

**Investigation into Compound(s) Exhibiting *Streptococcus mutans*
Anti-biofilm Activity**

This thesis is submitted by

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Declaration

I, Supanan Sucharit, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

A metagenomic library used in this study was constructed by Dr Liam Reynolds (Eastman Dental Institute, UCL).

An environmental bacterial isolate library was isolated by Dr Liam Reynolds under Swab and Send project.

Abstract

Streptococcus mutans is one of the main oral bacteria causing caries through biofilm formation. Bioactive compounds, derived from microorganisms, that disrupt *S. mutans* biofilms are a potential treatment for dental caries.

A high throughput microtitre plate biofilm assay was developed to screen an oral metagenomic library and an environmental isolate library for *S. mutans* clones that produced anti-biofilm compounds. Anti-biofilm activity was not found in 6,624 screened metagenomic clones. Two different kinds of anti-biofilm activity, biofilm detaching activity and biofilm inhibitory activity, were found to be produced by seven isolates from the environmental library.

The biofilm detaching activity was produced by all seven isolates, which were identified as *Bacillus velezensis* based on whole genome sequencing results. Isolate 6E produced the compound with the biofilm detaching activity in M9 minimal medium supplemented with sucrose. The activity disappeared after boiling and treatment with proteinase K, suggesting that the compound(s) responsible for the biofilm detaching activity was a protein. It was hypothesized that the activity might be due to levansucrase, which produce levan, however commercial levan had no effect on *S. mutans* biofilm formation. The biofilm detaching activity was not effective when *S. mutans* biofilms were allowed to form on saliva-coated surfaces. This suggests that the biofilm detaching activity might not be applicable for dental caries protection.

The biofilm inhibitory activity was produced by three isolates: 6E, 10G, and 11E. The biofilm inhibitory activity was not detected when the supernatant was

boiled. Many purification methods were used to separate the biofilm inhibitory however the compound has not yet been purified

The compound derived from *B. velezensis* 6E, 10G, 11E. with *S. mutans* biofilm inhibitory activity in this study may have potential to be applied as an anti-dental caries agent and should be investigated further in the future.

Impact statement

Dental caries is one of the most common oral diseases which can lead to lifelong effects. *S. mutans* is one of the main oral bacteria causing caries through adhesion, acid and biofilm formation. Research on identifying a way to inhibit biofilms produced by this major cariogenic pathogen might potentially lead to a means for the prevention and treatment of dental caries. Therefore, this study focused on identifying potential compounds that could inhibit biofilm formation by *S. mutans*.

The results presented in this thesis have identified new biological tools to target *S. mutans* biofilms. Two anti-biofilm activities were identified in novel strains of *Bacillus velezensis*, isolated from environmental samples in UK. The first anti-biofilm activity was a biofilm detaching activity that was detected in supernatants derived from seven strains of *B. velezensis*. The supernatants allowed *S. mutans* to grow and form biofilms but they caused the biofilms to easily detach. However, the biofilm detaching activity was not effective when *S. mutans* biofilms were allowed to form on saliva-coated surfaces. This finding provides knowledge for targeting *S. mutans* biofilm formation and paves the way for future studies to fully elucidate the mechanism of this biofilm detaching activity.

The second anti-biofilm activity detected in this study was a biofilm formation inhibitory activity. The supernatants derived from three strains of *B. velezensis* could inhibit biofilm formation from *S. mutans* on saliva-coated surfaces. The compound responsible for the biofilm formation inhibitory activity was subject to a number of purification methods but the compound has not been purified.

This compound may have the potential to be used as an agent for the prevention of dental caries and further attempts should be made to isolate the agent to allow further investigation in the future.

Currently dental caries is still widespread, even in developed countries, and compounds that could prevent or reduce *S. mutans* biofilms would be of huge benefit. The work presented in this thesis has identified bacteria that produce molecules that can inhibit *S. mutans* biofilm formation and which may lead to the development of novel treatment for dental caries.

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List of important abbreviations

| | |
|--------------------|--------------------------------------|
| α | Alpha |
| β | Beta |
| \pm | Plus or minus |
| \leq | Less than or equal to |
| \geq | Greater than or equal to |
| $^{\circ}\text{C}$ | Degree Celsius |
| ANI | Average nucleotide identity |
| BHI | Brain heart infusion medium |
| BLAST | Basic Local Alignment Search Tool |
| bp | Base pair |
| CFU | Colony forming unit |
| cm | Centrimetre |
| DDH | DNA-to-DNA hybridization |
| DIFP | Diisopropylfluorophosphate |
| DNA | Deoxyribonucleic acid |
| DW | Deionised water |
| eDNA | Extracellular DNA |
| EPS | Extracellular polymeric substances |
| GGDC | Genome-to-genome-distance calculator |
| h | Hour |
| HSP | High-scoring segment pairs |
| Kb | Kilobase |
| KDa | Kilo dalton |
| LB | Luria-Bertani |
| min | Minute |
| M | Molar |
| mM | Milli molar |
| MIC | Minimum Inhibitory Concentration |
| ml | Millilitre |

| | |
|----------|--|
| MWCO | Molecular weight cut off |
| M9 | M9 minimal medium |
| M9+G | M9 minimal medium supplemented with 1% glucose |
| M9+S | M9 minimal medium supplemented with 1% sucrose |
| nm | Nanometer |
| OD | Optical density |
| rpm | Round per minute |
| PBS | Phosphate buffered saline |
| PCR | Polymerase chain reaction |
| PMSF | Phenylmethylsulfonyl fluoride |
| SDS-PAGE | Sodium dodecyl sulfate polyacrylamide gel electrophoresis |
| TH | Todd Hewitt |
| x g | Times gravity |
| 1D-LCMS | One dimension - Liquid chromatography - Mass spectrometry |
| µm | Micrometre |
| µl | Microlitre |

CHAPTER 1

General Introduction

Chapter 1 General introduction

1.1 Dental Caries

The global economic burden of dental diseases cost \$544.41 billion in 2015 (Righolt *et al.*, 2018). Seventy-nine percent of dental diseases are due to severe tooth loss and untreated dental caries (Righolt *et al.*, 2018). The prevalence of dental caries has significantly increased, in many countries caries is reported to be the most common chronic disease. According to the report on the Global Burden of Diseases, Injuries, and Risk Factors Study 2016 (GBD 2016), the greatest globally prevalent disease was caries of permanent teeth (Vos *et al.*, 2017). The prevalence of dental caries in developed countries has also been reported to be high. For example, in the United Kingdom, children aged 5-15 had a 55-72% prevalence of dental caries in 2003 (Bagramian *et al.*, 2009). In the United States, children aged of 5-17 had a 50-78% prevalence of dental caries; five times more than asthma in 2009 and, recently in 2011-2012, nearly three in five of adolescents aged 12-19 had experienced dental caries (Bagramian *et al.*, 2009).

The definition of dental caries, as defined by Selwitz *et al.* (2007), is a destruction of hard tooth tissue by acid from bacterial fermentation in the oral cavity. The development of caries is a continuum of demineralization of hard tooth tissue which is initiated within the bacterial biofilm and causes a carious lesion, which can be cavitated or non-cavitated. If the caries progresses, it might cause severe tooth pain and /or tooth loss. Dental caries is a pathogenic disease affecting people throughout their lifetime once they have teeth in early-childhood. It is affected by multiple factors including cariogenic bacteria, saliva flow, fluoride exposure, oral hygiene behaviour, and consumption of dietary sugar (Selwitz *et al.*, 2007, Fontana *et al.*, 2010).

Dental caries is demineralisation of hard tooth tissue by acidic by-products from oral bacterial fermentation of dietary carbohydrates. The caries process develops where oral biofilms are allowed to mature on the tooth surface for a long period of time. Because, within the biofilm structure on the tooth surface, lactic acid bacteria, which are mainly streptococci, produce acid as a by-product of dietary carbohydrate fermentation. This acid leads pH values to fall below a critical point ($\text{pH} < 5.5$) resulting in the demineralisation of the tooth enamel. This demineralisation causes diffusion of tooth components (hydroxyapatite) such as calcium, phosphate, and fluoride. If this demineralisation is allowed to continue, a carious lesion will develop (Selwitz *et al.*, 2007, Kidd & Fejerskov, 2016).

However, demineralisation at the early stage can be reversed by remineralisation through uptake of calcium, phosphate, and fluoride. In the remineralisation process, fluoride acts as a catalyst for calcium and phosphate diffusion back into the tooth and this remineralises the crystalline structure in the lesion. The rebuilt crystalline structure is composed of fluorapatite which is more resistant to acid attack than hydroxyapatite, the original structure. Dental caries is actually dependent on a balance between demineralisation and remineralisation as shown in Figure 1.1 . Remineralisation occurs frequently when the low pH value is restored by saliva, which acts as a buffer, and where the areas have less microporous structure and higher fluoride concentration than the original structure (Selwitz *et al.*, 2007).

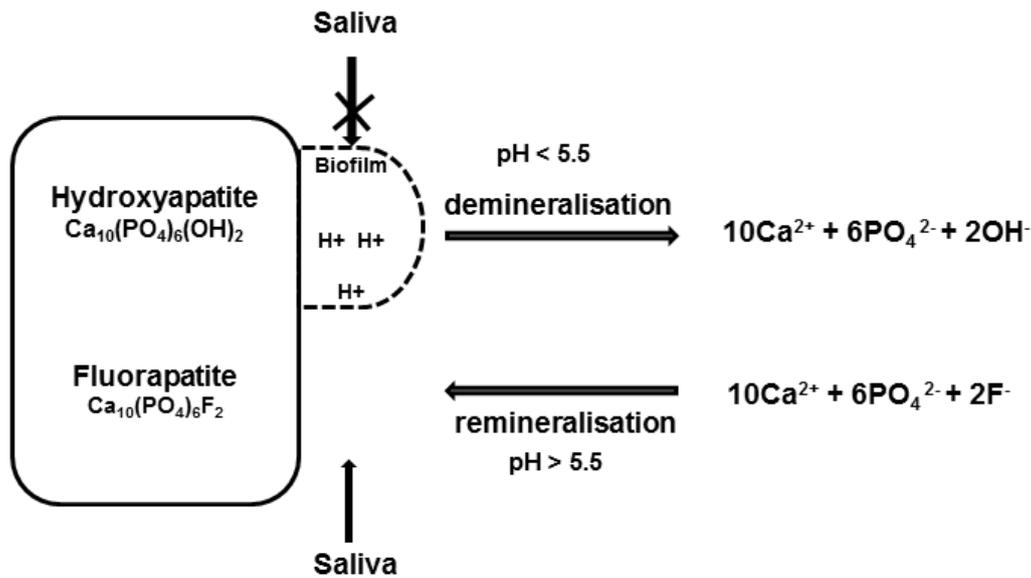


Figure 1.1 Cycle of demineralisation and remineralisation of enamel. The figure was drawn with ChemDraw Professional 16.0 and is based on an illustration by Winston & Bhaskar (1998).

Several risk factors for dental caries relate to the demineralisation of the tooth surface. The first factor is cariogenic bacteria. These bacteria mediate biofilm formation and/or the acid production such as *Streptococcus*, *Lactobacillus*, and *Fusobacterium*. The second factor is a dietary sugar which is used as a substrate to form the biofilm structure. Sucrose is a substrate of glucosyltransferases which catalyse the formation of glucan, which is a major component of the oral biofilms. Thus, appropriated oral hygiene behaviour can reduce the amount of biofilms on the tooth surface and minimise cariogenic bacteria in the oral cavity. The third factor is saliva flow. It increases pH in the oral cavity and leads to reduced demineralisation on the tooth surface (Selwitz *et al.*, 2007).

Moreover, human genetics has been investigated to be a contributor to dental caries. There are many genes which might be associated with dental caries, such as the gene responsible for enamel formation (*AMELX*), immune response (*DEFB1*), salivary

component related to saliva buffer capacity (CA6), and a taste receptor (TAS2R38) (Yildiz *et al.*, 2016).

The oral microbiome of each person is unique and plays a crucial role in maintaining oral health. Thus, the risk of dental caries to an individual depends on the oral microbiome (Grigalauskiene *et al.*, 2015, Tanner *et al.*, 2018).

Several methods are potential to use to prevent dental caries. The first method is the reduction of cariogenic bacteria using an antiseptic agent. For example, Chlorhexidine (C₂₂H₃₀Cl₂N₁₀) or 1,6-bis(4-chloro-phenylbiguanido) hexane has been used as an anti-cariogenic agent. At high concentration, its bactericidal effect occurs due to its ability to penetrate the bacterial cell wall resulting in precipitation of cytoplasm (Matthijs & Adriaens, 2002). At low concentrations, the bacteriostatic effect occurs by positively charged molecules of chlorhexidine binding to the negatively charged bacterial cell wall, phosphate groups and carboxyl groups (Matthijs & Adriaens, 2002). However, the ability of chlorhexidine to prevent dental caries is controversial among dental clinicians and educators (Autio-Gold, 2008). Some studies reported that chlorhexidine only temporarily reduced acidogenicity of oral microbiota (Andreadis *et al.*, 2015). This suggests that there must be some reservoir or retention sites where acidogenic bacteria were not affected by chlorhexidine. Moreover, using chlorhexidine causes some short-term side effects such as a yellow-brown staining of teeth and altered taste sensation. Due to lack of evidence for long-term clinical outcomes and reported side effects from using chlorhexidine, Autio-Gold (2008) suggests that chlorhexidine should not be recommended for dental caries prevention and also suggests alternative methods such as fluoride applications, diet modification, and good oral hygiene practices.

The second method of dental caries prevention is fluoride exposure. At a high concentration of fluoride, at a pH of more than 5.5, remineralisation of the surface of the hard tooth tissues occurs. At the areas which have a pore occurring from demineralisation, these pores can be filled by remineralisation of calcium, phosphate, and fluoride. Fluoride is an important molecule in the reaction of remineralisation as a catalyst. The crystal derived from remineralisation is much more acid resistant than the original structure. Fluoride is widely used in dental care products such as in fluoride toothpaste, fluoride gel, fluoride tablets and water-fluoridation. Marthaler (2004) indicates that even though there is wide spread use of fluoride in central and eastern Europe, caries prevalence is still high, because dental caries is a multifactorial disease.

The third method of dental caries prevention is the interruption of dental plaque or oral biofilm. There are many methods to reduce biofilm formation. Appropriate oral hygiene helps to prevent dental caries. Tooth brushing correctly can remove biofilm on the tooth surface. This leads to the reduction of demineralisation and also increased remineralisation exposing the tooth surface to fluoride and saliva. In addition, dental floss can disrupt the biofilm structure that cannot be reached by tooth brushing, especially, a deep area between the teeth and supragingival plaque.

The last method of dental caries prevention is the limitation of dietary carbohydrate consumption. Fermentable sugars are not only a major source for acid production by the bacterium but some are also important substrates for forming carbohydrate matrix in oral biofilms. For example, sucrose is a major substrate used in the synthesis of water-insoluble- α -1,3- glucan, which is a main structure of oral caries biofilms (Zeng & Burne, 2013). However, other dietary sugars might affect biofilm formation. Some

dietary carbohydrates (xylitol, sorbitol, mannitol) influenced the expression of genes related to biofilm formation by *S. mutans* e.g. *gtfB*, *gtfC*, *gtfD*, and *ftf* (Shemesh *et al.*, 2006).

In professional care, dental caries can be reduced efficiently by a dentist. At the primary occurrence of a carious lesion, the dentist can diagnose the white or discoloured area of early caries on the tooth surface and apply an appropriate treatment before it develops into severe dental caries especially in children. Tooth restoration has been used to treat severe caries. The area of caries is removed and the cavity is filled by material such as composites. In the worst case, the tooth might be removed (Selwitz *et al.*, 2007).

1.2 Oral microbial community

An adult human body contains approximately 3.8×10^{13} bacterial cells per 3.0×10^{13} human cells (Sender *et al.*, 2016). Secondary to the gut, oral cavity is the large community of commensal bacteria in a human body (Deo & Deshmukh, 2019). Oral cavity composes of soft and hard tissues such as the teeth, tongue, gingival sulcus, cheek, buccal mucosa, tonsils, hard palate and soft palate which provide suitable environment for bacterial growth (Dewhirst *et al.*, 2010). The oral microbiome consists of a core microbiome and a variable microbiome depending on healthy condition, lifestyle and physiological differences (Deo & Deshmukh, 2019).

The Human Oral Microbiome Project Database (HOMD) reported that more than 700 oral bacterial species were found in human oral cavities (Mark Welch *et al.*, 2016). Larger number of bacterial species in oral cavity may be detected using the modern technology for sequencing (Yoshizawa *et al.*, 2013). In a single human oral cavity, 75

to 150 species of oral bacteria have been identified from 16S rRNA sequencing (Peterson *et al.*, 2011, Yoshizawa *et al.*, 2013). Based on HOMD, there are 15 bacterial phyla: *Firmicutes*, *Proteobacteria*, *Bacteroidetes*, *Actinobacteria*, *Spirochaetes*, *Fusobacteria*, *Tenericutes*, *Chlamydiae*, *Synergistetes*, *TM7*, *Chlorobi*, *Chloroflexi*, GN02, SR1, and WPS-2 (Camanocha & Dewhirst, 2014).

In the oral community, microbiota influence host in the aspect of both health promotion and disease progression (Mark Welch *et al.*, 2016). Some bacteria play an important role to maintain oral homeostasis and prevent disease development by several mechanisms such as generation of energy, detoxification of environmental chemicals, promoting the growth of good bacteria and prevention of disease invasion (Kilian *et al.*, 2016). However, some bacteria are responsible for diseases progression such as in the pathogenesis of dental caries, periodontitis and gingivitis (Colombo & Tanner, 2019).

1.3 Cariogenic oral bacteria

There are about 700 taxa in the oral microbiota, thereby, it is difficult to specify the dental caries causative bacteria (Dewhirst *et al.*, 2010, Dewhirst, 2015). The association of microbiota in dental caries has been investigated (Deo & Deshmukh, 2019). Dental plaque, an oral biofilm, composes of microbial community, which is found to be cariogenic (Colombo & Tanner, 2019).

In particular, the major source of acid inducing demineralisation is acidogenic bacteria in the oral cavity. However acidogenic bacteria have been identified to be associated with dental caries including, *S. mutans*, low-pH non- *S. mutans* streptococci, *Veillonella* spp., *Actinomyces* spp., *Bifidobacterium* spp., *Lactobacillus* spp.,

Propionibacterium spp. and *Atopobium* spp. (Van Houte *et al.*, 1994, Peterson *et al.*, 2011, Tanner *et al.*, 2018).

However, several investigations have discovered that the proportion of *S. mutans* and *Lactobacillus* spp. is not always high when dental caries is found. In addition, dental caries can develop in the absence of these bacteria (Van Houte *et al.*, 1994, Tanzer *et al.*, 2001).

Therefore, the key factor of dental caries causation is the ecological balance of associated oral bacteria within the oral biofilm. At least two groups of bacteria are important, these are the acidogenic bacteria and the bacteria preventing high levels of alkalinity (Kleinberg, 2002).

Since *S. mutans* had been isolated from patients with dental caries, it has been recognized as a pathogenic cause of this disease. However, *S. mutans* is just one of microorganisms in oral cavity, therefore, it is unlikely to define as a main agent for cariogenesis. To clarify this, the cariogenic effect of *S. mutans* has been established in animal models and has developed to represent human diet styles. Recently, Ito *et al.* (2012) established an animal model using mice, which have long survival because they do not develop insulin-dependent diabetes mellitus (IDDM) and Sjögren's syndrome (SS), for oral bacterial colonization under humanized conditions. In the study, *S. mutans* UA159 culture was introduced to mice in many conditions such as (i) after or before mice were given water containing 1% sucrose, (ii) with and without human saliva treatment, (iii) in the present of adhesins (Ito *et al.*, 2012). After 24 h, samples were collected from labial surfaced of the maxillary incisor teeth with a sterile cotton ball to determine *S. mutans* cell number. The result showed the higher *S.*

mutans colonization on the tooth surface in the condition of (i) feeding 1% sucrose - containing water and diet, and (ii) pre- treated by human saliva. This confirmed the factors increase of the susceptibility of the mice to *S. mutans* (Ito *et al.*, 2012). In another study, *S. mutans* cell number was measured using qPCR from rat saliva sample (Yucesoy *et al.*, 2018). To assess carious lesion in rat model, teeth were assessed under optical microscopy and caries scoring was performed based on Keyes's method such as tooth surfaces were scored according to penetration depth of murexide as enamel or slight, moderate, or extensive dentin-type lesion (Yucesoy *et al.*, 2018). The result revealed that *S. mutans* cell number was increased when longer of incubation time and carious lesions were observed on the tooth surfaces and hemisectioned teeth from infected rats. Moreover, *S. mutans* has been established in other animal models such as hamsters (De Soet *et al.*, 1991) and monkeys (Lehner *et al.*, 1975, Jia *et al.*, 2006). These examples are enough to indicate that *S. mutans* can be established in animal model for studying dental caries and it is one of the main bacterium causing caries.

S. mutans is always present as part of a complex microbiota in oral cavity. The knowledge of the oral bacterial community has been being investigated. It is difficult to start studying dental caries based on the unknown information. Many information about *S. mutans* is available, particularly, complete genome sequence of *S. mutans* UA159 (Ajdic *et al.*, 2002), therefore *S. mutans* has been used as a model for studying biofilm and dental caries. Several studies used *S. mutans* monospecies biofilm model. Then, *S. mutans* has been found to be a key contributor to the formation of extracellular polysaccharide (Koo *et al.*, 2010) and one of the main acidogenic bacteria (de Soet *et al.*, 2000) for instance.

1.4 *Streptococcus mutans*

1.4.1 General information

Streptococcus mutans was first isolated from a dental caries patient (Clark, 1924). It is a Gram-positive bacterium and a facultative anaerobe. *S. mutans* is a non-motile, coccus-shaped bacterium, which arranges itself in chains. A rough, dry, light-yellow colony is formed on Todd-Hewitt (TH) agar, which adheres to the surface tightly.

S. mutans is a member of the phylum *Firmicutes*, which composed of Mutans group, Salivarius group, Anginosus group, Mitis group, Sanguinis group and Bovis group (Doern & Burnham, 2010). The Mutans streptococci group is divided into 9 serotypes (as shown in Table 1.1) based on sugar polymer composition (as shown in Figure 1.2) on the cell wall. In particular, *S. mutans* can be divided into 4 serotypes: *c*, *e*, *f*, and *k* based on the composition of rhamnose-glucose polymer on its cell surface. Among these four serotypes, serotype *c* is the most prevalent (Nakano *et al.*, 2004, Nakano & Ooshima, 2009, Shibata *et al.*, 2009, Braga *et al.*, 2013).

Table 1.1 Classification of mutans streptococci based on serotype. The table is based on an illustration by Nakano & Ooshima (2009)

| Species | Serotypes | Composed sugar | Origin |
|-------------------------------|--------------|--------------------|---------|
| <i>Streptococcus cricetus</i> | <i>a</i> | Galactose/glucose | Hamster |
| <i>Streptococcus rattus</i> | <i>b</i> | Galactose/rhamnose | Rat |
| <i>Streptococcus ferus</i> | <i>c</i> | Glucose/rhamnose | Rat |
| <i>Streptococcus macacae</i> | <i>c</i> | Glucose/rhamnose | Monkey |
| <i>Streptococcus mutans</i> | <i>c/e/f</i> | Glucose/rhamnose | Human |
| <i>Streptococcus mutans</i> | <i>k</i> | Rhamnose | Human |
| <i>Streptococcus sobrinus</i> | <i>d/g</i> | Galactose/glucose | Human |
| <i>Streptococcus downei</i> | <i>h</i> | Galactose/glucose | Monkey |

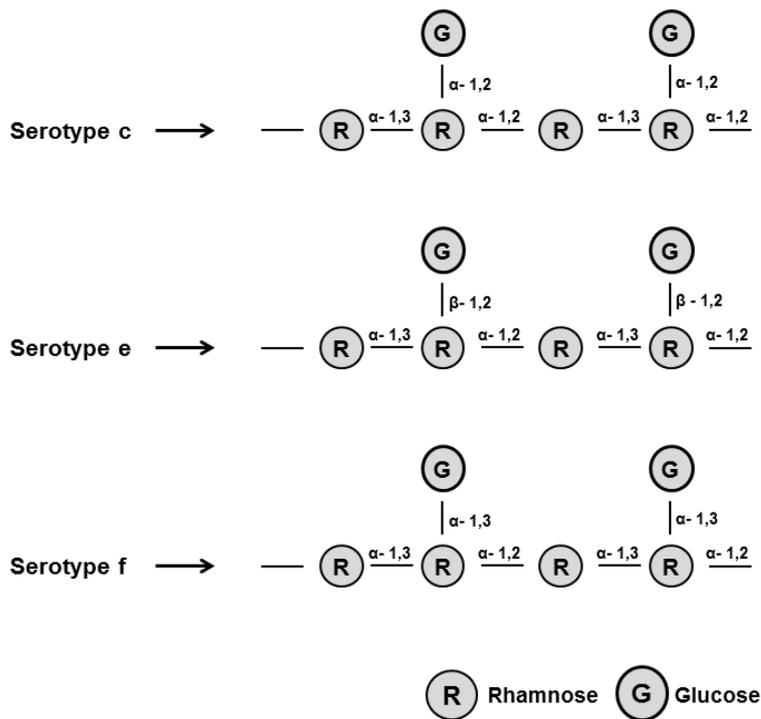


Figure 1.2 Structure of serotype specific polysaccharide in *S. mutans*

Figure was drawn with ChemDraw Professional 16.0 and is based on an illustration by Nakano *et al.* (2008), Shibata *et al.* (2009).

S. mutans UA159, used in this study, is grouped in serotype *c* and it was isolated originally from a child with active caries in 1982 in Oklahoma, United States (http://www.genome.jp/dbget-bin/www_bget?gn:T00100). The genome of *S. mutans* UA159 has been sequenced completely by Ajdic *et al.* (2002). The sequencing results show that the genome is composed of 2,030,936 base pairs with 1,963 ORFs (open reading frames) and a low GC content on average of 36.82%.

S. mutans is a part of the microbiota in the human oral cavity. Peterson *et al.* (2011) reported that the population of mutans streptococci found in human saliva is about 100 cell/ml (Peterson *et al.*, 2011). *S. mutans* is considered to be one of the major etiological agents of dental caries (Hamada & Slade, 1980, Yoshida & Kuramitsu,

2002, Peterson *et al.*, 2011). *S. mutans* has been also been found to be one of the bacteria that causes infective endocarditis (Gendron *et al.*, 2000). The crucial virulence properties of *S. mutans* are adhesion, biofilm formation, acid production and tolerance (Mitchell, 2003, Peterson *et al.*, 2011, Gowrishankar *et al.*, 2014).

1.4.2 Virulence factors

1.4.2.1 Adhesion

The adhesion of *S. mutans* cells on the tooth surface can be mediated via sucrose-dependent and sucrose-independent mechanisms. In sucrose-independent adhesion, in the absence of sucrose, the ability of *S. mutans* to adhere to and accumulate on the tooth surfaces is promoted by adhesins; proteins of the antigen I/II family such as: Ag I/II, P1, SpaP, Sr, AgB and PA (Taubman & Nash, 2006, Nobbs *et al.*, 2011). The proline-rich and central V domain of the adhesin are responsible for the interaction of the adhesins and salivary components (Nakai *et al.*, 1993, Hajishengallis *et al.*, 1994, Banas, 2004, Brady *et al.*, 2010).

Sortase a membrane-associated transpeptidase, is an enzyme which cell wall anchors several surface proteins on the cell surface of Gram-positive bacteria. The conserved region of a hexapeptide, LPXTG, in surface proteins is the consensus sequence identified by the sortase (Fischetti *et al.*, 1990). In *S. mutans*, six surface proteins have been identified with the consensus sequence LPXTG near the C-terminus of the proteins. This region is followed by hydrophobic region and charged tail. In the process of cell wall attachment of proteins, the sortase A cleaves the peptide bond between threonine (T) and glycine (G) residues in LPXTG motif and the enzyme links the threonine residue to the amino group of cell wall cross bridge as shown in Figure 1.3

(Selvaraj *et al.*, 2015). The surface proteins are attached by sortase A include P1 (also known as antigen I/II, SpaP, and Pac), glucan-binding protein C (GbpC), fructanase, (FruA), wall-associated protein A (WapA), wall-associated protein E (WapE), and dextranase (DexA) (Ajdic *et al.*, 2002).

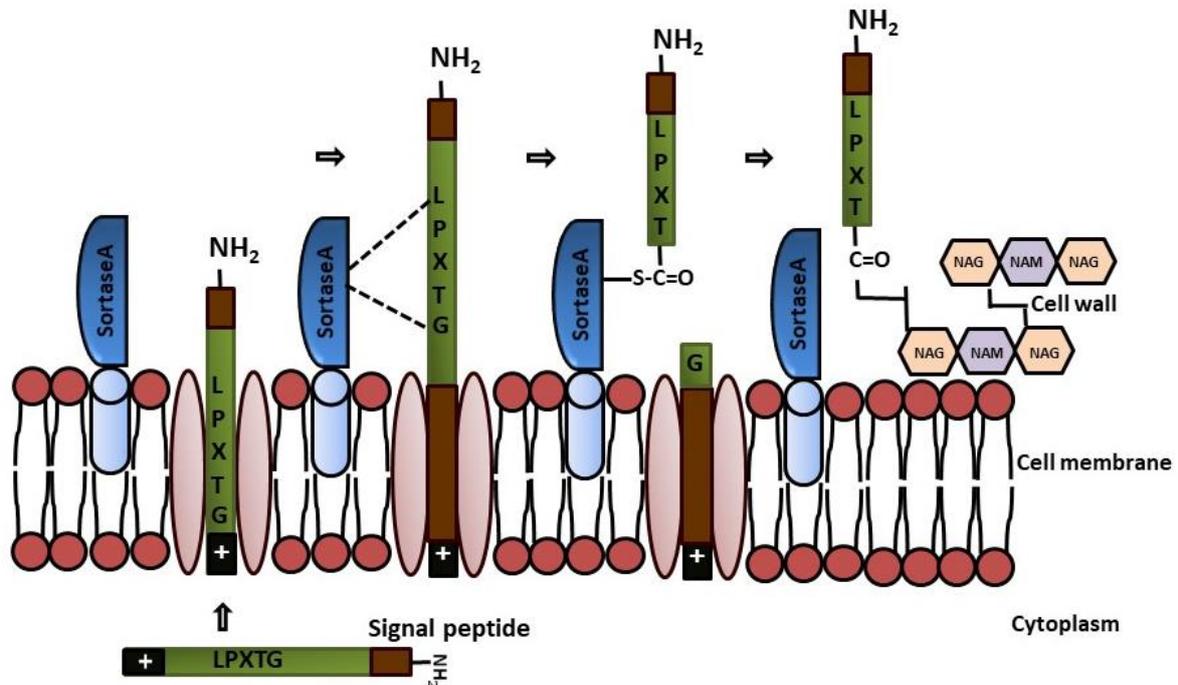


Figure 1.3 Cell wall sorting partway of surface proteins in *S. mutans*. Figure was drawn using PowerPoint 2013 and is based on an illustration by Selvaraj *et al.* (2015)

The function of the *srtA* gene in *S. mutans* UA159 has been investigated and found to be involved in biofilm formation (Lévesque *et al.*, 2005). *S. mutans* wild-type and *srtA* mutant, which was constructed by insertion-duplication mutagenesis, were examined for the ability to form biofilms. *S. mutans* deficient in SrtA had reduced biofilm formation. The reduction in biofilm due to the *srtA* mutation might be because of the six surface proteins containing LPXTG motifs not being linked to the cell wall. Three of the proteins which are FruA, WapA, and Wap E have been found to be involved with *S. mutans* biofilm formation. Lévesque *et al.* (2005) concluded that the reduction

in biofilm formed by the *srtA* mutant might be associated with *fruA*, *wapA*, and *wapE* (Lévesque *et al.*, 2005).

Sucrose-dependent adhesion plays a prominent role in the colonization of *S. mutans* on the tooth surface. In the presence of sucrose, glucosyltransferases (Gtfs) split the sucrose molecule into glucose and fructose. The glucose molecule is added to polymerise glucan. *S. mutans* possesses three types of Gtfs encoded by *gtfB*, *gtfC*, and *gtfD*. Gtfs synthesize both water-soluble glucan and water-insoluble glucan that play an important role in the colonization (Aoki *et al.*, 1986, Bowen & Koo, 2011). The ability of glucans to facilitate *S. mutans* adhesion is due to the interaction of the glucan polymer with either the salivary components or *S. mutans* cell surface (Banas, 2004). Khan *et al.* (2012) indicates that the water-insoluble glucan is more importance than the water-soluble glucan in the adhesion by *S. mutans* cells (Khan *et al.*, 2010). Gtfs not only catalyse glucan polymer formation but they also bind to glucan polymer. However, glucan cannot mediate *S. mutans* adhesion without glucan-binding proteins (Nobbs *et al.*, 2009).

Glucan binding proteins (Gbps) facilitate *S. mutans* adhering on the tooth surface. *S. mutans* possesses four types of Gbps: GbpA, B, C, D. Gbps cause cell aggregation and accumulation by binding to water-insoluble glucan (Lamont & Jenkinson, 2010). GbpC acts as a glucan receptor on the *S. mutans* cell surface (Sato *et al.*, 1997). GbpA and GbpD share a region of an amino acid repeat at C terminal. GbpD also acts as lipase and carboxylesterase (Shah & Russell, 2004).

Liao *et al.* (2014) reported that biofilm formation by *S. mutans* in the presence of DNase I was significantly reduced, and they hypothesised that extracellular DNA

(eDNA) affected biofilm formation. Then they demonstrated that the addition of chromosomal DNA from *S. mutans* and glucans, which were synthesised by glucosyltransferase B, played a crucial role in *S. mutans* adherence under sucrose-dependent conditions. However, the precise mechanism of the eDNA -glucan interaction in enhancing *S. mutans* adherence has not been reported thus far (Liao *et al.*, 2014).

1.4.2.2 **Biofilm formation**

S. mutans is considered to be one of the main bacteria forming an oral biofilm on the tooth surface. *S. mutans* possesses four Gtfs: Gtf A, B, C, D, most of these Gtfs are responsible for the production of water insoluble and water-soluble glucans. GtfB and GtfC catalyse an α -1, 3, glucan, water-insoluble glucan and GtfC and GtfD catalyse an α -1, 6- glucan, water-soluble glucan (as shown in Figure 1.4). These glucans are important components of the structure of the oral biofilm, particularly water-insoluble glucan (Gowrishankar *et al.*, 2014).

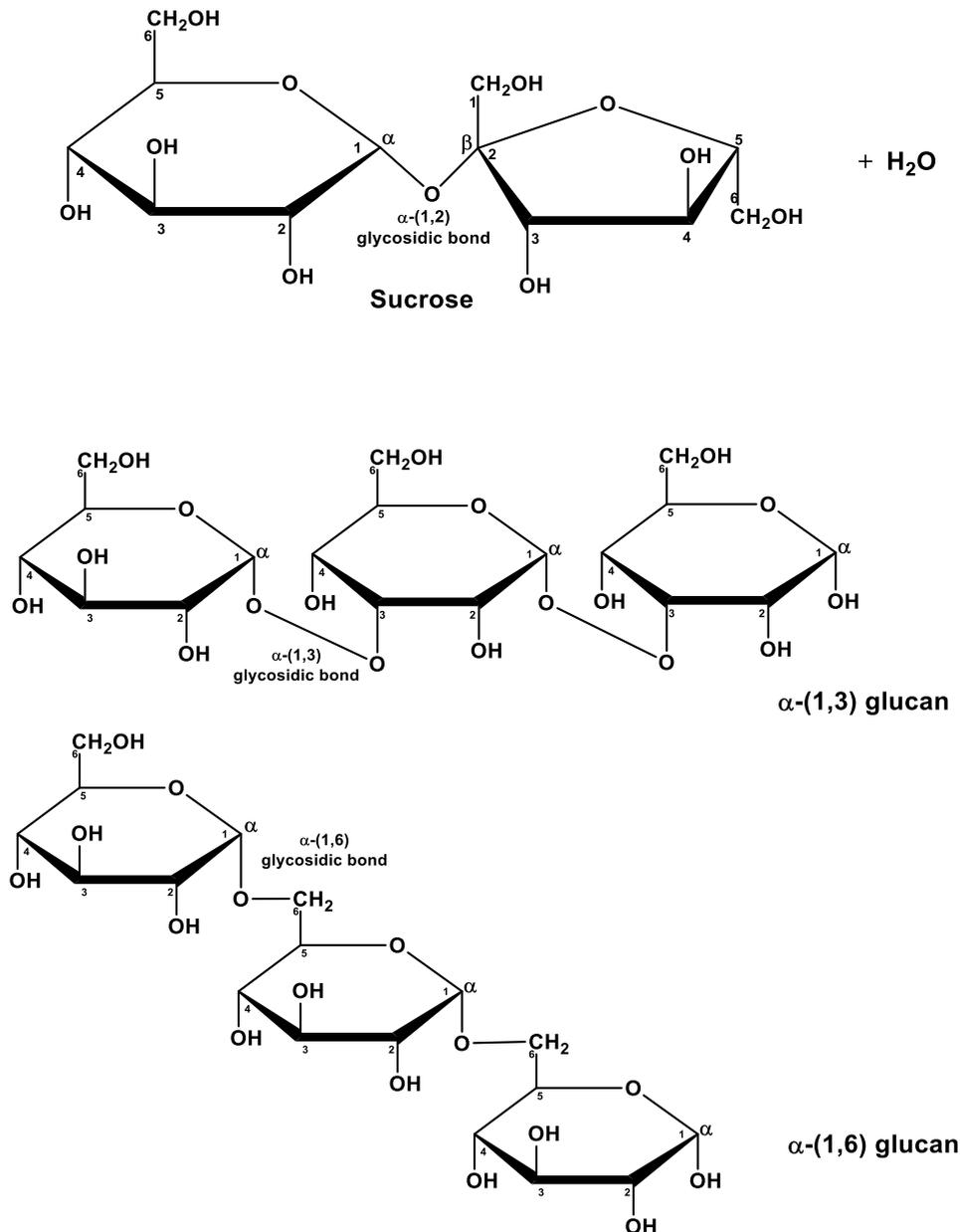


Figure 1.4 Schematic view of α -1, 3- glucan and α -1, 6- glucan. Figure was drawn with ChemDraw Professional 16.0 and is based on an illustration by Lara-Lemus *et al.* (2014).

After construction of a biofilm on the tooth surface, *S. mutans* uses fermentable dietary sugars as a carbon source and produces lactic acid via the glycolysis pathway. The acid produced by *S. mutans* when embedded in the biofilm structure cannot be neutralized by saliva resulting in a reduction of pH. The ecological changes within the biofilm structure lead to an increase in the proportion of *S. mutans* and other acidogenic and aciduric bacteria (Banas, 2004).

1.4.2.3 Acidogenicity and aciduricity

S. mutans generates ATP via the glycolysis pathway. Under glucose excess conditions, lactic acid is a major-end product of the glycolysis pathway. Lactic acid from the glycolysis pathway reduces the intracellular pH from 7.0 to 5.0 (Dashper & Reynolds, 1992). *S. mutans* constitutes an acid tolerance response (ATR) to survive in this low pH condition by several acid resistant mechanisms (as shown in Figure 1.5).

During glycolysis, lactic acid is synthesised as a by-product and accumulates in *S. mutans* cells. When the lactic acid concentration reaches a threshold (about 100 mM), a lactic acid efflux system is activated. This accumulation of intracellular lactic acid leads to a low pH level in the cytoplasm (Dashper & Reynolds, 1996). Intracellular lactic acid is translocated outside the *S. mutans* cells through transmembrane movement, an electroneutral process, as a result of higher intracellular pH and lower extracellular pH (Dashper & Reynolds, 1996).

The glycolysing cell of *S. mutans* increases the intracellular pH by translocating proton (H^+) to outside the cell. This proton translocation to the outside of the *S. mutans* cell across its cell membrane is by a proton-translocating ATPase (F_1F_0 -ATPase) (Dashper & Reynolds, 1996, Sasaki *et al.*, 2014). In the presence of ATP and a proton-motive force with an optimal potassium ion concentration in medium, potassium movement has been shown to be important to the translocation process of protons. The translocation of protons to outside *S. mutans* cell results in a low extracellular pH level and intracellular pH nearer neutrality (Dashper & Reynolds, 1992).

Water-insoluble glucan is crucial to proton accumulation on the *S. mutans* cell surface (Guo *et al.*, 2015). GtfB and GtfC synthesise surface-associated insoluble glucan. This

insoluble glucan not only facilitates *S. mutans* cell adhesion but also accumulates extracellular protons and results in a cell surface pH lower than in the surrounding medium. Although the exact mechanism of proton accumulation is not known, it is possible that water-insoluble glucans bind and recruit protons due to its negative charge (Guo *et al.*, 2015).

To survive in acid environment, *S. mutans* maintains intracellular pH at one unit higher than that of extracellular compartment by many mechanisms, including extrusion of proton, influx of cations, and generation of basic molecular species (Baker *et al.*, 2017). At an intracellular pH of 5.0, glycolysing *S. mutans* is able to maintain a transmembrane pH gradient (a different pH between intracellular and extracellular pH) of 1.37 ± 0.09 units (Dashper & Reynolds, 1992).

In addition, *S. mutans* avoids production of toxic environment by altering the end products of glycolysis pathway. This includes avoiding the generation of lactate, formate and acetate (Baker *et al.*, 2017). It also increases the import of other carbohydrate by encoding at least 14 phosphoenolpyruvate phosphotransferase systems (PTS) and multiple sugar metabolism (Msm) transporter (Ajdic *et al.*, 2002). The downregulation of a number of PTS during *S. mutans* growth in low pH environment has been reported (Len *et al.*, 2004, Baker *et al.*, 2015).

Several studies have revealed that acid tolerance in *S. mutans* involves expression of many genes such as *hk11* and *rr11* which code for a two-component signal transduction system (Li *et al.*, 2002, Guo *et al.*, 2015). Moreover, *S. mutans* encodes many enzymes to repair DNA and proteins which are damaged by acid, such as DnaK and GroES/GroEL chaperone systems (Lemos *et al.*, 2001, Baker *et al.*, 2017). The

acid tolerance response leads *S. mutans* to be one of the predominant bacteria among oral bacteria, with the most significant adaptive acid response (Svensater *et al.*, 1997).

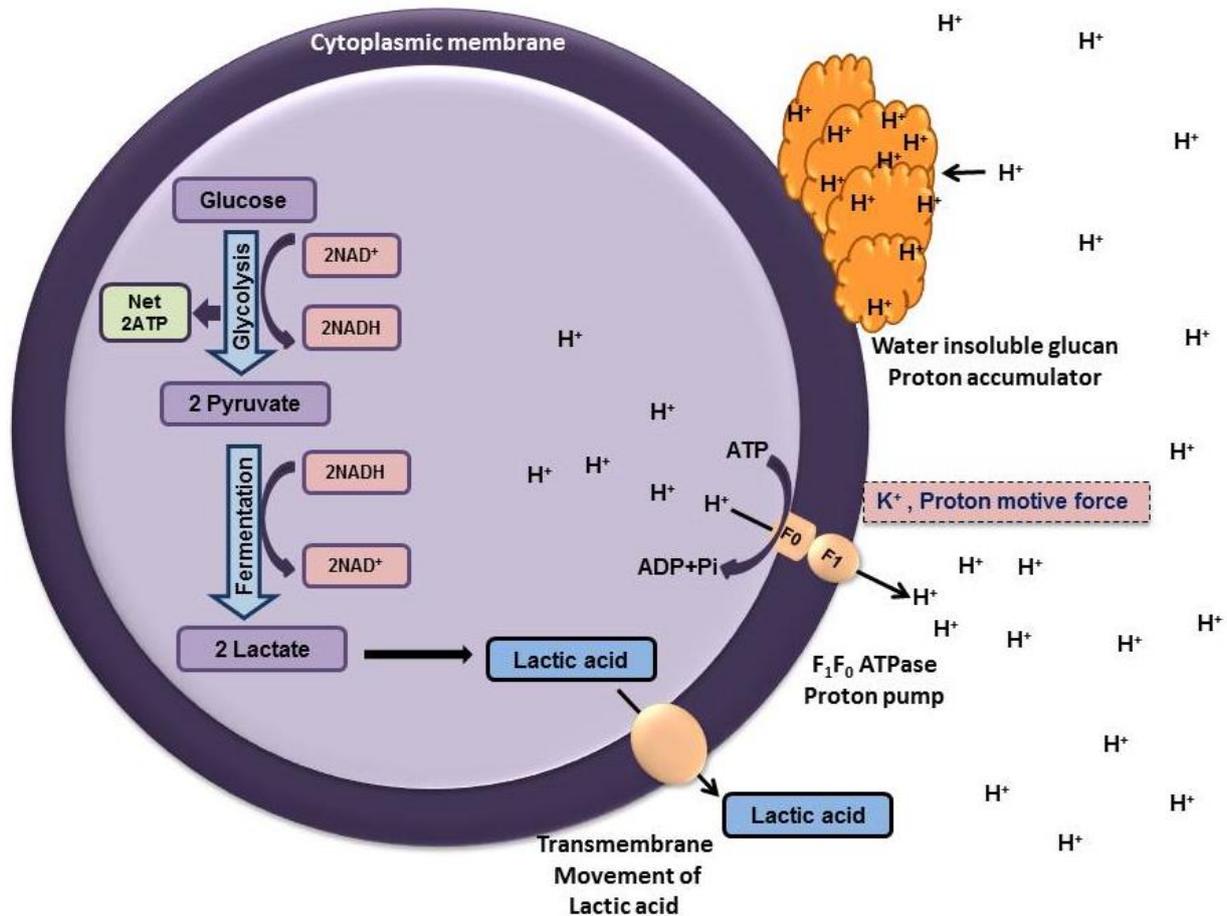


Figure 1.5 Acidogenicity and aciduricity of *S. mutans*

S. mutans produces lactate as a main by- product from the glycolysis and fermentation pathway. To reduce intracellular pH, lactic acid is translocated to the outside of the *S. mutans* cell through a transmembrane movement. In addition, intracellular protons are translocated by the F₁F₀-ATPase, proton pump. Extracellular protons are trapped and accumulated on the water insoluble glucan. These example mechanisms allow *S. mutans* to survive in acid condition. The figure was drawn using MS PowerPoint 2010 and is based on an illustration by Matsui & Cvitkovitch (2010).

1.4.2.4 Mutacins

S. mutans produces bacteriocins which are named mutacins (Hamada & Ooshima, 1975). These mutacins play crucial roles in the process of colonisation and establishment of *S. mutans* biofilm formation. Mutacins may help *S. mutans* to compete with other oral streptococcal colonizers. For example, mutacins have been reported to inhibit the growth of *S. gordonii* and *S. sanguinis* (Kuramitsu *et al.*, 2007). Two major classes of mutacins have been characterised as Class I, lantibiotic mutacins and Class II, non-lantibiotic mutacins (Merritt & Qi, 2012).

Lantibiotic mutacins have a wide spectrum activity against Gram-positive bacteria including non-producing strains of *S. mutans*. Lantibiotic refers to a bacteriocin molecule which has the characteristic dehydrated amino acid and intramolecular thioether bond (Qi *et al.*, 2001). These characteristics result in the formation of lanthionine and methyllanthionine residues within the bacteriocin molecule (Nes *et al.*, 2007). Merritt & Qi (2012) report that these unusual structures are important to the inhibitory activity of the lantibiotic mutacins and the structures increase their stability in extreme environments. Based on their structural features, lantibiotic mutacins are divided into Type A (more linear and flexible structure) and Type B (more rigid and globular structure) (Nes *et al.*, 2007). A number of lantibiotic mutacins have been characterized such as mutacin I, mutacin II, mutacin III/1140, mutacin B-NY266, mutacin Smb A/B, and mutacin K8 (Jung & Sahl, 1991, Hossain & Biswas, 2011, Merritt & Qi, 2012). Attempts to determine the mode of action of lantibiotic mutacins have been conducted, however the function of most mutacins have not been identified because the purification of the mutacins was not successful (Draper *et al.*, 2015). However, recent studies indicated that many mutacins are involved in cell membrane

pore formation and cell recognition of susceptible cells (Merritt & Qi, 2012, Biswas & Biswas, 2014).

Non-lantibiotic mutacins have a much narrower spectrum of activity than lantibiotic mutacins. They are mainly active against low G+C content Gram-positive bacteria, such as lactic acid bacteria, *Enterococcus*, *Listeria*, and *Clostridium* (Hécharad & Sahl, 2002). Unlike the lantibiotic mutacins, the non-lantibiotic mutacin genes are present in all *S. mutans* strains examined to date (Qi *et al.*, 2001). Peptides expressed from the non-lantibiotic mutacin genes are not post-translationally modified and the peptides are translocated to outside the *S. mutans* cell through a conserved peptide leader region, double-glycine (GG) motif. These leader peptides target a membrane-bound ABC transporter apparatus and the ABC transporter apparatus cleaves the GG sequence of the leader peptide before secreting the peptides to outside the *S. mutans* cell (Nes *et al.*, 1996). The mode of action of the non-lantibiotic mutacins mainly is as a result of the dissipation of the proton motive force, the force promoting movement of protons across membranes, of a cell through pore formation (Hécharad & Sahl, 2002). At least four of non-lantibiotic mutacins have been characterized: mutacin IV, mutacin V, mutacin VI, and mutacin N but *S. mutans* produces more non-lantibiotic mutacins which have not been characterized (Merritt & Qi, 2012).

1.5 Oral biofilm

1.5.1 Composition

Oral biofilms, or dental plaques, are formed on hard tissue surfaces in the oral cavity. The oral biofilms are composed of several microorganisms including those of the genera *Corynebacterium*, *Streptococcus*, *Haemophilus*, *Porphyromonas*, *Fusobacterium*, *Actinomyces*, *Prevotella*, *Veillonella* (Mark Welch *et al.*, 2016). These

microorganisms are embedded in extracellular polymeric substances (EPS). Microorganisms themselves are only a small part of the biofilm comprising less than 10% of dry mass and EPS is over 90% of dry mass (Flemming & Wingender, 2010). Dental plaque has been reported as composed of approximately 10-20% (dry weight) of glucan, 1-2% (dry weight) of fructan depending on dietary sugar intake. Approximately 40% of dry mass is proteins derived from oral bacteria and saliva (Bowen & Koo, 2011, Jakubovics & Burgess, 2015). eDNA is also found in dental plaque and eDNA plays an important role in cell aggregation and biofilm formation (Jakubovics *et al.*, 2013, Jakubovics & Burgess, 2015). Dental plaques contain lipid and minerals such as calcium, magnesium, phosphorus and iron and approximately 80% of *in situ* dental plaque is water (Bowen & Koo, 2011).

1.5.2 Biofilm formation

Oral biofilms on the tooth surface are formed in four main steps (as shown in Figure 1.6). Beginning with oral bacteria, especially pioneer colonizers, adhering to salivary components on tooth surfaces. Once irreversible adhesion occurs, early colonizers aggregate to adherent bacteria on the tooth surface. This cell-to-cell aggregation increases the density of cells binding to the tooth surface and also increases a high concentration of cell signalling molecules. These cell signalling molecules, or quorum sensing molecules, induce cells to form a biofilm. A part of the biofilm and/or some cells in the biofilm structure can be detached and some detached cells can re-attach to a new surface in order to form a biofilm.

Oral bacteria adhere to the tooth surfaces which are covered by salivary components, such as lysozyme, amylase, proline-rich proteins, and mucin. In the absence of sucrose, pioneer colonizers, such as *S. gordonii*, *S. sanguinis*, *S. mitis*, *S. oralis* and

Actinomyces spp. are able to bind to salivary components on the tooth surface. In this step, *S. mutans* can bind to salivary components directly using its adhesins (Ahn *et al.*, 2008, Brady *et al.*, 2010).

Then early colonizers, such as *S. mutans*, *Capnocytophaga ochracea*, *Haemophilus parainfluenza* and *Veillonella* spp. aggregate to previously bound pioneer colonizers (Kolenbrander *et al.*, 2010, Peterson *et al.*, 2011, Koo *et al.*, 2013). In the presence of sucrose, *S. mutans* synthesises Gtfs and these Gtfs produce glucans. Both Gtfs and insoluble glucan facilitate the adhesion of *S. mutans* and the co-aggregation of oral bacteria, because Gtfs can adhere to salivary components and insoluble glucan binds to Gbps (Nobbs *et al.*, 2009, Krzysciak *et al.*, 2014). After the primary colonizers and early colonizers adhere irreversibly on the tooth surface, other oral bacteria, such as other *Capnocytophaga* spp., *Actinomyces* spp., *Fusobacterium nucleatum*, *Prevotella intermedia*, *Porphyromonas gingivalis*, can adhere (Kolenbrander, 2000, Kolenbrander *et al.*, 2010).

Cell-to-cell communication plays an important role in biofilm formation using quorum-sensing system. For example, the adherent cells differentiate to become sessile cells and start forming biofilm (Leung *et al.*, 2015). In *S. mutans*, quorum-sensing is composed of competence stimulating peptide (CSP), a small signalling peptide, and ComDE two-component signal transduction system. The CSP-ComDE system of *S. mutans* is activated by stress such as acidic pH, amino acid starvation, and oxidative stress. After activation, the CSP-ComDE system regulates several phenotypes such as biofilm formation, acid tolerance, competence and bacteriocin production (Perry *et al.*, 2009, Leung *et al.*, 2015). Bacteria on tooth surfaces form the three-dimensional

structure by producing extracellular polymeric substances (EPS) such as polysaccharides, proteins, and eDNA.

1.5.3 Biofilm dispersion

Kaplan (2010) defined the mechanism of biofilm dispersion in three steps: detachment of biofilm cells, translocation of the detached biofilm cells to a new location, and attachment of the biofilm cells to the new location (Kaplan, 2010).

Biofilm dispersion can be divided into two types: passive dispersal and active dispersal (Kaplan, 2010, Fleming & Rumbaugh, 2017). The passive dispersal refers to biofilm dispersal caused by an external force such as mechanical intervention (tooth brushing, tongue movement, fluid and solid shear). The active dispersal refers to biofilm dispersal caused by the biofilm microbes (inside the biofilm structure). Active dispersal is mediated by many factors such as nutrient starvation, toxic by-products, and antimicrobial stress. Active dispersal is an important phase in the cycle of biofilms, which contributes to bacterial survival and disease transmission (Kaplan, 2010, Fleming & Rumbaugh, 2017).

