ADVANCEMENT OF BIOSURFACTANT PRODUCTION AND BIOSURFACTANT-AIDED POLLUTION REMEDIATION

by

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ABSTRACT

Biosurfactant enhanced soil washing and/or bioremediation have been proven as promising technologies for cleaning up petroleum hydrocarbon contaminants (PHCs)- and heavy metals-contaminated soil and groundwater. As environmentally friendly amphiphiles, biosurfactants display promising wetting, solubilization, and emulsification properties. Biosurfactant addition can enhance the mobility and bioavailability of entrapped PHCs in porous media, and finally improve their removal. Biosurfactants can also reduce the heavy metal toxicity and assist their removal through acting as metal complexing agents. The availability of economic biosurfactants, however, has become a major obstacle to their applications. In addition, little research has been conducted to investigate the role of biosurfactants, especially lipopeptides, in contaminated subsurface cleanup process and their impacts on oil degrading microbes.

To fill the knowledge gaps, a number of methodologies and mechanisms aimed at economical biosurfactant production and advanced biosurfactant enhanced subsurface co-contamination control have been investigated. Economical lipopeptide production by *Bacillus Substilis* N3-1P using fish waste as an unconventional medium was achieved. The lipopeptide production was further enhanced using immobilized robust biocatalysts on porous fly ash by *Bacillus Substilis* N3-1P, and the associated mechanisms were explored. The lipopeptide production by *Bacillus Substilis* N3-4P was optimized and its application for crude oil removal was examined. The impact of the generated biosurfactant on the biodegradation of PHCs in

presence of heavy metals was finally evaluated.

The newly developed lipopeptide production methodologies and the associated mechanisms helped to break down the barriers impeding economical biosurfactant production. The research outcomes (e.g., fish-waste-based hydrolysate, fly ash (FA) - based robust biocatalyst and optimized growth medium) could contribute to a cost-efficient biosurfactant production through proper selection of waste materials, advanced bioreactor design and medium optimization. This dissertation research was also a first attempt to identify the role of lipopeptides in cell surface associated biodegradation mechanisms in a co-contaminated environment. This research could help implement effective soil and groundwater remediation practices and bring short/long-term benefits to the governments, industries and communities at regional, national and international levels.

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LIST OF SYMBOLS AND ABBREVIATIONS

AES	Alcohol Ether Sulfates
ASTM	American Society for Testing and Materials
BATH	Bacterial Adherence to Hydrocarbons
BTEX	Benzene, Toluene, Ethylbenzene, Xylenes
CBPP	Corner Brook Pulp and Paper
CMC	Critical Micellar Concentration
CMD	Critical Micelle Dilution
ComA-P	Phosphorylated ComA
CSH	Cell Surface Hydrophobicity
CTAB	Cetyl Trimethylammonium Bromide
CTAC	Cetyl Trimethylammonium Chloride
DH	Degree of hydrolysis
DOSS	Dioctyl Sodium Sulfosuccinate
EI	Emulsification Index
EPA	Environmental Protection Agency

FA	Fly Ash
FCSAP	Federal Contaminated Sites Action Plan
FCSI	Federal Contaminated Sites Inventory
FH	Fish Head
FL	Fish Liver
FTIR	Fourier Transform Infrared
FTIR-ATR	Fourier transform infrared-attenuated total reflection
HLB	Hydrophilic-Lipophilic Balance
HTC	High Total Petroleum Hydrocarbon Contaminated
ICP-MS	Inductively Coupled Plasma Mass Spectrometry
ITOPF	International Tanker Owners Pollution Federation
LAB	Linear Alkylbenzene
LAS	Linear Alkylbenzene Sulfonates
LC 50	Lethal Concentration, 50%
MALDI-TOF-MS	Matrix Assisted Laser Desorption/Ionization Time of Flight-Mass Spectrometry
MSM	Mineral Salt Media

NL	Newfoundland and Labrador
NRPOP	Northern Region Persistent Organic Pollution Control
NRPS	Nonribosomal Peptide Synthetases
OD	Optical Density
OFAT	One Factor at A Time
ONP	o-Nitrophenol
ONPG	o-Nitrophenyl-β-D-Galactopyranoside
ORF	Open Reading Frame
PAH	Polycyclic Aromatic Hydrocarbons
PBS	Phosphate-buffered Saline
PFOS	Perfluorooctanesulfonate
PHCs	Petroleum Hydrocarbons
QS	Quorum Sensing
RSM	Response Surface Methodology
SAS	Secondary Alkane Sulfonates
SDS	Sodium Dodecyl Sulfate

ST	Surface Tension
TCA	Trichloroacetic acid
TLC	Thin Layer Chromatography
TPH	Total Petroleum Hydrocarbon

CHAPTER 1

INTRODUCTION

1.1 Background and Challenges

(1) Soil and Groundwater Contamination

In the past decades, the oil and gas industry in Canada has been boosted due to a growing energy demand. Oil exploration and production activities have encouraged regional expansion with thousands of job opportunities opened and billions of tax revenues generated (Banat et al., 2010; Deloitte, 2013; Verma et al., 2013). Setting as one of the starting points for the marine transportation of crude oil and petroleum products in Canada, new refineries have been constructed in southern Newfoundland, and expansion of transportation pipelines from Alberta, and Saskatchewan to Atlantic region have been proposed (Provencher, 2008; TransCanada, 2016; Verma et al., 2013). The storage, refining and transportation of petroleum hydrocarbons (PHCs) will pose a potential risk of oil spill along the involved regions including Newfoundland and Labrador (NL). Problems associated with soil and groundwater contamination, owing to the release of PHCs have been highlighted. To date, among 22,000 contaminated or suspected contaminated sites currently listed on the Federal Contaminated Sites Inventory (FCSI), 11,986 sites are contaminated with PHCs (e.g., aliphatic, aromatic, BTEX (benzene, toluene, ethylbenzene, xylenes) and polycyclic aromatic hydrocarbons (PAHs)) in urban, rural and remote areas across Canada (Treasury Board of Canada Secretariat, 2017). This problem has been acquiring growing attention of the public, governments and industries.

Spill accidents in the northern region (e.g., NL) are more damaging as the ecosystem is generally more fragile and sensitive, and the system recovery requires longer time than in warmer climates (Yang et al., 2012). The restoration of those petroleum contaminated sites is much more expensive and time-consuming for local industries and governments given the remote access, high energy costs and environmental conditions (e.g., low temperatures, low nutrients and soil heterogeneity). Furthermore, the co-existence of appreciable amounts of heavy metals in PHCs contaminated sites during oil spill has been widely acknowledged (Hussain and Gondal, 2008; Moreno et al., 2011), but very limited concerns have been expressed over the potential risk of co-existed carcinogenic metals in oils (Wise Jr et al., 2014). These contaminated sites adversely affect the human health and environmental compatibility, and lead to financial loss and reinvestment for local industries and governments.

Selection of an effective remediation strategy at the contaminated sites is extremely challenging in the northern region. The solubility of PHCs, also recognized as the controlling removing mechanism, is very limited in subsurface systems due to the hydrophobic nature (Bisht et al., 2015). A reduced permeability has also been identified due to the low subsurface temperature. Therefore, limited PHCs availability to oxidative and reductive chemicals, and/or microorganisms when applied to in-situ and/or ex-situ remediation techniques could lead to a poor recovery rate. This situation has hindered the efforts to effectively protect environments of this region. Hence, there

has been a growing recognition for the urgent need of more efficient, environmentally friendly and thoroughly tested technologies suited to soil remediation in northern regions and beyond (Lee et al., 2015).

(2) The importance of biosurfactants: advantages and applications

Biosurfactants, surface-active biomolecules produced by microbes, have attracted an increasing attention as amphipathic surface-active compounds (Muthusamy et al., 2008). Biourfactant enhanced soil water remediation is an emerging technology for enhanced removal of organic contaminants from the subsurface (Deshpande et al., 1999). Biosurfactants have diverse structures, and are capable of reducing the surface tension (ST) and interfacial tension (Singh and Cameotra, 2004). The addition of biosurfactants could allow well mixing of PHCs compounds and water, stimulate the entrapment of oil droplets into surfactant micelles, and enhance the apparent solubility and partitioning of PHCs compounds into water (Beal and Betts, 2000; Damrongsiri et al., 2013; Lanzon and Brown, 2013). Through prompting metal ion desorption from solid surfaces, forming metal-surfactant complexes, and reducing the interaction between heavy metals and microbes, biosurfactants could reduce the toxicity of heavy metals in contaminated sites (Gnanamani et al., 2010; Miller, 1995). Due to their distinctive surface activity character, biosurfactants have been widely used as detergents, emulsifiers, and foaming and dispersing agents in the fields of environmental, petroleum and pharmaceutical industries (Pacwa-Plociniczak et al., 2011).

Biosurfactants contain remarkably diverse chemical structures, such as glycolipids, lipopeptides, protein-polysaccharide complexes, phospholipids, and fatty acids. In comparison to the chemical counterparts, which are mostly synthesized from petroleum by-products, biosurfactants have high biodegradability, low toxicity, and a better foaming property and higher selectivity (Zhu et al., 2016). They are active even under harsh environmental conditions, such as extreme temperatures, pH and salinity. The development of biosurfactant-mediated remediation technique therefore needs to be greatly motivated (Bezza and Chirwa, 2016).

Despite the enormous potential for environmental applications, the high production cost and low productivity are major barriers in the economic competitiveness of biosurfactant production (Gudina et al., 2015a). Continuous research efforts have been spared to bring down the production costs for a wider commercial use. Muthusamy et al. (2008) pointed out that raw materials accounted for 10-30% of the overall production cost. An adapted microbial growth substrate or feed stock for low cost is anticipated. Furthermore, the complex regulation system during fermentation and limited effective production cells have also been identified to impact biosurfactant production rate (Chen and Chang, 2006). Last but not least, proper tailoring of growth substrate and optimized fermentation conditions can generate the desirable biosurfactant products to suit different applications (Benincasa et al., 2010).

Therefore, enhanced biosurfactant production can be achieved through the

development of efficient production bioprocesses, including the selection of the alternative waste substrates for economical biosurfactant production enhancement of cell density during fermentation, and optimization of cultural conditions (Makkar et al., 2011; Zhu et al., 2016).

(3) Biosurfactant enhanced soil and groundwater remediation: advantages and challenges

Biosurfactant enhanced remediation technologies (e.g., bioremediation and soil washing) have been proven as effective and reliable alternatives through both experimental studies and field applications for cleaning up PHCs-contaminated soil and groundwater (Zhang et al., 2011). **Bioremediation** is the process of using living microbes, usually bacteria, yeast and fungi, to degrade and convert hazardous contaminants into less toxic or nontoxic compounds (Portier, 2013). Bioremediation has been proven to be an effective, reliable, cost-efficient and eco-friendly substitute to traditional technologies (Zhang et al., 2011). The presence of appropriate pollutant-degrading microorganisms, proper environmental conditions, as well as the availability of PHCs are the key to a successful bioremediation (Khan et al., 2004; Sandrin and Hoffman, 2007). Biosurfactant addition could further enhance PHCs biodegradation through improved mobility and bioavailability. **Soil washing** has been another effective technology to remove contaminants from soil in recent years (Zhou et al., 2013). The integration of biosurfactants with washing solution could result in a more effective

washing solution. The washing solution can accelerate the desorption of contaminants from soil particles and enhance their solubilization into groundwater (Trellu et al., 2016).

Although applications of biosurfactant enhanced bioremediation and soil washing as effective remediation tools have been widely acknowledged, they are still facing challenges when applied in co-contaminated sites. Till now, the underlying mechanisms of biosurfactants enhanced desorption and PHCs biodegradation with the existence of heavy metals remain unclear. Extensive work has been carried out to explain the heavy metal toxicities to microorganisms, and their negative impacts on PHCs bioavailability (Sandrin and Hoffman, 2007; Thavamani et al., 2015). The activity of oil degrading microbes could be greatly affected by the metal stress (i.e., the existence of heavy metals), as a result of metal-cell surface interaction (Sandrin and Hoffman, 2007). The role of biosurfactants in PHCs or heavy metals contaminated systems has been investigated (Das et al., 2009a; Singh and Cameotra, 2004; Zhu et al., 2016). However, in a co-contaminated system, biosurfactant induced oil degradation, cell surface modification, cell activity stimulation, and the resulting PHCs solubilization and degradation remains unclear (Liu et al., 2016; Smułek et al., 2015). Therefore, an in-depth understanding of biosurfactant enhanced bioremediation and soil washing are highly required and would lead to a remarkable improvement of existing technologies.

1.2 Research Objectives

This dissertation research targeted the development of systematic experimental

approaches for the advancement of biosurfactants production and application in soil and groundwater. Environmentally friendly biosurfactant products were generated in an economically-efficient manner through using a waste-based substrate and an optimized fermentation process. Systematic experimental examination was conducted to develop technically feasible solutions for solving the challenging petroleum hydrocarbons (PHCs) and/or heavy metal contamination problems.

This dissertation research entailed the following tasks: 1) to produce lipopeptide biosurfactants by marine bacterium *Bacillus Substilis* N3-1P using fish waste as an unconventional medium; 2) to enhance lipopeptide productivity through immobilizing robust biocatalysts on porous fly ash generated by *Bacillus Substilis* N3-1P; 3) to optimize biosurfactant production by *Bacillus Substilis* N3-4P and the application for crude oil removal; and 4) to investigate the effect of a lipopeptide biosurfactant generated by *Bacillus Substilis* N3-1P on the biodegradation of hydrocarbons in presence of heavy metals.

1.3 Structure of the Thesis

Chapter 2 presents a comprehensive literature review of PHCs and heavy metal co-contamination, biosurfactants and their production, as well as biosurfactant aided remediation technologies.

Chapter 3 describes an enhanced biosurfactant production through using local

fishery waste streams as an alternative substrate. The enzymatic hydrolysis condition of fish waste was optimized using the response surface methodology (RSM). Enhanced biosurfactant production with fish-waste-based peptone was examined using marine originated five Bacillus strains.

Chapter 4 investigates a cost-effective and highly efficient biosurfactant production bioprocess through using fly ash (FA) as a solid carrier. The effects of FA on the growth of the biosurfactant producer, *Bacillus Substilis* N3-1P, and its biosurfactant production were evaluated. The effects of FA dosage on biosurfactant production were examined using parameters including surface tension, emulsification activity, and solution dilution as responses.

Chapter 5 further evaluates enhanced biosurfactant production by a *Bacillus Substilis* N3-4P strain through a manipulation of carbon and nitrogen sources. Biosurfactant productions with different media compositions was investigated. This lab generated biosurfactant product that was further tested as a washing agent for PHCs removal from soil.

Chapter 6 investigates the effect of a lipopeptide biosurfactant (generated in Chapters 4) on PHCs (i.e., diesel oil) biodegradation by *Rhodococcus erythropolis* M-25 under heavy metal stress. The interactions of lipopeptide biosurfactant with heavy metals and an oil degrading strain were observed. Performance of the lipopeptide on the distribution of diesel oil and its biodegradation were evaluated and their relationships

with cell surface properties were established.

Chapter 7 concludes this thesis with summarized research findings, contributions to knowledge, and recommendations for further studies.

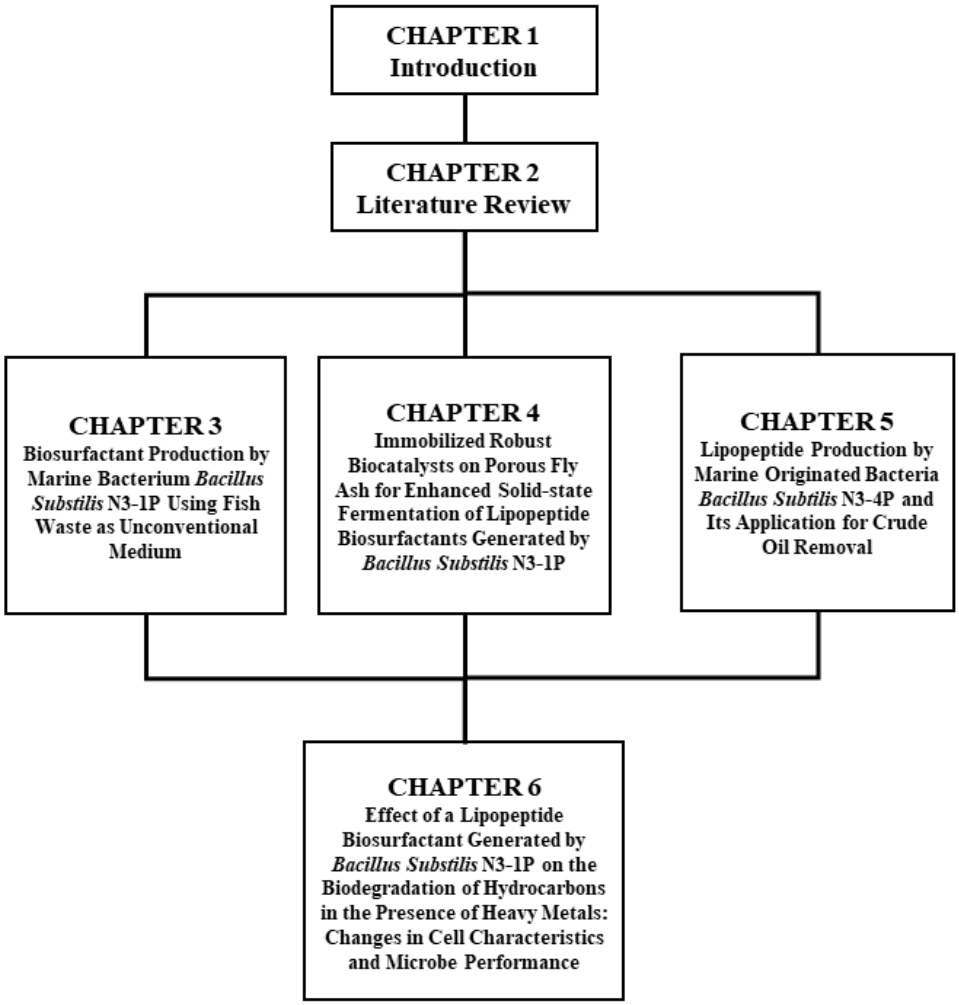


Figure 1-1 Schematic diagram of the thesis structure

CHAPTER 2

LITERATURE REVIEW

This chapter is based on the following manuscript:

Zhu, Z. W., Cai, Q., Zhang, B., Chen, B., and Lin, W. (2018). Advances in lipopeptide production and environmental application. *Biotechnology and Bioengineering.* (to be submitted)

Role: Zhiwen Zhu is the principal investigator of this study and acted as the first author of this manuscript under Dr. Baiyu Zhang and Dr. Bing Chen's guidance. Most contents of this paper were written by her and further edited by the other co-authors.

2.1 Subsurface Contamination of PHCs and Heavy Metals

There has been an increasing concern over the release of PHCs during industrial processes, such as oil exploration, drilling, refinement, transportation, oil processing and storage, stemming primarily from their complex structure and slow biodegradability. According to the International Tanker Owners Pollution Federation (ITOPF), approximately 5.73 million tonnes of oil have been released into the environment during 1970 to 2016 as a result of tanker incidents (ITOPF, 2017). PHCs are one of the most widespread soils contaminants in Canada, too. PHCs account for around 60% of the identified contaminants among the contaminated sites in Canada (Government of Canada, 2017). These released PHCs left unaddressed, will accumulate in the environment, and create a set of serious and long-lasting problems.

When released into soil and groundwater, the poorly soluble PHCs readily adsorb onto hydrophobic soil particles and soil organic matters, and significantly affect soil physical and chemical properties (Ren et al., 2018). The water and air diffusion in the soil pores are slowed and/or even blocked accordingly. Soil microbe activities and composition thus are affected (Williams et al., 2006). In addition, significant physical and chemical changes, such as PHCs composition, viscosity, and density, take place after spills (Annunciado et al., 2005, Lee et al., 2015). All these factors need to be accounted for in the cleanup strategy development.

The potential contaminants associated with PHCs include inorganic materials

such as trace metals, and in some cases naturally occurring radioactive materials (Williams et al., 2006). The metals present in the crude oils are mostly cobalt (Co^{2+}) and nickel (Ni^{2+}) (Khuhawar et al., 2012). Other reported metal ions in PHC compounds include Cu, Pb, Fe, Mg, Na, Zn, Cd, Ti, Mn, Cl, Na, Co, Ur, Al, and As. (Khuhawar et al., 2012). Different from PHCs, heavy metals are generally immobile and nonbiodegradable over time and thus persist in the subsurface for a long term, leaving adverse impact of heavy metals on the microbe activities. The bioavailability of heavy metals can be affected by the physical (e.g., temperature, phase association, adsorption, and sequestration), chemical (e.g., octanol/water partition coefficients, complexation kinetics, and thermodynamic equilibrium), and biological (e.g., species characteristics, trophic interactions, and biochemical/physiological adaption) factors (Tchounwou et al., 2012).

Soil and groundwater contaminated by a complex mixture of PHCs and heavy metals has become one of the major environmental concerns (Dong et al., 2013). The existence of cytotoxic heavy metals, even at low concentrations, may damage indigenous oil degrading microbes and inhibit PHCs biodegradation (Ojuederie and Babalola, 2017; Ramadass et al., 2016). Therefore, remediation strategies aimed at reducing the heavy metal toxicity and improving the PHCs biodegradability are desired.

2.2 Advancement of Biosurfactant Production

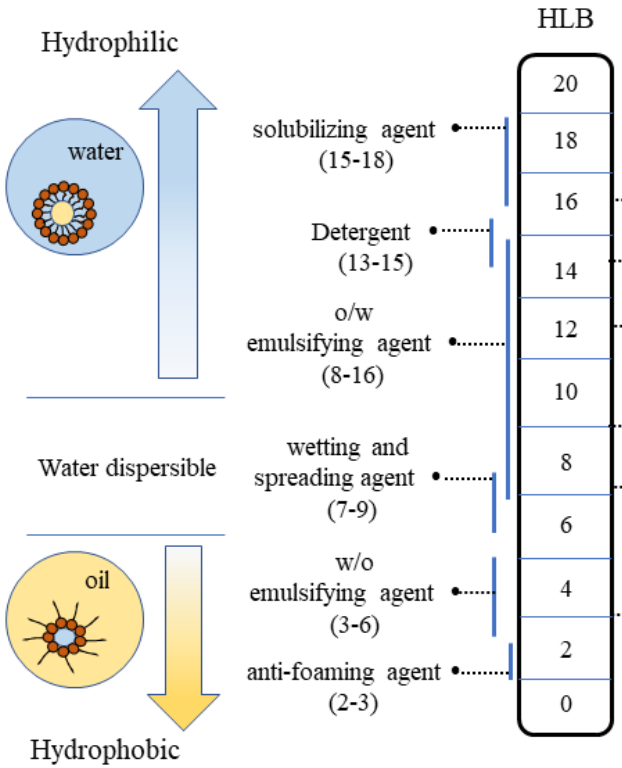
2.2.1 Surfactants and Biosurfactants

A surfactant is the amphiphilic surface-active agent containing a hydrophilic head and a hydrophobic tail (Takata and Ohshima, 2016). The non-polar hydrocarbon portion (e.g., $(CH_2)_n$, $(CF_2)_n$, $(SiR_2\text{-}O\text{-})_n$), also known as hydrophobic tail, interacts weakly with the water molecules. The hydrophilic portion (e.g., -COOH, $-SO_3H$, $-SO_3H$, $-NR_4^+$, $-CH_2\text{-}CH_2\text{-}O-$), formed by the polar or ionic groups, has a strong interaction with water molecules (Tadros, 2014).

Surfactants are normally classified on the basis of their hydrophilic groups, namely anionic, cationic, amphoteric, and nonionic ones, as Figure 2-1 illustrates. Anionic surfactants are the most widely used in industrial applications due to their highly potent detergency and low cost of manufacture (Che et al., 2003). They possess anionic functional groups at their head, such as carboxylates, sulfonate, phosphate, sulfate and isethionates. The most prominent anionic surfactants are linear alkylbenzene sulfonates (LAS), alcohol ether sulfates (AES), and secondary alkane sulfonates (SAS) (Steber, 2007). Other anionic surfactants include dioctyl sodium sulfosuccinate (DOSS), perfluorooctanesulfonate (PFOS), linear alkylbenzene (LABs). They tend to have a better foaming ability compared with other classes of surfactants (Williams, 2007). Cationic surfactants have positively charged ionic groups at the hydrophilic heads. Instead of using as an effective detergent, a cationic surfactant can be used as

antimicrobials, and anti-fungals (Rhein, 2007; Vieira and Carmona-Ribeiro, 2006). They are generally stable to pH changes, either in the acidic or alkaline environment. The most common cationic surfactants are the quaternary ammonium compounds such as cetyl trimethylammonium bromide (CTAB) and cetyl trimethylammonium chloride (CTAC). Amphoteric surfactants are the ones that have both ionic groups attached to the same molecule. They respond to the environment, and act as the anionic or cationic surfactant based on the environment pH. Nonionic surfactants do not dissociate when dissolved into water as they do not have any charge groups in the heads. A wide variety of surfactants belong to nonionic surfactants, such as Spans (sorbitan esters) and Tweens (Polysorbates), Brij, polyglycerol alkyl ethers, and glucosyl dialkyl ethers (Sonia et al., 2014).

The amphiphilic structure of a surfactant can also be characterized and classified by the **hydrophilic-lipophilic balance (HLB)** value (Figure 2-1). Selection of the proper surfactant for an environmental application on the basis of the HLB has been considered as one of the best-known methods (Tadros, 2006). HLB is a numerical system used to describe the relationship between the water soluble and oil soluble parts of a surfactant, giving a result on a scale of 1 to 20 (Williams, 2007). A low HLB value indicates a low water solubility, and stabilizes a water in oil (w/o) emulsion, whereas a high HLB value gives a better water solubility, thus easily forms an oil in water (o/w) emulsion.



SURFACTANT	HLB	CHARGE TYPE	CMC	MW (G/MOL)	REFERENCE
TWEEN 20	16.7	nonionic	0.08	1227	(Kim et al., 2001)
TWEEN 80	15.0	nonionic	0.01	429	(Wu et al., 2010)
TRITON X-100	13.4	nonionic	0.24	628	(Stavroudis, 2009)
CTAB	10	cationic	1.0	364.5	(Barut et al., 2005)
SPAN 20	8.6	nonionic	0.06	346	(Kim et al., 2001)
SPAN 80	4.3	nonionic	0.02	429	(Wu et al., 2010)

Figure 2-1 Introduction of commonly used chemical surfactants in the environmental field

Surfactants tend to adsorb at the interface between water and air or water and oil in an oriented fashion. By replacing the bulk molecules of higher energy, they can reduce the free energy of the system (Mulligan et al., 2001), thereby decreasing the surface and interfacial tension. The interfacial free energy, referred to as **surface or interfacial tension** γ, is given in $\mathbf{mNm^{-1}}$. As surfactant concentration increases, free surfactant monomers gradually accumulate and form spheroid or lamellar micelles. The surfactant concentration above which the micelle forms is named as the **critical micelle concentration (CMC)** (Rosen, 1978). It commonly used to express the efficiency of a surfactant, as micelle formation enables surface tension reduction, and organic contaminants solubility and bioavailability enhancement (Pacwa-Plociniczak et al., 2011). Surfactants can effectively adsorb onto soil particles, and increase the solubility of petroleum components or lower the interfacial tension to enhance mobility of the petroleum. The lower the petroleum concentration, the less quantities of surfactants that need to be applied to the system. Surface activity makes surfactants excellent emulsifiers, foaming and dispersing agents that have been widely used in industrial applications (Mulligan et al., 2001). However, the toxicity of some chemical surfactants poses as a growing concern for the environment, limiting their further applications. Therefore, it is desired to produce an environmentally friendly and highly efficient alternative for industrial applications.

Biosurfactants are surface-active compounds generated by microorganisms

during their growth (Banat et al., 2010). A biosurfactant usually consists of a hydrophilic tail formed by mono-, oligo- or polysaccharides, peptides or proteins; and a hydrophobic head formed by saturated, unsaturated, branched or hydroxylated fatty acids or fatty alcohols (Lovaglio et al., 2015; Pacwa-Plociniczak et al., 2011). This amphiphilic structure displays an affinity between two substances with different degrees of polarity, meanwhile, reduces the surface and interfacial tensions. Compared with chemical surfactants, they have higher biodegradability, less toxicity, better foaming properties and higher activity at extreme temperature, pH and salinity. The most active biosurfactants can lower the surface tension of water from 72 to 27 $mN \cdot m^{-1}$ and the interfacial tension between water and n-hexadecane from 40 to 1 $mN \cdot m^{-1}$ (Singh et al., 2018).

Bosurfactants are a group of diverse structural biomolecules produced by a variety of microorganisms. Compared with chemical surfactants, they have the following advantages:

- ***Great structure diversity.*** To date, over 2,000 biosurfactants have been described in the literature (Kosaric and Sukan, 2014) They have diverse structures in hydrophobic moiety, varying from short simple to long complex fatty acids. Functions and physiochemical properties vary with structures (Soberón-Chávez and Maier, 2011).

- ***Environmental Specificity.*** Being a complex organic molecule with diverse

specific functional groups and high selectivity, biosurfactants have been reported to have specific physico-chemical properties and to be active at extreme environmental conditions (e.g., temperature, pH and salinity) (De et al., 2015). They are reported to have better adaption to changes of the environment (Pacwa-Plociniczak et al., 2011). These properties therefore highlight their importance in contaminated sites cleanup, oil de-emulsification and recoveries, and pharmaceutical and food applications (Pacwa-Plociniczak et al., 2011).

- ***Low eco-toxicity. A*** study compared the acute toxicity of biosurfactants and synthetic surfactants used in oil spill remediation to two estuarine species, and a higher LC_{50} (lethal concentration, 50%) value of the chemical surfactant was reported (Edwards et al., 2003; Klosowska-Chomiczewska et al., 2011).

- ***Complete biological degradability.*** Compared with chemical surfactants mostly produced from petroleum products, biosurfactants are more easily biodegradable. The biological feature of biosurfactants, on the other hand, offers them the inherent feature of relatively high biodegradability. Studies have proved a high biodegradation rate of sophorolipid, surfactin, and arthrofactin (Klosowska-Chomiczewska et al., 2011).

The above-mentioned unique properties make the applications of biosurfactants

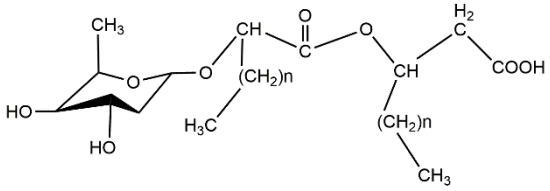
a very promising alternative for the application in the environmental industry. Both organic and inorganic contaminants can be removed through different processes (physical, chemical, and biological) in which biosurfactants are involved (Muthusamy et al., 2008).

Biosurfactants have been extensively produced and studied within recent years. Unlike chemical surfactants that are classified by the polar groups, biosurfactants are usually classified on the basis of their biochemical nature. Table 2-1 illustrates the classification of biosurfactants products and their origins, and Figure 2-2 exhibits the chemical structures of the representative biosurfactants. Usually, low molecular weight biosurfactants include glycolipids, phospholipid and lipopeptides. Their molecular weights generally range from 500D to 1500D (Mulligan, 2009), and are efficient in lowering surface and interfacial tensions. High molecular weight biosurfactants cover amphipathic polysaccharides, lipopolysaccharides, lipoproteins or complex mixtures of these biopolymers. They are recognized for their emulsion-stabilizing capabilities (Soberón-Chávez et al., 2011). Emulsans (Table 2-1) are the best known high-molecular-weight biosurfactants mainly produced by *Acinetobacter sp*. They are highly effective emulsifiers even in concentrations of 0.01-.0001% (Kosaric and Sukan, 2014).

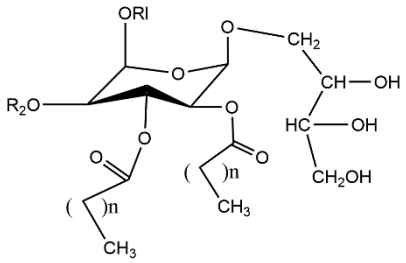
Table 2-1 Classification of surfactants by chemical structure

Chemical structure	Biosurfactants	Origins	References
Glycolipid	Rhmnolipid	*Pseudomonas Chlororaphis., Pseudomonas. Plantarii., Pseudomonas Putida., Pseudomonas Fluorescens., etc.*	(KK and Rahman, 2014; Wittgens et al., 2011)
	Sophorolipids	*Candida Bombicola., Candida Apicola., Candida. Batistae., Torulopsis. Bombicola., Candida. Lypolytica., Candida. Bombicola., Torulopsis.Apicola., Torulopsis.Petrophilum., Candida. Bogoriensis.*	(Van Bogaert et al., 2007)
	Cellobiolipids	*Ustilago Zeae., Ustilago Maydis.*	(Soberón-Chávez and Maier, 2011; Tran et al., 2014)
	Mannosylerythritol lipids	*Pseudozyma Antarctica. (yeast)., Candida Antartica., Ustilago Maydis.*	(Arutchelvi and Doble, 2011; Soberón-Chávez and Maier, 2011)
	Trehalolipids	*Mycobacterium Tuberculosis., Rhodococcus Erythropolis., Arthrobacter Paraffineus., Nocardia Erythropolis., Corynebacterium Lepus.*	(Franzetti et al., 2010)
Fatty acids, phospholipids and neutral lipids	Corynomycolic acid	*Corynebacterium Lepus.*	(Kosaric and Sukan, 2014)
	Spiculisporic acid	*Penicillium Spiculisporum.*	(Ishigami et al., 1983)
	Phosphati-dylethanolamine	*Acinetobacter*, *Rhodococcus Erythropolis.*	(Hirata et al., 1978; Singer and Finnerty, 1990)
Lipopeptides and lipoproteins	Surfactin, Fengycin and Iturin	*Bacillus Substilis., Bacillus. Licheniformis., Bacillus. Vallismortis., Bacillus. Mojavensis., Bacillus. Sonorensis., Bacillus. Thuringiensis., etc.*	(Mnif and Ghribi, 2015; Roongsawang et al., 2010)

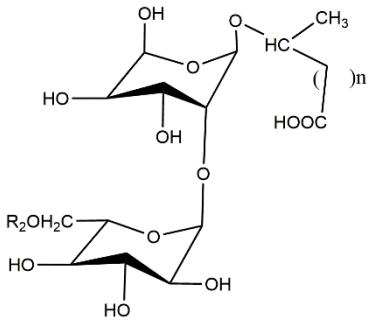
	Viscosin, Massetolide and Pseudophomin	*Pseudomonas Corrugate., Pseudomonas Fiuorescens., Pseudomonas Putida., Pseudomonas Tolaassii., Pseudomonas Syringae., Pseudomonas Entomophila., etc.*	(Mnif and Ghribi, 2015; Roongsawang et al., 2010)
Polymeric	Lipoheteropolysaccharide (Emulsan)	*Acinetobacter Calcoaceticus., Acinetobacter Venetianus., Acinetobacter Lwoffii., etc.*	(Nakar and Gutnick, 2001; Panilaitis et al., 2007)
	Alasan	*Acinetobacter Calcoaceticus., Acinetobacter Radioresistens.*	(Navon-Venezia et al., 1995)
	Liposan	*Candida Lipolytica.*	(Cirigliano and Carman, 1985)
	Manno-protein	*Saccharomyces Cerevisiae.*	(Cameron et al., 1988)
	Heteropolysaccharide (Biodispersan)	*Acinetobacter Calcoaceticus.*	(Dehghan–Noudeh et al., 2007)
	Carbohydrate-protein	*Candida Petrophillum, Endomycopsis Lipolytica.*	(Hardatt and Prakash, 2013; Kaur et al., 2010)
Particulate Biosurfactants	Membrane vesicles	*Acinetobacter Spp.*	(Käppeli and Finnerty, 1979)
	Fimbriae, whole cells	*Acinetobacter Calcoaceticus.*	(Kosaric and Sukan, 2014)



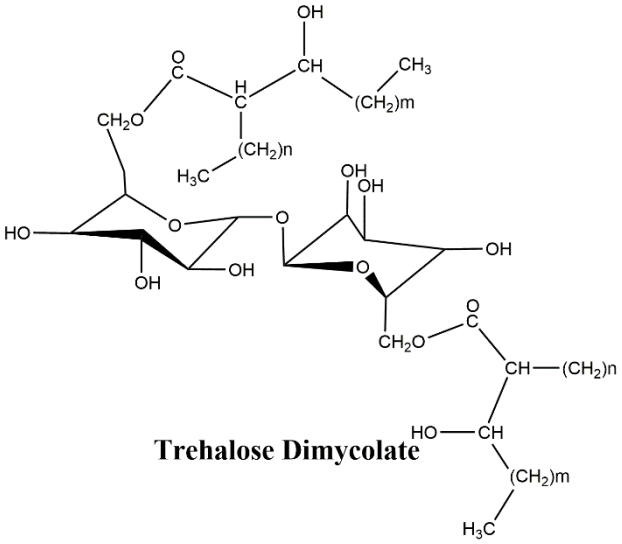
Monorhamnolipid



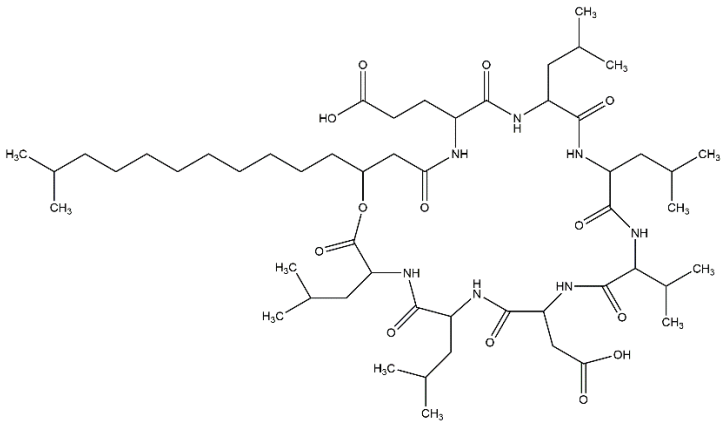
Mannosylerythritol lipids



Sophorolipid



Trehalose Dimycolate



Surfactin

Figure 2-2 Chemical structures of some reported biosurfactant active compounds

2.2.2 Lipopeptide Biosurfactants

Lipopeptides are a distinguished class of biosurfactants produced by a wide range of microbes. Owing to their diverse structural and functional characteristics, they have extremely low CMCs with surfactant, antimicrobial, or cytotoxic activities (Mulligan et al., 1999). Therefore, they attract interest in environmental, food, agricultural, pharmaceutical, and cosmetic industrial fields (Pacwa-Plociniczak et al., 2011).

