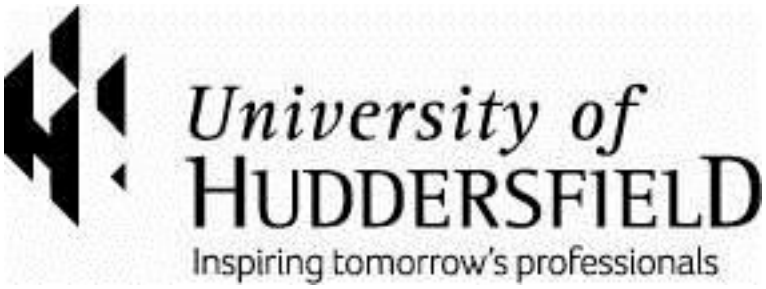
# Biofilm Formation and Biocide Resistance of *Acinetobacter baumannii*

Kamran Shoukat, M.Phil




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

**The University of Huddersfield**

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*Dedicated to my sweet mom, Dad and my uncle Abid Hussain (Late)*
*Thanks you for your moral and financial support through out of my life, off course you have made me where i am today, i just hope i have done you proud in return.*

*Love you.*

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## Publications

Shoukat. K., Humphreys, P., and Laws. A.P. (2012). Growing, analysing, Isolation and characterization of *Acinetobacter* Biofilm (Poster), in SGM Autumn conference March 26-29th 2012, conventional centre Dublin Ireland (conference proceedings).

Shoukat. K., Humphreys, P., and Laws. A.P. (2010). The impact of disinfectants on biofilm formation of *Acinetobacter sp* (Poster), in S***f***AM summer conference, 5th-8th July 2010 the Grand hotel Brighton(conference proceedings).

## Abstract

Multi drug resistant (MDR) strains of *Acinetobacter baumannii* have emerged as a major cause of nosocomial infections associated with significant morbidity and mortality. Over the last 20 years a worldwide expansion in *Acinetobacter* infections has been observed associated with intensive care units (ICUs), long term care facilities and wounded armed forces personnel. The developing resistance patterns seen in *Acinetobacter sp* suggest that the number of effective antibiotics may shortly be exhausted. The ability of *Acinetobacter sp* to form biofilms, resist desiccation and persist on hospital surfaces has played a critical role in the emergence of this bacterium as a human pathogen. The ability of clinical strains of *A. baumannii* to form strong adherent biofilms has also been recognised as a key virulence factor for this pathogen.

This thesis has investigated the ability of a range of *A. baumannii* strains to form biofilms and resist the impact of common biocides. In order to facilitate this research a carbohydrate free minimal media employing glucose or alcohols as sole carbon sources was developed. Considerable variations in the sensitivity of strains to ethanol or IPA was observed with the Type strain being more sensitive and less able to use alcohols to support growth than many of the clinical strains investigated. Alcohols as sole carbon sources had an impact on bacterial adherence, with 71% of strains being highly adherent when fed on alcohol as the sole carbon source. Scanning electron microscopy and fluorescent microscopy indicated that highly adherent strains were able to establish biofilms on both hydrophobic (plastic) and hydrophilic (glass) surfaces, forming carbohydrate based extracellular polymeric substances (EPS) during biofilm formation. EPS generation occurred alongside the transient generation of lactate, the latter being degraded during the stationary phase. Biofilm forming strains generated high MW EPS when grown on mineral media with ethanol as a sole carbon source, extracted EPS was shown to contain repeating units of both galactose and rhamnose sugars.

The Bioscreen system was used to determine the MICs of a range of quaternary ammonium compounds (QACs) and PHMB against the highly adherent strains. MIC values were below 35 mg/l for all biocides tested and MBC for planktonic cells were from 6 to 100 x greater than the MIC values. MBC values for biofilms were orders of magnitude greater than MBC values for planktonic cells with little variation between biocides or carbon source. Planktonic cells were able to form biofilms at concentration considerably greater than the 24 hour MBC for planktonic cells, demonstrating that biofilm formation provided additional protection against the biocides investigated.

A range of antimicrobial wound dressings (NSCD, ISCD, Honey and PHMB) were evaluated for their impact on commonly occurring wound pathogens i.e. *A. baumannii*, *P. aeruginosa* and *S. aureus* (MRSA). Dressings were evaluated against both planktonic cells and cells immobilised in a collagen matrix. In all cases there were significant differences ($p<0.05$) between strains of the same species when treated with the same dressing, indicating that significant variations in the susceptibility of wound pathogens to antimicrobial dressings were present at the sub species level. The diffusion barrier provided by the collagen matrix generated lower reduction values than the planktonic approach with a few exceptions. Broadly speaking the NSDC dressing was the most effective, PHMB least effective and the Honey dressing was most affected by the diffusion barrier provided by the collagen matrix.

# Contents

# 1 Introduction

Historically the clinical isolation of *Acinetobacter sp* was frequently ignored, being considered a low grade pathogen (Bergogne-Berezin *et al*. 1996). However, in recent years, multi drug resistant (MDR) strains of *A. baumannii* have emerged as a major cause of nosocomial infections associated with significant morbidity and mortality (McConnell *et al*. 2012). Over the last 20 years a worldwide expansion in *Acinetobacter* infections has been observed associated with intensive care units (ICUs), long term care facilities and wounded personnel (Sebeny et al., 2008, Sengstock *et al*. 2010). The developing resistance patterns seen in *Acinetobacter* sp suggest that the number of effective antibiotics may shortly be exhausted (Hanlon, 2005). The ability of *Acinetobacter sp* to resist desiccation and persist on hospital surfaces, materials and medical devices has played a critical role in the emergence of this bacterium as a human pathogen (Villegas and Hartstein, 2003).

## 1.1. Aims and Objectives

The overall aim of this research is to determine the impact of common biocides on the biofilm formation of clinical strains of *A. baumannii*.

The related objective being:

1. To determine the ability of *A. baumannii* strains to form biofilms when utilising a range of carbon sources including alcohols;
2. To extract and characterize the EPS generated by biofilm forming strains of *A. baumannii;*
3. To determine the Minimum Inhibitory Concentrations (MIC) of a range of biocides against clinical strains of *A. baumannii*;
4. To determine the Minimum Biocidal Concentrations (MBC) of a range of biocides against planktonic cells and biofilms of clinical strains of *A. baumannii*;
5. To determine whether or not *A. baumannii* strains are able to form biofilms in the presence of biocides;
6. To evaluate the antimicrobial efficacy of range of wound dressings against a variety of Gram-positive and Gram-negative wound pathogens.

# 2 Literature Review

## 2.1 *Acinetobacter*

### 2.1.1 The Genus *Acinetobacter*

In 1911 Beijerinck a Dutch Microbiologist, first isolated *Acinetobacter sp* from soil using a minimal media enriched with calcium acetate. Originally described as *Micrococcus calco-aceticus*, the genus *Acinetobacter* (from Greek word "akinetos", means non-motile) was proposed some 43 years later by Brisou and Prevot (Brisou and Prevot, 1954) to differentiate it from the motile organisms within the Genus *Achromobacter*. By 1968 the genus *Acinetobacter* was widely accepted after Baumann *et al* (Howard *et al*. 2012) published a comprehensive study of *Mirococcuscalco-aceticus, Alcaligenes hemolysans, Mima polymorpha, Moraxella lwoffi, Herellea vaginicola and Bacterium anitratum,* which concluded that they belonged to single genus and could not be further sub-classified (Howard et al., 2012, Lessel, 1971). Later, in 1971 the sub-committee on the taxonomy of Moraxella and Allied Bacteria officially acknowledged the genus *Acinetobacter* based on the results of Baumann's 1968 publication (Lessel, 1971). *Acinetobacter* may be easily identified presumptively to the genus level (Table 1) (Peleg *et al.* 2008) however; the organisms are often difficult to de-stain and, as such, are often incorrectly identified as Gram-positive (Howard *et al*. 2012). There is also a lack of a definitive metabolic test to distinguish *Acinetobacter* from other non-fermentating Gram-negative bacteria (Peleg et al., 2008). A widely used method to identify to the genus level relies on the ability of the mutant *A. baylyi* strain BD413 trpE27 to be transformed by the crude DNA of any *Acinetobacter sp* to a wild type phenotype (the transformation assay of Juni;) (Juni,1972).

### 2.1.2 *Acinetobacter* Species

For species level identification, the 28 available phenotype tests have proven to be 95.6% effective in identifying human skin-derived *Acinetobacter* (Seifert *et al*. 1997). However, phenotypic tests alone have proven to be ineffective in identifying more recently discovered genomic strains of *Acinetobacter* (Peleg *et al*. 2008). More advanced molecular diagnostic methods have been developed for the identification to the species level, these include: amplified 16S rRNA gene restriction analysis, high resolution fingerprints analysis by the amplified fragment length polymorphism, ribotyping, tRNA spacer fingerprinting, restriction analysis of the 16S-23S rRNA intergenic spacer sequences, sequence analysis of 16S-23S rRNA gene spacer region, and sequencing of the rpo*B* (RNA polymerase β subunit) gene and its

flanking spacers (Howard *et al*. 2012). DNA/DNA hybridisation studies have revealed that the genus now consists of 27 species and nine generic sub species (Di Nocera *et al*. 2011). The four species of *Acinetobacter* (*A. baumannii, A. calcoaceticus A.* genomic species 3 and *Acinetobacter* genomic species 13TU) are difficult to distinguish phenotypically and therefore are often referred to as the *A. calcoaceticus* complex (Howard *et al*. 2012). This nomenclature can be misleading as the environmental species *A. calcoaceticus* has not been implicated in disease, while the other three species in the complex have being implicated in both communities acquired and nosocomial infections (Peleg *et al*. 2008).

| **Characteristic** | |
|---|---|
| Gram reaction | -ve |
| Metabolism | Strictly aerobic |
| Fermentation | None |
| Motile | No |
| Pigmented | No |
| Oxidase | -ve |
| Catalase | +ve |
| Fastidious | No |
| DNA G+C content | 39-47% |
| Morphology | coccobacilli |
| Growth pattern | Diploid/chains |

Table 1. Characteristics of the Genus Acinetobacter (Peleg *et al*. 2008).

### 2.1.3 **Distribution of *Acinetobacter* sp.**

*Acinetobacter baumannii* has been recovered from soil, water, animals and humans (Paterson, 2006). On the basis of ecology, epidemiology and antibiotic phenotype of different isolates, Towner proposed three major *Acinetobacter* populations (Towner, 2009, Howard *et al*. 2012).

The first one consisting of *A. baumannii* and closely related members of the *A. baumannii* complex is represented by the strains isolated from hospitalised patients, medical environments, associated equipment's and medical personnel. Many of these isolates are resistant to multiple antibiotics; although strains such as the clinical isolate ATCC 19606$^{T}$ and ATCC 17978 remain sensitive. The second population include strains found on human and animal skin flora and those associated with food spoilage, members of this group include *A. johnsonii, A. lwoffii,* and *A. radio-resistens*. The last group consists of antibiotic-sensitive isolates obtained from environmental sources such as soil and wastewater and include *A. calcoaceticus* and *A. johnsonii*. Although most members of the last two groups are antibiotics sensitive, some isolates of *A. radio-resistens, A. calcoaceticus and A. johnsonii* have been found to be resistant to carbapenemase (Figueiredo *et al*. 2011).

## 2.2 *Acinetobacter* Infections

*A. baumannii* has become an increasingly important human pathogen, associated with infections acquired in hospitals, long term care facilities, in the community and in wounded military personnel (Sebeny *et al*. 2008, Anstey *et al*. 2002, Leung *et al*. 2006b, Scott *et al*. 2007, Sengstock et al., 2010, McConnell *et al*. 2012). In health care settings, it has become more difficult to treat *Acinetobacter* infections, because of their resistance to major groups of antimicrobial agents (Carling and Bartley, 2010, Kramer *et al*. 2006, Wagenvoort *et al*. 2011). *A. baumannii* infections are found across a wide range of anatomical regions and with varying severity and patient outcomes (Gordon and Wareham, 2010). There have been differences in opinion relating to the actual clinical impact of infection and associated impacts on patient mortality. Whilst most studies suggest that *Acinetobacter* infections results in detrimental effect on patient outcome, other studies have implied little or no impact (Grupper *et al*.2007, Wu et al., 2007, Sunenshine *et al*. 2007, Jang *et al*. 2009, Scott *et al*. 2007). One reason for this confusion is that the *Acinetobacter* isolates have only been identified to genus level, with many referring to infection with *A. calcoaceticus-baumannii* complex which could conceivably indicate colonization with the environmental species *A. calcoaceticus* coupled with a polymicrobial infection, rather than a mono-microbial infection with a virulent *Acinetobacter* species such as MDR *Acinetobacter* (Scott *et al*. 2007).

The most common types of infection caused by *A. baumannii* includes, but are not limited to pneumonia (both hospital and community-acquired), bacteraemia, skin and soft tissue infection, endocarditis, meningitis and urinary tract infections. Most of the cases are thought

to be acquired following exposure to *A. baumannii* that persists on contaminated hospital equipment or by contact with healthcare personnel that have been exposed through contact with colonized patients (Rodríguez-Baño *et al*. 2009, Maragakis *et al*. 2004, Crnich *et al*. 2005, Dijkshoorn *et al*. 2007, Asensio *et al*. 2008).

Hospital acquired pneumonia represents the most common clinical *A. baumannii* infection, generally associated with mechanical ventilation in the intensive care settings. It is thought that ventilators-associated pneumonia caused by *A. baumannii* results from the colonization of the airway via environmental exposure, followed by development of pneumonia (Dijkshoorn *et al*. 2007). It has been reported that 40% to 70% mortality rate is related to the ventilators-associated *Acinetobacter* pneumonia (Garnacho *et al*. 2003), however the mortality directly attributable to *A. baumannii* has been the subject of controversy (Falagas and Rafailidis, 2007, Abbo *et al*. 2007, Falagas *et al*. 2006, Lee *et al*. 2007). Community-acquired *A. baumannii* pneumonia is a less frequent infection with a 40% to 60% mortality rate (Leung *et al*. 2006a, Chen *et al*. 2001) often associated with underlying host factors such as alcohol abuse or chronic obstructive pulmonary disease (McConnell *et al*. 2012).

*A. baumannii* is also a common cause of bloodstream infections in the intensive care settings (Wisplinghoff *et al*. 2004) associated with the lower respiratory tract infections and intravascular devices (Jang *et al*. 2009, Jung *et al*. 2010). Risk factors associated with *A. baumannii* bloodstream infections included; immunosuppression, previous antibiotic therapy, ventilator use associated with respiratory failure, colonization with *A. baumannii* and invasive procedures (Jang *et al*. 2009, Jung *et al*. 2010). Crude mortality rates for *A. baumannii* bloodstream infections have been reported to be between 28% and 43% (Seifert *et al*. 1995). The emergence of drug resistance has contributed significantly to the burden presented by *A. baumannii* blood stream infection. A UK retrospective study (1998-2006) identified an increase in carbapenem resistance from 0% in 1998 to 55% in 2006 in *A. baumannii* isolates causing bacteraemia (Wareham *et al*. 2008). *A. baumannii* bacteraemia also has an associated economic burden, for example bacteraemia caused by MDR *A. baumannii* strains generated $3758 additional medical cost and 13.4 additional days of hospitalization per patient compared with none MDR strains in a tertiary care hospital in Taiwan (Lee *et al*. 2007). *A. baumannii* is an important cause of burn infections, although it can be difficult to differentiate between colonization of burn sites and infection. Because of the poor penetration of some antibiotics into burns and the high rates of multi drug resistance, these infections can be

extremely challenging for clinicians (Chim *et al*. 2007, Keen Iii *et al*. 2010, Keen *et al*. 2010, Albrecht *et al*. 2006).

*A. baumannii* is also responsible for meningitis, with the majority of cases occurring in patients recovering from neurosurgical procedures (Katragkou *et al*. 2006, Ng *et al*. 2006, Ho *et al*. 2007, Huttova *et al*. 2007, Metan *et al*. 2007, Paramythiotou *et al*. 2007, Sasar *et al*. 2007, Guardado *et al*. 2008, Krol *et al*. 2009, Cascio *et al*. 2010), however rare cases of community-acquired *A. baumannii* meningitis have been reported (Chang *et al*. 2000, Taziarova *et al*. 2007, Lowman *et al*. 2008, Ozaki *et al*. 2009). The clinical features of *A. baumannii* meningitis are similar to other bacterial meningitis, i.e. fever, altered consciousness, headache, and seizure (Rodri 2008). The mortality rates associated with *A. baumannii* meningitis are difficult to estimate due to the limited number of studies available and the lack of adequately sized study populations, however one retrospective study identified 51 cases of postsurgical *A. baumannii* meningitis in two tertiary care hospitals between 1990 and 2000 (Guardado *et al*. 2008). These cases represented 10.9% of all meningitis cases at these intuitions and had a crude mortality of 33%, other authors have reported much higher (71%) crude mortality (Metan *et al*. 2007) after evaluating the postsurgical *A. baumannii* meningitis in 28 patients.

### 2.2.1 **Outbreaks/clone involved**

*A. baumannii* has been associated with a number of global outbreaks with the emergence of a number of epidemic strains. Three major epidemic European clones Clone I, Clone II and Clone III been recognised. Clone I and Clone II were responsible for the outbreaks in hospitals of countries of North Western Europe. Clone I has also been isolated from Italy, Poland, Czech republic, South Africa and Spain, whereas Clone II was isolated from France, Greece, Turkey, South Africa, Spain and Portugal. Clone III was isolated from Italy, Spain, France and Netherlands. The latter data suggest that these clones are being virulent and MDR, responsible for outbreaks that are difficult to control and therefore becoming endemic in hospitals (Van Dessel *et al*. 2004).

### 2.2.2 **Military experience**

*A. baumannii* is also a major cause of burn infections in military personnel, and in some cases it has been identified as the most common cause of burn site infection (22%), with 53% of isolates being MDR (Keen *et al*. 2010, Keen Iii *et al*. 2010). Since the onset of conflict in Iraq and Afghanistan, a high of infection associated with MDR *A. baumannii-calcoaceticus* complex has also been reported amongst non-US military casualties evacuated to tertiary medical

centres (O'Shea, 2012). In UK MDR *A. baumannii* infections amongst British personnel returning from operational combat theatres was first reported following the survey of 30 hospitals that had received patients directly from Iraq between March and October 2003 (Jones *et al*. 2006). While 11 (37%) of the receiving hospitals reported the presence of MDR *A. baumannii* infections, their origins pre-dated the admission of military patients. However, several other strains of *A. baumannii* both outbreak and sporadic, had been isolated from patients evacuated from Iraq and were proving a persistent problem. From the onset of the conflict in Iraq in April 2003 and over the following 18 months, *A. baumannii* was isolated from 27 casualties evacuated from Gulf region and admitted to the military medical facility in Birmingham. Out of these 27 patients, 23 were military personnel and the rest of them were civilians (Jones *et al*. 2006). *A. baumannii* isolated from personnel associated with Iraq were PFGE typed and identified as a prominent strain, the T strain, first isolated in May 2003, which was different to the major circulating UK clones [South east (SE) clone, OXA-23 clone I and OXA-23 clone II]. The T strain isolated was MDR, susceptible to amikacin and tobramycin, but generally resistant to ampicillian, amoxicillin/clavulanic acid, gentamycin, ceftazidime, chloramphenicol, ciprofloxacin, cefotaxime, nitrofurantoin and trimethoprim.

Whilst the original T strain isolates were carbapenem-susceptible, they possessed the gene for an OXA-51 like carbapenemase and since April 2005 some members of the T strain clone have been found to be carbapenem-resistant due to activation of the $bla_{\text{OXA-51}}$.gene by ISA*ba*1 (Turton *et al*. 2006b). In a further analysis, a comparison of PFGE profiling of *A. baumannii* isolates from US and British Iraq causalities revealed that 25 British isolates includes representatives of the T strain, the OXA-23 clone II, and comparatively minor outbreak strain (Turton *et al*. 2006a). The 15 representative isolates of *A. baumannii,* isolated from inpatients at WRAMC, LRMC and US army field hospital in Baghdad included representatives of the T strain, OXA-23 clone II and the minor outbreak strain. The lack of the integrase gene in the rest of the nine US isolates indicated their sporadic origin. Both US and UK isolates were distinct from the most prevalent strains of *A. baumannii*, in hospitals in UK, namely representatives of the SE clone and OXA-23 clone I (Turton *et al*. 2004). Moreover, US personnel coming back from the Iraq war have shown the presence of multi-drug resistance *A. baumannii. A. baumannii* is also responsible for the multi-facility outbreaks described in France and USA, probably as a result of the transfer of colonised and infected patients from one facility to another (O'Shea, 2012).

A retrospective study investigated Canadian forces soldiers critically injured in Afghanistan requiring mechanical ventilation and repatriation to Canadian hospitals (Tien *et al*. 2007). Between January 2006 and September 2007, six Canadian soldiers were admitted to field hospital in Kandahar after significant combat-related traumatic injuries requiring mechanical ventilation; these soldiers were evacuated from Afghanistan through LRMC to five different Canadian hospitals, four of them developed MDR *A. baumannii* VAP *A. baumannii* isolated from Canadian hospitals and from LRMC were found identical antibiotic susceptibility after typed by PFGE (Tien *et al*. 2007). Furthermore, a variety of environmental samples collected from in and around the field hospital in Kandahar, including soil samples and those from critical care areas (ICUs and operation room) in late 2005 were analysed. *A. baumannii* was not isolated from any of the environmental samples except from a ventilator air intake filter sample; the isolate was identical to the MDR *A. baumannii* isolated from all four patients in LRMC and Canada.

### 2.2.3 *UK Acinetobacter spp.* bacteraemia statistics

In the years between 2008-12, the incidence of *Acinetobacter* spp. bacteraemia fell by 33% from 1.8-1.2 per 100,000 populations (Figure 2.1). Total number of bacteraemia reported in the UK increased by 1% and *Acinetobacter sp* accounted 0.6% of mono-microbial blood stream infection in 2011 making it the 20$^{th}$ most commonly reported mono-microbial blood stream infection causing organism (HPA, 2013). However, in 2012 the *Acinetobacter* spp. bacteraemia cases reduced from 1026 to 710 reports, which is the lowest number of reports in one year over a decade. In fact this is the first year that less than 25% of *Acinetobacter* spp. bacteraemia were identified as *A. calcoaceticus/baumannii,* having decreased each year since 2008. However the *Acinetobacter sp* reported as *A. lwoffii* have increased each year since (27-37% in 2008-12). The incidence of *Acinetobacter* spp. bacteraemia was higher in males than females across all the age groups, and the 75 and over were the most vulnerable, closely followed by the infections in children <1 year of age (Figure 2.2). The overall rate of infection in England, Wales and Northern Ireland was 1.2 per 100,000 populations in 2012, with Northern Ireland having the highest reported incidence with 1.5 per 100,000 populations, followed by England (1.3) and Wales (0.3). Within England there were wide variations in reports from 0.8/100,000 in the North East region to 2.1/100,000 in the London region (Figure 2.3) (HPA, 2013).

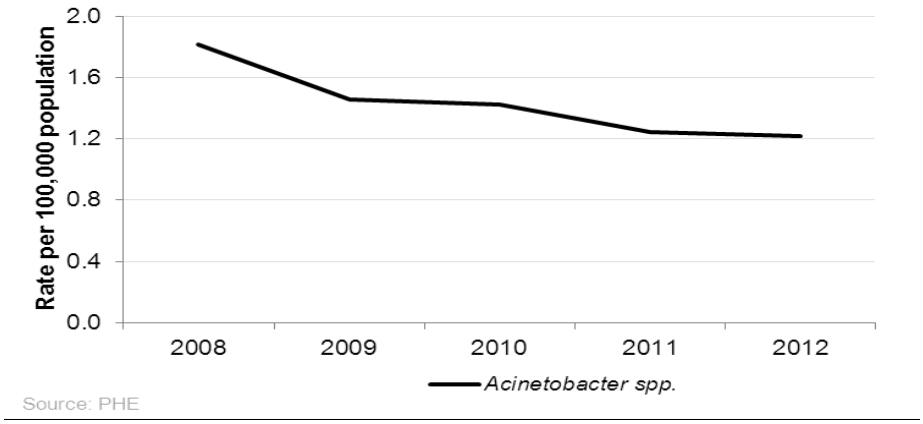


**Figure 2.1. *Acinetobacter sp* bacteraemia rates per 100,000 populations (England, Wales and Northern Ireland 2008-2012).**

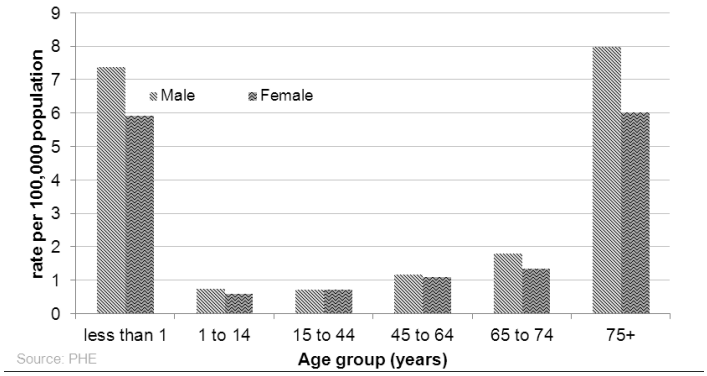


**Figure2.2.*Acinetobacter sp* bacteraemia age and sex rates per 100,000 populations (England, Wales and Northern Ireland 2008-2012)(HPA, 2013).**

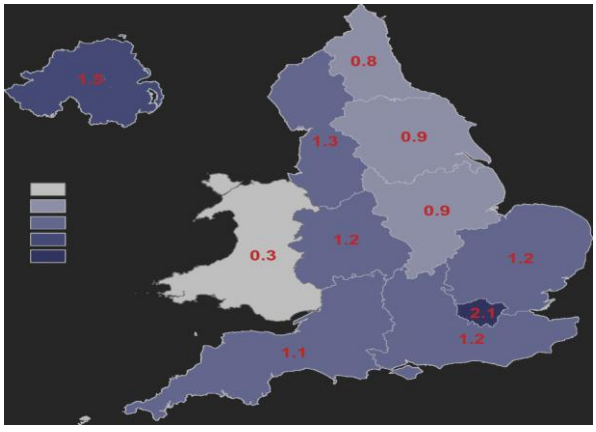


**Figure 2.3. Geographic distribution of *Acinetobacter sp* bacteraemia rates per 100,000 populations (England, Wales and Northern Ireland 2008-2012).**

### 2.2.4 **Antimicrobial Resistance**

The emergence of multi-antibiotic resistant *A. baumannii* strains has been attributed not only to its ability to rapidly develop resistance mechanisms but also to the fact that it is well suited to genetic exchange.

Therefore *Acinetobacter* belongs to a unique group of Gram negative bacteria characterised as "naturally transformable" (Perez *et al*. 2007). *Acinetobacter* have been reported to be resistant to *β*-lactams (Corvec et al., 2003, Fournier et al., 2006, Iacono et al., 2008, Perez *et al*. 2007, Ruzin *et al*. 2007), aminoglycosides, quinolones (Vila *et al*. 1995; Vila *et al*. 1997; Robicsek *et al*. 2005), tetracyclines (Perez et al., 2007, Ruzin et al., 2007), and polymyxins (Corvec et al., 2003, Fournier et al., 2006, Iacono et al., 2008, Perez et al., 2007, Ruzin *et al*. 2007, Giamarellou, 2007). Despite the environmental distribution of *Acinetobacter spp* in nature, MDR resistant *Acinetobacter* has no habitat other than hospital environment (Espinal *et al*. 2011).

MDR *A. baumannii* has been reported from different hospitals in Europe, USA, Japan, China, Hong Kong and Korea (McConnell *et al*. 2012). The surveillance data obtained by British Society for Antimicrobial Chemotherapy (BSAC) revealed the increasing resistance trends since 2002 in *A. baumannii*, with >30% of bacteraemic isolates in 2005 being resistant to piperacillin/tazobactam, gentamicin and with non-bacteraemic isolates being even more resistant (British Society for Antimicrobial Chemotherapy, 2007). MDR clones of *A. baumannii* have been isolated from 24 hospitals in the UK (Mostly in London area), which are resistant to piperacillin/tazobactam, gentamicin, ampicillin, piperacillin, ceftazidime, cefotaxime and ciprofloxacin, with most isolates also resistant to carbapenem (Turton *et al*. 2006b). The resistance of 226 strains found in the MYSTIC (Meropenem Yearly Susceptibility Test Information) collection when tested against carbapenems and comparators revealed that meropenem was the most active compound (76.1% susceptibility), followed by imipenem (74.7%) > gentamicin (51.9%) > ciprofloxacin (40.5%) >piperacillin/tazobactam (39.8%) > ceftazidime (38.1%) (Turton *et al*. 2006a).

A variety of risk factors have been associated with MRD *A. baumannii* infections (Falagas and Kopterides, 2006, Carbonne *et al*. 2005). According to a multivariate analysis of a 20 case-control study by Falagas and Kopterides (2006), antibiotics use was the most common risk factor identified in >50% of cases. Third generation cephalosporins and carbapenems were the most commonly implicated antibiotics, followed by fluroquinolones, amino-glycoscides

and metronidazole. However, Landman *et al* (Landman et al., 2002), found that the use of cephalosporins plus aztreonam, but no other antibiotics, was associate with the presence of MRD *A. baumannii*. Other risk factors include stays in ICU, length of ICU and hospital stay, gender, severity of illness and therapeutic interventions such as hydrotherapy, tracheostomy, transfusions, and placement of arterial and central venous catheters, Foley catheters, etc (D'Agata et al., 2000, Navon-Venezia et al., 2005, Falagas et al., 2006).

The ability of *A. baumannii* to acquire antibiotic resistance mechanism helps this organism to grow and persist in the hospital environment which resulted in the emergence of global MDR strains especially the alarming reports describes infections caused by pan drug-resistant strains with resistance to all clinically used antibiotics (Taccone et al., 2006, Valencia *et al*. 2009). These MDR strains proved to be challenge for the clinicians treating these infections and necessitate the development of novel strategies for preventing and treating infections caused by these strains. There are number of reviews which provide the comprehensive information on antibiotics resistance mechanism and clinical aspects of *A. baumannii* (Chopra et al., 2008, Peleg *et al*. 2008, Vila and Pachón, 2008, Gordon and Wareham, 2010, Fishbain and Peleg, 2010). The major reported resistance mechanism are summarise in Table. 2.2. *A. baumannii* have shown the remarkable capacity of acquire and rearrange genetic determinants that play a critical in pathobiology, the first that kind of report explaining the acquisition of genetic characteristics is the acquisition of the 86-kb AbaR1 resistance island, the acquisition of this island, which includes 45 resistance genes as well genetic traits coding for DNA mobilization functions could be explained by horizontal gene transfer from unrelated sources (Fournier *et al*. 2006). Another *A. baumannii* strains i.e. European Clone II strain ACICU harbour the AbaR2 resistance island (Iacono *et al*. 2008). More recently, a comparative study on genome wide analysis of ACICU and three strains belonging to A, B and C types determined by pulse field gel electrophoresis isolated during an outbreak at the National Institute of Health Clinical Centre. Snitkin et al (Snitkin *et al*. 2011), reported that the AbaR1 resistance island responsible for the antimicrobial resistance. This report also revealed that *A. baumannii* has the ability to adapt to hospital environments not only by horizontal acquiring genetic traits responsible for the evolution of non-MRD ancestors into MRD outbreaks strains, but also by rearranging pre-existing genes. *A. baumannii* strains has the ability to shuffle, add and or delete genes which are responsible for the coding for important virulence factors, specially cell surface proteins and O-antigens and the expression of the functions needed to acquire essential nutrients such as iron (Snitkin *et al*. 2011).

| Drug Class | Resistance mechanism | Examples |
|---|---|---|
| **B-lactams** | Inactivating enzymes | β-Lactamases (AmpC, TEM, VEB, PER,CTX-M, SHV)<br>Carbapenemases (OXA-23, -40, -51, -143-like, VIM, IMP, NDM-1, -2 ). |
| | Decreased outer membrane protein expression | CarO, 33-36kDa protein, OprD-like protein |
| | Altered penicillin-binding protein expression | PBP2 |
| | Efflux pumps | AdeABC |
| **Fluoroquinolones** | Target modification<br>Efflux pumps | Mutations in *gyr*A and *parC*<br>AdeABC, AdeM |
| **Aminoglycosides** | Aminoglycoside modifying enzymes<br>Efflux pumps<br>Ribosomal methylation | AAC, ANT, APH<br>AdeABC, AdeM<br>ArmA |
| **Tetracyclines** | Efflux pumps<br>Ribosomal protection | AdeABC, TetA, TetB<br>TetM |
| **Glycylcylines** | Efflux pumps | AdeABC |
| **Polymyxins (Colistin)** | Target modification | Mutation in the PmrA/B two components system (LPS modification), mutation in LPS biosynthesis genes. |

**Table 2.2: Major resistance mechanism found in *A. baumannii*.**

These finding suggest that non-MDR strains may serve as a source of antigenic variants that could play a critical role in the diversification and emergence of MDR *A. baumannii* clinical isolates, Imperi and his colleagues (Imperi *et al*. 2011), findings supported that report, they found that *A. baumannii* has relatively small sized core genome and a rather large accessory genome that hosts numerous antibiotic resistance and virulence determinants and is possible could be acquired by horizontal gene transfer process. A. *baumannii* also possess an intrinsic class D oxacillinase belonging to the OXA-51-like group of enzymes responsible for over 40 sequence variants (Alsultan *et al*. 2009), that gene helps the bacteria to hydrolyse penicillin's (ampicillin, ticarcillin, benzylpencillin and peperacillin) and carbapenems (imipenem and meropenm), but do so only weakly (Peleg *et al*. 2008). A significant contribution to lactam resistance by OXA-51 like enzymes therefore requires the presence of an insertion element ISAbal upstream of the gene, which act as a strong transcriptional promoter (Turton *et al*. 2006a). The most common enzymatic mode of carbapenem resistance is the production of oxacillinases encoded by genes of the blaOXA-23, blaOXA-40 and blaOXA-58 like lineage.

In Europe the spread of MDR *A. baumannii* not restricted to hospitals in cities but also occurs on a national scale, mostly through inter-hospital patient transfer's e.g the spread of Southeast

clone and the OXA-23 Clones I and II in southeast England (Coelho *et al*. 2006). International transfer of colonised patients has led to the introduction and subsequent epidemic spread of MRD *A. baumannii* strains from southern into northern European countries such as Belgium and Germany (Schulte *et al*. 2005). In a surveillance data (MYSTIC), from48 European hospitals from 2002-04, just 69.8% *A. baumannii* were susceptible to imipenem 73.1% susceptible to meropenem, however susceptibility to other antibiotics was very low i.e ceftazidime (32.4%), ciprofloxacin (34%) and gentamicin (47.6%) (Unal and Garcia-Rodriguez, 2005). There is also a long history of *A. baumannii* outbreaks in USA, outbreaks of carbapenem resistant *A. baumannii* were observed in New York city in 1991 and 1992 (Go *et al*. 1994). In recent years, the industry supported surveillance data includes isolates of *A. baumannii* collected between 2004-05 from 76 centres throughout the USA revealed that only 60.2% were susceptible to imipenem (Halstead *et al*. 2007). The outbreaks of *A. baumannii* also reported in Asia and Middle Eastern hospitals, rates of non- susceptibility (Anti-microbial surveillance program 2001-04) increased 25% for impienem and meropenem, 40% for cefepime and ceftazidime, 40% for ampicillin sulbactam, 35% for amikacin and 45% for ciprofloxacin(Gales *et al*. 2006).

### 2.2.5 **Mechanisms of resistance**

*Acinetobacter sp* show a wide range of antibiotic resistance mechanisms including:

- *β*-lactamase activity;
- alterations in outer membrane proteins (OMPs) (porins);
- alteration of penicillin-binding proteins (PBPs);
- increased activity of efflux pumps. (Corvec *et al*. 2003, Fournier *et al*. 2006, Iacono et al., 2008, Perez *et al*. 2007, Ruzin *et al*. 2007).

#### *2.2.5.1* ***β-lactamase activity***

*I. Class A β-lactamases*

Although TEM-1 β-lactamase is known to occur in *A. baumannii*, class A extended spectrum β-lactamases (ESBLs) have been reported recently (Vila et al., 1993, Poirel et al., 2005) , *A. baumannii* strains having PER-1, an ESBL demonstrate high level resistance to penicillins and extended spectrum cephalosporins and been reported in the organism responsible for outbreaks in France, Belgium and Bolivia hospitals (Celenza *et al*. 2006, Naas *et al*. 2006, Poirel *et al*. 2005) it has also been reported in USA (Hujer *et al*. 2006). *A. baumannii*, CTX-M-2 an ESBL characterised by enhanced hydrolysis of cefotaxime and ceftriaxone.

*II. Class B β-lactamases*

The increase in the number of metallo-beta-lactamases (MBLs) in *A. baumannii* results in the global emergence of resistance to β-lactams (Walsh, 2005, Walsh *et al*. 2005). These are Class B β-lactamases has the ability to hydrolyse carbapenems as well as every other beta-lactam antibiotic with the exception of aztreonam. They differ from Class A and D carbapenemases by having a metal ion in the active site, usually zinc which helps in catalysis (Walsh *et al*. 2005).

*III. Class C β-lactamases*

*Acinetobacter sp*, like other Gram negative organisms have a chromosomally encoded class C beta-lactamase. According to recent phylogenetic analysis chromosomal *ampC* genes in *Acinetobacter spp*, likely descend from a common β-lactamases gene ancestor and are more closely related to each other than to *ampC* genes present in other species of bacteria, it is proposed that these represent a distinct family of β-lactamases, the *Acinetobacter-derived cephalosporinases* (ADCs) (Hujer *et al*. 2005). The *bla* genes code for class C cephalosporinases that hydrolyse penicillins and narrow and extended spectrum cephalosporins, but not cefepime or carbapenems. Thus many clinical isolates of *A. baumannii* are resistant to ceftazidime (Hujer *et al*. 2005).

*IV. Class D β-lactamases*

Class D β-lactamases are usually contains penicillinase (oxacillinases). Some OXAs (OXA ESBLs), are also able to hydrolyse extended spectrum cephalosporinases (Aubert *et al*. 2001, Walther-Rasmussen and Høiby, 2006).

### *2.2.5.2 Changes in outer membrane proteins (OMPs) and penicillin-binding proteins (PBPs)*

The contribution of porins or outer membrane proteins (OMPs) and penicillin-binding proteins PBPs to antibiotics resistance in *A. baumannii* is not fully understood, laboratory studies however revealed the variability in the number of observed OMPs and PBPs (Cuenca *et al*. 2003, Fernández-Cuenca *et al*. 2003). The epidemic MDR *A. baumannii* from New York city revealed the presence of carbapenem resistant isolates with reduced expression of 37-, 44-, and 47kDa OMPs results in the increased expression of class C cephalosporinases (Quale *et al*. 2003), relatively similar results have been reported from Madrid, where there was the loss of 22-kDa and 33-kDa OMPs combined with the production of OXA-24 resulted in the resistance to carbapenems (Bou *et al*. 2000). The resistance of *A. baumannii* to carbapenemsis also explained by reduced expression of PBP-2 as described for isolates from Seville, Spain (Cuenca *et al*. 2003, Fernández-Cuenca *et al*. 2003).

#### *2.2.5.3 Efflux pumps*

Efflux pumps are a unique phenomenon in drug resistance, which is a single mechanism resulting in resistance against range of different classes of antibiotics (Perez et al., 2007). These efflux pumps mediate the efflux of compounds toxic to the bacterial cell, including antibiotics, i.e. coupled exchange with protons. Distinct families of efflux pumps found in different *spp* of bacteria namely the major facilitator superfamily, the resistance-nodulation-cell division family the multidrug and toxic compounds extrusion superfamily and the small multidrug resistance superfamily. In *A. baumannii,* the Ade/ABC efflux pump which is a member of resistance-nodulation-cell division family and has been well characterised, Ade/ABC efflux pump, pumps aminoglycosides, tetracyclines, chloramphenicol, fluoroquinolones, cefotaxime, erythromycin and trimethoprim (Magnet et al., 2001). The over expression of Ade/ABC efflux pump results in high level of resistance to carbapenem in conjunction with carbapenem-hydrolysing oxacillinases (Marqué et al., 2005).

### 2.2.6 **Additional Virulence Factors**

When compared with other Gram-negative bacteria, relatively few virulence factors have been identified for *A. baumannii*. The best characterised virulence factor of *A. baumannii* is OmpA. Evidence that *A. baumannii* OmpA contributes to virulence was obtained in a random transposon mutagenesis screen where deficient strains were unable to induce apoptosis in a human laryngeal epithelial cell line (Choi et al., 2005). A purified OmpA localised to the mitochondria and induced apoptosis through the release of the proapoptotic molecules cytrochrome *C* and apoptosis-inducing factor suggesting that this may be one possible pathway by which *A. baumannii* induces damage to human airways cells during infection. The role of OmpA in adherence and invasion of epithelial cells may also contribute to the spread of *A. baumannii* during infection, as the bacterial load in the blood of experimental induced *A. baumannii* pneumonia were significantly higher in mice infected with wild type strain than in mice infected with an equal amount of an isogenic OmpA mutant (Choi et al., 2008). The OmpA protein also helps *A. baumannii* persist and grow in human serum as it has been shown that OmpA interacts with soluble inhibitors of the alternative complement pathway and allow the bacteria to avoid complement-mediated killing (Kim et al., 2009). However, OmpA is unlikely to be the one factor that contributes to serum resistance since other *A .baumannii* strains all with putative OmpA genes, have significantly different capacities for growth and survival in human serum (Antunes et al., 2011b).

*A. baumannii* have also been shown to secrete OmpA in response to alcohols found in sanitation products (Smith et al., 2004, Edwards et al., 2007a) this may be associated with the emulsifying activity of OmpA, which could be useful in scavenging carbon for growth and survival under low nutrient conditions (Walzer et al., 2006). These bio-emulsifying proteins may also be helpful in bacterial adhesion, quorum sensing and the development of biofilms (Ron and Rosenberg, 2001). Exposure to ethanol also serves as an environmental signal that controls the salt tolerance and increased pathogenicity when tested in *Caenorhaditis elegans* (Smith et al., 2004). The genomic and mutagenic analysis of the strain ATCC 17978 proved that enhanced ethanol mediated virulence response in *Caenorhaditis elegans* worms and *Dictyostelium didcoideum* amoebae are due to the presence of genes located in pathogenicity islands, some of which code for novel gene products (Smith et al., 2007). The latter findings suggest that ethanol could play a global regulatory function; a hypothesis supported by the findings obtained using global RNA-sequencing (Camarena et al., 2010). This study revealed the identification of 49 ethanol-induced genes coding for metabolic functions, stress responses and virulence functions, suggests that ethanol affects the pathobiology of *A. baumannii.* These findings correlates with the findings of another study which states the presence of ethanol in the clinical settings may have an impact on pathobiology of *A. baumannii* (Edwards et al., 2007a). According to Leung *et al* (Leung et al., 2006a), the highly clonal hospital-acquired, multi drug resistant strains of *A. baumannii* existing in the UK appear to be less virulent than drug-sensitive community strains. Therefore, any enhanced effect on virulence due to alcohols used in health care setting may have major clinical implications.

Another virulence factor is lipopolysaccharide (LPS), *A. baumannii* LPS contains a lipid A moiety, the carbohydrate core, and the receptor O-antigen. Recently the role of *A. baumannii* LPS was investigated by Luke *et al* (Luke et al., 2010), who used mutants lacking the LpsB glycotransferase that results in a highly truncated LPS glycoform containing only two carbohydrate residues bound to lipid A. This mutant showed decreased resistance to human serum and decreased survival in a rat model of soft tissue infection compared with the isogenic parent strain, indicating the role for the surface carbohydrate residues of LPS in pathogenesis.

In addition to the LPS, the capsular polysaccharide has also been identified as a virulence factor in *A. baumannii.* The structure of capsular polysaccharide isolated from two clinical isolates revealed that a linear amino polysaccharide consisting of three carbohydrate residues

in one strain and a branched pentasaccharide in the other (Fregolino et al., 2011). Mutants that were deficient for growth in human ascites (peritoneal cavity) fluid because of transposon insertion in the *ptk* or *eps* A gene failed to produce a capsular-positive phenotype and showed decreased growth in both human serum and ascites compared with the wild type counterpart. Additionally, mutant strain (without capsule) cleared after 24 h post-infection in a rat soft tissue infection, whereas the isogenic parental strain persisted with > $10^7$cfu/ml of exudative fluid (Russo et al., 2010); demonstrating that the capsular polysaccharide appears to play an important role in protecting bacteria from the host innate immune response.

Lypolytic enzymes have also been implicated in *A. baumannii* pathogenicity. Lipolytic enzymes e.g phospholipases catalyse the cleavage of phospholipids, these enzymes are thought to contribute the pathogenesis of Gram-negative bacteria by helping in the lysis of host cells via cleavage of phospholipids present in the host cell membrane and by degrading phospholipids present at mucosal barriers to facilitate bacterial invasion. Removal of the two phospholipase D genes present in *A. baumannii* genome results in survival in serum and a reduced capacity for invading epithelial cells (Jacobs et al., 2010).

Iron is abundant in biological systems however; the availability of ferric iron is relatively poor due to its poor solubility under aerobic conditions and its chelation by low molecular weight compounds e.g. heme, and by high iron-binding proteins such as lactoferrin and transferrin. In iron limitation conditions, most of the aerobic bacteria express high affinity iron acquisition system which assist the production, export and uptake of $Fe^{3+}$chelators known as siderophores (McConnell et al., 2012). Some bacteria have the ability to utilize heme or haemoglobin as an iron source and some are able to remove iron from transferrin or lactoferrin (Crosa et al., 2004, Wandersman and Delepelaire, 2004). *A. baumannii* does not bind transferrin and does not carry genetic determinants coding for the proteins involved in the acquisition of iron from transferrin or lactoferrin (Smith et al., 2007). However, ATCC $19606^T$ uses heme as an iron source (Zimbler et al., 2009). In strain ATCC 17978 a chromosomal cluster annotated as AIS_1608-AIS_1614, is a polycistronic operon involved in the transport of heme from the periplasm into the cytoplasm (Smith et al., 2007). In a more recent genetic analysis (Antunes et al., 2011b, Eijkelkamp et al., 2011), different *A. baumannii* strains can use this compound as an iron source suggesting the presence of a heme uptake and utilization system. These observations indicate that the *A. baumannii*, genome contains genes coding for products devoted to the capture and utilizing of heme, a host product that

could be available at sites where there is an extensive cell and tissue damage (Brachelente et al., 2007, Charnot-Katsikas et al., 2009, Corradino et al., 2010), or in severely injured patients (Peleg et al., 2008).

*A. baumannii* may also require ferrous iron, available under low-oxygen conditions, because fully sequenced and annotated genomes show the presence of genes coding for Fe (II) transport systems (Antunes et al., 2011a), the functions of which remains to be tested. *A. baumannii* also have the ability to form siderophores, the best characterised system is that mediated by the siderophores acinetobactin, initially described in the ATCC 19606$^{T}$ strain and has a highly related molecular structure to anguibactin, which is a high affinity iron chelator produced by *Vibrio anguillarum* 775 (Fish pathogen) (Yamamoto et al., 1994, Yamamoto et al., 1999, Dorsey et al., 2004, Mihara et al., 2004). The only difference in these two siderophores is that anguibactin has a thiazoline group derived from cysteine, while acinetobactin contains oxazoline ring derived from threonine. In spite of this difference, the siderophores which are produced by these two different bacterial pathogens found in environments are also functionally related (Dorsey et al., 2004). The recent genomic analysis revealed that *A. baumannii* could contain more than one locus involved in siderophores biosynthesis (Eijkelkamp et al., 2011, Antunes et al., 2011a). In short, the available experimental and in *silico* observation indicate that *A. baumannii* can acquire iron either by using heme as an iron source or by capturing the metal with acinetobactin and/or more additional siderophores-mediated systems.

However, many of the environmental and physiological factors affecting the virulence phenotype of *A. baumannii* are unidentified and uncharacterized. Some *Acinetobacter* mutants harbour mutations impairing the expression of ABC transporters, an uncharacterised urease activity, and transcriptional regulators. Recently, it was reported that *A. baumannii* also sense and respond to the light an unexpected observation considering that of *A. baumannii* is non-photosynthetic microorganism (Mussi et al., 2010). In addition, these observations lead to the hypothesis that the outcome of certain infections, such as surface exposed wound infections could depend on the exposure of bacteria to the light and temperature lower than 37°C.

## 2.3 **Healthcare Associated Infections (HAI)**

Generally speaking the pathogens that cause Healthcare associated infections (HAI) have two specific properties i.e. not only do they cause disease but they also persist in hospital environments for long periods (Dancer, 1999, Kramer et al., 2006), e.g. methicillin-resistant

*Staphylococcus aureus* (MRSA), *Acinetobacter* and vancomycin-resistant *enterococci* (Carling and Bartley, 2010, Kramer et al., 2006, Wagenvoort et al., 2011). Viruses such as novovirus and influenza, and fungi such as *Candida albicans*, also persist in hospitals for several weeks (Kramer et al., 2006). Gram-negative coliforms, e.g. *E.coli* and *Klebsiella sp*, are less robust but survive on dry, as well as wet surfaces, although this tend to be for shorter periods of time than *Acinetobacter* (Kramer et al., 2006).

Despite the ubiquity of *Acinetobacter* spp in nature, multidrug resistant *Acinetobacter* has no particular habitat other than hospital environment. *Acinetobacter* has the ability to survive on dry, inanimate surfaces, acquire resistance genes and tolerate biocides and antibiotics, *A. baumannii* is capable of spreading within health care facilities and its impact in combat zones and natural disaster areas throughout the world is increasingly evident. Since *A. baumannii* has the ability to grow on fingertips and inanimate objects such as glass, and plastic surfaces, even after exposure to dry conditions, during extended period of time and its ability to form biofilm it shows much resistance to antimicrobial stressors, antibiotics or cleaning Products (Espinal et al., 2011). The emergence of pathogenic *Acinetobacter* infections in military personnel sustaining injuries during Iraq and Afghanistan conflicts have provided an important contribution to the epidemiology of infections with *Acinetobacter spp* (O'Shea, 2012). *Pseudomonas aeruginosa* is one of the most of important nosocomial pathogens, especially in intensive care units (ICUs) (Henrichfreise et al., 2007). *Pseudomonas aeruginosa* is one of the most difficult organisms to treat because of intrinsic and acquired antibiotic resistance. In Europe, *P. aeruginosa* was found to be the third most common isolate from nosocomial infections in (ICUs) (Henrichfreise et al., 2007).

### 2.3.1 **Contamination of Surfaces in Healthcare Environments**

Environmental screening has demonstrated that pathogens can survive on a variety of hospital surfaces (Getchell-White et al., 1989, Bhalla et al., 2004, Lemmen et al., 2004, Dancer et al., 2008, Kaatz et al., 1988, Wu et al., 2005, Kerr and Snelling, 2009). They can attached to droplets, skin scales or dust partials which may be intermittently dispersed through the atmosphere, ultimately settling on floors and other surfaces (Kramer et al., 2006). These include general surfaces such as shelves, and ledges, curtain, linen and cloths, telephone, computers, furniture and all items of clinical equipment, but some pathogens such as *Pseudomonas spp*, persist in damp places such as showers, baths and sinks, others e.g *C. difficile* and VRE, contaminate toilet areas or commodes (Lemmen et al., 2004, Kerr and

Snelling, 2009, Alfa et al., 2008, Noble et al., 1998). Dust associated microbes such as *Acinetobacter* spp. and MRSA settle on rarely cleaned or inaccessible surfaces, such as shelves, highly place equipment and computer keyboards, while coliforms such as *Klebsiella* and *Serratia sp*. are spread through air currents especially in hot dry summers (Dancer, 1999). Some items, e.g. lockers, sheets, beds and patient tables tend to host pathogens more frequently than others (Dancer et al., 2008, Malnick et al., 2008). This may be due to patients shedding from colonised sites, proper cleaning and/or frequent handling, but that will increase the risk of the infection for all the patients, especially patients with weak immune system, or those receiving antibiotics, in dwelling devices and/or surgery. The greatest infection risk for patients comes from surfaces beside or on beds (Bhalla et al., 2004, Dancer et al., 2008, Dancer et al., 2009, Wilcox et al., 2003). Furthermore, there are a number of studies that have an increased risk of acquiring a specific pathogen if a patient is admitted into a room previously occupied by a patient colonised or infected with the same organism (Carling and Bartley, 2010, Huang et al., 2006, Shaughnessy et al., 2011, Wilks et al., 2006).

The ability of *A. baumannii* to resist desiccation and persist on hospital surfaces, materials and medical devices has played a critical role in the emergence of this bacteria as a HAI (Villegas and Hartstein, 2003). *A. baumannii* has the ability to survive several days on dry surfaces and inanimate objects in hospitals, attached to the body parts of patients and can also survive on dust particles in dry conditions (Bergogne-Berezin and Towner, 1996, Davis et al., 2005). *Acinetobacter* has also be found on ventilators, mattresses, pillows, suctioning equipment, humidifiers, bed rails, bedsides, container of distilled water, nutrition equipment's, urine collection jugs, intravenous, portable water, reusable arterial pressure transducers, the nodes of electrocardiographs, wash basins, sinks, infusion pumps, hygroscopic bandages, showers, stainless-steel trolleys, resuscitation equipment and tables, portable radiology devices, bed linen, soap dispensers, spirometers, temperature probes and soap dispensers (Paterson, 2006).

### 2.3.2 **Biofilms**

It is difficult to describe biofilms briefly, as they have many characteristics and mechanisms, ecologies, physiological and genetic heterogeneities, resistance to disinfectants, sanitizers and antimicrobials (Bales et al., 2013). Biofilms are defined as highly self-organized, three-dimensional structure D community enclosed in a polymeric matrix or exopolymeric substances (EPS), constituting a protective mechanism to survive in harsh environments and

during host infection (Espinal et al., 2011, Jahid and Ha, 2012). Biofilms are composed of microorganisms attached to either each other, to living or non-biotic surfaces and may be embedded within a complex matrix of polysaccharides, proteins, nucleic acids and glycoproteins (Donlan and Costerton, 2002).

For the effective eradication of microorganisms within a biofilm, higher concentrations of antimicrobial agent are often required when compared to their planktonic or free floating non- biofilm counterparts (Thomas et al., 2011), and often the antimicrobial concentrations required for the removal can be 100-fold of that required for removal microorganisms in the planktonic state (Rasmussen and Givskov, 2006). In the context of human health 80% of infections are suggested to be biofilm related (Davies, 2003, Blackwell, 2005). For example in chronic wounds, e.g. diabetic foot ulcers, pressure ulcers and venous leg ulcers biofilms are a central part of the evolution of the infection (James et al., 2007). The effective and efficient use of medical devices such as catheters, artificial valves etc. are often compromised by the formation of biofilms (Strelkova et al., 2012).

*2.3.2.1 Biofilm Formation*

Biofilm formation is not a single step process, it is a stepwise, dynamic process and different physical, chemical, genetic and biological processes are involved in the maturation of biofilm. Although it is not clear but more or less five to six steps are involved in biofilms formation (Jahid and Ha, 2012). These steps are:

- reversible attachment to a produce surface;
- irreversible attachment through producing quorum sensing ;
- EPS production;
- micro-colony formation;
- colonization or maturation step;
- dispersal.

*2.3.2.2 Attachment*

There are few genetic mechanisms known to be involved in the attachment steps to produce biofilm however, several studies have suggested that the bacterial cellular surface charge, surface hydrophobicity, produce hydrophobicity, van der Waals forces and electrostatic forces, simultaneously interact and adhere to the surface (Palmer et al., 2007, Ukuku and Fett,

2006). However, different environmental conditions triggers diverse pathways to which help the bacterial colonization. In general, force-generating movements might be required to form attachments to produce (Jahid and Ha, 2012). The attachment also depends on the pathogenic and nutritional conditions of the host, hydrophobicity, cell surface charge and even bacteria to bacteria interactions (Ukuku and Fett, 2002).

*2.3.2.3 Quorum-sensing (QS) signal*

Bacteria initiating biofilm formation express a range of molecules which allow cell to cell communicate and coordination, a process known as Quorum-Sensing (QS) (Sperandio et al., 2003). There are four identified QS system. Of these, Gram negative bacteria have autoinducer-1 (AI-1) that secretes *N-acylhomoserine* lactones (AHLs) (Miller and Bassler, 2001) , and autoinducer-3 (AI-3) (Sperandio et al., 2003), while Gram positive bacteria have auto-inducer peptide (AIP) signalling pathways for intra-species communication. Both Gram positive and negative microorganisms express autoinducer-2 (AI-2), QS molecules furanosyl-borate-diester to facilitate inter-species communication (Miller and Bassler, 2001).

*2.3.2.4 EPS formation*

The exopolymeric substances (EPS) present in biofilms may include polysaccharides, proteins, glycoproteins, glycolipids, extra cellular DNA, metal ions, divalent cations and other surface active components (Morris et al., 1997, Yadav et al., 2012). When microorganisms secrete a critical concentration of auto-inducer molecules, they form EPS at the surface of bacterial aggregates (Abee et al., 2011). Bacterial aggregates generate EPS to provide protection against environmental stresses e.g., antibiotics, disinfectants and irradiation (Van Houdt and Michiels, 2010). The generation of EPS facilitates adherence to biotic or abiotic surfaces, micro-colony formation, and the 3-dimensional surface of a mature biofilm (Danese et al., 2000, Van Houdt and Michiels, 2010).

*2.3.2.5 Micro-colony formation*

Following attachment bacteria begin to multiply and initiate communication by the formation of quorum-sensing molecules. At a certain level of quorum-sensing molecules, environmental cues stimulate the formation of EPS and bacteria continue to multiply within the EPS. Analysis of biofilms has revealed that once EPS production has been initiated, bacteria focus on the maturation of the biofilm and produce pili, flagella and fimbriae (Davey and O'Toole, 2000).

*2.3.2.6 Colonization or maturation steps*

The final stage of biofilm formation is maturation; here biofilms develop into a self-organised complex structure containing a range of microenvironment. The final arrangement is in monolayers, a three dimensional structure consisting of bacteria surrounded by EPS, which has channels for nutrients and water flow (de Beer et al., 1994).

*2.3.2.7 Dispersal*

Dispersal or detachment of biofilms are due to various reasons, such as presence of QS molecules, nutrient accessibility, surface character changes, as well as physical forces from the surface (Kaplan, 2010). Another studied revealed that bacterial growth, different enzymes produced by bacteria, external environmental influences, nutrient deprivation, bacterial autocidal activity and human interactions are some of the factors results in dispersal or detachments of cells from biofilms.

### 2.3.3 ***Acinetobacter*** **Biofilms**

*A. baumannii* is able to form strong adherent biofilms that help the bacteria to survive for several weeks on abiotic surfaces resulting in contamination of hospital and medical instruments, e.g. incubator tubes, water lines, cleaning instruments, pillows and linen (Harrison et al., 2008, Donlan, 2008, Villegas and Hartstein, 2003, McConnell et al., 2012). Wroblewska *et al* (Wroblewska et al., 2008) reported that clinical strains of *A. baumannii* have the ability to form biofilms which act as a virulence factor. They used 34 clinical strains of *A. baumannii* isolated from patients hospitalized in two tertiary care hospitals for their studies. The isolates demonstrated a wide range of biofilm forming ability, with 12% high, 41% medium and 47% demonstrating a low level of biofilm production.

Pirog *et al* (Pirog et al., 2002), reported that *Acinetobacter* spp.12S has the ability to grow and synthesize EPS on different carbohydrate substrates like mono and disaccharides, molasses and starch. They reported that the *Acinetobacter* spp. was grown on carbohydrate media containing no pantothenic acid (Vitamin B3), which is required for growth on $C_2$ substrates. They used mixture of carbohydrate sources (0.01, 0.1, 0.5 and 1% v/v ethanol and 1% w/v glucose) and found that bacterial growth and EPS generation was higher when 0.01% ethanol with 1% glucose used. The EPS production was intensified as the content of the C2 substrate in the medium increased. He also reported that addition of low concentration (0.02% v/v) of acetate to the glucose-containing medium also stimulated the EPS synthesis. The biofilm

formation and attachment is variable among clinical isolates of *A. baumannii*; for example strains like ECII are more adherent than ECI to human bronchial tissue (Lee et al., 2006). Generally, *A. baumannii* adheres to biotic and abiotic surfaces via the same steps described for general biofilm formation. The associated EPS being composed of carbohydrates, proteins, nucleic acids and other macromolecules (McConnell et al., 2012). Donlan & Costerton (Donlan and Costerton, 2002, Gaddy and Actis, 2009), hypothesized that *A. baumannii* persistence in medical environments, resistance to antimicrobials, and disease generation is closely associated with its ability to form biofilm on solid surface. Some of the *A. baumannii* clinical isolates also form complex biofilm structures on the surface of liquid media know as pellicles (McQueary and Actis, 2011, Marti et al., 2011), the pellicles and biofilm formation on abiotic surfaces are variable among *A. baumannii* clinical strains and there is a clear correlation established between the nature of substrate and bacterial surface properties (McQueary and Actis, 2011, Marti et al., 2011). Furthermore, there are significant variations in the amount of biofilm formation and type of cell arrangements formed on the abiotic surfaces. Some cell arrangements are highly organised, multi-layered and complex structures encased within a biofilm or EPS matrix, while some others are simple monolayers of bacteria attached in an organized or random manner (McQueary and Actis, 2011).

A number of gene products have been proven to play a role in attachment and biofilm formation on abiotic surfaces e.g. pilus production mediated by the CsuA /BABCDE usher-chaperone assembly system is required for the attachment and biofilm formation on the abiotic surfaces by the *A. baumannii* ATCC 19606$^T$ strain. This operon seems to be wide spread among clinical isolates and an indication that it is a common factor among different clinical isolates (Tomaras et al., 2003). The *A. baumannii* ATCC 19606$^T$ strain also has ability to produce alternative pili that may help in the interaction of this pathogen with bronchial epithelial cells (De Breij et al., 2010). Loehfelm *et al* (Loehfelm et al., 2008) reported that biofilm-associated protein (Bap), conserved in the clinical isolates and appears to be associated with the cell-cell interactions that support the development and maturation of the biofilm.