Three modes of biofilm dispersal have been reported: erosion, sloughing, and seeding. Erosion refers to the release of small clusters or single cells from a biofilm. Sloughing refers to the sudden release of large portions of biofilm. Seeding dispersal refers to the release of a large number of single cells from hollow cavities of biofilm structure (Kaplan, 2010).

In *S. mutans*, the role of biofilm dispersion is possibly as a means for *S. mutans* to spread to a new sites (Lee *et al.*, 1996). In the oral cavity, biofilm dispersion is

mediated by salivary flow, mastication and tongue movement (Das *et al.*, 2011). Active *S. mutans* biofilm dispersion has been reported to be a result of an endogenous surface protein releasing enzyme (SPRE) activity obtained from *S. mutans* (Vats & Lee, 2000). Liu *et al.* (2013) have shown the difference in physiological properties of biofilm detached cells compared to planktonic cells and sessile cells (biofilm cells). These detached cells can attach to other surfaces, developing a new biofilm (Simões *et al.*, 2010, Hwang *et al.*, 2014).

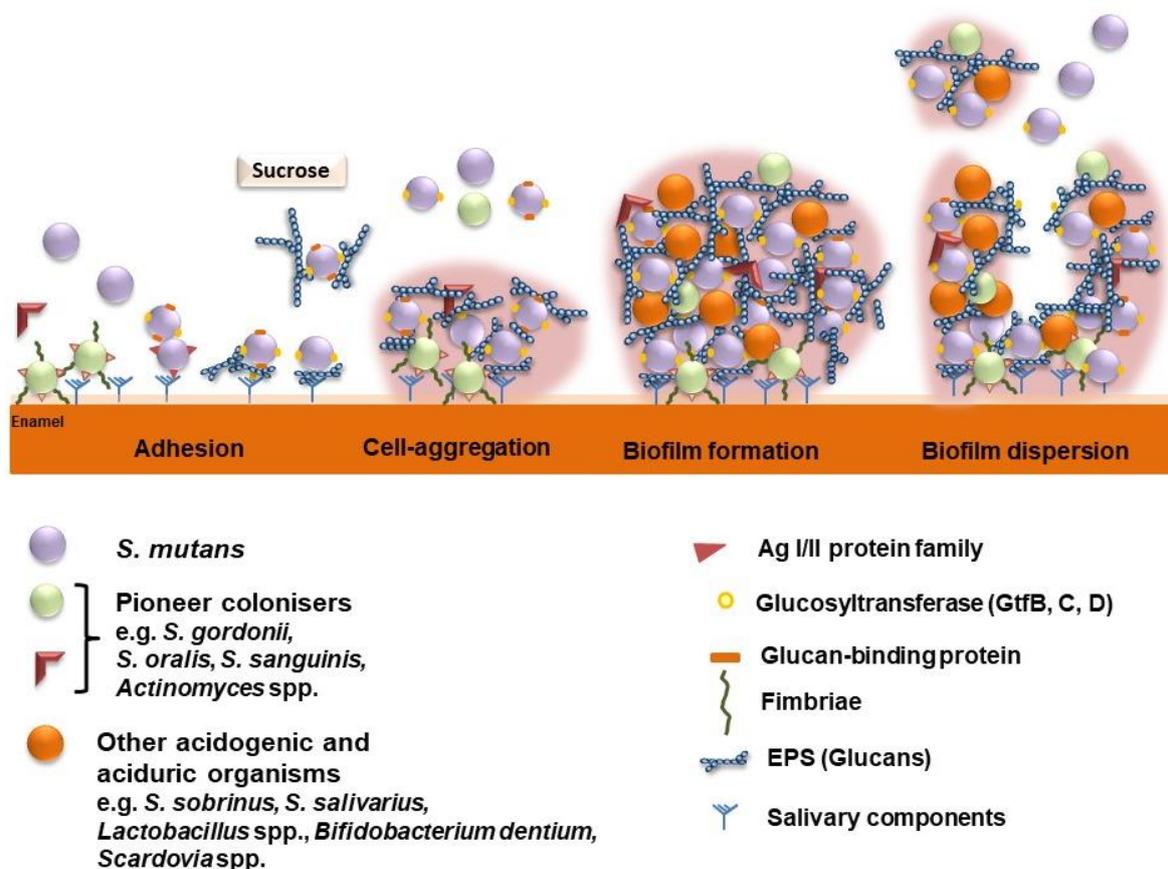


Figure 1.6 Oral biofilm formation

The formation of oral biofilm starts by the adhesion of pioneer colonizers to the abiotic surface. In the absence of sucrose, proteins such as adhesins, fimbria, and pili interact with salivary components on the enamel surface. While, in the presence of sucrose, Gtfs from streptococci produce glucans and these glucans together with Gbps, facilitate cell-to-cell-aggregation, especially aggregating to the adherent cells. An increase of cell density and quorum sensing leads to the differentiation of planktonic cells into sessile cells. Sessile cells mature and form multi-layered clusters and at the last step of the cycle, sessile cells will detach from the biofilm community. These

biofilm- detached cells will then adhere to a new surface to begin the colonisation and the formation of biofilm on new surface and the cycle starts again. The figure was drawn using MS PowerPoint 2010 and modified from Nobbs *et al.* (2009) and Koo *et al.* (2013).

1.6 Possible strategies to target *S. mutans* biofilm

Several lines of evidence suggest possible strategies to combat biofilms, including those produced by *S. mutans*, by targeting the stages of biofilm formation, such as the inhibition of bacterial adhesion on the surfaces, the inhibition of biofilm development and differentiation, the inhibition of biofilm cells, and the induction of biofilm dispersion (Yang *et al.*, 2012, Scharnow *et al.*, 2019)

1.6.1 Inhibiting the initial adhesion of *S. mutans*

The adhesion of *S. mutans* cells is one of the important process to form biofilms on the tooth surfaces. Many studies have focused on inhibiting *S. mutans* biofilm formation at the adhesion step. *S. mutans* planktonic cells adhere on the tooth surfaces through a sucrose-dependent and sucrose-independent mechanism which are targeted differently.

1.6.1.1 Sucrose-dependent mechanism

In the presence of sucrose, *S. mutans* glucosyltransferases (GtfB, GtfC, GtfD) and glucan binding protein (Gbps) are essential for attachment and biofilm formation. Molecules obstructing adhesion of *S. mutans* planktonic cells have been widely studied. Piceatannol and resveratrol have been discovered to be anti-adhesion molecules (Newbrun *et al.*, 1983, Kwon *et al.*, 2010). Piceatannol and resveratrol inhibit *S. mutans* biofilm formation by targeting GtfB and GtfC. A quinoxaline derivative

has been investigated to inhibit GtfC activity and reduce the adhesion of biofilms (Ren *et al.*, 2015). A small molecule, derived from a structure-based discovery study of the GtfC catalytic domain, inhibits GtfC activity and biofilm formation from *S. mutans*. The small molecule does not affect overall growth of *S. mutans* and commensal oral bacteria (Zhang *et al.*, 2017).

Scharnow *et al.* (2019) have revealed two Gtfs inhibitors which are (i) hydroxychalcones, that have an aromatic ketone and enone forming central core, and (ii) molecules with high heteroatom density, an atom other than carbon or hydrogen, especially nitrogen. These findings might help to guide the development of the new compounds that are potent GTF inhibitors for use against *S. mutans* biofilms (Scharnow *et al.*, 2019).

In the community of microbial species in natural biofilms, microorganisms coexist and interact with each other. The competition in the community drives microorganisms to evolve survival mechanisms (Xavier & Foster, 2007, Xavier *et al.*, 2009). Some bacteria might produce substances to combat other bacteria, especially during adhesion on the same area. For instance, biosurfactants produced by a strain of *Lactobacillus casei* (ATCC39392) reduces gene expression of *gtfB*, *gtfC*, and *ftf* in *S. mutans* and this reduced expression might reduce the adhesion of *S. mutans* due to the amount of glucan (Savabi *et al.*, 2014).

1.6.1.2 **Sucrose-independent mechanism**

In the absence of sucrose, sortase A facilitates the attachment of Agl/II, GbpC, FruA, WapA, Wap E and Dex A on *S. mutans* cell wall and these surface proteins have been confirmed to play important roles in adhesion, colonization and biofilm formation (Ajdic

et al., 2002, Lévesque *et al.*, 2005). These reasons lead sortase A to be an attractive target for *S. mutans* anti-biofilm activity. Hu *et al.* (2013) found that curcumin could inhibit the sortase A of *S. mutans* without affecting *S. mutans* viability. It was hypothesised that enone functionalities in curcumin are specific sites for irreversible modification by Cys184 in sortase A of *S. mutans*. This inhibition resulted in the release of Pac protein (antigen I/II, P1, and Spa P1) on *S. mutans* cell surfaces to supernatant and reduce the ability to form biofilms by *S. mutans*. A *trans*-chalcone, a chalcone flavonoid, inhibits sortase A and also inhibits biofilm formation by *S. mutans* (Wallock-Richards *et al.*, 2015). Mass spectrometry revealed that the *trans*-chalcone forms an adduct with the cysteine at the active site of sortase A (Wallock-Richards *et al.*, 2015).

Eugenol, a major component of essential oil from clove buds, inhibited biofilm formation from *S. mutans* (Adil *et al.*, 2014). Eugenol reduces the adhesion of *S. mutans* cells by reducing the expression of AgI/II.

An active compound from *Trachyspermum ammi* seeds was found to inhibit biofilm formation from *S. mutans* through a variety of mechanisms such as reducing adhesion and glucan synthesis and inhibiting the reduction in pH (Khan *et al.*, 2010). The most dominant mechanism seems to be the inhibition of adhesion and destroy the integrity of cell wall due to the reduction of hydrophobicity (Scharnow *et al.*, 2019).

1.6.2 Targeting EPS

As mentioned previously, the accumulation of EPS matrix in biofilms provides a protective barrier against antimicrobial agents. Targeting the EPS of *S. mutans* biofilms has been investigated in many studies.

1.6.2.1 Inhibition of EPS production

Since glucans are major components in EPS, inhibiting *gtfs* expression and Gtfs activity reduces EPS accumulation (Falsetta *et al.*, 2012, Bueno-Silva *et al.*, 2013, Wang *et al.*, 2016). Many compounds have been reported to inhibit *gtfs* expression such as a novel combination therapy (2 mM myricetin, 4 mM tt-farnesol, 250 ppm of fluoride) reduced the expression of *gtfB* (Falsetta *et al.*, 2012) and glass-ionomer cements (GIC) containing a quaternary ammonium monomer (dimethylaminododecyl methacrylate, DMADDM) reduced the expression of *gtfB*, *gtfC* and *gtfD* (Wang *et al.*, 2016).

Targeting to EPS synthetic components has been reported in a number of studies. 7-epiclusianone, a novel compound isolated from *Rheedia brasiliensis*, inhibits the activity of GtfB and GtfC resulting in the reduction of insoluble glucan synthesis (Murata *et al.*, 2010). Neovestitol-vestitol (NV), isolated from Brazilian red propolis, inhibits GtfD activity resulting in decrease amount of soluble glucan (Bueno-Silva *et al.*, 2013). This soluble glucan increases GtfB activity to synthesise insoluble glucan. Therefore, the inhibition of GtfD reduces the EPS accumulation (Venkitaraman *et al.*, 1995).

1.6.2.2 Disruption of EPS structure

The disruption of EPS structure is an attractive means to break *S. mutans* biofilms, which allows access of antimicrobial agents to kill microbes within biofilm. A previous

study by Gao *et al.* (2016) generated catalytic nanoparticles (CAT-NP) with peroxidase-like activity, containing Fe₃O₄. CAT-NP produced free-radicals, which could degrade the EPS matrix, particularly the water-insoluble glucan, and kill the embedded bacterial cells by causing DNA damage. The CAT-NP also reduced the acid dissolution of hydroxyapatite.

1.6.3 Direct interfering the survival of *S. mutans* biofilm cells

1.6.3.1 Killing *S. mutans* within biofilm structure

The clinical plaque disclosing/visualising agent erythrosine, when used as a photosensitiser in a photodynamic system, was found to kill *S. mutans* cells in a 200 µm-thick biofilm in a constant-depth film fermenter. The excited-state of erythrosine could react with biomolecules in *S. mutans* cells to produce free radicals or could react with an oxygen molecule to produce singlet oxygen. The free radicals or singlet oxygen can cause oxidation of cellular constituents such as cell membrane, and DNA, causing cell death (Wood *et al.*, 2006).

1.6.3.2 Inhibition of quorum sensing and two-component system

Quorum sensing systems modulate virulence factors of *S. mutans* including biofilm formation, genetic competence, bacteriocin production, and acid tolerance response (Perry *et al.*, 2009, Leung *et al.*, 2015). Therefore, targeting quorum sensing systems has been widely studied. For example, specifically (or selectively) targeted antimicrobial peptides (STAMPs) and other selective antimicrobial peptides (e.g. C16G2, M8G2, C16-33, and M8-33) were constructed based on the CSP-derived peptides. Beneficially, a low concentration of these molecules is required to target the quorum sensing system, but did not affect other oral streptococci (*S. gordonii* and *S. sanguinis*) (Eckert *et al.*, 2006).

Challisin, a protease derived from *S. gordonii*, has been reported to strongly inhibit mutacin production in *S. mutans* (Wang & Kuramitsu (2005). This result suggests that challisin interferes with the competence stimulating peptide (CSP) signaling system. In addition, He *et al.* (2012) demonstrated that a synthetic furanone C-30 inhibits biofilm formation by *S. mutans* (i.e. the reduction in thickness and integrity of biofilms), possibly by repressing transcription of the quorum sensing coding gene *luxS*, which could affect the expression of genes responsible for *S. mutans* biofilm formation including *gtfB* (Merritt *et al.*, 2003).

1.6.3.3 Interruption of acidogenicity and aciduricity

Within the biofilm structure lactic acid produced by *S. mutans* is a major cause of demineralisation of the enamel surface. Several previous studies have focused on targeting either acidogenicity or aciduricity to inhibit biofilm formation. Pandit *et al.* (2015) found that a fraction of hexane-dissolved extract from *Withania somnifera* composed of palmitic, linoleic and oleic acids, exhibits the strong anti-acidogenic, activity against *S. mutans*. The reduction in acid production and secretion might be due to an inhibitory activity of linoleic and oleic acids on F₁F₀-ATPase (Pandit *et al.*, 2015).

Arginine can be utilised by arginolytic bacteria, such as *S. gordonii* and *S. sanguinis*, through an arginine deiminase system (ADS) with ammonia being one of the end products resulting in increase of pH value (Sharma *et al.*, 2014). Arginine has been shown to affect the production of water insoluble glucan by reducing *gtfB* expression resulting in the reduction of biofilm formation and adhesion (He *et al.*, 2016). Arginine also mediates *S. gordonii* to produce H₂O₂ which is toxic to *S. mutans*. Toothpaste

containing arginine has been found to mediate a healthy change in microbiome (Nascimento *et al.*, 2014).

Fozo *et al.* (2004) reported that the proportion of monounsaturated fatty acids in cell membranes of *S. mutans* could affect its viability in low pH environment. They hypothesised that the unsaturated bonds of monounsaturated fatty acid might serve as a sink for protons. The increased activity of F₁F₀ ATPase, accompanied with larger amount of monounsaturated fatty acids in cell membrane, is observed in cells that grow under acid conditions. In addition, a putative FabM responsible for the production of monounsaturated fatty acid is identified. *S. mutans* that lacks *fabM* is extremely sensitive to low pH conditions (Fozo & Quivey, 2004).

Rhamnosyltransferase (encoded by *rgpF*) synthesizes the core polyrhamnose backbone of rhamnose-glucose polysaccharide (RGP) found in the cell wall of *S. mutans* (Kovacs *et al.* (2017). Deletion of *rgpF* disrupts the proton gradient across the membrane and prevents induction of F₁F₀ ATPase activity, leading to reduced ability of *S. mutans* to tolerate acid challenge and form biofilm on saliva-coated hydroxyapatite surfaces (Kovacs *et al.*, 2017).

1.6.4 Induction of biofilm dispersal

Many studies have developed biofilm dispersal agents by focusing on how to trigger active dispersal (Fleming & Rumbaugh, 2017). A biofilm dispersing molecule, cis-2-denoic acid (C2DA), has been shown to disperse pre-established biofilms of *S. mutans* (Rahmani-Badi *et al.*, 2015, Fleming & Rumbaugh, 2017). The combination of C2DA with chlorohexidine could disperse of dual-species biofilms of *S. mutans* and *C. albicans*. The activity of C2DA is detected at nano-molar concentration, suggesting

that it may induce the transition of *S. mutans* from a biofilm cell to a planktonic cell through a signaling mechanism (Rahmani-Badi *et al.*, 2015).

1.7 *Bacillus* spp.

1.7.1 General information

Bacilli are Gram-positive, rod-shaped and endospore-forming. The cell wall usually is composed of a meso-DAP direct murein cross-linkage type of peptidoglycan. Some bacilli are motile using peritrichous flagella but some bacilli are nonmotile. *Bacilli* are aerobes or facultative anaerobes (e.g. *B. anthracis*). *Bacilli* are typically found in soil and water. Most of the *Bacillus* have little pathogenic potential. However, many species such as; *Bacillus anthracis*, anthrax producer, *Bacillus cereus* and *Bacillus thuringiensis* are pathogenic bacteria.

There are over 200 named species of *Bacilli* which have been identified using several methods including genetic approaches, biological assays and matrix assisted-laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS). However, the identification methods have some limitations (Alcaraz *et al.*, 2010, Shu & Yang, 2017). For instance, 16S rRNA gene-based taxonomy cannot identify some *Bacillus* species due to the high degree of similarity of the 16S rRNA gene sequences (Jeyaram *et al.*, 2011). Identification using MALDI-TOF MS is affected by culture conditions such as the incubation period and temperature (Shu & Yang, 2017). In general, *Bacilli* are divided into five groups based on phylogeny analysis of 16S rRNA gene sequences: *B. subtilis*, *B. megaterium*, *B. cereus*, *B. circulans* and *B. brevis* groups (Berkeley *et al.*, 2008, Liu *et al.*, 2013).

1.7.2 A source of bioactive compounds

Among bacterial genera, *Bacillus* is one genus which has received a lot of attention because of their diversity and as a source of bioactive substances (Roongsawang *et al.*, 2010). *Bacillus* is a source of antimicrobial substances exhibiting several activities affecting many microorganisms, including against Gram-negative and Gram-positive bacteria and yeast (Abriouel *et al.*, 2010). The antimicrobial substances derived from *Bacilli* are a variety of peptide and lipopeptide antibiotics, and bacteriocins (Abriouel *et al.*, 2010).

1.7.2.1 Biosurfactants

Lipopeptide biosurfactants have been isolated from *Bacillus* and exhibit many biological activities such as reducing surface and interfacial tensions, anti-microbial and anti-enzymatic activities. The lipopeptide biosurfactant molecule consists of a hydrophobic fatty acid and a hydrophilic peptide chain (Roongsawang *et al.*, 2010). Lipopeptide biosurfactants from *Bacilli* are divided into three families: surfactin, fenycin, and iturin (Raaijmakers *et al.*, 2010). A study has reported that a biosurfactant derived from *S. mitis* reduces the adhesion of *S. mutans* cells on saliva-coated surface (Van Hoogmoed *et al.*, 2000).

1.7.2.2 Antibiotic substances

Stein (2005) reviewed antibiotic substances derived from *B. subtilis*. The antibiotics from *B. subtilis* can be divided into two groups based on their synthesis process: (i) ribosomally synthesised and post-translationally modified molecules (lantibiotics and lantibiotic-like peptides), and (ii) non-ribosomal generated molecules (polyketide, aminosugar, and phospholipid) (Stein, 2005). The examples of group (i) antibiotics are subtilin, ericin, mersadin, sublancin 168, and subtilosin A (Stein, 2005). The examples

of group (ii) antibiotics are surfactin, iturin, bacillomycin, mycosubtilin, fengycin, and corynebactin (Stein, 2005).

1.7.2.3 Enzymes

Due to the biodiversity and the high capacity for protein secretion of *Bacillus*, *Bacillus* is one of a major enzyme producers (Contesini *et al.*, 2018). Many enzymes have been discovered from *Bacillus* spp. Several commercial proteases are obtained from *Bacillus* species such as Proteinase®, Neutrase® and Optimase® PR (Contesini *et al.*, 2018). An extracellular alkaline amylase is produced by *Bacillus* sp. NRRL B-3881 (Boyer & Ingle, 1972). Chitinase, an enzyme catalysing chitin degradation, was isolated from *Bacillus* spp. (Thakkar *et al.*, 2017). A novel extracellular metalloprotease was isolated from *B. subtilis* GP227 (Rufo *et al.*, 1990). Alkaline lipase was identified from marine *B. smithii* BTMS 11 (Lailaja & Chandrasekaran, 2013). A novel thermotolerant endoglucanase from *Bacillus* sp. Strain C1 was isolated from cow dung (Sadhu *et al.*, 2013). Levansucrase has been isolated from many strains of *Bacilli* (Ben Ammar *et al.*, 2002, Tian *et al.*, 2011, Li *et al.*, 2015).

1.7.2.4 Levansucrase

Levansucrase (EC2.4.1.10) synthesizes levan from sucrose by transfructosylation. Levansucrase is a member of the glycosyltransferase family, based on the phylogenetic analysis of the amino acid sequences of glycosidases (Li 2015). Levansucrase is also grouped into glycoside hydrolase (GH) family 68 (Meng & Fütterer, 2003). Goncalves *et al.* (2015) reported that levansucrase has three different activities: (i) fructosyltransferase, (ii) hydrolase, and (iii) polymerase (Gonçalves *et al.*, 2015). The fructosyltransferase activity of levansucrase is the ability of the enzyme to transfer fructosyl units from sucrose to the fructan chain and the growth of the fructan

chain is due to the polymerase activity (Gonçalves *et al.*, 2015, Mendez-Lorenzo *et al.*, 2015). At low sucrose concentrations, hydrolysis occurs partially when levansucrase hydrolyses low molecular weight levans at β -2, 1 branches (Mendez-Lorenzo *et al.*, 2015).

In the presence of sucrose, levansucrase hydrolyses a sucrose molecule and provides a glucose and a fructose molecule as shown in Figure 1.7. Levansucrase transfers the fructosyl molecule to the acceptor which can be mono-, di-, oligosaccharide, or levan during the polymerisation process. Levan is a fructose polymer with β -2, 6 linkages and β -2, 1 branching, or β -2, 1-linked inulin (Meng & Fütterer, 2003, Gonçalves *et al.*, 2015).

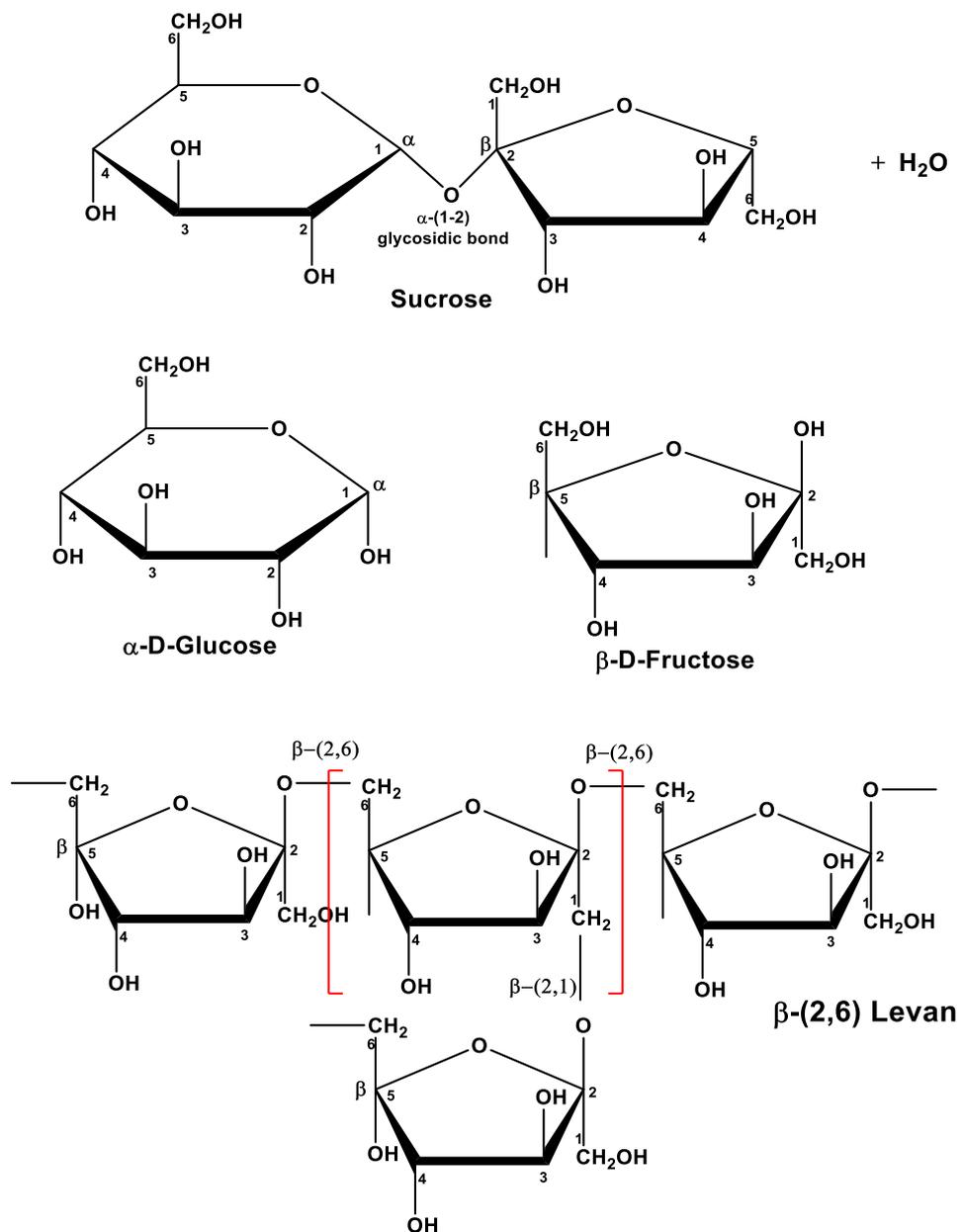


Figure 1.7 Structure of levan with β -2, 6 linkages and β -2, 1 branching

Sucrose molecule is hydrolysed at α -(1,2) glycosidic bond to provide glucose and fructose molecule. This fructose molecule is polymerised to synthesise levan. The figure was drawn with ChemDraw Professional 16.0 and is based on an illustration by Santos-Moriano *et al.* (2015) and Srikanth *et al.* (2015).

Levan is a fructose polymer which is produced from sucrose by levansucrase. The structure of levan (as shown in the Figure 1.7) consists of β -2, 6 linkages and β -2, 1 branching (Santos-Moriano *et al.*, 2015).

In nature, levan is synthesized, in the presence of sucrose, by many microorganisms (Archaea, fungi and bacteria) and a few plants such as *Pachysandra terminalis* (family Buxaceae), the Japanese pachysandra. The bacterial levans have a molecular weight of over 500 KDa, low intrinsic viscosity, and stability. Levans are commonly branched and form a compact nanosphere. This compact nanosphere leads levan to have a broad spectrum of applications such as anti-inflammatory, anti-oxidant, as a prebiotic, a whitener and in weight loss (Oner *et al.*, 2016).

In association with biofilm, levans are a dominant polysaccharide in EPS of *B. subtilis* biofilms which were grown in a sucrose-rich medium (SYM). Although levan is not essential for biofilm formation by *B. subtilis*, it is possibly important as a nutrient source, and stabilising component of the biofilms (Dogsa *et al.*, 2013). Levan was found to be a component of EPS of *S. mutans* biofilms (Corrigan & Robyt, 1979).

1.7.2.5 Serine proteases

Serine proteases, or serine peptidases, are a member of protease enzymes. This proteases are divided into five groups based on their catalytic mechanisms: serine proteases, metalloproteases, cysteine proteases, threonine proteases, and aspartic acid proteases (Barrett *et al.*, 2003). In previous studies, over 26,000 serine proteases were grouped into 13 clans and 40 families according to Rawlings *et al.* (2007) and Page & Di Cera (2008).

Among the proteolytic enzymes, serine proteases are the most abundant and functionally diverse in organisms (Page & Di Cera, 2008, Ruiz-Perez & Nataro, 2014). Serine proteases hydrolyse peptide bonds and occur in many biological systems including the digestive system of humans (chymotrypsin in small intestine), in bacteria, in preventing human blood clots and facilitating human sperm to enter egg cells (Neitzel, 2010).

Serine proteases contain a catalytic triad structure at the active site in order to break the peptide bonds. In Figure 1.8, the catalytic triad structure is composed of three amino acid residues: serine, histidine, and aspartic acid. A catalytic serine residue, serine 195, cleaves peptide bonds, whereas the histidine residue activates serine to cleave the peptide bond by working as a base catalyst and acid catalyst. An aspartic acid residue provides proton to histidine residue (Carter & Wells, 1988, Hedstrom, 2002, Wahlgren *et al.*, 2011).

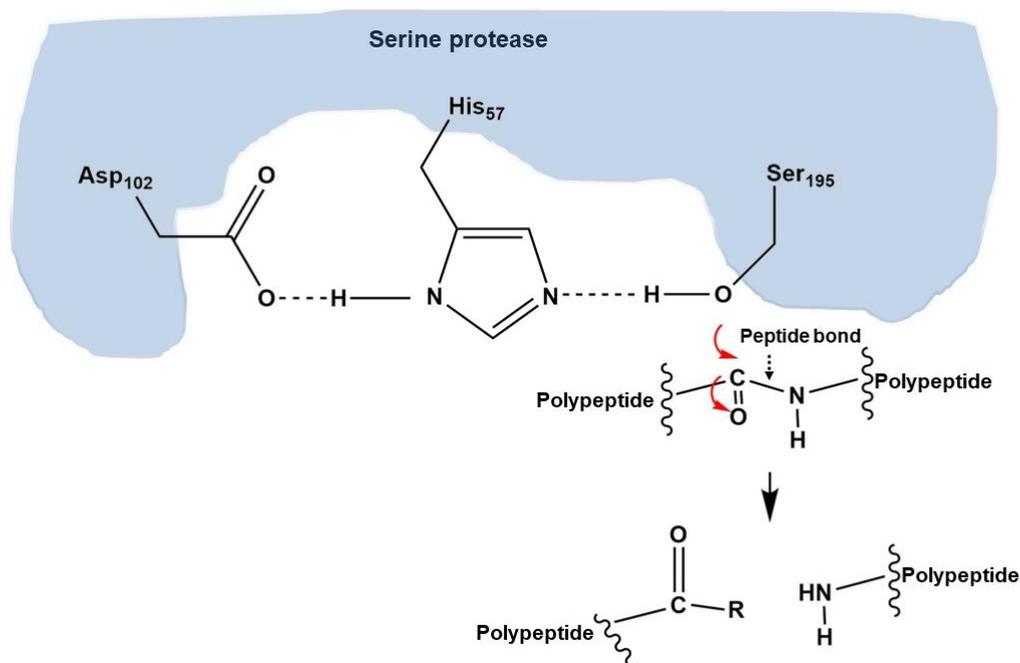


Figure 1.8 Schematic diagram of Ser-His-Asp catalytic triad of serine protease

In chymotrypsin, the hydroxyl molecule of serine residue 195 attacks the carbonyl carbon of polypeptide to start the process of cleaving peptide bond. The position of the catalytic triad in bacterial proteins can be different, such as Ser221, His64, and Asp32. The figure was drawn with ChemDraw Professional 16.0 and is based on an illustration by Neitzel (2010).

The subtilisin family of serine proteases have been investigated in *Bacilli* and have been reported to be involved in biofilm formation in many studies. Two serine proteases (subtilisin-like protease and glutamyl endopeptidase) derived from *B. pumilus* inhibit biofilm formation from *Serratia marcescens*. The two enzymes digest proteins of EPS structure from 7-day old biofilms (Mitrofanova *et al.*, 2017).

Serine proteases derived from other bacteria have been investigated for anti-biofilm activity. A serine protease related to biofilm detachment in *S. aureus* (Boles & Horswill, 2008). A serine protease, PKF (protease required for resistance to complement killing and suppression of biofilm formation), of *Acinetobacter baumannii* suppresses biofilm

formation (King *et al.*, 2013). A serine protease Esperase HPF (subtilisin) inhibits biofilm formation by four bacteria: *Microbacterium phyllosphaerae*, *Shewanella japonica*, *Dokdonia donghaensis* and *Acinetobacter Iwoffii*, which all bacteria were isolated from green alga *Ulva australis* (Hangler *et al.*, 2009).

1.8 Aim and objectives

The main aim of this study was to discover and investigate compounds with *S. mutans* anti-biofilm activity.

The specific aims were:

1. To screen for anti-biofilm activity from a metagenomic library and an environmental bacteria.
2. To investigate a compound responsible for anti-biofilm activity.

CHAPTER 2

Materials and Methods

Chapter 2 Materials and Methods

This chapter describes general materials and methods used throughout this thesis.

The specific methods are outlined at the beginning of each chapter.

2.1 Bacterial strains

Streptococcus mutans UA159 (ATCC 700610, US) (Ajdic *et al.*, 2002) used in this study was derived from American Type Culture Collection (ATCC) (US) and it was confirmed by 16S rRNA gene sequencing. *S. mutans* was cultured on Todd-Hewitt (TH) (Sigma Aldrich, UK) agar and incubated in an atmosphere of 5% CO₂ /95% air at 37°C for 24 h or on a rotary shaker at 200 rpm when grown in broth.

2.2 *S. mutans* biofilm formation

S. mutans cultures from 18 h of incubation in TH broth were diluted to approximately 3x10⁶ CFU/ml. The biofilm assay in this study used a 96-well oxygen plasma treated polystyrene flat-bottom microtitre plate (Sarstedt, Germany). The biofilm assay was started by adding 100 µl of the *S. mutans* culture and 100 µl of TH broth supplemented with 4% sucrose in order to obtain a final working concentration of 2% sucrose. The 96-well microtitre plate was covered by a breathable sealing film (Anachem, UK) and incubated in an atmosphere of 5% CO₂ /95% air at 37°C for 24 h.

2.3 Crystal violet quantification of biofilm formation

The amount of *S. mutans* biofilms in each well of a microtitre plate was determined after 24 h of incubation for biofilm formation. OD_{590nm} of biofilms with planktonic cells was measured using MRX Microplate Reader (Dynex Technologies, USA). To determine the amount of biofilms, planktonic cells were discarded from the incubated

plates using a micropipette and biofilms at the bottom of a microtitre plate well were gently washed twice with 200 µl of PBS (Sigma, UK) to remove the non-adherent cells. Then, the microtitre plate was air-dried in an incubator at 80°C for 30 min. The dried biofilms in each well were stained by adding 200 µl of 0.5% crystal violet (ProLab Diagnostics, UK) and incubated at room temperature for 1 min before removal. Then, non-bound crystal violet was washed twice with 200 µl of deionised water using a micropipette. To dissolve the bound-crystal violet, 200 µl of 33% acetic acid (Sigma, UK) was added into each well and mixed by gently pipetting up and down. For *S. mutans* biofilms in this study, the resulting, dissolved crystal violet solution was measured at OD_{590nm} using the microplate reader. Due to the very high concentration of dissolved crystal violet solution, it was diluted in deionised water. The optimal dilution in this study was 1:10 dilution which contained 20 µl of dissolved crystal violet solution and 180 µl of deionised water.

2.4 Screening for anti-biofilm activity from an environmental isolate library.

2.4.1 Primary screening

Environmental bacteria have been isolated from the Swab and Send project, which was initially started in UCL and then moved to Liverpool School of Tropical Medicine (LSTM). This website provides more information of the project, <https://www.lstmed.ac.uk/research/public-engagement/swab-send>). A total of 89 isolates were screened for *S. mutans* anti-biofilm activity. The biofilm formation assay was conducted in a 96-well microtitre plates. One-hundred microlitres of *S. mutans* culture in TH broth supplemented with 4% sucrose was used for biofilm formation assays. In the primary screening, isolates were cultured in 200 µl of BHI broth in a microtitre plate and incubated at 37°C for 18 h with shaking at 150 rpm. Then 18 h

cultures were centrifuged at 1,500 x *g* for 10 min (Jouan C412, Thermo Scientific) and 100 µl of supernatant was added to wells containing *S. mutans*. The microtitre plates were incubated in an atmosphere of 5% CO₂ /95% air at 37°C for 24 h. The amount of biofilm was determined by crystal violet biofilm assay.

2.4.2 Secondary screening

The environmental isolates exhibiting *S. mutans* anti-biofilm activity from the primary screening were grown in a volume of 4 ml of BHI broth at 37°C 200 rpm for 18 h. The 18 h cultures were centrifuged at 4,500 x *g* for 10 min at 4°C. The supernatants were filtered through a 0.22 µm pore size membrane filter. Filtered supernatants were tested for *S. mutans* anti-biofilm activity.

2.5 Heat stability assay

To determine the heat stability of a compound responsible for the anti-biofilm activity, 0.22 µm filtered supernatants contained in a 1.5 ml Eppendorf tube were boiled in water at 100°C for 15 min and then cooled on ice. The boiled- filtered supernatants were tested for the anti-biofilm activity.

2.6 *S. mutans* biofilm formation over a period of 24 h

To monitor biofilm formation by *S. mutans* over a period of 24 h, approximately 3x10⁵ *S. mutans* cells, in 200 µl of TH broth supplemented with 2% sucrose, were added into wells of a 96-well microtitre plate at hourly intervals for 24 h. To do this, two microtitre plates were used and a volume of 200 µl of *S. mutans* culture was added to each of the first column of the microtitre plate at time one (8.00 am) and the plates were incubated in an atmosphere of 5% CO₂ /95% air at 37°C for 24 h for the first plate and 11 h for the second plate. This provided biofilms formed over 24 h for the first plate

and biofilms formed for 11 h for the second plate. In the next hour, (time 2, 9.00 am), another 200 µl of *S. mutans* culture was added to the wells in the second column of the plates and incubated at the same condition. This provided biofilms formed for 23 h for the first plate and biofilms formed for 10 h for the second plate. The process was repeated until time 11 which provided biofilms formed for 14 h for the first plate and biofilms formed for 1 h for the second plate as shown in Table 2.1. Biofilms with planktonic cells were measured at OD_{600nm} using microplate reader, while the amount of biofilms was measured using crystal violet biofilm assay.

Table 2. 1 *S. mutans* biofilm formation over a period of 24 h

| Column of a microtitre plate | Time | | Biofilm incubation time (h) | |
|------------------------------|------|----------|-----------------------------|-------------------|
| | | | The first plate | The second plate |
| 1 | 1 | at 8.00 | 24 | 11 |
| 2 | 2 | at 9.00 | 23 | 10 |
| 3 | 3 | at 10.00 | 22 | 9 |
| 4 | 4 | at 11.00 | 21 | 8 |
| 5 | 5 | at 12.00 | 20 | 7 |
| 6 | 6 | at 13.00 | 19 | 6 |
| 7 | 7 | at 14.00 | 18 | 5 |
| 8 | 8 | at 15.00 | 17 | 4 |
| 9 | 9 | at 16.00 | 16 | 3 |
| 10 | 10 | at 17.00 | 15 | 2 |
| 11 | 11 | at 18.00 | 14 | 1 |
| 12 | - | at 19.00 | - | 0 |
| Biofilms measurement | | | Next day at 8.00 | Same day at 19.00 |

2.7 Capacity of filtered supernatants exhibiting anti-biofilm activity to disrupt early steps *S. mutans* biofilm formation

To determine the capacity of a compound(s) with anti-biofilm activity to disrupt the initial steps of *S. mutans* biofilm formation, a 0.22 µm filtered supernatant exhibiting *S. mutans* anti-biofilm activity was added to wells forming *S. mutans* biofilms at different times. To do this, *S. mutans* was grown in the wells and allowed to form biofilms for 1 h, 2 h, 3 h, 4 h, 5 h, 6 h and 7 h in an atmosphere of 5% CO₂ /95% air at 37°C. The planktonic cells and all the liquid were removed gently using a pipette before adding a volume of 100 µl of the filtered supernatant supplemented with 100 µl of fresh TH broth. A volume of 200 µl of fresh TH broth was used as a control group. The plate was incubated in the same conditions for a total of 24 h. Biofilms were measured by crystal violet biofilm assay.

2.8 Capacity of filtered supernatant exhibiting biofilm inhibitory activity to disrupt mature biofilms

To determine the capacity of a compound (s) with anti-biofilm activity to disrupt mature biofilms, a 0.22 µm filtered supernatant exhibiting *S. mutans* anti-biofilm activity was added to wells containing *S. mutans* mature biofilms. *S. mutans* formed biofilms in a microtitre plate for 7 h and 24 h in an atmosphere of 5% CO₂ /95% air at 37°C. The planktonic cells and all the liquid were removed gently and non-adherent *S. mutans* cells were washed twice with 200 µl of PBS. A volume of 100 µl of the filtered supernatant supplemented with 100 µl of fresh TH broth was added to the wells containing *S. mutans* biofilms. A volume of 200 µl of fresh TH broth was used as a control group. The plate was incubated in the same condition for 24 h. Biofilms were measured by crystal violet biofilm assay.

2.9 Determination of anti-biofilm activity on saliva-coated surface

Saliva samples were collected from 20 healthy people in the Microbial Diseases Department, UCL Eastman Dental Institute. Ethical approval for the saliva sampling was obtained from University College London (UCL) Ethics Committee and UCL Ethics Project ID Number 5017/001. Saliva samples were pooled together and food debris removed by centrifuging at 4,500 x *g* for 10 min at 4 °C. Saliva supernatant was filtered using a 0.22 µm filter. A volume of 1 ml filtered saliva was aliquoted into Eppendorf tubes and stored at -20 °C until used. To prepare saliva-coated surfaces for *S. mutans* biofilm assays, 50 µl of saliva was added into the wells of a microtitre plate and incubated at 37 °C for 18 h. Then, saliva was discarded and non-bound salivary molecules were removed by washing with 200 µl of PBS twice.

In the biofilm formation assay, filtered supernatants were added to the wells with saliva- and without saliva-coated surfaces. *S. mutans* culture was inoculated to each well. The plate was incubated in an atmosphere of 5% CO₂ /95% air at 37°C for 24 h.

2.10 Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

Protein samples in a volume of 40 µl were mixed with 10 µl of Pierce™ Lane Marker Reducing Sample Buffer 5X (ThermoFisher, UK) by pipetting and boiled at 100°C in boiling water for 3 min to denature proteins. To complete the reaction of Dithiothreitol (DTT) reducing di-sulfide bonds of the protein samples, samples were left at room temperature for 1 h after boiling.

Separating gels consisted of 10 % of resolving gel formulation which were prepared in a volume of 10 ml by adding 3.33 ml of 33% ProtoGel (National Diagnostic), 2.5 ml of 4 x ProtoGel Resolving buffer (0.375 M Tris-HCl and 0.1% SDS, pH 8.8) (National

Diagnostic), 4.06 ml of deionised water, 100 µl of 10% ammonium persulfate (APS) (National Diagnostic). The gel was polymerised chemically by adding 10 µl of TEMED (National Diagnostic). A volume of 7.5 ml of the solution was poured into the gel cassette and the gel was overlaid with isopropanol immediately to exclude O₂ and ensure a flat interface between separating gel and stacking gel. The gel was allowed to polymerise for 1 h (or 16 h for protein identification). When the gel was polymerised completely, isopropanol was discarded and washed by deionised water.

The stacking gel solution which contained 650 µl of 30% ProtoGel, 1.25 ml of 4 x ProtoGel of stacking buffer, 0.125 M Tris-HCl and 0.1% SDS, pH 6.8, (National Diagnostic), 3.05 ml of water, 25 µl of 10% APS and 10 µl of TEMED, was poured on the top of the separating gel. The comb was inserted immediately and the gel was allowed to polymerise for at least 1 h. The SDS-PAGE tank was filled with 1X Tris/Glycine SDS, prepared from 10X Tris/Glycine/SDS (0.25M Tris, 1.92 Glycine and 1% SDS) (National diagnostics) and samples were loaded into the wells of the SDS-PAGE gel. A volume of 5 µl Prestained Protein Marker Broad Range (7–175 KDa) (New England Biolabs, UK) was loaded into a well of the SDS-PAGE gel. Electrophoresis was started with an initial current of 60 mA and maintained at this current until the loading dye reached the separating gel. When this occurred, electrophoresis was continued at a current of 40 mA until the dye reached 0.5 cm from the bottom of the gel. SDS-PAGE gels were stained with QC Colloidal Coomassie Stain (Biorad, UK) for 16 h and de-stained in water for 1 h.

2.11 Genomic DNA extraction

Genomic DNA of seven isolates were extracted using Puregene Yeast/Bact Kit for Gram-positive bacteria. A volume of 500 µl of 16 –18 h old bacterial cultures was

aliquoted into 1.5 ml Eppendorf tubes and centrifuged at 14,680 x *g* (Eppendorf centrifuge 5415D) for 1 min. The pellets were extracted to obtain genomic DNA according to manufacturer's protocol with some modifications. The genomic DNA was eluted using 100 µl of molecular grade water (Sigma Aldrich) instead of 400 µl of the elution buffer supplied by the manufacturer.

2.12 Polymerase Chain Reaction (PCR)

Polymerase chain reactions of the 16S rRNA gene of bacteria producing *S. mutans* anti-biofilm activity were performed using a Biometra T3000 Thermocycler (Biometra, UK). BioMix Red (Bioline, UK), a complete ready-to-use 2X reaction mix composed of a stable *Taq* DNA polymerase, dNTPs, 2.5 mM Mg²⁺ and an inert red loading dye, was used in this study. A total volume of 30 µl of standard PCR reactions contained 15 µl of 2X Biomix Red, 2 µl of each primer (10 pmol/µl), 10 µl of molecular grade water (Sigma Aldrich, UK), 1 µl of 50-100 ng/µl DNA template. PCR conditions were started with initial denaturation at 94 °C for 3 min, and 35 cycles of (i) denaturation at 94 for 1 min, (ii) annealing at 55 °C for 30 sec, (iii) extension at 72 °C for 2 min. The final step in PCR reaction was a final extension at 72 °C for 5 min and stored PCR samples at 4 °C.

To amplify 16S rRNA gene of bacteria producing *S. mutans* anti-biofilm activity, 27 forward primer (27F) (5' AGAGTTTGATCMTGGCTCAG 3') (M= A or C) and 1492 reverse primer (1492R) (5' TACCTTGTTACGACTT 3') (Lane, 1991) were used.

2.13 Agarose gel electrophoresis

DNA products were visualised using agarose gel electrophoresis. DNA products were separated using 1-1.5% (w/v) agarose gels (Bioline, UK) which were prepared in 1X tris acetate EDTA (TAE) electrophoresis buffer (40mM Tris, 20mM Acetate and 1mM EDTA), prepared from 50 X TAE, and GelRed (1:10,000 dilution) (Biotium, Cambridge, UK). The DNA products were mixed with 5X loading dye (Qiagen, UK) and loaded into wells of the prepared gel. HyperLadder 1 kb in a volume of 1 μ l was loaded into the well as a marker. The gel was electrophoresed in 1 X TAE electrophoresis buffer and run at 50-100 V for 60-90 min. The gel was visualised under UV illumination using an Alpha Imager (Alpha InnoTech, UK) and the image of DNA bands on the gel was captured using Alpha View version 1.0.1.10 (Alpha InnoTech, UK).

2.14 PCR purification

PCR products were purified using a QIAquick PCR Purification Kit (Qiagen, UK) to remove primers, enzymes, salts and other impurities. The purifications were conducted according to the manufacturer's protocols. All centrifugation steps were done at 14,680 x *g* (13,000 rpm, Eppendorf centrifuge 5415D). Five volumes of buffer PB was added to one volume of the PCR products and mixed by inverting. The mixture was applied to the QIAquick spin column and centrifuged for one min to bind the DNA. The flow-through was discarded and 700 μ l of buffer PE was added to wash the column before centrifuging for one min. The flow-through was discarded and the column was centrifuged for an additional one min to remove the residual wash buffer. The spin column was transferred to a clean 1.5 ml Eppendorf tube and DNA was eluted by adding 30 μ l of molecular grade water to the centre of the QIAquick membrane, left to stand for three min and then centrifuged for one min. The purified DNA

concentration was measured using NanoDrop (Thermoscientific, US) and stored at -20 °C until use.

2.15 DNA extraction from agarose gel

A specific DNA band of interest on an agarose gel was extracted and purified using QIAquick Gel Extraction Kit (Qiagen, UK). All centrifugation steps were done at 14,680 x *g* (13000 rpm, Eppendorf centrifuge 5415D). After the DNA was subjected to agarose gel electrophoresis as described in section 2.13, the gel was viewed briefly under UV light to see the separation of DNA bands. The desired DNA fragment was excised from the gel using a clean scalpel blade and transferred in a 1.5 ml Eppendorf tube and weighed. Three volumes of buffer QG was added to one volume of gel and incubated in a heating block at 50°C with occasionally vortexing until the gel slice completely dissolved. One gel volume of isopropanol (300 µl isopropanol for 300 mg gel) was then added and mixed by inverting.

The mixture was applied to the QIAquick spin column and centrifuged for one min to bind the DNA. The flow-through was discarded and the spin column placed back into the same tube. A volume of 500 µl of buffer QG was added to the column and centrifuged for one min. The flow-through was discarded and 700 µl of buffer PE was added to wash the DNA in the column before centrifuging for one min. The flow-through was discarded and the column was centrifuged for an additional one min to remove the residual wash buffer.

The column was transferred to a clean 1.5 ml Eppendorf tube and DNA was eluted by adding 30 µl of molecular grade water to the centre of the QIAquick membrane, left to stand for three min and then centrifuged for one min. The purified DNA concentration

was measured using a NanoDrop and stored at -20 °C until use. The purity and integrity of the extracted DNA was checked again in 1% agarose gel.

2.16 DNA sequencing

DNA sequencing of the 16S rRNA gene was performed by Genewiz, formerly known as Beckman Coulter Genomics (UK) using Sanger sequencing method (ABI 3730XL). Whole genome sequencing was performed on Illumina MiSeq platform by MicrobesNG (<https://microbesng.uk/>).

2.17 *In silico* sequence analysis

The 16S rRNA gene sequences were analysed using BioEdit software version 7.2.0 (<http://www.mbio.ncsu.edu/bioedit/bioedit.html>) (Hall *et al.*, 2011) and aligned using the National Centre for Biotechnology Information (NCBI) megablast algorithm (<http://www.ncbi.nlm.nih.gov>). DNA sequences were compared by Clustal Omega version 1.2.1 (<https://www.ebi.ac.uk/services/teams/clustal-omega>) (Sievers & Higgins, 2014) and MEGA7 version 7.0.14 for phylogenetic tree analysis (Kumar *et al.*, 2016).

CHAPTER 3

Screening for *Streptococcus mutans* anti-biofilm activity

Chapter 3 Screening for *Streptococcus mutans* anti-biofilm activity

3.1 Introduction

S. mutans is one of the main cariogenic bacteria as a result of its ability to form biofilms, acid production, and cell-cell adhesion (Selwitz *et al.*, 2007). *S. mutans* forms biofilm structures and within the biofilm structure acid is produced which causes demineralisation of the tooth surface. Much research has focused on the inhibition of *S. mutans* growth and clinically, chlorhexidine has been used as an antiplaque agent (Matthijs & Adriaens, 2002) but the prevalence of dental caries in some countries has not diminished (Kassebaum *et al.*, 2015). This suggests that the efficiency of the protocol for prevention and control of dental caries is insufficient.

It has been hypothesised that the ability to form biofilms by *S. mutans* is an important cause of dental caries. The biofilm limits the diffusion of both external molecules into the biofilm structure and of internal molecules to the outside (Dodds *et al.*, 2000). Acid as a by-product of bacterial fermentation is accumulated within a biofilm on a tooth surface and causes a pH value less than 5.5. This promotes demineralisation of tooth enamel and if it is allowed to continue, caries will develop (Xiao *et al.*, 2017). Without biofilm, acid derived from *S. mutans* and other lactic acid bacteria in the oral cavity are neutralised by saliva. Thus, biofilm inhibition is a potential prevention of dental caries. Bioactive compounds derived from microorganisms might be a major source of biofilm disrupting compounds. Therefore, this chapter focuses on the screening for anti-biofilm activity from two different sources: a metagenomic library constructed from DNA from the human saliva and an environmental isolate library.

3.1.1 Human saliva metagenomic library

Reynolds (2017) constructed a metagenomic library from human saliva DNA and the library was created consisted of 27,000 clones. The human saliva metagenomic library contained both oral bacterial and human DNA. About 97,074 Kb of bacterial DNA was identified from the metagenomic library and the bacterial DNA consisted mainly *Prevotella* spp., *Streptococcus* spp. and *Veillonella* spp. based on Illumina 16S rRNA sequencing (Reynolds, 2017).

In the metagenomic library, DNA fragments from human saliva were cloned to CopyControl pCC1BAC vector, which has chloramphenicol resistance gene as a selective marker, and transformed to *E. coli* EPI300 as a host (Reynolds, 2017). Chloramphenicol is required for culturing recombinant *E. coli* in order to maintain the vector. Chloramphenicol is a broad-spectrum antibiotic which inhibits protein synthesis by preventing peptide-bond transformation of peptidyltransferase of 50s ribosome (Schwarz *et al.*, 2004). Chloramphenicol resistance gene in pCC1BAC vector expresses chloramphenicol acetyltransferase. This enzyme transfers acetyl groups to chloramphenicol molecules which inactivates chloramphenicol. In the screening for anti-biofilm activity of metagenomic clones in this chapter, chloramphenicol was added for recombinant *E. coli* growth.

3.1.2 Swab and Send project

Swab and Send project aims to identify new antibiotics. Swab samples from participants were sent to Dr Adam Roberts's laboratory in Liverpool School of Tropical Medicine. The swabs were cultured on BHI agar to isolate bacteria and fungi. Then,

single colonies were chosen and kept in -20 °C until used. More information are available at <https://www.lstmed.ac.uk/public-engagement/swab-send>.

