MULTI-SCALE BIOSURFACTANT PRODUCTION AND BIOSURFACTANT-AIDED SOIL WASHING

by

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ABSTRACT

Biosurfactants are widely used in oil and environmental industries due to their unique properties. The thesis first focused on examining tuna fish waste as low-cost substrates for microbial growth through an enzyme hydrolyzation process to produce the biosurfactant, surfactin. After enzyme hydrolyzation, the biosurfactant fermentation experiments were conducted at the bench-scale, batch-scale, and pilot-scale to determine optimal conditions for potential industrial production operations. Tuna fish waste was demonstrated to be a feasible substrate for supporting the growth of *Bacillus subtilis* (ATCC® 21332™) for biosurfactant synthesis. Optimal parameters for surfactin production (274 mg/L) were determined by conduct of the multi-scale fermentation tests.

The thesis also tackled evaluating the potential of biosurfactant-aided soil washing under different temperatures and salinity levels for oil removal. Rhamnolipids biosurfactant and Dioctyl sulfosuccinate sodium salt (DOSS) were mixed at several ratios in shaken flasks to conduct the research. Analysis of the surfactant and crude oil emulsions were developed based on their microscopic images and interfacial tension (IFT). The causal effect of IFT on crude oil removal efficiency was estimated by the structural causal model (SCM). Results of SCM showed that IFT has a minor suppression to the removal efficiency from an overall perspective but can enhance the removal efficiency under the high salinity.

The findings of this thesis shed light on biosurfactant production using fish wastes as an economical substrate and biosurfactant-aided soil washing for solving shoreline oil pollution problems. The thesis also demonstrated the applicability of causal inference analysis in environmental engineering.

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LIST OF SYMBOLS AND ABBREVIATIONS

Abs_{340}	absorbance at 340 nm
Abs_{370}	absorbance at 370 nm
Abs_{400}	absorbance at 400 nm
ASE	accelerated solvent extractor
ATE	average treatment effect
BOD	biological oxygen demand
CATE	conditional average treatment effect
CHO	conventional heavy crude
CMC	critical micelle concentration
CMD	critical micelle dilution
DH	degree of hydrolysis
DOSS	dioctyl sulfosuccinate sodium salt
DAG	directed acyclic graphs
ESI-MS	electro spray ionization mass spectrometry
GC-MS	gas chromatography-mass spectrometry
HPLC	high-performance liquid chromatography
HLD	hydrophilic-lipophilic deviation
IFT	interfacial tension
PT	placebo treatment
RCC	random common cause
s	variables in the sufficient set

SDS	sodium dodecyl sulfate
SCM	structural causal model
SR	subset replacement
ST	surface tension
TCA	trichloroacetic acid
UCC	unobserved common cause
vvm	air volume/culture volume/min
x	observation variable
y	conditional distribution

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CHAPTER 1

INTRODUCTION

1.1 Background

Fisheries and aquaculture has been playing a pivotal role in increasing food production for human nutrition and food security (Béné et al. 2016). Since 1990, global fishery production has increased sixfold, with an average annual growth rate of 5.8% during the period 2000-2016 (FAO, 2018). In 2016, global aquaculture production reached 80 million tons, with 54.1 million tons (68%) of finfish, 17.1 million tons (21%) of mollusks, 7.9 million tons (10%) of crustaceans, and 0.9 million tons (1%) of other aquatic animals (FAO, 2018). Fishery industries have been well developed in tropical and subtropical regions such as China, India, Indonesia, Vietnam, Bangladesh, Egypt, Norway, Chile, Myanmar, and Thailand. As a result of increased production, the fishery industry is now confronted with the problems associated with the accumulation of fish waste. The organic components of the fish waste have a high biological oxygen demand (BOD) and nitrogen content (Boopathy et al., 2007). If not treated properly, they can pose significant environmental and health problems (Ghaly et al., 2005). Recognition of the increasing environmental problem has highlighted the need for cost-efficient and more value-added utilization of fish wastes.

Surfactants are the active ingredients found in soaps and detergents with the ability to concentrate at the air-water interface and are commonly used to separate oily materials. Surfactants are widely used in household and industrial cleaners, personal care products, various types of manufacturing, including food processing and the production of plastics, paints and coatings, textiles, pulp and paper, and agricultural products (Soberon & Maier, 2011). In 2006, surfactant industries in the United States, Canada, Western Europe, and China was valued at around $20 billion. The demand for effective

surfactants in the agriculture, cosmetic, food, pharmaceutical, and environmental industries is steadily increasing. Since these surfactants must be both effective and environmentally compatible, it is natural to turn to the microbial world to try to meet this demand. However, the complexity and high cost of production limit the development of biosurfactants application on a large scale. Although there are limitations to the commercial production of biosurfactants, there is still great interest in these materials since they are considered to be environmentally friendly alternatives to synthetic surfactants.

Oil spills are of global concern due to their damage to the marine environment. After marine oil spills, the treatment of contaminated shoreline soils is a matter of widespread concern (Hamouda & Karoussi, 2008). Oil pollution of shorelines can cause remarkable damage to ecosystems, which can be the unique habitats for a variety of animals, including endangered species (Huettel, 2022). Once crude oil residues get weathered under coastal conditions, it can be challenging to remove them effectively. For example, asphaltenes would adsorb on mineral surfaces, alter their wettability and thus be more environmentally persistent (Gharbi et al., 2017). Therefore, effective remediation methodologies such as the potential application of biosurfactions to enhance the removal of oil stranded on shorelines should now be given consideration.

1.2 Statements of Problems

During fish processing, compared with traditional treatment technology, which converts waste material to fish meal, enzymatic hydrolysis is a promising alternative to recover biomass from aquatic products and generate high-value-added products known as fish protein hydrolysate (Araujo et al., 2021; Batista et al., 2009; Nilsang et al., 2005). The

processing wastes have been reported to be a good source of proteins, including enzymes and fats (Muzaifa et al., 2012; See et al., 2011; Wisuthiphaet et al., 2015). The hydrolysis of fishery by-products using enzymes in controlled conditions allows the release of nitrogen in a more soluble form of amino acids, making the hydrolyzed biomass the most available amino acid source. Studies indicated that generated hydrolysates could be used as an effective nutrient source for microbial growth to produce biosurfactants by fermentation (Marti-Quijal et al., 2020; Mo et al., 2018). However, the hydrolysis and fermentation processes need to be precisely controlled to retain the physicochemical properties of the generated hydrolysates and biosurfactants, avoiding the formation of peptides that completely lack functional properties. Therefore, the choice of enzyme, pH, hydrolysis time, enzyme/substrate ratio need to be carefully studied.

Soil washing is a water-based process for mechanically scrubbing soils ex-situ to remove undesirable contaminants, which is a promising technology to remediate oil-contaminated shorelines (Saeki et al.,2009). During the past decades, techniques of soil washing like mechanical stirring (Haba et al., 2014; Steenland et al., 2004) and mechanical shaking (Lin et al., 2017) have been developed as effective remediation methodologies with the application of sorbents (Chen et al., 2021; Gluhar et al., 2020; Saleem et al., 2018) and surfactant-based dispersants (Cai et al., 2021; Zhu et al., 2020). In the process of soil washing, once surfactant solutions are mixed with crude oil, emulsions can be formed because of the amphipathicity of the surfactant. By studying the formation of emulsions, the mechanism of surfactant-enhanced soil washing can be better understood. However, the ratio of different surfactant solutions, temperature and salinity can affect the formation of emulsion, interfacial tension and washing efficiency

(Hamouda & Karoussi, 2008; Urum et al., 2004; Urum et al., 2005). The causal inference between interfacial tension and removal efficiency has not been clarified. Thus, for the sake of shoreline environment and sustainability while enhancing the efficiency of surfactant-enhanced soil washing, the aforementioned affecting parameters should be studied.

To fill the research gaps, this research explored the possibility of using tuna fish waste as a substrate for generating biosurfactants in multi-scale, which would not only enhance the utilization of fish waste but also provide an environmentally friendly biosurfactant. Moreover, this research studied the affecting factors and causal inference in soil washing, which could support the stakeholders for decision making and application of surfactant-enhanced soil washing.

1.3 Objectives

This research has the following objectives:

(1) Characterization of hydrolyzed fish waste peptones;

(2) Examination of the operation conditions for biosurfactant production;

(3) Optimization of biosurfactant fermentation parameters in multi-scale;

(4) Evaluation of biosurfactant-aided soil washing and analysis of emulsions generated by surfactant solutions and crude oil;

(5) Comparison of interfacial tension and crude oil removal efficiency under multiple environmental conditions; and

(6) Investigation of causal inference between interfacial tension and crude oil removal efficiency.

1.4 Thesis Structure

This thesis is in manuscript-based format and that Chapter 3 is a manuscript that has been published in Catalysts, and Chapter 4 is another manuscript that is under preparation. This thesis consists of five chapters. **Chapter 1** demonstrated the research scope, research objectives and thesis structure. **Chapter 2** was the literature review of the thesis relevant topics, including (1) substrates and strains for biosurfactant production, (2) surfactant-enhanced soil washing, and (3) causal inference analysis. **Chapter 3** presented multi-scale biosurfactant production by *Bacillus subtilis* using tuna fish waste as substrate. **Chapter 4** presented affecting parameters and causal inference analysis of surfactant-enhanced cleaning technology in the oiled shoreline. **Chapter 5** presented the conclusions of this research and proposed some recommendations for future study.

CHAPTER 2

LITERATURE REVIEW

2.1 Biosurfactant Production

2.1.1 Biosurfactants

Biosurfactants, also known as microbial surfactants, are generally produced at the microbial cell surface or excreted (Costa et al., 2018). Biosurfactants are amphiphilic compounds that contain hydrophobic and hydrophilic moieties (Cunha et al., 2004). Therefore, they can exist at the interface between polar and nonpolar media. They possess structures of different chemical and surface properties, such as remaining active at extreme pH and salinity, dropping surface tension, stabilizing emulsions, promoting foaming (Saharan et al., 2011). These components have wide-ranging applications in agriculture, food, cosmetics, medicine, and the petroleum industries (Md, 2012). Figure 2.1 shows the summary of the diverse application of biosurfactants. This section introduced several typical biosurfactants, reviewed their properties and characteristics, and compared them with chemical surfactants.

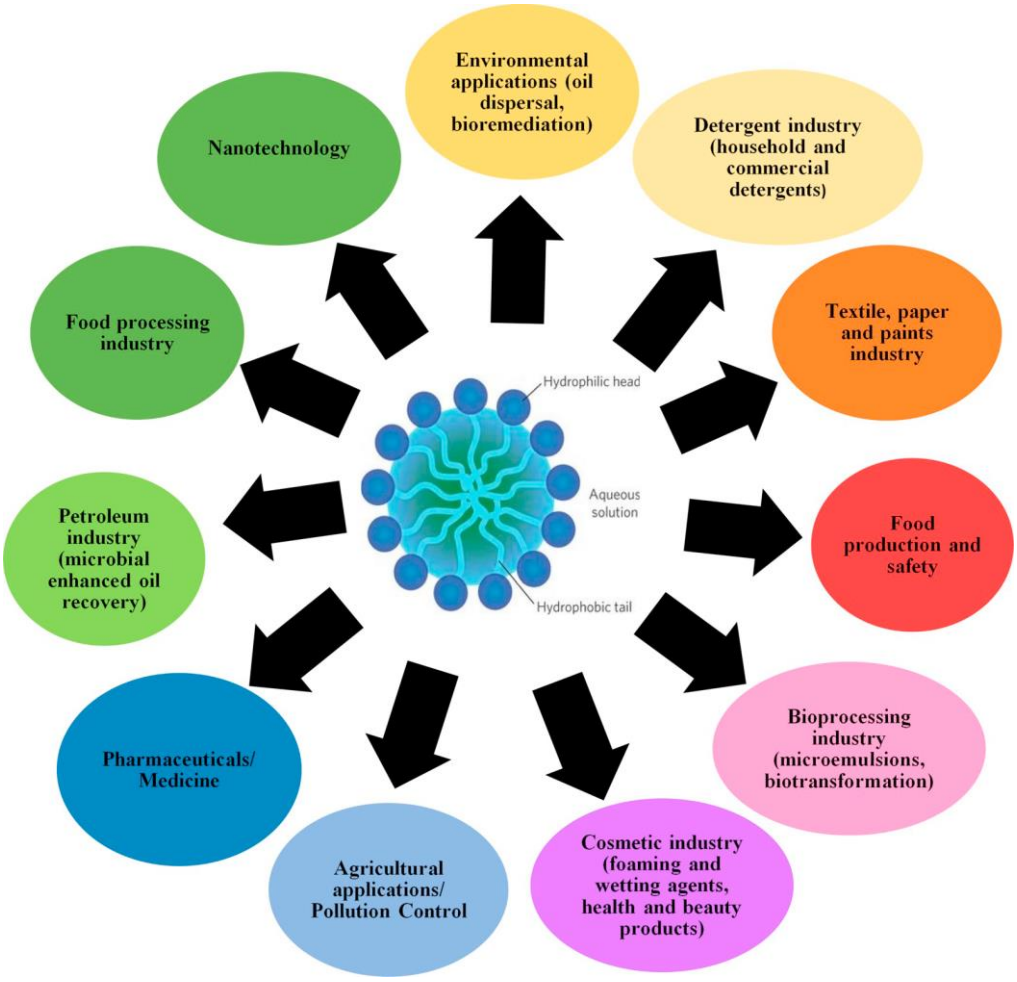


Figure 2.1 The summary of diverse applications of biosurfactants (Jimoh & Lin, 2019; Sharma & Oberoi, 2017).

2.1.1.1 Properties and Characteristics

Biosurfactants have unique structures. According to the chemical structure and microbial origin of biosurfactants, there are four main classes: glycolipids, lipopeptides, phospholipids, and polymeric biosurfactants (Md, 2012).

(1) Glycolipids

Glycolipids, containing a monosaccharide or oligosaccharide bound to a lipid moiety, are microbial surface-active compounds produced by various microorganisms (Mnif & Ghribi, 2016). Because of the physicochemical properties, biological activities, biocompatibility and biodegradability of glycolipids, they were the most widely used biosurfactants (Desai & Banat, 1997; Kitamoto et al., 2002). Among glycolipids, rhamnolipids are the most frequently studied, and they have been most applied by industry. (Lourith & Kanlayavattanakul, 2009)

Rhamnolipids were produced by *Pseudomonas aeruginosa* with glucose, glycerol or triglycerides as substrates (Jarvis & Johnson, 1949). They consist of one or two rhamnose molecules and are bonded to up to three molecules of hydroxyl fatty acids of varying chain length (Suresh Kumar et al., 2007). Figure 2.2 displays the chemical structure of rhamnolipids, namely Mono-rhamnolipids and Di-rhamnolipids. They can lower the surface tension between water and air from 72 to 27 mN/m with a critical micelle concentration (CMC) of 110–150 mg/L (Abalos et al., 2001; Lang, 2002). Besides the surface properties, rhamnolipids also showed excellent antimicrobial activities. Therefore, they have been used in many health care applications, especially in cosmetics (Benincasa et al., 2004). Although rhamnolipids have been the most popular biosurfactants introduced on the market, the scaled-up production of them is

still limited because the cost of substrates is relatively high (Müller et al., 2012). The development of next-generation rhamnolipids production strains and more suitable substrates is thus highly desired (Choi et al., 2011; Müller & Hausmann, 2011).

(2) Lipopeptides

Lipopeptides incorporate one or more lipid chains attached to a peptide headgroup (Hamley, 2015). They were first discovered in the secondary metabolites of *Bacillus subtilis* in 1968 (Arima et al., 1968). Because of their biological activities, such as interactions with biofilms, and anti-fungal, anti-inflammatory, anti-tumor, anti-virus, and anti-platelet properties, they have been widely used as antibiotics, feed additives, anti-tumor agents, urgent thrombolytic therapeutic agents, and drug delivery systems (Zhao et al., 2017). Surfactin, a typical lipopeptide, was regarded as one of the most active biosurfactants (Rosenberg & Ron, 1999). It can lower the surface tension to a range of 26.7–54.4 mN/m with an interfacial tension of 0.36–34 mN/m at the CMC of 1–240 μM (Kanlayavattanakul & Lourith, 2010).

(3) Phospholipids

The molecules of phospholipids contain phosphorus, a polar potion and non-polar potion, and the hydrophilic head group and hydrophobic acyl chains are linked to the alcohol (Li et al., 2015). They can be produced by bacteria like Thiobacillus thiooxidans in alkane medium (Knickerbocker et al., 2000). Phospholipids can act as emulsifiers, enabling oils to form a colloid with water; for example, lysolecithins are typically used for water–oil emulsions like margarine. As one of the components of lecithin, phospholipids were found in egg yolks and soybeans, which were used as a food additive in many products and can be purchased as a dietary supplement (Rydhag &

Wilton, 1981).

(4) Polymeric biosurfactants

Polymeric biosurfactants are characterized by their high molecular weight, ranging from 50,000 to greater than 1,000,000. These polymers contain lipids, carbohydrates and proteins. However, in some cases, the polymer can be a mixture of carbohydrates, proteins and lipids. These polymers are very heterogeneous, making it difficult to classify as accurately as low molecular weight biosurfactants. (Desai & Desai, 1993). The best characterization of polymeric biosurfactants is the emulsifier produced by *Acinetobacter calcoaceticus* RAG1 (Satpute et al., 2010). To date, emulsan, lipomanan, alasan, liposan and other polysaccharide protein complexes have been well studied (Santos et al., 2016). Known for their emulsifying abilities, these compounds are produced by many bacteria, archaea, and yeast (Shoeb et al., 2013). The fact of commercial importance is that each polymeric biosurfactant has a different hydrocarbon specificity with respect to the degree of emulsification. Generally, polymeric biosurfactants do not significantly reduce surface or interfacial tension (Ron & Rosenberg, 2001).