The lipopeptide biosurfactant product was first produced from the Gram-positive strain *Bacillus Substilis* IAM1213 (Roongsawang et al., 2010). Since then, the discovery of novel lipopeptide compounds has been exponentially growing. Over 263 lipopeptides have been produced by 11 different genera of bacteria and fungi with significant surface activities and/or anti-microbial activities (Coutte et al., 2017). Structurally, they are constituted by a fatty acid (saturated, unsaturated, or hydroxylated) in combination with a hydrophilic peptide moiety (peptides) and correspond to an isoform group that differs by the composition of the peptide moiety, the length of the fatty acid chain, and the link between the two parts. Several isoforms can be produced by the same strain (Mnif and Ghribi, 2015). Table 2-2 reviews and represents the structures of lipopeptides and their isoforms reported to date. Among the considered genera, *Bacillus* and *Pseudomonas* are the most studied, mainly due to a relatively higher productivity of natural strains (several hundred mg L^{-1}) (Coutte et al., 2017).

As Table 2-2 illustrates, the hydrophobic head of the lipopeptide is composed of diverse types of fatty acid (FA) with a β-OH or β-NH_2 group. The β-OH or β-NH_2 group forms an ester or peptide bond with the carboxyl group of the C-terminal amino acid. Surfactin, iturin, fengycin and lichenysin are among the most documented lipopeptides produced by *Bacillus* isolates. Also, viscosin, tensin, arthrofactin, pseudofactin and syringomycin are widely described lipopeptides mainly produced by *Pseudomonas* isolates (Table 2-2) (Mnif and Ghribi, 2015). These antibiotics are either cyclopeptides (iturin) or macrolactones (fengycin and surfactin) characterized by the presence of L and D amino acids and variable hydrophobic tails (Wang et al., 2004). Surfactin has a cyclic lactone ring structure consisting of a C_{12}-C_{16} β-hydroxy fatty acid attached to a heptapeptide with a variable amino acid at positions 2, 4 and 7 (Bonmatin et al., 2003). As a cyclic lipodecapeptides, fengycin contains a β-hydroxy fatty acid with a side chain length of 16 to 19 carbon atoms. Four D-amino acids and ornithine (a nonproteinogenic residue) are in the peptide portion of fengycin (Koumoutsi et al., 2004). Fengycin A and fengycin B are the two variants with Val and Ala respectively, at position 6 (Vanittanakom et al., 1986; Villegas-Escobar et al., 2013). Iturin has a C_{14}-C_{17} β-amino fatty acid moiety linked to a cyclic heptapeptide moiety with Asp or Asn at position 1 (Bonmatin et al., 2003).

Table 2-2 Structures of Lipopeptides family and their isoforms

Name	Structure	Origins	References
Surfactin Family			
Surfactin	FA-β-OH-l-Glu-l-Leu-d-Leu-l-Val-l-Asp-d-Leu-l-Leu	*Bacillus spp.*	(Cochrane and Vederas, 2016; Inès and Dhouha, 2015; Jacques, 2011)
Lichenysin	A/D FA-β-OH-l-Gln-l-Leu-d-Leu-l-Val-l-Asp-d-Leu-l-Ile	*Bacillus spp.*	
Lichenysin B	FA-β-OH-l-Glu-l-Leu-d-Leu-l-Val-l-Asp-d-Leu-l-Leu	*Bacillus spp.*	
Lichenysin C	FA-β-OH-l-Glu-l-Leu-d-Leu-l-Val-l-Asp-d-Leu-l-Ile	*Bacillus spp.*	
Pumilacidin	G FA-β-OH-l-Gln-l-[A_2]-d-Leu-l-[A_4]-l-Asp-d-Leu-l-[A_7] A_2 = Leu/Ile, A_4 = Val/Ile, A_7 = Ile/Val	*Bacillus spp.*	
Pumilacidin	FA-β-OH-l-Glu-l-Leu-d-Leu-l-Leu-l-Asp-d-Leu-l-[A_7] A_7 = Ile/Val	*Bacillus spp.*	
Fengycin Family			
Fengycin	FA-β-OH-l-Glu-d-Orn-d-Tyr-d-*a*Thr-l-Glu-d-[A_6]-l-Pro-l-Gln-l-Tyr-l-Ile A_6 = Ala/Val	*Bacillus spp.*	(Cochrane and Vederas, 2016)
Plipastatin	FA-β-OH-l-Glu-D-Orn-l-Tyr-D-*a*Thr-l-Glu-D-[A_6]-l-Pro-l-Gln-D-Tyr-l-Ile A_6 = Ala/Val	*Bacillus spp.*	(Roongsawang et al., 2010)
Agrastatin1	N/A	*Bacillus spp.*	(Patel et al., 2011)
Iturin Family			
Iturin A	FA-β-NH_2-l-Asn-d-Tyr-d-Asn-l-Gln-l-Pro-d-Asn-l-Ser	*Bacillus spp.*	(Inès and Dhouha, 2015)
Iturin C	FA-β-NH_2-l-Asp-d-Tyr-d-Asn-l-Gln-l-Pro-d-Asn-l-Ser	*Bacillus spp.*	(Roongsawang et al., 2010)
Bacillomycin L	FA-β-NH_2-l-Asn-d-Tyr-d-Asn-l-Ser-l-Glu-d-Ser-l-Thr	*Bacillus spp.*	(Cochrane and Vederas, 2016)
Bacillomycin D	FA-β-NH_2-l-Asn-d-Tyr-d-Asn-l-Gln-l-Pro-d-Asn-l-Thr	*Bacillus spp.*	(Cochrane and Vederas, 2016)

Bacillomycin F	FA-β-NH_2-l-Asn-d-Tyr-d-Asn-l-Gln-l-Pro-d-Ser-l-Asn	*Bacillus spp.*	(Cochrane and Vederas, 2016)
Mycosubtilin	N/A	*Bacillus spp.*	(Cochrane and Vederas, 2016)
Viscosin Family			
Viscosin	FA-β-OH-l-Leu-d-Glu-d-*a*Thr-d-Val-l-Leu-d-Ser-l-Leu-d-Ser-l-Ile	*Pseudomonas spp.*	(Roongsawang et al., 2010)
Viscosinamide	FA-β-OH-l-Leu-d-Gln-d-*a*Thr-d-Val-l-Leu-d-Ser-l-Leu-d-Ser-l-Ile	*Pseudomonas spp.*	(Geudens et al., 2013)
Massetolide A	FA-β-OH-l-Leu-d-Glu-d-*a*Thr-d-*a*Ile-l-Leu-d-Ser-l-Leu-d-Ser-l-Ile	*Pseudomonas spp.*	(Rokni-Zadeh et al., 2013)
Pseudophomin A	FA-β-OH-l-Leu-d-Glu-d-*a*Thr-d-Ile-d-Leu-d-Ser-l-Leu-d-Ser-l-Ile	*Pseudomonas spp.*	(Dahiya, 2013)
Pseudodesmin A	FA-β-OH-l-Leu-d-Gln-d-*a*Thr-d-Val-d-Leu-d-Ser-l-Leu-d-Ser-l-Ile	*Pseudomonas spp.*	(Geudens et al., 2013)
Syringomycin Family			
Syringomycin A	FA-β-OH-l-Ser-d-Ser-d-Dab-l-Dab-l-Arg-l-Phe-z-Dhb-l-$Asp_{(3\text{-}OH)}$-l-$Thr_{(4\text{-}Cl)}$	*Pseudomonas spp.*	(Hamley, 2015)
Syringostatin A	FA-β-OH-l-Ser-d-Dab-l-Dab-d-Hse-l-Orn-l-*a*Thr-z-Dhb-l-$Asp_{(3\text{-}OH)}$-l-$Thr_{(4\text{-}Cl)}$	*Pseudomonas spp.*	(Kahlon, 2016)
Syringotoxin B	FA-β-OH-l-Ser-d-Dab-l-Gly-d-Hse-l-Orn-l-*a*Thr-z-Dhb-l-$Asp_{(3\text{-}OH)}$-l-$Thr_{(4\text{-}Cl)}$	*Pseudomonas spp.*	(Menestrina et al., 2013)
Pseudomycin A	FA-β-OH-l-Ser-d-Dab-l-Asp-d-Lys-l-Dab-l-*a*Thr-z-Dhb-l-$Asp_{(3\text{-}OH)}$-l-$Thr_{(4\text{-}Cl)}$	*Pseudomonas spp.*	(Menestrina et al., 2013)
Cormycin A	FA-β-OH-l-Ser-d-Orn-l-Asn-d-Hse-l-His-l-*a*Thr-z-Dhb-l-$Asp_{(3\text{-}OH)}$-l-$Thr_{(4\text{-}Cl)}$	*Pseudomonas spp.*	(Strano, 2014)
Amphisin Family			
Anikasin	N/A		(Götze et al., 2017)
Arthrofactin	FA-β-OH-d-Leu-d-Asp-d-*a*Thr-d-Leu-d-Leu-d-Ser-l-Leu-d-Ser-l-Ile-l-Ile-l-Asp	*Pseudomonas spp.*	(Lange et al., 2012)
Amphisin	FA-β-OH-d-Leu-d-Asp-d-*a*Thr-d-Leu-d-Leu-d-Ser-l-Leu-d-Gln-l-Leu-l-Ile-l-Asp	*Pseudomonas spp.*	(Hamley, 2015)

Lokisin	FA-β-OH-d-Leu-d-Asp-d-*a*Thr-d-Leu-d-Leu-d-Ser-l-Leu-d-Ser-l-Leu-l-Ile-l-Asp	*Pseudomonas spp.*	(Schlusselhuber et al., 2018)
Pholipeptin	FA-β-OH-d-Leu-l-Asp-l-Thr-d-Leu-d-Leu-d-Ser-D-leu-d-Ser-d-Leu-l-Ile-d-Asp	*Pseudomonas spp.*	(Schlusselhuber et al., 2018)
Tensin	FA-β-OH-d-Leu-d-Asp-d-*a*Thr-d-Leu-d-Leu-d-Ser-l-Leu-d-Gln-l-Leu-l-Ile-l-Glu	*Pseudomonas spp.*	(Schlusselhuber et al., 2018)
Tolaasin Family			
Tolaasin I	FA-β-OH-Dhb-Pro-Ser-Leu-Val-Ser-Leu-Val-Val-Gln-Leu----Val-Dhb-*a*Thr-Ile-Hse-Dab-Lys	*Pseudomonas spp.*	(Hamley, 2015)
Fuscopeptin	FA-β-OH-Dhb-Pro-Leu-Ala-Ala-Ala-Ala-Val-Gly-Ala-Val-Ala---Val-Dhb-*a*Thr-Ala-Dab-Dab-Phe	*Pseudomonas spp.*	(Grgurina, 2013)
Corpeptin	FA-β-OH-Dhb-Pro-Ala-Ala-Ala-Val-Val-Dhb-Hse-Val-aIle-Dhb-Ala-Ala-Ala-Val-Dhb-*a*Thr-Ala-Dab-Ser-Ile	*Pseudomonas spp.*	(Huang et al., 2015)
Syringopeptin Family			
SP22	FA-β-OH-Dhb-Pro-Val-Val-Ala-Ala-Val---Val-Dhb-Ala-Val-Ala-Ala-Dhb-*a*Thr-Ser-Ala-Dhb-Ala-Dab-Dab-Tyr	*Pseudomonas spp.*	(Roongsawang et al., 2010)
SP25	FA-β-OH-Dhb-Pro-Val-Ala-Ala-Val-Leu-Ala-Ala-Dhb-Val-Dhb-Ala-Val-Ala-Ala-Dhb-*a*Thr-Ser-Ala-Val-Ala-Dab-Dab-Tyr	*Pseudomonas spp.*	(Roongsawang et al., 2010)
SP25[Phe_{25}]	FA-β-OH-Dhb-Pro-Val-Ala-Ala-Val-Leu-Ala-Ala-Dhb-Val-Dhb-Ala-Val-Ala-Ala-Dhb-*a*Thr-Ser-Ala-Val-Ala-Dab-Dab-Phe	*Pseudomonas spp.*	(Roongsawang et al., 2010)
Others			
Antiadhesin	FA-β-OH-l-Asp-l-Leu-l-Leu-l-Val-l-Val-l-Glu-l-Leu	*Bacillus spp.*	(Liu et al., 2007)
Bamylocin A	FA-β-OH-x-Glu-x-Leu-x-Met-x-Leu-x-Pro-x-Leu-x-Leu	*Bacillus spp.*	(Lee et al., 2007a)
Circulocin 1	*g*FA-β-OH-x-Thr-x-Phe-x-Ile-x-Dba-x-Asp	*Bacillus spp.*	(He et al., 2001)
Circulocin 3	*g*FA-β-OH-x-Thr-x-Leu-x-Ile-x-Thr-x-Asn-x-Ala	*Bacillus spp.*	(He et al., 2001)

Fusaricidin	*g*FA-β-OH-l-Thr-d-Val-l-Tyr-d-*a*Thr-d-Asn-d-Ala	*Bacillus spp.*	(Bionda et al., 2013)
Kurstakins	FA-x-Thr-x-Gly-x-Ala-x-Ser-x-His-x-Gln-x-Gln	*Bacillus spp.*	(Béchet et al., 2012)
Entolysin	FA-β-OH-d-Xle-d-Glu-d-Gln-d-Val-d-Xle-d-Gln-d-Val-d-Xle-d-Gln-d-Ser-l-Val-l-Xle-d-Ser-x-Xle	*Pseudomonas spp.*	(Rokni-Zadeh et al., 2013)
Ofamide	FA-β-OH-l-Leu-d-Glu-d-*a*Thr-d-*a*Ile-l-Leu-d-Ser-l-Leu-l-Leu-d-Ser-l-Val	*Pseudomonas spp.*	(Inès and Dhouha, 2015)
Pseudofactin	$CH_3(CH)_{14}CO$-x-Gly-x-Ser-x-Thr-x-Leu-x-Leu-x-Ser-x-Leu-x-Leu/Val	*Pseudomonas spp.*	(Janek et al., 2016)
Syringafactin	FA-β-OH-d-Leu-d-Leu-d-Gln-l-Leu-d-Thr-l-Val-d-Leu-l-Leu	*Pseudomonas spp.*	(Pauwelyn et al., 2013)
Pontifactin	Palmitic acid-Ser-Asp-Val-Ser-Ser	*Pontibacter korlensis*	(Balan et al., 2016)
Mixirin	FA-l-Asn-d-Tyr-d-Asn-l-Gln-l-Ser-d-Asn-l-Pro	*Bacillus subtilis and Bacillus amyloliquefaciens*	(Cochrane and Vederas, 2016)

2.2.3 Lipopeptide Synthesis and Genetic Regulation

Lipopeptides belong to the subgroup of nonribosomally produced peptides (Schwarzer et al., 2003). Their synthesis is modularly controlled and carried out by a series of large sized multi-enzyme complexes called nonribosomal peptide synthetases (NRPS) (Martínez-Núñez and y López, 2016). In this modular organization, a section of the polypeptide chain in NRPS is regarded as a module and sometimes can be divided into domains. Each module incorporates one amino acid into the final group, and the domains in the modules are responsible for catalysis of individual peptides synthesis steps (Schwarzer et al., 2003).

The gene clusters directing NRPS for lipopeptides synthesized by *Bacillus sp.* have been identified and characterized. Research findings have demonstrated the evolutionary lineages of this strain. The genetic regulation of the biosynthesis of lipopeptide by *Bacillus* has been reviewed by Roongsawang et al (2010). A summary of genetic machinery involved in the synthesis of surfactin, a widely used lipopeptide, is presented in Table 2-3. Surfactin synthesis involves NRPS with four open reading frames (ORFs) in the *srf*A operon, namely *srf*AA*, srf*AB, *srf*AC and *srf*AD (Lee et al., 2007b; Nakano et al., 1991). Each frame is responsible for one amino acid addition. For instance, surfactin synthetase I and II are encoded by *srf*AA and *srf* B, respectively. The *srf*AA contains three amino acid activating domains for glutamate, leucine and D-leucine, while *srf*AB contains the peptide synthesizing domain for activating valine,

aspartate, and D-leucine. In addition to activating leucine, their ORF SrfAC also encodes a thioesterase for peptide termination. Gene sfp, located downstream of the *srf*A operon, plays a significant regulatory role in lipopeptide synthesis (Das et al., 2008).

Those involved genes for lipopeptide synthesis are reported to be closely regulated by the quorum sensing (QS) system in response to cell density. This is a cell-cell communication system for assisting collective behavior within a community (Fletcher and Mullins, 2010). In this system, signal molecules, also referred as auto-inducers, are secreted to coordinately initiate complex dynamic behaviors. If properly regulated, strains are expected to outpace monocultures in performing complicated tasks (Scott and Hasty, 2016).

Bacillus spp. represent a typical QS system of Gram-positive bacteria. Four proteins are involved in the system, namely the ComQ isoprenyl transferase, the ComX signal peptide, the ComP histidine kinase, and the ComA response regulator (Oslizlo et al., 2014). Like the acyl-HSL in Gram-negative bacteria, ComX serves as the QS regulatory signal molecule extracellularly secreted by *Bacillus Substilis.* This peptide is then processed and modified by the isoprenyl transferase ComQ. Once ComX is accumulated to a critical concentration, it is apperceived by the membrane bounded receptor ComP. A phosphate group can be donated to the response regulator ComA by the receptor ComP. The phosphorylated ComA (ComA-P) then activates the gene

expression of *srf*A operon for the nonribosomal synthesis of surfactin (reference). Oslizlo et al. (2014) evaluated the influence of additional exogenous ComX on surfactin production using a signal deficient mutant strain. Their study confirmed the contribution of specific ComX–ComP interaction to *srf*A expression, which led to an overproduction of surfactin. At the exponential growth stage, a low expression of *srf*A was identified. A constantly secrete of ComX increases the growth of *Bacillus* cells. When cells approach the stationary phase, *srfA* is found actively involved in surfactin synthesis (Schneider et al., 2002). The concentration of ComX pheromone therefore is important in determining the expression of quorum-responsive genes (*srf*A). On the other hand, the *Rap-Phr* quorum-sensing pairs exhibit inhabitation to the important response regulators (e.g., ComA, Spo0F, or DegU) on basis of the cell growth rate (Bendori et al., 2015). Similarly, the expression of the *srf*A operon is also directed by regulators, such as DegU, AbrB, and CodY, in the system. Therefore, a continuous surfactin synthesis is limited by the cell-dependent QS regulation system.

In addition to the complex regulation system, poor surfactin production also contributed to limited effective *Bacillus Substilis* cells (approximately 10%) that was capable of sensing ComX pheromone for further surfactin production. Furthermore, studies recently indicated that surfactin could also serve as an extracellular signaling molecule that triggers the production of subpopulation of *Bacillus* cells (Zhi et al., 2017a). This process led to a communication interruption between ComX and ComP.

Table 2-3 Genetic machinery involved in surfactin synthesis from *Bacillus spp*.

Operon/Genes/Operator/ Promotear/Protein	Function	References
Genetic regulation of NRPSs for lipopeptide synthesis		
***Srf*AA*, *Srf* AB**	Efficient sporulation Amino acid activating domain for Glu, Leu, D -Leu Expression of *com*S gene	(Stachelhaus et al., 1999)
Srf*AC	Encodes a thioesterase of a Type I motif responsible for peptide termination	(Roongsawang et al., 2010)
***Srf*AD**	Encodes for the thioesterase domain (TE domain), responsible for cyclization of linear surfactin	(Pathak et al., 2014)
sfp	Surfactin production act in trans to promote surfactin production.	(Nakano et al., 1992)
sfp*	Activation of surfactin synthetase by post translational modification	(Nakano et al., 1992)
Involved quorum sensing system in regulating lipopeptide synthesis		
ComQ	Modification of comX to form signal peptide ComX	(González-Pastor, 2017)
ComP (Membrane receptor)	Sense ComX when critical concentration achieves, and autophosphorylates and activates the cognate response regulator ComA	(Ohsawa et al., 2006; Roongsawang et al., 2010)
ComA-	Phosphorylated ComA bind to ComA boxes, acting as a positive regulator to initiate the transcription of surfactin synthetase	(Lazazzera et al., 1997)
ComX (Signal peptide)	Controls expression of *srf*A and interaction with membrane bound histidine kinase ComP; respond regulator ComA	(Satpute et al., 2010)
SpoOK (Oligopeptide permease) RapC	Transfer of Competence stimulating factor (CSF) through the cell membrane; Phosphotransferase activity	(Das et al., 2008; Satpute et al., 2010)
ComR (Polynucleotide phosphorylase)	Enhances *srf*A expression posttranscriptionally	(Fabret et al., 1995)
SinR (Transcriptional regulator)	Negatively controls *srf*A possibility by regulating *com*R	(Jacques, 2011)

*** Part of peptide synthetase; ⁑ Multifunctional subunit of surfactin synthetase**

Instead of response to ComX, original surfactin production *Bacillus* cells commenced extracellular matrix production (López et al., 2009). Surfactin production was therefore reduced.

Unveiling the surfactin synthetic processes could contribute to a better design of biosurfactant production methods for cost-effective production of lipopeptides. Surfactin synthesis, highly dependent on cell density, faced the challenging constant production and limited overall production rate under the regulation of QS system. Technology development in promoting effective biosurfactant production cells is highly desired.

2.2.4 Economical Lipopeptide Production through Using Waste Substrates

Comparing the expected global surfactant market of US$ 44.9 billion by the end of 2022, the biosurfactant market is estimated to be US$ 36 billion, at the end of 2017, with an annual production of 340,000 tons per year (Cision, 2018; Reuters, 2018). On the other hand, the focus on sustainable production and “green” product standards worldwide has led to an increasing demand for environmentally friendly biosurfactant products (Marchant and Banat, 2012). Till now, commercialization of lipopeptide biosurfactants remains to be a problem owning to the high production cost (Marin et al., 2015). The expected breakthrough in terms of their applications as a substitution of chemical surfactants remains to be achieved. Continuous research efforts need to be

further devoted to proper medium manipulation through utilization of waste streams as substrates (Mukherjee et al., 2006).

Raw materials generally account for 10% to 30% of the total production costs in most biotechnological processes (Mukherjee et al., 2006). Research attention has been directed to the utilization of cheaper and renewable substrates for biosurfactant production (Makkar et al., 2011). Millions of tons of hazardous and non-hazardous wastes are generated each year worldwide. The treatment and disposal of these wastes therefore, not only create financial burden to various industries but also lead to environmental concerns. Those wastes, such as the ones generated from vegetable processing industries (e.g., plant oil, corn steep liquor), dairy and sugar (e.g., sugars, molasses) wastes, starchy substances (e.g., potato, rice mill), and other food processing industries have been widely evaluated in terms of their applicability for biosurfactant production.

A wide spectrum of carbon sources, ranging from petroleum derivatives (e.g., diesel, hexadecane, glycerol), to natural originated substrates (e.g., sucrose, glucose), have been evaluated for lipopeptide production. As cheaper substitutes, the effects of waste streams on lipopeptide production by *Bacillus spp.* have been reviewed in Table 2-4. All these studies aimed to cut the lipopeptide production costs by using cheaper raw materials and have gained various successes.

Vegetable processing wastes have attracted most of the attention as raw material

for lipopeptide production (Makkar et al., 2011). Plant derived wastes have shown promising application in effective lipopeptide production. For example, the toxic compounds in olive oil mill wastes, such as polyphenols, makes them unsuitable for human consumption, however, the nutrient compounds, such as sugars, organic acids, and free fatty acids in the raw material can serve as a carbon source for lipopeptide biosurfactant production (Maass et al., 2016). Nowadays, enzymatic hydrolysis has been increasingly examined and evaluated as a pretreatment methodology to obtain the bioavailable active compounds present in waste materials to promote biosurfactant production. Pre-hydrolysis of olive oil mill waste (Ramírez et al., 2016), lignocellulosic waste (Faria et al., 2014), peat (Sheppard and Mulligan, 1987), corncob (Chen et al., 2017), wheat straw (Prabu et al., 2015), grape marc (Rivera et al., 2007), soybean hull (Marti et al., 2015), and sisal pulp (Marin et al., 2015) to release sugars present in the cellulose and hemicellulose fractions for biosurfactant production have been validated.

Vegetable oil: Particularly, vegetable oils generated from the vegetable processing industry have served as the other carbon candidates for biosurfactant production. Palm oil (Khondee et al., 2015), sunflower oil (Hazra et al., 2015), and corn oil (Chander et al., 2012; Ghribi et al., 2012; Ghribi and Ellouze-Chaabouni, 2011) have all been tested as alternative carbon sources for biosurfactant production. It is worth being mentioned that, in addition to the application of palm oil as a substitute carbon source, Khondee et al. (2015) immobilized a biosurfactant producing strain onto a solid

material. A final biosurfactant production was screening to 10.9 g L^{-1}.

Dairy and sugar industry wastes: Molasses, generated after a series of evaporation, crystallization and centrifugation of sugarcane juice, is a by-product of sugar production from cane and/or the sugar beet industry. This dark viscous fluid is rich in sugars, suspended colloids, amino acids, vitamins, metal ions, and salts. Whey, the liquid by-product of cheese production, contains high levels of lactose (75% of dry matter) and 12-14% protein. Besides, organic acids, minerals, and vitamins are presented inside it. Given that only half of the produced cheese whey can be recycled, the disposal of the rest becomes a major problem for the industry. The waste effluent from this industry, however, supports good microbial growth and thus can be used as a cheap raw material for lipopeptide production (Gomaa, 2013; Reis et al., 2004). Nitschke and Pastore (2004) used molasses, and milk whey wastewater for their initial production of biosurfactant. Compared to a synthetic medium, lactic whey wastes might be a better substrate for biosurfactant production. Furthermore, the use of dairy wastewaters shed light on a stratagem for the economical lipopeptide production and efficient dairy wastewater management.

Starch rich substrates: Starchy waste materials are also potential alternatives for biosurfactant production. In addition to abundant carbohydrate, starch wastes mostly contain protein, fat, vitamins, inorganic minerals and trace metals too, which makes lipopeptide production applicable (Fox and Bala, 2000; Noah et al., 2005; Zhi et al.,

Table 2-4 Lipopeptide Biosurfactant production by *Bacillus* strains from waste/renewable substrates

Waste/by-Product	Pre-treatment	Biosurfactant-Producing Microorganism	Product	Highest Reported Productivity	References
Vegetable processing by-products					
Olive oil mill waste	Hydrolysis	*Bacillus Substilis*	Surfactin	0.0265 g L^{-1}	(Ramírez et al., 2016)
Olive oil mill waste	N/A	*Bacillus Substilis*	Crude Lipopeptide/ Surfactin	0.249/0.0077 g L^{-1}	(Maass et al., 2016)
Palm oil mill waste	Filtration	*Bacillus Substilis*	Surfactin	0.03-0.035 g L^{-1}	(Abas et al., 2013)
Lignocellulosic waste	Hydrolysis	*Pseudozyma antarctica*	Mannosylerythritol lipids	2.5 g L^{-1}	(Faria et al., 2014)
Peat hydrolysate	Hydrolysis	*Bacillus Substilis*	Surfactin	N/A	(Sheppard and Mulligan, 1987)
Corncob	Hydrolysis	*Bacillus Substilis*	Surfactin	0.523 g L^{-1}	(Chen et al., 2017)
Corn steep liquor	N/A	*Bacillus Substilis*	Surfactin	1.3 g L^{-1}	(Gudina et al., 2015a)
Grape mac	Hydrolysis	*Lactobacillus pentosus*	Surfactin	0.0048 g L^{-1}	(Rivera et al., 2007)
Soybean hull	Hydrolysis	*Bacillus Substilis*	Surfactin	0.235-0.312 g L^{-1}	(Marti et al., 2015)
Soybean curd	N/A	*Bacillus Substilis*	Iturin A	3.3 g kg^{-1}	(Mizumoto et al., 2006)
Sisal pulp	Hydrolysis	*Bacillus Substilis*	Surfactin	0.136 g L^{-1}	(Marin et al., 2015)
Vegetable oils					
Sunflower oil	N/A	*Bacillus clausii*	Surfactin	2.6 g L^{-1}	(Hazra et al., 2015)
Palm oil	N/A	*Bacillus sp* (immobilized)	Crude lipopeptide	10.9 g L^{-1}	(Khondee et al., 2015)
Corn oil	N/A	*Bacillus Substilis*	Lipopeptide	N/A	(Chander et al., 2012)

Corn oil + glucose	N/A	*Bacillus Substilis*	Lipopeptide	~ 1 g L^{-1}	(Ghribi and Ellouze-Chaabouni, 2011)
Coconut oil +glucose	N/A	*Bacillus Substilis*	Lipopeptide	< 0.8 g L^{-1}	(Ghribi and Ellouze-Chaabouni, 2011)
Dairy and sugar industry wastes					
Cheese whey	N/A	*Bacillus licheniformis*	lipopeptide	0.048 g L^{-1}	(Gomaa, 2013)
Whey distillery waste	N/A	*Bacillus Substilis*	Surfactin	N/A	(Kiran et al., 2010)
Sugarcane juice	N/A	*Bacillus Substilis*	Lipopeptide	N/A	(Reis et al., 2004)
Sugarcane bagasse + Okara	N/A (solid state)	*Bacillus pumilus*	Surfactin	0.809 g L^{-1}	(Slivinski et al., 2012)
Brown sugar	N/A	*Bacillus atrophaeus*	Lipopeptide	0.95 ± 0.071 g L^{-1}	(Zhang et al., 2016a)
Date molasses	N/A	*Bacillus Substilis*	N/A	2.29 g L^{-1}	(Al-Bahry et al., 2013)
Molasses	N/A	*Bacillus Substilis*	Surfactin	3.56 g L^{-1}	(Saimmai et al., 2011)
Starch rich substrates					
Potato waste	Filtrate	*Bacillus Substilis*	Surfactin	2.7 g L^{-1}	(Fox and Bala, 2000; Noah et al., 2005)
Cassava wastewater	Heat and centrifuge	*Bacillus Substilis*	Lipopeptide	3.0 g L^{-1}	(Nitschke and Pastore, 2006)
Distillers' grains	N/A	Co-cultures (*Bacillus Substilis* and *B. amyloliquefaciens*)	Surfactin	3.4 g L^{-1}	(Zhi et al., 2017b)
Rice mill polishing residue	Preheat	*Bacillus Substilis*	Surfactin	4.17 g kg^{-1}	(Gurjar and Sengupta, 2015)
Mixture of rice straw, starch and soybean flour	Milled (Solid state)	*Bacillus amyloliquefaciens*	Lipopeptide	0.0499 g gds^{-1} *	(Zhu et al., 2012)
Other unconventional substrate sources					

Soybean oil waste	N/A	*Bacillus pseudomycoides*	Lipopeptide	N/A	(Li et al., 2016)
Sunflower oil soapstock	N/A	*Bacillus clausii*	Surfactin	2.6 g L^{-1}	(Hazra et al., 2015)
Frying oil	N/A	*Bacillus pumilu*	N/A	5.7 g L^{-1} (crude)	(Oliveira and Garcia-Cruz, 2013; Shah et al., 2007)
Vinasse	Filtration	*Bacillus pumilu*	N/A	27.7 g L^{-1} (crude)	(Oliveira and Garcia-Cruz, 2013)
Orange peel		*Bacillus licheniformis*	Lipopeptide	1.28 g L^{-1}	(Kumar et al., 2016)
Cashew apple	Compress and filtration	*Bacillus Substilis*	Surfactin	0.319 g L^{-1}	(de Oliveira et al., 2013)

*gram of initial dry substrates (gds)
N/A: data not available

2017b). Fox and Bala (2000) highlighted the feasibility of biosurfactant production from potato wastes. In their research, solid and liquid potato waste mediums were prepared for the growth of *Bacillus Substilis* ATCC 21332. Their biosurfactant production was compared with the ones generated from established mineral salt medium. The stimulated solid potato medium reported a highest cell concentration, with a fewer additional nutrients requirement for the biosurfactant production. Potato waste possessed a better lipopeptide production efficiency than the commercially available potato starch.

Another attractive carbohydrate-rich waste substitute, cassava wastewater, is generated during the preparation of cassava flour in large amounts (Nitschke and Pastore, 2006). Lipopeptide biosurfactants were generated by *Bacillus substilis*, at a rate of 3.0 g L^{-1} using cassava wastewater. This biosurfactant was reported to have a high tolerance of elevated temperatures (100°C), high salinity (20% NaCl) and a wide range of pH.

Biosurfactant with excellent surface activity could be produced by adding proposed waste substrates. Further research is needed to continuously explore the candidate and demonstrate their suitability in an industrial-level biosurfactant production process. The problem of proper waste control and management has surfaced for fishery industries worldwide. Substantial amounts (approximately 50 wt.% of harvest) of flesh, skin, bones, entrails or liquid stick water could be generated during

fishery and aquaculture activities. Those waste materials, on the other hand, generally have oily nature and are rich in nutrients (e.g., proteins) (Arvanitoyannis and Kassaveti, 2008). Therefore, they can be used as a substrate for biosurfactant production and could add up to an enormous sum of economic and ecological benefit to the fishery industries worldwide.