3.2 Aims of the work described in this chapter

3.2.1 To develop a microtitre plate biofilm assay for testing metagenomic libraries for anti-biofilm activity

3.2.2 To screen for *S. mutans* anti-biofilm activity from a metagenomic library and an environmental isolate library

3.3 Materials and Methods

3.3.1 Development of biofilm assay

3.3.1.1 Effect of additional LB or BHI medium on *S. mutans* biofilm formation in THB

One hundred microliters of *S. mutans* cultures in TH broth supplemented with sucrose at a final concentration of 2% were allowed to form biofilms in the presence of two different media: 100 µl LB broth or 100 µl BHI broth. This was because LB broth was used for culturing the metagenomic library and BHI broth was used for culturing the environmental isolate library. The microtitre plate was incubated in an atmosphere of 5% CO₂ /95% air at 37°C for 24 h. Biofilms were measured using the crystal violet biofilm assay as described in section 2.3 in Chapter 2.

3.3.1.2 Effect of *E. coli* EPI300 containing pCC1BAC vector on biofilm formation by *S. mutans*

The metagenomic library was constructed from human saliva in *E. coli* EPI300 using the pCC1BAC vector (Reynolds, 2017). To determine the effect of this *E. coli* EPI300

containing the vector on biofilm assays of *S. mutans*, the assay was conducted by growing *E. coli* EPI300 containing pCC1BAC vector in 200 µl of LB broth supplemented with 12.5 µg/ml chloramphenicol in a 96-well microtitre plate at 37°C 150 rpm for 18 h. In Figure 3.1, to determine the effect of *E. coli* cells on *S. mutans* biofilm formation, a volume of 100 µl of *E. coli* culture was tested in the biofilm assay. To determine the effect of supernatant on *S. mutans* biofilm formation, *E. coli* cultures were centrifuged at 1,500 x g for 10 min and supernatant was used in the biofilm assay. To determine the effect of filtered supernatant on *S. mutans* biofilm formation, the supernatant was filtered using a 0.22 µm filter and the filtrate that passed through the filter membrane was tested in the biofilm assay (as shown in the section 2.2). To compare the *E. coli* culture, the supernatant and the filtered supernatant in the biofilm assay in a microtiter plate, 100 µl of these three samples was added into 100 µl of *S. mutans* culture in TH broth supplemented with 4% sucrose to a final working concentration of 2% sucrose. TH broth without *S. mutans* was used as control. The microtitre plate was incubated in an atmosphere of 5% CO₂ /95% air at 37°C for 24 h and biofilms were measured using crystal violet biofilm assay.

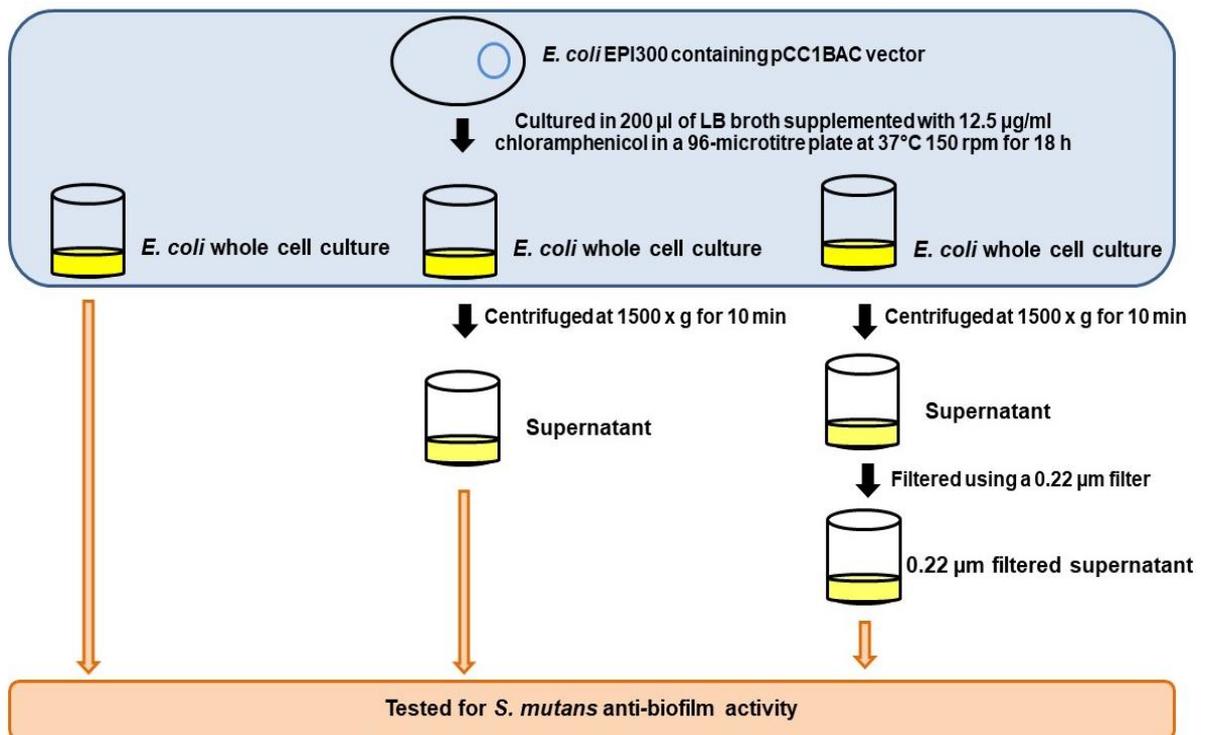


Figure 3.1 Schematic presentation of a method to determine the effect of *E. coli* EPI300 containing pCC1BAC vector on *S. mutans* biofilm assay. *E. coli* cell cultures, supernatants of *E. coli* cultures, and 0.22 µm filtered supernatants were tested for *S. mutans* anti-biofilm activity.

3.3.1.3 Determination of the MIC of polymyxin B against *E. coli* and *S. mutans* in the biofilm assay

Polymyxin B (Sigma, UK) was added to inhibit *E. coli* growth. A suitable concentration of polymyxin B was determined by adding it to microtitre plate wells containing a total volume of 200 µl of: (i) *S. mutans*, (ii) *E. coli*, and (iii) co-cultures of *S. mutans* and *E. coli*. A polymyxin B concentration ranged from 0 to 120 µg/ml was tested. The microtitre plate was incubated in an atmosphere of 5% CO₂ /95% air at 37°C for 24 h. The growth of *S. mutans* and *E. coli* were determined at OD_{590nm} of biofilm with planktonic cells and the biofilms were measured using crystal violet biofilm assay.

3.3.1.4 The effect of sucrose on *S. mutans* biofilm formation

To determine a suitable sucrose concentration for *S. mutans* biofilm formation, five sucrose concentrations of 0, 0.5, 1, 2 and 4% were tested in *S. mutans* biofilm assays. A 20% sucrose solution was sterilised by filtering with a 0.22 µm filter and was subsequently diluted in TH broth to make the concentrations of 1, 2, 4 and 8% and a volume of 100 µl of each concentration was added to 100 µl of *S. mutans* culture to make the final concentrations at 0.5, 1, 2 and 4%. The microtitre plate was incubated in an atmosphere of 5% CO₂ /95% air at 37°C for 24 h and biofilms were measured using crystal violet biofilm assay.

3.3.2 Screening for anti-biofilm activity in the metagenomic library

A total of 6,624 metagenomic clones were screened for *S. mutans* anti-biofilm activity. Biofilm formation by *S. mutans* was conducted in a 96-well microtitre plate by adding 100 µl of *S. mutans* in TH broth supplemented with 4% sucrose. To determine the anti-biofilm activity of metagenomic clones, 100 µl of supernatant of overnight culture of metagenomic clones in LB broth was centrifuged at 1,500 x g for 10 min. Polymyxin B at a concentration of 30 µg/ml was added to inhibit *E. coli* biofilm formation. The 96-well microtitre plate was incubated at 5% CO₂ /95% air at 37°C for 24 h and biofilms were measured using crystal violet biofilm assay.

3.3.3 Screening for anti-biofilm activity from an environmental isolate library

3.3.3.1 Primary screening

A total of 89 environmental bacteria from the Swab and Send project were screened for *S. mutans* anti-biofilm activity. The biofilm formation assay was conducted in a 96-well microtitre plate. One-hundred microliter of *S. mutans* suspended in TH broth

supplemented with 4% sucrose was used for biofilm formation. In the primary screening, the isolates were cultured in 200 µl of BHI broth at 37°C 150 rpm for 18 h. Then 18 h old cultures were centrifuged at 1,500 x g for 10 minutes and 100 µl of supernatant was tested for *S. mutans* anti-biofilm activity. The microtitre plates were incubated in 5% CO₂ /95% air at 37°C for 24 h and biofilms were measured.

3.3.3.2 Secondary screening

The environmental isolates exhibiting anti-biofilm activity from the primary screening were grown in 4 ml of BHI broth at 37°C 200 rpm for 18 h. Overnight cultures were centrifuged at 4,500 x g for 10 minutes at 4°C. Their supernatants were filtered using a 0.22 µm filter. The 0.22 µm filtered supernatants were tested for anti-biofilm activity.

3.3.3.3 Production of a compound (s) exhibiting biofilm detaching activity over time during growth in BHI medium

Isolates with biofilm detaching activity were cultured on BHI agar for 18 h and a single colony of each isolates was inoculated in 4 ml BHI broth. Then, they were incubated at 37 °C for 18 h with shaking at 200 rpm. The 18 h old bacterial cultures were inoculated into fresh 20 ml BHI broth at a final OD_{600nm} equal to 0.05 and incubated at 37 °C for 18 h with shaking at 200 rpm. Bacterial cultures were sampled at 5, 8, 24, 48, 72, and 96 h incubation times. The bacterial cultures were measured at OD_{600nm} and tested for biofilm detaching activity.

3.3.3.4 Production of biofilm detaching activity over time during growth in M9 minimal medium

Isolates with biofilm detaching activity were cultured on BHI agar for 18 h and a single colony of each isolate was inoculated in 4 ml BHI broth. Then they were incubated at

37 °C for 18 h with shaking at 200 rpm. The 18 h bacterial cultures were inoculated into 20 ml of fresh M9 medium to an initial OD_{600nm} of 0.05. The M9 was supplemented with one of the carbon sources: 1% glycerol, 1% sucrose, 1% sorbitol, 1% mannitol, and 0.5% methyl cellulose and incubated at 37 °C for 18 h with shaking at 200 rpm. Bacterial cultures were sampled at 5, 8, 24, 48, 72, and 96 h incubation time. The samples were measured at OD_{600nm} and tested for biofilm detaching activity.

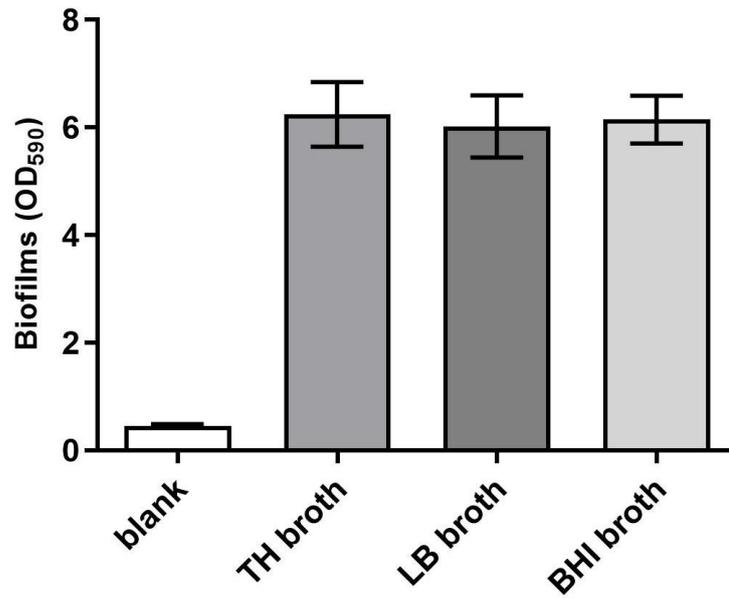
3.4 Results

3.4.1 Development of biofilm assay

3.4.1.1 Effect of growth medium on *S. mutans* biofilm formation

To develop the biofilm formation assay, *S. mutans* was grown in TH broth supplemented with sucrose at a final concentration of 2%. After 24 h, OD_{590nm} of biofilms with planktonic cells in TH broth alone was 0.467 ± 0.023 (mean \pm SD), and the OD_{590nm} of biofilms diluted 1:10 dilution was 0.642 ± 0.063 (mean \pm SD) (Figure 3.2). To screen for anti-biofilm activity, *S. mutans* culture in TH broth were cultured in the presence of one of two different media: LB broth for the metagenomic library, and BHI broth for the environmental isolate library. The OD_{590nm} of biofilms with planktonic cells cultured in LB and BHI were 0.394 ± 0.026 (mean \pm SD) and 0.490 ± 0.020 (mean \pm SD), respectively. The OD_{590nm} of biofilms diluted 1:10 dilution in the presence of LB was 0.602 ± 0.058 (mean \pm SD) and BHI was 0.662 ± 0.047 (mean \pm SD), respectively.

A.



B.

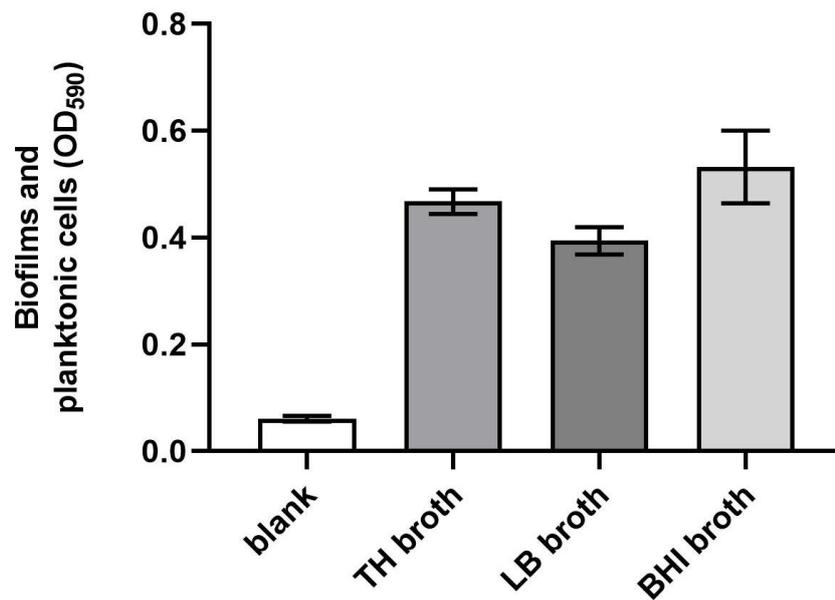


Figure 3.2 Reproducibility of *S. mutans* biofilm formation in three different media; TH broth, TH broth supplemented with LB broth and TH broth supplemented with BHI broth (A = biofilms, B = biofilms with planktonic cells.). The results are expressed as the mean \pm standard deviation of three independent experiments.

3.4.1.2 Effect of *E. coli* EPI300 containing pCC1BAC vector on the biofilm formation by *S. mutans*

After culturing *E. coli* EPI300 containing pCC1BAC vector in a 96-well microtitre plate, three samples were added to *S. mutans* biofilm assays: (i) *E. coli* whole cell cultures, (ii) supernatants of *E. coli* whole cell culture which could still have some *E. coli* cells left, and (iii) 0.22 μm filtered supernatants. *S. mutans* formed biofilms in the presence of these three samples. The results in Figure 3.3 show that no significant difference was observed for biofilm formation by *S. mutans* in the presence of these three samples. However, in the absence of *S. mutans*, biofilms were also observed from wells containing *E. coli* whole cell culture and supernatant.

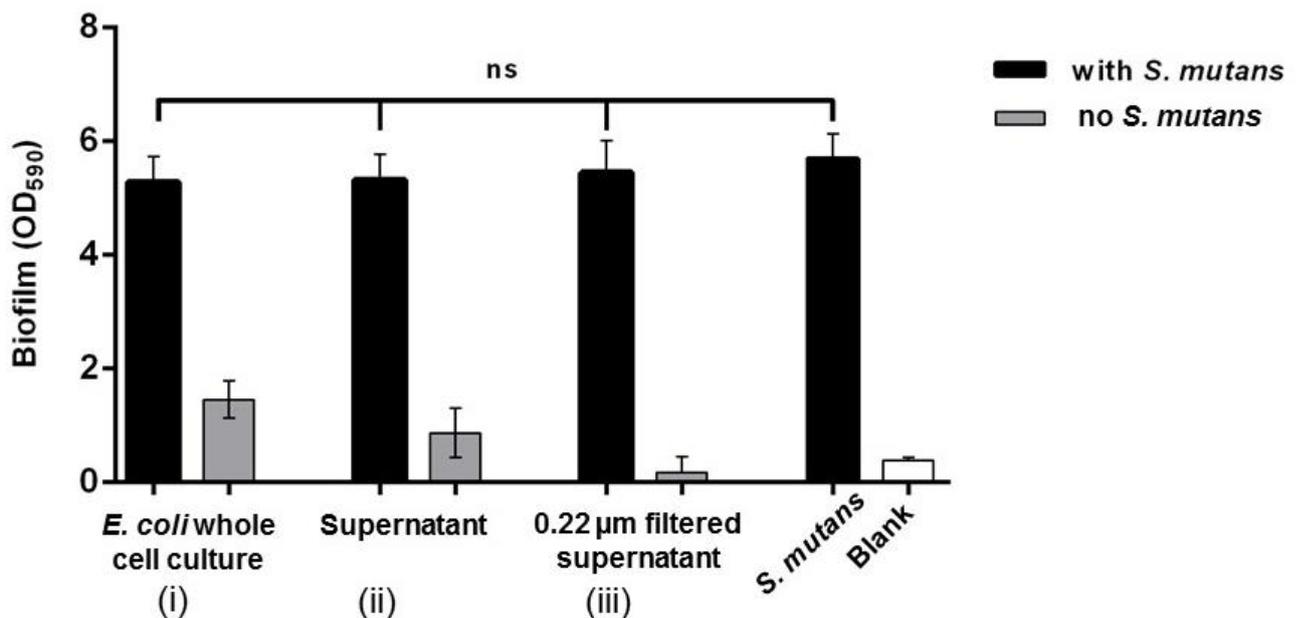


Figure 3.3 Effect of *E. coli* EPI300 containing pCC1BAC vector on the biofilm formation by *S. mutans*. *S. mutans* formed biofilms in the presence of (i) *E. coli* whole cell culture, (ii) supernatant of *E. coli* and (iii) 0.22 μm filtered supernatants (dark). The ability of these three samples to form biofilms in the absence of *S. mutans* was also determined (grey). The results were analysed by one-way-ANOVA, Bonferroni post hoc test ($p < 0.05$), and expressed as the mean \pm standard deviation of three independent experiments.

3.4.1.3 Determination of the MIC of polymyxin B against *E. coli* and *S. mutans* in biofilm assays

Figure 3.4 shows that *E. coli* EPI300 containing pCC1BAC vector or its supernatant could form biofilms under the conditions used in the *S. mutans* biofilm assay. To inhibit *E. coli* forming biofilms, polymyxin B, an antibiotic compound against Gram-negative bacteria, was selected to inhibit *E. coli* growth during *S. mutans* biofilm formation. To determine the concentration of polymyxin B that could completely inhibit *E. coli* biofilm formation, different concentrations of polymyxin B from 0 to 120 µg/ml were tested. Figure 3.4 shows the relationship between polymyxin B concentration and the amount of biofilm formed and indicates that 25 and 30 µg/ml of polymyxin B could completely inhibit biofilms formed by *E. coli* and had no effect on the biofilms formed by *S. mutans* on its own, or in combination with *E. coli*.

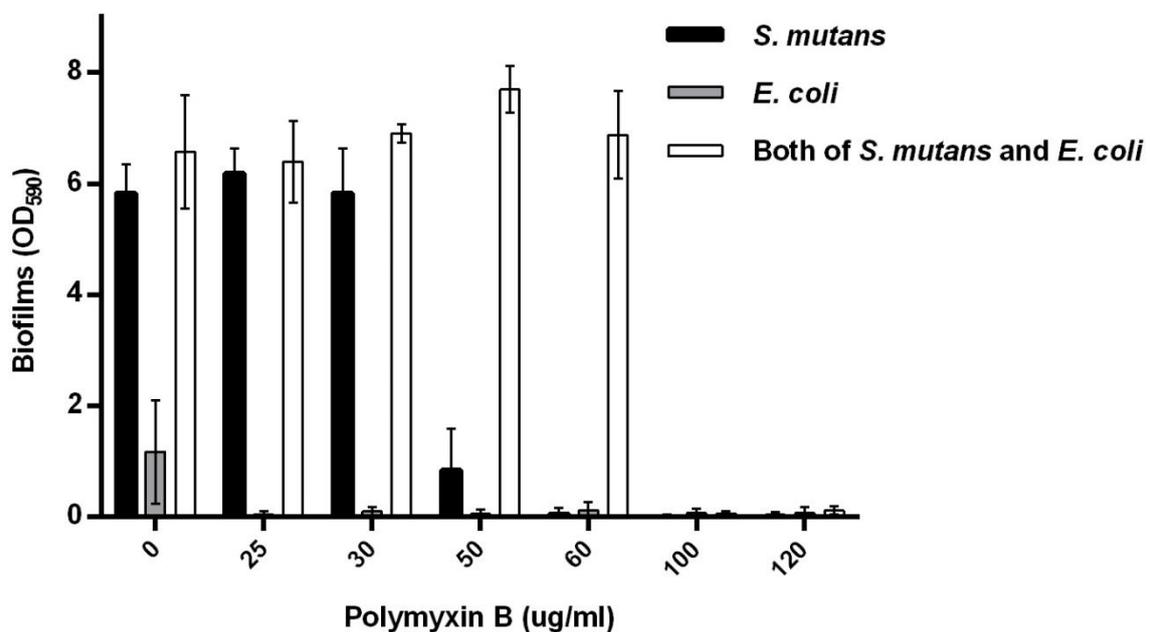


Figure 3.4 Effect of polymyxin B on biofilms in three conditions (1) *S. mutans* (black), (2) *E. coli* (grey), and (3) *S. mutans* and *E. coli* (white). The results were expressed as the mean ± standard deviation of three independent experiments.

3.4.1.4 Effect of sucrose on *S. mutans* biofilm formation

S. mutans formed biofilms in the presence of sucrose. The results from three biological replicates are shown that *S. mutans* produced maximum biofilm at 0.5 - 2% sucrose (as shown in Figure 3.5).

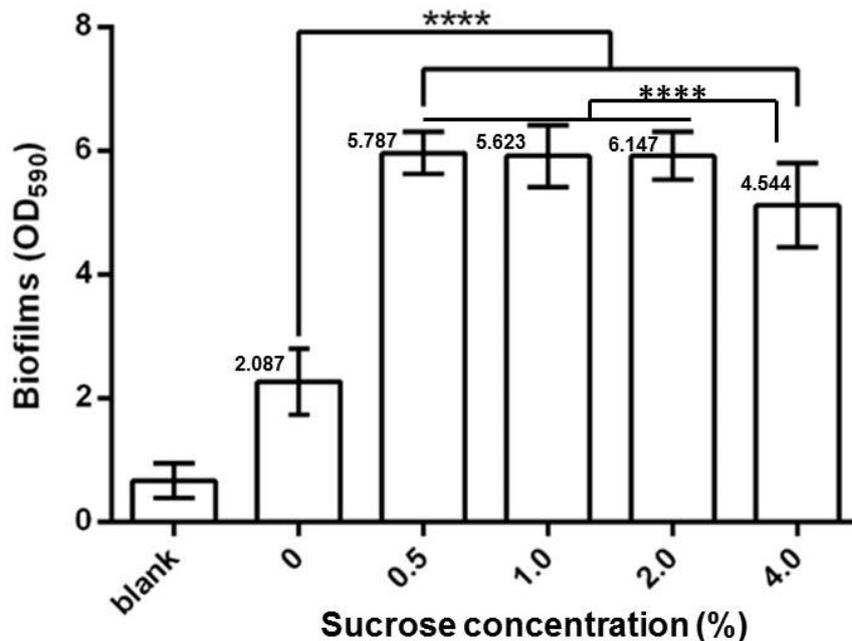


Figure 3.5 Biofilm formation in the presence of different sucrose concentrations. The results were analysed by one-way-ANOVA, Bonferroni post hoc test **** ($p < 0.0001$), and expressed as the mean \pm standard deviation of three independent experiments.

3.4.1.5 *S. mutans* biofilm formation over a period of 24 h

To monitor biofilm formation by *S. mutans* over a period of 24 h, *S. mutans* was cultured in TH broth supplemented with 2% sucrose and biofilm formation measured in a microtitre plate. The amount of biofilms and the OD_{600nm} of biofilm with planktonic cells are shown in the Figure 3.6. The amount of biofilms was steady between 0 h and 4 h incubation time and increased between 5 h and 8 h when the biofilms reached the highest OD_{590nm} value of approximately 0.6. The OD_{590nm} of biofilms remained in a range of 0.5 to 0.6 up until 24 h incubation time.

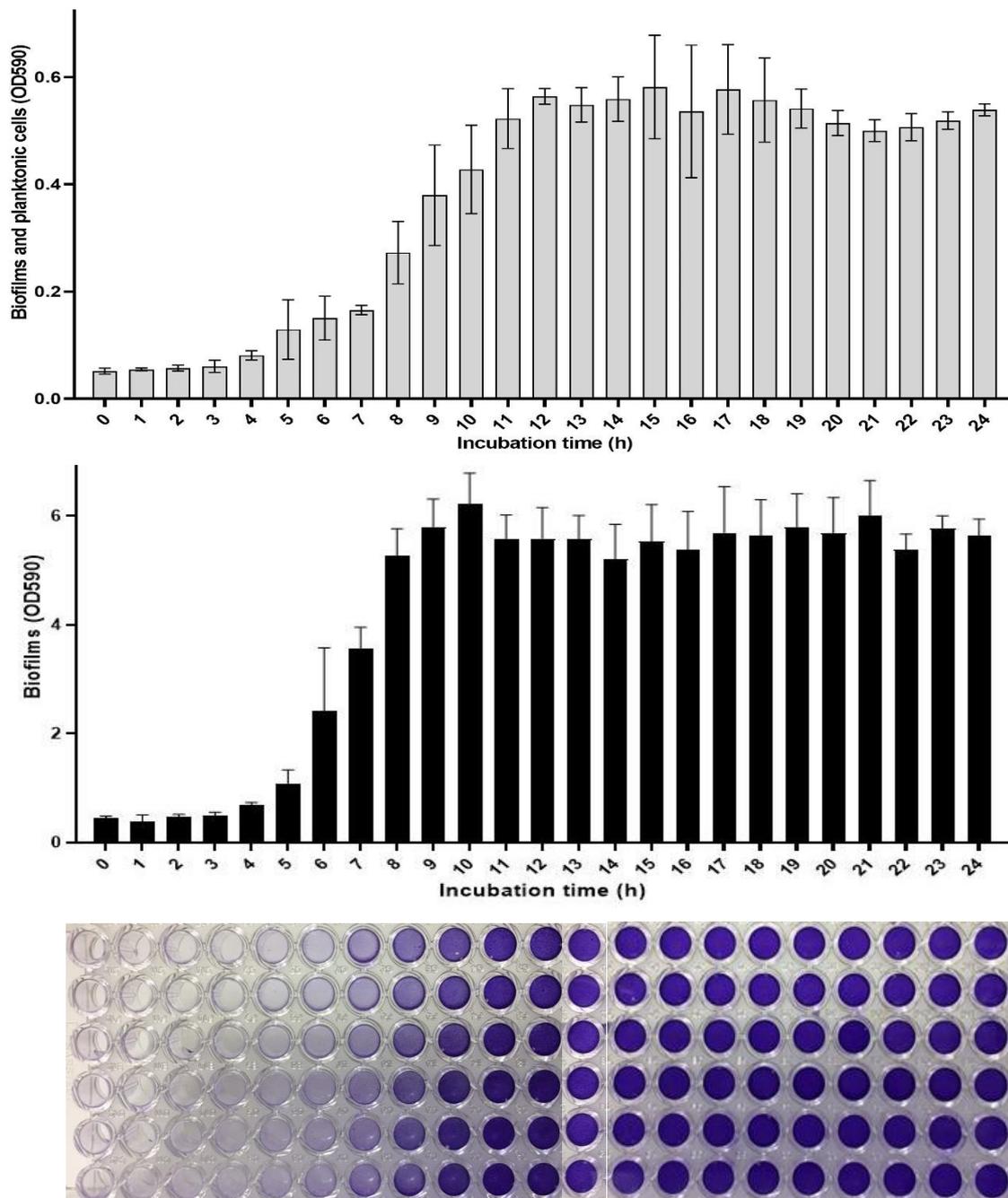


Figure 3.6 Biofilm formation by *S. mutans* over a period of 24 h *S. mutans* formed biofilms over a period of 24 h. Biofilms and biofilms with planktonic cells were measured. Biofilms are shown as 1:10 dilution (black) and biofilms with planktonic cells suspension (grey). The results are expressed as the mean \pm standard deviation of three independent experiments.

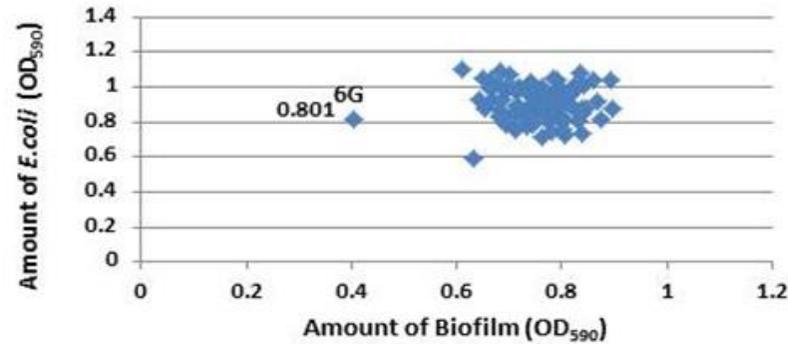
3.4.2 Screening for anti-biofilm activity from metagenomic library

Anti-biofilm activity of the metagenomic clones was screened and analysed by scatter plot (MS Excel 2010) and heatmap analysis, using a program developed by Dr

Thanapong Intara, University College London (unpublished). An example of the analysis of 96 metagenomic clones in plate number W5 is shown in Figure 3.7. The scatter plot shows the distribution of the relationship between OD_{590nm} of *S. mutans* biofilms and the OD_{590nm} to which the *E. coli* of each metagenomic clone grew. In Figure 3.7 A, 96 dots in the graph represented the relationship between *S. mutans* biofilms (X-axis) and *E. coli* overnight culture (OD_{590nm}) (Y-axis) of 96 metagenomic clones. The majority of the distribution was in the range of *S. mutans* biofilms from 0.6 to 0.9 and in the range of *E. coli* overnight culture from 0.7 to 1.1. The lowest *S. mutans* biofilm was caused by clone 6G. In addition, the clone 6G (yellow highlighted in the numerical plate) could be identified using heatmap analysis (as shown in Figure 3.7B). The Heatmap represents 96 data points in different tones of colour depending on the OD_{590nm} of either *E. coli* growth or *S. mutans* planktonic and biofilm cells or *S. mutans* biofilms as shown in Figure 3.7 B, C and D. Although supernatant from the clone 6G reduced *S. mutans* biofilm, it also reduced *S. mutans* biofilms with planktonic cells optical density (as shown in Figure 3.7 C). These results suggested that the clone 6G produced a compound exhibiting *S. mutans* growth inhibitory activity. In the anti-biofilm activity screening, a total of 6,624 metagenomic clones were analysed and no specific anti-biofilm activity was identified.

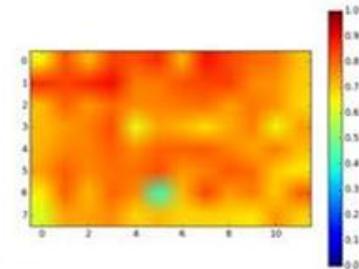
W5

A. Scatter plot



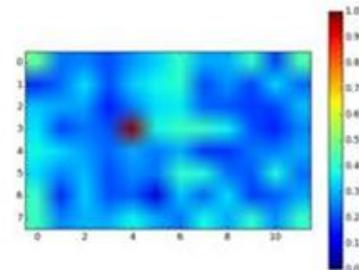
B. Biofilm

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|
| A | 0.65 | 0.821 | 0.714 | 0.843 | 0.824 | 0.861 | 0.719 | 0.892 | 0.835 | 0.797 | 0.784 | 0.702 |
| B | 0.867 | 0.838 | 0.873 | 0.897 | 0.779 | 0.779 | 0.828 | 0.838 | 0.846 | 0.763 | 0.751 | 0.692 |
| C | 0.708 | 0.806 | 0.746 | 0.8 | 0.765 | 0.773 | 0.784 | 0.795 | 0.739 | 0.787 | 0.757 | 0.681 |
| D | 0.714 | 0.745 | 0.774 | 0.83 | 0.632 | 0.742 | 0.717 | 0.668 | 0.729 | 0.776 | 0.642 | 0.745 |
| E | 0.72 | 0.767 | 0.771 | 0.807 | 0.762 | 0.802 | 0.807 | 0.815 | 0.771 | 0.733 | 0.779 | 0.709 |
| F | 0.742 | 0.812 | 0.762 | 0.79 | 0.79 | 0.827 | 0.749 | 0.77 | 0.817 | 0.794 | 0.693 | 0.663 |
| G | 0.655 | 0.805 | 0.713 | 0.802 | 0.762 | 0.405 | 0.712 | 0.838 | 0.743 | 0.78 | 0.695 | 0.809 |
| H | 0.611 | 0.738 | 0.735 | 0.76 | 0.684 | 0.716 | 0.682 | 0.685 | 0.682 | 0.663 | 0.751 | 0.684 |



C. Biofilm and planktonic cell

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|
| A | 0.472 | 0.241 | 0.257 | 0.2 | 0.274 | 0.33 | 0.411 | 0.292 | 0.294 | 0.412 | 0.193 | 0.44 |
| B | 0.169 | 0.227 | 0.351 | 0.203 | 0.328 | 0.368 | 0.4 | 0.199 | 0.27 | 0.194 | 0.338 | 0.23 |
| C | 0.32 | 0.291 | 0.284 | 0.149 | 0.248 | 0.346 | 0.356 | 0.226 | 0.208 | 0.18 | 0.194 | 0.309 |
| D | 0.351 | 0.19 | 0.24 | 0.209 | 0.978 | 0.369 | 0.418 | 0.416 | 0.365 | 0.204 | 0.156 | 0.254 |
| E | 0.369 | 0.342 | 0.281 | 0.228 | 0.293 | 0.245 | 0.294 | 0.173 | 0.173 | 0.246 | 0.247 | 0.293 |
| F | 0.34 | 0.257 | 0.312 | 0.198 | 0.262 | 0.24 | 0.408 | 0.394 | 0.235 | 0.208 | 0.384 | 0.238 |
| G | 0.416 | 0.167 | 0.331 | 0.2 | 0.22 | 0.085 | 0.32 | 0.223 | 0.345 | 0.187 | 0.215 | 0.287 |
| H | 0.417 | 0.257 | 0.302 | 0.29 | 0.365 | 0.29 | 0.245 | 0.374 | 0.291 | 0.399 | 0.266 | 0.408 |



D. E. coli

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|
| A | 1.042 | 0.973 | 0.981 | 0.996 | 0.999 | 1.034 | 0.974 | 1.031 | 1.071 | 0.981 | 1.044 | 1.062 |
| B | 0.908 | 0.867 | 0.81 | 0.864 | 0.737 | 0.872 | 0.859 | 0.841 | 0.867 | 0.877 | 0.896 | 0.976 |
| C | 0.836 | 0.903 | 0.771 | 0.791 | 0.89 | 0.799 | 0.817 | 0.823 | 0.851 | 0.861 | 0.874 | 1.004 |
| D | 0.966 | 0.787 | 0.904 | 0.799 | 0.578 | 0.89 | 0.887 | 0.922 | 0.813 | 0.943 | 0.922 | 0.999 |
| E | 0.992 | 0.872 | 0.92 | 0.941 | 0.89 | 0.74 | 0.808 | 0.889 | 0.913 | 0.969 | 0.905 | 0.981 |
| F | 1.019 | 0.942 | 0.986 | 1.027 | 0.971 | 0.978 | 0.959 | 0.961 | 0.969 | 0.915 | 0.966 | 0.976 |
| G | 0.865 | 0.719 | 0.74 | 0.8 | 0.708 | 0.801 | 0.769 | 0.726 | 0.815 | 0.842 | 0.773 | 0.949 |
| H | 1.086 | 0.948 | 0.767 | 0.871 | 0.857 | 0.88 | 0.82 | 0.92 | 0.824 | 1.034 | 0.968 | 1.082 |

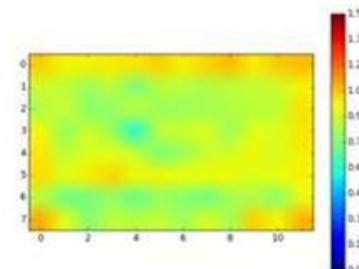


Figure 3.7 Analysis of 96 metagenomic clones from plate number W5. Data distribution scatter graph (A) and heat map analysis of biofilm (B), biofilms and planktonic cells (C) and *E. coli* growth (D). This example of metagenomic clone 6G (highlighted yellow) shows less biofilm was formed by *S. mutans* (B), but *S. mutans* growth was also inhibited (C). There was normal growth of the *E. coli* clone (D).

3.4.3 Screening for anti-biofilm activity from the environmental isolate library

3.4.3.1 Primary screening

Bacteria were isolated from environmental samples of the Swab and Send project by Dr Liam Reynolds (University College London). It was hypothesised that some environmental bacteria may produce a compound (s) which exhibit *S. mutans* anti-biofilm activity. To test the hypothesis, 89 isolates were cultured in 200 µl of BHI broth in a microtitre plate and centrifuged at 1,500 x g for 15 min. Supernatants were added to the biofilm assay. Ten supernatants were found to exhibit anti-biofilm activity, they came from isolates 3C, 6E, 8E, 9C, 9E, 9H, 10G, 11E, 12F and 12G.

3.4.3.2 Secondary screening

The ten isolates from the primary screening were cultured in 4 ml of BHI broth and 18 h bacterial cultures were centrifuged, filtered using a 0.22 µm filter and tested for anti-biofilm activity. In Figure 3.8, seven out of ten supernatants continued to exhibit anti-biofilm activity in the secondary screen. These seven isolates were isolates 3C, 6E, 8E, 9C, 9E 10G and 11E. These filtered supernatants allowed *S. mutans* to growth and form biofilms but they caused the biofilms to easily detached during gentle removal of planktonic cells using PBS. Thus, the activity was termed biofilm detaching activity.

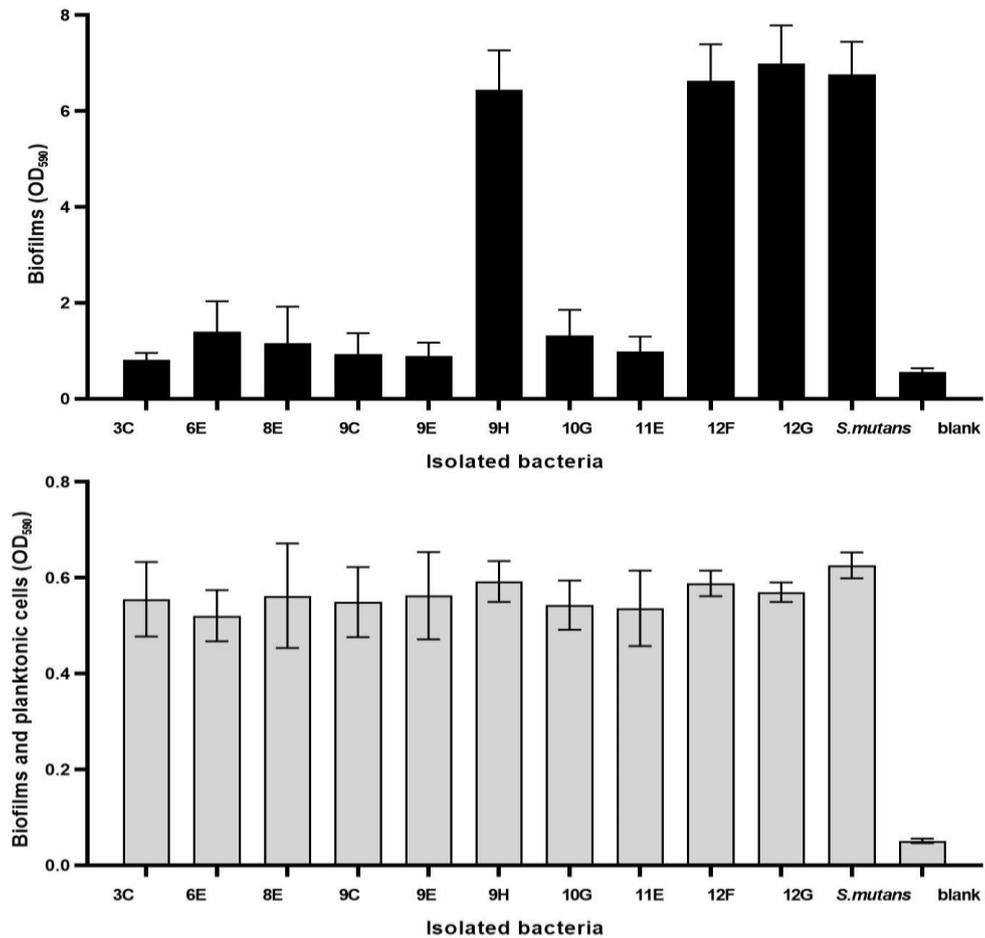


Figure 3.8 Biofilm formation by *S. mutans* in the presence of filtered supernatants. *S. mutans* formed biofilms in the presence of 0.22 μm filtered supernatants from ten isolates. Biofilms with planktonic cells (white) and biofilms (1:10 dilution) (black) were measured at OD_{590nm}. Results are expressed as the mean ± standard deviation of three independent experiments.



Figure 3.9 *S. mutans* biofilm detaching activity. After *S. mutans* was incubated for 24 h, biofilms formed at the bottom of microtitre plate well. This figure shows the biofilms after removing planktonic cells and washing the biofilms gently by adding PBS. *S. mutans* biofilm formed in TH broth (i), TH broth without *S. mutans* (ii), *S. mutans* biofilm formed in the presence of 6E-filtered supernatant showing the biofilm detaching activity (iii) and (iv).

3.4.3.3 Classification of isolates exhibiting biofilm detaching activity

To classify the seven isolates exhibiting biofilm detaching activity, the 16S rRNA gene of the seven isolates was amplified covering nine hypervariable regions (V1-V9) and sequenced. The amplicon size of 16S rRNA genes was between 1,289 bp and 1,365 bp long. Megablast analysis showed that the 16S rRNA gene sequences from the seven isolates were highly similar to many *Bacillus* species as shown in the Table 3.1.

Table 3.1 BLAST results for seven isolates producing *S. mutans* biofilm detaching activity

| Isolate | Size (bp) | Possible bacteria | Cover (%) | Identity (%) |
|---------|-----------|---|-----------|--------------|
| 3C | 1313 | <i>B. amyloliquefaciens</i> strain JK6, BG2, SN-9, HD 34, B19a, B9, B3, A30, A28, A12a, A9a, RJ2 <i>B. subtilis</i> strain YB-6, NW-4, SBB16 <i>B. methylotrophicus</i> strain Bacteria V, JJ-D34 <i>Bacillus sp.</i> M5(2015), M4(2015), AMARUN1, FJAT-25761, LB002, DY26-014, DY26-020, YJ11-7, YJ11-1-2, JJ-D16, JJ-D8 | 100 | 100 |
| 6E | 1347 | <i>B. methylotrophicus</i> strain VD18S15, JS25R, HQB246 <i>B. amyloliquefaciens</i> strain MKB04, w-2, Ab-525 <i>B. subtilis</i> strain B-065, BA-013, WX1, IHB B 1516, xw-2, CD-3 <i>Bacillus sp.</i> CBE 330AAn.1, XY3, DB14312, w-1(2014), CZB2, SE16, SE15,cs-2, BIHB 332, INBio 4503E Bacterium HJQ-4 | 100 | 100 |
| 8E | 1351 | <i>B. amyloliquefaciens</i> strain JK6, BG2, SN-9, B19a, B9, B3, A30, A28, A12a, A9a, RJ2, VD21R27, VD18S27, VX4S18, VX4S09 <i>B. subtilis</i> strain YB-6, NW-4, SBB16, Ku <i>B. methylotrophicus</i> strain Bacteria V, JJ-D34 <i>Bacillus sp.</i> AMARUN1, FJAT-25761, LB002, DY26-014, DY26-020, YJ11-7, YJ11-1-2, JJ-D16, JJ-D8 | 100 | 100 |
| 9C | 1314 | <i>Bacillus sp.</i> FJAT-25761, AMARUN1, LB002, DY26-014, DY26-020, YJ11-7, YJ11-1-2, JJ-D16, JJ-D8 <i>B. amyloliquefaciens</i> strain BG2, SN-9, CanL-23, B19a, B9, B3, A30, A28, A12a, A9a, RJ2, VD21R27, VD18S27, VX4S18, VX4S09, VX2S02 <i>B. subtilis</i> strain YB-6, NW-4, SBB16, Ku <i>B. methylotrophicus</i> strain Bacteria V, JJ-D34 | 100 | 100 |
| 9E | 1339 | <i>Bacillus sp.</i> FJAT-25761, LB002, DY26-014, DY26-020, YJ11-7, YJ11-1-2, JJ-D16, JJ-D8 <i>B. amyloliquefaciens</i> strain BG2, SN-9, B19a, B9, B3, A30, A28, A12a, A9a, RJ2, VD21R27, VD18S27, VX4S18, VX4S09, VX2S02 <i>B. subtilis</i> strain YB-6, NW-4, SBB16, Ku <i>B. methylotrophicus</i> strain Bacteria V, JJ-D34 | 100 | 100 |

| | | | | |
|-----|------|--|-----|-----|
| 10G | 1365 | B. amyloliquefaciens strain 213, 50, S2S95, 157,ZZ32, MKB04, w-2, Ab-525 B. subtilis strain CS1.03 B. siamensis strain LGB11,LGB1, LGB7, LGB8, LGB9, B. velezensis strain PR-2, CG-2, SCGB1, SCDB291, JS14C, JS25R B. methylotrophicus strain CL12, VD18S15 Bacillus sp. IP50, IP23, Ant-2a, CC-CY916, CBE 330AAn.1, DB14312, w-1(2014) | 100 | 100 |
| 11E | 1289 | B. methylotrophicus strain VD18S15, JS25R B. amyloliquefaciens strain MKB04, w-2, Ab-525 B. amyloliquefaciens subsp. Plantarum strain SOW2b-63 B. subtilis stain B-065, BA-013, WX1, IHB B 1516, xw-2, CD-3, TPSB6 Bacillus sp. CBE 330AAn.1, XY3, DB14312, w-1(2014), CZB2, SE16, SE15,cs-2, cna9, BIHB 332, INBio 4503E Bacterium HJQ-4 | 100 | 100 |

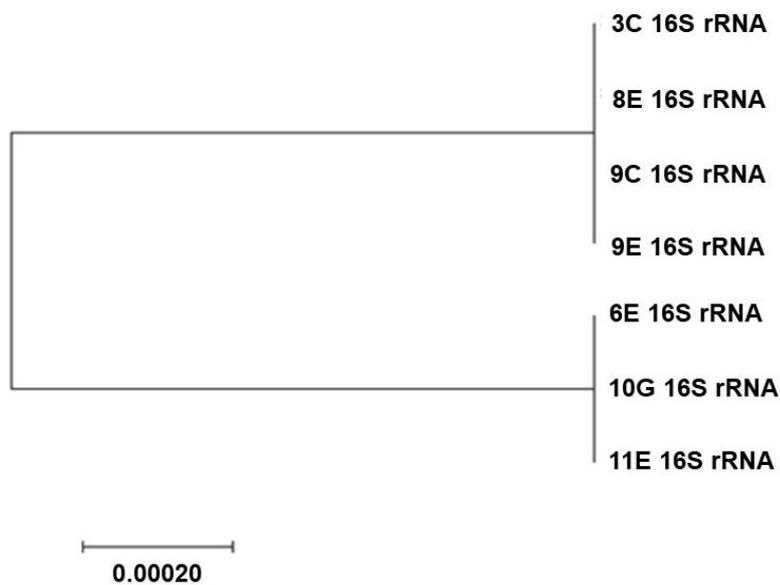
The 16S rRNA gene sequences of seven isolates were analysed using MEGA version 7.0.14. The phylogenetic tree of seven isolates indicated that they could be divided into two groups (as shown in Figure 3.10a).

However, 16S rRNA genes of *Bacillus* spp. shows a high similarity. Therefore using 16S rRNA might not be the best way to classify these bacteria producing anti-biofilm activity (Wang *et al.*, 2007). Thus, whole genome sequences were used for bacterial identification.

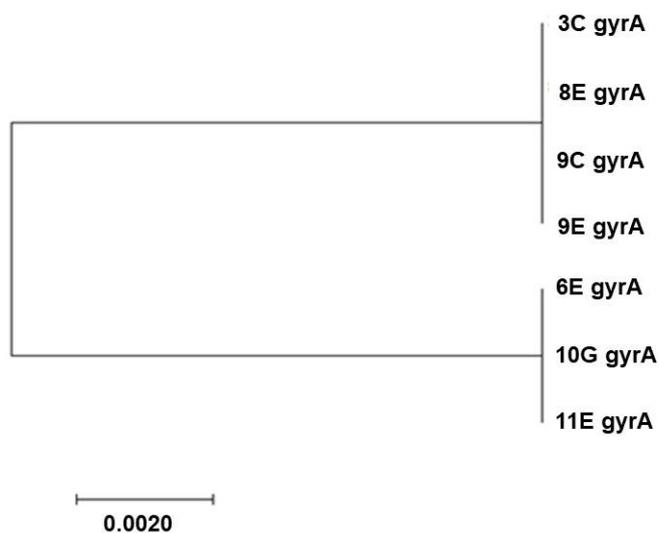
Figure 3.10 shows the phylogenetic tree of 16S rRNA (a), *gyrA* (b), and *rpoB* (c) gene sequences of seven isolates analyzed by the Neighbour-Joining method of MEGA 7.0.14. All phylogenetic trees showed similar results, with the seven isolates dividing into two groups. The first group was 3C, 8E, 9C, and 9E, and second group was 6E, 10G, and 11E. However, the phylogenetic tree of 16S rRNA gene sequences had the

smallest scale of evolutionary distance (0.00020) compared to the sequences of *gyrA* gene (0.0020) and *rpoB* gene (0.0010). Sequences of the 16S rRNA gene of seven isolates were analysed for evolutionary relationship with some closely related *Bacillus* spp. The phylogenetic tree of seven isolates and some *Bacillus* spp. is shown in Figure 3.11.

a. 16S rRNA gene sequences



b. *gyrA* (gyrase A) gene sequences



c. *rpo* β (RNA polymerase β subunit) gene sequences

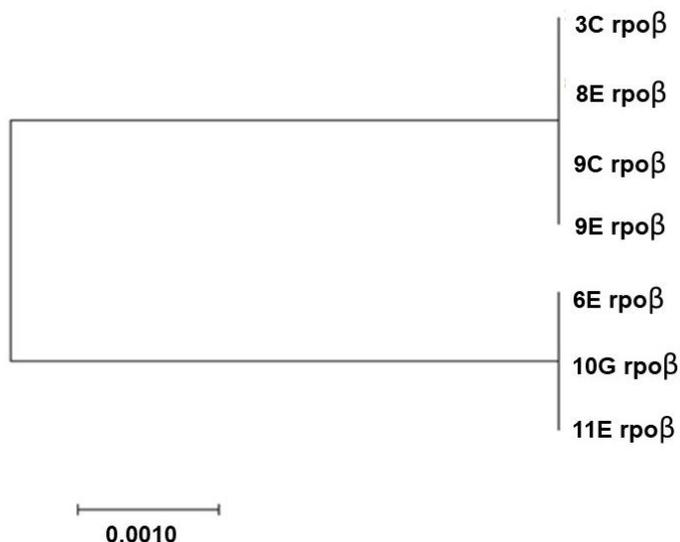


Figure 3.10 Phylogenetic trees using 16S rRNA (a.), *gyrA* (b.), and *rpo* β (c.) gene sequences of seven isolates analyzed by the Neighbour-Joining method of MEGA version 7.0.14.

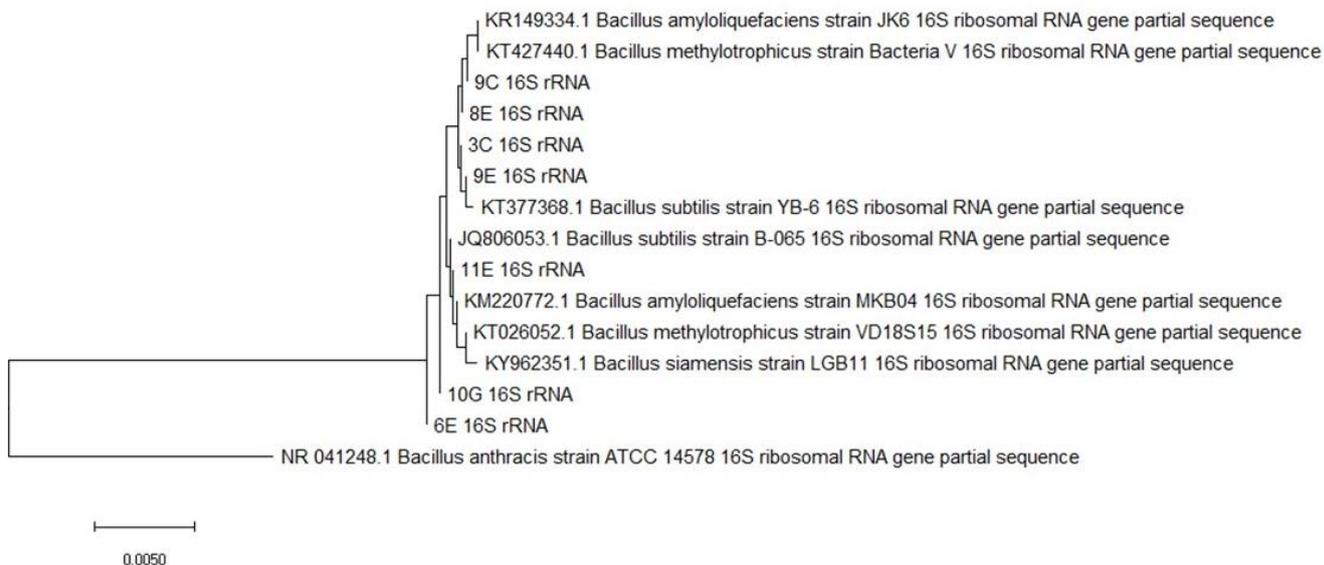


Figure 3.11 The phylogenetic tree of 16S rRNA gene sequences of 7 isolates and *Bacillus* spp. analyzed by the Neighbour-Joining method of MEGAX version 7.0.14.