In addition to (Bap), the *A. baumannii* clinical isolates also produce poly-β-1-6-N-acetylglucosamine (PNAG) for the development and maturation of the biofilm on glass surfaces by the cells cultured (Bentancor et al., 2012, Choi et al., 2009b). A two component regulatory system also reported in *A. baumannii* ATCC 19606$^T$ strain comprised of: a sensor

kinase encoded by *bfm*S, and a response regulator encoded by *bfm*R involved in bacteria-surface interaction (Tomaras et al., 2008). The insertional inactivation of *bfm*R results in the loss of expression of *cusA/BABCDE* operon resulted in the lack of pili production and biofilm formation on plastic surfaces when they are grown in rich medium; however the inactivation of *bfm*S sensor kinase gene resulted in diminishment but not abolishment of biofilm formation (Tomaras et al., 2008). In the absence of BfmRS system the composition of culture media still influence the interaction of cells with abiotic surfaces, these finding indicates that the BfmRS system cross talks with other sensing components and suggests instead of one, there are multiple and different stimuli which could control the biofilm formation via BfmRS regulatory pathway (Tomaras et al., 2008). When compared to adherence to abiotic surfaces, much less is known about the factors that influence adherence and biofilm formation on biotic surfaces. Gaddy *et al* (Gaddy et al., 2009), reported that *A. baumannii* attaches to the human epithelial cells and *C. albicans* filaments, in a process that involve at least one OmpA, although OmpA play a vital role in biofilm development on plastic surfaces, this outer membrane also critical for the interaction of *A. baumannii* with human and *Candida* cells when the *Candida* are in its filamentous form. *Candida*, *A. baumannii* (ATCC 19606$^{T}$) strain interactions are independent of pili assembled by the *csu* usher-chaperone system and lead to apoptotic death of the fungus filaments (Gaddy et al., 2009). These studies suggest that there is no direct correlation between biofilm formation on abiotic and biotic surfaces, and a wide variation exists in the cell-surface and cell-cell interactions that result in the adherence and biofilm formation by different *A. baumannii* clinical isolates. In addition, to that the role of pili in bacterial virulence and the pathogenesis of *A. baumannii* infections remains to be confirmed using appropriate derivatives and experimental infection models.

Adherence and biofilm formation also responds to a wide range of environmental and cellular factor (Stanley and Lazazzera, 2004), e.g. it depends upon the presence and expression of antibiotics resistant traits, such as the *bla*$_{\text{PER-1}}$ gene and a positive correlation exist between the presence and the expression of this gene and the amount of biofilm formed on the plastic surface and the adhesiveness of the bacteria to human epithelial cells (Lee et al., 2008), however another study revealed that only two out of 11 isolates carrying the *bla*$_{\text{PER-1}}$ gene formed strong biofilms when compared with isolates lacking the genetic determinant (Rao et al., 2008). Other environmental factors, such as temperature and extracellular free iron concentration, which are relevant for the interaction of *A. baumannii* with the host, also effect biofilm formation on abiotic surfaces (McConnell et al., 2012).

Another mechanism controlling bacterial adherence and biofilm formation is cell population density. Accordingly, environmental and clinical isolates produce quorum sensing signalling molecules (Gonzalez et al., 2001, Gonzalez et al., 2009), these studies proved that a large number of isolates produce quorum sensing and signalling molecules which seem to belong to three types of molecules. Although none of these sensors belongs to a particular species, however the Rf1-type sensor is more frequently found in isolates belonging to the *A. calcoaceticus-baumannii* complex. Niu et al (Niu et al., 2008) proved that the *A. baumannii* M2 clinical isolates produce an *N*-acyl-homoserine lactone [*N*-3-hydroxydodecanoyl-homoserinem lactone], the product of the *aba*I auto-inducer synthase gene, which is vital for the fully developed biofilm on abiotic surfaces, *aba*I auto-inducer also helps this isolate to move in semisolid media.

Another factor which influences biofilm formation on abiotic surfaces is light, although unexpected since *A. baumannii* is a chemotroph not know to conduct photosynthesis (Mussi et al., 2010). This response is mediated by BlsA photoreceptor protein, which contains a BLUF domain and uses flavin adenine dinucleotide (FAD) to sense light, however the mechanism by which BlsA transduces the light signal and controls gene expression are not well known (Mussi et al., 2010). The *A. baumannii* response to light seems to have a global effect on the physiology of *A. baumannii*, affecting not only biofilm formation, but also motility and virulence. In addition, the differential response to light is mediated by changes in temperature, which result in differential transcription of blsA at 28 and $37^0$C and hence differentially affect light controlled phenotypes (Mussi et al., 2010). *A. baumannii* biofilm formation and adherence depends on range of bacterial factors and multiple signals or cues. However, the medical relevance of the data obtained using *in vitro* models is not clear, considering the lack of correlation between the biofilm phenotype of different clinical isolates and their outbreak, epidemic and antibiotic resistance nature (de Breij et al., 2009).

### 2.3.4 **EPS characterisation**

The polysaccharides present in EPS can be present as hetero-polysaccharides or homo-polysaccharides. Hetero-polysaccharides are made up of a variety of monosaccharide's arranged in repeating units, a number of these structures have been characterised in recent years (Leroy and De Vuyst, 2004, Laws and Marshall, 2001). Furthermore, the variations in heteropolysaccharides is increased by the monomeric units being present in either α or β configuration, in the pyranose (*P*) or furanose (*f*) forms or in either D-or L-absolute

configuration (De Vuyst et al., 2001). The repeating structure is the simplest form of polysaccharide and the first was isolated from *St. thermophilus* and characterised by Doco *et al,* (Doco et al., 1990) as: →3)-β-D-Gal*p*-(1→3)-[α-D-Gal*p*-(1→6)]-β-D-Glc*p*-(1→3)-α-D-Gal*p*NAc-(1→.

*2.3.4.1 High performance anion exchange chromatography (HPAEC)*

In order to identify the primary structure of the EPS repeat unit it is firstly necessary to identify the monomer composition. In recent years, a number of methods have been used for monosaccharide analysis including High performance anion exchange chromatography (HPAEC), which has been widely used as an alternative to GC analysis due to a number of advantages over the GC method. This technique was first highlighted by Rocklin and Pohl (Rocklin and Pohl, 1983) for the carbohydrate analysis, since than the application and effectiveness of that technique have been reviewed by number of scientist (Lee, 1990, Cataldi et al., 2000). The main advantage of that method is that it allows the determination of intact monosaccharide's (initial depolymerisation by acid hydrolysis is required) without pre or post column derivatisation, which greatly reduces analysis times whilst eliminating the decrease in recovery often observed due to incomplete derivatisation (Currie and Perry, 2006).

*2.3.4.2 NMR analysis*

NMR spectroscopy is used to determine the overall secondary structure of EPS by providing information on the basis on ring size (pyranose/furanose) and anomeric configuration (α/β) of the individual monomers and also determining the relative orientations of the monomeric units to each other. The NMR spectroscopy as a tool for the determination of carbohydrates structures is underlined by number of reviews published in recent years (Lee flang et al., 2000, Bush et al., 1999). A key issue with NMR analysis is the removal of interfering substances. These can be classified as monomeric media components such as glucose and EPS equivalent substances that are often present in common media compositions such as yeast extract. Interference by these materials underlines the need for effective dialysis, the use of none interfering carbon sources and the use of media that are optimised for NMR analysis.

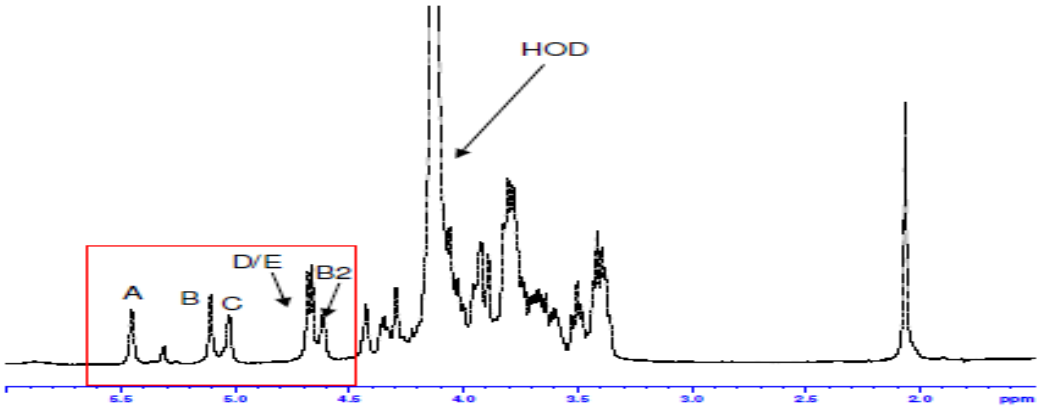


**Figure. 2.4.$^{1}$H NMR spectra of EPS from *Lactobacillus helvetics Rosviski* (Laws *et al.* 2009).**

2.3.4.2.1 *$^{1}$H NMR* analysis

When analysing $^{1}$H NMR the spectra should be split and viewed as having structural reporter signals (up filed and down field) and a bulk region (Vliegenthart et al., 1983). This approach breaks down the spectra enabling the similar identification of individual sugars along with their structural features and linkage compositions. The up field, reports region contains resonances from structural motifs which include ring substituents such as acyl, alkyl and acetyl, ring substitutions such as N-acetyl amino groups and H6 signals of 6-dexoy sugars. The anomeric proton resonances are located in the down field, reporter region of the spectra between 4.4-5.5 ppm (Figure 2.4). Integration of the anomeric resonances can be used to estimate the number of different monosaccharide units present in the repeat unit structure. The remaining shift protons are situated in the bulk region located between 4.3-3ppm (Vliegenthart et al., 1983).

2.3.4.2.2 *$^{13}$C NMR* analysis

$^{13}$C NMR provides information on the carbon atoms that are connected to hydrogen atoms in the molecule (attached protons). The $-CH_3$ and –CH groups both generates positive signals in the spectra whilst $-CH_2$ groups display a negative signal. As with $^{1}$H NMR spectra, specific regions of interest relating to carbohydrate can also be identified in the $^{13}$C NMR emanating the ring carbons ($C_2$-$C_5$) are located between 65-85 ppm, while carbons substituted with either an amino or methyl group are shifted up filed. The anomeric carbons ($C_1$) are shifted down field between 95-105 ppm due to glycosylation. The signals for the $C_6$ atoms appear as $-CH_2$ and are therefore represented as negative signals between 60-70 ppm.

*2.3.4.3 Weight-average Molecular Weight Determination (MALLS analysis)*

EPS of the bacterial species usually possess molecular mass ranges from 4 x $10^4$ to $10^6$ g $mol^{-1}$ (Vuyst and Degeest, 1999).The molecular mass is commonly recognised as one of the factors contributing in EPS functionally (Vuyst and Degeest, 1999). In general, the size of a polysaccharide is expressed by the number of monosaccharide units it contains, which is termed as the degree of polymerisation (DP). Previously, gel permeation chromatography (GPC) has been used to indicate a range of *MW* by comparison with controlled standard of a particular MW; however, this method needs large sample sizes and relies on the comparisons with standards. The current method used to determine *MW* and sizes of polysaccharides is high performance size exclusion chromatography with multi-angle laser light scattering (HP-SEC-MALLS) (Wyatt, 1993). HP-SEC-MALLS works with the same principle of weight and size separation with GPC but instead of preparative columns the sample is run through smaller analytic scale columns. The sample then pass through an online ultraviolet (UV), light detector which provides information on the presence of any residual proteins and 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 reference materials (Badel et al., 2011).

*2.3.4.4 Fourier transform infrared spectrocopic (FTIR)*

Fourier transform infrared (FTIR) spectroscopy is a rapid nondestructive method that has been applied to many biological systems (Schmitt and Flemming, 1998, Gómez-Zavaglia and Fausto, 2003). The techique is based on the principle that atoms in the molecules are not held rigidly apart and when subjected to infrared radiation (between 500 and 4000 $cm^{-1}$), the molecule will absorb energy and the bond will subject to a number of vibrations. Hence the absorbtion spectrum contains information regarding the molecular structure of the sample. The region between 4000 and 500$cm^{-1}$ holds the characteristic bands and is sutiable for the characterisation of microorganisms (Dittrich and Sibler, 2005, Schmitt and Flemming, 1998).The wide and intensive carbohydrate or EPS bands are foundat wave number 950-1200$cm^{-1}$ which can be attributed to –C-O-C- group vibrations in the cyclic structure (Gómez-Zavaglia and Fausto, 2003, Dittrich and Sibler, 2005, Lin et al., 2005).

## 2.4 **Cleaning and disinfection**

Cleaning is a process that removes foreign material e.g. soil, organic material, microorganism from an object, while disinfection is a process that reduces the number of pathogenic microorganism but not necessary bacterial spores from inanimate objects or skin to a level,

which is not harmful to health. Detergents are surfactants or a mixture of surfactants with cleaning properties in dilute solutions and they are composed of both hydrophobic and lipophilic parts, while disinfectant are substances that are applied to non-living objects to destroy microorganism that are living on the objects. Due to the environmental persistence of pathogens it is necessary to remove them through cleaning and/or disinfection (Dancer, 1999), since they may contaminate hands or be transported by air currents to be deposited on patient or surfaces (Casewell and Phillips, 1977, Bhalla et al., 2004). There are numerous guidelines emphasizing the importance of cleaning but these generally offer little practical advice on how to achieve effective cleaning, or how often sites should be cleaned (Dancer, 1999, Malik et al., 2003). There remains debate over cleaning in hospitals, because the link between cleaning and infection is hard to prove unequivocally. One reason being that there are other risk factors within the hospital environment e.g. hand hygiene, isolation facilities and antimicrobial prescribing (Dettenkofer et al., 2004). These factors mean that it is impossible to study cleaning in an independent scientific study and consequently determine the importance of cleaning towards overall infection rates (Dancer, 1999). The visual inspection of hospitals is not a reliable assessment of the infection risk for the patients (Malik et al., 2003, Dancer, 2004), as microbial contamination and visual dirt are not necessarily connected. In addition, cleanliness is often confused with cluttered cramped facilities and old and poorly maintained facilities (Dancer, 1999). There is still however a consensus that environmental cleaning is an essential component of infection controls (Carling and Bartley, 2010) and there are some studies that support the importance of cleaning in the reduction of HAIs (Carling and Bartley, 2010). Enhanced cleaning is generally included in any infection control strategy in response to an outbreak. Cleaning has been identified as a major control component for outbreaks of MRSA, drug resistant *Acinetobacter*, VRE and *C. difficile* (Wu et al., 2005, Morter et al., 2011, Wilks et al., 2006, Denton et al., 2004).

Since contaminated hand touch sites are considered to constitute an infection risk for patients, cleaning schedules generally focus on these sites (Dancer, 2004). However it is known that staff, patients and relatives release MRSA into the hospital environment, despite significant efforts for its removal (Hardy et al., 2007). One study that targeted hand touch sites on two surgical wards for a year with high frequency detergent based approach reduced the number of acute MRSA infections by 50% (Dancer et al., 2009, Wilson et al., 2011). Dry or detergent based cleaning can remove microbes, but will not kill them. Consequently, the risk of cleaning transporting microorganisms is high. There are numerous examples of

contaminated cleaning cloths and equipment's spreading contamination rather than removing it (Moore and Griffith, 2006, Barker et al., 2004, Scott and Bloomfield, 1990, Dharan et al., 1999, Bergen et al., 2009).

Disinfectants on the other hand can kill microbes but they are often expensive and may have environmental impacts (Dettenkofer et al., 2004, Kammerer, 2003). However, where there is a high risk to patients there is no alternative but to instigate disinfection rather than simple cleaning. This has led to the introduction of disinfectants into cleaning regimes in healthcare environments. One study that introduced a copper-based disinfectant and microfiber regimen for cleaning of high-risk sites in intensive care units. A further study in an Irish intensive care unit focused on the frequency that MRSA was recovered from locations around non-infected and infected patients. Level 2 cleaning (detergent followed by 1% hypochlorite) was effective against MRSA initially, but sites soon become re-contaminated (Wilson et al., 2011, Aldeyab et al., 2009). Disinfectant based cleaning is routinely performed for healthcare environments containing *C. difficile* positive patients, as both part of the management of sporadic cases or as outbreak control (Wilcox et al., 2003, McMullen et al., 2007).

There are now a wide range of disinfectants and disinfection technologies available to the healthcare sector however it is recognized that these are not alternative to traditional cleaning (Page et al., 2009), since no single process will remove all the relevant microbes from a healthcare environment. As new approaches have been utilized in healthcare settings concerns have been raised over the efficiency of methods such as, ozone, steam cleaning, microfibre, hydrogen peroxide and high-intensity light irradiation (Hardy et al., 2007, Nerandzic et al., 2010, Moore and Griffith, 2006, Berrington and Pedler, 1998, Falagas et al., 2011, Davies et al., 2011, Griffith and Dancer, 2009, Diab-Elschahawi et al., 2010, Memarzadeh et al., 2010, Sweeney and Dancer, 2009). There are also significant doubts over the activity of disinfectants in the field, since laboratory testing does not necessary predict in situ use (Sattar, 2010). There are always toxicity and cost issues which require consideration and issues regarding cross-resistance between biocides and antimicrobial agents (Russell, 2004a, Kammerer, 2003).

## 2.5 **Biocides and disinfectants**

Biocides are chemical agents having a broad spectrum of antimicrobial activity, commonly used as environmental disinfectants or antiseptics for skin decontamination (Cozad and Jones, 2003, Sopwith et al., 2002). There is a wide range of biocides commonly used in healthcare

settings, with many being combined to form commercial products. Some of the important biocides are discussed below.

### 2.5.1 **Alcohol**

Alcohol is commonly used as ethyl or isopropyl alcohol. Both of these are rapidly bactericidal against vegetative organism as well as being tuberculocidal, viricidal and fungicidal but have no activity against spores (Fraise et al., 2012). They are most active when used at a concentration of 60-90% (Morton, 1950, Boyce and Pittet, 2002). The activity of alcohol is probably due to its ability to denature proteins. The disadvantage of alcohols is their flammability and need to be used with care and stored appropriately. Alcohols are most commonly used in hands sanitizers and as a surface disinfectant.

### 2.5.2 **Quaternary ammonium compounds (QACs)**

The first commercially available QACs having antimicrobial property were benzalkonium chlorides. However, due to its inactivation by organic material and hard water and lower virucidal and tuberculocidal activity it lost favour as a disinfectant. It is believed that that their activity is due to inactivation of cell metabolic pathways and denaturation of proteins. More recently developed QACs have better tolerance of hard water and are bactericidal and virucidal against lipophilic viruses. QACs are used extensively in general disinfectant products for surfaces and for hand sanitation.

QAC are cationic surface active detergents widely used for the control of microorganism in clinical and industrial environments and used in the disinfection of hard surfaces (Labuschagne and Albertyn, 2007). They are amphoteric surfactants and contains one quaternary nitrogen that is associated with at least one major hydrophobic substituent such as alkyl groups or substituted alkyl groups, represented with R, and an anion such as CI or Br, represented with X (Fig 2.5) (Gilbert and Moore, 2005).

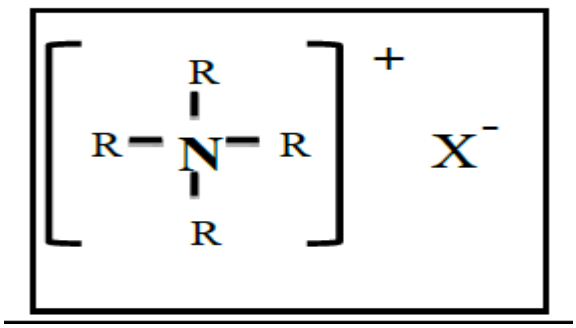


**Figure 2.5.The general structure of QACs.**

Jacobs and co-workers (Jacobs et al., 1916), published a paper describing the antimicrobial activity of quaternary ammonium compounds and later in 1935 it was shown that aliphatic groups with 8 – 18 carbons possesses antibacterial activity (Hegstad et al., 2010).The primary target of QACs seems to be the cytoplasmic (inner) membrane of bacteria (Hegstad et al., 2010). QACs are thought to adsorb to the relatively anonic bacterial cell walls, diffuse through the cell wall and binds to the cytoplasmic membrane (Hegstad et al., 2010, Sandt et al., 2007, Ioannou et al., 2007). Here they cause the disorganisation of cytoplasmic membrane which is thought to result in the leakage of intracellular material and ultimately causing cell death (Ioannou et al., 2007). The positively charged nitrogen group interacts with the phospholipids followed by the hydrophobic tail that integrates into the hydrophobic membrane core (Ioannou et al., 2007, Hegstad et al., 2010). Here they cause the disorganisation of the cytoplasmic membrane resulting in the release of intracellular molecules such as potassium ions and other intracellular low molecular weight material. QACs cause leakage of the cellular material purely because they adsorb to the cell membrane in large amounts causing damage (Ioannou et al., 2007). Bacterial cells surface carries a negative charge which are stabilized by cations such as $Mg^{2+}$ and $Ca^{2+}$ and many of the QACs also have the cationic property which helps them to exploit the interactions of $Ca^{2+}$ and phospholipids with the cell membrane (Gilbert and Moore, 2005), they proposed a model for the adsorption of the QACs to bacterial cell membrane. The positively charged quaternary ammonium chloride with the head groups of acidic phospholipids and subsequently the hydrophobic tail integrates into the hydrophilic membrane core (Figure. 2.6).

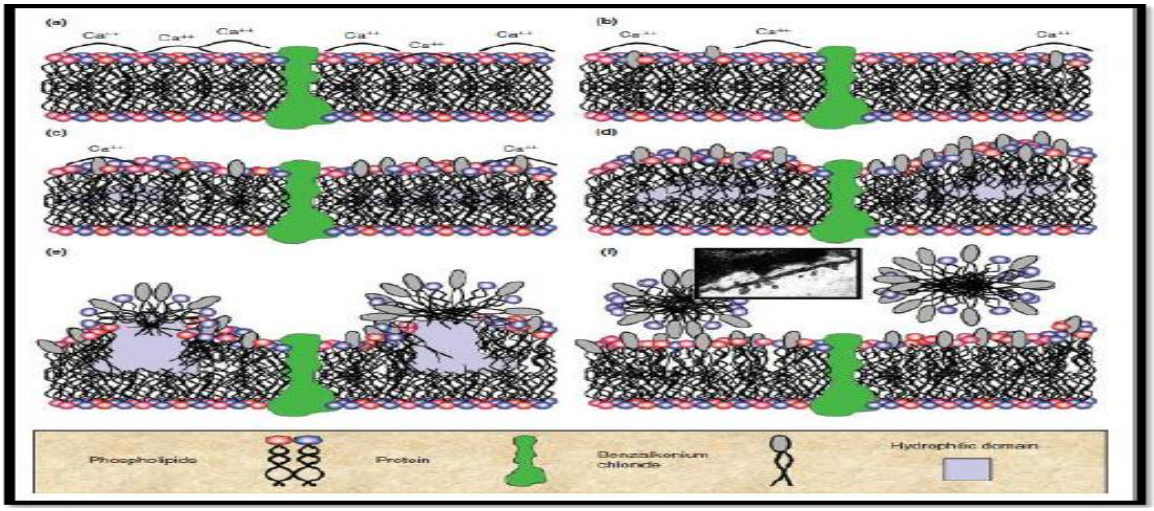


**Figure.2.6. Mechanism of action of quaternary ammonium chloride disinfectants (Gilbert and Moore, 2005).**

### 2.5.3 **Bi-guanides**

The antiseptic agent polyhexamethylene biguanide also known as polihexanide or PHMB has been used for over 60 years with no evidence of the development of resistance (Moore and Gray, 2007). There are no commercially available PHMB antiseptic/antimicrobial solutions or gel available on the UK Drug Tariff. However, in Europe Sterasept (Serag-Wiesner KG), is the only solution available as an approved finished drug product with antiseptic/antimicrobial effects. The common recommendation for infections with Gram negative pathogens is to use the higher concentration (0.1% v/v) (Dissemond et al., 2010). PHMB has little toxicity and has been found safe and effective in applications as treatment of eye infections and sanitising swimming pools (Motta et al., 2004, Motta and Trigilia, 2005). Studies in 1998 and in 2005 (total of 3,529 patients), revealed that skin sanitising to PHMB is low (~0.5%), even though the tested drug concentrations (2.5-5%) were five to ten times the concentration normally used in wound applications (Schnuch et al., 2007). In short, PHMB has good clinical safety (Disch et al., 2007, Mulder et al., 2007, Bruckner et al., 2008), targeted action on bacterial cell (Sørensen et al., 2003, Ousey and McIntosh, 2009), biocompatibility index >1 (Müller and Kramer, 2008), no know risks of resorption (Kaehn, 2010), low risk of contact sensitisation (Schnuch et al., 2007) and sustainability of the active pharmaceutical ingredient (Lee et al., 2004a). It is a fast acting biguanide sharing smilarities with antimicrobial peptides AMPs produced by many cells within wound, such as keratinocytes and inflammatory neutrophils, where they are thought to help the cells against infection (Sørensen et al., 2003, O'Hanlon and Enright, 2009, Ousey and McIntosh, 2009). However, PHMB does not interfere with the protein that makes up the animal cell membranes therefore; it has some specific antimicrobial action that does not affect the animal cell integrity. It is thought PHMB when adhered to the target cell membrane causes them to leak potassium ions and other dissolved ions from the cytoplasm causes cell death (Yasuda et al., 2003, Gilbert, 2006). PHMB has the effect on both the planktonic and biofilm bacteria (Seipp et al., 2005, Pietsch and Kraft, 2006, Harbs and Siebert, 2007) and its action on the bacterial cell membrane also means that the efflux pump is unable to remove that so intracellular bactericidal concentrations are maintained (Kingsley et al., 2009). Once PHMB inside the cell, it has been proved that PHMB binds to DNA and other nucleic acids, suggesting it may also damage or inactive bacterial DNA (Allen et al., 2004).

### 2.5.4 **Chlorine releasing agents**

Commonly used chlorine releasing agents include hypochlorites and sodium dichloroisicyanourate (NaDCC). They are rapidly bactericidal, virucidal, tuberculocidal and sporicidal (Rutala and Weber, 1997, Griffiths et al., 1999). Chlorine releasing demonstrate poor activity at low concentrations under dirty conditions because they are relatively easily inactivated by organic matter, it is therefore important that they are used on clean surfaces. The use of chlorine based disinfectants is specified in UK guidance (DoH and HPA, 2009, Pratt et al., 2007) where products delivering 1,000ppm free available chlorine are specified for cleaning associated with patients with *C. difficile* infections, in the presence of blood 10,000 ppm FAC is recommended (Boyce and Pittet, 2002).

### 2.5.5 **Aldehydes including glutaraldehyde.**

Aldehydes are used in healthcare settings predominantly as formaldehyde or glutaraldehyde. Formaldehyde has excellent antimicrobial activity including cidal activity against vegetative bacteria, mycobacteria. Viruses, fungi and bacterial spores. However, due to its toxicity it is no longer used except for fumigation of high-risk areas such as category 3 rooms. Glutaraldehyde is widely used in healthcare settings as an endoscope disinfectant. It is bactericidal and virucidal but only slowly sporicidal requiring three hours to produce a greater reduction. Concerns' regarding its toxicity and potential carcinogenicity has reduced its use in healthcare environments (Boyce and Pittet, 2002).

### 2.5.6 **Hydrogen peroxide**

Hydrogen peroxide acts by the production of free hydroxyl radicals which denatures cell walls and essential bacterial enzymes resulting in bactericidal activity. It is bactericidal including sporicidal, virucidal, fungicidal and tuberculocidal. Hydrogen peroxide is an irritant chemical and has been implicated in corneal damage therefore, it is not used widely (Fraise et al., 2012).

### 2.5.7 **Peracetic acid**

Peracetic acid is a strong oxidizing agent and has rapid bactericidal activity against range of vegetative organisms and spores. It is also veridical, fungicidal and tuberculocidal. Peracetic acid can be used as a liquid or generated in situ through aqueous reaction of tetraacetylethylenediamine (TAED) and a peroxide generator (Pan et al., 1999, Davies and Deary, 1991). Its mode of action is same as other peoxidases and oxidizing agents. Its

disinfectant property based on the release of active oxygen, the sensitive sulfhydryl and sulphur bonds in proteins, enzymes and other metabolities are oxidised and the double bonds are reacted results in the rupture of the cell wall, so it is effective against outer membrane lipoproteins, and facilitating its action against Gram-negative bacteria (Kitis, 2004).

### 2.5.8 **Iodophors**

These agents are complexes of iodine and a carrier resulting in a product, which allows sustained release of iodine. It is the free iodine, which mediates this agent's antimicrobial activity by disrupting protein and nucleic acid synthesis. The mostly used iodophor is povidone iodine and it is free iodine, which gives this product its antibacterial activity. Iodophors are bactericidal, virucidal and mycobactericidal but have poor sporicidal activity and variable anti-fungal activity (Fraise et al., 2012).

### 2.5.9 **Phenolics**

Carbolic acid (Phenol) was used by Lister as an antiseptic and its antibacterial activity are well known. Halogenated derivatives of carbolic acid are known as phenolics and tend to have improved antibacterial properties compared with the parent compound. Phenolic disinfectants are bactericidal, fungicidal and tuberculocidal although different compounds vary in their activity. However, the poor activity against blood borne viruses has limited the use of these disinfectants (Fraise et al., 2012).

### 2.5.10 **Biocidal Surfaces and Coatings**

Since high frequency touch sites are rapidly re-contaminated, surfaces or coating with prolonged biocidal activity might be useful in preventing recontamination (Aldeyab et al., 2009, Page et al., 2009, Brady et al., 2003). The problem with coating constituents is that it can wear off over time, degrade or simply fail due to the accumulation of organic soil, which might also encourage unforeseen long-term health problems in exposed persons or additional toxic effects on the environment (Page et al., 2009). There are also antimicrobial coatings for textiles (clothes, sheets and curtains), furniture, equipment (computers and catheters), hand touch sites (handles, pens etc.) and surfaces (floors and doors) (Page et al., 2009, De Muynck et al., 2010). There has also been interest in the use of metal-based surfaces e.g. copper, zinc, silver or titanium (Nanda and Saravanan, 2009, Weaver et al., 2008, Taylor et al., 2009, O'Hanlon and Enright, 2009, Casey et al., 2010, D'Arcy, 2001).

There are also electrostatic and inhibitory surfaces that repel microbial adhesion and even products marked as self-cleaning coatings (Shepherd et al., 2010, Page et al., 2009, Parkin and Palgrave, 2005). Further examples include coatings composed of nano-silver particles combined with titanium dioxide to form highly reactive $TiO_2$ Ag particles (Page et al., 2009), these particles can be applied on range of surfaces under low temperatures, which means that virtually all environmental surfaces in a hospital could be treated (Su et al., 2009). It is also possible to incorporate organic biocides into surfaces to provide some antimicrobial activity. By far the commonest organic based treatment is the use of Triclosan, a variety of Triclosan impregnated materials has been available in the supermarkets since 1997 (Page et al., 2009). One concern that has been raised regarding the use of these surfaces in healthcare settings is that they may provide false assurance if not tested properly and may lead to staff reducing their normal cleaning regimes (Dancer, 2010).

## 2.6 **Microbial Responses to Biocides**

Despite the increase in the usage and variety of biocidal products available, there has not been a significant increase in understanding of how bacteria respond to biocides. In particular, the bacterial response to the post use residues that persist at concentrations below recommended level in healthcare environments remain largely unknown. Another issues is the fact that the preferred mode of living for most microorganisms is as surface-adherent communities or biofilms (Leung et al., 2012), which are known to be more resistant to biocides (Potera, 1999). This contrast with the fact that most biocide testing is carried out against planktonic microorganism (Leung et al., 2012).

Bacteria have a variety of mechanisms at their disposal to reduce the cytoplasmic concentration of biocides (Maillard, 2007). However, it has been difficult to producing stable bacterial resistance to high biocide concentrations (Suller and Russell, 1999, Fitzgerald et al., 1992). The use of step wise biocide concentration increases have resulted in bacteria with increased minimum inhibitory concentrations (MIC), but rarely at in-use concentrations (Thomas et al., 2005, Suller and Russell, 1999, Lear et al., 2006, Walsh et al., 2003b). Alternative approaches which more closely mirror the way biocides are employed i.e. the exposure of high inoculums to high biocidal concentrations have produced some adaption but not as effectively as stepwise training (Walsh et al., 2003b, Thomas et al., 2000).

Although it is not easy to develop resistant mutants to high biocide concentrations, exposure to low concentrations may induce low-level resistance in bacteria. The induction of bacterial

resistance to almost all biocides has been documented, but particularly none-oxidizing ones such as phenolics, bis-biguanides and quaternary ammonium compounds (Russell, 2004a, Moken et al., 1997, McMurry et al., 1998). Although, the induction of *oxyR* and s*oxRS* regulons following exposure to oxidising agents has been described (Chapman, 2003).

In a range of studies the biochemical basis of tolerance has been identified e.g. benzalkonium chloride induced the expression of *qacA* and *qacB* genes (Paulsen et al., 1998), Aase *et al* (2000) observed that repeated benzalkonium chloride exposures resulted in the expression of pmf-driven efflux pumps, which allowed the bacteria to reduce the cytoplasmic concentration of the biocide (Aase et al., 2000). There have also been a number of studies demonstrating bacterial resistance to bisphenol, triclosan and chlorhexidine (McMurry et al., 1998, Russell, 2004b, Sanchez et al., 2005). Chuanchuen *et al* (Chuanchuen et al., 2001), reported the induction and expression of an efflux pump in *P. aeruginosa* following triclosan exposure, resulting in high-level resistance to both triclosan and ciprofloxacin. However, such induced bacterial strains have not been observed in practice (Russell, 2003). The induction of efflux pump mechanisms following low concentration, biocide exposures appears to be the primary mechanism of biocide resistance (Lear et al., 2002, Poole, 2005). A point emphasized by Paulsen et al (Paulsen et al., 1998), who suggested that the emergence of the *qacA* gene resulted from the use of chlorhexidine.

The expression of a range of *E. coli* genes was modified by exposure to low concentration of polyhexamethylenebiguanides (PHMB) (Allen et al., 2006). Firstly, at the ultra-structure level (outer membrane, periplasm and cytoplasmic membrane), which is not surprising due to the action mechanism of PHMB. Secondly, exposed bacteria lost their ability to produce repair pili and flagella, presumably due to increased exposure to the biocide. Thirdly, bacteria expressed the heat shock response, DNA damage (SOS) response and other DNA metabolism associated genes, presumably to repair significant DNA damage.

However, the most frequent mechanism for the biocide resistance is the use of efflux pumps which reduce intracellular concentrations to sub-toxic levels (Borges-Walmsley and Walmsley, 2001, Hegstad et al., 2010). Gilbert and Moore ((Gilbert and Moore, 2005), reported that efflux pumps are capable of removing QACs from the membrane core and effectively reducing the effectiveness of the QACs. These pumps operated by ATP driven transporters and proton pump antiporters.

The proton pump antiporters contains one of the three classes of antiporters, the resistance nodulation division family (RND), small multidrug resistance family (SMR) and the major facilitator superfamily (MF), while ATP driven antiporters contains the ATP binding cassette (ABC) (Putman et al., 2000, Borges-Walmsley and Walmsley, 2001, Kumar and Schweizer, 2005). Efflux pumps found in both the Gram positive and Gram negative bacteria but the efflux mediated resistance is more complex in Gram negative bacteria because of the complex nature of the cell wall (Sidhu et al., 2002, Kumar and Schweizer, 2005). The ABC transporters are rare in bacteria and are involved in uptake as well as efflux systems where energy is provided by hydrolysis of ATP (Putman et al., 2000, Borges-Walmsley and Walmsley, 2001, Kumar and Schweizer, 2005). The proton pump antiporters function by transporting biocides out of the cells by the help of transmembrane electrochemical gradient of protons or sodium ions, proton motive force and only differ in size (Putman et al., 2000). The MF transporters found in both Gram positive and Gram negative bacteria and composed of about 400 amino acids that are arranged into 12-14 membrane spanning helices (Putman et al., 2000, Borges-Walmsley and Walmsley, 2001, Kumar and Schweizer, 2005) and the *Staphylococcus Q*acA and *Q*acB proteins are part of this family proteins (Kumar and Schweizer, 2005, Gaze et al., 2005). The RND transporters are composed of around 1000 amino acids and have similar helix structures as the MF (Kumar and Schweizer, 2005), they operate with periplasmic membrane fusion proteins and an outer membrane protein, allowing the transport of toxic compounds out through both inner and the outer membrane of Gram negative bacteria(Putman et al., 2000). QACs resistant genes in the *Staphylococcus* genus are also widely spread amongst the clinical isolates (Bjorland et al., 2005). The small multidrug resistant family (SMF) includes, *qacH, qacG, qacJ* and *smr* and is found on non-conjugated and large conjugated (Bjorland et al., 2001, Bjorland et al., 2003), and this protein consist of 4 predicted transmembrane segments (Fig. 1.7). The multidrug transport pumps do not have specificity for a specific product which could potentially mediate the cross-resistance to a number of antimicrobial agents (Bowler et al., 2001). Putman et al (Putman et al., 2000), reported that several multidrug transporters can be present within the same bacterium and the availability of these different transporters may contribute to bacterial resistance against wide range of biocides.

### 2.6.1 **Biocide resistance in clinical settings**

The emergence of bacterial resistance to biocides is not a new phenomenon and has been investigated since the introduction of biocides in clinical settings. Clinical isolates

demonstrating biocide resistance have been associated with triclosan baths and hand washes, the use of chlorhexidine and QACs, and silver and silver sulfadiazine (Webster et al., 1994). There have also been a number of reports highlighting the failure of disinfectants used for clinical application (Anderson, 1989, Russell, 2004a, O'Rourke et al., 2003) although it is not clear if these are associated with resistance or poor application.

The resistance characteristic of environmental isolates (Lear et al., 2002)collected from biocide manufacturing sites demonstrated that apart from intrinsically resistant *Pseudomonas*, few genera showed resistance to triclosan or para-chloro-meta-xylenol. Although increased MIC was observed, the bacterial isolates remained susceptible to practical concentration (Lear et al., 2002).

Griffiths *et al* (Griffiths et al., 1997), demonstrated that *Mycobacterium chelonae* isolated from endoscope washer disinfector had developed resistance to the in-use (2% v/v) concentration of glutaraldehyde, but remained susceptible to other aldehydes (Fraud et al., 2001). Glutaraldehyde resistance was associated with minor modifications to the outer cell walls of these strains (Walsh et al., 1999, Fraud et al., 2001). Further *in-vitro* studies indicated that changes in the cell wall arabinogalactan/arabinomannan content of *M. chelonae* was responsible for the glutaraldehyde resistance observed in these strains (Manzoor et al., 1999). There have also been a number of studies demonstrating bacterial resistance to bisphenol, triclosan and chlorhexidine (McMurry et al., 1998, Russell, 2004b, Sanchez et al., 2005). Chuanchuen *et al* (Chuanchuen et al., 2001), reported the induction and expression of an efflux pump in *P. aeruginosa* following triclosan exposure, resulting in high-level resistance to both triclosan and ciprofloxacin. However, such induced bacterial strains have not been observed in practice (Russell, 2003). The induction of efflux pump mechanisms following low concentration, biocide exposures appears to be the primary mechanism of biocide resistance (Lear et al., 2002, Poole, 2005). A point emphasized by Paulsen et al (Paulsen et al., 1998), who suggested that the emergence of the *qacA* gene resulted from the use of chlorhexidine.
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concentration, biocide exposures appears to be the primary mechanism of biocide resistance (Lear et al., 2002, Poole, 2005). A point emphasized by Paulsen et al (Paulsen et al., 1998), who suggested that the emergence of the *qacA* gene resulted from the use of chlorhexidine.

### 2.6.2 **Biofilms and Biocide Resistance**

Bacterial growth as a biofilm often contributes to the failure of biocides and disinfection (Espinal et al., 2011). The highly organized structure and polymeric matrix of a biofilm provides a protective mechanism allowing bacteria to survive the harsh environments created by biocides and biofilm formation represents an important virulence factor (Espinal et al., 2011). Effective disinfection in the presence of a biofilm often requires higher concentrations of antimicrobial agents when compared to planktonic bacteria (Thomas et al., 2011). The antimicrobial concentrations required for biofilm inactivation can be 100 times that required for inactivation of removal planktonic cells (Rasmussen and Givskov, 2006).

These impacts may be species specific for example the efficacy of ortho-pthalaldehyde was reduced against Mycobacterial biofilms but not against *P. aeruginosa* ones (Berkelman et al., 1984). Survival of bacteria in biofilms has been identified as contributing to a number of outbreaks, e.g. *P. aeruginosa* resistance to iodophores, *Serratia marcescens* resistance to benzylkonium chloride and chlorhexidine. Recently a major outbreak of *Pseudomonas* infections in a neonatal unit in Northern Ireland was associated with biofilms in water distribution system(RQIA, 2012).

However many hospital associated infections are caused by bacteria associated in biofilms and yet most laboratories are not using biofilm test to assess the efficacy of biocides (Cookson, 2005), and there are currently no European standards for the testing of disinfectants against biofilm for healthcare applications.

## 2.7 **Evaluation of biocides**

The testing of biocides has received considerable attention for many years with a range of formal standard published world wide (Fraise et al., 2012). However, many of these techniques are designed for rapid determination of basic bactericidal activity and as such are not particularly useful for the generation of research data. However, more sophisticated approaches for evaluating the impact of biocides on both both planktonic cells and biofilm forming cells are available, specifically the Bioscreen C technology system (by Growth Curves Ab Ltd) and the MBEC systems (Innovotech Inc, Canada).

### 2.7.1 **Bioscreen C technology system**

The Bioscreen system monitors the live growth of microorganism by measuring turbidity of the liquid growth medium in the 100 honey comb wells at specific temperature and with or without shaking. These meaurments are done by realtime recording of the optical density of the cultures under investigation. The mesurment interval can be set from minute to hours. The reader consist of three inter-related systems i.e. mechanical transport, incubator and optical system (Figure 2.7). The incubator tray assembly holds the honeycomb plates in the correct position. The assembly shuttles left from the plate loading section into the measurment compartment, where light is passed through each well of the plate and the detector makes the OD readings. Incubator maintains the plates at the chosen temperature by circulating the heating-cooling liquid continously through the incubator. A halogen lamp produces light which then passes through the chopper wheel. The light path is turned 90 degrees by a mirror, than pass through the filter wheel. The correct filter is chosen by making the appropiate entry during the experiments set up (Wide band filter 480-520nm used for the turbidity meaurment). Filtered light moves through an optical fibre to the lens assembly in the measurement compartment, below the honeycomb plates. Light passes through the bottom of each well and results are collected by the detector and trasfered to PC attached with the system (Figure 2.7).

The Bioscreen technology generates large amounts of data which requires processing. A number of modelling approaches have been developed to transform Bioscreen data into key characteristics such as minimum inhibitory concentrations (MIC) (Lambert and Lambert, 2003, Lambert and Pearson, 2000b) and minimum biocidal concentrations (MBC). The MIC being the minimum concentration of a biocide required to prevent microbial growth, whilst the MBC is the the minimum concentration of a biocide required to kill microbial cells.

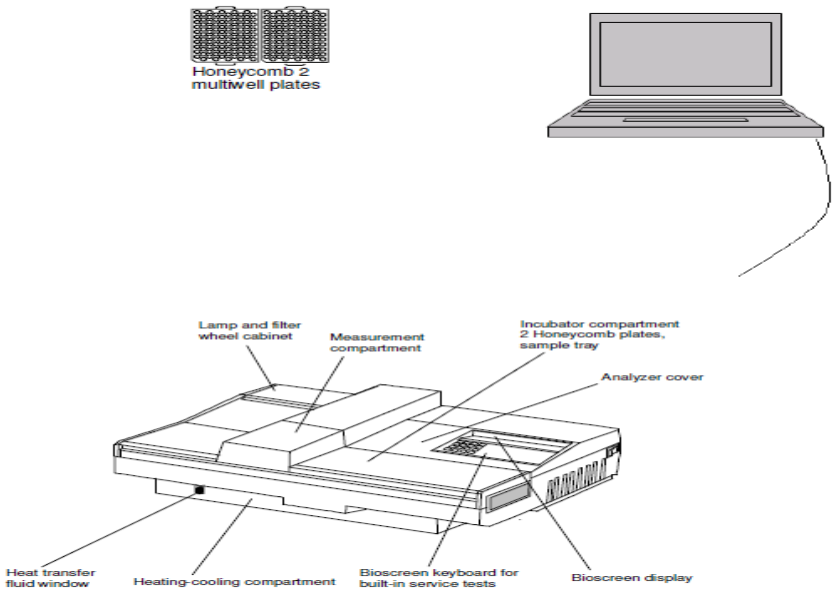



**Figure 2.7. The Bioscreen system.**

### 2.7.2 **MBEC system**

Biofilm forming bacteria are phenotypically distinct from suspended planktonic cells of the same genotype. Biofilm growth reactors are engineered to produce biofilms with specific characteristics, however by altering the engineered system or operating conditions characteristic of biofilm can be changed. Microscopically a biofilm is a sheet like structure with some architectural details and the purpose of MBEC system is to grow consistent, representative biofilms amenable to testing and evaluation (Harrison et al., 2010). The MBEC system consists of a polystyrene lid, with 96 downward-protruding pegs (Figure 2.8), that exactly fit into the wells of a 96-well plate and was originally designed for the rapid and reproducible assay for evaluating of biofilm susceptibility to antibiotic (Ceri et al., 1999a, Smith et al., 2012) and have been integrated into the testing of biocides particularly for *Pseudomonas* sp. The biofilm is established on pegs under batch conditions (no flow of nutrients in and out of the individual well) with gentle mixing. The established biofilm is then transferred into a new receiver plate for disinfectant efficiency testing. Additional efficiency is added by including the neutralizer controls within the assay device, and due to small volume of samples used (200µl) for testing of expensive biocides it is more economical and efficient than other approaches.

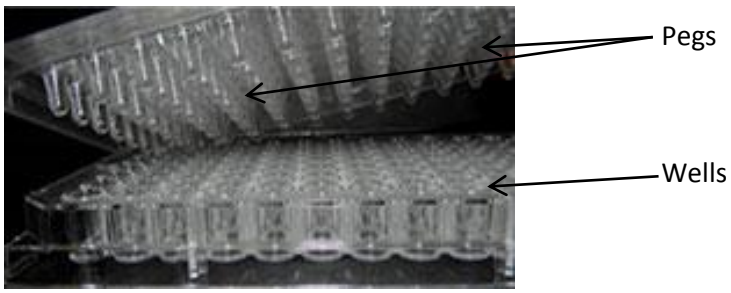



**Figure.2.8. The MBEC biofilm growth system (Innovotech Inc, Canada).**

## 2.8 **Wound dressing**

Chronic wounds (e.g. diabetic lower limb and pressure ulcers) are generally heavily colonised with pathogenic bacteria. The healing of these wounds depends on adjusting the equilibrium between the host-immune system and the pathogens present in the wound environment (Stephen-Haynes, 2004). Within wound microorganisms exist in either a free floating/planktonic state or as part of a biofilm associated with the wound bed (Thomas et al., 2011).

Many multi drug resistant organisms (MDROs) are also often associated with chronic and acute wounds. Topical antimicrobials are often the first approach applied to bio burden control, in wounds where there are clear signs of a progressive infection however; systematic antibiotics are generally applied (Bowler et al., 2012). Several factors determine the efficiency of systematic antibiotics such as: the extent of blood flow to the wound, the extent of antibiotic-resistance, the bacterial species present, the presence of biofilms (Zubair et al., 2011). Where MDROs have colonised the wound, the efficacy of systematic antibiotics treatment is uncertain. In this case the topical application of antiseptics and disinfectants may present a viable alternative due to their broader spectrum of activity and lack of bacterial resistance. By combining antiseptic and disinfectant agents with wound dressings it is possible to achieve a managed delivery of antimicrobial agents into the infected wound bed (Ovington, 2007).

There is large range of antimicrobial dressings available with varying claims of antimicrobial efficacy. Current approaches for the evaluation of antimicrobial wound dressings vary significantly in terms of media used, inoculum and sample size (Tkachenko and Karas, 2012), which may make direct comparison between dressing difficult (Chopra, 2007). In addition the presence of biofilms within wounds complicate the testing of these dressings, particularly when of our current knowledge of bacterial biofilm is based on *in vitro* observations of bacterial adherence to solid surfaces. This is a marked contrast to the situation in a chronic

wound where bacteria reside within a wound beds rather than attached to well defined solid surface (Werthan et al., 2010).

### 2.8.1 **Wound dressings**

There are lot of different dressing available for treatment of wounds, the use of wound dressing depend upon the characteristics of wound and the mode of dressing itself, below are some example of different wound dressing.

*a) Semi-permeable film dressings*

These dressing consist of a thin polyurethane type film, which is coated with adhesive layer, which help the dressing to adhere with the skin. These dressing are semi permeable which allow transmitting of moisture but do not adsorb the exudates, these film dressing provide a protective environment which is impermeable to bacteria and liquids and can stay in place for up to 7 days (Abdelrahman and Newton, 2011).

*b). Non-adherent contact layer dressings*

Non-adherent dressings applied directly onto the wounds to provide an interface with the secondary dressing or pad. They are usually made up of fine woven mesh, which allow exudates to pass through. Some of the non-adherent dressing contains paraffin or silicone to improve the non-adherence (Abdelrahman and Newton, 2011). Due to increasing concerns of non-adherent dressing about pain on dressing removal and risk of sensitivities the use of non-adherent dressing are less in modern day wound management (Lewis et al., 2001).

*c). Hydrocolloid dressings*

These are described as interactive as they use wound fluid to form a moist gel at the wound interface, these dressing consist of gelatins, pectins and carboxymethylcellulose which form the hydrocolloid base, which is then secured onto a backing of polyurethane film or foam, hydrocolloids, have a low moisture transmission rate of less than 300mg/$m^2$/24 hours (Seaman, 2002). Depending upon the level of exudates these dressing can stay up to 6 days.

*e). Hydrofibre dressings*

These dressing consist of insoluble polymers with high water contents making them ideal dressings to facilitate autolytic debridement of necrosis and slough. These are better for dry necrotic wound that require hydrating and debridement, whilst hydrocolloids debride tissues in sloughy, exuding wounds. A new sliver coating hydrofibre dressing Aquacel Ag and Acticoat is widely used now days due to the broad-spectrum antimicrobial activity of ionic silver to the proven exudate handling technology of hydrofibre (Harding et al., 2001).

*f). Foam dressings*

Foam dressing is made up of polyurethane or silicone, which helps them to handle large volumes of wound fluid. There properties vary from adhesive to non-adhesive formulation with varied thickness (Abdelrahman and Newton, 2011). Some modern foam dressing proved to be effective as cavity filters, when applied on wounds foam has the ability to adsorb exudates, preventing pooling and skin leakage, and maintain the low adherence to facilitate easy painless removal (Flores and Kingsley, 2007).

### *2.8.1.1* ***Antimicrobial dressings***

The **s**pread of infection in the individual has a serious implications for patient wellbeing and act as a pathogenic reservoir results in increasing the risk of cross contamination. Accurate differentail diagnosis and treatment with appropiate systemic antibiotics is essential (EWMA, 2006, WUWHS, 2007), bacteria resistant to topical antibiotics should be avoided (WUWHS, 2008), adjuvant topical antimicrobial dressings may be used to help reduce the wound bioburden (EWMA, 2006, WUWHS, 2007). However, critical colonisation and localised, subclinical infection remain an issue and are significant contributors for wound healing (Edwards and Harding, 2004, Warriner and Burrell, 2005). In recent years, dressing that contains andrelaese the antimicrobial agents at the wound surface has been in use (White and McDermott, 2001, Cooper, 2004), these dressing has the ability to provide continues relaese of the antimicrobial agents at the wound surface to provide a long lasting antimicrobial action in combination with maintenance of physiologically mosist enviornment for healing (Ovington, 2007). Table 2.3 explain the range of antimicrobial wound dressing available in the market.

| Dressing Name | Antimicrobial ingredient | Dressing Format | Manufacturer |
|---|---|---|---|
| Acticoat absorbent | Ionic silver | Calcium alginate | Smith & Nephew, Inc, Largo, FL, USA |
| Actisorb Silver 220 | Ionic silver and activated charcoal | Silver impregnated activated charcoal cloth | Johnson and Johnson Wound Management, Somerville, NJ, USA |
| Arglaes | Ionic silver | Transparent film or powder | Medline Industries, Inc, Mundelein, IL, USA |
| Aquacel AG | Ionic silver | Hydrofiber | Convatec, Skillman, NJ, USA |
| Contreet H | Ionic silver | Hydrocolloid | Coloplast Corp, Marietta, GA, USA |
| Contreet F | Ionic silver | Foam | Coloplast Corp, Marietta, GA, USA |
| Iodosorb | Molecular iodine | Gel or paste | HealthPoint Ltd, Ft. Worth, TX, USA |
| Silvasorb Antimicrobial Silver Dressing | Ionic silver | Hydrogel sheet or amorphous gel | Medline Industries, Inc, Mundelein, IL, USA |
| Kerlix AMD Gauze | PHMB | Gauze | Tyco Healthcare/Kendall, Mansfield, MA, USA |

PHMB indicates polyhexamethyl biguanide.

**Table.2.3 Examples of antimicrobial dressings (Ovington, 2007).**

*2.8.1.1.1 Iodine dressings*

Iodine has been used as antimicrobial agent for a long time, iodine mixed with polymeric cadexomer starch vehicle has been used as a topical gel or paste, the cadexomer moiety provides exudate adsoption from the wound which results in the slow release of free iodine (Ovington, 2007). Like all the other antiseptics, iodine effects multiple sites in microbial cells, resulting in cell distruption and cell death (WUWHS, 2007). However, its antimicrobial efficiancy, chemical stability, toxicity to host tissues and the ensuing effect on patient comfort has been debated (Wilson et al., 2005). It has been reported that povidone-iodine is not as effective as some other biocides against *Staphylococcous epidermis* within *in vitro* bioflims (Presterl et al., 2007). Iodine mixed with polymeric cadexomer proved to be a biofilm supression without significant damage to the host (Akiyama et al., 2004, Rhoads et al., 2008), but pain has been reported as a side effect of its use (Hansson, 1998).

2.8.1.1.2 Silver dressings

Silver (Aquacel Ag) based dressing are widley used in wound care (Klasen, 2000b, Klasen, 2000a, Demling and Desanti, 2001, Ip et al., 2006), the only side effects reported is skin discolouration and irritation (White and Cooper, 2005). A new sliver coating hydrofibre dressings Aquacel Ag and Acticoat is widely used due to the broad-spectrum antimicrobial activity of ionic silver to the proven exudate handling technology of hydrofibre (Harding et al., 2001). Questions have been concerns for the long use of silver on wounds especially in infants (Denyer, 2009), but to date no pathological consequences of silver used dressing has been reported, except some reports about the systemic uptake and deposition of silvers in organs (Denyer, 2009, Wang et al., 2009).

2.8.1.1.3 Honey dressings

Manuka honey dressing also used for the treatment of wound care. These dressing has been successfully used for the treatment of recalcitrant wounds within the maxillofacial unit and proved resistant to antibiotics. These honey coated dressing has been used directly on the wound and can be used for 2-3 days without changing (Visavadia et al., 2008). The exact mode of action of honey dressings not fully understood, however it is hyperosmolar and thus restricts the availability of environmental water to bacteria and other organisms (Molan, 2001), which results to cell disruption and death. Another property of honey is the release of hydrogen peroxide as the honey is diluted by exudates (Molan, 2004). However some honeys varieties particularly the Manuka and *Leptospermum* has the ability to retain their

antimicrobial property even without the release of hydrogen peroxide (Cooper et al., 2002b, Cooper et al., 2002a), which is thought to be phytochemical component (Molan, 2002).

#### 2.8.1.1.4 PHMB dressings

Recently polyhexamethylenebiguanide (PHMB) has been introduced which has effect on the microbial cell metabolism. PHMB is a fast acting biguanide compounds composed of synthetic mixture of polymers, having structural similarities to the antimicrobial peptides AMPs produced by many cells within the wounds, such as keratinocytes and inflammatory neutrophils, where they are thought to help the cells against infection (Sørensen et al., 2003, Ousey and McIntosh, 2009). AMP has a broad spectrum of activity against bacteria, viruses and fungi including cell death by disrupting cell membrane integrity (Moore and Gray, 2007). They are proved to be effective against *Pseudomonas aeruginosa* and *Staphylococcus aureus* which are common in many chronic wounds (Abdelrahman and Newton, 2011). PHMB product ranges includes Telf™ AMD drain, Kendall AMD antimicrobial foam having higher percentage of PHMB impregnated 0.5%, Biocellulose PHMB-donating dressings e.g Suprasorb X+PHMB, which contains 0.3% PHMB. In vitro and in vivo studies have proved that dressing or products containing PHMB reduces wound pain rapidly and effectively (Daeschlein et al., 2007, Galitz et al., 2009), reduces wound malodour (Daeschlein et al., 2007), reduces MMP-induced pre-wound breakdown (Cazzaniga et al., 2007, Werner et al., 2004), reduces slough within wound (Mueller and Krebsbach, 2008), increases keratinocyte and fibroblast activity (Wiegand et al., 2007), increases formation of granulation tissue (Mueller and Krebsbach, 2008), and helps remove non-viable tissue (Kaehn, 2009).

#### *2.8.1.1.5 Evaluation of antimicrobial dressings*

Current approaches for the evaluation of antimicrobial wound dressings vary significantly in terms of media used, inoculum and sample size (Tkachenko and Karas, 2012), which may make direct comparison between dressing difficult (Chopra, 2007). In addition the presence of biofilms within wounds complicate the testing of these dressings, particularly when our current knowledge of bacterial biofilm is based on *in vitro* observations of bacterial adherence to solid surfaces. This is a marked contrast to the situation in a chronic wound where bacteria reside within a wound beds rather than attached to well defined solid surface (Werthan et al., 2010).

# 3 METHODOLOGY

## 3.1 Materials

### 3.1.1 Chemicals

Unless otherwise stated all chemical used in this study were sourced from either Fisher Scientific Ltd or Sigma-Aldrich Ltd. Biological grade chemicals were used whenever possible.

#### *3.1.1.1 Biocides*

A range of biocides and biocidal products were employed in this investigation and are outlined below:

- Ethanol ($CH_3CH_2OH$)(Absolute alcohol 99%, Fisher Scientific Ltd);
- Iso-propylalcohol (IPA, propan-2-ol, $CH_3CHOHCH_3$, 99.8%, Fisher Scientific Ltd);
- Methanol ($CH_3OH$, 99.8+%, Fisher Scientific Ltd );
- Benzethonium Chloride (≥98%, Sigma-Aldrich Ltd);
- Benzalkonium Chloride (>95%, Sigma-Aldrich Ltd);
- Barquat MB-50 (Alkyl (C14 50%, C16 10%, C12 40%) Dimethyl Benzyl Ammonium Chloride, 80% w/v, Lonza Ltd);
- Bardap 26 (N,N-Didecyl-N-methyl-poly(oxyethyl) ammonium propionate, 80% w/v, Lonza Ltd);
- PHMB (Polyhexamethylenebiguanide, 50% w/v, Arch Chemicals Ltd).

### 3.1.2 Microbiological media

The following microbiological media were used throughout this study:

- Tryptone Soy Agar (TSA, LabM Ltd) Prepared by dispersing 37g in one litre of distilled water prior to autoclaving.
- Tryptone Soy Broth (TSB, LabM Ltd) Prepared by dispersing 30g in one litre of distilled water prior to autoclaving.
- Maximum Recovery Diluent (MRD, LabM Ltd) Prepared by dispersing 9.5g in one litre.
- DE neutraliser broth (DE, LabM Ltd) Prepared by dispersing 39g in one litre.

Unless otherwise stated all media and solutions were prepared in double distilled water.

#### *3.1.2.1 Mineral media*

To enable successful EPS characterisation a carbohydrate free mineral media was developed (Table 3.1).

| Components | $K_2HPO_4$ | $(NH_4)_2SO_4$ | $MgSO_4.7H_2O$ | NaCl | $CaCl_2$ | $FeCl_3$ | Casein hydrolysate |
|---|---|---|---|---|---|---|---|
| g/L | 4.5 | 1 | 0.2 | 0.1 | 0.1 | 0.02 | 0.05 |
| Final pH of the media adjusted to pH 7.0. | | | | | | | |

**Table: 3.1 Mineral media supplemented with Casein.**

### 3.2 Standard microbiology techniques

Unless otherwise stated all microbiology investigations were carried out via a set of standard microbiological techniques. Growth and enumeration of bacteria was carried out employing Maximum Recovery Diluent (MRD, LabM Ltd) in conjunction with Tryptone Soya Broth (TSB, LabM Ltd) or Tryptone Soya Agar (TSA, LabM Ltd). All cultures were incubated at 37$^{o}$C for 24 hours under aerobic conditions. Cultures were plated out via either pour, spread or spiral plating (Wasp II Spiral Plater, Don Whitley Scientific Ltd). All culture media, diluents and heat stable components were sterilised via autoclaving at 121$^{o}$C for 15 minutes, heat labile components were sterilised via filtration through sterile 0.45µm filters (Sartorius Ltd).

### 3.3 Microbial Cultures

A wide range of microbial cultures, both type strains and isolated strains were employed in this investigation. These cultures are outlined in Table 3.2, cultures were preserved at -80$^{o}$C on Microbank beads (Prolab Diagnostics Ltd). These beads were revived on TSA under standard conditions.

| Species | Culture Collection | Short Identifier | Source |
|---|---|---|---|
| *Acinetobacter baumannii* | NCIMB 19606 | Type | Oxoid Ltd |
| | ACCB 63608 | UK-HS | Strain provided by Dr M. Wren UCLH, London. |
| | CIP 105742 | 742 | Pasteur Institute, Paris, France. |
| | CIP 106882 | 882 | |
| | CIP 107292 | 292 | |
| | OXA-23 Clone 1 | Clone 1 | Strains provided by Dr J. Turton, LHCAI, HPA Colindale, London. |
| | OXA-23 Clone 2 | Clone 2 | |
| *Staphylococcus aureus (MSSA)* | ATCC 6538 | 6538 | Oxoid Ltd |
| *S. aureus (*MRSA) | ATCC 4300 | 4300 | Oxoid Ltd |
| | NCTC 13142 | 13142 | HPA, London, UK. |
| | N/A | 7F/C7 | Isolated from mobile phones (White et al 2013). |
| | | 9B/F6 | |
| *Pseudomonas aeruginosa* | ATCC 15442 | 15442 | Oxoid Ltd. |
| | N/A | I | Isolated from dressing removed from infected wounds. |
| | | II | |
| | | III | |
| | | IV | |
| | | V | |

**Table 3.2 Bacterial strains used in this investigation**

## 3.4 Chemical Analysis

### 3.4.1 Ethanol and IPA analysis

The quantitative determination of ethanol and IPA was performed via a QuantiChrom Ethanol Assay Kit (Universal Biologicals (Cambridge) Ltd). Sample ethanol and IPA concentrations were determined by comparison with a standard curve (Figure 3.1).

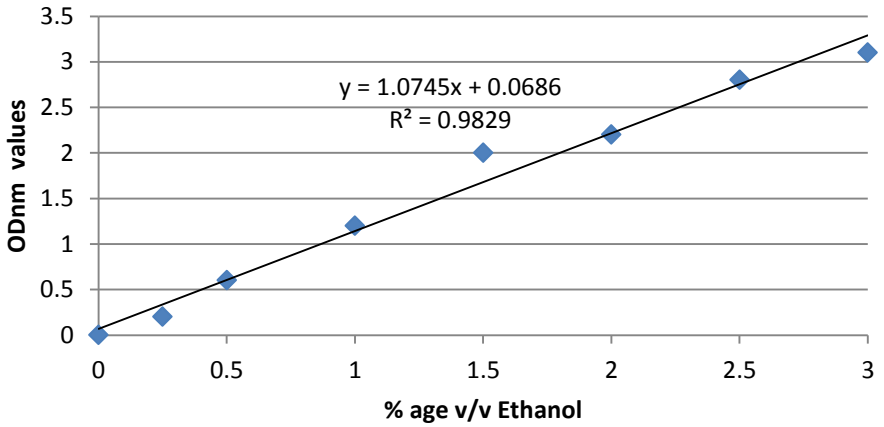



**Figure 3.1. Ethanol Assay Standard curve.**

### 3.4.2 Glucose and lactate analysis

The glucose and lactate concentration in samples was determined using a PicoTrace analyser (Trace Analytics GmbH).