2.2.5 System Optimization for Production Enhancement

Carbon and nitrogen sources, metal ions, selected biosurfactant producers, and culture conditions such as pH, temperature, agitation rate, and oxygen availability are the dominant factors in biosurfactant production. Biosurfactants can be produced through using both water soluble substrates (e.g., glycerol, glucose and ethanol) and water immiscible substrates (e.g., vegetable oils, diesel and hexadecane). Biosurfactant productivity is closely related with the type of biosurfactant producers and their preference metabolisms of carbon and nitrogen sources.

Carbon sources: It should be noted that biosurfactant production kinetics, production rates and product structures among different strains vary significantly (Mulligan et al., 2014). The most widely used carbon sources for *Bacillus spp.* are glucose (Najafi et al., 2010), sucrose (Joshi et al., 2008a), glycerol (de Faria et al., 2011), and some water immiscible carbon sources such as alkanes (Joshi et al., 2008a) and food oils (Anjum et al., 2016). For some *bacillus* strains, the biosurfactant production

mechanism can only be triggered when oil is added. Other biosurfactant producers That belong to *Bacillus spp*, generally have a better performance in a water-soluble carbon source. Yeh et al. (2005) evaluated lipopeptide production on glucose by *Bacillus Substilis* ATCC 21332. The importance of glucose was highlighted. After the depletion of glucose, lipopeptide production was decreased. An oversupply of glucose, on the other hand, may lead to a pH reduction of growth medium, as carbohydrate stimulates the production of secondary acid metabolites, such as uronic acid (Zhu et al., 2016). Lipopeptide production thus might be hindered.

Nitrogen sources: The type of nitrogen source is crucial to cell growth and biosurfactant production. Both inorganic and organic nitrogen sources have been studied in biosurfactant production, nevertheless, the most frequently used ones in terms of lipopeptide production have been nitrate salts and ammonia. In general, nitrate ions reported to have a better lipopeptide production. Abdel-Mawgoud et al. (2008) pointed out that the most favorable nitrogen source would be sodium nitrate. It was proposed that addition of nitrate, acting as a terminal electron acceptor under anaerobic conditions, could lead to a nitrogen limited environment, which promotes biosurfactant production. Nitrate utilization can be prolonged, followed by an increased lipopeptide production. Organic compounds such as urea and yeast extract were also determined as potential nutrient sources for biomass simulation. Zhu et al. (2016) highlighted the importance of organic nitrogen in biosurfactant production. In their study, *Bacillus spp* were unable

to synthesis lipopeptide without the existence of organic nitrogen. It was believed that the amine groups in the yeast extract either triggered the biosynthesis of peptide-containing biosurfactants like lipopeptide or stimulated the growth of the enzymes regulating the biosynthesis of biosurfactants (Qazi et al., 2013).

Other than carbon and nitrogen sources, trace elements such as iron and manganese, incubation temperature and the agitation are all important to lipopeptide production. Maximizing lipopeptide productivity or minimizing production costs demands the use of process-optimization strategies that involve multiple factors. One factor at a time (OFAT) is one of the classical methods to optimize the culture condition. However, its features like labor and time consuming, and missing of interaction effects urge the development of advanced optimization tools. Therefore, a statistical optimization strategy, such as response surface methodology (RSM) and taguchi methods could be increasingly used to optimize the culture conditions, and medium composition for lipopeptide production (Zhang et al., 2016b). Through the introduction of the optimization tools, the significant factors can be screened and, the interactions between different factors will be provided. Optimization process assists the industrial design for biosurfactant production, improvements such as an economical medium composition and favorable environmental conditions could be realized.

2.3 Biosurfactant Aided Contaminant Remediation

2.3.1 Biosurfactant Enhanced Soil Washing

Soil washing is an ex-situ soil treatment technique that removes hazardous contaminants from soil by scrubbing soil particles with a liquid (i.e., washing agent). When integrated with pumping activities, this technique can also be carried out to cleanup contaminants in the deeper subsurface. Chemicals are usually added to promote the release of contaminants with low solubility from soil. The use of surfactants to enhance the removal efficiency during soil washing is well documented (Mulligan et al., 2001; Zhou et al., 2013). Particularly, as an environmentally friendly alternative, biosurfactants have attracted increasing attention. It is believed that there are two mechanisms in biosurfactant enhanced soil washing, namely mobilization and solubilization. When the concentration of a biosurfactant is below its CMC value, it is in contact with the soil/oil system, and it reduce the surface and interfacial tension between air/water, oil/water, and soil/water systems. Consequently, the capillary force holding contaminants and soil can be reduced. In addition, the contact angle between soil and contaminants and the mobility of contaminants are both increased (Hernández-Espriú et al., 2013). With the increase of the biosurfactant concentration in the washing solution, the monomers aggregate to form micelles. Released hydrocarbons are incorporated into the chamber formed by the hydrophobic end of biosurfactant when the hydrophobic molecules clustered together. The process, also known as solubilization,

enhances the solubility of PHCs. The linear relationship between the solubilization of contaminants, and the concentration of surfactant has been noted (Mulligan et al., 2001).

Lai et al. (2009) compared the TPH removal efficiency in an oil refinery plant using biosurfactants (rhamnolipid, surfactin), and chemical surfactants (Tween 80 and Triton X-100). When adding 0.2% (w/w) of (bio)surfactant solution, biosurfactants exhibited remarkable removal rates from both slightly (3,000 mg kg^{-1}), and highly (9,000 mg kg^{-1}) TPH contaminated soils. Highly contaminated soil reported a better removal efficiency (63% with rhamnolipid), compared to that of slightly contaminated soil (23% with rhamnolipid). This result shed light on the potential industry application of biosurfactants as soil washing agents.

Bezza and Nkhalambayausi-Chirwa (2015) assessed lipopeptide enhanced PAHs desorption from contaminated soils. The enhanced PAHs desorption was in proportion to the lipopeptide concentration. No significant PAHs desorption was observed at a lipopeptide concentration below to 150 mg L^{-1}. It might due to the reduction of the effective micelle concentration as a result of biosurfactant sorption onto soil surface. Therefore, larger amounts of biosurfactants might be required in contaminated soil samples than in contaminated water samples. Generally, the removal of organic contaminants can be stimulated by the emulsification process, nevertheless, the contaminant volume is increased during this process as well. However, the soil washing process may be hindered once the emulsion is in a relatively immobile and highly

viscous form (Urum and Pekdemir, 2004).

When applied into heavy metal contaminated soil samples, lipopeptides could reduce interfacial tensions between heavy metals and soil particles and form aqueous complexes and/or micelles to enhance the mobility of heavy metals, the removal rates of heavy metal therefore are also improved. Till now, glycolipid biosurfactants have been extensively evaluated as a washing agent in PHCs contaminated soil whereas there have been limited investigations into the performance of lipopeptide biosurfactants as a washing agent.

2.3.2 Biosurfactant Enhanced Bioremediation

Acting as electron donors and carbon sources, PHCs can be readily degraded by microorganisms under aerobic conditions (Meckenstock et al., 2014). Biodegradation is considered as the ultimate mechanism to cleanup PHCs in the environment, and is economical effectively and environmentally friendly (Atlas and Hazen, 2011; Prince, 2005). Microbes can uptake PHCs through direct attachment. This process however, usually hindered by the low solubility and high hydrophobicity of hydrocarbons, and their strong sorption to soil (Zhu and Aitken, 2010). Biosurfactants, therefore, were produced by the microbes to overcome the diffusion-related mass transfer limitations and assist the biodegradation process (Szulc et al., 2014). Extensive research has been focused on the biosurfactant enhanced bioremediation. A positive response of oil degradation to an enhanced biosurfactant concentration has been reported. The role of

biosurfactants in enhanced biodegradation has been assessed. During the bioremediation process, biosurfactant addition facilitates the solubility and bioavailability of hydrocarbons through solubilization, mobilization, and emulsification. Furthermore, biosurfactants have been found to promote the accessibility of microbes to oil droplets owing to the enhanced cell surface hydrophobicity (Pacwa-Plociniczak et al., 2011). At the subcritical concentration, the mobilization mainly takes place through a surface and interfacial tension reduction between air/water and water/soil system. Consequently, the capillary force holding oil and soil was reduced, and the trapped oil droplets were released. At the supercritical concentration, the biosurfactant molecules rapidly formed micelles. The hydrophobic head formed micelle interior created an environment compatible for the hydrophobic organic contaminants. The solubilization of hydrocarbons thus was greatly stimulated (Urum et al., 2006). Furthermore, the HLB of surfactants is of great importance in the bioremediation process. Torres et al. (2005) indicated the beneficial effect of surfactants with low HLB value in enhanced bioremediation of an aged diesel contaminated soil. Biosurfactants, especially the ones with high molecular weight, primarily assist the solubilization oil into waters through effective emulsion formation. The small oil droplets stimulate the biodegradation process, and thus enhance the oil removal rate.

Franzetti et al. (2010) proposed the role of a biosurfactant in the interaction between oil degrading microbes and hydrocarbon contaminants. High cell surface

hydrophobicity eases the direct oil degrading microbes-oil droplets contact whereas a low cell surface hydrophobicity allows the attachment between microbes and micelles or emulsified oils. Through the assistance of biosurfactants, the oil accession mode of the oil degrading strains could be adjusted during their growth.

The study conducted by Cameotra and Singh (2009) further revealed the role of biosurfactants during the hexadecane uptake and its biodegradation by *Pseudomonas* species. The biosurfactant-assisted-dispersion of hexadecane increased the bioavailability to microbes. Under an electron microscope, the uptake of biosurfactant coated hydrocarbons was identified, similar to active pinocytosis. This "internalization" mechanism was firstly reported in this study.

Shin et al. (2004) reported the effect of pH on the solubilization and biodegradation of phenanthrene with a rhamnolipid at a fixed concentration of 240 ppm. Within a tested pH range of 4-8, the optimum solubilization and biodegradation were achieved at pH 4.5-5.5.

Kang et al. (2010) compared the biodegradation of a crude oil contaminated soil using sophorolipid and chemical surfactants (i.e., Tween 80, Tween20, Span 80, Span 20). The addition of nutrients and mineral salts together with sophorolipid achieved a dramatical increase in the biodegradation rate (from 32.6% ± to 80.7% ± 1.14). Though very limited polar compounds were removed, sophorolipid biosurfactant and chemical surfactant all reported a promising biodegradation rate for aromatics and saturates. In

eight weeks. the highest biodegradation rates were achieved using sophorolipid. The removal rates for saturates and aromatics were 80.7% ± 1.14 and 71.7% ± 1.24, respectively.

Bezza and Chirwa (2017b) investigated the pyrene biodegradation enhancement potential of lipopeptide in their study. Owning to the increased solubility, biosurfactant addition significantly increased the uptake of phenanthrene, pyrene and fluorene in all bacterial cultures. The growth of bacteria was stimulated by the addition of lipopeptide, till the optimum concentration was achieved at 600 mg L^{-1}. The optimum biodegradation rate (83%) thus was reported at a biosurfactant concentration of 600 mg L^{-1}. A further biosurfactant addition, nevertheless, exhibited an inhibitory effect to the bacteria growth and biodegradation rate.

Sajna et al. (2015) shared a similar research finding, pointing out that the optimum bacterial growth (four times higher than the control) and oil biodegradation occurred at a biosurfactant concentration of 2.5 mg L^{-1}. Thereafter, bacteria cells decreased rapidly with a further biosurfactant supplement. An average of 23.5% improvement in the degradation of C10- C24 alkanes was observed. Dodecane toped the biodegradation rate, while tetracosane and hexadecane were least degraded. The rate of degradation decreased with an increase in the chain length of hydrocarbon. The preferential pattern of hydrocarbon utilization by microbes when growing in a mixture of complex hydrocarbons suggested that lower chain molecules were utilized in the

initial stage and higher chain alkanes towards the later stage of growth. Nevertheless, the rate of hexadecane utilization was low even though it was not a higher chain length alkane such as hexadecane, which could possibly explain this observation.

Metals, such as nickel, lead, cadmium, etc., are frequently found in oil contaminated sites. The toxic nature of heavy metals, even at trace concentrations, as well as their non-biodegradability, make them a long-term threat in the environment. Though bioremediation is still regarded as a viable solution to those co-contaminated sites, the stress of heavy metals to ingenious microbes has been well documented. Nevertheless, other than the substantial information concerning the mechanisms of metal toxicity, the effect of metals on organic pollutant biodegradation, especially their effect on the response of oil degrading microbes during biodegradation, are poorly characterized.

Biosurfactants have been reported to be capable of selectively complexing cationic metal species, such as cadmium, lead, zinc and copper (Mulligan et al., 1999; Sandrin et al., 2000; Torrens et al., 1998). Ochoa-Loza et al. (2001) use an ion-exchange resin technique to identify the rhamnolipid complex selectivity of heavy metals in a contaminated soil system. The heavy metals, such as Cu (II), Pb (II), and Cd (II) had a higher affinity to complex compared with other ions. Besides the complexation, biosurfactants were able to reduce the toxicity of heavy metals by entrapping metal ions into their micelles. It was therefore believed that the tolerance and resistance of

indigenous microbes in a heavy metal contaminated soil system would be enhanced, and the bioavailability of organic contaminants to indigenous microbes was increased. Anionic biosurfactants, such as surfactin, rhamnolipid, and sophorolipids have all been reported to have a higher efficiency for the removal of copper and zinc from a hydrocarbon-contaminated soil (Singh and Cameotra, 2004).

Gnanamani et al. (2010) evaluated the removal efficiency of chromium (VI) using a biosurfactant producer *Bacillus sp.* MTCC 5514. Their study demonstrated the production of biosurfactants and extracellular enzymes of *Bacillus* could reduce 10-2000 mg L^{-1} of Cr (VI) to Cr (III) within 24-96 h. The extracellular enzyme produced by *Bacillus* helped to reduce highly toxic Cr (VI) to Cr (III). Cr (III) was quickly entrapped by biosurfactant micelles, and kept the activity of bacteria finally achieved a high removal rate. This research provided the possibility of clean up co-contaminated sites with biosurfactant enhanced bioremediation.

Jalali and Mulligan (2007) investigated the biosurfactant enhanced bioremediation potential in an aged petroleum and heavy metal co-contaminated site. By the end of the 50-day experiment, a stimulation of microbe growth was identified. The removal of the total petroleum reached to 36%. The injection of biosurfactant solution increased the heavy metal and hydrocarbon concentration from 2.2% and 2.1% to 4.4% and 8.3%, respectively. This result shed light on the feasibility of using biosurfactant to enhance the bioremediation of co-contaminated soil.

Another batch experiment was conducted by Song et al. (2008) by using saponin to enhance the removal of phenanthrene and cadmium from contaminated soils. When the concentration of saponin reached 3,750 mg L^{-1}, 87.7% and 76.2% of phenanthrene and cadmium could be removed, and this was greater than the use of Triton X100 and citric acid.

Till now, though promising discoveries have been reported, the use of biosurfactant enhanced bioremediation on the biodegradation of PHCs revealed many contradictory reports (Cameotra and Singh, 2009). Biosurfactant enhanced solubilization does not always lead to enhanced biodegradation. The mass transfer of oil droplet from the micellar chamber to the water phase are likely to affect the biodegradation rate. The inhibitory effect of a biosurfactant at a high concentration has also been widely reported (Bezza and Chirwa, 2017b; Sajna et al., 2015). The pore forming abilities and membrane permeabilizing properties possessed by biosurfactants such as iturin, fengycin and lichenysin, can permit the formation mixed micelles with membrane lipids, which may trigger the impairment of membrane integrity, and finally lead to necrosis and lysis of microbe cells (Inès and Dhouha, 2015). Furthermore, the enhanced solubilization of contaminants into the water phase may also be attributed to the decreased microbial activity and biodegradation rate (Obayori et al., 2008; Silva et al., 2014).

Biosurfactant enhanced bioremediation has the advantage of using indigenous

bacteria which can produce biosurfactants that take up hydrocarbons as substrates, and meanwhile remove the co-existing heavy metals. The injection of biosurfactants produced by organisms found to be already present at the contaminated site is another proposed strategy. It is more environmentally compatible and economically available than using modified clay complexes or metal chelators such as EDTA (Jalali and Mulligan, 2007).

Although bioremediation is a promising technology, remediation of sites co-contaminated with PHCs and metal pollutants is an intricate predicament, as two components needed to be treated differently. Research of biosurfactant applications in co-contaminated system are still in an early stage. The role of biosurfactants in this complex subsurface system needs to be identified, and their effects on microbes and contaminants needs to be further investigated.

CHAPTER 3

LIPOPEPTIDE PRODUCTION BY MARINE BACTERIUM *BACILLUS SUBSTILIS* N3-1P USING FISH WASTE AS UNCONVENTIONAL MEDIUM

This chapter is based on the following manuscript:

Zhu, Z. W., Zhang, B.Y., Chen, B. Cai, Q. (2018) Lipopeptide production by marine bacterium bacillus substilis N3-1P using fish waste as unconventional medium. Submitted to *Waste Management.* (submitted)

Role: Zhiwen Zhu is the principal investigator of this study and acted as the first author of this manuscript under Dr. Baiyu Zhang and Dr. Bing Chen's guidance. Most contents of this paper were written by her and further edited by the other co-authors.

3.1 Introduction

Surface-active agents or surfactants are an important class of chemical compounds widely used in different areas, such as environmental, petroleum, pharmaceutical and cosmetics industries (Mulligan, 2009). Surfactants can reduce surface and interfacial tensions, and in the meantime, form water-in-oil or oil-in-water emulsions (Hsu and Nacu, 2003). With increasing environmental awareness and emphasis on a sustainable development, biosurfactants recently have received increasingly attention as an alternative to the chemical ones. Biosurfactants are natural surface-active products produced by microorganisms during their growth (Thavasi et al., 2011). They have some desirable properties such as low toxicity, high biodegradability, high specificity, and strong effectiveness at extreme temperature, salinity and pH conditions (Pacwa-Plociniczak et al., 2011). Nevertheless, only a few biosurfactants are commercialized due to their high production costs. Around 10-30% of the total biosurfactant production cost arises from the raw material (Mukherjee et al., 2006). To decrease this cost and facilitate a wider commercial use, efforts have been devoted to the identification of proper waste medium.

The seafood and marine products industry is one of the major exporters in NL. The cod production has reached $9.4 million in 2012 (Dave, 2014). The industrial fish processing operation, however, also generates large numbers of solid wastes, which has accounted for 30-80% of the body weight of processed fish. If not properly treated, the

wastes will pose significant environmental and health problems. On the other hand, those marine originated wastes can provide proteins with high nutritional properties and a good pattern of essential amino acids. Enzymatic hydrolysis has been recognized as an effective approach to add value to fish wastes. The enzyme method could generate protein hydrolsates with specified functional and nutritional properties without nutrient loss (Kristinsson and Rasco, 2000; Liaset et al., 2000). The high nutrient and hydrocarbon content, as well as the negligible cost make fish waste a good candidate for microbe growth. Therefore, attempts to explore the use of fish peptone as nutrient additives have been reported. Safari et al. (2012) proved that fish head generated peptones promoted the growth of lactic acid bacteria. The growth of lactic acid bacteria on fish viscera was also confirmed (Vázquez et al., 2008). It is therefore hypothesized that fish waste hydrolysate could be a potential candidate to support biosurfactant production.

The identification and optimization of the hydrolysis conditions that affect the fish peptone production represent key points for the development of a cost-competitive biosurfactant production process. Fish wastes compounds, temperature, hydrolysis time, and the enzyme dose are of prime importance in controlling the hydrolysis processes (Bhaskar et al., 2008). The experiment based response surface methodology (RSM) has been widely used for experiment design and model setup by using statistical techniques (Kasiri et al., 2008). The factorial designs and regression analysis have been used to

evaluate multifactor interactions, and further define the desired optimized condition. Therefore, RSM could be employed to investigate the interaction among the controlling factors, and optimize the hydrolysis condition.

Enzyme hydrolysis of fish waste was used prior to the fermentation process, aiming to offer a more bioavailable form of carbon/nitrogen for biosurfactant production. In this study, the hydrolysis condition of fish waste was optimized using RSM and the generated product was used as unconventional substrates for biosurfactant production using marine originated bacterium *Bacillus Subtilis* N3-1P. The production rate was evaluated using critical micelle dilution (CMD). The generated biosurfactant products were characterized using parameters including ST, CMC, emulsification activity, and stability. The chemical composition was examined with thin layer chromatography (TLC). The biosurfactant was further characterized using Fourier Transform Infrared (FTIR) spectroscopy. Additionally, generated biosurfactant products were further characterized using matrix assisted laser desorption/ionization time of flight- mass spectrometry (MALDI-TOF-MS).

3.2 Materials and Methods

3.2.1 Materials

Cod livers and heads were provided by a local store in NL, Canada. Each of them was minced twice using a food processor at a medium speed for 120 seconds. Fresh

samples were taken for proximate composition analysis, and the results are illustrated in Table 3-1. The rest of the wastes were quickly stored in a refrigerator under -20°C for further analysis. Alcalase® 2.4L (endoproteinase from Bacillus licheniformis) (Sigma-Aldrich, Canada) was selected as the hydrolysis enzyme.

3.2.2 Optimization of Enzymatic Hydrolysis

Enzymatic hydrolysis conditions were optimized by employing the RSM with Central Composite design (CCD). Four independent variables (i.e., temperature (A,°C), hydrolysis time (B, hr), enzyme doze (C, %v/w) and different fish wastes (D)) were examined. The final response was defined as degree of hydrolysis (DH). Experiments were separated into three blocks to wave the effects from testing equipment. The parameters, levels and sequences of experimental treatments are summarized in Table 3-2.

The experimental procedures for enzymatic hydrolysis are illustrated in Figure 3-1. In brief, 50 g of waste sample were added into a 125mL Erlenmeyer flask and mixed with equal volumes (50 mL) of distilled water (1:1 w/v). Before Alcalase addition, the flasks were heated in a water bath at 90°C for 10 min to deactivate the endogenous enzymes in the fish wastes. After reactions (as Table 3-2 illustrates), Alcalase was desaturated by heating at 95 °C in a water bath for 10 min. The reaction mixtures were then centrifuged at 6,000 rpm for 20 min. The supernatants were collected and subjected to DH measurement.

Table 3-1 Proximate composition of fish wastes

Constituent	Fish Liver	Fish Head
Moisture (%)	71.3	58.9
Ashes (%)	4.45	10.05
Protein (%)	16.51	13.47

Table 3-2 CCD experiment design of fish waste hydrolysis

Block	Time (hr)	Enzyme dose (%v/w)	T (°C)	Fish waste	Block	Time (hr)	Enzyme dose (%v/w)	T (°C)	Fish waste
Block 3	3	2	45	Head	**Block 3**	3	2	45	Liver
Block 3	3	2	45	Head	**Block 3**	3	2	45	Liver
Block 1	2	1	50	Head	**Block 1**	2	1	50	Liver
Block 2	4	1	50	Head	**Block 2**	4	1	50	Liver
Block 2	2	3	50	Head	**Block 2**	2	3	50	Liver
Block 1	4	3	50	Head	**Block 1**	4	3	50	Liver
Block 3	3	0	55	Head	**Block 3**	3	0	55	Liver
Block 3	3	0	55	Head	**Block 3**	3	0	55	Liver
Block 3	1	2	55	Head	**Block 3**	1	2	55	Liver
Block 3	1	2	55	Head	**Block 3**	1	2	55	Liver
Block 1	3	2	55	Head	**Block 1**	3	2	55	Liver
Block 1	3	2	55	Head	**Block 1**	3	2	55	Liver
Block 2	3	2	55	Head	**Block 2**	3	2	55	Liver
Block 2	3	2	55	Head	**Block 2**	3	2	55	Liver
Block 3	3	2	55	Head	**Block 3**	3	2	55	Liver
Block 3	3	2	55	Head	**Block 3**	3	2	55	Liver
Block 3	5	2	55	Head	**Block 3**	5	2	55	Liver
Block 3	5	2	55	Head	**Block 3**	5	2	55	Liver
Block 3	3	4	55	Head	**Block 3**	3	4	55	Liver
Block 3	3	4	55	Head	**Block 3**	3	4	55	Liver
Block 2	2	1	60	Head	**Block 2**	2	1	60	Liver

Block 1	4	1	60	Head	**Block 1**	4	1	60	Liver
Block 1	2	3	60	Head	**Block 1**	2	3	60	Liver
Block 2	4	3	60	Head	**Block 2**	4	3	60	Liver
Block 3	3	2	65	Head	**Block 3**	3	2	65	Liver
Block 3	3	2	65	Head	**Block 3**	3	2	65	Liver

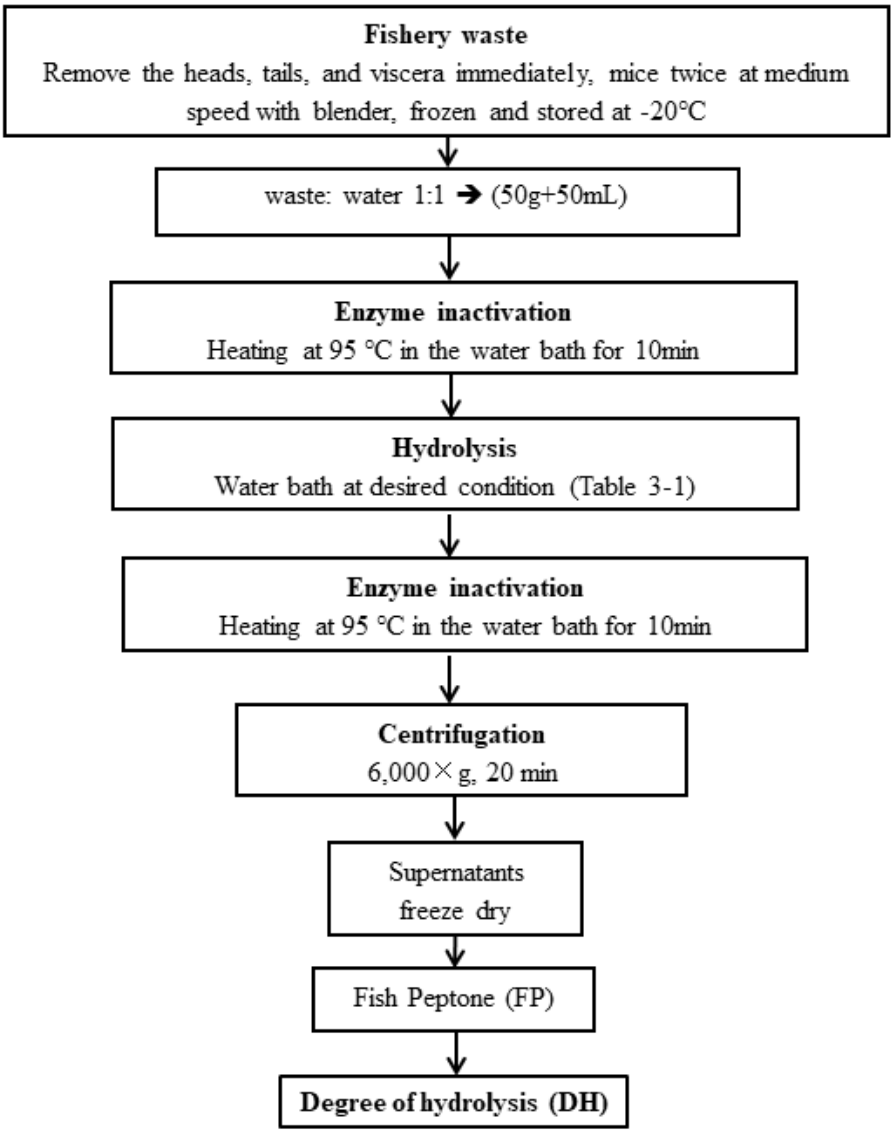


Figure 3-1 Flow chart of enzyme hydrolysis

3.2.3 Biosurfactant Producing Microorganisms

The biosurfactant producers used in this study were screened in northern region persistent organic pollution control (NRPOP) lab from oily contaminated seawater samples (Cai et al., 2014). *Bacillus* strains are a group of well-known biosurfactant producers, whose products can effectively lower the water surface tension to below 30mN/m. Among the screened *Bacillus* strains in the NRPOP lab, *Bacillus Substilis* N3-1P, N3-4P, N2-6P were identified as promising and economic lipopeptide producers (Cai et al., 2014). The commercialized lipopeptide production strain *Bacillus Substilis* 21332 was also selected in this research. The preparation of seed culture followed the method described by Zhu et al. (2016).

3.2.4 Biosurfactant Production and Purification

C/N source substitution: The feasibility of using fish waste generated head and liver peptones as carbon and/or nitrogen sources for biosurfactant production was evaluated. Glycerol (10 g L^{-1}) and NH_4SO_4 (10 g L^{-1}) were used as the carbon and nitrogen sources in control samples. They were replaced with fish head or liver peptones respectively at a concentration of 10 g L^{-1}. The concentrations of the supplemented mineral salts were (g L^{-1}): NaCl (15); $FeSO_4 \cdot 7H_2O$ (2.8×10^{-4}); KH_2PO_4 (3.4); $K_2HPO_4 \cdot 3H_2O$ (4.4); and $MgSO_4 \cdot 7H_2O$ (1.02). The composition of the trace element solution was as follows (g L^{-1}): $ZnSO_4$ (0.29); $CaCl_2$ (0.24); $CuSO_4$ (0.25); and $MnSO_4$

(0.17). The two portions were sterilized separately. Trace element solution was prepared and added at 0.5 ml L^{-1} of distilled water. To evaluate the feasibility of the fish-waste-based growth medium, the strains selected in this study (Section 3.2.3) were inoculated at a ratio of 2%. After incubated in a shaking incubator (200 rpm) at 30 °C for seven days, and each culture broth was centrifuged at 6,000 g for 15 minutes. The cell-free culture broth was then collected. Biosurfactant production was evaluated with ST, emulsification index (EI_{24}) and CMD values.

Alternative comprehensive medium: Biosurfactant productions using fish head and liver peptones as comprehensive growth medium were further investigated. The *Bacillus Substilis* strains able to use fish peptones as carbon and nitrogen sources were selected in this study. Fish head and liver peptones were added into distilled water at a series of concentrations (g L^{-1}): 10, 20, 30, 40 and 60. Key supplement minerals were added as follows (g L^{-1}): $FeSO_4 \cdot 7H_2O$ (2.8×10^{-4}), and $MnSO_4$ (0.17). After incubation in a shaking incubator (200 rpm) at 30 °C for seven days, each culture broth was centrifuged at 6,000 g for 15 minutes. The cell-free culture broth was then collected. Biosurfactant production was evaluated with ST, EI_{24} and CMD values. Biosurfactant products with the highest productivity using lab strains were further characterized.

3.2.5 Characterization of Generated Biosurfactants

Biosurfactant purification: The culture broths were centrifuged at 12,000×g for

10 min. The cell free supernatant was then adjusted to pH 2.0 with HCl and stored overnight at 4 °C. The sediments were then harvested by centrifuging at 12,000×g for 10 minutes. Acidified biosurfactant pellets were dissolved into 100 mL distilled water again. Sodium hydroxide was added to adjust the pH value to 7. Biosurfactant products were recovered with organic solvent extraction. An equal volume of chloroform–methanol (2:1 v/v) were used separately to extract the target biosurfactant products separately. The organic solvents were removed by rotary evaporation.

Characterization: The purified fish waste generated biosurfactants (i.e., liver- and head- based) from section 3.2.5 were subjected to physical-chemical properties characterization. Their water ST reductions and CMC values were examined. The stability of produced biosurfactants was evaluated. The chemical compositions were characterized with TLC. Their structures were determined using FTIR spectroscopy and MALDI-TOF-MS.

3.2.6 Sample Analysis

Proximate composition of fish peptone: Ash content was determined by AOAC 942.05 (AOAC, 2005). Crude protein was measured by AOAC 2001.11 (AOAC, 2005).

$$\mathbf{ash}\%(w/w) = \frac{weight\ of\ ash}{weight\ of\ sample,g} \times 100\% \quad \textbf{(3-1)}$$

***Degree of hydrolysis (DH)*:** DH was estimated using trichloroacetic acid (TCA) method (Holy et al., 1994). Generally, 50mL hydrolysate sample was mixed with 50 mL

20% TCA to create 10% TCA-soluble and TCA-insoluble fractions. The mixtures were centrifuged at 10,000 rpm and the supernatants were analyzed for nitrogen by the macro-Kjcldahl method (AOAC, 1980). The degree of hydrolysis (DH) was calculated as:

$$\boldsymbol{DH = \frac{10\%\ TCA\text{-}soluble\ N\ in\ sample}{Total\ N\ in\ sample} \times 100\%} \quad \textbf{(3-2)}$$

ST: The surface tension (ST) was measured by the ring method using a Du Nouy Tensiometer (CSC Scientific). Fifteen milliliter liquid was subjected to the determination of ST in a petri dish. To ensure the reliability of tested results, the average of three independent measurements was taken.

***CMC and CMD*:** CMC is defined as the surfactant concentration necessary to initiate micelle formation. The CMC of generated biosurfactants was determined by plotting the surface tensions as a function of biosurfactant concentration and it was found from the intercept of two straight lines extrapolated from the concentration-dependent and concentration-independent sections (Figure 3-2) (de Oliveira et al., 2013; Sheppard and Mulligan, 1987). CMD indicates the concentration of biosurfactant in the medium. It corresponds to the dilution this medium required to reach its CMC (Shavandi et al., 2011). It was determined following the method described by Cai et al. (2015).

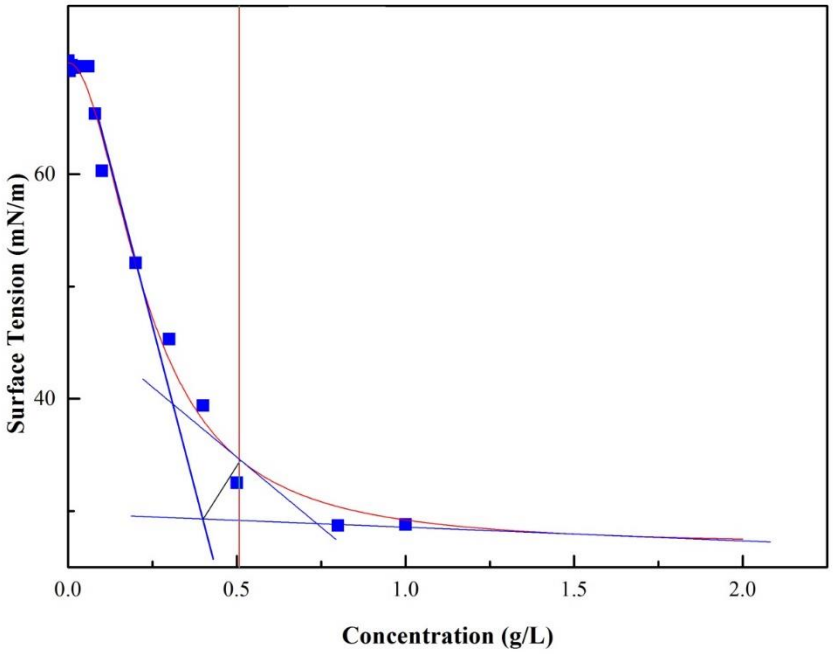


Figure 3-2 Methodology for CMC determination

***EI_{24}*:** The emulsification activity of the culture broth was determined by addition of 2 mL culture aliquot to 2 mL hexadecane and vortexed for 2 min to create an optimum emulsion. Tests were performed in duplicate for quality assurance purposes and the results were expressed using the average of two measurements.

$$\mathbf{EI_{24}} = \frac{\textbf{the height of the emulsified layer}}{\textbf{the height of the total liquid phase}} \times \mathbf{100\%} \qquad \textbf{(3-3)}$$

By repeating the reading after 24 hours, an indication of the stability of the emulsions is obtained. EI_{24}= 0 indicates no emulsification and El_{24} = l means 100% emulsification.

