Master Thesis

Optimization of Biosurfactants Production by *Bacillus subtilis* B37 Isolated from Palm Oil-Contaminated Soil

Thesis submitted in partial fulfillment of the requirements
for the degree of Master of Science
at the University of Natural Resources and Life Sciences Vienna

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Vienna, August 2017

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Zusammenfassung

Der Bakterienstamm *Bacillus subtilis* B37 wurde aus Palmöl kontaminierter Erde in Thailand isoliert und auf die Fähigkeit Biotenside zu produzieren untersucht. Die Kohlenstoff- und Stickstoffquelle im Medium wurde variiert sowie unterschiedliche pH-Werte und Rührgeschwindigkeiten verglichen, mit dem Ziel die Produktivität zu erhöhen. Die Eigenschaft des Stammes Tenside herzustellen, wurde mit Hilfe der Oberflächenspannung (ST) sowie Emulgierfähigkeit (EI_{24}) getestet. Die besten Ergebnisse erzielte die Kultivierung mit Melasse und Natriumnitrat als Medium Komponenten in einem Kohlenstoff zu Stickstoff (C/N)-Verhältnis von 20:1. Bei der Kultivierung im Schüttelkolben mit dem bereits optimierten Nährmedium und bei optimalen Umgebungsbedingungen (pH= 7,0 bei 30°C und 200 U/min), konnte die Oberflächenspannung des Mediums von 57 auf unter 30 mN/m reduziert werden. Neben geeignetem Medium und Kultivierungsbedingungen wurde auch die optimale Methode zur Rückgewinnung des bakteriellen Produktes bestimmt. Ein Chloroform-Methanol Gemisch im Verhältnis 2:1 erwies sich dabei als besonders wirkungsvoll. Die einzelnen Komponenten wurden mittels Dünnschichtchromatographie getrennt und die Ergebnisse zeigten, dass der untersuchte *Bacillus subtilis* Stamm B37 zur Gruppe der Glycolipide zugehörig ist. Das Glycolipid-Tensid weist zudem eine sehr gute Oberflächenaktivität auf und blieb auch bei hohen Temperaturen bis zu 121°C (Einwirkzeit 15 Minuten) und einem weiten pH-Bereich (pH= 3,0-10,0) stabil. Der Einfluss verschiedener Parameter auf die Stabilität des Biotensids wurde mit einem herkömmlichen, synthetischen Tensid (SDS) verglichen. Außerdem wurde die Kritische Mizellbildungskonzentration des untersuchten Bakterienstammes ermittelt (CMC= 1,5 g/L).

Die Untersuchung der Wachstumskinetik verdeutlicht, dass es sich bei dem in der vorliegenden Arbeit untersuchten Tensid um einen wachstumsabhängigen Metaboliten handelt. Die maximale Wachstumsrate wurde nach 36 Stunden Kultivierung erreicht (OD_{600}= 1,5) und das höchste Zelltrockengewicht lag am Ende der exponentiellen Wachstumsphase bei 3,2 g/L. Nach Optimierung der Medium Komponenten, Kultivierungsbedingungen und Rückgewinnungsverfahren, wurde eine Ausbeute von über 26 % erreicht. Es zeigte sich, dass *Bacillius subtilis* B37 eine bessere Oberflächenaktivität aufweist als kommerziell erhältliche chemische Tenside und mit dieser Eigenschaft ist dem Biotensid ein großes Verwendungspotential für mikrobiell begünstigte Anwendungsbereiche wie die Öl-Rückgewinnung, Bioremediation oder in der Medizin zuzuschreiben.

