The Purification and Functional Study of Compounds Produced by *Escherichia coli* BP that Induce and Inhibit Sulphate Reducing Bacteria Growth

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A thesis submitted in partial fulfilment of the requirements for the award of degree of Doctor of Philosophy of the University of Portsmouth

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AUTHOR'S DECLARATION

I declare that whilst registered as a candidate for the degree of Doctor of Philosophy of the University of Portsmouth, I have not been registered for any other research award. The results and conclusions embodied in this thesis are the work of the named candidate and have not been submitted for any other academic award.

Oluwafemi Adebayo Oyewole

ABSTRACT

The induction and inhibition of sulphate reducing bacteria (SRB) growth is of academic and biotechnological interests due to their involvement in corrosion, hydrocarbon degradation and reservoir souring. The purification and functional study of SRB growth enhancer (SGE) and SRB growth inhibitor (SGI) produced by *Escherichia coli* BP is reported in this research. The SGE can be used as a supplement in the media for rapid SRB detection while the SGI can be used for the prevention of biocorrosion mainly induced by SRB.

The optimum growth conditions for highest SGE production were determined. The enhancer and inhibitor were purified using ion exchange and size exclusion chromatography. SRB cultures in the presence of SGE showed two order of magnitude higher growth than untreated SRB while with SGI they showed growth with two order of magnitude less.

Scanning electron microscopy (SEM), atomic force microscopy (AFM) and confocal laser scanning microscopy (CLSM) were used to study the effects of the enhancer and inhibitor on SRB growth. The SRB treated with the enhancer had growth enhancement while the inhibitor caused changes of SRB morphology and revealed the bactericidal effects on SRB growth.

The structural analysis of purified SGE and SGI were carried out using nuclear magnetic resonance (NMR) spectroscopy and matrix-assisted laser desorption ionisation–time of flight (MALDI-TOF) spectrometry. The NMR spectra of SGI and SGE were assigned to methylene, methine, alkynes, ketones, esters, aromatics and amides functional groups.

The MALDI-TOF spectra in linear mode showed the compounds to be an oligomeric series with repeat ~ 213 m/z unit between each of the peaks. The data revealed that the molecular mass of SGE is around 1700 Da while that of SGI is estimated as 2400 Da.

DEDICATION

This thesis is dedicated to my late father, Samuel Oyewole Asalu, who sacrificed so much to ensure I have a good education but could not live to see the fulfilment of his dream.

And

To my beloved wife Helen, daughter Emmanuella, and son Timi.

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LIST OF ABBREVIATIONS

ADC	Analog-to-digital converter
ADP	Adenosine diphosphate
APS	Adenosine 5′ -phosphosulphate
AMP	Adenosine-5'-monosulphate
ANX	Diethylaminopropyl
AFM	Atomic force microscopy
ATP	Adenosine triphosphate
CFS	Cell free supernatant
CFU/ML	Colony forming unit per milliliter
CGA	Community genome arrays
CHCA	α-cyano-4-hydroxycinnamic acid
cHSQC	Carbon heteronuclear single quantum correlation
CLSM	Confocal laser scanning microscopy
CM	Carboxymethyl
cAMP	cyclic adenosine monophosphate
dH_2O	Distilled water
DNA	Deoxyribonucleic acid
DGGE	Denaturing gradient gel electrophoresis
DEAE	Diethylaminoethyl
Dsr	Dissimilatory sulphite reductase
EPS	Extracellular polymeric substances
FeS	Iron sulphide
FGA	Functional gene arrays

FID	Free induction decay
FNR	Ferredoxin-$NADP^+$ reductase
GF	Gel filtration
h	Hour
HSA	Human serum albumin
HPLC	High performance liquid chromatography.
HW-40S	Toyopearl
H_2S	Hydrogen sulphide
LB	Lysogeny or Luria Broth
LP	Low pressure
LPS	Lipopolysaccharides
MALDI- TOF	Matrix-assisted laser desorption ionisation–time of flight
MIC	Microbiologically influenced corrosion
MS	Mass spectrometry
MWCO	Molecular weight cut off
NMR	Nuclear magnetic resonance
NRB	Nitrate reducing bacteria
PCR	Polymerase chain reaction
pI	Isoelectric point
PLFA	Phospholipid fatty acids
POA	Phylogenetic oligonucleotide arrays
ppm	Parts per million
PVC	Polyvinyl chloride
Q	Quaternary ammonium
QPCR	Quantitative PCR

QS	Quorum sensing
RNA	Ribonucleic acid
rRNA	Ribosomal RNA
S	Methyl sulphonate
SO_4^{2-}	Sulphate
S^{2-}	Sulphide
SEM	Scanning electron microscopy
SGE	SRB growth enhancer
SGI	SRB growth inhibitor
SP	Sulphopropyl
SRB	Sulfate reducing bacteria
TEAB	Triethylammonium bicarbonate
THPS	Tetrakis-hydroximethyl phosphonium sulphate
UPLC-QToF	Ultra-performance liquid chromatography quadrupole time of flight
Vo	Void volume
VM	Vitamin medium
VMI	Vitamin medium with iron
VMR	Vitamin medium with reduced iron

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DISSEMINATION

Graduate Student Book Award, NACE International Foundation. NACE International corrosion conference CORROSION 2016– Vancouver Canada 6-10 March 2016.

The development of a novel inhibitor from a bacterium for the control of sulfate reducing bacteria (SRB). Book of Abstract - NACE International corrosion conference CORROSION 2016– Vancouver Canada 6-10 March 2016.

Inhibition of sulphate reducing bacteria (SRB) growth in oilfield environments. The 3 Min Thesis presentation - Faculty of Science University of Portsmouth 1 July 2015.

The development of an inhibitor for the control of sulphate reducing bacteria (SRB). Poster Presentation - Society for General Microbiology (SGM) conference - Birmingham 30 March - 2 April 2015.

Purification of sulphate reducing bacteria (SRB) growth inhibitor from BP bacterium. Book of Abstract and Presentation - NACE International corrosion conference CORROSION 2015 – Dallas USA 15-19 March 2015.

The induction and inhibition of sulphate reducing bacteria (SRB) growth by the compounds produced by EBP bacterium. Oral Presentation - Institute of Biomedical and Biomolecular Science (IBBS) University of Portsmouth Research Day, Portland Building 15 May 2015.

Purification of sulphate reducing bacteria (SRB) growth enhancer (SGE) and SRB growth inhibitor (SGI) from bacterium EBP. Poster Presentation - 'Institute of

Biomedical and Biomolecular Science (IBBS) Postgraduate Research Day, Eldon Building University of Portsmouth, 16th May 2014.

Purification of microbial growth enhancer (MGE) from bacterium EBP. Book of Abstract and Poster Presentation- Science Faculty PhD/MPhil Research Day, Spinnaker Building University of Portsmouth, 4th July, 2013.

Microbial influenced corrosion and its control measures. Oral Presentation-Corrosion of Infrastructure' - Present Knowledge and Future Solutions IOM3, London. 20 February 2013.

CHAPTER 1

GENERAL INTRODUCTION

1.1 General Overview

In this study, a facultative anaerobic bacterium (coded as *Escherichia coli* BP) was detected to have activities that could induce and inhibit sulphate reducing bacteria (SRB) growth. SRB growth enhancer (SGE) is a compound purified from *E. coli* BP that can be developed as a rapid detection tool for SRB. The bacterium also secretes an extracellular molecule that inhibits the growth of SRB and is referred to as SRB growth inhibitor (SGI). The purification of the compounds from the cell free supernatants (CFS) of *E. coli* BP was carried out using ion exchange and size exclusion chromatography. The functional activities of the SGE and SGI were studied using microbiological culture techniques and microscopy. In addition, nuclear magnetic resonance (NMR) spectroscopy and matrix-assisted laser desorption ionisation–time of flight (MALDI-TOF) spectrometry were used to analyse the compounds. The results obtained from this study were discussed based on secondary data reviewed from the literature.

1.2 Sulphate reducing bacteria (SRB)

Sulphate reducing bacteria (SRB) are a taxonomically unrelated group of microorganisms that acquire energy for their growth by oxidising organic substrates and hydrogen. They utilise sulphur compounds, as final electron acceptors during anaerobic growth (Guan, 2000; Zhang *et al*., 2011; Keller *et al*., 2011; Kakooei *et al*., 2012; Fichtel *et al*., 2012; AlAbbas *et al*., 2013). SRB encompass 60 genera of bacteria, accounting for 220 species (Barton & Fauque, 2009). These include proteobacteria e.g. *Desulfovibrio,* firmicutes e.g. *Desulfotomaculum* (Keller *et al*., 2011; Kakooei *et al*., 2012),

archaebacteria e.g. *Archaeoglobus* (Guan, 2000; Sherry *et al*., 2013) and thermodesulfobacteria e.g. *Thermodesulfobacter* (Sherry *et al*., 2013).

The SRB are widely distributed (Plugge *et al*., 2011), phylogenetically diverse (Castro *et al*. 2000) and thrive well in a wide range of environmental conditions (Grein *et al*., 2013). They grow well in anaerobic niches where sulphate reduction is the principal biomineralisation pathway (Dolla *et al*., 2006). They have been found in soil sediments, storage tanks, water sediments, oil-fields, buried steel piping, shipping industries, ships, nuclear waste containers, water and wastewater plants, marine sediments, hydrocarbon seeps, oil and gas production and storage facilities (Jack, 2002; Dolla *et al*., 2006; Muyzer & Stams, 2008; AlAbbas *et al*., 2013). They also use sulphate as a final electron acceptor for the breakdown of the organic nutrients. This results in sulphide production, or if iron is available, as black iron sulphide (Muyzer & Stams, 2008; Barton & Fauque, 2009). The ribosomes of SRB are characterised by specific sequence of nucleotides in 16S rRNA (ribosomal ribonucleic acid chains) which helps in their identification. SRB have previously been thought to be strictly anaerobic, although, some genera can tolerate some levels of oxygen (Hardy & Hamilton, 1981; Wargin *et al*., 2007; Lodowska *et al.,* 2012; Zhang-Sun *et al*., 2015).

1.3 Growth Requirements of SRB

SRB are chemolithotrophic and physiologically distinctive group of anaerobic bacteria. They obtain energy for growth by oxidising organic compounds and use sulphate as the terminal electron acceptor (Widdel, 1988; Jhobalia *et al*., 2005; Barton & Fauque, 2009). To allow electron flow, they have a diversity of proteins with redox active metal groups (Barton & Fauque, 2009). SRB are capable of utilising different organic compounds, but

with a preference for low molecular weight organic materials as carbon sources, such as organic acids (lactic, pyruvic, formic, acetic) or alcohols (ethanol, propanol) (Rzeczycka & Baszczyk, 2005). They grow at temperatures from 25 to 55°C, with cell forms ranging from cocci, rods, curved, cell aggregates to multicellular gliding filaments (Keller *et al.*, 2011). SRB vary in cell size for example, the average width of *Desulfovibrio* ranges from 0.5-1.3 x 0.8-5 μm and *Desulfotomaculum,* 0.5-2 x 2-9 μm (Wargin *et al*., 2007).

SRB utilise sulphate, thiosulphates or sulphites as a terminal electron acceptor, however in the absence of sulphate, some strains can utilise some inorganic sulphur compounds or organic compounds such as pyruvate, butyrate, propionate and lactate to produce acetate, hydrogen and carbon dioxide (Videla, 1996; Jhobalia *et al*., 2005; Rzeczycka & Baszczyk, 2005; Almeida *et al*., 2006; Muyzer & Stams, 2008) (Figure 1.1). In addition, fumarate, sulphonates (Muyzer & Stams, 2008), dimethylsulphoxide (Lie *et al*., 1996) and 3-chlorobenzoate (Dolfing & Tiedje, 1991) can act as electron acceptors for SRB. The activities and population of SRB often depend on the presence of organic compounds in the environment. The organic nutrients can be supplied either through primary production or sedimentation, however, the numbers and activities of SRB generally increase towards the anoxic layers (Jørgensen, 1982; Dolla *et al*., 2006).

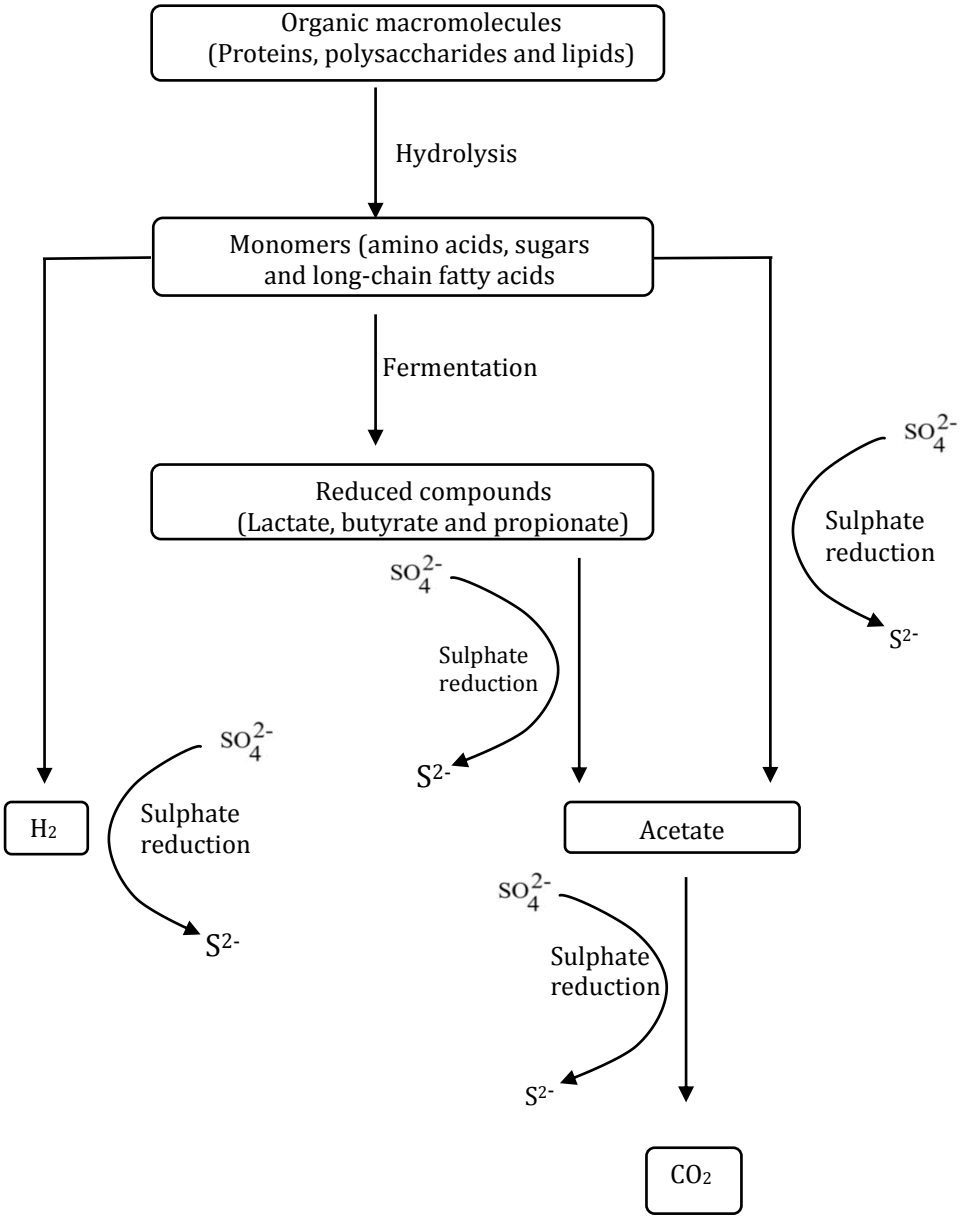

Figure 1.1. Degradation of sulphate containing organic matter in anoxic environments (Muyzer & Stams, 2008).

During hydrolysis, macromolecules are reduced by facultative anaerobes or anaerobic bacteria (such as *E. coli*, *Bacillus, Bacteroides* and *Clostridium*) to their monomers using their intracellular enzymes such as proteases, cellulases and lipases. During fermentation or acidogenesis, the monomeric products of hydrolysis are utilised by microorganisms to form such as lactate, butyrate propionate, acetate, ethanol, carbon dioxide, and hydrogen by enzymatic activities that vary tremendously with microbial species. In the presence of sulphate, the SRB reduce sulphate to sulphide through the dissimilatory sulphur reduction pathway.

1.4 Interaction of SRB with other microorganisms

In nature, SRB relate with other microorganisms in the environment and the products from these microorganisms. SRB often form a symbiotic relationship with other microorganisms, for example, the methanogens to degrade complex organic materials (Boetius *et al.,* 2000). SRB utilise lactate and acetate produced by methanogens to form hydrogen while methanogens utilise hydrogen to reduce carbon dioxide to methane (Almeida *et al*., 2006; Stolyar *et al.*, 2007). According to Dunne (2002), bacteria naturally occur in surface-bound and often community-based lifestyles, encouraging symbiosis among the biofilm microbial communities. Biofilms are described as complex functional microbial community embedded within an exopolymeric matrix often attached to a biological or abiotic surface (Dunne, 2002; Beech, 2003; Oh *et al*., 2007; Oyewole, 2011; Wikieł *et al*., 2014; Everett & Rumbaugh, 2015), thus microbial life often exists as a biofilm (Wikieł *et al*., 2014).

Biofilm structure ranges from a single layer to thick layers of microbial cells (Wimpenny *et al*., 2000). It may comprise a single or multiple microbial species that may include fermentative bacteria, aerobes, facultative anaerobes, and anaerobic bacteria, fungi, Archaea, and eukaryotes held together with extracellular polymeric substances (EPS). In addition, these organisms form synergistic and competing relationship (Usher *et al*., 2014) and transfer genetic materials among one another (Annous *et al*., 2009). This relationship can influence electrochemical processes and redox reactions during their co-operative metabolisms (O'Toole *et al*., 2000; Usher *et al*., 2014; Cote *et al*., 2014). Formation of a biofilm is an organized process facilitated by bacterial cell-to-cell communication identified as quorum sensing (QS). QS systems often regulate traits involving interactions between different microorganisms, both mutualistic and

detrimental (Taga & Bassler, 2003; Wang *et al*., 2005). Bacteria in biofilms often communicate using extracellular signalling molecules called autoinducers or pheromones using QS process (Rashmi *et al*., 2013). Autoinducers are produced extracellularly during specific stages of bacterial growth, recognised by a specific receptor, under certain physiological conditions or in response to environmental changes or dangers (Winzer *et al*., 2002). Small-molecule, chemical and nutrient signals (siderophore) as well as metabolic activities of microorganisms in biofilms induce alterations of gene expression that facilitates communications in QS (Blackledge *et al*., 2013; Everett & Rumbaugh, 2015).

Siderophores are metabolites that have low-molecular-mass (less than 2000 Da), have strong affinity for ferric ion (Fe^{3+}) and are often excreted and exported by microorganisms during low iron conditions (Brandel *et al*., 2012; Zheng & Nolan, 2012; Saha *et al*., 2013; Ahmed & Holmström, 2014). Biofilms often comprise heterogeneous microbial community with heterogeneous food chains, thus the food of one community is the by-product from another and the metabolic products from one community can help the growth of another. Therefore, biofilms can be termed as a strategy for microbial survival (Wikieł *et al.*, 2014). The three main stages of biofilm development are: the attachment of single layer of microbial cells to a biological or abiotic surface, maturation of the biofilm to a complex multifarious microbial community, and the dispersal of planktonic cells (Stoodley & Dirckx, 2003; Langer *et al*., 2014) (Figure 1.2).

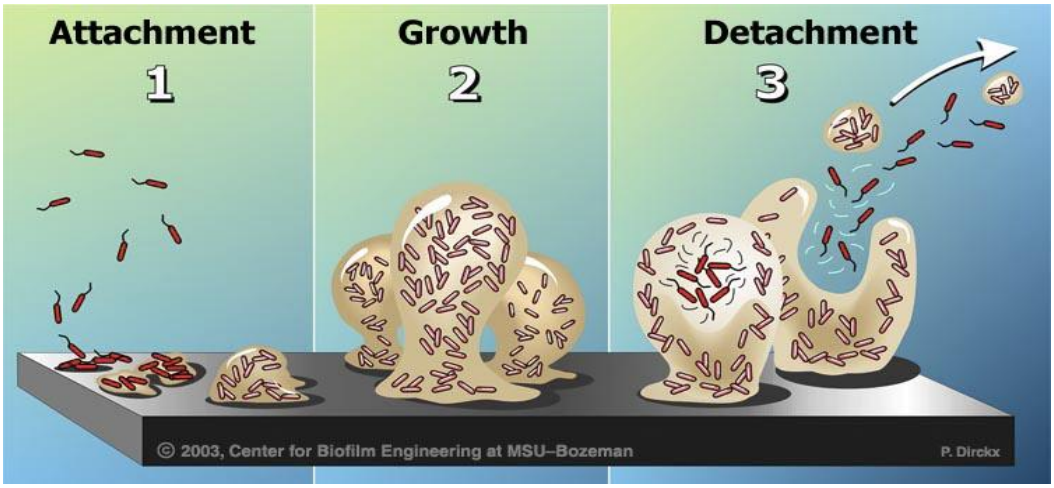

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Figure 1.2. Biofilm formation in three steps (Stoodley & Dirckx, 2003).
1. Attachment: This is the first stage in biofilm development where single bacteria colonies (in red) adhere irreversibly to a biotic or abiotic surface using structures such as pili, EPS and fimbriae. Once attached, the bacteria present in biofilms (coloured pink) initiate growth (Wikieł *et al*., 2014). **2.** Growth: This is the stage in which bacteria replicate actively to form a complex structure referred to as glycocalyx (Dunne, 2002). **3.** Detachment. This involves the dispersion of planktonic cells from biofilm matrix (Langer *et al*., 2014).

1.5 Environmental Impact of SRB

SRB are known to play a vital role in the sulphur cycle and are capable of reducing oxidised sulphur compounds. This leads to the production of sulphide (Almeida *et al*., 2006; Barton & Fauque, 2009). The sulphide produced by SRB is often encountered in the oil and gas industries during the exploration, oil and gas wells completion and during piping and storage of fuel products containing hydrogen sulphide (H_2S) (NACE, 2012) and are often isolated in injection waters and produced waters (Eden *et al*., 1993). Hydrogen sulphide is toxic, volatile, corrosive and inhibits the respiratory system. Its presence contaminates and makes it a more difficult task to improve the quality of crude oil and gas; thereby reducing its economic value (Schwermer *et al*., 2011).

In oil-field systems, SRB cause serious problems that include corrosion of metals (Guan, 2000; Kakooei *et al*., 2012; Fichtel *et al*., 2012), oil plugging of injection wells (Zarasvand & Rai, 2014), hydrocarbon degradation (Sherry *et al*., 2013; Wei *et al.*, 2010), fuel contamination with sulphide (Onyekpe & Dania, 2001), reservoir souring (Everett & Rumbaugh, 2015), and biofouling (Schwermer *et al*., 2011; Sherry *et al*., 2013), resulting in financial losses and environmental health and safety hazards.

1.5.1 Sulphidogenesis

During dissimilatory reduction metabolism of SRB, hydrogen sulphide (H_2S) is produced as the end product (Tang *et al*., 2009) that contributes to the maintenance of an anaerobic atmosphere suitable for SRB growth (Wikieł *et al*., 2014). SRB have been involved in reduction of oxidised sulphur compounds such as polythionate (SnO_6^{2-}), sulphite (SO_3^{2-}), thiosulphate ($S_2O_3^{2-}$) and sulphate (SO_4^{2-}) to sulphide (S^{2-}), through the dissimilatory sulphur reduction pathway (Figure 1.3) (Fauque & Barton, 2012; Stipanicev *et al*., 2013). Dissimilatory sulphur reduction metabolism has been the earliest energy yielding process that sustains life (Grein *et al*., 2013). Sulphate metabolism by SRB often involves the following enzymes:

(i) Hydrogenases are a key enzyme class involved in the metabolism of hydrogen (Martins & Pereira, 2013). Hydrogenases are integral in the energy-yielding mechanism of SRB (Almedia *et al*. 2006). The presence of a hydrogenase may influence corrosion mediated by SRB (Kakooei *et al*., 2012) and it is involved in expression or repression of protein control mechanisms (Bryant *et al*., 1991; Almedia *et al*. 2006; Rashamuse *et al*., 2008).

ii) Cytochrome *c*3, which is often referred to as the electron carrier of hydrogenease enzymes in SRB (Almedia *et al*. 2006; Rashamuse *et al*., 2008).

iii) Adenosine 5'- phosphosulphate (APS) reductase, which catalyzes the reduction of APS to form adenosine-5'-monosulphate (AMP) and sulphite is a unique enzyme present in all SRB (Wagner *et al*., 1998). Thus, the APS genes are involved in the energy metabolism of SRB and can be used for their identification (Almedia *et al*., 2006, Christophersen *et al*., 2011).

iv) Bisulphite reductase, also referred to as dissimilatory sulphite reductase (DSR), is another enzyme present in all SRB. DSR catalyses sulphite reduction to sulphide, trithionite ($S_3O_6^{2-}$) or thiosulphate ($S_2O_3^{2-}$). The presence of sulphite reductase can also serve as a gene marker in the identification of SRB (Stroupe & Getzoff, 2001; Wargin *et al*., 2007, Hsieh *et al*., 2010; Christophersen *et al*., 2011).

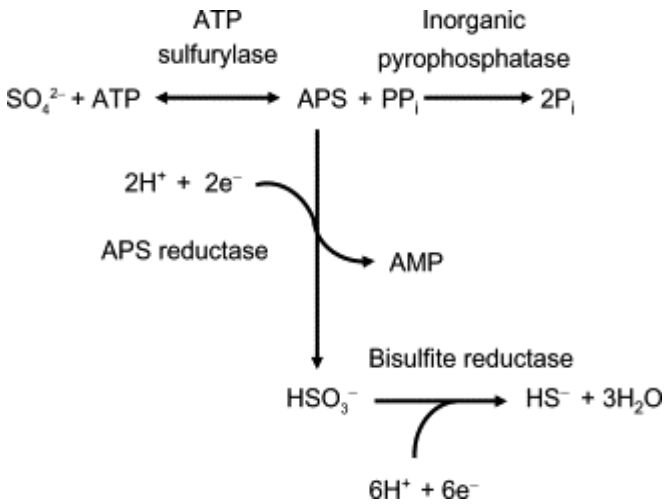


Figure 1.3. Dissimilatory sulphate reduction pathway (Fauque & Barton, 2012).
Prior to the use of sulphates as electron acceptors, they are first activated by ATP-sulphurylase. ATP-sulphurylase catalyses the reduction of sulphate to adenosine 5'-phosphosulphate (APS). APS is then reduced to SO^{3-} (sulphite) and AMP (adenosine monophosphate) by APS reductase. Sulphite is subsequently reduced to S^{2-} (sulphide) and AMP converted to ADP (adenosine diphosphate) by bisulphite reductase. In this process, two molecules of ATP are invested, the first is in the conversation of ATP to APS and second is from AMP to ADP (Muyzer & Stams, 2008).

Sulphide production in various industrial situations and various natural environments have ecological and economic implications due to its toxic properties (Almeida *et al*., 2006). SRB contamination of crude oil may contribute to its instability through biofouling of pipework, which may cause blockage of filters and pipelines (Marc, 2006). Hydrogen sulphide (H_2S) causes contamination of fuel, reduces crude oil quality and its economical values and also makes the crude oil more difficult to purify (Schwermer *et al*., 2011). The high toxicity of sulphide gas also represents significant hazards to the surrounding environment and may cause high risks for the field workers and other humans (Almeida *et al*., 2006).

The environments containing hydrogen and sulphur are hazardous to metals. In addition, H_2S is an inhibitor of respiratory system; it is unstable, toxic and corrosive and can have adverse effects in oil production and transportation in pipelines, storage tanks, platforms and ships (Almeida *et al*., 2006). Corrosion caused by H_2S is localised, and this can cause stress-orientated hydrogen induced cracking, hydrogen embrittlement and sulphide stress corrosion cracking. Although, H_2S may not essentially enhance the rate of corrosion, its presence in oil production system can set vulnerable materials to disastrous breakdown, thereby leading to increased operational costs and may result in the shut-down of oil-wells (Eden *et al*., 1993).

1.5.2 Hydrocarbon Degradation

Hydrocarbons are excellent sources of carbon and act as an electron donor for a wide variety of microorganisms (AlAbbas *et al*., 2013). Apart from the oil reservoirs souring due to the production of H_2S by metabolism of SRB, they degrade the hydrocarbons in fuel pumps, storage tanks, pipelines and injection wells thereby reducing the overall quality of fuel (Wikiel *et al*., 2014). Hydrocarbons are utilised by several kinds of

bacteria including SRB in several ways (Figure 1.4). The mineralisation of hydrocarbons by SRB further results in the production of sulphide (Almeida *et al*., 2006). Hydrocarbon-degrading SRB are widely distributed with respect to metabolism of substrates and their phylogeny. Crude oil comprises about 75 % aliphatic and aromatic hydrocarbons (Fukui *et al*., 1999). SRB have been reported to degrade hydrocarbons that include *n*-alkanes, *n*-alkenes (Jaekel *et al*., 2013) or aromatic hydrocarbons such as benzene, toluene or naphthalene (Fukui *et al*., 1999; Widdel *et al*., 2010). Oil and gas systems are considered suitable environments for the growth of SRB because of the presence of essential components necessary for their growth and metabolism (Almeida *et al*., 2006) such as electron acceptors, electron donors, carbon and water (Madigan, 2009).

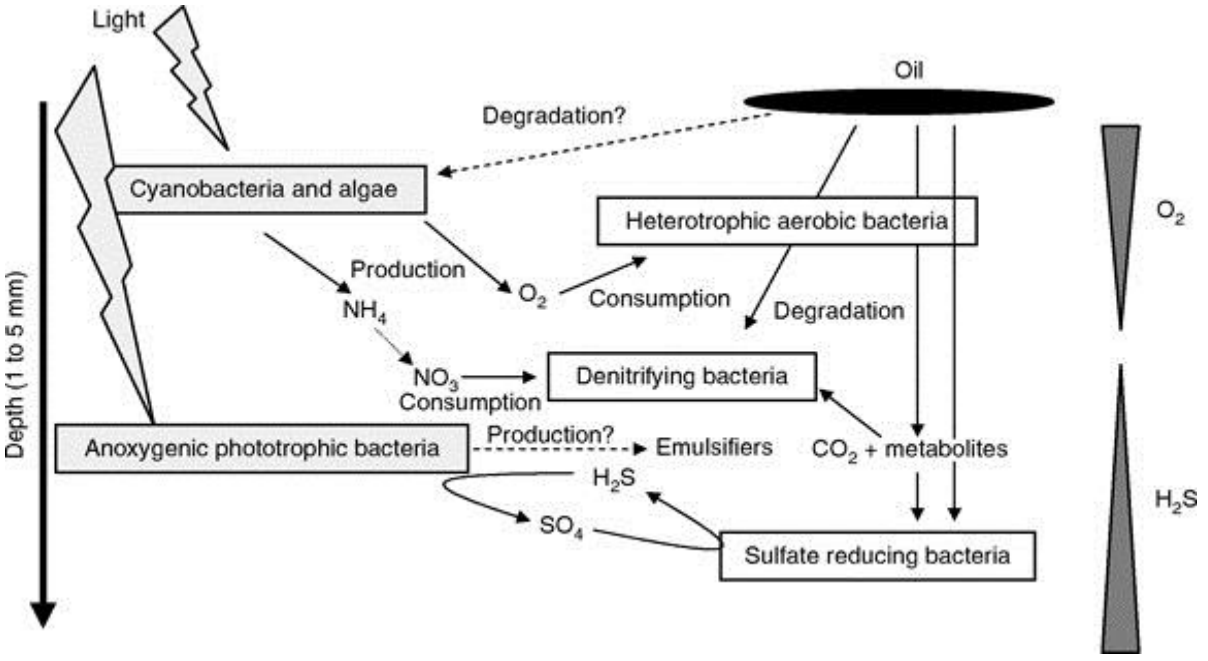


Figure 1.4. Degradation of Petroleum by a Microbial Mat (Duran & Goňi-Urriza, 2010). During anoxic conditions, crude oil that has been mineralised by heterotrophic bacteria and facultative anaerobes is utilised by SRB with a release of huge amount of sulphide. There is a syntrophic food association among the various bacterial groups.

