Production of Biosurfactants Using Industrial Waste Streams as Substrates

by

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Abstract

Surfactants have a widespread household and industrial applications such as pharmaceutical, cosmetic, petroleum, agriculture, environmental, health care, and food industries. Chemical surfactants can be toxic and non-biodegradable. Biosurfactants which are produced with microorganisms are biodegradable and potential substitute to chemically synthesized surfactants. Large-scale production of biosurfactants is limited due to high costs associated with production. These costs could be decreased by using industrial wastes and by-products as substrates in the growth medium and additionally reduce the environmental impacts of the wastes. Another useful method for achieving economic viability for biosurfactant production is optimization of the cultural conditions such as temperature, pH, agitation, aeration, and medium compositions.

The effectiveness of different local industrial waste streams for biosurfactant production was assessed using some indigenous *Bacillus subtilis*, *Rhodococcus erythropolis* and *Acinetobacter calcoaceticus* strains. The potentiated waste streams regarding appropriateness for microbial growth and biosurfactant production were obtained including brewery waste, glycerol from the conversion of fish oil to biodiesel, fish wastes, waste cooking oil and produced water. The waste streams were treated, centrifuged and filtered through membrane filters. According to the appropriate medium composition for each strain, which was derived from other studies, different mineral salts and trace elements were added to the waste streams. The cultivations were performed in flasks containing 50 mL medium and stirred in a rotary shaker at 30 °C and 200 rpm for several days.

Biosurfactant productivity was evaluated by surface tension and emulsification index measurement. After running numerous tests for biosurfactant production, low production rate sources of carbon were omitted from further studies, and suitable levels or concentrations of effective waste streams for each strain, as well as some other cultivation conditions, were identified. Also, the appropriate composition for the medium was derived through these pre-tests. Eventually, indigenous *Bacillus subtilis* N3-1P strain with the brewery waste and *Acinetobacter calcoaceticus* P1-1A strain with the refined waste cooking oil as the sole carbon sources yielded the best results and were chosen for further studies. Appendix I demonstrates a comprehensive explanation of the initial tests.

The medium and cultivation conditions optimizations were conducted in a series of experiments. Different factors have been chosen to facilitate a higher production rate of the biosurfactant such as carbon source concentration, nitrogen source concentration, NaCl concentration, agitation speed, temperature, and initial pH. Finally, response surface methodologies employing the Design Expert software were used to optimize different parameters. The optimizations were performed separately on the two selected strains with their appropriate waste streams as carbon sources. The predicted responses were validated experimentally under the optimum conditions.

The indigenous *Bacillus subtilis* N3-1P and the brewery waste as carbon source were used to model the biomass growth, biosurfactant production, and substrate utilization by fitting the experimental data to the Logistic, Contois and Luedeking-Piret models using the MATLAB software and regression analysis. The achieved models can be used for simulating the large-scale production of biosurfactants.

The results of these studies confirm that the local brewery waste and the refined waste cooking oil can be used as the sole carbon sources for biosurfactant production by indigenous *Bacillus subtilis* N3-1P and *Acinetobacter calcoaceticus* P1-1A, respectively. Using these sustainable and inexpensive carbon sources reduces costs associated with biosurfactant production and helps to generate an environmentally friendly way for waste treatment and disposal. Also, finding the optimum conditions for different parameters and using the simulated models can decrease the production costs and be useful tools for understanding the cultivation process and scaling up the biosurfactant production. The economically produced biosurfactants would have the ability to be used as an effective method to minimize the impacts of spilled oil in offshore Newfoundland and Labrador.

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Table of Contents

List of Tables

List of Figures

Chapter One: Introduction

Surfactants are amphiphilic molecules, defined as compounds which contain both hydrophilic and hydrophobic groups. The presence of these two groups in the same compound translates to robust partitioning behavior where, depending on the medium, they can act as emulsifiers, foamers, detergents, wetting agents and the key component of dispersants by decreasing the super facial and interfacial tension between solids, liquids, and gases. They have used widely in various household and industrial applications such as pharmaceutical, cosmetic, petroleum, agriculture, environmental, health care, and food industries. The world market of surfactants was 31 billion US-dollars in 2016. It is predicted that it will reach about $40 billion by the end of 2024 (Edsar 2018). Most of the surfactants are synthesized chemically from petroleum compounds. They are not easily biodegradable and can be toxic to the environment. Also, hazardous materials can be produced during the synthesizing of surfactants. These drawbacks have increased the importance of an environmentally friendly substitute for these chemicals during past decades.

Biosurfactants (BS), are biologically produced by microorganisms, and they have some advantages over chemically synthetic surfactants such as low toxicity, biodegradability, ability to be effective at a wide range of pH and temperature, a low critical micelle concentration, and a widespread industrial application such as bioremediation, health care, food and oil processing. However, large-scale production and utilization are limited because of the high cost of production and the narrow knowledge about their interactions with cells and abiotic environment. Cost-effective production of biosurfactants could be achieved by using industrial wastes and by-products as substrate or additives, thereby decreasing expensive medium costs and reducing the environmental impacts of the wastes (Desai and Banat 1997; Mulligan

2005; Makkar et al. 2011). Another useful method for achieving economic viability for biosurfactant production is optimization of the cultural conditions such as temperature, pH, agitation, aeration, and medium compositions (carbon, nitrogen and metal ions). Experimental design techniques like factorial design and response surface methodology (RSM) can be used for this purpose by defining the relationship between inputs and response factors. RSM is used to design experiments, to fit models by multiple regression analysis, to analyze the effect of several experimental variables, and to determine the variable conditions for an optimum response (Khuri and Cornell 1996; Myers and Montgomery 2002).

The objective of this research is to test different local industrial/commercial waste streams as substrates for the growth of various indigenous surfactant producing bacteria and then optimize and model the growth of bacteria and biosurfactant production. This project aims to reduce costs associated with biosurfactant production and reduce environmental impacts of industrial waste streams through utilization of these streams. The generated biosurfactants have the potential to treat oil spills in marine environments, especially the North Atlantic which the used bacteria are indigenous to.

This thesis consists of the first chapter of a brief introduction to the background of biosurfactants as well as the description of research objectives. Chapter two gives a comprehensive literature review of biosurfactant structure and classification, properties and production, factors influencing the production and methods for lowering the production cost, as well as the kinetic modeling of biosurfactant production. Chapter three has been published in the Journal of Environmental

Technology. It presents the optimization of biosurfactant formation by the indigenous *Bacillus subtilis* N3-1P using the brewery waste as the carbon source for the first time. Chapter four evaluates the biosurfactant generation by the local *Acinetobacter calcoaceticus* P1-1A using industrial waste streams and optimizes the biosurfactant production using the waste cooking oil as the sole carbon source. There was no previous work on optimizing the biosurfactant production using the waste cooking oil and an *Acinetobacter calcoaceticus* strain. Chapter five develops kinetic models of biosurfactant production by *Bacillus subtilis* N3-1P using the brewery waste as the sole carbon source. No research has been so far published on modeling biosurfactant production using the brewery waste and a *Bacillus subtilis* strain. Chapter six of this thesis contains a summary and recommendations.

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Chapter Two: Literature review

2.1 Biosurfactant structure and classification

Biosurfactants are structurally diverse and classified based on their chemical structure, molecular weight and the organisms that produce them. The vast majority are anionic or neutral. A typical biosurfactant is made up of a hydrophilic polar head, (such as an amino acid, peptide anion or cation or protein, mono/disaccharides and polysaccharides) and a hydrophobic non-polar tail (such as saturated or unsaturated hydrocarbon chains or fatty acids) (Makkar et al. 2011).

Biosurfactants are divided into low and high molecular weight compounds based on molecular weight. Low molecular weight compounds act by decreasing surface and interfacial tensions efficiently, while high molecular weight compounds are polymer like and “stick” firmly to surfaces. Glycolipids and lipopeptides are examples of low molecular weight biosurfactants. Rhamnolipid has been studied extensively and is produced by *Pseudomonas* species (Maier and Soberon-Chavez 2000; Abdel-Mawgoud et al. 2009; Jadhav et al. 2011). The high molecular weight biosurfactants (biodispersants or bioemulsifiers) are produced from polysaccharides, proteins, lipopolysaccharides, lipoproteins or complex mixtures of these biopolymers or in general, high molecular weight polymeric biosurfactants. They act to stabilize oil emulsions in water rather than decreasing interfacial tension and are efficient emulsifiers with the ability to work at low concentrations (0.01%-0.001%). Bioemulsans are the most studied bioemulsifiers and are produced by various species of *Acinetobacter*. Different biosurfactant-producing microorganisms, mainly bacteria, fungi, and yeasts, have been isolated and characterized from a wide diversity of environments such as soil, seawater, marine sediments and marine sites contaminated

with oil, petroleum or their by-products (Ron et al. 2002; Banat et al. 2010; Smyth et al. 2010). Different types of biosurfactants and their producers are shown in table 2-1.

Table 2-1. Biosurfactants classification, their origin and applications

Biosurfactant group (Type of Biosurfactant)	**Microorganism**	**Application**	**References**
Glycolipids (Rhamnolipids)	*Pseudomonas aeruginosa, Pseudomonas chlororaphis, Serratia rubidaea*	Oil bioremediation, metals removal from soil, antimicrobial properties	Tan et al. 1994; Maier and Soberon-Chavez 2000; Benincasa et al. 2004; Zhang et al. 2005; Chen et al. 2007; Mulligan 2009; Pirollo et al. 2008; Whang et al. 2008; Abdel-Mawgoud et al. 2009; Jadhav et al. 2011
Glycolipids (Trehalolipids)	*Rhodococcus erythropolis, Arthrobacter sp., Nocardia erythropolis, Corynebacterium sp., Mycobacterium sp., Micobacterium tuberculosis*	Oil bioremediation	Peng et al. 2007; Muthusamy et al. 2008; Franzetti et al. 2010
Glycolipids (Sophorolipids)	*Candida bombicola, Candida antartica, Candida petrophilum, Candida botistae, Candida apicola, Candida riodocensis, Candida stellata, Candida bogoriensis, Candida lipolytica*	Environmental applications, enhancement of oil recovery, antimicrobial properties, heavy metal removal	Felse et al. 2007; Whang et al. 2008; Daverey and Pakshirajan 2010
Lipopeptides and lipoproteins (Surfactin)	*Bacillus subtilis, Bacillus amyloliquefaciens, Bacillus pumilus*	Oil remediation, enhanced oil recovery, pesticides biodegradation, heavy metal removal, antimicrobial properties, cosmetics use	Awashti et al. 1999; Abdel-Mawgoud et al. 2008; Sen 2008; Seydlova and Svobodova 2008; Whang et al. 2008; Haddad et al. 2009; Mulligan 2009
Lipopeptides and lipoproteins (Lichenysin)	*Bacillus licheniformis*	Enhancement of oil recovery	Yakimov et al. 1997
Lipopeptides and lipoproteins (Rhodofactin)	*Rhodococcus sp.*	Bioremediation of marine oil pollution	Peng et al. 2008
Lipopeptides and lipoproteins (Viscosin)	*Pseudomonas fluorescens, Leuconostoc mesenteriods*	Bioremediation and biomedicine	Banat et al. 2010; Janek et al. 2010

Lipopeptides and lipoproteins (*subtilisin*)	*Bacillus subtilis*	Antimicrobial properties	Sutyak et al. 2008
Fatty acids/ neutral lipids (corynomycolic acids, spiculisporic acids)	*Penicillium spiculisporum, Corynebacterium lepus, Arthrobacter paraffineus, Talaramyces trachyspermus, Nocardia erythropolis,*	Metal recovery, bioemulsifiers	Gerson and zajic 1978; Hong et al. 1998; Ishigami et al. 2000
Phospholipids	*Acinetobacter sp., Rhodococcus erythropolis*	Bioremediation	Kosaric 2001
Polymeric biosurfactants (Emulsan)	*Acinetobacter calcoaceticus*	Bioemulsifier, enhancement of oil recovery	Zosim et al. 1982; Choi et al. 1996; Johri et al., 2002; Suthar et al. 2008
Polymeric biosurfactants (Alasan)	*Acinetobacter radioresistens*	Bioemulsifier	Navon-Venezia et al. 1995; Barkay et al. 1999
Polymeric biosurfactants (Biodispersan)	*Acinetobacter calcoaceticus*	Limestone dispersion	Rosenberg et al. 1988
Polymeric biosurfactants (Liposan)	*Candida tropicalis Candida lipolytica*	Bioemulsifier	Cirigliano et al. 1985
Polymeric biosurfactants (Mannoprotein)	*Candida tropicalis, Saccharomyces cerevisiae*	Bioemulsifier	Cameron et al. 1988
Particulate biosurfactants (Vesicles and fimbriae)	*Acinetobacter calcoaceticus, Pseudomonas marginilis, Pseudomonas Maltophila*	Bioremediation	Desai and Banat 1997
Particulate biosurfactants (Whole cells)	*Cyanobacteria*	Bio-flocculent	Levy et al. 1990

2.2 Biosurfactant properties

Biosurfactant activities can be evaluated by measuring the surface and interfacial tensions, stabilization of emulsions, critical micelle concentration (CMC), and hydrophilic-lipophilic balance (HLB). There are published data outlining these properties, and these properties are a function of the type of microorganism, substrate, pH, temperature, aeration, etc.

Surface tension is a measure of the surface free energy per unit area required to bring a molecule from the bulk phase to the surface. The surface tension of water without surfactant is 72 mN m^{-1} and with surfactant can decrease to 30 mN m^{-1} (Desai and Banat 1997; Soberon et al. 2011). In addition to surface tension, the interfacial tension can decrease; for example, the interfacial tension between water and n-hexadecane decreases from 40 to 1 mN m^{-1} (Soberon et al. 2011). During the adding of a surfactant, surface tension is reduced until the critical micelle concentration (CMC) is reached. One useful method for evaluating the efficiency of a surfactant is critical micelle concentration measurement. When CMC is low, less biosurfactant is required to reduce surface tension, showing the efficiency of the biosurfactant. Above the CMC, the mechanism shifts and surfactants molecules associate to form micelles, bilayers, and vesicle. The solubility and bioavailability of hydrophobic organic compounds are increased by reducing surface and interfacial tensions which can be achieved by micelle formation (Whang et al. 2008). Biosurfactants usually have the CMCs between 1 to 200 mg L^{-1}, and their molecular mass is between 500 and 1500 Daltons (Lang and Wagner 1987; Mulligan 2005).

An emulsion is created when one liquid phase is dispersed as microscopic droplets in another liquid phase. Emulsifiers can stabilize, and deemulsifiers can destabilize the emulsion. The emulsification activity is tested by the ability of the biosurfactants to generate turbidity due to suspended hydrocarbons in a liquid system. The deemulsification activity is derived by determining the effect of surfactants on a standard emulsion by using a synthetic surfactant (Zajic et al. 1977; Rosenberg 1986).

The hydrophilic-lipophilic balance (HLB) value expresses whether a biosurfactant can form a water-in-oil or an oil-in-water emulsion. Emulsifiers with low HLB are lipophilic and stabilize water-in-oil emulsification, whereas emulsifiers with high HLB have the opposite effect and enhance oil in water solubility (Desai and Banat 1997).

Hydrophobic organic chemicals such as hydrocarbons have low solubility in water resulting in limited bioavailability to microorganisms, especially in cold environment. Accumulation of biosurfactants at surface and interfaces results in the reduction of surface and interfacial forces between dissimilar phases and allows two phases to mix and interact more easily. Also, bioemulsifiers can enhance the apparent water solubility of hydrophobic compounds by stabilizing oil droplets in water. Therefore, biosurfactants can enhance hydrocarbon bioremediation by increasing the surface area of hydrocarbons, leading to an increase in mobility, solubility, and bioavailability of hydrocarbons. Many studies have been done to examine the effect of biosurfactants to enhance hydrocarbon degradation, and they reported high degradation rate (Barkay et al. 1999; Ron et al. 2002; Das et al. 2007; Peng et al. 2008; Whang et al. 2008; Mulligan 2009; Banat et al. 2010; Magdalena et al. 2011). Figure 2-1 shows a typical

biosurfactant at the interface of water and air and oil stabilization by micelle formation.

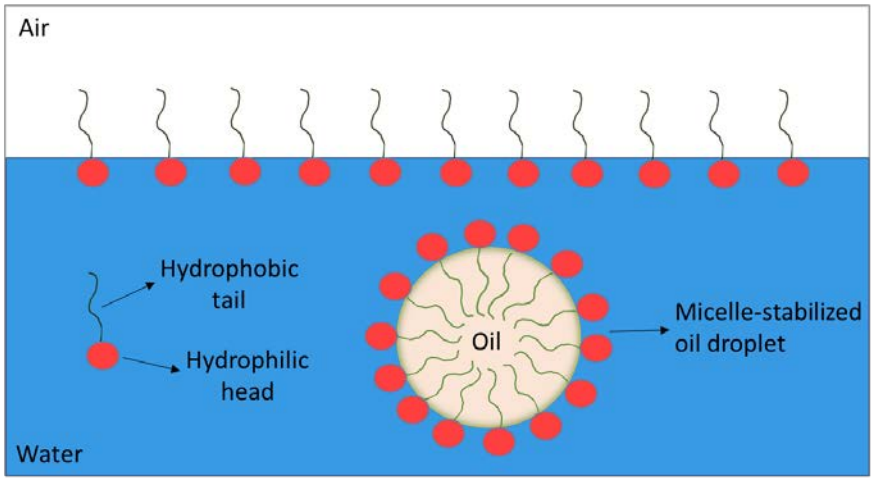

Figure 2-1. Biosurfactant accumulation at the interface of two phases and oil stabilization by micelle formation.

2.3 Biosurfactant production

Although biosurfactants have some advantages over synthetic surfactants, large-scale production and commercialization remain a challenge due to cost. For example, the cost of 95% pure rhamnolipid from AGAE Technologies is around $25000 for 1 kg, while the cost of chemical surfactants is approximately $2.2 for 1 kg. Biosurfactants in the range of 6-11 dollar kg^{-1} would be economical (Makkar et al. 2011). Approaches to lower cost or increase production volume include: (i) development of more effective bioprocess by optimizing the cultural conditions and downstream separation and purification process, (ii) use of low cost or waste substrates, (iii) development and use of overproducing strains (Mukherjee et al. 2006; Marchant and Banat, 2012). There are several publications in the optimization of cultural conditions

and the use of cost-efficient substrates (Joshi et al. 2008; Mukherjee et al. 2008; Pal et al. 2009; Saikia et al. 2012). Experimental design techniques like factorial design and response surface methodology (RSM) which study the relationships between several explanatory variables and one or more response variables are useful in optimizing different parameters on biosurfactant production.

2.3.1 Bioprocess optimization

Cultural conditions such as temperature, pH, agitation, aeration, dilution rate (for continues process), and medium composition and characterization, including carbon, nitrogen and metal ions can affect the production of biosurfactants and the type, cost, quality and quantity of them. There are several studies related to biosurfactant generation and the optimization of process properties (Abdel-Mawgoud et al. 2008; Joshi et al. 2008; Mukherjee et al. 2008; Pal et al. 2009; Najafi et al. 2010; Silva et al. 2010; Ghribi and Ellouze-Chaabouni 2011; Rashmi et al. 2012; Saikia et al. 2012; Luo et al. 2013; Rocha et al. 2014; Hu et al. 2015; Kumar et al. 2015; Ebadipour et al, 2016 ; Eswari et al. 2016; Sellami et al. 2016; Almeida et al. 2017). In order to obtain large quantities of biosurfactants with low cost, it is essential to optimize the process conditions.

2.3.2 Design of experiments

Statistical design of experiment (DOE) has been applied widely to give a better sense of experimental inputs and responses and the interaction between them. The advantages of the factorial design over traditional experiments are reduction of number of experiments, times and materials, elucidating the interaction between

variables, statistical analyses of data, and building a mathematical model. The replication involved in DOE allows an estimate of experimental error, and randomization averages out the effects of extraneous factors and reduces bias and systematic errors.

A simple method for estimating a first-degree polynomial or a linear model is the two-level factorial design which is useful for screening significant variables (explanatory variables which have an impact on the response variable(s)). Analysis of variance (ANOVA) is used to analyze the statistical significance, and the regression analysis is applied for model prediction. A more complicated and practical design, such as Response Surface Methodology (RSM) can be employed to design experiments and estimate a second-degree polynomial model for optimization purposes or when there is curvature in the system, which has linear, interaction, and quadratic terms. RSM allows determining the optimum combination of factors that yield the desired response(s) and predicting the response(s) at different amounts of the variables over the specified levels of interest (Khuri and Cornell 1996; Myers and Montgomery 2002). Central Composite Design (CCD) is a factorial design and has been used in design of experiments in biosurfactant production. The CCD is the most popular class of designs used for fitting second-order models. The total number of tests required for CCD is $2^{k-1} + 2k + n_c$, which includes 2^{k-1} fractional factorial points with its origin at the centre, 2k points fixed axially at a distance α from the centre to generate the quadratic terms, and replicate tests at the centre (n_c); where k is the number of independent variables. Experimental design should include enough replications at the centre point to provide an independent estimate of the experimental error allowing it to be tested for the lack of fit of the model (Khuri and Cornell 1996). For example, for

five variables, the recommended number of tests at the centre is six. Hence the total number of tests required is 32. For statistical calculation, the experimental variables x_i have been coded as X_i as the transformation equation (Eq. (1))

$$X_i = \frac{(X_i - X_0)}{\Delta X_i} \quad (1)$$

where X_i is the dimensionless coded value of the *i*th independent variable, x_i is the uncoded value of the *i*th independent variable, x_0 is the value of x_i at the centre point, and Δx_i is the step change value.