A single colony of the seven isolates were submitted to MicrobesNG (Birmingham, UK). Whole genome sequencing was performed on the Illumina Miseq platform and contigs were assembled using SPAdes. General properties of genomes of seven isolates are summarised in Table 3.2.

Table 3.2 Metadata for seven isolates producing a compound (s) exhibiting biofilm detaching activity

| Isolate name | Source | Total length (bp) | No. of contigs | Largest contig (bp) | N ₅₀ | G+C content (%) | Most frequent Family (%) | Most frequent Family | Most frequent Species (%) | Most frequent Species |
|--------------|---|-------------------|----------------|---------------------|-----------------|-----------------|--------------------------|----------------------|---------------------------|----------------------------------|
| 3C | Cats claws, teeth, mouth and paws | 4,102,777 | 69 | 469,884 | 262,849 | 46.32 | 98.42 | <i>Bacillaceae</i> | 63.21 | <i>Bacillus methylotrophicus</i> |
| 6E | Bedford station machine touchscreen | 3,984,077 | 53 | 976,586 | 527,016 | 46.30 | 98.12 | <i>Bacillaceae</i> | 33.48 | <i>Bacillus methylotrophicus</i> |
| 8E | Ice skating boots | 4,100,871 | 62 | 820,921 | 262,849 | 46.32 | 98.23 | <i>Bacillaceae</i> | 60.72 | <i>Bacillus methylotrophicus</i> |
| 9C | Ice skating boots | 4,099,026 | 59 | 820,921 | 262,849 | 46.31 | 98.04 | <i>Bacillaceae</i> | 59.28 | <i>Bacillus methylotrophicus</i> |
| 9E | Hot water bath 50°C in a laboratory | 4,116,474 | 90 | 820,921 | 262,639 | 46.30 | 97.90 | <i>Bacillaceae</i> | 59.10 | <i>Bacillus methylotrophicus</i> |
| 10G | Egyptian money of different currency values | 3,983,309 | 51 | 976,586 | 527,016 | 46.32 | 97.54 | <i>Bacillaceae</i> | 30.94 | <i>Bacillus methylotrophicus</i> |
| 11E | Compost | 4,020,633 | 109 | 976,586 | 527,016 | 46.27 | 97.78 | <i>Bacillaceae</i> | 34.97 | <i>Bacillus methylotrophicus</i> |

Remark N₅₀ is defined as the sequence length at the half of the summation of the total length of all contigs

MicrobesNG provided a clue that the seven isolates might be *B. methylotrophicus*, now reclassified as *B. velezensis* (Fan *et al.*, 2017), but the percentages of nucleotide similarity using Kraken were very low as shown in Table 3.2. Taxonomy identification of seven isolates was investigated based on their genome using Megablast against all microbial completed genomes (<http://www.ncbi.nlm.nih.gov>). The results indicated that isolate 6E, 10G and 11E were closest to *B. velezensis* strain SRCM100072 and isolate 3C, 8E, 9C and 9E were closest to *B. velezensis* strain NKG-1.

Then the genome of seven isolates were analysed by Average Nucleotide Identity using reciprocal best hit (two-way ANI) (Rodriguez-R & Konstantinidis, 2014) and DNA-to-DNA hybridization (DDH) using the Genome-to-Genome Distance Calculator (GGDC) version 2.1 (Meier-Kolthoff *et al.*, 2013) and compared to *B. velezensis* strain SRCM100072 and *B. velezensis* strain NKG-1. In Table 3.3, the results show the seven isolates all have pairwise DDH values above the recommended threshold of 70% for species delineation (Wayne *et al.*, 1987, Meier-Kolthoff *et al.*, 2013). The ANI values of isolate 3C, 8E, 9C and 9E produced the highest similar to *B. velezensis* NKG-1 (98 %) and the ANI values of isolate 6E, 10G and 11E produced the highest similar result to *B. velezensis* SRCM100072 (98 %). The recommended cut-off point of 70% DDH for species delineation corresponds to approximately 95 % ANI (Goris *et al.*, 2007) and 79% DDH for subspecies delineation. These results suggested that isolate 3C, 8E, 9C and 9E belong to the same species with the *B. velezensis* NKG-1 and isolate 6E, 10G and 11E belong to the same species with *B. velezensis* SRCM100072.

Table 3.3 Result of genome-to-genome distance comparisons and ANI values of closely related type strains from *B. velezensis*

| Reference | Query | Tools | | | | |
|--|-------|---|------------------|------------------------------|------------------------------|---------------------------------------|
| | | Genome-to-Genome Distance Calculator 2.1 Recommended Formula 2 (Identities/HSP length) | | | | ANI calculator |
| | | Distance | DDH estimate (%) | Probability (%) that DDH>70% | Probability (%) that DDH>79% | Average Nucleotide Identity (ANI) (%) |
| <i>B. velezensis</i> strain SRCM100072 | 6E | 0.0191 | 83.70 | 93.03 | 54.58 | 98.26 |
| | 10G | 0.0191 | 83.70 | 93.03 | 54.58 | 98.26 |
| | 11E | 0.0191 | 83.70 | 93.03 | 54.57 | 98.19 |
| | 3C | 0.0198 | 83.00 | 92.65 | 53.40 | 97.34 |
| | 8E | 0.0198 | 83.00 | 92.65 | 53.39 | 97.64 |
| | 9C | 0.0198 | 83.00 | 92.65 | 53.39 | 97.33 |
| | 9E | 0.0198 | 83.10 | 92.66 | 53.43 | 97.63 |
| <i>B. velezensis</i> strain NKG-1 | 6E | 0.0191 | 83.70 | 93.03 | 54.58 | 97.53 |
| | 10G | 0.0191 | 83.70 | 93.03 | 54.58 | 97.53 |
| | 11E | 0.0191 | 83.70 | 93.03 | 54.58 | 97.53 |
| | 3C | 0.0149 | 87.50 | 94.86 | 61.18 | 98.18 |
| | 8E | 0.0149 | 87.50 | 94.86 | 61.16 | 98.21 |
| | 9C | 0.0149 | 87.50 | 94.86 | 61.16 | 98.23 |
| | 9E | 0.0148 | 87.50 | 94.87 | 61.20 | 98.13 |

ANI stands for Average nucleotide identity
 DDH stands for DNA-to-DNA hybridization
 HSP stands for high-scoring segment pairs
 70% DDH is a cut-off point for species delineation
 79% DDH is a cut-off point for subspecies delineation

Phylogenomic analysis of the seven isolates compared to references genomes from four clades of the *Bacillus subtilis* group and one *Bacillus* outgroup, *B. cereus*, using the Neighbour-Joining method of MEGA 7.0.14 and Reference sequence Alignment based Phylogeny (REALPHY) 1.12 (Bertels *et al.*, 2014). The phylogenomic tree is shown in Figure 3.12. The tree suggested that the seven isolates were in clade II and they were *B. velezensis* in the *B. amyloliquefaciens* operational group.

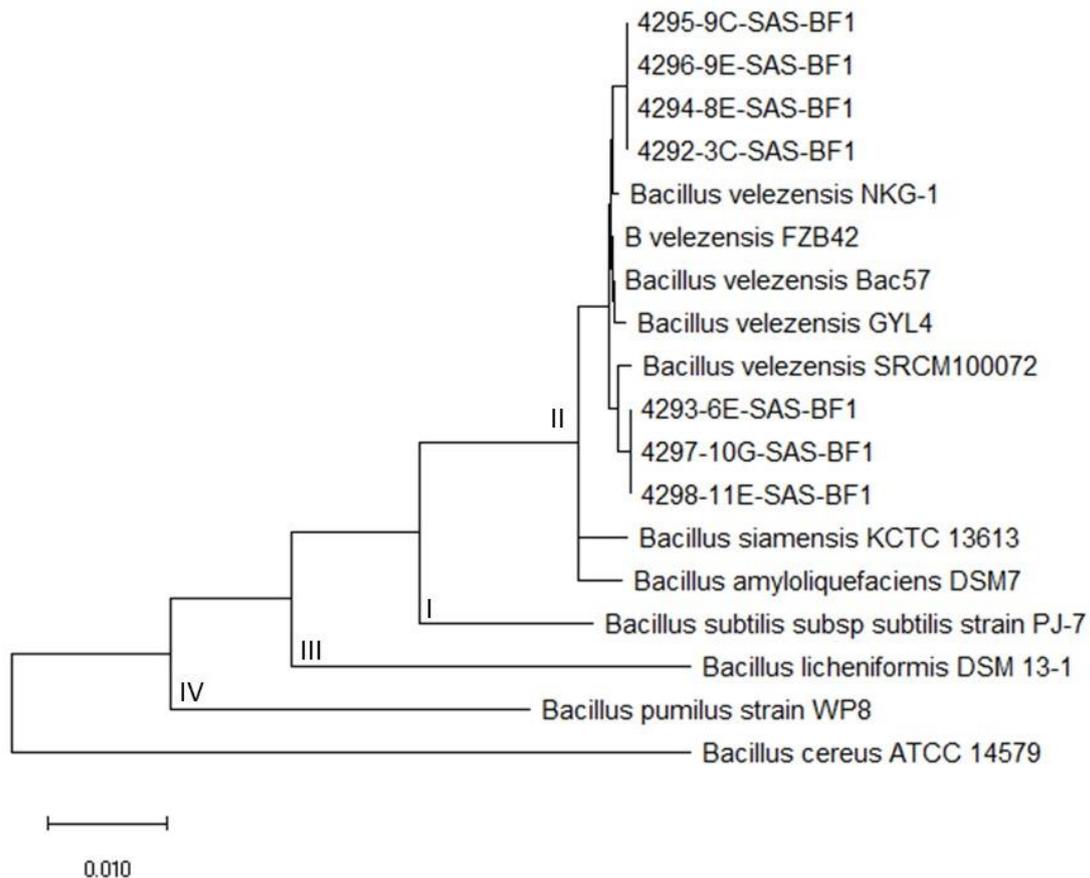


Figure 3.12 Phylogenomic Tree of seven isolates based on their genomes compared to other members of the *B. subtilis* group using Neighbour-Joining method of MEGA 7.0.14 and Reference sequence Alignment based Phylogeny (REALPHY 1.12 (Bertels *et al.*, 2014).

The genome of *B. cereus* ATCC14579 was used as outgroup. The roman letters at the branching points demonstrate the four clades of *B. subtilis* group: *subtilis* (I), *amyloliquefaciens operational group* (II), *licheniformis* (III), and *pumilus* (IV). Isolate 3C is shown as 4292-3C-SAS-BF1 and other isolates are shown as the same pattern as 3C in different initial number.

3.4.3.4 Heat stability assay

To evaluate the heat stability of compounds exhibiting biofilm detaching activity, 0.22 µm filtered supernatants of 3E, 6E, 8E, 9C, 9E, 10G, and 11E were boiled at 100°C for 15 min. The boiled- filtered supernatants were tested for biofilm detaching activity by adding 100 µl of each boiled-filtered supernatant to 100 µl of *S. mutans* cell culture. As shown in Figure 3.13, none of the boiled filtered supernatants demonstrated biofilm

detaching activity. Thus, the compounds responsible for the biofilm detaching activity in the filtered supernatants were heat sensitive at 100°C for 15 min.

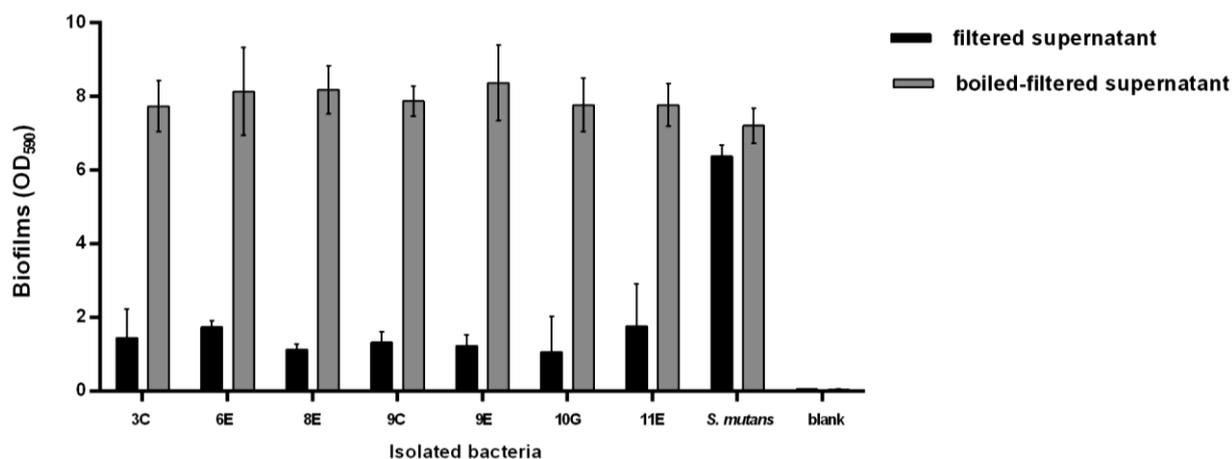


Figure 3.13 Biofilm formation by *S. mutans* in the presence of boiled filtered supernatants.

The graph shows the amount of biofilm in the presence of heat-treated supernatants from the isolates. Filtered supernatants from seven isolates were boiled at 100°C for 15 min. The boiled filtered supernatants were tested for biofilm detaching activity. The results show the mean \pm standard deviation of three independent experiments. *S. mutans* biofilms in the presence of boiled-filtered supernatants (grey), filtered supernatants (black).

3.4.3.5 Capacity of filtered supernatants exhibiting biofilm inhibitory activity to disrupt biofilms

To measure the ability of filtered supernatants to disrupt *S. mutans* biofilms, filtered supernatants were added to wells of a microtitre plate at the start of the experiment (0 h) and to other wells at 7 h and 24 h post incubation. For the 7 h and 24 h times, *S. mutans* was allowed to form biofilms for 7 h and 24 h before planktonic cells were discarded, the filtered supernatants were then added and incubated for 24 h. Figure 3.14 shows that biofilm detaching activity was detected when the filtered supernatants were added to the wells at the start of experiment (0 h) only but it was not detected when the supernatants were added at either at 7 h or 24 h post incubation. These

results suggested that a compound responsible for the biofilm detaching activity in the filter supernatants affected the *S. mutans* biofilms at the start of biofilm formation.

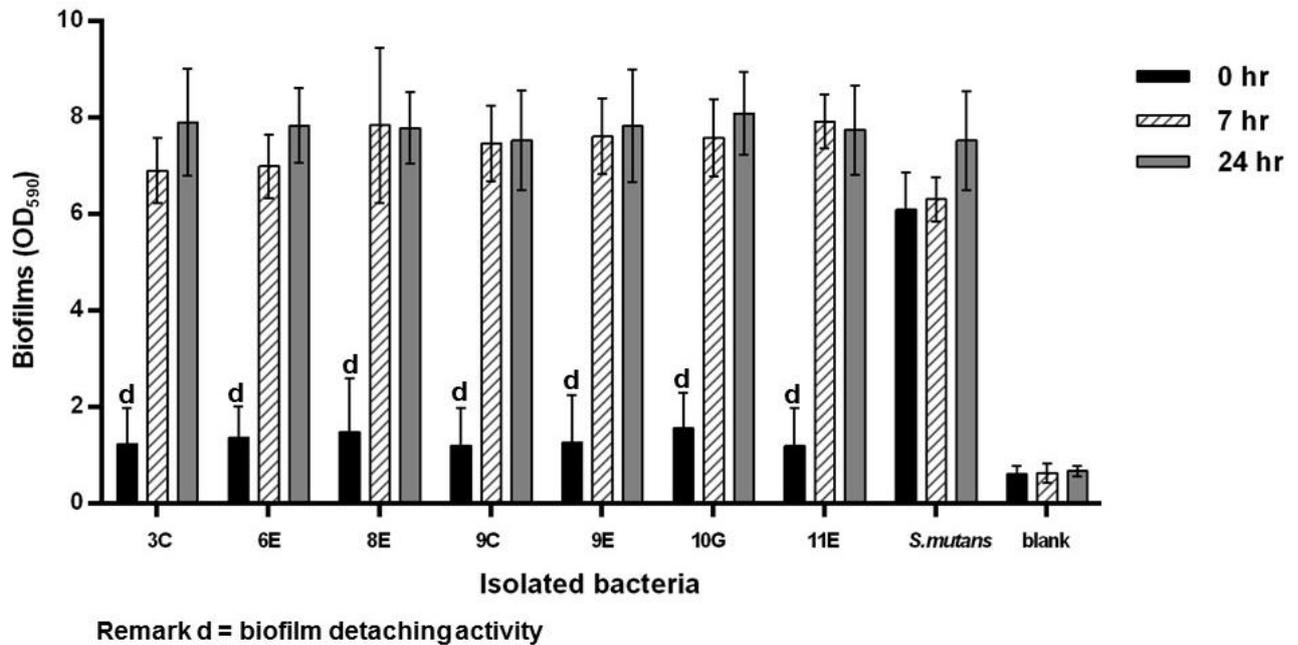


Figure 3.14 Biofilm formation by *S. mutans* after adding filtered supernatants to 7 h and 24 h old biofilms and at the start of biofilm assay (0 h) as a control group. *S. mutans* formed biofilms in microtitre plates for 7 h and 24 h. After that, planktonic cells were removed before adding the 0.22 μ m filtered supernatants from isolate 3C, 6E, 8E, 9C, 9E, 10G and 11E. Control group was *S. mutans* biofilm formation in the presence of the filtered supernatant at the start of biofilm assay (0 h). Biofilms were measured using crystal violet biofilm assay and the results are expressed as the mean \pm standard deviation of three independent experiments.

3.4.3.6 Evaluation of the ability of isolate 6E and 9C to produce biofilm detaching activity when grown in M9 medium

Seven isolates were found to produce biofilm detaching activity when cultured in BHI medium. However, BHI medium contains many biomolecules, particularly proteins. This might be an obstacle to separating and identifying a compound responsible for biofilm detaching activity in the supernatant. M9 medium is a minimal medium containing only minimal nutrients necessary for bacterial growth (1 g/L ammonium chloride, 6 g/L disodium hydrogen phosphate, 3 g/L potassium dihydrogen phosphate,

0.5 g/L sodium chloride, 2 ml/l of 1 M magnesium sulfate solution and 20 ml/l 20% solution from a carbon source). It contains identified molecules and this composition might be useful to separate compounds derived-from bacteria. Isolate 6E and 9C were chosen as representatives of phylogenetic group 2 and group 1, respectively. Isolate 6E and 9C were cultured in M9 medium supplemented with 1% glucose (M9+G). Bacterial cultures were collected at 24, 48, and 72 h and measured for biofilm detaching activity. The results shown in Figure 3.15, show that none of the filtered supernatants from M9+G demonstrated biofilm detaching activity. This result suggests that the isolates could not produce any compound exhibiting biofilm detaching activity in M+G.

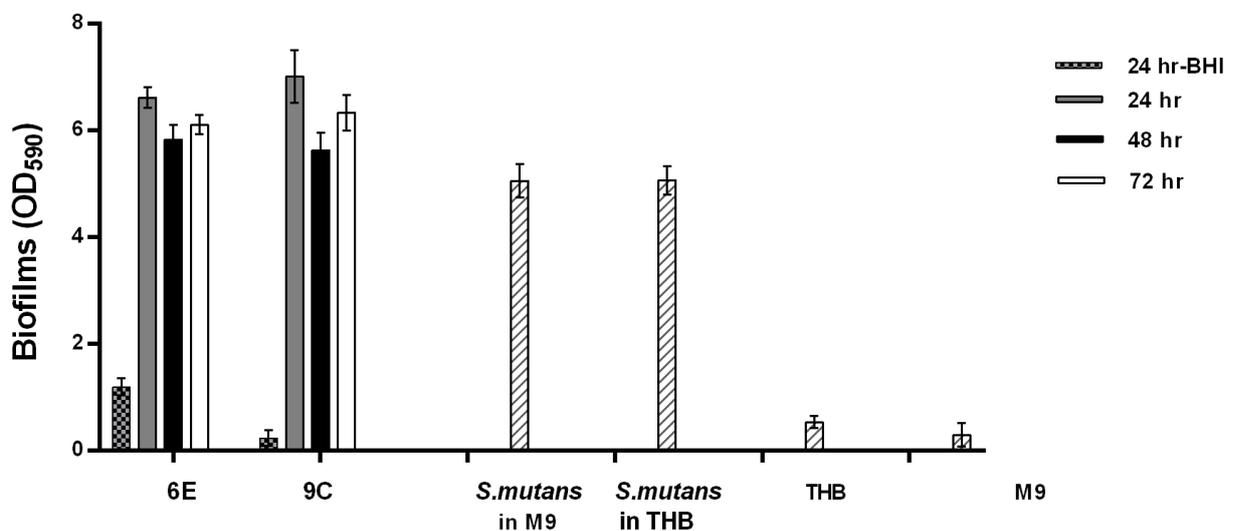


Figure 3.15 Biofilm formation by *S. mutans* in a presence of filtered supernatants when isolate 6E and 9C were grown in M9+G.

Isolate 6E and 9C were grown in M9 medium supplemented with 1% glucose. Bacterial cultures were centrifuged and their supernatants were filtered. The 0.22 μm filtered supernatants from 24 h (grey), 48 h (black), and 72 h (white) of incubation were tested for biofilm detaching activity. Positive controls of 6E and 9C were grown in BHI medium for 24 h (dots) and negative control of *S. mutans* were grown in M9 and THB (stripes). The results are expressed as the mean \pm standard deviation of two independent experiments.

3.4.3.7 Growth curves of isolate 6E and 9C grown in BHI and M9 minimal medium

In the previous experiment the filtered supernatant of 6E and 9C did not demonstrate biofilm detaching activity when they were grown in M9+G. At different phases of bacterial growth, compound (s) responsible for the biofilm detaching activity might be produced at different levels. To test this hypothesis, 6E and 9C were grown in 20 ml of BHI broth and 20 ml of M9+G. Bacterial cultures were collected at different incubation times and the OD_{600nm} measured. Filtered supernatants from different incubation times were tested for biofilm detaching activity.

3.4.3.7.1 Isolate 6E

In Figure 3.16, the growth curves of 6E in BHI broth medium and M9+G were observed for 96 h. The growth curves were different when growing 6E in these media. In BHI medium, isolate 6E started the log phase at 3 h after incubation and rapidly reached the highest OD_{600nm} of 3.27 ± 0.06 at 11 h. Then after that OD_{600nm} decreased steadily to 0.88 ± 0.21 at 48 h and was about OD_{600nm} of 1.01 ± 0.06 at 96 h. In M9+G the optical density was less than in BHI medium. Isolate 6E entered the log phase at 3 h after incubation and reached early stationary phase at 11 h with an optical density of 0.65 ± 0.01 . The highest optical density was 0.83 ± 0.05 at 24 h and it was steady until 0.67 ± 0.01 at 96 h.

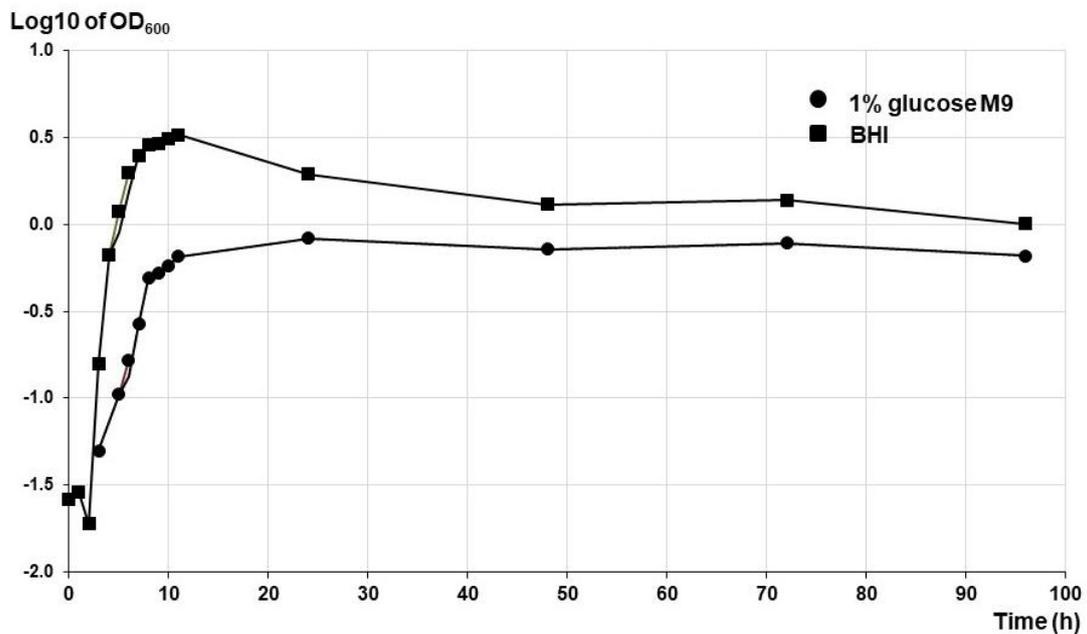
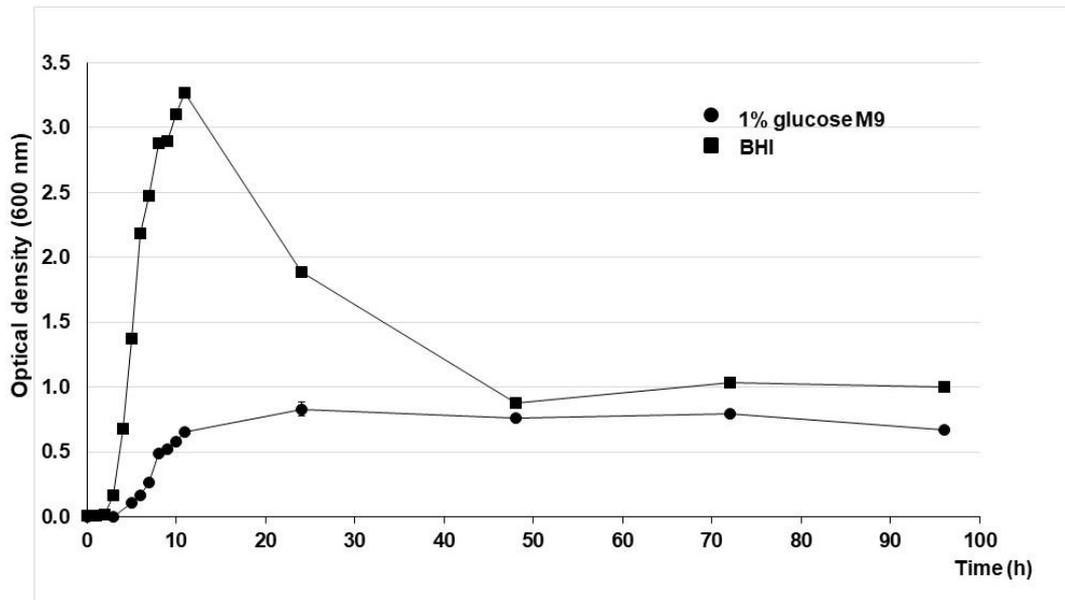


Figure 3.16 Growth curve of 6E in BHI and M9 minimal medium supplemented with 1% glucose.

Isolate 6E was cultured in BHI broth and M9 medium supplemented with 1 % glucose (M9+G). Bacterial cultures, in BHI medium (■) and M9+G (●) were measured at OD_{600nm}. The results are expressed as the mean and the range of the data points are within 12% in average of each other of at least two independent experiments.

Bacterial cultures of 6E in BHI and M9 medium at 5, 8, 24, 48, 72 and 96 h were tested for biofilm detaching activity. Bacterial cultures were centrifuged (4,500 x g for 10 min

at 4°C) and their supernatants were filtered using a 0.22 µm filter. The filtered supernatants were tested for biofilm detaching activity. Figure 3.17 shows biofilm formation by *S. mutans* in the presence of the filtered supernatants from 6E grown in BHI and M9+G, at six-time points. Biofilm detaching activity was observed from filtered supernatants in BHI medium only but not in the filtered supernatants from M9+G cultures. Interestingly, two anti-biofilm activities were observed from BHI medium. The first activity was the biofilm detaching activity which was found in 24 h and 48 h bacterial cultures as found in previous experiments. Another activity detected was biofilm inhibitory activity. In the presence of this filtered supernatant, *S. mutans* grew very well (data not shown) but *S. mutans* could not form biofilm (as shown in Figure 3.18). This biofilm inhibitory activity was observed from filtered supernatant in BHI medium at 72 and 96 h.

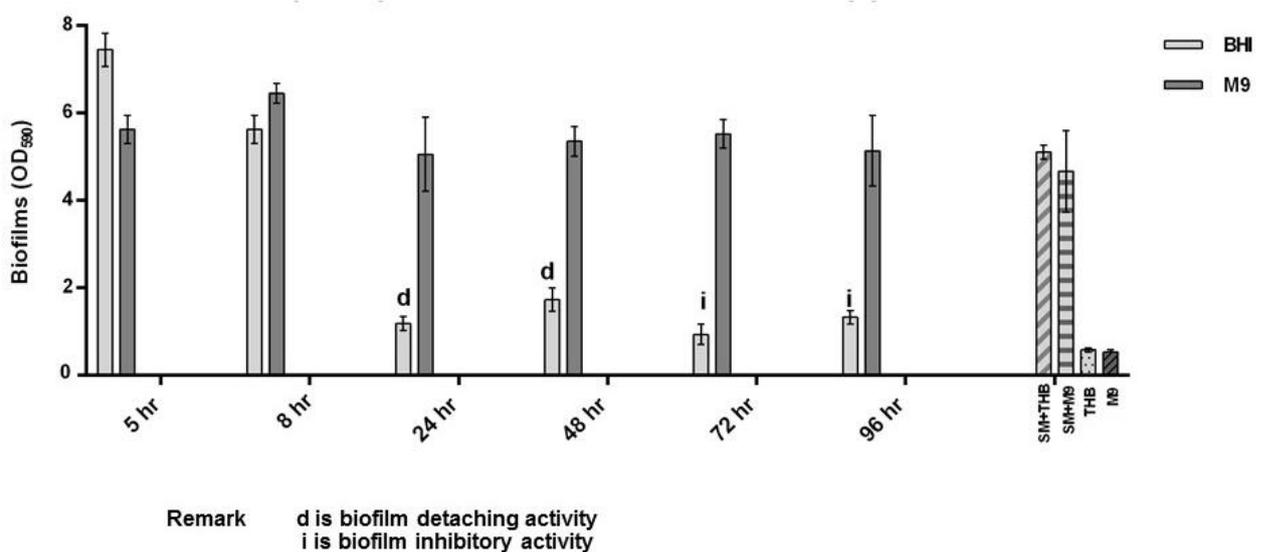


Figure 3.17 Biofilm formation by *S. mutans* in the presence of filtered supernatants of 6E in BHI and M9 + glucose medium during a period of 96 h. Isolate 6E was grown in BHI and M9 medium supplemented with 1% glucose (M9+G). Bacterial cultures were collected at 5, 8, 24, 48, 72 and 96 h post incubation and centrifuged at 4,500 x g for 10 min at 4°C. Their supernatants were filtered using a 0.22 µm filter. The filtered supernatants in BHI medium (light grey) and M9+G medium (dark grey), were tested for anti-biofilm activity. The results are expressed as the mean ± standard deviation of at least two independent experiments.

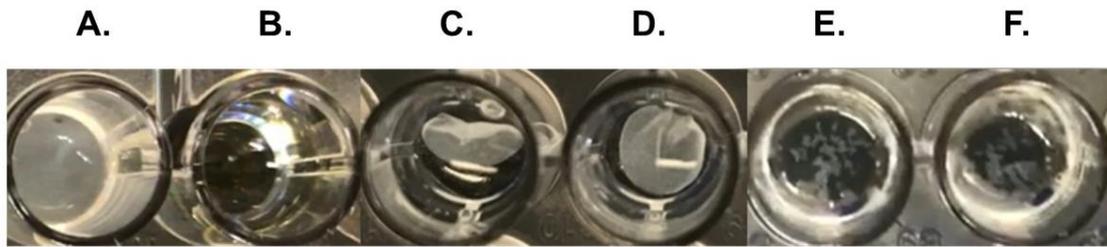


Figure 3.18 Biofilm formation by *S. mutans* in the bottom of a microtitre plate after removing planktonic cells.

S. mutans biofilms in the presence of TH broth alone (A) and without *S. mutans* (B) as a blank. *S. mutans* biofilms formed in the presence of filtered supernatants from 24 h (C) and 48 h (D) cultures of isolate 6E. *S. mutans* biofilms formed in the presence of filtered supernatants from 96 h (E and F) cultures of isolate 6E.

3.4.3.7.2 Isolate 9C

In Figure 3.19, the growth curves of 9C in BHI broth medium and M9+G were observed for 96 h. The growth curves were different when growing 9C in these media. In BHI medium, 9C started the log phase at 3 h after incubation and rapidly reached the highest OD_{600nm} of 2.85 ± 0.21 at 11 h. Then after that OD_{600nm} decreased rapidly to 0.81 ± 0.09 at 24 h and maintained OD_{600nm} of about 0.35 ± 0.08 at 48 h until 0.37 ± 0.05 at 96 h. In M9+G the OD_{600nm} was less than in BHI medium. Isolate 9C entered the log phase at 5 h after incubation and reached early stationary phase at 11 h with an optical density was 0.85 ± 0.22 . The highest optical density was 0.97 ± 0.15 at 24 h and it was reduced to 0.38 ± 0.01 at 96 h.

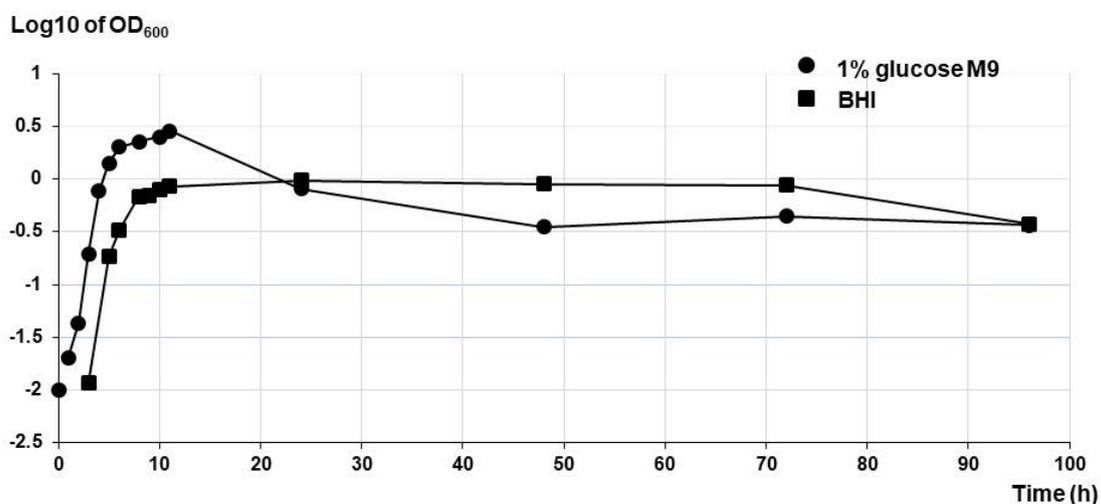
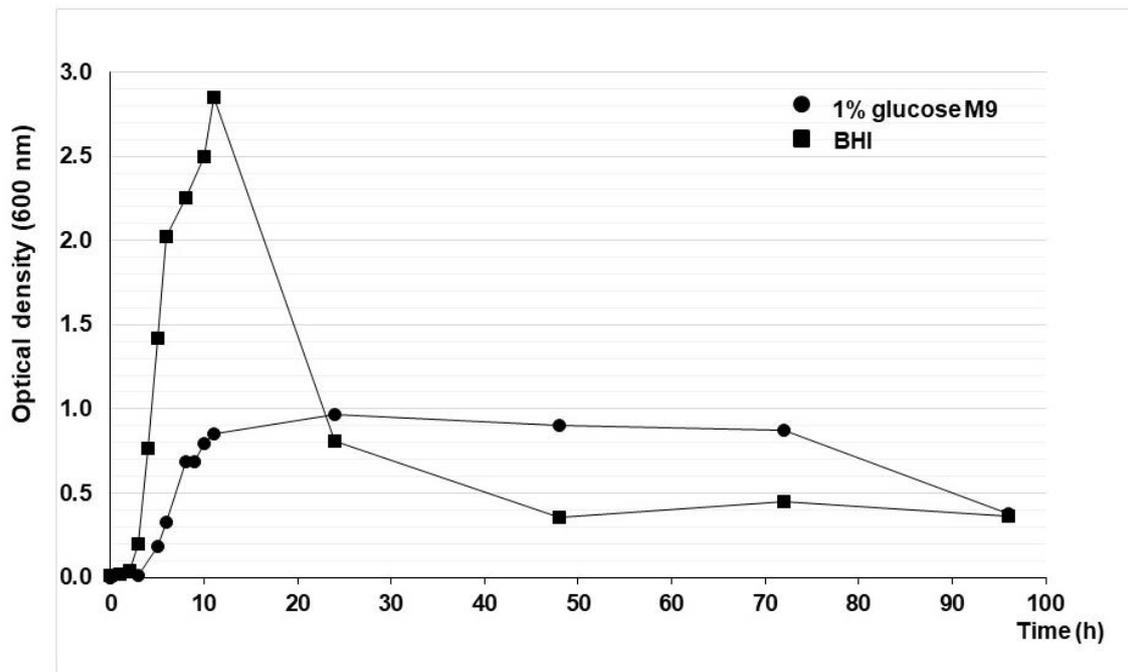


Figure 3.19 Growth curve of 9C in BHI and M9 minimal medium supplemented with 1% glucose.

Isolate 9C was cultured in BHI broth and M9 medium supplemented with 1 % glucose (M9+G). Bacterial cultures, in BHI medium (■) and M9+G (●) were measured at OD_{600nm}. The results are expressed as the mean and the range of the data points are within 10 % of each other of at least two independent experiments.

Bacterial cultures of isolate 9C in BHI and M9 medium at 8, 24, 48, 72 and 96 h were tested for biofilm detaching activity. Bacterial cultures were centrifuged (4,500 x g for 10 min at 4 °C) and their supernatants were filtered using a 0.22 µm filter. The filtered supernatants were tested for biofilm detaching activity. Figure 3.20 shows biofilm

formation by *S. mutans* in the presence of filtered supernatants from 9C grown in BHI and M9+G, at six-time points. Biofilm detaching activity was observed from filtered supernatants in BHI medium only but not in the filtered supernatants from M9+G cultures.

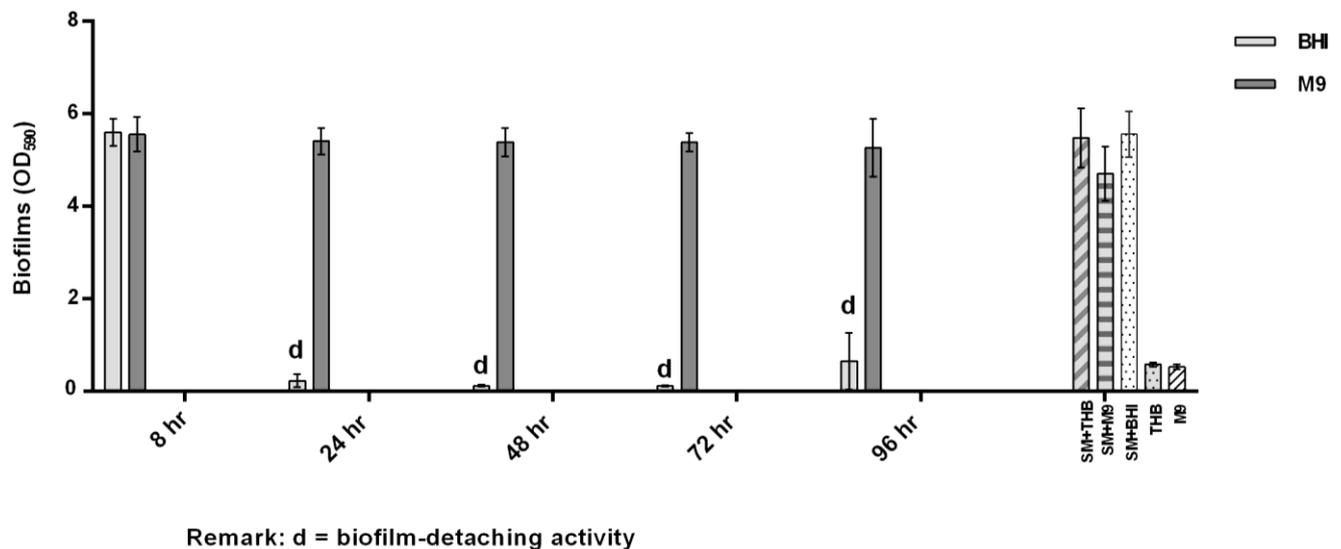


Figure 3.20 Biofilm formation by *S. mutans* in the presence of filtered supernatants from 9C grown in BHI and M9 minimal medium supplemented with 1% glucose during a period of 96 h.

Isolate 9C was grown in BHI and M9 medium supplemented with 1% glucose (M9+G). Bacterial cultures were collected at 8, 24, 48, 72 and 96 h post incubation and the 0.22 μ m filtered supernatants in BHI medium (light grey) and M9 +G (dark grey), were tested for biofilm detaching activity. The results are expressed as the mean \pm standard deviation of at least two independent experiments.

3.4.3.8 Production of a compound (s) exhibiting biofilm detaching activity over time during growth in M9 minimal medium

In previous experiments, isolate 6E and 9C produced anti-biofilm activity when grown in BHI medium but they did not produce any anti-biofilm activity when grown in M9+G medium. Bacteria can utilise different carbon sources via different metabolic pathways. It was hypothesised that 6E and 9C might produce anti-biofilm activity when grown in M9 medium supplemented with other carbon sources. To test this hypothesis, five carbon sources were added to M9 medium instead of 1% glucose. These were 1%

glycerol, 1% sucrose, 1% sorbitol, 1% mannitol, and 0.5% methyl cellulose. Isolate 6E and 9C were cultured in BHI medium and 18 h bacterial cultures were inoculated at an OD_{600nm} equal to 0.005 in 20 ml of M9 medium supplemented with one of those carbon sources. Bacterial cultures were collected when the biofilm detaching activity and biofilm inhibitory activity were detected at 24 h and 72 h after incubation, respectively. Bacterial cultures were centrifuged at 4,500 x g at 4°C for 10 min. Supernatants were filtered using a 0.22 µm filter. Filtered supernatants were tested for *S. mutans* anti-biofilm activity.

3.4.3.8.1 Isolate 6E

Figure 3.21 shows biofilm formation by *S. mutans* in the presence of filtered supernatants from isolate 6E grown in M9 medium supplemented with five different carbon sources compared to growth in BHI medium. Biofilm detaching activity was observed from filtered supernatant of isolate 6E grown in M9 medium supplemented with 1% sucrose at 24 h of incubation. *S. mutans* biofilm formation was not affected in the presence of filtered supernatants from M9 medium supplemented with five carbon sources without 6E. Therefore, this experiment confirmed that growing 6E in M9 medium supplemented with 1% sucrose could produce biofilm detaching activity.

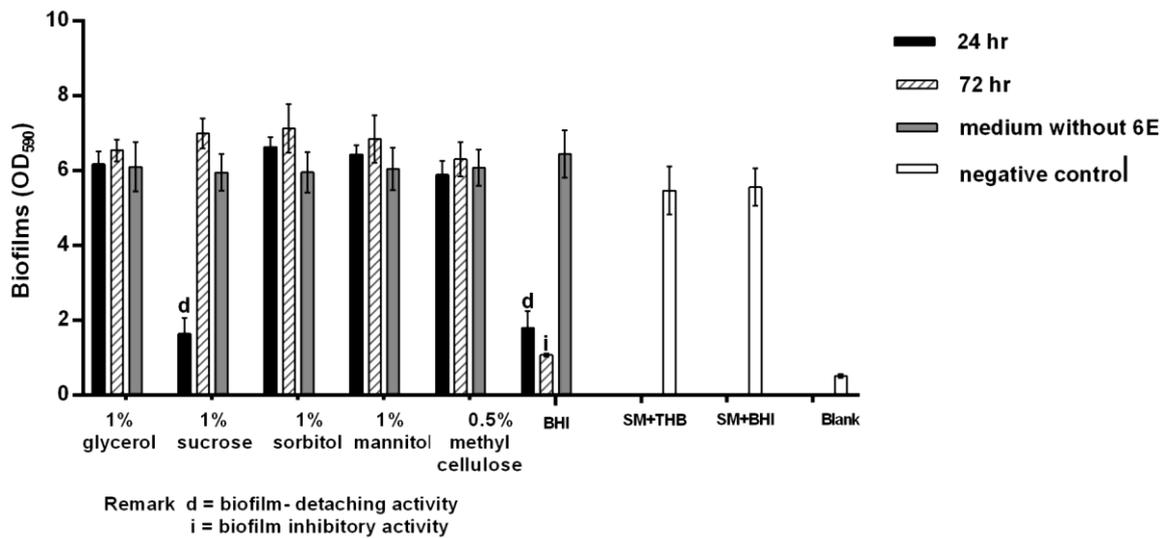


Figure 3.21 Biofilm formation by *S. mutans* in the presence of filtered supernatants from 6E growing in M9 minimal medium supplemented with five carbon sources

: 1% glycerol, 1% sucrose, 1% sorbitol, 1% mannitol, and 0.5% methyl cellulose for 24 h (black) and 72 h (stripes). The 0.22 μm filtered supernatants were tested for biofilm detaching activity. Biofilm formation by *S. mutans* in the presence of M9 medium with the five carbon sources but without 6E being grown in it (grey). The results are expressed as the mean \pm standard deviation of at least two independent experiments.

3.4.3.8.2 Isolate 9C

Figure 3.22 shows biofilm formation of *S. mutans* in the presence of filtered supernatants from isolate 9C grown in M9 medium supplemented with five different carbon sources compared to the growth of 9C in BHI medium. Biofilm detaching activity was observed in the filtered supernatant from BHI medium but none of the other filtered supernatants demonstrated biofilm detaching activity.

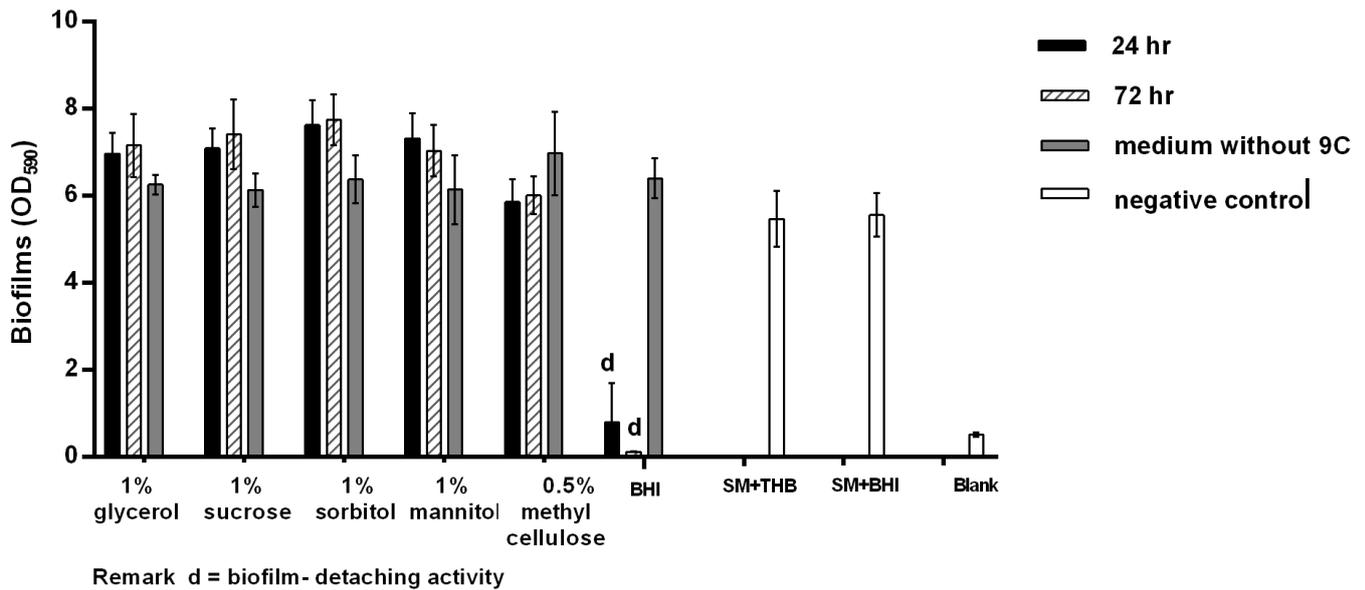


Figure 3.22 Biofilm formation by *S. mutans* in the presence of filtered supernatants from isolate 9C grown in M9 minimal medium supplemented with five carbon sources:

1% glycerol, 1% sucrose, 1% sorbitol, 1% mannitol, and 0.5% methyl cellulose for 24 h (black) and 72 h (stripes) of incubation. The 0.22 μm filtered supernatants were tested for biofilm detaching activity. Biofilm formation by *S. mutans* in the presence of M9 medium with the five carbon sources without growth of 9C (grey). The results are expressed as the mean \pm standard deviation of at least two independent experiments.

3.5 Discussion

In the development of the microtiter plate biofilm assay to screen for *S. mutans* anti-biofilm activity from both the metagenomic library, cultured in LB broth, and the environmental library, cultured in BHI broth, the effect of both LB and BHI media on biofilm formation were determined. The results in Figure 3.2 demonstrated that the $\text{OD}_{590\text{nm}}$ of biofilms and biofilms with planktonic cells in both BHI and LB media were similar to that the $\text{OD}_{590\text{nm}}$ of biofilms and biofilms with planktonic cells in TH broth. These results indicated that the microtitre plate biofilm assay was reproducible. Thus, the microtitre plate biofilm assay could be used in a screening assay to identify *S.*

mutans anti-biofilm activity using both the metagenomic clones and the environmental bacterial isolates.

Co-culture of *S. mutans* and *E. coli* EPI300 in the biofilm assay was developed in order to test the metagenomic library for clones producing biofilm inhibitory compounds. To test the metagenomic library three assays were carried out: (1) *E. coli* whole cell culture, (2) *E. coli* supernatants, and (3) 0.22 µm filtered supernatants. The results that show these filtered supernatants could be used to test for anti-biofilm activity because there was no interference from *E. coli* biofilm formation. However, the filtering process was too expensive to screen the 27,000 clones in the metagenomic library. Supernatants and *E. coli* whole cell cultures could also be used by adding polymyxin B in the biofilm assay. Polymyxin B belongs to a cyclic peptide antibiotic group. It affects cell membrane permeability and inhibits against Gram-negative bacteria. Polymyxin B shows affinity towards lipid A of lipopolysaccharide of the Gram-negative outer membrane (Jones *et al.*, 2005, Kicielinska *et al.*, 2016, Liu *et al.*, 2016). The results in the section of 3.4.1.3 indicated that a 30 µg/ml of polymyxin B was used for the biofilm assay to inhibit *E. coli* biofilm formation with no effect on *S. mutans* biofilm formation.

In *S. mutans* sucrose is a substrate for producing glucans by glucosyltransferases, especially for synthesising water-insoluble glucan, which plays an important role in the process of forming the three-dimensional structures of biofilms on the tooth surfaces. Many studies have studied *S. mutans* biofilms in the presence of sucrose concentrations in the range of 0.5 to 5% (Islam *et al.*, 2008, Liu *et al.*, 2013, Decker *et al.*, 2014, Angius *et al.*, 2015, Pan *et al.*, 2015, Veloz *et al.*, 2015, Zhang *et al.*, 2015).

In this study, different sucrose concentrations were tested in a biofilm assay. The results indicate that *S. mutans* biofilms were increased significantly in the presence of sucrose compared to the control which was no sucrose. However, there was no significant difference in biofilm formation between the concentration of 0.5%, 1.0% and 2.0% ($p < 0.0001$). Biofilms formed at these three concentrations were significantly more than biofilms formed in the presence of sucrose at a concentration of 4%. These results were similar to Zhou *et al.* (2014). They measured the expression of *gtfB* and *gtfC* and sucrose-dependent adhesion of *S. mutans* in the presence of different sucrose concentrations. They found that expression of both genes and cell adhesion increased significantly with increasing sucrose concentrations from 1% to 10%, but no significant difference was observed between 10% and 20% of sucrose concentration.

Sucrose is an important carbon source for *S. mutans*, especially for forming biofilms. Zeng & Burne (2013) revealed sucrose-metabolizing pathways in *S. mutans*. Sucrose can be used extracellularly and intracellularly. In Figure 3.23, extracellular sucrose is utilised by glucosyltransferases (*GtfB*, *GtfC*, *GtfD*) to produce glucans and levansucrase uses sucrose to produce levan (Zeng & Burne, 2013). Intracellularly, sucrose from outside *S. mutans* cells is transferred to the inside of the cells through four main channels: two of phosphotransferase systems (EII^{ScrA} and EII^{Tre}), a multisugar metabolite system (MSS) and a maltose/maltodextrin ABC transporter.