1.5.3 Equipment Failure

The activities of SRB can cause a decrease in the carrying capacity of pipelines (Water in Corrosion, WC, 2010), system malfunction (Akpabio *et al.*, 2011), damage to vessels, pipes and storage tanks (Beech & Gaylarde, 2008; Priha *et al*., 2013), increase equipment failure (Kakooei *et al.,* 2012), loss of injectivity, plugging of filters, and malfunctioning of heat exchanger (Priha *et al*., 2013). These can eventually lead to breakdown of oil and gas industrial facilities.

1.5.4 Biofouling

Biofouling is the gradual accumulation of unwanted microbiological deposit on a surface, which contributes to microbiologically influenced corrosion (MIC), clogging of filters, and production of H_2S and pyrite scale (Borenstein, 1994; Choudhary, 1998). It usually comprises biofilms, inorganic particles and corrosion products, for example iron sulphide (FeS) (Gu *et al*., 2011).

1.5.5 Biocorrosion

SRB have long been identified as the key contributor to corrosion in oil and gas fields (von Wolzogen Kühr & van der Vlugt, 1934; Hamilton, 1985; Thomas *et al.,* 1988; Bryant *et al*., 1991; Little & Edyrena, 1992; Beech *et al*., 1999; Jack, 2002; Kjellerup *et al*., 2005; Beech & Gaylarde, 2008; Cetin & Aksu, 2009; Zhang *et al*., 2011; Oyewole, 2011; Kakooei *et al*., 2012; Zhao *et al*., 2012; Guan *et al*., 2013) due to their ability to reduce oxidised sulphur compounds to form sulphide that attacks metal and leads to corrosion and pitting (Cetin & Aksu, 2009; Guan *et al*., 2013). SRB generally influence corrosion by shifting the electrochemical processes at the boundary between the metal and the surrounding bulk fluid and this could occur through electrochemical or chemical reactions (Videla & Herrara, 2005; Muthukumar *et al*., 2007). This may be mediated by

the production of iron binding EPS, iron sulphides, iron phosphides and cathodic depolarisation or anodic depolarisation (Beech *et al*., 1999; Kjellerup *et al*., 2005; Çetin *et al*., 2007; Zhang *et al*., 2011; Kakooei *et al*., 2012).

Biocorrosion by SRB has the following characteristics: it occurs in anaerobic environments when water is present; forms tubercles on metal surfaces; the SRB are present and provides anaerobic conditions necessary for corrosion leading to the pitting of the metal. There is a direct proportionality between the rate of pitting and time, indicating the autocatalytic nature of the process thereby causing the corroded metals to perforate instead of disintegrating (Barton, 1997). In oil and gas environment, microbiologically influenced corrosion (MIC) can occur anywhere which may include water injection, system, downhole tubulars, topside equipment, pipelines, storage tanks and oil production systems (Augustinovic *et al*., 2012). In some cases, corrosion pits lead to large areas of metal loss sufficient enough to cause stoppage of vessels through rupture and hassled piping that can eventually impede operation (Jack, 2002; Augustinovic *et al*., 2012). This is more risky than leak formation as a result of steel perforation due to pitting (Jack, 2002).

The cost linked to MIC is huge and its influence on the economy of a nation is significant. MIC is responsible for over 20 % of the total corrosion damage and up to 50 % for total damage worldwide (Jack *et al*., 1992; Flemming, 1996; Graves & Sullivan, 1996; Schwermer *et al*., 2011). MIC is perhaps the only method by which stainless steel piping can perforate in a few months (Jack, 2002). The activities of SRB may be responsible for over 75 % of the corrosion in production oil wells, and more than 50 % of buried pipelines and cables observed failures (Walch, 1992). During the last 100 years, oil-field environments have experienced contaminated soil, surface and groundwater due to

leakages from corroded facilities (Augustinovic *et al*., 2012). According to Amuda *et al*. (2008), about 5 % of industrialised nations' income is used for the control of corrosion problems.

The 2006 Alaskan oil-spill in pipelines carrying crude oil operated by British Petroleum provides additional confirmation of enormous financial losses and environmental hazards due to MIC (Beech & Gaylarde, 2008). The overall damage cost to biocorrosion and biofouling in the United States has been projected to be at least $200 billion yearly (Microbion, 2014) and $6 billion in Australia (Jones *et al.*, 2008). Amuda *et al*. (2008) estimated the annual cost of corrosion in Nigeria to be about $3.2 billion. Beech & Gaylarde (2008) reported the replacement costs for biocorroded gas mains in the United Kingdom to be £0.25 billion per annum. Aside from financial losses, leaks from oil pipelines and storage vessels can cause environmental tragedy due to the release of toxic chemicals, hydrocarbons, H_2S and flammable liquids (Zhang *et al*., 2015).

1.6 Identification of SRB

There are several methods that have been used for the identification of SRB, they include visualising SRB biofilms using microscopy, biochemical and genetic assays and microbiological culture techniques, as discussed below.

1.6.1 Microscopy

Light microscopy, scanning electron microscopy (SEM), atomic force microscopy (AFM) and/or confocal laser scanning microscopy (CLSM) can be used to view SRB biofilms. Chemical spectroscopy and surface chemical analysis provide information on the nature of the FeS due to the presence of SRB (Beech *et al*., 1999; Kjellerup *et al*., 2005; Videla & Herrera, 2005; Little *et al*., 2006).

1.6.2 Biochemical assay

Hydrogenase and Adenosine-5-phosphosulfate (APS) reductase have been used to determine the population of SRB. (i) ATP has been used to detect microbial activity but it is also useful in detecting the presence of SRB. ATP (adenosine triphosphate) assays are commonly used to determine the number of viable cells in a given sample and are based on the reaction between luciferin and luciferase. In this reaction, ATP provides the energy required for the oxidation of luciferin by luciferase to form oxyluciferin resulting in light emission. The amount of light emitted during luciferin degradation corresponds to the level of ATP present that is;

$$\text{Luciferin} + \text{ATP} \xrightarrow{\text{Luciferase}/O_2} \text{Oxyluciferin} + \text{AMP} + \text{PPi} + \text{Light}$$

(ii) APS reductase is an enzyme present in all SRB, unlike hydrogenase that is found only in hydrogenase positive SRB. Identification of APS reductase and hydrogenase begins by extraction from any sample e.g. sludge, corrosion products and produced water. This is followed by filtration, lysis of cells, addition of antibody reagent and exposure to colour developing solution. If APS reductase is present a blue colour appears whose intensity corresponds to the amount of APS reductase present in the sample. Unlike measuring APS reductase, if hydrogenase activity is to be measured, samples are exposed to hydrogen anaerobically. All SRB-hydrogenase positive are able to produce hydrogen in the cathode and reduces an indicator dye in solution which is indicated by the development of blue colour. Thus, measurement of the intensity of hydrogen produced helps to quantify the level of hydrogenase enzyme present (Beech & Gaylarde, 1999; Videla & Herrera, 2005; Kjeelerup *et al*., 2005; Little & Lee, 2007).

1.6.3 Genetic assay technique

Genetic assays have two approaches: Sequence analysis that involves extraction of DNA, amplification of extracted DNA copies using polymerase chain reaction (PCR) in the sample followed by evaluation of the PCR products by a community fingerprint technique, such as denaturing gradient gel electrophoresis (DGGE) and or sequence analysis. The target probe is specific to SRB such as DSR and APS reductase. Each band produced by DGGE corresponds to a distinct bacterial population and represent their diversity. The products obtained from the PCR reaction can be sequenced and compared with sequences from the Genbank database. This will allow the identification of the organism (Videla & Herrera, 2005; Little *et al*., 2006).

Another approach to genetic assay is hybridisation. DNA microarray or hybridisation is a rapid technique useful in analysing the level of gene expression when a large number of genes are collected in a given cell samples of known and unknown function. A DNA microarray is made of a pre-designed collection of nucleic acid probes immobilised on a solid matrix (Call, 2001; Palka-Santini *et al*., 2009). Probes specific for sulfate reduction by SRB are used in each strip and include the DSR and APS reductase gene. It is developed from a method called Southern blotting where DNA fragments are added to a substrate after which the fragments are probed using an identified gene sequence (Grigoryev, 2011). The analysis can be carried out one time in a high throughput and parallel manner (Simon *et al*., 2002; Van Nostrand *et al*., 2010). Schena *et al*. (1995) reportedly designed the first microarray to examine *Arabidopsis thaliana*. This gives rise to the development of microarrays for the study of gene expression of other microorganisms found in the environment (Tiquia *et al*., 2004).

Three types of arrays are currently utilised for the study of gene expression of environmental samples, they include (i) Phylogenetic oligonucleotide arrays (POA) (ii) Functional gene arrays (FGA) and (iii) Community genome arrays (CGA). The POA contains probes obtained from conserved genes e.g. 16S rRNA and it is useful in examining the microbial community of a given sample or determine the phylogenetic relationship among microorganisms (Van Nostrand *et al*., 2010). Unlike the POA and as the name implies, the FGA is made of genes that code for a specific function in an organism e.g. the H_2S production in SRB (Gentry *et al*., 2006; Van Nostrand *et al*., 2010) whereas the CGA is concerned with determining gene expression of a whole microbial genomic DNA so as to identify an organism or a gene of interest (Wu *et al*., 2004).

1.6.4 Culture techniques

Culture medium used to grow SRB include Postgate medium (Postgate, 1984) and other modified media such as vitamin medium (VM) (Zinkevich & Beech, 2000). After preparation of the culture medium, it is de-oxygenated, inoculated with the test samples and incubated anaerobically at 37°C for up to 28 days. The presence of SRB is indicated by the production of black ferrous sulphide due to the reduction of sulphate to sulphide, and the eventual reaction of sulphide with iron in the medium (Postgate, 1984; Beech *et al*., 1999; Kjellerup *et al*., 2005; Little *et al*., 2006; Muyzer & Stams, 2008; Barton & Fauque, 2009). Culture techniques are the only technique that are not expensive nor do they require specialist training. However, growth of SRB with the use of culture media is often very slow and therefore will benefit from additional growth supplement.

1.7 Induction of Bacterial Growth

The synthesis of metabolites is influenced by many factors, as a result of interactions with other organisms in a community. The production of specific metabolites as a result

of co-culture or mixed fermentation is such an example. For instance, emericellamide A and B, which are cyclodepsipeptides derived from an *N*-β-hydroxyacyl glycyl-L-valyl-L-leucyl-L-alanyl-L-alanine by the formal condensation of the alcoholic hydroxyl group with the *C*-terminal carboxyl group were induced during mixed fermentation of the marine-derived fungus *Emericella* sp. and actinomycete *Salinispora arenicola* (Bertrand *et al*., 2013).

Another way bacterial growth is enhanced is through the addition into their culture medium growth supplements such as small molecules or heavy metals that function as growth inducers or by challenging a given microorganism with biotic stress that can be induced through interaction with other microbial partners (Agostini-Costa *et al*., 2012; Bertrand *et al.*, 2014). For example, growth inducers purified from *Spirulina platensis* has been shown to promote the growth of *Lactococcus lactis*, *L. casei*, *L. acidophilus*, *L. bulgaricus* and *Streptococcus thermophilus*, (Parada *et al*., 1998). Folic acid synthesised by probiotic bacteria has been used as growth supplement in bacterial growth media (Rossi *et al*., 2011). An autoinducer purified from *E. coli* and *Hafnia alvei* was shown to promote the growth of *Acinetobacter lwoffii, Enterobacter cloacae, Enterobacter sakazaki, Klebsiella pneumonia, Morganella morganii, Proteus mirabilis, Serratia marcescens* and *Staphylococcus albus* (Freestone *et al*., 2003).

1.8 Inhibition of Bacterial Growth

Many compounds have been shown to have inhibitory effects against bacterial growth. Some of these compounds are chemically derived, while others are derived from other microorganisms. The compounds that have been used to control SRB in the environment are discussed below:

1.8.1 Microbial derived SRB inhibitors

The formation of a corrosion inhibitor generated by biofilms attached to metal surfaces could provide excellent protection on metal against SRB. Thus, the use of beneficial biofilms or their products is one of the more promising approaches in the development of more effective SRB control (Guan, 2000; Little & Lee, 2009; Videla & Herrera, 2009).

Some microbial-derived products from biofilm have been shown to have inhibitory effects on SRB. For example Jayaraman *et al*. (1999) reported that antimicrobials produced within biofilms of *Bacillus subtilis* and *B. licheniformis* inhibit SRB growth. *B. brevis* biofilm produces a compound referred to as gramicidin-S that caused a reduction in the corrosion rates of mild steel by inhibiting *Desulfovibrio orientis, D. vulgaris* and *D. gigas* (SRB) (Jayaraman et *al*., 1999; Morikawa, 2006; Zuo, 2007). γ-polyglutamate and polyaspartate production by *Bacillus licheniformis* biofilm had some protective layer on aluminum against corrosion causing SRB (Mansfeld *et al*., 2002; Ornek *et al*., 2002; Zuo, 2007). Indolicidin, bactenecin, and polymyxin produced by *Paenibacillus polymyxa* are capable of reducing the growth of SRB (Jayaraman et *al*., 1999; Zuo, 2007). EPS produced by *Vibrio* sp. offered some protection against mild steel (Majumdar *et al*., 1999) and Garcia *et al.* (2012) showed that biofilms comprising *Enterobacter cloacae, Pantoea agglomerans, Bacillus cereus, Alcaligenes faecalis, Pseudochrobactrum asaccharolyticum, Brucellaceae bacterium* and *Delftia tsuruhatensis* inhibited the corrosion rate on a copper surface.

Various mechanisms which biofilms use to inhibit corrosion have been suggested. They include the growth inhibition of corrosion causing microorganisms (Jayaraman *et al*., 1999; Zuo, 2007); the prevention of microbial adhesion by bacterial surface active agents (Korenblum *et al*., 2012)**;** the production of antimicrobial agents (Jayaraman *et*

al., 1999; Zuo, 2007; Zarasvand & Rai, 2014), the production of a biofilm protective layer (Majumdar *et al*., 1999; Grooters *et al*., 2007), attack on the APS and DSR enzymes responsible for hydrogen sulphide production in SRB (Tsygankova *et al.*, 2014; Zarasvand & Rai, 2014), and the removal of corrosion agents e.g. chloride (Dubiel *et al.*, 2002).

1.8.2 Biocompetitive Exclusion

The biocompetitive exclusion strategy is in use by oil industries to inhibit MIC and reservoir souring caused by SRB (Almeida *et al*., 2006). Biocompetitive exclusion is the use of nutrients that encourage the growth of competing bacteria populations such as nitrate reducing bacteria (NRB). Studies have reported a shift in SRB population by NRB *via* biocompetitive exclusion (Halim *et al*., 2012; Ranjan *et al*., 2012). The use of nitrate to encourage the growth and activity of NRB and to repress the production of H_2S by SRB was first proposed by Postgate (1952). An example of microorganism that can be used is nitrate reducing *Sulphurospirillum* species. They outcompete SRB for organic electron donors (Hubert & Voodour, 2007). The addition of nitrate encourages NRB growth and prevents SRB growth thereby eliminating H_2S production. The sulphide produced by SRB is oxidised by NRB. The oxidised sulphur compounds formed help in maintaining the reduction potential that is inhibitory to SRB growth (Halim *et al*., 2012). Thus, when nitrate is added, this can cause a shift in the microbial population from SRB to NRB. This method of nitrate addition is effective in SRB control both in the field and in laboratory (Stott *et al*., 2008).

Biocompetitive exclusion may not always work. Apart from the high cost of nitrate, (i) addition of nitrate to produced water can result in ageing of oil and eutrophication. This may cause an increase in biomass, which may contribute undesirable effects such as

down-hole plugging of disposal well by nitrate utilising bacteria (Eckford & Fedorak, 2002), (ii) some SRB are capable of reducing nitrate and once the level of nitrate is reduced, SRB resumes their growth resulting in pit corrosion, (iii) it could cause an increase in localised corrosion due to the production of polysulphide and thiosulphate as a result of simultaneous oxidation of sulphide and reduction of nitrate (Videla, 2002; Halim *et al*., 2012), (iv) due to the increased activity of nitrate reducing bacteria, topside biofouling may result especially in ships and offshore platforms (Stott *et al*., 2008).

1.8.3 Chemical inhibitors

The commonly used method for the control of SRB influenced corrosion in petrochemical industries is the application of chemical inhibitors and / or antifouling agents. Corrosion inhibitors (for example phosphate, polyphosphates, benzoates) are perhaps the most common methods used to protect petrochemical facilities against corrosion. Corrosion inhibitors are substances / chemicals that combine with either the corroding (anode) or protective (cathode) metal thereby stopping the corrosion process. This is achieved by the formation of a barrier layer that prevents electron and ionic flow across the barrier membrane (Koch *et al.,* 2001; Videla & Herrera, 2009). Chemical inhibitors are compounds that have the ability to reduce the rate of corrosion in aqueous solution, oil, fuels and the atmosphere; some of them are incorporated in the formulation of a protective coating (Kopeliovich, 2012). The nature of both corrosion and corrosion inhibition is electrochemical (Videla & Herrera, 2009). However, microorganisms can utilise the inhibitors as sources of carbon and energy for their growth and metabolism, thereby causing an alteration of the molecule structure of the inhibitor, or its removal. This will affect the specific performance of corrosion inhibition and can cause the corrosion to resume (Obuekwe, 1983; Jack, 2002; Videla & Herrera, 2009). The majority of the antifouling agents are not environmentally friendly; this

accounts for the reasons some of these agents have been banned e.g. tributyltin compounds (Gittens *et al*., 2013).

1.8.4 Biocides

Biocides are commonly used for the control of SRB mediated corrosion (Zuo, 2007). Biocides are chemical agents that are able to kill living organisms. Biocides can either be oxidising or non-oxidising toxicant (Lin & Ballim, 2012). Examples of oxidising biocides used in industries are chlorine, ozone and bromine. Non-oxidising biocides e.g. formaldehyde, gluteraldehyde, isothiazolones, and Tetrakis-hydroxymethyl phosphonium sulphate are more effective than oxidising biocides for the control of bacteria, fungi and algae because they are more persistent and pH independent (Videla & Herrera, 2005). Photodisinfection has also been shown to have bactericidal effects on SRB on metal substrates *via* lipid peroxidation and membrane damage (Street & Gibbs, 2010). Chemical biocides have the potential of destroying planktonic cells, but biofilms located on the surfaces of pipeline and the substratum often prevent the effects of toxic biocides, even at increased concentration. Most biocides are toxic, persistent in the environment and are able to accumulate in a variety of matrices (Videla *et al*., 2004). Some biocides are ineffective due to bacterial tolerance and resistance (Sanders, 2003). Application of biocides to large industrial systems have achieved limited success, perhaps due to frequency of application and decrease concentration with increase distance of travel in the system (Sanders, 2003).

1.8.5 Coatings

Paints, resins, oil, ceramic lacquer, or grease and polyvinyl chloride (PVC) can be used to completely cover metals (Jack, 2002). Protective coatings may be organic e.g. bitumen, vinyls, chlorinated rubber, acrylics and inorganic coatings (e.g. galvanising and

metalising (Zuo, 2007)). These afford excellent protection as long as the coating can be maintained intact. Coating of metals with a less expensive metal over expensive ones, provides a sacrificial protection for the noble metals, for example, when zinc is coated over iron or steel (i.e. galvanising). Galvanising either serves as a barrier thereby preventing access to the surface of metal or reduces the film of electron by introducing a highly resistant corrosion cell circuit. Resistant coating types are additives, which forms inhibitory agents when water and oxygen are present (Videla, 2002; Koch *et al.,* 2001).

Lubricants have been used to keep corrosion and wear from metal components. However, lubricants may contain hydrocarbons that support microbial growth in the presence of moisture and other favourable conditions (Jack, 2002).

Metals may be coated using chemical action. This involves the use of an impervious film e.g. an oxide coating. Also oxidising agents e.g. nitric acids enable iron to become chemically unreactive or passive due to the production of a film of stable oxide. In addition, the use of epoxy based primer coatings on a properly washed and cleaned surface has shown good results. However, the level of salt on the surface should be kept low so as to obtain a good protective measure (Jack, 2002; Oyeleke *et al.,* 2005).

1.9 Purification and Identification of Metabolites Involved In Bacterial Growth Induction and Inhibition

In the previous sections different mechanisms by which SRB's growth is inhibited and enhanced have been discussed, this section describes strategies used to isolate compounds which inhibit or induce their growth from producing organisms. Microbial products are important sources of novel bioactive compounds (Bertrand *et al*., 2014). According to Giddings & Newman (2013), at least 33 % of all natural products are

derived from microorganisms. Several methods can be used to purify and identify these compounds from microorganisms. Separation and isolation of bioactive compounds are based on appropriate biological, chemical and analytical methods, such as (i) ion exchange chromatography (ii) gel filtration or size exclusion chromatography, and (iii) high performance liquid chromatography (HPLC). For identification of bioactive compounds, (i) nuclear magnetic resonance (NMR) and (ii) matrix-assisted laser desorption ionisation–time of flight (MALDI-TOF) mass spectrometry may be used.

1.9.1 Ion Exchange chromatography

The most popular method for the purification of charged molecules especially proteins is ion exchange chromatography. It is a form of absorptive liquid chromatography that allows for the separation of ionisable molecules based on how they differ in their charge properties (Xu, 2005; Cummins *et al*., 2011; Khan, 2012). It is also useful in desalination and concentration of molecules (Xu, 2005). Chromatographic techniques have been used for the purification of bioactive compounds, for example, Zhou *et al.* (2014) have purified marine bacterial inhibitors from a sponge-derived fungus *Aspergillus* sp. using repeated chromatographic techniques.

Typically, membrane matrices used in ion exchange chromatography are either cellulose or agarose-based matrices with covalently bonded charged functional groups e.g. polyimides, polyether ketone, polysulphone, polyethersulphone, poly p-phenyleneoxide, poly p-phenylene sulphide. Ion exchange chromatography can either be cationic or anionic (Nakatani *et al*., 2012; Rathore & Gupta, 2012). Anionic exchangers e.g. quaternary ammonium (Q), diethylaminoethyl (DEAE), methyl sulphonate (S), diethylaminopropyl (ANX) are resins with positively charged functional groups (e.g. $-NR_3^+$, $-NH_3^+$, $-NR_2H^+$,$-NRH_2^+$,$-PR_3^+$,) and cationic exchangers e.g.

sulphopropyl (SP) and carboxymethyl (CM) are resins with negatively charged functional group e.g. $–COO^-$, $–SO_3^-$,$–PO_3^{2-}$ (Xu, 2005; Rathore & Gupta, 2012). Buffers commonly used for anionic exchange chromatography include Tris-NaCl, phosphate and ethanolamine, while buffers for cationic chromatography are lactic acid, acetic acid, phosphate in concentrations of 10-50 mM and pH range of 6-8 (Cummins *et al*., 2011; Rathore & Gupta, 2012).

The first stage in ion exchange chromatography is equilibration in which exchanger is brought to a starting state in terms of pH and ionic strength. The second stage is sample application and adsorption, in which the solute molecules carrying the appropriately charge displace counter-ions and bind reversibly to the resins. The extent of adsorption of the molecules to the resin is determined by the ionic interaction between the oppositely charged ionic groups and in the functional group on the resin. The strength of the interaction is driven by the number and location of the charges on the molecule and on the functional group. Once the samples are applied to the column, an increasing salt concentration is then applied to elute the sample component from the column either by stepwise or by linear gradient or by changing its pH (Khan, 2012). The next stage is regeneration and it involves the removal of molecules that were not eluted from the column. The ions separated are depicted as chromatographic peaks, each peak representing a separate ion from the sample, the height and breath of each peak represents the concentration of ions moving through the column at a given time (Cummins *et al*., 2011; Bruckner, 2012; Rathore & Gupta, 2012).

1.9.2 Gel filtration

Gel filtration (GF) is a form of non-absorptive chromatography that separates molecules on the basis of their shape and molecular size (Hagel, 2001). Before use, the column is

first equilibrated with appropriate buffer. Biomolecules are eluted from the GF column in order of size, with larger molecules eluting first (GE Healthcare, 2011). The basic components of the gel filtration experiment are the matrix (stationary phase) in a chromatography column and the elution buffer (mobile phase). Elution is often isocratic in which a constant solvent ratio is used throughout the purification. Examples of commonly used matrix are Sephadex™, Superdex™, and Superose™ with 0.05 M sodium phosphate, Tris-HCl or 0.15-0.25 M NaCl as the buffers (Hagel, 2001; EDVOTEK, 2011). Gel filtration is suitable for biomolecules that are sensitive to variation in environmental conditions (Hagel, 2001; EDVOTEK, 2011).

1.9.3. High performance liquid chromatography (HPLC)

HPLC is a form of column chromatography that pumps a sample mixture (mobile phase) at high pressure of up to 400 atmospheres through a column with chromatographic packing material (stationary phase). The sample is carried by a moving carrier gas stream of helium or nitrogen and elution may be isocratic or by gradients (van Wyk & Britz, 2010; Szymanowska-Powałowska *et al*., 2013).

1.9.4. Nuclear magnetic resonance (NMR) spectroscopy

Nuclear magnetic resonance (NMR) spectroscopy, first developed in 1946 by a group of scientists at Stanford and M.I.T. in the USA, is a powerful and complex analytical technique widely used in chemical research for determining structures and molecular identity of small to medium-sized molecules and to study their dynamics, interactions and physical properties. For example, NMR can be used to deduce structures of proteins or nucleic acids based on spectral lines of different atomic nuclei that are excited when a strong magnetic field and a radiofrequency transmitter are applied (Gunther, 2013).

NMR spectroscopy has grown into an indispensable tool for chemical analysis and structural determination of natural products (Witkowski & Wawer, 2013).

The principle of NMR is anchored on the spinning of nuclei with an odd mass or atomic number for example, ^{1}H and ^{13}C. This generates a small magnetic field around the nucleus and yields chemical information as it relates to the structure of the compounds (Brunning, 2015). The NMR spectrometer consists of the following components: (i) a strong and stable static magnetic field that generate macroscopic magnetization in an NMR sample, (ii) an inductive radio-frequency pickup coils that detect the signals known as free induction decay (FID) from the magnetic field, (iii) a high-power radio-frequency transmitter capable of delivering short pulses that interact with all of the nuclei of one isotope in the sample, (iv) a sensitive receiver to amplify the NMR signals, (v) an analogue to digital converter (ADC), which converts the detected signals from a voltage to a binary number that can be stored in computer memory, (vi) a "pulse programmer" that carries out data acquisition and produces precisely timed pulses and delays, and (vii) a computer for data processing (Gunther, 2013; Teng, 2013).

1.9.5. Mass spectrometry

Mass spectrometry (MS) is an accurate and sensitive tool used to determine the mass of biomolecules (Erba & Petosa, 2015). The components of a mass spectrometer consist of the ionisation source, mass analyser and detector. The mass spectra signal is often generated by a computer system connected to the mass spectrometry component (Kicman *et al.*, 2007). The source can be achieved by electron ionisation, chemical ionisation, electrospray, matrix laser desorption (MALDI) and atmospheric chemical ionisation. Different types of analysers are used such as Quadrupole, Sector, Linear Quadrupole Ion-Trap and Time of Flight (TOF). If the sample has functional groups that

readily accept a proton (H^+) then positive ion detection is used e.g. amines $R\text{-}NH_2 + H^+ = R\text{-}NH_3^+$ as in proteins or peptides. If the sample has functional groups that readily lose a proton then negative ion detection is used e.g. carboxylic acids $R\text{-}CO_2H = R\text{-}CO_2^-$ and alcohols R-OH = R-O- as in saccharides or oligonucleotides.

The general principle of mass spectrometry is the production, separation and detection of gas-phase ions (Jurinke *et al*., 2004). Mass spectrometry can be used to determine the molecular weight of intact non-covalent biomolecule complexes, their structure and dynamics (Erba & Petosa, 2015), their interaction between subunits and stoichiometry (Madler *et al*., 2013), their hierarchy and relative position (core *vs*. periphery) as well as the strength of inter-subunit interactions (Erba & Petosa, 2015).

One of the benefits in the use of mass spectrometry is that only small amounts of samples are needed (Madler *et al*., 2013). As mass spectrometry relies on molecules being converted into ions in the gas-phase and due to the involatile nature of large polar biomolecules such as proteins and peptides some mass spectrometry-based approaches are used to overcome ionisation problems. One such method is the MALDI. MALDI-MS involves a non-destructive vapourisation and ionisation of both large and small biomolecules that are embedded in a solid organic matrix and subsequently desorbed and ionised by a pulse of laser light (Erba & Petosa, 2015). MALDI-MS is commonly combined with the TOF mass analysers (Kicman *et al*., 2007; Tran *et al*., 2011; De Carolis *et al*., 2014).

MALDI-TOF spectrometry is a relatively new method for the structural analysis of biomolecules such as peptides, carbohydrates, oligonucleotides, natural products, glycoconjugate drugs and lipids (Ernst & Gunter, 2009). It was first introduced in 1988

by Karas and Hillenkamp (Karas & Hillenkamp, 1988). It produces mostly single-charged ions (z = 1) such that the mass-to-charge ratio (m/z) of the analyte corresponds to its mass value (De Carolis *et al*., 2014). Some of the advantages of the use of MALDI-TOF spectrometry are (i) it works for compounds >500 Da and up to several 100 kDa (ii) the absence of overlapping high charge state distributions (iii) it is more tolerance to salts, detergents and other contaminates and (iv) the acquisition and interpretation of MALDI data are quite straight forward (Signor & Erba, 2013). However, the accuracy of mass determination by MALDI is dependent on the matrix compound, sample purity, preparation and concentration (Lewis *et al.*, 2000; Jurinke *et al*., 2004).

1.10. Justification and Aims of this Study

SRB have been implicated in biocorrosion, biofouling and hydrocarbon degradation. Early identification of SRB is difficult because they are capable of operating at low cell densities. This implies that rapid or early detection is important to their control (Horacek, 1992; Kondo *et al*., 2008). Qi *et al*. (2014) supported this statement by emphasising the difficulty involved in fast enumeration of SRB population in corrosion and environmental studies. Historically, SRB have been detected and enumerated in oil-field systems by the use of culture media that detect only a fraction of the SRB population (Ingvorsen & Vester, 1998). Brink *et al.* (1994) emphasised the lack of comprehensive microbiological bioassays methods in oil-fields. Cowan (2005) asserted that it takes 28 days to cover the whole SRB detection process and sometimes false reports are obtained. Rapid detection tools available are complex and expensive e.g. quantitative PCR and SRB Rapid Detection Test Kit using APS reductase as a marker (Horacek, 1992; Eden *et al*., 1993; Kondo *et al*., 2008). Therefore, due to the difficulty in rapid detection of SRB population and the importance of early identification, current

studies on SRB are directed to their early detection using simple and quick approaches especially by supplementing small molecules that can induce microbial growth into microbiological culture media (Bertrand *et al*., 2014).

Another technique central to the control of SRB is the inhibition of their growth or activity. Many approaches have been tried to limit SRB growth in industries so that the production of hydrogen sulphide is prevented. These include the use of nanofiltration, biocompetitive exclusion, chemical inhibitors, biocides and coatings. However, the use of these methods has proved to be ineffective, or impracticable in the field and too expensive. Microorganisms and the products of their metabolism are able to degrade most biocides, coatings and chemical inhibitors, utilising them for their growth and metabolism (Muthukuma *et al*., 2007). This will decrease the effectiveness of the treatment compounds and eventually lead to increased microbial population sizes (Little & Lee, 2009), often with greater negative impacts (Zuo, 2007). In addition, incidences of equipment failure have been reported with the use of some biocides and most of the biocides pose danger to the environment due to their toxicity (Gittens *et al*., 2013; Zarasvand & Rai, 2014). Also many of the chemically-derived antifouling agents are not environmentally friendly (Zarasvand & Rai, 2014). This accounts for why urgent research is directed towards the use of microbial metabolites to inhibit the growth of fouling organisms through the production of antifouling enzymes, antimicrobial agents and chemical signals (Gittens *et al*., 2013; Zarasvand & Rai, 2014).

In nature, SRB have the ability to interact with other microorganisms in the environment and the products from these microorganisms (Oh *et al*., 2007; Wikieł *et al*., 2014). When SRB was co-cultured with *E. coli* BP, it was observed to induce the growth and increase the detection sensitivity of the SRB cultures. Similarly, the addition of cell

free supernatant (CFS) from *E. coli* BP has a similar effect on the growth / detection of SRB. In order to identify the compounds responsible for the growth induction of *E. coli* BP, it is important that they be purified and characterised.