### 3.4.3 Total carbohydrate analysis

The phenol/sulphuric acid procedure employed for total carbohydrate analysis was based on that described by (Dubois et al., 1956). The carbohydrate content of a sample is determined by comparison with a glucose standard curve. In order to carry out the analysis 1 ml of phenol solution (5% w/v) was added to 1ml of the test solution (unknown or standard). Immediately after the addition of phenol, 5ml of concentrated sulphuric acid is added rapidly (~15-30sec) and mixed. The reaction mixture is incubated at 70°C for 20 minutes, mixed then and placed in a 10°C water bath for a further 10 minutes. Finally the absorbance of the solution at 490nm is measured and compared against a glucose standard curve (25-300ppm) to determine the carbohydrate content of the unknown sample (Figure 3.2).

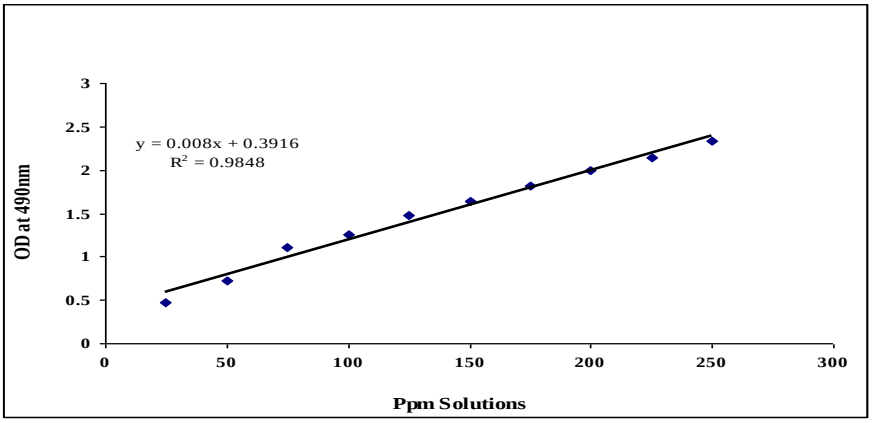



**Figure 3.2. Carbohydrate Assay Standard curve.**

### 3.4.4 Exopolysaccharide (EPS) extraction

Three methods for the extraction and characterization of *A. baumannii* EPS were employed during this study:

1. Poly-β-(1-6)-*N*-Acetylglucoseamine (PNAG) method(O'Toole and Kolter, 1998);
2. EDTA method (Sheng et al., 2005);
3. Trichloroacetic acid method (Marshall et al., 2001).

#### *3.4.4.1 Poly-β-(1-6)-N-Acetylglucoseamine method*

To determine if *A. baumannii* has the ability to produce PNAG, the bacteria strains were grown on plates containing Congo red agar composed of brain heart infusion agar (Oxoid, UK) supplemented with 5% sucrose and 0.8 mg/ml of Congo red (Sigma Chemical ltd. UK) as described by (Handke et al., 2004). On these plates PNAG synthesizing cells produced red colonies, whereas PNAG deficient cells produced white colonies. After screening, PNAG producing strains were grown in a 6-litre culture of mineral media supplemented with 1% v/v ethanol and incubated at 37°C for 72 hours with continuous shaking at ~90-110rpm. Bacterial cells were collected by centrifugation at 9000g for 15 minutes and resuspended in 100ml of 20mM EDTA buffer (pH 8.0) containing lysozyme (500mg), and incubated at room temperature for 30min. DNAase I (25mg) and RNAase A(100mg) were then added, and the suspension incubated at room temperature for 1hour and then 37°C for 2 hours. The cells were then removed by centrifugation and the EPS precipitated with 3 volumes of ethanol at 4°C for 24-48 hours. The precipitate was recovered by centrifugation at 9000g for 15 minutes and then suspended in ultrapure water and dialysed using 2000 MWCO dialysis tubes (Sigma Aldrich Ltd) against ultrapure water for 72 hours at 4°C, with three changes of water per day to remove the small natural sugars. The extracted material was freeze dried and stored for further analyse by $^{1}$H NMR.

#### *3.4.4.2 EDTA method*

EPS can be classified by its proximity to the cell surface. Capsular or cell bound-EPS tightly linked via a covalent or non-covalent association with cells and free EPS which is not directly attached to the cell surfaces (Wingender et al., 1999). Bacteria were grown and harvested as described for the PNAG method. In this case however, the cell pellets were used for the extraction of the bound EPS and the supernatant was used for the extraction of free EPS. Cells were harvested from the culture media by centrifugation at 5000rpm for 15min at 4°C. Bound EPS was extracted from the cell pellet using EDTA method as described by Sheng *et al* (2005) with some modification, which reduced the release of nucleic acids by preventing cell lysis. Cells were washed twice with 0.9% w/v NaCl to remove the traces of the media. The washed cells were resuspended in 1:1 volume of solution 0.9% w/v NaCl and 2% w/v EDTA and incubated for 60min at 4°C. The supernatant used for the extraction of bond EPS was centrifuged at 10,000xg for 60min at 4°C and then filtered through a nitrocellulose membrane.

To determine the free EPS the supernatant was recentrifuged at 10,000*g* for 30min at 4°C to remove residual cells and then the EPS was precipitated by mixing the supernatant 3 volumes of ethanol at -20°C for 18h. Precipitated EPS was recovered by centrifugation at 10,000*g* for 15min at 4°C. The extract was resuspended in ultrapure water and dialysed against ultrapure water to removed ethanol using 2000 MWCO dialysis tubes (Sigma Aldrich Ltd). The extracted material was freeze dried and stored for further analysis e.g. $^{1}H$ NMR, FTIR and MALLS analysis.

#### *3.4.4.3 Trichloroacetic acid method*

The isolation of exopolysaccharide (EPS) was carried out by the method described by (Marshall et al., 2001), with some modifications. The procedure involves a series of ethanol precipitations, centrifugation and dialysis. Samples of overnight bacterial culture (5 litres) in mineral media were heated at 80°C for 20-30min and then left to cool at room temperature. Following cooling trichloroacetic acid (TCA, Sigma Aldrich, UK) was added until the TCA concentration of the sample reached 14% (w/v) and the sample was then left at 4°C overnight. The samples were then centrifuged at 25,000g (Avanti J-26 XPI centrifuge, Coulter ltd UK, High Wycombe, UK) for 35 minutes at 4°C to remove cells and proteins. An equal volume of chilled absolute ethanol was then added to the supernatant which was then left for 48 hours at 4°C to allow the precipitation of the crude EPS. After precipitation the sample was centrifuged, as above, and the pellet retained. To remove the traces of DNA, RNA and other nucleic acids remnants from the pellets, the pellet was re-dissolved in 10 ml of phosphate buffer saline (PBS) containing 10mM $MgCl_2$, 1mM $CaCl_2$, 100µg/ml of RNAase (Sigma Aldrich, UK), and 100µg/ml of DNAase (Sigma Aldrich, UK), incubated at 37°C for 2-3 hours. To remove protein contamination 100ml of protease (20µg/ml) (Sigma Aldrich, UK), was added and again incubated at 37°C for 2-3 hours. The solution was then heated to 80°C for 30mins to inactivate the enzymes and finally centrifuge at 25,000g, 4°C for 30min. The resulting supernatant was then subjected to ethanol precipitation. After 24 hours of precipitation at 4°C the sample was centrifuged, as above, and the pellet retained. The pellet was then dissolved in ultra-pure water and dialysed against ultra-pure water for 72 hours at 4°C, with three changes of water per day to remove the small natural sugars. The extracted EPS was then characterized by solid state NMR, FTIR, MALLS analysis and further characterised by HPEAC.

#### *3.4.4.3.1 Preparation of dialysis tubing*

Dialysis tubing was prepared by boiling in deionised water (500ml) containing EDTA (0.186g) (Sigma Aldrich, UK), and sodium carbonate (10g) for 10 minutes. The tubing was then rinsed before boiling again with deionised water and store at 4°C in deionised water.

### 3.4.5 Structural characterisation of Exopolysaccharides

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

#### *3.4.5.1 NMR analysis*

All the $^{1}H$ NMR spectra of extracted EPS were generated on either a Bruker Avance DPX4000 400.13 or a Bruker Avance DPX500 500.13MHz by Dr. Neil Mclay. All the samples of EPS were prepared either in deuterium dioxide ($D_2O$) or DMSO (GOSS Scientific Instruments Ltd, Nantwich, UK) and the spectra were acquired using Bruker pulse sequences at a temperature of 70°C unless otherwise stated. Chemical shifts were expressed in ppm relative to an internal standard of acetone.

The solid state $^{13}C$ NMR were performed by Department of chemistry, University of Durham, UK using a Varian VNMRS spectrometer operating at 100.56 MHz for $^{13}C$. The peaks between 170 and 180 ppm are typical of carboxylic acid or ester carbons. Those between 50 and 70 ppm are in the region for ether or alcohol. Signals in the range 60 to 110 ppm are typically polysaccharide or EPS. Cellulose for example would have a C1 signal around 105 ppm, C4 80-90 ppm (crystalline ~88, amorphous ~84 ppm and broad), C2, C3 and C5 around 75 ppm and C6 around 63 ppm. The 130-140 ppm band is probably C=C (possibly, =CH at 130, CH2= at 135 ppm).

#### *3.4.5.2 Molecular Weight Determination (MALLS analysis)*

The weight average molecular weight (Mw) and polydispersity (Mw/Mn) of the pullulan standard and extracted EPS were determined by HP-SEC-MALLS analysis. It should be noted that dn / dc values for control samples have previously been determined at University of Huddersfield. Solutions of 1mg/ml were prepared in deionised water. Upon complete dissolution samples were filtered through 0.2µm PTFE Puradisc syringe filters (Whatman UK Ltd). Filtered samples (200µm) were injected (using a 7125i injection port, Rheodyne LLC, California, USA) onto an analytic size exclusion column (Polymer Labs Aquacel-OH-Mixed-H 8µm particle size, 300 x 7.5mm Polymer Laboratories, UK). Ultrapure water delivered by

HPLC pump (Prominence LC-20 AD, Shimadzu, Milton Keynes, UK) at 1mL/min, neutral analytes were eluted and passed through by series of detectors. The samples first pass through a UV detector (Prominence SPD-20A, Shimadzu) with a wavelength set to 260nm; this identifies the presence of any residual DNA present in the sample. The concentration of sample was then detected by a refractive index (RI) detector (Optilab Rex, Wyatt technology, Santa Barbara, USA) and finally the weight-average molecular weight is measured using multi-angle laser light scattering (MALLS) photometer with the laser set to 690nm (Dawn OES, Wyatt Technology, Santa Barbara, USA). The chromatographic conditions are outlined below:

- Pump -Prominence LC-20AD;
- Flow rate -1mL/min;
- Mobile Phase -Ultrapure water;
- Injection Volume-200µL;
- Column-Plaquagel-OH-Mixed-H 8µm, 300x 7.5mm;
- Detector Calibration Constant-2.0x$10^{-5}$ ($V^{-1}$);
- Detectors -UV, RI and MALLS;
- Run Time-45mins.

#### *3.4.5.3 FTIR spectra of EPS*

Fourier transform infrared spectroscopic (FTIR) method has been used for EPS characterisation (Nicolet 380 FTIR). For this purpose 1mg of the sample was used at room temperature. Trasmission spectra was recorded using at least 32 scans with 4$cm^{-1}$ rsolution in the spectral range 4000-5004$cm^{-1}$. (FTIR) spectroscopy is a rapid nondestructive method that has been to many biological systems (Schmitt and Flemming, 1998, Gómez-Zavaglia and Fausto, 2003). The techique is based on the principle that atoms in the molecules are not held rigidly apart and when subjected to infrared radiation (between 500 and 4000 $cm^{-1}$), the molecule will absorb energy and the bond will be subject to number of vibrations. Hence the absorbtion spectrum contains information regarding the molecular structure of the sample. All the absorption spectra of the extracted samples were recorded between 4000 and 500$cm^{-1}$ with a FTIR spectrometer (Magna-IR 750, Nicolet Instrument, USA). The region between 4000 and 500$cm^{-1}$ holds the characteristic bands and is sutiable for the characterisation of microorganisms (Dittrich and Sibler, 2005, Lin et al., 2005). The wide and intense carbohydrate or EPS bands are found at wave number 950-1200$cm^{-1}$ which can be attributed

to –COC- group vibrations in the cyclic structure (Gómez-Zavaglia and Fausto, 2003, Dittrich and Sibler, 2005, Schmitt and Flemming, 1998).

#### *3.4.5.4 Monomer analysis*

Monomer analysis was carried out using High Performance Anion Exchange Chromatography with a Pulsed Amperometric Detector (HPAEC-PAD, Dionex now Thermo Fisher Scientific Inc). Isolated EPS (3 mg) was suspended in 2 mL of 4M trifluoroacetic acid (TFA) (Sigma Aldrich, UK) in a pressure tube and heated at 120$^{o}$C for 2 hours. After 2 hours the samples were cooled to room temperature and evaporated to dryness under a constant stream of nitrogen at (60$^{o}$C). The dried sample was reconstituted with ultra-pure water 1-1.5ml and was used directly for HPAEC-PAD.

Reconstituted, hydrolysed EPS samples were the injected (AS50 Autosampler, Dionex Co.) into the HPAEC. Sodium hydroxide (8mM, Isocratic) delivered by a gradient pump (GS50 Gradient pump, Dionex Co.) at 0.5ml/min was used to elute the monosaccharide's through the PAD detector (ED50 Electrochemical detector, Dionex Co.). The monosaccharide standards used to obtain the linear calibration data were prepared to the specific concentrations in deionised water and were run using the chromatographic conditions listed below:

- Pump -GS50 Gradient pump;
- Flow rate-0.5mL/min;
- Mobile Phase - 8mM Sodium Hydroxide;
- Injection Volume - 200µL;
- Column-CarboPac PA203 x 150mm;
- Detector – PAD;
- Run Time-20mins.

## 3.5 Experimental procedures

### 3.5.1 Determination of the Minimum Bactericidal Concentration (MBC) of alcohols and containing hand gels

Initially the MBC of ethanol and IPA was determined in 96 well plates, each well containing 200µl µl of TSB, 50µl of a 2x10$^{3}$ cfu/ml *A. baumannii* culture and 50µl of alcohol solution to generate a concentration gradient 80-40% v/v. Following a 5min contact time the wells were sub-cultured under standard culturing conditions. The MBC being identified as the lowest concentration from which no viable cells could be cultured. Further investigations utilised

lower concentration of alcohols and also included had gels. In this case 10ml of MRD containing 0.1-5% v/v of ethanol, ethanol based hand rub (Salvo gel), IPA, or IPA based hand rub (Purell) and 50µl of $2x10^3$ cfu/ml *A. baumannii* were incubated under standard conditions (n = 4). After 24 hours incubation the number of bacteria present were enumerated.

### 3.5.2 Alternative media for the EPS production

In the search for carbohydrate free media for the growth and production of EPS from *A. baumannii* mineral media (Table 3.2) was supplemented with (0.01- 0.5% w/v) Lactalbumin (Oxoid Ltd), casein (Oxoid Ltd) or casein + lactalbumin. These milk derived complex nitrogen sources were chosen because the EPS isolation and characterisation methodologies employed in this work have already been applied to bacteria grown in milk based media (Laws et al., 2008). Media was inoculated with 50µl of $2x10^3$ cfu/ml. *A. baumannii* and incubated under standard conditions After 24 hours incubation the number of bacteria present were enumerated (n = 4).

### 3.5.3 Assessment of ethanol and IPA as sole carbon sources

The impact of ethanol and IPA on the growth of *A. baumannii* was investigated using mineral media supplemented with 0.001, 0.01, 0.03 and 0.05% (v/v) ethanol or IPA. Controls were also run with 1% w/v glucose. Media was inoculated with 50µl of $2x10^3$ cfu/ml *A. baumannii* and incubated under standard conditions. After 24 hours incubation the number of bacteria present were enumerated. To confirm that alcohol was being metabolised, the batch growth experiments described above were repeated with either 1% (v/v) ethanol or IPA under standard conditions. After 24 hours incubation the concentration of alcohol remaining was determined (n = 4).

### 3.5.4 Determination of MICs using the Bioscreen automated growth instrumentation

The Bioscreen technology (Oy Growth Curves Ab Ltd) is a computer-controlled incubator/reader/ shaker able to monitor bacterial growth via increases in optical density (OD) in real time. It employs a 100 well micro-plate format, with two plates being utilised per run, allowing up to "200 tests being run simultaneously". In this investigation the Bioscreen instrument was employed to determine the MIC or alcohols and other biocides.

To determine the MIC of a particular biocide a dilution series was created down the plate using the biocide prepared in mineral media, a typical concentration series would be 10% - 0.625% (v/v). Once prepared 50µl of a $10^3$cfu/ml suspension of the desired bacteria were

added in all of the wells and shaken at 37°C for 24 hours with reading taken at 1 hour intervals. In order to determine the minimum biocidal concentration at the end of the Bioscreen run 100µl was transferred from each well to another Bioscreen plate containing DE neutralising broth (LabM Ltd). The DE plate was then incubated under standard conditions for 24 hours after which each well was plated out onto TSA to determine the presence of absence of live organisms.

In order to generate MIC values from the Bioscreen output the approach described by Lambert and Pearson (2000) was employed. Firstly the area under the OD time curve (Figure 3.3.) is determined using the trapezoidal rule performed in Microsoft Excel. Comparison of the test curve with that of the positive and negative control allows the calculation of a fraction area (fa) (Equation 3.1). Once a range of fractional areas have been calculated they can be plotted against the log of the biocide concentration (Figure 3.4) to allow analysis via a modification of the Gompertz function (Equation 3.2). This function was fitted to the data using the solver function in Excel; this allowed the MIC to be calculated from the constants M and B (Equation 3.3).

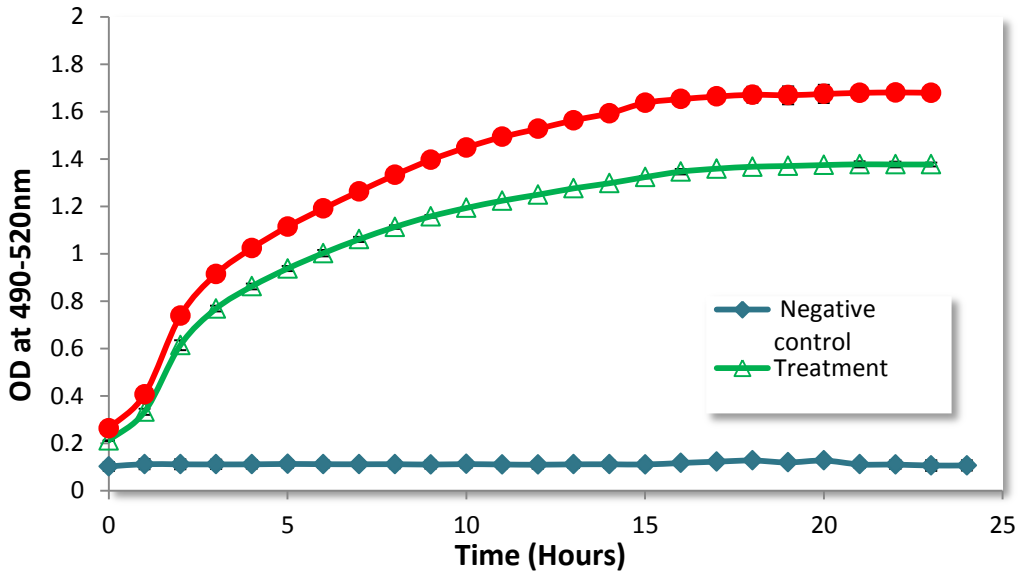



**Figure 3.3. A typical Bioscreen OD vs. time curve**

$$fa = \left(\frac{Area_T - Area_{NC}}{Area_{PC} - Area_{NC}}\right) \qquad \text{Eqn 3.1}$$

Where: $Area_T$ = Area under the test curve, $Area_{NC}$ = Area under the negative control and $Area_{PC}$= Area under the positive control.

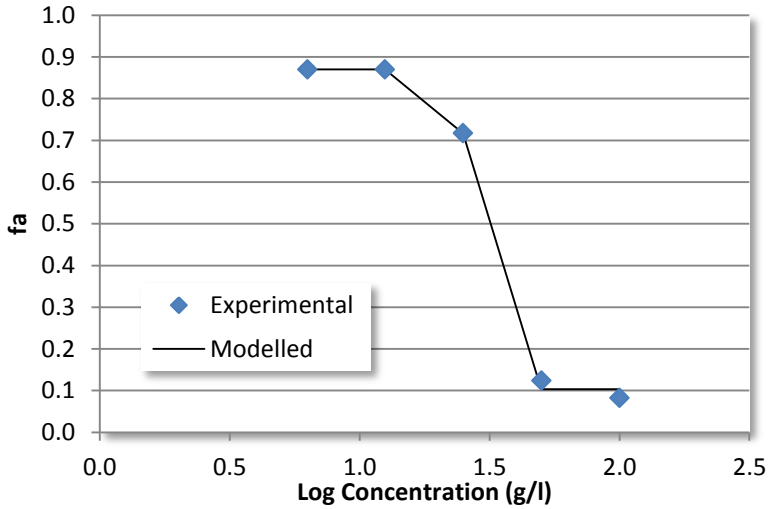



**Figure 3.4. A typical *fa* vs. Log concentration curve**

$$y = A + Ce^{-e^{B(x-M)}} \qquad \text{Eqn 3.2}$$

Where: $A$ = Lower asymptote of y, $C$ is Distance between upper and lower asymptote (Approx. 1), $B$ = Slope parameter $X$ = Biocides concentration and $M$ = Log Concentration of the inflexlon point.

$$MIC = 10^{(M+\frac{1}{B})} \qquad \text{Eqn 3.3}$$

Where: $B$ = slope function, $M$ = is the log concentration at the inflexion point.

### 3.5.5 Hydrophobicity of planktonic cells

Evaluation of hydrophobicity of planktonic cells was carried out using the microbial adhesion to n-hexadecane (MATH) test (Mattos-Guaraldi et al., 1999). In short, overnight cultures grown at 37°C in mineral media were centrifuged in a bench micro centrifuge to generate a pellet. The pellet was dispersed in MRD to an OD at 550nm of 0.8. Five millilitre of this bacterial suspension was overlaid with 100ml of Tween 80 (5mg/ml, Sigma Aldrich Ltd). After 1-2 min of agitation by vortexing, the phases were allowed to separate for 15minutes at room temperature. The absorbance of the aqueous phase was then measured at 550nm. The hydrophobicity test was performed directly on planktonic cells or after three washing in MRD in order to remove EPS (Campanac et al., 2002). Results were expressed as the percentage of cells excluded from the aqueous phase determined by the equation (Equation 3.4):

$$\%\ Hydrophobicity = \frac{(A_0 - A)}{A_0} - 100 \qquad Eqn\ 3.4$$

Where $A_0$ and A are, respectively, the initial and final ODs of the aqueous phase.

Strains were considered as: strongly hydrophobic when the values obtained were >50%, moderate hydrophobic for values ranging between 20% and 50% and hydrophilic when values were <20%.

### 3.5.6 ATP dependent test for hydrophobicity confirmation

An alternative approach to the MATH test was developed employing ATP content by using the ATP dependent biomass detection kit (3M-clean trace, 3M health care, Germany). The test was performed as described for the MATH test except that the ATP content of the aqueous phase was used instead of the absorbance at 550 nm.

### 3.5.7 Quantitative estimation of Biofilm

The method of O'Toole (O'Toole et al., 2000), was employed for the quantitative estimation of biofilms, this method uses crystal violet to stain biofilms attached to a surface, with the extent of the biofilm being proportional to the amount of crystal violet retained. A single colony of *A. baumannii* was removed from a stock plate and grown on in 20ml of TSB under standard conditions to a level of $10^7$ cfu/ml. 50µl of this overnight culture was placed in the wells of a 96 well plate along with 50µl of mineral media and incubated under standard conditions. Following incubation the wells were washed four times with sterile distilled water and allowed to air dry for 5-10 minutes. Following drying 100µl of crystal violet was added in each well and the plate was left at room temperature for 30min. The plate was again washed four times with sterile distilled water and air dried for 5-10min. following drying 200µl of ethanol was added to each well to extract the retained crystal violet, 125µl of this ethanol was then transferred to a clean 96 well plate. The amount of crystal violet extracted was then determined by absorbance at 540nm on a plate reader (Multiskan EX, Thermo Labsystems). All the strains were classified into the following categories: non-adherent (0), weakly adherent (+), moderate adherence (++) and strongly adherent (+++) based on the absorbance (Ab) of the bacterial film using the approach outlined by Stepanovi et al (Stepanovi et al., 2000). The classification process is outlined below:

| | |
|---|---|
| $Ab_{test} \leq Ab_{control}$ | Non-adherent |
| $Ab_{control} \leq Ab_{test} \leq 2 \times Ab_{control}$ | weakly adherent |
| $2 \times Ab_{control} \leq Ab_{test} \leq 4 \times Ab_{control}$ | Moderately adherent |
| $4 \times Ab_{control} \leq Ab_{test}$ | Strongly adherent |

### 3.5.8 Drip flow reactor (DFR) for the continuous growth of biofilm

A drip-flow biofilm reactor (DFR) (BioSurface Technologies Corporation) was used to grow *A. baumannii* biofilms under low shear conditions close to the air-liquid interface using the approach described by Stewart et al (Stewart et al., 2001, Werner et al., 2004).This system was used to grow biofilms on glass slides using minimal media enriched with both ethanol and glucose. These biofilms were established by operating the reactors in batch mode for 6h at room temperature, these were allowed to mature for an additional 48h with a continuous flow of minimal medium over inclined glass coupons set at a 10° angle as described by Werner et al, 2004 (Werner et al., 2004).

After 48 hours the glass coupons were removed, drained to remove any planktonic cells and fixed with 2.5% v/v formalin and stained with ethidium bromide (500mg/ml) (Sigma Aldrich, UK) for 15min. Slides then washed with d.$H_2O$ and stained with fluorescent brighter 28 or calcoflour white (0.1% w/v) (Sigma Aldrich, UK) and observed under a fluorescent microscope. Fluorescent brighter 28 or calcoflour white stain (Sigma Aldrich, UK), reacts with the carbohydrates in EPS to give a blue colour whilst the bacterial cells are stained red by the ethidium bromide.

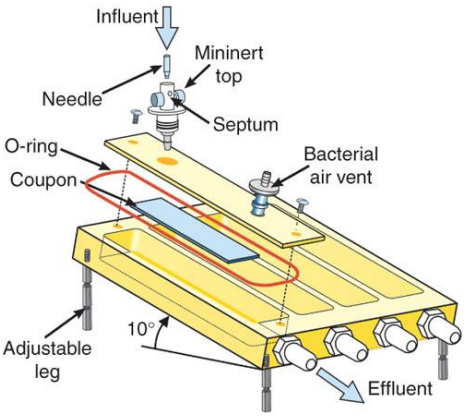



**Figure 3.5. Drip flow biofilm reactor (Goeres et al., 2009)**

### 3.5.9 MBEC Biofilm cultivation

The MBEC systems (InnovotechInc, Canada) as originally described by (Ceri et al., 1999b) was used to test biofilms against biocides. The MBEC system consists of a polystyrene lid, with 96 downward-protruding pegs, that exactly fit into the wells of a 96-well plate (Figure 3.6). In order to grow *A. baumannii* biofilms on the MBEC system an inoculum was prepared by suspending bacteria from overnight TSB broth cultures in 0.9% w/v NaCl to match a 1McFarland standard. The standard inoculum was then diluted 3 folds in growth media to

achieve an initial viable count of approximately $1x10^7$cfu/ml, 150µl of this inoculum was then transferred into each well of a 96 well plate. The inoculated plate was then incubated overnight at 37°C on a shaker at 5 rpm. Following this initial incubation, biofilms were rinsed with 0.9% w/v NaCl to remove the loosely attached planktonic cells. Biofilm formation was evaluated by breaking 2-3 pegs from the MBEC system and recovering the associated biofilm into 200µl of 0.9% w/v NaCl via 5 minutes of ultra-sonication. The number of recovered bacteria was determined by plating out under standard conditions.

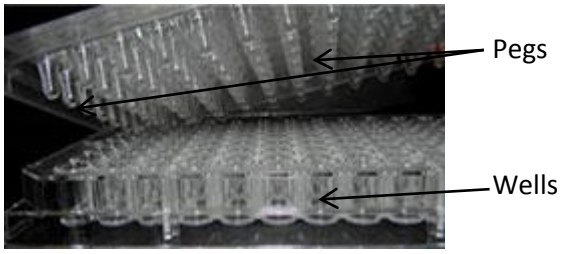



**Figure 3.6. The MBEC biofilm growth system (Innovotech Inc, Canada).**

The method of (Harrison et al., 2008) (Figure 3.7), was followed to determine the $MBC_{b,}$ using *A. baumannii* biofilms prepared as described above. Serial dilutions of the biocides of interest were prepared in 200µl mineral media using 96-well microtiter plate. After rinsing the biofilm plate was placed into the biocide wells and incubated overnight at 37°C on a shaker with speed of 5 rpm. After incubation the biofilm plate was transferred into a 96-well recovery plate containing DE neutraliser broth (LabM Ltd) for 20 minutes. Biofilms were then recovered by sonication into MRD and then serially diluted and plated out on TSA and incubated under standard conditions.

*3.5.9.1 Biofilm formation in the presence of biocides*

Serial dilutions of the biocides of interest were prepared in 200 µl mineral media using 96-well micro titer plate, 50µl of a $10^3$ cfu/ml *A. baumannii* suspension was added to each well and a sterile MBEC plate attached, the completed plate was the incubated overnight at 37°C on a shaker with speed of 5 rpm. After incubation the MBEC plate was rinsed and then transferred to a 96-well recovery plate containing DE neutraliser broth (LabM Ltd) for 20 minutes neutralisation. After neutralisation the whole MBEC plate was sonicated for 5mins. Minimum bactericidal concentrations (MBCb) for the biofilms were then determined by reading the optical density at 650nm of the recovery plates, using Thermomax micotitre plate reader with Softmax pro data analysis software or via ATP determination.

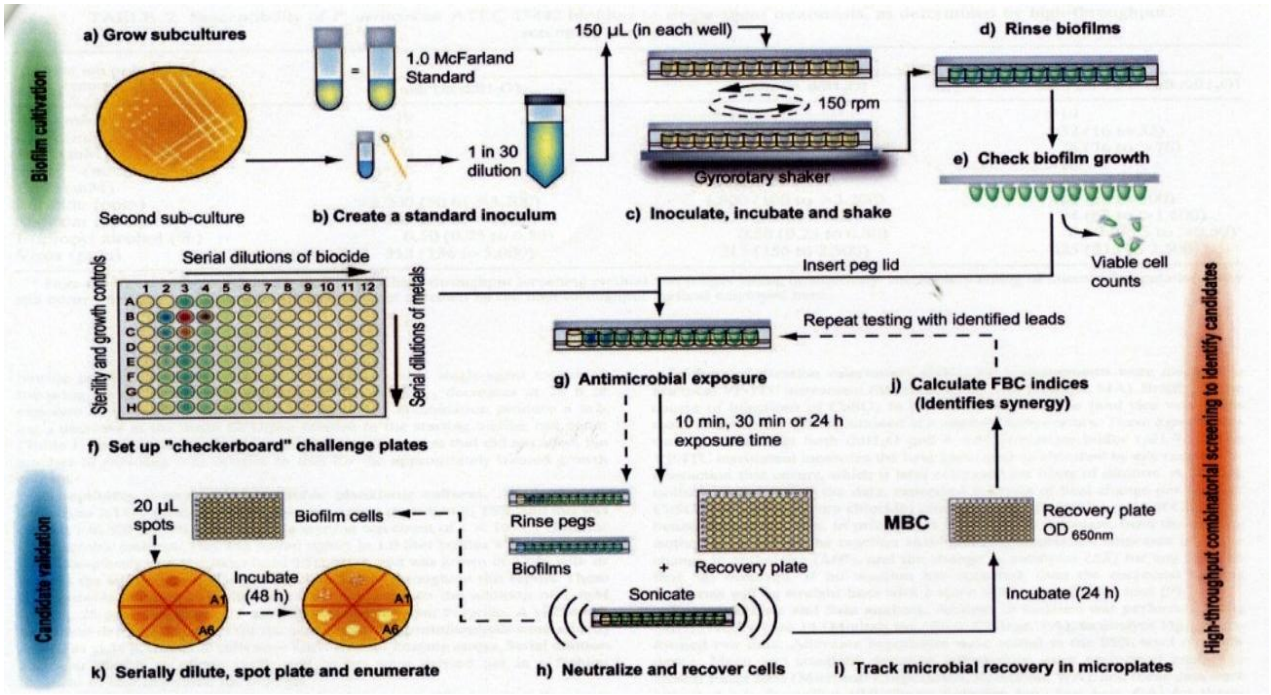



**Figure. 3.7. Determination of minimum bactericidal concentrations for biofilms (Harrison *et al*, 2008).** High-throughput screening may be used to identify synergistic antimicrobial interactions that kill microbial biofilms. Starting from cryogenic stocks, the desired bacterial strain was streaked out twice on TSA (a), and colonies from the second subcultures were suspended ingrowth medium to match a 1.0 McFarland optical standard (b). This standardized suspension, diluted 30-fold in TSB, served as the inoculum for the CBDs. The inoculated devices were assembled and incubated on a gyrorotary shaker (c), which facilitated the formation of 96 statistically equivalent biofilms on the peg surfaces (data not shown). Biofilms were rinsed with 0.9% NaCl (d), and surface-adherent growth was verified by viable cell counting (e). Antimicrobials were set up in "checkerboard" arrangements in microtiter plates (f), and the rinsed biofilms were inserted into these challenge plates for the desired exposure time (g). Following antimicrobial exposure, biofilms were rinsed and inserted into recovery plates. Biofilm cells were disrupted into the recovery medium by sonication (h), and the recovery plates were incubated for 24 h before the OD650 values of recovered cultures were read in a microtiter plate reader (i). This allowed the FBC index to be calculated, and this was used to identify "candidate" synergistic interactions (j). Candidates were validated by repeating the testing process (as outlined in steps a-h), but instead of qualitative measurements, biofilm cell survival was quantified by viable cell counting on agar plates (k).

### 3.5.10 . Evaluation of antimicrobial wound dressings

#### *3.5.10.1 Bacterial strains*

All strains of *S. aureus and P. aeruginosa* identified in Table 3.2, along with six strains of *A. baumannii* (Type, UK-HS, Clone I and Clone II, 882 and 292) were used to evaluate the effectiveness of the antimicrobial wound dressings.

#### *3.5.10.2 Culture conditions*

Stock suspensions were prepared by taking cultures from TSA stock plates and dispersing these in sterile conical flask containing 10 ml of MRD and 5 g of glass beads by gentle shaking on an orbital shaker. The OD of the suspension was adjusted to be equivalent to a 0.5 McFarland standard at 600 nm in a spectrophotometer. This OD approximated to a 1-1.5x$10^8$cfu/ml suspension. A working suspension of 1-1.5x$10^5$cfu/ml was then prepared in a simulated wound fluid (SWF) composed of a 50:50 suspension of MRD and foetal calf serum (Sigma-Aldrich Ltd).

#### *3.5.10.3 Antimicrobial dressings*

Four commercially available dressings were selected for evaluation:

- A Manuka honey based dressing (Actilite, Advancis medical, UK);
- A PHMB based dressing (Suprasorb, Lohmann and Rauscher, Germany);
- A nanocrystalline silver coated dressing (NSCD) (Acticoat, Smith & Nephew Medical ltd, UK);
- Anionic silver coated dressing (ISCD) (Aquacel Ag, Convatec, UK).

#### *3.5.10.4 Antimicrobial activity against planktonic cells*

This approach is based on the international standard for the evaluation of antimicrobial plastics (BS ISO22196:2007). A 16$cm^2$ (4x4 cm) portion of dressing was placed on a sterile plastic sheet in the base of a sterile Petri dish. The dressing was then inoculated with 400 µl of test suspension before being covered with another layer of sterile plastic sheet. The inoculated dressings were then incubated for 24 hours at 37°C at 90 % humidity. After 24 hours, the number of surviving bacteria was determined by stomaching the inoculated dressings in 10 ml of a validated neutraliser and plating out the neutraliser on TSA under standard conditions. In addition, the dressing, base layer and top layer of plastic sheet were overlaid with TSA to determine the number of surviving bacteria remaining on these surfaces.

The fraction of surviving bacteria was then determined by means of comparison with controls as described in BS ISO22196:2007.

*3.5.11.5 Antimicrobial activity against immobilised cells*

An *in vitro* collagen wound model (CWM) mimicking conditions in chronic wounds and soft tissue infections (Brackman et al., 2011) were employed. Matrices of polymerized rat-tail collagen type I (BD Biosciences UK) were prepared in 15-ml tubes following the manufacturer's protocol for eukaryotic cell culturing. For 10 ml of collagen matrix solution (3.8mg/ml), 1ml of phosphate buffer saline (PBS) was mixed with 0.019 ml of NaOH (1M) and 0.7 ml of bacterial suspension (1-1.5x$10^5$ cfu/ml) in SWF and kept on ice until required. Finally 8.27 ml of collagen from cold collagen stock (4mg/ml) was added and, after mixing, 1ml aliquots of collagen matrix were transferred to the wells of a 24 well polystyrene plate (Fisher Scientific, UK). To polymerise the collagen, the microtiter plate was placed in an incubator at 37 °C for 1 hour.

To test the antimicrobial activity of the test dressings, dressing were cut aseptically to fit the well diameter and placed on top of polymerized collagen gel. The plates were then incubated for 24 hours at 37°C at 90 % humidity. Following incubation the dressings were removed and the number of surviving bacteria was determined by dissolving the collagen matrices by adding 1ml of collagenase solution (1mg/ml in PBS from *Clostridium histolyticum* (Sigma Aldrich, UK) to the collagen gel followed by incubation at 37°C for 30-60min. The suspension was then mixed thoroughly and re-incubated for 60-80min, until complete digestion. The solution from each well was then transferred to 10 ml of a validated neutraliser and the fraction of surviving bacteria determined by plating out the neutraliser on TSA under standard conditions. In addition, the dressings were overlaid with TSA to determine the number of surviving bacteria remaining on these surfaces. The fraction of surviving bacteria was then determined by means of comparison with controls as described in BS ISO22196:2007.

## 3.6 Data processing and statistical analysis

All general data processing was carried out employing Microsoft Excel. Statistical analysis was carried out via SPSS V.20 (IBM Corporation, USA). Due to the large number of comparisons, which increases the risk of Type I error, and the difference in variance, the Games-Howell *post hoc* test was used to identify significant differences between variables (Field, 2013).

# 4 Results and discussions

## 4.1 The impact of alcohol on the growth, survival and EPS generation of *A. baumannii*

A range of alcohols e.g. ethanol and isopropanol (IPA) are employed in healthcare settings as antiseptics, disinfectants and components of biocidal blends. The presence of these compounds in healthcare settings potentially represents a carbon source for the growth of *Acinetobacter sp* if present at sub-lethal concentrations. There is a considerable body of data indicating that *Acinetobacter sp* have a range of metabolic responses to alcohols (See section 2.2.6). Consequently the growth response of *Acinetobacter sp* to alcohols provides an insight into the survival of these pathogens in healthcare environments. Initial investigations focussed on the Type strain (ATCC 19606) and the UK Hospital Strain (UK-HS) using a range of media and alcohol concentrations.

### 4.1.1 Effect of alcohols and alcohol containing hand gels on bacterial growth

Initial investigations focussed on the impact of alcohols and alcohol containing hand gels on the growth of Type and UK-HS strains. Concentration profiles of ethanol and IPA from 80-40% were prepared in 96 well plates containing TSB and a bacterial inoculum of 10µl of a $2x10^3$ cfu/ml culture. Following a 5 minute contact time the wells were sub-cultured under standard conditions for 24 hours, the minimum biocidal concentration (MBC) being identified as the lowest concentration from which no viable cells could be cultured (Table 4.1). In the case of the Type strain the MBC was 50% for both ethanol and IPA, however in the case of the UK-HS the MBC for IPA was higher at 55%.

| **Bacterial Strain** | **Ethanol** | **IPA** |
|---|---|---|
| | **Concentration (v/v)** | |
| Type | 50% | 50% |
| UK-HS | 50% | 55% |

**Table . 4.2. MBC for Ethanol and IPA.**

Further investigation of the ability of *A. baumannii* strains to grow and survive in the presence of alcohols were carried out in Maximum recovery diluent (MRD), since this provided a lower organic load than the Trypton soya agar (TSB) employed previously. The impact of alcohol, alcohol based hand rub (Salvo gel), isopropanol alcohol (IPA) and isopropanol alcohol based

hand rub (Purell gel) on both the Type strain and UK Hospital Strain (UK-HS) was investigated by growing them in MRD supplemented with the alcohols and gels at 0.1%-5% v/v under standard conditions. The amount of hand gel employed was adjusted to provide the required alcohol concentration. Both strains exhibited a decrease in growth as the concentration of both ethanol and IPA increased (Figure 4.1). This impact on growth was most pronounced in the case of the Type strain with inhibitory impacts becoming obvious at 1% v/v, in the case of UK-HS growth was not obviously impacted until the concentration reached 3% v/v (Table 4.2). Both strains failed to grow when the concentration of either ethanol or IPA was increased above 5%. The impact of the alcohol based hand gels was less consistent (Figure 4.2), particularly the case with UK-HS where the impact of Purell gel was significantly lower than that seen with the equivalent concentration of ethanol. These differences are likely to be due to the additional components present in alcohol gels e.g. glycerol.

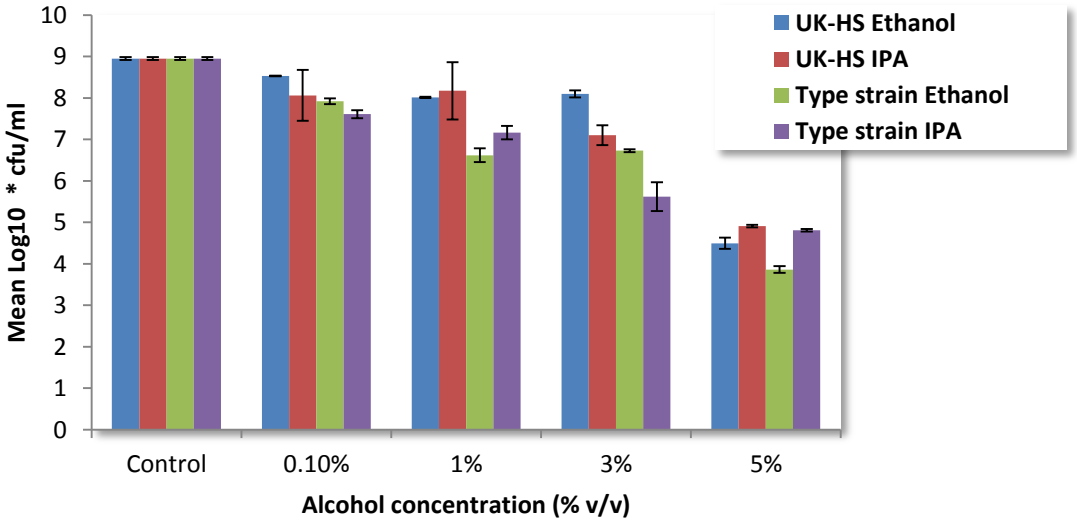



**Figure: 4.1. Effect of alcohols on *Acinetobacter* growth.**

*Cfu/ml= Colony forming units/ml, Standard deviation, n=4.

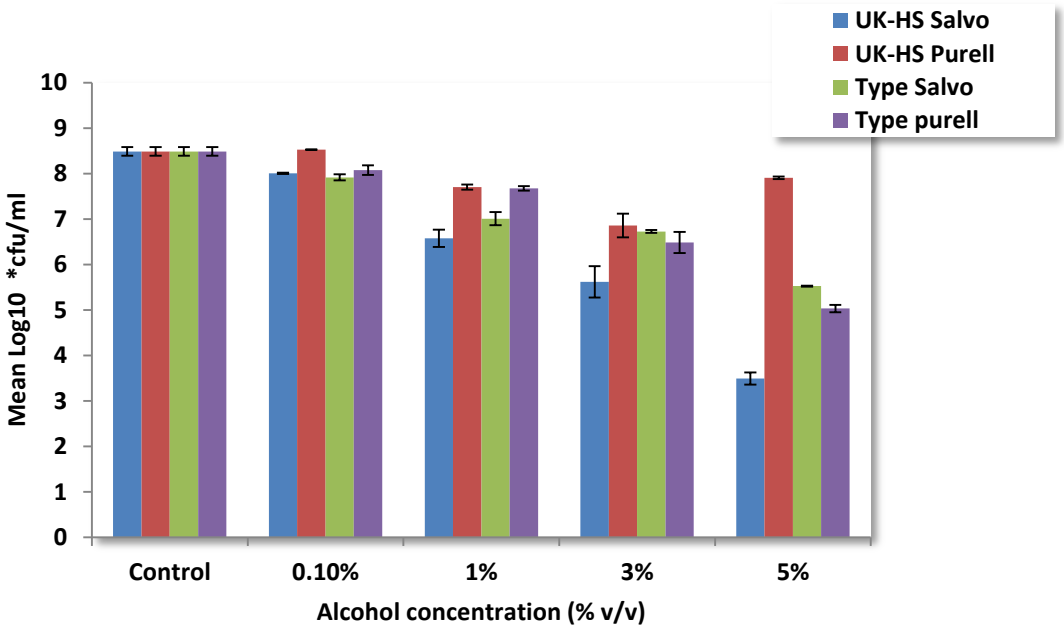



**Figure: 4.2. Effect of different alcohol gels on *Acinetobacter* growth.** *Cfu/ml= Colony forming units/ml, Standard deviation, n=4.

| Treatment | Significance at 95% CL | |
|---|---|---|
| | UK-HS | Type |
| **Ethanol** | Cont>0.1%>1%=3%>5% | Cont>0.1%>1%=3%>5% |
| **IPA** | Cont=0.1%=1%=3%>5% | Cont>0.1%>1%>3%=5% |
| **Salvo** | Cont>0.1%>1%>3%>5% | Cont>0.1%>1%=3%>5% |
| **Purell** | Cont>0.1%>1%>3%<5% | Cont>0.1%>1%>3%>5% |

**Table 4.2. Significance differences in alcohol growth data.**

### 4.1.2 Alternative media for *Acinetobacter sp* growth

The growth of *Acinetobacter sp* in TSB and MRD allowed preliminary investigations of the impact of alcohols on their growth. However in order to study the response of these bacteria to biocides and their generation of EPS a defined media which supported the growth of *Acinetobacter sp* without interfering with biocidal impacts or the analysis of EPS was required. Initially a basic mineral media with 1% glucose (See section.3.1.2.1) was chosen. In order to provide nitrogen source casein, lactalbumin or casein + lactalbumin were evaluated. These

milk derived complex nitrogen sources were chosen because the EPS isolation and characterisation methodologies employed in this work have already been applied to bacteria grown in milk based media (Laws et al., 2008). These initial tests were carried out on the Type strain and UK-HS. When casein and/or lactalbumin were employed at concentrations ranging from 0.01% to 0.5% w/w all strains were able to grow to between log 7.5 to 8.25cfu/ml in overnight cultures which equates to between a 4.1 to 5.2 cfu/ml increase over and above the mineral media control (Figure 4.3). This indicates that although, a complex nitrogen source is required for growth, 0.01 % w/w of either lactalbumin or casein was sufficient to stimulate extensive growth in the presence of a suitable carbon source.

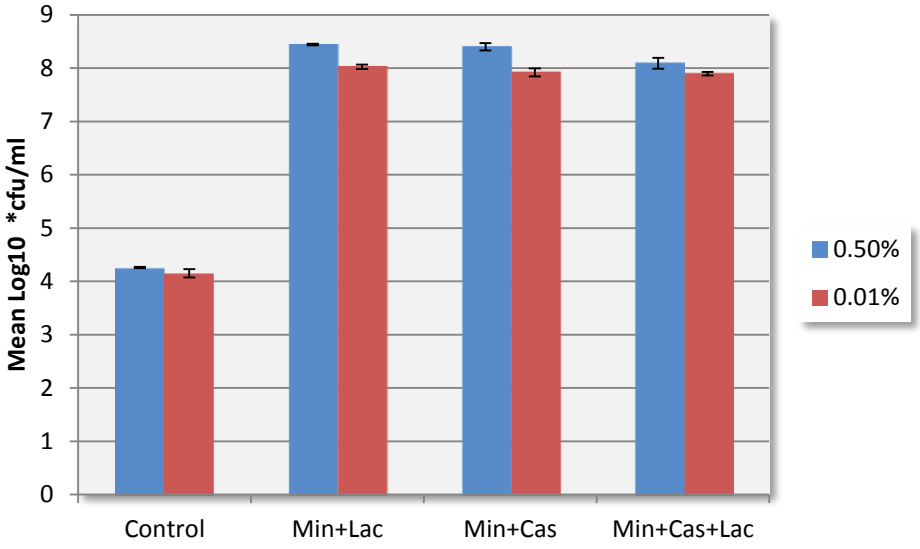



**Figure: 4.3. Impact of complex nitrogen sources on bacterial growth.** *Cfu/ml= Colony forming units/ml, Standard deviation, n=4, Control = Mineral media, Cas = Casein, Lac = lactalbumin.

### 4.1.3 Impact of ethanol and IPA as sole carbon sources

The impact of ethanol and IPA on the growth of the Type strain and UK-HS was investigated using mineral media supplemented with 0.001, 0.01, 0.03 and 0.05% (v/v) ethanol or IPA. Controls were also run with 1% w/w glucose. Under these conditions both bacteria were able to grow using both alcohols as a sole carbon source. However, as the alcohol concentrations increased the growth of the Type strain decreased significantly indicating inhibition (Figure 4.4, Table 4.3), whilst the UK-HS was able to maintain growth across the range of alcohol concentrations tested, with some significant changes evident across the alcohol concentrations (Figure 4.5, Table 4.3).

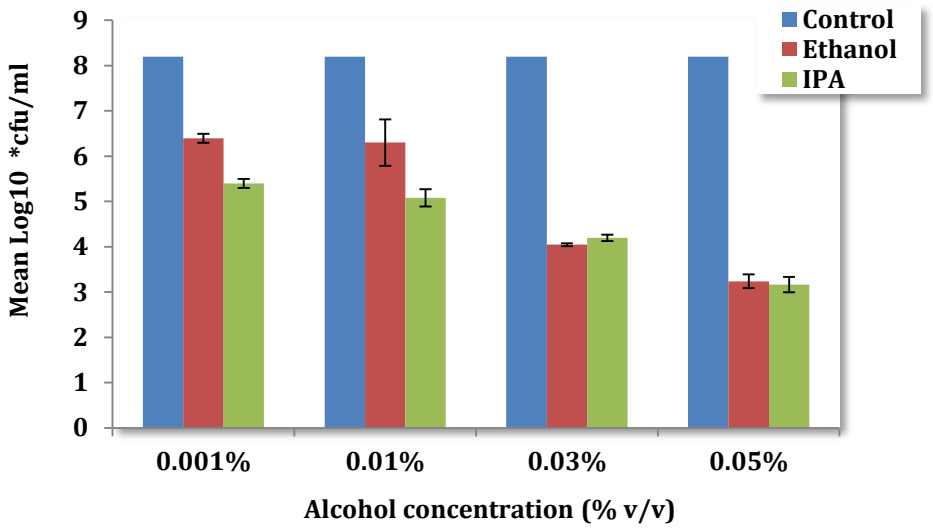



**Figure: 4.4. Effect of carbon source on the Type strain.** *Cfu/ml= Colony forming units/ml, Standard deviation, n=4.

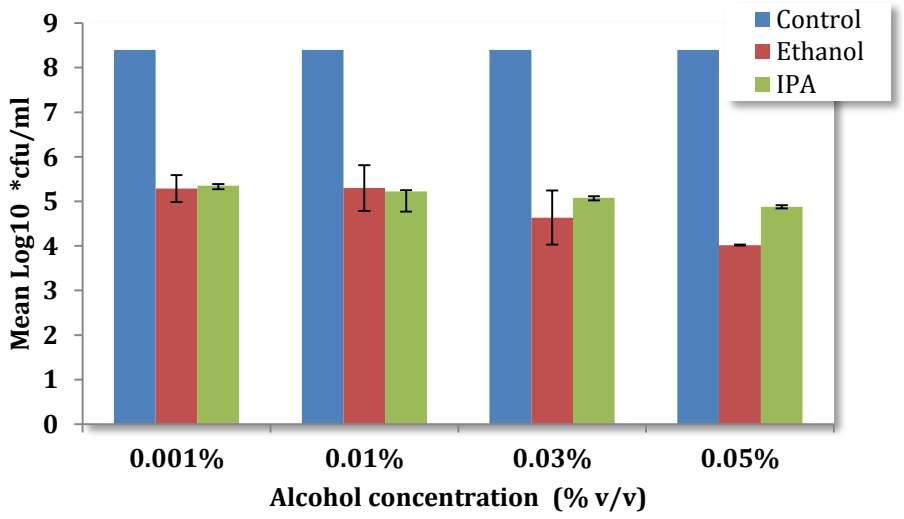



**Figure: 4.5. Effect of carbon source on UK-HS.** *Cfu/ml= Colony forming units/ml, Standard deviation, n=4,

| Treatment | Significance at 95% CL | |
|---|---|---|
| | Type | UK-HS |
| **Ethanol** | Cont>0.001%=0.01%>0.03%>0.05% | Cont>0.001%=0.01%=0.03%=0.05% |
| **IPA** | Cont>0.001%=0.01%>0.03%>0.05% | Cont>0.001%=0.01%=0.03%>0.05% |

**Table 4.3 Significance differences in growth at low alcohol concentrations.**

To establish a broader understanding of the consumption of alcohol across all *Acinetobacter* strains available and to confirm that alcohol was being metabolised, batch growth

experiments with mineral media supplemented with either 1% v/v ethanol or IPA were run and monitored from alcohol removal (Figure 4.6). These experiments confirmed the inability of the Type strain to utilise alcohols at these concentrations. All other strains were able to remove over 90% of both ethanol and IPA in 24 hours with the exception of the UK-HS which only consumed approximately 40% of either alcohol during this period.

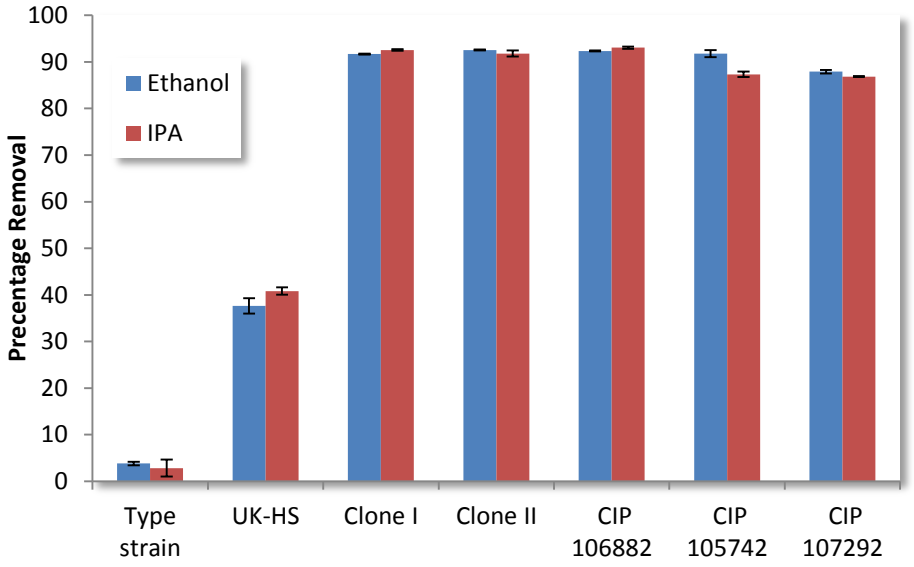



**Figure: 4.6. Percentage removal of 1% v/v ethanol and IPA during batch growth (Bioassay Kit).** Standard deviation, n=4,

### 4.1.4 Determination of the MIC of alcohols

In order to provide a more sophisticated understanding of the impact of alcohols on the growth of *A. baumannii* the Bioscreen technology was employed to determine the relevant MICs (See section 3.5.4). All seven *A. baumannii* strains were employed using mineral media supplemented with methanol, ethanol, IPA or glucose at a range of concentrations (0.63-10% v/v). The concentration range was selected to sit between the MBC (Table 4.1) and the concentrations able to support independent growth (Figures 4.4 to 4.6). Methanol was included to determine if the number of carbons in an alcohol had an impact on its biocidal efficiency and glucose was included as a positive control. MIC values were calculated from Bioscreen outputs using the approach described by (Lambert, 2001). Typical Bioscreen outputs can be found in Figure 4.7 along with the associated fractional area relationships (Figure 4.8), the remaining outputs can be found in the appendix. In all but one case (Clone 1) methanol generated the highest MIC of the three alcohols investigated. The response to the two alcohols in general use within Healthcare environments varied across the range of strains

tested, with Clone 2 being most sensitive to both alcohols and 882 being the least sensitive (Table 4.4).

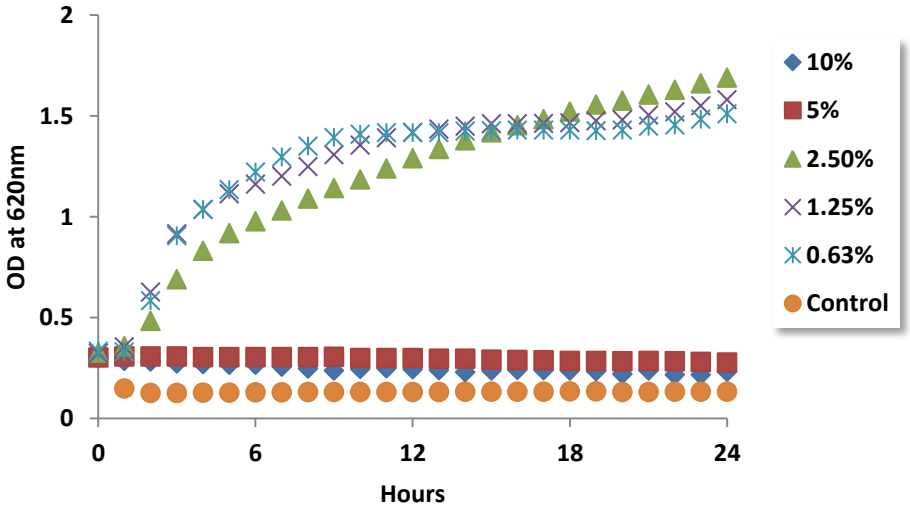



**Figure.4.7. Bioscreen output for UK-HS grown on ethanol.**

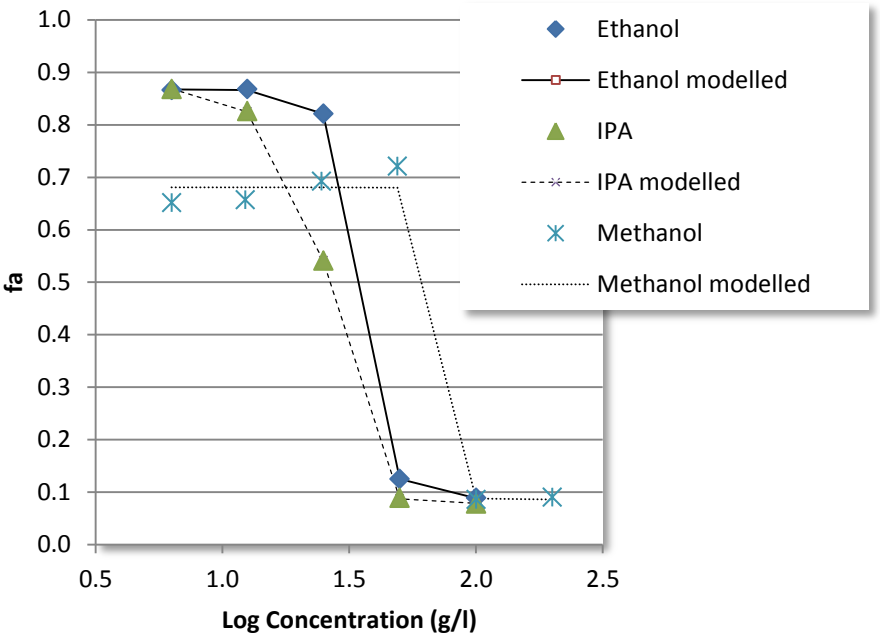



**Figure 4.8. Fractional area and concentration plot for UK-HS.**

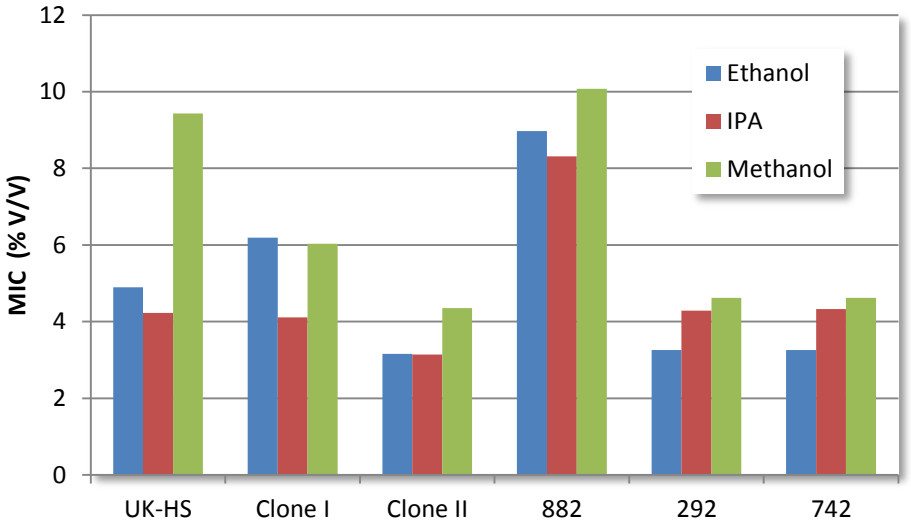



**Figure 4.9. MIC data for all *Acinetobacter* strains.** Standard deviation, n=3.

| Ethanol | | | | IPA | | | |
|---|---|---|---|---|---|---|---|
| Min | | Max | | Min | | Max | |
| Strain | Conc (% v/v) | Strain | Conc (% v/v) | Strain | Conc (% v/v) | Strain | Conc (% v/v) |
| Clone 2 | 3.2 | 882 | 9.0 | Clone 2 | 3.1 | 882 | 8.3 |

**Table 4.4. Minimum and maximum MIC data for Ethanol and IPA.** Standard deviation, n=3.

### 4.1.5 **Discussion**

The MBC's determined here are below the in use concentrations recommended for alcohols (60-70%) (Fraise et al., 2012) but are at the concentration below which antimicrobial activity is known to decrease (McDonnell and Russell, 1999). The impact of ethanol in the presence of other carbon sources has been investigated by a number of authors (Pirog et al., 2002, Smith et al., 2004), a situation that mirrors the experiments reported above employing TSB and MRD. Pirog *et al* (Pirog et al., 2002) reported on *Acinetobacter* strains that were unable to grow solely on alcohols without supplementation with pantothenic acid (Vitamin B3), a situation not reflected in the growth of *Acinetobacter* strains investigated here where most of those investigated were able to grow on either ethanol or IPA as the sole carbon source. However, the Type strain grew poorly when provided with significant amounts of alcohol as a sole carbon source (Figure 4.7) suggesting in this case supplementation may be necessary. The growth of *Acinetobacter* strains on minimal media with ethanol as a sole carbon source without supplementation is well established (Navon-Venezia et al., 1995, Walzer et al., 2006) a situation reflected in the growth of the clinical strains investigated here.