Biosurfactant composition: Fish wastes generated biosurfactants were further analyzed for its chemical constitution with TLC. The biosurfactant sample was dissolved in 1mL of methanol and analyzed on TLC silica gel plates (Sigma Aldrich). The developing solvent used for the chromatography was chloroform:methanol:acetic acid (60:25:5, v/v). The spots were visualized with standard spray reagent as follows: 1) The TLC plate was sprayed with ninhydrin reagent and then heated at 105 for 5 min. The amino acid content will be visualized as a dark purple color with ninhydrin reagent. 2) The plate was sprayed with phenol-sulfuchromic acid and heated at 105 ºC for 5 min. The sugar content on the plate could be spotted with a dark orange or brown color. 3) Insert the plate into an iodine chamber for the characterization of lipid containing spots (purple color).

***Stability of biosurfactant*:** The effect of several environmental parameters on the surface activity of the biosurfactant was determined. NaCl at different concentrations was mixed with the cell free broth for the determination of stability of salinity at 1, 2, 3, and 4%. The pH effect was determined by adjusting the pH value of the cell free broth to different values of 2, 4, 6, 8, and 10 using 1 N NaOH or 1 N HCl. To determine the heat stability of the surface-active compounds, the cell free broth was incubated for 0, 4, 25, 50, 75, and 100 °C for 120 min.

FTIR analysis: Both FA particles and biosurfactant products were examined with FTIR (Bruker Tensor). FA particles were characterized with the KBr-pellet method. Spectral measurements were performed in the transmittance mode. Crude biosurfactant products were directly characterized with Fourier transform infrared-attenuated total reflection (FTIR-ATR) spectroscopy. IR was traced over the range of 400–4000 cm^{-1}. All data were corrected for background spectrum.

MALDI-TOF-MS analysis: The chemical structure of FL and FH based biosufactant products were examined with MALDI-TOF mass spectra by a SCIEX MALDI TOF/TOF System. Each purified biosurfactant sample was dissolved into 10 mL distilled water and then passed through 0.2 μm filter before test. For mass spectrometric analysis of isolated lipopeptide biosurfactants, 2 μL portion of biosurfactant solution was mixed with an equal volume of matrix medium (a saturated solution of α-cyano-4-hydroxycinnamic acid in 50% aqueous acetonitrile containing 0.1%

(v/v) trifluoroacetic acid). The positive-ion detection and the reflector mode were used. The acceleration and reflector voltages were 20 and 23.4 kV in the pulsed ion extraction mode. Postsource decay mass spectra were obtained with the same sample.

3.2.7 Statistical Analysis

Optimizations of the enzymatic hydrolysis were designed and analyzed using Design-Expert® 8.0.6. Design at center points in each factorial block, axial point, and axial (star) points were performed in duplicate (as Table 3-2illustrates). Each enzymatic sample was determined in triplicate for DH. Biosurfactant production experiments were performed in triplicate and analyzed using OriginPro® 9.

3.3 Results and Discussion

3.3.1 Optimization of Fish Waste Hydrolysis

The influences of hydrolysis time (factor A), enzyme-to-substrate ratio (factor B), temperature (factor C) and waste material (factor D) on the enzymatic hydrolysis were determined using RSM. Among the four independent variables, the enzyme-to-substrate ratio (B) ($p < 0.0001$) and hydrolysis temperature (C) ($p < 0.0001$) had a higher impact on the hydrolysis result. The effect of hydrolysis time (A) ($p = 0.0132$), though less than factors B and C, was also considered to be significant ($p<0.05$). The impact of waste composition to the final DH results was little ($p = 0.6450$). The interactions among the

different variables were also limited ($p > 0.05$).

The response surface graphs for DH listed in Figure 3-3 (A)-(C) proved the ANOVA analysis result. Alcalase was employed in previous fish hydrolysis studies because of its high degrees of hydrolysis. Hydrolyzation can be achieved in a relatively short time under moderate conditions (Benhabiles et al., 2012). In this study, Alcalase was found to possess broad specificity to achieve a high DH. The DH of both waste materials (i.e., head and liver) has a positive response to enzyme-to-substrate. The optimized enzyme-to-substrate ratios were estimated at 2.72% for fish liver and 2.92% for fish head, respectively (Table 3-3). A continuous increase of enzyme dose could further improve the DH of fish waste, however, at a slower rate. The Catla (*Catla catla*) hydrolysis study conducted by Bhaskar and Mahendrakar (2008) drew the same conclusion. The growth of DH rate slowed down with an increase of Alcalase dose.

The optimized temperature for two wastes was estimated at 52.51°C and 54.07°C for liver peptone and head peptone, respectively (Table 3-3). The DH was then gradually reduced at a continuous temperature increase. It was believed that the Alcalase slowly become thermally denatured above 55°C. This result was in accordance with the conclusion drew by Ovissipour et al. (2009). Though hydrolysis time has less significance than the enzyme dose and hydrolysis temperature, an increase of this factor could also contribute to a higher DH, as observed in Figure 3-3. Similar to an increase of enzyme dosage, the prolonged hydrolysis time could further improve DH, though at

a slower increase rate.

Following the optimized enzymatic hydrolysis conditions, the verification results are illustrated in Table 3-3. The experiment varication results showed a good agreement between the experimental results and the RSM models.

The characterization of hydrolyzed peptones is listed in Table 3-4. As observed, the nitrogen content was 98.14 and 128.92 mg g^{-1} for fish liver and fish peptones, respectively. These results are in accordance to the values found for the widely used commercial peptones (Table 3-4). Similarly, the C/N ratios for both peptones fell into the range of commercial peptones. Fish head peptone had a relatively higher C/N ratio than the liver peptones. As observed, both peptones possessed high concentrations of TC, TN and C/N contents and thus could be used as good substitutes for traditional biosurfactant production mediums.

3.3.2 Production of Biosurfactants Using Fish Peptones

The feasibility of using fish peptones to support bacteria growth and biosurfactant production as carbon and/or nitrogen sources was investigated. The results are listed in Figure 3-4. As predicated, both peptones could be used as nitrogen sources for all the examined biosurfactant producers. Surface tension reductions were observed in all the samples (i.e., FH (N) and FL (N)). It has been proven that organic nitrogen (e.g., yeast extract or protein hydrolysates) is required as an inducer to stimulate biosurfactant

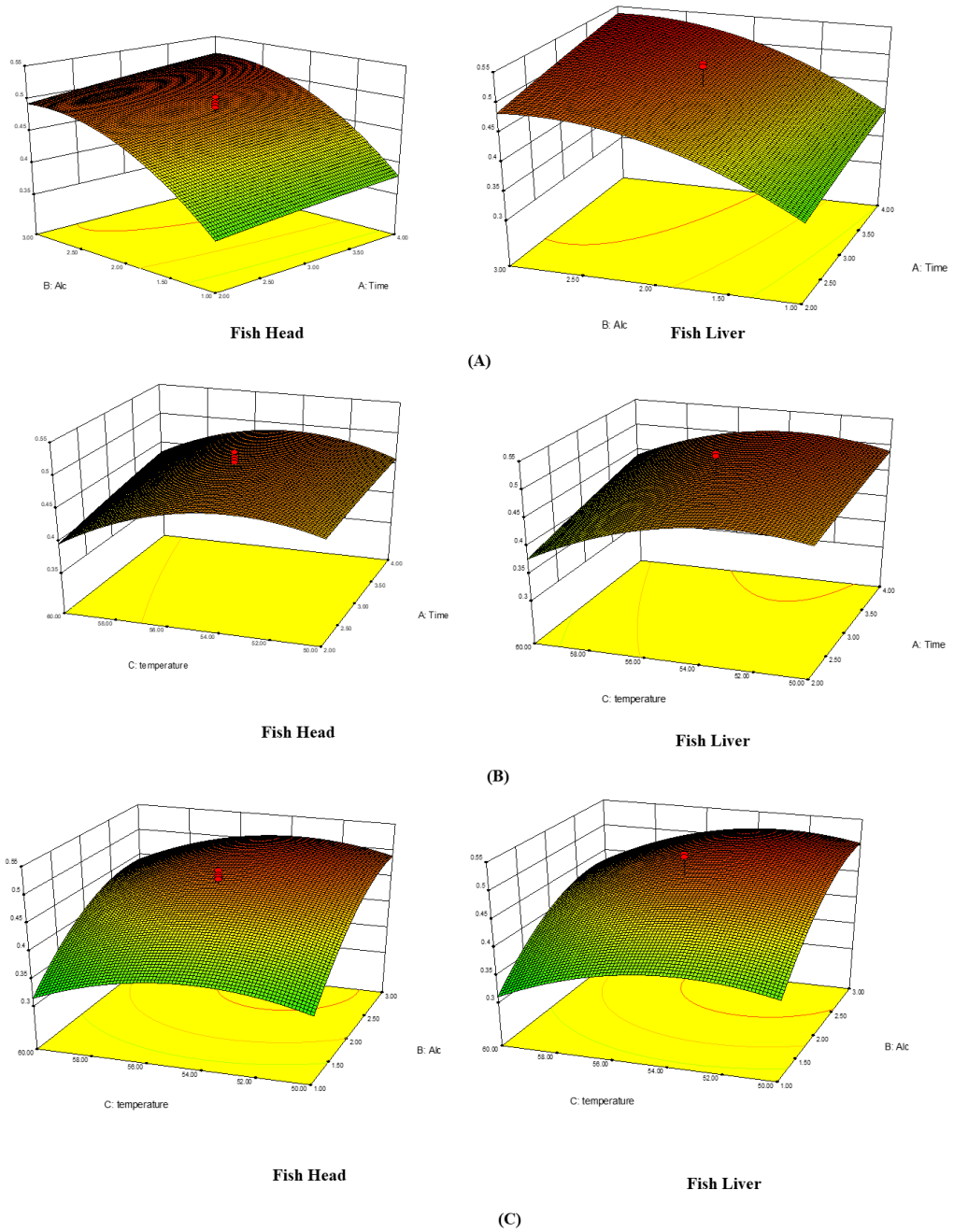


Figure 3-3 Response surface graphs for DH as a function of (a) time and enzyme dose; (b) temperature and time; (c) temperature and enzyme dose

Table 3-3 Optimization of fish waste hydrolysis

Fish Waste	Time (hr)	Alc (%)	Temperature (°C)	Estimated DH (%)	Validated DH (%)
Liver	4	2.72	52.51	53.39	51.61
Head	4	2.92	54.07	52.35	49.37

Table 3-4 Characterization of fish waste generated peptones

Peptones	Total carbon (mg g^{-1})	Total organic carbon (mg g^{-1})	Total nitrogen (mg g^{-1})	Ash (%)	C/N	Ref
Fish head	405.06	73.35	98.14	5.8	4.12	
Fish liver	399.89	66.24	128.92	6.3	3.1	
Tryptone	N/A	N/A	133	6.6	3.4	(Aspmo et al., 2005)
Soytone	N/A	N/A	94	12.0	4.4	(Aspmo et al., 2005)
Yeast extract	N/A	N/A	114	13.1	3.9	(Aspmo et al., 2005)

production (Zhu et al., 2016). The developed fish-waste-based peptones (i.e. fish liver and fish head) in this study could be used as a cheap nitrogen alternative for biosurfactant production. However, the *Bacillus* strains have a different response to fish peptones as carbon sources. Biosurfactant productions, as reflected from surface tension reduction, were only observed in *Bacillus Subtilis* N3-1P and *Bacillus Subtilis* 21332 samples (Figure 3-4).

This study proved that *Bacillus* strains could effectively metabolize the hydrolyzed peptones for biosurfactant production. The highest biosurfactant production rate (20 CMD) was reported by *Bacillus Subtilis* N3-1P using fish liver peptone as the nitrogen source. Among the tested strains, *Bacillus Subtilis* N3-1P and 21332 had a better response to fish peptone than the others. Higher biosurfactant production rates were observed in fish-liver-based samples than fish-head-based medium (both as carbon and nitrogen sources). Lipopeptide synthesis was directly regulated by NRPS, that can directly incorporate some amino acid to the final lipopeptide product (Schwarzer et al., 2003). Therefore, the hydrolyzed amino acids in fish liver samples might be more suitable for biosurfactant production. Interestingly, the cell-free culture mediums were not able to form emulsions as the control medium did (data not shown). It was assumed that the hydrolyzed medium might inhibit the emulsification formation.

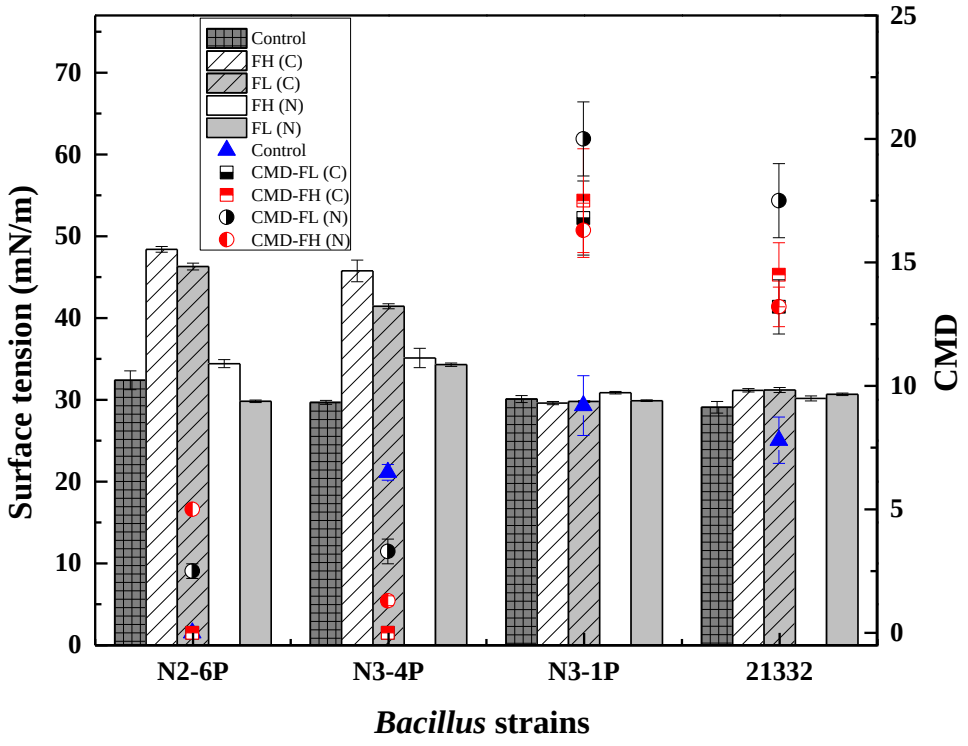


Figure 3-4 Feasibility of biosurfactant production using fish head (FH) and fish liver (FL) peptones.

Fish waste peptones were further investigated as comprehensive mediums for biosurfactant production using *Bacillus Subtilis* N3-1P and *Bacillus Subtilis* 21332. The results are presented in Figure 3-5. Both strains shared a similar biosurfactant production trend. Higher biosurfactant production rates were obtained when using fish-liver-based peptone as the sole medium. The highest biosurfactant production rates were 54.72 and 59.33 CMD for *Bacillus Subtilis* N3-1P and *Bacillus Subtilis* 21332, respectively. The productivities were 47.59 and 49.24 CMD for *Bacillus Subtilis* N3-1P and *Bacillus Subtilis* 21332 using fish-head-based growth medium.

The different amino acid composition in two peptones may contribute to the varied final productivity. For example, higher concentrations of valine and lysine could dramatically increase biosurfactant production whereas alanine and arginine could inhibit the production process (Makkar and Cameotra, 2002). Nevertheless, at a high fish peptone concentration (over 30 g L^{-1}), the biosurfactant production was inhibited. The medium composition is a key factor affecting the structural diversity and productivity of biosurfactants. Insufficient nitrogen environment could facilitate biosurfactant production (Reis et al., 2013). Under a nitrogen limiting condition, continuous cell growth and dividing was inhibited. A microbial metabolism favoring production of secondary metabolites was then promoted and the expression of biosurfactant production gene was then stimulated（Nurfarahin et al., 2018). It was believed that the hydrolysis pretreatment greatly increased the bioavailable carbon and nitrogen

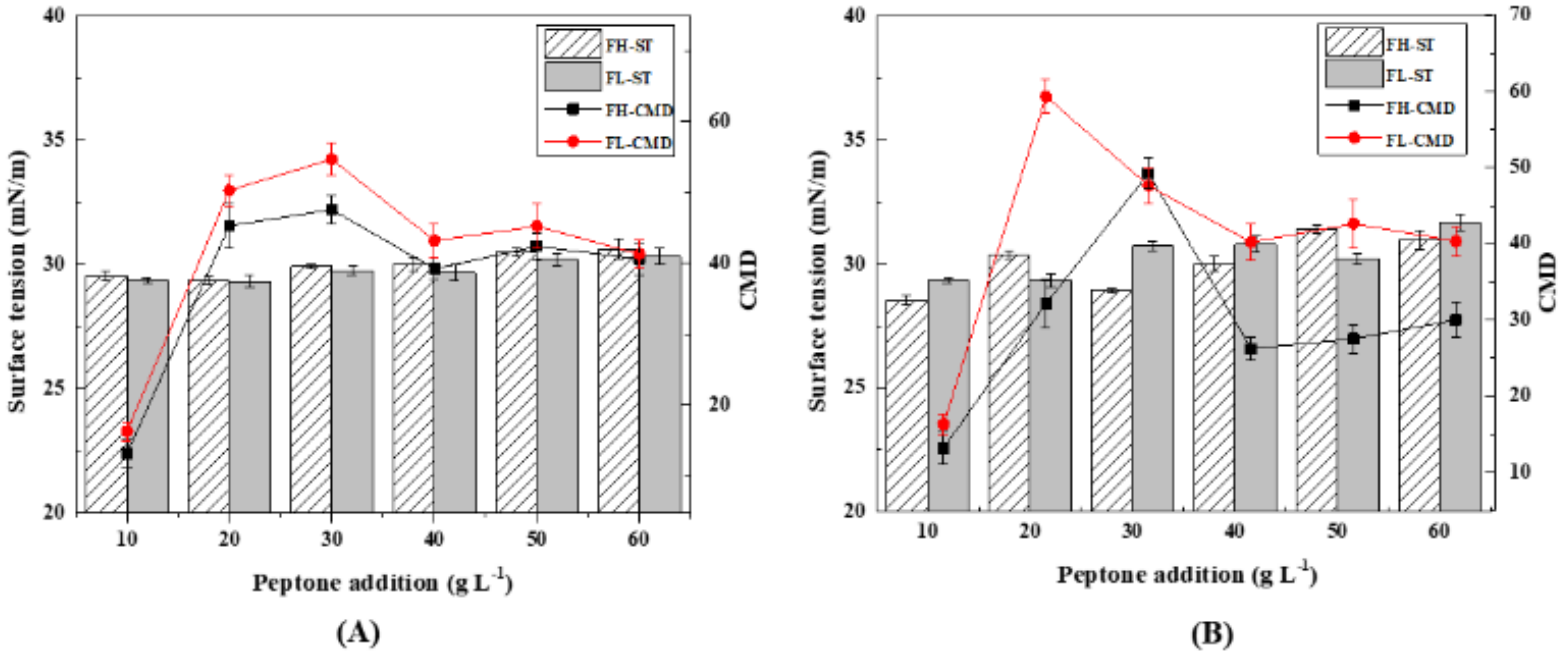


Figure 3-5 Feasibility of biosurfactant production using fish head (FH) and fish liver (FL) peptones as raw medium. (A) *Bacillus Substilis* N3-1P; (B) *Bacillus Substilis* 21332

concentration, thus stimulating biosurfactant production by *Bacillus Substilis*.

The growth of *Bacillus spp* on waste substrates and the biosurfactant production has been previously reported. Serving as an alternative for a carbon source, agro-wastes such as brewery effluents, molasses, fruit, and vegetable decoctions have been confirmed as biosurfactant producing substrates (de Oliveira et al., 2013; Gomaa, 2013; Płaza et al., 2011). Waste oil such as frying oil (Oliveira and Garcia-Cruz, 2013; Yañez-Ocampo et al., 2017), and olive oil mill waste (Ramírez et al., 2015) were also evaluated. The recognition of proper nitrogen substitutes is desired. This was the first of a few investigations to identify cheap nutrient replacements for biosurfactant production.

3.3.3 Physic-Chemical Characterization of Generated Biosurfactants

The physic-chemical properties of a biosurfactant generated by *Bacillus Substilis* N3-1P were determined. The CMC values were 0.18 g L^{-1} and 0.3 g L^{-1} respectively for the crude biosurfactant generated from fish liver and fish-head-based peptones (Figure 3-6). This value is compatible with the biosurfactant products generated by other *Bacillus Substilis* strains (Cavalcante Barros et al., 2008; Das and Mukherjee, 2007). Purified biosurfactant products were able to form a stable emulsion with diesel oil (EI_{24} of 65%). The emulsification process could be inhibited by the fish-based culture medium. The TLC analysis revealed that the biosurfactant product produced by *bacillus*

is primarily consisting of lipid and protein.

The stability of biosurfactant products generated from fish wastes (i.e., liver and head) was assessed under a wide range of environmental conditions (i.e., temperature, salinity and pH). As Figure 3-7 illustrates, the surface activities of generated biosurfactants were positively correlated with temperature. The surface tension remained in a narrow window of 35.2 – 27.3 mN/m from 0˚C to 100˚C. The results proved that the generated biosurfactants had Kraft temperatures (also known as the critical micelle temperatures) below 0˚C. This Kraft point is closely related with their structure and ionic character (Lee et al., 2013). The thermostable natures of produced biosurfactants were confirmed by other studies. Biosurfactant products produced by four different *Bacilli* isolates could be kept stable for nine days at 80°C (Joshi et al., 2008a). Salinity also had a limited effect on the stability of generated biosurfactants (Figure 3-7). This behavior may be predicable, since high salt concentrations can considerably reduce the size and shape of the micelle, thus affecting the functional properties of a biosurfactant (Al-Bahry et al., 2013). These results highlighted the applicability of the crude biosurfactant produced by *Bacillus Substilis* N3-1P in a cold

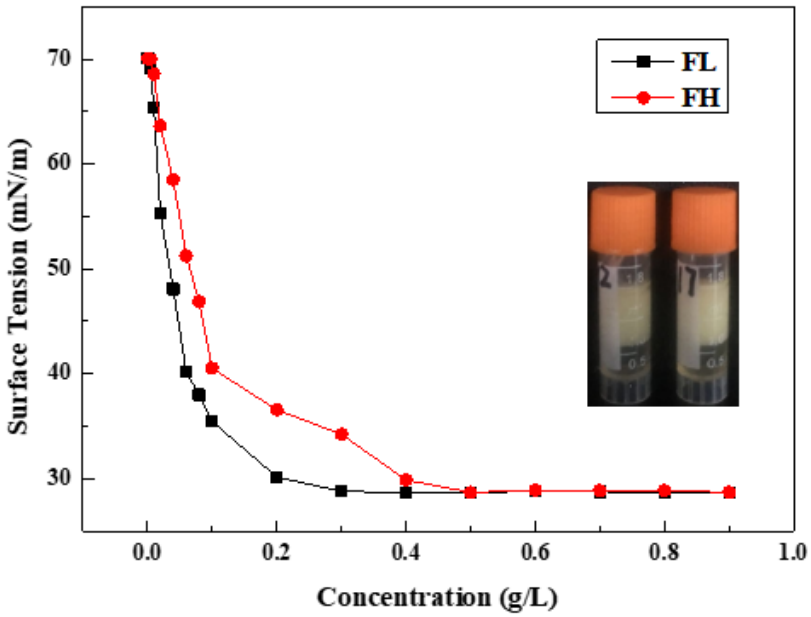


Figure 3-6 CMC values of fish-waste-based biosurfactants generated by *Bacillus Substilis* N3-1P

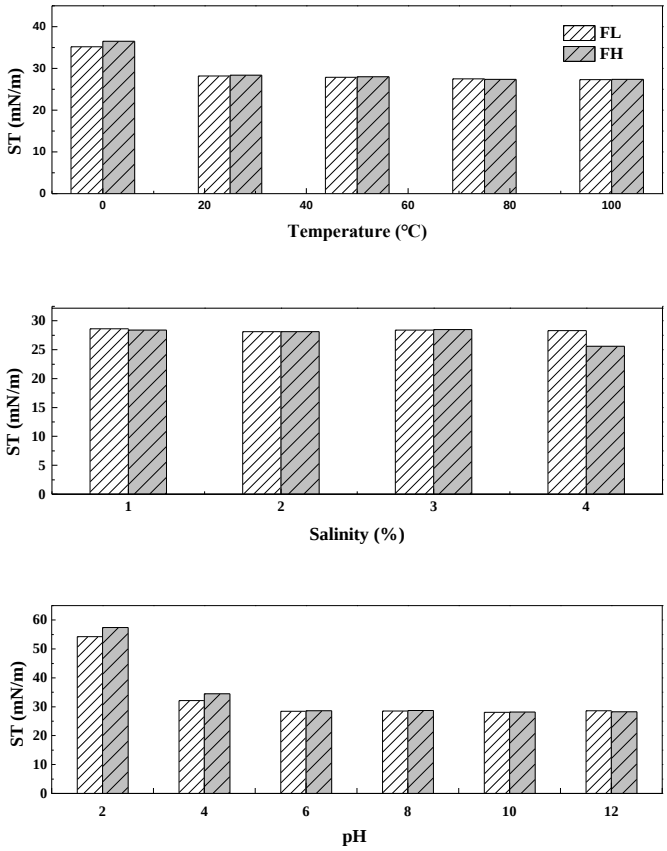


Figure 3-7 Stability of generated fish waste biosurfactants

coastal environment. It has been reported that the structure and size of the micelles in a water–oil system could be affected by the environmental pH (Das et al., 2009b). The environmental behaviors of the biosurfactants would then be affected. The surface activities of fish waste generated biosurfactants were inhibited at low pH (i.e., pH = 2) (Figure 3-7) due to the formation of precipitates. The precipitated and structurally distorted biosurfactants lost their capabilities of reducing surface tension. Also, the presence of negatively charged groups at the end of the biosurfactant molecule in an acid environment might also lead to such an instability (Gudina et al., 2010). The surface tensions of fish waste generated biosurfactants remained almost constant at a pH range from 4 to 10.

3.3.4 Structure Characterization of Generated Biosurfactants

The FTIR spectrums were examined to obtain the information on the functional groups of generated biosurfactant products, and the results are illustrated in Figure 3-8 (A). Two biosurfactants (i.e., fish liver and fish-head-based biosurfactants) showed an apparent similarity with stretched intense peaks in the region of 500 - 4500 cm^{-1}. The stretching absorption between 1050 – 1150 cm^{-1} may denote a C-O stretch, and could be primary, secondary or tertiary alcohol. The absorbance peaks at 1350 – 1650 cm^{-1} evidenced the presence of amide groups. Another broad stretched peaks between 2850-3050 could be contributed by the $-CH_3$, $-CH_2$ or -CH groups. The presence of a board O-H band (3300 to 2600 cm^{-1}) and the strong C=O stretching (1600-1700 cm^{-1})

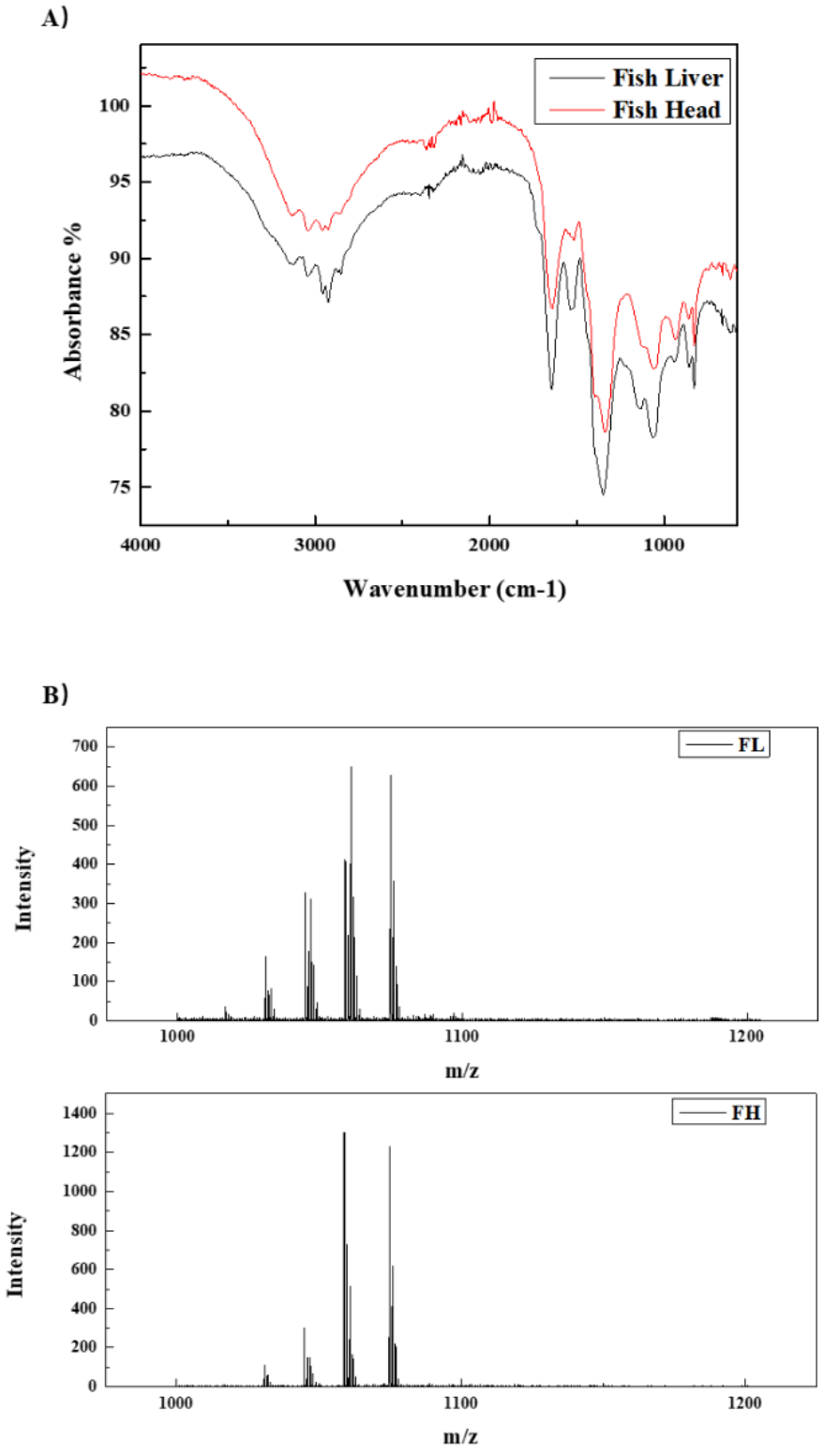


Figure 3-8 Characterization of biosurfactants generated by FL (fish liver) and FH (fish head) peptones. (A) FTIR analysis; (B)MALDI-TOF analysis

evidenced the existence of carboxylic acid groups. The FTIR spectrums suggested that the biosurfactant products were lipopeptides.

Figure 3-8 (B) presents the structure of generated biosurfactants analyzed by MALDI-TOF. This result clearly indicates that the two products have very similar composition. Two groups of lipopeptides, namely surfactin (m/z 1016, 1030, 1044, 1058, and 1060) and itruin (1026, 1043, 1065, 1079) were identified in both fish waste generated biousrfactants.

3.4 Summary

The application of RSM identified the individual and interactive effects of the hydrolysis condition on fish waste peptone yields. The validity of the model was confirmed by the close agreement between the experimental and predicted values. Fish waste hydrolysates (i.e., fish liver and fish head) could serve as carbon and nitrogen sources to support biosurfactant production. They achieved a higher production rate than the control medium. A maximum biosurfactant productivity could reach 54.72 CMD for *Bacillus Subtilis* N3-1P when using fish liver peptone as the only substrate. The CMC values were 0.18 g L^{-1} and 0.3 g L^{-1} respectively for crude biosurfactant generated from fish liver and fish-head-based peptones. The FTIR and MALDI-TOF results proved the final products belonged to lipopeptides.

CHAPTER 4

IMMOBILIZED ROBUST BIOCATALYSTS ON POROUS FLY ASH FOR ENHANCED SOLID-STATE FERMENTATION OF LIPOPEPTIDE BIOSURFACTANTS GENERATED BY *BACILLUS SUBSTILIS* N3-1P

This chapter is based on the following manuscript:

Zhu, Z. W., Zhang, B.Y., Chen, B. Husain. T, Cai, Q. (2018). immobilized robust biocatalysts on porous fly ash for enhanced solid-state fermentation of lipopeptide biosurfactants generated by *bacillus substilis* N3-1P. Submitted to *Journal of Environmental Chemical Engineering*.

Role: Zhiwen Zhu is the principal investigator of this study and acted as the first author of this manuscript under Dr. Baiyu Zhang and Dr. Bing Chen's guidance. Most contents of this paper were written by her and further edited by the other co-authors. (submitted)

4.1 Introduction

Surfactants are a group of amphiphilic substances that can reduce the surface or interfacial tension of a liquid. The surface active agents, such as lipopeptides, glycolipids, phospholipids, fatty acids, and neutral lipids, if produced by microorganisms during their growth, are named as biosurfactants (Shekhar et al., 2015). Biosurfactants, produced by microorganisms during their growth, exhibit high surface activities and low CMC and are, therefore, attracted much attention in recent years (Cai et al., 2014; Zhu et al., 2016). Compared to synthetic surfactants, biosurfactants offer the advantages of keeping a stable and effective performance even under extreme environment conditions, in the meantime; possess little or no environmental impact due to the low toxicity and high biodegradability (Pacwa-Plociniczak et al., 2011). Therefore, their applications acting as detergents, emulsifiers, and foaming, wetting, and dispersing agents in the fields of environmental, oil and pharmacy industries are highly expected (Mulligan et al., 2001). Despite their environmentally favorable characteristics, the economic feasibility of biosurfactants remains to be problematic owning to the poor production rate, arising primarily from the complex regulation system during fermentation and limited effective production cells (Chen and Chang, 2006). Zhi et al. (2017a) indicated that biosurfactant producers such as *Bacillus Substilis* can generate surfactin, a lipopeptide, through biosynthetic regulation of a quorum sensing system. In this system, surfactin synthesis, competence development and

sporulation are cross-linked within a complex network of pheromones and pleiotropic regulators. As a consequence of quorum sensing, surfactin synthesis is dependent on cell density, preventing constant production and limiting overall yields. Therefore, methodologies for increasing cell density, thus enhancing biosurfactant productivity need to be further studied.

Compared with suspended cell cultures, whole cell immobilization in the form of biofilm has been suggested as an effective approach due to higher cell density, shorter fermentation time, and less chance of contamination (Todhanakasem, 2017; Zhou et al., 2008). Biofilm is an assemblage of microorganisms embedded in a self-produced matrix of extracellular polymeric substances (Kokare et al., 2009). They can self-immobilize and self-regenerate on all kinds of interfaces with well-organized metabolism and, in the meantime, accelerate the fermentation process (Karande et al., 2016; Todhanakasem, 2017). Biofilm hence are regarded as promising biocatalysts for organic synthesis, due to their robust and long-lasting feature, as well as the accelerated fermentation process with their existence (Halan et al., 2012; Liu and Li, 2007). A proper selection of a solid carrier can greatly improve the growth rate of biofilm, and thereby effectively increase the density of cells and stimulate the production of target metabolites (El-Fattah et al., 2013). Recent findings indicated that the harness of porous solid carriers with larger surface areas such as activated carbon and expanded clay could promote gas exchange, and provide a larger cell attachment and more immobilization sites for microbes (Chen

and Chang, 2006; Rebah et al., 2002). The solid carriers could also provide a larger buffer capacity under extreme culturing conditions and hence protect the microbes from biotic and abiotic stresses (Chen and Chang, 2006; El-Fattah et al., 2013). Fly ash (FA) is a municipal solid waste produced worldwide due to the combustion of coal at high temperature. Owing to the concentrated toxic heavy metal in the ash, FA is regarded as a hazardous waste. Research has been centered on the treatment of FA through detoxification and potential resource recovery. For example, the treated FA can be beneficially used as a natural absorbent after proper treatment given its porous structure. However, no attempt has ever been made to use FA as a solid carrier for microbial growth.

