of nuclear waste are wastes which contain relatively lower levels of radioactivity and includes all wastes that have radioactivity level below 4 GBq (gigabecquerels) per tonne of alpha or 12 GBq per tonne of beta/gamma activity [3]. Most of the LLW arise from the operation of decommissioning nuclear facilities whereas a few of them including (Figure 1.2) metal scraps, paper and plastics that come from hospitals and Universities that can be safely disposed

of either directly or after incineration with household, commercial or industrial wastes [4].

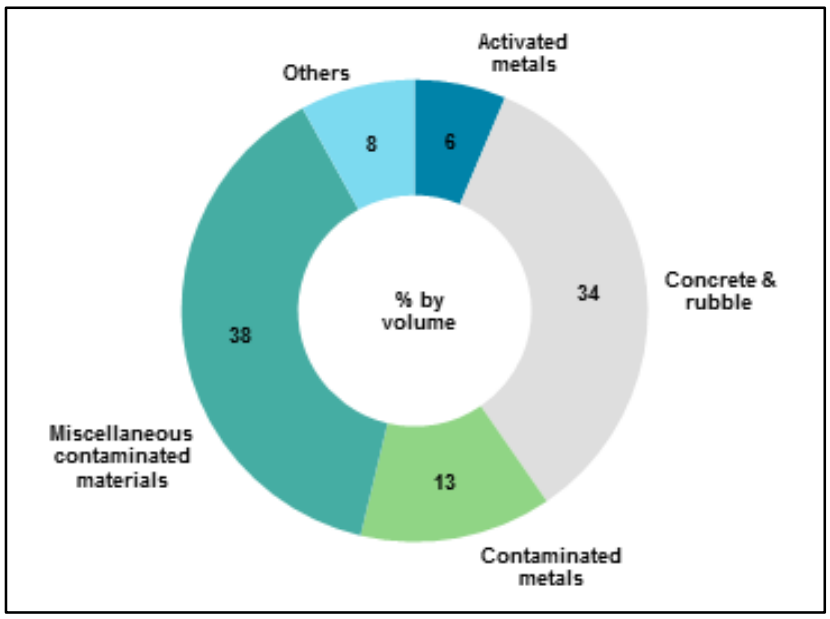


Figure 1.2 Different kinds of wastes that make up LLW taken from [2]

There is also a sub-category of LLW referred to as Very Low Level Waste (VLLW). This type of waste includes low volume loads which can be safely disposed of alongside municipal, commercial or industrial wastes for which each 0.1 cubic metre of waste contains less than 400 kBq of total activity or for which a single item from the waste inventory will contain less than 40 kBq of total activity of the waste. The VLLW also includes high volume bulk disposals with maximum concentration of 4 MBq activity per tonne of total activity which is disposed of in specified landfills.

1.1.2. The scope of nuclear wastes in the UK

As of 1st April, 2016, nuclear waste comprised of all radioactive materials that had been declared as waste and are being kept up to this date [5]. The total mass of nuclear waste and forecast in the future up to 2125 is 4.9 million tonnes occupying a volume of 4.77 million cubic metres [2] (Table 1.1)

The packaged volume is the total or 'final' volume of the waste including the waste itself, the immobilising medium and the waste container. The packaged volumes and the total number of packages is essential for the planning of the waste storage facilities.

Nuclear waste especially, the ILW is accumulating in storage due to lack of a safe disposal route. Approximately 74% of the total reported volume as at 1st April, 2016 was housed at Sellafield. The rest are stored at Magnox and Advanced Gas-cooled Reactor (AGR) power stations, Dounreay, Aldermaston and Harwell [6].

Table 1.1 Total packaged volume for all nuclear waste forecast up to 2125

Values calculated after waste packaging; adapted from [5].

Waste type	**Total cubic metres**
VLLW	2,720,000
LLW	1,600,000
ILW	449,000
HLW	1,500
Totals	**4,770,000**

1.2. Long-term management plan for LLW and ILW in the UK

The current plan for long-term management of LLW and ILW which constitute the largest volume of the radioactive waste of the national inventory is the 'geological disposal' [7] whereby nuclear waste would be placed in deep underground in a Geological Disposal Facility (GDF; nuclear waste repository) with no intention to retrieve the waste once the facility is closed [2]. The GDF

(Figure 1.3) seeks to achieve two high-level safety objectives namely, Isolation and containment of the waste. Isolation describes the system of elimination of the waste from the surface environment and away from people. This would reduce the risk of human interference and the impact of climatic conditions. The containment describes the propensity of the engineered facility to keep possession of the radionuclides over a long period of time. It is envisaged that radioactive decay will continuously reduce the amount of radionuclides within the waste, however the engineered facility has a limited ability of containment. At this time, further containment will be expected to be provided by the geosphere [7].

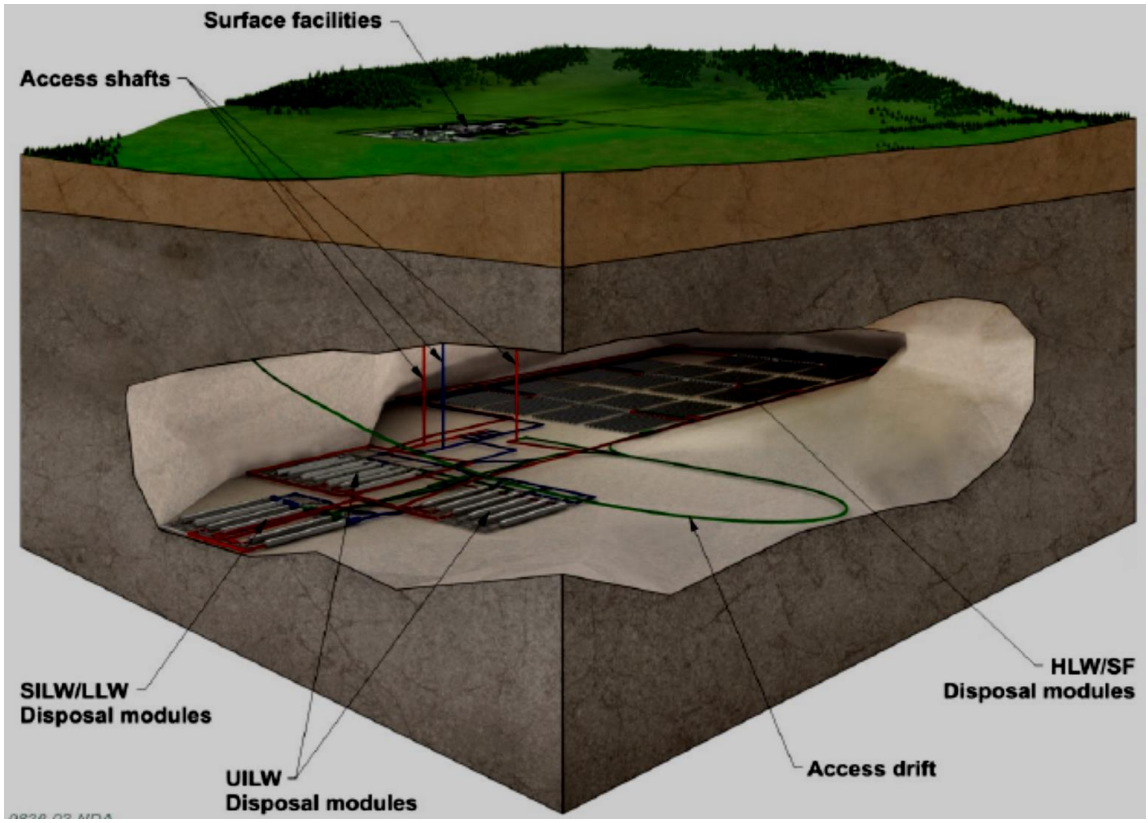


Figure 1.3 Schematic representation of the generic GDF

The generic GDF; adapted from Geological disposal - Gas Status report 2010

1.2.1. The Generic Safety Functions of the multi-barrier system

The multi-barrier system involves designing a GDF with a system of barriers that will combine with the natural barrier systems of its geological environment to prevent the migration of the radionuclides associated with the waste. This conceptual role of the GDF is referred to as the safety function [8]. The different parts of the multi-barrier geological disposal facility are expected to function effectively at different timescales in ensuring the achievement of the high level safety objectives [9]. The various components include the wasteform, waste container, backfill and the geosphere (Figure 1.4).

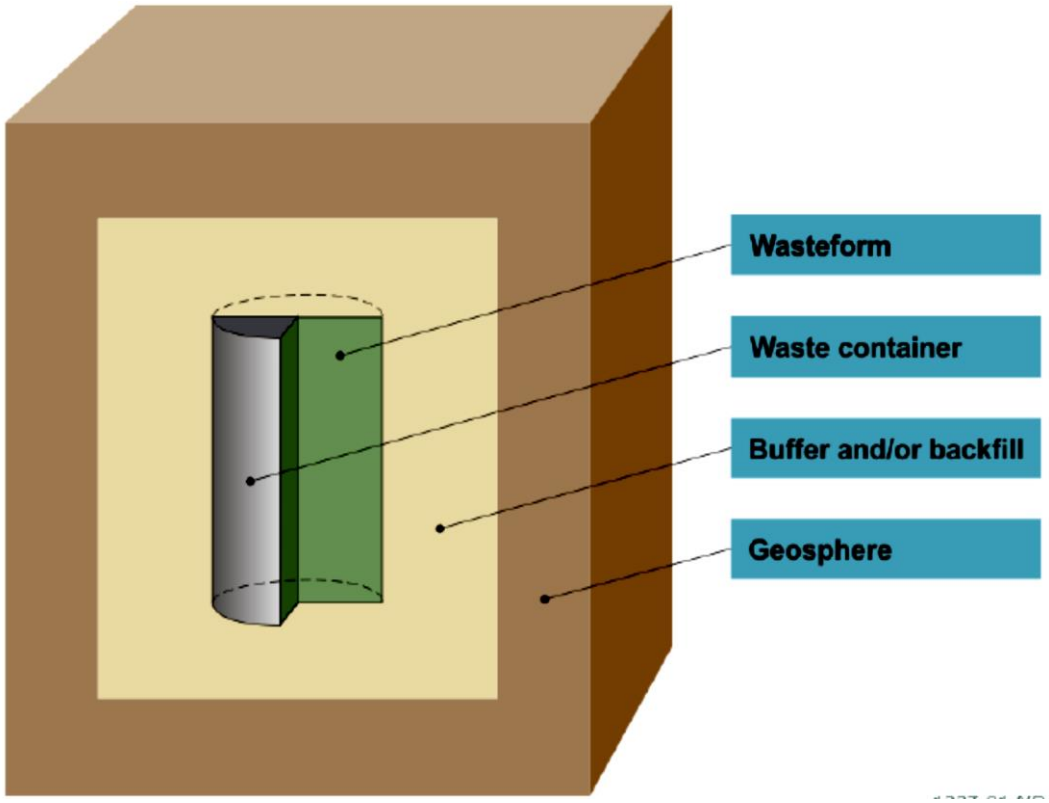


Figure 1.4 Schematic representation of the generic multi-barrier system.

Multi-barrier system; Adapted from the Geological disposal - Gas status report 2010

1.2.2. The Cement-based disposal concept for LLW/ILW

The LLW/ILW is a heterogeneous wastes consisting of homogeneous sludge, liquids and slurries immobilised in a solid matrix [7, 10, 11]. One option under consideration for the disposal of the ILW is the cement-based disposal concept. Within this concept, physical containment of the radioelements would be provided by the immobilisation of the waste and packaging in stainless steel canisters. Emplacement of the waste packages in the GDF would provide geological isolation of the radionuclides associated with the waste. The waste packages and the vaults of the repository would be backfilled with cementitious material – a Nirex Reference Vault Backfill (NRVB) after all the waste packages have been emplaced (Figure 1.5). This backfill would provide chemical conditioning and sorption capacity which are important for combating radionuclide migration from the near-field [7].

Figure 1.5. **Packaged and backfilled waste in a 500 litre ILW disposal steel drum (cutaway) taken from [12]**

After the wastes are emplaced in the GDF, sealed and the facility is closed, the entire disposal system is expected to evolve. This will enable some of the safety functions of the engineered barriers to function. Resaturation of the facility with

groundwater will generate a high pH from alkali metal hydroxides including $Ca(OH)_2$, NaOH and KOH. The temperature is expected to rise from the radioactivity of the high level wastes and environmental anaerobic condition would prevail from oxygen removal associated with the processes of metal corrosion involving the wasteform canisters and metallic structural components of the facility [13, 14].

The NRVB which is to be used for the backfill, is composed of fine aggregate of ordinary Portland cement, limestone filler and hydrated lime or Portlandite [$Ca(OH)_2$] and when dissolved in water, various hydration products including calcium-silica-hydrate (CSH) will form. Immediately after closure, the pH of the facility is expected to rise to values greater than 12.5 from the dissolution of cementitious material such as Portlandite in the ingressing groundwater thereby producing hydroxyl ions [14]. The reaction is given as follows:

$$\mathbf{Ca(OH)_{2(s)} + H_2O_{(l)} = Ca_{(aq)} + 2OH^-_{(aq)}}$$

Figure 1.6 illustrates the evolution of pH at room temperature in the cement pore fluid due to dissolution of cementitious materials.

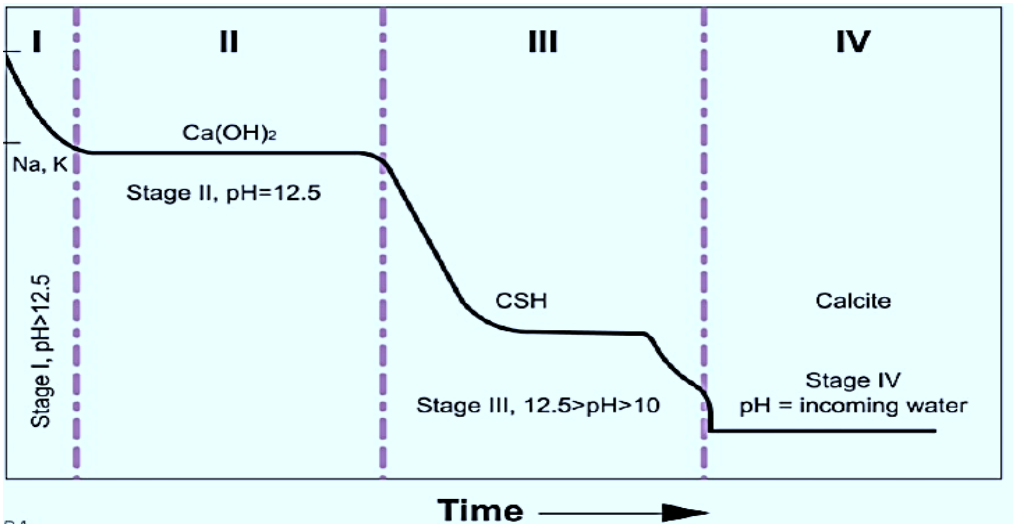


Figure 1.6 Sequential dissolution of cementitious materials in porewater and the expected pH evolution at room temperature

Image adapted from Wang *et al*. [14])

Stage I would be marked by leaching of soluble alkali hydroxides to raise the pH to above 12.5. The dissolution of Portlandite in the groundwater produces hydroxyl ions to buffer the pore water to approximately 12.5 (stage II). At stage III, further interactions of the groundwater with the hydration products of the cement such as hydrated calcium silicates (CSH) are expected to buffer the pH of the facility to establish a 12.5>pH>10.0. The pH buffering is expected to continue beyond stage III but at this stage would be mediated by lower solubility minerals such as calcite ($CaCO_3$). The pH at stage IV is expected to be about 10.0 [8].

At the early post-closure period, a number of processes are expected to generate heat which would increase the overall temperature of the facility. These processes include radioactivity of high level wastes, hydration of cementitious materials, corrosion of metals and microbial activities. In the cement-based disposal system for ILW, the most significant heat generation would result from cement hydration. The reaction of cementitious materials with porewater proceeds through a range of exothermic reactions, some of which occur rapidly and release significant amount of heat. This would increase the surrounding temperature for a number of years. In the UK ILW disposal designs using the cement backfill, once the GDF is closed, temperature is expected to reach a high of about 80^{o}C for a period of five years [15, 16]. The temperature will gradually fall as heat-producing processes diminish. Furthermore, as heat transfer occurs via the processes of conduction, convection and radiation the temperature of the facility will fall.

These chemical and thermal changes that are expected to occur within the GDF will significantly impact on the integrity of cellulosic materials present in the waste [17].

1.3. Structure of Cellulose and the Degradation of Cellulosic Materials Relevant to the Cement-based disposal concept of ILW

The cellulosic materials present in LLW and ILW include wood from contaminated furniture, cotton, paper and cloth from contaminated disposables and laboratory wear [17, 18]. Cellulose consists of chains of cellobiose units which are linked together in a repeating fashion. Cellobiose (Figure 1.7) units are disaccharides which are composed of units of D-glucose in a β-(1-4) glycosidic linkages such that each D-glucose unit is rotated through about 180^{o} compared to neighbouring molecule [18, 19].

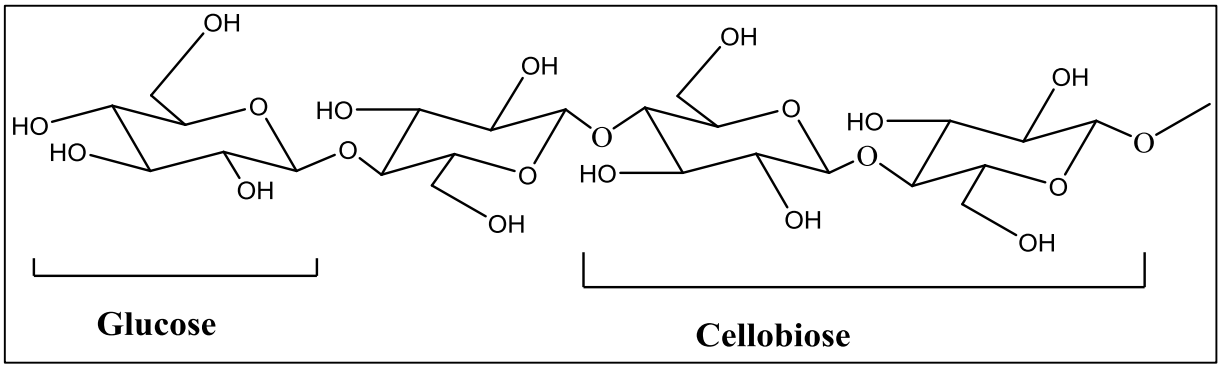


Figure 1.7 Molecular structure of cellulose

Modified structure from Zugenmaierad, (2001) [20]

Under anaerobic alkaline conditions relevant to the cement-based radioactive repository, cellulosic materials undergo degradation to form cellulose degradation products (CDP) [18]. Studies have shown that these materials decompose into a range of smaller molecules or short-chain hydrocarbons [21, 22]. The main mechanism of cellulose decomposition described in literature include physical (radiolytic) decomposition, chemical (alkaline) decomposition and biological (Microbial) decomposition [23]. The post closure events of the cement-based radioactive waste disposal concept provide a set of relevant conditions for consideration in investigation of decomposition of cellulose in those three areas. These conditions include anoxic conditions, a rise in temperature to an initial high of about 60^{o}C and falling subsequently to room temperature (25^{o}C). The

saturation of the cementitious materials with groundwater ingression creates hyperalkaline condition (pH 13.5) initially but later dropping to 12.5 as the cementitious materials decompose [22].

1.3.1. Radiolytic Degradation of Cellulose

Radiolysis is the dissociation of chemical compounds by ionising radiation [24]. Radiolytic decomposition of a polymer results from the cleavage of one or more chemical bonds within the polymer when it is exposed to a beam of high energy flux. The initial effect of ionising radiation on a polymer, in spite of its chemical structure, is the removal of electron thereby forming a positive ion [25]. When this ion is neutralised by the electron, an excited molecule is formed which later breaks up forming free radicals [25].

The exposure of gamma radiation on materials composed primarily of cellulose showed degradation of the cellulose where mid-chain scission was indicated as the dominant process of the cellulose rupture [26-28]. The results of irradiation by electron beam on cellulosic materials showed degradation mechanisms that were consistent with earlier findings [29] but alpha radiolysis of cellulose, rubber and plastic wastes appeared to be limited to measurements relating to gas evolution in the Waste Isolation Pilot Plant (WIPP) in the USA [30]. The main gaseous products from earlier work on cellulose radiolysis in the WIPP experiments included hydrogen and oxygen [31-33]. Through the process of mid-chain scission occurring at the glycosidic linkages, dry cellulose radiolysis in a nitrogen atmosphere has been shown to generate products ranging from cellobiose to celloheptose [18, 34]. Units of glucose can also degrade directly when irradiated to form carbonyl and carboxylic group and associated gases such as carbon dioxide, carbon monoxide and hydrogen [34]. Another related study has also indicated the formation of a mixture of acetone, arabinose, formic acid, glucuronic acids, gluconic acids, oxalic acid, malonaldehyde, acetaldehyde, formaldehyde, deoxysaccharides, glucose and xylose during irradiation of dry

cotton cellulose in either inert or oxygen-rich environment [35]. In these studies, no differences were noted in the components of the radiolytical products in both environments however, the oxygen-rich environment enhanced the yields of all the products especially the formic acid, malonaldehyde and other carboxy groups.

1.3.2. Anaerobic Alkaline Degradation of Cellulose

Cellulosic materials hydrolyse chemically into a range of cellulose degradation products (CDPs) under alkaline conditions [17, 36]. The major CDPs include α-isosaccharinic (α-3-deoxy-2-C-(hydroxymethyl)-erthyro-pentanoic) acid, β-Isosaccharinic (β-3-deoxy-2-C-(hydroxymethyl)-threo-pentanoic) acid (Figure 1.8), Xylo-isosaccharinic acid, α- and β-Metasaccharinic acids, hydroxybutyric acids, octanedioic acids and a range of volatile free fatty acids (VFAs). The VFAs include acetic acid, propionic acid, isovaleric acid and butyric acid. The two major CDPs that are of special relevance to geological disposal of LLW and ILW are the α-Isosaccharinic and β-Isosaccharinic acids due to their interactions with radionuclides [17, 37]**.** Their decomposition pathways, mechanisms and rates of decomposition are of special interests to researchers in order to provide information on performance of the radioactive waste repository [38-40].

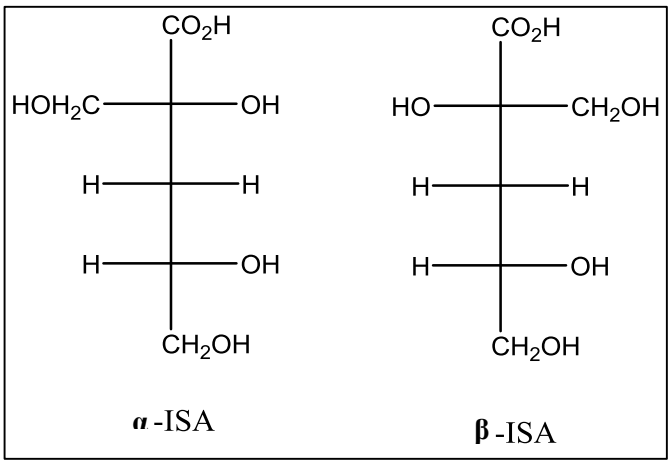


Figure 1.8 ISA stereoisomers

Three main reaction pathways have been described which underlie the end-wise degradation of cellulose under alkaline conditions [17]. These three mechanisms include the peeling reaction, stopping reaction and mid-chain scission reaction. The peeling reaction which involves a series of chemical steps, occurs at the reducing end group of the cellulose chain such that the terminal anhydroglucose unit is removed one at a time to form α/β forms of ISA. The formation of ISA exposes another reducing glucose unit at the terminus, which repeats the peeling reaction. It appears that peeling reaction can result in complete decomposition of cellulose if it is allowed to continue, however, investigation into the kinetics of the reaction has showed that due to the interference of a stopping reaction, complete dissolution does not occur [41]. The stopping reaction blocks the chemical peeling reaction and stabilises the molecular chain through the formation of alkali-stable terminal groups. These alkali-stable intermediate compounds include α/β substituted forms of glucometasaccharinic acids (MSA) [42, 43]. MSA was first identified by acid hydrolysis of alkali-stable cellulose end groups [44]. The third mechanism of cellulose hydrolysis is the mid-chain scission, which involves a hydroxide, catalysed random cleavage of the glycosidic links along the molecular chain resulting in the generation of new reducing terminal groups. The two reducing terminal groups can then proceed to a peeling reaction.

The formation of ISA from the degradation of cellulose in the cement-based GDF has received a considerable attention because firstly, it is identified as the most abundant CDP [45] and secondly due to its ability to form complex with a range of radionuclides thereby influencing their transport. For instance, ISA and associated CDPs generated during cellulosic decomposition were able to form soluble complexation reactions with thorium, uranium (IV) and plutonium [39]. The results of this study suggested that ISA concentration is proportional to plutonium solubility. For instance, ISA concentration of 10^{-5} M was found to

increase the solubility of plutonium even above pH 12.0. Moreover, ISA concentration of 1.5 x 10^{-3} M could increase solubility from 10^{-5} to 10^{-4} M. Similarly, ISA concentration of 2.0 x 10^{-3} could increase the solubility of plutonium by a factor of 2.0 x 10^{5}. Similar studies on properties of complexation reactions between ISA in alkaline solutions and radioactive elements such as thorium, americium, europium and nickel have been carried out [23, 46-50]. Although the calcium salt of alpha ISA is only sparingly soluble, it could form soluble complexes with thorium and europium at pH between 10.7 and 13.3 [51].

The ability of α-ISA to reduce sorption capacity of radionuclides was supported by the work of Tits *et al*. [23] which noted that α-ISA obstructed the uptake of thorium in a hardened cement. Warwick *et al* [48, 49] have also noted the ability of ISA to influence solubility of uranium and nickel whereas the work of Tits *et al*. [23] have shown that europium, americium and thorium are able to sorb to calcite in an ILW GDF in the absence of ISA [23]. The findings of the study suggested that ISA concentration above 10^{-5} ML^{-1} hampers the sorption of thorium (2 x 10^{-5} ML^{-1}) onto calcite quite significantly and that thorium would rather prefer to form a complex with the ISA instead of calcite.

The effects of CDPs on the solubility of radionuclides was investigated in which CDP leachates were generated from wood, rad wipes or cotton wool under anaerobic conditions over a period of 103 days, using calcium hydroxide of pH 12.7. ISA was found to be the primary product of the leachates from the decomposition of the cellulosic materials. More importantly, ISA and xylose-ISA (X-ISA) were able to enhance the solubility of europium at pH 12. ISA however showed an overpowering effect on the solubility of thorium compared to that of X-ISA which had a lesser effect [52].

1.3.3. Microbial degradation of cellulose

Direct microbial cellulose utilisation within the GDF will depend on the availability of water and nutrient, pH and temperature. As the GDF resaturates with groundwater ingress and an ambient pH of about 10.5 is generated whilst the temperature continuously declines to about 30^{o}C, direct microbial decomposition of cellulose will occur to generate gases and other short-chain soluble organic compounds [10].

In a GDF simulation experiment, Basil *et al*. [53] indicated that chemical hydrolysis of cellulose could lead to the generation of hydrolysis products, under alkaline conditions envisaged in an ILW repository. In an abiotic experiment, samples containing autoclaved tissues and cotton wool were incubated at 25 and 50^{o}C in a saturated solution of $Ca(OH)_2$ at pH 12 for 30 days. Analysis of the filtrate from these samples confirmed chemical hydrolysis of the cellulose polymers through accumulation of ISA and other soluble products. At 50^{o}C significant decrease in pH was observed in both set of experiments (from 11.8 to 9.4 and 12.1 to 9.9 respectively for tissue and cotton wool experiments). ISA concentration also accumulated in both experiments (10.4mM and 3.5mM respectively for tissue and cotton wool). At 25^{o}C however, almost a stable pH was observed in both experiments (12 to 11.8 for tissue and 12.3 to 12.0 for cotton) whereas small ISA concentration accumulated in both experiments (6.5 mM and 1.5 mM respectively for tissue and cotton wool). In the biotic experiment, when samples were inoculated with sediments from a hyperalkaline site at 50^{o}C, the pH and ISA profiles were similar to their abiotic counterparts indicating that the decomposition of the cellulose polymers were through chemical hydrolysis. However, at 25^{o}C, microbial activity was observed through enzymatic hydrolysis of cellulose with accumulation of acetate. The microcosms became acidified which marked a decrease in pH and ISA concentration. Analysis of microbial DNA from the biotic experiments supported the claim that activities of microflora

can influence cellulose decomposition and formation of organic acids including ISA through interplay between direct and indirect mechanisms [35]. Consistent with earlier studies, these findings imply that microbial activities can have a significant role in attenuating radionuclide mobility in the near-field of the LLW and ILW disposal sites.

1.4. Prevalence of ISA and other CDPs in the natural Environment

Although the distribution of ISA in the natural environment is quite uncommon it is expected to be present within sites contaminated with effluents of Kraft paper mills [54]. In the Kraft process, wood is shredded in a chipper to increase surface area and then treated with white liquor that contains sodium hydroxide and sulphides at elevated temperatures of between 160 and 180^{o}C. The alkaline conditioning causes the lignin bonds to cleave which leads to solubilisation and subsequent degradation of the lignin [55, 56]. The celluloses and hemicelluloses also solubilise in the alkaline medium and chemically decomposed through the peeling reaction [56, 57] which generates a range of CDPs including ISA.

In a recent study, Rout *et al* demonstrated that both apha (α) and beta (β) stereoisomers of ISA could be generated *in-situ* depending on the prevailing environmental conditions [58]. They generated the ISA from an uncontaminated soil and alkaline leachate (pH13.6) at 4, 10 and 20°C. The authors also detected ISA, acetic acid and other VFAs from both soil and pore water obtained from the hyperalkaline soil at Harpur Hill, Buxton. Further analysis also revealed the presence of Fe(II) and Fe(III), nitrates and sulphates. Together, these findings suggest that ISA, although not common in the natural environment, it could still be available in some areas depending on the conditions prevailing there.

1.4.1. Evidence of microbial degradation of ISA under repository conditions

Greenfield *et al*. [59] have noted that even though the distribution of ISA within the natural environment is quite rare, there is no microbiological evidence to indicate its resistance to biodegradation. Studies by Strand *et al*. [54] and Bailey *et al*. [60] suggested that bacteria that are able to utilise ISA were not widely distributed but they were found particularly on sites contaminated with wastes rich in ISA from paper pulping industries.

Earlier studies on microbial ISA degradation began with Williams and Morrison who investigated the metabolism of α-D-glucoisosaccharic acid in rumen bacteria and holotrich protozoa. They generated saccharinic acids, which included metasaccharinic and isosaccharinic acids as well as lactic acids from treatment of timothy grass stem tissues with sodium hydroxide. Using the saccharinic acids as carbon sources for the bacterial and protozoal isolates from the rumen of sheep, their principal findings were that under anaerobic conditions, alkali breaks down forage material and produces saccharinic acids which were degraded by the rumen microorganisms but were poorly utilised as compared to D-glucose [61].

In another study, Horiko *et al*. [62] isolated microorganisms from soil and described a novel method that could be used to cultivate them in media that contained black liquor. The study also indicated that those microorganisms could survive and grow under high alkaline conditions of up to pH 10.0, which relates to repository hyperalkaline condition. In spite of those findings, a major denunciation of this study is that the authors could not determine the source of the carbon, which was being utilised by the microorganisms. Black liquor, a waste product from kraft process contains other carbon compounds in addition to ISA and MSA, it is therefore possible that those microorganisms were utilising carbon sources present in the media other than the saccharinic acids.

Strand *et al*. [54] investigated into the potentials of microorganisms to degrade ISA under aerobic conditions. α-Glucoisosaccharnic (GISA) was synthesised and

used a carbon source alongside glucose in mineral media containing Bacto-Peptone and yeast extract and pH adjusted to 7.2 or 9.5. In addition to laboratory strains of Gram-positive and Gram-negative bacteria, isolates obtained from aerobic wastewater treatment pond of a kraft paper mill, swabs from pulp washers, sludge of municipal treatment plant and bottom of surface litter layer of forest soil were tested on the media for their GISA biodegradability at 20^{o}C to 30^{o}C for a period of one week. The main findings were that neither the laboratories aerobic bacteria nor the facultative anaerobic bacteria from the forest soil were capable of biodegrading GISA. However, a number of microorganisms obtained from the pulp mill pond could degrade GISA. The authors therefore deduced from the findings that common forest soil microorganisms were unable to utilise GISA as a carbon source and that unlike the breakdown of other carbohydrates, GISA metabolism perhaps requires unusual or modified enzyme(s) that is present in microorganisms living in environments contaminated with ISA such as the paper mill sites [63]. Strand and co-workers identified a pond isolate capable of GISA biodegradation as *Ancylobacter aquaticus* and that growth was enhanced in all the isolated strains when the media was enriched with vitamins, peptone and phosphates. They suggested that organisms capable of ISA degradation may require special growth factors [63].

Bailey [60] also demonstrated that GISA could be metabolised by a bacterium that grew well on media enriched with GISA as the sole carbon source but at acidic and near-neutral conditions. The isolate was obtained from mud at the bottom of lake that had been contaminated by effluents from a pulping industry. The absolute limit of growth for the isolate was found to be between 5.1<pH<7.2 however, optimum growth was achieved at pH 6.0 ± 0.5. Initial GISA concentration of 2 gL^{-1} was completely consumed within 24 hours in batch laboratory fermentation. The isolate was found to be incapable of utilising glucose and appeared to be independent of organic nitrogen [60]. A major criticism of this

investigation is the acidic pH at which this isolate biodegrade ISA as GISA is a product of an alkaline attack on cellulose and so found mainly in alkaline environments [62]. It is therefore probable that this isolate may not be functional under conditions expected to dominate the ILW disposal facility.

Pekarovicova *et al*. [64] also recovered eight isolates from an environment polluted with sulphate pulp mill and reported that all were capable of utilising GISA. They identified the best GISA utilising bacterium as *Microccocus lylae* and reported that *M. lylae* was capable of growth and GISA utilisation within a range of pH between 5.0-9.0 with the optimum being pH 8.0 [64]. In a subsequent study, the authors reported that *M. lylae* completely utilised GISA within 48 hours, whereas 67%, 45% and 38% respectively for 2-hydroxubutanoic acid, lactic acid and glycolic acid were consumed under the same experimental condition [65].

In an investigation into anaerobic degradation of GISA, Wang *et al*. [66] indicated that α-GISA could be used effectively as a source of energy for anaerobic bacterial growth. The study explored the potentials of microbial consortia within a bleach plant earmarked for the treatment of kraft waste effluents to consume either glucose or GISA with or without chlorinated phenols. In those experiments, the microorganisms within the inoculum were capable of utilising both glucose and GISA as carbon sources and generated methane. The authors observed that the two carbon sources (glucose and GISA) produced different methanogenic consortia that led to variation in the rates of production of methane [66].

One of the pioneering works regarding the effects of microbiology on ISA in terms of radioactive disposal in the UK was that of Grant *et al*. [67] where a range of microbial consortia obtained from natural and alkaline contaminated sites were investigated for their ISA biodegradability potential. The study showed microbial growth was limited at pH 10.5 within ISA enrichment seeded with sample from the natural, uncontaminated soil. However, when the same sample was inoculated

into media that contained carbon-14 labelled ISA, and nitrate as a source of nitrogen, the ISA was found to have oxidised and a net reduction of nitrate to nitrite and further reduction from nitrite to nitrogen gas observed. A carbon-14 labelled carbon dioxide gas was also detected indicating that the carbon dioxide had been generated from the carbon-14 labelled ISA. In a continuous flow chemostat experiment in which planktonic cells were inoculated, a variable rate of ISA degradation between 5.6 and 0.2 $molyr^{-1}g^{-1}$ was observed and when a range of biofilms were cultivated on concrete blocks made from NRVB, the rate of degradation of ISA was between 40.6 and 350 $mol^{-1}yr^{-1}m^{-2}$ [67]. Interestingly, these investigations on biofilms demonstrated the ability of microbial consortia within biofilm to degrade ISA under hyperalkaline condition of pH 12.5.

Francis *et al*. [68] in their investigation into biodegradation of organic ligands in transuranic wastes (TRU) predicted that the biotransformation of radionuclides that had complexed with organic ligands should precipitate the radionuclides and retard their migration. The authors isolated an aerobic bacterium from an enrichment media inoculated with soil sample and demonstrated that the aerobic bacterium was capable of ISA biodegradation from an actinide-ISA complex.

In another study relevant to nuclear waste, Maset *et al*. [69] investigated into the effect of organic co-contaminants including ethylenediaminetetraacetic acid (EDTA), nitrilotriacetic acid (NTA) and ISA on the speciation and solubility of technetium and Rhenium under reducing conditions. They noted from their microcosm experiment that under nitrate reducing conditions at pH 6.25, microbial populations within silty loam soil appeared to have removed the organic complexes including ISA from the porewater that originally contained pertechnetate-organic (TcO_4^- - organic) complexes. However, the authors did not observe any reduction in the perrhenate-organic (ReO_4^- - organic) complexes within the period of incubation [69].

A study by Basil *et al*. [70] focused on the degradation of ISA under hyper-alkaline conditions. They produced a range of enrichment cultures with calcium salt of α-ISA being the sole carbon source and described the biodegradation profiles of ISA under aerobic and nitrate reducing conditions by microbial community within sediments obtained from legacy lime works site in Buxton. Ecological analysis of their consortia suggested that Proteobacteria was the dominant genus of the aerobic culture while the nitrate reducing cultures were dominated by the facultative anaerobic denitrifiers from the genus, *Azoarcus*. Consistent with earlier findings with Grant *et al*. [71] the authors attributed the oxidation of ISA to bioreduction of nitrate.

The generation of ferric iron from the corrosion of the GDF materials made of steel and the presence of sulphates due to groundwater ingress that may support microbial bioreduction processes have been investigated [10, 72]. Against that backdrop, Rout *et al*. [73] determined the potential of microbial communities within anaerobic near-surface sediments to utilise CDP and associated ISAs under iron and sulphate reducing conditions as well as under methanogenic conditions. They investigated into the microbial processes that may occur at the interface between ungrouted cellulosic wastes and the cementitious backfill as well as between the facility and the host rock that is receiving ISA leachate. Using sediment samples from Leeds/Liverpool canal, the microcosms amended with haematite showed reduction of Fe (III) to Fe (II) which coincided with biodegradation of ISA. The fermentation of ISA in the iron reducing system led to the production of acetic acid which was also subsequently biodegraded. Formation of methane was observed which indicated the presence of active methanogens within fermentative and iron-reducing communities. Community analysis of the microcosm showed that iron reduction was due to activities of *Geobactor* species and organisms from sulphate reducing bacteria (SRB) groups that were present in the microcosm. Methanogenic bacteria that are capable of

aceticlastic and hydrogenotrophic metabolism were also observed within the microcosm. In the sulphate reducing microcosm, the presence of sulphate favourably selected SRBs to dominate the redox process. Sulphide was produced while acetic acid was accumulated alongside ISA degradation from CDP. Sulphate reducing bacteria were observed alongside Clostridia from the microcosm through direct PCR method. Within the methanogenic microcosm, the degradation of both isomers of ISA resulted in the production of acetic acid (which was later utilised) and the generation of methane. The community profile by direct and nested PCR confirmed the presence of Clostridia and methanogens. Alongside bioreduction of iron and sulphate and methanogenic conditions fermentation processes were observed which led to the production of volatile fatty acids including acetic acid, propionic acid, isobutyric acid, butyric acid and isovaleric acids [73]. The authors concluded that although both stereoisomers of ISA are not naturally observed in the wider environment, however, microorganisms present in anoxic sediments are able to utilise both forms of ISA as carbon source through redox processes at approximately neutral pH.

Furthermore, the authors seeded soil sample contaminated with the leachate into a CDP-driven microcosm that operated at pH11, microorganisms within the soil were able to utilise ISAs and generated methane, carbon dioxide and hydrogen. Microbial community analysis indicated the dominance of genus *Alkaliphilus* from Clostridia in the fermentation systems. Methanogenesis was attributed to the activities of dominant species from *Methanobacterium* and *Methanomassiliicoccus*. The authors concluded that at hyper-alkaline (pH11.0) anoxic conditions, sediments at the legacy lime kiln site, Harpur Hill, Buxton, Derbyshire, contain active microbial communities that are capable of utilising both α and β forms of ISA as a carbon sources to generate acetic acid, hydrogen and methane. They suggested that ISAs may act as key electron donor to support the growth of microorganisms. Based on the findings of their study, they predicted

that microorganisms that are capable of ISA degradation will potentially evolve within the cementitious ILW disposal facility decades after closure if the ambient pH falls within pH11. Providing that microbial activities are not limited by pH, their results suggest that microbial consortia similar to that which has evolved at the legacy lime kiln site in Harpur Hill Buxton, Derbyshire will establish within the cementitious GDF and be able to metabolise all forms of ISAs to mitigate the impact of ISA-radionuclide complexation reactions [73].

Kuippers *et al*. [74] reported the fate of ISA under circumneutral conditions including direct oxidation of ISA under aerobic and nitrate-reducing conditions and under anaerobic conditions where ISA was fermented to acetate, propionate and butyrate under Fe(III) and sulphate reduction. The authors reported the microbial communities of the aerobic cultures were dominated by Betaproteobacteria whereas majority of the fermentation reactions were dominated by Firmicutes maily from class Clostridia. The authors noted that addition of electron acceptors minimised methanogenesis within within those communities [74].

Microorganisms usually exist in biofilms in mixed communities within the natural environment and that biofilms exist in a wide range of environments [75, 76]. Some components of microbial biofilm such as extracellular polymeric substance including polysaccharides, proteins, lipids and nucleic acids enhance the survival and growth of microorganisms [77] and offer stronger resistance to environmental stress including temperature fluctuations, desiccation, pH and ultra-violet radiation [78-82]. In relation to the microbiology of the ILW GDF, the migration and adherence of microorganisms to establish niches in order to survive and grow is important to radionuclide migration. In view of this, Charles *et al*. [83] cultivated biofilm forming consortium *in situ* that colonised cellulosic materials under anaerobic conditions and subsequently described the propensity of the microbial consortium to degrade CDP. The researchers incubated cellulosic

materials (cotton) in an inert plastic liner with an open lower base placed in a 0.5 m deep borehole in an area inundated with alkaline leachate (pH12.0) at Harpur Hill, Buxton, UK. Following three months of incubation samples were retrieved and were observed to have undergone partial hydrolysis and colonised by a Clostridia-dominated biofilm community. The presence of hydrogenotrophic, alkaliphilic methanogens was also indicated. When these samples were seeded into a microcosm driven by CDP under alkaline anaerobic conditions, the microbial communities within the sample were able to utilise all forms of ISA from the CDP. The researchers observed a shift in the dominance of the microbial community from Clostridia and methanogens (which appeared to have been masked) to an established flocculate community which was dominated by *Alishewanella* species. The flocs consisted of bacteria embedded in polysaccharides and proteins fixed by extracellular DNA [83]. Consistent with earlier studies, this study demonstrated additional evidence of biodegradation potential of alkaliphilic microbial communities under alkaline, manmade environments. However, the survival and growth of microorganisms within the ILW cementitious GDF is largely dependent on the ability of the microbial communities to tolerate the calcium dominated, high pH environment established from the dissolution of cementitious materials in percolating groundwater. Following on from their previous study on flocculate producing microcosm operating at pH11, the authors investigated how floc formation reduces the stress experienced by microorganisms living in alkaline environments. Flocs from this microcosm were isolated and their structural morphology, EPS composition and pH profiling as well as response to alkaline conditions were investigated. The findings of this study demonstrated that through flocculation, microbial communities within a hyperalkaline environment are able to survive and grow which supports earlier findings that the success of microorganisms in the environment is determined by the microenvironment that they create in lieu of the bulk environmental chemistry [84, 85]. Charles and his co-workers concluded that

multi-species floc formation composed of a complex mixture of EPS has shown the ability to maintain a lower internal pH and provide protection for the survival and propagation of microorganisms against hyper-alkaline environment similar to that which is expected to occur in the cementitious ILW-GDF [86].

1.5. Methods for the identification and characterisation of microbial populations within an environmental sample

1.5.1. Culture-based techniques

In order to isolate and enumerate microorganism from real sites, or from model systems associated with radioactive waste disposal, researchers have adopted the culture-based techniques [87-90]. These techniques involve growing microorganisms present in a sample in broth or on a solid media such that the kind of microorganism that is isolated is determined by the components of the media and conditions in which incubation is done. Culture-based techniques allow the enumeration of either the total or a specific group of culturable microorganisms to be estimated by counting them directly on solid media or by adopting the Most Probable Number (MPN) statistical technique.

In estimating bacteria numbers by the direct counting (spread plate) method, bacterial colonies on solid media can be grown by the classical streaking method, pour-plate or spiralling method while the counting is done manually or aided by colony counter machines or spiral machine readers. Vreeland *et al*. [87] adopted the culture-based approach to determine the size of the bacterial population as well as the heterogeneous distribution of halophilic microorganism in a hypersaline environment earmarked for the disposal of transuranic wastes in the US [87]. Farkas *et al*. [89] also used this approach to characterise and enumerate the subterranean bacteria in the Hungarian Upper Permian Siltstone (Aleurolite) formation in order to assess the safety of future nuclear waste facility.

In the MPN enumeration method, a 10-fold serial dilution of sample is prepared

followed by inoculation of 1 mL of each dilution in triplicates in broth cultures for incubation. After incubation, the culture tubes are examined for growth (turbidity). The pattern of growth in the culture tubes is compared with a standard MPN table [90, 91]. The MPN method has been used to estimate the microbial populations that are likely to potentially present within the geological disposal sites U.S, Japan and Scandinavia [92-94].

The main advantages of the culture-based method include firstly, allowing the isolation of culturable microorganisms, which could be subject to further investigations. Secondly, it is well established and thirdly, the cost involved is relatively low as well as technically undemanding. However, not all microorganisms are culturable under laboratory conditions thus some important microbes within an environment of interest may not be isolated [95, 96]. Developing a model that matches the conditions of the specific geological environment from where the sample has been obtained (in terms of organic source and culture conditions) and allowing a long incubation time are essential to the recovery of a more representative microbial populations [95]. However, matching the geochemical needs of the environment to that of the model may be too difficult whereas allowing long period of incubation is time consuming and susceptible to contamination.

1.5.2. Microcosm investigations

Microcosms are models or representations that simulate the characteristics of interest within the context of a wider environment. Microcosms may be set up using a defined or undefined community of microorganisms or a specific bacterium and operated according to the objectives of the study. The operation of the microcosm may be carried out through a batch process in which the system is completely closed until the end of the experimental procedure or may be run as a continuous flow process in which nutrients are regularly fed into the system. Single-isolate operated microcosms have been used to investigate the direct

interactions between radionuclides and microorganisms such as *Microbacterium, Geobactor* and *Shewanella* spp. [97, 98]. Horn *et al*. [99] investigated microbial effects on corrosion of metals for nuclear waste packaging using single-species operated microcosms [99]. Most studies have adopted batch microcosms for the investigation of gas generation from microbial metabolism of the organic components of nuclear wastes [100-103]. The continuous flow system which is suitable for the investigation of the impact of microorganism on soluble chemical species was employed by Horn *et al*. [99] in a study of the denitrification potential of the indigenous microflora of Yucca Mountains rock samples. Usually operated through a column, the continuous flow microcosm has been used by researchers such as Kelly *et al*. [104] in the investigation of radionuclide (^{241}Am) release from a LLW, Nikolova *et al*. [105] for the investigation of bioclogging [105], and in the investigation of the potential interactions between biofilm formation, minerals and corrosion of repository materials [106-108].

Some microcosms are enriched with specific nutrients or growth factors to form enrichment cultures and incubated under specific conditions that promote the growth of microorganisms of interest [109]. Enrichment cultures select only a small number of microorganisms of the original sample to dominate the population and hence are not representative of the entire bacterial population. This method cannot therefore be used to enumerate different groups of microorganisms. Beadle *et al*. [100] employed the enrichment culture technique to assess the processes by which terminal electrons are transferred within radiological sites**.** The enrichment approach has also been used to investigate the impact of iron reduction on the behaviour of uranium [110], the recovery of sulphur reducing bacteria (SRB) and acetogenic bacteria from bentonite [111]**,** factors influencing microbial growth and activities in geological samples from Yucca Mountain [112] as well as the extent to which cellulose will degrade under the WIPP conditions [87]. In their recent studies, Bassil *et al*. Rout *et al*. Charles

et al. and Kuippers *et al*. [70, 74, 83, 113] used either $Ca(ISA)_2$ or CDP enrichment approach in investigating microbial community degradation of ISA.

1.5.3. Biochemical/Physiological characterisation

The kind of substrate that microorganisms utilise provides information about their functional activities based on the type of enzymes they have. The differences in specific enzymatic activities that an organism can undertake also provide indications about the physiology and the natural habitat of the organism [114].

Physiological characterisation is the identification of microorganism on the basis of utilisation of specific substrate and subsequent generation of metabolic products [115, 116]. Garland and Mills [117] proposed pattern analysis of carbon-source utilization as simple and rapid method for characterising heterotrophic microbial communities. The microtitre plates developed by Biolog Inc., have been extensively used for the characterization of microbial communities.

Some researchers have used the physiological approach to identify a range of microorganisms that live particularly, at geological disposal sites. For instance, Vreeland *et al*. [87] used this approach to assess the distribution and diversity of halophilic bacteria in a subsurface salt formation in the US, whereas Nedelkova *et al*. [97] adopted the approach to identify *Microbacterium* isolates recovered from a Siberian radioactive waste repository which could tolerate high levels of uranium, lead, copper, silver, cadmium, nickel and chromium [87, 97]. Other researches have also used radio-labelled carbon compound techniques in conjunction with the physiological method to investigate biodegradation of organic substrates and sulphates reduction in studies relating to radioactive waste disposal [112, 118].

1.5.4. Biochemical markers Based methods

The use of some biochemical markers such adenosine tri-phosphate (ATP) and Phospholipid fatty acids (PLFA) can provide information about the type of microorganisms living in a particular geological site. ATP is an essential coenzyme for the transfer of energy within cells hence its presence is an indication of biological activity [119]. Total ATP from a particular soil sample may be considered a function of the active microbial population within the sample. Investigation into the amounts of ATP in both shallow and deep groundwaters in comparison with viable and direct microbial counts in the Fennoscandian Shield demonstrated the robustness and reliability of the method within a detection limit of 2 X 10^3 cells mL^{-1} [120].

PLFA are membrane components of microorganisms which degrade rapidly with cellular membranes following cell death. A measure of PLFA is therefore a measure of active microbial populations in a sample and provides information about the total microbial biomass levels. In addition, it indicates the community structure of the microorganisms present in a particular location since some PLFA biomarkers are specific to a particular group of microorganisms. Estimation of microbial populations within samples associated with radioactive disposal such as opalinus clay and buffer materials from *in-situ* experiments as well as samples from long term corrosion experiments using PLFA has been successfully carried out in the US, Canada and Switzerland [93, 121, 122].

1.5.5. Molecular based methods of microbial community analysis

The vast majority of microbial communities within the natural environments are yet to be cultured with the classical laboratory based methods. As a result, the primary source of information for these microorganisms is their biomolecules which include nucleic acids, lipids and proteins. The culture–independent nucleic acid techniques for microbial community analysis involves a comparative

analysis of either whole genome or a selected gene such as 16S rRNA leading to structural and functional diversity of microorganisms [123]. These approaches can be broadly be put into two main categories, namely, whole community analysis and partial community analysis, depending on their ability to provide detailed information on microbial diversity structure and functions [124]. The different approaches are shown in Figure 1.9.

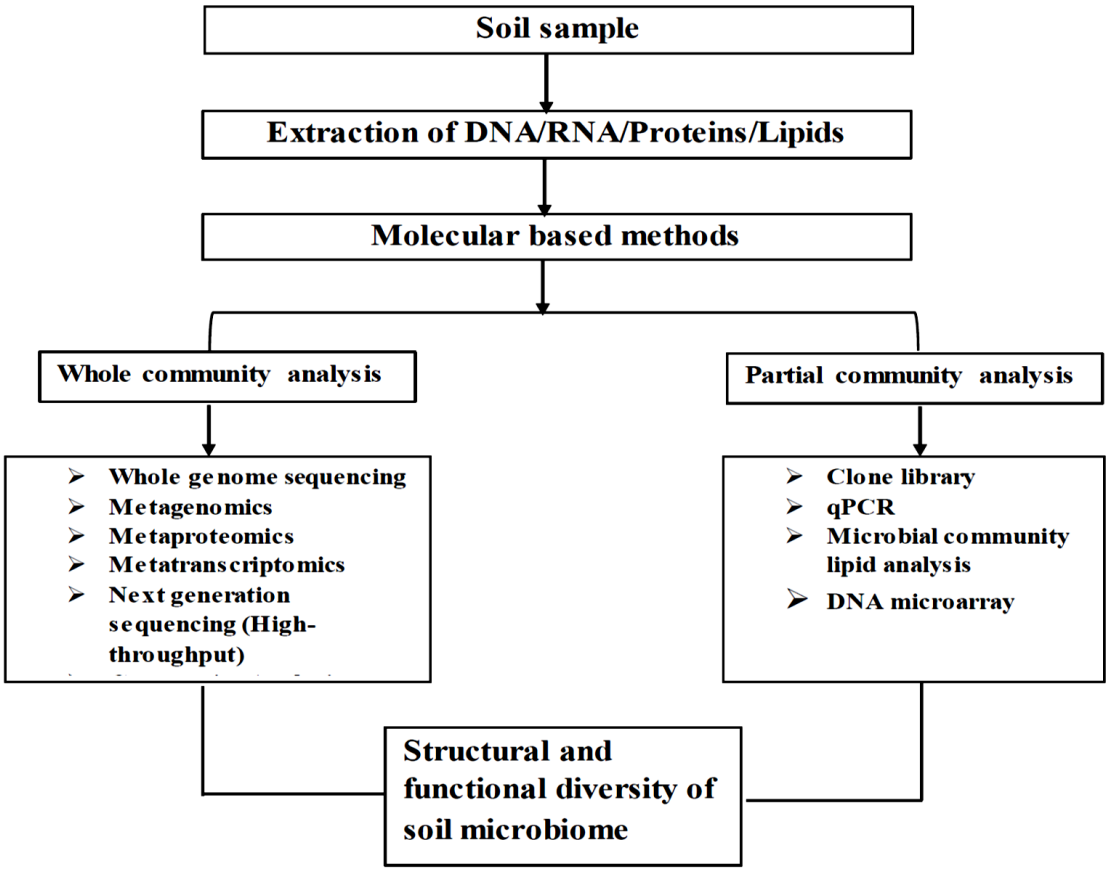


Figure 1.9 Culture-independent molecular techniques for the characterisation of structural and functional diversity of soil microbiome

1.5.6. Partial community Analysis

Approaches involving the use of partial community analysis generally include polymerase chain reaction (PCR) where total DNA/RNA is extracted from the soil sample and is used as the template for microbial characterisation. The principle behind this method is that the PCR products reflect a representative microbial signatures from all microorganisms present within the sample. In these

methods, PCR amplification of conserved genes such as 16S rRNA from a sample is widely used for microbial characterisation. The 16S rRNA is present in all species of bacteria but varies in some regions among different species. It is this variation among individual species which are exploited through molecular technological advancement to identify an organism or show its phylogenetic lineage. The choice of this gene for ecological studies is primarily due to its ubiquity in all prokaryotes, the fact that it contains structurally and functionally conserved regions as well as the variable and highly conserved regions [123]. Moreover, the suitability of the approximately 1500 bp gene size and the availability of the 16S rRNA gene sequences on sequence databases for comparison make it a good choice for ecological characterisation of soil microorganisms. In these methods, by evaluating the phylogenetic relatedness to known microorganisms on the basis of 16S rRNA sequences homology, the closest affiliation of the unknown isolate is assigned [125]. Other genes which are also conserved and are used in microbial investigations include RNA polymerase beta subunit (*rpoB*), gyrase beta subunit (*gyrB*), recombinase A (*recA*) and heat shock protein (*hsp60*) [126]. The amplified PCR products from environmental sample are usually analysed using a clone library method [127], genetic fingerprinting [128] or DNA microarrays [129].

In spite of its specificity, PCR is criticised for biasness in environmental samples where primers tend to bind strongly to certain species more than others [130, 131]. Also PCR does not provide information about the size of the populations from which the DNA has been extracted. However, the development of quantitative PCR (qPCR, [132, 133] provides information about the initial quantity of DNA templates present in the sample so that the overall quantification of groups within the sample can be estimated either by relative abundance or by the number of gene copies. During qPCR, the generation of PCR product is detected by a fluorescent marker added to the PCR mix which binds to PCR products as they generate and

fluoresce. The increase in intensity of the fluorescence is proportional to the concentration of amplicons during each PCR cycle [134]. The qPCR quantification is achieved by comparing the sample with a standard. A study into the abundance of nitrate reductase genes and the recovery of *Rhodanobacter* sp from site sediments contaminated with nitrates and uranium adopted the qPCR approach [135]. Similarly, Lear *et al* [136] estimated the abundance of microbial populations of the family *Geobacteriaceae* in columns during an investigation into the interaction between iron-reducing microorganisms and technetium solubility.

Some nuclear waste disposal programmes have successfully adopted the nucleic acid based methods in their studies. Analysis of samples obtained from groundwaters by Pedersen *et al*. [137] (1996) using the nucleic acid method identified a number of microorganisms of the genera *Acinetobacter*, *Bacillus Desulfovibrio* or *Thiomicriospira* as well as some sulphate- and iron-reducing bacteria including *Desulfomicrobium*, *Desulfovibrio* and *Shewanella* species. Also during an investigation into the behaviour of uranium and technetium under iron-reducing conditions in microcosms seeded with samples from far-field of UK LLW disposal site, Wilkins *et al*. [138] adopted the nucleic acid base approach to describe shifts that occurred in microbial populations [138]. Similarly, Fox *et al*. [110] employed this method to detect changes in microbial population during an investigation into the biogeochemical behaviour of uranium (IV) in a simulated study of near-field of UK LLW disposal site at Drigg, Cumbria. Investigation into the microbial diversity present within uranium deposits and their potential role in the biogeochemical cycle and bioremediation in Jaduguda and Bagjata uranium mines in India, Islam *et al*. [139] adopted the nucleic acid technique which revealed the predominance of microorganisms belonging to the genera *proteobacteria*, *Acidibacteria*, *Bacteroidetes* and *Firmicutes*. Rout *et al*. [113]

and Charles *et al*. [83] used the nucleic acid technique to investigate the diversity of microorganisms in ISA degrading communities.

1.5.7. Microbial lipid analysis

The use of nucleic acid-independent microbial biomolecules such as lipids in the characterisation of soil micro fauna has been adopted without the need for classical laboratory culturing [140]. Fatty acids signatures exist in microbial biomass as they are present in relatively constant proportions that can be used to distinguish between different taxonomic groups in a given community. In this method, fatty acids are extracted via saponification which is followed by methylation to yield the respective fatty acid methyl ester (FAME). The FAMEs are analysed using chromatographic methods where the pattern of chromatograms are compared with a reference FAME database to identify the fatty acids and the microbial signatures using statistical methods [140]. FAME samples can also be analysed by Gas Chromatography Mass Spectrometer peaks generated by the GC and their corresponding mass spectra are compared FAME references in a National Institute Standard and Technology (NIST) library [141].

Fatty acids are one of the most important structural components of the cellular materials of microorganisms and they occur mainly within the cell membranes where they primarily function as the acyl constituents and hydrophobic components of the membrane phospholipids [142]. On the basis of biosynthetic relationships, membrane fatty acids may be classified into two major families namely straight-chain fatty acids or branched-chain fatty acids [143]. The straight-chain fatty acids, which occur quite commonly in bacteria, are synthesized from acetyl-CoA and malonyl-CoA where the former is the primer while the latter is the chain extender. The family of the straight-chain include palmitic acid, stearic acid, hexadonic acid, octadecanoic acid and cyclopropanic acids. The family of branched-chain fatty acids which includes iso- and anteiso-fatty acids are not

common in bacteria. They are synthesised from the iso or anteiso primer and malonyl-CoA [143] which may later undergo modification.

The type and the relative abundances of fatty acids that a cell produces (to a large extent), is determined by the genetic constitution of the organism [144]. Several dozens of genes are responsible for the encoding of enzymes that synthesize fatty acids, phospholids and lipid A which may or may not be conserved [145] and can be used as biomarkers for the purposes of microbial identification. The presence of branched-chain and the straight chain fatty acids in microbial cell membranes may be used to identify the microorganism as Gram-positive or Gram-negative bacteria. For example, in Gram-negative bacteria such as *E. coli* β-ketoacyl-Acyl Carrier Protein Synthase III (FabH; the determining factor in the biosynthesis of branched-chain fatty acids) uses straight chain fatty acids as substrate whereas in Gram-positive bacteria such as *B. subtilis* the FabH mainly uses larger branched-chain fatty acids substrates. As a result, the branched-chain fatty acids have been shown to be present only in Gram-positive bacteria and not in Gram-negative bacteria [145-147]. Similarly, 3-hydroxyl fatty acids (a component of endotoxin, Lipid A) are synthesized only in Gram-negative bacteria and can used in the differentiation of Gram-negative bacteria from Gram-positive ones [145].

FAME analysis can also be used to classify microorganisms as aerobes, anaerobes or facultative anaerobes. Quezada *et al*. [144] demonstrated that the saturated and hydroxy $C_{:10}$, $C_{:12}$ and $C_{:18}$ are potential FAME biomarkers for the determination of aerobic bacteria whereas branched and unsaturated FAME are biomarkers for anaerobes. For facultative anaerobic bacteria characterisation, the FAME signature includes unsaturated, branched-chain and hydroxyl $C_{:14}$. FAME analysis can also be used to identify an unknown microorganism even down to the species level [148]. For example, specific FAME signature of *Norcardia amarae* ($C_{19:1\omega8\,OH}$, *cis*-$C_{16:1\omega6}$, $iC_{15:0\,2OH}$) and their relative abundance has been used as a

biomarker to demonstrate their potential in the quantitative determination of the abundance of the species in activated sludge [149].

In addition to using fatty acid profiling as a biomarker in the identification of unknown microorganisms, specific patterns of FAME can be indicative of physiological or environmental stress in a particular species [150]. Changes that occurs within an organism as result of exposure to a toxic substance has been evaluated using FAME analysis. Pinkart *et al*. [151] showed that the exposure of *Pseudomonas pudica* P8 to incremental concentration of phenol led to a corresponding increase of its trans-unsaturated fatty acids [150].

In the event of cold-shock, bacteria adopt a fatty acid desaturation mechanism to modulate membrane fluidity whereby saturated fatty acids are converted to unsaturated fatty acid [152]. As a common occurrence, $C_{:16:0}$ is converted to $C_{16:1\omega9}$ whereas $C_{18:0}$ converts to $C_{18:1\omega9}$ with the help of acyl-lipid desaturases [153]. This mechanism of unsaturation is crucial to the conversion of the normal gel-like physiological state of the membrane to a liquid-crystalline state for the tolerance and acclimatization to cold [154]. Similarly, chain length modification of fatty acids may be used as a survival mechanism to low temperatures. The shorter the chain length, the lower the melting point and the longer the chain length, the higher the melting point [155]. Longer chain fatty acids are able to span the width of the bilayer better than shorter chain and aid in the packing of acyl chains thereby making the membrane environment more gel-like. Short chain fatty acids (< 12 carbon atoms) on the other hand can neither span the lipid bilayer nor establish hydrophobic interactions with other lipids and proteins hence, maintain the normal fluid state of the membrane [156].

1.5.8. Whole community analysis

Analysis of 16S rRNA gene sequences which is commonly adopted in ecological studies does not provide sufficient resolution at species level because of being a highly conserved molecules [157]. As a result, a more comprehensive techniques including metagenomics, transcriptomics, metaproteomics and Next Generation Sequencing (NGS) of the whole community analysis are adopted to provide holistic information on the total DNA extracted from the soil sample

1.5.8.1. Metagenomics

Metagenomics is the application of genomic technologies and bioinformatics tools to investigate genetic content of entire microbial communities [158]. The principle of metagenomics is the analysis of the entire genetic composition of environmental microbial communities as would be analysed for a pure isolate. The construction of metagenomic libraries involves extraction of total DNA from the soil sample, cloning of random DNA fragments in suitable vectors, transformation of the clones into a host bacterium followed by the selection of positive clones. While metagenomics libraries of small DNA fragments of about 2-3 kb provide better coverage of metagenome, large-fragment metagenomics libraries of about 100-200 kb is suitable for investigating multigene biochemical pathways and to obtain the genome of rare members of the microbial community, it is estimated that genome clone of about 10^{11} would be required [159]. The screening of metagenomics libraries could be done either by sequence-driven or function-driven metagenomics analysis. The sequence-driven metagenomics screening which involves high-throughput sequencing highlights important genomic features including genomic organisation, acquisition of traits through horizontal gene transfer as well as redundancy of functions in the community. In the case of functional screening metagenomic analysis, libraries are screened by expressing a selected phenotype in a particular medium [160]. A number of biochemical benefits have been reaped from environmental metagenomics

libraries including novel antibiotics (turbomycin, terragine) and microbial enzymes such as cellulases, lipases and amylases [161]. A major limitation of the metagenomics approach is the under level or no expression of many environmental genes in heterologous host such as *E. coli*. As a result, any strategy that can enhance heterologous expression of unknown genes will be desirable to the advancement of metagenomics.

1.5.8.2. Metatranscriptomics

Transcriptomics involves the techniques applied to the study of the transcriptome (total RNA transcripts) encoded by the genome of an organism at a specific time or under a specific set of conditions [162]. The DNA within the genome of an organism contains the all the genetic information of the organism and is expressed through transcription where mRNA is the transient intermediate molecule. The steps involved in transcriptomics include RNA isolation, Expressed Sequence Tags (EST) where RNA is copied as cDNA by reverse transcriptase followed by sequencing and serial analysis of gene expression (SAGE). Genes are transcribed within an organism to produce mRNA transcripts which are isolated from the organism. Using reverse transcriptase, the mRNA is copied into stable double stranded cDNA. The ds-cDNA is then digested by restriction enzyme in the SAGE to produce 11-nucleotide tag fragments which are concatenated and sequenced by Sanger sequencing. The sequences are then deconvoluted to find the frequency of each tag. The tag frequency is used to report on the transcription of the gene that produced the tag [162].

In metatranscriptomics, microbial gene expression profiles within the natural environment such as soil is investigated by monitoring the transcription of gene through random sequencing of mRNA transcripts extracted from microbial communities at a particular time and place [163]. This approach is particularly useful when investigating changes that occur during gene expression and their

regulation in relation to changes in environmental conditions [124]. Urich *et al* combined transcriptomic profiling with pyrosequencing techniques where the rRNA tags revealed the phylogenetic composition of soil microbial communities and showed the relative abundances of Actinobacteria, Proteobacteria and Crenarchaeota. The authors also used information from the mRNA tags to report key metabolic enzymes including monooxygenase (*amoA and amoC*), nitrite reductase (*nirk*) all of which were involved in ammonia oxidation. They also reported on gene transcripts that code for the enzymes involved in CO_2 fixation in Crenarchaeota namely methyl-manonyl-CoA mutase and 4-hydroxybutyryl-CoA dehydratase [164]. Perazzolli *et al*. [165] also used transcriptomic techniques to reveal complex adaptations of soil microorganisms to harsh conditions of the soil matrix as well as the competition and cooperation that occurs between soil microrganisms.

1.5.8.3. Metaproteomics

Proteomics refers to the set of techniques and procedure for studying the entire complement of proteins expressed in the genome of an organism [166]. Proteomics offer alternative biomarkers of clearer gene functions to lipids and nucleic acids by reflecting on the actual metabolic activity and regulatory cascades of genes thereby providing direct information about microbial activity [167]. In metaproteomics, the proteins expressed by environmental microbial communities at a given point in time is studied on a large scale [168]. Metaproteomics offer a comprehensive approach to quantitative and qualitative treatments of the physiology of microbial communities to provide information relating to protein abundances as well as protein-protein interractions. The identification of proteins provides the opportunity to link to the corresponding metagenomics sequences and the associated metabolic functions to the individual microorganism. During metaproteomics, total proteins are extracted from environmental sample and separated by one-dimensional and two-dimensional

electrophoresis to generate a community proteofingerprint. Protein spots are then digested and are identified by analytical methods such as chromatographic and mass spectroscopy. Benndorf *et al*. [169] used a metaproteomic approach to identify proteins that were involved in bioremediation of chlorophenoxy acid in soil samples.

1.5.8.4. Next Generation Sequencing (NGS) technology

Next Generation Sequencing (NGS) technology also referred to as High-throughput sequencing involves a massive parallel sequencing with millions of sequences in one experiment without requiring bacterial cloning of DNA fragments and electrophoresis [170]. The NGS libraries are prepared in cell-free system and the sequence output is directly detected. The major limitations include the production of shorter reads with higher error rate. Also, the PCR amplification is affected by the secondary structure of the genomic fragment and thermal stability of the genome [171]. NGS involves sample and metadata collection, DNA extraction, library construction, sequencing and read preprocessing followed by quantitative analysis and functional binning. A range of NGS platforms are available which including Illumina/Solexa, Pacific Biosciences, Ion Torrent, SOLiD, Life/APG, HeliScope/Helicos BioSciences and Roche/454Life Sciences which are much faster and less expensive than the traditional Sanger sequencing of cloned amplicons [172]. The 454Life Sciences pyrosequencing technique allows high-throughput sequencing of hypervariable region of 16S rRNA gene and provide a much higher coverage of microbial diversity. Bassil *et al*. [70] and Kuippers *et al*. [74] used the 454 Life Sciences pyrosequencing to analyse microbial diversity within ISA biodegrading communities.

1.6. Overview of sampling sites analogous to the GDF for microbial investigations

In radioactive waste management, natural and anthropogenic analogues have been used in support of arguments for long-term safety of radioactive waste repositories [173, 174]. The most important factors for consideration are the heterogeneity and complexity of the natural systems as well as the timescales for the assurance of safety of the disposal facility. The natural and anthropogenic analogues have vital role to play in the simulation of potential evolution of the disposal facility. Thus, a good understanding and modelling of natural and anthropogenic system evolution using current tools and data demonstrates ability to predict future development of the facility [175].

Across the spectrum of studies that relate to geological disposal, samples have been taken from sites that are analogous to the repository conditions because such locations could be colonized by microbial communities that are able to survive utmost conditions including extremes of pH, temperature, radiation, salt, drought, oxygen and nutrients [176-178]. For example natural hyperalkaline environments that have resulted from the interaction of water with salts within the geological formations such as the mono lake in California, soda lake in Kenya and high cement pore water in Maqarin, Jordan have been described in a number of studies [179-181]. A number of novel species of microorganisms have been recovered from these environments. In the California meromictic Mono lake (alkaline, hypersaline water body, pH 10) for example, due to the differences in the physical and chemical properties along the depth of the lake, different subtypes of bacteria including α, β and ɣ Proteobacteria have been described [179]. Moreover, two Gram-positive alkaliphilic anaerobic bacteria *Bacillus arsenicoselenatis* (strain E1H) and *Bacillus selenitireducens* (strain MLS 10) were isolated from the anoxic muds of the Mono Lake rich in arsenic and both isolates were able to dissimilatory reduce arsenic and selenium with associated oxidation of lactate to acetate [182].

Hoover *et al*. (2003) also isolated a novel obligately anaerobic halophilic spirochaete, *Spirochaete americana* from the Mono Lake that was capable of utilising a range of carbohydrates including D-glucose, fructose and starch. The strain ASpGT was capable of growth between 10 to 44^{o}C with the optimum being 37 ^{o}C, pH 8.0 to 10.5 (pH 9.5 optimum) and at sodium chloride concentration between 2-12% with optimum growth occurring at 3% [183]. Gorlenko *et al*. [184] also isolated a novel obligatory anaerobic alkalithermophilic, chemo-organotrophic bacterium from the sediments of the Mono Lake hot spring that was capable of growth in the presence of elemental sulphur, polysulphide and thiosulphate with associated reduction to H_2S. The isolate, *Anaerobranca californiensis* could also reduce Fe (III) and Se (IV). Further studies have also described isolations of extremophiles that are capable of sulphate and arsenic reduction from sediments of the Mono lake [185-187]. Similar studies on another natural analogue, the Soda Lakes in the Kenyan-Tanzanian Rift Valley have also described the presence of α, β and ɣ Proteobacteria in addition to very diverse group of microorganisms [180, 188].

The Cyprus Natural Analogue Project (CNAP) has studied the Troodos mountain range at the centre of the island of Cyprus and the Mamonia terrain in the Paphos distric of Cyprus. Of special relevance to the geodisposal concept is the widespread occurrence of bentonite/bentonite analogue deposits and alkaline springs which have resulted from serpentinisation of ophiolites [189]. Investigations into the microbiology of the alkaline springs and the sediments have shown a diverse group of microorganisms some of which were capable of reducing ferric iron under high pH conditions (pH 9.5) [190].

In the UK, an anthropogenic analogue at Harpur Hill, Buxton, Derbyshire was formed by a legacy lime kiln operations in the early 1830's. Inefficient processing of the lime led to the generation of large amount of waste which became liable to leaching by percolating rain and ground waters. The percolation of groundwater

has led to the formation calcium hydroxide leachates, which emerges, from its base into a valley known as the Brook Bottom valley where together with the dissolution of atmospheric carbon dioxide has generated calcium carbonate tufa. This is what has led to the formation of the current day 'lagoon' of calcium carbonate precipitate, highly alkaline leachates and calcium carbonate tufa deposits with the *in situ* pH being 12.0 [191]. A survey conducted at the site has shown that a wide range of materials including including metals, plastics, rubbers, cellulosic materials such as wood and grass are present. Alluvial clays and different kinds of exogenic rocks such as sandstones, siltstones and shales have also been identified at the site. The presence of some of these materials at the site may expand our knowledge on their behaviour in hyperalkaline environments potentially analogous to the cement-based nuclear waste repository [191].

Several studies relating to geological disposal have been carried out using samples from this site. For instance, Burk *et al*. [178] investigated into the geochemical processes that occurs within the buried, saturated organic-rich soil layer that has been trapped under the calcite precipitate of the this site. They found that microbial populations within the soil were able to reduce nitrates and iron whereby the electron donor was unknown. Their study further showed that the pH of the site was 12.3 and community analysis of the sample described the presence of anaerobic alkaliphilic microbial population from the Comamonadaceae family of the β-proteobacteria. The dominant species was however, unknown. They noted that the identified microbial population was potentially capable of reducing nitrate through the use of donors obtained from the organic matter content of the soil. They also found that underneath the region of nitrate reducing activities, a significant amount of Fe (II) was present which suggested the occurrence of microbial iron reduction. However, no reduction process occurred above pH 11 [178].

As previously discussed, sediments from this site has been used by Basil *et al*. [70] as an inoculum to assess the ISA biodegradation potential of the local microbial communities via redox reactions. Rout *et al*. [73] detected the presence of ISA and VFAs from samples of soil taken from the site and subsequently showed that using the alkali leachates from the site, ISA could be generated from soil sample previously not contaminated with leachates from the site. Charles *et al*. [83] showed ability of the local microbial community at the site to colonise and partially hydrolyse a cellulosic material (cotton) through the formation of biofilm and reported later that the local microbial consortia within the biofilm could degrade all forms of ISA. In these studies, microbial community analyses have described a diverse group of microorganisms including methanogenic bacteria capable of acetogenic and hydrogenotrophic metabolism.

1.7. Biochemical processes relevant to anaerobic microbial degradation of carbohydrate substrates

Under anaerobic system of complex microbial community, mutualistic behaviour of various anaerobic microorganism results in the biodegradation of complex organic substances into simple, stable products such as CH_4, CO_2 and H_2. This bioconversion process is possible due to activities of fermentative, syntrophic, acetogenic and methanogenic bacteria. These four groups of microorganisms gives rise to the main biochemical processes that occur within anaerobic microbial community systems.

1.7.1. Fermentation

Under anaerobic conditions, fermentation processes occur whereby microorganisms utilise carbon sources to produce lactic acids, volatile fatty acids (VFA), alcohols and gas. Fermentation involving a single species of microorganism is often used to produce a specific product usually for commercial purposes but in a complex microbial community, the products are usually a

mixture of VFAs, alcohol and gases [192]. Generally, under anaerobic alkaline conditions fermentation processes reduce with increasing pH up to 11.0 [193] however, addition of carbonate to a batch reactor increased acetic acid and hydrogen production in a pH range of 8.0-11.0 [194]. In most microbial fermentations, glucose dissimilation occurs through glycolysis where pyruvate or any of the compounds produced enzymatically from pyruvate such as acetaldehyde is generated. Acetaldehyde may be reduced by NADH to ethanol which the cell excretes. Lactic acid fermentation, which occurs in organisms such as streptococci (*Streptococcus lactis*) and lactobacilli including *Lactobacillus casei*, *L. pentosus* produces lactic acid and organisms that produce only lactic acid from carbohydrate substrate fermentation are homofermenters which dissimilate glucose exclusively through the glycolytic pathway. On the contrary, heterofermentative organisms ferment carbohydrates to generate multiple products, including VFAs, ethanol and gases maily CO_2 and H_2. This type of fermentation occurs among members of the family Enterobacteriaceae (*Escherichia coli*, *Salmonella*, *Shigella*, and *Proteus* species). Other microorganisms including *Enterobacter aerogenes*, *Aeromonas*, and members of the genera *Serratia*, *Erwinia*, and *Bacillus* have also been noted to generate other neutral products such as ethanol, acetylmethylcarbinol (acetoin) and 2,3-butylene glycol in addition to CO_2 and H_2. Many obligate anaerobic bacteria belonging to the class clostridia including *Clostridium saccharobutyricum*, *C. thermosaccharolyticum* have also been reported to ferment carbohydrates to produce butyrate, acetate, CO_2, and H_2. Other species of *Clostridum* such as *C. acetobutylicum* and *C. butyricum* also generate these end products of fermentation in addition to butanol, acetone, isopropanol, formate, and ethanol. Similarly, members of the genera *Propionibacterium* and *Veillonella* ferment glucose and produce CO_2, propionate, acetate, and succinate [195].