Schlagwörter: Biotenside, Optimierung, *Bacillus subtilis*, Oberflächenaktivität

Abstract

The production of biosurfactants produced by *Bacillus subtilis* strain B37 isolated from palm oil-contaminated soil in Southern Thailand was studied. Culture conditions involving variations in carbon and nitrogen sources as well as pH-value and agitation speed were evaluated, with the aim of increasing productivity in the process. Biosurfactant production of the strain was tested by measuring surface tension (ST) and emulsifying index (EI_{24}). The best results for the production of biosurfactants by *B. subtilis* B37 were obtained when using molasses and sodium nitrate as carbon and nitrogen sources respectively, with a C/N ratio of 20:1. Cultivation in a shake-flask containing optimized medium composition and initial pH of 7.0 at 30°C and 200 rpm could reduce the surface tension from 57 to below 30 mN/m. Besides optimized medium composition and cultivation conditions, the best method for the recovery of biosurfactants was investigated. A chloroform/methanol mixture in a 2:1 ratio was thereby the most effective. The composition of obtained biosurfactant was studied by thin-layer chromatography and demonstrated the glycolipid nature. It had a good surface activity and showed stability during exposure to high temperatures up to 121°C (15 min exposure time), as well as a wide range of pH (pH= 3.0-10.0). The influence of different parameters on the stability of the biosurfactant was compared with a synthetic surfactant (SDS). Furthermore, the critical micelle concentration of obtained biosurfactant was 1.5 g/L.

In the present study, the production of biosurfactants was growth associated as indicated by the growth and biosurfactant production kinetics. Maximum growth occurred after 36 hours of cultivation (OD_{600}= 1.5) and the highest cell dry weight of 3.2 g/L was reached at the end of the exponential growth phase. After optimized cultivation conditions and recovery method an overall yield of above 26 % could be obtained. The results indicate that *Bacillus subtilis* B37 have better surface activity than a common chemical surfactant, thus have potential for microbial enhanced applications such as oil recovery, bioremediation or in medicine.

Keywords: biosurfactants, optimization, *Bacillus subtilis*, surface tension

Acknowledgment

First of all, special thanks go out to Univ.Prof. Dipl.-Ing. Dr. Dietmar Haltrich who made this collaboration possible and gave me the opportunity to work abroad for this project under his supervision. Next I want to thank Assoc.Prof. Dr. Suppasil Maneerat, who welcomed me warmly at his department, introduced me to this fascinating issue and supported me during my whole stay in Thailand. Other great thanks go to Theerawat Onlamool for his patience to teach me many things in the laboratory and his guidance on how to survive in this totally different environment. I also thank everybody at Prince of Songkla University from the Department of Industrial Biotechnology, Faculty of Agro-Industry, who introduced me to their culture, showed me delicious Thai food and always welcomed me with a smile.

Finally, I want to thank my family, my partner and all my friends for their motivation and enormous support.

Contents

Chapter 1 Introduction

Thailand is with 2.3 million tons of crude palm oil in 2016 the third largest producer of palm oil worldwide, following Indonesia and Malaysia (USDA). Most factories are located in the southern part of the country generating large quantities of waste such as residual palm oil that can contaminate soil and water (Chavalparit *et al.*, 2006). These oil residues can be utilized by some microorganisms that produce biosurfactants in order to adsorb, emulsify, wet and disperse or solubilize water-immiscible material (Nerurkar *et al.*, 2009).

Biosurfactants are natural products synthesized by various microorganisms that exhibit surface and emulsifying activities. In recent years biosurfactants have gained much attention as possible replacement for some existing products which mainly derived from petroleum. The properties of biosurfactants exceed many synthetic surfactants in terms of structural diversity, greater biodegradability, lower critical micelle concentration and higher surface activity (Banat *et al.*, 2000). Other important advantages are their low toxicity profiles to freshwater, marine and terrestrial ecosystems. Hence, biosurfactants are promising candidates for a variety of environmental friendly applications, not only in food, cosmetic and pharmaceutical industries but in environmental protection and energy-saving technology as well (Kitamoto *et al*., 2002).

However, the major concern regarding the use of biosurfactants on a large scale is the complexity and relatively high production and recovery costs. In order to make biosurfactants capable of high-yield production, it is necessary to select efficient strains of microorganisms with the potential of producing surface-active compounds. Another important factor to reduce the costs is the optimization of the medium composition and usage of alternative inexpensive renewable substrates such as agro-industrial wastes (Saisa-ard *et al*., 2014).

1.1 Nature of biosurfactants

Biosurfactants are amphiphilic compounds with both hydrophilic and hydrophobic moieties. They are produced mainly by aerobically growing microorganisms and are either neutral or anionic type (Banat *et al.*, 2010). The hydrophobic or non-polar moiety is generally made up of fatty acid or hydroxyl fatty acid with a size ranging from C8 to C18. The hydrophilic or polar head group appears in many variations such as carbohydrate, peptide, cyclic peptide, carboxylic acid, alcohol or phosphate group (Mulligan *et al.*, 2001). The structure of biosurfactants allows them to exist preferentially at the interface between two immiscible fluids. These properties make surfactants capable of reducing surface (air/water) and interfacial (oil/water) tension and to enhance water solubility of hydrocarbons to form emulsions (Desai & Banat, 1997).