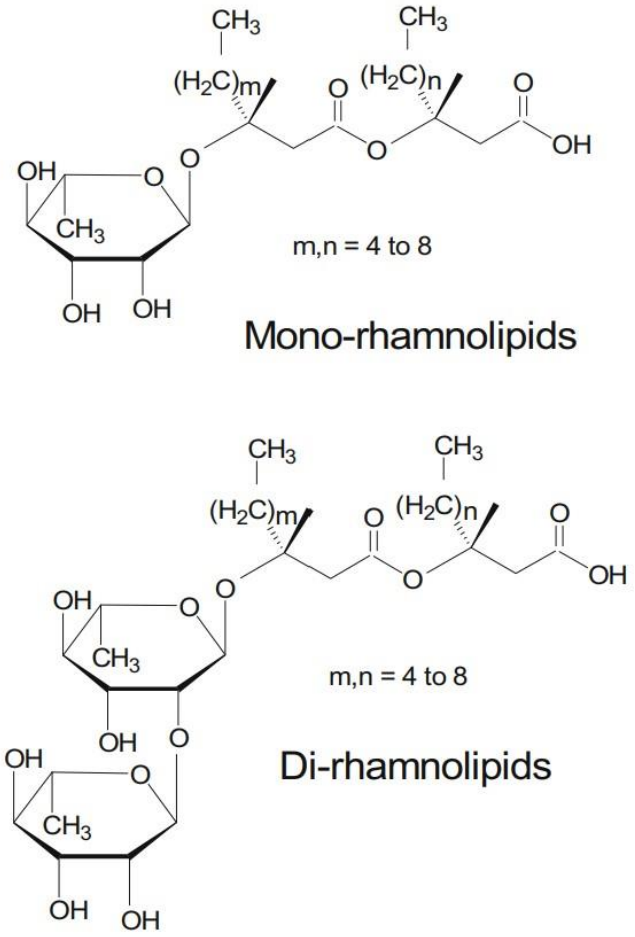


Figure 2.2 Chemical structure of Mono-rhamnolipids and Di-rhamnolipids (Soberón-Chávez et al., 2005).

2.1.1.2 Comparation of Chemical Surfactants and Biosurfactants

At present, most of the surfactants on the market are produced by chemical synthesis. When they are produced and used, they can cause irreparable damage to the ecological environment and cause severe pollution, which is bound to bring hidden dangers to human health. With greater awareness of environmental protection in the past few decades, biosurfactants have gradually become popular in public. While chemical surfactants are generally produced by synthesis methods, production methods of biosurfactants mainly include the microbial fermentation method, enzyme synthesis method, and natural biological extraction method (Zhang et al., 2018b). Compared with chemically synthesized surfactants, biosurfactants have many advantages: They have various types of structures and massive molecular weight, some with unique functional groups and superior surface properties; they have a wide range of applications, almost covering various fields; environmentally friendly, biodegradable; they can be used under extreme temperature, pH and salinity conditions; they are biocompatible, virtually no allergic reactions (Al-Wahaibi et al., 2014; Aparna et al., 2012; Khaje Bafghi & Fazaelipoor, 2012; Liu et al., 2017). Due to these properties of biosurfactants, they are increasingly popular in the petroleum industry and environmental engineering applications.

However, the problems of low yield, high cost of production, and purification exist in the fermentative production of surfactant, hindering the industrialization of its products, resulting in insufficient supply of their products. To address these problems, various researchers have suggested the use of biologically derived waste materials with a high content of carbohydrates or lipids as a substrate for biosurfactant production. This

would both lower biosurfactant production costs and provide of means of reducing pollution from waste materials released into the environment.

2.1.2 Substrates and Strains for Biosurfactant Production

2.1.2.1 Wastes as Substrates for Biosurfactant Production

This section described the use of a number of waste materials currently used in biosurfactant production, all of which can be utilized to decrease the economic cost and control the possible pollution by waste. Based on the literature, examples of representative waste materials include: olive mill waste, corn steep liquor and sugarcane molasses waste, animal fat waste, buttermilk and poultry-transforming waste, shrimp shell waste, and fish waste.

(1) Olive mill waste

After the first extraction of olive oil, olive mill waste is a by-product of the procedure (Tortosa et al., 2012). The carbon source is a critical factor in the procedure of biosurfactant production. After knowing olive mill waste has high potential as a carbon source for biosurfactant production by testing the content, Ramírez and Vaz demonstrated the effectiveness of hydrolysis pretreatment of olive mill waste by using three hydrolysis methods: enzymatic hydrolysis, acid pretreatment plus enzymatic hydrolysis, and acid hydrolysis (Ramírez et al., 2016b). *Pseudomonas aeruginosa* and *Bacillus subtilis* were the two bacterial species used for the fermentation. Ramírez and Vaz used a kinetic study to analyze the results, and results showed that enzymatic hydrolysis is the best method to pretreat the raw material, *Pseudomonas aeruginosa* and *Bacillus subtilis* yielded 29.5 and 13.7 mg/L of rhamnolipids and surfactin, respectively.

Compared with those non-hydrolysed olive mill waste, the yield of hydrolysed olive mill waste is much higher. Therefore, the application of olive mill waste as a carbon source during biosurfactant production can improve the yield. Moreover, the concentration of the olive mill waste is a significant factor, because the biosurfactant production would increase with a higher concentration of olive mill waste. All in all, these studies proved hydrolysis of olive mill waste enhanced biosurfactant yield.

(2) Corn steep liquor and sugarcane molasses waste

Compared to corn and sugarcane, some corn steep liquor and sugarcane molasses are low-value products, which cannot be directly used in food production. Chaprão et al. (2018) found that *Bacillus methylotrophicus*, isolated from seawater, can utilize the 3% corn steep liquor and sugarcane molasses as the substrate to produce biosurfactants. After 144 h of cultivation, the result showed in the proton nuclear magnetic resonance spectrum, demonstrating the maximum concentration of biosurfactant can be 10.0 g/L, with outstanding performance in the bioremediation of oil-contaminated environments. Although the detailed information regarding the biosurfactant is not clear yet, it can be a lipopeptide based on the results. Furthermore, they concluded that this biosurfactant showed low biomolecule toxicity, so it was safe to apply it to the environment. This study demonstrates the possibility of making full use of industrial waste (corn steep liquor and sugarcane molasses) to produce a lipopeptide biosurfactant, which can contribute to the petroleum-contaminated areas.

(3) Animal fat waste

Animal fats and oils are lipid materials derived from animals. After meat processing, plenty of animal fat is left without further use. In order to process the animal fat waste

as well as obtain the valuable product, Santos et al. (2014) set *Candida lipolytica* UCP0988 as the strain for the fermentation, 5% animal and 2.5% corn steep liquor as the substrate, 5.3 as the pH value and 28 ℃ as the temperature. The 2^3 design analyzed the responses of surface tension, biomass and yield, demonstrating the possibility to use animal fat to produce sophorolipids. According to Santos et al., the new biosurfactant was promising for application in the bioremediation field because of the reduction of surface tension after 144 h of cultivation. In conclusion, the possibility of using animal fat waste to produce biosurfactants is feasible.

(4) Buttermilk and poultry-transforming waste

Large volumes of agricultural wastes, such as buttermilk and poultry-transforming waste are often discarded directly or processed into low-value products. Using the Box–Behnken Design and response surface methodology, Zouari et al. conducted a series of experiments to find the optimal condition in submerged fermentation (Zouari et al., 2021). The strain used was *Bacillus subtilis* SPB1 (HQ392822), isolating from the laboratory from Tunisian soil contaminated by hydrocarbons. By setting various concentrations of the buttermilk and poultry-transforming waste, they got the production yield and then plotted them. The result showed that the best production yield is about 12.61 ± 0.7 g/L of crude lipopeptide biosurfactant when the ratio of milk /distilled water was 1.5, poultry-transforming wastes was 23 g/L, and the inoculum size was 0.12, which was three times the reported data. In conclusion, the use of agro-industrial residues such as buttermilk and poultry-transforming waste can obtain a high biosurfactant production yield.

(5) Shrimp shell waste

Shrimp shells are formed of chitin, which is ordinarily indigestible and a bit uncomfortable to try to chew and swallow. During the processing of shrimp, plenty of shrimp shells may be useless. However, it can improve the value of the shrimp shell waste if it can be processed as biosurfactants. Kadam and Savant (2019) used the strain called *Pseudomonas stutzeri* strain L1 isolated from a marine fishing port in Mumbai to experiment and various substrates such as de-oiled cakes of soybean, sunflower and coconut, fish waste, shrimp shell waste, sugarcane and mosambi waste. The results showed that the isolate exhibited emulsification activity using shrimp shell waste is the highest, which proves the feasibility of using shrimp shell waste to produce biosurfactants. Although this study demonstrated few about the type of biosurfactant, which can be conducted by the Gas Chromatography-Mass Spectrometry (GC-MS) method, the optimal condition of the fermentation is given. Furthermore, the authors appealed to use multivariate analysis and response surface methodology to obtain the most abundant yield.

(6) Fish waste

During the fish processing, 40% to 60% of the total weight of the fish head, fish skin, fish bones, red meat, viscera, and other wastes would be produced (Jia et al., 2013). Most companies choose to process these wastes directly into fishmeal for animal feed, and some companies even choose to discard the waste directly. Fish waste and wastewater from processing are likely to cause environmental pollution, and it would cause a series of health problems because wastewater and fish waste are rich in suspended solids, dissolved oxygen, and nitrogen (Boopathy et al., 2007). At the same time, it also causes a waste of resources. Because these substances contain a large

amount of crude protein, which can be used as a good source of protein, if the waste is fully utilized, polyunsaturated fats, minerals, enzymes, chitin, and other substances can be obtained. Therefore, if industries can make full use of fish wastes such as bonito wastes through technology, they can reduce environmental pollution and turn wastes into treasure by producing value added substances that people need. Kazemi et al. (2016) used fish waste compost and a strain isolated from the North Atlantic Ocean called *Rhodococcus erythropolis* sp. P6-4P as the material for fermentation and produced biosurfactant. Therefore, it was proved feasible to utilize fish waste as substrates to produce biosurfactants.

2.1.2.2 Biosurfactant Production Strategies

There are some strategies to increase the yield of biosurfactant production. Generally, the selection of the target strains is the most important thing, which decides the type of the biosurfactant. Besides, fermentation conditions and the sources of carbon and nitrogen also affect the quality and quantity of the production.

(1) Carbon sources and nitrogen sources

During biosurfactant production, carbon sources in substrates play an important role in influencing the yield both in quality and quantity (Lee et al., 2018). The use of hydrophobic carbon sources was proved to triple the yield of biosurfactant production after optimization (Burgos-Díaz et al., 2013). The use of low molecular weight carbohydrates, especially glycerol has been proved to further enhance the yield of lipopeptides (Chakraborty et al., 2015). Therefore, adding additional carbon sources to

the waste-based substrates is an alternative strategy in biosurfactant production. In addition, nitrogen sources also have an effect on the yield of biosurfactant production by affecting the microbial growth and the synthesis of bioactive metabolites (Jimoh & Lin, 2019; Silva et al., 2010). By adding organic sources of nitrogen to the microbial medium, some *Bacillus* isolates increased the yield of biosurfactant production significantly (Elazzazy et al., 2015). However, the ratio of carbon and nitrogen sources is the key factor in the optimization of fermentation (Xia et al., 2012). When the ratio was relatively high, the yield of production may decrease because the bacterial growth are inhibited (Patil et al., 2014). Considering the cost and efficiency of the fermentation, nitrogen sources should be in minimal supply to the medium.

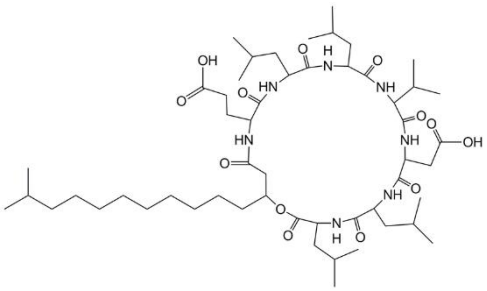
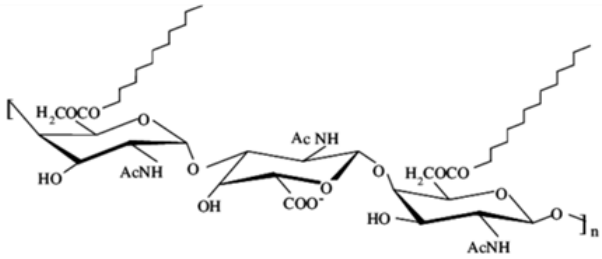
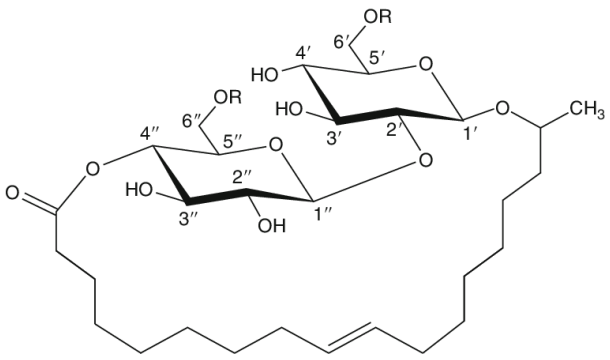
(2) Fermentation conditions

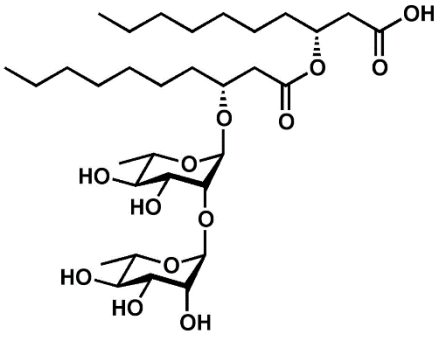
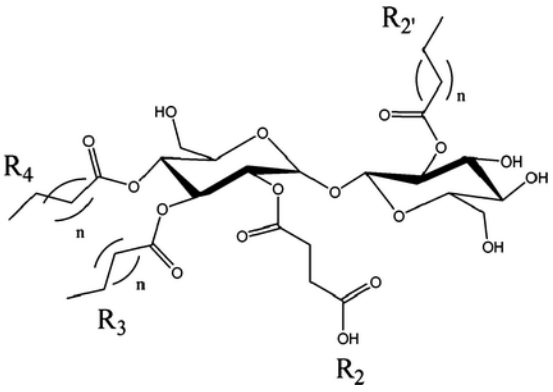
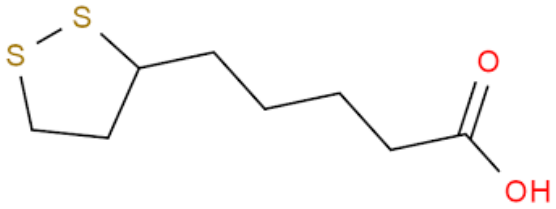
There are several fermentation conditions in biosurfactant production, such as temperature, pH, agitation speed, aeration, and fermentation time. Temperature is a critical factor in the optimization of the fermentation yield. While plenty of strains can get the maximum yield when the temperature is around 30 ℃, there are still some strains increasing the yield when the temperature is 37 ℃ (Kannahi & Sherley, 2012). Similarly, the optimum pH, agitation speed, aeration of the fermentation settings should be suitable for the strains. In addition, the fermentation time plays a significant part in the biomass yield, and the cell density, and thus should be set after knowing the change of biomass growth and the formation of the production (Nalini & Parthasarathi, 2018). For example, once the fermentation time is relatively long, the nutrient constituents and oxygen can be limited, which would inhibit the metabolism activities. Therefore, the optimal fermentation conditions require plenty of experiments.

2.1.2.3 Potential of Biosurfactant Production Using Fish Waste

As aforementioned, fish waste was studied as a raw material and can be used as a good source of protein to obtain polyunsaturated fats, minerals, enzymes, chitin, and other substances by hydrolysates. Enzymatic hydrolysis was usually selected for the raw fish waste with high value-added (Araujo et al., 2021). The list of bacteria in Table 2.1 are all characteristic strains of different biosurfactants. Characteristic media and isolation methods have been established. It has obvious reference significance to produce biosurfactants using fish waste as substrates.

Table 2.1 Typical strains for biosurfactant production

Types of strains	Target production and references	Molecular structural formula
ATCC 55033 ATCC 21332 ATCC 6633	Surfactin (Johri et al., 2002; Mousavian & Rahimi, 2010; Shete et al., 2006; Smyth et al., 2014)	 (https://en.wikipedia.org/wiki/Surfactin)
ATCC 31012	Emulsan (Mukherjee et al., 2006; Randhawa, 2014; Shete et al., 2006; Yoneda et al., 2006)	 (https://www.researchgate.net/figure/Scheme-of-emulsan-structure)
ATCC 22214	Sophorolipids (Raheb et al., 2005; Rispoli et al., 2010; Saerens et al., 2011; Van Bogaert et al., 2007)	 (https://www.researchgate.net/figure/Structure-of-a-classic-sophorolipid-lactonic)

ACTT 9027 ATCC 15453	Rhamnolipids (Bandyopadhyay et al., 2013; Henkel et al., 2017; Shete et al., 2006) (Mulligan et al., 2014; Randhawa, 2014; Walter et al., 2010)	 (https://en.wikipedia.org/wiki/Rhamnolipid)
ATCC 53968	Trehalolipids (Amin et al., 2013; Shete et al., 2006)	 (https://link.springer.com/chapter/10.1007/978-3-642-14490-5_5)
ATCC 8662	Liposan (Amaral et al., 2010; Pinchuk et al., 2000; Cirigliano et al., 1985)	 (http://www.chemspider.com/Chemical-Structure.841.html)

2.1.3 Multi-scale Biosurfactant Production

The application of various multi-scale biosurfactant production methods have provided data on the optimal conditions for the production of high-value biosurfactant products for use by various industries (Mohanty et al., 2021). As the fermentation procedure is tough to control, it remains challenging to develop this technology on a large scale. The conduct of in-depth multi-scale biosurfactant production studies are needed to improve the yield of biosurfactants on a large scale.

In general, large-scale production is based on the optimal conditions of the lab scale. Velioglu and Urek (2015) utilized several industrial wastes as substrates, and selected *Pleurotus djamor* as the strain to produce biosurfactants in multi-scale. Results showed that the determination of the best substrate for biosurfactant production could be helpful to the whole fermentation process. Large-scale biosurfactant production was proved to be an economical and environmentally friendly way to make use of wastes. In addition, with the help of kinetic studies, target strains had the potential to produce biosurfactants having lower surface tension and high yield on a large-scale (Heryani & Putra, 2017).

Moreover, in large-scale production, recovery, purification, and downstream processing are three key factors accounting for more than 60% of the total costs (Sarachat et al., 2010). Besides the production yield, these factors should be considered as well. Thus, a multi-step technology should be applied to them according to the production scale (Alcantara et al., 2014).