Therefore, the aim of this study is to purify and identify two bioactive compounds derived from *E. coli* BP observed to influence the growth of SRB. One of the compounds referred to as SRB growth enhancer (SGE) induces the growth of SRB and the other compound, known as SRB growth inhibitor (SGI), inhibits SRB growth. The addition of SGE to commonly used media for growing SRB would increase SRB detection sensitivity, enhance rapid re-activation of dormant SRB and will allow isolation of SRB which otherwise would stay undetected due to low cell density. SRB growth inhibitor (SGI) may be developed as an anti-corrosion and anti-fouling agent when incorporated to crude oil processing. This would prevent problems of corrosion, reservoir plugging, hydrocarbon degradation, biofouling and fuel contamination with sulphide facilitated by SRB.

The specific objectives of this study are as follows:

1. Design the procedure for preparation of SGE for testing on SRB growth
2. Optimise growth conditions for production of SGE
3. Purify the SGE in preparative quantity.
4. Characterisation of SGE and SGI: Novel versus established compounds
5. Study of SRB growth in the presence of SGI and SGE using microscopy
6. Analyse SGE and SGI by NMR spectroscopy and MALDI-TOF spectrometry

CHAPTER 2

MATERIALS AND METHODS

2.1 Microbiological Techniques

2.1.1 Bacterial strains

The bacterial strains used for this study were *E. coli* BP; *D. indonesiensis* NCIMB 13468; recovered from a corroded carbon steel in an Indonesia marine environment (Feio *et al.,* 1998; Feio *et al.,* 2000), *D. alaskensis* NCIMB 13491; isolated from a soured oil field in Purdu Bay Alaska (Feio *et al*., 2004) and *D. vulgaris* NCIMB 8303. The bacterial strains were obtained from the Microbiology Research Laboratory at the University of Portsmouth.

2.1.2 Media

The growth media used for this study were VM medium with 500 mg/l of $FeSO_4$ (VMI) and VM medium with 50 mg/l of $FeSO_4$ (VMR) (Zinkevich & Beech, 2000), M9 medium (Miller, 1972), LB (Lysogeny Broth) medium and MacConkey agar. The composition of VM media and methods of preparation is shown in Section 2.1.2.1. The preparation of M9 medium is explained in Section 2.1.2.2, preparation of LB medium is in Section 2.1.2.3, preparation of MacConkey Agar medium is in Section 2.1.2.4 and preparation of 0.9 % NaCl for enumeration of *E. coli* BP culture is listed in Section 2.1.2.5.

2.1.2.1 Preparation of VM media

The compounds in Table 2.1 were measured into a 1 L bottle;

Table 2.1. Composition of VM medium

Compounds	**Weight**
KH_2PO_4	0.5 g
NH_4Cl	1 g
Na_2SO_4	4.5 g
$CaCl_2.2H_2O$	0.04 g
$MgSO_4.7H_2O$	0.06 g
$Na_3C_6H_5O_7.2H_2O$	0.3 g
NaCl	25 g
Casamino acids	2 g
Tryptone	2 g
$Na_2C_3H_5O_3$	6 g

Up to one litre of distilled water (dH_2O) was added and was dissolved by stirring using a magnetic stirrer. The pH was adjusted to 7.5 using 5 M NaOH. 1 ml vitamin stock solution (Table 2.2) and trace elements (1 ml stock) (Table 2.3) were added. The solution was de-oxygenated with nitrogen gas for 25 min ensuring that the sparger was located at the bottom of the solution. $FeSO_4.7H_2O$ (VMI - 0.5 g, VMR - 0.05 g) was added

and the solution was stirred until solute was dissolved. The pH was again adjusted to 7.5. The solution was purged/de-oxygenated for another 25 min, the cap/dispenser tightly fastened to prevent the entrance of oxygen. Using the two-way nitrogen valve, 9 ml of VMI solution was dispensed into a 10 ml vial and 4.5 ml of VMR solution was dispensed into a 5 ml vial. The vial was immediately covered with a stopper (20 mm) (Fisher Scientific) and crimp seals (20 mm) (Fisher Scientific) and autoclaved at 121°C for 15 min.

Table 2.2. Composition of vitamin solution for VM medium

Vitamins	**Weight**
Vitamin B1 (Thiamine)	0.6 mg
Vitamin B2 (Riboflavin)	0.2 mg
Vitamin B3 (Nicotinic acid)	0.5 mg
Vitamin B5 (Pantothenic acid)	0.6 mg
Vitamin B6 (Pyridoxal phosphate)	0.6 mg
Vitamin B12 (Cobalamin)	0.05 mg
Vitamin C (L-ascorbic acid)	100 mg
Vitamin H (Biotin)	0.01 mg
dH_2O	Up to 1 litre

Table 2.3. Composition of trace elements solution (Modified Wolff's mineral solution)

Trace elements	Weight
$C_6H_9NO_6$	1.5 g
$MgSO_4 \cdot 7H_2O$	3.0 g
$MnSO_4 \cdot H_2O$	0.5 g
NaCl	1.0 g
$FeSO_4 \cdot 7H_2O$	0.1 g
$CoSO_4 \cdot 7H_2O$	0.1 g
$NiCl_2 \cdot 6H_2O$	0.1 g
$CuCl_2 \cdot 2H_2O$	0.1 g
$ZnSO_4 \cdot 7H_2O$	0.1 g
$CuSO_4 \cdot 5H_2O$	0.01 g
$KAl(SO_4)_2 \cdot 12H_2O$	0.01 g
H_3BO_3	0.01 g
$Na_2MoO_4 \cdot 2H_2O$	0.01 g
Na_2SeO_3	0.001 g
dH_2O	Up to 1 litre

2.1.2.2 Preparation of M9 medium

To prepare a 1 L of M9 medium, 750 ml of dH_2O was added into a 1 L bottle. It was sterilised by autoclaving at 121°C for 15 min and allowed to cool, 100 µl sterile 1 M $CaCl_2$ (stock) was added and swirled to mix. To a sterile 250 ml cylinder was added 200 ml of sterile M9 salts 5x concentrated (stock), 2 ml of sterile 1 M $MgSO_4$ (stock) and 20 ml of sterile 20 % glucose (stock). Sterile dH_2O was added to make up the volume to 250 ml and the mixture was added into 1 L bottle containing 100 µl sterile 1 M $CaCl_2$ and 750 ml sterile dH_2O, which constitutes M9 medium. All the chemicals for M9 preparation were obtained from Fisher Scientific.

The M9 medium was filtered into a sterile 1 L bottle by passing it through a 0.22 µm Millipore Durapore™ (Stericup) *in vacuo*. 10 ml vials capped with stoppers (20 mm) and crimp seals (20 mm) and 100 ml vials were autoclaved at 121°C for 15 min and oven-dried at 60°C. 9.5 ml of M9 medium was dispensed into the sterile 10 ml vials through 0.2 µm filter using sterile syringe while 95 ml of M9 medium was dispensed into the sterile 100 ml vials using a sterile cylinder after which it was capped with sterile stoppers (20 mm) and crimp seals (20 mm). The various stock solutions from which M9 medium was prepared is as follows:

(i) Preparation of 5 x M9 salts stock solution:

The compounds in Table 2.4 were measure into a 1 L bottle

Table 2.4. Composition of M9 salts 5x concentrated

Compounds	**Weight**
$Na_2HPO_4.7H_2O$	64 g
KH_2PO_4	15 g
NaCl	2.5 g
NH_4Cl	5 g
dH_2O	Up to 1 litre

The compound was dissolved by stirring using a magnetic stirrer and sterilised by autoclaving at 121°C for 15 min.

(ii) Preparation of 1 M $MgSO_4$: To a 200 ml bottle containing 80 ml dH_2O was added 24.65 g of $MgSO_4.7H_2O$ and was dissolved by stirring. The volume was made up to 100 ml and autoclaved at 121°C for 15 min.

(iii) Preparation of 20 % glucose: To a 100 ml bottle containing 80 ml of dH_2O was added 20 g of glucose, it was dissolved by stirring and the volume was made up to 100 ml using dH_2O and filter sterilised into a sterile bottle *via* a 0.22 μm Millipore filter *in vacuo*.

(iv) Preparation of 1 M $CaCl_2$: The 14.7 g of $CaCl_2$. $2H_2O$ was added into 80 ml of dH_2O. It was dissolved by stirring. The volume was made up to 100 ml using dH_2O and filter sterilised into a sterile bottle *via* a 0.22 µm Millipore filter *in vacuo*.

2.1.2.3 Preparation of LB medium

The 10 g of tryptone, 5 g yeast extract and 10 g NaCl were weighed into 1 L bottle. 800 ml of dH_2O was added and the nutrients were dissolved by stirring. The volume was made up to 1 L and sterilised by autoclaving at 121°C for 15 min. All the chemicals for LB medium preparation were obtained from Fisher Scientific.

2.1.2.4 Preparation of MacConkey Agar medium

The 52 g of MacConkey agar (Fisher Scientific) was weighed into 1 L bottle. 800 ml of distilled water was added and the nutrients were dissolved by stirring. The volume was made up to 1 L and sterilised by autoclaving at 121°C for 15 min.

2.1.2.5 Preparation of 0.9 % NaCl for enumeration of *E. coli* BP culture

To a 100 ml bottle was added 0.9 g of NaCl (Fisher Scientific), 80 ml of dH_2O was added. The nutrients were dissolved by stirring. The volume was made up to 100 ml and sterilised by autoclaving at 121°C for 15 min.

2.1.3 Standard growth conditions for *E. coli* BP cells

The *E. coli* BP culture was refreshed by inoculating a stock culture in freshly prepared M9 or LB medium (1:20) and grown at 37°C for 24 h. The culture was serially diluted ten-fold, plated on MacConkey agar and incubated at 37°C for 24 h to check for purity and for counting of viable bacterial cells expressed as colony forming unit per millilitre

(cfu/ml). The culture of *E. coli* BP streaked on MacConkey agar is shown in Figure 2.1. The procedure for enumeration is described in Section 2.3.2.

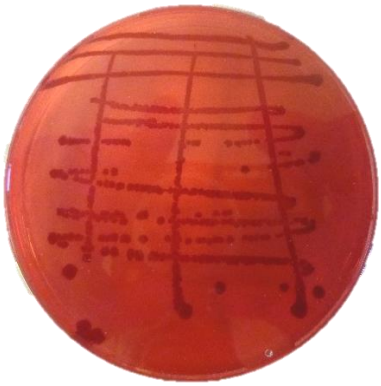


Figure 2.1. *E. coli* BP streaked on MacConkey agar plate

2.1.4 Growth procedure for SRB

The 1 ml stock culture of each SRB strain (*D. indonesiensis, D. alaskensis* and *D. vulgaris*) was inoculated into 9 ml of VMI medium at 1:10 (v/v) ratio using a syringe and needle to prevent introduction of oxygen into the vial (Figure 2.2). The cultures were incubated at 37°C for 7 days. The presence of SRB was revealed by the change of the growth medium from light yellow to black colour at the base of the vial as a result of formation of insoluble iron sulphide (FeS) due to reaction of hydrogen sulphide (H_2S) produced by metabolising cells with iron sulfate in the medium.

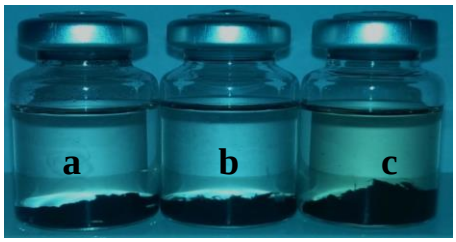


Figure 2.2. Example of SRB Growth in VMI medium
(a) *D. indonesiensis*, (b) *D. vulgaris* and (c) *D. alaskensis*

2.2 Development of an SGE Purification Procedure

The CFS obtained from an overnight culture of *E. coli* BP grown in 100 ml of LB medium under aerobic conditions was diluted 1:3 with 10 mM Tris-HCl, pH 8 and loaded on 5 ml of Q Sepharose Fast Flow™ resin with protein binding capacity of 120 mg HSA/ml (GE Healthcare, 2014) in 1.0 × 10 cm Econo-Column® (Bio-Rad). The procedure of obtaining CFS from *E. coli* BP is listed in Section 2.3.1 and the procedure for preparation of 10 mM Tris-HCl, pH 8 is listed in Section 2.4.1. The CFS was diluted 1 in 3 with 10 mM Tris-HCl, pH 8.0 to lower the molarity and allow binding of the materials to the Q Sepharose Fast Flow™ resins. The column was connected to Bio-Rad Biologic Low Pressure (LP) system and a computer system installed with Bio-Rad Biologic LP system software so as to monitor the purification process. The UV optics of Bio-Rad LP system measures at 280 nm to detect protein and a conductivity cell to monitor gradient (Bio-Rad, 2016). The diluted CFS was pumped into the column using 1 ml/min flow rate. The column was washed with 10 volumes of 50 mM NaCl in 10 mM Tris-HCl, pH 8.0 and the bound materials in the column were eluted with step gradients of 125, 250 and 500 mM NaCl in 10 mM Tris-HCl, pH 8.0. The partially purified SGE fractions were tested for activity on the growth of *D. indonensiensis* in VMR medium following the procedure in Section 2.7.

2.3 Optimisation of *E. coli* BP Growth Conditions for SGE Production

The schematic diagram for optimising the growth conditions for SGE production is presented in Figure 2.3. This involves preparation of growth media, inoculation of *E. coli* BP cells into M9 medium, growth of culture in M9 medium at various incubation time, centrifuging and filtration of culture to obtain a cell free supernatant (CFS), purification of CFS by ion exchange chromatography and testing of each fraction for activity.

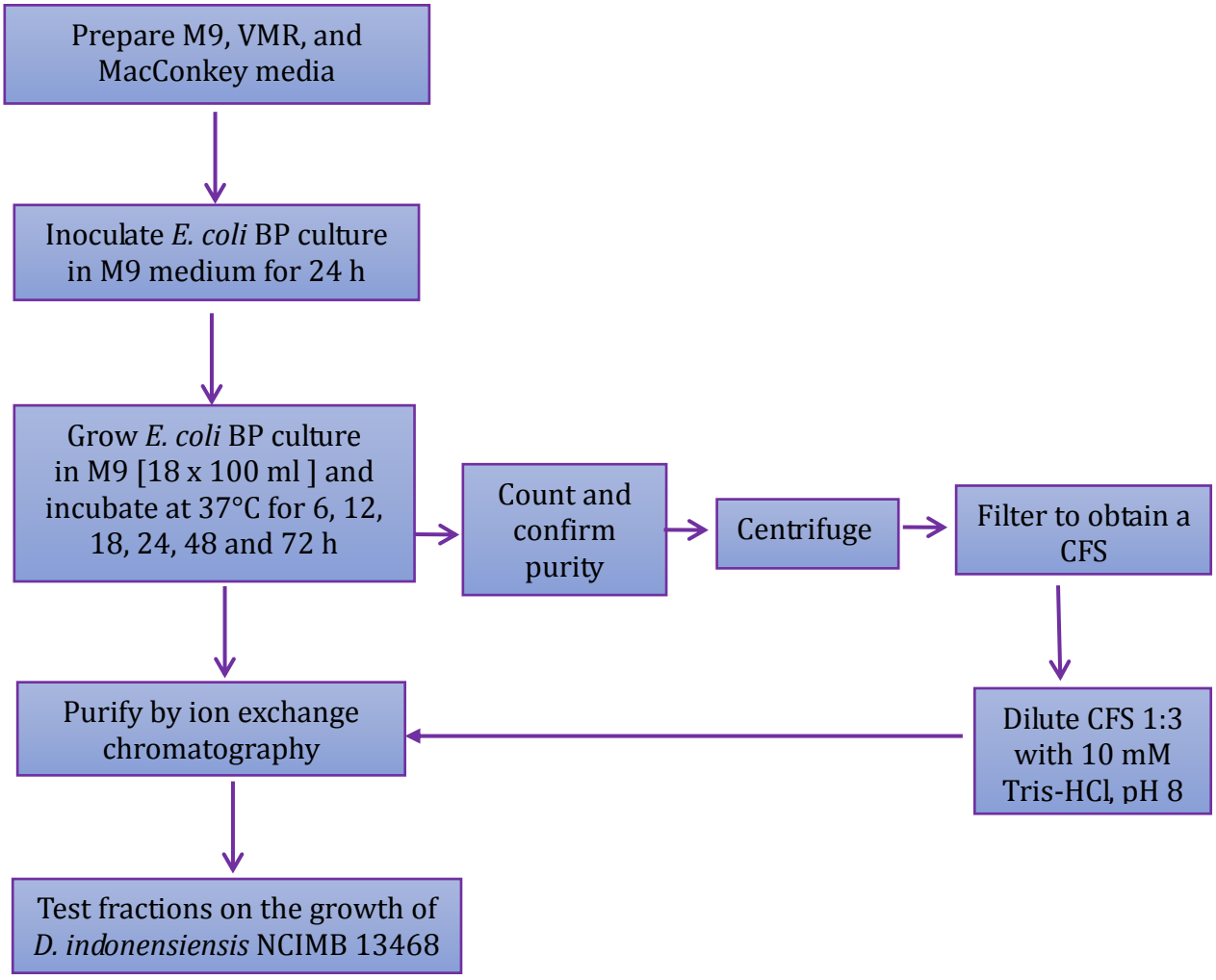


Figure 2.3. Optimising growth conditions for SGE production

2.3.1 Procedure of obtaining cell free supernatant (CFS)

To obtain a CFS, an overnight culture of *E. coli* BP (5 ml) was inoculated into 95 ml of M9 medium at 1:20 (v/v) ratio (18 x 100 ml vials) and incubated at 37°C for 6, 12, 18, 24, 48 and 72 h in aerobic condition using an Innova™ 4000 incubator shaker at 220 rpm (New Brunswick Scientific), limited oxygen conditions (Figure 2.4) and anaerobic conditions. After incubation, 0.1 ml each of the 100 ml *E. coli* BP culture was serially diluted tenfold and loaded on MacConkey agar to check for purity of the culture and determine the viable counts expressed as colony forming unit per millilitre (cfu/ml) (Section 2.3.2). Each of the 100 ml cultures were spun in SORVALL RC 5C Plus at 18,000rpm (38,828 g) for 35 min using rotor SS-34, 4°C. The supernatant obtained was filter-sterilised *via* a 0.22 µm Millipore filter unit (Stericup) *in vacuo* to obtain a CFS and stored at 4°C for further purification.

Figure 2.4. *E. coli* BP grown in M9 medium under limited oxygen condition

2.3.2 Enumeration of *E. coli* BP cultures

Enumeration of *E. coli* BP cultures was performed in a laminar flow cabinet. Due to high concentrations of bacteria cells in the original samples, serial dilution was used to lower the concentration of samples to obtain a visible isolated colonies that can be counted on the MacConkey agar plates. To achieve this, 10 sterile Eppendorf tubes were placed in a

rack and labeled 10^{-1} to 10^{-10}, each of the tubes was filled with 0.9 ml of 0.9 % NaCl solution using 1 ml micropipette. Using a syringe, the first tube was injected with 0.1 ml of *E. coli* BP culture from the original vial. The solution was mixed well to ensure an even distribution of bacterial cells. 0.1 ml of sample was then taken from the first tube to the second tube. This was also thoroughly mixed. The procedure was repeated until all ten tubes have been inoculated. Each of the dilution was then mixed and loaded in triplicate on freshly prepared MacConkey agar plates. The culture was allowed to dry, covered and incubated in an inverted position at 37°C overnight. Cultures that developed were counted and expressed as colony forming unit per ml of sample (cfu/ml).

2.4 Biochemistry Techniques

2.4.1 Buffers used for ion exchange chromatography

The buffers used for purification by ion exchange chromatography are listed below.

i. 10 mM Tris-HCl, pH 8.0 (Buffer 0)
ii. 10 mM Tris-HCl pH 8.0, 50 mM NaCl (Buffer 1)
iii. 10 mM Tris-HCl pH 8.0, 125 mM NaCl (Buffer 2)
iv. 10 mM Tris-HCl pH 8.0, 250 mM NaCl (Buffer 3)
v. 10 mM Tris-HCl pH 8.0, 500 mM NaCl (Buffer 4)
vi. 10 mM Tris-HCl pH 8.0, 700 mM NaCl (Buffer 5)
vii. 10 mM Tris-HCl pH 8.0, 1 M NaCl (Buffer 6)
viii. 10 mM Tris-HCl pH 8.0, 50 mM NaCl, 20 % absolute ethanol (Buffer 7)

To prepare these buffers, a stock solution of 50 mM Tris-HCl, pH 8 was made by measuring 4.44 g of Tris-HCl (Sigma-Aldrich) and 2.65 g of Tris-Base (Sigma-Aldrich) and dissolved in up to 1 L of dH_2O while 5 M NaCl was prepared, 58.44 g of NaCl (Sigma-

Aldrich) dissolved in 200 ml of dH_2O. **A litre of 10 mM Tris-HCl, pH 8.0 (Buffer 0)** was prepared by adding 200 ml of 50 mM Tris-HCl, pH 8.0 into a 1 L cylinder. The volume was made up to 1 L using dH_2O. It was sterilised *in vacuo* by passing through a Millipore Durapore™ 0.22µm (Stericup) filter. To prepare a litre of **10 mM Tris-HCl pH 8.0, 50 mM NaCl (Buffer 1)**, 200 ml of 50 mM Tris-HCl, pH 8.0 and 10 ml of 5 M NaCl was made up to 1 L with dH_2O. It was sterilised *in vacuo* by passing through a Millipore Durapore™ 0.22 µm (Stericup) filter. To prepare a **litre of 10 mM Tris-HCl pH 8.0, 250 mM NaCl (Buffer 2)**, 200 ml of 50 mM Tris-HCl, pH 8.0 and 25 ml of 5 M NaCl was made up to 1 L with dH_2O. It was sterilised *in vacuo* by passing it through a Millipore Durapore™ 0.22 µm (Stericup) filter. For a **litre of 10mM Tris-HCl pH 8.0, 250 mM NaCl (Buffer 3)**, 200 ml of 50 mM Tris-HCl, pH 8.0 and 50 ml of 5 M NaCl was made up to 1 L with dH_2O. It was sterilised *in vacuo* by passing it through a Millipore Durapore™ 0.22 µm (Stericup) filter. **A litre of 10 mM Tris-HCl pH 8.0, 500 mM NaCl (Buffer 4)** was made by adding 200 ml of 50 mM Tris-HCl, pH 8.0 and 100 ml of 5 M NaCl up to 1 L with dH_2O. It was sterilised *in vacuo* by passing it through a Millipore Durapore™ 0.22 µm (Stericup) filter. **A litre of 10 mM Tris-HCl pH 8.0, 700 mM NaCl (Buffer 5)** was prepared by adding 200 ml of 50 mM Tris-HCl, pH 8.0, 140 ml of 5 M NaCl up to 1 L with dH_2O. It was sterilised *in vacuo* by passing it through a Millipore Durapore™ 0.22 µm (Stericup) filter. **A litre of 10 mM Tris-HCl pH 8.0, 1 M NaCl (Buffer 6)** was prepared by addition of 200 ml of 50 mM Tris-HCl, pH 8.0 and 200 ml of 5 M NaCl was made up to 1 L with dH_2O. It was sterilised *in vacuo* by passing it through a Millipore Durapore™ 0.22 µm (Stericup) filter. **A litre of 10 mM Tris-HCl pH 8.0, 50 mM NaCl, 20 % absolute ethanol (Buffer 7)** was prepared by addition of 200 ml of 50 mM Tris-HCl, pH 8.0, 10 ml 5 M NaCl in 800 ml of dH_2O and sterilised *in vacuo* by passing it through a

Millipore Durapore™ 0.22 µm (Stericup) filter. The volume was made up to 1 L by adding 200 ml of absolute ethanol (Fisher Scientific).

2.4.2 Purification of CFS by ion exchange chromatography

The first stage for the purification of the CFS was ion exchange chromatography. This procedure was carried out using 5 ml of Q Sepharose Fast Flow™ resin in 1.0 × 10 cm Econo-Column® (Bio-Rad). The column was equilibrated with 10 volumes of 50 mM NaCl in 10 mM Tris-HCl, pH 8.0 using 1 ml/min flow rate. The CFS was diluted 1 in 3 with 10 mM Tris-HCl, pH 8.0. The diluted CFS was pumped into the column using 1 ml/min flow rate. The column was washed with 10 volumes of 50 mM NaCl in 10 mM Tris-HCl, pH 8.0 and the bound materials in the column were eluted with step gradients of 125, 250 and 500 mM NaCl in 10 mM Tris-HCl, pH 8.0. The partially purified SGE fractions were tested for activity on the growth of *D. indonensiensis* in VMR medium following the procedure in Section 2.7.

2.5 Preparative Purification of SGE using Ion Exchange Chromatography

The *E. coli* BP was inoculated in 100 x 100 ml (10 L) of M9 medium using 1:20 dilution of cells and incubated at 37°C for 18 h in limited oxygen condition. The *E. coli* BP cultures were spun at 13000 rpm (22278 g) for 35 min at 4°C in SORVALL RC 5C Plus centrifuge using rotor SLA-1000. The supernatant was sterilised using a 0.22 µm Millipore filter unit (Stericup) to obtain a CFS, and was stored at 4°C for further purification. The absence of any *E. coli* BP cells in the CFS was checked by plating of 0.1 ml of the CFS on freshly prepared MacConkey agar plates and following incubation at 37°C overnight. The protein concentration of the CFS was measured using nanodrop

2000 spectrophotometer (Thermo Scientific). To purify the 10 CFS, ion exchange chromatography was used and elution was by step gradient (Figure 2.5).

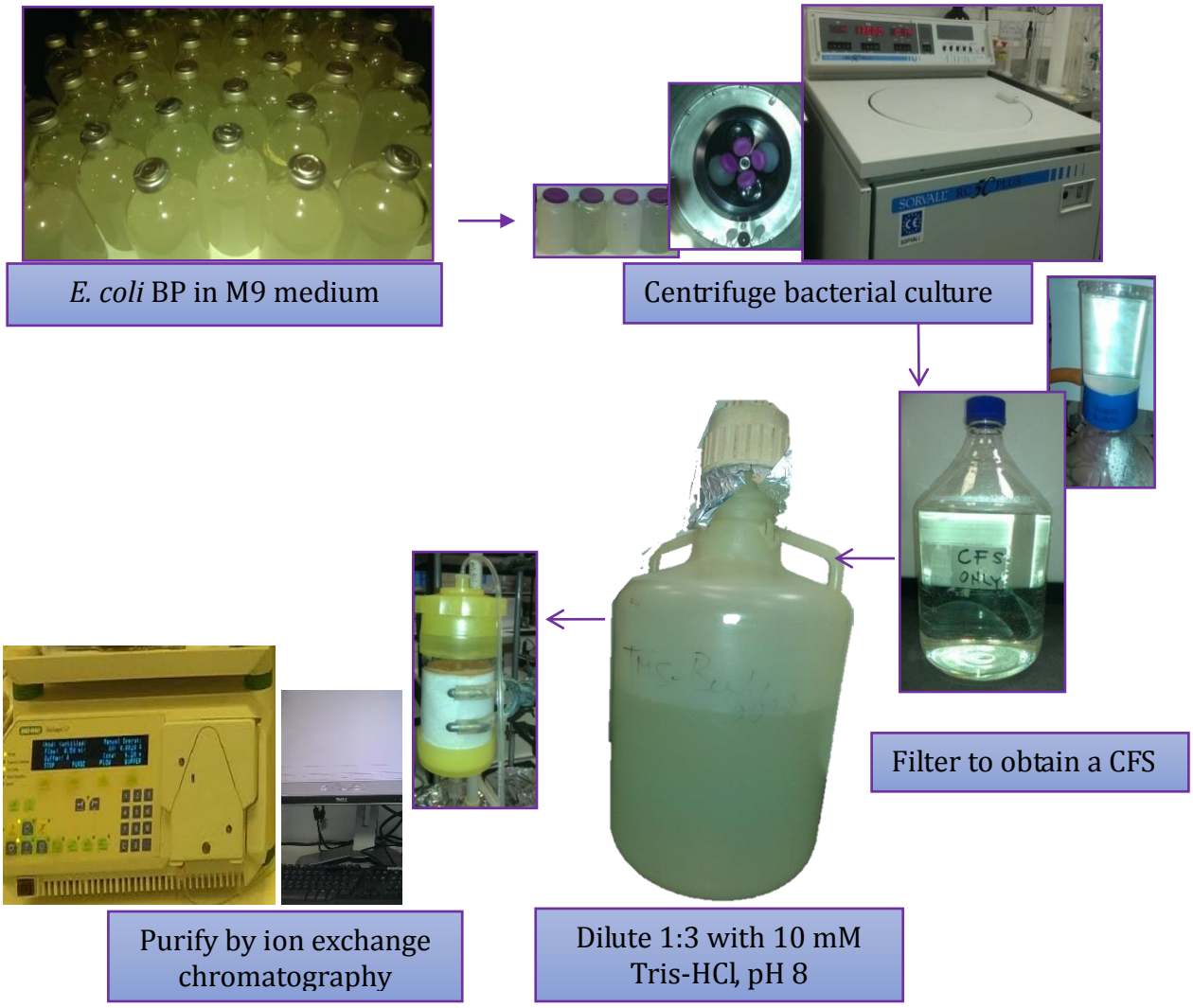


Figure 2.5. Preparative purification of SGE

The column (5.0 × 10 cm Econo-Column® (Bio-Rad)) contains 100 ml of Q Sepharose Fast Flow™ (GE Healthcare) resin. The column was equilibrated with 20 volumes of 50 mM NaCl, 10 mM Tris-HCl, pH 8.0 using 10 ml/min flow to ensure washing of the unbound materials. Prior to loading, the CFS was diluted 1 in 3 with 10 mM Tris-HCl, pH 8.0 and applied by pumping onto the column. The column was washed with 10 volumes

of 50 mM NaCl, 10 mM Tris-HCl, pH 8.0 and the bound materials in the column were eluted with step gradients of 125, 250 and 500 mM NaCl in 10 mM Tris-HCl, pH 8.0 using Bio-Rad Biologic LP system. The chromatographic fractions were collected, and stored at 4°C for testing and for further purification.

2.6 Purification of SGE using Size Exclusion Chromatography

The step 3 fraction (~ 100 ml) obtained from preparative purification was concentrated to ~ 3 ml by ion exchange chromatography before purification by size exclusion chromatography. This involves 1:10 dilution of the step 3 fraction with 10 mM Tris-HCl, pH 8 to lower the NaCl concentration to 50 mM, so as to ensure the fraction has the same NaCl concentration as the equilibration buffer (10 volume of 50 mM NaCl in 10mM Tris-HCl, pH 8) thus, allowing the materials to bind onto the Q Sepharose Fast Flow™ resin. The column (Econo-Column® (Bio-Rad) 1.5 x 10 cm) containing 3 ml of Q Sepharose Fast Flow™ resin was equilibrated with 10 volume of 50 mM NaCl in 10 mM Tris-HCl, pH 8. The diluted fraction was loaded onto the column using 3 ml/min flow rate. It was washed with 10 volumes of 50 mM NaCl in 10mM Tris-HCl, pH 8 and eluted with one step gradient of 700 mM NaCl in 10 mM Tris-HCl, pH 8.

The column used for the purification by size exclusion chromatography (gel filtration) contains Toyopearl (HW-40S) (TOSOH Bioscience LLC) with a fractionation range of 100 - 7000 Da. The 2.5 x 75 cm Econo-Column® (Bio-Rad) with cross-sectional area of 4.91 cm^2 was prepared with 200 ml of HW-40S resin and was equilibrated with 10 volumes of 50 mM NaCl in 10 mM Tris-HCl, pH 8.0 using 0.5 ml/min flow rate. In all the purifications by size exclusion, 50 mM NaCl in 10 mM Tris-HCl, pH 8 was used as the mobile phase.

