Gram Negative Bacterial Biofilm Formation and Characterisation of Extracellular Polymeric Substances

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A thesis submitted to the University of Huddersfield in partial fulfilment of the requirements for the degree of Doctor of Philosophy

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February 2016

Copyright Statement

Acknowledgments

Words are almost inadequate to express my thanks to my supervisors Dr. Paul Humphreys, for his help, guidance, encouragement and support throughout the project period. He has been patient and kind, and gave me freedom to explore my ideas and to gain new knowledge. I would like to thank my co-supervisor Professor Andrew Laws and his research group for their assistance in chemistry lab work and interpretation of NMR analysis. I want to specially thank my colleague Dr. Simon Rout for his immense help in various aspects during whole my study that I will never ever forget his massive help. I would also like to thank Dr. Neil McLay for running NMR analysis and all technicians' staff Felix Owusu-Kwarteng, Margaret Craven, Sophie Sinclair and Elena Tillotson for their helps during the project period. A special thanks to all my colleagues in Microbiology and Chemistry research laboratory specially Dr. Kamran Shoukat and Ammar Al Jasim for their help during my research project. Thanks to Eva Garret and Emily Woodhead for their helps during bacterial pathogenicity analysis (wax worms injection).

Dedication

To my parents, my brothers and my sister

Abstract

Gram negative bacteria such as *Stenotrophomonas maltophilia, Pseudomonas aeruginosa* and *Citrobacter freundii* are often associated with multiple drug resistance and the generation of nosocomial infections. In the current study several clinical strains of theses bacteria (Ps 1, Ps 3, Ps 5, St 18, St 51, St 53 and *C. freundii*) and two culture collection strains Ps 10421 and St 9203 were evaluated for their ability to generate biofilms and the characteristics of the associated extracellular polysaccharides they produced.

The ability of these strains to develop biofilms on a range of media and with a number of carbon sources was investigated. A range of mineral media employing glucose, ethanol and glycerol were developed in such a way as to ensure they did not contain compounds that interfere with extracellular polysaccharide analysis allowing a more in depth analysis of the extracellular polysaccharide generated by the bacteria under investigation.

Following an assessment of the biofilm forming potential of all the strains under consideration, three were singled out for particular attention, i.e. Ps 3, St 53 and *C. freundii* strain isolated during this investigation. Two of strains were chosen for the strength of their biofilm forming potential (Ps 3 and St 53), on the other hand *C. freundii* was chosen because the scientific literature contains very little published information regarding its extracellular polysaccharide and its biofilm forming characteristics. These bacteria were able to produce biofilm on both hydrophobic (plastic) and hydrophilic (glass) surfaces. In order to get a broader understanding of the biofilm forming capabilities of these bacteria their whole genomes were sequenced and subsequently published. These genomes demonstrated that St 53 and *C. freundii* both contained the *pgaABCD* which is known to be associated with biofilm formation. Whilst Ps 3 contains a full complement of pel (PA3058-PA3064), psl (PA2231-2245) and alginate biosynthesis operons (PA3540-3548) related to biofilm formation. In addition all three species contained genes associated with virulence, pathogenicity and antibiotic resistance.

The generation and extraction of extracellular polymeric substance generated by these three bacteria underwent a period of optimisations which included an optimisation of both the media and the growth conditions and the extraction process. In particular the use of trichloroacetic acid (TCA) was found to be critical with 0-5% TCA considered optimum for the removal of proteins prior to polysaccharide extraction. This is far less than has been previously employed in studies on lactic acid bacteria, however when used with the Gram negative bacteria investigated here, high levels of TCA degraded the polysaccharide that was being generated preventing its extraction in the quantities required for analysis.

Analysis of the polysaccharides produced by St 53, Ps 3, and *C. freundii*, all demonstrated typical NMR spectra associated with bacterial extracellular polysaccharide. However, the NMR spectra from these polysaccharides also contained peaks typical of the presence of dextran. The use of a fungal dextranase confirmed the presence of a dextran like polymer in the polysaccharide generated by these bacteria. This indicated that all three of these bacteria generated complex polysaccharides with at least two components one mannose rich and the second a dextran like glucose rich polymer. This is the first report of a dextran being associated with the EPS of these bacteria and suggests that the Pel polysaccharide of *P. aeruginosa* is a dextran.

Investigation of bacterial pathogenicity focussed on Ps 3 since *P. aeruginosa* is the most pathogenic of the three species investigated. The culture collection strain Ps 10421 failed to produce outer membrane vesicles (OMV) without antibiotic treatment, however Ps 3 generated OMV under normal growth conditions generating more when grown on ethanol rather than glucose. In order to investigate the impact of ethanol vs glucose grown culture a wax worm pathogenicity model was employed. This model revealed that ethanol grown cells were more pathogenic than glucose grown cells. This difference could be attributed to the effects of type of carbon sources that induce virulence genes to generate more toxins. Transcriptomic analysis of Ps 3 grown with ethanol vs growth on glucose revealed large differences in gene expression but no definitive evidence of which cellular processes were responsible for this enhanced pathogenicity associated with grown on ethanol.

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Words count excluding appendices - 58,318

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1. Introduction

Mortality and morbidity rates are high in intensive care units worldwide due to infections caused by multidrug resistant organisms (MDRO), in recent years there has been a significant increase in multidrug resistance amongst Gram negative bacteria (De Angelis et al., 2014). Gram negative bacterial species such as *Stenotrophomonas maltophilia*, *Pseudomonas aeruginosa* and *Citrobacter freundii* are opportunistic pathogens which are often multidrug resistant and have been shown to be problematic in clinical settings (Chaudhry et al., 2014, Kim et al., 2003, Brooke, 2012, Lister et al., 2009).

Treatment of the infections caused by these bacteria is complicated by biofilm formation which increase their antibiotic resistance, prevalence and survival (de Abreu et al., 2014). The Extracellular Polymeric Substance (EPS) generated by bacteria when they form a biofilm acts as a diffusion barrier against antimicrobial agents e.g. antibiotics, reducing the effective concentration that the bacteria is exposed to (Kokare et al., 2009).

The persistence and survival of Gram negative bacteria on surfaces is longer than that see in in Gram positive bacteria (Kramer et al., 2006), Gram-negative bacteria such as *Pseudomonas aeruginosa* and *Stenotrophomonas maltophilia* can survive for months on biotic (living) and abiotic (non-living) surfaces such as epithelial cells, glass and plastic surfaces (Kramer et al., 2006, Brooke, 2007) and this is due to biofilm formation and the outer membrane structure of Gram negative bacteria (Martinez, 2011).

The research reported in this thesis is focussed on the biofilm formation, EPS generation and associated pathogenicity of several Gram negative bacteria associated with human infections.

2. Literature Review

2.1. Bacteria

Bacteria can be defined as small, unicellular prokaryotic organisms with simplest life form. Prokaryotes are living cells without nucleus and other membrane-bound structures. Generally microorganisms are classified in to two major groups as eukaryotes and prokaryotes based on cellular structure and function. The prokaryotes are also classified in to eubacteria and archaebacteria. Eubacteria and archaebacteria are commonly referred to as bacteria (Gerardi, 2006). Based on morphology and cell wall response to chemical stain (Gram stains), bacteria are commonly divided into several groups, one of them is Gram negative bacilli (Schleifer and Kandler, 1972).

2.2. Gram negative bacilli bacteria

This group of bacteria are considered a significant cause of infection in the community and nosocomial settings (Wong et al., 2014). Several studies have shown an increasing in incidence and resistance to antibiotics among Gram negative bacilli including *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Acinetobacter* spp., *Escherichia coli*, *Stenotrophomonas maltophilia*, *Citrobacter freundii* with multidrug resistance being common (McGowan, 2006, Lockhart et al., 2007). In the USA, an analysis by the National Nosocomial Infection Surveillance System for diseases such as pneumonia and UTI acquired during stays in intensive care units between 1986 and 2003 showed that Gram negative bacilli were the most frequently isolated bacteria. The review also highlighted the difficulty of treating these intensive care units infections due to the increased incidence antimicrobial resistance in this group of bacteria (Rahal, 2009).

2.2.1. *Stenotrophomonas maltophilia*

Stenotrophomonas maltophilia (*S. maltophilia*) is a bacterium that was first isolated in 1943 and identified as *Bacterium booker* and then classified in 1961 by (Hugh and Ryschenkow, 1961) as a member of the genus *Pseudomonas* (*Pseudomonas maltophilia*). It was then renamed as *Xanthomonas maltophilia* (Swings et al., 1983) and then transferred in 1993 (Palleroni and Bradbury, 1993) into the new *Stenotrophomonas* genus. Another 3 species, *Pseudomonas geniculata*, *Pseudomonas betle* and *Pseudomonas hibiscicola* were also reclassified as *Stenotrophomonas maltophilia* (Singer et al., 1994, Van den Mooter and Swings, 1990, Anzai et al., 2000). *S. maltophilia* is an obligate aerobic non-fermentative, Gram-negative, bacilli or curved rods, 0.5 to 1.5 µm, catalase positive and mostly oxidase negative, belonging to the Gammaproteobacteria class, *Xanthomonadaceae* family (Denton and Kerr, 1998, Brooke, 2012). *S. maltophilia* has a broad distribution and can be found in various environments and geographical regions, It has been isolated from a wide range of both institutional and environmental habitats e.g. hospitals, water (rivers, wells, and bottled water), sewage, soil, plant and food (Youenou et al., 2015, Denton and Kerr, 1998, Hoefel et al., 2005).

According to Svensson-Stadler et al. (2012) of the 12 identified species within the genus *Stenotrophomonas* only *S. maltophilia* is isolated from human and can cause disease, while the others are isolated from the environment and are non-pathogenic. One of the most important features of *S. maltophilia* is its ability to form biofilms on biotic and abiotic surfaces such the epithelial cells of the lung, implanted medical devices (catheters and respiratory therapy equipment), glass and plastic (Brooke, 2014, Denton and Kerr, 1998).

S. maltophilia is listed by the World Health Organization (WHO) as one of the most important multidrug resistant bacteria in hospitals worldwide being resistant to β-lactam drugs, chloramphenicol, tetracyclines and cephalosporins. It is considered an opportunistic pathogen causing nosocomial infections especially

in intensive care units amongst the immunocompromised, cancer patients and those with central venous catheters (Brooke, 2014). *Stenotrophomonas maltophilia* can cause various serious infections in humans. *S. maltophilia* is usually associated with respiratory tract infection in persons with cystic fibrosis (CF) (Waters et al., 2013) but recently the prevalence of infection even in normal patients (non-cystic fibrosis) has increased, such infections include bloodstream infections (Garazi et al., 2012), meningitis (Papadakis et al., 1997), endocarditis (Rodero et al., 1996), bacteraemia (Friedman et al., 2002), pneumonia (Hanes et al., 2002, Irifune et al., 1994), septicaemia and urinary tract infection (Brooke, 2012).

2.2.2. *Pseudomonas aeruginosa*

In 1882, Gessard isolated *Pseudomonas aeruginosa* from a cutaneous wound (Wilson and Dowling, 1998) to later described by Migula (1894), as such this species was part of the early history of bacteriology (Peix et al., 2009). *Pseudomonas aeruginosa* (*P. aeruginosa*) is a Gram-negative aerobic or (facultative anaerobic), non-spore forming bacteria, bacilli or slightly curved shape, 0.5–1.0 to 1.5– 5.0 µm, motile with polar flagella or rarely non-motile and belonging to the Gamma proteobacteria class, family *Pseudomonadaceae* (Frangipani et al., 2008, Brenner et al., 2005). The species grows well in moist environments but can grow in a wide variety of environments including soil, waters, plants and hospitals (Wilson and Dowling, 1998). The ability of *P. aeruginosa* to; grow with minimal nutritional requirements on a wide variety of carbon source, it's tolerance and resistance to a variety of physical and environmental condition, resistance to a wide range of antimicrobial agents and its ability to produces many virulence factors such as (exotoxin A, exoenzyme S, phospholipase C, rhamnolipid and alkaline protease) make it a highly successful species (Van Delden and Iglewski, 1998). These characteristics also enable its long-term persistence and transmission in a variety of environmental niches

including hospitals where it has become one of the most common opportunistic pathogenic bacteria (Persson, 2010, Lanini et al., 2011).

P. aeruginosa is responsible for both community-acquired and hospital acquired infections. In the community it is associated with eye infections, ulcerative keratitis, skin infections (Fujitani et al., 2011, LaBauve and Wargo, 2012) and diabetic wounds infections (Edmonds et al., 1986). It is also responsible for many hospital-acquired infections (Fujitani et al., 2011, LaBauve and Wargo, 2012) especially in immunocompromised patients being responsible for cystic fibrosis lung infections (Oliver et al., 2000), acute pneumonias (Arancibia et al., 2002) burn and open wounds infection (Rumbaugh et al., 1999), acute and chronic urinary tract infection especially in patients with catheter device (Nicolle and Committee, 2005), bacteraemia and septicaemia (Kang et al., 2003).

P. aeruginosa is considered one of major causes of nosocomial infections accounting for 10–15% of nosocomial infections worldwide (Strateva and Yordanov, 2009). This is not surprising since it is one of the most frequently isolated bacteria in hospitals (Kramer et al., 2006). Hospital environmental transmission is considered the major source of most *Pseudomonas* outbreaks, rather than cross transmission between patients. Hospital environmental isolates of *P. aeruginosa* have been shown to possesses higher levels of resistance to anti-pseudomonal antibiotics when compared to *Pseudomonas* isolates from patients (Fujitani et al., 2011).

Infections caused by *P. aeruginosa* are very difficult to treat, which is due to the natural resistance of *P. aeruginosa* to a wide range of antimicrobial agents (Strateva and Yordanov, 2009). This is partially due to the fact that *P. aeruginosa* forms extensive biofilms on a variety of surfaces (O'Toole and Kolter, 1998, Tielker et al., 2005, Soto, 2014).

2.2.3. *Enterobacteriaceae*

Enterobacteriaceae are a large heterogeneous family of bacteria in the Gammaproteobacteria class. The *Enterobacteriaceae* members are Gram-negative rods, which range between 1.0–6.0 μm in length, grow well at 22–35°C, and live in environments such as soil, water, fruits, meats, eggs, vegetables, and as a commensal in the intestines of humans and animals. Many members of the family *Enterobacteriaceae* have the ability to cause disease in human and animal such as *Citrobacter* spp., *Enterobacter* spp., *Escherichia* spp., *Klebsiella* spp (Brenner et al., 2005). Among the wide range of Gram-negative bacteria that are responsible for nosocomial infections, member of the family *Enterobacteriaceae* is considered the most commonly identified group (Peleg and Hooper, 2010). Increasing multidrug resistance among the *Enterobacteriaceae* is considered a significant risk to public health. *Enterobacteriaceae* that excrete Extended Spectrum β-lactamases (ESBL) and those producing carbapenemases have spread worldwide and have significantly reduced the usefulness of cephalosporins and carbapenems as treatment options (Delgado-Valverde et al., 2013, Gupta et al., 2011a).

2.2.3.1. *Citrobacter freundii*

The genus *Citrobacter* was described for the first time by Braak and nominated as *Bacterium freundii* in 1928, and then later in 1932, the bacterium was re named as *Citrobacter freundii* by Werkman and Gillen. *Citrobacter* spp. are Gram negative rod shape, 2-6μm, facultative anaerobes, with peritrichous flagella, they are oxidase negative and catalase positive (Borenshtein and Schauer, 2006, Brenner et al., 2005). *Citrobacter freundii* (*C. freundii*) is a member of the family *Enterobacteriaceae* that can be found in environments such as soil, water and food (Samonis et al., 2009) it is also a commensal of the intestinal tracts of both humans and animals (Bai et al., 2012). It is also considered an opportunistic pathogen that can cause several infection especially in high-risk groups (Zhang et

al., 2008). It is typically associated with diarrhoea (Bai et al., 2012), wound infections (Rao et al., 2013), bacteremia (Shih et al., 1996), neonatal meningitis and brain abscess (Doran, 1999) (Badger et al., 1999), necrotizing pancreatitis (Lozano-Leon et al., 2011) and UTI and blood stream infections (Ranjan and Ranjan, 2013, Pepperell et al., 2002). *C. freundii* is resistant to several antibiotics such as ceftriaxone, ceftazidime, piperacillin cephalosporins and penicillins (Lavigne et al., 2007, Kim et al., 2003), ampicillin (Doran, 1999). There is also evidence of resistance to carbapenems which were considered to be one of the few remaining treatments for infections caused by multidrug resistant isolates (Zhang et al., 2008). *C. freundii* produces several virulence factors such as Shiga-like toxins and heat stable toxin type III and has the ability to produce biofilms to colonize and attach to surfaces and devices such as catheter (Shirtliff and Leid, 2009, Bai et al., 2012).

2.3. Bacterial Pathogenicity

Pathogenicity is the ability of an agent to cause disease (Beceiro et al., 2013) and a number of mechanisms are used by the pathogenic bacteria to cause disease in humans. Common mechanisms amongst pathogens include; adherence, invasion, toxin and enzymatic damage to host cells and tissues, avoidance and disruption of host defences (Wilson et al., 2002), all of which contribute to the establishment and propagation of an infection. Most pathogenic bacteria use of a combination of toxin generation and tissue/cell invasion to cause disease (Beceiro et al., 2013), these processes are summarised in (Figure 2.1).

As mentioned previously, Gram-negative pathogens have received considerable attention in recent years due to the emergence of (MDRO). Key mechanisms driving this multi-drug resistance include the presence of bacteria efflux pumps and the generation of antibiotic degrading enzymes such as β-lactamases, the genes associated with these capabilities can be either genomic or plasmid associated and transferred by various gene transfer mechanisms. Recently, the

reported cases of MDR associated infections due to *P. aeruginosa S. maltophilia and C. freundii* have increased (Kumar et al., 2013, Brooke, 2012).

In the case of *S. maltophilia,* pathogenicity is associated with their ability to:

- Form biofilm on different surfaces including (Teflon, glass, and plastics and on host tissues). Biofilm formation is considered a significant feature of *S. maltophilia* pathogenesis with 65% of hospital-acquired infections being associated with biofilms.
- Antibiotic resistance.
- The production of hydrolytic enzymes.
- Adherence to and invasion of host cells.
- Lipopolysaccharide endotoxins (Brooke, 2012).

In the case of *C. freundii* common virulence factors include:

- Biofilm formation (Pereira et al., 2010).
- Toxin generation including a Shiga-like enterotoxins.
- A type III secretion system (T3SS) (Bai et al., 2012).
- Hydrolytic enzyme (Gunasekaran et al., 2006).
- Resistance to a wide range of antimicrobial agents (Lavigne et al., 2007).

The pathogenicity of *P. aeruginosa* depends on a wide range of virulence factors which all combine to produce a highly effective pathogen (Figure 2.2) (Gellatly and Hancock, 2013) these include:

- Ability to form biofilms on biotic or abiotic surfaces.
- Extracellular factors including lipases and phospholipase.
- Outer membrane vesicles.
- Type III secretory systems.
- Toxins such as pyocyanin, pyoverdine, hydrogen cyanide and Exotoxin A.
- Enzymes such as neutral and alkaline proteases, elastase, rhamnolipid and haemolysin.

- Siderophores for iron sequestration.
- Cellular factors including lipopolysaccharide (LPS), flagella and pili.
- The control of virulence and associated pathogenicity through cell-to-cell communication mechanism (Quorum Sensing) (Li et al., 2007, Davies et al., 1998, Wagner and Iglewski, 2008, Lyczak et al., 2000, Ballok and O'Toole, 2013, Ledizet et al., 2012, Gellatly and Hancock, 2013).
- Antimicrobial resistance associated with the expression of efflux pumps, the low permeability of the outer-membrane and the acquisition of β-lactamases.

The pathogenicity of *P. aeruginosa* and its ability to resist antibiotics and disinfectants has been attributed to the large size and the versatility of its genome (Mesaros et al., 2007).

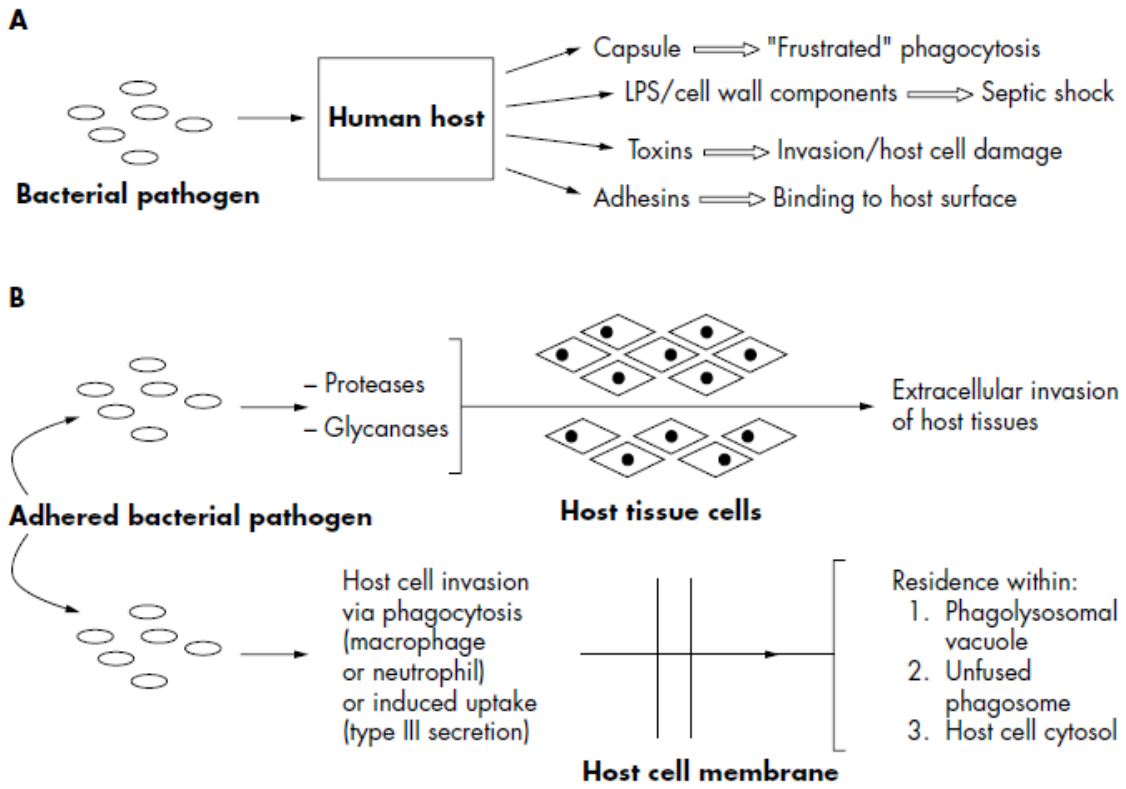


Figure 2.1 An overview of bacterial mechanisms for pathogenicity (Wilson et al., 2002). (A) The bacterial components that interact with the host include: (i) Capsules (protection) (ii) Lipopolysaccharide and cell wall (septic shock), (iii) Toxins (damage and invasion) and (iv)

Adhesions (attachment). (B) Once adhered to a host surface, a bacterial pathogen may further invade host tissues. (i) Proteases and glycanases (digest host and invasion), (ii) natural phagocytosis mechanisms of macrophages and neutrophils (invasion) or type III secretion system (T3SS) by induced uptake (adhesion). Within the host cell, the pathogen may reside within a phagolysosomal vacuole (a phagosome which has fused with a lysosome), a phagosome which has not fused with a lysosome, or within the host cell cytosol.

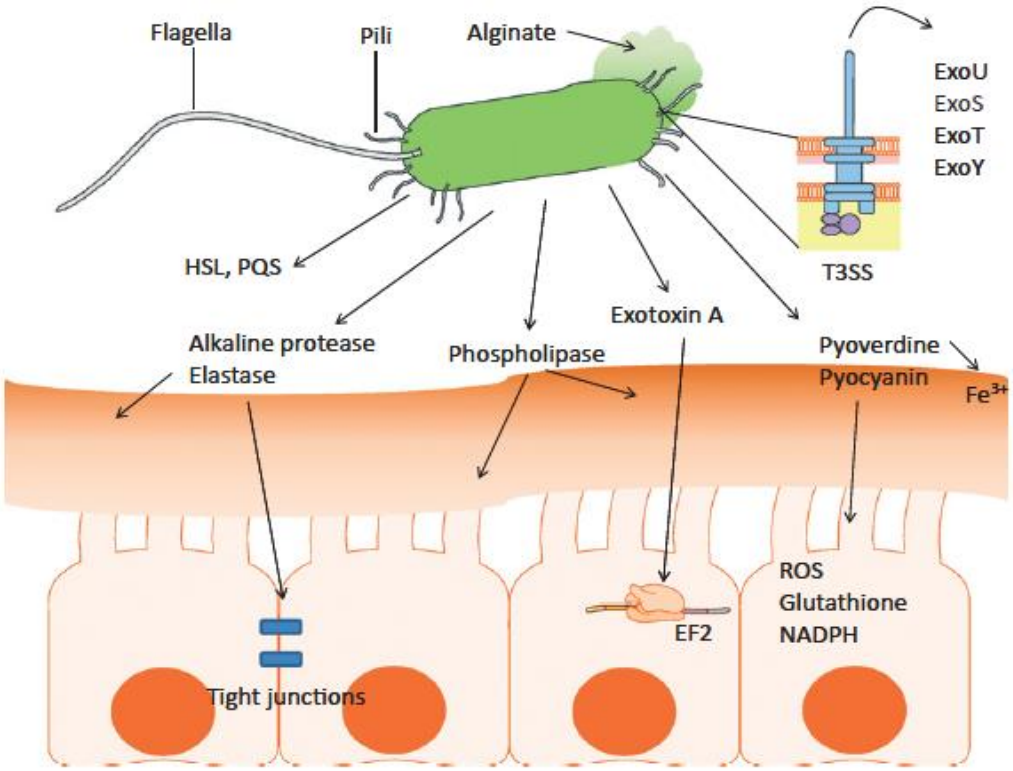


Figure 2.2 A summary of *Pseudomonas aeruginosa* virulence factors (Gellatly and Hancock, 2013). Attachment and adhesion through flagella, pili and lipopolysaccharide that cause highly inflammatory. Activation of T3SS which is able to insert cytotoxins into the host cell. Secretion of several virulence factors such as proteases (degrade host complement factors, mucins, and disrupt tight junctions between epithelial cell), Exotoxin A (host elongation factor EF2 inhibitor), Lipases and phospholipases (lipids), pyocyanin (interfere with host cell electron transport pathways) and pyoverdine (chelating agent).

2.4. Biofilms

2.4.1. Bacterial biofilm formation

Generally, bacteria exist in nature in two form, either planktonic where the bacteria are free living and growing in suspension or in a biofilm. In biofilms

bacteria are attached to each other and/or a surface as a community where they form a network of cells surrounded by a self-produced matrix of EPS (Lear and Lewis, 2012, Hall-Stoodley et al., 2004). It is estimated that more than 99% of microorganisms on the earth live as a biofilm (Vu et al., 2009, Garrett et al., 2008).

Biofilm formation occurs as a sequential process and one key aspect of biofilm formation is (QS). In the case of *Pseudomonas* spp. biofilms, five stages of development have been identified (Figure 2.3) (Marić and Vraneš, 2007, Renner and Weibel, 2011).;

1. Reversible attachment: Interaction between planktonic bacteria and substrate surface is considered the initial event in biofilm development. This phase is called reversible adsorption because some bacteria attach to the substrate surface only for a brief period and then detach from it (Figure 2.3). The contact between planktonic bacteria and the surface may be either random or controlled due to (chemotaxis and motility) (Dunne, 2002).
2. Irreversible attachment: After binding to the surface, bacterial cells start the process of irreversible adhesion by the formation of multi-layered cell clusters and the production of EPS which provide the developing biofilm with structural support. Production of the EPS also secures the adhered bacterial biofilm more firmly to the surface. In this phase bacteria lose their mobility and bacterial cells attach to each other on the substrate surface and start to form microcolonies (Stoodley et al., 2002, Cegelski et al., 2008).

3. Maturation I (microcolonies formation): After irreversibly attaching to a surface, bacterial cells undergo phenotypic changes, and the process of biofilm maturation I begins. In this phase, a matrix of EPS is produced and microcolonies become multi-layered (Figure 2.3). This matrix is an essential part of the biofilm, which contributes to its antibiotic resistance (Cegelski et al., 2008).

4. Maturation II: In this stage, microcolonies of bacteria grow to their maximum size and thickness. Once the biofilm has matured to this stage, it becomes more resistant and very difficult to treat or eradicate with antimicrobial agents and this is due to the failure of the agents to penetrate the full depth of this matured biofilm (Cegelski et al., 2008, Aslam, 2008).
5. Detachment and dispersion: Microcolony structure changes to a shell-like structure having an inner empty cavity. In this stage, bacterial cells recover their mobility, start to detach, migrate and adhere to other surfaces where they form another biofilm. In this manner biofilm formation becomes a cyclical process (Stoodley et al., 2002).

Biofilms are composed of microcolonies that consist of distinct communities of bacterial cells surrounded by EPS. Depending on the type of bacteria, microcolonies are composed of 10-25% of cells and 79-90% of EPS (Kokare et al., 2009, Marić and Vraneš, 2007, Garrett et al., 2008).

Life in a biofilm has several advantages for the microbial communities involved, including:

a) Protection from environmental factors such as desiccation, radiation and toxic compounds.
b) The provision of ideal environmental conditions and nutrients.
c) Opportunities for the transfer of genetic material between the individual cells within the bacterial communities.
d) Protection from antimicrobial agents such as antibiotic, biofilms can increase resistance by more than a hundred fold in some species (Kokare et al., 2009, Garrett et al., 2008).

Bacterial biofilms cause problems across a wide range of economic sectors such as food, dairy, water systems and oil industry. In hospitals, biofilms are associated with a wide range of nosocomial infections, since both commensal and pathogenic microorganisms form biofilm on a wide range of surfaces and medical devices especially implanted medical device including prosthetic heart valves, orthopedic

implants, intravascular catheters, artificial hearts, urinary catheters and contact lenses (Bryers, 2008). Currently, biofilms formation are estimated to be responsible for over 65% of nosocomial infections and 80% of all microbial infections (Soto, 2014). Biofilms are also associated with several conditions such as otitis media, cystic fibrosis pneumonia, intensive care unit pneumonia, contact lens related infections, urinary catheter cystitis, central venous catheter infections and dental caries (Costerton et al., 1999).

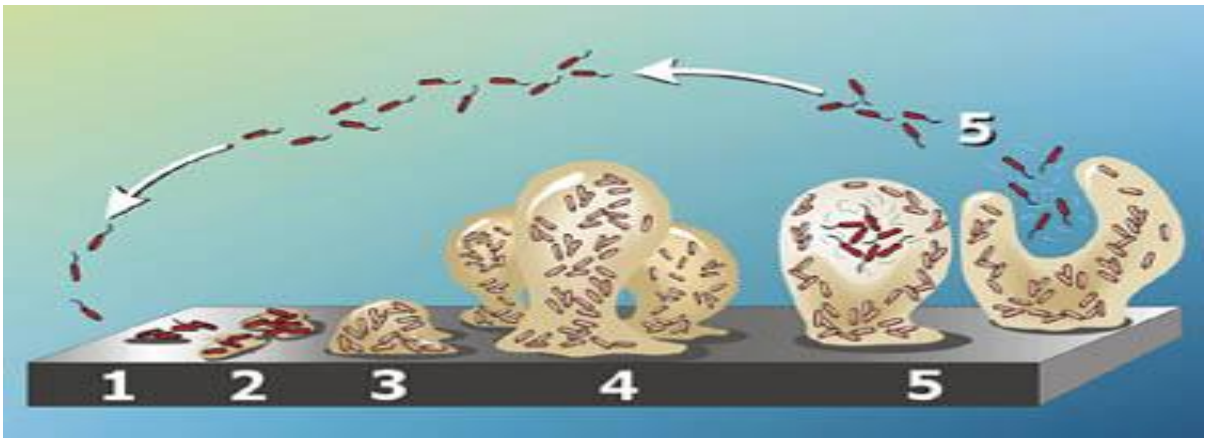


Figure 2.3 *P. aeruginosa* biofilm formation stages on glass slide under continuous flow medium starts with 1: initial attachment or reversible attachment of bacteria to the surface. 2: irreversible attachment and EPS production 3: early biofilm architecture I. 4: maturation of biofilm architecture II, and finally 5: biofilm dispersion and single cells spreading (Stoodley et al., 2002).

2.4.2. Biofilm analysis

A wide range of quantitative and qualitative techniques have developed for the investigation of biofilms (Pantanella et al., 2013, Taj et al., 2011). The simplest of these approaches are staining methodologies such as crystal violet or safranin staining for biofilm biomass quantification tissue culture plate (TCP) (Christensen et al., 1985, Stepanović et al., 2000, Pantanella et al., 2007), tube methods (TM) or tube adherence (TA) (Freeman et al., 1989, Christensen et al., 1982) Congo red agar method (CRA) (Freeman et al., 1989), bioluminescent

assays (Donlan et al., 2001, Angell et al., 1993), air-liquid interface coverslip assay (Ludwicka et al., 1985, Merritt et al., 2005), molecular techniques (PCR) (Melo et al., 2013). A wide range of microscopy approaches have been used to visualize biofilms from such as light or fluorescent microscopic examination (Juan C et al., 2012), confocal laser scanning microcopy, scanning electron microscope (SEM) and transmission electron microscopy (TEM) (Zahller and Stewart, 2002, Wallace et al., 2011).

Due to complexity of biofilm, an extensive study of biofilm requires a combination of different experimental methods (Pantanella et al., 2013). Currently, several qualitative methods (TA, CRA) or quantitative method (TCP) are used as routine test in medical areas for the detection of biofilm production, as well as molecular techniques (PCR), which amplify the genes involved in biofilm production as a complement these methods (Oliveira and Maria de Lourdes, 2010).

2.4.3. Quorum sensing (QS)

Cell to cell communication in bacterial communities is called Quorum sensing (QS). Both Gram-positive and Gram-negative bacteria are capable of QS through the production and release of chemical signalling molecules which are called autoinducers (AIs). The mechanism of QS starts by producing AI molecules inside the bacterial cells and their distribution throughout the inner and outer membranes, As the bacterial population density builds the AIs bind to transcription factors or activators (cytoplasmic receptors) forming AI-bound receptors that stimulate the bacteria to alter the expression of target genes e.g. genes that regulate various physiological activities include virulence factor secretion, antibiotic production, motility, sporulation, and biofilm formation (De Kievit and Iglewski, 2000, Miller and Bassler, 2001, Rutherford and Bassler, 2012).

In general, the process or types of QS are different between Gram-negative and Gram-positive bacteria. Gram-negative bacteria use small molecules N-acyl or acylated homoserine lactones (AHL) as autoinducers, while Gram-positive bacteria use peptides or autoinducing peptides (AIP), as signalling molecules to communicate (Miller and Bassler, 2001, Rutherford and Bassler, 2012). QS systems are considered key to the regulation of virulence in both Gram-negative and Gram-positive pathogens and its function is different in Gram negative and Gram-positive bacteria. In *P. aeruginosa* for example QS is considered essential for the development of biofilm-associated infections, while in *Staphylococcus* species, biofilm formation is not promoted by QS (Sifri, 2008).

In *P. aeruginosa,* beside the two major autoinducers (Las and Rhl), A third autoinducer molecule was identified and designated as Pseudomonas quinolone signal (PQS) which is structurally very different from the former autoinducers (Las and Rhl). While the AI in *S. maltophilia* are called diffusible signal factor (DSF) that regulate antibiotic resistance and virulence (Fouhy et al., 2007). A wide range of pathogens use QS to regulate the production of virulence factors (Rutherford and Bassler, 2012). For example, pathogenicity of *P. aeruginosa* usually depend on its ability to produce several extracellular virulence factors such as proteases, exotoxin A, haemolysins, pyocyanin, rhamnolipid and biosurfactants. Regulation of the genes that encode these exoproducts is primarily under the control of the bacteria's signalling system (Whitehead et al., 2001), The AI's of *P. aeruginosa* also have iron chelating properties (Antunes et al., 2010) and regulate gene expression during biofilm formation (De Kievit, 2009). In some other bacteria AI are used as signalling inhibitors during competition between species or strains. For example different strains of *Staphylococcus aureus* are able to generate peptides that are able to inhibit gene expression of other strains (Antunes et al., 2010).

2.5. Extracellular polymeric substance (EPS)

The ‘‘EPS’’ abbreviation has been used to identify a range of biofilm associated materials such as Exopolymers (Omoike et al., 2004), Extra cellular polysaccharides (Kiraly et al., 1997), Exopolysaccharides (Roca et al., 2015b) (McSwain et al., 2005) and extracellular polymeric substances (Dogsa et al., 2005) (McSwain et al., 2005). The term extracellular polymeric substances (EPS) covers a much wider range of materials, including, polysaccharides, proteins, phospholipid and nucleic acids (Czaczyk and Myszka, 2007, Hall-Stoodley et al., 2004, Vu et al., 2009) as such this use of the term covers the full range of extracellular polymers produced by bacteria during biofilm formation. EPS production has a wide variety of roles includes adhesion to surfaces, bacterial cell aggregation, protection from engulfment by predatory protozoa and (phagocytes), protection from desiccation and antimicrobial agents (Nwodo et al., 2012).

Carbohydrates are generally classified as monosaccharaides, disaccharides, oligosaccharides and polysaccharides (Harvey and Ferrier, 2011). Polysaccharides are defined as compounds that are composed of a high number of monosaccharides (more than ten) linked together by glycosidic bonds (McNaught and McNaught, 1997) and include compounds such as cellulose and chitin which are very common in nature (Karunaratne, 2012). A wide range of Extracellular polysaccharides (EPS) are produced by bacteria (Staudt, 2009).
Generally polysaccharide excreted by bacteria can be divided to two groups on the basis of their chemical composition:

a) Homopolysaccharides are defined as a polymer that are composed of one type of sugar e.g. dextrans and cellulose which are composed of series of D-glucose units and produced by *Streptococcus bovis* (Bailey and Oxford, 1958) and *Acetobacter xylinum* (Matsuoka et al., 1996), respectively.
b) Heteropolysaccharides which are composed of two or more different sugars, examples include the polysaccharides that formed by lactic acid bacteria (Sutherland, 1990, Hutkins, 2008).

2.5.1. Extraction and characterisation of Extracellular Polymeric Substances (EPS)

In order to understand the role played by EPS in biofilm formation and persistence it is necessary to extract, purify and characterise individual EPS components. The selection of an appropriate extraction method is very important for studying the physical and chemical properties of EPS (Comte et al., 2006, Flemming et al., 2000) since different extraction methods often lead to very different results (Sun et al., 2014). There are three common approaches for the extraction of EPS: physical, chemical, and a combination of the two (Nguyen et al., 2012). Physical methods generally include centrifugation, ultra sonication and heating, while chemical methods employ chemical substance such as EDTA, formaldehyde, NaCl, ethanol, NaOH and NaOH-formamide for the extraction of EPS (Flemming et al., 2000, Liu and Fang, 2002, More et al., 2014). Combinations of physical and chemical methods have been used in the study of sludge sample such as (centrifugation with chemical substance), (heating +centrifugation + chemical substance) (Liu and Fang, 2002, Bales et al., 2013), lactic acid bacteria (Chadha, 2009, Laws et al., 2001) and *Bifidobacteria* spp. (Leivers, 2011).

Quantity, quality, composition, physical and chemical properties of the extracted EPS depends on both the interaction between the EPS and the cells surfaces and the extraction method employed (Flemming et al., 2000, Pan et al., 2010). A study by Liu and Fang (2002), indicated that EPS yield depends on the method of extraction, for example using (formaldehyde + NaOH) yields more EPS than (formaldehyde + cation exchange resin + EDTA) and the composition of the extracted EPS was the same regardless of the methods used i.e. carbohydrate, protein, humic substance, and small quantities of uronic acid and DNA. Other studies by Comte et al. (2006) and Pan et al. (2010), indicated that generally the quantity of EPS extracted by chemical methods is greater than that extracted by physical methods. However with chemical approaches the extracted

EPS is often contaminated by the chemical reagents employed, especially in the case of EDTA. The presence of chemical contaminants may alter the characteristic of the extracted EPS (Comte et al., 2006). Comte et al. (2006) also indicated that heating (80˚C) may induce the hydrolysis of EPS. Centrifugation is often used as a control for EPS extraction against which other physical and chemical methods are compared (Pan et al., 2010, Liu and Fang, 2002, Comte et al., 2006). Overall a combination of physical and chemical methods is now considered to be more effective in terms of yield and purity than either approach as can be seen in most recent studies (Nguyen et al., 2012).

The isolation and characterisation of the EPS produced by bacteria is important, since it allows the understanding of the roles played by this class of molecules in a wide range of functional applications. In order to find the primary structure of the crude EPS, it is necessary to identify or determine the monomer composition (repeat unit) and their relative ratio in the repeating unit as well as determine how each monosaccharide is arranged (linked) in the repeating unit. Beside these, Nuclear Magnetic Resonance (NMR) spectroscopy, vibration spectroscopic methods (FTIR) and Multi angle Laser Light Scattering (MALLS) have a great value for the study and characterisation of the structure of EPS (Synytsya and Novak, 2014, Judd, 2010). In addition to polysaccharide content EPS is also characterized for its protein and DNA content (Judd, 2010).

2.5.1.1. Total carbohydrate and protein content

Initial analysis of bacterial EPS generally involves an estimation of the total carbohydrate and/or protein content. The phenol sulphuric acid method by (Dubois et al., 1956) is often used for total carbohydrate content and the Bradford assay (Bradford, 1976) provides a good estimate for the total protein content.

2.5.1.2. Nuclear Magnetic Resonance (NMR) Analysis

NMR spectroscopy is used for the elucidation of glycosidic linkages. It is an important non-destructive standard technique for the investigation of polysaccharides (Wingender et al., 2012). NMR spectroscopy determines the structure of the EPS by providing information on ring protons which are frequently crowded and are present in a narrow region (3.0 to 4.0 ppm) (pyranose/furanose) and the anomeric protons (alpha and beta) 4 to 6 (4.5-5.5) ppm are usually distinct from this region. Integration of the anomeric signals gives a preliminary indication of the number and relative orientations of sugars in the repeating unit. The popularity of the structural investigation of carbohydrates by NMR spectroscopy is highlighted by the large number of reviews published in recent years (Bush et al., 1999, Leeflang et al., 2000, Synytsya and Novak, 2014, Sadiq, 2015).

Several type of NMR analysis are combined to analyse EPS such as one-dimensional (1D) proton NMR analysis (^{1}HNMR) and Two-dimensional NMR Analysis (2D). Combination of 1D-NMR with 2D-NMR analysis can be used to determine the structure of the oligosaccharide unit by establishing how the monosaccharide units are linked together and how each carbon and hydrogen are positioned. Combinations of the following 2D- experiments are often required for unequivocal structural determination of the repeating unit structure polysaccharide units:

- COSY - Correlation Spectroscopy.
- TOCSY - Total Correlation Spectroscopy.
- HSQC - Heteronuclear Single Quantum Coherence.
- HMBC - Heteronuclear Multiple Bond Correlation.

NMR (1D and 2D) analysis of EPS is often combined with other analysis including mass spectrometry or chemical analysis (monosaccharide composition or methylation analysis) to determine the complete structure (Gruter et al., 1993).

2.5.1.3. Fourier transform infrared spectroscopy (FTIR)

Fourier transform-infrared spectroscopy (FTIR) spectroscopy is a powerful tool for the structural analysis of polysaccharides. FTIR is a non-destructive technique for the structural analysis of polysaccharides which works by determining of certain chemical groups on the surface of an internal reflection element. FTIR enables the simultaneous collection of information on the chemical and structural features of adherent bacteria within a minute (Wingender et al., 2012, Synytsya and Novak, 2014). Signals in the spectral region of 800-1200 cm^{-1} are important for the structural characterisation of polysaccharides (Coimbra et al., 2002, Černá et al., 2003).

2.5.1.4. Monosaccharide Analysis

As mentioned previously, monosaccharide units attached together through alpha or beta glycosidic linkages form repeating oligosaccharide units (polysaccharides) ranging from linear homopolysaccharides to highly branched heteropolysaccharides (Poli et al., 2011). Cleavage of the glycosidic linkages of the repeating unit structures is required in order to determine the type and the amount of monosaccharides that are present in a sample. Gas chromatography (GC) and high performance liquid chromatography (HPLC) are the most frequently used methods and provide qualitative and quantitative estimation of monosaccharide content. These techniques are preferred to enzymatic methods due to the latter being highly specific and only able to determine a limited number of monosaccharide (Preedy, 2012).

In order to determine the monosaccharide constituents of the polysaccharide, the polysaccharide is firstly hydrolysed with the aid of an acid catalyst (HCl, H_2SO_4 or TFA) and heating to form sugar monomers, these are then analysed by High Performance Anion Exchange Chromatography with Pulsed Amperometric Detection (HPAEC-PAD) or assayed by (GC) after derivatization (premethylation, hydrolysis and acetylated) to alditol acetates. In recent years

HPAEC-PAD has become increasingly popular as an analytical method for monomer analysis in EPS (Leivers, 2011, Cataldi et al., 2000) being first introduced by (Rocklin and Pohl, 1983).

2.5.1.5. Linkage Analysis

Since bacterial EPS may contain a great number of possible monosaccharide combinations and each monomer can also possess a number of different linkages within the structure. It is clearly necessary to determine how each of the monomeric units are linked together in order to get a full appreciation of EPS structure. The most widely used method for linkage analysis is methylation followed by GC-MS (Biermann and McGinnis, 1988, Laine et al., 2002, Synytsya and Novak, 2014).

2.5.1.6. Weight-average Molecular Weight (Mw) Determination

Generally, the size of a polysaccharide is expressed by the number of monosaccharide units it contains; which is termed the degree of polymerisation (DP). The determination of polysaccharide molecular weight is not simple, because it cannot be attributed to one distinctive molecular weight as with monodispersed molecules (Leivers, 2011, Chadha, 2009). Polysaccharides are considered polydispersed molecules because they have a range of molecular weights rather than just one i.e. a polymer is composed of macromolecules of differing molar masses. Polysaccharides are generally characterized by measuring their polydispersity, which is the ratio of the weight-average molecular weight (Mw/Mn) divided by the number-average molecular weight (Gilbert et al., 2009). A polymer is considered monodispersed if the Mw/Mn is equal to one. Weight-average molecular weights of several bacterial exopolysaccharide structures such as *Pseudomonas sp*, *Lactobacillus sp* have been determined by Multi-angle Laser Light Scattering (MALLS)(Fishman et al., 1997, Chadha, 2009).

Currently the method of choice for MW estimation for polysaccharides is High Performance Size Exclusion Chromatography combined with Multi-angle Laser Light Scattering (HP-SECMALLS) (Wyatt, 1993). HP-SEC-MALLS works on the principal of weight and size separation by passing the sample through a smaller analytical scale columns (Oliva et al., 2001, Ritter et al., 2010). Generally, the sample is then passed through an inline ultraviolet (UV) light detector which provides information on the presence of any residual proteins or nucleic acids which may be present in the sample. The system is then coupled with both a MALLS and differential refractive index (RI) detectors which allow for the determination of the accurate molecular mass of the EPS without the requirement of reference materials (Badel et al., 2011).

2.5.2. EPS of Gram Negative Bacteria

2.5.2.1. *Stenotrophomonas maltophilia* EPS structure

Stenotrophomonas maltophilia EPS has been described as a highly charged EPS due to the presence of three uronic acids and an ether-linked D-lactate substituent. Methylation analysis suggested 4-linked Glc, 4-linked GlcA and 3, 4-linked GalA in equal molar amounts in the native polymer as shown in (Figure 2.4) (Cescutti et al., 2011). Data collected by Cuzzi (Cuzzi, 2012) using gas-chromatographic analysis confirmed that *Stenotrophomonas maltophilia* EPS is composed of Glucose (Glc), glucuronic acid (GlcA), galacturonic acid (GalA) and 3-Lac-Hex A residues.

The molecular mechanisms of *Stenotrophomonas maltophilia* biofilm formation have not been well studied (Pompilio et al., 2011). Several genes like rmlA, rmlC, and xanB are necessary for *Stenotrophomonas maltophilia* WR-C biofilm formation and twitching motility, and any mutations in rmlA, rmlC and xanB genes display contrasting biofilm phenotypes (Huang et al., 2006). Pompilio et al. (2011), indicated that the presence of rmlA, spgM or rpfF genes did not significantly affect the biofilm formed by *Stenotrophomonas maltophilia*.

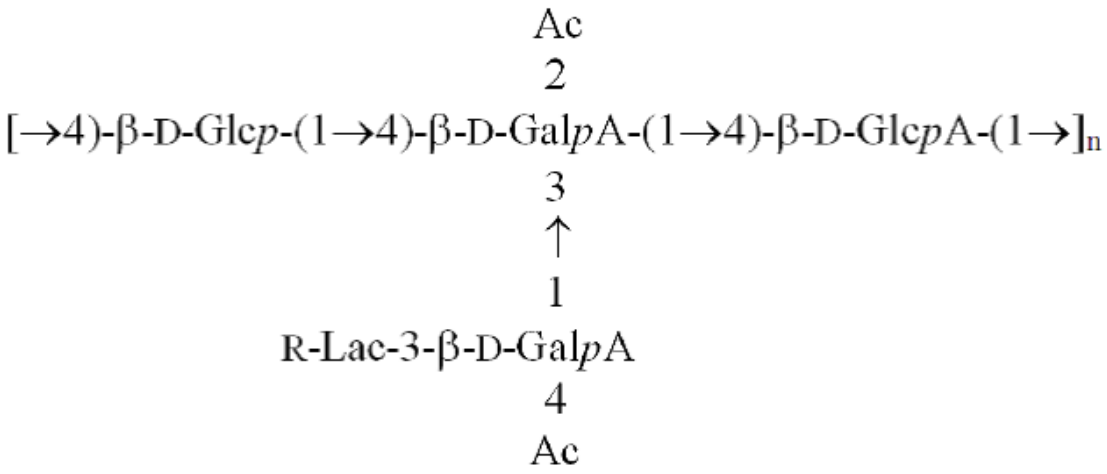


Figure 2.4 Primary structure of the repeating unit of *Stenotrophomonas maltophilia* EPS contains one 4-linked D-Glc, one 4-linked D-GlcA, one 3,4-linked D-GalA and one terminal non reducing D-GalA substituted on C3 with a R lactate group (Cescutti et al., 2011).

2.5.2.2. *Pseudomonas aeruginosa* EPS structure

An additional survival strategy of *P. aeruginosa* is its ability to produce several types of EPS during environmental change (Franklin et al., 2011). Generally *P. aeruginosa* has the ability to produce several EPS compounds such as alginate, Pel and Psl, rhamnolipid and lipopolysaccharide (LPS) (Byrd et al., 2009). *Pseudomonas* spp. produce EPS that is composed of glucose, rhamnose, galactose, and mannose (Bajpai, 2015). Alginate is the predominant EPS in mucoid strains, while in non-mucoid strains such as (PA01 and PA14) Pel and Psl are predominant (Colvin et al., 2012). Alginate and Psl EPS have different chemical structures (Figure 2.3 and 2.4), while the structure of Pel has not been fully characterized. Physiological properties of the cells and the biofilm matrix depend on the type of EPS. In general, at any given time, *P. aeruginosa* strains produce predominantly one secreted polysaccharide EPS, although these strains have the genetic capacity to produce all three polysaccharides EPS (Franklin et al., 2011). Rugose small colony variant (RSCV) isolates of *P. aeruginosa* from lung tissue from a cystic fibrosis sufferer are characterized by wrinkled, small colonies may be an exception to this rule, since they have been shown by

transcriptional analysis to express both the Psl and Pel operons simultaneously (Starkey et al., 2009).

Alginate is a high molecular weight acetylated polymer composed of non-repetitive monomers of β-1, 4 D-Mannuronic acids linked to α-1, 4 L-Guluronic (Mittal et al., 2009, Mann and Wozniak, 2012) (Figure 2.5). Biosynthesis of alginate required 12 genes which are encoded by the algD operon (algD/8/44/K/E/G/X/L/I/J/F/A) while another protein, AlgC, is encoded elsewhere (Rehman et al., 2013, Franklin et al., 2011).

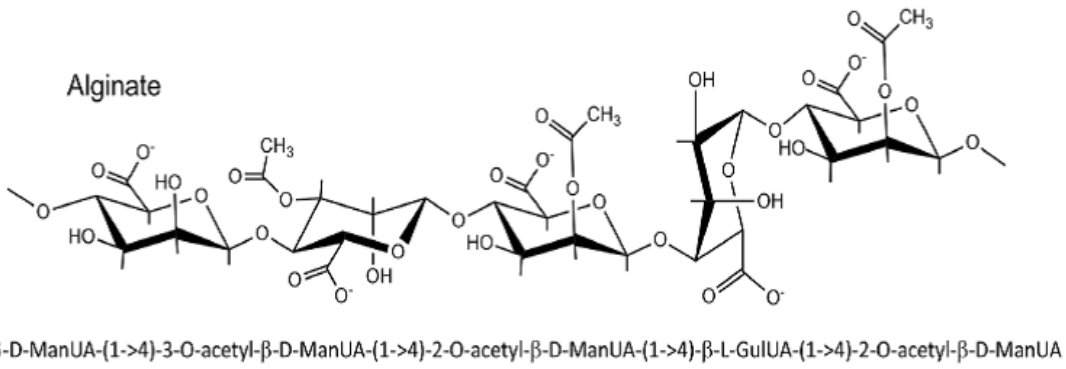


Figure 2.5 Alginate structure (Franklin et al., 2011)

Non-alginate producing strains of *P. aeruginosa* may produce the other polysaccharides Pel and Psl during biofilm formation (Mittal et al., 2009). The Psl polysaccharide is composed of a repeating sub-unit of a neutral pentamer containing mannose, rhamnose and glucose (Byrd et al., 2009, Franklin et al., 2011) and possibly galactose as seen in (Figure 2.6). Some studies have mentioned that the Psl EPS is a mannose rich polysaccharides (Friedman and Kolter, 2004b, Branda et al., 2005) whilst other studies have indicated that the Psl EPS is both galactose and mannose-rich with some glucose, and trace amounts of xylose, rhamnose, and *N*-acetylglucosamine (GlcNAc) (Ma et al., 2007). Twelve genes are required for the biosynthesis of Psl EPS (psl A, B, C, D, E, F, G, H, I, J, K, L) (Franklin et al., 2011, Colvin et al., 2012, Branda et al., 2005) with Psl of

the biofilm forming PA01 strain receiving considerable attention (Ma et al., 2009).

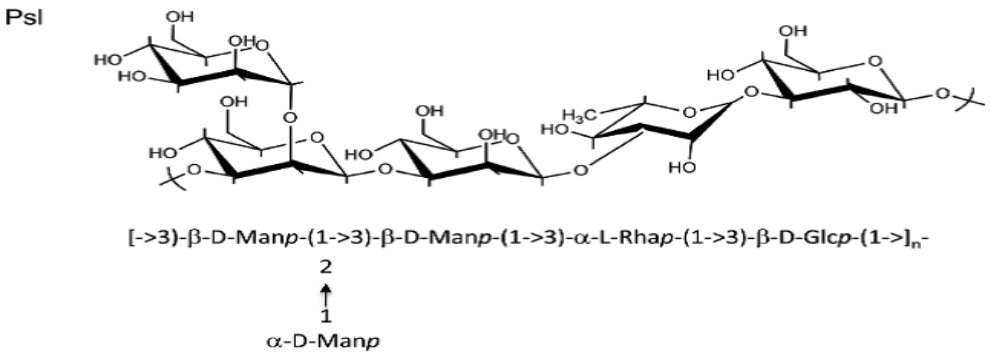


Figure 2.6 Psl polysaccharide structure. Composed of a repeating pentamer containing D-mannose, L-rhamnose, and D-glucose (Franklin et al., 2011).

The Pel EPS is formed at the air–liquid interface and is controlled by seven genes (pelA, B, C, D, E, F, and G) which are essential for biofilm formation in the *P. aeruginosa* PA14 strain. At present, the structure of Pel EPS has not been determined (Franklin et al., 2011, Wei and Ma, 2013, Colvin et al., 2011a, Colvin et al., 2012, Whitfield et al., 2015) but carbohydrate and linkage analyses provide evidence that Pel EPS is a glucose-rich matrix polysaccharide that is distinct from cellulose (Ryder et al., 2007, Ma et al., 2009, Lembre et al., 2012a). This glucose-rich polysaccharide (Pel EPS) is also essential for the formation of a wrinkled colony and a surface-associated biofilm (Colvin et al., 2011a, Sakuragi and Kolter, 2007).

2.5.2.3. *Enterobacteriaceae* family and *Citrobacter freundii* EPS structure

Some members of the clinically important *Enterobacteriaceae* such as *Citrobacter* spp. and *Enterobacter* spp. have the ability to produce cellulose EPS and or curli fimbriae (csg) during biofilm formation. Cellulose biosynthesis required genes that are encoded by the bcsA, B, Z, C operon. Among these genes, bcsA encodes the cellulose synthase while the exact functions of the other genes remain unknown, but curli fimbriae synthesis are encoded by the csgB, A, C

operon. Cellulose and curli fimbria biosynthesis is commonly regulated, either through adrA or directly by csgD (Zogaj et al., 2003).

Zogaj et al. (2003) also indicated that *Citrobacter* spp. and *Enterobacter sakazakii* displayed an EPS, cell clumping, pellicle formation, and biofilm formation associated with the expression of cellulose and curli fimbriae. *Citrobacter* spp. produce curli fimbriae and cellulose while *Enterobacter* spp. produced cellulose. In both bacteria the *csgD-csgBA* region and the cellulose synthase gene *bcsA* were conserved emphasising the importance of biofilm formation for these bacteria that are frequently associated with biofilm related catheter-associated UTI (Zogaj et al., 2003). According to (Bajpai, 2015), all slime bacteria EPS contain uronic acid, and *Citrobacter* spp. EPS mainly consist of galactose, glucose and mannose and is similar in characteristics to chitosan (Jang et al., 2005). While in another study (Rättö et al., 2006), *Citrobacter* spp. produced EPS that was composed mainly of fucose, rhamnose, and other sugars such as galactose, glucose, mannose, galacturonic acid, glucuronic acid and pyruvate.

2.6. Hydrophobicity

One key factor associated with the ability of bacteria to adhere to a surface (e.g. medical implants hydrocarbons, oral cavity, food products) is the surface hydrophobicity of the cells. Hydrophobicity or microbial adhesion studies are widespread in the biofilm research since this process is essential in the establishment of microbial biofilms on virgin surfaces e.g. medical implants, catheters, etc. The surfaces of bacteria are composed of numerous macromolecules such as polysaccharides, lipopolysacchrides (LPS), lipo-proteins, lipids and lipoteichoic acids, and surface appendages such as fimbriae, pili and flagella. These surface structures generate the physicochemical properties of the bacteria such as its surface charge. Bacterial surface structures vary with ambient environmental conditions, mutations or gene expression (Saini, 2010).

Bacterial cell surface lipopolysaccharides (LPS) are a major component in Gram-negative bacterial outer membrane (Moran, 2009). The LPS molecule is an amphiphilic molecule composed of a hydrophobic glycolipid (Lipid A), a hydrophilic heteropolysaccharide (core polysaccharide), and an O-antigen or O chain which is a long chain polysaccharide (Saini, 2010).

Bacterial surface hydrophobicity is an important determinant in the adherence of the bacteria to both living and non-living surfaces, and it may assist bacterial colonization on epithelial tissues and medical devices, consequently surface hydrophobicity is closely related to biofilm formation and pathogenicity (Polaquini et al., 2006, Mattos-Guaraldi et al., 1999). Adherence is considered a critical stage because once adhered bacteria produce biofilms and eradication becomes very difficult (Krepsky et al., 2003). Polaquini et al (2006) indicated that hydrophobic bacteria are more adherent to the surfaces of inanimate objects and have enhanced phagocytosis resistance than hydrophilic bacteria (Polaquini et al., 2006).

There are a wide range of methodologies for the measurement of microbial hydrophobicity, such as contact angle measurement, microbial adhesion to hydrocarbons (MATH), salt aggregation test (SAT) (Saini, 2010). Unfortunately, many of these methods are highly variable and inconsistent, making effective hydrophobicity measurements problematic.

2.7. Outer membrane vesicles (OMV)

Outer membrane vesicles (OMV) are spherical and bi-layered nano-vesicles with a diameter of 20~250 nm that are secreted by a large variety of Gram-negative bacteria either during normal bacterial growth or as a stress response (MacDonald and Kuehn, 2013). OMV are part of the membranous structure in all Gram negative bacteria including pathogenic and non-pathogenic strains (Lim and Yoon, 2015). OMV contain much of the biological content that found within the parent bacterium including lipopolysaccharide (LPS),

phospholipids, periplasmic and membrane-bound proteins. Furthermore, several virulence factors, such as adhesions enzymes, toxins, DNA, RNA and peptidoglycan can all be found in OMV (Kaparakis-Liaskos and Ferrero, 2015, Lee, 2012, MacDonald and Kuehn, 2013, Kulp and Kuehn, 2010). OMV provide an advantage to the parent bacterium by facilitating bacterial communication and transferring enzymes, DNA, and proteins, as well as promoting the survival and maintenance of bacterial communities (Kaparakis-Liaskos and Ferrero, 2015). These characteristics make OMV important during the initial stages of infection (Li et al., 1998) (Figure 2.7).

OMV are produced from the outer membrane and periplasmic membrane, so they reflects these components of the bacteria concerned. *S. maltophilia*, *P. aeruoginosa* and *C. freundii* have all been shown to secrete OMV (Devos et al., 2015, Kadurugamuwa and Beveridge, 1995, MacDonald and Kuehn, 2013). In *P. aeruoginosa* OMV are composed of alkaline phosphatase, phospholipase C, protease, and peptidoglycan hydrolase (Li et al., 1998). OMV plays a role in nutrient transformation, biofilm formation and resistance to antimicrobial (Lim and Yoon, 2015). OMV also play a role in communication between cells, the lysing and killing of competing bacteria and the modulation of host immune response, and as such they contribute to bacterial survival and virulence. For example OMV of *Pseudomonas aeruoginosa* PA01 are capable of lysing *Staphylococcus aureus* and *Escherichia coli*, while those generated by *Citrobacter* strains can lyse *Mycobacterium* strains (Li et al., 1998, Lee, 2012, Kulp and Kuehn, 2010).

The small size and membrane component of OMVs provide protection from host proteases and may facilitate their dissemination into tissues beyond the area that the further bacterium can reach (MacDonald and Kuehn, 2013). In nosocomial associated *S. maltophilia*, the generation of OMV increases after treatment with β-lactam antibiotic carbapenem. In this case the OMV generated contained two chromosomes encoding for a β-lactamase (Devos et al., 2015).

OMV help *P. aeruoginosa* in pathogenesis due to their association with other virulence factors such as peptidoglycan hydrolase, hemolysin, phospholipase C and alkaline phosphatase. These OMV effect host cells by disrupting the electron transport chains and trafficking pathways of the cells. Due to these factors *P. aeruoginosa* OMVs can kill both Gram-negative and Gram-positive competing bacteria in co culture or in the environment especially during infection (MacDonald and Kuehn, 2013). OMV production is affected by growth conditions such as media type, temperature and nutrient levels, for example an increase in temperature will increase OMV production (Kulp and Kuehn, 2010). In *P. aeruginosa* planktonic bacteria release OMV during normal growth, providing this bacterium with an highly potent host cell lysis mechanism (Eddy et al., 2014, Avila-Calderón et al., 2011). In common with other Gram negative bacteria the generation of OMV by *P. aeruginosa* is enhanced by the presence of stress factors such as the presence of antibiotics or nutrient limitations. OMV produced under stress condition are different from those that produced under normal growth condition (Chatterjee and Chaudhuri, 2012). This stress response explains why OMV production in *P. aeruginosa* increases following exposure to antibiotics such as gentamicin and carbenicillin. This increase in OMV secretion is mirrored by an increase in biofilm formation (Kadurugamuwa and Beveridge, 1995, Yoon et al., 2011).

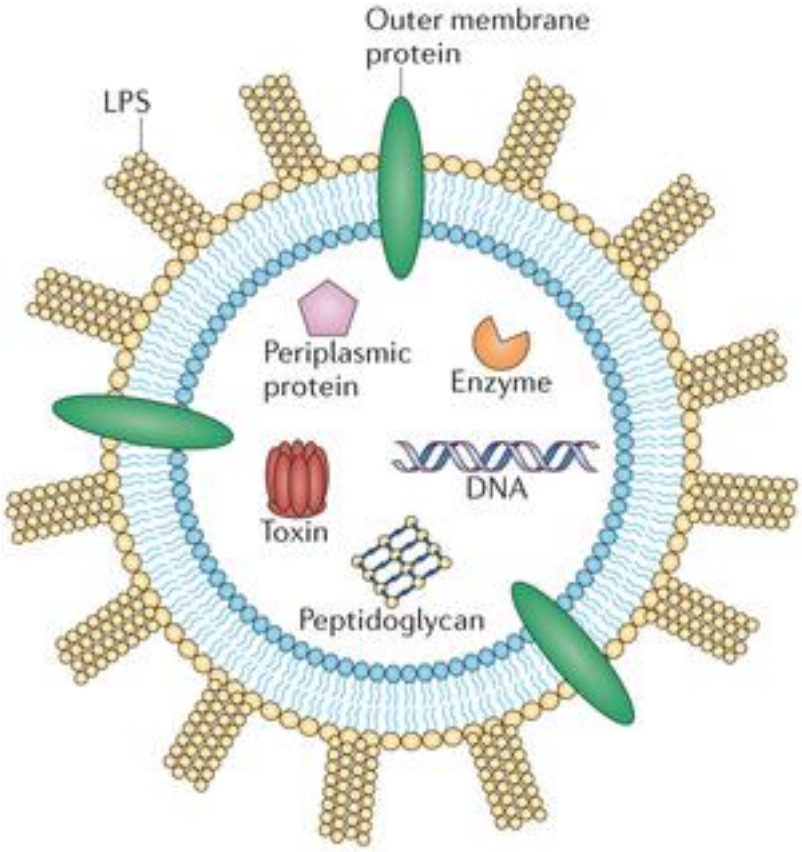


Figure 2.7 Gram negative bacterial outer membrane vesicle (OMV) containing biological contents that found within the parent bacterium (Kaparakis-Liaskos and Ferrero, 2015).

2.8. The *Galleria mellonella* model for bacterial pathogenicity

Recently the larvae of the wax moth (*Galleria mellonella*) has increasingly being used to study the virulence and pathogenicity of bacteria and fungi (Harding et al., 2013). In addition to the obvious ethical advantages there are a number of practical advantages to the use of the *Galleria mellonella* (*G. mellonella*) larvae including the fact that they are easy to handle, can be studied in large numbers and the experiments are cost-effective (Bender et al., 2013). Furthermore presence of similar tissues, anatomy, and physiological functions in insect and mammalian gastrointestinal tracts make them an ideal model for human pathogenicity. For example, the *G. mellonella* mid gut microvilli contain microbes that resemble those found associated with the mammalian intestinal microvilli

(Mukherjee et al., 2013). Another advantage of the *Galleria* model is that it can be maintained very easily, and can be injected and infected without anaesthesia. Although insects do not have the adaptive immune system found in mammals, the hemolymph of *G. mellonella* contains haemocytes that behave similarly to phagocytic cells in response to bacterial infection (Harding et al., 2013).

G. mellonella has been used to study a wide range of bacterial pathogens and their virulence including:

- The effects of inoculum size and pathogenicity of *Acinetobacter baumannii* versus the less pathogenic species *Acinetobacter baylyi* and *Acinetobacter lwoffii* (Peleg et al., 2009);
- Wand et al. (2012) used *G. mellonella* to study pathogenicity of biofilm formation by *Acinetobacter baumannii* and its relation to virulence;
- Whiley et al. (2013) investigated virulence of the Liverpool Epidemic Strain (LES) of *P. aeruginosa* isolated from Cystic Fibrosis patients;
- Miyata et al. (2003) studied the pathogenesis of the type III secretion system in *P. aeruginosa*;
- The pathogenicity of *Klebsiella pneumoniae* was comparing with the pathogenicity of none pathogenic *Klebsiella* strains (Insua et al., 2013).
- *Helicobacter pylori* virulence (Giannouli et al., 2014);
- Effects of inoculum size, antibacterial agents against *Staphylococcus aureus* and methicillin-resistant *S. aureus* (MRSA) infections (Desbois and Coote, 2011);
- The study of OMV biogenesis in *Serratia marcescens* (McMahon et al., 2012) and *Campylobacter jejuni* (Elmi et al., 2012).

From this list of investigations it is clear that the wax worm model has gained some level of acceptance as a pathogenicity model for bacterial infections.

2.9. Bacterial gene expression study

Gene expression can be used to study any changes in the physiological states and functional activities of a cell under environmental stresses. It has been observed that major differences in gene expression occur during the transition from the planktonic to the biofilm mode of growth (Sauer et al., 2002, Stoodley et al., 2002). In a study of *Escherichia coli*, biofilm gene were differentially expressed by at least two-fold between biofilm and planktonic cells (Prigent-Combaret et al., 1999). Physiological activity studies of bacteria growing in biofilms are difficult due to the diversity of processes occurring simultaneously and temporally as the biofilm develops. The activities of a particular biofilm will be effected and shaped by the specific chemical and physical environment in which it grows. Consequently it is difficult to develop a consensus picture of the physiology of the biofilm as it developed, for example Folsom et al, (2010) found little overlap in the lists of genes differentially expressed between the planktonic and biofilm states of *P. aeruginosa* (Folsom et al., 2010).

Several methods have been used for the study of bacterial physiology such as proteomic and transcriptomic analysis. The proteome is defined as the characterisation of the complete or whole protein complement of a genome (Graves and Haystead, 2002). Proteomic analysis is an alternative approach to the analysis of differential gene expression (Sauer et al., 2002, Monteoliva and Albar, 2004). Proteomic analysis of an organism not only provides a list of all proteins encoded by the genome but also data on protein expression under defined conditions (Graves and Haystead, 2002). Proteomic analysis has been used to investigate biofilm-specific gene expression in the biofilms of *P. aeruginosa* (Sauer et al., 2002). The results obtained from this approach indicated that a large number of genes are differentially expressed during biofilm development which differed from that seen in the planktonic profile, possibly correlating with the expression of a different biofilm phenotypes (Sauer et al., 2002). Proteomics is still faced with a number of challenges such as the protein purification

requirements demanded by the study of low-abundance proteins such as transcription factors, protein kinases and regulatory proteins (Steyn, 2005).

A transcriptome is a complete set of transcripts recovered from a cell (Nagalakshmi et al., 2010) and is essential for interpreting the functional elements of the genome and revealing the molecular constituents of cells and tissues, as well as understanding the development and disease. The key aims of transcriptomics are:

- The cataloguing of all types of transcript, including (mRNAs, non-coding RNAs and small RNAs).
- The determination of the transcriptional structure of genes.
- The quantification of the changing expression levels of each transcript during development and under different conditions (Wang et al., 2009).

The analysis of transcriptomes is of great value for the investigation of gene function, expression and regulation. Gene expression provides dynamic changes in information regarding the physiological state and functional activities of a cell under different environmental conditions (Zhou et al., 2004).

A number of approaches are available for the analysis and quantification of the transcriptome, such as the hybridization sequence method (Sanger), microarray hybridization base methods and RNA sequencing (Nagalakshmi et al., 2010). hybridization sequence method (Sanger) are relatively low throughput, more qualitative and expensive. On the other hand hybridization based methods (Microarray) are high throughput, and relatively inexpensive however they do have several limitations, including reliance on existing knowledge about genome sequence. Also they require complicated normalisation methods because of the difficulty of comparing expression levels between experiments. These disadvantages limited the use of traditional sequencing technology in annotating the structure of transcriptomes. RNA sequencing methods have now been developed which allow the mapping and quantifying transcriptomes, this has a clear advantage over existing methods. In RNA sequencing a population of RNA

is converted to cDNA fragments with adaptors attached to one or both ends. Each molecule, is then sequenced to obtain short sequences from one end or both ends. Typically, the reads are 30–400 bp (Wang et al., 2009).

RNA sequencing offers several advantages for the study of complex transcriptomes (Table 2.1). Unlike hybridization based methods (Microarray), sequencing is not limited to the detection of transcripts that correspond to defined genomic sequences. RNA sequencing can reveal the precise location of transcription limitations, to a single base resolution. RNA sequencing has no or very low background and does not have an upper limit for quantification of number of sequences. This means that this technology has a large dynamic range of expression levels over which transcripts can be detected. By contrast, DNA microarrays lack sensitivity for genes expressed either at low or very high levels (100 to a few-hundredfold). RNA sequencing has also been shown to be highly accurate for quantifying expression levels. The results of RNA sequencing also show high levels of reproducibility, for both technical and biological replicates. Consequently, RNA sequencing is the first quantitative sequencing based method with a very high-throughput allowing the analysis of the entire transcriptome. This allow annotation and the quantification of gene expression with lower costs than other methods (Wang et al., 2009).

Due to these advantages, RNA sequencing is a highly useful approach for the investigation of bacterial transcriptomic dynamics during development and normal physiological changes, for the analysis of biomedical samples, and the comparison between normal and diseased tissues, as well as the classification of diseases (Wang et al., 2009). RNA sequencing has been used successfully in a number of studies such as monitoring gene expression during yeast vegetative growth, yeast meiosis and mouse embryonic stem cell differentiation, *Sac charomyces cerevisiae*, *Schizosaccharomyces pombe, Drosophila melanogaster* and *Bacillus halodurans* (Nagalakshmi et al., 2010).

Table 2.1 Summary of RNA sequencing advantage comparing to the other methods

Technology	Hybridization sequence methods (EST)	Hybridization based methods (Microarrays)	RNA sequencing
Principle	Sanger sequencing	Hybridization	High throughput sequencing
Resolution	Single base	Several -100 base	Single base
Throughput	Low	High	High
Background noise	Low	High	Low
Dynamic range to quantify gene expression	Not practical	Up to few Hundredfold	>8000 fold
Required amount of RNA	High	High	Low
Large genome transcriptome mapping cost	High	High	Low

Expressed sequence tag (EST); collection of partial cDNA sequences

3. Research Aims

There are four research aims associated with this project:

1. To determine the ability of Gram negative pathogenic bacteria to form biofilms on inanimate surfaces such as glass and plastic;
2. To determine the chemical structure of the EPS generated by the Gram negative pathogens under investigation;
3. To evaluate the pathogenicity of biofilm forming Gram negative pathogens on different carbon sources using the *Galleria mellonella* wax worm model;
4. To employ genomic and transcriptomic approaches to determine the molecular basis of biofilm formation and associated pathogenicity of the Gram negative pathogens growth on different carbon sources under investigation.

4 Material and Methods

4.1 Bacterial strains

A wide range of bacterial strains including both type or lab and isolated strains were investigated in this study. These bacteria are outlined in (Table 4.1). Several hospital isolates of *Stenotrophomonas maltophilia* were kindly provided by Dr Jane Turton, Laboratory of Health Care Associated Infection (LHCAI), Health Protection Agency (HPA), London, UK. *Pseudomonas aeruginosa* strains had been previously isolated from a discarded wound dressing provided anonymously from a local skin integrity practitioner (Shoukat et al., 2015).

All strains used in this investigation were preserved at -80˚C using the Cryobank bacterial preservation systems. Cultures were recovered from Cryobanks by sub culturing onto TSA and grown aerobically overnight at 37˚C. Stock plates were then kept in the fridge at 4˚C and grown overnight at 37˚C on TSA prior to use. Prior to media inoculation, overnight fresh bacterial cultures were grown on TSA from stock plates. Bacterial suspensions of 10^8 or 10^9 CFU/ml were then prepared from the stock plates in small conical flask containing Maximum recovery dilution (MRD) and glass beads by adjusting the OD at 620nm (EN, 2009).

Table 4.1 Bacterial strains used in this study

Species	Type of strain	Culture collection or patient (ID)	Short identifier	Source
Stenotrophomonas maltophilia	Clinical strain	H09*****18	St 18	Strains provided by Dr J. Turton, LHCAI, HPA London.
	Clinical strain	H08*****51	St 51	
	Clinical strain	H09*****53	St 53	
	Lab strain	NCIB 9203 (ATCC 13637)	St 9203	ATCC
Pseudomonas aeruginosa	Clinical strain	N/A	Ps 1	Isolated from dressing removed from infected wounds.
	Clinical strain		Ps 3	
	Clinical strain		Ps 5	
	Lab strain	NCIB 10421 (ATCC 15442)	Ps 10421	ATCC
Acinetobacter baumannii	Clinical strains	OXA-23 Clone 1	Clone 1	Strains provided by Dr J. Turton, LHCAI, HPA London.
	Culture collection	CIP 106882	882	Pasteur Institute, Paris, France

4.2. Media, reagent and other preparations

Several microbiological media and modified media were used throughout this study. Mineral media with a range of carbon sources (MM Glc, MM Gly and MM Eth) were prepared according (Shoukat, 2014) with modification as shown in (Table 2.2). All material in mineral media are from Fisher Scientific Ltd, except casein which is from Fluka.

Table 4.2 Mineral media with different carbon source

No.	Components	Formula	MM Glc g/l	MM Gly g/l	MM Eth g/l
1	Dipotassium phosphate	K_2HPO_4	4.5	4.5	4.5
2	Ammonium sulphate	$(NH_4)_2SO_4$	1	1	1
3	Magnesium sulphate heptahydrate	$MgSO_4.7H_2O$	1	1	1
4	Sodium chloride	NaCl	0.1	0.1	0.1
5	Calcium chloride	$Cacl_2$	0.1	0.1	0.1
6	Ferric chloride	$Fecl_3$	0.02	0.02	0.02
7	Casein	Casein	1, 2 or 3	1	1
8	Glucose	Glucose	5,10,15, 20	-	-
9	Glycerol	Glycerol	-	5,10,15, 20	-
10	Ethanol 99%	Ethanol 99%	-	-	5.29, 10.57 ,15.86
		pH	7	7	7

Mineral medium glucose (MM Glc), Mineral medium glycerol (MM Gly), Mineral medium ethanol (MM Eth)

Note: Glucose and glycerol were added prior to autoclaving, while ethanol were added after autoclaving

Note: Different concentration of carbon source and casein were used according to the purpose of the experiment in order to optimise the quantification of biofilm.

Note: All ethanol volume (ml/l) converted to (g/l) by using density of ethanol which is 0.789 g/cm^3.

A range of other media were also used in this investigation including:

- Tryptone Soy Agar - TSA (LabM Ltd), prepared by dissolving 37g in 1liter of deionised water.
- Tryptone Soy Broth - TSB (LabM Ltd), Prepared by dissolving 30g in 1liter of deionised water.
- Maximum Recovery Diluent - MRD (LabM Ltd), Prepared by dissolving 9.5g in 1liter of deionised water.
- Simulated Wound Fluid (SWF) prepared by mixing equal volume of filtered Foetal calf serum (Sigma-Aldrich Ltd) and sterilised MRD.
- Phosphate Buffer Saline (PBS) prepared by dissolving 8g/l NaCl, 0.2g/l KCl, 1.44 g/l Na_2HPO_4 and 0.24g/l KH_2PO_4 (Fisher) in UPW with the pH adjusted according to the purpose of experiment.
- Luria Bertani Broth (LB) prepared by dissolving 10g Tryptone, 5g Yeast Extract, and 5g Sodium Chloride in 1liter of deionised water.

(Note: TSA, TSB, MRD, LB and PBS were autoclaved at 121˚C for 15 min).

- Agarose gel (1%) was prepared by dissolving and melting 1g agarose gel powder (Bio line) in 100ml 1x Tris-acetate-EDTA (TAE). 1µl gel stain was added before pouring the gel.
- Primers for PCR reactions were prepared by mixing 10µl (100pmol) forward primer (F) with 10µl (100pmol) reverse primer (R) and making up to 100µl with diethylpyrocarbonate (DEPC) water.