2.2 Surfactant-enhanced Soil Washing

2.2.1 Soil Washing for Oil Removal

Because of human activities, liquid petroleum hydrocarbon could be released into the environment, especially the marine ecosystem. The oil spill has a negative impact on human beings, animals, and plants, which is likely to break the ecological balance and cause inevitable damage to the environment (Mäkitie et al., 2018). Crude oil contains many pollutants and poisonous substances including polar hydrocarbons, n-alkanes, unresolved complexes of alkanes, as well as aromatics, resin and asphaltene residuals (Killops & Al-Juboori, 1990). Once the spilled oil gets weathered because of the natural influence such as sunshine, wind, and waves, it can be more challenging to remove (Fingas, 2016). Therefore, to control this pollution, soil remediation technology is needed. There are three main methods dealing with contaminated oil: physical remediation, chemical remediation, and bioremediation.

(1) Physical remediation

Physical remediation uses physical principles and specific engineering techniques to remove or transform contaminants from the soil into harmless forms. It mainly includes soil replacement, gas-phase extraction, extraction elution, electric repair, thermal desorption, and biochar adsorption (Dhaka & Chattopadhyay, 2021).

(2) Chemical remediation

Chemical remediation method is to use chemical reaction principles and engineering technology to decompose oil pollutants in soil into non-toxic small molecules to use to achieve the purpose of soil remediation. It is generally applicable to the treatment of

high concentration polluted sites. The main restoration technologies include chemical oxidation, plasma degradation, and photocatalytic degradation (Koul & Taak, 2018).

(3) Bioremediation

It is a controlled or spontaneous process of using organisms, especially microorganisms, to catalyze the degradation of organic pollutants to repair contaminated environments or eliminate pollutants from the environment. Among them, microbial remediation technology uses microorganisms, indigenous bacteria, foreign bacteria, and genetically engineered bacteria to transform and degrade pollutants through metabolism. By changing various environmental conditions, such as nutrition, redox potential, co-metabolic matrix, to enhance microbial degradation to achieve the purpose of treatment (Adams et al., 2015).

In addition, soil washing is a remediation process that removes contaminants from the soil (Mao et al., 2015a). Because surfactants are soluble both in water and oil, they showed potential in soil washing by the mechanism of mobilization and solubilization (Mulligan et al., 2001). Mobilization occurs at a concentration below the surfactant CMC, which depends on the ionic charge of the surfactant solution. Solubilization depends on the combination of surfactant and oil residuals. Considering the significance of sustainable development, *Ex-situ* soil washing allows redevelopment and more treatment methods (Elgh-Dalgren et al., 2009). After adding washing solution to the oil-contaminated soil, high-energy mixing, mechanical shearing, and dispersion are applied to remove contaminated oil residues in *Ex-situ* soil washing (Ceschia et al., 2014). Figure 2.3 and Figure 2.4 show the schematic diagram of *Ex-situ* soil washing and *In-situ* soil washing, respectively.

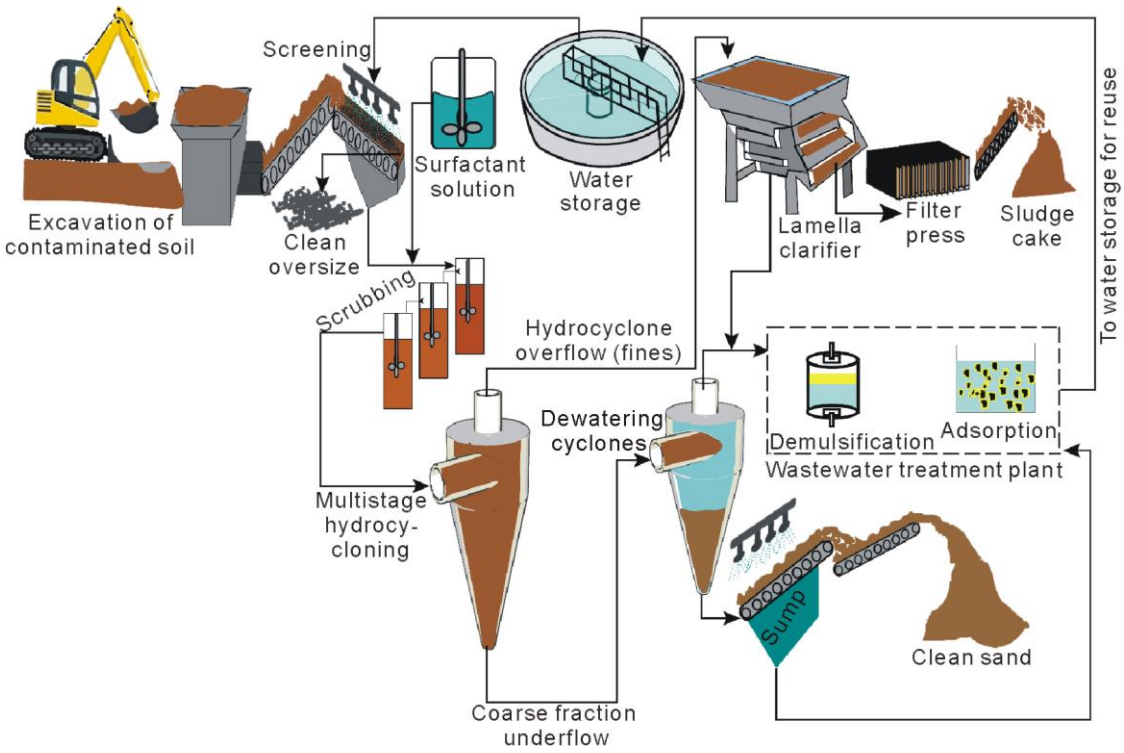


Figure 2.3 Schematic diagram of an Ex-situ soil washing system (Befkadu & Quanyuan, 2018)

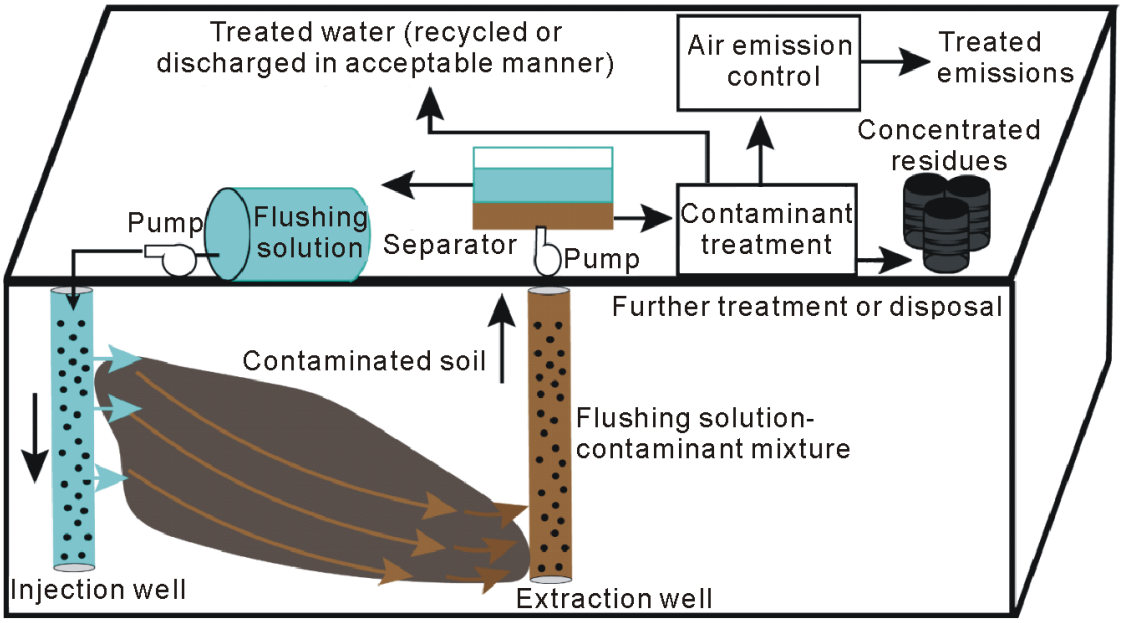


Figure 2.4 Schematic diagram of In-situ soil washing (Befkadu & Quanyuan, 2018)

2.2.2 Factors Affecting Soil Washing Efficiency

In general, factors affecting soil washing include: soil properties, contaminant properties, and process-based parameters.

(1) Soil properties

Soil physical properties mainly include texture, structure, porosity, density, consistence, and aggregate stability (Li et al., 2016; Young, 2012). They affect oil retention capacity of the soil. In addition, studies reported that pH of the soil is an important factor determining the washing efficiency (Akpoveta et al., 2012). It can be explained as pH can influence the composition of surfactant solutions. When the same type of surfactant was used in different types of soil, the results showed a significant difference in the oil removal efficiency (Li et al., 2016). Therefore, the selection of surfactants should depend on the properties of the soil properties.

(2) Contaminant properties

Contaminant properties refer to the type, physicochemical form, and degree of weathering of the oil residues (Befkadu & Quanyuan, 2018). Studies reported that by increasing the oil concentration from 1000 to 2000 mg/kg, the removal efficiency increased from 35% to 45% with the same concentration of surfactant. When the initial level was relatively high, oil removal efficiency tended to be high as well because water in the solution also can remove a part of the contaminant. Due to the influence of the mechanism of mobilization, heavy crude oil in the soil tends to be more difficult to remove because the viscosity of heavy crude oil is greater than the light crude oil. Additionally, the physicochemical form and the degree of weathering of the oil have

great impact on the efficiency. It can be more challenging to remove the aged and weathered oil than the fresh oil in the soil (Chen et al., 2007). It suggests that once the contaminated area is found, remediation should be conducted immediately.

(3) Process-based parameters

By adjusting the surfactant concentration, temperature, operation time, and stirring speed, soil washing efficiency can be enhanced (Peng et al., 2011). Because of the reduction in interfacial tension and the increase in the hydrophobic attraction of the surfactant micelles, soil washing efficiency has the tendency to increase with the increasing surfactant concentration (Zhu et al., 2005). While some research showed that with the increase of temperature, washing efficiency tended to increase, other research reported it had no significant influence (Peng et al., 2011; Urum et al., 2004). It suggests that not all soil washing processes are affected by temperature. Operation time also should be considered in an economical way since the removal efficiency would not increase with additional washing process time (Chang et al., 2000). However, with a high stirring speed, the strong collision between soil particles can inhibit the oil residuals to combine with surfactants, and thus decrease the removal efficiency (Peng et al., 2011). In addition, interfacial tension would be decreased with the addition of salt (Bera et al., 2012). Comparing the removal efficiency using distilled water, seawater, and surfactant together with seawater to wash the oil-contaminated soils, it is shown that solution consisting of surfactant and seawater had better performance, which can be attributed to the synergistic effect of salt and surfactants (Huang et al., 2015; Urum et al., 2005). In conclusion, optimize the process-based parameters could help to increase the washing efficiency and save the process cost.

2.2.3 Surfactant-enhanced Oil Removal

According to their hydrophilic head groups, surfactants are classified as cationic, anionic, nonionic, and zwitterionic surfactants (Rosen, 2012). Because cationic surfactants tend to sorb to the soil when the surfactant concentration is high, they are not suitable for soil washing compared with anionic and nonionic surfactants (Ishiguro & Koopal, 2016). Moreover, cationic surfactants also are less environmentally compatible. By using anionic surfactants as washing solution, it was found that sodium dodecyl sulfate (SDS) achieved high removal efficiency when removing phenanthrene from kaolinitic soil (López-Vizcaíno et al., 2012). Due to the feature of their hydrophilic group, nonionic surfactants are environmentally friendly and can be compatible with other types of surfactants (Singla et al., 2009). It was also found that during soil washing, nonionic surfactants were effective under extreme conditions regardless of salinity or hardness. It can be concluded that they are less sensitive to electrolytes and the presence of divalent cations (Elsayed et al., 2013). Similarly, zwitterionic surfactants also showed potential to be used in soil washing since they are compatible with other classes of surfactants and biodegradable (Rios et al., 2017).

Biosurfactants-enhanced soil washing is becoming popular as it is considered as more environmentally friendly and effective than chemical surfactants. Ivshina et al. (2016) found that the removal efficiency of using biosurfactants produced by *R.ruber* IEGM 231 was 2.5 times more than chemical surfactants when the soil was contaminated by petroleum. In biosurfactants, rhamnolipids, fructose lipids, sophorolipids, surfactin, polymyxin and humic substances have been mostly used to enhance the oil removal

(Mao et al., 2015b). Results showed that rhamnolipids produced by *P. aeruginosa* and surfactin produced by *Bacillus subtilis*, had better performance compared with chemical surfactants such as Tween 80 and Triton X-100 (A et al., 2009).

Some mixed surfactants showed better synergistic properties, such as considerable improvement in solubilization capacity, decrease on the surface tension and CMC value, high cloud point, and low Krafft point (Abayneh et al., 2018). These features can help to increase the removal efficiency, especially the improvement in solubilization capacity. Moreover, the total amount of surfactants can be reduced because of the synergism effects (Shi et al., 2015). However, few studies explained the mechanism behind the synergistic properties (Lai et al., 2009).

2.3 Causal Inference Analysis

2.3.1 Introduction to Causal Inference

What is the real cause of an outcome, and how should we quantify its effect? In the field of scientific research, answering a causal question is both easy and difficult. It is easy because researchers have already deployed various statistical approaches and protocols looking for causal evidence. The methods including correlation analysis, time series analysis and regression analysis. On the other hand, answering the causal questions is hard since there are some issues emerging while researchers applying the methods in their research. The first issue is the confusion between correlation and causation (Holland, 1986). Due to ambiguous hypotheses and similarities between the two concepts, misidentifying the correlations as causalities is common. Another issue is the inappropriate use of conventional methods without the support of prior knowledge, which was constantly being overlooked in the existing studies. Those methods include time series analysis such as Granger causal test (Damette & Goutte, 2020; Delnevo et al., 2020; Mele & Magazzino, 2020) and machine learning models (Magazzino et al., 2020; Mele et al., 2021) Missing essential confounders is also a quite common issue (Bates et al., 2020; Coccia, 2020; Pearl, 2000; Varian, 2016). Many spurious correlations could emerge due to such omission (Imbens & Rubin, 2015). Finally, among all the studies that attempted to estimate the causal effects quantitatively, few incorporated methods to refute the relationships or falsify the assumptions. The step is quite essential, especially when the ground truth of the causal links is unknown (Sharma & Kiciman, 2020). Thus, causality in the real world can hardly be identified nor quantified with only the classic statistical methods aforementioned, especially when

only observational data are the only available key to the problem. Thanks to the growing research on causal inference in the statistics and artificial intelligence field during the past few decades (Butcher et al., 2021; Glymour et al., 2019; Prosperi et al., 2020), two effective causal inference paradigms emerged from a group of attempts to mitigate the challenge during the last few decades and have evolved to two general theories for causal reasoning from observational data. One is the potential outcomes framework carried forward by Imbens and Rubin (2015), the other is the Structural Causal Model (SCM) by Pearl (2000). Both methods are essential and valuable tools and can contribute significantly to the field of environmental studies.

2.3.2 Structural Causal Models

The Structural Causal Model (SCM) based on the Bayesian Network and Structural Equation Model (Pearl, 2000) is one of the most established causal inference methods. The main improvement of SCM compared with its predecessors is SCM uses a causal diagram as part of the input. In this way, the prior knowledge is introduced into the system in a causal directional manner rather than bidirectional probability distribution in BN and SEM. Hence, the relationships in SCM can more accurately represent real-world causal links. It makes SCM a suitable tool for performing pseudo experiments and extracting causal insights from observational data. Intervention is the first concept that needs to be elaborated. It means to change the value of a causal variable X on purpose then observe the changes in the corresponding variable Y. The effect of an intervention operation expressed in the form of a probability distribution is given as $P(y|do(x))$. It can be explained as "the conditional distribution of Y when the

observation variable X is set to x." The primary goal of do-calculus is to estimate based on observed data outside of a controlled randomized experiment if no access to the measurement can be directly acquired (Pearl, 2000). For such a purpose, SCM uses directed acyclic graphs (DAG) to reflect the causal relationship between different variables. A variable in the dataset will be a vertex in the graph, and a directed edge (arrow) indicates a causal link. This causal diagram explicitly introduces prior knowledge regarding the data-generating process to the system. Given a causal diagram of a problem based on a series of mathematically proven graphic-based operations, a set of variables in the graph can be picked from all the given variables while following the graph-based operations. The selected variables are then sufficient to calculate the causal effects of interest. The set of these variables is hence called the sufficient set. Another important assumption is that if a causal effect can be estimated, all the variables in the sufficient set have to be observable. With the causal relationships confirmed through the causal diagram and an appropriate sufficient set has been selected through the graph-based operations, an estimator needs to be picked to calculate the causal effects.

This study selected SCM as our causal inference engine due to its versatility and lucidity. Its definition of causal effects is more generalized. Besides, due to the open-source DoWhy framework, SCM has now been wrapped with a programming interface and thus can better adapt to various scientific problems. In the environmental science field, SCM has been used to investigate the causal effect of oil dispersants on microbial communities (Cao et al., 2022) and test the robustness of potential causal relationships between COVID-19 severity and environmental factors (Kang et al., 2021), indicates its capability of evaluating the causal factors under multiple scenarios.

2.4 Summary

In this chapter, section 2.1 reviewed several typical biosurfactants and the production of biosurfactants. Section 2.1.1 introduced properties and characteristics of biosurfactants like glycolipids, lipopeptides, phospholipids, and polymeric biosurfactants, most of which showed excellent surface properties. Moreover, biosurfactants are environmentally friendly and biodegradable, making them can be safer and less harmful to the environment when used. However, problems such as low yield, high cost of production and purification existed in the process of production, leading to the limitation of large-scale production. Section 2.1.2 reviewed substrates and strains for biosurfactant production. Waste such as olive mill waste, corn steep liquor and sugarcane molasses waste, animal fat waste, buttermilk and poultry-transforming waste, shrimp shell waste, and fish waste had the potential to be used as substrates in biosurfactant production. By adjusting the carbon and nitrogen sources, the yield of production can be increased. Fermentation conditions like temperature, pH, agitation speed, aeration, and fermentation time could be optimized to increase the biosurfactant yield. Section 2.1.3 reviewed several key factors in the multi-scale biosurfactant fermentation, the utilization of waste materials was proven to be feasible in large-scale production. Besides, parameters optimized in the lab-scale production are important for scaling-up production to meet operational needs. However, there are few studies that explored the multi-scale biosurfactant production using fish waste as substrates. Therefore, multi-scale biosurfactant production studies, especially on a large scale are warranted.