Regression analysis is a general approach to fit the empirical model with the collected response variable data. Once the experiments were performed, the relationship between the independent variables and their responses were fitted to a predictive second-order polynomial equation (Eq. (2)).

$$Y_i = \beta_0 + \sum_{i=1}^{k} \beta_i X_i + \sum_{i=1}^{k} \beta_{ii} X_i^2 + \sum_{i<j}^{k} \sum \beta_{ij} X_i X_j \quad (2)$$

where Y_i is the predicted response, subscripts i and j take values from 1 to k of the number of factors, β_0 is a constant, β_i's are the linear coefficients, β_{ii}'s are the quadratic coefficients, β_{ij}'s are the interaction coefficients, and X_i and X_j are the coded dimensionless values of the investigated variables. It should be noted these techniques are limited in application (i.e. to the specific system) however useful in experimental design to minimize time and costs.

Design Expert software is a convenient method for applying the CCD and calculating the coefficients of the second-order polynomial equation. The adequacy of the model can be evaluated through analysis of variance (ANOVA) and the normal plot of

residuals. The graphical representations of the results are used to describe the effect of the variables on the response(s) and to determine the interactions between the factors.

2.3.3 Cultural medium influence on biosurfactant production

The composition and characteristics of biosurfactants are influenced by the nature of the carbon and nitrogen source as well as the presence of iron, magnesium, manganese, phosphorus, and sulfur in the media.

2.3.3.1 Carbon source

The carbon sources can be divided into three categories; carbohydrate, hydrocarbon and vegetable oils which can be used in combination or individually (Gautam and Tyagi 2006). It is essential to find the best type and amount of carbon source for each strain.

Bacillus subtilis strains can grow on different substrates such as carbohydrate substrates including commercial sugar, molasses, glycerol, milk whey, starch, glucose, sucrose, lactose, mannitol, cassava wastewater, cashew apple juice, potato process effluent; vegetable oils such as soybean oil and waste frying oils, and hydrocarbons such as waste lubricating oil, crude petroleum, oil hydrocarbons and oily sludge (Mercade et al. 1996; Kim et al. 1997; Makkar and Cameotra 1997; Nitschke et al. 2004; Reis et al. 2004; Abdel-Mawgoud et al. 2008; Das et al. 2009; Rocha et al. 2009; Faria et al. 2011; Ghribi and Ellouze-Chaabouni 2011; Vedaraman and Venkatesh 2011; Pemmaraju et al. 2012).

According to Kim et al. (1997), a high yield of C9-BS was obtained from a culture of *Bacillus subtilis* C9, using a carbohydrate substrate, while a hydrocarbon substrate

inhibited the production of the biosurfactant. Abdel-Mawgoud et al. (2008) found vegetable oils (soybean oil and olive oil), hydrocarbon (hexadecane and paraffin oil) and lactose and galactose have an inhibitory effect on bacterial growth by *Bacillus subtilis* isolate BS5, and glucose and molasses are the best carbon sources. Ghribi and Ellouze-Chaabouni (2011) claimed the addition of hydrocarbons into the culture medium enhanced biosurfactant formation by *Bacillus subtilis* SPB1 strain. However, Cooper et al. (1981) found that although the addition of hexadecane increased the biomass, it inhibited the surfactin production by *Bacillus subtilis* ATCC 21332.

Both water-soluble (glucose, glycerol, fructose, whey waste, cassava wastewater and molasses), and water-insoluble carbon sources (vegetable oils, soap stock and hydrocarbons) have been utilized for production of rhamnolipids (Syldatk et al. 1985; Dubey and Juwarkar 2001; Santa Anna et al. 2001; Benincasa et al. 2002; Zhang et al. 2005; Raza et al. 2006; Chen et al. 2007). However, hydrophobic carbon sources such as vegetable oils are especially effective at promoting the production of rhamnolipids. It should be noted that in some studies (Koch et al. 1991; Zhang et al. 2005) no rhamnolipid production was observed when hexadecane, individual alkanes with chain lengths ranging from C12 to C19 and crude oil were used as carbon sources; but the addition of small amounts of purified rhamnolipids facilitates the rhamnolipid production.

Acinetobacter calcoaceticus strains can grow and produce biosurfactants on a variety of carbon sources such as aliphatic hydrocarbons, ethanol, acetate, whey and naphthalene (Choi et al., 1996; Johri et al., 2002; Phetrong et al., 2008; Zhao and Wong, 2009; Amoabediny et al., 2010).

Rhodococcus erythropolis strains can grow on hydrophobic substrates such as liquid paraffins, hexadecane, n-alkanes, kerosene, diesel fuel, residual sunflower frying oil and waste lubricating oil, and hydrophilic substrates such as glycerol, ethanol, glucose, molasses, sucrose, sorbitol and mannitol (Kretschmer et al. 1982; Pirog et al. 2004; Peng et al. 2007; Gogotov and Khodakov 2008; Sadouk et al. 2008; Pal et al. 2009). When grown on hydrophilic substrates, the strain produces mainly the emulsifier; on hydrophobic substrates, substances with surface-active properties are synthesized.

These studies indicate that different microbes respond differently to the carbon sources, demonstrating the available carbon source has great relevance to the type of biosurfactant produced (Desai and Banat 1997; Saharan et al. 2011).

2.3.3.2 Nitrogen source

Nitrogen is essential for building the proteins required for microbial growth. Several sources of nitrogen have been used for biosurfactant production, such as urea, peptone, ammonium sulfate, ammonium nitrate, sodium nitrate, meat extract, and malt extract. Yeast extract is widely used as a nitrogen source for generating biosurfactants, but according to the nature of microorganisms and the culture medium, a different amount is required (Saharan et al. 2011).

Ammonium salts and urea are the best sources of inorganic nitrogen sources for biosurfactant production by *Arthrobacter paraffineus* ATCC 19558 (Duvnjak et al. 1983), while nitrate is preferred by *Pseudomonas aeruginosa* 44T (Robert et al. 1989) and *Rhodococcus* sp. ST-5 (Abu-Ruwaida et al. 1991).

The production of biosurfactants typically occurs when the nitrogen source is limited during the stationary phase of cell growth. The increase due to the nitrogen limitation occurred in *Rhodococcus* sp. ST-5 (Abu-Ruwaida et al. 1991) *Pseudomonas aeruginosa* CFTR-6 (Venkata Ramana and Karanth 1989), *Candida tropicalis* IIP-4 (Singh et al. 1990), and *Nocardia* strain SFC-D (Kosaric et al. 1990). Another study showed that nitrogen limitation changes the composition of the biosurfactant produced by *Pseudomonas* spec. DSM 2874 (Syldatk et al. 1985). The C:N ratio strongly influence total rhamnolipid productivity. Maximum rhamnolipid production occurred at C:N ratio of 16:1 to 18:1 and there was no surfactant production below C:N ratio of 11:1 (Guerra-Santos et al. 1986). C:N ratio of 22.8 led to the greatest production of rhamnolipids in a study by Santa Anna et al. (2001). Another study indicates that it is the absolute quantity of nitrogen and not its relative concentration that appears to be important for the optimum biosurfactant generation (Hommel et al. 1987; Desai and Banat 1997). The production of biosurfactant by *Pseudomonas fluorescens* Migula 1895-DSMZ using olive oil as a substrate and ammonium chloride, sodium nitrate and ammonium as nitrogen sources was studied by Abouseoud et al. (2008), and the best result was obtained when ammonium nitrate was used, with a C:N ratio of 10. Heryani and Putra (2017) found the optimum C:N ratio for biosurfactant production by *Bacillus* species BMN 14 is 12.4, reaching the surface tension of 27 mN m^{-1}.

2.3.3.3 Metal ion concentration

Metals are important cofactors in enzymes and therefore affect the production of biosurfactants. The overproduction and modified properties of surfactin biosurfactant

occur in the presence of Fe^{2+} and inorganic cations in mineral salt medium (Thimon et al. 1992).

In a study of surfactin production by *Bacillus subtilis* ATCC 21332 by Cooper et al. (1981), only three salts, $MnSO_4$, $FeSO_4$, and $Fe_2(SO_4)_3$ were positive for production. Other salts such as $MgSO_4$, $CaCl_2$, Na_2HPO_4, KH_2PO_4, $NaNO_3$, $ZrOCl_2$, UO_2-$(C_2H_3O_2)_2$, or $VOSO_4$ did not affect either biomass or surfactin concentration. $ZnSO_4$ suppressed the growth of *Bacillus subtilis* and several others, such as $CuSO_4$, $NiSO_4$, $CoSO_4$, and $A1_2(SO_4)_3$ completely inhibited growth. Sousa et al. (2011) found that the high salt concentration may be the cause of the lower production of rhamnolipids when using the crude glycerin (derived from the transesterification process) as a substrate by a new strain of *Pseudomonas aeruginosa.*

In a study by Wei et al. (2007), trace element composition was optimized to improve surfactin production from *Bacillus subtilis* ATCC 21332. Mg^{2+}, K^+, Mn^{2+}, and Fe^{2+} were considered, and Mg^{2+}, K^+, and interaction of Mg^{2+} and K^+ were significant, suggesting that Mg^{2+} and K^{2+} ions were more critical factors when a certain level of Fe^{2+} or Mn^{2+} was provided in the medium.

2.3.4 Environmental factors influence on biosurfactant production

Environmental factors, such as pH, and growth conditions are extremely critical in biosurfactant production.

2.3.4.1 pH

The impact of acidity was studied in the production of glycolipids by *Candida Antarctica* ATCC 20509 and *Candida apicola* ATCC 96134. The production of

glycolipids was maximum when pH was kept at 5.5 and when pH was not controlled, the synthesis of the biosurfactant decreased (Bednarski et al. 2004).

The effect of pH in the generation of biosurfactants by *Candida antarctica* T-34 was investigated using phosphate buffer with pH values in the range of 4 to 8, and all conditions used resulted in a reduction of biosurfactant yield when compared to neutral conditions (distilled water) (Kitamoto et al. 2001). The effect of initial pH in the production of a biosurfactant by *Yarrowia lipolytica* NCIM 3589 showed that the best synthesis occurred at a pH of 8.0, which is the natural pH of seawater (Zinjarde and Pant 2002).

The pH of the culture also is important in the sophorolipid production by a *Torulopsis bombicola* strain (Gobbert et al. 1984). Production of rhamnolipid by *Pseudomonas aeruginosa* DSM 2659 was high in a pH range from 6.0 to 6.5 and reduced sharply above pH 7.0 (Guerra-Santos et al. 1986).

2.3.4.2 Temperature

Most studies suggest that the optimum biosurfactant production occurs over a temperature range of 25 to 30˚C and there is an optimal temperature for each microbial strain. Higher temperatures increase the enzymatic activity and as several enzymes have essential roles in biosurfactant formation and microbial growth, can increase the rate of metabolism and reproduction (Zhang et al. 2015).

2.3.4.3 Aeration and agitation

Agitation of the nutrient broth enhances mass transfer; however, high agitation speed may result in lower yield due to shear effects.

The optimized air flow rate and dissolved oxygen levels of biosurfactant generated by *Candida antarctica* was determined to be 1 vvm and 50% respectively (Adamczak and Bednarski 2000).

2.3.5 Cost-efficient and waste derived substrates for biosurfactant production

Utilization of waste has not only environmental benefits regarding resource recovery and a decrease in the volume and toxicity of waste streams but also has economic benefits through possibly lower biosurfactant production costs. Various renewable sources for biosurfactant generation have been studied, including agroindustrial products, industrial wastes from frying oils, oil refinery wastes, molasses, starch-rich wastes, cassava wastewater, potato waste and distilled grape marc, and these substrates have been proven to be effective for biosurfactant formation (e.g. Fox and Bala 2000; Makkar and Cameotra 2002; Maneerat 2005; Nitschke and Pastore 2006; Rivera et al. 2007; Sobrinho et al. 2008; Makkar et al. 2011; Almeida et al. 2017; Das and Kumar 2018).

2.3.5.1 Vegetable oils and oil wastes

Vegetable oils are lipid carbon sources and contain saturated and/or unsaturated fatty acids (C_{16}-C_{18}). A variety of vegetable oils of olive, grapeseed, canola, corn, palm, coconut, sunflower, rapeseed, fish and soybean oil have been used in biosurfactant production (Makkar and Cameotra 1999; Mukherjee et al. 2006; Makkar et al. 2011; Saharan et al. 2011; Chooklin et al. 2013; Radzuan et al. 2017). The processing of crops and other plants produce wastes rich in nutrients, and their disposal is a challenge due to the high nutrient levels and volumes of waste (Haba et al. 2000).

There are several studies related to biosurfactant formation using oil wastes from vegetable oil refineries and the food industry, industrial oil wastes such as soap-stock, tallow, marine oils, waste oils generated from domestic uses, lard and free fatty acids. Ideally, one would take advantage of local industrial wastes to minimize costs related to transportation; however, this must be balanced by the target bacteria and biosurfactants.

2.3.5.2 Waste frying oil

Frying oils are produced in large quantities in the food industries and are effective and inexpensive raw materials for biosurfactant production. Cooking oil changes its composition and contains more polar compounds after being used, depending on the food, the type of frying and the number of times it has been used. Haba et al. (2000) tested waste olive and sunflower cooking oil as carbon sources for biosurfactant generation with 36 isolated bacteria. The most important differences between the composition of used and standard unused oil are the presence of fatty acids of low chain length (<C10), myristic acid and lauric acid in the used oil. Most of the *Pseudomonas* strains tested showed good results with both oils with olive oil performing better regarding production. Most of the *Pseudomonas* strains reduced the surface tension to 34-36 mN m^{-1}, and the production of rhamnolipid was 2.7 g L^{-1} with a yield of 0.34 g g^{-1}. Rhamnolipid production by *Pseudomonas aeruginosa* ATCC 9027 was also studied by Luo et al. (2013) with waste frying oil as the sole carbon source using response surface method. Maximum rhamnolipid production of 6.6 g L^{-1} was achieved at the optimal condition of temperature, NO^{3-} and Mg^{2+}. Feeding the oil in two batches enhanced rhamnolipid production to 8.5 g L^{-1} after 72

h. Biosurfactant production by *Pseudomonas aeruginosa* OG1 was optimized yielding 13.31 g L^{-1} of the product with 52 g L^{-1} of waste frying oil as the source of carbon (Ozdal et al. 2017). Kitchen waste oil was used for producing biosurfactant by a strain of *Pseudomonas aeruginosa* and reached to better results than glucose, molasses, glycerol and rapeseed oil as carbon sources with 2.47 g L^{-1} of generated biosurfactant (Chen et al. 2018).

A biosurfactant producing strain was able to generate 3.7 g L^{-1} of biosurfactant when 2% (v v^{-1}) of waste cooking oil was used as the source of carbon (Yanez-Ocampo et al. 2017). Vedaraman and Venkatesh (2011) studied the production of surfactin by *Bacillus subtilis* MTCC 2423 using waste frying oils. Surface tension decreased by 56.32%, 48.5% and 46.1% with glucose, waste frying sunflower oil and waste frying rice bran oil, respectively, with a biomass formation of 4.36 g L^{-1}, 3.67 g L^{-1} and 4.67 g L^{-1} and product yields (g product g $substrate^{-1}$) of 2.1%, 1.49% and 1.1%. Sadouk et al. (2008), in an approach to reducing the cost of production of glycolipids by *Rhodococcus erythropolis* 16 LM.USTHB utilized residual sunflower frying oil as the carbon source. With substrate concentration of 3%, they could achieve surface tension of 31.9 mN m^{-1} and emulsion index of 63% by the produced biosurfactant.

2.3.5.3 Soap stock

Soap stock is produced during the extraction of oil from oil seeds by hexane and other chemicals and has been used to produce emulsan and biodispersant. *Pseudomonas aeruginosa* LBI, isolated from petroleum contaminated soil, produced surface-active rhamnolipids biosurfactant by batch fermentation in a mineral salt medium with soapstock as the sole carbon source (Benincasa et al. 2002). The biosurfactant

production was increased by limiting nitrogen, with a maximum rhamnolipids concentration of 15.9 g L^{-1} and stable emulsions.

2.3.5.4 Glycerol

Sousa et al. (2011) examined the production of rhamnolipids by *Pseudomonas aeruginosa* MSIC02 using glycerol/glycerin (the by-product of biodiesel production). Among the different carbon sources, the highest rhamnolipid concentration was achieved when hydrolyzed glycerin was used. Glycerin from biodiesel production can be a heterogeneous mixture, including glycerol, the original grease (esters), triglycerides, fatty acids and soaps, alcohol (ethanol or methanol) and hydroxides. The glycerol was the predominant carbon source present in hydrolyzed glycerin and had better potential for rhamnolipid production compared to other sources (such as triglycerides, fatty acids, and esters of fatty acids). Carbon (as glycerol) and nitrogen concentrations and cultivation conditions for biosurfactant generation by *Pseudomonas aeruginosa* UCP0992 were optimized by Silva et al. (2010). A biosurfactant concentration of 8.0 g L^{-1} was achieved after 96 h, and the medium surface tension was reduced to 27.4 mN m^{-1}. Biosurfactant formation by two strains of *Pseudomonas* species was evaluated using carbon sources such as different vegetable oils (corn and sunflower oils) and glycerol. The produced biosurfactants reduced surface tension of all culture media tested, although it was more effective when glycerol was used (Santos et al. 2010). The potential biodegradation of crude oil was examined by Zhang et al. (2005) with a strain of *Pseudomonas aeruginosa*, which produced 15.4 g L^{-1} rhamnolipids using glycerol and showed better performance when compared to glucose, vegetable oil, and liquid paraffin.

The production of biosurfactant by *Rhodococcus erythropolis* ATCC 4277 was examined by Ciapina et al. (2006), with glycerol. Approximately 1.7 g L^{-1} of biosurfactant was produced after 51 h of cultivation with surface and interfacial tension values of 43 and 15 mN m^{-1}, respectively. The potential of utilizing different carbon substrates like sucrose, lactose, starch, sodium gluconate, glycerol, sodium acetate, sodium carbonate, sodium oxalate, lactic acid, acetic acid, trisodium citrate, yeast extract and beef extract for the generation of biosurfactant by a strain of *Bacillus circulans* was evaluated by Das et al. (2009). The production of the crude biosurfactant was found to be highest with glycerol (2.9 g L^{-1}). Faria et al. (2011) examined the production of surfactin by *Bacillus subtilis* LSFM-05 using raw glycerol as the sole carbon source, with the surface tension reduction to 29.5 from 47.5 mN m^{-1}. Sousa et al. (2012) also studied the production of biosurfactants by several *Bacillus subtilis* strains using glycerol from the biodiesel synthesis and reached to the surface tension of 27.1 mN m^{-1} with *Bacillus subtilis* LAMI009.

2.3.5.5 Starch based substrates

The potato processing industry is a major source of low-cost starchy substrate. The waste is rich in carbon (in the form of starch and sugars), nitrogen and sulfur, inorganic minerals, trace elements and vitamins (Saharan et al. 2011). Fox and Bala (2000) evaluated potato substrates for surfactant production by a *Bacillus subtilis* ATCC 21332 strain. Surface tension of 28.3 mN m^{-1} was achieved with the simulated solid potato medium. Thompson et al. (2000) studied the effect of high solids (HS) and low solids (LS) potato effluents as substrates for surfactin production by *Bacillus subtilis* 21332. Jain et al. (2013) used potato peel powder, corn powder, Madhuca

indica, and sugarcane bagasse as carbon sources for biosurfactant production by *Klebsiella* species strain RJ-03 with the best yield of 15.40 g L^{-1} by corn powder.

2.3.5.6 Molasses and sugars

Molasses is produced in the sugar industry as a co-product and is a rich source of carbon. Molasses and corn-steep liquor were used as the primary carbon and nitrogen source to produce rhamnolipid biosurfactant using *Pseudomonas aeruginosa* GS3 (Patel and Desai 1997). The biosurfactant yield reached at maximum value with a combination of 7% (v v^{-1}) molasses and 0.5% (v v^{-1}) corn-steep liquor waste. Aparna et al. (2012) studied *Pseudomonas* species 2B for biosurfactant production. Five different low-cost carbon substrates, including molasses, whey, glycerol, orange peelings, and coconut oil cake were evaluated for biosurfactant formation. The maximum generated biosurfactant (4.97 g L^{-1}) occurred at 96 h when the cells were grown on modified medium containing 1% (v v^{-1}) molasses. Molasses was used to produce biosurfactant by some *Bacillus* species, and acceptable results were achieved (Nitschke et al. 2004; Reis et al. 2004).

Soy molasses which contain high fermentable carbohydrate was used to produce sophorolipids by *Candida bombicola* ATCC 22214 with the yield of 21 g L^{-1} (Solaiman et al. 2004). The same group has also shown that soy molasses can act as the carbon and nitrogen source with the yields of 53 g L^{-1} (Solaiman et al. 2007).

Cassava wastewater is also a carbohydrate-rich residue produced in large amounts during the preparation of cassava. Nitschke and Pastore (2006) used this waste for surfactin production by *Bacillus subtilis* LB5a. Cassava wastewater has also been

used as a substrate for the simultaneous production of rhamnolipids and poly hydroxyl alkanoates using *Pseudomonas aeruginosa* L2-1 by Costa et al. (2009).

The cashew apple juice has been used for biosurfactant production and optimization by *Pseudomonas aeruginosa* MSIC02, and surface tension of 28 mN m^{-1} was achieved (Rocha et al. 2014). Biosurfactant (6.9 g L^{-1}) was generated by *Yarrowia lipolytica* IMUFRJ 50682 and clarified cashew apple juice (CCAJ) by Fontes et al. (2012). Pineapple juice used as an alternative carbon source by *Pseudomonas fluorescens* MFS03 with the yields of 9.43 g L^{-1} (Govindammal and Parthasarathi 2013).