The vitamin B12 (1,355 Da) (Desrousseaux *et al*., 2012) and Blue Dextran - 2,000,000 Da (Sigma-Aldrich, 1997) were used as molecular weight markers. The vitamin B12 was used to identify low molecular weight compounds while the Blue Dextran - 2,000,000 Da was used to identify the void volume. Concentrated step 3 fraction was applied to the gel filtration column using 0.5 ml/min flow rate. The fractions without the void volume were then pooled and again concentrated to 1 ml using Q Sepharose Fast Flow and eluted by one step gradient of 700 mM NaCl in 10 mM Tris-HCl, pH 8. The HW-40S column was washed with 10 volume of 50 mM NaCl in 10 mM Tris-HCl, pH 8 after which the concentrated fraction was applied to the column using 0.5 ml/min flow rate. During gel filtration, two distinct peaks were detected. These peaks subsequently named as SGI (Peak 1) and SGE (Peak 2) were pooled separately, and sterilised by filtration into a sterile vial (Fisher Scientific) using Minisart 0.22 µm syringe filter (Fisher Scientific) and de-oxygenated for 30 min by sterile nitrogen gas. It was immediately covered with a sterile injection stopper (Fisher Scientific) and sealed using an aluminum crimp seal (Fisher Scientific). Both fractions were tested for SRB inhibitory and enhancing activities following the procedure in Section 2.7.

2.7 Procedure for testing SGI and SGE on SRB Growth

Prior to testing, the SGI fractions were filter-sterilised using a syringe driven Millex^{R} sterile filter unit with MF-Millipore^{TM} membrane (0.22 µm) and de-oxygenated by sterile nitrogen for 30 min. The fractions were tested by serially diluting *D. indonesiensis*, *D. vulgaris* and *D. alaskensis* in VMI up to 10^{-5} dilution (test for inhibition) and VMR (test for enhancement) and incubated at 37°C. Prior to testing, the test organisms were grown in VMI medium at 1:10 (v/v) ratio in anaerobic condition at 37°C for 7 days. The SRB initial inoculum size was determined using a haemocytometer.

Fractions were introduced into each of the dilutions at 1:20 (v/v) ratio. Controls were set up without SGI or SGE. Growth was examined and recorded daily by observing the presence of insoluble black iron sulfide at the base of the vials. All the tests were done under anaerobic conditions due to the anaerobic nature of SRB. Multiple tests were also carried out to confirm results.

2.8 Dialysis of SGI and SGE Fractions

Dialysis of the SGI and SGE fractions was carried out by placing 2 ml of each fraction into the 5 ml dialysis chamber (Harvard Apparatus) The 1 kDa molecular weight cut off (MWCO) cellulose acetate membrane (Harvard Apparatus) was inserted into the dialyser body. The chamber was sealed by tightening cap and immersed horizontally into 2 L of sterile de-ionise water in a sterile beaker. It was placed on a magnetic stirrer at 4°C and left for 4 h. This was followed by four successive changes. The final change was done using de-ionised water molecular biology grade (SIGMA). After dialysis, the samples were tested for activities following the procedure in Section 2.7.

2.9 Procedure for testing SGI and SGE on *E. coli* BP Growth

An overnight culture of *E. coli* BP was inoculated into a 9.5 ml of M9 medium under limited oxygen condition using 1:20 v/v ratio and treated with 1:10 v/v ratio of SGI. The same was repeated for SGE. Total viable count was determined daily for three days by serially diluting 0.1 ml of culture and plated on a freshly prepared MacConkey agar with incubation at 37°C. The colonies that developed were counted and expressed as colony forming unit per ml of samples following the procedure in Section 2.3.2.

2.10 SEM Analysis on SGI and SGE treated SRB

The effect of SGI and SGE on the morphology of SRB cells and biofilm formation was investigated using a SEM, JEOL 6060LV. Glass coupon (7 x 10 mm) was placed inside 9 ml of VMR medium in a 10 ml vial. The medium was de-oxygenated using nitrogen gas for 30 min and sterilised by autoclaving for 15 min. An aliquot of the 7 days old culture of *D. indonesiensis* was added into the medium using 1:10 (v/v) ratio and SGI was added into the medium at 1:20 (v/v) ratio. The culture was incubated at 37°C for 1, 4 and 7 days. All above procedures were carried out using aseptic technique. Two controls were set up one with pure SGI and another comprising a culture of *D. indonesiensis* only. This procedure was repeated for SGE.

Glass coupons containing biofilms were fixed with Gluteraldehyde (4 %) in phosphate buffer (0.1 M) overnight at 20 (±2)°C and briefly rinsed in phosphate buffer (0.1 M) twice at ten min interval. The glass coupons were then fixed with 3 drops of osmium tetroxide (1 %) in sodium cacodylate buffer (0.1 M) at 20 (±2)°C for 1 hour and mixed gently using B7925 rotator to ensure uniform distribution of the solvent. The coupons were rinsed twice in 0.1 M phosphate buffer at ten min interval. This was followed by 10 min sequential exposures to 30, 50, 70, 90 % (v/v) aqueous solutions of absolute ethanol (reagent grade) and final immersion for 10 min in 100 % acetone (reagent grade). Following dehydration, two drops of hexamethyldisilazane were placed on each coupon to cover the slides and the specimens were incubated overnight at 20 (±2)°C. Prior to SEM imaging viewing, the glass coupons were placed on aluminum stubs and sputter coated with gold using Q150R ES for 5 min. The Au-coated specimens were viewed at 15 kV accelerating voltage value.

2.11 AFM Analysis on SGI and SGE treated SRB

The effect of SGI and SGE on the morphology of SRB cells and biofilm formation was also carried out using Multi-Mode/NanoScope IV scanning probe microscope, Bruker, Santa Barbara, CA, USA. Glass coupon (7 x 10 mm) was placed inside a 9 ml of VMR medium in a 10 ml vial. The medium was de-oxygenated using nitrogen gas for 30 min and sterilised by autoclaving for 15 min. An aliquot of the 7 days old culture of *D. indonesiensis* was added into the medium using 1:10 (v/v) ratio and SGI was added into the medium at 1:20 (v/v) ratio. The culture was incubated at 37°C for 7 days. All above procedures were carried out aseptically. A control was untreated culture of *D. indonesiensis*. This procedure was repeated for SGE. The glass coupon was removed from the medium by using forceps and placed on a surface of freshly-cleaved muscovite mica (1 cm^2; Agar Scientific, Stansted, Essex, UK), left for 2 min, rinsed with dH_2O and dried in a N_2 stream. The surface was then attached to a nickel disk mounting assembly (1 cm^2) using double-sided adhesive tape and placed on top of the AFM scanner. AFM studies were performed in air under ambient conditions (T = 23°C, RH = 21 %) using the J-scanner (max. xy = 200 µm). Scanning was performed in contact mode using a V-shaped, silicon nitride cantilever with an integrated tip (lever D: t = 0.6 µm, l = 205 µm, w = 25 µm, k = 0.06 N m^{-1}; model: NP-10, Bruker, France). The images were subsequently processed and dimensions measured using NanoScope Analysis software (V 1.4, Bruker).

2.12 CLSM Analysis on SGI and SGE treated SRB

The test organisms were stained with live/dead bacterial viability kit and viewed with confocal laser scanning microscope, LSM 710 (Zeiss). The SYTOX 9 stains live cells green and the propidium iodide stains dead cells red. Microscope cover slip (7 x 10 mm) fixed

onto rubber stoppers was placed inside a 9 ml of VMR medium in a 10 ml vial. The medium was de-oxygenated using nitrogen gas for 30 min and sterilised by autoclaving for 15 min. An aliquot of the 7 days old culture of *D. indonesiensis* was added into the medium using 1:10 (v/v) ratio and SGI was added into the medium at 1:20 (v/v) ratio. The culture was incubated at 37°C for 7 and 37 days. The cover slip was removed using forceps, washed by dipping thrice in 2.5 % of NaCl. The excess of washing solution was removed using filter paper and the cover slip was placed in a Petri dish. The biofilm was stained with live/dead bacterial viability kit (1.5 µl of propidium iodide and 1.5 µl SYTOX 9 in 997µl of sterile de-ionize water) and incubated for 30 min in the dark at room temperature. The coupon was washed by dipping thrice in 2.5 % NaCl and placed on a microscope slide in an inverted position. The excess of washing solution was removed using filter paper, sealed by nail varnish and after drying, the surface of the coupon was cleaned with 98 % of alcohol to remove the biofilm and sterilise the surface. The coupon was viewed with confocal microscopy (LSM 710 Zeiss) using fluorophores and excitation wavelengths of Argon 488 nm and Helium-Neon (He-Ne) 543 nm.

2.13 NMR Analysis on SGI and SGE

Samples were dialysed against water to remove buffer (Section 2.8). After dialysis, the SGI and SGE fractions were frozen with liquid nitrogen in a sterile round bottom flask and lyophilised to dryness using SENTRY 2.0 (VirTis SP Scientific) freeze dryer. Ten milligram (10 mg) of each SGI and SGE fraction containing the bioactive constituents was dissolved in 600 µl of 99.9 % D_2O (Sigma Aldrich) and placed into a Wilmad 535 NMR tube (Sigma Aldrich). Spectra were recorded at least 2 h after dissolution, at 25°C and at a pH of 7.5 to 8.0.

NMR spectroscopy was performed on a Varian Inova 600 MHz spectrometer, equipped with 5-channels, a 5 mm triple resonance ($^{1}H/^{13}C/^{15}N$) coldprobe and actively shielded pulse field z-axis gradients. One dimensional proton (^{1}H) spectra were acquired as 2048 complex points, with 128 transients and a spectral width of 12.0 ppm. Water suppression was achieved through use of the wet sequence. ^{13}C-^{1}H gradient heteronuclear single quantum coherence (gHSQC) spectra were acquired as 1024 and 280 complex points using spectral widths of 12.0 and 140 ppm for the F2 (^{1}H) and F1 (^{13}C) dimensions respectively. The direct dimension (F2) used 64 transients. The ^{13}C transmitter offset was initially set at 70 ppm, but other combinations of offset and sweep width were used to focus onto the aliphatic and aromatic regions of the spectrum. Water suppression was achieved using sensitivity-enhanced gradient coherence selection Marion *et al.*, 1989; Sklenar *et al.*, 1993) and States quadrature detection was employed in the indirect dimension (States *et al.*, 1982). Spectral processing was performed using NMRPipe (Delaglio *et al.*, 1995) and visualised with NMRView (Johnson & Blevins, 1994). Proton chemical shifts were referenced to the HDO signal at 4.773 ppm and 25°C with ^{13}C chemical shifts indirectly referenced from the proton by taking into account the gyromagnetic ratio. NMR was also performed with JEOL JNM-LA400 NMR spectrometer with broadband probe and auto tuning.

2.14 Matrix-Assisted Laser Desorption Ionisation–Time of Flight (MALDI-TOF) Analysis on SGI and SGE

Samples preparation for MALDI experiments involved preparation of 50 mM of Triethylammonium bicarbonate (TEAB) buffer (SIGMA-ALDRICH) and dissolving lyophilised SGI and SGE fractions in 50 µl of TEAB buffer. The 50 µl of TEAB buffer was placed in a spin cartridge and 100 µl of TEAB was added. This was spun six times at

13000 rpm for 3.5 min at room temperature using Galaxy 14 D centrifuge (VWR). This is to ensure complete buffer exchange to TEAB buffer to improve resolution. Mass spectrometry plate reader was prepared by washing with isopropanol, water and methanol and was allowed to dry. The 3 µl of SGI samples was mixed with 3 µl of α-cyano-4-hydroxycinnamic acid (CHCA) matrix, spotted on the microplate reader and allowed to dry. The MALDI micro MX™ (MICROMASS MS TECHNOLOGIES) was calibrated using a mixture of des-Arg-Bradykinin, Angiotensin I, Glu-Firbopeptide B and Neurotensin (Applied Biosystems). The screening of SGI and SGE fractions was made for mass-charge-ratio (m/z) range of 500 – 6000 Da in positive modes. Control used were 10 mM Tris-HCl, pH 8, matrix (α-cyano-4-hydroxycinnamic acid (CHCA)), TEAB buffer, mixture of TEAB buffer and matrix.

CHAPTER 3

DEVELOPMENT OF AN SGE PURIFICATION PROCEDURE

3.1 Introduction

It was shown previously that *E. coli* BP isolated from a clinical sample induced the growth of SRB at low cell density. To study the functional activity of SGE, a procedure for partial purification was designed. The first target was to determine whether ion exchange chromatography can be used for purification and the type of resin suitable. Ion exchange chromatography also known as salt gradient elution is a form of absorptive liquid chromatography that allows for the separation of ionisable molecules based on how they differ in their charge properties (Xu, 2005; Cummins *et al*., 2011; Khan, 2012). If SGE possesses at least one negatively charged group, Q Sepharose Fast Flow™ media can be used for its purification.

3.2 Results

To find out if it is possible to use ion exchange chromatography for purification of the SGE, Q Sepharose Fast Flow™ media was used. The CFS obtained from an overnight culture of *E. coli* BP grown in 100 ml of LB under aerobic condition was loaded on Q Sepharose Fast Flow™ column. Elution was carried out using 3 step gradients of 125, 250 and 500 mM NaCl in 10 mM Tris-HCl, pH 8.0. Five repeat experiments were undertaken, which generated the same chromatogram of elution (Figure 3.1). The partially purified SGE fractions and void volume were tested for activity on the growth of SRB in VMR medium. Growth was examined and recorded as either positive (+) or negative (-), which corresponds to the presence or absence of black colour due to

reaction of the hydrogen sulphide produced by metabolising SRB cells with iron sulphate in the medium to form insoluble black iron sulphide (Muyzer & Stams, 2008; Barton & Fauque, 2009).

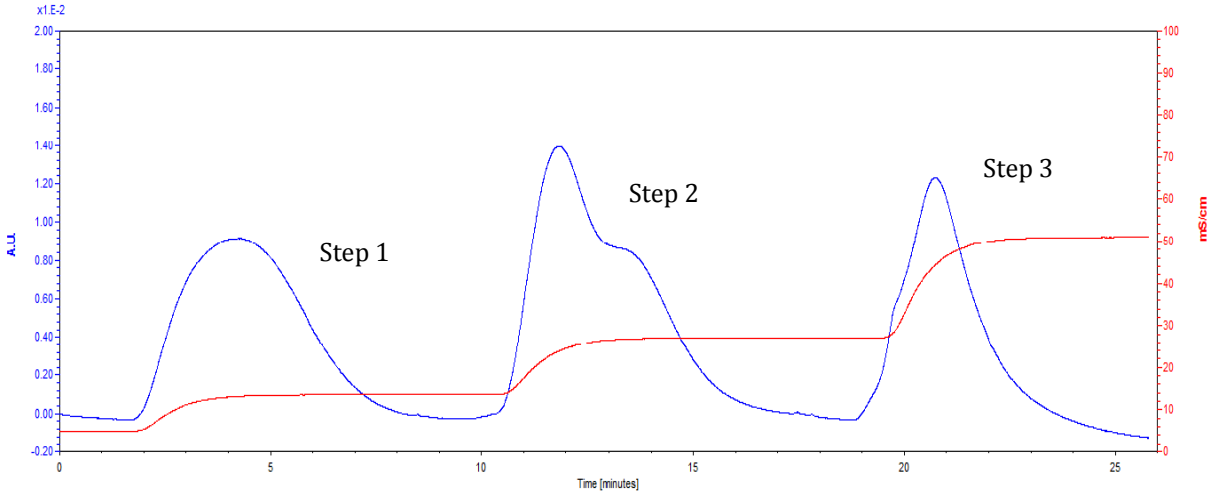


Figure 3.1. Chromatogram of elution of CFS produced by *E. coli* BP culture grown overnight in aerobic condition

Step 1: 125 mM NaCl, Step 2: 250 mM NaCl, step 3: 500 mM NaCl in 10 mM Tris-HCl, pH 8.0. The blue peak is UV peaks measured at 280 nm while the red line indicates conductivity (mS/cm).

The result obtained from testing the chromatographic fractions (SGE) on the growth of *D. indonesiensis* is shown in Table 3.1. The main SGE activity was found in the fraction eluted with 500 mM NaCl, 10 mM Tris-HCl, pH 8 (step 3). There was no activity in the void volume. This revealed that SGE can be purified by ion exchange chromatography using Q Sepharose Fast Flow™. It also revealed that SGE carries a negative charge on it because it binds to the positively charged quaternary amino functional group of the resin.

Table 3.1. The effects of step gradient fractions on SRB growth in VMR medium.
The SGE was purified from 100 ml of CFS obtained from *E. coli* BP grown in aerobic condition and eluted using step gradient: step 1 (125 mM NaCl), step 2 (250 mM) and step 3 (500 mM NaCl) in 10 mM Tris-HCl, pH 8.0. SRB: *D. indonesiensis*; SRB growth (+), no SRB growth (-), SRB initial inoculum size: 3.5 x 10^7 cfu/ml. Void volume: unbound materials. Five independent experiments were done.

	Day 1			**Day 2**				**Day 3**			
	10^{-1}	10^{-2}	10^{-3}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-1}	10^{-2}	10^{-3}	10^{-4}
Untreated SRB	+	+	-	+	+	-	-	+	+	-	-
+Step 1	+	+	-	+	+	-	-	+	+	+	-
+Step 2	+	+	-	+	+	-	-	+	+	+	-
+Step 3	+	+	-	+	+	+	-	+	+	+	+
+Void volume	+	+	-	+	+	-	-	+	+	-	-

3.3 Conclusions

It was determined that SGE possesses at least one negatively charged group and therefore can be purified by ion exchange chromatography using Q Sepharose Fast Flow™ media. The main SGE activity was eluted using 500 mM of NaCl. It was also shown that the amount of purified SGE is sufficient for testing on the growth of SRB.

CHAPTER 4

OPTIMISATION OF GROWTH CONDITIONS FOR THE PRODUCTION OF SGE

4.1 Introduction

The second objective of this study was to optimise the growth conditions for the highest production of SGE by *E. coli* BP. Although good results were obtained when LB medium was used for *E. coli* BP growth, the presence of tryptone and yeast extract in the medium may interfere with purification and structural analysis. Different microbial products have been purified using M9 medium such as L-asparaginase from *Staphylococcus* sp. (Prakasham *et al*., 2007) and n-butanol from *E. coli* (Saini *et al*., 2015). Therefore, medium for culturing *E. coli* BP was changed to M9 minimal medium, which lacks tryptone and yeast extract.

Several authors have reported that different growth conditions can change the level of production of microbial products (Cruz Ramos *et al*., 1995; Nakano *et al*., 1997; Spiro & Guest, 1990; Park & Leppla, 2000; Madigan & Martinko, 2006). *E. coli* BP is a facultative anaerobe, therefore in order to achieve the highest level of SGE production, the different growth conditions were tested, such as growth in aerobic, limited oxygen and anaerobic conditions at 37°C for 6, 12, 18, 24, 48 and 72 hours.

4.2. Results

4.2.1 Growth of *E. coli* BP under various conditions

Each set of *E. coli* BP cultures grown in M9 medium and incubated at 37°C under different condition was serially diluted and inoculated on freshly prepared MacConkey

agar for enumeration. The results presented in Table 4.1 show the count of *E. coli* BP culture grown in 100 ml of M9 medium under aerobic, limited oxygen and anaerobic conditions**.** Cultures that developed were counted and expressed as colony forming unit per ml of sample (cfu/ml). In all the three incubation conditions, the *E. coli* BP culture incubated for 18 h had the highest viable mean counts and 72 h had the least mean count.

For cultures incubated in aerobic conditions, the least mean bacterial count (8.8 x 10^5 cfu/ml) was obtained after 72 h incubation period followed by 48 h incubation period (2.5 x 10^8 cfu/ml), while the highest mean count (1.1 x 10^9 cfu/ml) was obtained in *E. coli* BP culture incubated for 18 h followed by 24 h incubation period (7.3 x10^8 cfu/ml). For *E. coli* BP cultures grown in limited oxygen condition, the highest mean count (7.5 x 10^8 cfu/ml) was obtained in 18 h incubation period closely followed by 24 h incubation with the mean count of 4.0 x10^8 cfu/ml, while the least mean count (1.2 x 10^6 cfu/ml) was obtained after 72 h growth and 48 h incubation period had the mean count of 8.7 x 10^7 cfu/ml. However, for *E. coli* BP cultures incubated in anaerobic condition, the 18 h had the highest mean count (3.5 x 10^8 cfu/ml) while 72 h *E. coli* BP growth had the least mean count of 3.3 x 10^3 cfu/ml (Table 4.1, Figure 4.1).

Table 4.1. Mean count of *E. coli* BP grown in aerobic, limited oxygen and anaerobic conditions

±: standard error of the mean. Cfu/ml: colony forming unit per milliliter

Incubation period (h)	Cell counts (cfu/ml) of *E. coli* BP grown at 37°C under		
	Aerobic condition	**Limited oxygen condition**	**Anaerobic condition**
6	6.0×10^8 ±5773.71	2.8×10^8 ±11547	3.6×10^6 ±230.94
12	7.7×10^8 ±36055.53	3.8×10^8 ±49103.09	7.2×10^7 ±2027.59
18	1.1×10^9 ±88191	7.5×10^8 ±52387.47	3.5×10^8 ±17638.35
24	7.3×10^8 ±15275.26	4.0×10^8 ±54873.62	3.3×10^8 ±17638.35
48	2.5×10^8 ±5773.51	8.7×10^7 ±1452.97	1.9×10^7 ±2962.73
72	8.8×10^5 ±14.53	1.2×10^6 ±57.74	3.3×10^3 ±0.15

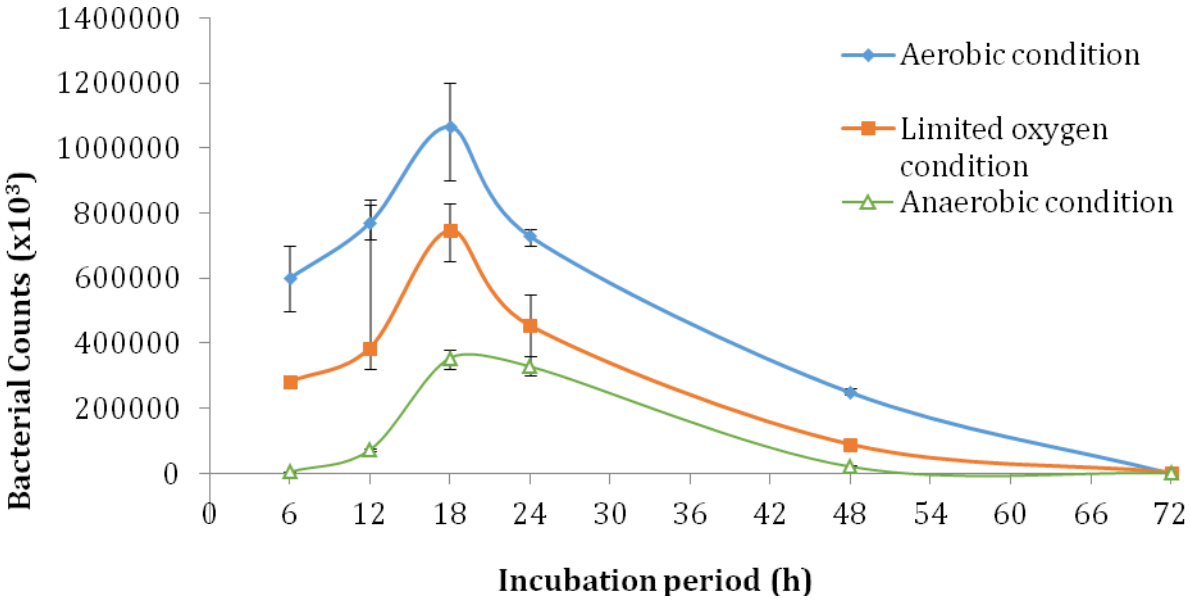


Figure 4.1. *E. coli* BP growth in aerobic, limited oxygen and anaerobic conditions

4.2.2 Results of testing fractions on SRB Growth

It was determined that Q Sepharose Fast Flow™ media can be used for SGE purification (Chapter 3). Therefore, each set of CFS obtained from *E. coli* BP cultures grown at different conditions was partially purified using Q Sepharose Fast Flow™ media. The representation of ten elution profiles of CFS from *E. coli* BP grown at 37°C for 18 h in limited oxygen condition is presented in Figure 4.2. Fractions obtained were tested for activity on the growth of *D. indonensiensis* in VMR medium.

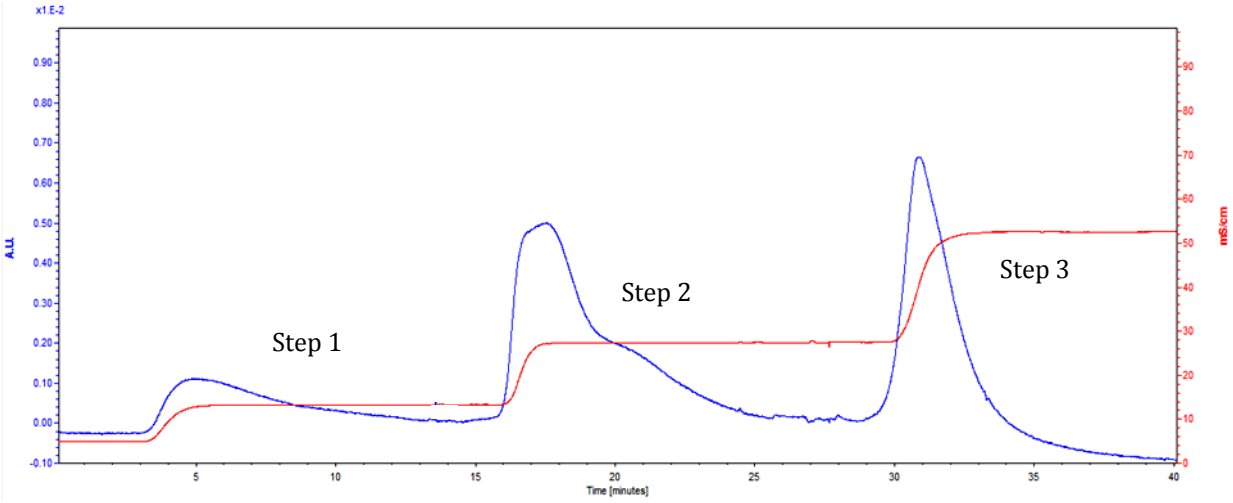


Figure 4.2. Chromatogram of elution of CFS produced by *E. coli* BP grown in limited oxygen conditions
Step 1: 125 mM NaCl, Step 2: 250 mM NaCl, Step 3: 500 mM NaCl in 10 mM Tris-HCl, pH 8.0. The blue peak is UV peaks measured at 280 nm while the red line indicates conductivity (mS/cm).

The results obtained from testing fractions eluted with 125 mM NaCl (step 1), 250 mM NaCl (step 2) and 500 mM NaCl 10 mM Tris-HCl, pH 8 (step 3) on the growth of *D. indonesiensis* is shown in Table 4.2. The representation of SRB growth in VMR medium in the presence of step 3 gradient is shown in Figure 4.3. The control *D. indonesiensis* had growth only in 10^{-1} and 10^{-2} dilution after 1 day. Similarly, there was growth only in 10^{-1} and 10^{-2} dilution after fractions obtained when CFS from *E. coli* BP culture grown in aerobic, limited oxygen and anaerobic conditions for 6, 12, 18, 24, 48 and 72 h were

purified by ion exchange chromatography and eluted with step 1 and step 2 gradients. This shows there was no growth induction of *D. indonesiensis* at day 1 with all the tested fractions from Step 1 and Step 2 gradients. *D. indonesiensis* grown in aerobic and anaerobic condition and treated with fraction eluted with step 3 gradient also had no influence on *D. indonesiensis* growth when compared with the control *D. indonesiensis*. However, for 18 h *E. coli* BP grown in limited oxygen condition and eluted with step 3 gradient, there was growth in 10^{-3} dilution after 1 day. This shows a growth induction of *D. indonesiensis* with one order of magnitude difference when compared with control *D. indonesiensis*. Thus, activities were observed only in *E. coli* BP grown for 18 h in limited oxygen condition and eluted by 500 mM NaCl 10 mM Tris-HCl, pH 8 after 1 day growth. Buffer controls in all the tests did not influence the growth of *D. indonesiensis*.

Table 4.2. The effects of step gradient fractions on SRB growth in VMR medium after 1 day

The fractions were obtained using 3 steps gradient (125, 250 and 500 mM NaCl), (A): aerobic condition, (LO): Limited oxygen condition, (An): Anaerobic condition. SRB: *D. indonesiensis*; SRB growth (+), no SRB growth (-). SRB initial inoculum size: 2.8 x 10^7 cfu/ml. The buffers were used as supplement in control experiment. Several independent experiments were done in triplicate and produced identical results.

	Fraction from Step 1 gradient			**Fraction from Step 2 gradient**			**Fraction from Step 3 gradient**			
	10^{-1}	10^{-2}	10^{-3}	10^{-1}	10^{-2}	10^{-3}	10^{-1}	10^{-2}	10^{-3}	10^{-4}
SRB	+	+	-	+	+	-	+	+	-	-
+ SGE 6 h (A)	+	+	-	+	+	-	+	+	-	-
+ SGE 6 h (LO)	+	+	-	+	+	-	+	+	-	-
+ SGE 6 h (An)	+	+	-	+	+	-	+	+	-	-
+ SGE 12 h (A)	+	+	-	+	+	-	+	+	-	-
+ SGE 12 h (LO)	+	+	-	+	+	-	+	+	-	-
+ SGE 12 h (An)	+	+	-	+	+	-	+	+	-	-
+ SGE 18 h (A)	+	+	-	+	+	-	+	+	-	-
+ SGE 18 h (LO)	+	+	-	+	+	-	+	+	+	-
+ SGE 18 h (An)	+	+	-	+	+	-	+	+	-	-
+ SGE 24 h (A)	+	+	-	+	+	-	+	+	-	-
+ SGE 24 h (LO)	+	+	-	+	+	-	+	+	-	-
+ SGE 24 h (An)	+	+	-	+	+	-	+	+	-	-
+ SGE 48 h (A)	+	+	-	+	+	-	+	+	-	-
+ SGE 48 h (LO)	+	+	-	+	+	-	+	+	-	-
+ SGE 48 h (An)	+	+	-	+	+	-	+	+	-	-
+ SGE 72 h (A)	+	+	-	+	+	-	+	+	-	-
+ SGE 72 h (LO)	+	+	-	+	+	-	+	+	-	-
+ SGE 72 h (An)	+	+	-	+	+	-	+	+	-	-
+ Step 1 Buffer	+	+	-	+	+	-	+	+	-	-
+ Step 2 Buffer	+	+	-	+	+	-	+	+	-	-
+ Step 3 Buffer	+	+	-	+	+	-	+	+	-	-

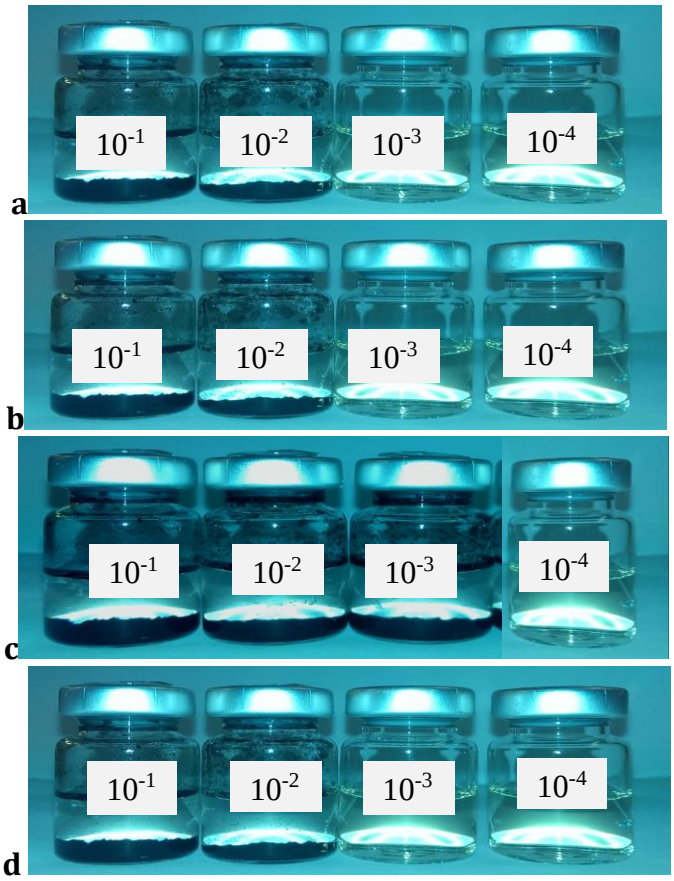


Figure 4.3: 1 day growth of *D. indonesiensis* in VMR medium with and without the presence of step 3 gradient fractions

(a) Growth of control *D. indonesiensis*. (b) Growth of *D. indonesiensis* with Step 3 gradient fraction when *E. coli* BP culture was grown in aerobic condition for 18 h (c) Growth of *D. indonesiensis* with Step 3 gradient fraction when *E. coli* BP culture was grown in limited oxygen condition for 18 h (d) Growth of *D. indonesiensis* with Step 3 gradient fraction when *E. coli* BP culture was grown in anaerobic condition for 18 h. The black colour at the base of the vials indicates *D. indonesiensis* growth.