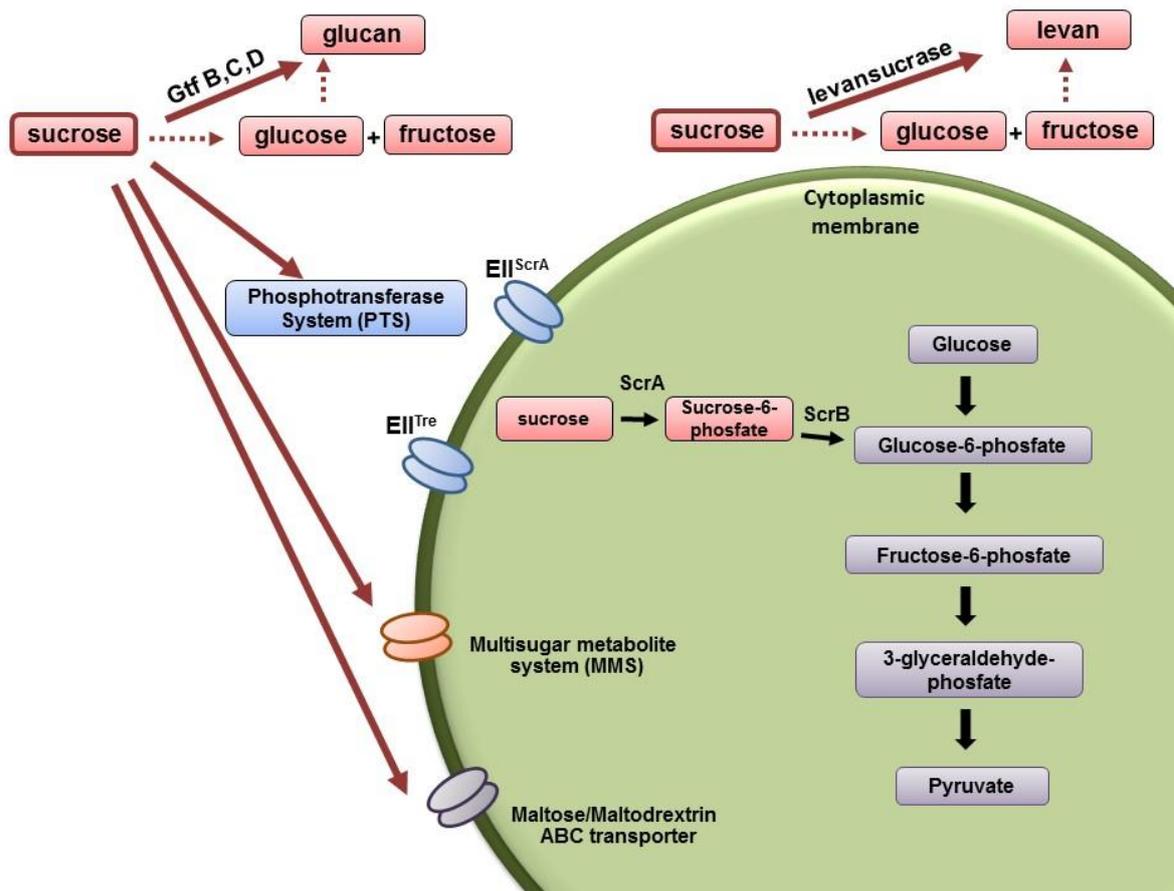


Figure 3.23 Schematic representation of sucrose utilisation by *S. mutans*. In extracellular environment, sucrose is a substrate of Gtfs to synthesise glucan and it is also a substrate of levansucrase to synthesise levan. Sucrose is transported to inside *S. mutans* cells by (i) phosphotransferase system (PTS): EII^{ScrA} and EII^{Tre}, (ii) multisugar metabolite system (MMS), and (iii) maltose/maltodextrin ABC transporter. The figure was drawn using MS PowerPoint and based on an illustration by Hamada & Slade (1980), Bowen & Koo (2011) and Zeng & Burne (2013).

Metagenomic based strategies are powerful to investigate microorganisms in many niches, such as the oral cavity (Roberts & Kreth, 2014, Sukumar *et al.*, 2016), and human gut (Qin *et al.*, 2010). Metagenomic based strategies extract the genomes of microorganisms in an ecosystem for several purposes such as determining species diversity or for screening functional genes of interest (Méndez-García *et al.*, 2018). Metagenomic based strategies have increased the opportunity to discover enzymes with novel biocatalytic activities from unculturable microorganisms (Kennedy *et al.*, 2008, Arivaradarajan *et al.*, 2015, Tansirichaiya *et al.*, 2016, Thies *et al.*, 2016). Thus,

in this study, a metagenomic library constructed in *E. coli* was screened for clones with *S. mutans* anti-biofilm activity.

In the metagenomic library, DNA fragments from human saliva were cloned into CopyControl pCC1BAC vector, which contains a chloramphenicol resistance gene as a selective marker and the construct was transformed into *E. coli* EPI300. In the screening for anti-biofilm activity of metagenomic clones, chloramphenicol was added to the growth medium of recombinant *E. coli*. There was apparently no active concentration of chloramphenicol remaining in the supernatant after cultivation of *E. coli* for 24 h as no inhibition of *S. mutans* growth by these supernatants was observed in the biofilm assay.

The results of screening the metagenomic clones for *S. mutans* anti-biofilm activity in the section of 3.4.2 were visualised using heatmap analysis which identified both *S. mutans* anti-biofilm activity and antibacterial activity. Heatmap analysis converted the absorbance values of each well in 96-well plate into a visual map where they were shown in a colour ranging scale from low to high absorbance, from blue to red (as shown in Figure 3.7). If an *E. coli* clone resulted in a low level of *S. mutans* growth, this meant that the clone may produce *S. mutans* growth inhibitory activity. If a clone resulted in a small amount of *S. mutans* biofilm but did not inhibit *S. mutans* growth, this indicated that this clone may produce *S. mutans* anti-biofilm activity. The results from the screening of 6,624 metagenomic clones showed no clones producing anti-biofilm inhibitory activity.

To screen for anti-biofilm activity from the environmental isolate library, a total of 89 environmental bacteria were screened for *S. mutans* anti-biofilm activity. In the primary screening, anti-biofilm activity was detected in the presence of supernatants from ten isolates. It was possible that the culture supernatants from ten isolates tested in the

biofilm assay contained some residual bacterial cells and these cells might affect *S. mutans* biofilm during biofilm assay. To ensure that any activity found was due to extracellular compounds derived from the isolates in the secondary screening, the supernatants were filtered using a 0.22 µm filter to remove any residual cells and the filtrate were tested in the biofilm assay. The results showed that the filtered supernatants from seven of ten isolates exhibited biofilm detaching activity. These results suggested that the extracellular compound (s) from seven isolates was responsible for the biofilm detaching activity.

The biofilm detaching activity observed in this study indicated that the tested agent allowed *S. mutans* to grow and form biofilms on the surface of microtiter plate. However, the cohesive forces between these biofilms and the microtiter plate surface was weak. As a consequence, floating of biofilms was observed while washing planktonic cells.

To determine the capacity of the compounds in the filtered supernatants to disrupt *S. mutans* biofilms, a biofilm disruption assay was conducted on biofilms of different ages, which were at start of biofilm formation (0 h), 7 h and 24 h. The filtered supernatants from seven isolates showed biofilm detaching activity when the supernatants were added at the start of *S. mutans* biofilm formation, at 0 h, but not at 7 h or 24 h. These results might be due to the 7 h and 24 h biofilms being less susceptible. Previous studies by Dodds *et al.* (2000), Yang *et al.* (2012) indicated that older biofilms are less susceptible to antimicrobial agents than the younger biofilms because of the higher complexity of the EPS structure reduces the diffusion of anti-biofilm compounds into the biofilm layers. This might be the case in this study, that the

compound responsible for biofilm detaching activity in the filtered supernatants could not penetrate through the barrier of the biofilm structure/layers.

Another possibility for the lack of biofilm detaching activity in the 7 h and 24 h aged biofilms could be that there might not be a target for the compound responsible for the activity after attachment. The compound might have a specific target for the biofilm detaching activity which is present at 0 h but not at 7 h or 24 h. In addition, the compound in the supernatants might inhibit *S. mutans* cell adherence to the microtitre plate surface. Recent studies indicated that a peptide molecule reduced *S. mutans* adhesion when the peptides were coated on titanium surfaces (Maddikeri *et al.*, 2008, Bruellhoff *et al.*, 2010).

In the bacterial classification described in this chapter, 16S rRNA gene sequencing showed that seven isolates were members of *Bacillus* genus. Although this bacterial identification was based on 16S rRNA gene sequences, 16S rRNA gene sequences do not always discriminate against some closely related *Bacillus* spp. Wang *et al.* (2007) found that 16S rRNA gene sequence is not very good at determining the *B. subtilis* group at the species level. Nukamura *et al.* (1999) also found about 99.5% nucleotide identity of the 16S rRNA gene sequences of two clusters of the *B. subtilis* group. Moreover, the 16S rRNA gene sequence of the genus *Bacillus* is closely related with more than 99% similarity between species as reported by Starostin *et al.* (2015).

Several alternative methods have been used to classify *Bacillus* sp. Liu *et al.* (2013) used seven housekeeping genes; *gyrB*, *rpoB*, *pycA*, *pyrE*, *mutL*, *aroE* and *trpB* to classify 76 marine strains. The genes *gyrB* and *pyrE*, showed comparatively high-resolution power for this classification (Liu *et al.*, 2013). Hotta *et al.* (2011) classified *B. subtilis* at subspecies and strain levels using MALDI-TOF MS at 8 or 20 selected

ribosome subunit proteins (Hotta *et al.*, 2011). Some core genes, such as *gyrA*, *gyrB*, and *rpoB*, have been used to discriminate the members of *Bacillus* (Folmsbee *et al.*, 2006, Wang *et al.*, 2007, Vos *et al.*, 2012). Thus, in this study, after whole genome sequencing, *gyrA* and *rpoB* of the seven isolates were used for classification. The phylogenetic tree (as shown in Figure 3.10) could divide the seven isolates into two groups, similar to the tree based on the 16S rRNA gene sequence. These results suggested that using 16S rRNA, *gyrA* and *rpoB* gene sequences provided similar results but they were not enough to be used to identify the species of the seven isolates in this study.

MicrobesNG results using Kraken indicated that the most closely related bacterium to the seven isolates was *B. methylotrophicus* but the nucleotide similarities were very low about 59.10 % to 63.21 %. Kraken has slightly lower sensitivity than Megablast (<https://ccb.jhu.edu/software/kraken/>). Using Megablast, the results showed that the seven isolates were *B. velezensis*, which is a synonym of *B. methylotrophicus*. The phylogenomic tree showed that they were in clade II of *B. amyloliquefaciens* operational group.

The genome of seven isolates were analysed using DDH and ANI. The results confirmed that the seven isolates were members of *B. velezensis*. The seven isolates might be divided into two groups due to their high similarity of ANI value (>99%). This includes group 1: 3E, 8E, 9C and 9E, and (separately) group 2: 6E, 10G and 11E, were clonal within the group, which is similar to the results from phylogenetic data of 16S RNA, *gyrA* and *rpoB*. The information of isolation process such as Gram stain might be useful to classify them, although it was not performed in this study. Here, we could indicate that the seven isolates were isolated from different samples and different places. However, these are not enough to ensure that they are not clonal

strain. Therefore, the results in this study suggest that seven isolates were *B. velezensis* and could be divided into two closely related groups.

To separate the compound responsible for the biofilm detaching activity, the isolates were cultured in M9 medium, which is a defined medium in which some bacteria can grow. M9 medium contains disodium hydrogen phosphate and potassium dihydrogen phosphate as buffering agents. Ammonium chloride provides a nitrogen source and sodium chloride provides ions and osmotic support. In this study, because I found that the seven isolates clustered into two groups based on nucleotide identity due to the nucleotide similarity, only isolate from each group, 6E and 9C, were selected to grow in M9 medium. The medium was supplemented with glucose as a carbon source. Both isolates were observed to grow well in the medium but the biofilm detaching activity was not observed. It was speculated that growth with different carbon sources might result in different biological activity (Tejera *et al.*, 2004). Thus, both isolates were grown in M9 medium supplemented with five different carbon sources: sucrose, glycerol, mannitol, sorbitol and cellulose. The biofilm detaching activity was observed when isolate 6E was cultured in M9 medium supplemented with sucrose.

Recent studies have reported on the influence of media compositions on phenotypic features. For example, *Bacillus licheniformis* was grown in different media supplemented with different types of poultry feather waste, with different chemical compositions (Parrado *et al.*, 2014). In another example, the *Gluconacetobacter diazotrophicus* exolevanase gene (*IsdB*) was expressed in the presence of fructose or glycerol but not in the presence of sucrose or glucose (Menéndez *et al.*, 2009, Velazquez-Hernandez *et al.*, 2011). The effect of carbon source on gene expression might be a reason for the different phenotype seen in my studies.

In this study, the reason why sucrose could induce the production of a compound responsible for the biofilm detaching activity has not been investigated. A clue might be provided, if the compound with the activity was identified.

3.6 Conclusion

In summary, a high throughput, microtitre plate biofilm assay was developed to enable testing of metagenomic libraries for members producing biofilm inhibitory compounds. The metagenomic library and the environmental isolate library were screened for *S. mutans* anti-biofilm activity. Biofilm detaching activity was observed from seven isolates. Based on whole genome sequences, they were members of *B. velezensis* and they were divided into two groups. Seven isolates produced the biofilm detaching activity when they were cultured in either BHI medium or M9 minimal medium supplemented with 1% sucrose as a carbon source.

CHAPTER 4

**Investigation into compound(s) exhibiting *Streptococcus mutans* biofilm
detaching activity**

Chapter 4 Investigation into compound exhibiting *S. mutans* biofilm detaching activity

4.1 Introduction

In chapter 3, seven isolates were observed to produce *S. mutans* anti-biofilm activity. All of them produced *S. mutans* biofilm detaching activity when they were cultured in BHI medium. Due to the high similarity of 16S rRNA gene sequences from seven isolates, isolate 6E was chosen as a representative. Strain 6E was found to produce the biofilm detaching activity when it was grown in M9 minimal medium supplemented with 1% sucrose (M9+S). This chapter describes work focusing on identifying a compound or compounds that had biofilm detaching activity. In particular, a compound in the supernatant derived from 6E which was grown in M9+S medium is discussed here.

It was hypothesised that the compound responsible for the biofilm detaching activity might be a protein because it was inactivated by boiling. Proteinase K was used to test the hypothesis. However, the proteinase K would be carried over into the biofilm assay and could possibly digest proteins involved in *S. mutans* biofilm formation. So a proteinase K inhibitor was needed to stop proteinase K activity before testing in the biofilm assay.

Proteinase K, or endopeptidase K, is a serine protease which belongs to the subtilisin family. Serine proteases contain a catalytic triad active site that facilitates peptide bond hydrolysis. The catalytic triad is composed of three amino acid residues which are serine, histidine, and aspartic acid as described in chapter 1 (Figure 1-7). The proteinase K used in this study is a commercial enzyme isolated from a fungus,

Engyodontium album (formerly *Tritirachium album*). Two proteinase K inhibitors which are commonly used are diisofluorophosphate (DIFP) and phenylmethanesulfonyl fluoride (PMSF). DIFP was chosen as it is more stable.

4.2 Aims of the work described in this chapter

The aim of the work presented in this chapter was to identify the compound(s) responsible for biofilm detaching activity from isolate 6E, which was cultured in M9 minimal medium supplemented with 1% sucrose. Once it was confirmed that the compound(s) belonged to a protein family, the proteins in the fractions, exhibiting biofilm detaching activity, were identified.

4.3 Materials and Methods

Results in chapter 3 showed that filtered supernatants from 6E, 10G and 11E were heat sensitive when they were boiled at 100°C for 15 min. Therefore, it was possible that the compound(s) responsible for biofilm detaching activity might be a protein. To test this, proteinase K was used to determine if it could inactivate the compound responsible for the biofilm detaching activity. Following treatment of the supernatant with proteinase, DIFP was added as a proteinase K inhibitor to prevent any effect of proteinase K on *S. mutans* biofilm formation.

4.3.1 Optimisation and determination of the effect of DIFP

A range of final concentrations from 0.05 mM to 0.20 mM DIFP were added to 100 µl aliquots of the 0.22 µm filtered supernatant from isolate 6E, which had been cultured in M9 minimal medium supplemented with 1% sucrose (M9+S), to determine the effect of DIFP on *S. mutans* biofilm detaching activity. A positive control group was

conducted by adding this final concentration range of DIFP to 100 μ l TH broth supplemented with 0.1 mg/ml proteinase K and a negative control group was a 100 μ l TH broth alone. After 15 min of incubation at 37°C in a water bath, 100 μ l aliquots of the mixtures were tested for *S. mutans* biofilm assay as shown in Figure 4.1. Then, *S. mutans* were allowed to form biofilms for 24 h in the presence of three samples and biofilm was measured using crystal violet biofilm assay.

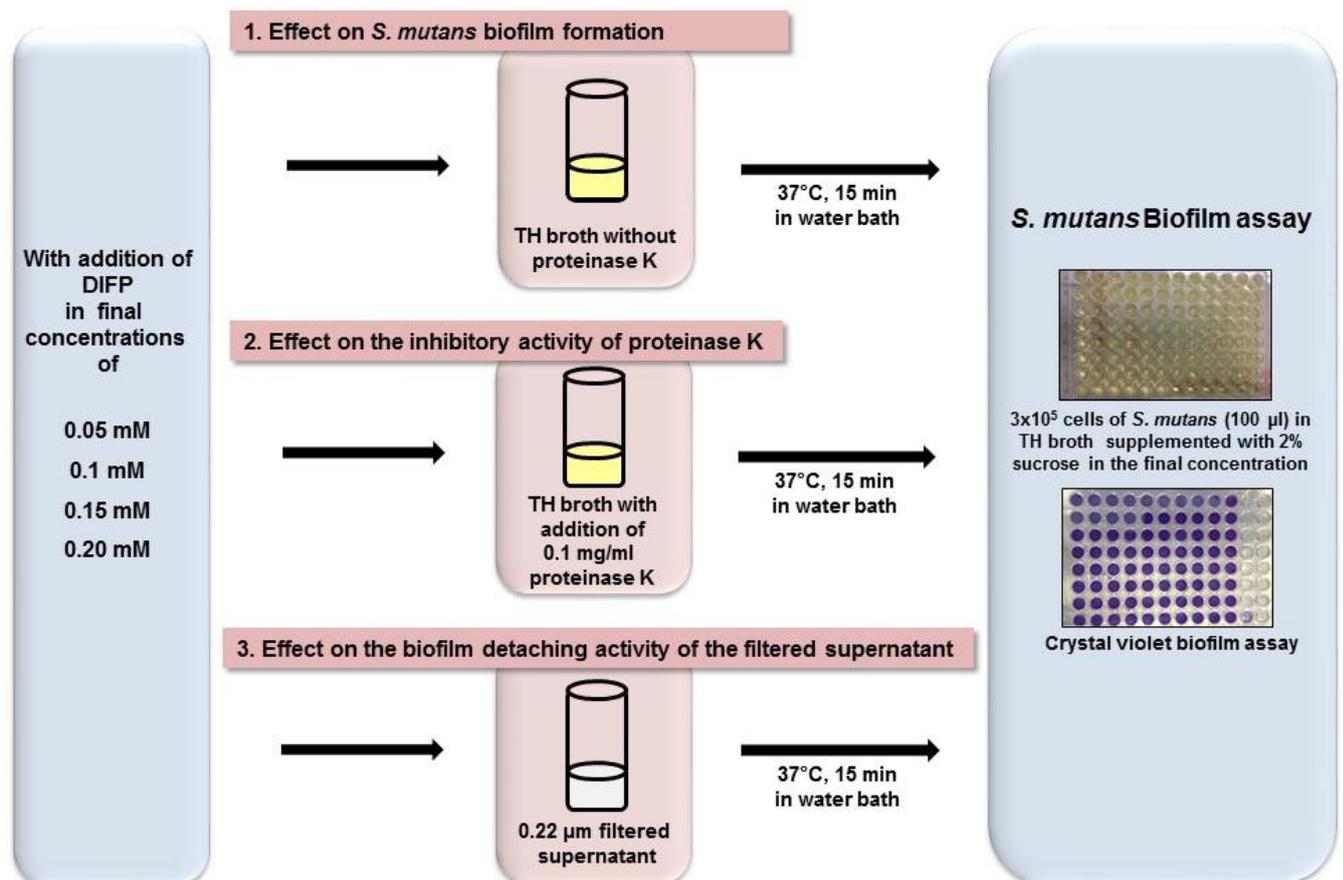


Figure 4.1 Flow chart of experiments used to optimise and determine the effect of DIFP on (1) *S. mutans* biofilm formation, (2) the inhibitory activity of proteinase K, and (3) *S. mutans* biofilm detaching activity of filtered supernatant.

4.3.2 Determination of the effect of proteinase K on *S. mutans* biofilm detaching activity of culture supernatant from isolate 6E

A final concentration of 0.1 mg/ml proteinase K was added to 100 µl of the 0.22 µm filtered supernatant from isolate 6E, which was cultured in M9 minimal medium supplemented with 1% sucrose (M9+S). Then, proteinase K was allowed to digest proteins in the filtered supernatant for 16 h of incubation at 37 °C in a water bath. After that a final concentration of 0.1 mM DIFP was added into the mixture which was further incubated in the same condition for 30 min to stop the proteinase K activity. The mixture was then tested for biofilm detaching activity by adding to *S. mutans* biofilm formation. After 24 h, *S. mutans* biofilm was measured using crystal violet biofilm assay. In Figure 4.2, control groups, labelled (1) to (4), were used in the biofilm assay in addition to the test sample, labelled (5). Figure 4.2 shows the four control groups in this experiment with (1) 100 µl fresh filtered supernatant, (2) 100 µl filtered supernatant incubated at 37°C for 16 h, (3) 100 µl filtered supernatant supplemented with 0.1 mg/ml of proteinase K incubated at 37°C for 16 h, and (4) 100 µl filtered supernatant supplemented with 0.1 mM of DIFP incubated at 37°C for 3 h.

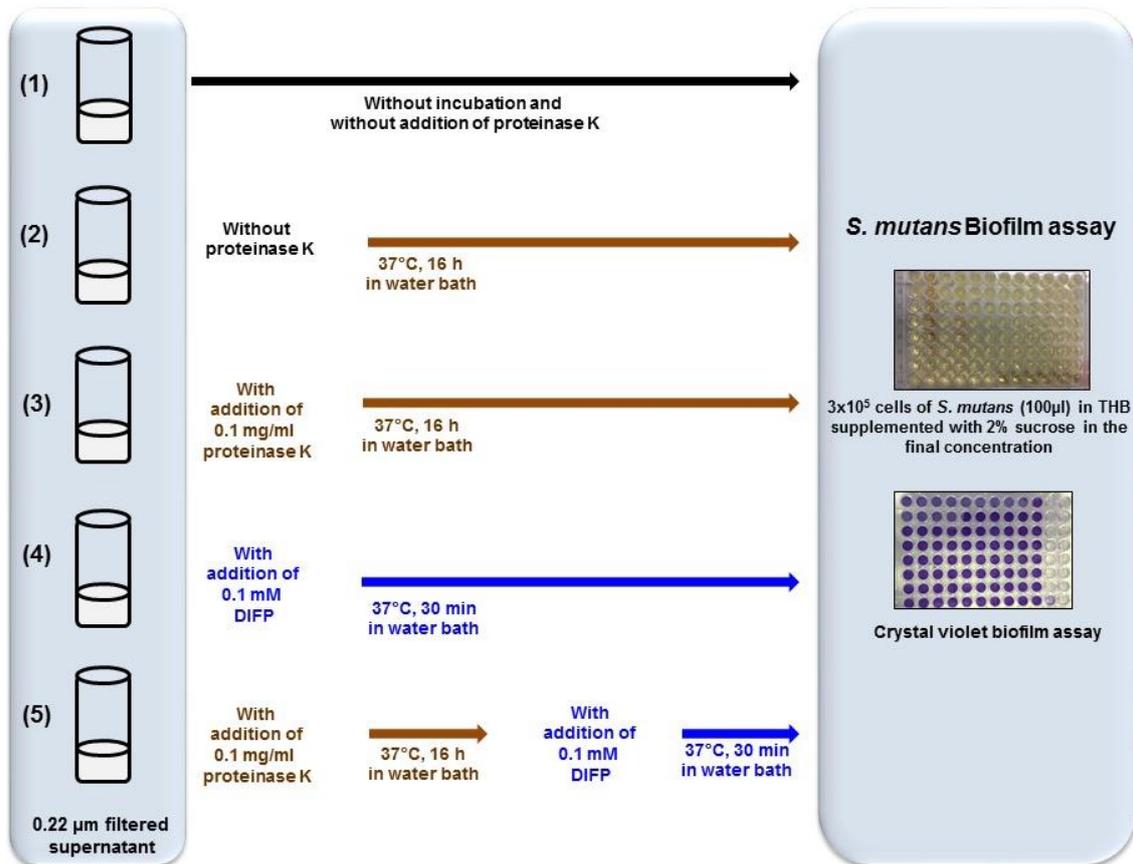


Figure 4.2 Flow chart of the experiments used to determine the effect of proteinase K on *S. mutans* biofilm detaching activity.

4.3.3 Estimation of the size of the compound exhibiting biofilm detaching activity using centrifugal ultrafiltration

To estimate the size of the compound responsible for biofilm detaching activity in the 0.22 µm filtered supernatant from 6E, which was grown in M9+S, a volume of 500 µl of the filtered supernatant was filtered using two different sizes of centrifugal ultrafiltration devices, 50kDa and 100kDa (Amicon® Ultra-0.5 Centrifugal Filters, Millipore, Germany) (as shown in Figure 4.3). The compounds were separated by centrifuging (14,000 x g, 30 min 4°C) and two fractions were separated from the ultrafiltration devices. The first fraction was a filtrate fraction which passed through the filter membrane containing molecules smaller than 50 KDa, when using the 50KDa filter, and 100 KDa, when using the 100KDa filter. The other fraction was the retentate

fraction which contained molecules that did not pass through the filter membrane due to their sizes being equal to or bigger than 50 KDa when using the 50KDa filter, and 100 KDa, when using the 100KDa filter.

To recover the retentate fraction remaining on the filter membrane, a volume of 500 μ l of molecular grade water was added to the filter and pipetted up and down. The device was left at room temperature for 3 min and pipetted up and down again prior to being turned over into a clean Eppendorf tube and centrifuged (14,000 x g, 10 min 4°C). The filtrate and retentate fractions from both ultrafiltration devices were tested for biofilm detaching activity and the supernatant was used as a positive control group.

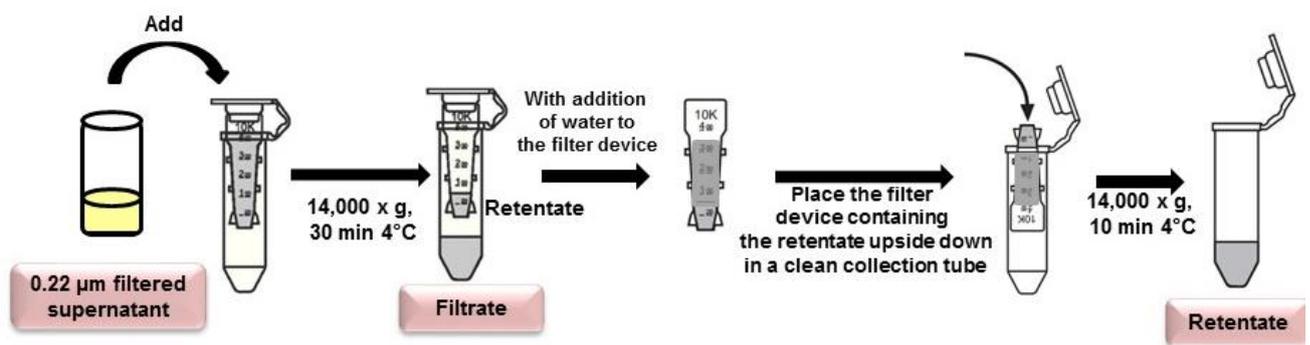


Figure 4.3 Flow chart of the method for centrifugal ultrafiltration (Amicon Ultra-0.5 Centrifugal Filter Devices, Millipore, Germany).

4.4 Results

4.4.1 The effect of DIFP on *S. mutans* biofilm formation and biofilm detaching activity

Diisofluorophosphate (DIFP), an irreversible inhibitor of proteinase K, was selected to inhibit proteinase K activity prior to the biofilm assay. The optimum concentration of DIFP was determined by examining a range concentrations from 0.05 mM to 0.20 mM. Figure 4.4 shows the results of three conditions. In the first condition, labelled (1) in Figure 4.4, biofilm formation in the presence of DIFP at four different

concentrations was similar to the biofilm formation in the absence of DIFP. This suggests that DIFP at these concentrations did not affect *S. mutans* biofilm formation. The second condition, labelled (2) in Figure 4.4, was *S. mutans* biofilm formation in the presence of DIFP and proteinase K. The amount of biofilm formed in the second condition was similar to the amount of biofilm formed in the absence of DIFP and proteinase K. This suggests that DIFP at these concentrations inhibited the activity of proteinase K. In the third condition, labelled (3) in Figure 4.4, of biofilm formation in the presence of DIFP and 0.22 μm filtered supernatant, biofilm detaching activity was observed. This suggests that DIFP at these concentrations did not affect the biofilm detaching activity of the filtered supernatant.

These experiments demonstrate that DIFP could inhibit the activity of proteinase K. Moreover, DIFP did not affect *S. mutans* biofilm formation and biofilm detaching activity of the filtered supernatant. Therefore, DIFP was used to inhibit proteinase K in the next experiment.

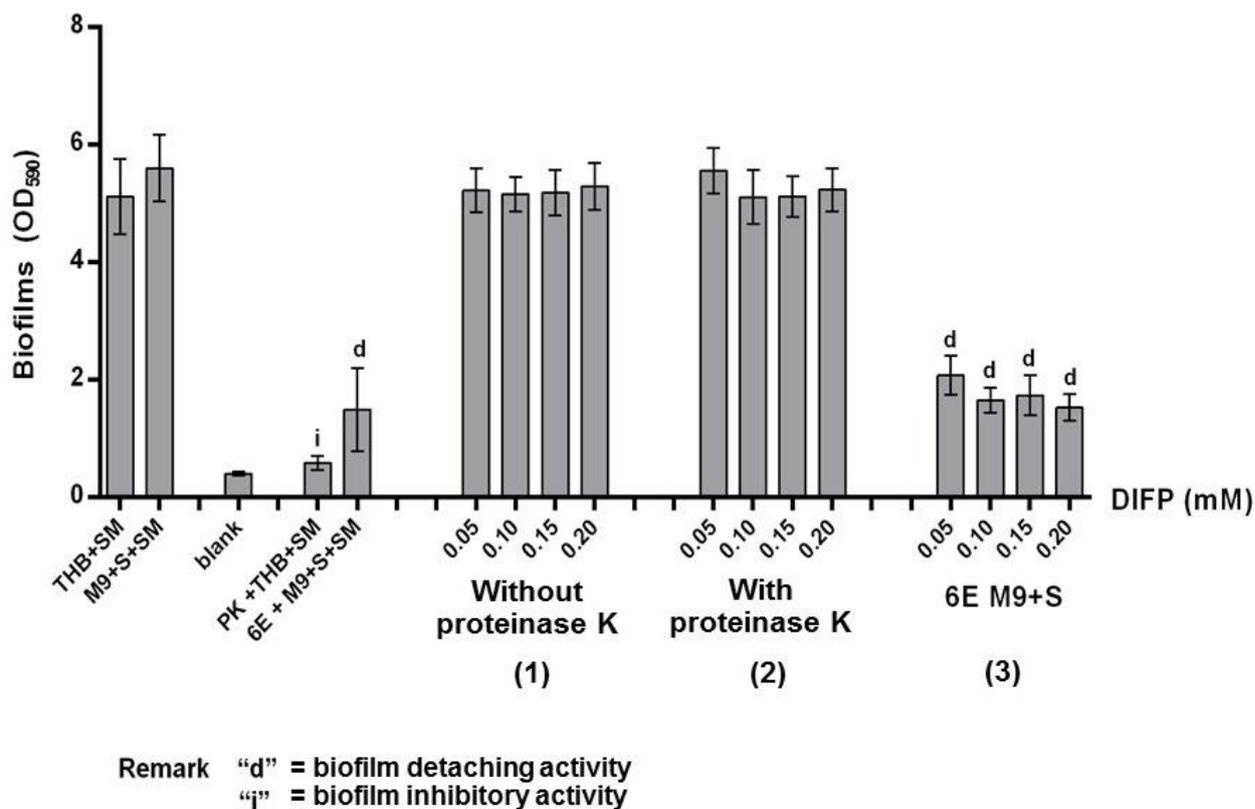


Figure 4.4 Effect of DIFP and proteinase K on the *S. mutans* biofilm detaching activity of supernatant from isolate 6E. Three conditions were examined in the *S. mutans* biofilm assay: (1) in the presence of DIFP only, (2) in the presence of DIFP and proteinase K, and (3) in the presence of DIFP and the filtered supernatant derived from isolate 6E. The amount of biofilm was measured and the results are expressed as the mean \pm standard deviation of three independent experiments.

4.4.2 The effect of proteinase K on *S. mutans* biofilm detaching activity

It was hypothesised that the compound in the filtered supernatant, which was responsible for the biofilm detaching activity, may be a protein, because it was shown to be heat sensitive as described in chapter 3.

Proteinase K, which is an enzyme capable of digesting many different proteins, was selected to test the hypothesis. If the compound was inactivated by proteinase K, it was likely to be a protein or a molecule containing a peptide bond. However, a problem with using proteinase K is that it may also inhibit *S. mutans* biofilm formation and cause detachment by digesting *S. mutans* proteins involved in biofilm formation. To counter

this a proteinase K inhibitor was required to be added before conducting the biofilm assay. Diisofluorophosphate (DIFP) was shown in the experiments described in section 4.4.1 (as shown in Figure 4.4) not to affect *S. mutans* biofilm formation and biofilm detaching activity of the filtered supernatant. DIFP at a final concentration of 0.1 mM was selected to inhibit proteinase K activity before conducting the biofilm assay.

The results in Figure 4.5 show reduction of biofilms in the test groups (labelled 1, 2, 3 and 4). This demonstrates the biofilm detaching activity of filtered supernatant (labelled 1, 2 and 4), the biofilm inhibitory activity of proteinase K (labelled 3). In the test group number 5 (labelled 5), the biofilm detaching activity of the filtered supernatant was not detected when the filtered supernatant was treated with proteinase K followed by DIFP. Control groups (labelled C1 to C7) were conducted in this experiment. *S. mutans* formed biofilms in TH broth supplemented with M9+S and BHI broth (labelled C1, C2 and C3) and labelled C4 is a blank without *S. mutans* culture. *S. mutans* formed biofilms normally in the presence of DIFP alone (labelled C5) or DIFP with proteinase K (labelled C7). This demonstrates that adding DIFP alone or DIFP with proteinase K did not affect *S. mutans* biofilm formation. The biofilm inhibitory activity was detected in the presence of proteinase K alone (labelled C6). This demonstrates that proteinase K inhibited *S. mutans* biofilm formation.

In conclusion, these results suggest that the biofilm detaching activity in the filtered supernatant from 6E grown in M9+S was proteinaceous.

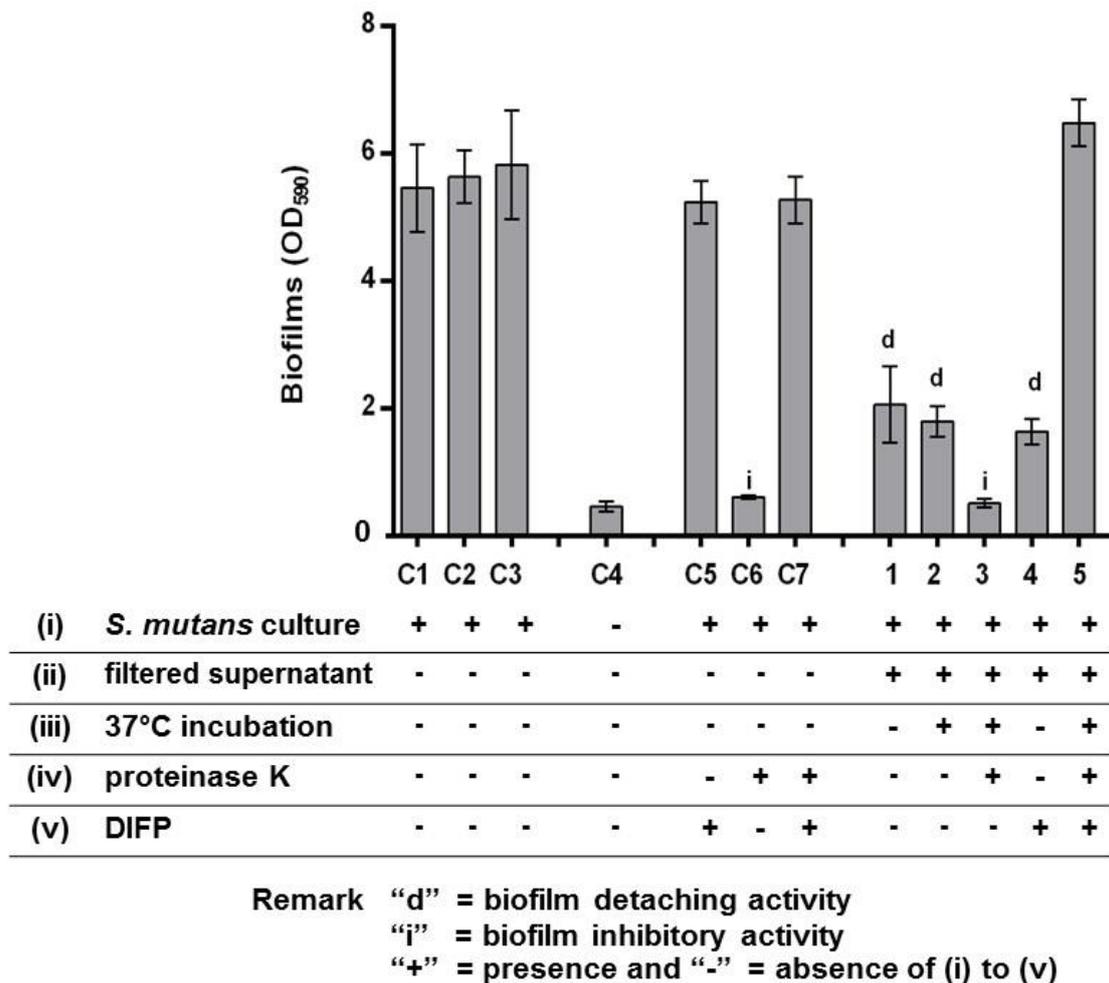


Figure 4.5 Effect of proteinase K on the *S. mutans* biofilm detaching activity of the filtered supernatant from isolate 6E. Proteinase K was added to the filtered supernatants from 6E. The mixtures of the filtered supernatants and the proteinase K were incubated to digest the protein molecules in the filtered supernatants and the proteinase K activity was stopped by adding DIFP. The mixture of the filtered supernatants, proteinase K, and DIFP were tested for *S. mutans* biofilm detaching activity. In the graph, seven control groups were conducted in the *S. mutans* biofilm assay: C1) TH broth, C2) M9+S, C3) BHI broth, C4) blank without *S. mutans*, C5) DIFP only, C6) proteinase K only, C7) both DIFP and proteinase K. Five test groups were examined in the *S. mutans* biofilm assay in the presence of 1) the filtered supernatants, 2) the filtered supernatants incubated at 37°C for 16 h without proteinase K, 3) the filtered supernatants incubated with proteinase K at 37°C for 16 h, 4) the filtered supernatants incubated with DIFP at 37°C for 30 min, 5) the filtered supernatants with proteinase K incubated at 37°C for 16 h and proteinase K activity was stopped by adding DIFP. Biofilm formation was measured and the results are expressed as the mean ± standard deviation of three independent experiments.

4.4.3 Size estimation of the compound(s) with biofilm detaching activity using centrifugal ultrafiltration

Biofilm detaching activity was observed in the 0.22 μm filtered supernatant from isolate 6E. It was established that the compound responsible for the activity was probably a proteinaceous molecule. The size of the compound in the supernatant was determined using a 50 KDa and a 100 KDa centrifugal ultrafiltration devices.

Figure 4.6 shows biofilm formation by *S. mutans* in the presence of the fractions obtained from the 50 KDa and the 100 KDa filters. The biofilm detaching activity was present only in the retentate fractions from the 50 KDa and the 100 KDa filters. This suggests that the size of the protein responsible for the activity was possibly equal to or bigger than 100 KDa.

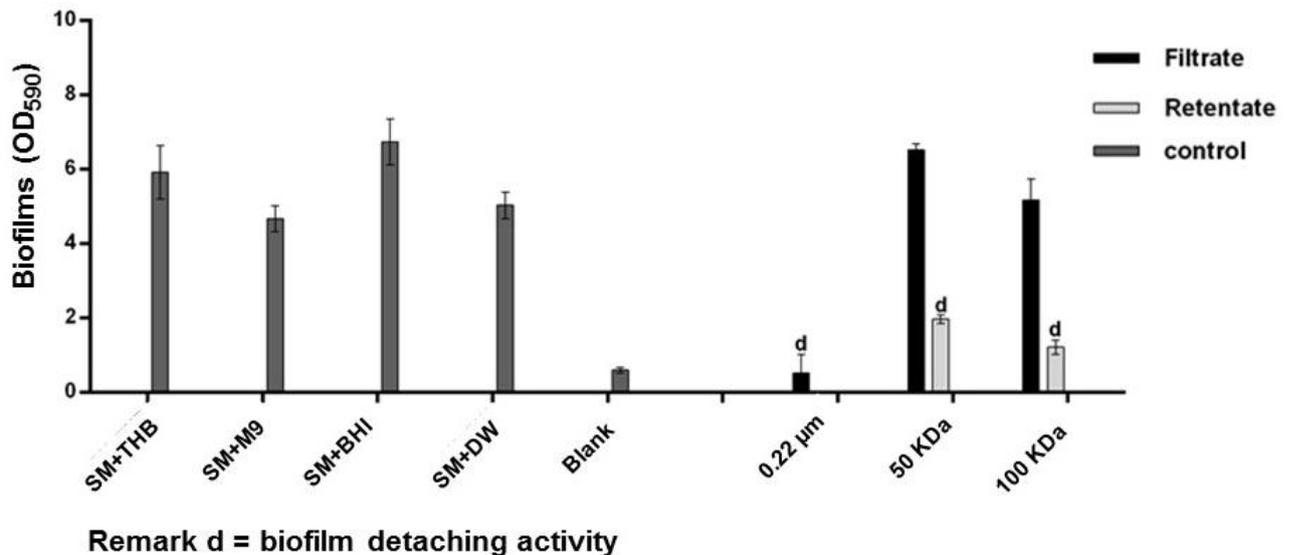


Figure 4.6 Size estimation of the protein responsible for the biofilm detaching activity. Biofilm formation by *S. mutans* in the presence of the fractions: retentate (light grey) and filtrate (black) from 50 KDa and 100 KDa filters. The control groups (dark grey) of *S. mutans* (SM) biofilm in the presence of THB (Todd Hewitt Broth), M9 minimal medium supplemented with 1% sucrose (M9+S), BHI (Brain Heart Infusion broth), Distilled water (DW) and Blank without *S. mutans*. Biofilms were measured and the results are expressed as the mean \pm standard deviation of three independent experiments.

4.4.4 SDS-PAGE analysis of filtered supernatant exhibiting biofilm detaching activity

To analyse the proteins secreted by isolate 6E, SDS-PAGE was performed on supernatants of bacteria grown in 20 ml of BHI medium and M9 minimal medium supplemented with 1% of sucrose or 1% of glucose. The 0.22 μm filtered supernatants of bacterial cultures were concentrated using 100 KDa centrifugal ultrafiltration devices (Amicon® Ultra-15 Centrifugal Filters, Millipore, Germany). The retentate fractions, which could not pass through the membrane filter were used for SDS-PAGE analysis.

Figure 4.7 shows a picture of the SDS-PAGE gel revealing the different protein bands of the fractions derived from 6E grown in different media. There were several different bands of protein when 6E was grown in BHI medium compared to growth in M9 medium. In 6E grown in M9 minimal medium, the protein bands from the medium supplemented with sucrose (M9+S) were different to the protein bands from the medium supplemented with glucose (M9+G), which does not have biofilm detaching activity. Three clear protein bands were present in M9+S of molecular weights of 28 KDa, 48 KDa and 56 KDa and some not so clear bands between 30 KDa and 46 KDa but these bands were strong bands of M9+G. Thus, these protein bands from M9+S were cut out as they could potentially be responsible for the biofilm detaching activity.

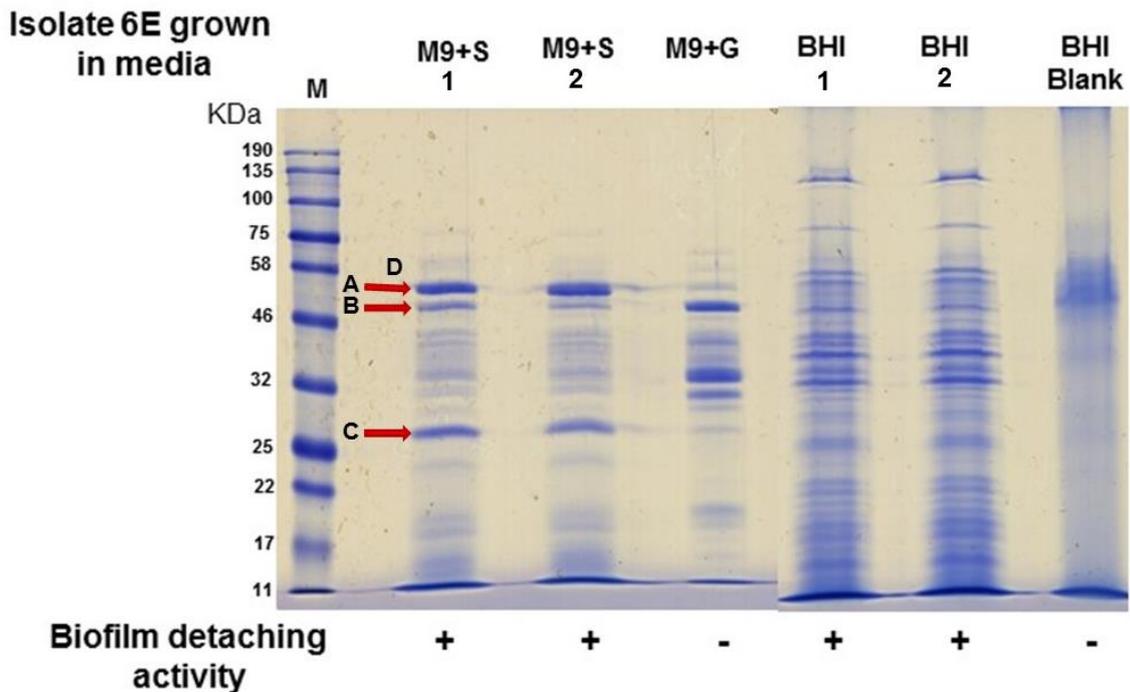


Figure 4.7 Coomassie blue stained SDS-PAGE analysis of the filtered supernatant from isolate 6E grown in different media on a 10% polyacrylamide gel. The filtered supernatants were concentrated using a 100 KDa centrifugal ultrafiltration device. Where, M9+S = M9 minimal medium supplemented with 1% sucrose, M9+G = M9 minimal medium supplemented with 1% glucose, BHI = Brain Heart Infusion medium, 1 and 2 = technical replicates. Red arrows indicate three clear protein bands at the molecular weights of 28 KDa, 48 KDa and 56 KDa. A, B, C and D were selected for protein identification. Biofilm detaching activity of each fraction is shown at the bottom of the gel, where “+” represents the presence of activity while “-” represents no activity. This SDS-PAGE gel is a representative of three experiments.

4.4.5 Identification of protein responsible for biofilm detaching activity

SDS-PAGE analysis revealed protein bands from the filtered *S. mutans* supernatant exhibiting biofilm detaching activity which were not present in proteins from M9+G cultures. Four gel bands from isolate 6E cultured in M9+S: A, B, C and D, (as shown in Figure 4.7) were cut and submitted to FingerPrints Proteomics (University of Dundee, UK) (<http://proteomics.lifesci.dundee.ac.uk/protein-identification-1d-nlc-ms-ms>). Proteins contained in the gel bands were eluted and digested using trypsin. The tryptic fragments of four bands were conducted to LC-MS/MS and generated peptide

mass fingerprint spectra. These unknown spectra were searched in the Unimod (Creasy & Cottrell, 2004) database using Mascot (<http://www.matrixscience.com>).

The results from the peptide fingerprinting are shown in Table 4.1. The top three matching proteins in the gel bands are recorded in the table giving the protein description, protein score, coverage and molecular mass. The most abundant protein in the gel bands A, B, C, and D were catalase, levansucrase, TasA, and alpha-amylase like protein, respectively.

Table 4.1 Protein identification of SDS-PAGE gels bands using LC-MS/MS analysis

| SDS-PAGE gel band | Protein Description | Protein score | Coverage (%) | MW (KDa) | Predicted isoelectric point |
|-------------------|--|---------------|--------------|----------|-----------------------------|
| A | 1. Catalase | 40018.70 | 96.88 | 54.5 | 6.95 |
| | 2. Uncharacterized protein | 4499.34 | 78.26 | 59.2 | 6.02 |
| | 3. Pectin lyase like protein | 3244.77 | 82.26 | 50.9 | 5.92 |
| B | 1. Levansucrase | 24811.83 | 91.12 | 53.0 | 6.64 |
| | 2. Catalase | 2989.70 | 83.58 | 54.5 | 6.95 |
| | 3. Enolase | 2629.67 | 70.47 | 46.6 | 4.77 |
| C | 1. TasA | 15525.43 | 84.67 | 28.1 | 6.83 |
| | 2. Serine alkaline protease (subtilisin E)(EC:3.4.21.62) | 1807.26 | 55.50 | 39.1 | 9.25 |
| | 3. Extracellular serine | 1441.49 | 44.96 | 85.8 | 7.33 |
| D | 1. Alpha-amylase like (EC | 1597.75 | 49.77 | 72.3 | 5.82 |
| | 2. Hypothetical protein | 1489.05 | 26.87 | 83.8 | 5.68 |
| | 3. Catalase | 1111.44 | 49.90 | 54.5 | 6.95 |

Remark

- **Protein Description** When available provides the available protein names from the 'Protein names' section of the corresponding protein's UniProt entry.
- **Protein Score** The sum of the ion scores of all peptides that were identified.
- **Coverage** The percentage of the protein sequence covered by identified peptides.

4.4.6 Effect of levan on the formation of biofilms by *S. mutans*

The most abundant protein in SDS-PAGE gel band B was levansucrase, an enzyme that catalyses the formation of levan: β -2, 6-fructan from sucrose (as shown in Figure 4.8). Levan might affect *S. mutans* biofilm formation or it might mediate biofilm detaching activity. Thus, the ability of levan to cause biofilm detachment was examined. Commercial levan from *Erwinia herbicola* in a range concentration, from 0.0 to 5.0 mg/ml, was added to the *S. mutans* biofilm formation assay.

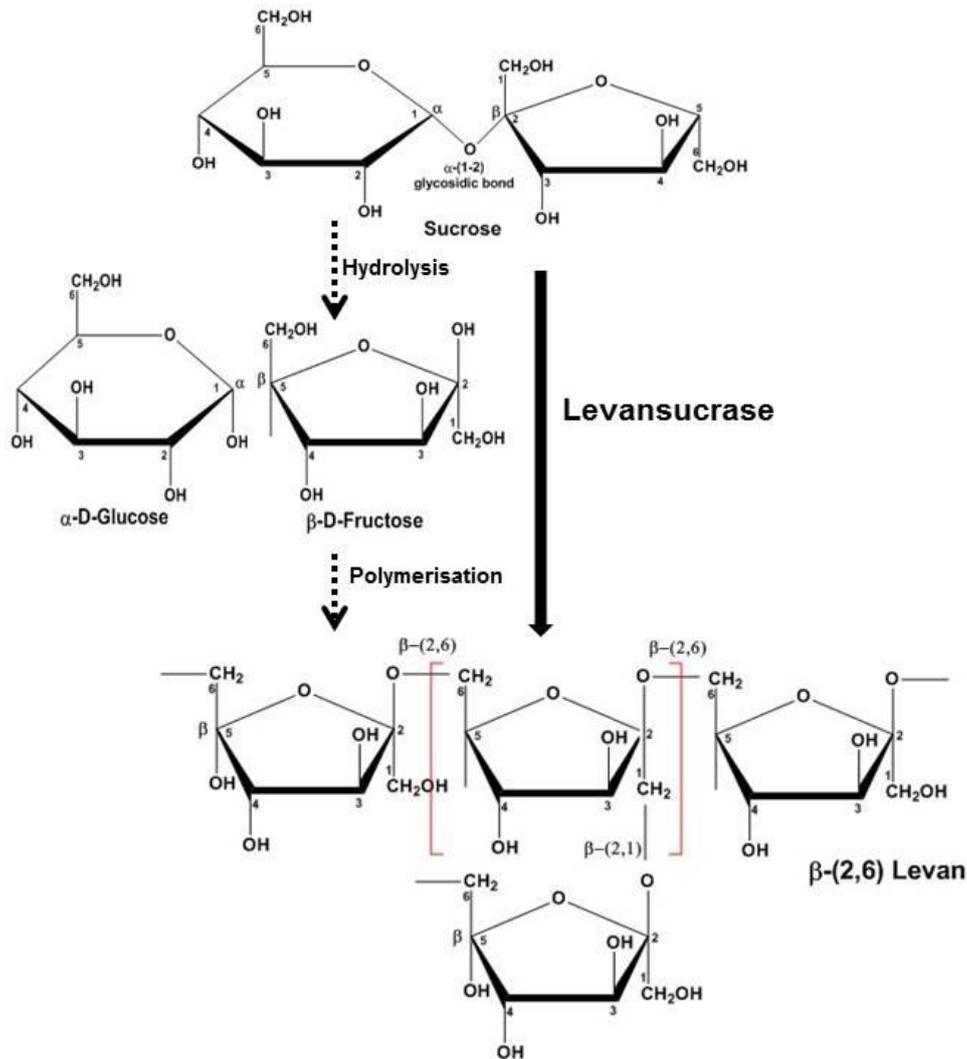


Figure 4.8 Formation and structure of levan with β -2, 6 linkages. The figure was drawn with ChemDraw Professional 16.0 and modified from Santos-Moriano *et al.* (2015), (Srikanth *et al.*, 2015).

Biofilm formation by *S. mutans* in the presence and absence of levan as shown in Figure 4.9. Detached biofilms were not observed at any concentration of levan. However, biofilms were reduced significantly at levan concentrations of 4.00 and 5.0 mg/ml. This suggests that levan at a high concentration could reduce *S. mutans* biofilm formation but levan did not cause biofilm detaching activity.