- Dialysis tubing (14000 typical molecular weight cut-off (MWCO) cellulose membrane dialysis tubing, 33 mm width (Sigma Aldrich Ltd)) was prepared by washing the tubing under flowing tap water for at least 4 h or overnight, and then washed in 0.3% (w/v) sodium sulphide solution at 80˚C for 1 min followed by washing in hot deionized water at 60˚C for 2 min, followed by washing in 0.2% (v/v) sulphuric acid solution for 1 min and then finally rinsing with hot deionized water at 60˚C for 2 min. Finally the dialysis tubing was rinsed with deionized water and stored at 4˚C in a container containing deionized water.
- To prepare a protein dye stock 100mg Coomassie Blue G–250 was dissolved in 50 ml of 95% Ethanol and then mixed vigorously with 100 ml of 85% (w/v) phosphoric acid (H_3PO_4) and then made up to 200ml with UPW. The dark red solution were formed and stored in a dark bottle at 4˚C. The assay reagent was prepared by mixing 1 volume of dye stock with 4 volume of UPW, and filtering through Whatman paper before use.
- 4 M sulfamic acid (H_3NSO_3) solution was prepared by dissolving 3.884g of sulfamic acid in 5 ml UPW under continuous mixing whilst adding potassium hydroxide (KOH) until completely dissolved. The final volume was completed with UPW (10ml final volume).
- 0.15% (w/v) m-hydroxydiphenyl reagent was prepared by dissolving 15 mg of m-hydroxydiphenyl in 10 ml of 0.5% (w/v) sodium hydroxide (NaOH) in the dark bottle and stored in the fridge.
- 2.86% (w/v) borax reagent was prepared by dissolving sodium tetraborate 2.86g in 100 ml of concentrated sulfuric acid.
- 50mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer (HEPES) was prepared by dissolving 11.915g in 1litter of sterilised UPW and then sterilised by filteration.
- Congo Red agar (CRA) media were prepared according to (Freeman et al., 1989). By weighting 37 g/l brain heart infusion broth, 50 g/l sucrose, 10 g/l

agar and 8 g/l Congo red indicator. Congo red stain was prepared as a concentrated solution and sterilized separately. It was then added to the autoclaved brain heart infusion agar before solidifying.

4.3 Bacterial identification

After recovering bacterial isolates from storage, several tests were performed in order to confirm the identification of the bacterial strains concerned.

4.3.1. Bacterial morphology and Gram stain

Overnight fresh cultures were used for Gram staining and cell morphological investigations. The bacterial film or smear was prepared by spreading pure bacterial colonies mixed with a drop of sterilised distilled water on a clean slide and then fixed by heating. After that the slide stained (crystal violet, iodine, alcohol and safranin) and washed by water after each stain (Estridge et al., 2000). Finally, the slide left to dry and observed under oil immersion objective lens (100x).

4.3.2. Biochemical tests

4.3.2.1 Oxidase Test

The test was performed according to manufacturer's instruction (Prolab Diagnostics Ltd). Briefly, 1-2 drops of oxidase reagent was added to the filter paper and left to dry for 30-60 seconds. A loop full of bacteria from an overnight fresh bacterial plate culture was then mixed with the reagent on the filter paper. A positive result was recorded if a deep blue/purple colour developed within 30 seconds.

4.3.2.2 Catalase test

The catalase test was performed by smearing samples taken from fresh overnight bacterial cultures grow on TSA to the centre of the clean slide. A few drops of H_2O_2 were then added to the smear. The rapid evolution of bubbles was taken as evidence of a positive result (catalase positive).

4.3.2.3 Triple sugar iron agar (TSI) test

TSI medium was prepared by mixing 20g/l peptone, 3g/l beef extract, 3g/l yeast extract, 10g/l lactose, 10g/l sucrose, 1g/l dextrose, 0.2g/l ferrous sulphate, 5g/l sodium chloride, 0.3 sodium thiosulphate, 0.025g/l phenol red and 12 g/l agar. The mixture was dissolved in distilled water and the pH adjusted to 7.4± 0.2 and sterilised by autoclaving at 15 lbs. at (121°C) for 15 min. The tubes were inoculated with a fresh overnight culture using straight loop. Interpretation of result were checked after incubation at 37°C by monitoring bottom of the tubes (acid, acid and gas or alkaline), slant surface (acid, acid and gas, alkaline or no change) and also observing the black precipitate in the bottom.

4.3.3 Molecular identification of bacteria

4.3.3.1 DNA extraction

Bacterial DNA was extracted from pure cultures via the UltraClean Microbial DNA isolation kit (Cambio Ltd, USA) following the manufacturer's instructions. Briefly, several colonies from fresh overnight cultures were added to approximately 1.8ml of PCR grade water, mixed and then centrifuged at 10,000xg for 30 seconds. The supernatant was then discarded and re-centrifuged under the same condition and the remained supernatant removed. The pellet was suspended in 300µl MicroBead solution and vortexed gently and then transferred to a MicroBead tube. 50µl of solution MD1 were added and the MicroBead tube, vortexed at a maximum speed for 15-20 min using the MO BIO vortex adapter. After that the MicroBead tube was centrifuged at 10000xg for 30 seconds at room temperature and the supernatant transferred to a new clean 2ml Eppendorf tube. 100µl of solution MD2 were then added to the supernatant, vortexed and incubated for 5 min at 4°C. The tubes were centrifuged at 10000xg for 1 min at room temperature then the supernatant carefully transferred to a new Eppendorf tube with a pipettes without touching the pellet. 900µl MD3 were added to the supernatant and vortexed for 5 seconds, after that approximately 700µl transferred

into the Spin Filter and centrifuged at 10000xg for 30 seconds. Flow through were discarded and remaining supernatant were added to the spin filter once more and centrifuge at 10000xg for 30 seconds at room temperature. 300µl Solution MD4 were then added and centrifuged at room temperature for 30 seconds at 10000xg and the flow through were discarded then centrifuged at room temperature for 1 min at 10000xg. The spin filter was then transferred carefully to a new clean Eppendorf tube and 50µl of solution MD5 were added to the centre of the white filter membrane, centrifuged at 10000xg for 30 seconds. Finally, the spin filter was discarded and the DNA concentration measured by micro volume spectrophotometer (Jenway, Genova Nano) against solution MD5 as a blank. The amount of genomic DNA extracted was measured by agarose gel electrophoresis using 1% (w/v) agarose gel. The extracted DNA was mixed with 5xDNA Loading Buffer Blue (Bioline) and run against 5µl of a 1kb HyperLadder (Bioline).

4.3.3.2 Polymerase Chain Reaction PCR

Amplification of the 16S rRNA gene was performed by using a Techne Thermocycler (Bibby Scientific Ltd., Stone, UK). A 50µl final volume PCR mixture was prepared by mixing 25µl Biomix red, 17µl PCR grade water, 3µl primer (F&R) and 5µl template. PCR was completed under the following conditions: heated lid at 100°C, 95˚C for 5 min followed by 35 cycles of 94˚C for 1 min, 60˚C for 45 seconds, and 72˚C for 1 min and then by a temperature period of 72˚C for 2 min, and holding at 4˚C. PCR products were analysed directly by 1% (w/v) agarose gel electrophoresis alongside 5µl of a 1kb HyperLadder.

4.3.3.3 PCR products purification.

PCR products were purified using a QIAquick PCR Purification Kit according to manufactures protocol. Briefly 5 volumes of Buffer were added to 1 volume of the PCR sample product and mixed. Then 10µl of 3M sodium acetate was added and mixed. The mixture was transferred into the QIAquick spin column

and centrifuged for 30–60 seconds at 13000 rpm, and the flow through decanted. After that 750µl of Buffer PE were added into the QIAquick column and centrifuged at 13000 rpm for 30–60 seconds, the flow though is discarded and the sample re-centrifuged for 1 min. The DNA was eluted by adding 50µl Buffer EB to the centre of the QIAquick membrane and centrifuged at 13000 rpm for 1 min. The concentration of DNA was measured by micro volume spectrophotometer and analysed once more by agarose gel electrophoresis under the same condition outlined above. Finally, 15µl of of 10µg/ml purified PCR product was mixed with 2µl forward (F) or reverse primer (R) in an Eppendorf tube and sent for 16S rRNA sequencing at (Eurofins Genomics-UK).

4.3.3.4 Analysis of bacterial sequences

After the 16S rRNA genes were sequenced, the sequences were processed and analysed by several online databases. Initially the software provided by the Ribosomal Database Project (RDP) (RDP Release 11, Update 4: May 26, 2015) (Cole et al., 2013) was used, this is a free online gene sequence database analyses tool. Initially the RDP - classifier program (RDP Naive Bayesian rRNA Classifier Version 2.10, October 2014) was employed to classify the bacteria concerned in the higher order bacterial taxonomy (domain and genus) (Wang et al., 2007). The programme provides a 95% confidence threshold for the analysed sequences.

The RDP sequence match program (version 3) was used to find closest direct sequence matches to the gene sequences generated by this research. Here, 10000 sequences are included in the search with the ability to selecting up to 20 match bacteria (Cole et al., 2003). After uploading unknown sequenced gene data in this program, the top 4 nearest neighbours in the system were selected for further analysis.

4.3.3.5. Construction of phylogenetic trees

Initially the 16s rRNA gene sequence was subjected to chimeric testing using the mothur software (Schloss et al., 2009). Both the RDP and the MEGABLAST programmes were used to construct phylogenetic trees of the bacterial strains under investigation. The MEGA BLAST software (version 5.05) is a reliable and commonly used tool for the generation phylogenetic tree from bacterial 16s rRNA gene sequences (Tamura et al., 2011). BLAST is designed to look for local alignments or finding matches to a query sequence within a large sequence database, such as Gene bank (Altschul et al., 1990). Phylogenetic analysis is performed by selecting all sequence close to the sequence from the unknown sample, these are then aligned using the muscle software (Edgar, 2004). For pairwise distances computation, the Kimura 2-parameter model (Kimura, 1980) was used with 1000 replicate bootstraps. After the application of the neighbour joining method (Saitou and Nei, 1987) to generate the relative evolutionary history, the phylogenetic tree is plotted.

4.4. Whole Genome Sequencing (WGS)

In order to perform whole genome sequencing overnight cultures were grown in TSB at 37˚C for 24 h. Bacterial DNA was then extracted by the same method previously described (See Section 4.3.3.1), however in this case a greater concentration and purity was required. Greater than 100µl of DNA were prepared at >50 µg/ml with the purity of 1.8-2.0 at OD 260/280. The draft genome sequences were generated by BaseClear Ltd (NL) using an Illumina High sequencing 2500 system and Paired-end 125 cycles sequence reads were generated. FASTQ sequence files were generated using the Illumina Casava pipeline version 1.8.3 and the assembly prepared using CLC Genomics Workbench version 7.0.4. The contigs were linked and placed into scaffolds or supercontigs. The orientation, order, and distance between the contigs was estimated using the insert size between the paired-end and/or meta pair reads

using the SSPACE Premium scaffolder version 2.3 (Boetzer et al., 2011). The WGS were then analysed or annotated by the rapid annotation using subsystem technology (RAST) (Aziz et al., 2008), National Centre for Biotechnology Information (NCBI) and the SEED viewer which provides a comparative analysis using the latest curated data sets (Overbeek et al., 2005).

4.5 Biofilm formation

4.5.1 Congo red agar assay for biofilm formation (CRA)

CRA medium (Freeman et al., 1989) was prepared as described in (Section 4.2). CRA medium were inoculated with overnight bacterial culture and incubated at 37˚C for 24h. Black colonies indicated a biofilm producing strain.

4.5.2 Estimation and quantification of biofilm formation

The ability of bacteria to form biofilms was tested using the approach described by (Stepanović et al., 2007) with slight modifications. Briefly, instead of adding media to the 96 well plate and then inoculated the wells, the test suspensions were made up in Eppendorf or universal tubes and then transferred to the wells. This approach allowed a wider range of parameters to be investigated such as inoculum size, carbon source, nitrogen source and growth media. After 24 h incubation at 37˚C, the contents of each well were discarded and each well was washed 4 times with 200- 220µl of sterile distilled water (SDW) and for left 5-10 min to dry, then 190-200µl of 0.1% crystal violet stain was added to each well. After 30 min, the stains were decanted, and the plate was rinsed off 4 times with 200- 220µl SDW and left 5-10 min to dry. After that 200µl of 95% (v/v) ethanol was added to each well for 20 min then the optical densities (OD) of stained adherent bacterial films were read using a Multiscan microtiter-plate reader (Thermo Labsystems -Finland) at OD 570 nm.

Each isolate was tested in triplicate. Non inoculated medium controls (ODc) were included. The cut-off value (ODc) was established which was defined as three

standard deviations above the mean ODc i.e. (ODc=average OD of negative control + (3×SD of negative control). The final OD value of a tested strain was expressed as average OD value of the strain reduced by ODc value (OD=average OD of a strain - ODc). The ODc value was calculated on each microtiter plate separately. Any negative value were presented as zero, while any positive value indicates biofilm production. For easier interpretation of the results, strains were divided into the following categories (Stepanović et al., 2000):

OD ≤ ODc= Non biofilm producer;

ODc<OD ≤ 2xODc= weak biofilm producer;

2xODc<OD≤ 4xODc=moderate biofilm producer;

4xODc ≤ OD=strong biofilm producer.

4.6 Bacterial growth measurements, carbon source residue, total carbohydrate and polysaccharide estimation

Bacterial growth curve were performed using two methods. The first being the measurement of OD at 450-580nm (wideband) using a Bioscreen C automated growth curve monitor (OY, Finland). Growth was measured using a 10^7 CFU/ml inoculum for 72h at two different temperatures 30˚C and 37˚C. The average of 5 replicates per each strain in a medium were used to generate the growth curves for each strain studied, media without inoculation was used as a control (Tauk-Tornisielo et al., 2007). In addition growth curves were also measured via viable counts (CFU/ml) in shake flask cultures. Growth in these cultures was also assessed via the degradation of the carbon source, total carbohydrate profiles and polysaccharide levels. This was carried out on a range of carbon sources (glucose, glycerol and ethanol) at different temperatures (30˚C and 37˚C). In these investigations 99ml of fresh sterilised medium (MM Eth 10.57g/l, and MM Glc 10g/l) were prepared in 250ml conical flask (Ethanol and glucose were added separately after autoclaving), and then inoculated with 1ml from adjusted fresh stock culture (overnight bacterial culture prepared in MRD) 10^9 or 10^6 CFU/ml,

respectively. The cultures were then incubated with shaking 100-120 rpm/min at (30˚C and 37˚C) and samples were taken at various time points during incubation and analysed.

4.6.1 Bacterial viable count

Samples were serially diluted in MRD and then cultured on TSA using an automated spiral plater (Don Whitely Ltd, England). The plates were then inverted and incubated at 37˚C for 24 h. After incubation the colonies were counted electronically using a colony counter (DW Scientific Ltd, UK) and the result were expressed as colony forming units (CFU/ml).

4.6.2 Quantitative estimation of ethanol

Quantitative determination of ethanol from was performed by using Quanti Chrom Ethanol Assay Kit (DIET-500) from Bio assay systems (Harvard USA). According to the manufactures protocol, samples were treated by mixing 250µl of samples with 500µl 10% TCA, centrifuged at 14000 rpm for 5 min then the supernatant were carefully transferred to an Eppendorf tubes. For standard curve, 2%, 1.5%, 1% and 0.5% (v/v) standards ethanol was prepared from the 10% (v/v) ethanol standard provided and sterilised distilled water used as a blank control (Figure 4.1). 400µl of each diluted standard and sample were transferred to separate Eppendorf tubes and then 400µl of reagent A were added to each tube and mixed quickly. The tubes were incubated at room temperature for 30 min. Then 400µl reagent B were added quickly and mixed well. Finally 1ml of mixture was transferred to cuvettes and read by spectrophotometer (Cary 50 Bio UV, Varian Inc. Corporation, Palo Alto, CA, USA) at a wave length 570-600 nm (OD 580nm).

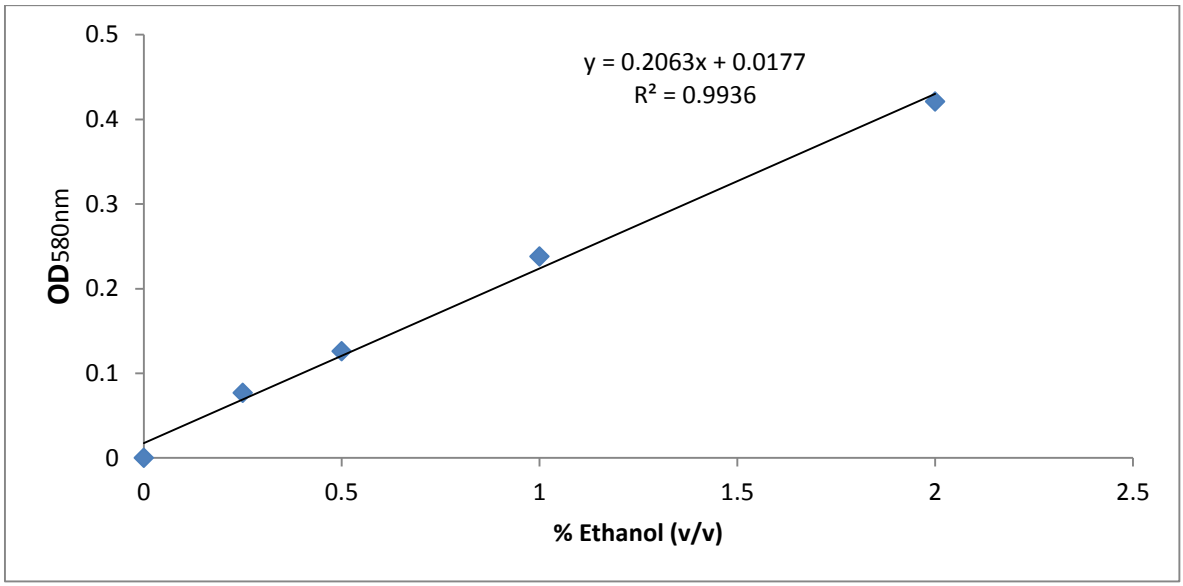


Figure 4.1 Ethanol standard curve for quantification of ethanol.

4.6.3 Quantitative estimation of glucose

Glucose was determined using a Pico trace analyser (Freital-Germany). Briefly 10µl of filtered or centrifuged supernatant culture were added to 500µl buffer, mixed and read by Pico trace.

4.6.4 Total carbohydrate estimation

Total carbohydrate and sugar content was estimated spectrophotometrically via the phenol sulphuric acid method described by (Dubois et al., 1956). The test was calibrated using a series of glucose standards (0, 20, 40, 60, 80 and 100 ppm) prepared in deionized water. 1ml of standards and 0.1 ml of sample were transferred to clean universal tubes and adjusted to 1ml with deionized water. 1ml 5% (w/v) phenol solution was then added to each tube and mixed, 5ml of concentrated sulphuric acid was then added quickly to the surface of the mixture, and then the tubes were tighten and mixed. The tubes were placed in the water bath at 70- 80˚C for 20 min and then placed in a cool water bath at 10°C for a further 10 min. The absorbance was measured spectrophotometrically (Cary 50 Bio UV) at 490nm against a blank (deionised water mixed with phenol and sulphuric acid). The unknown samples absorbance

were compared to a graph generated from the results obtained from the glucose standards (Figure 4.2).

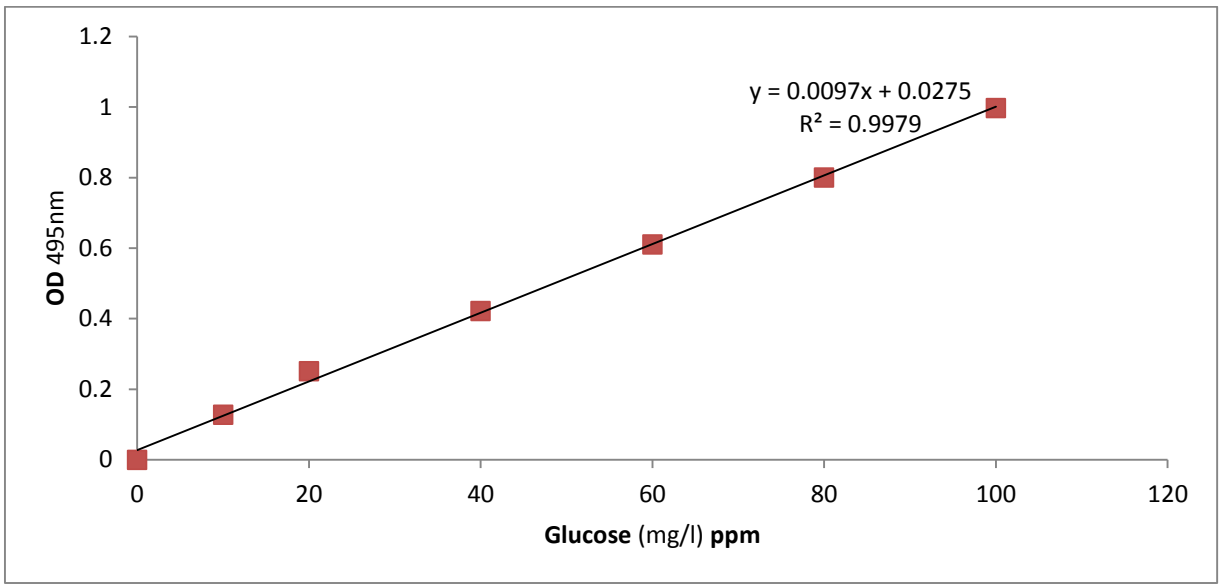


Figure 4.2 Total carbohydrate assay standard curve for quantification of carbohydrate.

4.6.5 Protein estimation

The Bradford test method (Bradford, 1976) was used to estimate the total protein concentrations. Cultures or media were pre-treated by centrifugation and the supernatant retained for analysis. A standard curve was constructed by using a dilution series (50, 100, 150, 200, 250 and 300 mg/l or µg/ml) of a Bovine Serum Albumin protein (BSA) dissolved in ultrapure water. 1ml of each sample, standards and water (blank) were added to a glass tube and then 5ml of protein reagent (Coomassie Brilliant Blue G-250) was added to the each test tube and the content were mixed by vortexing. Absorbance readings were taken spectrophotometrically within 60 min at a wavelength 595nm against a blank. The standard curves were plotted according to series standards and the unknown protein weight determined according to plotted standard curve (Figure 4.3).

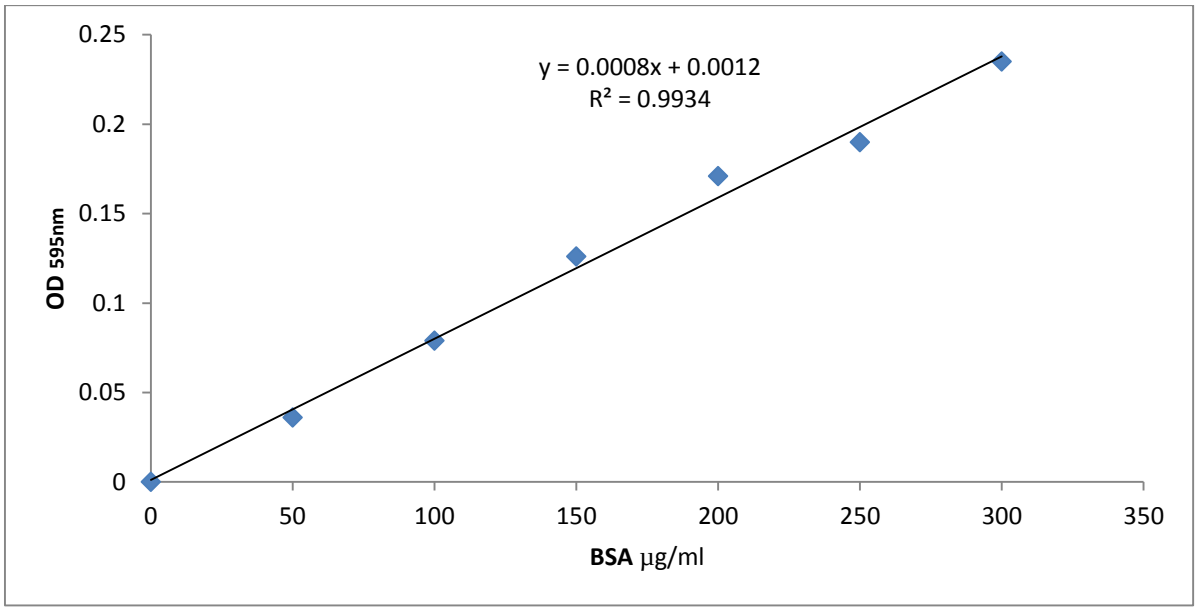


Figure 4.3 Bovine Serum Albumin (BSA) protein standard curve for quantification of protein.

4.6.6 Uronic Acid Estimation

The colorimetric meta-hydroxydiphenyl assay (Bhaskar, 2003) was used to estimate the uronic acid content of samples. Briefly, a standard stock solution was prepared by dissolving 10mg of galacturonic acid in 100ml of ultrapure water. The stock solution was serially diluted to prepare a series of standards (20, 40, 60, 80 and 100 mg/l). 0.4 ml of the standards, sample or water (blank) was transferred to a tube, and then 40 µl of 4 M sulfamic acid was added and mixed. Then 2.4 ml of 0.5% (w/v) Sodium tetraborate or borax were added to each tube, vortexed thoroughly and heated at 100°C in a water bath for 20 min, after that the tubes were immediately cooled on ice. Once cool 80µl m-hydroxydiphenyl reagent was added and mixed. Finally the absorbance was measured spectrophotometrically (Cary 50 Bio UV) at 520nm against a blank and compared to the galacturonic acid standard curve (Figure 4.4).

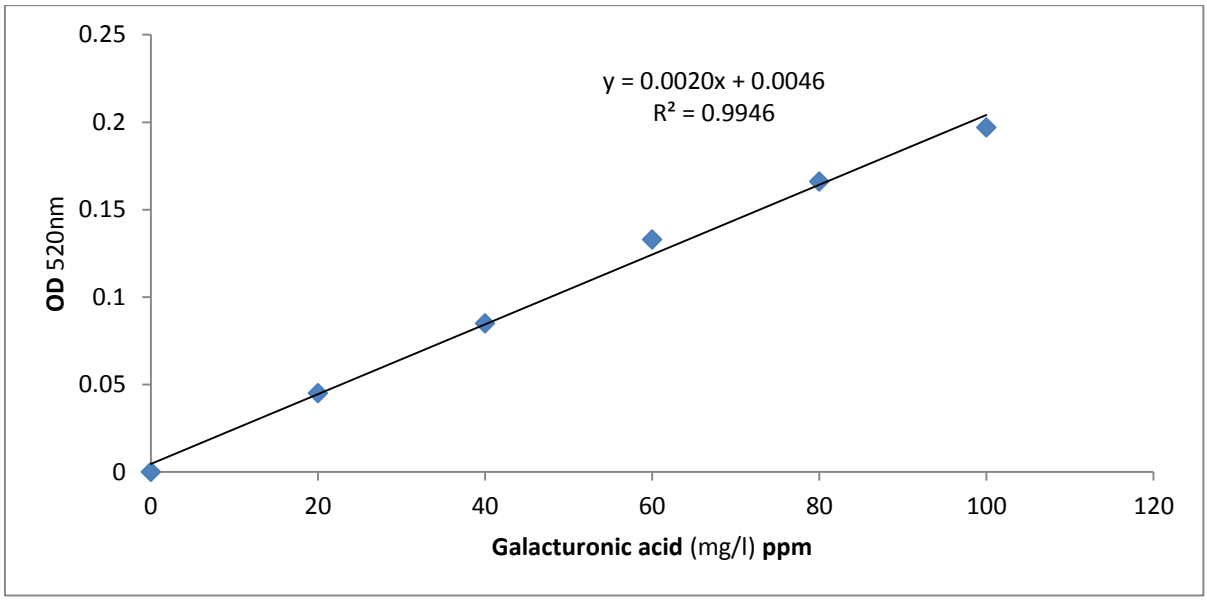
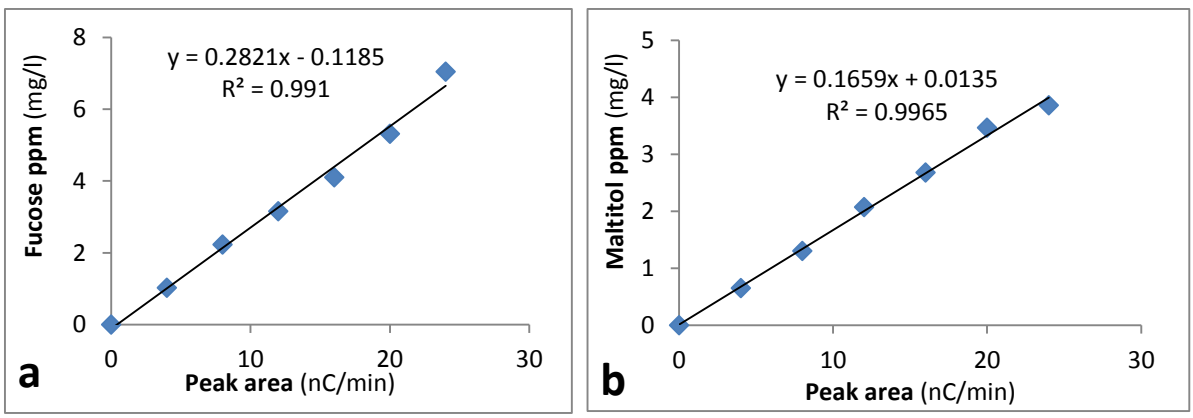


Figure 4.4 Galacturonic acid standard curve for quantification of uronic acid.

4.6.7 EPS sugars estimation using HPAEC

The quantification of EPS sugar monomers were performed by HPAEC. Serial concentrations of each sugar D(+)-fucose 99%, maltitol 99%, 2-Deoxy-D-glucose 99%, D(+)-galactosamine 99%, L-rhamnose 99%, D(+)-glucosamine 99%, D-(+)-galactose 99%, D-(+)-glucose 99% and D-(+)-mannose 99%, were prepared in UPW and measured by HPEAC allowing a standard curve to be plotted for each sugar. EPS was hydrolysed to the individual sugar components, the concentration of which was determined via HPAEC and reference to the appropriate standard curve (Figure 4.5).



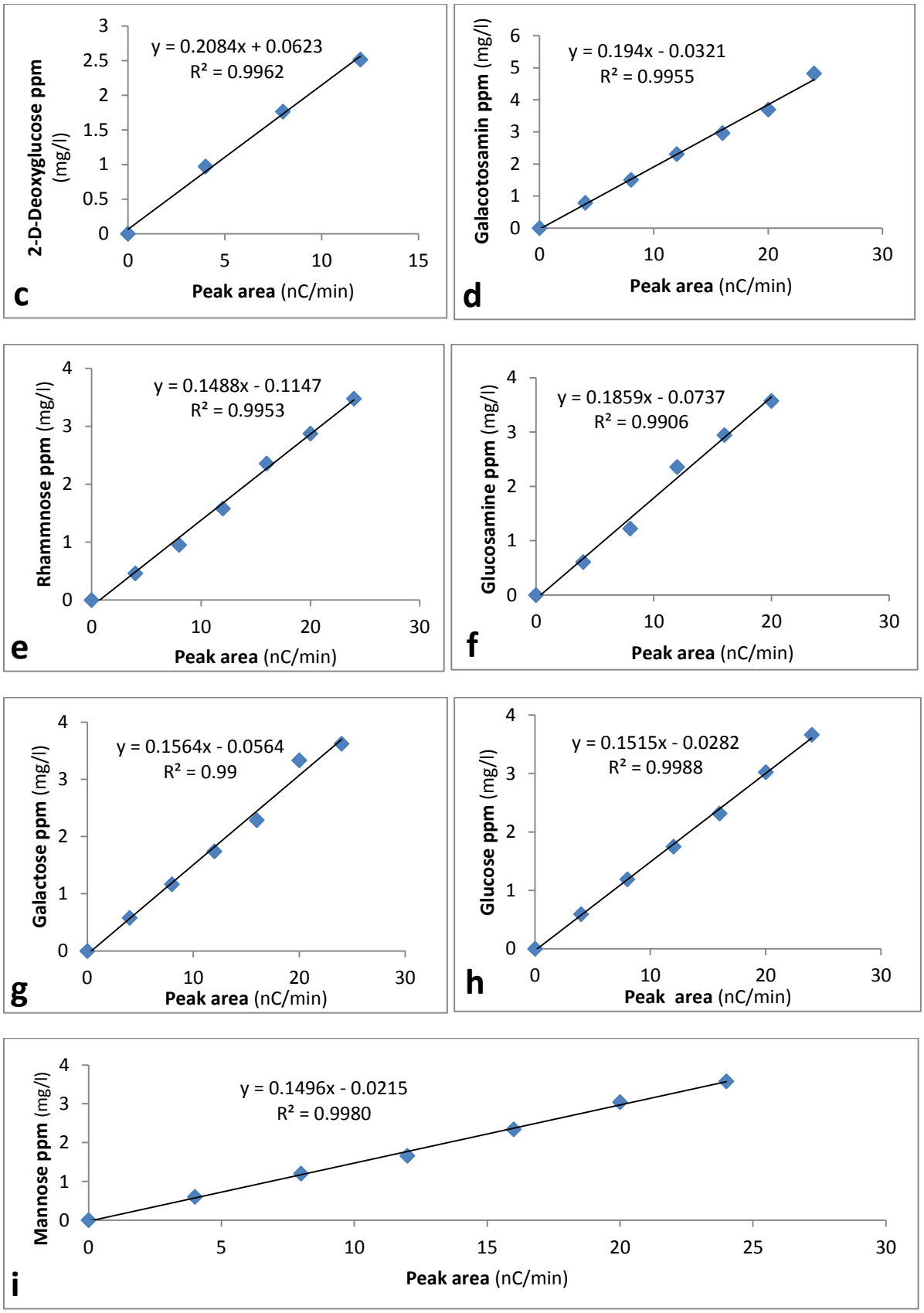


Figure 4.5 HPAEC monomer analysis standard curve for sugar quantification. a) fucose, b) maltitol, c) 2-Deoxy-D-glucose, d) galactosamine, e) rhamnose, f) glucosamine, g) galactose, h) glucose and i) mannose

4.7 Hydrophobicity tests

Several methods of hydrophobicity determinations were performed with different hydrocarbons. In all hydrophobicity test the MM Glc media employed contained 10 g/l glucose and 1 g casein while MM Eth media contained 13.4 ml/l (10.57 g/l) ethanol and 1 g casein.

4.7.1 Microbial Adhesion to Hydrocarbons (MATH) Test

The MATH test was used as described by (Saini, 2010). Overnight cultures plate cultures (TSA) were used to inoculate a series of media; TSB, MM Glc and MM Eth. These were incubated overnight at 30˚C, and then 100µl of each culture were transferred to another set of medium and incubated overnight at 30C. The cultures were the centrifuged at 8500xg for 7 min and the supernatant discarded. The bacterial pellets were washed with either TSB or 10mM potassium chloride (KCl) and resuspended in the same medium (two sets for each medium) while the other bacterial pellets from MM Glc and MM Eth were washed with phosphate buffer saline (PBS) and resuspended in two sets of PBS for each as shown in table (Figure 4.6). All suspensions were mixed and the OD (wavelength 600 nm) and volume of the resuspended pellet were adjusted to close to 0.8 (Initial absorbance) and 8ml, respectively. The resuspension media was used as a blank. The suspensions were then transferred to sterilised clean universal tube. A range of hydrocarbons were evaluated in the test, i.e. 1ml 0.8% tween 80 (w/v) (fisher) or 1ml hexadecane. The hydrocarbons were added separately to each set of cell suspension (TSB, KCl, PBS (MM Eth) and PBS for (MM Glc)) in a universal tube and vortexes for 2 min and then the suspensions were allowed to stand at room temperature for 15 min to allow the hydrocarbon and aqueous phases to separate. After that 1ml of the aqueous phase at or near the bottom of the tube was carefully removed by pipette and transferred to a cuvette. The absorbance (Final absorbance) of the removed media was measured against the media blank. Finally the cell surface hydrophobicity was calculated and interpreted as follows:

(%) = (Initial absorbance - Final absorbance)/ Initial absorbance x 100

Interpretation:

20 ≥ % Hydrophobicity = Hydrophilic

20≥ % Hydrophobicity ≤ 50 =Moderate Hydrophobicity

50≤ % Hydrophobicity = Strong Hydrophobicity

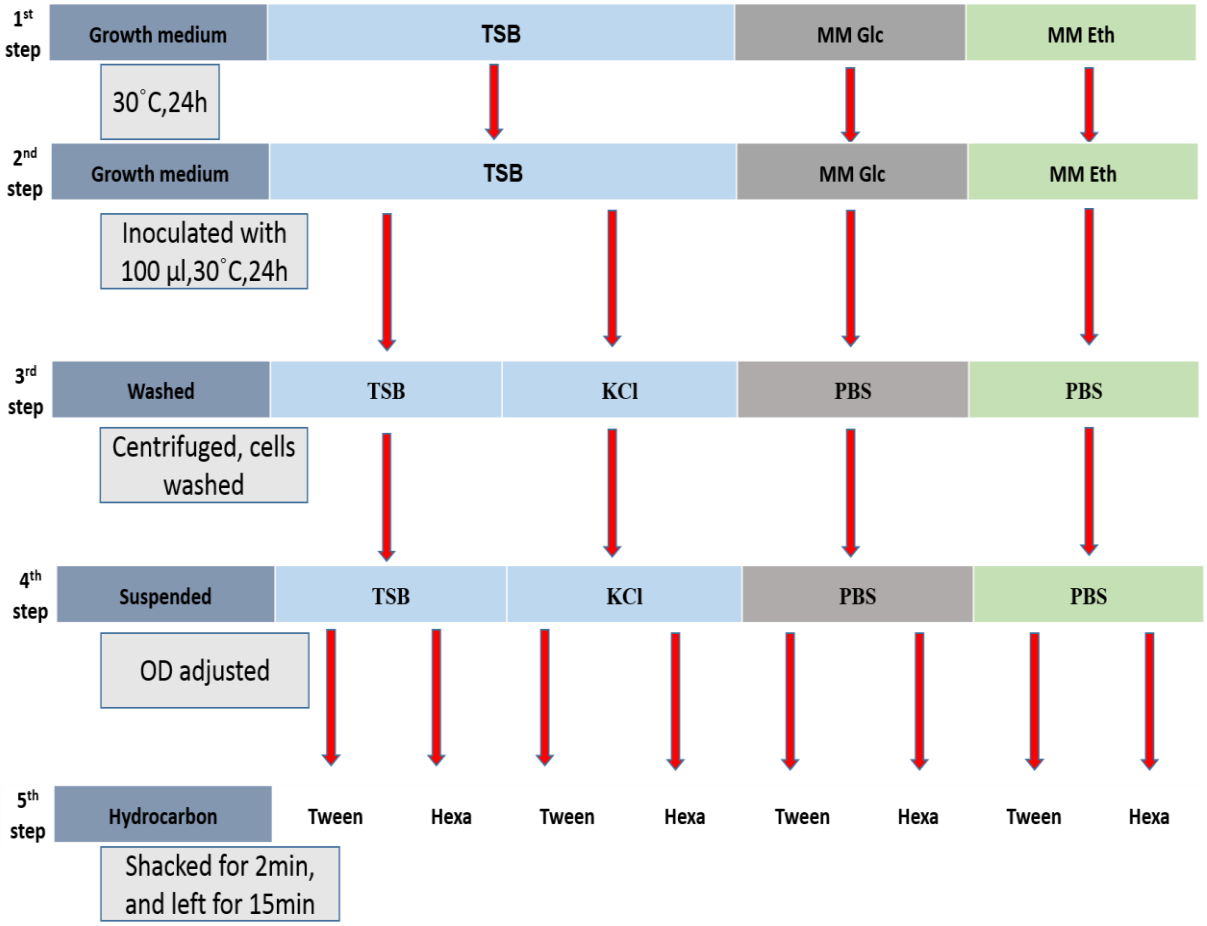
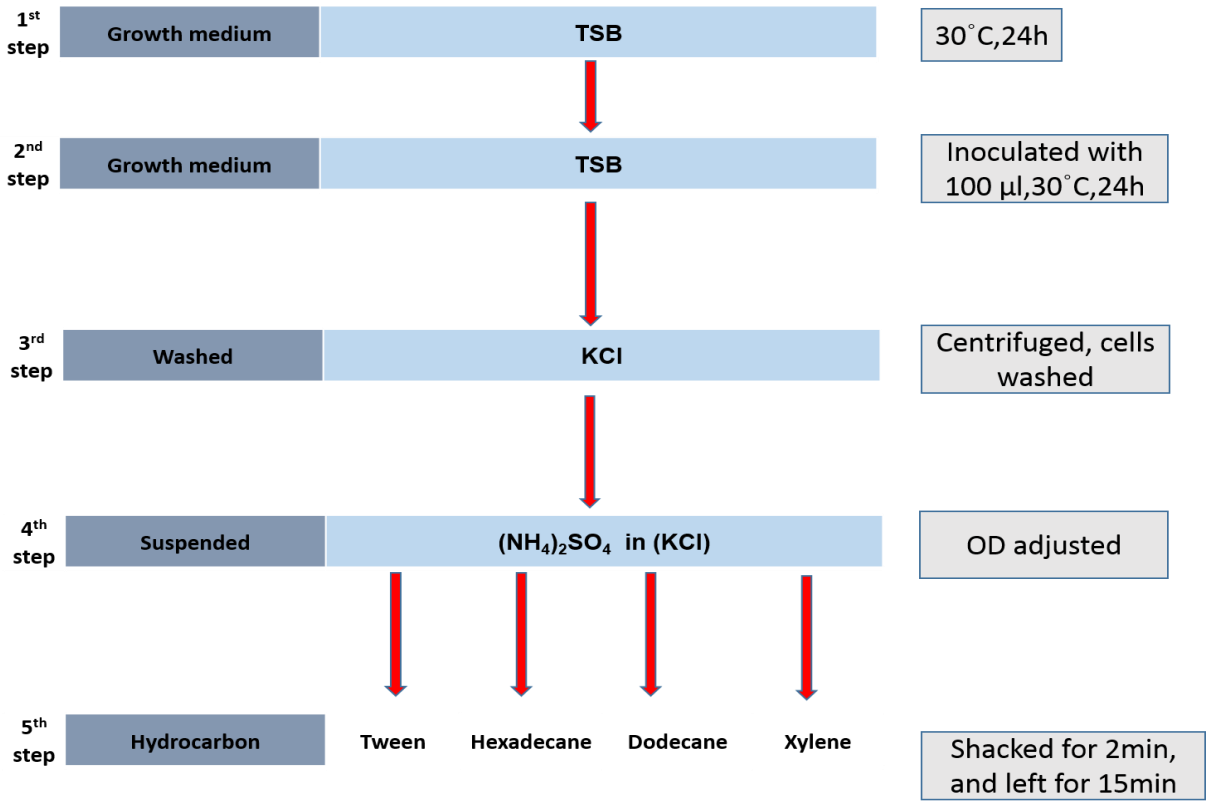


Figure 4.6 Microbial Adhesion to Hydrocarbons (MATH) test steps

4.7.2 MATH test salting out

The MATH test protocol as outlined by (Saini, 2010) was employed in this study. After two growth cycles on TSB, the bacterial culture was centrifuged and the supernatant were discarded. The pellet was resuspended in 10mM potassium chloride solution and centrifuged. The pellet was resuspended in 2mM ammonium sulphate in 10mM potassium chloride buffer solution and transferred

to clean tube as shown in (Figure 4.7) The absorbance (Initial absorbance) was adjusted at 600 nm to be close to 0.8 against the same solution as a blank. A range of hydrocarbons were evaluated e.g. 1ml 0.8% Tween 80 (w/v), 1ml hexadecane, 1ml dodecane and 1ml p-xylene. These were added to the cell suspension in a glass culture tube and vortexes for 2 min and then allowed to stand at room temperature for 15 min. After that 1ml from the aqueous phase near the base of the tube was taken and transferred to cuvette. At a wavelength of 600 nm, the OD were measured (Final absorbance) against the blank control. The cell surface hydrophobicity was calculated by the same equation that outlined above.



*2mM ammonium sulphate prepared in 10mM potassium chloride buffer solution.

Figure 4.7 MATH test salting out steps

4.7.3 Hydrophobicity-the modified adherence and aggregation test (MAA).

The hydrophobicity test as previously described by (Rosenberg et al., 1986) was evaluated with the some slight modifications. A range of media (TSB, SWF, MM (without carbon and casein), 2mM ammonium sulphate in KCl, MM Glc and MM Eth) were used to make stock culture from an overnight culture grown on TSA. The OD of each stock culture was adjusted to approximately 0.8 at OD_{600} (Initial OD) and the volume was adjusted to 4ml in each tube. Then 0.5 ml 0.8% Tween 80 (w/v) and 0.5ml hexadecane were added to each set of tubes as shown in (Figure 4.8). The tubes were vortexed for 2 min and left to stand for 15 min to allow phase separation. Then 1ml of the aqueous phase was withdrawn and measured at OD_{600} (Final OD). Surface hydrophobicity was expressed as a percentage according to the following formula: [1- (Final OD / Initial OD)] x 100 with the same interpretation as mentioned above.

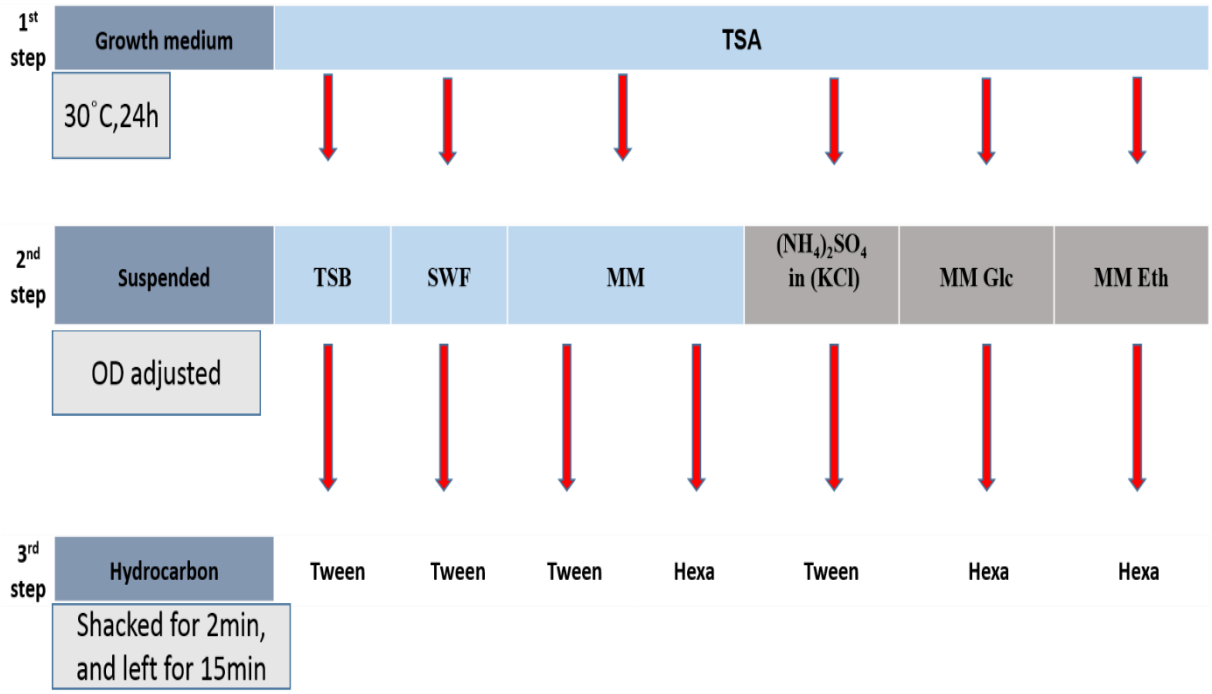


Figure 4.8 Modified adherence and aggregation steps

4.7.4 Salt aggregation test-SAT

The Salt Aggregation Test (SAT) was performed as outlined by (Krepsky et al., 2003). Serial dilutions of ammonium sulphate $(NH_4)_2SO_4$ (Fisher) (4, 3, 2, 1.5, 1, 0.75, 0.5, 0.25, 0.125, 0.0625, 0.031 and 0.0078 mM) were prepared in Phosphate buffer saline (PBS) at pH 7. Bacterial cultures were prepared in three different way; 1) overnight culture on TSA medium, 2) overnight bacterial culture in MM Eth and 3) overnight bacterial culture in MM Glc Both bacterial broth cultures (MM Eth and MM Glc) were centrifuged and the pellet suspended in PBS, while bacterial culture on TSA were picked off and suspended in PBS, centrifuged and re suspended in PBS. 15µl of bacterial suspension (OD620=1.00) were mixed with equal volume of different concentrations of ammonium sulphate and mixed on the slide. For more visibility 5µl 0.1% crystal violet (filtered twice through a filter paper and 0.45µm filter) was also added to the mixture. The lowest concentration of ammonium sulphate giving bacterial aggregation or clumping was recorded as an indication of the level of bacterial surface hydrophobicity. surface hydrophobicity intensity was classified according to the approach outlined by (Lee and Yii, 1996) and expressed as:

< 1 M = Strong hydrophobic,
1 – 2 M = Moderate hydrophobic,
$>2 \leq 4$ M = Weak hydrophobic,
≥ 4 M = Hydrophilic

4.8 Bacterial pellicle formation

This assay were performed according to that described by (Friedman and Kolter, 2004b). In this procedure 9.9ml of either MM Eth, MM Glc or TSB were placed in glass tubes (18- 150mm) and inoculated with 0.1ml of a 10^9 CFU/ml overnight culture and incubated at room temperature in a stable condition, without shaking. Pellicle formation was determined by checking the air-liquid interface of the standing culture. Pellicle formation was considered positive when the surface of the broth was completely covered by a dense or solid layer of bacteria and

matrix material. The same protocol was also used for pellicle formation at a larger scale using a 2 liter (270-130mm) container and 1 litter of culture media at 30˚C.

4.9 Drip Flow Reactor (DFR)

A four chamber drip flow biofilm reactor system was used in this study with a range of coupons, media and bacteria. The DFR was prepared according to the operators manual provided by the centre for biofilm engineering, standardized biofilm methods laboratory, Bio surface technologies, Inc. (2007). The method described by (Hassett et al., 1999) was employed. All equipment and media used were sterilised and two different coupon were used (glass slides and plastic). The first two chambers were filled with 10ml MM Eth (Ethanol 10.57g/l) and the others with MM Glc (Glucose 10gm/l). The chamber were then inoculated with the relevant overnight culture and incubated at room temperature for 4-6 h on a flat surface i.e. zero position. After that each chamber was connected to a continuous flow media (same media in the chamber) reservoir at a flow rate of approximately 0.75ml/min. During drip flow operation the system was set at a 10° angel to allow the media to flow across the surface. The biofilms were grown under a continuous flow of media and incubated at room temperature for 24 and 48 h. After incubation, the coupons washed with SDW then fixed with 2.5% formalin or by passing through a Bunsen burner flame three times. The slides were then stained with diluted ethidium bromide 1mg/ml and left for 15 min. The coupons were then washed in distilled water and stained with 0.1% calcofluor or fluorescent brighter 28 (Sigma Aldrich, UK) for 15min. finally the coupons were observed under the fluorescence microscope. Fluorescent brighter 28 stains exopolysaccharide as blue, while ethidium bromide stains bacterial cell (DNA) as red (Hill et al., 2010).

4.10 RNA sequencing (Transcriptome)

4.10.1 Total RNA extraction and purification from bacteria

RNA was extracted and purified from bacteria by using an ISOLATE- II Mini Kit (Bioline) and RNeasy MinElute Cleanup Kit (Qiagen). The extraction or isolation was performed according to manufacturer's instruction, briefly, two mineral media, one with glucose (10g/l) and other with ethanol (10.57g/l), were inoculated with a stock culture from fresh overnight bacterial culture (Ps 3). After incubation for 24 h, both cultures were mixed very well and centrifuged at 8000xg for 10 min, the supernatant was discarded and the pellet were suspended in 100µl TE buffer containing 1mg/ml lysozyme and then mixed well and incubated for 10 min at 37˚C. After incubation, 350µl lysis buffer and 3.5 µl β-ME were added to the suspension and mixed vigorously. The mixture transferred to ISOLATE II Filter (violet) and filtered at 11000xg for 1 min. 350 µl 70% (v/v) ethanol added to the filtrate and mixed and then transferred to ISOLATE II RNA mini filter (blue) and centrifuged at 11000xg for 30 seconds. After discarding the supernatant, 350 µl Membrane Desalting Buffer (MEM) were added and centrifuged for 1 min at 11000xg then the supernatant were discarded. 95µl 10% DNase I reaction mixture (10µl DNase I in 90µl RDN buffer) added directly in to the centre of the filter and incubated for 15 min at room temperature. The mini filter was then washed by adding 200µl wash buffer RW1 and centrifuged at 11000xg for 30 seconds and then the filtrate decanted and the filter tube transferred to new Eppendorf tube. After that the mini filter was washed for a second time by adding 600µl of wash buffer RW2 and centrifuged for 30 seconds. The filtrate was discarded and the mini filter washed for a last time with 250µl wash buffer RW2 and centrifuged for 2 min at 11000xg. Finally the mini filter was transferred to a new tube and the RNA eluted by adding 60µl RNase free water and centrifugation at 11000xg for 1 min. The concentration of the total RNA

was measured by micro volume spectrophotometer and RNase free water used as a blank.

4.10.2 RNA clean-up

A RNeasy MinElute Cleanup Kit (Qiagen) was used for the purification and clean-up of the extracted RNA. According the manufacturers protocol, the volume of extracted RNA sample was adjusted to 100 µl with RNase-free water, mixed and topped up with 350µl Buffer RLT. At this point 250µl of 99% (v/v) ethanol was added to the diluted RNA, and mixed and transferred to a RNeasy MinElute spin column and centrifuged at 8000xg for 15 seconds with the flow-through being discarded. 500µl Buffer RPE was then added to the spin column and centrifuged at 8000xg for 15 seconds and once again the flow-through was decanted. This was followed by the addition of 500µl of 80% (v/v) ethanol to the RNeasy MinElute spin column which was then centrifuged for 2 min at 8000xg. After that the flow-through was decanted off and the RNeasy MinElute spin column was transferred to new Eppendorf tube which was centrifuged at maximum speed for 5 min. The spin column was then placed in a sterilised Eppendorf tube and 25µl RNase-free water added directly to the centre of the spin column membrane, the tube was centrifuged for 1 min at full speed to elute the RNA. The concentration and purity of RNA was measured by a spectrophotometer and running gel. For RNA sequencing, more than 350µl of each pure RNA sample were prepared with >170 µg/ml or ng/µl and purity 1.8-2.0 at OD 260/280 nm and approximately 2.0 at OD 260/230 nm.

4.10.3 RNA sequencing (Next Generation Sequencing).

After the preparation of the RNA, the samples were sent for RNA sequencing via Next Generation Sequencing (NGS) (Baseclear Ltd, Netherlands). The standard protocol for NGS of RNA starts with rRNA depletion using Epicentre bacteria Ribo-zero rRNA depletion kit to deplete Ribosomal RNA from

total RNA. This is followed by Illumina strand-specific mRNA-seq library preparation and sequencing. Once the purity of the RNA has been confirmed (RIN value >8.0), strand-specific mRNA-seq libraries are generated by the "dUTP method" that includes barcoding which is compatible with either single-read or paired end sequencing (Parkhomchuk et al., 2009, Levin et al., 2010). Then purified mRNA is then fragmented and converted to double-stranded cDNA. DNA adapters with sample-specific barcodes are ligated and a PCR amplification is performed with the outputs being subjected to further quality control including purity and concentration measurements. The Illumina libraries are then diluted, clustered and sequenced on the Illumina HiSeq 2500 or MiSeq instruments. The Illumina sequencing data produced is processed removing the sequence reads that are of too low quality (only PF "passing filter" reads are used) and are demultiplexed based on the sample-specific barcodes. An additional filtering step using in house scripts was applied to remove reads containing adaptor sequences or the Illumina PhiX control sequences. The sequence data are provided to the customer in FastQ format and the final step is bioinformatics. The sequence reads were filtered and trimmed and these reads are aligned against the reference sequences using the CLCbio "RNA-Seq" software. In addition expression values are calculated and compared between the samples. Quality control is performed through boxplot analysis, principle component analysis (PCA) and hierarchical clustering of the normalized expression values.

For a two-group experiment the 'Fold Change' means how many times bigger the mean expression value in group 2 (MM Eth) is relative to that of group 1 (MM Glc). If the mean expression value in group 2 (MM Eth) is bigger than that in group 1 (MM Glc) this value is the mean expression value in group 2 (MM Eth) divided by that in group 1 (MM Glc). If the mean expression value in group 2 (MM Eth) is smaller than that in group 1 (MM Glc) the fold change is the mean expression value in group 1 (MM Glc) divided by that in group 2 (MM Eth) with a negative sign. In terms of gene expression profiles, fold changes and their

significance (*P* values), representing differential expression, those mRNAs with *p* values < 0.01 were considered to be differentially expressed (Rumbo-Feal et al., 2013).

4.11 Isolation and quantification of outer membrane vesicles (OMV)

The isolation of OMV was performed using the protocol described by Kadurugamuwa and Beveridge (Kadurugamuwa and Beveridge, 1995) (Figure 4.9), while the quantification was performed by Bradford test. Both Ps 3 and Ps 10421 grown on both MM Glc and MM Eth were examined for OMV production. Overnight cultures grow on MM Eth (Ethanol 10.57g/l) and MM Glc (Glucose 10g/l) broth were inoculated (10^7 CFU/ml as final inoculum size) and incubated at 30^{o}C for 18-24 h with shaking 100 rpm. After the incubation, the broth cultures were centrifuged at 10000xg for 10-15 min (Avanti J-26 XPI centrifuge, using with JSP F250 rotor, Beckman Coulter Ltd UK, High Wycombe, UK) and the cells discarded. The supernatants were filtered through Millipore filters (EMD Millipore Steritop Sterile Vacuum Bottle-Top Filters -0.22µm pore size – Fisher) to remove residual cells. The filtrate was ultra-centrifuged at 40000xg for 90 min at 5°C using Optima L- look Ultra Centrifuge with 50.2 Ti rotor (Beckman Coulter Ltd UK, High Wycombe, UK), the supernatant was decanted and the pellets suspended in 50mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer (HEPES) at pH 6.8 and recentrifuged for 60-90min at 40000xg at 5˚C. The supernatant was then discarded and the pellet suspended in 50mM HEPES buffer (pH 6.8) and filtered through 0.45µm syringe filter. A 100µl aliquot of the filtrate was cultured on TSA media to confirm purity and the concentration of vesicles quantified by the Bradford assay before storage at 4˚C (Figure 4.9).

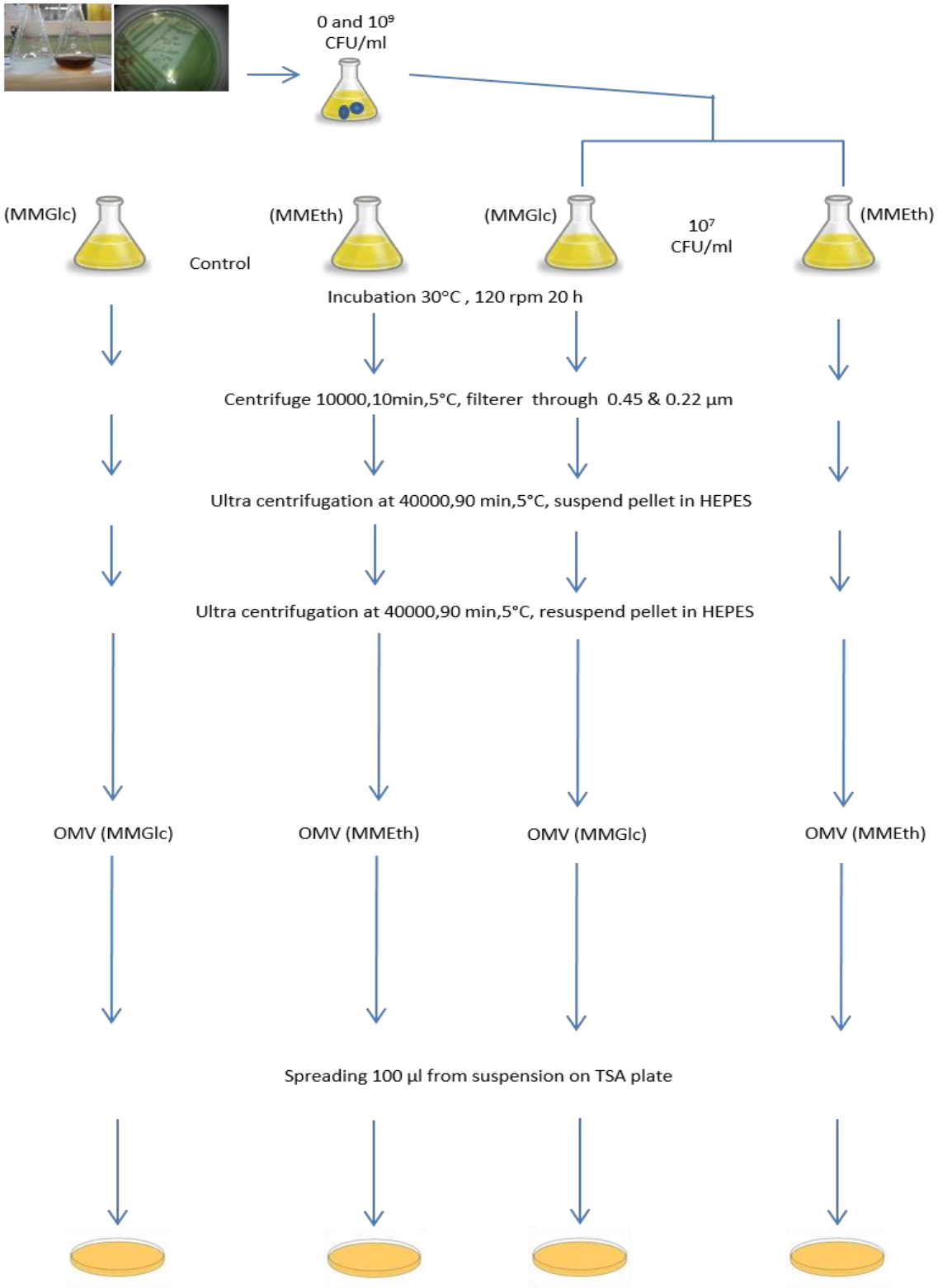


Figure 4.9 OMV production and extraction diagram on mineral media containing different carbon source.

4.12 Isolation and characterisation of bacterial EPS

A range of analytical techniques were employed to characterise the EPS recovered from the bacteria under investigation.

4.12.1 Developing an alternative media for EPS production (Control)

A range of specific media such as MM Glc (10g/l glucose, 1g/l casein), MM Gly (10g/l glycerol) and MM Eth (10.57g/l ethanol) were used for to study bacterial EPS production and characterisation alongside a range of more conventional media (TSB, SWF and LB broth).

4.12.2 Optimisation of bacterial EPS production and extraction

Several culture media were tested for EPS production with the effects of different inoculum size, media, incubation period, and trichloroacetic acid (TCA) concentrations on the quantity and the quality of the extracted EPS evaluated (Figure 4.10). Generally speaking 495ml of media was inoculated with 5ml of fresh overnight bacterial culture adjusted to 10^9 CFU/ml and then incubated at 30°C with shaking at 100-120 rpm/min for 72-80 h. The extraction of EPS was carried out by the method described by (Marshall et al., 2001). After incubation, the culture were heated in water bath at 60-65°C for 30 min (optional) and then centrifuged at 10000xg (Avanti J-26 XPI centrifuge, Beckman Coulter Ltd UK, High Wycombe, UK) (F250 Rotor) for 10 min at 5˚C to remove the cell. 5% TCA (w/v) (sigma-Aldrich) were added to the supernatant with shaking and left incubated at 5˚C overnight for protein precipitation. The solution was then centrifuged at 20000xg (Avanti J-26 XPI) for 20 min at 4˚C to remove the protein and then 2 volumes of chilled Ethanol (Methylated spirit industrial-fisher) were added and mixed well and then left at 4˚C for two days to precipitate the EPS. The EPS was then recovered by centrifugation at 25000xg (Avanti J-26 XPI) for 30-35 min at 4˚C. The supernatant was discarded and the pellet (EPS) dissolved in the ultra-pure water (UPW) and dialysed (dialysis tubing cellulose membrane –

sigma) in UPW for 48-72 h at 4˚C, with 2-3 changes of water per day to remove small sugars. After dialysis, the samples were taken and analysed by Pico trace to detect glucose residues. The EPS solution was then freeze-dried (frozen and lyophilised) in a pre-weighed bottle or round-bottom flask for 24-48 h using liquid nitrogen and a freeze-drier (Christ Alpha 2-4 LD plus- SciQuip Ltd, UK). Once a freeze dried product had been obtained several tests such as EPS composition, NMR, monomer analysis using HPAEC, FTIR and MALLS analysis were performed.

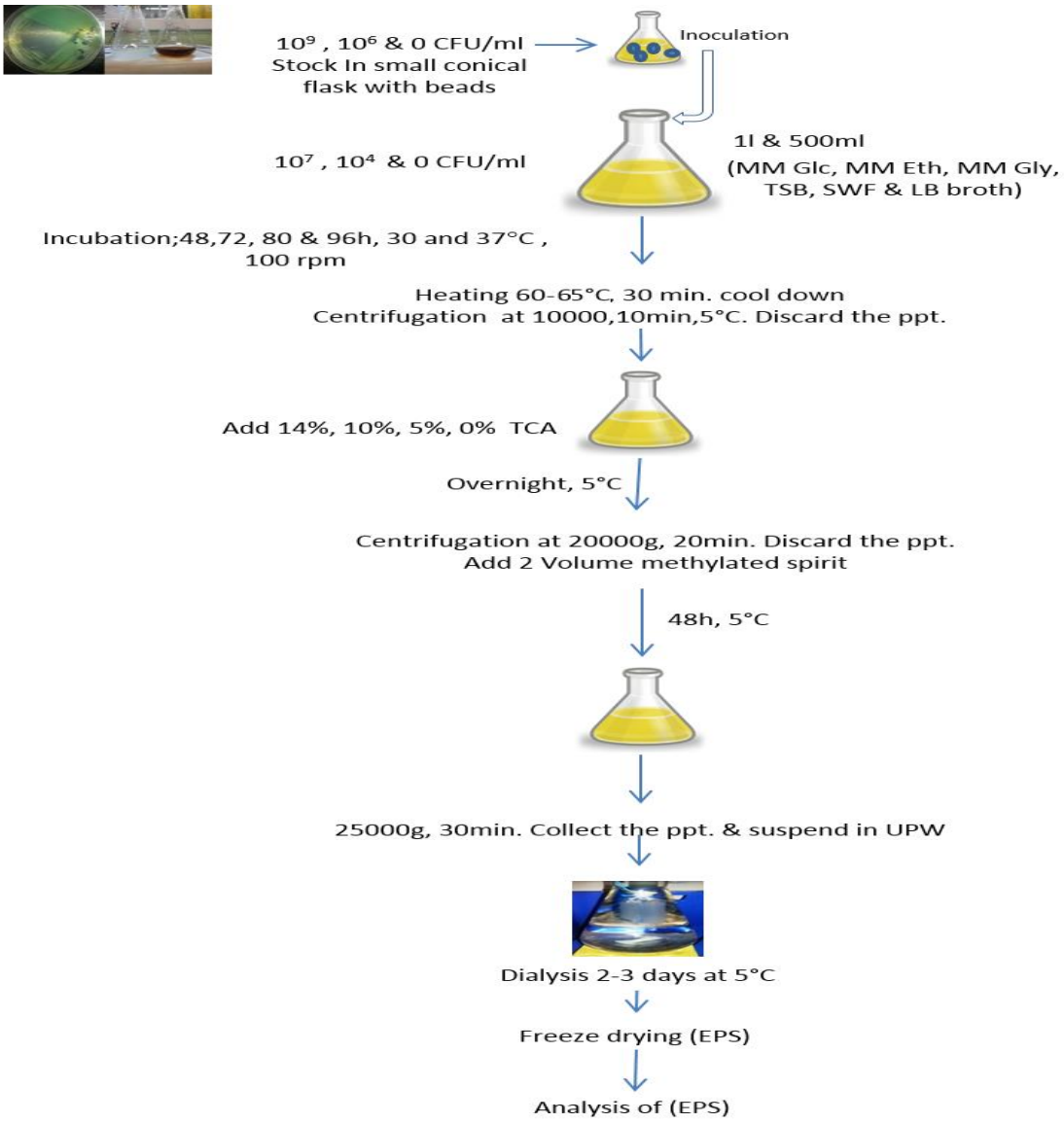


Figure 4.10 EPS production and extraction optimisation diagram.

4.12.3 EPS dry weight

After freeze drying, The EPS dry weight was determined either through removal and weighing or by weighing the container with and without the EPS material.

4.12.4. Estimation of glucose content

Glucose was measured by dissolving 1mg of extracted EPS in 1ml UPW. The suspension were then filtrated or centrifuged and the supernatant analysed using the Pico trace analyser (Section 4.6.3).

4.12.5. Estimation of total carbohydrate content

This test were performed by dissolving 1mg of extracted EPS in 1ml of UPW and then analysed by the phenol sulphuric acid method as described by Dubois et al (1956)(Section 4.6.4).

4.12.6 Estimation of protein content

The Bradford assay was used to determine the protein content of the EPS. In this analysis, 1mg of extracted EPS was dissolved in 1ml UPW, mixed and analysed as mentioned in (Section 4.6.5)

4.12.7 Estimation of uronic acid content

In this analysis 1mg of extracted EPS was dissolved in 1ml of UPW and then analysed colorimetrically by m-hydroxydiphenyl assay as described in (Section 4.6.6).

4.12.8 Nuclear Magnetic Resonance (NMR) analysis of EPS

Nuclear magnetic resonance (NMR) spectra of the extracted EPS and media controls were analysed by Dr. Neil McLay, University of Huddersfield using a Bruker Avance DPX500 500.13MHz. NMR spectra were recorded at an elevated probe temperature of 70˚C. Proton NMR (^{1}HNMR) were achieved by

dissolving 5-7 mg of EPS in 550µl deuterium dioxide > 99.9 % D (D_2O) (Goss Scientific Instruments Limited, UK) with heating or sonicating if required. Top spin 3.1 software was used to process the resulting data.

4.12.9 Monosaccharide composition or monomer analysis of EPS.

In order to determine the monosaccharide constituents of the extracted EPS via High Performance Anion Exchange Chromatographic with a Pulsed Amperometric Detector (HPAEC-PAD) the EPS was firstly treated (hydrolysed) with an acid catalyst such as trifluoroacetic acid (TFA) (Sigma Aldrich, UK). In this case 3mg of extracted EPS was dissolved in 2ml of 2M TFA in a pressure tube and then heated for 2 h at 120˚C. After heating, the sample was left to cool at room temperature. The pressure tube was then opened and the sample evaporated to dryness under a constant stream of nitrogen gas at 50-60˚C or the samples were transferred to a small tube and dried by a TurboVap LV Concentration Workstation (Caliper Life Sciences, USA).

Following drying 3ml of UPW was added to the dried sample and mixed via vortexing or sonicating to make a 1000ppm (stock) which was then filtered through either a 0.45 or 0.22µm filter. This sample was then compared with several standard sugar solutions (20ppm) of: D-(-)-Arabinose 98%D, (+)-glucosamine 99%, D(+)-galactosamine 99%, D(+)-fucose 99%, 2-Deoxy-D-glucose 99%, maltitol 99%, L-rhamnose 99%, D-(+)-glucose 99%, D-(+)-galactose 99%, D-(+)-mannose 99%, D-(+)-raffinose 98%, D-(+)-Galacturonic acid monohydrate 97% (Sigma Aldrich & Fisher Scientific, UK) and hydrolysed alginate (mannuronic acid and guluronic acid).

All sample and standards were analysed by HPAEC-PAD (Dionex ICS -3000, Dionex Corporation, CA, USA or Dionex ICS -5000, Dionex Corporation, CA, USA) using a Sodium hydroxide (10mM, Isocratic) mobile phase at 0.45ml or 0.5ml/min. Analysis, separation and quantification of neutral monosaccharide sugars and uronic acid sugars derived from the extracted bacterial EPS was

performed according to (Wang et al 2012). Briefly, three eluents were prepared 1) 10mM NaOH (Mobile phase), 2) 200mM NaOH and 3) 500mM NaOAc + 200mM NaOH, then the Isocratic elution program was used to analysis neutral and uronic acid sugars using ICS -500 at 0.5ml/min flow rate. After the run, the generation time of the standard peaks and the samples peaks were compared. To quantify each sugar within the EPS, the peak area of any sugar were compared to a graph generated by HPAEC from a series of sugar standards.

4.12.10 Bacterial EPS treatment with enzyme dextranase

After the optimisation of EPS production the bacterial EPS were treated by dextranase enzyme from *Chaetomium erraticum* (Sigma Aldrich, UK) to confirm the presence of dextran. The effects of the enzyme dextranase was first tested on a commercial dextran diluting to 100mg of dextran in a 25 ml conical flask volume containing 10 ml D_2O. The mixture were divided in two and 0.1ml of the dextranase were added to one of them and then both mixture were incubated at 50ºC for 3 h in a shaking incubator. After that 0.25g of TCA was added to each mixture and processed as described in (Section 4.12.2), after dialysis, the solution were analysed by proton NMR. Dextranase enzyme interference were also investigated by mixing 1ml dextranase enzyme with 9 ml UPW and incubated at 50˚C for about 3 h. Prior and post dialysis were analysed by proton NMR.

In order to investigate the bacterial EPS, a broth culture grown on 990ml of MM Eth was inoculated with 10ml of $\approx 10^9$ CFU/ml stock culture prepared with MRD from an overnight culture and then incubated at 30 ºC for about 80h with 120 rpm in shaking incubator. After that the broth was incubated at 60-65˚C in a water bath for 30 min and then cooled and centrifuged at 10000xg to remove cells. The supernatant was then divided in two, one portion was treated with dextranase (1% v/v final concentration) and the other half left without any enzyme treatment. Both broths were then incubated at 50˚C for 3-4 h in shaking incubator, after which both conical flasks were treated with trichloroacetic acid 5% (w/v)

overnight at 4ºC before being centrifuged at 20000xg to remove proteins and any residual cells. Then 2 volumes of ice-cold ethanol (Methylated spirit- fisher) were added to the supernatant and left at 4°C for two days, following a further centrifugation (25000xg) at 4°C for 30 min to precipitate EPS. The supernatant was discarded and the EPS pellet was reconstituted in ultra-pure water and dialysed against ultra-pure water for 72 h with 2-3 water changes daily. The contents of the dialysis tubing were then freeze dried. The crude EPS was dissolved in D_2O and ^{1}H-NMR carried out. 3mg of crude EPS was boiled in 2 ml trifluoroacetic acid (TFA) for 2 h prior to monomer analysis via HPAEC-PAD eluting 10mM sodium hydroxide at a flow rate of 0.5 ml/min, while for HPSEC-MALLS analysis, 1000ppm of EPS were prepared by dissolving 3mg EPS in 3ml UPW.

4.12.11 EPS characterisation by FTIR analysis

The bacterial EPS and other crude substances were characterised using a Fourier transform infrared spectrophotometer (FTIR) (Magna-IR 750, Nicolet Instrument, USA). The most commonly region used for analysis is 400-4000 cm^{-1} as all the molecules of interest possess characteristic absorbance frequencies and primary molecular vibrations in this region. Small amount of sample or crude EPS were subjected to IR spectra measurement in the frequency range of 400 and 4000 cm^{-1} (Castro et al., 2014).

4.12.12 Molecular Weight Determination of EPS

High-performance size exclusion chromatography (HPSEC) and a multi-angle laser light scattering detection (MALLS) was used to determine the weight-average molecular weight (Mw) and polydispersity (Mw/Mn) of bacterial EPS and dextran standard control (BDH, Grade A) MW 200000-270000 Da. A dextran or pullulan 800000 Da polysaccharide was used as a control to check the HPSEC-MALLS was working correctly.

Bacterial EPS and dextran solutions were prepared by dissolving 3mg in 3ml of UPW and mixing overnight on magnetic stirrer. Solutions were filtered through 0.2 or 0.45μm syringe filters and then approximately 0.5ml of solutions were injected into the SEC-MALLS through a 7125 injection port (Rheodyne LLC, California, USA) onto an analytic size exclusion column composed of three Aqua gel-OH-mixed columns (40,50 and 60 with 15μm particle size, 250 x 4mm, Varian, Oxford, UK). The samples were first passed through a UV detector (Prominence SPD-20A, Shimadzu, Milton Keynes, UK) to detects the presence of any DNA residue in the sample at a wavelength 260 nm. The concentration of sample (EPS, dextran or pullulan) was then detected by a refractive index detector (RI) (Optilab Rex, Wyatt technology, Santa Barbara, USA) while a multi-angle laser light scattering (MALLS) photometer with the laser set to 690 (Dawn HELEOS, Wyatt Technology, Santa Barbara, USA) was used to measure the weight-average molecular weight (Mw). Astra -6 software was used to processing the outcome data.

4.13 Bacterial virulence (Pathogenicity)

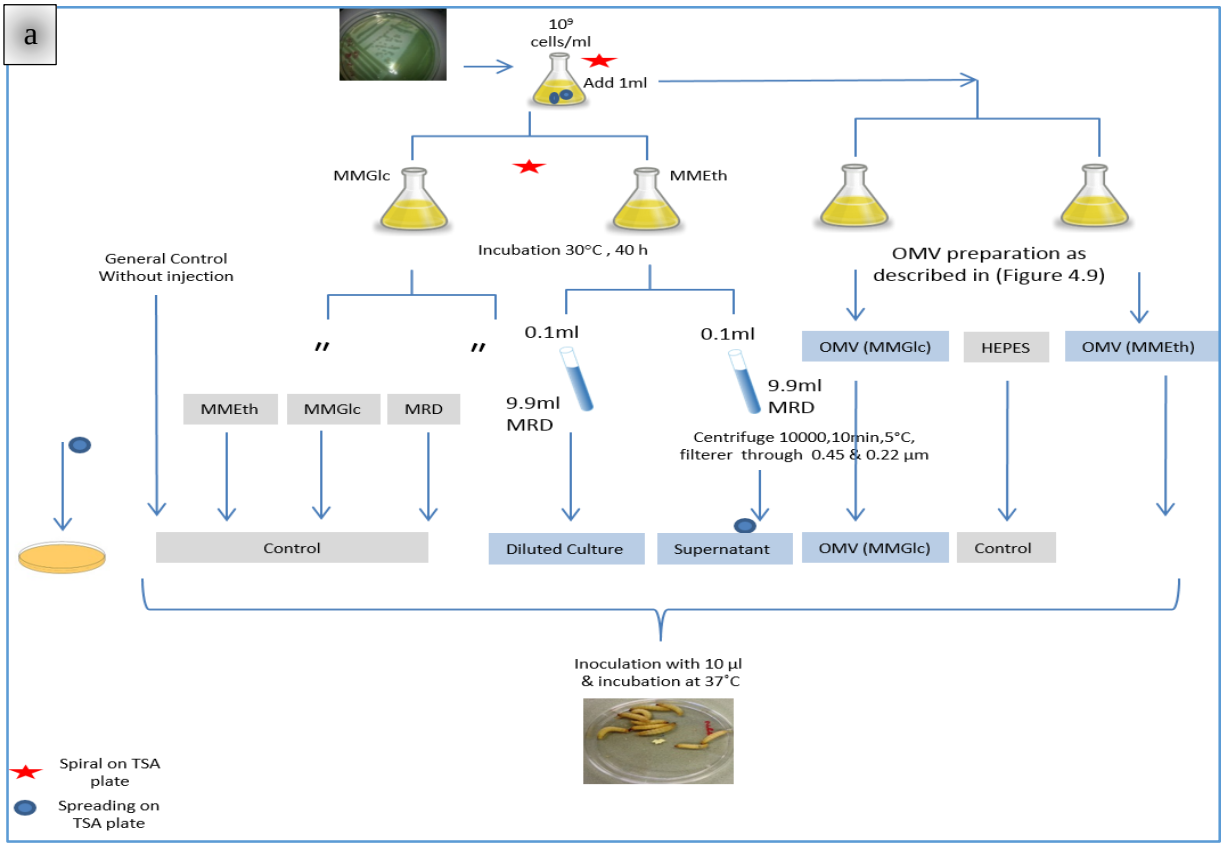
The greater wax moth larvae *Galleria mellonella* (*G. mellonella*) were used to study bacterial pathogenicity. In this study, the impact of bacterial biofilm formation, bacterial culture composition and the production of outer membrane vesicle on pathogenicity. *G. mellonella* larvae were purchased from a commercial supplier, maintained in the dark on wood chips at 4˚C and used within 1 week. Before starting the experiments the larvae were left at room temperature for several hours. At the time of inoculation, larvae were selected on the basis that they were active, ≥ 2 cm in length, cream coloured with no visible signs of melanisation and pupation. These larvae were then placed in a sterilised tube or flask and put in the ice. The larvae were then held between the thumb and forefinger and injected very carefully with 10μl of each experimental condition through the end rear pro leg into the hemocoel using a 10μl GC syringe. The

syringe needle was cleaned and sterilised between each experiment by washing twice with 70% isopropyl alcohol, followed by 3 washings with sterilised DW. The injected larvae were put in labelled petri dishes and then incubated at 37˚C and monitored each hour (Figure 4.11 a). Larvae were examined for pigmentation and their survival recorded every hour. Larvae were considered dead when they showed no movement in response to touch.