2.3.5.7 Dairy industry whey

Whey is a by-product of cheese production, and a large amount of whey from the dairy industry is disposed of through the effluent treatment systems. Whey contains valuable nutrients such as lactose and protein (Saharan et al. 2011). Dubey and Juwarkar (2001) investigated the biosurfactant production from a synthetic medium and industrial wastes, such as distillery and whey wastes to isolate *Pseudomonas aeruginosa* BS2 strain and the generated biosurfactants effectively reduced the surface tension to 27 mN m^{-1}. Daverey and Pakshirajan (2010) studied the production of sophorolipids by the yeast *Candida bombicola* NRRL Y-17069 on a medium containing mixed hydrophilic substrate (deproteinized whey and glucose), yeast extract and oleic acid. A yield of 34 g L^{-1} was achieved under experimental conditions. Medium containing agro-industrial wastes whey and corn steep liquor were used to produce biosurfactant by *Candida glabrata* UCP 1556 reaching to the surface tension of 28.8 mN m^{-1} (Lima et al. 2017). Milk whey has also been used as a

substrate for biosurfactant formation by some *Bacillus* species, but the other examined sources, including molasses and cassava flour wastewater, have shown better results (Nitschke et al. 2004)

2.3.5.8 Animal fat

Animal fat was used for the production of sophorolipids biosurfactant using yeast *Candida bombicol*a ATCC 22214 by Deshpande and Daniels (1995). The growth was poor when fat was provided as the sole carbon source, but a mixture of glucose and fat leads to the highest level of growth. In another study animal fat with corn steep liquor and glucose used as a low-cost substrate for glycolipid production by the yeast *Candida lipolytica* UCP0988 and a surface tension of 28 mN m^{-1} was achieved after 6 days (Santos et al. 2013).

2.3.5.9 Hydrocarbons, diesel oil and Lubricating (lube) oils

In a study by Pirollo et al. (2008) the *Pseudomonas aeruginosa* LBI isolated from hydrocarbon-contaminated soil was examined as a biosurfactant producer. The strain was able to produce biosurfactant and grow in all the carbon sources under study, including kerosene, diesel oil, crude oil and oil sludge from the bottom of a storage tank, except benzene and toluene. The highest quantities (9.9 g L^{-1}) of biosurfactant were generated when diesel oil was used at 30% (w v^{-1}). The biosurfactant could emulsify all the hydrocarbons tested. Raza et al. (2006) also investigated the production of biosurfactant using different hydrocarbon substrates such as *n*-hexadecane, paraffin oil and kerosene oil by *Pseudomonas aeruginosa* EBN-8. The surface tension decreased to 29 mN m^{-1} when the carbon sources were paraffin oil and *n*-hexadecane.

Peng et al. (2007) isolated a biosurfactant-producing bacterium by *Rhodococcus erythropolis* 3C-9 strain and found that apart from n-octane, all tested n-alkanes (from C_5 to C_{36}) can be used as the carbon sources. The use of C_{14}-C_{36} n-alkanes, resulted in good growth, while slight growth occurred when C_5, C_6, C_7 and C_9 n-alkanes were used. Another study of biosurfactant formation by *Rhodococcus erythropolis* SH-5 using glucose, molasses, sucrose, ethanol, alkanes (kerosene or diesel fuel), and oil as carbon sources, achieved the highest biosurfactant yield by 2% kerosene (Gogotov and Khodakov 2008). The nutritional requirements and growth characteristics of a biosurfactant produced by *Rhodococcus* species ST-5 was examined when carbon sources were glucose, kerosene, yeast extract, n-paraffin, peptone, and tetradecane. Hydrocarbons, especially n-paraffin resulted in the best surface tension and biosurfactant yield. The optimum nitrogen source between NH_4OH, $NaNO_3$ and $(NH_4)_2SO_4$ was sodium nitrate, and best C:N ratio was found to be 22 (Abu-Ruwaida et al. 1991).

The efficiency of *Bacillus subtilis* DM-04 and *Pseudomonas aeruginosa* M and NM strains in utilizing crude petroleum oil hydrocarbons as carbon source was compared by Das et al. (2007). These bacterial strains could degrade crude petroleum oil hydrocarbons as the sole source of carbon and energy, while *Pseudomonas aeruginosa* strains were more efficient than *Bacillus subtilis* strain in utilizing the TPH. The degradation of N-alkanes (C_{14}-C_{30}) was favored over PAHs present in crude petroleum-oil by all the bacteria. Pemmaraju et al. (2012) examined the biosurfactant production by *Bacillus subtilis* DSVP23 utilizing aliphatic, aromatic, and polar components from the oily sludge as the sole source of carbon and energy.

Maximum biosurfactant generation (6.9 g L^{-1}) was achieved after 5 days of cultivation.

Waste or used lubricating (lube) oils are a major environmental problem. The waste oil can bind to organic matter, mineral particles and organisms in the environment (Shale et al. 1989). Mercade et al. (1996) investigated the screening and selection of microorganisms capable of using waste lube oil as the substrate for generating biosurfactants and 10% of the isolated strains produced biosurfactants (*Rhodococcus* and *Bacillus* species).

These studies verify the ability of the biosurfactant-producing microorganisms to generate biosurfactants on different waste streams as substrates, which can decrease the cost of the production.

2.4 Product recovery

Purification and downstream processing costs can account for 60% of the total production costs (Saharan et al. 2011). Table 2-2 shows several processes for improving the recovery of biosurfactants (Gautam and Tyagi 2006). Recovery processes are based on biosurfactant properties such as their surface activity or their ability to form micelles and/or vesicles (Saharan et al. 2011). The most widely used processes are extractions with various solvents, including chloroform-methanol, dichloromethane-methanol, butanol, ethyl acetate, pentane, hexane, acetic acid, ether, etc. Less costly and less toxic solvents such as methyl tertiary-butyl ether (MTBE) can reduce the recovery expenses substantially and minimize the environmental hazards (Desai and Banat 1997).

Table 2-2. Different techniques for improving the recovery of biosurfactants

Mode of process	Technique
Batch	Ammonium sulfate precipitation Acetone precipitation Acid precipitation Solvent extraction Crystallization
Continuous	Centrifugation Adsorption Foam separation and precipitation Tangential flow filtration Diafiltration and precipitation Membrane Ultrafiltration

2.5 Kinetic modeling

Kinetic models can be used to correlate the microbial growth with the substrate concentration and product formation of biotechnological processes.

Typically, when a microbial culture grows in a batch medium, the cell density will change with time through four distinct stages as is illustrated in figure 2-2: lag phase, logarithmic (exponential) growth phase, stationary phase, and death phase. In lag phase, the population is adjusting to the environment. In the log or exponential phase, the cells are rapidly growing and dividing. Stationary phase occurs due to nutrient depletion and/or formation of toxic or inhibitory compounds. In Death phase, cells lose their ability to reproduce and die.

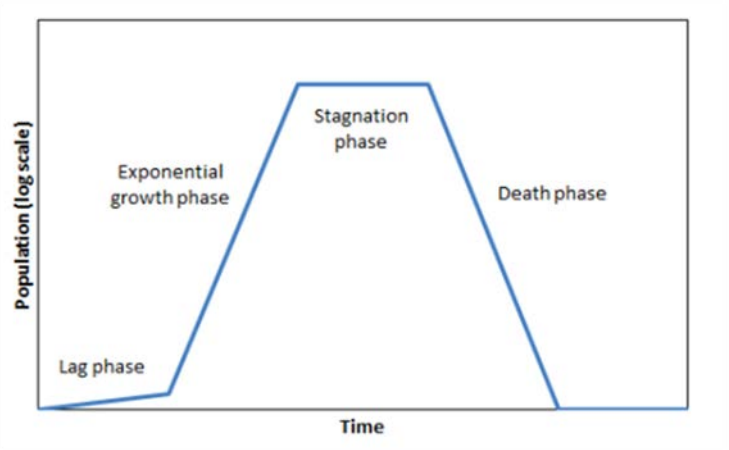

Figure 2-2. Typical growth curve for a microbial culture

2.5.1 Microbial growth kinetics

The Malthusian model or exponential law (Eq. (3)) is a simple model which shows the relation of biomass production concerning time in exponential phase (Malthus 1830).

$$\frac{dX}{dt} = \mu X \tag{3}$$

where, X is the cell mass concentration (g L^{-1}), t is the time (hr), and μ is the specific growth rate (hr^{-1}) which shows the ability of a microbial population to grow. Integrated form of Eq. (3) is Eq. (4).

$$\ln \frac{X}{X_0} = \mu t \tag{4}$$

where, X_0 is the initial biomass concentration.

The other useful model which relates the biomass concentration and the time in both exponential and stationary phases is the logistic model as expressed in Eq. (5) (Verhulst 1838).

$$\frac{dX}{dt} = \mu_{max} X \left(1 - \frac{X}{X_{max}}\right) \quad (5)$$

where μ_{max} is the maximum specific growth rate coefficient (hr^{-1}), and X_{max} is the carrying capacity or the maximum concentration of cells ($g\ L^{-1}$). By integrating Eq. (5), Eq. (6) yields:

$$X = \frac{X_0 e^{(\mu_{max}\, t)}}{1 - \frac{X_0}{X_{max}}\left(1 - e^{(\mu_{max} t)}\right)} \quad (6)$$

Also, some kinetic models have been developed to describe the relation between the cell growth and substrate concentration including Monod, Teissier, Contois, and Mosser (Teissier 1936; Monod 1949; Moser 1958; Contois 1959).

The most commonly used model is developed by Monod (Eq. (7)) which relates the growth rate to the concentration of a single source of energy which is called growth-limiting substrate.

$$\mu = \mu_{max} \frac{S}{K_S + S} \quad (7)$$

where, S is the substrate concentration ($g\ L^{-1}$), and K_s is the half saturation constant for that substrate, or the substrate concentration at which one-half the maximum specific growth rate is achieved.

The Contois model which is a derivation from the Monod equation describes by Eq. (8) (Contois 1959).

$$\mu = \mu_{max} \frac{S}{KX + S} \quad (8)$$

where K is the Contois constant. As for each organism, the coefficients vary with environmental conditions, they have to be estimated for each strain and under constant conditions of temperature, pressure and medium composition.

When there are double limiting substrates, a double Monod model can be considered as Eq. (9) (McGee et al. 1972):

$$\mu = \mu_{max} \frac{S_1}{K_{S_1} + S_1} \frac{S_2}{K_{S_2} + S_2} \quad (9)$$

where 1 and 2 represent different substrates. However, some limitations of this model lead to other more complicated multi-substrate models (Mankad and Bungay 1988; Yoon et al. 1977).

A mathematical model known as Monod modified or Haldane-Andrews model proposed for describing the growth inhibition kinetics and is the most widely used model with substrate inhibition (Eq. (10)) (Andrews 1968).

$$\mu = \mu_{max} \frac{S}{K_S + S + \frac{S^2}{K_i}} \quad (10)$$

where K_i is the inhibition constant.

Also, biomass yield ($Y_{x/s}$) relates the specific rate of biomass growth and substrate consumption (Eq. (11)).

$$\frac{dS}{dt} = -\frac{1}{Y_{X/S}}\frac{dX}{dt} \quad \text{where} \quad Y_{X/S} = \frac{dX}{dS} \tag{11}$$

2.5.2 Product formation modeling

The kinetics of product formation can be modeled using Luedeking- Piret model (Eq. (12)), depending on three classifications and based on their production in different growth phases (Luedeking and Piret 1959; Kumar et al. 2015).

$$\frac{dP}{dt} = a\frac{dX}{dt} + bX \tag{12}$$

where P is the product formation concentration, ‘a’ is the growth associated term, and ‘b’ is the non-growth associated term.

When product formation is associated with microorganism growth, b=0. In the stationary phase when product formation is partially connected to microorganism growth, a=0. When product formation is not related to microorganism growth or when there is mixed growth associated production, both constants exist.

Eq. (13) is derived from integrating Eq. (12).

$$P = aX_0\left(\frac{e^{(\mu_{max}\,t)}}{1-\left(\frac{X_0}{X_{max}}\left(1-e^{(\mu_{max}\,t)}\right)\right)} - 1\right) + b\frac{X_{max}}{\mu_{max}}\ln\left(1-\frac{X_0}{X_{max}}\left(1-e^{(\mu_{max}t)}\right)\right) \tag{13}$$

$Y_{P/S}$ is the product yield coefficient based on the substrate, and $Y_{P/X}$ is the specific product yield coefficient as Eqs. (14,15).

$$\frac{dS}{dt} = -\frac{1}{Y_{P/S}}\frac{dP}{dt} \quad \text{where} \quad Y_{P/S} = \frac{dP}{dS} \tag{14}$$

Also:

$$\frac{dP}{dt} = Y_{P/X}\frac{dX}{dt} \quad \text{where} \quad Y_{P/X} = \frac{dP}{dX} \tag{15}$$

2.5.3 Substrate utilization modeling

By extending material balance, substrate utilization can be determined by Eq. (16).

$$-\frac{dS}{dt} = c\frac{dX}{dt} + dX \tag{16}$$

where ‘c’ and ‘d’ are the kinetic constants.

The integration of Eq. (16) leads to Eq. (17).

$$S = S_0 - cX_0\left(\frac{e^{(\mu_{max}t)}}{1-\left(\frac{X_0}{X_{max}}\left(1-e^{(\mu_{max}t)}\right)\right)} - 1\right) - d\frac{X_{max}}{\mu_{max}}\ln\left(1-\frac{X_0}{X_{max}}\left(1-e^{(\mu_{max}t)}\right)\right) \tag{17}$$

Proposed models and constants can be estimated by fitting the experimental data with the equations using regression analysis. If the fitting for an equation is not reasonable and the model is not significant, other models will be used to find the best fitted model. They have been used to evaluate the behavior of the microbial system and develop the process or industrial application, thereby achieving maximum product output. There have been several studies on modeling the biosurfactant production and microbial growth, which are shown in table 2-3.

Table 2-3. Different studies on biosurfactant modeling

Microorganism	Substrate	Model	Parameters	R^2-value	References
Gordonia alkanivorans CC-JG39	Diesel	Monod	μ_{max} = 0.158 h^{-1} K_S = 3.196 g L^{-1}	0.963	Young et al. 2005
Lactococcus lactis 53	Cheese whey	Logistic	μ_{max} = 0.372 h^{-1}	0.984	Rodrigues et al. 2006
			$Y_{P/S}$ = 0.06 g g^{-1} $Y_{X/S}$ = 0.23 g g^{-1} $Y_{P/X}$ = 0.24 g g^{-1}	0.961	
Streptococcus thermophiles A	Cheese whey	Logistic	μ_{max} = 0.447 h^{-1}	0.968	
			$Y_{P/S}$ = 0.06 g g^{-1} $Y_{X/S}$ = 0.27 g g^{-1} $Y_{P/X}$ = 0.22 g g^{-1}	0.973	
Nocardia amarae NRRL B-8176	Olive oil	Logistic	μ_{max} = 0.0177 h^{-1}	0.8518	Moussa et al. 2010
			K_e = 0.245 g $g^{-1}h^{-1}$ $Y_{P/S}$ = 0.0828 g g^{-1} $Y_{X/S}$ = 0.039 g g^{-1}	0.9551	
			a = 2.1242	0.916	
A consortium contains six bacterial strains	Diesel oil	Andrews inhibitory	μ_{max} = 0.022 h^{-1} K_S = 18.68 g L^{-1} K_I = 29.02 g L^{-1}		Sadouk-Hachaichi et al. 2014
Pseudomonas aeruginosa 2297	Sawdust	Logistic	μ_{max} = 0.047 h^{-1} $Y_{P/X}$ = 1.02 g g^{-1}		Kumar et al. 2015

2.6 Summary

This chapter reviewed the biosurfactant structure, properties, and production. Biosurfactants are effective and environmentally compatible alternatives to chemically synthesized surfactants in widespread fields of application. However, the high production cost of biosurfactants has made application challenging at a large-scale. Costs could be reduced by replacing the expensive carbon sources with low cost renewable substrates such as locally based industrial waste streams or by-products. An understanding of the factors affecting the bioprocess including culture medium compositions and environmental factors can enhance production yield. With respect to kinetic modeling, it was noted that there are limited studies on modeling the biosurfactant production. The capability of utilizing each substrate for biosurfactant

generation by the microorganisms and the optimum conditions depend on the desired strain. There was no published work in utilizing and optimizing the local brewery waste and the waste cooking oil for generating biosurfactants using the *Bacillus subtilis* N3-1P and *Acinetobacter calcoaceticus* P1-1A strains native to North Atlantic Canada. The biosurfactants produced with the indigenous strains are potential compounds for oil spill removal in the offshore Atlantic Ocean. The review provided the basis for the subsequent work.

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Chapter Three: Optimization of biosurfactant production by *Bacillus subtilis* N3-1P using the brewery waste as the carbon source

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Abstract

Biosurfactants are biologically produced by microorganisms and therefore biodegradable, making ideal substitutes to chemical surfactants for various applications. Large scale production of biosurfactants is limited because of the high cost. The production cost could be reduced by optimizing cultural conditions and using wastes as substrates. In this work, the response surface methodology (RSM) was used to optimize biosurfactant production by *Bacillus subtilis* N3-1P strain using brewery waste as the sole carbon source. Five independent variables were varied; carbon and nitrogen concentration, agitation speed, temperature and initial pH. Surface tension and emulsification index were used to measure biosurfactant production. Results indicated that the best surface tension and emulsification index were 27.31 mN m^{-1} and 63.11%, respectively, under optimized cultural conditions (7% (v v^{-1}) brewery waste, 6.22 g L^{-1} ammonium nitrate, initial pH of 6.41, 150 rpm, and 27°C). The predicted responses were validated experimentally under the optimum conditions, and 657 mg L^{-1} of biosurfactant was produced with a critical micelle concentration of 107 mg L^{-1}.

3.1 Introduction

Surfactants are amphiphilic molecules which contain both hydrophilic and hydrophobic groups. They can act as emulsifiers, foamers, detergents, wetting agents or dispersants, based on chemical structures and surface properties. A surfactant can promote a decrease in the surface and interfacial tension between solids, liquids and gases. Biosurfactants are surfactants biologically produced by microorganisms, including bacteria, filamentous fungi and yeast. They are classified based on their

chemical structure, molecular weight and the organisms that produce them. Biosurfactants are structurally classified as glycolipids; lipopeptides and lipoproteins; fatty acids, phospholipids and neutral lipids; as well as polymeric and particulate biosurfactants. They have improved technical characteristics over synthetic surfactants such as low toxicity, high biodegradability, and high effectiveness over a wide pH and temperature range. Biosurfactants have had widespread industrial applications in bioremediation, health care, food and oil processing (Desai and Banat 1997; Mulligan 2005; Makkar et al. 2011).

Bacillus subtilis strains are among the most effective biosurfactant producers and can generate biosurfactants for enhancing bioremediation of contaminated sites and oil recovery, and in food, cosmetic and pharmaceutical industries (Abdel-Mawgoud et al. 2008; Wang et al. 2008; Haddad et al. 2009). An indigenous *Bacillus subtilis* strain that was isolated from oily contaminated marine environment in Atlantic Canada was used in this research (Cai et al. 2014).

Despite the advantages of the biosurfactants produced by *Bacillus subtilis* strains, large scale production and utilization of them are limited due to high cost of production (Desai and Banat 1997; Makkar et al. 2011). Approaches for lowering cost or increasing production include optimization of culturing conditions and the downstream separation and purification process, and use of low cost and/or waste substrates (Mukherjee et al. 2006; Marchant and Banat 2012).

Wastes generated from food processing and other sources could serve as the sustainable carbon source for biosurfactant production, using a waste that would otherwise be a cost with respect to treatment and disposal. The utilization of various

waste streams has been studied previously including frying oils, oil refinery wastes, molasses, starch rich wastes, cassava waste water, potato waste and distilled grape marc (Fox and Bala 2000; Makkar and Cameotra 2002; Maneerat 2005; Nitschke and Pastore 2006; Rivera et al. 2007; Sobrinho et al. 2008; Silva et al. 2010; Makkar et al. 2011; Luo et al. 2013; Rocha et al. 2014; Lan et al. 2015). Examples of studies of using different carbon sources for biosurfactant production with different *Bacillus subtilis* strains are summarized in Table 3-1. The carbon source used in this study is the waste of the first stage (mash and lauter tun) of beer production at Quidi Vidi brewing Company. Mashing is the process of combining a mix of milled barely grain and water, known as liquor, and heating this mixture in a vessel called a mash tun. Mashing allows the enzymes in the malt to break down the starch in the grain into sugars, typically maltose to create malty liquid called wort. Lautering is the separation of the wort from the grains. The waste stream from mash and lauter tun, usually consist of spent grain and sugars, including maltose and glucose.