1.7.2. Acetogenesis

Acetogenesis involves the conversion of H_2 and CO_2 into acetic acid by acetogenic bacteria such as *Clostridium aceticum* [196] or the direct conversion of carbohydrate substrate such as glucose into acetic acid by bacteria such as *Moorella thermoacetica* (a *Clostridium* in the family Thermoanaerobacteriaceae) [197]. In a system of biodegration of carbohydrate by a complex microbial community, acetogenic bacteria such as *C. aceticum* and those of the genera, *Syntrophomonas* and *Syntrophobacter* convert the acid phase products (syngas; H_2 and CO_2) into acetate and hydrogen (equation 1) which may be utilised by methanogenic bacteria [198].

$$2CO_2 + 4H_2 \longrightarrow CH_3COO^- + H^+ + 2H_2O \quad \text{..... equation 1.}$$

The activities of versatile acetogenic bacteria such as *M. thermoacetica* capable of energy conservation by both autotrophic (acetogenesis) and heterotrophic (homoacetogenesis) modes of metabolism, generate stoichiometrically 3 moles of acetic acid from from carbohydrates (eg. glucose) through the acetyl CoA pathway (equation 2) [199].

$$C_6H_{12}O_6 \longrightarrow 3CH_3COO^- + 3H^+ \quad \text{..... equation 2.}$$

As a result of acetogenesis in these systems, hydrogen is released, which in turn produces toxic effects on the microorganisms which carry out this process. Therefore, to allow the process to continue, a symbiotic association between acetogenic and hydrogenotrophic methanogenic bacteria becomes necessary [198, 200]. This symbiotic association is referred to as syntrophy.

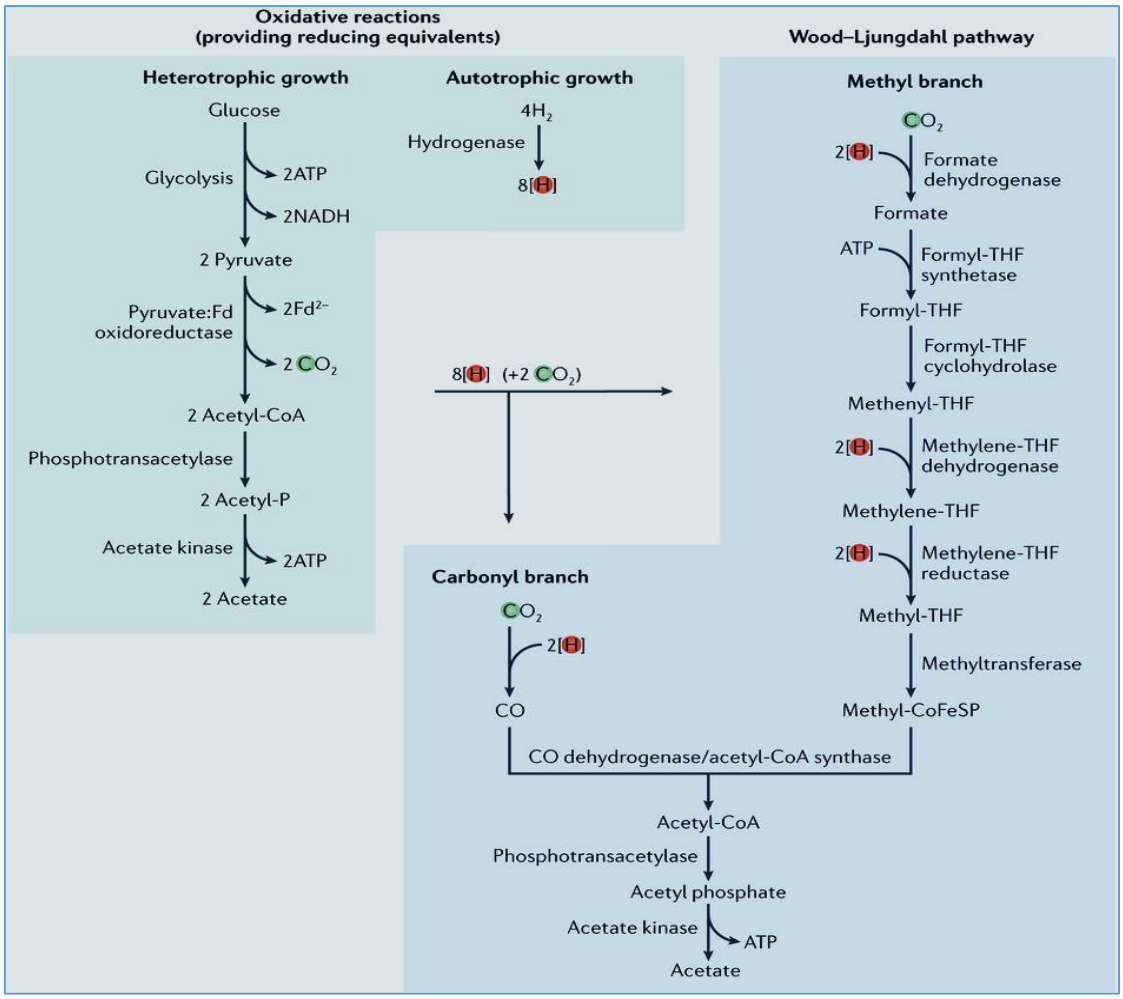


Figure 1.10 Acetogenic Pathways

The pathways illustrate heterotrophic acetogenesis (direct conversion from glucose), autotrophic acetogenesis (reduction of $2CO_2$ with $4H_2$) and Wood–Ljungdahl pathway (WLP; reduction of $2CO_2$ with 4H2 the taken from Schuchmann and Müller [201].

Figure 1.10 illustrates how acetogenic bacteria generate acetate (CH_3COOH) from $2CO_2$ through the Wood–Ljungdahl pathway (WLP). In heterotrophic acetogenesis, bacteria oxidize a molecule of glucose generating two molecules of CH_3COOH and two molecules of CO_2 through a series of ezyme catalized reactions involving glycolysis, pyruvate:ferredoxin oxidoreductase, phosphotransacetylase and acetate kinase. In the reducing equivalent two molecules of CO_2 are reduced with $2H_2$ to CH_3COOH via Wood–Ljungdahl pathway (WLP). During the process of autotrophic acetogenesis, CH_3COOH is

formed from $4H_2$ and $2CO_2$. At the carbonyl end of the WLP, a molecule of CO_2 is reduced to CO which is catalysed by carbon monoxide (CO) dehydrogenase/acetyl-CoA synthase (CODH/ACS). The methyl branch of the WLP on the other hand, a molecule of CO_2 is reduced to formate catalysed by formate dehydrogenase and is attached to the cofactor tetrahydrofolate (THF) therby producing formyl-THF catalysed by formyl-THF synthetase. The formyl-THF is utilised by formyl-THF cyclohydrolase which result in the production of methenyl-THF. The methenyl-THF with the help of methylene-THF dehydrogenase becomes reduced to methylene-THF which undergoes a further reduction via methylene-THF reductase to methyl-THF. A final transfer of the methyl group from methyl-THF by a methyltransferase with the help of corrinoid iron-sulphur protein (CoFeSP) yields CODH/ACS. The bifunctional CODH/ACS enzyme reduces CO_2 to CO at the carbonyl branch which merges with the methyl group from the methyl branch. Together with CoA they all form acetyl-CoA. The presence of phosphotransacetylase converts acetyl-CoA to acetyl phosphate, which is further converted into acetate catalysed by acetate kinase [201].

1.7.3. Syntrophism

Syntrophism is a form of symbiotic association within a complex microbial community where two metabolically unrelated groups of microorganisms enable the degradation of complex organic substrates where one group breaks down the substrate for the other group to utilise the product [202]. Within microbial community the conversion of complex organic compounds to produce CH_4 and CO_2 is possible owing to the cooperation between syntrophic microorganism. Without syntrophism, activities of some microorganism could be limited by the toxic effects of their own products while others would be deprived of essential substrates hence, syntrophism occurs in the natural environment to achieve various roles during the process of anoxic biodegradation of organic substrate [203]. The syntrophy between hydrogen-producing acetogenic bacteria such some

species of the genera *Clostridium* and *Acetivibrio* allows for the selection of hydrogenotrophic methanogens or members of the genera *Syntrophomonas*, *Syntrophospora*, and *Syntrophobacter* which obligatorily use hydrogen as terminal electron acceptor.

1.7.4. Methanogenesis

Methanogenesis within a system of anoxic microbial degradation of organic substrate is the process by which metanogenic bacteria (members of the archaeal domain) produce methane (CH_4) mainly from acetic acid and or H_2 and CO_2. In a typical anaerobic methanogenic digestion process, although only few bacteria are capable of producing CH_4 from acetic acid, the bulk of the CH_4 arise from the conversion of acetic acid by heterotrophic methanogenic bacteria [204]. Only about 30% of CH_4 generated during anaerobic digestion comes from CO_2 reduction by autotrophic methanogenic microorganism where H_2 is used up. This creates good environmental conditions for the growth of acidophilic bacteria which generate short-chain organic acids in acidification phase of the anaerobic digestion process and consequently lower the production of H_2 in acetogenic phase. The result of such a conversion is the production of gas rich in CO_2 [205, 206]. On the basis of their 16S rRNA sequences, methanogens fall under the seven orders namely Methanobacteriales, Methanococcales, Methanomicrobiales, Methanosarcinales, Methanocellales, Methanopyrales and Methanomassiliicoccales [207, 208]. On the basis of substrate for methanogenesis which inlcudes CH_3COOH, CO_2, H_2, and methylated compounds (methyl alcohol) methanogens may be put into three main groups namely hydrogenotrophic methanogens, acetogenic methanogens and methylotrophic methanogens [203, 209]. Hydrogenotrophic methanogens use H_2 and CO_2 to synthesize CH_4 while the acetogenic methanogens use acetic acid to produce CH_4 (Acetogenic methanogenesis). The groups that fall under methylotrophic methanogens use compounds such as methanol and methylamines to synthesize CH_4. Members of

the family *Methanobacteriaceae* consisting of the genera *Methanobacterium*, *Methanobrevibacter*, *Methanosphaera* and *Methanothermobacter* are noted for hydrogenotrophic methanogenesis. In addition to reduction of CO_2 with H_2, formate is used by many species. Species of the genus *Methanosphaera* however, reduces methanol with H_2 instead of CO_2 [210]. Members of the genus *Methanosaeta* are capable of utilizing acetate specifically in the aceticlatic methanogenic pathway to produce methane [211]. The *Methanosarcina* are noted for the all-round capability of producing CH_4 through the three known methanogenic pathways. They are able to use both the aceticlastic and the hydrogenotrophic methanogenesis pathways thereby making them more tolerant to specific inhibitors of the pathway including fluoroacetate and methyl fluoride [212]. In addition, they are also known to produce CH_4 from one-carbon compounds include methylamines, methanol, and methyl thiols[213]. The methylotrophic methanogens fall under the orders Methanosarcinales, Methanobacteriales and Methanomassiliicoccales. They are sub-grouped into two depending on the presence or absence of cytochromes. Those with cytochromes are capable of oxidizing methyl groups to CO_2 through electron transport chain while those without cytochromes are obligatorily H_2-dependent.

Figure 1.10 illustrates the three main methanogenic pathways and their associated genes. In hydrogenotrophic methanogenesis (shown in blue lines in Figure 1.11), through a series of enzyme catalysed reactions and intermediates including formyl, methylene, and methyl levels, CO_2 is successively reduced and at the mrethyl level, the methyl group is transferred to Coenzyme M, forming methyl-CoM. The methyl-CoM is then reduced to CH_4 through methyl coenzyme M reductase (Mcr). In the aceticlastic methanogenic pathway (shown in red lines in figure 1.11), with the help of low-affinity acetate kinase (AK)-phosphotransacetylase (PTA) system, acetate is firstly converted to acetyl-CoA in the case of *Methanosarcina* whereas *Methanosaeta* uses the high-affinity

adenosine monophosphate (AMP)-forming acetyl-CoA synthetase. In each case, the acetyl-CoA formed is converted to a methyl group which is further converted into CH_4 using key enzymes such as Cdh, Mtr and Mcr. In the case of methylotrophic pathway (shown in Green in Figure 1.11), the methyl-groups from methylated compounds are transferred to a methanol-specific corrinoid protein which produces Methyl-CoM via Coenzyme M methyltransferase. The Methyl-CoM subsequently is later converted to CH_4 via Mcr reductase.

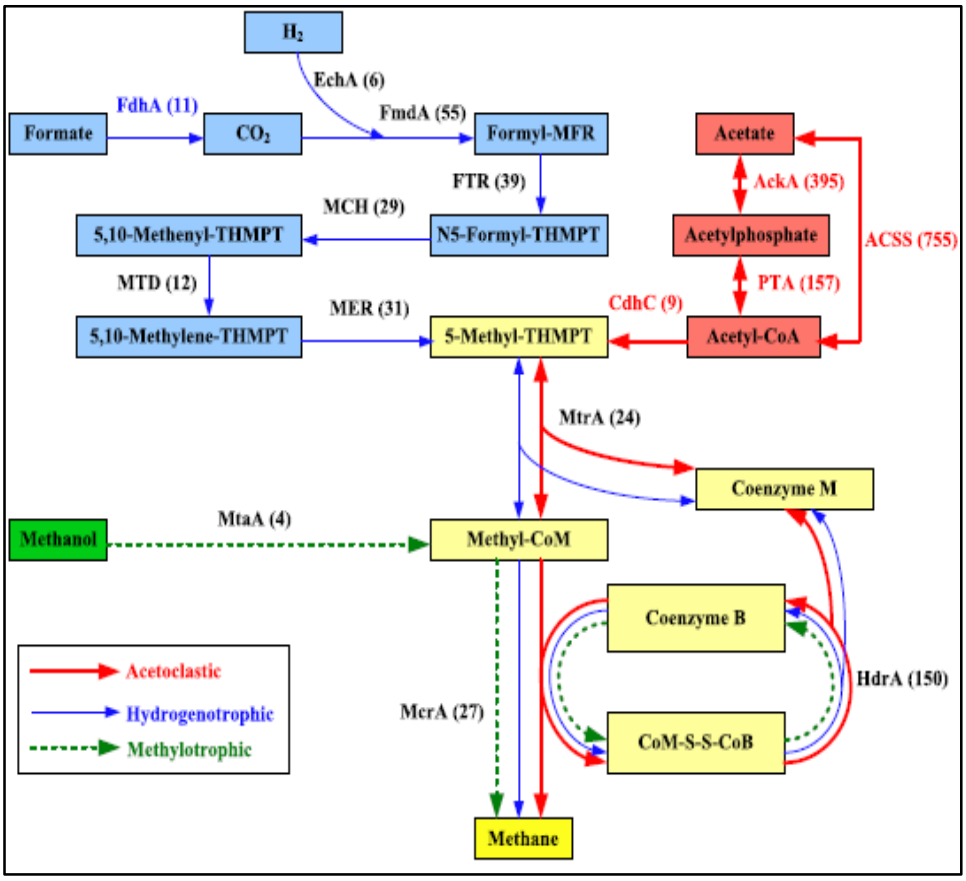


Figure 1.11 The three main methanogenic pathways and associated genes;

The pathway was adapted from Guo *et al*. [214]. FdhA, glutathione-independent formaldehyde dehydrogenase; EchA, hydrogenase subunit A; FmdA, formylmethanofuran dehydrogenase subunit A; FTR, formylmethanofuran tetrahydromethanopterin N-formyltransferase; MCH, methenyltetrahydromethanopterin cyclohydrolase; MTD, methylenetetrahydromethanopterin dehydrogenase; MER, coenzyme F420-dependent N5, N10-methenyltetrahydromethanopterin reductase; MtrA, tetrahydromethanopterin S-methyltransferase; MtaA, [methyl-Co(III) methanol-specific corrinoid protein]:coenzyme M methyltransferase; McrA, methyl-coenzyme M reductase alpha subunit; AckA, acetate kinase; ACSS, acetyl-CoA synthetase; PTA, phosphate acetyltransferase; HdrA, heterodisulfide reductase subunit A; CdhC, acetyl-CoA decarbonylase/synthase complex subunit beta.

Chapter 2

2. Aims and Objectives

In the current plan for the UK's geological disposal of ILW, the formation of water soluble complexants in the form of CDP including ISAs is an issue of particular concern due to their role in radionuclide migration. Recent studies have shown that the metabolic capacity of microbial consortia indigenous to anthropogenic sites can potentially degrade these ISAs. In most of these studies, the focus has been on coupling the calcium salt of α-ISA ($Ca[(\alpha\text{-}ISA)_2]$) metabolism with biogeochemical processes involving a range of terminal electron acceptors where the $Ca[(\alpha\text{-}ISA)_2]$ is used as an analogue for CDP. Furthermore, studies on the characteristics and degradation profiles of ISAs under alkaliphilic anaerobic conditions by pure microbial isolates is lacking in the literature.

Against this background, this study aimed at investigating the microbial metabolism of ISA under a range of anoxic, hyperalkaline conditions and assess the impact of using the calcium salt of α-ISA as an analogue for CDPs on microbial community evolution. Most studies have used different ammonium compounds as part of the growth media to provide nitrogen for microbial growth. As nitrogen had been predicted to be lacking in the GDF, this study aimed at investigating the chemical and microbial community evolutions resulting from ISA degradation in systems amended with NH_4^+ against NH_4^+ free systems.

In addition, the isolation and characterization of alkaliphilic microorganisms with the potential to degrade ISAs would provide the basis for further investigation into the rates of degradation and the genes that are associated with ISA metabolism.

By characterizing ISA degrading bacteria and communities, this study aims at addressing gaps in our knowledge regarding ISA degradation, identify key points where interventions may be most effectively applied to improve the safety of the waste repository and to inform policy and surveillance relating to nuclear waste disposal.

Chapter 3

3. Materials and Methods

3.1. General Reagents

Unless otherwise indicated, all reagents used during the investigations were obtained from Fisher Scientific (Loughborough, Leicestershire, UK), LabM Limited (Haywood, Lancashire, UK) and Sigma-Aldrich Ltd (Gillington, Dorset, UK).

3.1.1. Growth media Preparation
3.1.2. Mineral media

Mineral media was prepared using the reagents shown in Tables 1 and 2 in deionised water as previously described [215]. The pH of the media was adjusted as required using either 2M NaOH or 2M HCl and flushed with oxygen free nitrogen for 20 minutes to create anoxia [216].

Table 3.1 Mineral media components per litre of oxygen free water

Reagent	**Chemical formula**	**Mass(gL^{-1})**
Anhydrous potassium dihydrogen phosphate	KH_2PO_4	0.27
Disodium hydrogen phosphate dodecahydrate	$Na_2HPO_4.12H_2O$	1.12
Ammonium chloride	NH_4Cl	0.53
Calcium chloride dihydrate	$CaC_{l2}.2H_2O$	0.075
Magnesium chloride hexahydrate	$MgC_{l2}.6H_2O$	0.1
Iron(II) chloride tetrahydrate	$FeC_{l2}.4H_2O$	0.02
Resazurin (oxygen indicator)	$C_{12}H_7NO_4$	0.001
Disodium sulphide	$Na_2S.9H_2O$	0.1
Trace elements solution	See Table 2	10mL
*Vitamin solution (as required)	See Table 3	20mL

Table 3.2 Components of trace elements solution per litre of oxygen free water

Reagents	**Chemical formula**	**Mass (gL^{-1})**
Manganese chloride tetrahydrate	$MnCl_2.4H_2O$	0.05
Boric acid	H_3BO_3	0.005
Zinc chloride	$ZnCl_2$	0.005
Copper chloride	$CuCl_2$	0.003
Disodium molybdate dihydrate	$Na_2MoO_4.2H_2O$	0.001
Cobalt chloride hexahydrate	$CoCl_2.\ 6H_2O$	0.1
Nickel chloride hexahydrate	$NiCl_2.\ 6H_2O$	0.01
Disodium selenite	Na_2SeO_3	0.005
Disodium tungstate dihydrate	$Na_2WO_4.2H_2O$	0.002

Table 3.3Components of vitamin solution

Vitamin solution	**Chemical formula**	**Mass (mgL^{-1})**
Folic acid (B9)	$C_{19}H_{19}N_7O_6$	2
Pyridoxine hydrochloride (B6),	$C_8H_{12}CINO_3$	10
Riboflavin hydrochloride (B2),	$C_{17}H_{20}N_4O_6$	5
Thiamine hydrochloride (B1),	$C_5H_6N_2O_2$	5
Biotin (B7)	$C_{10}H_{16}N_2O_{3S}$	2
Nicotinic acid (B3),	$C_6H_5NO_2$	5
Calcium pantothenate (B5),	$C_{18}H_{32}CaN_2O_{10}$	5
Cobalamin (B_{12})	$C_{63}H_{88}CoN_{14}O_{14}P$	0.1
P-Aminobenzoic acid	$H_2NC_6H_4CO_2H$	5
Thiotic (lepoic) acid	$C_8H_{14}O_2S_2$	5
Monopotasium phosphate	K_2HPO_4	900

3.2. Preparation and characterization of Cellulose Degradation Products (CDP)

Cellulose degradation Products (CDP) were prepared using laboratory tissue (Pristine Paper, London, UK) as described by Cowper *et al* (2011). The tissue (200g) was added to 0.1 M NaOH (1.8 L) and $Ca(OH)_2$ (10 gL^{-1}) in a pressure vessel and purged with nitrogen for 30 minutes. The vessel was then sealed and the headspace was flushed with nitrogen for another 30 minutes to remove any residual oxygen. The vessel was then incubated for 30 days at 80ºC. The resultant liquor (the CDP) was filtered into a sterile and nitrogen-flushed 1 L Schott bottle using 0.22µm filter unit (Millipore, Watford, UK) under nitrogen atmosphere in sealed glove box. The CDP was stored in ambient conditions in the dark [217]. The components of the CDP was characterised by means of Gas Chromatography-Mass Spectrometry (GC-MS) and High Performance Anion Exchange Chromatography (HPAEC).

3.3. Preparation and characterization of Calcium alpha-Isosaccharinic Acid (Ca[(α-ISA)$_2$])

The calcium salt of alpha-Isosaccharinic acid (Ca[(α-ISA)$_2$]) was prepared as described by Whistler and BeMiller [218] whereby α-lactose monohydrate (50 g) was dissolved in ultrapure water (500 mL) in a 1 L round-bottom flask. $Ca(OH)_2$ (13.5 g) was dissolved in the mixture and sonicated for 10 minutes to enhance chemical processing and flushed with oxygen-free nitrogen for 30 minutes. By means of a magnetic stirrer and paraffin oil bath, the mixture was heated at 90ºC under constant stirring and nitrogen reflux for 16 h after which it was filtered while hot into another 1 L round-bottom flask. The filtrate (500 mL) was concentrated to 150 mL through rotary evaporation and the concentrate transferred into a 500 mL conical flask before being incubated at 4ºC for seven days. The white precipitate that settled at the bottom of the conical flask was filtered under vacuum and the white crystalline residue was washed first with 15

mL cold ultrapure water, followed by 15 mL cold ethanol and 15 mL cold acetone. The resulting product, $Ca(\alpha\text{-}ISA)_2$ was dried in an oven overnight at 120^{o}C [218]. The identification and purity of the product was analysed with High Performance Anion Exchange Chromatography (HPAEC) and Nuclear Magnetic Resonance (NMR) spectroscopy respectively.

3.4. Analytical Methods

3.4.1. Identification and quantification α- and β-Isosaccharinic acids using high performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD)

The identification and quantifications of α- and β-Isosaccharinic acids contained in samples was achieved by analysing the samples with HPAEC-PAD employing the use of a Dionex 5000 ion chromatographic system (Dionex, Camberley, UK). The system has the associated software package - Chromeleon 7.0 version 7.1.3.2425 (Dionex Corporation) which integrates and processes chromatograms. In addition, the IC5000 is equipped with auto-sampler (AS50), a gradient pump (GS50) and electrochemical detector (ED50) which uses gold working and Ag/AgCl reference electrodes in amperometric detection mode and utilizes a preprogrammed quadrupole wave form. Elution of analytes was achieved by an isocratic mobile phase using NaOH (50 mM) eluent at a flow rate of 0.5 $mLmin^{-1}$ and analytes separated by a 250 mm long Dionex CarboPac PA20 column with internal diameter of 3 mm and a ≤10Å pore size containing 6 μm non-porous beads covered with latex Dionex micro Bead resin. There was also a CarboPac PA20 guard column (3 x 150 mm) as part of the system. The column automatically regenerates between sample analyses by eluting with NaOH (200 mM) for 20 minutes. Each sample was amended with a 40 ppm D-ribonic acid internal standard before analysis and 10 μL of each sample was injected onto the column for analysis. A range of standards were prepared using pure α- and β-ISA to enable the determination of the concentrations of analytes [219]

3.4.2. Identification and quantification of volatile free Fatty acids using gas chromatography with flame ionisation detection (GC-FID)

The identification and determination of the concentrations of volatile Fatty Acids (VFAs) present in the samples were achieved by using HP GC6890 (Hewlett Packard, UK) gas chromatographic system equipped with Flame Ionisation Detector (FID). The GC-FID system also had Chemstation Rev.A.10.02 [1757] software (Agilent Technologies, USA) which allowed for integration, processing and visualization of chromatograms. Adopting a previously described method [90], each samples (900 μL) was mixed with 85% phosphoric acid (100 μL) in a 1.5 mL Eppendorf tube and 500 uL of the mixture transferred into 2 mL screw cap vial. An injection volume of 1 μL was taken by the auto sampler and passed through HPFFAP column (30 m x 0.535 m x 1.00 μm; Agilent Technologies) with helium as the carrier gas. The VFAs were detected by flame ionization detection (FID) using hydrogen/air detector gas with initial temperature of 95°C for 2 minutes and increasing to 140°C at a ramp rate of 10°C min^{-1} without a hold, followed by a second ramp to 200°C at a ramp rate of 40°C min^{-1} with a hold of 10 minutes and later dropping to a post run temperature of 50°C. A range of VFAs standards were prepared from a mixed stock solution of VFAs (Supelco analytical, Pennsylvania, US) to generate a calibration curve for each acid.

3.4.3. Identification and quantification of gaseous products using Gas Chromatography equipped with Thermal Conductivity Detection (GC-TCD)

Gaseous products including hydrogen, carbon dioxide and methane were identified and quantified using an Agilent 6850 gas chromatographic system with a Thermal Conductivity Detector (TCD) and a GS-Q column (30m x 0.53mm ID, Agilent technologies, Berkshire, UK). The carrier gas was 4 mL min^{-1} helium. Sampling was carried out manually by sampling 100 μL of microcosm headspace gas by means of a lockable gastight syringe and injected into the GC system port

at 30°C. The detector temperature was 200°C. The instrument was calibrated using known concentrations of each gas.

3.4.4. Identification of major components of Cellulose Degradation Products

The identification of the major products of chemical cellulose degradation was carried out by Innovative Physical Organic Solutions (IPOS) service (University of Huddersfield). Identification was done using a High Resolution TOF Mass Spectrometer equipped with Dual Electrospray Ionisation (HR MS-ESI; Agilent 6210; Agilent Technologies). The ionisation source details used for both positive and negative modes included a VCap voltage of 300V, Fragmentor voltage of 150V, Gas temperature of 350^{o}C, an octopole OCT 1 RF Vpp: 250 V and a Nebuliser Pressure: 40 psi. The Drying Gas Flow Rate was adjusted to 10Lmin^{-1} and skimmer voltage of 65V. The masses generated from the spectra were exported onto National Institute of Standards and Technology (NIST) database for identification.

3.4.5. Nuclear Magnetic Resonance (NMR) Spectrometry for checking the purity of Ca(α-ISA)$_2$ and for identification of exopolysaccharides (EPS) of biofilms

The purity of Ca(α-ISA)$_2$ and the identification of EPS was carried by Nuclear Magnetic Resonance (NMR) Spectrometry Service (University of Huddersfield) whereby Bruker Avance 500 MHz spectrometer (Bruker 400’54 Ascend) with Bruker pulse sequences at ambient temperature was used.

3.5. Microbiological methods

3.5.1. Microcosm investigations

3.5.2. Aerobic microcosm for biodegradation of ISA from Ca(α-ISA)$_2$ and CDP

Soil microcosms were prepared in duplicate by adding 72 mL of mineral media described in Tables 1 and 2 but without resazurin and reducing agents, to fresh soil samples (5g) obtained from Harpur Hill, Buxton (Derbyshire, UK) in 250 mL conical flasks. To each reactor, CDP (8 mL) was added [73]. Similar microcosms driven by Ca(α-ISA)$_2$ (2 mM) were also prepared in duplicate. Here soil (5g) from the same site was diluted in 80 mL of mineral media plus the required amount of Ca(α-ISA)$_2$. The pH of the microcosm was adjusted to pH 10.0 with filter-sterilized 4M NaOH or HCl. The reactors were wrapped in foil to exclude light and closed with sterile foam bung to allow for gas exchange while preventing contamination. To cater for sorption, a sterile control whereby the media with the inoculum for both reactor types were double-autoclaved under standard conditions were also set up before being incubated at 25^{o}C in an orbital incubator (Stuart Orbital Incubator SI500) at 100 rev min^{-1} for 7 days. Reactor fluid (2 mL) was sampled daily, 100 µL of which was tested with 3M Clean-Trace Biomass Detection Kit (3M, UK) in conjunction with a luminometer for ATP/biomass detection. Following biomass analysis, CFU/mL of microbial concentration was calculated against a standard curve of based on *E. coli* K12 [83]. The remaining sample was centrifuged and the supernatant filter-sterilized with 0.45 µL filters (filtropur; Sarstedt AG & Co. Germany) and stored at -20^{o}C for ISA/VFA analysis. Sampling continued until the stationary growth phase of the enrichment culture assessed by ATP activity was reached.

3.5.3. Anaerobic batch culture for biodegradation of Ca(α-ISA)$_2$ and CDP

Anaerobic batch cultures under constant stirring at 25^{o}C at pH 10 were prepared in duplicates using the same composition of mineral medium and concentration of carbon sources as described previously for aerobic cultures except for the addition of reducing agents. 0.05% of L-cysteine and sodium thioglycolate was added to the medium along with 0.001 g/L resazurin as oxygen indicator. Total volume (80 mL) was dispensed into 100 mL Schott bottles and covered with three-holed screw caps equipped with rubber septa before autoclaving under standard conditions to ensure an anaerobic environment within the bottles. 2 mL samples were taken every two days, assessed for ATP/biomass detection, and processed as previously described until the stationary growth phase of the enrichment culture was reached. The samples were stored at -20^{o}C for component analysis.

3.5.4. Batch/Fed culture to assess the the impact of nitrogen source and the form of ISA on microbial community evolution

Cellulose degradation product (CDP) and Ca(α-ISA)$_2$ microcosms were set up in duplicates for the two carbon sources with and without added NH_4^+ under anaerobic conditions (10% H_2, 10% CO_2 , 80% N) using the Buxton soil sample (5g) mentioned in the earlier section as seed. The total concentration of ISA in all microcosms for the different carbon sources were made equal (i.e. 8 mL CDP leachate in 72 mL mineral media and 71.89mg Ca(α-ISA)$_2$ in 80 mL mineral media respectively for CDP- and Ca(α-ISA)$_2$-driven microcosms). The pH of all the reactors was adjusted using sterile 4M NaOH, or 4M HCl before the entire volume was flushed with nitrogen for 20 minutes. Microcosms were wrapped in foil to exclude light before being incubated at a constant temperature of 25°C and stirred at 100rpm. In order to employ a batch/feed regimen on the reactors, 8mL of the total volume was removed at the end of 7 days of sampling. This removed volume was then replaced with a 10% of the total volume solution of CDP diluted in mineral media (CDP reactors) or 7.18 mg of Ca(α-ISA)$_2$ diluted in mineral

media. The headspace was also de-pressurized and purged with nitrogen at the end of each cycle. This feed cycle continued on a weekly basis for ten weeks, such that the entire microcosm volume had been replaced. During this tenth week, 1 mL of reactor fluid was taken from each microcosm on a daily basis for 7 days. The pH of the sample was measured before being centrifuged and filtered through a 0.45μm syringe filter unit and frozen for downstream analysis. The total ISA concentration, volatile fatty acid content and headspace gas composition were all determined using methods previously described in Rout *et al*. [73]. The pH of samples was measured using a pH meter and calibrated electrodes (Mettler Toledo, UK) and total biomass was estimated using previously described methods [83].

3.5.5. Batch/Fed culture for microbial community ISA biodegradation under elevated pH conditions

The set up described in the previous section was repeated normally (using the full mineral media described in section 3.1.2) for a range of pH (pH10.0-11.5). The feed cycle regimen continued for 10 weeks, which was proceeded, with 5 daily sampling for a period of 45 days. All samples were treated as described in the previous section and stored at -20°C prior to analysis.

Generally, the rates of ISA degradation under anoxic conditions was determined from the half-life first order equation:

$$t_{1/2} = ln2/k$$

Where t1/2 is the timescale on which initial concentration had reduced to half of its original value

3.5.6. Determination of ammonium ion (NH_4^+) concentration within microcosm background soil extract

The ammonium ion (NH_4^+) concentration within the microcosm background soil extract was Spectrophotometrically determined using a modified indophenol method described by Bolleter *et al*. [220].

3.5.7. Stock Reagents

Phenol-alcohol reagent was prepared by dissolving 10 g of phenol in 95% ethyl alcohol to a final volume of 100 ml while sodium nitroprusside (nitroferricyanide) reagent was prepared by adding 1 g in deionised water to a final volume of 200 mL. In order to prepare an alkaline complexing reagent, 100 g of trisodium citrate ($Na_3C_6H_7O_5$) and NaOH (5 g) were mixed in deionised water to a final volume of 500 ml. Oxidizing solution was made by mixing 25 mL of sodium hypochlorite in the alkaline complexing reagent to a final volume of 100 mL.

Two calibration solutions – A and B were prepared. Calibration solution A (NH_4-N stock) was prepared by dissolving 4.72 g of dry $(NH_4)_2SO_4$ in 900 ml of deionised water and made up to 1 L. Calibration solution B (100 ppm NH_4-N stock) was also prepared by transferring 10 mL of solution A to deionised water and made up to 100 mL volume and used as freshly prepared.

3.5.8. Soil extracts preparation

Soil samples (1 g) were suspended in 20 mL deionised water, mixed thoroughly and centrifuged. The supernatant was pipetted used freshly prepared.

3.5.9. The Indophenol Assay

Different concentrations of stock solution B (1 mL, 750 µL, 500 µL, 200 µL, 100 µL, 50 µL, 0 µL) were prepared and to each, 400 uL of the phenol solution, 0.4 mL of the nitroferricyanide and 1 mL of the oxidising reagent was added in a test tube. Samples were mixed well and allowed for an hour for colour development.

The absorbance were recorded using spectrophotometer and used to generate a standard curve. The reagents were added to the soil extracts (1 mL) in triplicates and the absorbance determined. The concentration of NH_4^+ in the background soil extract was deduced from the standard curve.

3.5.10. Microbial Community Analysis

The microbial community analysis was carried out by ChunLab (South Korea) employing Next Generation Sequencing (NGS) technology through MiSeq platform. Identification of individual microorganisms was done using EzTaxon-e database.

3.5.11. Isolation and cultivation of alkaliphilic microorganisms from reactors

Solid mineral media plates (pH 10) enriched with either CDP or $Ca(\alpha\text{-ISA})_2$ were prepared on which samples from the reactors were plated and incubated under anaerobic conditions at 25°C. Following growth, distinct colonies were cultivated on CDP/$Ca(\alpha\text{-ISA})_2$ plates from where pure cultures were obtained for 16s rRNA sequencing.

3.5.12. DNA extraction and purification

3.5.12.1. The co-extraction of DNA method

DNA from microcosms was extracted using a modified version of a method described by Griffiths *et al*. [221]. Microcosm fluid was concentrated by centrifugation ($5x10^3$ g; 20 min) and washed in 10 M of 0.1 M sodium phosphate buffer (pH 7.4; 3.1g of $NaH_2PO_4.H_20$ and 10.9 g of Na_2HPO_4 in 1 L deionised water) The sample (0.5 g) was mixed with 0.5 mL of extraction buffer (cetyl-trimethylammonium bromide; CTAB, 5% wt/vol prepared in 0.35 M sodium chloride/240 mM phosphate buffer, pH 8.0), 0.5 mL phenol-chloroform-isoamyl alcohol (ratio; 25:24:1) and 100 µL betamercaptoethanol in a bead tube containing

0.1mm diameter glass beads. Tubes were then bead-beaten for 15 minutes by attachment to vortex adapter (MO-BIO vortex adapter, MO-BIO, Carlsbad, CA, US) set to full speed. The resultant suspension was then centrifuged at 13,500 rpm for 5 minutes and the top aqueous layer (~450 µL) was transferred to a sterile, DNase/RNase free 1.5 mL tube. Chloroform-isoamyl alcohol in the ratio 24:1; 500 µL) was added to the tube and the mixture was vortexed to mix and centrifuged at 13,500 rpm for 5 minutes. The aqueous top layer (~450 µL) was transferred to a fresh 1.5 mL tube. To this tube, 1 mL polyethylene glycol (PEG; 30% PEG-6000; wt/vol) solution was added in 1.6M sodium chloride). The mixture was vortexed to mix and incubated overnight at 4°C.

Following overnight incubation, sample tubes were centrifuged for 10 minutes at 14,000 rpm. The supernatant was pipetted and discarded while DNA remained as a pellet which was washed with 70% ice cold ethanol (l200 µL). The alcohol was pipetted out and any residual ethanol was allowed to air-dry. The DNA pellet was re-suspended in 30 µL of sterile deionised water.

3.5.12.2. UltraClean® Microbial DNA Isolation Kit

Genomic DNA from pure cultures were extracted using the UltraClean microbial DNA isolation kit (MO-BIO, Carlsbad, CA, US) according to the manufacturer's instructions.

3.5.13. Polymerase chain reaction (PCR) for the amplification of DNA

In order to amplify the concentration of the extracted DNA, direct PCR of the ~1,500 bp eubacteria 16S rRNA gene was performed using the broad specificity primers pA: AGAGTTTGATCCTGGCTAG; pH: AAGGAGGTGATCCAGCCGCA (Bioline, UK). The *Taq* polymerase used was BIOTAQ™ being part of Biomix™ (Bioline, London, UK) which also consisted of reaction buffer, magnesium (5mM) and dNTPs. The PCR reaction mix consisted of microbial DNA (5 µL), each primer (1.5 µL of 10 pmol), Biomix

PCR master mix (25 µL, BIOLINE, UK) and made up to 50 µL PCR reaction volume with PCR grade water. To allow for effective primer binding and to increase PCR product concentration, a 1:10 dilution DNA template was prepared in PCR grade water before adding to the PCR reaction mix. Samples in PCR reaction tubes were subjected to PCR programme with thermal cycler (TECHNE TC, Staffordshire, UK) which began with initial incubation at 94^{o}C for 5 min. This was then followed by 35 cycles of 3 steps; denaturing at 94^{o}C for 1 min, then annealing at 60^{o}C for 1 min, primer extension at 72^{o}C for 1min and final extension at 72^{o}C for 5 min

3.5.14. Gel electrophoresis for the visualisation of PCR product

A concentrated solution of 50X concentrate of tris-acetate EDTA (TAE) buffer (pH 8) was prepared by dissolving tris base (242 g), 57.1 mL glacial acetic acid and 100 mL 0.5M EDTA in a litre of ultrapure water. Gel electrophoresis on 1% molecular grade agarose gel was prepared in 1X TAE buffer in which the agarose solution in a 250 mL conical flask was heated in a microwave for 3 minutes to completely melt and allowed to cool to ~45°C before 1 µL of SYBR® safe stain Life Technology, Paisley, UK) was added and mixed by gently swirling the conical flask. Following casting and setting of the gel in the tank, the gel was submerged in a TAE buffer and 5 µL of DNA template (PCR product) which had been mixed with loading dye (1 µL; Bioline, London, UK) was loaded into each well alongside a 1kb DNA ladder (hyperlader, I kb, Bioline, London, UK). Electrophoresis was run for 60 minutes at 90V before being visualised under UV light using PCR BioDoc-It® 210 imaging system (UVP LLC, Upland, CA, US).

3.5.15. Purification of PCR product with Qiaquick PCR purification kit

The purification of PCR products for downstream applications was carried out with the Qiaquick PCR purification kit (Qiagen, Surrey, UK) in accordance with the manufacturer's instructions. In Brief, the PCR product was salted by the addition of 5 volumes of buffer PB to 1 volume of the product at a pH 7.5. Sodium acetate (3M) was added to the mixture to reduce the pH before the samples were loaded into Qiaquick silica spin column. To allow the binding of the DNA onto the silica membrane of the column, the sample was centrifuged at 10,000 rpm for 60 seconds. The PCR product was then washed to remove associated contaminants such as primers, dNTPs and polymerases with the addition of an ethanol based buffer before a further centrifugation step. Elution buffer EB (30 µL; 10 mM Tris·Cl, pH 8.5) was added to the centre of the silica membrane followed by a final centrifugation step into a clean DNase free 2 mL collection tube [222].

3.5.16. Determination of DNA concentration

The concentration of DNA was determined by means of a spectrophotometer (Genway, Genova Nano, Stone, UK) at 260nm UV wavelength exposure where the extinction co-efficient of double stranded DNA is 0.020 $(\mu g/mL)^{-1}cm^{-1}$. The optical density of 1 therefore corresponds to a concentration of 50 ng µL-1 of DNA. The purity of DNA was determined by the ratio of optical densities at 260:280nm, whereby a ratio within a range of 1.8-2.0 was considered to have a lower protein contamination.

3.5.17. DNA sequencing

Following a successful PCR, the DNA was submitted to Eurofins Genomics (Wolverhampton, UK) for sequencing according to sample submission guide in which a premix sample of total volume (17 µL) containing 15µL of purified DNA

(10 ngμL^{-1}), 2 μL of either a forward or reverse primer (10pmolμL^{-1}) and made up to volume with PCR grade water.

3.5.18. DNA Sequence analysis for microbial identification

Sequence results were combined into a fasta file and checked for chimeras against a reference sequence using UCHIME function in the MOTHUR project [223, 224]. Mega suite version 5 for Windows was used to trim the sequences of primer artefacts and other irrelevant sequences [225] before submitting to Genbank where *E. coli* (Genbank accession number J01695) 16S rRNA gene sequence was used as the reference sequence [226]. Sequences were then analysed using the NCBI database with the help of MegaBlast, a basic alignment search tool for 16S rRNA sequence for Bacteria.

3.5.19. Construction and inference of phylogenetic trees

The construction of phylogenetic trees was carried out using MEGA version 5 suite for Windows where the MUSCLE (Multiple Sequence Comparison by Log-Expectation) function of the MEGA suite was used to align the sequences with references obtained from Ribosomal Database project (RDP: Classifier – RDP Naïve Bayesian rRNA Classifier version 2.10; Taxonomical Hierarchy RDP 16S rRNA training set 14) at a confidence threshold of 95%. Phylogenetic estimates were completed employing the neighbour joining tree method utilising bootstrap test of 1000 replications to generate consensus tree.

3.5.20. Whole genome sequencing of pure bacterial isolate

The whole genome sequencing of the extracted microbial DNA from the pure bacterial isolate – *Oceanobacillus* sp. strain Hud, was carried out by Baseclear Netherlands using whole genome sequencing (WGS) strategy.

3.5.21. Catalase and oxidase tests

Catalase test was carried out by adding a drop of 3% H_2O_2 on a clean glass slide and by means of a sterile loop, a colony was taken and smeared in the drop of the peroxide. Bubble formation indicated the presence of catalase enzyme.

Oxidase test was performed on test strips (Thermo Fisher Scientific, Oxoid Ltd, Basingstokes, UK) impregnated with NNN'N'tetramethyl-p-phenylene-diamine dihydrochloride for the detection of bacterial cytochrome oxidase enzyme. By means of a sterile plastic loop, a colony was taken and smeared on the test strip. The appearance of a purple colour (indophenol) within 10 seconds indicated a positive results.

3.5.22. Motility Test

Test for motility was carried out by hanging drop method under X40 of Olympus microscope (Olympus Optical BX 40F, Japan)

3.5.23. Batch fermentation with pure cultures for ISA Biodegradation

A batch fermentation experiment was set up using minimal medium (pH 9) previously described by Basil, *et al*. [70]. Briefly, the composition of the minimal media included g/L $NaHCO_3$ (2.5), NH_4Cl (4.7), NaH_2PO_4 (0.53), KCl (0.1) and 2% v/v vitamin solution and mineral solution. The vitamin solution is as shown in Table 2 whereas the mineral solution consisted of g/L $MgSO_4$.$7H_2O$ (3), $MnSO_4.2H_2O$ (0.5), NaCl (1), $FeSO_4.7H_2O$ (0.1), $ZnSO_4$ (0.1), $Na_2MoO_4.2H_2O$ (0.001g), $CoCl_2.6H_2O$ (0.1), $CuSO_4.5H_2O$ (0.01), $Na_2MnO_4.2H_2O$ (0.01). No electron acceptors were added. CDP and Ca(α-ISA)$_2$ were added as carbon sources similar to procedure previously described for anaerobic microbial consortia. The setup was prepared in duplicate in 30 mL Wheaton bottles with a working volume of 25 mL with an initial inoculum of 10^4 CFU/mL of the bacterial suspension. A control experiment containing no live bacteria was set up. Sampling was carried out every 2 days whereby 1 mL of reactor fluid was taken by means

of syringe and hypodermic needles and pH measured by means of micro pH probe (Mettler Toledo, Leicester, UK).

Bacterial growth was determined by spectrophotometer by measuring absorbance at 620nm wavelength after which samples were filtered using 0.4 µm filter unit before storing at -20°C for ISA/VFA analysis.

3.5.24. Biochemical characterization with API 20 A and Biolog PM/Ecoplates

The biochemical characterization of the pure isolates using API 20 A test strips and PM/Ecoplates was carried following manufacturer's instructions. All strips and plates were incubated at 25°C.

3.5.25. Assessing microbial tolerance to heavy metals and salt concentration using the Bioscreen automated growth curve

The tolerance of isolates to a range of pH, temperature, heavy metal concentrations and salt was assessed using bioscreen-C growth curve analyser (Bioscreen PF-1100 C; USA). For pH tolerance, microbial cells (10^3 $CFUmL^{-1}$) were inoculated into 9 mL iso-sensitest (Oxoid, UK) broth for different pH (pH 4-12) of which 300 µL was pipetted into honeycomb multiwall plates and incubated for 24 hours at 25°C in the bioscreen-C under constant shaking and wideband filter (450-580nm). To assess the tolerance of the isolates to heavy metals (Zn, Cu, Pb, Ni, Cd and Co), the same set up as used for pH tolerance was used except that different concentrations of the metals (1mM – 5mM) were incorporated into the growth medium. Similarly, tolerance to salt concentration was achieved as described. The determination of optimum pH and temperature was deduced from the area under the microbial growth curve while resistance to heavy metals was assessed by modelling MIC using a modified Gompertz function [227].

In order to compare the area under the growth (measured as optical density, OD)/time curve of samples duplicate readings were averaged and the control well

reading was removed from the data. Adopting the method by Shi-Tao Yeh [228] the trapezoidal rule was used to calculate the area under the microbial growth curve (AUC) as follows:

AUC (FA) = (C3 + C4) / 2 * (t4 – t3)

Where;

AUC = Area under the curve

FA = Fractional Area,

C3, C4 = OD in cell 3 and cell 4 (on excel spreadsheet)

t4,t3 = time 3 and 4

The Fractional areas (FA) were calculated from the ratio of the test area sum to that of the control area sum in order to obtain the relative amounts of growth. Using the calculated FAs and a modified Gompertz function in conjunction with the solver tool of Microsoft Excel, the Minimum Inhibitory Concentration (MIC) and Non Inhibitory Concentration (NIC) were modelled from the equations below:

$$\mathbf{Y = A + Ce^{-eB(x-M)}}$$

$$MIC = 10^{(M-1/B)}$$

$$NIC = 10^{(M-1.718/B)}$$

where,

Y = fractional area of the Gompertz function

The values of A, B, C and M are first selected as ‘dummy’ variables and fitted into the Gompertz function and are later used in solver to reduce the errors between the function and experimental values.

3.5.26. Extraction and identification of Fatty Acids Methyl Esters (FAME)

FAME analysis was performed using a modified version of the Sherlock Microbial Identification (MIDI) System. Fresh bacterial cells (40 mg) were placed in clean sterile 13x100 culture tubes. To separate the fatty acids materials from non-fatty acids materials such as sterols, sugars and microbial proteins, samples were saponified with the addition of 1.0 mL of Reagent 1 containing sodium hydroxide (45 g), methanol (150 mL) and distilled water (150 mL) to the tubes containing the cells. Tubes were then vortexed briefly to mix and heated for 5 minutes in a boiling water bath after which they were vortexed vigorously for 10 seconds and returned to the water bath for additional 25 minutes. In order to derivatize and make the fatty acids more amenable for analysis, samples were cooled to room temperature before the addition of 2 mL reagent 2 consisting of 325 mL 6.0N HCl and 275 mL methyl alcohol and heated to 80°C (± 1°C) for 10 minutes (± 1 min.). To extract the fatty acid methyl esters, the tubes were allowed to cool to room temperature before the addition of 1.25 mL of Reagent 3 made of 200 mL hexane and 200 mL methyl-tert-butyl ether was added which extracted the fatty acid methyl esters into the organic phase. The tubes were tumbled gently in a rotator for 5 minutes and allowed to settle. The aqueous lower phase was pipetted and discarded. Finally, 3 mL of Reagent 4 consisting of 10.8 g of NaOH dissolved in 900 mL of distilled water was added to sample as a base wash to remove contaminants. The sample was allowed to settle after which 2/3 of the organic phase was pipetted for analysis. The identification of microbial Fatty Acid Methyl Esters (FAME) was carried out by Gas Chromatography-Mass Spectrometer (GC-MS). Mass spectra of the peaks was compared in the order of percentage similarity to FAME standards in the NIST library associated with the GC-MS software.

3.5.27. Extraction and identification of microbial isoprenoids (Menaquinones)

Microbial isoprenoids/menaquinones were extracted using the method described by Collins *et al*. [229]. Cells were grown on fastidious anaerobic agar and incubated at 25°C for 18 h before being dried at 70°C overnight after which 100 mg were mixed with 20 mL chloroform/methanol (2:1 v/v). The suspension was stirred continuously overnight after which biomass was filtered. The extract was then evaporated to dryness at 36°C.

The identification of menaquinone was carried out against MK-7 standard (Sigma-Aldrich, UK) using thin layer chromatography (TLC) was on 0.20 mm pre-coated TLC-sheets (DC-Fertigfolien Alugram®, Germany) and a developing solvent consisting of petroleum ether (b.p 60-80°C)/diethyl ether (85:15, v/v). Menaquinones were detected by irradiation of short-wave ultraviolet light on the TLC sheet. The values of retention factor (R_F) were calculated from the ratio of distance moved by the sample to the distance moved by the solvent (solvent front).

R_F = distance moved by sample / distance moved by sample

3.5.28. Biofilm formation and adherence assay for *Exiguobacterium* sp. strain Hud and *Oceanobacillus* sp. strain Hud

Isolates were plated on solid mineral media containing $Ca(ISA)_2$ containing (4mM) for 72 hours under obligate anaerobic conditions (80% N_2, 10% CO_2, 10% H_2) in an anaerobic workstation (Don Whitley, Shipley, UK) at 25°C after which a colony was from each isolate was smeared on a clean glass slide. A 100 µL solution of Calcofluor white (1 gL^{-1}) was added to sample and covered with a clean cover slip. Sample were observed under fluorescence laboratory microscope (Olympus BX41, Olympus, USA).

To carry out biofilm adherence assay, 10 universal bottles (for each isolate) containing sterile mineral media (10 mL) were inoculated with the isolates and incubated for 18 hours in orbital incubator at 25°C , 120 rpm after which 1 in 10 dilution of each sample was done in another set of mineral media. From this suspension, 10 µL was dispensed into microtiter plates containing 150 µL of sterile mineral media octuplicates. Negative controls containing only mineral media were also set up. Plates were incubated at 25°C for 18 hours anaerobically. Following incubation, microtiter plates were carefully tipped and washed (3X) with phosphate buffered saline (200 µL) after which 200 µL of 99% methyl alcohol was added to the wells for 15 minutes to fix the biofilm. Plates emptied and allowed to air-dry after which 200 µL of crystal violet solution was added to the wells to stain adherent biofilm. Plates were tipped carefully, washed with distilled water and air-dried. Glacial acetic acid (160 µL; 33%) was added to the wells to solubilize the biofilm and quantified at 492 nm using plate reader (Fluostar Optima, BMG Labtech). Actual biofilm of the isolates was determined by deducting the absorbance of controls.
Biofilm adherence classification by Stepanovic *et al*. [230] was adopted as follows:

$OD \leq ODc$……non adherent biofilm

$ODc < OD \leq 2XODc$ ……..weakly adherent biofilm

$2XODc < 4XODc$ ……...moderately adherent biofilm

$4XODc < OD$ ……..strongly adherent biofilm

(OD = optical density of sample; ODc = optical density of control wells)

3.5.29. Extraction and characterization of exopolysaccharides of *Exiguobacterium* sp. strain Hud and *Oceanobacillus* sp. strain Hud

Fresh inoculum suspensions of the two isolates in 10 mL minimal media incubated for 18 hours anaerobically at 25°C were made. From this suspension, 1 mL was inoculated into 1 L minimal media and incubated for 72 hours. Cells were then separated via centrifugation using Avanti® J-26 XPI Centrifuge (Avanti J- 26S XPI-Beckman Coulter, Inc., USA) equipped with F250 Fiberlite™ F14-6 x 250y Fixed-Angle Rotor at 10,000 x g, 15 min, 4°C. The supernatant containing the EPS was kept for downstream application. The EPS which was thought to be attached to the cells was also extracted by resuspending the cell pellets in 500 mL NaoH (1.0 M) and kept under constant stirring overnight by means of a magnet stirrer. The detached EPS was recovered from the alkali solution via centrifugation as described.

Following centrifugation, EPS was precipitated from the resultant supernatant by mixing with two volumes of chilled ethanol and kept under 4°C for 48 hours The precipitate was recovered via centrifugation (25,000 x g, 4 °C, 30 min.). To purify the EPS, the precipitate was redissolved in deionised water and dialysed against deionised water with dialysis tubes (12–14 kDa molecular mass cut-off; Sigma, UK) for three continuous days at 4°C under constant stirring and with three changes per day. Finally, the dialysed fractions were poured into 100 mL Shott bottles and lyophilised in a freeze dryer (Modulyo 4K Freeze Dryer, Edwards, Northern Scientific, York, UK).

EPS extraction was also carried out on agar-based culture whereby cell biomass was recovered from the surface of solid minimal media following 72 hours of anaerobic incubation. Two millilitre (2 mL) of deionised water was pipetted onto the culture plates and by means of a sterile loop cells were harvested into a Shott bottle. One volume of NaOH (2 M) was added to the cell suspension and placed on a magnetic stirrer and gently stirred overnight at ambient temperature to release the EPS attached to the cells surface. The detached EPS was extracted following the procedure described above.

Proton NMR (1H-NMR) analysis on the EPS was carried out by the University of Huddersfield NMR service. Lyophilised EPS (6.5mg) was dissolved in deuterium oxide

(D2O; 0.65 mL; Goss Scientific Instruments Ltd, Essex, UK) prior to analysis. The NMR spectra were recorded at elevated probe temperature (70°C) which did not only shift the HOD signal into a clearer region of the spectrum, but also increased the spectral resolution by reducing the viscosity of the sample. All NMR spectra were recorded on a Bruker Avance 500.13 MHZ 1H (125.75 MHZ 13C) spectrometer (bruker - biospin, Coventry, UK) using Bruker's pulse programme. Spectra were referenced using internal acetone (δ= 2.22 ppm).

Chapter 4

4. Results and discussion

4.1. Preparation and Characterization of $Ca(ISA)_2$ and Cellulose Degradation Products (CDP)

4.1.1. The rationale for the study

The broad aim of this study was to investigate microbial biodegradability potential of ISAs that are expected to generate within the nuclear waste repository after the facility is closed. The aim of this section was to prepare and characterize the $Ca(ISA)_2$ and the CDP that was be used as feedstock in the study.

On the basis of the method described in the previous section (section:3.3), analysis of the $Ca(ISA)_2$ with the ^1H NMR using Bruker Avance 500 MHz spectrometer with pulse sequences at ambient temperature showed spectra (Figure 4.1) that conformed to all the protons that were directly bonded to a carbon as per a molecule of ISA [231]. High Performance Anion Exchange Chromatography (HPAEC) analysis of the sample also showed ISA peak and retention times (Figure 4.2) which were the similar to the standard $Ca(ISA)_2$ obtained from a manufacturer (Carbosynth Ltd, Berkshire, UK).

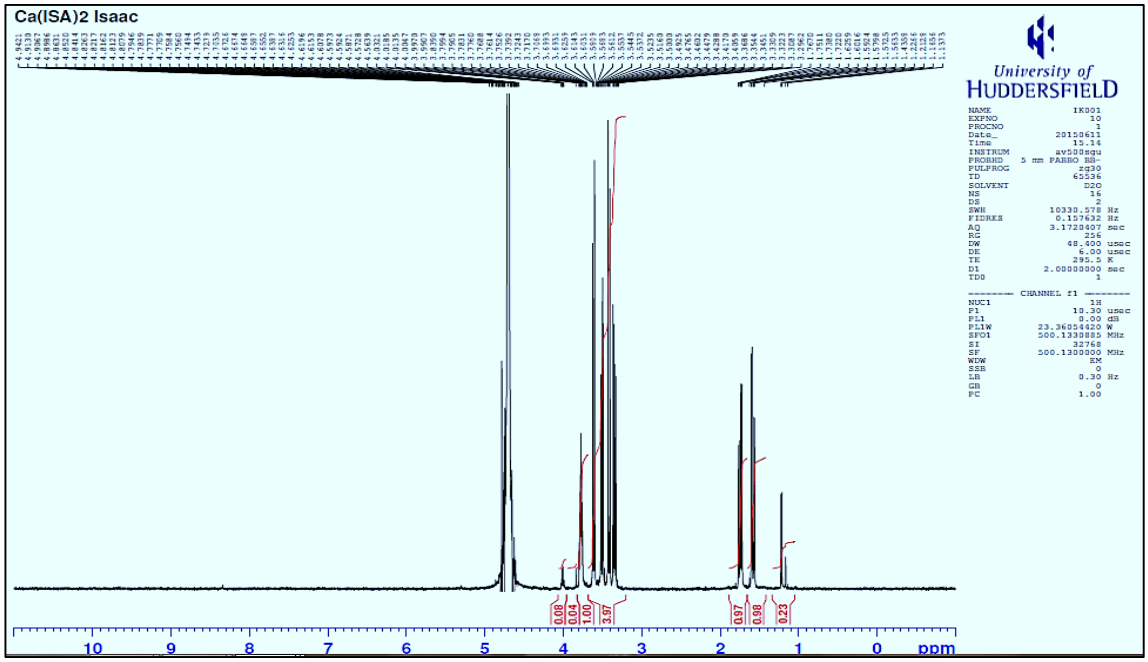


Figure 4.1 ^1H NMR spectra for Ca(α-ISA)$_2$ characterization

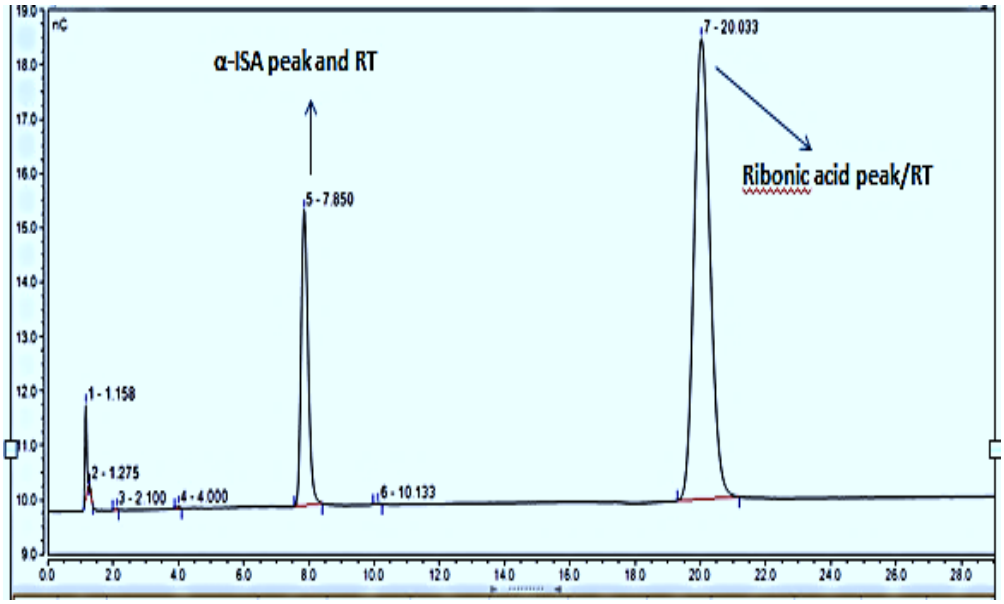


Figure 4.2 α-ISA peak and Retention Time measured from HPAEC during $Ca(ISA)_2$ characterization

The analytical procedures for the characterisation of the CDP revealed that the preparation of CDP from laboratory tissue paper yielded a complex mixture of compounds including α- and β-Isosaccharinic acid, xylose, isovaleric (pentanoic) acid and a range of volatile fatty acids (VFA) including acetic, formic and butyric acids. These components were also recorded in the study of Rout *et al*. [73] during an investigation into microbial degradation of CDP. Analysis of Total Organic Carbon (TOC) indicated that the CDP contains approximately 4000 mg/L of Organic Carbon.

4.1.2. Key findings

- The $Ca(ISA)_2$ prepared from α-lactose monohydrate was analysed to have characteristics that conformed to a standard $Ca(ISA)_2$
- The CDP prepared from laboratory tissue paper composed of a mixture of organic compounds including ISAs and volatile fatty acids including acetic acid
- The total organic carbon of the CDP was found to be approximately 4000mg/L

4.2. Microbial community degradation of ISA under alkaliphilic aerobic and anaerobic conditions

4.2.1. The rationale for the study

Some earlier studies focused on biodegradation of ISA under aerobic conditions [54, 60] the results of which offered insights into microbial ISA bioremediation. Recent studies have demonstrated that microbial populations within organic rich soil layer from Harpur Hill (Buxton, Derbyshire, UK) are capable of biodegrading ISA within 15 days under aerobic and nitrate reducing conditions [70] and within 14 days at circumneutral pH [74]. Investigation into microbial diversity have also shown the presence of alkaliphilic, anaerobic microorganisms indigenous to the site [232]. As anoxic conditions are likely to dominate post closure of the ILW-GDF due to corrosion processes while nitrate and iron reducing conditions are likely to be limited, fermentative and methanogenic processes are therefore expected to play a significant role in microbial metabolism of ISA. In this vein, although aerobic conditions are not expected to dominate the GDF, an investigation into the potential of microbial consortia within sediments from this site to utilize ISA under alkaliphilic, aerobic and anaerobic condition will expand our knowledge on the behaviour of the GDF when it is finally closed.

This section of the study aimed at assessing the ISA biodegradability potentials of the microorganisms present within Harpur Hill organic rich soil layer at high pH of 10.0 under both aerobic and anaerobic conditions. The experimental set up is discussed in sections 3.5.2 and 3.5.3.

4.2.2. Results/discussion

Under aerobic conditions, both microcosms driven by $Ca(ISA)_2$ and CDP observed complete degradation of ISA which occurred within 6 days of incubation (Figure 4.3A and Figure 4.4A) where the first order rates of degradation were $1.8 \times 10^{-1} \pm 0.00\ d^{-1}$ for the $Ca(ISA)_2$ system and $3.2 \times 10^{-1} \pm 0.00\ d^{-1}$ for total ISA in the CDP system. The biochemical pathway for the degradation within the two systems appeared similar whereby the removal of ISA was accompanied by

increase in biomass (Figure 4.3B and Figure 4.4B) and a drop in pH from the initial of pH10.0 to a final of pH 8.9 in the $Ca(ISA)_2$ system (Figure 4.3A) and pH 10.0 - pH 8.8 in the CDP system (Figure 4.4A). The drop in pH in these experiments could be attributed to the formation of acidic products such as CO_2 (although not measured) from the degradation of ISA.

Contrasting with previous findings, no terminal electron acceptors (TEA) were included in the media however, ISA degradation was possible in both microcosms within fewer days as compared with the study by Bassil *et al*. [70] which took 9 days and that of Kuippers *et al*. [74] which took 14 days for a complete biodegradation of ISA to occur.

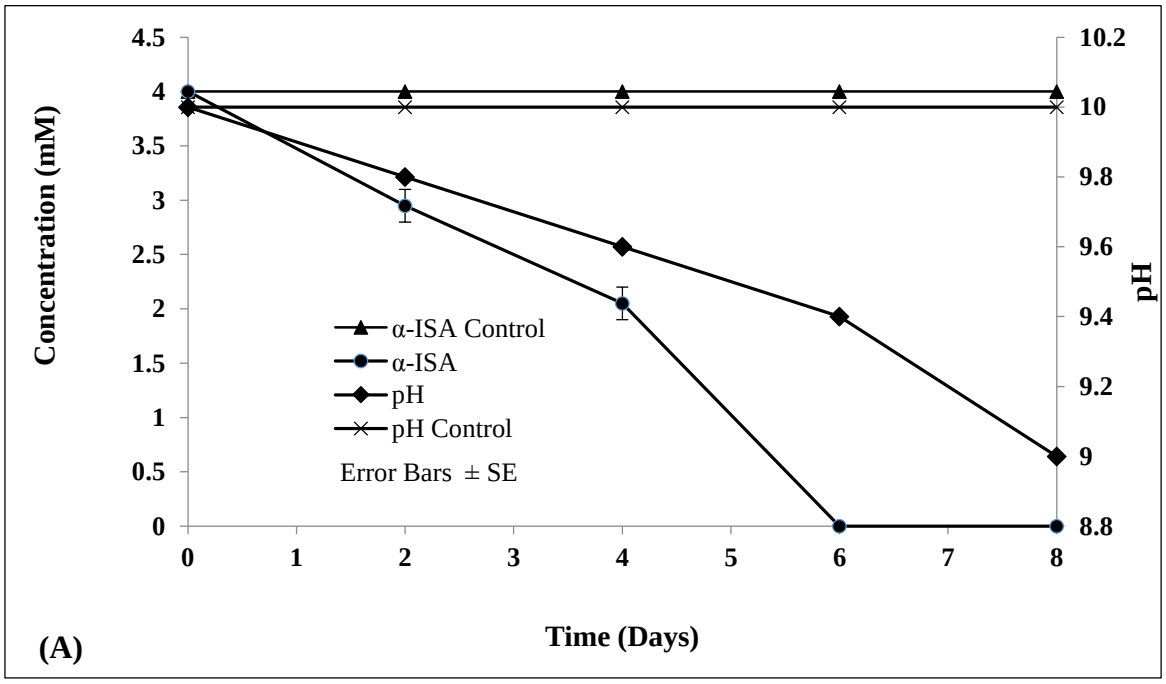


Figure 4.3 Aerobic microbial degradation of α-ISA

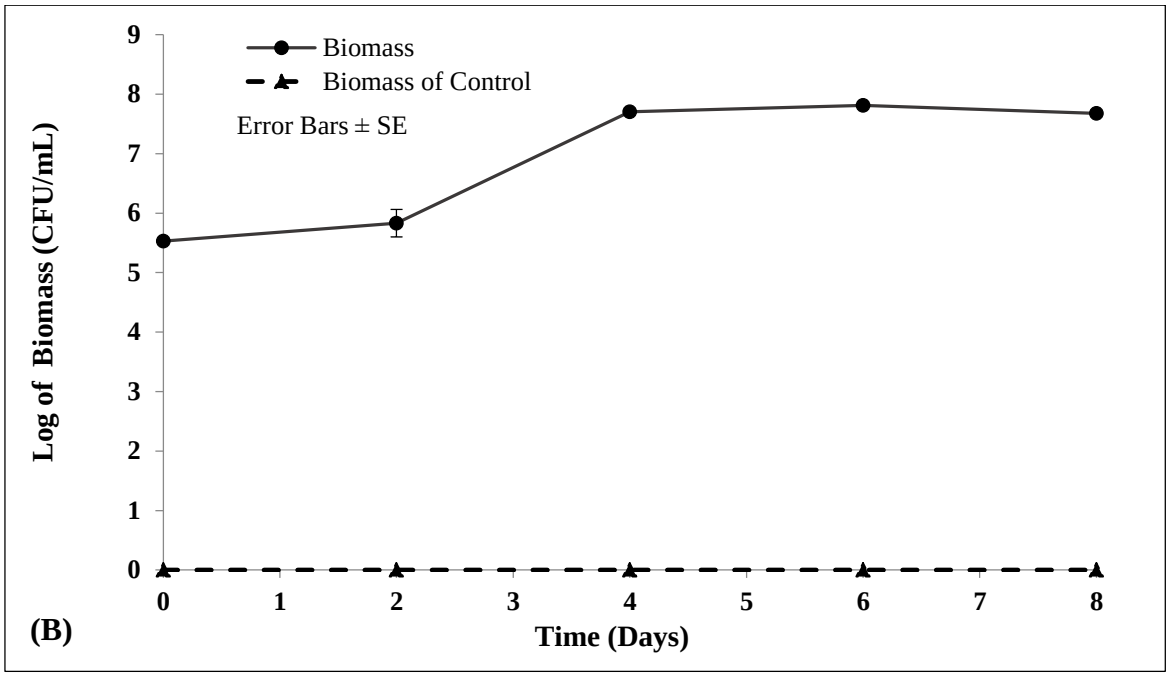


Figure 4.3 continues: $Ca(\alpha\text{-ISA})_2$ microcosm seeded with soil sample from Harpur Hill site showing (A) ISA removal and pH drop with time, (B) Microbial biomass in CFU/mL

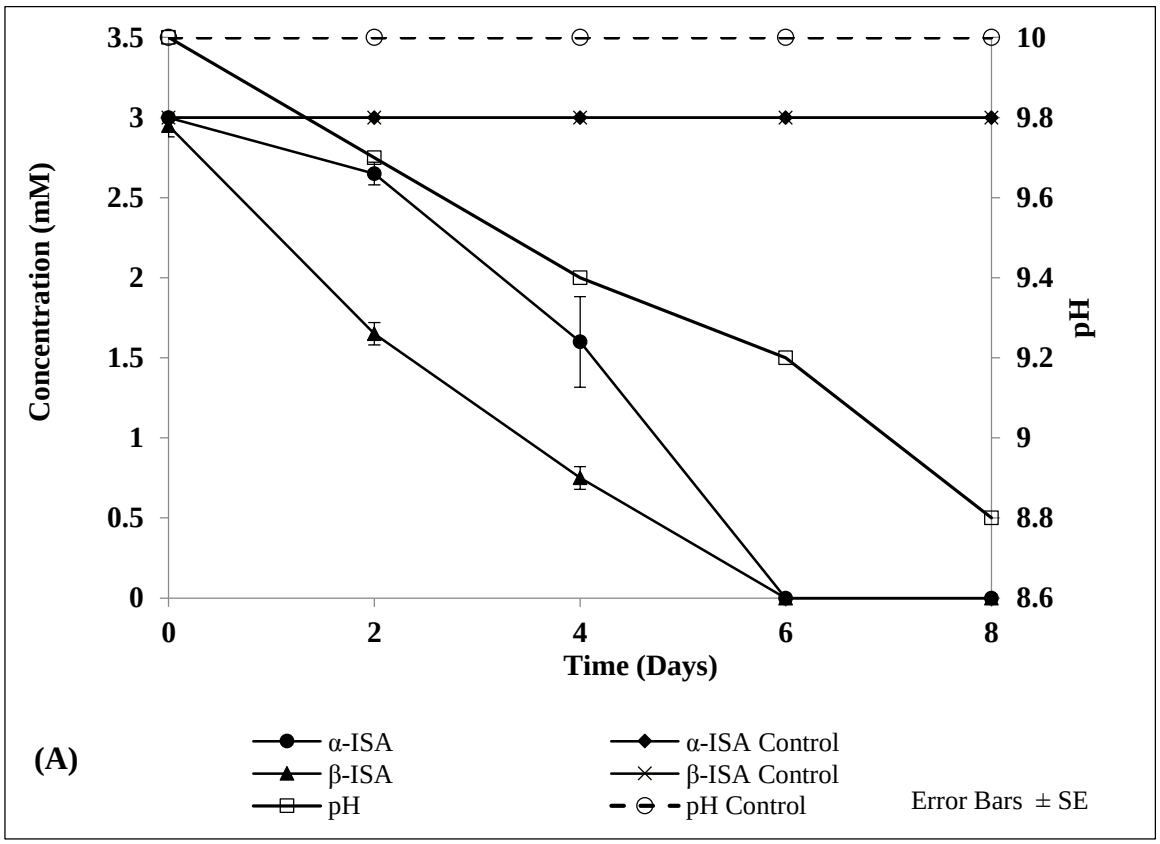


Figure 4.4 Aerobic microbial degradation of α-, β-ISA from CDP

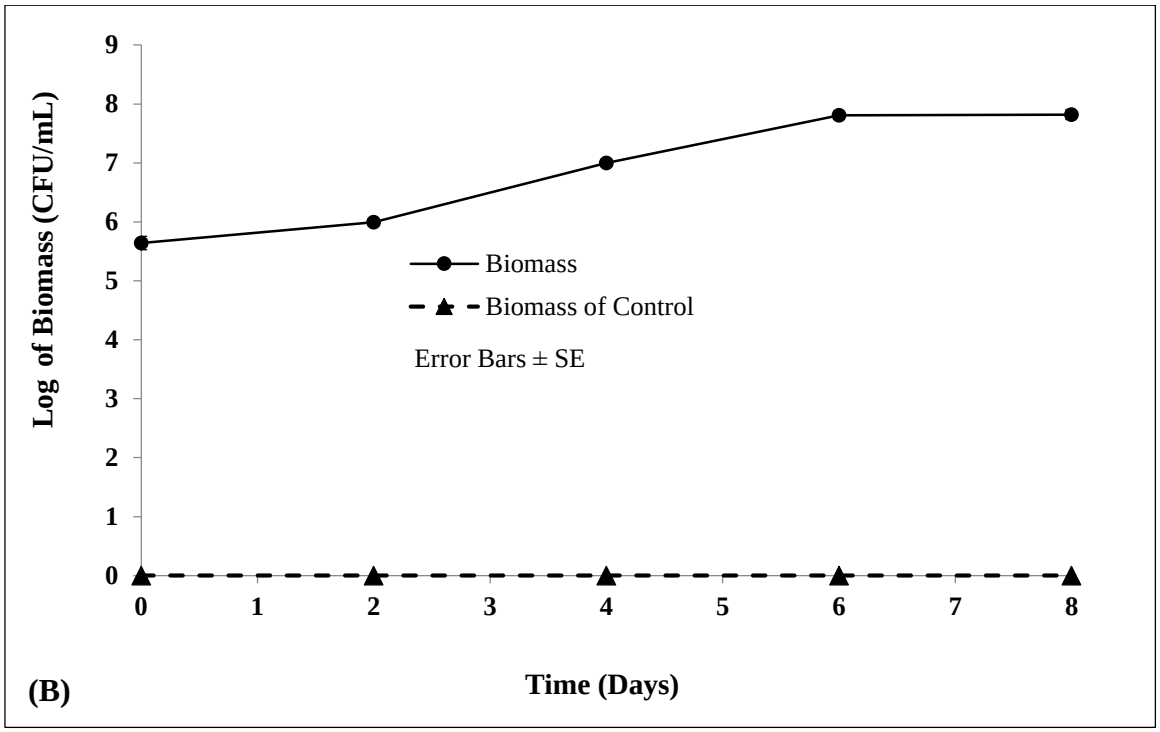


Figure 4.4 continues: CDP microcosm seeded with soil sample from Harpur Hill site showing (A) ISA removal and pH drop with time, (B) Microbial biomass in CFU/mL

Under anaerobic conditions, the microcosm enriched with $Ca(ISA)_2$ demonstrated a complete degradation of ISA within twelve (12) days. ISA removal occurred quite steadily for the first six (6 days) and proceeded sharply until no ISA could be detected. The microcosm showed a mean first order rate of degradation of 1.0 x 10^{-1} ± 0.00 d^{-1}. The degradation of ISA was accompanied by the production and accumulation of acetic acid whereby 2.6 mM representing a yield of 65% of the total degraded ISA accumulated by the end of the sampling period. A drop in pH from the initial pH 10.0 to a final pH of 7.9 was observed. An increase in biomass accompanied the removal of ISA.