Initial investigations were performed according to (Harding et al., 2013) with some modification. Here the bacterial of interest were grown in conical flasks incubated in a shaking incubator at 30°C with 100 rpm for 40 h. In these experiments 99ml of media was placed in a 250 ml conical flaks and inoculated with 1ml of a 10^9 CFU/ml stock culture. In order to investigate the impact of the bacterial cells 0.1ml culture was diluted in 9.9 ml of sterile MRD prior to injection. To investigate the impact of the supernatant 0.1ml of culture was mixed with 9.9ml MRD and then centrifuged at 10000xg for 10 min at room temperature, the pellet was discarded and the supernatant filtered twice through 0.45 & 0.22µm sterile filters prior to injection. In order to study the effect of OMV, the OMV were prepared as described in Section 4.11. In all these initial investigations 7 larvae were used per experiment.

Further studies were performed according to (Desbois and Coote, 2011) with slight modification. In these studies bacterial cultures were grown in the same manner as described above with different incubation times i.e. 24 and 80 h. In this case lethal dose (LD50) experiments were carried out by using a dilution series (10^8,10^7,10^6 and 10^5 CFU/ml) prepared in sterile PBS. Cultures were diluted to an absorbance of 1.115 at 620 nm to give an approximate suspension of 10^9 CFU/ml. Supernatant and OMV investigations were carried out as outlined above but with sterile PBS replacing sterile MRD. In all these investigations 10 larvae were used per experiment (Figure 4.11b).

Alongside each experiment, a range of control larval incubations were prepared, these were either injected with 10 µl of each media or solution used e.g. MRD, PBS, MM Glc, MM Eth and HEPES to determine if these solutions had any negative impacts on the larvae. There was also, always a set none injected worms used to ensure that the incubation conditions did not impact on the viability of the larvae.



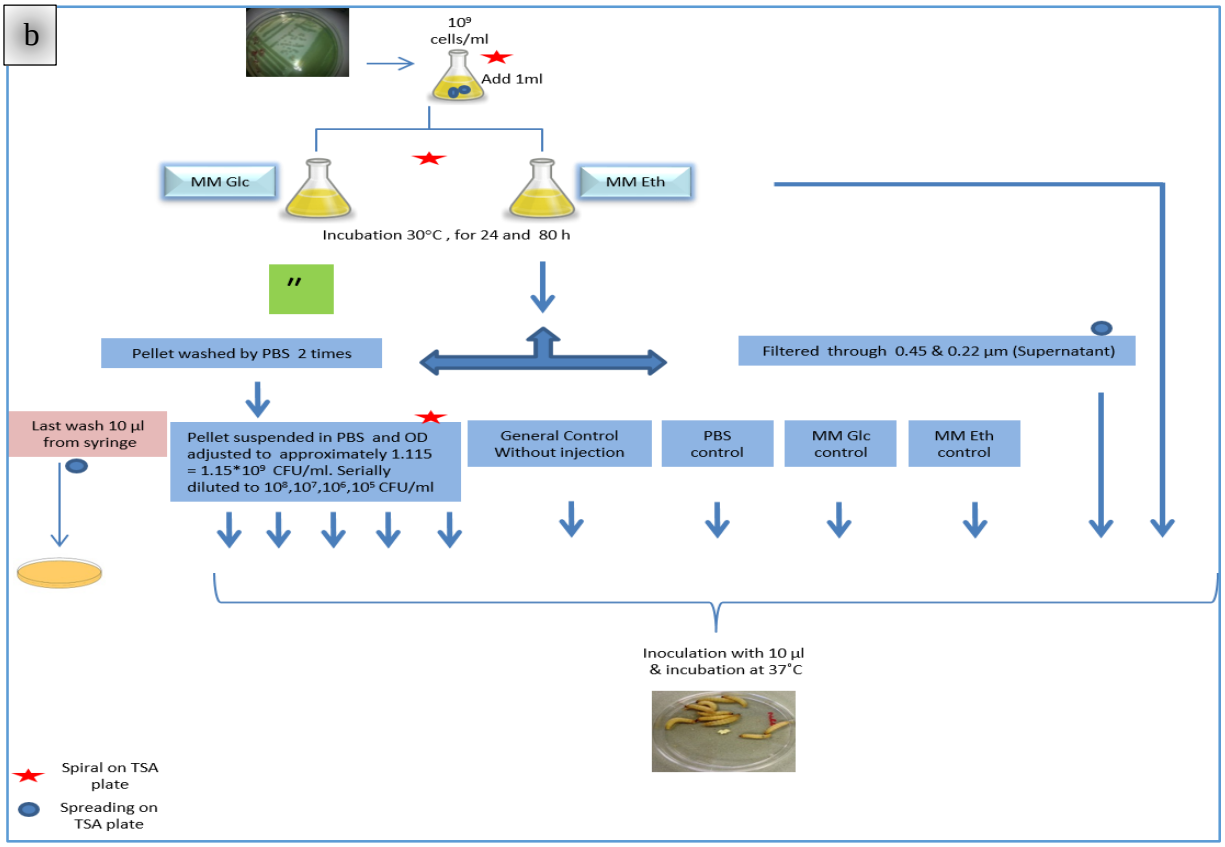


Figure 4.11 Bacterial pathogenicity diagram in wax worms a) pathogenicity of bacterial culture dilution directly, supernatant and OMV at 40h b) pathogenicity of bacterial culture directly, supernatant and washed bacterial cells at 24 and 80h.

4.14 Bacterial survival in Ultra-Pure Water (UPW)

Bacterial survival in sterilised UPW was investigated by preparing suspensions of each bacterial strain in UPW using overnight cultures. Before autoclaving, conical flasks were well washed and then filled with 99 ml of UPW, after autoclaving, the UPW was inoculated with a 1ml of bacterial suspension of approximately 10^6 CFU/ml that prepared in sterilised UPW. These were then incubated at 30˚C and then monitored daily for 40 days. The same experiment were repeated focusing on (St 53, St 10421, Ps 3, Ps10421, and *C. freundii*) but with 10^7 CFU/ml as a final inoculum size and incubated at 30°C for about 1 year.

5. Results

5.1. Characterisation and re-identification of bacteria.

The bacterial isolates used in this study originated from various sources, see section 4.1. The identity of the strains used in this study was confirmed by both conventional and molecular techniques. In addition two culture collection strain were included to act as controls, these were *Stenotrophomonas maltophilia* 9203 (St 9203) and *Pseudomonas aeruginosa* 10471 (Ps 10471) from National Collection of Industrial Bacteria (NCIB).

5.1.1 Colony morphology, Gram stains and biochemical tests of bacteria

Fresh overnight cultures were prepared on TSA, individual colonies were selected and Gram stained (Figure 5.1), followed by the oxidase and catalase tests. During this identification process, isolate Ps 5 originating from a wound dressing that was presumed to be a *Pseudomonas* spp., was shown to be contaminated. Streak plating was used to obtain pure cultures of the two bacteria present. Both isolates were Gram negative but had different oxidase and triple sugar iron agar (TSI) results (Table 5.1). With the exception of the contaminant found in Ps 5 all the isolates conformed to what would be expected for *Stenotrophomonas* spp. and *Pseudomonas* spp. isolates. The unknown contaminant had characteristics that suggested it was a member of the *Enterobacteriace* family.

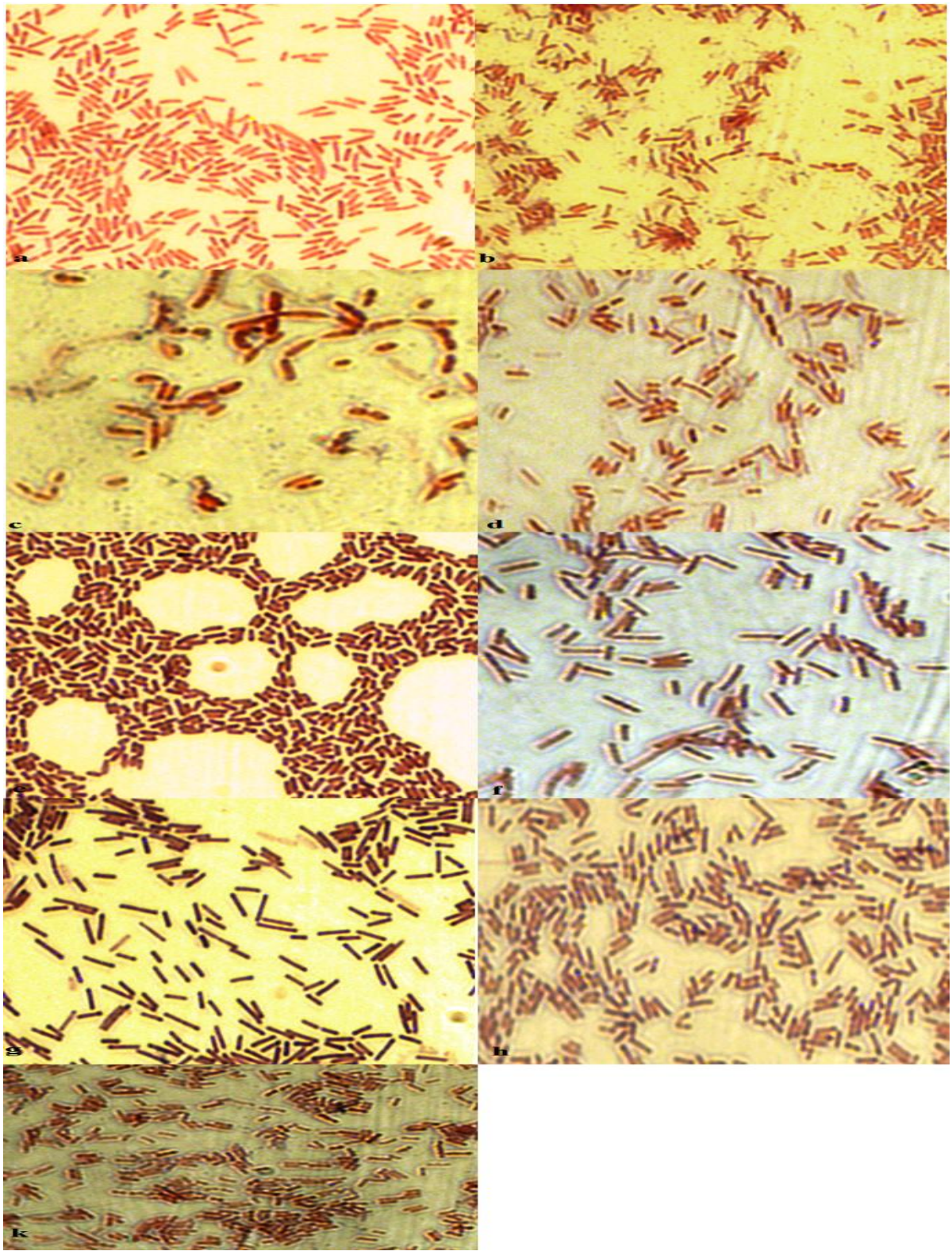


Figure 5.1 Gram stained bacterial smears (1000x). a)St 18 b)St 51 c)St 53 d)St 9203 e)Ps 1 f)Ps 3 g)Ps 5 h)Ps 10421 and k) Unknown. All bacterial strains (a-k) are Gram negative bacilli.

Table 5.1 Culture characterisation, Gram reaction and biochemical test of isolate bacteria.

Bacteria	*Colony morphology		Gram reaction	Shape	Oxidase Test	Catalase Test	TSI Slant/Butt
St 18	**Less than 1mm, circular, creamy yellow, smooth, entire margin, slightly convex, glistening, no odour**		-ve	Bacilli	-	+	K/K
St 51	**2 mm ,circular, creamy yellow, smooth, entire margin, slightly convex, glistening, odour**		-ve	Bacilli	-	+	K/K
St 53	**Less than 1mm,circular, creamy yellow, smooth, entire margin, slightly convex, glistening, no odour**		-ve	Bacilli	-	+	K/K
St 9203	**Less than 1mm,circular, creamy yellow, smooth, entire margin, slightly convex, glistering, no odour**		-ve	Bacilli	-	+	K/K
Ps 1	**3 mm, wrinkled, dark green pigmentation irregular margins, opaque, odour**		-ve	Bacilli	+	+	K/K
Ps 3	**4 mm, wrinkled, green pigmentation irregular margins, opaque, odour**		-ve	Bacilli	+	+	K/K
Ps 5	**Different**	****Ps 5**	-ve	Bacilli	+	+	K/K
		*****Unknown**	-ve	Bacilli	-	+	A/A
Ps 10421	**4 mm, circular, lemon to green pigmentation, smooth entire margins, convex, opa-que, no odour glistening**		-ve	Bacilli	+	+	K/K

(A: Acid) the colour changed to yellow. (K; Alkaline) the colour remains red. *Colony morphology at 24hours. **3-4mm in size, wrinkled, yellow, convex, irregular margins, opaque, no odour. ***3-4mm in size, smooth, creamy white, convex, entire margins, glistening, strong odour.

5.1.2. Molecular identification of bacterial

The identification of the individual isolates was confirmed by the sequencing of the 16s rRNA gene and subsequent comparison with the relevant data bases (Section 4.3.3.1). Taxonomy of bacterial sequences were estimate via the RDP-classifier program as shown in (Table 5.2).With the exception of the unknown contaminating isolate, all the other isolates were identified as either *Stenotrophomonas maltophilia* or *Pseudomonas aeruginosa* while the unknown isolate was identified as *Citrobacter freundii* (Table 5.3). The phylogenetic trees

(figure 5.2) illustrated that the clinical and lab strains *Pseudomonas aeruginosa* (Ps1, Ps3, Ps5 and Ps 10421) and unknown bacteria were closely related to *Pseudomonas aeruginosa* and *Citrobacter freundii* groups, respectively. While *Stenotrophomonas maltophilia* (St 18, St 51, St 53 and St 9203) sequences were related to *Stenotrophomonas maltophilia* groups that containing also *Pseudomonas hibsicula* and *Pseudomonas geniculate,* both of which are now reclassified as *Stenotrophomonas maltophilia*.

Table 5.2 Taxonomy of bacterial gene sequences analysed by Ribosomal Database Project (RDP) - classifier program

Taxonomy	ST 18 ST 51 ST 53	Ps 1 Ps 3 Ps 5	Unknown
Domain	Bacteria	Bacteria	Bacteria
Phylum	Proteobacteria	Proteobacteria	Proteobacteria
Class	Gammaproteobacteria	Gammaproteobacteria	Gammaproteobacteria
Order	Xanthomonadales	Pseudomonadales	Enterobacteriales
Family	*Xanthomonadaceae*	*Pseudomonadaceae*	*Enterobacteriaceae*
Genus	*Stenotrophomonas*	*Pseudomonas*	*Citrobacter*

Table 5.3 Tope four nearest neighbours for each bacterial sequences analysed by Ribosomal Database Project (RDP) – sequence match program, showing ; S_ab score, unique common oligomers and sequence full name with its passport number

Bacteria	S_ab score	Oligomers	Match bacteria	Strain Passport
St 18, 863 unique oligomers	0.988	1444	*Stenotrophomonas maltophilia*	IAM 12423
	0.962	1392	*Stenotrophomonas pavanii*	ICB 89
	0.946	1400	*Pseudomonas hibiscicola*	ATCC 19867T
	0.943	1388	*Pseudomonas beteli*	ATCC 19861T
St 51, 861 unique oligomers	0.988	1444	*Stenotrophomonas maltophilia*	IAM 12423
	0.962	1392	*Stenotrophomonas pavanii*	ICB 89
	0.950	1388	*Pseudomonas beteli*	ATCC 19861T
	0.945	1345	*Pseudomonas geniculata*	ATCC 19374T
St 53, 920 unique oligomers	0.983	1444	*Stenotrophomonas maltophilia*	IAM 12423
	0.964	1392	*Stenotrophomonas pavanii (T)*	ICB 89
	0.959	1388	*Pseudomonas beteli (T)*	ATCC 19861T
	0.941	1400	*Pseudomonas hibiscicola*	ATCC 19867T
Ps 1, 924 unique oligomers	0.973	1456	*Pseudomonas aeruginosa*	DSM 50071; X06684
	0.897	1444	*Pseudomonas otitidis*	MCC10330
	0.895	1460	*Pseudomonas aeruginosa*	DSM 50071; HE978271
	0.870	1344	*Pseudomonas panipatensis*	Esp-1
Ps 3, 901 unique oligomers	0.997	1460	*Pseudomonas aeruginosa*	DSM 50071; HE978271
	0.921	1444	*Pseudomonas otitidis*	MCC10330
	0.908	1344	*Pseudomonas panipatensis*	Esp-1
	0.906	1456	*Pseudomonas aeruginosa*	DSM 50071; X06684
Ps 5, 463 unique oligomers	0.976	1460	*Pseudomonas aeruginosa*	DSM 50071; HE978271
	0.916	1444	*Pseudomonas otitidis*	MCC10330
	0.911	1427	*Pseudomonas nitroreducens*	IAM 1439
	0.903	1417	*Pseudomonas indica*	AF302795
Unknown, 942 unique oligomers	0.989	1410	*Citrobacter freundii*	DSM 30039
	0.975	1434	*Citrobacter braakii*	CDC 80-58
	0.966	1434	*Citrobacter murliniae*	CDC 2970-59
	0.938	1397	*Citrobacter werkmanii*	CDC 0876-58

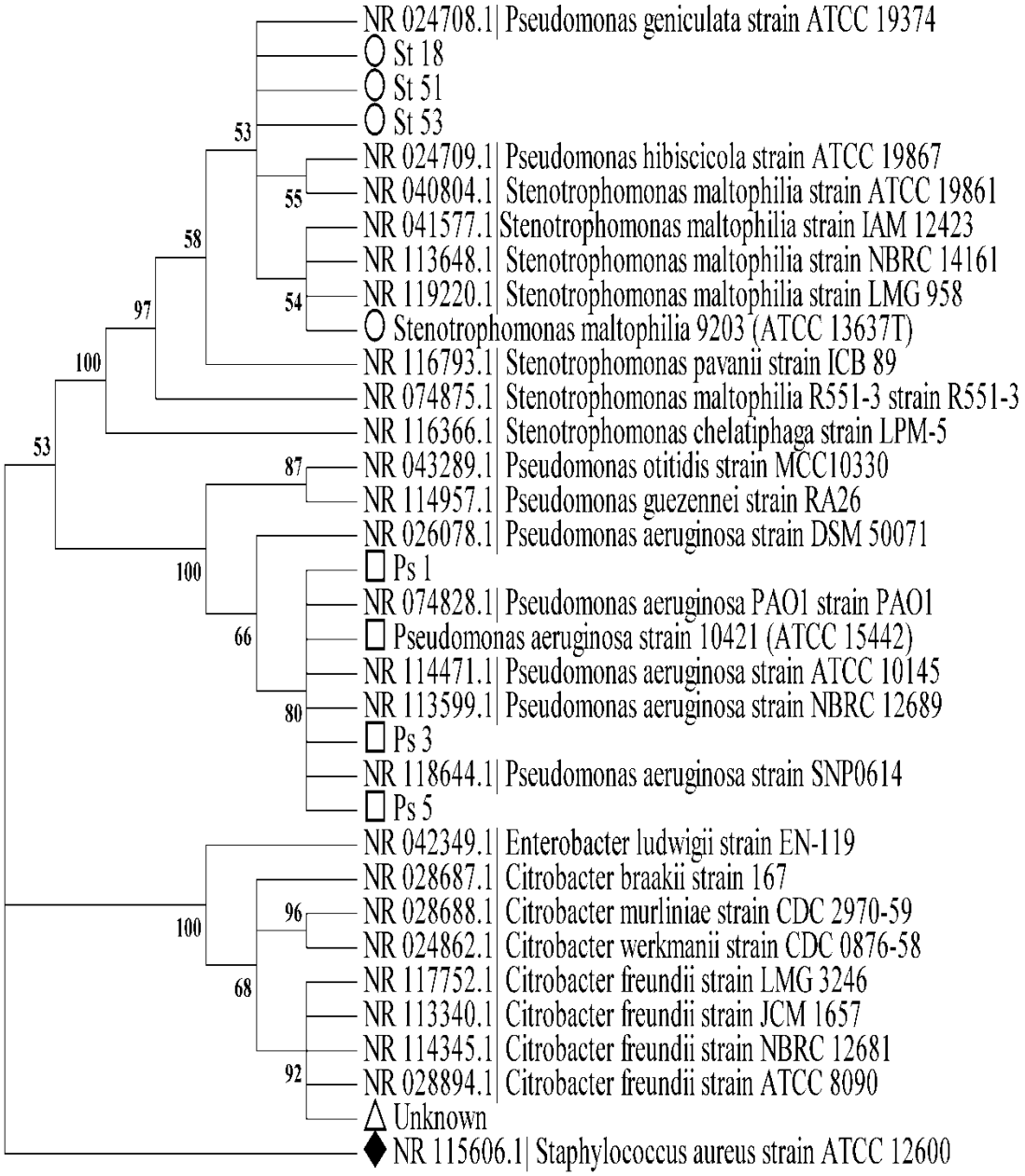


Figure 5.2 Phylogenetic relationships dendrogram between sequences of bacterial samples strains marked as ○□△◆ (St 18, 51, 53, 9203, Ps 1, 3, 5, 10421 and unknown bacteria) and (*Staphylococcus aureus* as a control) and closely related type or lab strains of the genera Stenotrophomonas, Pseudomonas and Citrobacter based on 16S rRNA gene sequence similarity using Neighbour-joining method with 1000 replicates boots trap. Branches reproduced in >50% of the replicates are collapsed.

5.1.3. Whole genome sequencing (WGS)

Three isolates (St 53, Ps 3 and *C. freundii*) were chosen for further investigation and whole genome sequencing based on their strong biofilm formation in the case of both St 53 and Ps 3 while *C. freundii* was chosen because it's not a well characterised EPS producer (See chapter 6). A draft whole genome sequence was obtained for *Stenotrophomonas maltophilia* (St 53), *Pseudomonas aeruginosa* (Ps 3) and *Citrobacter freundii* (SA79) by the application of a whole genome shotgun sequence strategy. RAST and SEED programs showed that the St 53 genome sequencing generated 128 contigs with a draft genome 4,637,878 bp in length and a G_C content of 66.3%. The draft genome contained a total of 4,082 coding sequences (CDS), where 77 pseudogenes, 3 genes coding for rRNA (5S, 16S, 23S), 64 genes coding for tRNAs, and 1 for noncoding RNA. RAST annotation (Figure 5.3) showed that 31 genes were present encoding multidrug-resistance efflux pumps, 21 were involved in cobalt-zinc-cadmium resistance and 10 genes were involved in the production of β-lactamases. Also present were genes involved in the resistance to arsenic and fluoroquinolones, as well as tripartite efflux pumps and a single gene encoding an aminoglycoside adenylyl transferase. In addition, genes were present that were responsible for encoding proteins involved in the synthesis of biofilms, which are likely to enhance its pathogenicity (Liaw et al., 2010). Overall, the strain demonstrates the potential for resistance to a wide range of antimicrobial strategies within the clinical setting.

The draft genome sequencing of strain Ps 3 generated 165 contigs, with a sequence length of 6,799,547 bp (66.2% G_C content). The draft genome contained a total of 6,161 coding sequences (CDSs), where 35 pseudogenes, 2 genes coding for rRNA (16S, 23S), 57 genes coding for tRNA, and 1 noncoding RNA (ncRNA) gene. *Citrobacter freundii* strain SA79 was 4,870,483 bp in length across 19 contigs with a G_C content of 51.7%. The draft genome contained a total of 4,480 CDSs, 36 pseudogenes, 3 genes coding for rRNA (5S, 16S, 23S), 72 genes coding for tRNA, and 9 ncRNAs were present. Further analysis of the

two genomes (*Pseudomonas aeruginosa* strain PS3 and *Citrobacter freundii* strain SA79) using RAST indicated that both organisms carried genes encoding resistance to antibiotics and toxic compounds such as beta lactamase (BLI and BLc) genes and multi drug resistance efflux pump. In addition, the presence of genes involved with exopolysaccharide and biofilm synthesis such as Pel genes in Ps 3 and pga genes in *C. freundii* suggests that these organisms may be of further clinical interest as shown in (Figure 5.3). These sequences (whole-genome shotgun projects) were submitted to Genbank under the accession numbers;

-JRJA00000000 (*Stenotrophomonas maltophilia* strain 53)
-JRGP00000000 (*Pseudomonas aeruginosa* strain PS3)
-LAZI00000000 (*Citrobacter freundii* strain SA79).

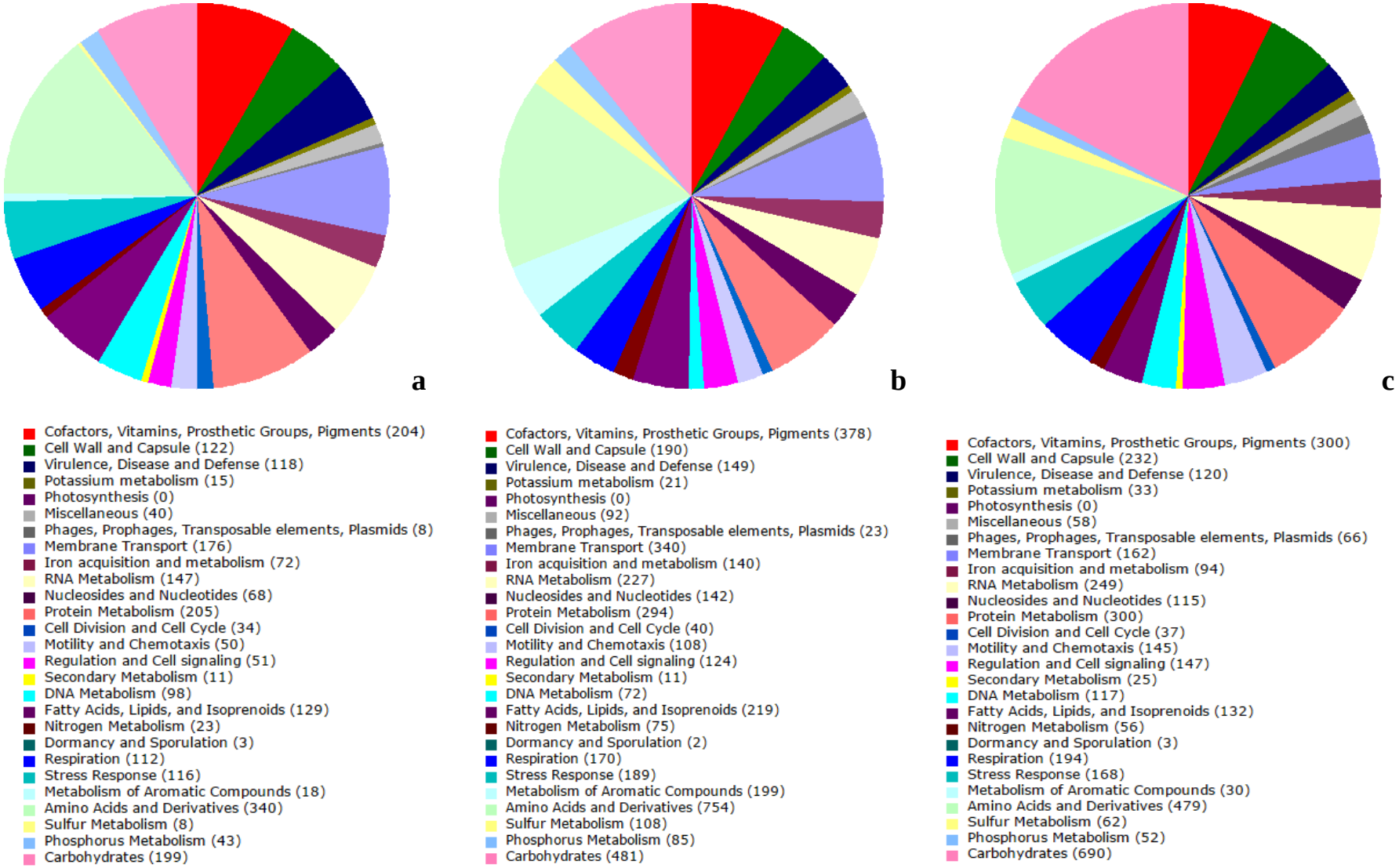


Figure 5.3 Subsystem category distribution (pie chart) and its subsystem feature count (table) using RAST program; a) *Stenotrophomonas maltophilia* 53, b) *Pseudomonas aeruginosa* ps3 and c) *Citrobacter freundii* SA79.

5.1.4. Discussion

Culture characteristic and biochemical test proved to be a good starting point to ensure that the isolates used for the entirety of the study were pure and correctly identified. Confirmation of isolate identification by 16s rRNA gene sequencing indicated that isolate *Pseudomonas aeruginosa* Ps 5 was contaminated with *Citrobacter freundii* SA79. All the *P. aeruginosa* isolates and *Citrobacter freundii* were clustered around *P. aeruginosa* and *Citrobacter freundii* group, respectively. While *Stenotrophomonas* isolates (St 18, 51, 53, and 9203) were clustered among *Stenotrophomonas maltophilia* groups that also containing *Pseudomonas geniculate* and *Pseudomonas hibiscicola*, however these species have been re-classified as a member of genus *Stenotrophomonas* (Anzai et al., 2000).

Bacterial whole genome sequencing is an important tool for mapping the genomes of novel bacteria, bacterial identification and comparing genomes with other bacteria. Whole genome sequencing provides an indication of the ability of the bacteria under investigation to form biofilms formation and antimicrobial resistance, (biofilm formation by these bacteria were further studied in next chapter).

The St 53 genome revealed the presence of 5 genes previously identified as being involved in biofilm formation, 118 features for virulence, disease and defence, 95 genes for resistance to antibiotic and toxins including 31 Multidrug Resistance Efflux Pump (MREP) genes, and 9 genes encoding invasion and intracellular resistance. This was similar to *S. maltophilia* K29a (Taxonomy ID 40324.1) (http://rast.nmpdr.org/) (Overbeek et al., 2014), which has fewer biofilm associated genes (4) but a greater number of resistance and virulence genes (109 and 124 respectively) and a lower number of MREP genes (21) and similar number of genes encode invasion and intracellular resistance (11).

Pseudomonas aeruginosa Ps 3 contains a full complement of pel (PA3058-PA3064), psl (PA2231-2245) and alginate biosynthesis operons (PA3540-3548) associated with *Pseudomonas aeruginosa* PA01 (Sharma et al., 2014) (http://www.pseudomonas.com). In addition Ps 3 contains 149 genes associated with virulence, disease and defence, 131 genes for resistance to antibiotic and toxins including 22 Multidrug Resistance Efflux Pump (MREP), and 13 genes encode invasion and intracellular resistance. This suggests that Ps 3 is more virulent and has greater resistance potential than well studied pseudomonas such as PA01 and UCBPP-PA14. For example PA01 (Taxonomy ID 208964.1) (http://rast.nmpdr.org/) (Overbeek et al., 2014) has 122 genes associated with resistance to antibiotic and toxins including 18 MREP, and 11 genes encode invasion and intracellular resistance. In the case of *Pseudomonas aeruginosa* UCBPP-PA14 (Taxonomy ID 208963.3) (http://rast.nmpdr.org/) (Overbeek et al., 2014) there are 113 antibiotic resistance features present, 11 genes are associated with MREP, and invasion and intracellular resistance are encoded by 11 genes.

Whole genome sequencing showed the presence of the entire *pgaABCD* locus in St 53 and *C. freundii*. This locus which is recognised to encode the synthesis of a polysaccharide poly-β-1,6-N-acetyl-D-glucosamine (β-1,6-GlcNAc, PGA) which is present in the extracellular matrix of biofilms in a wide range of bacteria. It also mediates cell-to-cell and cell-to-surface adhesion and stabilization of biofilm formation in a variety of bacteria including *E. coli* (Wang et al., 2004), the cystic fibrosis pathogen *Achromobacter xylosoxidans* (Jakobsen et al., 2013), *Pectobacterium atrosepticum* (Pérez-Mendoza et al., 2011), *Acinetobacter baumannii* (Choi et al., 2009), *Klebsiella pneumoniae*, *Enterobacter cloacae*, *S. maltophilia*, and *Burkholderia cepacia* (Skurnik et al., 2012).

The presence of these genes in those bacteria suggesting that they may synthesize related polysaccharides that contribute to biofilm formation and potentially biofilm-mediated diseases. Surprisingly, there is increasing evidence that many of the MDR bacterial species involved in both community and hospital acquired infections share the genes that involved in surface polysaccharide PNAG synthesis (pga loci) (Roux et al., 2012). None of the *pga* genes have been identified in *P. aeruginosa* (Wang et al., 2004). Chromosomal deletions of each of the *pga* genes role have been performed in *E. coli* in order to determine their purpose. These investigations have shown that all the *pga* genes are required for optimal biofilm formation. The cytoplasmic membrane proteins (PgaC and PgaD) are necessary for PGA synthesis and the outer membrane proteins (PgaA and PgaB) are needed for PGA export (Jakobsen et al., 2013)

5.1.5 Key findings

- All of bacteria chosen for this study were confirmed to belong to the species expected.
- *Stenotrophomonas maltophilia* 53, *Pseudomonas aeruginosa* ps3 and *Citrobacter freundii* SA79 were all identified as novel biofilm forming strains.
- Whole genome sequencing revealed that *Stenotrophomonas maltophilia* 53, *Pseudomonas aeruginosa* ps3 and *Citrobacter freundii* SA79 all possessed a number of genes related to biofilm formation, antimicrobial resistance and pathogenicity.

The works represented in this chapter are published as follows:

Akbar, S., Rout, S. P. & Humphreys, P. N. 2015a. Draft genome sequence of the biofilm-forming *Stenotrophomonas maltophilia* strain 53. *Genome announcements,* 3, e00312-15.

Akbar, S., Rout, S. P. & Humphreys, P. N. 2015b. Draft genome sequences of *Pseudomonas aeruginosa strain* PS3 and *Citrobacter freundii* strain SA79 obtained from a wound dressing-associated biofilm. *Genome announcements,*3, e00561-15.

5.2 Bacterial biofilm formation and hydrophobicity

In this chapter, the ability of the bacterial strains under investigation to form biofilms was tested using a range of methodologies. In a number of cases a period of method development was required to ensure the tests could be performed in a reproducible manner.

5.2.1 Strength of biofilm formation (96 well plate)

All bacterial strains were tested for their ability to form biofilms using the standard 96 well plate method by (Stepanović et al., 2007) as described in Section 4.5.2 (Figure 5.4). Initial work focussed on the impact of inoculum size and the concentration of carbon and nitrogen sources on biofilm formation. In order to do this bacterial numbers (CFU/ml) were calibrated against optical density to remove the need to count bacterial numbers prior to setting up the experiment (Figure 5.5).

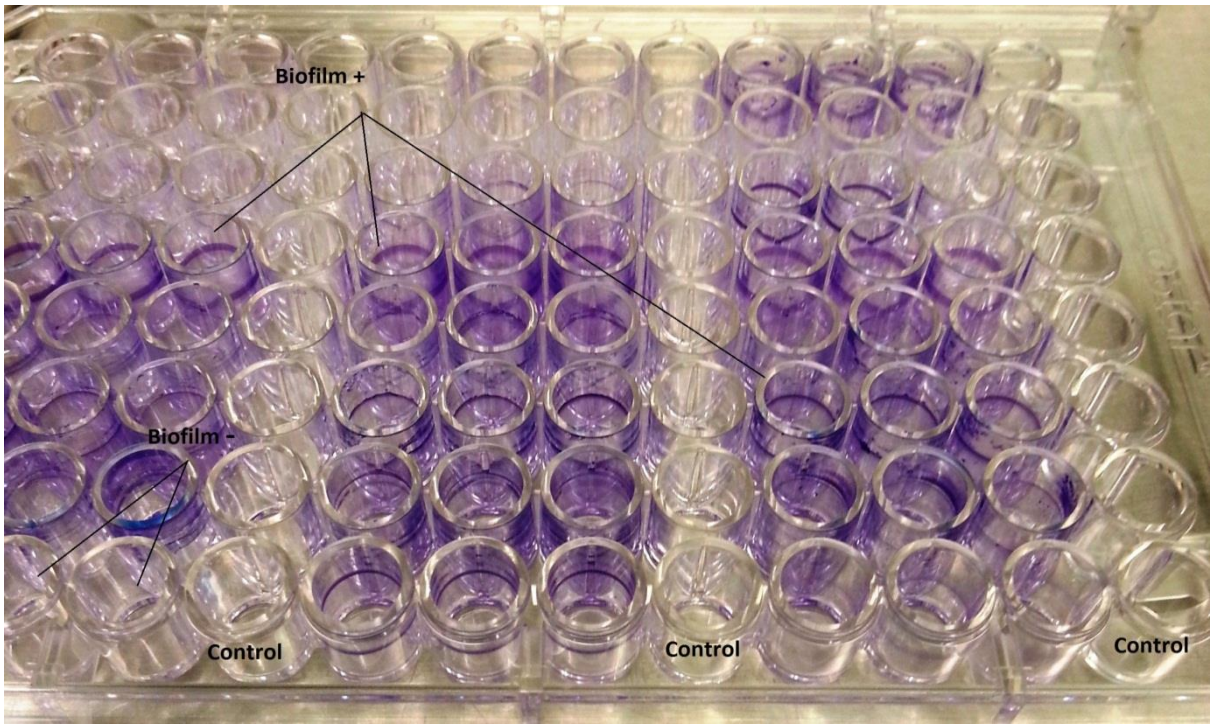


Fig 5.4 Bacterial biofilm formation in 96 well plates.

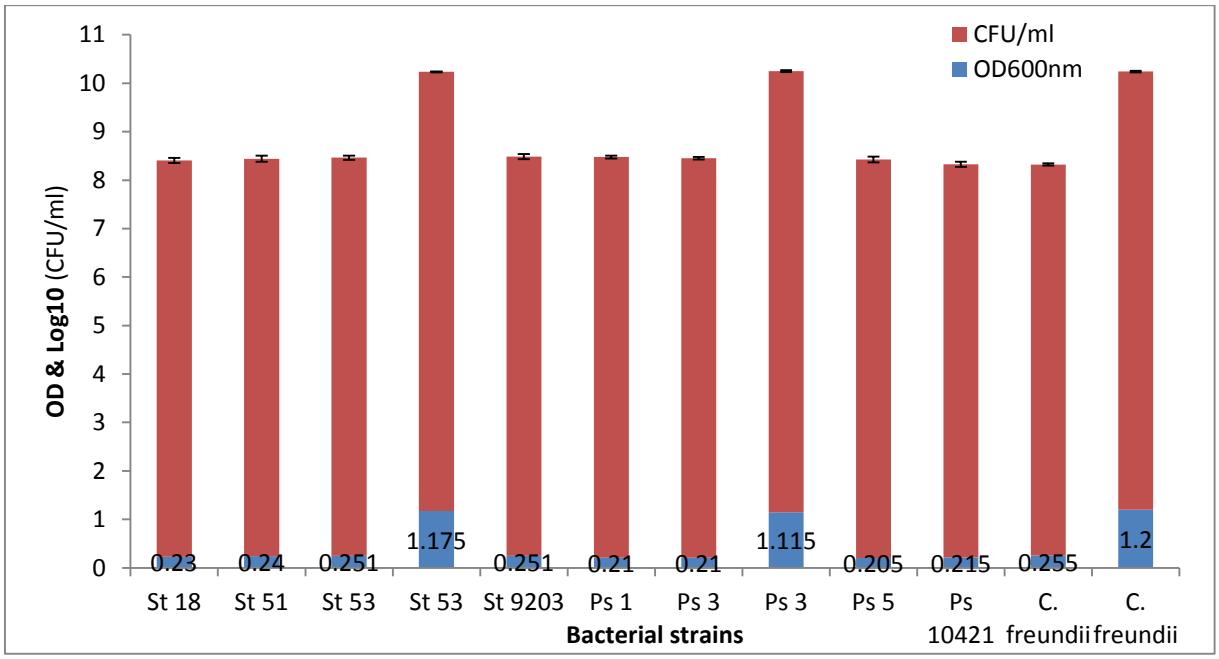


Figure 5.5. Calibration of optical density against bacterial numbers at approximately 10^8 or 10^9 (CFU/ml). Error bars represent standard error of the mean of three replicates.

5.2.2. Effect of inoculum size on biofilm formation

In those strains that biofilms were formed, the strength of biofilm formation increased with inoculum size particularly once the inoculum was greater than $1.0x10^7$ CFU/ml i.e. $2.0x10^6$ cells per 200µl in the well (Figure 5.6). Of all the strains tested only St 18, Ps 5 and *C. freundii* were unable to form biofilms under the some of the conditions investigated (Figure 5.6). In view of the lack of research into *C. freundii*, this strain was included in further biofilm investigations despite its poor biofilm formation under some conditions.

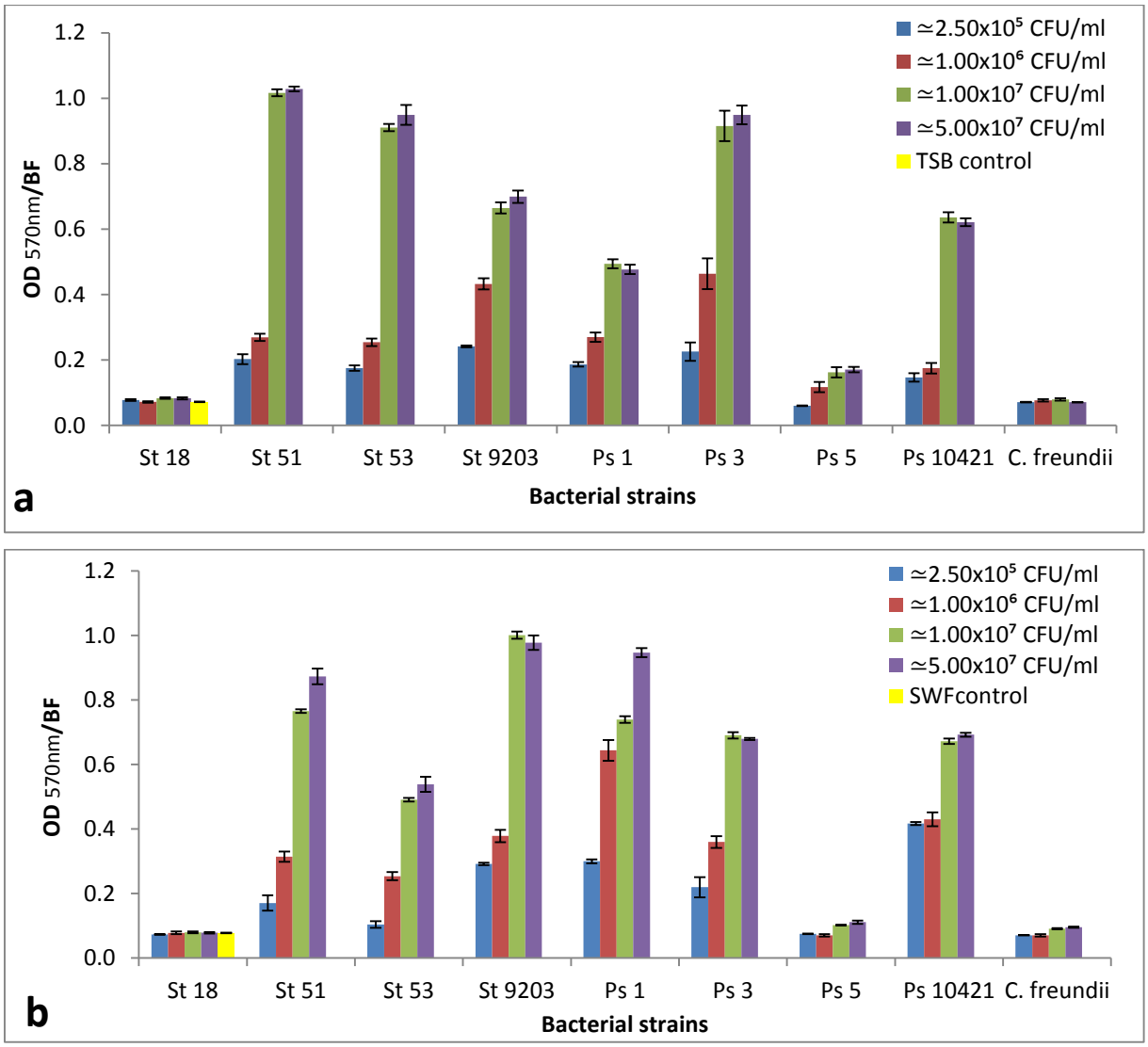
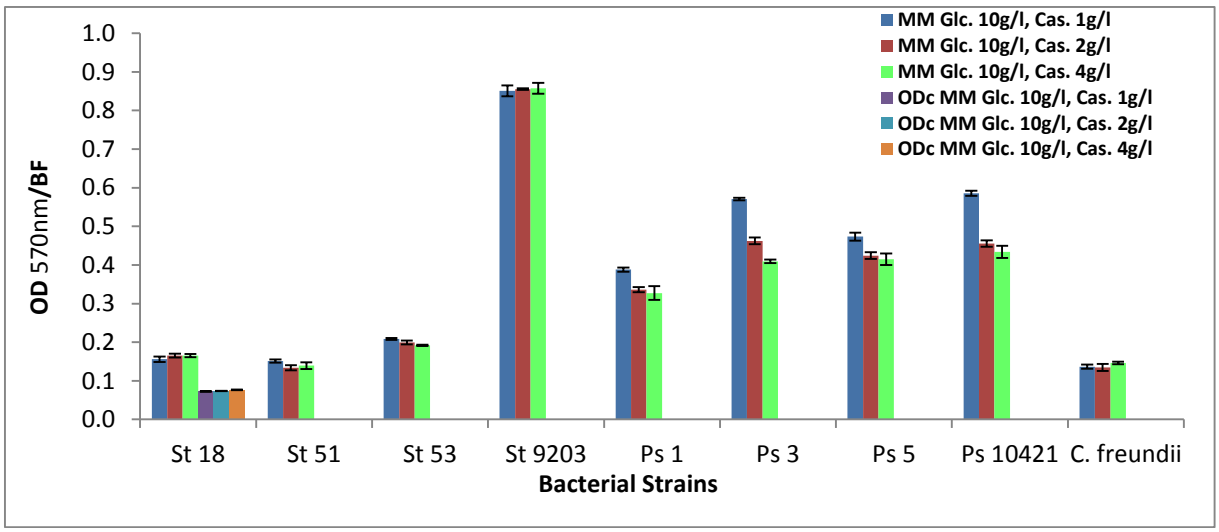


Figure 5.6 Effects of inoculum size on biofilm formation in different media a) Tryptone soya broth (TSB) and b) Simulated wound fluid (SWF). Error bars represent standard error of the mean of three replicates.

5.2.3. Media development, the impact of carbon and nitrogen sources on biofilm formation.

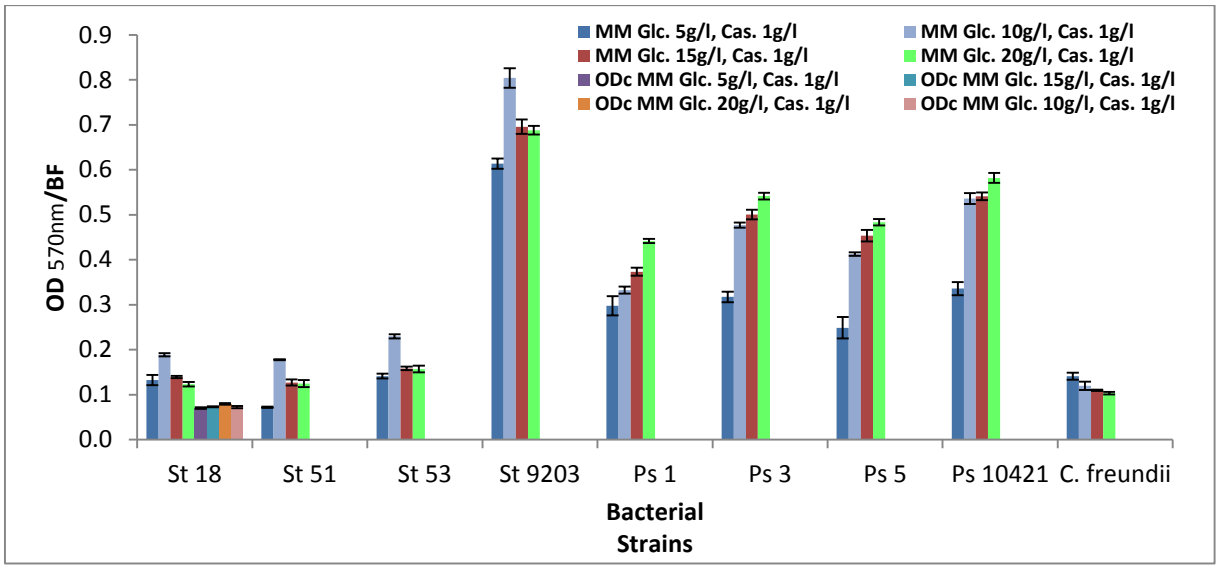
After optimising inoculum size, three media formulations were developed based on a range of primary carbon (glucose, glycerol and ethanol) (MM Glc, MM Gly and MM Eth) and nitrogen (casein) sources in order to allow a reliable analysis of EPS. These media were formulated in order to exclude components such as yeast extract, beef extract or any other substance that may contain compounds that will interfere with EPS analysis via NMR (Alhudhud et al., 2014).

Increasing the casein concentration led to decreasing biofilm formation among some *P. aeruginosa* strains (Figure 5.7), which contrasted with *S. maltophilia* and *C. freundii* where casein concentrations had no observable impact on biofilm formation. Consequently the casein concentration was set at 1g/l for all the future biofilm investigations. As illustrated in (Figure 5.8), *S. maltophilia* and *P. aeruginosa* had quite distinct responses to increasing glucose concentrations. Biofilm formation by *S. maltophilia* increased up to 10g/l of glucose after which biofilm formation began to decline. Biofilm formation by *P. aeruginosa* on the other hand, increased as glucose concentration increased across all concentrations investigated. Finally, biofilm formation by *C. freundii* decreased as glucose concentration increased with the strength of biofilm formation being lower at all concentrations than any of the other bacteria investigated.



MM Glc. Cas.: Mineral media containing glucose and casein
ODc MM Glc. Cas. : Optical density control of mineral media containing glucose and casein

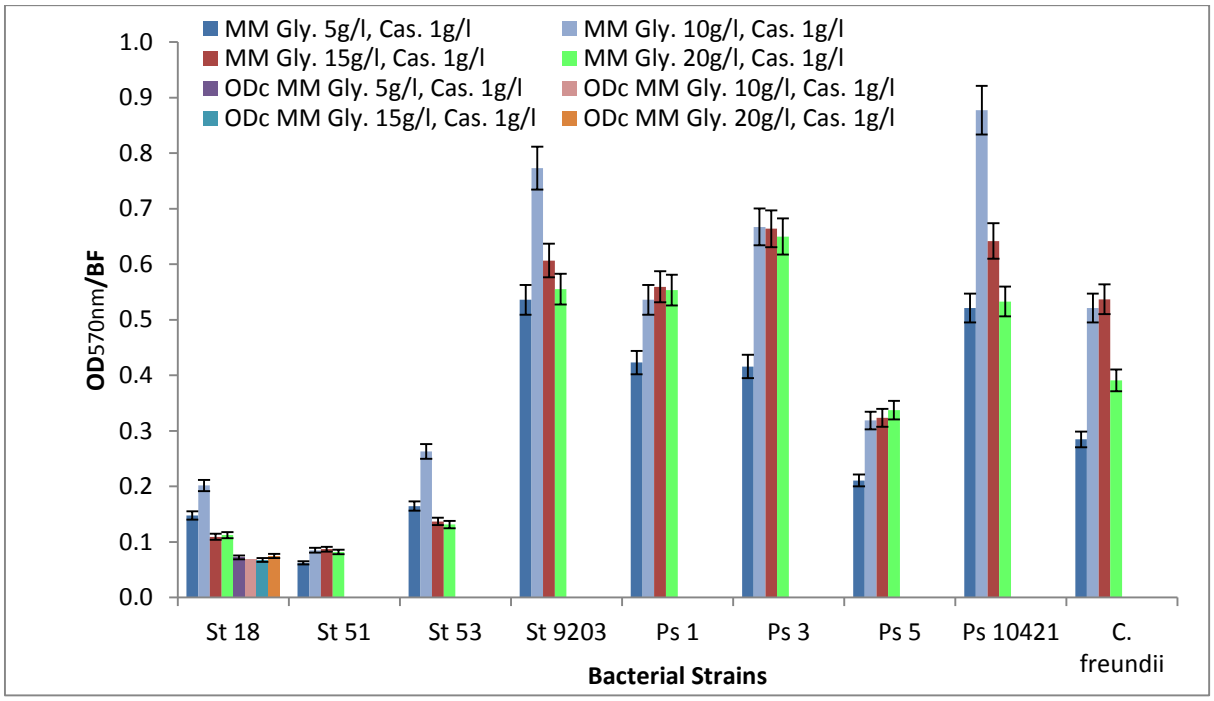
Figure 5.7 The effects of casein concentration on bacterial biofilm formation on 96 well plates inoculated with approximately 10^7 cells/ml in the presence 10 g/l glucose. Error bars represent standard error of the mean of three replicates.



MM Glc. Cas.: Mineral media containing glucose and casein
ODc MM Glc. Cas. : Optical density control of mineral media containing glucose and casein

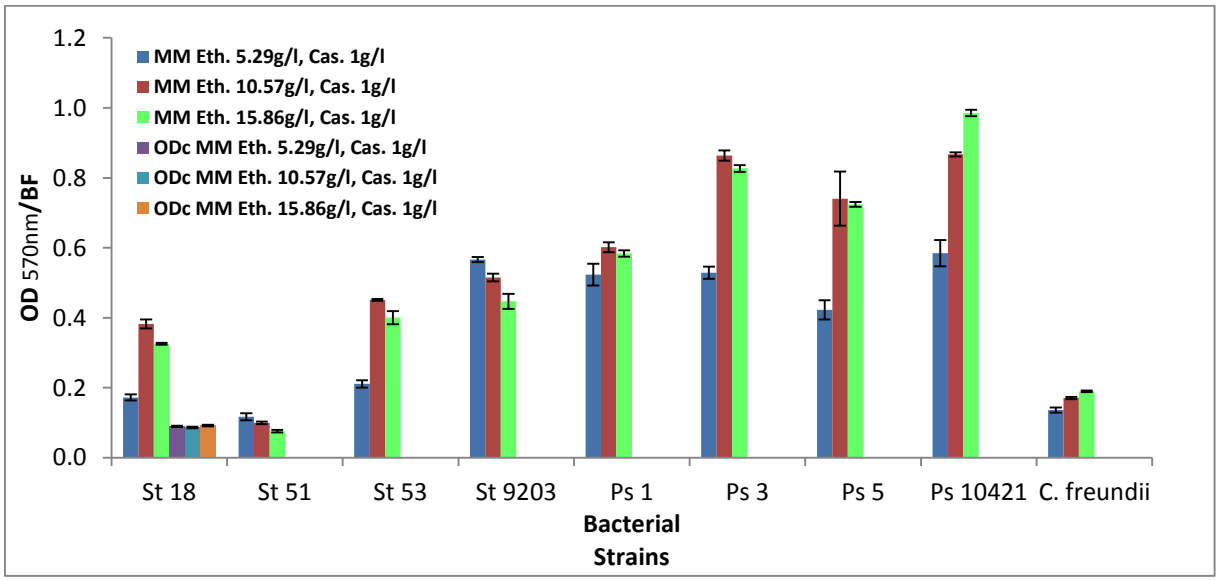
Figure 5.8 Effects of glucose concentration on bacterial biofilm formation with fixed concentration of casein 1g/l inoculated with approximately 10^7 cells/ml. Error bars represent standard error of the mean of three replicates.

The response to increasing glycerol concentrations was similar to that seen with glucose (Figure 5.9) i.e. *S. maltophilia* demonstrated an increase followed by a decrease in biofilm formation and biofilm formation by *P. aeruginosa* increased as glycerol concentration increase. The situation with *C. freundi* was however quite different with the bacteria exhibiting much greater biofilm formation than observed with glucose (Figure 5.8). Biofilm formation by *C. freundi* increased with increasing glycerol concentration up to 15 g/l glycerol (Figure 5.9) beyond which biofilm formation was reduced. In general the response in biofilm formation seen with increased ethanol concentration was similar to the observed with glucose and glycerol (Figures 5.8 to 5.10), that is an initial increase followed by a decrease at the higher concentrations. There were exceptions however, in the case of St 51 and 9203 biofilm formation declined with increasing ethanol concentration which contrasts with Ps 10421 where biofilm formation increased across all ethanol concentrations (Figure 5.10).



MM Gly. Cas. : Mineral media containing glycerol and casein
ODc MM Gly. Cas. : Optical density control of mineral media containing glycerol and casein

Figure 5.9 Effects of different glycerol concentration on bacterial biofilm formation after inoculation with approximately 10^7 cells/ml. Error bars represent standard error of the mean of three replicates.

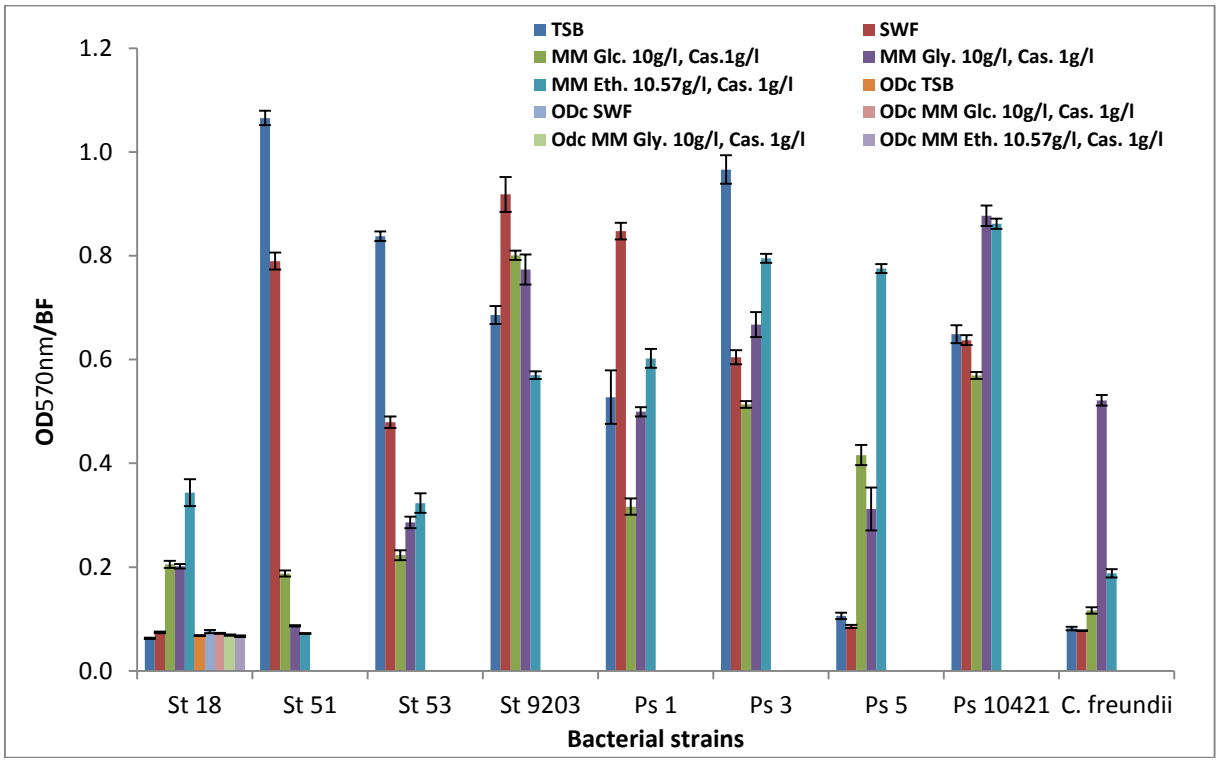


MM Eth. Cas.: Mineral media containing ethanol and casein.
ODc MM Eth. Cas. : Optical density control of mineral media containing ethanol and casein.

Figure 5.10 Effects of series concentration of ethanol on bacterial biofilm formation after inoculation with approximately 10^7 cells/ml. Error bars represent standard error of the mean of three replicates.

5.2.4 Impact of media on bacterial biofilm formation

Alongside the evaluation of mineral media two other media (TSB and SWF) were also evaluated for their impact on biofilm formation. All bacteria produced strong biofilm when grown in both TSB and SWF except St 18, Ps 5 and *C. freundii*. Ps 3 was a strong biofilm producer in all media as was St 53 except in MM Glc in which a moderate biofilm was produced (Figure 5.11).

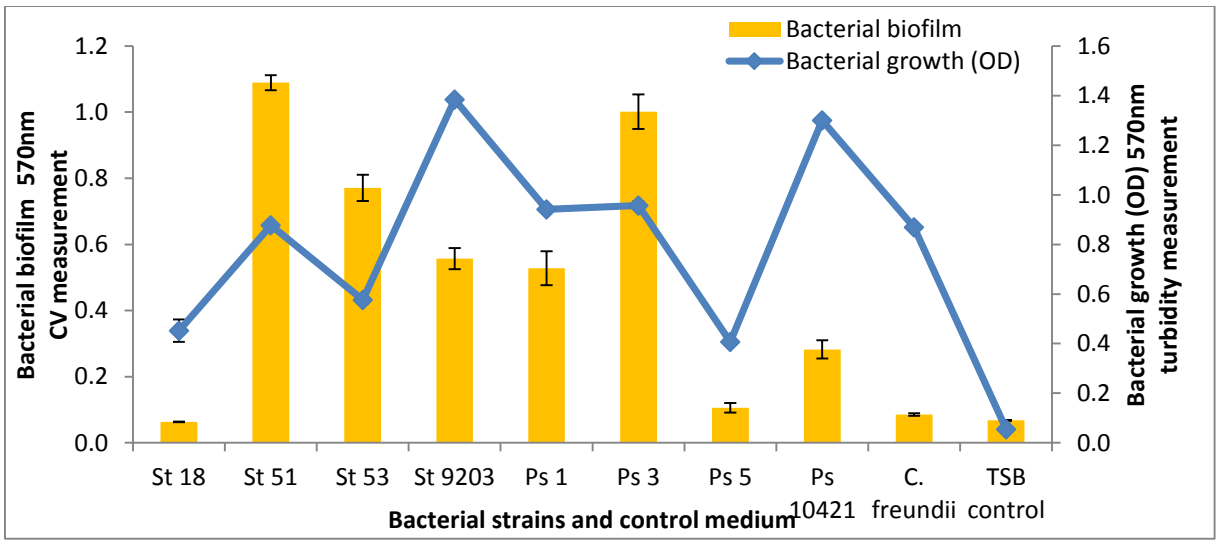


TSB: Trypton soy broth. SWF: Simulated wound fluid,
MM Glc. Cas.: Mineral media containing glucose and casein,
MM Gly. Cas. : Mineral media containing glycerol and casein,
MM Eth. Cas.: Mineral media containing ethanol and casein,
ODc: Optical density control for each media.

Figure 5.11 Effects of different media (TSB, SWF, MM Glc, MM Gly and MM Eth) on bacterial biofilm formation using 96 well plates after inoculation with approximately 10^7 cells/ml. Error bars represent standard error of the mean of three replicates.

5.2.5 Bacterial growth vs biofilm formation

The relationship between bacterial growth and their biofilm formation was investigated in both TSB and SWF by analysis of the OD of bacterial growth prior biofilm quantification using a multiscan microtiter-plate reader. As shown in Figure 5.12, St 53 and Ps 5 grown in SWF reached an OD of about 0.6 and 0.8, while after staining or biofilm quantification, St 53 and Ps 5 produced strong and weak biofilm, respectively. The growth (OD) of St 51 and St 9203 were about 0.8 and 1.4 in TSB, respectively (Figure 5.13), while after quantification of biofilm, production of biofilm were stronger in St 53 than in St 9203. Although all bacterial strains grew well in TSB, SWF and the other media as indicated by the increase in optical density detected using the bioscreen growth technology (Bioscreen Growth Curve C) (OY, Finland) (Appendix). However, strong or high growth was not correlated with strong biofilm formation, in fact no obvious correlation between bacterial growth and biofilm formation could be found (Figure 5.12 and 5.13).



CV: Crystal violet

Figure 5.12 Bacterial growth (turbidity measurement) relation to biofilm formation (CV measurements) in SWF medium. Error bars for both CV and turbidity measurements represent standard error of the mean of three replicates measurement.

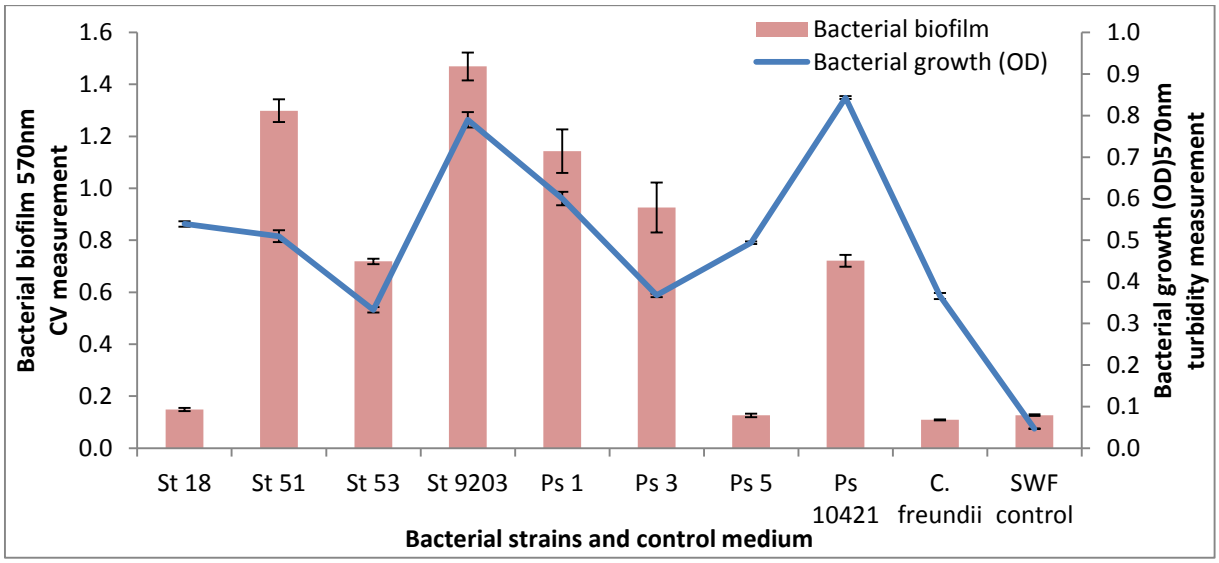


Figure 5.13 Bacterial growth (turbidity measurement) relation to biofilm formation (CV measurements) in TSB medium. Error bars for both CV and turbidity measurements represent standard error of the mean of three replicates measurement.

5.2.6 Evaluation of bacterial biofilm formation using Congo Red Agar (CRA).

The CRA method is often used to screen bacteria for biofilm formation and EPS production (Freeman et al., 1989) (Section 4.5.1). However, after growing all bacterial strains on CRA media, only *S. maltophilia* 53, 9203, and *C. freundii* were identified as biofilm producers (Positive) (Figure 5.14) an observation at odds with the data generated by the 96 well biofilm assay (See Section 5.2.1). An observation that suggests this approach is not universally applicable to screening for biofilm formation.

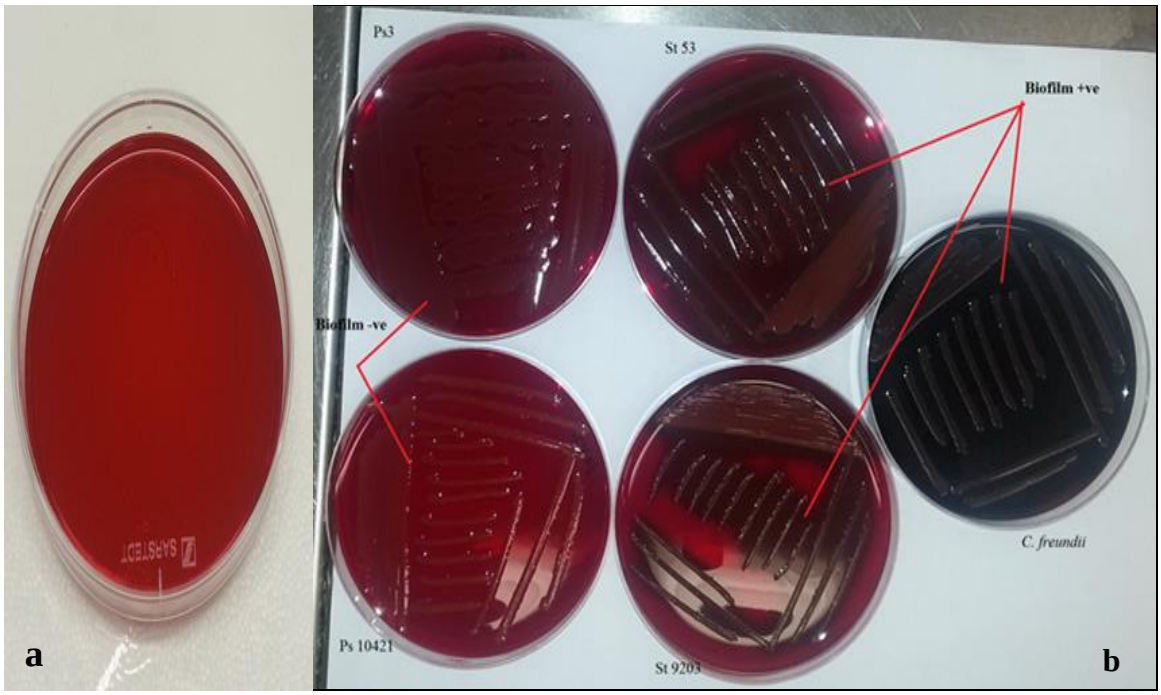


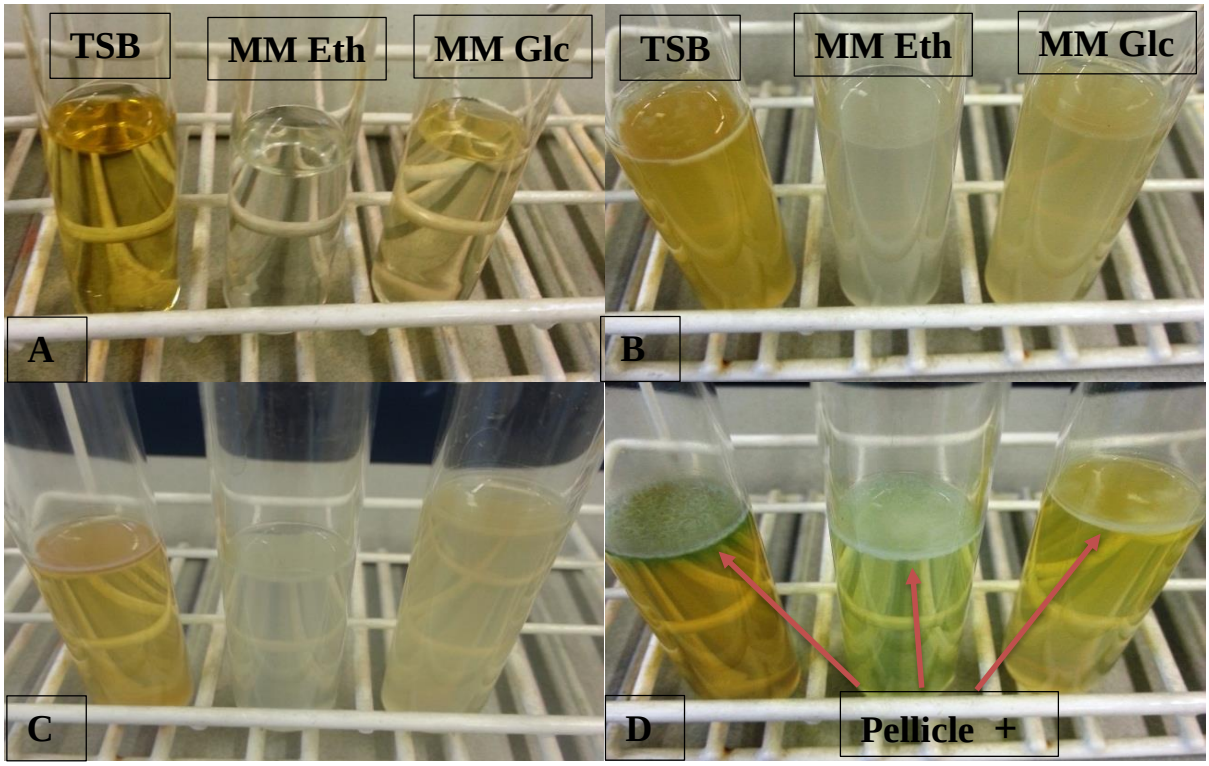
Figure 5.14 Qualitative analysis of bacterial biofilm production by (CRA) method. a) CRA medium without inoculation (control). b) Black colonies biofilm production (+ve) such (St 53, 9203, *C. freundii*), while non-biofilm production (-ve) such as (Ps 3 and 10421).

5.2.7 Bacterial pellicle formation

Bacterial pellicles are biofilms formed at the air liquid interface (Wu et al., 2012, Martí et al., 2011), the evaluation of pellicle formation was performed as described in (Section 4.8) using a range of media (Figure 5.15). All the *P. aeruginosa* strains investigated demonstrated the ability to form a pellicle irrespective of the media employed (Table 5.4). Pellicle formation in *S. maltophilia* was media dependent with a variety of responses depending on strain involved (Table 5.4). The largest contrast was seen between St 18 that did not form a pellicle and St 53 which formed a pellicle in all media. In the case of *C. freundii* a pellicle was only formed in the presence of ethanol.

Table 5.4 Pellicle biofilm formation in different media (TSB, MM Eth 10.57 g/l and MM Glc 10 g/l) at room temperature for different incubation times.

Bacterial strains	TSB		MM Eth.		MM Glc.	
	3 Days	7 Days	3 Days	7 Days	3 Days	7 Days
St 18	-	-	-	-	-	-
St 51	+	+	-	-	-	-
St 53	+	+	+	+	+	+
St 9203	+	+	-	-	-	-
Ps 1	+	+	+	+	+	+
Ps 3	+	+	+	+	+	+
Ps 5	+	+	+	+	+	+
Ps 10421	+	+	+	+	+	+
C. freundii	-	-	+	+	-	-



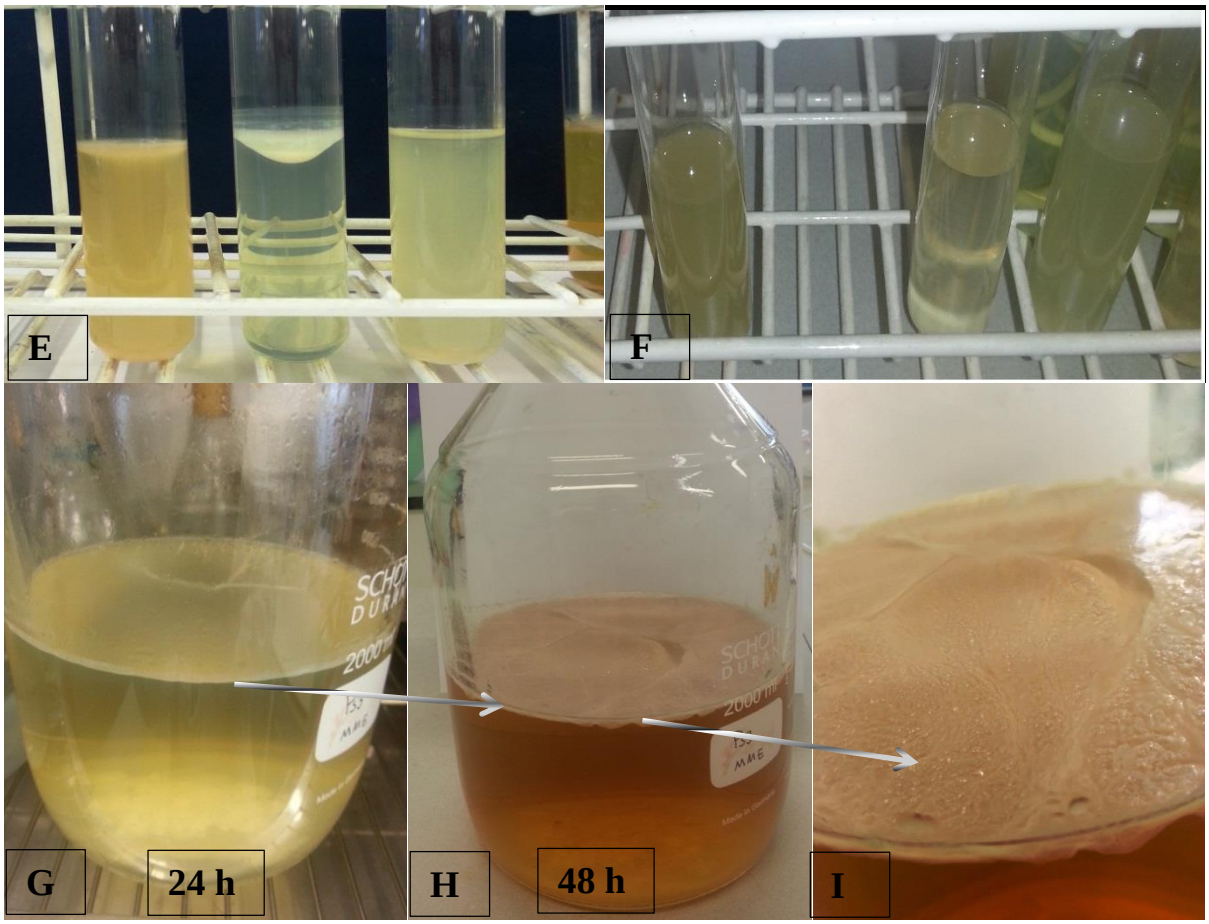
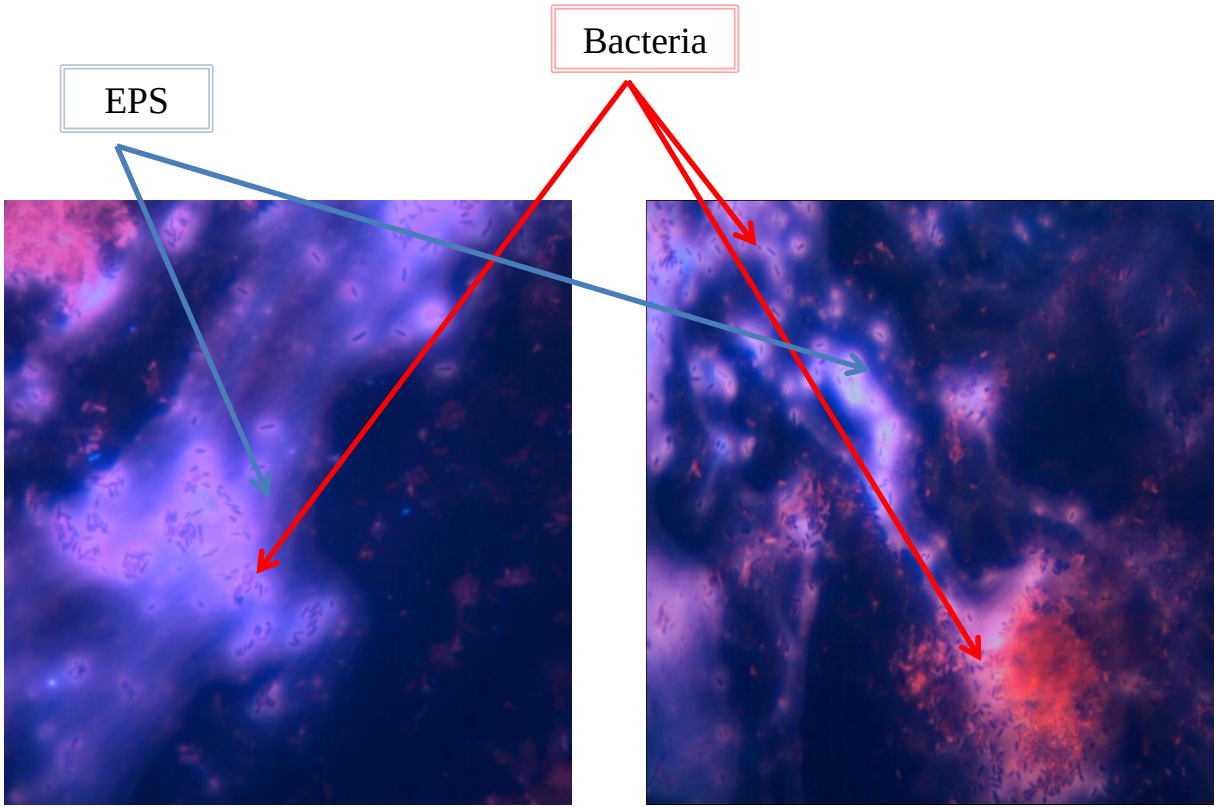


Figure 5.15 Pellicle biofilm formation during growth in different media (TSB, MM Eth 10.57 g/l and MM Glc 10 g/l) at room temperature A) TSB, MM Eth and MM Glc media control, B) St 53, C) St 9203, D) Ps 3, E) Ps 10421, F) *C. freundii* G) H) I) Ps 3 pellicle biofilm formation grow in MM Eth using 2 litter bottle after 24 and 48 h.