Table 3-1. A summary on using different carbon sources for biosurfactant production by *Bacillus subtilis*

Carbon Sources	**Best Response**	**Reference**
Glucose, Soybean oil, Hexadecane, Glucose + Soybean oil, Glucose + Hexadecane	Surface tension = 28.2 mN m^{-1} with glucose	Kim et al. 1997
Molasses, milk whey and cassava flour wastewater	Surface tension = 26 mN m^{-1} with cassava flour wastewater	Nitschke et al. 2004
Glucose, fructose, sucrose, maltose, lactose, galactose, mannose, sorbitol, glycerol, glucose syrup, molasses, malt extract, soybean oil, olive oil, hexadecane and paraffin oil	Biosurfactant = 1.12 g L^{-1} with molasses	Abdel-Mawgoud et al. 2008
Glucose, sucrose, starch, and glycerol	Biosurfactant = 720 mg L^{-1} with glucose	Ghribi and Ellouze-Chaabouni 2011
Glucose, waste frying sunflower oil, and waste frying rice bran oil	Surface tension reduction of 56.32% for glucose, 48.5% for waste frying sunflower oil and 46.1% for waste frying rice bran oil	Vedaraman and Venkatesh 2011
Raw glycerol from a biodiesel plant	Surface tension of the foam = 29.5 mN m^{-1}	Faria et al. 2011
By-product glycerol from biodiesel production	Biosurfactant = 441.06 mg L^{-1} Surface tension = 28.8 mN m^{-1}	Sousa et al 2012
Soluble starch, beer wastewater, sucrose, and liquid paraffin	Biosurfactant = 1.26 g L^{-1} Surface tension = 22.8 mN m^{-1} with beer wastewater	Liu et al. 2014
Corn steep liquor	Biosurfactant = 4.8 g L^{-1} Surface tension = 29.1 mN m^{-1}	Gudina et al. 2015

Experimental design techniques like factorial design and response surface methodology (RSM) have been used to optimize experiments by defining the relationship between inputs and response factors. RSM is an effective and useful method to design experiments, to fit models by multiple regression analysis, to analyze the effect of several experimental variables, and to determine the variable conditions for an optimum response (Khuri and Cornell, 1996; Myers and Montgomery, 2002). To achieve economic viability for biosurfactant production, it is essential to optimize the cultural conditions such as temperature, pH, agitation, aeration, dilution rate (for continuous process), and medium compositions (carbon, nitrogen and metal ions) affecting the production. There are several studies related to biosurfactant production and the optimization of process parameters (Sen and Swaminathan 1997; Jacques et al 1999; Joshi et al. 2008; Mukherjee et al. 2008; Pal et al. 2009; Najafi et al. 2010; Ghribi and Ellouze-Chaabouni 2011; Ghribi et al. 2011; Saikia et al. 2012; Kim and Kim 2013; Luo et al. 2013; Hu et al. 2015; Kumar et al. 2015; Eswari et al. 2016; Sellami et al. 2016; Ebadipour et al. 2016). Biosurfactant production optimization using *Bacillus subtilis* are outlined in table 3-2.

Table 3-2**.** A review on optimizing biosurfactant production by *Bacillus subtilis*

Optimum conditions	**Best Response**	**Method**	**Reference**
pH = 7.2, temperature = 42.1 °C, inoculum concentration = 5.2% (v v^{-1}) rotate speed = 163 rpm	Surface tension = 22.8 mN m^{-1}, Biosurfactant = 1.26 g L^{-1}	Box-Behnken Design	Liu et al. 2014
Glucose = 30 g L^{-1}; NH_4NO_3 = 6.0 g L^{-1}; K_2HPO_4 = 1.1 g L^{-1}; $MgSO_4 \cdot 7H_2O$ = 0.3 g L^{-1}; KH_2PO_4 = 2.8×10^{-2} g L-1, $FeSO4.7H_2O$ = 6×10^{-2} g L^{-1}	Biosurfactant = 2.42 g L^{-1}	Plackett Burman Design	Mukherjee et al. 2008
Orange peels = 15.5 g L^{-1}, soya bean = 10 g L^{-1}, diluted sea water = 30%	Biosurfactant = 4.45 g L^{-1}	Central Composite Design	Ghribi et al. 2011
Sucrose = 70 g L^{-1}, yeast extract = 5 g L^{-1}, $FeSO_4.7H_2O$ = 0.055 g L^{-1} KH_2PO_4 = 0.15 g L^{-1}	Biosurfactant = 10 g L^{-1}	Central Composite Design	Kumar et al. 2015
Temperature = 37.4 °C, agitation speed = 140 rpm, pH = 6.75, aeration = 0.75 vvm	Biosurfactant = 1.1 g L^{-1}	Central Composite Design	Sen et al. 1997
Soluble starch = 1.55% (w v^{-1}), skim milk = 0.477% (w v^{-1}), KNO_3 = 0.096% (w v^{-1}), T = 37.145 °C	Surface tension reduction = 45.353%.	Central Composite Design	Kim and Kim 2013
Temperature = 30°C; pH = 7.0; shaking = 200 rpm; KH_2PO_4 = 1.9 g L^{-1}; trace elements = 1 mL L^{-1}; sucrose = 20 g L^{-1}; peptone = 30 g L^{-1}; yeast extract = 7 g L^{-1}	Biosurfactant = 0.826 g L^{-1}	Plackett Burman Design	Jacques et al. 1999

CCD, one type of RSM, is a factorial design and used in this study to design the experiments of biosurfactant production by *Bacillus subtilis* N3-1P. The CCD is the most popular class of designs used for fitting second-order models. The total number of tests required for CCD is $2^{k-1} + 2k + n_c$; where k is the number of independent variables and n_c is replicate tests at the centre (Khuri and Cornell 1996)

The objectives of this research are to optimize biosurfactant production by a *Bacillus subtilis* strain, using RSM, to reduce production cost through using a brewery waste as the sole carbon source. This is the first study assessing local brewery waste as the sole carbon source in producing biosurfactant by the indigenous *Bacillus subtilis* N3-1P strain. This has the potential to be a low cost and sustainable alternative for biosurfactant production to treat oil spills in marine environments, especially the North Atlantic which this bacterium is indigenous to.

3.2 Materials and Methods

3.2.1 Microorganism

The biosurfactant producer strain, *Bacillus subtilis* N3-1P, was isolated in the Northern Region Persistent Organic Pollution Control (NRPOP) laboratory from petroleum hydrocarbon contaminated aquatic sources of oil and gas platforms offshore Atlantic Canada. This strain is one of the most effective isolates with promising ability to decrease surface tension and raise emulsification index (Cai et al. 2014).

3.2.2 Obtaining and treating the brewery waste

The carbon source which was used in this study is the waste of the beer production at Quidi Vidi brewing Company. After centrifugation and microfiltration of the sample through 0.2 µ membrane filter, total carbohydrate was analyzed using the anthrone method and Agilent 8453 spectrophotometer, and it was found to be 469.6 g L^{-1}. The sterilized brewery waste was stored at –18 °C until needed.

3.2.3 Inoculum preparation

The microbial strain was first cultured in a 50-mL nutrient salt medium and 8 g L^{-1} glucose as the carbon source containing a trace metal solution in a 125 mL sterilized Erlenmeyer flask. The nutrient salt medium is composed of (g L^{-1}) NH_4NO_3, 4; Na_2HPO_4, 5; NaCl, 3.3; KH_2PO_4, 4; $FeSO_4$•7H2O, 0.0012; yeast extract, 0.5. A 0.5-ml element solution containing $ZnSO_4$, 0.29; $CaCl_2$, 0.1; $MnSO_4$, 0.4 (g L^{-1}) was added to 1 L of the nutrient salt medium. The medium was inoculated with a loopful of bacteria colony from the agar plate. The bacteria were further cultured in the Innova 43 incubator shaker for 24 h at 30 ºC and 200 rpm. The optical density of the broth was then measured by a spectrophotometer, and as it varied for each experiment, the amount equal to 2% (v v^{-1}) at OD_{660} = 0.5 were used as the inoculum for the production process. This amount was found to be effective through performing some pre-tests and applied to standardize the inoculum size to add the same number of bacteria to each flask.

3.2.4 Production of biosurfactants

The bacteria were inoculated into 125 mL flasks containing 50 mL medium, as described above, and stirred in a rotary shaker for 3 days. Carbon and nitrogen sources were added to the medium according to the experimental design given in table 3-4. The initial pH, temperature and agitation speed were also adjusted accordingly; using 1 M NaOH and H_3PO_4 for pH adjustment. The biosurfactant also was produced using 16 g L^{-1} glucose as the sole carbon source to compare the effectiveness of the brewery waste with a commercial carbon source. This value was derived from other studies and measuring the total carbohydrate of the brewery waste. All experiments

were done in duplicate and medium without inoculum was used as the negative control.

3.2.5 Optimization of biosurfactant production using RSM

CCD was used in this study as a mathematical method to design experiments of biosurfactant production. For five variables, the recommended number of tests at the centre is six. Hence the total number of tests required is 32. For statistical calculation, the experimental variables x_i have been coded as the dimensionless X_i by dividing the difference values between each uncoded variable and the variable at the centre point by the step change value.

Regression analysis is a general approach to fit the empirical model with the collected response variable data. Once the experiments were performed, the relationship between the independent variables and their responses were fitted to a predictive second-order polynomial equation.

In this study, the five independent process variables chosen were carbon source, nitrogen source, agitation speed, temperature and initial pH, at five levels ($\pm\alpha$, $\pm$ 1, 0: where α is the distance from the design centre) and six replicates at the central points were used to design the experiments. The levels of the lowest, low, centre, high and the highest for the design variables are stated in Table 3-3.

Table 3-3. Independent variables and their levels used in CCD

Independent variables	Code	Coded values and the corresponding values of parameters				
		-2	**-1**	**0**	**1**	**2**
Carbon source % (v v^{-1})	A	2	4	6	8	10
Nitrogen source (g L^{-1})	B	2	4	6	8	10
pH	C	4.75	5.75	6.75	7.75	8.75
Agitation speed (rpm)	D	0	100	200	300	400
Temperature (°C)	E	18	24	30	36	42

The response variables measured were surface tension (ST) and emulsification index (EI).

Experiments were randomized in order to maximize the effects of unexplained variability in the observed responses due to extraneous factors.

3.2.6 Biosurfactant recovery and purification

In order to produce cell free supernatant, the culture broth was centrifuged at 8000 rpm for 15 min. This crude biosurfactant was used to measure surface tension, critical micelle dilution and emulsification index to evaluate the biosurfactant production. All measurements were conducted in triplicate.

The crude biosurfactant solution was purified by acidifying to pH 2.0 with 6 N HCl and keeping at 4°C overnight. Then the precipitated biosurfactant was collected by centrifuging at 1000 rpm for 15 min. The precipitate was then dissolved in distilled water and adjusted to pH 7.0 using 1N NaOH. Solvent extraction with chloroform-methanol (2:1) was performed on the solution to separate the organic layer, which was subjected to the rotary evaporator to achieve dried purified biosurfactant. The

biosurfactant concentration was calculated by dividing the weight of the dried product by the total volume of the crude biosurfactant solution.

3.2.7 Responses determination

3.2.7.1 Surface tension measurement

The surface tension of the crude biosurfactant was measured by the Du Nuoy ring method using a tensiometer (CSC Scientific Company), at room temperature.

3.2.7.2 Emulsification index measurement

In this research the emulsification index (EI_{24}) was measured to evaluate the emulsification capacity of the produced biosurfactant. For this purpose, 2 ml hexadecane was added to the same amount of the crude biosurfactant and the mixture was vortexed at high speed for 2 min. After 24 hrs, EI_{24} was calculated by dividing the height of the emulsion layer by the total height of the liquid, multiplying by 100 (Silva et al. 2010; Sousa et al. 2012; Gudina et al. 2015).

$$EI_{24} = \frac{Height\ of\ the\ emulsion\ layer}{Height\ of\ the\ total\ liquid} \times 100 \quad (1)$$

3.2.7.3 Critical micelle concentration measurement

The crude biosurfactant was diluted several times, and the surface tension of each diluted product was measured. The concentration at which the surface tension raises sharply can be determined by plotting a graph of ST against biosurfactant concentration. CMC is the corresponding biosurfactant concentration at the intercept of the best two lines which can be fitted to the data (Morais et al. 2017).

3.3 Results and Discussion

3.3.1 Experimental design and statistical model analysis

The experiments were conducted according to the CCD, which is designed by Design-Expert 10.0.1 software. The results were listed in Table 3-4.

Table 3-4. Experimental central composite design run sheet with actual independent and response variables

Run	Factor 1 A: Carbon (% (v v^{-1}))	Factor 2 B: Nitrogen (g L^{-1})	Factor 3 C: pH	Factor 4 D: Agitation speed (rpm)	Factor 5 E: Temperature (°C)	Response 1 ST (mN m^{-1})	Response 2 EI (%)
1	4	4.00	5.75	100	36	30.19	53.61
2	6	6.00	6.75	200	30	27.30	62.71
3	2	6.00	6.75	200	30	29.72	62.37
4	4	8.00	7.75	100	36	32.18	51.4
5	6	6.00	6.75	200	30	28.24	61.63
6	4	4.00	5.75	300	24	28.91	53.12
7	8	8.00	5.75	300	24	28.54	57.64
8	6	6.00	6.75	0	30	27.88	57.53
9	4	8.00	5.75	100	24	29.69	59.19
10	8	8.00	7.75	300	36	31.09	46.29
11	4	4.00	7.75	300	36	32.84	44.66
~~12~~	~~6~~	~~6~~.00	~~8.75~~	~~200~~	~~30~~	~~37.45~~	~~57.41~~
13	6	6.00	6.75	200	30	27.52	64.03
14	6	6.00	6.75	200	30	28.09	60.75
15	6	6.00	6.75	200	30	27.21	63.11
16	8	4.00	5.75	300	36	29.16	50.19
17	6	6.00	6.75	200	18	28.31	56.49
18	10	6.00	6.75	200	30	28.56	63.11
19	4	8.00	7.75	300	24	31.31	50.6
20	8	8.00	5.75	100	36	28.95	56.21
21	6	10.00	6.75	200	30	29.91	61.37
22	6	6.00	6.75	200	42	30.85	51.49
23	8	4.00	5.75	100	24	28.24	59.52
24	6	2.00	6.75	200	30	30.16	60.47
25	6	6.00	6.75	200	30	27.48	61.3
26	6	6.00	6.75	400	30	28.61	54.01
27	8	8.00	7.75	100	24	30.95	55.45
28	8	4.00	7.75	100	36	32.08	48.99
29	8	4.00	7.75	300	24	31.47	53.38
30	4	8.00	5.75	300	36	29.30	52.51
~~31~~	~~6~~	~~6~~.00	~~4.75~~	~~200~~	~~30~~	~~45.00~~	~~42.86~~
32	4	4.00	7.75	100	24	31.60	53.47

The coefficients of the second-order polynomial equation were calculated by fitting the experimental data to the model by the software.

In the derivation of the models, two runs (at low pH = 4.75 and high pH = 8.75) were removed as they were outliers due to low biosurfactant production.

3.3.1.1 RSM results with surface tension as the response

For ST as the response variable, A (Carbon), C (pH), E (Temperature), A^2, B^2, C^2, E^2 which show nonlinearities between the response and indipendant variables are significant model terms. However, B (Nitrogen) should be in the model as B^2 is significant. The main significant factors are C and C^2. Hence, there is no interaction between factors and agitation speed has no effect on ST. Figure 3-1 summarizes the effect of all factors on the surface tension. The final equation in terms of coded factors is as equation 2.

$$ST = + 27.79 - 0.33 \times A - 0.12 \times B + 1.28 \times C + 0.42 \times E + 0.34 \times A^2 + 0.56 \times B^2 + 1.27 \times C^2 + 0.45 \times E^2 \quad (2)$$

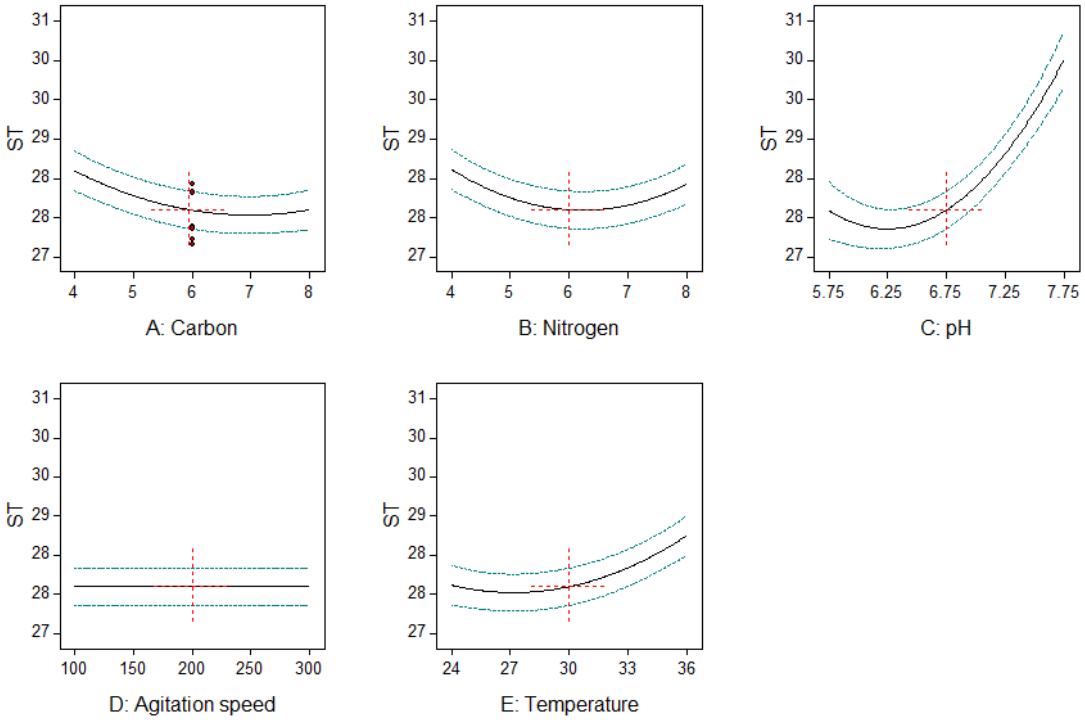

Figure 3-1. Effect of all variable factors on ST of the produced biosurfactants. The dotted lines show the 95% confidence band on the mean prediction.

The results of this study show that the brewery waste which has a high amount of carbohydrate, is an applicable carbon source for the *Bacillus subtilis* N3-1P growth and biosurfactant production. As Figure 3-1 illustrates, the addition of the brewery waste from 4 to 6.9 % (v v^{-1}), decreased the ST and when the brewery waste increased from 6.9 to 8 % (v v^{-1}), ST rose slightly. Each bacterial strain has a limit and optimum for carbon source concentration, after which due to effects such as inhibition of secondary metabolites, low pH, or increases in viscosity (reducing oxygen transfer and homogeneity), bacterial growth or biosurfactant production can be negatively impacted.

The ammonium nitrate concentration shows a similar trend to the carbon source. The ST decreased when ammonium nitrate concentration rose from 4 to 6.2 g L^{-1} and

further addition of ammonium nitrate increased ST. One important factor for bacterial growth and biosurfactant production is the carbon to nitrogen ratio. Each strain has an optimum amount of C:N, the lower or higher ratio leads to lower production rate (Guerra-Santos et al. 1986; Santa Anna et al. 2001; Abouseoud et al. 2008). In this work the carbon to nitrogen ratio varied from 2.7 to 10.2 and changes in the surface tension could be related to this ratio; however, further work is required to verify.

Higher temperatures increase the enzymatic activity and as several enzymes have essential roles in biosurfactant production and microbial growth, can increase the rate of metabolism and reproduction (Zhang et al. 2015). However, there is an optimal temperature for each microbial strain. According to this study, the temperature increase to 27 °C, enhance biosurfactant production, after which biosurfactant production decreased.

Among different variables, pH has the most effect on ST. The pH can cause changes in permeability and electric charge of the membrane and affects the activity of enzymes involved in the microbial growth and reproduction (Zhang et al. 2015). The best response was obtained at pH slightly below neutral. The ST decreased from pH 5.75 to 6.4, and increased considerably at higher pH. In a study by Abdel-Mawgoud et al., a similar trend was observed where the optimum range of pH was between 6.5 and 6.8 (Abdel-Mawgoud et al. 2008). Further, in a study of biosurfactant production by a *Bacillus subtilis* strain the optimum pH was 6.75 (Sen and Swaminathan 1997).

3.3.1.2 RSM results with emulsification index as the response

When the response variable is the emulsification index (EI), C (pH), D (Agitation speed), E (Temperature), C^2, D^2, E^2 are significant model terms. Again, there is no important interaction between factors. The effects of all factors on EI are shown in figure 3-2. The final equation in terms of coded factors is shown in equation 3.

$$EI = +62.09 - 2.36 \times C - 1.52 \times D - 2.02 \times E - 5.59 \times C^2 - 1.58 \times D^2 - 2.02 \times E^2 \quad (3)$$

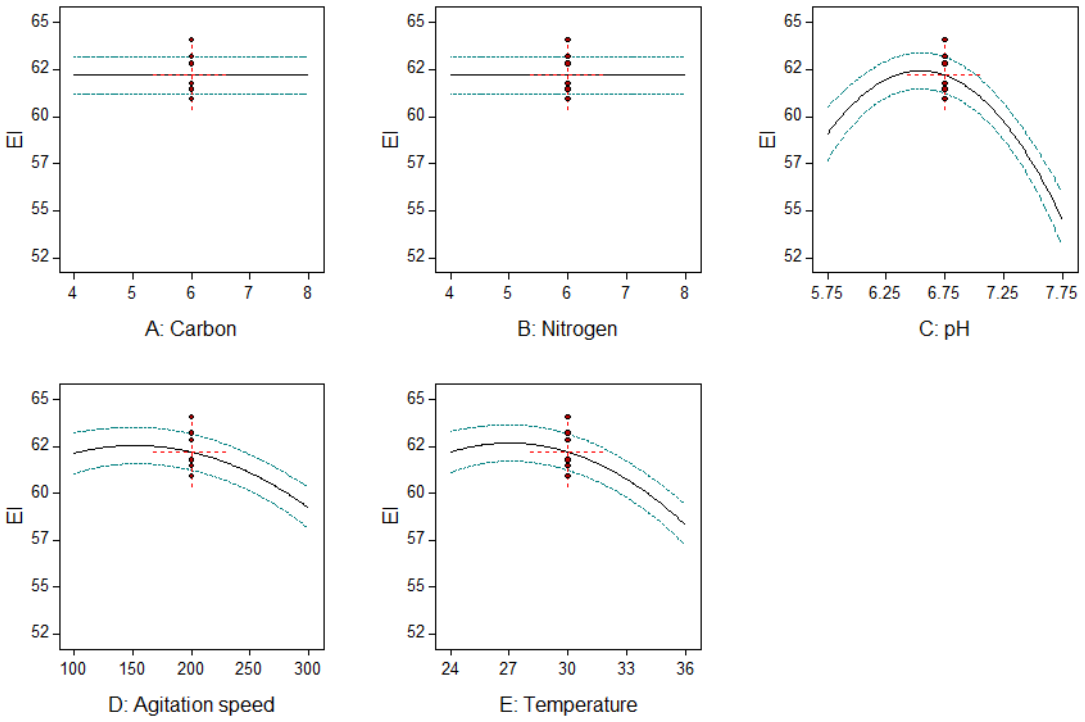

Figure 3-2. Effect of all variable factors on EI of the produced biosurfactants. The dotted lines show the 95% confidence band on the mean prediction

The equation indicates the relationship between pH and emulsification index is not linear (C^2) and the rate of change of EI with pH is higher than temperature (E^2) and agitation rate (D^2). This is consistent with the results of a previous study which optimized pH, temperature, glucose concentration and salinity for biosurfactant

production by a *Bacillus mycoides* strain (Najafi et al. 2010). However, an interaction between pH and temperature was observed in this work (Najafi et al. 2010) which was not observed in our work. A study which was done to optimize biosurfactant production by *Bacillus licheniformis*, with temperature, pH and beetroot (substrate) concentration showed similar results to our work where the rate of change of emulsification index was most impacted by temperature followed by pH (Amodu et al. 2014). Temperature, pH, incubation period and glucose concentration were optimized in another study and again the rate of change of emulsification index was most impacted by temperature squared when *Bacillus brevis* was used for biosurfactant production. The pH and temperature were the next factors with high effects (Mouafi et al. 2016).