Section 2.2 reviewed surfactant-enhanced soil washing technologies. Section 2.2.1 reviewed the risk of oil spills and three main methods dealing with the contaminated

soil. Surfactants showed potential in soil washing because of the mechanism of mobilization and solubilization. Section 2.2.2 summarized factors affecting soil washing efficiency. The properties of soil like texture, structure, porosity, density, consistence, and aggregate stability can affect the capacity of oil retention in soil. The type, physicochemical form, and the degree of weathering of the oil residues have great impacts on the removal efficiency. By adjusting the surfactant concentration, temperature, operation time, and stirring speed, soil washing efficiency can be enhanced. Section 2.2.3 summarized the application of surfactants in soil washing. Biosurfactants like rhamnolipids had higher removal efficiency than one chemical surfactant. Some mixed surfactants have good synergistic properties, such as considerable improvement in solubilization capacity and decrease in the surface tension and CMC value. However, few studies explored the mechanism of these synergistic properties.

Section 2.3 gave a review of causal inference and structural causal model. Section 2.3.1 introduced causal inference and reviewed the issues in this field. Section 2.3.2 introduced the properties of the structural causal model. To date, causal inference analysis between interfacial tension and removal efficiency in soil washing has not been studied.

CHAPTER 3

MULTI-SCALE BIOSURFACTANT PRODUCTION BY BACILLUS SUBTILIS USING TUNA FISH WASTE AS SUBSTRATE[1]

[1] The research finding in Chapter 3 has been accepted for publication by Catalysts (Impact factor: 4.146) https://doi.org/10.3390/catal11040456

Hu, J., Luo, J., Zhu, Z., Chen, B., Ye, X., Zhu, P., & Zhang, B. (2021). Multi-scale biosurfactant production by Bacillus subtilis using tuna fish waste as substrate. Catalysts, 11(4), 456.

Role: Conceptualization, J.H., B.C., and P.Z. Methodology, J.H. and J.L. Validation, B.Z. and Z.Z. Resources, B.C. and P.Z. Data curation, J.H. and X.Y.Writing original draftpreparation, J.H.Writing review and editing, J.L., Z.Z., and B.Z. Visualization, B.C. and X.Y. Supervision, B.Z., B.C., and P.Z. Project administration, B.Z. and Z.Z.

3.1 Introduction

Biosurfactants are surface-active macromolecules secreted by microorganisms through their secondary metabolism (Zhu et al., 2019). A biosurfactant has an amphoteric molecular structure with a hydrophilic head and a hydrophobic tail (Georgiou et al., 1992). Biosurfactants have many advantages over chemical ones as surface-active agents, such as a wider diversity of molecules with unique functional groups, higher biodegradability, better biocompatibility, and wider application under extreme temperature, pHs, and salinity conditions (Al-Wahaibi et al., 2014; Aparna et al., 2012; Khaje Bafghi & Fazaelipoor, 2012; Liu et al., 2017; Unás et al., 2018). These environmentally friendly macromolecules have recently been considered as potential candidates of biocatalysts grounded on their diverse and complementary functional groups and surface activities. Biosurfactants as biocatalysts to facilitate the phytoremediation of hydrocarbons in soils have been reported (Almansoory et al., 2015). They could catalyze and promote the natural gas hydrate formation in seawater saturated sand/clay (Arora et al., 2016; Rogers et al., 2003). Emerging trending also focuses on the formation of biosurfactant-based hybrids for the biocatalysis process. Biosurfactant–inorganic hybrid nanoflower was synthesized with catalytic activity in degrading cationic dyes (Jiao et al., 2017). Lipopeptides are a group of the most effective biosurfactants that offer promising biocatalytic activities (Castelletto et al., 2019). They are crystalline extracellular products mostly produced by *Bacillus subtilis* (Jing & Qian, 2008; Sen & Swaminathan, 1997). As one of the most widely studied lipopeptides, surfactin was first discovered in the fermentation broth of *Bacillus subtilis* IFO3039 (Arima et al., 1968). They possess high surface activity, emulsification, foaming ability, and biocatalytic activity (Altenbuchner, 2016; Mandal et al., 2013;

Peypoux et al., 1999). The surface tension of water could be reduced from 72 mN/m to 27 mN/m with surfactin addition at a concentration of 0.005%. Because of these excellent properties, surfactin has been widely used in oil, environmental, pharmaceutical, food processing industries, and beyond (Fei et al., 2020; Liu et al., 2015; Nitschke et al., 2009; Santos et al., 2018).

Surfactin can be synthesized via microbial fermentation. However, low yield and high production cost hinder the industrialization of surfactin, resulting in an insufficient supply of this product with relatively high prices, and limited industrial application. To solve the previously mentioned problems, researchers try to explore the utilization of organic wastes as a rich source of hydrocarbons and nutrients for biosurfactant production. In the meantime, the pollution caused by the waste materials could be minimized. Till now, olive mill wastes (Ramírez et al., 2016), corn steep liquor, and sugarcane molasses wastes (Chaprão et al., 2018), animal fat and oil wastes (Santos et al., 2014), buttermilk and poultry-transforming wastes (Zouari et al., 2021), and shrimp shell wastes (Kadam & Savant, 2019) have demonstrated the feasibility to support biosurfactant production. Some of the previously mentioned waste materials proved to be feasible substrates for surfactin production with varied yields.

Fish wastes, such as fish head, fish skin, fish bones, red meat, and viscera generated from fish processing operations, account for 40% to 60% of the total weight of the fish (Nemati et al., 2017). Those wastes are likely to cause environmental pollution and even a series of health problems as a rich source of suspended solids, organic carbon, and nitrogen (Boopathy et al., 2007). At the same time, such a high organic content (e.g., proteins, polyunsaturated fats, and minerals) can be utilized as nutrients before being

discarded. Their utilization as fishmeal for animal feed has been commonly adopted with low economic returns and high environmental pollution. Previous studies indicated that fish peptones extracted from fish wastes exhibited the potential to support microbe growth (Safari et al., 2012; Vázquez et al., 2008; Zhu et al., 2020b). To date, few attempts have been made on biosurfactant production from fish wastes compared to that of other waste materials. Therefore, further in-depth investigation on fish waste-based biosurfactant production as an environmentally friendly alternative to make full use of these fish wastes is highly desired.

Lab-scale investigations on biosurfactant production are necessary as preliminary investigations of the fermentation conditions. However, they cannot reflect the system complexity when produced on a large scale. The problem raised by the change of configuration of reactors, air input, and agitation type could lead to various operational challenges. For example, in the pilot-scale experiment, there would be plenty of foam in the production process. To further confirm the commercial application of fermentation production, technology practice on biosurfactant production on a large and pilot-scale is a clear necessity toward their industrialization and commercialization, yet it is tackled in a limited way (Xu et al., 2020b). A full-scale demonstration of biosurfactant production using fish wastes as the substrate is, thus, highly desired.

Bonito (*Katsuwonus pelamis*), which is a tribe of medium-sized, ray-finned predatory fish in the family Scombridae, belongs to the tuna family. Though easily caught, bonitos are not popular because of the meat quality and the fishy smell and, thus, cheaper than other tuna in the East China Sea. Therefore, they are commonly processed to produce fish products. The proper utilization and treatment of the generated fish waste become

a challenge. We tried to solve this problem by using tuna fish wastes as a substrate for biosurfactant production. Scale-up studies were also conducted to facilitate the industrialization of biosurfactants. To achieve the objectives, tuna fish wastes were processed using the enzymatic hydrolysis method to generate the fish peptones. These generated fish peptones were served as the substrate for biosurfactant production. *Bacillus subtilis* (ATCC® 21332™) was selected as a representative lipopeptide producer (Fox & Bala, 2000; Wei & Chu, 1998; Wei et al., 2007). Surface tension (ST) and critical micelle dilution (CMD) were evaluated for monitoring biosurfactant production. Electro Spray Ionization Mass Spectrometry (ESI-MS) and high-performance liquid chromatography (HPLC) were used to evaluate the production. Three scales (20 ml, 7 L, and 100 L) of production were conducted to achieve the system scale-up.

3.2 Materials and Methods

3.2.1 Materials

Samples of tuna red meat wastes were from Ningbo Today Food Co. LTD, a fish processing plant in Zhejiang, China. Each sample was minced three times using a food processor at medium speed for 120 s. Fresh tuna red meat waste samples were taken for composition analysis prior to storage at -20 °C for subsequent experimentations. The red meat, which accounted for 13% to 15% of the weight of fish, was collected from tuna fish and subjected to proximate composition analysis. The results indicated that this meat had a moisture content of 58.6%, protein content of 18.1%, and fat content of 7.6 g/100 g. Freeze dried samples of *Bacillus subtilis* (ATCC® 21332™) used in this study were reactivated and cultured in both nutrient agar medium and/or nutrient broth

at 30 ℃. After 24 h of culture on a solid medium, milk-white bacterial colonies can be seen. For preservation, ATCC 21332 were freeze-dried in tubes for cryogenic Security Level 1 storage storage at -80 ℃.

The surfactin standard sample was purchased from Sigma. Sodium chloride, protease peptone, beef extract, agar, ferrous sulfate, manganese sulfate, sodium hydroxide, concentrated hydrochloric acid, and methyl alcohol was purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). PB05 basic protein (200,000 U/g) and PB02 animal protein (100,000 U/g) were purchased from Naning Pangbo Biological Engineering Co., Ltd. (Guangxi, China). The anti-foaming agent Foamdoctor® F2875 was purchased from Shenzhen Dayang New Material Co., Ltd. (Guangdong, China).

3.2.2 Enzymatic Hydrolysis for Generating a Fish Waste-Based Substrate

The lab-scale enzyme hydrolysis of fish wastes followed the method developed by a local laboratory. PB05 basic protein and PB02 animal protein were selected as the hydrolysis enzymes. Generally, 200 g of the waste sample was added into a 1000 mL Erlenmeyer flask and mixed with equal volumes (200 mL) of distilled water (1:1 w/v). The ratio of animal protease and alkaline protease was 2:1(w/w), and the volume of the enzyme was 1.5%. The pH was 7, which was regulated by HCl (2 mol/L) and NaOH (2 mol/L). The hydrolysis time was 6 h. The temperature was 50 °C with heating in the water bath. After finishing the hydrolysis period, the Erlenmeyer flask was put into another water bath at 90 °C for 15 min. The mixture in the flask was then centrifuged at 8,000 g for 10 min. The supernatants were collected for the degree of hydrolysis (DH) measurement, and some were concentrated to one-third by the rotary evaporator, after which fish waste peptone was produced by the freeze dryer for three days. Figure 3.1

shows procedures during the enzyme hydrolysis of fish waste. Fish waste peptone was then stored at 4 °C for subsequent experiments in the laboratory. Procedures of enzyme hydrolysis generation for the scale-up testing were the same as those in the laboratory with enlarged amounts of all involved materials proportionally.

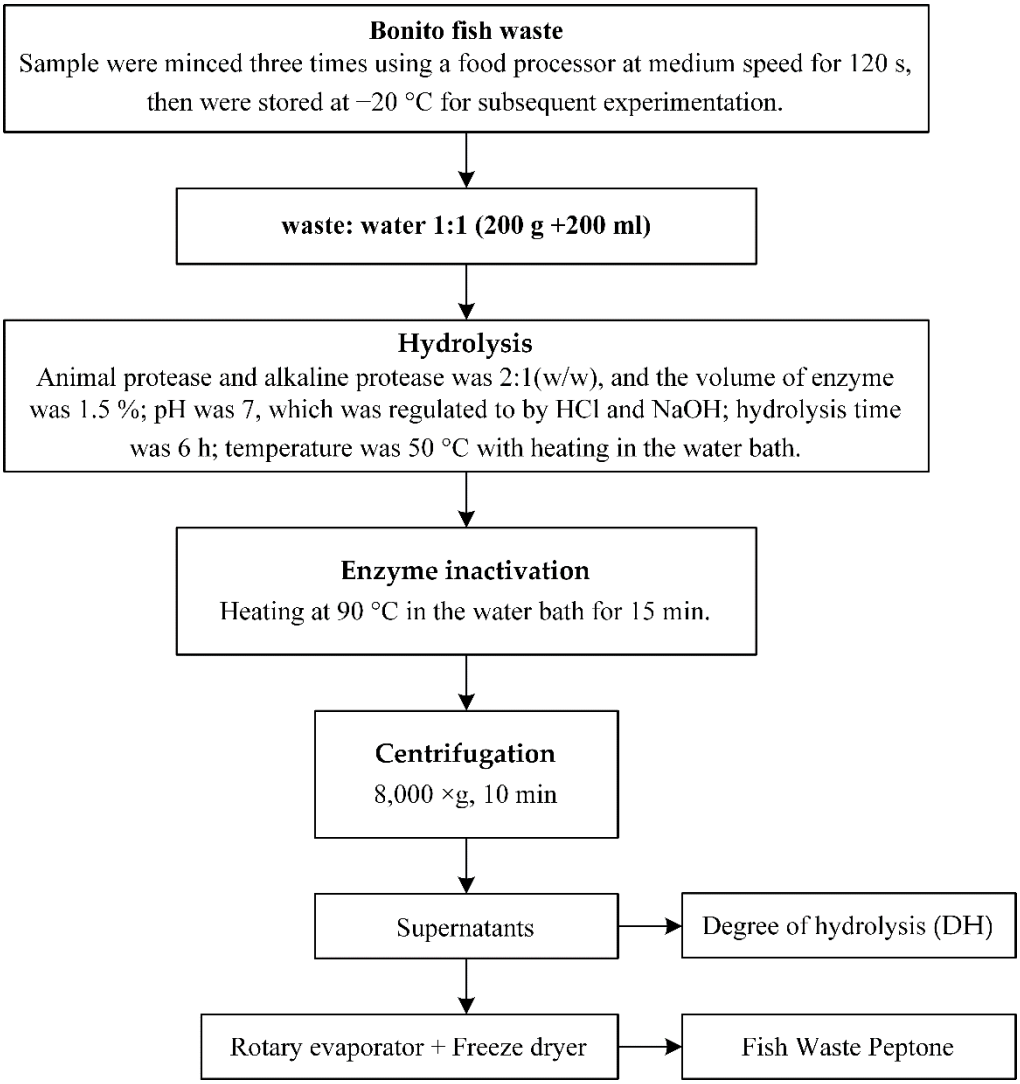


Figure 3.1 Flow chart of the enzyme hydrolysis in the laboratory

3.2.3 Bench-Scale Biosurfactant Production (20 mL)

After strain activation, bacteria were first inoculated in a Petri dish and incubated at 30 °C for 24 h. The materials of the inoculation broth followed as: NaCl (5 g/L), protease peptone (5 g/L), beef extract (3 g/L), and distilled water. After autoclaving at 121 °C for 20 min, a selected single strain colony was transferred into the Erlenmeyer flask.

The Erlenmeyer flask was incubated in a rotatory shaker at 120 rpm under 30 °C for 24 h. All experiments in this study used 2% (v/v) inoculum as a seed culture level during fermentation. After freeze-drying for 3 days, fish waste peptones were prepared as comprehensive medium with distilled water and key supplement minerals: $FeSO_4$ and $MnSO_4$.

To obtain the optimized fish waste peptone concentration, these lab-scale experiments were conducted with a series of concentrations (g/L): 20, 30, 40, 50, and 60. Key supplement minerals were added as follows (g/L): $FeSO_4$ (5×10^{-4}) and MnSO4 (0.15). Twenty milliliters of distilled water were added into each 50 mL Erlenmeyer flask. The strain in the flask was used as inoculum at the 2% (v/v) ratio. Erlenmeyer flasks were then incubated in a shaking incubator (130 rpm) at 30 °C for 7 days. After incubation, the supernatant was collected after centrifuging at 8000 g for 10 min. Biosurfactant production was evaluated with ST and CMD values. Each concentration had three parallel runs and all evaluations of the experiments were triplicate.

3.2.4 Batch-Scale Biosurfactant Production (7 L)

BioFlo 120 (Eppendorf, Germany) was the fermentation tank used in the batch-scale

experiments (14 L total volume, 7 L working volume). Figure 3.2 shows the batch-scale experimental set-up for biosurfactant production. The fermenter was equipped with a paddle mixer, a heater band, and a set of sensors (i.e., foam, pH, temperature, dissolved oxygen, and revolving speed of stirring paddles), which could be controlled to set the experiments to desired conditions through the control panel under the screen. Air was injected through the gas pump during the fermentation. The antifoaming agent was added into a container through the pump after foaming. Optimized concentrations of fish waste peptones and manganese followed the results from the previous experiments. The key supplement minerals were added as follows (g/L): $FeSO_4$ (5×10^{-4}) and $MnSO_4$ (0.15). After autoclaving at 121 °C for 20 min and cooling down to room temperature, 2% inoculum of strains were added into the tank. The temperature was set as 30 °C, dissolved oxygen was set as 50%, and the revolving speed of stirring paddles was set as 120 rpm. Samples were taken through the outlet every 4 h before 24 h to obtain detailed biomass change. After 24 h, samples were taken through the outlet every 12 h until the end of the fourth day. Biosurfactant production was evaluated with ST and CMD values. Samples at each time had two parallels and all evaluations of the experiments were triplicate.

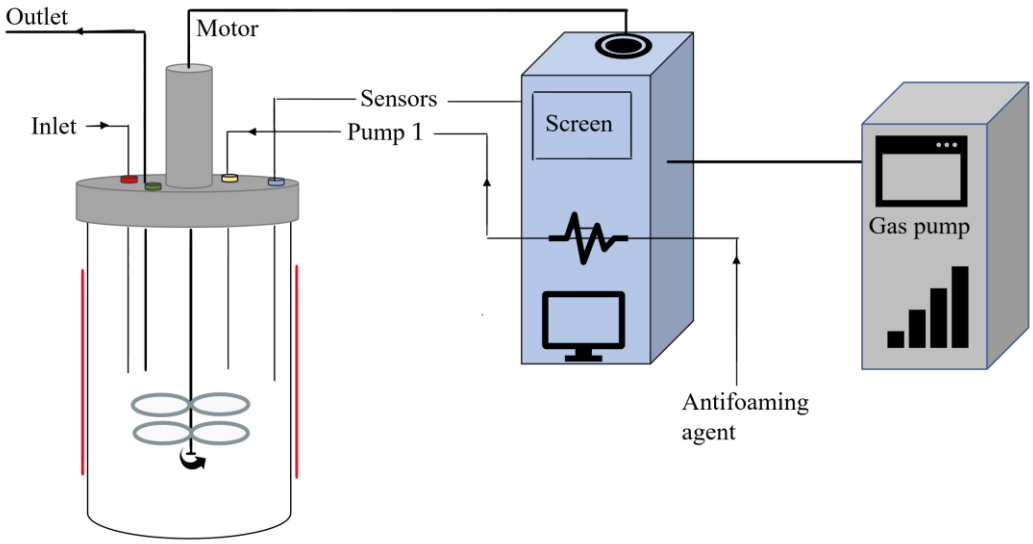


Figure 3.2 Lab-scale experimental set-up (7 L)

3.2.5 Pilot-Scale Biosurfactant Production (100L)

The pilot-scale experiments were conducted in a 200 L fermentation tank (Zhen-jiang Dongfang, Jiangsu, China) at a working volume of 100 L. Figure 3.3 shows the fish waste processer in pilot-scale experiments. Figure 3.4 shows the pilot-scale experimental set-up for biosurfactant production. As shown in the figure, this fermentation set-up was comprised mainly of a fermentation tank, a seed tank, and an agitator motor for agitation. There were several pumps connecting the seed tank with the fermentation tank: feeding and discharging production. Parameters, such as temperature, pH, and ventilation capacity, were controlled by a control system. The anti-foaming agent was added into the container through the pump near the outlet after foaming. Optimized concentrations of fish waste peptones and manganese followed the results of bench-scale experiments. The key supplement minerals were added as follows (g/L): $FeSO_4$ (5×10^{-4}) and $MnSO_4$ (0.15). After autoclaving at 121 °C for 20 min and cooling down to room temperature, 2% inoculum of strains were added into the fermentation tank through the pump. Temperature was set as 30 °C, dissolved oxygen was set as 50%, and the revolving speed of stirring paddles was set as 120 rpm. The ventilation rate was 1 vvm. Samples were taken through the outlet after 0, 10, 24, and 48 h. Biosurfactant production was evaluated with ST, biomass, ESI-MS, and HPLC.