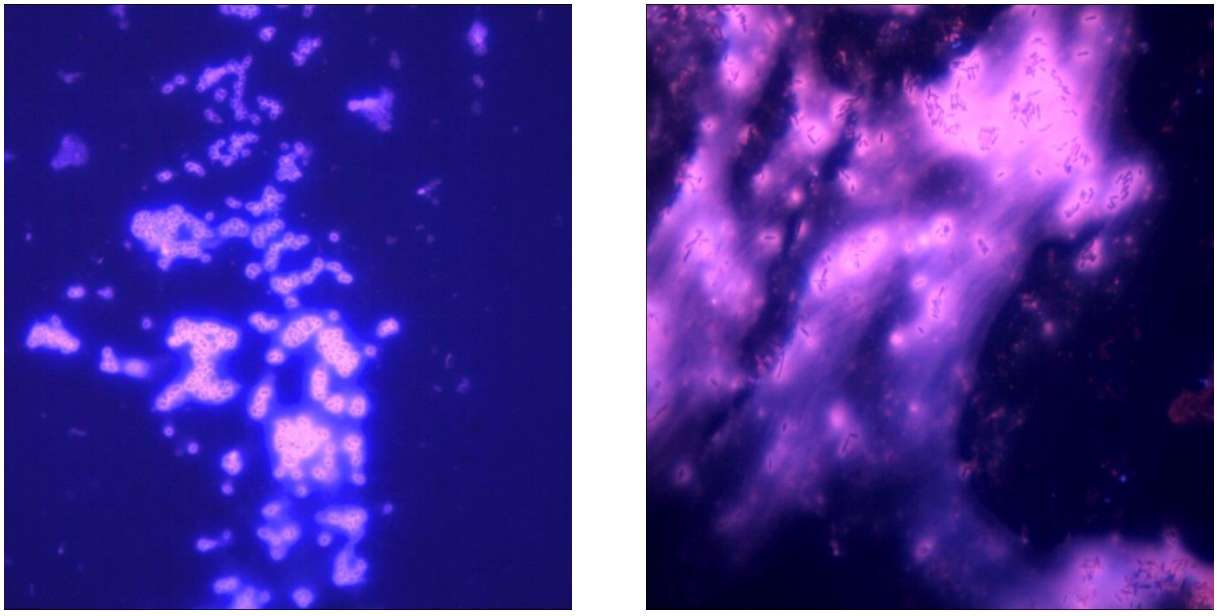
5.2.8 Bacterial biofilm formation under low shear condition

The strains demonstrating the greatest biofilm formation among each hospital groups (St 53, Ps 3) and *C. freundii* as a bacterium with very little biofilm related research (Section 5.2.1) were investigated for their ability to produce biofilms under low shear condition using the DFR method (See section 4.9) (Hassett et al., 1999). The DFR was used to simulate moist aerobic environments such as those found around sinks etc. In addition to the clinical strains two lab strains St 9203 and Ps 10421 were also tested. Under fluorescence microscope, bacteria or nucleic acid appeared as red colour, while biofilm (EPS) appeared as a blue colour (Hill et al., 2010). As shown in (Figure 5.16 a-t), all 5 bacterial

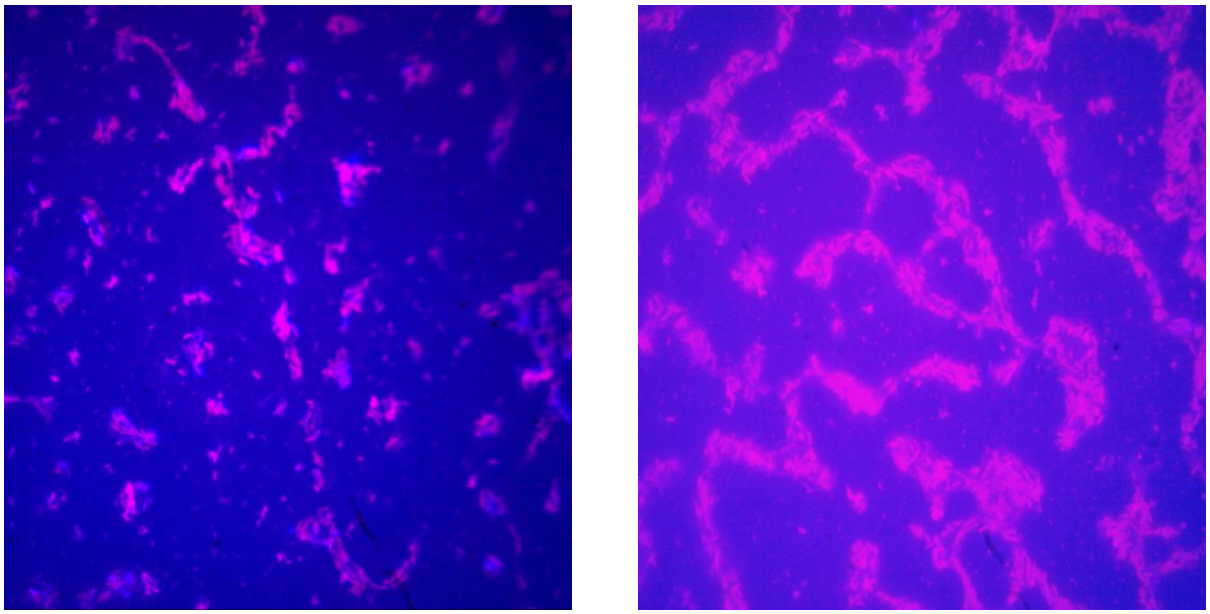
strains (St 53, St 9203, Ps 3, Ps 10421 and *C. freundii*) were able to produce biofilms on glass (hydrophilic) and plastic polystyrene (hydrophobic) slides after 24 h in the presence glucose or ethanol as sole carbon source.



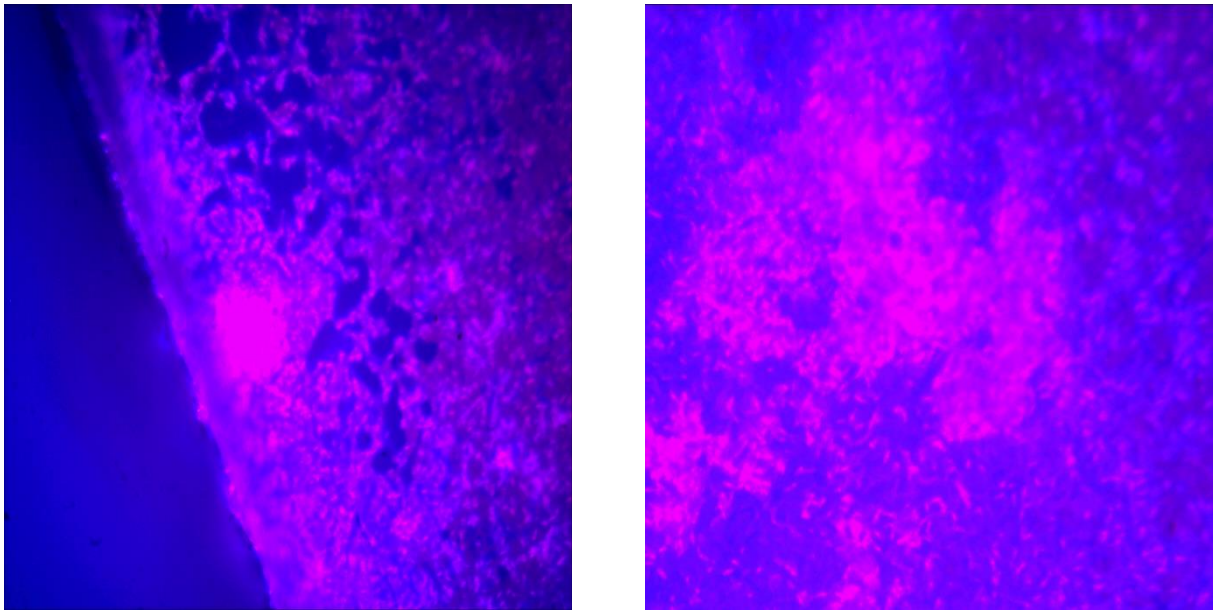
a) St 53 biofilm formation in (MM Glc) on glass slide after 24 and 48 h



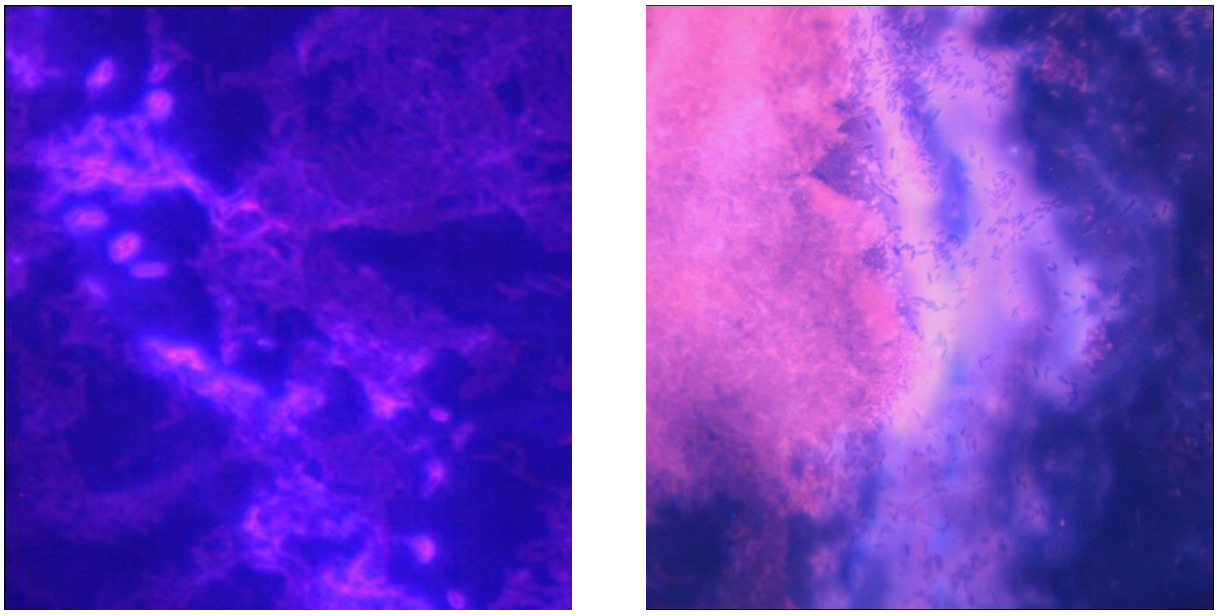
b) St 53 biofilm formation in (MM Eth) on glass slide after 24 and 48 h



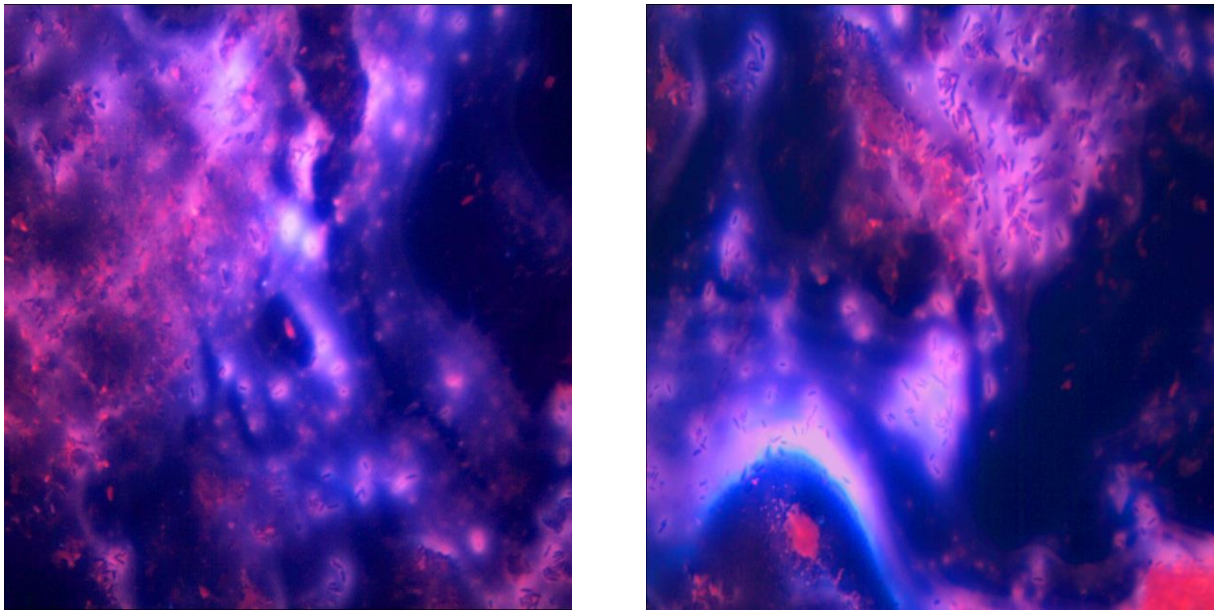
c) St 53 biofilm formation in (MM Glc) on plastic slide after 24 and 48 h



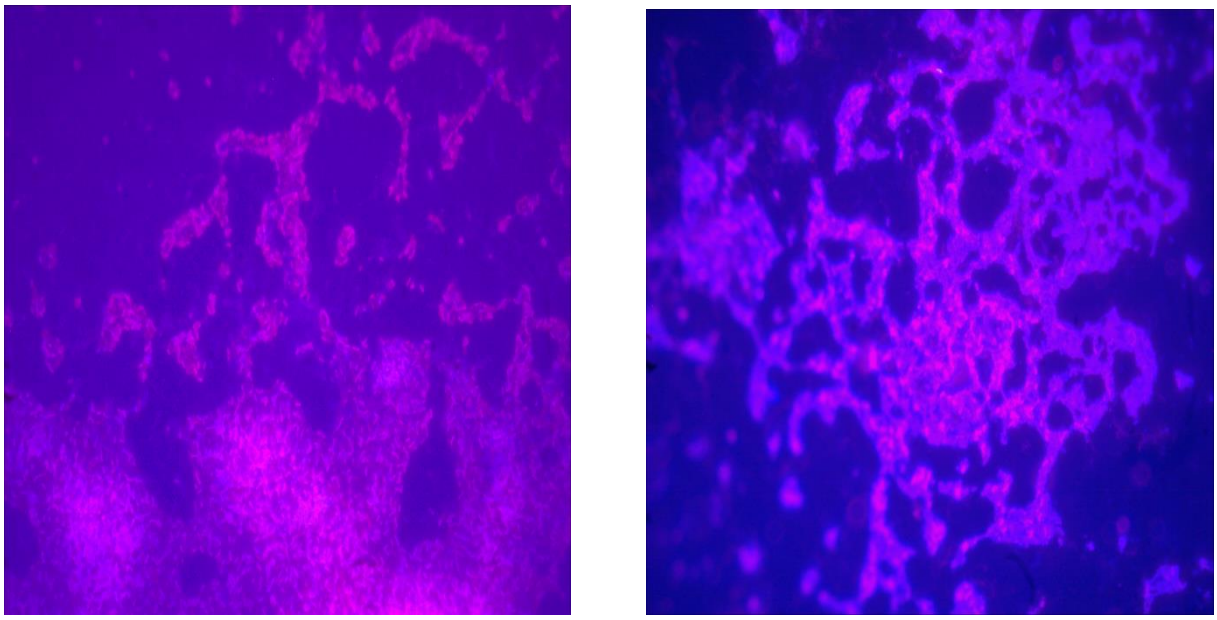
d) St 53 biofilm formation in (MM Eth) on plastic slide after 24 and 48 h



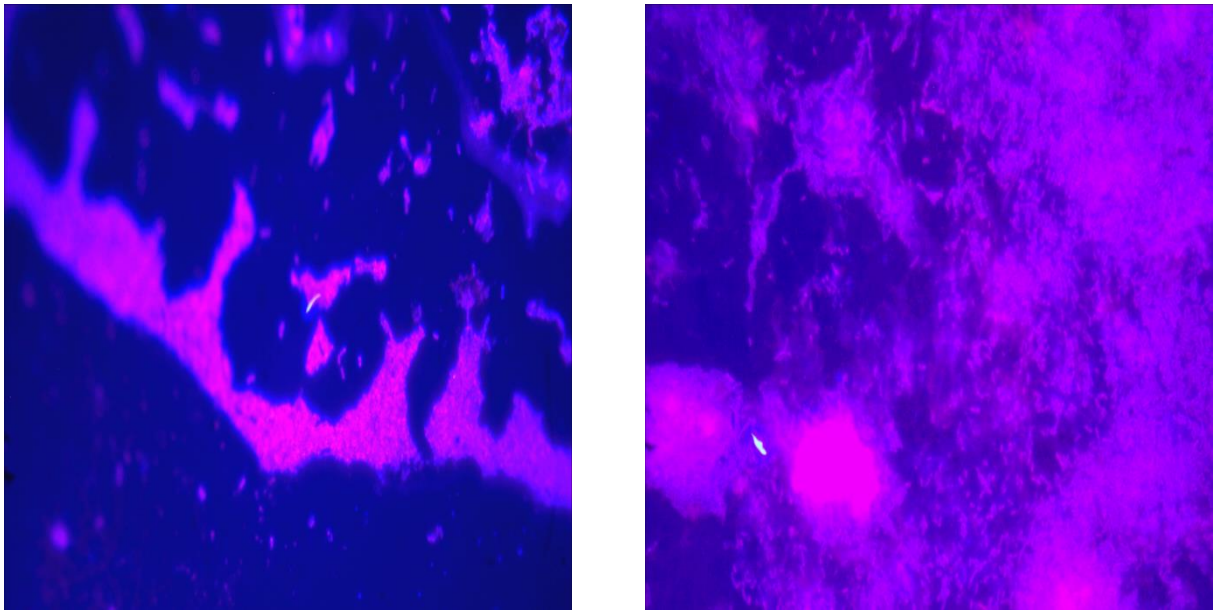
e) Ps 3 biofilm formation in (MM Glc) on glass slide after 24 and 48 h



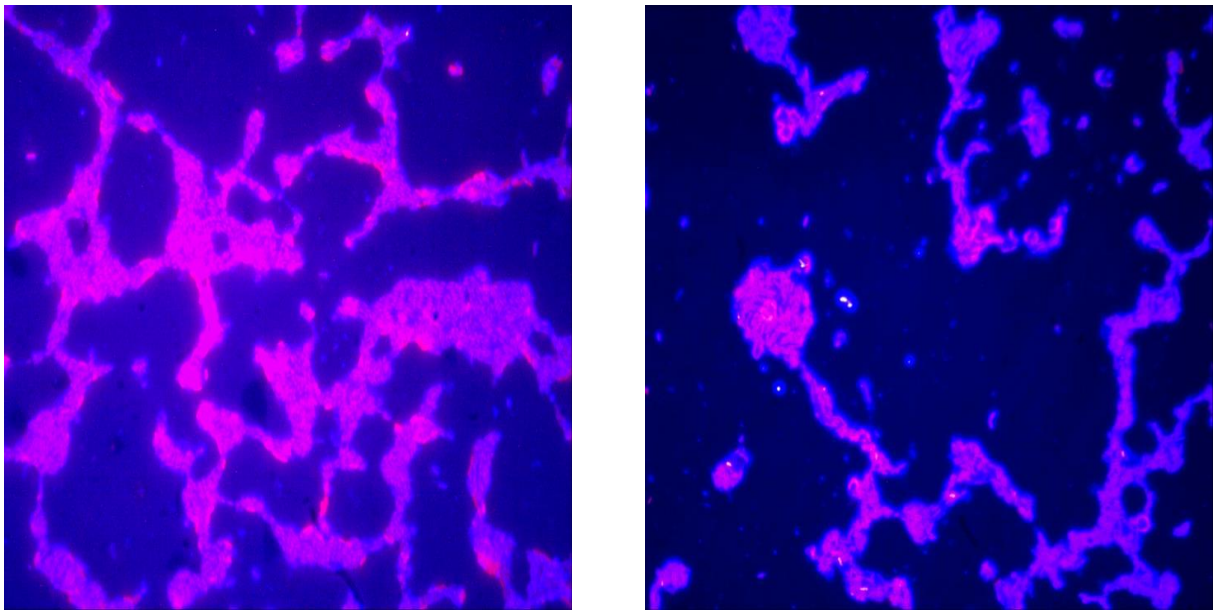
f) Ps 3 biofilm formation in (MM Eth) on glass slide after 24 and 48 h



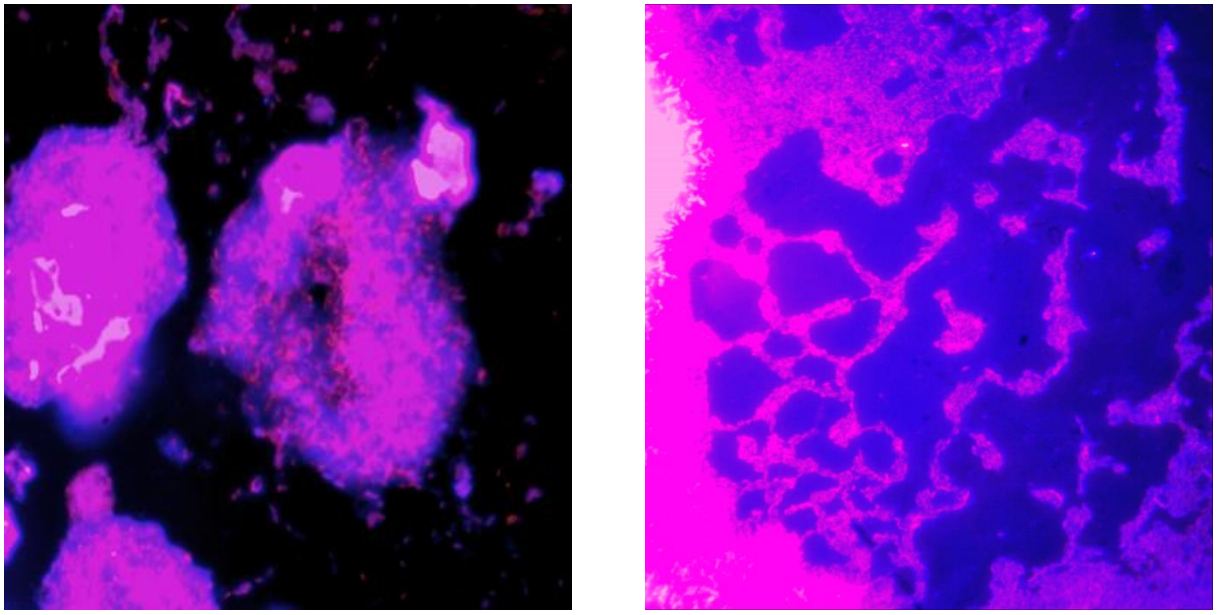
g) Ps 3 biofilm formation in (MM Glc) on plastic slide after 24 and 48 h



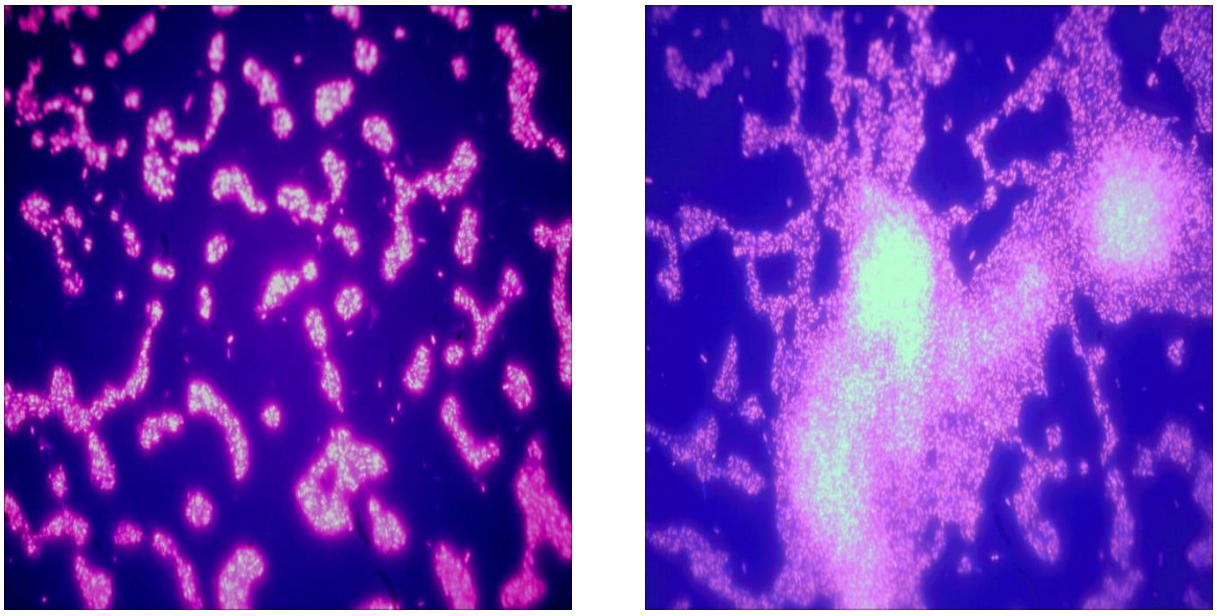
h) Ps 3 biofilm formation in (MM Eth) on plastic slide after 24 and 48 h



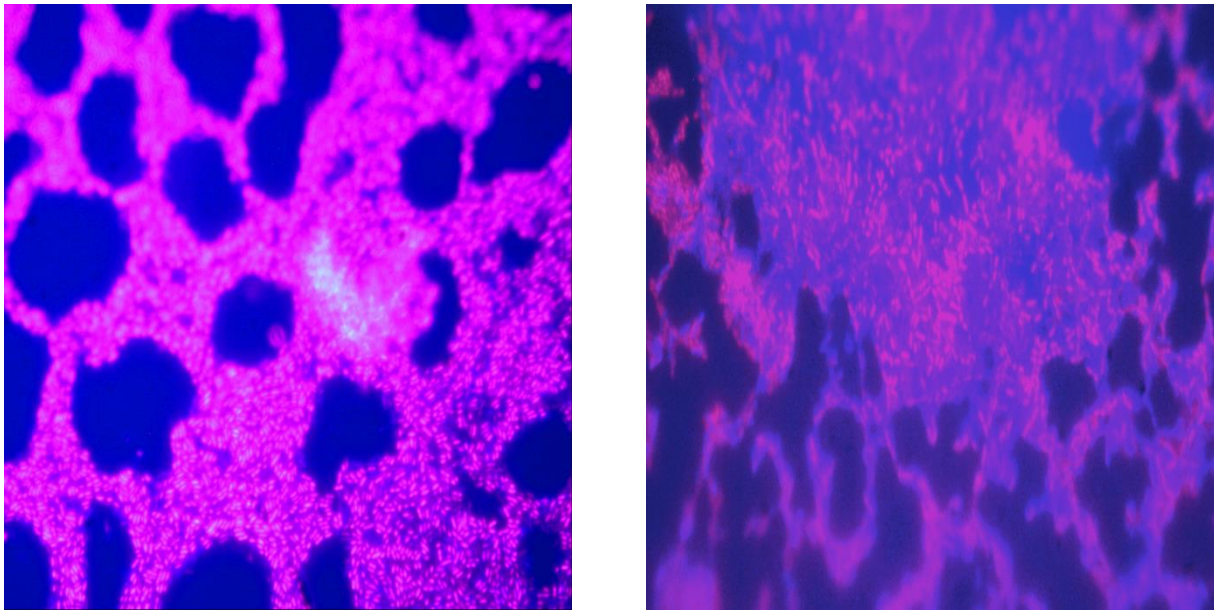
i) St 9203 biofilm formation in (MM Glc) on glass slide after 24 and 48 h



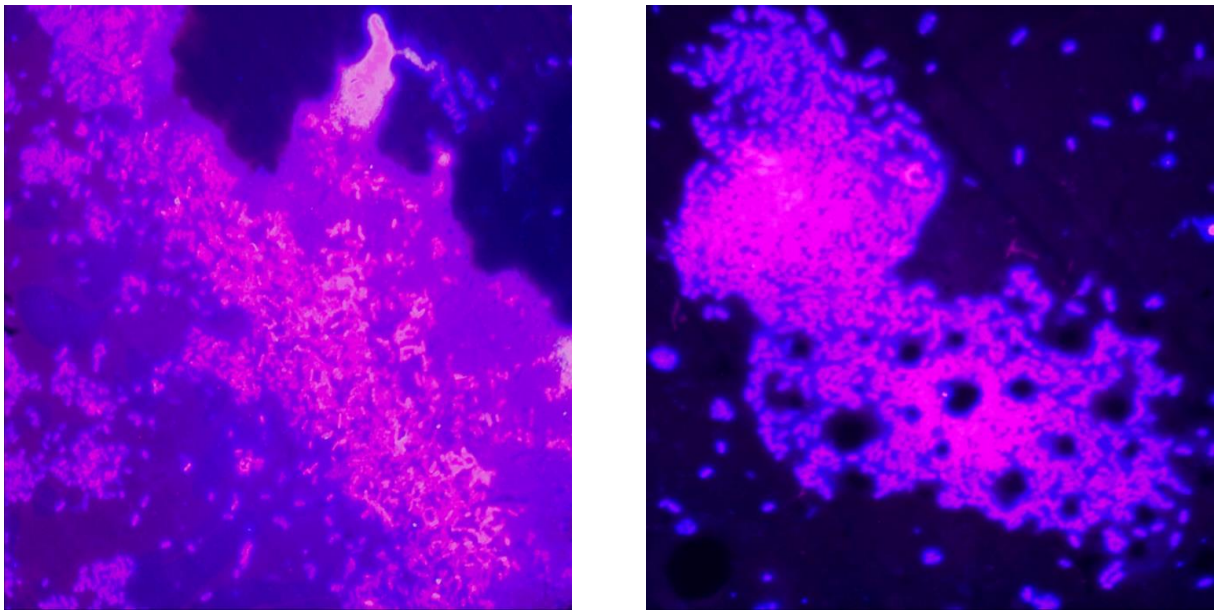
j) St 9203 biofilm formation in (MM Eth) on glass slide after 24 and 48 h



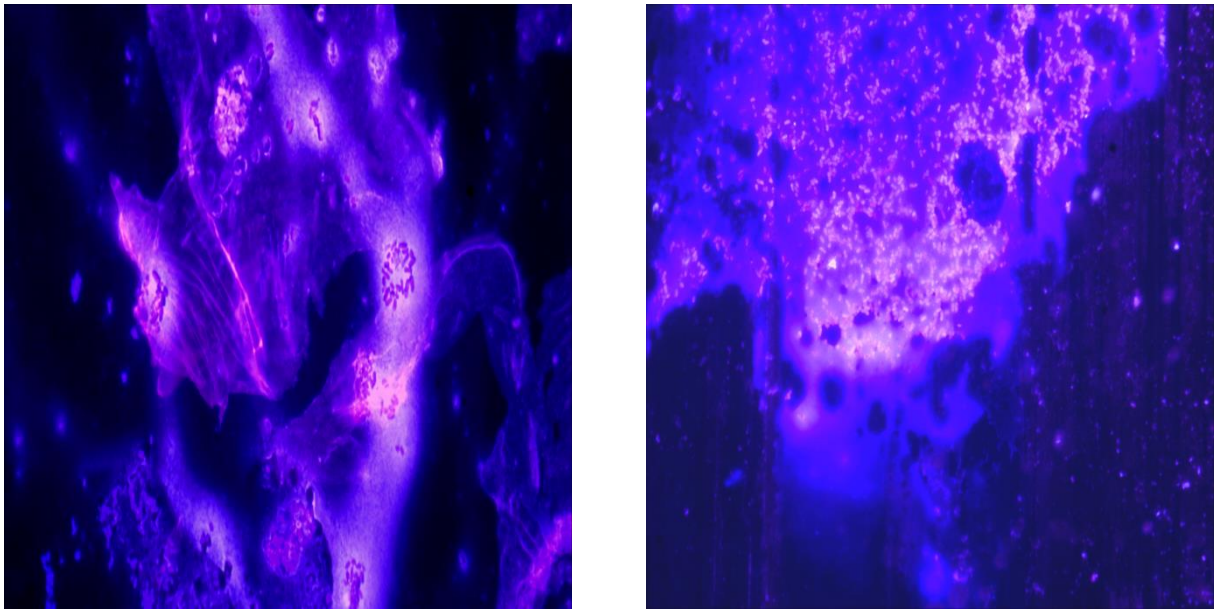
k) St 9203 biofilm formation in (MM Glc) on plastic slide after 24 and 48 h



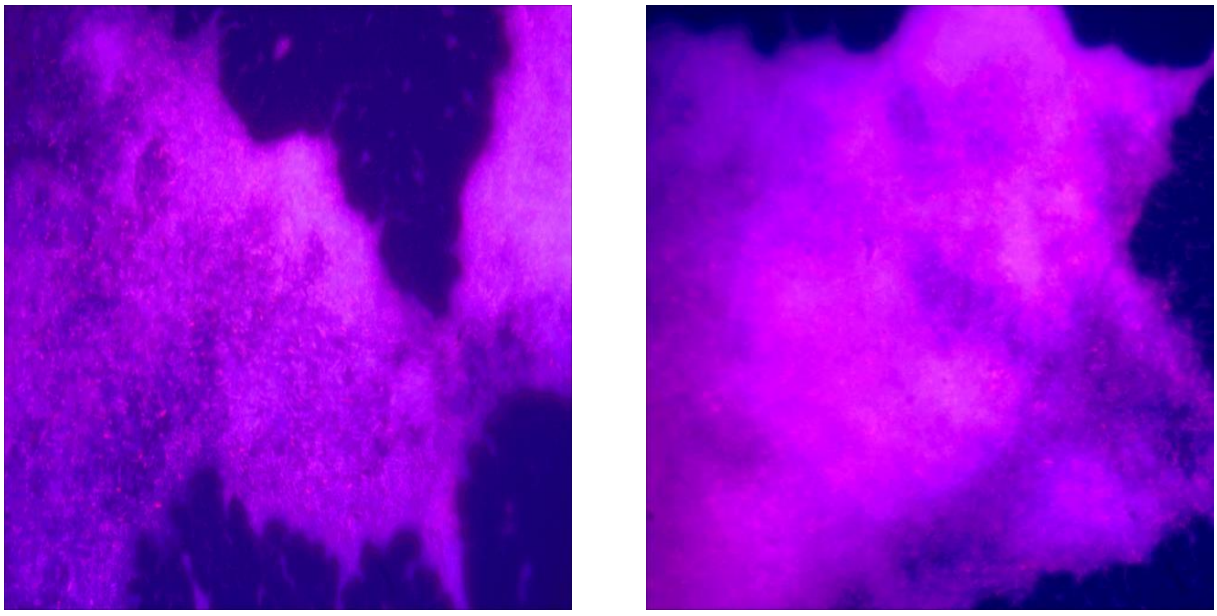
l) St 9203 biofilm formation in (MM Eth) on plastic slide after 24 and 48 h



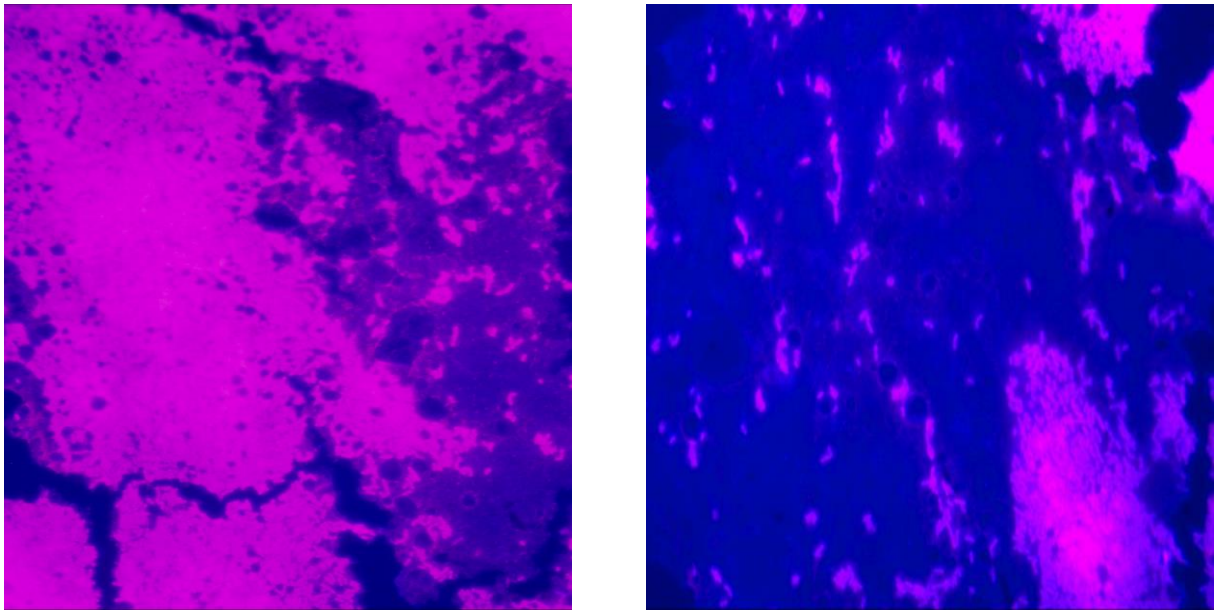
m) Ps 10421 biofilm formation in (MM Glc) on glass slide after 24 and 48 h



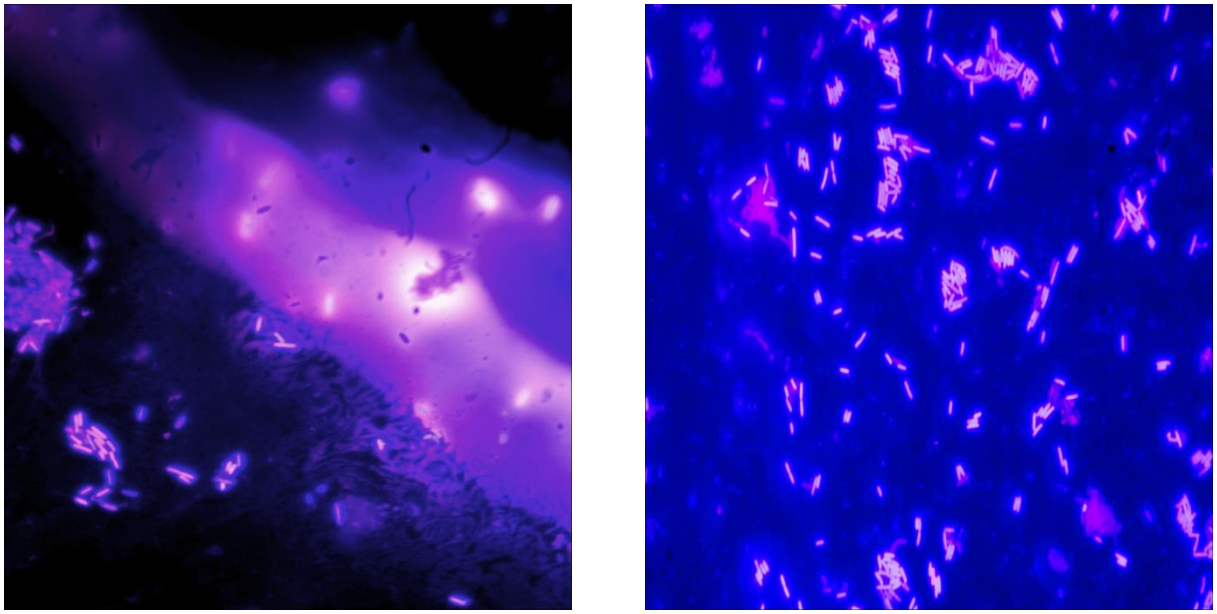
n) Ps 10421 biofilm formation in (MM Eth) on glass slide after 24 and 48 h



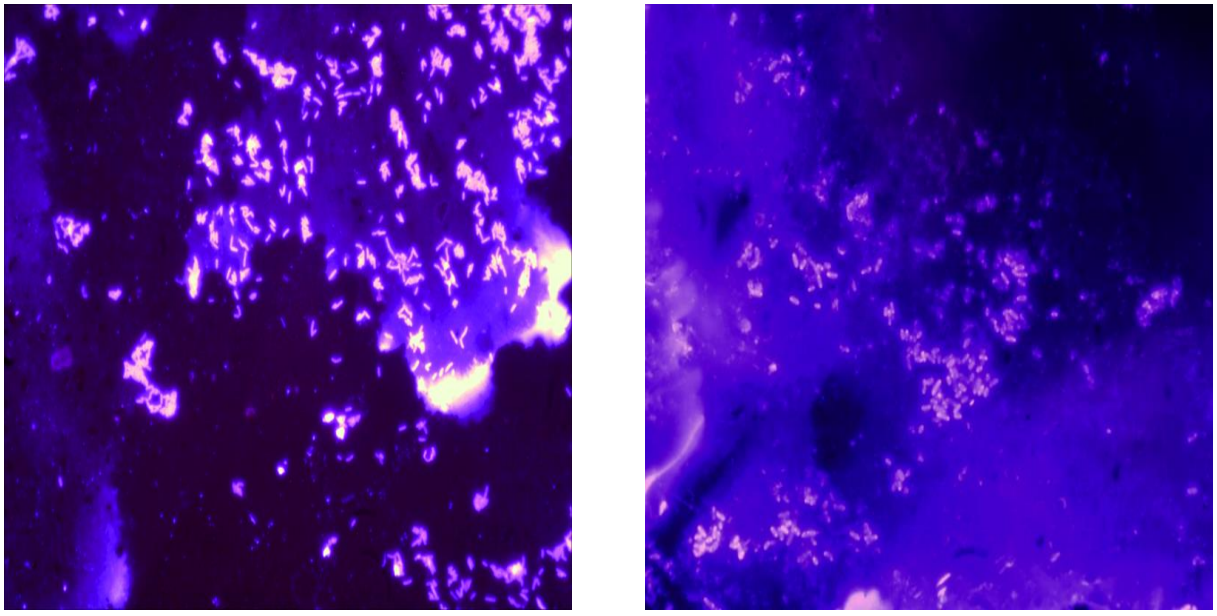
o) Ps 10421 biofilm formation in (MM Glc) on plastic slide after 24 and 48 h



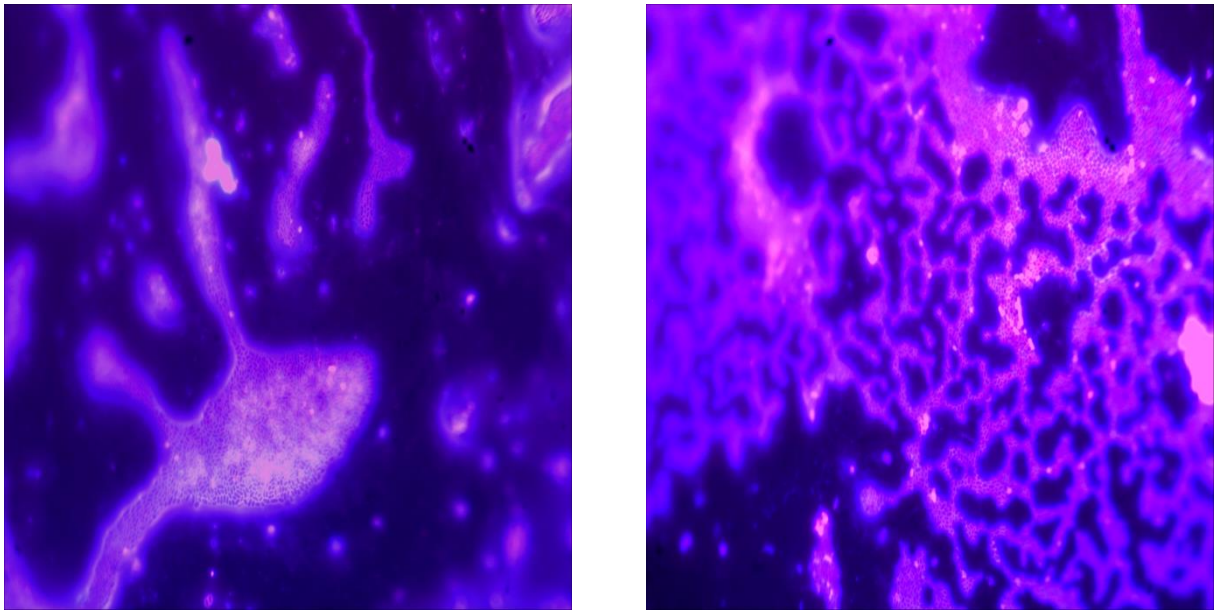
p) Ps 10421 biofilm formation in (MM Eth) on plastic slide after 24 and 48 h



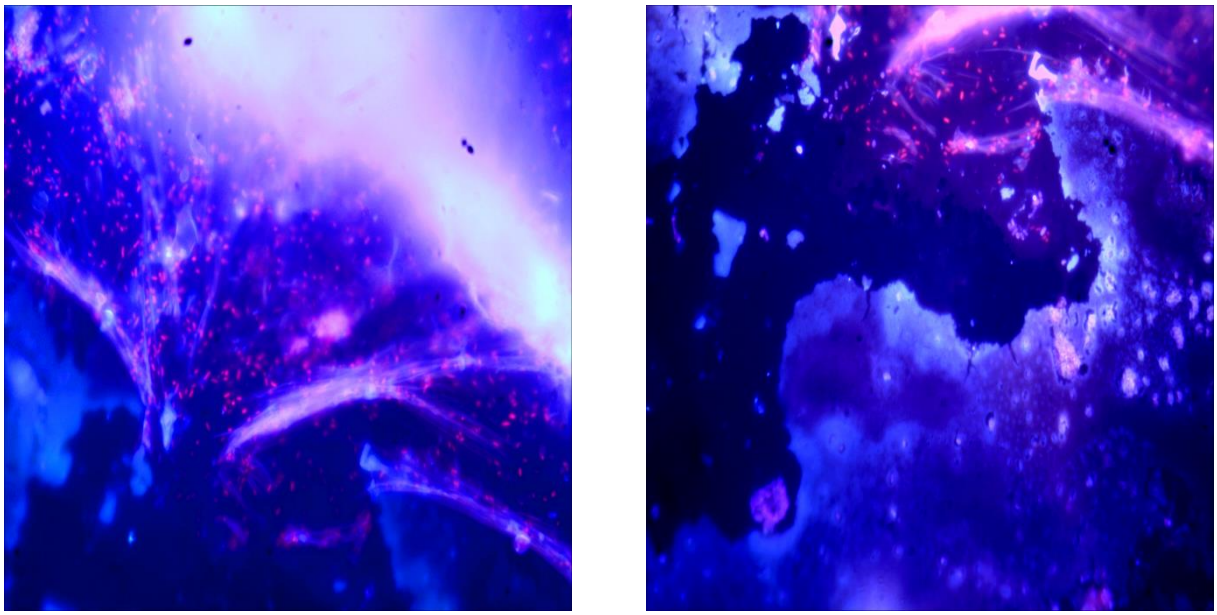
q) *C. freundii* biofilm formation in (MM Glc) on glass slide after 24 and 48 h



r) *C. freundii* biofilm formation in (MM Eth) on glass slide after 24 and 48 h



s) *C. freundii* biofilm formation in (MM Glc) on plastic slide after 24 and 48 h



t) *C. freundii* biofilm formation in (MM Eth) on plastic slide after 24 and 48 h

St: *Stenotrophomonas maltophilia*, Ps: *Pseudomonas aeruginosa* and *Citrobacter freundii*

Figure 5.16 Bacterial growths in MM Eth and MM Glc and biofilm formation on glass microscope slide and plastic slide (polystyrene) under low shear condition using DFR. a-d) St 53, e-h) Ps 3, i-l) St 9203, m-p)Ps 10421 and q-t) *C. freundii*.

5.2.9 Bacterial hydrophobicity

Bacterial hydrophobicity has been associated with bacterial adhesion by a number of authors (Jones et al., 1996, Soo, 2012, Rosenberg and Doyle, 1990, Rozgonyi et al., 1985, Ljungh and Wadström, 1982). Several methods were used to analysis the hydrophobicity as described in section 4.7, Figure 5.17 illustrates the difference between hydrophobic and hydrophilic bacteria.

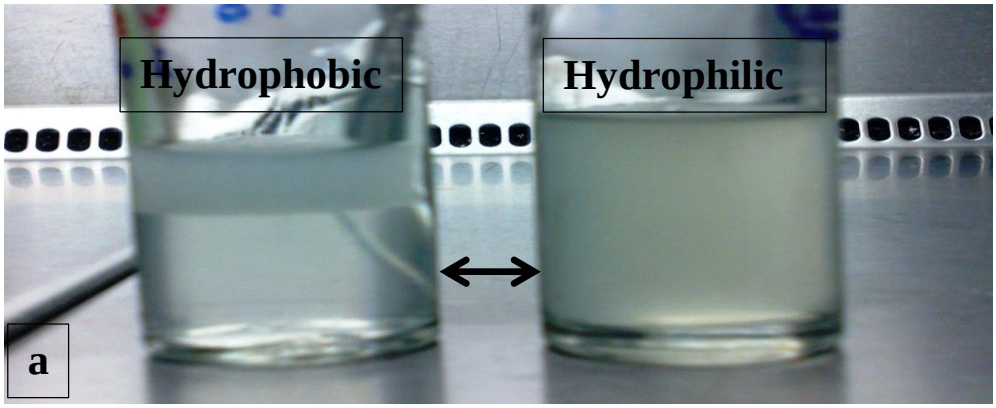


Figure 5.17 Hydrophobic and hydrophilic bacteria after adding hydrocarbon and mixing. Phase separation in hydrophobic bacteria.

5.2.9.1 MATH test

The MATH test (Section 4.7.1) was used to study the effects of several factors (effects of growth media, suspension solution, and hydrocarbons) on the hydrophobicity of the cells. Results showed that after centrifugation and washing, suspension of the cells in PBS or KCl was more effective than TSB, also the use of hexadecane generated higher estimates of hydrophobicity than Tween 80 (Figure 5.18a). Bacteria also demonstrated a greater hydrophobicity when grown in MM Eth. than when grown in MM Glc (Figure 5.18b). Overall the MATHs test for hydrophobicity was more effective when strains were grown in MM Eth, washed and resuspended in PBS or KCl, and hexadecane is used as the partitioning agent (Figure 5.18c). All strains were moderately hydrophobic with the exception of Ps 10421 which was strongly hydrophobic (Figure 5.18c)

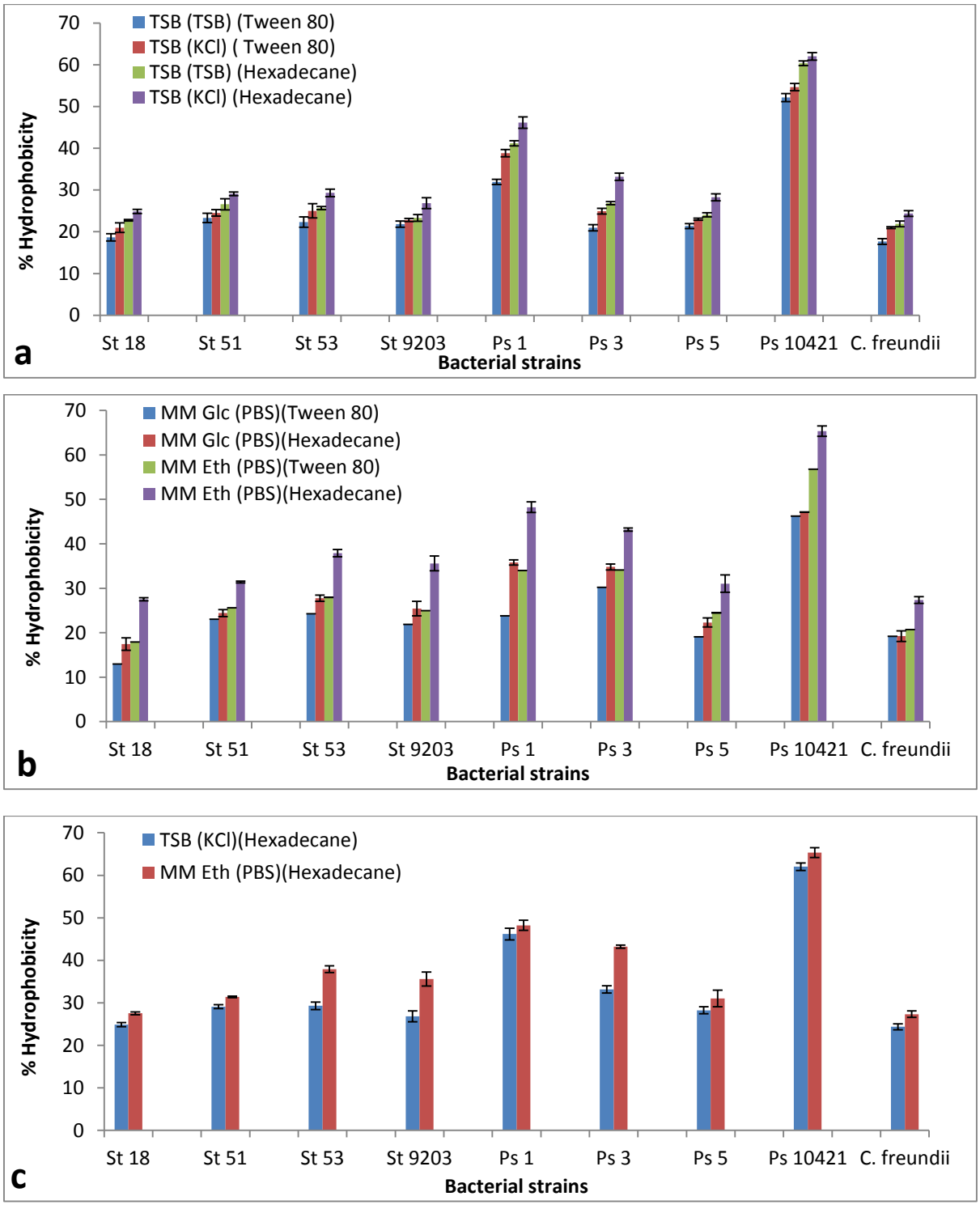


Figure 5.18 Effects of growth media, suspension solution, and hydrocarbons (Tween 80 and hexadecane) on bacterial hydrophobicity. Error bars represent standard error of the mean of two replicates. a) Bacterial growth in TSB, washed and suspended in TSB and KCl, b) Bacterial growth in MM Glc and MM Eth, washed and suspended in PBS and KCl. c) Bacterial growth in MM Eth and TSB, washed and suspended in KCl and PBS, respectively.

5.2.9.2 MATH test with salting out

This approach was used to evaluate a range of partitioning substrates i.e. dodecane, Tween 80, xylene, and hexadecane. The tests showed that after washing cells in 10mM KCl and suspension in 2mM ammonium sulphate (prepared in 10mM KCl buffer solution) hexadecane was the best of the three hydrocarbons evaluated (Figure 5.19). When hexadecane was used in the MATH test with salting out two strains (*Pseudomonas aeruginosa* 1 and 10421) were identified as strongly hydrophobic and the rest moderately hydrophobic.

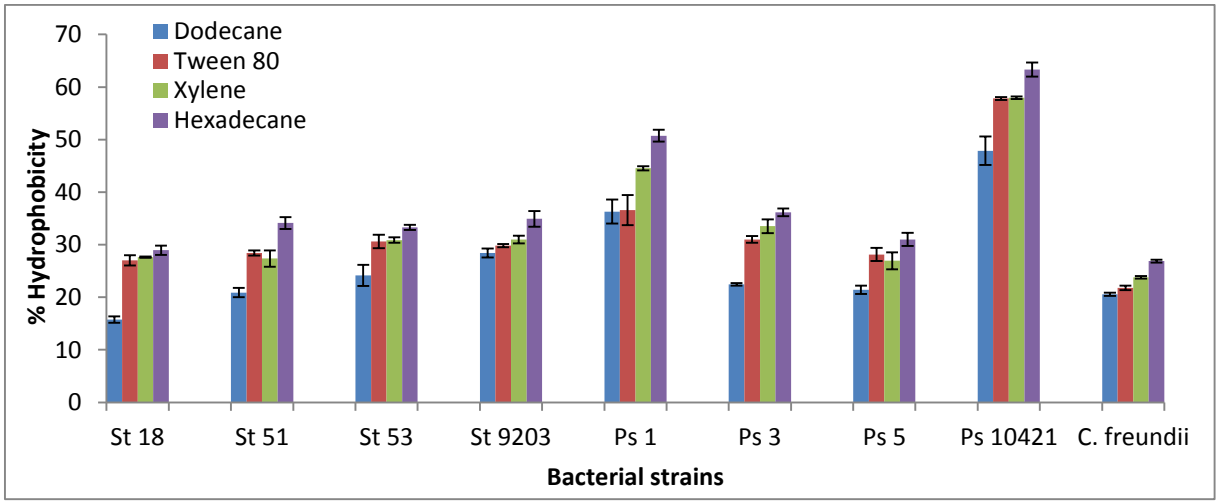
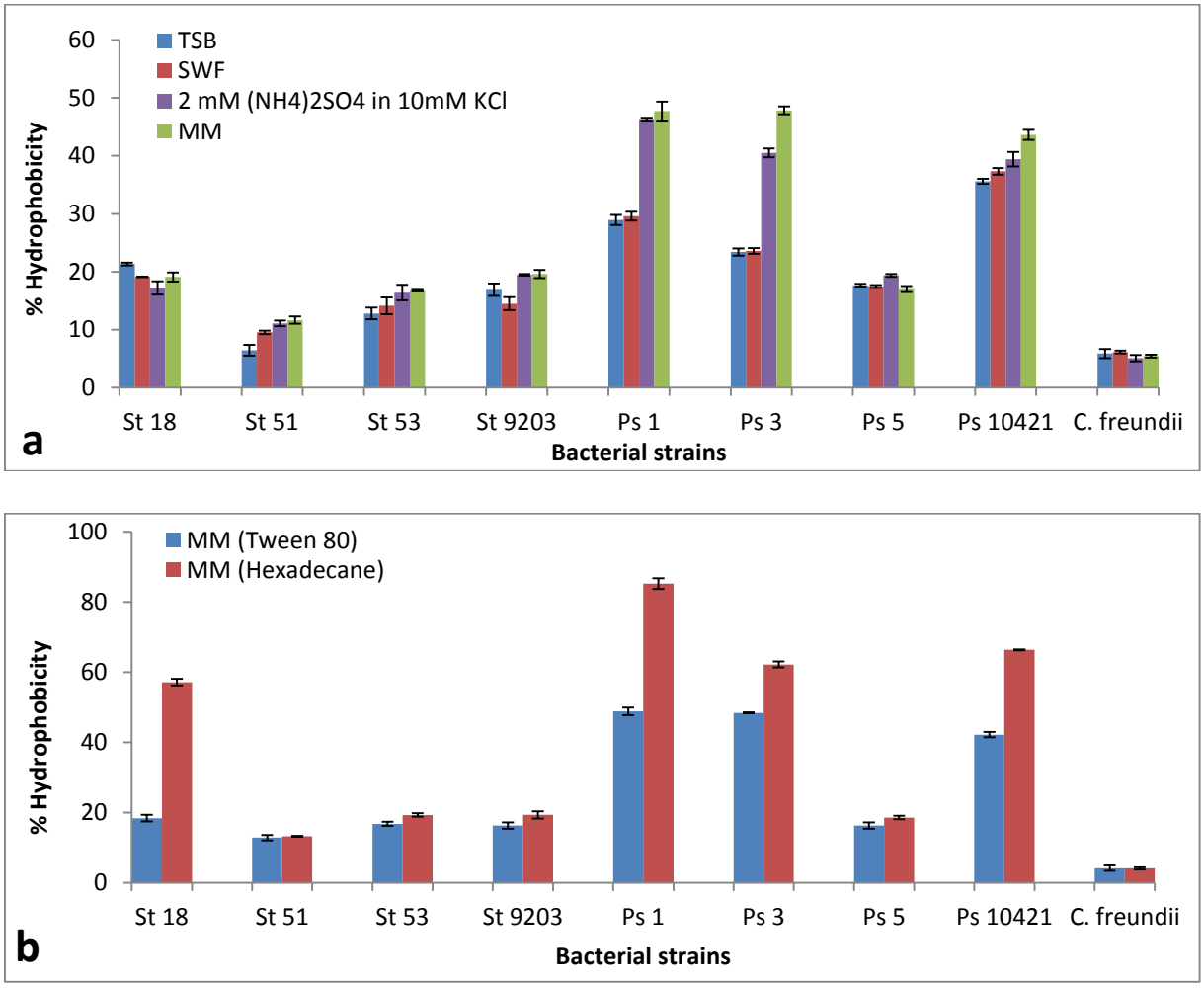


Figure 5.19 Comparing four different hydrocarbons in MATH salting out test after bacterial strains grown in TSB, washed by KCl and suspended in 2mM ammonium sulphate (prepared in 10mM potassium chloride buffer solution). Error bars represent standard error of the mean of two replicates.

5.2.9.3 The modified microbial adherence and aggregation (MAA) test

As with previous methods this approach was optimised by using several suspension agents and hydrocarbons. As shown in (Figure 5.20), suspension in mineral media without carbon source and casein (MM) led to increase bacterial adherence to the hydrocarbon than other suspension solutions (Figure 5.20a). Hexadecane was more effective than Tween 80 for the evaluation of adherence by bacterial (Figure 5.20b). The effects of MM, MM Glc. and MM Eth. as

suspension solution were also studied, in some strains *Stenotrophomonas maltophilia* 9203 and *Pseudomonas aeruginosa* 5 there wasn't any obvious difference between any of the suspension media, while in the other strains except *C. freundi*, MM led to increase adherence to hexadecane hydrocarbon and increase hydrophobicity. MM Glc. had a significant effect on bacterial adherence generating hydrophilic behaviour (0% hydrophobicity) in *Stenotrophomonas maltophilia* 51 and *C. freundii* (Figure 5.20c).



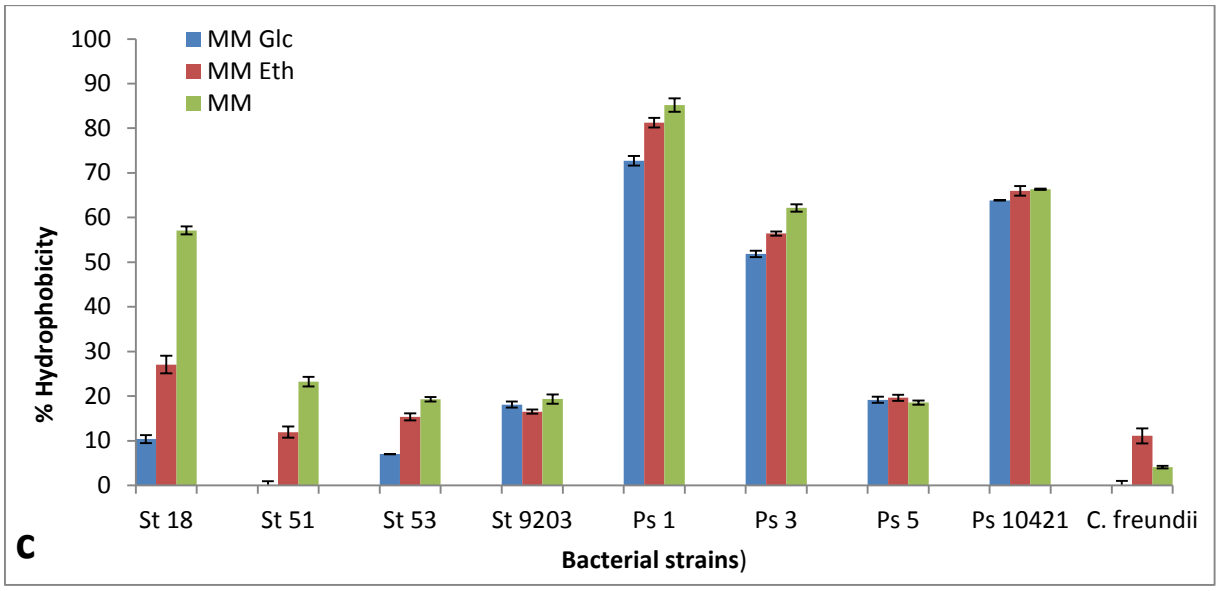


Figure 5.20 Modified MATH test, comparing suspension media and hydrocarbon. a) Effects of different suspension solution on bacterial adherence to Tween 80. b) Comparison between Tween 80 and hexadecane hydrocarbons on bacterial hydrophobicity. c) Effects of MM Glc, MM Eth and MM as a suspension on bacterial adherence to hexadecane. Error bars represent standard error of the mean of two replicates.

5.2.9.4 Bacterial hydrophobicity by SAT test

The salt aggregation test (SAT) were performed as described in Section 4.7.4. In this test positive and negative results are recorded on the basis of aggregation or clumping of bacteria in the presence of ammonium sulphate (Figure 5.21). There were no obvious difference between SAT results in bacteria grown in MM Glc or MM Eth and the suspension of TSA grown bacteria in PBS. With one exception (Ps 5 grown on TSA) all *P. aeruginosa* strains demonstrated strong hydrophobicity using this method. Results for the *S. maltophilia* were more diverse with the type strain (St 9203) and St 18 demonstrating strong hydrophobicity, St 53 giving moderate results and St 51 being weakly hydrophobic. *C. freundii* was also weakly hydrophobic under all test conditions (Table 5.5).

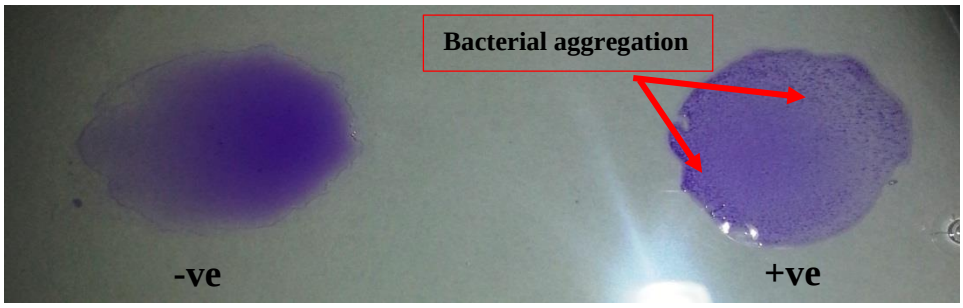


Figure 5.21 SAT test represents positive and negative reaction of bacterial aggregation.

Table 5.5 Hydrophobicity intensity comparisons between bacterial strains grow in MM Glc, MM Eth and bacterial suspension from TSA culture.

Bacterial strains	MM Glc	MM Eth	TSA
St 18	**0.75 (S)**	**0.75 (S)**	**0.75 (S)**
St 51	**3 (W)**	**3 (W)**	**3 (W)**
St 53	**2 (M)**	**2 (M)**	**2 (M)**
St 9203	**0.007 (S)**	**0.007 (S)**	**0.007 (S)**
Ps 1	**0.007 (S)**	**0.007 (S)**	**0.007 (S)**
Ps 3	**0.007 (S)**	**0.007 (S)**	**0.007 (S)**
Ps 5	**0.007 (S)**	**0.007 (S)**	**0.007 (S)**
Ps 10421	**0.007 (S)**	**0.007 (S)**	**0.007 (S)**
C. freundii	**4 (W)**	**3 (W)**	**4 (W)**

≤1 = (S) Strong hydrophobic, >1-≤2 = (M) Moderate hydrophobic, >2-≤4 = (W) Weak hydrophobic, >4= (H) Hydrophilic

5.2.10 Comparison of hydrophobicity results with biofilm formation

Having compiled all the different approaches to hydrophobicity alongside the biofilm formation data it is clear that there is no consistency between the hydrophobicity tests employed and that there is limited correlation between these tests and the ability of the bacteria studied to form biofilms (Table 5.6). Attempts to make correlations between hydrophobicity and biofilm formation are complicated by the fact that across the bacteria studied growth media had a major impact on biofilm formation (Table 5.6) particularly in the case of *S. maltophilia*. Except biofilm formation in (MM Glc and MM Eth) and SAT test (MM Glc and Eth) were showed very high correlation.

Table 5.6 Comparison and relation bacterial hydrophobicity tests and biofilm formation on 96 well plates using different methods and media.

Bacterial strains	Biofilm formation 96 well plate (different media)					Hydrophobicity test (different methods)			
	TSB	SWF	MM Glc	MM Gly	MM Eth	MATH test	MATH test Salting out	Modified MAA	SAT (MM Eth or Glc)
St 18	**N**	**W**	**M**	**M**	**S**	**M**	**M**	**S**	**S**
St 51	**S**	**S**	**M**	**W**	**W**	**M**	**M**	**M**	**W**
St 53	**S**	**S**	**M**	**S**	**S**	**M**	**M**	**M**	**M**
St 9203	**S**	**S**	**S**	**S**	**S**	**M**	**M**	**H**	**S**
Ps 1	**S**	**S**	**S**	**S**	**S**	**M**	**S**	**S**	**S**
Ps 3	**S**	**S**	**S**	**S**	**S**	**M**	**M**	**S**	**S**
Ps 5	**W**	**W**	**S**	**S**	**S**	**M**	**M**	**M**	**S**
Ps 10421	**S**	**S**	**S**	**S**	**S**	**S**	**S**	**S**	**S**
C. freundii	**W**	**W**	**W**	**S**	**M**	**M**	**M**	**H**	**W**

H: Hydrophilic, N: No Biofilm W: Weak Biofilm or Hydrophobic, M: Moderate Biofilm or Hydrophobic and S: Strong Biofilm or Hydrophobic.

5.2.11 Discussion

After confirming the identification of the bacterial strains under investigation (Section 5.1) the ability of these bacteria to form biofilms was investigated. The process of bacterial biofilm formation is influenced by various factors including nutrients level, temperature, incubation period, culture concentration. etc. (O'Toole et al., 2000). The microtiter plate assay employed in this study is a popular approach for the study of biofilm formation (O'Toole et al., 2000). The use of this approach in this study showed that all bacterial strains produced biofilms in all media (TSB, SWF, MM Glc, MM Gly and MM Eth) with the exception of St 18 that failed to produce biofilm in TSB. In general the response in biofilm formation seen with increased ethanol concentration was similar to that observed with glucose and glycerol (Figures 5.8 to 5.10), which is an initial increase followed by a decrease at the higher concentrations. There were exceptions however, in the case of St 51 and 9203 biofilm formation declined with increasing ethanol concentration, which contrasts with Ps 10421 where biofilm formation increased across all ethanol concentrations (Figure 5.10). The strength of these biofilms varied, however, from weak to strong (Figure 5.11). This approach also demonstrated that biofilm formation by the isolates under investigation was affected by inoculums size, nutrient concentration and composition (Figure 5.6). In most studies using the 96 well plate method for biofilm formation no data on the inoculum size is reported e.g. biofilm formation by Gram negative bacteria (Zubair et al., 2011), or *Stenotrophomonas maltophilia* (Huang et al., 2006), or *Pseudomonas aeruginosa* (Head and Yu, 2004).

As demonstrated in this study, the strength of the biofilm rises with increasing initial inoculum size which agreed with previous studies on *Staphylococci* (Stepanović et al., 2007, Stepanović et al., 2003). The impact of inoculum size on biofilm formation may be in part explained by quorum sensing, which has been shown to be a key process in biofilm formation in *Pseudomonas aeruginosa* (Sifri,

2008). This observation indicates that an optimisation phase is needed prior to the presentation of results from this approach especially when comparing several bacteria. The strains used here also changed their biofilm formation behaviour in response to nutrient concentrations (carbon and nitrogen source) and the type of media being used (Figure 5.7-5.11) this observation agreed with previous studies on *Citrobacter* spp. (Allan et al., 2002) and *Escherichia coli* (Reisner et al., 2006, Davey and O'toole, 2000).

The CRA assay was not successful in identifying biofilm formation, or the strength of that formation with a third of strains (33%) failing to produce biofilms (Figure 5.14). This finding shows that the CRA test produces a high number of false negatives. This observation has also been made in a number of previous studies (Mathur et al., 2006, Melo et al., 2013, Hassan et al., 2011). Consequently the CRA method was not used in the remainder of the study.

When the bacterial strains were screened for pellicle formation only St 18 failed to produce a pellicle (Table 5.4) indicating that pellicle formation was a common capability of the Gram negative wound and mucous membrane associated pathogens. The ubiquity of pellicle formation amongst Gram negative pathogens has been reported in previous studies (Veeranagouda et al., 2011, Zogaj et al., 2003).

The major risk factor for *Stenotrophomonas maltophilia, Pseudomonas aeruginosa* and *Citrobacter freundii* infections in hospitalized patients is the implantation of medical devices such as urinary tract catheters, central venous catheters and prosthetic heart valves, and wound infections (Hill et al., 2010, Harrison-Balestra et al., 2003, Di Bonaventura et al., 2004, Chen et al., 2013, Song et al., 2013). Evaluation of the biofilm formation capability by *Pseudomonas aeruginosa, Stenotrophomonas maltophilia* and *Citrobacter freundii* in this study indicated that these bacteria possess the capacity for biofilm formation on glass and plastic surfaces (Figure 5.16) as reported by (Jucker et al.,

1996, Huang et al., 2006, Tran et al., 2014, Pereira et al., 2010). This indicates that these strains are able to adhere to implants and surfaces in healthcare environments and maintain a level of contamination through the formation of biofilms.

Attachment to surface such as plastics has been associated with microbial surface hydrophobicity. Bacteria with the highest hydrophobicity usually show maximum capability of microbial adhesion on both hydrophobic and hydrophilic support surfaces (Liu et al., 2004). However, a screening of the various hydrophobicity tests available (Table 5.6) indicated that there is an issue with the reproducibility between methods and the correlation with biofilm formation. These issues have been raised by previous studies such as that of Krepsky et al. (2003) and Mattos-Guaraldi et al. (1999). Selecting proper method for hydrophobicity may be consider key point to make correlation to biofilm formation. Among several methods for hydrophobicity only SAT test (MM Eth or MM Glc) were showed high correlation with biofilm formation in MM Eth and MM Glc. In view of this the use of hydrophilic and hydrophobic surfaces in drip flow biofilm reactors (see section 5.2.8) may be provide further approach for the investigation of surface hydrophobicity.

5.2.12 Key finding

According to parameters were used in this study:

- The optimum inoculum size for the screening of biofilm formation is 1-5x10^7 cells/ml;
- The optimum carbon source concentration for the screening of biofilm formation is 10 g/l of glucose (Except for Pseudomonas which is 20g/l), 10 g/l of glycerol and 13.4 ml/l (10.57 g/l) of ethanol with 1g/l casein as a nitrogen source;
- Bacteria can grow and form biofilms (weak to strong) on a wide range of carbon source such as glucose, glycerol and ethanol;
- Bacterial growth does not always lead to biofilm formation;
- The Congo red agar test underestimates the ability to form biofilms amongst bacteria;
- Pellicle formation is effected by the type of media
- Pellicle formation is a common capability of Gram negative pathogens;
- Bacterial strains (St 53, 9203, Ps 3, 10421 and *C. freundii*) produce biofilms on both glass and polystyrene under low shear condition when grown on MM Glc. and MM Eth. Media;
- Most of the approaches available to determine bacterial surface hydrophobicity are unreliable and fail to correlate with biofilm formation except (SAT test).

5.3 EPS production and characterisation

This chapter focuses on the generation of EPS by those bacteria identified as the strongest biofilm producers (See section 5.2). It also covers media development and the characterisation of the EPS produced. The research reported on media development and associated growth and EPS production was carried out on *Stenotrophomonas maltophilia* 53 and *Pseudomonas aeruginosa* 3.

5.3.1 Media selection

A range of media were investigated for their ability to support EPS investigations. In order to do this media were processed via the EPS extraction and analysis procedure outlined in section 4.12 before they were used to grow bacteria. As shown in (Figure 5.22), the anomeric region of the proton NMR showed that TSB and LB broth both contained EPS equivalent components that would have interfered with the analysis of bacterial EPS. In addition to the presence of significant amounts of tryptone in both media, TSB also contained soybean meal while LB contained yeast extract. SWF on the other hand did not generate any EPS signals during proton NMR analysis, however it did generate a large amount of crude substance (3.55 g/l) that could mask EPS production by the bacteria under investigation. Analysis of this crude extract for carbohydrates and proteins indicated that it contained 9.3% w/w carbohydrate and 28.6 % w/w protein (Table 5.7). In addition, monomer analysis of the SWF crude substance (EPS) showed the presence of several sugars (Figure 5.22). In view of these results TSB, LB and SWF were rejected as candidate media for EPS characterisation studies.

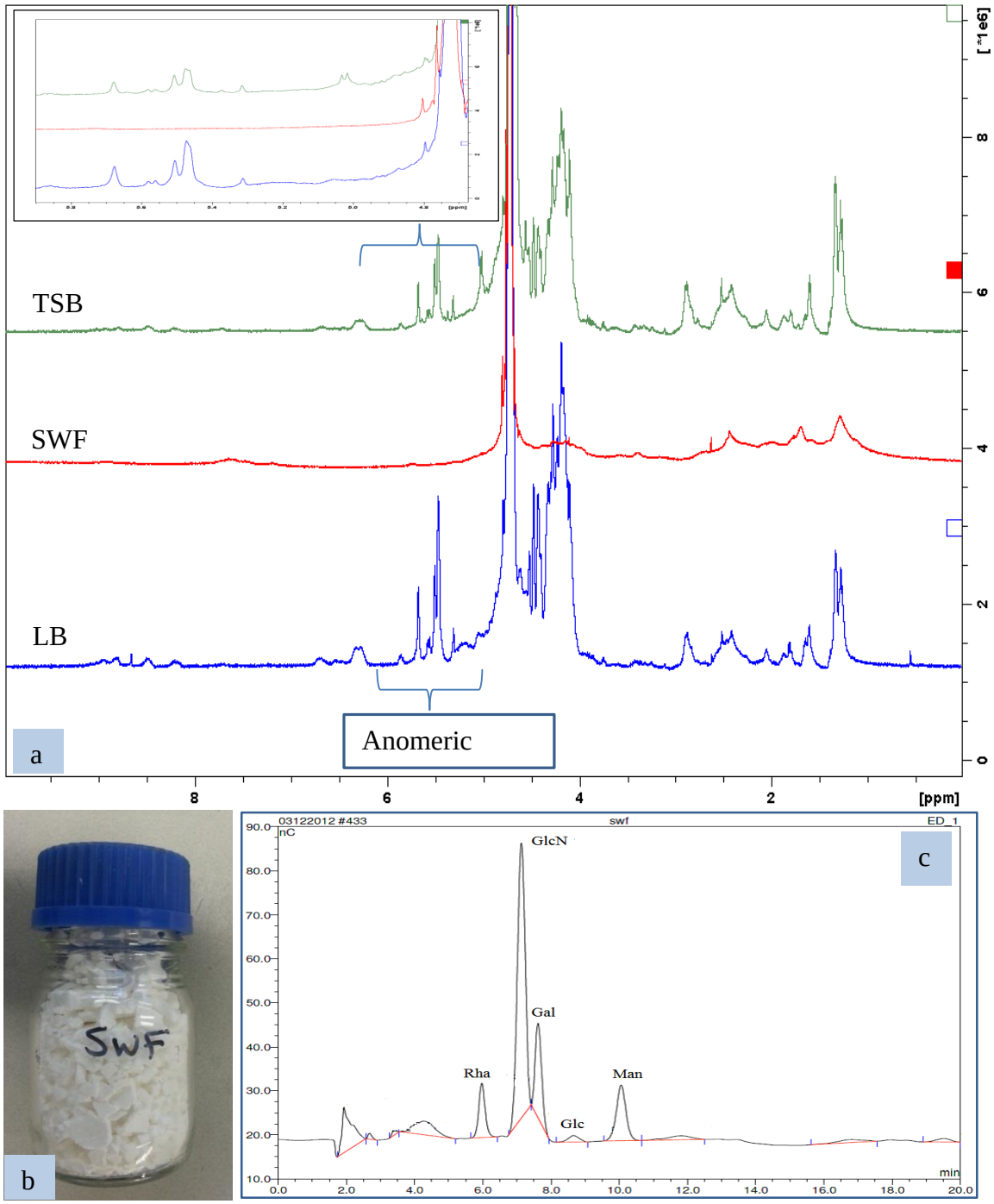


Figure 5.22 NMR analysis and monomer analysis of different media. a) Proton NMR analysis and monomer analysis of different media (TSB, SWF and LB) showing the signal spectra of EPS in the anomeric region in TSB and LB broth media b) Crude substance (EPS) extracted from SWF medium c) monomer analysis of EPS extracted from SWF medium shows present of Rhamnose (Rha), Glucosamine (GlcN), Galactose (Gal), Glucose (Glc) and Mannose (Man).