The ability of biosurfactants to reduce the surface or interfacial tension increases with its increasing concentration until it reaches a critical concentration, known as critical micelle concentration (CMC) (Mulligan, 2005). Figure 1 shows the surface tension (mN/m) as a function of biosurfactant concentration (g/L). Above the CMC there is no further reduction in surface or interfacial tension. At the CMC, biosurfactant molecules spontaneously self-assemble to structures such as vesicles, bilayers or micelles. The CMC is specific to each surfactant and is dependent on the surfactant structure as well as the pH, ionic strength and temperature of the solution (Soberón-Chávez, 2011). A lower CMC indicates that less surfactant is required to decrease surface tension. The CMC of biosurfactants ranges from 1 to 2000 mg/L, whereas interfacial and surface tension are approximately 1 and 30 mN/m respectively (Santos *et al.*, 2016). In general, biosurfactants exhibit low CMC values which therefore make them more efficient than their synthetic counterparts (Bognolo, 1999).

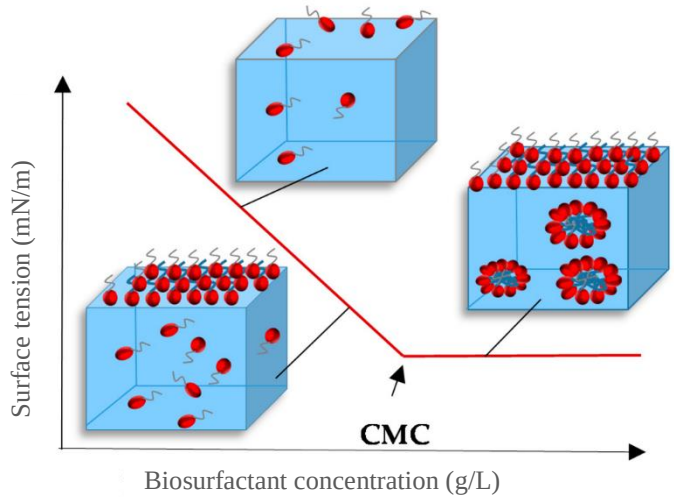

Figure 1: Illustration of regions in which micelle formation occurs (CMC represents critical micelle concentration). (Santos *et al.*, 2016)

1.1.1 Biosurfactant classification

Surface-active agents are mainly categorized into low-molecular weight biosurfactants and high-molecular weight bioemulsifiers. The former states are known for their excellent surface activity which involves lowering the surface tension between different phases. In contrast, they are less effective at emulsifying two immiscible liquids compared to high-molecular weight bioemulsifiers (Uzoigwe *et al.*, 2015). The major classes of low-molecular mass biosurfactants include lipopeptides, glycolipids and phospholipids, whereas high-molecular mass surfactants include polymeric and particulate surfactants (Ron & Rosenberg, 2001). Biosurfactants are further divided into six groups based on their chemical composition and microbial origin (Table 1).

Table 1: Examples of the main classes of biosurfactants and their microbial origin, modified from (Mulligan, 2005)

Biosurfactant class	Type of surfactant	Microorganism
Glycolipids		
	Rhamnolipids	*Pseudomonas aeruginosa,* *Pseudomonas* sp., *Serratia rubidea*
	Trehalose lipids	*Arthrobacter paraffineus,* *Corynebacterium* spp., *Mycobacterium* spp., *Nocardia* sp.
	Sophorose lipids	*Candida apicola, Candida bogoriensis,* *Candida bombicola, Candida lipolytica*
Fatty acids		*Arthrobacter paraffineus,* *Capnocytophaga* sp., *Corynebacterium lepus,* *Nocardia erythropolis*
Phospholipids		*Acinetobacter* sp., *Aspergillus* sp.
Lipopeptides		
	Surfactin	*Bacillus pumilus, Bacillus subtilis*
	Lichenysin A, B	*Bacillus licheniformis*
	Ornithine	*Thiobacillus thiooxidans*
	Viscosin	*Pseudomonas fluorescence*
Polymeric		
	Alasan	*Acinetobacter radioresistens*
	Emulsan	*Acinetobacter calcoaceticus*
	Liposan	*Candida lipolytica,*
Particulate		*Acinetobacter* spp., *Pseudomonas marginalis*