Figure 3.3 Fish waste processer in pilot-scale experiments

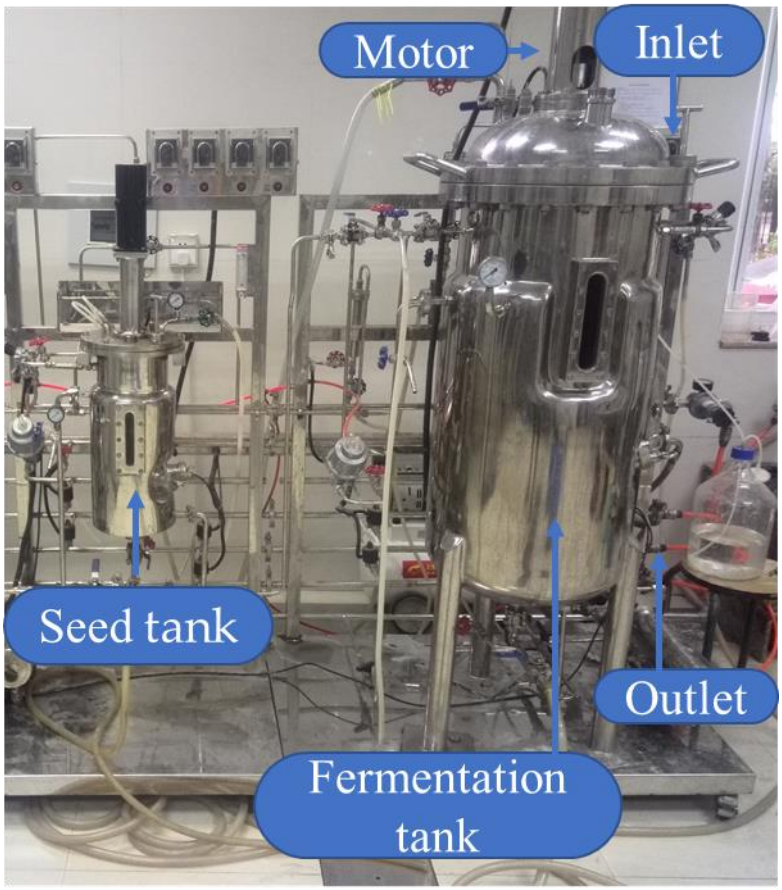


Figure 3.4 Pilot-scale experimental set-up (100 L)

3.2.6 Evaluation of Biosurfactant Production Performance

Surface tension: Surface tension was measured by the plate method using Sigma 700 surface tension meter (Biolin Scientific, Sweden). Twenty-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.

Critical micelle dilution: Critical micelle dilution was measured by the plate method using a SIGMA 700 surface tension meter. Critical micelle dilution could reflect the concentration of biosurfactants in the medium and be determined following the method described by Cai (2017). When the ST just exceeded 40 mN/m, the dilution process stopped, and the dilution ratio was recorded as the CMD value for this culture broth. All the measurements were performed in triplicate.

Biosurfactant Purification and Characterization: Put the 7 L of fermentation broth in the centrifuge for 10 min at the rate of 8000 × g to eliminate the thallus. The volume of the supernatant was defined as 30 mL. Then, 6 mol/L HCl was used to adjust pH to 2.0 as white flocculent precipitates formed. The supernatant was placed still for a while for more precipitates to gather. Then, put the supernatant with 5 ml in a centrifuge for 15 min at the rate of 10,000 × g to collect precipitates. The supernatant was shaken with 5 mL methyl alcohol and extracted for 1 h. Thereafter, a surfactin standard substance and supernatant were sent for inspection using qualitative and quantitative analysis through ESI-MS (Ningbo Institute of Oceanography, Ningbo, China) and HPLC (Ningbo Boao Bioengineering Co. LTD, Ningbo, China), respectively (Lingyan et al., 2014).

3.3 Results and Discussion

3.3.1 Characterization of Hydrolyzed Peptones

The degree of fish waste enzymatic hydrolysis was around 44.2% using the trichloroacetic acid (TCA) method. The amino acid analysis proved the existence of Phe, Ala, Met, Pro, Gly, Glu, Arg, Lys, Tyr, Leu, Ser, Thr, Asn, Val, Ile, and His in generated fish meat peptone, and the results were shown in Figure 3.5. The freeze-drying process for each batch of concentrated tuna fish head hydrolysate required 3 to 4 days. A total of 89% weight loss was reported in the freeze-drying process, and, accordingly, 22.96% of tuna red meat can be converted to fish peptone through an enzymatic process.

Characterization of hydrolyzed peptones can be meaningful for the production analysis. The characteristics of different raw materials could cause significant effects on the properties of substrates (Zhang et al., 2018). In this research, the yield of the process related to hydrolysis was around 44.2%. The generated result was in accordance with the ones generated as 42.9% using the same method. The hydrolyzed peptones contained materials to be a kind of comprehensive substrate.

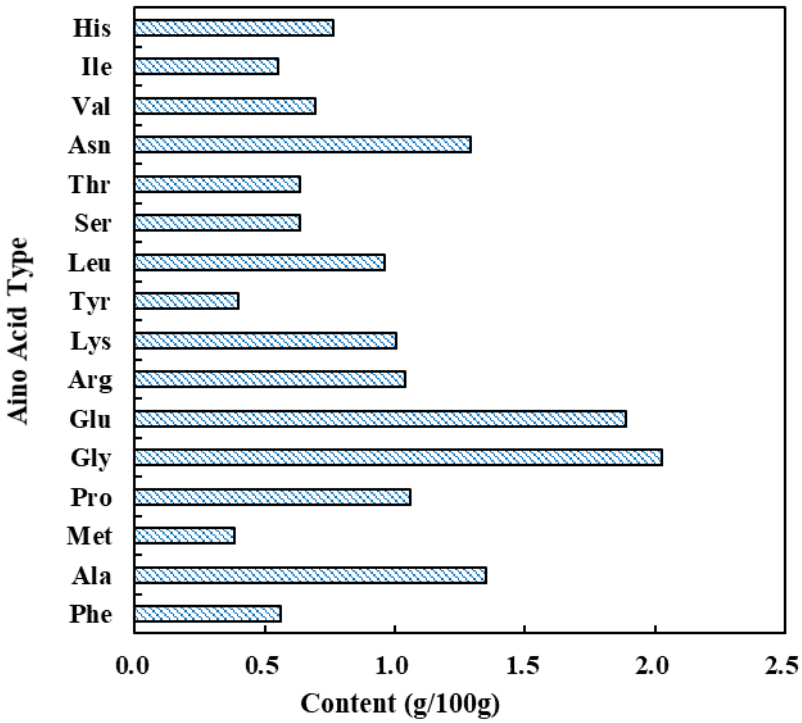


Figure 3.5 Composition of amino acid in tuna waste-based peptone after hydrolysis

3.3.2 Bench-Scale Production of Biosurfactants

The results of batch-scale experiments of biosurfactant production using different fish waste peptone concentrations are shown in Figure 3.6. The ST of all the substrates were reduced to around 28 mN/m, which proved that ATCC 21332 could produce biosurfactants with comprehensive fish waste broth (with key supplement minerals). Moreover, the highest biosurfactant production rate was reported at a substrate concentration of 20 g/L, whose CMD values reached around 60. Thus, 20 g/L was selected as the optimized concentration.

An optimized fermentation medium at a concentration of 20 g/L proved that superfluous nutrients could have an inhibiting effect on surfactin production (Varley, 1999). Furthermore, Pepi et al. (2013) indicated that some fatty acids (e.g., palmitic acid and oleic acid) in fish peptone could also inhibit the biosurfactant production by *Bacillus* strains. Adding a small amount of manganese ion as a trace element was beneficial for the production of biosurfactants because the manganese ion could be the most important cofactor of glutamine synthetase, and glutamine synthetase is very important for the assimilation of inorganic nitrogen by organisms (Deshpande et al., 1981). Huang et al. (2015) found that a manganese ion could have a positive effect on nitrogen use and surfactin production by *Bacillus subtilis* ATCC 21332. Biosurfactant production by *Bacillus subtilis* ATCC 21332 using cod liver and head wastes was evaluated in our previous work (Zhu et al., 2020a). The results showed that medium composition could significantly affect the structure and yield of produced biosurfactants. The generated fish waste peptones could substantially vary among different fish species and waste sources and affect biosurfactant production accordingly. The ST and CMD (i.e., 29.4

mN/m and 60.7, respectively) generated in this study were comparable to the ones generated by cod liver and head wastes peptones (i.e., 59.3 and 49.2, respectively). By using glycerol and waste frying oil as comparative carbon sources with *Bacillus subtilis* to produce biosurfactant, Ramirez et al. (2016) found olive mill wastes were potential substrates for biosurfactant production, which produced surfactin at a maximum concentration of 3.12 mg/L with 2% w/v of olive mill wastes in the medium. The substrate can be optimized by adding additional nitrogen sources or carbon sources by conducting this step from bench-scale to pilot-scale. Sufficient carbon could facilitate the biosurfactant production process (Reis, 2013). Therefore, additional carbon sources into this system, such as glucose and glycerol, or a continuous exploration of waste carbon sources (e.g., olive mill wastes) would be appealing and likely to improve the yield of biosurfactant production. Bench scale studies proved the feasibility to use tuna red meat wastes as a comprehensive substrate. Optimized fermentative conditions (e.g., concentrations of peptone and key supplement minerals) were determined.

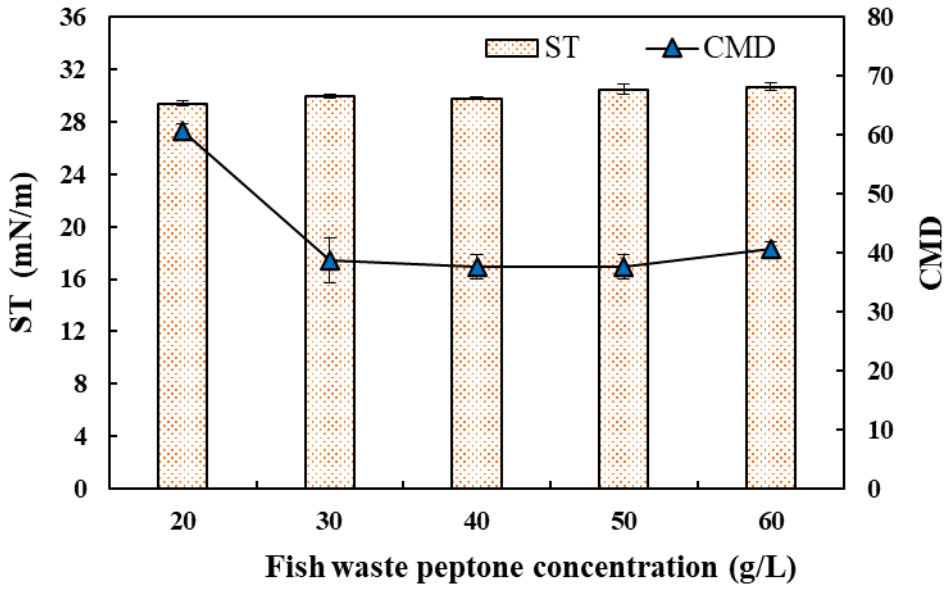


Figure 3.6 Bench-scale experiments exploring different fish waste peptone concentrations

3.3.3 Batch-Scale Production of Biosurfactants

As shown in Figure 3.7, a reduction of ST occurred in the first 12 h, indicating a gradual secretion of biosurfactant products. A rapid ST drop and a CMD increase were found between the 12–24 h, implying a surge of biosurfactant production during this period. The ST and CMD values remained the same after 36 h. The highest biosurfactant concentration in the fermentation medium was achieved between 24–36 h. This result shed light on the fermentation time selected for pilot-scale experiments and a 48-hour fermentation period was selected for the pilot-scale production.

To date, demonstration of biosurfactant production at scales (e.g., batch-scale and pilot-scale) using waste streams as substrate has been rarely explored, yet of great importance on their way to industrialization. Therefore, the antifoaming agent was added through the pump after foaming began. After screening available antifoaming agents through a performance evaluation, the Foamdoctor® F2875 was chosen as the product applied in batch/pilot studies. While ST values were similar between bench-scale and batch-scale experiments, a reduction of biosurfactant production (i.e., CMD values decreased from around 60 to 50) was reported during system scale-up.

In an amplification system, changes in the fermentation conditions such as pH, dissolved oxygen, and defoaming agents would affect the yield of surfactants (Shaligram & Singhal, 2010; Yeh et al., 2010). These results generated by other studies could help explain the different CMD values between bench-scale and batch-scale products. Moreover, in a larger system, defoaming agents were essential because of the foam formation. All conditions that change in a larger system could affect the metabolism activities and, thus, affect the yield of surfactants. Generally, the ventilation

rate was 1 air volume/culture volume/min (vvm), which made strains carry out cell metabolism activities in a suitable batch-scale condition. The volume of defoaming agents depended on the foaming situation, which was usually controlled by sensors on the fermenter. In addition, for continuous cell growth, the effect of inoculum age should also be considered (Abdullah et al., 2000).

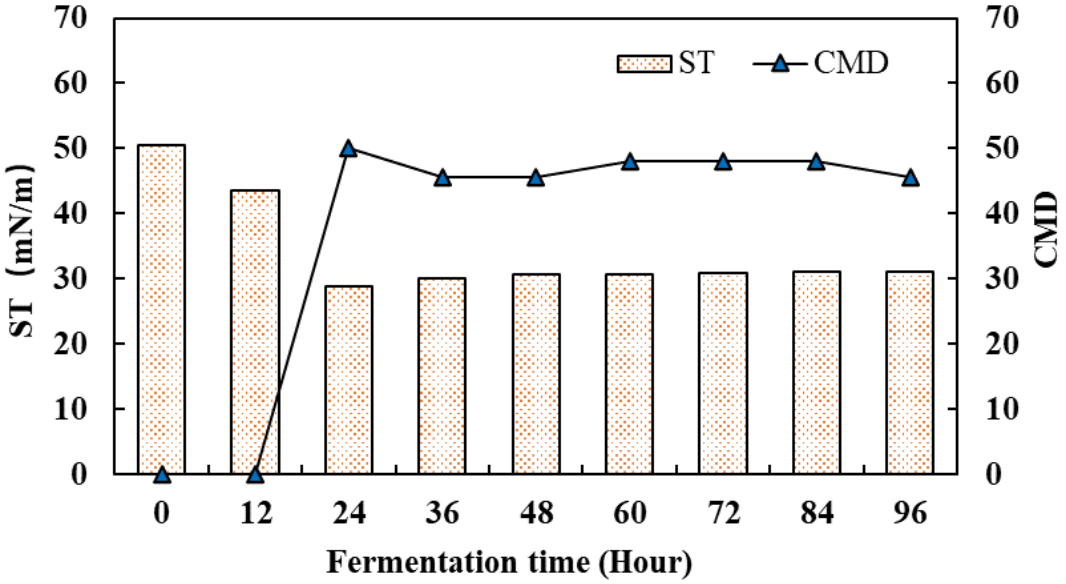


Figure 3.7 ST and CMD values in batch-scale experiments (7 L) with optimized concentration

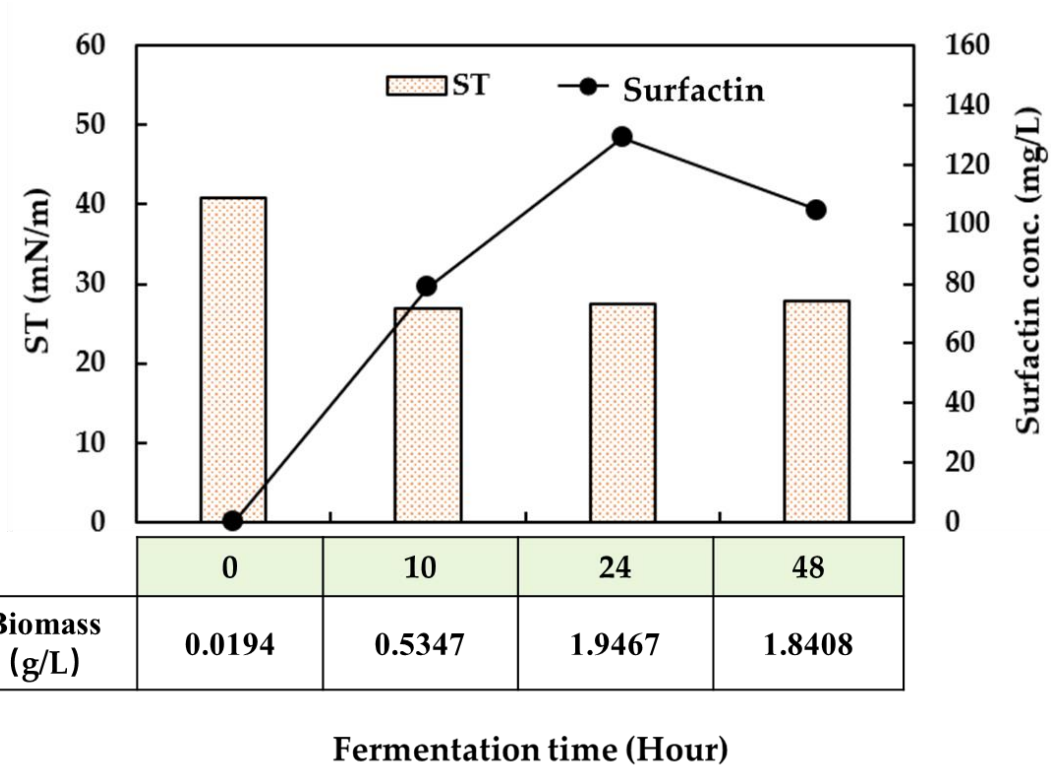
3.3.4 Pilot-Scale Biosurfactant Production Experiments

The results of fish wastes-based biosurfactant production in a pilot-scale reactor were shown in Figure 3.8, with a biomass result illustrated. The highest concentration was 129 mg/L in 24 h. The growth of bacteria was boosted from 6 h to 24 h and reached a peak after 24 h. The growth status of Bacillus subtilis ATCC 21332 in the culture medium was inferred because of sufficient materials. The fast metabolization of Bacillus strains led to an increasing bacterial colony concentration. Nutrient demand exceeded the supply after 24 h and limited the metabolic activities of strains and their reproduction. The content of bacteria went through a transitional plateau period, and the bacterial growth curve's plateau period existed between 12 h and 36 h. Compared with the results of batch-scale, the trends of ST values and biosurfactant contents were similar.