There is no significant change in EI values with carbon and nitrogen concentrations. This can be related to the presence of some impurities in the cell-free supernatant which could interfere with emulsion formation and the EI values. Besides, other levels of these independant variables may be more appropriate for the EI as the response variable.

The main factor affecting the EI is pH squared. EI increased with the pH from 5.75 to 6.5, and dropped rapidly until maximum pH studied (7.75). There was a slight increase in EI value by raising temperature to 27 °C, while further increase resulted in lower EI. Agitation of the nutrient broth enhances mass transfer; however, high agitation speed may result in lower yield due to shear effects. In this research, the EI value reached a maximum by increasing the agitation speed from 100 to 150 rpm, and higher rates decreased EI. This value is in good agreement with previous studies

where 140 and 163 rpm were the optimum agitation speed for biosurfactant production by a *Bacillus subtilis* strain (Sen and Swaminathan 1997; Liu et al. 2014).

3.3.1.3 Model validation

In order to validate the model and investigate the effect of factors, the analysis of variance (ANOVA) was performed on both response variables, ST and EI. Results of ANOVA analysis are illustrated in Tables 3-5 and 6. Values of 'Prob > F' less than 0.05 indicate model terms are significant. For both response variables, the most appropriate model which is suggested by Design Expert (p-value < 0.0001) is the quadratic model.

Table 3-5. ANOVA Summary for the quadratic model when ST is the response variable

Source	**Sum of Squares**	**df**	**Mean Square**	**F Value**	**p-value Prob > F**	
Model	70.90	8	8.86	47.55	< 0.0001	significant
A-Carbon	2.57	1	2.57	13.81	0.0013	
B-Nitrogen	0.37	1	0.37	1.99	0.1735	
C-pH	26.37	1	26.37	141.48	< 0.0001	
E-Temperature	4.30	1	4.30	23.08	< 0.0001	
A^2	2.91	1	2.91	15.62	0.0007	
B^2	8.06	1	8.06	43.22	< 0.0001	
C^2	9.83	1	9.83	52.72	< 0.0001	
E^2	5.12	1	5.12	27.47	< 0.0001	
Residual	3.91	21	0.19			
Lack of Fit	3.01	16	0.19	1.04	0.5292	not significant
Pure Error	0.90	5	0.18			
Cor Total	74.81	29				
Std. Dev.	0.43		R-Squared		0.9477	
Mean	29.54		Adj R-Squared		0.9278	
			Pred R-Squared		0.8792	

Table 3-6. ANOVA Summary for the quadratic model when EI is the response variable

Source	**Sum of Squares**	**df**	**Mean Square**	**F Value**	**p-value Prob > F**	
Model	774.42	6	129.07	54.46	< 0.0001	significant
C-pH	89.07	1	89.07	37.58	< 0.0001	
D-Agitation speed	55.48	1	55.48	23.41	< 0.0001	
E-Temperature	98.05	1	98.05	41.37	< 0.0001	
C^2	208.55	1	208.55	87.99	< 0.0001	
D^2	66.47	1	66.47	28.04	< 0.0001	
E^2	109.22	1	109.22	46.08	< 0.0001	
Residual	54.51	23	2.37			
Lack of Fit	46.86	18	2.60	1.70	0.2910	not significant
Pure Error	7.66	5	1.53			
Cor Total	828.93	29				
Std. Dev.	1.54		R-Squared	0.9342		
Mean	56.22		Adj R-Squared	0.9171		
			Pred R-Squared	0.8285		

For ST and EI as response variables, the r-squared values are 0.9477 and 0.9342 respectively, which shows good fitness of the model. The predicted r-squared of 0.8792 and 0.8285 are in reasonable agreement with the adjusted r-squared of 0.9278 and 0.9171 respectively; as the difference is less than 0.2, indicating a good agreement between the experimental and predicted values.

Normal plots of residuals are used to check the appropriateness of the model used. It should produce an approximately straight line to consider normal distribution. In figure 3-3, residuals follow a straight line, so we can conclude normality and good model fit. Based on this analysis, these models can be used to predict ST and EI of the produced biosurfactant within the range of the independent variables.

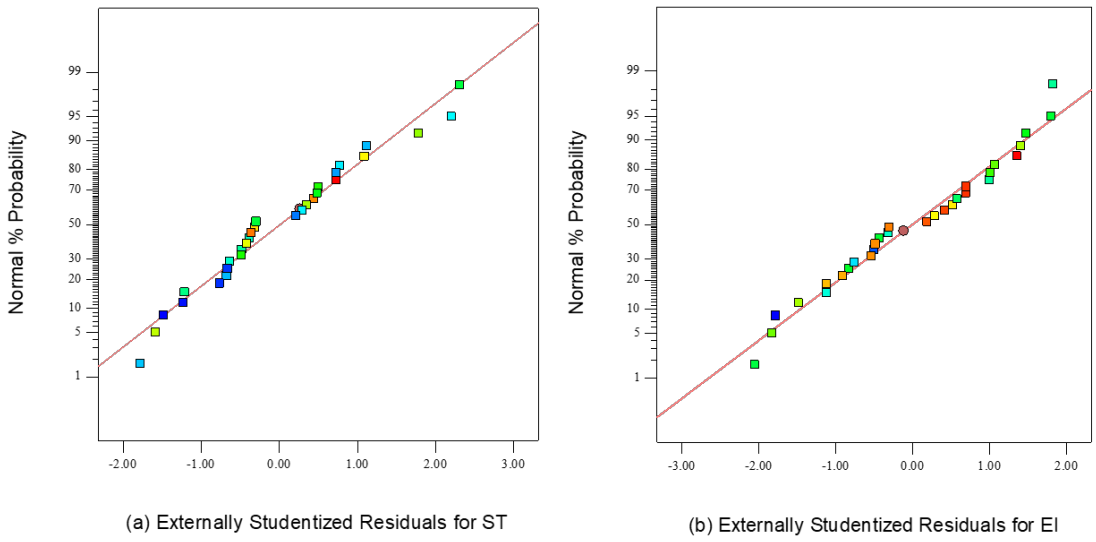

Figure 3-3. Normal plot of residuals for (a) ST and (b) EI as response variables

3.3.2 Optimization of the response variables

The best combination of factors to minimize ST is 7% (v v^{-1}) brewery waste, 6.22 g L^{-1} ammonium nitrate, pH of 6.24, and 27°C, resulting in a predicted surface tension of 27.28 mN m^{-1}. Maximum predicted Emulsification index was found to be 63.20% with pH of 6.54, 152 rpm and 27°C. Minimizing ST and simultaneously maximizing ET is also found with surface tension of 27.31 mN m^{-1}, and the emulsification index of 63.11%, according to the results of the software. The optimum results were occurred in a solution of 7% (v v^{-1}) brewery waste, 6.22 g L^{-1} ammonium nitrate, pH of 6.41, 150 rpm, and 27°C.

The response variables were experimentally measured at optimum points and the results closely matched the predicted values (ST = 27.26 mN m^{-1}, EI = 62.33%). The model was confirmed as the values are inside the prediction intervals, and the

difference between the model prediction and the experimental data was less than 0.5%.

The cultivation was also performed at optimum conditions with 16 g L^{-1} glucose as the sole carbon source instead of the brewery waste. After 72 hrs, the surface tension was found to be 30.8 mN m^{-1}, while when the brewery waste was used it was ~27 mN m^{-1}. The emulsification index was 63% with the glucose, which is almost the same as the emulsification index with the brewery waste. Comparing the brewery waste results with the glucose shows that the brewery waste is a sustainable substitution for a commercial carbon source for biosurfactant production by *Bacillus subtilis* N3-1P. In previous studies for biosurfactant production by *Bacillus subtilis* strains the surface tension was as low as 22.8 mN m^{-1} to 26 mN m^{-1} (Nitschke et al. 2004; Liu et al. 2014), and as high as 28.2 mN m^{-1} to 29.5 mN m^{-1} (Kim et al. 1997; Faria et al. 2011; Sousa et al. 2012; Gudina et al. 2015).

The concentration of the biosurfactant at optimum conditions was 657 mg L^{-1}, while 534 mg L^{-1} biosurfactant was produced with glucose as carbon source. The relationship between biosurfactant concentration and surface tension is shown in figure 3-4. Increasing the biosurfactant concentration decreases in surface tension until the CMC is reached and after that no significant changes are observed in ST. The critical micelle concentration at optimized situation was 107 mg L^{-1}. This CMC value is reliable and demonstrates the feasibility of producing biosurfactant using the brewery waste as the sole carbon source which lower the costs associated with biosurfactant production. The CMC of 136 mg L^{-1} was acquired when glucose was used as the sole carbon source. The obtained CMC value was camparable with the

results of other studies (100 mg L^{-1} and 160 mg L^{-1}) using the industrial wastes for biosurfactant production by some *Bacillus subtilis* strains (Fox and Bala 2000; Gudina et al. 2015).

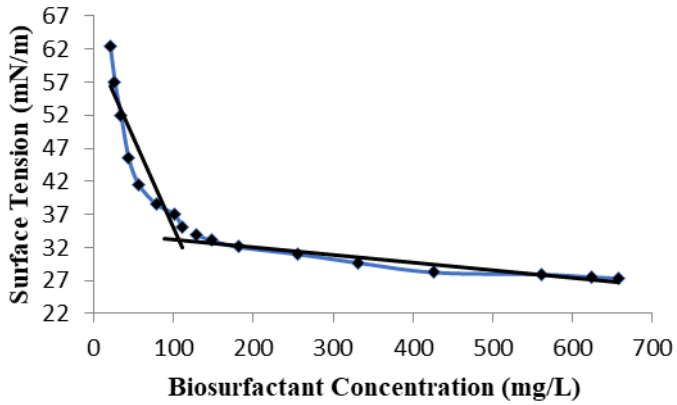

Figure 3-4. Surface tension at different biosurfactant concentrations for CMC measurement

3.4 Summary

This research demonstrated that the brewery waste, a sustainable and inexpensive carbon source can be used for biosurfactant production by local *Bacillus subtilis* N3-1P effectively. Results of CCD experiments indicated that all factors including carbon, nitrogen, pH, agitation speed and temperature influenced the biosurfactant production, with pH dominating. The optimum conditions for biosurfactant production were brewery waste of 7% (v v^{-1}), ammonium nitrate at 6.22 g L^{-1}, pH of 6.41, agitation speed of 150 rpm, and temperature at 27°C, leading to a surface tension of 27.31 mN m^{-1}, and the emulsification index of 63.11%. Biosurfactant production using brewery stream compared favourably with a commercial carbon source. Using the brewery waste as the sole carbon source for biosurfactant

production reduces costs associated with biosurfactant production and helps to generate an environmentally friendly way for waste treatment and disposal.

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Chapter Four: Evaluation and optimization of biosurfactant production by *Acinetobacter calcoaceticus* P1-1A using waste streams

This chapter is under review by the Environmental Progress & Sustainable Energy Journal. Bahareh Moshtagh is the primary author and the co-authors are Dr. Kelly Hawboldt and Dr. Baiyu Zhang. Ms. Moshtagh's contributions to this paper include writing the paper, performing all the laboratory testing and analyses, and interpreting the results. Dr. Hawboldt and Dr. Baiyu Zhang contributed in providing technical guidance, reviewing, and revising the manuscript.

Abstract

The high cost of biosurfactant production is an obstacle for widespread commercial applications. Cost-effective generation of biosurfactants could be achieved using industrial wastes and by-products as substrates and tailoring cultural conditions. In this work, waste streams including refined waste cooking oil and crude glycerol were compared to each other and to commercial carbon sources. Based on this assessment the waste cooking oil was selected for further studies. A response surface methodology (RSM) was then used to study biosurfactant production by *Acinetobacter calcoaceticus* P1-1A strain using the refined waste cooking oil as the sole carbon source. The concentrations of carbon, nitrogen, and NaCl, as well as the initial pH and temperature were varied. The emulsification index was measured as the response. The cultural conditions to reach the maximum emulsification index (68.17%) were 4.35% (v v^{-1}) refined waste cooking oil, 6.5 g L^{-1} ammonium sulfate, 13.5 g L^{-1} NaCl, initial pH of 7.7, and temperature of 34.8 °C. The experimental validation of the predicted response under optimum conditions was performed with 862 mg L^{-1} of the biosurfactant product generated. The product showed high thermal, pH and salinity stability. Producing biosurfactant with this sustainable alternative carbon source at optimum conditions reduces the cost of generating the valuable substance especially useful for treating oil spills in the harsh marine environment.

4.1 Introduction

Biosurfactants are amphiphilic molecules biologically produced by microorganisms. They can decrease surface and interfacial tensions between different phases. Biosurfactants have various industrial applications, and they have enhanced technical

features over chemically synthetic surfactants such as high biodegradability, low toxicity, and high effectiveness over a wide pH and temperature range (Desai and Banat 1997).

Acinetobacter species can produce biosurfactants, and among them, *Acinetobacter calcoaceticus* has been studied extensively. Most studies focused on their chemical composition, physical properties and metabolic control (Goldman et al. 1982; Johri et al. 2002; Panilaitis et al. 2002; Patil and Chopade 2001; Phetrong et al. 2008; Pines and Gutnick 1986; Rosenberg and Ron 1997; Sar and Rosenberg 1983). The high emulsifying activity of the produced biosurfactants by *Acinetobacter* species make them suitable for a wide variety of commercial applications including oil spill response, microbial enhanced oil recovery, and those in cosmetics, food, pharmaceutical and agricultural industries (Choi et al. 1996; Gutnick et al. 1991; Zhao and Wong 2009). *Acinetobacter calcoaceticus* strains can grow and produce biosurfactants on a variety of carbon sources such as aliphatic hydrocarbons, ethanol, acetate, whey and naphthalene (Amoabediny et al. 2010; Choi et al. 1996; Johri et al. 2002; Phetrong et al. 2008; Zhao and Wong 2009). Some studies related to producing biosurfactants with *Acinetobacter calcoaceticus* species are summarized in table 4-1. An indigenous *Acinetobacter calcoaceticus* strain isolated from an oily contaminated site in the marine environment of Atlantic Canada was used in this research (Cai et al. 2014).

Table 4-1. Review of studies on biosurfactant production with *Acinetobacter calcoaceticus* species

Strain	Carbon source	Result	Reference
Acinetobacter calcoaceticus NS6	Ethanol (2%, v v^{-1})	Biomass = 2.6 g L^{-1}	(Sar and Rosenberg 1983)
Acinetobacter calcoaceticus A2	Ethanol (16 g L^{-1})	Biosurfactant = 4 g L^{-1}	(Rosenberg et al. 1988)
Acinetobacter calcoaceticus RAG-1	Soap stock	Biosurfactant accumulation = 25 g L^{-1}	(Shabtai 1990)
Acinetobacter calcoaceticus RAG- 1	Ethanol (6.5 g L^{-1})	Biosurfactant = 4.7 g L^{-1}	(Choi et al. 1996)
Acinetobacter calcoaceticus MM5	Glucose, gasoline, jet fuel, tetradecane, citrate, acetate and ethanol	No growth on glucose, gasoline and jet fuel. EI = 60% (with tetradecane) EI = 64% (with citrate) EI = 60% (with acetate) EI = 60% (with ethanol)	(Marin et al. 1996)
Acinetobacter calcoaceticus RAG-1 and its transposon mutants	Ethanol and fatty acids of different chain length	Transposon mutants produced structural variants biosurfactants with enhanced emulsifying activity	(Johri et al. 2002)
An *Acinetobacter calcoaceticus* strain	Cashew apple juice	EI = 85.7%	(Rocha et al. 2006)
Acinetobacter calcoaceticus subsp. *Anitratus* SM7	n-Heptadecane (0.3%, v v^{-1})	Biosurfactant = 2.94 g L^{-1}	(Phetrong et al. 2008)
Acinetobacter calcoaceticus NRRL B-59191	Glycerol (2%, v v^{-1})	Biosurfactant = 2.2 g L^{-1}	(Rooney et al. 2009)
Acinetobacter calcoaceticus BU03	Glucose (10 g L^{-1})	CMC = 152. 4 mg L^{-1}	(Zhao and Wong 2009)
Acinetobacter calcoaceticus PTCC 1641	Whey	Surface tension = 30.52 mN m^{-1}	(Amoabediny et al. 2010)
Acinetobacter calcoaceticus PTCC1318	Crude oil, soy oil and ethanol	Biomass = 3 g L^{-1} (with crude oil) Biomass = 3.4 g L^{-1} (with soy oil) Biomass = 2.9 g L^{-1} (with ethanol)	(Chamanrokh et al. 2010)
Acinetobacter calcoaceticus K-4	Ethanol and organic acids	Biosurfactant = 5 g L^{-1}	(Pirog et al. 2012)
Acinetobacter sp. YC-X 2	Beef extract and n-hexadecane	EI = 71.3%	(Chen et al. 2012)
Acinetobacter calcoaceticus BS	Crude oil (1%, v v^{-1})	EI = 64%	(Hassanshahian et al. 2012)
Acinetobacter calcoaceticus B-59190	Sodium citrate (20 g L^{-1})	Biosurfactant = 1.54 g L^{-1}	(Hošková et al. 2014)

Acinetobacter calcoaceticus IMV B-7241	Technical glycerol (2%, v v^{-1})	Biosurfactant = 5.6 g L^{-1}	(Pirog et al. 2015)
Acinetobacter calcoaceticus IMV B-7241	Glycerol as a by-product of biodiesel production and waste sunflower oil	Biosurfactant = 5 g L^{-1} (with glycerol) Biosurfactant = 4.3 g L^{-1} (with waste oil)	(Pirog et al. 2017)

Commercialization of biosurfactants is restricted due to the high cost of production (Desai and Banat 1997; Makkar et al. 2011). The cost of production could be decreased through optimization of fermentation conditions and the operational conditions during downstream biosurfactant separation and purification, as well as the utilization of inexpensive and/or waste substrates (Mukherjee et al. 2006; Marchant and Banat 2012).

Industrial wastes could be used as the sustainable carbon sources for biosurfactant production to reduce the cost of generating biosurfactants, which also provides promising options for waste treatment and disposal. Various renewable sources for biosurfactant production have been studied and proven to be effective, such as agro-industrial wastes, frying oils, oil refinery wastes, molasses, starch-rich wastes, cassava wastewater, potato waste and distilled grape marc (Fox and Bala 2000; Makkar and Cameotra 2002; Makkar et al. 2011; Maneerat 2005; Nitschke and Pastore 2006; Rivera et al. 2007; Sobrinho et al. 2008). However, the utilization of a waste stream as the carbon source for growth of *Acinetobacter calcoaceticus* has not been studied widely. Cashew apple juice has been studied as a viable carbon source for biosurfactant formation by an *Acinetobacter calcoaceticus* strain, with a maximum emulsion index of 85.7% (Rocha et al. 2006). In another study, *Acinetobacter calcoaceticus* IMV B-7241 was used to obtain 5 g L^{-1} and 4.3 g L^{-1} biosurfactant product when the carbon source was glycerol as a by-product of biodiesel production and waste sunflower oil, respectively (Pirog et al. 2017).

In this study waste cooking oil and glycerol (byproduct of biodiesel production) were used as the sole carbon sources for the generation of biosurfactant. Glycerol, a tribasic

alcohol, is a byproduct of the biodiesel generation process. In general, 1 kg of crude glycerol is produced during the generation of every 10 kg of biodiesel (Ciriminna et al. 2014). The glycerol market has been saturated already. Thus, any alternative consumption of this byproduct is advantageous to the larger scale production of biodiesel. Crude glycerol has been used widely as a carbon source for biosurfactant production (Zhang et al. 2005; Ciapina et al. 2006; Das et al. 2009; Santos et al. 2010; Silva et al. 2010; Faria et al. 2011; Sousa et al. 2012). Waste cooking oil has been utilized as a carbon source for biosurfactant production previously (Haba et al. 2000; Sadouk et al. 2008; Vedaraman and Venkatesh 2011; Luo et al. 2013). Various polar compounds and polymers are produced during the frying process. Adsorption has been used for improving the quality of used frying oil (Miyagi and Nakajima 2003; Wadekar et al. 2012; Asri and Puspita Sari 2015).