In the microcosm amended with CDP, a similar ISA degradation profile was observed whereby both α and β forms of ISA were completely utilized. The β-ISA was found to have been degraded within sixteen (16) days whereas α-ISA completely degraded within 24 days. A total of 3.7 mM acetic acid accumulated

which represented 54% of the total degraded ISA. A drop in pH of the medium from an initial of pH10.0 to a final pH of 7.6 was observed by the end of sampling. Increased biomass was also observed throughout the experimental period.

In both microcosms, ISA removal was accompanied by acetic acid production, a drop in pH and increase in biomass. These suggest that microbial populations within the organic rich soil layer may be using a similar biochemical pathway for ISA degradation. ISA removal from CDP took a longer time compared to it $Ca(ISA)_2$ counterpart probably due to the presence of other carbon substrates present in the CDP which microorganisms can utilize.

These results presented here expand knowledge on the potentials of bacteria indigenous to a particular environment to degrade ISA as sole carbon source.

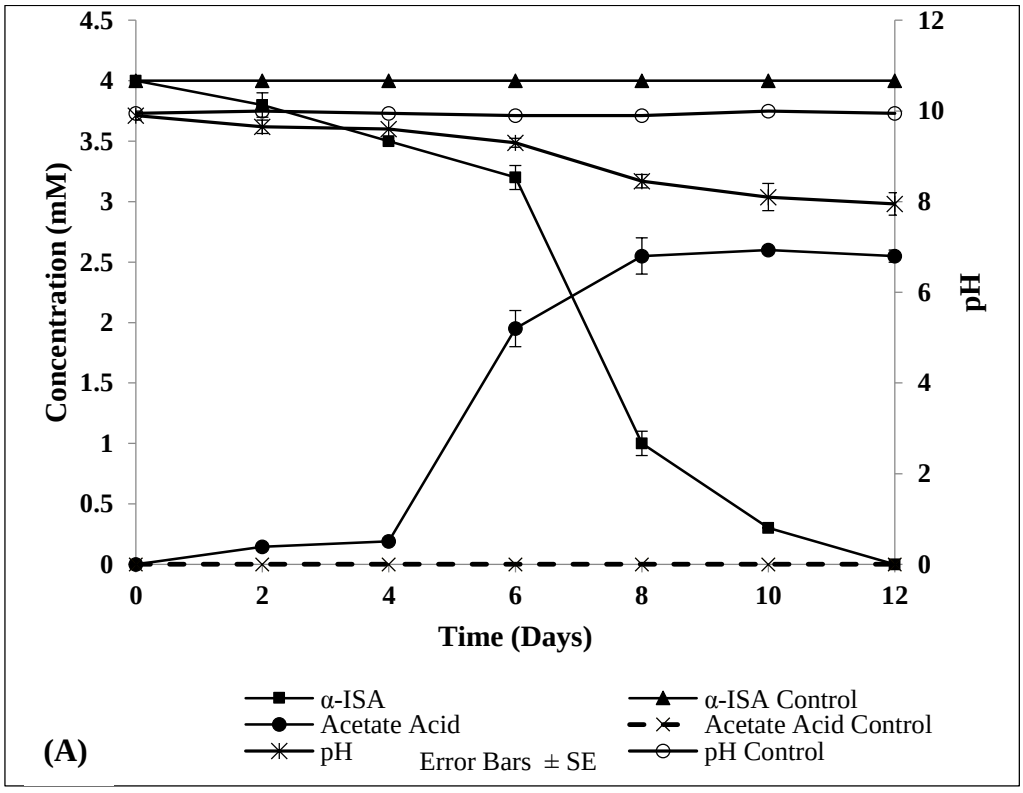


Figure 4.5 Anaerobic microbial degradation of α-ISA

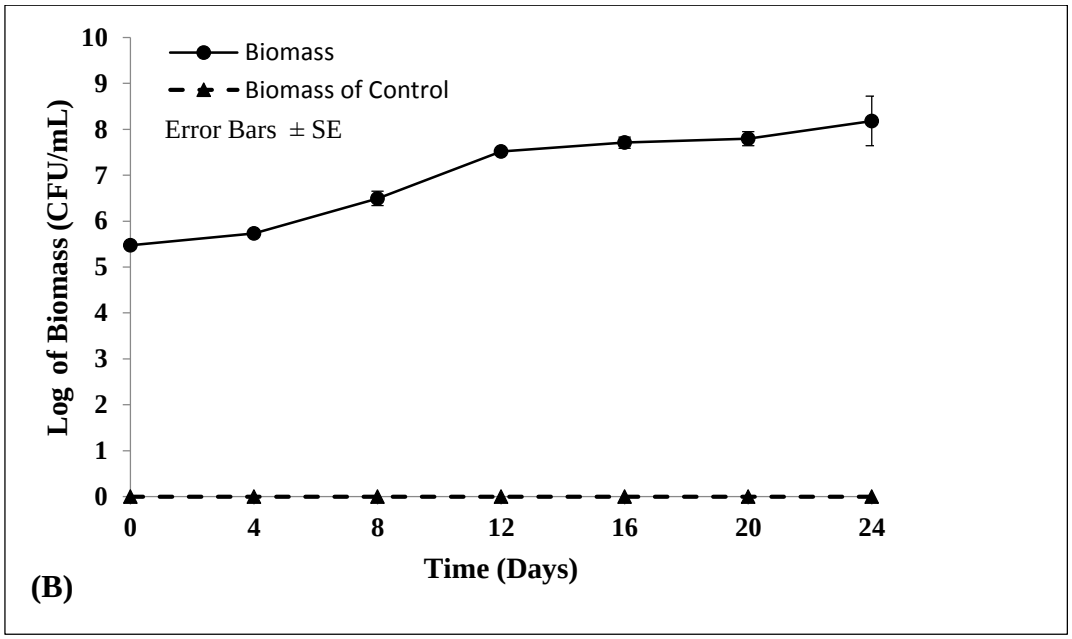


Figure 4.5 continues: $Ca(ISA)_2$ microcosm seeded with soil sample from Harpur Hill site showing (A) ISA removal and pH drop with time, (B) Microbial biomass in CFU/mL

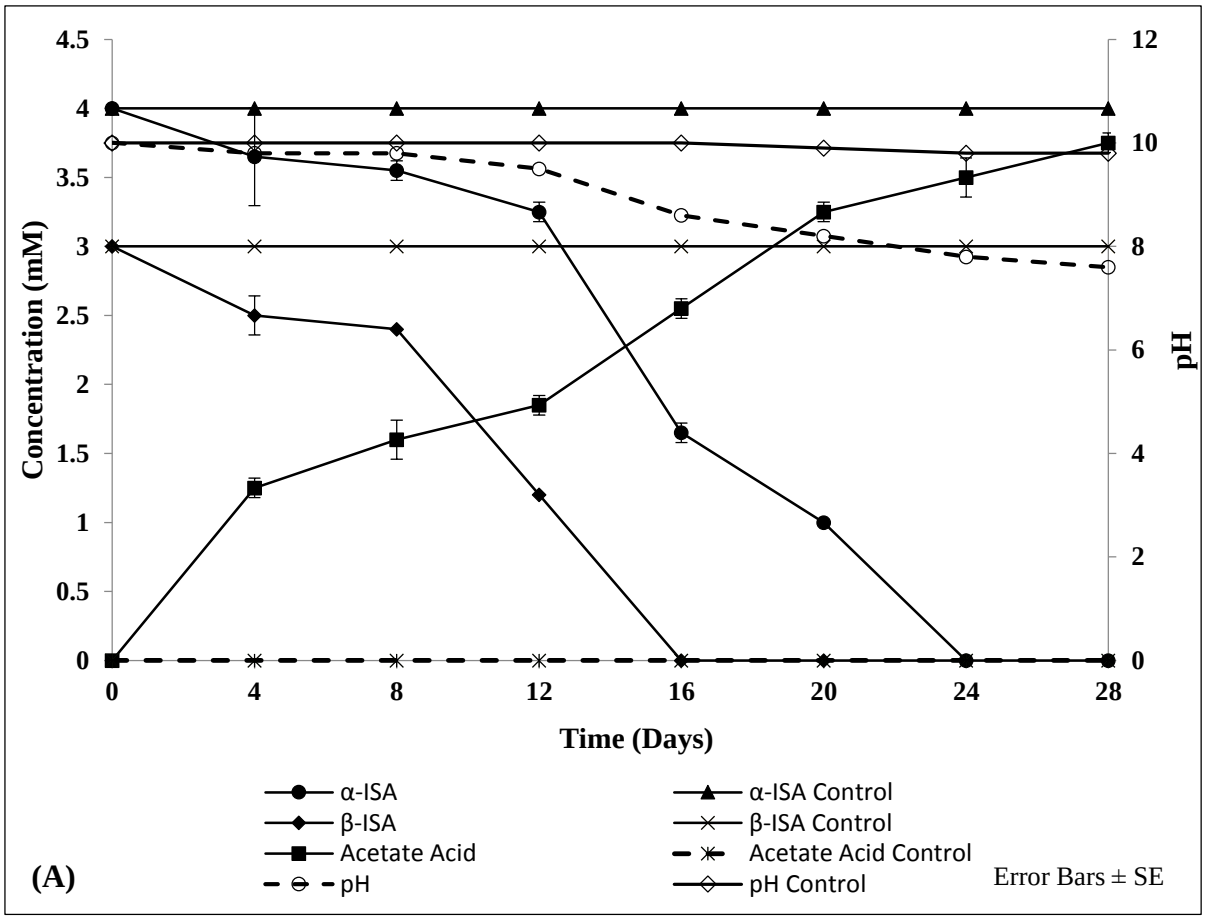


Figure 4.6 Anaerobic microbial degradation of α-/β-ISA from CDP

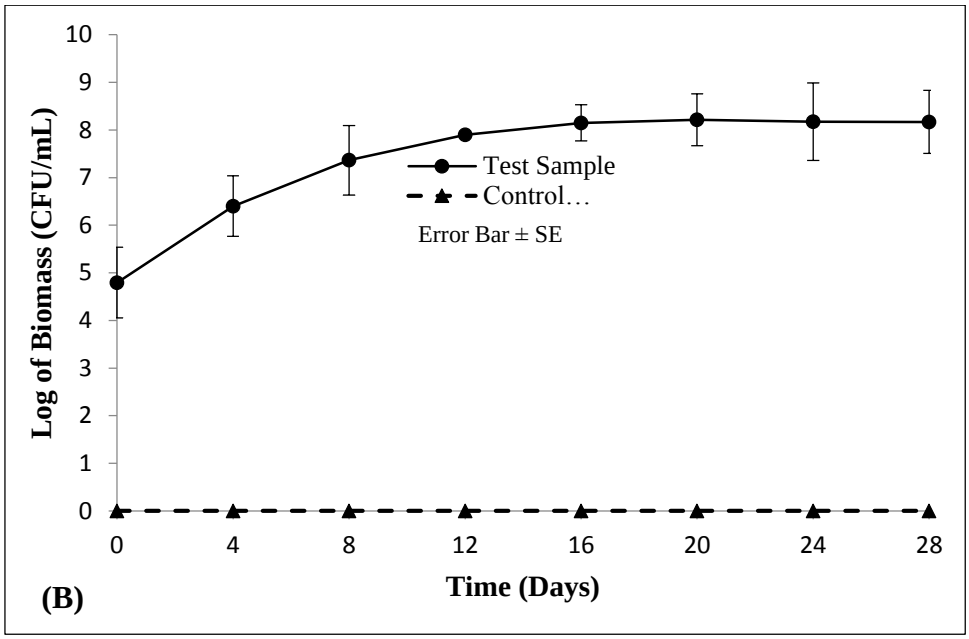


Figure 4.6 continues: CDP microcosm seeded with soil sample from Harpur Hill site showing (A) ISA removal and pH drop with time, (B) Microbial biomass in CFU/mL

4.2.3. Key findings

- Microorganisms present within Harpur Hill organic rich soil layer are able to degrade both α and β forms of ISA under both aerobic and anaerobic conditions
- In both $Ca(ISA)_2$ and CDP systems of experiment under aerobic and anaerobic conditions, the pH of the media dropped indicating the formation of acidic products
- The degradation of ISA under anaerobic conditions was accompanied by the production of acetic acid

4.3. The impact of nitrogen source and the form of ISA on microbial community evolution.

4.3.1. The rationale for the study

Microbial degradation of CDP will have an impact on the evolution of an ILW-GDF, consequently, a number of studies have investigated the degradation of CDP and associated ISA's. Two contrasting approaches have been employed, Rout *et al*. [113] and Charles *et al*. [83] used CDP containing a mixture of a range of ISA's, whilst Bassil *et al*. [70] and Kuippers *et al*. [74] used the calcium salt of α-ISA as an analogue of CDP.

In most ISA biodegradation studies, researchers have introduced different kinds of ammonium compounds in different concentrations as part of growth media components [70, 73, 74]. This reflects the fact that ammonium compounds are the preferred nitrogen source for most microorganisms [233, 234]. However, deep geological environments such as nuclear waste repositories are often marked by very low concentrations or complete lack of biodegradable molecules [235]. In particular, nitrogen and or phosphorus have been assessed to be growth-limiting nutrients in geological disposal repositories [236] with nitrogen fixation being the most likely source of nitrogen.

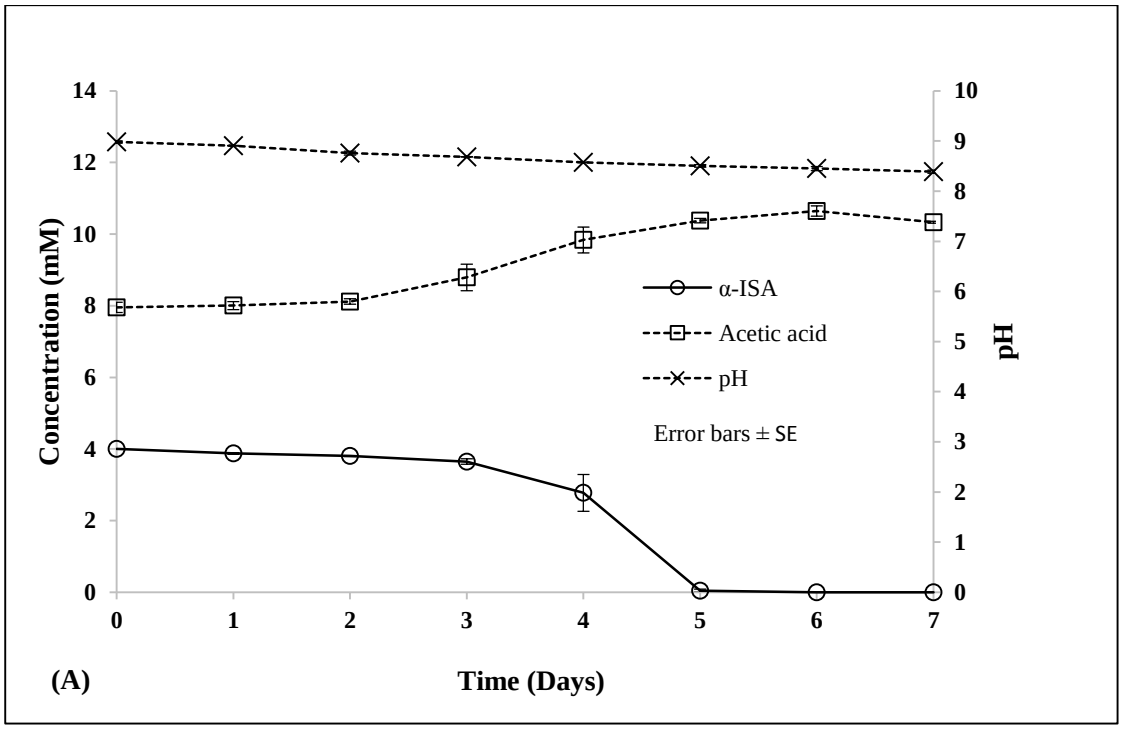
The aim of this section of the study was to assess the impact of nitrogen source and the form of ISA employed, on the evolution of ISA degrading microbial communities in relation to a nitrogen free system. The operation of the microcosms used in this section is described in section 3.5.1.

4.3.2. Results/Discussion

4.3.3. Chemical analysis and biomass of the established communities

In the microcosm fed solely on α-ISA (from $Ca(ISA)_2$) , complete degradation of ISA occurred within 6 days of incubation irrespective of the presence of NH_4^+ (Figure 4.7A and B). The mean first order rates of degradation were found to be $1.8 \times 10^{-1} \pm 0.00\ d^{-1}$ and $2.3 \times 10^{-1} \pm 0.01\ d^{-1}$ in respect of NH_4^+ amended and NH_4^+ free systems. Within the CDP-fed microcosms the β-ISA was completely removed within 5 days followed by α-ISA on day 6 of sampling (Figure 4.7C and D) in the presence and absence of NH_4^+. The rate of total ISA removal in these systems was found to be $1.7 \times 10^{-1} \pm 0.00\ d^{-1}$ in the presence of NH_4^+ and $2.2 \times 10^{-1} \pm 0.00\ d^{-1}$ in the NH_4^+ free system.

In all systems, the degradation of ISA coincided with the generation and accumulation of acetic acid (Figure 4.7). In the NH_4^+/α-ISA fed systems (Figure 4.7A) the consortia generated 2.37 mM acetic acid over the sampling period, representing a 59% yield from α-ISA. In the microcosm without NH_4^+ (Figure 4.7B) however, α-ISA degradation generated a relatively reduced amount of acetic acid whereby the yield was 2.22 mM across the batch/fed cycle, representing a percentage yield of 56%. In the NH_4^+ amended CDP fed microcosm (Figure 4.7C), the net acetic acid accumulation was 2.73 mM which represents a yield of 63% while its counterpart without NH_4^+ (Figure 4.7D) yielded relatively lower amounts of acetic acid (1.47 mM) representing a 37% yield. In all systems the pH was not amended during the sampling period, however a decrease in pH of about 1.0 pH unit was observed.



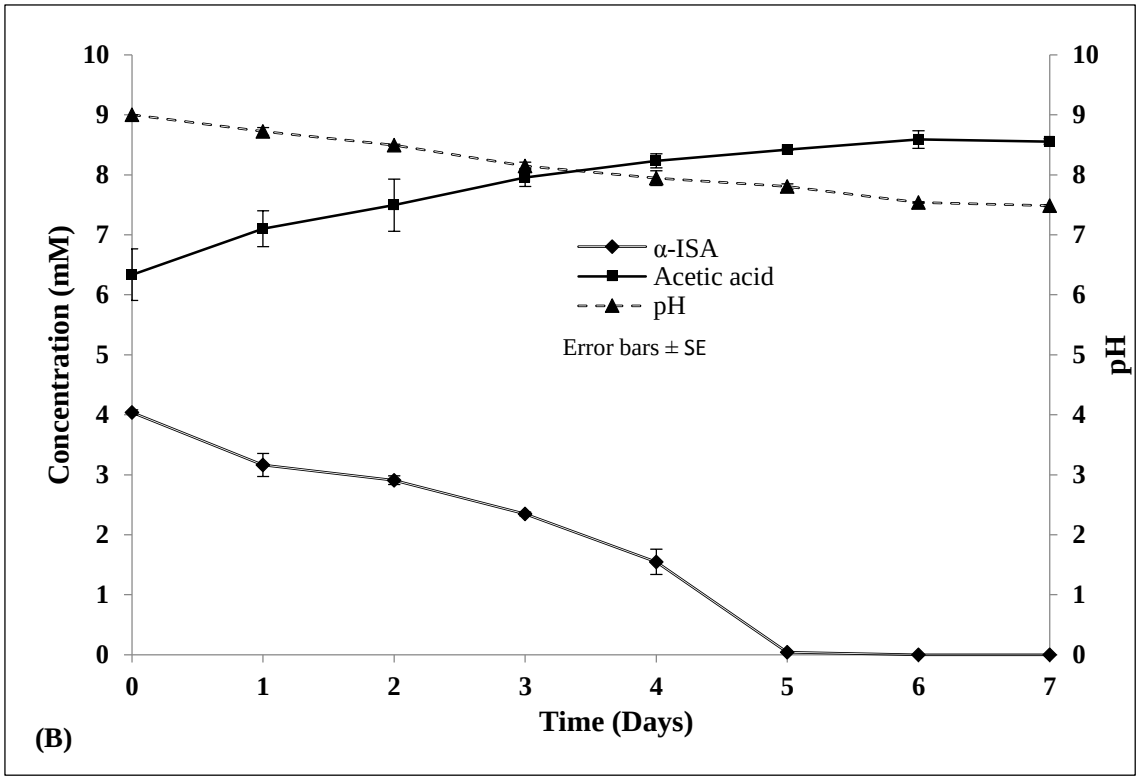
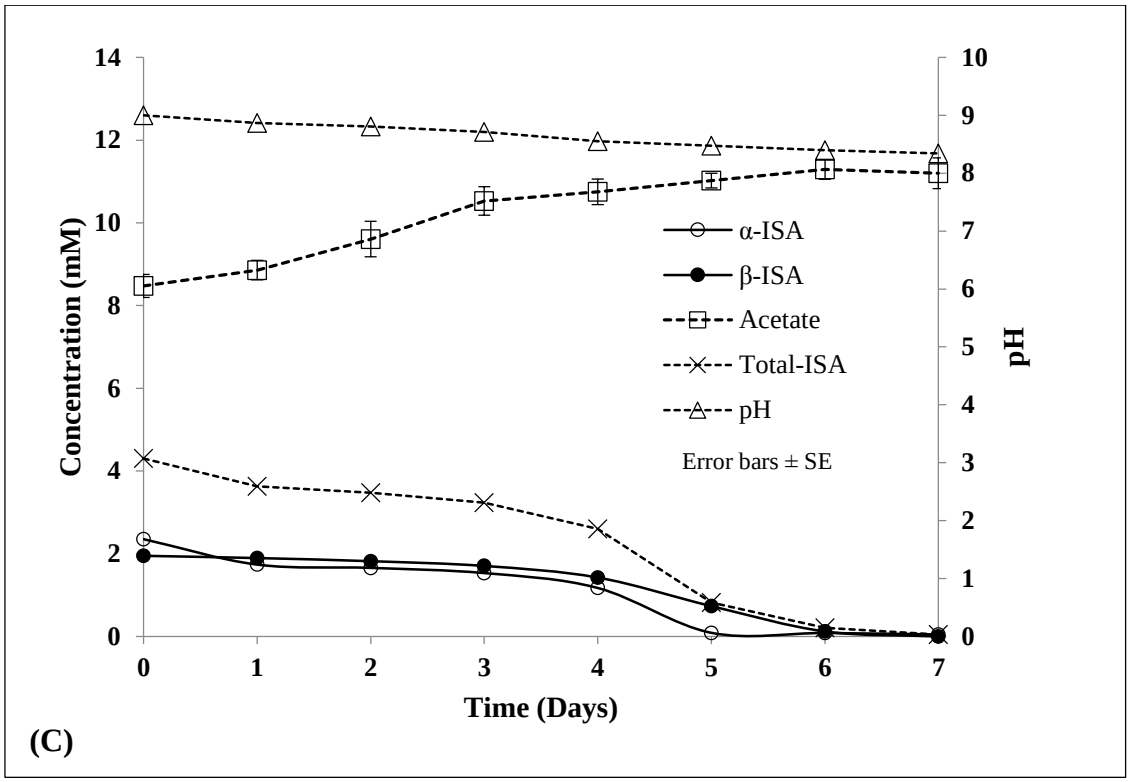


Figure 4.7 ISA biodegradation in model systems driven by $Ca(ISA)_2$ with/witout NH_4^+



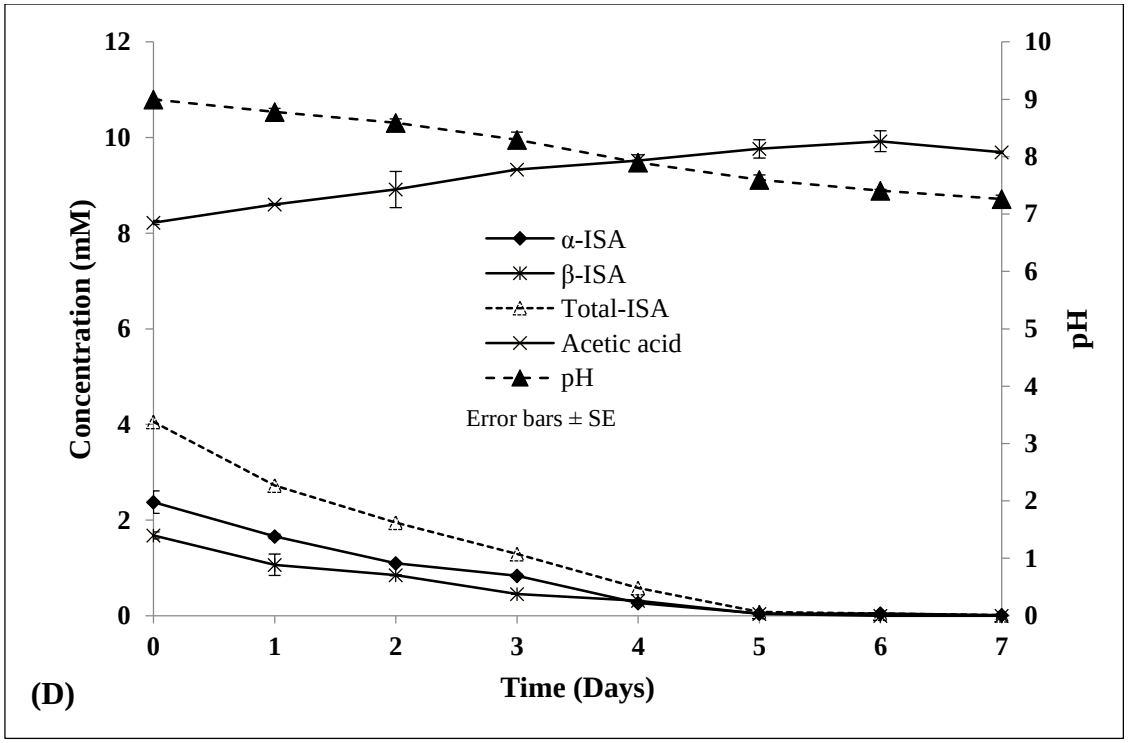


Figure 4.7 continues: (A) $Ca(ISA)_2$ driven system amended with NH_4^+, (B) $Ca(ISA)_2$ driven system without NH_4^+, (C) CDP driven system amended with NH_4^+, (D) CDP driven system without NH_4^+

The biomass generated during the degradation process are presented in Figure 4.8 whereby the profiles appear similar in all systems. Throughout these systems, increased biomass was observed by the stationary growth phase indicating that microorganisms within these systems were able to assimilate ISA regardless of the source of carbon or presence or absence of NH_4^+. However, the standing biomass at the beginning of the feed cycle was markedly different. The microcosms run in the absence of NH_4^+ had a ten-fold greater standing biomass than the NH_4^+ fed systems suggesting that the presence of ammonia was reducing the overall level of biomass supported by the system. This agrees with the review by Yenigün and Demirel [237] which reported the toxicity of ammonia in a methanogenic system. The effect of ammonia on the growth of *Bacillus* species and some other bacteria have also been investigated by Leejeerajumnean *et al*. [238].

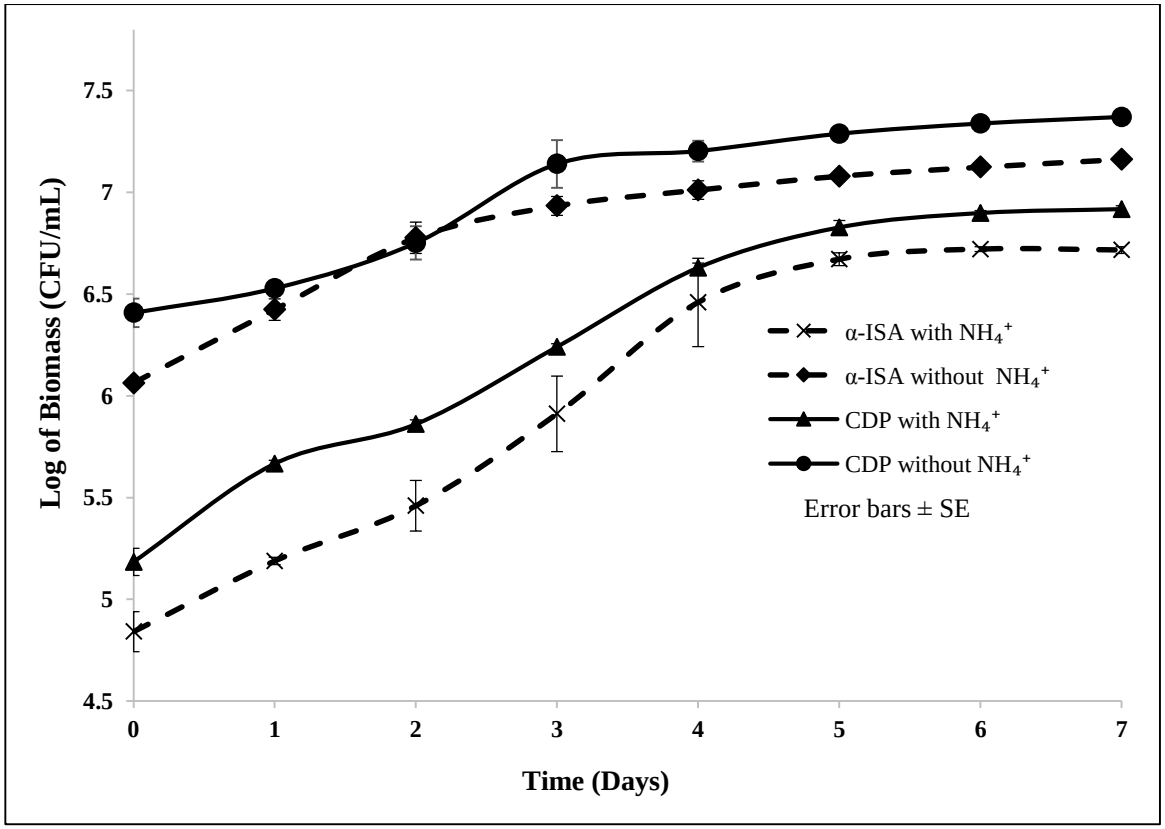
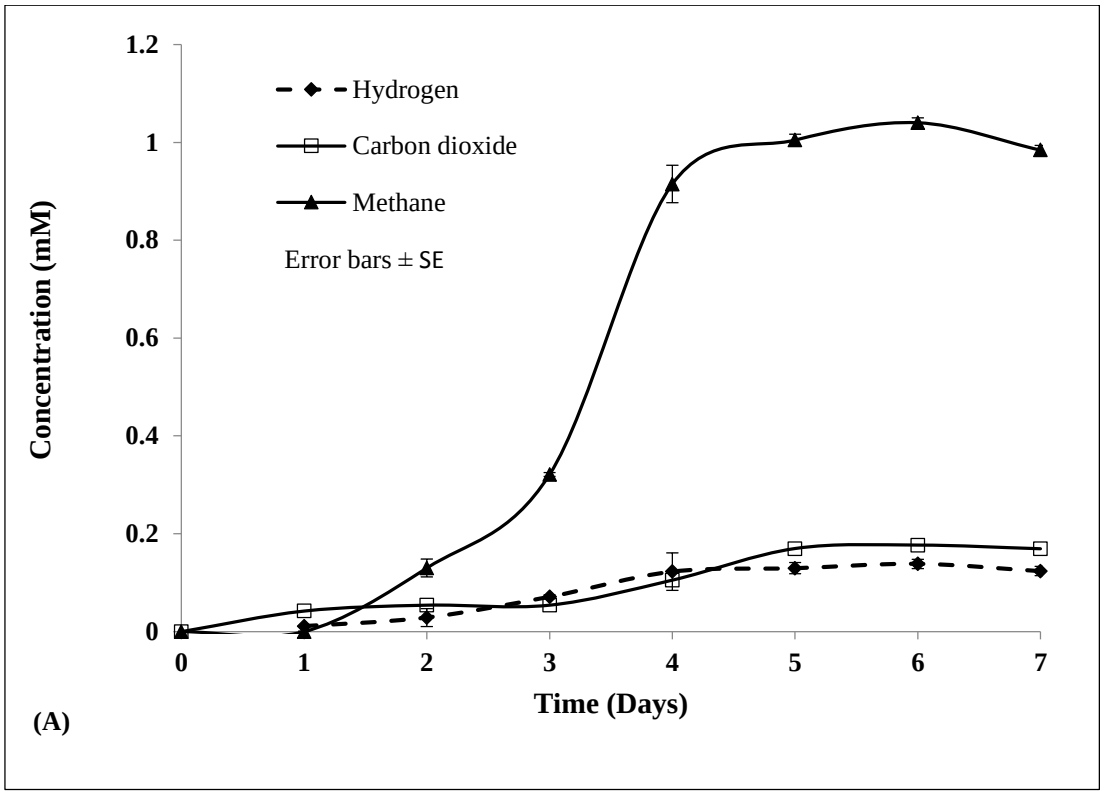


Figure 4.8 Biomass produced in the different systems

Biomass from $Ca(ISA)_2$ and CDP systems amended with NH_4^+ or in NH_4^+ free media

4.3.4. Gas evolution within the microcosms

The generation of gas was observed within the headspace of each system, where methane (CH_4), carbon dioxide (CO_2) and hydrogen (H_2) were evolved (Figure 4.9). Within the headspace of the α-ISA system amended with NH_4^+, the total CH_4 generation was $9.8x10^{-1}$ mM (Figure 4.9A) which represents a yield of 24.6% of the total degraded carbon substrate. In the same microcosm, CO_2 generation was $1.7x10^{-1}$ mM whereas H_2 generation was $1.2x10^{-1}$ mM representing percentage yields of 4% and 3% respectively. The CO_2 yields however, may have been underestimated due to the possibility of alkaline pH enhancing its solubility. In the case of α-ISA system without NH_4^+, the headspace gas evolution included a total of 1.25 mM (Figure 4.9B) constituting 31% yield of CH_4, $4.5x10^{-2}$ mM of CO_2 representing 1% yield whereas $1.0x10^{-1}$ mM generation of H_2 made up a net yield of 2.5%. Within the CDP-fed microcosms, a total of 1.07 mM methane gas was generated within the headspace of the system amended with NH_4^+ (Figure 4.9C). This concentration represented 24.88% yield of the CH_4 gas. The production of $2.58x10^{-1}$ mM CO_2 constituted 6.01% while $8.5x10^{-2}$ mM H_2 constituted $1.97x10^{-1}$% yield from the carbon source. In a similar fashion, the generation of 1.33 mM CH_4 gas yielded 33.3%; $1.4x10^{-1}$ CO_2 yielded 3.0% while $2.5x10^{-1}$ mM H_2 yielded 6.25% within the headspace of microcosm driven by CDP but without added NH_4^+ (Figure 4.9D). The relative percent gas yields across the systems are presented in Figure 4.10. Generally, CH_4 appeared to be the major gas generated in these systems. Paired T-test analysis showed significant difference between the production and accumulation of CH_4 in the NH_4^+ amended and the NH_4^+ free systems (P = 0.0006; for both $Ca(ISA)_2$ and CDP driven microcosms)



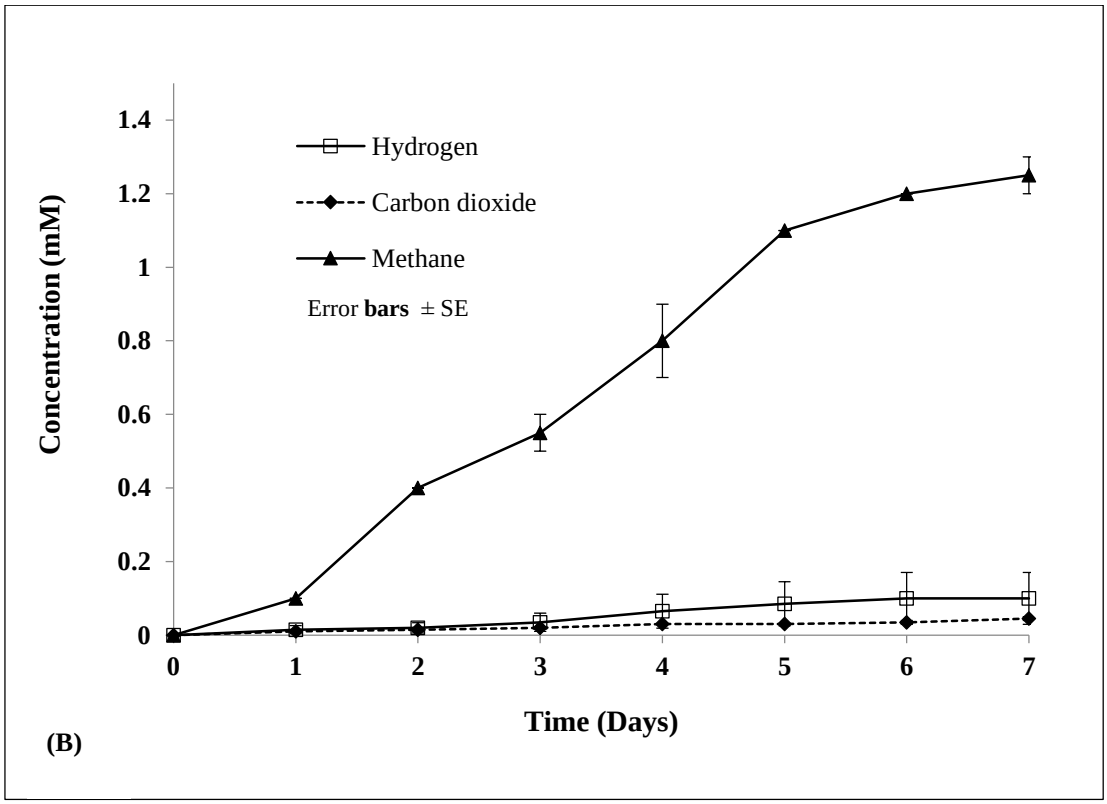
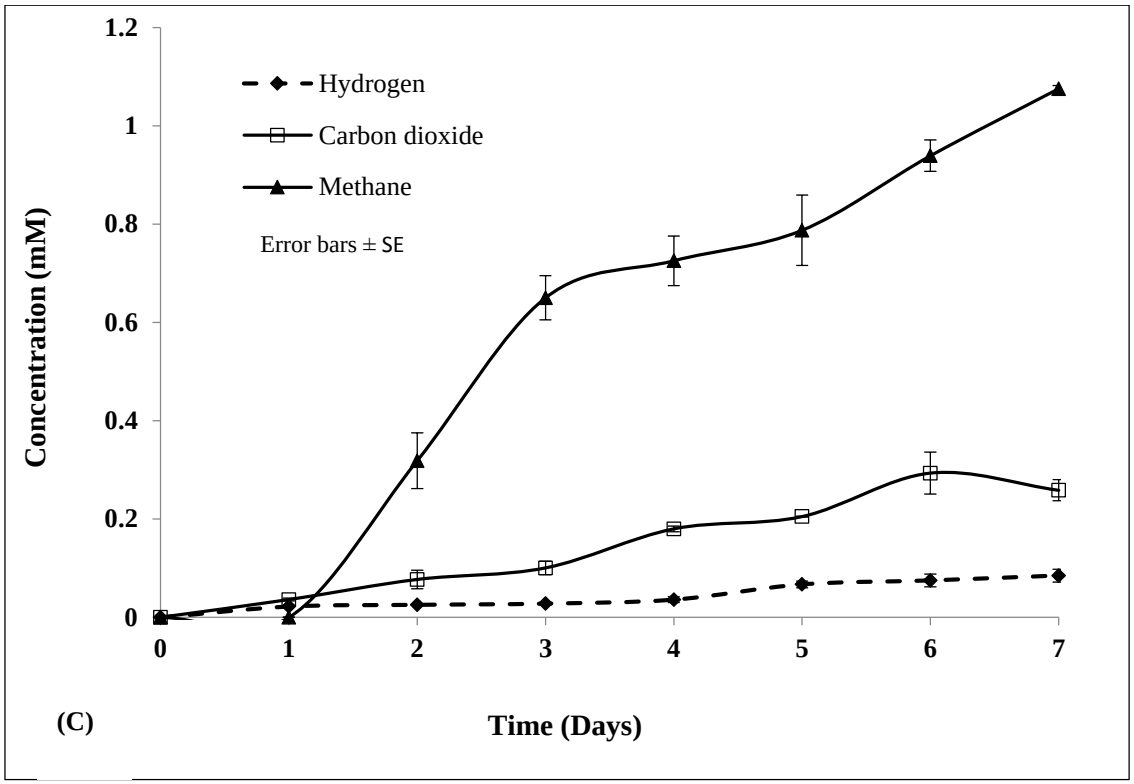


Figure 4.9 Headspace gas evolution in model systems



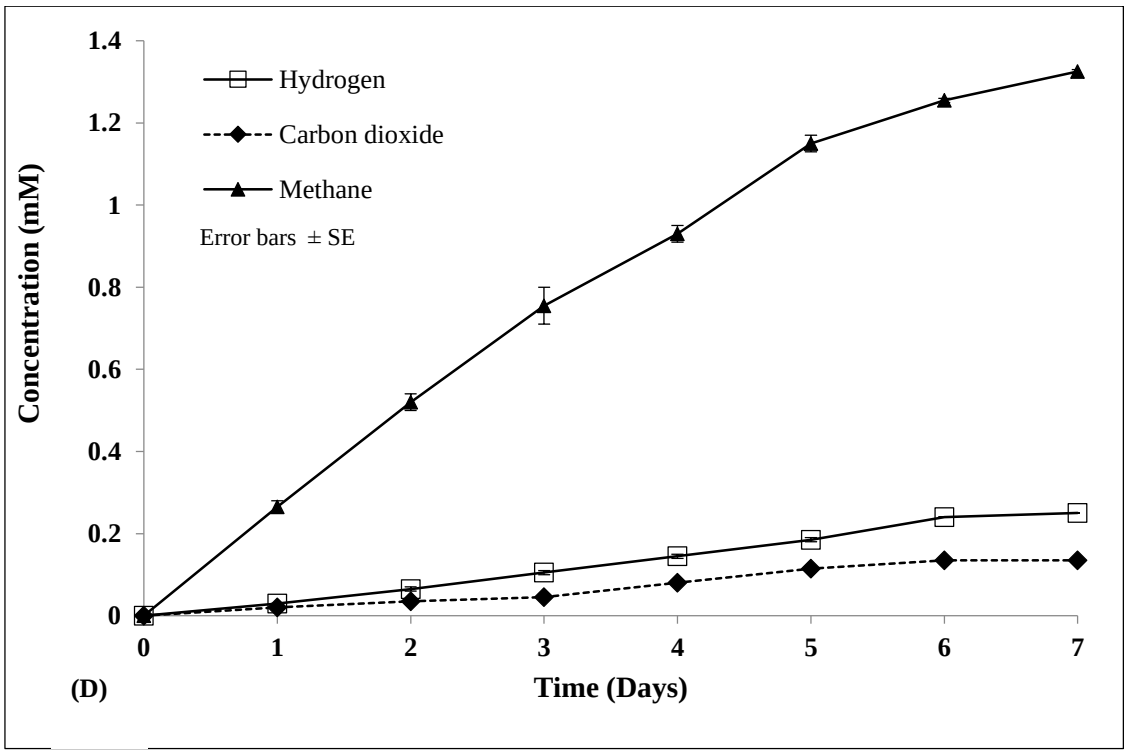


Figure 4.9 continues: Headspace gas measured from microcosms - (A) α-ISA driven system amended with NH_4^+, (B) α-ISA driven system without NH_4^+, (C) CDP driven system amended with NH_4^+, (D) CDP driven system without NH_4^+

Figure 4.10 shows the relative amounts of fermentative product (acetic acid) headspace gas evolution across all four methanogenic systems. The CDP system amended with NH_4^+ appeared to have generated and accumulated more acetic acid that the other systems but on the average, the $Ca(ISA)_2$ systems seemed to have a greater yield. The reason for this however requires further investigation.

The NH_4^+ amended systems generated more CH_4 than the NH_4^+ free system. This suggests that although at higher concentrations NH_4^+ become toxic and inhibits the growth of some microorganisms, in a complex methanogenic polymicrobial system, it still remains one of the essential nutrients for microbial growth and activities. Many researchers including Koster and Lettinga [239], Angelida and Ashring [240] have reported that aceticlastic methanogens are more sensitive to NH_4^+ inhibition compared to hydrogenotrophic methanogens. In a recent study, Hao *et al*. [241] noted that total ammonia nitrogen impacted significantly on methanogenesis. They however noted that CH_4 production was recovered by the synthrophic oxidation of acetate in association with Hydrogenotrophic Methanogenesis performed by acetate oxidizing syntrophs or through Acetoclastic Methanogenesis catalyzed by members of the Methanosarcinaceae family. It can therefore be demonstrated from the present study involving complex system of microorganisms that the increased generation of CH_4^+ in the NH_4^+ amended system could be the result of activities of hydrogenotrophic methanogens.

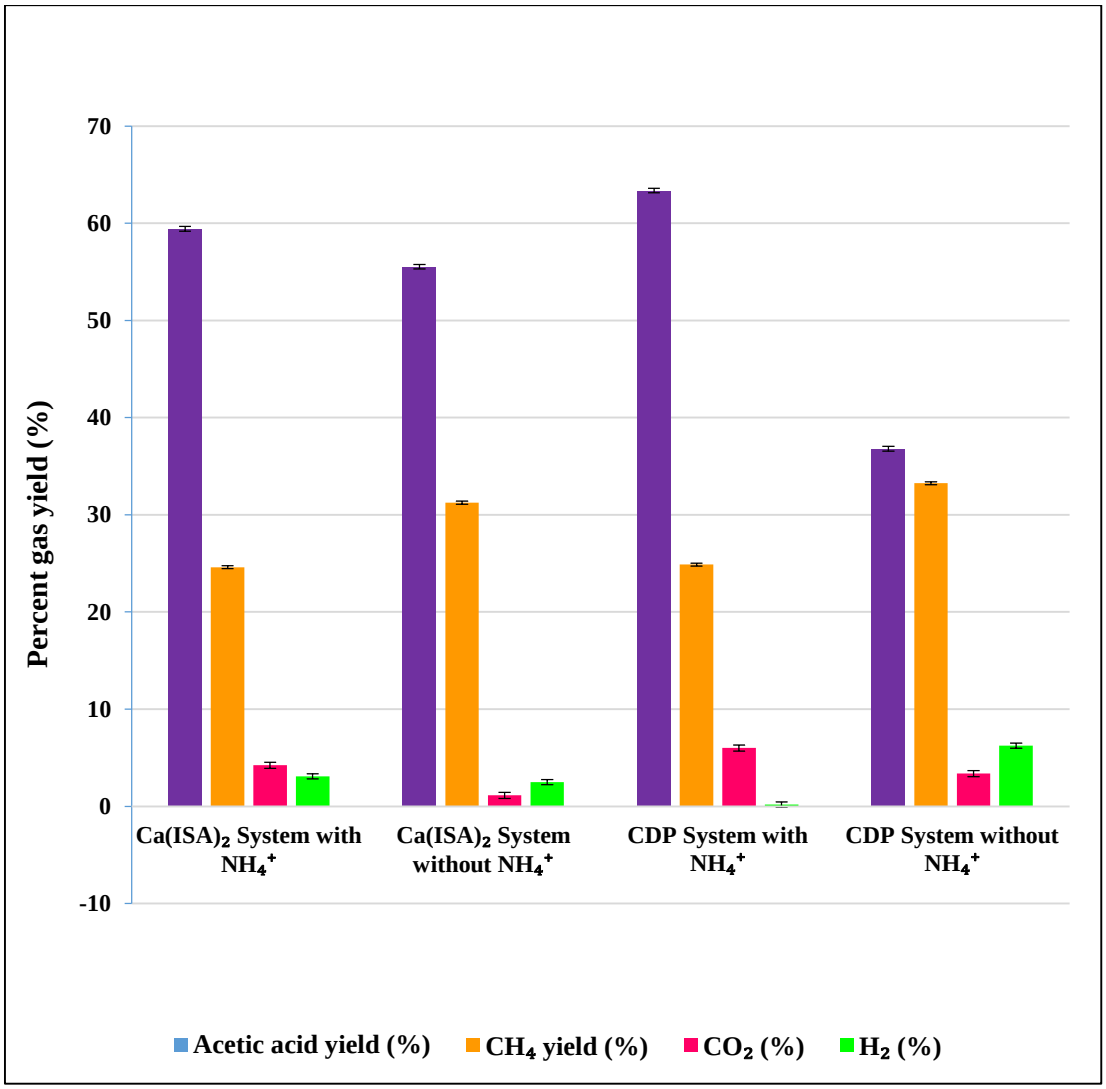


Figure 4.10 Percentage yields of acetic acid and headspace gas evolution across the established consortia

4.3.5. Microbial community profiling based on 16S rRNA gene sequencing

Analysis of the four established communities and a sample of the seeding (background) soil based on 16S rRNA gene sequencing showed that these communities were very diverse where several thousands of microbial species belonging to both archaea and bacteria were present. The total valid reads for the background soil was 2,822 out of which a bulk of 2819 was made up of bacterial domain and 3 from the domain Eukarya which were not considered for the purposes of this study. There were no reads for Archaeal taxa within the background soil where their presence appeared to have been masked by the dominating bacterial domain. The bacterial domain was composed of a diverse community of bacterial groups where Proteobacteria (1,360), Firmicutes (446), Actinobacteria (377) and Bacteroidetes were prominent. Actinobacteria (100), Planctomycetes (71) and Chloroflexi were also present in relatively higher proportions.

In the established consortia fed with $Ca(ISA)_2$ and amended with NH_4^+, out of the total of 14,421 OTUs, 14,030 were of the bacterial domain whereas 391 composed of members of the Archaeal domain. Compared to its NH_4^+ free counterpart, the bacterial domains were 33,708 whereas the Archaea were 1,55. In both systems, the Archaeal domain consisted mainly members of the phylum Euryarchaeota. In the bacterial domains, the dominant phylum for both systems, was Firmicute where the system with NH_4^+ consisted of 10,644 clones of its totals but 21,221 for its counterpart without NH_4^+. Members of the phyla Proteobacteria and Bacteroidetes were present but their dominance varied between the two systems. While Proteobacteria (1,779) was the second predominant phylum in the $Ca(ISA)_2$ system amended with NH_4^+, this phyla was the third highest (2,135) in the system without NH_4^+ even though it appeared more in numbers than its counterpart. Bacteriodetes was the second in prominence (8,611) in the system without NH_4^+ while it was the third (1,547) in the other system with NH_4^+. Other phyla which

occurred in relatively higher proportions in both systems respectively for $Ca(ISA)_2$ with and without NH_4^+ included Actinobacteria (10; 54), Chloroflexi (5; 56) and Acidobacteria (3; 23). Unique phyla of the system without NH_4^+ included Planctomycetes (26), Tenerricutes (15), Chlorobi (15), Verrucomicrobia (8), Gemmatimonadetes (6) and Saccharibacteria (1).

In the case of the CDP driven systems, the total number of OTUs identified were 14,900 for the system with NH_4^+ and 42,090 for the other system without NH_4^+. Out of these totals, the bacterial domain (14,427) constituted 97% in the system with NH_4^+ while the system without NH_4^+ was made up of 99.9% of bacterial domain (42,090). The Archaeal group of organisms which was mainly composed of members of the phylum Euryarchaeota, were also present in smaller proportions in these systems where only $1.72x10^{-2}$% of the totals were present in the consortia amended with NH_4^+ and only $6.41x10^{-4}$ % in the ammonia free system. In both CDP systems, in the bacterial domain, the phylum Firmicutes dominated representing 71% of the total reads in the presence of NH_4^+ which fell to 47% in the absence of NH_4^+. Proteobacteria made up the second largest group in both systems representing 18% with NH_4^+ and 47% without NH_4^+. Members of the phylum Bacteroidetes were also present in both systems but were much more prevalent in the system with NH_4^+ (11%) than that without ($2.2x10^{-1}$ %). Other minor phyla present in both systems included Actinobacteria, Chloroflexi, and Planctomycetes. While the phyla Armatimonadetes and Chlamydiae were unique to the system amended with NH_4^+, Saccharibacteria and Spirochaetes were unique to the system without NH_4^+.

Across the CDP and $Ca(ISA)_2$ fed microcosms, the established consortia at the phylum level produced some differences and similarities whereby taxa associated with the phylum Acidobacteria were conspicuously missing in both systems fed with CDP. Also, the phylum Saccharibacteria, although smaller in numbers, appeared to be associated with only the systems without NH_4^+. The shared and

unique phyla between the background soil communities and the established consortia across all systems are shown in Figure 4.11. In general, the NH_4^+ free systems were more diverse than the systems enriched with NH_4^+ which reflects on the lower biomass recorded in the previous section.

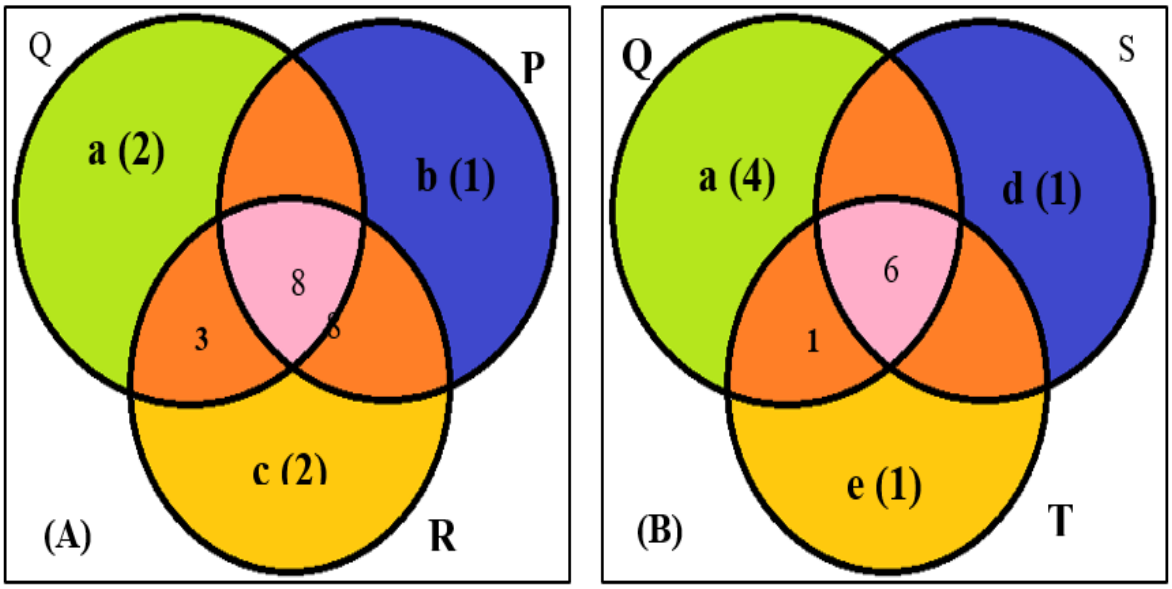


Figure 4.11 Shared and unique phyla within the background soil and the established consortia

(A) $Ca(ISA)_2$-fed microcosm and (B) CDP-fed microcosm. Q; background soil, P $Ca(ISA)_2$ with NH_4^+, R; $Ca(ISA)_2$ without NH_4^+, S; CDP with NH_4^+, T; CDP without NH_4^+. Number in parenthesis represents the number of phyla. In (A), a; Fibrobacteres and Spirochaetes, b; Chlamydiae, c; Tenericutes and Saccharibacteria, a∩b; nil, b∩c; nil, a∩c; Nitrospirae, Chlorobi, Verrumicrobia, a∩b∩c; Proteobacteria, Chloroflexi, Firmicutes, Planctomycetes, Actinobacteria, Acidobacteria, Gemmatimonadetes and bacteriodetes. In (B) a; Chlorobi, verrumicrobia, Gemmatimonadetes and Nitrospirae, d; Clamydiae, e; Saccharibacteria a∩d; Spirochaetes, a∩e; Armatimonadetes, d∩e; nil, a∩d∩e; Proteobacteria, Chloroflexi, Firmicutes, Planctomycetes, Actinobacteria and bacteriodetes.

At the genus level, within the $Ca(ISA)_2$ system, out of the total taxa under consideration, 12 were unique to $Ca(ISA)_2$ without NH_4^+, another 12 were unique to the system with NH_4^+ while 32 were unique to the background soil. Whereas 10 taxa were shared between the two $Ca(ISA)_2$ fed systems, a unit taxon was shared between each of the $Ca(ISA)_2$ and the background soil. Four genera were shared between all three communities (Figure 4.12A). Within the CDP communities, a similar trend occurred whereby 24 taxa at the genus level, were unique to the system fed with CDP plus NH_4^+ whereas 10 taxa were unique to the system fed with CDP but without NH_4^+. Here, the background soil consisted of 30 unique taxa. All three communities shared only one taxon (Tissierella) between them. While the CDP with NH_4^+ driven system shared a unique taxon with the CDP counterpart without NH_4^+, the former also shared only one taxon with the background soil. There was no common taxon between the background soil and the CDP system without ammonia (Figure 4.12B). Collectively, these findings suggest that NH_4^+ impacted on the selection of taxa in these systems.

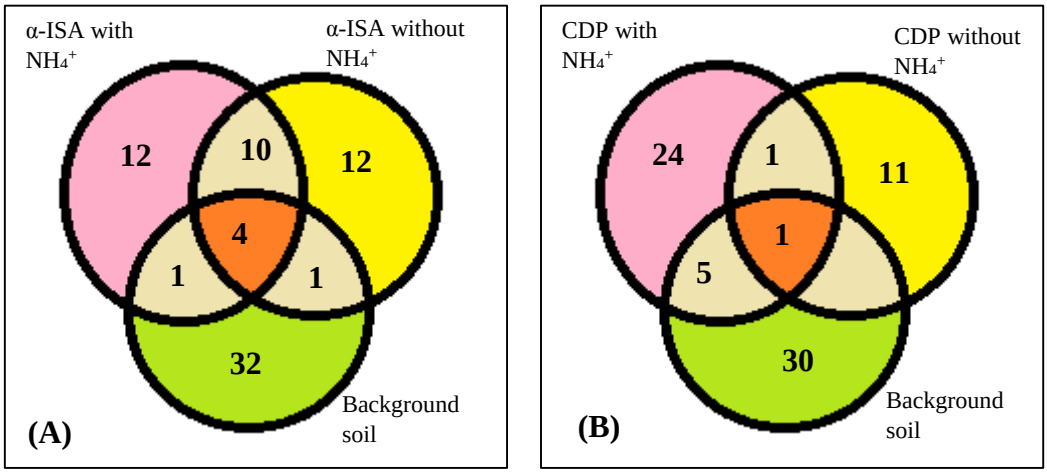


Figure 4.12 Effect of NH_4^+ on evolution of (A) $Ca(ISA)_2$ driven system and (B) CDP driven system.

The Venn diagrams show unique and shared genera across the systems and the background soil

The Alpha-diversity indices of OTUs of the established microbial communities compared with the background soil (Figure 4.13) appeared to show that the consortia fed with CDP and amended with NH_4^+ was most diverse representing 32% of the total OTUs followed by its $Ca(ISA)_2$ counterpart (29%). The community fed with $Ca(ISA)_2$ but without NH_4^+ appeared as the next diverse representing 16% and followed by its CDP counterpart (12%). Although the overall results suggest that NH_4^+ played a significant role in the selection of taxa in these communities, it appears microorganisms that were selected in the $Ca(ISA)_2$/CDP enrichment systems were very low in numbers in the background soil. This could be due to the harsh environmental conditions such as lower nutrient availability and pH stress (site pH *in situ* was 12.5) which may have caused taxa associated with class such as Clostridia to sporulate. The high number of bacteria therefore appeared to have skewed the DNA extraction and the subsequent alpha diversity indices in favour of the enrichment cultures against the initial soil. Furthermore, the alpha-diversity indices (Figure 4.13) appeared to show that NH_4^+ enrichment supports a more diverse microbial population than it NH_4^+ free counterpart but it could be due to the skewing of the DNA extraction in favour of the NH_4^+ enrichment which has been found to have a lesser diversity in the previous section.

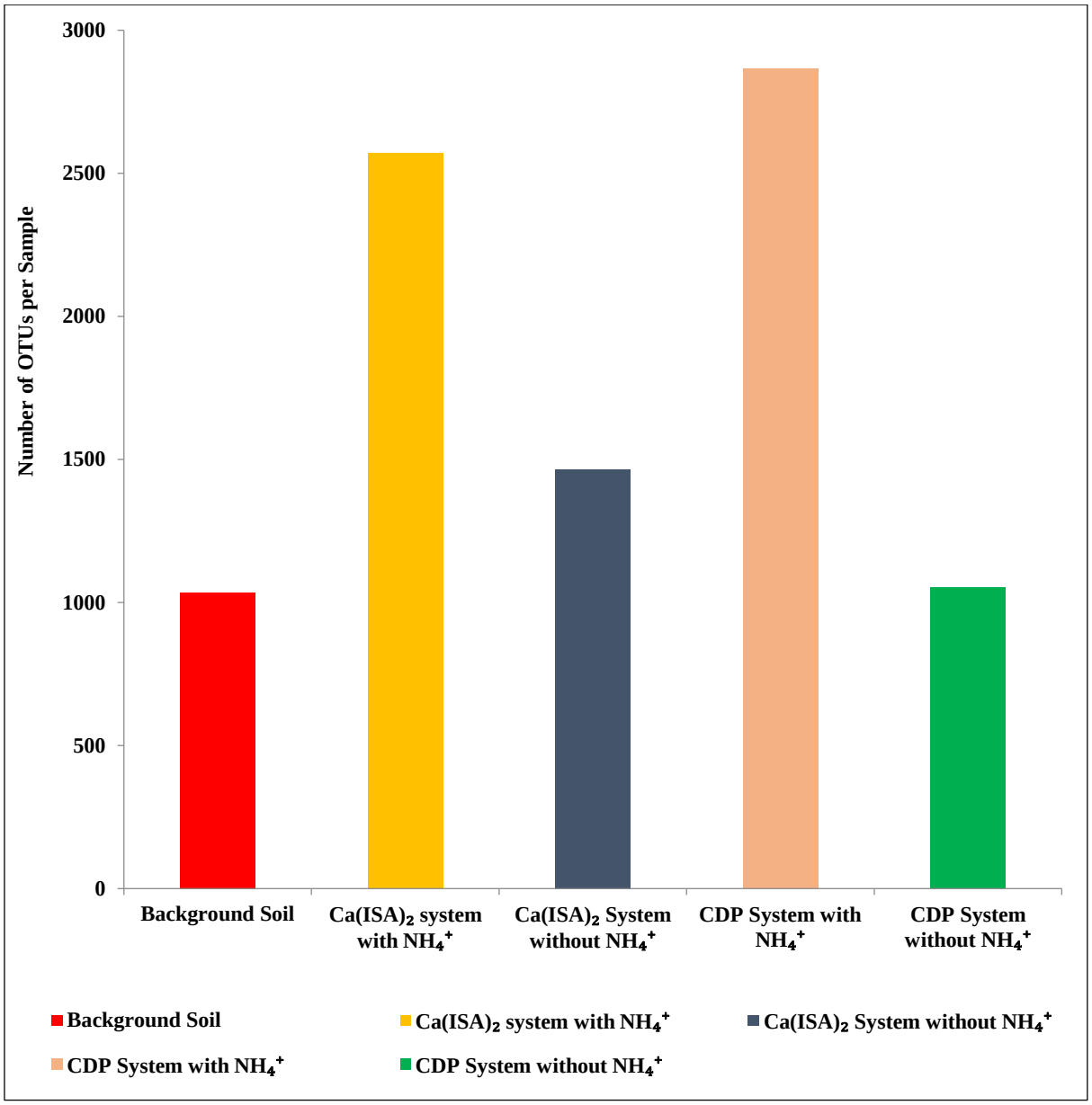


Figure 4.13 α-diversity indices of the OTUs of the background soil and the established microbial communities.

The evolutionary relationship using the taxonomic assignments of the established taxa across the different consortia and that of the background soil was also investigated where beta-analysis by Unifrac distance matrix and Principal Coordinate Analysis (PCA) [242] showed that irrespective of the carbon source, the taxa within these consortia amended with NH_4^+ were much more clustered together indicating a phylogenetic relationship between them (Figure 4.14). The systems without NH_4^+ however, did not cluster together indicating that they were not as phylogenetically related as those selected by the NH_4^+. This further suggests that ammonia rather than the carbon source had a bigger impact the community evolution.

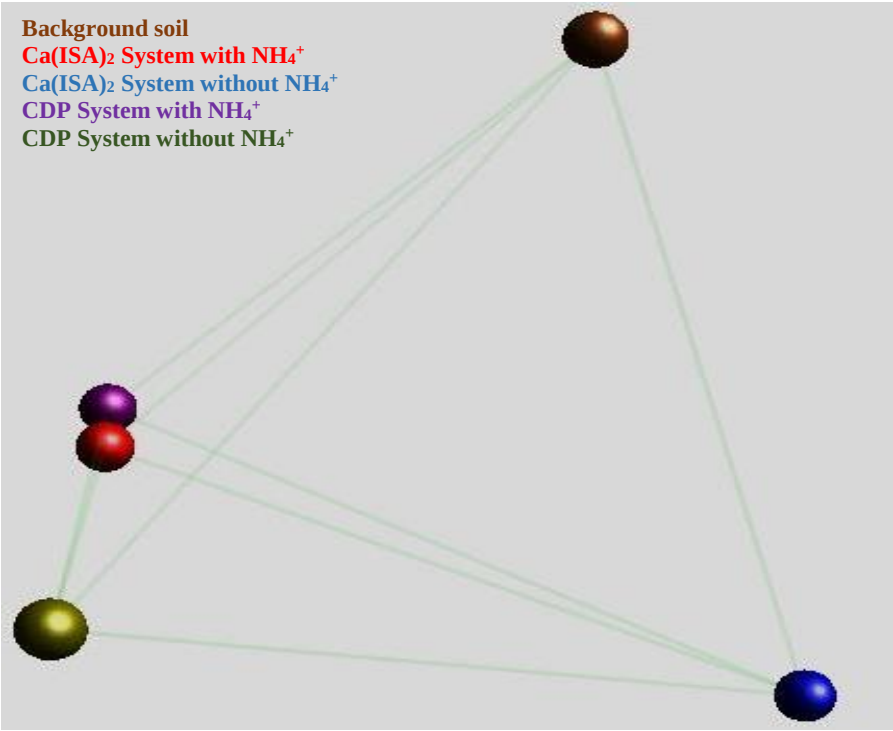


Figure 4.14 β-diversity by Unifrac distance matrix and PCA of the established microbial community

The microbial communities were from $Ca(ISA)_2$ and CDP systems amended with/without NH_4^+. The diagram shows the phylogenetic relationship between taxa associated with the established consortia and the background soil. Lines indicate the distance between communities

At the genus level, in both systems (CDP and $Ca(ISA)_2$) amended with NH_4^+, taxa associated with unclassified genus with the designation AB630534 were most prevalent, representing 19% of the total $Ca(ISA)_2$ driven system and 11% of the CDP-driven system (Figure 4.15). In both systems while taxa associated with Clostridiales dominated, representing 59% and 64% of the totals respectively, the systems without NH_4^+ were composed of 22% and 44% in respect of $Ca(ISA)_2$ and CDP driven communities (Figure 4.15). The $Ca(ISA)_2$ and CDP driven systems without NH_4^+ were dominated by taxa associated with the orders Tissierellales (27%) and Burkhoderiales (46%) respectively.

Within the order Clostridiales present in both $Ca(ISA)_2$ and CDP driven communities, clones from the genus *Clostridium* showed greatest sequence homology to *Clostridium clariflavum* and *Clostridium geopurificans*. *Clostridium clariflavum* has been implicated in the production of acetic acid from carbohydrates [243] while *Clostridium geopurificans* has been associated with fermentative bioremediation of cyclic nitramine explosives (Research Department eXplosive (RDX)) [244]. The genus *Ercella* were also prevalent with the NH_4^+ rich systems where they constituted 3% of the total reads in the $Ca(ISA)_2$ system and 6% of the CDP system. Although the clones associated with this genus at the species level were unclassified, earlier studies have shown that some species are associated with the production of succinate [245]. Members of the genus *Acetobacterium* were unique to both systems driven by $Ca(ISA)_2$ where they consisted of 2% of total reads within the system amended with NH_4^+ but only 1% of the system without NH_4^+. The genome analysis showed that members of this genus exhibit greatest sequence homology with *Acetobacterium wieringae*, *Acetobacterium carbinolicum* and *Acetobacterium woodii*. *Acetobacterium wieringae*, described as an anaerobic, chemlithotrophic, acetogenic bacterium, has been shown to be capable of chemolithotrophical conversion of molecular hydrogen and carbon dioxide into acetic acid as the only product [246]. The study

by Eichler and Schink [247] demonstrated that *Acetobacterium carbinolicum*, obligate, anaerobic bacterium is capable of utilising H_2/CO_2, aliphatic alcohols, glucose, fructose, lactate, pyruvate and other organic compounds to generate acetate and other VFAs. *Acetobacter woodii* has been associated with the utilisation of CO to produce acetate [248]. Furthermore, the study by Balch *et al*. [249] demonstrated that *Acetobacterium woodii* was capable of oxidising hydrogen and reducing carbon dioxide to generate acetate and in a co-culture with methanogens methane is generated from those substrates. Another genus from Clostridiales that was present and unique to the systems without NH_4^+ was the genus *Desulfitobacterium* which made up 0.9% of the totals in the $Ca(ISA)_2$ system and 0.8% of the total reads in the CDP system. The clones within this genus exhibited highest sequence homology with *Desulfitobacterium hafniense*. This organism has been described by Prat *et al*. [250] as anaerobic, spore-forming, ubiquitous environmental microorganism with a broad metabolic versality and is capable of adapting its physiology during adverse conditions. The authors demonstrated that *Desulfitobacterium hafniense* was capable of utilising tetrachloroethane through organohalide respiration from which genome investigation revealed the presence of 57 proteins involved in stress response and associated regulatory pathways. Proteins involved in carbon and energy metabolism were also noted [250]. Members of the genus *Acetovibro* from the order Clostridiales were also present within the CDP enriched with NH_4^+ where they represented 7.8% of the totals. A small proportion of this genus was also present within the $Ca(ISA)_2$ counterpart where they made up to only 0.4% of the total reads in the system. The clones within the *Acetovibro* showed greatest sequence homology to *Clostridium straminisolvens* which has been described previously to be associated with cellulolytic processes [251]. Also present within the two systems with NH_4^+ was the genus *Anaerotruncus colihominis* which represented 3.50% of the totals within the CDP system and only 0.30% in the $Ca(ISA)_2$ system. This organism is reported to be capable of growth up to up to a

pH of 11 and can ferment a range of sugars to acetic acid and gas [252]. The genus *Anaerovorax* was found only in the CDP-driven system with NH_4^+ where it represented 1.3% of the total reads. Although the species under this genus were unclassified, other reported species within the same genus are noted to be capable of producing acetate, butyrate, molecular hydrogen and ammonia from organic substrates [253]. The genera *Anaerosporobacter* and *Desulfitispora* were also found in smaller proportions within the CDP systems. While *Anaerosporobacter* represented only 0.62% of the total reads within $Ca(ISA)_2$ system with NH_4^+, a similar percentage of 0.69% was represented by *Desulfitispora* within the $Ca(ISA)_2$ system without NH_4^+. While some species of *Anaerosporobacter* are noted to be associated with the metabolism of a range of carbohydrates with the end products being formate, acetate and hydrogen [254] some species of *Desulfitispora* are described as anaerobic halophilic microorganisms capable of sulfidogenic metabolism [255].

The genus *Alcaligenes* from the order Burkholderiales which dominated the system fed with CDP without NH_4^+ was unique to that that system (Figure 4.15). Members of this genus showed greatest sequence homology with *Alcaligenes aquatilis, Alcaligenes faecalis* and one other unclassified species with the designation AKMR. While *Alcaligenes aquatilis*, was also found in very small amounts in the $Ca(ISA)_2$ driven systems, is noted for tolerance to high pH and toxic concentrations of heavy metals such [256, 257], *Alcaligenes faecalis* is associated with anoxic degradation of phenol as sole carbon source and has demonstrated the ability to denitrify [258]. A subspecies of *Alkaligenes* found in CDP without NH_4^+ fed system was *Alcaligenes faecalis* subsp. *phenolicu* which has previously been associated with phenol degradation and denitrification [259]. Another genus from the order Burkholderiales found within the $Ca(ISA)_2$ system with NH_4^+ was *Delftia* which represented 3.18% of the total reads. Some reported species of *Delftia* have been noted to degrade extracellular peptidoglycan and

various kinds of carbohydrates [260]. Some species from the order Burkholderiales were present in small fractions across the systems. For instance, there were clones which showed greatest sequence homology with *Herbaspirillum lusitanum* which was found only among the two communities with NH_4^+ which has been reported previously to be involved in nitrogen fixation [261]. Clones that showed greatest sequence homology to *Herbaspirillum hiltneri* and *Herbaspirillum frisingense* were also prevalent within the $Ca(ISA)_2$ system with NH_4^+ and CDP without NH_4^+ respectively. While *Herbaspirillum hiltneri* is reported to possess proteins that are involved in the transport and assimilation of nitrates [262], *Herbaspirillum frisingense* is reported to be involved in nitrogen fixation [263]. The presence of clones that showed 99% sequence identity with *Hydrogenophaga flava*, 96% sequence similarity with *Rodoferax saidenbachensis* and 87% sequence homology with *Paraburkholderia metalliresistens* were present in the community fed with $Ca(ISA)_2$ without NH_4^+. Previous studies have shown that while *Hydrogenophaga flava* is capable of autotrophic hydrogen oxidation [264], *Rodoferax saidenbachensis* is reported as a psychrotolerant slow growing bacterium capable of utilising various kinds of carbohydrate substrates including glucose and lactate [265]. Earlier studies by Guo *et al* showed the multiple heavy-metal resistance capabilities of *Paraburkholderia metalliresistens* including resistance to Cd^{2+}, Pb^{2+} and Zn^{2+} [266].

Within the Firmicutes, the order Tissierellales was prevalent only in the microcosms without NH_4^+ where taxa associated with this genus represented 27% of the entire community fed on $Ca(ISA)_2$ and only 2% that of the CDP counterpart. The genus *Tissierella* which consisted of 11.26 % of the total reads in the $Ca(ISA)_2$ system, also prevailed in a small proportion of 5% in the CDP counterpart. Clones of the genus *Tissierella*, showed the greatest sequence homology to *Tissierella creatinine*, previously reported to be associated with the with the utilisation of nitrogen-containing compounds such as creatinine [267].

In the systems without NH_4^+, the predominant taxa at the genus level included *Sedimentibacter* which represented 19% of the $Ca(ISA)_2$ population. The genus *Sedimentibacter* was also present in other systems including both systems with added NH_4^+. Members of this genus were observed to show greatest sequence homology with *Sedimentibacter hydroxybenzoicus* and two other unclassified species. *Sedimentibacter hydroxybenzoicus*, strictly anaerobic and capable of growth up to 41°C at circumneutral pH has been noted for fermentative degradation of organic pollutants with major VFAs being acetate and butyrate [268]. Other known species of this genus are associated with the fermentation of amino acids and pyruvate to generate acetate, butyrate and lactate [269].