Therefore, in this work, the FA was tried to serve as the solid carrier for facilitating cost-effective and highly efficient biosurfactant production for the first time. Two hypotheses were examined: (1) the porous structure of FA could provide a larger surface area for the attachment of biocatalyst, thereby greatly stimulating biosurfactant production and (2) the immobilized bacterial biofilm may have a positive effect on the detoxification of FA by means of a bioleaching process. The biosurfactant producing microorganism applied was *Bacillus Substilis* N3-1P, which was isolated from the Atlantic Ocean. The performance of FA on cell growth and biosurfactant production was investigated. The effect of FA dosage on biosurfactant production was examined using parameters including ST, emulsification activity, and solution dilution as

responses. The generated biosurfactant product was characterized through determining ST and CMC. Its structure was further characterized using FTIR spectroscopy and MALDI-TOF-MS.

4.2 Material and Methods

4.2.1 FA as Solid Carrier

FA to be used as a platform for biosurfactant production was obtained from Corner Brook Pulp and Paper (CBPP) plant, Newfoundland and Labrador, Canada. Bunker C oil had been mixed with wasted pulpwood as a burning fuel during the thermal mechanical pulp process. Generated fly ash was then collected from the power boiler and subjected to air drying. The properties of fly ash are listed in Table 4-1. FA was characterized before and after incubation with FTIR by the KBr pellet method and scanning electron microscopy (SEM).

4.2.2 Biosurfactant Producing Microorganisms

Bacillus. subtilis is a motile, Gram-positive, rod shaped endospore-forming bacteria widely studied in biofilm formation. They are famous for producing biosurfactants, especially effective lipopeptide biosurfactants. The bacterium used in this study, *Bacillus. subtilis* N3-1P, was screened from oily contaminated seawater samples (Cai et al., 2014). This strain was identified as a promising and economic biosurfactant producer among the screened bacteria, whose product possessed strong

Table 4-1 Characteristics of CBPP FA

Property	
pH	12
Density (g cm^{-3})	0.45
Moisture content (%)	0.89
Surface area (m^2 g^{-1})	249.4
C/N ratio	572.95
Element content in solid (Unit: mg Kg^{-1})	
Mg	511.65
Al	947.03
Fe	784.20
P	114.33
Cl	11634
Zn	11.72
Cu	7.28
Pb	2.25
V	15.46
Cr	4.73
Ni	15.96
Ca	2656.36

surface activity and high emulsification capacity.

The composition of the culture medium for *Bacillus Substilis* N3-1P was as follows: BD Difco™ Marine Broth (Fisher Scientific, Canada) 37.4 g in 1L of distilled water. A loopful of a bacteria colony was transferred into a 125 mL Erlenmeyer flask containing 50 mL inoculum broth. This seeded culture medium was initially grown on a rotary incubator shaker (VWR, Canada) at 200 rpm for 24 h under room temperature to reach its exponential growth phase. The biosurfactant production medium was comprised of sucrose (30), NH_4NO_3 (10), NaCl (15), KH_2PO_4 (3.4), $K_2HPO_4 \cdot 3H_2O$ (4.4), $MgSO_4 \cdot 7H2O$ (1.02), and yeast extract (0.5) (g L^{-1}).

4.2.3 Biosurfactant Production with FA

Effect of FA on biocatalyst immobilization: FA was added into the production medium (section 4.2.2) at a 1% level to assist the attachment of the biofilm-based biocatalyst and the one without FA was used as a control. A seeded culture medium (section 4.2.2) was used as inoculum at 1% (v/v) level. Samples were collected every six hours for a total of 24 hours. FA particles in the culture broth were removed through a filtration process. The filtrate containing culture broth was centrifuged at 12,000 rpm for 10 min to remove the remaining cells. Filtrated FA particles were gently washed three times with Phosphate-buffered saline (PBS) buffer solution and then subjected to zeta potential measurement. The growth behavior of the biofilm-based biocatalyst was quantified by the variation of zeta potential. The immobilized biocatalyst on FA

particles was further characterized with FTIR and SEM. All the samples were collected and analyzed in triplicate. The cell-free filtrate was analyzed for biosurfactant productivity using CMD as an indicator.

Effect of FA dosage on biosurfactant production: FA particles were added into biosurfactant production medium (section 4.2.2) at a level of 0.5%, 1% and 2% (w/v), respectively. Medium without FA particles was used as control. The seeded culture medium (section 4.2.2) was used as inoculum at 1% (v/v) level. Samples were collected at six-hour intervals for the first day and then every 24 hours for next 6 days. Each sample was subjected to filtration to remove the FA particles and then centrifuged at 12,000 rpm for 10 min to remove the remaining cells. The fermentation process was monitored by measuring parameters such as ST, pH, and CMD. The FA particles were collected and further examined to determine the effect of biosurfactant adsorption on the final productivity. The residue FA particles were treated to separate the adsorbed biosurfactant following the method described by Dubey et al. (2005). The optimum FA level and incubation time obtained from the above tests were selected for batch scale biosurfactant production. All the analysis in this study was performed in triplicate.

4.2.4 Characterization of Generated Biosurfactant Product

The optimum FA addition level and incubation time derived from section 4.2.2 was selected during batch scale biosurfactant production. Biosurfactants in the FA particles were collected using the method described in section 4.2.2. The FA free culture

broth was centrifuged at 12,000 rpm for 10 min to remove the remaining cells. Cell-free culture broth was extracted using an equal volume of chloroform–methanol (1:2 v/v) solvent. The solvent was removed by rotary evaporation. Combined biosurfactant products extracted from the FA particles and culture medium, and subjected to ST and CMC measurement. Its structure was further characterized using FTIR spectroscopy and MALDI-TOF-MS. All the characterization results were compared with the ones generated by the control.

4.2.5 Bioleaching of Heavy Metals from FA

Metal leachability from the FA particles during biosurfactant production process was estimated. Cell-free culture mediums at a FA concentration of 1% and 2% collected from section 4.2.3 were further examined for the bioleaching behavior. Samples were collected and analyzed in duplicate, and a triplicate analysis was performed when the deviation was greater than 5%. The concentrations of leached heavy metals were examined using inductively coupled plasma mass spectrometry (ICP-MS). The differences of FA based medium and control samples were recorded.

4.2.6 Sample Analysis

pH and moisture content: The pH of FA from CBPP was measured following ASTM D1512-15b, and the moisture content was determined by American Society for Testing and Materials (ASTM) D1512−05 (2012).

ST: The determination of ST was followed by the method described in section

3.2.6.

CMC and CMD: The determination of CMC and CMD was followed by the method described in section 3.2.6.

Zeta potential analysis: The determination of the FA zeta potential was modified from the methods described by Akgün (2005) and Li et al. (2011). FA samples collected from incubation samples during the first 24 hours were gently washed three times with 10 mM PBS buffer solution (pH 7.4) and then dissolved into this PBS buffer solution to reach a final concentration of 1 mg mL^{-1}. Each sample was gently shaken for 12 hours before measuring with a Malvern Zetasizer.

Trace metals: The trace metals in the FA samples were analyzed by the modified Environmental Protection Agency (EPA) method 3050 using inductively coupled plasma mass spectrometry (ICP-MS, PerkinElmer ELAN DRCII, USA). A 100 ± 10 mg of FA sample was weighed in a 15 mL Teflon vial with a screw cap. Then 3 mL of 8N HNO_3 was added and heated on a hot plate at 70°C for two days. The sample was then cooled again.; Afterwards, 1 mL of HNO_3 and 1 mL of H_2O_2 were added and the sample was heated at 70°C for two days to remove organic matters. The sample was then dried and cooled. An additional 2 mL of 8N HNO_3 and one mL HF were added to the sample and heated at 70°C for two days. After drying and cooling, 3mL of aqua regia (V_{HCl}: V_{HNO3}= 3:1) was added to the sample and heated at 70°C for one day. The sample was eventually dried, cooled, and dissolved in 2% HNO_3. The solution was then diluted and analyzed by ICP-MS.

FTIR analysis: Both FA particles and biosurfactant products were examined with FTIR (Bruker Tensor). FA particles were characterized with the KBr-pellet method. Spectral measurements were performed in the transmittance mode. Crude biosurfactant products were directly characterized with Attenuated Total Reflection -FTIR spectroscopy in the absorbance mode. IR was traced over the range of 400–4000 cm^{-1}. All data were corrected for background spectrum.

MALDI-TOF-MS analysis: Biosufactant product analysis in filtrate, FA, and blank samples were examined with MALDI-TOF mass spectra by a SCIEX MALDI TOF/TOF System as section 3.2.7 described.

4.3 Results and Discussion

4.3.1 Effect of Biocatalyst Immobilization on Microbe Growth

Previous studies have proved the cell growth stimulation and product promotion through certain types of porous carrier, such as â-cyclodextrin, filter paper, and silica gel (Yeh et al., 2005). The mechanism of FA enhanced biosurfactant production through the self-produced biocatalyst is presented in Figure 4-1(A). Biofilms, also act as a biocatalyst in this study, and are microbial communities encased in a layer of self-produced matrix extracellular polymeric substances and they adhered to various surfaces. Those free-floating biosurfactant producers started to flow into the channels of FA particles and initially attached to their surface within the first few hours. Those pioneers then quickly anchored themselves to the matrix via the production of pili,

fimbriae, and exopolysaccharides (Rendueles and Ghigo, 2012). Following initial attachment, proliferation and building of microcolonies on FA surface spontaneously occurred through the production of an extracellular matrix. It is believed that bisurfactant production was stimulated through a quorum sensing system at this stage, not only to enhance the swarming motility of biosurfactant producer, but also to alter the wettability and potential of the platform surface to facility their residence (Gélis-Jeanvoine et al., 2016; Ribeiro et al., 2012; Yeh et al., 2005). The role of lipopeptide biosurfactant as the signaling molecules triggering robust biofilm formation for bacillus strains under laboratory conditions has been identified (Zeriouh et al., 2014). Additionally, recent researches confirmed that microbes tend to reside in biofilms, rather than the free-floating forms (Frederick et al., 2011). Biofilm was able to provide biosurfactant producers a stable environment under external stress (e.g., disinfectants and antibiotics) by reducing the diffusion of those compounds (Berlanga and Guerrero, 2016), and thereby promoting their growth rate, and the follow-up biosurfactant production.

Previous studies recognized that the pore structure and surface charge of FA were major contributors to the adsorption process (Rendueles and Ghigo, 2012). Visual, elemental and spectroscopic analyses (i.e., with SEM and FTIR) were carried out to provide multiple disciplines of evidence on the microstructure and surface chemistry of FA before and after incubation. The results are presented in Figure 4-1(B). The SEM image of raw CBPP FA demonstrated its highly porous, platelet and fiber shaped

structure. Its Energy Dispersion X-ray spectroscopy analysis revealed a dominant amount of carbon (C) on the surface. Surface properties play an important role in initial cell attachment (Goller and Romeo, 2008). The rough surface of FA as Figure 4-1(B) presented, has been considered as an excellent solid carrier to promote cell settlement and biofilm growth, owing to the enhanced cell-surface interactions and strengthened protection from shear force (Li et al., 2007). Additionally, the sorbent properties of FA offered the biosurfactant producer a better access to the localized nutrients, providing a higher metabolic activity with those free-living ones. After incubation, a layer of biofilm was identified on the FA surface from the SEM image. According to the Dispersion X-ray spectroscopy result, the composition and abundance change of dominant components on the surface of the FA further proved the growth of biofilm on the FA surface.

The FTIR spectra (Figure 4-1(b)) provided qualitative characterization about the surface of FA, primarily through providing the information of functional groups. The major peaks acquired from raw FA material was in accordance with the one reported by Martins et al. (2007). Carbonate (870-1400 cm^{-1}) group was recognized in this study. Its presence in wood-based FA was widely acknowledged, as the combustion process mineralized the organic compounds, and transformed the basic cations to their oxide forms. They are lately hydrated and subsequently convert to the forms of carbonates and phosphates (Demeyer et al., 2001). The alkane C-H bond stretch (2700-3000 cm^{-1}) and carboxylic and/or hydroxyl groups (3200-3600 cm^{-1}) were identified from the FTIR

analysis (Weber et al., 2006). The presence of Al and Fe as oxygen functional groups within Al-OH and Fe-OH (800-900 cm^{-1}) can be confirmed from Putra et al. (2009). The FTIR spectrum of FA samples after the incubation process was also presented in Figure 4-1(b). This result presented an increased intensity of carboxylic and/or hydroxyl groups in 3200-3600 cm^{-1} and 800-1000 cm^{-1} and a new N-H bond stretch at 1700-1800 cm^{-1}, verifying the existence of biofilm on its surface. It was recognized that biofilm was composed of 90% of water, and 10% of polysaccharide, protein, and DNA, etc. The abundant carboxylic group in FA and biofilm surface may form a chemical bond structure with carbonate groups on FA surface, and thus led to the disappearance of stretching bond at 1450 cm^{-1}.

Figure 4-1(c) provided biosurfactant productivity with the addition of FA. This result further proved the enhanced production mechanism described in Figure 4-1(a). Acting as a signal molecular, biosurfactant was initially secreted to simulate EPS production and biofilm formation at a relatively slow rate (Zeriouh et al., 2014). A rocketing biosurfactant production rate was observed after incubation for 18 hours. The assembled biocatalyst on FA particles accelerated the reaction by well over ten-fold. Biosurfactant concentration was increased from 9 CMD in control sample to 100 CMD in FA sample after incubation for 24 hours. The intercellular communications within a biofilm further stimulated the up-and-down regulation of gene expression, enabling temporal adaptation such as phenotypic variation and the ability to survive in nutrient deficient conditions (Garrett et al., 2008), and thereby promote the biosurfactant

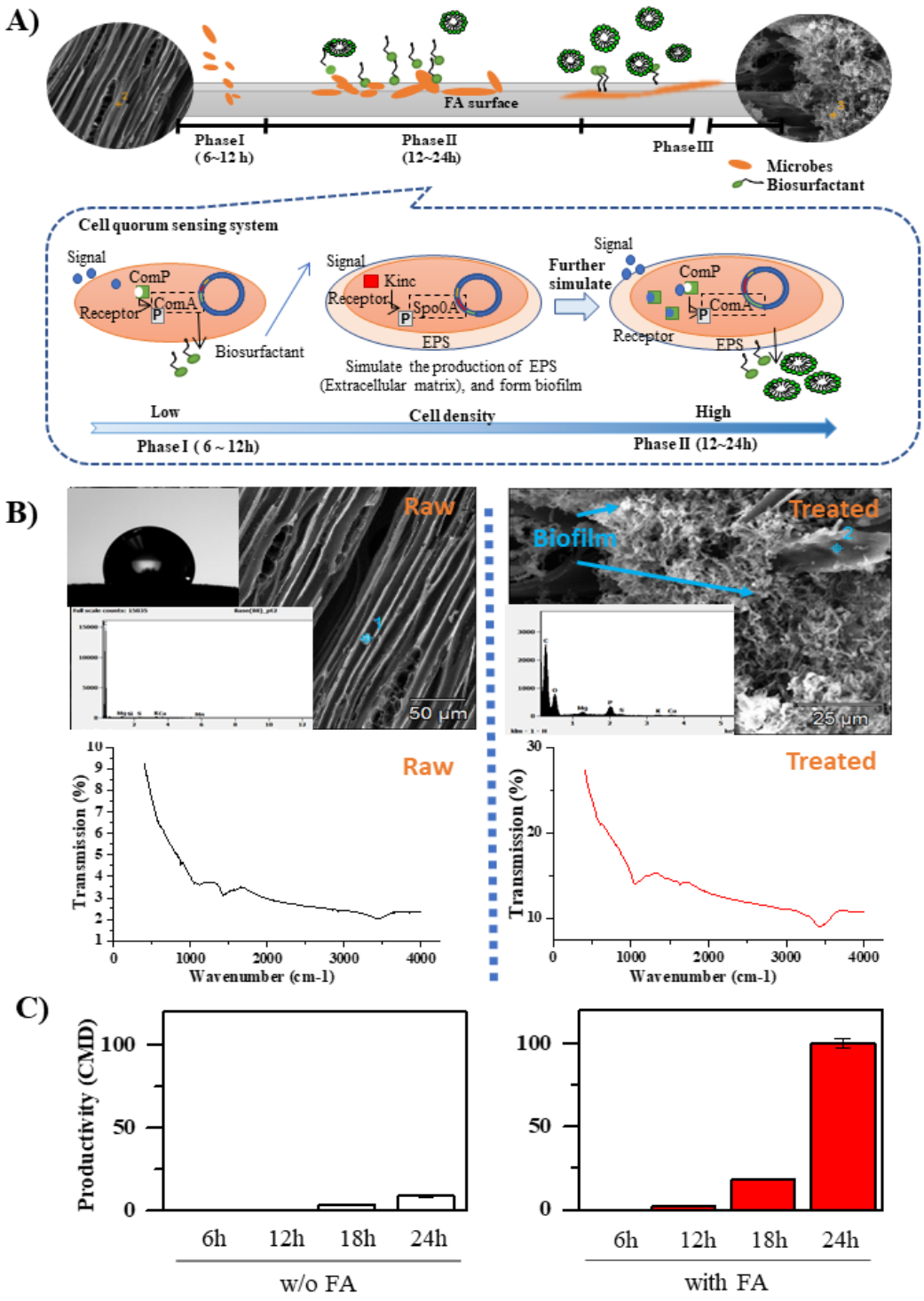


Figure 4-1 A) Mechanism of immobilized biocatalyst enhanced biosurfactant production on FA particles; B) Characterization of FA with SEM and FTIR; and C) Biosurfactant production with and w/o the existence of FA based platform

production. This study demonstrated a successful application of FA as a platform to stimulate the biosynthesis of lipopeptide through biofilm encased cells and the findings in this study was similar to the one generated by Wigneswaran et al. (2016).

It is acknowledged that cell attachment and biofilm formation will alter the physiochemical properties of porous medium. The surface electrostatic charge of porous medium will be affected by the attached biosurfactant production cells and the EPS matrix accordingly. Zeta potential measurement has been widely used to characterize the solid-liquid interface, obtaining the nature and charge information of solid surface, and exhibiting the electrokinetic behavior of solid-liquid interface. Therefore, the zeta potential variation was investigated to shed light on the attachment of microbes and growth of biofilm on FA surface. Their results are listed in Table 4-2. Zeta potentials of all FA particles were below zero in the provided neutral buffer solution. The ones with 2% FA dosage had the lowest starting zeta potential value, followed by 1% and then 0.5%. This negative zeta potential might be due to the initial conditioning process, resulting from the attraction of mineral groups in the growth medium such as PO_4^{3-}, and SO_4^{2-} (Julien et al., 1998). A sharp decrease of zeta potential was identified during the first six hours, confirming the strong attachment of negatively charged biosurfactant producer to FA surface. Hydrophobic FA surface tended to enhance bacterial attachment onto its surface through removing those adsorbed surface water, and attracting bacteria with hydrophobic properties (Van Loosdrecht et al., 1987). Microbes were then embedded into self-produced extracellular matrix. When biofilm was gradually

Table 4-2 Zeta Potential of FA as a function of incubation time at different dosage

Time (h)	0.5% FA		1% FA		2% FA	
	Zeta Potential (ξ)	SD (%)	Zeta Potential (ξ)	SD (%)	Zeta Potential (ξ)	SD (%)
0	-3.45	6	-9.54	6	-10.89	2
6	-13.78	5	-15.05	5	-13.43	5
12	-14.48	4	-15.05	3	-14.08	3
18	-17.17	3	-15.08	6	-14.48	6
24	-19.13	1	-16.52	1	-14.77	5

produced, its majority component, namely neutral polysaccharide, shielded and/or neutralized the negatively charged surface functional groups, such as DNA and protein, and thereby slowed the decrease of zeta potential (Liu and Li, 2007). This explained the relative stable zeta potential during next few hours. The higher the concentration of FA particles, the longer the biosurfactant producer took to finish biofilm assembling. After that, a continuous decrease of zeta potential was observed due to the production and adsorption of produced biosurfactants on FA particles. At pH 7.4, most functional groups at the hydrophilic moiety of the produced anionic lipopeptide molecule were protonated or compensated by a counter ion, leaving limited acidic residues (e.g., Glu and Asp) that worked as effective negatively-charged carriers (Fan et al., 2014). The continuous accumulation of those produced anionic lipopeptide biosurfactant thus decreased the zeta potential of FA surface.

4.3.2 Effect of FA Dosage on Biosurfactant Production

Effect of FA supplement as a platform for stimulating the growth of biocatalyst upon the enhancement of biosurfactant production was assessed. The medium was supplemented by a fixed amount (0.5%, 1% and 2%, respectively) of FA carrier. Biosurfactant production rate was obtained using the selected *Bacillus* strain, and the correlation between FA dosage and biosurfactant production rate was evaluated as shown in Figure 4-2. The results clearly demonstrated the remarkable advantage of using FA to promote biosurfactant production. As Figure 4-2 (A) shows, the lag phase of biosurfactant production was clearly affected by FA dosage. Production acceleration

was firstly taken place in incubation samples with 0.5% FA dosage and last found in the samples with 2% dosage. The biosurfactant production was accelerated with CMD increased from 9 to 110, 100 and 70 after 24 hours, respectively. Afterwards, a reduction of biosurfactant production was recognized, accompanied by a pH reduction (Figure 4-2 (B)), which was due to the generation of secondary acid metabolites such as uronic acid when using sugar as the carbon source (Zhu et al., 2016). Biosurfactant was produced and led to the surface tension reduction (Figure 4-2 (C)).

A biosurfactant production reduction (i.e., Figure 4-2) in culture media was also observed after using activated carbon as solid carrier (Yeh et al., 2005). A possible explanation to this observation is the depletion of carbon source in culture media, and an assimilation of biosurfactant as alternative carbon source for cell growth occurred. On the other hand, the adsorption of biosurfactant onto solid carrier could be another reason to biosurfactant concentration drop in culture media (Dubey et al., 2005). The underlying mechanism is trying to be identified in this study. The biosurfactant content in culture media and FA carrier were examined separately following the method described in Figure 4-2 (D). The biosurfactant concentration in culture broth (0.5% L, 1% L, 2% L) and in FA particles (0.5% D, 1%D, 2%D) were shown in Figure 4-2 (E). Study result proved that production reduction was mainly owning to the adsorption process. The adsorption of biosurfactant on FA particles was gradually increased until the maximum adsorption capacity of FA was filled at fifth day. The addition of 2% of FA gave the highest final biosurfactant yield, namely 305 CMD. The yield with the

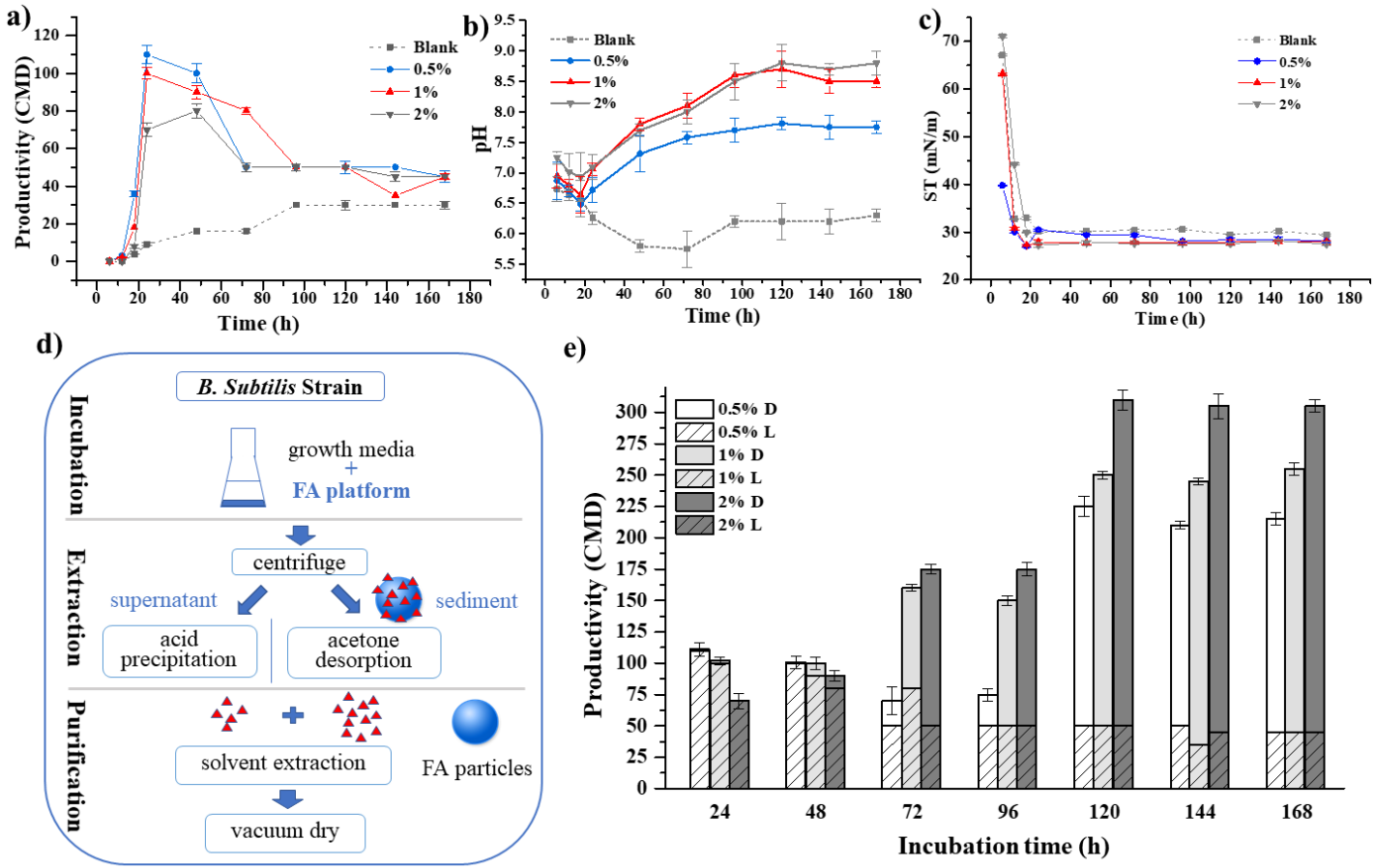


Figure 4-2 Biosurfactant production with FA based platform. A) Productivity of growth medium; B) pH of growth medium; C) ST of growth medium, D) Flow chart of biosurfactant production process and E) Biosurfactant production in culture broth (0.5% L, 1% L, 2% L) and in FA particles (0.5%D, 1%D, 2%D)

addition of 1% FA and 0.5% FA were 255 CMD and 170 CMD, respectively. A positive relationship between FA dosage and biosurfactant production yield was observed. The higher dosage of FA provided a larger surface area of the biocatalyst. Through serving as a cell growth booster for the growth of *Bacillus Substilis* in the media, a higher biosurfactant production yield was achieved. In addition, a higher FA dosage increased the iron content (Table 4-1), which also contributed to the increase of the biosurfactant production yield (Wei et al., 2004).

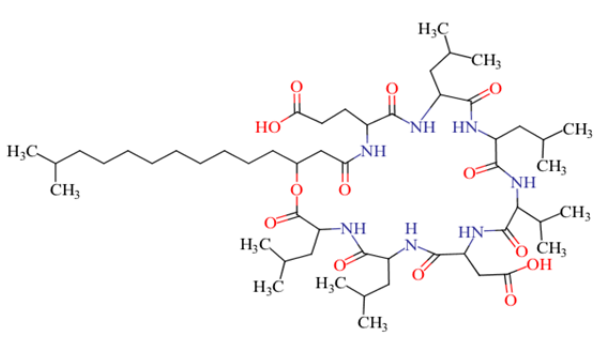
4.3.3 Characterization of Produced Biosurfactant

The surface activity properties of generated biosurfactant product were characterized in this study through measuring ST and CMC values. Results indicated that both biosurfactant products generated by FA based medium and control sample could reduce the ST of water from 75 to 27.8 mN/m. The CMC value of the FA based medium was 0.407 g L^{-1}, lower than the one generated by control (0.524 g L^{-1}). It was assumed that the attachment of biosurfactant production cells on the FA surface eased the purification process. Biosurfactant was more easily desorbed from FA particles than other impurities. Its purity therefore was enhanced (Dubey et al., 2005).

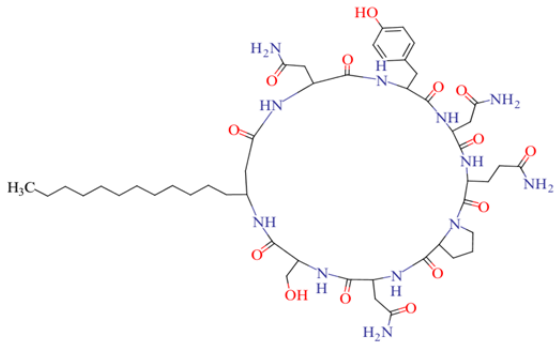
FTIR was further examined in this study to acquire the chemical bond (functional groups) information of generated biosurfactant product. Figure 4-3 presented the FTIR spectra of biosurfactant products generated by FA based medium and control samples in the region of 400-4500 cm^{-1}. Both products had similar spectrum, indicating they

shared same functional groups. The characteristic absorbance peaks at 700-950 cm^{-1} (peaks 1, 2, and 3) revealed the presence of long-chain aliphatic fatty acid. The stretching mode identified from the band range 1000-1250 cm^{-1} evidenced the presence of carbonyl (peak 6) and amide groups (amide I band) (peaks 4 and 5). The peaks corresponded to the linkage group between the amine and carboxylic groups of amino acids and to the carboxylic group of the fatty acid (Gordillo and Maldonado, 2012). FTIR results displayed absorbance in the range of 1,600 to 1,700 cm^{-1} (peaks 9 and 10), due to the deformation mode of the N-H bond combined with C-N stretching mode, indicating the existence of amide II band. A typical CH stretching vibration in the alkyl chain was identified from band range 2700-2900 cm^{-1} (peaks 11 and 12). The bands to indicate the presence of C=O stretching was not identified in this figure. The biosurfactant products were characterized after acid precipitation. Through exerting an effect on the acyl chains at the peptide terminus (e.g. the formation of hydrogen bond) the secondary structure of the lipopeptide could be affected by such an acidic environment. Therefore, the band might have some shrift and was covered by other strong bond in the diagnosis fingerprinting area. FTIR results confirmed that the biosurfactant was lipopeptide in nature.

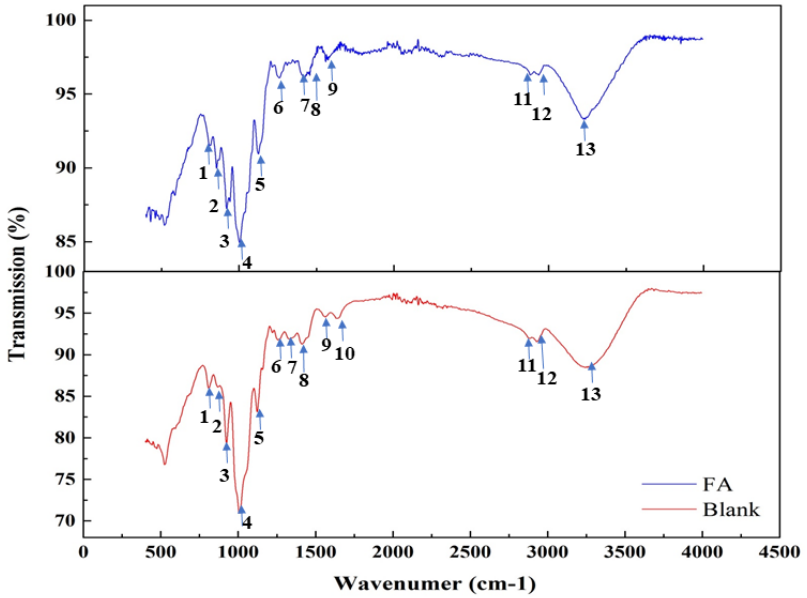
The structure of the lipopeptide biosurfactant was elucidated based on MALDI-TOF spectral analysis and the results (Figure 4-4) were in accordance with the one generated by Yang et al. (2005). This study compared the biosurfactant products generated in a FA based culture medium with and without the desorption process, and



Surfactin



Iturin A



Peak	Functional group
1	-CH 1,3 Disubstitution (meta) (810–750 and 900–860) 1,4-Disubstitution (para) Peroxides (-COOC-) Vinyl CH (995–985 and 915–890)
2	
3	
4	Primary amine, CN stretch
5	Alkyl-substituted ether, (-CO) Cyanate (-OCN and C-OCNstretch)
6	Primary or secondary (-OH)
7	Vinyl (-CH)
8	Methylene CH bend
9	Secondary amine, NH bend (R-NH-R)
10	Alkenyl (C=C); Secondary amine, NH bend; Open-chain imino (-C=N-)
11	Methyl (-CH3)
12	Methylene (=CH-)
13	Hydroxyl (R-OH)

Figure 4-3 FTIR analysis of biosurfactant produced by *Bacillus* strains with FA based platform

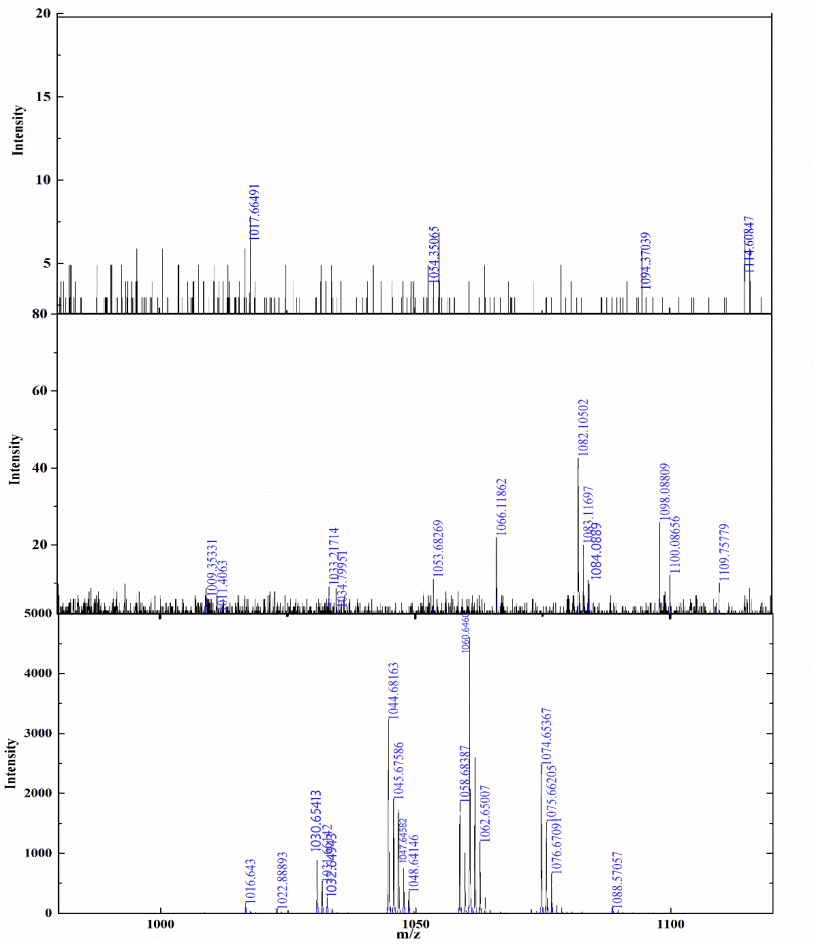


Figure 4-4 Characterization of biosurfactant with MALDI-TOF

the one generated in the control sample using FA free growth medium. The results were in accordance with CMD result generated in Chapter 4.3.2. Comparing the low intensity and limited identified biosurfactant product in FA free culture broth, the FA based medium showed a higher productivity. The productivity of biosurfactant product was increased almost 100 times after the desorption process. This result clearly indicated the existence of two groups of lipopeptide biosurfactants, namely surfactin (m/z 1008, 1016, 1030, 1044, 1058, and 1060) and itruin (1026, 1043, 1065, 1079, 1093).