Response surface methodology (RSM) is an effective and useful alternative to design experiments. It has been widely used to fit experimental data to models by multiple regression analysis, to investigate the effect of several experimental variables, and to discover the conditions for the optimized response(s) (Khuri and Cornell 1996; Myer and Montgomery 2002). To economically produce a biosurfactant, the “best” cultural conditions such as temperature, pH, agitation, aeration, and medium compositions (e.g., carbon, nitrogen, and metal ions) must be determined. Biosurfactant production and optimization have been extensively studied previously (Joshi et al. 2008; Mukherjee et al. 2008; Pal et al. 2009; Najafi et al. 2010; Ghribi and Ellouze-Chaabouni 2011; Saikia et al. 2012; Luo et al. 2013; Hu et al. 2015; Kumar et al. 2015; Ebadipour et al. 2016; Sellami et al. 2016; Eswari et al. 2016; Bertrand et al. 2018). However, there are fewer studies on optimal biosurfactant generation using

Acinetobacter calcoaceticus. Amoabediny et al. (2010) looked at the fermentative conditions for biosurfactant production by *Acinetobacter calcoaceticus* (PTCC 1641) and determined that using a 5 vol.% inoculum size, 300 rpm agitation speed, and 15 mL of volume, the surface tension of 30.52 mN m^{-1} in solution was achieved (Amoabediny et al. 2010). RSM was used to optimize the media formulation for biosynthesis of a biosurfactant produced by *Acinetobacter* sp. YC-X 2, resulting in a formula with 3.12 g L^{-1} beef extract; 20.87 g L^{-1} peptone; 1.04 g L^{-1} NaCl; and 1.86 g L^{-1} n-hexadecane (Chen et al. 2012). The most popular RSM design for fitting second-order models, CCD was adopted in this study to design the experiments of biosurfactant formation by *Acinetobacter calcoaceticus* P1-1A.

The objectives of this research are to investigate the potential use of the local waste streams for biosurfactant production by the indigenous *Acinetobacter calcoaceticus* P1-1A strain and to achieve CCD based optimization of biosurfactant generation. The research targets on reducing the cost of biosurfactant production through using less expensive waste streams as the sole carbon source. The literature was reviewed extensively and only one paper (Pirog et al. 2017) used an *Acinetobacter calcoaceticus* strain for biosurfactant production with the waste cooking oil and glycerol (byproduct of biodiesel production), as the sole carbon source. In this study there was no work in optimizing the biosurfactant production.

4.2 Materials and Methods

4.2.1 Microorganism

The biosurfactant producer strain, *Acinetobacter calcoaceticus* P1-1A, was used in this study. It was isolated from an oily contaminated sample obtained from an offshore oil and gas platform in Atlantic Canada. The isolation was performed in the Northern Region Persistent Organic Pollution Control (NRPOP) laboratory (Memorial University). This strain was confirmed as a promising biosurfactant producer and showed a great potential of raising emulsification index and decreasing surface tension (Cai et al. 2014).

4.2.2 Waste streams as the sole carbon source

Waste cooking canola oil from a local restaurant was examined as the carbon source in this study. Silica gel was used as an adsorbent to remove thermal decomposition compounds from the waste oil to make it suitable as a source of carbon for microbial growth and biosurfactant production. A mixture of 50 g of filtered waste oil and 15 g of silica gel in a beaker was heated to 100 °C while stirring at the speed of 60 rpm for 1 hour. The mixture was filtered through a 0.45 µm pore size filter paper to separate the adsorbent from the solution and obtain the refined waste cooking oil.

A glycerol product generated from the conversion of fish oil to biodiesel was provided from Marine Bioprocessing Unit, Centre for Aquaculture and Seafood Development, Fisheries and Marine Institute, Memorial University. The glycerol was centrifuged and then sterilized by filtration through 0.45-pm membrane filters. After that, it was neutralized using 1 M H_2SO_4 and heated at 110 ºC under agitation for 90

minutes to remove the methanol content and again filtered to obtain the crude glycerol for biosurfactant production. The pretreated wastes (i.e., waste cooking canola oil, refined waste cooking oil and crude glycerol) were stored at –18 °C until needed.

4.2.3 Inoculum preparation

The *Acinetobacter calcoaceticus* P1-1A strain was cultured in a 50-mL nutrient salt medium, with 2 % (v v^{-1}) n-hexadecane as the carbon source in a 250 mL sterilized Erlenmeyer flask. After that, a loopful of bacteria colony from the agar plate were transferred to the media. The nutrient salt medium is composed of (g L^{-1}) $(NH_4)_2SO_4$, 5; NaCl, 15; KCl, 1; KH_2PO_4, 3; Na_2HPO_4, 1.2; $FeSO_4$ $7H_2O$, 0.002; $MgSO_4$, 0.2; yeast extract, 0.5. A 0.5-mL element solution containing $ZnSO_4$, 0.29; $CaCl_2$, 0.1; $CuSO_4$, 0.25; $MnSO_4$, 0.4 (g L^{-1}) was added to 1 L of the nutrient salt medium. The cultures were grown at 30 ºC and 200 rpm in the Innova 43 incubator shaker for 48 hrs.

4.2.4 Biosurfactant production and optimization using RSM

The experiments were conducted in 250 mL flasks containing 50 mL medium with 4% (v v^{-1}) of the inoculums at OD_{660} = 0.5 to ensure that each flask received an equal amount of the bacterial suspensions. The cultivation was performed at 200 rpm for six days. Multiple waste carbon sources including refined waste cooking oil, waste cooking oil, and crude glycerol were used and compared to commercial sources of carbon such as pure glycerol, diesel, ethanol, and n-hexadecane. Based on these tests the waste carbon source that showed the best support for bacterial growth was selected and added to the medium as outlined in table 4-2.

Table 4-2. Independent variables and their levels used in CCD

Independent variables	Code	Coded values and the corresponding values of parameters				
		-2	-1	0	1	2
Carbon % (v v^{-1})	A	1	3	5	7	9
Nitrogen (g L^{-1})	B	1	3	5	7	9
NaCl (g L^{-1})	C	5	10	15	20	25
pH	D	5	6	7	8	9
Temperature (°C)	E	21	26	31	36	41

To design experiments for optimization of biosurfactant production, CCD of response surface methodology (RSM) was applied. The difference values between each variable and the centre point value were divided by the step change to obtain the coded dimensionless X_i, to be used for statistical calculations. The CCD was applied, and the data were fitted to a predictive second-order polynomial equation using the relationship between the independent variables and their responses.

The selected independent process variables for this study were the concentration of carbon source (refined waste cooking oil), the nitrogen source ($(NH_4)_2SO_4$), sodium chloride, pH, and temperature, at five levels, with six replicates at the central points. The response variable was the emulsification index. The levels of the design variables are stated in Table 4-2. 1 M NaOH and 1 M HCl were used for pH adjustment.

Experiments were randomized to overcome any possible biases. All experiments were performed in duplicate. A flask without inoculum was used as the negative control.

To produce cell-free supernatant (crude biosurfactant), the culture broth was centrifuged at 10,000 rpm for 15 min and then passed through a 0.45 µm pore size filter paper. Emulsification index, surface tension (ST), concentration of the

biosurfactant, and critical micelle concentration were measured to evaluate performance and productivity of the crude biosurfactant. All measurements were conducted in triplicate.

4.2.5 Surface tension measurement

The amount of the energy used per unit area to transfer a molecule from the bulk phase to the surface is called surface tension. The surface tension of water without surfactant is 72 mN m^{-1}. With a surfactant, it can be decreased to 30 mN m^{-1} (Desai and Banat 1997; Soberón-Chávez and Maier 2011). A tensiometer (CSC Scientific Company) was used to measure the surface tension of the crude biosurfactant by the Du Nuoy ring method at the ambient temperature.

4.2.6 Emulsification index measurement

The emulsification activity is assessed by the ability of a biosurfactant to generate turbidity due to suspended hydrocarbons in a liquid system. In order to evaluate the emulsification capacity of the produced biosurfactant in this research, the emulsification index (EI) was measured. A mixture of 2 mL hexadecane and 2 mL of the crude biosurfactant was vortexed at high speed for 2 min. The mixture was kept at the room temperature for 24 hrs. Dividing the height of the emulsion layer by the total height of the liquid, multiplying by 100 provided the EI as illustrated in Eq. (1) (Silva et al. 2010; Sousa et al. 2012).

$$EI = \frac{Height\ of\ the\ emulsion\ layer}{Height\ of\ the\ total\ liquid} \times 100 \quad (1)$$

4.2.7 Critical micelle concentration measurement

During the addition of a surfactant, surface tension is reduced until the critical micelle concentration (CMC) is reached. CMC is commonly used to measure the efficiency of a surfactant. An efficient biosurfactant has a lower CMC, so less amount of the biosurfactant is required to reduce surface tension (Mulligan 2005). Above the CMC, the mechanism shifts and biosurfactant molecules associate to form micelles, bilayers, and vesicles, which enable biosurfactants to reduce the surface and interfacial tension (Soberón-Chávez and Maier 2011). Biosurfactants usually have CMCs between 1 to 200 mg l^{-1} (Mulligan 2005).

In this study, surface tensions of several dilution samples of the crude biosurfactant were measured. Then a graph of ST against biosurfactant concentration was plotted. The biosurfactant concentration at the intercept of the best two lines which can be fitted to the data leads to the CMC (Morais et al. 2017).

4.2.8 Emulsification stability test

The crude biosurfactant solution was maintained at constant temperatures in the range of 4–90 °C for 2 hrs and then reached to room temperature to test its thermal stability. The pH of the crude biosurfactant was adjusted to values of 4 to 10 to test its pH stability. For studying the resistance of the produced biosurfactant to salt, different concentrations of NaCl from 2 to 10 % (w v^{-1}) were added to the crude biosurfactant. EI was measured to check the stability of the produced biosurfactant at different conditions.

4.2.9 Biosurfactant recovery

The biosurfactant was recovered from the crude biosurfactant solution using the modified Folch mixture to calculate the concentration of produced biosurfactant (Pirog et al. 2017). Twenty mL of 1 M HCl was added to 100 mL of the crude biosurfactant in a 500-mL cylindrical separatory funnel. The funnel was shaken for 3 min. Then an additional 15 mL of 1 M HCl and 65 mL of chloroform–methanol mixture (chloroform:methanol = 2:1) were added, and the funnel was shaken for 5 min. The mixture in the funnel was set aside until phase separation. The bottom phase was released, and the water phase was extracted once more with 35 mL of 1 M HCl and 65 mL of chloroform–methanol mixture and again 100 mL of chloroform–methanol mixture was used for the third time. All three extracts were combined and evaporated in a rotary evaporator at 50°, until completely dry. The biosurfactant concentration was determined by dividing the weight of the dried product by the whole volume of the crude biosurfactant solution.

4.3 Results and Discussion

4.3.1 Evaluation of different carbon sources

The *Acinetobacter calcoaceticus* P1-1A strain can consume different kinds of carbon sources to produce biosurfactants. The refined waste cooking oil, waste cooking oil, crude glycerol, pure glycerol in 4 % (v v^{-1}), n-hexadecane, diesel and ethanol in 2 % (v v^{-1}) were used as the carbon sources in this study to examine the associated effect on biosurfactant production by *Acinetobacter calcoaceticus* P1-1A. The results using the waste streams were then compared with those with the commercial carbon

sources. The emulsification index and surface tension of the produced biosurfactant are presented in table 4-3.

Table 4-3. Effect of different carbon sources on biosurfactant production by *Acinetobacter calcoaceticus* P1-1A. Results represent the average of independent experiments ± standard deviation.

Carbon source	Refined waste cooking oil	Waste cooking oil	Crude glycerol	Pure glycerol	n-Hexadecane	Diesel	Ethanol
EI (%)	59.3±2.56	57.9±3.13	40.1±4.22	54.7±1.52	60.1±2.63	58.7±1.55	53.4±3.26
ST ($mN\ m^{-1}$)	37.5±0.33	38.4±0.56	49.5±0.61	44.7±0.44	36.6±0.42	38.1±0.56	42.1±0.6

Table 4-3 indicates that of the waste streams, the crude glycerol was not an efficient source for biosurfactant generation. The refined waste cooking oil led to slightly higher emulsification index and lower surface tension than the unrefined waste cooking oil and the refined oil will have lower contaminants that could impact longer term effectiveness. The emulsification index for the refined waste cooking oil was between the values obtained with some commercial sources of carbon (n-hexadecane and diesel) and higher than the value for ethanol. The surface tension values also showed that the refined waste cooking oil was the best waste stream studied and was comparable with the commercial sources. The refined waste cooking oil was then selected for further evaluation of the impacts of fermentation conditions on biosurfactant production by *Acinetobacter calcoaceticus* P1-1A.

4.3.2 Statistical model analysis

The optimization experiments were conducted according to the CCD (Design-Expert 10.0.1 software). The run orders and results are listed in Table 4-4.

Table 4-4. Experimental central composite design run sheet with actual independent and response variables

Run	Factor 1 A: Carbon (% (v v^{-1}))	Factor 2 B: Nitrogen (g L^{-1})	Factor 3 C: NaCl (g L^{-1})	Factor 4 D: pH	Factor 5 E: Temperature (°C)	Response EI (%)
1	5	5	15	5	31	31
2	1	5	15	7	31	50.8
3	7	3	20	6	36	39.1
4	7	3	10	8	36	58.8
5	7	7	20	8	36	61.8
6	3	3	20	8	36	52.6
7	5	1	15	7	31	48.9
8	5	5	15	9	31	56.9
9	3	3	10	6	36	45.5
10	5	5	5	7	31	56.1
11	5	5	15	7	31	65.7
12	5	5	15	7	31	60.3
13	7	7	10	8	26	51.5
14	5	5	15	7	31	61.9
15	5	5	15	7	31	67.2
16	3	7	10	8	36	68.5
17	3	3	10	8	26	53.8
18	7	3	10	6	26	33.6
19	7	7	10	6	36	48.1
20	5	5	15	7	31	66.7
21	3	7	20	6	36	54.5
22	5	5	15	7	41	57.3
23	5	5	25	7	31	52.5
24	3	3	20	6	26	41
25	5	9	15	7	31	59.2
26	3	7	20	8	26	55.6
27	3	7	10	6	26	46.2
28	7	3	20	8	26	45.5
29	9	5	15	7	31	48.7
30	5	5	15	7	21	48.3
31	5	5	15	7	31	57
32	7	7	20	6	26	35.6

The software was used to fit the experimental data to the model and find the coefficients of the second-order polynomial equation. The analysis of variance (ANOVA) was performed to validate the model and investigate the effect of factors on the response variable (i.e., EI). Results of ANOVA analysis are shown in Table 4-5. Values of "Prob > F" less than 0.05 specify model terms are significant. In this study, Design Expert suggested the quadratic model (p-value < 0.0001).

Table 4-5. ANOVA Summary for the quadratic model

Source	**Sum of Squares**	**df**	**Mean Square**	**F Value**	**p-value Prob > F**
Model	2740.53	20	137.03	10.65	0.0001
A-Carbon	95.60	1	95.60	7.43	0.0197
B-Nitrogen	219.01	1	219.01	17.03	0.0017
C-NaCl	31.51	1	31.51	2.45	0.1458
D-pH	1017.90	1	1017.90	79.14	< 0.0001
E-Temperature	294.70	1	294.70	22.91	0.0006
AB	8.85	1	8.85	0.69	0.4244
AC	5.625E-003	1	5.625E-003	4.373E-004	0.9837
AD	20.03	1	20.03	1.56	0.2380
AE	18.28	1	18.28	1.42	0.2583
BC	2.81	1	2.81	0.22	0.6496
BD	0.14	1	0.14	0.011	0.9186
BE	29.98	1	29.98	2.33	0.1551
CD	12.08	1	12.08	0.94	0.3534
CE	1.89	1	1.89	0.15	0.7087
DE	1.27	1	1.27	0.098	0.7596
A^2	275.32	1	275.32	21.40	0.0007
B^2	116.00	1	116.00	9.02	0.0120
C^2	108.83	1	108.83	8.46	0.0142
D^2	597.61	1	597.61	46.46	< 0.0001
E^2	155.33	1	155.33	12.08	0.0052
Residual	141.49	11	12.86		
Lack of Fit	58.47	6	9.75	0.59	0.7331
Pure Error	83.01	5	16.60		
Cor Total	2882.02	31			
Std. Dev.	3.59		R-Squared		0.9509
Mean	52.51		Adj R-Squared		0.8616
			Pred R-Squared		0.4295

After removing those model terms which are not significant from the model, the good fitness of the model can be concluded. The r-squared value is 0.9178, and the predicted r-squared of 0.8036 is in reasonable agreement with the adjusted r-squared of 0.8787; as the difference is less than 0.2, specifying an acceptable agreement between the experimental and predicted values.

The appropriateness of the model was checked using normal plots of residuals (Figure 4-1). The normality and good fit are shown by the straight-line plot. Therefore, the proposed model can be employed for prediction of the EI of the generated biosurfactant inside the range of the independent variables studied.

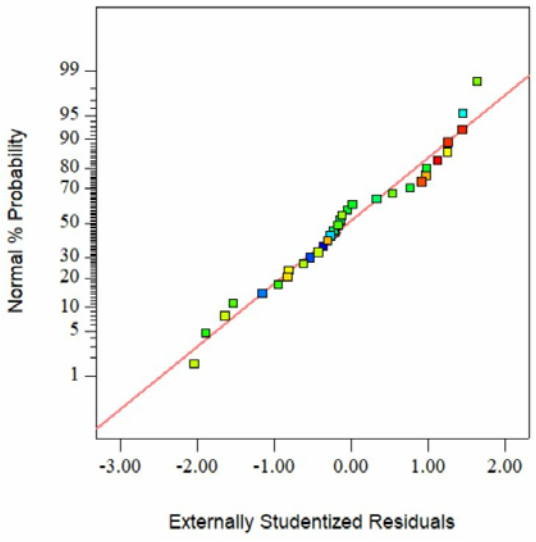

Figure 4-1. Normal plot of residuals

4.3.3 RSM results

Significant model terms are A (Carbon), B (Nitrogen), D (pH), E (Temperature), A^2, B^2, C^2, D^2, E^2 which express nonlinearities between the response and independent variables. The C (NaCl) should be in the model as C^2 is significant. The main significant model terms are D and D^2. The model does not indicate any interaction between factors. The final equation in terms of coded factors is as Eq. (2).

$$EI = + 62.85 - 2.00 \times A + 3.02 \times B - 1.15 \times C + 6.51 \times D + 3.5 \times E - 3.06 \times A^2 - 1.99 \times B^2 - 1.93 \times C^2 - 4.51 \times D^2 - 2.3 \times E^2 \quad (2)$$

Figure 4-2 summarizes the effect of factors on the emulsification index. There is a maximum carbon source concentration and any concentration over this maximum value negatively impacted bacterial growth or biosurfactant production. As Figure 4-2(A) illustrates, the addition of the refined waste cooking oil from 3 % (v/v) to 4.5% (v/v) improved the emulsification index, but higher values decreased the emulsification index.

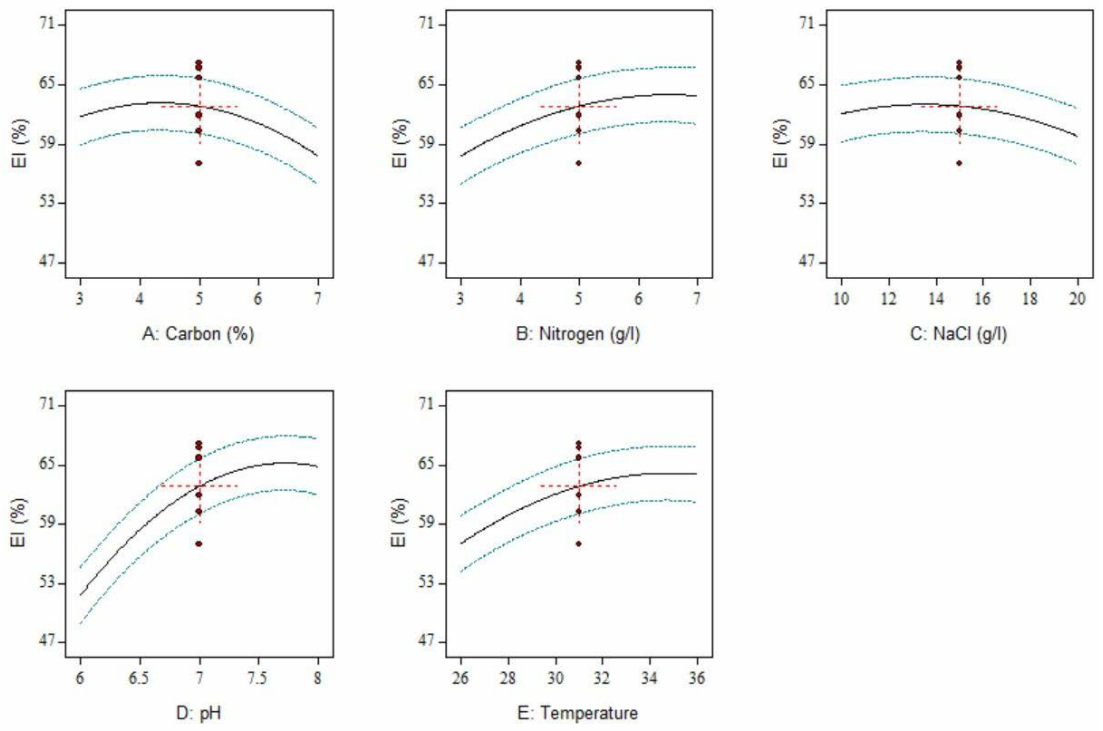

Figure 4-2. Effect of (A) Carbon source concentration (%), (B) nitrogen concentration (g L^{-1}), (C) NaCl concentration (g L^{-1}), (D) pH and (E) temperature (°C), on the emulsification index (EI) of the produced biosurfactant. The dotted lines show the 95% confidence band on the mean prediction

The nitrogen source is an important factor for bacterial growth and strains have an optimum carbon to nitrogen ratio. Lower or higher ratios lead to lower production rates (Guerra-Santos et al. 1986; Abouseoud et al. 2008). As can be seen in figure 4-

2(B), the emulsification index increased with the addition of ammonium sulfate until approximately the maximum level of nitrogen concentration in this study and then decreased slightly. The interaction between carbon and nitrogen sources were reported in some biosurfactant formation studies (Mutalik et al. 2008; Kim and Kim 2013; Ebadipour et al. 2016); however, it was not significant in this experiment in addition to some other works (Joshi et al. 2008; Kumar et al. 2015; Ozdal et al. 2017). The interaction between carbon and nitrogen can be related to the power of the carbon to nitrogen ratio in biomass growth and biosurfactant production. Although the carbon to nitrogen ratio is an important factor for biosurfactant generation, here the exact amounts of them are more critical than their ratio.