5.3.2 Development of EPS free media and growth optimisation

As described in Chapter 6, several mineral media were prepared and optimised for biofilm formation as well as EPS production and characterisation. Mineral media with different carbon source were investigated for compounds that would generate NMR signals in the anomeric (EPS) region. In order to do this 500ml of each media was processed for EPS analysis (Section 4.12) with and without TCA treatment. The resulting crude substance ranged from 1 to 2 mg and the associated proton NMR analysis showed no interfering peaks in the anomeric region (Figure 5.23). The extracted crude substance was also checked for carbohydrate and protein content with the exception of MM Glc all media extracts were soluble carbohydrate and protein free. In the case of MM Glc, 0.001% w/w protein was detected (Table 5.7).

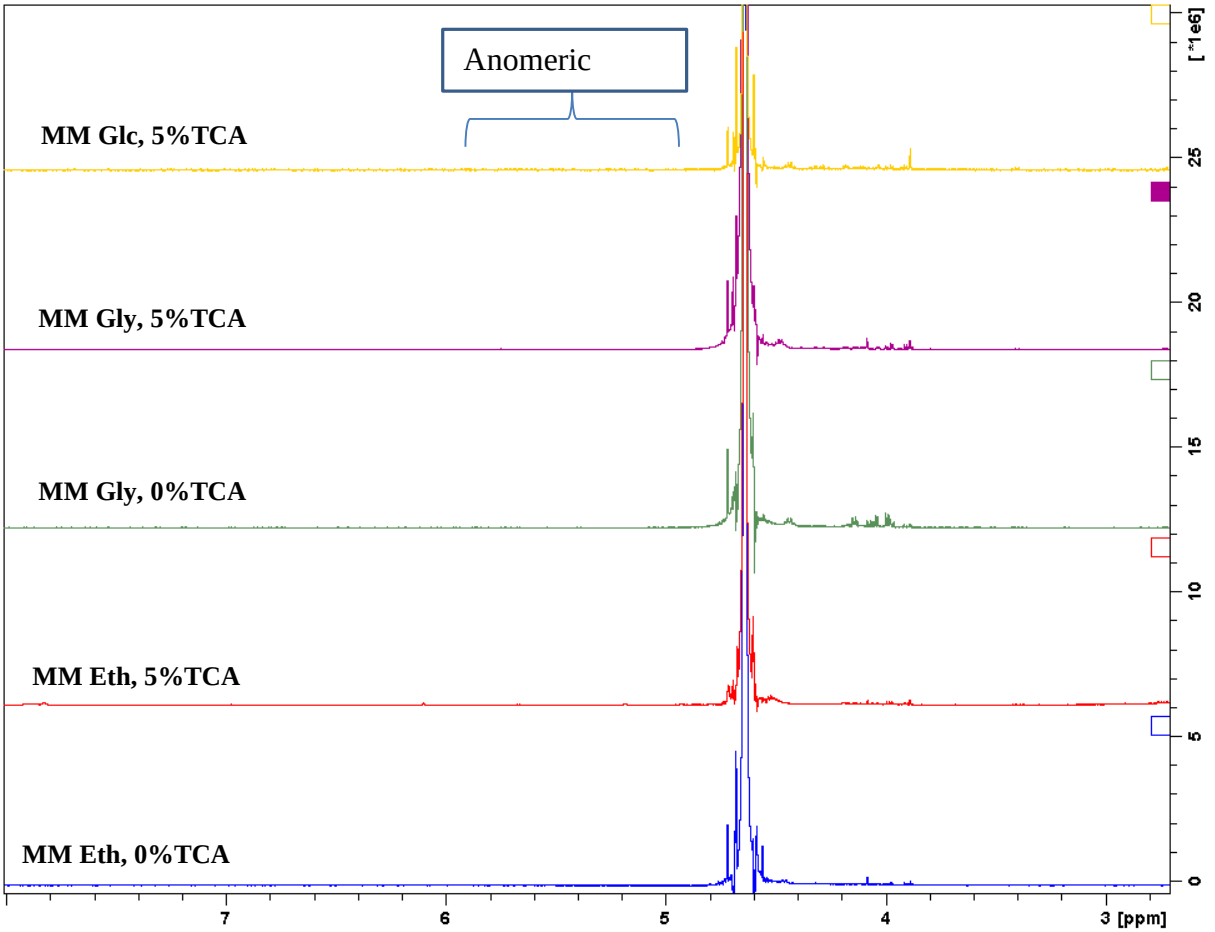


Figure 5.23 Proton NMR analysis of different modified carbon source mineral media treated with 0 and 5% TCA shows free EPS spectra in the anomeric region.

Table 5.7 Carbohydrate and protein analysis of the crude substance extracted from several media (without bacterial inoculation).

Media	TCA%	Carbohydrate content (%)	Protein content (%)
***TSB**	5	-	-
SWF	5	9.3	28.6
***LB**	5	-	-
MM Glc	5	0	0.001
MM Gly	5	0	0
MM Gly	0	0	0
MM Eth	5	0	0
MM Eth	0	0	0

*Carbohydrate and protein test were not performed

EPS production was optimised on the basis of inoculum size, incubation temperature, incubation time and the concentration of TCA used in the EPS extraction process (TCA is employed to remove proteins). When bacteria were grown on MM Eth, the total carbohydrate concentration was equal to the polysaccharide (EPS) concentration, however when grown on MM Glc, the polysaccharide (EPS) concentration was calculated by subtracting the total carbohydrate concentration from the glucose concentration.

5.3.3 *Stenotrophomonas maltophilia* 53 EPS production optimisation.

When grown at 37˚C for more than 80 h in MM Glc broth using a 10^4 CFU/ml as starting concentration. St 53 demonstrated poor growth with >60% of glucose remaining unused and approximately 74.07 mg/l of polysaccharide (EPS) produced (Figure 5.24a). When the initial inoculum was increased to 10^7 CFU/ml and incubated at 37 and 30˚C for 80h all the glucose was metabolised and approximately 558 and 948 mg/l polysaccharide was produced (Figure 5.24 b & c).

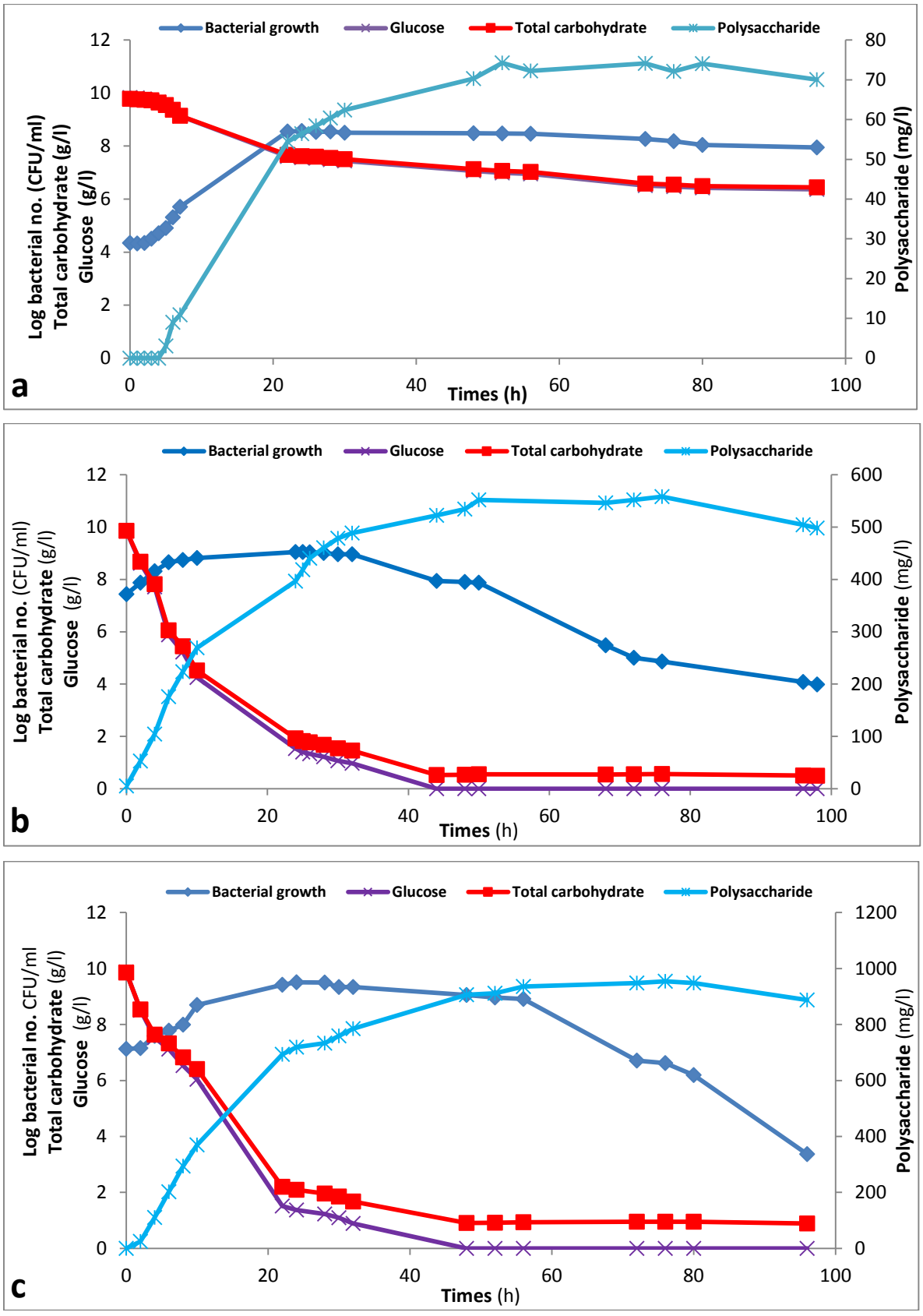
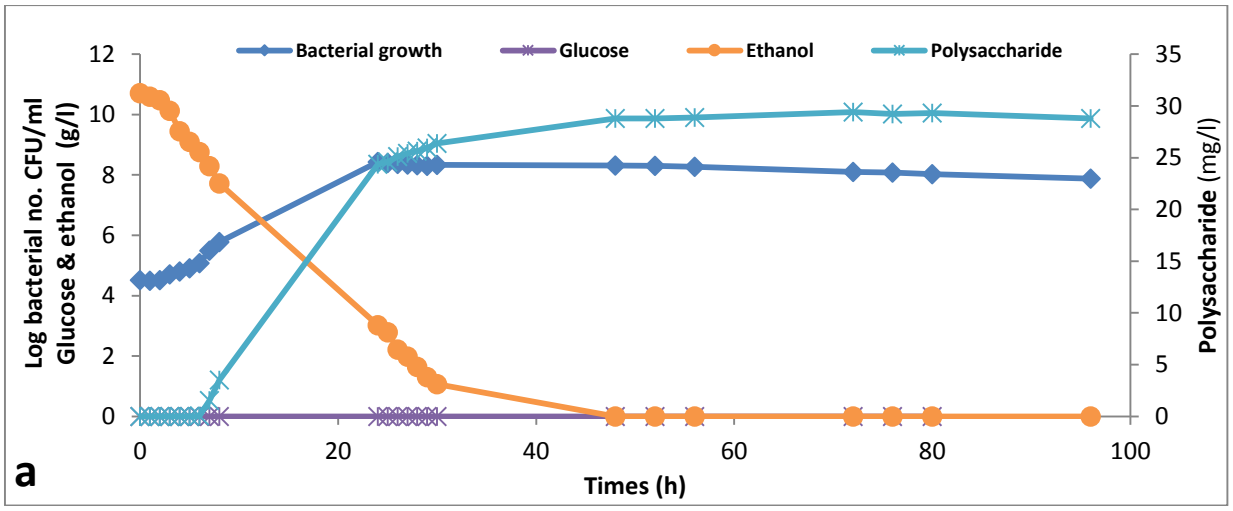
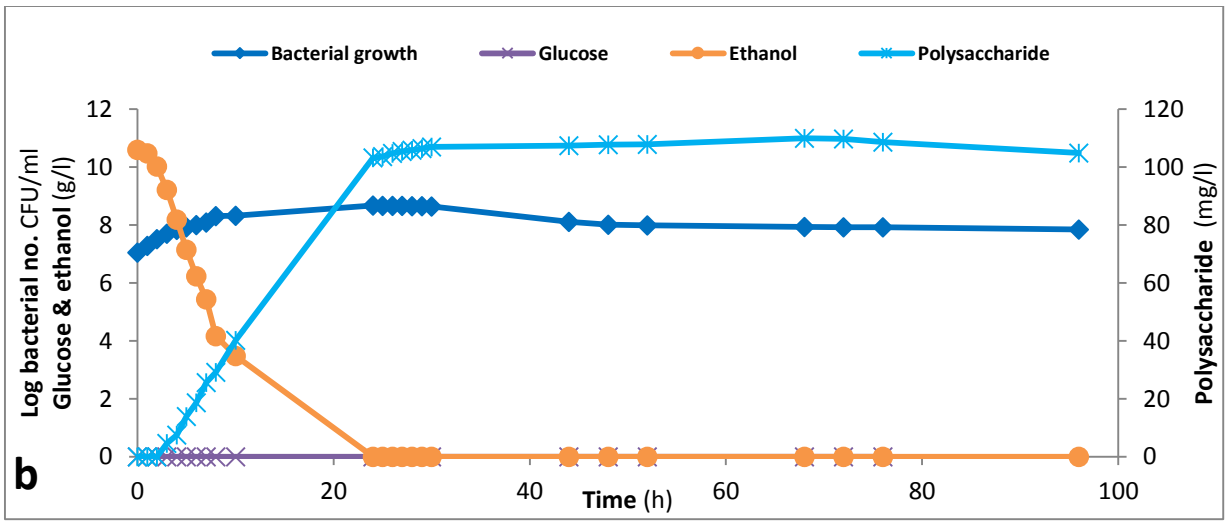


Figure 5.24 Glucose utilisation and polysaccharide formation during growth of *Stenotrophomonas maltophilia* 53 on MM Glc (10g/l) at different inoculum culture size and temperature a) 10^4 CFU /ml, 37˚C, b) 10^7 CFU /ml, 37˚C, c) 10^7 CFU /ml, 30˚C.

When grown on MM Eth, St 53 (37˚C, 80 h) generated approximately 29.4 mg/l polysaccharide during growth when a 10^4 CFU /ml starting inoculum was employed. Increasing the inoculum size to 10^7CFU/ml resulted in an increase of polysaccharide production to 109 mg/l after 72-96 h at 37°C (Figure 5.25 a & b). Using the same starting inoculum but a reducing the incubation temperature to 30°C resulted in 125 mg/l polysaccharides being produced after 80 h of incubation which increased to 129.8 mg/l after 96 h of incubation (Figure 5.25 c). Under all condition (10^4 or 10^7 CFU/ml starting inoculum and incubation temperature at 30 or 37˚C) ethanol were completely consumed by the bacteria within 48 h.





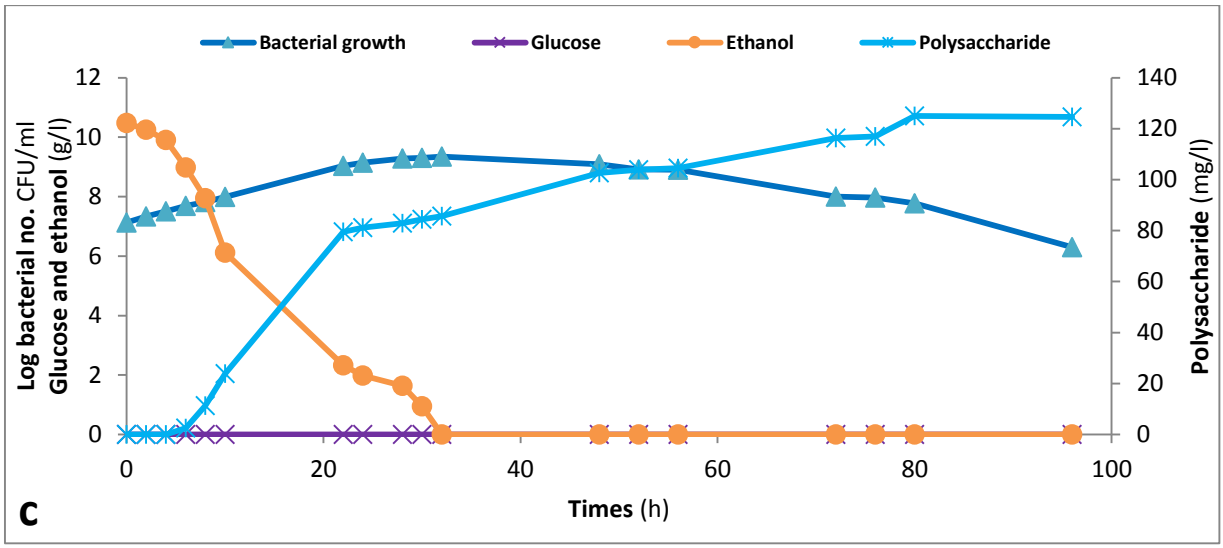
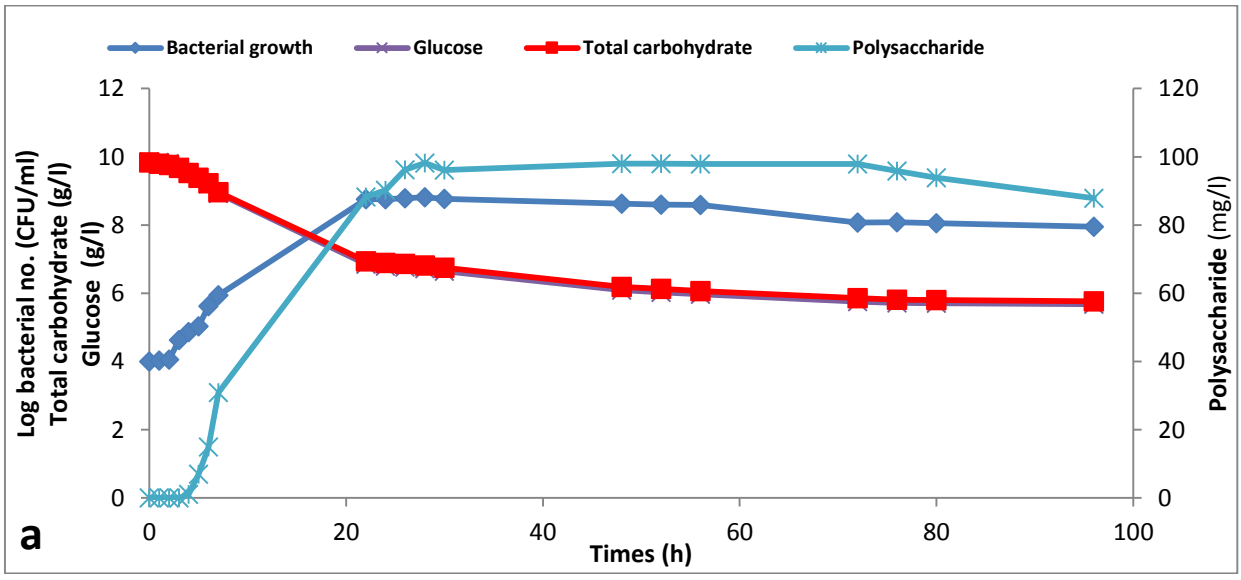


Figure 5.25 Ethanol utilisation and polysaccharide formation during growth of St 53 on MM Eth (10.57 g/l) at different inoculum culture size and temperature a) 10^4 CFU /ml, 37˚C, b) 10^7 CFU /ml, 37˚C, c) 10^7 CFU /ml, 30˚C.

5.3.4 *Pseudomonas aeruginosa* 3 EPS production optimisation

Ps 3 also produced more polysaccharides (594.0 mg/l) at 30˚C using a 10^7 CFU/ml inoculum size (Figure 5.26c) than when grown at 37˚C using either a 10^4 (97.9 mg/l) or 10^7 CFU/ml (498.2 mg/l) (Figure 5.26 a & b). Approximately 55% of the available glucose remained un-degraded after 96 h of incubation when an initial inoculum of 10^4 CFU/ml was used.



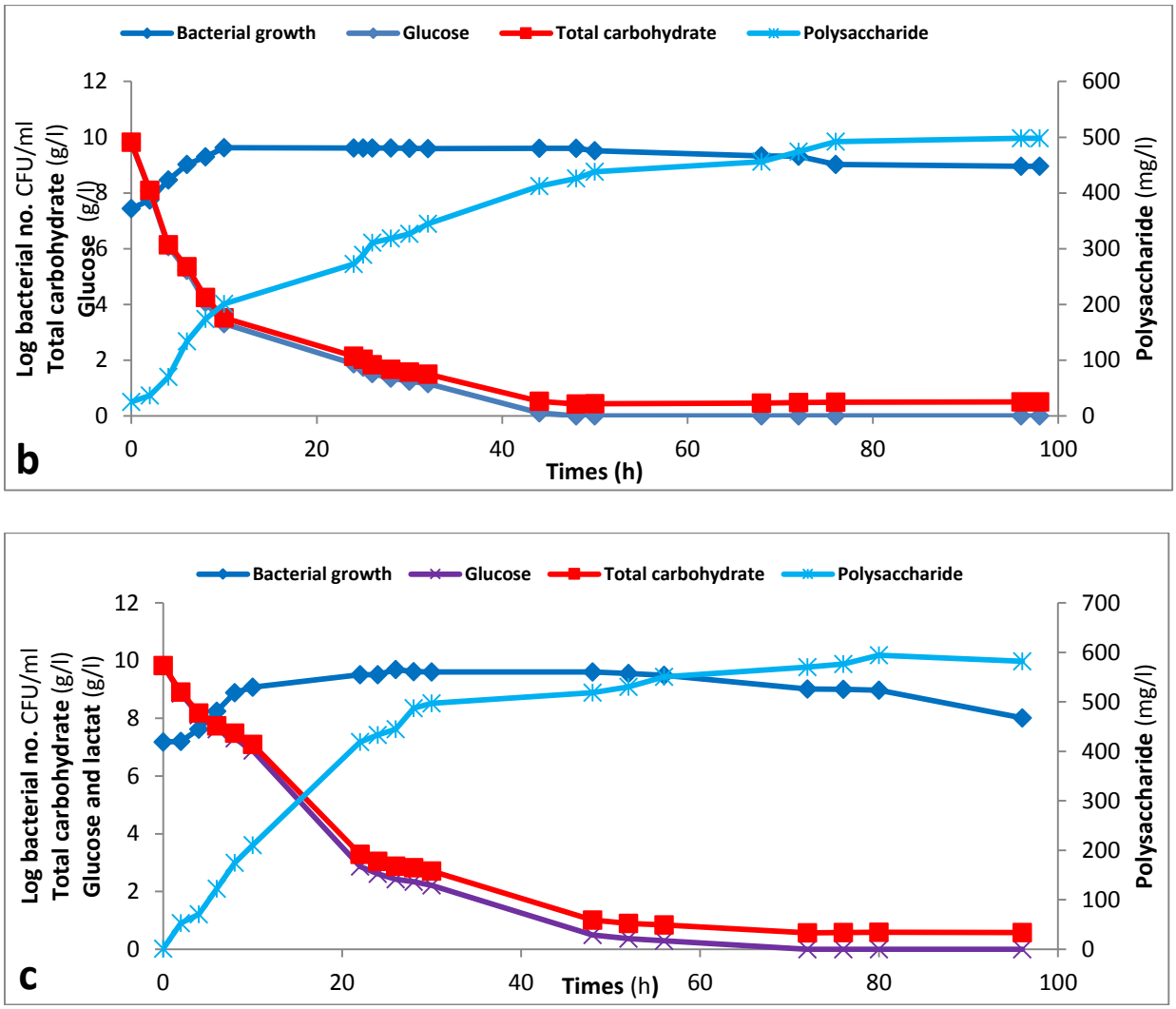


Figure 5.26 Polysaccharides production and glucose utilisation during Ps 3 growth on MM Glc (10g/l glucose) at different initial inoculum size and temperature a) 10^4 CFU /ml, 37˚C, b) 10^7 CFU /ml, 37˚C, c) 10^7 CFU /ml, 30˚C.

When grown on MM Eth at 37˚C and an initial inoculum of 10^4 CFU/ml Ps 3 generated 77.0 mg/l after 72 h of incubation. As before increasing the initial inoculum size to 10^7 CFU/ml increased the amount of polysaccharide generated at both 37˚C (95.0 mg/l) and 30˚C (119 mg/l) (Figure 5.27). In all cases the ethanol feed was depleted within 48 h of incubation.

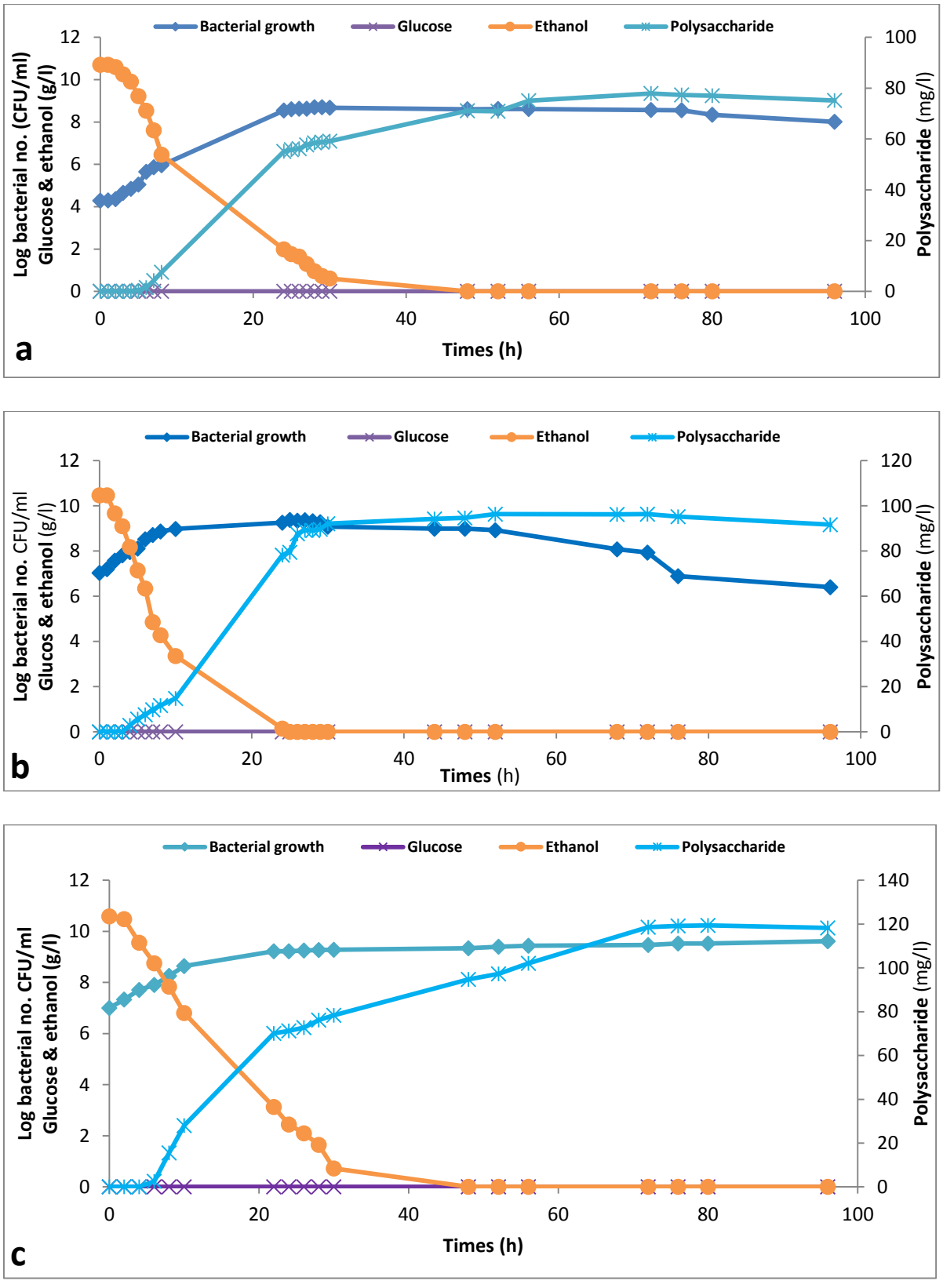


Figure 5.27 Ethanol utilisation and polysaccharide formation during growth of Ps 3 on MM Eth (10.57 g/l) at different inoculum culture size and temperature a) 10^4 CFU /ml, 37˚C, b) 10^7 CFU /ml, 37˚C, c) 10^7 CFU /ml, 30˚C.

5.3.5 MM Glc and MM Eth control

Sterile media control incubations were established to ensure there were no abiotic losses of glucose or ethanol from the two media employed (Figure 5.28).

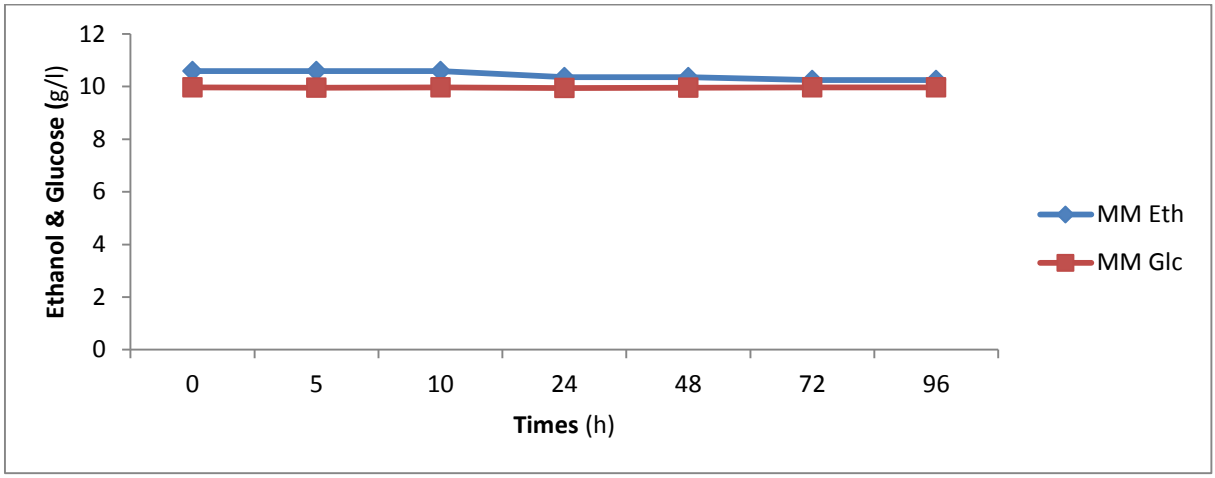


Figure 5.28 Control curve of glucose and ethanol estimation within MM Glc (10 g/l glucose) and MM Eth (10.57 g/l ethanol) without any bacterial inoculation at 37˚C for 96 h.

5.3.6 Optimisation and analysis of bacterial EPS production

After the extraction of EPS from the broth cultures the material was analysed for the presence of carbohydrate, protein and uronic acid. The material was then subjected to structural analysis via proton NMR and HPAC-PAD as described in Sections 4.12.8 and 4.12.19 respectively.

5.3.6.1 NMR analysis of *Pseudomonas aeruginosa* 3 EPS

EPS production and extraction were optimised by varying a range of growth and extraction parameters (Figure 5.29). When MM Glc was inoculated with 10^4 CFU/ml of Ps 3 and incubated for 48 h at 37°C and then treated with 14% TCA (to precipitate protein), 18 mg/l of crude product was recovered. However, when subjected to proton NMR analysis the material failed to generate any signals in the anomeric region (figure 5.29a). When the incubation period was increased to 72 h or the TCA concentration decreased to 10%, peaks in the anomeric region indicative of the presence of EPS were seen (Figure 5.29 b & c).

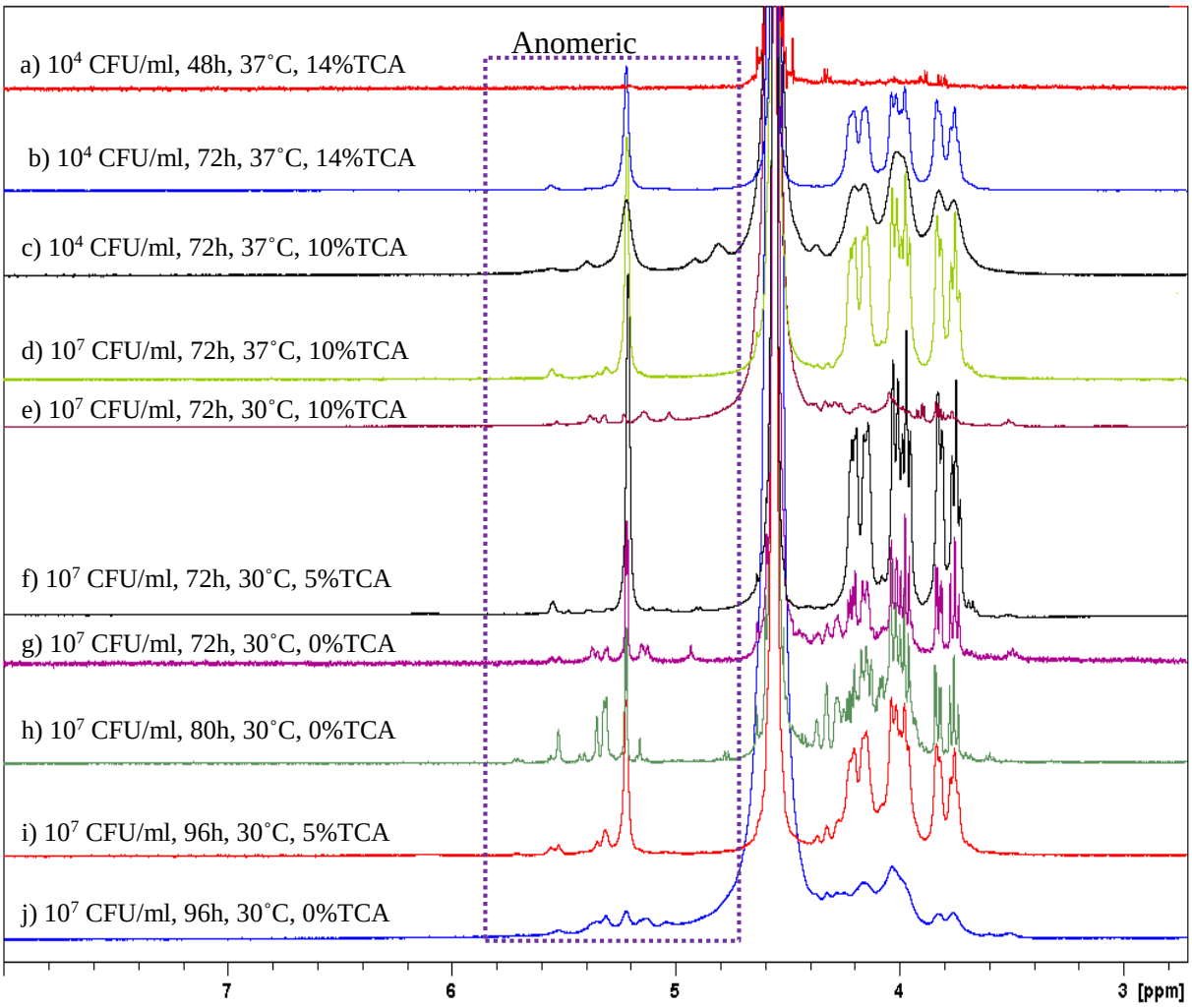


Figure 5.29 Proton NMR analysis of extracted EPS from Ps 3 grow on MM Glc at different inoculum size, incubation period, incubation temperature and TCA concentration treatment. All NMR recorded in D_2O at 70˚C.

By increasing the initial inoculum size to 10^7 CFU/ml and employing 10% TCA (w/v) in the extraction process, similar EPS spectra were generated from broths grown at both 30 and 37˚C (Figure 5.29 d & e). However, more product was generated at 30˚C than 37˚C (Table 5.8). Further increases in incubation time up to 96 h at 30˚C with 0 and 5% TCA concentrations resulted in a degradation of the NMR spectra and a reduction in the amount of crude substance recovered (Figure 5.29 f, g, i & j) (Table 5.8). Consequently, the optimum growth and extraction conditions for Ps 3 grown on MM Glc (Figure 5.29 h) (Table 5.8) were 30˚C, 72h and 5% TCA.

Table 5.8 Conditions, characteristics and analysis of the crude substance (EPS) produced by Ps 3 on different carbon source mineral media.

Batch Culture	Media	Temperature (°C)	Incubation period (h)	TCA %	Amount of crude substance mg/l	Carbohydrate content per mg %	Protein content per mg %	Glucose (g/l) or (mg/dl)	Uronic acid per mg %
a	MM Glc	37	48	14	18	16.13	0.35	0	0.00
b		37	72	14	22.2	19.33	0.60	0	0.07
c		37	72	10	29.4	24.38	0.85	0	0.62
d		37	72	10	35	29.23	0.85	0	0.92
e		30	72	10	33	32.11	0.98	0	1.17
f		30	72	5	44	53.56	0.98	0	1.17
g		30	72	0	56.4	49.12	1.73	0	0.52
h		30	80	0	59.6	50.15	1.98	0	1.22
i		30	96	5	26.4	25.10	1.23	0	1.12
j		30	96	0	36.4	26.55	2.35	0	0.52
k	MM Gly	30	72	5	29	25.10	3.60	0	2.02
l		30	72	0	55	34.48	4.98	0	1.12
m	MM Eth	30	72	5	30.2	21.80	2.48	0	1.42
n		30	72	0	51.6	33.76	3.10	0	1.02

The process carried out for Ps 3 grown on MM Glc was repeated with MM Gly and MM Eth (Figure 5.30, Table 5.8). There are not any obvious difference in the presence of peaks when 0 or 5% TCA were used to treat EPS extracted from MM Gly grown cultures although the peaks were stronger in the absence of TCA (Figure 5.30 k & l). In the case of MM Eth grown cultures the use of 5% TCA resulted in a less diverse spectra when compared to treatment without TCA

(Figure 5.30 m & n, Table 5.2). In both cases (MM Gly and MM Eth) the absence of TCA treatment increased the amount of crude substance and the amounts of protein and carbohydrate present in the material recovered from the broths (Table 5.8).

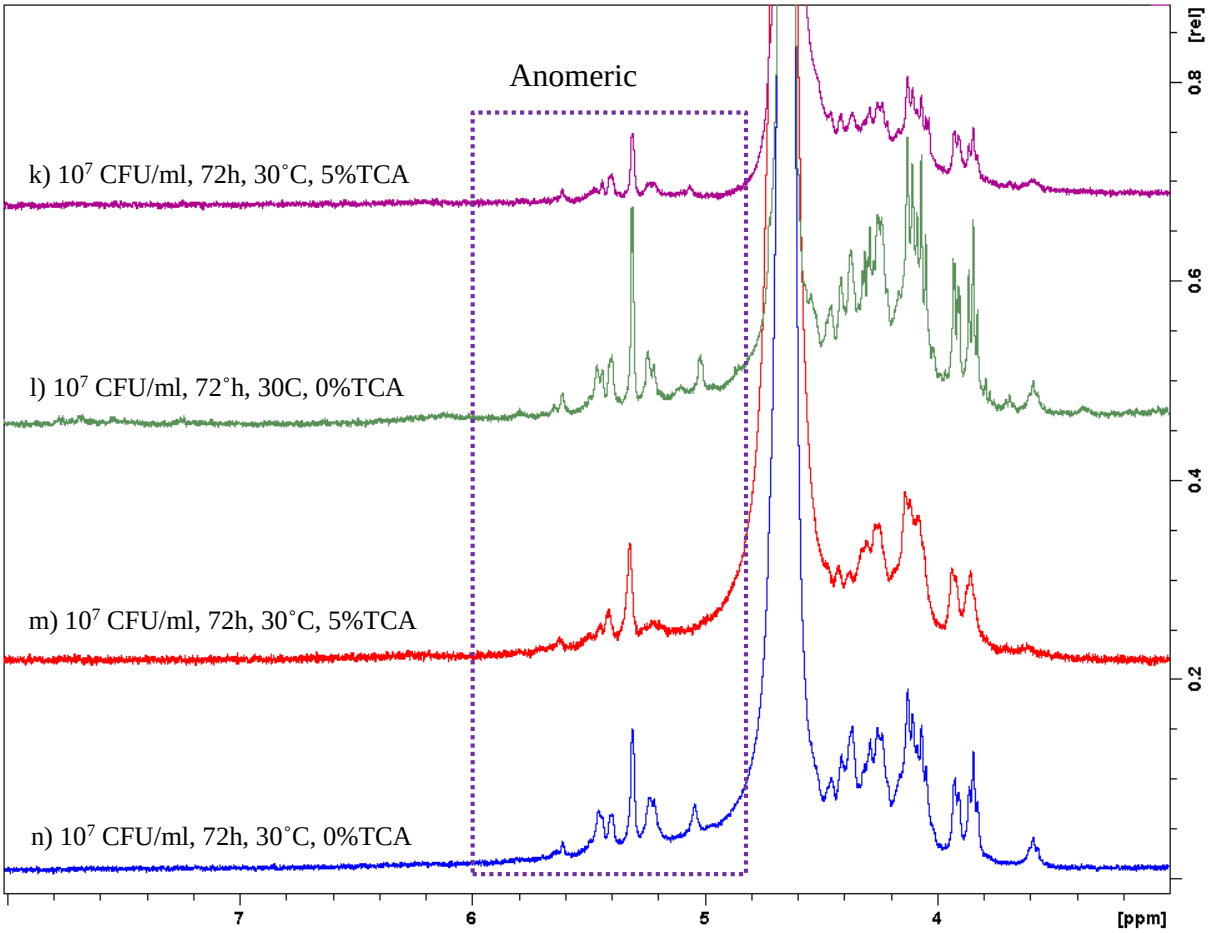


Figure 5.30 Proton NMR analysis of extracted EPS from Ps 3 grow on different media for 72h with different TCA concentration treatment. k & l) MM Glycerol and m & n) MM Ethanol. All NMR recorded in D_2O at 70˚C.

5.3.6.2 NMR analysis of *Stenotrophomonas maltophilia* 53 EPS

The optimisation of EPS production and extraction from St 53 was carried out in the same manner as that outlined above for Ps 3. However, in this case (Figure 5.31) when grown on MM Glc the use of 10% TCA reduced the diversity of the spectra when compared to the extracts generated when 0 or 5% TCA was used (Table 5.9). Also the amount of crude extract generated with 10% TCA was lower than that seen with 5 and 0%, in addition the amount of carbohydrate

present in the extract was actually lower (Table 5.9). The amount of EPS recovered decreased when the incubation period was extended to 96 h and this decrease was associated with a loss of diversity in the NMR signal spectra in anomeric region. When MM Gly and MM Eth were used the only main observation was the loss of spectral diversity when 5% TCA was used along with MM Gly (Figure 5.31 f). Generally speaking the spectra and compositions of the EPS did not differ markedly between EPS treated with and without TCA when the same growth conditions were used.

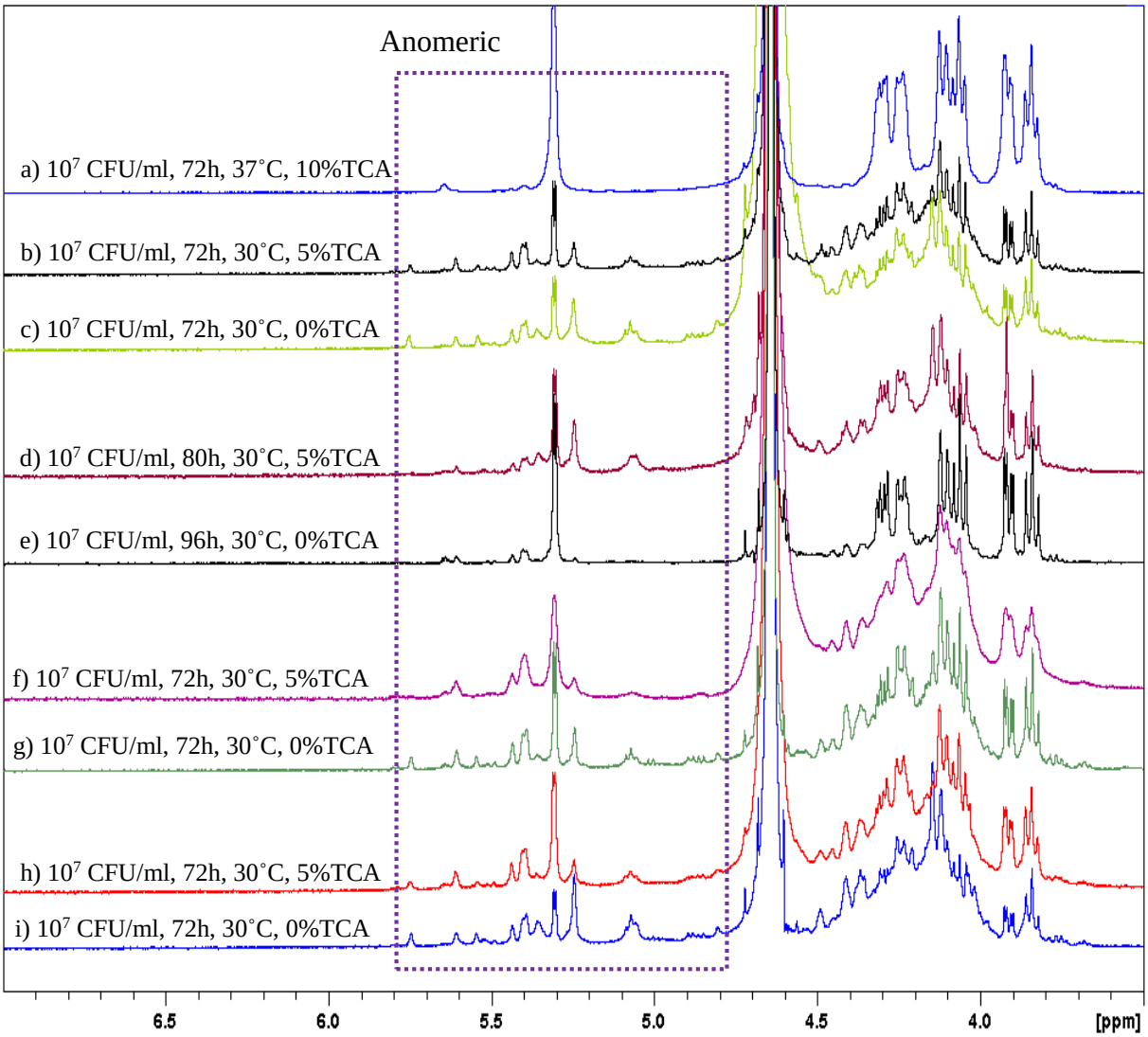


Figure 5.31 Proton NMR analysis of extracted EPS from St 53 grow on different carbon source mineral media, inoculum size, incubation period, incubation temperature and TCA concentration treatment. a-e) MM Glc, f & g) MM Gly and h & i) MM Eth. All NMR recorded in D_2O at 70˚C.

Table 5.9 Conditions, characteristics and analysis of the crude substance (EPS) produced by St 53 on different carbon source mineral media.

Batch Culture	Media	Temperature (°C)	Incubation period (h)	TCA %	Amount of crude substance mg/l	Carbohydrate content per mg %	Protein content per mg %	Glucose (g/l) or (mg/dl)	Uronic acid per mg %
a	MM Glc	37	72	10	20.05	28.40	0.35	0	1.87
b		30	72	5	31	41.91	0.73	0	1.97
c		30	72	0	38.8	47.16	2.35	0	1.42
d		30	80	5	43.8	56.55	0.85	0	2.02
e		30	96	0	39	42.42	2.73	0	1.37
f	MM Gly	30	72	5	30	43.97	4.10	0	1.27
g		30	72	0	42.2	47.37	4.98	0	1.42
h	MM Eth	30	72	5	24.4	45.31	1.10	0	1.87
i		30	72	0	36.4	51.91	1.98	0	2.22

5.3.6.3 NMR analysis of *C. freundii* EPS

The optimisation of EPS production and extraction from *C. freundii* was carried out in the same manner as that outlined above for Ps 3 and St 53. However in the case of *C. freundii* successful NMR spectra were only generated when the bacteria was grown on MM Glc (Figure 5.32) even though crude extract with a significant carbohydrate content was generated with other media (Table 5.10). This poor NMR performance may be due to the crude extract having a low solubility and does not necessarily mean that no EPS was generated. EPS production and extraction on MM Glc was more effective after 80 h than 96 h an observation that is common across all three bacteria investigated. The use of 5%

TCA caused the disappearance of some peaks in anomeric region (Figure 5.32, Table 5.10).

Table 5.10 Conditions, characteristics and analysis of the crude substance (EPS) produced by *C. freundii* on different carbon source mineral media

Batch Culture	Media	Temperature (°C)	Incubation period (h)	TCA %	Amount of crude substance mg/l	Carbohydrate content per mg %	Protein content per mg %	Glucose (g/l) or (mg/dl)	Uronic acid per mg %
a	MM Glc.	30	80	5	28.2	46.55	1.73	0	1.47
b		30	80	0	46.2	52.73	2.48	0	1.62
c		30	96	5	27.6	33.66	2.10	0	1.47
d		30	96	0	44.2	35.82	3.48	0	1.42
e	MM Gly.	30	80	5	22	49.23	1.23	0	0.82
f		30	80	0	30	54.28	1.85	0	1.02
g	MM Eth.	30	80	5	30.6	39.54	0.98	0	0.72
h		30	80	0	38	42.73	1.60	0	0.67

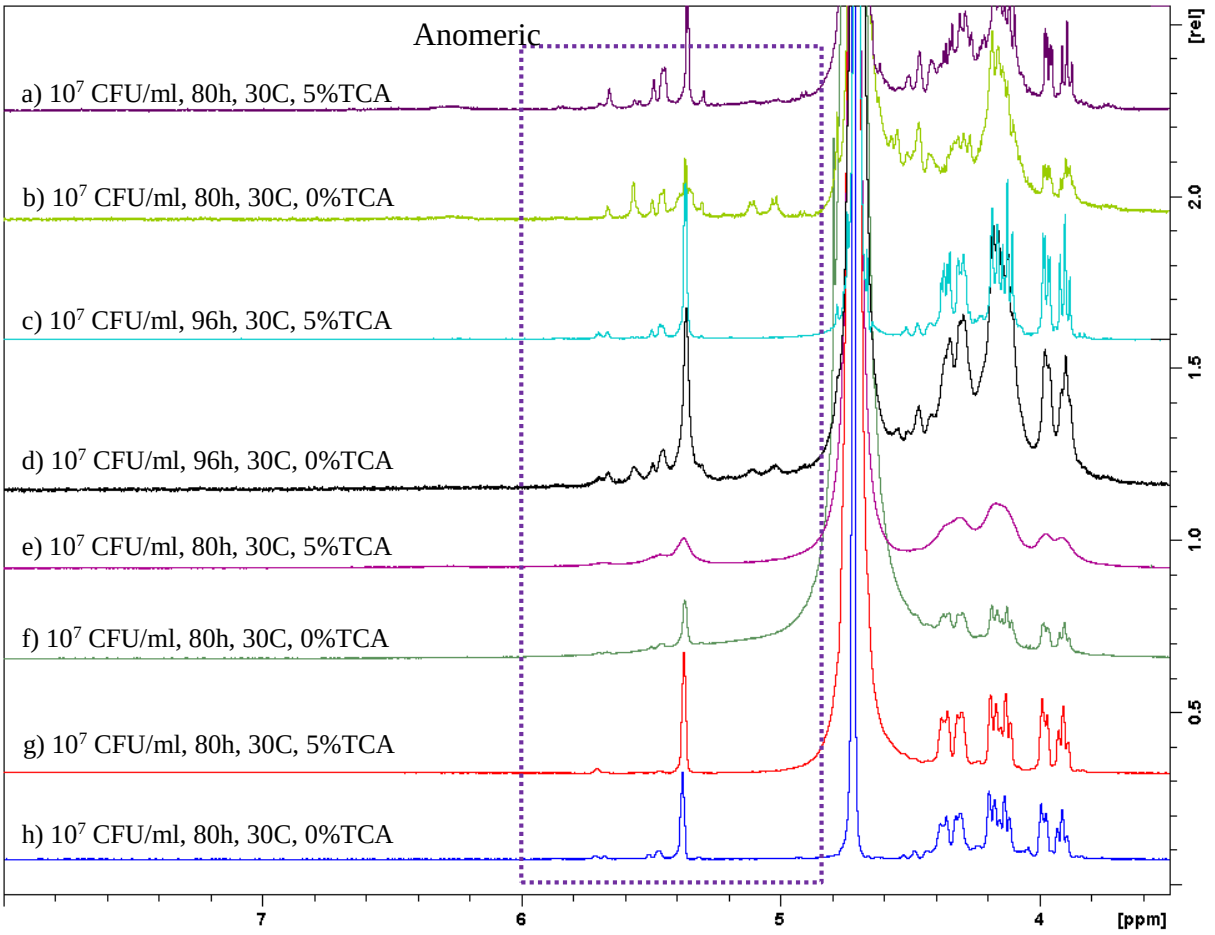


Figure 5.32 Proton NMR analysis of extracted EPS from *C. freundii* grow on different carbon source mineral media, incubation period, and TCA concentration treatment. a-d) MM Glc, e & f) MM Glc and g & h) MM Eth. All NMR recorded in D_2O at 70°C.

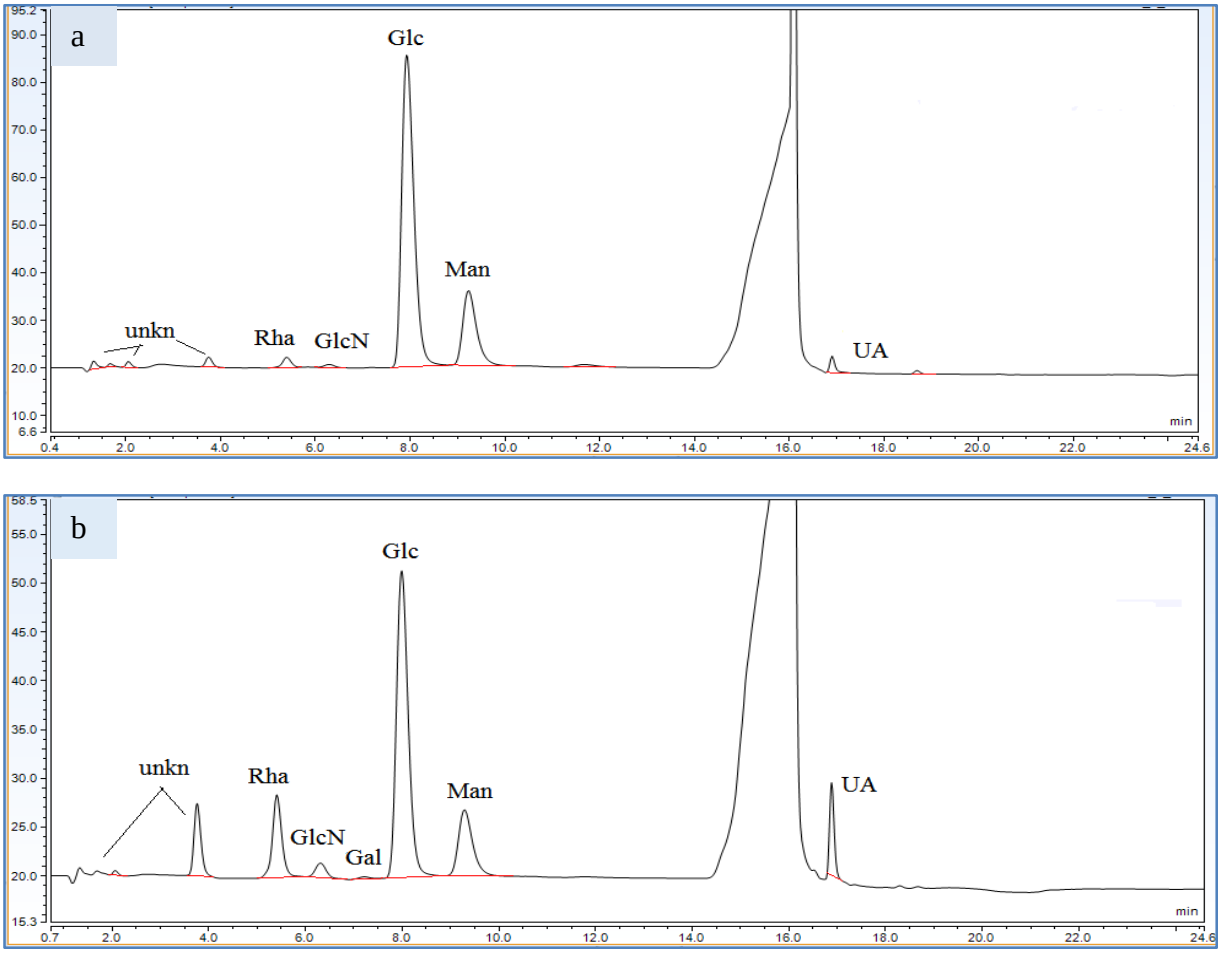
5.3.7. EPS monomer analysis

5.3.7.1 Ps 3 EPS monomer analysis

After hydrolysis, the monosaccharide composition of the EPS was analysed by HPAEC-PAD as described in Section 4.12.9 (Figure 5.33). Ps 3 EPS grown on MM Glc (10^7 CFU/ml, 30 C, 72 h, 5% TCA) was 67.49 % neutral sugar with some uronic acid and an additional unidentified neutral sugar. While the polysaccharide content in the EPS not treated with TCA was about 52.38% neutral sugar. These overall sugar compositions are greater than the carbohydrate estimates generated by the phenol sulphuric acid method (Table 5.8) this reflects

the greater accuracy of the monomer analysis and the fact that the phenol method can only be calibrated against a single carbohydrate.

The composition of TCA treated EPS was dominated by glucose (61.18%) with lesser amounts of rhamnose, glucosamine and mannose while the non TCA treated EPS also contained the same composition plus trace amounts of galactose (Figure 5.34).



Unknown (unkn), Rhamnose (Rha), Glucosamine (GlcN), Galactose (Gal), Glucose (Glc), Mannose (Man) and Uronic acid (UA).

Figure 5.33 HPAEC-PAD monomer analysis of Ps 3 extracted EPS on MM Glucose (10^7CFU/ml as initial culture) after 72 h incubation at 30˚C treated with a) 5%TCA b) 0%TCA.

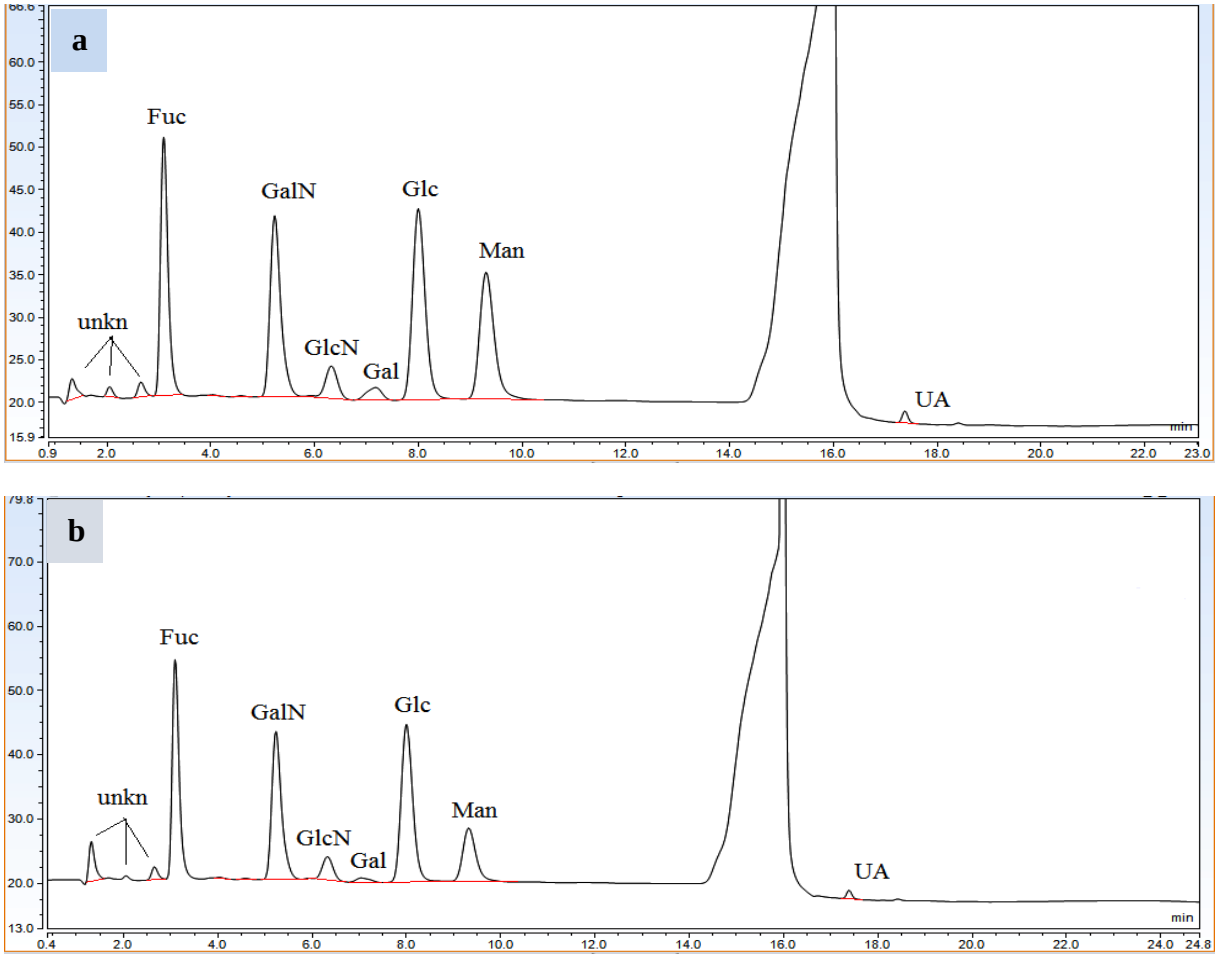
Under same condition but with an increased incubation period (10^7 CFU/ml, 30 ˚C, 80 h, without TCA treatment) on MM Glc, Ps 3 produced more crude substance than 72 h (Table 5.8) and with the same general composition (Figure 5.36).

The EPS neutral sugar content of the material extracted from MM Gly and MM Eth with 5% TCA were 28.6 and 25.42% with additional uronic acids and an additional unidentified neutral sugar. The neutral sugar content of the EPS extracted without TCA treatment increased to 40.95% on MM Gly and 60.95% on MM Eth. This is the opposite to that seen when Ps 3 was grown on MM Glc, suggesting that the TCA treatment was degrading the EPS generated when Ps 3 was grown on MM Gly and MM Eth. This is supported by the fact that the glucose and mannose concentrations within EPS extracted from MM Gly cultures decreased after treatment with TCA from 20.62 and 11.15% to 13.58 and 5.67% respectively, while the rhammnose and galactose contents remain the same. The same trend is seen in MM Eth grown cultures where all sugar components within the EPS decreased with TCA treatment.

5.3.7.2 St 53 EPS monomer analysis

In the case of St 53 the use of 5% TCA in the extraction process had a significant impact on the amount and composition of the EPS extracted (Figure 5.34 and 5.35). In all cases the use of 5% TCA increased the overall amount of sugars recovered on all mineral media. The use of TCA changed the relative compositions of the recovered EPS for example in the presence of TCA glucose (21.08%) and mannose (16.34) were the main components on all mineral media (Figure 5.34). When the TCA treatment was excluded glucose (23.19%) and galactosamine (14.27%) became the main components of the EPS extracted MM Glc grown cultures. This changed with the MM Eth grown cultures where the absence of TCA resulted in an EPS dominated by approximately equal amounts

of glucose (15.44%) and mannose (15.26%). The analysis of the EPS from MM Gly grown cultures extracted in the absence of TCA also showed glucose and mannose to be the main components (Figure 5.34). When incubation for 80 h (10^7 CFU/ml, 30°C, 5%TCA treatment) on MM Glc, St 53 (Figure 5.36) produced more crude substance than 72 h (Table 5.8) and with the same general composition.



Unknown (unkn), Fucose (Fuc), Galactosamine (GalN), Glucosamine (GlcN), Galactose (Gal), Glucose (Glc) Mannose (Man) and Uronic acid (UA)

Figure 5.35 HPAEC-PAD monomer analysis of extracted EPS from St 53 growth on MM Glucose (10^7 CFU/ml as initial culture) after 72 h incubation at 30˚C treated with a) 5%TCA b) 0%TCA.

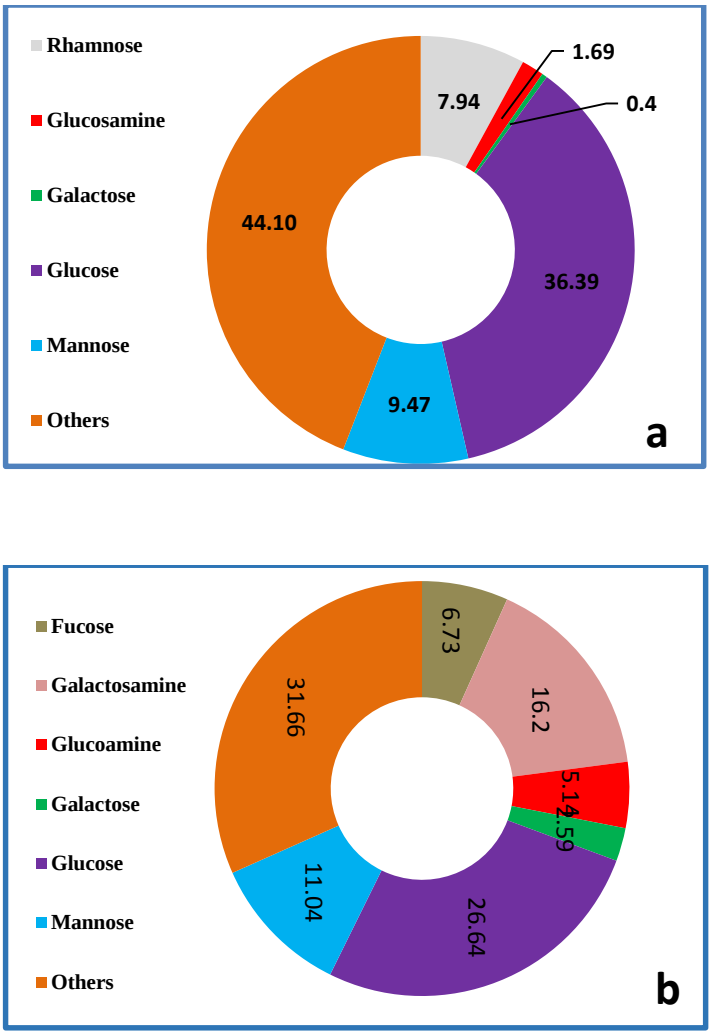


Figure 5.36 Bacterial EPS composition analysed by HPAEC-PAD after bacterial growth in MM Glc for 80 h at 30˚C with 10^7 CFU/ml as initial culture. a) Ps 3 EPS treated with 0% TCA and b) St 53 EPS treated with 5% TCA.

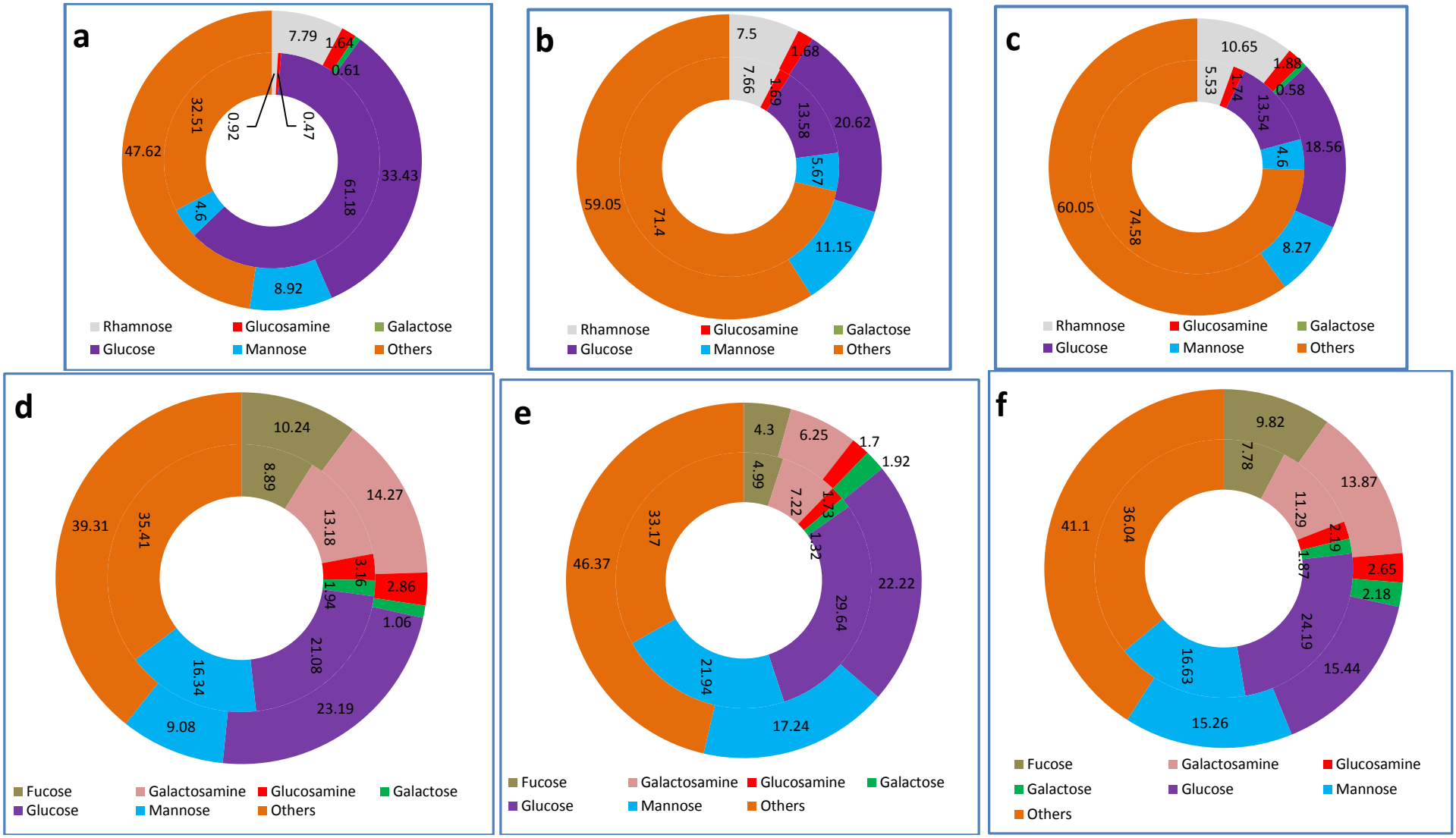
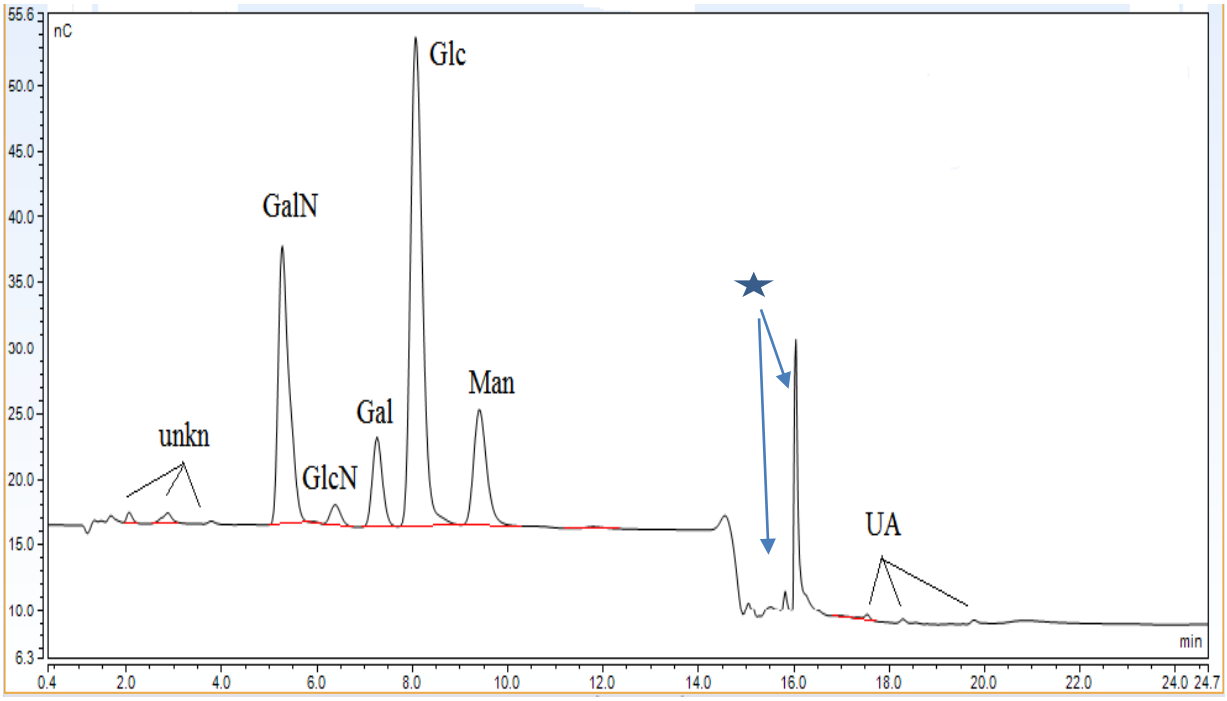


Figure 5.34 HPAEC-PAD monomers analysis of Ps 3 and St 53 extracted EPS (after 72 h incubation at 30 C) on different carbon source mineral media treated with 5% TCA (inner circle) and 0% TCA (outer circle). a) Ps 3 EPS on MM Glc b) Ps 3 EPS on MM Gly c) Ps 3 EPS on MM Eth d) St 53 EPS on MM Glc e) St 53 EPS on MM Gly f) St 53 EPS on MM Eth.

5.3.7.3 *C. freundii* EPS monomer analysis

Monomer analysis showed several sugars within the EPS of *C. freundii* (Figure 5.37). Glucose and mannose were the major components present in extracts from all media (MM Glc, MM Gly and MM Eth) (Table 5.11). The total sugar content of EPS extracted from MM Gly cultures (with the exception of unknown peaks) were higher at 66.56% than seen on the other media (Table 5.11). The use of TCA slightly changed some of compositions of the recovered EPS from MM Eth but with the same amounts (Table 5.11).



Unknown (unkn), Galactosamine (GalN), Glucosamine (GlcN), Galactose (Gal), Glucose (Glc) Mannose (Man) and Uronic acid (UA). * Peaks from program (not sugars).

Figure 5.37 HPAEC-PAD monomer analysis of extracted EPS from *C. freundii* growth on MM Glucose (10^7 CFU/ml as initial culture) after 80 h incubation at 30˚C treated with 0% TCA.

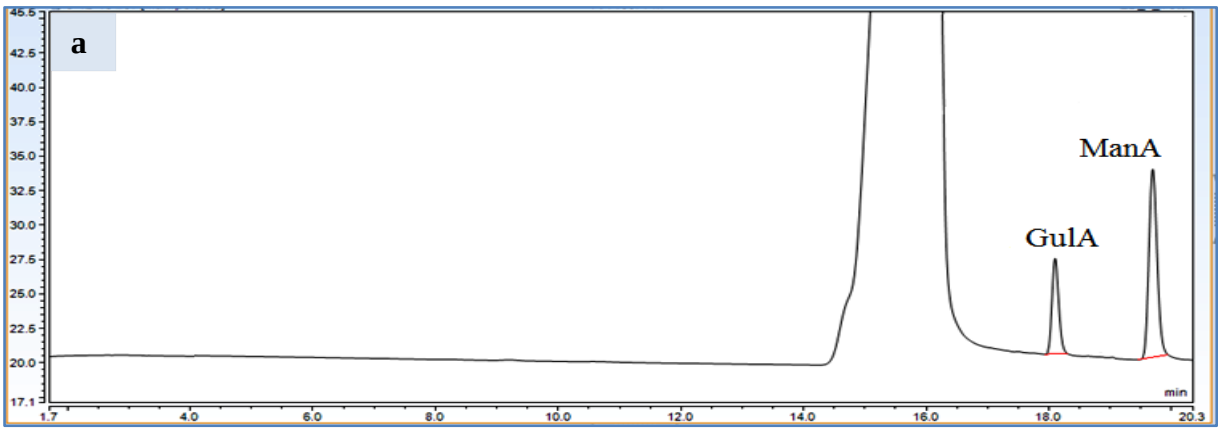
Table 5.11 HPAEC-PAD monomers analysis of *C. freundii* extracted EPS (%) on different carbon source mineral media.