Among these groups, most detailed studied biosurfactants are surfactin synthesized by *Bacillus subtilis* (Mulligan, 2005) and glycolipids produced by *Pseudomonas aeruginosa* (Soberón-Chávez *et al.*, 2005) and different *Burkholderia* species (Dubeau *et al.*, 2009). The chemical structure of glycolipids is composed of a hydrophobic fatty acid chain in combination with a hydrophilic moiety of carbohydrate (Müller *et al.*, 2011). The most intensively studied glycolipids are rhamnolipids, consisting of a glycon (mono- or di-rhamnose) and an aglycon part (fatty acid chain) linked to each other via O-glycosidic linkage (Figure 2). Rhamnolipids display high surface activities and are produced in relatively high yields with short incubation periods. Besides that, they are contributing to the pathogenesis of *P. aeruginosa* infections and therefore it is essential to understand and control their production and effect (Soberón-Chávez, 2011).

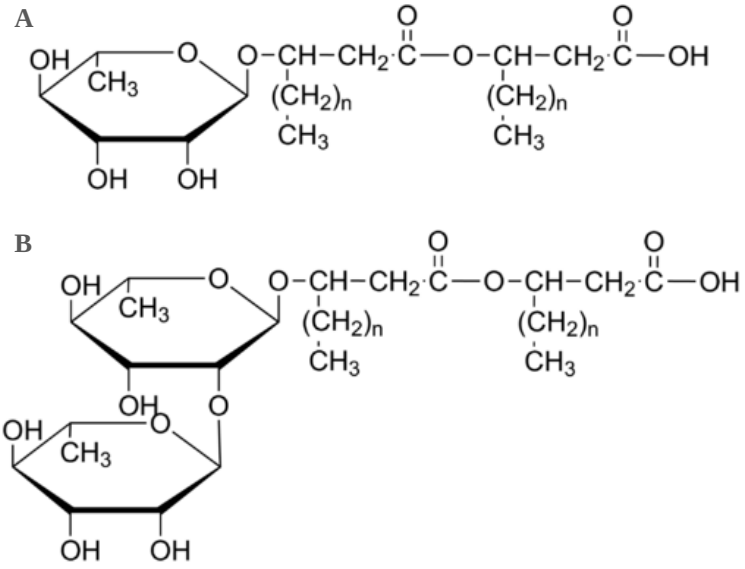

Figure 2: Chemical structure of mono-rhamnolipid (A) and di-rhamnolipid (B). (Magalhães & Nitschke, 2012)

Another effective biosurfactant is surfactin, a non-ionic lipopeptide produced by various strains of *B. subtilis*. It lowers the surface tension of pure water from 72 mN/m to less than 30 mN/m with a critical micelle concentration of as low as 24 µM (Desai & Banat, 1997). Surfactin consists of a β-hydroxyl fatty acid and a cyclic peptide, shown in Figure 3. The peptide is made out of seven amino acids, which always composes of five lipophilic amino acids and two negatively charges hydrophilic ones (Kakinuma *et al.*, 1969).

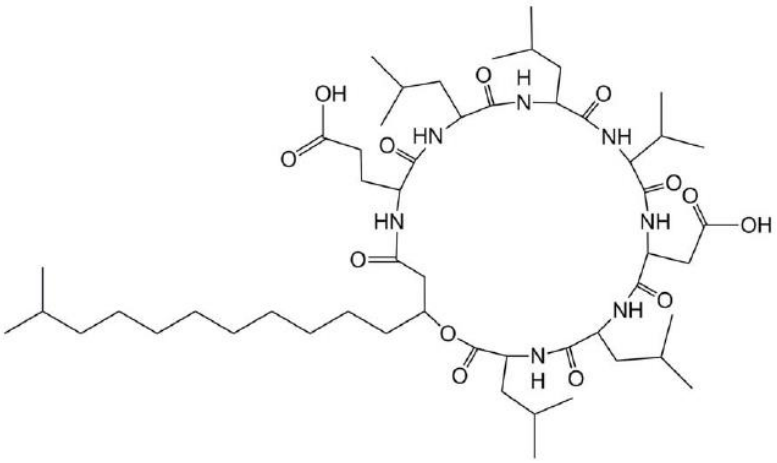

Figure 3: Chemical structure of a C15 surfactin molecule (Liu *et al*., 2012)

However, the “generally recognized as safe” (GRAS) status of *B. subtilis* according to the American Food and Drug Administration (FDA) makes the use of this organism preferable for large scale bioprocesses.