Addition of sodium chloride to the medium from 10 g L^{-1} to near 15 g L^{-1} had almost no effect on the emulsification index. However, a reduction in the emulsification index was observed with sodium chloride concentrations higher than 15 g L^{-1} (figure 4-2(C)). This could be due to plasmolysis of the cells at hypertonic conditions. The NaCl concentration is an important factor for biosurfactant production, given the strain is isolated from the marine environments. The optimal NaCl concentration was found to be 20 g L^{-1} for biosurfactant formation by a marine *Vibrio* species (Hu et al. 2015). Sodium chloride at a concentration of 20–30 g L^{-1} is required for emulsifier production by a marine strain of *Yarrowia lipolytica* (Zinjarde and Pant 2002). Another marine strain of *Brevibacterium casei* produced the maximum amount of biosurfactant in the presence of 20 g L^{-1} NaCl in the culture medium (Kiran et al. 2010). A *Pseudoalteromonas agarovorans* strain was shown to produce a high amount of biosurfactant at NaCl concentration of 30 g L^{-1} (Choi et al. 2009).

In this study, the most significant factor that affected the emulsification index was pH. As figure 4-2(D) shows, the emulsification index increased sharply as pH increased from 6 to 7.5 and then decreased slightly at higher values. The value of pH can affect the microbial growth and reproduction acutely.

Higher temperatures would increase the enzymatic activity which can increase the rate of metabolism and reproduction (Zhang et al. 2015). However, there is an optimal temperature for each microbial strain. According to this study (figure 4-2(E)), the temperature has the same trend as the nitrogen source concentration and rising it to near 35 °C, resulted in the emulsification index increase. Temperatures higher than 35 °C, decreased emulsification index slightly.

4.3.4 Response variables under optimized conditions

The best combination of independent variables to maximize the emulsification index were carbon concentration of 4.35% (v v^{-1}), the nitrogen concentration of 6.5 g L^{-1}, NaCl concentration of 13.5 g L^{-1}, pH of 7.7, and 34.8 °C. Under such optimized conditions, a maximum emulsification index of 68.17% was predicted.

The conditions outlined above were used in experiments to validate the model, and an emulsification index of 67.7% was achieved. As this value was within the prediction intervals, and the difference between the predicted value and the experimental data was less than 0.5%, the model is validated. This value is close to the maximum EI of 71.3% achieved in a study by *Acinetobacter* sp. YC-X 2 (Chen et al. 2012). The surface tension, the concentration of the biosurfactant and CMC at these conditions were 32.4 mN m^{-1}, 862 mg L^{-1}, and 156 mg L^{-1}, respectively.

The results were compared to cultivation at the conditions above using 2 % (v v^{-1}) n-hexadecane as the sole carbon source. The emulsification index was found to be 64.35%, and the corresponding surface tension, the concentration of the biosurfactant, and CMC were 35.1 mN m^{-1}, 743 mg L^{-1}, and 137 mg L^{-1} respectively. In this study the refined waste cooking oil outperformed the commercial carbon source terms of EI, biosurfactant produced, and CMC and on the similarly regarding surface tension.

4.3.5 Emulsification stability

The results of the stability tests are illustrated in figure 4-3. The biosurfactant obtained from this study showed a high level of thermal stability as the emulsification index was stable over the temperature range (4 - 90 °C). The emulsification index of the produced biosurfactant was steady in the NaCl concentration range of 2 to 10 % (w v^{-1}), showing stability in high salinity. The emulsification index was constant under neutral-basic conditions (pH=5.5-10). However, the emulsification index decreased from 63.5% at the pH of 5.5 to 50% at the pH of 4. Overall the biosurfactant is stable at alkaline conditions, but not under acidic conditions. The high stability of the produced biosurfactant by *Acinetobacter calcoaceticus* P1-1A using refined waste cooking oil demonstrates its capability for various commercial applications.

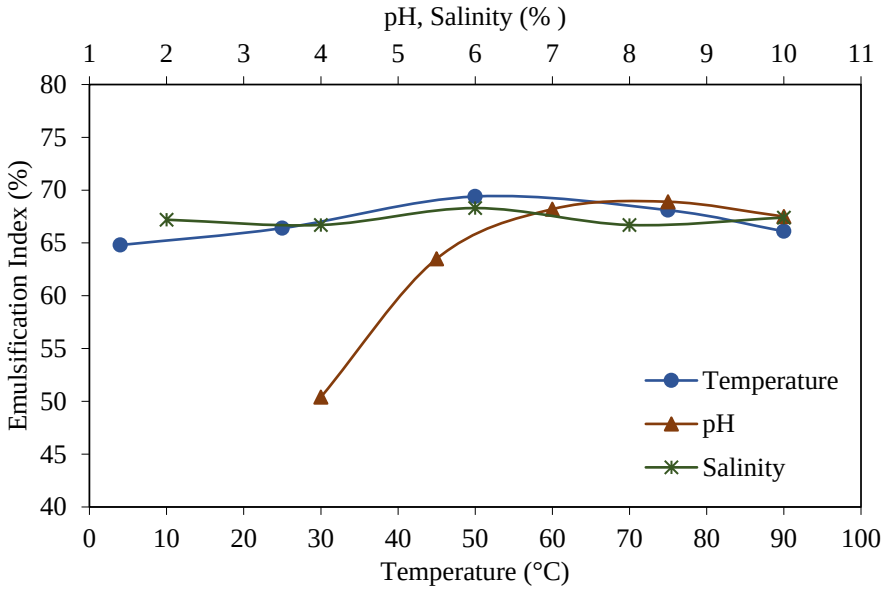

Figure 4-3. Effect of temperature, pH, and salinity on the emulsification index of the produced biosurfactant

4.4 Summary

The results of this research show the effectiveness of using local waste cooking oil as a cost-efficient carbon source for biosurfactant production using the indigenous *Acinetobacter calcoaceticus* P1-1A. Five factors including the concentration of carbon, nitrogen and NaCl, pH and temperature were studied using RSM. All factors influenced the biosurfactant generation, while pH had the most effect. The optimum emulsification index was found to be 67.7%, and the comparison of the results with a commercial carbon source showed the sustainability of using the refined waste cooking oil for the biosurfactant production. The produced biosurfactant showed high stability at different temperatures, pHs and salinities. Using this low-cost source of carbon instead of the expensive commercial products would have the environmental

advantage of converting the waste stream into the valuable product and reducing the costs associated with biosurfactant formation.

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Chapter Five: Kinetic modeling of biosurfactant production by *Bacillus subtilis* N3-1P using brewery waste

This chapter is under review by the Applied Biochemistry and Biotechnology Journal. Bahareh Moshtagh is the primary author and the co-authors are Dr. Kelly Hawboldt and Dr. Baiyu Zhang. Ms. Moshtagh's contributions to this paper include writing the paper, performing all the laboratory testing and analyses, and interpreting the results. Dr. Hawboldt and Dr. Baiyu Zhang contributed in providing technical guidance, reviewing, and revising the manuscript.

Abstract

The indigenous *Bacillus subtilis* N3-1P strain and a brewery waste as the source of carbon were used to produce a biosurfactant. The batch cultivation was performed under the optimum conditions. Models describing the biomass growth, biosurfactant production, and substrate utilization were developed by fitting the experimental data to the Logistic, Contois and Luedeking-Piret models using MATLAB software and regression analysis. The kinetic parameters including the maximum specific growth rates (μ_{max}), the Contois constant (K), parameters of the Luedeking-Piret models, $Y_{X/S}$, $Y_{P/S}$, and $Y_{P/X}$ were calculated. The experimental and predicted model showed good agreement. The developed models are a key step in designing reactors for scale up of biosurfactant production.

5.1 Introduction

Surfactants are compounds which decrease surface and interfacial tensions between different phases. Biosurfactants are more readily biodegradable than their chemically synthesized alternatives, and highly efficient. Biosurfactants are useful in industries including bioremediation, health care, food, cosmetic and oil processing as emulsifiers, dispersants, foamers, detergents and wetting agents (Desai and Banat 1997; Makkar et al. 2011).

Various species of microorganisms show the ability to produce biosurfactants, particularly *Bacillus subtilis* strains (Abdel-Mawgoud et al. 2008). However, the high cost of biomass production is a barrier to large-scale biosurfactant production. One effective strategy to lower the cost is replacing commercial substrates in the growth

media with the wastes or by-product streams from different industries, such as food processing. Different waste streams have been used in biosurfactant production with *Bacillus subtilis* strains including potato waste, cassava wastewater, milk whey, waste frying oils, by-product glycerol from biodiesel production, molasses, and corn steep liquor (Fox and Bala 2000; Nitschke et al. 2004; Nitschke and Pastore 2006; Faria et al. 2011; Vedaraman and Venkatesh 2011; Sousa et al. 2012; Gudiña et al. 2015; Liu et al. 2015). In this work we used an indigenous *Bacillus subtilis* strain (isolated from the oily contaminated marine environment in Atlantic Canada) (Cai et al. 2014) for biosurfactant formation and modeling the rate of production to facilitate the larger scale biosurfactant generation. Brewery waste from the first stage of beer production at Quidi Vidi Brewing Company was used. This material is composed of spent grain and sugars, including maltose and glucose, which was used as the carbon source.

Another option for reducing the cost is optimizing the cultivation conditions and medium compositions using experimental design techniques such as factorial design and response surface methodology (RSM) (Khuri and Cornell 1996; Myer and Montgomery 2002). Biosurfactant formation optimization using *Bacillus subtilis* strains has been studied previously (Sen 1997; Jacques et al. 1999; Mukherjee et al. 2008; Ghribi et al. 2011; Kim and Kim 2013; Kumar et al. 2015; Liu et al. 2015). In a previous work by the authors, RSM was used in biosurfactant production with the above strain and brewery waste as the sole carbon source. The optimum growth conditions determined in this work was used in this study (Moshtagh et al. 2018).

Kinetic models are used to predict microbial growth, substrate and bioproduct formation during fermentation and hence bioreactor design and downstream

processing requirements. There are several studies on modeling the biosurfactant generation and microbial growth (Young et al. 2005; Rodrigues et al. 2006; Moussa et al. 2010; Kumar et al. 2014; Sadouk-Hachaïchi et al. 2014). The equations used to in this study to model the growth and production are outlined below, and it is assumed that the carbon source is the only growth limiting substrate.

The equation relating biomass growth as a function of time t in the exponential growth phase is outlined in Eq. (1) (Villadsen et al. 2011).

$$\frac{dX}{dt} = \mu . X \tag{1}$$

where X is the cell mass concentration (g L^{-1}), t is the time (hr), and μ is the specific growth rate (hr^{-1}) for the particular strain. The integrated form of Eq. (1) is:

$$\ln \frac{X}{X_0} = \mu . t \tag{2}$$

where X_0 is the initial biomass concentration.

The relationship between biomass concentration with time in both exponential and stationary phases can be represented using the logistic model in Eq. (3) (Villadsen et al. 2011).

$$\frac{dX}{dt} = \mu_{max} X \left(1 - \frac{X}{X_{max}}\right) \tag{3}$$

where μ_{max} is the maximum specific growth rate coefficient (hr^{-1}), and X_{max} is the maximum concentration of cells (g L^{-1}). By integrating Eq. (3), Eq. (4) yields:

$$X = \frac{X_0 e^{(\mu_{max} t)}}{1 - \frac{X_0}{X_{max}}\left(1 - e^{(\mu_{max} t)}\right)} \tag{4}$$

The growth rate is a function of the substrate concentration. Typical kinetic models have been used to describe this relationship include; Monod, Tessier, Contois, and Mosser (Villadsen et al. 2011). The Contois model relates the growth rate to the concentration of a single source of energy and is a derivation from the Monod equation:

$$\mu = \mu_{max} \cdot \frac{S}{KX+S} \quad (5)$$

where S is the substrate concentration, and K is the Contois constant. Among the mentioned models, the best result was obtained with the Contois model, so it was used in this study. As for each organism, the coefficient varies with environmental conditions thus needs to be estimated for each strain, under defined conditions of temperature and medium composition.

The biomass yield ($Y_{x/s}$) relates the specific rate of biomass growth and substrate consumption:

$$\frac{dS}{dt} = -\frac{1}{Y_{X/S}} \cdot \frac{dX}{dt} \quad \text{where} \quad Y_{X/S} = \frac{dX}{dS} \quad (6)$$

For biosurfactant production, the Luedeking- Piret model (Luedeking and Piret 2000; Kumar et al. 2014) was used.

$$\frac{dP}{dt} = a\frac{dX}{dt} + bX \quad (7)$$

where P is the product formation concentration, ‘a’ is the growth associated term, and ‘b’ is the non-growth associated term.

Eq. (8) is derived from integrating Eq. (7).

$$P = aX_0\left(\frac{e^{\mu_{max}t}}{1-\left(\frac{X_0}{X_{max}}(1-e^{\mu_{max}t})\right)}-1\right)+b\,\frac{X_{max}}{\mu_{max}}\ln\left(1-\frac{X_0}{X_{max}}(1-e^{\mu_{max}t})\right) \quad (8)$$

$Y_{P/S}$ is the product yield coefficient based on the substrate and $Y_{P/X}$ is the specific product yield coefficient as Eqs. (9,10).

$$\frac{dS}{dt} = -\frac{1}{Y_{P/S}}.\frac{dP}{dt} \qquad \text{where } Y_{P/S} = \frac{dP}{dS} \quad (9)$$

$$\frac{dP}{dt} = Y_{P/X}.\frac{dX}{dt} \qquad \text{where } Y_{P/X} = \frac{dP}{dX} \quad (10)$$

The substrate utilization can be determined by Eq. (11).

$$-\frac{dS}{dt} = c\frac{dX}{dt} + dX \quad (11)$$

where, ‘c’ and ‘d’ are the kinetic constants.

The integration of Eq. (11) leads to Eq. (12).

$$S = S_0 - c\,X_0\left(\frac{e^{\mu_{max}t}}{1-\left(\frac{X_0}{X_{max}}(1-e^{\mu_{max}t})\right)}-1\right)-d\,\frac{X_{max}}{\mu_{max}}\ln\left(1-\frac{X_0}{X_{max}}(1-e^{\mu_{max}t})\right) \quad (12)$$

There is little research on kinetic modeling of the bioprocess of the biosurfactant production with several *Bacillus subtilis* strains and equations (Heryani and Putra 2017; Sakthipriya et al. 2018) and no work was found on modeling the biosurfactant formation, biomass growth or substrate utilization with the *Bacillus subtilis* N3-1P. The biosurfactant produced with this strain is a potential means for oil spill removal in the harsh marine environment. The dependency of the kinetic models to the specific

microorganisms and environmental conditions (Tan et al. 1996), requires an understanding of the biosurfactant production kinetics under these conditions.

The aim of this study is to identify the kinetic parameters of the indigenous *Bacillus subtilis* N3-1P strain, using experimental data obtained from a batch bacterial cultivation. The models for biomass growth, consuming of substrate and production of biosurfactant were developed.

5.2 Materials and Methods

5.2.1 Microorganism

The indigenous *Bacillus subtilis* N3-1P isolated from petroleum hydrocarbon contaminated sources in cold marine environment of Atlantic Canada was used in this work. The isolation was performed in the Northern Region Persistent Organic Pollution Control (NRPOP) laboratory (Cai et al. 2014).

5.2.2 Obtaining and treating the brewery waste

The waste of the first stage of the beer production at Quidi Vidi Brewing Company was chosen for this study. The obtained waste was centrifuged and micro-filtered through a 0.2 µ membrane filter. The treated brewery waste was stored at –18 °C until needed.

5.2.3 Inoculum preparation

The nutrient salt medium is composed of (g L^{-1}) NH_4NO_3, 4; Na_2HPO_4, 5; NaCl, 3.3; KH_2PO_4, 4; $FeSO_4$ 7H2O, 0.0012; yeast extract, 0.5. A 0.5-ml element solution

containing $ZnSO_4$, 0.29; $CaCl_2$, 0.1; $MnSO_4$, 0.4 (g L^{-1}) was added to 1 L of the nutrient salt medium. The inoculation was performed in a 50-mL nutrient salt medium and 8 g L^{-1} glucose as the carbon source in a 125 mL sterilized Erlenmeyer flask with a loopful of bacteria colony from the agar plate. The Innova 43 incubator shaker was used for culturing the bacteria for 24 h at 30 ºC and 200 rpm. The optical density of the broth was then measured by a spectrophotometer, and the amount equal to 2% (v v^{-1}) at OD_{660} = 0.5 was used as the inoculum for the cultivation process.

5.2.4 Production of biosurfactants

The cultivation was conducted into 125 mL flasks containing 50 mL medium under optimum conditions which were identified in the previous study (7% (v v^{-1}) brewery waste, 6.22 g L^{-1} ammonium nitrate, initial pH of 6.41, 150 rpm, and 27°C). Therefore, the medium was composed of (g L^{-1}) NH_4NO_3, 6.22; Na_2HPO_4, 5; NaCl, 3.3; KH_2PO_4, 4; $FeSO_4$ 7H2O, 0.0012; yeast extract, 0.5 and 7% (v v^{-1}) brewery waste with element solution as described above. The samples were taken from the rotary shaker at different time intervals up to 72 hrs. The initial pH was adjusted to 6.41 using 1 M NaOH and H_3PO_4. All experiments were conducted in duplicate.

Cell-free supernatant was attained by centrifuging the culture broth at 8000 rpm for 15 min and this broth was used to measure surface tension, to evaluate the biosurfactant production and the substrate concentration. All measurements were performed in triplicate.

5.2.5 Biomass measurement

Bacterial growth of samples was monitored by the dry weight method. To measure the dry weight of biomass, 10 mL of culture broth was filtered through a pre-weighted 0.2 µm filter paper and washed two times with 10 mL distilled water. The filter paper was then left in an oven to dry for 24 h. After that, it was cooled down in desiccators before reading the final weight. The biomass was expressed as cell dry weight (g L^{-1}).

5.2.6 Substrate measurement

In this study substrate refers to the source of carbon (brewery waste). As such, the substrate concentration was monitored by measuring the total carbohydrate of the cell-free supernatant, analyzed using the anthrone method and Agilent 8453 spectrophotometer and was demonstrated as g L^{-1}.

5.2.7 Surface tension measurement

The Du Nuoy ring method was used for determining the surface tension of the cell-free supernatant. A tensiometer (CSC Scientific Company) was used for this purpose and measurements were condcuted at room temperature.

5.2.8 Evaluation of biosurfactant production

The biosurfactant concentration (g L^{-1}) was determined using the curve of biosurfactant concentration vs. surface tension obtained under the optimum conditions (Moshtagh et al. 2018).

5.2.9 Modeling

The biomass, substrate utilization and product concentration were measured as a function of time. The sampling time intervals were every 8 hours and each time two samples were taken. Kinetic parameters were first calculated by linearization using Excel software, and these values were used as initial conditions for modeling to determine the parameters values by non-linear regression using MATLAB R2017b software.

5.3 Results and Discussion

5.3.1 Biomass, biosurfactant production and substrate utilization as a function of time

In a previous work (Moshtagh et al. 2018), the *Bacillus subtilis* N3-1P strain production of biosurfactant using brewery waste as the sole carbon source compared favorably with a commercial carbon source. The response surface methodology (RSM) was used to optimize biosurfactant production, with a concentration of brewery waste of 7% (v/v), ammonium nitrate of 6.22 g L^{-1}, pH of 6.41, agitation speed of 150 rpm, and temperature of 27°C.

The profiles of biomass growth, biosurfactant production, and carbon consumption in substrate are presented in figure 5-1. As shown in figure 5-1, after a lag phase of 8 hrs, the bacterial growth increased during the exponential phase of the cultivation, followed by a rise in biosurfactant generation and a sharp decline in the concentration of the substrate. In the stationary phase (~ 50 hrs), the concentration of biomass was

maximum at 3.42 g L^{-1} (X_{max}). Biosurfactant production started increasing after 24 hours of cultivation and reached a maximum of 0.679 g L^{-1} after 64 hrs.

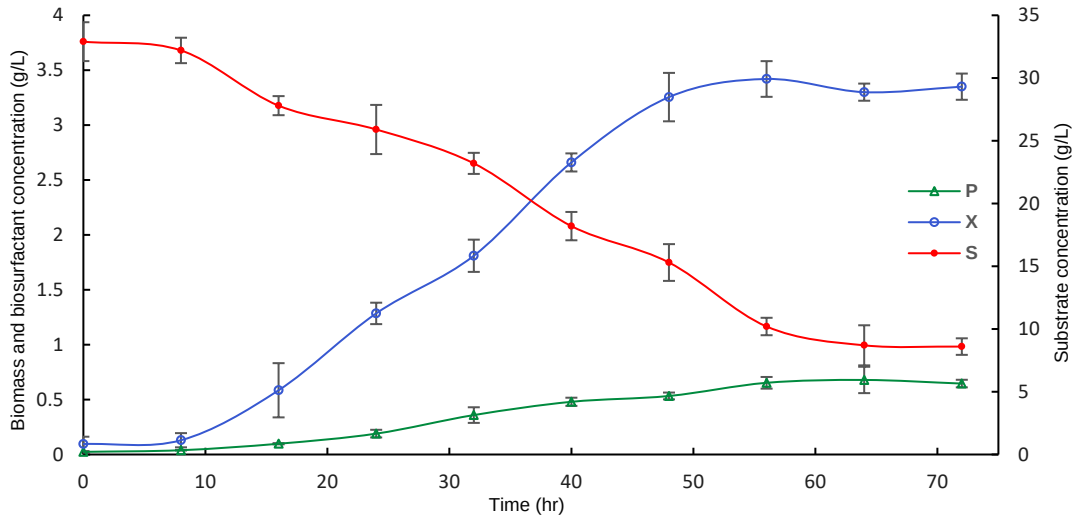

Figure 5-1. Time course profile for experimental data of biomass growth (X), biosurfactant production (P), and substrate utilization (S). Error bars represent standard deviation from the mean value.