Enhanced pathogenicity due to the presence of alcohol has been reported for *Acinetobacter* spp (Smith et al., 2004) again in the presence of other carbon sources. The positive impact of ethanol was reduced as the concentration was raised to 5% and became negative above 5% (Smith et al., 2004), a trend that is reflected in the data collected here (Figures 4.1 and 4.2). Enhanced growth of *Acinetobacter* strains at alcohol concentrations at or around 1% has been reported by a number of authors (Smith et al., 2004, Edwards et al., 2007a). Edwards *et al* (Edwards et al., 2007b), reported the enhancement of growth and virulence in the presence of ethanol and found the significant increase in growth when the minimal media is supplemented with ≤1% of four commercially available hand rubs i.e Purell, Spirogel, Softalind and Skinman. They reported that the unknown factor which enhanced pathogenicity and virulence to *A. baumannii* was secretion of proteins in response to alcohols. One of these proteins was identified as OmpA, which was recognized as having emulsifying activity, which could be useful in scavenging carbon for growth from complex energy sources such as hydrocarbons. The secretion of OmpA by *A. baumannii* following exposure to 0.5% v/v ethanol in minimal media has also been described by Walzer *et al* (Walzer et al., 2006), suggesting that it may be the important response to growth and survival under low nutrient conditions. These bioemulsifiers protein may also be helpful in bacterial adhesion, quorum sensing and the development of biofilms (Ron and Rosenberg, 2001).

The MIC determined for ethanol and IPA for the clinical strains investigated are towards the upper end of published values for alcohol MICs on bacteria in general that range from 1 to 5% (Oh and Marshall, 1993, Mazzola et al., 2009, Wadhwani et al., 2008). One published value for *A. calcoaceticus*of4.4% (Mazzola *et al*., 2009) is broadly similar to the values generated during this study with the exception of 882 which had an MIC of approximately double this value (Table 4.4). Interestingly these authors indicated that the presence of glycerine, a common component of alcohol hand gels increased the MIC by ≈50%, an observation that aligns with the reduced impact of Purell gel observed here.

### 4.1.6 **Key Findings**

- A carbohydrate free minimal media employing alcohols as sole carbon sources has been developed.
- The MBC of ethanol and IPA for all strains ranged from 50 to 55% v/v.
- The MIC of ethanol and IPA for all clinical strains ranged from 3.1 to 9.0% v/v.
- The Type strain was significantly more sensitive to alcohols and less able to use ethanol or IPA as their sole carbon source.
- Strain 882 was the most resistant strain when exposed to ethanol and IPA.

## 4.2 A Biofilm formation

### 4.2.1 Quantitative determination of biofilm formation

The ability of all available strains of *A. baumannii* to form biofilms was assessed via the crystal violet/ethanol method (See section. 3.5.7). All strains were grown in mineral media supplemented with either 1% v/v of glucose, ethanol or IPA. For the purpose of comparative analysis of test results, the classification of (Stepanovi et al., 2000) was followed. The crystal violet/ethanol method was supplemented by direct enumeration of adhered bacteria. Of the strains investigated the Type strain proved to be none adherent, followed by the UK-HS which was uniform weakly adherent (Table 4.5). All other strains were strongly adherent when grown on ethanol and IPA but Clone 1, 2 and strain 742 had reduced adherence when grown on glucose (Table 4.5). Adherence and biomass content were positively correlated (Figure 4.10) with highly adherent strains having two orders of magnitude greater biomass than weakly adherent strains.

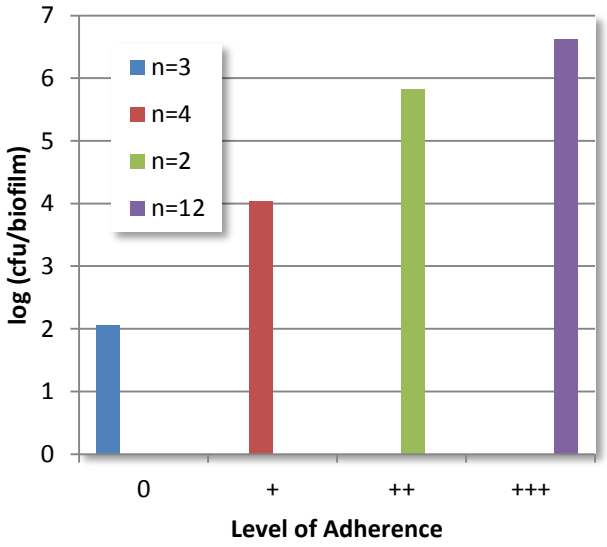



**Figure 4.10. Biofilm formation and associated levels of biomass of seven strains of *A. baumannii* on different carbon sources i.e. 1% v/v Ethanol, IPA and Glucose in mineral media.** *Cfu/ml = Colony forming units/ml, No adherence (0), Weak adherence (+), Moderate adherence (++), Strong adherence (+++).

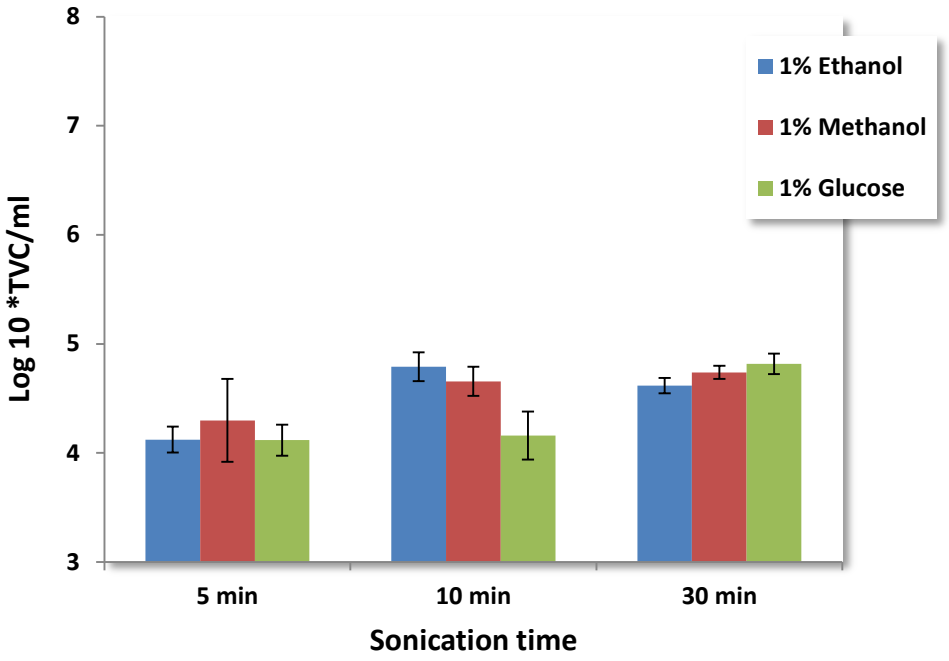
### 4.2.2 Formation of biofilms on MBEC surfaces

The ability of the MBEC plastic surfaces to support *Acinetobacter* biofilms and the ease with which cells can be recovered from these surfaces was assessed using UK-HS, Clone 1, Clone 2 and 882 strains grown in mineral media supplemented with either 1% v/v ethanol, methanol and glucose. The biofilms formed by these bacteria were removed from the MBEC pegs via sonication (5, 10 and 30minutes) (Figure 4. 11 a-d). All strains investigated generated

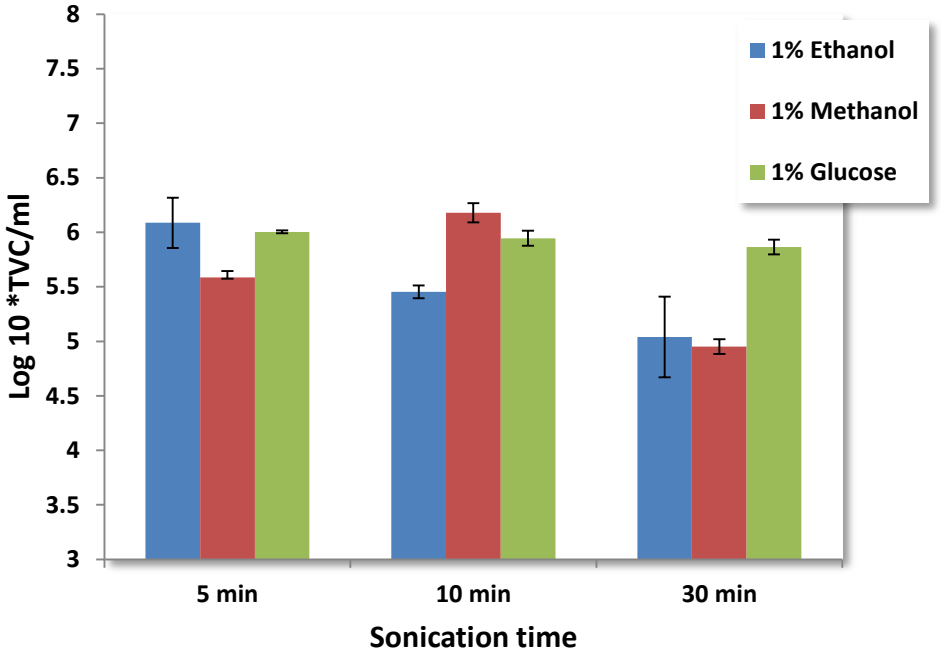
significant biofilms on pegs (>log 5 cfu/ml) irrespective of the carbon source. The only exception being the UK-HS which generated an order of magnitude lower recoverable bacteria. Across all the strains investigated 5 minutes sonication being sufficient to recover adhered cells.

| Bacterial strains | Carbon source | CV test | $Log_{10}$ (cfu/ml) |
|---|---|---|---|
| **Type** | **Ethanol** | 0 | 2.06 |
| **UK-HS** | | + | 4.10 |
| **882** | | +++ | 7.08 |
| **Clone 1** | | +++ | 6.75 |
| **Clone 2** | | +++ | 6.92 |
| **742** | | +++ | 6.50 |
| **292** | | +++ | 6.45 |
| **Type** | **IPA** | 0 | 2.03 |
| **UK-HS** | | + | 4.06 |
| **882** | | +++ | 6.06 |
| **Clone 1** | | +++ | 6.63 |
| **Clone 2** | | +++ | 6.65 |
| **742** | | +++ | 6.83 |
| **292** | | +++ | 6.05 |
| **Type** | **Glucose** | 0 | 2.08 |
| **UK-HS** | | + | 3.88 |
| **882** | | +++ | 6.88 |
| **Clone 1** | | ++ | 5.83 |
| **Clone 2** | | ++ | 5.83 |
| **742** | | + | 4.11 |
| **292** | | +++ | 6.66 |
| No adherence (0), Weak adherence (+), Moderate adherence (++), Strong adherence (+++). | | | |

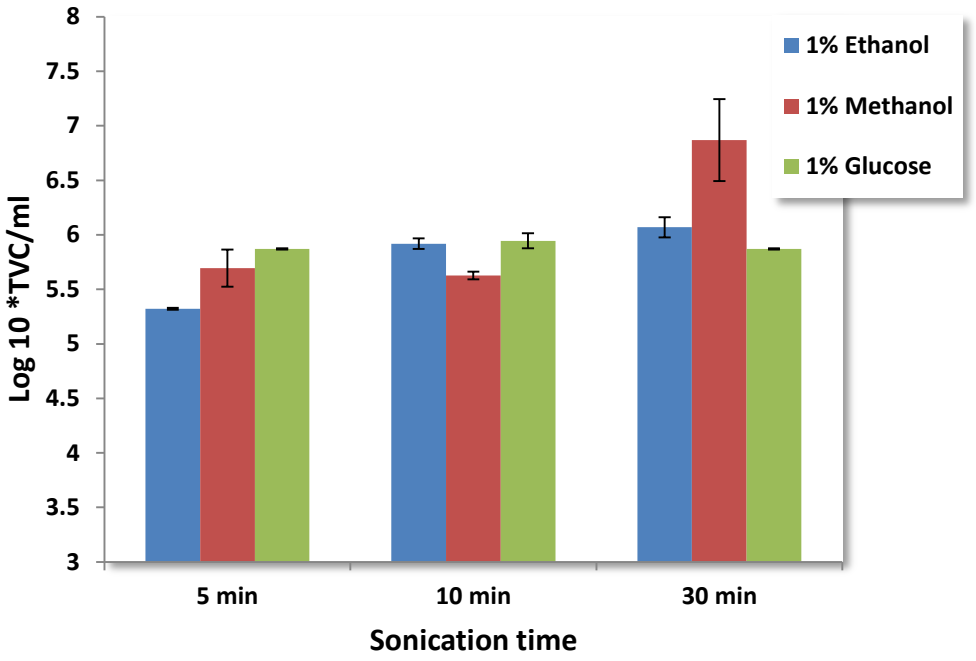
**Table.4.5. Effect of different carbon sources i.e. 1% v/v Ethanol, IPA and Glucose in mineral media on biofilm forming capacity of seven strains of *A. baumannii* using the crystal violet/ethanol method.** (See section. 3.5.7). *Cfu/ml = Colony forming units/ml, Standard deviation, n=3.



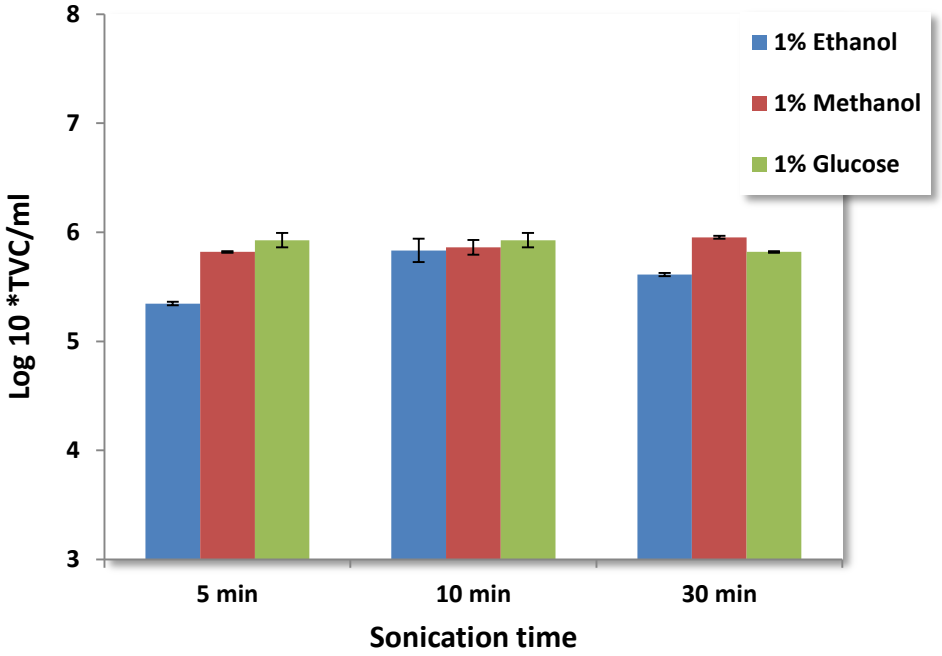

(a). UK-HS, ACCB 63608




(b). OXA-23 Clone 1




(c). OXA-23 Clone II



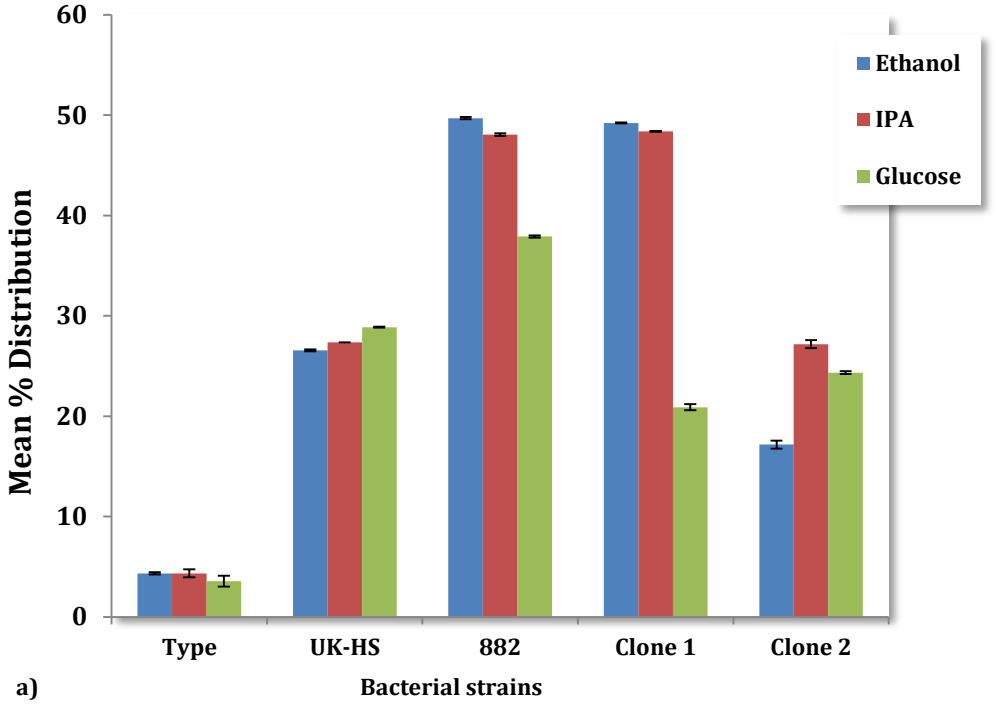

(d). CIP 106882

**Figure: 4.11. Biofilm growth on MBEC surfaces and effect of different sonication time or biofilm removal from MBEC surfaces of different *A. baumannii* strains.** *TVC = Total viable counts.

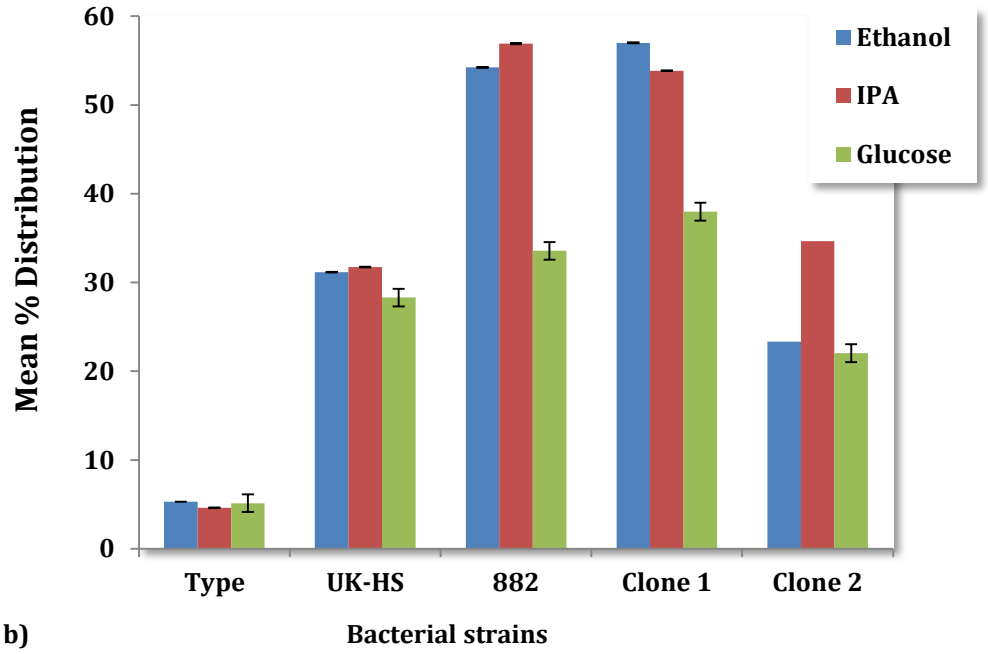
### 4.2.3 **Assessment of hydrophobicity**

Hydrophobicity has been associated with the ability of bacteria to adhere to surfaces and form biofilms. An evaluation of the hydrophobicity of *A. baumannii* was carried out using the microbial adhesion to n-hexadecane (*MATH*) test (Mattos-Guaraldi et al., 1999). All strains were cultured in mineral media with ethanol, IPA or glucose as carbon sources. Two different methods were employed to evaluate the *MATH* of all the strain; a qualitative method (spectroscopic method OD 550 nm) and quantitative methods (Total viable counts (TVC) and ATP determination). These tests demonstrated that all the strains were hydrophobic with the exception of the Type strain (Figure 4.11). Of the strains tested Clone 1 and 882 were strongly hydrophobic (> 50%), UK-HS and Clone 2 were moderately hydrophobic (>20%<50%) and Type stains being hydrophilic (<20%) (Figure 4.11). However, when grown on glucose all the strains became moderately hydrophobic (>20%<50%) while Type strain remained hydrophilic (<20%) (Figure. 4.12).

Hydrophobicity determinations were also performed, to determine the impact of media composition on culture hydrophobicity. Three different media were investigated i.e. mineral media with ethanol, trypton soya broth (TSB) and a simulated wound fluid (SWF) containing foetal calf serum (FCS). In this case, the TVC method was replaced with an ATP determination. Both of the tests proved that Clone 1 and 882 were strongly hydrophobic (>50%), UK-HS and Clone 2 were moderately hydrophobic (>20%<50%) and the Type strain was hydrophilic (<20%) (Figure. 4.13) when grown on mineral media with ethanol as a carbon source. However when grown on TSB and SWF Clone 1, 882,UK-HS and Clone 2 become moderately hydrophobic (>20%<50%) whilst the Type strain remained hydrophilic (<20%).

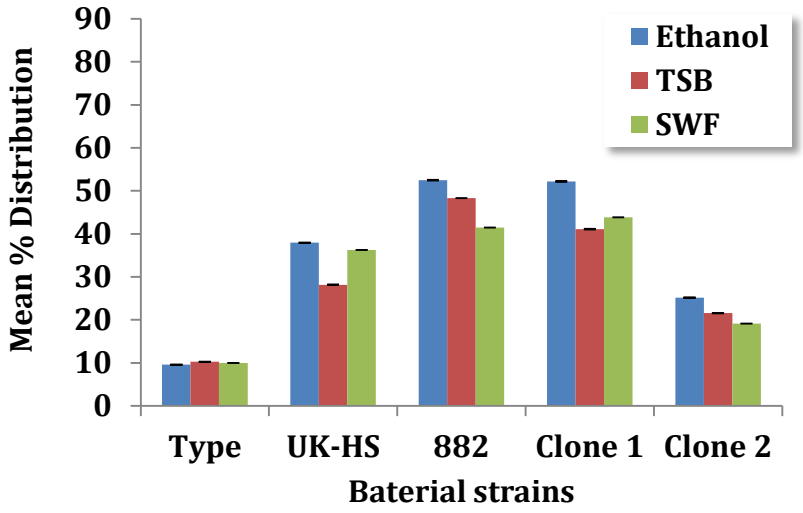



a)

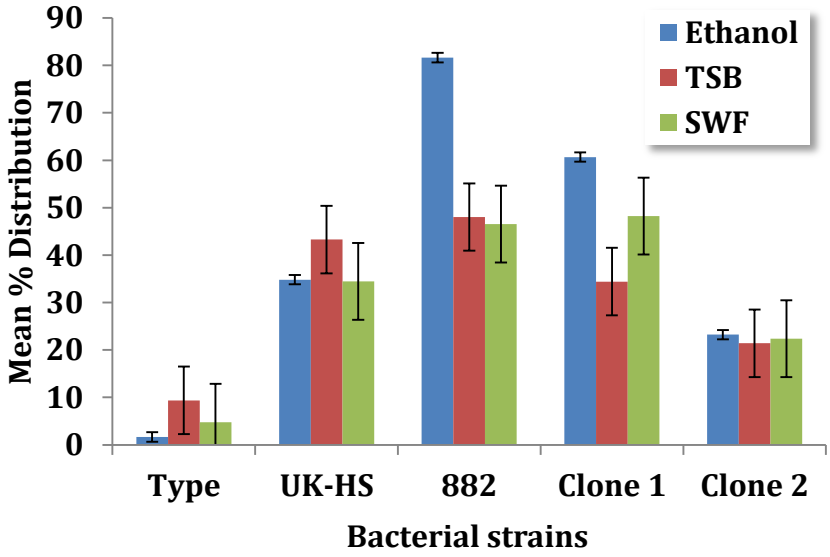



b)

**Figure. 4.12. Hydrophobicity determination of different *A. baumannii* strains on different carbon sources i.e. 1% v/v Ethanol, IPA and Glucose in mineral media using *Microbial adhesion to hydrocarbon* (**n-hexadecane) **test** (*MATH*). **(a). Spectroscopic method OD 550nm. (b).** Standard deviation, n=3.




a)




b)

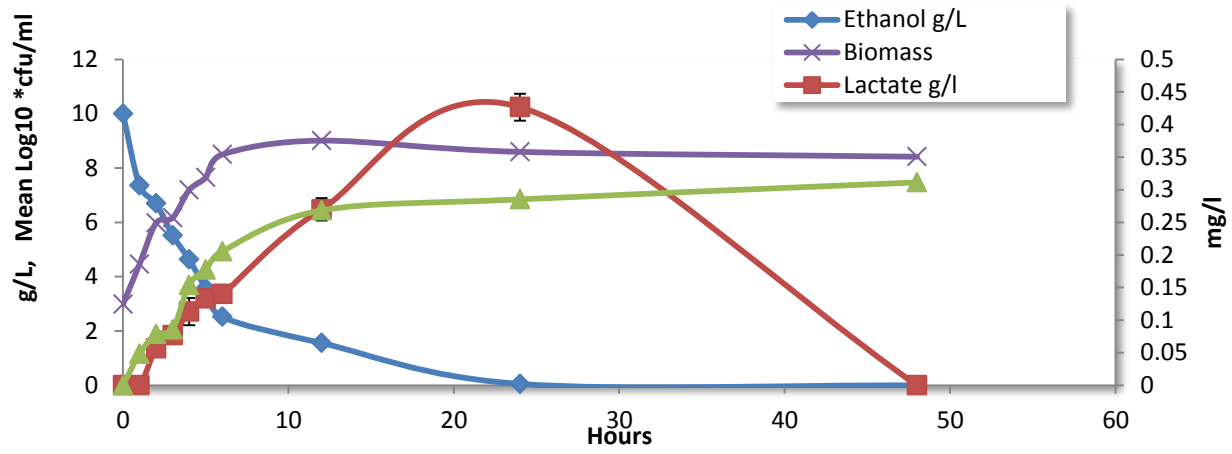
**Figure. 4.13. Comparisons of hydrophobicity of different *A. baumannii* strains on different media i.e. Mineral media with 1% v/v Ethanol, TSB and SWF using *Microbial adhesion to hydrocarbon* (**n-hexadecane) **test** (*MATH).* (a). Spectroscopic method OD 550nm. (b). ATP dependent test**.** Standard deviation, n=3.

### 4.2.4 **Total carbohydrate estimation**

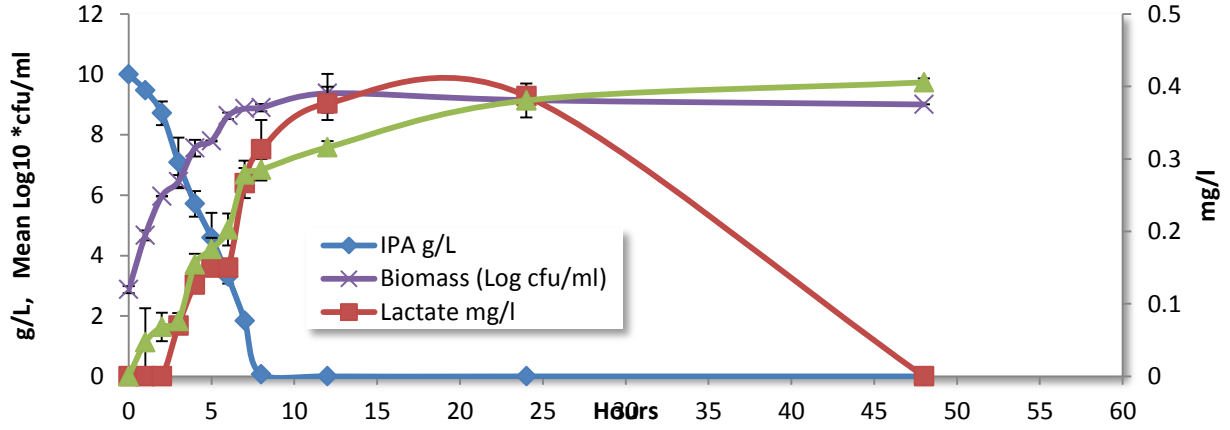
Batch growth experiments were performed to determine if biofilm forming strains were generating EPS with a total carbohydrate estimation being used as a measure of this generation. A range of carbon sources (glucose, ethanol and IPA) were used along with the two strong biofilm forming strains 882 and Clone 1 identified in Section 4.2. The procedure used for total carbohydrate contents was based on that described by (Dubois et al., 1956). Alongside EPS generation the consumption of the carbon sources, biomass production and lactate production were also monitored. When glucose was employed as the carbon source glucose concentration was deducted from the total carbohydrate measurements to determine the free EPS concentration. Both strains demonstrated a classic batch growth curve on all three carbon sources with a limited lag phase. The incubation period was not long enough for a death or decline phase to occur.

In all cases (882 and Clone 1) EPS generation was associated with the exponential phase of biomass growth and although EPS generation stopped once biomass generation had ceased, there was no evidence that this carbohydrate was utilised as a carbon source during the stationary phase (Figures 4.14 and 4.15). This contrasts with the primary carbon sources, which reduced to zero during the exponential growth phase or the lactate evolution which increased during exponential growth and then was metabolised back to zero during the stationary phase (Figures 4.14 and 4.15).

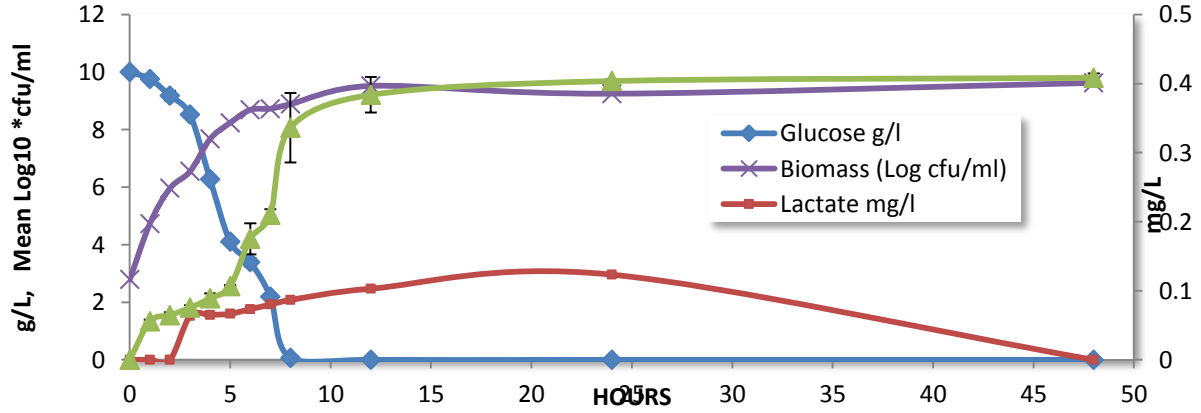
The amount of EPS produced across all carbon sources and strain was broadly similar (0.35 to 0.4 mg/l) as was the amount of biomass produced (Log 9 to 9.5 cfu/ml). On a weight-by-weight basis EPS generation accounted for ≈ 0.004% of the carbon source consumed regardless of the nature of the carbon source. The only major difference in the batch growth profiles was in the lactate profiles with 882 producing approximately four times as much as that produced by 882 on glucose or that produced by Clone 1 on any of the carbon sources (≈ 0.4-0.45 mg/l c.f. 0.1-0.15 mg/l). (Figures. 4.14 )




**(a). 882 ethanol**

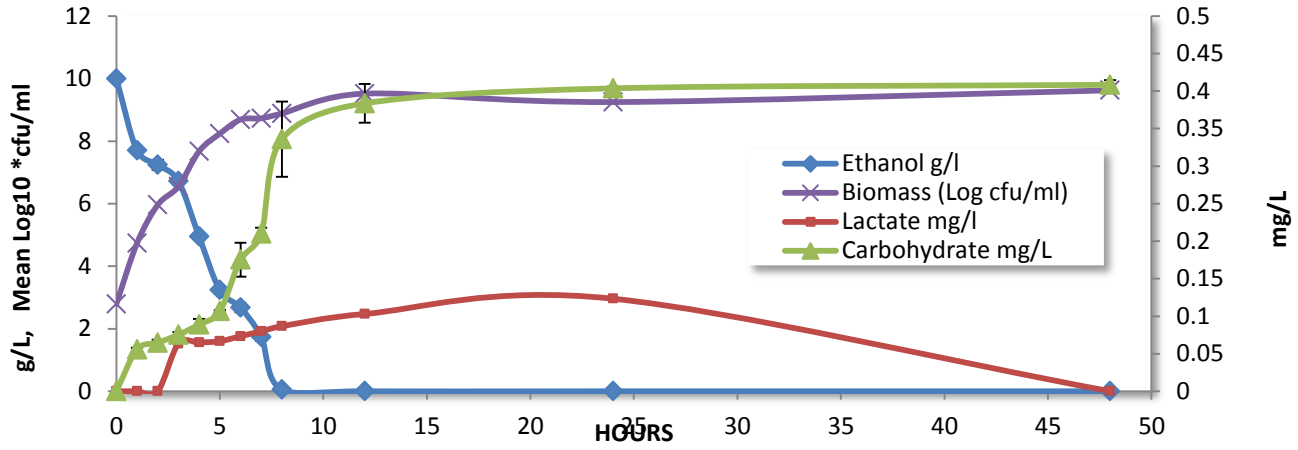



**(b). 882 IPA**

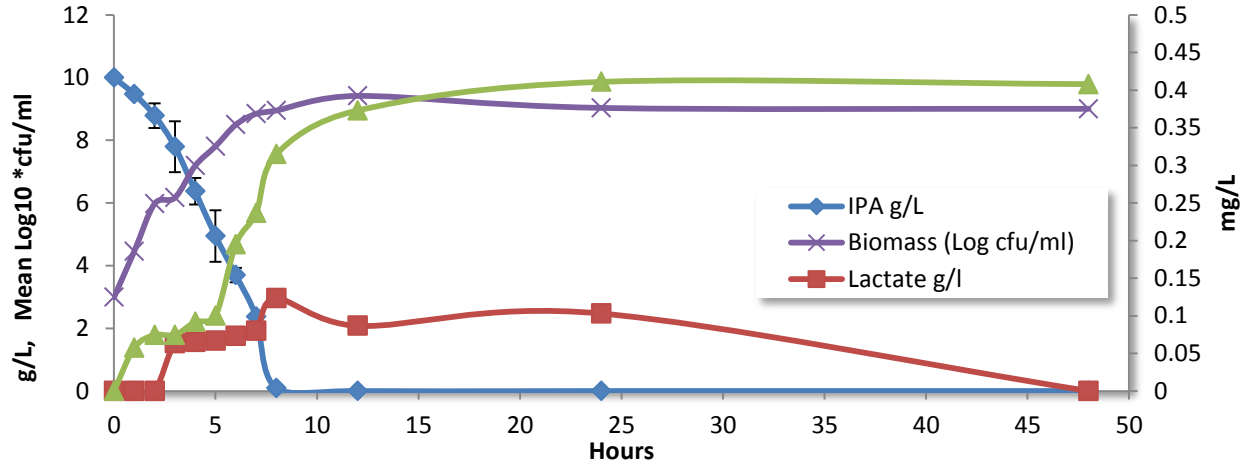



**(c). 882 Glucose**

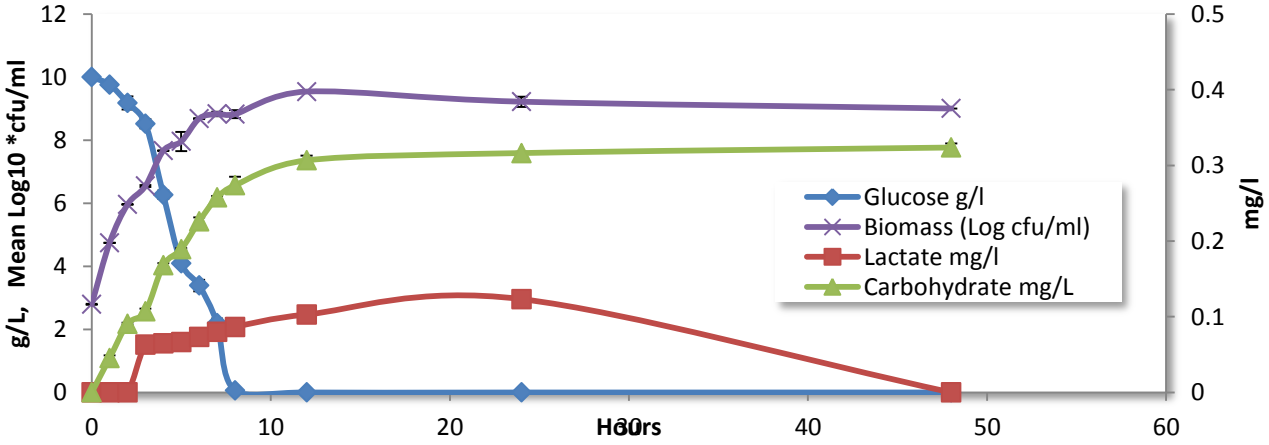
**Figure. 4.14. Total carbohydrate estimation, ethanol removal and biomass production by 882 on carbon source (a). 1% v/v Ethanol. (b). 1% v/v IPA. (c). 1% v/v Glucose.** ). *Cfu/ml = Colony forming units/ml, Standard deviation, n=3.




**(a). Clone 1 Ethanol.**




**(b). Clone 1 IPA.**

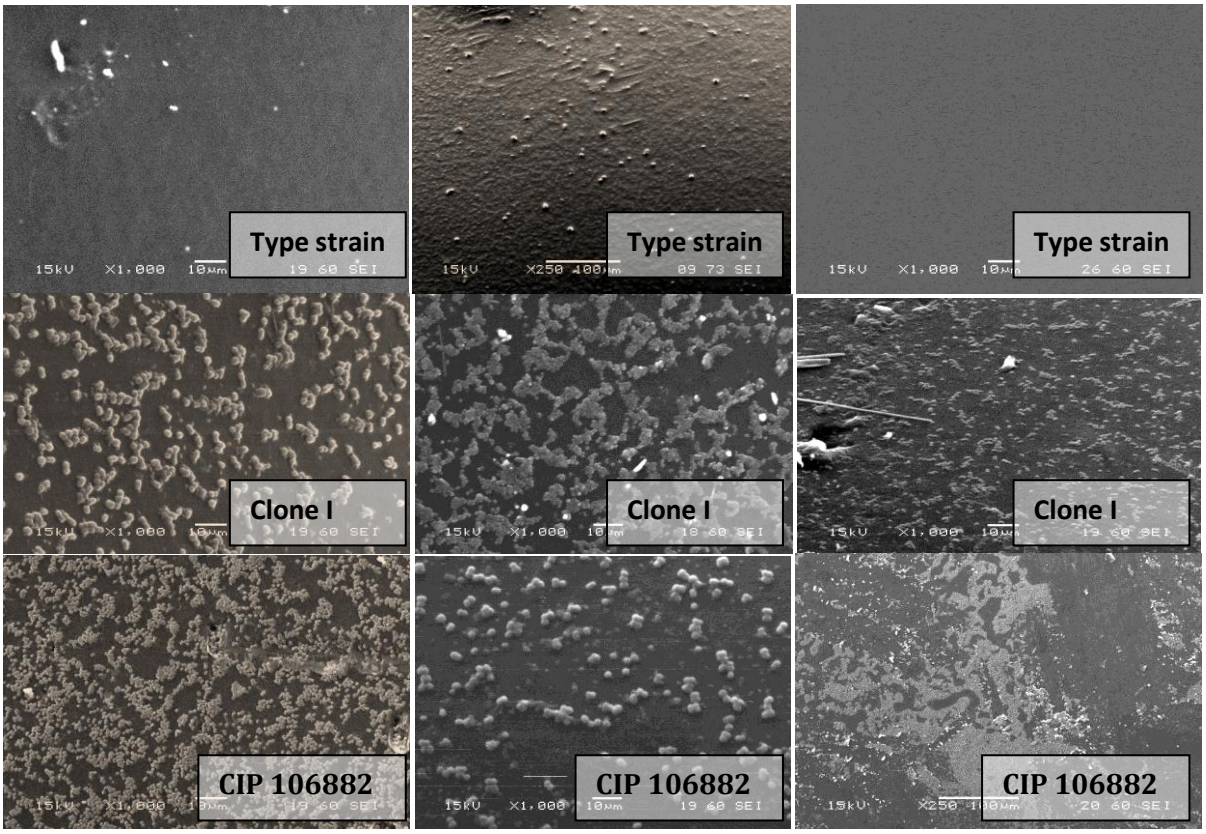



**(c). Clone 1 Glucose.**

**Figure.4.15. Total carbohydrate estimation, ethanol removal and biomass production by Clone 1 on carbon source (a). 1% v/v Ethanol. (b). 1% v/v IPA. (c). 1% v/v Glucose.** ). *Cfu/ml = Colony forming units/ml, Standard deviation, n=3.

### 4.2.5 **Scanning electron micrograph (SEM) of *A. baumannii* strains**

In order to directly visualise *A. baumannii* biofilms the high biofilm forming strains i.e.882 and Clone 1 were grown on plastic surfaces using mineral media enriched with either glucose, IPA or ethanol as a carbon source, with the Type strain being used as a control. SEM revealed that both 882 and Clone 1 formed biofilms on the plastic surfaces on all the carbon sources. However the Type strain did not form any obvious biofilm (Figure 4.16), an observation which supports the results of the crystal violet/ethanol method for biofilm formation.

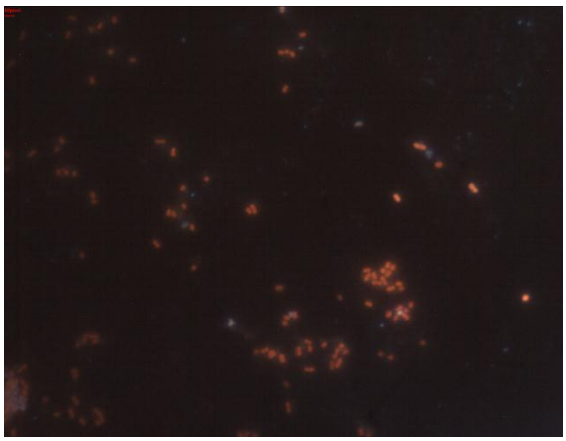



**ETHANOL** **IPA** **GLUCOSE**

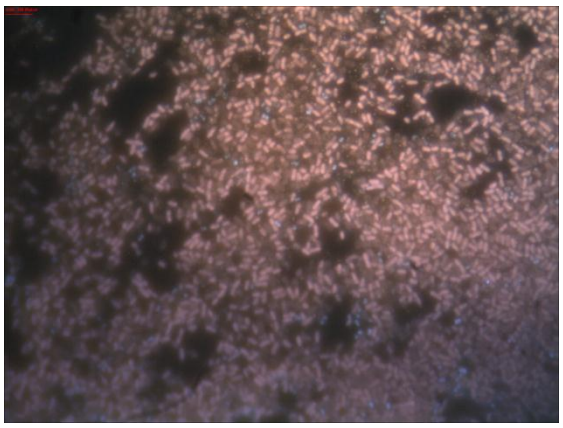
**Figure. 4.16: Scanning Electronic Micrograph of biofilm forming strains of *A. baumannii* grown in mineral media supplemented with 1 % v/v ethanol, IPA and glucose.**

### 4.2.6 **Drip flow reactor (DFR) and fluorescent microscopy**

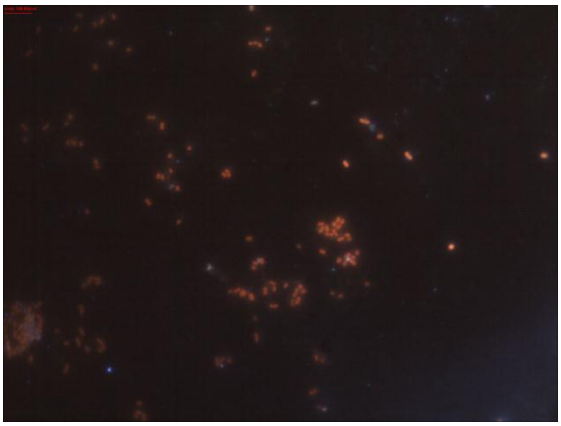
*A. baumannii* biofilms (Type strain, 882 and Clone 1) were grown on glass slides using the drip-flow reactor fed on minimal medium with ethanol, IPA or glucose as a carbon and energy source. Glass coupons were after either 24 or 48hours, drained and fixed for fluorescent microscopy. Consistent with the SEM observations both the strains were able to form biofilm on all the carbon sources. i.e. ethanol, IPA and glucose. In this case the generation of EPS could be directly visualised by the blue/white fluorescence of the calcofluor white carbohydrate stain. The formation of the biofilm and associated EPS is seen as a blue colour around the red bacterial cells. The biofilm starts forming after 24 hours; however biofilm was more obvious after 48 hours of continuous growth in the drip flow reactor. Type strain did not form any biofilm however; both the biofilm forming strains i.e. 882 and Clone 1developed stronger biofilms with more obvious EPS when the carbon source was ethanol and IPA as compared to the glucose (Figures 4.17 - 4.19). Biofilms with a more compact structure were seen with 882 than Clone 1 on all the carbon sources (Figure 4.17 - 4.19).



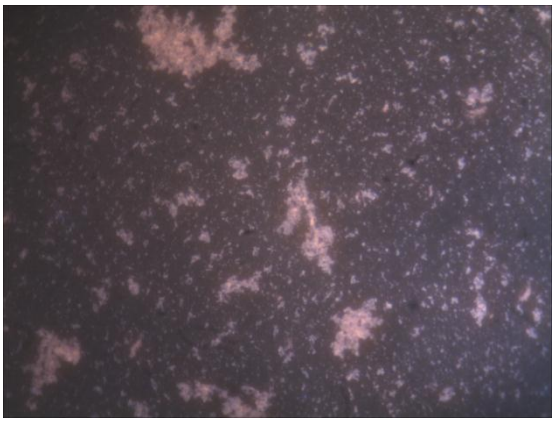
(A)



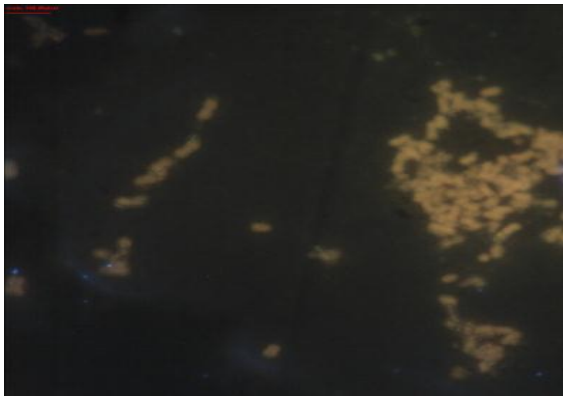
(B)



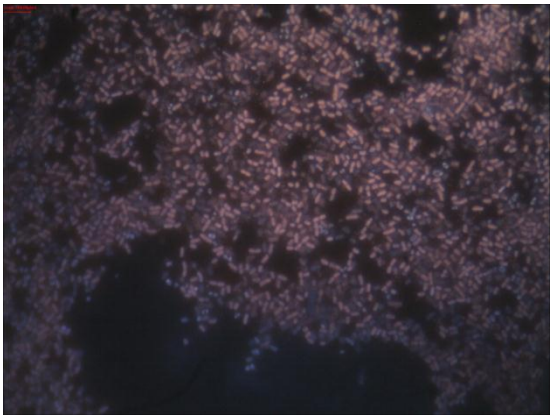
(C)



(D)

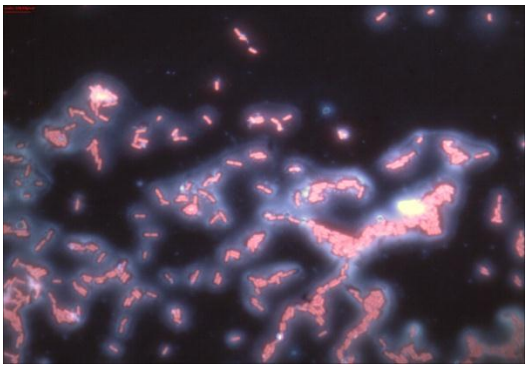


(E)



(F)

**Figure. 4.17. Fluorescent microscopy (x 1000) of *A. baumanii* Type strain grown in mineral media supplemented with 1 % v/v ethanol, IPA and glucose.** (A). Ethanol 24hours. (B). Ethanol 48hours. (C). IPA 24hours. (D). IPA 48hours. (E). Glucose 24hours. (F). Glucose 48hours. **EPS or biofilm visualised by the blue/white colour around the red *A. baumannii* strain.**



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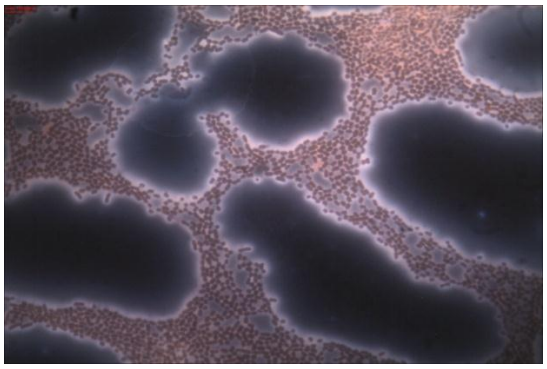

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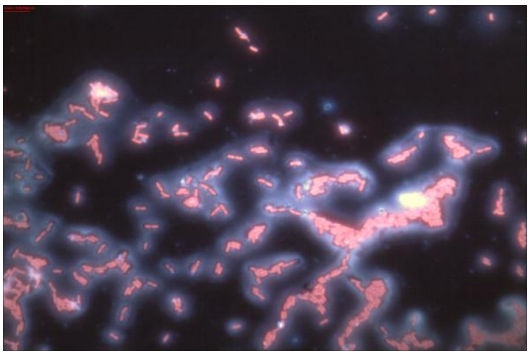

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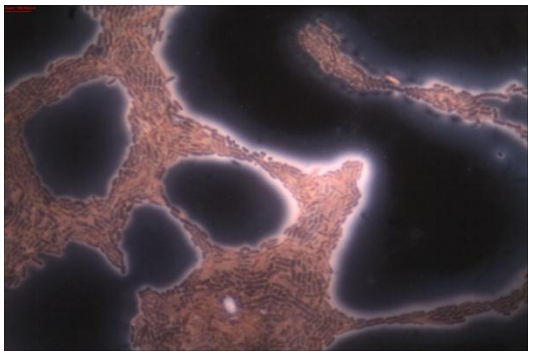
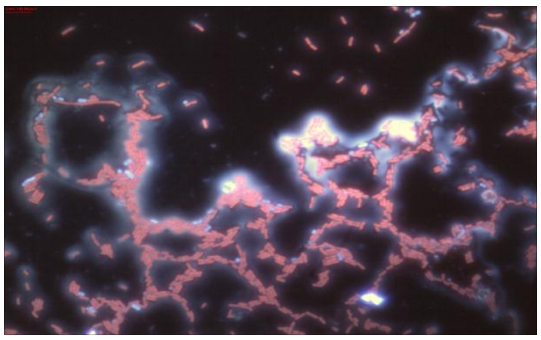
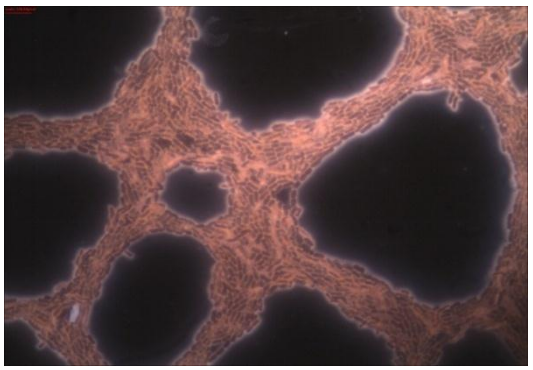


**(D)**

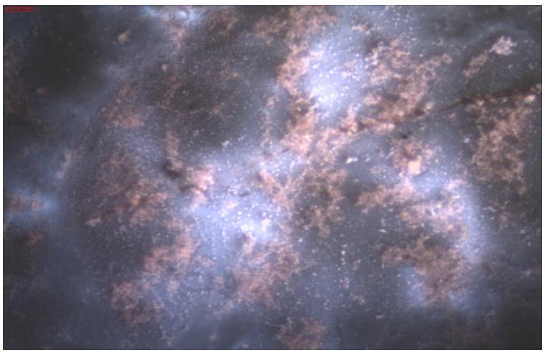


**(E)**

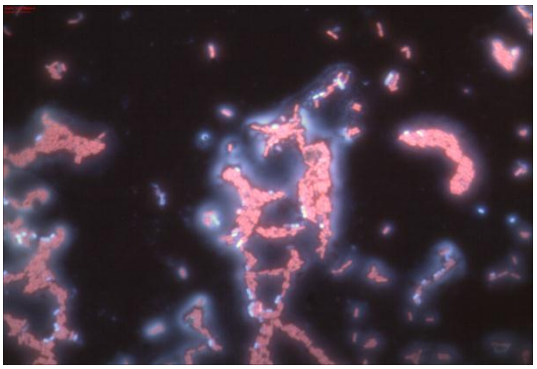


**(F)**

**Figure. 4.18. Fluorescent microscopy (x 1000) of *A. baumannii* CIP 106882 strain grown in mineral media supplemented with 1 % v/v ethanol, IPA and glucose.** (A). Ethanol 24hours. (B). Ethanol 48hours. (C). IPA 24hours. (D). IPA 48hours. (E). Glucose 24hours. (F). Glucose 48hours. **EPS or biofilm visualised by the blue/white colour around the red *A. baumannii* strain.**



**(A)**



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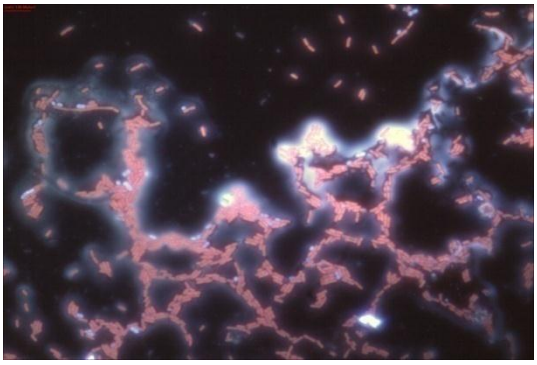
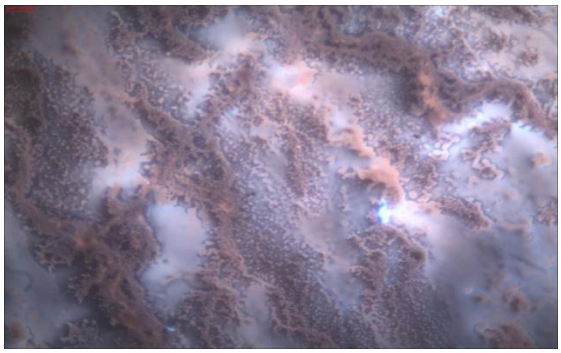
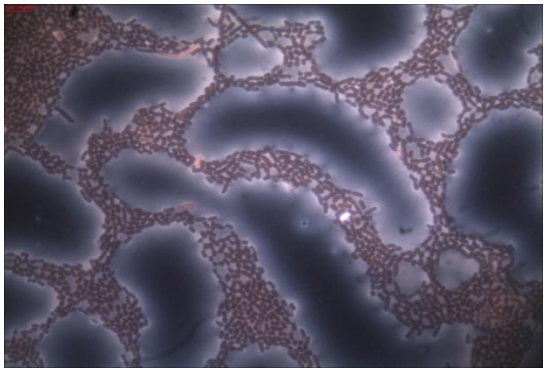


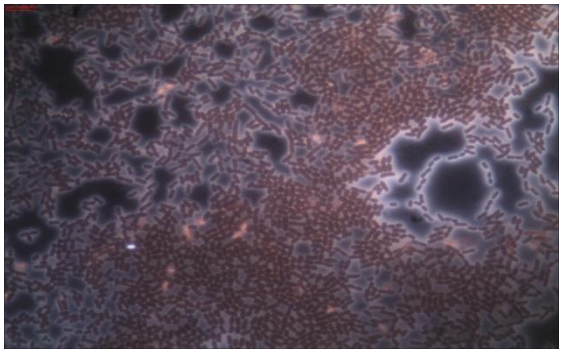
**(C)**



**(D)**



**(E)**



**(F)**

**Figure. 4.19. Fluorescent microscopy (x 1000)of *A. baumannii* OXA-24 Clone I strain grown in mineral media supplemented with 1 % v/v ethanol, IPA and glucose.** (A). Ethanol 24hours. (B). Ethanol 48hours.(C). IPA 24hours. (D). IPA 48hours. (E). Glucose 24hours. (F). Glucose 48hours. **EPS or biofilm visualised by the blue/white colour around the red *A. baumannii* strain.**

### 4.2.7 **Discussion**

*A. baumannii* is known to form strong adherent biofilms that allow the bacteria to survive for several weeks on abiotic surfaces in healthcare settings (Harrison et al., 2008, Donlan, 2008, Villegas and Hartstein, 2003, McConnell et al., 2012). Biofilm formation amongst clinical strains has been identified as a virulence factor (Wroblewska et al., 2008). However, as observed here, biofilm formation amongst *A. baumannii* strains is not consistent; Wroblewska *et al* (Wroblewska et al., 2008) investigated 34 clinical strains from patients hospitalized in two tertiary care hospitals. The isolates demonstrated a wide range of biofilm forming ability, with 12% high, 41% medium and 47% demonstrating a low level of biofilm production. A similar variation in adherence was observed when the adherence of clinical isolates to human bronchial tissue was investigated (Lee et al., 2006). In the case of the strains investigated here (Table 3.1) a greater number demonstrated a high level of adherence with the strength of adherence being dependent on the carbon source. Wroblewska *et al* (2008) did not consider the impact of carbon source on biofilm formation, but the data collected by current study clearly shows that alcohol based carbon sources increase adherence, with 71% of strains being highly adherent when fed on alcohol as the sole carbon source with only the Type strain and UK-HS remaining poorly adherent regardless of carbon source. Lee et al (2006) observed lower levels of adherence for Clone 1 than Clone 2 onto epithelial cells, when tested here for adherence to plastic surfaces no differences between Clones 1 and 2 was seen. Other authors (McQueary and Actis, 2011) have shown the Type strain to be comparable in its ability to form biofilms but in this case TSB was employed as a growth media, emphasising the importance of the carbon source on biofilm formation.

The variations between strains and the impact of carbon source on biofilm formation was mirrored in the hydrophobicity data, where there were clear differences between strains (Figures. 4.12) and the use of alcohol as a sole carbon sources generated greater hydrophobicity than glucose, SWF or TSB (Figure. 4.13). This correlation between biofilm formation and hydrophobicity is to be expected since plastic surfaces are hydrophobic in nature (McQueary and Actis, 2011). Authors such as McQueary and Actic (2011) found no correlation between hydrophobicity and biofilm formation on plastic commenting that this was at variance with observations on *Listeria* and *Neisseria*

*spp*. Pompilio *et al* (Pompilio et al., 2008), tested 40 clinical isolates of *Stenotrophomonas maltophilia* strains to investigate the correlation between the adherence and the biofilm formation on polystyrene and cell surface hydrophobicity and motility, they found that most of the strains were able to adhere and form biofilm and there is a positive correlation was observed between the hydrophobicity and levels of both adhesion and biofilm formation. Out of eleven (27.5%), of the strains proved to be hydrophobic, with hydrophobicity greatly increased as *S. maltophilia* attached to the substratum.

The MATH test has also been used to monitor the cell surface hydrophobicity of nosocomial pathogen *Pseudomonas aeruginosa* as it grew in the presence of benzalkonium chloride (Machado et al., 2011). They reported that the strain OO14 increased in hydrophobicity as the cells grew in resistance to the disinfectant. Bos et al (Bos et al., 1999) used the *MATH* test on dental colonisers such as *Streptococcus* and *Actinomyces* and found that the presence of divalent calcium cations increased the adhesion to hexadecane and chloroform. Costa, et al (Guimaraes et al., 2006) and Di Bonaventura, *et al* (Di Bonaventura et al., 2008), reported that hydrophobicity of the bacterial surface is an important factor for adherence and colonization of bacteria to both living (epithelial mucous tissues) and non-living surfaces (medical devices). Umamaheshwari and Jain, (Umamaheshwari and Jain, 2004), reported that the hydrophobic cell surface components may serve as a binding target for antibacterial lipobeads. Rosenberg (Rosenberg, 2006), reported that hydrophobic surface property of the bacteria play a vital role in growth on hydrophobic materials, initial biofilm formation, adhesion to host cells, aggregation and flocculation and it is the one of many parameters which determines the ability of a cell to adhere, invade and cause damage. However, McQueary and Actic (2011) employed TSB as a growth media which as can be seen here does not favour biofilm formation in some *A. baumannii* strains. When the Type strain and more hydrophobic strains such as 882 and clone 1 were grown on plastic surfaces and visualised via SEM, the less hydrophobic Type strain was unable to maintain a significant biofilm suggesting that when grown on a more minimal media the Type strain was unable to maintain a biofilm on a hydrophobic surface, indicating a direct correlation between hydrophobicity and biofilm formation.

A number of gene products have been proven to play a role in attachment and biofilm formation on abiotic surfaces e.g. pilus production mediated by the CsuA/BABCDE usher-chaperone assembly system is required for the attachment and biofilm formation on the abiotic surfaces by the *A. baumannii* Type strain. This operon seems to be wide spread among clinical isolates and an indication that it is a common factor among different clinical isolates (Tomaras et al., 2003). However McQueary and Actic (2011) demonstrated that even in the presence of this gene significant strain to strain variations in biofilm formation were evident. The Type strain also has ability to produce alternative pili that may help in the interaction of this pathogen with bronchial epithelial cells (De Breij et al., 2010). Loehfelm *et al* (Loehfelm et al., 2008) reported that biofilm-associated protein (Bap), conserved in the clinical isolates and appears to be associated with the cell-cell interactions that support the development and maturation of the biofilm. In addition to (Bap), the *A. baumannii* clinical isolates also produce poly-β-1-6-N-acetylglucosamine (PNAG) for the development and maturation of the biofilm on glass surfaces by the cells cultured (Bentancor et al., 2012, Choi et al., 2009b). A two component regulatory system also reported in *A. baumannii* Type strain comprised of: a sensor kinase encoded by *bfm*S, and a response regulator encoded by *bfm*R involved in bacteria-surface interaction (Tomaras et al., 2008). The insertional inactivation of *bfm*R results in the loss of expression of *cusA/BABCDE* operon resulted in the lack of pili production and biofilm formation on plastic surfaces when grown in rich medium (LB broth), however the inactivation of *bfm*S sensor kinase gene resulted in diminishment but not abolishment of biofilm formation (Tomaras et al., 2008). In the absence of BfmRS system the composition of culture media still influence the interaction of cells with abiotic surfaces, these finding indicates that the BfmRS system cross talks with other sensing components and suggests instead of one, there are multiple and different stimuli which could control the biofilm formation via BfmRS regulatory pathway (Tomaras et al., 2008). However, all Tomaras et als (2008) work was carried out using the Type strain with glucose as a carbon source, consequently the differences seen here with ethanol and other strains was not investigated.