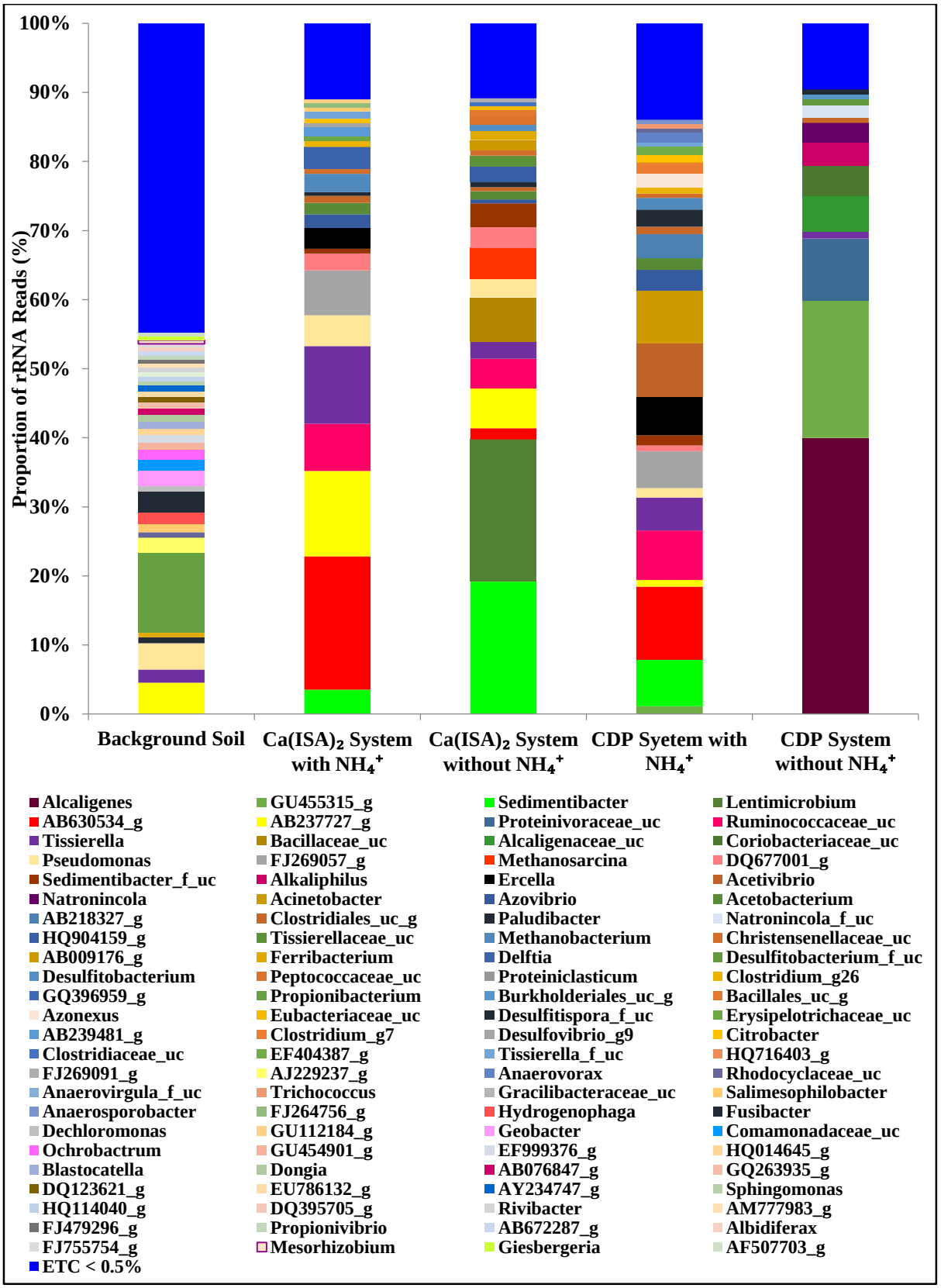


Figure 4.15 System community profiling at the genus level

Microbial community profiling at the order level showed quite a diverse group of microorganisms within the background soil from where the various enrichment systems have evolved (figure 4.16). The order Burkhoderiales were dominant and they represented 13% of the total community within the background soil. Also present were taxa associate with the orders Xanthomonadales, Rhodospirillales, Rhodobacteraes, Rhizobiales and Sphingomonadales within the phylum Betaproteobacteria. Together these taxa represented 21% of the total reads. Taxa associated with the order Cytophagales were also present within the soil sample but could not be found in any of the established communities. The absence of methanogens was quite conspicuous within the soil sample, which may be attributed partly to the high pH of the site of the soil as well as a lack of substrates to support the growth methanogens.

Several taxa associated with different orders were present but could not be shown in the figures 4.16 and 4.17 because their reads fell below the inclusion criterion of 0.5%. These taxa are therefore included in the sector designated as 'ETC < 0.5' of each figure.

The order Bacteroidales was common to all systems except the CDP-driven system without NH_4^+ where it was conspicuously missing (Figures 4.16 and 4.17). Its prevalence in the $Ca(ISA)_2$ system with NH_4^+ was only 10% of the total community but 25% of the $Ca(ISA)_2$ without NH_4^+ where it was the second most prevalent order to the order Tissierellales. The genus *Paludibacter* from the order Bacteroidales was quite common to those systems which supported the selective growth of members of this order including the two $Ca(ISA)_2$ systems and their CDP counterpart enriched with NH_4^+. Clones of the genus *Paludibacter* showed greatest sequence similarity with *Paludibacter propionicigenes* which has previous been described as strictly anaerobic bacterium capable of producing propionic acid [270]. The genus *Flavobacteria* was also prevalent across the systems where clones showed highest homology to *Flavobacterium reichenbachii*

the functional activities of which have not been well characterized. Other clones from this order were present in the in the CDP systems with NH_4^+ which showed a high degree of similarities to *Prevotella malaninogenica*, *Alistipes shahii* and *Barnesiella intestinihominis* all of which are not very well characterised in terms of their functional roles.

Members of the taxa associated with Pseudomonadales also consisted of a significant proportion of the background soil and total established consortia across the systems except CDP system without NH_4^+. It comprised 5% of the $Ca(ISA)_2$ driven system with NH_4^+, 3% of the $Ca(ISA)_2$ driven system without NH_4^+ and 9% of the CDP driven system with NH_4^+. At the genus level, systems comprised of *Pseudomonas* associated taxa representing 4%, 2.64% and 1% respectively of the total communities driven by $Ca(ISA)_2$ with NH_4^+, $Ca(ISA)_2$ without NH_4^+ and CDP with NH_4^+. The genus *Acinetobacter* were present in the CDP fed community the represented the most prevalent Pseudomonadales (8%). Here, the clones within the communities showed greatest sequence homology with *Acinetobacter Iwoffi* group. The order Rhodocyclales were also more prevalent within the CDP driven system with NH_4^+, representing 6% of the total community than 2% and 2% in respect of $Ca(ISA)_2$ system with NH_4^+ and without NH_4^+. While the order Rhodocyclales was not found in the CDP system without NH_4^+, the taxa with this order were dominated by members of the genera *Azonexus* and *Azovibrio* which have both been implicated in diazotrophism (nitrogen fixation) [271].

The order Erysipelotrichales were the second most prevalent taxa within the $Ca(ISA)_2$ driven system without NH_4^+ where the order represented 13% of the total community. In the other $Ca(ISA)_2$ system however, the order represented 6% of the total community. While the order was missing in the CDP system without NH_4^+, it represented only 1% of the total community within the CDP consortia

enriched with NH_4^+. Most of the members of this order fell into the unclassified genus with the designation AB237727 (Figure 4.15).

The order Coriobacteriales of Actinobacteria was unique to the CDP system without NH_4^+ where the percent prevalence was 6% of the total community. Taxa belonging to this order fell into the unclassified genus designated GQ396959 where members showed highest sequence similarity to GU565251.

Another order that was unique to the $Ca(ISA)_2$ driven system without NH_4^+ was the order Thermoanaerobacteriales where taxa associated with this order composed of 0.6% of the established community. At the genus level, members within this order belonged to *Thermoanaerobacterium*. Although clones belonging to this genus were unclassified, previous studies have shown that members are thermophilic, anaerobic bacteria with some species capable of fermenting xylan and biomass-derived sugars to produce ethanol at high yields [272].

The order Bacillales was present in the $Ca(ISA)_2$ driven system with NH_4^+ where it represented 7% of the total community and also in the system fed with CDP with NH_4^+ where it consisted of 0.6% of the total reads. Taxa associated with this order fell mainly into the genus *Bacillus* (Figure 4.15) where the clones present expressed highly similar sequence homology with a number of *Bacillus* species. For instance, some clones shared greatest sequence homology (99%) with *Bacillus endoradicis*, (97%) with *Bacillus cereus*, (99%) with *Bacillus pumilus*, (95%) with *Bacillus litoralis*, (99%) with *Bacillus zhaugzhouensis*, (99%) with *Bacillus crassostreae*, (97%) with *Bacillus aquimari* and (97%) with *Bacillus nealsonii* previously reported to produce spores that are resistant to radiation such as gamma radiation [273].

The order Enterobacteriales of Gammaproteobacteria were present within the established consortia fed with NH_4^+-enriched $Ca(ISA)_2$ and CDP (Figure 4.17). The percent prevalence of the order in these communities were 0.7% for the

$Ca(ISA)_2$ driven community and 1% of the CDP community. Within this order, members of the genus *Citrobacter* were most prevent where the clones showed high sequence similarity (99%) to *Citrobacter gillenii*.

Desulfovibrionales from Gammaproteobacteria were present in the community fed with NH_4^+-rich $Ca(ISA)_2$ representing 0.6% of the total reads (Figure 4.13B). The dominant genus of this order was *Desulfovibrio* where clones belonging to this genu expressed greatest sequence homology (99%) with *Desulfovibrio putealis*. *Desulfovibrio putealis* have been previously reported to be involved in sulphur reduction [274].

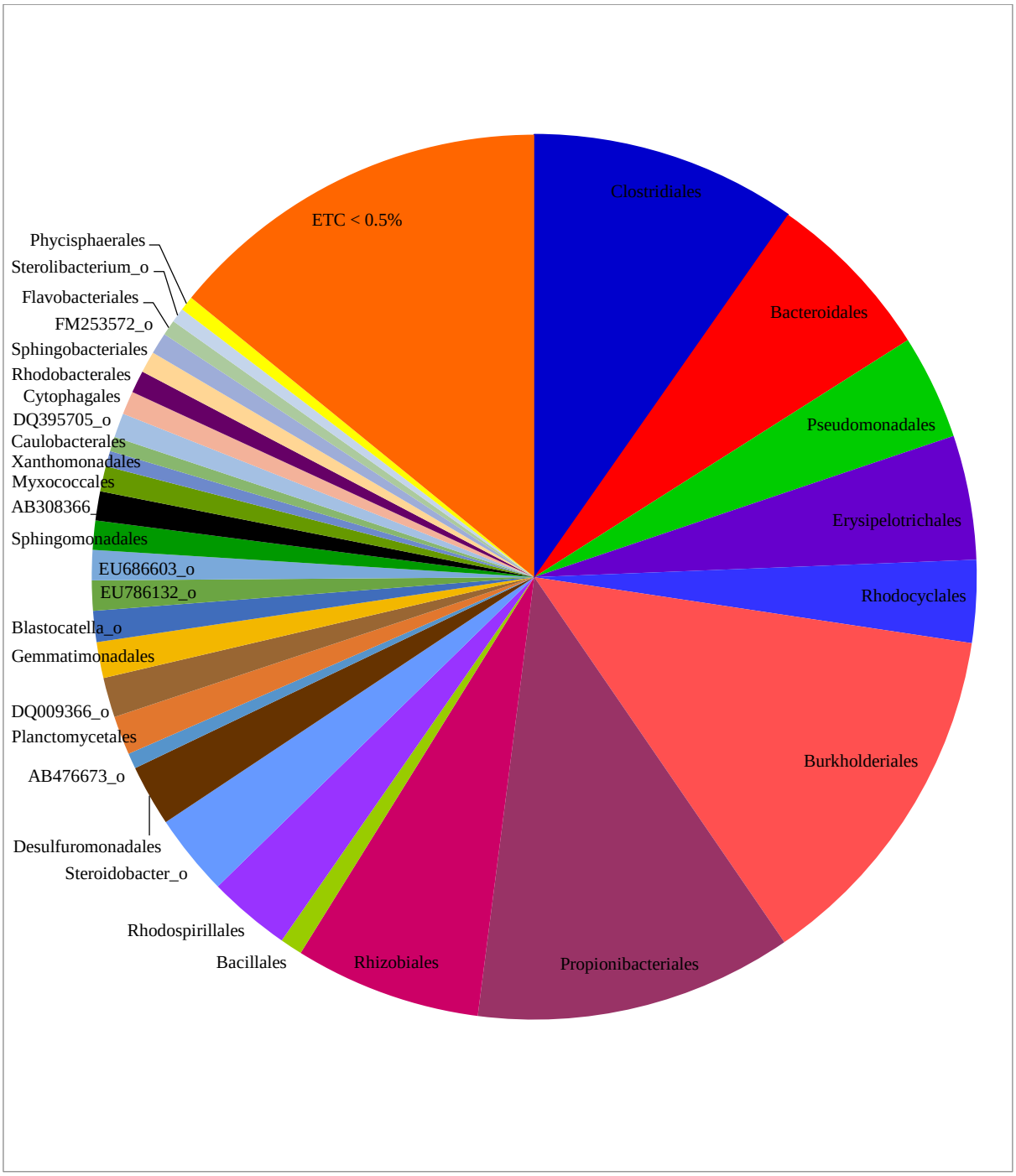
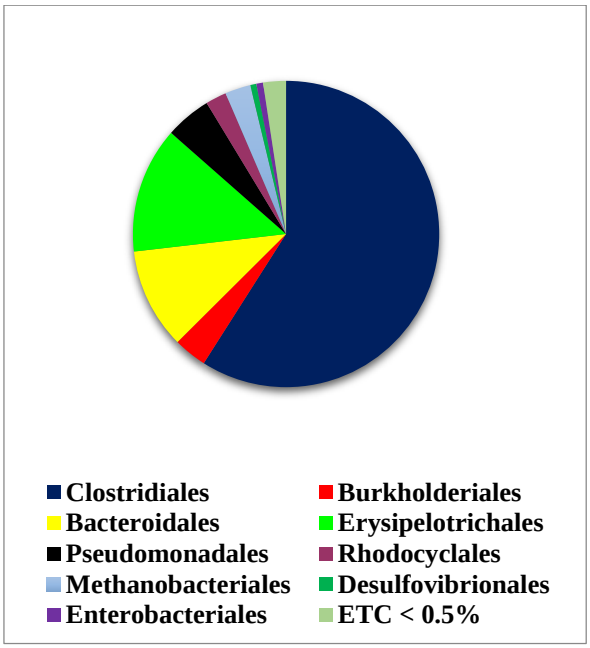
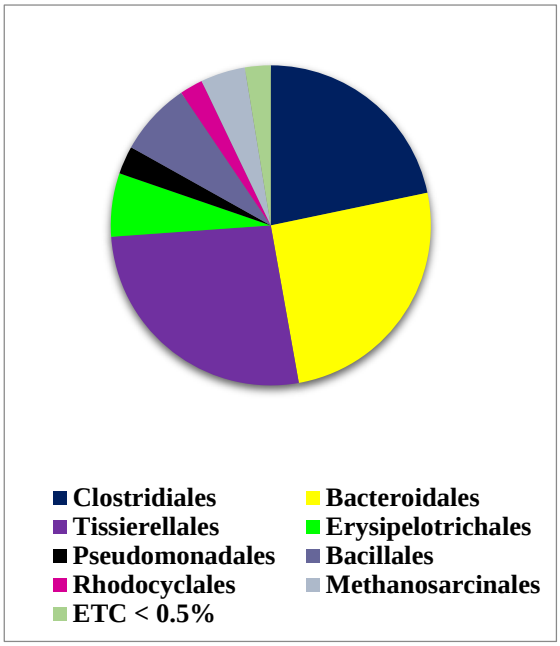


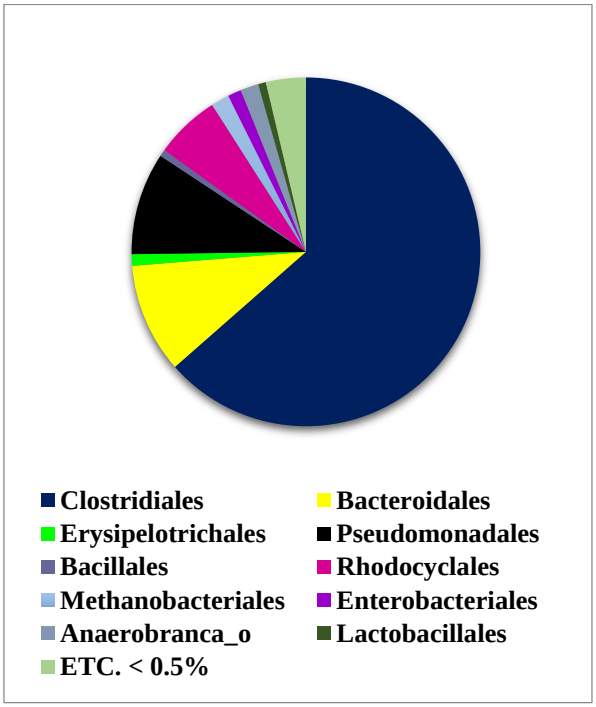
Figure 4.16 Microbial community profiling of the systems' background soil at the Order level



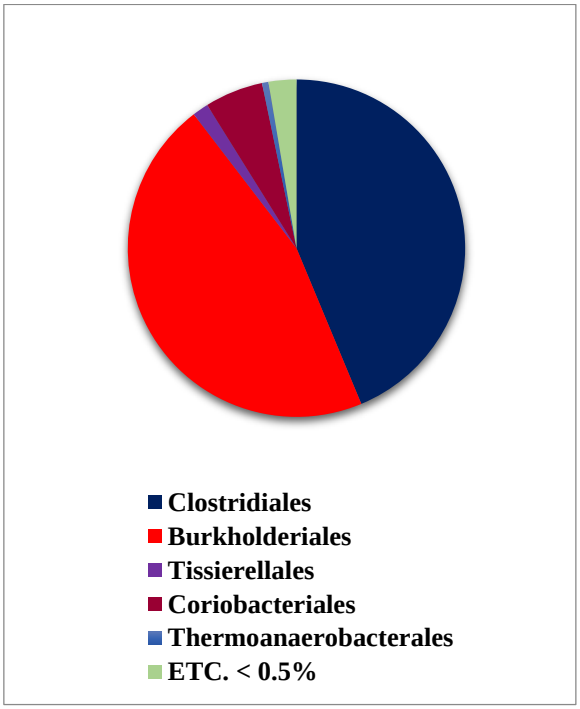
B. $Ca(ISA)_2$-driven system with NH_4^+



C. $Ca(ISA)_2$-driven system without NH_4^+



D. CDP-driven system with NH_4^+



E. CDP-driven system without NH_4^+

Figure 4.17 ISA degrading systems community profiling at the order level

Archaeal taxa of the orders Methanobacteriales and Methanosarcinales were present within the community profiles. The Methanobacteriales were present in both $Ca(ISA)_2$ and CDP systems amended with NH_4^+ where they represented 2% of the communities. Taxa associated with Methanobacteriales were also present in the community fed with CDP but without NH_4^+ however, the fraction of their prevalence fell below the 0.5% mark selected for further analysis in this work. The Archaeal taxa associated with Methanosarcinales prevailed within the community driven by $Ca(ISA)_2$ but without NH_4^+ where these taxa composed of 5% of the total reads. At the genus level, the Methanobacteriales were represented almost exclusively by the genus *Methanobacterium* where the clones of this genus showed the greatest sequence homology with *Methanobacterium alcaliphilum*, a hydrogenotrophic methanogen, capable of growth up to pH 9.9 [275]. Some of the clones also showed high sequence similarity with *Methanobacterium movens* which is reported to generate methane from H_2 and CO_2 [276]. High degree of sequence similarity of clones of Methanobacteriales from the CDP with NH_4^+ driven system to *Methanobacterium flexile* was also observed whereby similar to *M. movens*, this archaeal species is also noted by the same authors to produce methane solely from H_2 and Carbon dioxide [276]. On the other hand, clones within the order Methanosarnales showed greatest degree of sequence similarity to *Methanosarcina mazei* and *Methanosarcina soligelidi* which are all capable of acetogenic methanogensis. The *Methanosarcina mazei* has previously been described by Deppenmeier *et al*. [277] as obligate anaerobic metabolically versatile methanogen whereby detailed genomic analysis suggested an important evolutionary role played by lateral gene transfer in forging the physiology of this metabolic versatility. The authors noted that *M. mazei* is capable of utilising acetate, H_2/CO_2 and methanol to generate methane and carbon dioxide and when methylamines are used as substrate, they can also generate ammonia [277]. The description and functional activities of *Methanosarcina soligelidi* were noted by Wagner *et al*. [278] as desiccation- and freeze-thaw-resistant methanogenic

archaeon capable of both hydrogenotrophic and aceticlastic methanogenesis using H_2/CO_2, acetate and methanol as substrates.

Although ammonia is essential for microbial growth, the addition or omission of NH_4^+ from the cultivating media did not affect the degradation of ISAs or their products. As a result, a similar chemical evolution occurred in both systems with and without NH_4^+. The presence NH_4^+ however, impacted on the microbial community evolution in these systems. This is evidenced by the differences in the community structures. The β-diversity indicated by the Unifrac distance matrix and PCA showed that both $Ca(ISA)_2$ and the CDP systems amended with NH_4^+ selected for taxa that clustered together and hence, were phylogenetically related as compared to the background soil and the NH_4^+ free systems (Figure 4.14). The NH_4^+ free systems did not cluster suggesting that NH_4^+ was the main factor for the selection and clustering of the taxa.

These results for the first time, demonstrated methanogenesis from α-ISA as sole carbon source at pH values relevant to the alkaline disturbed zone of the ILW GDF. The findings of this study coupled to recent findings by Kuippers *et al*. [74] and Rout *et al*. [58, 113] indicate that either α-ISA alone or as a part of mixed carbon source in the form of CDP are likely to support a methanogenic system within the operational period of such a facility. Irrespective of the source of ISA (single or mixed diastereomers), the degradation rates were similar. Together, these findings suggest that the metabolism of ISA diastereomers occur through the same metabolic pathways. The outcome of this study however, contrasts with previous studies which appeared to suggest that α-ISA is not degradable without the presence of terminal electron acceptors [70].

The generation of methane was due to presence of Methanobacteriales and Methanesarcinales supported by the background soil which is consistent with previous studies in which soil sample from the same site at Harpur Hill has supported the growth of methanogens [58, 83]. The volumes of methane generated

across these systems appear similar indicating that ISA was the main carbon source that generated the substrates for methanogenesis. The other carbon sources within the CDP are likely to be recalcitrant to these methanogens. The recalcitrance of some of the components of CDP was previously reported [73, 113].

According to Krich *et al*. [279], in a biphasic anaerobic digestion 4 mM of a carbohydrate substrate would yield 8 mM acetic acid. The generation and accumulation of acetic acid within these systems fell below the theoretical values whereby the $Ca(ISA)_2$ amended with NH_4^+ system yielded 30% of the theoretical values, the NH_4^+ free $Ca(ISA)_2$ system; 28%, CDP amended with NH_4^+; 54% and NH_4^+ free CDP 18% of theoretical values. This observation in addition to the presence of both acetogenic and hydrogenotrophic methanogens indicate that the major biochemical pathway for methanogenesis within these systems was both aceticlastic and hydrogenotrophic methanogenesis. It is also important to note that the reduction in acetate concentration with respect to the theoretical values in these polymicrobial systems could not only be limited to Archaeal processes since earlier findings have reported syntrophic interaction between acetate-oxidizing bacteria and autotrophic methanogens within methanogenic environment [280]. The authors indicated that syntrophic acetate-oxidizing bacteria such as *Clostridium ultunense* are capable acetate oxidation to H_2 and CO_2 only when their products could be utilised by hydrogenotrophic methanogens such as *Methanoculleus*.

4.3.6. Determination of NH_4^+ concentration in the background soil

Apart from potentially playing a role in the selection of some taxa, the lack of NH_4^+ did not seem to deprive microorganisms present within the microcosms of any essential growth factor. This was evidenced by both chemical and community evolutions discussed in the previous sections. As a result, an investigation into the cause of this similar profiles became necessary, one of which was to examine the level of soil NH_4^+. The aim was to investigate the concentration of NH_4^+ within the background soil in order to determine the possibility of microbial community to resource ammonia from their surroundings.

Spectrophotometric determination of ammonium ion (NH_4^+) concentration in the background soil extract using a modified indophenol method described by Bolleter *et al*. [220] showed that the background soil contained a residual 0.57 mM NH_4^+ per gram of soil. This suggests that the hyperalkaline background soil offered a source of NH_4^+ that could potentially support the local microbial community. Figure 4.18 shows the calibration curve used to deduce the concentration of the soil NH_4^+.

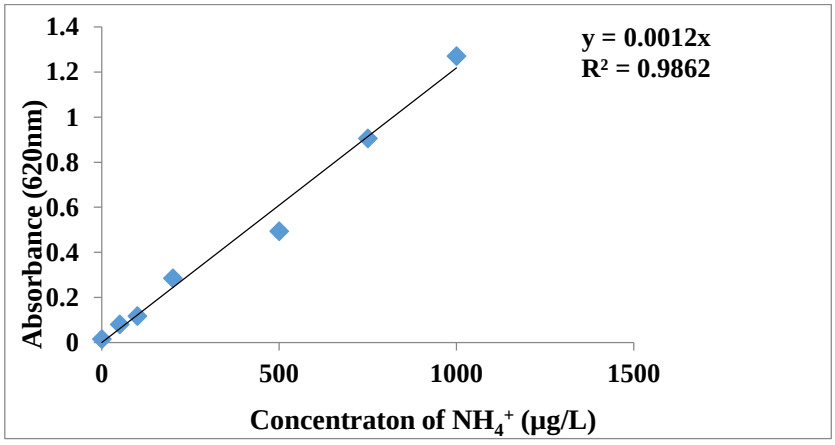


Figure 4.18 Calibration curve for spectrophotometric determination of soil NH_4^+ concentration.

Absorbance of soil sample @ 620nm = 1.241

In the previous section, although some nitrogen fixers were encountered during the community analysis, they were found in both systems with and without NH_4^+ and with similar percentage prevalence. As a result, their presence could not demonstrate that they had been purposely selected due to the absence of nitrogen source in the media. The determination of 0.57 mM NH_4^+ per gram of the background soil meant that indigenous microorganism could resource NH_4^+ from their environment which potentially was utilised for growth in the NH_4^+ free systems. Within the NH_4^+ amended systems, nitrogen levels appeared to be elevated as there were more sources that could generate nitrogen including the background soil, the cultivating media itself and from the nitrogen fixing bacteria such as *Azonexus* and *Azovibrio* which were encountered within the community analysis of these systems. This accounted for nitrogen toxicity which evidently reduced the overall biomass in those systems.

4.3.7. The impact of using $Ca(ISA)_2$ and CDP as a feedstock on microbial community evolution

The use of calcium salt of α-ISA ($Ca(ISA)_2$) and the mix of ISAs present in CDP are the two commonest sources of carbon used as feedstock in studies relating to nuclear waste disposal in literature. However, the review of Humphreys *et al*. [18] on cellulose degradation and the fate of degradation products under repository conditions indicated that under anaerobic alkaline conditions relevant to the cement-based radioactive repository, cellulosic materials will undergo degradation to form cellulose degradation products (CDP) [18]. This suggest that between $Ca(ISA)_2$ and CDP, the latter would most likely form as the major source of carbon within the nuclear waste repository.

The aim of this part of the section of the project was to investigate the impact of $Ca(ISA)_2$ and CDP as feedstock on the evolution of the respective communities. In order to achieve this, the two established communities driven by NH_4^+-rich $Ca(ISA)_2$ and CDP feedstock were selected for further investigations.

The results of the ISA degradation profiles appeared similar whereby both the $Ca(ISA)_2$ and CDP were able to support the growth of microbial populations present within these two systems, evidenced by the removal of ISAs and formation of fermentative products. Analysis of the established consortia revealed that within the CDP driven system, 10 unique taxa were present at the genus level while 6 unique genera present within the $Ca(ISA)_2$ system. Overall, there were 16 shared taxa between the two systems (Figure 4.19). Comparing the two systems with the background soil sample, all three shared 4 taxa at the genus level between them while 32 genera were unique to the background soil sample. Furthermore, following the comparison of the two communities via Cramer von Mises-type statistics, the *P* value for Monte Carlo test was 0.004 indicating that the communities were significantly different.

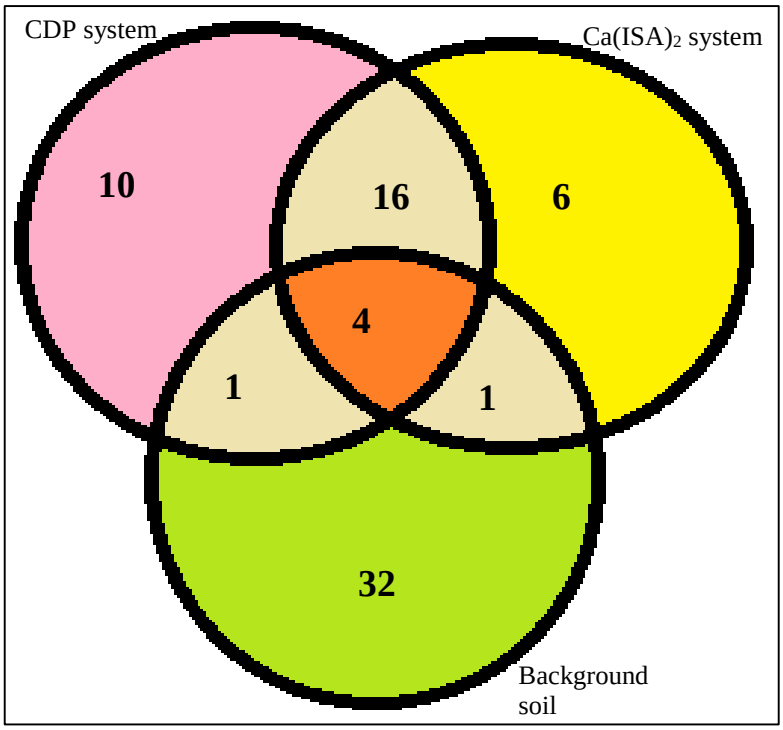


Figure 4.19 Impact of Ca(ISA)2 and CDP on community evolution.

Venn diagram of shared and unique genera across the three systems

The presence of different taxa within the hyperalkaline-contaminated site suggests, in addition to previous studies, that microorganisms within the soil sediments of this site represent a diverse pool of metabolic potential for the bioremediation of organic polutants within the environment. The use of ISAs as a carbon source for growth within a fermentative system appears to have selected for Clostridiales, Bacteroidales and Burkholderia associated taxa. The prevalence of these taxa was unsurprising considering their strong affiliation with anaerobic fermentation of carbohydrates in previous studies [258, 281-283].

In terms of the impact of $Ca(ISA)_2$ and CDP across these system, differences and similarities were observed on the community evolution. Between the two systems, there were 16 shared taxa 11 of which were of the order Clostridiales. In spite of the similarities, quantitative comparisons between the two community profiles showed a significant difference ($p = 0.004$) between the two systems. This was

further confirmed by beta diversity by PCA, which indicated that the two system communities were significantly different from each other and from the original soil sample.

The difference between the two communities fed with $Ca(ISA)_2$ and CDP is the result of the more complex mixture of carbonaceous compounds present within the CDP. The method of generation of CDP has been previously shown to contain various organic acids of which ISA represents more than 70% of the total organic carbon [113]. A significant proportion of organic carbon could not be characterised within this study due to the detection limits of the equipment used in view of previous study which showed that the number of organic acids generated from alkaline hydrolysis of cellulose can be extensive [17]. This probably reflects the increased prevalence of members of the genus *Acinetobacter* within the CDP driven system, where previous studies have reported of their ability to grow under anaerobic conditions utilising a range of short chain organic acids [284, 285].

4.3.8. Key findings

- Microorganisms present within the microcosm were able to degrade ISA from both $Ca(ISA)_2$ and CDP
- The use $Ca(ISA)_2$ and CDP as a carbon and the presence or absence of NH_4^+ did not affect the chemical evolution but the community evolution
- The presence of NH_4^+ appeared to reduce the overall biomass of those systems amended with NH_4^+ thereby suggesting ammonia toxicity
- The background soil presents a source NH_4^+ that could support the microbial growth
- Microorganisms within the soil sediments of this hyperalkaline site represent a diverse pool of metabolic potential for the bioremediation of environmental organic polutans

4.4. Microbial community ISA biodegradation under elevated pH conditions

4.4.1. Rationale for the study

Previous authors have shown the degradation of ISA associated with $Ca(ISA)_2$ or as part of a CDP mixture at either circumneutral pH or higher pH up to pH 11.0 [70, 73, 74, 83]. In these studies, however, the impact of pH on the evolution of associated communities has not been described. The aim of this section of the study was to investigate the degradation profiles of microbial communities associated with ISA degradation at elevated pH and the impact of high pH on the evolution of these communities. As a result, batch microcosms between pH 10.0 and 11.5 driven by either $Ca(ISA)_2$ or CDP were set up as described in the method section from which ISA biodegradation, headspace gas evolution and microbial community analysis data could be obtained after a 10-week feed/waste cycle at regular intervals up to day 30.

4.4.2. Results/discussion

4.4.3. Chemical analysis of $Ca(ISA)_2$ driven microcosm

Across the microbial consortia of the different pH ranges (pH 10.0-11.5), significant amount of ISA was degraded when $Ca(\alpha\text{-}ISA)_2$ was employed as the sole carbon source. At the beginning of the sampling period, the initial ISA concentration within the microcosms varied due to the residual ISA from the waste/feed cycle. In the pH 10.0 microcosm, at the end of sampling 3.46 mM representing 76% of the initial ISA had degraded. Within the microcosms in which pH was amended to 11.0 and 11.5, ISA degradation was affected due to the harsh alkaline environments. For instance, in the pH 11.0 microcosm, while 2.85 mM (56%) of the initial 5.26 mM ISA degraded, a total of 2.98 mM representing 53% of the initial ISA degraded from the pH11.5 system by the end of sampling (Figure 4.20). The first order mean rate of degradation of ISA in these systems was the same at $4.0 \times 10^{-2} \pm 00\ d^{-1}$. It is worthy of note that in the pH10.0 system, ISA removal occurred gradually from the beginning of sampling until day 20

when a sharp drop was observed from that point until day 30 when the sampling ended. This observation coincided with a significant 1 unit drop in pH from the initial of 10.0 to 9.0 (Figure 4.20). The final pH in the pH 10.0 system was 8.5. A similar observation was made in the pH11.5 systems. There was no significant ISA degradation until day 5 probably due to the harsh conditions imposed on the system by the initial ambient pH. However, a sharp drop in ISA concentration (from 5.52 mM to 3.77mM) was observed when the pH dropped from 11.5 to 11.1 between day 5 and day 15 suggesting evolution of the microcosm and formation of niches during the first few days.

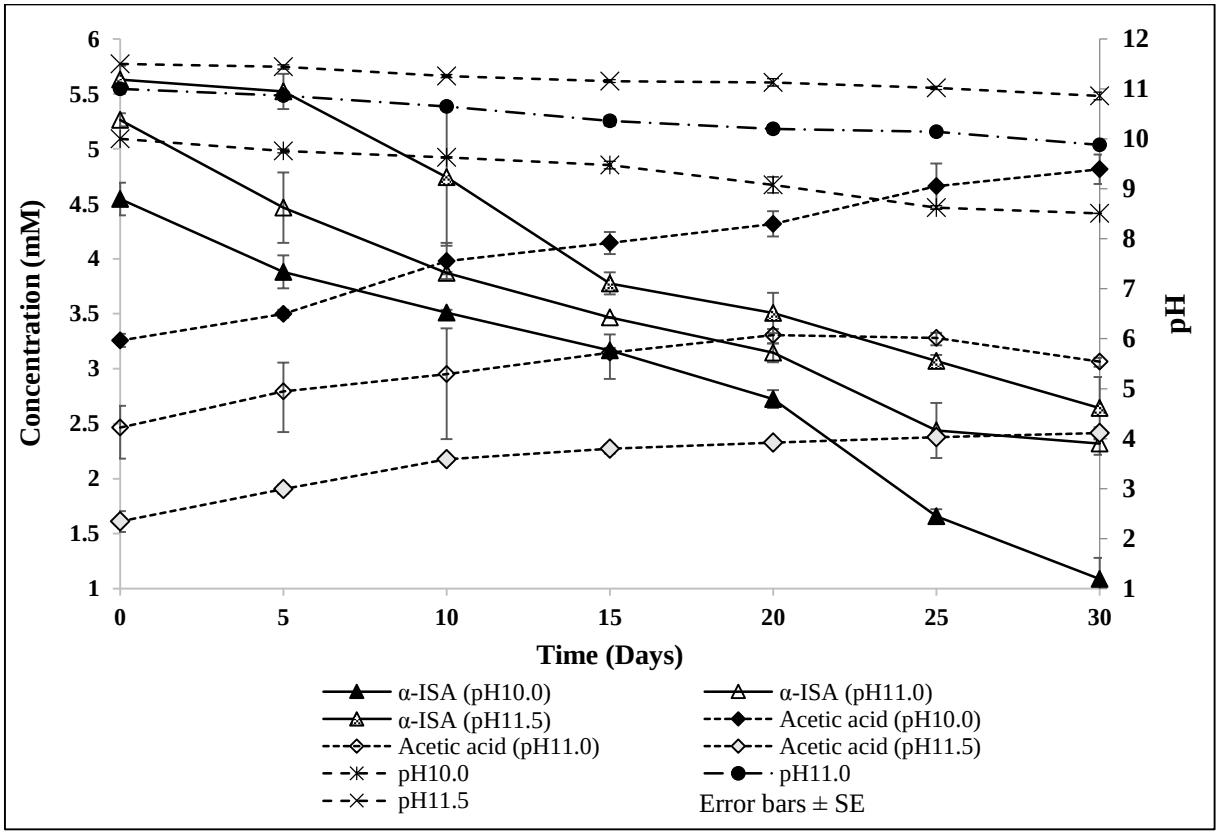


Figure 4.20 ISA degradation with concomitant acetic acid generation and pH drop across different pH ranges

In all three systems, acetic acid was the main volatile fatty acid detected from the fermentation of the carbon substrate. A net acetic acid accumulation of 1.55 mM representing a percentage acetic acid yield of 45% from the degradable ISA was detected in the pH10.0 system at the end of the sampling period. The net acetic acid yield detected from the pH11.0 microcosm at the end of sampling was 0.60 mM representing a yield factor of 20%, was lower than that detected from the pH11.5 system where a net 0.80 mM (26% of degradable ISA) was detected.

ISA degradation was accompanied by increase in cell biomass (Figure 4.21) as a measure of ATP activity within the microcosms. The growth kinetics appeared to be similar across the systems. Across the systems, increased biomass was observed by the end of the sampling period.

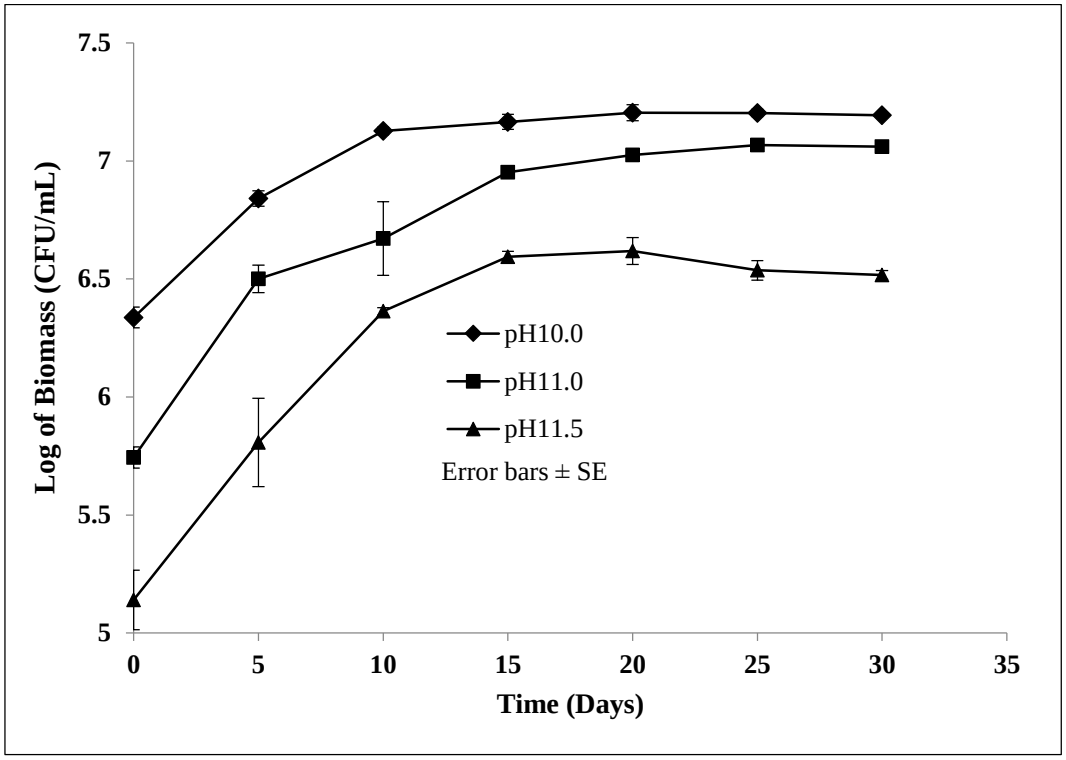


Figure 4.21 Microbial biomass within microcosms at elevated pH

4.4.4. Gas evolution in headspace of $Ca(ISA)_2$ driven microcosms

Gas generation was evident in the headspace of each microcosm where methane, hydrogen and carbon dioxide were detected (Figure 4.22). Within the pH10.0 microcosm, a substantial amount of methane was generated when compared with the other two systems. Thus, at the end of sampling, the concentration of gases generated within the headspace of the pH10.0 microcosm was such that as 4.61 x 10^{-2} mM representing 13.33% of the total degradable ISA was converted into CH_4 whereas the net yield of CO_2 and H_2 detected were 0.03 mM (1%) and 0.12 mM (3%) respectively. In the pH11.0 microcosm, a total net yield in respect of CH_4, CO_2 and H_2 were 0.15 mM (5%), 0.1 mM (0.04%) and 0.05 mM (2%). Methane gas could not be detected from the headspace of the pH11.5 microcosm throughout the sampling period (Figure 4.22). However, the yields for CO_2 and H_2 detected were 0.14 mM (4.68%) and 0.04 mM (2%). The outstanding carbon is likely to have been transformed into biomass, dissolved inorganic carbon and possibly into the formation of carbonate.

The percentage yield of acetic acid and all gases generated across the three system are summarised in Table 4.1.

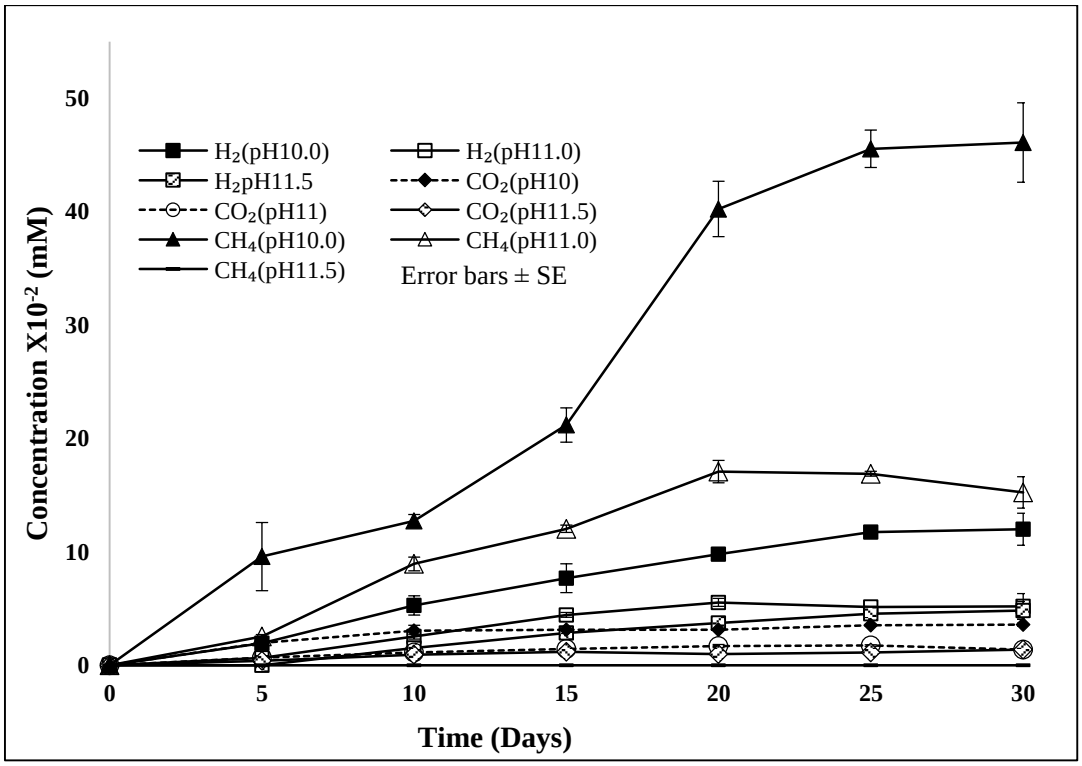


Figure 4.22 Gas evolution within the headspace of the $Ca(ISA)_2$ microcosms

Table 4.1 Percentage yields for acetic acid and headspace gas evolution in $Ca(ISA)_2$-driven microcosms

	Acetic acid (%)	CH_4 (%)	H_2 (%)	CO_2 (%)	Total (%)
pH10.0	45	13.33	3.47	1.04	**62.84**
pH11.0	20	5.09	1.69	0.47	**26.82**
pH11.5	26	-	1.62	4.68	**32.32**

4.4.5. Chemical analysis of the CDP driven microcosm

The CDP driven microcosms also demonstrated ISA degradation across the three systems whereby ISA degradation was accompanied by acetic acid production (Figure 4.23) and the generation of CH_4, H_2 and CO_2 in the headspace of these systems. There was also a significance increase in biomass in all the microcosms. In the pH10.0 microcosm, all the β-ISA (1.58 mM) was completely degraded within 20 days whereas $4.86x10^{-2}$ mM α-ISA could still be detected in the sample at the end of sampling (Figure 4.23). A total ISA of 3.61 mM that is 82% of ISA component of the CDP substrate degraded from the pH10.0 microcosm at a first order mean rate of total ISA degradation of $1.7 \times 10^{-1} \pm 00$ d^{-1}. When compared with the pH10.0 system driven by $Ca(ISA)_2$ where 76% of the total ISA degraded, the CDP system appeared to favour ISA degradation. The ISA removal was accompanied by a net acetic acid yield of 46% which is similar to the percentage yield in the $Ca(ISA)_2$-driven system. The pH dropped from the initial of 10.0 to pH 8.0 (Figure 4.23). In the microcosm in which the pH was elevated to 11.0, a total of 77% of the overall ISA within the microcosm was degraded (Figure 4.23) which is another improvement over its $Ca(ISA)_2$ driven counterpart. The mean rate of total ISA degradation was $1.4 \times 10^{-1} \pm 0.00$ d^{-1}. The net acetic acid yield in this microcosm was 1.15 mM representing a percentage yield of 48%. This acetic acid yield is much higher than that observed in the $Ca(ISA)_2$ driven microcosm partly due to the breakdown of a more amenable β-ISA. As shown in Figure 4.23, the pH of this system dropped from the initial of 11 to a final of 9.78. A similar trend of events occurred in the microcosm that was operated at an elevated pH of 11.5. In spite of the harsh ambient pH, 66% of the original ISA degraded at first order mean rate of $1.4 \times 10^{-1} \pm 0.00$ d^{-1} yielding a net acetic acid of 0.67 mM (23% yield).

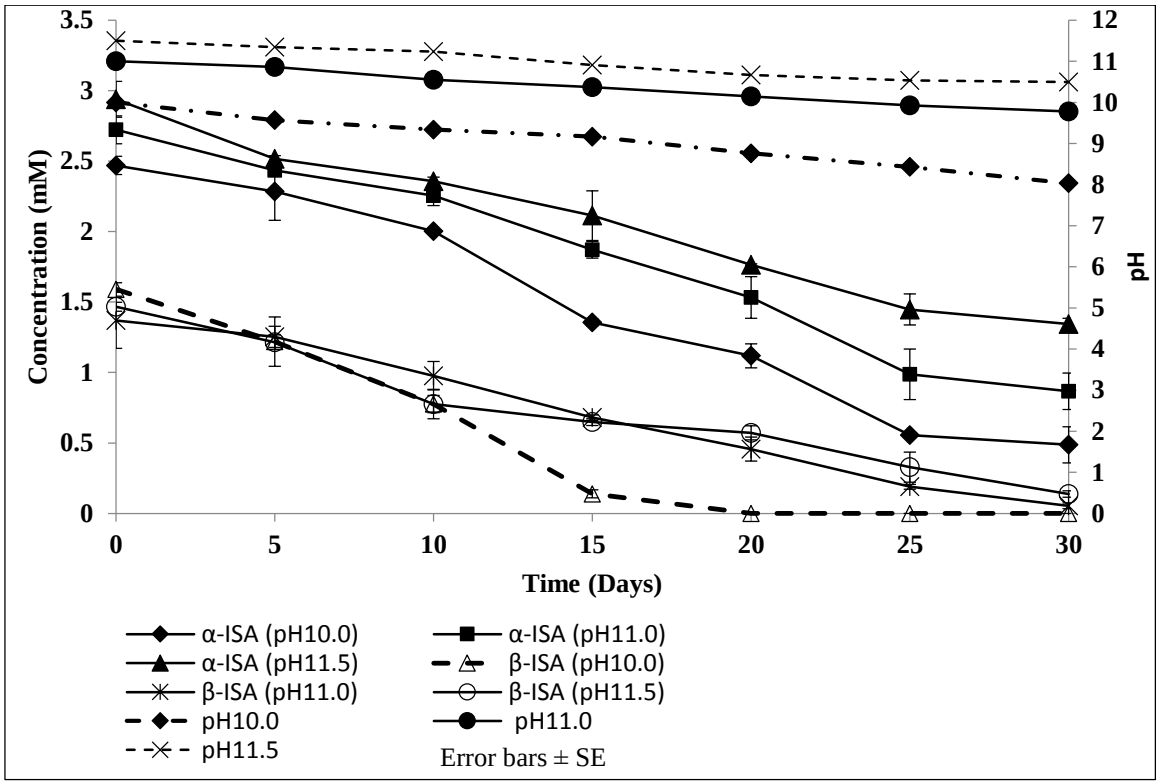


Figure 4.23 α/β-ISA degradation profiles with accompanying pH drop in the CDP driven microcosms

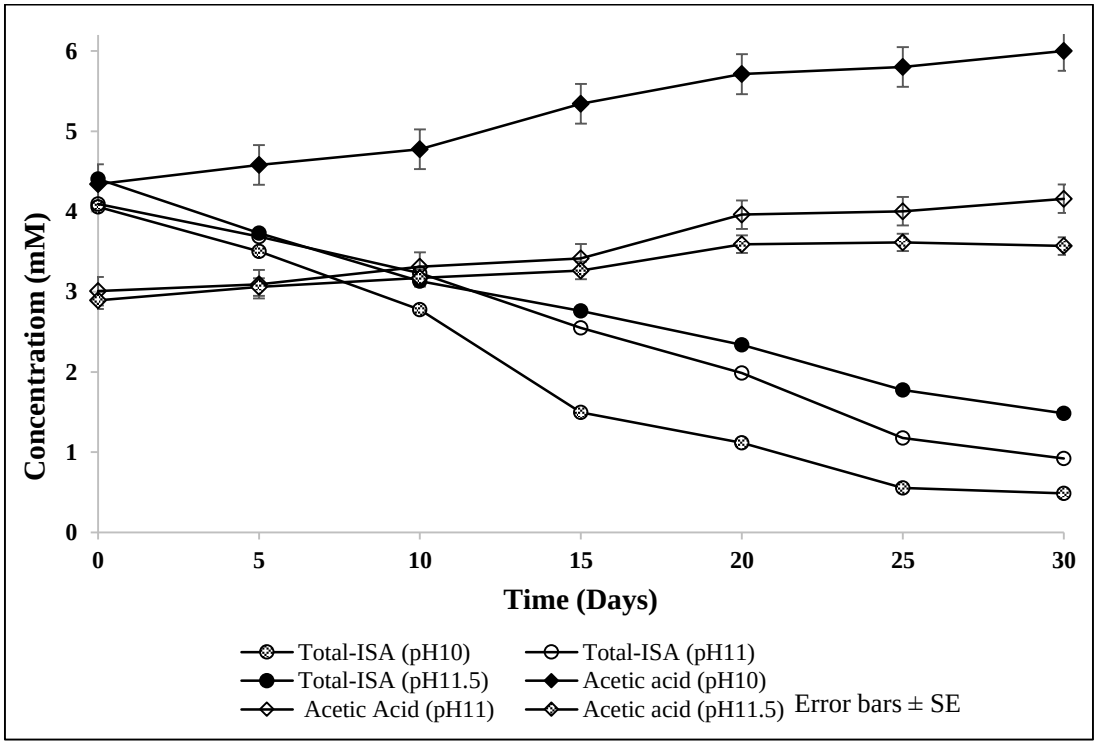


Figure 4.24 Total ISA and net acetic acid yields in the CDP-driven microcosm

Across the three systems, the increase in biomass, which accompanied ISA degradation, appeared to show a similar profile (Figure 4.25).

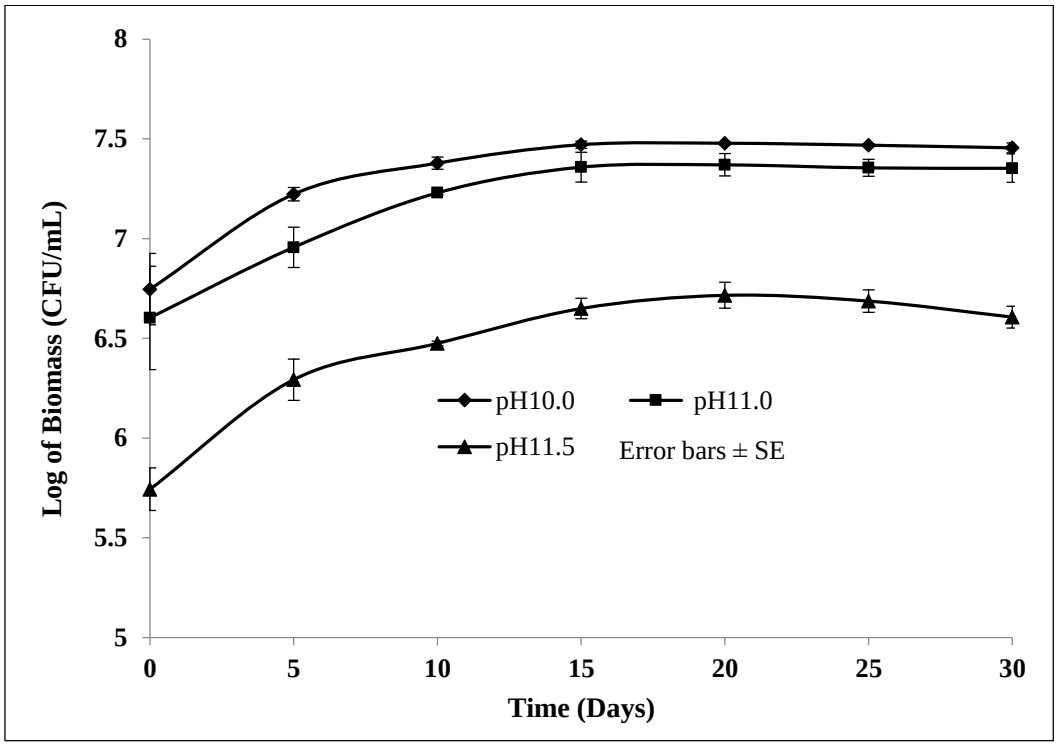


Figure 4.25 Microbial biomass production in CDP-driven systems

4.4.6. Gas evolution in headspace of CDP-driven microcosms

Similar to the $Ca(ISA)_2$-driven microcosm, gases including CH_4, CO_2 and H_2 were generated and could be detected within the headspace of the microcosms (Figure 4.26). In the headspace of the pH10.0 microcosm, a total of $4.6x10^{-1}$ mM of CH_4 could be detected at the end of the experiment. This made a percentage CH_4 yield of 13%. At the same time, CO_2 and H_2 gases were generated whereby the percentage yields were 1% and 3% respectively. In the case of the pH11.0 system, the percent yields in respect of the three detectable gases – CH_4, CO_2 and H_2 were 5%, 1.63% and 0.5%. No CH_4 could be detected in the headspace of the pH11.5 microcosm however, 0.1% and 2% yields were obtained respectively for CO_2 and H_2 (Figure 4.26). Table 4.2 provides a summary of the percent yields of acetic acid and headspace gas evolution.

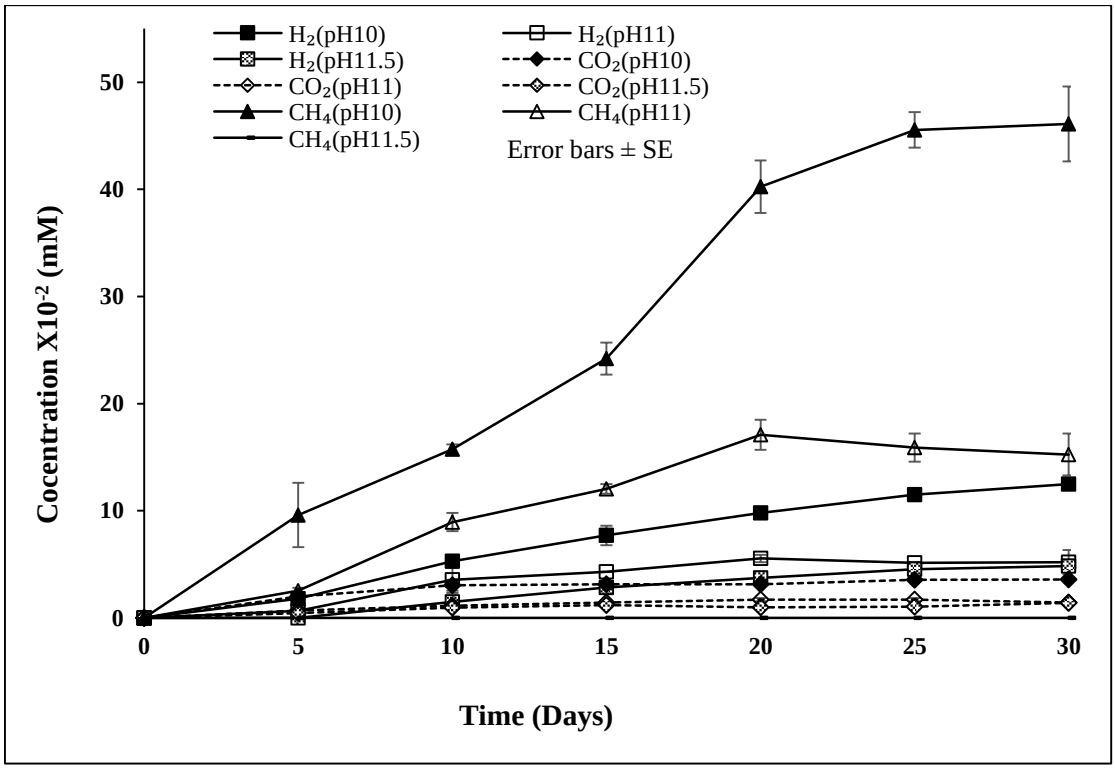


Figure 4.26 Gas evolution within the headspace of CDP microcosms

Table 4.2 Percentage yields for acetic acid and headspace gas evolution in CDP driven microcosms

	Acetic acid (%)	CH_4 (%)	H_2 (%)	CO_2 (%)	Total (%)
pH10.0	46	12.90	3.49	1.00	63.39
pH11.0	47.94	4.79	1.63	0.45	54.81
pH11.5	23.10	-	1.65	0.47	25.22

The acetic acid yield from both $Ca(ISA)_2$ and CDP driven microcosms varied whereby the CDP system appeared to have produced more acetic acid than its $Ca(ISA)_2$ counterpart. Meanwhile, the headspace gas evolution produced similar profiles that require further investigation.

4.4.7. Microbial community profiling based on 16S rRNA gene sequences

The diversity of microbial communities across the six microcosms sampled after 30 days following a 10-week waste/feed cycle are compared based on 16S rRNA gene sequences. Although the prevailing environmental conditions were quite harsh, these microcosms maintained quite a diverse microbial consortia reflecting on the capabilities of some species of microorganisms to survive adverse environmental conditions [286, 287]. In total, over fifty thousand reads were obtained for each sample where a total of 33,708 consisting of 1,551 Archaeal and 32,157 bacterial clone libraries were obtained by CD-HIT. The alpha-diversity analysis of the communities (Figure 4.27) shows that the pH10.0 CDP-driven microcosm was the most diverse community in terms of OTUs followed by its $Ca(ISA)_2$ counterpart. While the two sets of pH11.0 and pH11.5 microcosms appeared to have similar taxonomic diversity, the latter was the least diverse. These findings suggest that pH was a key factor in the selection of taxa in these systems.

The phylogenetic relationship between the selected taxa across the different microcosms was also investigated where beta-analysis by Unifrac distance matrix [242] showed that each of the $Ca(ISA)_2$ and CDP systems selected for taxa that were phylogenetically related to each other (Figure 4.28).

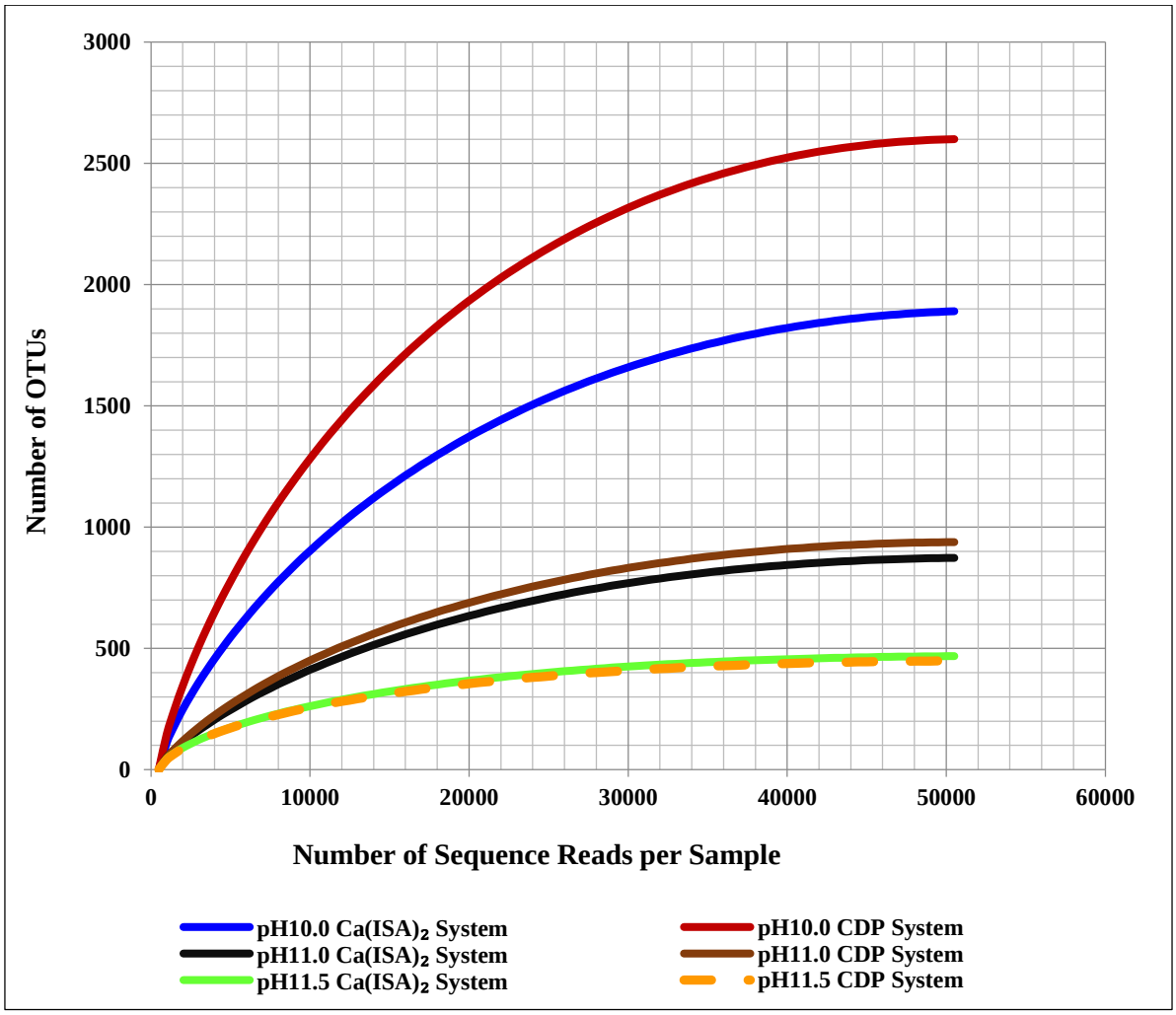


Figure 4.27 Alpha-diversity rarefaction curve by CD-HIT

The different consortia sampled for the alpha diversity analysis 30 days after a 10-week waste/feed cycle

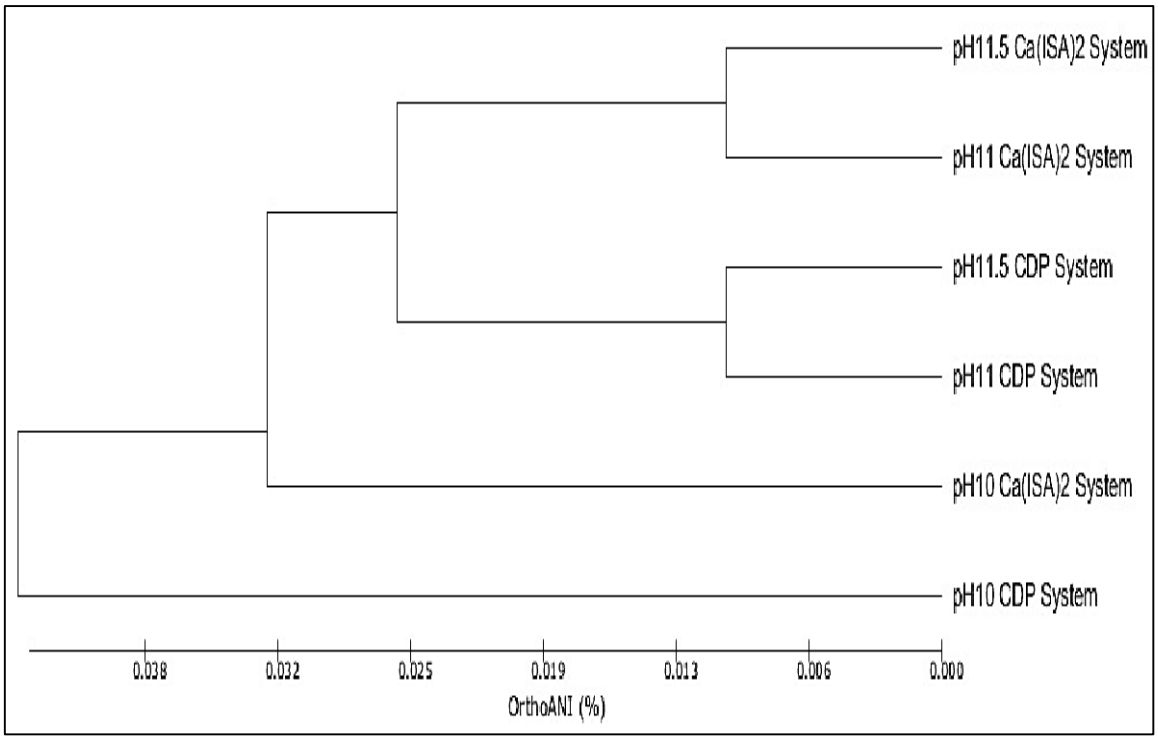


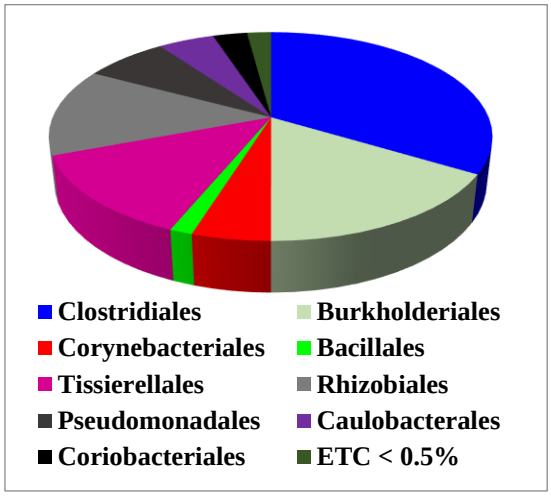
Figure 4.28 Beta-diversity of sample from across the systems

The beta-diversity shows the phylogenetic relationship between sets of taxa within the consortia of the different microcosms by Unifrac distance matrix [242] sampled after 30 days following 10-weeks waste/feed cycle

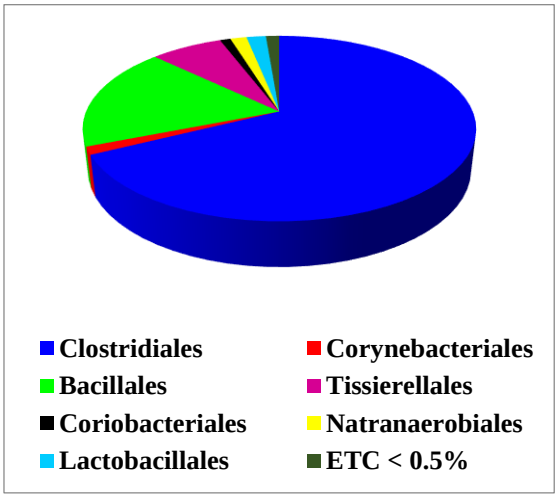
The microbial community profiling showed established consortia under the pH stress conditions where taxa associated with the order Clostridiales dominating in all systems except pH 11.0 and pH11.5 CDP systems (Figure 4.29). In pH10.0, for both $Ca(ISA)_2$ and CDP systems, Clostridiales dominated both community profiles representing 34% and 68% of the totals in respect of $Ca(ISA)_2$ and CDP fed systems. In microcosms with elevated pH, the prevalence of Clostridiales was evident whereby the order consisted of 41% of the entire community profile of the pH11.0 $Ca(ISA)_2$-fed microcosm, and 33% of its CDP counterpart. Similarly, in the pH11.5 microcosms, the taxon represented 38% and 32% in respect of the total community within the $Ca(ISA)_2$ and CDP-fed consortia. At the genus level, one of the associated taxa of the Clostridiales which was prevalent across all microcosms was the unclassified genus with the designation GU455315_g which

represented 14% of the pH10.0 consortia fed with $Ca(ISA)_2$ and dominated the CDP counterpart with a percentage composition of 29%. This genus also constituted 4% and 14% (respectively for pH11.0 $Ca(ISA)_2$ and CDP) and; 6% and 19% for pH11.5 $Ca(ISA)_2$ and CDP-fed consortia suggesting that this organism may have played a critical role in the chemical evolution of these microcosms. Another genus associated with this order and which was prevalent to all the systems was the genus *Alkaliphilus* which made a total of 6.17% (pH10.0. $Ca(ISA)_2$), 11.23% (pH10.0 CDP), 6.71% (pH11.0 $Ca(ISA)_2$), 7.63% (pH11.0 CDP), 2.04% (pH11.5 $Ca(ISA)_2$) and 4.44% (pH11.5 CDP). Members of the genus *Alkaliphilus* have been previously described as endospore-forming, alkaliphilic, strictly anaerobic chemo-organotrophic microorganism capable of utilising variety of carbohydrates and capable of growth up to 45°C [288]. Other members of this genus have also been implicated in bioreduction of Fe(III)-citrate, Fe(III)-EDTA, Co(III)-EDTA, or Cr(VI) as electron acceptors which are all associated with the nuclear waste disposal concepts. In view of their capabilities, members of the genus *Alkaliphilus*, have been exploited for bioreduction or immobilization of toxic metals including cobalt, chromium, uranium and technetium [289]. The community analysis further suggests that the clones share greatest homology with *Alkaliphilus peptidifermentans* previously isolated from cellulolytic consortium and is described as aerotolerant, endospore-forming halotolerant anaerobe and obligate alkaliphile, capable of producing acetate and formate as the only major products of fermentation from carbohydrate metabolism [290]. The genus *Proteiniclasticum* was also found in smaller proportions across the community profiles of all the systems but made 2% of the pH10.0 $Ca(ISA)_2$ system. Members of the genus *Proteiniclasticum* have been previously implicated in cellulose degradation (but not saccharides) where their major fermentation products were acetate, propionate and iso-butyrate [291] and in activated sludge where high protein and polysaccharide content led to their dominance in the consortium [292]. Also prevalent in all systems but in relatively higher proportion

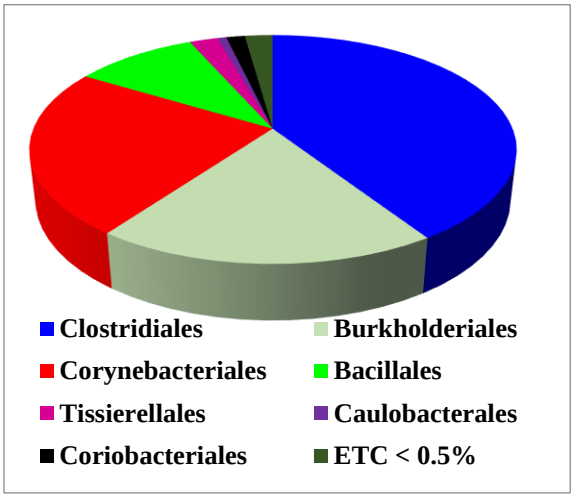
in the pH10.0 CDP fed consortia was the genus *Desulfitispora* which have previously been described as sulfidogenic, haloalkaliphilic, spore-forming anaerobic bacteria capable of fermenting a range of sugar substrates [293]



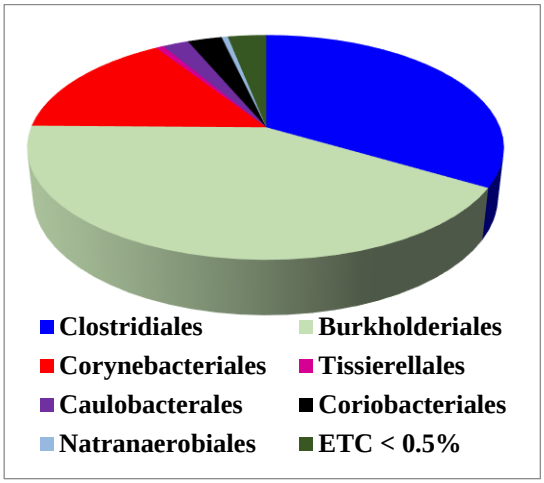
pH10.0 Ca(ISA)$_2$ system



pH10.0 CDP system

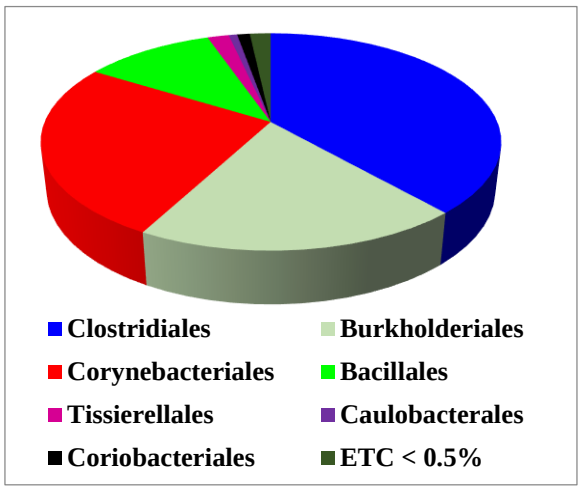


pH11.0 Ca(ISA)$_2$ system

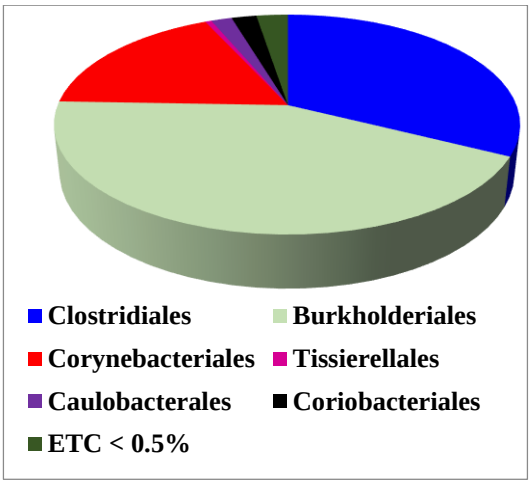


pH11.0 CDP system

Figure 4.29 Elevated pH systems community profiling at the order level



pH11.5 $Ca(ISA)_2$ system



pH11.5 CDP system

Figure 4.29 continues: The elevated pH systems taxonomic analysis at the order level was profiled 30 days after fed/waste cycle

The order Burkholderiales (Figure 4.29) was conspicuously absent in the pH10.0 CDP system however, 16% of the total microbial consortia of the $Ca(ISA)_2$-fed microcosm was made of taxa associated with this order. In other systems, the composition of this order were 19% (pH11.0 $Ca(ISA)_2$), 42% (pH11.0 CDP), 19% (pH11.5 $Ca(ISA)_2$), and 43% pH11.5 CDP. Within this order, the mos prevalent taxon across all the elevated pH system systems was the genus *Alkaligenes*. This genus made a total of 15% of the pH10.0 $Ca(ISA)_2$ system, 17% of the pH11.0 $Ca(ISA)_2$ system, 36% of the pH11.0 CDP system, 19% of pH11.5 $Ca(ISA)_2$ system and 43% of the pH11.5 CDP system (Figure 4.30). Within this community profile, the genus *Alcaligenes* consisted mainly of two species that shared closest homology with *Alcaligenes aquatilis* and an unclassified species with the designation *Alcaligenes*_uc. Some species of this genus such as *Alcaligenes aquatilis* in addition to metabolising a range of carbohydrate substrates, have been associated with tolerance of high pH (up to pH10.0), high temperature (up to 42°C) and tolerance to toxic concentrations of heavy metals such as Cr^{2+}, As^{2+}, Pb^{2+} and Cu^{2+} [256, 257] all of which are associated with the nuclear waste disposal concept.