To date, no pilot-scale studies on lipopeptide production by *Bacillus substilis* ATCC 21332 have been reported using waste streams as substrate, so it was meaningful to explore the optimum conditions for surfactin fermentation scale-up. The surfactin production reduction was also reported in pilot-scale reactors compared to that of batch-scale studies using *Bacillus substilis* B006 for surfactin production (Wang et al., 2017), whose surfactin productivity reached 314.73 mg/L. The larger the system is, the more complicated the operation conditions are. Therefore, more emphasis should be given to the performance investigation for surfactin synthesis on a large scale.

The drop of surfactin content between 24 to 48 h could be explained by a rapid biosurfactant production and a spontaneous foam overflow occurred after that. To solve this problem, a recovery tank was connected to the fermenter for foam collection. In this study, the foam collected in the recovery tank contained a surfactin concentration

of around 274 mg/L, doubled its concentration in the sample collected at 24 h. Moreover, although the antifoaming agent was used during the fermentation period, there was still a lot of foam before 24 h. A novel bioreactor system based on integrated foam-control and a repeated fed-batch fermentation strategy has been applied to rhamnolipids production (Xu et al., 2020a), which could help enhance biosurfactant production.



	0	10	24	48
Biomass (g/L)	0.0194	0.5347	1.9467	1.8408

Figure 3.8 ST values, surfactin concentrations, and biomass in pilot-scale experiments (100 L)

3.3.5 Characterization of Biosurfactant Production

Electro Spray Ionization Mass Spectrometry (ESI-MS) analysis was conducted to characterize the structures of generated biosurfactant products. Results are shown in Figure 3.9. The surfactin standard exhibited five anion peaks around the mass-to-charge ratio of 1000. When compared to purified samples corresponding to the standard substance, surfactin was proven to be the product of fermentation.

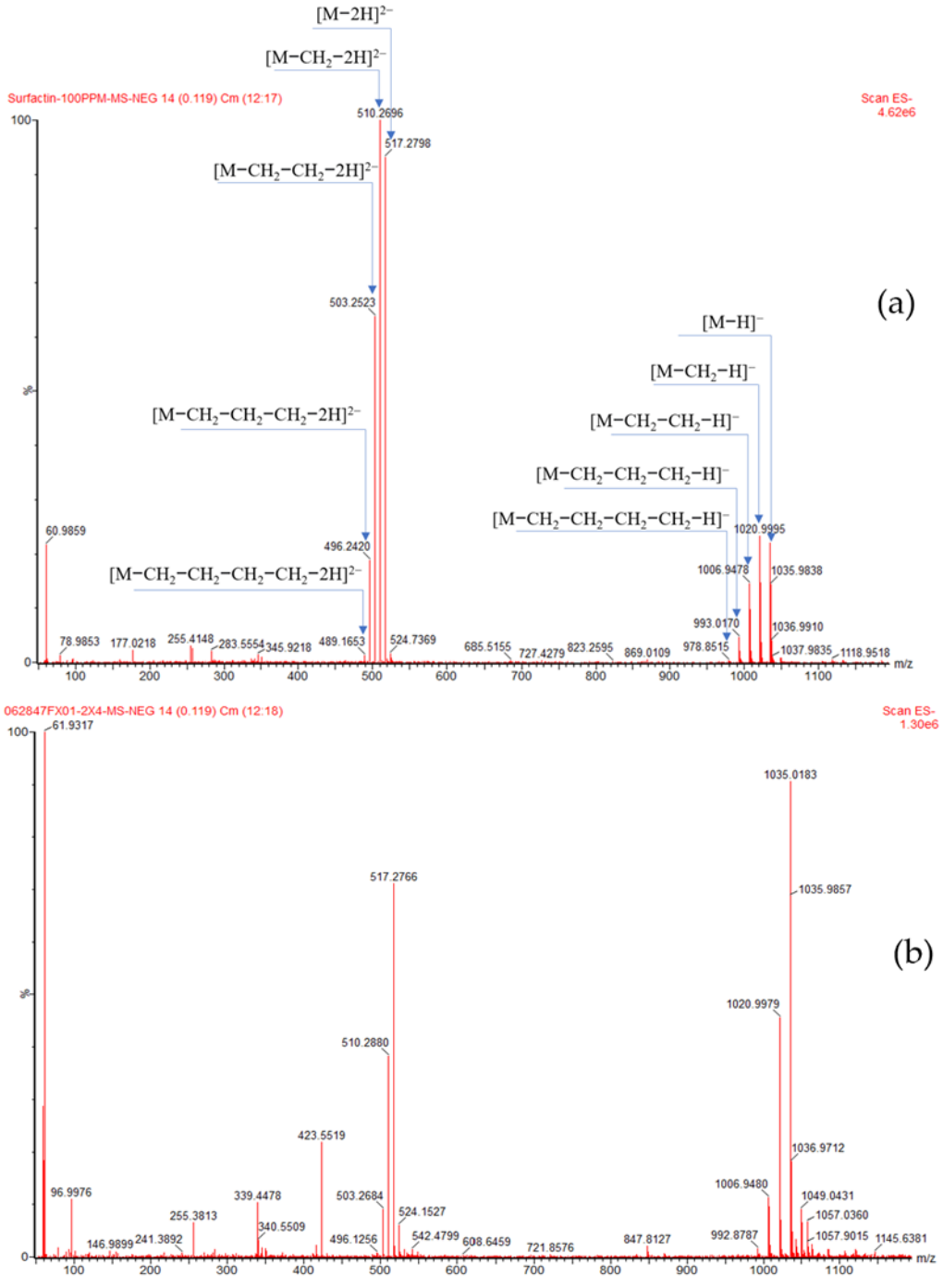


Figure 3.9 ESI-MS analysis (a) Surfactin standard sample ESI-MS. (b) Biosurfactant production ESI-MS

3.4 Summary

This study explored the conditions of using tuna fish wastes to generate surfactin. The research could help the local factories to dispose of their organic waste, thus reducing the environmental issues associated with the discharge of wastewaters. Fish wastes were first evaluated as a comprehensive substrate for strain growth and surfactin synthesis. The scale-up validation of surfactin production was attempted with a surfactin productivity of 274 mg/L in the fish-waste-based fermentation medium. Further works will be needed to further optimize the comprehensive fish waste substrate with a proper supplement of carbon or nitrogen source. This study demonstrated a cost-efficient approach for surfactin synthesis and paved the way for the industrialization of their production through an understanding of the metabolic mechanism and production kinetics of surfactin produced by strain ATCC 21332.

CHAPTER 4

BIOSURFACTANT-ENHANCED CLEANING OF OILED SHORELINE: AFFECTING PARAMETERS AND CAUSAL INFERENCE ANALYSIS

4.1 Introduction

Oil spills could cause catastrophic impacts on human life and aquatic ecosystems (Maes, 2004; McNicoll, 1995). Under natural influence such as wind and waves, along with increasing human activities, the spilled oils can easily reach the shoreline (Geng & Boufadel, 2015; Geng et al., 2020; Lee et al., 2015). After weathering, they can be more environmentally persistent and challenging to be removed (Gharbi et al., 2017), urging the need for effective oiled shoreline cleanup methodologies. The use of approved surface washing agents is acceptable for mitigating shoreline crude oil contaminations in some nations. Surfactants are the most used component in washing fluids due to their capacities for solubilizing hydrophobic organic compounds (Befkadu & Quanyuan, 2018; Ishiguro & Koopal, 2016). In addition to chemical surfactants, which may be harmful to the shoreline environment, the application of green biosurfactants is emerging (Hu et al., 2021; Jiang et al., 2020). After adding washing solution to the oil-contaminated soil, high-energy mixing, mechanical shearing and dispersion are applied in this process to remove contaminated oil residues (Ceschia et al., 2014). Biosurfactants like rhamnolipids, surfactin and humic substances have shown the potential in soil washing (Mao et al., 2015). Exploring and optimizing the biosurfactant application for soil washing could expand the the choice of options within our remediation toolbox (Chen et al., 2020).

Due to surfactants' amphipathicity, emulsions can be easily formed when crude oil and surfactant solutions are mixed. The continuous aqueous phase and the dispersed oleic phase can be immiscible in some emulsions, that will result in a s three phase system. Nanoemulsion is one classic aforementioned emulsions, which is kinetically stable in the form of small droplets size (< 500 nm), and usually produces products with high

surface area per unit volume, robust stability, optically transparent appearance, and tunable rheology (Gupta et al., 2016). The formation of nanoemulsion can promote petroleum mobilization, and indicates low interfacial tension is between the oil-water interface. Using a biosurfactant as the emulsifier, a nanoemulsion system can be prepared through a high or low energy method (Bai & McClements, 2016; Lovaglio et al., 2011). However, in terms of research on surface washing technologies we are still in the preliminary stage of research on the the generation and application of biosurfactant-based nanoemulsion systems. There is need for a greater understanding of the microstructures and phase behaviors under various shoreline conditions (e.g., temperature, salinity) (Nawavimarn et al., 2021; Rongsayamanont et al., 2017). To better understand the mechanism of surfactant-enhanced soil washing, research efforts are urgently needed to contribute to the knowledge in constituents and phase behavior of emulsions.

Factors influencing the effectiveness of surfactant-aided soil washing are also an interesting topic. Temperature and salinity are also two important factors, especially during the shoreline soil washing scenarios. In terms of temperature, Urum and Pekdemir found that soil washing efficiency tends to grow along with temperature increases (Urum & Pekdemir, 2004). However, some other studies reported that the impact of temperature is insignificant (Peng et al., 2011). Among the discussions, interfacial tension as a mediator has drawn our attention. It is a factor that connects to many conditions such as temperature and salinity. Being reported by previous studies, it seems that interfacial tension can be reduced along with the addition of salt to surfactant solutions (Kumar & Mandal, 2016). Besides, both temperature and salinity can theoretically have effects on the interfacial tension and crude oil removal efficiency.

However, the causal relationship between interfacial tension and crude oil removal efficiency in the process of shoreline soil washing has never been discussed nor estimated. As a potential mediator (a factor passing causal effect from other variables to the target variable), such ambiguity about interfacial tensions can lead to inconsistent estimation and biased discussion of the whole nanoemulsion soil washing process. Thus, in this study, from the perspective of surfactant-enhanced shoreline soil washing, we are interested in answering the following questions: Will the change of interfacial tension directly affect the crude oil removal efficiency? If yes, what is the strength of such a causal effect? Due to the presence of the synergistic effect that existed in the complex oil-soil-surfactant solution system, the question aforementioned can hardly be answered with only the observational data available, even with the assistance from conventional analysis tools. For such investigation, new tools with the capability to conduct causal inference based on experimental data are desired.

Causal inference, a topic that has been long discussed, is drawing growing research attention due to the recent methodology breakthrough along with the rise in computing power and data-driven approaches. Among the emerging methods, Structural Causal Model (SCM) is considered to be a more versatile and robust approach (Aliprantis, 2015; Markus, 2021). Based on a series of graph-based operations within the data generating process, it explicitly includes all the causal factors in the analytical procedure. Most importantly, quasi-experiment can be conducted through SCM, which enables the SCM to provide causal interpretations rather than correlations. Potential causal relationships in SCM can undergo a series of refutation tests to check their robustness with the support of classic statistical tools. Therefore, we chose SCM as the causal inference method in our study to investigate the role of interfacial tension during the process of

soil washing.

The current study experimentally investigated the formation of biosurfactant-involved emulsion under various temperatures and salinities. After testing the interfacial tension between the designed surfactant solutions and crude oil, surface washing was then conducted to determine the efficiency of crude oil removal. Causal inference analysis was then conducted with the help of SCM, to quantitatively estimate the causal links between the interfacial tension and crude oil removal efficiency.

4.2 Materials and Methods

4.2.1 Materials

The crude oil (Conventional heavy crude, CHO) was provided by Multi-Partner Research Initiative (MPRI) in Canada. The density of this crude oil was tested as 0.8 g/cm^3 and the reflective index was 1.66. Rhamnolipids were obtained from AGAE Technologies (Corvallis, USA). Dioctyl sulfosuccinate sodium salt (DOSS) was purchased from Alfa Aesar (Tewksbury, USA). Certified ACS grade n-hexane and HPLC grade n-hexane were both obtained from Fisher Scientific (Montreal, Canada). The properties of the soil used in this study are listed in Table 4. 1 (Li et al., 2018).

4.2.2 Formation of Emulsions

Preparation of emulsions was conducted as previously described (Acosta et al., 2008). The emulsions prepared through mixing 1 mL crude oil and 1 mL surfactant solution (concentration: 0.25M) in 7 mL vials. The mass ratios of rhamnolipids and DOSS of diverse surfactant solutions were set at: 0:10, 2:8, 5:5, 8:2 and 10:0 under the salinity of 0, 1.5 and 3% NaCl. After shaking vials on the vortex mixer for 3 min, samples were

incubated at 5, 15 and 25 ℃. After 14 days, the phase behavior of emulsions was identified, and the microscopic image of emulsions was taken through Swift SW380T (SWIFT Microscopes, USA). Nano ZS90 ZEN3690 Zetasizer (Malvern, UK) was used to obtain the emulsion particle size.

4.2.3 Soil Washing for Oil Removal

The oil-contaminated soil was prepared by adding 1 g crude oil to 1 kg sands. Briefly, crude oil was dissolved in hexane, mixed with sands, and sonicated for 10 mins. Then, samples were put in the fume hood at 25 ℃ for 72 h to evaporate hexane. The sand samples were collected in the amber glass at 5 ℃ refrigerator for storage.

All the Shaken Flask tests were conducted in 50 mL Erlenmeyer flasks with 10 g prepared sand sample and 20 mL diluted surfactant solution. All the surfactant solutions used in this process were diluted 50 times using distilled water. Flasks were put into shakers at 200 rpm under designated temperature (5, 15 and 25 ℃) for 24 h to wash crude oil. Each condition had three parallel runs and all evaluations of the experiments were in triplicate.

Crude oil was extracted using Dionex ASE 350 accelerated solvent extractor (ASE) system (Thermo Scientific, USA) following the manufacturer's procedures. To avoid the influence of surfactant solutions on the extraction process, supernatant was gently removed after the shaken flask test, and soil samples were put into the oven at 50 ℃ for 36 h. After samples cooled down to the room temperature and the leaking check of the ASE system, HPLC-grade hexane was used as a solvent to extract crude oils. Treated soil samples were filled into cells using the funnel after installing cell filters (27-mm cellulose filter) in the center of the cell bottom end cap. To reduce the amount of solvent

used during the extraction, any void volume in the cell with an inert material was filled. After loading the cell trays, collecting vessels, and rinsing cubes, system method was set up as: temperature: 100 ℃; heat period: 5 min; static time: 5 min; rinse volume: 60 %; purge time: 90 s; cycles: 3 times. The sequence of this extraction system was from 1 to 24. This operation lasted 14 h under the designed methods. Samples were collected in the collection vessels for further tests.

4.2.4 Sample Analysis

Interfacial tension: Interfacial tension between prepared surfactant solutions and crude oil was measured by the drop shape method using DSA25S drop shape analyzer (KRUSS, Germany) following the manufacturer's procedures. Figure 4.1 shows the experimental setup of the tension meter. Briefly, to assure the accuracy of measurements, the B factor was controlled between 0.4 to 0.6. All samples were tested three times. To set the temperature at 5, 15 and 25 ℃, iso-temp 6200 R28 refrigerated/heated bath circulator (Fisher Scientific, USA) was used in this process.

Crude oil removal efficiency: The oil dissolved in hexane was quantified by UV-Vis spectrophotometer (Thermo Scientific, USA). Briefly, 340, 370 and 400 nm were selected and calculated to measure the oil concentration for dissolved oil concentration in hexane (Chandrasekar et al., 2006). The area was determined by following Eq. (4.1). The calibration standards are shown in Figure 4.2.

$$Area = \frac{(Abs_{340} + Abs_{370} \times 2 + Abs_{400} \times 30) \times 30}{2} \quad (4.1)$$

Samples collected after ASE system were added into 50 mL volumetric flasks for crude

oil residue quantification. The crude oil removal efficiency can be calculated as Eq. (4.2).

$$\text{Removal efficiency} = \frac{(\text{Oil contained in sands} - \text{Oil residue})}{\text{Oil contained in sands}} \quad (4.2)$$

To ensure the quality assurance and quality control, each group of experiment in the soil washing process was conducted in triplicate. The average values were applied in the data analysis, with the standard deviation displayed as error bars in related figures.

4.2.5 Causal Inference Analysis

In a causal process, the causal effect of a variable to a specific target variable can be explicitly calculated. It can be expressed in the following probability distribution: $P(y|do(x))$. The distribution can be interpreted as "the conditional distribution of Y when the observation variable X is set to x". The ultimate goal of causal estimation is to estimate the aforementioned probability distribution. However, in most scenarios where only observed data is available, such probability can hardly be explicitly calculated due to many reasons, including ethical reasons and infeasibility to re-conduct a past experiment with only a few conditions changed. To tackle the counterfactual challenge, a set of graphic operations is supported. Such operations and criteria can help users identify a "sufficient set" of variables in the graph. Using only the variables in the sufficient set instead of every variable in the causal diagram, the target causal effect can then be estimated. In other words, if the sufficient set contains no unobserved or unmeasurable variable, $P(y|do(x))$ can later be calculated (Pearl, 2009).