4.3 Conclusions

The optimum conditions for the highest production of SGE is growth of *E. coli* BP for 18 h in limited oxygen condition. There is a correlation between the counts of *E. coli* BP and the production of SGE, thus the highest count of *E. coli* BP correlates with the highest induction of *D. indonensiensis* growth. This indicates that the level of SGE produced is dependent on the quantity of *E. coli* BP.

CHAPTER 5

PREPARATIVE PURIFICATION OF SGE

5.1 Introduction

The optimum growth conditions obtained for the production of SGE described in Chapter 4 were used for the preparative purification of the enhancer. This involves the growth of *E. coli* BP culture in limited oxygen condition at 37°C for 18 h and elution using 3 step gradients of NaCl in 10 mM Tris-HCl, pH 8.0. The preparative purification of the compounds is essential to obtain sufficient quantity for further testing and analysis.

Many approaches that have been employed for analytical and small scale purification of SGE, such as ion exchange chromatography, gel filtration and HPLC, have also been used for large scale purification (Hagel, 2001; Cummins *et al*., 2011; Szymanowska-Powałowska *et al*., 2013). In order to obtain an improved resolution and visible spectra or peaks, fractions are often concentrated. Methods used for concentration include ion exchange chromatography with one step gradient and lyophilisation. After concentration by lyophilisation, samples are dissolved in a preferred quantity of buffer for further purification.

5.2 Results

It was challenging to estimate the quantity of CFS sufficient for the laboratory preparative scale purification of SGE by ion exchange chromatography. Q Sepharose Fast Flow™ media has a binding capacity of 120 mg HSA/ml (GE Healthcare, 2014). The total protein content in 1 L of CFS from *E. coli* BP was 147 mg. 20 ml of resin should have been sufficient for purification of proteins present in the 10 L CFS, but 100 ml of resin was used because bacteria produce a number of biomolecules that are negatively

charged and will compete with the positively charged amino group of the resins. This is supported by findings from Cooper (2000) who stated that bacterial cells are made up of negatively charged macromolecules including nucleic acids, lipids, carbohydrates, polysaccharides, lipopolysacharides, glycoproteins and proteins that constitute 80 to 90 % of the cell constituents. Cooper (2000) further explained that the macromolecules contain thousands of low-molecular-weight precursors such as fatty acids and some amino acids, which also will compete with the positively charged amino group of the resins. Therefore, various scale up volumes including 5, 7, 10, 15 and 20 L of CFS from *E. coli* BP cultures were tested and finally 10 L of CFS was selected for the large scale purification of SGE without overloading the column.

The first stage of purification was carried out by ion exchange chromatography. The bound materials in the column were eluted by step gradients. It was carried out using 125 (step 1), 250 (step 2) and 500 mM NaCl (step 3) in 10 mM Tris-HCl, pH 8 as described in Section 2.5. The representation of twenty five different elution profiles is shown in Figure 5.1. The step gradients of 125 and 250 mM NaCl were used to remove main quantity of the high molecular weight compounds such as DNA, protein, polysaccharides and glycoproteins present in the CFS. Fraction of step 3 gradient (~ 100 ml) was used for further purification by gel filtration.

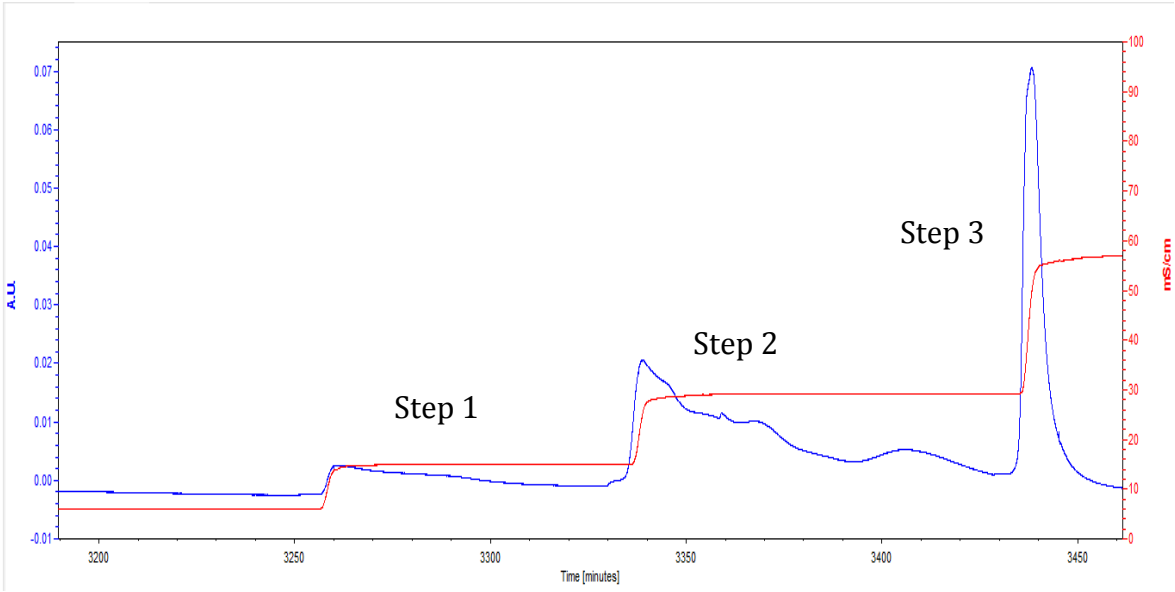


Figure 5.1. Chromatogram of elution of CFS from *E. coli* BP using step gradients
Step 1: 125 mM NaCl in 10 M Tris-HCl, pH 8, Step 2: 250 mM NaCl in 10 mM Tris- HCl, pH 8, step 3: 500 mM NaCl in 10 mM Tris-HCl, pH 8.0. The blue line is UV (A.U.) absorbance measured at 280 nm while the red line indicates conductivity (mS/cm).

In order to use gel filtration efficiently, the volume of loading fraction for this size of column should not be more than 3 ml. To achieve this, the ~ 100 ml of fraction was concentrated to ~ 3 ml as described in Section 2.6. The representation of the chromatogram of elution is shown in Figure 5.2.

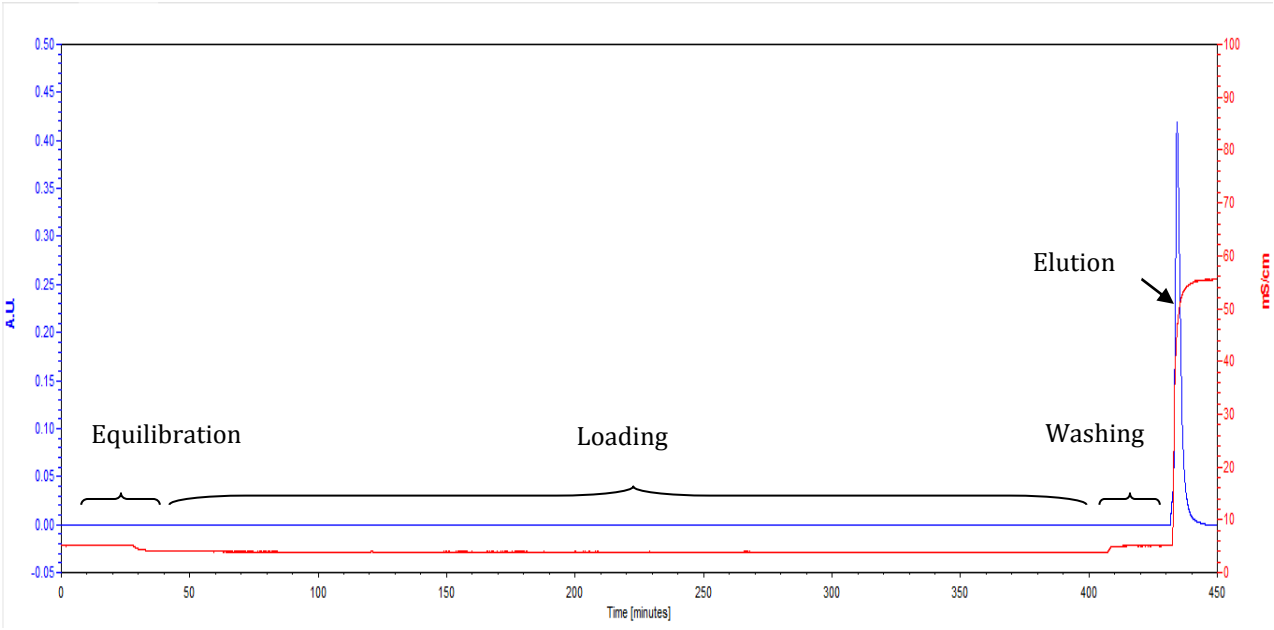


Figure 5.2. Chromatogram of elution of step 3 fraction
The 100 ml of step 3 fraction was diluted and loaded on 3 ml of Q Sepharose Fast Flow™. The blue line is UV absorbance measured at 280 nm, the red line indicates conductivity (mS/cm).

Toyopearl (HW-40S) resins (TOSOH Bioscience LLC) with a fractionation range of 100 - 7000 Da was used for gel filtration of the Q Sepharose fraction. This range was used because the features of SGE observed in Chapter 4 indicate that it is a secondary metabolite and most secondary metabolites are lower than 3000 Da (Demain, 1998; Ruiz *et al.*, 2010; Agostini-Costa *et al.*, 2012; Sugar *et al.*, 2014). The column of HW-40S resin was calibrated with blue dextran (2,000,000 Da) and vitamin B12 (1,355 Da). The representation of the chromatogram of the two standards on HW-40S column after ten repeat experiments is shown in Figure 5.3.

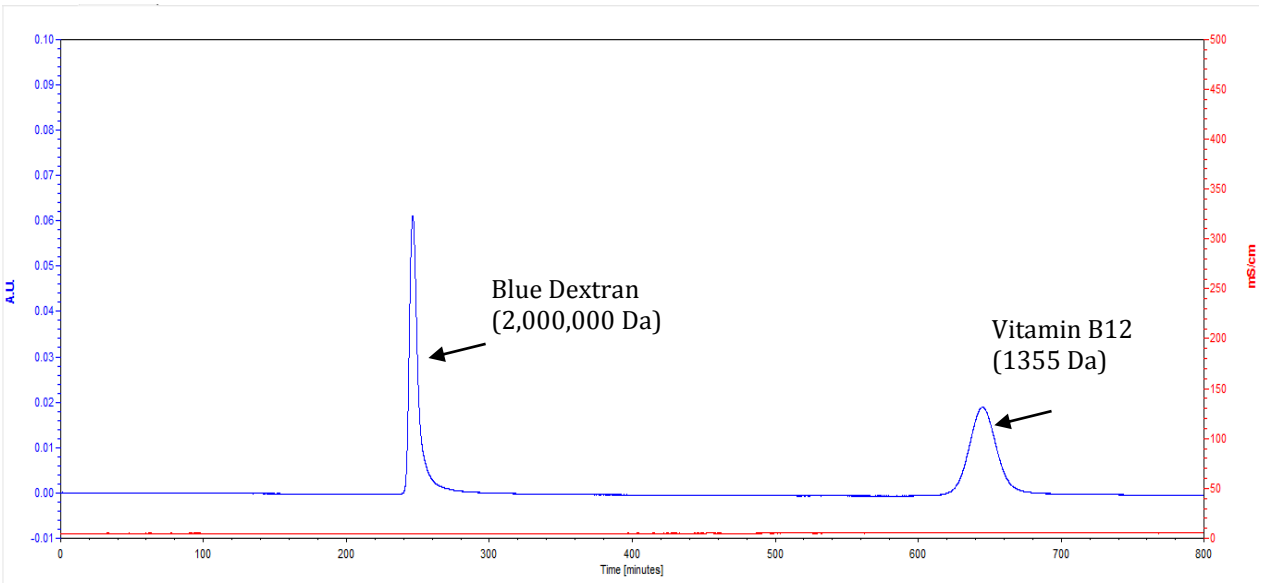


Figure 5.3. Calibration of HW-40S column with Blue Dextran and vitamin B12
The blue line on the chromatogram shows UV absorbance measured at 280 nm, the red line indicates conductivity (mS/cm). Column: 2.5 x 75 cm with cross-sectional area 4.91 cm^2

The aim of the first gel filtration was to remove compounds that were either greater than 7000 Da or below 100 Da in mass, and thus provide a more efficient purification in subsequent gel filtration run. The void volume was tested for activities using *D. indonensiensis* cultures. No activity was detected. The fractions without the void volume (~ 30 ml) were then pooled and concentrated to ~ 1 ml using Q Sepharose Fast Flow™ as described in Section 2.6. The concentrated fraction was applied to the HW-40S column. During gel filtration, two distinct peaks were detected (Figure 5.4). At least seventy-five repeat experiments were done and generated the same results as shown in Figure 5.4. In order to estimate the molecular size of the compounds in these peaks, the position of vitamin B12 (1355 Da) and Blue Dextran (2,000,000 Da) were compared with the position of the peaks on the gel filtration chromatogram. The position of vitamin B12 and blue dextran eluted is shown on the gel filtration chromatogram of Figure 5.4. The peaks 1 and 2 were eluted before vitamin B12, therefore, the molecular mass of the peak 1 and 2 compouds is higher than 1355 Da.

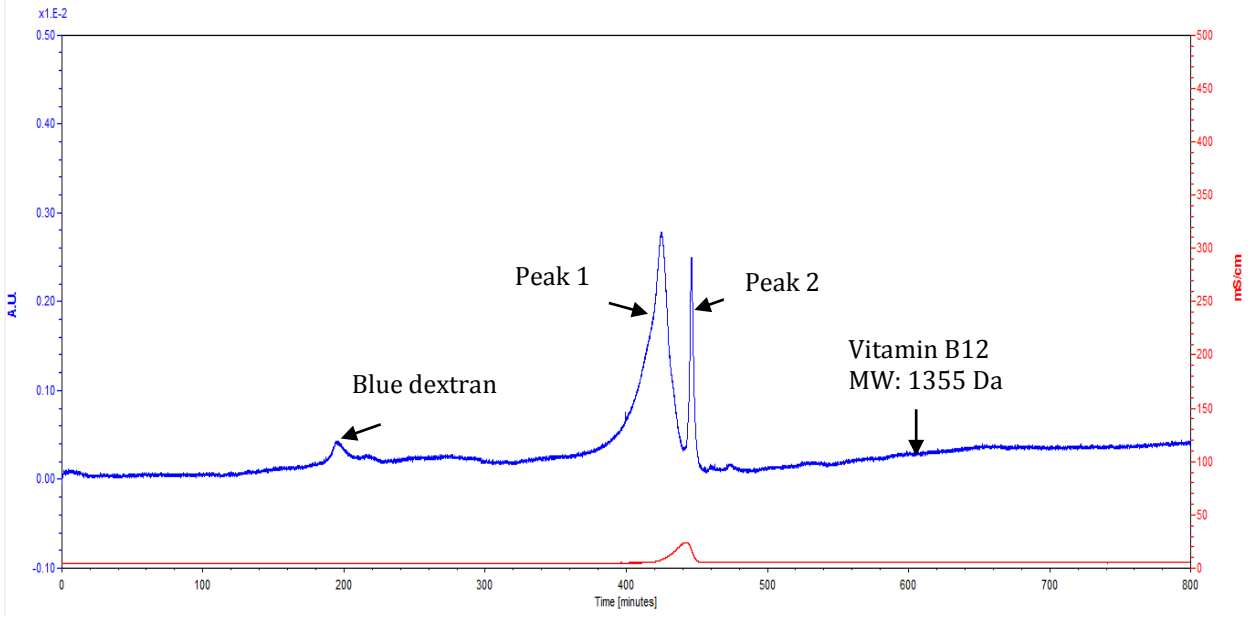


Figure 5.4. Chromatogram of concentrated step 3 fraction on HW-40S column
Peak 1 is subsequently referred to SGI and peak 2, SGE. The blue line on the chromatogram is UV absorbance measured at 280 nm, the red line indicates conductivity (mS/cm). Multiple experiments were done.

5.2.1 Results of testing fractions from gel filtration on SRB growth

The results of testing fractions from gel filtration on SRB growth showed that compounds present in the peak 2 had inducing effect, while peak 1 exhibit slight inhibitory effect on the growth of SRB strains (Table 5.1). When serially diluted cultures of each SRB strains (*D. indonensiensis*, *D. vulgaris* and *D. alaskensis*) in VMR medium were tested with peak 2 fraction, growth was observed up to 10^{-4} dilution after two days of incubation (Table 5.1, Figure 5.5 b) whereas untreated SRB culture had growth only up to 10^{-2} dilutions (Table 5.1, Figure 5.5 a). This is a two order of magnitude difference between growth of untreated SRB culture and SRB treated with peak 2 fraction.

Table 5.1. SRB growth in VMR medium in the presence and absence of peak 2 fraction (+) SRB growth, (-) no SRB growth. Several independent experiments were done in triplicate. SRB: *D. indonensiensis*, *D. vulgaris* and *D. alaskensis*. SRB initial inoculum size: 1.0 - 2.5 x 10^7 cfu/ml.

	Day 1			**Day 2**				**Day 3**			
	10^{-1}	10^{-2}	10^{-3}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-1}	10^{-2}	10^{-3}	10^{-4}
SRB	+	+	-	+	+	-	-	+	+	-	-
+ Peak 2 fraction	+	+	+	+	+	+	+	+	+	+	+
+ Peak 1 fraction	+	-	-	+	-	-	-	+	±	-	-

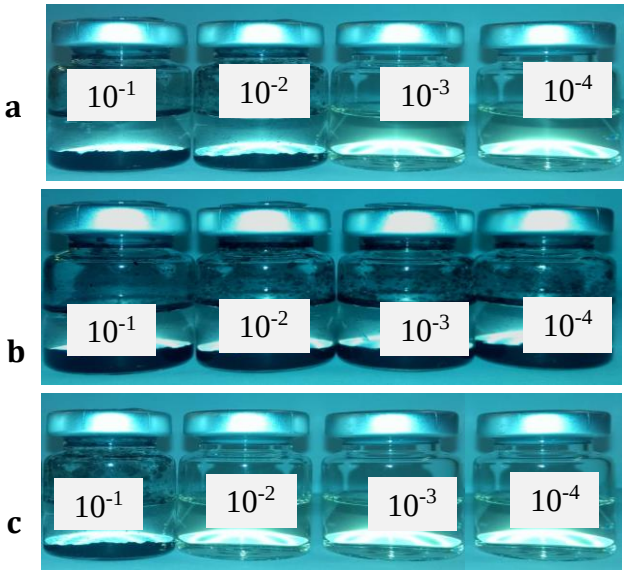


Figure 5.5. 2 days growth of SRB in the presence and absence of peak 2 fraction (a) Growth of control SRB, (b) Growth of SRB with peak 2 fraction, (c) Growth of SRB with peak 1 fraction. The black precipitate at the base of the vials indicates SRB growth.

To optimise bioactivity test of peak 1 fraction for inhibitory effects on SRB growth, VMI medium was used. The VMI medium contains 500 mg/l of $FeSO_4.7H_2O$, which is optimum for SRB growth. The idea here was to check if the medium will show a better inhibition of SRB growth by peak 1 fraction. The results of testing peak 1 fraction on SRB growth are shown in Table 5.2 and Figure 5.6. It revealed that SRB strains tested with peak 1 fraction had growth up to 10^{-2} dilution after 3 days incubation (Table 5.2,

Figure 5.6 b) when compared with untreated SRB that had growth up to 10^{-4} (Table 5.2, Figure 5.6 a). This shows a two order of magnitude growth inhibition of each SRB strain by the peak 1 fraction, when compared with untreated SRB.

Thus, the peak 2 fraction induces the growth of the SRB while the peak 1 fraction inhibits their growth and are referred to as SGE and SRB growth inhibitor (SGI) respectively.

Table 5.2. SRB growth in VMI medium the presence and absence of peak 1 fraction. (+) SRB growth, (-) no SRB growth. Several independent experiments were done in triplicate. SRB: *D. indonensiensis*, *D. vulgaris* and *D. alaskensis*. SRB initial inoculum size: 1.0 - 3.5 x 10^7 - cfu/ml.

	Day 1			**Day 2**				**Day 3**			
	10^{-1}	10^{-2}	10^{-3}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-1}	10^{-2}	10^{-3}	10^{-4}
SRB	+	+	-	+	+	+	-	+	+	+	+
+ SGI	+	+	-	+	+	-	-	+	+	-	-

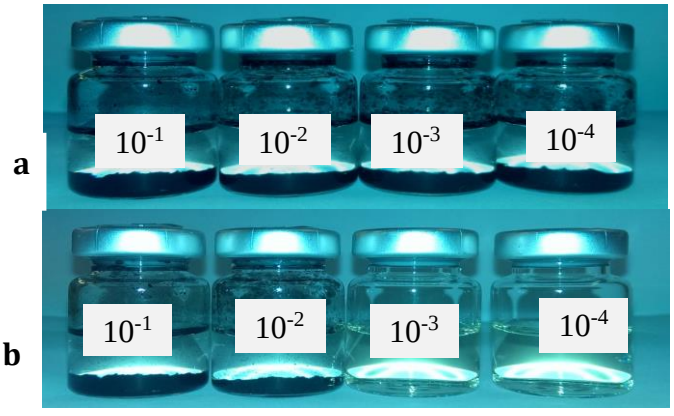


Figure 5.6. 3 days growth of SRB in VMI medium in the presence and absence of SGI (a) Growth of control SRB, (b) Growth of SRB with SGI. The black precipitate at the base of the vials indicates SRB growth.

5.3 Conclusions

The large scale purification of SGE was considered. It was also observed that the *E. coli* BP produced a compound that inhibits SRB growth and is referred to as SGI. Both SGE and SGI have molecular weight greater than 1355 Da.

CHAPTER 6

CHARACTERISATION OF SGE AND SGI: NOVEL VERSUS ESTABLISHED COMPOUNDS

6.1. Introduction

The peaks obtained after purification of CFS from *E. coli* BP in Chapter 5 showed that fractions from peak 2 (SGE) had inducing effects on the growth of SRB whereas the fraction from peak 1 (SGI) had inhibitory effect on their growth. Certain microorganisms have been reported to secrete compounds that have effects on the microorganisms that produce them, for example *Hafnia alvei* produces ferrioxamine G (672 Da) and ferrioxamine E (653.53 Da) that induce the growth of the parent organism as well as other bacteria (Reissbrodt *et al.,* 1990; Gledhill & Buck, 2012). *N*-acyl homoserine lactone (101.10 Da) a class of autoinducers generally involved in bacterial quorum sensing has been found in *E. coli* (Barrios & Achenie, 2010; Rumbaugh & Kaufmann, 2012). Freestone *et al*. (2003) also reported an autoinducer purified from *E. coli* with a molecular mass of 500 Da, which was shown to promote the growth of other bacteria such as *Enterobacter cloacae, Klebsiella pneumonia, Morganella morganii, Proteus mirabilis, Serratia marcescens* and *Staphylococcus albus.*

Some compounds have also been secreted and exported by microorganisms in iron deficient media and are called siderophores. They have low-molecular-weight (less than 2000 Da) (Brandel *et al*., 2012; Ahmed & Holmstroin, 2014; Dux *et al*., 2014). The most aerobic and facultative anaerobic bacteria produce at least one siderophore (Neilands,

1995; Ali & Vidhale, 2013). A hydrophobic siderophore known as enterobactin (669.55 Da) is produced by *E. coli* (Neilands, 1993). *Pseudomonas aeruginosa* produces pyoverdin (1365.42 Da) and pseudobactin (1353 Da) (Mohandass, 2004).

The M9 medium used to culture *E. coli* BP is also devoid of iron (Section 2.1.2.2). However, the molecular weight of a reported siderophore produced by *E. coli* is less than 1000 Da. In this Chapter, an attempt was made to confirm that the molecular weight of the SGE and SGI is higher than 1000 Da and, therefore, not the known autoinducers and siderophore produced by *E. coli*.

6.2 Results

To investigate if SGE and SGI have influence on the growth of *E. coli* BP, a fresh culture of *E. coli* BP with initial inoculum size of 2.8×10^8 cfu/ml was inoculated in M9 medium under limited oxygen condition and tested with SGE and SGI as described in Section 2.9. This was done in order to find out if the compounds are known autoinducers produced by *E. coli*. The total viable count of *E. coli* BP was determined daily for three days as described in Section 2.3.2. The results obtained showed there was no influence of both the SGE and SGI on the counts of *E. coli* BP when compared with the count of control cultures of *E. coli* BP (Table 6.1).

Table 6.1. Counts of *E. coli* BP in the presence and absence of SGE and SGI.
SGE: SRB growth enhancer, SGI: SRB growth inhibitor, cfu/ml: colony forming unit per milliliter. Five independent experiment was done.

	Day 1 (cfu/ml)	**Day 2 (cfu/ml)**	**Day 3 (cfu/ml)**
***E. coli* BP**	2.5×10^8	3.0×10^7	3.5×10^6
+ SGE	2.4×10^8	2.8×10^7	3.0×10^6

+ SGI	2.0 x 10^8	2.5 x 10^7	2.5 x 10^6

From the chromatogram of gel filtration of step 3 fraction (Section 5.2), the molecular weight of SGE and SGI is estimated to be above 1355 Da because it is eluted before vitamin B12 with molecular weight of 1355 Da. To exclude the possibility that well known siderophores and autoinducer of *E. coli* are present in the gel filtration fraction, SGI and SGE fractions were dialysed against dH_2O using 1000 Da MWCO membrane (Section 2.8). The dialysed fractions were sterilised by filtration and de-oxygenated to provide anaerobic condition and the activities were tested on the growth of *D. indonensiensis* as described in Section 2.7. The results revealed that there was growth induction of *D. indonensiensis* with SGE (Table 6.2; Figure 6.1) and growth inhibition with SGI (Table 6.3; Figure 6.2) as previously observed in non-dialysed fractions (Section 5.2). This suggests that both SGI and SGE are not significantly smaller than 1000 Da.

Table 6.2. SRB growth in VMR medium in the presence and absence of dialysed SGE SRB growth (+), no SRB growth (-). Several independent experiments were done in triplicate. SRB: *D. indonensiensis.* SRB initial inoculum size: 2.4 x 10^7 cfu/ml

	Day 1			**Day 2**				**Day 3**			
	10^{-1}	10^{-2}	10^{-3}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-1}	10^{-2}	10^{-3}	10^{-4}
SRB	+	+	-	+	+	-	-	+	+	-	-
+ SGE	+	+	-	+	+	+	+	+	+	+	+

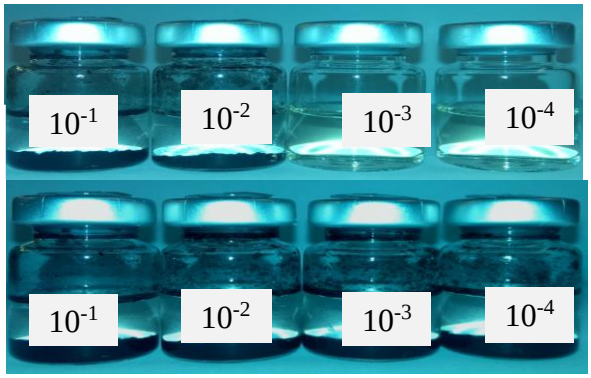


Figure 6.1. Growth of SRB in VMR medium in the presence and absence of dialysed SGE (a) Growth of untreated SRB, (b) Growth of SRB with dialysed SGE. The black precipitate at the base of the vials indicates SRB growth.

Table 6.3. SRB growth in VMI medium in the presence and absence of dialysed SGI. SRB growth (+), no SRB growth (-). Several independent experiments were done in triplicate. SRB: *D. indonensiensis.* SRB initial inoculum size: 1.2 x 10^7 cfu/ml

	Day 1			**Day 2**				**Day 3**			
	10^{-1}	10^{-2}	10^{-3}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-1}	10^{-2}	10^{-3}	10^{-4}
SRB	+	+	-	+	+	+	-	+	+	+	+
+ SGI	+	+	-	+	+	-	-	+	+	-	-

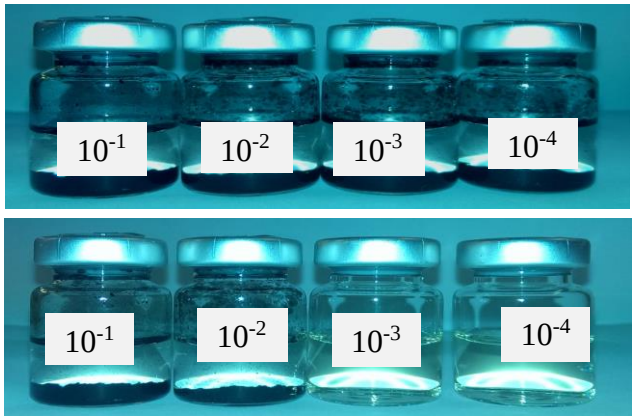


Figure 6.2. Growth of SRB in VMI medium in the presence and absence of dialysed SGI (a) Growth of untreated SRB, (b) Growth of SRB with dialysed SGI. The black precipitate at the base of the vials indicates SRB growth.

6.3. Conclusions

The SGE and SGI induce and inhibit SRB growth respectively. The results obtained do not suggest that SGE is an autoinducer or a known siderophore produced by *E. coli* as the molecular weight is greater than the molecular weight of any known siderophore and autoinducer produced by *E. coli.* Also the SGI has not been documented in literature as a compound produced by *E. coli.*

CHAPTER 7

MICROSCOPIC STUDIES OF SRB GROWTH IN THE PRESENCE OF SGE AND SGI

7.1 Introduction

Microscopy techniques provide information about the form and structure of microbial cells, their biofilms or physiological changes due to the presence of exogenous substances. This involves the use of light microscopy, scanning electron microscopy (SEM), atomic force microscopy (AFM) or confocal laser scanning microscopy (CLSM) (Beech *et al*., 1999; Kjeelerup *et al*., 2005; Videla & Herrera, 2005; Little *et al*., 2006).

SEM in particular provides valuable information regarding the cells distribution within the biofilms and helps to study the structure of biological samples (Gubner & Beech, 1999). In addition, SEM helps to visualise the composition, distribution and biofilms development and how they relate to the substratum (El Abed *et al*., 2012). SEM has also been used to study adhesion in formation of biofilm. It provides a high resolution and plane images of biological specimens with its large depth of field (Kirk *et al*., 2009) and uses a beam of electrons to create an image of a sample and can allow imaging and if necessary quantification of surfaces (El Abed *et al*., 2012). The AFM offered a three-dimensional surface topography of the image in a single scan and thereby enabled more detailed information of roughness, physical properties and surface area variations of the bacterial cells due to differences in deposition parameters (Russell & Batchelor, 2001). Likewise the CLSM has been explored to view structural features of biofilms. CLSM enables non-invasive, optical sectioning of the biofilm structure by the provision of three dimensional images (Adair *et al*., 2000; McLean *et al*., 2008). To differentiate dead

from live cells, staining with SYTOX 9 and propidium iodide have been used. The live cells retain the green colour of SYTOX 9 while the dead cells are stained red due to propidium iodide. SYTOX 9 and propidium iodide stains have been used for bacterial nonspecific viability stains in confocal study because of the ability to disperse passively through cell membranes (Peyyala *et al*., 2011).

There are limited reports available on the deformity of SRB cells due to microbial metabolites. However, Korenblum *et al.* (2005) stated that the surfactin-like molecule produced by *Bacillus* sp. exhibited antimicrobial activities against *D. alaskensis* NCIMB 13491. Transmission electron micrograph of the treated cells revealed damaged cell membranes compared with untreated cells.

The study of the effects of SGI and SGE on SRB growth was carried out using SEM, AFM, and CLSM. The aim of this section is to utilise microscopy to examine the impact of SGI and SGE on the morphology and integrity of the SRB cultures. Although, cell counts are not the best method used because of the morphological change in growth. Biomass might be better. However, the use of biomass also has some draw backs (which include does not exclude cell debris) so it was decided to move onto looking at cell viability. This is to substantiate the bioassay results obtained when SGI and SGE were tested on serially diluted cultures of *D. indonensiensis*, *D. alaskensis* and *D. vulgaris* (Chapter 5).