Another order which was prevalent across the systems but made very small numbers within the profiles was Rhizobiales. However, within the pH10.0 of the $Ca(ISA)_2$ system, individual taxa associated with this order was the third most abundant order, representing 14% of the total community profile (Figure 4.29). This order comprised 11% of the genus *Brucella*, 0.9% *Ochrobactrum* and 0.24% of *Pseudochrobactrum* both of which have been previously associated with the metabolism of a range of sugars [294].

Taxa associated with the order Natranaerobiales were only present in the pH10.0 CDP-fed microcosm where they represented 2% of the total community profile. This order consists of the genus *Natranaerobius* where a particular species *Natranaerobius thermophilus* has been described previously as halophilic (up to 19% NaCl), alkaliphilic (up to pH10.6), and thermophilic (up to 57°C) and have been noted to degrade a variety of carbohydrates [295].

At the genus level most of the taxa were unclassified (Figure 4.30) but in addition to *Alcaligenes* taxa associated with *Dietzia*, *Tissierella*, *Alkaliphilus*, *Brevundimonas*, *Trichococcus* and *Anaerobacillus* were prominent in these systems.

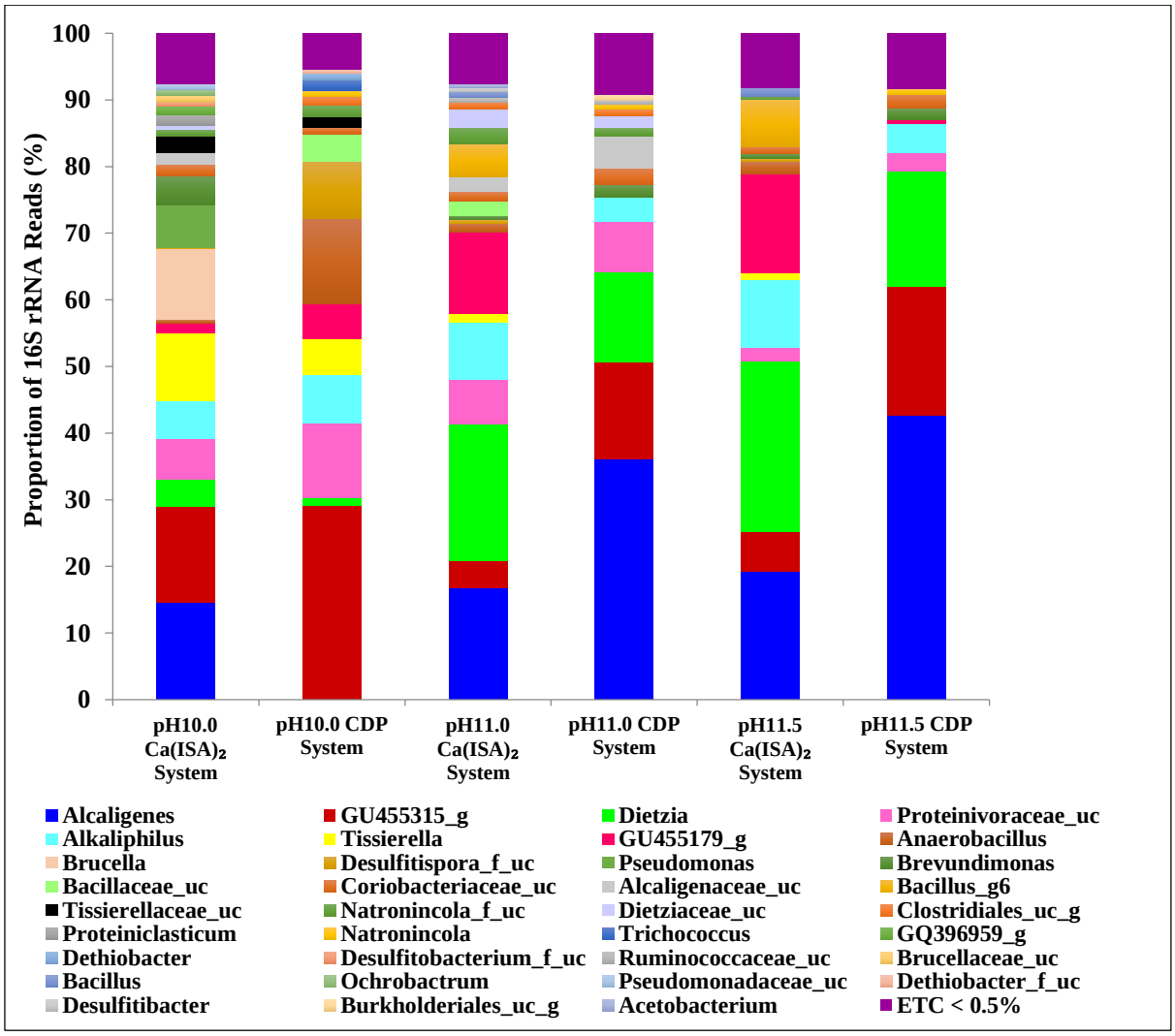


Figure 4.30 System community profiling of the taxonomic analysis at the genus level

The suborder Corynebacteriales of the order Actinomycetales was prevalent within the profiles of all the consortia whereby a significant proportions of the profiles at higher pH such as pH11.0 and pH11.5 consisted of taxa associated with this order. The order represented 5% of the pH10.0 $Ca(ISA)_2$-driven microcosm while the proportion in its CDP counterpart was just 1%. In the higher pH microcosms, the representation of taxa within Corynebacteriales increased suggesting ability to survive and out-compete others in higher pH values. For instance, in the pH11.0 microcosm fed with $Ca(ISA)_2$, the prevalence was 21% which was second to Clostridiales. In its CDP-fed counterpart, the prevalence was 15%. In the pH11.5 microcosms, the prevalence were 26% and 18% respectively

for the $Ca(ISA)_2$- and CDP-driven systems. In all these systems, the order comprised mainly members of the genus *Dietzia*. The prevalence of *Dietzia* in these microcosms of high pH values is consistent with the findings of Charles *et al*. [86] where members of this genus co-dominated with those of *Alishewanella* in a microbial consortium inundated with floc formation in pH values up to pH13.0. In that study, the authors noted that the flocs provided protection and reduced the pH stress experienced by the indigenous microbiota of the anaerobic alkaline environment.

The Archaeal communities present consisted mainly of the orders Methanosarcinales and Methanobacteriales which explains the methanogenic activity of these communities. At the species level, these clones shared greatest homology with *Methanosarcina mazei* (CP009512), *Methanosarcina soligelidi* (JQLR01000001), *Methanobacterium movens* (EU366499) and an unclassified methanogen of designation JN397647_S. They were present in all microcosms however, methane could not be detected in the elevated pH11.5 irrespective of the source of carbon. This observation, in line with the previous findings of Gutierrez *et al* [296] suggests that the activities of methanogenic bacteria could be suppressed by elevated pH levels. Members of the order Methanobacteriales have been previously implicated in hydrogenotrophic methanogenesis where *M. movens* have been shown to utilise H_2 and CO_2 [276, 297] whereas those of the order Methanosarcinales are implicated in aceticlastic methanogenesis [298]. The biochemical pathway for the formation of methane in these systems can therefore be related to acteticlastic and hydrogenotrophic methanogeneis.

The results presented in this body of work extends our knowledge that alkaliphilic environmental bacteria living within anaerobic sediments possess the metabolic capacity to degrade ISA although ISA is not usually found in the natural environment [73]. Through a fermentative, hydrogenotrophic or acetoclastic methanogenesis, microbial consortia present within these anaerobic microcosms

operating between pH10.0 and pH11.5 and driven by either $Ca(ISA)_2$ or products of cellulose degradation, have been able to degrade ISA from these sources . Both sources of ISA produced similar degradation profiles suggesting that microbial metabolism of ISA occurred through the same pathway irrespective of the source. Both forms of ISA (α-/β-ISA) were degraded even at higher pH which contrasts with a previous study where the β-forms accumulated due to the effect of high pH [113].

The formation of major fermentative products including acetic acid and gases such as CH_4, H_2 and CO_2 which accumulated in the headspace of these systems agrees with previous findings of Rout *et al*. [73] and Kuipers *et al*. [74] where microbial consortia driven by either ISA on its own or in a mixture as CDP resulted in methanogenesis. The production of acetic acid and especially CO_2 led to pH drop in these systems, which in some cases augmented ISA substrate degradation by the resident microbial community. This observation supports the claim by Humphreys *et al*. [299] that the degradation of cellulose degradation products under repository conditions would be controlled by the ambient pH with degradation being confined to low pH niches. The ambient pH therefore was a major rate limiting factor in ISA degradation as suggested by Rout *et al*. [113]. This is even more evident in the present study whereby irrespective of the carbon source, the percentage of ISA degradation decreased with increasing pH as follows - pH10.0>pH11.0>pH11.5.

Krich *et al* [279] proposed an ideal stoichiometry for a biphasic anaerobic digestion for a simple case of carbohydrate breakdown as:

$$\mathbf{C_2H_{12}O_2 + 2H_2O \rightarrow 4H_2 + 2C_2H_4O_2 + 2CO_2} \quad \textbf{……(1)}$$

$$\mathbf{2C_2H_4O_2 \rightarrow 2CH_4 + 2CO_2} \quad \textbf{……... (2)}$$

Figure 4.31 shows the percentage yield of acetic acid and headspace gas yield with respect to theoretical values across the $Ca(ISA)_2$ and CDP methanogenic

systems. Both acetic acid and headspace gas generated within these systems fell short of the theoretical values suggesting that acetate removal was not only limited to activities of methanogenic microorganisms but may also be due to activities of acetate-utilising bacteria described previously by Ito *et al*. [300]. Previous authors have also noted that *Methanobacterium* sp. identified with these microcosm can grow on H_2 and CO_2 in an anaerobic digester [301]. It can therefore be extrapolated from those findings that the low levels of H_2 and CO_2 detected from the headspace of the microcosms may be due to activities of these methanogens.

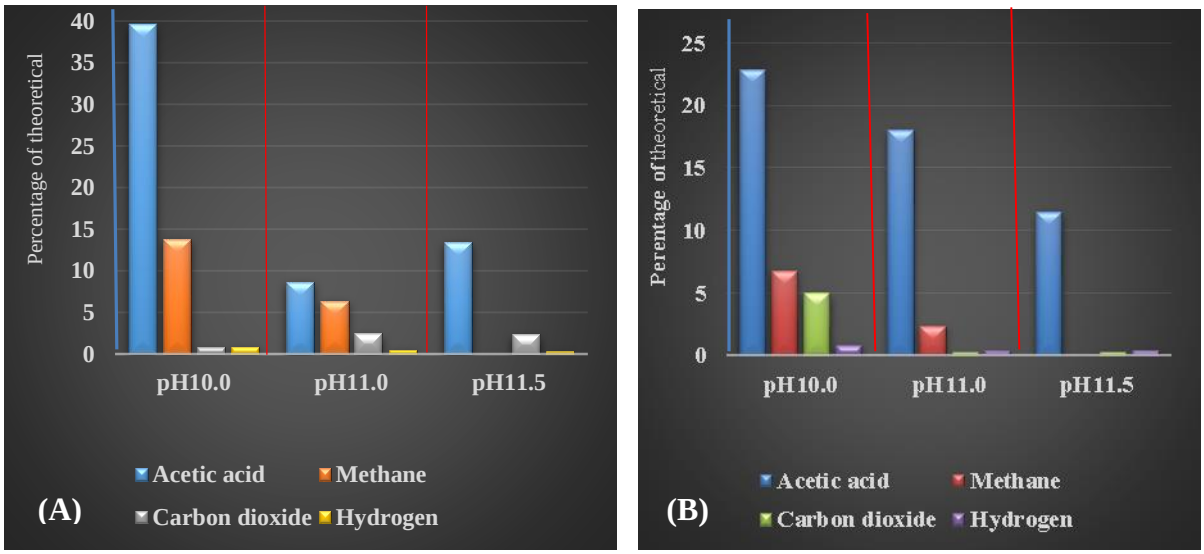


Figure 4.31 Products of elevated pH systems compared with theoretical values

Percentage yield of fermentation product including acetic acid, CO_2, CH_4 and H_2 with respect to theoretical vales in (A) Ca(ISA)2 system; (B) CDP system

4.4.8. Key findings

- Microorganisms within natural systems are capable of high pH stress within which they degrade ISA under anaerobic conditions
- Both pH and type carbon of carbon source may play a role in the selection of taxa under in these systems
- The metabolic pathway of ISA biodegradation appeared the same irrespective of the source of ISA
- Environmental pH may play an important role in activities of methanogens and ISA biodegradation

4.5. Characterisation of bacterial Isolates 1

4.5.1. *Exiguobacterium* sp. strain Hud

4.5.2. Overview

The genus *Exiguobacterium* consists of members that are non-sporing Gram-positive rods of the phylum Firmicutes and order Bacillales from the family of Bacillales Inserta Sedis XII. The group was first classified into the genus *Exiguobacterium* by Collins *et al*. [302]. Different species from the group have been isolated from various environments including soils, freshwater, salt waters, brine shrimps, permafrost, glaciers and hydrothermal vents [303] which is indicative of their ability to survive and grow in extremes environments. For instance, some species have been recovered from environments extreme temperatures and pH while others have been found to show high level of resistance to antibiotics, heavy metals and UV radiations [304, 305]. Some species are also noted for their wide metabolic capacity which allows them to utilise a range of organic substrates. This metabolic capability has generated a considerable interest among researchers in the area of bioremediation [306].

Within the Siberian tundra, temperatures could be as low as -40°C where water and carbon substrates are usually limited however, Rodrigues *et al*. [307] isolated three strains of the genus from this site of which one of them was classified as *E. undae* while the other two were proposed as novel species designated as a novel *E. sibiricum* based on phenotypic dissimilarities and differences in DNA-DNA reassociation values. The researchers indicated that the temperature range within which members of the genus could grow was -2.5 – 40.0°C and reported the presence of *csp* (cold shock protein) and *hsp70* (Class I-heat shock protein which enable them to tolerate such temperature extremes). This indicates that some members are psychrophilic while others are thermophilic. For instance, *E. profundum* was isolated from a hydrothermal vent at deep sea at a depth of 1600m and could tolerate a wide span of temperature between 12 and 49°C [308]. The tolerance of *Exiguobacterium* species in hyperalkaline environment was

described by Ueno *et al*. [309] where *E. aurantiacum* was found to be capable of growth above pH 10.0 and shows ATPase activity at 30°C [309]. Similarly, *E. oxidotolerance* isolated from a drain of a fish processing plant which is often contaminated with H_2O_2 as a bleaching agent was found to be of high catalase activity and capable of growth at pH 10.0 [310]. At lower pH conditions, the survival and growth of the genus is shown in the works of La Duc and his colleagues as well as the group led by Pandit where *E. acetylicum* strain KSC_Ak2F was shown to be capable of growth at pH 5.1 and novel species *Exiguobacterium* (JX028147) isolated from Indian state of Gujarat that was capable of growth at pH 5.0 respectively [311, 312]. In terms of resistance to radiation, Gutiérrez-Preciado and his colleagues isolated *E. chiriqhucha* str. N139, from a high-altitude Andean lakes and showed that the strain was resistant to high doses of UV-B radiation [313]. The high altitude Andean lakes (HAALs) site has been described by earlier researchers as a site inundated with high UV radiation and extremes of temperature and salt [314, 315]. This suggests that earlier species isolated by Ordoñez *et al*. [316] and Flores *et al*. [317] are potentially tolerant to UV radiation. On the basis of their tolerance of toxic metals, *E. aestuarii* strain CE1 recovered from municipal waste rich soil from the banks of Kestopur canal (Kolkata), West Bengal, India, was shown to tolerate high concentration of heavy metals including Co, Ni, Pb, Cr and Fe with the capacity to remove Ni^{2+} and Cr^{6+} [318]. Other studies have also shown the propensity of some members of the genus for the bioreduction of arsenate and tolerance towards mercury [319, 320]. The pathogenicity of members belonging to this genus is not well described however, molecular identification of *E acetylicum* implicated it as the aetiological agent of bacteraemia in an elderly individual whereas *E. sibiricum* has been implicated in skin infection [321, 322]. Pitt *et al*. [323] also isolated *E. aurantiacum* from six patients suffering from bacteraemia, three of whom had myeloma.

Rout *et al*. [324] in a study to investigate the biodegradation of anaerobic alkaline cellulose degradation products isolated a strain of *Exiguobacterium* designated, *Exiguobacterium* sp. strain Hud from a fermentative alkaliphilic methanogenic polymicrobial microcosm that was operating at pH 10 for which they carried out a draft genome sequencing using a Whole Genome Shotgun (WGS) sequence strategy. The genomic sequencing generated 826 contigs with a total coding sequences of 3,484 (CDS). The total length of the draft genome was 3,359,295-bp in length where majority of the genes identified with Rapid Annotation using Subsystem Technology (RAST; Figure 4.32) were responsible for cellular functions and support. For instance, 98 proteins were shown to engage in DNA repair indicating the strains ability to survive and grow in a range of environments whereas 75 were involved in stress response, reflecting on the ability of the strain to tolerate different kinds of stress including carbon starvation and oxidative stress.

Particularly of interest was the presence of genes that code for the metabolism of carbohydrates under both aerobic and anaerobic conditions and that the strain could utilise a range of carbohydrate substrates including disaccharides such as lactose, fructose and trehalose and monosaccharides including galactose, xylose and mannose. The genome further suggests the presence of genes that code for biochemical processes for substrate metabolism including glycolysis and tricarboxylic acid cycle (TCA) of substrates to release their stored energy.

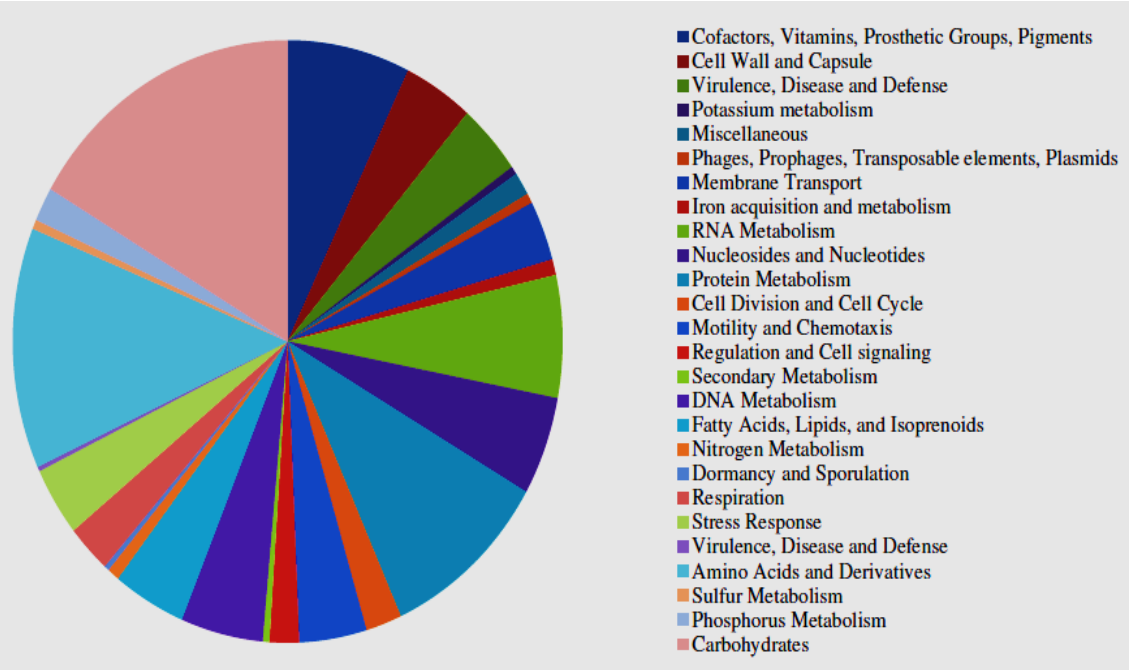


Figure 4.32 RAST annotation for the draft genomic sequencing of *Exiguobacterium* sp. Strain Hud

RAST annotation, taken from Rout *et al*. [324].

4.5.3. Rationale for the study

The current genome annotation for this strain showed the ability to utilise a wide range of carbon substrate which is consistent with the findings of previous studies [306]. This suggests that the genes present may code for multifunctional proteins that could support ISA metabolism. Furthermore, the ability to tolerate stresses imposed by extreme conditions and being able to tolerate carbon starvation are hallmarks for any microorganism that can thrive under repository conditions. Additionally, having been recovered from a polymicrobial consortium that was capable of ISA degradation, makes this organism a good candidate for ISA biodegradation.

4.5.4. Results and Discussion

Exiguobacterium sp. strain Hud was capable of growth on solid mineral media supplemented with either CDP or Ca(α-ISA)$_2$ at pH 10.0 after 48 hours suggesting ability to utilise ISAs under alkaliphilic anaerobic conditions. The colony morphology within 18 h following sub-culturing on FAA (pH 10.0) appeared round, shiny, irregularly elevated and orange in colour on both media whereas the Gram reaction indicated Gram-positive rods. Figure 4.33 shows the electron micrograph of the strain. In addition the strain was found to be motile and positive for both catalase and oxidase.

Comparing growth response under aerobic and anaerobic conditions, growth was observed to be slightly retarded on the same medium under anaerobic condition suggesting that cell development under alkaliphilic anaerobic condition may need higher growth requirements [325]. When culture plates were left for about three weeks on FAA under anaerobic conditions, a Gram reaction showed a reshape of the cells from the normal Gram-positive rods to morphology between Gram-positive coccobacilli and Gram-positive cocci. This suggests that the strain may be capable of bacterial morphological plasticity (pleomorphism) as observed in the work of Chaturvedi *et al*. [326] on *Exiguobacterium soli* sp. nov. isolated from McMurdo dry valleys, Antarctica whereby *E. soli* was found to be rod-shaped (1.9 mm x and 0.95 mm) at the beginning of growth but at the stationary-phase was found to be cocobacillary shaped (1.5 mm in length and 1.15 mm in diameter).

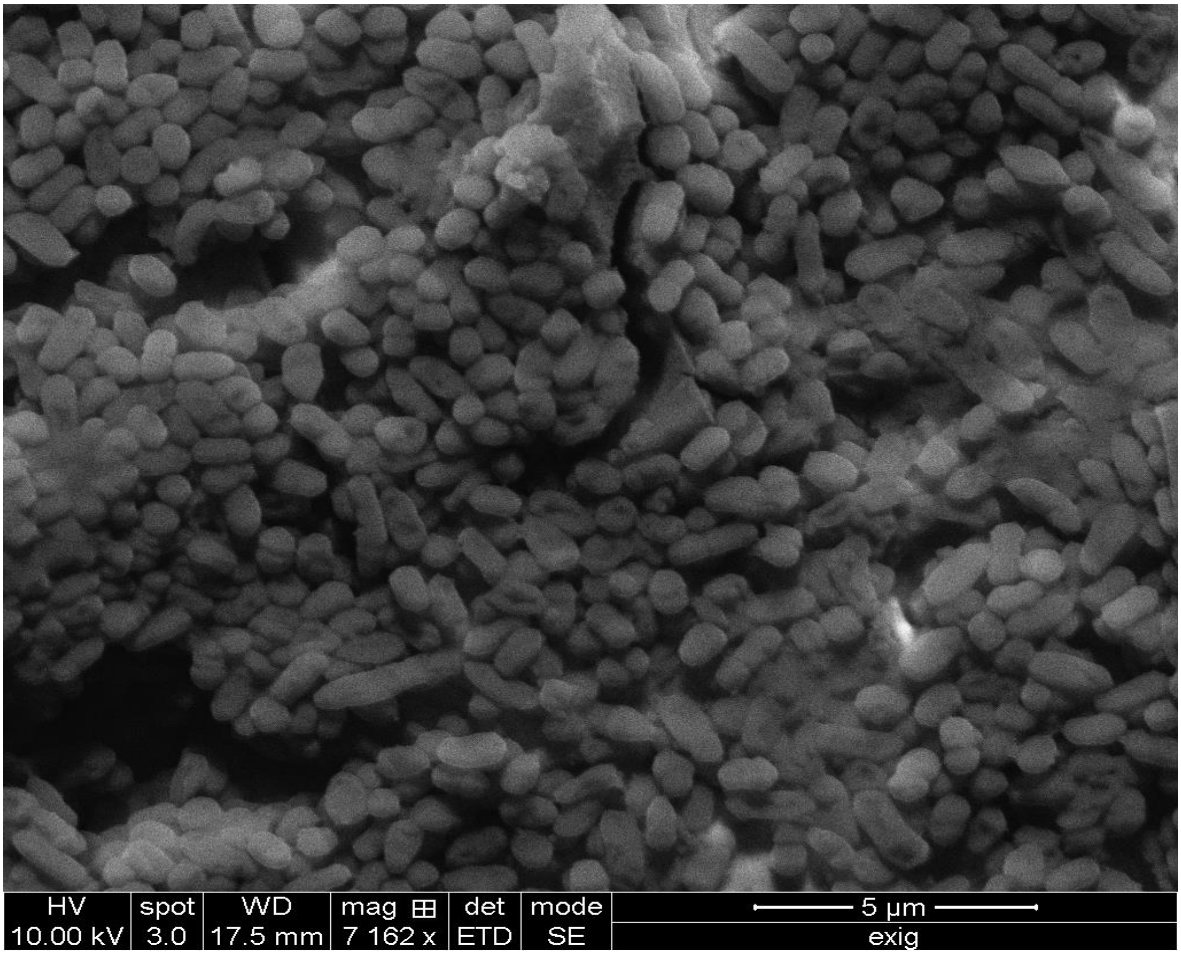


Figure 4.33 Electron micrograph of *Exiguobacterium* sp. strain Hud.

4.5.5. Phylogenetic inference of *Exiguobacterium* sp. strain Hud

Comparison of the 16S rRNA gene sequence indicated that the closet match was to *Exiguobacterium mexicanum* strain 8N (99% identity; Accession number NR042424.1). In order to obtain the evolutionary relationship between the strain and other species of the genus, MEGA 6 analysis was carried out using sequence from the NCBI database. A pairwise distance matrix was calculated to determine whether the sequence obtained for the strain was a duplicate of any of the sequence matches.

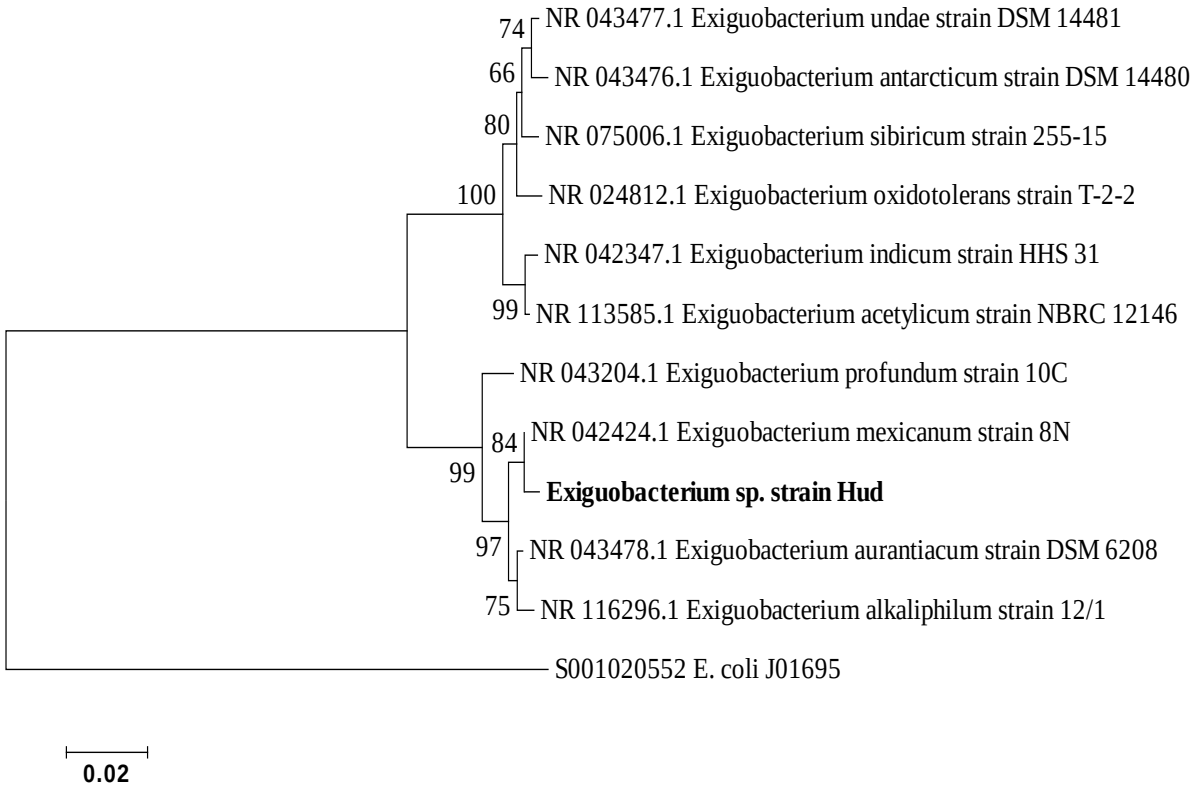


Figure 4.34 Neighbour-Joining tree based on 16S rRNA gene sequences

The inferred tree shows the phylogenetic relationship between *Exiguobacterium* sp. strain Hud and other species of the genus. *E. coli* J01695 was used as outgroup. Bar, 2 nucleotide substitution per 100 nucleotides

The evolutionary relationship of the isolate was inferred using the Neighbour-Joining method [327] of MEGA 6 programme and the optimal phylogenetic tree was obtained (Figure 4.34). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test of 1000 replicates are shown next to the branch points [328]. The analysis that involved 12 nucleotide sequences included *E. coli* L01696 as the bacterial outgroup to root the tree. The inferred phylogeny shows that the isolate clusters with *Exiguobacterium mexicanum* strain 8N in a bootstrap test of 84%. The phylogenetic tree also showed that two strains; *Exiguobacterium aurantiacum* strain DSM 6208 and *Exiguobacterium alkaliphilum* strain 12/1 were closely related in a bootstrap test

of 98%. All four strains showed a close phylogenetic relationship with *Exiguobacterium profundum* strain 10C.

4.5.6. ISA biodegradation

In broth media in which *Exiguobacterium* sp. strain Hud was inoculated into mineral medium with Ca(α-ISA)$_2$ at pH 9 under anaerobic condition at 25°C, no significant cell growth was observed even after 28 days hence, there was no obvious ISA removal, acetate generation or pH reduction in these cultures (Figure 4.35).

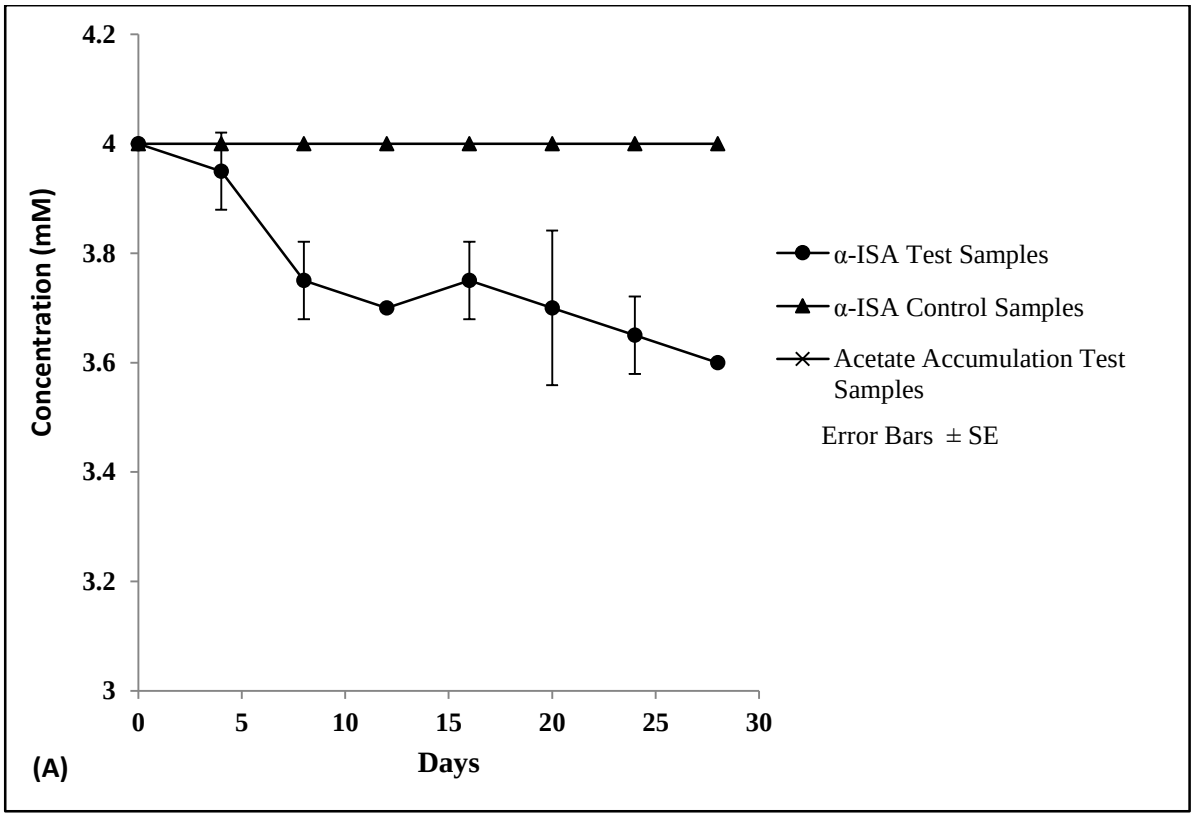
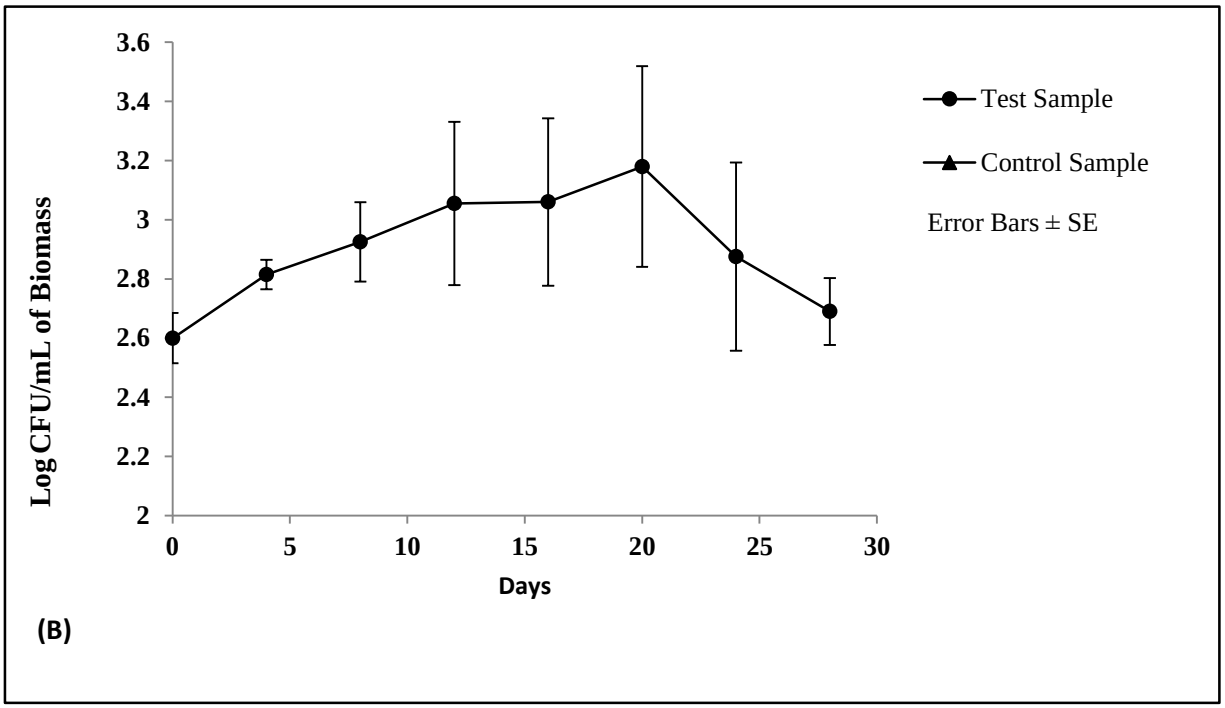


Figure 4.35 *Exiguobacterium* sp. strain Hud degradation of ISA



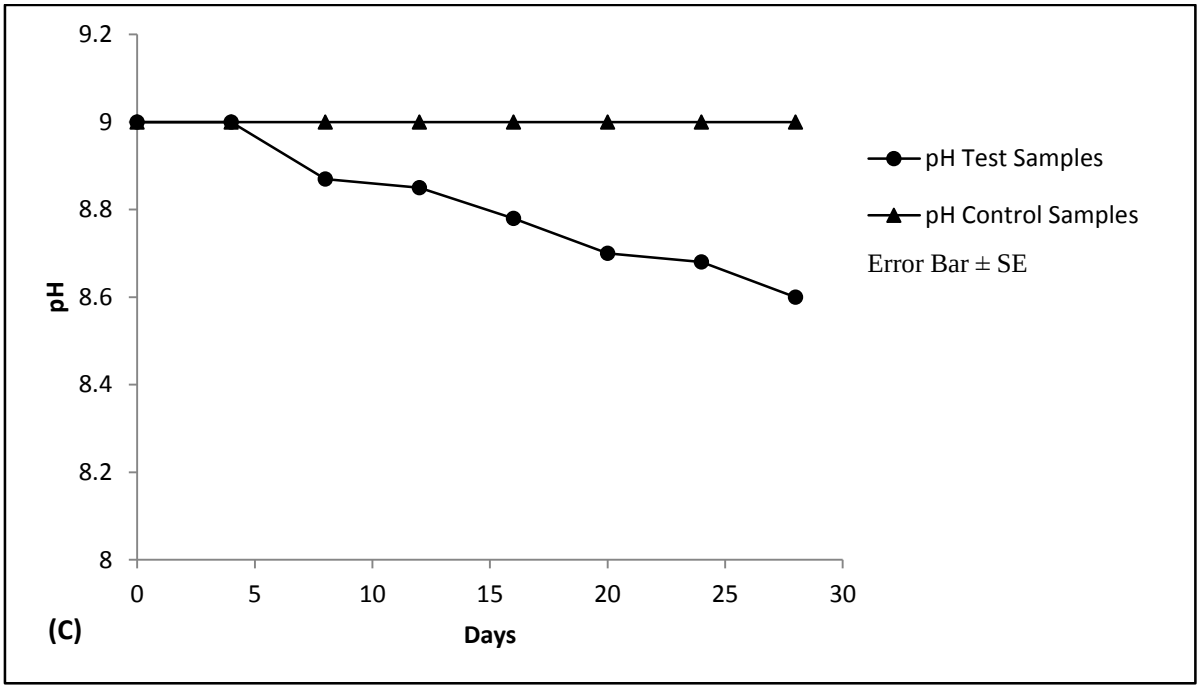


Figure 4.35 continues: ISA biodegradation was investigated in mineral medium showing (A) α-ISA removal and acetate accumulation, (B) microbial growth and (C) pH drop with time

The experiment was repeated in minimal media supplemented with 2% vitamin and trace elements solution at pH 8 where 4 mM NaISA and 2 mM $Ca(ISA)_2$ were added as carbon substrates. Within 14 days ISA in all sets of experiments were either completely or significantly consumed by the strain (Figures 4.36/4.37). There was a concomitant production and accumulation of acetic acid which was detected in the test samples but not in the control.

In the NaISA experiment, ISA degradation occurred continuously but gradually within the first four days but increased sharply afterwards, until day 10 when the total ISA was observed to have been completely degraded. The observable lag in the removal of ISA within the first four days may suggest bacterial adaptation to the culture environment [329]. The first order mean rate of degradation was 1.0 x 10^{-1} ± 0.00 d^{-1}. Concomitant with the ISA degradation, substantial amounts of acetate (3.60 mM) was generated which accumulated from the onset of ISA degradation to the end of the experimental period (Figure 4.36A). Out of initial NaISA concentration 4 mM, a total of 3.60 mM acetate was generated and accumulated representing a percentage yield of 90%. The production of acetate suggests that the strain was utilising ISA via a fermentative acetogenesis.

Schroder *et al*. [330] proposed that 1 mole of glucose was stoichiometrically fermented to 2 moles of acetate, 2 moles of CO_2 with the evolution of 4 moles of H_2 which is consistent with the acetate phase of the biphasic anaerobic digestion of glucose proposed by Krich *et al*. [279] Thus:

$$\mathbf{C_6H_{12}O_6 \longrightarrow 2CH_3COOH + 2CO_2 + 4H_2}$$

This indicates that acetate generated from the substrate was 45.0% of the theoretical. Coincidentally, the turbidity of the medium increased suggesting an increase in biomass (Figure 4.36B). This indicates that part of the carbon substrate (after acetate generation) was converted into biomass generation which increased by 1000 fold. Also as shown in Figure 4.36C, the pH of the inoculated medium

but not the control experiment dropped from the initial pH 8.0 to pH 6.7 indicating that the medium was acidified during the fermentation process.

In the abiotic control the ISA concentration remained almost the same throughout the experimental period and no acetate generation was observed (Figure 4.36).

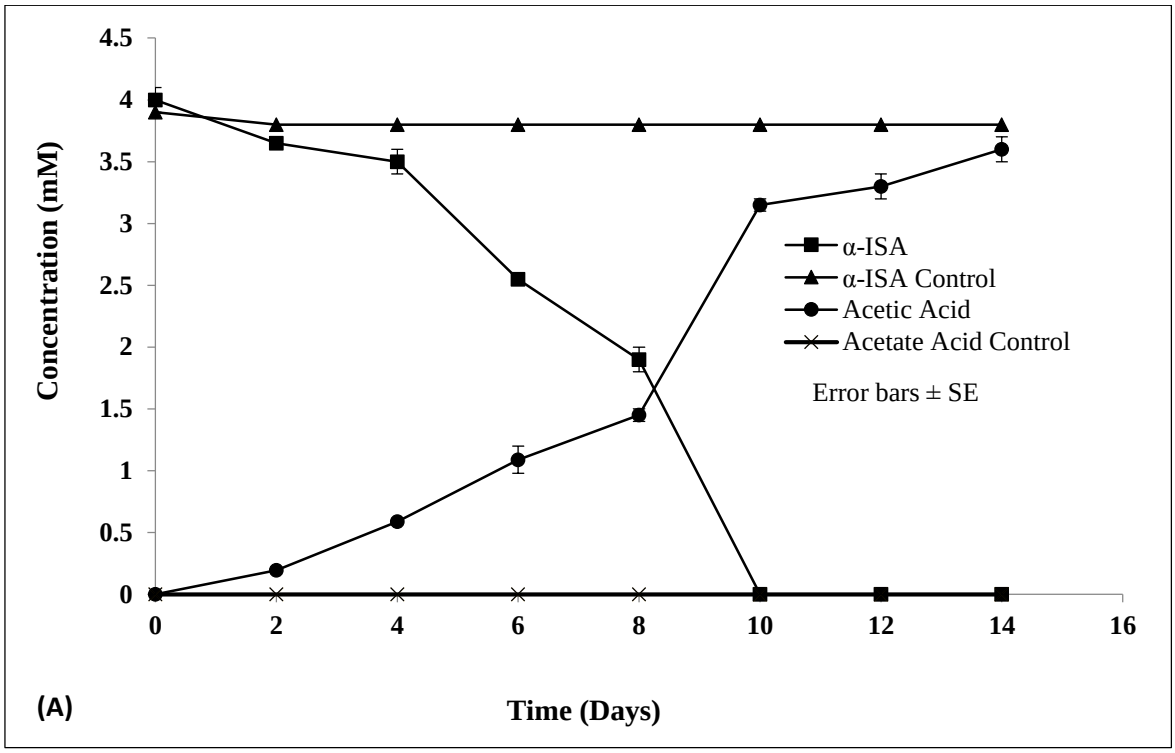
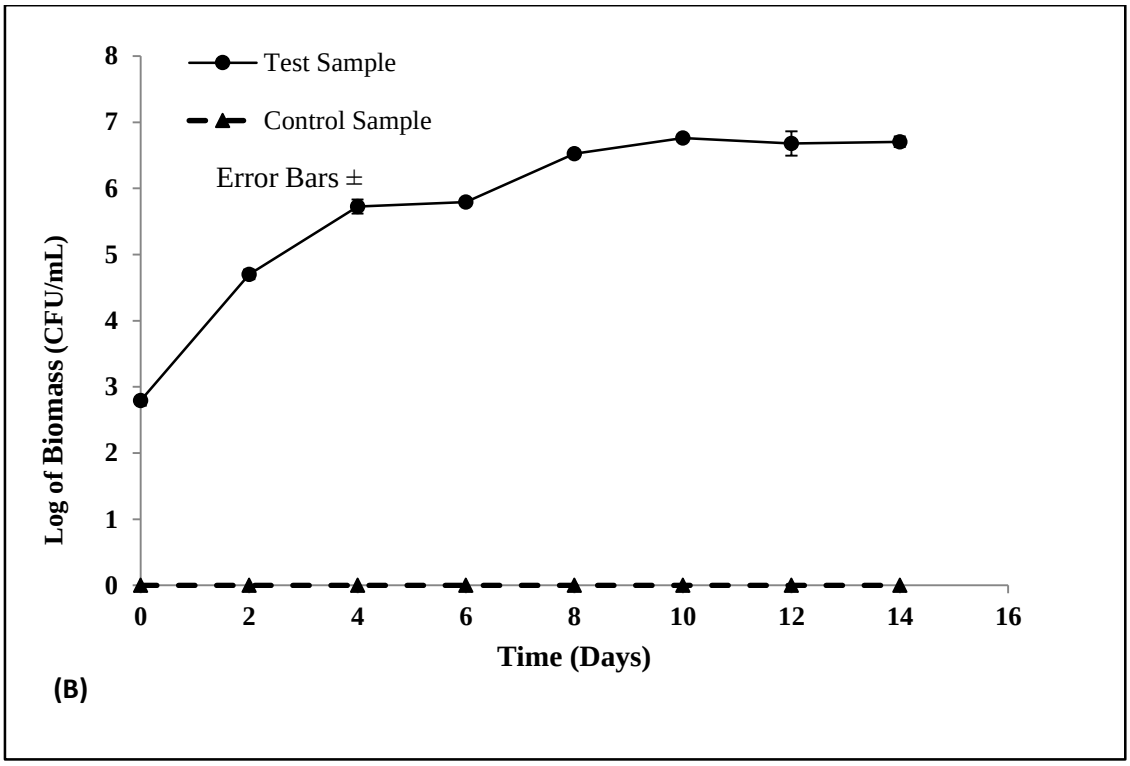


Figure 4.36 *Exiguobacterium* sp. strain Hud degradation of ISA from NaISA



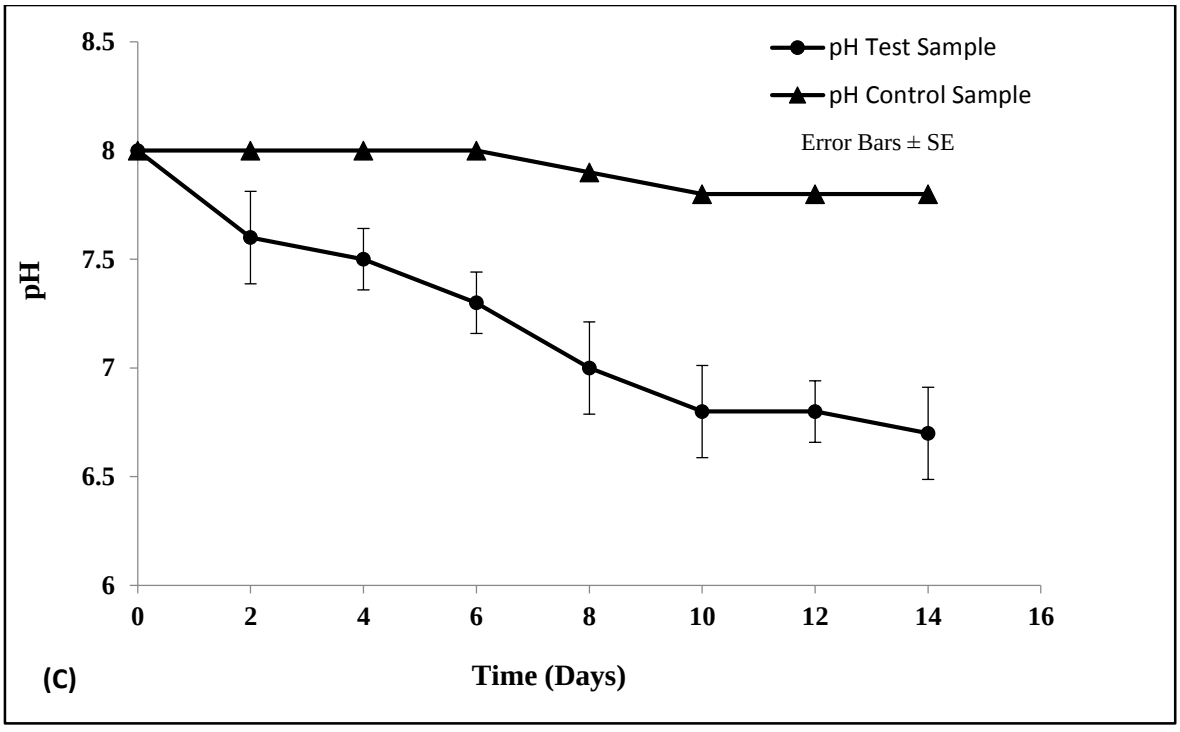
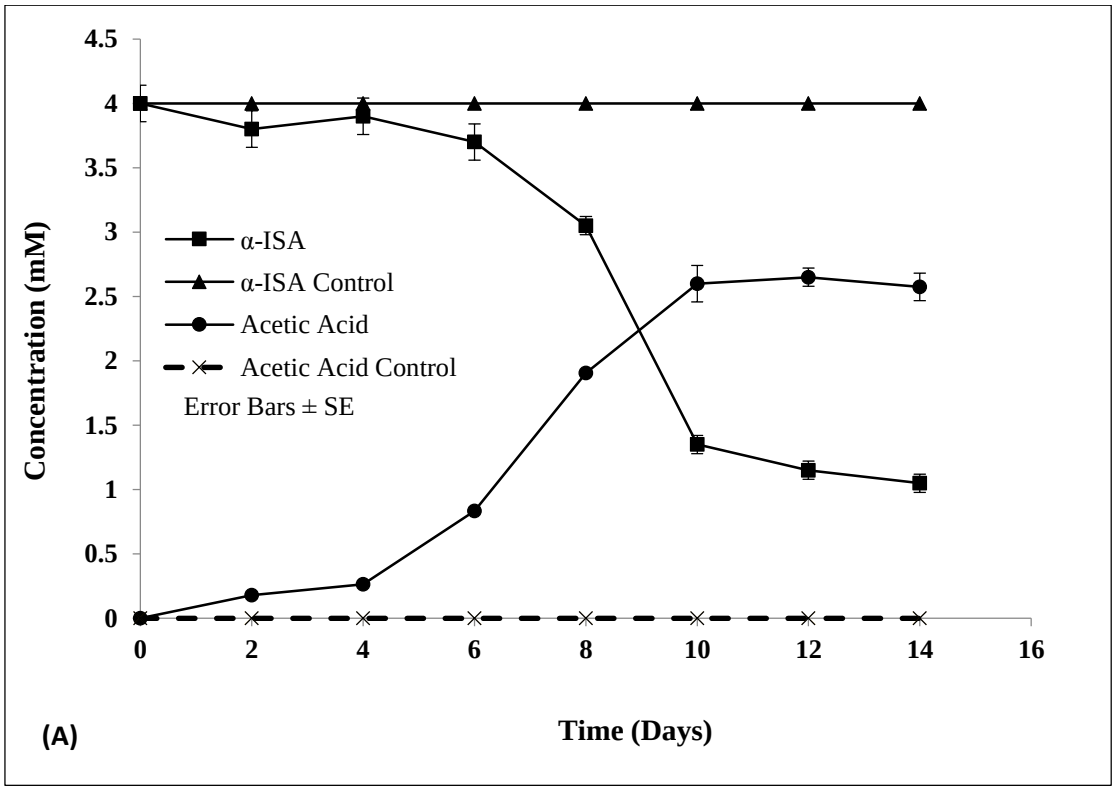


Figure 4.36 continues: ISA degradation experiment set up in minimal media supplemented with vitamin and trace elements solution at pH 8.0 showing (A) α-ISA removal and acetate accumulation, (B) microbial growth and (C) Drop in pH against time

Similar degradation profiles were observed in the $Ca(ISA)_2$ experiment except that ISA degradation occurred at a slower rate compared to the NaISA counterpart (Figure 4.37). The first order mean rate was $7.0 \times 10^{-1} \pm 0.00\ d^{-1}$. The ISA removal occurred sharply between day 8 and day 10 after which it appeared to level off between day 10 and day 14. This corresponded to the stationary-phase of the bacterial growth (Figure 4.37B). By the end of the sampling period, 0.8 mM $Ca(ISA)_2$ representing 20% of the original concentration, could still be detected in the medium. A similar incomplete degradation of ISA from $Ca(ISA)_2$ was observed in the study by Kuippers *et al*. [74] where 18% of the original substrate remained undegraded under nitrate reducing condition. The authors attributed the incomplete degradation of ISA to nitrite toxicity but the present study contained no added nitrate in the medium which means that further investigation is required to look into the incomplete degradation of $Ca(ISA)_2$. The degradation of ISA coincided with acetate accumulation (2.57 mM) representing a yield of 80% but 76% of the theoretical. Approximately 1000 fold increase in cell biomass was observed which suggests that part of the carbon from the substrate was converted into biomass (4.37B). A pH drop of 1.8 (Figure 4.37C) suggests acidification growth medium similar to that which occurred in the NaISA experiment.



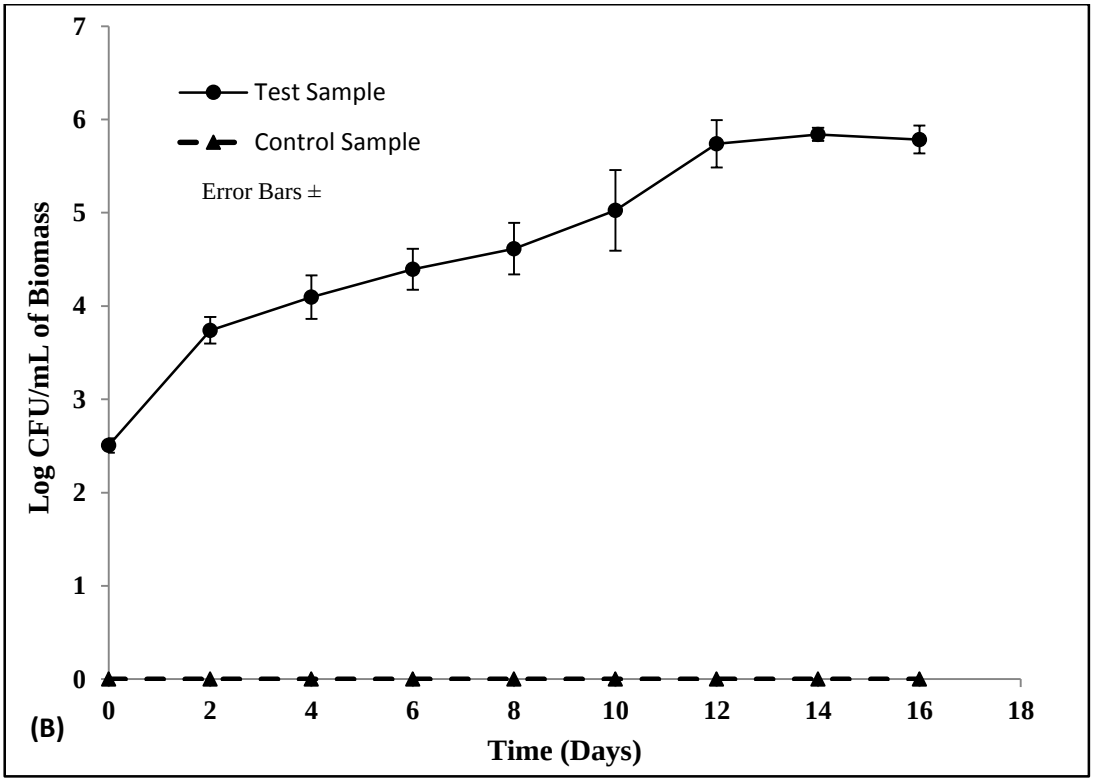


Figure 4.37 *Exiguobacterium* sp. strain Hud degradation of ISA from $Ca(ISA)_2$

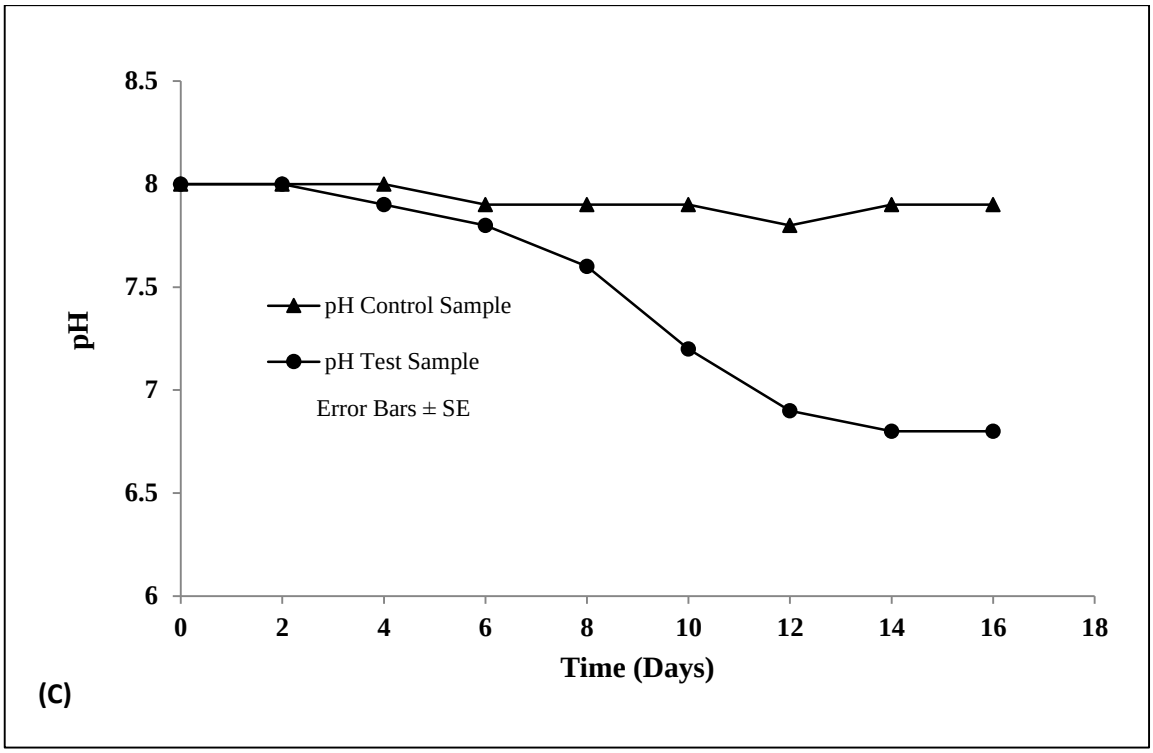


Figure 4.37 continues: ISA degradation in minimal media supplemented with vitamin and trace elements solution at pH 8.0 showing (A) α-ISA removal and acetate accumulation, (B) microbial growth and (C) drop in pH against time

The batch fermentation experiment in minimal media supplemented with vitamin solution was repeated for pH9.0 whereby NaISA, $Ca(ISA)_2$ and CDP were added as carbon substrates. However, due to an initial slower microbial activity, the incubation time was extended to 45 days instead of the 20 days for the pH8.0 system. Across the three systems, ISA degradation was observed from the beginning to the end of the sampling period. For instance, in the culture fed with NaISA, ISA degradation was faster between day 15 and day 35 before a complete degradation occurred on day 40 (Figure 4.38). The overall first order mean rate of degradation was $2.9 \times 10^{-1} \pm d^{-1}$. The degradation of the ISA was accompanied by the generation and accumulation of acetate (2.86 mM) however, in this experiment, the concentration of acetate could not be detected until day 10. This may be due to low amount of ISA that had degraded at that time. In total, the

percentage acetic acid yield was 72%, which is 19% lower than its counterpart in pH 8.0. However, this yield is only 35% of the theoretical indicating that most of the carbon substrate went into biomass generation which was up by almost 1000 fold (Figure 4.38). The pH of the growth medium dropped from 9.0 to a final pH of 7.7 (Figure 4.38A) suggesting acidification of the media.

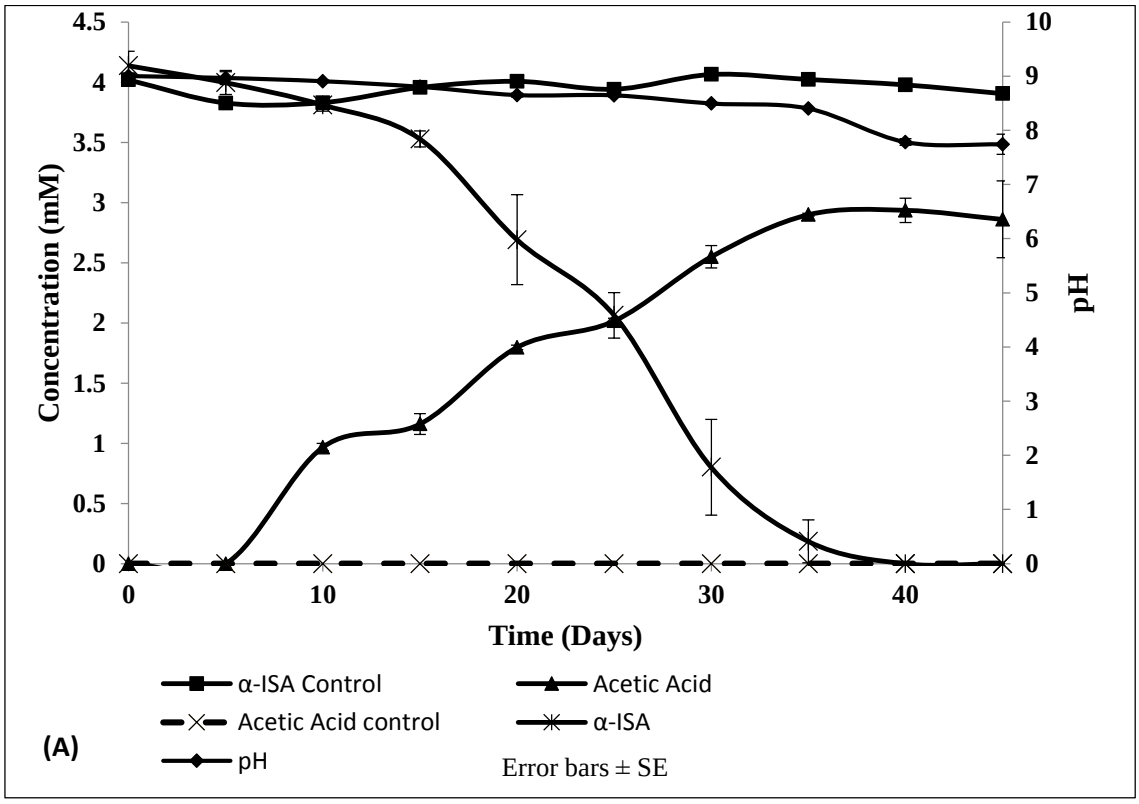


Figure 4.38 *Exiguobacterium* sp. strain Hud degradation of ISA from NaISA

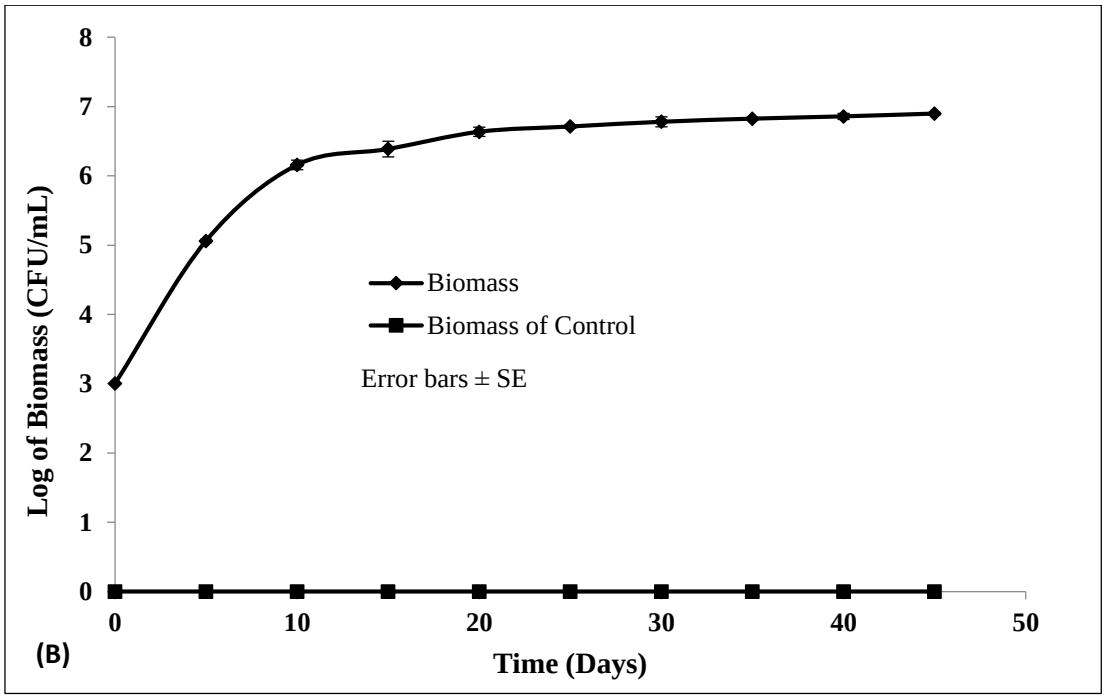


Figure 4.38 continues: ISA degradation in minimal media supplemented with vitamin and trace elements solution at pH 9.0 showing (A) ISA removal, acetate accumulation and drop in pH and (B) microbial growth curve

A similar degradation profile occurred in the system driven by $Ca(ISA)_2$ where the first order mean rate of degradation was 2.6 x 10^{-1} ± 0.00 d^{-1}. A striking difference between the NaISA and $Ca(ISA)_2$ was observed between day 25 and day 40 where ISA appeared to be degraded sharply in the $Ca(ISA)_2$ driven system (Figure 4.39A). It took 5 additional days for complete degradation of ISA to occur in the $Ca(ISA)_2$ cultures compared to its NaISA counterparts. The percentage acetic acid yield, which accompanied ISA removal, was 92%, which is 28% in in yield compared to its pH 8.0 counterpart. This yield in pH 9.0 is however, 45% of the theoretical yield. The increase in turbidity of the medium due to an increased biomass of almost 1000 fold (Figure 4.39B) suggests utilization of the carbon source for biomass generation. The biomass generation in the pH 9.0 system (4.39B) was lower than that of the pH 8.0, which may be due to the ambient pH imposing stress on the system. Acidification of the medium by acid products was evident by the pH drop from the initial of 9.0 to a final 7.3 (Figure 4.39A).

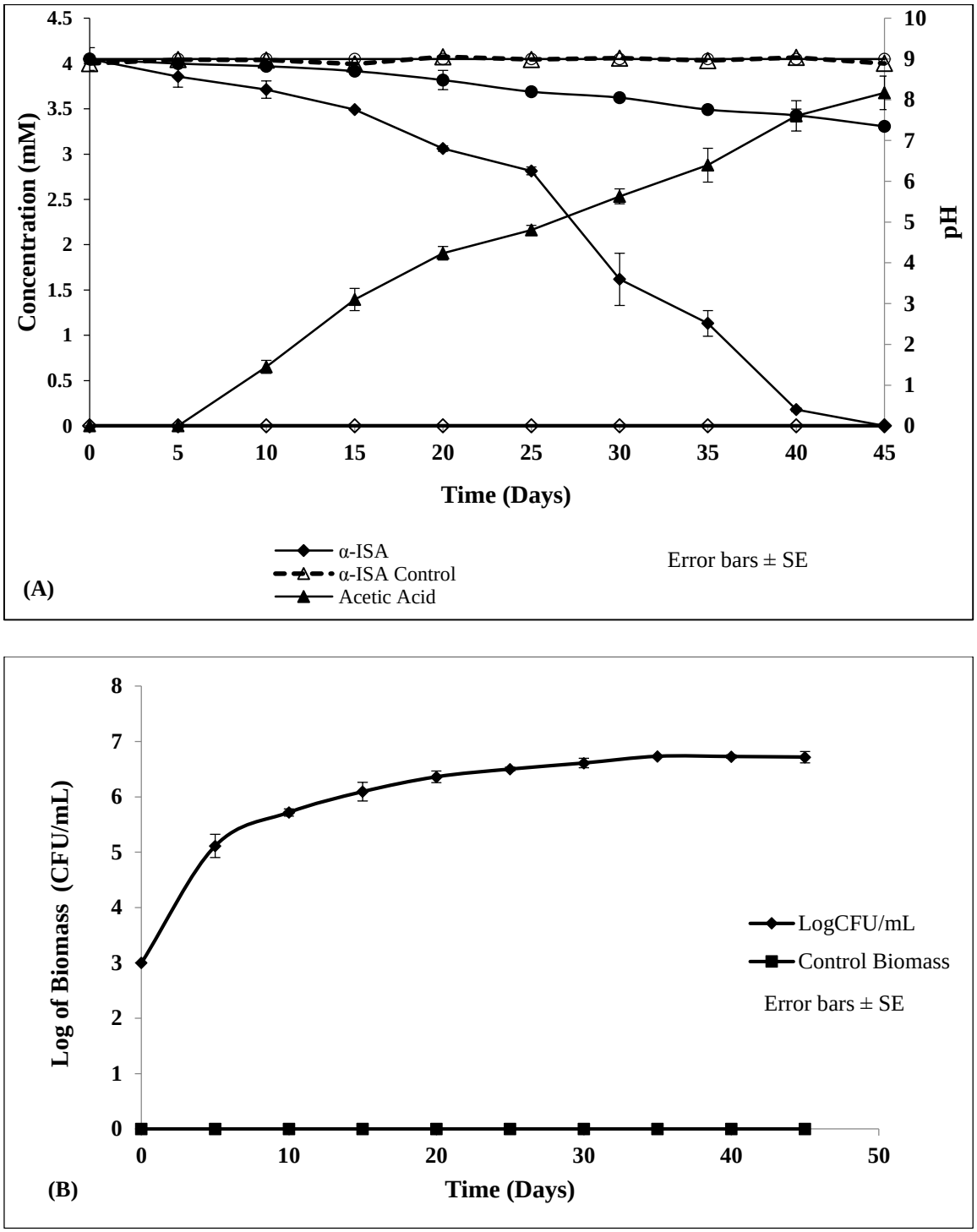


Figure 4.39 *E.* sp. strain Hud degradation of ISA from $Ca(ISA)_2$

ISA degradation in minimal media supplemented with vitamin and trace elements solution at pH 9.0 showing (A) ISA removal, acetate accumulation and drop in pH and (B) Biomass

In the CDP-fed cultures, degradation of both α- and β-ISA occurred concurrently however, within 25 days of incubation, β-ISA was completed degraded (Figure

4.40A) at first order mean rate of 1.3 x 10^{-1} ± 0.00 d^{-1}. By the end of sampling, 0.08 mM (2%) of the initial Ca(α-ISA)$_2$ concentration could still be detected. The net acetate generation within the system was found to be 1.62 mM, which represented a percentage yield of 42.52% of the initial carbon source but 21.25% of the theoretical value (Figure 4.40A). Cell biomass was found to have also increased almost 2.5-folds (Figure 4.40B). The pH dropped from initial pH of 9.0 to a final of 7.1.

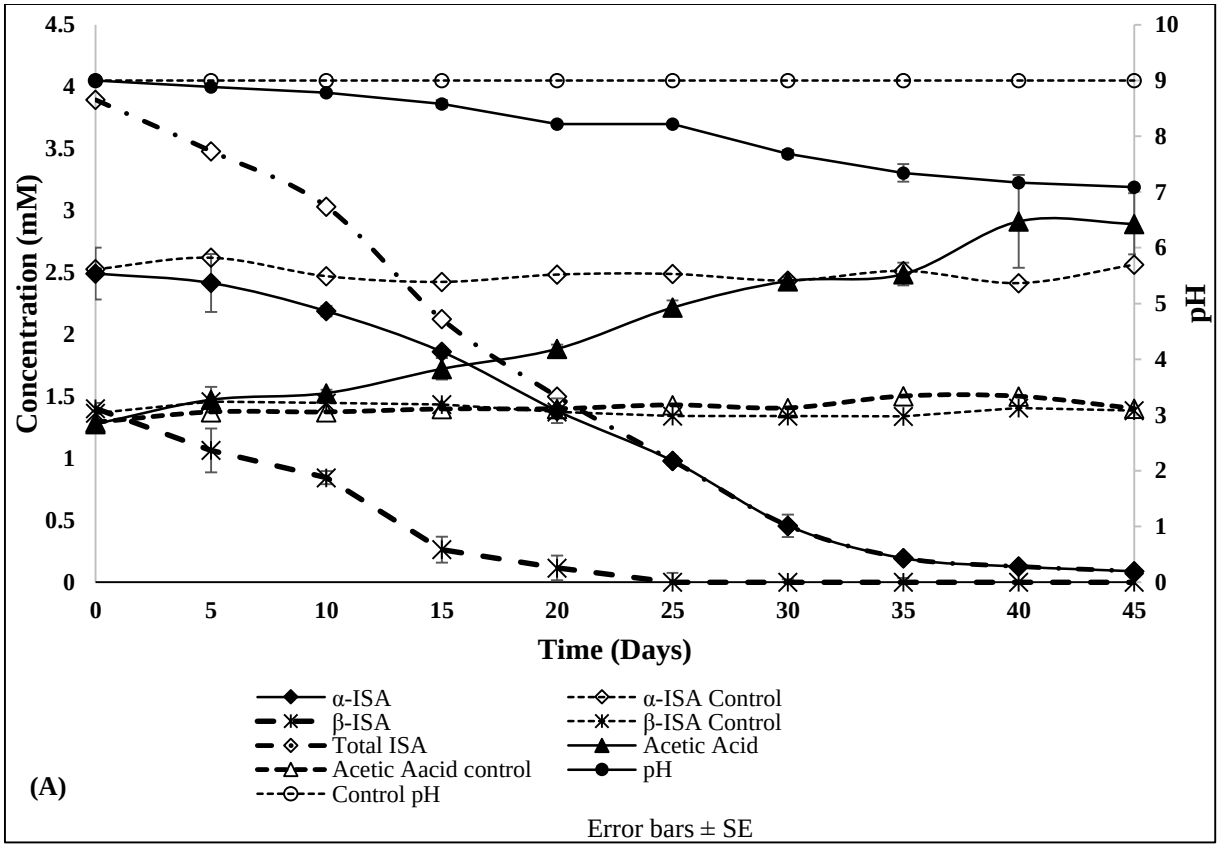


Figure 4.40 *Exigubacterium* sp. strain Hud degradation of ISA from CDP

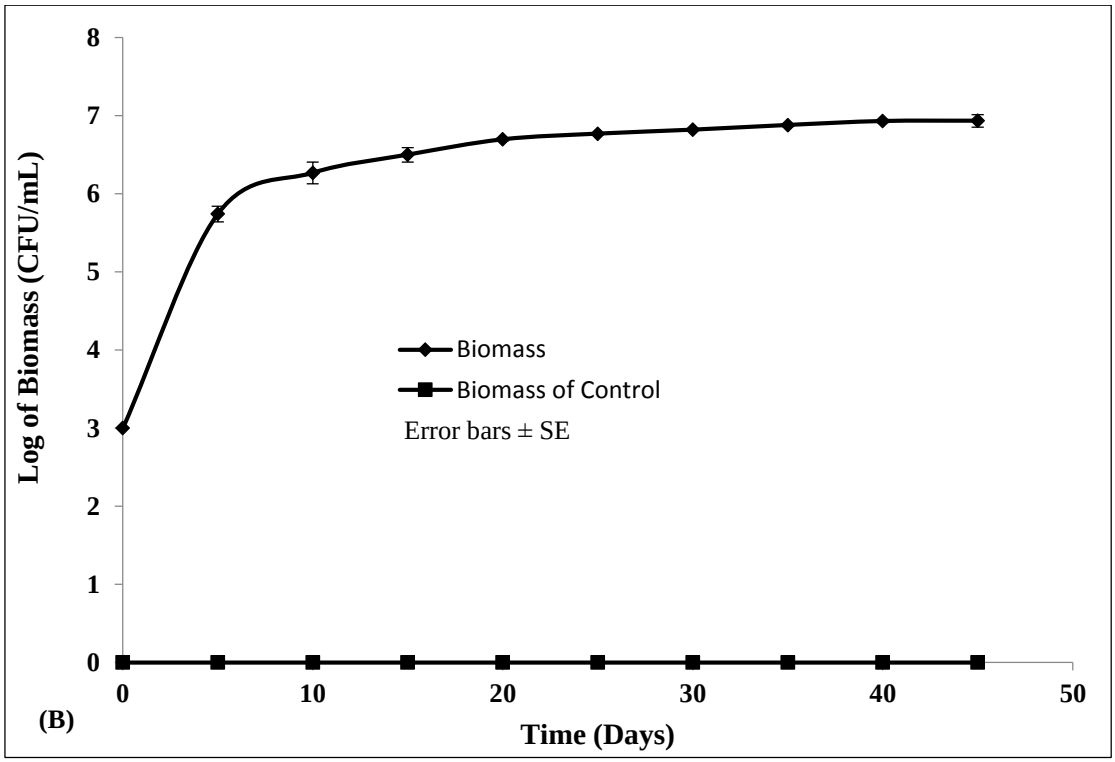


Figure 4.40 continues: ISA degradation in minimal media supplemented with vitamin and trace elements solution at pH 9.0 showing (A) ISA removal, acetate accumulation and drop in pH and (B) microbial biomass

ISA degradation profiles involving pure cultures under alkaliphilic anaerobic conditions relevant to the GDF are unavailable in literature. The results presented in this study show for the first time that *Exiguobacterium* sp. Strain Hud is capable of biodegradation of NaISA, $Ca(ISA)_2$ and both the α and β forms of ISA present in CDP. This suggest that irrespective of the source, microorganisms are capable of ISA biodegradation. The outcome of this body of work agree with earlier assertion by Greenfield *et al*. [59], that although ISA is not commonly encountered in the natural environment there is no reason for it to resist microbial degradation. ISA degradation with the strain however, did not occur either in mineral media or in Horikoshi (I) media which was either supplemented with or without yeast extract. It is possible that the mineral media and the modified Horikoshi (I) contained chemicals that were inhibitory to the growth of the strain, or rather did not have the correct proportions of chemicals or growth factors that

could ‘trigger’ those proteins required for the production of appropriate enzymes for ISA metabolism. On a favourable note, microbial growth and degradation involving the strain could occur in minimal media supplemented with vitamins which suggests that the metabolism of ISA probably requires some special microbial growth factors or it may be in support of the claim by Strand *et al*. [54] that the degradation of ISA requires some unusual or modified enzymes than those ordinarily required for other carbohydrate substrates.