The causal effect can be expressed based on a sufficient set S:

$$P\big(Y\big|\, do(X = x)\big) = \sum_{s} P(Y = y|\, X = x, S = s)P(S = s) \tag{4.3}$$

Where $do(\cdot)$ indicates the intervention operation, X, Y indicate the treatment and the outcome variables, respectively. S indicates the variables in the sufficient set. In contrast, x, y and s indicate the individual value in corresponding variables (Judea, 2010). Using the intervention and other graph operations, causal relationships can be distinguished from the correlations.

In a causal inference application, when a specific set of variables has been confirmed to be sufficient to calculate a causal effect, we can choose a model (estimator) from a series of regression techniques such as linear regression and machine learning models and then fit the model to the sufficient dataset, i.e., the sub-dataset with only the chosen causal variables from the sufficient set above mentioned. With the fitted causal estimator, the causal relationships between different variables can then be calculated. The causal relationship strengths between each treatment and outcome can be quantitatively evaluated in two metrics: average treatment effect (Elsayed et al.) and conditional average treatment effect (CATE) (Imbens, 2004).

ATE was calculated using the following equation:

$$\mathrm{ATE} = \frac{1}{\mathrm{N}}\sum\big(\mathrm{y}_1(\mathrm{i}) - \mathrm{y}_0(\mathrm{i})\big) \tag{4.4}$$

Where $y_1(i) - y_0(i)$= is the difference between outcomes for individual i under treatment (y_1) and without treatment (y_0). N is the total amount of individuals. Another metric, CATE, is the ATE value calculated under different conditions. The

corresponding condition combinations will be further elaborated in the next section.

To increase the chance of catching both linear and non-linear causal effects, the machine-learning-based CausalForestDML (Microsoft Research, 2020) algorithm and a linear estimator were selected as the causal effect estimators. The refutation methods will be further introduced in the next section.

Causal inference methods used in the study were acquired from the DoWhy packages, a Python package specialized in providing SCM causal inference interfaces (Sharma & Kiciman, 2020). The CausalForestDML (DMLOrthoForest in the previous version) algorithm is included in EconML, which is a Python package dedicated to providing machine learning-based causal estimators (EconML, 2019).

Table 4. 1 Soil particle size analysis

Particle type	Diameter (mm)	Percentage (%)
Coarse sand	0.5–2	4.6
Medium sand	0.25–0.5	36.4
Fine sand	0.125–0.25	47.1
Very fine sand	0.0625–0.125	10.8
Silt	<0.0625	1.1

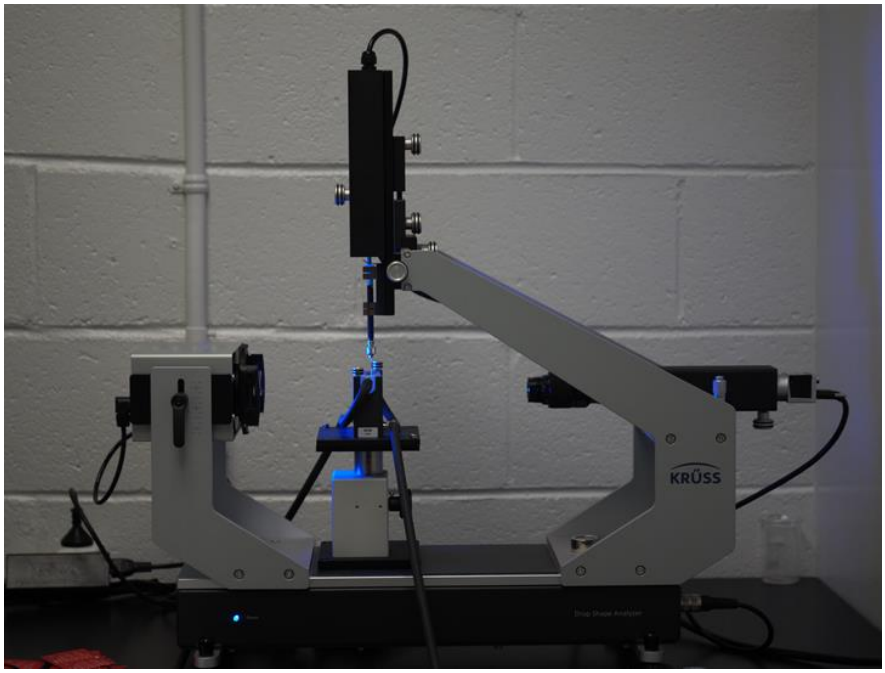


Figure 4.1 Drop shape analyzer

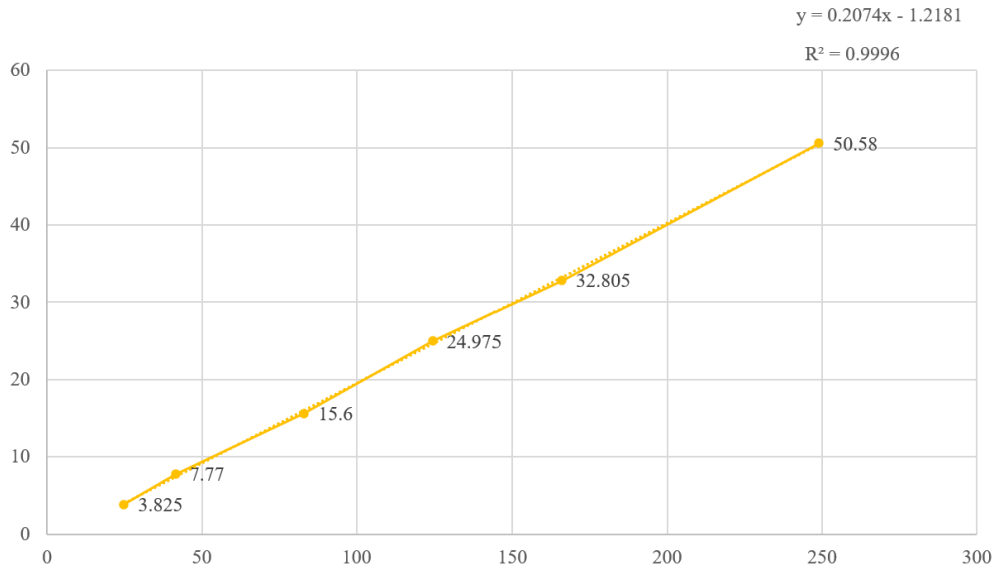


Figure 4.2 Conventional Heavy Crude Oil standard curve in Hexane

4.3 Results and Discussion

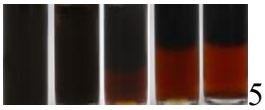
4.3.1 Formation of Emulsions Under Various Temperature, Salinity, and Surfactant Ratio

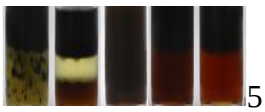
After storage at designed temperature for 14 days, emulsions fabricated from the crude oil and surfactant solutions under different saline conditions were observed, as in Figure 4. 3. Phase behavior study of emulsions found that the three-phase nanoemulsion systems were established under several conditions (e.g., temperature: 15 ℃, salinity: 1.5 %, R:D=2:8). Figure 4.4 shows the schematic diagram of three kinds of emulsions phase behavior and microscopic images of emulsion phase (10×40) when the temperature was 15 ℃ and salinity was 1.5 %. Being more specifically, Figure 4.4 (a) shows a three-phase oil-in-water nanoemulsion system, where the nanoemulsion phase had an optically transparent appearance. Because of low interfacial tension, crude oil tends to partition within the nano-micellar structure of nanoemulsion spontaneously (Koroleva & Yurtov, 2012). The oil droplets in the nanoemulsion phase were easy to be observed. As shown in Figure 4.4 (b), when the ratio of Rhamnolipids to DOSS is 5:5, water-in-oil emulsion forms. In Figure 4.4 (c), the microscopic image shows blurred oil droplets, which indicates this oil-in-water emulsion was not a fine nanoemulsion.

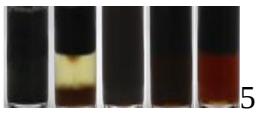
In this study, salinity was seemed to be more important in forming nanoemulsion. For example, when the temperature was 15 ℃, the addition of salt (1.5 % salinity) on surfactant solution (R:D=0:10 and R:D=2:8) made the dispersed oleic phase be formed at the bottom. It suggests that cations in salt may be adsorbed on the nano-micelle surface, leading to the formation of nanoemulsion. Comparing the phase behavior when DOSS was the only component of surfactant solution, the solution contained

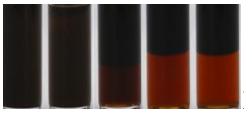
rhamnolipids (R:D=2:8), creating more volume of nanoemulsion with crude oil. This phenomenon proved the synergistic effect of the emulsifier mixtures could benefit nanoemulsion preparation by decreasing the interfacial tension (Qadir et al., 2016). However, not all synergistic effect of emulsifier mixtures works in the formulation of nanoemulsion because only when the ratio is R:D=2:8, the synergistic effect was significant. In terms of temperature, when R:D=2:8 and salinity=1.5 %, nanoemulsion with optically transparent appearance was only observed when the temperature was 15 ℃, which indicates temperature can affect the formation of nanoemulsion as well. These results proved both temperature and salinity of the system are important parameters influencing the phase behavior (Ren et al., 2019).

To characterize the oil-in-water nanoemulsion, dynamic light-scattering instruments were used to obtain the droplet size (temperature: 15 ℃, salinity: 1.5 %, R:D=2:8). Droplets with a size smaller than 500 nm were observed. Results showed that the sample contains one major population by volume with an average size of 459.4 nm (Figure 4.5), which was assumed with desirable measurement quality. Since shaking by mixer was the only method used in the preparation of emulsions, large sizes of oil droplets were thus obtained as reported by other studies (Haba et al., 2014; Nitschke et al., 2010). These findings proved that the mixture ratio of rhamnolipids and DOSS at 2:8 can form stable nanoemulsions with heavy crude oil.


5 ℃, 0 % salinity, Rhamnolipids: DOSS= 0:10 2:8 5:5 8:2 10:0


5 ℃, 1.5% salinity, Rhamnolipids: DOSS= 0:10 2:8 5:5 8:2 10:0


5 ℃, 3 % salinity, Rhamnolipids: DOSS= 0:10 2:8 5:5 8:2 10:0


15 ℃, 0 % salinity, Rhamnolipids: DOSS= 0:10 2:8 5:5 8:2 10:0


15 ℃, 1.5 % salinity, Rhamnolipids: DOSS= 0:10 2:8 5:5 8:2 10:0


15 ℃, 3 % salinity, Rhamnolipids: DOSS= 0:10 2:8 5:5 8:2 10:0


25 ℃, 0 % salinity, Rhamnolipids: DOSS= 0:10 2:8 5:5 8:2 10:0


25 ℃, 1.5 % salinity, Rhamnolipids: DOSS= 0:10 2:8 5:5 8:2 10:0

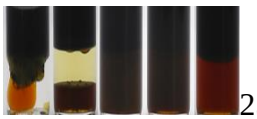

25 ℃, 3 % salinity, Rhamnolipids: DOSS= 0:10 2:8 5:5 8:2 10:0

Figure 4. 3 Emulsion appearance in vials

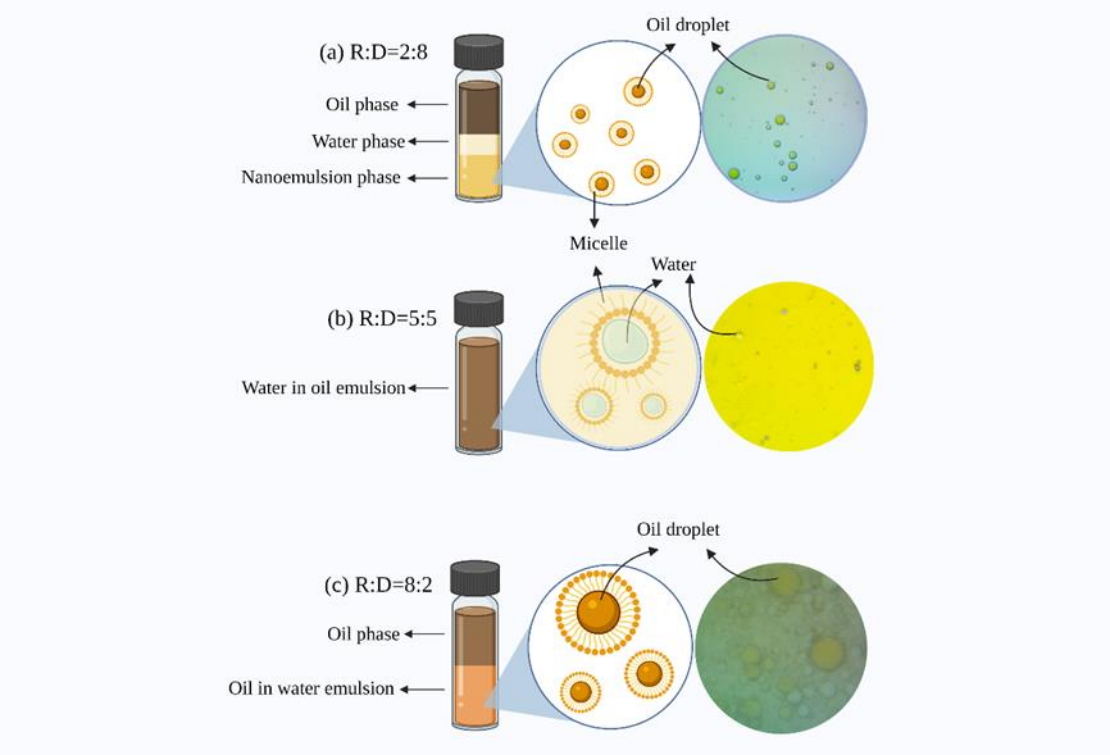
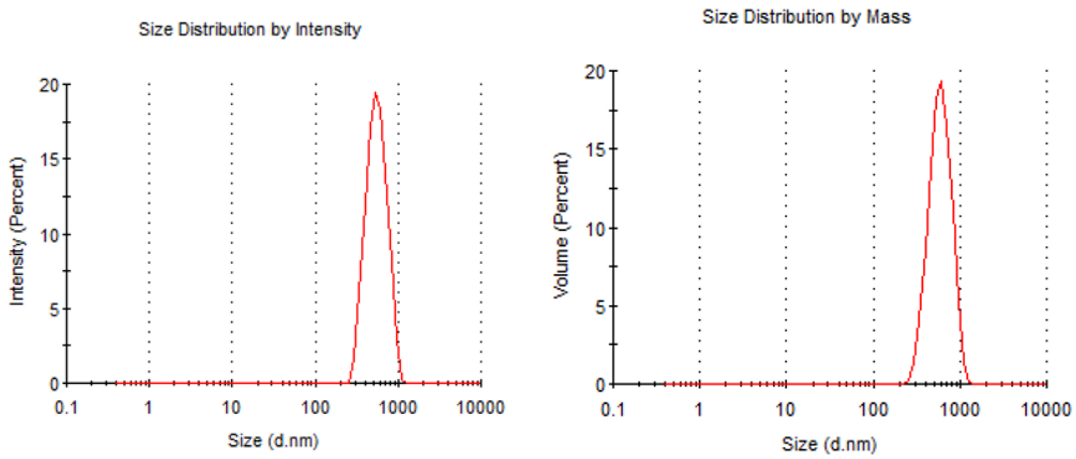


Figure 4.4 Schematic diagram and microscopic images of emulsion behavior (15 ℃, 1.5% salinity, R:D=Rhamnolipids: DOSS). a. R:D=2:8; b. R:D=5:5; c. R:D=8:2



(a) (b)

Figure 4.5 Nanoemulsion size distribution by (a) Intensity and (b) Mass

4.3.2 Influence of Temperature, Salinity, and Surfactant Ratio on Interfacial Tension

The interfacial tension was further measured by a drop shape analyzer in this study. IFT plays an important role in determining the characteristics of surfactant solutions (Bai & McClements, 2016). The effect of temperature and salinity on IFT using different ratios of diluted surfactant solutions was studied and the results are shown in Figure 4.6. As shown in the figure, the lowest IFT (0.59 mN/m) was measured when the temperature was 5 ℃, and salinity was 3 % and R:D=2:8. Moreover, when the temperature was 15 ℃ and other conditions were the same, interfacial tension was also less than 1 mN/m. However, high interfacial tension was almost measured when rhamnolipids and DOSS were not mixed up. It showed when there was only a single surfactant consisting of the solution, interfacial tension measured was relatively higher than the mixed solution did.

Salinity seems to be the most influential factor on IFT. The interfacial tension decreased with the increase of salinity in all groups. This decrease can attribute to the reduction in electrostatic repulsion, and surfactant molecules adsorbed to the oil-water interfaces (Kumar et al., 2021). Effects of temperature and surfactant ratio on interfacial tension were not as significant as salinity made. To elaborate, when there was not any addition of salt in the system, interfacial tension measured at surfactant solution with R:D=0:10 and R:D=2:8 increased along with the temperature. However, with the increase of rhamnolipids in the solution, the tendency was interfacial tension reached the lowest when the temperature was 15 ℃ or the values were very close at 15 ℃ and 25 ℃. The addition of salt made this tendency change since the lowest interfacial tension measured was at low temperature. Besides, the synergistic effect of different anionic surfactant

solutions can lead to a lower interfacial tension was proved.

These effects can be further explained by the hydrophilic-lipophilic deviation (HLD). According to HLD equation, the IFT of mixed anionic surfactants without solvent is related to characteristics of surfactants, salinity, and temperature. The lowest IFT would be measured when the HLD value is zero (Rongsayamanont et al., 2017). With the decrease of IFT, crude oil is more mobile and soluble in the surfactant solution, which is desirable in the soil washing process (Kumar & Mandal, 2018).