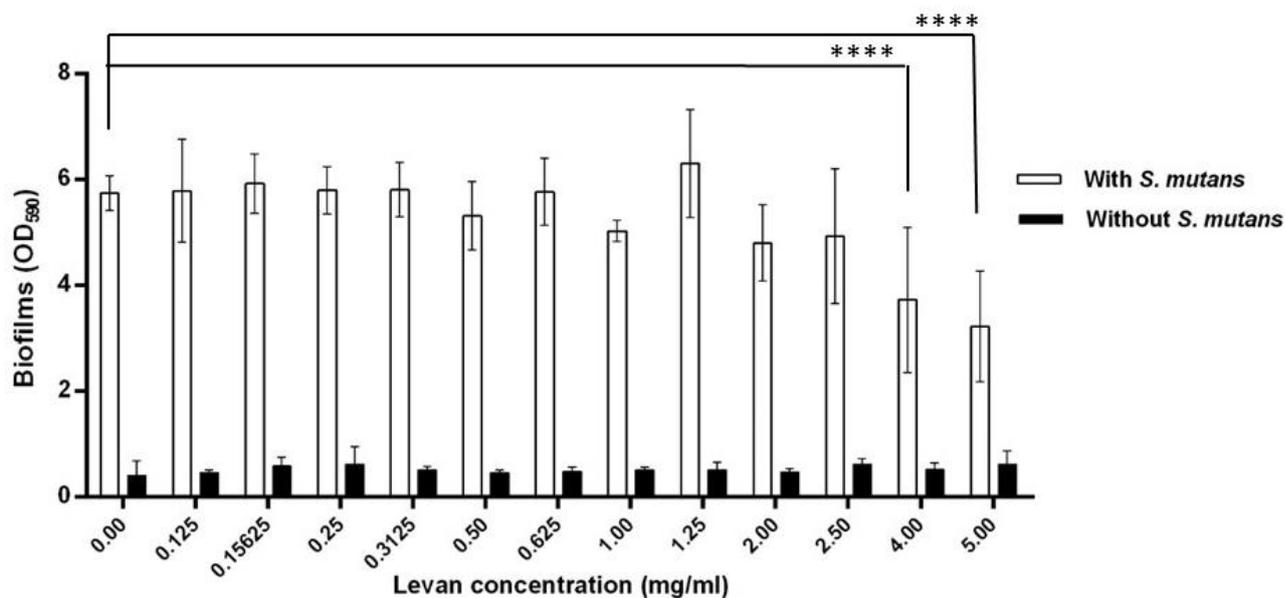


Figure 4.9 Biofilm formation by *S. mutans* in the presence of levan. Levan was added to the *S. mutans* biofilm formation assay at the start of the assay. The figure shows biofilm formation by *S. mutans* in the presence of levan (white) and the control without *S. mutans* (black) as a blank. Biofilms were measured at an OD_{590nm}. The results are expressed as the mean ± standard deviation of four independent experiments. The data were analysed by One-way ANOVA using Dunnett's multiple comparisons test. Statistical significance is indicated by **** representing p < 0.0001.

4.4.7 Determination of biofilm detaching activity on saliva-coated surfaces

4.4.7.1 Determination of biofilm detaching activity from 6E grown in M9 minimal medium supplemented with sucrose (M9+S) on saliva-coated surfaces

The biofilm assay was conducted on a saliva-coated polystyrene surface in order to simulate the coating on the tooth surface in the oral cavity. *S. mutans* formed biofilms on saliva-coated surfaces in the presence of the filtered supernatant from isolate 6E grown in M9+S. The control group was *S. mutans* forming biofilms on surfaces which had not been coated with saliva as described in section 2.2 in Chapter 2. In Figure 4.10, the biofilm detaching activity of the filtered supernatant was not detected when the biofilm assay was conducted on saliva-coated surfaces. However, the activity was still observed when the biofilm assay was conducted on surfaces without a saliva

coating. These results that the biofilm detaching activity from 6E grown in M9+S is not effective on biofilms on saliva coated surfaces.

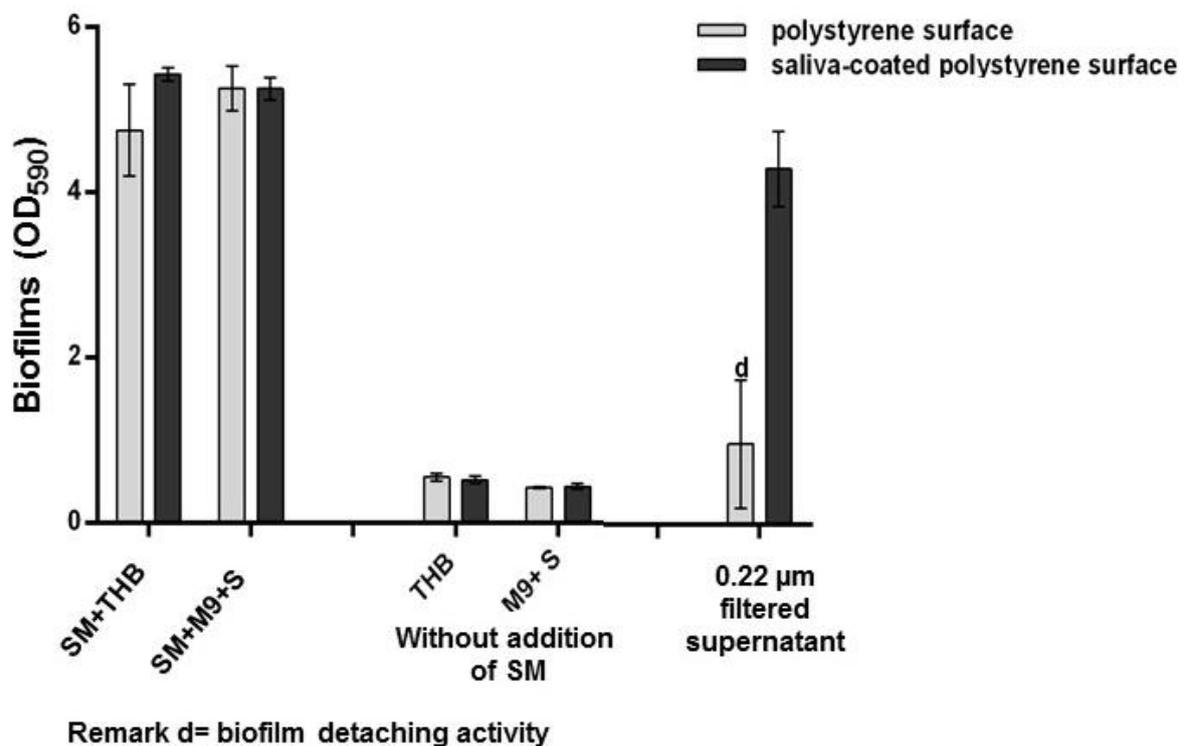


Figure 4.10 Biofilm formation by *S. mutans* on uncoated or saliva-coated well surfaces. In the biofilm assay, 0.22 µm filtered supernatant from 6E grown in M9 medium supplemented with 1% sucrose was added to saliva-coated well surfaces (black) or wells that were not coated (grey) as a positive control. Negative controls were 100 µl of Todd Hewitt broth (SM+THB) or 100 µl of M9 medium supplemented with 1% sucrose (SM+M9+S). Biofilms were measured at an OD_{590nm} after crystal violet staining. The results are expressed as the mean ± standard deviation of three independent experiments.

4.4.7.2 Determination of biofilm detaching activity from supernatants of isolate 3C, 6E, 8E, 9C, 9E, 10G, and 11E grown in BHI medium on saliva-coated surfaces

The biofilm detaching activity produced by isolate 6E grown in M9 medium supplemented with sucrose could be different from that produced by growth in BHI medium. The 0.22 µm filtered supernatants from all seven isolates, which were grown in BHI broth, were tested for the capacity to detach biofilms on the saliva-coated surfaces. Figure 4.11 shows the biofilms in the presence of the filtered supernatants

from seven isolates. The biofilm detaching activity was only observed when the biofilm assay was conducted on polystyrene surfaces but not on the saliva-coated polystyrene surfaces. These results show that the biofilm detaching activity from seven isolates grown in BHI broth is not effective on biofilms formed on saliva coated surfaces.

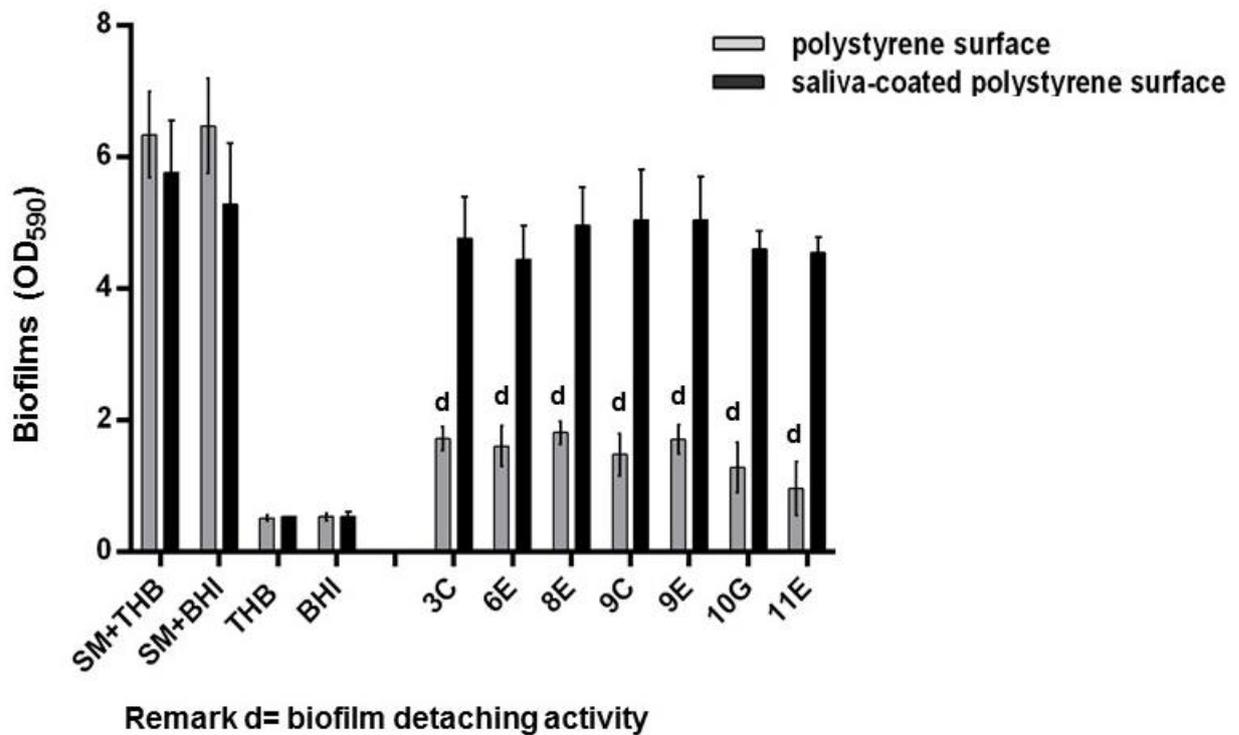


Figure 4.11 Biofilm formation by *S. mutans* on uncoated or saliva-coated well surfaces in the presence of filtered supernatants from seven isolates grown in BHI broth. The 0.22 μm filtered supernatants from 3C, 6E, 8E, 9C, 9E, 10G, and 11E which were grown in BHI broth were added to the wells with saliva-coated surfaces (black) and were not coated (grey) as a positive control. Negative controls were 100 μl of Todd Hewitt broth (SM+THB) or 100 μl of BHI broth (SM+BHI). Blanks without the addition of *S. mutans* were conducted. Biofilms were measured at an $\text{OD}_{590\text{nm}}$ after staining with crystal violet. The results are expressed as the mean \pm standard deviation of three independent experiments.

4.5 Discussion

The results detailed in chapter 3 showed that the 0.22 μm filtered supernatant from isolate 6E lost biofilm detaching activity after boiling in water at 100°C for 15 min. This result suggested that a protein might be responsible for the biofilm detaching activity. Proteinase K, a serine endopeptidase (or serine protease), is an enzyme that

hydrolyses peptide bonds of protein molecules (Jany *et al.*, 1986). Proteinase K is stable under a broad range of conditions and can completely digest many proteins to dipeptides. Thus many studies use proteinase K to digest proteins (Link *et al.*, 1999, Washburn *et al.*, 2001, Wu *et al.*, 2003, Shukla & Rao, 2013).

In this study proteinase K was used to determine if it could digest the compound responsible for biofilm detaching activity in the filtered supernatant. The data shown in Figure 4.4 indicated that biofilm inhibitory activity was observed in the presence of proteinase K alone in *S. mutans* biofilm assays. This result suggested that proteinase K inhibited *S. mutans* biofilm formation. This result is consistent with findings in some other bacteria (Fleming & Rumbaugh, 2017). The biofilm inhibitory activity of proteinase K could be because several *S. mutans* proteins are crucial in the process of biofilm formation such as adhesins, glycosyltransferases, and glucan binding protein (Nobbs *et al.*, 2009, Lamont & Jenkinson, 2010, Krzysciak *et al.*, 2014).

To overcome the problem with proteinase K masking inhibition of the biofilm detaching activity, the proteinase K in the mixture with the filtered supernatant needed to be inhibited before testing in the biofilm assay. To inhibit the activity of proteinase K, a serine protease inhibitor, Diisofluorophosphate (DIFP) was used. DIFP is an irreversible inhibitor of serine proteases. An example of the inhibitory mechanism of DIFP against serine proteases is depicted in Figure 4.12. The hydroxyl group of the active site serine residue of the catalytic triad is fluorophosphorylated by the fluorophosphate group of DIFP upon catalytic processing of DIFP by the enzyme. This reaction mimics the catalytic mechanism of the serine proteases and does not affect the other 27 serine residues of chymotrypsin, a serine protease. The

diisopropylphosphoryl derivative of the serine protease is stable and results in a totally inactive enzyme (Oda *et al.*, 1998).

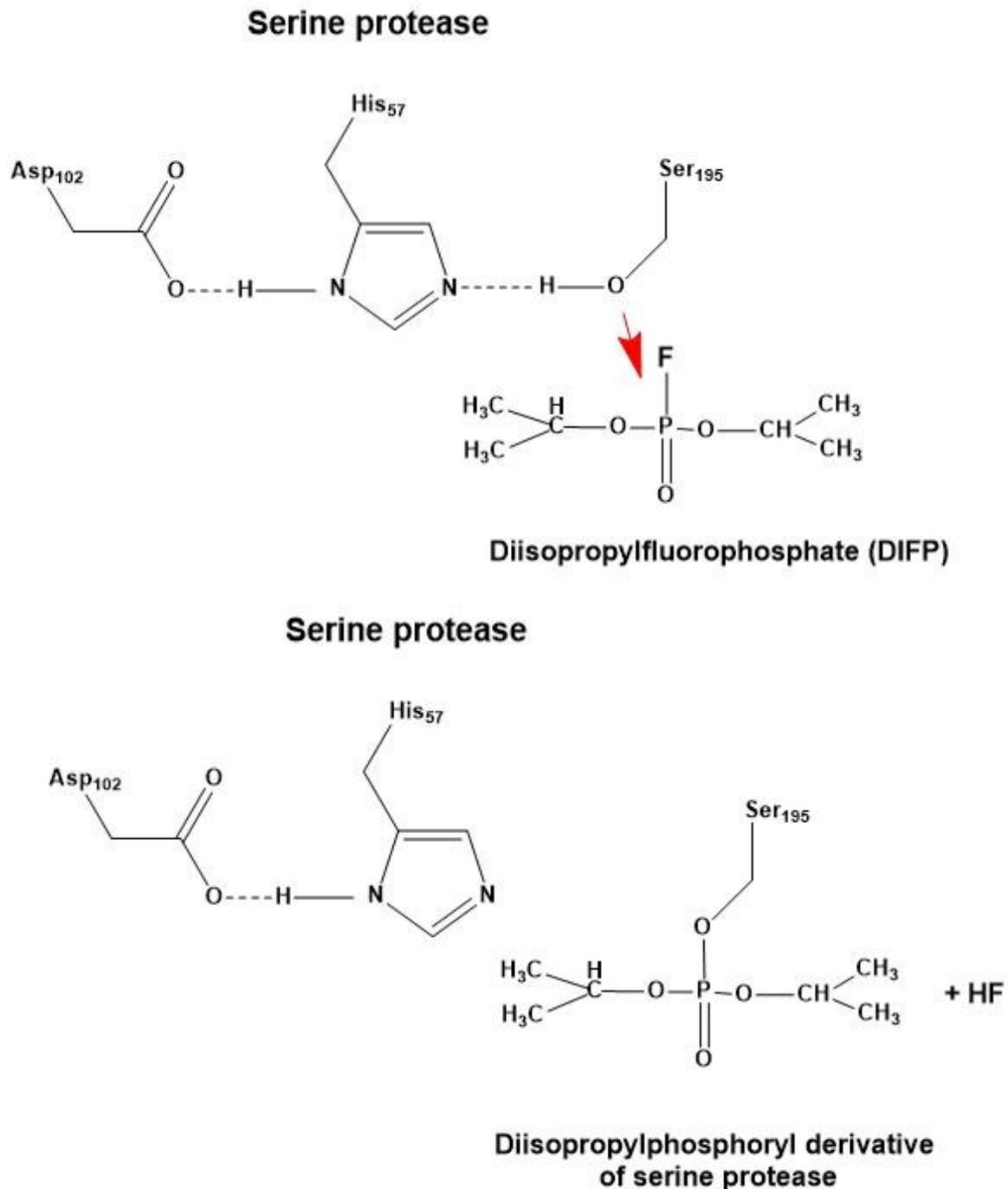


Figure 4.12 Inhibitory reaction of DIFP against the catalytic triad active site of the serine protease chymotrypsin. The hydroxyl group of active site serine residue of the serine protease reacts with the fluorophosphate group of DIFP. The product of this reaction is a diisopropylphosphoryl derivative of the serine protease which is an inactive serine protease. The figure was drawn using ChemDraw Professional 16.0 and modified from Bachovchin (1986).

After the filtered supernatant was treated with proteinase K for 16 h and the proteinase K activity was inhibited by DIFP, the biofilm detaching activity of the filtered supernatant was not detected. These results suggested that the compound responsible for biofilm detaching activity was digested by proteinase K. Thus, this experiment established that the compound was a protein or a molecule containing peptide bonds.

The size of the compound responsible for biofilm detaching activity was estimated in this study. The results in section 4.4.3 suggested that the compound responsible for biofilm detaching activity may be greater than 100 KDa, However, it is possible for a protein which did not pass through the 100 KDa filters to have a size less than 100 KDa. For instance, protein-protein interaction might occur during filtration.

Protein identification conducted by FingerPrints Proteomics (University of Dundee) revealed possible proteins in the SDS-PAGE gels of a fraction exhibiting biofilm detaching activity (as shown in Table 4.1). In band A, the major component was catalase but other proteins were present such as an uncharacterized protein YqcC and a pectin lyase like protein. Catalase is an enzyme, which hydrolyses hydrogen peroxide (H_2O_2) to water and oxygen. Some commensal oral bacteria (*S. gordonii* and *S. sanguinis*) that compete with *S. mutans* produce H_2O_2 (Ryan and Kleinberg (1995). *S. mutans* does not produce catalase but it produces other ROS detoxification enzymes such as superoxide dismutases, alkyl hydroperoxidase reductase and glutathione oxidoreductase (Gonzalez et al., 2012). Alkyl hydroperoxidase reductase (EC1.11.1.15) is a member of the peroxidase family of enzymes (<http://www.enzyme-database.org/class.php>). It is unlikely that catalase identified from band A is involved in biofilm detaching activity.

Pectin lyase like protein has not been reported to be involved with *S. mutans* biofilm formation. Pectin is not identified to be one of the components of EPS of *S. mutans* biofilms. A recent study (Kawarai 2016) reported that pectin, galacturonic acid, in green tea could enhance *S. mutans* biofilm formation.

The gene *yqcC*, an uncharacterized gene encoding a putative extracytoplasmic protein, has not been reported in *S. mutans* but it has been reported that it was overexpressed in biofilm versus exponential growth phase of *E. coli* K-12 (Beloin et al., 2004).

One of the major proteins identified in band C was TasA which is an extracellular protein associated with the sporulation of *Bacillus subtilis* (Stöver & Driks, 1999). Recently TasA has been found to be an important protein in the exopolysaccharide matrix of floating biofilms of *B. subtilis* (Branda et al., 2006, Romero et al., 2010). TasA has been proposed to form amyloid fibres and these fibres are responsible for cell aggregation and the integrity of the extracellular matrix in the biofilm structure (Romero et al., 2010). TasA also exhibits antimicrobial activity against Gram-positive and Gram-negative bacteria including *E. coli* BL21(DE3), *B. subtilis* PY79, *Enterococcus aerogenes* W31851-1, *Agrobacterium tumefaciens* GV3101 and *Erwinia amylovora* EG321 (Stöver & Driks, 1999). Thus, it is possible that TasA might be response for the biofilm detaching activity.

In band B, the major protein was identified as levansucrase, a fructosyltransferase enzyme which synthesises levan from sucrose. The properties of levan such as low intrinsic viscosity, water solubility and high tensile strength result in levan being a compact polymer (Velazquez-Hernandez et al., 2011). Levansucrase has been

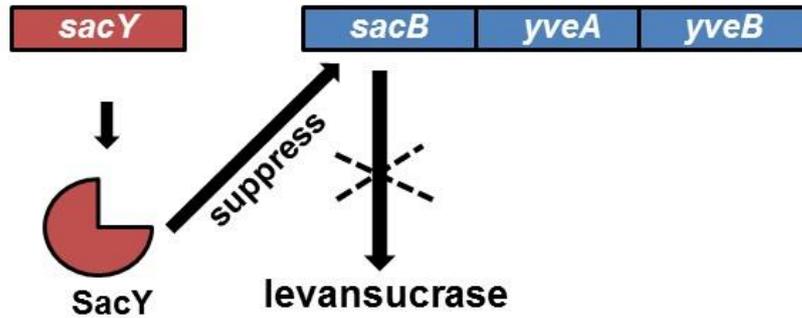
reported to be involved in biofilm formation by *Gluconacetobacter diazotrophicus*. A study reported that levansucrase might be involved in reducing cell adherence on glass surfaces and it might be involved in the reduction of cell density of mature biofilms on inert or abiotic surfaces (Velazquez-Hernandez *et al.*, 2011).

S. mutans produces levansucrase (Ajdic *et al.*, 2002) and the levan might be an extracellular storage compound produced during periods of nutrient starvation in *S. mutans* biofilms (Burne *et al.*, 1996, Laue *et al.*, 2006). It was reported that levan plays an important role in the adhesion and colonisation of *S. mutans* and pioneer colonisers of saliva-coated hydroxyapatite surfaces (Rozen *et al.*, 2001). Interestingly the biofilm detaching activity was present when isolate 6E was grown in the M9 minimal medium supplemented with sucrose only and the levansucrase gene in *B. subtilis* is controlled by a sucrose-inducible anti-termination mechanism (Crutz *et al.*, 1990, Oner *et al.*, 2016). Levansucrase is encoded by *sacB* which is a part of *sacB yveB yveA* levansucrase tricistronic operon (as shown in Figure 4.13). In the absence of sucrose, SacY inhibits the levansucrase operon and represses *sacB*. In the presence of sucrose, sucrose binds to SacY and this allows the transcription of the *sacB* gene.

It is possible that the effect of levansucrase was caused by depletion of sucrose in the biofilm assay medium. Sucrose is not only a substrate for levansucrase but also a substrate of glucosyltransferases (GTFs) to synthesise glucans. The reduction of sucrose in the growth medium could lead to less production of glucans which are important for *S. mutans* biofilm formation such as adhesion on surface. In addition, in the biofilm assay levansucrase could use the sucrose present in the medium to synthesise levan. Thus, levan might play a role in the biofilm detaching activity. However, the results in this chapter demonstrated that levan did not show biofilm

detaching activity. Levan produced from Gram-positive and Gram-negative bacteria are different (Oner *et al.*, 2016). The different characteristics of levan, from different sources, might cause levan to have different biological activities (Li *et al.*, 2015). For example, a commercial levan was used in this study derived from *E. herbicola*. A study reports that molecular weight of levan produced from *E. herbicola* ATCC 15552 was $1.1-1.6 \times 10^6$ Da (Keith *et al.*, 1991), which was higher when it was compared to levan produced from *B. subtilis* CCT 7712 (568 KDa) (Dos Santos *et al.*, 2013), and *B. subtilis* (natto) Takahashi (1794 KDa) (Shih *et al.*, 2005). Thus, due to the different size, the levan used in this study might not represent levan produced from isolate 6E. The better experiment to determine the effect of levansucrase on *S. mutans* biofilm formation is the addition of levan from *Bacillus* sp. or levansucrase enzyme into the biofilm assay.

A. Without sucrose



B. With sucrose

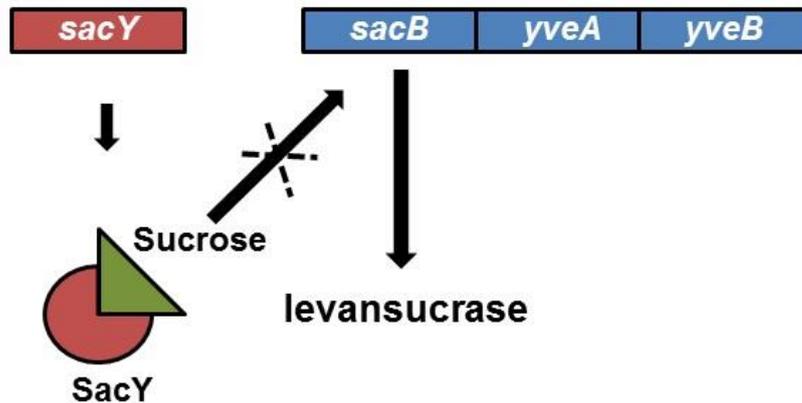


Figure 4.13 Schematic representation of *sacB-yveB-yveA* levansucrase tricistronic operon in *Bacillus* sp. In the absence of sucrose (A), *sacB* gene of tricistronic operon is suppressed by SacY. In the presence of sucrose (B), sucrose binds to SacY and this allows the transcription of *sacB* gene. The figure was drawn using MS PowerPoint and is based on an illustration by Dogsa *et al.* (2013).

In the oral cavity, tooth surfaces are covered by salivary pellicles most of the time. The biofilm assay was conducted on a saliva-coated surface in order to simulate the coating on the tooth surface in the oral cavity and it was found that the supernatant for 6E could not detach the *S. mutans* biofilm.

Saliva contains proteins, electrolytes and water (De Almeida *et al.*, 2008). Some of these salivary pellicles have been reported to be involved in mediation of bacterial adhesion. For example, salivary agglutinin (or gp340) is a glycoprotein that plays an

important role in mediating the adhesion and aggregation of *S. mutans*. Salivary agglutinin binds to an adhesin P1 on *S. mutans* cell surface (Ahn *et al.*, 2008). Mucin is a high-molecular-weight glycoprotein. Carpenter (2013) reported that the terminal sialic acid of this glycoprotein plays an important role in binding to bacterial cells. These salivary proteins which would be adhered to the polystyrene surface might affect the nature of the biofilm attachment to the surface and therefore the ability of compounds derived from isolate 6E to cause biofilm detachment. Several studies have investigated proteolytic activity from human saliva such as cysteine proteases (Isemura *et al.*, 1984) and serine protease (Meiser *et al.*, 2010). It is possible that the compound, which was a protein, responsible for biofilm detaching activity might be affected by these proteases.

4.6 Conclusion

This chapter describes the characterisation of a compound secreted by isolate 6E which had the ability to detach *S. mutans* biofilms formed on uncoated polystyrene surfaces. This compound could be produced in M9 minimal medium supplemented with sucrose. The compound was found to be a protein and could possibly be levansucrase, TasA or an uncharacterised protein YqcC. The biofilm detaching activity was absent when conducting the biofilm assay on saliva-coated polystyrene surfaces. Therefore, the compound might be applicable to dental caries prevention.

CHAPTER 5

Investigation and purification of the compound with *S. mutans* biofilm inhibitory activity

Chapter 5 Investigation and purification of the compound with *S. mutans* biofilm inhibitory activity

5.1 Introduction

Beside the biofilm detaching activity mentioned in previous chapters, biofilm inhibitory activity was also observed in the 0.22 μm filtered supernatant of isolate 6E, 10G and 11E. In the work described in this chapter, the culture volume was scaled up for one isolate, isolate 6E, to provide large quantities of the biofilm inhibitory activity for purification of the compound responsible.

5.2 Aims of the work described in this chapter

To investigate and purify the compound responsible for the biofilm inhibitory activity present in the supernatant from growth of isolate 6E.

5.3 Materials and Methods

5.3.1 Size estimation of the compound with biofilm inhibitory activity using centrifugal ultrafiltration

A volume of 500 μl of 0.22 μm filtered supernatant was added to 0.5 ml Amicon centrifugal filters (Amicon® Ultra-0.5 Centrifugal Filters, Millipore, Germany) with different pore sizes; 3 KDa, 30 KDa, 50 KDa, and 100 KDa, and they were centrifuged at 14,000 x g for 30 min. Deionised water (500 μl) was added to the retentate, which had not passed through the filter membrane, and pipetted up and down. The filter housing was then turned upside down in a clean tube and centrifuged again at 14,000 x g for 10 min. The retentates and filtrates from all of the filters were tested for biofilm inhibitory activity.

5.3.2 Scale-up production of the compound with biofilm inhibitory activity

A single colony was inoculated into 4 ml of BHI broth, cultured at 37°C for 18 h and used to inoculate 200 ml of BHI broth in a one litre Duran bottle and incubated at 37°C with shaking at 200 rpm for 24 h. A volume of 3 ml of bacterial culture at 3 h, 5 h, 8 h after inoculation was taken to measure the optical density at a wavelength of 600 nm and to measure the biofilm inhibitory activity. The bacterial cultures were centrifuged at 5,000 x g for 10 min and the supernatant was filtered using a 0.22 µm filter. The filtrate was tested for biofilm inhibitory activity.

5.3.3 Effect of DIFP on the biofilm inhibitory activity of culture supernatant from isolate 6E

A concentration of 0.1 mM of DIFP was added to 4 ml of 0.22 µm filtered supernatant from isolate 6E culture and supernatant without DIFP was used as a control. After 3 h of incubation, 3 ml of the 0.22 µm filtered supernatant treated with DIFP and the untreated control were injected into individual 10 KDa dialysis cassettes, and dialysed against 400 ml of deionised water at 4°C. Deionised water was changed three times after 3 h and then left for 16 h overnight. After dialysis the volume of supernatants had increased so the samples were concentrated using Amicon Ultra 0.5 ml centrifugal filters with a molecular weight cut-off of (MWCO) 3 KDa (Sigma Aldrich, UK). The retentate fractions from the filter were recovered by adding deionised water to make a volume of 500 µl. The solution in the filter was pipetted up and down. The filter housing was then turned upside down in a clean tube and centrifuged again at 14,000 x g for 10 min. The retentates and filtrates from all of the filters were tested for biofilm inhibitory activity.

5.3.4 Azocasein protease assay

To measure the proteolytic enzyme activity of the supernatants, filtered supernatants were tested at dilutions of 1, $\frac{1}{2}$, $\frac{1}{4}$, $\frac{1}{8}$, and $\frac{1}{16}$. A volume of 100 μl of the supernatants and dilutions were added to wells of a 96 well plate containing 50 μl of 6 % azocasein (w/v) in 0.5 M Tris pH 7.0. The microtitre plate was incubated at 37°C for 30 min in the dark. To stop the reaction, 150 μl of 20 % acetic acid was added to each well and the denatured protein was pelleted by centrifugation at 1,500 x g for 15 min. A volume of 75 μl of supernatants was removed gently in triplicate. The absorbance was read at 450 nm using a Dynex plate reader. A 100 μl volume of BHI broth was used as a blank and 0.1 mg/ml of Proteinase K was used as a positive control.

5.3.5 Bradford protein assay of fractions with biofilm inhibitory activity

Samples from the purification process of the compound responsible for the biofilm inhibitory activity were measured to estimate the amount of protein using a Bradford protein assay kit (Thermo Scientific, UK). A volume of 5 to 10 μl of samples was added to a well of a microtitre plate and mixed with 250 μl of Bradford reagent. The microtitre plate was shaken at 100 rpm for 30 s and incubated at room temperature for 30 min before measuring the absorbance at 590 nm. Protein concentration was determined based on a bovine serum albumin (BSA) standard curve.

5.3.6 Ammonium sulfate protein precipitation

To precipitate protein with biofilm inhibitory activity, solid ammonium sulfate was added to the 0.22 μm filtered supernatant at 4°C. Proteins in the filtered supernatant were fractionated by precipitating at two different ammonium sulfate saturations which

were 30% and 30-75%. After incubating for 18 h at 4°C, the 30% saturated ammonium sulfate solution was centrifuged at 12,000 x g at 4°C for 20 min. The protein pellet was resuspended in 21 ml of HPLC water and kept at 4°C until use. Solid ammonium sulfate was added to the supernatant fraction to bring it to 75% saturation. After incubating for 18 h at 4°C the 75% saturated ammonium sulfate solution was centrifuged at 12,000 x g at 4°C for 20 min. The protein pellet was resuspended in 80 ml of HPLC water and kept at 4°C until use. The amount of solid ammonium sulfate added to achieve the correct saturations were determined using an online calculator at <http://www.encorbio.com/protocols/AM-SO4.htm>. The 30% and 30-75% precipitated proteins were tested for biofilm inhibitory activity, protease activity, and the amount of protein by Bradford assay.

Hydrophobic interaction column chromatography using HiTrap Phenyl Fast Flow (High Sub) Column preparation: A 1 ml HiTrap Phenyl FF column was washed using 10 ml of binding buffer (1.0 M ammonium sulfate, 50 mM sodium phosphate pH 7.0) with a 1 ml/min flow rate.

Sample preparation: A volume of 1 ml of double strength binding buffer (2.0 M ammonium sulfate, 100 mM sodium phosphate pH 7.0) was added to 1 ml of the 30-75% ammonium sulfate protein fraction and centrifuged at 4,500 x g at 4°C for 20 min. The resulting supernatant was filtered using a 0.22 µm filter. The 0.22 µm filtrate was injected onto the Phenyl FF column at a flow rate of 1 ml/min. The solution which drained out while loading the 2 ml of sample was collected in 1 ml fractions and named unbound 1 (UB1) and unbound (UB2). Then the column was washed using 10 ml of binding buffer and ten fractions of 1 ml each were collected and labelled W1 to W10. After that, bound proteins were eluted using 10 ml of elution buffer (50 mM sodium phosphate pH 7.0) and ten fractions of 1 ml each were collected and labelled F1 to

F10. The Phenyl FF column was washed again using 10 ml of binding buffer. The column was stored in 20% ethanol by washing with 10 ml of 20% ethanol.

5.3.7 HiTrap benzamidine Fast Flow column chromatography

Column preparation: A 1 ml HiTrap Benzamidine FF (USA) column was washed using 10 ml of binding buffer (20 mM Tris-HCl, 0.5 M NaCl pH 7.4) with a 1 ml/min flow rate.

Sample preparation: A volume of 1 ml of double strength binding buffer (40 mM Tris-HCl, 1.0 M NaCl pH 7.4) was added to 1 ml of the 30-75% ammonium sulfate protein fraction and centrifuged at 4,500 x g at 4°C for 20 min. The resulting supernatant was filtered using a 0.22 µm filter. The 0.22 µm filtrate was injected onto the benzamidine affinity column at a flow rate of 1 ml/min. The solution which drained out while loading the 2 ml of sample was collected in 1 ml fractions and named unbound 1 (UB1) and unbound 2 (UB2). Then the column was washed using 10 ml of binding buffer and ten fractions of 1 ml each were collected and labelled W1 to W10. After that, bound proteins were eluted using 10 ml of elution buffer (50 mM glycine pH 3.0) and ten fractions of 1 ml each were collected and labelled F1 to F10 fraction. The benzamidine column was washed again using 10 ml of binding buffer. The column was stored in 0.05 M acetate buffer, pH 4.0 containing 20% ethanol by washing with 10 ml of the storage buffer.

5.3.8 Ion exchange column chromatography

Two types of ion exchange column chromatography, cation and anion exchange chromatography were conducted to attempt to separate the protein responsible for biofilm inhibitory activity. A High S cation exchange column buffered at pH 5.0 and 6.5

was used for cation exchange and a Bio-Scale Mini Macro-Prep DEAE Cartridge (Bio-Rad, UK) buffered at pH 7.0 and 8.0 was used for anion exchange.

Column preparation: The High S and DEAE columns were equilibrated with 25 ml of 50 mM sodium phosphate buffer of the appropriate pH at a flow rate of 5 ml/min.

Sample preparation: A volume of 30 ml of 0.22 μm filtered supernatant from isolate 6E which was grown in 200 ml BHI medium was mixed with 90 ml of deionised water to dilute the salt concentration of BHI. Proteins in this sample were desired to be captured by DEAE or sulfur trioxide molecules in ion exchange columns. Some proteins were adjusted pH to be negative charge or positive charge before applying to the anion and cation columns, respectively. To do this, a volume of 120 ml of 0.1 M sodium phosphate buffer pH 5.0 or 6.5 for the High S column and pH 7.0 or 8.0 for the DEAE column, was added to the mixture of filtered supernatant and deionised water.

A volume of 240 ml of the mixture of the filtered supernatant, deionised water and 50 mM sodium phosphate buffer was applied to the column at a flow rate of 5 ml/min. In this step, some unbound proteins passed through the column. The remainder of the unbound proteins were eluted with 25 ml of 50 mM sodium phosphate buffer at a flow rate of 5 ml/min. Bound proteins were eluted with 10 ml of 1.0 M sodium chloride in 50 mM sodium phosphate buffer at a flow rate of 5 ml/min. The columns were regenerated with 10 ml of 2.0 M sodium chloride in 50 mM sodium phosphate buffer

pH 6.7. The columns were washed again using 25 ml of deionised water and stored in 20% ethanol.

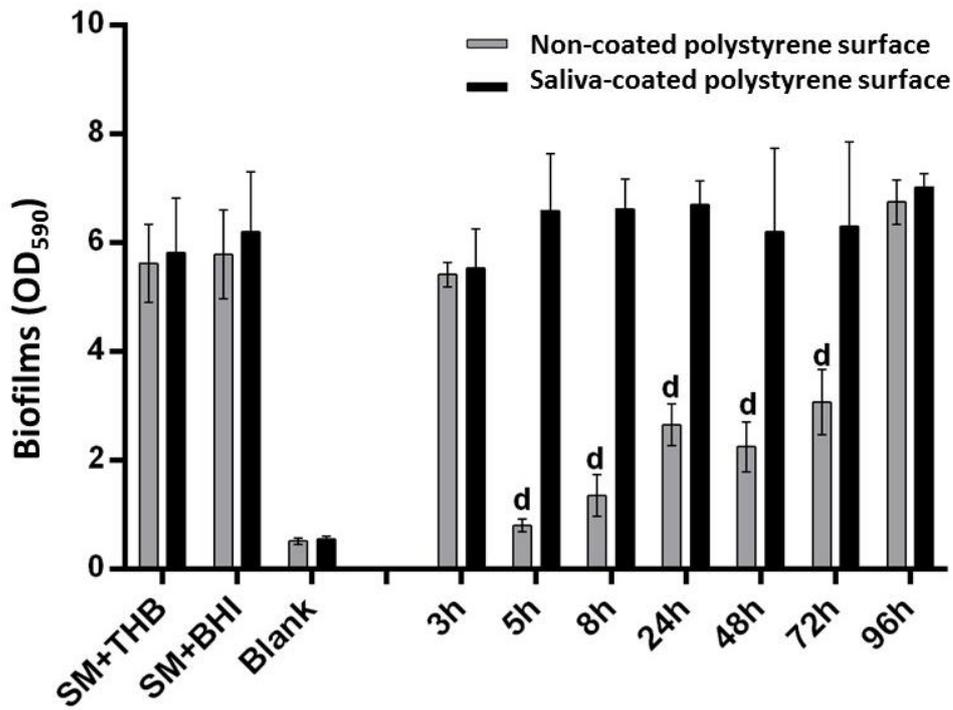
5.4 Results

5.4.1 Determination of ability of isolate 6E to produce biofilm inhibitory activity

In chapter 3, seven isolates were found to produce a compound(s) exhibiting biofilm detaching activity when they were grown in 20 ml of BHI broth for 24 h at 37 °C. In addition to the detaching activity, the supernatants of isolate 6E obtained from 72 h and 96 h cultures had biofilm inhibitory activity (as shown in Figure 3.17). It was hypothesised that the other six isolates might also be able to produce a with biofilm inhibitory activity. The seven isolates were screened for the production of biofilm inhibitory activity in culture supernatants at 3 h, 5 h, 8 h, 24 h, 48 h, 72 h and 96 h of incubation. Their supernatants were collected and centrifuged, filtered using a 0.22 µm filter and tested for *S. mutans* biofilm inhibitory activity using uncoated and saliva-coated polystyrene 96-well microtitre plates.

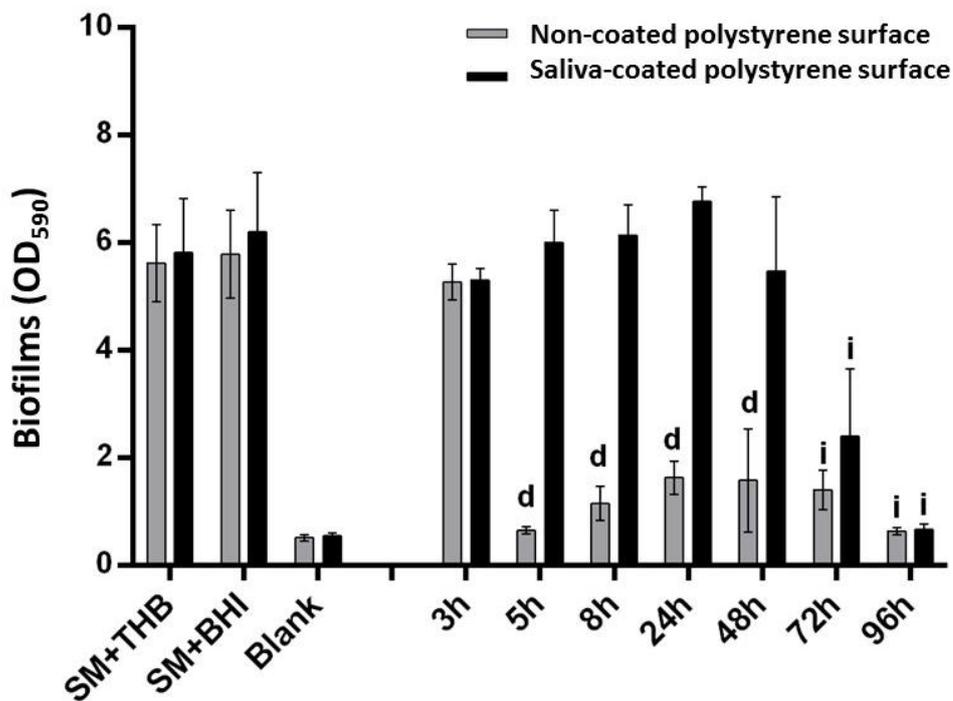
Figure 5.1 shows biofilm formation by *S. mutans* in the presence of supernatants from isolates 3C, 6E, 8E, 9C, 9E, 10G and 11E at 3 h, 5 h, 8 h, 24 h, 48 h, 72 h and 96 h of incubation. Biofilm inhibitory activity was produced in the bacterial cultures, at 72 h and 96 h of isolates 6E, 10G and 11E but not in isolates 3C, 8E, 9C and 9E. The biofilm inhibitory activity was found to be effective against *S. mutans* biofilms upon growth on non-coated and saliva-coated polystyrene microtitre plates.

1a) Isolate 3C



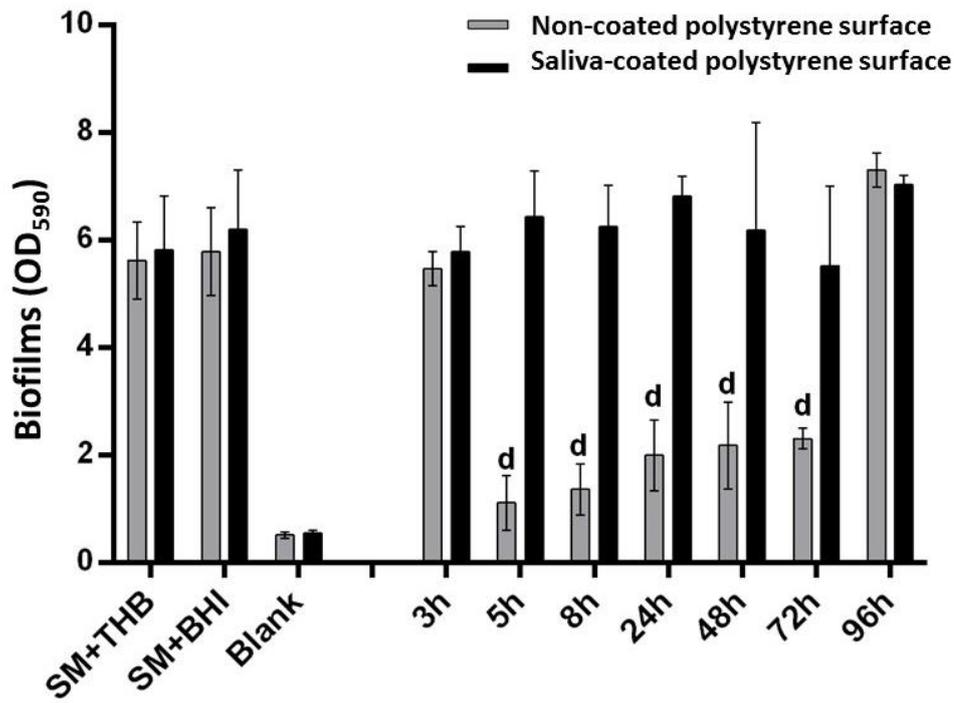
Remark: d is biofilm detaching activity

1b) Isolate 6E



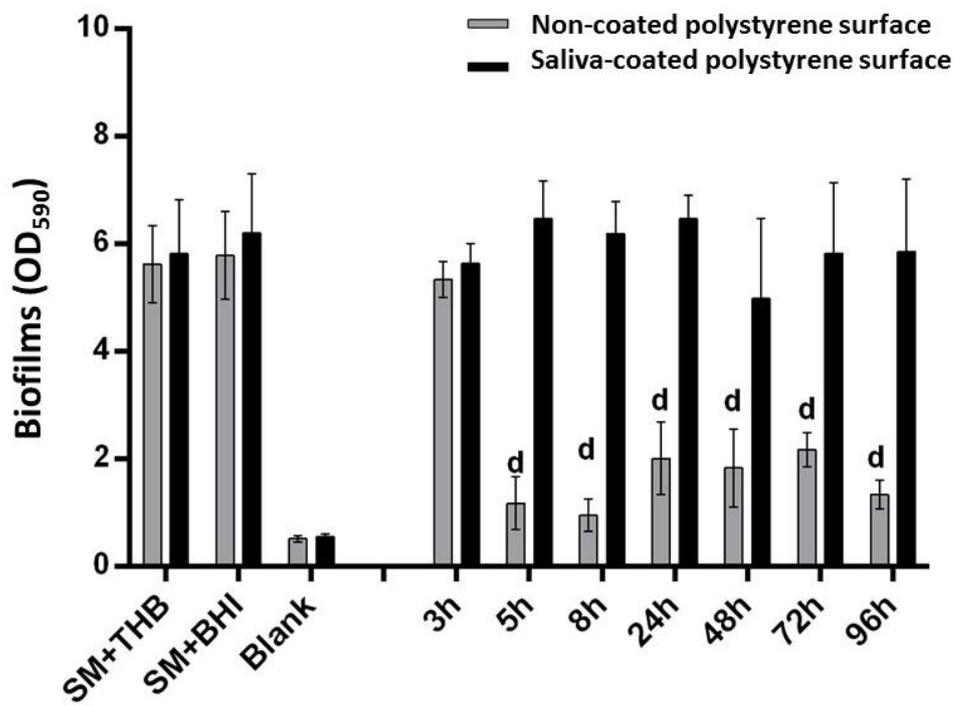
Remark: d is biofilm detaching activity
i is biofilm inhibitory activity

1c) Isolate 8E



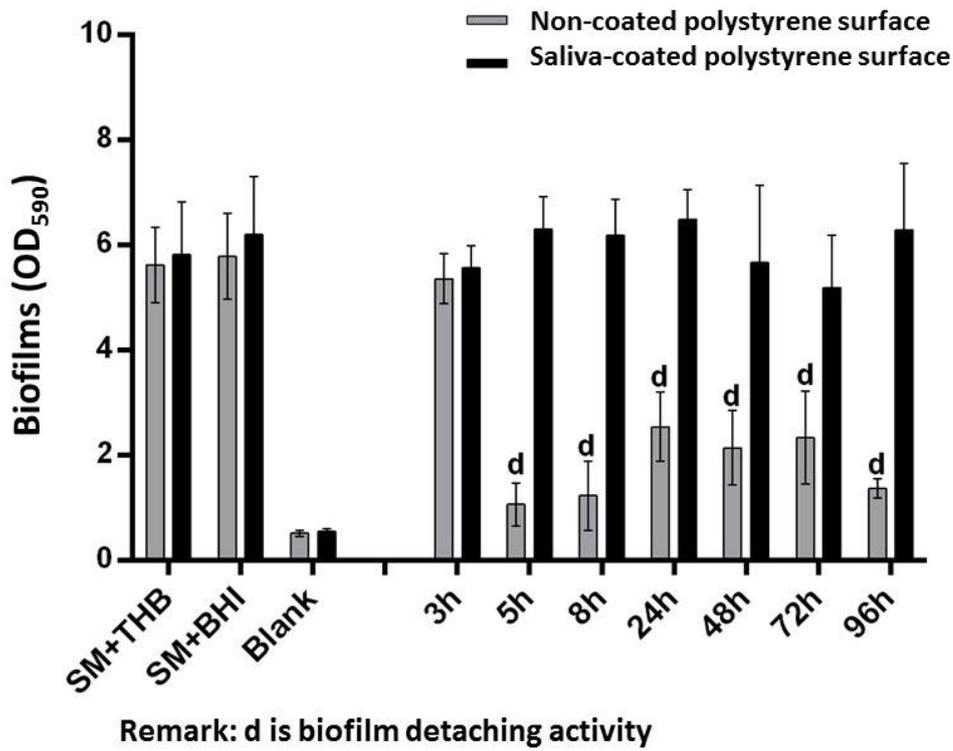
Remark: d is biofilm detaching activity

1d) Isolate 9C

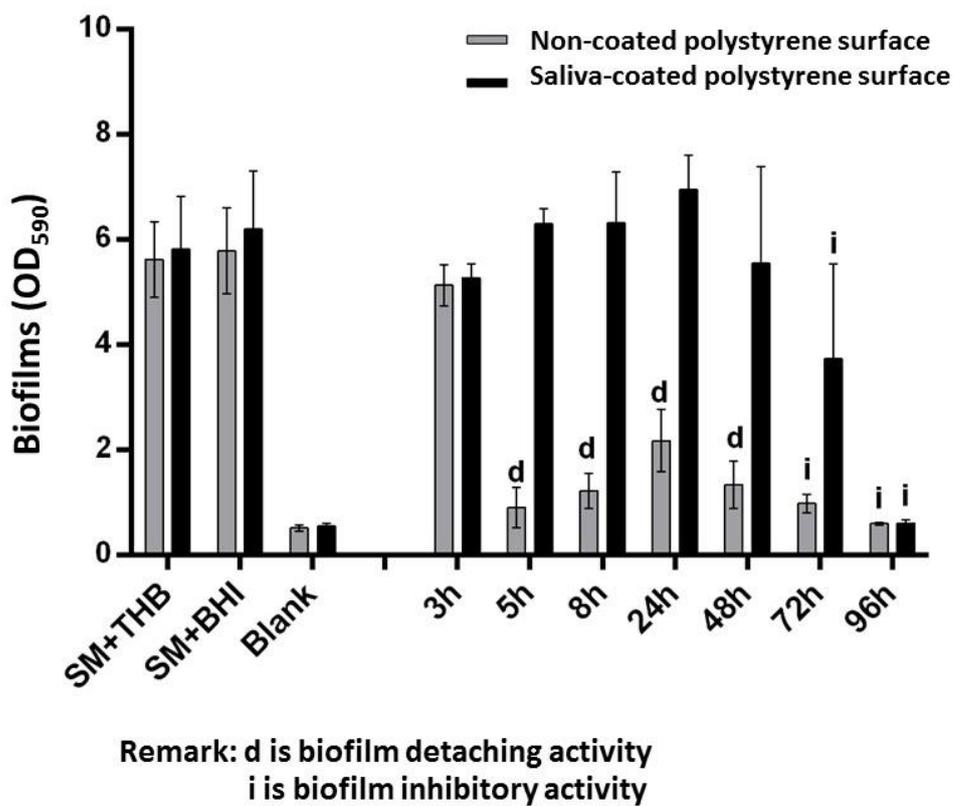


Remark: d is biofilm detaching activity

1e) Isolate 9E



1f) Isolate 10G



1g) Isolate 11E

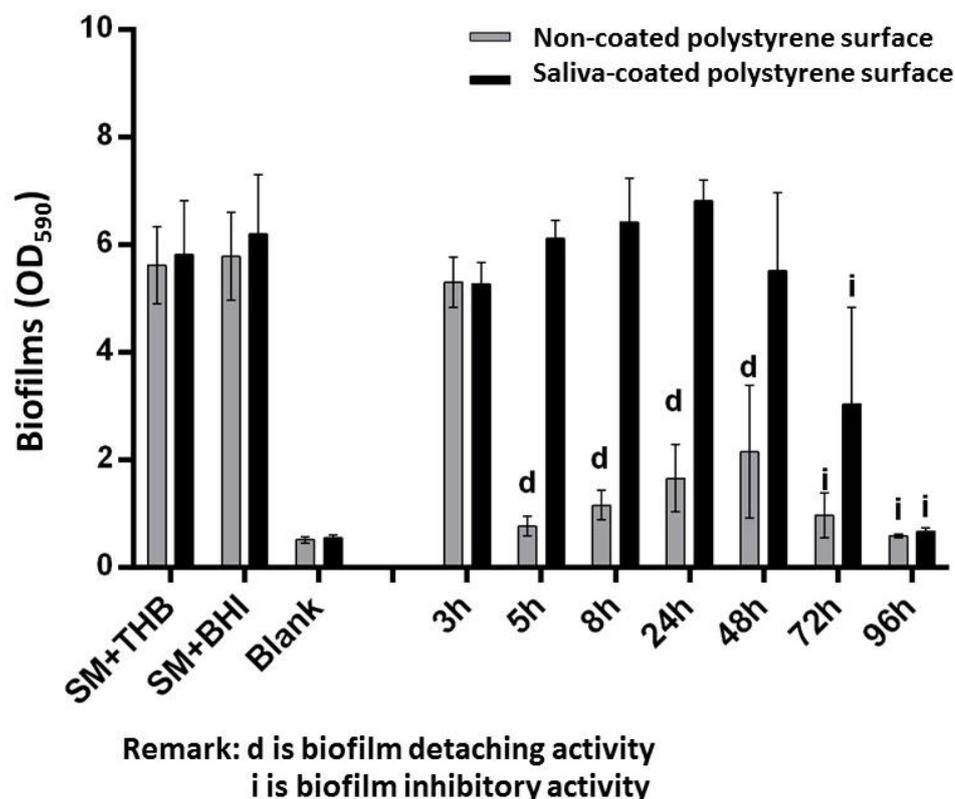


Figure 5.1 Biofilm formation by *S. mutans* in the presence of filtered culture supernatants of seven isolates grown for different times. Figures 1a to 1g show *S. mutans* biofilm formation in the presence of culture supernatants of isolates 3C, 6E, 8E, 9C, 9E, 10G, and 11E, respectively. The seven isolates were grown in BHI broth at 37°C. Bacterial cultures were collected at 3 h, 5 h, 8 h, 24 h, 48 h, 72 h and 96 h after inoculation and centrifuged. Supernatants were filtered through a 0.22 µm filter. Filtered supernatants were tested for *S. mutans* biofilm inhibitory activity on polystyrene surfaces (grey) and saliva-coated polystyrene surfaces (black). Biofilms were measured using the crystal violet staining method. The results are expressed as the mean ± standard deviation of at least two independent experiments.