7.2 Results

7.2.1 The effects of SGI and SGE on SRB growth using SEM

The representation of images obtained when cultures of *D. indonensiensis* in VMR medium was supplemented with SGI and viewed using SEM is shown in Figure 7.1. SGI fraction with no addition of bacterial cells is in Figure 7.1 a. There were no major

changes observed between the morphology of cells, and biofilm formation of untreated (Figure 7.1 b) and SGI-treated SRB cells (Figure 7.1 c) after 1 day of growth. After 4 days of growth, the SGI treated cells showed deformity in morphology and stunted growth due to their interaction with SGI (Figure 7.1 e) but untreated cells appeared normal (Figure 7.1 d). This suggests that the SGI had a negative impact on the morphology and integrity of the SRB cultures that may eventually lead to the impairment of their cellular functions resulting in the cell death. At the 7th day of incubation, the untreated *D. indonensiensis* cells appeared normal (Figure 7.1 f) but the SGI treated cells showed a pronounced deformity, making the negative impact of SGI on *D. indonensiensis* cultures more obvious (Figure 7.1 g). In this case, the integrity of the cell was compromised and the cell content released due to the interaction of SRBs with the inhibitor. Meanwhile, the cell deformity, bulge and elliptical changes observed in SGI treated SRB cells are similar to reports obtained when Mn^{2+} and Cd^{2+} were incubated with cultures of SRB strain *Salmonella daqing,* isolated from Daqing oil-field (Liu *et al.*, 2011). Similarly, Mishra & Malik (2013) reported changes in the physiology and morphology of bacterial cells when exposed to heavy metal.

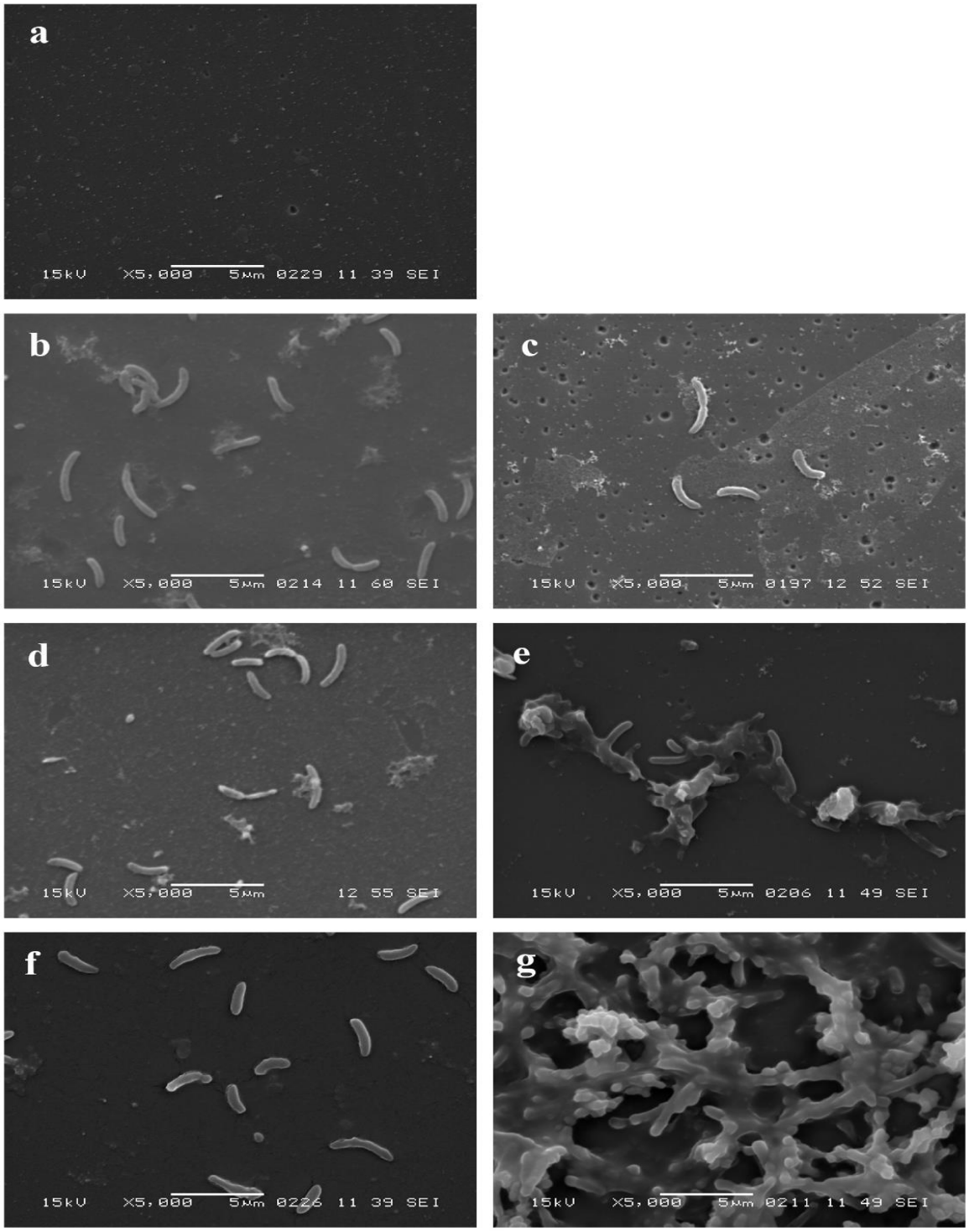


Figure 7.1. Scanning electron micrographs of SRB treated with SGI
(a) SGI fraction only, (b) untreated *D. indonensiensis* after 1 day of growth, (c) *D. indonensiensis* treated with SGI after 1 day of growth, (d) untreated *D. indonensiensis* after 4 days of growth, (e) *D. indonensiensis* treated with SGI after 4 days of growth, (f.) untreated *D. indonensiensis* after 7 days of growth, (g) *D. indonensiensis* treated with SGI after 7 days of growth. The same SRB inoculum size of 2.5 x10^7 cfu/ml was used for the experiment.

The representation of scanning electron micrographs obtained when cultures of *D. indonensiensis* in VMR medium was supplemented with SGE and viewed using SEM (Figure 7.2). The micrograph of SGE fraction incubated without SRB is presented in Figure 7.2 a. *D. indonensiensis* supplemented with SGE showed increased cell numbers and enhanced growth at each incubation period due to the presence of SGE (Figure 7.2 c, e and g) when compared to the control (Figure 7.2 b, d and f). Thus, the SGE induced the growth rate of the SRB cultures perhaps due to a reduction in time for their cell division. After 4 days of incubation, there was a notable interaction between the SGE fractions and the *D. indonensiensis* culture, which resulted in the growth induction of the SRB (Figure 7.2 e) unlike the untreated cultures (Figure 7.2 d). After 7 days incubation of *D. indonensiensis* with SGE in Figure 7.2 g, the EPS matrix encapsulates the SRB cells making them appear much healthier compared to the control cultures (7.2 f). According to Liu *et al.* (2011), the cell membranes of bacteria are semi permeable and this may allow the inflow of the growth promoter thereby enriching the cells and encouraging biofilm formation. The more abundant viable cells observed with SGE correlates to the more rapid growth observed in liquid medium of the bioassay result (Chapter 5).

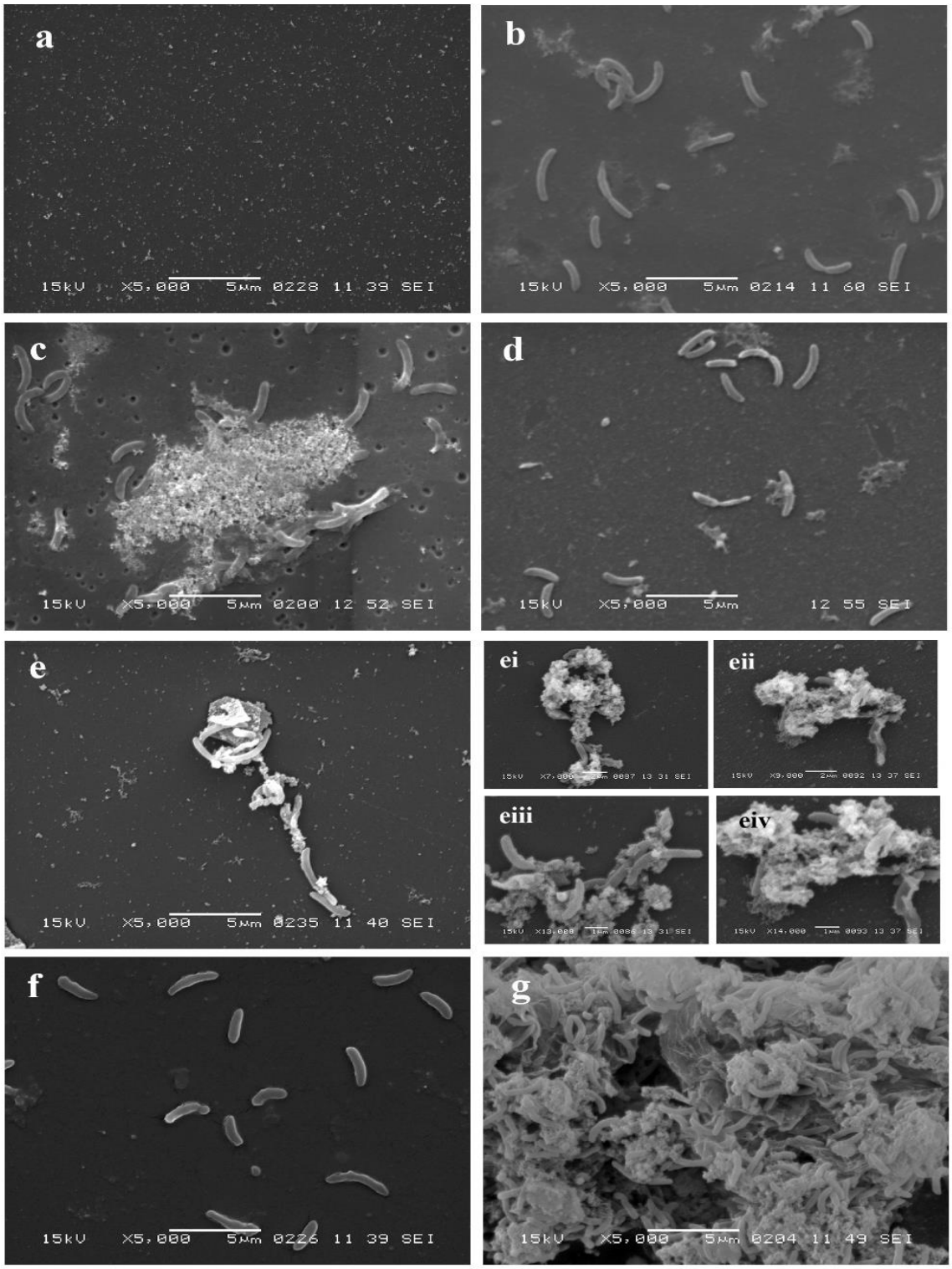


Figure 7.2. Scanning electron micrographs of SRB treated with SGE
(a) SGE fraction only, (b) untreated *D. indonensiensis* after 1 day of growth, (c) *D. indonensiensis* treated with SGE after 1 day of growth (d) untreated *D. indonensiensis* after 4 days of growth (e) *D. indonensiensis* treated with SGE after 4 days of growth; (ei) x7000, (eii) x9000, (eiii) x13000, (eiv) x14000, (f.) Untreated *D. indonensiensis* after 7 days of growth, (g) *D. indonensiensis* treated with SGE after 7 days of growth. The same SRB inoculum size of 2.5 x10^7 cfu/ml was used for the experiment

7.2.2 The effects of SGI and SGE on SRB growth using AFM

The representation of atomic force micrographs of *D. indonensiensis* grown for 7 days with SGI and SGE are presented in Figure 7.3. *D. indonensiensis* treated with SGI had deformed cells (Figure 7.3 b). In contrast, *D. indonensiensis* treated with SGE had increased number of bacterial cells and enhanced growth (Figure 7.3 c) while the growth of untreated *D. indonensiensis* appeared normal (Figure 7.3 a).

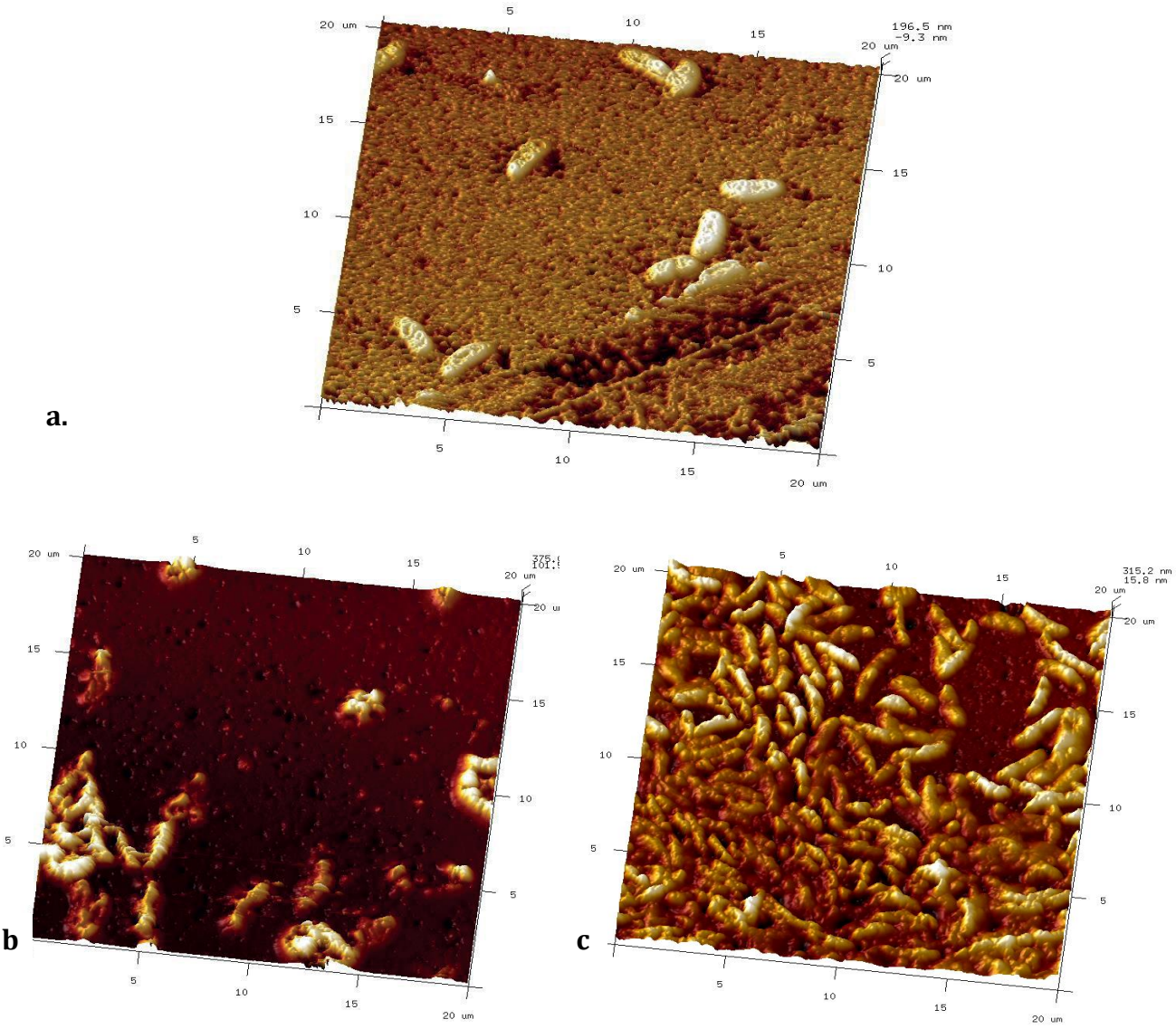


Figure 7.3. The atomic force micrographs of treated and untreated SRB
(a) Untreated *D. indonensiensis* after 7 days of growth, (b) *D. indonensiensis* treated with SGI after 7 days of growth, (c) *D. indonensiensis* treated with SGE after 7 days of growth. The same SRB inoculum size of 3.5 x10^7 cfu/ml was used for the experiment

7.2.3 The effects of SGI and SGE on SRB growth using CLSM

To further confirm the effects of SGI and SGE on the growth of *D. indonesiensis* and *D. vulgaris*, the SRB cultures were stained with live/dead bacterial viability kit and viewed with confocal laser scanning microscope. The results obtained revealed a greater number of *D. indonensiensis* were stained red, which indicates dead cells due to the presence of SGI after 7 days of growth (Figure 7.4 b) compared with untreated cells (Figure 7.4 a). This is similar to the observations with SGI treated *D. vulgaris* (Figure 7.4 e, f). After a prolonged incubation period (37 days), significant proportion of *D. indonensiensis* was dead (Figure 7.4 d) when compared with the control (Figure 7.4 c). In contrast, the treatment of *D. indonensiensis* and *D. vulgaris* with SGE showed more viable cells and the number of viable cells is more abundant at 7 days compared to the controls (Figure 7.5 a-f).

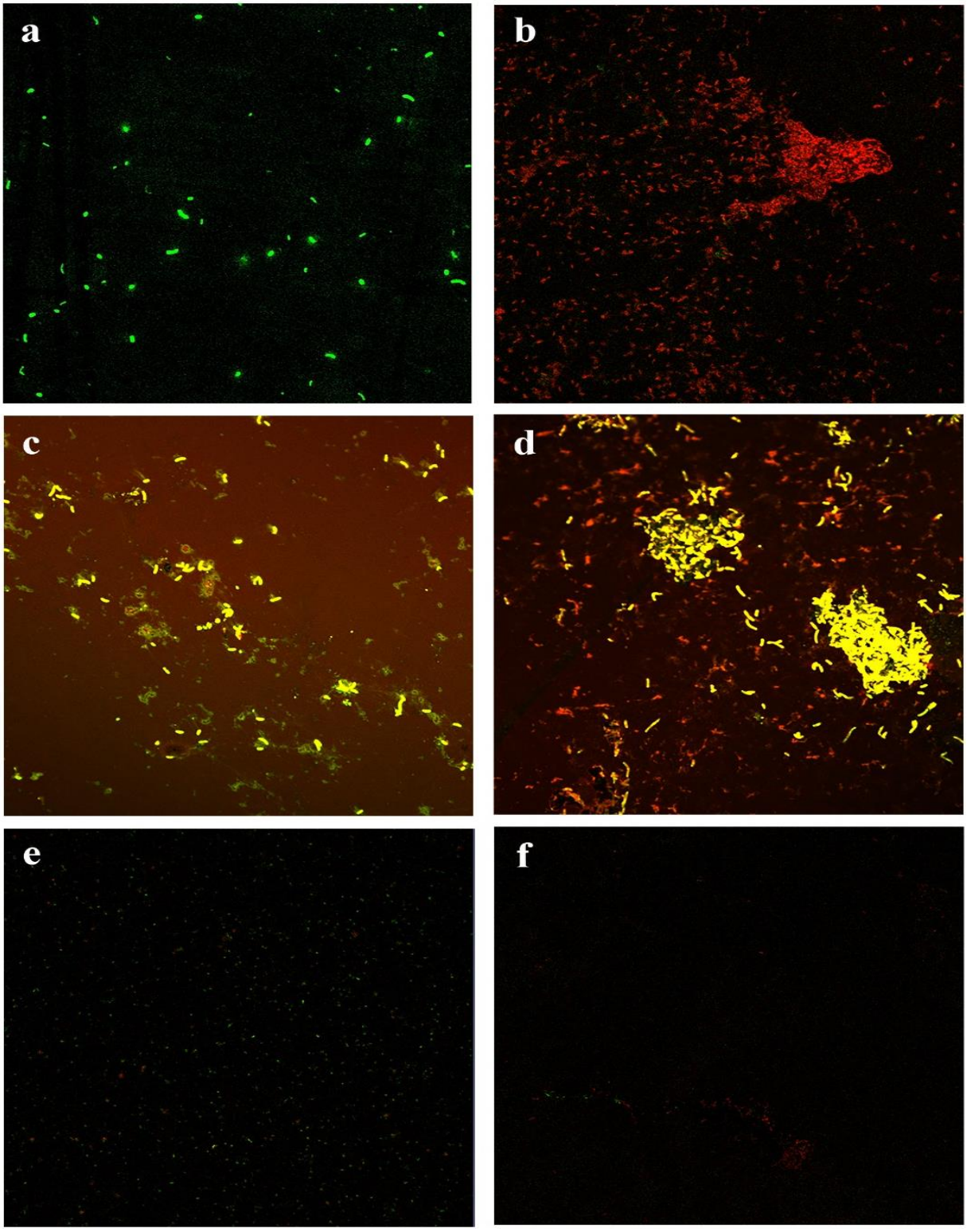


Figure 7.4. Confocal laser scanning micrographs of SRB treated with SGI (a) Untreated *D. indonensiensis* after 7 days of growth, (b) *D. indonensiensis* treated with SGI after 7 days of growth, (c) untreated *D. indonensiensis* after 37 days of growth, (d) *D. indonensiensis* treated with SGI after 37 days of growth, (e) untreated *D. vulgaris* after 7 days of growth, (f) *D. vulgaris* treated with SGI after 7 days of growth. The same SRB inoculum size of 2 x10^7 cfu/ml was used for the experiment. Live cells are stained green whle dead cells are stained red.

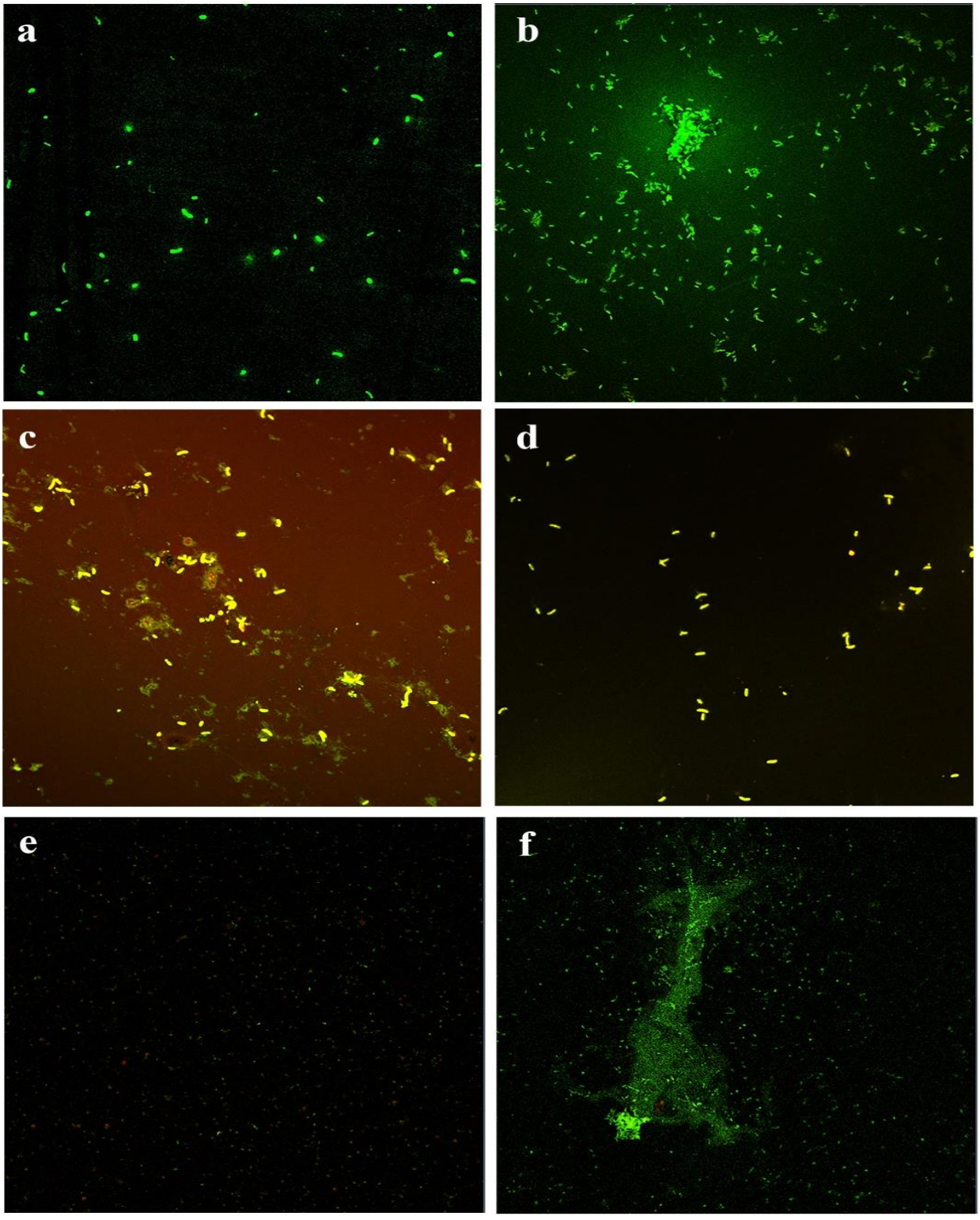


Figure 7.5. Confocal laser scanning micrographs of SRB treated with SGE (a) Untreated *D. indonensiensis* after 7 days of growth, (b) *D. indonensiensis* treated with SGE after 7 days of growth, (c) untreated *D. indonensiensis* after 37 days of growth, (d) *D. indonensiensis* treated with SGE after 37 days of growth, (e) untreated *D. vulgaris* after 7 days of growth, (f) *D. vulgaris* treated with SGE after 7 days of growth. The same SRB inoculum size of 2 x 10^7 cfu/ml was used for the experiment. Live cells are stained green.

7.3 Conclusions

The results obtained revealed that the SGE enhanced the growth of the SRB strains as revealed by the SEM and AFM whereas the SGI changed the morphology and caused a stunted growth of the SRB. The results from the confocal study showed that the growth of the SRB cells was not only inhibited by the SGI but the compound also had bactericidal effects on the cells, while the enhancer showed an enhanced live population of SRB cells.

CHAPTER 8

ANALYSIS OF SGI AND SGE BY NMR AND MALDI-TOF SPECTROMETRY

8.1 Introduction

The characteristic components of SGI and SGE were screened using NMR and MALDI-TOF spectrometry. NMR was used in order to gain insight into the functional groups present in the two compounds, which can give a clue to their structure (Singh, 2015). The applications of NMR include analysis of compound mixtures as well as determination of unknown compounds by inferring the basic structure directly or matching spectra to known libraries (Gerothanassis *et al*., 2002). When the sample is excited with a radio frequency pulse, a nuclear magnetic resonance response known as free induction decay (FID) is obtained and is mathematically manipulated (Fourier transformation) to detect the individual frequencies that produce the actual spectrum. The frequency of the sample signal is referred to as chemical shift designated by the symbol δ and expressed as parts per million (ppm) (Reusch, 2013; Reich, 2016).

MALDI-TOF spectrometry is a relatively new method for the structural analysis of biomolecules such as peptides, carbohydrates, oligonucleotides, natural products, glycoconjugate drugs and lipids (Ernst & Gunter, 2009). In TOF analysis, a set of ions that are given the same amount of energy laser radiation are accelerated to a detector. Due to their difference in mass, the ions reach the detector, through the drift space, at different times based on the mass, charge, and kinetic energy of each ion (Lewis *et al.*, 2000). Ions with smaller mass-to-charge ratio (m/z) value and more highly charged

ions move faster until they reach the detector. Consequently, the time-of-ion flight is proportional to the m/z value of the ion. The most frequently used matrices for MALDI-TOF spectrometry are 2,5-dihydroxybenzoic acid, a-cyano-4-hydroxycinnamic acid, sinapinic acid, ferulic acid, and 2,4-hydroxyphenyl benzoic acid (Croxatto *et al.,* 2012; Signor & Erba, 2013; De Carolis *et al*., 2014).

It has been shown that SGI and SGE have inhibitory and inducing effects on the growth of SRB respectively (Chapter 5 and Chapter 6). Therefore, there is the need to characterise the components of the SGE and SGI. This chapter describes the steps taken to attempt the structural characterisation of SGI and SGE, which includes the use of NMR and MALDI-TOF spectrometry.

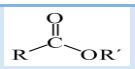
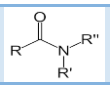
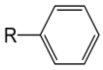
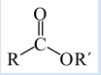
8.2 Results

8.2.1 The NMR spectra

Each dialysed and lyophilised SGI and SGE fraction was dissolved in D_2O and analysed on a Varian Inova 600 MHz spectrometer and JEOL JNM-LA400 NMR spectrometer with Broadband probe and Auto tuning. The NMR peaks assignments were performed by comparison to reference spectra (SDBS http://sdbs.db.aist.go.jp/sdbs/cgi-bin/direct_frame_top.cgi). The spectra are presented in Figure 8.1, Figure 8.3, Figure 8.4 and Figure 8.6. By comparison to reference spectra, the large peak at 3.6 ppm is attributed to Tris (Pouchert & Behnke, 1993). This also corresponds to proton attached to carbon atom at 60 ppm (Figure 8.2 and Figure 8.6). The peak at 4.6:60 ppm is solvent residual HDO (Gottlieb *et al*., 1997). The functional groups observed in the spectra are listed in Table 8.1. They are methylene (R_2CH_2) between 1-1.3 ppm, methine (R_3CH) between 1.3-1.4 ppm and alkyne ($RC \equiv CR'$) 1.4-1.6 ppm. According to Fox (2008) aliphatic carbon bond protons are found in 0-5 ppm (CH 4-5 ppm, CH_2 2-4 ppm, CH_3 0.2

ppm) (Figures 8.1, 8.3, 8.4 and 8.6). In relating the bioactivity and chemical structure of sesquiterpene lactone, it is believed that the exomethylene group on the sesquiterpene lactone is responsible for its cytotoxicity because, whilst the methylene group structure was modified, the cytotoxicity was lost (Chaturvedi *et al.,* 2010).

Table 8.1. Functional groups found on the NMR spectra

	Name	General Formula	General structure	Positions on the NMR Spectra (ppm)
1	Methylene	R_2CH_2	$H_2C{=}CH{-}R$	1-1.3
2	Methine	R_3CH		1.3-1.4
3	Alkyne	RC ≡ CR’		1.4-1.6
4	Ketones	$RCOCH_3$		2.1 and 2.6
5	Amide	RCONR2		6 - 8.5
6	Aromatics	Ar-H		6 - 8.5
7	Ester	HC-COOR, RCOO-CH		2-2.22 and 3.9-4.1

Chaturvedi *et al.* (2010) demonstrated that α-methylene-γ-lactone and α, β-unsaturated cyclopentenone ring (or α epoxycyclpentenone) present in sesquiterpene lactone are essential for their *in vivo* activities. They further confirmed that the versatile biological activities exhibited by sesquiterpene lactone are due to the presence of either an α-methylene-γ lactones and α, β-unsaturated cyclopentenone ring. Chen *et al*. (1996) also reported that the presence of dicentrine methine showed strong inhibition of collagen-induced platelets aggression indicating the methine also has some bioactive properties. In addition, terminal alkyne has been used in organic synthesis, pharmaceutical science, material science and bio-orthogonal chemistry due to their functionality and bioactivities (Zhu *et al*., 2015). Also, ketones ($RCOCH_3$) were observed in 1D H-NMR

between 2.1 and 2.6 ppm. According to Plyuta *et al*. (2014), ketones secreted by bacteria are able to interact with bacterial quorum sensing mechanisms. This may regulate a wide range of physiological activities such as growth and coordinate gene expression.