5.3.2 Microbial growth kinetics

The Contois model (Eq. (5)) was used to relate the biomass growth rate to the concentration of the substrate. To determine Contois kinetics, the data after the lag phase until the stationary phase was considered. The μ_{max} was 0.1213 h^{-1} and K was 3.1766 $g_{substrate}$ $g_{biomass}^{-1}$. The coefficient of determination (R^2) value is generally used to describe the goodness of fit for the experimental and calculated data. The R^2 value for fitting the contois model was found to be 0.96. In a previous study Contois model was applied for modeling the biomass used to model growth of *Bacillus subtilis* YB7 with waxy crude oil as a carbon source. The μ_{max} was 0.64 h^{-1}, with a R^2 of 0.81. Using the Monod model, the μ_{max} was 0.1 h^{-1} with R^2 of 0.97 (Sakthipriya et al. 2018).

The Monod model was also used to describe the growth of *Gordonia alkanivorans* CC-JG39 and diesel as the source of carbon giving a μ_{max} of 0.158 h^{-1} (Young et al. 2005).

The logistic equation (Eq. (3)) was also used to relate the biomass concentration to time. The μ_{max} was 0.1242 h^{-1}, and X_0 was 0.0857 g L^{-1}, with R^2 of 0.9941, which shows good prediction (Figure 5-2). The μ_{max} was relatively close to the one derived from the Contois model. The calculated μ_{max} is less than 0.447 h^{-1} from a *Streptococcus thermophiles* strain using cheese whey for biosurfactant production (Rodrigues et al. 2006), and more than 0.0177 h^{-1} and 0.0467 h^{-1} for *Nocardia amarae* and *Pseudomonas aeruginosa* strains where olive oil and sawdust were used as carbon sources for biosurfactant formation, respectively (Moussa et al. 2010; Kumar et al. 2014).

The biomass yield was 0.143 $g_{biomass}$ $g_{substrate}^{-1}$. In biosurfactant production studies using *Streptococcus thermophiles* and *Nocardia amarae* strains, values of 0.27 and 0.039 were observed (Rodrigues et al. 2006; Moussa et al. 2010).

5.3.3 Biosurfactant formation kinetics

The kinetics of the biosurfactant production was evaluated using Eq. (7). The "a" parameter was 0.1695 $g_{biosurfactant}$ $g_{biomass}^{-1}$ and 'b' was 0.0008 $g_{biosurfactant}$ $g_{biomass}^{-1}$ h^{-1} with R^2 value of 0.9916. In a study of biosurfactant production using a *Pseudomonas aeruginosa* strain and sawdust as the carbon source the 'a' value was neglible and 'b' was calculated to be 0.186 $g_{biosurfactant}$ $g_{biomass}^{-1}$ (Kumar et al. 2014). This means the biosurfactant production is not associated strongly with growth, but rather the non-

growth parameter ('b'). The calculated data against the experimental are compared in figure 5-2. In this work the growth associated constant, 'a' is much greater than the non-growth associated constant 'b', and therefore the biosurfactant production is dominated by the growth of the bacteria. Eqs (9,10) were used to calculate the product yield coefficient based on the substrate (0.026 $g_{biosurfactant}\ g_{substrate}^{-1}$) and biomass (0.188 $g_{biosurfactant}\ g_{biomass}^{-1}$). In the studies noted above values of 0.06 $g_{biosurfactant}\ g_{substrate}^{-1}$ (Rodrigues et al. 2006) and 0.0828 $g_{biosurfactant}\ g_{substrate}^{-1}$ (Moussa et al. 2010) were reported for $Y_{P/S}$, and $Y_{P/X}$ was found to be 0.24 $g_{biosurfactant}\ g_{biomass}^{-1}$ (Rodrigues et al. 2006) and 1.02 $g_{biosurfactant}\ g_{biomass}^{-1}$ (Kumar et al. 2014).

5.3.4 Substrate utilization kinetics

The initial substrate concentration used in this study was 32.9 g L^{-1}, and the value of μ_{max} from the logistic equation (0.1242 h^{-1}) was used to fit the experimental data to the Eq. (12) due to improved fit of the logistic model. The values of the constants 'c' and 'd' were 4.79 $g_{substrate}\ g_{biomass}^{-1}$ and 0.0656 $g_{substrate}\ g_{biomass}^{-1}\ h^{-1}$, respectively. The R^2 value was 0.9808. Again, the simulated model for substrate utilization was compared to the experimental data in figure 5-2.

The developed kinetic values reflect the medium used, given this is a waste stream, the medium composition could vary. The variability of the medium means additional tests with waste from different times/batches (e.g., brewery waste) to confirm the kinetic parameters fall within an acceptable range, but recognizing there will be variability. In short, the outputs of the simulated models are in accordance with the experimental results, proving the accuracy and adequate representation of the proposed models at the optimum conditions.

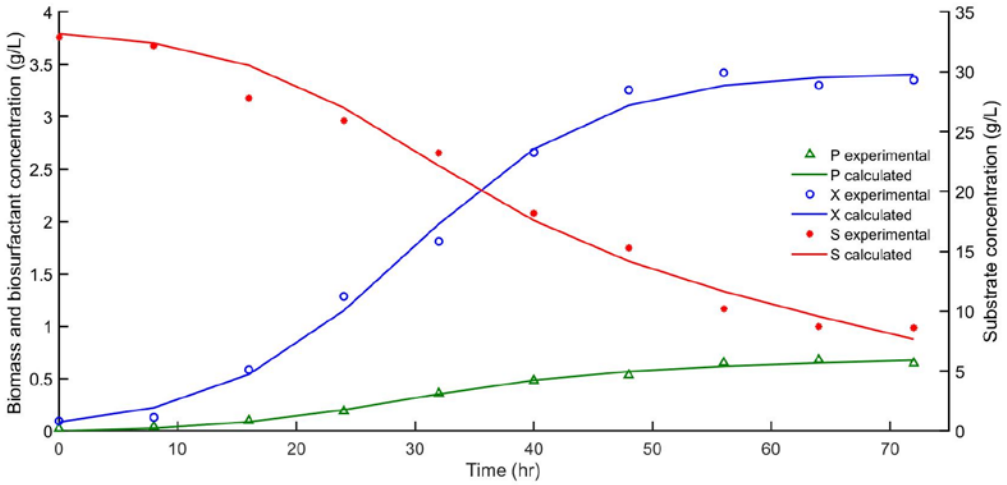

Figure 5-2. Time course profile for experimental and calculated data of biomass growth (X), biosurfactant production (P), and substrate utilization (S)

5.4 Summary

The kinetic and model equations for the production of biosurfactant using indigenous *Bacillus subtilis* N3-1P strain and a brewery waste as carbon source were determined in this study. The logistic model was used for growth pattern in both exponential and stationary phases. Contois model was also applied to relate the biomass growth rate to the concentration of the substrate in the exponential phase. The logistic model selected due to better fit. The biosurfactant production was modeled by the Luedeking-Piret model, and it was observed that biosurfactant formation was growth associated. The high values of the coefficient of determination and the graphs of the experimental and simulated results showed the accuracy of the developed models and parameters. The developed models would provide an understanding of bacterial cultivation and controlling the biomass growth, biosurfactant production and substrate utilization kinetics in large-scale production.

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Chapter Six: Research conclusions and recommendations

6.1 Conclusions

Surfactants have used widely in various household and industry applications. The global surfactant market was $31 billion in 2016, and the demand is increasing. From an environmental presepective it is desireable to develop alternatives to the chemically synthesized surfactants due to non-biodegradability and toxicity of some surfactants. Biosurfactants which are produced with microorganisms can be used for this purpose. However, the high cost of biosurfactant production is an obstacle to large-scale production and commercialization of them. The most effective methods to lower the production cost include the use of low cost or waste substrates and the development of more effective bioprocess by optimizing the culture conditions and downstream separation process. As shown in the literature review, several low cost non-synthetic and industrial wastes can be used as substrates for biosurfactant generation. Using these substrates is important from both environmental and economical perspective and can be considered not only as a waste to be treated but also a resource to be recovered. It is widely recognized that experimental design techniques like response surface methodology (RSM) are useful in optimizing different conditions on biosurfactant production, and several studies have been done in this regard. These two methods were applied in this study as the aim of this research is reducing the costs associated with biosurfactant production to justify its commercialization. Also, kinetic models for biomass growth, substrate utilization and biosurfactant formation were derived to have a better insight into the process and to enhance the large-scale production.

For this purpose, several local industrial waste streams were assessed for biosurfactant generation using bacterial strains indigenous to Atlantic waters. The selected waste streams were brewery waste, glycerol from the conversion of fish oil to biodiesel, fish wastes, waste cooking oil and produced water. The initial tests revealed that the glycerol, fish wastes and produced water were not suitable for biosurfactant production with the selected strains. The appropriate carbon sources were selected for further experiments.

Chapter three investigates the use of the local brewery waste for biosurfactant formation with indigenous *Bacillus subtilis* N3-1P for the first time. Initial screening showed there was sufficient biosurfactant production to continue with more detailed experiments to optimize the biosurfactant generation with different parameters including carbon and nitrogen concentrations, pH, agitation speed and temperature. pH had the most signifcant effect on production. The optimum conditions for generating biosurfactant were determined, and reliable surface tension, emulsification index, biosurfactant concentration, and critical micelle concentration were obtained (ST = 27.26 mN m^{-1}, EI = 62.33%), which compared favourably with a commercial carbon source.

In chapter four a local waste cooking oil was used as a carbon source for biosurfactant production from indigenous *Acinetobacter calcoaceticus* P1-1A. The independent variables were carbon, nitrogen and NaCl concentrations, pH and temperature, and the emulsification index were measured as the response variable. It was the first study in optimizing the biosurfactant production using the waste cooking oil and an *Acinetobacter calcoaceticus* strain. All chosen parameters, especially pH, affected the

EI which reached 67.7% at optimum conditions. The biosurfactant produced using a commercial carbon source was compared and demonstrated the capability of the refined waste cooking oil for the biosurfactant production with the chosen strain. The produced biosurfactant was exposed to different temperatures, pHs and salinities to test its effectiveness at harsh conditions, and it showed high stability, ensuring its potential for oil spill remediation at the cold marine environment.

It is essential to increase our knowledge of the whole process of biosurfactant production and to improve the commercial design and monitoring of biomass growth, biosurfactant production, and substrate utilization. For this purpose, the kinetic parameters and models were developed by fitting the experimental data to the models utilizing the MATLAB software and regression analysis in chapter five. The experiments were conducted using the brewery waste as the carbon source and the indigenous *Bacillus subtilis* N3-1P strain under the optimum conditions which were found in chapter three. Modeling the biosurfactant formation, biomass growth and substrate utilization with a *Bacillus subtilis* strain and the brewery waste was investigated for the first time in this study. For biomass growth, the logistic model was applied, and the Contois model was used to describe the biomass growth rate and the concentration of the substrate. The Luedeking-Piret model was employed for modeling the biosurfactant formation, and the product formation was found to be growth associated. The proposed models and kinetic parameters were validated by comparing the graphs of the experimental and predicted results and acceptable values of coefficients of determination.

The most effective technique for lowering the costs associated with biosurfactant production is using sustainable and inexpensive carbon sources which were investigated in this thesis. Two local strains and waste streams were found to be effective for biosurfactant production. Other useful methods for reducing the costs and understanding the cultivation process and scaling up the biosurfactant production were also analyzed in this work by applying the response surface methodology to find the optimum conditions for biosurfactant production, and developing the models for the biosurfactant generation, biomass growth and substrate utilization. The local bacterial strains would have the best capability to be used for the treatment of the oil spills in marine environments which the used bacteria are indigenous to. As each stain respond differently to the substrate and environmental conditions during the cultivation process, it is necessary to conduct the appropriate experiments to find the optimum substrate and conditions to produce the useful substances with the reasonable costs.

6.2 Recommendations for future work

- Further work is required in assessing the economics around waste as a substrate, and cost evaluation of all the processes. The results need to be compared to the costs of commercial substrates for biosurfactant production to find out how efficient the process is.
- All parts of this study were performed at the laboratory scale. More studies are required to produce biosurfactants on a pilot scale. For economic performance, the optimized conditions and model parameters need to be supported at a larger scale.
- Further studies should be done on the application of the produced biosurfactants for oil spill response in the simulated harsh conditions. For this purpose, appropriate solvents should be modified to generate biodispersants.
- The composition and structure of the produced biosurfactants can be characterized in another study.
- It might be helpful to genetically engineer the bacteria to develop over-producer strains.

Appendix I: Initial and failure experiments

Industrial waste streams, including brewery waste, glycerol from the conversion of oil to biodiesel, fish wastes, waste cooking oil and produced water were chosen to be tested as substrates (nutrient source) for microbial growth and biosurfactant production. Waste streams were treated to remove all solid particles and microbial production inhibitors. All waste streams were stored at –18 °C until needed.

Some strains of *Bacillus subtilis*, *Rhodococcus erythropolis*, and *Acinetobacter calcoaceticus* which were isolated from petroleum hydrocarbon contaminated sources in NL and a commercial *Bacillus subtilis* strain were chosen for further studies.

Inoculum preparation

Each microbial strain was enriched using 50 mL nutrient salt medium and a carbon source in a 250 mL Erlenmeyer flask. The nutrient broth was sterilized in an autoclave for 45 min. After that trace metal solution was added and a colony of bacteria was transferred to the medium under the biosafety cabinet with fire and loop. The culture was grown on a rotary incubator shaker for 24 to 48 hrs at 30 ºC and 200 rpm.

The nutrient salt medium for *Rhodococcus erythropolis* is composed of (g L^{-1}) $(NH_4)_2SO_4$, 10; KCl, 1.1; NaCl, 1.1; $FeSO_4$ $7H_2O$, 2.8* 10^{-4}; KH_2PO_4, 3.4; K_2HPO_4 $3H_2O$, 4.4; $MgSO_4$, 0.5; yeast extract, 0.5, and 2% (v/v) n-hexadecane as the sole carbon source.

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The nutrient salt medium for *Bacillus subtilis* is composed of (g L^{-1}) NH_4NO_3, 4; Na_2HPO_4, 5; NaCl, 2.2; KH_2PO_4, 4; $MgSO_4$, 0.2; FeSO4 $7H_2O$, 0.0012; yeast extract, 0.5, and 8 g L^{-1} glucose as the sole carbon source.

The nutrient salt medium for *Acinetobacter calcoaceticus* is composed of (g L^{-1}) $(NH_4)_2SO4$, 5; NaCl, 15; KCl, 1; KH_2PO_4, 3; Na_2HPO_4, 1.2; $FeSO_4$ $7H_2O$, 0.002; $MgSO_4$, 0.2; yeast extract, 0.5, and 2 % (v v^{-1}) n-hexadecane as the carbon source

A 0.025-mL trace metal solution containing $ZnSO_4$, 0.29; $CaCl_2$, 0.1; $CuSO_4$, 0.25; $MnSO_4$, 0.2 (g L^{-1}) was added to each flask.

Biosurfactant production

Cultivations were performed in 250 mL flasks containing aliquots and 50 mL medium at 30 °C and stirred in a rotary shaker at 200 rpm for several days.

Different waste streams were pre-tested for biosurfactant generation, and low production rate waste streams were removed from further studies. A commercial medium and strain were used to compare biosurfactant production. As a result, the best waste stream and suitable concentration for each strain were determined.

Different nitrogen sources such as NH_4NO_3 (0-6 g L^{-1}), $(NH_4)_2SO_4$ (0-10 g L^{-1}) and yeast extract (0-5 g L^{-1}) were also tested at different concentrations.

Biosurfactant productivity was evaluated by surface tension measurement (using a tensiometer) and emulsification index (E_{24}) determination.

The industrial waste streams used as the substrate and the obtained results are as follows:

Glycerol waste

Glycerol waste from the conversion of fish oil to biodiesel was provided by the Marine Bioprocessing Unit, Centre for Aquaculture and Seafood Development, Fisheries and Marine Institute, Memorial University of Newfoundland (CASD). The glycerol stream was centrifuged and then filtered through 0.45 µ membrane filters. After that, it was neutralized with 1 M H_2SO_4 and heated at 110 ºC under agitation for 90 minutes to remove the methanol content and again filtered to reach the crude glycerol sample. Also, another waste glycerol sample was provided from Midland Biofuels, which was produced during biodiesel production from waste vegetable oils, and the same treatment procedure was done on it.

The crude glycerol samples and nitrogen sources at different concentrations were used to generate biosurfactant with all bacterial strains; however, the results were not acceptable as the surface tension was more than 48 mN m^{-1} and the emulsification index was less than 42% with unstable emulsions. The glycerol wastes were removed from optimization studies as a result of the low production rate.

Fish waste

In regions of intensive fish harvesting and processing, a significant amount of waste is produced which contains valuable products such as proteins, pigments, and lipids. The high amount of fish waste is produced in Atlantic Canada, and most of them are discharged to the ocean and/or sent to a landfill after treatment. The use of these materials has the advantage of reducing the cost and waste utilization which reduce the environmental impacts of wastes.

Two fish waste samples were provided from CASD (Centre for Agriculture and Seafood Development) of the Marine Institute, and a left-over sample from supercritical carbon dioxide extraction for recovering oil from fish waste. For using these wastes as a substrate for biosurfactant production, the carbon and nitrogen of it should be extracted. Extraction was done by different steps; at first, the samples were ground using a pilot scale grinder with a plate size of 3 mm. Then they were heated to 80 °C for 20 minutes. After that, they were centrifuged at 1500 rpm for 10 minutes, and hot water at 90 °C was added to them. The next step was the centrifugation at 4000 rpm for 10 minutes. Finally, the liquid layer was filtered with 0.2 µ filter papers.

Also, a liquid shrimp waste from Shellex Company was provided which was filtered with 0.2 µ filter papers.

All wastes were used as the carbon and/or nitrogen sources with all strains for biosurfactant formation. The best result showed the surface tension of 36 mN m^{-1} and the emulsification index of 40%. Because of the low emulsification index and unstable emulsions, the fish wastes and the shrimp waste were not used for further studies.

Brewery waste

Brewery waste which is the waste of the first stage (mash and lauter tun) of beer production was obtained from the Quidi Vidi Brewing Company. A mixture of milled barely grain and water (liquor), is heated in a vessel called a mash tun. By this process, the starch in the grain is broken down with the enzymes in the malt into sugars, typically maltose. In Lauter tun, the grain is separated from the liquid. The

waste stream from the mash and lauter tun, usually consist of spent grain and sugars, including maltose and glucose. After centrifugation and microfiltration of the sample through 0.2 µ membrane filter, total carbohydrate was analyzed using the anthrone method and Agilent 8453 spectrophotometer, and it was found to be 469.6 g L^{-1}.

Brewery waste was used as the carbon source with different nitrogen sources for biosurfactant production. Acceptable results were observed with *Bacillus subtilis* strains, but the surface tension and the emulsification index measured with the other strains were more than 40 mN m^{-1} and less than 54%, respectively. So, the brewery waste production with the *Bacillus subtilis* strains was chosen to be used for the rest of the studies.

Produced water

Produced water is the largest waste stream generated during oil and gas recovery operations. The produced water sample was obtained from the Suncor Energy Company. The sampling points were just after separator train before the produced water treatment system, mid-point in the produced water treatment system and discharge point. The polycyclic aromatic hydrocarbons (PAH) of the produced water was analyzed using GC/MS, and it shows the presence of phenol and naphthalene.

The results of benzene, toluene, ethylbenzene and xylene (BTEX) and total petroleum hydrocarbon (TPH) analyses using GC/MS are shown in table A-1.

Table A-1. The results of BTEX and TPH analyses on the produced water

Petroleum hydrocarbons	Units	Produced water
Benzene	mg/L	6.5
Toluene	mg/L	3.6
Ethylbenzene	mg/L	0.28
Xylene (total)	mg/L	1.2
>C6 - C10 (less BTEX)	mg/L	Not detected
>C10-C16 Hydrocarbons	mg/L	7.5
>C16-C21 Hydrocarbons	mg/L	5.0
>C21-C32 Hydrocarbons	mg/L	7.9

The results show that the TPH or carbon content of the produced water is much lower than the studies that paraffin or hexadecane were used for biosurfactant generation, and the biosurfactant production with this waste led to poor results.

Waste cooking oil

Waste cooking canola oil from a local restaurant was used as a carbon source, and adsorption with silica gel was used to remove thermal decomposition compounds from the waste oil. A mixture of 50 g of filtered waste oil and 15 g of silica gel in a beaker was heated to 100 °C while stirring at the speed of 60 rpm for 1 hour. The mixture was filtered through a 0.45 µm pore size filter paper to separate the adsorbent from the solution and obtain the refined waste cooking oil. Both waste cooking oil and refined waste cooking oil were used with different concentrations and nitrogen sources. When biosurfactant was produced with the *Acinetobacter calcoaceticus* strain, the results were acceptable; however, the surface tension was more than 44 mN m^{-1}, and the emulsification index was less than 51% with other strains. So, the

biosurfactant production was further examined with the *Acinetobacter calcoaceticus* strain and waste cooking oil as the sole carbon source.