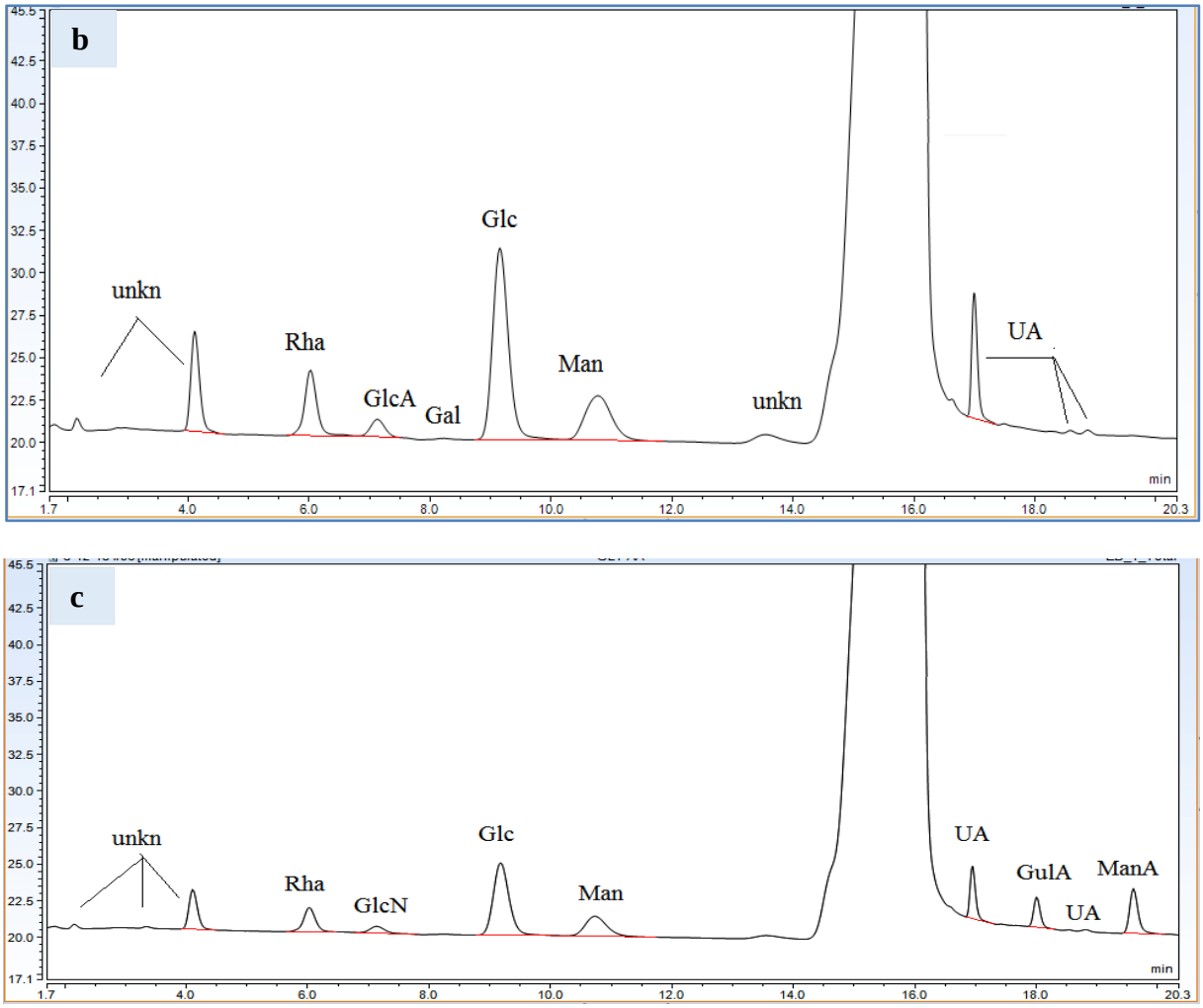
Media	TCA %	Galactosamine	Rhamnose	Glucosamine	Galactose	Glucose	Mannose	Others
***MM Glc**	0	5.4	0.00	0.59	6.49	33.03	13.34	41.14
***MM Gly**	0	2.92	0.46	0.53	0.91	38.44	23.31	33.44
MM Eth	0	3.59	0.41	0.44	5.15	31.66	10.08	48.66
MM Eth	5%	5.39	0.61	0.67	7.72	47.50	15.13	48.67

*The data for 5% TCA are not studied

5.3.8 Presence of alginate within the Ps 3 EPS

Alginate is a common EPS generated by some *Pseudomonas* species. In order to check to see if Ps 3 generated an alginate the Ps 3 EPS was compared with an alginate (Protanal LF 240 D from algae source) via monomer analysis using HPAEC-PAD. In addition hydrolysed alginate and hydrolysed Ps 3 EPS were mixed and analysed via monomer analysis. The monomer analysis indicated that the Ps 3 EPS did not share any peaks with the alginate and therefore did not contain any alginate components (Figure 5.38).



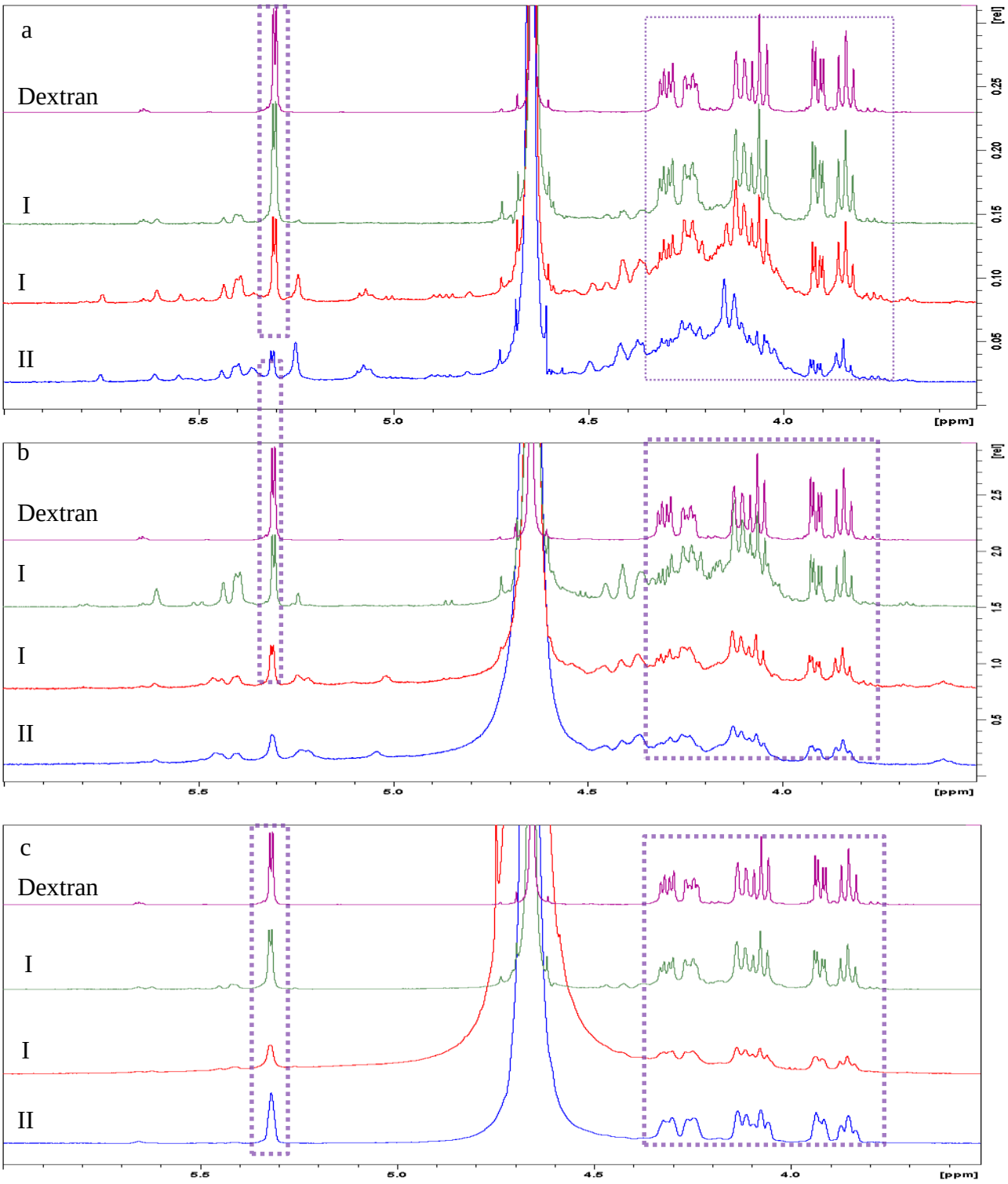


Unknown (unkn), Rhamnose (Rha), Glucosamine (GlcN), Galactose (Gal), Glucose (Glc) Mannose (Man), Uronic acid (UA), Guluronic acid (GulA) and Mannuronic acid (ManA)

Figure 5.38 HPAEC-PAD monomer analysis of extracted Ps 3 EPS compared and spiked with alginate a) Alginate b) Ps 3 EPS c) Ps 3 EPS mixed with alginate and the peaks referred to unknown UA because they are not spiked with alginate components peaks.

5.3.9 Dextran production by St 53, Ps3 and *C. freundii*

Whilst bacterial EPS generally generate peak in the anomeric region of a proton NMR spectra, it was noticed that the EPS spectra produced by St 53, Ps3 and *C. freundii* contained peaks in other regions of the spectra that were consistent with polysaccharide dextran (Figure 5.39).



I) MM Glc, II) MM Gly and III) MM Eth

Figure 5.39 Proton NMR analysis spectra of the dextran as control compared to extracted EPS from different bacteria after growing on different carbon sources mineral media a) St 53, b)Ps 3 and c) *C. freundii* . All NMR recorded in D_2O at 70˚C.

In order to confirm the presence of dextran, a dextranase treatment process was employed (Section 4.12.10). Initially the dextranase enzyme was investigated via proton NMR to ensure no interfering compounds were present in the enzyme powder. Analysis of the dextranase before and after dialysis found no interfering spectral peaks in the region associated with dextran polymer (Figure 5.40).

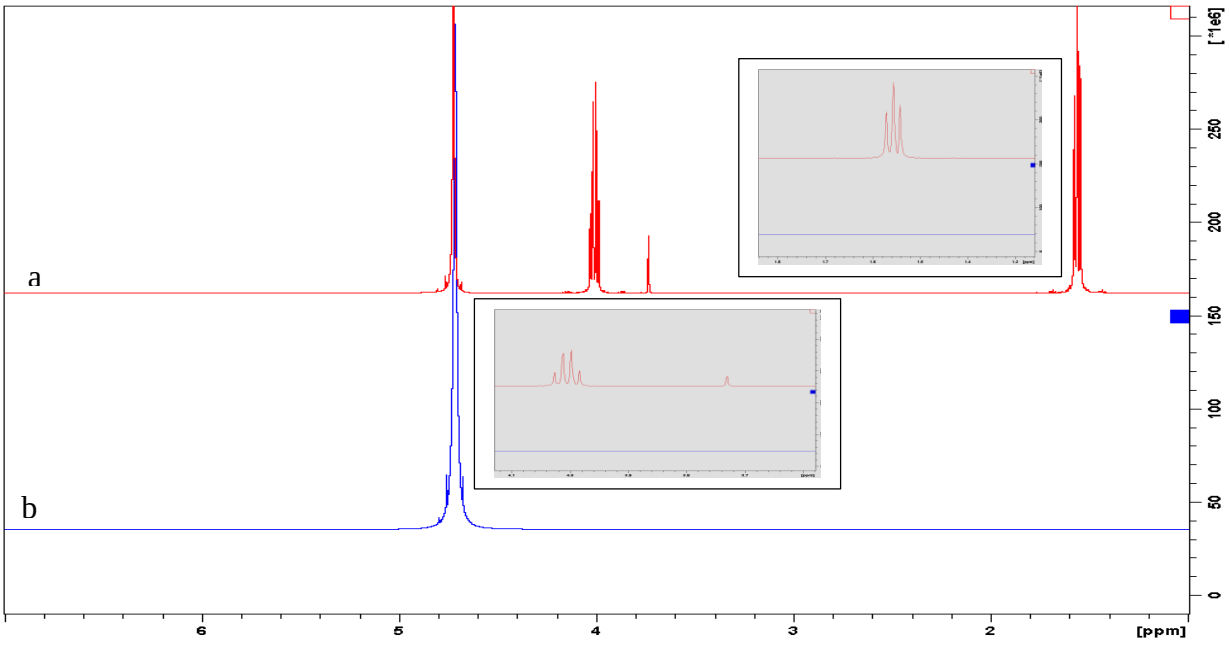


Figure 5.40 Proton NMR analysis spectra of the dextranase enzyme. a) Before dialysis b) after dialysis. All NMR recorded in D_2O at 70˚C.

Once the possibility of interference had been ruled out, the impact of the dextranase was first tested on a commercial dextran, as shown in Figure 5.41 the enzyme treatment removed all the peaks associated with the dextran polymer. Proton NMR spectra of dextranase treated EPS from Ps 3 grown on MM Eth and MM Gly, and St 53 and *C. freundii* grown on MM Eth resulted in the loss of NMR spectral peaks associated with dextran (Figure 5.42 and 5.43). This treatment did not completely remove all indications of an EPS from the NMR spectra, rather the remaining spectral peaks provided evidence that these bacteria (Ps 3, St 53 and *C. freundii*) produced a complex EPS that may be composed of two polysaccharides and that one of them has dextran like properties (Figure 5.42 and 5.43).

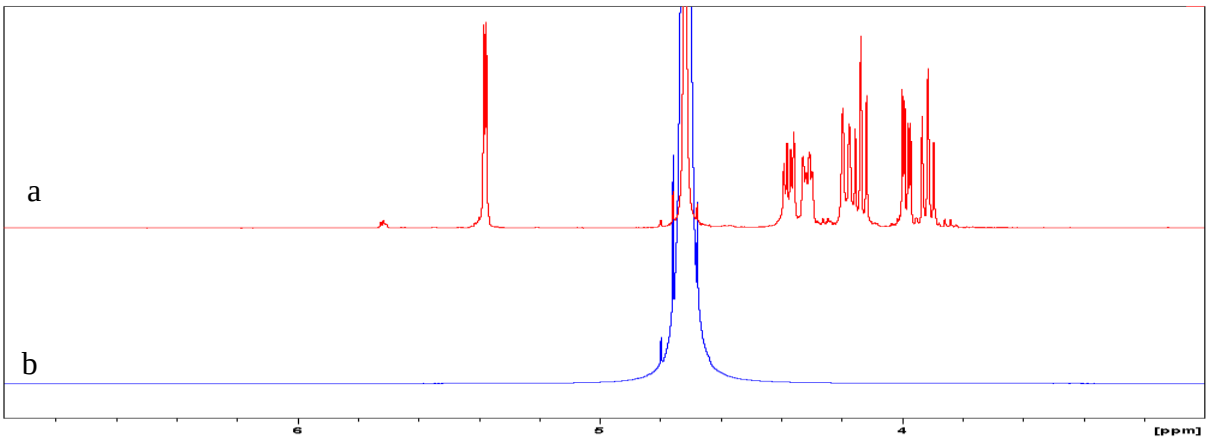
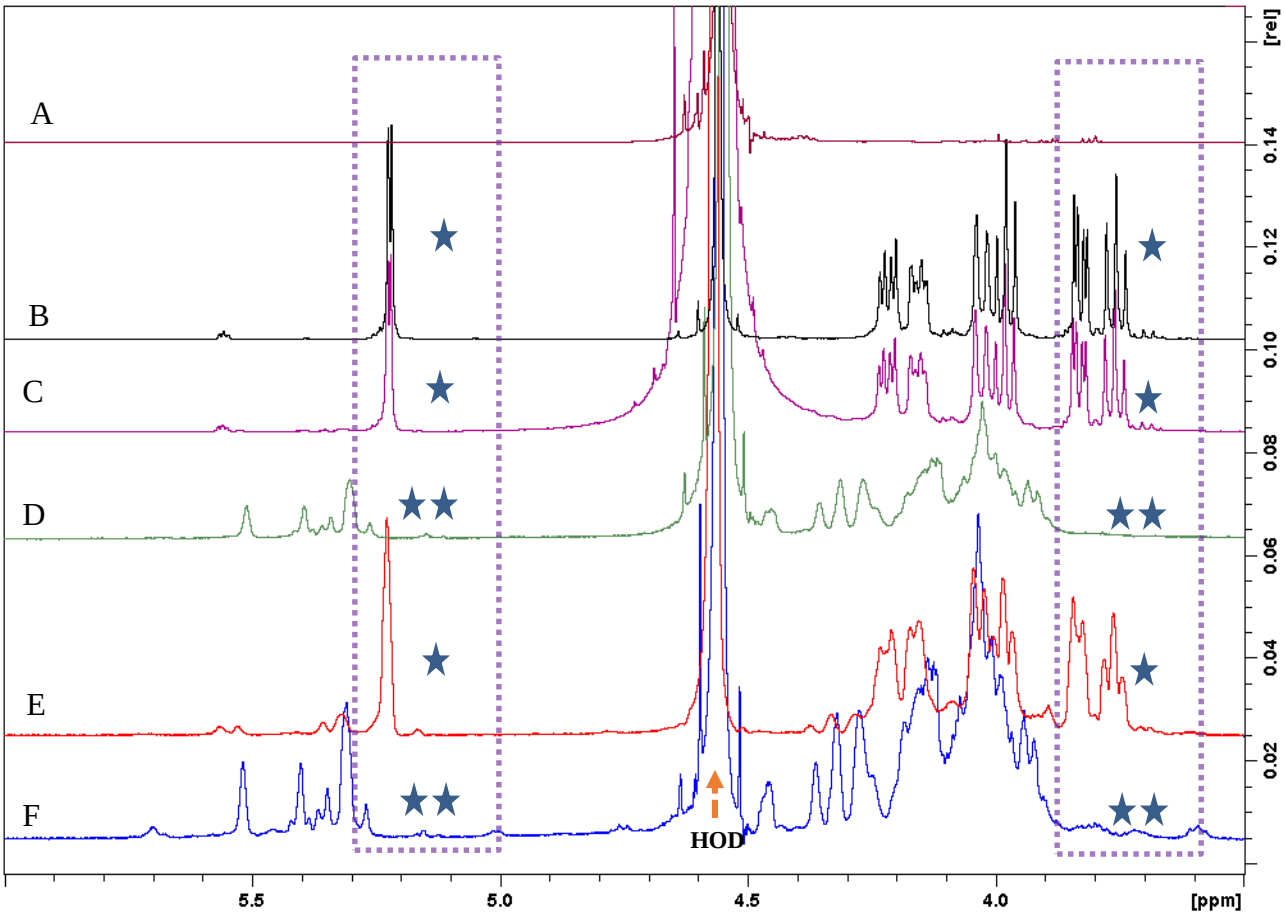


Figure 5.41 Proton NMR analysis spectra of the dextranase enzyme effects on dextran after dialysis. a) Dextran prior to enzyme treatment b) Dextran after enzyme treatment. All NMR recorded in D_2O at 70˚C.

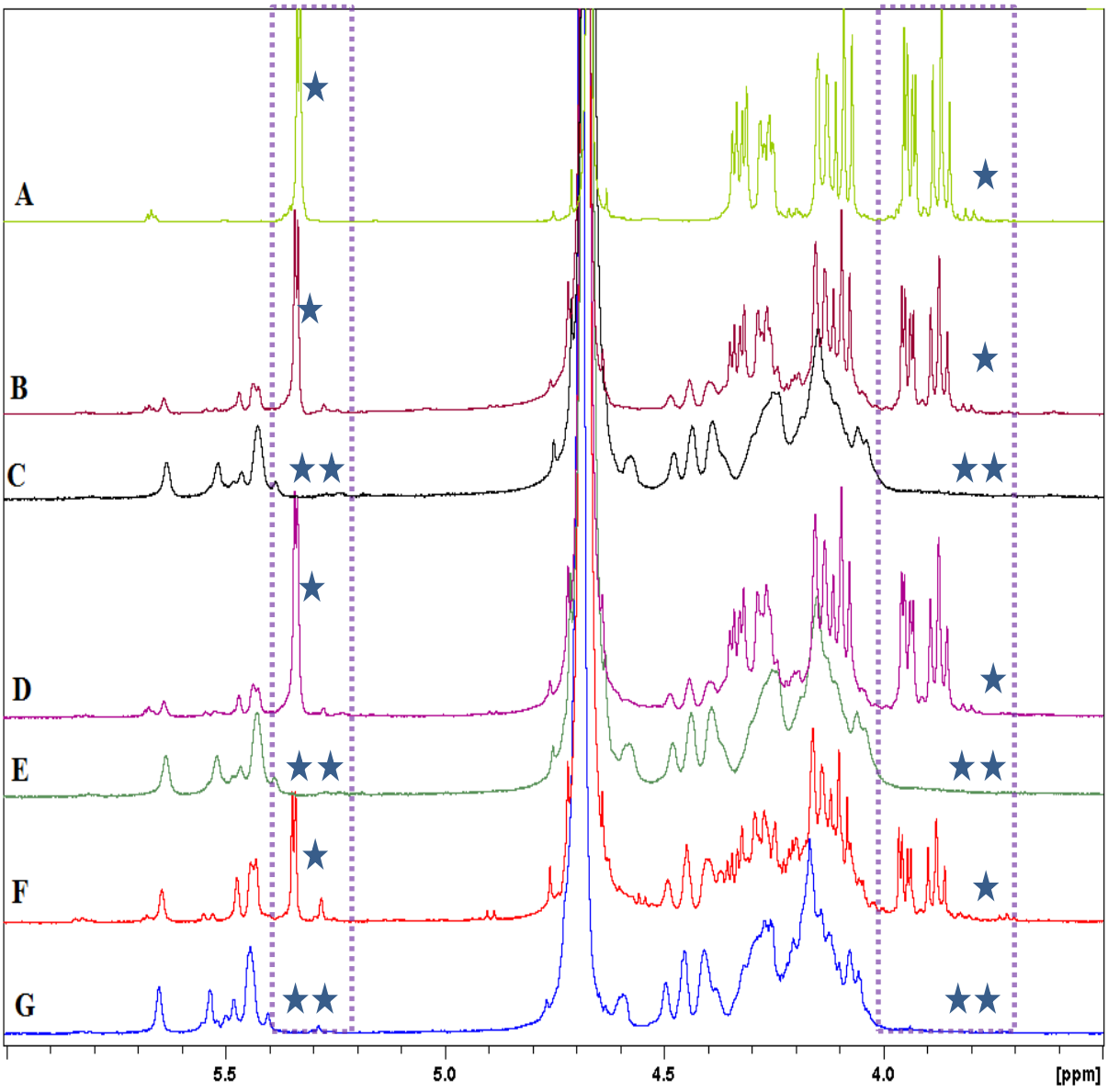


* EPS Dextran peaks.

** Post EPS dextran treatment with dextranase shows vanishing dextran peaks.

Figure 5.42 ^{1}H-NMR spectra of Gram negative bacterial EPS in mineral media containing ethanol as a sole carbon source with and without dextranase treatment after incubation for 80h. Spectra are representative of: un-inoculated control media (A), analytical grade

dextran (B), *Stenotrophomonas maltophilia* strain 53 (C&D) and *Citrobacter freundii* strain SA79 (E&F). All NMR recorded in D_2O at 70˚C.



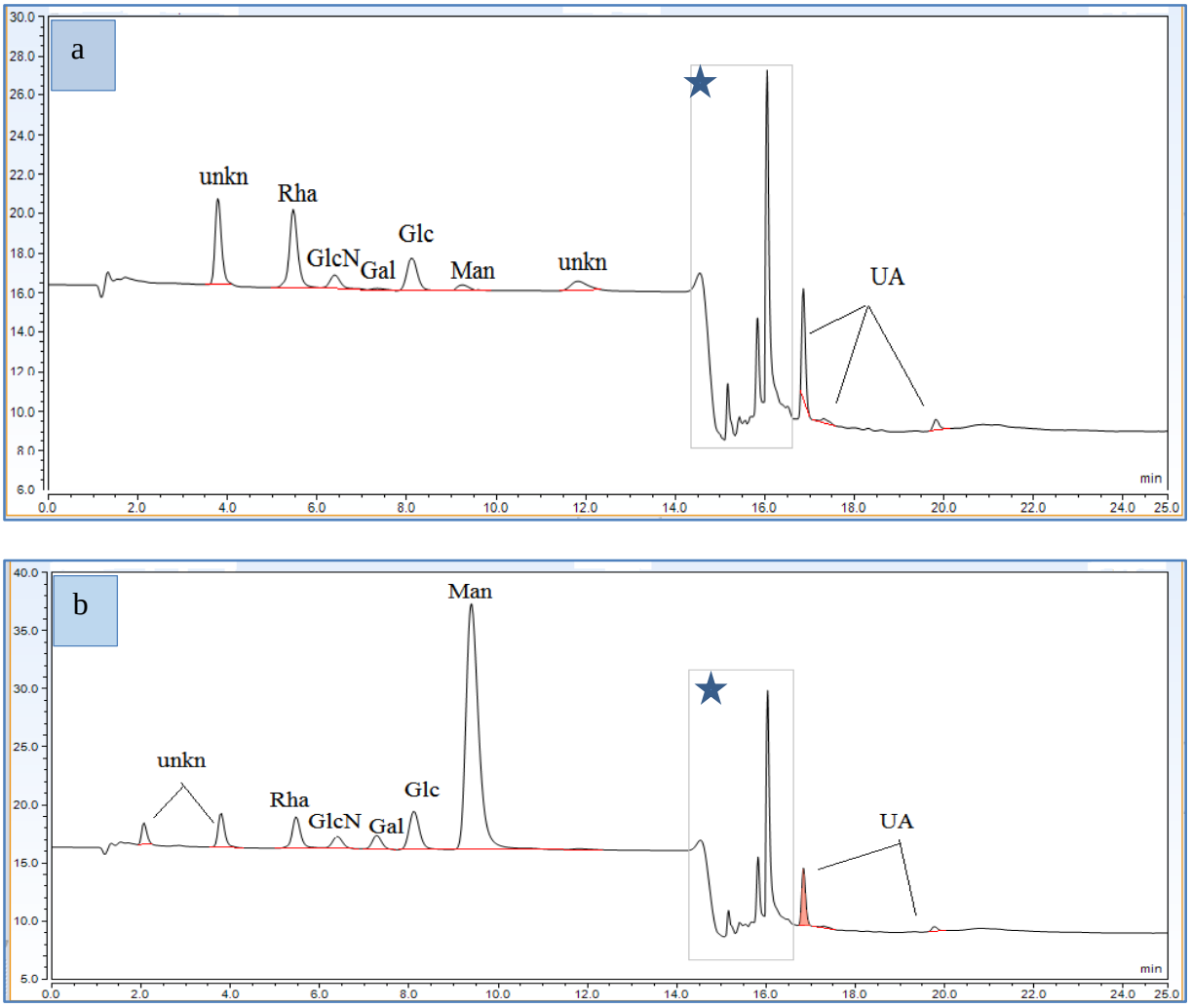
* EPS Dextran peaks.

** Post EPS dextran treatment with dextranase shows vanishing dextran peaks.

Figure 5.43 ^{1}H-NMR spectra of *Pseudomonas aeruginosa* strain (Ps3) EPS in mineral media containing ethanol, glycerol or glucose as a sole carbon source with and without dextranase treatment after incubation for 80h. Spectra are representative of: analytical grade dextran (A), Ps3 EPS in MM Eth (B&C), Ps3 in MM Gly (D&E) and Ps3 in MM Glc (F&G). All NMR recorded in D_2O at 70˚C.

5.3.9.1 Monomer analysis of dextranase treated EPS

Monomer analysis of the dextranase treated EPS generated by Ps 3 grown on MM Eth, MM Gly and MM Glc and St 53 and *C. freundii* grown on MM Eth showed that the removal of the dextran like polymer resulted in a shift from a glucose rich polymer to one high in mannose (Figure 5.44, Table 5.12).



Unknown (unkn), Rhamnose (Rha), Glucosamine (GlcN), Galactose (Gal), Glucose (Glc) Mannose (Man), Uronic acid (UA), Guluronic acid (GulA) and Mannuronic acid (ManA). *Peaks of program (not sugar peaks).

Figure 5.44 HPAEC-PAD monomer analysis of extracted EPS from Ps 3 growth on MM Glycerol after dialysis. a) prior to dextranase treatment b) after dextranase treatment.

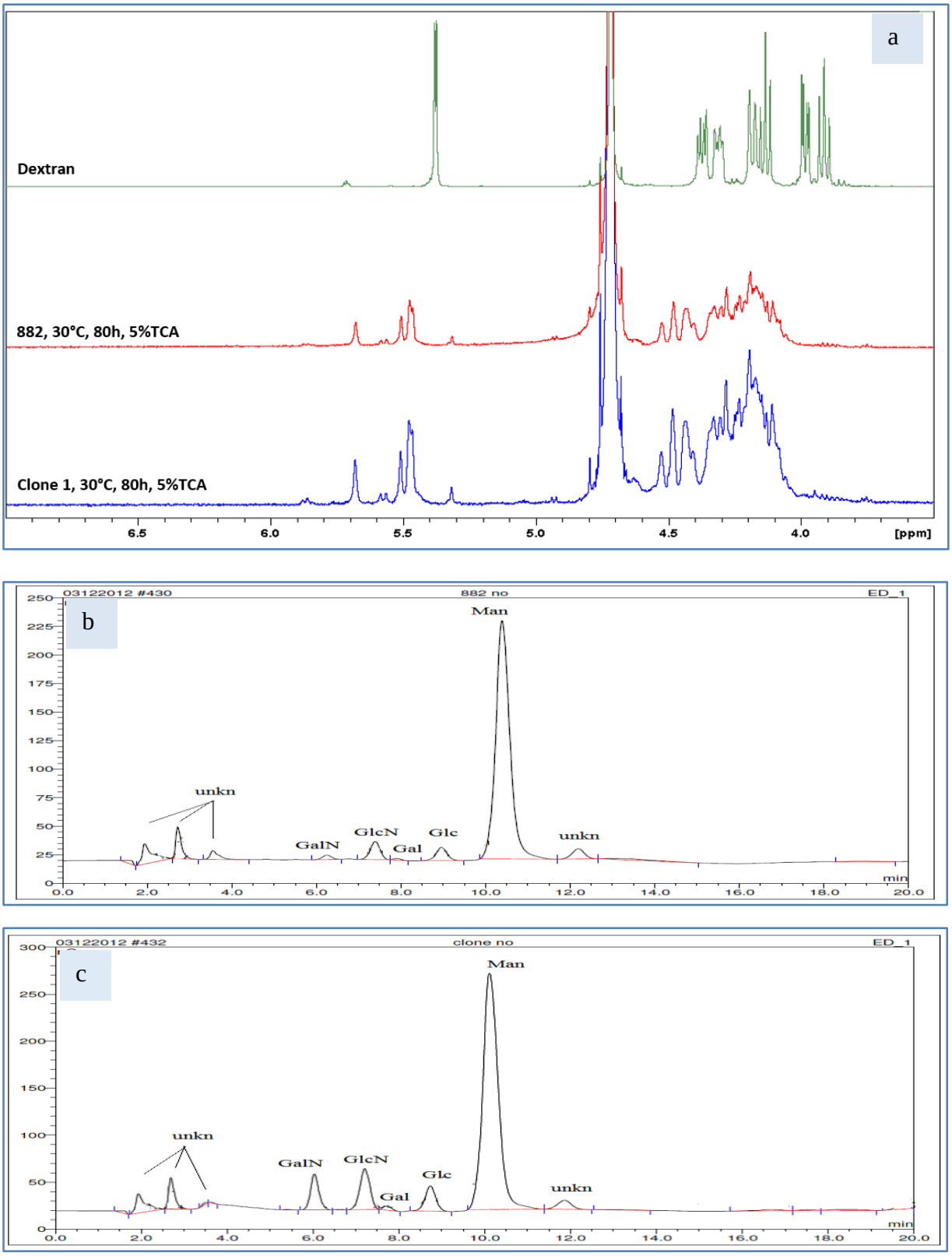
Table 5.12 HPAEC-PAD monomer analysis of prior and post enzyme dextranase treatment of extracted EPS from Ps 3, St 53 and *C. freundii*.

Bacterial strains	Media	Dextranase	Neutral sugar composition of EPS (%)						
			Fucose	Galactosamne	Rhamnose	Glucosamine	Galactose	Glucose	Mannose
Dextranase enzyme	UPW *	-	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Ps 3	MM Eth	Untreated	0.00	0.00	7.67	0.99	0.00	64.71	26.62
		Treated	0.00	0.00	11.37	2.73	0.45	5.99	79.46
Ps 3	MM Gly	Untreated	0.00	0.00	27.78	6.12	0.43	44.03	21.56
		Treated	0.00	0.00	8.81	2.50	2.91	8.01	77.76
Ps 3	MM Glc	Untreated	0.00	0.00	1.37	0.70	0.00	91.08	6.85
		Treated	0.00	0.00	5.94	2.11	0.44	7.28	84.24
St 53	MM Eth	Untreated	19.79	19.09	0.00	2.75	1.98	28.89	27.50
		Treated	7.06	6.48	0.00	1.78	0.57	2.22	81.89
C. freundii	MM Eth	Untreated	0.00	7.00	0.79	0.86	10.03	61.68	19.64
		Treated	0.00	0.58	1.10	4.14	1.08	5.40	87.71

* After dialysis analysed directly by HPAEC

5.3.9.2 *Acinetobacter baumanni* EPS analysis (negative control)

In contrast to Ps 3, St 53 and *C. freundii* (Figure 5.42 and 5.43), proton NMR analysis showed that the EPS generated by both *Acinetobacter baumanni* 882 and Clone 1 strains showed no evidence of dextran indicating that not all Gram negative bacteria generated dextran like polymers in their EPS. Additionally monomer analysis showed that mannose was the dominant sugar (Figure 5.45) rather than glucose.

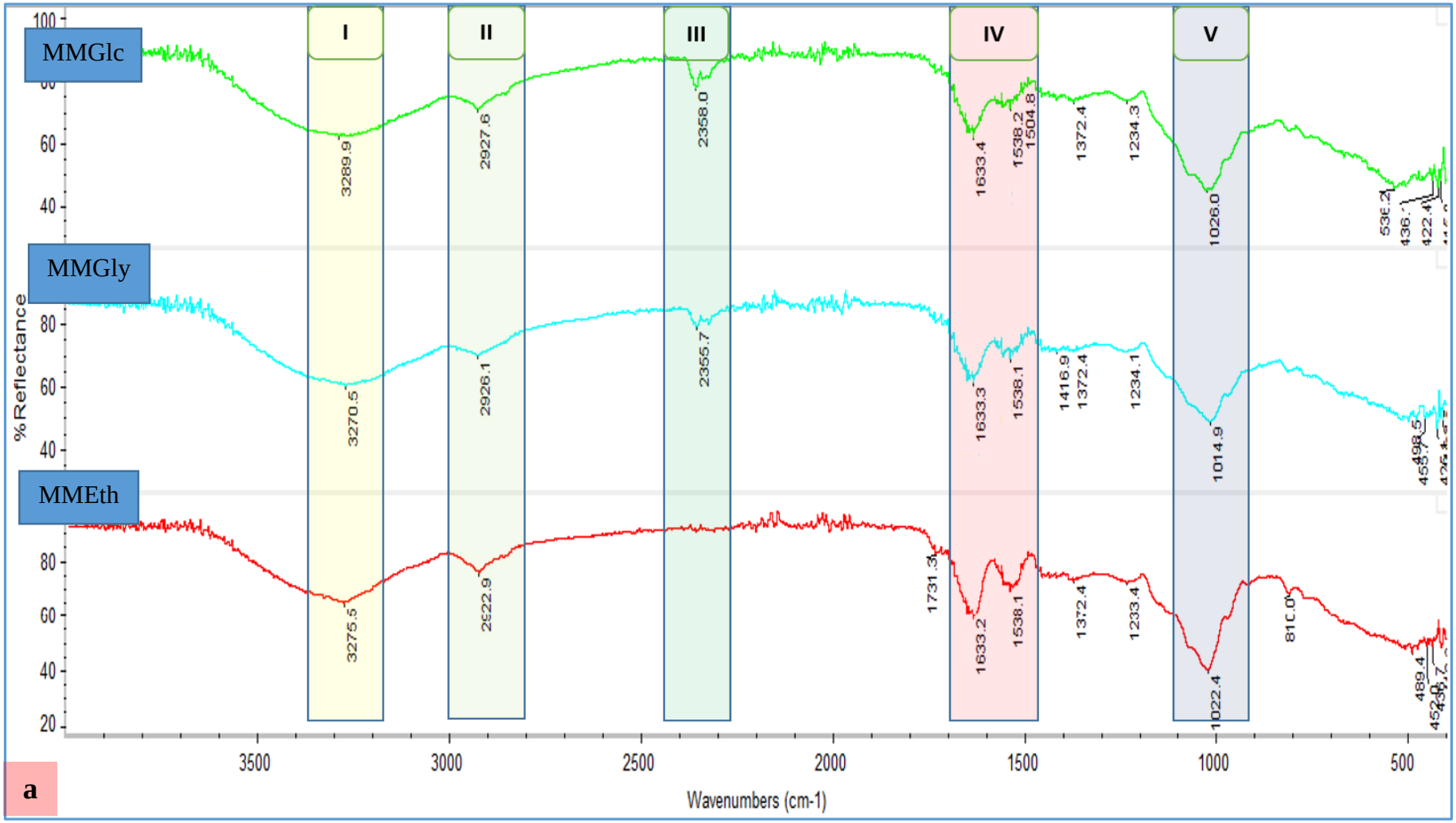


Unknown (unkn), Galactosamine (GalN), Glucosamine (GlcN), Galactose (Gal), Glucose (Glc) and Mannose (Man).

Figure 5.45 Proton NMR and HPAEC-PAD monomer analysis of extracted *Acinetobacter baumanni* EPS without dextranase treatment. a) Proton NMR analysis of 882 clone 1 strains EPS compared to dextran control, b) Monomer analysis of 882 strain EPS c) Monomer analysis of clone 1 strain EPS.

5.3.10 FTIR spectra of EPS

EPS crude extracts from St 53, Ps 3 and *C. freundii* were analysed by FTIR with the focus on the characteristic spectral region of 4000–400 cm^{-1} (see appendix). As shown in Figure 5.46 all samples contained absorption bands indicative of the presence of hydroxyl group (–OH), amine (N-H), carboxylic acid (COOH) or (C (O) OH), amide groups (C=O) and ether (C-O or C-O-C). Spectra around 3300 indicate the presence of (O-H) and (N-H) groups (Batool et al., 2015, Mangwani et al., 2014, Mahapatra and Ramachandra, 2013) while the weak band near 2925 cm^{-1} is attributed to methylene (C–H) vibrations (Batool et al., 2015, Manuzon, 2009, Mangwani et al., 2014, Sathiyanarayanan et al., 2015, Mahapatra and Ramachandra, 2013). Another spectral brand around 2357 & 2330 cm^{-1} indicated to the presence of atmospheric CO_2 and adsorbed water (Xueref and Dominé, 2003, McGoron et al., 2009, Mahapatra and Ramachandra, 2013). Bands between ~ 1740–1640 cm^{-1} are due to the presence of (C=O) vibrations in esters or fatty acids of lipids (Mahapatra and Ramachandra, 2013). Two signals near 1633 cm^{-1} (amide I) and 1538 cm^{-1} (amide II) represents (N-H) bending of protein (Batool et al., 2015, Manuzon, 2009, Mahapatra and Ramachandra, 2013, Lembre et al., 2012b). A small band near 1240 cm^{-1} may be indicated to (P=O) stretch by the phosphodiester bond suggesting the presence of nucleic acids in the crude extract (Mahapatra and Ramachandra, 2013, Lembre et al., 2012b). The spectral region from 1200 -900 cm^{-1} especially near 1000 cm^{-1} is due to (C-O-C or C-O) which is caused by ring-stretching vibrations of carbohydrates and polysaccharides (Lembre et al., 2012b, Batool et al., 2015, Manuzon, 2009, Mangwani et al., 2014, Mahapatra and Ramachandra, 2013, Naumann, 2000). The few small bands found ~ 800–550 cm^{-1} are attributed to aromatic C–H bending (Mahapatra and Ramachandra, 2013).


MMGlc
MMGly
MMEth
I
II
III
IV
V
%Reflectance
Wavenumbers (cm-1)
3289.9
2927.6
2358.0
1633.4
1538.2
1504.8
1372.4
1234.3
1026.0
3270.5
2926.1
2355.7
1633.3
1538.1
1416.9
1372.4
1234.1
1014.9
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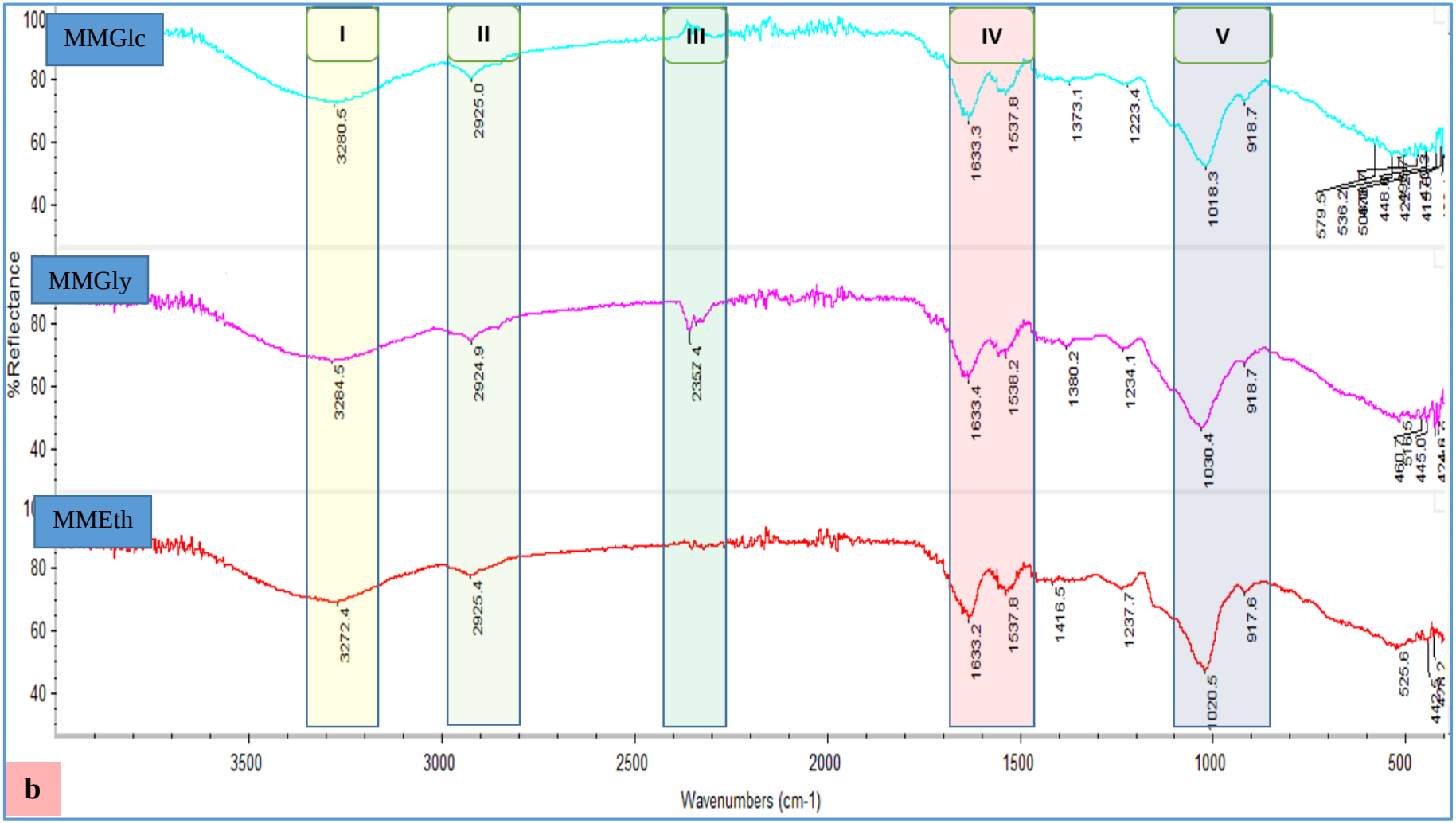
810.0
a


MMGlc
MMGly
MMEth
I
II
III
IV
V
% Reflectance
Wavenumbers (cm-1)
3500
3000
2500
2000
1500
1000
500
3280.5
2925.0
1633.3
1537.8
1373.1
1223.4
1018.3
918.7
3284.5
2924.9
2357.4
1633.4
1538.2
1380.2
1234.1
1030.4
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917.6
525.6

b

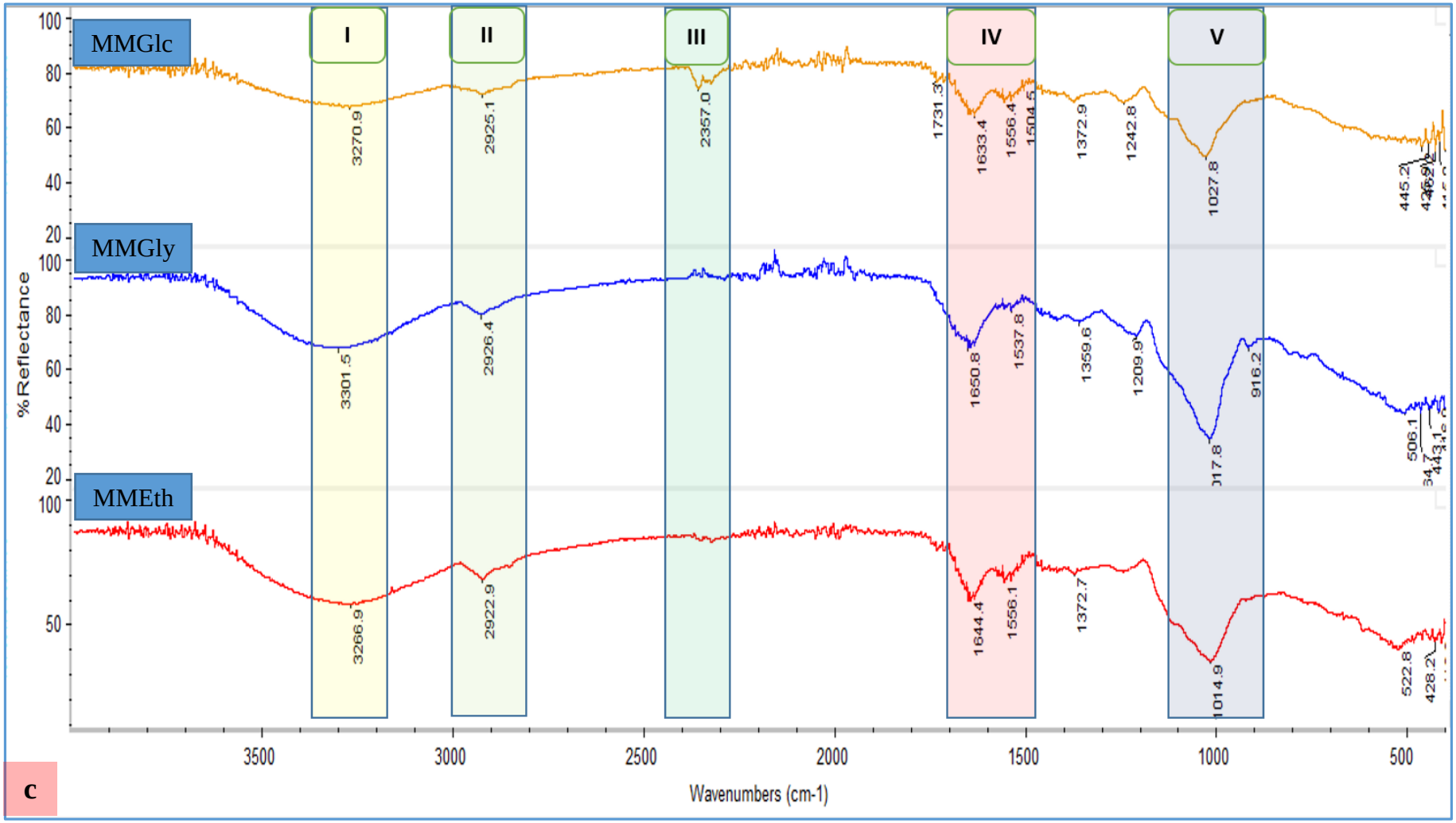
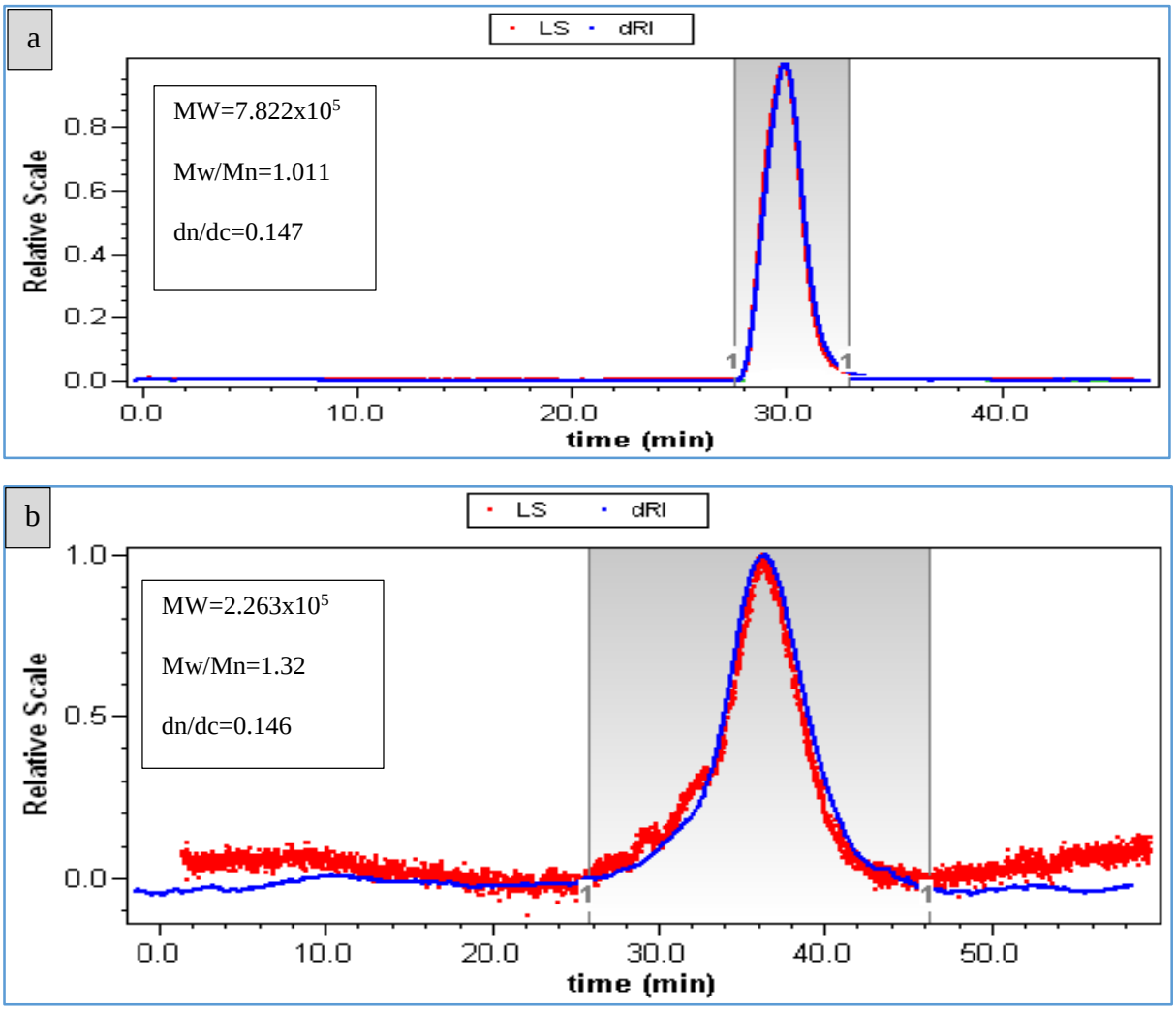


Figure 5.46 FTIR analysis of EPS produced by different bacteria a) St 53 b) Ps 3 and c) *C. freundii* showing different functional group and band assignment. I) O-H or N-H group II) C-H group III) CO2 or water IV) Protein and V) Polysaccharide.

5.3.11 Determination of Weight-average Molecular Weight for EPS

The weight-average molecular weight (Mw) of the EPS produced by Ps3, St 53 and *C. freundii* on MM Eth was determined using the HP-SEC-MALLS. As mentioned in Section 4.12.12, prior to the analysis of EPS, the accuracy of the HP-SEC-MALLS instrument was evaluated using a pullulan standard of known Mw 800,000 or/and dextran 200000-270000 Dalton (Da) with low polydispersity (DP) (Mw/Mn = ~1.23 or 1.25) respectively. The results generated showed that the HP-SEC-MALLS provided good accurate and precise results for the pullulan and dextran (Figure 5.47).

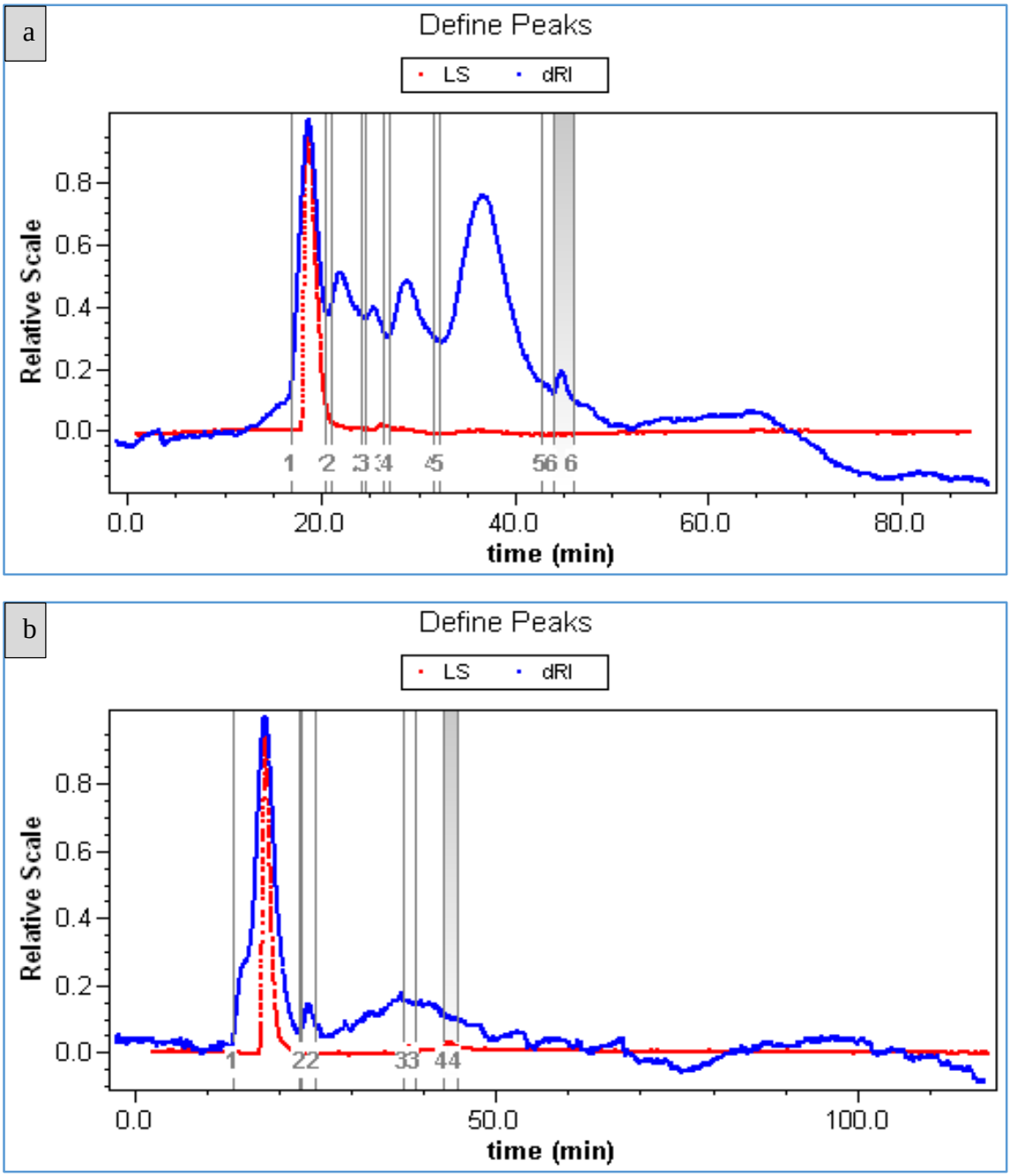


LS: Light scattering dRI: Differential refractive index

Figure 5.47 Chromatogram of standard polysaccharides (1000 ppm, flow rate 0.7ml/min, mobile phase UPW solvent RI 1.330, laser wavelength 690.0 nm, dn/dc 0.147 ml/g).
a) Pullulan and b) Dextran.

Ps3 EPS (with and without dextranase treatment) was shown to be a high molecular weight compound. In the case of Ps 3 EPS without dextranase treatment, MALLS analysis (Figure 5.48) indicated that the sample comprises of a number of different molecular weight EPSs. The SEC chromatogram trace shows 6 distinct peaks with different molecular weight ranged from ($1.1x10^5$ to $9.5x10^5$) Da with a low DP ranged from (1.025 to 1.573) while the MALLS analysis of dextranase treated Ps 3 EPS showed one main peak with very small peaks. The Mw and DP of the main peak was $6.9x10^5$ Da with 1.320 while Mw and DP of the rest were not available potentially due to very small molecular weight (Table 5.13).

MALLS of St 53 EPS without dextranase treatment showed three distinct peaks indicative of high molecular weight compounds while treated EPS showed two high Mw peaks and one with non-available data .The MALLS of treated and non-treated EPS of *C. freundii* showed high molecular weight molecules ranging from 10^5 to 10^6 Da. In all bacterial strains (Ps 3, St 53 and *C. freundii*), the MALLS of the treated EPS, showed that the concentration of the first peak increased while the rest of peaks were decreased comparing to the non-treated EPS (Table 5.13).



LS: Light scattering dRI: Differential refractive index

Figure 5.48 HP-SEC-MALLS analysis of Ps 3 EPS a) EPS without dextranase treatment b) EPS treated with dextranase.

Table 5.13 HP-SEC-MALLS analysis of dextranase treated and non-treated extracted EPS from Ps 3, St 53 and *C. freundii*.

Bacterial EPS	MALLS analysis	Peak 1	Peak 2	Peak 3	Peak 4	Peak 5	Peak 6
Ps 3 EPS (Without dextranase treatment)	^Mw & Dist.	$5.7x10^5$ (20.41%)	$1.1x10^5$ (12.11%)	$3.1x10^5$ (6.32%)	$4.7x10^5$ (15.65%)	$3.9x10^5$ (42.99%)	$9.5x10^5$ (2.52%)
	Polydispersity	1.573	1.139	1.102	1.016	1.130	1.025
Ps 3 EPS (Dextranase treatment)	Mw & Dist.	$6.9x10^5$ (97.4%)	n/a (0.87%)	n/a (0.99%)	n/a (0.74%)	-	-
	Polydispersity	1.320	n/a	n/a	n/a	-	-
St 53 EPS (Without dextranase treatment)	Mw & Dist.	$3.6x10^5$ (15.53%)	$3.0x10^6$ (45.35%)	$8.5x10^5$ (39.12%)	-	-	-
	Polydispersity	1.593	1.222	1.187	-	-	-
St 3 EPS (Dextranase treatment)	Mw & Dist.	$2.7x10^5$ (87.98%)	$6.2x10^5$ (6.69%)	n/a (5.53%)	-	-	-
	Polydispersity	1.040	1.009	n/a	-	-	-
C. freundii EPS (Without dextranase treatment)	Mw & Dist.	$2.3x10^6$ (30.10%)	$2.2x10^6$ (21.70%)	$1.0 x10^6$ (48.2%)	-	-	-
	Polydispersity	1.198	1.028	1.029	-	-	-
C. freundii EPS (Dextranase treatment)	Mw & Dist.	$4.7x10^5$ (85.35%)	$3.0x10^5$ (6.89%)	$8.4x10^5$ (7.76%)	-	-	-
	Polydispersity	1.153	1.009	1.640	-	-	-

^ Mw ; Molecular average molecular weight (Dalton) Dist; Distribution of the molecule (%)

5.3.12 Discussion

One of the most important aspects of the study of bacterial EPS production and its associated characterisation, is the selection of a media that is free of any interfering substances e.g. yeast extract (Alhudhud et al., 2014). In this study, several broth media (TSB, LB and SWF media) were ruled out due to the presence of these interfering substances (Figure 5.22). However TSB and LB broth have both been used in previous study (Subramaniam et al., 2007, Yilmaz et al., 2012, García et al., 2015), throwing into doubt the accuracy of currently published data. During the selection of a media for EPS production and characterisation, proton NMR analysis is not sufficient on its own to determine the presence of interfering substances since proton NMR analysis depends on the dissolution of the sample in the solvent i.e. solubility is one limiting factor in liquid state proton NMR analysis (de Alvarenga, 2011). For example the crude substance extracted from SWF medium was poorly soluble in D_2O and consequently generated no spectra in the anomeric region during proton NMR analysis which would suggest that it might be a good candidate for EPS investigations. However, monomer analysis by HPAEC-PAD of the same sample (SWF crude substance) showed several sugar peaks providing evidence for the presence of polysaccharides (Figure 5.22). Beside proton NMR and HPAEC, the phenol sulphuric acid (Dubois et al., 1956) and Bradford (Bradford, 1976) tests showed the presence of sugars and significant amounts of protein in the SWF extract (Table 5.7). In order to avoid the problem associated with interfering substances, several mineral media with different carbon sources (MM Glc, MM Gly and MM Eth) were developed and modified. These media were free of interfering polysaccharides as demonstrated by proton NMR, the phenol sulphuric acid and Bradford tests and monomer analysis (Figure 5.23 and Table 5.7).

EPS production was optimised in MM Glc and MM Eth by studying the effects of inoculum size (10^4 or 10^7 CFU/ml), incubation temperature (30 or 37˚C) and

incubation time on the utilisation of the carbon source, total carbohydrate and polysaccharide (EPS) production during bacterial growth. According to this study, polysaccharide (EPS) production was optimised when a starting inoculum of 10^7 CFU/ml was employed with an incubation temperature of 30˚C and an incubation period of 72-80 h (≈ 80 h). This was considered optimum for polysaccharide (EPS) production in St 53 (Figure 5.24c and 5.25c) and Ps3 (Figure 5.26c and 5.27c). Lower initial inoculum sizes (10^4 CFU/ml) and higher incubation temperatures (37˚C) generated less EPS in broth culture (Figure 5.24, 5.25, 5.26 and 5.27 a & b). These observations were confirmed in both growth curve experiments (Sections 5.3.3 and 5.3.4) and through the extraction and analysis of the EPS via proton NMR analysis (Section 5.3.6.1). During the latter stage an additional bacteria (*C. freundii*) and an additional media (MM Gly) were added to the study.

Optimisation based on the extraction and characterisation of the EPS via proton NMR confirmed the observations made during the growth curve experiments that the optimum conditions were an initial inoculum size of 10^7 CFU/ml, an incubation temperature of 30˚C and an incubation period of for 72-80 h. Whilst this optimisation stage confirmed the growth conditions required for good EPS generation it also optimised the extraction methodology used in the process. This was focussed on the amount of TCA used in the extraction process, which is included for the precipitation of proteins, DNA and lipids (Bales et al., 2013). However the data presented here suggests that in some cases too much TCA may be altering the quantity and quality of the EPS extracted. When used at 14 and 10 % TCA caused a reduction in EPS yield and the disappearance of some peaks from the proton NMR spectra suggesting that it may be either degrading the EPS or precipitating some EPS components with the proteins (Figure 5.29 and 5.31) (Alhudhud et al., 2014, Madhuri and Prabhakar, 2014). The impact of TCA varied between bacteria and in some cases between carbon sources used by the same bacteria (5.3.6.1 to 5.3.6.3). Several TCA concentrations have been used in

previous studies, ranging from 4% (Kılıç and Dönmez, 2008), 13% (Silva et al., 2012), 14% (Leivers, 2011) to 20% (Bales et al., 2013). The data presented here suggests that the use of TCA should be optimised on a case by case basis to ensure an effective EPS extraction process.

The three bacteria that are the focus of this section strains (Ps 3, St 53 and *C. freundii*) all produced an EPS with a significant component with a strong homology with dextran (Figure 5.39). The presence of this dextran like component was independent of the carbon source and was susceptible to digestion by dextranase (Sections 5.3.9). Removal of this dextran component revealed a second more complex EPS component that was mannose dominated with a range of additional sugars (See Table 5.12).

Dextran is an extracellular homopolysaccharide characterised by a backbone of α- (1→6) linked D- glucose and variable amounts of α- (1→2), α- (1→3), α- (1→4) branching. Dextran based extracellular polysaccharides are commonly associated with Gram positive lactic acid bacteria such as *Streptococcus, Leuconostoc, Lactobacillus* and *Weissella* species (Burmølle et al., 2014, Monsan et al., 2001) Within a clinical setting, the ability of *Streptococcus mutans* to generate extracellular dextran to aid adherence through biofilm formation upon dental plaques has also been described (Bowen and Koo, 2011). Evidence of dextran production by Gram negative bacteria within the literature is scarce, however Milintawisamai and colleagues observed the production of dextran by *Klebsiella pneumoniae* and *Enterobacter cloacae* isolated from a sugarcane factory (Milintawisamai et al., 2009). More recently, the work of Jowiya et al showed that α-dextran containing exopolysaccharide production was stimulated in *Campylobacter jejuni* ssp when in the presence of pancreatic amylase (Jowiya et al., 2015).

P. aeruginosa is associated with the generation of a range of extracellular polymeric substances (EPS) including three polysaccharides identified as

Alginate, Psl and Pel (Franklin et al., 2011, Bazaka et al., 2011, Sharma et al., 2014). The formation of alginate is associated with mucoid growth and respiratory tract infection in cystic fibrosis patients (Whitfield et al., 2015). The clinical importance of alginate formation has resulted in this being the most studied of the *P. aeruginosa* polysaccharides. However, Ps 3 which is the focus of this investigation did not generate an alginate, as has been shown by the monomer analysis studies outlined in section 5.3.8 This is despite the fact that Ps 3 genome contains a full complement of alginate biosynthesis operons (PA3540-3548) associated with *Pseudomonas aeruginosa* PA01 (Sharma et al., 2014) (http://www.pseudomonas.com).

The Psl polysaccharide has been characterised as a neutral, branched polysaccharide with repeating D-mannose, D-glucose, and L-rhamnose sub units (Byrd et al., 2009). The Pel polysaccharide on the other hand is associated with pellicle formation and remains uncharacterised, it is generally referred to as a glucose rich polymer (Friedman and Kolter, 2004a, Whitfield et al., 2015). Ps 3 contains a full complement of the pel (PA3058-PA3064) and psl (PA2231-2245) biosynthesis operons identified in *Pseudomonas aeruginosa* PA01(Sharma et al., 2014) (http://www.pseudomonas.com). Ps 3 generated a pellicle and by inference the Pel polysaccharide under the conditions investigated here (See section 5.2.7). Analysis of the EPS generated by Ps 3 suggests that the Pel polysaccharide is a dextran rich polymer which is amendable to degradation by fungal dextranases. This analysis is consistent with the description of the Pel polysaccharide as a glucose rich polymer (Ryder et al., 2007, Ma et al., 2009).

Previous studies have suggested that the pel was cellulose-like exopolysaccharide and its biosynthesis is the least characterised *P. aeruginosa* exopolysaccharide (Ghafoor et al., 2013). Franklin and colleagues suggested that Pel biosynthesis is more similar to that of alginate and cellulose (Franklin et al., 2011). Once the dextran polymer has been removed the remaining EPS resembles the well

characterised Psl polysaccharides as indicated by the presence of mannose as a major component (Friedman and Kolter, 2004b, Branda et al., 2005) (Table 5.12). The generation of mannose rich EPS has been identified in a wide range of both Gram positive and negative bacteria (Bales et al., 2013). In this study mannose represented over 75% of the EPS once the dextran had been removed among all the bacterial strains (Ps 3, St 53 and *C. freundii*). This is similar to the results from a similar study where 80-90% of EPS that was extracted from *Pseudomonas aeruginosa* and *Acinetobacter baumannii* was composed of mannose, while *Klebsiella pneumoniae* and *Staphylococcus epidermidis* strains containing 40–50% mannose (Bales et al., 2013).

The EPS generated *by S. maltophilia* has not been as extensively researched as that of *P. aeruginosa*. The data that has been generated as part of this study does not align with what little data is available in the literature. For example Cescutti et al (2011) described the *S. maltophilia* EPS as a highly charged polymer due to the presence of three uronic acids and an ether-linked D-lactate substituent. Methylation analysis suggested 4-linked Glc, 4-linked GlcA and 3, 4-linked GalA in equal molar amounts in the native polymer (Cescutti et al., 2011). Cuzzi (Cuzzi, 2012) also detected uronic acids (glucuronic acid (GlcA), galacturonic acid (GalA)) in *S. maltophilia* EPS alongside glucose.

The data presented in this research suggests that *S. maltophilia* in common with *P. aeruginosa* is capable of synthesising more than one EPS depending on the environment and that pellicle formers such as Ps 3 and St 53 respond to an environments that favour pellicle formation by generating an EPS that is glucose rich.

Biofilm formation amongst pathogenic *Enterobacteriaceae* such as *Citrobacter* spp. has been associated with the production of a cellulose based EPS and/or curli fimbre that are common in catheter-associated UTI (Zogaj et al., 2003). Other authors such as Jang et al (2005) have suggested that *Citrobacter* spp. EPS mainly

consists of galactose, glucose and mannose (Jang et al., 2005). While in another study (Rättö et al., 2006), *Citrobacter* spp. produced EPS that was composed mainly of fucose, rhamnose, and other sugars such as galactose, glucose, mannose, galacturonic acid, glucuronic acid and pyruvate. The compositions in these latter studies are closer to composition determined in this study, which once again underlines the fact that EPS generation can be highly varied even within species.

The genome of St 53 and *C. freundii* showed the presence of the entire *pgaABCD* locus. This locus which is recognised to encode the synthesis of a polysaccharide poly-β-1,6-N-acetyl-D glucosamine (β-1,6-GlcNAc, PGA). This polysaccharide (PGA) is present in the extracellular matrix of biofilms generated by a wide range of bacteria including *Escherichia coli*, *Actinobacillus pleuropneumoniae*, *Yersinia pestis*, *Bordetella* spp. In these bacteria, PGA has been ascribed to mediating surface attachment, aggregation and adhesion for stabilizing biofilm formation as well as protecting against environmental stresses and antimicrobial resistance (Wang et al., 2004, Shanmugam et al., 2015).

Numerous analytical techniques were used in this study for the analysis of bacterial EPS. One of these methods is FTIR spectroscopy that measures the vibrations of functional groups the mixtures (Castro et al., 2014). FTIR spectroscopy showed the presence of EPS with all its component like polysaccharide (carbohydrate), protein and may be nucleic acid also. However, the NMR showed very similar spectra between extracted EPS from all 3 bacteria (Ps 3, St 53 and *C. freundii*) on different carbon sources media but the fingerprint region 900– 1400 cm^{-1} showed the difference between these EPS and this due to the band shifts in these regions. Different EPS were characterised by FTIR in previous study such as EPS from acidophilic microbial biofilm (Jiao et al., 2010) and *Bacillus subtilis* (Omoike and Chorover, 2004).

5.3.12 Key finding

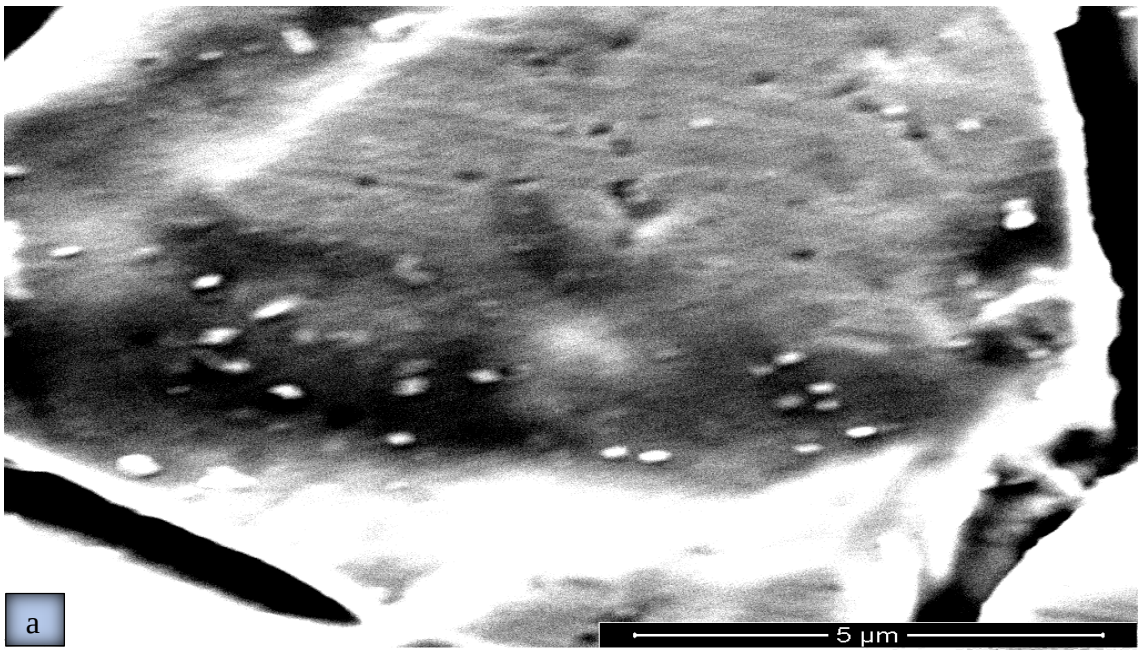
- TSB, LB and SWF were not suitable media for the investigation of EPS production and characterisation due to the presence of interfering substances.
- A range of modified mineral media with different carbon source (MM Glucose, MM Glycerol and MM Ethanol) were developed that were suitable for the study EPS production and characterisation due to the absence of interfering substances.
- 10^7 CFU/ml of bacteria as initial inoculum, and incubation at 30˚C for approximately 80h with 0-5% TCA for protein treatment, represent the optimum conditions for EPS production and extraction for Ps3, St 53 and *C. freundii*.
- During growth and biofilm formation on MM Glucose, MM Glycerol and MM Ethanol, Ps3, St 53 and *C. freundii* produced a complex EPS that was composed of at least two polysaccharides in which one of them is a dextran like polymer.
- EPS sugar components from Ps3, St 53 and *C. freundii* were very similar and differed in only 1 or 2 sugars.
- The Pel polysaccharide of Ps 3 is composed of dextran and the Psl polysaccharide has a mannoses rich composition.

5.4.Pathogenicity of biofilm forming *Pseudomonas aeruginosa* strains

This chapter discusses the pathogenicity and virulence factors of *Pseudomonas aeruginosa* (Ps 3 and Ps 10421). The pathogenicity of these strain is discussed in terms of their OMV and their impact on the wax worms' *G. mellonela* infection model. Also studying bacterial survival in water.

5.4.1 Production and extraction of OMV

OMV were extracted as described in (Section 4.11). After overnight incubation, Ps 10421 (lab strain) failed to produce OMV on both MM Glc and MM Eth while Ps 3 (hospital strain) produced OMV on both media. The absence of bacterial growth on TSA after incubation with 100 µl of extracted OMV (suspended in 3 ml HEPES) demonstrated the purity (no contaminant) of the Ps 3 OMV. Protein determination via the Bradford test showed the concentration of OMV extracted to be 346 and 538 µg/ml on MM Glc and MM Eth respectively. Ps 3 OMV were also visualised via SEM as a pure extract and during biofilm formation (Figure 5.49).



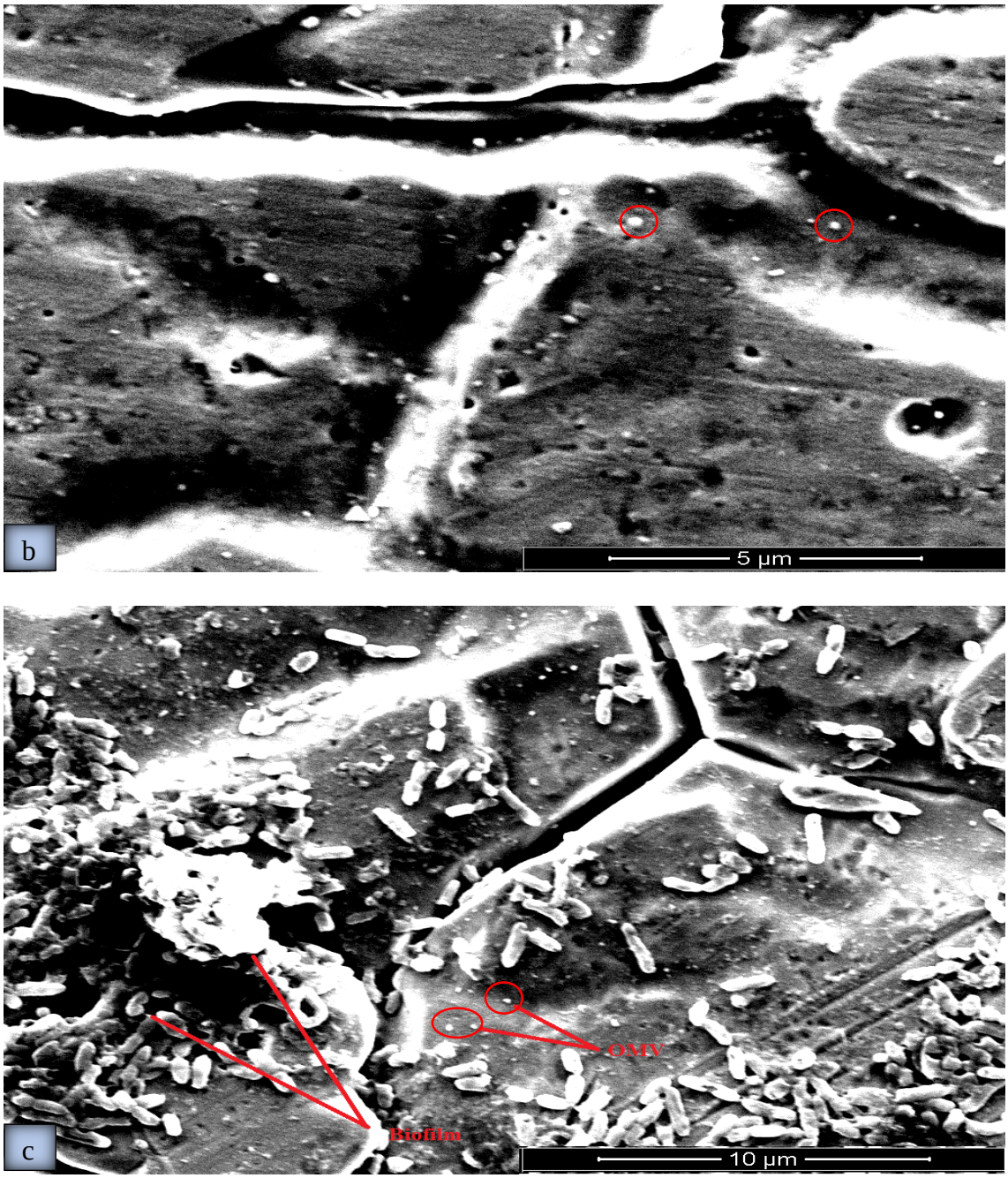


Figure 5.49 SEM of Ps 3 of Biofilm and extracted OMV a) OMV MM Glc, b) OMV MM Eth and c) Biofilm and OMV on coupon MM Eth.

5.4.2 Bacterial growth and biofilm formation

Bacterial growth on both MM Glc and MM Eth at different interval 24, 40, and 80 h showed different colour formation due to carbon sources, the age of the culture and the extent of biofilm formation (Figure 5.50).