Another mechanism controlling bacterial adherence and biofilm formation is cell population density. Accordingly, environmental and clinical isolates produce quorum sensing signalling molecules (Gonzalez et al., 2001, Gonzalez et al., 2009), these studies

proved that a large number of isolates produce quorum sensing and signalling molecules which seem to belong to three types of molecules. Although none of these sensors belongs to a particular species, however the Rf1-type sensor is more frequently found in isolates belonging to the *A. calcoaceticus-baumannii* complex. Niu et al (Niu et al., 2008) proved that the *A. baumannii* M2 clinical isolates produce an *N*-acyl-homoserine lactone [*N*-3-hydroxydodecanoyl-homoserinem lactone], the product of the *aba*I auto inducer synthase gene, which is vital for the fully developed biofilm on abiotic surfaces, *aba*I auto inducer also helps this isolate to move in semisolid media.

Glass represents a hydrophilic rather than a hydrophobic surface for biofilm production and has been used by some authors (McQueary and Actis, 2011) as a contrast to polystyrene in biofilm studies. In this investigation glass slides in a drip flow reactor were employed to investigate biofilm formation by the high biofilm forming strains 882 and Clone 1 and the low biofilm forming Type strain. The drip flow system is a more representative approach for assessing biofilm formation on glass since the bacteria have to adhere to the substrate in order to stay in the system. In contrast to McQueary and Actic's (2011) these hydrophobic strains were able to form and maintain significant biofilms on glass under low shear conditions with no obvious difference between glucose or alcohol as a carbon source (Figures. 4.17, 4.18). The hydrophilic Type strain was also able to form biofilms on glass, these were less extensive when fed alcohols, particularly IPA than the two hydrophobic strains. Significant variations in the amount of biofilm formed and the cell arrangements present on abiotic surfaces have been reported for *A. baumannii* (McQueary and Actis, 2011). Some cell arrangements are highly organised, multi-layered and complex structures encased within a biofilm or EPS matrix, while some others are simple monolayers of bacteria attached in an organized or random manner (McQueary and Actis, 2011). In the case of the strains investigated here only monolayers were formed under either static (Figures. 4.17, 4.18) or low shear environments.

Edwards *et al* (Edwards and Harding, 2004) reported that *A. baumannii* is able to readily metabolized low concentrations of ethyl alcohol and secreted a range of proteins including OmpA, which is recognized as having emulsifying activity. The secretion of OmpA by *A. baumannii* following exposure to 0.5% ethanol in minimal media has also been described by Walzer *et al* (Walzer et al., 2006), suggesting that it may be the

important response to growth and survival under low nutrient conditions. These bioemulsifying proteins also assist in bacterial adhesion, quorum sensing and the development of biofilms (Ron and Rosenberg, 2001). Generally, *A. baumannii* adheres to biotic and abiotic surfaces via the same steps described for general biofilm formation. The associated EPS being composed of carbohydrates, proteins, nucleic acids and other macromolecules (McConnell et al., 2012). Pirog *et al* (Pirog et al., 2002), reported that *Acinetobacter* spp.12S has the ability to grow and synthesize EPS on different carbohydrate substrates like mono and disaccharides, molasses and starch. He reported that the *Acinetobacter* spp. were grown on carbohydrate media containing no pantothenic acid (Vitamin B3), which is required for growth on $C_2$-substrates. He used mixture of carbohydrate sources (0.01, 0.1, 0.5 and 1% ethanol and 1% glucose) and found that bacterial growth and EPS generation was higher when 0.01% ethanol with 1% glucose used. The EPS production was intensified as the content of the C2 substrate in the medium increased. The author also reported that the addition of low concentration (0.02%) of acetate to the glucose-containing medium also stimulated the EPS synthesis. The growth of the *Acinetobacter* strains at the expense of alcohol as the sole carbon source without any complex media supplements has already been identified as a characteristic of hydrophilic, strongly adherent strains such as 882 and Clone 1 (Section 4.1.3).

The formation of polysaccharide based EPS by these strains at the expense of alcohols as well as glucose is evident from the drip flow biofilms where the EPS is stained by the carbohydrate selective stain calcofluorwhite (Figures. 4.18- 4.19), indicating that these strains are able to synthesise complex carbohydrates from C2 and C3 alcohols in minimal media. Interestingly the Type strain did not generate any obvious EPS suggesting that the hydrophobic biofilm forming strains adhere to glass through the generation of EPS, whilst the hydrophilic Type strain employs an alternative approach such as the use of pili as outlined by McQueary and Actic (2011). The formation of biofilm was observed as a blue colour around the red bacterial cells that starts forming after 24 hours, however biofilm was more obvious after 48 hours of continuous growth in a drip flow reactor. Both the strains developed strong biofilms when the carbon source was ethanol or IPA as compared to the glucose and can be seen easily by the fluorescent microscopy. In addition *A. baumannii* strains 882 developed more compact

biofilm as compared to Clone 1 on all the carbon sources (Figures 4.17 - 4.19). The generation of EPS is associated with cell growth as can be seen from the batch growth of these strains, the resulting EPS is not utilised as a carbon source after formation and is produced during growth along with lactate. The formation of acid from glucose is a common observation for *A. calcoaceticus-baumannii* complex as is the consumption of lactate as a sole carbon source (Nemec et al., 2011), in this case acid is also produced from C2 and C3 alcohols.

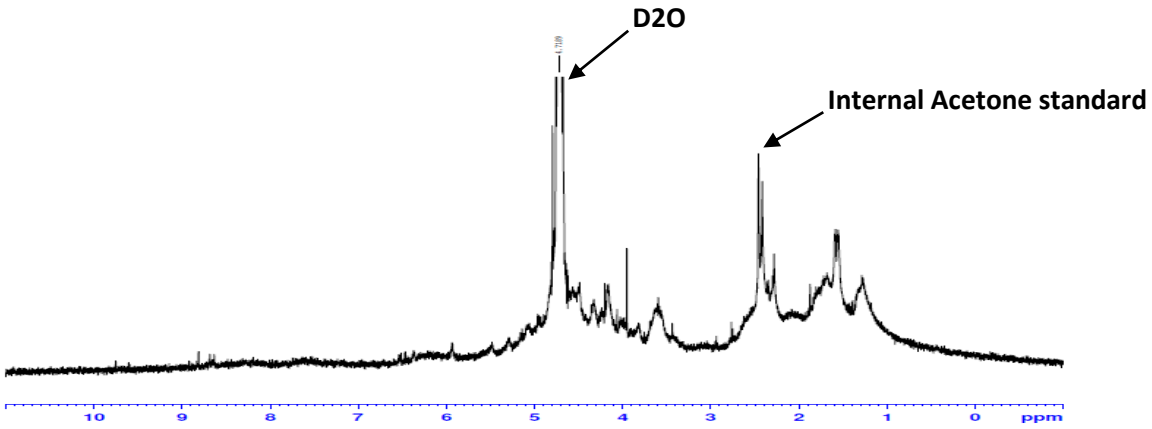
### 4.2.8 **Key findings**

- There is significant variation in biofilm forming capacity amongst the strains investigated;
- Growth on alcohols as sole carbon increased the biofilm forming capacity of some strains over media containing glucose as a sole source of carbon;
- Strong biofilm formers (882 and clone 1) were also strongly hydrophobic and able to establish biofilms on both plastic and glass surfaces;
- Carbohydrate based EPS was generated during biofilm formation and growth on alcohols;
- The EPS generated during growth was produced alongside lactate during the growth phase but was not degraded during the stationary phase.

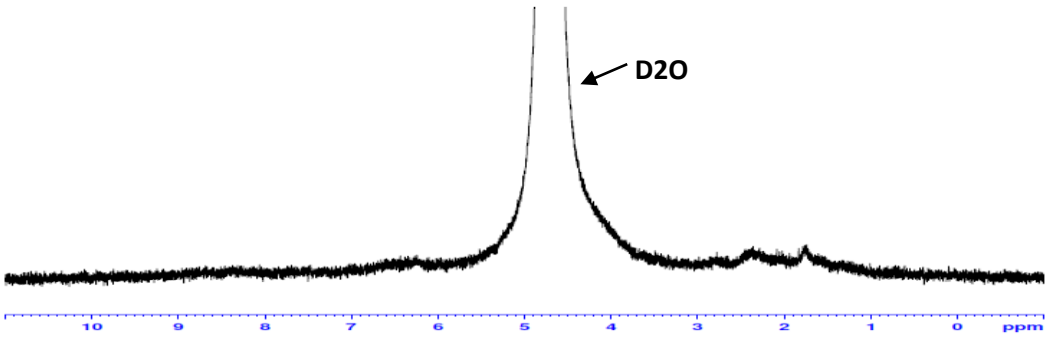
## 4.3 EPS Characterisation

### 4.3.1 Poly-β-(1-6)-N-Acetylglucoseamine (PNAG) method

Growth on Congo red plates was used as an indication of the generation of PNAG by *A. baumannii.* All the available strains (Table. 3.2) were screened with only Clone 1 and 882 generating red colonies indicating the ability to produced PNAG. This is consistent with the identification of these strains as strong biofilm formers (See Table. 3.2). These two strains were then taken forward for a more detailed isolation and characterization of PNAG (See section 3.4.4.1) by the $^{1}$H NMR. However, $^{1}$H NMR spectra did not contain any evidence for the presence of PNAG (Figure 4.20, a & b) suggesting that these strains produced PNAG at a levels below that extractable by the approach (please see Figure 4.21 for comparison).




(A)




(B)

**Figure: 4.20: $^{1}$H NMR spectrum of PNAG or EPS from (a). 882 (b). Clone 1.** $^{1}$H NMR spectra on either a Bruker Avance DPX4000 400.13 or a Bruker Avance DPX500 500.13MHz. Spectra was acquired using Bruker pulse sequences at a temperature of 70°C.

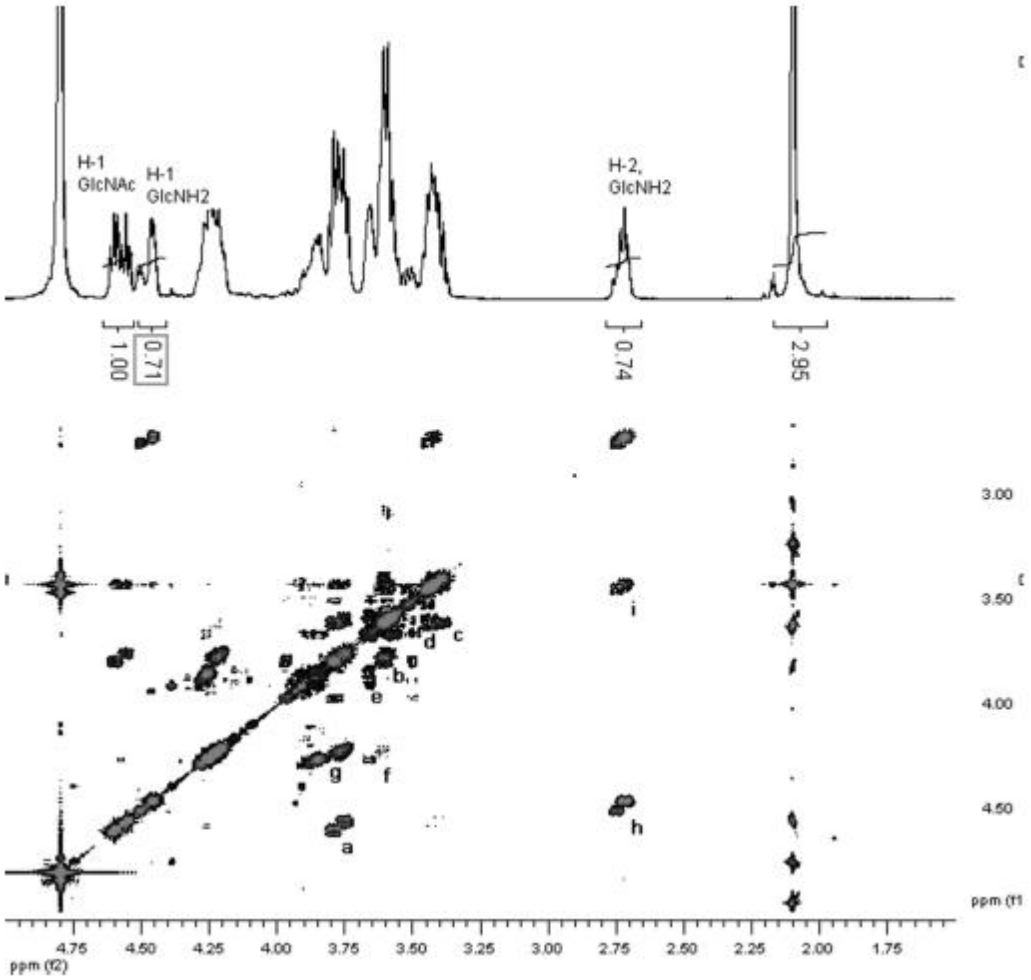



**Figure: 4.21: $^{1}$H NMR spectrum of PNAG (Choi et al., 2009a).**

### 4.3.2 **EDTA method**

EPS can be classified by its proximity to the cell surface. Capsular or cell bound-EPS is tightly linked via a covalent or non-covalent association whereas free EPS is not directly attached to the cell surfaces (Fig. 4.22) (Wingender et al., 1999). Clone 1 and 882 grown in 6-litre culture of mineral media supplemented 1% ethanol and incubated at 37°C for 48-72 hours with continuous shaking at ~90-110 rpm. EPS was separated using the method of Sheng *et al* (Sheng et al., 2005) with some modification (See section. 3.4.4.2). The resulting EPS was characterised via $^{1}$H NMR along with weight average molecular

weight determinations using HP-SEC-MALLS and FTIR.

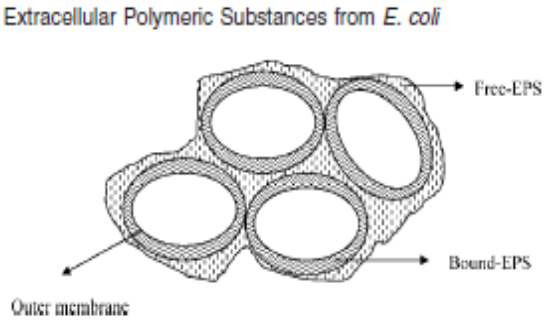



**Figure. 4.22. Distribution of Bond and free EPS surrounding bacteria.**

#### *4.3.2.1 Weight-Average molecular Weight Determination (MALLS)*

A standard material (pullulan) of known molecular weight and low polydispersity was chosen to ensure the accuracy of the instrumentation. Pullulan is a linear homopolysaccharide consisting solely of glucose units. The $M_w$ of the pullulan standard is 800,000g $mol^{-1}$(Brittain, 1997) with a polydispersity ($M_w/M_n$) of 1.23 and a *dn/dc* value of 0.148ml/g (Figure 4.23). Both the strains i.e. Clone I and 882 revealed the high molecular weight compounds. In case of CIP 106882 MALLS analysis (Figure 4.24a) indicated a *Mw* of 62,333Da and a polydispersity (the size of a polysaccharide is expressed by the number of monosaccharide units it contains, which is termed as the degree of polymerisation (DP) ($M_w/M_n$) of 1.632. In the case of Clone 1 (Figure 4.24b) the *Mw* was 96,500Da while the polydispersity ($M_w/M_n$) was recorded as 2.24.

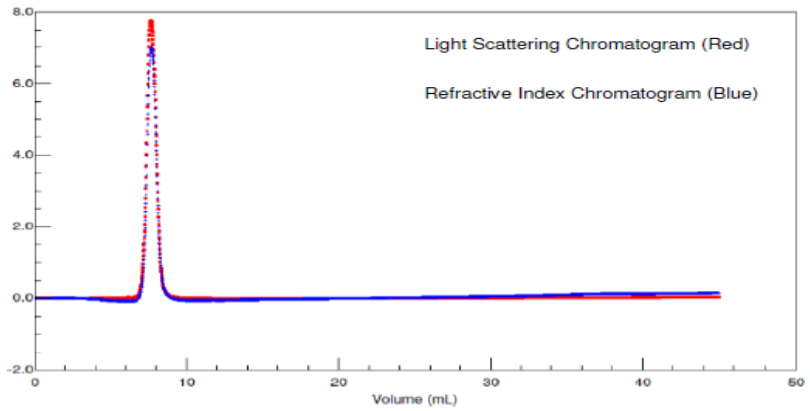
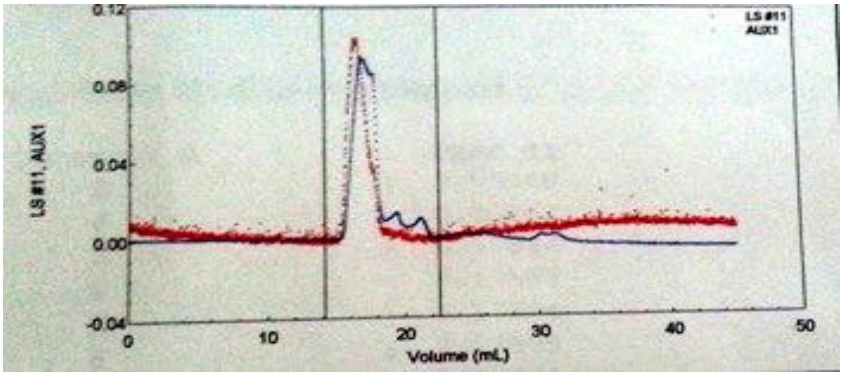
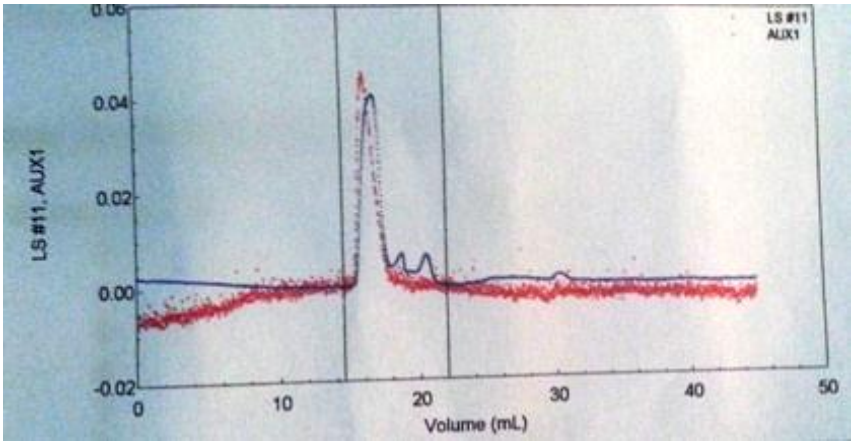



**Figure. 4.23. A chromatogram of the Pullulan standard (800,000 $M_w$).**




**(A)**

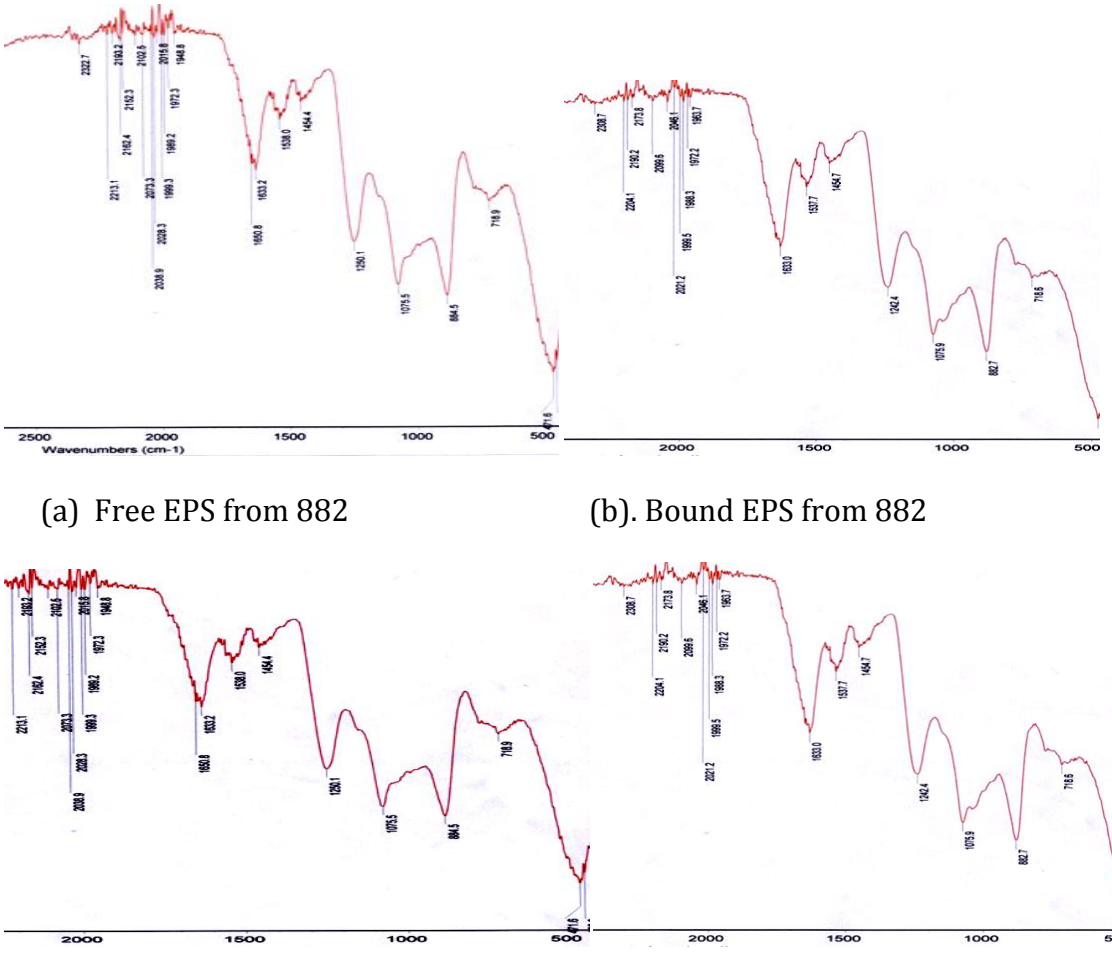



**(B)**

**Figure. 4.24. A chromatogram of EPS produced by (a). 882 (b). Clone 1.**

*4.3.2.2* ***FTIR spectra of EPS***

The region between 4000 and 500cm$^{-1}$ holds the characteristic bands for carbohydrates and is suitable for the characterisation of microorganisms (Schmitt and Flemming 1998). The wide and intensive carbohydrate or EPS bands were found at wave length 950-1200cm$^{-1}$ which were attributed to –COC- group vibrations in the cyclic structure(Gómez-Zavaglia and Fausto, 2003). FTIR spectra for free and bound EPS from Clone 1 and 882 have signals in the region of 950-1200cm$^{-1}$ which is attributed to the vibrations of C-0-P and C-O-C stretching of a diverse polysaccharide groups, and the bands at 1260cm$^{-1}$ and 1080cm$^{-1}$ exhibit the strecthing of P=O of phophoryl and phosphodiester groups from phosphorylated proeins, plyphosphate products and nucleic acids. There are also several bands in the region of 1450-1700 cm$^{-1}$ which reffers to the amide groups of the proteins (Figure 4.25. a-d).




(a) Free EPS from 882 (b). Bound EPS from 882

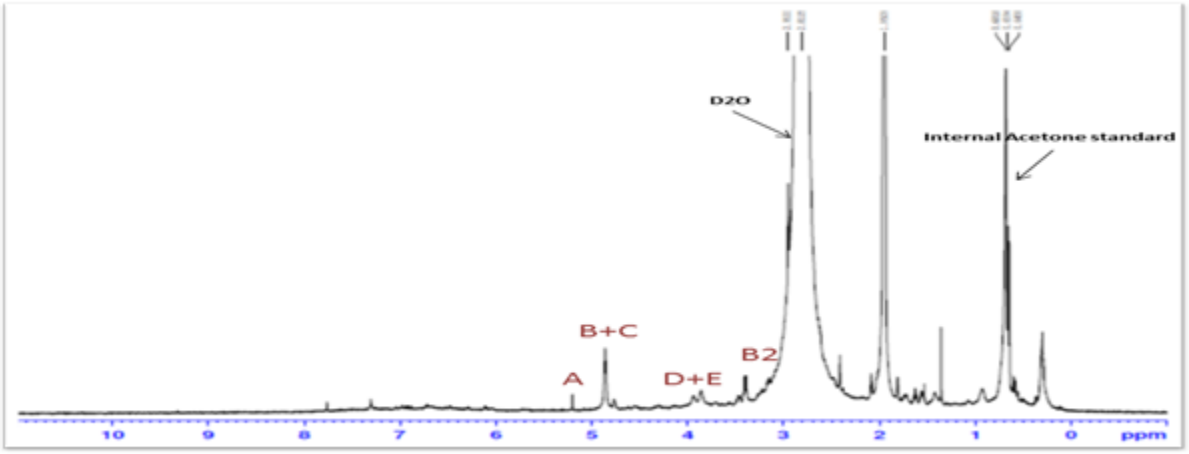
(c). Free EPS from Clone I. (d). Bound EPS from Clone I.

**Figure. 4.25 (a-d): FTIR spectra of free and bound EPS from 882 and Clone 1.** Fourier transform infrared spectrocopic (FTIR) . Trasmission spectra was recorded using at least 32 scans with 4cm$^{-1}$ rsolution in the spectral range 4000-5004cm$^{-1}$. Carbohydrate or EPS bands are found at wave number 950-1200cm$^{-1}$.

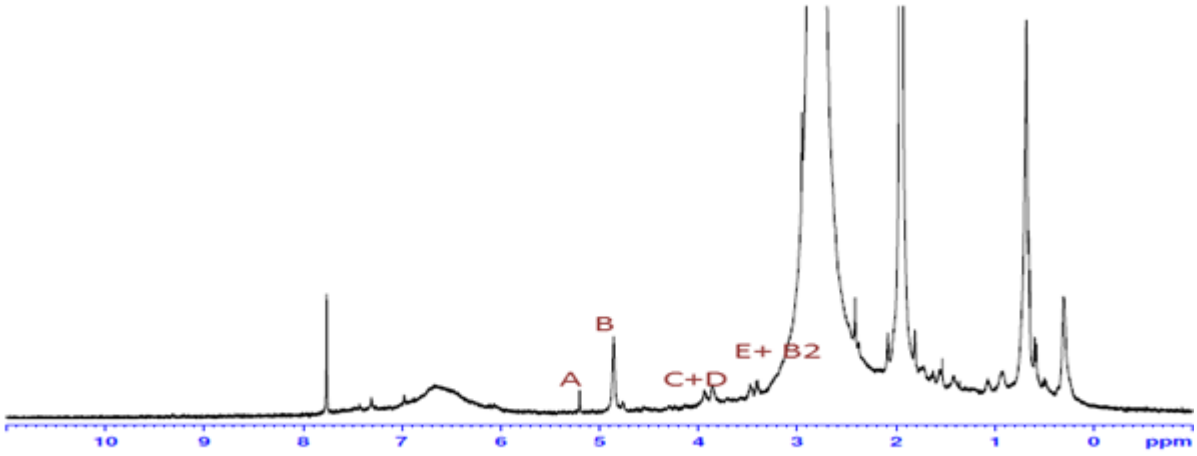
### *4.3.2.3 The $^{1}$H NMR*

Signals that can be attributed to EPS lie between the regions of 4-5.5ppm. Spectra of the free EPS from both the strains does not show any signals for EPS or carbohydrate, however the bound EPS results revealed that bacterial strain 882 generate weak EPS signals in the designated region, there were five anomeric protons (arbitrarily designated A-E from left to right) and one ring proton (B2). The B and C overlapping each other while D and E next to each other and B2 peak is next to the D2O peak. Similarly in case of Clone 1, $^{1}$H NMR revealed weak EPS signals in the designated region, there were five anomeric protons (arbitrarily designated A-E from left to right) and one

ring proton (B2). The A and B were clear and C+D AND E+B2 next to each other (Figure 4.24. a, b). The $^{1}H$ NMR results are consistence with the MALLS analysis (4.3.2.1) that the EPS extracted from both species by this approach were not of high molecular weight consequently they generated weak NMR signals(Figure 4.26. a, b).




**(A)**




**(B)**

**Figure: 4.26: $^{1}H$ NMR spectrum of Bound EPS from (a). 882 (b).Clone 1.** $^{1}H$ NMR spectra on either a Bruker Avance DPX4000 400.13 or a Bruker Avance DPX500 500.13MHz. Spectra was acquired using Bruker pulse sequences at a temperature of 70°C.

### 4.3.3 **Trichloroacetic acid (TCA) method**

The isolation of exopolysaccharide (EPS) was carried out by the method described by (Marshall et al., 2001), with some modifications. The additional steps include the use of RNAase, DNAase and protease to minimize impurities in the EPS which interfere with NMR spectra and lead to the false positive results. The method is referred to as the ethanol method and it involves a series of ethanol precipitations, centrifugation, dialysis and freeze drying. .

#### *4.3.3.1* ***Weight-Average molecular Weight Determination (MALLS)***

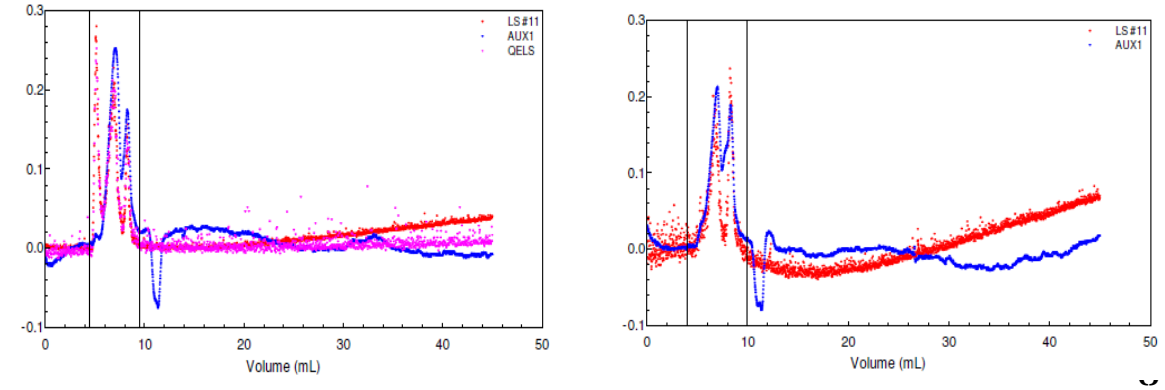
Both Clone 1 and 882 generated high molecular weight compounds and there were two distinct peaks other than the standard (Figure 4.25. a,b). In the case of Clone 1 MALLS (Figure 4.27b) analysis produced a *Mw* of 2,620,000Da while the polydispersity ($M_w/M_n$) was recorded as 2.62, whilst in the case of 882 (Figure 4.27 a) the *Mw* was 1,270,000 Da while the polydispersity ($M_w/M_n$) was recorded as 1.87. The MALLS analysis indicates that TCA method extracts high molecular weight materials than the EDTA method by both of the strains i.e. Clone 1 and 882 using TCA method as compared to EDTA method. In case of Clone 1, the TCA method produced products with molecular weight of 2,620,000Da rather 96,500 Da by EDTA and 882 produced products with molecular weight of 2,620,000 Da rather than 96,500 Da by the EDTA method.

#### *4.3.3.2* ***FTIR spectra of EPS***

The region between 4000 and 500cm$^{-1}$ holds the characteristic bands and is sutiable for the characterisation of microorganisms (Schmitt and Flemming, 1998). Intensive carbohydrate or EPS bands were found at wave number of 950-1200cm$^{-1}$ which were attributed to –COC- group vibrations in the cyclic structure (Gómez-Zavaglia and Fausto, 2003). In EPS extracted from both Clone 1 and 882 strong signals were generated in the region of 950-1200cm$^{-1}$ (Figure 4.28) which is attributed to the vibrations of -C-0-P- and --C-O-C- stretching of a diverse polysaccharide groups, which revealed that there are carbohydrate present in the both of EPS samples.

#### *4.3.3.3 The $^{1}H$ NMR and $^{13}C$ NMR*

Signals in the regions (4-5.5ppm) associated with the presence of EPS were present in the $^{1}H$ NMR spectra of both strains investigated. In both the cases A and B were clear and C+D AND E+B2 next to each other (Figure 4.29 a, b). In both the strains i .e. 882 and Clone 1, $^{13}C$ NMR spectra also revealed signals in the polysaccharide region i.e. 60 to 110 ppm. Starting from left of the spectra, we have first signal at 105 ppm for C1, than at 87ppm for C4, 75ppm for C2, C3 and C5 carbons and C6 has signal in the range of 63ppm (Figure 4.30 a, b). The results of our $^{1}H$ NMR consistence with our previous results MALLS and FTIR (See sections. 4.3.3.1 and 4.3.3.2) that EPS were of high molecular weight resulting in better signals with $^{1}H$ NMR.




**(a)** **(b)**

**Figure. 4.27. MALLS chromatogram of EPS produced by (a). 882 (b).Clone 1.**

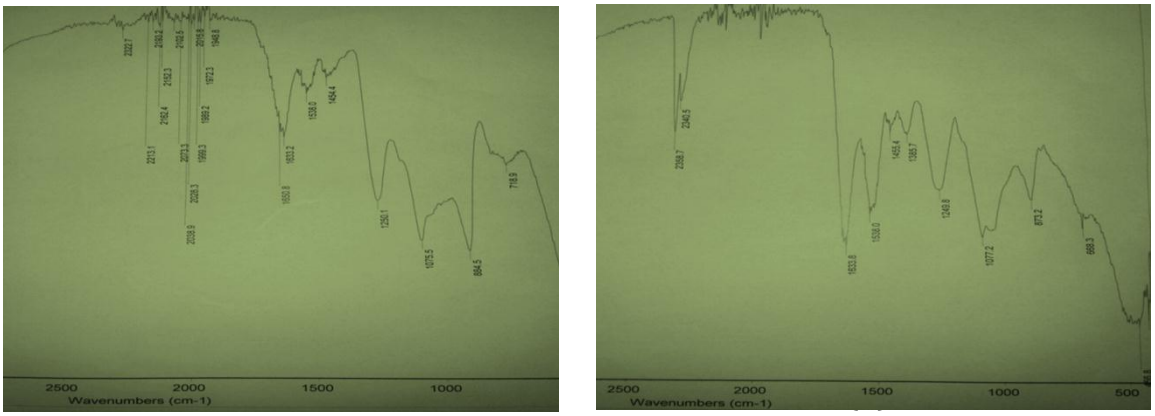
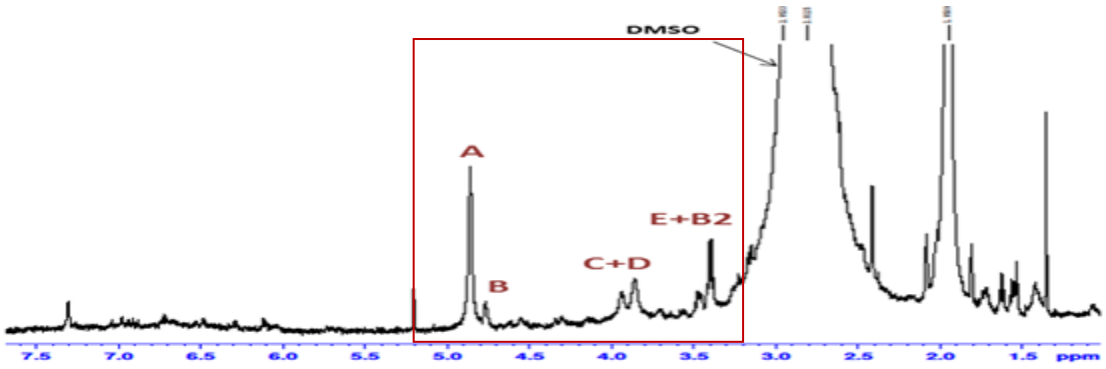
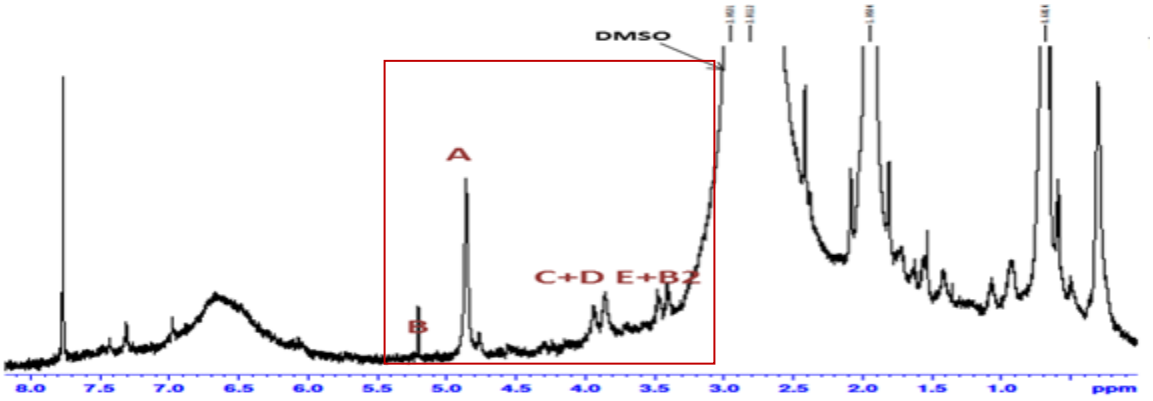



**Figure. 4.28. FTIR spectra of EPS from (a). 882 (b). Clone 1.** Fourier transform infrared spectrocopic (FTIR) . Trasmission spectra was recorded using at least 32 scans with 4cm$^{-1}$ rsolution in the spectral range 4000-5004cm$^{-1}$. Carbohydrate or EPS bands are found at wave number 950-1200cm$^{-1}$.

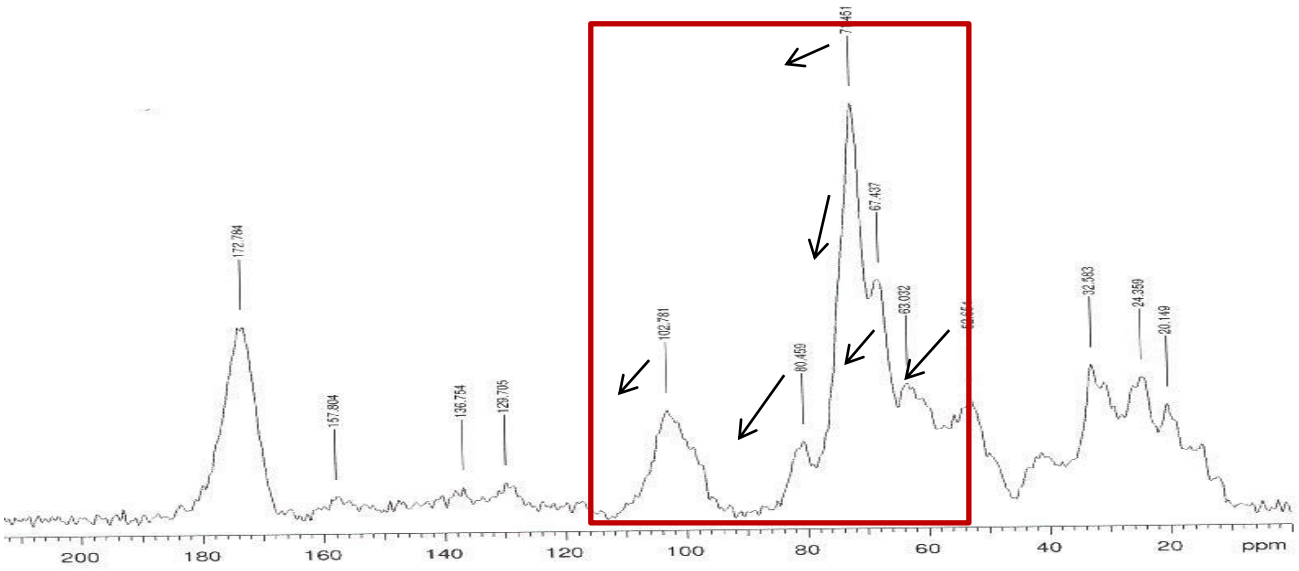



**(a)**

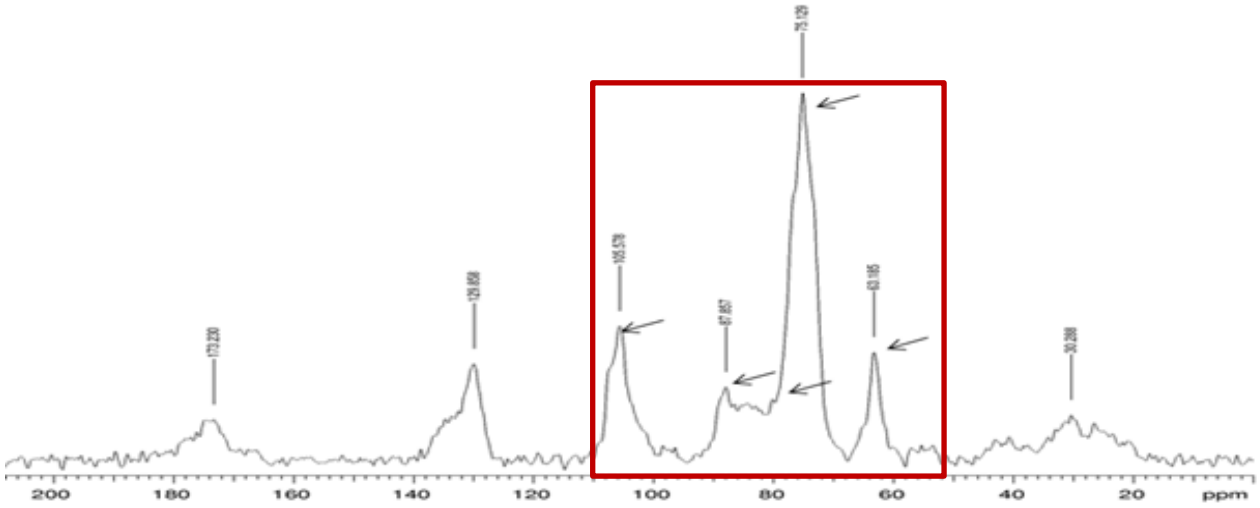



**(b)**

**Figure: 4.29. $^{1}$H NMR spectrum of EPS from (a). 882 (b). Clone 1.** $^{1}$H NMR spectra on either a Bruker Avance DPX4000 400.13 or a Bruker Avance DPX500 500.13MHz. Spectra was acquired using Bruker pulse sequences at a temperature of 70°C.




**(a)**

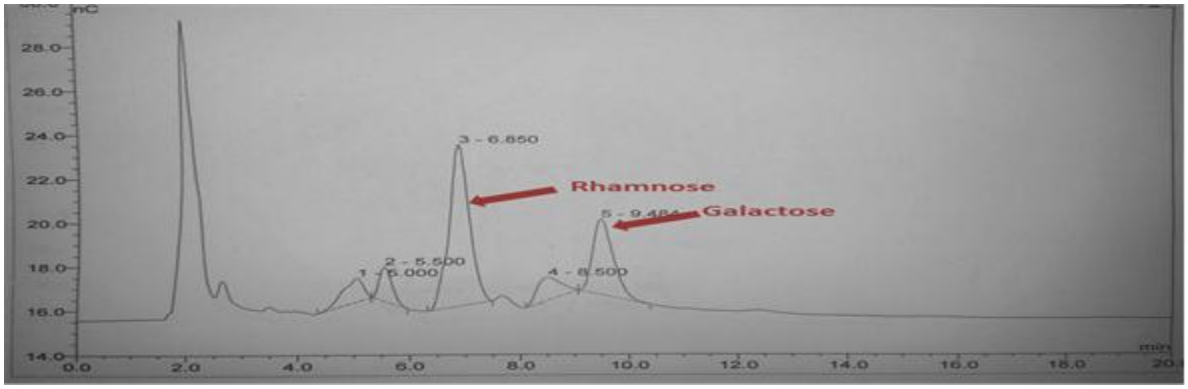



**(b)**

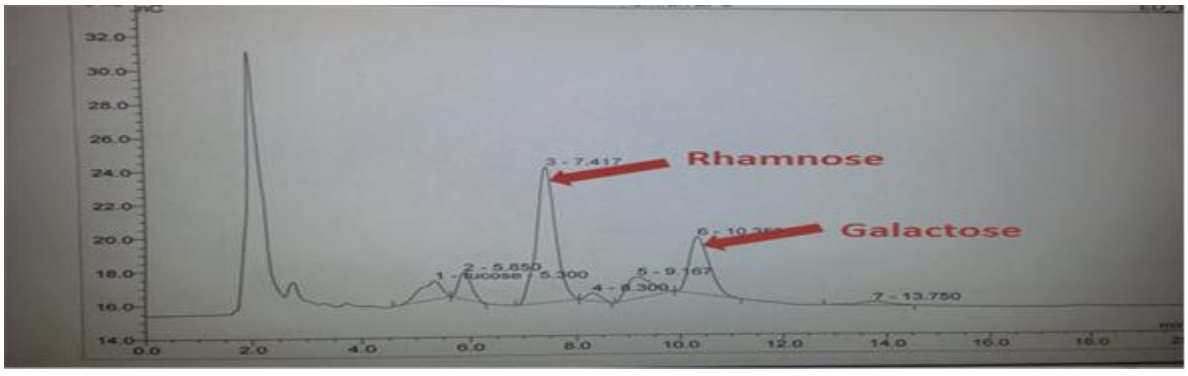
**Figure: 4.30. $^{13}$C NMR spectrum of EPS from (a). 882 (b). Clone 1.** $^{13}$C NMR were performed using a Varian VNMRS spectrometer operating at 100.56 MHz for $^{13}$C. Signals in the range 60 to 110 ppm are typically polysaccharide or EPS. C1 signal around 105 ppm, C4 80-90 ppm, C2, C3 and C5 around 75 ppm and C6 around 63 ppm.

#### *4.3.3.4* ***Monomer analysis using HPAEC-PAD***

After the confirmation from MALLS, FTIR and NMR (Section 4.3.3.1-3) of the presence of EPS, HPAEC was employed to provide an insight into the composition of the polysaccharide. Monomer analyst using HPAEC-PAD is frequently utilised as a alternative option to the GC-MS method of detection. HPAEC-PAD is often used as it involves a simple, quick and efficient one step acid hydrolysis procedure. As the monomer gives different responses upon their interaction with the PAD in the HPEAC system (dependent of the pKa of the sugars), so the three different sugars standard i.e galactose, mannose and rhamnose were run to compare the test results. The similarities of the peaks observed in the HPAEC-PAD chromatogram (Fig. 4.31 a, b) were determined by comparing of the sample with standard compounds. Peaks from both the strains i.e. 882 and Clone 1 were identified as rhamnose and galactose.




**(a)**




**(b)**

**Figure. 4.31. HPAEC- chromatogram of EPS. (a). 882 (b). Clone 1.** EPS samples were injected (AS50 Autosampler, Dionex Co.) into the HPAEC. Sodium hydroxide (8mM, Isocratic) delivered

by a gradient pump (GS50 Gradient pump, Dionex Co.) at 0.5ml/min was used to elute the monosaccharides through the PAD detector (ED50 Electrochemical detector, Dionex Co.).

### 4.3.4 **Discussion**

The formation of EPS is a key step in the ability of *A. baumannii* to adhere to biotic and abiotic surfaces, and is implicated in increased virulence, antibiotic resistance, reduced phogocytosis, disinfection tolerance and extended environmental persistence. For the isolation and characterization of EPS from two strains of *A. baumannii* known to be strong biofilm formers (See section 4.1) three different extraction techniques were employed. In order to prevent interference from the growth media a carbohydrate free mineral media with an alternative carbon source (ethanol) known to support the biofilm formation of these strains was employed.

*A. baumannii* strains able to produce PNAG have been identified (Choi et al., 2009b), along with a cluster of four genes, *pga A, B, C,* and *D*. The authors were able to transfer the *pga* locus from *A. baumannii* to a PNAG negative *E. coli* strain which results in the synthesis of PNAG in the transformed strain. The presence of PNAG like molecules has also been reported in some *E. coli* and *S. epidermidis* (Wang, X. *et al*., 2004, Maira-Latran.T. *et al*., 2002). Analysis of Clone 1 and 882 for PNAG was inconclusive, whilst its presence was indicated by growth on Congo red attempts to isolate PNAG was unsuccessful due to either its absence or the generation of low concentrations. These results indicate that if Clone 1 and 882 do generate PNAG they do so at a level that suggests it is not a major component of the EPS they generate. Loehfelm *et al* (Loehfelm *et al.* 2008) reported that the *A. baumannii* clinical isolates also produced poly-β-1-6-N-acetylglucosamine (PNAG) for the development and maturation of the biofilm on glass surfaces (Bentancor *et al.* 2012, Choi *et al.* 2009b). Given the importance of PNAG in biofilm maturation it is likely that Clone 1 and 882 are able to produce PNAG but at concentrations below that detectable by this analysis.

Both the EDTA and TCA methods for EPS extraction were able to generate high MW material with Clone 1 producing the higher MW material in both cases. The TCA method isolated much higher MW material than the EDTA method suggesting that the EDTA method may be degrading the EPS resulting in smaller molecules, which may in turn result in the loss of material during dialysis which will contribute to the small amount of material available for NMR analysis.

The TCA method has been successfully applied to EPS characterisation of Lactobacilli and *Bifidobacteria* (Salazar *et al.* 2009, Laws *et al.* 2008). These EPS recovered from the *A. baumannii* are within the range of MWs recorded for *Bifidobacterium* sp using the same approach (Leivers *et al.* 2011). The MALLS and FTIR data indicate the presence of high molecular weight compounds in the extracted EPS from the two strains under investigation. The $^{1}H$ NMR and $^{13}C$ NMR and HPAEC analysis indicates that this EPS is carbohydrate based and contains both galactose and rhamnose. These results correlate with the finding of (Yadav *et al.* 2012), who reported FTIR analysis of *A. baumannii* sugar and sugar derivative peaks falls in the region of 1000-1100$cm^{-1}$. They also reported that the EPS produced by biofilm forming strains of *A. junii* (BB1) were primarily composed of neutral sugars (73.21%), amino acids (.23%), α-amino acids (11-13%), uronic acid (10%) and aromatic amino acids (1.23%) with three main sugars residues being present i.e. galactose, mannose and arabinose. The presence of galactose being consistent with the results reported here for Clone 1 and 882.

### 4.3.5 **Key findings**

- PNAG could not be extracted from EPS material generated by the two strong biofilm forming strain of *A. baumannii* i.e. Clone 1 and 882;
- Clone 1 and 882 are able to generate high MW EPS when grown on mineral media with ethanol as a sole carbon source;
- Of the EPS extraction methods employed the TCA method was most successful from the perspective of the amount of material recovered and it's MW;
- In all cases analysis, in particular NMR, was hampered by the low solubility of the extracted material with the use of solid state NMR partially overcoming these problems;
- In both cases the EPS extracted contained both galactose and rhamnose sugars.

## 4.4 Impact of QAC and PHB

### 4.4.1 Determination of MIC and MBC concentration of QAC and PHMB

The Bioscreen system was used to determine the MICs of a range of quaternary ammonium compounds (QACs) and PHMB against the strong biofilm forming strains Clone 1 and 882. These strains were grown in mineral media supplemented with either ethanol or glucose. MIC values were calculated from Bioscreen outputs using the approach described by Lambert and Pearson (2002) (See Appendix). When grown on glucose both strains (Clone 1 and 882) were least sensitive to PHMB (Figure 4.30) and most sensitive to Barquat. In the case of ethanol as sole carbon source the differences between biocides was less pronounced (Figure 4.32), in the case of Clone 1 Benzathonium chloride was the most effective, in the case of 882 there were no major differences between the biocides.

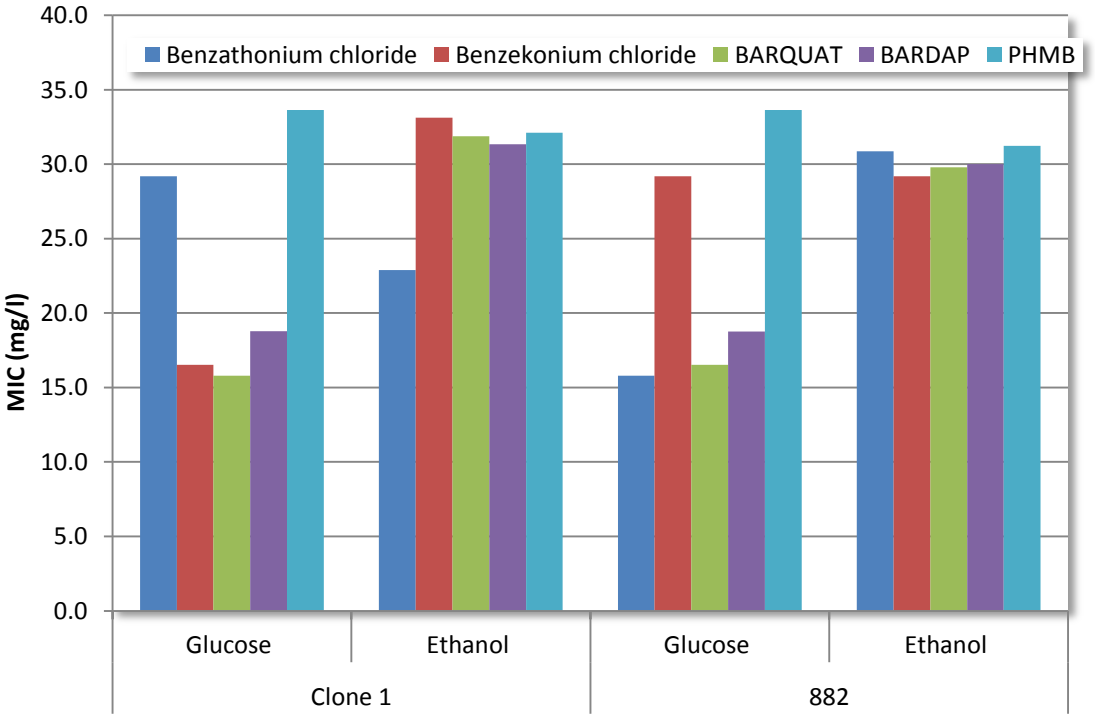



**Figure. 4.32. MIC data for QACs and PHMB.**

By plating out the wells at the end of the Bioscreen it was possible to generate data on the Minimum Biocidal Concentration (MBC) as well as the MIC. MBC were again determined using the approach of Lambert and Pearson (2002) but in this case (log TVC $_{test}$ / LogTVC$_{Control}$) was plotted against the Log of the biocide concentration to generate

the required inhibition profile (e.g. Figure 4.33). The resulting MBC (Figure 4.34) were consistently >80% greater than the associated MIC (Figure 4.32), indicating that these two strains of *Acinetobacter* are able to survive for significant lengths of time exposed to inhibitory concentrations of biocides. The greatest difference (≈100x greater) was seen with Clone 1 grown on glucose and exposed to Benzethonium chloride and the smallest difference was (≈6x greater) seen when 882 was grown on ethanol and exposed to Benzethonium chloride. In the case of the QATs ethanol grown cultures had lower MBC than glucose grown cultures (Figure 4.34).

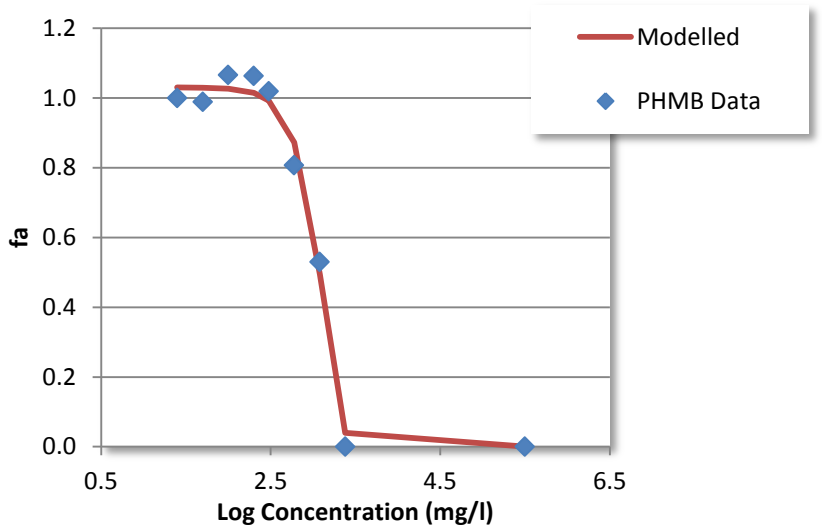



**Figure. 4.33. MBC inhibition profile for PHMB.**

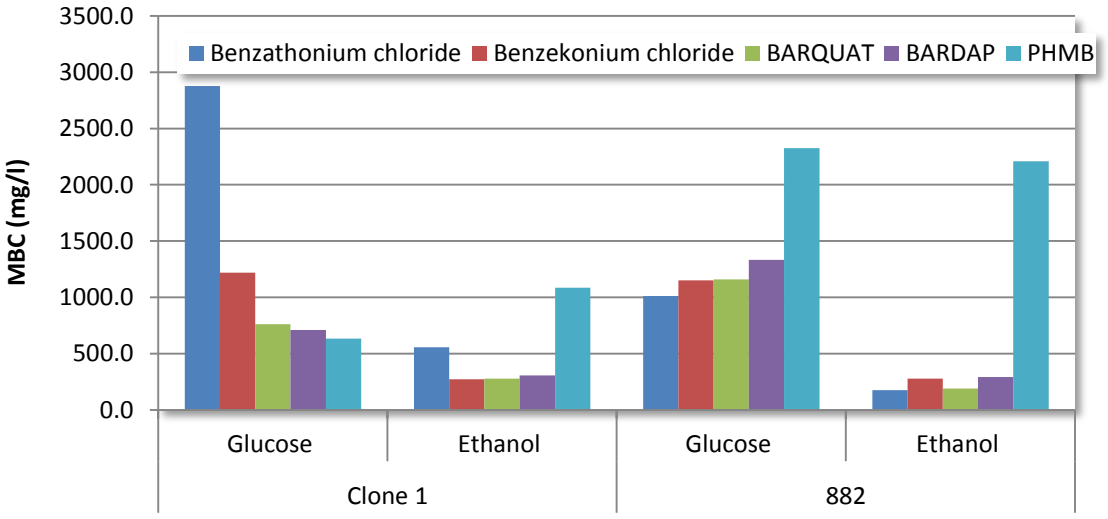



**Figure. 4.34. MBC data for QACs and PHMB.**

### 4.4.2 Determination of MBC of QACs and PHMB using MBEC systems

The MBEC system was used to determine the response to pre-grown biofilms to the biocides under investigation. These biofilms were exposed to biocides for 15 minutes prior to neutralisations and the enumeration of the numbers of bacteria surviving on the pegs. As previously describes a MBC was determined by the Lambert and Pearson (2002) approach with (log $TVC_{test}$ / $LogTVC_{Control}$) being plotted against the Log of the biocide concentration to generate the required inhibition profile. The resulting MBC's were significantly greater than those calculated for planktonic cells grown in the Bioscreen (Figure 4.35). It should be noted that in these 24 hour MIC experiments no growth was detected above the MIC values (Figure 4.36) from time zero, demonstrating that the biocides were having an immediate effect on the replication of these bacteria.

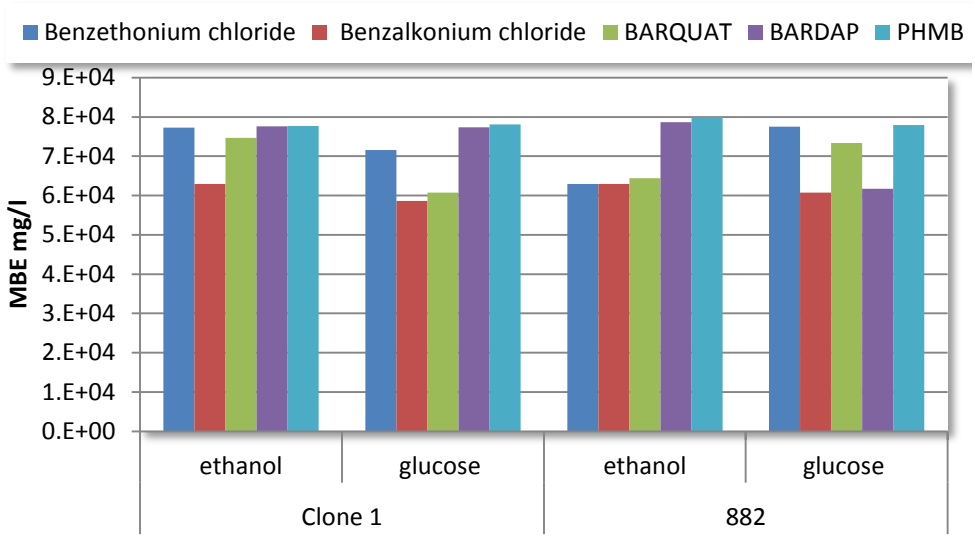



**Figure. 4.35. Biofilm MBC data for QACs and PHMB.**

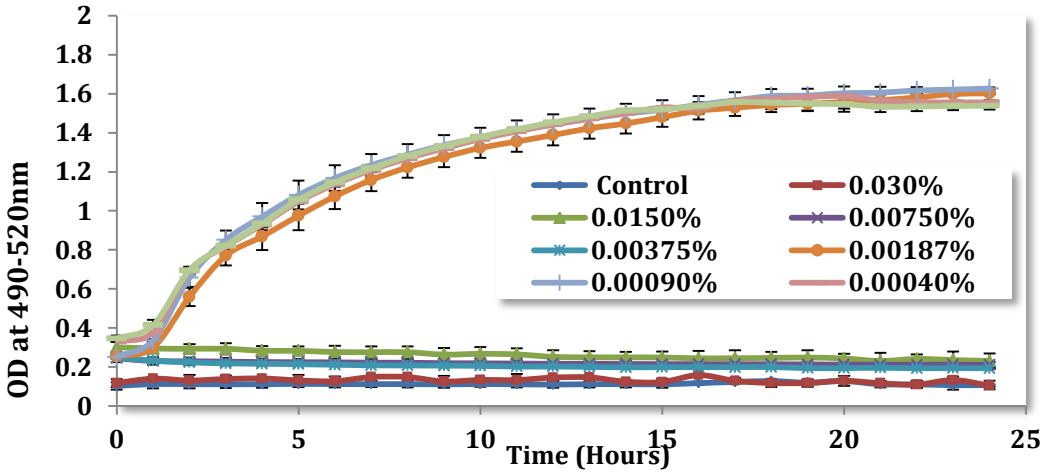
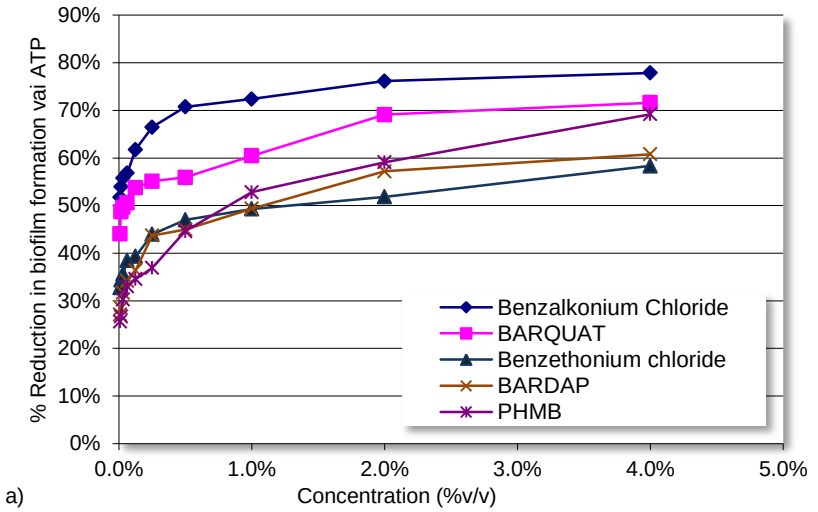



**Figure 4.36. Bioscreen output for Clone 1 grown on ethanol.**

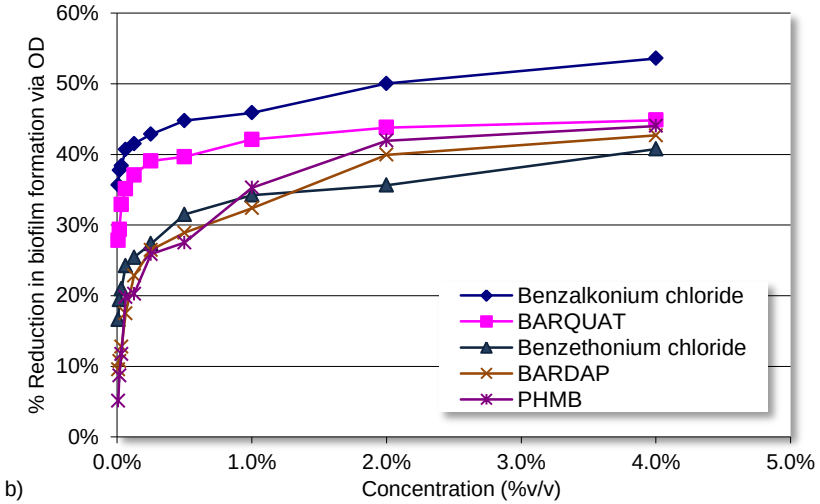
### 4.4.3 **Determination of the impact of QACs and PHMB on biofilm formation**

The MBEC system was used to determine the impact of QACs and PHMB on the ability of Clone 1 and 882 to form biofilms, which is an approximation of a MIC for a biofilm. This was carried out by placing the MBEC biofilm plates into 96 well plates that contained different levels of biocide along with the bacteria and growth media. Biofilm formation was assessed by the number of bacteria recoverable from rinsed pegs after 24 hour incubation. Two approaches were used to determine biomass concentration firstly a semi quantitative method based on optical density and secondly a quantitative method using ATP content (See section.2.5.6).