5.4.2 Effect of heat on the biofilm inhibitory activity

A heat sensitivity test is a rapid method for determining if an activity is due to a protein because most proteins are denatured by heating as a result of the structural changes (Harris *et al.*, 2010). However, some proteins can renature after cooling (Tani *et al.*, 1997). To evaluate heat sensitivity, filtered supernatants of 6E, 10G and 11E, which exhibited biofilm inhibitory activity, were heated at 100°C for 15 min. After cooling for 15 min at room temperature, the heat-treated supernatants were tested for *S. mutans*

biofilm inhibitory activity. The biofilm inhibitory activity was not detected in the heat-treated supernatants of the three isolates (as shown in Figure 5.2). This result may indicate that the compound responsible for the biofilm inhibitory activity is a protein.

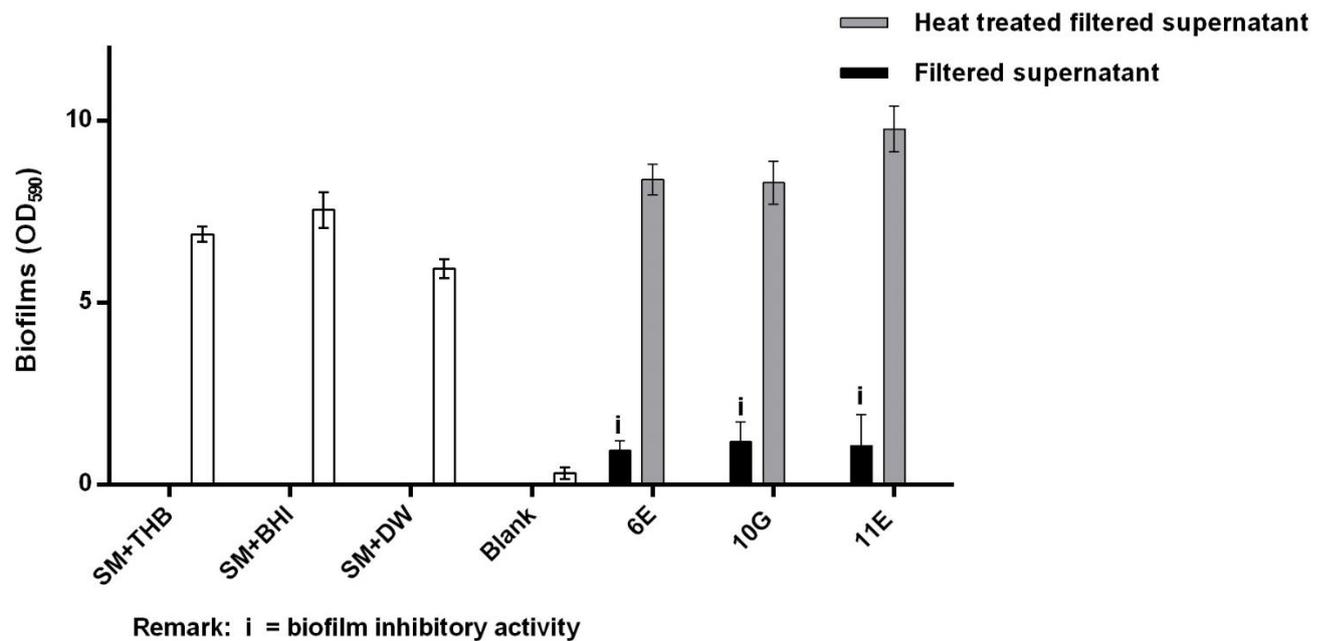


Figure 5.2 Biofilm formation by *S. mutans* in the presence of heat-treated filtered supernatants. Isolates 6E, 10G and 11E were grown in BHI broth at 37°C for 96 h. The 0.22 µm-filtered supernatants with (grey) and without heat treatment (black) were tested for biofilm inhibitory activity. Biofilms were measured using the crystal violet staining method and the amount of crystal violet was measured at an absorbance of 590 nm. The results are expressed as the mean ± standard deviation of three independent experiments.

5.4.3 Effect of proteinase K on the biofilm inhibitory activity

Since it was possible that the compound responsible for the biofilm inhibitory activity was a protein or polypeptide we used proteinase K, which digests peptide bonds, to provide further evidence that the compound responsible for the biofilm inhibitory activity was due to a protein. However, proteinase K itself can inhibit *S. mutans* biofilm formation. Thus DIFP was used as a proteinase K inhibitor. In the experiments, proteinase K at a final concentration of 100 µg/ml was added to 500 µl of filtered supernatant and the mixture was incubated at 37°C in a water bath for 16 h. Then,

DIFP at a final concentration of 0.1 mM was added to inhibit proteinase K activity and incubated at 37°C for 30 min. The filtered supernatants treated with proteinase K and DIFP were tested for biofilm inhibitory activity.

Figure 5.3 shows the amount of biofilm formed by *S. mutans* in the presence of filtered supernatants from 6E, 10G, and 11E before and after treatment with proteinase K. Biofilm inhibitory activity was observed in the untreated filtered supernatants but was absent in the filtered supernatants either treated with both proteinase K and DIFP or DIFP alone. These results confirm that the compound responsible for biofilm inhibitory activity was a protein or polypeptide, since DIFP alone could inhibit the biofilm inhibitory activity, it suggested that the compound might be a serine protease.

5.4.4 Size estimation of compound exhibiting biofilm inhibitory activity using centrifugal ultrafiltration

A volume of 500 μ l of filtered supernatants of 6E, 10G and 11E, which exhibited biofilm inhibitory activity, was added to centrifugal filters with four different pore sizes; 3 KDa, 30 KDa, 50 KDa, and 100 KDa, and centrifuged at 14,000 x g for 30 min. Filtrates and retentates were tested for anti-biofilm activity. The results showed that the biofilm inhibitory activity was present in only retentates from the three isolates and was not present in any filtrate. This demonstrates that the compound responsible for biofilm inhibitory activity cannot not pass through 3 KDa, 30 KDa, 50 KDa, and 100 KDa filters.

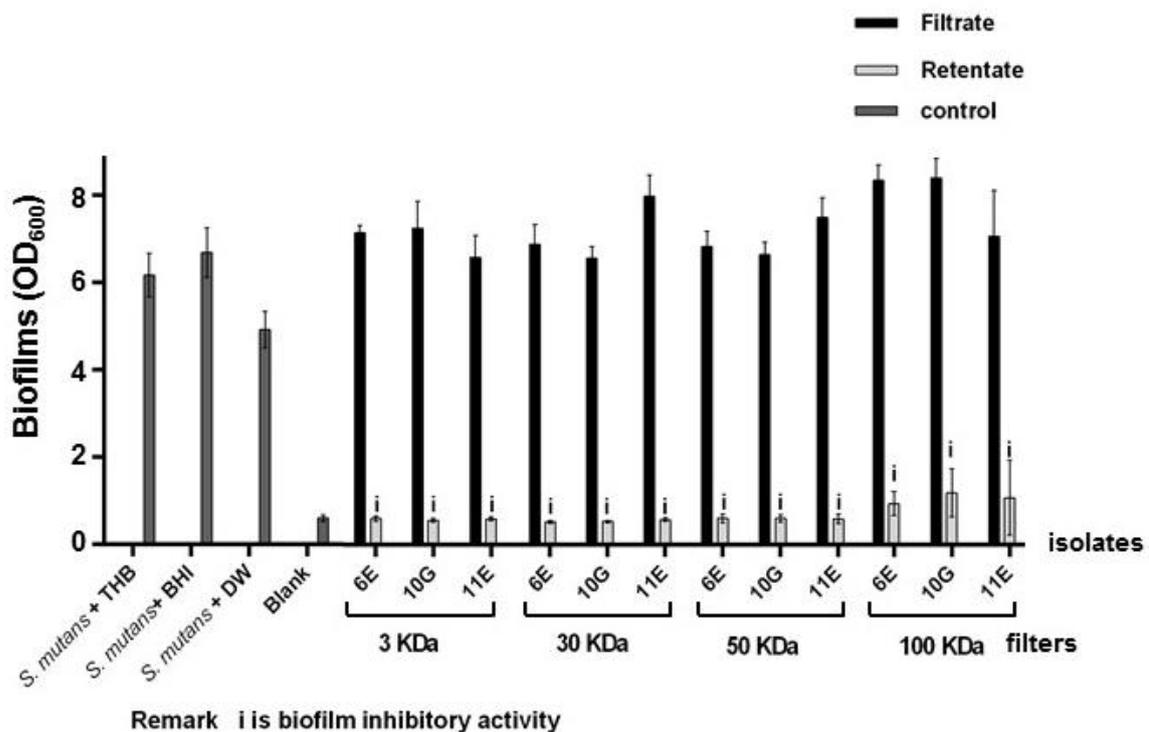
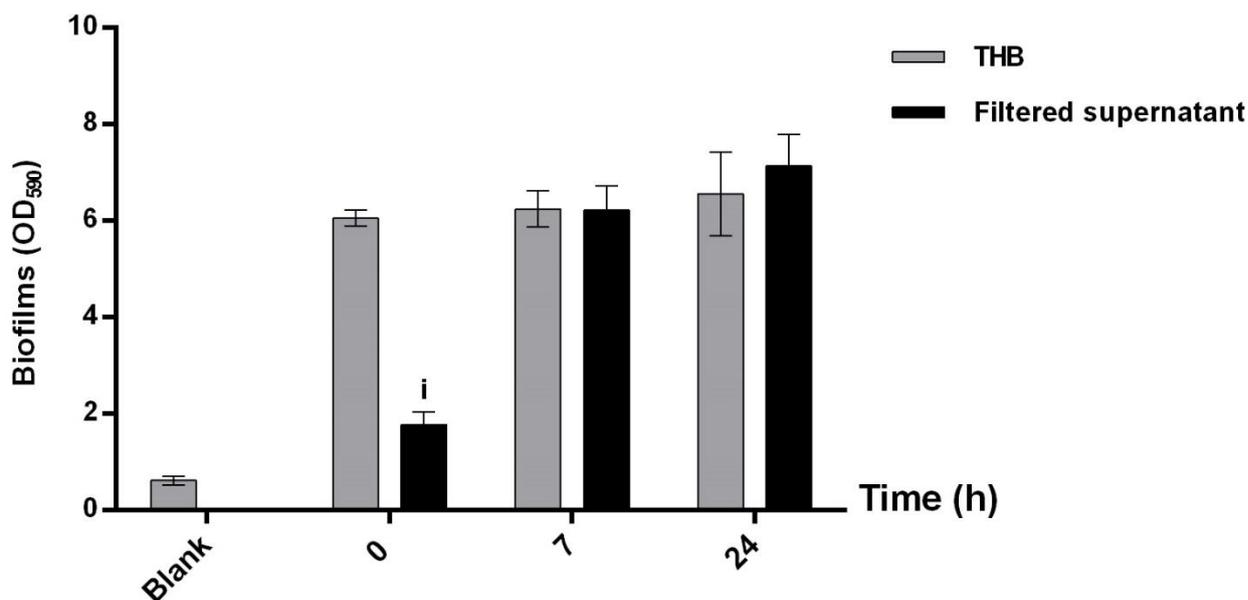


Figure 5.4 Size estimation of the compound responsible for biofilm inhibitory activity. Biofilm formation by *S. mutans* in the presence of the ultrafiltration fractions of supernatants from isolates 6E, 10G and 11E: retentates (light grey) and filtrates (black) from 3 KDa, 30 KDa, 50 KDa and 100 KDa filters. The control groups (dark grey) of *S. mutans* in the presence of THB (Todd Hewitt Broth), BHI (Brain Heart Infusion broth), Distilled water (DW) and blank without *S. mutans*. Biofilms were measured and the results are expressed as the mean \pm standard deviation of three independent experiments.

5.4.5 Biofilm inhibitory activity and biofilm disrupting activity of filtered supernatants

5.4.5.1 Capacity of filtered supernatants to disrupt an early step in *S. mutans* biofilm formation

The 0.22 μm filtered supernatant from 6E grown in BHI broth with biofilm inhibitory activity was added to microtitre wells containing *S. mutans* culture after incubation for 1 h, 2 h, 3 h, 4 h, 5 h, 6 h, and 7 h and the microtitre plate was incubated for a total of 24 h. Addition of the filtered supernatant at 0 h, that is at the same time as inoculation with *S. mutans* was a control group. Another control group was the addition of THB (Todd Hewitt Broth) at each time point. In Figure 5.5, the highest biofilm inhibitory activity was found by adding the filtered supernatant at the same time as inoculation with *S. mutans* (0 h). Biofilm inhibitory activity was also significant when the filtered supernatant was added at 1 h and 2 h after inoculation with *S. mutans*. However, biofilm inhibitory activity was not detected after 4 h following inoculation.



Remark: i = biofilm inhibitory activity

Figure 5.6 Biomass of *S. mutans* after adding the filtered supernatant from isolate 6E to biofilm assays at different times. *S. mutans* formed biofilms in microtitre plates for 7 h and 24 h. After that, planktonic cells were removed before adding the 0.22 μ m. Biofilms were measured and the results are expressed as the mean \pm standard deviation of three independent experiments.

5.4.6 Scale-up production of the compound exhibiting biofilm inhibitory activity

Isolate 6E was found to produce a compound with biofilm inhibitory activity when it was grown in a volume of 20 ml BHI broth in a 50 ml tube as shown in 5.4.1. The results shown in Figure 5.7 (top) demonstrate that biofilm inhibitory activity was present in 24 h and 48 h filtered supernatants which was faster than when 6E was grown on a smaller scale. In addition, *S. mutans* growth inhibitory activity, a new activity in this study, was firstly found from 72 h and 96 h filtered supernatants as shown in the bottom of the Figure 5.7.

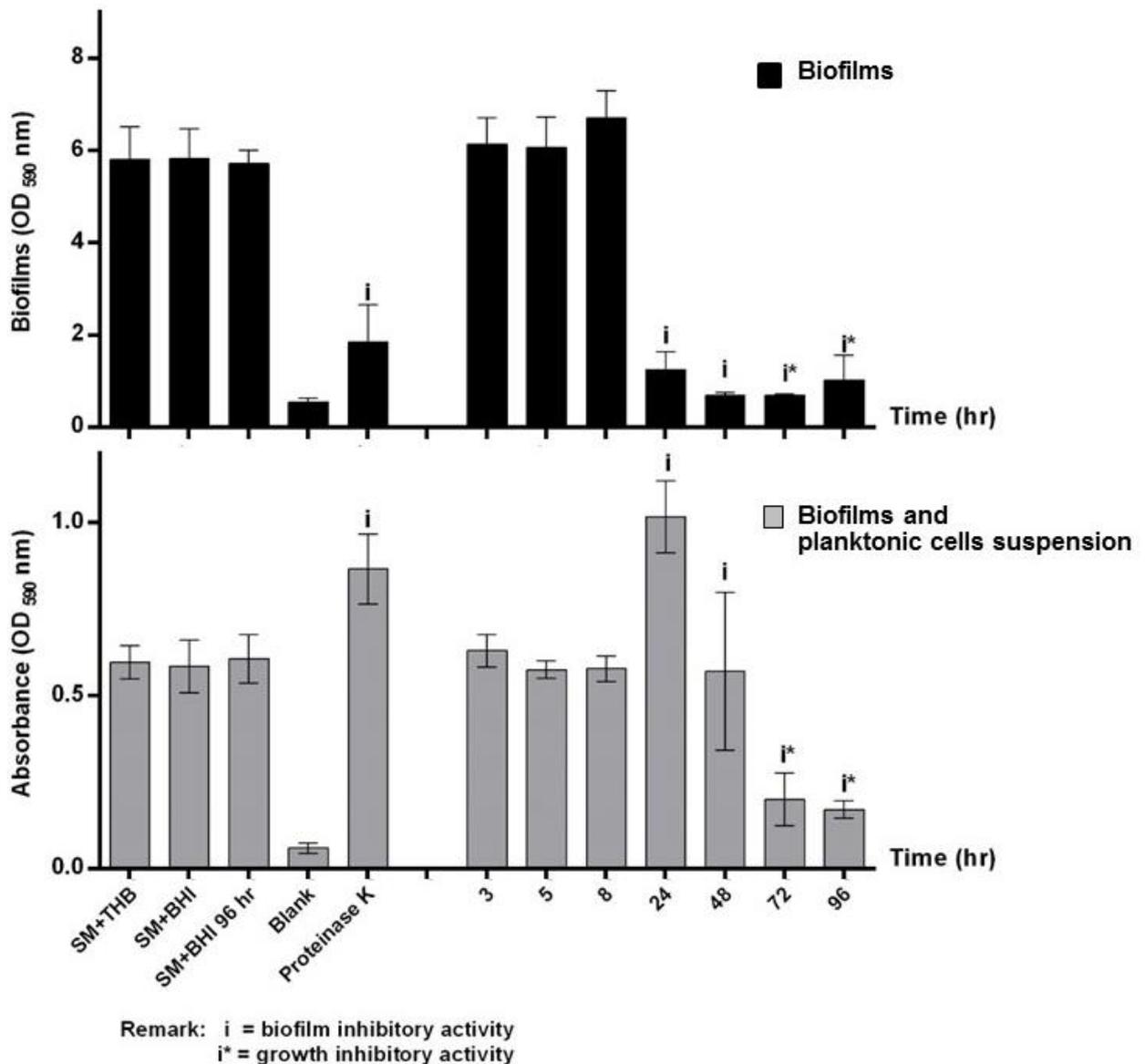


Figure 5.7 Biofilm formation by *S. mutans* in the presence of filtered supernatants from scale-up production. The 0.22 μ m filtered supernatant from isolate 6E grown in 200 ml BHI broth for different amounts of time: 3 h, 5 h, 8 h, 24 h, 48 h, 72 h and 96 h. The filtered supernatants were measured for biofilm inhibitory activity. The top graph shows *S. mutans* biofilms (black) in the presence of the filtered supernatants and *S. mutans* growth (OD_{590nm}) (grey) is shown in the bottom graph. The results are expressed as the mean \pm standard deviation of at least three independent experiments.

5.4.7 Ammonium sulfate precipitation of the compound responsible for biofilm inhibitory activity

Table 5.1 shows the partial purification of the compound responsible for the biofilm inhibitory activity in the filtered supernatant from six bottles of 200 ml cultures of isolate

6E using ammonium sulfate precipitation. The results from biofilm inhibitory activity assays, protease activity assays and the amount of protein are shown in the table. The biofilm inhibitory activity was present in all bottles. The supernatants from the six bottles were pooled together and filtered using 0.45 μm filters. The filtered supernatant was precipitated by adding solid ammonium sulfate at two saturations, 30% and then 30-75%. The protein pellets derived from these saturations were tested for biofilm inhibitory activity.

The biofilm inhibitory activity of the 30 – 75 % protein fraction was 13850.67 units with a yield of 42.99%. The specific biofilm inhibitory activity of this 30-75% protein fraction was 537.47 units/mg, which was about two times higher than the 0.45 μm filtered supernatant (266.74 unit/mg). Thus, the 30-75% protein fraction was selected for further purification.

Table 5.1 Partial purification of the compound responsible for biofilm inhibitory activity from isolate 6E using ammonium sulfate precipitation

| Fractions | Total volume (ml) | Biofilm inhibitory activity | | Protease activity | | Protein | | Specific activity | |
|---|-------------------|-----------------------------|-----------|-------------------|-----------|-------------|-----------|---------------------------------------|-----------------------------|
| | | Activity (unit) | Yield (%) | Activity (unit) | Yield (%) | Amount (mg) | Yield (%) | Biofilm inhibitory activity (unit/mg) | Protease activity (unit/mg) |
| Bottle A | 188 | 4809.67 | - | 4742.61 | - | 20.33 | - | 236.58 | 233.28 |
| Bottle B | 188 | 7324.17 | - | 4812.80 | - | 23.12 | - | 316.79 | 208.17 |
| Bottle C | 188 | 4148.53 | - | 3358.93 | - | 18.10 | - | 229.20 | 185.58 |
| Bottle D | 188 | 4292.67 | - | 4431.79 | - | 17.96 | - | 239.01 | 246.76 |
| Bottle E | 188 | 7104.83 | - | 5023.36 | - | 23.53 | - | 301.95 | 213.49 |
| Bottle F | 188 | 4537.07 | - | 4206.19 | - | 19.50 | - | 232.67 | 215.70 |
| Total | 1128 | 32216.93 | 100.00 | 26575.68 | 100 | 122.55 | 100 | 262.89 | 216.86 |
| 0.45 µm filtered supernatant | 1020 | 31237.50 | 96.96 | 20304.00 | 76.40 | 117.11 | 95.56 | 266.74 | 173.38 |
| Ammonium sulfate protein precipitation | | | | | | | | | |
| 30% | 21 | 3055.50 | 9.48 | 45.92 | 0.17 | 10.63 | 8.67 | 287.44 | 4.32 |
| 30-75% | 35.5 | 13850.67 | 42.99 | 531.81 | 2.00 | 25.77 | 22.00 | 537.47 | 20.64 |

5.4.8 Hydrophobic interaction column chromatography using HiTrap Phenyl Fast Flow (High Sub)

To purify the compound responsible for biofilm inhibitory activity in the 30-75% protein fraction derived from ammonium sulfate precipitation, double strength binding buffer (2.0 M ammonium sulfate, 100 mM sodium phosphate pH 7.0) was added to this 30-75% protein fraction. To prevent the Phenyl FF column from becoming blocked by protein which did not dissolve, the mixture was centrifuged and filtered through a 0.22 μm filter. The filtrate was applied to the Phenyl FF column and 1 ml fractions collected. The column was washed with 10 ml of 1.0 M ammonium sulfate in 50 mM sodium phosphate pH 7.0. Then bound protein was eluted using 50 mM sodium phosphate buffer pH 7.0. The eluants from the column, while loading the filtrate (non-bound protein 1 and 2, NB1 and NB2), while washing the non-bound proteins from the column (W1-10), and while eluting the bound proteins (F1-10), were tested for biofilm inhibitory activity, protease activity, and the amount of protein. The data are shown in Table 5.2.

The results shown in the Table 5.2, demonstrate that during purification total protein decreased as expected, however the yield of biofilm inhibitory activity also decreased. The protein in the 30-75% protein fraction which did not re-dissolve in 2 ml of buffer, labelled (4) in Table 5.2, and which was centrifuged out, had the highest specific biofilm inhibitory activity (769.04 unit/mg). It seems that the proteins responsible for biofilm inhibitory activity were lost from the purification process in the centrifugation step. In addition, more biofilm inhibitory activity was lost on filtering the supernatant from this centrifugation step, labelled (5) in Table 5.2. This led to a reduction in the yield of biofilm inhibitory activity to 22.05 %. The 0.22 μm filtrate, labelled 5 in Table 5.2, had the lowest biofilm specific inhibitory activity (81.97 unit/mg). Further purification on the Phenyl FF column resulted in fraction F2 and F3, labelled (6) and

(7), respectively, in Table 5.2, having higher specific biofilm inhibitory activity, 264.84 unit/mg and 336.43 unit/mg, respectively, than in the previous step. These results indicate that the Phenyl FF column could separate some protein responsible for the biofilm inhibitory activity in the 0.22 μm filtrate (5). Figure 5.8 shows the biofilm inhibitory activity, protease activity and the amount of protein in fractions obtained from the Phenyl FF column.

Table 5.2 *S. mutans* biofilm inhibitory activity, total protein and specific activity of protein in fractions obtained from HiTrap Phenyl FF column

| | Volume (ml) | Total protein (µg) | Total biofilm inhibitory activity (unit) | The yield of biofilm inhibitory activity (%) | Specific activity (unit/mg) |
|---|-------------|--------------------|--|--|-----------------------------|
| (1) Protein from the 30-75% ammonium sulfate precipitation | 1 | 629.46 | 237.33 | 100 | 377.04 |
| Phenyl FF column | | | | | |
| <i>Added double strength of binding buffer (2.0 M ammonium sulfate in 100 mM sodium phosphate pH 7.0)</i> | | | | | |
| (2) Protein from the 30-75% ammonium sulfate precipitation redissolved in double strength of the binding buffer | 2 | 775.50 | 234.00 | 104.71 | 301.74 |
| <i>Centrifuged at 4500 x g at 4°C for 20 min</i> | | | | | |
| (3) Supernatant of the protein from 30-75% ammonium sulfate precipitation redissolved in double strength of the binding buffer | 1.9 | 781.06 | 116.80 | 52.27 | 149.54 |
| (4) Precipitated protein of the protein from 30-75% ammonium sulfate precipitation redissolved in double strength of the binding buffer | ~0.1 | 30.83 | 23.71 | 10.61 | 769.04 |
| <i>The supernatant was filtered using a 0.22 µm filter</i> | | | | | |
| (5) 0.22 µm filtrate | 1.9 | 601.09 | 49.27 | 22.05 | 81.97 |
| <i>Eluted by 50 mM sodium phosphate pH 7.0</i> | | | | | |
| (6) F2 | 1 | 158.58 | 42.00 | 18.79 | 264.84 |
| (7) F3 | 1 | 67.47 | 22.70 | 10.16 | 336.43 |

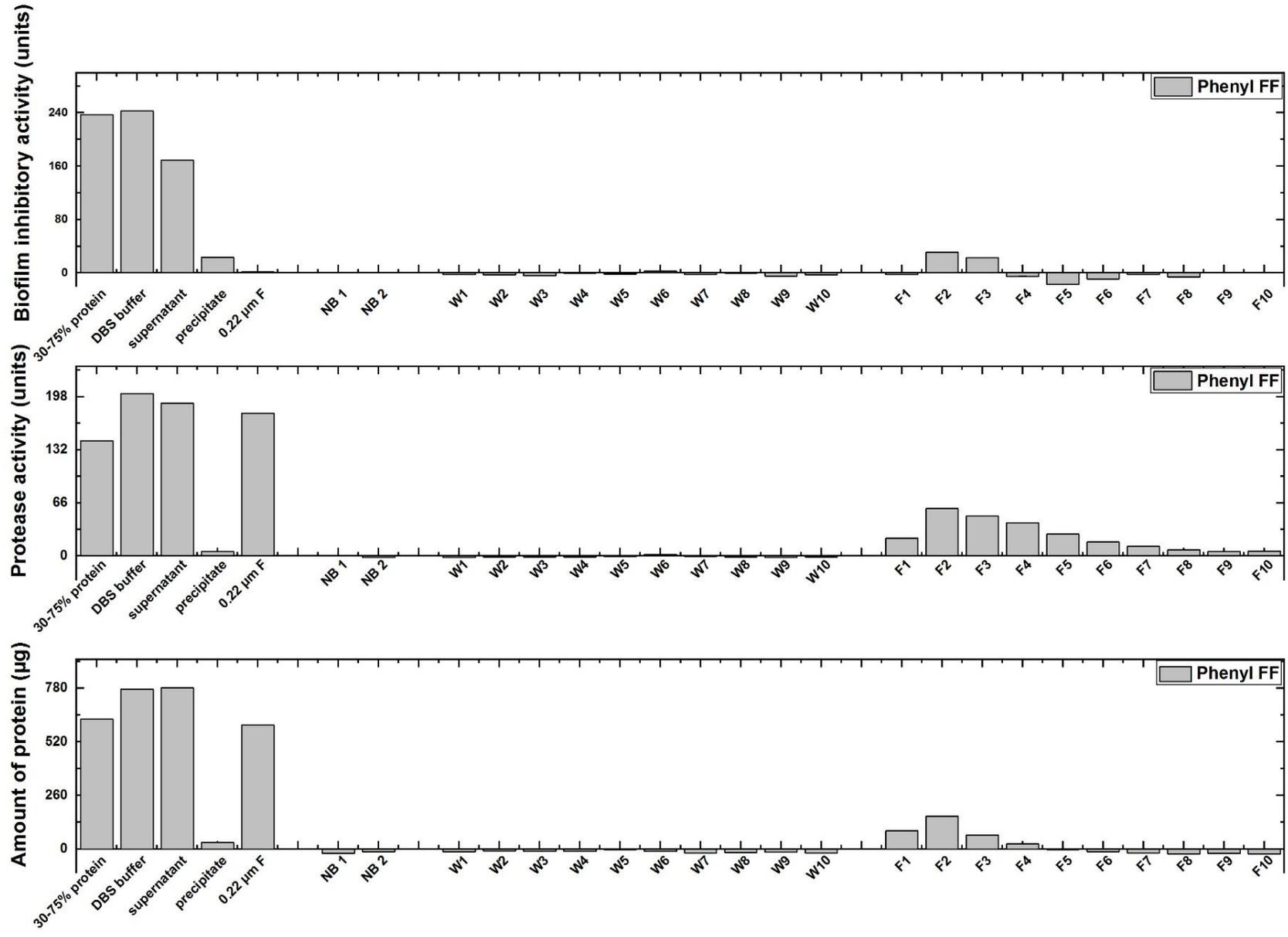


Figure 5.8 Biofilm inhibitory activity, protease activity and the amount of protein in fractions obtained from the Phenyl FF column where “30-75% protein” refers to the proteins in the 30-75% ammonium sulfate precipitation, “DBS buffer” refers to the mixture of the protein in the 30-75% ammonium sulfate precipitation and double strength of binding buffer (2.0 M ammonium sulfate, 100 mM sodium phosphate pH 7.0), “supernatant” refers to protein in the 30-75% ammonium sulfate precipitation redissolved in the double strength of binding buffer, “precipitate” refers to protein in the 30-75% ammonium sulfate precipitation that was not re-dissolved in the double strength of binding buffer, “0.22 μ m F” refers to the redissolved proteins in the double strength of binding buffer and which passed through a 0.22 μ m filter, “NB” is non-bound protein in eluants from the Phenyl FF column while loading the 0.22 μ m F, “W1 to W10” are non-bound protein in the eluants from the column while washing, “F1 to F10” are the bound protein in the eluants from the column while elution.

5.4.9 HiTrap benzamidine Fast Flow column chromatography

The biofilm inhibitory activity produced by isolate 6E was lost when the supernatant was treated with DIFP (as shown in Figure 5.3). DIFP is a serine protease inhibitor, so it is possible that the compound responsible for the biofilm inhibitory activity is a serine protease. To purify a potential serine protease, benzamidine sepharose was selected to bind the serine protease. In this experiment, the 30-75% ammonium sulfate fraction was resuspended in 40 mM Tris-HCl, 1.0 M NaCl pH 7.4, the mixture was filtered using a 0.22 μm filter before applying to the HiTrap Benzamidine column.

The results shown in Table 5.3, demonstrate that during purification total protein decreased as expected, however the yield and specific biofilm inhibitory activity also decreased. The protein in the 30-75% protein fraction after adding double strength binding buffer and filtering through a 0.22 μm filter, labelled (2) and (3) in Table 5.3, had a slightly decreased specific biofilm inhibitory activity (293.74 unit/mg and 301.33 unit/mg, respectively). Further purification on the benzamidine column resulted in fraction W1, labelled (4) in Table 5.3, increasing in specific biofilm inhibitory activity, 200.12 unit/mg. During elution of the bound protein from the benzamidine column, no fraction had high biofilm inhibitory activity but F1 and F2 had high protease activity, labelled F1 to F10 in the Figure 5-10. These results indicate that the benzamidine column could separate some protease but not separate the compound with biofilm inhibitory activity.

Table 5.3 *S. mutans* biofilm inhibitory activity, total protein and specific activity of protein in fractions obtained from benzamidine FF column

| | Volume (ml) | Total protein (µg) | Total biofilm inhibitory activity (unit) | Yield of biofilm inhibitory activity (%) | Specific activity (unit/mg) |
|---|-------------|--------------------|--|--|-----------------------------|
| (1) Protein from the 30-75% ammonium sulfate precipitation | 1 | 629.46 | 237.33 | 100 | 377.04 |
| Benzamidine FF column | | | | | |
| <i>Added double strength binding buffer (40 mM Tris-HCl, 1.0 M NaCl pH 7.4)</i> | | | | | |
| (2) Protein from the 30-75% ammonium sulfate precipitation redissolved in double strength of binding buffer | 2 | 747.17 | 219.47 | 98.21 | 293.74 |
| <i>0.22 µm filter</i> | | | | | |
| (3) 0.22 µm filtrate of the protein from the 30-75% ammonium sulfate precipitation redissolved in double strength of binding buffer passed through 0.22 µm filter | 1.9 | 722.17 | 217.61 | 97.38 | 301.33 |
| <i>Washed by binding buffer (20 mM Tris-HCl, 0.5 M NaCl pH 7.4)</i> | | | | | |
| (4) W1 | 1 | 136.92 | 27.40 | 12.03 | 200.12 |

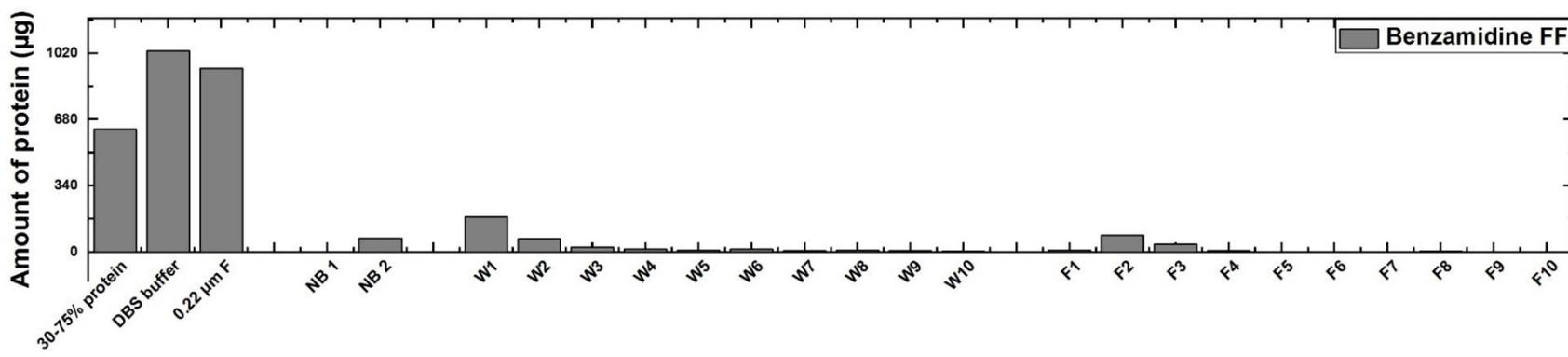
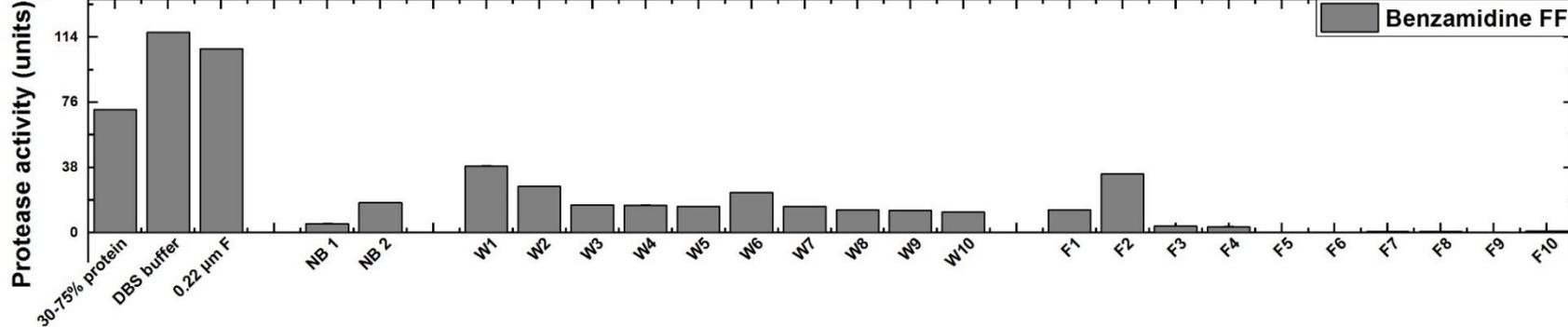
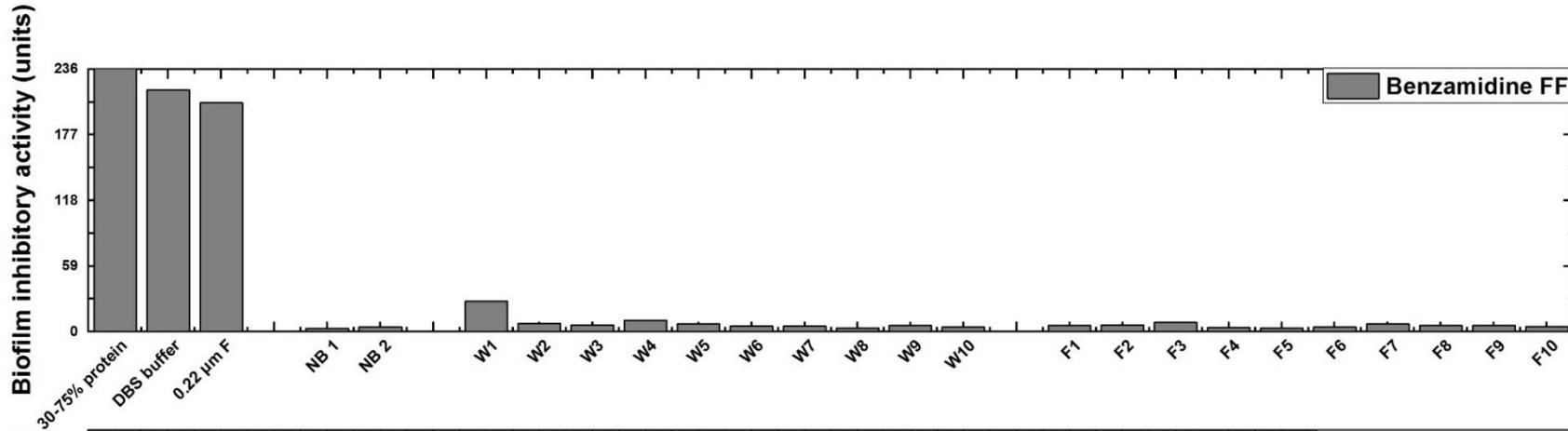
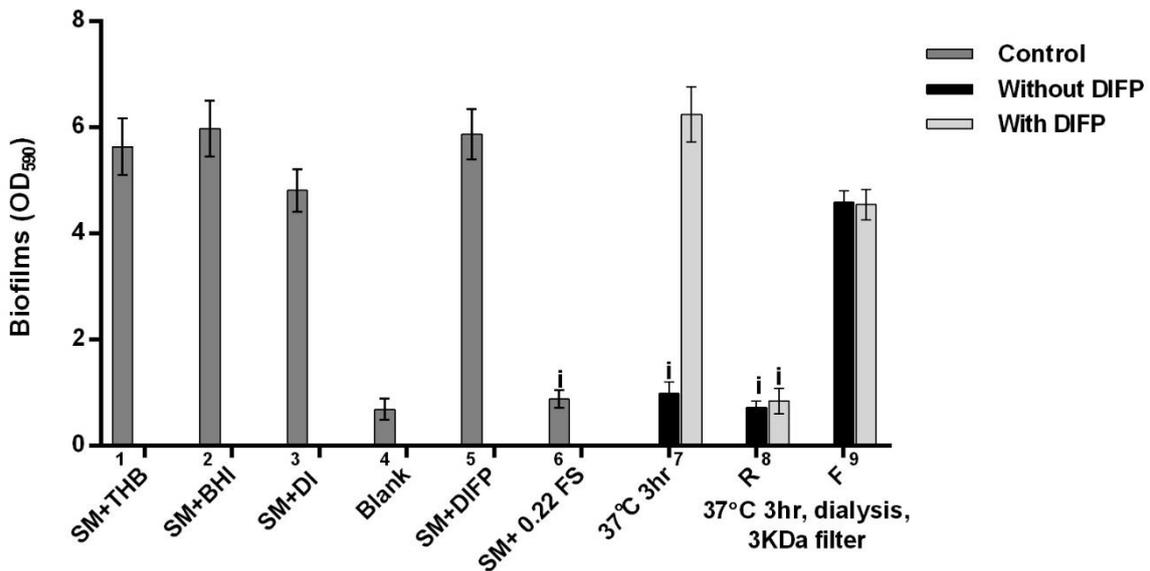


Figure 5.9 Biofilm inhibitory activity, protease activity and the amount of protein in fractions obtained from benzamidine FF column where “30-75% protein” refers to the proteins in the 30-75% ammonium sulfate precipitation, “DBS buffer” refers to the mixture of the proteins in the 30-75% ammonium sulfate precipitation and double strength of binding buffer (40 mM Tris-HCl, 1.0 M NaCl pH 7.4), “0.22 μ m F” refers to the proteins in the 30-75% ammonium sulfate precipitation redissolved in the double strength of binding buffer and which passed through a 0.22 μ m filter, “NB” is non-bound protein in eluants from the benzamidine FF column while loading the 0.22 μ m F, “W1 to W10” are non-bound protein in the eluants from the column while washing, “F1 to F10” are the bound protein in the eluants from the column while elution.

5.4.10 Effect of DIFP on biofilm inhibitory activity

In section 5.4.3, the data suggested that the biofilm inhibitory activity produced by isolate 6E in 20 ml cultures was lost after adding DIFP. The experiments described in the current section aimed to determine the effect of DIFP on the biofilm inhibitory activity of the filtered supernatant of 6E grown in 200 ml of BHI broth. Filtered supernatants with and without DIFP were incubated for 3 h at 37°C. Both samples were dialysed using 10 KDa dialysis cassettes to remove DIFP. The samples were measured for biofilm inhibitory activity.

The results shown in Figure 5.10 demonstrate that biofilm inhibitory activity was not detected when the filtered supernatant was treated with DIFP, labelled 7 and light grey in the Figure 5.10. However, this filtered supernatant treated with DIFP had restored biofilm inhibitory activity after it was dialysed to removed DIFP using a 10 KDa dialysis cassette, labelled 8 and light grey in the Figure 5.10.



Remark: i = biofilm inhibitory activity

Figure 5.10 Biofilm formation by *S. mutans* in the presence of filtered supernatant treated with DIFP. The 0.22 μ m filtered supernatants (as shown as “0.22 FS” in the graph) from isolate 6E were treated with or without DIFP at 37°C for 3 h. Both samples were dialysed using 10 KDa dialysis cassettes to remove the DIFP. The graph shows biofilm formation by *S. mutans* in the presence of the filtered supernatant with (light grey) and without DIFP (black). Control groups (dark grey) were *S. mutans* (SM) biofilms in the presence of: (1) Todd Hewitt Broth (SM+THB), (2) Brain heart infusion broth (SM+BHI), (3) deionised water (SM+DI), (4) Blank without *S. mutans*. (5) DIFP (SM+DIFP), and (6) the 0.22 μ m filtered supernatant (SM+0.22 FS). The test groups were *S. mutans* biofilms in the presence of: (7) the filtered supernatant with (light grey) and without DIFP (black) incubated at 37°C for 3 h (37°C 3 h), (8) and (9) retentate (R) and filtrate (F), respectively, of the filtered supernatant with (light grey) and without DIFP (black) after dialysed the volume of dialysis cassettes were increased and they were adjusted to an initial concentration using a 3 KDa filter (37°C 3 h, dialysis, 3 KDa filter). Biofilms were measured and the results are expressed as the mean \pm standard deviation of three independent experiments.

5.4.11 Ion exchange chromatography

Two types of ion exchange column chromatography were conducted in these experiments. The anion and cation exchangers were a DEAE column and a High S column, respectively.

Anion exchange chromatography binds and allows separation of net-negatively charged proteins. Proteins, which have a pI less than the pH value of the buffer used in the experiment, would have a net-negative charge and would bind to the $N^+(C_2H_5)_2$ molecule of the DEAE column, theoretically.

Figure 5.11 illustrates the procedure used with the DEAE column. Due to a high salt concentration in the BHI medium, 30 ml filtered supernatant was diluted to decrease the salt concentration by adding deionised water. Then it was mixed with 0.1 M sodium phosphate buffer pH 7.0 and 8.0 before loading to the DEAE column. The sample was applied to the DEAE column and 5 ml fractions collected. The column was washed with 50 mM sodium phosphate buffer and the bound protein was eluted with 1.0 M sodium chloride in 50 mM sodium phosphate pH 6.7. The loaded sample, the unbound protein eluted while loading the sample and the eluted fractions from the DEAE column at pH 7.0 and pH 8.0 were tested for biofilm inhibitory activity and amount of protein as shown in Table 5.4.

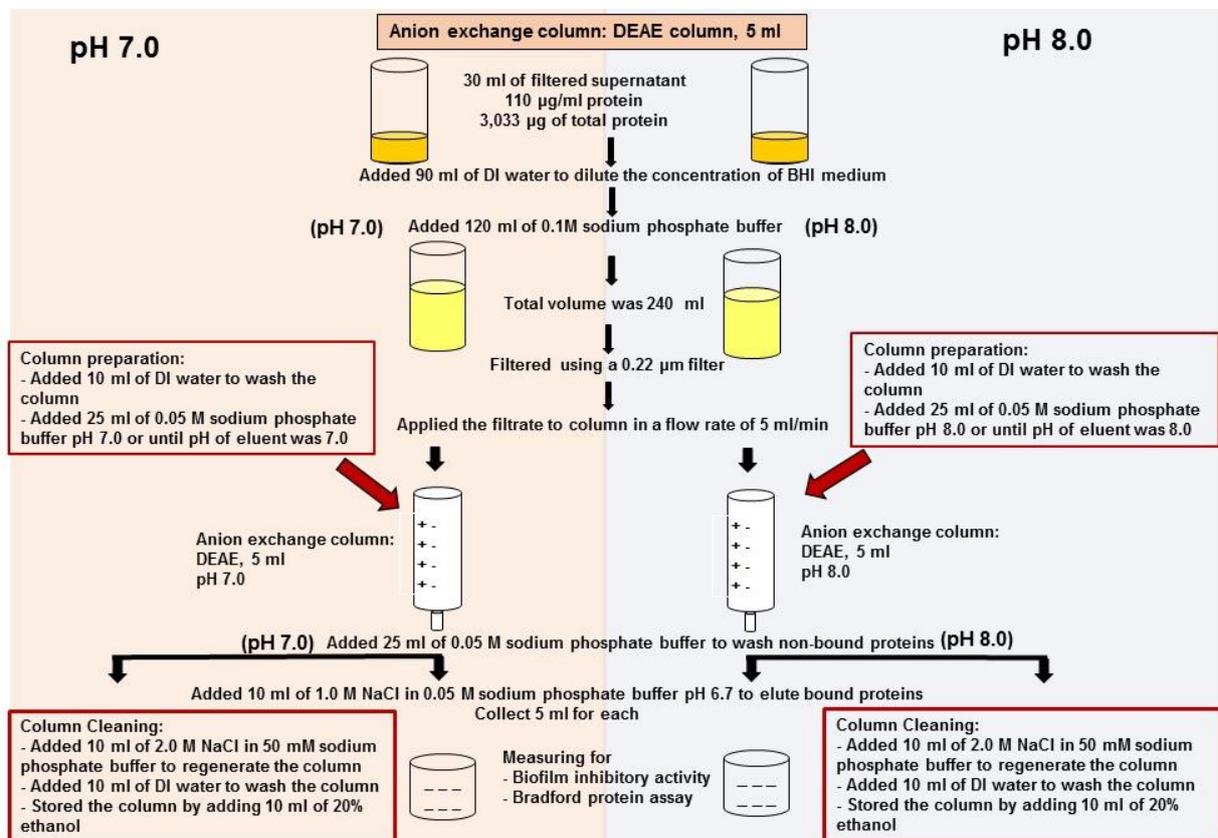


Figure 5.11 Flow chart of anion exchange column chromatography using the DEAE column

The results in Table 5.4 demonstrate that none of the eluted fractions from either pH showed biofilm inhibitory activity, labelled (4), (5), (8) and (9) in the Table 5.4.

Table 5.4 *S. mutans* biofilm inhibitory activity, total protein and specific activity of protein in fractions obtained from DEAE column

| Samples | Volume (ml) | Total protein (µg) | Total biofilm inhibitory activity (unit) | The yield of biofilm inhibitory activity (%) | Specific biofilm inhibitory activity (unit/mg) |
|---|-------------|--------------------|--|--|--|
| (1) 0.22 µm filtered supernatant | 30 | 3033.33 | 259.90 | 100 | 81.17 |
| DEAE column pH 7.0 | | | | | |
| <i>Added 90 ml of deionised water and 120 ml of 0.1M sodium phosphate buffer pH 7.0</i> | | | | | |
| (2) Loaded sample | 240 | 2533.33 | 27.50 | 10.58 | 5.43 |
| (3) Unbound protein | 240 | 1566.67 | 12.70 | 4.89 | 4.05 |
| <i>Eluted by 1.0 M NaCl in 50 mM sodium phosphate pH 6.7</i> | | | | | |
| (4) E1 | 5 | 280.56 | ND | - | - |
| (5) E2 | 5 | 84.49 | ND | - | - |
| DEAE column pH 8.0 | | | | | |
| <i>Added 90 ml of deionised water and 120 ml of 0.1M sodium phosphate buffer pH 8.0</i> | | | | | |
| (6) Loading sample | 240 | 2561.11 | 34.60 | 13.31 | 6.45 |
| (7) Unbound protein | 240 | 1383.33 | 29.60 | 11.39 | 10.70 |
| <i>Eluted by 1.0 M NaCl in 50 mM sodium phosphate pH 6.7</i> | | | | | |
| (8) E1 | 5 | 256.94 | ND | - | - |
| (9) E2 | 5 | 41.55 | ND | - | - |

Remark ND means none detection

Cation exchange chromatography columns, bind and allow separation of net-positively charged proteins. Proteins, which have a pI more than pH value of the buffer used in the experiment, would have a net-positive charge and would bind to the sulphur trioxide (SO³⁻) molecule of the High S column, theoretically. Figure 5.12 illustrates the procedure used with the High S column. To do this, deionised water and double

strength buffer were added to the filtered supernatant, labelled (1) in the Table 5.5. The loaded sample, the unbound protein eluted while loading the sample and the elution fractions from High S column at pH 5.0 and pH 6.5 were tested for biofilm inhibitory activity and amount of protein as shown in Table 5.5.

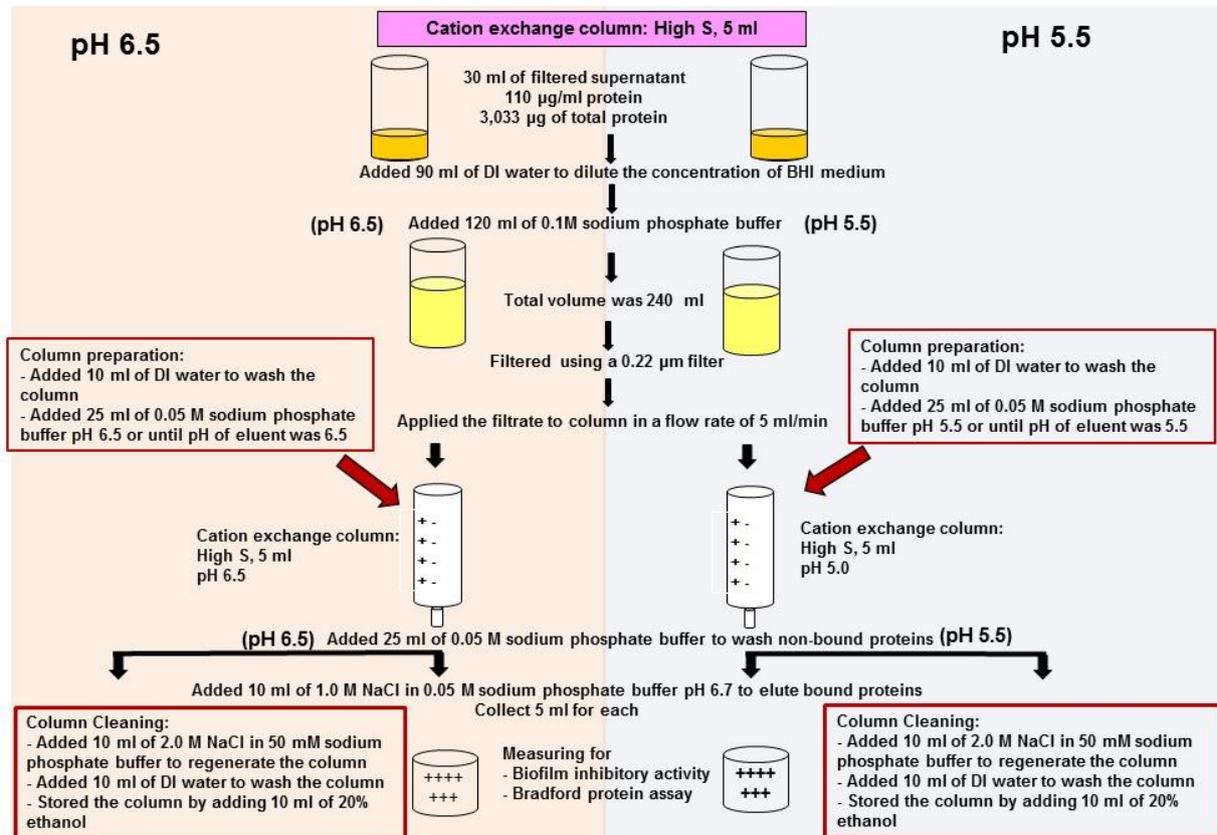


Figure 5.12 Flow chart of cation exchange column chromatography using the High S column

The results in Table 5.5 demonstrate that none of the eluted fractions from either pH showed biofilm inhibitory activity, labelled (4), (5), (8) and (9) in the Table 5.5.