The functional groups between 6 and 8.5 ppm in the spectra belong to the amide and aromatics (Figures 8.1b, 8.3, 8.4b and 8.6). Stierle and Stierle (2000) reported that biologically active compounds purified from *Penicillium* with a high degree of aromatic character are found in ^{1}H-NMR in the region of 6.5 and 8.5 ppm. Several authors assert that in one dimensional ^{1}H-NMR spectroscopy, amide region have been indicated to have chemical resonances that range from 6 - 11 ppm but central at 8 - 8.5 ppm (Rizo & Bruch, 1996; Silverstein *et al*., 2014). Most of the NH groups obtained in this study are between 8.4 and 8.7 ppm, which suggests the presence of amino acids that includes Valine (Hα 4.8; Hβ 2.13, Hγ 0.94), Leucine (Hα 4.39; Hβ 1.65, Hγ 1.65, Hδ 0.94), Aspartate (Hα 4.77; Hβ 2.75), Lysine (Hα 4.36; Hβ 1.75, Hγ 1.47, Hδ 1.71, Hε 3.02), Asparagine (Hα 4.76; Hβ 2.76), glutamine (Hα 4.30; Hβ 1.97, Hγ 2.28), methionine (Hα 4.51; Hβ 2.0, Hγ 2.63, Hδ , Hε 2.13), and histidine (Hα 4.36; Hβ 3.20, Hγ 8.14, Hε 8.13) (Reid *et al*., 1997). Reid *et al.* (1997), Rehm *et al*. (2002) and Lee *et al*. (2006) reported that the presence of amide proton resonances between 7.5 and 8.5 indicates the presence of unfolded proteins.

The spectra (Figures 8.1b, 8.3, 8.4b and 8.6) also revealed the presence of aromatics (Ar-H) in the region of 6-8.5 ppm Ju and Parales (2010) explained that the vast majority of nitroaromatic compounds are derived from bacteria, for example *Streptomyces*. *Streptomyces* strains have also been reported to produce a variety of siderophores that have a *o*-nitrosophenol with functional groups at the *p* position, for example *S. murayamaesis* produces a compound with a carbonyl amine substitution while *S. griseus*

produces actinoviridin, which is a carboxylic acid substitution. Aside from *Streptomyces* strains, gram negative bacteria such as *Pseudomonads* and *Burkholderia* strains produce pyrrolnitrin (257.07 Da) -a chloro-nitroarene metabolite with antifungal activity. *Actinosporangium* produces pyrrolomycins A, B and E, which are active against gram negative and gram positive bacteria (Ju & Parales, 2010).

Another functional group seen at 2-2.22 ppm (HC-COOR) and 3.9-4.1 ppm (RCOO-CH) was ester. Several esters containing compounds have been reported by several authors to have varying bioactivity against a wide range of microorganisms, for example, tetracyclic diterpenoids phorbol esters (Goel *et al*., 2007). Goel *et al.* (2007) further argued that the C-3, C-4 oxygens and the C-20 hydroxyl of the phorbol play critical roles for their biological activity. MubarakAli *et al*. (2012) also reported antimicrobial activity of fatty acid methyl esters purified from *Scenedesmus bijugatus* var *bicellularis* biomass against *Staphylococcus aureus*, *E. coli* and *Candida albicans.*

The 2D proton-detected heteronuclear shift correlation experiment was carried out using carbon Heteronuclear Single Quantum Correlation (cHSQC) and spectra are shown in Figure 8.2 and Figure 8.5. However, the concentration of the compounds is very low. The results suggest that the protons may belong to saturated carbon atoms (methyl carbon C-C) at 1.3:22, 2.8:26, 9.4:28, 5.6:50 and a C-X at 4.42:82 and 4.6:92 where X implies an electronegative heteroatom. The fractions of SGI and SGE have similar spectra but with minor differences in 1D ^{1}H-NMR at 6.2 and 8.1 ppm, and ^{1}H-^{13}C Correlation (5.8:2). These peaks are present in SGE but not in SGI. This difference may be responsible for the dissimilar bioactive nature of the two fractions. This difference may be due to the absence of an amino acid that may give SGE the enhanced function while SGI exhibited inhibitory activities.

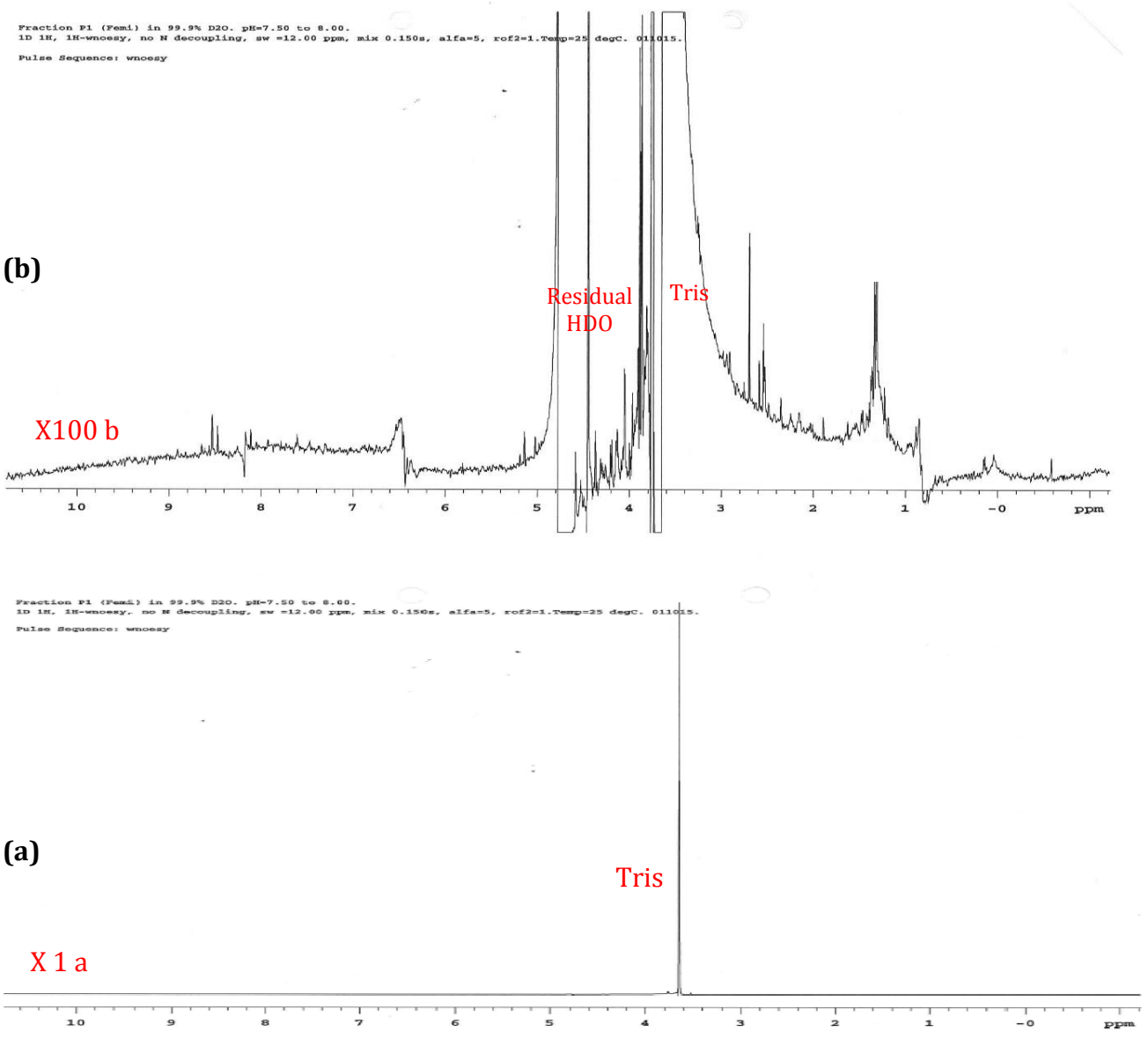


Figure 8.1. Proton NMR spectra of SGI using varian inova 600 MHz spectrometer
(a) The spectra of proton NMR of SGI. According to report from Pouchert & Behnke (1993), it reveals the spectra are mainly of Tris. However, when enlarged to x100 in (b), it reveals the presence of residual HDO at 4.6 ppm and some other small molecules in the region of 0-8.5 ppm, which are assigned to methylene (1-1.3 ppm), methine (1.3-1.4 ppm), alkynes (1.4 -1.6 ppm), ketones (2.1-2.6), esters (2.0-4.1 ppm), aromatics and amides (6 - 9 ppm) functional groups.

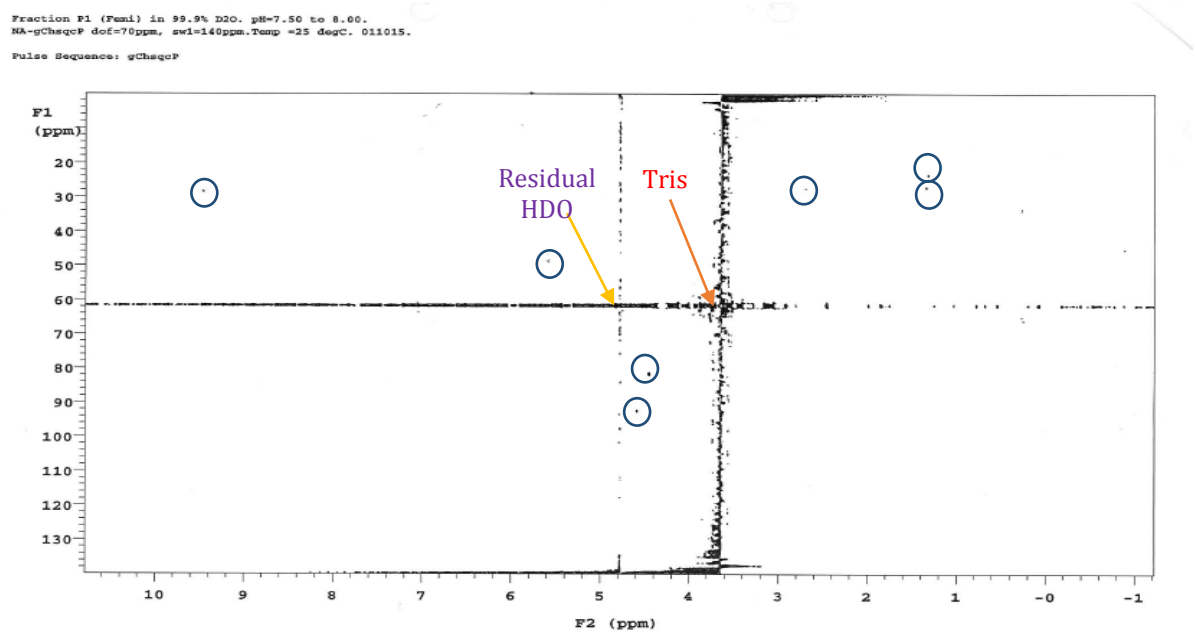


Figure 8.2. ^{1}H-^{13}C Correlation (cHSQC) of SGI using Varian Inova 600 MHz spectrometer
This is a 2D proton-detected heteronuclear shift correlation carried out using carbon Heteronuclear Single Quantum Correlation (cHSQC) on Varian Inova 600 MHz spectrometer. The marked arrow at 3.6 ppm, which corresponds to proton attached to carbon atom at 60 ppm is assigned to Tris (Pouchert & Behnke, 1993). The purple arrow at 4.6:60 ppm is residual HDO. The smaller molecules marked in blue circles may correspond to unsaturated carbon atoms.

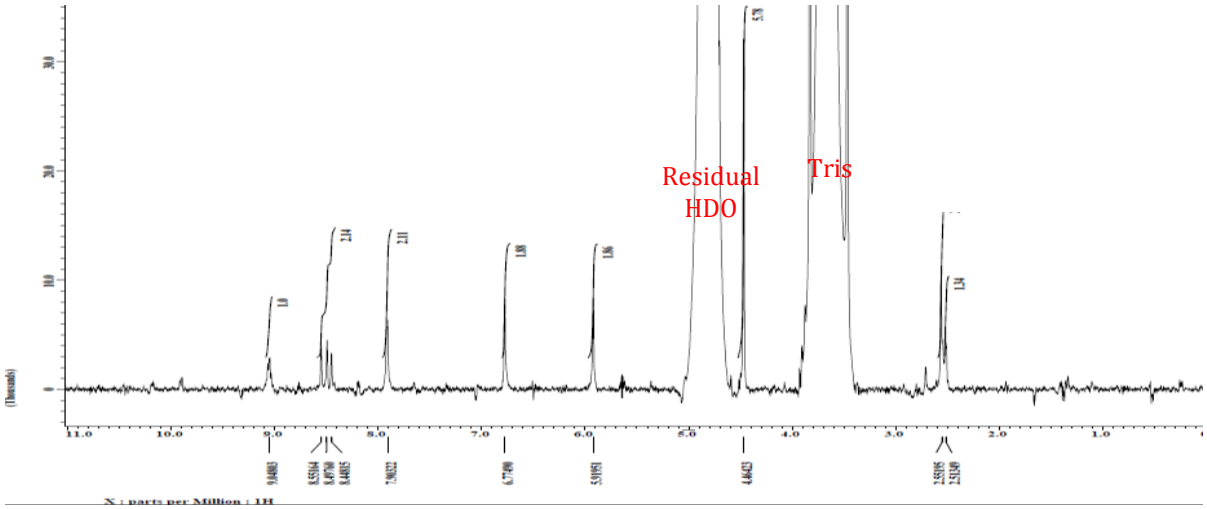


Figure 8.3. Proton NMR spectra of SGI using JEOL JNM-LA400 NMR spectrometer
The presence of Tris and HDO is indicated at 3.6 and 4.6 ppm respectively. The spectra also reveals the presence of amides or aromatics (6 - 9 ppm), alkynes (2.5 ppm), secondary amines (2.6 ppm), ketones (2.5) and alcohol or phenolic compounds (4.5 ppm).

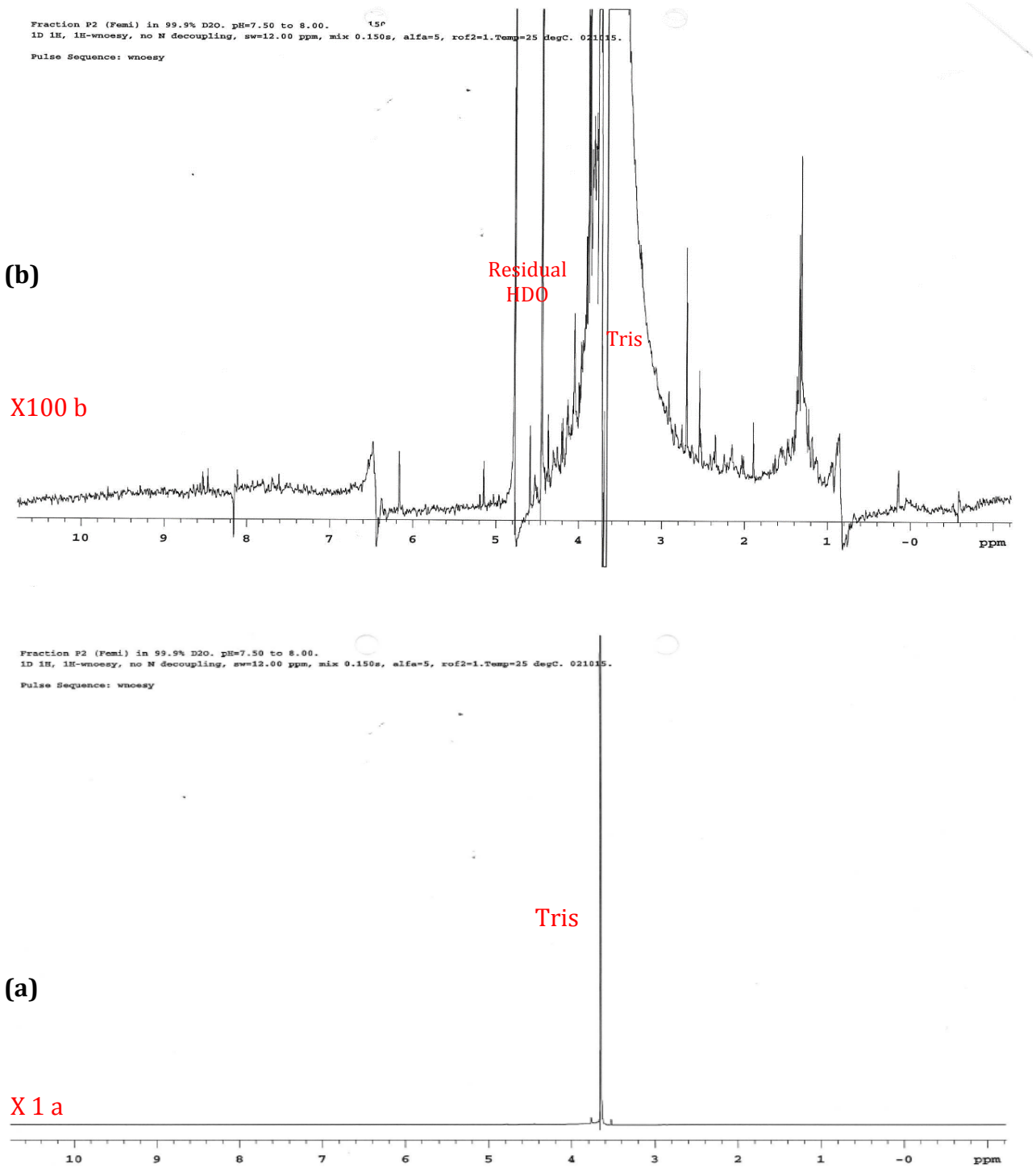


Figure 8.4. Proton NMR spectra of SGE using Varian Inova 600 MHz spectrometer

(a) The spectra of proton NMR of SGE. According to report from Pouchert & Behnke (1993), it reveals the spectra are mainly of Tris. The residual HDO is indicated at 4.6 ppm (Gottlieb *et al*., 1997). However, when enlarged to x100 in (b), it reveals some other small molecules in the region of 0-8.5 ppm, which are assigned to methylene (1-1.3 ppm), methine (1.3-1.4 ppm), alkynes (1.4 -1.6 ppm), ketones (2.1-2.6), esters (2.0-4.1 ppm), aromatics and amides (6-9 ppm) functional groups. The main difference between Figure 8.1 b and 8.4 b is the presence of a peak at 6.2 ppm in Figure 8.4 b.

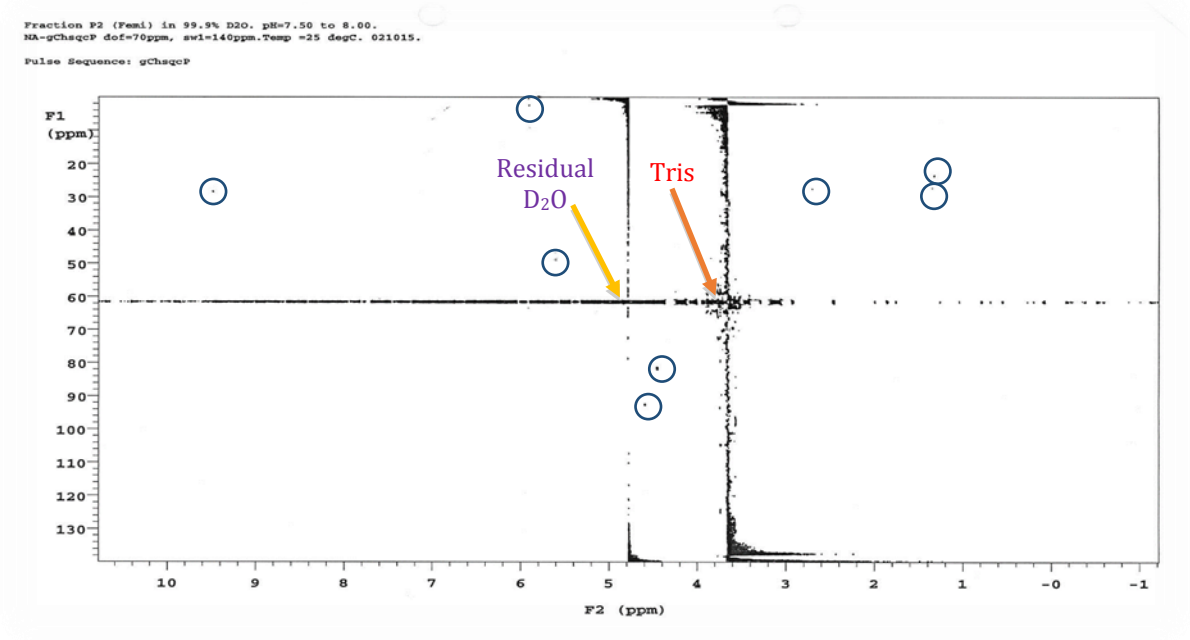


Figure 8.5. ^{1}H-^{13}C Correlation (cHSQC) of SGE
This is a 2D proton-detected heteronuclear shift correlation carried out using carbon Heteronuclear Single Quantum Correlation (cHSQC). The marked red arrow at 3.6 ppm, which corresponds to proton attached to carbon atom at 60 ppm is assigned to Tris (Pouchert & Behnke, 1993) while the purple arrow at 4.8:60 ppm indicates the residual D2O. The smaller molecules marked in blue circles may correspond to unsaturated carbon atoms. The correlation data of SGE differ from that of SGI by the presence of proton at 6.0 which correspond to carbon at 4.0.

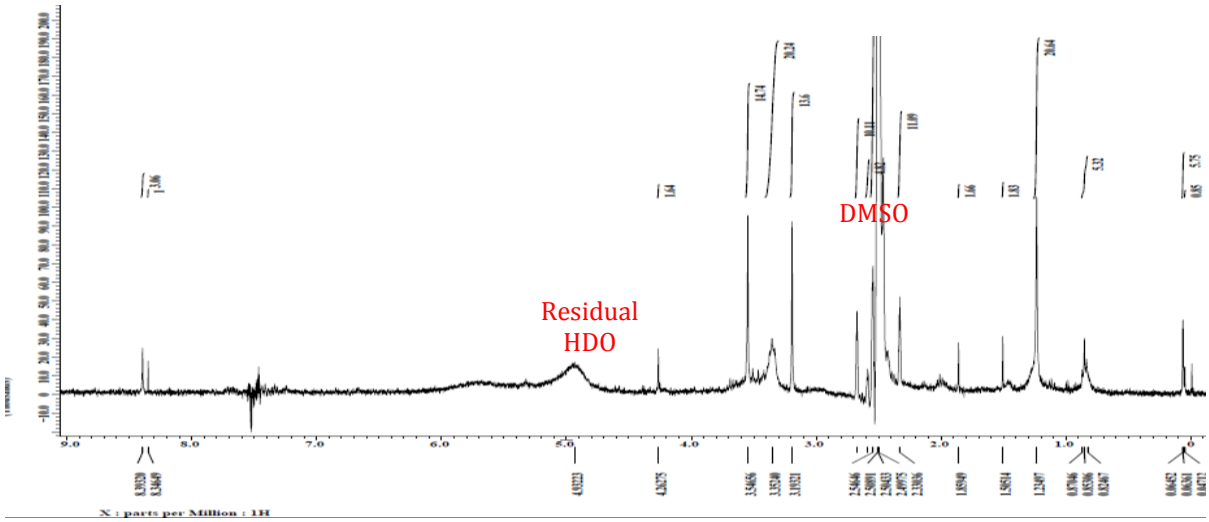


Figure 8.6. Proton NMR spectra of SGE using JEOL JNM-LA400 NMR spectrometer
The presence of DMSO and HDO is indicated at 2.5 and 4.8 ppm respectively. The spectra also reveal the presence of amides or aromatics (8.5- 8.3 ppm), alkynes (2.5 ppm), secondary amines (1.5 -2.6 ppm), alcohol or phenolic compounds (4.2 ppm) and primary amines (1.2-1.5 ppm).

8.2.2 The MALDI-TOF spectra

MALDI-TOF analysis was carried out on the SGI and SGE dialysed and lyophilised fractions. At first no signal was observed on the MALDI-TOF possibly due to the presence of Tris buffer. In order to improve resolution of the signal, buffer exchange was carried out with Triethylammonium bicarbonate (TEAB) buffer.

Five controls were used in this study, namely (i) a calibration mixture of des-Arg-bradykinin (905.05 Da), angiotensin I (1298.51 Da), Glu-fibrinopeptide B (1571.61 Da) and neurotensin (1673.96 Da) measured as average of $(M+nH)n^{+}$ (Applied Biosystems)(Figure 8.7), (ii) α-cyano-4-hydroxycinnamic acid (CHCA) matrix with molecular weight of 189.17 Da (Applied Biosystems) (Figure 8.8), (iii) TEAB buffer (SIGMA) (Figure 8.9), (iv) mixture of TEAB buffer and α-cyano-4-hydroxycinnamic acid (CHCA) matrix (Figure 8.10), (v) 10 mM Tris-HCl, pH 8 (Figure 8.11). The spectra reveal the relative abundance against the m/z in linear mode. The intensity of the controls signals are low compared to that of the SGI and SGE.

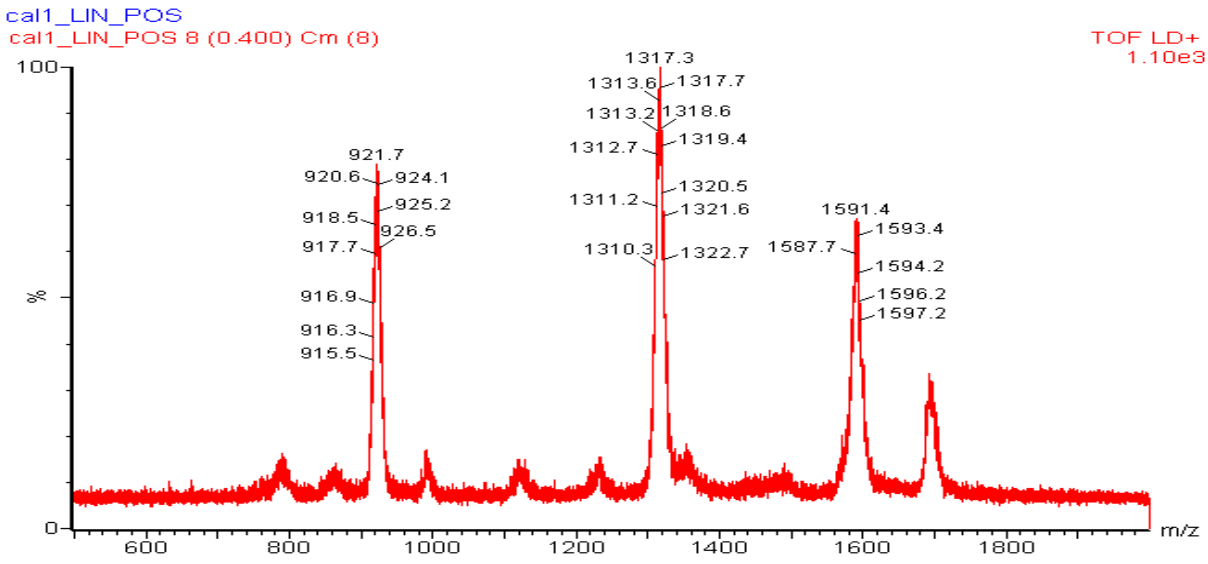


Figure 8.7. Mixture of des-Arg-bradykinin, angiotensin I, Glu-F fibrinopeptide B and neurotensin in positive mode
The mixture has the following m/z ratio: 1690.96 (neurotensin), 1591.4 (Glu-fibrinopeptide B), 1317.3 (Angiotensin I) and 921.7 (des-Arg-bradykinin)

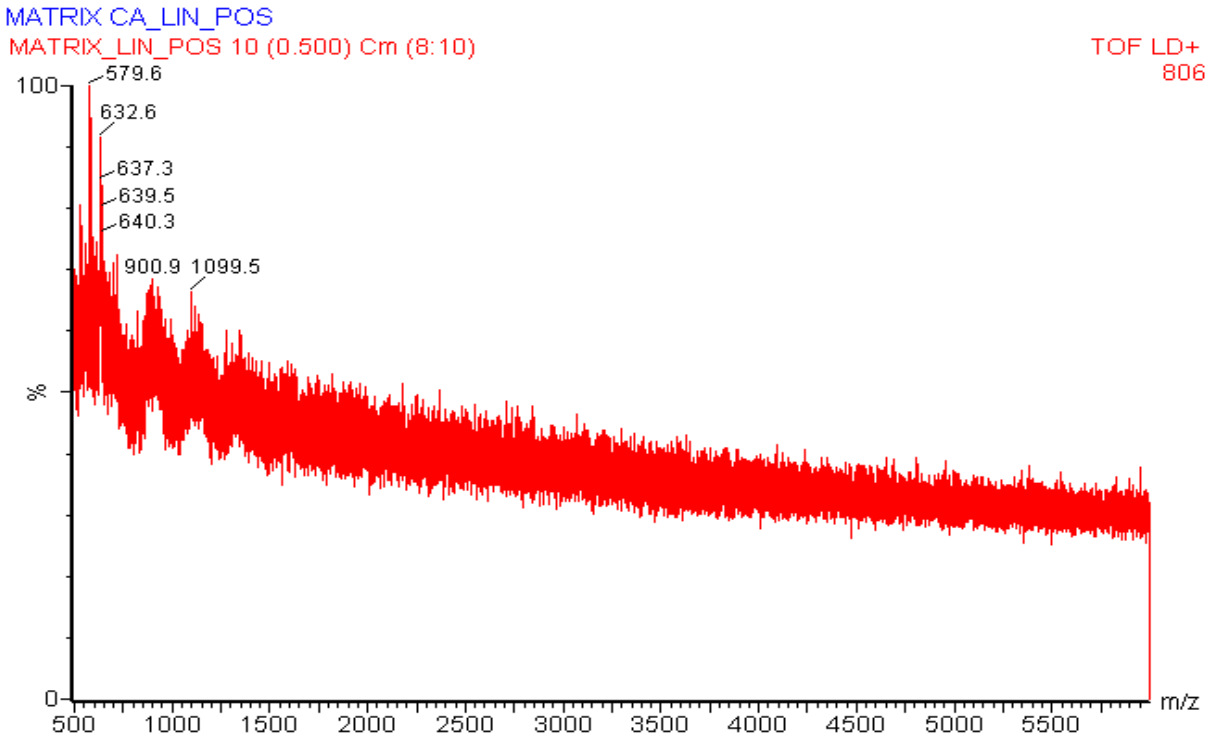


Figure 8.8. CHCA matrix in positive mode

CHCA: α-cyano-4-hydroxycinnamic acid in m/z of 500-6000. Signals are observed in m/z 1099.5, 900.9, 632.6, 579.6

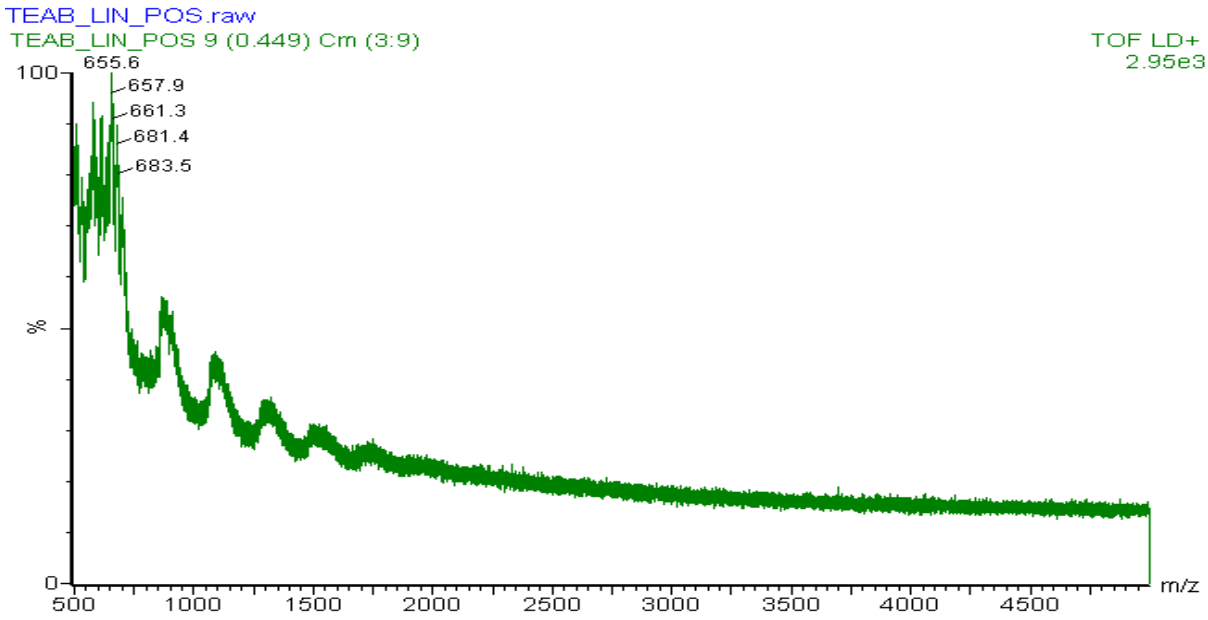


Figure 8.9. TEAB buffer in positive mode

TEAB: Triethylammonium bicarbonate buffer in mz of 500-5500. Signals are observed in m/z of 1523.5, 1313.9, 1150 and 850