Rout *et al*. [324] noted in their draft genome sequencing of this strain that the strain possesses genes that encode proteins involved in glycolysis and tricarboxylic acid cycle (TCA). These important findings suggest that the biochemical pathways involved in the metabolism of ISA by this strain is similar to the key biochemical pathways described by Schroder *et al*. [331] whereby *Thermotoga maritima* was shown to biodegrade glucose to synthesise acetate with the evolution of both carbon dioxide and hydrogen gas. Although the yields fell short of the theoretical, the stoichiometries that relate to the biodegradation of the ISA substrate in the different systems are shown below:

pH8.0 systems

NaISA-driven system

$4C_6H_{12}O_6 \longrightarrow 3.6CH_3COOH + XCO_2 + XH_2$

$Ca(ISA)_2$-driven system

$4C_6H_{12}O_6 \longrightarrow 2.5CH_3COOH + XCO_2 + XH_2$

pH9.0 systems

NaISA-driven system

$4C_6H_{12}O_6 \longrightarrow 2.8CH_3COOH + XCO_2 + XH_2$

$Ca(ISA)_2$-driven system

$4C_6H_{12}O_6 \longrightarrow 3.6CH_3COOH + XCO_2 + XH_2$

CDP-driven system

$$4C_6H_{12}O_6 \longrightarrow 1.6CH_3COOH + XCO_2 + XH_2$$

(Note: the amounts CO_2 and H_2 were not determined in these studies due to experimental limitations. Hence, their stoichiometric coefficients are represented by X)

4.5.7. Biochemical Characterisation of *Exiguobacterium* sp. strain Hud

A wide range of biochemical analysis were employed to characterise *Exiguobacterium* sp. strain Hud. In order to profile the metabolic capabilities of the strain, Biolog Ecoplate, Phenotypic Microarray (PM1 and PM2, Biolog Inc. Hayward, USA), Analytical Profiling Indext (API, bioMérieux) 20A test strips and traditional biochemical tests were used. The organic substrates utilised by the strain are listed in Table 4.3.

Table 4.3 Biochemical profile of *Exiguobacterium* sp. strain Hud

API 20A	Biolog Ecoplate/PM 1 & 2 Plates		
D-glucose	Pyruvic acid methyl ester	Dulcitol	Adonitol
D-mannose	B – methyl-D-glucoside	Uridine	Maltotriose
D-lactose	1,2-propanediol	Glycerol	2-deoxyadenosine
D-saccharose	A – cyclodextrin	D-fructose	Inosine
D-maltose	N-acetyl-D-glucosamine	L-lactic acid	Glyceryl-L-glutamic acid
Salicin	A-hydroxybutyric acid	D-manitol	L-alanyl-glycine
Xylose	D-gluconic acid	D-melibiose	Acetoacetic acid
L-arabinose	α-Ketobutyric acid Dihydroxyacetone	D-ribose	N-acetyl-β-D-mannosamine
Glycerol	Putrescine	L-proline	Glyceryl-L-proline
D-cellubiose	N-acetyl-D-glucosamine	Acetic acid	L-lyxose
D-mannose	Tween 20, 40 and 80	Glycogen	Chondroitin sulphate
D-melizitose	L-aspartic acid	Tween 40	α-,β- and ɣ-cyclodextrin
D-raffinose	D-glucosaminic acid	Lactulose	Dextrin
D-sorbitol	B-methyl-D-glucoside	Sucrose	Gelatin
L-rhamnose	L-glutamic acid	D-galactose	Laminarin
D-trehalose	Thymidine	D-alanine	Mannan
	Pectin	Gentiobiose	Turanose
	Amygdalin	Palatinose	α-ketovaleric acid
	A-&β-methyl-D-glucoside	Salicin	D-L-octopamine

A table showing substrates that could be degraded by *Exiguobacterium* sp. strain Hud using API 20A and Biolog PM1 and PM2

4.5.8. Extraction and identification of Fatty acid methyl esters (FAME) of *Exiguobacterium* sp. strain Hud

The objective of this part of the research was to isolate and characterize the membrane fatty acid component of *Exiguobacterium* sp. strain Hud and to compare with those obtained from four other selected species of the same genus.

The FAME results presented in Table 4.4 shows FAMEs percentage composition of the five species of the genus, *Exiguobacterium* from which extraction have been made. Only FAMEs of 12 or more carbon chains and their percentage compositions greater than 1% are shown and percentages greater than 10% are shown in bold. While $iC_{12:0}$ appeared as the most abundant FAME in three species namely *Exiguobacterium* sp. strain Hud, *Exiguobacterium undae* and *Exiguobacterium alkaliphilum*, $aiC_{12:0}$ and was the most abundant in *Exiguobacterium aurantiacum* and *Exiguobacterium oxidotolerans*. While individual fatty acids of the original environmental type species of *Exiguobacterium aurantiacum* isolated by Collins *et al*. [302] was not determined the major fatty acids obtained for the species isolated from blood cultures of patients were found to be isoC13:0, anteisoC13:0, C16:0, isoC17:0, and C18:0 [323]. In the case of *Exiguobacterium undae* (type strain $L2^T$) isolated from surface water of garden pond, the major fatty acids included $iC_{13:0}$, $aiC_{13:0}$, $iC_{15:0}$, $C_{16:1\omega11c}$, $C_{16:1\omega7c}$, $C_{16:0}$ and $C_{17:0}$ [332]. Similarly, the fatty acids of *Exiguobacterium alkaliphilum* (type strain $12/1^T$) isolated from alkaline wastewater drained sludge by Kulshreshtha *et al*. [333] included iso-$C_{13:0}$, anteiso-$C_{13:0}$, iso-$C_{15:0}$, iso-$C_{16:0}$, iso-$C_{17:0}$ and anteiso-$C_{17:0}$. Yumoto *et al*. [310] also characterized the fatty acids of *Exiguobacterium oxidotolerans* (type strain $T\text{-}2\text{-}2^T$) isolated from drain of fish processing plant as iso-$C_{13:0}$, anteiso-$C_{13:0}$, iso-$C_{15:0}$, iso-$C_{16:0}$, iso-$C_{17:0}$, anteiso-$C_{17:0}$ and iso-$C_{17:1}$.

Table 4.4 Fatty acid percentage composition of *Exiguobacterium* species.

Fatty acid	*E.* strain Hud	*E. aurantiacum*	*E. undae*	*E. alkaliphilum*	*E. oxidotolerans*
$C_{12:0}$	2.3	1.5	1.6	**22.4**	
$iC_{12:0}$	**32.4**	**12.9**	**15.2**	**23.8**	
$aiC_{12:0}$		**16.6**			**20.5**
$C_{13:0}$	**14.3**		9.6		**13.7**
$iC_{13:0}$					3.2
$C_{14:0}$			1.7		
$iC_{14:0}$					24
$aiC_{14:0}$	3.2	3.4	19.7	4.9	
$C_{15:0}$					
$C_{16:0}$			1.5		1.1
$iC_{16:0}$					**10.1**
$aiC_{16:0}$			2.2		1.5
$C_{17:0}$	7.2			2.8	
$iC_{17:0}$			11.2	1.7	
$C_{17:1\ (9)}$				4.3	4.3
$C_{18:0}$			5.5		
$C_{18:1\ (9)}$		3.2			

Only percentage compositions greater than 1% are shown. Percentages greater than 10% are shown in bold

The results of the present study and earlier findings from literature show that the longest carbon chain fatty acid obtained for all five species of the genus was C18 (isomers of octadecanoic acid) which can undergo changes in chain lengths in the event of temperature changes [153]. This biochemical property may play a role in the ability of members of this genus to survive and grow in extremely cold or hot environments. The propensity with which bacteria are able to control their membrane phospholipid biophysical properties allows them to thrive in extremes of physical environment [153]. In order to achieve fatty acid homeostasis, microorganisms precisely adjust the composition of the phospholipids within their membranes by modifying the type and alter the structure of pre-existing ones [334]. This biochemical survival strategy could play a role in enhancing the chances of survival of this strain in the radioactive waste repository.

4.5.9. Extraction and identification of isoprenoid quinones of *Exiguobacterium* sp. strain Hud

Isoprenoids (terpenoid quinones/menaquinone) are membrane bound compounds in almost all living organisms. Currently, the only known microorganisms that do not have isoprenoid quinones are some obligatory fermentative bacteria that lost the ability to biosynthesise them [229], parasitic *Rickettsia* and *Mycoplasma* bacteria that lack the both MEP and MVA biosynthetic pathways [335] and some methanogenic Archaea of the order Methanosarcinales [336]. Isoprenoids consists of a polar head group and a hydrophobic side chain which provides the lipid soluble property and anchors the molecule within the lipid bilayer of the microbial cell membrane [337].

Microbial isoprenoids are categorised into two major groups on the basis of structural differences. These are naphthoquinones and benzoquinones. The benzoquinones consists of menaquinones (MK; vitamin K_2) and ubiquinones (Coenzyme Q) [338]. Menaquinones (Figure 4.41) are the most ancient and abundant isoprenoids found in bacteria [339].

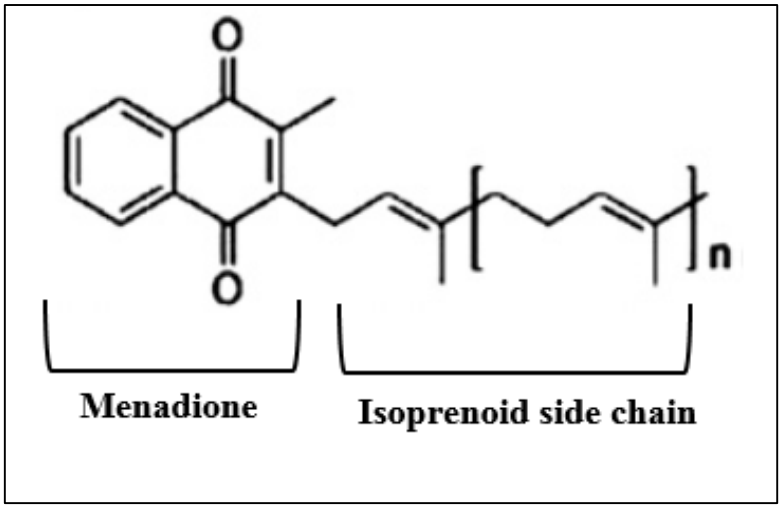


Figure 4.41 Structure of menaquinone (MK)

The structure shows menadione (2-methyl-1,4-naphthoquinone) ring and the isoprenoid side chain

In microorganisms, isoprenoids are synthesised via two distinct biochemical pathways known as mevalonate (MVA) and Methylerythritol 4-phosphate (MEP) pathway both of which are well elaborated elsewhere [340-342]. Generally, through the pathway used by lactic acid bacteria (LAB) menadione ring is synthesized from chorismate, which is derived from shikimate pathways through a series of enzymes-catalysed reactions. The enzymes are coded by the *men* genes. The isoprenoid side chain is biosynthesised separately but later joins onto the ring to form dimethyl menaquinone (DMK) which later methylate to form menaquinone (MK) [343]. However, according to Collins *et al*. [229], most species of microorganism lack the requisite methylase enzyme for the conversion of DMK to MK and as a result exclusively produce DMK. The structure of menaquinone can be altered through chemical modification of the menadione ring or the side chain depending on the microorganism involved and the growth conditions. A common modification results from demethylation of menadione ring which regenerates DMK or saturation of the unsaturated side chain [343]. Gram-positive bacteria are generally noted for the production of MK whereas Gram-negative bacteria produce MK, DMK and ubiquinones [229]. The key function of MKs in microorganisms is their role in respiratory electron transport chain where they transport electrons in the cytoplasmic membrane [344]. Furthermore, the reduced form of MK (DMK) have been found to exhibit antioxidant properties and is implicated in protection of cellular membrane against lipid oxidation [341]. They are also noted to be involved in active transport of molecules across the cell membranes [345]. In *Bacillus subtilis*, MKs have been noted to play a role in sporulation [346]. Even though composition of quinone within the cell membrane of a group of bacteria may vary occasionally, depending on culture conditions, the predominant quinone type remains generally unchanged among single species. As a result, MKs are used as biochemical markers for the identification of microorganisms.

The aim of this part of the study was to characterize *Exiguobacterium* sp. strain Hud on the basis of the major MKs. MKs were extracted from the isolates and characterized as described in the method section.

The identification of menaquinone was carried out against MK-7 standard (Sigma-Aldrich, UK) using thin layer chromatography (TLC) (see methods section). The result of TLC is presented in Figure 4.42 The solvent, petroleum ether (b.p 60-80°C)/diethyl ether (85:15, v/v) was capable of eluting the standard MK-7 wholly leaving no spot at the baseline which suggests that the standard MK-7 was neat and soluble in the solvent. Conversely, dark spots were left on the baseline of the five samples indicating presence of insoluble constituents. All samples from the five species of the genus *Exiguobacterium* contained MK-7 in addition to other components which could not be identified because they were not present in the standard. The presence of MK-7 in all samples is consistent with earlier findings where this respiratory quinone was detected as the major isoprenoid component of the microbial cytoplamic membrane in addition to minor components including MK-6, MK-5 (only for *E. undae*) and MK-8 [310, 332, 333, 347]. The detection of MK-7 has also been reported as a major menaquinone in many other Gram-positive bacteria including *Bacillus, Kurthia* and *Staphylococcus* [229].

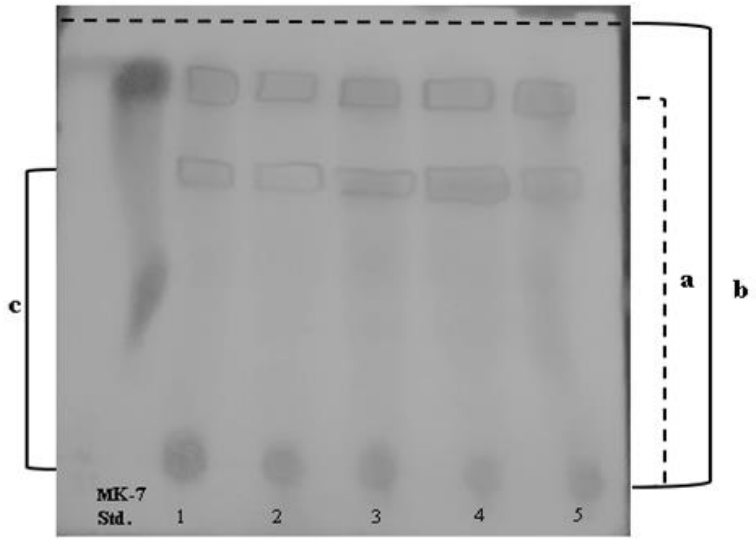


Figure 4.42 UV irradiated *Exiguobacterium* spp menaquinone samples on chromatographic plate

The diagram shows chromatograms for MK-7 std. = standard MK-7; 1 = *Exiguobacterium* strain Hud; 2 = *Exiguobacterium aurantiacum*, 3 = *Exiguobacterium undae*; 4 = *Exigobacterium alkaliphilum*; 5 = *Exiguobacterium oxidotolerans*. a = distance moved by sample; b = solvent front; c = distance moved by unknown sample

The R_F values MK-7 and the unidentified MK were approximately 0.84 and 0.64 respectively.

Due to the presence of unknown MKs, further analysis of the respiratory quinones for *Exiguobacterium* strain Hud was carried out by the Identification Service, Leibniz-Institut (DSMZ) – Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany. The outcome shows that MK-7 is the major menaquinone (85%), followed by MK-9 (9%) and MK-6 (6%).

4.5.10. *Exiguobacterium* sp. strain Hud tolerance to pH

The tolerance of the strain to a wide range of pH (pH 4 – pH 12) was assessed by growth characteristic in a Bioscreen-C automated growth curve analyser following overnight incubation at 25°C in iso-sensitest broth (Oxoid). The growth curves are shown in Figure 4.43.

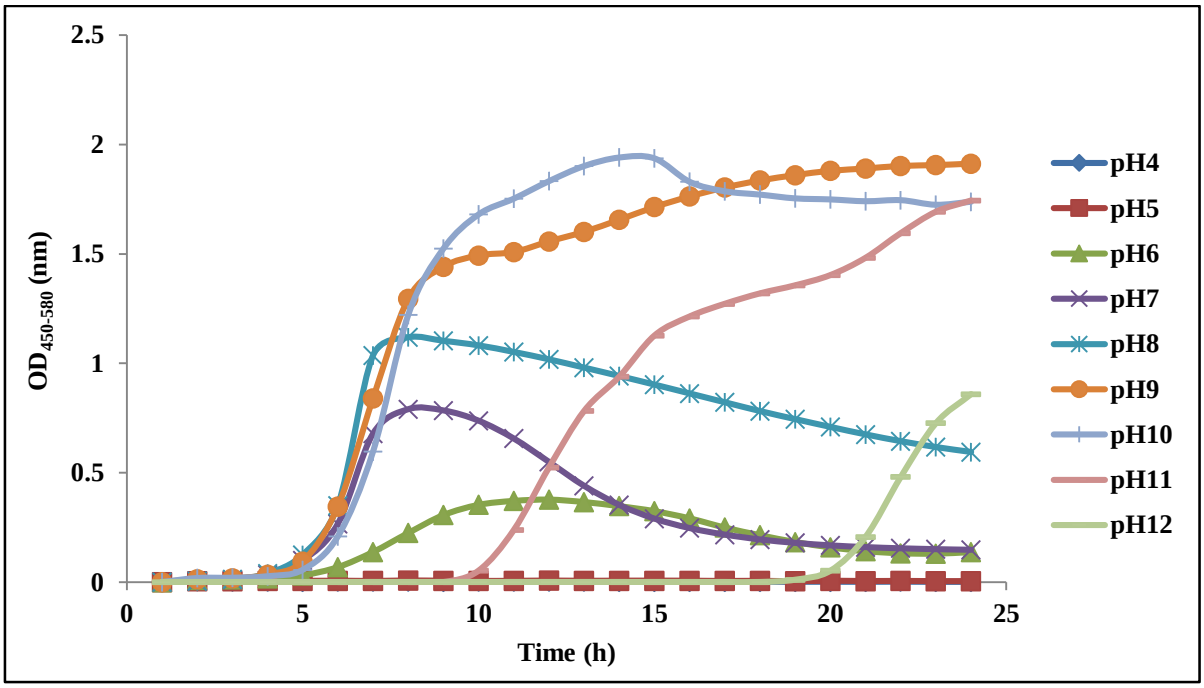


Figure 4.43 Growth curves showing the tolerance of *Exiguobacterium* sp. strain Hud towards pH

No growth was observed in pH 4.0 suggesting the inability of the strain to function in very low acidic conditions. Growth was possible at pH 6.0 and at neutral pH however, the exponential phase was quite short while stationary phase progressed across the period suggesting poor growth. The strain appeared to survive better within a pH range of 9.0 -11.0. Growth at pH 11.0 and 12.0, which followed a lag phase of 10 and 20 hours respectively, is quite striking, suggesting ability of the strain to adapt and grow at pH values relevant to the GDF. This is consistent with

earlier studies where other species have been found to grow at higher pH. In order to estimate the optimum pH for growth, the absorbance (OD values) were expressed as a function of pH and plotted against pH to indicate the optimum pH (Figure 4.44).

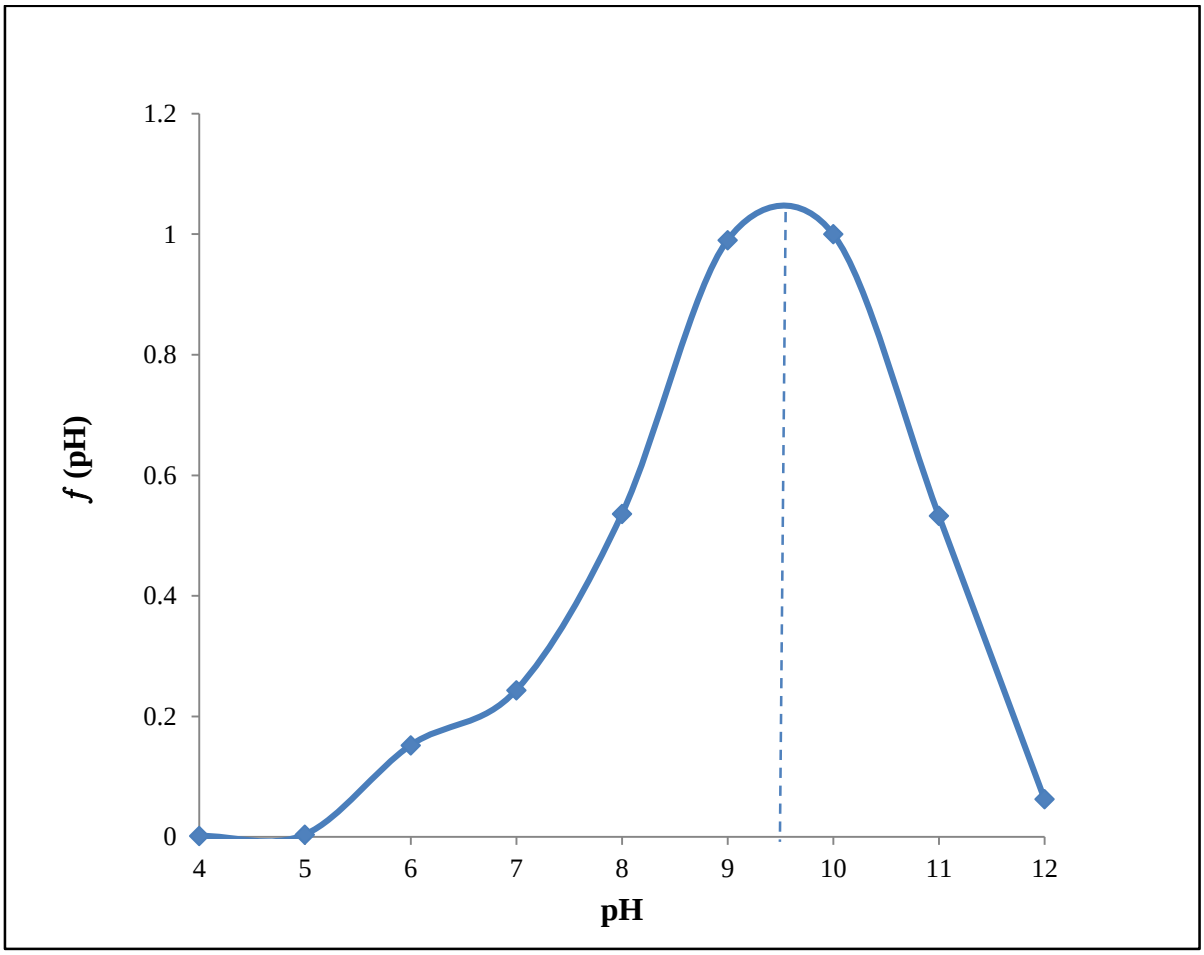


Figure 4.44 A pH profiling curve showing the optimum pH for growth of *Exiguobacterium* sp. strain Hud

4.5.11. *Exiguobacterium* sp. strain Hud tolerance to heavy metals

The tolerance of the strain to heavy metals ions including Zn^{2+}, Co^{2+}, Cu^{2+}, Ni^{2+}, Cd^{2+} and Pb^{2+} was assessed based on growth response to different concentrations of the metals between 0.5 to 5 mM. Growth was assessed as a measure of absorbance at 450-580 nm for 18-hour incubation in the bioscreen-C microbiological Growth Analyser (Labsystems, Helsinki, Finland) under constant shaking at 25°C. The minimum inhibitory concentration (MIC) and the No inhibitory Concentration (NIC) for each metal was then calculated as outlined in the methods section. Across the inhibition profiles for all the metals, no growth was observed at concentrations above 3 mM reflecting on the toxicity of the metals to the strain at those concentrations. The individual analysis can be found in Appendix with the MIC and NIC for the six metal ions used against *Exiguobacterium* sp. strain Hud shown in Table 4.5.

Table 4.5 The MIC and NICs of heavy metals against *Exiguobacterium* sp. strain Hud

Metal	MIC (mM)	NIC (mM)
Zn^{2+}	2.1	1.42
Ni^{2+}	1.92	1.18
Cu^{2+}	5.10	1.82
Co^{2+}	3.01	1.02
Cd^{2+}	19.96	5.92
Pb^{2+}	undetermined	undetermined

This method could not be used to determine the tolerance of the strain to lead (Pb^{2+}) because increasing the concentration of the lead generated precipitation of the media within the wells. This increased the opacity of the media especially at higher concentration which gave a false optical density (OD) readings at the wavelength used. In addition, although MIC and NIC values could be calculated for Cobalt the shape of the curve requires further investigation.

Heavy metal ions such as Zn^{2+}, Ni^{2+}, Cu^{2+}, Co^{2+}, Cd^{2+}, and Pb^{2+} are common environmental pollutants usually in areas with high anthropogenic pressure and their presence in the atmosphere, soil or water pose serious problems all life forms [348]. The main sources of these metals in the soil include erosion, natural weathering of earth crust, leaching, mining activities and industrial effluents [349]. In view of the presence of ions of these metals in the GDF the ability of any ISA-degrading microorganism to tolerate these metal ions is important.

The results presented here show the tolerance of *Exiguobacterium* sp. strain Hud to six common environmental metallic pollutants which generate toxic effects in living organisms. Consistent with the findings of Karami *et al*. [319] and Anderson *et al*. [320] where members of the Genus *Exigubacterium* showed ability to tolerate or remove these heavy metals, this study has shown the resistance capacity of the strain to Zn, Ni, Cu, Co and Cd. The tolerance of the strain to Pb^{2+} however, could not be determined within the limits of the experimental parameters. Future study should therefore look into the use of classical method such as broth/agar dilution or the method described by Mann and Markham [350] in which redox dye resazurin was used to determine the MIC of oil-based compounds in lead tolerance for this strain.

The MICs and NICs of the metals were determined for the strain and between these two principal concentrations, microbial growth inhibition was observed. This inhibition was attributed to toxification of the microbial system which slowed down growth in a dose dependent manner [351]. Moreover, at higher

levels of concentration for a specific microorganism, heavy metal ions form unspecific complex compounds within the intracellular milieu and produces toxic effects which affect various physiological processes that culminate into malfunctioning of heavy metal homeostasis system within the cell [352].

The NIC was defined as the concentration above which the inhibitory effect of the metal ions begins to impact on the growth of microorganism and below which microbial growth proceeds exponentially like the control experiment. The MIC on the other hand was defined as the lowest concentration of the metal ion above which no growth relative to the control occurred [351].

In this study however, three of the metals examined including Zn, Ni and Cu, the relationship between FA and metal concentration is linear which indicates a single mechanism of inhibition due to the metal. In the cobalt experiment however, it appears that a complex inhibition mechanism is involved where growth was inhibited even below the MIC. Further investigation is therefore required to analyse the mechanisms involved in cobalt inhibition against this microorganism. In line with earlier findings, this study further reiterates that some members of the genus, *Exiguobacterium* are tolerant to heavy metals and that *Exiguobacterium* sp. strain Hud could be employed for bioremediation [306, 318].

4.5.12. *Exiguobacterium* sp. strain Hud tolerance to salt (NaCl)

The use of salt formations for the disposal of nuclear waste has been embraced for decades especially in the United States where the concept has been successfully demonstrated for over ten years at the operation site at Waste Isolation Pilot Plant (WIPP) near Carlsbad, New Mexico [353]. In the UK, evaporates which includes anhydrite ($CaSO_4$ - anhydrous), and halite (rock salt - NaCl) have been considered among other host rocks for geological disposal [354]. Generally, for repository considerations, domal and bedded formations of salt (sodium chloride) interspersed with anhydrite, shale, dolomite and other salts such

as potassium chloride are preferred [353]. Against this background, it is necessary to assess how microorganisms especially those with ISA degrading potentials respond to elevated levels of salinity. This is so because sodium chloride and its derivatives can be toxic to some bacteria which reflects their use in food industries as an antimicrobial and antibotulinal agents where they have been potent in preventing microbial growth. For instance it is incorporated as an additive to meat and meat products or brine solutions and also in sun-dried fish and meat [355] to prevent microbial contamination and spoilage [356-358].

In environments dominated by high pH and salt, microbial life is impacted in a number of ways. Microbial communities in such areas are affected by stresses imposed by the high alkaline environment which is often much higher than the internal pH of the microorganism. As a result, alkaliphilic microorganism must maintain a stable homeostasis for survival [359] and in addition, Krulwich *et al* [360] noted that they require a high energy drive for the biosynthesis of ATP while they have to retain water in the cell and maintain osmotic homeostasis. Alkaliphic microorganism can achieve this through the use of osmoregulatory or osmoprotectant compounds [360] where salt ions such as K^+ plays important role in the synthesis of counter ions such as glutamate [361]. Na^+ fluxes have also been implicated in pH homeostasis in marine microbiology [362]. In halophilic microorganisms, NaCl is used to regulate osmotic pressure between internal and external environment where it is noted to prevent swelling, deformation, bursting and cell lysis [363]. Lytic processes of halophiles are controlled by enzymes that are inhibited by Na^+ and this inhibition prevents the destruction of the mucopeptide portion of the cell walls of bacteria [364]. NaCl has been implicated in enhancing metabolic activity where increase in sodium chloride concentration in a growth medium that was used to culture moderately halophilic bacteria resulted in increased uptake of metabolites [363]. The increase in metabolic activity was linked to increase in enzymatic activities.

The aim of this section of study was to examine the tolerance of *Exiguobacterium* sp. strain Hud to different concentration of NaCl and to estimate the MIC and NIC of NaCl for the strain. The method used was the same for the tolerance of metal ions described in the previous section. The fractional areas plotted against the log concentrations of the NaCl are shown in Figure 4.45. Visible growth relative to the control was observed between 1% and 10%. Concentrations of NaCl above 10% were inhibitory to the strain. The MIC and NIC were calculated to be 9% and 4% respectively.

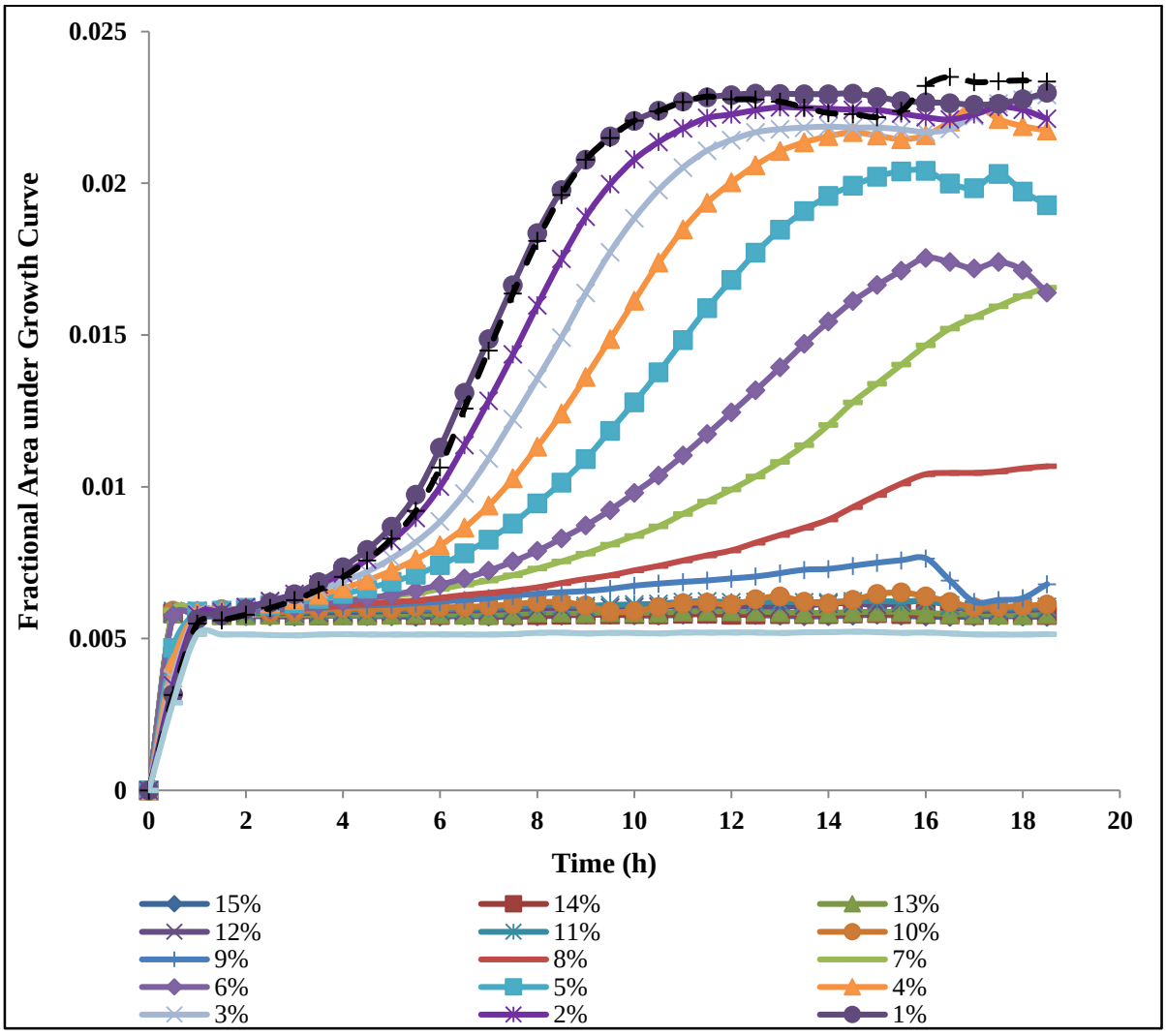


Figure 4.45 The tolerance of E*xiguobacterium* sp. strain Hud in a range of salt concentration

The ability of microorganisms to survive and grow in a range of salt concentration has received increased attention because of the prospective potentials of such microbes in bioremediation of pollutants in environments of fluctuating salinity [365]. Some of those microorganisms are noted for their essential role in the degradation of organic pollutants to generate alternative energy source [366].

In this body of research, the tolerance of *Exiguobacterium* strain Hud to a range of salt concentration has been investigated where for the first time, the MIC and NIC of salt for the strain has been estimated. Consistent with the studies of Kulshreshtha, *et al*. [333], Yumoto *et al*. [367] and many others where members of the genus *Exiguobacterium* were found to be tolerant to salt.

4.5.13. Combined effects of pH and Temperature on the growth of *Exiguobacterium* sp. strain Hud

Environmental factors such as pH, temperature, moisture, nutrient and oxygen have profound effect on the growth of bacteria. The GDF is expected to be dominated by some environmental factors, which will impose stress on microbial communities. Two of such factors are pH and temperature. Since these factors will occur concurrently and exert full synergistic effect on indigenous microflora, the combined effect of these two parameters on the growth of *Exiguobacterium* sp. strain Hud was investigated alongside four other species of the genus including *Exiguobacterium undae, Exiguobacterium aurantiacum, Exiguobacterium alkaliphilum* and *Exiguobacterium oxidotolerans* which were obtained from DSMZ.

The relationship between pH and microbial growth (transformed into fractional areas) across the different temperatures (10 – 45°C) is presented in a scatter the plot in Figure 4.46. Generally, pH and temperature produced a synergistic effect on the growth of the organism. It can be seen from the Figure 4.46 that no growth occurred at three different temperatures including 10°C, 15°C, 20°C and 45°C across the different range of pH used. At 25°C, growth lagged behind from pH 4

until around neutral pH where growth commenced and proceeded exponentially until maximum growth was reached at pH 9.0, attaining a mean FA of 1.34. Growth then declined sharply from pH 10.0 to pH 11.0 until no growth was observed at pH 12.0.

At 30°C, the combined effect of the two parameters was felt by the strain whereby growth lagged behind until pH 6.0 when exponential growth progressed across pH 7.0 and 8.0 until pH 9.0 where maximum growth was attained at FA value of 1.63. Growth deceleration began sharply at pH 10.0 and continued until pH 11 where minimal amount of growth (FA value of 0.01) was observed. No microbial growth occurred at this temperature at pH 12.0. It is striking that maximum growth of the strain occurred at 30°C over a wider range of pH (between pH 6.0 – 11.0).

At 35°C, stimulation of growth began at pH 5 where the onset of exponential growth increased until pH 9.0 where it reached its maximum (FA value of 1.52). Growth then declined from pH 10.0 to pH 12.0 where no growth was observed.

Growth at high temperature of 40°C was stimulated at pH 6 where exponential growth began and continued to pH 9.0. Between pH 9.0 and 10.0, the plot shows progress of growth inhibition up to pH 11.0 and 12.0 where no growth occurs.

Optimum growth for the strain within the limits imposed by the synergistic effect of the two parameters occurred at pH 9.0 across the various temperatures that supported growth. Temperature of 30°C was the most favourable temperature where 45°C, 20-10°C were unconducive for growth within the limits of this experiment. In order to show how growth responds to the effects of the two parameters a 3D plot was used Figure 4.47.

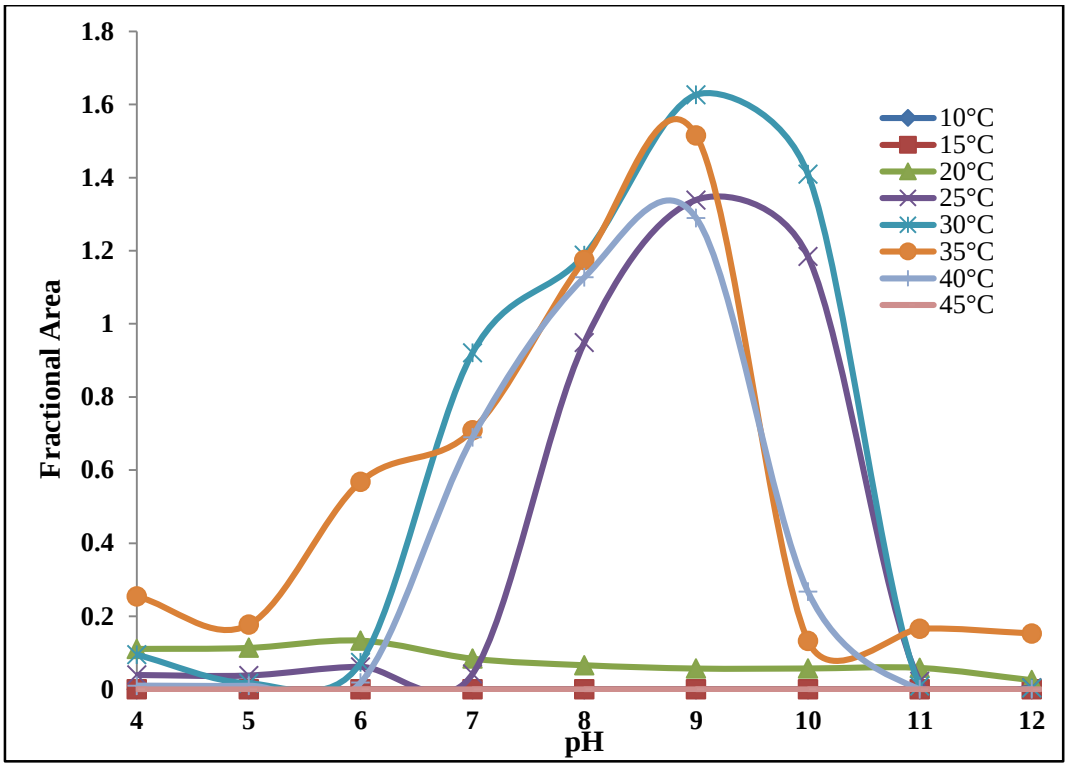


Figure 4.46 Effect pH on growth of *Exiguobacterium* sp. strain Hud across a wide temperature range.

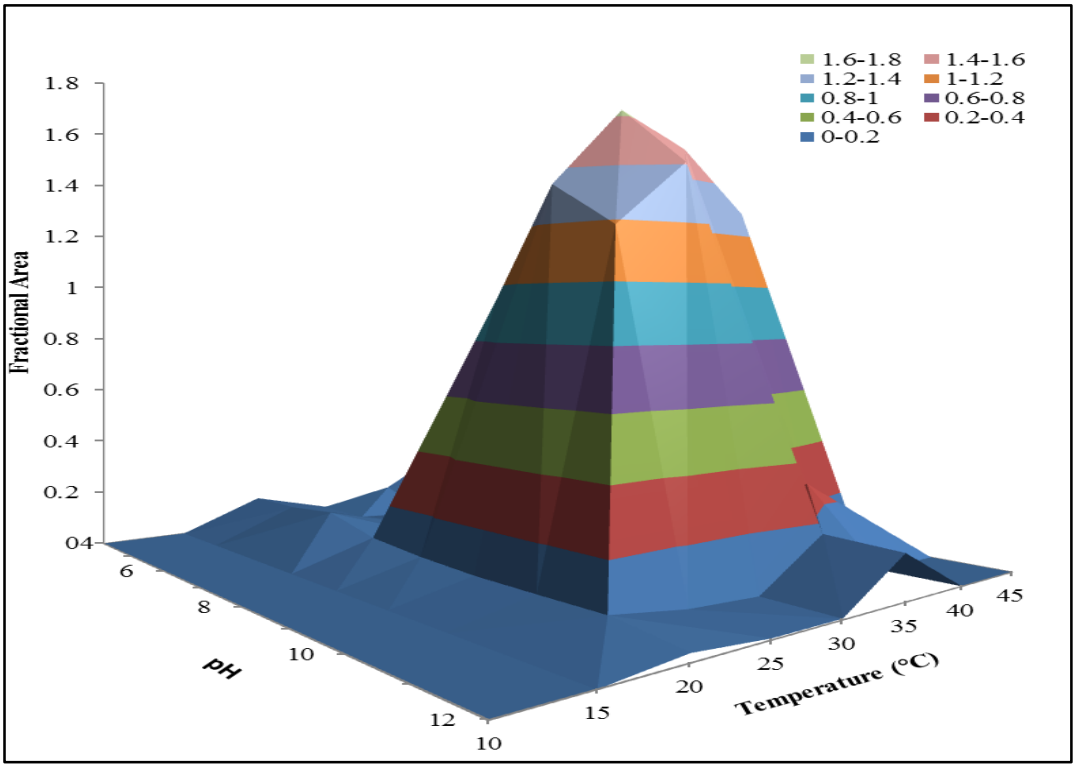


Figure 4.47 A 3D surface plot showing the relationship between pH and temperature

The 3D plot shows the synergistic effect of the two parameters on the growth of *Exiguobacterium* sp. strain Hud

Figure 4.47 shows the relationship between pH and temperature settings used to incubate *Exiguobacterium* sp. strain Hud and the growth response generated by the growth analyser for a period of 18 h. The different colour schemes represent growth while the continuous surface represents the response values of growth. The incubation at high temperature of 45°C in low pH (between 4 and 6) and high pH (between 10 and 12) inhibited growth (FA between 0.0 - 0.2) are indicated by dark blue colour at the base of the growth pyramid. The peak (FA 1.63) of the 3D surface plot which is highlighted by dark green colour corresponds to the highest growth recorded which occurred at 30°C in pH 9.0. The middle of the 3D growth pyramid, coded with the different colour schemes, indicates growth (FA between 0.4 – 1.6) at 25, 35 and 40°C in pH range of 6.0 and 11.0.

In the work involving *Exiguobacterium aurantiacum*, the highest growth ocurred at pH 9.0 at a temperature of 20°C (Figure 4.48). At 30°C, growth of the strain occurred over a wide range of pH between 5.0 and 11.0. This indicates the tolerance of the strain to a wide range of pH. It is quite striking to note that the strain was capable of growth even at pH 11.0 at 25°C, 30°C and 40°C. No growth however, occurred at 10°C and 15°C across the pH range. Similarly no growth occurred ocurred in pH values between 4 and 5 and 11 and 12 over the range of temperatures.

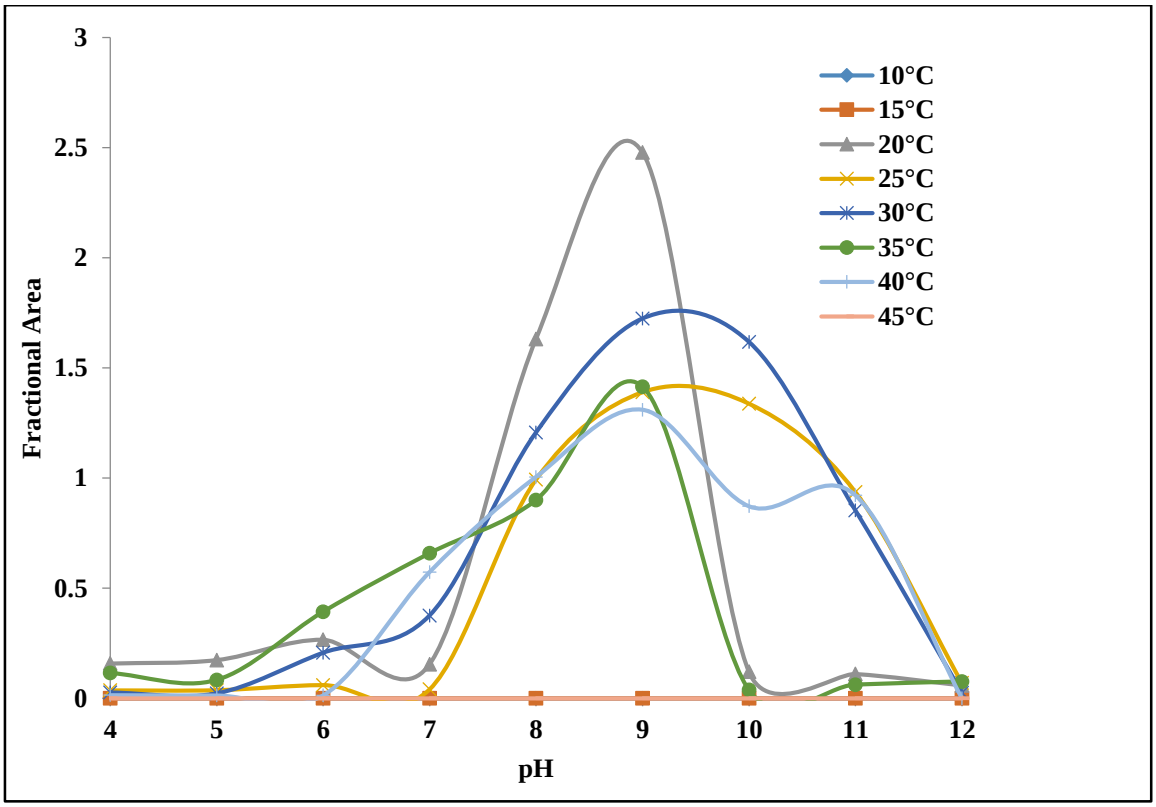


Figure 4.48 Effect pH on growth of *Exiguobacterium aurantiacum* across a wide temperature range

Figure 4.49 represents the 3D surface plots of growth response of *Exigubacterium aurantiacum* to temperature and pH. The dark blue colour represents the maximum growth (FA values between 2 – 2.5) at 25°C. The base of the pyramid which is colour coded with light blue represents the areas where the combined effect of pH and temperature inhibited the growth of the microorganism.

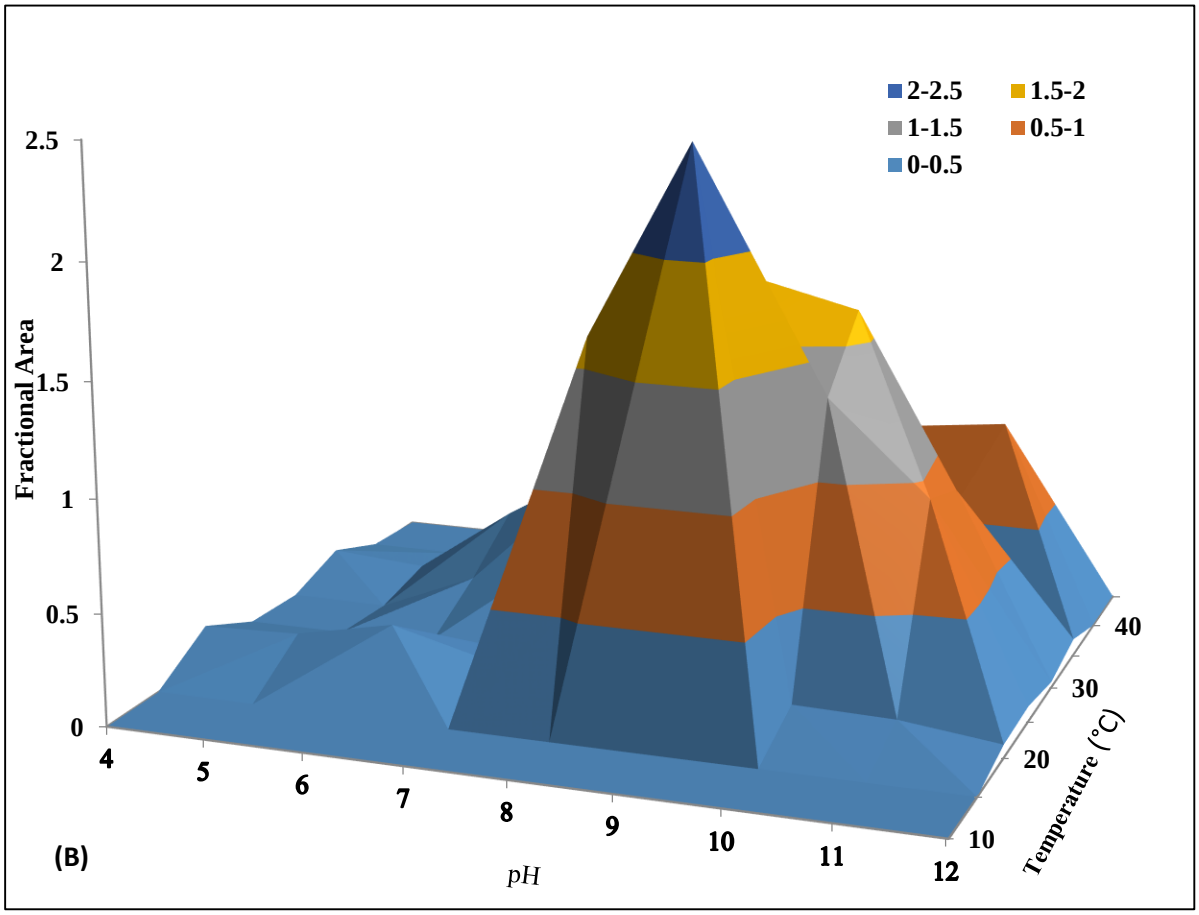


Figure 4.49 The 3D scatter plot showing synergistic effect pH and temperature on the growth of *Exiguobacterium aurantiacum*

The effect of pH on growth of *Exiguobacterium undae* is shown in Figure 4.50 where temperature at 20°C supported the strain optimum growth (FA 2.6) at pH 20°C. At the same time, growth was observed within a wide range of pH between 5.0 and 10.0). No growth was observed in 10°C, 15°C, 40°C and 45°C over the range of pH. Similarly, no growth occurred at pH 4.0 and 5.0 and 11.0 and 12.0.

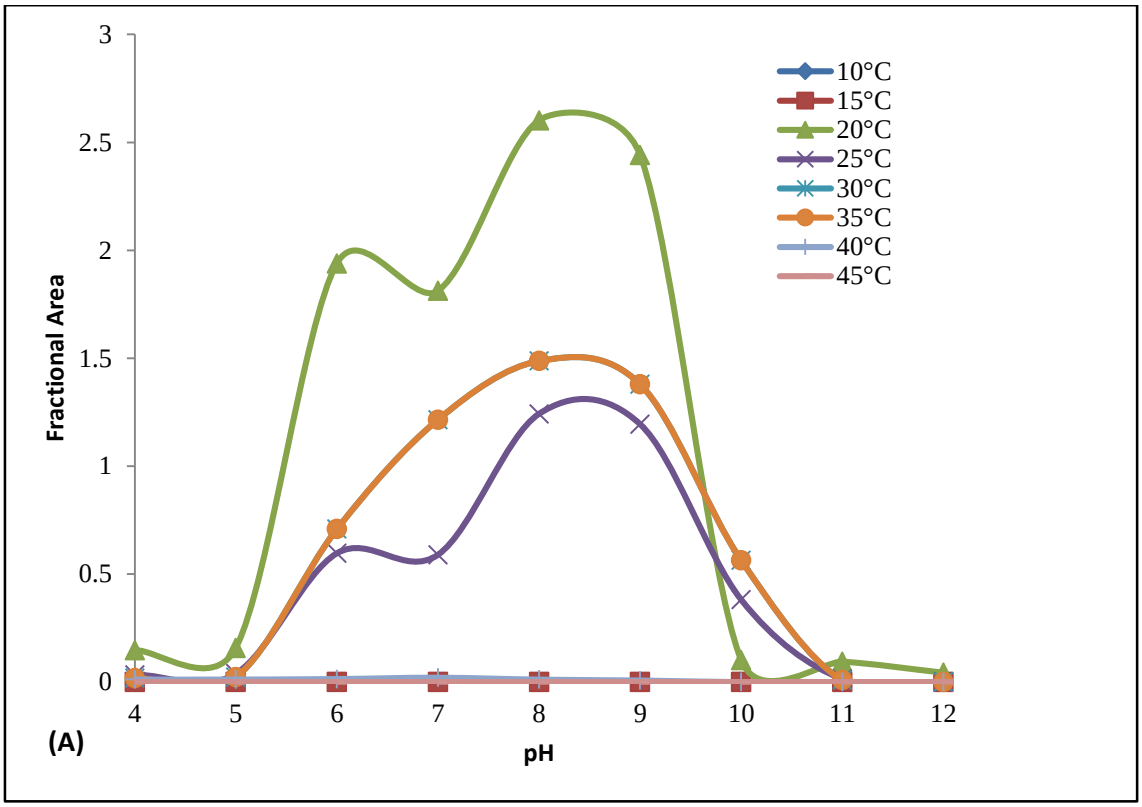


Figure 4.50 Effect pH on growth of *Exiguobacterium undae* across a wide temperature range

The surface plot (Figure 4.51) shows the effect of both pH and temperature on the growth of *Exiguobacterium undae*. The peak of the growth pyramid (Green colour, FA 2.5 – 3) represents the maximum growth of the strain which occurred at 20°C at pH 8.0. No growth was observed at pH 4.0, 5.0, 11.0 and 12.0 as well as in 10°C, 15°C, 40°C and 45°C and these are indicated in blue colour at the base of the pyramid.

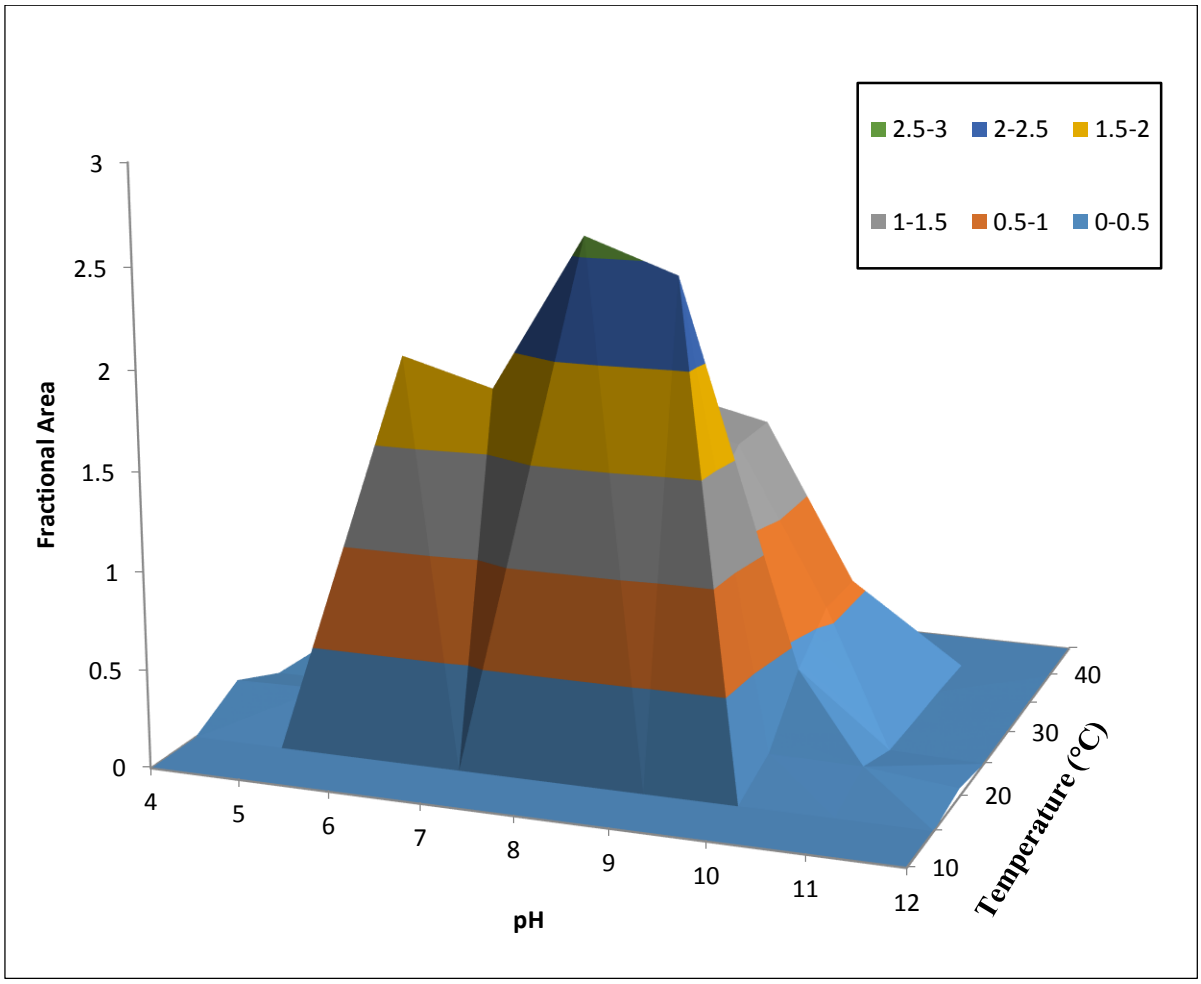


Figure 4.51 The 3D scatter plot showing synergistic effect pH and temperature on the growth of *Exiguobacterium undae*

The pH profiles of *Exiguobacterium alkaliphilum* under a range of temperatures is presented in Figure 4.52. The results show that the strain responded favourably to growth between neutral pH and pH 10.0 where optimum growth (FA 2.75) occurred at pH 9.0 in 20°C. At 30°C, growth occurred over a wide range of pH between 6.0 and 11.0 in which maximum growth at pH 9.0.

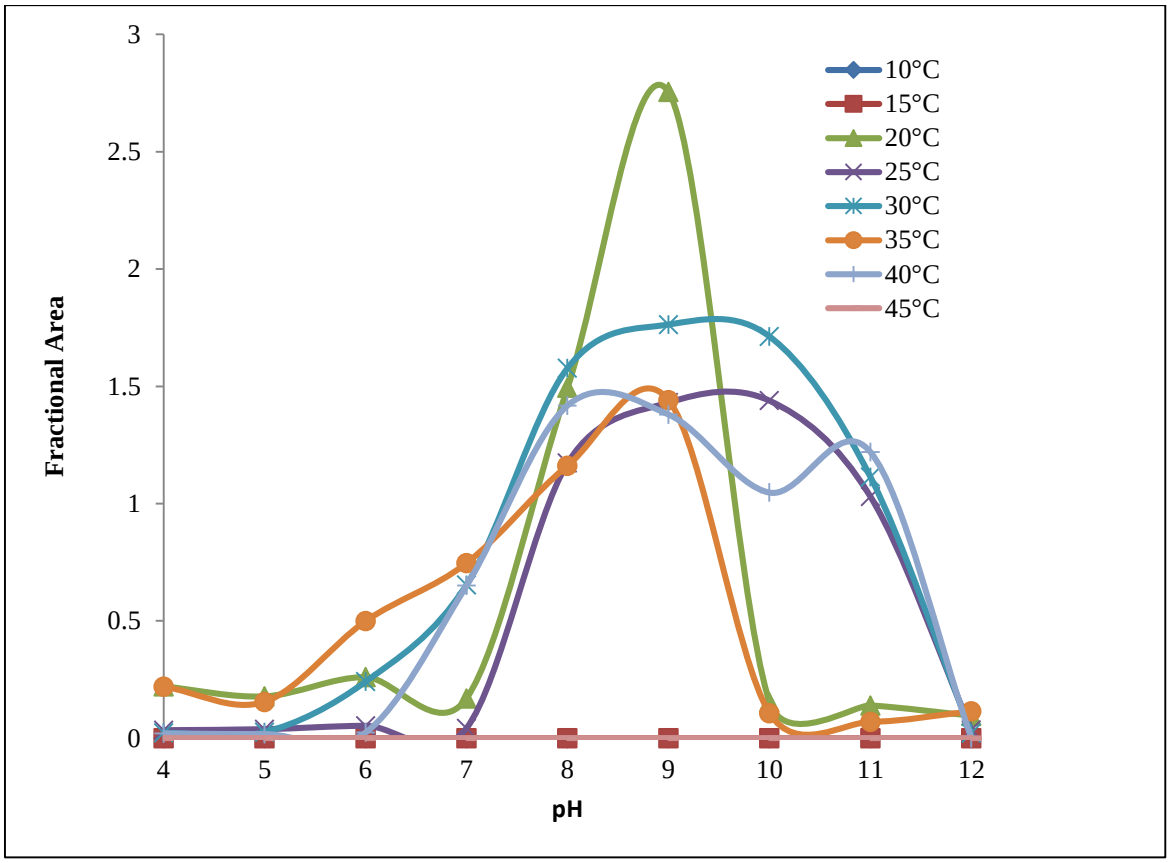


Figure 4.52 Effect pH on growth of *Exiguobacterium alkaliphilum* across a wide temperature range

The surface plot (Figure 4.53) shows how temperature and pH synergistically affect the growth of *E. alkaliphilum*. The peak (FA 2.5 – 3.0) denoted by green colour shows the optimum growth of the strain which occurred at 20°C at pH 9.0.

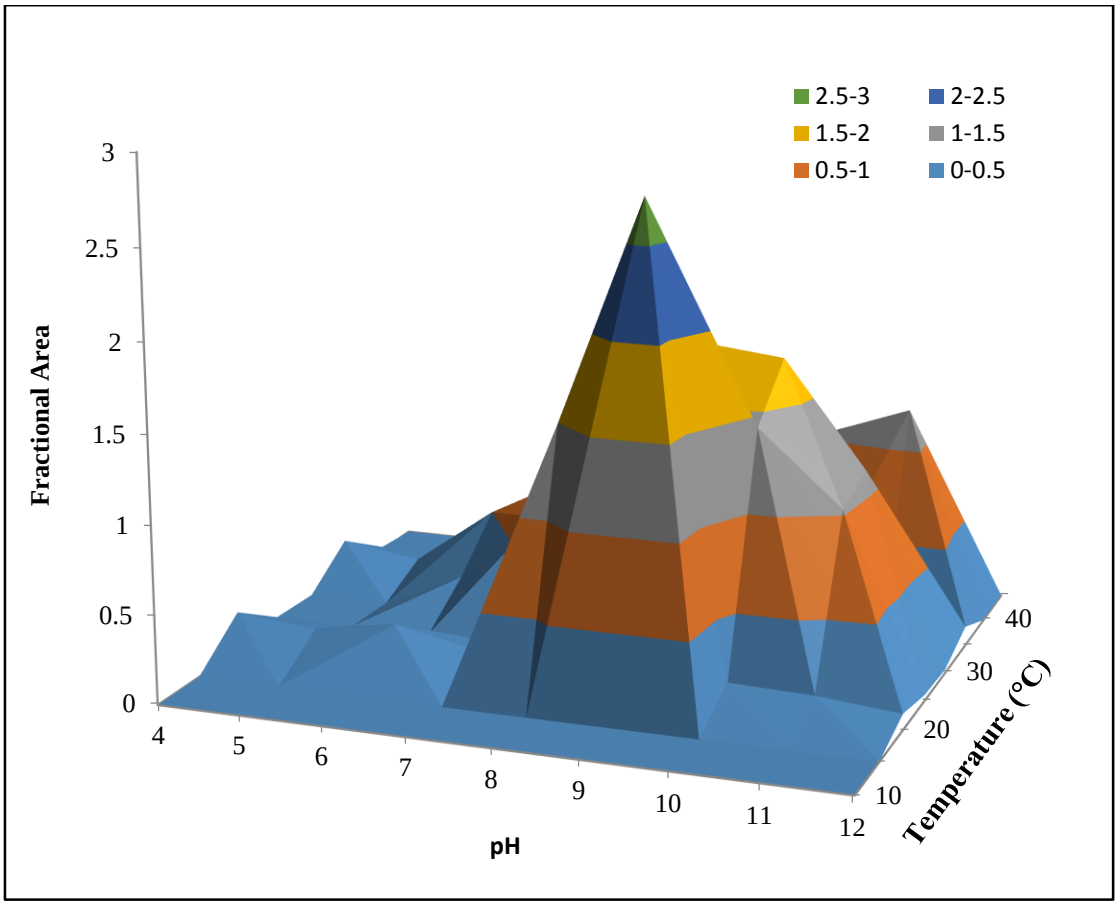


Figure 4.53 The 3D scatter plot showing synergistic effect pH and temperature on the growth of *Exiguobacterium alkaliphilum*

The effect of pH on the growth of *Exiguobacterium oxidotolerans* over a range of temperatures is presented in Figure 4.54 where the tolerance of the strain to a wide range of pH and temperature was demonstrated. Growth was observed in all temperatures except 10°C, 15°C and 45°C. Similarly, the strain was able to grow in all the pH except in pH 4.0, 5.0 and 12.0.

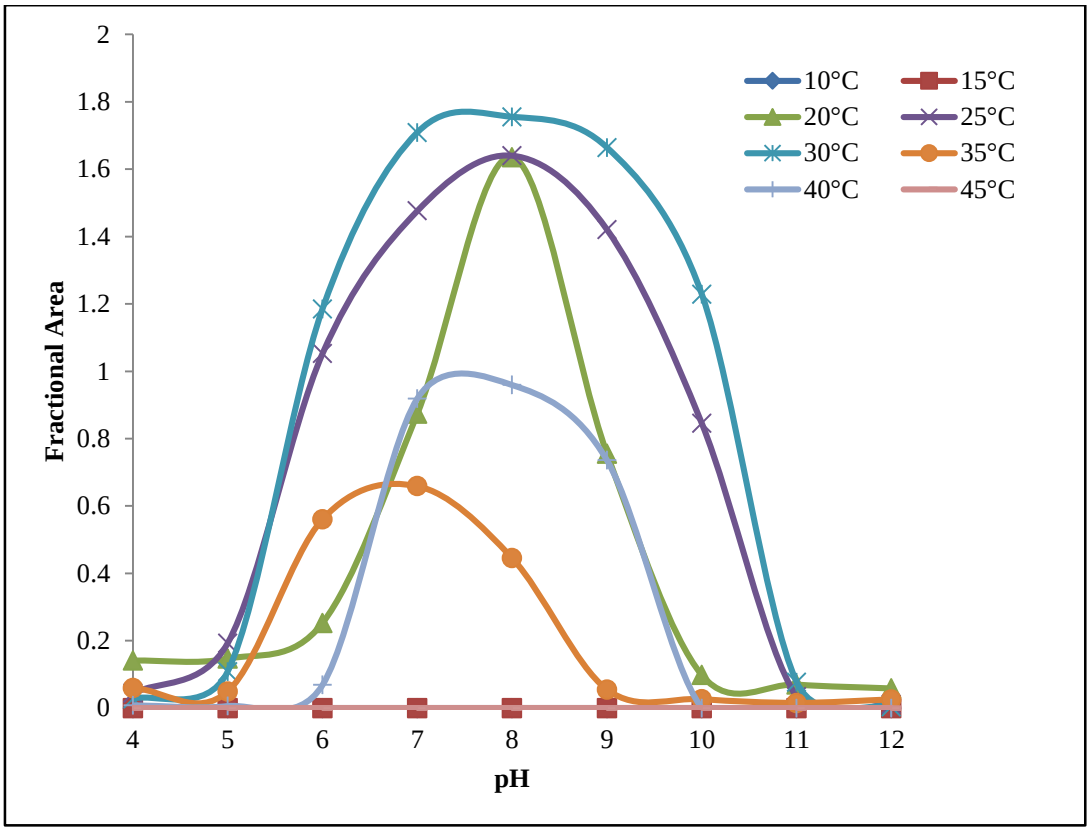


Figure 4.54 Effect pH on growth of *Exiguobacterium oxidotolerans* across a wide temperature range

The influence of pH and temperature on the growth of *Exiguobacterium oxidotolerans* is shown in the surface plot (Figure 4.55). The optimum growth which occurred at 30°C at pH 9.0 is shown at the peak of the pyramid denoted by grey colour.

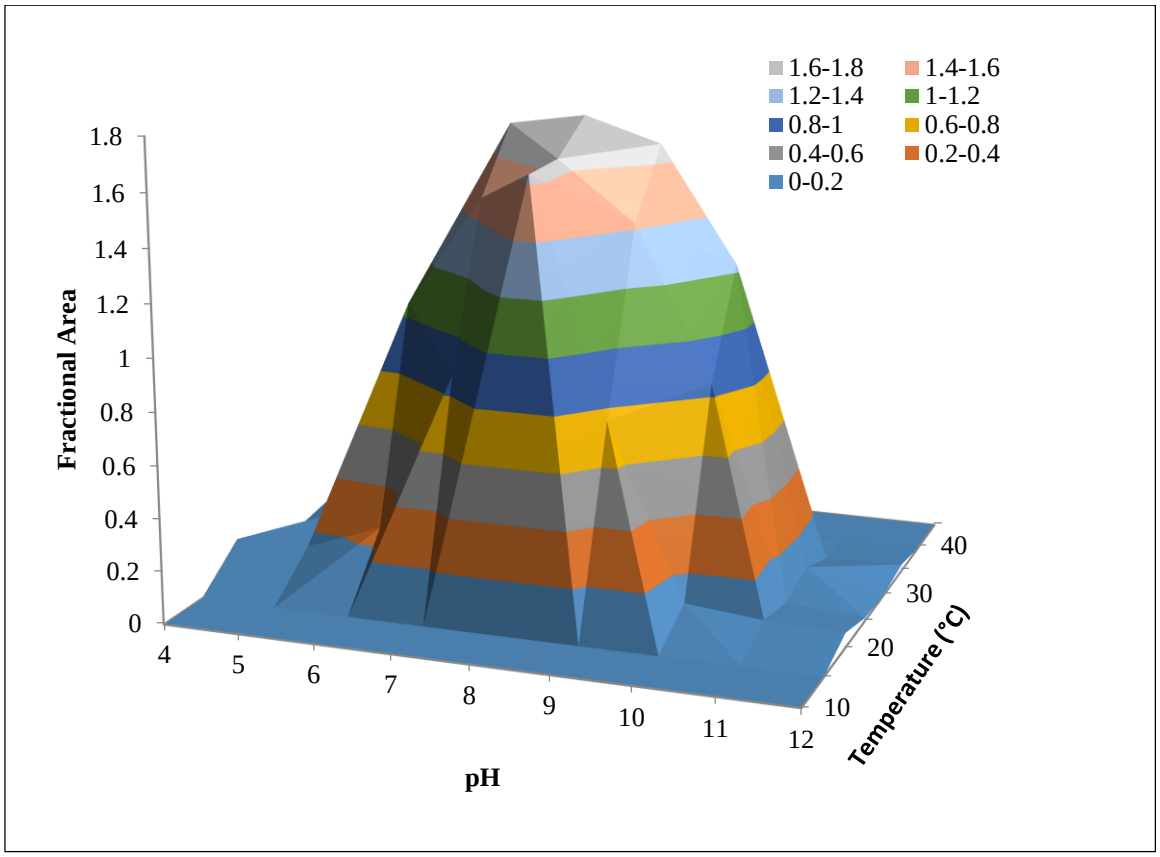


Figure 4.55 The 3D scatter plot showing synergistic effect pH and temperature on the growth of *Exiguobacterium oxidotolerans*

Consistent with earlier findings, the outcome of this study demonstrates the tolerance of the isolates to a wide range of temperature and pH whereby all strains were able to grow between pH 6.0 and 11.0. Optimum growth for all the strains occurred at higher pHs such as pH 8.0 and 9.0 reflecting on the alkaliphilic properties of the genus. The genus *Exiguobacterium* is described as a group with diverse origin [303, 368]. Although some species of this genus have been recovered from extremes of hot and cold temperatures none of the five isolates tested in this study could grow at lower temperatures below 15°C. This observation may be partly be attributed to the impact of the two parameters on the isolates under laboratory conditions. All the strains except *Exigubacterium undae* attained optimum growth at pH 9.0 and could grow in higher pH values up to pH 11.0. These findings are generally supportive evidence of the thermophilic and

alkaliphilic characteristics of some members of this genus already described in literature [308, 368].

These findings together suggest that within the GDF, the growth of microorganism could be impacted by the combined effect of pH and temperature that would evolve and could potentially affect ISA biodegradation.

4.5.14. *Exiguobacterium* sp. strain Hud biofilm formation and characterisation

In view of the harsh conditions that that are expected to prevail within the GDF, the formation of biofilms will be an important survival strategy for microoganisms and to establish niches where the organisms would be confined.

The aim of this part of the research was to assess the biofilm-forming potentials of *Exiguobacterium* sp. strain Hud and investigate the adherence of the biofilm to abiotic surface. The exopolysaccharide of the biofilm was also extracted and investigated.

4.5.15. Calcofluor White staining

Calcofluor white is a non-specific polysaccharide-binding fluorochrome dye used in staining polysaccharides such as cellulose and chitin contained in the cell walls of fungi [369] and many other cellulose-containing microorganisms as well as extracellular matrix of microbial biofilm [370]. Calcofluor white staining is a rapid procedure for the detection of pathogenic microorganisms including *Pneumocystis carinii*, *Microsporidium*, *Acanthamoeba*, *Naegleria*, and *Balamuthia* species [371].

Maximum excitation and fluorescence with aqueous solutions of cacofluor white occurs with ultraviolet, violet or blue light whereby Fungi, *Pneumocystis* cysts and parasites fluoresce with brilliant apple-green [372]. In biofilm assays, the use of calcofluor white staining has been employed. In one such studies, when 135 µM calcofluor white was applied to the biofilm formed by *Pseudomonas*

aeruginosa displayed blue under scanning laser fluorescence microscope (Zeiss 510 META Laser Scanning microscope; Zeiss, Oberkochen, Germany [373]). Daniëlle *et al*. [374] also used calcofluor staining in a study to investigate *Pseudomonas aeruginosa* biofilm formation and slime excretion on antibiotic-loaded bone cement where the biofilm stained blue under confocal scanning laser microscopy.