4.3.4 Bioleaching of Heavy Metals from Fly Ash

Leachability of heavy metals from FA by biosurfactant producer was illustrated in Figure 4-5 A-C), and the principal component analyses of the behaviors of FA contained elements were illustrated in Figure 4-5 D). The behaviors in culture medium can be attributed into two groups. One group was bacterial growth-related elements, such as Cl, P, Mg, Ca, and Al, while the other was the heavy metals such as Cr, Pb and Zn. The increased leachability was identified from all three heavy metals (Cr, Pb and Zn). Previous research revealed that Zn extraction process was faster than the others (Xin et al., 2012). This trend was also proved in this study. A higher concentration of Zn was reported in the sample of Day 4. This increase is likely due to the heterogeneous heavy metal distribution in the fly ash particles (Provis et al., 2009). The slow extraction process for Pb and Cr might be due to the relatively high pH value in growth medium, as a bio-acidic dissolution was preferred in the bioleaching process. The bioleaching

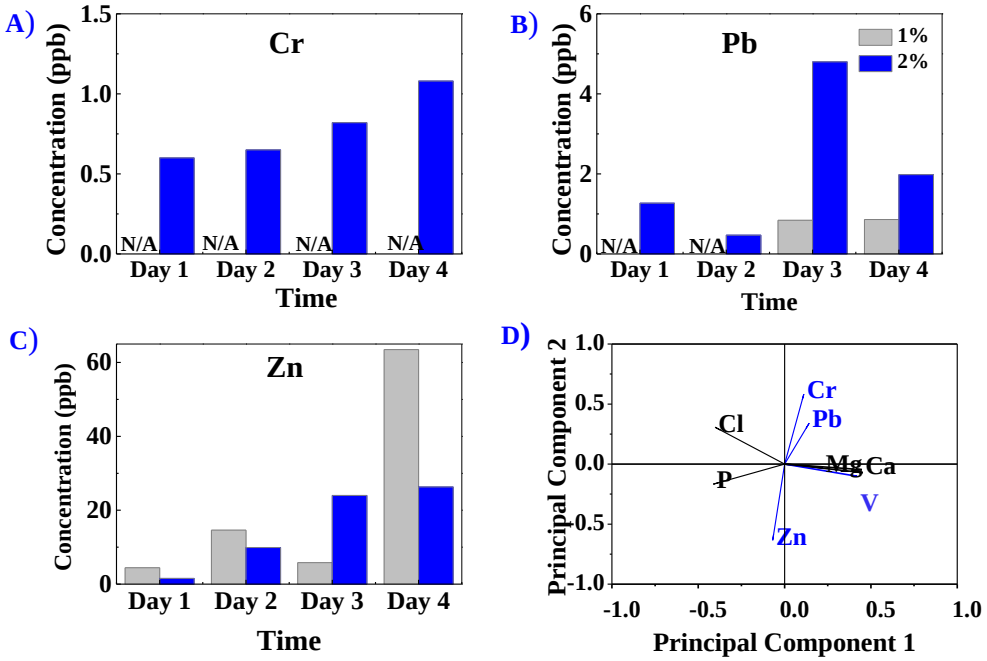


Figure 4-5 Bioleaching of heavy metal from FA surface. A)-C) Bioleaching of Cr, Pb and Zn into medium; D) Principal component analysis of leachate metals

attempt in this study indicated that FA could be detoxicated after several runs of the incubation process and thus ease its disposal and treatment process (Zeng et al., 2015).

4.4 Summary

This study examined an environmental-friendly and cost-effective way to produce biosurfactant through applying self-produced biocatalyst immobilized on FA surface. Results indicated that the addition of FA particles at 2% w/v ratio triggered the growth of biofilm thus remarkably increased the biosurfactant production rate. The application of FA further enhanced biosurfactant purity, resulting in a lower CMC value. The FTIR and MALDI-TOF characterized the product as a lipopeptide. The findings improved the understanding of cultivation setups and shed light on the application of fixed bed biofilm reactor for catalyzing bioproduct generation.

CHAPTER 5

LIPOPEPTIDE BIOSURFACTANT PRODUCTION BY MARINE ORIGINATED BACTERIA *BACILLUS SUBTILIS* N3-4P AND ITS APPLICATION FOR CRUDE OIL REMOVAL

This chapter is based on the following paper:

Zhu, Z.W., Zhang, B., Chen, B., Cai, Q. and Lin, W. 2016. Biosurfactant Production by Marine-Originated Bacteria Bacillus Subtilis and Its Application for Crude Oil Removal. *Water, Air, & Soil Pollution*, 227(9):328

Role: Zhiwen Zhu is the principal investigator of this study and acted as the first author of this manuscript under Dr. Baiyu Zhang and Dr. Bing Chen's guidance. Most contents of this paper were written by her and further edited by the other co-authors.

5.1 Introduction

Biosurfactants are a group of surface active molecules synthesized by microorganisms (Cai et al., 2015). They have amphipathic molecules that tend to accumulate at the interfaces between fluid phases with different polarities (e.g., oil-water or air-water), thus they are capable of reducing surface tension (ST) and interfacial tensions (Ghribi et al., 2012) between individual molecules. In addition, they are able to form emulsions where hydrocarbons can solubilize in water or where water can solubilize in hydrocarbons (Desai and Banat, 1997; Joshi et al., 2008b). In recent years, much attention has been directed towards biosurfactants due to their advantages such as lower toxicity, higher biodegradability, better environmental compatibility, stronger foaming ability and greater selectivity than chemical surfactants (Pacwa-Plociniczak et al., 2011). They exhibit stable performance even at extreme temperatures, pH and salinity, and have the ability to be synthesized from renewable feed stocks (Ilori et al., 2005). Furthermore, biosurfactants have high surface-activities together with low CMC, in some cases even lower than most of the traditional chemical surfactants (Mulligan, 2005). The aforementioned advantages allow their use and possible replacement of chemically synthesized surfactants in environmental and petro-chemical industries, and as antimicrobial agents in health care and food processing industries (Banat et al., 2000; Gudina et al., 2015a). In recent years, the application of biosurfactants has been regarded as a cost-effective and eco-friendly approach in

environmental remediation such as soil washing (Mulligan et al., 2001; Silva and Sarubbo, 2015; Urum et al., 2003). Though soil washing has been widely applied to remediate soil contaminated with crude oil, only a few studies focused on the removal of crude oil from contaminated soil through a soil washing process with biosurfactants (Uhmann and Aspray, 2012; Urum and Pekdemir, 2004).

Biosurfactants display a wide variety of chemical structures including small molecular weight biosurfactants such as glycolipids, phospholipids and lipopeptides; and high molecular weight biosurfactants such as amphipathics, polysaccharides, proteins, lipopolysaccharides, and lipoproteins (Pacwa-Plociniczak et al., 2011). Surfactin is a lipopeptide biosurfactant produced by *Bacillus Substilis* strains. Kuyukina et al. (2005) examined the enhanced crude oil desorption and dispersion through in a soil system with the injection of biosurfactant solution. Gudina et al. (2015b) and Pereira et al. (2013) reported the enhanced solubilization of crude oil from soil with the injection of biosurfactant, and the reduction of ST to 27 mN/m. Surfactin also shows a high emulsifying activity and high antimicrobial, antiviral, and antitumor activities (Gudina et al., 2013). However, the biosurfactants produced by *Bacillus Substilis* strains were not well commercialized mainly due to the high production cost (Marin et al., 2015). The expected breakthrough in terms of their applications remains to be achieved. Research has indicated that proper selection of culture conditions for biosurfactant production, especially the carbon and nitrogen sources, can promote its production rate

thus reduce its production costs (Fonseca et al., 2007). Aiming at production cost reduction and effectiveness improvement, selection of nutrient sources was suggested to be further explored (Daverey and Pakshirajan, 2009; Reis et al., 2004; Saikia et al., 2014).

In this study, biosurfactant production by a *Bacillus Substilis* strain previously isolated from the Atlantic Ocean (Cai et al., 2014) was studied through a proper manipulation of carbon and nitrogen sources. Biosurfactant productions with different media compositions were investigated using parameters including ST, emulsification activity, and solution dilution. Biosurfactants generated by the selected growth media were characterized for the composition with thin layer chromatography (TLC). The ionic charge of generated biosurfactants and their stability were further studied. Finally, the effectiveness and applicability of the biosurfactant product in enhanced oil removal was evaluated.

5.2 Materials and Methods

5.2.1 Biosurfactants Producing Microorganism

The bacterium used in this study were screened in the NRPOP lab from oily contaminated seawater samples, named as *Bacillus Substilis* N3-4P (Cai et al., 2014). Bacillus strains are a group of well recognized biosurfactant producers, which can lower the water ST below 27 mN/m. Among screened bacillus strains in the NRPOP lab,

Bacillus Substilis N3-4P was identified as a promising and economic biosurfactant producer whose products possessed strong surface activity and high emulsification capacity. *Bacillus Substilis* 21332 is a well-known commercialized biosurfactant producer for the product of surfactin. It was obtained from the American Type Culture Collection (ATCC) as a performance comparison with lab generated bacteria.

5.2.2 Media and Cultivation Conditions

The composition of the inoculum broth used was as follows: BD Difco™ Nutrient Broth 23400 (Fisher Scientific Company, Ottawa, Canada) 8.0 g and NaCl 5.0 g in 1L of distilled water. A loopful of a bacteria colony was transferred to a 125 mL Erlenmeyer flask containing 50 ml inoculum broth. The culture was initially grown on a BUCHI® R-215 rotary incubator shaker at 200 rpm for 24 h under room temperature. This seeded culture media was used as inoculum at the 1% (v/v) level. For biosurfactant production, a mineral salt medium modified from Cai et al. (2014) was listed as follows (g L^{-1}): hexadecane (1%), glucose (0.5) and sucrose (0.5), $(NH_4)_2SO_4$ (10), NaCl (15), $FeSO_4 \cdot 7H_2O$ (2.8×10^{-4}), KH_2PO_4 (3.4), $K_2HPO_4 \cdot 3H_2O$ (4.4); $MgSO_4 \cdot 7H_2O$ (1.02); yeast extract (0.5) and trace element solution, 0.5 ml L^{-1} of distilled water. The trace element solution contained (in g L^{-1}) $ZnSO_4$ (0.29); $CaCl_2$ (0.24); $CuSO_4$ (0.25); $MnSO_4$ (0.17) g L^{-1} of distilled water and was sterilized separately.

Carbon and nitrogen sources were added separately. In order to study the effect

of carbon source on biosurfactant production, the carbon source in this growth media was replaced by sodium acetate (SA), sodium citrate (SC), glycerol (GLY), glucose (GLU), sucrose (SUC), starch (STA), n-hexadecane (HEX), and diesel (DIE) separately at a concentration of 10 g L^{-1}, or 1% (v/v). Similarly, while exploring the nitrogen effect on biosurfactant production, the nitrogen sources in original recipe ($(NH_4)_2SO_4$ and yeast extract) were replaced by ammonium sulfate (AS), yeast extract (YE), and sodium nitrate (SN) at a concentration of 10 g L^{-1} separately.

The effect of different carbon and nitrogen sources on the production of biosurfactants was evaluated using ST, emulsification index (EI), and series dilution as productivity, respectively. Medium without bacteria was used as the abiotic control. The selected carbon and nitrogen sources were further used for biosurfactant production by lab screened bacteria *Bacillus Substilis* N3-4P. The biosurfactant product was purified and freeze dried for characterization. The characterizations of biosurfactants include composition content using TLC and its chemical composition content, and ionic character. Lab generated biosurfactants were further validated for their application in the soil washing system to clean up crude oil contaminated soils.

5.2.3 Biosurfactant Production and Purification

Selected biosurfactant production medium are listed as follows (g L^{-1}) based on the results from section 5.2.2: Selected carbon source glycerol (10), nitrogen source

$(NH_4)_2SO_4$ (10), NaCl (15), $FeSO_4 \cdot 7H_2O$ (2.8×10^{-4}), KH_2PO_4 (3.4), $K_2HPO_4 \cdot 3H_2O$ (4.4), $MgSO_4 \cdot 7H_2O$ (1.02), and yeast extract (0.5) as additive and trace element solution, 0.5 ml L^{-1} of distilled water. The trace element solution, as described in section 5.2.2 was sterilized separately. Fermentation was performed in 1L flasks containing 600 mL production medium. The medium was incubated in a shaking incubator at 200 rpm for five days. The culture broth was centrifuged at 12,000 rpm for 10 min to remove all cells. Afterwards, biosurfactant solution was further purified through solvent extraction with an equal volume of chloroform–methanol (1:2 v/v) solvent. The solvent was removed by rotary evaporation and then freeze dried.

5.2.4 Biosurfactant Enhanced Soil Washing

Lab scale biosurfactant enhanced soil washing experiments were carried out in a bench-scale column as Figure 5-1 illustrated. The soil was air dried, homogenized and kept in an oven overnight at 105°C. Physical and chemical characterization of the soil was performed in accordance with methods of soil analysis (Page, 1982). The results presented in Table 5-1 suggested that the soil is a fine silty loam. Five grams of crude oil was spiked into 1 kg of soil and well mixed before use. Five hundred gram of crude oil contaminated soil sample was layered into a cylindrical column with a diameter of 3.8 cm and height of 30 cm. To prevent soil from being washed from the column the bottom was covered with a layer of fiberglass. Additionally, a layer of glass beads with a 6 mm diameter were laid at the bottom and top of the soil column. Soil washing

experiments were conducted with lab generated biosurfactant solutions at two different concentrations of 4 g L^{-1} and 8 g L^{-1}. A control experiment was run in parallel where the soil was treated with distilled water. The biosurfactant solution or water was continuously pumped through the column for 8.5 hours. The washing effluent from the column was collected and analyzed for flushed crude oil concentration. Soil samples were collected before and after the experiment for the removal rate.

5.2.5 Sample Analysis

ST and CMC: The determination of ST and CMC was followed by the method described in section 3.2.6.

Dilution of biosurfactant solution: The biosurfactant concentration was estimated by measuring the ST for varying dilutions (2, 5 and 10-fold) of the sample. The dilution at which the ST began to increase indicated the effective biosurfactant concentration exceeded the CMC (Ghurye et al., 1994). A higher ST indicated a lower concentration of the biosurfactant solution.

EI_{24}: The determination of EI24 was followed by the method described in Chapter 3.2.6.

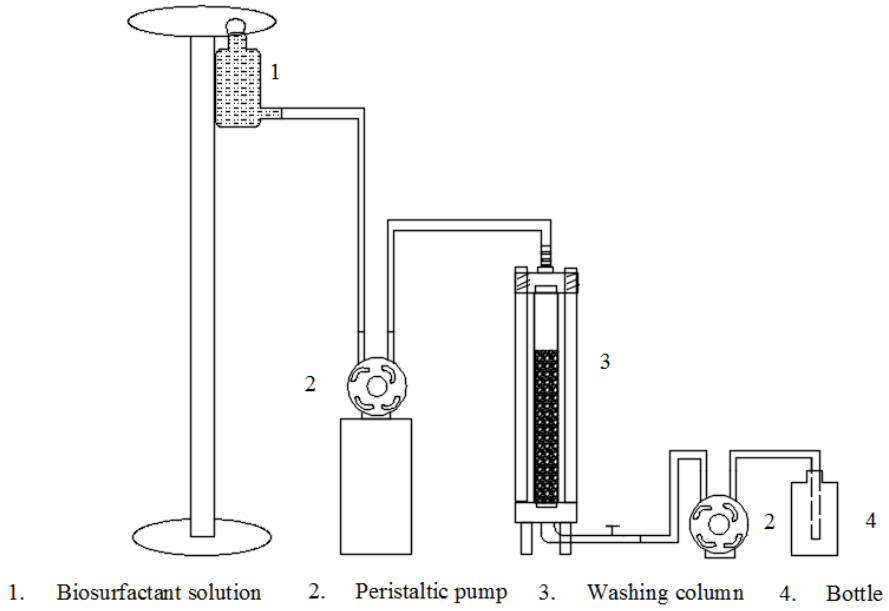


Figure 5-1 Bench scale soil washing system

Table 5-1 Physical properties of soil

SOIL PROPERTIES	VALUE
PARTICLE SIZE DISTRIBUTION (%)	
SILT (<0.06 MM)	43
SAND (0.06-2 MM)	52
GRAVEL (>2 MM)	5
MASS OF CRUDE OIL PER GRAM OF SOIL (MG)	4.8
BULK DENSITY (G·CM-3)	1.53
POROSITY (%)	36.5
PH	7.43

TLC: A 0.01 g of purified biosurfactant sample was dissolved in 1 mL methanol and was subjected to TLC analysis. Ten microliter sample was applied to a silica gel TLC plate (Sigma Aldrich). Carbohydrate and lipid were developed in a chloroform: methanol: acetate acid (95:15:2) solvent system and protein was developed in an n-butanol: acetic acid: water (4:3:0.5) solvent system. The spots were revealed with colour reagents. For detection of amino acids, the dry plates were sprayed with a solution of 0.5 g ninhydrin in 100 mL acetone and kept at 105 ºC for 5 min. Lipid content was visualized by iodine chamber. Carbohydrates were visualized by spraying phenol-sulfuchromic acid and heating at 105 ºC for 5 min.

Composition analysis: The chemical composition of biosurfactant was determined at a concentration of 10mg mL^{-1}. The protein content was determined by the method of Bradford (1976). Total carbohydrate content was estimated using the phenol–sulfuric acid method by Dubois et al. (1956). Lipid content was determined based on the method described by Pande et al. (1963).

Stability characterization: The stability of generated biosurfactants was determined at different temperature, pH, and salinity following Abouseoud et al. (2008). Generally, 1 CMC of biosurfactant solution was prepared and maintained at a constant temperature of 0, 25, 50, 75, and 100 °C for 120 min and cooled at room temperature. Similarly, pH stability was determined by adjusting the pH value of biosurfactant solution to 2, 4, 6, 8, 10, and 12 using HCl or NaOH. The effect of salinity on the

stability of the biosurfactant was investigated by adding NaCl at a concentration of 1, 2, 3, and 4% (w/v). Stability was determined by the change of ST values in duplicate.

Ionic charge determination: The ionic charge of generated biosurfactants was characterized using the agar double diffusion tests (Meylheuc et al., 2001). This test was based on the passive diffusion of two compounds bearing the same or opposite type charges in an agar plate. A low hardness agar plate (1%) was prepared with two regularly-spaced rows of wells. The bottom hole was filled with lab generated biosurfactant solution, and the upper well was filled with selected pure compound with known ionic charge. The appearance of precipitation lines with known compounds indicated the ionic character of lab generated biosurfactants. The selected anionic compounds, sodium dodecyl sulphate (SDS) (Sigma-Aldrich) was prepared at a concentration of 20 mmol L^{-1}. The cationic compounds barium chloride (Sigma-Aldrich) and cetyltrimethylammonium bromide (CTAB) (Sigma-Aldrich) were prepared at 50 mmol L^{-1} and 20 mmol L^{-1} respectively following Meylheuc et al. (2001).

Chemical analysis of crude oil in soil: Soil samples were collected before and after the soil washing process to test the crude oil concentration. Generally, soil samples were taken from three spots in the reactor, and mixed well before the test. The concentration of crude oil in collected soil was determined using the method adapted from Urum and Pekdemir (2004) and Han et al. (2009). Generally, 10 mL of hexane was mixed with 5g of collected soil sample. The mixture was shaken laterally

for 5 min, and all the n-hexane/crude oil extract was removed by centrifugation for 10 min at 3,000 rpm. The extraction process was repeated for four times until the final extract had the same absorbance as that of the pure n-hexane. All four times the extract was collected into a volumetric flask and made up to 50 mL with n-hexane. Concentration of crude oil was determined by measuring the absorbance of extract at the wavelength of 229 nm at room temperature with a Sigma spectrophotometer. The test was performed in duplicate. The concentration of crude oil in the soil system was determined using Equation 5-2 as follows:

$$\mathbf{O = 2.25A/m\ (mg \cdot g^{-1})} \qquad \textbf{(5-1)}$$

where O is the concentration of crude oil in soil ($mg \cdot g^{-1}$ dry soil);

A is the absorbance of the diluted crude oil/n-hexane solution at 229 nm; and

m is the weight of soil collected (g).

The crude oil removal efficiency was determined using the Equation 5-3 as follows:

$$\mathbf{Removal}\ (\%) = \frac{O_i - O_r}{O_i} \times 100\% \qquad \textbf{(5-2)}$$

where O_i is the initial crude oil concentration in the crude oil contaminated soil ($mg \cdot g^{-1}$ dry soil) before washing and O_r is the residual crude oil concentration in the soil ($mg \cdot g^{-1}$ dry soil) after washing.

Chemical analysis of crude oil in washing solution: A 10 mL of soil washing solution was collected every 30 min and analyzed for flushed crude oil concentration. Ten milliliters of n-hexane were added into the washing solution and were shaken laterally for 30 min. Samples were then centrifuged at 3,000 rpm for 10 min. The centrifuged supernatant was analyzed for crude oil content using a spectrophotometer at 229 nm. The concentration of crude oil was then determined following the method mentioned previously in section 5.2.5.

5.2.6 Statistical Analysis

All the tests were performed in duplicated to ensure the reliability of results, and the results were expressed as the average of two measurements. Biosurfactant production and its performance were analyzed using OriginalPro® 9.0 with paired t-tests for the statistical evaluation of differences between treated groups and the control. A P-value of less than 0.05 indicated a significant difference between the tested groups.

5.3 Results and Discussion

5.3.1 Effects of Carbon and Nitrogen Sources on Biosurfactant Production

Different water miscible and immiscible carbon substrates were investigated for their capacity to support bacteria growth and biosurfactant production by lab screened marine origin bacteria *Bacillus Substilis* N3-4P and one commercial bacterium *Bacillus*

Substilis 21332 in this work. Hexadecane and diesel were employed as water-insoluble carbon sources, and glucose, sucrose, starch and glycerol were selected as water-soluble carbon sources. An important indication for the production of biosurfactant is the reduction of surface tension of growth medium (Youssef et al., 2004). The surface tension reduction ability of generated biosurfactants is illustrated in Figure 5-2. From the figure it can be found that no biosurfactant production was detected by *Bacillus Substilis* 21332 in the medium using diesel and hexadecane as carbon sources. Instead, water soluble carbon sources, such as glucose, sucrose, starch, and glycerol were more preferred for biosurfactant production. The surface tension of starch-based growth media could be reduced to as low as 28 mN/m. Biosurfactants produced by *Bacillus Substilis* 21332 mostly displayed a good EI value, and the one generated by glucose could reach to a value of 55.2%. For lab screened strain *Bacillus Substilis* N3-4P, the addition of glycerol, hexadecane and diesel were found to promote the production of biosurfactants. The lowest surface tension of glycerol-based cell free culture broth that could be achieved was 27.8 mN/m, with an EI of 38.3%.

The effect of nitrogen sources on biosurfactant production rate was also investigated in the study, and the results are listed in Figure 5-2. The studied nitrogen sources were classified into organic (yeast extract) and inorganic (sodium nitrate and ammonium sulfate) sources. From this figure it can be found that the role of nitrogen source in influencing biosurfactant production is quite evident. The organic nitrogen

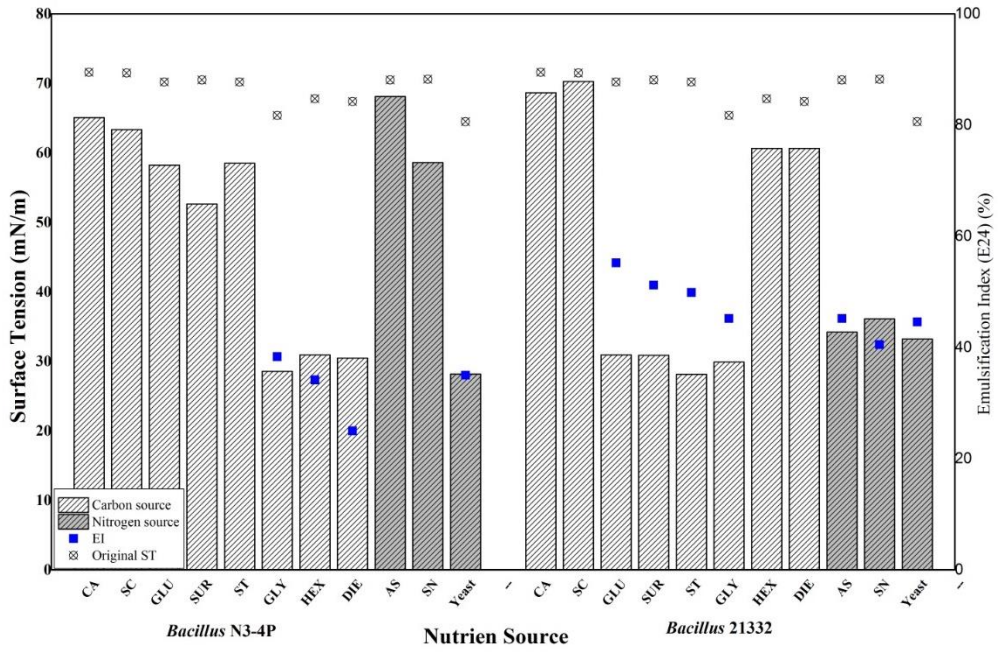


Figure 5-2 Effect of carbon sources (CA, SC, SUR, ST, Gly, Hex, Die) and nitrogen sources (AS, SN, Yeast) on surface tension reduction and emulsification index of lipopeptide biosurfactant generated by *B.Substilis* N3-4P and *B.Substilis* 21332

source yeast extract was a promising nitrogen source for both bacteria. A decrease in surface tension of the culture broth was observed for *Bacillus Substilis* 21332 using AN and SN based growth media. However, neither of them assisted biosurfactant production by *Bacillus Substilis* N3-4P. In addition, comparing the biosurfactant production by *Bacillus Substilis* N3-4P using different carbon and nitrogen sources, it was found that a biosurfactant production was observed in GLY, HEX, DIE based media using AN and yeast extract as the nitrogen source, and Yeast based media using hexadecane as the carbon source, yet AN as a sole nitrogen source and hexadecane resulted in a poor biosurfactant production. Therefore, it can be concluded that a mixture of both organic and inorganic nitrogen sources can greatly promote the biosurfactant production.

The selection of proper carbon sources is highly related with biosurfactant production rate and the final products (Panilaitis et al., 2007). Therefore, cell free growth media was further diluted 2, 5, and 10 times and examined for surface tension reduction as an indirect measurement of the relative biosurfactant concentration in the growth media. The recorded results are illustrated in Figure 5-3. From the figure it can be found that the effect of carbon source on biosurfactant production by *Bacillus Substilis* N3-4P was as follows: glycerol > hexadecane > diesel. After 10 times dilution, the concentration of biosurfactant in the solution was able to reduce its surface tension to lower than 40 mN/m. Sucrose, starch and glycerol can serve as promising carbon

sources for *Bacillus Substilis* 21332. Those carbon source-based cell free growth media were able to reduce surface tension to lower than 35 mN/m after 10 times dilution. The most effective carbon source for *Bacillus Substilis* 21332 was sucrose, whose surface tension remained unchanged even after 10 times dilution. Yeast was identified as a favorable nitrogen source for both bacillus strains.

The mechanisms of carbon source utilization for biosurfactant production are closely related with selected bacteria. This study indicated that for strain *Bacillus Substilis* N3-4P, biosurfactant production rate is much higher when using glycerol or hydrocarbon as carbon sources. Healy et al. (1996) reported that the addition of carbohydrate was capable of stimulating the production of secondary acid metabolites such as uronic acid, which hindered the synthesis of biosurfactants. This may explain the inhibition of biosurfactant production by *Bacillus Substilis* N3-4P using carbohydrate carbon source such as starch and sucrose in this study. On the other hand, bacteria *Bacillus Substilis* 21332 was found to have an opposite preference on the selected carbon source. A poor biosurfactant production was discovered using hydrocarbon as their carbon sources yet this rate was much higher on water soluble carbon substrate. The ability of using water soluble carbon sources for the production of biosurfactants was reported by previous studies (Fox and Bala, 2000; Patel and Desai, 1997). Research conducted by Abdel-Mawgoud et al. (2008) and Das et al. (2009b) reported an inhibitory effect on the use of hydrocarbons (including n-hexadecane and

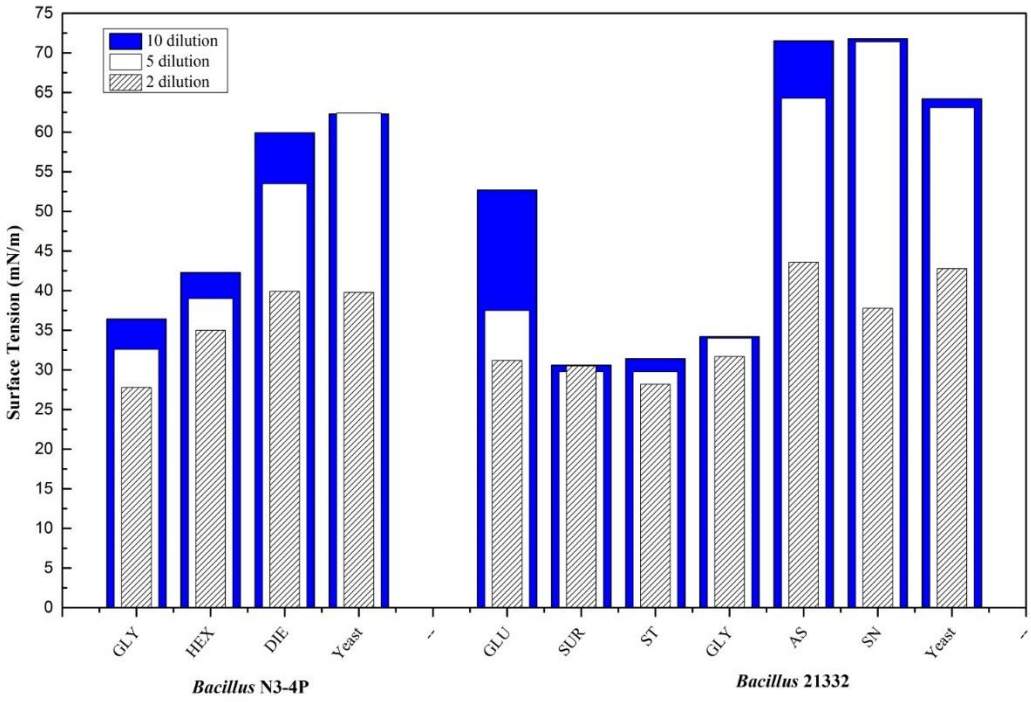


Figure 5-3 Effect of carbon source (Gly, Hex, Die) and nitrogen source (AS, SN, Yeast) on lipopeptide biosurfactant production generated by *B.Substilis* N3-4P and *B.Substilis* 21332

diesel) for bacterial growth and biosurfactant production. In their study, glucose and sucrose-based growth media had a better bioisurfactant production rate. Similarly, the uses of hydrocarbons as the sole carbon source resulting in no biosurfactant production was also proved by Joshi et al. (2008b). Das and Mukherjee (2007) utilized potato as substrate, and the production rate can reach high up to 80.0 ± 9 mg gds^{-1} (per g of dry substrate). Though the generation of biosurfactants with hydrocarbons as the substrate was also being reported (Gudina et al., 2013), they are mainly used for in-situ remediation purposes instead of for direct biosurfactant production. Besides that, addition of minerals is also important for biosurfactant production, and the addition of nutrients such as yeast extract will stimulate the production of biosurfactant even with the presence of hydrocarbons (Cai et al., 2014). In conclusion, various cheaper carbon sources can be used as an alternative to support the growth of lab screened bacillus strains for biosurfactant production. They can even be identified as an industry waste. For instance, glycerol is a by-product of the biodiesel industry, starch and glucose can be widely found in agro-industrial waste, and sucrose is commonly existing in sugar processing industry waste (Das et al., 2009b). A proper selection of corresponding industrial waste can further reduce the production cost.

Research has indicated that the conditions of nitrogen metabolism have played an important role in surfactin production (Davis et al., 1999). Many different sources of nitrogen had been investigated for biosurfactant production, and the most frequently

used substrates were nitrate salts and ammonia. Among the inorganic salts tested, nitrate ions supported maximum biosurfactant production in *Bacillus Substilis* (Makkar and Cameotra, 1998). A recent research conducted by Abdel-Mawgoud et al. (2008) indicated that sodium nitrate was the best nitrogen source for surfactin production while other tested nitrogen sources decreased surfactin production with different degrees. This conclusion was verified in this study as well. Utilization of $NaNO_3$ as a nitrogen source presented the best reduction of surface tension by *Bacillus Substilis* 21332. In contrast to the carbon source used in biotechnological processes, complex or less well-defined sources of nitrogen (e.g., yeast extract or protein hydrolysates) are relatively less researched, yet have proved to have promising productivity. The utilization of protein hydrolysates as an alternative nitrogen source is attractive for biosurfactant production.

5.3.2 Characterization of Generated Biosurfactant Product

Lab screened bacteria *Bacillus Substilis* N3-4P was further characterized for its chemical composition and stability using glycerol as the selected carbon source, and $(NH_4)_2SO_4$ and yeast extract as nitrogen sources. The biosurfactant product was able to reduce the surface tension of distilled water from 72 mN/m to 27 mN/m. The CMC value of the product was determined by separately measuring the surface tension of different concentrations of the product, and the value was 0.507 g L^{-1}.

A TLC analysis indicated the biosurfactant product was a mixture of carbohydrate,

lipid and protein. This mixture contained 21% (w/w) of protein, a 35% (w/w) of lipid and 18% of carbohydrate (w/w). This result indicated that the product was very likely to be a mixture of lipopeptide and glycolipid biosurfactant. No precipitation lines were observed between lab generated biosurfactants and selected chemical compounds (barium chloride, CTAB, SDS). Therefore, lab generated biosurfactant product was proved have non-ionic character. Mulligan (2005) and Cameotra and Makkar (1998) also proved that most of the biosurfactants were either with neutral or anionic character.