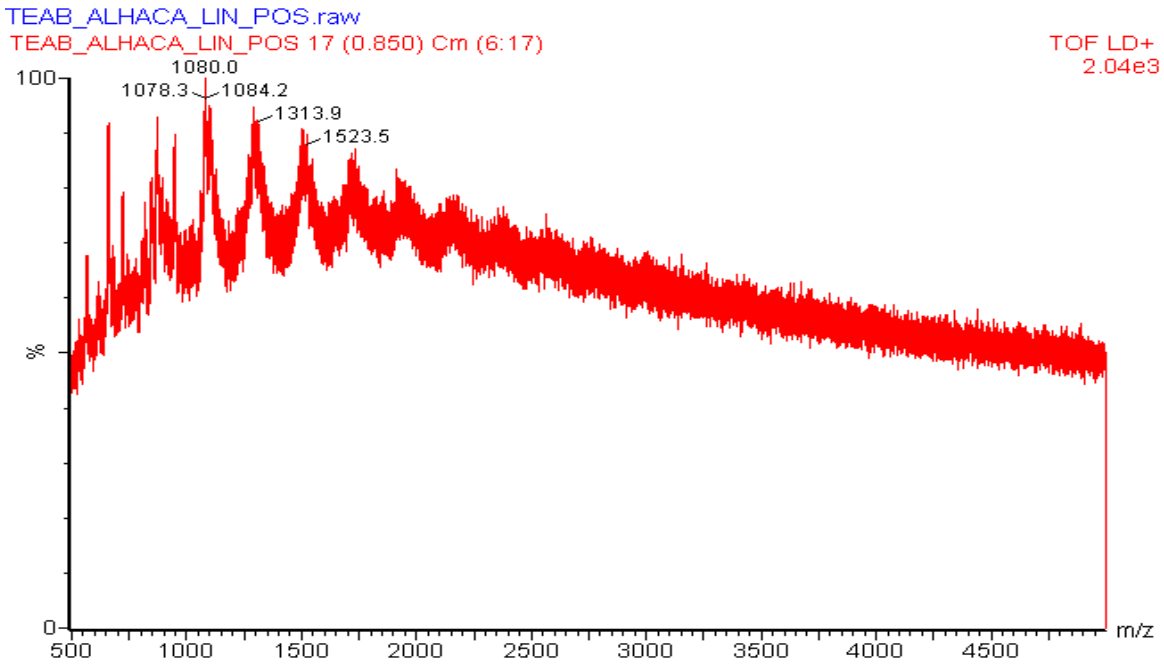


Figure 8.10. Mixture of TEAB and CHCA matrix in positive mode
TEAB: Triethylammonium bicarbonate buffer and CHCA: α-cyano-4-hydroxycinnamic acid in m/z of 500-5500. Signals are observed in m/z of 1523.5, 1313.9, 1084.2, 1080.0, 1078.3, 632.6, and 579.6

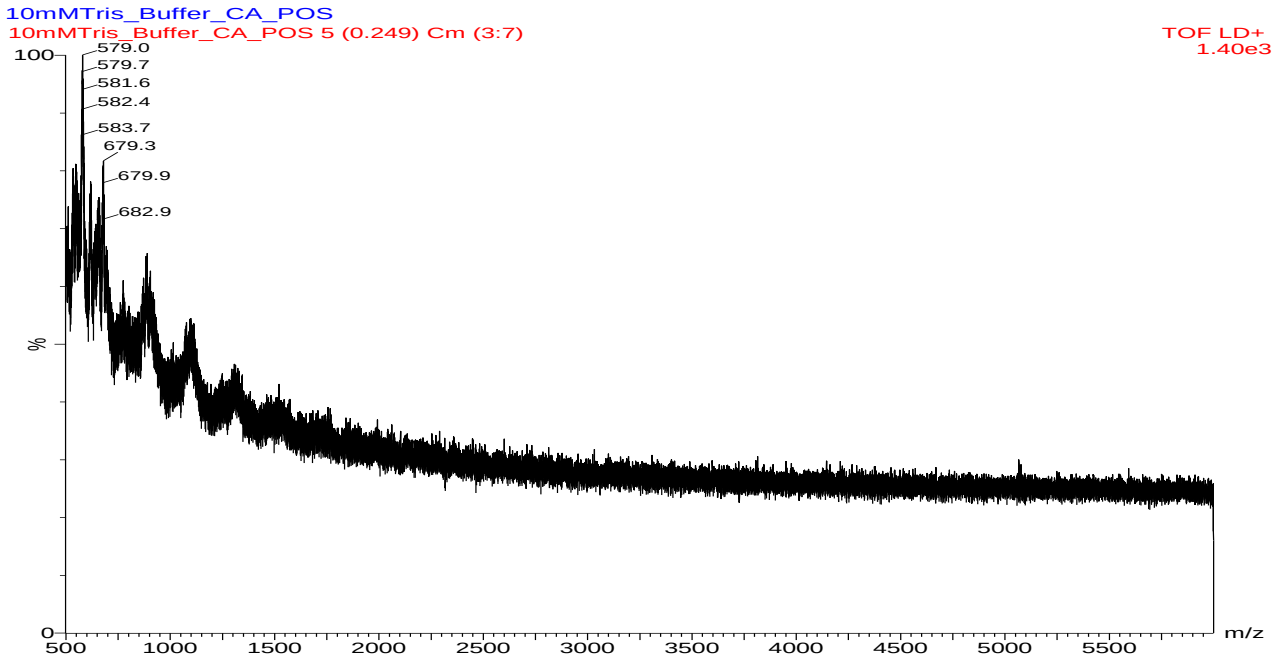


Figure 8.11. 10 mM Tris HCl, pH 8 in positive mode
Peak in m/z of 500-5500 is shown. Signals are observed in m/z of 579.0, 679.3, 900, 1150, and 1350.

The spectra of SGI and SGE in positive mode are shown in Figure 8.12 and Figure 8.13 respectively. The spectra suggest the compounds to be an oligomeric series with repeat unit of ~ 213 m/z. Wallace & Guttman (2002) posit that this is a characteristic spectrum of a condensation homopolymer. Several polymers have been found in bacterial cells such as carbohydrates, lipids, proteins, lipopolysaccharides, glycoproteins and nucleic acids (Cooper, 2000). In both spectra, there is a gradual increase in the relative abundance as the m/z decreases. The dominant peaks in the Figure 8.12 spectrum are observed at m/z 674.3, 888.3, 1100.3, 1313.3, 1526.3 and 1739.3 with the least m/z value giving the highest relative abundance. However in Figure 8.13 spectrum, dominant peaks are observed in m/z 673.1, 886.3, 1099.7, 1312.7, 1525.7, 1738.7, 1951.7, 2164.7 and 2400. According to Pavia *et al.* (2013), the heaviest significant m/z value often represents the molecular ion in mass spectrum, therefore, m/z value of 2400 in SGI indicates the molecular mass of SGI to be around 2400 Da (Figure 8.12). Similarly, the m/z value of 1700 in SGE indicates the molecular mass of SGE to be around 1700 Da (Figure 8.13).

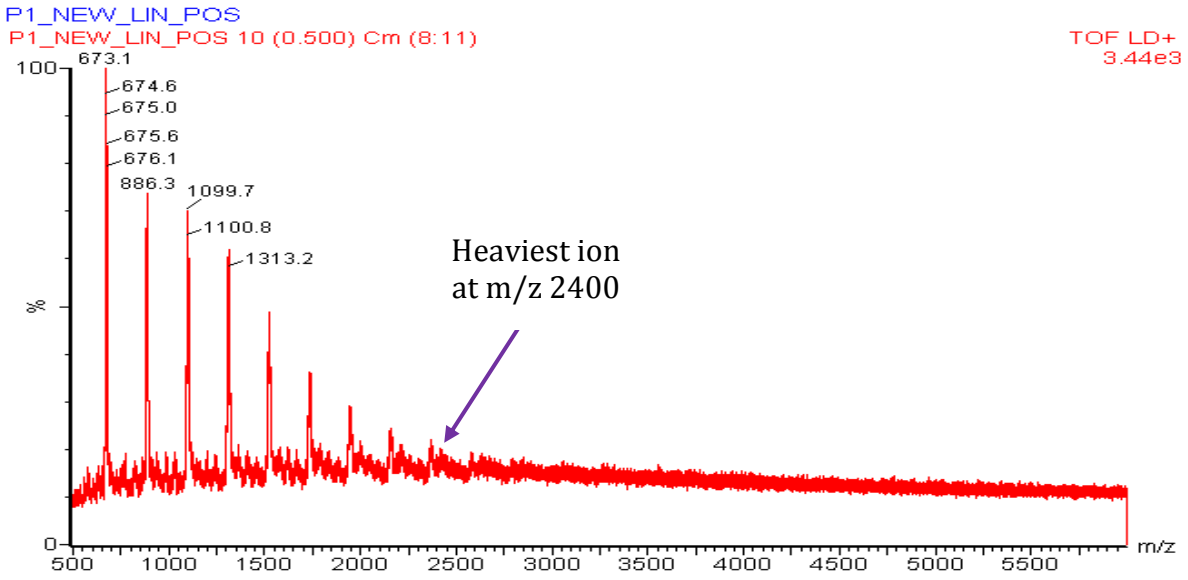


Figure 8.12. SGI in positive mode

The spectrum contains fragments with a repeating unit of ~ 213 m/z between each peak. The heaviest significant m/z value of 2400 indicates the molecular weight to be around 2400 Da.

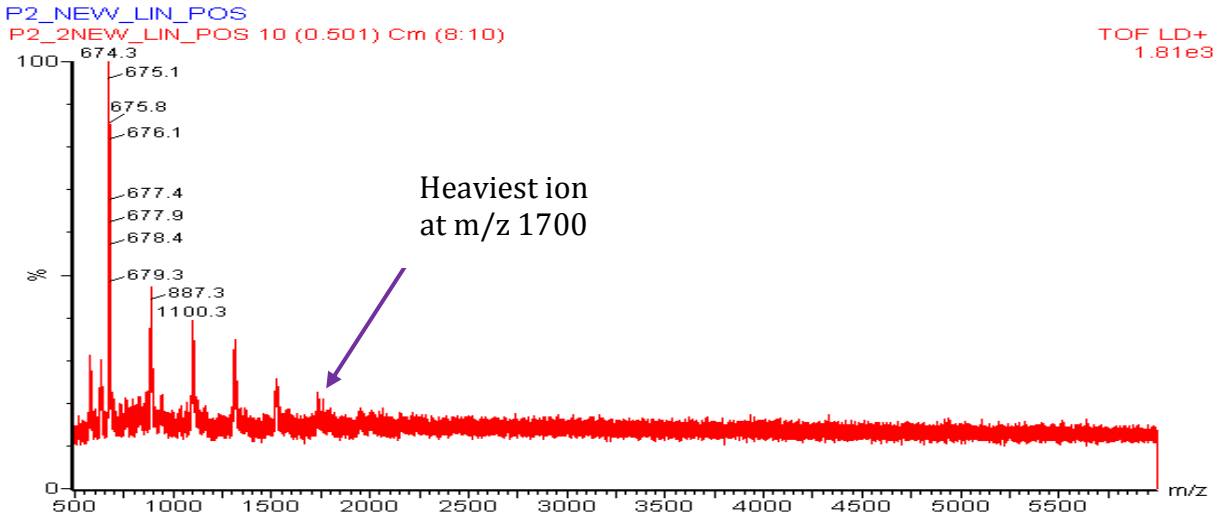


Figure 8.13. SGE in positive mode

The spectrum contains fragments with a repeating unit of ~ 213 m/z.The heaviest significant m/z value of 1700 indicates the molecular weight to be around 1700 Da.

8.3 Conclusions

In the NMR spectra, the chemical shifts (ppm) in the region of 0–8.5 ppm are assigned to methylene, methine, alkynes, ketones, esters, aromatics and amides functional groups. This suggests the presence of carbohydrate and proteins in SGE and SGI. In the MALDI-TOF spectrum, the heaviest ion of SGI has a m/z ratio value of 2400, this indicates the molecular mass of SGI to be around 2400 Da. Similarly, the heaviest ion of SGE has a m/z ratio value of 1700 and indicates the molecular mass of SGE to be around 1700 Da.

CHAPTER 9

GENERAL DISCUSSION

The CFS of *E. coli* BP purified by ion exchange chromatography produced fractions that influenced the growth of SRB. The *E. coli* BP incubated for 12, 18 and 24 h had the highest induction on the growth of *D. indonesiensis*, although optimum at 18 h. This indicates that the compounds are secondary metabolites produced at the stationary phase of growth of the bacterium. This is similar to the explanations of Monteiro *et al*. (2005) and Carvalho *et al*. (2010) who argued that microorganisms have long been known to produce secondary metabolites during their stationary phase of growth, mostly in response to a growth-limiting nutrient and the metabolites have been harnessed in the production of useful industrial products. Similarly, Sarkar *et al.* (2010) and Rolfe *et al*. (2012) explained that in stationary bacterial growth phase, there is usually a buildup of inhibitory metabolites and exhaustion of space and nutrients because of the limited batch nature of the medium and because growth rate is balanced by the number of dead cells.

The optimum production of SGE was found under limited oxygen condition. According to the reports of Spiro and Guest (1990), different regulatory systems have been devised by bacteria that allow them to cope with different environmental conditions and to utilise these conditions for their own interest. Nakano *et al*. (1997) explained that facultative anaerobes are able to utilise anaerobic growth for respiration during a low oxygen composition by fermenting sugars. This process may induce the production of useful metabolites. Similarly, Cruz Ramos *et al*. (1995) reported that anaerobiosis strongly induce expression of ferredoxin-$NADP^+$ reductase (fnr) gene in *B. subtilis*. Furthermore, Nakano *et al*. (1997) reported the activity of fnr is excited by anaerobiosis.

According to Madigan and Martinko (2006), the energy released during anaerobic respiration (2-ATP) is less compared to aerobic respiration (36 ATP), however anaerobic respiration results in the production of alcohol, lactic acids, food products and other compounds derived from microorganisms. In anaerobic conditions, microbial cells utilise glucose for the production of energy by anaerobic respiration and fermentation pathways. For example, when *E. coli* is in anaerobic conditions, a transcriptional regulator ArcB-A inhibits aerobic enzymes and ferredoxin-$NADP^+$ reductase (fnr) allows it to undergo anaerobic respiratory process.

Based on the combined results from gel filtration chromatography, dialysis by 1000 Da MWCO membranes, NMR spectroscopy and MALDI-TOF spectrometry, the molecular weight of the two compounds is estimated to be around 1700 Da for SGE and SGI, 2400 Da. This shows that these compounds have low molecular weight properties. Their low molecular weight property suggest that the compounds being siderophore cannot be ruled out. Brandel *et al*. (2012), Zheng and Nolan (2012); Saha *et al*. (2013) and Ahmed and Holmström (2014) described siderophores as metabolites that have low-molecular-mass, have strong affinity for ferric ion (Fe^{3+}) and are often excreted and exported by microorganisms during low iron conditions. In this study, M9 medium was utilised for the production of SGE and SGI from *E. coli* BP, but M9 medium is devoid of iron. However, according to Neilands (1995), iron is one of the most essential elements required for the growth of microorganisms. Similarly, when there is none/low iron availability in a medium, most bacteria devise a means to scavenge iron from the environment using siderophores which have a very high affinity for Fe^{3+}, thereby making iron available to the microbial cell (Brandel *et al*., 2012; Zheng & Nolan, 2012; Saha *et al*., 2013; Ahmed & Holmström, 2014).

Mohandass (2004) reported that siderophores are mostly novel compounds and contain many modified amino acids that are naturally not found elsewhere. Also, Martinez *et al*. (2003), Ali and Vidhale (2013) and Ahmed & Holmström (2014) added that siderophores have several biotechnological, agricultural, environmental and medicinal applications. According to Martinez *et al*. (2003), most bacteria synthesize one or more types of siderophores, which are often secreted into the growth medium. For example, in iron deficient medium *Hafnia alvei* produces ferrioxamine G (672 Da) and ferrioxamine E (653.53 Da) that are capable of inducing the growth of self and other bacteria (Reissbrodt *et al.,* 1990; Gledhill & Buck, 2012), *Ustilago sphaerogena* produces ferrichrome (687.70 Da) (Messenger & Ratledge, 1985; Andrews *et al*., 2003), a marine bacterium *Alteromonas haloplanktis* synthesises bisucaberin with molecular weight of 400.47 Da (Takahashi *et al*., 1987; Mansson *et al*., 2011), acinetobactin (346.34 Da) is produced by *Acinetobacter calcoaceticus* and *Pseudomonas aeruginosa* produces two siderophores namely pyoverdin (1365.42 Da) and pseudobactin (1039.82 Da) (Faraldo-Gómez *et al*., 2003; Mohandass, 2004; Vandenende *et al*., 2004; Balado *et al*., 2015).

The other possibility that the compounds are not siderophore is related to the size. The results obtained does not suggest that SGE is a siderophore produced by *E. coli* because the molecular weight is greater than the molecular weight of enterobactin, a siderophore produced by *E. coli* with a molecular weight of 669.55 Da (Neilands, 1993; Albrecht *et al*., 2009). Similarly, the autoinducer purified from *E. coli* has a molecular weight of 500 Da (Freestone *et al*., 2003). In addition, the SGI has not been documented in literature as a compound produced by *E. coli*. Thus, the SGE and SGI to my knowledge have not been previously described.

Although, iron can be acquired by bacterial cells using other means aside from siderophore. Messenger and Barclay (1983) argued that iron can be acquired by the cell *via* the binding of iron to less specific cell wall components, for example Gram-negative bacteria uses lipopolysaccharides (LPS) as the most important non-specific cation-binding element. Lipopolysaccharides are the principal component of Gram negative bacteria and they give them their endotoxic and antigenic properties. The outer membrane also contains a protein-porins that serves as a substrate transport channel and helps in the semi-permeability of the small molecules (Banoub *et al*., 2010).

The results obtained from the count of *E. coli* BP and the activities of SGE indicate that there is a correlation between the total viable bacterial counts and the production of the SGE. Similarly, Carvalho *et al*. (2010) reported the optimum production of bioactive compounds from *Bacillus subtilis* R14 during stationary phase under oxygen limitation. In the same way, Rattanachuay *et al*. (2010) grew *Pseudomonas* sp. W3 for 18 h to produce extracellular compounds that have inhibitory effects on pathogenic vibrios.

The SGE can be used as a rapid detection tool for SRB. If SGE is incorporated to commonly used media for detecting and isolating SRB, it can help to detect SRB with low cell densities, it can also enhance SRB growth and detection. This is similar to the argument of Bertrand *et al.* (2014) who stated that microbiological culture media can be supplemented with small molecules that can induce or inhibit microbial growth. This detection sensitivity is comparable with complex rapid techniques for example SRB Rapid Detection Test Kit using APS reductase as a marker (Horacek, 1992). However, the advantages of this approach is that this method (i) allows the use of culture method often used for SRB enumeration and detection, (ii) rapid SRB growth and detection sensitivity, (iii) living and metabolically active SRBs can be seen, (iv) SRB which cannot

be recovered using standard culture procedures and therefore, often wrongly thought as dead can be reactivated.

During purification of SGE the inhibitor of SRB growth (SGI) was discovered. The development of biofilms follows three stages and include irreversible attachment of single bacteria colonies on biotic or abiotic surfaces using structures such as pili, extracellular polymeric substances (EPS) and fimbriae. The next stage is growth. Once bacterial colonies are attached to surfaces, the bacteria replicate actively to form a complex structure referred to as glycocalyx (Dunne, 2002) and the cycle continues following dispersion of planktonic cells from the biofilm matrix (Langer *et al*., 2014). However, if the initiation of SRB colonisation on surfaces is prevented or if their growth is impeded, this will inhibit biofilm formation and eventually lead to the limitation of damage such as biocorrosion, equipment failure, biofouling, hydrocarbon degradation and reservoir souring facilitated by SRB.

Several studies have described some inhibitors of SRB growth that are derived from bacteria; for example, Jayaraman *et al*. (1999) and Zuo (2007) reported that indolicidin, bactenecin, and polymyxin produced by *Paenibacillus polymyxa* are capable of inhibiting SRB growth. *Bacillus brevis* produces a compound referred to as gramicidin-S that inhibits the growth of *Desulfovibrio orientis, D. vulgaris* and *D. gigas* (Jayaraman *et al*., 1999; Morikawa, 2006; Zuo, 2007) and thereby reduced corrosion caused by the SRB. In addition, *Bacillus licheniformis* secretes γ-polyglutamate and polyaspartate that reduce SRB growth (Mansfeld *et al*., 2002; Ornek *et al*., 2002; Zuo, 2007). The mechanism of SRB growth prevention by these organisms have been suggested and include either the production of antimicrobial agents (Jayaraman *et al*., 1999; Zuo, 2007; Zarasvand & Rai, 2014), or attack on the adenosine 5'- phosphosulphate (APS) and bisulfate reductase

(DSR) responsible for hydrogen sulfide production in SRBs (Muyzer & Stams, 2008; Tsygankova *et al.*, 2014; Zarasvand & Rai, 2014). Similarly, the SGE may function in SRB induction by increasing their growth rate while the SGI may function by causing damage in the cells as observed in this study.

The MALDI-TOF spectra showed the presence of low molecular weight compounds in the range of 1700 Da for SGE and 2400 Da for SGI. The spectra showed equal and repeating units of ~ 213 m/z between the peaks. According to Wallace & Guttman (2002), the equal and repeating units are characteristic spectra of condensation homopolymers.

The NMR spectra do not give unambiguous detailed information regarding the structure of the SGE and SGI due to low concentration and signal interference in the experiments performed. Malet-Martino & Martino (1991), Gadian *et al.* (1993) and Chatham & Blackband (2001) argued that analysis by NMR spectroscopy is limited by lack of sensitivity, and means that analytes must be present at fairly high concentrations in order to produce detectable signals. This also prevents the combination of the MALDI-TOF spectra with the NMR for conclusive results on the structural components of SGE and SGI. However, the NMR and MALDI-TOF spectra revealed that the compounds are small molecular weight biomolecules and that the two molecules are very closely related.

CHAPTER 10

GENERAL CONCLUSIONS AND FUTURE STUDIES

10.1 General Conclusions

Two low molecular weight compounds have been isolated and purified from *E. coli* BP and are referred to as SRB growth enhancer (SGE) and SRB growth inhibitor (SGI). The biomolecules possess at least one negatively charged group. There was optimum production of SGE when *E. coli* BP was grown in M9 medium under limited oxygen condition at 37°C for 18 hours. The results obtained from purification of SGE revealed the presence of an inhibitor of SRB growth. Functional analysis showed inhibitory effects of the inhibitor on the growth of SRB and SGE revealed their growth induction.

Based on the results from gel filtration chromatography and dialysis, the molecular weight of the two compounds is estimated to be above 1000 Da. It is unlike that of the autoinducers purified from *E. coli* with molecular weight less of 500 Da, neither is it enterobactin (669.55 Da), a siderophore produced by *E. coli.* Also the SGI has not been reported in the literature as a compound produced by *E. coli*.

The results obtained from microscopy showed that the SGI changed the morphology and caused a stunted growth of the SRB strains whereas the SGE enhanced their growth as revealed by the SEM and AFM. In addition, the results from the confocal study showed that the large proportion of the SGI treated cells were dead while the enhancer showed more live SRB cells.

The activity of the SGI on SRB growth and morphology is similar to that observed by other inhibitors; for example, gramicidin-S (Jayaraman *et al*., 1999; Morikawa, 2006), γ-

polyglutamate (Mansfeld *et al*., 2002; Ornek *et al*., 2002), and indolicidin (Jayaraman *et al*., 1999; Zuo, 2007), so that it can be developed as an alternative for the control of SRB colonisation. In contrast, the enhancer produced by the same bacterium induced SRB growth and may be used as a tool for their rapid detection.

The data obtained from the analysis of SGE and SGI by NMR spectroscopy showed signals consistent with methylene, methine, aromatic and amide functional groups in the region of 0–8.5 ppm although this alone is insufficient in suggesting a structure. However, the most interesting results from MALDI-TOF spectrometry showed a spectra with equal and repeating units of ~ 213 m/z between the peaks. The heaviest ion of SGI was at m/z 2400 and indicates the molecular weight of SGI to be around 2400 Da, while the heaviest ion of SGE was at m/z 1700 and indicates the molecular weight of SGE to be around 1700 Da.

10.2 Future Research

The results of this study have shown that SGI and SGE had *in vitro* inhibitory and inducing effects respectively on the growth of SRB. However, investigating the effects of these compounds on the growth of SRB in the field is suggested. Structural characterisation of the biomolecules followed by large scale chemical synthesis of the characterised molecules is thought worth pursuing.

Definitive experiments demonstrating siderophore activity would be essential. Appropriate mass spectrometry may be carried out to determine coordination with Fe^{3+} (Ertl *et al*., 2004). Possibly Mossbauer spectroscopy to reveal possible coordination (Kamnev et *al*., 1999; D'Antonio *et al*., 2009) and capillary electrophoresis (Rodriguez *et al*., 2006; Unger, 2009).

While purifying SGE and SGI, the use of other elution buffers (for example phosphate buffer) may be considered instead of Tris. In the data obtained from NMR, Tris interfered with the spectra hindering structural identification. In addition, increasing the purity and quantity of the SGE and SGI may aid in the detection sensitivity of the signal during NMR spectroscopy. This may be achieved if several rounds of 10 L CFS of *E. coli* BP is used for the purification.

While testing the SGE and SGI for bioactivity, the amount of FeS produced by the SRB could be weighed for further analysis. This may give additional data to the use of +/- for representing the growth difference beween SRB control and treated cells.

Additional analytical techniques such as ultra-performance liquid chromatography quadrupole time of flight (UPLC-QToF) mass spectrometry and x-ray photoelectron spectroscopy may also be used to obtain more information for structural characterisation of the SGE and SGI.

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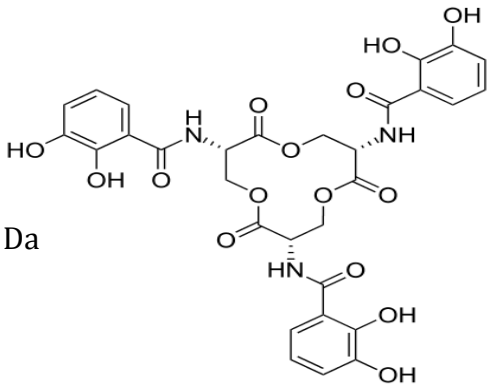
APPENDIX

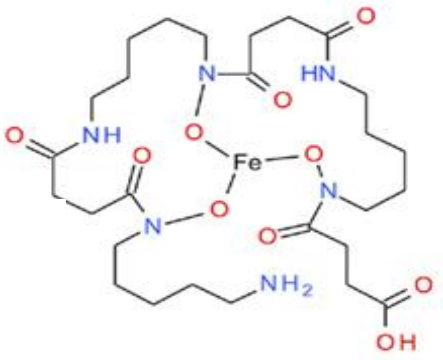
Appendix 1. Structures of siderophores mentioned in the text

MW: 669.55 Da



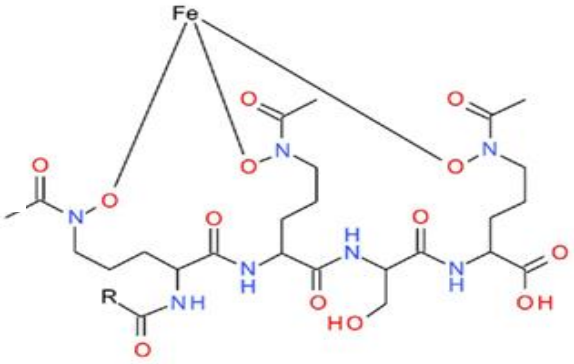
(i) Enterobactin (Neilands, 1993; Albrecht *et al.*, 2009)

MW: 672 Da



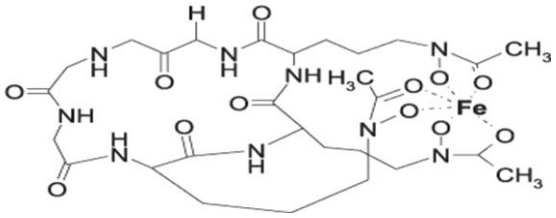
(ii) Ferrioxamine G (Reissbrodt *et al.,* 1990; Gledhill, & Buck, 2012).

MW: 653.53 Da



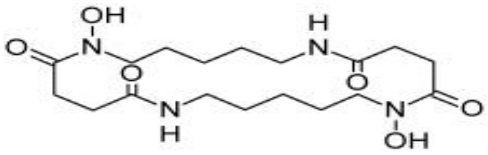
(iii) Ferrioxamine E (R= $C_{15}H_{29}$) (Reissbrodt *et al.,* 1990; Gledhill & Buck, 2012).

MW: 687.70 Da



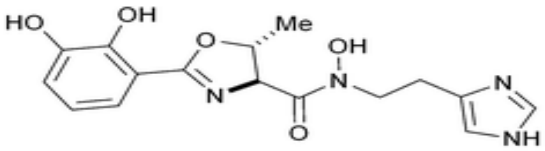
(iv) Ferrichrome (Messenger & Ratledge, 1985; Andrews *et al*., 2003)

MW: 400.47 Da



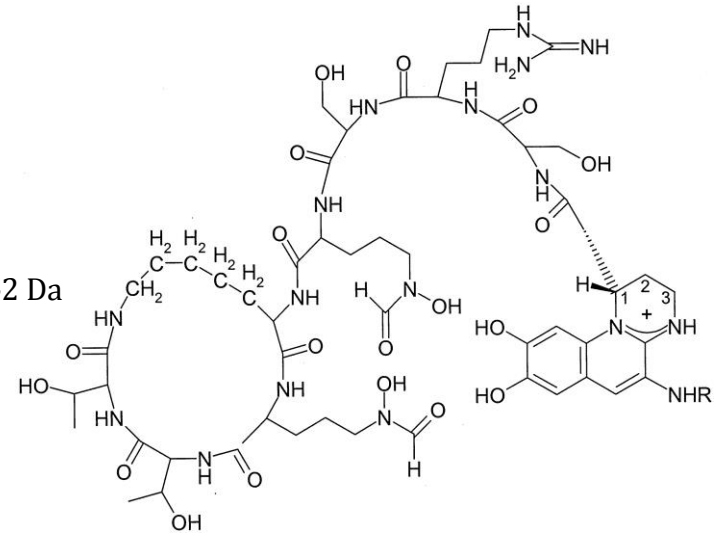
(v) Bisucaberin (Takahashi *et al*., 1987; Mansson *et al*., 2011)

MW: 346.34 Da



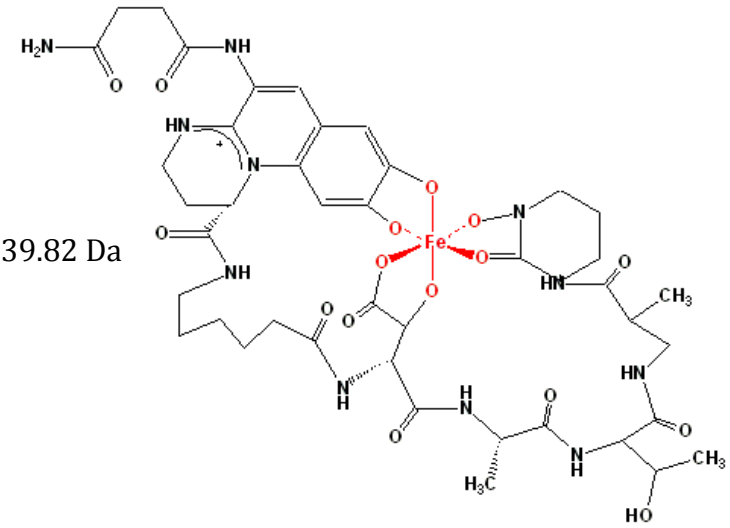
(vi) Acinetobactin (Mohandass, 2004; Balado *et al*., 2015)

MW: 1365.42 Da



(vii) Pyoverdin (Vandenende *et al*., 2004; Mohandass, 2004)

MW: 1039.82 Da



(viii) Pseudobactin (Faraldo-Gómez *et al*.,2003; Mohandass, 2004)