Table 5.5 *S. mutans* biofilm inhibitory activity, total protein and specific activity of protein in fractions obtained from High S column

| Samples | Volume (ml) | Total protein (µg) | Total biofilm inhibitory activity (unit) | The yield of biofilm inhibitory activity (%) | Specific activity (unit/mg) |
|--|-------------|--------------------|--|--|-----------------------------|
| (1) 0.22 µm filtered supernatant | 30 | 3033.33 | 259.90 | 100 | 81.17 |
| High S column pH 5.0 | | | | | |
| <i>Added 90 ml of deionised water and 120 ml 0.1 mM sodium phosphate pH 5.0</i> | | | | | |
| (2) Loading sample | 240 | 2477.78 | 49.20 | 18.93 | 12.10 |
| (3) Unbound protein | 240 | 1577.78 | 37.70 | 14.51 | 11.54 |
| <i>Eluted by 1.0M NaCl in 50 mM sodium phosphate pH 6.7</i> | | | | | |
| (4) E1 | 5 | 26.85 | ND | - | - |
| (5) E2 | 5 | ND | ND | - | - |
| High S column pH 6.5 | | | | | |
| <i>Added 90 ml of deionised water and 120 ml of 0.1 mM sodium phosphate pH 6.5</i> | | | | | |
| (6) Loading sample | 240 | 2561.11 | 22.60 | 8.70 | 4.61 |
| (7) Unbound protein | 240 | 2150 | 3.00 | 1.15 | 0.70 |
| <i>Eluted by 1.0 M NaCl in 50 mM sodium phosphate pH 6.7</i> | | | | | |
| (8) E1 | 5 | 116.67 | ND | - | - |
| (9) E2 | 5 | 1.62 | ND | - | - |

Remark ND mans none detection

5.5 Discussion

This chapter describes the screening of seven isolates for the production of a compound (s) exhibiting biofilm inhibitory activity over time during growth. The biofilm inhibitory activity was detected from three isolates: 6E, 10G and 11E but was not detected from four isolates: 3C, 8E, 9C and 9E. The compound responsible for the biofilm inhibitory activity was produced at 72 h and reached the highest level of activity

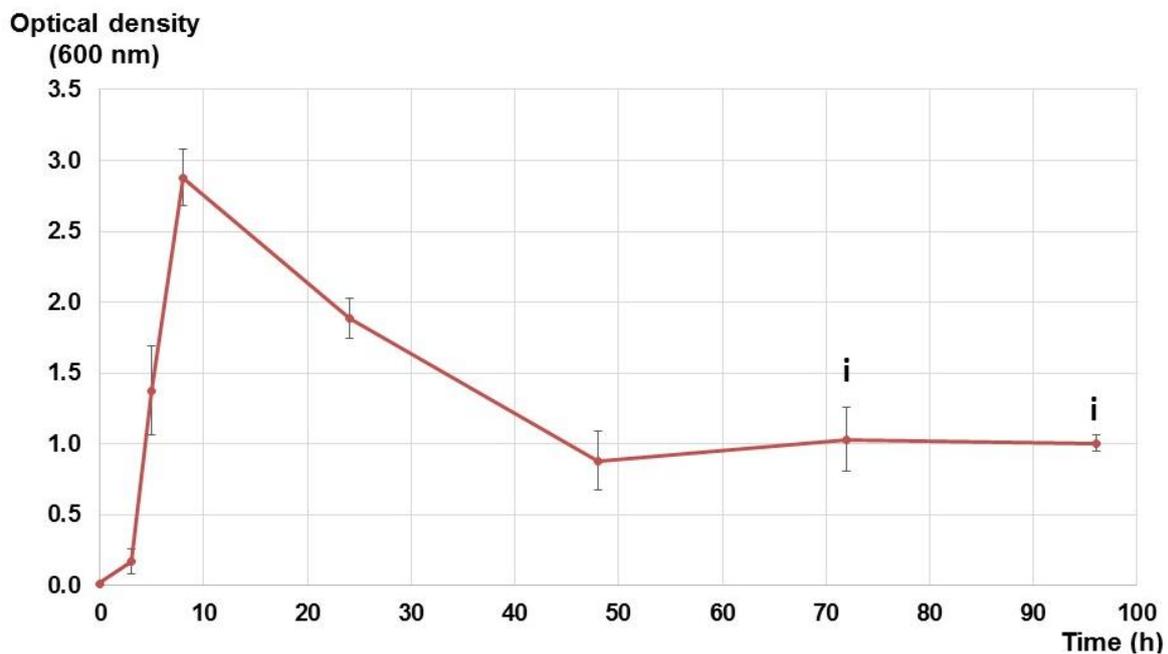
at 96 h of incubation time. The seven isolates could be divided into two groups based on the phenotype of the ability to produce a compound with biofilm inhibitory activity. The phenotypic result correlated with the divide between the seven isolates found using genotypic molecular markers such as 16S rRNA, *rpoβ* and *gyrA* gene sequences as shown in Chapter 3. Given this it was possible that a gene (s) related to the production of the compound might be present in isolate 6E, 10G and 11E and absent in isolate 3C, 8E, 9C, 9E. Thus, analysis of whole genome sequences might narrow down and lead to the identification of a gene related to the production of the compound with biofilm inhibitory activity (Lechner *et al.*, 2011, Gupta *et al.*, 2014).

-To try and determine the nature of the molecule (s) responsible for the biofilm inhibitory activity produced by isolates 6E, 10G, and 11E, the filtered supernatants were heated at 100 °C 15 min. All of the supernatants lost activity suggesting that the compound responsible for the activity was heat sensitive and it might be a protein.

To establish that the compound might be a protein, proteinase K was used. Due to the fact that proteinase K itself has biofilm inhibitory activity, DIFP was used to inhibit the proteinase K activity after treating the supernatants from isolate 6E, 10G and 11E. Unexpectedly the biofilm inhibitory activity was lost when treated with DIFP alone. Thus, it was hypothesised that the compound responsible for the activity might be a serine protease or a compound containing a serine protease like catalytic triad structure.

The large reduction in the optical density of isolate 6E cultures was found, as shown in Figure 5.13, in post-exponential phase. This reduction was probably caused by cell lysis and could have resulted in the release of many intracellular enzymes including proteases (Stephenson *et al.*, 1999, Westers *et al.*, 2004). *B. subtilis* has also been reported to produce many extracellular proteins including serine proteases, called

subtilisins (Westers *et al.*, 2004, Stein, 2005). Subtilisin production is very low during exponential growth phase and the production is increased after growth phase to induce the sporulation of *B. subtilis* cells (Stein, 2005). This might be a reason that the biofilm inhibitory activity was detected from culture supernatants from 72 h and 96 h incubation times. These incubation times were similar to a study by Gençkal & Tari (2006) who investigated an alkaline protease derived from *Bacillus* sp. cultured for 96 h. Moreover, Gençkal & Tari (2006) also found that total protease activities were increased when culturing the *Bacillus* sp. for 73 h to 96 h, similar to my findings (as shown in Figure 5.1). These findings could suggest that a protease was possibly produced from isolate 6E in my study.



Remark: i = Biofilm inhibitory activity

Figure 5.13 Optical density of isolate 6E grown in BHI broth over time. The biofilm inhibitory activity (i) was detected in cultures at 72 h and 96 h when grown in 20ml of BHI broth.

To scale up the production of the compound responsible for biofilm inhibitory activity, isolate 6E was selected to grow on a larger scale using 200 ml of BHI broth in a 1L Duran bottle. The biofilm inhibitory activity was produced under these conditions.

Interestingly, isolate 6E produced the activity earlier in growth compared to growth in 20 ml of BHI broth in a 50 ml tube.

Different environmental conditions between the 20 ml and 200 ml cultures probably account for the difference in the time required for the production of the compound with the inhibitory activity. For instance, the amount of oxygen might influence the production of the compound with the biofilm inhibitory activity. The air space volume in the 1L Duran bottle (800 ml) was more than in the 50 ml centrifugal tube (30 ml). Moreover, the air-liquid interface area and the air space volume to bacterial culture volume ratio of the Duran bottle were higher than the 50 ml tube, suggesting a difference in oxygen availability between both conditions. Thus, oxygen could be one of the factors causing an early production of the compound responsible for biofilm inhibitory activity in the Duran bottle.

Recent studies have demonstrated that oxygen is involved in an increase in the production of surfactin and lipopeptides from *Bacillus* sp. (Jacques *et al.*, 1999, Yeh *et al.*, 2006, Fahim *et al.*, 2012). Yeh *et al.* (2006) showed that the volumetric oxygen transfer coefficient has a positive effect on surfactin production by *B. subtilis* ATCC2133, however, the exact mechanism of how oxygen promotes surfactin production has not been reported yet.

A 0.5 ml centrifugal ultrafiltration filter was used to concentrate the compound responsible for biofilm inhibitory activity in the 0.22 filtered supernatants of isolate 6E, 10G and 11E. The results in 5.4.4 demonstrated that the biofilm inhibitory activity was present in the retentate fractions of a 3 KDa, 30 KDa, 50 KDa, and 100 KDa filters. This indicates that the compound responsible for the activity did not pass through the filter membrane. Thus, the size of the compound was possibly greater than 100 KDa.

In the scale up production of the compound with biofilm inhibitory activity from isolate 6E grown in a volume of 200 ml BHI broth, the compound was separated using a 400 ml Amicon stirred ultrafiltration cell (model 8400, Millipore, USA). The filtered supernatant (0.45 μm) was subjected to the ultrafiltration using a 100 KDa filter housed in the Amicon stirred ultrafiltration cell and the apparatus was pressurized with nitrogen gas (as shown in Figure 5.14). It was expected that the biofilm inhibitory activity would remain in the retentate fraction, as it had previously been found in the retentate of a 100 KDa filter when using a 0.5 ml Amicon centrifugal ultrafiltration unit. However, the activity was not found in the retentate fraction using the 400 ml Amicon centrifugal ultrafiltration unit. This might be the result of the different systems of ultrafiltration. In this larger volume unit, the mass transfer across the membrane was driven by the pressure of nitrogen gas. A stirrer inside the apparatus minimised the boundary layer of proteins on the membrane surface and that reduces the likelihood of clogging of the membrane pores with proteins (Simon *et al.*, 2000). The experiments were also conducted using a 50 KDa, 30KDa and 10 KDa filters to separate the compound responsible for the biofilm inhibitory activity. The activity was not found in the retentate of 100 KDa (> 100KDa) nor in the 10 KDa filtrate (<10 KDa) but it was found in both the retentate and the filtrate when using 30 KDa or 50 KDa filters. This suggested that the size of the compound with the activity was more than 10 KDa and it was less than 100 KDa. Thus, it was difficult to separate the compound using ultrafiltration in this study.

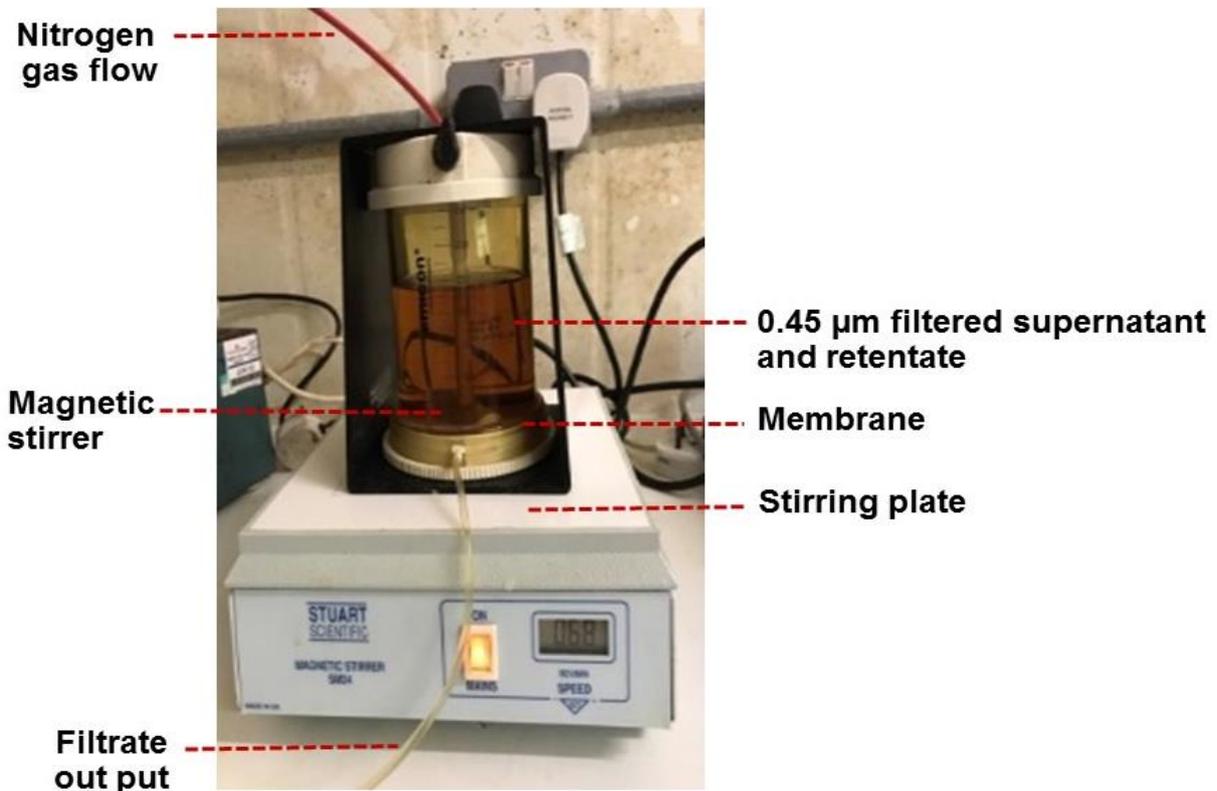


Figure 5.14 Amicon stirred ultrafiltration cell model 8400 (Millipore, US)

The failure of using ultrafiltration to purify proteins has been reported by Georgiou *et al.* (2001). A 30 KDa molecular weight cut-off centrifugal ultrafiltration unit was used to separate albumin and proteins in whole human plasma. Protein bands in both filtrate (<30 KDa) and retentate (>30 KDa) fractions of this ultrafiltration unit showed similar results as protein bands of the whole plasma.

The capacity of the filtered supernatants to disrupt *S. mutans* biofilm was examined on early steps of biofilm formation and mature biofilms. The results as shown in Figure 5.10 that the biofilm inhibitory activity was active at the early stage of biofilm formation. The highest activity was found when the filtered supernatant was added at the start of biofilm formation. Biofilm inhibitory activity was observed when the supernatant of isolate 6E was applied to the *S. mutans* biofilm formation assays at 1 h and 2 h but the activity was not effective on 7 h and 24 h old biofilms. This result suggests that the compound affects the process of biofilm formation. It is possible that the compound

affected adhesion or cell-to-cell attachment or communication. Some substances have been shown to affect *S. mutans* biofilm formation at the step of adhesion to the surface. For example a novel compound from *Trachyspermum ammi* (Khan *et al.*, 2010), a high molecular weight coffee fraction (cHMW) (Stauder *et al.*, 2010) and sphinganine and phytosphingosine (Cukkemane *et al.*, 2015). Moreover, a surface-protein-releasing enzyme (SPRE), an endogenous protease, produced from *S. mutans* BM71 causes the detachment of its own adherent cells (Lee *et al.*, 1996). Further experiments might provide additional information on the mode of action of the compound responsible for the biofilm inhibitory activity and should be conducted using the pure compound.

The attempts to separate the compound(s) responsible for biofilm inhibitory activity in this study were conducted using several methods, including hydrophobic interaction chromatography (HIC), benzamidine affinity chromatography and ion exchange chromatography.

HIC using a Phenyl Sepharose Fast Flow column is based on the binding of hydrophobic regions of macromolecules to hydrophobic ligands on chromatography adsorbents (McCue, 2009). In water, which is a poor solvent for nonpolar molecules, water molecules form around hydrophobic molecules leading to the increase of entropy. In this condition, hydrophobic molecules attempt to decrease the entropy by self-association or aggregation which is driven by the increased entropy. The polarity of solvent can be controlled by the addition of salt or organic solvents. In this study, ammonium sulfate was selected because ammonium ions and sulfate ions promote the strongest cation and very strong anion hydrophobic interactions, respectively.

This study demonstrated that the Phenyl FF column could separate the compound responsible for the biofilm inhibitory activity as the activity was detected in the eluted

fractions F2 and F3. This suggests that the compound responsible for the activity contains hydrophobic regions which could bind to a hydrophobic region of the phenyl molecule. However, it was not suitable for purification because the data indicates that the double strength binding buffer (2.0 M ammonium sulfate in 100 mM sodium phosphate pH 7.0), which was added in the sample preparation, led the active compound to aggregate.

Affinity chromatography, when possible, is one of the most powerful purification methods to separate a specific molecule or a group of complex molecules from complex mixtures. The principle of this chromatography is based on highly specific interactions between two molecules such as antigen and antibody, enzyme and substrate (Urh *et al.*, 2009). In this chapter, the results from the determination of the effect of proteinase K on the biofilm inhibitory activity (as shown in Figure 5.3) demonstrated that the biofilm inhibitory activity itself was lost when DIFP, a serine protease inhibitor, was added to the filtered supernatant of isolate 6E. Suggesting that DIFP inhibited the active compound in the filtered supernatant. It was therefore hypothesised that the compound responsible for this activity might be a serine protease. To isolate the compound, Benzamidine Fast Flow column chromatography was selected because benzamidine is a reversible inhibitor of serine proteases. However, the biofilm inhibitory activity was not detected from any fractions during the elution of bound-protein from the column. This result suggested that the compound responsible for the activity could not bind to the benzamidine molecule in the column and the results led to a hypothesis that the compound responsible for the biofilm inhibitory activity was possibly not a serine protease and that DIFP was not acting on the compound from isolate 6E but was acting on a *S. mutans* protease produced in response to the compound from 6E. To test this DIFP was added to the filtered

supernatant of 6E and then any unreacted DIFP which was not irreversibly bound was removed by dialysis. The results as shown in Fig 5-10 demonstrated that the filtered supernatant of 6E, which was treated with DIFP and then dialysed had biofilm inhibitory activity. This result suggested that the compound from 6E was not inhibited directly by DIFP. Thus, the compound was not a serine protease. However a *S. mutans* serine protease might be involved in the process.

The last chromatography techniques which were used to attempt to separate the compound responsible for biofilm inhibitory activity were ion exchange chromatography steps, cation and anion exchange chromatography. The experiments aimed to separate compounds with a wide range of pI values. The DEAE column buffered at pH 7.0 and pH 8.0 should bind and allow separation of molecules which have a pI less than 7.0 or 8.0, respectively, as they will have a net negative charge. The High S cation exchange column buffered at pH 5.0 and pH 6.5 was expected to bind to net positively charge molecules which have a pI more than 5.0 or 6.5, respectively.

The results as shown in the Table 5.4 and Table 5.5 demonstrated that both DEAE and High S columns had not separated any fraction with biofilm inhibitory activity. Much of the activity was lost after adding 0.1 M sodium phosphate buffer at pH 5.0, or 6.5, or 7.0 or 8.0 in preparing the sample for the columns. The exact reason of the activity loss has not been identified in this study. However, recent studies reported that the pH alteration affect enzyme activity. For example, pH causes enzyme aggregation (Cromwell *et al.*, 2006) and rotates enzyme structure (Das & Balasubramanian, 2018) leading to the reduction of active site and substrate interaction. The change of pH also leads to the alteration of electric field of the region around active site and this results in the effect on chemical bonds of molecules responsible for activity (Fried *et al.*, 2014).

5.6 Conclusion

The results in this chapter demonstrate that isolates 6E, 10G and 11E produced a compound (s) during growth which had the ability to inhibit *S. mutans* biofilm formation on saliva-coated polystyrene surfaces. Thus, the compound might be a potential treatment to apply in dental caries prevention. The attempts to separate the compound (s) responsible for the biofilm inhibitory activity based on physiochemical properties such as size, hydrophobicity, benzamidine affinity and ion exchange chromatography could not separate the compound responsible for the activity. The finding also suggested that the compound was not a serine protease as it was hypothesised in the beginning of this chapter but that a *S. mutans* serine protease may be induced or activated by the compound from 6E.

CHAPTER 6

Final Conclusion

Chapter 6 Final conclusion

S. mutans is considered as one of the major bacteria causing dental caries due to it causing demineralisation when growing as a biofilm on the tooth surface. The main aim of this study was to isolate sources of, and compound(s) exhibiting *S. mutans* anti-biofilm activity. Two sources of bacteria were screened for anti-biofilm activity. The first source was a metagenomic library constructed from human saliva. The results of screening of 6,624 out of the 27,000 clones in the library were that *S. mutans* anti-biofilm activity was not detected from this subset of the human saliva metagenomic library.

It has been shown in previous studies that the human saliva metagenomic library used in my study was useful in isolating a novel ABC transporter for tetracycline and tigecycline (Reynolds *et al.*, 2016). In addition, it aided in identifying a bacterial clone with reduced susceptibility to cetyl-trimethylammonium bromide (CTAB) (Tansirichaiya *et al.*, 2018). These other studies demonstrate that the human saliva metagenomic library is a potential source of genes encoding multiple biologically-active molecules which can be used in functional screens. The negative results found in this study could be due to multiple factors. Firstly, the screening covered only 24.53% of the total metagenomic library and one or more of the other 20,376 clones (75.47%) might exhibit *S. mutans* anti-biofilm activity.

Secondly, obviously foreign genes require transcription machinery from host cells and the different transcription machinery between host and foreign cells may result in compatibility problems that affect the transcription of foreign genes (Warren *et al.*, 2008). The transcription of most *E. coli* genes are controlled by the binding of the *E.*

coli polymerase σ^{70} subunit to the recognition sequences of promoter regions (Paget & Helmann, 2003). Therefore, the promoter regions of foreign genes would need to contain significant matches to the *E. coli* polymerase σ^{70} binding site for transcription (Warren *et al.*, 2008). Recognition sequences of promoter regions in *E. coli* are AT rich. Thus, the promoter region of foreign genes that contain high GC may reduce the binding of *E. coli* polymerase σ^{70} subunit (Warren *et al.*, 2008).

The initial process of mRNA translation in *E. coli* requires ribosomal protein S1 to bind to a specific sequence, called the Shine-Dalgarno sequence, these are purine rich sequences upstream of the initiation codon (Boni *et al.*, 1991). The protein S1 of *E. coli* is composed of six domains, with the two first domains responsible for binding the ribosome and another four domains involve with binding mRNA (Salah *et al.*, 2009). The composition of protein S1 of Gram-positive bacteria is similar to Gram-negative bacteria which is composed of six domains. However, the function of the first domain of Gram-positive bacteria protein S1 is different to Gram-negative bacteria (Salah *et al.*, 2009). The difference of S1 proteins might affect the translation of foreign mRNA of Gram-positive bacteria in *E. coli* host cells.

Codon usage might influence the expression of foreign proteins in *E. coli* host cells. Recent studies have reported that the use of synonymous codons, which are different codons encoded the same amino acid, is different in organisms (Ermolaeva, 2001, Mitra *et al.*, 2016). Bias in codon usage influences the translation speed and protein folding as a result of many factors including tRNA concentration, mRNA secondary structure, mRNA degradation, and ribosome loading and recycling (Mitra *et al.*, 2016). Particularly, in *E. coli*, codon content is shown to modulate mRNA stability and

translation efficiency, which could be affected by ribosomal elongation and tRNA concentration (Boël *et al.*, 2016).

Thirdly, it has been demonstrated that the diversity of genes from microorganisms in the metagenomic library used in this study was, 43.2 % Bacteroidetes and 42.1 % Firmicutes represent the most abundant populations (Reynolds, 2017). This number demonstrates that many Gram-positive bacterial DNA fragments were inserted into the pCC1BAC vector in *E. coli* EPI300 host cells. The difference in structure of the cell envelope between Gram-positive and Gram-negative bacteria may affect the transport of extracellular proteins. In Gram-positive bacteria, extracellular proteins are secreted across one phospholipid bilayer of cell membrane and a thick layer of peptidoglycan-rich cell wall, whereas, in Gram-negative bacteria, the proteins are secreted across an inner cell membrane, a thin layer of peptidoglycan and outer membrane with lipopolysaccharide (Green & Meccas, 2016). Therefore, extracellular proteins that are encoded by foreign genes from Gram-positive bacteria may have reduced capacity to be transport out of the *E. coli* host cell.

At least nine secretory pathways have been identified in bacterial protein translocation (Lasica *et al.*, 2017). Gram-negative bacteria use seven pathways: type I to VI and type VIII (T1SS to T6SS, and T8SS), to secrete proteins (Green & Meccas, 2016, Lasica *et al.*, 2017). However, only five of these pathways: type I to III, type V and type VIII (T1SS to T3SS, T5SS and T8SS), have been successfully used to secrete recombinant proteins (Burdette *et al.*, 2018).

The Type VII system (T7SS) was first found in Gram-positive bacteria such as *Mycoplasmata tuberculosis*, *Staphylococcus aureus*, *Streptococcus agalactiae*, *Bacillus*

subtilis. A recent study (Unnikrishnan *et al.*, 2016) reported that type VII systems are also found in some Gram-negative bacteria, *Helicobacter pylori*, *Pseudomonas aeruginosa*, and *Vibrio cholerae*, but a type VII system has not been identified in *E. coli*.

Type II and type V pathways require two steps to transport proteins (i) crossing the inner membrane into the periplasm is by the general secretory (Sec) and twin arginine translocation (Tat) pathways and (ii) crossing the outer membrane to the extracellular space through type II and type V pathways (Depluvere *et al.*, 2016). Signal peptides of secretory proteins play an important role in the secretory process of both Sec and Tat pathways (Freudl, 2018). The specific requirements of the secretory pathway of some foreign proteins might limit the translocation of the foreign proteins in *E. coli* host cells. A recent study by Lasica *et al.* (2017) reported a novel secretory pathway, type IX, T9SS, only found in the *Bacteroidetes* phylum. Type IX has been shown to play an important role in the translocation of protein virulence factors across the outer membrane. Without the type IX in *E. coli* translocation of proteins derived from *Bacteroidetes* which is the most abundant in the metagenomic library in this study may be affected. Together, these factors could reduce the chances of foreign proteins being secreted by *E. coli* and thereby the chances of discovering compounds with *S. mutans* biofilm inhibitory activity.

The second bacterial source screened for antibiofilm activity was a library of environmental bacteria from the Swab and Send project. A subset of 89 isolates from the library were screened for activity and seven isolates were found to produce either one or two anti-biofilm activities: (i) biofilm detaching activity and (ii) biofilm formation inhibitory activity. All seven isolates produced compound(s) exhibiting biofilm

detaching activity but only three isolates produced compound(s) with biofilm formation inhibitory activity.

The compound(s) with biofilm detaching activity allowed *S. mutans* to produce biofilms but the biofilms could be detached easily. The discovery of this activity suggested that it might be useful for dental caries prevention. Thus, an attempt to identify the compound responsible for the biofilm detaching activity was conducted as described in Chapter 3. Two isolates (isolate 6E and 9C), which were representatives of the seven isolates based on genetic molecular markers: 16S rRNA, *gyrA*, and *rpoβ* gene sequence nucleotide similarity, were cultured in M9 minimal medium supplemented with different carbon sources instead of culturing them in the rich medium, Brain Heart Infusion. Isolate 6E was found to produce a compound(s) exhibiting biofilm detaching activity when it was cultured in M9 medium supplemented with sucrose (M9+S) but not with others carbon sources including glucose. The culture supernatant from isolate 6E cultured in M9+S was used to attempt to identify the compound with biofilm detaching activity. After treatment of the supernatant, containing the biofilm detaching activity, with proteinase K the activity was inactivated. This result suggested that the compound was a protein or a molecule containing peptide bonds. The filtered supernatants with and without biofilm detaching activity, which were derived from isolate 6E culturing in M9 supplemented with sucrose and glucose, respectively, were analysed using SDS-PAGE. The results revealed that the filtered supernatants with biofilm detaching activity showed four different bands on SDS-PAGE gel relative to supernatants that lacked the biofilm detaching activity. These four bands were identified, by FingerPrints Proteomics using LC-MS/MS, as catalase, levansucrase, TasA and an alpha-amylase like protein. Two proteins, TasA and levansucrase, were hypothesized to be possibly involved with the biofilm detaching activity.

The production of the compound with the biofilm detaching activity was dependent on sucrose supplementation in the culture medium. Thus, it was possible that levansucrase might be the protein responsible for the activity because it has been reported that this enzyme is produced in the presence of sucrose. A tricistronic operon in *Bacillus* sp. is composed of three genes and one of them, *sacB*, encoding for levansucrase has been shown to be induced by sucrose (Dogsa *et al.*, 2013). Levansucrase hydrolyses sucrose and synthesises levan. Therefore, levan was a possible candidate for the biofilm detaching activity. Commercial levan, derived from *Erwinia herbicola*, was used in the *S. mutans* biofilm assay but it did not have any biofilm detaching activity. This result suggests that levan may not be responsible for the activity. Because the levan derived from Gram-positive and Gram-negative bacteria have different characteristics (Oner *et al.*, 2016), levan from isolate 6E, which is Gram-positive bacterium, might be different from the levan from *E. herbicola*, a Gram-negative bacterium. A better experiment would be using the levan from strain 6E in the *S. mutans* biofilm assay. To simulate the tooth surface in the oral cavity, *S. mutans* biofilm assays were conducted using saliva-coated surfaces. The culture supernatant, exhibiting the biofilm detaching activity on uncoated surfaces, did not exhibit the activity on saliva-coated surfaces. This result suggested that the compound might not be useful for dental caries prevention.

Aside from the biofilm detaching activity, biofilm formation inhibitory activity was detected from three of seven isolates (isolate 6E, 10G and 11E) and the compound with the activity inhibited *S. mutans* producing biofilms on saliva-coated surfaces. This activity was discovered while investigating the optimum conditions for production of the compound(s) with the biofilm detaching activity during growth of isolate 6E. Several attempts to purify the compound were conducted in this study. The results suggested

that the activity was heat sensitive and it might be a protein. After proteinase K treatment, the biofilm inhibitory activity of the supernatant from isolate 6E was not detected. Also, DIFP inhibited the biofilm inhibitory activity, leading to the hypothesis that the compound responsible for the biofilm inhibitory activity might be a serine protease.

Because the biofilm inhibitory activity was produced in BHI medium only and not in M9 medium, the purification of the compound with the activity was conducted from isolate 6E cultured in BHI medium. Purification based on size did not separate the compound because the activity was detected in both the fractions that passed through and that did not pass through 30 KDa and 50 KDa filters. The compound in culture supernatants was separated using ammonium sulfate protein precipitation at 30-70 % saturation. The 30-70 % protein fraction with biofilm inhibitory activity was applied to a Phenyl Fast Flow column but the majority of the activity was lost during the preparation processes. Thus, the Phenyl FF column could not be used to separate the compound with the biofilm inhibitory activity in this study.

Because of the hypothesis that the compound might be a serine protease, the 30-70 % protein fraction was applied to Benzamidine-sepharose column which is an affinity chromatography step specific to serine proteases. The benzamidine column could not separate the compound and this suggested that the hypothesis that the compound might be a serine protease could be incorrect or that this serine protease had weak or no binding to benzamidine. Moreover, determination of the effect of DIFP on the biofilm inhibitory activity using a dialysis cassette to remove excess DIFP, suggested that the DIFP did not inhibit the biofilm inhibitory activity directly. This finding strongly suggests that the compound responsible for the activity was not a serine protease.

Then, the culture supernatant was separated using ion exchange chromatography, both cation and anion exchangers were used, a High S column and a DEAE column, respectively. However, the compound with the biofilm inhibitory activity could not be separated using either of the columns due to the loss activity in the preparation processes. Thus, the compound with biofilm inhibitory activity was not isolated and identified in this study.

Based on whole genome sequencing of seven isolates exhibiting biofilm detaching activity as described in Chapter 3 the isolates could be divided into two groups: (i) isolates 6E, 10G and 11E and (ii) isolates 3C, 8E, 9C and 9E. This result was similar to the divide using the molecular markers: 16S rRNA, *rpoB* and *gyrA*, and corresponded to the phenotype of the capacity to produce a compound with biofilm formation inhibitory activity as described in Chapter 5. Genome-to-genome distance and ANI values suggested that the seven isolates were *B. velezensis* according to the highest similarity to the two reference *B. velezensis* in this species (*B. velezensis* NKG-1 and *B. velezensis* SRCM100072) in Chapter 3.

B. velezensis belongs to clade II of the *B. amyloliquefaciens* operational group (Fan *et al.*, 2017). Members of clade II have been isolated from a variety of environments such as plant roots (Krebs *et al.*, 1998, Meng *et al.*, 2016) and marine environments (Liu *et al.*, 2010). They are able to produce many bioactive compounds, especially those of benefit to plants (Meng *et al.*, 2016). One of them, *B. velezensis* strain FZB42 is a model for Gram-positive bacteria that are plant-growth-promoting and used for biocontrol of rhizobacteria (Fan *et al.*, 2018). A recent study by Yoo *et al.* (2018) reports that *B. velezensis* K68 produces 1-deoxynojirimycin (1-DNJ), a known GTF expression inhibitor, and inhibits *S. mutans* biofilm formation by reducing expression

of *gtfBCD* and cell adhesion. *B. velezensis* K68 produced 1-DNJ when it was cultured in Tryptic Soy medium supplemented with 1% glucose. The medium used for culturing *B. velezensis* K68 are different to my study which used BHI medium.

A molecular approach might be required to identify the gene(s) responsible for the biofilm inhibitory activity. Transposon mutagenesis is one strategy that has been used to identify genes of interest. For example, the screening for genes related to riboflavin production in *B. subtilis* (Tännler *et al.*, 2008) and genes involving the biosynthesis of bacilysin from *B. subtilis* PY79 (Yazgan *et al.*, 2001). Therefore, transposon mutagenesis might be useful to identify genes involved in the biosynthesis of a compound responsible for the biofilm inhibitory activity.

Real time PCR of genes related to *S. mutans* biofilm formation might provide a clue of the mechanism of action of the biofilm inhibitory activity. The expression level of genes related biofilm formation have been studied such as, (i) those involved in polysaccharide synthesis *gftB*, *gftC*, *gftD*, *ftf* (Savabi *et al.*, 2014), (ii) acid tolerance: *fabM* (Fozo & Quivey, 2004), *rgbF* (Kovacs *et al.*, 2017), (iii) Quorum sensing system: *vicR*, *comCDE*, *cipB*, and *sigX* (Palmer *et al.*, 2013, Gowrishankar *et al.*, 2014, Leung *et al.*, 2015), (iv) cell adhesion: *gpbB* (Gowrishankar *et al.*, 2014), *srtA* (Lévesque *et al.*, 2005).

To conclude, this study investigated *B. velezensis* strains that produced at least two compounds with *S. mutans* anti-biofilm activities. One of the activities, the biofilm formation inhibitory activity might have the potential to be used in the prevention of dental caries, because the compound responsible for this activity inhibited *S. mutans*

biofilm formation on saliva-coated surfaces. However, the compound responsible for the biofilm inhibitory activity needs to be isolated and identified in the future.

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APPENDIX

Appendix

Appendix 1

Table A.1 Cell wall-anchored or surface-associated adhesins of *S. mutans* adapted from Nobbs *et al.* (2009)

| Protein group | Protein(s) | Function(s) and/or substrate(s) |
|------------------------------|--|--|
| Agl/II family | SpaP/P1/PAc/Sr/AgB | Coaggregation; multiple substrates |
| Saliva-binding proteins | Agl/II family | Salivary components (gp340, salivary glycoproteins, proline-rich proteins) |
| Fibrinogen-binding proteins | SmFnB FBP-130a PavA-likea Agl/II family | Fibrinogen |
| Glucan-binding proteins | GbpABCD | Dextran-dependent aggregation;glucan |
| Collagen-binding proteins | WapA Cnm Agl/II familya | Collagen |
| Plasminogen-binding proteins | Enolase | Plasminogen, plasmin |
| Laminin-binding proteins | Agl/II family Cnm | Laminin |
| Fibrinogen-binding proteins | SpaP/P1/PAc/Sr/AgB | Fibrinogen |
| Host cell-binding proteins | FBP-130a Agl/II family | Endothelial cells Epithelial/endothelial cells (alphah5 beta1 integrin) |
| Enzymes | HtrA FruA Dex, DexA | Serine protease Beta-D-Fructosidase Dextranase |

Appendix 2

Table A.2 Protein identification using LC-MS/MS analysis

| Band | Protein Description | Score | Coverage | # proteins | # unique peptides | # peptides | # PSMs | # AAs | MW (KDa) | Calc.pI |
|------|--|----------|----------|------------|-------------------|------------|--------|-------|----------|---------|
| A | Catalase | 40018.70 | 96.88 | 1 | 72 | 72 | 2002 | 481 | 54.5 | 6.95 |
| | Uncharacterized protein yqcC | 4499.34 | 78.26 | 1 | 43 | 43 | 211 | 529 | 59.2 | 6.02 |
| | Pectin lyase like protein | 3244.77 | 82.26 | 1 | 30 | 30 | 176 | 468 | 50.9 | 5.92 |
| B | Levansucrase | 24811.83 | 91.12 | 1 | 62 | 62 | 1355 | 473 | 53.0 | 6.64 |
| | Catalase | 2989.70 | 83.58 | 1 | 39 | 39 | 163 | 481 | 54.5 | 6.95 |
| | Enolase | 2629.67 | 70.47 | 1 | 27 | 27 | 116 | 430 | 46.6 | 4.77 |
| C | TasA | 15525.43 | 84.67 | 1 | 40 | 40 | 710 | 261 | 28.1 | 6.83 |
| | Serine alkaline protease (subtilisin E)(EC:3.4.21.62) | 1807.26 | 55.50 | 1 | 14 | 14 | 104 | 382 | 39.1 | 9.25 |
| | Extracellular serine protease | 1441.49 | 44.96 | 1 | 31 | 31 | 70 | 803 | 85.8 | 7.33 |
| D | Alpha-amylase like (EC 3.2.1.1) | 1597.75 | 49.77 | 1 | 28 | 28 | 120 | 659 | 72.3 | 5.82 |
| | Hypothetical protein | 1489.05 | 26.87 | 1 | 20 | 20 | 78 | 774 | 83.8 | 5.68 |
| | Catalase | 1111.44 | 49.90 | 1 | 27 | 27 | 50 | 481 | 54.5 | 6.95 |

Protein Description When available provides the available protein names from the 'Protein names' section of the corresponding protein's UniProt entry.

Protein Score The sum of the ion scores of all peptides that were identified.

Coverage The percentage of the protein sequence covered by identified peptides.

Proteins The number of identified proteins in a protein group, that is, the number of proteins displayed in the Protein Group Members view.

Unique Peptides The number of peptide sequences that are unique to a protein group. These are the peptides that are common to the proteins of a protein group, and which do not occur in the proteins of any other group. The number of unique peptides that determine a protein group can be set in Proteome Discoverer.

Peptides The total number of distinct peptide sequences identified in the protein group.

PSM's The number of peptide-spectrum matches. The number of PSM's is the total number of identified peptide spectra matched for the protein. The PSM value may be higher than the number of peptides identified for high-scoring proteins because peptides may repeatedly be identified.

AAs Number of amino acids

Calc.pI calculator of protein isoelectric point

Appendix 3

Amino acid sequence of protein of SDS-PAGE gel band A

Catalase

MSSNKLTTSWGAPVGDNQNSMTAGDRGPALIQDVHLLLEKLAHFNRERVPERVVHAKGAGA
HGYFEVTNDVTKYTKAAFLSEVGKRTPLFIRFSTVAGELGSSDTPRDPGRFAVKFYTEEG
NYDIVGNNTPVFFIRDIAIKFPDFIHTQKRDPRTLKNPTAVWDFWLSPELHQVTILMS
DRGIPATLRHMHGFGSHTFKWTNDKGEVWIKYHFKEQGVKNLDVNTAAKIAGENPDYH
TEDLFNAIENGDFPAWKLYVQIMPLEDANTYRFDPFDVTKVWSQKDYPLIEVGRMVLNRN
PENYFAEVEQATFSPGTLVPGVDVSPDKMLQGRLFAYHDAHRYRVGANHQALPINRSRNE
VKNYQRDGMRFDDNGGRSVYYEPNFSGGPKESPEDKQAAYPVSGFADSVSYNHHDHYTQ
AGDLYRLMSEERARLVANIVSAMKPVKEEIKLRQIGHFYKADPEYGRRVAEGLGLPSPK

Uncharacterized protein yqcC

AYEEKTDWLPDDPINEDDVNRWEKGIKDAHTDLAAHKNDMNNPHKTTKAQIGLGNVDNV
QQAAKKDFDRHNQDLDRHVTKEERQKWNNGQLTKLTDDSGKYLISIQDGLDFHQIVDQLN
QSFFFYTNNTGVHTPPLSARGLYIGFKSYGEALAMDYEGGTWRKTLKASGWDWVQLETS
EGAQKKVDAHTKQTDIHVTKAEKDTWNAGQLFKITADNGTQKINLTSGSFYDSLKDVGSV
TFYGTNAVTDNPSNTSLRGMQLVGQPGIGNGYAVDVKGNAWWFYNSNQTAINWYPIESI
AGAQSKVDAHAIDSSIHISPIERDKWNGGQLAKLTKDDGKRTRLESGSDILALSSGFYYG
MGAQLKNNPVENDNSFFNYDVIESESGRKSILAWRSYDNNVWIATVHTDGVFKGWKRLFT
EEEFNKMTWYDLTLINGHKAGARNPQYAVWGNQVFTTRGEVVPDFDSIFAVLPSEAHPST
QKSMAAPLFGTTGITKYAEPTEGELRINGKYAKIEGNITGISIDNNFTL

Pectin lyase like protein

MKIKLSILSAAVLAAGITAFVWPKTEANEKMQTDALYVSPAGSDQNEGTKEKPFRTLKHA
AEKAEAGTTVLIREGTYDETFEVKHSHTAEKPITFRNYQNEHVSISGKSAPKSDSETPLI
QIRNKQYITIHGVTLENLSVSSSEDATAMGIFVTGSSSHINIDGNHIRNIKTADAEGNAHG
IAFYGTGAMKDV SITNNTVEKLT LGASEAVVLNGNV DGFKIAGNTIRDNNNIGIDVIGYE
GTSKQNDYARNGVIENNTVSHNSSYGNPAYGDEYSAGGIYVDGAEHVDIKNNTVYNNDLG
IEATSEHKGYARDIRITDNKVYGNAYTGISIGGYDTKRGGTINSVIAHNIMYRNDTKDL
DGGQLLLQYGTGNTIEKNIMTASGSRIFIANDYTKNEGNTVNHNVYHKEAGKDG IWNWK
NREYDSFTAYQKGTANDLDSIYADPMYRDESSYDFTLKP GSPALPVIQ

Amino acid sequence of protein of gel band B

Levansucrase

MNIKKFAKQATVLTFTTALLAGGATQAFKENTQKPYKETYGVSHITRHDMLQIPKQQQS
EKYQVPQFDQSTIKNIESAKGLDVWDSWPLQNADGTVAEYNGYHVVFALAGSPKDADDS
IYMFYQKVGDNSIDSWKNAGR VFKDSDKFDANDEILKEQTQEWSGSATFTSDGKIRLFYT
DFSGTHYGKQSLTTAQVNVSKSDDTLKINGVEDHKTIFDGDGKTYQNVQQFIDEGNYTSG
DNHTLRDPHYVEDKGHKYLVFEANTGTDNGYQGEESLFNKAYYGGSTNFFRKESQKLQQS
AKKRDAELANGALGIVELNDDYTLKKVMKPLITSNTVTDEIERANVFKMNGKWYLF TDSR
GSKMTIDGINSNDIYMLGYVSNLSLTGPYKPLNKTGLVLQMGLDPNDVTFTYSHFAVPQAK
GNNVVITSYMTNRGFFEDKKATFAPSFLNKNKTSVVKNSILEQQQLTVNN

Catalase

(See sequences in band A)

Enolase

MPYIVDVYAREVLDSRGNPTVEVEVYTETGAFGRALVPSGASTGEYEAVELRDGDKDRYL
GKGVLTAVNNVNEIISPELLGFDVTEQNAIDQLLIELDGTENKGGKLGANAILGVSMACAR
AAADFLQIPLYQYLGGFNSKTLVPVMMNIVNGGEHADNNVDIQEFMIMPVGAPNFREALR
MGAQIFHSLKSVLSAKGMNTAVGDEGGFAPNLGSNEEALQTIVEAIEKAGFKPGEEVKLA
MDAASSEFYNKEDGKYHLSGEGVVKTS AEMVDWYEEMVSKYPIISIEDGLDENDWEGHKL
LTERLGKKVQLVGDDL FVTNTKKLAEGIKNGVGN SILIKVNQIGTLTETFD A IEMAKRAG
YTAVISHRSGETEDSTIADIAVATNAGQIKTGAPSRTRVAKYNQLLR IEDQLAETAQYH
GINSFYNLNK

Amino acid sequence of protein of gel band C

TasA

MGMKKKLSLGVASAALGLALVGGGTWAAFNDVKSTDATFASGTLDSLAKESANVNLSNL
KPGDKLTKDFEFRNNGSLAIKEVLMALNFTDFKGAKKGNESAEDFLSQFEITVLTVGKEG
NGYYPKNIILKAASLKDLYLMSTKQDKAAAE AISKHIDPKFLSESGKVVN VATINGKTAPE
YDGVPKTPADYDQVRMEIQFKNDTAKTADGLSVQNKFGNAISLQFSFEATQWNGLTITK
DHTDKDGYVKENEKAHSEDKN

Serine alkaline protease (subtilisin E)

MRGKKVWISLLFALALIFTMAFGSTSPAHAAGKSNGEKKYIVGFKQTMSTMSAAKKKDDVI
SEKGGKVQKQFKYVDAASATLNEKAVKELKKDPSVAYVEEDHVAQAYAQSVPYGVSQIKA
PALHSQGFTGSNVKVAVIDSGIDSSHPDLKVAGGASMVPSETNPFQDNNSHGTHVAGTVA
ALNNSVGLVGVAPSASLYAVKVLGADGSGQYSWIINGIEWAIANNMDVINMSLGGPSGSA
ALKAAVDKAVASGIVVAAAGNEGTSSTVGYPGKYPSVIAVGAVNSSNQRASFSSVG
SELDVMAPGVSIQSTLPGNKYGAYNGTSMASPHVAGAAALILSKHPNWTNTQVRSSLENT
TTKLGDAFYYGKGLINVQAAAQ

Extracellular serine protease

MKKGIIRYLLPAFVLSFTLSTSSQAAPASKPQTPDLEKAEVFGDIDMTTGKQTTVIVELK
EKSLAEAKELGKAQTKSKLKSERSKVKKKALKTIKHGKINREYEQVFSGFMSKLPANEIP
KLLSDQDVKAVYPNVYHTDQLKDKDITLSKDAVSPQMDDSAPYIGANDAWKLGYTGKGV
KVAIIDTGVEYKHPDLKKNFGQYKGYDFVDNDYDPEETPSGDPRGASTDHGTHVAGTVAA
NGTIKGVAPDATLLAYRVLGPGSGTTENVIAGIERAVQDGADVMNLSLGNVNNPDWAT
STALDWAMSEGVTAVTSNGNSGPNWTVGSPGTSREISVGATQLPLNEYAVSFGSYSSA
KVMGYNKEDDIKALNKKETELIEAGIGEQQDFEGKDLKGVAVVKRGSIAFVDKADNAKK
AGAIGMVVYNNAPGEIEANVPGMSVPTVKLSSEDGEKLVSQLKAGGKATFHLSVAKSLT
EQMADFSSRGPVMDTWMIKPDVSAPGVNIVSTIPTHDPADPYGYGSKQGTSMASPHVAGA
AAVIKQAKPKWSPEQIKAALMNTAETLTDADGDVYPHNAQGAGSIRIMKAIKADSLVAPG
SYSYGTFMKDKGNETKKETFTIENQSSIRKSYQLEYSFNGTGITVSGTDRVVIPAHQTGK
VNAKVKVNAKKVAGTYEGTVTVREGGKTVAKVPTLLIVKEPDYPRVTSIDVQDGTQGT
YQIETYLPAGAEELAFVYDSNLDVFGQAGIYKKQDKGYQYFDWNGKVNNGDTALPAGEYY
MLAYAANKGKSSQVLTEKPFIE

Amino acid sequence of protein of gel band D

Alpha-amylase like

MFKKRFKTSLLPLFAGFLLLFHLVLSGPAGANAETANKSNEVTDSSVKNGTILHAWNWSF
NTLTENMKEIRDAGYAAIQTSPINQVKEGNQGDKMSMNWYWLYQPTSYQIGNRYLGTQE
FKDMCAAAEKYGVKIVDAVVNHTTSDYGAISDEIKRISNWITHGNTQIKNWSDRWDITQN
ALLGLYDWNTQNTEVQAYLKGFLERALNDGADGFRYDAAKHIELPDDGNYGSQFWPNITN
TSAEFQYGEILQDSASRDYAYANYMNVNTASNYGHSIRSALKNRNLSVSNISHYASDVSA
KLVTVVESHDTYANDDEESTWMSDDDIRLWAVIGSRSGSTPLFFSRPEGGGNGVRFPGK
SQIGDRGSALFKDQAITAVNQFHNEMAGQPEELSNPNGNNQIFMNQRGSKGVLANAGSS
SVTINTSTKLPDGRYDNRAGAGSFQVANGKLTGTINARSAVLYPDDIGNAPHVFLNYQ
TEAVHSFNDQLTVTLRANAKTTKAVYQINNGQETAFKDGDRLTIGKEDPIGTTYNVKLTG
TNGEGASRTQEYTFVKKDPSQTNIIQYQNPDHWGNVNAIYKHDGGGAIELTGSWPGKAM
TKNADGIYTLTPANADTADAKVIFNNGSAQVPGQNHPGFDYVQNGLYNNSGLNGYLPH

Hypothetical protein

MTNYTTFLGLYKPDKAEGIEVESIAQNFEAIDSKIGAALSDGTSTYENLNERLNMYENRF
 DNVTERNVMDFGAKGDGVTDDTQAFHDAMADGGYVVTVPAGVFRTSGLFVPSNTMLVGAG
 KKRTVIKLLDETPVGHSLVLTNSDYTNNGENIYVGHLLTDWNKDTRPAGWKIPKGPSTSSCL
 LFANVDYSFVEHVFAKNAGLHGFDSTSPNYNRHGEKDDPTYYPDGCNFVTFSSHCEATGS
 GDDNFTCHFHFHTTFSHCVSYHPMGNNGTSNSNCFEIDDGSQDVFVDSCVAIGGARGFE
 IKAHDYAPAAKRVQLANCRAYENAIGFCIRHIGHHLSAEPTSKTAHDVQLVNCQATNPKQ
 NSIYKGVGASALVSGYDSVNIVNFQAICEDRSYDYSNDTIIMIYYKGRYVNLKNITIRG
 FRSAHDLYVLGGENAVGKCMIDGVFLVDGGTTGLTFGGGVDNCTVSNVYGHRLGASSGT
 VGLYSSGPDNLISNIDFQGYETTFHVGGKTPYELFKHKGSSVMASKDSHVTGTNGFVIG
 TVRGQAAGGSQSGVIGSYNTINPNANSTVWGWSGSPSSANRKIELHAKEGTIKATGGV
 TGSSTFSDYAEYFESLSGEKIPTGTLVTLEGDKIRQAEKGDLMLGVISETAGVILGESSF
 HWSGRYVKNEFFGGYVYEEQKDANGHTVMAPKENPDFRPEEDYVSREERDEWNIVGLIGQV
 YVRCDETVKAGDFIHAHNGIATKSDAPNQRWQVMKVINEFDADKGFVALVFIR

Catalase

(see sequence in band A)

Appendix 4

Table A-3 Average nucleotide identity based on BLAST+ of seven isolates and two closely related type strains using JSpeciesWS

| | Average nucleotide identity based on BLAST+ (ANIb) | | | | | | | | |
|---|--|-------|-------|-------|-------|-------|-------|--------|-------|
| | (i) | (ii) | (iii) | (iv) | (v) | (vi) | (vii) | (viii) | (ix) |
| (i) 6E | | 99.95 | 99.94 | 97.94 | 97.94 | 97.94 | 97.94 | 98.26 | 97.81 |
| (ii) 10G | 99.94 | | 99.96 | 97.94 | 97.94 | 97.94 | 97.94 | 98.26 | 97.80 |
| (iii) 11E | 99.90 | 99.90 | | 97.9 | 97.88 | 97.89 | 97.9 | 98.19 | 97.73 |
| (iv) 3C | 97.72 | 97.72 | 97.76 | | 99.94 | 99.94 | 99.94 | 97.34 | 98.18 |
| (v) 8E | 97.71 | 97.72 | 97.72 | 99.95 | | 99.95 | 99.96 | 97.28 | 98.21 |
| (vi) 9C | 97.73 | 97.73 | 97.78 | 99.94 | 99.98 | | 99.96 | 97.33 | 98.23 |
| (vii) 9E | 97.64 | 97.65 | 97.68 | 99.88 | 99.88 | 99.88 | | 97.18 | 98.13 |
| (viii) <i>B. velezensis</i> SRCM100072 | 98.73 | 98.32 | 98.32 | 97.34 | 97.64 | 97.33 | 97.63 | | 97.36 |
| (ix) <i>B. velezensis</i> NKG-1 | 97.53 | 97.53 | 97.53 | 98.09 | 98.09 | 98.09 | 98.09 | 97.36 | |

Appendix 5

Script for visualize heatmap of absorbance values from microplate reader

```
fig = plt.imshow([[0.0, 0.1, 0.3],  
                 [0.5, 1, 0.6],  
                 [0.3, 0.6, 1]])  
fig.show()
```

plt is a python package tool visualize data