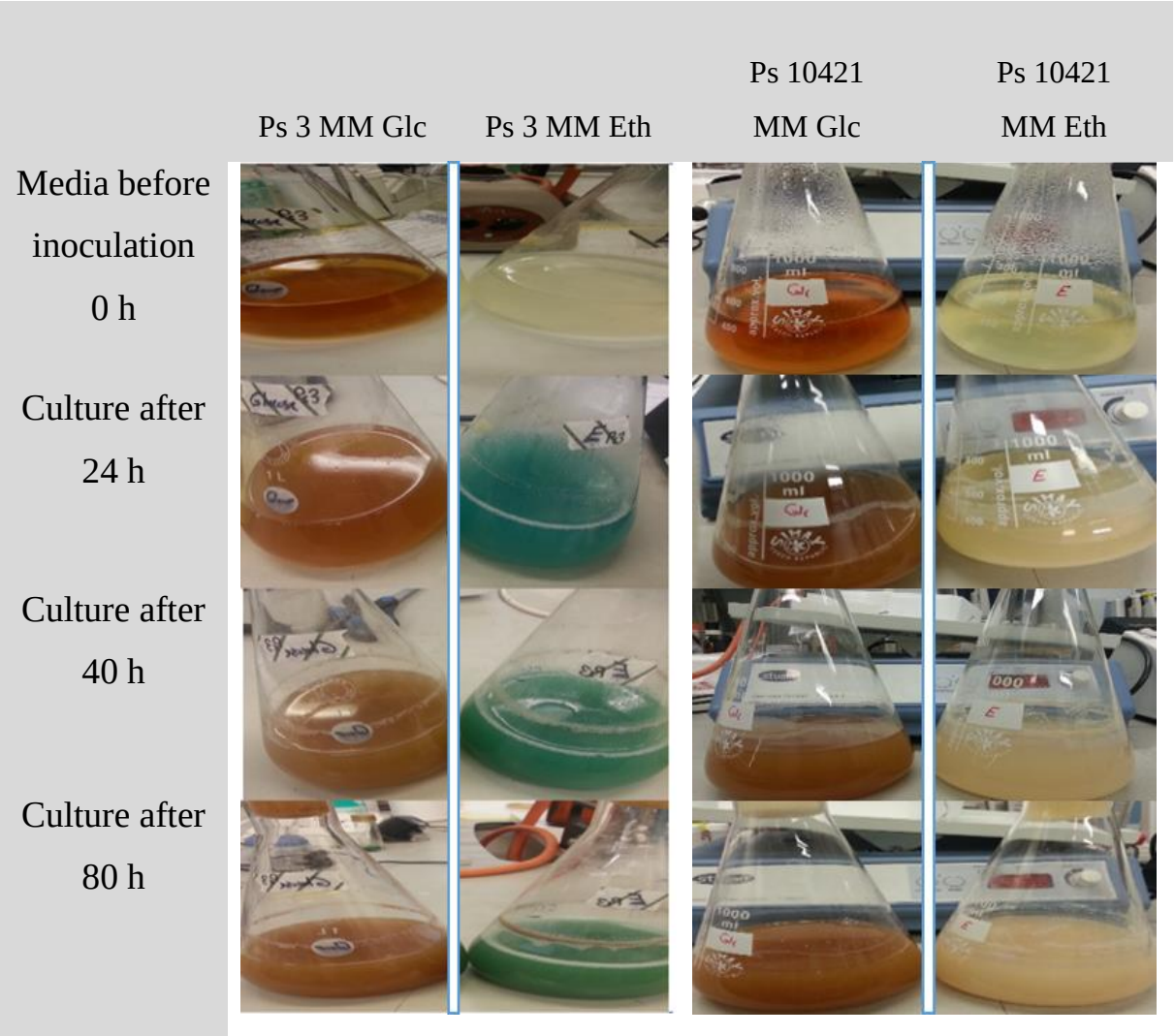


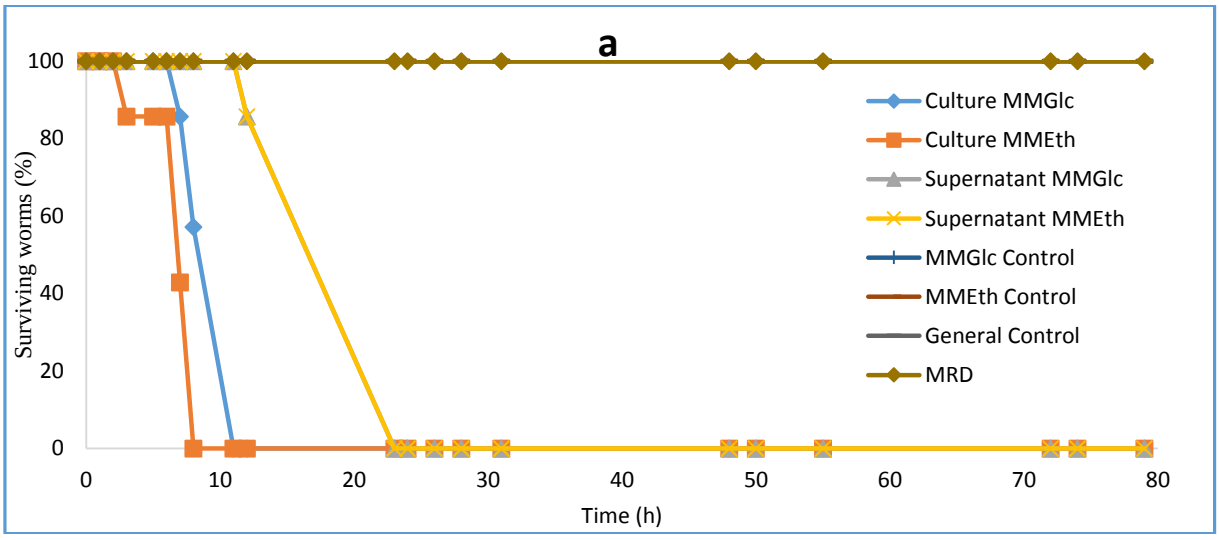
Figure 5.50 Bacterial growth and biofilm formation of Ps 3 and Ps 10421 on different carbon sources for 80 h incubation at 30˚C.

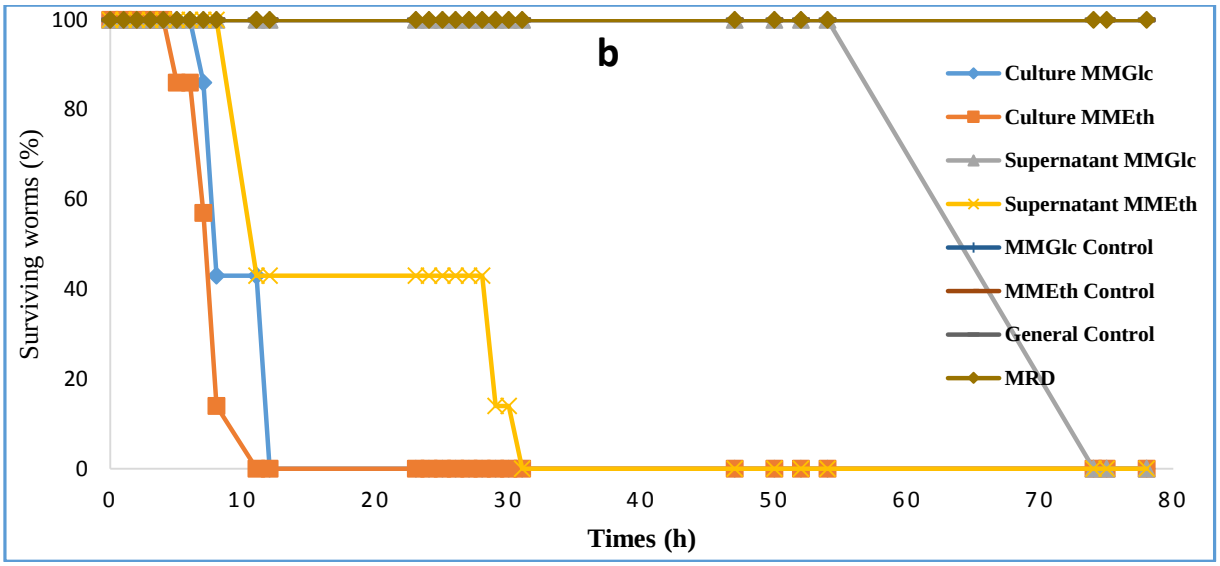
5.4.3 Preliminary pathogenicity investigations

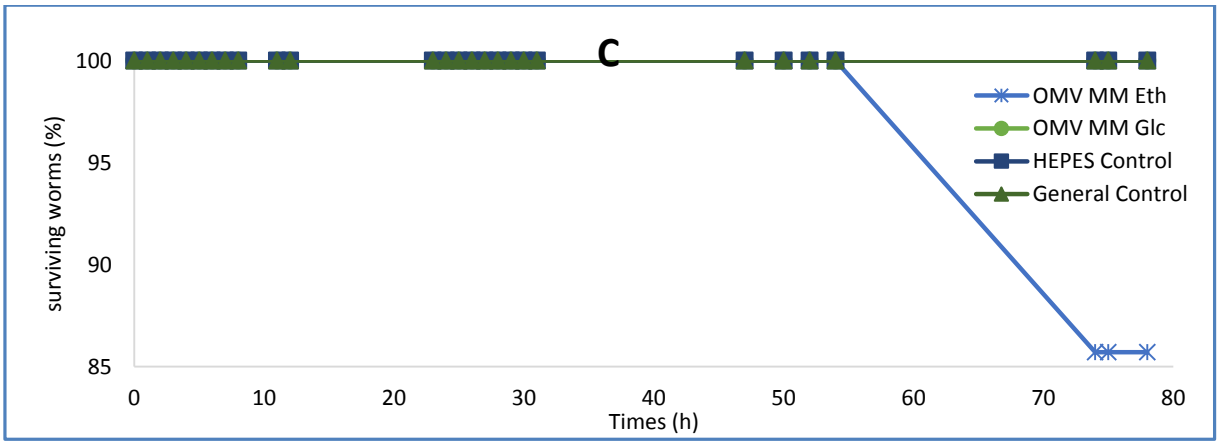
Preliminary pathogenicity investigations focussed on proving the method and developing the necessary controls. Initially worms were infected with 10 µl of a 40 h culture of the lab strain (Ps 10421) grown on either MM Eth or MM Glc and diluted to 10^7 CFU/ml in MRD (100000 cells per larvae). Under these conditions no worms survived 8 h after exposure to MM Eth grown bacteria but survived up to 11 h when exposed to MM Glc grown bacteria. Compared to 10^7 CFU/ml, the worms demonstrated a greater survival when exposed to the same

volume of cell free culture supernatants, under these conditions there was 86% survival after 12, but 100% mortality after 24 h, (Figure 5.51a). This indicates that the bacteria were responsible for the mortality rather than any toxins that the bacteria may produce or the OMV.

When this investigation was repeated with Ps 3 the worms had a better survival rate suggesting that Ps 3 was less pathogenic than the lab strain. When grown on MM Eth, Ps 3 generated a 0% survival within 8-11 h, however when grown on MM Glc there was a 43% survival at 11 h and a total kill at 12 h post infection. The cell free supernatant taken from Ps 3 grown on MM Eth and MM Glc required 31 and 54 h respectively to generate a complete kill (Figure 5.51b). This data again support the observation that the lab strain is more pathogenic than Ps 3. The pathogenicity of extracted OMV from Ps 3 growth on both MM Glc and MM Eth was also studied, with 100% survival in both cases for up to 54 h. (Figure 5.51c). In addition to the experiments outlined above a range of controls were run to check the toxicity of media and diluents such as MRD, MM Glc, MM Eth and HEPES buffer (figure 5.51). In all cases there was 100% survival of the worms up to 3 days post exposure, indicating the absence of toxicity of any of these media. At the same time uninoculated controls (General control) survived also survived for more than 3 days, these control indicated that the incubation conditions (temperature etc.) had no deleterious impact on the worms.







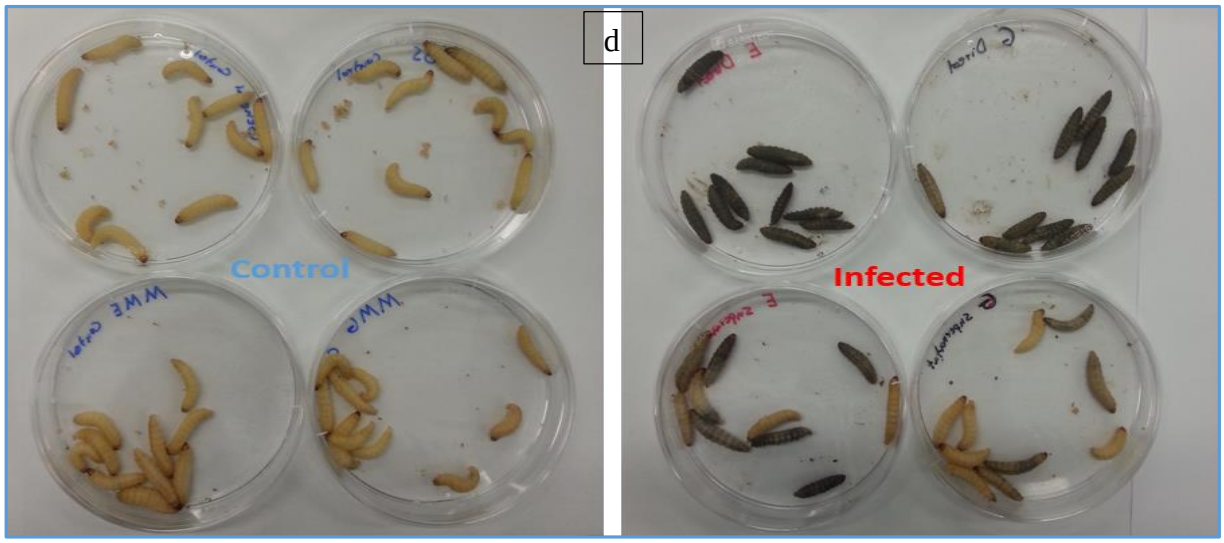


Figure 5.51 Pathogenicity of bacterial growth on different carbon sources using wax worms infection model. The symbol represents pathogenicity of bacterial culture and supernatant free CFU from a) 40 h old culture Ps 10421 b) 40 h old culture Ps 3 c) OMV and d) Control and infected worms.

5.4.5 Pathogenicity of Ps 3 after 24 and 80 h incubation

Following the previous experiments, a number of additional investigations were carried out. In these experiments a slightly improved methodology was employed and the age of the cultures was aligned with the EPS generation observed in previous experiments (Section 5.3.4).

The direct injection of 24 h Ps 3 cultures grown in MM Glc with $1.82x10^9$ CFU/ml or $1.82x10^7$ cells per larva (no washing or dilution) generated a complete kill within 9h post injection, the same bacteria grown on MM Eth with ($1.64x10^9$ CFU/ml or $1.64x10^7$ cells per larvae) generated a complete kill after 7 h. The injection of cell free supernatants further illustrated the difference between the MM Glc and MM Eth grown cultures with the glucose supernatant being considerable less toxic (Figure 6.4). The data showed obvious difference between the pathogenicity of bacterial culture and supernatant of the Ps3 that grow on MM Glc and MM Eth (Figure 5.52)

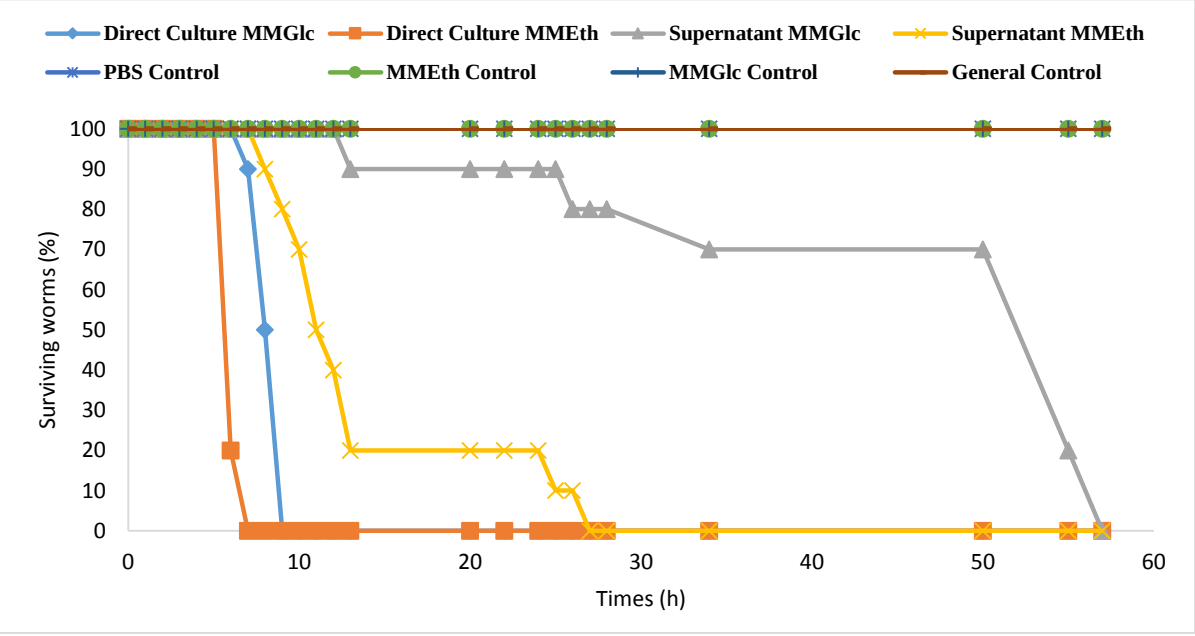


Figure 5.52 Ps 3 bacterial culture and free cells supernatant pathogenicity in wax worms after infection from 24 h old culture.

When 80 h Ps 3 cultures were employed for the same investigation, the direct injection of MM Glc ($9.65x10^8$ CFU/ml or $9.65x10^6$ cells per larvae) and MM Eth ($1.05x10^9$ CFU/ml or $1.05x10^7$ cells per larvae) cultures generated complete kills after 8 and 6 h respectively. The culture supernatant (cell free) also showed the same pattern as shown by the fact that the survival of worms infected with MM Glc supernatant was 40% after 24h while the survival worms that infected with MM Eth was 0% after 12 h. Worms inoculated with sterile PBS, MM Glc and MM Eth as controls, demonstrated 100% survival and exhibited no change in shape, colour or activity over the incubation period (Figure 5.53).

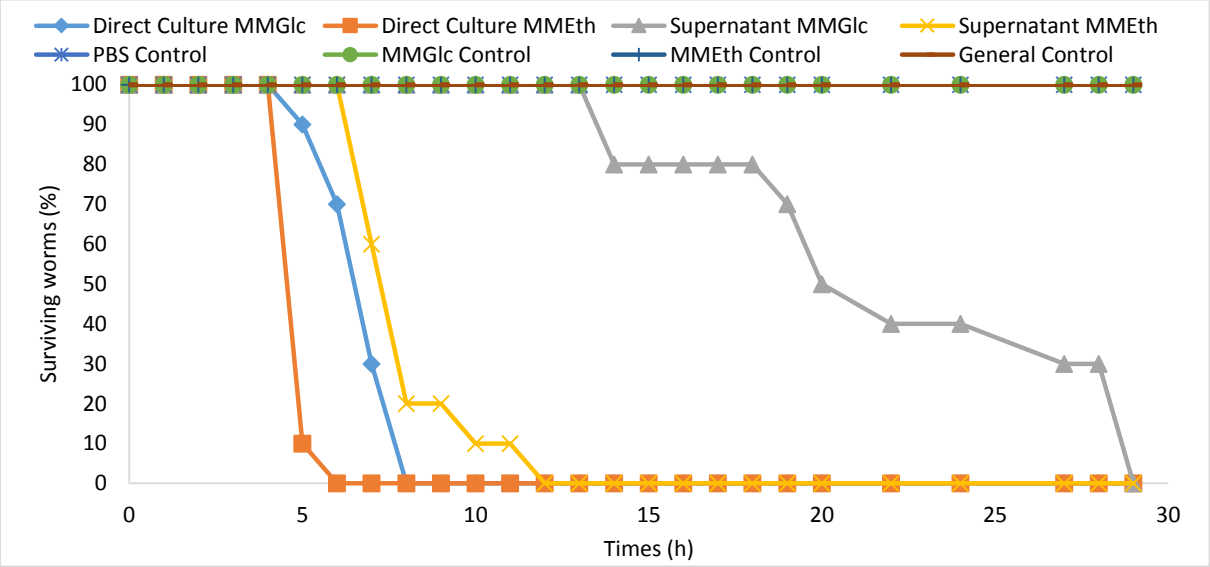


Figure 5.53 Ps 3 bacterial culture and free cells supernatant pathogenicity in wax worms after infection from 80 h old culture.

5.4.6 Lethal Dose (LD50) estimations for Ps 3 obtained from 24 and 80 h old cultures

In order to determine LD50 values for Ps 3, cells were harvested, washed in PBS and then serially diluted prior to worm injection to generate a dose response curve. This was completed for Ps 3 grown on MM Glc and MM Eth over 24 and 80 h incubation times. As expected the data indicated that the mortality rates amongst the worms were decreased with decreasing bacterial inoculum size on both media (Figure 5.54). The LD50 values were calculated after 12 h of

infection (Figure 5.54 c & d) and the data showed that LD50 for Ps 3 grown on MM Glc was $3.16x10^8$ CFU/ml ($3.16x10^6$ CFU/larvae) whilst that for Ps 3 grown on MM Eth was an order of magnitude lower at $1.36x10^7$ CFU/ml ($1.36x10^5$ CFU/larvae).

Direct injection of 24 h culture media containing $1.24x10^9$ CFU/ml (MM Eth, $1.24x10^7$ CFU/larvae) and $1.62x10^9$ CFU/ml (MM Glc, $1.62x10^7$ CFU/larvae) (Figure 5.52) killed the worms faster than injections of PBS washed cells containing the same overall levels of bacteria (MM Eth $1.15x10^9$ CFU/ml, $1.15x10^7$ CFU/larvae and MM Glc $1.63x10^9$ CFU/ml, $1.63x10^7$ CFU/larvae).

LD50 determinations using 80 h cultures (Figure 5.55) again demonstrated the greater pathogenicity of ethanol grown Ps 3 (Figure 5.55 a & b) with the LD50 for glucose grown bacteria being $1.0x10^7$ CFU/ml ($1.0x10^5$ CFU/larvae) and the LD50 for ethanol grown Ps 3 being once again an order of magnitude lower $1.73x10^6$ CFU/ml ($1.73x10^4$ CFU/larvae). These LD50's are also an order of magnitude lower than those obtained from 24 h culture grown on the same media (Figure 5.56).

Direct injection of 80 h MM Eth culture media containing $1.05x10^9$ CFU/ml ($1.05x10^7$ CFU/larvae) and MM Glc media containing $9.65x10^8$ CFU/ml ($9.65x10^6$ CFU/larvae) (Figure 5.53) killed the worms faster than the injections of PBS washed cells containing approximately the same overall levels of bacteria (MM Eth $9.5x10^8$ CFU/ml, $9.5x10^6$ CFU/larvae and MM Glc $1.25x10^9$ CFU/ml, $1.25x10^7$ CFU/larvae) respectively (Figure 5.55).

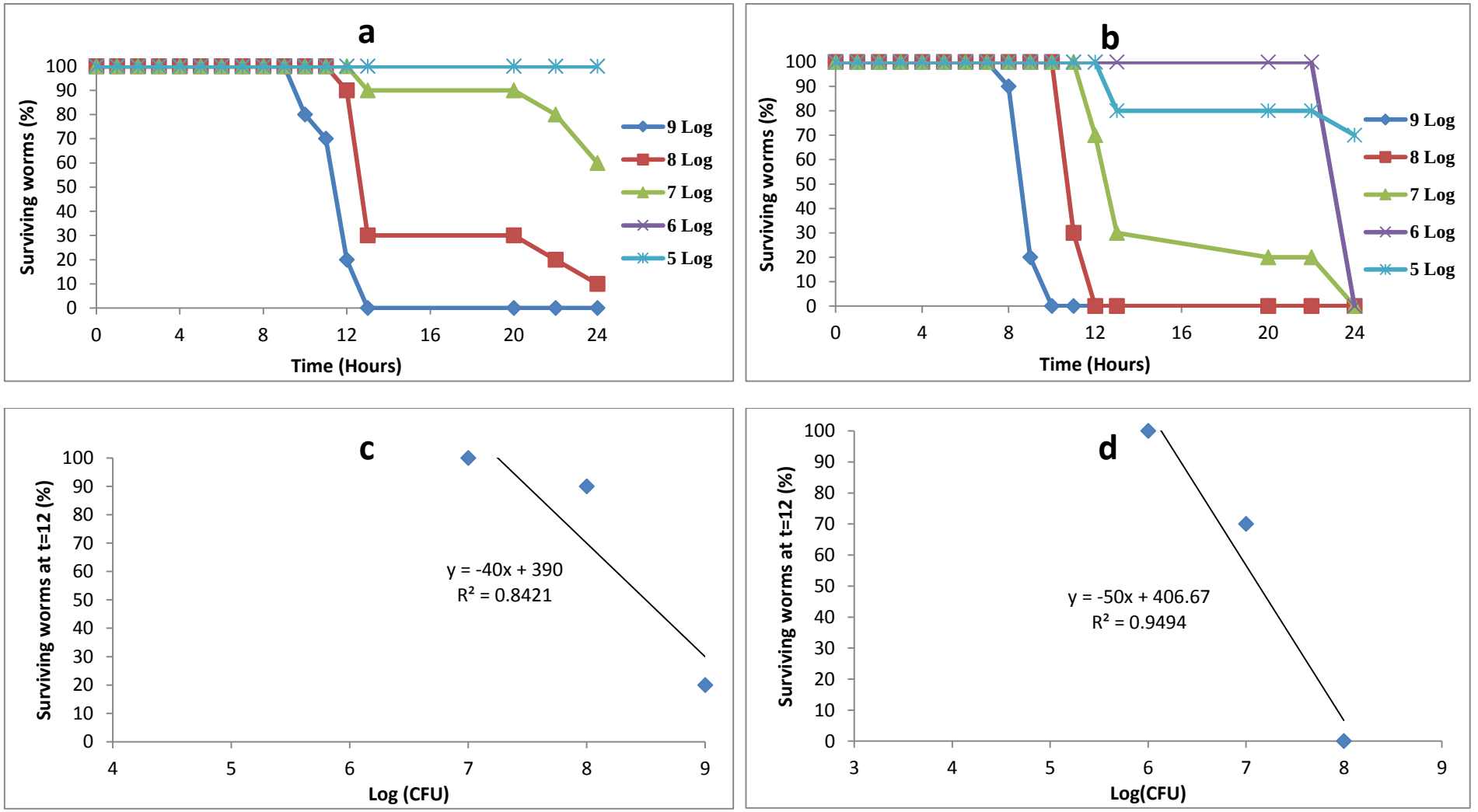


Figure 5.54 Pathogenicity of different inoculum size and LD50 curve from (24 h Ps 3 old culture) in wax worms. a) MM Glc b) MM Eth c) LD50 curve on MM Glc d) LD50 curve on MM Eth.

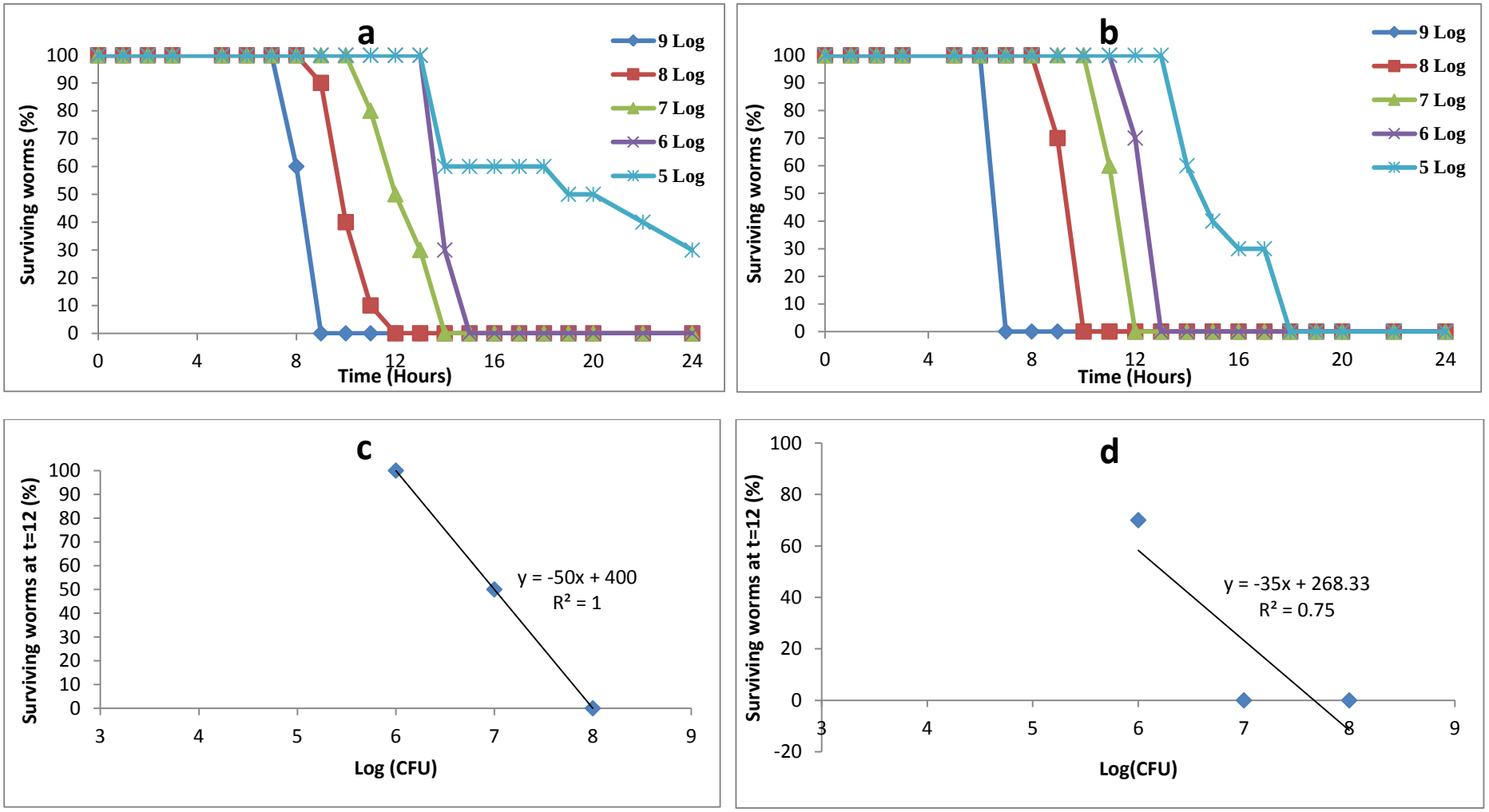
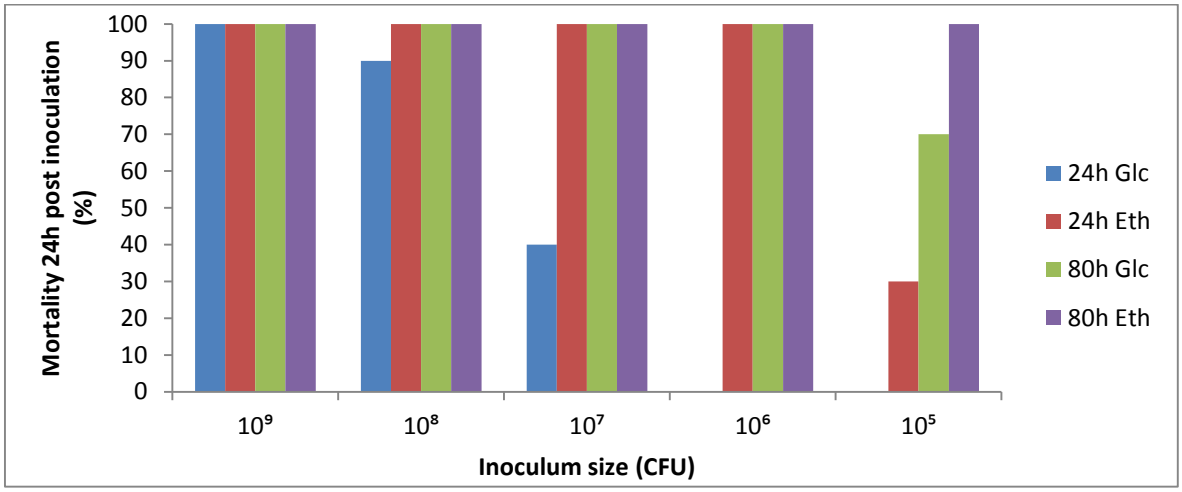
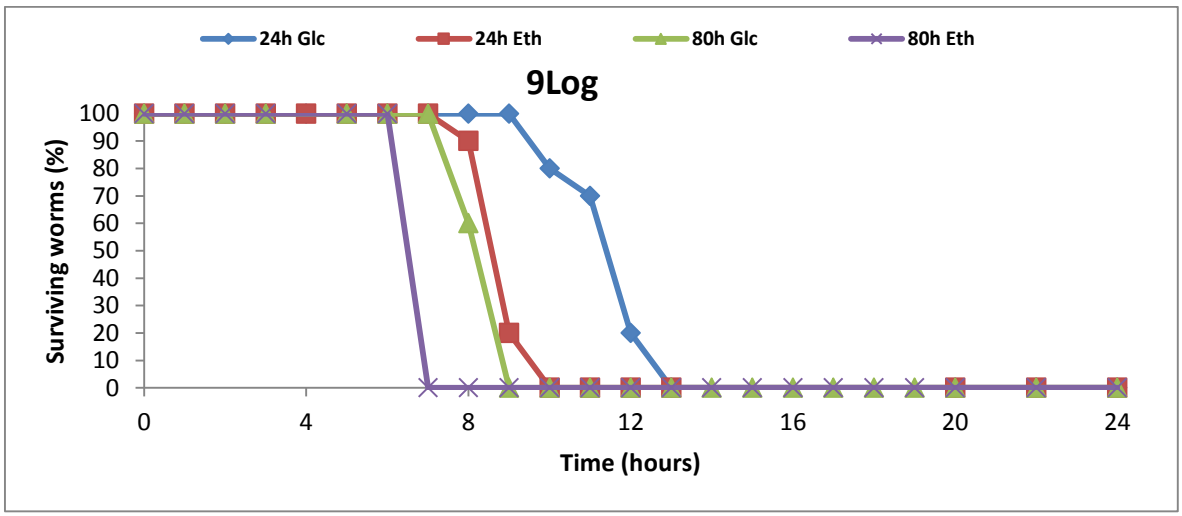
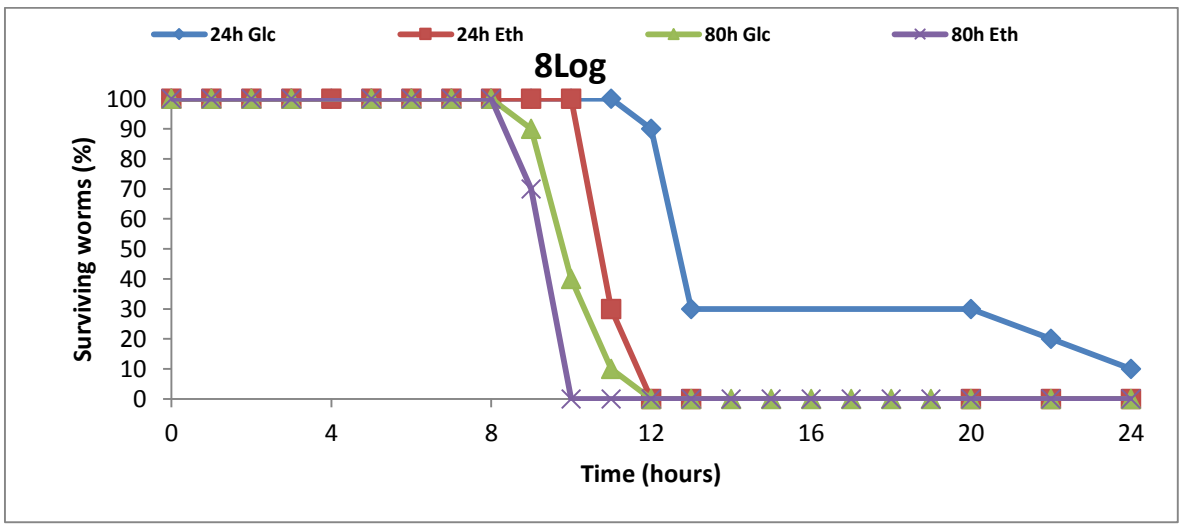


Figure 5.55 Pathogenicity of different inoculum size and LD50 curve from (80 h Ps 3 old culture) in wax worms. a) MM Glc b) MM Eth c) LD50 curve on MM Glc d) LD50 curve on MM Eth.


Mortality 24h post inoculation (%)
100
90
80
70
60
50
40
30
20
10
0
10^9
10^8
10^7
10^6
10^5
Inoculum size (CFU)
24h Glc
24h Eth
80h Glc
80h Eth


24h Glc
24h Eth
80h Glc
80h Eth
9Log
Surviving worms (%)
100
90
80
70
60
50
40
30
20
10
0
0
4
8
12
16
20
24
Time (hours)


24h Glc
24h Eth
80h Glc
80h Eth
8Log
Surviving worms (%)
100
90
80
70
60
50
40
30
20
10
0
0
4
8
12
16
20
24
Time (hours)

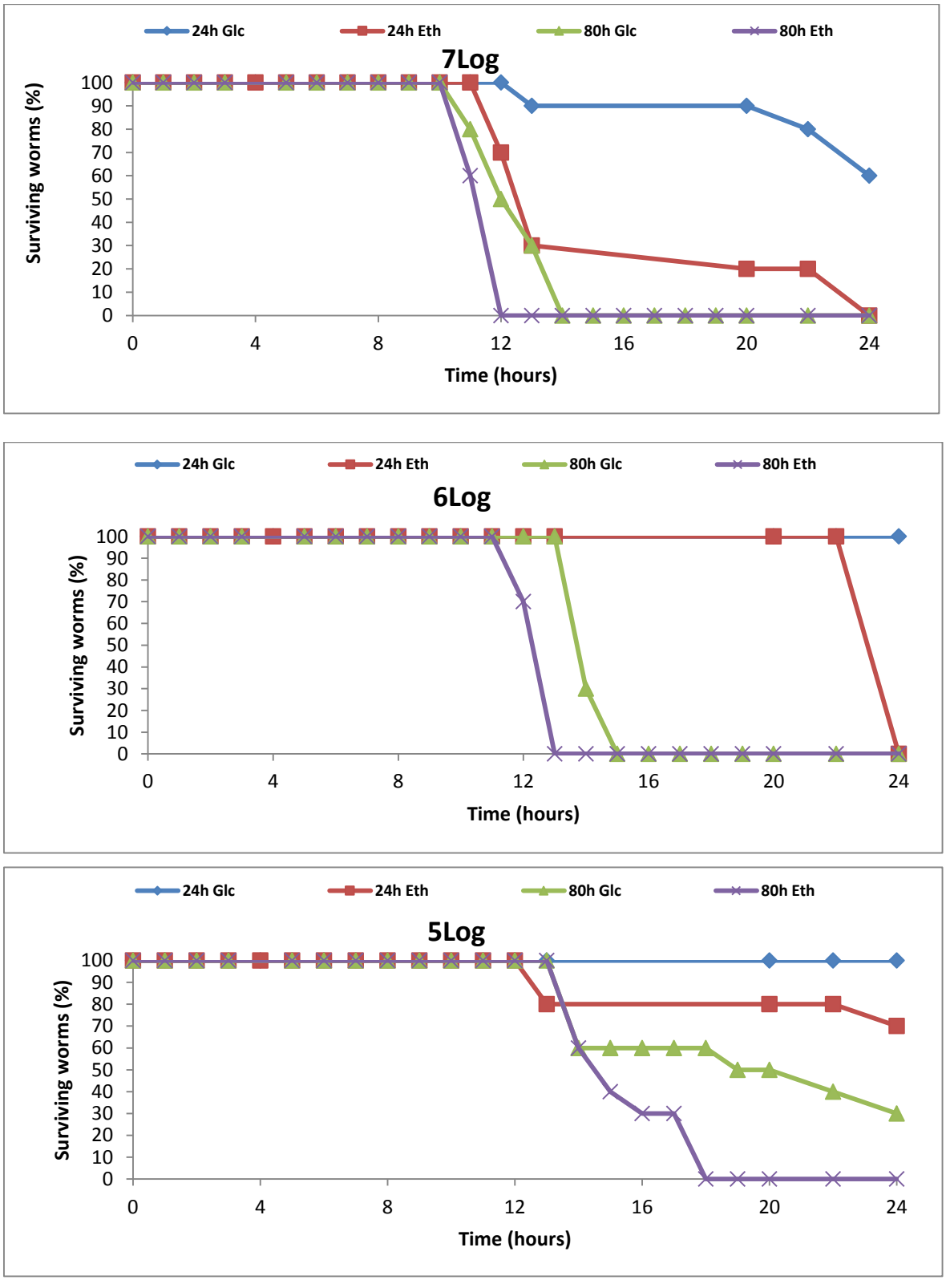


Figure 5.56 Comparison between the pathogenicity of different inoculum size taken from (24 and 80 h Ps 3 old culture) on MM Glc and MM Eth using wax worms models.

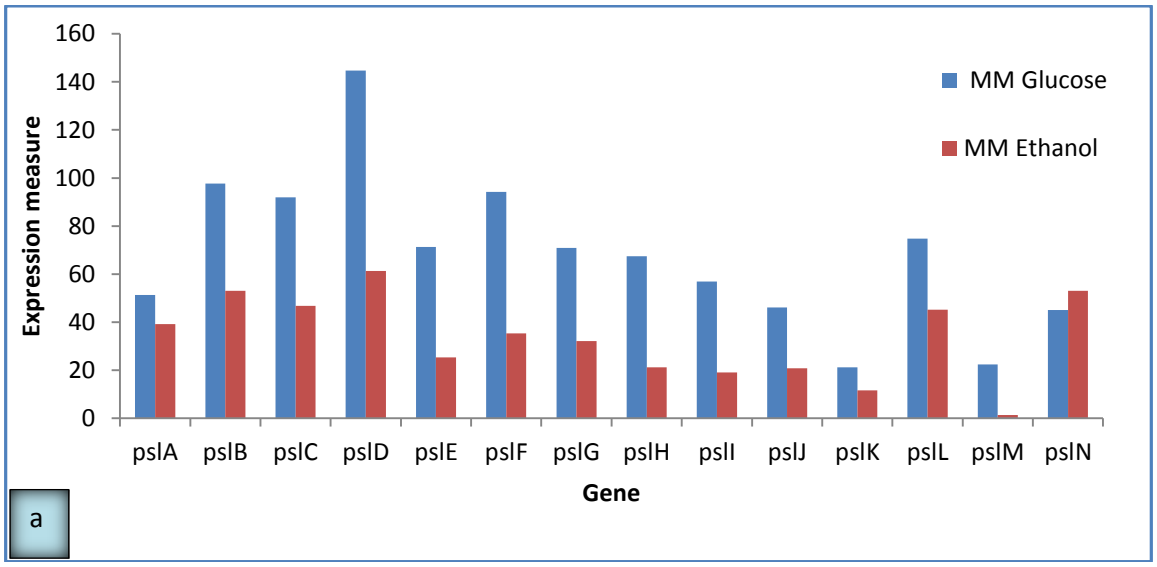
5.4.7 Gene expression (Transcriptomic studies)

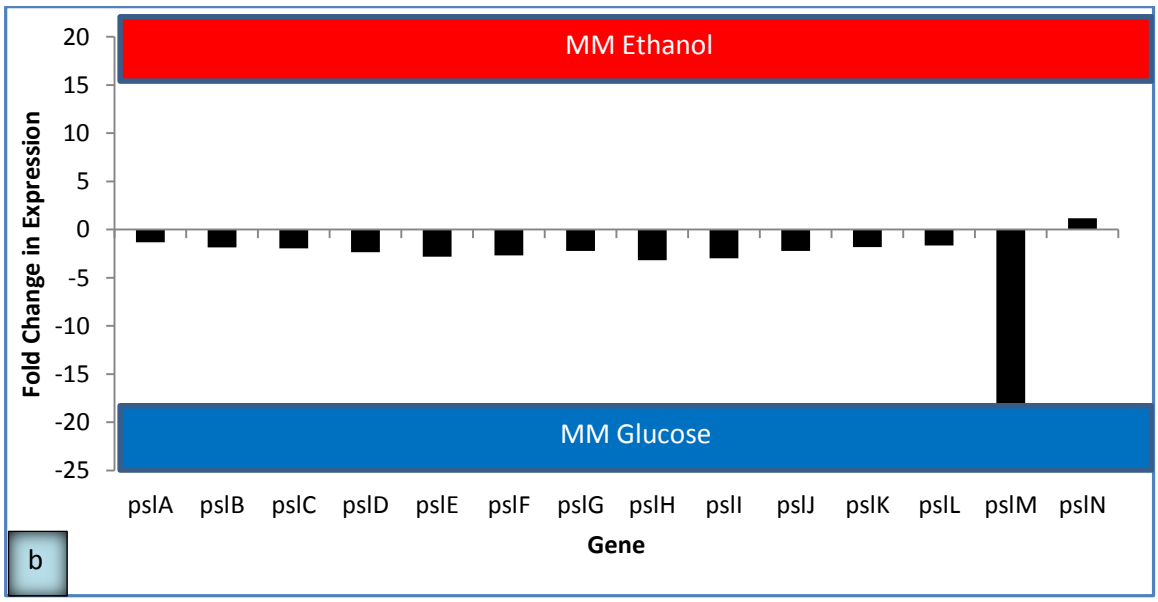
In order to gain a more detailed understanding of the difference in pathogenicity observed between glucose grown and ethanol grown Ps 3 a transcriptomic study was performed. In order to do this the mRNA derived from 24 h incubations of Ps 3 grown on both MM Glc and MM Eth were sequenced and the relative expression quantified.

In terms of the biofilm genes, all psl genes present were expressed on both carbon sources (Figure 5.57), only psl N was slightly more expressed (1.2 fold) on MM Eth than MM Glc. All the other psl genes demonstrated greater expression on MM Glc (1.6-3.0 fold) with the exception of psl M which had a 18 fold increased expression when comparing to expression on MM Eth (Figure 5.57).

Of the other biofilm gene, the pel genes demonstrated low levels of expression when compared to the psl genes, a surprising observation since Ps 3 is a pellicle forming bacteria. The expression of all pel genes except pel D and F was greater on MM Eth than MM Glc (Figure 5.57). The sequence data also showed that all alginate genes present in the genome were expressed but not extensively (1-5 fold) on both media. Some alg genes were expressed more on MM Eth while the others expressed more MM Glc. A range of other genes demonstrated greater expression on MM Eth than MM Glc (Table 5.14, 5.15). Although a number of gene demonstrated high levels of over expression when grown on ethanol (Table 5.14) many of these did not have a direct link to increased pathogenicity with the possible exception of the hypothetical proteins and tonb-dependent receptors. A number of other genes that were more closely related to pathogenicity showed lower but significant levels of increased expression (all genes showed significant difference-gene expression between MM Eth and MM Glc $P < 0.01$) (Table 5.15) including Exotoxin A, T3SS, protease, LPS, lipoprotein, OMV and pyocyanin (Ramsey and Wozniak, 2005). Other genes that

expressed very high such as peptidase which is also called protease (Barrett and McDONALD, 1986) and Acyl-homoserine lactone acylase subunit beta is a QS system that control the virulence of *P. aeruginosa* PA01 (Table 5.15).





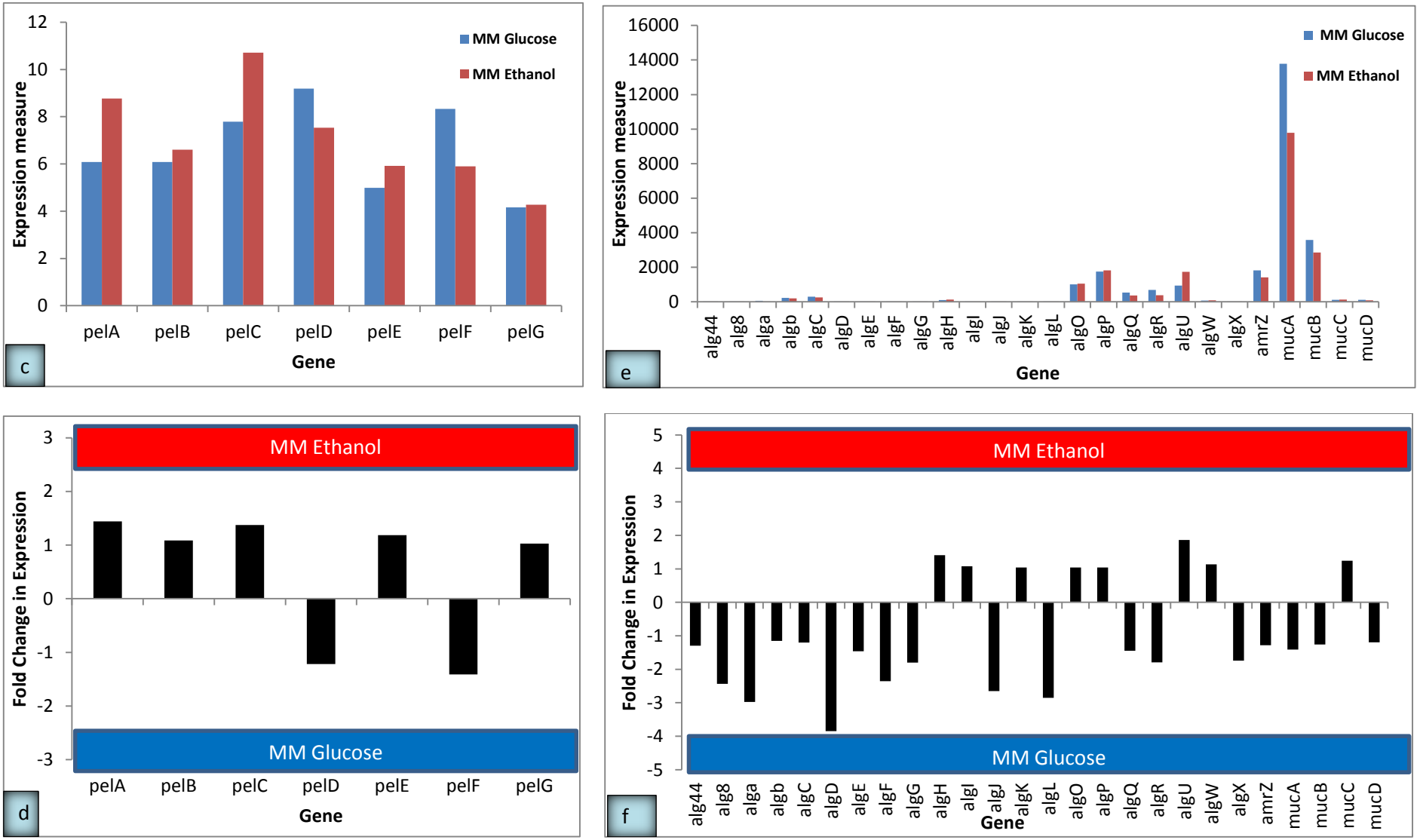


Figure 5.57 Ps 3 biofilm gene expression and fold change on both MM Glc and MM Eth. a) pel genes expression b) Fold change expression in pel genes c) psl genes expression d) Fold change expression in psl genes e) alg genes expression f) Fold change expression in alg genes.

Table 5.14 Expression of some genes with high fold change on both MM Glc and MM Eth.

Fold change	Glucose Expression	Ethanol Expression	Product
100.7679	2.515006	253.4318	Hypothetical protein
95.08242	3.087868	293.602	Thiamine pyrophosphate-binding protein
94.53701	3.303803	312.3317	Hypothetical protein
93.69668	1.061799	99.48703	Zinc abc transporter permease
90.70073	1.546175	140.2392	Abc transporter substrate-binding protein
83.11379	1.357305	112.8107	Manganese abc transporter atp-binding protein
67.75152	0.116182	7.871531	Atpase
60.45531	20.1512	1218.247	5-methyltetrahydropteroyltriglutamate--homocysteine methyltransferase
50.16423	2.871032	144.0231	Cyclic peptide transporter
47.66241	1.198741	57.13489	Class v aminotransferase
43.61641	44.83348	1955.475	Transporter
42.22653	1.875224	79.1842	Chromophore maturation protein pvdo
40.96604	4.5713	187.2681	Biopolymer transporter exbd
33.04537	7.2566	239.797	Hypothetical protein
31.99378	29.25349	935.9296	Rna 3'-terminal-phosphate cyclase
30.9771	22.67754	702.4842	Ornithine monooxygenase
29.91421	4.890289	146.2892	Metal abc transporter substrate-binding protein
27.08849	2.179672	59.04402	Thioesterase
26.26187	69.72777	1831.182	Rna ligase
24.84973	4.427303	110.0173	Acetyltransferase
24.53562	5.895424	144.6479	Tonb-dependent receptor
24.21095	7.536326	182.4616	Hypothetical protein
24.01025	6.600788	158.4866	Membrane protein

Fold change: Fold (times) change is calculated as the ratio of the difference between expression value on MM Eth and the expression value on MM Glc i.e. fold change = expression value on MM Eth / expression value on MM Glc.

Table 5.15 Expression of some genes and fold change on both MM Glc and MM Eth that related to virulence.

Fold Change	Glucose Expression	Ethanol Expression	Products
2.61	271.67	710.32	(2Fe-2S) ferredoxin
23.11	3.50	80.96	Acyl-homoserine lactone acylase subunit beta
2.61	271.67	710.32	(2Fe-2S) ferredoxin
3.69	146.13	539.43	Anthranilate synthase component 2, Pyocyanine specific

2.12	19.77	41.99	Exoenzyme S synthesis protein C
10.79	2.14	23.06	Exotoxin A regulatory protein
14.89	9.84	146.49	Lipoprotein
1.55	45.34	70.12	Lipoprotein
1.38	147.01	202.32	LPS biosynthetic protein lpxo
2.43	29.40	71.37	LPS core heptose(I) kinase rfap
5.75	219.02	1260.23	Outer membrane protein
1.40	7.84	10.99	Outer membrane protein
1.89	153.91	291.03	Outer membrane protein assembly factor bamb
1.43	139.58	199.83	Outer membrane protein assembly factor bamd
2.48	2.55	6.31	Outer membrane receptor fepa
25.90	7.63	197.366	Peptide synthase
78.50	5.45	427.7052	Peptidase
26.40	3.67	96.7725	Peptidase m19
1.86	39.62	73.62	Protease
1.63	246.79	401.70	Protease lasa
1.49	673.51	1003.19	Protease pfpi
2.21	427.44	944.63	Protease subunit hflk
2.19	401.16	877.50	Protease subunit hflk
3.22	249.65	802.68	Type II secretion system protein F
1.92	4.85	9.32	Type III export apparatus protein
1.37	7.38	10.13	Type III export protein psci
1.13	3.40	3.85	Type III secretion outer membrane protein popn
1.83	9.76	17.82	Type III secretion system chaperone yscw
1.36	3.31	4.51	Type III secretion system protein
1.70	18.14	30.89	Type III secretion system regulator exsd

Fold change: Fold (times) change is calculated as the ratio of the difference between expression value on MM Eth and the expression value on MM Glc i.e. fold change = expression value on MM Eth / expression value on MM Glc.

5.4.8 Discussion

As previously mentioned, *P. aeruginosa* causes a wide range of disease in humans and is an opportunistic pathogen in a range of different hosts, including mammals, insects, nematodes and plants (Jander et al., 2000). The application of infection models to pathogen is an important step in the understanding of the pathogenesis of the associated diseases and infections. Recently, insects have become commonly used infection models due to the similarity of their immune system to that of mammals (Insua et al., 2013). A variety of *P. aeruginosa* strains are extremely pathogenic to insects, including the wax worms or *G. mellonella* (Miyata et al., 2003). Previous reports have shown that non-pathogenic microbes such as *E coli* OP50, *Saccharomyces cerevisiae* and some strains of *Aspergillus fumigatus* were unable to kill the worms with up to 10^7 CFU per larvae. This data shows the viability of wax worms as an infection model to study the pathogenicity of *Pseudomonas aeruginosa* (Peleg et al., 2009). In the current study, wax worms were used to study the pathogenicity of *P. aeruginosa*, with a particular focus on Ps 3.

Initial work focussed on the OMV of *P. aeruginosa* since these have been implicated in virulence and disease generation (MacDonald and Kuehn, 2013). However in this case the lab strain *P. aeruginosa* (NCIMB 10421) failed to produce OMV on both MM Glc and MM Eth, however in contrast Ps 3 produced OMV on both carbon sources (Figure 5.49). It is generally thought that both pathogenic and non-pathogenic *Pseudomonas* species, release OMV during normal growth (Eddy et al., 2014, Avila-Calderón et al., 2011) so the lack of OMV from the lab strain is contrary to accepted thinking. However, a major controlling factor for the production of OMV is the presence of stress such as antibiotics (Chatterjee and Chaudhuri, 2012). OMV production is also regulated by other factors including growth conditions, media type, temperature and nutrient levels (Kulp and Kuehn, 2010). Chatterjee and Chaudhuri (2012) indicated that pathogenic bacteria produce more OMV than non pathogenic bacteria and that

bacteria under stress produce more OMV. Proteomic analysis of OMV produced by *Xanthomonas campestris* on two different culture media indicated that the culture conditions have an intensive effect on the protein composition (Sidhu et al., 2008b). It is possible that the culture conditions and media used did not generate sufficient stress on the lab strain to initiate OMV production. However, this particular strain of *Pseudomonas aeruginosa* (Ps 10421) is considered to be none pathogenic (Wang et al., 2014) being neither invasive or cytotoxic (Aristoteli and Willcox, 2003) and lacking in siderophore and pyocyanin production genes (Wang et al., 2014). Consequently, it may be that it is not an OMV producer in the absence of external stress factors such as antibiotics.

The current study demonstrated that Ps 3 generated more OMV and was more pathogenic when grown on ethanol rather than glucose as sole carbon source (Figure 6.3 c). However, when cell free extracts were injected into the worms the mortality of the worms significantly decreased indicating that the OMV are not in themselves especially pathogenic since they would have remained in the supernatant after the bacterial cells had been removed. The *P. aeruginosa* strain employed in this study (Ps 3) appears to continuously generate OMV, with structures appears to be OMV visible in SEM images of Ps 3 biofilms (Figure 5.49 c). OMV are produced by a wide variety of Gram-negative bacteria during all stages of bacterial growth as well as in a variety of growth environments including liquid culture, solid culture, and in biofilms as a survival strategies and damage the host cells (Kuehn and Kesty, 2005, Ellis and Kuehn, 2010). Multiple virulence factors are packaged in *P. aeruginosa* OMV including alkaline phosphatase, β-lactamase and haemolytic phospholipase C. These secreted virulence factors are important because they play important roles in host colonization, for example alkaline phosphatase promotes biofilm formation, β-lactamase degrades host antimicrobial peptides, haemolytic phospholipase C is cytotoxic and promotes *P. aeruginosa* virulence (Bomberger et al., 2009).

Although Ps 10421 is not considered pathogenic (Wang et al., 2014) when cultured on both glucose and ethanol mineral media for 40 h it was more pathogenic than Ps 3 (Figure 5.51 a & b). This suggests that the injection process has assisted Ps 10421 in overcoming its lack of invasion mechanisms. Preliminary studies on *P. aeruginosa* pathogenicity successfully demonstrated the value of the wax worm as a pathogenicity model.

Wax worms have been used by several authors for the study of pathogenicity of *P. aeruginosa*. These studies have focussed on the role of Type III Secretion System (Miyata et al., 2003), the effects of LPS O Antigens on Type III Secretion (Augustin et al., 2007). Jander et al. (2000) indicated that larvae of wax worms are quite sensitive to *P. aeruginosa*. After infection of wax worms with inoculum size up to 10000 of *P. aeruginosa* strains PA14, larvae survived until 48 h and then died within 2 h after rapid melanisation. Both Ps 10421 and Ps 3 strains were more virulent during growth on MM Eth than MM Glc than published research on PA14.

One of the more striking aspects of this section of the work is the enhanced pathogenicity associated with growth on ethanol as sole carbon source. Both Ps 10421 and Ps 3 were more pathogenic when fed on ethanol as the sole carbon source suggesting that ethanol is enhancing the expression of bacterial virulence factors that is promoting the death of the worms. Alternatively, the bacteria may be responding to the presence of ethanol by altering their physiology (Smith et al., 2004) which in turn is having a deleterious impact on the survival of the worms. Control experiments have indicated that the ethanol in the media is not harmful to the worms. In addition cell free extracts of ethanol fed broth cultures were considerable less toxic to the worms than washed and re-suspended ethanol grown cells. This data indicates that growth on ethanol has sustained impact on the pathogenicity of the bacterial cells. The mechanism of carbon source preference by *P. aeruginosa* is poorly understood, despite the fact that the carbon source has

intensive effects on bacterial virulence, including toxin production, antibiotic resistant and biofilm formation (Palmer et al., 2007). In a study on several clinical *P. aeruginosa* strains, environmental factors such as the carbon and nitrogen sources were shown to influence in vitro biofilm formation and quorum sensing (Palmer et al., 2007). In another study of growing *P. aeruginosa* and the yeast *C. albicans* , Chen et al. (2014) found that ethanol produced by *C. albicans* it influence biofilm formation and phenazine production in *P. aeruginosa*. Smith et al. (2004) Reported that low doses of ethanol acts as a signalling molecule that affects bacterial physiology and survival. In *Acinetobacter* species, ethanol stimulated growth, pathogenicity, as wells increase the survival against toxic salts (Smith et al., 2004).

Extended incubation times (80 h) of bacterial culture promoted EPS production, the cultures became even more pathogenic when compared to the 24 h old cultures (Figure 5.52 and 5.53). The LD50 of the 80 h old culture was approximately 1 log lower than the equivalent 24 h LD50. The same observation was also seen between MM Eth and MM Glc grown cultures (Figure 5.54 and 5.55). Consequently there was a 2 log difference between the least pathogenic cultures i.e. MM Glc cultures grown over 24 h, and the most pathogenic cultures i.e. MM Eth cultures grown over 80 h.

The direct injection of bacterial cultures were more virulent than PBS washed bacteria (24 and 80 h old culture) adjusted to the same inoculum size. This indicates that the broth contained additional toxic compounds which enhanced the toxic impact. However, although the supernatant did have a toxic impact on the worms, in isolation this impact was considerably lower than that generated by the bacterial cells. This data suggests that EPS production and by inference biofilm formation increases virulence over and above that associated with ethanol as a sole carbon source and that the pathogenicity of the bacteria is associated with cellular processes rather than the excretion of toxins.

In several studies, the soluble virulence factors released by bacteria during growth have been investigated. These soluble factors include: LPS or endotoxin in the culture supernatant of *E. coil* (Zhang et al., 1998); serine protease in *S. maltophilia* liquor as a nematocide (Huang et al., 2009); putative virulence factors (lipase, protease and phospholipase) in *S. maltophilia* culture supernatants with haemolytic, enzymatic activities and cytotoxic impacts (Figueiredo et al., 2006); the generation of a variety of extracellular enzymes by *S. maltophilia*, including DNase, RNase, protease, fibrinolysin, lipases, and elastase (Denton and Kerr, 1998); quorum sensing signals, such as acyl homoserine lactones (AHLs) in *P. aeruginosa* (Gupta et al., 2011b); elastase and pyocyanin generation by *P. aeruginosa* (Frank, 2012); rhamnolipid secretion by *P. aeruginosa* (Zulianello et al., 2006). *P. aeruginosa* secretes a wide range of virulence factors that contribute to its pathogenicity such as LPS, alginate, alkaline protease, exotoxin A, DNase, and phospholipase C (Kadurugamuwa and Beveridge, 1995). The accumulation of these toxin in the bacteria and the culture supernatant at 80 h incubation may be more than 24 h and this was another reason that for more virulence.

In the current study, biofilm formation and EPS generation has been investigate as a virulence factor, however the transcriptome analysis of gene expression after 24 h incubation showed quite varied expression of EPS associated genes, some of which are more expressed on MM Glc while others are more expressed during growth on MM Eth (Figure 5.57). However, the transcriptome provides no clear evidence that biofilm/EPS generation is the factor that enhances the pathogenicity of Ps 3 when grown on ethanol.

A large number of genes appeared to be enhanced in terms of their expression when Ps3 was fed on ethanol. The genes in (Table 5.14) do not appear to be directly relevant to virulence with the exception of hypothetical protein and tonb-dependent receptor. Many of these gene products are related to membrane transport and include Thiamine pyrophosphate-binding protein , Zinc abc transporter permease, Abc transporter substrate-binding protein, and Manganese

abc transporter atp-binding protein transport (Hawkins et al., 1989, Maret, 2001, Berntsson et al., 2010, Garmory and Titball, 2004). Other transport related genes include 5-methyltetrahydropteroyltriglutamate-homocysteine methyltransferase (zinc transporter) (Mládková et al., 2014), Class V aminotransferase (transfer of amino groups) (Yoshikane et al., 2008), Other genes highly expressed included the genes for the Chromophore maturation protein pvdo which is related to pyoverdine (pvdMNOP) production (Ye et al., 2014).

A number of highly expressed genes were referred to as hypothetical proteins. These genes are predicted to be expressed from an open reading frame which have not been experimentally characterised (no experimental evidence of translation) and whose functions cannot be presumed from simple sequence comparisons alone (no identifiable function) (Kolker et al., 2004, Desler et al., 2009).

The TonB-dependent receptors which is found in Gram-negative bacteria functions as an iron chelator (siderophores) transporting iron into the periplasm. Bacteria use (siderophores) to establish commensal and pathogenic relationships with hosts and to survive as free-living organisms (Ferguson and Deisenhofer, 2002), so this gene may be related to increased virulence.

The genes in outline in Table 5.15 are more easily associated with increased pathogenesis. These includes genes associated with redox processes and electron transport such as the (2Fe-2S) ferredoxin which is a bacterial thioredoxin (Meyer, 2001). These ferredoxin indicated in the secretion of cytotoxic necrotizing factor 1 in *E. coli* k1 (Yu and Kim, 2010).

Several other genes that have a relation to pathogenicity include Exotoxin A, Type III secretion, Protease, LPS, lipoprotein, OMV and pyocyanin. Exotoxin A or Pseudomonas Exotoxin (PE) is considered one of the major virulence factor of *Pseudomonas aeruginosa*. This toxin has the ability to binds to a specific receptor on animal cells, and entering the cells then blocks protein synthesis causing cell death (Morlon-Guyot et al., 2009). Some Gram-negative bacteria

such as *P. aeruginosa* and *Neisseria meningitidis* secrete lipoproteins that have a significant role in the pathogenesis of infection (Godlewska et al., 2009).

Type III secretion (T3SS) is a complex apparatus that is used by *P. aeruginosa* to inject proteins (effector proteins) into the host cells extracellular environment. T3SS have been associated with an increase in disease severity during acute pneumonia, bacteremia, burn infections and sepsis (Hauser, 2009). Protease is another virulence factor secreted by *P. aeruginosa* that directly attack host tissues through hydrolysing peptide bonds (Hoge et al., 2010). *P. aeruginosa* biofilms are also able to secrete proteases to the environment surrounding the biofilms (Ołdak and Trafny, 2005). Another factor in *P. aeruginosa* that mediates both bacterial virulence and host responses and consequently contributes to pathogenesis in burn patients and immunocompromised hosts such as those with cyctic fibrosis is lipopolysaccharide (LPS) (Pier, 2007).

Most strains of *P. aeruginosa* produce pyocyanin complexes which are redox-active phenazine compounds which have a range of functions such as the suppression and damaging of mammalian cells. Phenazines can also benefit *P. aeruginosa* by serving as signalling molecules, regulating persister cell formation, influencing colony morphology, promoting iron acquisition and biofilm development (Glasser et al., 2014, Mavrodi et al., 2001). The presence of high concentrations of pyocyanin in the sputum of patients with CF indicated that this complex plays a role in pulmonary tissue damage. In some model pathogenesis systems, pyocyanin synthesis by *P. aeruginosa* is necessary for causing disease symptoms in plants and for effective killing of the nematode and the insect (*G. mellonella*) (Mavrodi et al., 2001). Glasser et al. (2014) Indicated that endogenous phenazines (such as pyocyanin) support the anaerobic survival of *P. aeruginosa,* however the physiological mechanism behind survival was unknown. Glasser et al. (2014) Also suggest that phenazines can increase the metabolic adaptability of microorganisms by facilitating energy generation ATP synthesis.

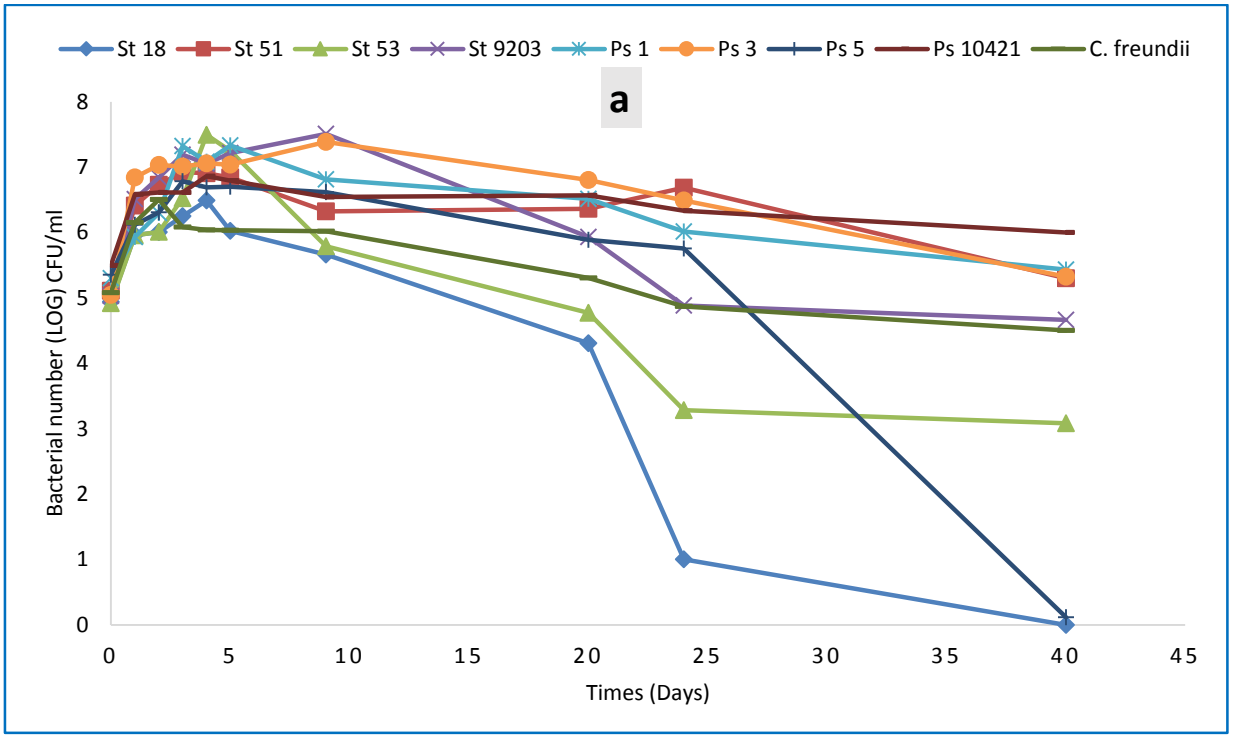
8.4 Key finding

- The Ps 3 strain of *P. aeruginosa* was able to produce OMV in the absence of any external stressors on both glucose and ethanol as sole carbon source.
- The Ps 10421 strain of *P. aeruginosa* was unable to produce OMV in the absence of any external stressors on both glucose and ethanol as sole carbon source.
- When grown on ethanol Ps 3 generated a greater quantity of OMV than generated when grown on glucose.
- Both Ps 3 and Ps 10421 were more virulent when grown on ethanol than when grown on glucose.
- Regardless of carbon source, 80 h cultures were more virulent than 24 h old culture of Ps 3.
- The LD50 of both 24 and 80 h old culture were approximately 1 Log lower when grown on ethanol rather than when grown on glucose.
- PBS washed bacteria were less virulent than bacterial culture with same inoculum size.
- Biofilm genes (psl, pel and alg) genes were expressed on both carbon source media but it's not clear if it is related to the virulence of the Ps3.

5.5 Survival of Gram negative bacteria under low nutrient conditions

After inoculation of sterilised ultra-pure water (UPW) with 10^5 CFU/ml (final inoculum size), all bacterial strains were able to survive for more than 40 days except St 18 and Ps 5 (Figure 5.58). As a general observation all bacteria multiplied during first 2 days, this continued at a reduced rate up to 9 days after which a reduction in cell numbers occurred. Increasing the inoculum size to 10^7 CFU/ml for St 53, St 9203, Ps 3, Ps 10421 and *C. freundii,* resulted in survival for more than one year. The only exception being *C. freundii,* in this case the number

of cells was 4.02 x10^2 and 0.00 CFU/ml after 52 and 119 days respectively. Colony morphology and Gram stains showed that all strains remained pure during whole years except Ps 3 that was pure up to 277 days but was seen to be contaminated at 365 days.



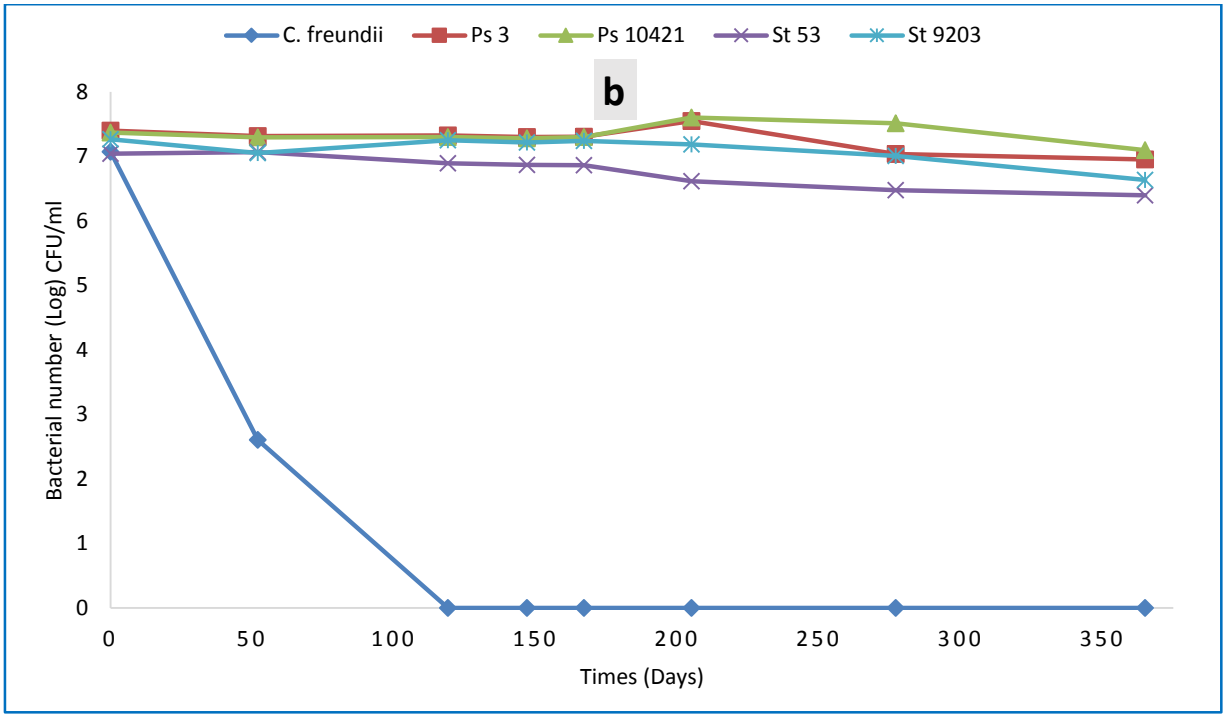


Figure 5.58 Bacterial survival in sterilised UPW with different inoculum size a) 10^5 CFU/ml b) 10^7 CFU/ml.

5.5.1 Discussion

In our study. Ps 3, 10421, St 53, 9203 and *C. freundii* survived for extended periods in sterilised UPW with no obvious carbon source. In a previous study by Brooke (2012) it was reported that *P. aeruginosa* was able to survive for months on dry surfaces and even able to persist and grow in contaminated antimicrobial hand soap containing triclosan. *S. maltophilia* also prefers wet surfaces and aqueous solutions. *S. maltophilia* has the ability to survive in drinking water, UPW, and treated water with filtration, UV exposure, or deionization. Brooke (2012) also indicated that in response to starvation such as in tap water, mineral water or reverse osmosis water, these bacteria produce ultra-micro cells (UMC) 0.1 to 0.2 μm in size that can pass through a 0.2 μm filter. This adaption is thought to reduce the energy costs of chemotaxis. These UMC have been recovered from chlorinated drinking water samples and were capable to cultivation and biofilm formation on PVC pipe walls (Brooke, 2012). *P. aeruginosa* was able to survive in DW with 5.45-6.15 Log for about 128-145 days (Farber and Todd, 2000). In another study, to understand the survival and multiplication *P. solanacearum* in pure water, (Sunaina et al., 1998) indicated that the bacteria was able to multiply for the first day of incubation at 10 and 20˚C then bacterial number remained stable for the rest of the period but at 30˚C, all strains multiplied for 2 days and bacterial number decreased gradually reaching non detectable level by the tenth day of incubation. While in the present study, all strains were able to multiply at the beginning of incubation and remains detectable for long period except St 18 and Ps 5 (Figure 5.58). Liao and Shollenberger (2003) reported that *P. fluorescens* was able to survive for 16 years in DW while *P. aeruginosa* PA01 with approximately 9 Log inoculum size, was able to survive in DW for about 30 weeks (2.67 Log) (Liao and Shollenberger, 2003).

5.5.2 Key finding

- The bacterial strains Ps3, Ps 10421, St 53, St 9203 and *C. freundii* were able to survive for extended time periods in sterile UPW.

6. General Discussion

Gram-negative bacterial pathogens have a significant impact on human health and wellbeing particularly since they have developed resistance to a range of antibiotics. The increase in infections caused by MDRO bacteria has resulted in efforts to examine the survival strategies and explain the mechanisms used by these pathogens during infection and disease. These pathogens investigated in this study, *P. aeruginosa*, *S. maltophilia* and *C. freundii* are all identified as MDRO (Rosenberger et al., 2011, Brooke, 2012) and this study has focused on their ability to form biofilms since biofilm formation is a key characteristic of pathogenic organisms in healthcare settings.

This investigation focussed on a range of clinical isolates and a smaller number of laboratory strains obtained from culture collections. All clinical strains of *S. maltophilia* that were kindly provided by Dr Jane Turton, Laboratory of Health Care Associated Infection, Health Protection Agency, London, UK. The clinical *P. aeruginosa* strains had been previously isolated from discarded wound dressings provided anonymously from a local skin integrity practitioner. The strain of *C. freundii* investigated in this study also originated from these discarded wound dressings and was found to be a contaminant of one of the original *P. aeruginosa* isolates. Initially the identity of all the strains employed was confirmed by 16s rRNA gene sequencing along with established culture based biochemical tests.

All three candidates species (*P. aeruginosa, S. maltophilia* and *C. freunii*) were known to produce biofilms on polystyrene surfaces (Pompilio et al., 2015, Allan et al., 2002, O'Toole, 2011). This was confirmed in the present study using 96 well plates (polystyrene). However, in this study biofilm formation was found to be variable and media dependent (Figure 5.11). All bacterial strains of *Stenotrophomonas, Pseuodomonas* and *Citrobacter* genera formed different biofilms ranged from no biofilm formation to strong biofilm formation on

different media, which agrees with a range of previous studies (Di Bonaventura et al., 2004, Pompilio et al., 2008, Müsken et al., 2010, Pereira et al., 2010). Davey and O'toole (2000) reported that the microbial adherence and biofilm formation is effected by a number of variables including species of bacteria and nutrient availability.

The optimum inoculum size for biofilm formation was in the region of 1-5x10^7 CFU/ml (Figure 5.6). It was shown that the size of inoculum significantly influence the amount of biofilm formation, i.e. biofilm density increased with the increasing of inoculum size, thus standardised inoculums should be used. In most studies dealing with biofilm quantification, the exact size of the inoculums has not been determined (Stepanovic *et al.*, 2007). In some studies the initial inoculum is specified as a 1:100 dilution of an overnight culture, this approach has been used for the quantification of biofilm production in Gram negative bacteria (Zubair et al., 2011), *S. maltophilia* (Huang et al., 2006), *P. aeruginosa* (Head and Yu, 2004, Rogan et al., 2004, Abidi et al., 2013) *E. coli* O157:H7 (Ryu and Beuchat, 2005). However, this does not reflect the fact that different bacteria will generate different cell densities when grown over night. In other studies several inoculum size were used for biofilm formation for example in *S. maltophilia*, 5x10^5-1x10^6 CFU/ml (Di Bonaventura et al., 2004), 1x10^8 CFU/ml (Di Bonaventura et al., 2007), 10^7 CFU/ml (Pompilio et al., 2011) 5x10^7-1x10^8 CFU/ml (De Rossi et al., 2007), 1x10^6 CFU/ml (de Rossi et al., 2009, Roscetto et al., 2015), in *P. aeruginosa* 10^7 CFU/ml (Cady et al., 2012) 10^6 CFU/ml (Tavernier and Coenye, 2015) 1.5x10^8 CFU/ml (Kazemian et al., 2015), *Burkholderia cenocepacia* 1x10^6-1x10^7 CFU/ml (Caraher et al., 2007), *Acinetobacter spp., Burkholderia spp., Mycobacterium spp.* 1x10^8 CFU/ml (Simões and Simões, 2013), *Staphylococcus pseudintermedius* 10^5 CFU/ml (Field et al., 2015). This wide range of initial inoculum sizes prevents the cross comparison of biofilm formation data between publications. The data generated here suggests that a range of inoculum sizes should be used to determine the

optimum for the conditions investigated before the strength of biofilm formation for a particular strain is reported. This will prevent to study variation in the reported strength of biofilm formation.