Both approaches generated a similar biofilm formation vs concentration relationship indicating that ATP and OD measurements were both acceptable indications of biofilm formation (Figure 4.37-4.40). In all cases (both bacteria and both carbon sources) no biofilm formation occurred above a concentration of 5% for all biocides under investigation. This sharp cut off meant that the application of the Lambert and Pearson (2002) approach was not appropriate since all the MIC's calculated were at or around 5% (data not shown). Between 5% and 0.0078% there was a linear reduction in biofilm formation when plotted against the log of the biocide concentration (Figure 4.41 to 4.44).

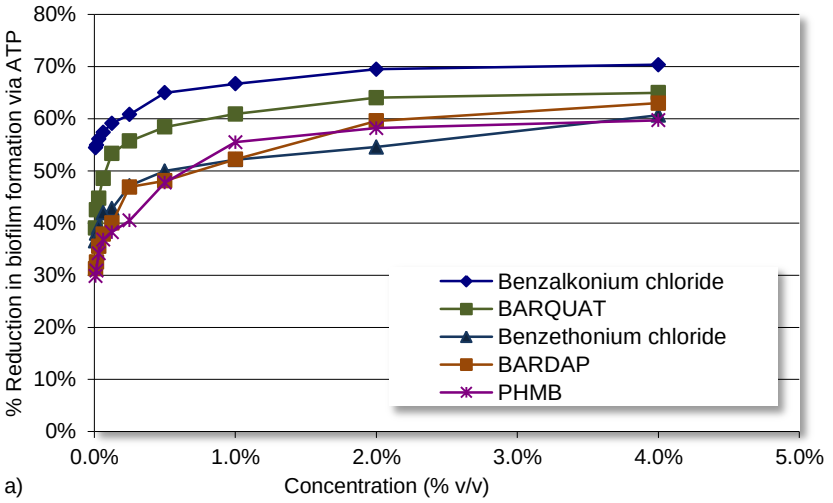



a)

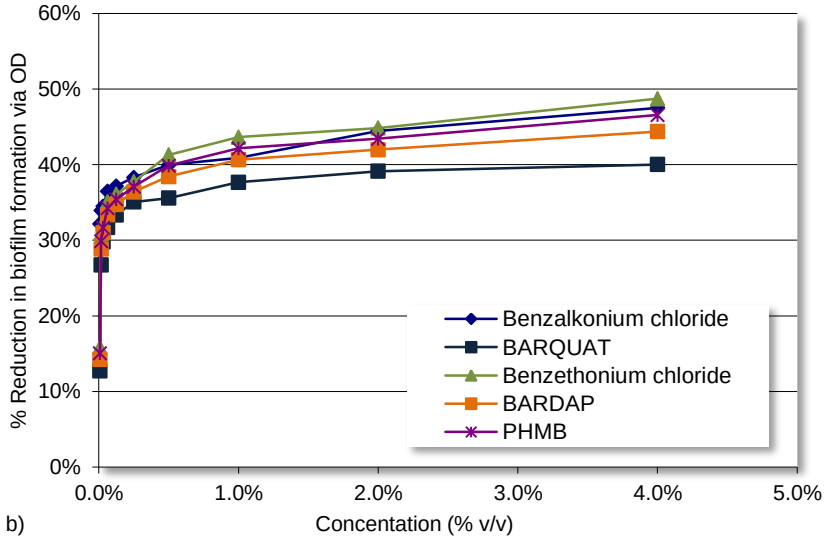



b)

**Figure. 4.37. Biofilm formation: Clone 1 on Glucose a). ATP, b). OD.**

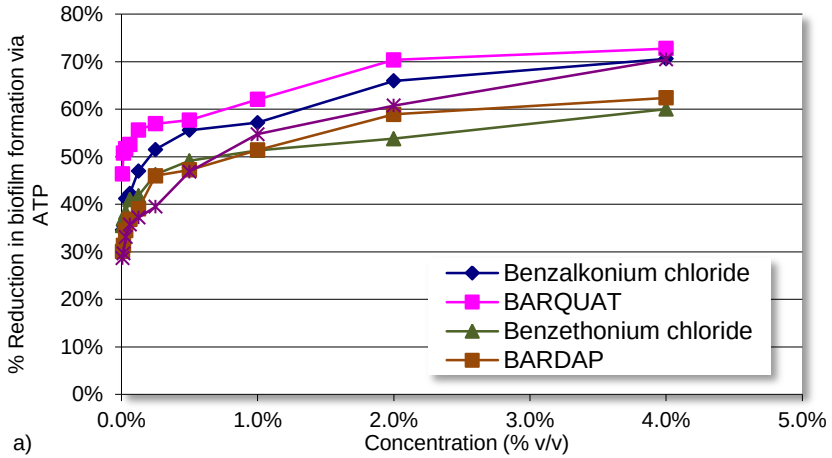



a)




b)

**Figure. 4.38. Biofilm formation: Clone 1 on Ethanol a). ATP, b). OD.**



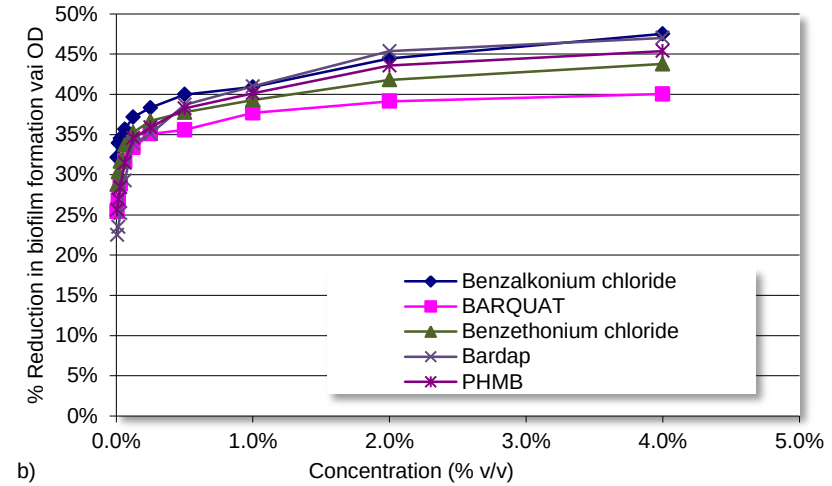
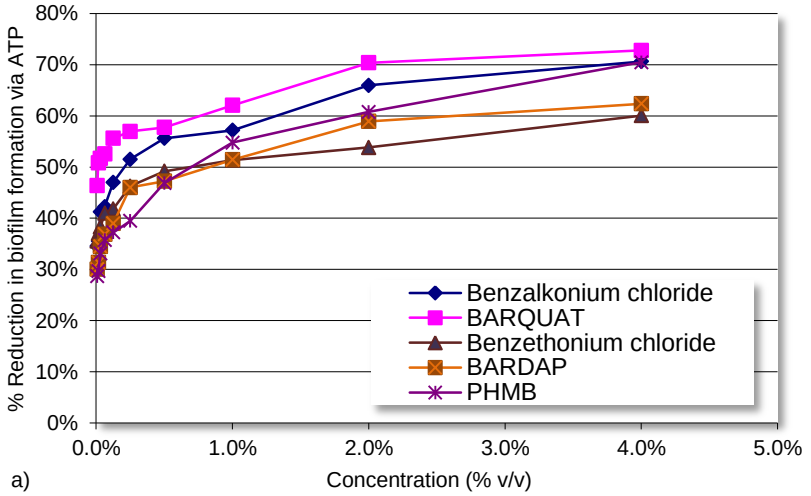




**Figure. 4.39. Biofilm formation:882 on Glucose a). ATP, b). OD.**



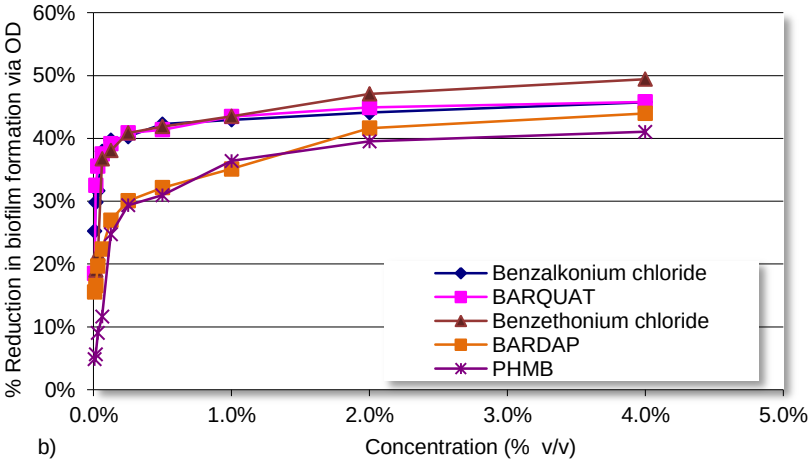
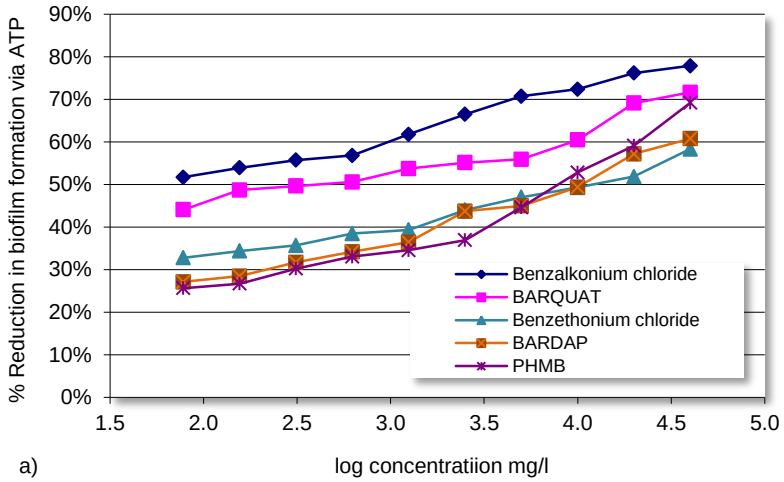
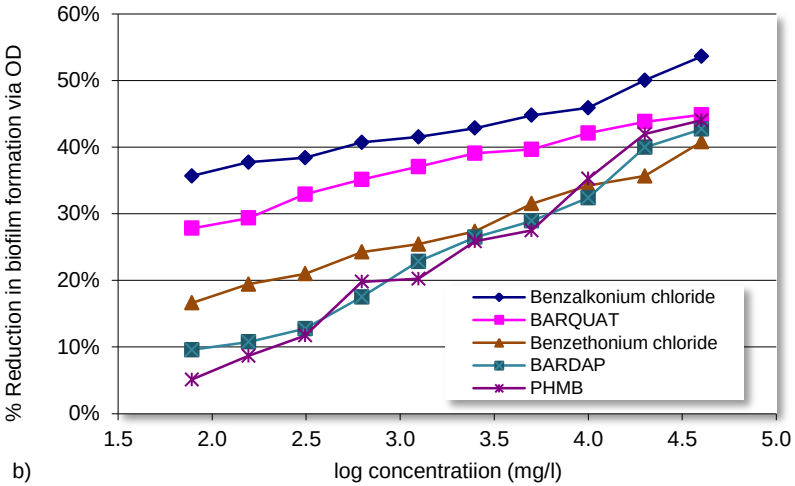




**Figure. 4.40. Biofilm formation: 882 on Ethanol a). ATP, b). OD.**

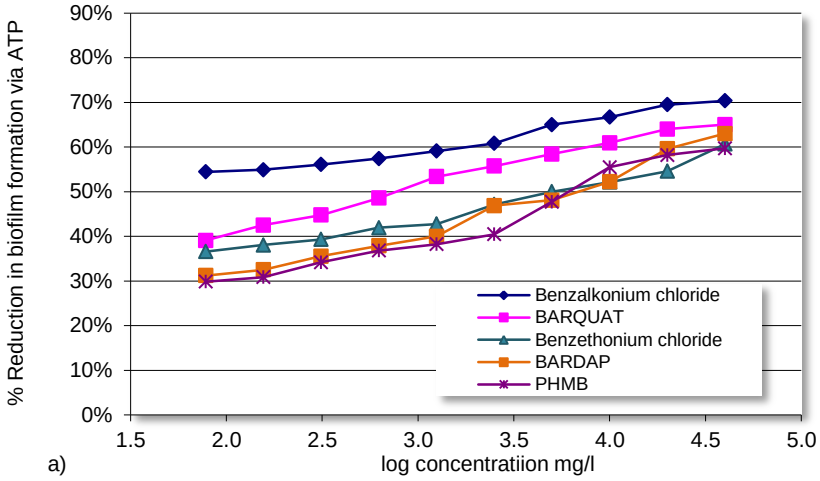



a)

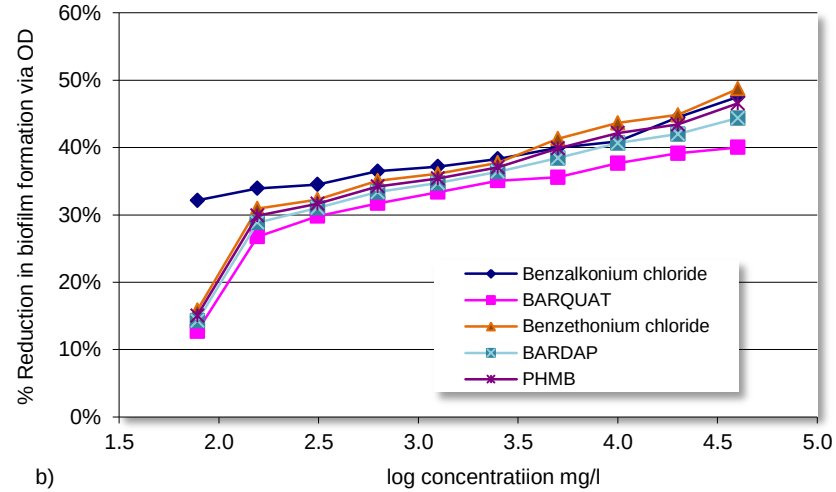



b)

**Figure. 4.41. Concentrations vs biofilm formation: Clone 1 on Glucose a) ATP, b). OD.**

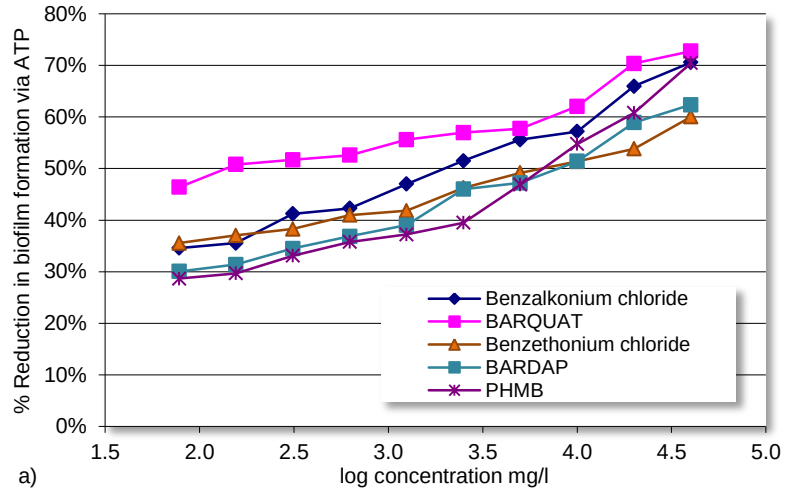



a)




b)

**Figure. 4.42. Concentrations vs biofilm formation: Clone 1 on Ethanol a) ATP, b). OD.**



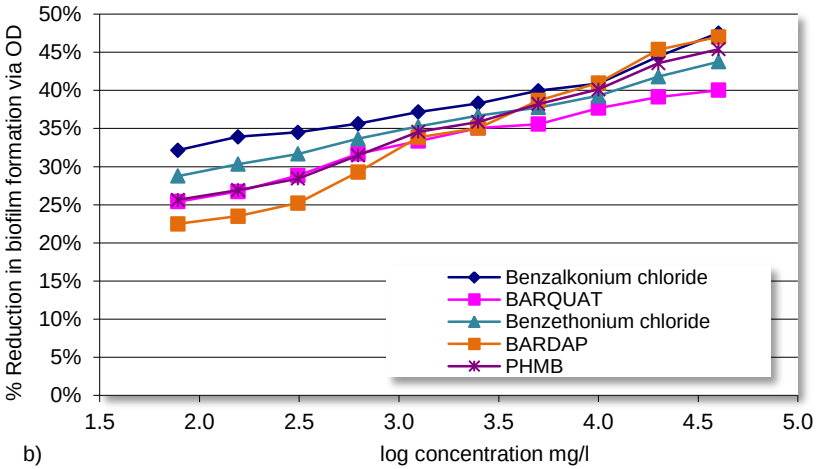
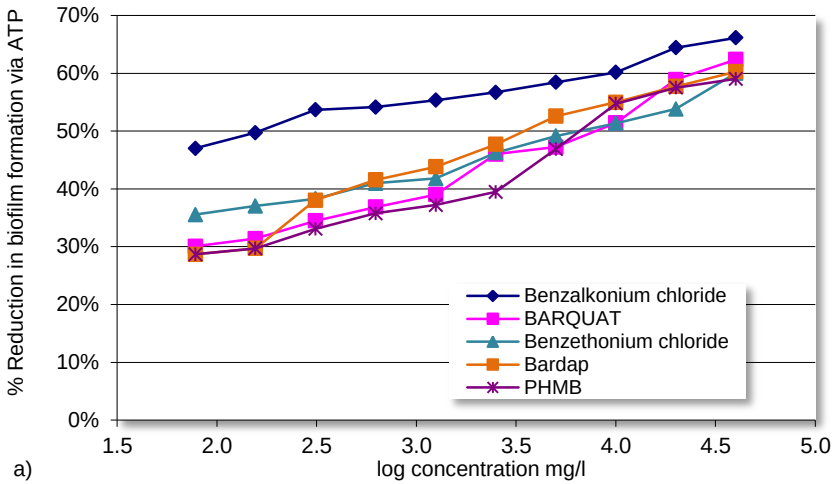




**Figure. 4.43. Concentrations vs biofilm formation: 882on Glucose a). ATP. b). OD.**



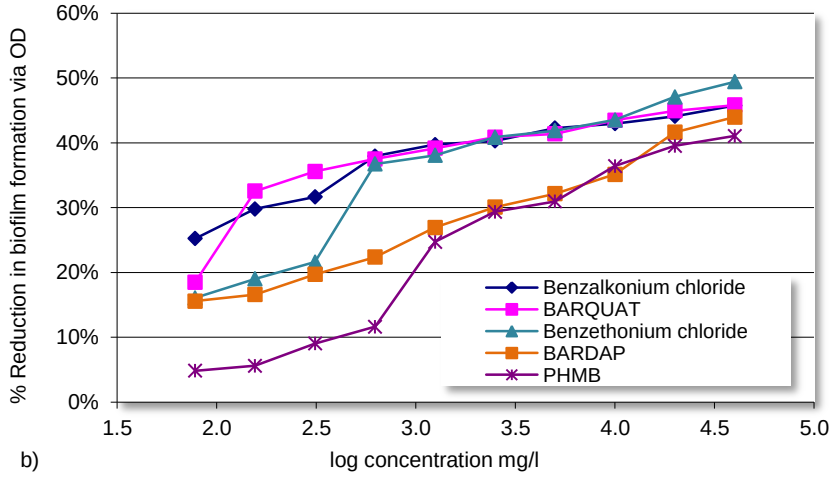




**Figure. 4.44. Concentrations vs biofilm formation: 882 on Ethanol a) ATP. b). OD.**

### 4.4.4 **Discussion**

The MIC and MBC of a range of quaternary ammonium compounds (QACs) and PHMB has been determined for the two strong biofilm forming strains i.e. Clone 1 and 882 on different carbon sources i.e. ethanol, glucose using the Bioscreen technology. In the case of glucose both strains (Clone 1 and 882) were least sensitive to PHMB and most sensitive to Barquat, however in the case of ethanol there were no major differences observed between biocides in 882 and in case of Clone 1 Benzethonium chloride was the most effective as compared to other biocides.

The MBC determined for both strains demonstrated that they have the ability to survive concentrations 80% greater than the MIC. The greatest difference (≈100x greater) was seen with Clone 1 grown on glucose and exposed to Benzethonium chloride and the smallest difference was (≈6x greater) seen when 882 was grown on ethanol and exposed to Benzethonium chloride. In the case of the QACs ethanol grown cultures had lower MBC than glucose grown cultures, which may be due to the solubilisation of QACs in alcohols may facilitate the entry of these biocides into the bacterial cells.

MBC for biofilms were also determined using pre-grown biofilms on MBEC pegs. MBC for biofilms were an order of magnitude greater ($10^4$ mg/l) than those calculated for planktonic cells ($10^2$ to $10^3$ mg/l) indicating that the formation of biofilms increased the ability of *Acinetobacter spp* to survive treatment with QAT's and PHMB. Given that biofilm formation provided such a significant improvement in survival, the ability of *Acinetobacter spp.* to form biofilms in the presence of these biocides becomes important. In all cases (both bacteria and both carbon sources) no biofilm formation occurred above a concentration of 5% ($5x10^4$ mg/l) for all biocides under investigation and between 4% and 0.0078% there was a linear reduction in biofilm formation when plotted against the log of the biocide concentration (Figure 4.4139 to 4.44). This indicates that planktonic *A. baumannii* cells can form biofilms at concentrations above the MBC for planktonic cells and close to or equal to the MBC for pre-grown biofilms. These results correlate with the findings of Kuwamura-Sato *et al* (Kawamura-Sato *et al.* 2008) who determined the MICs and MBCs values of different biocides i.e. chlorhexidinegluconate, benzethonium chloride bezalkonium chloride and alkyl diaminoethylglycine hydrochloride (ADH) against a range of clinical isolates of

*Acinetobacter*. $MIC_{90s}$ obtained by the broth micro-dilution method for benzethonium chloride and bezalkonium chloride were ≤25mg/L which is consistent with the results obtained with glucose grown isolates and are generally lower than the ethanol grown cultures. However, maximum MIC for specific strains were 50 mg/l for bezalkonium chloride and 100 mg/l for benzethonium chloride values greater than any recorded in this study. They also determined the MBC values of the four disinfectant and found that the MBC for the majority of strains were <64 mg/l, although the presence of organic material (3% BSA) generated higher MBC values (512 mg/L) which were closer to the values generated here. Although the methods employed to determine MIC and MBC values are different to those employed here the major difference is that in this study there is a greater difference between the MBC and the MIC values. Other authors have assessed the susceptibility of *Acinetobacter sp* to disinfectants (Martro *et al.* 2003, Wisplinghoff *et al.* 2007), however, they did not consider the disinfectants considered here. In both cases they did not find any correlation between antibiotic resistance and biocide susceptibility. One study that did consider Benzalkonium and Benzethonium chloride (Kawamura-Sato *et al.* 2010) found some correlation between antibiotic resistance and a reduced susceptibility to these biocides amongst a small number of clinical isolates.

The impact of these biocides on other bacteria has been more extensively investigated. Joynson et al (Joynson et al., 2002), reported that the MICs of Benzalkonium chloride against *Pseudomonas aeruginosa* NCIMB 10421 was 25.4mg/L. Penna and his collegues (Penna *et al.* 2001), worked with 10% w/v Benzalkonium chloride and monoquaternary mixture of alkyldimethylbenzylammonium chlorides and found that MICs of 156mg/L against *Bacillus stearothermophilus* ATCC 7953, 59mg/L against *E.coli* ATCC 25922, 78mg/L against *Enterobacter cloacae* IAL 1976 and 59mg/L against *Serratia* and *Staphylococcus aureus* ATCC 25923. Walsh et al (Walsh et al., 2003a), checked the MICs of Didecyldimethylammonium chloride (DDDMAC or DDAC) and reported a MIC of 5mg/L against *Staphylococcus aureus* ATCC 9518 and *E.coli* ATCC 10536 but 500mg/L against *P. aeruginosa* ATCC 15442. Ioannou et al (McBain et al., 2004) used Didecyldimethylammonium chloride (DDDMAC or DDAC) and found the MICs against *Staphylococcus aureus* ATCC 6538 was 0.4mg/L. They also determined the MICs of N-alkyldimethylbenzylammonium chloride against *Staphylococcus aureus* ATCC

6538 and found a MIC of 0.7mg/L. Lambert and Pearson (Lambert and Pearson, 2000a) worked on the MICs of N-alkyltrimethylammonium bromide ($C_8$, $C_{10}$, $C_{12}$, $C_{14}$, $C_{16}$, $C_{18}$) against *Staphylococcus aureus* ATCC 6538 and *P. aeruginosa* ATCC 2730 and reported that MICs of *Staphylococcus aureus* ATCC 6538 against $C_8$ was 594mg/l, $C_{10}$ was 79.4mg/L, $C_{12}$ was 7.9 mg/L, $C_{14}$ was 1.22 mg/L, $C_{16}$ was 0.51 mg/L and $C_{18}$ was 1.02 mg/L. However, in case of *P. aeruginosa* ATCC 2730 the MICs values were higher as compared to *Staphylococcus aureus* ATCC 6538 against all the of N-alkyltrimethylammonium bromide i.e $C_8$ (4844 mg/L), $C_{10}$ (1462 mg/L), $C_{12}$ 346 (mg/L), $C_{14}$ (83.7mg/L) and more than 1000 mg/L against both the $C_{16}$ and $C_{18}$. McBain *et al* (McBain et al., 2004), determined the MICs of Bardac against different bacteria and reported that MICs against *Pseudomonas* sp strain MBRG 4.7 was 15.6mg/L, *Enterococcus saccharolytics* strain MBRG 20.9 was 31.2mg/L, *Aeromonas hydrophila* MBRG 4.3 was 15.6, *Citrobacter sp*. strain MBRG 20.9 was 7.8mg/L and against *Sphingobacterium multivorum* MBRG 30.1 was 3.9mg/L. (Espinal et al., 2011).

The prolonged survival of clinical isolates of *Acinetobacter spp.* at the MIC has been reported by other authors (Kawamura-Sato et al., 2008, Kawamura-Sato *et al.* 2010), this agrees with the observations in this study that *A. boumannii* strains can survive inhibitory concentrations of biocides for prolonged periods prior to the formation of biofilms. Thomas *et al* (Thomas *et al.* 2011) reported that effective disinfection in the presence of a biofilm often requires higher concentrations of antimicrobial agents when compared to planktonic bacteria. The antimicrobial concentrations required for biofilm inactivation can be 100 times that required for inactivation of removal planktonic cells (Rasmussen and Givskov, 2006). A similar observation was made here where the increase in MBC was between 10 and 100x the MBC concentration for planktonic cells. These impacts may be species specific for example the efficacy of ortho-pthalaldehyde was reduced against *Mycobacterial* biofilms but not against *P. aeruginosa* ones. Survival of bacteria in biofilms has been identified as contributing to a number of outbreaks, e.g. *P. aeruginosa* resistance to iodophores, *Serratia marcescens* resistance to benzylkonium chloride and chlorhexidine. Recently a major outbreak of *Pseudomonas* infections in a neonatal unit in Northern Ireland was associated with biofilms in water distribution system (RQIA, 2012).

### 4.4.5 **Key findings**

- MIC values for both strains were below 35 mg/l for all biocides tested;
- There was a greater variability in MIC values apparent for the glucose grown bacteria rather than the ethanol grown bacteria;
- MBC for planktonic cells were from 6 to 100 x greater than the MIC values;
- MBC were lower for ethanol grown bacteria rather than glucose grown bacteria;
- MBC values for biofilms were orders of magnitude greater than MBC values for planktonic cells with little variation between biocides or carbon source;
- Planktonic cells were able to form biofilms at concentrations up to a concentration of 4% for all biocides, a concentration above the 24 hour MIC and MBC for planktonic cells.

## 4.5 Evaluation of antimicrobial wound dressing

A range of common antimicrobial wound dressings (NSCD, ISCD, Honey and PHMB) were evaluated for their ability to prevent the growth of a range of commonly occurring wound pathogens i.e. *A. baumannii*, *P. aeruginosa* and *S. aureus*. Two approaches were used to evaluate these dressings the first employing planktonic cells and the second cells immobilised in a collagen matrix.

### 4.5.1 Planktonic cells

In the majority of cases there were significant differences ($p<0.05$) between strains of the same species when treated with the same dressing. The only exception being *S. aureus* treated with the Honey dressing where ANOVA suggested that there were no significant difference at 95% confidence limits between species (See Appendix). This meant that it was not possible to combine the data on a species by species basis. It also indicated that significant variations in the susceptibility of wound pathogens to antimicrobial dressings were present at the sub species level (Table 4.6) in a number of cases the difference between minimum and maximum impacts were many orders of magnitude.

| Dressing | Mean Log Reductions (cfu) | | | | | | *MaximumDifference* |
|---|---|---|---|---|---|---|---|
| | *A. boumannii* | | *P. aeruginosa* | | *S. aureus* | | |
| | Min | Max | Min | Max | Min | Max | |
| NSCD | 7.2 | 9.9 | 6.8 | 8.4 | 6.6 | 7.4 | 2.7 |
| ISCD | 6.0 | 8.5 | 5.3 | 8.3 | 2.5 | 7.2 | 4.7 |
| Honey | 2.8 | 9.4 | 5.3 | 8.3 | 6.3 | 6.9 | 6.6 |
| PHMB | 2.7 | 4.9 | 3.3 | 9.1 | 5.3 | 7.0 | 5.8 |

**Table 4.6 Variations in response to antimicrobial wound dressings-planktonic cells.**

The NSCD dressing demonstrated a high level of antimicrobial activity generating kills close to the maximum possible across all strains investigated (Figure 4.45 a). Total kills i.e. no recoverable bacteria are indicated by the diagonal hatching on the histogram bars. After NSCD the Honey dressing performed best with maximum kills for a number of *A. baumannii* and *P. aeruginosa* strains (Figure 4.45 c) and a consistent level of kill for *S. aureus* with no significant differences (ANOVA, $P>0.05$) between the impacts on all strains tested. However, the Honey dressing did have some significant dips in

performance particularly against *A. baumannii* 882 and 292 and the Type strain of *P. aeruginosa.* In all these three cases the log reduction achieved was significantly lower ($P<0.05$) than that achieved with the majority of the other strains.

The ISCD dressing had a relatively consistent performance across the *A. baumannii* and *P. aeruginosa* strains tested (Figure 4.45 b) but failed to generate a complete kill in all but two occasions. It was significantly poorer ($P<0.05$) in performance when compared to NSCD for 4 out of the 5 *A. baumannii* strains, however with the exception of the Type strain there was no significant difference ($P>0.05$) between ISCD and NSCD for the other *P. aeruginosa* strains. In the case of *S. aureus* ISCD was again significantly ($p<0.05$) poorer in performance than NSCD for the majority of strains. A similar picture emerges when ISCD is compared with the Honey dressing with the latter being generally more effective. The only exception being *A. baumannii* strain 882 where the Honey dressing has a significantly poorer performance ($p<0.05$).The PHMB dressing had the poorest overall performance of the four dressings failing to generate a complete kill for any of the bacterial strains tested. It was particularly poor against *A. baumannii* where is had significantly poorer performance ($P<0.05$) than any of the other dressings against any of the strains with the exception of 882 and the Honey dressing. PHMB performed better against the strains of *P. aeruginosa* and *S. aureus* tested, performing as well as the other dressings in the majority of cases although it did have particularly poor performance against the *P. aeruginosa* type strain.

### 4.5.2 **Collagen immobilised cells**

As was the case with the planktonic cells there were significant differences (ANOVA, $p<0.05$) between strains of the same species when treated with the same dressing, consequently it was not possible to collate data on a species level (See Appendix). It also indicated that collagen wound model reflected the significant sub-species levels variations in the susceptibility to antimicrobial dressings seen in the planktonic model (Table 4.7) in a number of cases the difference between minimum and maximum impacts were many orders of magnitude. Unlike the planktonic model the NSCD and ISCD produced broadly similar results (Figure 4.46 a & b) with the major differences being seen with the *P. aeruginosa* strains where NSCD generated greater log reductions than ISCD in 4 out of the 6 strains tested ($p<0.05$, See Appendix). There were no differences between the two dressings when tested against *S. aureus.*

| Dressing | Mean Log Reductions (cfu) | | | | | | *Maximum Difference* |
|---|---|---|---|---|---|---|---|
| | *Acinetobacter* | | *Pseudomonas* | | *S. aureus* | | |
| | Min | Max | Min | Max | Min | Max | |
| NSCD | 6.6 | 8.9 | 6.3 | 8.3 | 4.8 | 8.6 | 3.8 |
| ISCD | 6.2 | 8.9 | 4.8 | 8.3 | 5.1 | 8.6 | 3.5 |
| Honey | 2.0 | 6.2 | 1.8 | 4.9 | 4.9 | 5.6 | 4.2 |
| PHMB | 2.6 | 6.4 | 3.6 | 8.3 | 3.6 | 4.2 | 3.8 |

**Table 4.7 Variations in response to antimicrobial wound dressings-immobilised cells.**

The NSCD performed significantly better than the Honey dressing for all strains with the exception of *S. aureus* 4330 and significantly better than the PHMB dressing for all strains with the exception of the *P. aeruginosa* Type strain and Type 5. The ISCD performed significantly better than the Honey dressing for all strains with the exception of *S. aureus* 4330 and *P. aeruginosa* Type 2 and significantly better than PHMB with the exception of three *P. aeruginosa* strains (Type, Type 3 and 5) (See Appendix). Comparison between the Honey and the PHMB dressing was variable with Honey being generally better against *S. aureus*, whilst PHMB was generally better against *P. aeruginosa,* with a broadly similar picture against *A. baumannii* (See Appendix).

### 4.5.3 **Comparison between the two methods**

The use of collagen potentially provided a diffusion barrier to the antimicrobials present in the dressing. Consequently, you might expect that reduction measured through the collagen approach to be less than that seen for the planktonic approach where there is no barrier between the dressing and the bacteria. This is generally the case across all the dressing with more strains showing no difference between the two approaches or the collagen approach generating a lower reduction in viable counts than the planktonic approach (Table 4.8, See appendix). However, in the case of the ISCD, Honey and PHMB dressings there are exceptions to this expectation suggesting that the collagen did not generate a consistent barrier to the diffusion of the active ingredients.

| | NSCD | | | ISCD | | | Honey | | | PHMB | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | C > P | C=P | C<P | C>P | C=P | C<P | C>P | C=P | C<P | C>P | C=P | C<P |
| *A. baumannii* | | All | | | 882, 292 | All others | 882 | 292 | All others | 882, 292 | | All others |
| *P. aeruginosa* | | Type, T4 | All others | Type | T4 | All others | | | All | Type | T3,4 & 5 | T1,2 |
| *S. aureus* | | 6538, 9B/F6 | All others | 6538 4300 | 9B/F6 | 7F/C7 13142 | | 6538 9B/F6 | All others | | | All |

C>P: Collagen reduction greater than planktonic reduction, C=P: No difference between methods, C<P: Collagen reduction less than planktonic reduction. Differences at 95% CLs (See appendix).

**Table 4.8 Comparison of the two assessment methods**

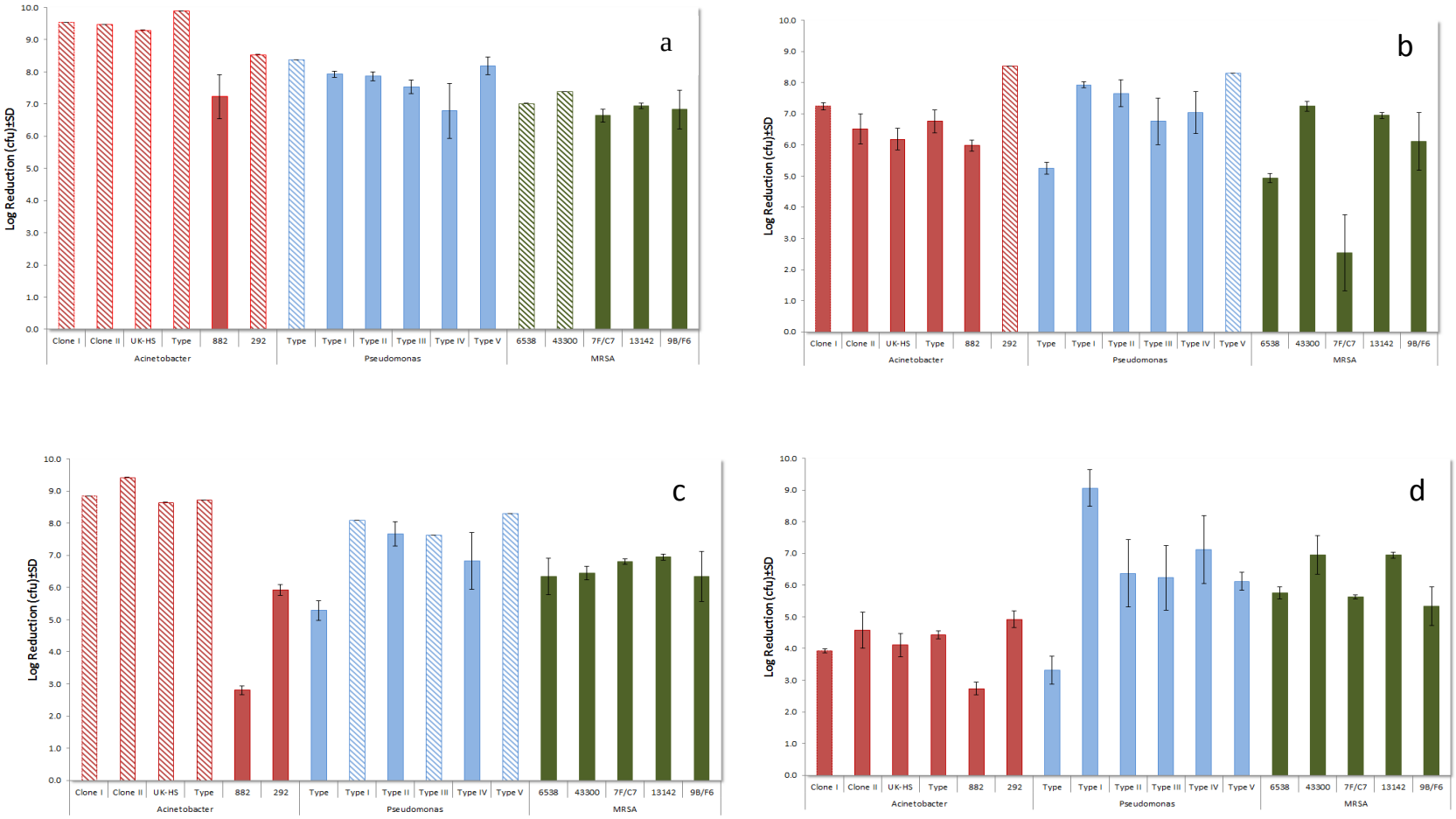



**Figure 4.45. Biocidal impact of antimicrobial wound dressings against planktonic cells (a) NSCD (b) ISCD(c) Honey (d) PHMB.**

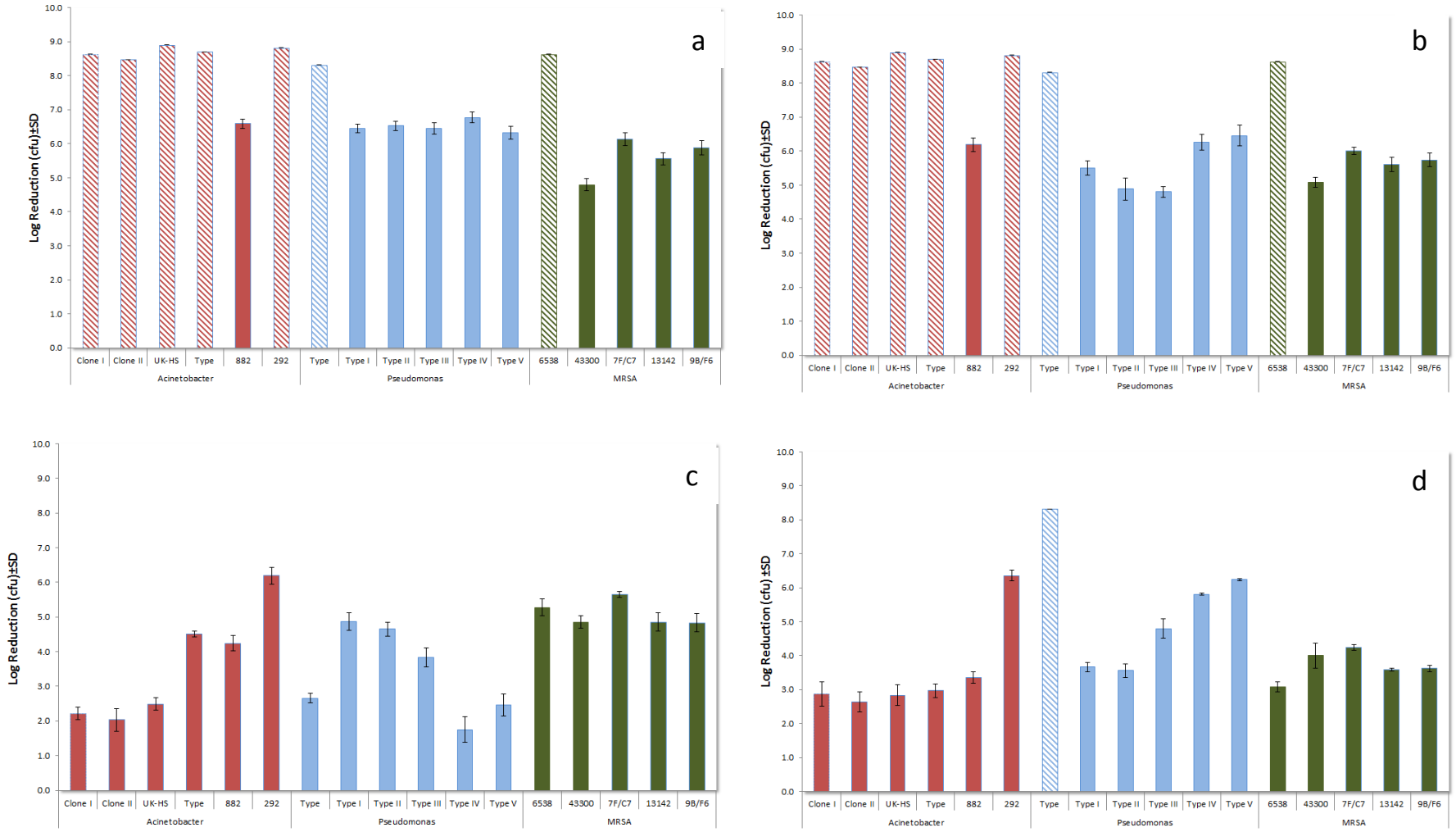



**Figure 4.46. Biocidal impact of antimicrobial wound dressings against immobilised cells (a) NSCD (b) ISCD(c) Honey (d) PHMB.**

### 4.5.4 **Discussion**

A range of antimicrobial wound dressings (NSCD, ISCD, Honey and PHMB) were evaluated for their ability to prevent the growth of a range of commonly occurring wound pathogens i.e. *A. baumannii*, *P. aeruginosa* and *S. aureus*. Two approaches were used to evaluate these dressings i.e. planktonic cells and the second cells immobilised in a collagen matrix. In both approaches there were significant differences ($p<0.05$) between strains of the same species when treated with the same dressing indicating that significant variations in the susceptibility of wound pathogens to antimicrobial dressings were present at the sub species level (Table 4.6). The diffusion barrier provided by the collagen matrix generated lower reduction values than the planktonic approach with a few exceptions. Generally speaking the Honey dressing was most impacted by the use of a collagen matrix than the other dressings.

The pathogens investigated included both Gram positive and Gram negative organisms known to colonise and infect a variety of dermal wounds (Bowler et al., 2012). In a recent study conducted by (Lipova et al., 2010), it was reported that, out of a total 777 bacterial strains isolated from burn patients, 65% of these strains were identified as Gram-Positive and 35% as Gram-negative. The most commonly isolated Gram-Positive opportunistic pathogens were coagulase-negative *Staphyloccous and Bacillus sp* and most common among the Gram-negative opportunistic pathogens includes *E. coli, A. baumannii* and *P. aeruginosa* (Lipova et al., 2010).Since bacteria are capable of forming biofilm on living tissues e.g. a wound bed, which enhances their tolerance to antimicrobial agents (Zubair et al., 2011) and the host immune cells, it was important to evaluate the susceptibility of these pathogens to the variety of dressing in both of their free living and immobilised forms. The results of NSCD and ISCD dressings correlate with the finding of other studies that showed greater antimicrobial activity of NSCD than ISCD dressings (Tkachenko and Karas, 2012, Bradshaw, 2011, Thomas *et al.* 2011). Silver has been used medically for thousands of years (Thomas *et al.* 2011). Dressings containing silver has recently been strongly marketed and has increased in usage by ~200% since 1996 and now used extensively in the care of chronic wounds (Bradshaw, 2011). The antimicrobial properties of Silver are due to its ability to form ionic salts ($Ag^+$) in the presence of acids. Positively charged silver ions are attracted by the negatively charged structures of cell membrane which allows the silver to bind and

enter into the bacterial cell (Michaels et al., 2009). The interaction of (Ag+) with the bacterial thiol (-SH) group leads to inactivation, blocking the key pathways such as cellular respiration structural changes in the bacterial membrane and blocking of enzyme and transport systems (Asavavisithchai *et al.* 2010). They may also act by denaturing bacterial RNA or DNA which may lead to the inhibition of transcription and replication (Fong and Wood, 2006). Unlike antibiotics, which are generally biochemically specific, metals are toxic to multiple components of the bacterial cells. This multi-system affect means it is less likely that bacteria will develop the resistance to silver, as multiple random mutations required. However, it might be possible that exposure of low level of silver may aid the development of resistance (Leaper, 2006). The different forms of silver has been tested and it has been proved that the elemental silver (Ag) has little or no antibacterial activity; however in its ionic cation form (Ag+), it is highly active, in the presence of wound exudates, silver readily ionizes. Different variation in the form of silver between dressings may therefore affect the ability to release ions (Lansdown, 2004). Silver is effective against some antibiotic resistance bacteria, including MRSA and Vancomycin-resistant Enterococci (Jones et al., 2004). In addition, MRSA isolated from wound for known silver resistant genes were tested and were found to be susceptible to the silver dressing used (Loh *et al.* 2009). Silver based dressing are widley used in wound care (Klasen, 2000b, Klasen, 2000a, Demling and Desanti, 2001, Ip *et al.* 2006), the only side effects reported is skin discolouration and irritation (White and Cooper, 2005). Qquestions have been raised regarding the long term use of silver on wounds especially in infants (Denyer, 2009), but to date no pathological consequences of silver used dressing has been reported, except some reports about the systemic uptake and deposition of silvers in organs (Denyer, 2009, Wang *et al.* 2009).

The results with Manuka honey dressing correlates with the findings of other studies that showed antimicrobial activity of honey (Bradshaw, 2011, Stephen-Haynes, 2004). These dressings have been successfully used for the treatment of recalcitrant wounds containing pathogens resistant to antibiotics; the dressings can be applied directly to the wound and can be used for 2-3 days without changing (Visavadia *et al.* 2008). However a large scale randomized trial showed no significant advantage for the use of

honey dressings over standard wound care in the treatment of chronic wounds (Du Toit and Page, 2009).

The exact mode of action of honey dressings is not fully understood, however it is hyperosmolar and thus restricts the availability of environmental water to bacteria and other organisms (Molan, 2001), which results in cell disruption and death. Another property of honey is the release of hydrogen peroxide as the honey is diluted by exudates (Molan, 2004). The production of hydrogen peroxide being associated with glucose oxidase activity (French *et al.* 2005). Although honey has a general broad-spectrum antimicrobial action, different honey e.g. Manuka (New Zeland), Heather (UK) and Khadikraft (India) vary substantially in their activity (Mullai and Menon, 2007). Some honeys varieties particularly the Manuka and *Leptospermum* honeys have the ability to retain their antimicrobial property even without the release of hydrogen peroxide (Cooper et al., 2002b, Cooper *et al.* 2002a), which is thought to be associated with a phytochemical component (Molan, 2002). These phytochemical factors (non-peroxidase factors) exert a high antimicrobial effect in some honeys (e.g. Manuka honey) that do not degrade even when treated with heat or light and remain effective following dilution (Olaitan *et al.* 2007). Consequently the antibacterial effect of honey has been attributed to a range of properties including pH, osmosis, hydrogen peroxide and phytochemical content (Stephen-Haynes, 2004).

Recently polyhexamethylenebiguanide (PHMB) has been introduced as a component of wound dressings. PHMB is a fast acting biguanide compound composed of synthetic mixture of polymers, having structural similarities to the antimicrobial peptides AMPs produced by many cells within the wounds, such as keratinocytes and inflammatory neutrophils, where they are thought to help the cells against infection (Sørensen et al., 2003, Ousey and McIntosh, 2009). AMP have a broad spectrum of activity against bacteria, viruses and fungi including cell death by disrupting cell membrane integrity (Moore and Gray, 2007) and are proven to be effective against *P. aeruginosa* and *S. aureus* which are common in many chronic wounds (Abdelrahman and Newton, 2011). PHMB products include Telf™ AMD drain, Kendall AMD antimicrobial foam (0.5 % PHMB), Biocellulose based PHMB-donating dressings e.g Suprasorb X+PHMB, which

contains 0.3% PHMB. In vitro and in vivo studies have proved that dressing or products containing PHMB reduces wound pain rapidly and effectively (Daeschlein et al., 2007, Galitz et al., 2009), reduces wound malodour (Daeschlein et al., 2007), reduces MMP-induced periwound breakdown (Cazzaniga et al., 2007, Werner et al., 2004), reduces slough within wound (Mueller and Krebsbach, 2008), increases keratinocyte and fibroblast activity (Wiegand *et al.* 2007), increases formation of granulation tissue (Mueller and Krebsbach, 2008), and helps remove non-viable tissue (Kaehn, 2009). Alblas et al (Alblas et al., 2011, Wild *et al.* 2012) reported the antimicrobial activity of PHMB against treatment or prophylaxis of local infections in burns and trauma wounds.

In wound care, PHMB has demonstrated the ability to block *P. aeruginosa* induced infections (Cazzaniga et al., 2007), and can also kill a range of bacteria and fungi (Lee *et al.* 2004b, Werthen *et al.* 2004). A number of German studies have shown that PHMB demonstrated a positive effect on bacterial biofilms (Harbs and Siebert, 2007, Butcher, 2012). A prospective, randomised study was conducted to directly compare the efficiency of two PHMB products on the eradication of MRSA infections proved that 6 out of 15 patients were MRSA free after one week of therapy, and 10 out of 15 were MRSA free by the end of week two. In Suprasorb X+PHMB dressing group 13 out of 15 were MRSA negative at the end of week one ($p<0.05$), and were all negative by the end of the week two ($p<0.05$) (Gray et al., 2010).

### 4.5.5 **Key findings**

- Broadly speaking the NSDC dressings was the most effective wound dressing overall, with the PHMB dressing being the least effective;
- The biocidal impact of the Honey dressing was the most effected by immobilising bacteria in a collagen matrix;
- There were significant differences in susceptibility to all dressings between strains of the same species.

# 5 General Discussion

Historically the clinical isolation of *Acinetobacter sp* was frequently ignored, since it was often considered a low grade pathogen (Bergogne-Berezin *et al*., 1996). However, in recent years, multi-drug resistant (MDR) strains of *A. baumannii* have emerged as a major cause of nosocomial infections associated with significant morbidity and mortality (McConnell *et al.* 2012). Over the last 20 years a worldwide expansion in *Acinetobacter* infections has been observed associated with intensive care units (ICUs), long term care facilities and wounded personnel (Sebeny et al., 2008, Sengstock *et al.* 2010). The developing resistance patterns seen in *Acinetobacter sp* suggest that the number of effective antibiotics may shortly be exhausted (Hanlon, 2005). The ability of *Acinetobacter sp* to resist desiccation and persist on hospital surfaces, materials and medical devices has played a critical role in the emergence of this bacterium as a human pathogen (Villegas and Hartstein, 2003). *A. baumannii* is able to form strong adherent biofilms that help the bacteria to survive for several weeks on abiotic surfaces resulting in contamination of hospital and medical instruments, e.g. incubator tubes, water lines, cleaning instruments, pillows and linen (Harrison et al., 2008, Donlan, 2008, Villegas and Hartstein, 2003, McConnell *et al.* 2012). The ability of clinical strains of *A. baumannii* to form strong adherent biofilms has now been recognised as a key virulence factor for this pathogen (Wroblewska *et al.* 2008).

In this study the MICs and MBCs of alcohols and alcohol containing products against clinical strains of *A. baumannii* were determined. It was observed that the MBC of ethanol and IPA for all strains ranged from 50 to 55% v/v and the MIC of ethanol and IPA for all clinical strains ranged from 3.1 to 9.0% v/v. A carbohydrate free minimal media employing alcohols as sole carbon sources has been developed and our results revealed that all *Acinetobacter* strains under investigation have the ability to grow on minimal media supplemented with either ethanol or IPA as a sole carbon source. However, the Type strain was significantly more sensitive to alcohols and had a reduced ability to utilise ethanol or IPA as its sole carbon source. The MBC's determined here are below the in use concentrations recommended for alcohols (60-70%) (Fraise *et al.* 2012). The impact of ethanol in the presence of other carbon sources has been investigated by a number of authors (Pirog *et al.* 2002, Smith *et al.* 2004), a situation

that mirrors the experiments reported here employing TSB and MRD. The growth of *Acinetobacter* strains on minimal media with ethanol as a sole carbon source without supplementation is well established (Navon-Venezia *et al.* 1995, Walzer *et al.* 2006) a situation reflected in the growth of the clinical strains observed here. Enhanced pathogenicity due to the presence of alcohol has been reported for *Acinetobacter sp* (Smith *et al.* 2004) again in the presence of other carbon sources. The positive impact of ethanol was reduced as the concentration was raised to 5% v/v and became negative above 5% v/v (Smith *et al.* 2004), a trend that is reflected in the data collected here (Figures 4.1 and 4.2). Enhanced growth of *Acinetobacter* strains at alcohol concentrations at or around 1% v/v has been reported by a number of authors (Smith *et al.* 2004, Edwards *et al.* 2007a). Edwards *et al* (Edwards *et al.* 2007b), reported the enhancement of growth and virulence in the presence of ethanol and found the significant increase in growth when the minimal media is supplemented with ≤1% v/v of four commercially available hand rubs i.e Purell, Spirogel, Softalind and Skinman. They reported that the unknown factor which enhanced pathogenicity and virulence to *A. baumannii* was secretion of proteins in response to alcohols. One of these proteins was identified as OmpA, which was recognized as having emulsifying activity, which could be useful in scavenging carbon for growth from complex energy sources such as hydrocarbons. The secretion of OmpA by *A. baumannii* following exposure to 0.5% v/v ethanol in minimal media has also been described by Walzer *et al* (Walzer *et al.* 2006), suggesting that it may be the important response to growth and survival under low nutrient conditions. These bioemulsifiers protein may also be helpful in bacterial adhesion, quorum sensing and the development of biofilms (Ron and Rosenberg, 2001). The MIC determined for ethanol and IPA for the clinical strains investigated are towards the upper end of published values for alcohol MICs on bacteria in general that range from 1 to 5% v/v (Oh and Marshall, 1993, Mazzola *et al.* 2009, Wadhwani *et al.* 2008). One published value for *A. calcoaceticus* of 4.4% v/v (Mazzola *et al.* 2009) is broadly similar to the values generated during this study with the exception of 882 which had an MIC of approximately double this value (Table 4.4). Interestingly these authors indicated that the presence of glycerine, a common component of alcohol hand gels

increased the MIC by ≈50%, an observation that aligns with the reduced impact of Purell gel observed here (Fig. 4.2).

Biofilms are defined as highly self-organized, three-dimensional structures where the microbial community is enclosed in a polymeric matrix of exopolymeric substances (EPS), constituting a survival mechanism for harsh environments (Espinal *et al.* 2011, Jahid and Ha, 2012). Biofilms are composed of microorganisms attached to either each other, to living or non-biotic surfaces and may be embedded within a complex matrix of polysaccharides, proteins, nucleic acids and glycoproteins (Donlan and Costerton, 2002). For the effective eradication of microorganisms within a biofilm, higher concentrations of antimicrobial agent are often required when compared to their planktonic or free floating non- biofilm counterparts (Thomas *et al.* 2011). The antimicrobial concentrations required for biofilm removal can be 100-fold of that required for removal microorganisms in the planktonic state (Rasmussen and Givskov, 2006). In the context of human health 80% of infections are suggested to be biofilm related (Davies, 2003, Blackwell, 2005),for example in chronic wounds, e.g. diabetic foot ulcers, pressure ulcers and venous leg ulcers biofilms are a central part of the evolution of the infection (James *et al.* 2007). The effective and efficient use of medical devices such as catheters, artificial valves etc. are often compromised by the formation of biofilms (Strelkova *et al.* 2012). A. *baumannii* is known to form strong adherent biofilms that allow the bacteria to survive for several weeks on abiotic surfaces in healthcare settings (Harrison *et al.* 2008, Donlan, 2008, Villegas and Hartstein, 2003, McConnell *et al.* 2012). However as observed here, biofilm formation amongst *A. baumannii* strains is not consistent (Fig. 4.9), Wroblewska *et al* (Wroblewska *et al.* 2008) investigated 34 clinical strains from patients hospitalized in two tertiary care hospitals. The isolates demonstrated a wide range of biofilm forming ability, with 12% high, 41% medium and 47% demonstrating a low level of biofilm production. A similar variation in adherence was observed when the adherence of clinical isolates to human bronchial tissue was investigated (Lee *et al.* 2006). In the current study a greater number of strains demonstrated a high level of adherence (Table 4.5) with the strength of adherence being dependent on the carbon source. Wroblewska *et al* (2008) did not consider the impact of carbon source on biofilm formation, but the data here clearly reveals that

alcohol based carbon sources increase adherence, with 71% of strains being highly adherent when fed on alcohol as the sole carbon source with only the Type strain and UK-HS remaining poorly adherent regardless of carbon source. Lee *et al* (2006) observed lower levels of adherence for Clone 1 than Clone 2 onto epithelial cells, when tested here for adherence to plastic surfaces no differences between Clones 1 and 2 was seen. Other authors (McQueary and Actis, 2011) have shown the Type strain to be comparable in its ability to form biofilms but in this case TSB was employed as a growth media, emphasising the importance of the carbon source on biofilm formation. The variations between strains and the impact of carbon source on biofilm formation was revealed in the hydrophobicity data, where there were clear differences observed between strains (Figures. 4.11) and the use of alcohol as a sole carbon sources generated greater hydrophobicity than glucose, SWF or TSB (Figure. 4.12). This correlation between biofilm formation and hydrophobicity is to be expected since plastic surfaces are hydrophobic in nature.

The MATH test has also been used to monitoring the cell surface hydrophobicity of nosocomial pathogen *Pseudomonas aeruginosa* as it grew in the presence of benzalkonium chloride (Machado *et al.* 2011). They reported that the strain OO14 increased in hydrophobicity as the cells grew in resistance to the disinfectant. Bos *et al* (Bos *et al.* 1999) used the *MATH* test on dental colonisers such as *Streptococcus* and *Actinomyces* and found that the presence of divalent calcium cations increased the adhesion to hexadecane and chloroform. Costa, et al (Guimaraes *et al.* 2006) and Di Bonaventura, et al (Di Bonaventura *et al.* 2008), reported that hydrophobicity of the bacterial surface is an important factor for adherence and colonization of bacteria to both living (epithelial mucous tissues) and non-living surfaces (medical devices). Umamaheshwari and Jain, (Umamaheshwari and Jain, 2004), reported that the hydrophobic cell surface components may serve as a binding target for antibacterial lipobeads. Rosenberg (Rosenberg, 2006), reported that hydrophobic surface property of the bacteria play a vital role in growth on hydrophobic materials, initial biofilm formation, adhesion to host cells, aggregation and flocculation and it is the one of many parameters which determines the ability of a cell to adhere, invade and cause damage.