Biosurfactant stability with different environmental conditions, such as variation temperatures, pHs and salinities are highly related with its applicability in the fields. Therefore, the stability of biosurfactant product generated by *Bacillus Substilis* N3-4P was tested over a wide range of temperature, pH value and salinity (Figure 5-4). Enhanced surface tension reduction ability during the heating process was observed in this study. An enhanced surface activity of biosurfactant product was observed as the temperature increased. A lab generated biosurfactant solution achieved the lowest surface tension when the temperature reached 100 °C, yet it still had a remarkable surface tension reduction capacity even at 0°C. Therefore, it can be concluded that this product maintains its surface properties unaffected in the range of temperatures between 0 and 100 °C. Similarly, the surface tension of generated biosurfactants decreased as the pH increased, indicating that they had a better stability under a relatively high pH condition. The unchanged surface tension of biosurfactant solution under various

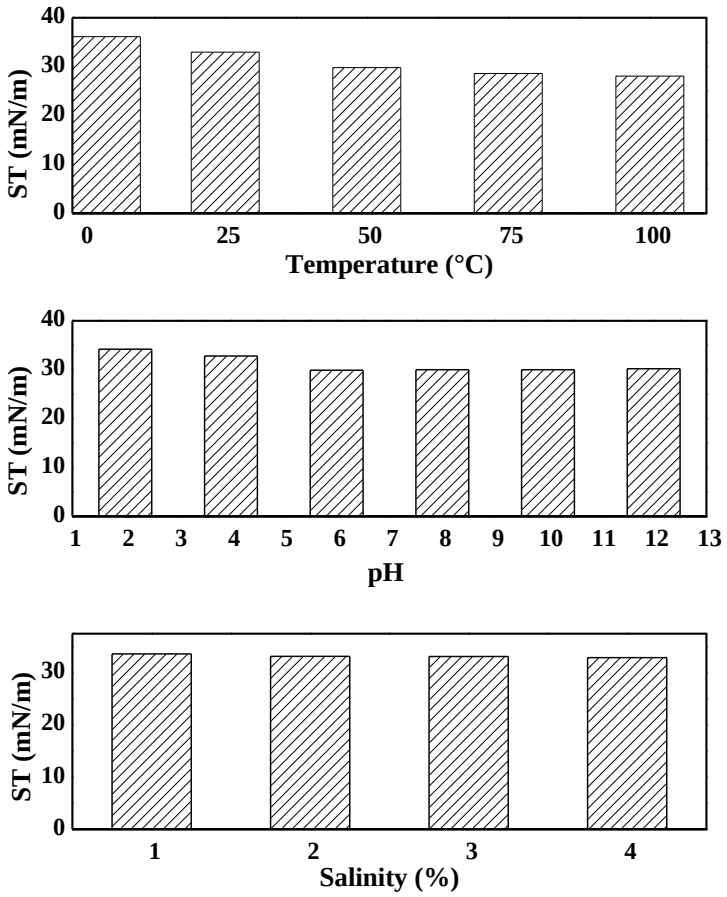


Figure 5-4 Stability of the biosurfactant product produced by *B.Substilis* N3-4P under various temperature, pH and salinity conditions

salinities demonstrated the generated biosurfactant had a stable performance between salinity of 1%-4%.

5.3.3 Biosurfactant Enhanced Soil Washing for Crude Oil Removal

Figure 5-5 illustrates the effect of biosurfactant concentration on enhanced crude oil removal in soil washing systems. From this figure it can be found that both biosurfactant solutions significantly enhanced the removal rate of crude oil as compared to a control column using distilled water as a washing agent. Biosurfactant concentration with 4g L^{-1} had a lower crude oil removal rate and a longer washing time than the one with 8g L^{-1}. It can also be found that biosurfactant- enhanced aqueous systems were much faster to reach saturation than the control. As the biosurfactant concentration was increased, an improved percentage of crude oil removal was observed. With the application of 4g L^{-1} and 8g L^{-1} crude biosurfactant solution, a 58% and 65.2% of crude oil could be removed from the soil systems respectively, while the control system (water only) could only cleanup 36.9% crude oil in the contaminated soil.

Given that the crude oil was complex in nature and composition with over 50-80% aliphatic hydrocarbons, the cleanup of crude oil contaminated soils requires more efforts compared with other petroleum contaminated soils (NRC, 1985). The result

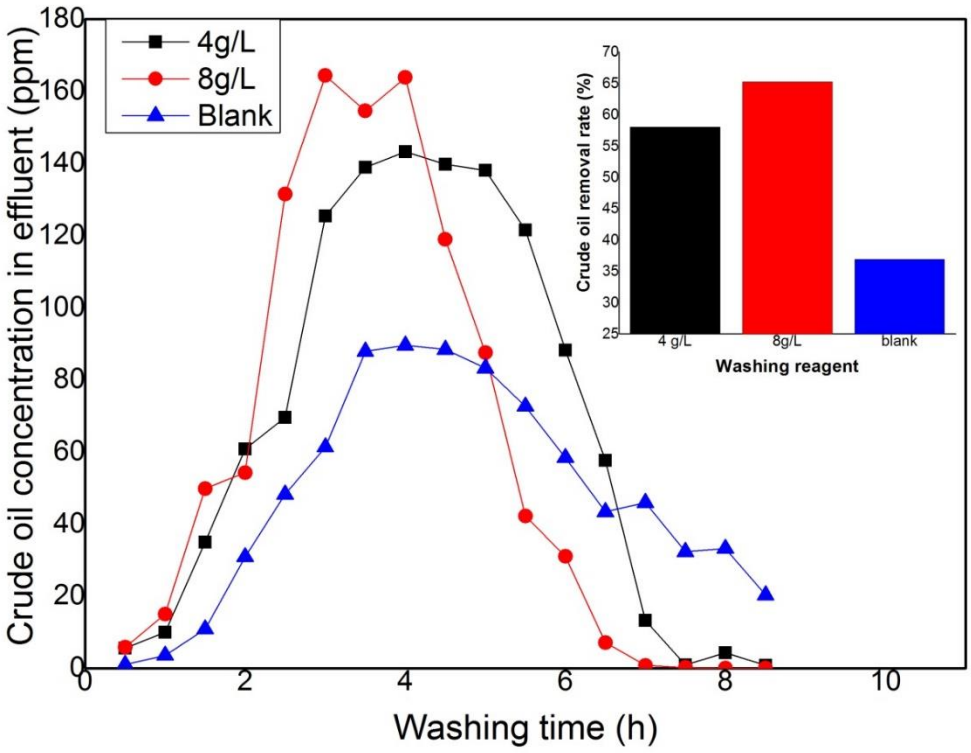


Figure 5-5 Effect of biosurfactant concentration on enhanced crude oil removal in soil washing systems

obtained in this study was expected, and in accordance with previous studies where Lai et al. (2009) examined biosurfactant enhanced TPH removal from low TPH contaminated soil (3 mg g^{-1}) and high TPH contaminated (HTC) soil (9 mg g^{-1}). Their result indicated that biosurfactant product had a better performance when treating high TPH contaminated soil. Addition of rhamnolipid and surfactin solution could increase the removal rate from 20.4% to over 60% from HTC soil. This study suggested that lab generated biosurfactants could be used as an effective washing agent to cleanup crude oil in a soil system. Urum et al. (2006) provided some insight on the removal of crude oil from soil systems. Their research indicated the preference for crude oil removal highly depended on the selected surfactant. A significant amount of oil compounds could be removed from the systems; however, high molecular weight aromatic hydrocarbons such as dibenzothiophenes, an organic compound occurring widely in heavier fractions of petroleum, can hardly be removed by the studied surfactants. A similar conclusion was confirmed by Zhang (2015) as well. This study helped to explain the relationship between residue oil in the soil column and limited oil concentration in the eluent in this study.

In this study, compared with using distilled water as washing agent, crude oil removal rate was significantly increased using biosurfactant based washing solution. The mechanism of biosurfactant enhanced crude oil removal is closely related with its concentration. When the concentration of biosurfactant solution was below its CMC

value, the mechanism of biosurfactant enhanced crude oil removal mainly relied on the reduced surface and interfacial tension at the water-air and crude oil-water interface due to its amphiphilic structure (Abdul et al., 1990). The lowered interfacial tension thus led to an increased contact angle and reduced capillary force holding the crude oil and soil particles and consequently enhanced the mobility of crude oil (Kavitha et al., 2014; Pacwa-Plociniczak et al., 2011). This is also known as the mobilization mechanism (Pacwa-Plociniczak et al., 2011). When the concentration of biosurfactant is above its CMC value, the formation of biosurfactant micelle can greatly increase the solubilization process, and help to solubilize the residue oil compounds left in the soil system and enhance the removal of organic contaminants (Urum and Pekdemir, 2004). Moreover, a recent research conducted by Zhang et al. (2014) indicated that biosurfactant enhanced solubilization and structural disjoining pressure in the wedge film is another reason for the oil droplet detachment from the soil surface. The extent of this pressure is correlated with the micelle size, particle size, and surface charge of particles (Zhang et al., 2014). Therefore, it can be concluded that the enhanced solubilization and the structural disjoining pressure was the major reason for crude oil removal in this study. The increased concentration of biosurfactant would accelerate the formation of micelles in system, and those micelles could replace the biosurfactant monomers adsorbed to the soil, and increase the effective biosurfactant concentration in the system. In this study, compared with using 4g L^{-1} of biosurfactant solution as

washing agent, a higher crude oil removal rate was reported using the biosurfactant solution at 8g L^{-1}.

Last but not least, the removal of hydrocarbon from soil system was reported to be closely related with soil texture and mineralogy (Lee et al., 2002). The reported removal rate in this study was lower than the ones treated with sandy soils. Researches also proved that biosurfactants had a better performance in sandy soils (Lee et al., 2001). The effectiveness of surfactant-based remediation can be limited by adsorption of surfactants to clay, silt, and organic soil contents (Lee et al., 2001). Furthermore, given that most of the soil surface was negatively charged; the adsorption process was even worse for the cationic surfactants. They tended to a higher affinity on the soil particles, thus affect its removal efficiency. Lab generated non-ionic biosurfactant by *B. Substilis* N3-4P were proved to have a non-ionic character, thus they were believed to have a better performance. This result was proved by other studies. Kavitha et al. (2014) and Zhang (2015) found that the solubility of crude oil was proved to be proportional to the concentration of biosurfactants with a non-ionic nature, such as rhamnolipid. Urum and Pekdemir (2004) found that with the injection of rhamnolipid at 25 CMC, the removal rate of crude rate could reach up to 80%. In this study, a 58% and 65.2% of crude oil was removed from soil system using biosurfactant solutions at 4g L^{-1} and 8g L^{-1} respectively. Considering that the lab generated biosurfactant is a non-ionic lipopeptide complex, a higher removal rate is expected when a higher concentration of biosurfactant

solution is adopted as a washing agent.

5.4 Summary

The enhanced biosurfactant production by marine originated bacteria *Bacillus Substilis* under different carbon and nitrogen sources was studied by comparing the ST, EI and CMD. The results proved the capability of marine bacteria *Bacillus Substilis* N3-4P in producing biosurfactants, which were a mixture of lipopeptide and glycolipid. The production rate and emulsion capacity were compatible with those generated by commercial strains *Bacillus Substilis* 21332. The highest production rate was achieved when using glycerol as the carbon source, and yeast extract and sodium nitrate as nitrogen sources. The biosurfactant solution could reduce the surface tension of distilled water to as low as 27 mN/m, with a CMC value of 500 mg L^{-1}. Even after dilution of 10 times, a surface tension of 36.4 mN/m was still observed. The biosurfactant product was found to have non-ionic character, and had a stable performance with a duration up to 24 hours at various temperatures (0-100 °C), pH (2-8) and salinity (1-4%) values.

This study further evaluated the effectiveness and applicability of the generated biosurfactants in crude oil soil washing. The results showed that the removal rates reached 58% and 65.2% by introducing the generated biosurfactants with the concentrations of 4g L^{-1} and 8g L^{-1}, respectively. In comparison, only 36.9% of crude oil was washed out with water only. Given the adsorption of anionic surfactant onto

negatively charged soil particles, the injection of non-ionic bacillus biosurfactant was consider as more suitable as a soil washing agent since it was less likely to be adsorbed to the soil and thus was mobile and effective. Overall, the results demonstrated the potential of bacillus biosurfactants for applications in petroleum contaminated site remediation. Ongoing studies are being carried out analyzing soil samples and effluent for residue components to better understand the removal mechanism by using biosurfactants as a soil washing agent.

CHAPTER 6
EFFECT OF A LIPOPEPTIDE BIOSURFACTANT GENERATED BY *BACILLUS SUBSTILIS* N3-1P ON THE BIODEGRADATION OF HYDROCARBONS IN THE PRESENCE OF HEAVY METALS: CHANGES IN CELL CHARACTERISTICS AND MICROBE PERFORMANCE

This chapter is based on the following manuscript:

Zhu, Z. W., Cai, Q., Zhang, B., Chen, B., Lee, K., and Lin, W. (2018). Effect of a lipopeptide biosurfactant generated by *bacillus substilis* N3-1P on the biodegradation of hydrocarbons in presence of heavy metals: changes in cell characteristics and microbe performance. *Environmental Pollution.* (to be submitted).

Role: Zhiwen Zhu is the principal investigator of this study and acted as the first author of this manuscript under Dr. Baiyu Zhang and Dr. Bing Chen's guidance. Most contents of this paper were written by her and further edited by the other co-authors.

6.1 Introduction

Industrial development and human activities have led to an increasingly concern over groundwater and soil contamination as a result of unexpected release of PHCs and associated pollutants (e.g., heavy metals) from petroleum and/or the refining products in recent decades (Chandra et al., 2013). Employing microbial processes (also known as bioremediation) to cleanup contaminants has proven to be effective and reliable due to the high ecological significance and cost-efficiency features. However, the low bioavailability of PHCs due to the hydrophobic nature (low water solubility) has hindered their biodegradation (Liu et al., 2017). The co-occurrence of toxic heavy metals such as nickel (Ni), cadmium (Cd) and lead (Pb) could further change and/or inhibit the metabolic activity and physiological processes of oil-degrading microbes, posing another major obstacle to PHCs biodegradation (Olaniran et al., 2013). Biosurfactants have attracted increasing attention as amphipathic surface-active compounds. They have high biodegradability, low toxicity, and specific activity at extreme environmental conditions (Mulligan et al., 2014). They can reduce the surface tension of water, and interfacial tension between two liquids (e.g., water and oil). The formation of surfactant micelles promotes the partitioning of PHCs into the aqueous phase (Beal and Betts, 2000; Damrongsiri et al., 2013; Lanzon and Brown, 2013). In the meantime, less soluble metal salts including phosphate and sulfide precipitates are formed through the metal-surfactant complexation process (Mosa et al., 2016). Cell

interactions with cations are reduced accordingly, and the heavy metal toxicity can be decreased in the co-contamination system (Gnanamani et al., 2010).

To date, the underlying mechanism of biosurfactants enhanced biodegradation (e.g., biosurfactant micellization behaviors, PHCs solubilization modifications and oil-microbe attachment) have been documented (Bai et al., 2017; Ivshina et al., 2016; Liu et al., 2016). Application of biosurfactants has demonstrated the capacity to overcome the diffusion-related mass transfer limitations of hydrocarbons; and biosurfactant-enhanced bioremediation technique have thus been developed (Bezza and Chirwa, 2016; Liu et al., 2016). Biosurfactant induced cell surface modifications have been spotted (Kuyukina et al., 2016; Sun et al., 2016). Cell surface properties (e.g., the cell surface hydrophobicity (CSH), cell surface permeability and zeta potential), have been reported to be of significant importance during a biodegradation process. Yet, they can be changed through biosurfactant induced removal of lipopolysaccharide from the cell surface, and/or the adsorption of biosurfactants onto the cell surface (Zhong et al., 2015). In addition, the membrane transportation of PHCs, also known as a key process in governing the PHCs biodegradation rate (Zhang et al., 2013), can be affected by biosurfactant-cell wall lipid bilayer interaction (Zeng et al., 2018). However, few studies regarding the cell surface associated mechanism of biosurfactant enhanced PHCs degradation in a co-contaminated environment have been reported (Liu et al., 2016; Smułek et al., 2015).

Therefore, the present study attempted to investigate the effect of a biosurfactant on the biodegradation of a PHC (i.e., diesel) in the presence of the heavy metal (i.e., Ni) with various concentrations within an acute (24hr) and long (14 day) term, respectively. The lipopeptides biosurfactant product generated in Chapter 4, with demonstrated high surface activities and extremely low CMCs, was selected in this study. It is hypothesized that in a co-contaminated environment, lipopeptide biosurfactant addition could not only reduce the toxicity of heavy metals, but also affect cell wall-associated PHCs degradation mechanism (e.g., biosorption and biodegradation). *Rhodococcus erythropolis*, a well-known PHCs degrader was selected in this study. The effects of the lipopeptide biosurfactant on the cell surface properties, including bacterial CSH, membrane permeability, cell zeta potential, and cell size distribution in the oil-metal co-contaminated environment were examined. Performance of the lipopeptide biosurfactant on diesel oil partitioning and biodegradation was evaluated. This study would provide a detailed insight into the mechanisms of lipopeptide aided PHCs biodegradation in a co-contaminated system through the interactions among lipopeptide, target contaminants (i.e., PHCs and Ni), and oil degrading microbes (i.e., *Rhodococcus erythropolis*).

6.2 Materials and Methods

6.2.1 Oil Degrading Microorganism and Growth Media

Oil biodegrading bacteria *Rhodococcus erythropolis* was isolated from the oily contaminated sea water and sediment samples from the coastal line (N43.9° to N47.8°) of Newfoundland, Canada (Cai et al., 2014). A loopful of a bacteria colony was transferred from agar plate into a 125 mL an Erlenmeyer flask containing 50 mL of inoculum medium. The inoculum medium was composed of 8.0 g L^{-1} BD Difco™ Nutrient Broth 23400 (Fisher Scientific Company, Ottawa, Canada) and 5.0 g L^{-1} NaCl. It was used as a seeded culture after 24h at an inoculation concentration of 2% (v/v).

The mineral salt media (MSM) used in the bacterial biodegradation assay was modified as described by Yu et al. (2007). The modified MSM recipe was as follows (mg L^{-1}): NaH_2PO_4 (500), KH_2PO_4 (850), K_2HPO_4 (1656), NH_4Cl (1000), $MgSO_4 \cdot 7H2O$ (1.0), $FeSO_4 \cdot 7H2O$ (1.0), $MnSO_4 \cdot H_2O$ (0.36), $ZnSO_4 \cdot 7H_2O$ (0.3), $CoCl_2 \cdot 6H_2O$ (0.1), $CaCl_2 \cdot 2H_2O$ (1.0) and marine nutrient broth (3.74). Diesel oil (Irving, NL, Canada) was added at 2% (v/v). $NiCl_2$ was added into the system to reach a final concentration of 25 ppm and 250 ppm respectively.

The lipopeptide biosurfactant used in this study was generated as described in Chapter 4. The CMC of the generated biosurfactant was 0.407 g L^{-1}. The lipopeptide biosurfactant was prepared at five levels (i.e., 0.5 CMC, 1 CMC. 2 CMC, 4 CMC, and

8 CMC). They were autoclaved and added into MSM medium separately.

6.2.2 The Effect of Biosurfactant on Microbe Activity in the Co-Contamination System

Effects of lipopeptide on the microbial activity in a co-contaminated system were examined in 50 mL Erlenmeyer flasks. Each system was prepared in triplicate, and incubated at 30 °C in an orbital shaker (150 rpm). The ones without lipopeptide were used as control systems. The reactions were terminated at 24 hours and 14 days respectively to evaluate the biosurfactant enhanced acute and long-term microbial effect. After centrifuging the samples at 6,000 rpm for 15 min, cell pellets were re-suspended into PBS buffer solution. The cell surface properties of each cell sample, namely bacterial CSH, membrane permeability, and cell zeta potential were determined.

6.2.3 The Effect of Biosurfactant on Ni^{2+} behaviour in the Co-Contamination System

The cell-free liquid samples collected form Task 6.2.2 were further analyzed for the effects of lipopeptide on the behavior of Ni^{2+}. The particle size, as an indicator of micellar behavior were evaluated. In the meantime, the heavy metal (Ni^{2+}) concentration in each sample was evaluated to reflect biosurfactant enhanced immobilization.

6.2.4 Distribution and Biodegradation of PHC

The distribution and biodegradation of the PHC (i.e., diesel) by *Rhodococcus erythropolis* were carried out in 125 mL Erlenmeyer flasks with 20 MSMs (section 6.2.1) in triplicate. Lipopeptide biosurfactant was added at a concentration of 0.5, 1, 2, 4, and 8 CMC, respectively. The flasks were incubated at 30 °C in an orbital shaker (150 rpm) for14 days. After treatment, the samples were collected and treated with the procedures illustrated in Figure 6-1. Each sample was passed through a filter paper (2 μm) to separate oil degrading cells and the culture medium. The filter paper was further washed with 2 mL MSM broth to collect the residue PHC on it. Two filtrate portions (L1 and L2) were collected to determine the PHC concentration in the aqueous phase. Oil degrading cells on the filter paper were re-suspended in 2.0 mL MSM and lysed by sonication. PHC in the cell debris (L3) was extracted with methanol following the method described by Li and Zhu (2014). The sorption ratio, defined as the ratio of the PHC amount on cell surface to total amount of PHC in the system, was introduced to evaluate the extent of PHC sorption in each system (Zhang and Zhu, 2012).

Biosurfactant enhanced PHC biodegradation was carried out in 125 Erlenmeyer flasks containing 20 mL of MSM contaminated with 2% (v/v) diesel oil. The concentrations of lipopeptide biosurfactant were used as follows: 0.5, 1, 2, 4, and 8 CMC. After incubation on a rotary shaker (150 rpm) at 30 ºC for 14 days, samples were collected. PHC in each sample was extracted with hexane. Five milliliters of hexane

were added into each sample and it was shaken at 200 rpm for 5 minutes to collect the organic phase. This procedure was repeated for three times and the final solution was added to 20 mL with hexane. The distribution and biodegradation of PHC in each sample, represented by the concentrations of alkanes, was measured by GC-MS.

6.2.5 Sample Analysis

Inner membrane permeability: Bacteria can produce a hydrolytic enzyme, named β-galactosidase, after being activated by lactose. Located in the cell membrane, β-galactosidase will be released into the culture medium when the cell membrane permeability increases. The o-Nitrophenyl-β-D-Galactopyranoside (ONPG) will be hydrolyzed into galactose and o-nitrophenol (ONP) (yellow in color). The optical density (OD) of the culture medium thus will be increased (Lehrer et al., 1989). Therefore, effects of lipopeptide biousrfactant on the membrane permeability of *Rhodococcus erythropolis* were determined by measuring the release of β-galactosidase as described by Zhang et al., (2013).

Each cell pellet was washed three times with 0.01 M PBS (pH 7.2) solution, and then re-suspended in to the MSM medium to reach an absorbance of 0.8 at OD_{600}. The bacterial suspension (5 mL) was mixed with 5 mL PBS buffer solution and 0.5 mL ONPG (30 mM). Samples without biosurfactant and contaminants were used as the blank control. After incubation at 30 °C for 2 h, samples were centrifuged (8,000×g) for

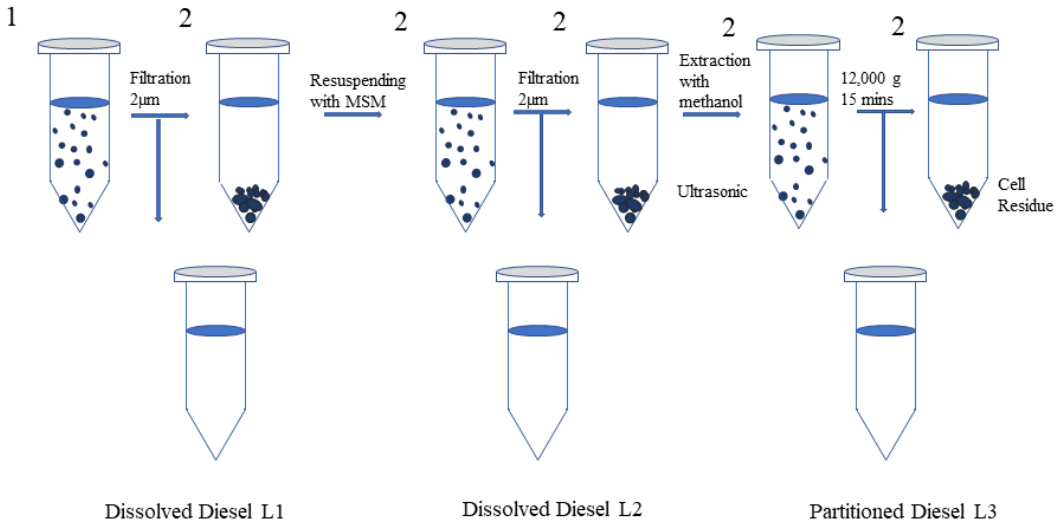


Figure 6-1 The analysis of diesel partition on oil degrading strain cells

10 min and the production of o-nitrophenol (ONP) was measured by UV-spectrophotometer at 415 nm. The release of β-galactosidase was evaluated based on the productivity

The absorbance of reaction and blank control system were represented by $A_{415,i}$, and $A_{415,0}$, respectively. The release of β-galactosidase was evaluated based on the productivity of o-nitrophenol (ONP) and calculated using the Equation 6-1 as follows:

$$\eta ONP = \frac{A_{415} \times \nu}{\nu_0 \times d \times t \times \xi} \quad \textbf{(6-1)}$$

where A_{415} ($A_{415}=A_{415,i}-A_{415,0}$) is the absorbance caused by the biosurfactant; ξ is the extinction coefficient (4.86 cm/mM) of ONP; ν,ν_0 and t are the sample volume (mL) and reaction time (h), respectively; d is the optical path of cuvette (cm).

Cell surface hydrophobicity: Bacterial adherence to hydrocarbons (BATH) method was used to determine the changes of bacterial cell surface hydrophobicity (Sokolovská et al., 2003). After harvest, the *Rhodococcus erythropolis* were washed with PUM buffer solution (22.2 g $K_2HPO_4 \cdot 3H_2O$, 7.26 g KH_2PO_4, 1.8 g urea, 0.2 g $MgSO_4 \cdot 7H_2O$ in 1000 mL distilled water, pH 7.1) two times to remove residue hydrocarbons and biosurfactants.

The cells were then resuspended in PUM buffer to an initial absorbance (O.D.400 nm) of between 1.4 and 1.6. Each cell suspension (1.2 ml) was dispensed into a round-bottom acid-washed test tube (i.d. 10 mm) and 0.2 ml sterile n-hexadecane added.

Following preincubation at 30°C for 10 min, the test tubes were uniformly vortexed for 120 s. After allowing 15 min for the phases to separate, the lower aqueous phase was carefully removed with a Pasteur pipette and its turbidity at 400 nm measured. Hydrophobicity was expressed as the percentage of adherence to n-hexadecane that was calculated as follows: 100 × (1 – O.D. of the aqueous phase/O.D. of the initial cell suspension). For a given sample, three independent determinations were made.

Heavy metal concentration: Each cell-free supernatant sample was digested to release the trapped Ni^{2+} in the lipopeptide-Ni complex. The concentrations of the target metals in each sample were measured by a Perkin–Elmer atomic absorption analyst 100 spectrophotometer. All tests were in triplicate and error bars based on the standard deviation were plotted.

Zeta potential analysis: The determination of cell zeta potential was modified from the methods described by Akgün (2005) and Li et al. (2011). Bacterial cells were gently washed three times with 10 mM PBS buffer solution (pH 7.4) and then dissolved into this PBS buffer solution (OD600 = 0.8). Each sample was then analyzed with Malvern NanoZetasier ZS.

GC–MS: GC–MS analysis was conducted on an Agilent 7890A gas chromatograph equipped with a DB-5MS column fused silica capillary column (30 m × 0.32 mm × 0.25 μm). The GC oven temperature was held isothermally for 5 min at 60 °C, programmed to sequentially step from 60 to 300 °C at 3 °C min^{-1}, and

then held isothermally for 45 min at 300 °C. Helium was used as carrier gas at a flow rate of 1.0 mL min^{-1}. The transfer line temperature was 250 °C and the ion source temperature was 200 °C. The ion source was operated in the electron ionization (EI) mode at 70 eV. Full scanning was used to identify the biomarker compounds and calculate molecular parameters. The scanning range was from *m*/*z* 50–580.

6.2.6 Statistical Analysis

The experiment was arranged in a completely randomized design. All data reported were averaged values of three independent replicates. Statistical analysis of the data was carried out using OrigniPro®. Differences were considered statistically significant at $p < 0.05$.

6.3 Results and Discussion

6.3.1 Effect of Biosurfactants on Effect of Cell Surface Properties

- ***Cell surface hydrophobicity***

Playing a key role in regulating the bacteria, PHCs and solid surface interactions, microbial CSH could provide valuable evidence for evaluating the ability of bacteria in up taking and biodegrading hydrocarbons (Bezza and Chirwa, 2017a). In general, high CSH allows better attachment of oil degrading strains to PHCs in a long run.

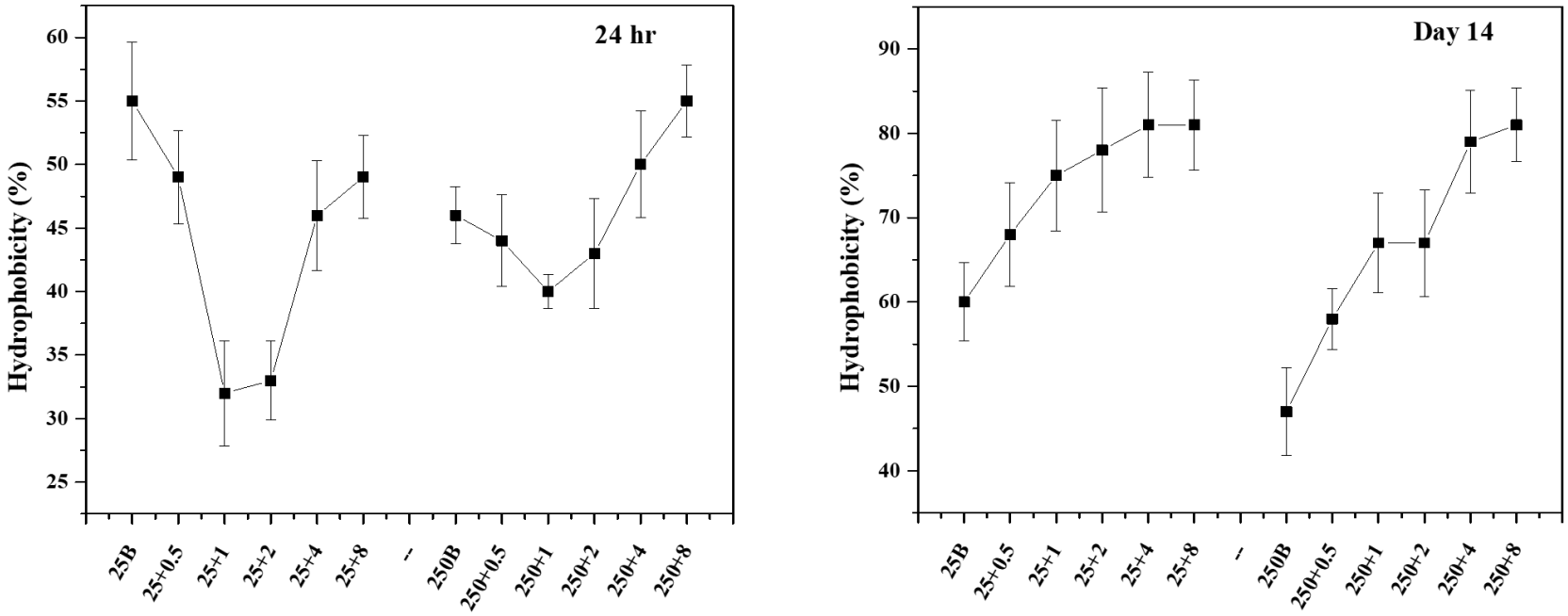


Figure 6-2 Analysis of the surface hydrophobicity of oil degrading cell at various Ni^{2+} (25 ppm and 250 ppm) and biosurfactant concentrations (blank, 0.5 CMC, 1CMC, 2CMC, 4CMC, 8CMC) in short (24h) and long (14 day) period

Biosurfactant addition could enable an increase of cell surface hydrophobicity, thus improve oil degrading microbe-PHCs droplet interaction. Concentration-dependent CHS changes of *Rhodococcus erythropolis*, after the exposure to a co-contaminant in a short (24hr) and long period (14 day), are presented in Figure 6-2. As shown in this figure, a decrease of bacteria CSH was observed after 24 hours at a relatively low CMC value (i.e., 0.5 and 1 CMC) under both heavy metal concentrations (i.e., 25 ppm and 250 ppm). In the same set, bacterial CSH was significantly increased as lipopeptide concentrations increased from 1 CMC to 8 CMC. The 14-day CSH value was found to be much higher than the ones in 24 hours. Lipopeptide addition exhibited a positive effect on strain CSH under both heavy metal concentrations (i.e., 25 ppm and 250 ppm).

- ***Membrane permeability***

Acting as a barrier against PHCs and metal ions uptake, an understanding about biosurfactant effect on the membrane permeability is important. To reveal possible lipopeptide induced changes of cell wall permeability in a co-contaminated system, the release of cytoplasmic β-galactosidase, a hydrolytic enzyme in the cell membrane, was evaluated and the results are presented in Figure 6-3.

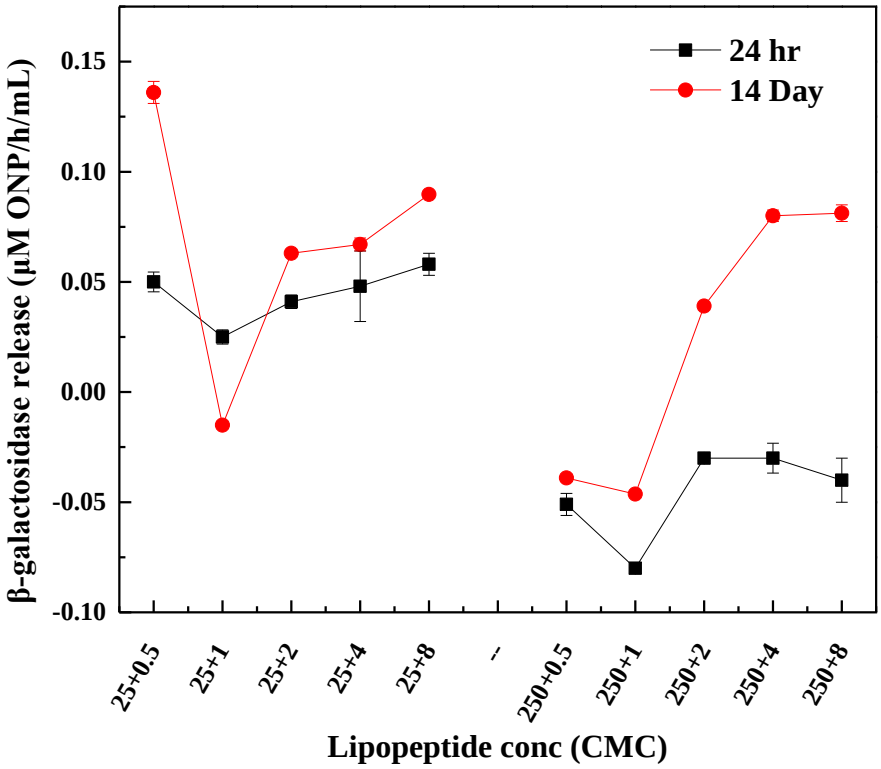


Figure 6-3 Analysis of the membrane permeability of oil degrading cell at various Ni^{2+} (25 ppm and 250 ppm) and biosurfactant concentrations (blank, 0.5CMC, 1CMC, 2CMC, 4CMC, 8CMC) in short (24h) and long (14 day) period

After 24h exposure to the co-contaminated environment, an increased cell membrane permeability was observed in low Ni^{2+} co-contaminated systems (25 ppm). However, in 250 ppm Ni^{2+} co-contaminated environments, the high Ni^{2+} stress and their biosorption on to cell surfaces significantly inhibited the cell membrane permeability. Though lipopeptide addition at a concentration above its CMC was able to reduce the adverse effect, the release of β-galactosidase was still less than the control sample (and showed as negative value). Generally, the cell surface permeabilities were promoted at a biosurfactant concentration over its CMC. The 14-day exposure experiment shared a similar result. When the lipopeptide concentration was above its CMC, the cell membrane permeability was notably promoted.

Lipopeptide induced permeability modification was believed to be a dynamic and slow process. However, after a 14-day exposure, there was not much difference on cell membrane permeability between two contamination sets (i.e., 25 ppm and 250 ppm Ni^{2+} co-contaminated environment, respectively). At a lipopeptide addition of 8 CMC, the highest β-galactosidase release were achieved at 0.0897 μM ONP/h/mL in 25 ppm Ni^{2+} co-contaminated environment and 0.0812 μM ONP/h/mL in 250 ppm Ni^{2+} co-contaminated environment, respectively.