In *P. aeruginosa* stains, increasing casein led to decrease biofilm production while increasing glucose up to 2% led to increase biofilm formation. This suggests that the carbon to nitrogen ratio influences biofilm formation and when excess carbon is present the bacteria divert this to the formation of extracellular polysaccharides. In *S. maltophilia* strains, increasing casein concentration showed no effects while glucose 1% was the optimum for biofilm formation (Figure 5.7 and 5.8). However in a study by Zgair and Chhibber (2011), 0.2% glucose generated the maximum adhesion to polystyrene when a range of glucose concentrations (0, 1, 2, 4, 8, 16 g/l) in LB broth was used (Zgair and Chhibber, 2011). Generally, the ratio of carbon source (glucose, glycerol and ethanol) to nitrogen source (casein) in our study was approximately 10:1 for all strains, however in the case of the *P. aeruginosa* strains 20:1 (glucose:casein) showed the maximum biofilm production. However 10:1 was used in EPS production investigations to avoid the glucose interfering with EPS analysis. This observation is in agreement with the previous study of (Sutherland, 2001) which indicated that the promotion of biofilm formation and associated EPS production was mainly affected by the nutrient content of the growth medium. EPS synthesis depends on the availability of carbon substrates and its balance with other limiting nutrients. The presence of excess carbon substrate and the limitation of other nutrients nitrogen, phosphate and potassium stimulate EPS production (Sutherland, 2001). In another study, Karatan and Watnick (2009) indicated that an increase in nutrients concentration of various carbon and/or nitrogen sources such as glucose, glutamate, citrate and ammonium chloride in the media leads to termination of biofilm formation in the *P. aeruginosa* (Karatan and Watnick, 2009) as was seen in this study when the casein concentration was increased.

In the present study, the strength of biofilm formation differed between bacterial strains of the same species, and this is confirming the fact that different strains have different biofilm potentials (Hancock et al., 2011). All bacterial strains except St 18 were able to produce a pellicle (an air-liquid biofilm) on at least one media at room temperature (Figure 5.14), similar result were found in previous study (Zogaj et al., 2003, Colvin et al., 2011b). Drip flow biofilm reactors (DFR) are designed for the study of biofilms grown under low shear conditions. DFR are considered an ideal method for general biofilm studies, evaluating and testing of medical material and indwelling medical device (Schwartz et al., 2010). DFR represent biofilm formation as seen in nature, for example DFR were successfully used for the study of *Actinobacillus pleuropneumoniae* biofilm formation which is associated with respiratory infections. This is because the DFR creates an environment with an air-liquid interface that closely resembles the lung environment (Tremblay et al., 2013). In this study, St 53, 9203, Ps 3, 10421 and *C. freundii* were able to produce biofilm on both hydrophilic (glass slides) and hydrophobic (plastic slides) surfaces (McQueary and Actis, 2011, Machado et al., 2011). No obvious differences were seen when either glucose or ethanol were used as the carbon source during biofilm formation on either glass or plastic under low shear conditions. Previous studies have demonstrated the ability of *P. aeruginosa, S. maltophilia and C. freundii* to form biofilms under flow environment (Habimana et al., 2010, Hunt et al., 2004, Stoodley et al., 1999) and on biotic and abiotic surfaces in hospitals devices used for patient care (catheters and respiratory therapy equipment) (Shirtliff and Leid, 2009, Brooke, 2012, O'Toole and Kolter, 1998, Soto, 2014).

The ability of *P. aeruginosa*, *S. maltophilia* and *C. freundii* to form biofilms using ethanol as a sole carbon source may be considered one of the strategies the results in the persistence of these bacteria in hospitals. These bacteria may be degrading antiseptic and detergent residues, which contain traces

of ethanol and that have contaminated the hospital environment as a carbon source.

Sequencing of the genomes of the three of the strains investigated in this study (Ps 3, St 53 and *C. freundii*) showed the presence of set of genes associated with biofilm formation such as the *pgaABCD* locus in both St 53 and *C. freundii* and a full complement of pel (PA3058-PA3064), psl (PA2231-2245) and alginate biosynthesis operons (PA3540-3548) in Ps 3.

Cell surface properties are recognized as the key factors that influence bacterial adhesion and colonization on biotic and abiotic surfaces (Zita and Hermansson, 1997, Di Bonaventura et al., 2008, Guimarães et al., 2006). The differences between strains and the effect of carbon source on biofilm formation was revealed by the hydrophobicity test data. Clear differences were observed between strains and the use of ethanol as carbon sources (MM Eth) generated greater hydrophobicity than MM Glc, SWF or TSB (except in SAT test). This correlation between biofilm formation and hydrophobicity is to be expected, since plastic surfaces are hydrophobic in nature (McQueary and Actis, 2011). Machado et al. (2011) also studied the cell surface hydrophobicity (MATH) to monitor *P. aeruginosa* growing in the presence of benzalkonium chloride and found that the hydrophobicity were increase as the cells grew in resistance to the disinfectant. Another study by (Norman et al., 2002) indicated that *P. aeruginosa* cells grown on glucose, produced both high molecular weight negatively charged B band LPS and low molecular weight neutral A band LPS that demonstrate low surface hydrophobicity (Norman et al., 2002). Ethanol promoted bacterial adherence may be species specific. For example *Streptococcus spp.* increased adherence to human cells after exposure to 1% ethanol (Vasiliou et al., 2015).

In the present study, different hydrophobicity result recorded according to using different Hydrocarbons (Section 5.2.9) a result that agrees with previous studies (Saini, 2010).The variability seen in the hydrophobicity results makes it

difficult to make any correlation with biofilm formation, however, it is noticeable that bacterial biofilms on (MM Eth and MM Glc) and SAT test on (MM Eth and MM Glc) showed a good correlation between biofilm formation and hydrophobicity (Table 5.6). After testing 40 clinical isolates of *S. maltophilia* strains to investigate the correlation between the adherence and the biofilm formation on polystyrene and cell surface hydrophobicity; Pompilio et al. (2008) found that most strains were able to adhere and form biofilms and that there was a positive correlation between hydrophobicity and biofilm formation, although some strong biofilm formers were hydrophilic (Pompilio et al., 2008).

Extracellular Polymeric Substance (EPS) are high molecular weight polymers secreted by microorganisms which contain polysaccharides, proteins, phospholipid, nucleic acids (DNA and RNA) and humic substance (Czaczyk and Myszka, 2007, Hall-Stoodley et al., 2004, Vu et al., 2009). The production of these polymeric substances has a wide variety of roles includes adhesion to surfaces, bacterial cell aggregation, protection from engulfment by predatory protozoa and (phagocytes), protection from desiccation and antimicrobial agents (Nwodo et al., 2012). In this study the focus was on the generation and analysis of EPS. Selection of a media is one of the most important aspects in the study of bacterial EPS production and characterisation since the media should be free of any interfering substances e.g. yeast extract (Alhudhud et al., 2014). In this study, several broth media (TSB, LB and SWF media) were ruled out due to the presence of these interfering substances (Figure 5.22). However TSB and LB broth have both been used in previous studies (Subramaniam et al., 2007, Yilmaz et al., 2012, García et al., 2015, Ngwai et al., 2006), throwing into doubt the accuracy of currently published data. During the selection of a media for EPS production and characterisation, proton NMR analysis is not sufficient on its own to determine the presence of interfering substances since proton NMR analysis depends on the dissolution of the sample in the solvent (de Alvarenga, 2011). Beside proton NMR HPAEC, phenol sulphuric acid carbohydrate test, Bradford protein test and uronic

acid test are all good parameters to determine the suitability of any media. In order to avoid the problems associated with interfering substances, several polysaccharides free mineral media such as MM Glc, MM Gly and MM Eth were developed and modified (Alhudhud et al., 2014, Shoukat, 2014).

The optimum conditions for the generation and extraction of EPS generated by Ps3, St 53 and *C. freundii* on mineral media was an initial inoculum of 10^7 CFU/ml, incubation at 30˚C for approximately 80h with 0-5 % TCA for the removal of proteins from the crude extract. For many bacteria the optimum condition for EPS production are different from their optimum growth. EPS production is effected by several factors such as temperature, pH and medium composition, and even the strain. It is also known that some bacterial EPS degrade after synthesis. For example some EPS by *Leuconostoc mesenteroides* and *Streptococcus thermophilus* showed a loss of EPS during the later stages of the growth cycle (Yang, 2011).

EPS produced by Ps3, St 53 and *C. freundii* on all carbon sources was a complex polymer that was composed of at least two polysaccharides one of which was a dextran like polymer. The production of this dextran like component was independent on the carbon source. Removal of this dextran component by dextranase treatment revealed a second more complex EPS that was mannose dominated with a range of additional sugars. Some researcher are convinced that the type of carbon sources cannot effect on EPS composition, while the other found that carbon source can have an effect on molecular weight, EPS yield and even chemical composition. Excess of carbon and limiting nitrogen will enhance EPS production, however in different bacteria, carbon source has different effect on EPS production and sugar composition (Yang, 2011). In some bacteria such as *Acidithiobacillus ferrooxidans*, the EPS composition varies and depends on the type of substrate or growth media on which the cells are grown. Different substrates show different mode of attachment and this leads to different EPS gene

expressions (Vu et al., 2009). In the current study the chemical composition of each bacterial EPS generated on different carbon sources was the same but with different quantities i.e. different proportions of the individual monosaccharide sugar. This result agrees with Petry et al. (2000) who reported that the chemical composition of EPS generated by *Lactobacillus delbrueckii* subsp. *bulgaricus* growth on different carbon sources was the same but with different relative proportions of the individual monosaccharides (Petry et al., 2000).

Dextran is a high molecular weight, extracellular homopolysaccharide of glucose that is produced by bacteria such as *Streptococcus, Leuconostoc, Lactobacillus Weissella* and *Campylobacter* species (Monsan et al., 2001, Burmølle et al., 2014, Sadiq, 2015). *Streptococcus mutans* produce extracellular dextran to adhere to teeth and form dental plaques (Bowen and Koo, 2011). The production of dextran by bacteria is linked to a range of hydrolytic glucansucrases (glucosyltransferase) (Monsan et al., 2001). Whole genome sequencing showed the presence of glucosyltransferases in all bacterial strains Ps 3, St 53 and *C. freundii*) (http://rast.nmpdr.org/) (Overbeek et al., 2014).

EPS sugar components from Ps3, St 53 and *C. freundii* were very similar differing in only 1-2 sugars (Figure 5.34 and Table 5.11). (Bales et al., 2013) analysed the EPS composition of several bacteria, like *Klebsiella pneumoniae, P. aeruginosa,* and *A. baumannii,* among all the sugars present, they only differed in 1-3 sugar. According to Liu (2012), the EPS in many Gram-negative bacteria are relatively simple, and the monomeric composition of Gram-negative bacterial EPS were either homopolysaccharides (polymers composed of D-glucose), or heteropolysaccharides, which compose of 2-4 types of monosaccharide residues. Several sugars including D-glucose, D-mannose, D-galactose, L-fucose and L-rhamnose are frequently found in microbial EPS (Liu, 2012).

Some of the polysaccharides in Gram-negative bacteria polyanionic due to the presence of uronic acid sugars or pyruvates, this leads to an increase in the

anionic properties of the EPS. This results in the EPS having an affinity for divalent cations such as calcium and magnesium which act to increase the binding force in a developing biofilm (Vu et al., 2009). The most common sugars in Gram negative EPS are glucose, galactose, ribose, fructose, xylose, and arabinose while the rarer sugars are fucose, rhamnose and uronic acid. The presence of rarer sugars in the EPS, make it more favorable for different fields of applications, such as anti-inflammatory substances, antiviral agents and antioxidant. An example of bacteria that produce rare sugars are the marine bacterium *Enterobacter cloacae* and *Streptococcus* spp. (Roca et al., 2015a).

P. aeruginosa is able to produce range of EPS such as Alginate, Psl and Pel (Franklin et al., 2011, Bazaka et al., 2011, Sharma et al., 2014). The Psl polysaccharide has been characterised as a neutral, branched polysaccharide with repeating D-mannose, D-glucose,and L-rhamnose sub units (Byrd et al., 2009) while the Pel polysaccharide that produce pellicle remains uncharacterised, but it is generally referred to as a glucose rich polymer (Friedman and Kolter, 2004a, Whitfield et al., 2015).

WGS showed that Ps 3 contained a full complement of alginate (PA3540-3548), pel (PA3058-PA3064) and psl (PA2231-2245) biosynthesis operons that also identified in *P. aeruginosa* PA01 (Sharma et al., 2014) (http://www.pseudomonas.com). One of the most significant outcomes of the project is the indication that the pel polysaccharide of *P. aeruginosa* is a dextran like polymer. The presence of a dextran being demonstrated by dextranase treatment and the removal of peaks associated with dextran from NMR spectra. This analysis is consistent with the description of the Pel polysaccharide as a glucose rich polymer (Ryder et al., 2007, Ma et al., 2009). When the pel EPS is removed by the dextranase the remaining EPS is a mannoses rich polysaccharide consistent with a Psl polysaccharide. Ps 3 failed to produce an alginate in the current study, even though it had the required alginate biosynthesis operons

(PA3540-3548) (Sharma et al., 2014) (http://www.pseudomonas.com). However, in this study the Ps 3 was grown in culture conditions ideal for pellicle formation rather than the cell surface biofilm environments consistent with mucoid growth and alginate generation. The current study suggests that the Psl and Pel polysaccharides may be synthesized at the same time by Ps 3 when grown under pellicle forming conditions. In a study by Karatan and Watnick (2009), sugar analysis of the EPS isolated from biofilms of non-mucoid strains of *P. aeruginosa* PA01, showed the presence of large amounts of both glucose (Pel) and mannose (Psl), while the absence of uronic acid indicating the lack of an alginate (Karatan and Watnick, 2009). This observation is highly consistent with the data generated here but with presence of some unknown uronic acid peaks.

In the present study both *S. maltophilia* St 53 and *C. freundii* produced complex polysaccharides. One of them being dextran like and the other one composed of Fucose, Galactosamine, Glucosamine, Galactose, Glucose, Mannose and some unknown uronic sugars in case of St 53. In the case of *C. freundii*, it was composed of Galactosamine, Glucosamine, Galactose, Glucose, Mannose and some unknown uronic sugars. Cescutti et al (2011) mentioned that the *S. maltophilia* EPS is composed of Glucose, Glucuronic acid, Galacturonic acid (Cescutti et al., 2011) while *Citrobacter* spp. has been associated with the production of a cellulose based EPS (Zogaj et al., 2003). Other authors such as (Rättö et al., 2006) have suggested that *Citrobacter* spp. EPS mainly consists of Fucose, Rhamnose, and other sugars such as Galactose, Glucose, Mannose, Galacturonic acid, Glucuronic acid and pyruvate. The compositions in these latter studies are closer to the composition determined in this study, which once again underlines the fact that EPS generation can be highly varied even within species. The genome of St 53 and *C. freundii* showed the presence of the entire *pgaABCD* locus that encodes the synthesis of a polysaccharide.

Gram-negative bacteria are able to produce OMV during normal bacterial growth or as a stress response (MacDonald and Kuehn, 2013). In this study The Ps 3 strain of *P. aeruginosa* was able to produce OMV in the absence of any external stressors when grown on both glucose and ethanol as sole carbon sources while Ps 10421 failed to produce OMV on both carbon sources. Sidhu et al. (2008a) were able to purified OMV from *Pseudomonas aeruginosa* PA01 on TSB (Sidhu et al., 2008a). When Ps 3 was grown on ethanol it generated a greater quantity of OMV than when grown on glucose. Both Ps 3 and Ps 10421 were more virulent when grown on ethanol than when grown on glucose. Sidhu et al. (2008a) investigated OMV purification on two different culture media and reported that culture conditions have an effect on the protein composition of the OMV (Sidhu et al., 2008a). Kulp and Kuehn (2010) also indicated that OMV production is affected by growth conditions such as media type, temperature and nutrient levels (Kulp and Kuehn, 2010). In a previous study, Sidhu et al. (2008a) reported that during growth of *Xanthomonas campestris* , this bacteria was able to release OMV into the culture supernatant that contained 31 proteins of which half are virulence-associated. Another reason for the pathogenicity of OMV are the presence of virulence factors found within the parent bacterium. For example *P. aeruoginosa* OMV are composed of alkaline phosphatase, phospholipase C, protease, and peptidoglycan hydrolase (Li et al., 1998) and these OMV play a role in nutrient transformation, biofilm formation and resistance to antimicrobial agents (Lim and Yoon, 2015). OMV also play a role in communication between cells, the lysing and killing of competing bacteria and the modulation of host immune response, and as such they contribute to bacterial survival and virulence. For example OMV of *Pseudomonas aeruoginosa* PA01 are capable of lysing *Staphylococcus aureus* and *Escherichia coli*, while those generated by *Citrobacter* strains can lyse *Mycobacterium* strains (Li et al., 1998, Lee, 2012, Kulp and Kuehn, 2010). OMV help *P. aeruoginosa* in pathogenesis due to their association with other virulence factors such as peptidoglycan hydrolase,

hemolysin, phospholipase C and alkaline phosphatase. These OMV effect host cells by disrupting the electron transport chains and trafficking pathways of the cells. Due to these factors *P. aeruginosa* OMVs can kill both Gram-negative and Gram-positive competing bacteria in co-culture or in the environment especially during infection (MacDonald and Kuehn, 2013).

In the current study *P. aeruginosa* Ps 3 grown on ethanol was more pathogenic than when grown on glucose. This suggests ethanol induced virulence gene expression more than glucose. This difference may be due to carbon sources that induce virulence genes to excrete toxins. Differences in virulence due to different grown media has been reported in *Listeria monocytogenes* (Park et al., 1992). These authors reported that expression of virulence genes was greater on Brain heart infusion than Tryptone soya broth and this may be due to either the presence of unknown inducers of gene expression (virulence) in BHI and their absence from other (Park et al., 1992). In another study on invasion of HeLa cells by *Streptococcus pyogenes* media composition was shown to have a significant impact on invasion (Edwards and Massey, 2011).

In order to gain a better insight into the impact of ethanol vs glucose, the associated transcriptomes were constructed. The Ps 3 transcriptome showed that biofilm (psl, pel and alg) genes were expressed on both carbon source media but it's not clear if it is related to the virulence of the Ps 3. To determine how ethanol affects psl and pel genes, Tashiro et al. (2014) studied the transcription of pslA and pelA in the presence and absence of ethanol. Surprisingly the results showed that there are no difference between the expression in both genes during presence and absence of ethanol however the 96 well plate showed induction of biofilm and pellicle formation. They reported that pslA and pelA expression is not effected by ethanol at the transcriptional level, rather ethanol increased Psl and Pel at the translational level, or increased biofilm and pellicle formation without affecting the synthesis processes (Tashiro et al., 2014).

Finally, bacterial strains Ps 3, 10421, St 53, 9203 and *C. freundii* were able to survive for long periods in sterilised ultrapure water (UPW). Kramer et al. (2006) indicated that *Pseudomonas* can survive for months on dry surfaces and non-living objects, they also noted that humidity can improve persistence (Kramer et al., 2006). Another author reported that *Pseudomonas* was able to growth and survive for months in distilled water (Leung et al., 2001) while Liao and Shollenberger (2003) reported that both *P. fluorescens* and *P. aeruginosa* PA01 were able to survive in distilled water for 16 years and 30 weeks respectively (Liao and Shollenberger, 2003). *S. maltophilia* were also studied by some author, Brooke (2012) mentioned that this bacteria prefers wet surfaces and aqueous solution and also it that it has the ability to survive in drinking water and ultrapure water (Brooke, 2012).

7. Future works

- Further studies on the production of Extracellular Polymeric Substances (EPS) and dextran by *S. maltophilia* (St 53) and *C. freundii* on a range of carbon sources.

- 2D NMR and Linkage analysis of the sugar monomers present in the EPS produced by *P. aeruginosa* (Ps 3) *S. maltophilia* (St 53) and *C. freundii* in order to determine the structure of the EPS produced by these bacteria.

- Proteomic analysis of Ps 3 cultures grown on glucose and ethanol in order to identify the basis of the enhanced pathogenicity observed in ethanol grown cultures.

- Study the links or relations between bacterial number (inoculum size), Quorum Sensing (QS), biofilm and pathogenicity.

- Determine the pathogenic characteristics of the Outer Membrane Vesicle (OMV) produced by St 53 and *C. freundii* grown on a range of carbon sources using the wax worm infection model.

8. References

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Statistics analysis

Anova: Two-Factor Without Replication				
SUMMARY	*Count*	*Sum*	*Average*	*Variance*
(2Fe-2S) ferredoxin	2	981.99	491.00	96206.9
Acyl-homoserine lactone acylase subunit beta	2	84.46	42.23	3000.0
(2Fe-2S) ferredoxin	2	981.99	491.00	96206.9
Anthranilate synthase component 2, Pyocyanine specific	2	685.56	342.78	77342.4
Exoenzyme S synthesis protein C	2	61.76	30.88	246.9
Exotoxin A regulatory protein	2	25.2	12.60	218.8
Lipoprotein	2	156.33	78.17	9336.6
Lipoprotein	2	115.46	57.73	307.0
LPS biosynthetic protein lpxo	2	349.33	174.67	1529.6
LPS core heptose(I) kinase rfap	2	100.77	50.39	880.7
Outer membrane protein	2	1479.25	739.63	542059.1
Outer membrane protein	2	18.83	9.42	5.0
Outer membrane protein assembly factor bamb	2	444.94	222.47	9400.9
Outer membrane protein assembly factor bamd	2	339.41	169.71	1815.0
Outer membrane receptor fepa	2	8.86	4.43	7.1
Peptide synthase	2	204.996	102.50	17999.9
Peptidase	2	433.1552	216.58	89149.7
Peptidase m19	2	100.4425	50.22	4334.0
Protease	2	113.24	56.62	578.0
Protease lasa	2	648.49	324.25	11998.6
Protease pfpi	2	1676.7	838.35	54344.5
Protease subunit hflk	2	1372.07	686.04	133742.7
Protease subunit hflk	2	1278.66	639.33	113449.9
Type II secretion system protein F	2	1052.33	526.17	152921.1
Type III export apparatus protein	2	14.17	7.09	10.0
Type III export protein psci	2	17.51	8.76	3.8
Type III secretion outer membrane protein popn	2	7.25	3.63	0.1
Type III secretion system chaperone yscw	2	27.58	13.79	32.5
Type III secretion system protein	2	7.82	3.91	0.7
Type III secretion system regulator exsd	2	49.03	24.52	81.3
Glucose Expression	30	3571.13	119.04	26992.6
Ethanol Expression	30	9266.4537	308.88	136021.5

ANOVA						
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Rows	3,850,812.64	29	132,786.64	4.3929	0.0001	1.8608
Columns	540,611.87	1	540,611.87	17.8848	0.0002	4.1830

Error	**876,597.96**	**29**	**30,227.52**			
Total	**5,268,022.48**	**59**				

Anova: Two-Factor Without Replication				
SUMMARY	***Count***	***Sum***	***Average***	***Variance***
Hypothetical protein	**2**	**255.95**	**127.97**	**31479.62**
Thiamine pyrophosphate-binding protein	**2**	**296.69**	**148.34**	**42199.23**
Hypothetical protein	**2**	**315.64**	**157.82**	**47749.12**
Zinc abc transporter permease	**2**	**100.55**	**50.27**	**4843.76**
Abc transporter substrate-binding protein	**2**	**141.79**	**70.89**	**9617.88**
Manganese abc transporter atp-binding protein	**2**	**114.17**	**57.08**	**6210.93**
Atpase	**2**	**7.99**	**3.99**	**30.07**
5-methyltetrahydropteroyltriglutamate--homocysteine methyltransferase	**2**	**1238.40**	**619.20**	**717716.77**
Cyclic peptide transporter	**2**	**146.89**	**73.45**	**9961.95**
Class v aminotransferase	**2**	**58.33**	**29.17**	**1564.43**
Transporter	**2**	**2000.31**	**1000.15**	**1825275.51**
Chromophore maturation protein pvdo	**2**	**81.06**	**40.53**	**2988.34**
Biopolymer transporter exbd	**2**	**191.84**	**95.92**	**16689.06**
Hypothetical protein	**2**	**247.05**	**123.53**	**27037.52**
Rna 3'-terminal-phosphate cyclase	**2**	**965.18**	**482.59**	**411030.78**
Ornithine monooxygenase	**2**	**725.16**	**362.58**	**231068.55**
Metal abc transporter substrate-binding protein	**2**	**151.18**	**75.59**	**9996.83**
Thioesterase	**2**	**61.22**	**30.61**	**1616.78**
Rna ligase	**2**	**1900.91**	**950.45**	**1551360.50**
Acetyltransferase	**2**	**114.44**	**57.22**	**5574.62**
Tonb-dependent receptor	**2**	**150.54**	**75.27**	**9626.12**
Hypothetical protein	**2**	**190.00**	**95.00**	**15299.43**
Membrane protein	**2**	**165.09**	**82.54**	**11534.65**
Glucose Expression	**23**	**248.93**	**10.82**	**284.37**
Ethanol Expression	**23**	**9371.45**	**407.45**	**306443.46**

ANOVA						
Source of Variation	***SS***	***df***	***MS***	***F***	***P-value***	***F crit***
Rows	**3566675**	**22**	**162121.59**	**1.1211**	**0.395**	**2.0478**
Columns	**1809135**	**1**	**1809135.07**	**12.5108**	**0.001**	**4.3009**
Error	**3181337**	**22**	**144606.24**			
Total	**8557147**	**45**				

Appendix

5.1 All sequences used in the current study

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ACCTACGGGTGAAAGCAGGGGATCTTCGGACCTTGCGCGATTGA
ATGAGCCGATGTCGGATTAGCTAGTTGGCGGGGTAAAGGCCCA
CCAAGGCGACGATCCGTAGCTGGTCTGAGAGGATGATCAGCCA
CACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCA
GTGGGGAATATTGGACAATGGGCGCAAGCCTGATCCAGCCATA
CCGCGTGGGTGAAGAAGGCCTTCGGGTTGTAAAGCCCTTTTGTT
GGGAAAGAAATCCAGCTGGCTAATACCCGGTTGGGATGACGGT
ACCCAAAGAATAAGCACCGGCTAACTTCGTGCCAGCAGCCGCG
GTAATACGAAGGGTGCAAGCGTTACTCGGAATTACTGGGCGTA
AAGCGTGCGTAGGTGGTCGTTTAAGTCCGTTGTGAAAGCCCTGG
GCTCAACCTGGGAACTGCAGTGGATACTGGGCGACTAGAGTGT
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GAGATCAGGAGGAACATCCATGGCGAAGGCAGCTACCTGGACC
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GGGAATATTGGACAATGGGCGCAAGCCTGATCCAGCCATACCG
CGTGGGTGAAGAAGGCCTTCGGGTTGTAAAGCCCTTTTGTTGGG
AAAGAAATCCAGCTGGCTAATACCCGGTTGGGATGACGGTACC
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CGTGCGTAGGTGGTCGTTTAAGTCCGTTGTGAAAGCCCTGGGCT
CAACCTGGGAACTGCAGTGGATACTGGGCGACTAGAGTGTGGT
AGAGGGTAGCGGAATTCCTGGTGTAGCAGTGAAATGCGTAGAG
ATCAGGAGAACATCCATGGCGAAGGCAGCTACCTGGACCAACA
CTGACACTGAGGCACGAAAGCGTGGGGAGCAAACAGGATTAGA
TACCCTGGTAGTCCACGCCCTAAACGATGCGAACTGGATGTTGG
GTGCAATTTGGCACGCAGTATCGAAGCTAACGCGTTAAGTTCGC
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ACGGGTGAAAGCAGGGGATCTTCGGACCTTGCGCGATTGAATG
AGCCGATGTCGGATTAGCTAGTTGGCGGGGTAAAGGCCCACCA
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TGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTG
GGGAATATTGGACAATGGGCGCAAGCCTGATCCAGCCATACCG
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CAAAGAATAAGCACCGGCTAACTTCGTGCCAGCAGCCGCGGTA
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CGTGCGTAGGTGGTCGTTTAAGTCCGTTGTGAAAGCCCTGGGCT
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AGAGGGTAGCGGAATTCCTGGTGTAGCAGTGAAATGCGTAGAG
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>Stenotrophomonas maltophilia 9203 (ATCC 13637T)
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TCCTGGTGTAGCAGTGAAATGCGTAGAGATCAGGAGGAACATC
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GCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAA
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GTCATCATGGCCCTTACGGCCAGGGCTACACACGTACTACAATG
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GAGGGAGAAAGTGGGGGATCTTCGGACCTCACGCTATCAGATG
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CTGGGAACTGCATCCAAAACTACTGAGCTAGAGTACGGTAGAG
GGTGGTGGAATTTCCTGTGTAGCGGTGAAATGCGTAGATATAGG
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CACTGAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACC
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AGATG

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>Pseudomonas aeruginosa strain 10421 (ATCC 15442)
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GGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGATGCA
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GAGATGCTTTGGTGCCTTCGGG

>gi|636559546|ref|NR_115606.1| Staphylococcus aureus strain ATCC 12600 16S ribosomal RNA gene, partial sequence
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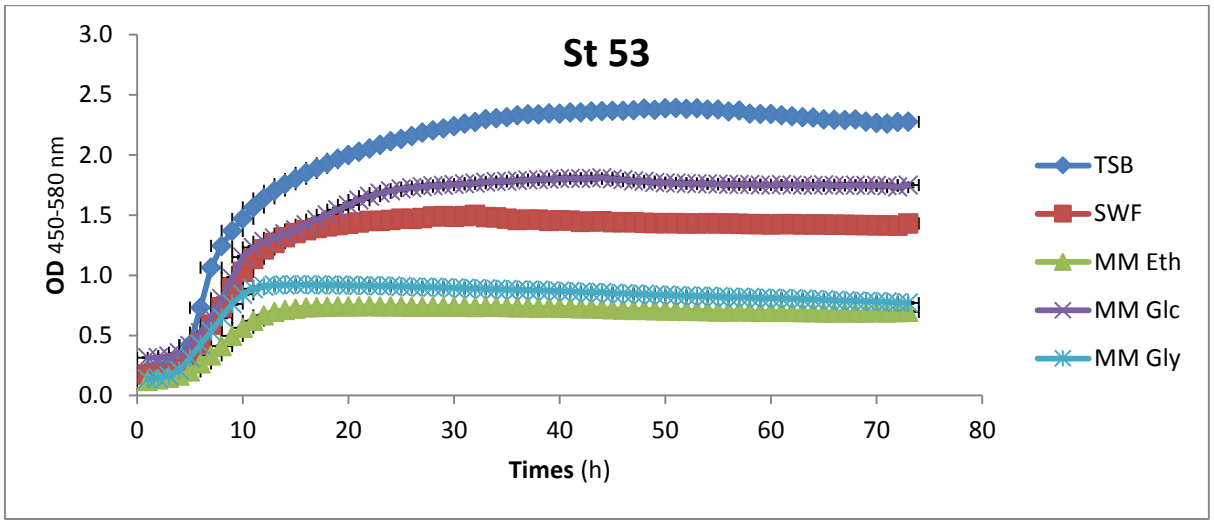
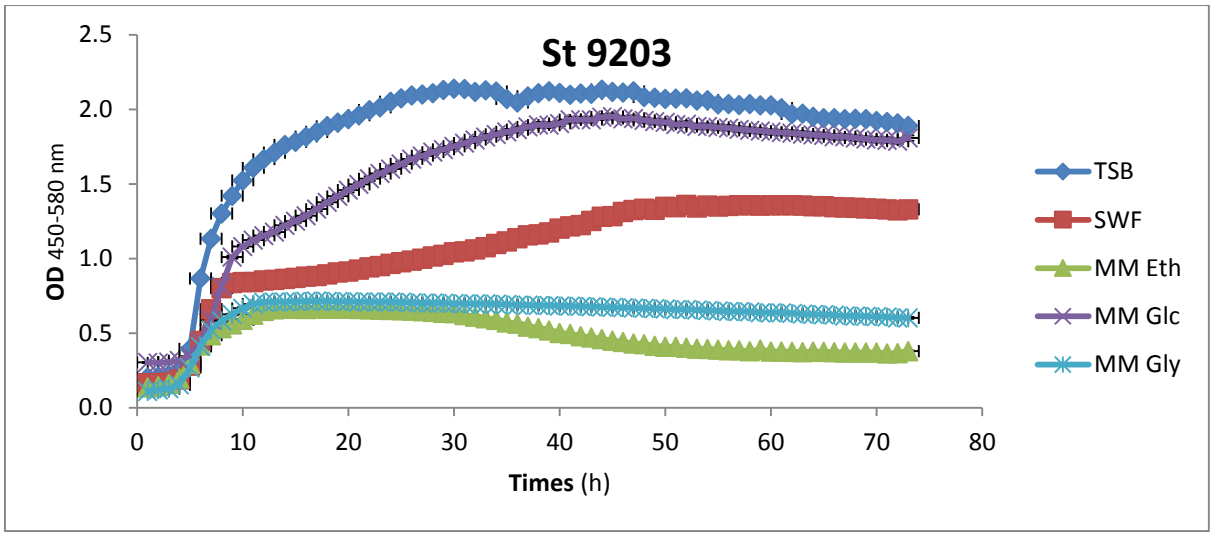
5.3 Growth curve


St 53
OD 450-580 nm
Times (h)
TSB
SWF
MM Eth
MM Glc
MM Gly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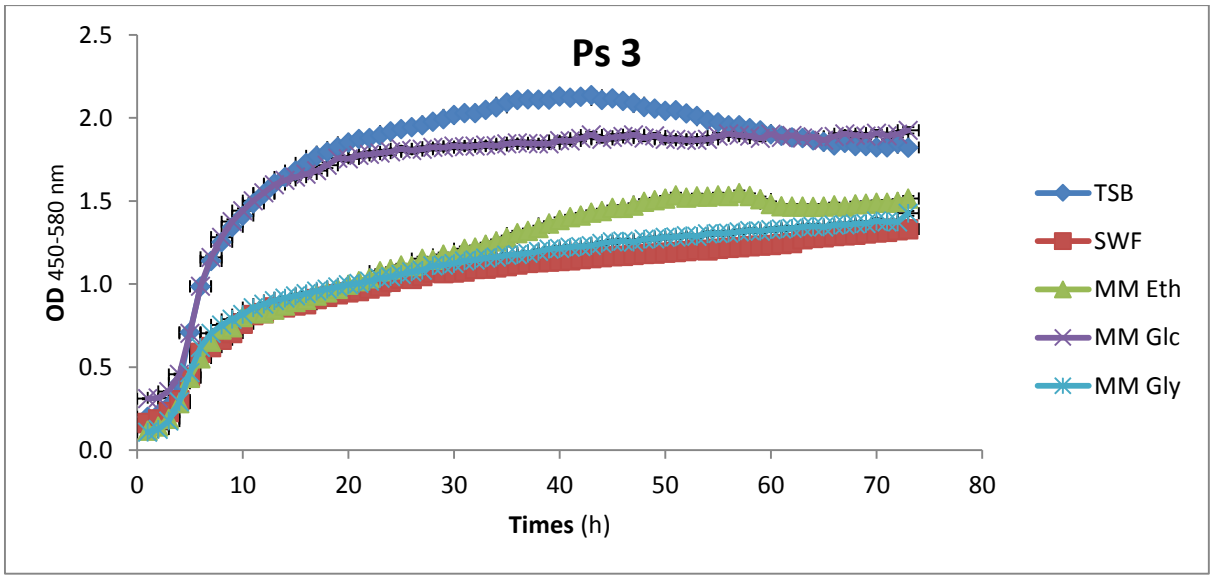
Times (h)
TSB
SWF
MM Eth
MM Glc
MM Gly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Times (h)
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SWF
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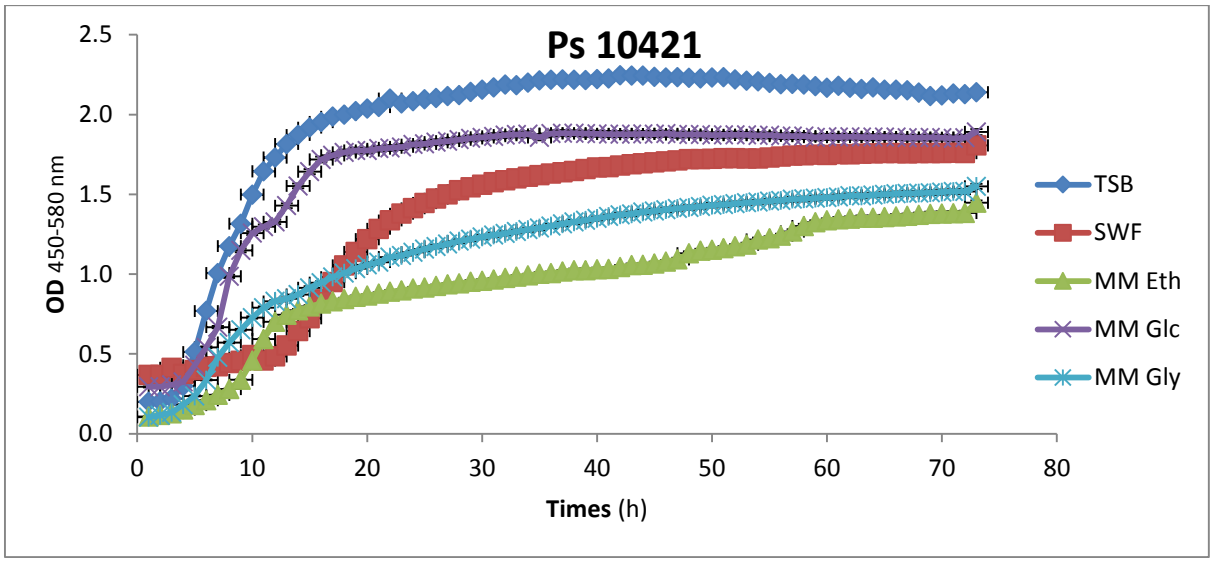
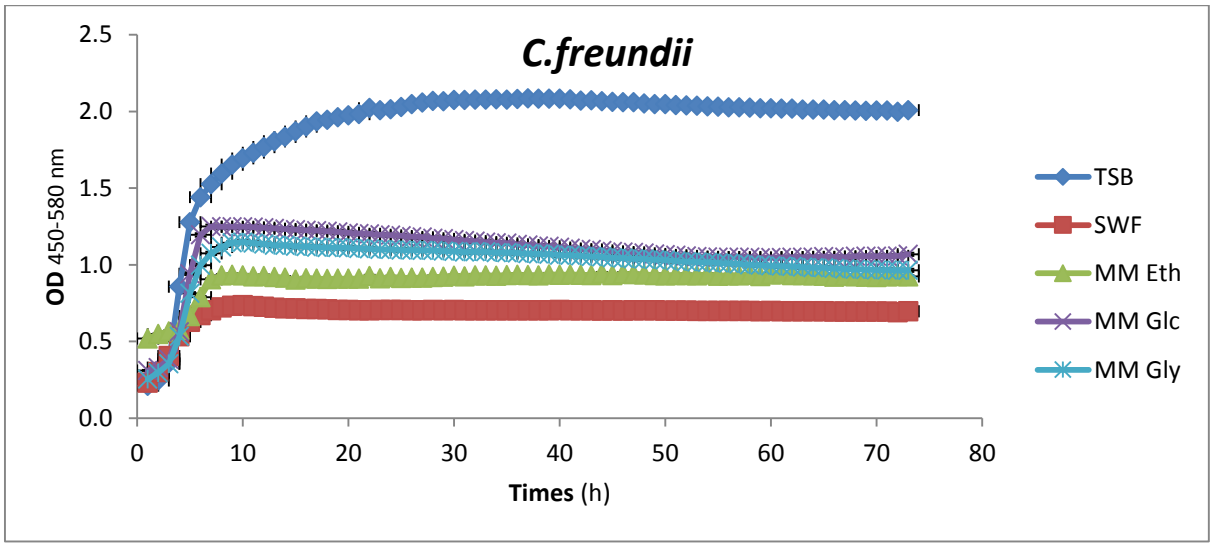
MM Glc
MM Gly





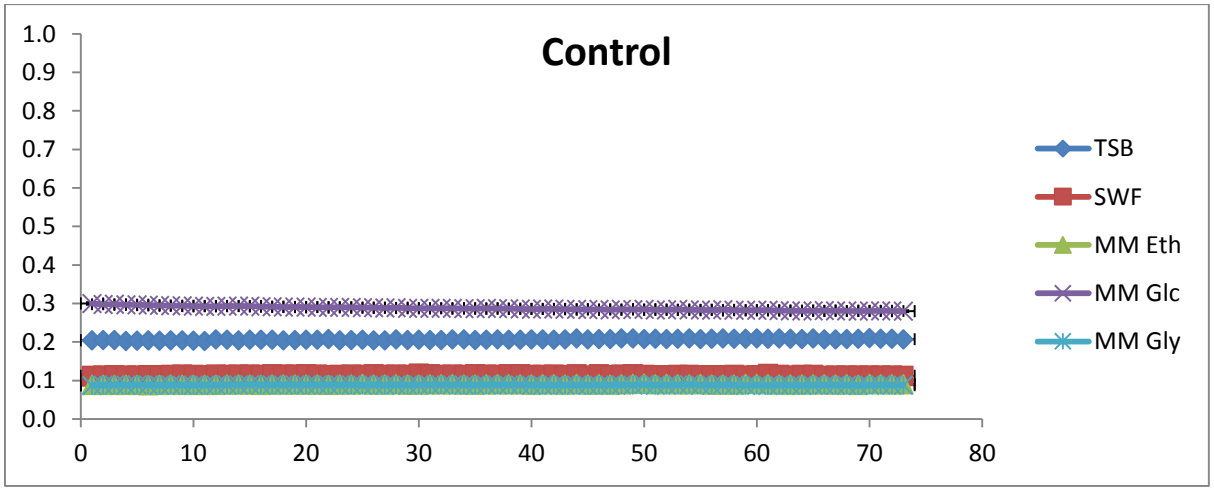
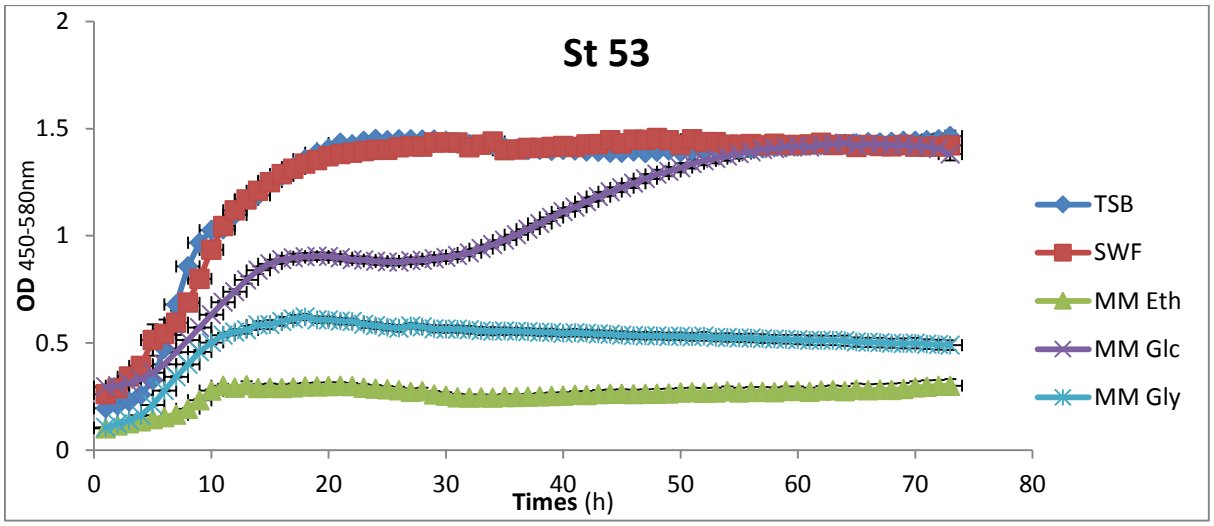
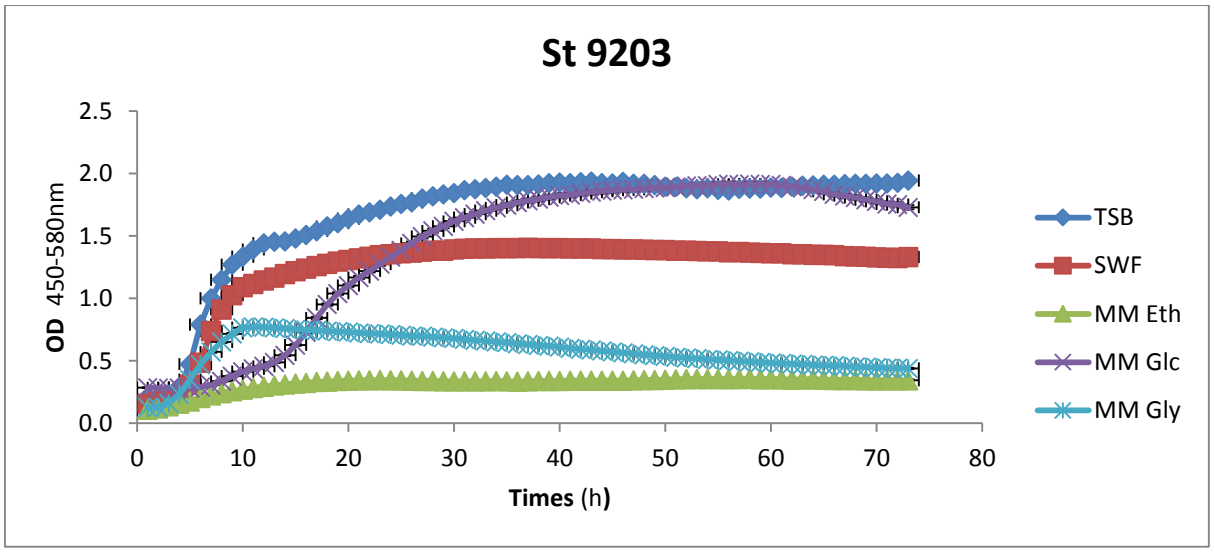


Figure 5.3A Growth curves of different bacteria St 53, St 9203, Ps 3, Ps 10421, *C. freundii* and control on different media at 30°C from time 0 until 72 h in an automated Bioscreen C system (O.D. wideband). Error bars represent standard error of the mean of five replicates.


St 53
OD 450-580nm
Times (h)
TSB
SWF
MM Eth
MM Glc
MM Gly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Times (h)
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SWF
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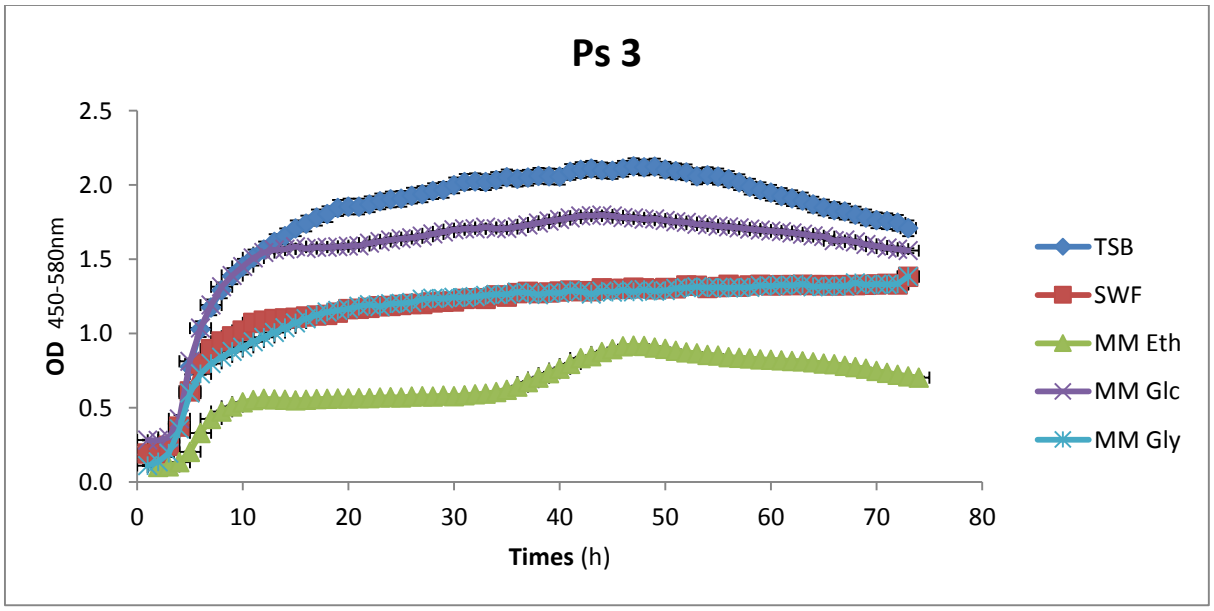
MM Glc
MM Gly


Ps 3
OD 450-580nm
Times (h)
TSB
SWF
MM Eth
MM Glc
MM Gly

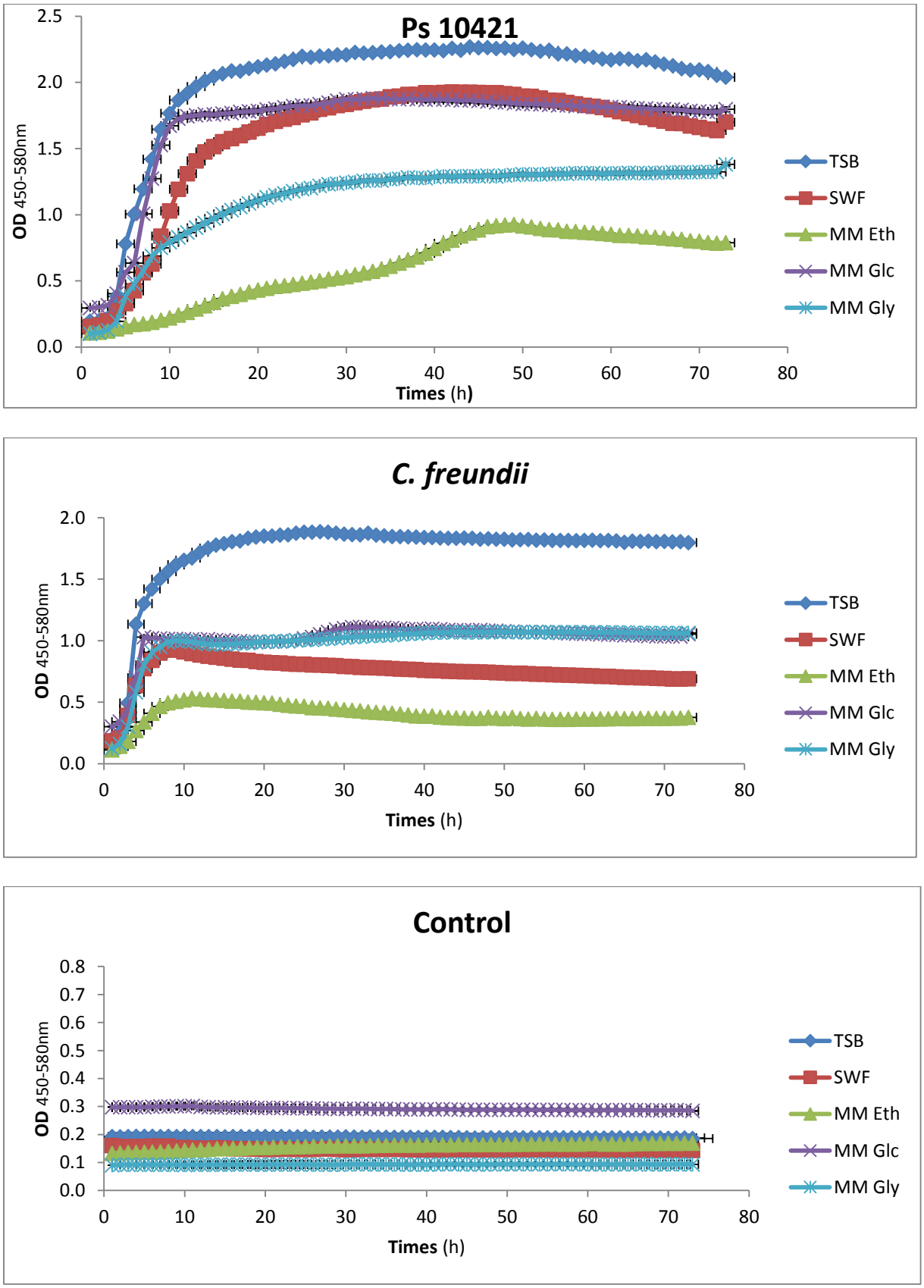


Figure 5.3B Growth curves of different bacteria St 53, St 9203, Ps 3, Ps 10421 *C. freundii* and control on different media at 37°C from time 0 until 72 h in an automated Bioscreen C system (OD: wideband). Error bars represent standard error of the mean of five replicates.

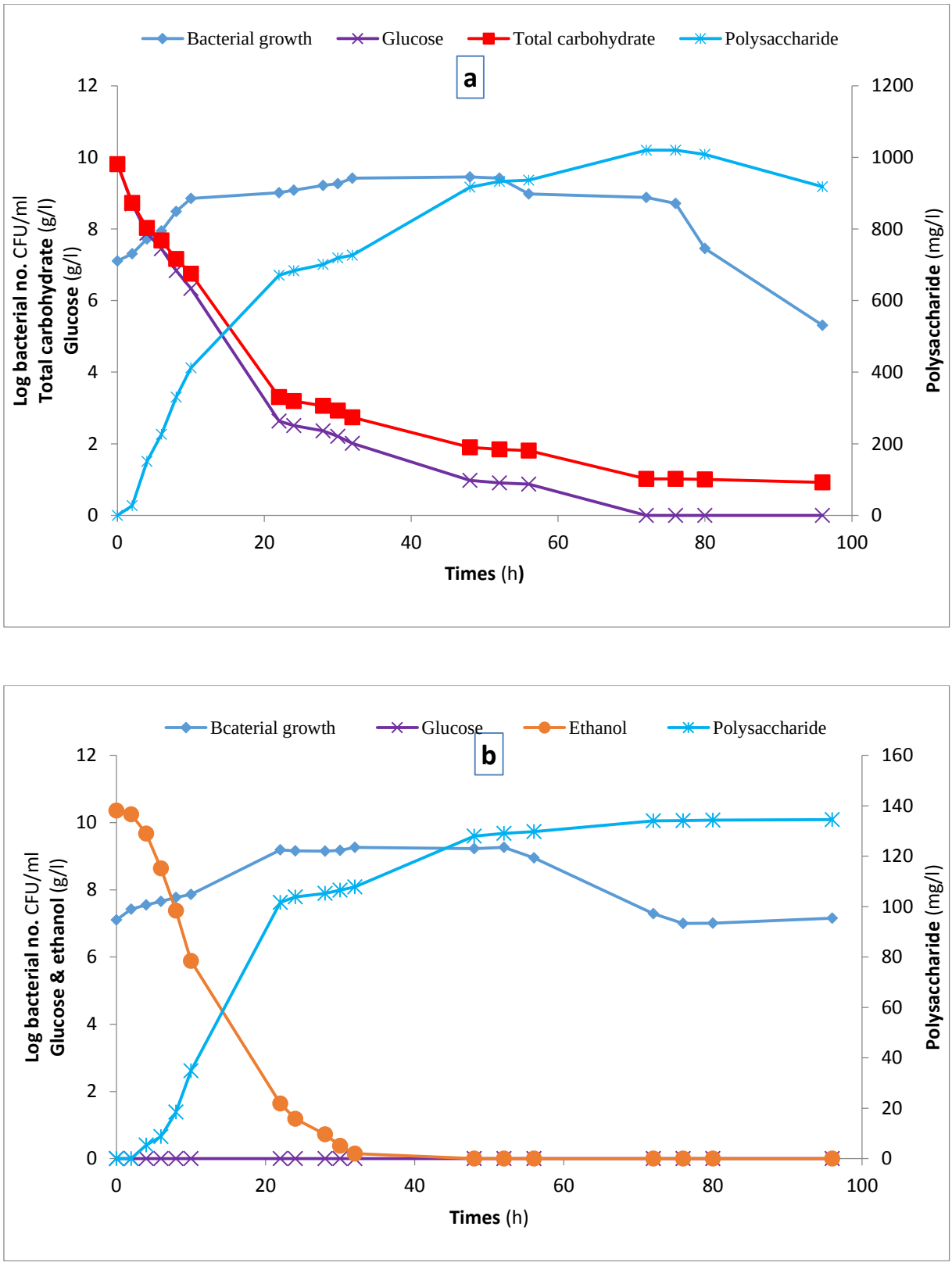
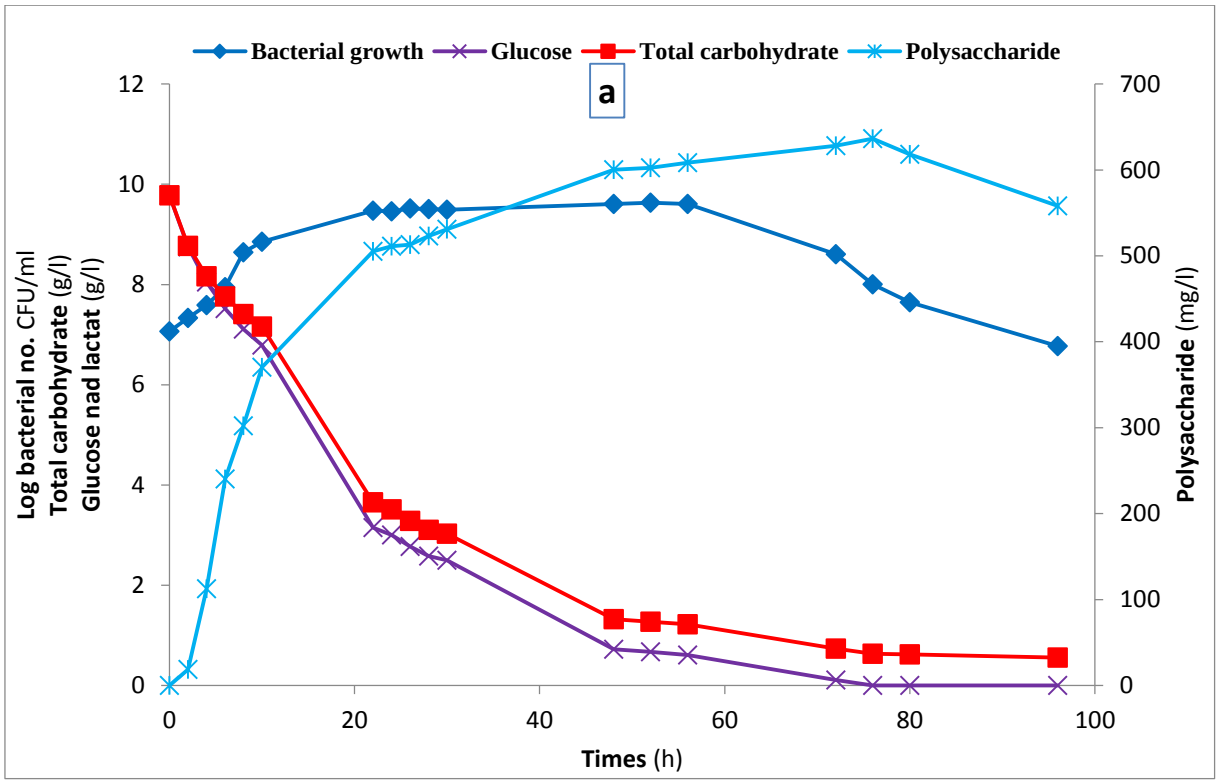


Figure 5.3 C Carbon utilisation and polysaccharide formation during growth of St 9203 on different carbon sources after inoculation with 10^7 CFU/ml at 30°C for 4 days a) MM Glc (10g/l) b) MM Eth (10.57 g/l).



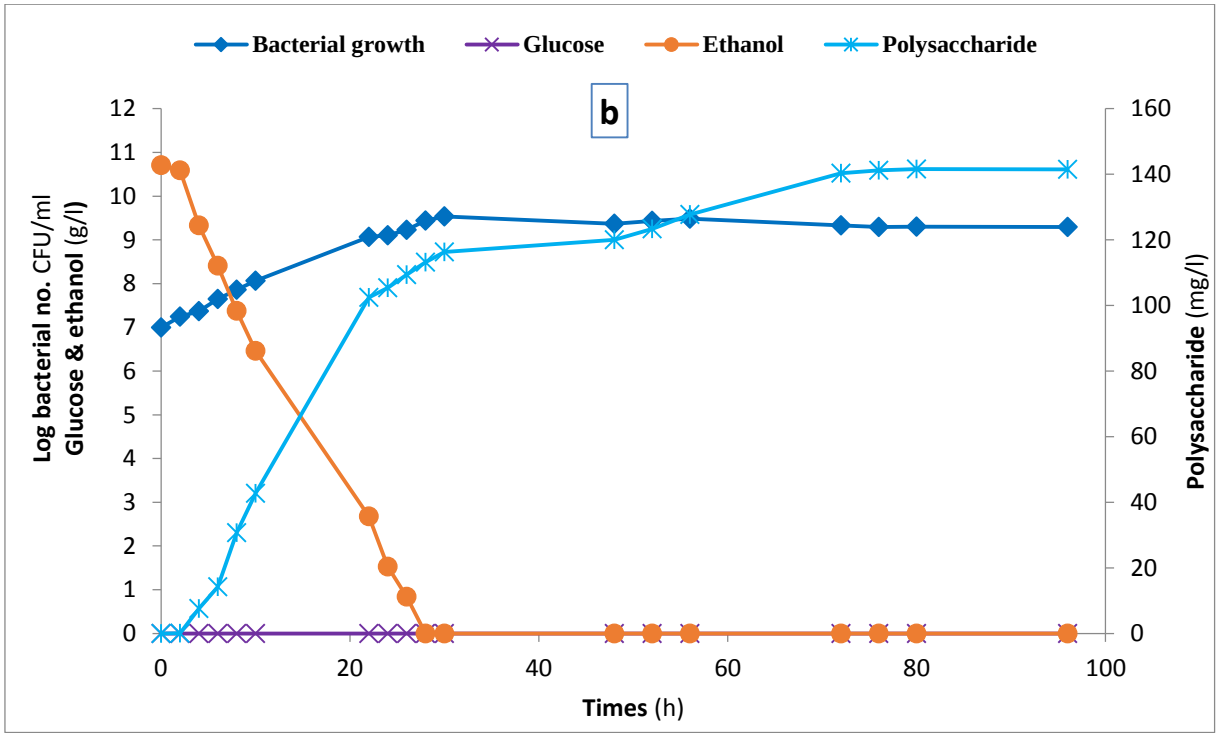


Figure 5.3 D Carbon utilisation and polysaccharide formation during growth of Ps 10421 on different carbon sources after inoculation with 10^7 CFU/ml at 30°C for 4 days a) MM Glc (10g/l) b) MM Eth (10.57 g/l).

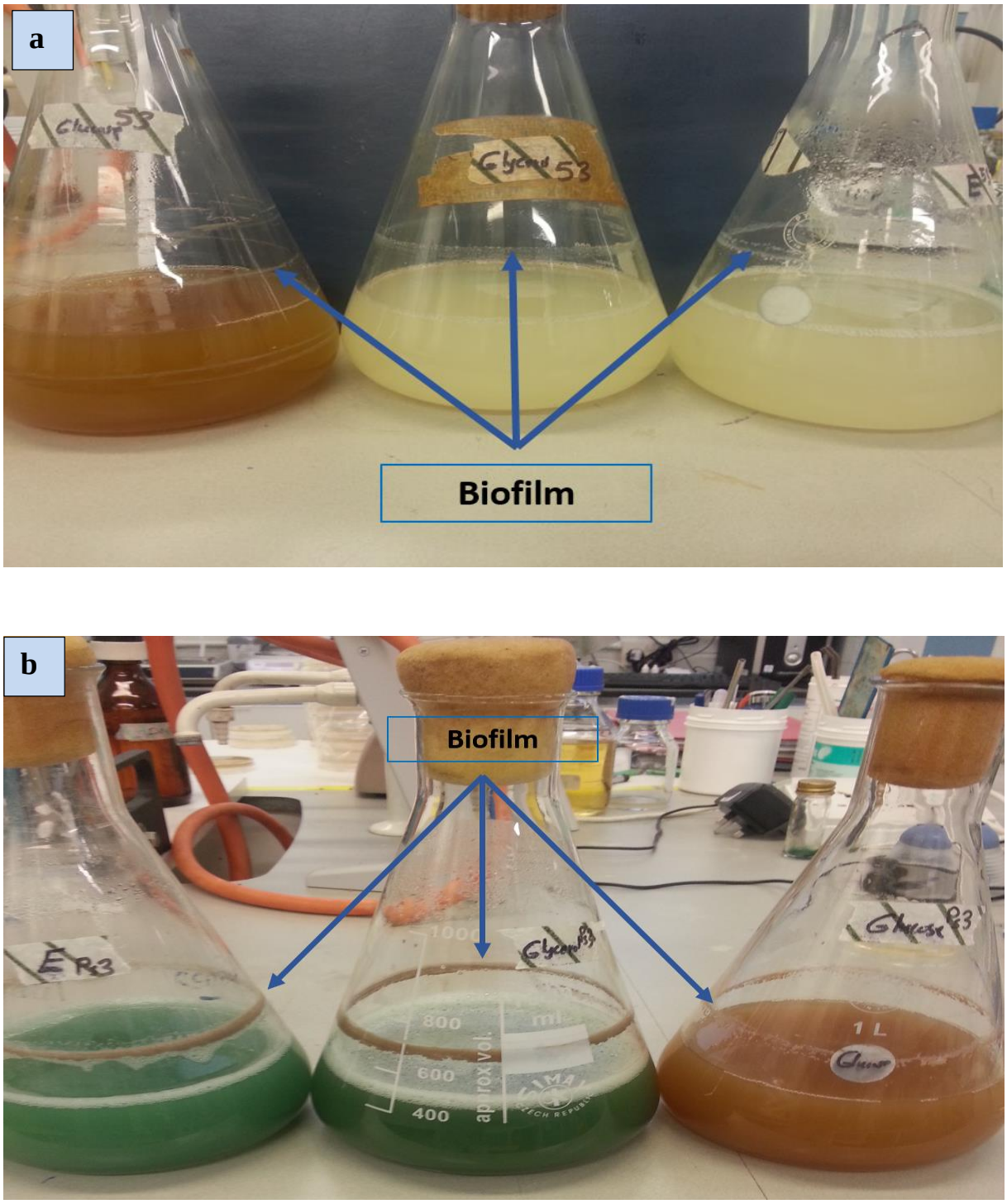


Figure 5.3 E Bacterial biofilm formation during growth on mineral media containing different carbon source (Glucose, Glycerol and Ethanol) a) St 53 and b) Ps 3**.**

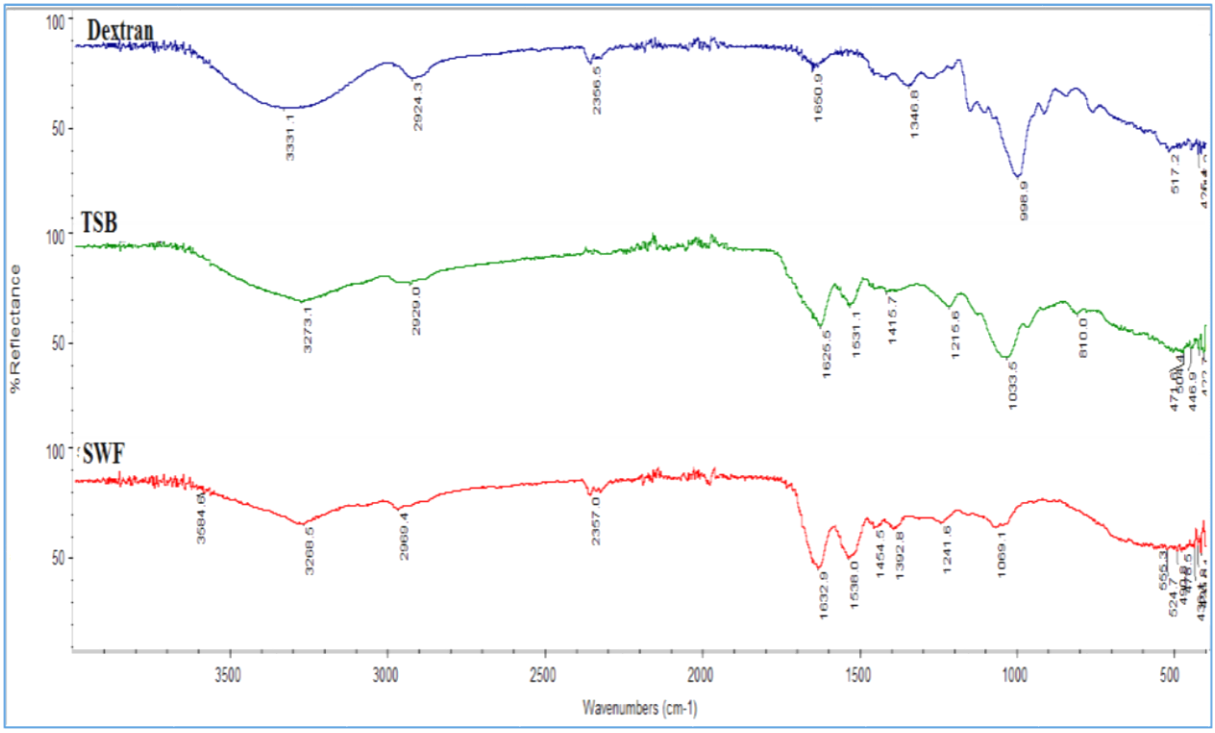


Figure 5.3 F FTIR analysis of dextran polysaccharide and some interfering crude substance in TSB and SWF medium without any bacterial cultivation showing different functional group and band assignment.

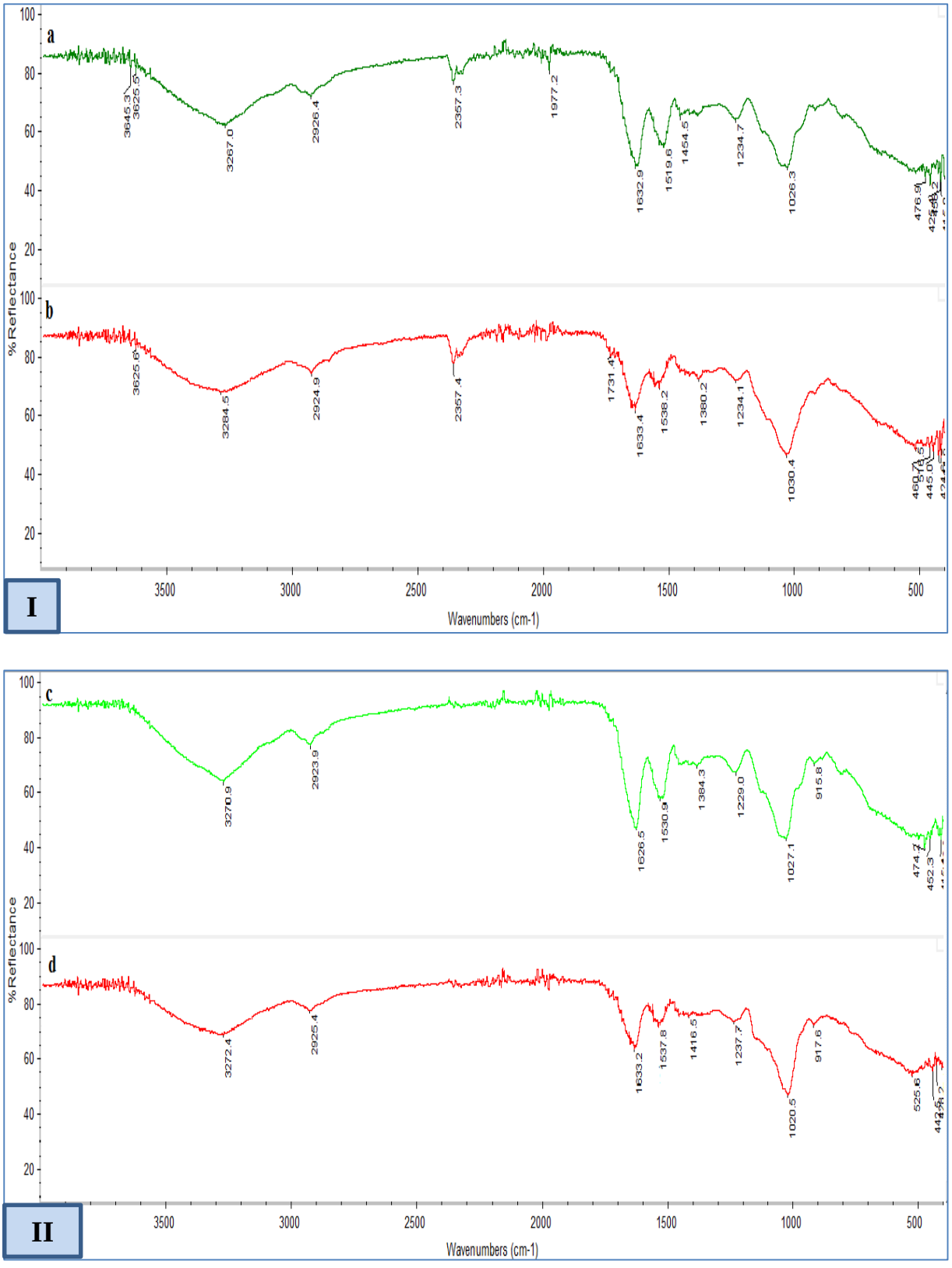


Figure 5.3 G FTIR analysis of EPS produced by Ps 3 on I) MM Eth and II) MM Glc. a and c) prior dextranase treatment b and d) post dextranase treatment .

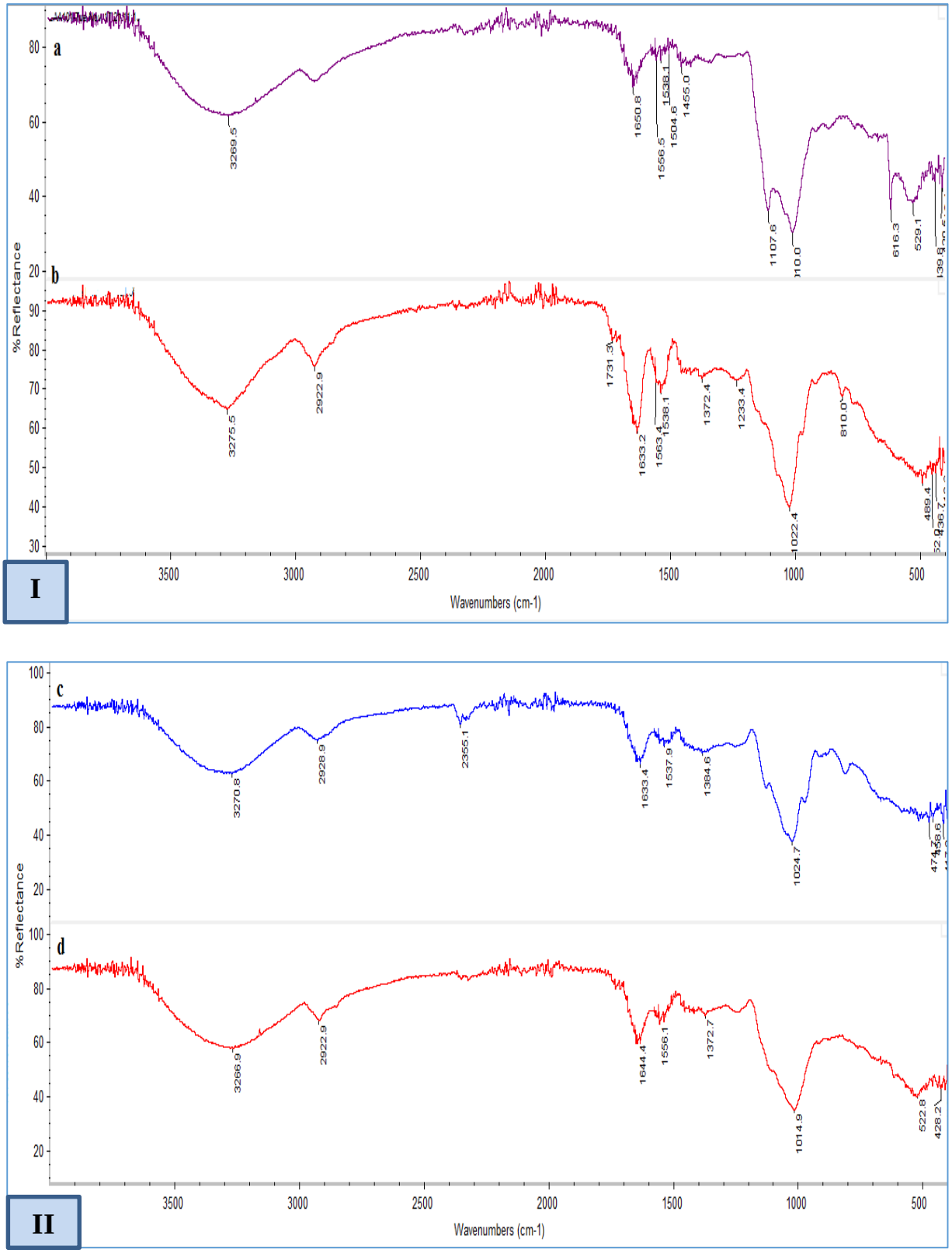


Figure 5.3 H FTIR analysis of EPS produced by different bacteria on MM Eth prior I) St 53 and II) *C. freundii*. a and c) prior dextranase treatment b and d) post dextranase treatment

5.4 Bacterial pathogenicity

Table 5.4 Determination of biofilm genes (psl, pel and alginate) in Ps 3 comparing to PA01

Ps3 Genbank accession	Locus tag	Gene Name	% similarity to PA01	Mismatches
LH31_26130	PA2231	pslA	100.00	0
LH31_26125	PA2232	pslB	99.59	2
LH31_26115	PA2233	pslC	99.67	1
LH31_26110	PA2234	pslD	99.61	1
LH31_26120	PA2235	pslE	99.70	2
LH31_26105	PA2236	pslF	99.75	1
LH31_26100	PA2237	pslG	100.00	0
LH31_26095	PA2238	pslH	98.51	6
LH31_16460	PA2239	pslI	98.94	3
LH31_16455	PA2240	pslJ	99.79	1
LH31_16450	PA2241	pslK	98.93	5
LH31_16445	PA2242	pslL	98.87	4
LH31_16440	PA2243	pslM	98.27	10
LH31_16435	PA2244	pslN	99.40	2
Absent	PA2245	pslO	-	-

Ps3 Genbank accession	Locus tag	Gene Name	% similarity to PA01	Mismatches
LH31_17590	PA3064	pelA	99.05	1
LH31_17595	PA3063	pelB	99.05	9
LH31_17600	PA3062	pelC	100.00	0
LH31_17605	PA3061	pelD	99.12	4
LH31_17610	PA3060	pelE	99.09	3
LH31_17615	PA3059	pelF	99.41	3
LH31_17620	PA3058	pelG	100.00	0

Ps3 Genbank accession	Locus tag	Gene Name	% similarity to PA01	Mismatches
LH31_22495	PA3542	alg44	100.00	0
LH31_22490	PA3541	alg8	100.00	0
LH31_22540	PA3551	algA	99.79	1
LH31_19975	PA5483	algb	99.33	3
LH31_13805	PA5322	algC	99.78	1
LH31_22485	PA3540	algD	100.00	0
LH31_22505	PA3544	algE	100.00	0

LH31_22535	PA3550	algF	99.54	1
LH31_22510	PA3545	algG	99.63	2
LH31_08110	PA0405	algH	99.47	1
LH31_22525	PA3548	algI	99.23	4
LH31_22530	PA3549	algJ	100.00	0
LH31_22500	PA3543	algK	99.79	1
LH31_22530	PA3547	algL	100.00	0
LH31_15795	PA3257	algO	100.00	0
LH31_30390	PA5253	algP	100.00	0
LH31_30380	PA5255	algQ	100.00	0
LH31_30350	PA5261	algR	100.00	0
LH31_22015	PA0762	algU	99.37	2
LH31_06145	PA4446	algW	99.23	3
LH31_22515	PA3546	algX	99.58	2
LH31_23600	PA3385	AmrZ	100.00	0
LH31_22020	PA0763	mucA	100.00	0
LH31_22025	PA0764	mucB	100.00	0
LH31_22030	PA0765	mucC	99.81	1
LH31_22035	PA0766	mucD	99.37	3