However, McQueary and Actic (2011) employed TSB as a growth media which as can be seen here does not favour biofilm formation in some *A. baumannii* strains. When the Type strain and more hydrophobic strains such as 882 and Clone 1 were grown on plastic surfaces and visualised via SEM, the less hydrophobic Type strain was unable to maintain a significant biofilm suggesting that when grown on a more minimal media the Type strain was unable to maintain a biofilm on a hydrophobic surface, indicating a direct correlation between hydrophobicity and biofilm formation. The Type strain was however able to form biofilms on hydrophilic surfaces (glass) under low sheer conditions, however, under these conditions the hydrophobic strains were also able to form biofilms through the generation of EPS.

A number of gene products have been proven to play a role in attachment and biofilm formation on abiotic surfaces e.g. pilus production mediated by the CsuA/BABCDE usher-chaperone assembly system is required for the attachment and biofilm formation on the abiotic surfaces by the *A. baumannii* Type strain. This operon seems to be wide spread among clinical isolates and an indication that it is a common factor among different clinical isolates (Tomaras et al., 2003). However McQueary and Actic (2011) demonstrated that even in the presence of this gene significant strain to strain variations in biofilm formation were evident. The Type strain also has the ability to produce alternative pili that may help in the interaction of this pathogen with bronchial epithelial cells (De Breij et al., 2010). Loehfelm *et al* (Loehfelm *et al.* 2008) reported that biofilm-associated protein (Bap), conserved in the clinical isolates and appears to be associated with the cell-cell interactions that support the development and maturation of the biofilm. In addition to (Bap), the *A. baumannii* clinical isolates also produce poly-β-1-6-N-acetylglucosamine (PNAG) for the development and maturation of the biofilm on glass surfaces by the cells cultured (Bentancor *et al.* 2012, Choi et al., 2009b). The strong biofilm forming strains was evaluated for the PNAG production both the strains (882 and Clone I) produced PNAG on Congo red plates, however no PNAG was detectable via $^{1}$H NMR suggesting that either Congo red plates is not a definitive screen for PNAG or the concentrations generated were too low to be detected following extraction.

A two component regulatory system has also been reported in the *A. baumannii* Type strain (ATCC 19606$^{T}$) which comprises of: a sensor kinase encoded by *bfm*S, and a response regulator encoded by *bfm*R involved in bacteria-surface interaction (Tomaras et al., 2008). The insertional inactivation of *bfm*R results in the loss of expression of *cusA/BABCDE* operon resulted in the lack of pili production and biofilm formation on plastic surfaces when grown in rich medium (LB broth), however the inactivation of *bfm*S sensor kinase gene resulted in diminishment but not abolishment of biofilm formation (Tomaras *et al.* 2008). In the absence of BfmRS system the composition of culture media still influence the interaction of cells with abiotic surfaces, these finding indicates that the BfmRS system cross talks with other sensing components and suggests instead of one, there are multiple and different stimuli which could control the biofilm formation via BfmRS regulatory pathway (Tomaras *et al.* 2008). However, all Tomaras *et al's* (2008) work was carried out using the Type strain with glucose as a carbon source, consequently the differences seen here with ethanol and other strains was not investigated.

Another mechanism controlling bacterial adherence and biofilm formation is cell population density. Accordingly, environmental and clinical isolates produce quorum sensing signalling molecules (Gonzalez et al., 2001, Gonzalez et al., 2009), these studies proved that a large number of isolates produce quorum sensing and signalling molecules which seem to belong to three types of molecules. Although none of these sensors belongs to a particular species, however the Rf1-type sensor is more frequently found in isolates belonging to the *A. calcoaceticus-baumannii* complex. Niu et al (Niu *et al.* 2008) proved that the *A. baumannii* M2 clinical isolates produce an *N*-acyl-homoserine lactone [*N*-3-hydroxydodecanoyl-homoserinem lactone], the product of the *aba*I auto inducer synthase gene, which is vital for the fully developed biofilm on abiotic surfaces, *aba*I auto inducer also helps this isolate to move in semisolid media.

Generally, *A. baumannii* adheres to biotic and abiotic surfaces via the same steps described for general biofilm formation. The associated EPS being composed of carbohydrates, proteins, nucleic acids and other macromolecules (McConnell *et al.* 2012). Pirog *et al* (Pirog *et al.* 2002), reported that *Acinetobacter* spp.12S has the ability

to grow and synthesize EPS on different carbohydrate substrates like mono and disaccharides, molasses and starch. He reported that the *Acinetobacter* spp. were grown on carbohydrate media containing no pantothenic acid (Vitamin B3), which is required for growth on $C_2$-substrates. He used a mixture of carbohydrate sources (0.01, 0.1, 0.5 and 1% v/v ethanol and 1% w/v glucose) and found that bacterial growth and EPS generation was higher when 0.01% v/v ethanol with 1% w/v glucose used. The EPS production was intensified as the content of the C2 substrate in the medium increased.

The growth of the *Acinetobacter* strains at the expense of alcohol as the sole carbon source without any complex media supplements has already been identified as a characteristic of hydrophilic, strongly adherent strains such as 882 and Clone 1 (Section 4.1.3). The formation of polysaccharide based EPS by these strains at the expense of alcohols as well as glucose is evident from the drip flow biofilms where the EPS is stained by the carbohydrate selective stain calcofluor white (Figures. 4.18-4.19), indicating that these strains are able to synthesise complex carbohydrates from C2 and C3 alcohols in minimal media. The formation of biofilms was observed as a blue colour around the red bacterial cells which starts forming after 24 hours, however biofilms were more obvious after 48 hours of continues growth in a drip flow reactor. Both the strains developed strong biofilm when the carbon sources were ethanol and IPA as compared to the glucose and can be seen easily by the fluorescent microscopy in addition *A. baumannii* strains 882 developed more compact biofilm as compared to Clone 1 on all the carbon sources (Figures. 4.18-19).

The generation of EPS is associated with cell growth as can be seen from the batch growth of these strains (Figures 4.14 & 4.15), the resulting EPS is not utilised as a carbon source after formation and is produced during growth along with lactate, however the Type strain does not produce any biofilm or EPS when grown on similar carbon sources. The formation of acid from glucose is a common observation for *A. calcoaceticus-baumannii* complex as is the consumption of lactate as a sole carbon source (Nemec *et al.* 2011), in this case acid is also produced from C2 and C3 alcohols. Both the strong biofilm forming strain of *A. baumannii* i.e. Clone I and 882 are able to generate high MW EPS when grown on mineral media with ethanol as a sole carbon

source. Of the EPS extraction methods employed the TCA method was most successful from the perspective of the amount of material recovered and it's MW. In all cases analysis, in particular NMR, was hampered by the low solubility of the extracted material with the use of solid state NMR partially overcoming these problems. In both cases the EPS extracted contained both galactose and rhamnose sugars.

Both the EDTA and TCA methods for EPS extraction were able to generate high MW material with Clone 1 producing the higher MW material in both cases. The TCA method isolated much higher MW material than the EDTA method suggesting that the EDTA method may be degrading the EPS resulting in smaller molecules, which may in turn result in the loss of material during dialysis which will contribute to the small amount of material available for NMR analysis. The TCA method has been successfully applied to EPS characterisation of *Lactobacilli* and *Bifidobacteria* (Salazar *et al.* 2009, Laws *et al.* 2008)with the EPS recovered from the *A. baumannii* are within the range of MWs recorded for *Bifidobacterium sp* using the same approach (Leivers et al., 2011). The MALLS and FTIR data indicate the presence of high molecular weight compounds in the extracted EPS from the two strains under investigation. The $^{1}H$ NMR and $^{13}C$ NMR and HPAEC analysis indicates that this EPS is carbohydrate based and contains both galactose and rhamnose (Fig. 4. 29). These results correlate with the finding of (Yadav *et al.* 2012), who reported FTIR analysis of *A. baumannii* sugar and sugar derivative peaks falls in the region of 1000-1100$cm^{-1}$. They also reported that the EPS produced by biofilm forming strains of *A. junii* (BB1) were primarily composed of neutral sugars (73.21%), amino acids (23%), α-amino acids (11-13%), uronic acid (10) and aromatic amino acids (1.23%) with three main sugars residues being present i.e. galactose, mannose and arabinose. The presence of galactose being consistent with the results reported here for Clone 1 and 882.

The MIC and MBC concentrations of a range of quaternary ammonium compounds (QACs) and PHMB has been determined for the strong biofilm forming strains i.e. Clone 1 and 882. In the case of glucose both strains (Clone 1 and 882) were least sensitive to PHMB and most sensitive to Barquat and Benzekonium chloride respectively, however in case of ethanol there were no appreciable difference observed between biocides in

the case of 882, in the case of Clone 1 Benzekonium chloride was the most effective as compared to other biocides. In the case of the MBC, both strains have the ability to survive for longer period of time and in case of QATs ethanol grown cultures proved to have lower MBC as compared to glucose.

Pre-grown biofilms were used to determine MBC for biofilms and to compare responses of planktonic cells and biofilm cells. MBC values for biofilms were orders of magnitude greater than those calculated for planktonic cells. The MBEC system was also used to determine the impact of QACs and PHMB on the ability of Clone 1 and 882 to form biofilms. In all cases (both bacteria and both carbon sources) no biofilm formation occurred at a concentration of 5% for all biocides under investigation and between 4% and 0.0078% there was a linear reduction in biofilm formation when plotted against the log of the biocide concentration (Figure 4.18 to 4.21).

Bacteria have a variety of mechanisms at their disposal to reduce the cytoplasmic concentration of biocides (Maillard, 2007). However, it has been difficult to producing stable bacterial resistance to high biocide concentrations (Suller and Russell, 1999, Fitzgerald et al., 1992). The use of step wise biocide concentration increases have resulted in bacteria with increased minimum inhibitory concentrations (MIC), but rarely at in-use concentrations (Thomas *et al.* 2005, Suller and Russell, 1999, Lear *et al.* 2006, Walsh *et al.* 2003b). Alternative approaches which more closely mirror the way biocides are employed i.e. the exposure of high inoculums to high biocidal concentrations have produced some adaption but not as effectively as stepwise training (Walsh *et al.* 2003b, Thomas *et al.* 2000). Although it is not easy to develop resistant mutants to high biocide concentrations, exposure to low concentrations may induce low-level resistance in bacteria. The induction of bacterial resistance to almost all biocides has been documented, but particularly none oxidizing ones such as phenolics, bis-biguanides and quaternary ammonium compounds (Russell, 2004a, Moken *et al.* 1997, McMurry *et al.* 1998). Although, the induction of *oxy R* and s*ox RS* regulons following exposure to oxidising agents has been described (Chapman, 2003). These results correlate with the findings of Kuwamura-Sato et al (Kawamura-Sato *et al.* 2008) who determined the MICs and MBCs values of different biocides i.e.

chlorhexidinegluconate, benzethonium chloride bezalkonium chloride and alkyl diaminoethylglycine hydrochloride (ADH) against a range of clinical isolates of *Acinetobacter*. $MIC_{90s}$ obtained by the broth micro-dilution method for benzethonium chloride and bezalkonium chloride were ≤25mg/L which is consistent with the results obtained with glucose grown isolates and are generally lower than the ethanol grown cultures. However, the maximum MIC for specific strains were 50 mg/l for bezalkonium chloride and 100 mg/l for benzethonium chloride values greater than any recorded in this study. They also determined the MBC values of the four disinfectant and found that the MBC for the majority of strains were <64 mg/l, although the presence of organic material (3% w/v BSA) generated higher MBC values (512 mg/L) which were closer to the values generated here. Although the methods employed to determine MIC and MBC values are different to those employed here the major difference is that in this study there is a greater difference between the MBC and the MIC values.

Other authors have assessed the susceptibility of *Acinetobacter sp* to disinfectants (Martro *et al.* 2003, Wisplinghoff *et al.* 2007), however, they did not consider the disinfectants considered here. In both cases they did not find any correlation between antibiotic resistance and biocide susceptibility. One study that did consider Benzalkonium and Benzethonium chloride (Kawamura-Sato *et al.* 2010) found some correlation between antibiotic resistance and a reduced susceptibility to these biocides amongst a small number of clinical isolates.

The prolonged survival of clinical isolates of *Acinetobacter sp* at the MIC has been reported by other authors (Kawamura-Sato *et al.* 2008, Kawamura-Sato *et al.* 2010), this agrees with the observations in the this study that *A. baumannii* strains can survive inhibitory concentrations of biocides for prolonged periods prior to the formation of biofilms.

Thomas *et al* (Thomas *et al.* 2011) reported that effective disinfection in the presence of a biofilm often requires higher concentrations of antimicrobial agents when compared to planktonic bacteria. The antimicrobial concentrations required for biofilm inactivation can be 100 times that required for inactivation of removal planktonic cells

(Rasmussen and Givskov, 2006). A similar observation was made here where the increase in MBC was between 10 and 100x the MBC concentration for planktonic cells.

These impacts may be species specific for example the efficacy of ortho-pthalaldehyde was reduced against Mycobacterial biofilms but not against *P. aeruginosa* ones. Survival of bacteria in biofilms has been identified as contributing to a number of outbreaks, e.g. *P. aeruginosa* resistance to iodophores, *Serratia marcescens* resistance to benzylkonium chloride and chlorhexidine. Recently a major outbreak of *Pseudomonas* infections in a neonatal unit in Northern Ireland was associated with biofilms in water distribution system (RQIA, 2012).

Chronic wounds (e.g. diabetic lower limb and pressure ulcers) are generally heavily colonised with pathogenic bacteria. The healing of these wounds depends on adjusting the equilibrium between the hosts-immune system and the pathogens present in the wound environment (Stephen-Haynes, 2004). Within wound microorganisms exist in either a free floating/planktonic state or as part of a biofilm associated with the wound bed (Thomas *et al.* 2011). Many MDROs are also often associated with chronic and acute wounds. Topical antimicrobials are often the first approach applied to bio burden control, in wounds where there are clear signs of a progressive infection however; systematic antibiotics are generally applied (Bowler *et al.* 2012). Several factors determine the efficiency of systematic antibiotics such as: the extent of blood flow to the wound, the extent of antibiotic-resistance, the bacterial species present, the presence of biofilms (Zubair *et al.* 2011). Where MDROs have colonised the wound, the efficacy of systematic antibiotics treatment is uncertain. In this case the topical application of antiseptics and disinfectants may present a viable alternative due to their broader spectrum of activity and lack of bacterial resistance. By combining antiseptic and disinfectant agents with wound dressings it is possible to achieve a managed deliver of antimicrobial agents into the infected wound bed (Ovington, 2007). There is a large range of antimicrobial dressings available with varying claims of antimicrobial efficacy. Current approaches for the evaluation of antimicrobial wound dressings vary significantly in terms of media used, inoculum and sample size (Tkachenko and Karas, 2012), which may make direct comparison between dressing difficult (Chopra, 2007). In

addition the presence of biofilms within wounds complicate the testing of these dressings, particularly when  of our current knowledge of bacterial biofilm is based on *in vitro* observations of bacterial adherence to solid surfaces. This is a marked contrast to the situation in a chronic wound where bacteria reside within a wound beds rather than attached to well defined solid surface (Werthan *et al.* 2010).

The antimicrobial wound dressings (NSCD, ISCD, Honey and PHMB) were evaluated for their ability to prevent the growth of a range of commonly occurring wound pathogens i.e. *A. baumannii*, *P. aeruginosa* and *S. aureus*. Two approaches were used to evaluate these dressings i.e. planktonic cells and the second cells immobilised in a collagen matrix. In case of planktonic cells, majority of cases there were significant differences ($p<0.05$) between strains of the same species when treated with the same dressing. The NSCD dressing proved to have a high level of antimicrobial activity (Figure 4.45 a), after NSCD the Honey dressing performed best with maximum kills for a number of *A. baumannii* and *P. aeruginosa* strains (Figure 4.45 c) and a consistent level of kill for *S. aureus* with no significant differences (ANOVA, $P>0.05$) between the impacts on all strains tested. The ISCD dressing had a relatively consistent performance across the *A. baumannii* and *P. aeruginosa* strains tested (Figure 4.45 b) but failed to generate a complete kill in all but two occasions. It was significantly poorer ($P<0.05$) in performance when compared to NSCD for 4 out of the 5 *A. baumannii* strains, however with the exception of the Type strain there was no significant difference ($P>0.05$) between ISCD and NSCD for the other *P. aeruginosa* strains. In the case of *S. aureus* ISCD was again significantly ($p<0.05$) poorer in performance than NSCD for the majority of strains. The PHMB dressing had the poorest overall performance of the four dressings failing to generate a complete kill for any of the bacterial strains tested. It was particularly poor against *A. baumannii* where it had significantly poorer performance ($P<0.05$) than any of the other dressings against any of the strains with the exception of 882 and the Honey dressing. PHMB performed better against the strains of *P. aeruginosa* and *S. aureus* tested, performing as well as the other dressings in the majority of cases although it did have particularly poor performance against the *P. aeruginosa* type strain.

In the case of collagen immobilised bacteria, as was the case with the planktonic cells, there were significant differences (ANOVA, $p<0.05$) between strains of the same species when treated with the same dressing, consequently it was not possible to collate data on a species level. It also indicated that collagen wound model reflected the significant sub-species levels variations in the susceptibility to antimicrobial dressings seen in the planktonic model (Table 4.7) in a number of cases the difference between minimum and maximum impacts were many orders of magnitude. Unlike the planktonic model the NSCD and ISCD produced broadly similar results (Figure 4.46) with the major differences being seen with the *P. aeruginosa* strains where NSCD generated greater log reductions than ISCD in 4 out of the 6 strains tested ($p<0.05$, See Appendix). However, there were no differences between the two dressings when tested against *S. aureus.* The NSCD performed significantly better than the Honey dressing for all strains with the exception of *S. aureus* 4330 and significantly better than the PHMB dressing for all strains with the exception of the *P. aeruginosa* Type strain and Type 5. The ISCD performed significantly better than the Honey dressing for all strains with the exception of *S. aureus* 4330 and *P. aeruginosa* Type 2 and significantly better than PHMB with the exception of three *P. aeruginosa* strains (Type, Type 3 and 5) (See Appendix). Comparison between the Honey and the PHMB dressing was variable with Honey being generally better against *S. aureus*, whilst PHMB was generally better against *P. aeruginosa,* with a broadly similar picture against *A. baumannii* (See Appendix). The use of collagen potentially provided a diffusion barrier to the antimicrobials present in the dressing. Consequently, you might expect that reduction measured through the collagen approach to be less than that seen for the planktonic approach where there is no barrier between the dressing and the bacteria. This is generally the case across all the dressing with more strains showing no difference between the two approaches or the collagen approach generating a lower reduction in viable counts than the planktonic approach (Table 4.8). However, in the case of the ISCD, Honey and PHMB dressings there are exceptions to this expectation suggesting that the collagen did not generate a consistent barrier to the diffusion of the active ingredients.

The multi-drug resistant organisms (MDROs) investigated in this study includes both Gram positive and Gram negative groups that are well known to colonise and

potentially infect the variety of dermal wounds (Bowler *et al.* 2012). In a recent study conducted by (Lipova *et al.* 2010) it was reported that, out of a total 777 bacterial strains isolated from burn patients, 65% of these strains were identified as Gram-Positive and 35% as Gram-negative. The most commonly isolated Gram-Positive opportunistic pathogens were coagulase-negative *Staphylococcus* and *Bacillus sp* and most common among the Gram-negative opportunistic pathogens includes *E. coli, A. baumannii* and *P. aeruginosa* (Lipova *et al.* 2010).Since bacteria are capable of forming biofilm on living tissues e.g. a wound bed which enhances their tolerance to antimicrobial agents (Zubair *et al.* 2011) and the host immune cells, it was important to evaluate the susceptibility of these MDROs to the variety of dressing includes silver coated dressings etc., in both of their living forms i.e. most natural tolerant form and less natural free living form.

# 6 Major Findings

The major findings of this study are outlined below:

- The ability of *A. baumannii* to utilise alcohols as sole carbon sources varies between strains with a number of clinical isolates being better suited to the utilisation of these compounds than the Type strain;
- The ability of *A. baumannii* strains to form biofilms on plastic surfaces varied between strains and between carbon sources;
- Strong biofilm forming strains were able to form biofilms on both hydrophobic (plastic) and hydrophilic (glass ) surfaces through the generation of carbohydrate based EPS;
- Strong biofilm forming strains were able to generate high MW EPS containing galactose and rhamnose sugars;
- When challenged with a range of biocides biofilm forming strains were able to form biofilms at concentrations above the MBC of planktonic cells;
- The formation of biofilms provided enhanced protection when exposed to a range of biocides with biofilm MBC being orders of magnitude greater than the MBC for planktonic cells.

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# 8 Appendix

## 8.1 Statistical analysis of ethanol growth data

| Descriptives | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | N | Mean | Std. Deviation | Std. Error | 95% Confidence Interval for Mean | | Min | Max |
| | | | | | | Lower Bound | Upper Bound | | |
| UK-HS Ethanol | Control | 4 | 8.9570 | .03302 | .01651 | 8.9045 | 9.0096 | 8.92 | 9.00 |
| | 0.1% | 4 | 8.5282 | .00928 | .00464 | 8.5134 | 8.5430 | 8.51 | 8.54 |
| | 1% | 4 | 8.0106 | .01393 | .00697 | 7.9884 | 8.0327 | 8.00 | 8.03 |
| | 3% | 4 | 8.0946 | .08403 | .04202 | 7.9609 | 8.2283 | 7.97 | 8.16 |
| | 5% | 4 | 4.4950 | .13492 | .06746 | 4.2803 | 4.7097 | 4.39 | 4.69 |
| | Total | 20 | 7.6171 | 1.64002 | .36672 | 6.8495 | 8.3846 | 4.39 | 9.00 |
| UK-HS IPA | Control | 4 | 8.9570 | .03302 | .01651 | 8.9045 | 9.0096 | 8.92 | 9.00 |
| | 0.1% | 4 | 8.0620 | .61545 | .30772 | 7.0826 | 9.0413 | 7.42 | 8.74 |
| | 1% | 4 | 8.1718 | .69169 | .34584 | 7.0712 | 9.2725 | 7.15 | 8.60 |
| | 3% | 4 | 7.0987 | .23922 | .11961 | 6.7180 | 7.4793 | 6.74 | 7.25 |
| | 5% | 4 | 4.9088 | .02911 | .01455 | 4.8625 | 4.9551 | 4.88 | 4.94 |
| | Total | 20 | 7.4397 | 1.48213 | .33141 | 6.7460 | 8.1333 | 4.88 | 9.00 |
| UK-HS Salvo | Control | 4 | 8.9570 | .03302 | .01651 | 8.9045 | 9.0096 | 8.92 | 9.00 |
| | 0.1% | 4 | 8.0106 | .01393 | .00697 | 7.9884 | 8.0327 | 8.00 | 8.03 |
| | 1% | 4 | 6.5774 | .19034 | .09517 | 6.2745 | 6.8803 | 6.30 | 6.71 |
| | 3% | 4 | 5.6211 | .34763 | .17381 | 5.0680 | 6.1743 | 5.15 | 5.97 |
| | 5% | 4 | 3.4950 | .13492 | .06746 | 3.2803 | 3.7097 | 3.39 | 3.69 |
| | Total | 20 | 6.5322 | 1.96037 | .43835 | 5.6147 | 7.4497 | 3.39 | 9.00 |
| UK-HS Purell | Control | 4 | 8.9570 | .03302 | .01651 | 8.9045 | 9.0096 | 8.92 | 9.00 |
| | 0.1% | 4 | 8.5282 | .00928 | .00464 | 8.5134 | 8.5430 | 8.51 | 8.54 |
| | 1% | 4 | 7.7042 | .05612 | .02806 | 7.6149 | 7.7935 | 7.64 | 7.77 |
| | 3% | 4 | 6.8591 | .25844 | .12922 | 6.4479 | 7.2703 | 6.52 | 7.15 |
| | 5% | 4 | 7.9088 | .02911 | .01455 | 7.8625 | 7.9551 | 7.88 | 7.94 |
| | Total | 20 | 7.9915 | .74635 | .16689 | 7.6422 | 8.3408 | 6.52 | 9.00 |
| Type Ethanol | Control | 4 | 8.9570 | .03302 | .01651 | 8.9045 | 9.0096 | 8.92 | 9.00 |
| | 0.1% | 4 | 7.9189 | .06776 | .03388 | 7.8111 | 8.0267 | 7.82 | 7.98 |
| | 1% | 4 | 6.6184 | .16649 | .08325 | 6.3535 | 6.8834 | 6.38 | 6.76 |
| | 3% | 4 | 6.7290 | .03333 | .01666 | 6.6760 | 6.7821 | 6.68 | 6.75 |
| | 5% | 4 | 3.8617 | .08375 | .04188 | 3.7284 | 3.9950 | 3.74 | 3.94 |
| | Total | 20 | 6.8170 | 1.75355 | .39211 | 5.9963 | 7.6377 | 3.74 | 9.00 |
| Type IPA | Control | 4 | 8.9570 | .03302 | .01651 | 8.9045 | 9.0096 | 8.92 | 9.00 |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | 0.1% | 4 | 7.6078 | .09334 | .04667 | 7.4593 | 7.7563 | 7.53 | 7.72 |
| | 1% | 4 | 7.1611 | .16175 | .08088 | 6.9037 | 7.4185 | 7.01 | 7.30 |
| | 3% | 4 | 5.6211 | .34763 | .17381 | 5.0680 | 6.1743 | 5.15 | 5.97 |
| | 5% | 4 | 4.8121 | .03099 | .01549 | 4.7628 | 4.8614 | 4.77 | 4.84 |
| | Total | 20 | 6.8318 | 1.51417 | .33858 | 6.1232 | 7.5405 | 4.77 | 9.00 |
| Type Salvo | Control | 4 | 8.9570 | .03302 | .01651 | 8.9045 | 9.0096 | 8.92 | 9.00 |
| | 0.1% | 4 | 7.9189 | .06776 | .03388 | 7.8111 | 8.0267 | 7.82 | 7.98 |
| | 1% | 4 | 7.0104 | .14475 | .07237 | 6.7801 | 7.2408 | 6.87 | 7.15 |
| | 3% | 4 | 6.7290 | .03333 | .01666 | 6.6760 | 6.7821 | 6.68 | 6.75 |
| | 5% | 4 | 5.5282 | .00928 | .00464 | 5.5134 | 5.5430 | 5.51 | 5.54 |
| | Total | 20 | 7.2287 | 1.18529 | .26504 | 6.6740 | 7.7835 | 5.51 | 9.00 |
| TypePurell | Control | 4 | 8.9570 | .03302 | .01651 | 8.9045 | 9.0096 | 8.92 | 9.00 |
| | 0.1% | 4 | 8.0805 | .10501 | .05250 | 7.9134 | 8.2476 | 7.93 | 8.15 |
| | 1% | 4 | 7.6769 | .04996 | .02498 | 7.5974 | 7.7564 | 7.60 | 7.71 |
| | 3% | 4 | 6.4888 | .23350 | .11675 | 6.1173 | 6.8604 | 6.15 | 6.64 |
| | 5% | 4 | 5.0341 | .08098 | .04049 | 4.9053 | 5.1630 | 4.99 | 5.16 |
| | Total | 20 | 7.2475 | 1.40166 | .31342 | 6.5915 | 7.9035 | 4.99 | 9.00 |

**ANOVA**

| | | Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|---|
| UK-HS Ethanol | Between Groups | 51.024 | 4 | 12.756 | 2394.466 | .000 |
| | Within Groups | .080 | 15 | .005 | | |
| | Total | 51.104 | 19 | | | |
| UK-HS IPA | Between Groups | 38.988 | 4 | 9.747 | 53.183 | .000 |
| | Within Groups | 2.749 | 15 | .183 | | |
| | Total | 41.737 | 19 | | | |
| UK-HS Salvo | Between Groups | 72.488 | 4 | 18.122 | 513.187 | .000 |
| | Within Groups | .530 | 15 | .035 | | |
| | Total | 73.018 | 19 | | | |
| UK-HS Purell | Between Groups | 10.368 | 4 | 2.592 | 180.087 | .000 |
| | Within Groups | .216 | 15 | .014 | | |
| | Total | 10.584 | 19 | | | |
| Type Ethanol | Between Groups | 58.299 | 4 | 14.575 | 1754.941 | .000 |
| | Within Groups | .125 | 15 | .008 | | |
| | Total | 58.424 | 19 | | | |
| Type IPA | Between Groups | 43.088 | 4 | 10.772 | 341.380 | .000 |
| | Within Groups | .473 | 15 | .032 | | |
| | Total | 43.562 | 19 | | | |
| Type Salvo | Between Groups | 26.610 | 4 | 6.652 | 1195.221 | .000 |
| | Within Groups | .083 | 15 | .006 | | |
| | Total | 26.693 | 19 | | | |

| | | | | | | |
|---|---|---|---|---|---|---|
| | Between Groups | 37.101 | 4 | 9.275 | 612.717 | .000 |
| Type Purell | Within Groups | .227 | 15 | .015 | | |
| | Total | 37.329 | 19 | | | |

**Multiple Comparisons**

Games-Howell

| Dependent Variable | (I) VAR00001 | (J) VAR00001 | Mean Difference (I-J) | Std. Error | Sig. | 95% Confidence Interval | |
|---|---|---|---|---|---|---|---|
| | | | | | | Lower Bound | Upper Bound |
| UK-HS Ethanol | Control | 0.1% | .42882* | .01715 | .000 | .3462 | .5114 |
| | | 1% | .94646* | .01792 | .000 | .8672 | 1.0258 |
| | | 3% | .86242* | .04514 | .000 | .6592 | 1.0657 |
| | | 5% | 4.46204* | .06945 | .000 | 4.1206 | 4.8034 |
| | 0.1% | Control | -.42882* | .01715 | .000 | -.5114 | -.3462 |
| | | 1% | .51764* | .00837 | .000 | .4846 | .5506 |
| | | 3% | .43360* | .04227 | .007 | .2132 | .6540 |
| | | 5% | 4.03322* | .06762 | .000 | 3.6769 | 4.3895 |
| | 1% | Control | -.94646* | .01792 | .000 | -1.0258 | -.8672 |
| | | 0.1% | -.51764* | .00837 | .000 | -.5506 | -.4846 |
| | | 3% | -.08404 | .04259 | .433 | -.3017 | .1336 |
| | | 5% | 3.51557* | .06782 | .000 | 3.1612 | 3.8700 |
| | 3% | Control | -.86242* | .04514 | .000 | -1.0657 | -.6592 |
| | | 0.1% | -.43360* | .04227 | .007 | -.6540 | -.2132 |
| | | 1% | .08404 | .04259 | .433 | -.1336 | .3017 |
| | | 5% | 3.59962* | .07948 | .000 | 3.2814 | 3.9178 |
| | 5% | Control | -4.46204* | .06945 | .000 | -4.8034 | -4.1206 |
| | | 0.1% | -4.03322* | .06762 | .000 | -4.3895 | -3.6769 |
| | | 1% | -3.51557* | .06782 | .000 | -3.8700 | -3.1612 |
| | | 3% | -3.59962* | .07948 | .000 | -3.9178 | -3.2814 |
| UK-HS IPA | Control | 0.1% | .89507 | .30817 | .215 | -.7329 | 2.5230 |
| | | 1% | .78519 | .34624 | .349 | -1.0454 | 2.6158 |
| | | 3% | 1.85834* | .12074 | .002 | 1.2345 | 2.4822 |
| | | 5% | 4.04819* | .02201 | .000 | 3.9652 | 4.1312 |
| | 0.1% | Control | -.89507 | .30817 | .215 | -2.5230 | .7329 |
| | | 1% | -.10988 | .46293 | .999 | -1.8542 | 1.6344 |
| | | 3% | .96327 | .33015 | .178 | -.5270 | 2.4535 |
| | | 5% | 3.15313* | .30807 | .008 | 1.5242 | 4.7820 |
| | 1% | Control | -.78519 | .34624 | .349 | -2.6158 | 1.0454 |
| | | 0.1% | .10988 | .46293 | .999 | -1.6344 | 1.8542 |
| | | 3% | 1.07315 | .36594 | .182 | -.6221 | 2.7684 |
| | | 5% | 3.26301* | .34615 | .010 | 1.4315 | 5.0945 |
| | 3% | Control | -1.85834* | .12074 | .002 | -2.4822 | -1.2345 |
| | | 0.1% | -.96327 | .33015 | .178 | -2.4535 | .5270 |
| | | 1% | -1.07315 | .36594 | .182 | -2.7684 | .6221 |
| | | 5% | 2.18985* | .12049 | .001 | 1.5638 | 2.8159 |
| | 5% | Control | -4.04819* | .02201 | .000 | -4.1312 | -3.9652 |
| | | 0.1% | -3.15313* | .30807 | .008 | -4.7820 | -1.5242 |
| | | 1% | -3.26301* | .34615 | .010 | -5.0945 | -1.4315 |
| | | 3% | -2.18985* | .12049 | .001 | -2.8159 | -1.5638 |
| UK- HSSalvo | Control | 0.1% | .94646* | .01792 | .000 | .8672 | 1.0258 |
| | | 1% | 2.37960* | .09659 | .000 | 1.8876 | 2.8716 |
| | | 3% | 3.33590* | .17460 | .001 | 2.4215 | 4.2503 |
| | | 5% | 5.46204* | .06945 | .000 | 5.1206 | 5.8034 |
| | 0.1% | Control | -.94646* | .01792 | .000 | -1.0258 | -.8672 |
| | | 1% | 1.43314* | .09543 | .002 | .9308 | 1.9355 |
| | | 3% | 2.38943* | .17395 | .003 | 1.4688 | 3.3100 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| | | 5% | 4.51557* | .06782 | .000 | 4.1612 | 4.8700 |
| | 1% | Control | -2.37960* | .09659 | .000 | -2.8716 | -1.8876 |
| | | 0.1% | -1.43314* | .09543 | .002 | -1.9355 | -.9308 |
| | | 3% | .95630* | .19816 | .028 | .1366 | 1.7760 |
| | | 5% | 3.08244* | .11666 | .000 | 2.6285 | 3.5364 |
| | 3% | Control | -3.33590* | .17460 | .001 | -4.2503 | -2.4215 |
| | | 0.1% | -2.38943* | .17395 | .003 | -3.3100 | -1.4688 |
| | | 1% | -.95630* | .19816 | .028 | -1.7760 | -.1366 |
| | | 5% | 2.12614* | .18645 | .002 | 1.2843 | 2.9680 |
| | 5% | Control | -5.46204* | .06945 | .000 | -5.8034 | -5.1206 |
| | | 0.1% | -4.51557* | .06782 | .000 | -4.8700 | -4.1612 |
| | | 1% | -3.08244* | .11666 | .000 | -3.5364 | -2.6285 |
| | | 3% | -2.12614* | .18645 | .002 | -2.9680 | -1.2843 |
| UK-HSPurell | Control | 0.1% | .42882* | .01715 | .000 | .3462 | .5114 |
| | | 1% | 1.25282* | .03256 | .000 | 1.1206 | 1.3850 |
| | | 3% | 2.09791* | .13027 | .002 | 1.4224 | 2.7734 |
| | | 5% | 1.04819* | .02201 | .000 | .9652 | 1.1312 |
| | 0.1% | Control | -.42882* | .01715 | .000 | -.5114 | -.3462 |
| | | 1% | .82400* | .02844 | .000 | .6786 | .9694 |
| | | 3% | 1.66909* | .12930 | .004 | .9845 | 2.3537 |
| | | 5% | .61937* | .01527 | .000 | .5474 | .6913 |
| | 1% | Control | -1.25282* | .03256 | .000 | -1.3850 | -1.1206 |
| | | 0.1% | -.82400* | .02844 | .000 | -.9694 | -.6786 |
| | | 3% | .84510* | .13223 | .024 | .1855 | 1.5047 |
| | | 5% | -.20462* | .03161 | .010 | -.3372 | -.0720 |
| | 3% | Control | -2.09791* | .13027 | .002 | -2.7734 | -1.4224 |
| | | 0.1% | -1.66909* | .12930 | .004 | -2.3537 | -.9845 |
| | | 1% | -.84510* | .13223 | .024 | -1.5047 | -.1855 |
| | | 5% | -1.04972* | .13004 | .014 | -1.7274 | -.3721 |
| | 5% | Control | -1.04819* | .02201 | .000 | -1.1312 | -.9652 |
| | | 0.1% | -.61937* | .01527 | .000 | -.6913 | -.5474 |
| | | 1% | .20462* | .03161 | .010 | .0720 | .3372 |
| | | 3% | 1.04972* | .13004 | .014 | .3721 | 1.7274 |
| Type Ethanol | Control | 0.1% | 1.03811* | .03769 | .000 | .8774 | 1.1988 |
| | | 1% | 2.33859* | .08487 | .000 | 1.9113 | 2.7659 |
| | | 3% | 2.22798* | .02346 | .000 | 2.1400 | 2.3160 |
| | | 5% | 5.09532* | .04501 | .000 | 4.8928 | 5.2978 |
| | 0.1% | Control | -1.03811* | .03769 | .000 | -1.1988 | -.8774 |
| | | 1% | 1.30048* | .08987 | .001 | .8992 | 1.7018 |
| | | 3% | 1.18987* | .03775 | .000 | 1.0292 | 1.3505 |
| | | 5% | 4.05721* | .05386 | .000 | 3.8522 | 4.2622 |
| | 1% | Control | -2.33859* | .08487 | .000 | -2.7659 | -1.9113 |
| | | 0.1% | -1.30048* | .08987 | .001 | -1.7018 | -.8992 |
| | | 3% | -.11061 | .08490 | .709 | -.5377 | .3165 |
| | | 5% | 2.75673* | .09318 | .000 | 2.3626 | 3.1508 |
| | 3% | Control | -2.22798* | .02346 | .000 | -2.3160 | -2.1400 |
| | | 0.1% | -1.18987* | .03775 | .000 | -1.3505 | -1.0292 |
| | | 1% | .11061 | .08490 | .709 | -.3165 | .5377 |
| | | 5% | 2.86734* | .04507 | .000 | 2.6650 | 3.0697 |
| | 5% | Control | -5.09532* | .04501 | .000 | -5.2978 | -4.8928 |
| | | 0.1% | -4.05721* | .05386 | .000 | -4.2622 | -3.8522 |
| | | 1% | -2.75673* | .09318 | .000 | -3.1508 | -2.3626 |
| | | 3% | -2.86734* | .04507 | .000 | -3.0697 | -2.6650 |
| Type IPA | Control | 0.1% | 1.34923* | .04951 | .000 | 1.1210 | 1.5775 |
| | | 1% | 1.79595* | .08254 | .001 | 1.3815 | 2.2104 |
| | | 3% | 3.33590* | .17460 | .001 | 2.4215 | 4.2503 |
| | | 5% | 4.14493* | .02264 | .000 | 4.0599 | 4.2300 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| | 0.1% | Control | -1.34923* | .04951 | .000 | -1.5775 | -1.1210 |
| | | 1% | .44672* | .09338 | .027 | .0657 | .8278 |
| | | 3% | 1.98667* | .17997 | .004 | 1.1137 | 2.8596 |
| | | 5% | 2.79570* | .04918 | .000 | 2.5660 | 3.0254 |
| | 1% | Control | -1.79595* | .08254 | .001 | -2.2104 | -1.3815 |
| | | 0.1% | -.44672* | .09338 | .027 | -.8278 | -.0657 |
| | | 3% | 1.53995* | .19171 | .005 | .7124 | 2.3675 |
| | | 5% | 2.34898* | .08235 | .000 | 1.9330 | 2.7649 |
| | 3% | Control | -3.33590* | .17460 | .001 | -4.2503 | -2.4215 |
| | | 0.1% | -1.98667* | .17997 | .004 | -2.8596 | -1.1137 |
| | | 1% | -1.53995* | .19171 | .005 | -2.3675 | -.7124 |
| | | 5% | .80903 | .17450 | .069 | -.1063 | 1.7243 |
| | 5% | Control | -4.14493* | .02264 | .000 | -4.2300 | -4.0599 |
| | | 0.1% | -2.79570* | .04918 | .000 | -3.0254 | -2.5660 |
| | | 1% | -2.34898* | .08235 | .000 | -2.7649 | -1.9330 |
| | | 3% | -.80903 | .17450 | .069 | -1.7243 | .1063 |
| Type Salvo | Control | 0.1% | 1.03811* | .03769 | .000 | .8774 | 1.1988 |
| | | 1% | 1.94658* | .07423 | .000 | 1.5784 | 2.3147 |
| | | 3% | 2.22798* | .02346 | .000 | 2.1400 | 2.3160 |
| | | 5% | 3.42882* | .01715 | .000 | 3.3462 | 3.5114 |
| | 0.1% | Control | -1.03811* | .03769 | .000 | -1.1988 | -.8774 |
| | | 1% | .90847* | .07991 | .001 | .5641 | 1.2529 |
| | | 3% | 1.18987* | .03775 | .000 | 1.0292 | 1.3505 |
| | | 5% | 2.39071* | .03419 | .000 | 2.2140 | 2.5675 |
| | 1% | Control | -1.94658* | .07423 | .000 | -2.3147 | -1.5784 |
| | | 0.1% | -.90847* | .07991 | .001 | -1.2529 | -.5641 |
| | | 3% | .28140 | .07427 | .105 | -.0865 | .6493 |
| | | 5% | 1.48224* | .07252 | .001 | 1.0998 | 1.8647 |
| | 3% | Control | -2.22798* | .02346 | .000 | -2.3160 | -2.1400 |
| | | 0.1% | -1.18987* | .03775 | .000 | -1.3505 | -1.0292 |
| | | 1% | -.28140 | .07427 | .105 | -.6493 | .0865 |
| | | 5% | 1.20084* | .01730 | .000 | 1.1174 | 1.2843 |
| | 5% | Control | -3.42882* | .01715 | .000 | -3.5114 | -3.3462 |
| | | 0.1% | -2.39071* | .03419 | .000 | -2.5675 | -2.2140 |
| | | 1% | -1.48224* | .07252 | .001 | -1.8647 | -1.0998 |
| | | 3% | -1.20084* | .01730 | .000 | -1.2843 | -1.1174 |
| Type Purell | Control | 0.1% | .87655* | .05504 | .001 | .6167 | 1.1364 |
| | | 1% | 1.28012* | .02994 | .000 | 1.1619 | 1.3984 |
| | | 3% | 2.46819* | .11791 | .001 | 1.8598 | 3.0766 |
| | | 5% | 3.92291* | .04372 | .000 | 3.7278 | 4.1180 |
| | 0.1% | Control | -.87655* | .05504 | .001 | -1.1364 | -.6167 |
| | | 1% | .40357* | .05814 | .008 | .1540 | .6531 |
| | | 3% | 1.59165* | .12801 | .001 | 1.0342 | 2.1491 |
| | | 5% | 3.04636* | .06630 | .000 | 2.7922 | 3.3005 |
| | 1% | Control | -1.28012* | .02994 | .000 | -1.3984 | -1.1619 |
| | | 0.1% | -.40357* | .05814 | .008 | -.6531 | -.1540 |
| | | 3% | 1.18808* | .11939 | .006 | .5916 | 1.7846 |
| | | 5% | 2.64279* | .04757 | .000 | 2.4519 | 2.8337 |
| | 3% | Control | -2.46819* | .11791 | .001 | -3.0766 | -1.8598 |
| | | 0.1% | -1.59165* | .12801 | .001 | -2.1491 | -1.0342 |
| | | 1% | -1.18808* | .11939 | .006 | -1.7846 | -.5916 |
| | | 5% | 1.45472* | .12357 | .002 | .8826 | 2.0268 |
| | 5% | Control | -3.92291* | .04372 | .000 | -4.1180 | -3.7278 |
| | | 0.1% | -3.04636* | .06630 | .000 | -3.3005 | -2.7922 |
| | | 1% | -2.64279* | .04757 | .000 | -2.8337 | -2.4519 |
| | | 3% | -1.45472* | .12357 | .002 | -2.0268 | -.8826 |

*. The mean difference is significant at the 0.05 level.

## 8.2 **Statistical analysis of low alcohol growth data**

| Descriptives | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | N | Mean | Std. Dev | Std. Error | 95% Confidence Interval for Mean | | Minimum | Maximum |
| | | | | | | Lower Bound | Upper Bound | | |
| UK-HS Ethanol | Cont | 4 | 8.2000 | .00000 | .00000 | 8.2000 | 8.2000 | 8.20 | 8.20 |
| | 0.001% | 4 | 5.2858 | .30034 | .15017 | 4.8078 | 5.7637 | 5.03 | 5.60 |
| | 0.01% | 4 | 5.2996 | .51239 | .25620 | 4.4843 | 6.1150 | 4.82 | 5.74 |
| | 0.03% | 4 | 4.6362 | .60691 | .30346 | 3.6705 | 5.6019 | 4.06 | 5.49 |
| | 0.05% | 4 | 4.0190 | .01200 | .00600 | 3.9999 | 4.0381 | 4.01 | 4.03 |
| | Total | 20 | 5.4881 | 1.51177 | .33804 | 4.7806 | 6.1956 | 4.01 | 8.20 |
| UK-HS IPA | Cont | 4 | 8.2000 | .00000 | .00000 | 8.2000 | 8.2000 | 8.20 | 8.20 |
| | 0.001% | 4 | 5.3543 | .08394 | .04197 | 5.2208 | 5.4879 | 5.31 | 5.48 |
| | 0.01% | 4 | 5.2192 | .44944 | .22472 | 4.5040 | 5.9344 | 4.76 | 5.78 |
| | 0.03% | 4 | 5.0811 | .06343 | .03171 | 4.9802 | 5.1820 | 5.02 | 5.15 |
| | 0.05% | 4 | 4.8767 | .03332 | .01666 | 4.8237 | 4.9297 | 4.85 | 4.91 |
| | Total | 20 | 5.7463 | 1.28238 | .28675 | 5.1461 | 6.3464 | 4.76 | 8.20 |
| Type Ethanol | Cont | 4 | 8.4000 | .00000 | .00000 | 8.4000 | 8.4000 | 8.40 | 8.40 |
| | 0.001% | 4 | 6.3971 | .09997 | .04998 | 6.2380 | 6.5562 | 6.31 | 6.49 |
| | 0.01% | 4 | 6.2996 | .51239 | .25620 | 5.4843 | 7.1150 | 5.82 | 6.74 |
| | 0.03% | 4 | 4.0444 | .03340 | .01670 | 3.9912 | 4.0975 | 4.00 | 4.08 |
| | 0.05% | 4 | 3.2365 | .15200 | .07600 | 2.9946 | 3.4783 | 3.01 | 3.32 |
| | Total | 20 | 5.6755 | 1.90098 | .42507 | 4.7858 | 6.5652 | 3.01 | 8.40 |
| Type IPA | Cont | 4 | 8.4000 | .00000 | .00000 | 8.4000 | 8.4000 | 8.40 | 8.40 |
| | 0.001% | 4 | 5.3971 | .09997 | .04998 | 5.2380 | 5.5562 | 5.31 | 5.49 |
| | 0.01% | 4 | 5.0822 | .19044 | .09522 | 4.7791 | 5.3852 | 4.97 | 5.37 |
| | 0.03% | 4 | 4.1967 | .07337 | .03668 | 4.0800 | 4.3135 | 4.12 | 4.28 |
| | 0.05% | 4 | 3.1648 | .16866 | .08433 | 2.8964 | 3.4332 | 3.01 | 3.32 |
| | Total | 20 | 5.2482 | 1.80582 | .40379 | 4.4030 | 6.0933 | 3.01 | 8.40 |

| ANOVA | | | | | | |
|---|---|---|---|---|---|---|
| | | Sum of Squares | df | Mean Square | F | Sig. |
| UK-HS Ethanol | Between Groups | 41.260 | 4 | 10.315 | 71.508 | .000 |
| | Within Groups | 2.164 | 15 | .144 | | |
| | Total | 43.423 | 19 | | | |
| UK-HS IPA | Between Groups | 30.603 | 4 | 7.651 | 178.612 | .000 |
| | Within Groups | .643 | 15 | .043 | | |
| | Total | 31.246 | 19 | | | |
| Type Ethanol | Between Groups | 67.771 | 4 | 16.943 | 285.464 | .000 |
| | Within Groups | .890 | 15 | .059 | | |
| | Total | 68.661 | 19 | | | |
| Type IPA | Between Groups | 61.718 | 4 | 15.430 | 963.225 | .000 |
| | Within Groups | .240 | 15 | .016 | | |
| | Total | 61.959 | 19 | | | |

| Multiple Comparisons | | | | | | | |
|---|---|---|---|---|---|---|---|
| Games-Howell | | | | | | | |
| Dependent Variable | (I) VAR00001 | (J) VAR00001 | Mean Difference (I-J) | Std. Error | Sig. | 95% Confidence Interval | |
| | | | | | | Lower Bound | Upper Bound |
| UK-HS Ethanol | Control | 0.001% | 2.91425* | .15017 | .001 | 2.1177 | 3.7108 |
| | | 0.01% | 2.90035* | .25620 | .006 | 1.5414 | 4.2593 |
| | | 0.03% | 3.56381* | .30346 | .005 | 1.9541 | 5.1735 |
| | | 0.05% | 4.18101* | .00600 | .000 | 4.1492 | 4.2128 |
| | 0.001% | Control | -2.91425* | .15017 | .001 | -3.7108 | -2.1177 |
| | | 0.01% | -.01390 | .29696 | 1.000 | -1.2209 | 1.1931 |
| | | 0.03% | .64956 | .33858 | .422 | -.7886 | 2.0877 |
| | | 0.05% | 1.26676* | .15029 | .014 | .4714 | 2.0621 |
| | 0.01% | Control | -2.90035* | .25620 | .006 | -4.2593 | -1.5414 |
| | | 0.001% | .01390 | .29696 | 1.000 | -1.1931 | 1.2209 |
| | | 0.03% | .66345 | .39714 | .512 | -.8403 | 2.1672 |
| | | 0.05% | 1.28066 | .25627 | .058 | -.0776 | 2.6389 |
| | 0.03% | Control | -3.56381* | .30346 | .005 | -5.1735 | -1.9541 |
| | | 0.001% | -.64956 | .33858 | .422 | -2.0877 | .7886 |
| | | 0.01% | -.66345 | .39714 | .512 | -2.1672 | .8403 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| | | 0.05% | .61720 | .30352 | .418 | -.9919 | 2.2263 |
| | 0.05% | Control | -4.18101* | .00600 | .000 | -4.2128 | -4.1492 |
| | | 0.001% | -1.26676* | .15029 | .014 | -2.0621 | -.4714 |
| | | 0.01% | -1.28066 | .25627 | .058 | -2.6389 | .0776 |
| | | 0.03% | -.61720 | .30352 | .418 | -2.2263 | .9919 |
| UK-HS IPA | Control | 0.001% | 2.84568* | .04197 | .000 | 2.6231 | 3.0683 |
| | | 0.01% | 2.98079* | .22472 | .004 | 1.7888 | 4.1728 |
| | | 0.03% | 3.11889* | .03171 | .000 | 2.9507 | 3.2871 |
| | | 0.05% | 3.32330* | .01666 | .000 | 3.2349 | 3.4117 |
| | 0.001% | Control | -2.84568* | .04197 | .000 | -3.0683 | -2.6231 |
| | | 0.01% | .13512 | .22860 | .968 | -1.0222 | 1.2925 |
| | | 0.03% | .27321* | .05260 | .014 | .0709 | .4755 |
| | | 0.05% | .47762* | .04516 | .002 | .2748 | .6804 |
| | 0.01% | Control | -2.98079* | .22472 | .004 | -4.1728 | -1.7888 |
| | | 0.001% | -.13512 | .22860 | .968 | -1.2925 | 1.0222 |
| | | 0.03% | .13810 | .22695 | .964 | -1.0331 | 1.3093 |
| | | 0.05% | .34250 | .22534 | .613 | -.8435 | 1.5285 |
| | 0.03% | Control | -3.11889* | .03171 | .000 | -3.2871 | -2.9507 |
| | | 0.001% | -.27321* | .05260 | .014 | -.4755 | -.0709 |
| | | 0.01% | -.13810 | .22695 | .964 | -1.3093 | 1.0331 |
| | | 0.05% | .20441* | .03582 | .016 | .0546 | .3542 |
| | 0.05% | Control | -3.32330* | .01666 | .000 | -3.4117 | -3.2349 |
| | | 0.001% | -.47762* | .04516 | .002 | -.6804 | -.2748 |
| | | 0.01% | -.34250 | .22534 | .613 | -1.5285 | .8435 |
| | | 0.03% | -.20441* | .03582 | .016 | -.3542 | -.0546 |
| Type Ethanol | Control | 0.001% | 2.00288* | .04998 | .000 | 1.7377 | 2.2680 |
| | | 0.01% | 2.10035* | .25620 | .015 | .7414 | 3.4593 |
| | | 0.03% | 4.35563* | .01670 | .000 | 4.2670 | 4.4442 |
| | | 0.05% | 5.16353* | .07600 | .000 | 4.7604 | 5.5667 |
| | 0.001% | Control | -2.00288* | .04998 | .000 | -2.2680 | -1.7377 |
| | | 0.01% | .09747 | .26103 | .994 | -1.2188 | 1.4138 |
| | | 0.03% | 2.35275* | .05270 | .000 | 2.1069 | 2.5986 |
| | | 0.05% | 3.16064* | .09096 | .000 | 2.8010 | 3.5203 |
| | 0.01% | Control | -2.10035* | .25620 | .015 | -3.4593 | -.7414 |
| | | 0.001% | -.09747 | .26103 | .994 | -1.4138 | 1.2188 |
| | | 0.03% | 2.25528* | .25674 | .012 | .9016 | 3.6089 |
| | | 0.05% | 3.06318* | .26723 | .003 | 1.7880 | 4.3383 |
| | 0.03% | Control | -4.35563* | .01670 | .000 | -4.4442 | -4.2670 |
| | | 0.001% | -2.35275* | .05270 | .000 | -2.5986 | -2.1069 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| | | 0.01% | -2.25528* | .25674 | .012 | -3.6089 | -.9016 |
| | | 0.05% | .80790* | .07781 | .005 | .4203 | 1.1955 |
| | 0.05% | Control | -5.16353* | .07600 | .000 | -5.5667 | -4.7604 |
| | | 0.001% | -3.16064* | .09096 | .000 | -3.5203 | -2.8010 |
| | | 0.01% | -3.06318* | .26723 | .003 | -4.3383 | -1.7880 |
| | | 0.03% | -.80790* | .07781 | .005 | -1.1955 | -.4203 |
| Type IPA | Control | 0.001% | 3.00288* | .04998 | .000 | 2.7377 | 3.2680 |
| | | 0.01% | 3.31785* | .09522 | .000 | 2.8127 | 3.8230 |
| | | 0.03% | 4.20325* | .03668 | .000 | 4.0087 | 4.3978 |
| | | 0.05% | 5.23517* | .08433 | .000 | 4.7878 | 5.6825 |
| | 0.001% | Control | -3.00288* | .04998 | .000 | -3.2680 | -2.7377 |
| | | 0.01% | .31496 | .10754 | .157 | -.1348 | .7647 |
| | | 0.03% | 1.20037* | .06200 | .000 | .9607 | 1.4400 |
| | | 0.05% | 2.23229* | .09803 | .000 | 1.8350 | 2.6296 |
| | 0.01% | Control | -3.31785* | .09522 | .000 | -3.8230 | -2.8127 |
| | | 0.001% | -.31496 | .10754 | .157 | -.7647 | .1348 |
| | | 0.03% | .88540* | .10204 | .005 | .4238 | 1.3470 |
| | | 0.05% | 1.91733* | .12720 | .000 | 1.4379 | 2.3968 |
| | 0.03% | Control | -4.20325* | .03668 | .000 | -4.3978 | -4.0087 |
| | | 0.001% | -1.20037* | .06200 | .000 | -1.4400 | -.9607 |
| | | 0.01% | -.88540* | .10204 | .005 | -1.3470 | -.4238 |
| | | 0.05% | 1.03192* | .09197 | .002 | .6280 | 1.4358 |
| | 0.05% | Control | -5.23517* | .08433 | .000 | -5.6825 | -4.7878 |
| | | 0.001% | -2.23229* | .09803 | .000 | -2.6296 | -1.8350 |
| | | 0.01% | -1.91733* | .12720 | .000 | -2.3968 | -1.4379 |
| | | 0.03% | -1.03192* | .09197 | .002 | -1.4358 | -.6280 |

*. The mean difference is significant at the 0.05 level.