- ***Cell zeta potential***

The electrostatic charge plays a primary role in sustaining cell activities and behaviors through influencing the overall cell polarity and maintaining the degree of

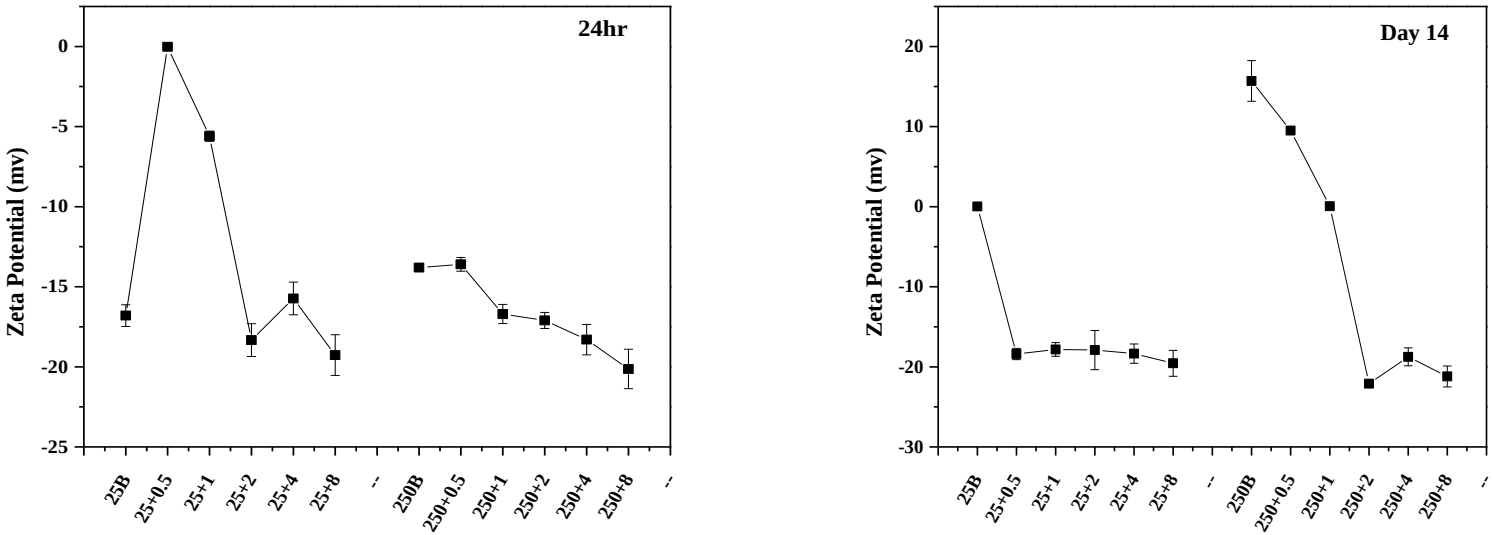


Figure 6-4 Analysis of the zeta potential (ζ) of oil degrading cell at various Ni^{2+} (25 ppm and 250 ppm) and biosurfactant concentrations (blank, 0.5 CMC, 1CMC, 2CMC, 4CMC, 8CMC) in short (1day) and long (14 day) period

surface hydrophilicity (Wilson et al., 2001). Zeta potential, the electrical potential of the interfacial region between the bacterial surface and the aqueous environment, has been widely used to assess the net cell surface charge (Wilson et al., 2001). Dissipation of zeta potential of *Rhodococcus erythropolis* biomasses were assessed and the results are presented in Figure 6-4. The results revealed a reduced absolute value of the negative cell surface zeta potential at a biosurfactant concentration below its CMC value in 24h. An increased biosurfactant concentration from 1 CMC to 8 CMC significantly increased the stability of oil degrading strain *Rhodococcus erythropolis*, as the absolute value of the negative cell surface zeta potential increased in both co-contaminated environments.

There was a significant linear correlation between surface zeta potential and the cell surface hydrophobicity. In the case of long-term exposure to a co-contaminated system, positive cell surface zeta potentials (0.031 mV and 15.7 mV for 25 ppm and 250 ppm, respectively) were observed in biosurfactant free systems. It is thus believed that a biosorption of Ni^{2+} occurred, and the cell wall was destabilized. The negative functional molecular groups on the cell surface served as a binding site and, Ni^{2+} therefore was adsorbed onto the cell surface. Interestingly, such changes in potential (within a co-contaminated system) can be correlated with the increased membrane permeability (Figure 6-3). This correlation further proved the adsorption of Ni^{2+} ions onto the cell surface. The cell surface charge was neutralized, and membrane permeability was altered accordingly. Lipopeptide addition exerted a positive impact on

the cell surface zeta potential of *Rhodococcus erythropolis*, as the zeta potential was gradually shifted towards neutrality and finally dropped to negative in both co-contaminated systems. It promoted the desorption of Ni^{2+} from the cell surface. For a low14-day Ni^{2+} co-contaminated system (25 ppm), lipopeptide concentration had no significant effects on zeta potential. The system achieved a stable status at a lipopeptide concentration of 0.5 CMC and above. It was assumed that the maximum amount of desorbed Ni^{2+} already was achieved at a lipopeptide concentration of 0.5 CMC. In a high 14-day Ni^{2+} co-contaminated system (250 ppm), cell surface zeta potential rapidly dropped with a lipopeptide concentration increase, till the most stable system (i.e., with the highest absolute zeta potential values) (-22.1 mv) was achieved at a lipopeptide concentration of 2 CMC.

It was believed that biosurfactant could facilitate the solubility enhancement of PHCs in the liquid phase, and further improve their biodegradation through the modification of cell surface properties (De et al., 2015). Figure 6-5 proposes a schematic diagram of the lipopeptide enhanced PHCs bioremediation of *Rhodococcus erythropolis* in a co-contaminated environment. The heavy metal (i.e., Ni^{2+}) in the environment quickly attached and adsorbed onto the cell surface of oil degrading strain *Rhodococcus erythropolis* (Figure 6-5-I). The increased cell surface zeta potential (Figure 6-4) and cell droplet size (Figure 6-6) shed light on this proposed assumption.

Lipopeptide addition was able to modify the cell surface properties of tested oil

degrading strain *R.erythropoli* through the adsorption process (Figure 6-5-II). These changes have been verified in other studies, especially with the application of rhamnolipid. Two mechanisms of rhamnolipid induced CSH variation have been reported: rhamnolipid adsorption onto cell surface (Zhong et al., 2007) and rhamnolipid induced cell surface composition changes (Owsianiak et al., 2009). Hou et al. (2017) evaluated the effect of rhamnolipids on CSH modification and believed that rhamnolipids resided on cell surface in an oriented manner, through the interaction between carboxyl or rhamnosyl groups and polar structures of cell surface by hydrogen bonding, dipolar, electrostatic, or short-term forces. The cell surface therefore become more hydrophobic. In general, the hydrophobic moiety of monomer rhamnolipids tended to contact strains with relatively high CSH, exposing the opposite to the environment (Zhong et al., 2015). The CSH therefore was changed. The hydrophobic surface of tested oil degrading strain *R.erythropoli* in this study (as shown in Figure 6-2, blank samples) made adsorption of lipopeptide on cell surface with its hydrophobic tail, occur. This helped to explain the decrease of CSH at a concentration below lipopeptide CMC at an early stage (i.e., 24h).

Other than biosurfactant adsorption, biosurfactant addition could further change the cell surface compositions (Shao et al., 2017; Xin et al., 2012). The changes on Gram negative bacteria, such as *Pseudomonas aeruginosa* strains have been widely reported. Their outer membrane components (e.g., proteins, polysaccharides and even trace

elements) could interact with rhamnolipids through a micellar capture, and lead to a removal of target cell surface components (Tang et al., 2016). Not only cell surface hydrophobicity could be changed, the cell surface permeability and their fluidity, as well as cell surface zeta potential could also be altered (Bhattacharjee et al., 2016; Zeng et al., 2018). However, the mechanisms responsible for the cell surface properties change of gram negative strains (e.g., the *R.erythropoli* in this study) by biosurfactants have not been studied yet. Distinguishing themselves from Gram-negative bacteria, Gram-positive bacteria have a larger fraction of negatively charged phosphatidylglycerol, as shown in Figure 6-5 instead of a layer of outer membrane. Therefore, nutrients and minerals may have a higher chance to go through this phosphatidylglycerol layer and contact with the inner cytoplasmic membrane (Lambert, 2002). The cell permeability investigation was conducted in this study (Figure 6-3). It was also suggested an increased cell membrane permeabilities at the lipopeptide concentration above its CMC values, especially after a long-term exposure to the co-contaminated environment (i.e., 14 days). This were probably due to the release of heavy metal originally adsorbed on the cell surface. This phenomenon tended to occur at a biosurfactant concentration above its CMC values (Sotirova et al., 2009).

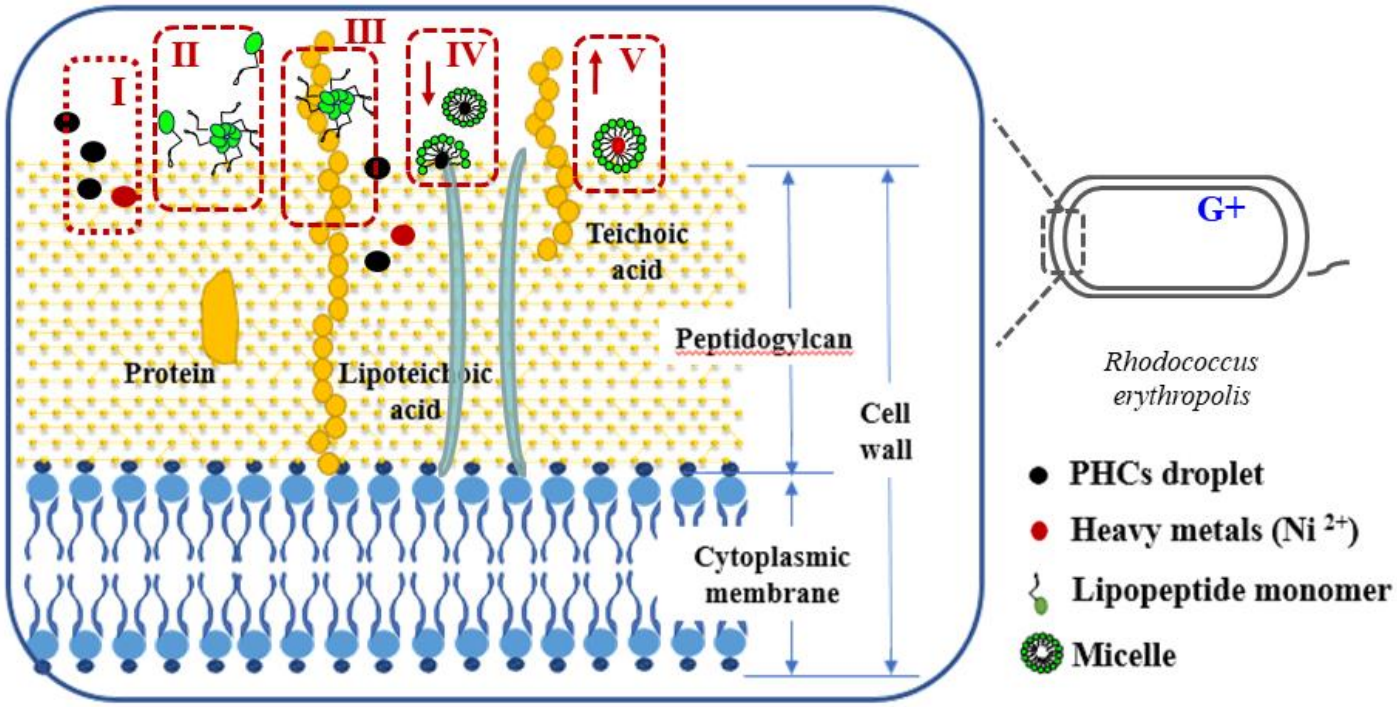


Figure 6-5 Schematic diagram describing the lipopeptide enhanced PHCs bioremediation in a co-contaminated environment: (I) Biosorption/bioaccumulation of Ni^{2+} onto cell surface; (II) Adsorption of lipopeptide onto cell surface with modified cell surface hydrophobicity; (III) Interaction with cell surface compounds (e.g., teichoic acid and protein compounds) to destabilize cell wall; (IV) enhanced mass transfer of micellar PHCs into cell; (V)interaction with Ni^{2+} to form lipopeptide- Ni complex and reduce its toxicity.

Lipopeptide then reduced heavy metal toxicity and enhanced PHCs biodegradation as shown in Figure 6-5 (IV and V). The formation of micelles on the bacterial cell surface have a strong influence on the surfactant enhanced metal desorption (da Rocha Junior et al., 2018; Mulligan, 2009) and PHCs bioavailability (Bezza and Chirwa, 2016). Particularly, hemi-micelle formation and its cell surface adsorption is a necessary requirement for biosurfactant enhanced PHCs biodegradation (Lin et al., 2017). This assumption was proven by the results listed in Figure 6-6 and Figure 6-7 (discussed below).

6.3.2 Effect of Biosurfactants on Ni^{2+} Behavior in the Co-contaminated System

It was believed that the heavy metal ions (i.e., Ni^{2+}) have a stronger affinity to lipopeptide than the cell surface in a form of lipopeptide-Ni complex. Lipopeptide biosurfactant could enhance Ni^{2+} desorption from *Rhodococcus erythropolis* cell surface (referred to Figure 6-5, mechanism III). To investigate the short and long-term (i.e., 24hr and 14 days) effect of lipopeptide on Ni^{2+} behavior, the metal ion concentrations in the MSM were determined with the result illustrated in Figure 6-6 (A) and (B). In 24hr-25 ppm-Ni contaminated systems, the highest Ni^{2+} concentration was reported in blank sample (15.78 ppm). Lipopeptide addition reduced the heavy metal concentration in the MSM medium. The effects of lipopeptide on 24hr-250 ppm-Ni contaminated systems were more complicated. The highest Ni concentration was

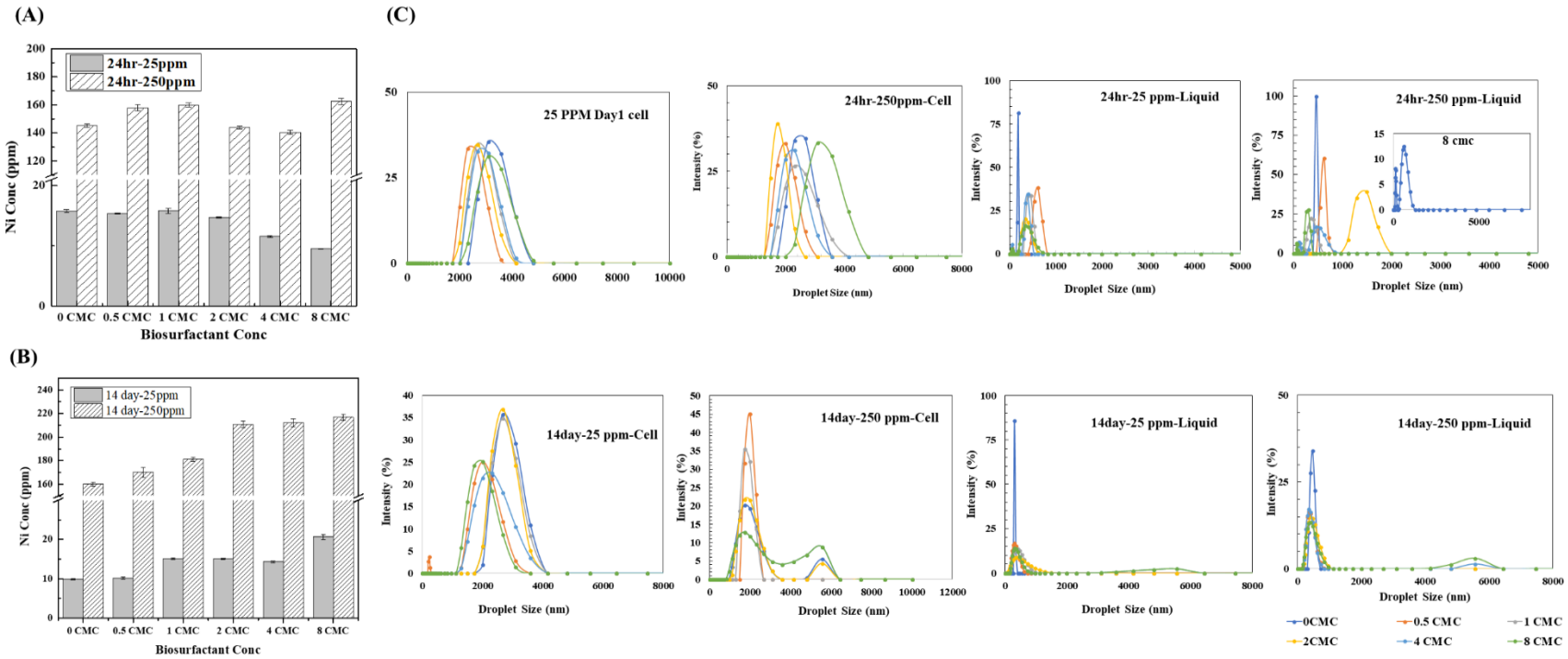


Figure 6-6 Effect of lipopeptide on Ni behavior in co-contaminated environment: A) Short-term lipopeptide effect on Ni^{2+} ion content in a co-contaminated system; B) Long-term lipopeptide effect on Ni^{2+} ion content in a co-contaminated system; c) Droplet size distribution of microbe cells and cell-free medium

reported in the sample with 1 CMC lipopeptide addition (159.95 ppm) It was believed that the initial Ni^{2+} biosorption process contributed to the decrease of metal ion, as can be verified from zeta potential result (Figure 6-4). An enhanced lipopeptide concentration, especially over the CMC value, somehow improved this sorption process. The lowest Ni^{2+} ion concentration in the medium was reported at 9.48 ppm (8 CMC) and 140.49 ppm (4 CMC) in low and high heavy metal co-contaminated systems, respectively. After a long-term exposure, however, a reverse trend was reported: the highest Ni^{2+} ion concentrations (22.58 ppm and 224.1 ppm) in the medium were recognized at the system with highest lipopeptide addition (8 CMC). Most of the adsorbed Ni^{2+} ions were released back into the growth medium again.

The droplet size distribution of cells and cell-free solution was examined to shed light on the behaviors of Ni^{2+} ion in the environment. The results are illustrated in Figure 6-6 (C). The sorption of biosurfactant monomers, micelles, and Ni^{2+} ions onto the cell surface was expected. The size distribution of *R.erythropoli* were mostly ranged between 2000-4000 nm, much larger than the reported regular droplet size (1000-2 000 nm). After 14 days, two peaks were identified in some 250 ppm Ni^{2+} co-contaminated samples (i.e., both cell and liquid samples with 2 CMC, 4 CMC and 8 CMC lipopeptide biosurfactant addition). This might be due to the formation of large sized Ni-lipopeptide micellar complex onto cell surface and its desorption from cell surface to the medium solution thereafter. In a short period (24 h), the droplet size distributions of cell-free

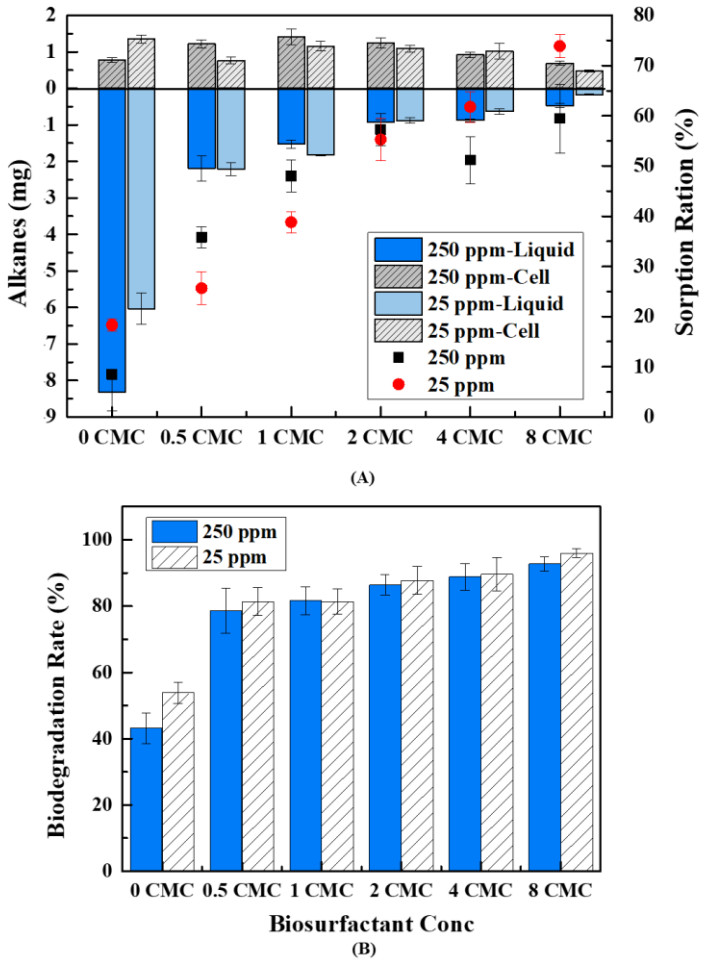


Figure 6-7 Effect of lipopeptide on PHCs biodegradation: (A) Partitioning of PHCs on cell surface in 25 ppm and 250 ppm Ni^{2+} co-contaminated environments; (B) Effect of lipopeptide on PHCs biodegradation in 25 ppm and 250 ppm Ni^{2+} co-contaminated environments

medium mostly ranged from 0-1000 nm, yet this distribution in controlled samples ranged from 0-100 nm. Particularly, in 250 ppm Ni^{2+} co-contaminated samples, two distribution peaks (0-100 nm and 100-1000 nm) were identified. These results again evidenced the formation of Ni-lipopeptide complex.

6.3.3 Distribution and Biodegradation of PHC in the Presence of Lipopeptide

The distributions of PHCs by *Rhodococcus.erythropolis* cells were shown in Figure 6-7(A) by presenting the total amount of alkanes absorbed by the cells and the solubilized portion in the medium solution. PHCs were believed to be easily accumulated on the cell surface, a primary barrier inhibited the further PHCs transfer. This transfer could be further inhibited by a high heavy metal stress. The high Ni^{2+} stress (i.e., 250ppm) control sample had a much lower total alkanes sorption (0.77 mg) and the sorption ratio (8.5%) than the low Ni^{2+} stressed (i.e., 25ppm) one (1.35 mg alkanes sorption and 18.3% sorption ratio). Lipopeptide dramatically influenced the alkanes sorption by *Rhodococcus.erythropolis* cells. Lipopeptide addition led to a rapid decrease of total alkanes in the aqueous phase in both Ni^{2+} co-contaminated systems (Figure 6-7(A)). The sorption ratio was also correspondingly increased with an enhanced lipopeptide concentration. The highest sorption ratios (59.45% and 73.89% for 25 ppm and 250 ppm Ni^{2+}co-contaminated systems, respectively) were achieved at a lipopeptide concentration of 8 CMC. Lipopeptide induced reduction of surface and interfacial

tension in aqueous phase greatly enhanced the solubilization of diesel, and diesel-cell surface interaction (Liu et al., 2017). The bioavailability of diesel in the medium therefore was increased. The change of alkanes distribution on cell surface was in accordance with the result of cell surface permeabilities (Figure 6-3).

The total alkanes on *Rhodococcus. erythropolis* cells was higher at 250 ppm- Ni^{2+} stress than the lower (i.e., 25 ppm) ones. Therefore, it was assumed that the accumulation and biosorption of heavy metals may alter the cell membrane structure, thus led to an enhanced PHC permeability. With the lipopeptide (from 0 to 8 CMC) enhanced PHC sorption and intermembrane transfer, their biodegradation rates were increased simultaneously (Figure 6-7(B)). When the CMC of lipopeptide was added at 8 CMC. the highest biodegradation rate was achieved at 92.7% and 96% for 5 ppm and 250 ppm Ni^{2+}co-contaminated systems, respectively.

The relationships between membrane properties and the PHC partitioning would enhance the understanding of lipopeptide-aided transmembrane transport behavior. The surfactant accumulated on the cell surface and started to form surfactant-lipid mixed micelles once the surfactant concentration rose beyond the lytic concentration level (Zhang et al., 2013). Therefore, chemical surfactant (i.e., Tween 80) concentration played a key role in PHCs transmembrane process. The research result in this study agreed well with previous conclusions that transmembrane transport of PHCs was a limiting step during the biodegradation process, which could be greatly improved by

surfactant addition (Liu et al., 2012; Ren et al., 2018; Zeng et al., 2018). The addition of lipopeptide, namely surfactin and fengycin from *Bacillus Substilis* A21, could effectively remove high concentrations of petroleum hydrocarbons (64.5% with an initial concentration of 1,886 mg kg^{-1}) and metals (cadmium, cobalt, lead, nickel, copper, and zinc), and soil phytotoxicity was reduced, too (Singh and Niven, 2013). However, this research was attempted to further unveil the underlying mechanism of lipopeptide enhanced PHCs bioremediation in a co-contaminated environment. The effects of lipopeptide on the PHCs mass transfer, even with the existence, showed a concentration-dependent pattern. It was believed the addition of lipopeptide enhanced the dissolution, sorption and biodegradation ratio of diesel oil by reducing the toxicity of heavy metal and modifying cell membrane permeability to enhance the sorption properties.

6.4 Summary

Although the effects of biosurfactant-influenced bioavailability and biodegradation of PHCs on cell debris have been widely reported, rhamnolipid mostly remains to the target biosurfactant. Little information is available regarding lipopeptide-enhanced PHCs distribution and biodegradation in a co-contaminated environment. In this study, the impact of lipopeptides on PHCs (i.e., diesel) biosorption and biodegradation in co-existence of heavy metal (i.e., Ni^{2+}) was evaluated. The cell properties of Gram-positive strain (i.e., *Rhodococcus*) was firstly investigated. In contrast to most Gram-negative bacteria, a higher concentration of lipopeptide,

especially above its CMC, assisted the cell microstructures modification, enhancing the cell membrane permeability and CSH. The PHCs biosorption by strain *Rhodococcus* thus is favored. Lipopeptide addition, could significantly improve the biosorption of PHCs on cell surface, and results in a higher degrade rate consequently. These findings advance the mechanistic understanding of lipopeptide-regulated biosorption and biodegradation of PHCs. This is the first time that the underlying mechanism of lipopeptide modified cell surface properties and the correlation to PHCs biodegradation was evaluated with the co-existence of heavy metal. Lipopeptide, as an effective biosurfactant, can be utilized as a novel additive to improve the microbial biodegradation of PHCs in the heavy metal co-contaminated environments.

CHAPTER 7

CONCLUSIONS AND RECOMMENDATIONS

7.1 Conclusions

This dissertation research targeted on the development of economical feasible biosurfactant production methodologies and advanced biosurfactant based remediation technologies. Lipopeptide biosurfactant products were generated and characterized. The biosurfactant production process was optimized and the associated fermentation mechanisms were explored. Finally, the mechanisms responsible for biosurfactant-enhanced PHCs biodegradation and heavy metal complexation were investigated. The key research activities and findings were summarized and stated below:

The economical biosurfactant production was achieved using the marine originated bacteria *Bacillus Substilis* N3-1P and a waste stream from local fishery industry as the substrate. Protein hydrolysate was prepared from cod waste proteins. Hydrolysis conditions (i.e., time, temperature, pH and enzyme to substrate level) for preparing protein hydrolysates from the fish waste proteins were optimized by RSM using a factorial design. An optimized DH of 51.61% was achieved after enzymatic treatment of fish liver waste for four hours. Biosurfactant production was studied by *Bacillus Substilis* N3-1P using generated fish protein hydrolysate as nutrient source. The biosurfactant product reduced the surface tension of water from 72 to 27 mN/m. The CMC value for generated biosurfactants was 0.2 g L^{-1}. Biosurfactant product exhibited a stable performance under extreme environmental conditions (pH, salinity and temperature).

The biosurfactant production process by marine originated bacteria *Bacillus Substilis* N3-1P was enhanced using immobilized biofilm on porous fly ash as the carrier. Enhanced biosurfactant production was examined by the addition of FA generated from local pulp mill. FA served as a solid platform for the immobilization of biofilm. The highest biosurfactant yield was boosted over ten times with the addition of 0.5% FA (concentration increased from 9 CMD to 110 CMD within 24 hours). Final concentration of biosurfactant collected from FA particles and growth medium reached 305 CMD, at a 2% FA dosage. Results of FTIR spectroscopy and MALDI-TOF analysis demonstrated that the final biosurfactant product belonged to lipopeptides.

The biosurfactant production by marine originated bacteria *Bacillus Substilis* N3-4P was optimized through manipulation of carbon and nitrogen sources, and the product was further applied for crude oil removal. The economic production medium using different carbon (i.e., n-hexadecane, diesel oil, glycerol, glucose, starch and sucrose) and nitrogen sources (i.e., $NaNO_3$, $(NH_4)_2SO_4$ and yeast extract) were studied. The best performance of biosurfactant production was achieved when using glycerol as carbon source, and sodium nitrate and yeast extract as the substrate. The production rate was enhanced five times compared with the original screening recipe. The CMC value of the product is 0.507 g L^{-1}. A thin layer chromatography (TLC) analysis indicated that the purified product is a mixture of protein, lipid and carbohydrate. The microbially produced biosurfactant product was

further examined as a soil washing agent to enhance crude oil removal in a soil column system. The removal rates of 58% and 65% was achieved using the biosurfactant solution with concentrations of 4 g L^{-1} and 8 g L^{-1}, respectively.

The role of the newly generated lipopeptide biosurfactant by *Bacillus Substilis* N3-1P on PHC (i.e., diesel) biodegradation in presence of the heavy metal (i.e., Ni) was evaluated. Effects of the biosurfactant product on the sorption and biodegradation of diesel, as well as its interactions with bacterial cell surface under heavy metal stress were investigated. Results proved that the lipopeptide biosurfactant product enhanced diesel biodegradation through forming complexation with Ni and exerting effects on the sorption of diesel onto bacterial cell. A positive correlation of liquid droplet size and Ni concentration in culture medium indicated the formation of Ni-lipopeptide complexation. A relatively high positive correlation was observed between biosurfactant enhanced biodegradation and enhancement of cell surface hydrophobicity for diesel in the presence of Ni. A continuous decreased cell surface zeta potential, and enhanced cell membrane permeability proved a biosurfactant induced microbe activity restoration. The final PHC removal rates were 92.7% and 96% for 5 ppm and 250 ppm Ni^{2+}co-contaminated systems, respectively.

7.2 Research Contributions

(1) This is the first attempt of using fish waste as substrate for biosurfactant

production. A standardized DOE-based enzymatic hydrolyzation methodology was developed and expected to provide a new trash-to-treasure solution across multiple industrials. The obtained hydrolysates could serve as low-cost substrate for microbe growth to biosynthesis high-added value fermentative products including biosurfactants.

(2) Biofilm acting as robust biocatalysts immobilized on waste fly ash surface has been firstly applied for enhanced biosurfactant production. A biocatalytic enhanced biosurfactant biosynthesis mechanism was proposed. The application of fly ash as the solid carrier could attribute to a cost-efficient fermentation process (i.e., higher productivity, less fermentation time and less undesirable by-products). The proposed mechanism of this biocatalytic process could advance the understanding of cultivation setup and be promisingly used for future bioreactor design to improve the biosurfactant productivity.

(3) This research is the first investigation regarding the optimum carbon and nitrogen sources for the potential lipopeptide production bacterium *Bacillus Substilis* N3-4P screened from Atlantic Ocean. The effects of carbon and nitrogen sources on the lipopeptide production were investigated. The results could contribute to an economical lipopeptide production in terms of medium optimization and raw material cost reduction.

(4) The thesis has filled the knowledge gap in lipopeptide enhanced PHCs biodegradation with the existence of heavy metals from the perspective of cell surface associated activates. A lipopeptides enhanced biodegradation mechanism for Gram-

positive bacterium (i.e., Rhodococcus.erythropolis) was firstly evaluated. The research outcomes advanced the mechanistic understanding of biosurfactant induced cell surface modification and biodegradation enhancement, thus contributed to the knowledge of biosurfactant enhanced biodegradation in a PHC and heavy metal co-contaminated environment.

7.3 Recommendations

Based on the research presented in this dissertation, further studies are suggested in the following areas:

(1) Innovative enhanced remediation technologies can be further developed to extend biosurfactant applications in harsh environments. Biosurfactant based nanoemulsion (Bio-NE) solution can result in a higher surface area and lower interfacial tension than a biosurfactant solution. The development of the Bio-NE solutions using biosurfactants produced in this thesis and their integrated with the existing flushing/washing system may lead to promising soil remediation methods.

(2) Only limited PHCs and heavy metals were evaluated regarding the biosurfactant induced cell surface modification and biodegradation enhancement. An in-depth understanding of the interaction among the lipopeptide, other PHCs compounds and heavy metals (especially multiple heavy metals in one system) needs to be further advanced.

(3) Multi-scale demonstrations of biosurfactant enhanced soil and groundwater remediation are important before filed trails. Therefore, the scale-up of the developed biosurfactant aided remediation methodologies/technologies is highly expected.

7.4 Selected Publications

Manuscript under preparation

Zhu, Z. W., Cai, Q., Zhang, B., Chen, B., Lee, K., and Lin, W. (2018). Effect of a lipopeptide biosurfactant generated by *bacillus substilis* N3-1P on the biodegradation of hydrocarbons in presence of heavy metals: changes in cell characteristics and microbe performance. *Environmental Pollution.* (to be submitted)

Zhu, Z. W., Cai, Q., Zhang, B., Chen, B., and Lin, W. (2018). Advances in lipopeptide production and environmental application. *Biotechnology and Bioengineering.* (to be submitted)

Zhu, Z. W., Cai, Q., Zhang, B., Chen, B. (2018) Microbial communities and their biodegradation of diesel contaminated site in northern Labrador (in preparation).

Manuscripts under review

Zhu, Z. W., Zhang, B.Y., Chen, B. Cai, Q. (2018) Lipopeptide production by marine bacterium bacillus substilis N3-1P using fish waste as unconventional medium.

Submitted to *Waste Management.* (submitted)

Zhu, Z. W., Zhang, B.Y., Chen, B. Husain. T, Cai, Q. (2018) immobilized robust biocatalysts on porous fly ash for enhanced solid-state fermentation of lipopeptide biosurfactants generated by bacillus substilis N3-1P. Submitted to *Journal of Environmental Chemical Engineering*. (submitted)

Peer review journal publications

Zhu, Z.W., Zhang, B., Chen, B., Cai, Q. and Lin, W. (2016). Biosurfactant Production by Marine-Originated Bacteria Bacillus Subtilis and Its Application for Crude Oil Removal. *Water, Air, & Soil Pollution,* 227(9):328.

Conference papers and oral presentations

Zhu, Z.W., Cai, Q.H., Chen, B., Liu, B., Zhang, B.Y. (2017). Microbial communities and their natural attenuation in diesel contaminated site in northern Labrador. 67th Canadian Chemical Engineering Conference (2017CSChe), October 22-25, 2017. Edmonton, Canada.

Zhu, Z.W., Zhang, B.Y., Chen, B., Ling, J.J., Lee, K. (2017). Enhanced biosurfactant production by marine originated bacteria Bacillus Subtilis and its application for oil removal with the existence of heavy metal. Symposium on persistent and emerging organic pollution in cold and coastal environments (PEOPLE 2017). October 16-17, 2017. St. John's. Canada.

Zhu, Z.W., Zhang, B.Y., Chen, B., Cai, Q.H. Husain, T. (2015). Production and characterization of biosurfactants by marine origin bacteria for oil spill remediation in harsh environments. 6th annual Arctic Oil & Gas North America Conference. April 14 – 15, 2015, St. John's. NL. Canada

Zhu, Z.W., Zhang, B.Y., Chen, B., Lin, W.Y., Song, X. 2015. Effect of biosurfactants on motility of bacteria under heavy metal stress. 25th Annual International Conference on Soil, Water, Energy, and Air. March 23-26, 2015, San Diego, California, US.

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