In order to determine whether the biofilm of *Exiguobacterium* sp. strain Hud can form biofilm, a calcofluor staining was performed on a sample of colonies obtained from the strain. The result of the calcofluor white staining of the biomass of *E.* sp. strain Hud is presented in Figure 4.56. Cells are stained apple-green whereas EPS are stained light-blue around the edges of the cells.

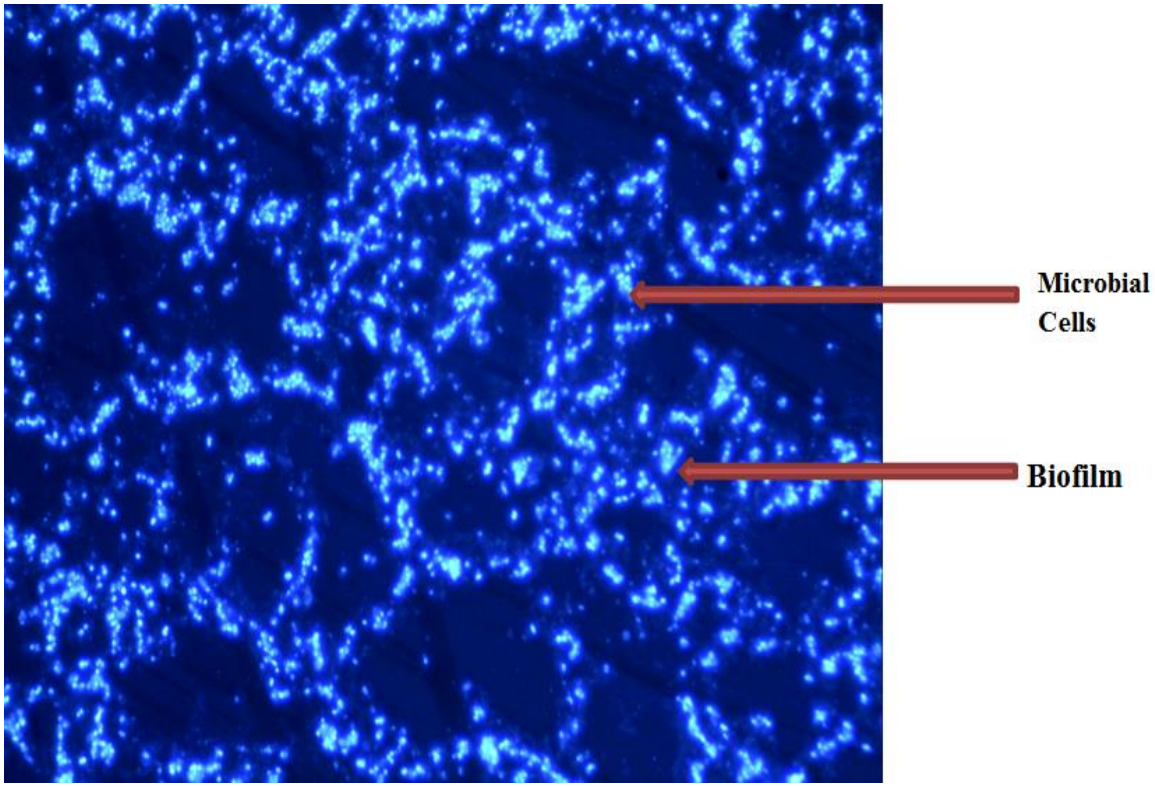


Figure 4.56 Calcofluor staining of biofilm produced by *Exiguobacterium* sp. strain Hud

Cells are stained apple-green whereas exopolysaccharides of the biofilm (around cells) are stained light-blue.

4.5.16. Biofilm formation and adherence assay

Following calcofluor white staining, Biofilm formation and adherence was assessed. In order to quantify the biofilm adherence, the method used by Stepanovic *et al*. [230] was adopted with a few modifications (See methods section).

The result presented in Figure 4.57 shows the biofilm formation and adherence onto the wells (abiotic surface). Statistical analysis with paired T-test showed significant difference between the mean absorbance of the control (ODc) and the sample (ODs; *P* value = 4.46 E-7) following 18 hours of incubation. At least, 10 fold increase in absorbance over ODc was observed for the sample which according to the biofilm adherence capability classification means that the strain produces a strong adherent biofilm [230].

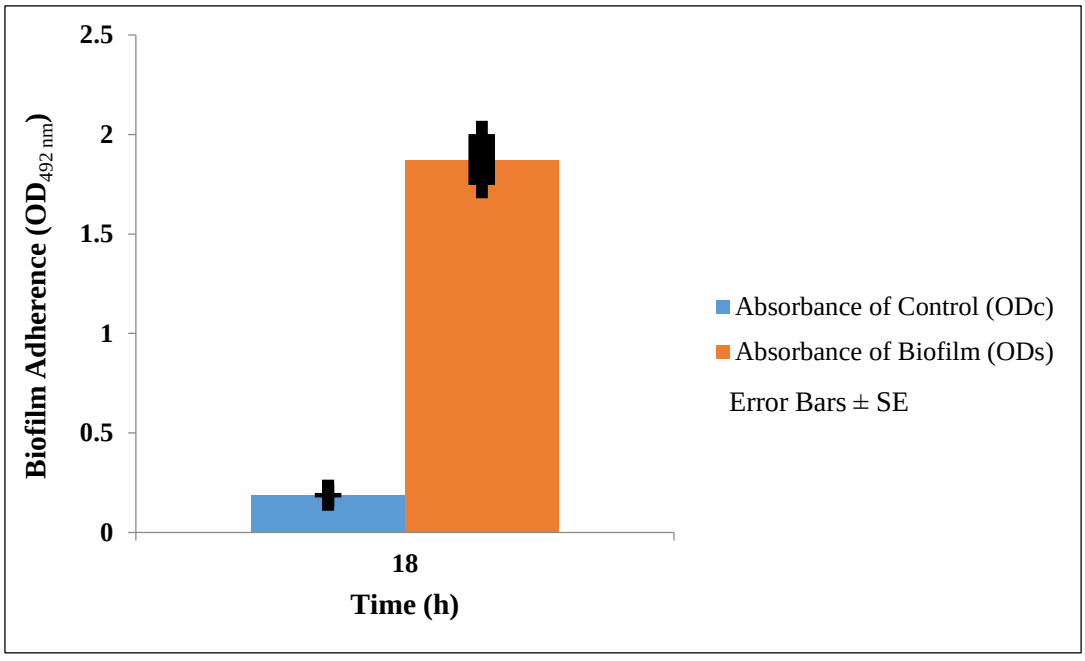


Figure 4.57 Biofilm adherence of *Exiguobacterium* sp. strain Hud

Biofilm adherence onto abiotic surface following 18 hours of incubation. ODc is the optical density of negative control; ODs is the optical density of the biofilm exclusively

To analyse the polysaccharides generated by *Exiguobacterium* sp. strain Hud which may be associated with biofilm production the test organism was incubated anaerobically in 1 L minimal media at 25^{o}C for 72 h. Following incubation, the EPS produced during microbial growth was extracted prior to analysis (see methods section). EPS extraction was also carried out on agar-based culture whereby cell biomass was recovered from the surface of solid minimal media following 72 h of anaerobic incubation. Proton NMR (^{1}H-NMR) analysis on the EPS was carried out by the University of Huddersfield NMR service. The Figure 4.58 represents ^{1}H-NMR spectra of the negative control (uninoculate minimal media) and the biofilm sample obtained from *Exiguobacterium* sp. strain Hud. It can be seen from the spectra that no signal was generated from both the negative control sample and that of the biofilm obtained from the strain which indicates that no polysaccharides were present in the samples analysed.

Analysis with Proton NMR (^{1}H-NMR) generate signals from all protons present within polysaccharide units which normally occur at 1-6 ppm chemical shift range. The anomeric proton in a unit of a polysaccharide (sugar) which is located next to two electronegative atoms becomes deshielded as the oxygen atoms draw electrons and leave the protons. In view of this, the signal of the proton occurs at the lower field (~ 4.5 – 5.8 ppm) as compared to the protons in the other units which is expected to occur at higher chemical shifts (~ 3.4 – 4.5ppm). Methyl protons appear at much higher fields (~ 1.2ppm).

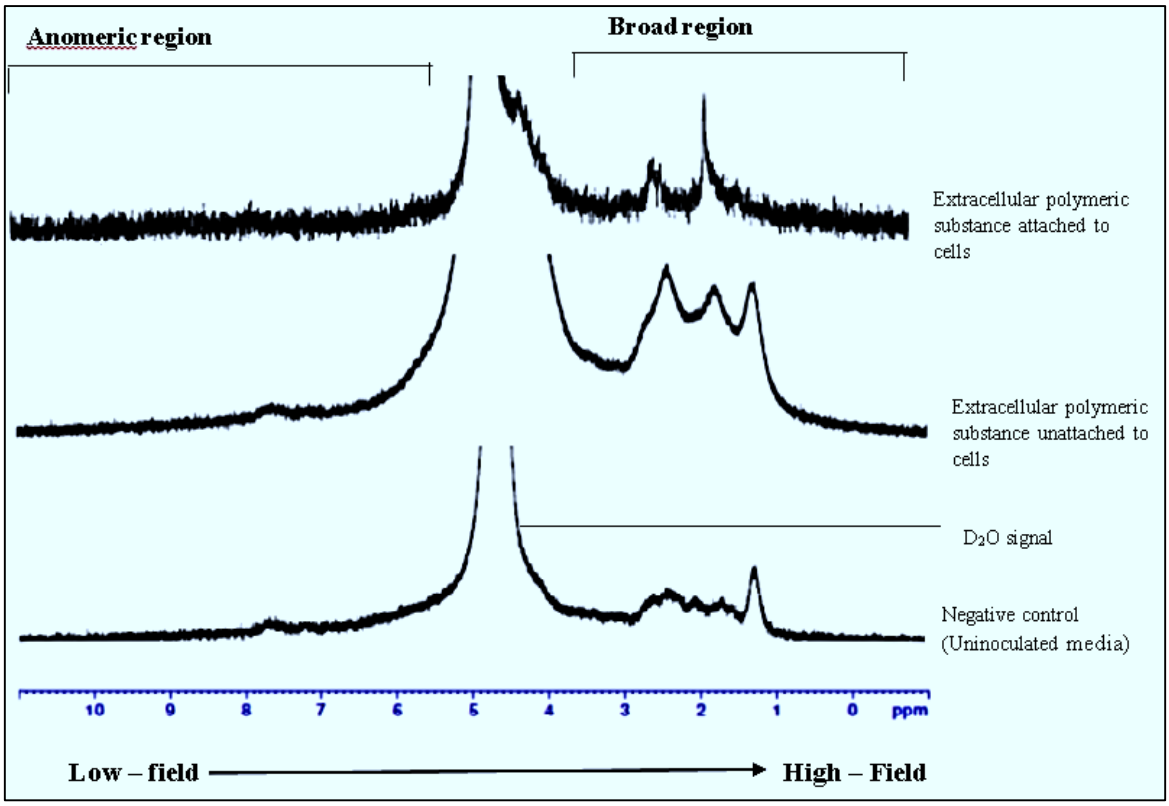


Figure 4.58 ^{1}H-NMR analysis of EPS samples of *Exiguobacterium* sp. strain Hud

Spectra obtained from control (uninoculated minimal media) and from biofilm sample from *Exiguobacterium* sp. Strain Hud. The spectra show no signals in the anomeric regions for both the control and biofilm samples

The results presented here show biofilm forming ability of *Exiguobacterium* sp. Strain Hud. Some species of this genus have been isolated from microbial biofilms [375] [376], from water and patients associated with peri-implantitis [377]. The present study expands our knowledge on the potential of *Exiguobacterium* sp. strain Hud to form a biofilm (confirmed by calcofluor white staining) and subsequent adherence of the biofilm onto the abiotic surface.

In the context of geological disposal of radioactive waste, biofilm formation is necessary for the survival of any microorganism that can utilise ISA as carbon source under repository conditions with special emphasis on high pH [59]. Within the biofilms microorganisms will be able to establish niches and obtain protection from the external repository conditions. Formation of biofilms on abiotic surfaces

within the repository will also increase sorption of radionuclides if the immobile microorganisms (Biofilm) actively take up the radionuclides onto cell surfaces or by intracellular uptake or through microbially induced precipitation [299]. The formation of biofilm within the repository is also likely to impact on radionuclide transport if biofilms clog up pore spaces and eventually block the pore system [378].

The EPS of biofilms usually contains polysaccharides, proteins, nucleic acids and lipids which may form part of the microbial capsular material or be released into the surrounding as dispersed slime. As part of this study, attempts to isolate the exopolysaccharides produced by *Exiguobacterium* sp. strain Hud was not successful. While it is possible that the biofilm of *Exigubacterium* sp strain Hud is eDNA or protein based [379], this inability to detect EPS within the biofilm may also be attributed to a number of factors including those required to trigger EPS production. EPS production is influenced markedly by the composition of media and fermentation parameters and that the relative monosaccharides ratios within EPS are affected by pH and carbon sources [380]. These conditions which are strain specific need to be optimised to trigger the production of the EPS and to increase yield [381-383]. It is therefore possible to demonstrate from these studies that the laboratory conditions needed to elicit EPS production in *Exiguobacterium* sp. strain Hud may not have been optimised rather than suggesting that the strain is non-EPS producing. Further studies on optimisation of conditions for effective production and chemical characterisation of the strain's EPS may improve our knowledge on biofilm formation in microorganisms associated with ISA biodegradation under repository conditions.

4.5.17. Key findings

- *Exiguobacterium* sp. strain Hud has shown the potential to degrade ISAs under anoxic alkaline conditions representative of the GDF
- The biodegradation of ISAs results in the generation of acetic acid suggesting an acetogenic pathway
- The degradation of ISAs may require some unusual enzymes compared to other carbohydrates such as glucose
- The main fatty acids of *Exiguobacterium* sp strain Hud $iC_{12:0}$, $C_{13:0}$, $aiC_{14:0}$ and $C_{17:0}$. The major menaquinone is MK7
- The strain demonstrated broad biochemical capabilities along with significant salt, pH and heavy metal tolerances.
- The main physiological and biochemical characteristics of *Exiguobacterium* sp. strain Hud are presented in Table 4.6 together with those of other four species from the literature

Table 4.6 Physiological and biochemical characteristics of *Exiguobacterium* sp. strain Hud compared with other related species

Characteristics and biochemical profile	***Exiguobacterium* species**				
	1	**2**	**3**	**4**	**5**
Colony colour	**Orange**	orange-yellow	orange	orange	Orange or yellow
Motility	**+**	*	+	*	+
Flagella	*****	*	Peritrichous	*	Peritrichous
Oxidase	**+**	-	+	-	+
Catalase	**+**	+	+	+	+
NaCl tolerance (%)	**9.4**	6	*	9.5	12 but not 15
Optimum growth temperature (°C)	**35**	37	37	35	34
Optimum pH	**9.5**	10	*	9.5	7-10
Carbohydrate utilization					
Glycogen	**+**	+	+	+	*
Mannan	**+**	*	+	-	*
N-Acetyl glucosamine	**+**	*	+	+	*
N-Acetyl mannosamine	**+**	*	W	+	*
Cellobiose	**+**	*	+	+	*
Melibiose	**+**	*	+	*	*
Raffinose	**+**	*	V	-	*
D-Glucose	**+**	+	+	+	*
D-Galactose	**+**	*	+	W	-
D-Mannitol	**+**	V	+	*	*
D-Raffinose	**+**	*	+	-	-
D-Ribose	**+**	+	+	+	+
D-Mannose	**+**	+	+	*	+
D-trehalose	**+**	*	*	*	*
D-Sorbitol	**+**	*	-	+	*
Acetic acid	**-**	*	+	-	*

Information on other four species; taken from Frühling *et al*., Kulshreshtha *et al*., Pitt *et al*. and Yumoto *et al*., [323, 332, 333, 367]. 1, *E.* sp. strain Hud, 2, *E. aurantiacum*, 3, *E. undae*, 4, *E. alkaliphilum*, 5, *E. oxidotolerans*. +, positive, -, negative, W' weak, V, variable, *, not determined

4.5.18. Characterisation of bacterial isolate 2

4.5.19. *Oceanobacillus* sp. strain Hud

4.5.20. Overview

The genus *Oceanobacillus* consisting of endospore-forming Gram-positive rods within the phylum Firmicutes were first described by Lu *et al*. in 2001 [384] where the novel strain (HTE831) was isolated from 1,050 m depth on the Iheya ridge, off the coast of Japan. Most species of this genus have been isolated from hypersaline environment such as the core of deep sea sediments and salt lakes [385] thereby reflecting their ability to survive and grow in hypersaline environments. This halophilic nature of some members of the genus has attracted the attention of researchers in their search for robust biocatalysts, as in other halotolerants [386]. Other species of the genus have also been recovered from fermented foods [387] and the human gut [388, 389]. Some species are obligatory aerobic, whereas others are facultative anaerobes [390].

Members of the genus in addition to their halotolerance are also noted for their tolerance to high pH [384] and temperature within which these isolates utilised a range of carbon substrates [385]. In the case of ability to grow at high temperatures, Whon *et al*. [387] and Raats *et al*. [391] demonstrated that *Oceanobacillus kimichii* and *Oceanobacillus chironomi* were capable of growth up to 46°C. In the bioremediation of heavy metals, Mulik and Bhadekar [392] in a study to assess heavy metal removal by bacterial isolates from the Antarctic oceanic region demonstrated that a member of this genus designated *Oceanobacillus* sp. 39 could tolerate high concentrations of Cd^{2+}, Cr^{2+} Ni^{2+} and Pb^{2+}.

In a research project aimed at isolating alkaliphilic bacteria from hyperalkaline soil, Almrabet (MSc student of the environmental Microbiology group, University of Huddersfield) recovered the strain *Oceanobacillus* sp. strain Hud from mineral media broth inoculated with 10 g of soil sample from Harpur Hill

site [393]. The solid mineral medium for the isolation was supplemented with 20% NaCl and CDP as carbon source at pH 9.0.

Following DNA extraction, a draft whole genome sequence was carried out using a whole genome shortgun (WGS) sequence strategy. The Illumina Hiseq 2500 system (BaseClear, NL) was employed to generate paired-end 125 cycles sequence reads while the Illumina Casava pipeline (version 1.8.3) generated FASTAQ sequence files. Quality assessment was carried out by the Illumina Chastity filter and reads containing adapter sequence were removed in-house. A second quality assessment was carried out by FASTQC tool (version 0.010.0). The FASTQ trimmed and assembled by employing CLC Genomic Workbench (version 7.0.4). The determination of the optimal k-mer size was done with KmerGenie [394]. The contigs were linked and placed into scaffolds or supercontigs. Estimation of the orientation, order and distance between the contigs were done using insert size between the paired-end and/or matepair reads using the SSPACE Premium scaffolder version 2.3 [395].

4.5.21. Genome Description

Whole genome sequencing generated 85 contigs with (PEGs), with total genome size 4,692,842 bp in length with 39.2% G-C content. The whole genome sequence which was annotated with the RAST server [396], produced an output of subsystem coverage of 48% which contained a total of 2,178 proteins. There were 2,058 non-hypothetical and 120 hypothetical proteins (Figure 4.56).

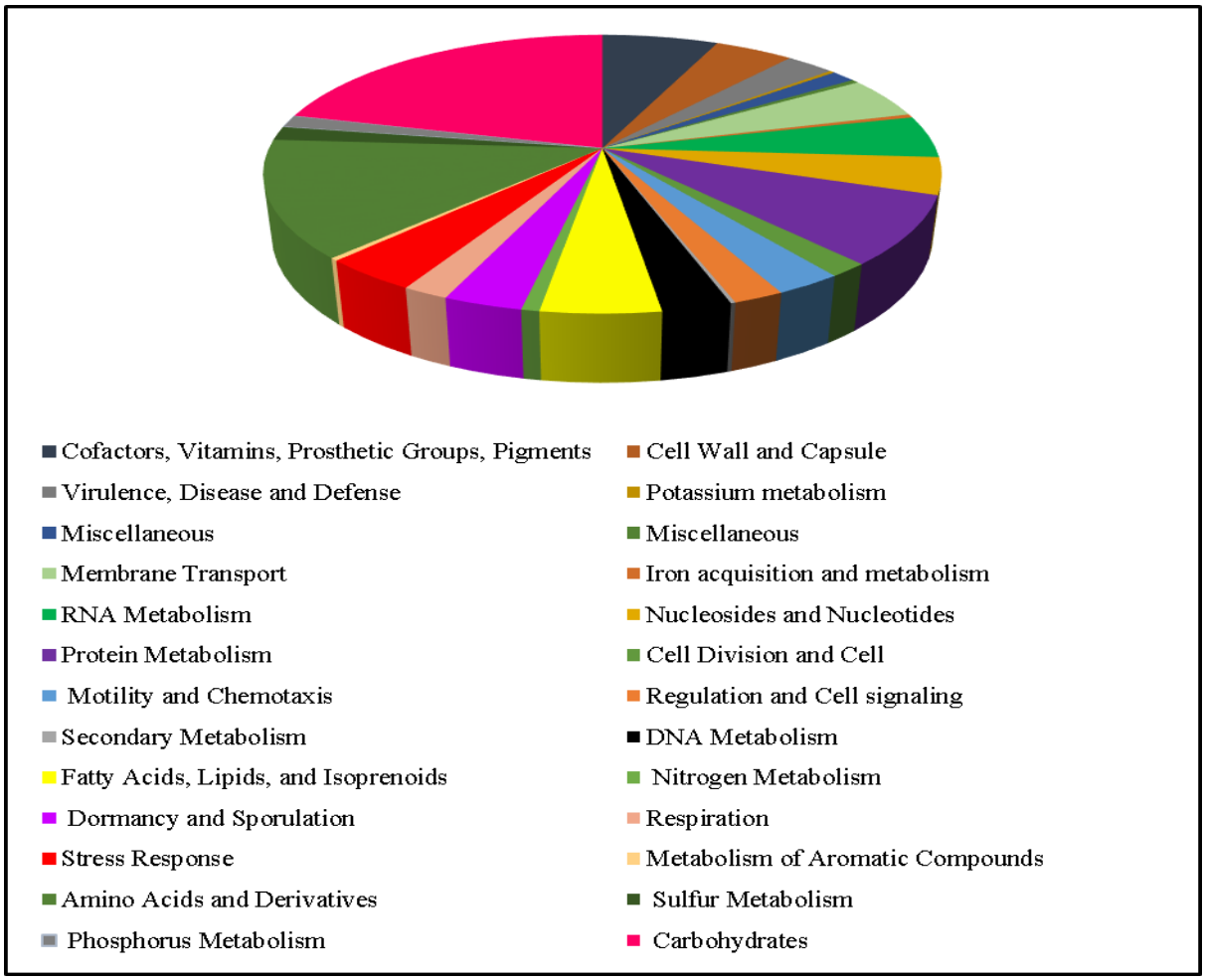


Figure 4.59 RAST annotation of WGS sequencing of *Oceanobacillus* sp. strain Hud Showing subsystem coverage and features with protein counts

Many of the proteins identified (Figure 4.59) were responsible for support and normal cell functioning including cell wall and capsule synthesis, membrane transport system and for the production of amino acids and its derivatives. A total of 89 and 155 proteins that were annotated were shown to be responsible for the metabolism of DNA and RNA respectively. Furthermore, a number of genes (124) were noted to be related to stress response, which included genes coding for proteins involved in resistance to antibiotics and toxic compounds, protection from reactive oxygen species and osmotic stress. The presence of these stress response genes underscores the ability of members of this genus to survive under

extreme conditions including high pH, temperatures and saline environment [390, 397].

In the case of resistance to toxic compounds, the genome annotation revealed the presence of 23 genes 22 of which coded for resistance to heavy metals while the remaining one coded for fosfomycin resistance. Cadmium-transporting ATPase (cadA), cadmium efflux system accessory protein (CadC) and cadmium resistance protein D (cadD) were noted to confer on the organism, resistance to cadmium toxicity. Genes were also present that coded for resistance to zinc toxicity where the sensor protein of zinc sigma-54-dependent two-component system (zraS) and response regulator of zinc sigma-54-dependent two-component system zraR were evident. In order for the strain to resist chromium toxicity, chromate resistance protein chrI and chrB as well as chromate transport protein A (chrA) were found within the genome. The genome annotation also revealed the presence of arsenic efflux pump-driving protein A (arsA), asenic efflux pump protein B (arsB) and arsenic reductase C (arsC) all of which confer ability of the strain to resist arsenic toxicity. The presence of these heavy metal resistance genes reflects on the ability of some members of this genus to be involved in bioremoval of some heavy metals [392].

In the case of resistance to osmotic stress, the presence of 14 genes were evident, 13 of which were involved in choline uptake and conversion to betaine clusters. Included in this group of genes was alcohol dehydrogenase protein B (GbsB) essential for the utilization of choline. The remaining genes coded for proteins involved in osmoregulation where outer membrane protein A (ompA), osmotically inducible protein Y (osmY) among other proteins were observed. For oxidative stress response, the presence of genes coding for superoxide dismutase A, B, C and F (sod A, sod B, sod C and sod F) were present among others. In order to survive extremes of temperature, the genes involved in responding to cold/heat shock such as the cold shock protein A (cspA) family and heat inducible

repressor protein A (HrcA) were present in the genome. Detoxification and periplasmic stress proteins were also present for conferring stress response to the organism.

The genome annotation also revealed that the subsystem category involving metabolism with the highest number of proteins was carbohydrate subsystem where 666 proteins (20.96% of the proteins annotated) were involved in the metabolism of carbohydrates (Figure 4.57). In this subsystem feature counts, protein associated with the metabolism of amino-sugars and sugar alcohols were present. Genes encoding proteins implicated in CO_2 fixation and mixed acid fermentations that generate products such as lactate, acetate, CO_2 and H_2 were also reported. A number of genes encoding proteins involved in central carbohydrate metabolism were present, where 25 proteins responsible for the pyruvate metabolism II: Acetyl-CoA, acetogenesis from pyruvate were evident. The presence of genes encoding proteins involved in glycolysis, gluconeogenesis, TCA and pentose phosphate pathway were also noted. In terms of utilisation of carbon sources, the genome showed that the isolate is capable of metabolism of a range of carbon substrates. Genes for the metabolism of monosaccharides such as mannose, galactose, D-ribose, Xylose and D-sorbitol; disaccharides such as sucrose, lactose, fructose, trehalose and polysaccharides such as glycogen were present. The percentages of the the subsystems associated with carbohydrate metabolism is shown in Figure 4.60.

Microorganisms are often faced with nutrient limitations in their surroundings but the ability of bacteria to survive such nutrient limitation via physiological processes is important for their survival in nutrient-limiting environments [398]. The genome annotation of this strain suggests the presence of carbon storage regulator gene (CsrA), which is reported to repress gluconeogenesis, glycogen biosynthesis, catabolic processes and biofilm formation [399-402] during nutrient limitation. This physiological behaviour reflects on ability of some species of this

genus to survive under limited-nutrient environment such as deep-sea sediments [385].

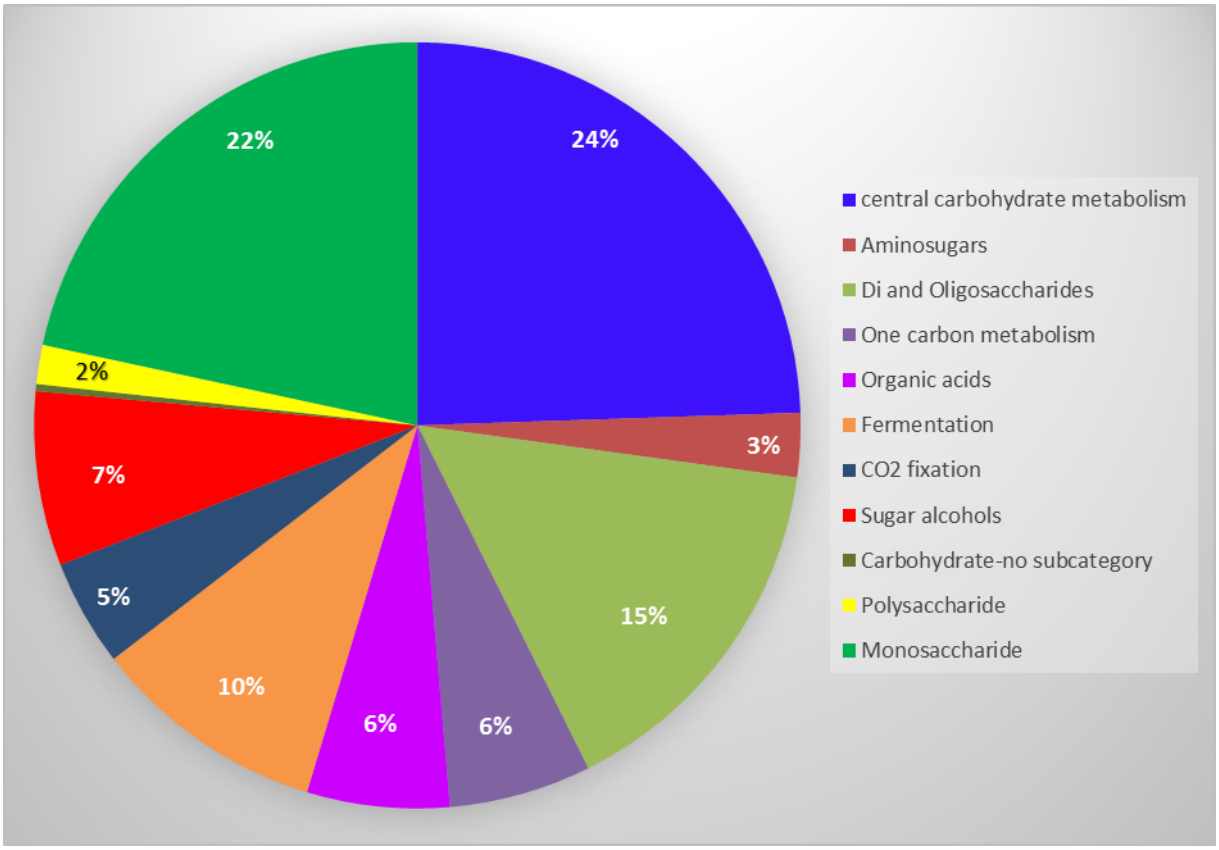


Figure 4.60 RAST subsystem annotation for *Oceanobacillus* sp. strain Hud showing percentages of sub-features associated with carbohydrate metabolism.

4.5.22. Rationale for using *Oceanobacillus* sp. strain Hud in ISA biodegradation experiment.

Most members of the genus *Oceanobacillus* have been described as halo-alkaliphic microorganisms and have been recovered from either salty or alkaline environments including fermented indigo [403, 404], fermented food [387, 405] and marine environments [384, 406, 407]. Moderately alkaliphilic microorganisms can survive and grow in alkaline environment with a pH range of 9-10 [408]. In order to survive such high pH environments, these microorganisms make use of homeostatic mechanisms such as Na^+/H^+ antiporterdependent to

neutralize cytoplasmic pH. Moderately halophilic microorganisms can survive and grow in salty environments within a range of 5 – 20% (w/v) NaCl through the regulation of osmotic concentration [409]. As a result of their osmoregulatory strategies, halophilic bacteria are able to use osmolytes including betaines, polyols and ectoines under hypersaline conditions to adapt and counter external osmostic pressure [410]. The collective haloalkaliphic properties of *Oceanobacillus* species suggest that these microorganisms may have biotechnological applications such as biodegradation of organic pollutants. In addition, having been isolated from mineral media in which CDP was the main carbon source, this strain was selected for ISA biodegradation on the basis of the presence of genes coding for the metabolism of a range of carbon substrates. Furthermore, the presence of genes involved in stress response and fermentation of carbon substrates to produce acetate, ethanol, and formate at pH values above 7 (according to the genome annotation) makes the strain potentially capable of surviving and growing under the expected conditions of nuclear waste repository.

4.5.23. Cultivation of *Oceanobacillus* sp. strain Hud

Oceanobacillus sp. strain Hud could grow on solid mineral media with either CDP or $Ca(\alpha\text{-ISA})_2$ as the sole carbon source at pH 10.0 within 48 hours indicating their ability to utilise ISAs under alkaliphilic [411] anaerobic conditions. The colony morphology appeared to be cream in colour, sticky, circular and opaque following 18 h of incubation at 25°C on FAA at pH 10.0 under anaerobic conditions. The cells were Gram-positive rods facultatively anaerobic and motile with hanging drop microscopy. The strain was catalase and oxidase positive. The electron microscopy of the cells are shown in figure 4.61.

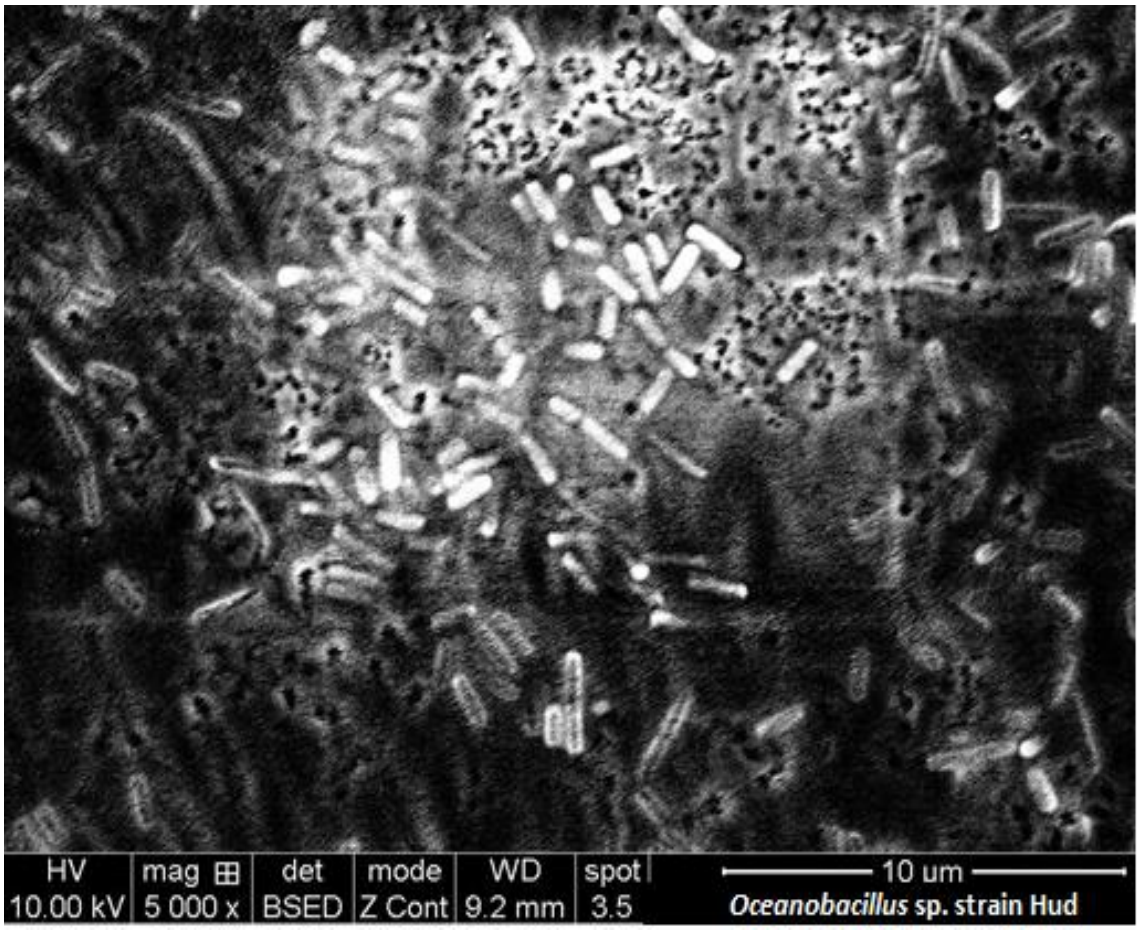


Figure 4.61 Electron micrograph of *Oceanobacillus* sp. strain Hud.

The 16S rRNA gene sequencing indicated that the strain was 99% identical to *Oceanobacillus aidingensis* strain AD7-25 and Oceanobacillus *oncorhynchi* strain 20AG. Phylogenetic analysis clustered the isolate with *Oceanobacillus aidingensis* (98%) via bootstrap analysis of 1000 replicate trees. Furthermore, both strains clustered 97% together with *Oceanobacillus oncorhynchi* strain 20AG and forms a clade thereby reflecting on evolutionary relationship between the three isolates (Figure 4.62). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test of 1000 replications are shown next to the branch points [328].

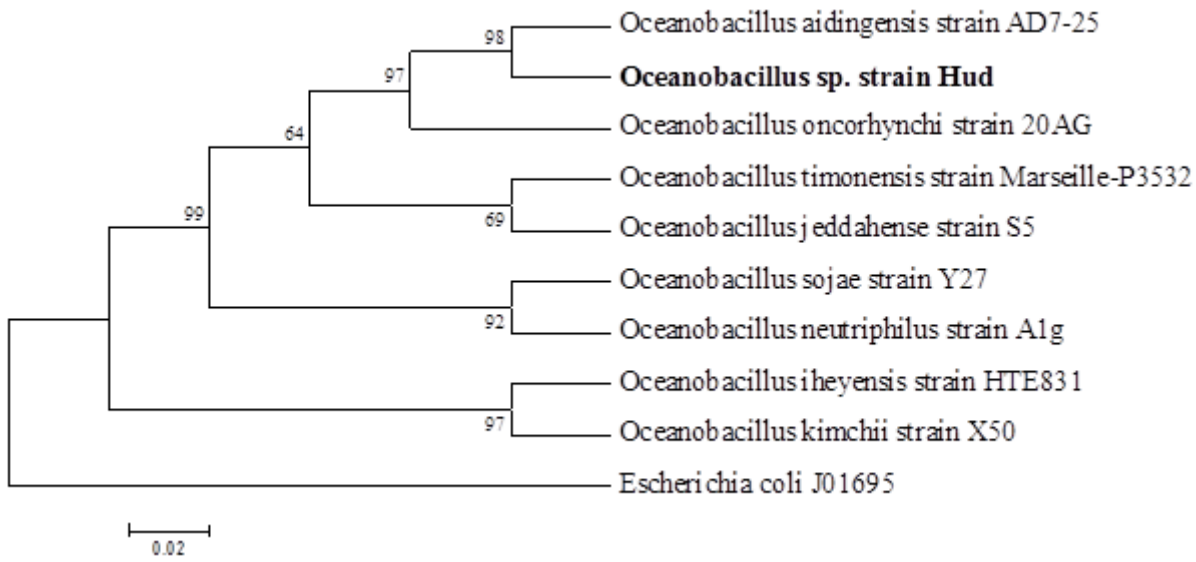


Figure 4.62 Neighbour-Joining tree based on 16S rRNA gene sequences of *Oceanobacillus* sp. strain Hud

The inferred tree shows the phylogenetic relationship between *Oceanobacillus* strain Hud and other species of the genus. *E. coli* J01695 was used as outgroup. Bar, 2 nucleotide substitution per 100 nucleotides

4.5.24. ISA biodegradation

In order to investigate whether *Oceanobacillus* sp. strain Hud could utilise ISA as carbon source, a batch fermentation experiment in minimal media supplemented with 2% vitamin and trace elements solution was set up whereby NaISA, $Ca(ISA)_2$ and CDP were added as carbon substrates following the method described previously. The pH was adjusted to 9.0. In all three experiments, ISA removal was accompanied by acetic acid production with a resultant increase in biomass. The generation of acetic acid resulted in the reduction in pH of each microcosm when compared to the abiotic control (Figures 4.61 and 4.62).

In the $Ca(ISA)_2$ driven system ISA removal began from the fifth (5^{th}) day of sampling and continued steadily until the end of sampling period at a first order mean rate of degradation of $1.3 \times 10^{-1} \pm 0.00$ d^{-1}. At the end of sampling, a mean concentration of 2.23 mM ISA remained, indicating that 56% of the original concentration could still be detected in the samples (Figure 4.63). The

biodegradation of ISA was accompanied by increase in biomass (Figure 4.63B) and accumulation of acetic acid up to a concentration of 1.69 mM at the end of the sampling period (Figure 4.63A). This represents a total acetic acid yield of 42% mM per mM ISA degraded but only 21% of the theoretical value. The detection of acetic acid lagged behind until day 15 probably because the concentration at that time was much below the detection limit. A drop in pH from the initial pH 9 to a final mean pH of 7.59 (Figure 4.63A) suggested acidification of the medium from the generation of acidic products of fermentation [412]. The parameters of the abiotic control system (i.e ISA concentration and pH) remained unchanged by the end of the experimental period and as a result, no acetic acid was detected.

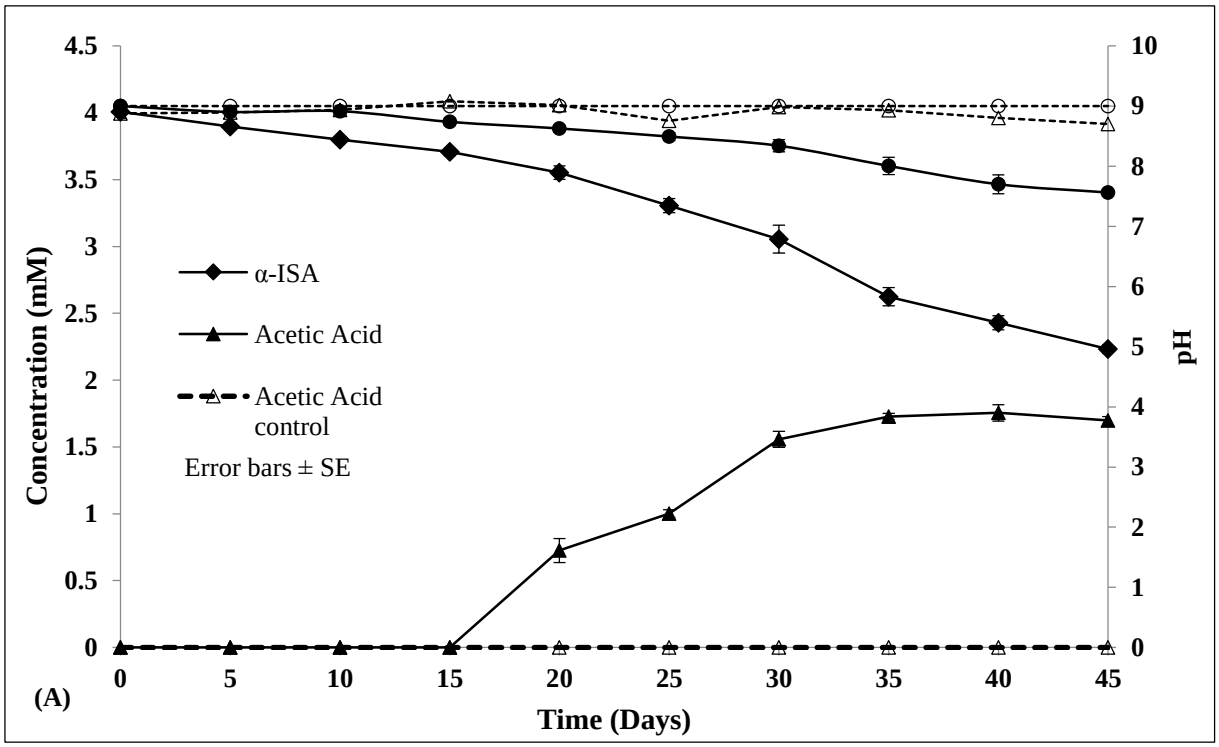


Figure 4.63 *Oceanobacillus* sp. strain Hud degradation of ISA from $Ca(ISA)_2$

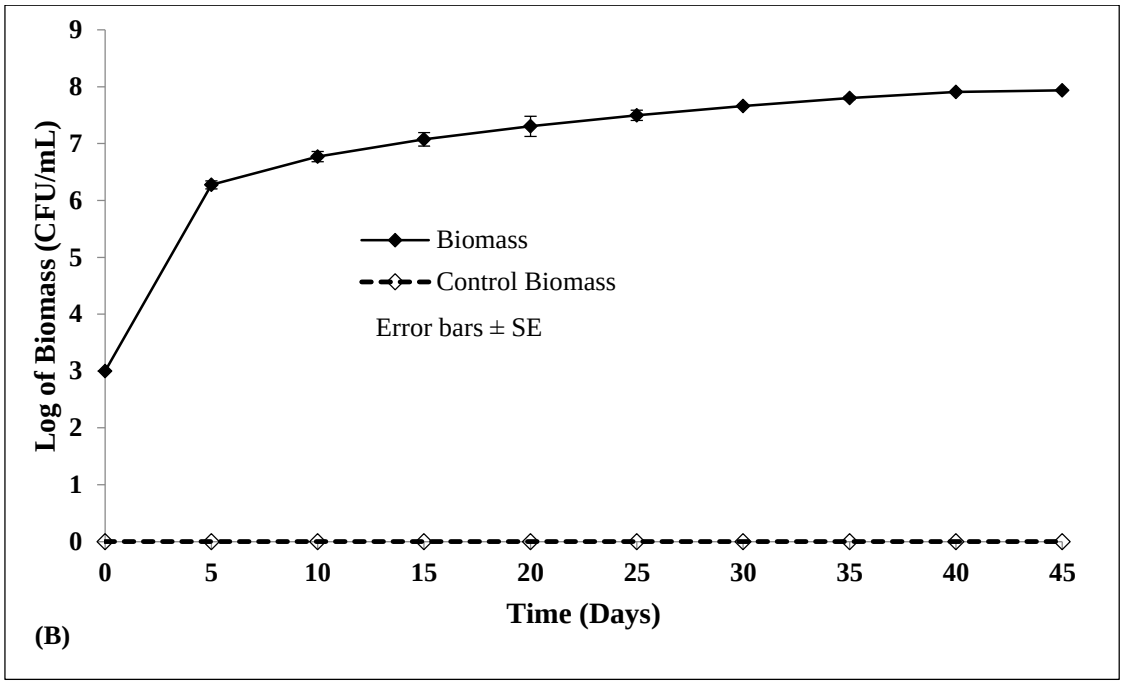


Figure 4.64 continues: ISA degradation in minimal media supplemented with vitamin and trace elements solution at pH 9.0 showing (A) ISA removal, acetate accumulation and drop in pH and (B) Biomass

The degradation profile observed in the NaISA experiment was similar to that observed with $Ca(ISA)_2$. As shown in Figure 4.65 partial removal of ISA (49%) was observed by the end of the experimental period where the first order mean rate of degradation was $1.5 \times 10^{-1} \pm 0.00\ d^{-1}$. Similar to the $Ca(ISA)_2$ degradation profile, the ISA removal from the NaISA culture was associated with a corresponding acetate accumulation as the main detectable VFA and an increase in biomass (Figures 4.65A And 4.65B). The acetic acid concentration of 1.81 mM could be detected 5 days earlier than in the $Ca(ISA)_2$ system at the end of the sampling period. Thus, out of 2.03 mM NaISA degraded, mM acetic acid was generated which represented acetic acid yield of 89%. The early removal of ISA in the NaISA system than in $Ca(ISA)_2$ system suggests Ca-ISA complex is harder to transport into cells than the Na-ISA complex. Biomass generation within the two systems as a result of the fermentation of ISA was essentially the same (Figures 4.62B). This suggests that the organism is capable of ISA assimilation

irrespective of the source. Similar to the $Ca(ISA)_2$ system, the pH drop from an initial of 9.0 to 7.68 (Figure 4.65A) could be attributed to acidification via CO_2 and acetic acid production.

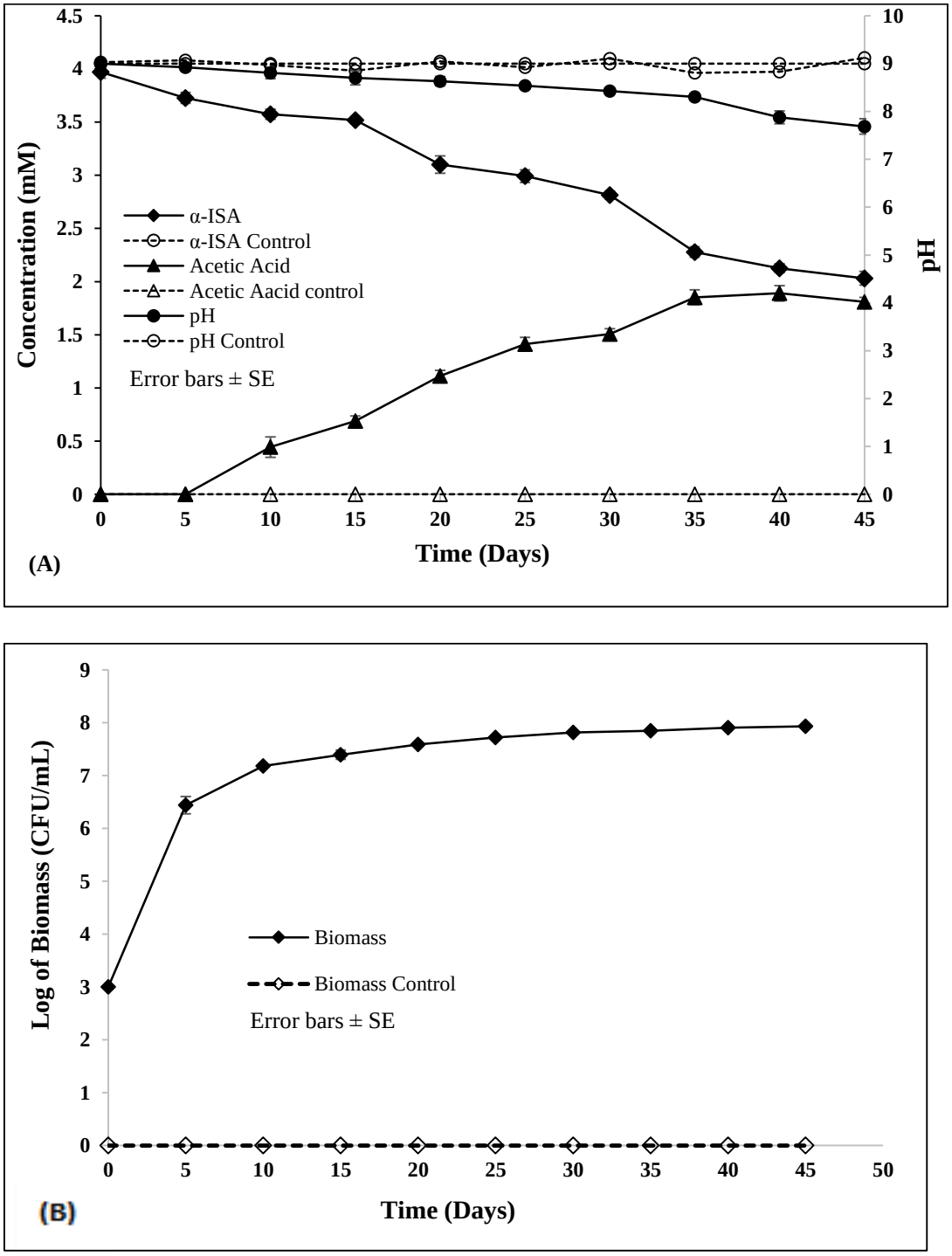


Figure 4.64 *Oceanobacillus* sp. strain Hud degradation of ISA from NaISA

ISA degradation in minimal media supplemented with vitamin and trace elements solution at pH 9.0 showing (A) ISA removal, acetate accumulation and drop in pH and (B) Biomass.

In the CDP system, both forms of ISA (α- and β-ISA) contributed to an initial total concentration of 3.98 mM in the CDP mixture (Figure 4.65A). Removal of β-ISA began after day 5 and was completed degraded by the end of day 30 at first order rate of total ISA degradation of 2.8 x 10^{-1} ± 0.00 d^{-1}. A total of 24% of the initial concentration of the α- form of ISA (2.4 mM) still remained in the system until the end of the experimental period. This suggest that β-ISA which is more soluble [413] is more amenable to microbial action than the α-ISA. Similar to the two systems discussed earlier, acetic acid accumulation was associated with the ISA degradation where a net 1.08 mM concentration was detected at the end of sampling. The degradation of ISA also coincided with pH reduction and increase in biomass as shown in Figures 4.65A and 6.5B.

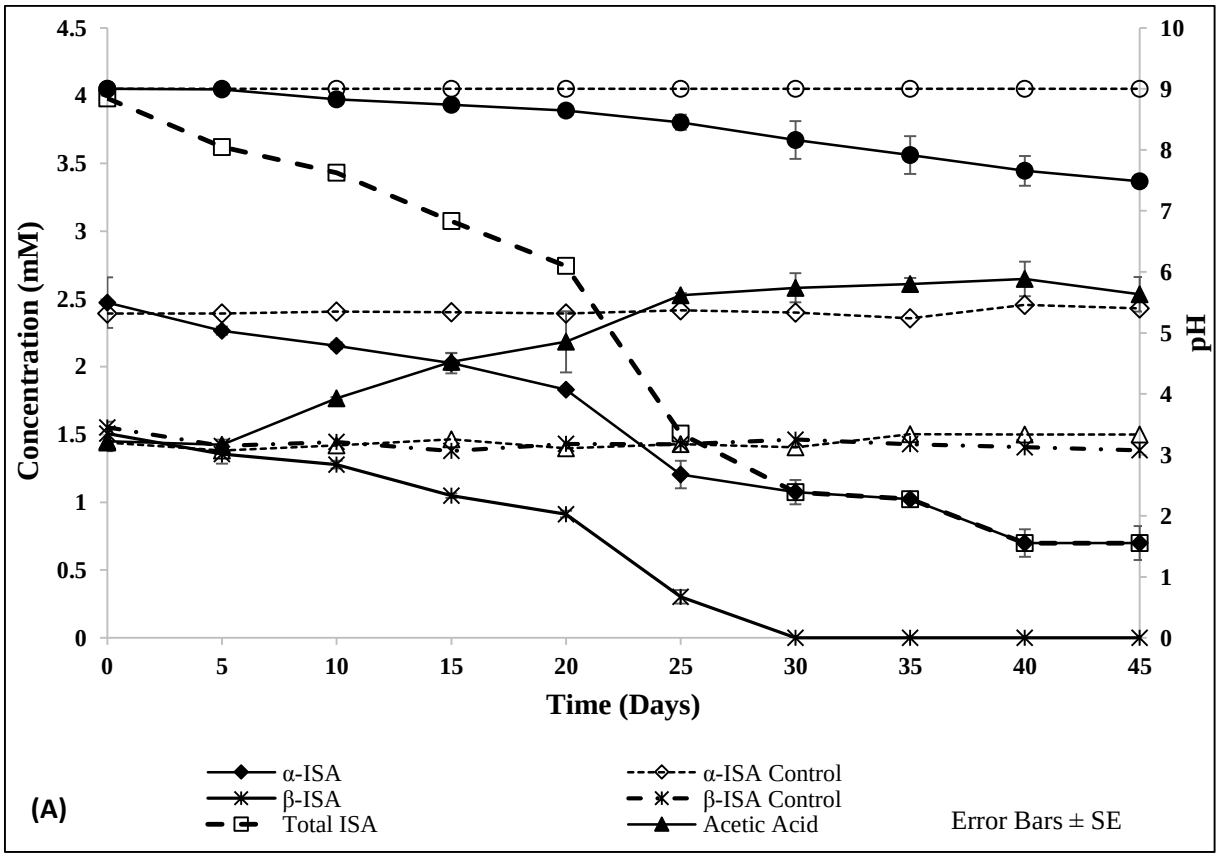


Figure 4.65 *Oceanobacillus* sp. strain Hud degradation of ISA from CDP

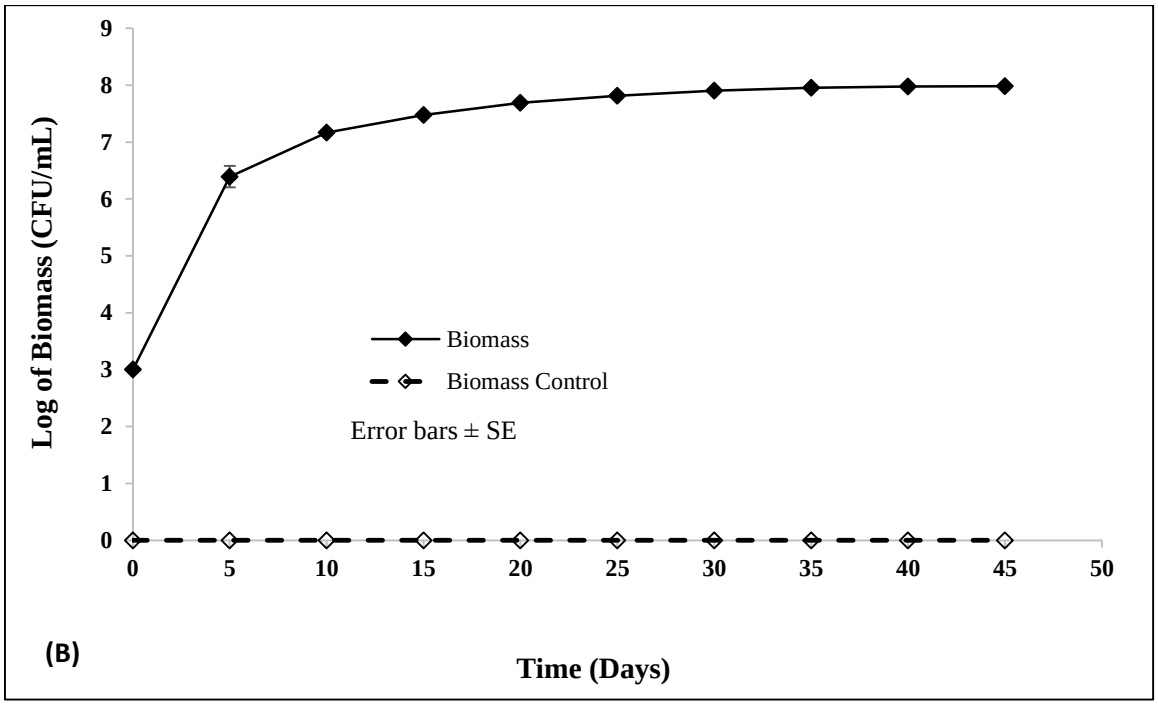


Figure 4.65 continues: ISA minimal media supplemented with vitamin and trace elements solution at pH 9.0 showing (A) ISA removal, acetate accumulation and drop in pH and (B) Biomass

Across all the systems enriched with either $Ca(ISA)_2$, NaISA and CDP, the ISA degradation profiles appeared similar. In these systems only partial α-ISA biodegradation occurred which agrees with earlier findings by Charles *et al*. [83] and Kuippers *et al*. [74]. In those ISA biodegradation studies, the authors noted that only partial α-ISA degraded in their microcosms. This observation with α-ISA microbial degradation may be attributed to the complex formation between α-ISA ligand and the metals involved, in relation to the different transport systems associated with those complexes within microbial cells.

Across the systems, evidence of fermentation processes could be observed from the generation of acetic acid as the main volatile fatty acid. This observation is linked to the presence of genes described in the genome annotation to be responsible for acetogenic activities of the strain. The drop in pH across all three systems may be associated with the production of carbon dioxide and acetic acid as products of fermentation. These observations when compared to the abiotic

control suggest that the degradation of ISA in these systems is due to microbial metabolism via an acetogenic biochemical pathway whereby CO_2 and H_2 may be produced as by-products.

4.5.25. Biochemical Characterisation of *Oceanobacillus* sp. strain Hud

Biochemical analysis of *Oceanobacillus* sp. strain Hud was carried out in order to profile the metabolic capabilities of the strain whereby Biolog, Phenotypic Microarray (PM1 and PM2, Biolog Inc. Hayward, USA), Analytical Profiling Index (API, bioMérieux) 20A test strips and traditional biochemical tests were used. The carbohydrate substrates utilised by the strain listed in Table 4.7. The ability of the strain to metabolise a wide range of carbohydrate substrates is a reflection of the presence of genes that code for the metabolism of carbohydrates as shown by the genome annotation.

The carbohydrate substrate utilisation profiles of *Oceanobacillus* sp. strain Hud with its closest neighbours, *Oceanobacillus aidingensis* (type strain AD7 25^T) and *Oceanobacillus oncorhynchi* shows both unique and shared characteristics (Figure 4.66). Whereas both *Oceanobacillus* sp. strain Hud and *aidingensis* were capable of hydrolysing Tween 80, mannitol, xylose and aesculin, *Oceanobacillus oncorhynchi* could hydrolyse only Tween 40 on its own. On the other hand, *Oceanobacillus oncorhynchi* shares similar metabolic capabilities with *Oceanobacillus* sp. strain Hud in its ability to utilise D-mannose, raffinose, and trehalose. All three species of the same clade could not hydrolyse gelatin but are similar in their ability to metabolise maltose, fructose, galactose, glucose melibiose and sucrose. *Oceanobacillus* sp. Strain Hud is biochemically unique from the other two neighbours in its capacity of utilising a range of carbohydrate substrates including *N*-acetyl-glucosamine, raffinose, arbutine, cellobiose, salicin, amygdalin, L-rhamnose, D-tagatose, D-arabinose, D-ribose.

Table 4.7 Biochemical profile of *Oceanbacillus* sp. strain Hud using API 20A and Biolog PM1 and PM2

API 20 A and Biolog PM1 and PM2					
L-Arabinose	D-Glucose	D-Ribose	α-D-Glucose	D-Glucose-1-Phosphate	D-Lactic Acid Methyl Ester
N-Acetyl-D-Glucosamine	Glycerol	D-Glucosaminic Acid	α-Keto-Butyric Acid	D-Fructose-6-Phosphate	Oxalomalic Acid
D-Galactose	D,L-Malic Acid	Tween 40	Maltose	Lactulose	Succinamic Acid
D-Trehalose	L-Fucose	L-Rhamnose	D-Mannitol	β-Methyl-D-Glucoside	L-Tartaric Acid
D-Mannose	D-Glucuronic Acid	D-Xylose	L-Glutamic Acid	Maltotriose	L-Alaninamide
Dulcitol	D-Gluconic Acid	D-Fructose	D-Melibiose	Sucrose	Hydroxy-L-Proline
D-Sorbitol	D-Fructose-6-Phosphate	Acetic Acid	Thymidine	Uridine	Sec-Butylamine
m-Inositol	Fumaric Acid	Propionic Acid	D-Cellobiose	Adenosine	Melibionic Acid
L-Alanine	D-Psicose	L-Galactonic Acid-γ-Lactone	Methyl Pyruvate	D-Galacturonic Acid	Oxalic Acid
L-Alanyl-Glycine	L-Lyxose	Inosine	L-Malic Acid	L-Galactonic	N-Acetyl-D-Glucosaminitol
Acetoacetic Acid	Glucuronamide	N-Acetyl-β-D-Mannosamine	Xylitol	Turanose	β-Hydroxy Butyric Acid
α-Cyclodextrin	β-Cyclodextrin	γ-Cyclodextrin	Dextrin	N-Acetyl-D-Galactosamine	N-Acetyl-Neuraminic Acid
Amygdalin	D-Arabinose	D-Arabitol	Arbutin	D-Fucose	3-0-β-D-Galacto-pyranosyl-D-Arabinose
D-Raffinose	Salicin	Sedoheptulosan	D-Glucosamine	Itaconic Acid	5-Keto-D-Gluconic Acid
Stachyose	D-Tagatose	aesculin			

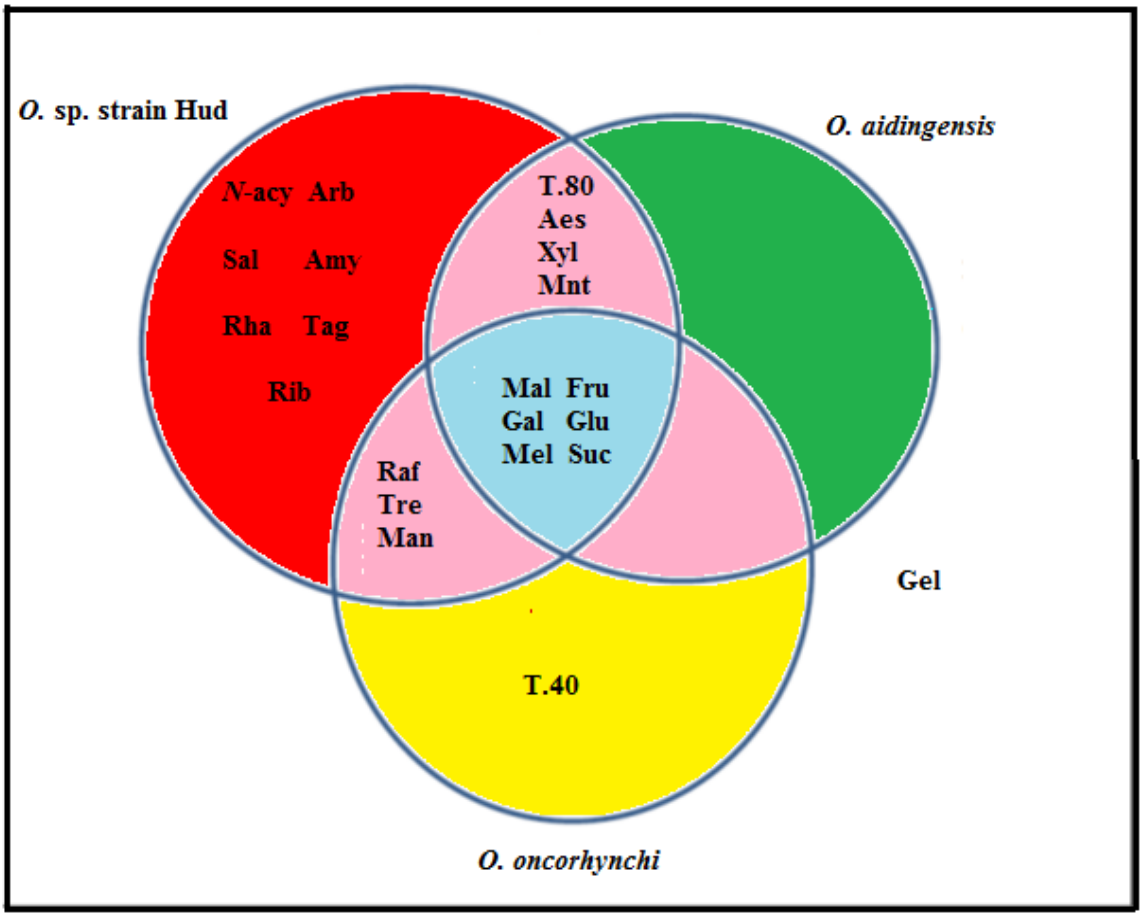


Figure 4.66 Unique and shared carbohydrate substrate utilisation among the 3 phylogenetically related species of *Oceanobacillus* sp. strain Hud

Oceanobacillus sp strain Hud (data from this study), *Oceanobacillus aidingensis* [414], *Oceanobacillus oncorhynchi* [390]. N-acy; *N*-acetyl-glucosamine, Arb; arbutine, Cell; cellubiose, Sal; salicin, Amy; amygdalin, Rha; rhamnose, Tag; tagatose, Ara; arabinose, Raf; raffinose, Rib; ribose, Tre; Trehalose, Man; mannose, T.80; Tween 80' Aes; easculin, Xyl; xylose; Mnt; mannitol; Mal; maltose, Fru; fructose, Gal; galactose, Glu; glucose, Mel; melibiose, T.20; Tween 20, T.60; Tween 60.

4.5.26. Extraction and identification of Fatty acid methyl esters (FAME) of *Oceanobacillus* sp. strain Hud

The results presented in Table 4.8 show the percentage composition of FAMEs from five species of the genus, *Oceanobacillus*. Only FAMEs of 12 or more carbon chains and their percentage compositions greater than 1% are shown and percentages greater than 10% are shown in bold. Interestingly, anteiso-pentadecanoic acid (ai$C_{15:0}$) is the major fatty acid among most species of this genus as observed in the report by Liu *et al*. [414]. In the present study, it was the second most abundant fatty acid (17.2%) after $C_{14:0}$ (49%) of the total fatty acid detected in *Oceanobacillus* sp. strain Hud. This difference in composition may be due to the difference in growth media and extraction method used or it may be a unique characteristic of this strain. The presence of long chain saturated fatty acids (17 carbons) is an indication that members of this genus may be capable of surviving in extremes of temperatures through fatty acid homeostasis [334, 415].

Table 4.8 Fatty acid percentage composition of *Exiguobacterium* species.

Fatty acid	*O.* sp. strain Hud	*O. aidingensis*	*O. oncorhynchi*	*O. kimchii*	*O. iheyensis*
$C_{14:0}$	**49.5**			5.6	
$iC_{14:0}$		**15.0**	**16.4**		**10.0**
$iC_{15:0}$		**17.5**	**13.1**	**23.9**	**28.1**
$aiC_{15:0}$	17.2	**44.5**	**28.3**	**36.2**	**32.1**
$C_{16:0}$	4.1	1.7	1.7		1.5
$iC_{16:0}$	1.2	9.2	**21.0**	6.0	8.6
$aiC_{16:0}$	5.2				
$C_{17:0}$		2.1	1.7		
$iC_{17:0}$		1.8	3.9	4.3	3.3
$aiC_{17:0}$		6.9	**12.6**	**10.8**	**17.0**

Only percentage compositions greater than 1% are shown. Percentages greater than 10% are shown in bold. Data for *Oceanobacillus* sp. Strain Hud (this study); *Oceanobacillus aidingensis* and *Oceanobacillus onchorhynchi* from [414]; *Oceanobacillus kimchi and Oceanobacillus. iheyensis* [387].

4.5.27. Extraction and identification of isoprenoid quinones (Menaquinone) of *Oceanobacillus* sp. strain Hud

The results in Figure 4.67 Show the TLC chromatographic plate showing spots of standard MK-7 and samples from *Oceanobacillus* sp. strain Hud after irradiation with UV. The result indicates that MK-7 and another menaquinone which could not be identified because it was not present in the standard were evident within the membranes of the strain. This outcome agrees with earlier findings where MK-7, has been reported as the major isoprenoid quinone with or without MK-6 [4, 9, 30].

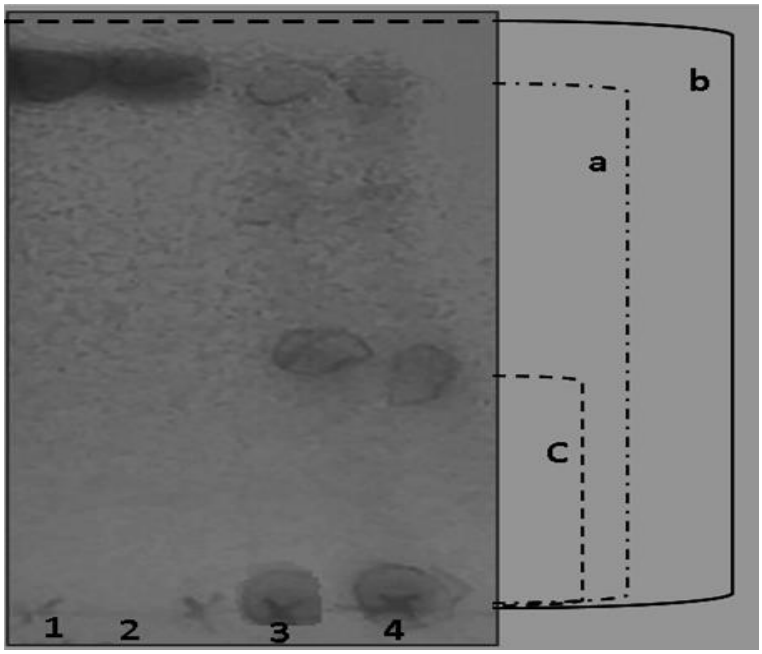


Figure 4.67 Menaquinone MK-7 standard and menaquinone sample from *Oceanobacillus* sp. strain Hud

UV irradiated chromatographic plate showing spots for 1 = MK-7 standard; 2 = MK-7 standard 3 = *Oceanobacillus* sp. Strain Hud sample 1, 4 = *Oceanobacillus* sp. Strain Hud sample 2. a = distance moved by sample; b = solvent front; c = distance moved by unknown sample

The R_F values of MK-7 and the unidentified MK were approximately 0.81 and 0.56 respectively

4.5.28. *Oceanobacillus* sp. strain Hud tolerance to pH

Some members of the genus *Oceanobacillus* have been described as either facultative or obligate alkaliphiles [416], the results presented in Figure 4.68 shows that *Oceanobacillus* sp. strain Hud is capable of growth in a wide range of pH between 6 and 12. Growth in pH 11 and 12 lagged behind for 16 and 20 hours respectively suggesting bacterial adaptation to higher pH values. This outcome is of particular significance to the nuclear waste disposal where pH is expected to rise to a high of 12.5 but later drop to pH 10.0 in the facility [8]. Optimum growth occurred at pH 9.0 suggesting that the strain is alkaliphic.

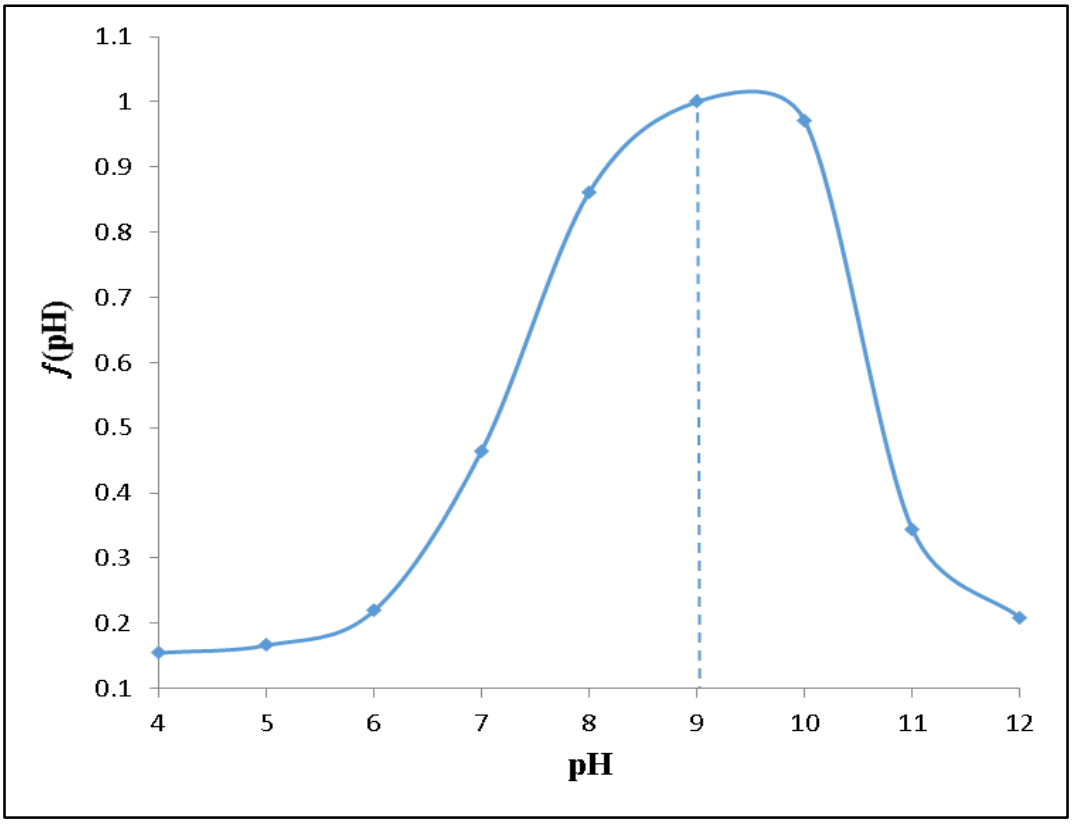


Figure 4.68 pH profiling curve showing the optimum pH for growth of *Oceanobacillus* sp. strain Hud

4.5.29. *Oceanobacillus* sp. strain Hud tolerance to heavy metals

Growth response to different concentrations (0.5-5 mM) of heavy metal ions (Zn^{2+}, Co^{2+}, Cu^{2+}, Ni^{2+}, Cd^{2+} and Pb^{2+}) was used as an indicator of tolerance of the strain to heavy metals. There is limited literature available on the metal tolerance of *Oceanobacillus sp*. In one study, Vela-Cano *et al*. reported that an isolate with 100% similarity to *Oceanobacillus sp*. FR667183.1 was recovered from a microcosm seeded with sewage sludge compost tea and amended with increasing concentrations of Pb, Zn, Cd and Cu metals [417]. Mulik and Bhadekar [392] also reported that an isolate recovered from Antarctic oceanic region designated *Oceanobacillus* sp. 39 was capable of tolerance and the bioremoval of heavy metals including Cd^{2+}, Cr^{2+}, Ni^{2+} and Pb^{2+}.

The present study extends the available knowledge regarding the tolerance of members of this genus to heavy metals. While nickel and copper appeared to be the most tolerable metals, cobalt seems to be most toxic. The first values for MIC and NIC of the strain within the genus *Oceanobacillus* for the respective metals are shown in Table 4.9. The MIC for Pb^{2+} could not be determined within the limits of this experiments.