## 8.3 **Comparison of wound models**

### 8.3.1 **Planktonic wound model**

| Descriptive | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | N | Mean | Std. Dev | Std. Error | 95% Confidence Interval for Mean | | Min | Max |
| | | | | | | Lower Bound | Upper Bound | | |
| clone1 | NSCD | 5 | 9.5420 | .00000 | .00000 | 9.5420 | 9.5420 | 9.54 | 9.54 |
| | ISCD | 5 | 7.2400 | .12298 | .05500 | 7.0873 | 7.3927 | 7.02 | 7.30 |
| | Honey | 5 | 8.8550 | .00000 | .00000 | 8.8550 | 8.8550 | 8.86 | 8.86 |
| | PHMB | 5 | 3.9270 | .06918 | .03094 | 3.8411 | 4.0129 | 3.83 | 4.02 |
| | Total | 20 | 7.3910 | 2.22475 | .49747 | 6.3498 | 8.4322 | 3.83 | 9.54 |
| clone2 | NSCD | 5 | 9.4790 | .00000 | .00000 | 9.4790 | 9.4790 | 9.48 | 9.48 |
| | ISCD | 5 | 6.5166 | .48625 | .21746 | 5.9128 | 7.1204 | 5.94 | 6.96 |
| | Honey | 5 | 9.4330 | .00000 | .00000 | 9.4330 | 9.4330 | 9.43 | 9.43 |
| | PHMB | 5 | 4.5754 | .56477 | .25257 | 3.8741 | 5.2767 | 3.91 | 5.30 |
| | Total | 20 | 7.5010 | 2.15319 | .48147 | 6.4933 | 8.5087 | 3.91 | 9.48 |
| ukhs | NSCD | 5 | 9.2960 | .00000 | .00000 | 9.2960 | 9.2960 | 9.30 | 9.30 |
| | ISCD | 5 | 6.1802 | .34764 | .15547 | 5.7485 | 6.6119 | 5.90 | 6.78 |
| | Honey | 5 | 8.6490 | .00000 | .00000 | 8.6490 | 8.6490 | 8.65 | 8.65 |
| | PHMB | 5 | 4.1078 | .36149 | .16166 | 3.6589 | 4.5567 | 3.82 | 4.56 |
| | Total | 20 | 7.0582 | 2.12846 | .47594 | 6.0621 | 8.0544 | 3.82 | 9.30 |
| Act_Type | NSCD | 5 | 9.9010 | .00000 | .00000 | 9.9010 | 9.9010 | 9.90 | 9.90 |
| | ISCD | 5 | 6.7566 | .37294 | .16678 | 6.2935 | 7.2197 | 6.26 | 7.09 |
| | Honey | 5 | 8.7240 | .00000 | .00000 | 8.7240 | 8.7240 | 8.72 | 8.72 |
| | PHMB | 5 | 4.4258 | .12898 | .05768 | 4.2657 | 4.5859 | 4.28 | 4.63 |
| | Total | 20 | 7.4519 | 2.13872 | .47823 | 6.4509 | 8.4528 | 4.28 | 9.90 |
| A882 | NSCD | 5 | 7.2280 | .69143 | .30922 | 6.3695 | 8.0865 | 6.48 | 8.29 |
| | ISCD | 5 | 5.9800 | .17564 | .07855 | 5.7619 | 6.1981 | 5.73 | 6.20 |
| | Honey | 5 | 2.8020 | .14220 | .06359 | 2.6254 | 2.9786 | 2.62 | 3.00 |
| | PHMB | 5 | 2.7300 | .20396 | .09121 | 2.4767 | 2.9833 | 2.51 | 3.01 |
| | Total | 20 | 4.6850 | 2.04992 | .45837 | 3.7256 | 5.6444 | 2.51 | 8.29 |
| A292 | NSCD | 5 | 8.5400 | .00000 | .00000 | 8.5400 | 8.5400 | 8.54 | 8.54 |
| | ISCD | 5 | 8.5400 | .00000 | .00000 | 8.5400 | 8.5400 | 8.54 | 8.54 |
| | Honey | 5 | 5.9220 | .15928 | .07123 | 5.7242 | 6.1198 | 5.76 | 6.18 |
| | PHMB | 5 | 4.9140 | .26359 | .11788 | 4.5867 | 5.2413 | 4.59 | 5.25 |
| | Total | 20 | 6.9790 | 1.64883 | .36869 | 6.2073 | 7.7507 | 4.59 | 8.54 |
| Ps_Type | NSCD | 5 | 8.3810 | .00000 | .00000 | 8.3810 | 8.3810 | 8.38 | 8.38 |
| | ISCD | 5 | 5.2588 | .18911 | .08457 | 5.0240 | 5.4936 | 5.01 | 5.50 |
| | Honey | 5 | 5.2822 | .30779 | .13765 | 4.9000 | 5.6644 | 4.96 | 5.80 |
| | PHMB | 5 | 3.3086 | .44059 | .19704 | 2.7615 | 3.8557 | 2.85 | 3.73 |
| | Total | 20 | 5.5577 | 1.88166 | .42075 | 4.6770 | 6.4383 | 2.85 | 8.38 |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| P1 | NSCD | 5 | 7.9268 | .09123 | .04080 | 7.8135 | 8.0401 | 7.89 | 8.09 |
| | ISCD | 5 | 7.9268 | .09123 | .04080 | 7.8135 | 8.0401 | 7.89 | 8.09 |
| | Honey | 5 | 8.0900 | .00000 | .00000 | 8.0900 | 8.0900 | 8.09 | 8.09 |
| | PHMB | 5 | 9.0560 | .57799 | .25848 | 8.3383 | 9.7737 | 8.39 | 9.91 |
| | Total | 20 | 8.2499 | .55363 | .12380 | 7.9908 | 8.5090 | 7.89 | 9.91 |
| P2 | NSCD | 5 | 7.8658 | .13461 | .06020 | 7.6987 | 8.0329 | 7.63 | 7.93 |
| | ISCD | 5 | 7.6508 | .42901 | .19186 | 7.1181 | 8.1835 | 6.95 | 7.93 |
| | Honey | 5 | 7.6698 | .38626 | .17274 | 7.1902 | 8.1494 | 7.00 | 7.93 |
| | PHMB | 5 | 6.3682 | 1.05817 | .47323 | 5.0543 | 7.6821 | 5.62 | 8.00 |
| | Total | 20 | 7.3887 | .82616 | .18473 | 7.0020 | 7.7753 | 5.62 | 8.00 |
| P3 | NSCD | 5 | 7.5326 | .21332 | .09540 | 7.2677 | 7.7975 | 7.15 | 7.63 |
| | ISCD | 5 | 6.7566 | .74230 | .33197 | 5.8349 | 7.6783 | 5.78 | 7.63 |
| | Honey | 5 | 7.6280 | .00000 | .00000 | 7.6280 | 7.6280 | 7.63 | 7.63 |
| | PHMB | 5 | 6.2278 | 1.02345 | .45770 | 4.9570 | 7.4986 | 5.70 | 8.06 |
| | Total | 20 | 7.0363 | .83406 | .18650 | 6.6459 | 7.4266 | 5.70 | 8.06 |
| P4 | NSCD | 5 | 6.7868 | .86229 | .38563 | 5.7161 | 7.8575 | 5.87 | 7.85 |
| | ISCD | 5 | 7.0330 | .67744 | .30296 | 6.1919 | 7.8741 | 6.38 | 7.85 |
| | Honey | 5 | 6.8266 | .88591 | .39619 | 5.7266 | 7.9266 | 5.83 | 7.85 |
| | PHMB | 5 | 7.1236 | 1.06871 | .47794 | 5.7966 | 8.4506 | 5.98 | 8.26 |
| | Total | 20 | 6.9425 | .82434 | .18433 | 6.5567 | 7.3283 | 5.83 | 8.26 |
| P5 | NSCD | 5 | 8.1816 | .26922 | .12040 | 7.8473 | 8.5159 | 7.70 | 8.30 |
| | ISCD | 5 | 8.3020 | .00000 | .00000 | 8.3020 | 8.3020 | 8.30 | 8.30 |
| | Honey | 5 | 8.3020 | .00000 | .00000 | 8.3020 | 8.3020 | 8.30 | 8.30 |
| | PHMB | 5 | 6.1198 | .27887 | .12471 | 5.7735 | 6.4661 | 5.79 | 6.47 |
| | Total | 20 | 7.7263 | .96943 | .21677 | 7.2726 | 8.1801 | 5.79 | 8.30 |
| St6538 | NSCD | 5 | 7.0210 | .00000 | .00000 | 7.0210 | 7.0210 | 7.02 | 7.02 |
| | ISCD | 5 | 4.9442 | .14727 | .06586 | 4.7613 | 5.1271 | 4.74 | 5.11 |
| | Honey | 5 | 6.3454 | .57441 | .25689 | 5.6322 | 7.0586 | 5.89 | 7.32 |
| | PHMB | 5 | 5.7550 | .18020 | .08059 | 5.5312 | 5.9788 | 5.54 | 5.94 |
| | Total | 20 | 6.0164 | .83393 | .18647 | 5.6261 | 6.4067 | 4.74 | 7.32 |
| St4300 | NSCD | 5 | 7.3960 | .00000 | .00000 | 7.3960 | 7.3960 | 7.40 | 7.40 |
| | ISCD | 5 | 7.2404 | .15093 | .06750 | 7.0530 | 7.4278 | 7.10 | 7.40 |
| | Honey | 5 | 6.4506 | .20853 | .09326 | 6.1917 | 6.7095 | 6.17 | 6.66 |
| | PHMB | 5 | 6.9596 | .61217 | .27377 | 6.1995 | 7.7197 | 6.12 | 7.40 |
| | Total | 20 | 7.0116 | .47858 | .10701 | 6.7877 | 7.2356 | 6.12 | 7.40 |
| St_7f_C7 | NSCD | 5 | 6.6444 | .19924 | .08910 | 6.3970 | 6.8918 | 6.44 | 6.84 |
| | ISCD | 5 | 2.5272 | 1.21978 | .54550 | 1.0126 | 4.0418 | .36 | 3.24 |
| | Honey | 5 | 6.8036 | .07916 | .03540 | 6.7053 | 6.9019 | 6.66 | 6.84 |
| | PHMB | 5 | 5.6288 | .05329 | .02383 | 5.5626 | 5.6950 | 5.56 | 5.68 |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Total | 20 | 5.4010 | 1.85340 | .41443 | 4.5336 | 6.2684 | .36 | 6.84 |
| St13142 | NSCD | 5 | 6.9438 | .09123 | .04080 | 6.8305 | 7.0571 | 6.90 | 7.11 |
| | ISCD | 5 | 6.9438 | .09123 | .04080 | 6.8305 | 7.0571 | 6.90 | 7.11 |
| | Honey | 5 | 6.9438 | .09123 | .04080 | 6.8305 | 7.0571 | 6.90 | 7.11 |
| | PHMB | 5 | 6.9438 | .09123 | .04080 | 6.8305 | 7.0571 | 6.90 | 7.11 |
| | Total | 20 | 6.9438 | .08372 | .01872 | 6.9046 | 6.9830 | 6.90 | 7.11 |
| St9B_F6 | NSCD | 5 | 6.8296 | .60463 | .27040 | 6.0788 | 7.5804 | 5.75 | 7.10 |
| | ISCD | 5 | 6.1060 | .92685 | .41450 | 4.9552 | 7.2568 | 5.25 | 7.10 |
| | Honey | 5 | 6.3418 | .78577 | .35141 | 5.3661 | 7.3175 | 5.36 | 7.10 |
| | PHMB | 5 | 5.3272 | .60912 | .27241 | 4.5709 | 6.0835 | 4.40 | 5.74 |
| | Total | 20 | 6.1511 | .88080 | .19695 | 5.7389 | 6.5634 | 4.40 | 7.10 |

**Multiple Comparisons**

Games-Howell

| Bacteria | (I) VAR00001 | (J) VAR00001 | Mean Difference (I-J) | Std. Error | Sig. | 95% Confidence Interval | |
|---|---|---|---|---|---|---|---|
| | | | | | | Lower Bound | Upper Bound |
| clone1 | NSCD | ISCD | 2.30200* | .05500 | .000 | 2.0781 | 2.5259 |
| | | Honey | .68700 | .00000 | . | .6870 | .6870 |
| | | PHMB | 5.61500* | .03094 | .000 | 5.4891 | 5.7409 |
| | ISCD | NSCD | -2.30200* | .05500 | .000 | -2.5259 | -2.0781 |
| | | Honey | -1.61500* | .05500 | .000 | -1.8389 | -1.3911 |
| | | PHMB | 3.31300* | .06310 | .000 | 3.0978 | 3.5282 |
| | Honey | NSCD | -.68700 | .00000 | . | -.6870 | -.6870 |
| | | ISCD | 1.61500* | .05500 | .000 | 1.3911 | 1.8389 |
| | | PHMB | 4.92800* | .03094 | .000 | 4.8021 | 5.0539 |
| | PHMB | NSCD | -5.61500* | .03094 | .000 | -5.7409 | -5.4891 |
| | | ISCD | -3.31300* | .06310 | .000 | -3.5282 | -3.0978 |
| | | Honey | -4.92800* | .03094 | .000 | -5.0539 | -4.8021 |
| clone2 | NSCD | ISCD | 2.96240* | .21746 | .001 | 2.0772 | 3.8476 |
| | | Honey | .04600 | .00000 | . | .0460 | .0460 |
| | | PHMB | 4.90360* | .25257 | .000 | 3.8754 | 5.9318 |
| | ISCD | NSCD | -2.96240* | .21746 | .001 | -3.8476 | -2.0772 |
| | | Honey | -2.91640* | .21746 | .001 | -3.8016 | -2.0312 |
| | | PHMB | 1.94120* | .33329 | .002 | .8684 | 3.0140 |
| | Honey | NSCD | -.04600 | .00000 | . | -.0460 | -.0460 |
| | | ISCD | 2.91640* | .21746 | .001 | 2.0312 | 3.8016 |
| | | PHMB | 4.85760* | .25257 | .000 | 3.8294 | 5.8858 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| | PHMB | NSCD | -4.90360* | .25257 | .000 | -5.9318 | -3.8754 |
| | | ISCD | -1.94120* | .33329 | .002 | -3.0140 | -.8684 |
| | | Honey | -4.85760* | .25257 | .000 | -5.8858 | -3.8294 |
| ukhs | NSCD | ISCD | 3.11580* | .15547 | .000 | 2.4829 | 3.7487 |
| | | Honey | .64700 | .00000 | . | .6470 | .6470 |
| | | PHMB | 5.18820* | .16166 | .000 | 4.5301 | 5.8463 |
| | ISCD | NSCD | -3.11580* | .15547 | .000 | -3.7487 | -2.4829 |
| | | Honey | -2.46880* | .15547 | .000 | -3.1017 | -1.8359 |
| | | PHMB | 2.07240* | .22429 | .000 | 1.3539 | 2.7909 |
| | Honey | NSCD | -.64700 | .00000 | . | -.6470 | -.6470 |
| | | ISCD | 2.46880* | .15547 | .000 | 1.8359 | 3.1017 |
| | | PHMB | 4.54120* | .16166 | .000 | 3.8831 | 5.1993 |
| | PHMB | NSCD | -5.18820* | .16166 | .000 | -5.8463 | -4.5301 |
| | | ISCD | -2.07240* | .22429 | .000 | -2.7909 | -1.3539 |
| | | Honey | -4.54120* | .16166 | .000 | -5.1993 | -3.8831 |
| Act_Type | NSCD | ISCD | 3.14440* | .16678 | .000 | 2.4654 | 3.8234 |
| | | Honey | 1.17700 | .00000 | . | 1.1770 | 1.1770 |
| | | PHMB | 5.47520* | .05768 | .000 | 5.2404 | 5.7100 |
| | ISCD | NSCD | -3.14440* | .16678 | .000 | -3.8234 | -2.4654 |
| | | Honey | -1.96740* | .16678 | .001 | -2.6464 | -1.2884 |
| | | PHMB | 2.33080* | .17648 | .000 | 1.6767 | 2.9849 |
| | Honey | NSCD | -1.17700 | .00000 | . | -1.1770 | -1.1770 |
| | | ISCD | 1.96740* | .16678 | .001 | 1.2884 | 2.6464 |
| | | PHMB | 4.29820* | .05768 | .000 | 4.0634 | 4.5330 |
| | PHMB | NSCD | -5.47520* | .05768 | .000 | -5.7100 | -5.2404 |
| | | ISCD | -2.33080* | .17648 | .000 | -2.9849 | -1.6767 |
| | | Honey | -4.29820* | .05768 | .000 | -4.5330 | -4.0634 |
| A882 | NSCD | ISCD | 1.24800* | .31904 | .047 | .0204 | 2.4756 |
| | | Honey | 4.42600* | .31569 | .000 | 3.1895 | 5.6625 |
| | | PHMB | 4.49800* | .32239 | .000 | 3.2776 | 5.7184 |
| | ISCD | NSCD | -1.24800* | .31904 | .047 | -2.4756 | -.0204 |
| | | Honey | 3.17800* | .10106 | .000 | 2.8511 | 3.5049 |
| | | PHMB | 3.25000* | .12037 | .000 | 2.8626 | 3.6374 |
| | Honey | NSCD | -4.42600* | .31569 | .000 | -5.6625 | -3.1895 |
| | | ISCD | -3.17800* | .10106 | .000 | -3.5049 | -2.8511 |
| | | PHMB | .07200 | .11119 | .913 | -.2941 | .4381 |
| | PHMB | NSCD | -4.49800* | .32239 | .000 | -5.7184 | -3.2776 |
| | | ISCD | -3.25000* | .12037 | .000 | -3.6374 | -2.8626 |
| | | Honey | -.07200 | .11119 | .913 | -.4381 | .2941 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| A292 | NSCD | ISCD | .00000 | .00000 | . | .0000 | .0000 |
| | | Honey | 2.61800* | .07123 | .000 | 2.3280 | 2.9080 |
| | | PHMB | 3.62600* | .11788 | .000 | 3.1461 | 4.1059 |
| | ISCD | NSCD | .00000 | .00000 | . | .0000 | .0000 |
| | | Honey | 2.61800* | .07123 | .000 | 2.3280 | 2.9080 |
| | | PHMB | 3.62600* | .11788 | .000 | 3.1461 | 4.1059 |
| | Honey | NSCD | -2.61800* | .07123 | .000 | -2.9080 | -2.3280 |
| | | ISCD | -2.61800* | .07123 | .000 | -2.9080 | -2.3280 |
| | | PHMB | 1.00800* | .13773 | .001 | .5442 | 1.4718 |
| | PHMB | NSCD | -3.62600* | .11788 | .000 | -4.1059 | -3.1461 |
| | | ISCD | -3.62600* | .11788 | .000 | -4.1059 | -3.1461 |
| | | Honey | -1.00800* | .13773 | .001 | -1.4718 | -.5442 |
| Ps_Type | NSCD | ISCD | 3.12220* | .08457 | .000 | 2.7779 | 3.4665 |
| | | Honey | 3.09880* | .13765 | .000 | 2.5385 | 3.6591 |
| | | PHMB | 5.07240* | .19704 | .000 | 4.2703 | 5.8745 |
| | ISCD | NSCD | -3.12220* | .08457 | .000 | -3.4665 | -2.7779 |
| | | Honey | -.02340 | .16155 | .999 | -.5659 | .5191 |
| | | PHMB | 1.95020* | .21442 | .001 | 1.1826 | 2.7178 |
| | Honey | NSCD | -3.09880* | .13765 | .000 | -3.6591 | -2.5385 |
| | | ISCD | .02340 | .16155 | .999 | -.5191 | .5659 |
| | | PHMB | 1.97360* | .24035 | .000 | 1.1825 | 2.7647 |
| | PHMB | NSCD | -5.07240* | .19704 | .000 | -5.8745 | -4.2703 |
| | | ISCD | -1.95020* | .21442 | .001 | -2.7178 | -1.1826 |
| | | Honey | -1.97360* | .24035 | .000 | -2.7647 | -1.1825 |
| P1 | NSCD | ISCD | .00000 | .05770 | 1.000 | -.1848 | .1848 |
| | | Honey | -.16320 | .04080 | .053 | -.3293 | .0029 |
| | | PHMB | -1.12920* | .26168 | .038 | -2.1697 | -.0887 |
| | ISCD | NSCD | .00000 | .05770 | 1.000 | -.1848 | .1848 |
| | | Honey | -.16320 | .04080 | .053 | -.3293 | .0029 |
| | | PHMB | -1.12920* | .26168 | .038 | -2.1697 | -.0887 |
| | Honey | NSCD | .16320 | .04080 | .053 | -.0029 | .3293 |
| | | ISCD | .16320 | .04080 | .053 | -.0029 | .3293 |
| | | PHMB | -.96600 | .25848 | .065 | -2.0183 | .0863 |
| | PHMB | NSCD | 1.12920* | .26168 | .038 | .0887 | 2.1697 |
| | | ISCD | 1.12920* | .26168 | .038 | .0887 | 2.1697 |
| | | Honey | .96600 | .25848 | .065 | -.0863 | 2.0183 |
| P2 | NSCD | ISCD | .21500 | .20108 | .722 | -.5404 | .9704 |
| | | Honey | .19600 | .18293 | .720 | -.4812 | .8732 |
| | | PHMB | 1.49760 | .47704 | .106 | -.4143 | 3.4095 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| | ISCD | NSCD | -.21500 | .20108 | .722 | -.9704 | .5404 |
| | | Honey | -.01900 | .25817 | 1.000 | -.8478 | .8098 |
| | | PHMB | 1.28260 | .51064 | .167 | -.5634 | 3.1286 |
| | Honey | NSCD | -.19600 | .18293 | .720 | -.8732 | .4812 |
| | | ISCD | .01900 | .25817 | 1.000 | -.8098 | .8478 |
| | | PHMB | 1.30160 | .50377 | .158 | -.5505 | 3.1537 |
| | PHMB | NSCD | -1.49760 | .47704 | .106 | -3.4095 | .4143 |
| | | ISCD | -1.28260 | .51064 | .167 | -3.1286 | .5634 |
| | | Honey | -1.30160 | .50377 | .158 | -3.1537 | .5505 |
| P3 | NSCD | ISCD | .77600 | .34540 | .237 | -.5356 | 2.0876 |
| | | Honey | -.09540 | .09540 | .759 | -.4838 | .2930 |
| | | PHMB | 1.30480 | .46754 | .142 | -.5247 | 3.1343 |
| | ISCD | NSCD | -.77600 | .34540 | .237 | -2.0876 | .5356 |
| | | Honey | -.87140 | .33197 | .176 | -2.2228 | .4800 |
| | | PHMB | .52880 | .56541 | .788 | -1.3227 | 2.3803 |
| | Honey | NSCD | .09540 | .09540 | .759 | -.2930 | .4838 |
| | | ISCD | .87140 | .33197 | .176 | -.4800 | 2.2228 |
| | | PHMB | 1.40020 | .45770 | .118 | -.4630 | 3.2634 |
| | PHMB | NSCD | -1.30480 | .46754 | .142 | -3.1343 | .5247 |
| | | ISCD | -.52880 | .56541 | .788 | -2.3803 | 1.3227 |
| | | Honey | -1.40020 | .45770 | .118 | -3.2634 | .4630 |
| P4 | NSCD | ISCD | -.24620 | .49040 | .956 | -1.8371 | 1.3447 |
| | | Honey | -.03980 | .55288 | 1.000 | -1.8106 | 1.7310 |
| | | PHMB | -.33680 | .61411 | .944 | -2.3238 | 1.6502 |
| | ISCD | NSCD | .24620 | .49040 | .956 | -1.3447 | 1.8371 |
| | | Honey | .20640 | .49875 | .974 | -1.4163 | 1.8291 |
| | | PHMB | -.09060 | .56587 | .998 | -1.9810 | 1.7998 |
| | Honey | NSCD | .03980 | .55288 | 1.000 | -1.7310 | 1.8106 |
| | | ISCD | -.20640 | .49875 | .974 | -1.8291 | 1.4163 |
| | | PHMB | -.29700 | .62080 | .962 | -2.3009 | 1.7069 |
| | PHMB | NSCD | .33680 | .61411 | .944 | -1.6502 | 2.3238 |
| | | ISCD | .09060 | .56587 | .998 | -1.7998 | 1.9810 |
| | | Honey | .29700 | .62080 | .962 | -1.7069 | 2.3009 |
| P5 | NSCD | ISCD | -.12040 | .12040 | .759 | -.6105 | .3697 |
| | | Honey | -.12040 | .12040 | .759 | -.6105 | .3697 |
| | | PHMB | 2.06180* | .17335 | .000 | 1.5065 | 2.6171 |
| | ISCD | NSCD | .12040 | .12040 | .759 | -.3697 | .6105 |
| | | Honey | .00000 | .00000 | . | .0000 | .0000 |
| | | PHMB | 2.18220* | .12471 | .000 | 1.6745 | 2.6899 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| | Honey | NSCD | .12040 | .12040 | .759 | -.3697 | .6105 |
| | | ISCD | .00000 | .00000 | . | .0000 | .0000 |
| | | PHMB | 2.18220* | .12471 | .000 | 1.6745 | 2.6899 |
| | PHMB | NSCD | -2.06180* | .17335 | .000 | -2.6171 | -1.5065 |
| | | ISCD | -2.18220* | .12471 | .000 | -2.6899 | -1.6745 |
| | | Honey | -2.18220* | .12471 | .000 | -2.6899 | -1.6745 |
| St6538 | NSCD | ISCD | 2.07680* | .06586 | .000 | 1.8087 | 2.3449 |
| | | Honey | .67560 | .25689 | .176 | -.3701 | 1.7213 |
| | | PHMB | 1.26600* | .08059 | .000 | .9379 | 1.5941 |
| | ISCD | NSCD | -2.07680* | .06586 | .000 | -2.3449 | -1.8087 |
| | | Honey | -1.40120* | .26519 | .015 | -2.4207 | -.3817 |
| | | PHMB | -.81080* | .10408 | .000 | -1.1472 | -.4744 |
| | Honey | NSCD | -.67560 | .25689 | .176 | -1.7213 | .3701 |
| | | ISCD | 1.40120* | .26519 | .015 | .3817 | 2.4207 |
| | | PHMB | .59040 | .26923 | .248 | -.4210 | 1.6018 |
| | PHMB | NSCD | -1.26600* | .08059 | .000 | -1.5941 | -.9379 |
| | | ISCD | .81080* | .10408 | .000 | .4744 | 1.1472 |
| | | Honey | -.59040 | .26923 | .248 | -1.6018 | .4210 |
| St4300 | NSCD | ISCD | .15560 | .06750 | .240 | -.1192 | .4304 |
| | | Honey | .94540* | .09326 | .002 | .5658 | 1.3250 |
| | | PHMB | .43640 | .27377 | .472 | -.6781 | 1.5509 |
| | ISCD | NSCD | -.15560 | .06750 | .240 | -.4304 | .1192 |
| | | Honey | .78980* | .11512 | .001 | .4127 | 1.1669 |
| | | PHMB | .28080 | .28197 | .760 | -.8073 | 1.3689 |
| | Honey | NSCD | -.94540* | .09326 | .002 | -1.3250 | -.5658 |
| | | ISCD | -.78980* | .11512 | .001 | -1.1669 | -.4127 |
| | | PHMB | -.50900 | .28922 | .388 | -1.5833 | .5653 |
| | PHMB | NSCD | -.43640 | .27377 | .472 | -1.5509 | .6781 |
| | | ISCD | -.28080 | .28197 | .760 | -1.3689 | .8073 |
| | | Honey | .50900 | .28922 | .388 | -.5653 | 1.5833 |
| St_7f_C7 | NSCD | ISCD | 4.11720* | .55273 | .005 | 1.9230 | 6.3114 |
| | | Honey | -.15920 | .09588 | .425 | -.5070 | .1886 |
| | | PHMB | 1.01560* | .09224 | .001 | .6625 | 1.3687 |
| | ISCD | NSCD | -4.11720* | .55273 | .005 | -6.3114 | -1.9230 |
| | | Honey | -4.27640* | .54665 | .005 | -6.4925 | -2.0603 |
| | | PHMB | -3.10160* | .54602 | .016 | -5.3202 | -.8830 |
| | Honey | NSCD | .15920 | .09588 | .425 | -.1886 | .5070 |
| | | ISCD | 4.27640* | .54665 | .005 | 2.0603 | 6.4925 |
| | | PHMB | 1.17480* | .04267 | .000 | 1.0336 | 1.3160 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| | PHMB | NSCD | -1.01560* | .09224 | .001 | -1.3687 | -.6625 |
| | | ISCD | 3.10160* | .54602 | .016 | .8830 | 5.3202 |
| | | Honey | -1.17480* | .04267 | .000 | -1.3160 | -1.0336 |
| St13142 | NSCD | ISCD | .00000 | .05770 | 1.000 | -.1848 | .1848 |
| | | Honey | .00000 | .05770 | 1.000 | -.1848 | .1848 |
| | | PHMB | .00000 | .05770 | 1.000 | -.1848 | .1848 |
| | ISCD | NSCD | .00000 | .05770 | 1.000 | -.1848 | .1848 |
| | | Honey | .00000 | .05770 | 1.000 | -.1848 | .1848 |
| | | PHMB | .00000 | .05770 | 1.000 | -.1848 | .1848 |
| | Honey | NSCD | .00000 | .05770 | 1.000 | -.1848 | .1848 |
| | | ISCD | .00000 | .05770 | 1.000 | -.1848 | .1848 |
| | | PHMB | .00000 | .05770 | 1.000 | -.1848 | .1848 |
| | PHMB | NSCD | .00000 | .05770 | 1.000 | -.1848 | .1848 |
| | | ISCD | .00000 | .05770 | 1.000 | -.1848 | .1848 |
| | | Honey | .00000 | .05770 | 1.000 | -.1848 | .1848 |
| St9B_F6 | NSCD | ISCD | .72360 | .49490 | .505 | -.9221 | 2.3693 |
| | | Honey | .48780 | .44340 | .700 | -.9538 | 1.9294 |
| | | PHMB | 1.50240* | .38383 | .019 | .2732 | 2.7316 |
| | ISCD | NSCD | -.72360 | .49490 | .505 | -2.3693 | .9221 |
| | | Honey | -.23580 | .54341 | .971 | -1.9868 | 1.5152 |
| | | PHMB | .77880 | .49600 | .451 | -.8686 | 2.4262 |
| | Honey | NSCD | -.48780 | .44340 | .700 | -1.9294 | .9538 |
| | | ISCD | .23580 | .54341 | .971 | -1.5152 | 1.9868 |
| | | PHMB | 1.01460 | .44463 | .186 | -.4298 | 2.4590 |
| | PHMB NSCD | NSCD | -1.50240* | .38383 | .019 | -2.7316 | -.2732 |
| | | ISCD | -.77880 | .49600 | .451 | -2.4262 | .8686 |
| | | Honey | -1.01460 | .44463 | .186 | -2.4590 | .4298 |

*. The mean difference is significant at the 0.05 level.

### 8.3.2 **Collagen wound model comparison**

| Descriptives | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | N | Mean | Std. Dev | Std. Err | 95% Confidence Interval for Mean | | Min | Max |
| | | | | | | Lower Bound | Upper Bound | | |
| clone1 | NSCD | 5 | 8.6300 | .00000 | .00000 | 8.6300 | 8.6300 | 8.63 | 8.63 |
| | ISCD | 5 | 8.6300 | .00000 | .00000 | 8.6300 | 8.6300 | 8.63 | 8.63 |
| | Honey | 5 | 2.2156 | .18713 | .08369 | 1.9833 | 2.4479 | 1.94 | 2.43 |
| | PHMB | 5 | 2.8694 | .36170 | .16176 | 2.4203 | 3.3185 | 2.22 | 3.05 |
| | Total | 20 | 5.5863 | 3.13738 | .70154 | 4.1179 | 7.0546 | 1.94 | 8.63 |
| clone2 | NSCD | 5 | 8.4670 | .00000 | .00000 | 8.4670 | 8.4670 | 8.47 | 8.47 |
| | ISCD | 5 | 8.4670 | .00000 | .00000 | 8.4670 | 8.4670 | 8.47 | 8.47 |
| | Honey | 5 | 2.0320 | .32237 | .14417 | 1.6317 | 2.4323 | 1.49 | 2.32 |
| | PHMB | 5 | 2.6350 | .29647 | .13258 | 2.2669 | 3.0031 | 2.20 | 2.99 |
| | Total | 20 | 5.4003 | 3.16041 | .70669 | 3.9211 | 6.8794 | 1.49 | 8.47 |
| ukhs | NSCD | 5 | 8.9030 | .00000 | .00000 | 8.9030 | 8.9030 | 8.90 | 8.90 |
| | ISCD | 5 | 8.9030 | .00000 | .00000 | 8.9030 | 8.9030 | 8.90 | 8.90 |
| | Honey | 5 | 2.4891 | .18698 | .08362 | 2.2569 | 2.7212 | 2.21 | 2.71 |
| | PHMB | 5 | 2.8346 | .31209 | .13957 | 2.4471 | 3.2221 | 2.60 | 3.36 |
| | Total | 20 | 5.7824 | 3.20846 | .71743 | 4.2808 | 7.2840 | 2.21 | 8.90 |
| Act_Type | NSCD | 5 | 8.7025 | .00000 | .00000 | 8.7025 | 8.7025 | 8.70 | 8.70 |
| | ISCD | 5 | 8.7025 | .00000 | .00000 | 8.7025 | 8.7025 | 8.70 | 8.70 |
| | Honey | 5 | 4.5008 | .08525 | .03813 | 4.3950 | 4.6067 | 4.43 | 4.65 |
| | PHMB | 5 | 2.9699 | .20385 | .09116 | 2.7168 | 3.2230 | 2.75 | 3.25 |
| | Total | 20 | 6.2189 | 2.60988 | .58359 | 4.9975 | 7.4404 | 2.75 | 8.70 |
| A882 | NSCD | 5 | 6.5900 | .13565 | .06066 | 6.4216 | 6.7584 | 6.46 | 6.81 |
| | ISCD | 5 | 6.1900 | .20248 | .09055 | 5.9386 | 6.4414 | 5.89 | 6.43 |
| | Honey | 5 | 4.2420 | .22443 | .10037 | 3.9633 | 4.5207 | 3.87 | 4.47 |
| | PHMB | 5 | 3.3542 | .15893 | .07108 | 3.1569 | 3.5516 | 3.23 | 3.63 |
| | Total | 20 | 5.0941 | 1.38601 | .30992 | 4.4454 | 5.7427 | 3.23 | 6.81 |
| A292 | NSCD | 5 | 8.8172 | .00000 | .00000 | 8.8172 | 8.8172 | 8.82 | 8.82 |
| | ISCD | 5 | 8.8172 | .00000 | .00000 | 8.8172 | 8.8172 | 8.82 | 8.82 |
| | Honey | 5 | 6.1889 | .24969 | .11166 | 5.8789 | 6.4990 | 5.87 | 6.47 |
| | PHMB | 5 | 6.3517 | .15893 | .07108 | 6.1544 | 6.5491 | 6.23 | 6.63 |
| | Total | 20 | 7.5437 | 1.31487 | .29401 | 6.9284 | 8.1591 | 5.87 | 8.82 |
| Ps_Type | NSCD | 5 | 8.3180 | .00000 | .00000 | 8.3180 | 8.3180 | 8.32 | 8.32 |
| | ISCD | 5 | 8.3180 | .00000 | .00000 | 8.3180 | 8.3180 | 8.32 | 8.32 |
| | Honey | 5 | 2.6574 | .14049 | .06283 | 2.4830 | 2.8318 | 2.48 | 2.85 |
| | PHMB | 5 | 8.3180 | .00000 | .00000 | 8.3180 | 8.3180 | 8.32 | 8.32 |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Total | 20 | 6.9028 | 2.51561 | .56251 | 5.7255 | 8.0802 | 2.48 | 8.32 |
| P1 | NSCD | 5 | 6.4490 | .12670 | .05666 | 6.2917 | 6.6063 | 6.29 | 6.55 |
| | ISCD | 5 | 5.5046 | .20808 | .09306 | 5.2462 | 5.7630 | 5.26 | 5.79 |
| | Honey | 5 | 4.8686 | .24635 | .11017 | 4.5627 | 5.1745 | 4.62 | 5.23 |
| | PHMB | 5 | 3.6640 | .13244 | .05923 | 3.4996 | 3.8284 | 3.52 | 3.78 |
| | Total | 20 | 5.1216 | 1.05223 | .23529 | 4.6291 | 5.6140 | 3.52 | 6.55 |
| P2 | NSCD | 5 | 6.5230 | .13843 | .06191 | 6.3511 | 6.6949 | 6.32 | 6.68 |
| | ISCD | 5 | 4.8846 | .32899 | .14713 | 4.4761 | 5.2931 | 4.39 | 5.17 |
| | Honey | 5 | 4.6484 | .20595 | .09210 | 4.3927 | 4.9041 | 4.36 | 4.90 |
| | PHMB | 5 | 3.5564 | .20387 | .09117 | 3.3033 | 3.8095 | 3.34 | 3.84 |
| | Total | 20 | 4.9031 | 1.10882 | .24794 | 4.3842 | 5.4220 | 3.34 | 6.68 |
| P3 | NSCD | 5 | 6.4492 | .16816 | .07520 | 6.2404 | 6.6580 | 6.36 | 6.75 |
| | ISCD | 5 | 4.8048 | .15562 | .06959 | 4.6116 | 4.9980 | 4.68 | 5.02 |
| | Honey | 5 | 3.8344 | .27864 | .12461 | 3.4884 | 4.1804 | 3.52 | 4.13 |
| | PHMB | 5 | 4.7966 | .28965 | .12953 | 4.4370 | 5.1562 | 4.34 | 5.13 |
| | Total | 20 | 4.9712 | .98758 | .22083 | 4.5090 | 5.4335 | 3.52 | 6.75 |
| P4 | NSCD | 5 | 6.7714 | .16073 | .07188 | 6.5718 | 6.9710 | 6.51 | 6.91 |
| | ISCD | 5 | 6.2560 | .23027 | .10298 | 5.9701 | 6.5419 | 5.85 | 6.40 |
| | Honey | 5 | 1.7500 | .36216 | .16196 | 1.3003 | 2.1997 | 1.51 | 2.37 |
| | PHMB | 5 | 5.8038 | .03410 | .01525 | 5.7615 | 5.8461 | 5.77 | 5.86 |
| | Total | 20 | 5.1453 | 2.05250 | .45895 | 4.1847 | 6.1059 | 1.51 | 6.91 |
| P5 | NSCD | 5 | 6.3242 | .18671 | .08350 | 6.0924 | 6.5560 | 6.19 | 6.63 |
| | ISCD | 5 | 6.4552 | .31122 | .13918 | 6.0688 | 6.8416 | 6.11 | 6.71 |
| | Honey | 5 | 2.4582 | .32410 | .14494 | 2.0558 | 2.8606 | 2.21 | 2.82 |
| | PHMB | 5 | 6.2410 | .03417 | .01528 | 6.1986 | 6.2834 | 6.21 | 6.30 |
| | Total | 20 | 5.3697 | 1.74082 | .38926 | 4.5549 | 6.1844 | 2.21 | 6.71 |
| St6538 | NSCD | 5 | 8.6300 | .00000 | .00000 | 8.6300 | 8.6300 | 8.63 | 8.63 |
| | ISCD | 5 | 8.6902 | .13461 | .06020 | 8.5231 | 8.8573 | 8.63 | 8.93 |
| | Honey | 5 | 5.2700 | .24321 | .10876 | 4.9680 | 5.5720 | 4.97 | 5.58 |
| | PHMB | 5 | 3.0858 | .14657 | .06555 | 2.9038 | 3.2678 | 2.88 | 3.26 |
| | Total | 20 | 6.4190 | 2.43636 | .54479 | 5.2787 | 7.5593 | 2.88 | 8.93 |
| St4300 | NSCD | 5 | 4.7926 | .17496 | .07824 | 4.5754 | 5.0098 | 4.60 | 4.99 |
| | ISCD | 5 | 5.0766 | .14231 | .06364 | 4.8999 | 5.2533 | 4.93 | 5.31 |
| | Honey | 5 | 4.8544 | .18075 | .08084 | 4.6300 | 5.0788 | 4.58 | 5.09 |
| | PHMB | 5 | 4.0042 | .36675 | .16402 | 3.5488 | 4.4596 | 3.58 | 4.46 |
| | Total | 20 | 4.6820 | .46778 | .10460 | 4.4630 | 4.9009 | 3.58 | 5.31 |
| St_7f_C7 | NSCD | 5 | 6.1322 | .17982 | .08042 | 5.9089 | 6.3555 | 5.92 | 6.34 |
| | ISCD | 5 | 6.0104 | .10662 | .04768 | 5.8780 | 6.1428 | 5.90 | 6.14 |
| | Honey | 5 | 5.6480 | .08818 | .03944 | 5.5385 | 5.7575 | 5.57 | 5.74 |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | PHMB | 5 | 4.2356 | .08095 | .03620 | 4.1351 | 4.3361 | 4.13 | 4.35 |
| | Total | 20 | 5.5066 | .78255 | .17498 | 5.1403 | 5.8728 | 4.13 | 6.34 |
| St13142 | NSCD | 5 | 5.5592 | .17982 | .08042 | 5.3359 | 5.7825 | 5.34 | 5.77 |
| | ISCD | 5 | 5.6002 | .20708 | .09261 | 5.3431 | 5.8573 | 5.40 | 5.93 |
| | Honey | 5 | 4.8542 | .26564 | .11880 | 4.5244 | 5.1840 | 4.53 | 5.17 |
| | PHMB | 5 | 3.5860 | .04684 | .02095 | 3.5278 | 3.6442 | 3.56 | 3.67 |
| | Total | 20 | 4.8999 | .85408 | .19098 | 4.5002 | 5.2996 | 3.56 | 5.93 |
| St9B_F6 | NSCD | 5 | 5.8806 | .20964 | .09376 | 5.6203 | 6.1409 | 5.66 | 6.22 |
| | ISCD | 5 | 5.7426 | .20808 | .09306 | 5.4842 | 6.0010 | 5.50 | 6.03 |
| | Honey | 5 | 4.8294 | .26555 | .11876 | 4.4997 | 5.1591 | 4.50 | 5.15 |
| | PHMB | 5 | 3.6202 | .08728 | .03903 | 3.5118 | 3.7286 | 3.54 | 3.74 |
| | Total | 20 | 5.0182 | .94463 | .21123 | 4.5761 | 5.4603 | 3.54 | 6.22 |

**Multiple Comparisons**

Games-Howell

| Dependent Variable | (I) VAR00001 | (J) VAR00001 | Mean Difference (I-J) | Std. Error | Sig. | 95% Confidence Interval | |
|---|---|---|---|---|---|---|---|
| | | | | | | Lower Bound | Upper Bound |
| clone1 | NSCD | ISCD | .00000 | .00000 | . | .0000 | .0000 |
| | | Honey | 6.41440* | .08369 | .000 | 6.0737 | 6.7551 |
| | | PHMB | 5.76060* | .16176 | .000 | 5.1021 | 6.4191 |
| | ISCD | NSCD | .00000 | .00000 | . | .0000 | .0000 |
| | | Honey | 6.41440* | .08369 | .000 | 6.0737 | 6.7551 |
| | | PHMB | 5.76060* | .16176 | .000 | 5.1021 | 6.4191 |
| | Honey | NSCD | -6.41440* | .08369 | .000 | -6.7551 | -6.0737 |
| | | ISCD | -6.41440* | .08369 | .000 | -6.7551 | -6.0737 |
| | | PHMB | -.65380* | .18212 | .043 | -1.2843 | -.0233 |
| | PHMB | NSCD | -5.76060* | .16176 | .000 | -6.4191 | -5.1021 |
| | | ISCD | -5.76060* | .16176 | .000 | -6.4191 | -5.1021 |
| | | Honey | .65380* | .18212 | .043 | .0233 | 1.2843 |
| clone2 | NSCD | ISCD | .00000 | .00000 | . | .0000 | .0000 |
| | | Honey | 6.43500* | .14417 | .000 | 5.8481 | 7.0219 |
| | | PHMB | 5.83200* | .13258 | .000 | 5.2923 | 6.3717 |
| | ISCD | NSCD | .00000 | .00000 | . | .0000 | .0000 |
| | | Honey | 6.43500* | .14417 | .000 | 5.8481 | 7.0219 |
| | | PHMB | 5.83200* | .13258 | .000 | 5.2923 | 6.3717 |
| | Honey | NSCD | -6.43500* | .14417 | .000 | -7.0219 | -5.8481 |
| | | ISCD | -6.43500* | .14417 | .000 | -7.0219 | -5.8481 |
| | | PHMB | -.60300 | .19586 | .060 | -1.2312 | .0252 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| | PHMB | NSCD | -5.83200* | .13258 | .000 | -6.3717 | -5.2923 |
| | | ISCD | -5.83200* | .13258 | .000 | -6.3717 | -5.2923 |
| | | Honey | .60300 | .19586 | .060 | -.0252 | 1.2312 |
| ukhs | NSCD | ISCD | .00000 | .00000 | . | .0000 | .0000 |
| | | Honey | 6.41394* | .08362 | .000 | 6.0735 | 6.7544 |
| | | PHMB | 6.06844* | .13957 | .000 | 5.5003 | 6.6366 |
| | ISCD | NSCD | .00000 | .00000 | . | .0000 | .0000 |
| | | Honey | 6.41394* | .08362 | .000 | 6.0735 | 6.7544 |
| | | PHMB | 6.06844* | .13957 | .000 | 5.5003 | 6.6366 |
| | Honey | NSCD | -6.41394* | .08362 | .000 | -6.7544 | -6.0735 |
| | | ISCD | -6.41394* | .08362 | .000 | -6.7544 | -6.0735 |
| | | PHMB | -.34550 | .16270 | .240 | -.8942 | .2032 |
| | PHMB | NSCD | -6.06844* | .13957 | .000 | -6.6366 | -5.5003 |
| | | ISCD | -6.06844* | .13957 | .000 | -6.6366 | -5.5003 |
| | | Honey | .34550 | .16270 | .240 | -.2032 | .8942 |
| Act_Type | NSCD | ISCD | .00000 | .00000 | . | .0000 | .0000 |
| | | Honey | 4.20166* | .03813 | .000 | 4.0465 | 4.3569 |
| | | PHMB | 5.73264* | .09116 | .000 | 5.3615 | 6.1038 |
| | ISCD | NSCD | .00000 | .00000 | . | .0000 | .0000 |
| | | Honey | 4.20166* | .03813 | .000 | 4.0465 | 4.3569 |
| | | PHMB | 5.73264* | .09116 | .000 | 5.3615 | 6.1038 |
| | Honey | NSCD | -4.20166* | .03813 | .000 | -4.3569 | -4.0465 |
| | | ISCD | -4.20166* | .03813 | .000 | -4.3569 | -4.0465 |
| | | PHMB | 1.53098* | .09881 | .000 | 1.1756 | 1.8863 |
| | PHMB | NSCD | -5.73264* | .09116 | .000 | -6.1038 | -5.3615 |
| | | ISCD | -5.73264* | .09116 | .000 | -6.1038 | -5.3615 |
| | | Honey | -1.53098* | .09881 | .000 | -1.8863 | -1.1756 |
| A882 | NSCD | ISCD | .40000* | .10900 | .032 | .0390 | .7610 |
| | | Honey | 2.34800* | .11728 | .000 | 1.9531 | 2.7429 |
| | | PHMB | 3.23575* | .09344 | .000 | 2.9348 | 3.5367 |
| | ISCD | NSCD | -.40000* | .10900 | .032 | -.7610 | -.0390 |
| | | Honey | 1.94800* | .13518 | .000 | 1.5141 | 2.3819 |
| | | PHMB | 2.83575* | .11512 | .000 | 2.4623 | 3.2092 |
| | Honey | NSCD | -2.34800* | .11728 | .000 | -2.7429 | -1.9531 |
| | | ISCD | -1.94800* | .13518 | .000 | -2.3819 | -1.5141 |
| | | PHMB | .88775* | .12299 | .001 | .4837 | 1.2918 |
| | PHMB | NSCD | -3.23575* | .09344 | .000 | -3.5367 | -2.9348 |
| | | ISCD | -2.83575* | .11512 | .000 | -3.2092 | -2.4623 |
| | | Honey | -.88775* | .12299 | .001 | -1.2918 | -.4837 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| A292 | NSCD | ISCD | .00000 | .00000 | . | .0000 | .0000 |
| | | Honey | 2.62824* | .11166 | .000 | 2.1737 | 3.0828 |
| | | PHMB | 2.46546* | .07108 | .000 | 2.1761 | 2.7548 |
| | ISCD | NSCD | .00000 | .00000 | . | .0000 | .0000 |
| | | Honey | 2.62824* | .11166 | .000 | 2.1737 | 3.0828 |
| | | PHMB | 2.46546* | .07108 | .000 | 2.1761 | 2.7548 |
| | Honey | NSCD | -2.62824* | .11166 | .000 | -3.0828 | -2.1737 |
| | | ISCD | -2.62824* | .11166 | .000 | -3.0828 | -2.1737 |
| | | PHMB | -.16278 | .13237 | .630 | -.6047 | .2791 |
| | PHMB | NSCD | -2.46546* | .07108 | .000 | -2.7548 | -2.1761 |
| | | ISCD | -2.46546* | .07108 | .000 | -2.7548 | -2.1761 |
| | | Honey | .16278 | .13237 | .630 | -.2791 | .6047 |
| Ps_Type | NSCD | ISCD | .00000 | .00000 | . | .0000 | .0000 |
| | | Honey | 5.66060* | .06283 | .000 | 5.4048 | 5.9164 |
| | | PHMB | .00000 | .00000 | . | .0000 | .0000 |
| | ISCD | NSCD | .00000 | .00000 | . | .0000 | .0000 |
| | | Honey | 5.66060* | .06283 | .000 | 5.4048 | 5.9164 |
| | | PHMB | .00000 | .00000 | . | .0000 | .0000 |
| | Honey | NSCD | -5.66060* | .06283 | .000 | -5.9164 | -5.4048 |
| | | ISCD | -5.66060* | .06283 | .000 | -5.9164 | -5.4048 |
| | | PHMB | -5.66060* | .06283 | .000 | -5.9164 | -5.4048 |
| | PHMB | NSCD | .00000 | .00000 | . | .0000 | .0000 |
| | | ISCD | .00000 | .00000 | . | .0000 | .0000 |
| | | Honey | 5.66060* | .06283 | .000 | 5.4048 | 5.9164 |
| P1 | NSCD | ISCD | .94440* | .10895 | .000 | .5780 | 1.3108 |
| | | Honey | 1.58040* | .12389 | .000 | 1.1510 | 2.0098 |
| | | PHMB | 2.78500* | .08197 | .000 | 2.5224 | 3.0476 |
| | ISCD | NSCD | -.94440* | .10895 | .000 | -1.3108 | -.5780 |
| | | Honey | .63600* | .14421 | .010 | .1712 | 1.1008 |
| | | PHMB | 1.84060* | .11031 | .000 | 1.4723 | 2.2089 |
| | Honey | NSCD | -1.58040* | .12389 | .000 | -2.0098 | -1.1510 |
| | | ISCD | -.63600* | .14421 | .010 | -1.1008 | -.1712 |
| | | PHMB | 1.20460* | .12508 | .000 | .7746 | 1.6346 |
| | PHMB | NSCD | -2.78500* | .08197 | .000 | -3.0476 | -2.5224 |
| | | ISCD | -1.84060* | .11031 | .000 | -2.2089 | -1.4723 |
| | | Honey | -1.20460* | .12508 | .000 | -1.6346 | -.7746 |
| P2 | NSCD | ISCD | 1.63840* | .15962 | .000 | 1.0650 | 2.2118 |
| | | Honey | 1.87460* | .11097 | .000 | 1.5073 | 2.2419 |
| | | PHMB | 2.96660* | .11020 | .000 | 2.6024 | 3.3308 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| | ISCD | NSCD | -1.63840* | .15962 | .000 | -2.2118 | -1.0650 |
| | | Honey | .23620 | .17358 | .559 | -.3449 | .8173 |
| | | PHMB | 1.32820* | .17309 | .001 | .7478 | 1.9086 |
| | Honey | NSCD | -1.87460* | .11097 | .000 | -2.2419 | -1.5073 |
| | | ISCD | -.23620 | .17358 | .559 | -.8173 | .3449 |
| | | PHMB | 1.09200* | .12960 | .000 | .6770 | 1.5070 |
| | PHMB | NSCD | -2.96660* | .11020 | .000 | -3.3308 | -2.6024 |
| | | ISCD | -1.32820* | .17309 | .001 | -1.9086 | -.7478 |
| | | Honey | -1.09200* | .12960 | .000 | -1.5070 | -.6770 |
| P3 | NSCD | ISCD | 1.64440* | .10246 | .000 | 1.3158 | 1.9730 |
| | | Honey | 2.61480* | .14554 | .000 | 2.1246 | 3.1050 |
| | | PHMB | 1.65260* | .14978 | .000 | 1.1447 | 2.1605 |
| | ISCD | NSCD | -1.64440* | .10246 | .000 | -1.9730 | -1.3158 |
| | | Honey | .97040* | .14273 | .002 | .4831 | 1.4577 |
| | | PHMB | .00820 | .14705 | 1.000 | -.4974 | .5138 |
| | Honey | NSCD | -2.61480* | .14554 | .000 | -3.1050 | -2.1246 |
| | | ISCD | -.97040* | .14273 | .002 | -1.4577 | -.4831 |
| | | PHMB | -.96220* | .17974 | .003 | -1.5380 | -.3864 |
| | PHMB | NSCD | -1.65260* | .14978 | .000 | -2.1605 | -1.1447 |
| | | ISCD | -.00820 | .14705 | 1.000 | -.5138 | .4974 |
| | | Honey | .96220* | .17974 | .003 | .3864 | 1.5380 |
| P4 | NSCD | ISCD | .51540* | .12558 | .018 | .1020 | .9288 |
| | | Honey | 5.02140* | .17720 | .000 | 4.3907 | 5.6521 |
| | | PHMB | .96760* | .07348 | .000 | .6804 | 1.2548 |
| | ISCD | NSCD | -.51540* | .12558 | .018 | -.9288 | -.1020 |
| | | Honey | 4.50600* | .19193 | .000 | 3.8652 | 5.1468 |
| | | PHMB | .45220* | .10410 | .038 | .0372 | .8672 |
| | Honey | NSCD | -5.02140* | .17720 | .000 | -5.6521 | -4.3907 |
| | | ISCD | -4.50600* | .19193 | .000 | -5.1468 | -3.8652 |
| | | PHMB | -4.05380* | .16268 | .000 | -4.7103 | -3.3973 |
| | PHMB | NSCD | -.96760* | .07348 | .000 | -1.2548 | -.6804 |
| | | ISCD | -.45220* | .10410 | .038 | -.8672 | -.0372 |
| | | Honey | 4.05380* | .16268 | .000 | 3.3973 | 4.7103 |
| P5 | NSCD | ISCD | -.13100 | .16231 | .849 | -.6782 | .4162 |
| | | Honey | 3.86600* | .16727 | .000 | 3.2980 | 4.4340 |
| | | PHMB | .08320 | .08488 | .768 | -.2518 | .4182 |
| | ISCD | NSCD | .13100 | .16231 | .849 | -.4162 | .6782 |
| | | Honey | 3.99700* | .20095 | .000 | 3.3533 | 4.6407 |
| | | PHMB | .21420 | .14002 | .498 | -.3492 | .7776 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| | Honey | NSCD | -3.86600* | .16727 | .000 | -4.4340 | -3.2980 |
| | | ISCD | -3.99700* | .20095 | .000 | -4.6407 | -3.3533 |
| | | PHMB | -3.78280* | .14574 | .000 | -4.3697 | -3.1959 |
| | PHMB | NSCD | -.08320 | .08488 | .768 | -.4182 | .2518 |
| | | ISCD | -.21420 | .14002 | .498 | -.7776 | .3492 |
| | | Honey | 3.78280* | .14574 | .000 | 3.1959 | 4.3697 |
| St6538 | NSCD | ISCD | -.06020 | .06020 | .759 | -.3053 | .1849 |
| | | Honey | 3.36000* | .10876 | .000 | 2.9172 | 3.8028 |
| | | PHMB | 5.54420* | .06555 | .000 | 5.2774 | 5.8110 |
| | ISCD | NSCD | .06020 | .06020 | .759 | -.1849 | .3053 |
| | | Honey | 3.42020* | .12431 | .000 | 2.9951 | 3.8453 |
| | | PHMB | 5.60440* | .08900 | .000 | 5.3189 | 5.8899 |
| | Honey | NSCD | -3.36000* | .10876 | .000 | -3.8028 | -2.9172 |
| | | ISCD | -3.42020* | .12431 | .000 | -3.8453 | -2.9951 |
| | | PHMB | 2.18420* | .12699 | .000 | 1.7564 | 2.6120 |
| | PHMB | NSCD | -5.54420* | .06555 | .000 | -5.8110 | -5.2774 |
| | | ISCD | -5.60440* | .08900 | .000 | -5.8899 | -5.3189 |
| | | Honey | -2.18420* | .12699 | .000 | -2.6120 | -1.7564 |
| St4300 | NSCD | ISCD | -.28400 | .10086 | .089 | -.6101 | .0421 |
| | | Honey | -.06180 | .11250 | .944 | -.4222 | .2986 |
| | | PHMB | .78840* | .18172 | .021 | .1499 | 1.4269 |
| | ISCD | NSCD | .28400 | .10086 | .089 | -.0421 | .6101 |
| | | Honey | .22220 | .10288 | .218 | -.1115 | .5559 |
| | | PHMB | 1.07240* | .17593 | .006 | .4318 | 1.7130 |
| | Honey | NSCD | .06180 | .11250 | .944 | -.2986 | .4222 |
| | | ISCD | -.22220 | .10288 | .218 | -.5559 | .1115 |
| | | PHMB | .85020* | .18286 | .015 | .2115 | 1.4889 |
| | PHMB | NSCD | -.78840* | .18172 | .021 | -1.4269 | -.1499 |
| | | ISCD | -1.07240* | .17593 | .006 | -1.7130 | -.4318 |
| | | Honey | -.85020* | .18286 | .015 | -1.4889 | -.2115 |
| St_7f_C7 | NSCD | ISCD | .12180 | .09349 | .591 | -.1941 | .4377 |
| | | Honey | .48420* | .08957 | .007 | .1711 | .7973 |
| | | PHMB | 1.89660* | .08819 | .000 | 1.5835 | 2.2097 |
| | ISCD | NSCD | -.12180 | .09349 | .591 | -.4377 | .1941 |
| | | Honey | .36240* | .06188 | .002 | .1626 | .5622 |
| | | PHMB | 1.77480* | .05987 | .000 | 1.5799 | 1.9697 |
| | Honey | NSCD | -.48420* | .08957 | .007 | -.7973 | -.1711 |
| | | ISCD | -.36240* | .06188 | .002 | -.5622 | -.1626 |
| | | PHMB | 1.41240* | .05353 | .000 | 1.2407 | 1.5841 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| | PHMB | NSCD | -1.89660* | .08819 | .000 | -2.2097 | -1.5835 |
| | | ISCD | -1.77480* | .05987 | .000 | -1.9697 | -1.5799 |
| | | Honey | -1.41240* | .05353 | .000 | -1.5841 | -1.2407 |
| St13142 | NSCD | ISCD | -.04100 | .12265 | .986 | -.4356 | .3536 |
| | | Honey | .70500* | .14346 | .007 | .2307 | 1.1793 |
| | | PHMB | 1.97320* | .08310 | .000 | 1.6542 | 2.2922 |
| | ISCD | NSCD | .04100 | .12265 | .986 | -.3536 | .4356 |
| | | Honey | .74600* | .15063 | .006 | .2569 | 1.2351 |
| | | PHMB | 2.01420* | .09495 | .000 | 1.6450 | 2.3834 |
| | Honey | NSCD | -.70500* | .14346 | .007 | -1.1793 | -.2307 |
| | | ISCD | -.74600* | .15063 | .006 | -1.2351 | -.2569 |
| | | PHMB | 1.26820* | .12063 | .001 | .7912 | 1.7452 |
| | PHMB | NSCD | -1.97320* | .08310 | .000 | -2.2922 | -1.6542 |
| | | ISCD | -2.01420* | .09495 | .000 | -2.3834 | -1.6450 |
| | | Honey | -1.26820* | .12063 | .001 | -1.7452 | -.7912 |
| St9B_F6 | NSCD | ISCD | .13800 | .13210 | .730 | -.2850 | .5610 |
| | | Honey | 1.05120* | .15130 | .001 | .5606 | 1.5418 |
| | | PHMB | 2.26040* | .10156 | .000 | 1.8949 | 2.6259 |
| | ISCD | NSCD | -.13800 | .13210 | .730 | -.5610 | .2850 |
| | | Honey | .91320* | .15087 | .002 | .4236 | 1.4028 |
| | | PHMB | 2.12240* | .10091 | .000 | 1.7597 | 2.4851 |
| | Honey | NSCD | -1.05120* | .15130 | .001 | -1.5418 | -.5606 |
| | | ISCD | -.91320* | .15087 | .002 | -1.4028 | -.4236 |
| | | PHMB | 1.20920* | .12501 | .001 | .7425 | 1.6759 |
| | PHMB | NSCD | -2.26040* | .10156 | .000 | -2.6259 | -1.8949 |
| | | ISCD | -2.12240* | .10091 | .000 | -2.4851 | -1.7597 |
| | | Honey | -1.20920* | .12501 | .001 | -1.6759 | -.7425 |

*. The mean difference is significant at the 0.05 level.

### 8.3.3 **Comparison between wound models (planktonic vs collagen)**

**Multiple Comparisons**

Games-Howell

| *Acinetobacter*sp | (I) VAR00001 | (J) VAR00001 | Mean Difference (I-J) | Std. Error | Sig. | 95% Confidence Interval | |
|---|---|---|---|---|---|---|---|
| | | | | | | Lower Bound | Upper Bound |
| Clone 1 | NSCD-P | NSCD-C | .91200 | .00000 | . | .9120 | .9120 |
| | ISCD-P | ISCD-C | -1.39000* | .05500 | .000 | -1.6757 | -1.1043 |
| | Honey-P | Honey-C | 6.63940* | .08369 | .000 | 6.2047 | 7.0741 |
| | PHMB-P | PHMB-C | 1.05760* | .16469 | .020 | .2336 | 1.8816 |
| Clone 2 | NSCD-P | NSCD-C | 1.01200 | .00000 | . | 1.0120 | 1.0120 |
| | ISCD-P | ISCD-C | -1.95040* | .21746 | .007 | -3.0801 | -.8207 |
| | Honey-P | Honey-C | 7.40100* | .14417 | .000 | 6.6521 | 8.1499 |
| | PHMB-P | PHMB-C | 1.94040* | .28526 | .006 | .7091 | 3.1717 |
| UK-HS | NSCD-P | NSCD-C | .39300 | .00000 | . | .3930 | .3930 |
| | ISCD-P | ISCD-C | -2.72280* | .15547 | .001 | -3.5304 | -1.9152 |
| | Honey-P | Honey-C | 6.15994* | .08362 | .000 | 5.7255 | 6.5943 |
| | PHMB-P | PHMB-C | 1.27324* | .21358 | .005 | .4233 | 2.1232 |
| Type | NSCD-P | NSCD-C | 1.19850 | .00000 | . | 1.1985 | 1.1985 |
| | ISCD-P | ISCD-C | -1.94590* | .16678 | .003 | -2.8123 | -1.0795 |
| | Honey-P | Honey-C | 4.22316* | .03813 | .000 | 4.0251 | 4.4212 |
| | PHMB-P | PHMB-C | 1.45594* | .10788 | .000 | 1.0075 | 1.9044 |
| 882 | NSCD-P | NSCD-C | .63800 | .31511 | .550 | -.9357 | 2.2117 |
| | ISCD-P | ISCD-C | -.21000 | .11987 | .662 | -.6869 | .2669 |
| | Honey-P | Honey-C | -1.44000* | .11882 | .000 | -1.9338 | -.9462 |
| | PHMB-P | PHMB-C | -.62425* | .11564 | .010 | -1.0892 | -.1593 |
| 292 | NSCD-P | NSCD-C | -.27717 | .00000 | . | -.2772 | -.2772 |
| | ISCD-P | ISCD-C | -.27717 | .00000 | . | -.2772 | -.2772 |
| | Honey-P | Honey-C | -.26693 | .13245 | .531 | -.8167 | .2828 |
| | PHMB-P | PHMB-C | -1.43771* | .13765 | .000 | -2.0153 | -.8601 |

*. The mean difference is significant at the 0.05 level.

**Multiple Comparisons**

Games-Howell

| *Pseudomonas sp* | (I) VAR00001 | (J) VAR00001 | Mean Difference (I-J) | Std. Err | Sig. | 95% Confidence Interval | |
|---|---|---|---|---|---|---|---|
| | | | | | | Lower Bound | Upper Bound |
| PS Type | NSCD-P | NSCD-C | .06300 | .00000 | . | .0630 | .0630 |
| | ISCD-P | ISCD-C | -3.05920* | .08457 | .000 | -3.4985 | -2.6199 |
| | Honey-P | Honey-C | 2.62480* | .15131 | .000 | 1.9525 | 3.2971 |
| | PHMB-P | PHMB-C | -5.00940* | .19704 | .000 | -6.0330 | -3.9858 |
| Ps Type 1 | NSCD-P | NSCD-C | 1.47780* | .06982 | .000 | 1.1939 | 1.7617 |
| | ISCD-P | ISCD-C | 2.42220* | .10161 | .000 | 1.9671 | 2.8773 |
| | Honey-P | Honey-C | 3.22140* | .11017 | .000 | 2.6491 | 3.7937 |
| | PHMB-P | PHMB-C | 5.39200* | .26518 | .000 | 4.0846 | 6.6994 |
| Ps Type 2 | NSCD-P | NSCD-C | 1.34280* | .08635 | .000 | 1.0010 | 1.6846 |
| | ISCD-P | ISCD-C | 2.76620* | .24178 | .000 | 1.7920 | 3.7404 |
| | Honey-P | Honey-C | 3.02140* | .19576 | .000 | 2.1791 | 3.8637 |
| | PHMB-P | PHMB-C | 2.81180* | .48193 | .029 | .4018 | 5.2218 |
| Ps Type 3 | NSCD-P | NSCD-C | 1.08340* | .12148 | .000 | .5956 | 1.5712 |
| | ISCD-P | ISCD-C | 1.95180* | .33918 | .029 | .2665 | 3.6371 |
| | Honey-P | Honey-C | 3.79360* | .12461 | .000 | 3.1463 | 4.4409 |
| | PHMB-P | PHMB-C | 1.43120 | .47568 | .231 | -.8600 | 3.7224 |
| Ps Type 4 | NSCD-P | NSCD-C | .01540 | .39227 | 1.000 | -1.9507 | 1.9815 |
| | ISCD-P | ISCD-C | .77700 | .31998 | .383 | -.7240 | 2.2780 |
| | Honey-P | Honey-C | 5.07660* | .42802 | .001 | 3.1331 | 7.0201 |
| | PHMB-P | PHMB-C | 1.31980 | .47818 | .308 | -1.1615 | 3.8011 |
| Ps Type 5 | NSCD-P | NSCD-C | 1.85740* | .14652 | .000 | 1.2581 | 2.4567 |
| | ISCD-P | ISCD-C | 1.84680* | .13918 | .002 | 1.1238 | 2.5698 |
| | Honey-P | Honey-C | 5.84380* | .14494 | .000 | 5.0909 | 6.5967 |
| | PHMB-P | PHMB-C | -.12120 | .12565 | .959 | -.7635 | .5211 |

*. The mean difference is significant at the 0.05 level.

| Multiple Comparisons | | | | | | | |
|---|---|---|---|---|---|---|---|
| Games-Howell | | | | | | | |
| *S. aureus* | (I) VAR00001 | (J) VAR00001 | Mean Difference (I-J) | Std. Error | Sig. | 95% Confidence Interval | |
| | | | | | | Lower Bound | Upper Bound |
| 6538 | NSCD-P | NSCD-C | -1.60900 | .00000 | . | -1.6090 | -1.6090 |
| | ISCD-P | ISCD-C | -3.74600* | .08923 | .000 | -4.0999 | -3.3921 |
| | Honey-P | Honey-C | 1.07540 | .27896 | .092 | -.1827 | 2.3335 |
| | PHMB-P | PNMB-C | 2.66920* | .10388 | .000 | 2.2535 | 3.0849 |
| 43300 | NSCD-P | NSCD-C | 2.60340* | .07824 | .000 | 2.1969 | 3.0099 |
| | ISCD-P | ISCD-C | 2.16380* | .09277 | .000 | 1.7964 | 2.5312 |
| | Honey-P | Honey-C | 1.59620* | .12342 | .000 | 1.1052 | 2.0872 |
| | PHMB-P | PNMB-C | 2.95540* | .31914 | .001 | 1.6146 | 4.2962 |
| 87F/C7 | NSCD-P | NSCD-C | .51220* | .12003 | .034 | .0359 | .9885 |
| | ISCD-P | ISCD-C | -3.48320* | .54758 | .024 | -6.3042 | -.6622 |
| | Honey-P | Honey-C | 1.15560* | .05299 | .000 | .9452 | 1.3660 |
| | PHMB-P | PNMB-C | 1.39320* | .04334 | .000 | 1.2143 | 1.5721 |
| 13142 | NSCD-P | NSCD-C | 1.38460* | .09018 | .000 | .9926 | 1.7766 |
| | ISCD-P | ISCD-C | 1.34360* | .10120 | .000 | .8908 | 1.7964 |
| | Honey-P | Honey-C | 2.08960* | .12561 | .000 | 1.5014 | 2.6778 |
| | PHMB-P | PNMB-C | 3.35780* | .04586 | .000 | 3.1589 | 3.5567 |
| 9B/F6 | NSCD-P | NSCD-C | .94900 | .28619 | .167 | -.3892 | 2.2872 |
| | ISCD-P | ISCD-C | .36340 | .42482 | .978 | -1.7351 | 2.4619 |
| | Honey-P | Honey-C | 1.51240 | .37093 | .084 | -.2292 | 3.2540 |
| | PHMB-P | PNMB-C | 1.70700* | .27519 | .025 | .3082 | 3.1058 |
| *. The mean difference is significant at the 0.05 level. | | | | | | | |