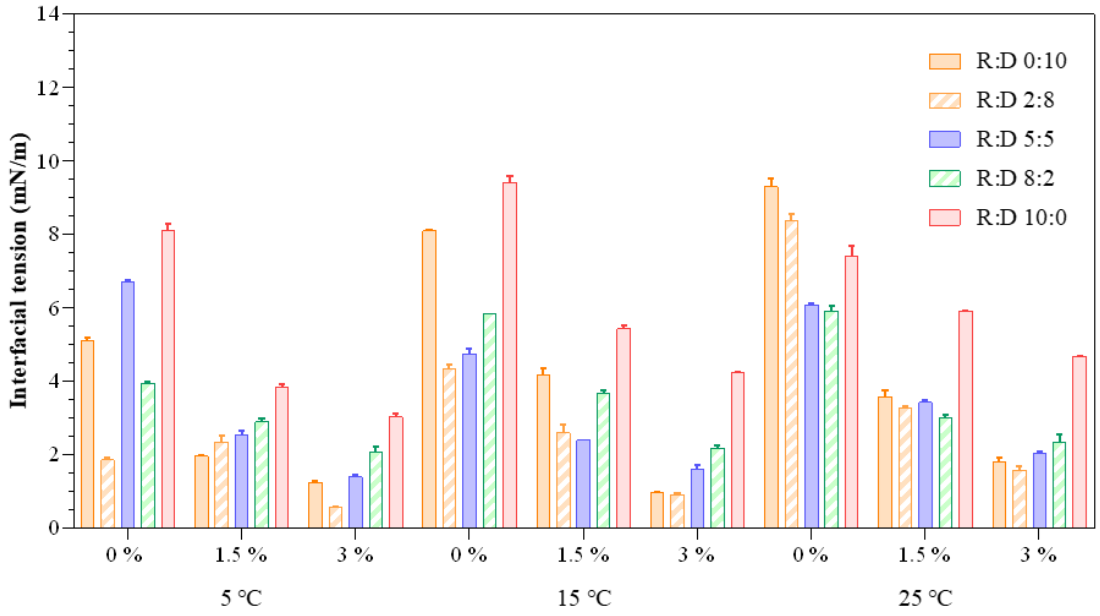


Figure 4.6 The effect of temperature and salinity on interfacial tension using different ratios of surfactant solutions

4.3.3 Oil Removal Efficiency

The performance of surfactant-enhanced crude oil removal was investigated at different treatments and the results are shown in Figure 4.7. According to the results, lower salinities considerably enhanced the crude oil removal efficiency when the ratio of surfactants was R:D= 0:10 and 2:8. However, this tendency was not the same when DOSS was not the primary surfactant added into the solution. Therefore, it proved that rhamnolipids are more potential to be applied in shoreline soil washing because it is less sensitive to saline conditions. Lai et al. (2009) also found that biosurfactants exhibit higher total petroleum hydrocarbon removal efficiency than the chemical surfactants measured. Chaprão et al. (2015) examined the oil removal efficiency of biosurfactants and chemical surfactants (Tween 80 and TX-100). Results showed that in the oil-contaminated sands, biosurfactants removed oil more than Tween 80 and TX-100 did. The highest removal efficiency was most measured when the ratio of R:D=8:2. These results suggest that the participant of one kind of chemical surfactant as co-surfactant in biosurfactant solution may further enhance the removal efficiency.

There were several possible explanations why temperature, salinity, and surfactant ratios can affect the crude oil removal efficiency in soil washing (Peng et al., 2011; Urum & Pekdemir, 2004; Urum et al., 2004; Wei et al., 2015). Synergetic effects can be one of the best explanations. It not only results in a reduction of the total amount of surfactants used but also reduces the cost and the environmental impact (Shi et al., 2015). When temperature increases, the viscosity of oil trapped in soil reduces with the result of increasing the oil-soil mobility and interaction with surfactants. However, the interfacial tension also increased when the temperature increased, which may be hard

to claim the mechanism from the experiment.

Because of the systematic differences between liquid-liquid to liquid-solid, interfacial tension may not be able to reflect the crude oil removal efficiency in many operations of soil washing. To thoroughly examine the relationship between interfacial tension and removal efficiency, SCM is needed for a more comprehensive causal analysis.

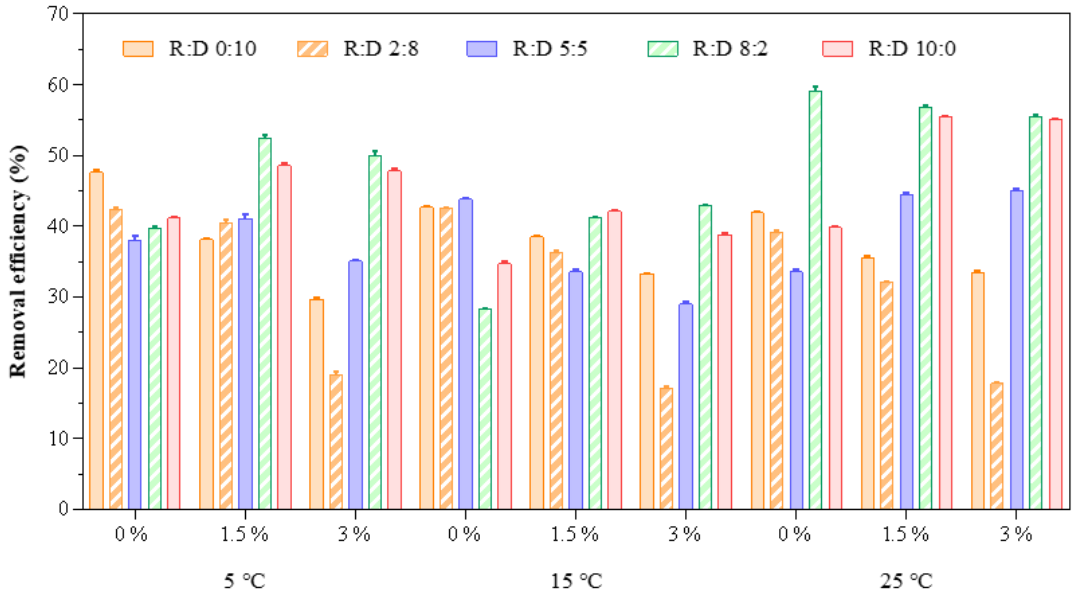


Figure 4.7 The effect of temperature and salinity on crude oil removal efficiency using different ratios of surfactant solutions

4.3.4 Causal Effect of Interfacial Tension on Crude Oil Removal Efficiency

Figure 4.8 is the causal diagram for this study. As shown in the figure, all the causal links among the variables have been reported in existing literature except for the one from interfacial tension to removal efficiency, which is our estimation target.

The SCM was further applied to clearly illustrate the relationships between IFT and removal efficiency. The causal effect of interfacial tension on the removal rate is -0.260, which means from the general perspective, one unit increase in interfacial tension will reduce the removal rate by -0.26%. Note that this estimation can be considered as bias reduced since it corresponds to the strength of the link from “Interfacial Tension” to “Removal Rate”. Considering the span of interfacial tension in the dataset, raising interfacial tension from the lowest value to the highest possible value will reduce the removal rate by 2.30%. Thus, it seems that interfacial tension can only slightly suppress oil removal efficiency. However, when considering the CATE from three different conditions of concern, the result became interesting. We regrouped the five compositions with different ratios of Rhamnolipids and DOSS to two categories: pure substance and mixture, as shown in Table 4. 2 to explore the synergetic effects. Though no significant difference was observed, it seems that the causal effect of interfacial tension under the mixture condition is slightly more stable because it has a lower variance (0.174) than the pure substance group (0.237), indicating a more predictable behavior under the condition. We also checked the CATE under two individual conditions, namely temperature and salinity, in each subgroup. The updated CATE was provided in Table 4. 3. A temperature of 15 ℃ can provide a minimum suppression to the causal effect brought by interfacial tension since, under 5 ℃ or 25 ℃, the causal

effect is negative. For different salinity conditions, CATE under 3 g/L concentration is the only positive treatment effect. It seems that at least within the salinity range given in our study, the treatment effect of interfacial tension under a higher salinity can enhance the removal rate. The aforementioned characteristics are essentially the same in both subgroups.

After the estimation, to test the robustness of the relationships, we applied four kinds of refutation methods to the causal link between interfacial tension and removal rate. Adding Random Common Cause (RCC), Adding Unobserved Common Cause (UCC), Placebo Treatment (PT) and Subset Replacement (SR) were selected as the refuters. RCC and UCC will both add common cases to the dataset to test if the target variable will change significantly. The difference is RCC provides a common cause with random values, and UCC provides a common cause whose values correlate to both the treatment and the target values. PT will replace the chosen treatment variable's value with some independent random values SR will replace the given dataset with a randomly selected subset. The estimated effects are expected to stay as close to the original estimation as possible stable under RCC, UCC and RS, and drop to zero under PT. Estimation under each refutation process is repeated 100 times. The proposed link successfully passed the refutation test, indicating the proposed causal relationship is likely to stand.

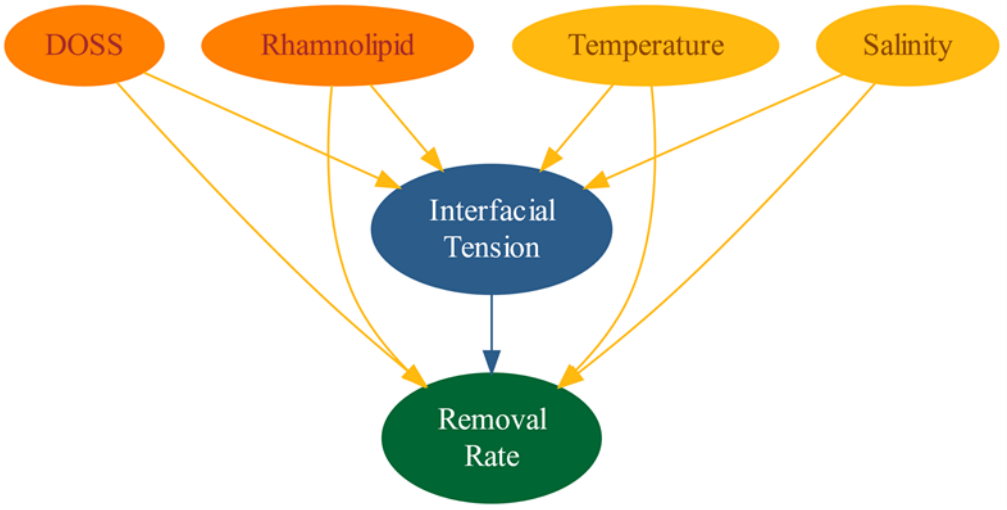


Figure 4.8 A causal diagram including all the variables in the emulsion system. Experimental conditions are in orange and yellow, the mediator variable (Interfacial Tension) is in blue, and the target variable is in green

Table 4. 2 CATE of interfacial tension on removal rate

Type	Salinity(g/L)	Temperature℃	CATE
Pure	0	5	-0.416
		15	-0.400
		25	-0.600
	1.5	5	-0.333
		15	-0.283
		25	-0.520
	3	5	0.533
		15	0.731
		25	0.230
Mixed	0	5	-0.427
		15	-0.420
		25	-0.474
	1.5	5	-0.217
		15	-0.216
		25	-0.301
	3	5	0.388
		15	0.660
		25	0.303

Table 4. 3 CATE of interfacial tension on removal rate

	Salinity (g/L)						Temperature (℃)					
	Pure			Mixture			Pure			Mixture		
	0	1.5	3	0	1.5	3	5	15	25	5	15	25
CATE	-0.470	-0.377	0.498	-0.440	-0.245	0.451	-0.072	0.017	-0.294	-0.085	0.008	-0.157

4.4 Summary

In this study, we found nanoemulsions can be formed using rhamnolipids and DOSS under various temperature and salinity conditions. After conducting soil washing in a shaken flask, the data related to interfacial tension and removal efficiency was collected and quantitatively estimated the causal effect through SCM, a causal inference method. SCM, a graph-based causal inference method was used to estimate the causal effect from interfacial tension to the crude oil removal rate with the aid of a machine learning-based estimator. CATE under multiple conditions was calculated and the result revealed that although interfacial tension has a minor suppression to the removal rate from an overall perspective, under high salinity, its suppression may turn into enhancement and moderate temperature can reinforce such characteristics. The proposed causal links were submitted to four different robustness check methods, and the results indicated that such a causal relationship is likely to hold. Unlike results from other common frameworks for evaluating a factor's contribution to the outcome, the causal estimations above can represent the strength of real-world causal relationships to a certain degree. We hope that our proposed method in the study could provide support to the stakeholders for decision making and application of surfactant-enhanced soil washing.

CHAPTER 5

CONCLUSION AND RECOMMENDATIONS

5.1 Conclusions

This study demonstrated multi-scale biosurfactant production using tuna fish waste as a substrate. Characterization of hydrolyzed fish peptones (degree of enzymatic hydrolysis: 44.2 %) verified that it was a comprehensive substrate. . Results from the bench-scale experiments showed that a fish peptone concentration as high as 20 g/L could be achieved under optimal conditions. The ideal fermentation time was determined to be 48 h in batch-scale experiments. Subsequent pilot scale results achieved a surfactin concentration of 129 mg/L with the foam contained surfactin at an even higher concentration of 274 mg/L. These results improved the understanding of fish waste utilization and provided guidance for the future production of surfactin production on a large scale.

In addition, this study explored the affecting factors in shoreline cleaning and analyzed the causal inference between interfacial tension and removal efficiency. The formation of emulsions generated by rhamnolipids, DOSS and crude oil was investigated. Results showed nanoemulsion could be formed with an average size of 459.4 nm. The ratio of surfactants, temperature, and salinity all had effects on interfacial tension and oil removal efficiency. After further analyzing the causal inference in this study, results showed that interfacial tension had a minor suppression to the removal efficiency from an overall perspective, but interfacial tension can also have an enhancement to removal efficiency when there was high salinity. These results improved the understanding of the synergetic effects in mixed surfactants and further explored the affecting factors in biosurfactant-enhanced soil washing technologies.

5.2 Research Contributions

(1) In this study, the biosurfactant production in multi-scale using fish waste as substrate was proved feasible. The research can help the fishery dispose of the fish waste and the environmental problem of wastewater. In addition, it sheds light on the large-scale production of biosurfactants in a cost-efficient way.

Role: Jiheng Hu worked on this study and acted as the first author of this manuscript under the guidance of Jie Luo, Dr. Zhiwen Zhu, Dr. Xudong Ye, Dr. Peng Zhu, Dr. Baiiyu Zhang and Dr. Bing Chen.)

(2) This study explored the causal inference between interfacial tension and oil removal efficiency in soil washing technologies for the first time. It helps to understand the synergetic effects in mixed surfactants and provides support to the stakeholders for decision making and application of surfactant-enhanced soil washing.

(3) Publication: Hu, J., Luo, J., Zhu, Z., Chen, B., Ye, X., Zhu, P., & Zhang, B. (2021). Multi-scale biosurfactant production by Bacillus subtilis using tuna fish waste as substrate. Catalysts, 11(4), 456.

5.3 Recommendations for Future Work

(1) Other fish waste obtained from the local industries should be further examinedfor theircapacity for use as substrates for the production of biosurfactants.

(2) *Bacillus subtilis* ATCC 55033 was demonstrated to have great potential for the production of biosurfactant compounds. Experimental results to date support the conduct of further studies to evaluate its use for the production of surfactin from tuna

fish waste.

(3) Additional carbon and nitrogen sources can be added to the fish waste-based substrate to optimize the yield. Temperature and pH in the pilot-scale were not explored in this study; future studies to determine optimal parameters for these factors will increase the yield.

(4) The causal inference between interfacial tension and removal efficiency has been studied in this research. This work on causal inference should be expanded to include surface tension.

(5) Studies on the removal efficiency of other crude oils in addition to weathered crude oils should be conducted.

(6) The other potential applications of mixed surfactants can be explored, for instance, the application of dispersants to facilitate enhanced oil recovery.

(7) Future work can also focus on the pilot-scale soil washing using the mixture of rhamnolipids and DOSS.

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APPENDICES

APPENDIX A: Batch-scale fermentation system

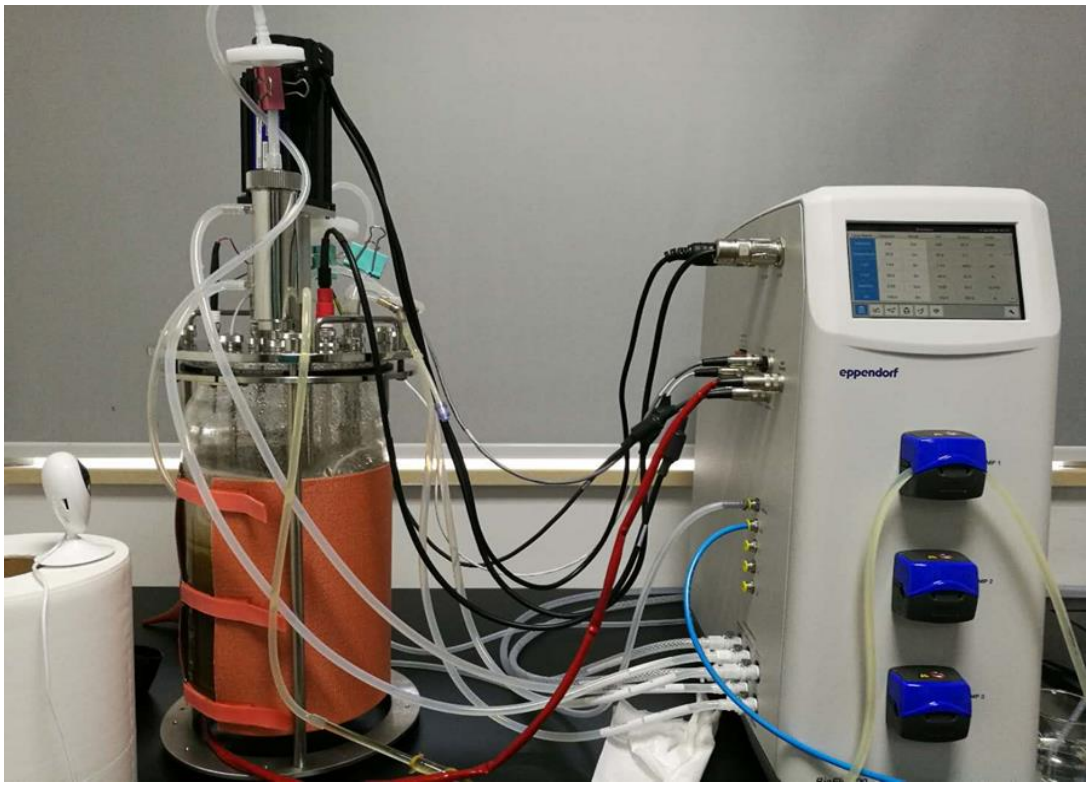

Figure A-1 Fermentation tank in batch-scale

APPENDIX B: Pilot-scale fermentation system

Figure B-1 Freezer dryer in pilot-scale fermentation