Table 4.9 The MICs and NICs of five heavy metals for *Oceanobacillus* sp. strain Hud

Metal	MIC (mM)	NIC (mM)
Zn^{2+}	1.41	0.09
Ni^{2+}	4.09	2.09
Cu^{2+}	4.50	2.32
Co^{2+}	0.57	2.3×10^{-3}
Cd^{2+}	2.78	0.31
Pb^{2+}	undetermined	undetermined

4.5.30. *Oceanobacillus* sp. strain Hud tolerance to salt (NaCl)

Most species of *Oceanobacillus* have been described as either halotolerant [390] or halophilic [414, 416] depending on the site where the strain was isolated. In these studies the isolates have been shown to grow within a wide range of salt concentration from 0-25%. In the current study, whilst the strain was isolated from a hyperalkaline calcium dominated environment, it still demonstrated tolerance to a wide range of salt concentration between 1 and 15% (Figure 4.69), with optimum growth occurring at approximately 5.5% salt concentration. The current results are consistent with earlier findings where *Oceanobacillus aidingensis* tolerated salt within a range of 0-21%, *Oceanobacillus oncorhynchi*; 0-22%, *Oceanobacillus neutrophilus*; 0-17%; *Oceanobacillus locisalsi*; 25% *Oceanobacillus sojae*; 0-15% [414].

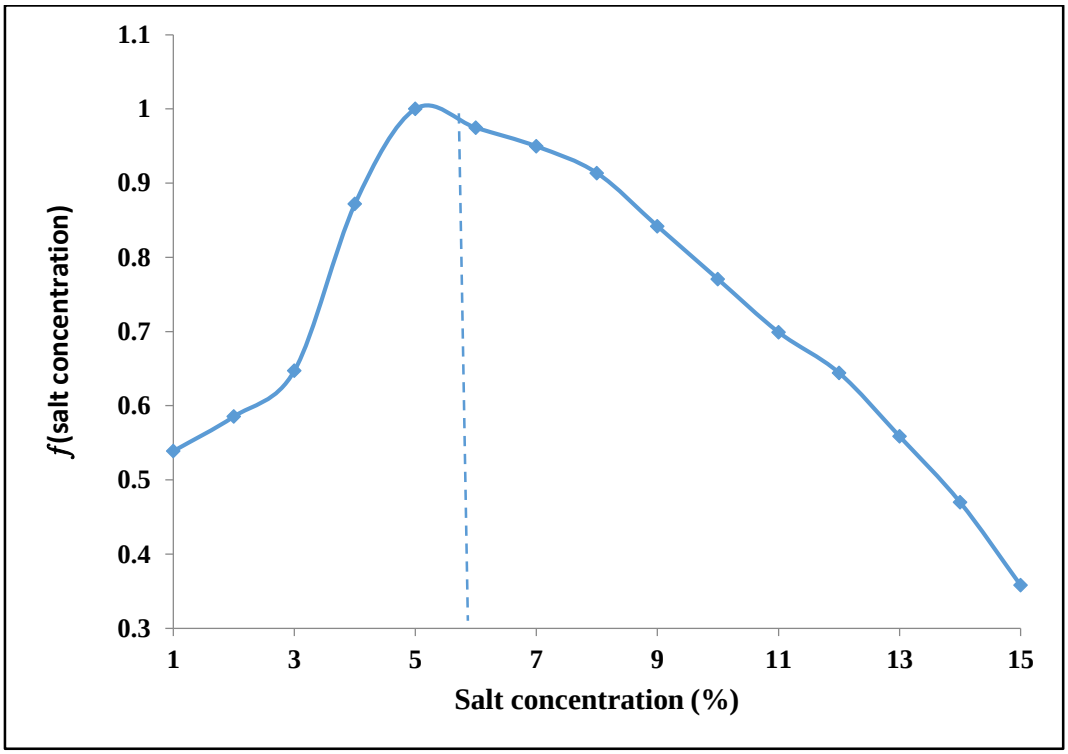


Figure 4.69 *Oceanobacillus* sp. strain Hud growth response to increased salt concentration

Growth was expressed as a function of salt concentration.

Having shown the high tolerance to salt and potential for biodegradation of ISA collectively, this data suggest that *Oceanobacillus* may be a useful candidate for consideration in the disposal of radioactive waste especially in those underground repositories based in salt formations.

4.5.31. Assessing the combined effect of pH and temperature on the growth of *Oceanobacillus* sp. strain Hud

The impact of pH on the growth of *Oceanobacillus* sp. strain Hud across a wide range of temperatures was investigated. The results indicated (Figure 4.70) that the isolate was incapable of growth at either 10°C or 45°C across a pH range of 4 to 12. However, having been shown to grow at high pH values and possessing genes coding for response to cold- and heat-shock may suggest that a reasonable time limit may be required for growth under these conditions. Under the synergistic effect of pH and temperature, optimum growth occurred at pH 9.5 at 30°C. Across all temperatures that supported growth, growth optimality occurred at pH 9.0 thereby highlighting on the alkaliphilic properties of the strain.

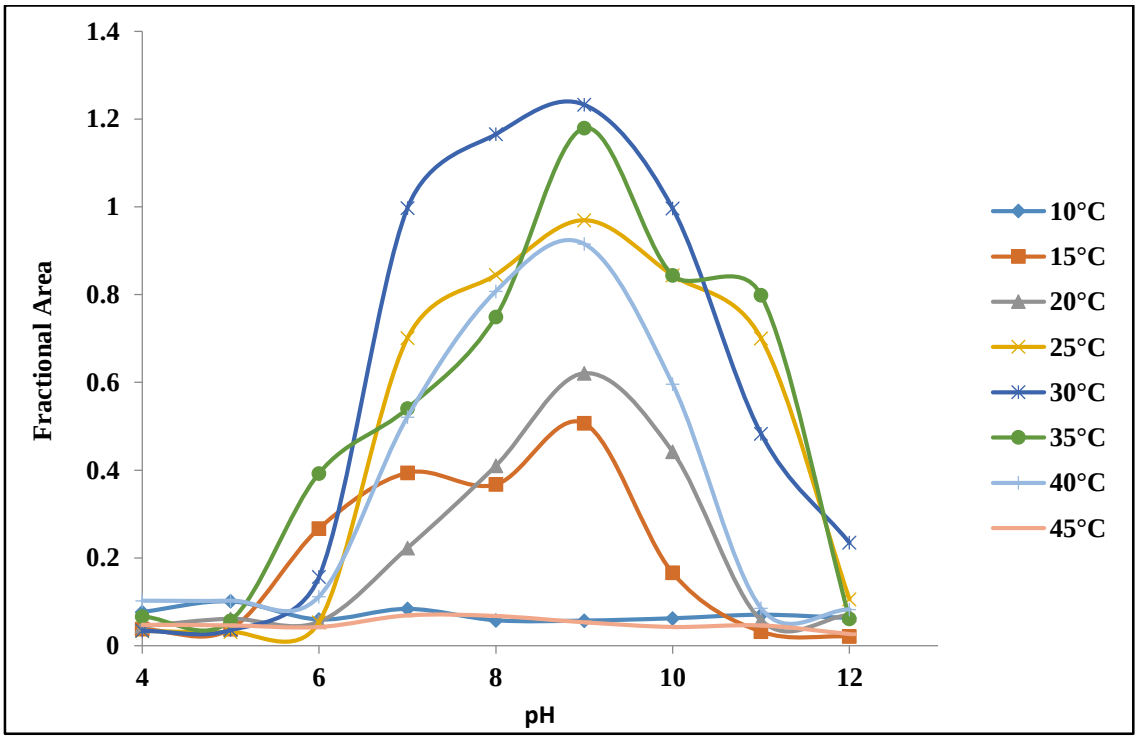


Figure 4.70 Effect pH on growth of *Oceanobacillus* sp. strain Hud across a wide temperature range

The combined effect of pH and temperature on the growth of *Oceanobacillus* sp. strain Hud is presented in Figure 4.71 where the peak of the 3D surface plot represents the highest growth attained by the strain which occurred at pH 9.0 at 30°C. The base represents those parameters within which no growth occurred such as pH 4 to pH 5 between 10 and 45°C.

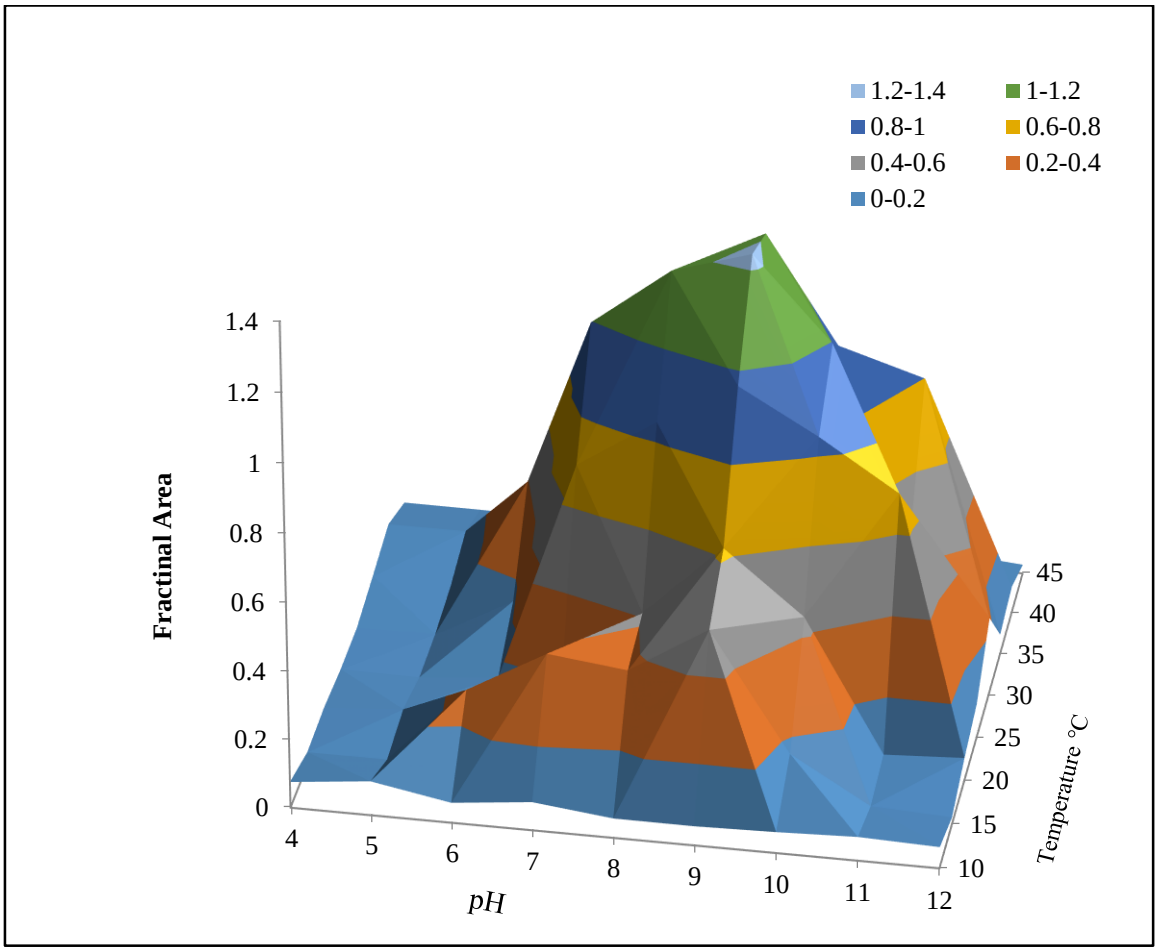


Figure 4.71 A 3D surface plot showing the synergistic effects of pH and temperature on the growth of *Oceanobacillus* sp. strain Hud

4.5.32. Biofilm formation by *Oceanobacillus* sp. strain Hud

4.5.33. Calcoflour white staining

Calcofluor white staining (Figure 4.72) of *Oceanobacillus* sp. strain Hud showed biofilm material which is stained light-blue around the edges of the cells.

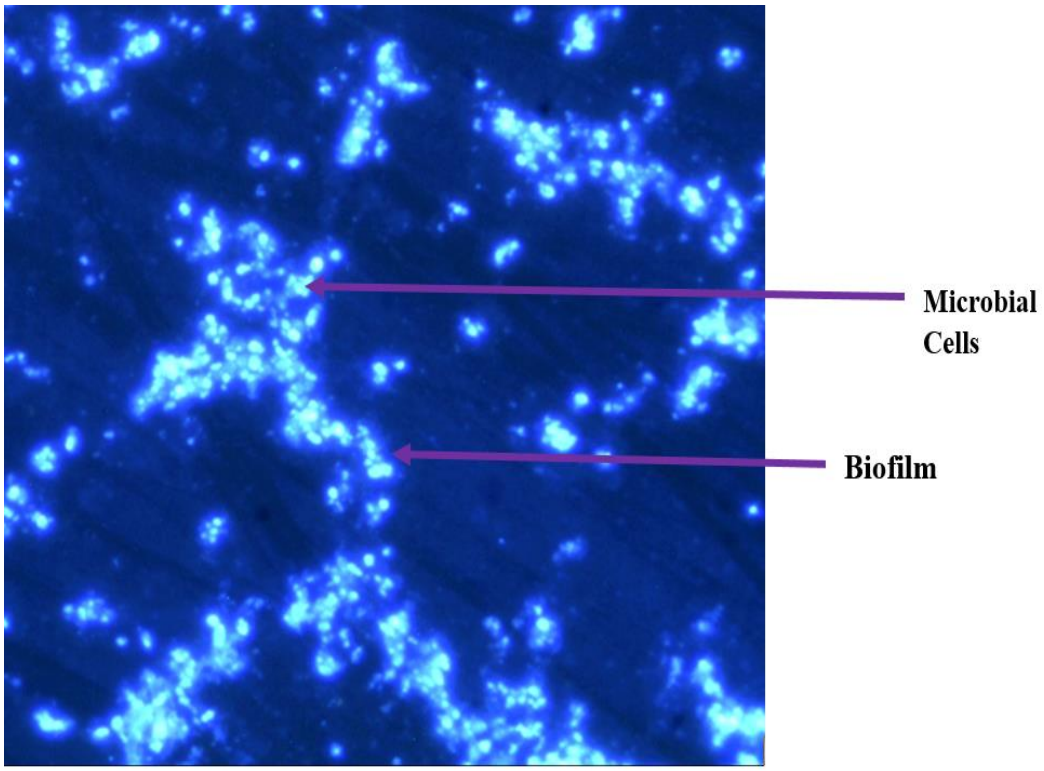


Figure 4.72 Calcofluor staining of biofilm produced by *Oceanobacillus* sp. strain Hud.
Cells are stained bright-green whereas the biofilm (around cells) are stained light-blue

The biofilm formation and adherence assay (Figure 4.73) was performed following 18 hours of incubation. The mean absorbance of the control (ODc) was 0.187 whereas that of the strain was 2.107. Analysis of paired T-test showed that there is a significant difference between the absorbance of the control and the experimental (P = 1.63 E-7). This difference represented about 11 fold increase in the experimental value over the control which according to the biofilm adherence capability classification meant that the strain produced a strong

adherent biofilm [230]. Isolation of exopolysaccharides from the biofilm was however unsuccessful.

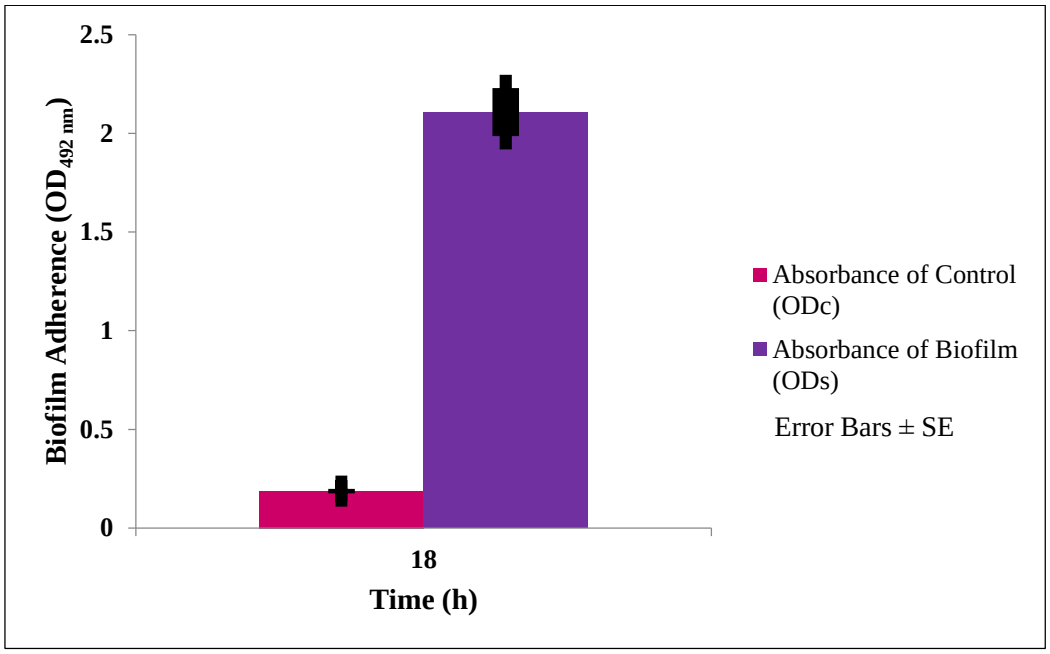


Figure 4.73 Biofilm adherence of *Oceanobacillus* sp. strain Hud

Biofilm adherence onto abiotic surface was observed following 18 hours of incubation. ODc is the optical density of control and ODs is the optical density of the biofilm exclusively.

4.5.34. Key findings

- The genome annotation of *Oceanobacillus* sp. strain Hud has showed the presence of genes that code for the metabolism of several carbohydrate
- The strain has shown the potential to degrade ISAs under anoxic, alkaline conditions representative of the GDF
- The biodegradation of ISAs results in the generation of acetic acid suggesting an acetogenic pathway similar to the one described by Eiteman and Altman [412] and Schroder *et al*. during a study on *Thermotoga maritima* [330]
- The main fatty acids of *Oceanobacillu* sp. strain Hud $C_{14:0}$, $aiC_{15:0}$, $C_{16:0}$ $iC_{16:0}$ and $aiC_{16:0}$
- The major menaquinone is MK7

- ❖ The strain demonstrated broad biochemical capabilities along with significant salt, pH and heavy metal tolerances
- ❖ The strain has been shown to be haloalkaliphilic and has demonstrated the capability as a strong adherent biofilm former
- ❖ The main physiological and biochemical characteristics of *Oceanobacillus* sp. strain Hud are presented in Table 4.10 together with those of other four species from the literature

Table 4.10 Physiological and biochemical characteristics of *Oceanobacillus* sp. strain Hud compared with other related species

Characteristics and biochemical profile	***Oceanobacillus* species**				
	1	**2**	**3**	**4**	**5**
Colony colour	**cream**	cream	white	Cream	Creamy white
Motility	**+**	+	+	+	+
Flagella	*****	Polar	Peritrichous	Polar	Peritrihous
Oxidase	**+**	-	+	+	+
Catalase	**+**	+	+	+	+
NaCl tolerance (%)	**15**	21	22	15	21
Optimum growth temperature (°C)	**30**	33-37	30-36	35	30
Optimum pH	**9.0**	7.0-7.5	9.0-10	9.0	7.0-9.5
Carbohydrate utilization					
N-Acetyl glucosamine	**+**	-	-	+	-
N-Acetyl mannosamine	**+**	-	-	*	-
Cellobiose	**+**	-	+	+	-
Melibiose	**+**	-	+	*	-
Raffinose	**+**	-	+	-	-
D-Glucose	**+**	+	+	+	+
D-Galactose	**+**	+	+		-
D-Mannitol	**+**	-	*	*	*
D-Ribose	**+**	-	*	+	*
D-Mannose	**+**	-	+	*	+
D-trehalose	**+**	-	+	*	-
D-Sorbitol	**+**	-	-	+	-

1. *Oceanobacillus* sp. strain Hud (this study), 2. *Oceanobacillus aidingensis* [414], 3. *Oceanobacillus oncorhynchi* [390], 4. *Oceanobacillus kimchii* [387] and 5. *Oceanobacillus iheyensis* [384]

4.5.35. Isolation of alkaliphilic bacteria from pH 9.0 methanogenic polymicrobial microcosms

4.5.36. Rational for the study

ISA degradation by bacterial communities from anthropogenic hyper-alkaline soil has been the focus of current research. As a result, ISA biodegradation profiles involving pure bacterial isolates is lacking in literature. The aim of this section of the study therefore was to isolate in pure cultures individual cultivable species which form part of the ISA degrading polymicrobial consortia within the microcosms fed with both ISA and CDP for investigation into their ISA degradability potentials. This will allow for subsequent study into the molecular mechanisms associated with ISA assimilation by microorganism which is currently unavailable in literature.

4.5.37. *Shewanella* sp. strain IK Hud

Members of the genus *Shewanella* comprises several species of Gram-negative, motile rods which are commonly found in marine and freshwater environments [418]. They belong to the y-subclass of the Proteobacteria within the family Vibrionaceae [419] with *S. putrefaciens* as type strain [419]. Strains are mostly facultative anaerobes but usually maintains respiratory metabolism where under anoxic biogeochemical conditions, oxidation of organic matter can be coupled to the reduction of NO^-, Fe^{3+} and sulphur compounds [418]. Most species have been associated with biodegradation of organic compounds using terminal electron acceptors including Fe^{3+}, Mn^{4+} and NO^- [420-423]. Some species have been reported to have the ability to utilize arsenate, thiosulphate in addition to nitrate, iron or manganite across a broad range of temperatures and pH under anaerobic conditions [424]. The authors also reported the ability of the strain to tolerate the presence of heavy metals including (Cd, Co, Cr, Cu, Mn, Mo, Se, V and Zn under anaerobic conditions within which they grow and utilize arsenate thereby producing arsenic (III) sulfide (As_2S_3).

In the present study, the isolate designated *Shewanella* sp. strain IK Hud was isolated from a CDP-driven methanogenic polymicrobial microcosm operated at alkaliphic anaerobic conditions which was under investigation of ISA biodegradation. Ability of the strain to grow on solid mineral media with CDP as sole carbon source coupled to its characteristics in literature suggest the strain may be useful in ISA degradation studies relating to nuclear disposal.

Analysis of the 16S rRNA gene sequences showed that the strain shared 99% sequence homology with *Shewanella putrfaciens* strain Hammer 95 and *Shewanella profunda* strain LT13a. Phylogenetic analysis (Figure 4.74) of associated taxa showed that the strain clustered 88% around *Shewanella putrfaciens* in a bootstrap test of 1000 replications indicating evolutionary relation between the two strains. The inferred phylogeny also suggested that the two strains were phylogenetically related to *Shewanella profunda* strain LT13a as both clustered 95% in the percentage replicate trees.

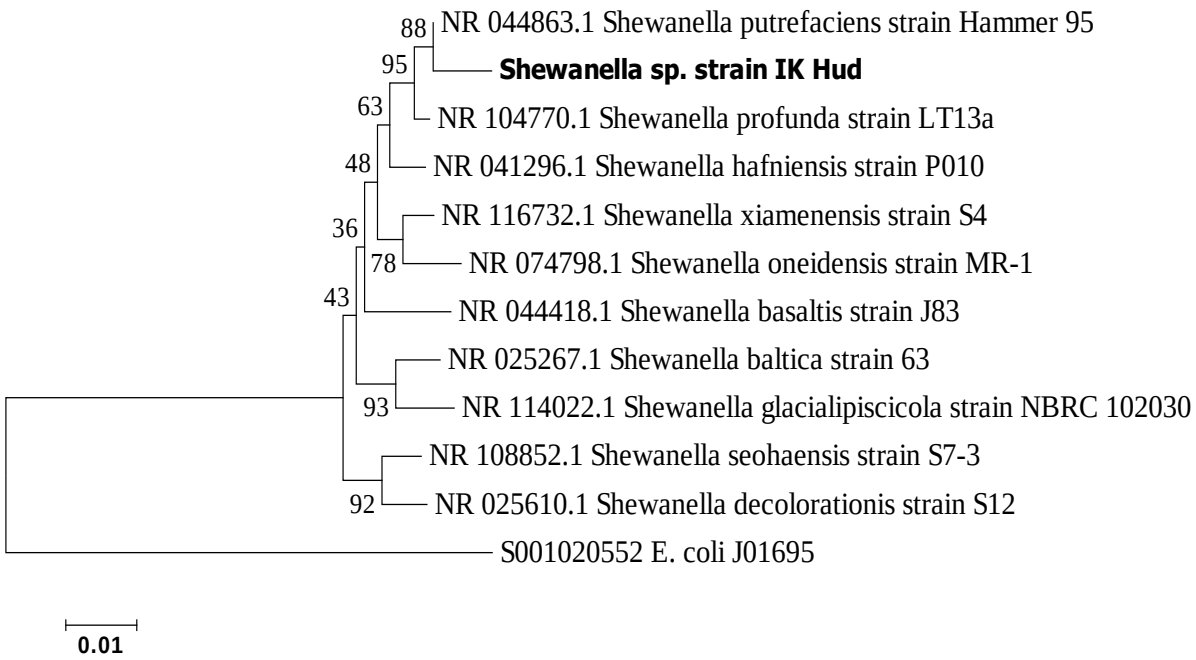


Figure 4.74 Phylogenetic tree of *Shewanella* sp. Strain IK Hud and other related species

Neighbour-Joining tree based on 16S rRNA gene sequences showing the phylogenetic relationship between *Shewanella* strain IK Hud and other species of the genus. *E. coli* J01695 was used as outgroup. Bar, 1 nucleotide substitution per 100 nucleotides

4.5.38. *Trichococcus* sp. strain IK Hud

The genus *Trichococcus* was first described in 1984 when some novel species associated with the genus were isolated from a bulking sludge [425]. The type strain is *T. flocculiformis*. To date, there are only five known species [426] namely *T. pasteurii, T. patagoniensis, T. collinsii, and T. palustris* in addition to the type strain. Members of the genus are filamentous Gram positive bacteria consisting of several hundred coccoid cells where these filaments tend to form small white pellet flocs during cultivation without shaking in broth media [425].

Members of the genus have been reported to show various features with the potentials for biotechnological applications including environmental bioremediation, production of chemicals with antifreeze properties, production of extracellular polysaccharides and production of lactic acid from a wide range of carbohydrate substrates [426, 427]. Van-Gelder *et al*. [428] described a novel

isolate from a methanogenic bioreactor sludge of the genus *Trichococcus* with metabolic capacity of fermenting glycerol as the sole energy and carbon source to generate 1,3-propanediol as main product, with lactate, acetate and formate as minor products. This further expands knowledge on the bioremediation potentials of the strain.

In this study the strain was isolated from a complex methanogenic polymicrobial ISA-degrading consortia that was operating under anaerobic condition at pH9.0. The strain was capable of growth on solid mineral media with $Ca(ISA)_2$ as the only carbon source suggesting its ISA biodegradation potential in pure culture.

Analysis of the 16S rRNA gene showed that the strain designated *Trichococcus* sp. strain IK Hud shares 99% sequence similarity with all the five known species. However, phylogenetic analysis (Figure 4.75) of the isolate showed that this strain was closely related more to *T. flocculiformis* as the percentage of the replicate trees in a bootstrap test showed that the two strain clustered together by 93%. The two organisms both clustered 51% with *T. pasteurii*.

Filamentous microorganism have been reported to play a significant role in floc and biofilm formation where they produce amyloid-like adhesins during floc formation and serve as backbone of biofilm matrix [429, 430]. Together, these characteristics suggest that *Trichococcus* sp. strain IK Hud played important role within the community that degraded ISA and that within the repository where the formation of niches are required members of this genus could be useful.

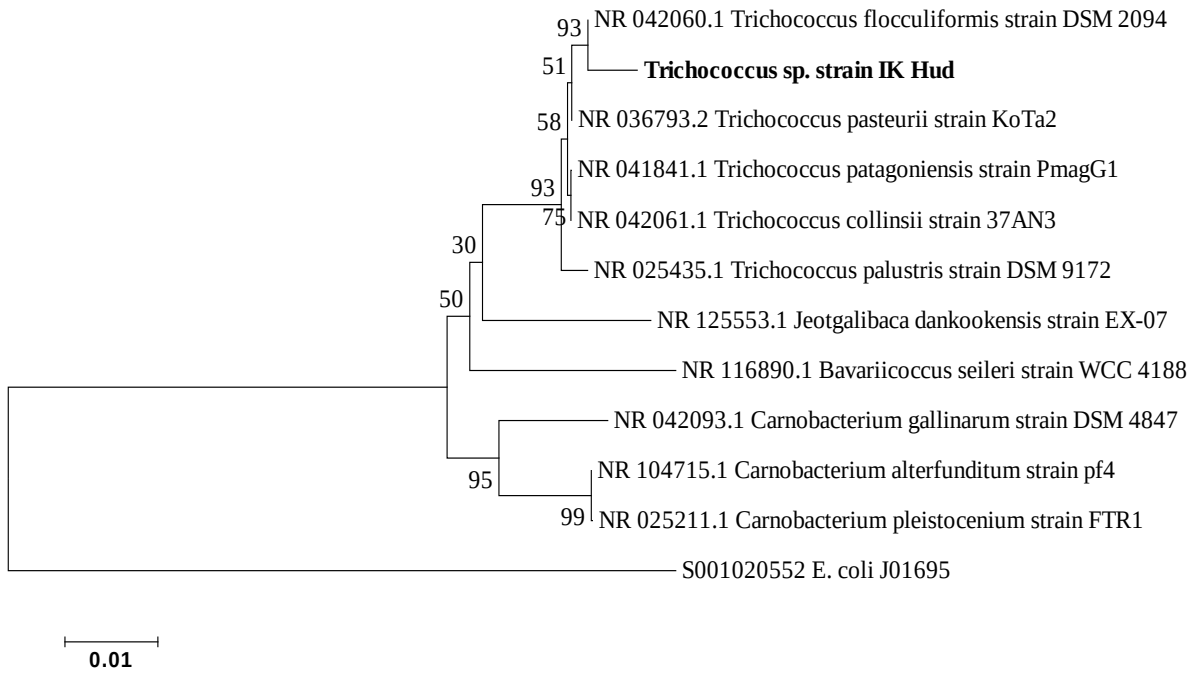


Figure 4.75 Phylogenetic tree of *Trichococcus* strain Hud and other related species

Neighbour-Joining tree based on 16S rRNA gene sequences showing the phylogenetic relationship between *Trichococcus* strain IK Hud and other species of the genus. *E. coli* J01695 was used as outgroup. Bar, 1 nucleotide substitution per 100 nucleotides

4.5.39. *Bacillus* sp. strain IK Hud

The genus *Bacillus* was first described by Cohn in 1872 when he renamed *Vibrio subtilis* that had been described by Ehrenberg in 1835 as *Bacillus mycoides* [431]. Currently, the genus represents one of the large and diverse groups associated with the family Bacillaceae. The most distinguishing feature of members of this genus the production of endospores resistant to both chemical and physical agents which underscores their ubiquity in the natural environment [432]. Earlier studies have reported of impressive physiological states which enable members of the genus to degrade a wide range of substrates produced by both plants and animal including cellulose [433], starch, hydrocarbons and proteins [432]. Some species have been found to be heterotrophic nitrifiers, denitrifiers, nitrogen fixers, as well as facultative chemolithotrophs. Some members of the genus are acidophiles, alkaliphiles and psychrophiles while others are thermophiles [434, 435]. Others

have been noted for their detoxification potentials of heavy metals, including lead, chromium, and copper, by biosorption [436].

The current strain designated *Bacillus* sp strain Hud was isolated from a CDP-driven methanogenic, polymicromicrobial community that was operating at a starting pH 9.0. It was found to be facultative anaerobe, Gram positive rods which formed radial filaments colonies and could grow on solid mineral media with only CDP as the sole carbon source; suggesting the ability to utilize ISA. 16S rRNA gene sequence showed that the strain shares 99% percent sequence homology with *Bacillus mycoides* and other related species. Phylogenetic analysis (Figure 4.76) however showed that the *B.* strain IK Hud is more closely related to *B. mycoides* than other species as the bootstrap test of 1000 replicate trees showed that the two strains clustered by 89%. The closest neighbours of the two were *B. thuringiensis* and *B. toyonensis*. Given the characteristics of members of the genus *Bacillus* coupled to the ability to form resistant endospores suggest that this strain is a potential candidate for further investigation in ISA degrading experiments.

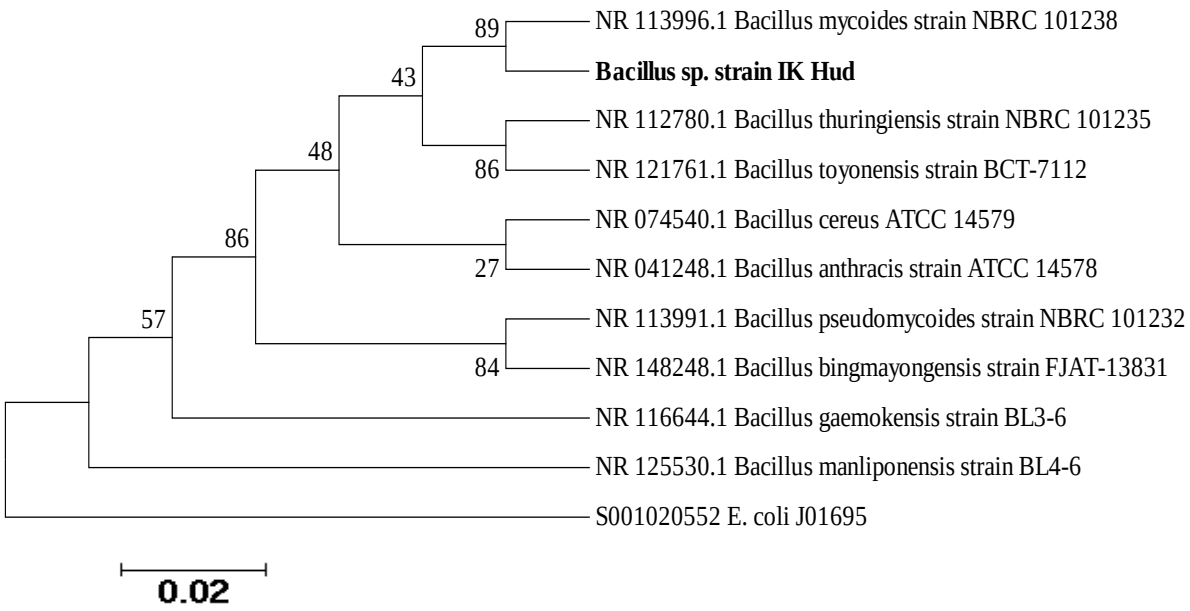


Figure 4.76 Phylogenetic tree of *Bacillus* sp. strain IK Hud and other related species

Neighbour-Joining tree based on 16S rRNA gene sequences showing the phylogenetic relationship between *Bacillus* sp. strain IK Hud and other species of the genus. *E. coli* J01695 was used as outgroup. Bar, 2 nucleotide substitution per 100 nucleotides

Chapter 5

5. Conclusions

5.1. Overview

The United Kingdom has a substantial amount of nuclear waste with LLW and ILW forming the bulk of the waste. These wastes need to be disposed of permanently and the Government's current plan is geological disposal in a GDF – an engineered facility in a deep vault underground to retain the waste without the intention to retrieve [2].

Upon closure of the facility, chemical evolution within the facility will lead to the degradation of cellulosic materials buried together with the waste thereby generating soluble complexants such ISA [18] which has been reported to enhance radionuclide migration through the formation of complexes. This will compromise the radionuclide retention capacity of the GDF.

As an organic compound, ISA represents a potential organic substrate for microbial biodegradation within the GDF which has the potentials to be colonised by microorganisms both during the constructional and operational phases [299]. However, a major limitation to microbial colonization of the GDF is the high pH that will prevail for a long time.

Model studies have suggested that ISA degradation is possible under the GDF conditions but these studies have focused on coupling the calcium salt of α-ISA ($Ca[(\alpha\text{-}ISA)_2]$) metabolism with biogeochemical processes involving terminal electron acceptors where the $Ca[(\alpha\text{-}ISA)_2]$ is used as an analogue for CDP [70, 74]. Furthermore, most studies have employed the use of compounds containing NH_4^+ in their growth media although earlier studies have noted that some essential growth nutrients including nitrogen and phosphorus will be lacking in the GDF [236]. Investigation into the characteristics and degradation profiles of ISAs under alkaliphilic anaerobic conditions by pure microbial isolates is lacking in the literature.

This study aimed at investigating microbial metabolic capacity to biodegrade ISAs under a range of alkaliphilic, anaerobic conditions, assessing the impact of $Ca(ISA)_2$, CDP and NH_4^+ on ISA degradation and resultant microbial community evolution. In addition, the isolation and characterization of alkaliphilic, microorganisms with ISA degradability potentials were also carried.

5.2. Conclusion 1

5.2.1. Microbial community degradation of ISA under aerobic and anaerobic conditions

The aim of this section of the study was to first investigate whether microbial consortia within an anthropogenic hyperalkline soil could degrade ISA at high pH of 10 under aerobic and anaerobic conditions where $Ca(ISA)_2$ and CDP were used as sole carbon source. Microorganisms within the seeding soil were able to degrade ISAs completely from the microcosms under both conditions. These findings agreed with earlier studies and highlighted on the potentials of microorganism in bioremediation of ISA.

5.3. Conclusion 2

5.3.1. The impact of nitrogen source and the form of ISA on microbial community evolution

In ISA degradation studies, the two major sources of ISA used are the $Ca(ISA)_2$ and CDP with different kinds of ammonium compounds at different concentrations being used as the preferred nitrogen source. However, these ammonium compounds are not likely to be present in the nuclear waste repository. The aim of this section of the study was to assess the impact of the form of ISA employed and the type of nitrogen source used on the evolution of ISA degrading microbial communities in relation to a nitrogen amended and nitrogen free system under alkaliphilic, anaerobic conditions.

The chemical analysis of the established consortia showed that in both NH_4^+ amended and NH_4^+-free systems, microorganisms within the consortia were capable of ISA biodegradation irrespective of the carbon source. Across the microcosms, ISA biodegradation was accompanied by the generation of acetic acid, CO_2 and H_2. The CO_2 yield were lower than the expected due to high pH enhancing the solubility of CO_2. Together with acetic acid products, the CO_2 acidified the growth media, which led to a drop in pH thereby creating a microbial niche for those microorganisms, which could not grow at high pH. The NH_4^+-free systems generated more biomass than the system amended with NH_4^+, which indicated that some level of ammonium toxicity was reducing the overall biomass in those systems. The yields of methane in the systems amended with NH_4^+ were higher than the NH_4^+-free systems but this was attributed to the activities of autotrophic methanogens.

Analysis of the established consortia showed a complex community of bacterial and archaeal domains, dominated by taxa associated with Firmicutes while members of Proteobacteria dominated the background soil. The enrichment cultures selected for a high number of bacteria, which led to alpha diversity analysis favouring them against the background soil. The presence of NH_4^+ was found to support a more diverse microbial population that were phylogenetically related.

The archaeal domain across the systems was composed mainly of the Methanobacteriales and Methanosarcinales, which were, involved in both acetogenic and hydrogenotrophic activities. In association with the bacterial domain, ISA was broken down by the archaea into CH_4^+, CO_2 and H_2 where the main biochemical pathway for the breakdown was a biphasic anaerobic digestion of ISA substrate involving aceticlastic and hydrogenotrophic methanogenesis.

The background soil was found to contain soil ammonium ions at concentrations that could potentially be a source of nitrogen for microorganisms. It also contained

nitrogen fixing bacteria hence, the presence or absence of NH_4^+ did not affect the degradation of ISA but it impacted on the evolution of the microbial community.

The type of carbon source ($Ca(ISA)_2$ or CDP) did not affect ISA degradation however, the established microbial communities under these carbon sources were significantly different from each other and from the background soil.

5.4. Conclusion 3

5.4.1. Microbial community ISA biodegradation under elevated pH conditions

Previous authors have reported on ISA biodegradation up to pH11.0 but the associated communities have not been described. The aim of this section of the study was therefore to investigate the degradation profiles of microbial communities associated with ISA degradation at higher pH (pH10.0-11.5) and how the high pH impacted the evolution of these microbial communities.

In the $Ca(ISA)_2$ system at pH10.0, complete degradation of ISA occurred by the end of the study. In the higher pH ranges however, partial but significant ISA degradation occurred. Across all systems, the degradation was accompanied by the generation of biomass, production and accumulation of acetic acid and evolution of gases including CH_4, CO_2 and H_2. There was no CH_4 production across both systems at pH11.5 but only CO_2 and H_2 due to the high pH suggesting that methanogenesis was limited by elevated pH.

The ISA degradation profile and the generation of other parameters were less with increasing pH which indicated that pH is a major barrier to microbial activities under repository conditions.

Microbial community analysis showed that in spite of the harsh pH stress, these microcosms maintained quite a diverse population of microorganisms which reflect the ability of some species to survive adverse environmental conditions.

Each of the carbon substrate selected for taxa that were phylogenetically related in spite of the pH indicating that the type of carbon source played a role in microbial community evolution. Complex microbial consortia were dominated mostly by Clostridiales where individual species have been reported to be capable of growth under harsh conditions and capable of fermenting a number of carbohydrate substrates.

The production of CH_4 was evidenced by the presence of archaeal community which was dominated mainly by Methanobateriales and Methanosarcinales. These groups of methanogens are noted for the acetogenic and hydrogen-scavenging methanogenesis. The presence of methanogens in the pH11.5 system but no production of methane indicates that activities of methanogenic bacteria are suppressed by high pH. While pH is a major limiting factor in microbial processes, alkaliphilic microbial consortia involving both bacterial and archaeal domains exist in the natural environmental that are capable of ISA degradation. The production of gaseous products is likely to impact on the pressurization of the nuclear waste disposal facility.

5.5. Conclusion 4

5.5.1. Characterization of *Exiguobacterium* sp. strain Hud.

Exiguobacterium sp. strain Hud was isolated from a fermentative alkaliphilic methanogenic polymicrobial microcosm that was operating at pH10.0. Several authors have reported of the ability of members of this genus to survive extreme conditions including temperature, pH, heavy metals and radiations. In addition, they have been noted for their ability to metabolise a range of carbon substrates. The annotation of the whole genome sequencing of *Exiguobacterium* sp. strain Hud showed the presence of genes that confirmed the afore-mentioned

characteristic. The aim of this section of the study was to characterize *Exiguobacterium* strain Hud and assess its ability to degrade ISA.

The strain was found to be phylogenetically related to *Exiguobacterium mexicanum* (99% sequence homology). It was identified to be facultative anaerobic, Gram positive pleomorphic rod and was capable of metabolising a wide range of carbon substrates. The organism was found to be motile and both catalase and oxidase positive. The major fatty acids being $C_{12:0}$, $iC_{12:0}$, $C_{13:0}$, $aiC_{14:0}$ and $C_{17:0}$. The main menaquinones are MK6, MK7 and MK9 with MK7 being the dominant one. The strain was found to be alkaliphilic tolerating pH values up to 12 with the optimum pH being 9.5, surviving and growing under multiple stress conditions including pH and temperature. This reflected on its ability to live in extremes of pH and temperature from where members of the genus are commonly isolated. The tolerance to salt concentration was also examined whereby it was found to tolerate salt concentration up to 10% with the optimum being 4.5%. The isolate was found to be a strong biofilm former. In terms of ISA biodegradability potentials, the strain was noted to ferment ISA at pH8.0 and 9.0 producing acetic acid and generating biomass.

5.6. Conclusion 5

5.6.1. Characterization of *Oceanobacillus* sp. Strain Hud.

Members of the genus *Oceanbacillus* have been described as endospore-forming, halotolerant facultative anaerobic bacterium. Most strains have been isolated from hypersaline environment and noted for their resistance to heavy metal toxicity. Some authors have described their ability to degrade a range of organic compounds and hence suggested its application in bioremediation of organic pollutants.

Oceanobacillus sp. strain Hud was isolated from soil samples from hyperalkaline, Harpur Hill site and in view of the characteristics presented in literature about members of this genus, the aim of this section of the work was to characterize and assess its ISA biodegradation.

The phylogenetic analysis showed that the strain shared 99% sequence homology with *Oceanobacilluis aidingensis* and *Oceanobacillus oncorhynchii.* The cells were Gram-positive rods, facultative anaerobe and positive for motility. The strain was both catalase and oxidase positive. While the major fatty acids included $C_{14:0}$, $aiC_{15:0}$, $aiC_{15:0}$, $C_{16:0}$, $iC_{16:0}$ and $aiC_{16:0}$, the major menaquinone was MK7.

The strain was found to be to be alkaliphilic surviving and growing in a wide pH range between 4 and 12 with pH9.0 being the optimum. The whole genome sequencing of the strain revealed the presence of genes that code for several stress responses including cold and heat shock, osmotic stress and resistance to heavy metals. There were also genes that were responsible for carbohydrate metabolism, which was evident in its ability to degrade a range of carbohydrate substrates during the biochemical analysis.

The strain was found to form strong adherent biofilm on abiotic surface suggesting ability to withstand adverse environmental condition. In terms of ISA degradation, the *Oceanobacillus* strain Hud was found to degrade ISA to acetic acid and generating biomass at pH9.0.

5.6.2. Conclusion 6

5.6.3. Isolation of alkaliphilic bacteria from methanogenic polymicrobial microcosms

Three microorganisms have been isolated from the polymicrobial consortia that degraded ISA. These isolates included taxa associated with the genera *Shewannella*, *Trichoccocus* and *Bacillus*. Having been able to grow on media in which CaISA or CDP were the sole carbon source coupled to the characteristics

of their respective genera suggest their potential for ISA degradation where the molecular mechanisms associated with ISA metabolism may be studied.

5.7. Impact of the study

Relating to nuclear waste repository, the findings of this study has shown that ISAs that are predicted to generate from alkaline degradation of cellulosic materials under repostory conditions could be biodegraded by microorganisms that would colonize the facility. The microbiology of the facility will play a significant role in its chemical evolution whereby gaseous products such as methane, carbon dioxide and hydrogen resulting from microbial biochemical activities are likely to contribute to the pressurization of the cement-based facility. The management of gases in the cement-based engineered barrier systems (EBS) however, has been extensively studied and noted in the Gas Status Report [354]. For instance, CO_2 is expected to dissolve in porewater and react with cementitous materials which is especeted to remove CO_2 from the gas phase [437]. Gas migration however, is likely to occur within the facility. The Nirex Reference Vault Backfill (NRVB) of the cement-based facilty is noted to have gas permeability of 10^{-16} m^2 to to assist with gas release from the EBS [438]. It is therefore important to consider the formulation of the NRVB to have properties with high porosity and gas permeability without compromising safety.

Although the presence of NH_4^+ and the type of carbon source ($Ca(ISA)_2$ or CDP) have been shown not to have significance on ISAs degradation, they have been shown to impact on the microbial community evolution which is important for the GDF safety assessment. It is therefore important to consider those factors in ISA degradation studies.

The isolation and characterization of novel ISA degrading microorganisms will allow for the study into the molecular basis of ISA biodegradation in terms of analysing genes that are involved. The study of these genes would provide the

opportunity for further investigation into how ISA degrading microorganisms may be engineered or genetically modified.

The organic rich soil surface layer of the Harpur Hill site has been shown to harbour indigenous methanogenic polymicrobial populations of microorganisms that are well adapted to high pH environment and capable of ISA degradation under anaerobic condition. Current ISA degradation studies have focussed on using sediments from the site. This suggests that the Harpur Hill anthropogenic hyperalkaline site may be a potential candidate site for a geological disposal facility.

5.8. Future works

The molecular characterization of ISA degrading communities has highlighted the vast populations of microorganisms that are present within the established microbial consortia. Further studies will focus on targeting organisms of interest based on these analysis for bioremediation of ISA.

The identification of *Exiguobacterium* sp. strain Hud and *Oceanobacillus* sp. strain Hud as novel ISA degrading bacteria and their subsequent molecular characterization will serve as a basis for the study into genes and biochemical pathways associated with ISA degradation. The isolation of alakliphilic, facultative anaerobic microorganisms from ISA degrading microcosms would also allow further studies into the potentials of pure isolates to degrade ISA.

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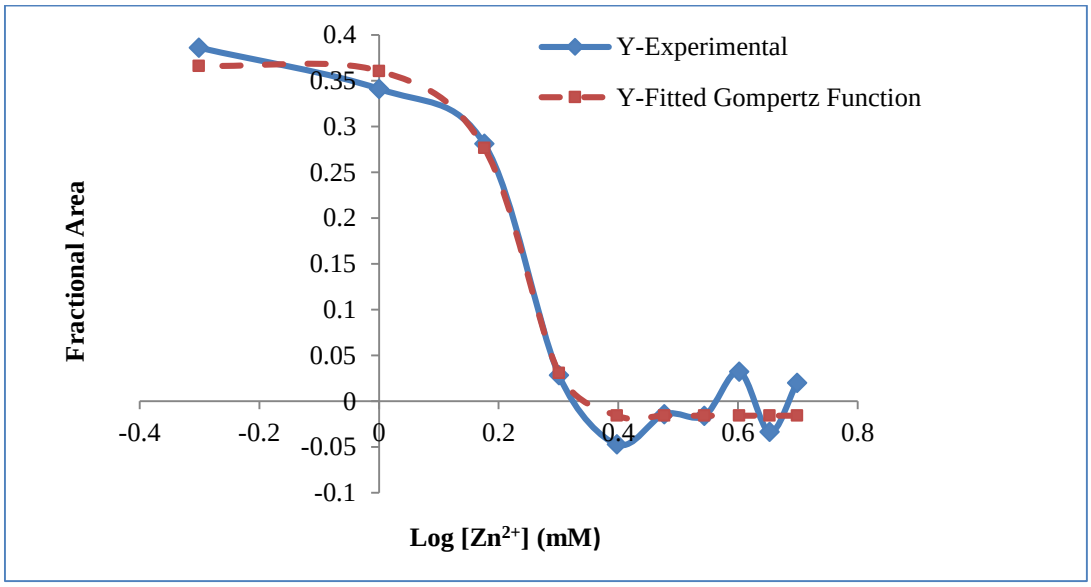
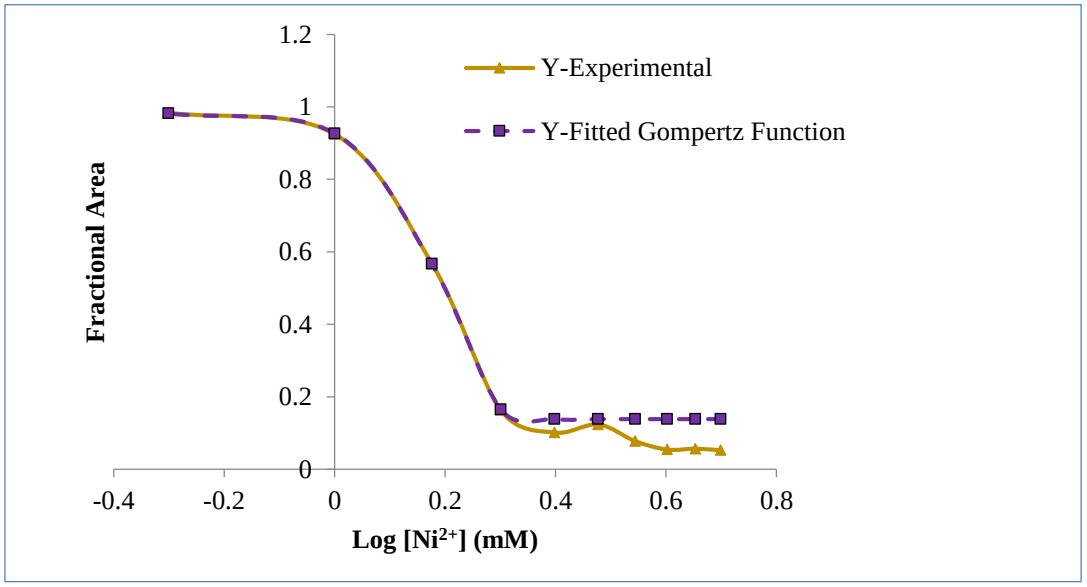
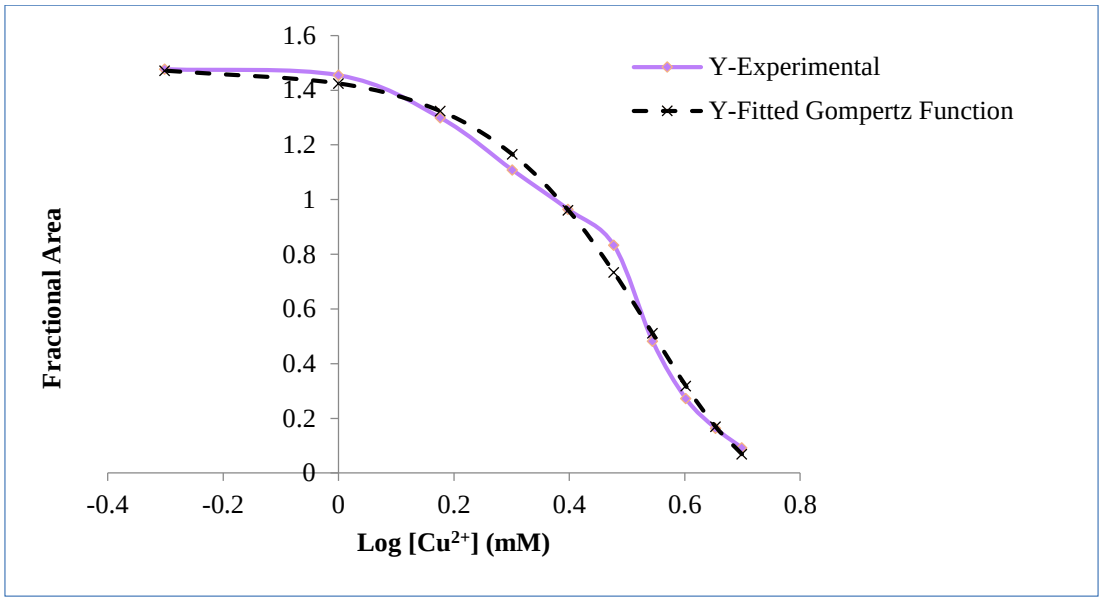
7. Appendix

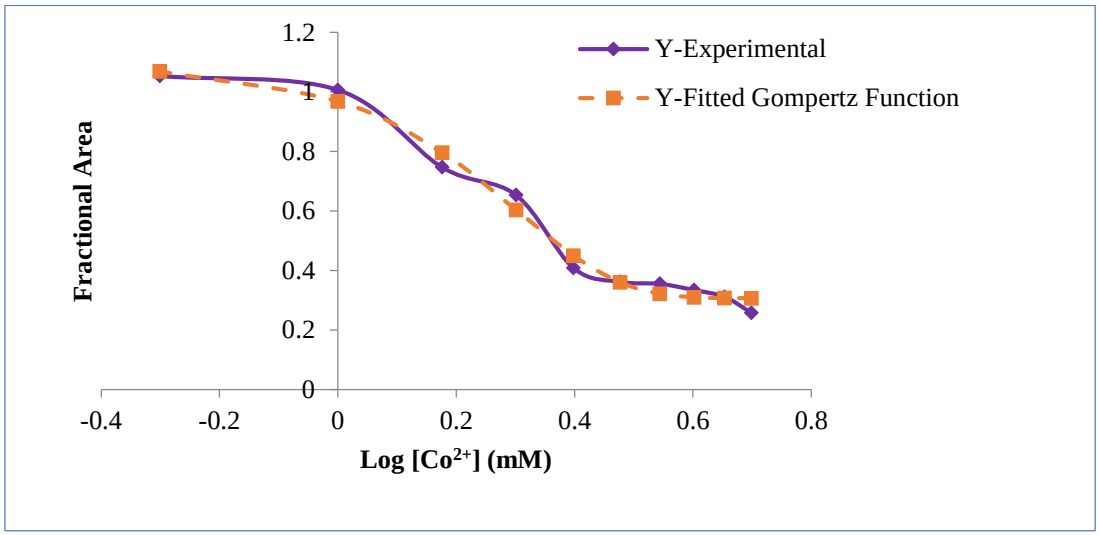
Exiguobacterium sp. strain Hud heavy metal tolerance

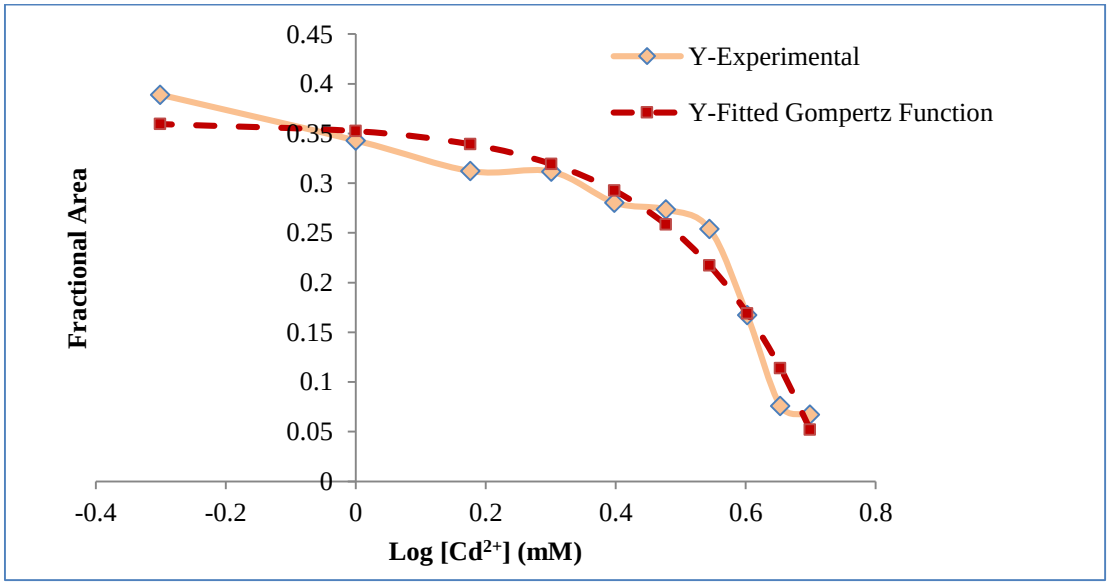
Graphs show a plot of fractional areas against log of metal ion concentration for experimental and fitted Gompertz function

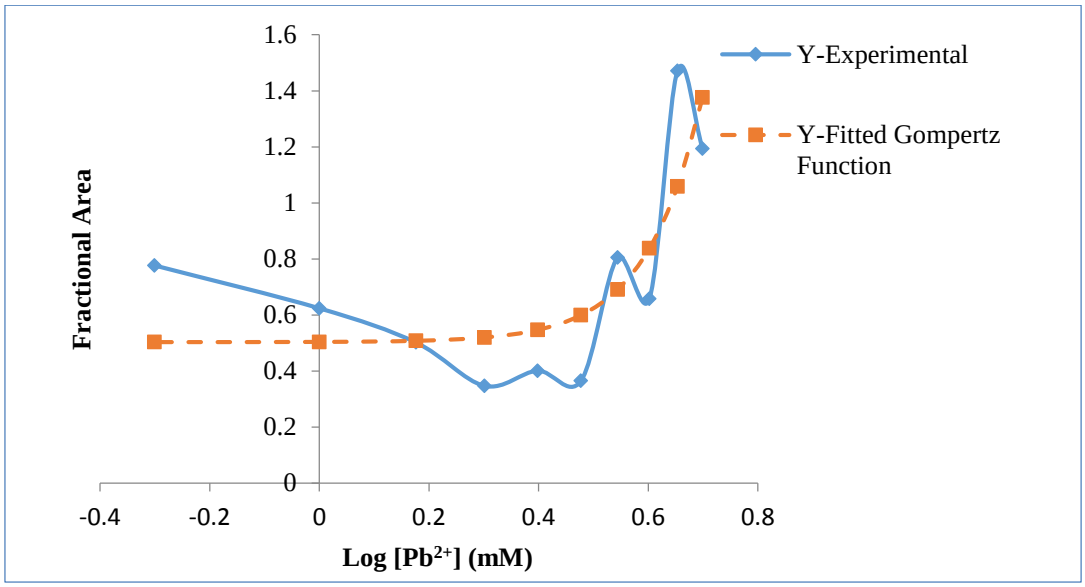





Y-Experimental
Y-Fitted Gompertz Function
Fractional Area
Log [Cu2+] (mM)
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
-0.4
-0.2
0
0.2
0.4
0.6
0.8

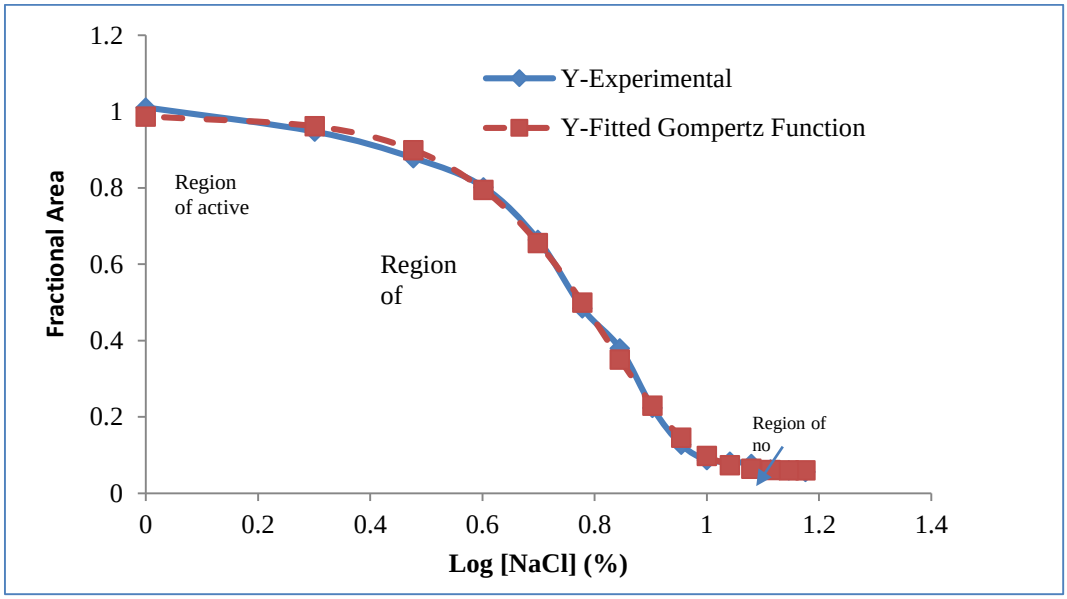

Y-Experimental
Y-Fitted Gompertz Function
Fractional Area
Log [Co2+] (mM)
1.2
1
0.8
0.6
0.4
0.2
0
-0.4
-0.2
0
0.2
0.4
0.6
0.8


Y-Experimental
Y-Fitted Gompertz Function
Fractional Area
Log [Cd2+] (mM)
0.45
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
-0.4
-0.2
0
0.2
0.4
0.6
0.8



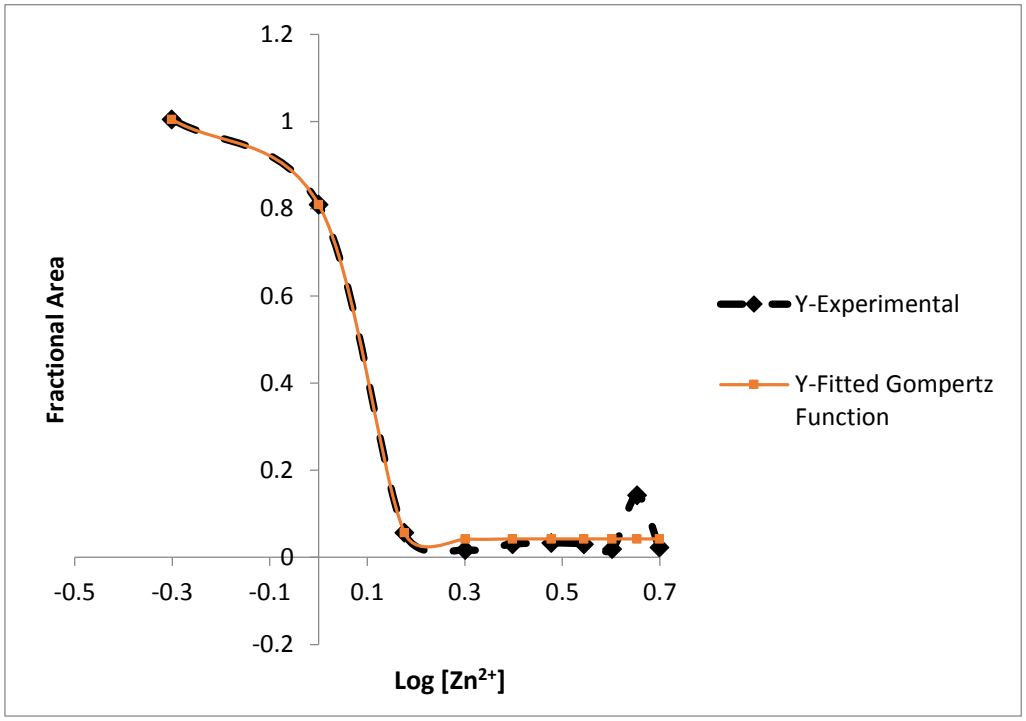
Exiguobacterium sp. strain Hud NaCl tolerance

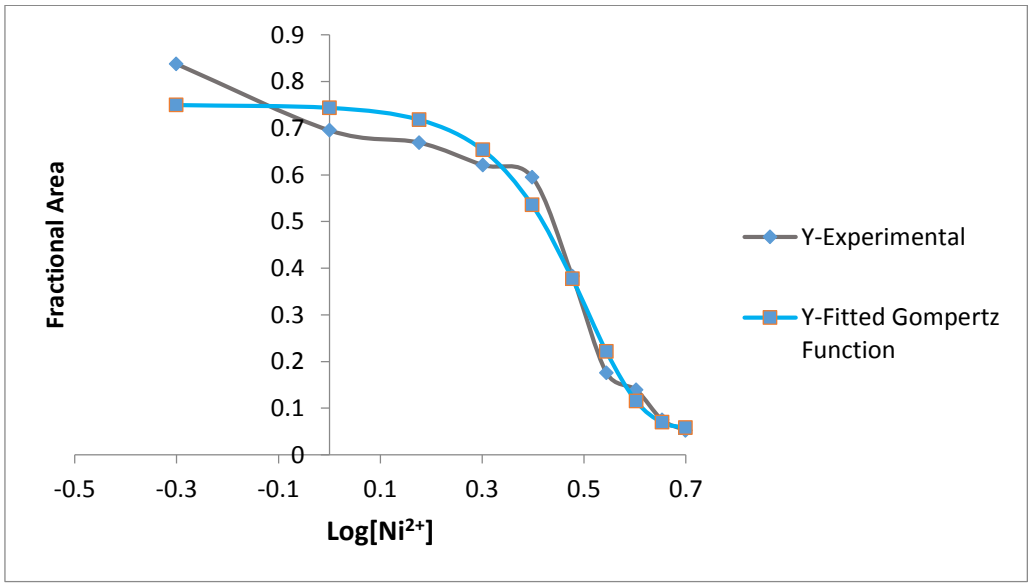
Graphs show a plot of fractional areas against log of NaCl concentration for experimental and fitted Gompertz function

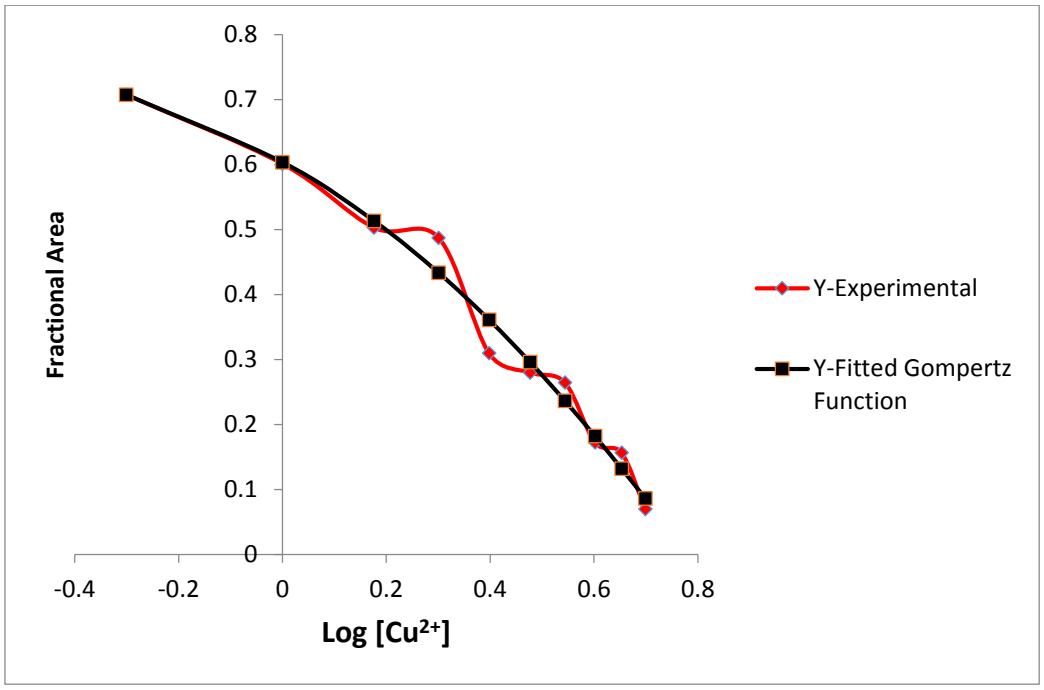


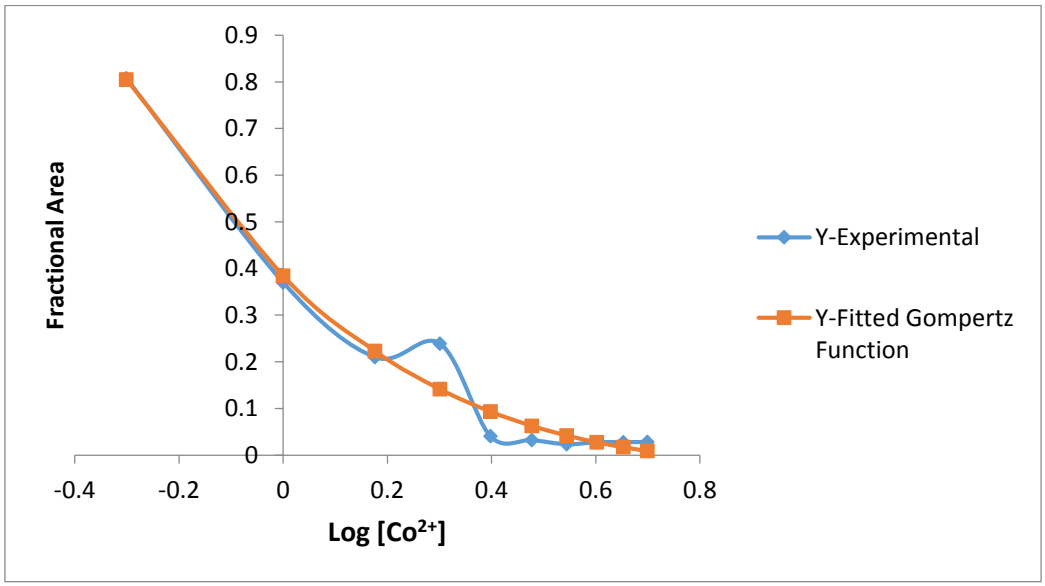
Oceanobacillus sp. strain Hud heavy metal tolerance

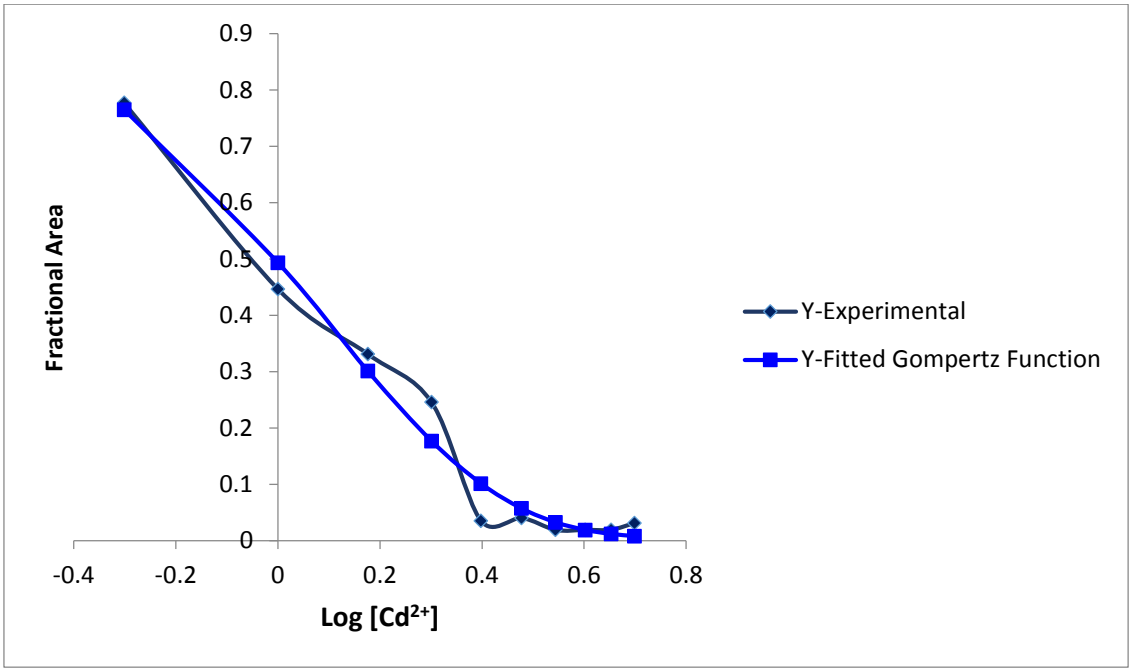
Graphs show a plot of fractional areas against log of metal ion concentration for experimental and fitted Gompertz function






0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Fractional Area
-0.4
-0.2
0
0.2
0.4
0.6
0.8
Log [Cu²⁺]
Y-Experimental
Y-Fitted Gompertz Function


0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Fractional Area
-0.4
-0.2
0
0.2
0.4
0.6
0.8
Log [Co²⁺]
Y-Experimental
Y-Fitted Gompertz Function



Statistical analysis

T-test for CDP with and without NH_4^+ systems

t-Test: Paired Two Sample for Means		
	CDP System with NH_4^+	*CDP System without NH_4^+*
Mean	0.55	0.7875
Variance	0.168571429	0.235535714
Observations	8	8
Pearson Correlation	0.971450972	
Hypothesized Mean Difference	0	
df	7	
t Stat	-5.157518783	
P(T<=t) one-tail	0.000656526	
t Critical one-tail	1.894578605	
P(T<=t) two-tail	0.001313052	
t Critical two-tail	2.364624252	

T-test for $Ca(ISA)_2$ with and without NH_4^+ systems

t-Test: Paired Two Sample for Means		
	$Ca(ISA)_2$ Syetem with NH_4^+	*$Ca(ISA)_2$ Syetem without NH_4^+*
Mean	0.525	0.6875
Variance	0.216428571	0.255535714
Observations	8	8
Pearson Correlation	0.96130805	
Hypothesized Mean Difference	0	
df	7	
t Stat	-3.264606815	
P(T<=t) one-tail	0.006888242	
t Critical one-tail	1.894578605	
P(T<=t) two-tail	0.013776484	
t Critical two-tail	2.364624252	

Statistical analysis for *Exiguobacterium* sp strain Hud biofilm formation

t-Test: Paired Two Sample for Means		
	Absorbance of Control (ODc)	*Absorbance of Biofilm (ODs)*
Mean	0.1875125	2.06195
Variance	0.001065504	0.131446014
Observations	8	8
Pearson Correlation	0.978413126	
Hypothesized Mean Difference	0	
df	7	
t Stat	-16.03244804	
P(T<=t) one-tail	4.46101E-07	
t Critical one-tail	1.894578605	
P(T<=t) two-tail	8.92201E-07	
t Critical two-tail	2.364624252	

Statistical analysis for *Oceanobacillus* sp. strain Hud biofilm formation

t-Test: Paired Two Sample for Means		
	Absorbance of Control (ODc)	*Absorbance of Biofilm (ODs)*
Mean	0.1875125	2.2950375
Variance	0.001065504	0.118588908
Observations	8	8
Pearson Correlation	0.738977243	
Hypothesized Mean Difference	0	
df	7	
t Stat	-18.57004013	
P(T<=t) one-tail	1.62891E-07	
t Critical one-tail	1.894578605	
P(T<=t) two-tail	3.25783E-07	
t Critical two-tail	2.364624252	

16srRNA sequencing for microbial identification

>IK130220152_premix -- 23..949 of sequence ***Exiguobacterium* sp. strain Hud**

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TCCTACGGGAGGCAGCAGTAGGGAATCTTCCACAATGGACGAAAGTCTGATGGAGCAACG
CCGCGTGAACGATGAAGGCCTTCGGGTCGTAAAGTTCTGTTGTAAGGGAAGAACAAGTGC
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>IK2706201603_premix -- 44..965 of sequenceIK2706201603 ASR003D253
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>IK2704201605_premix -- 51..994 of sequence ***Shewanella* sp strain IK Hud**
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