1.2 Properties of biosurfactants

The properties of biosurfactants such as surface and emulsifying activity, pH and thermal stability as well as solubility make them very attractive for industrial use. Moreover, biosurfactants are non-toxic and biodegradable, both important properties in times of increasing environmental awareness and interest in sustainable processes (Holmberg, 2001; Kim *et al.*, 1997).

Surface tension: The measurement of the surface tension reduction is a common method to detect biosurfactant production. The surface tension is a property of liquid surfaces that causes them to behave like an elastic sheet. This behavior can be evaluated using a ring-tensiometer, consisting of a platinum ring hanging from a balanced hook and immersing into the examined liquid (Özdemir *et al.*, 2004). The maximum force (F) needed to detach the ring from the liquid surface is recorded and equivalent to the surface tension (Figure 4).

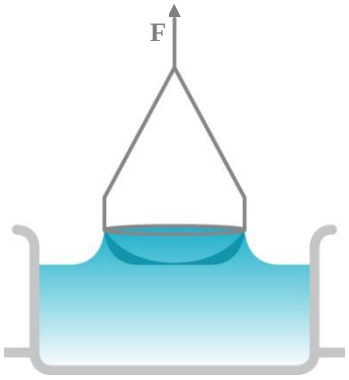

Figure 4: Scheme of surface tension measurement by ring method, modified from (Biolin Scientific)

Whereas surface tension is related to the effectiveness of surfactants, the efficiency is measured by the critical micelle concentration (CMC). The CMC is the concentration limit after which the addition of more biosurfactant will not further reduce the surface tension (Pacwa-Plociniczak *et al.*, 2011). Therefore, a low CMC is more efficient in lowering the surface tension than a biosurfactant with a high CMC.

Emulsification activity: Another important factor to determine biosurfactant production is the estimation of the emulsification activity. The definition of an emulsion is that of a dispersion of one immiscible liquid (dispersed phase) in another (continuous phase). There are two basic types of emulsions: oil-in-water (o/w) and water-in-oil (w/o). The addition of biosurfactants to an emulsion can increase the stability of the generally instable mixture (Uzoigwe *et al.*, 2015). To measure the emulsifying potential of the biosurfactant-producing strain, an equal volume of the sample is mixed with hydrocarbons such as crude oils, kerosene, diesel or petrol. After a specified time, the emulsification index can be calculated through the percentage of the height of emulsion layer (he) divided by the total height (hT) of the liquid column (Patel & Desai, 1997).

$$EI_{24} = \frac{he\ (mm)}{hT\ (mm)} * 100$$

Tolerance to temperature, pH and ionic strength: Many biosurfactants show activity at temperatures as high as 90°C and pH-values ranging from 2.0 to 12.0. Moreover, biosurfactants can be used at salt concentrations up to 10 %, whereas 2 % NaCl is enough to inactivate most synthetic surfactants (Cameotra & Makkar, 1998). The stability of biosurfactant at such extreme physical parameters make them attractive for different industrial and environmental applications.

1.3 Factors affecting biosurfactant production

The preferred environmental conditions are unique to each microorganism and are essential for achieving maximum growth and product synthesis. Thus, the optimization of the medium composition is an important factor to increase the efficiency of biosurfactant production in terms of higher yields and lower production costs.

Carbon source: Biosurfactants are produced from a wide variety of carbon substrates which therefore plays a key role in the optimization process. These substrates can be divided into two main categories: water-soluble carbohydrates and water-insoluble hydrocarbons (Kim *et al.*, 1997). Ghribi and Ellouze-Chaabouni (2011) have reported the effect of medium components on biosurfactant production by *Bacillus subtilis*. They found that among carbohydrates, glucose achieved the best biosurfactant production with an optimum concentration of 40 g/L. In general, carbohydrates achieve higher cell and biosurfactant

concentrations than hydrocarbons. However, the utilization of hydrocarbons as a feedstock is of increasing importance in terms of bioremediation of oil-polluted ecosystems. Consequently, recent studies have focused on the evaluation of insoluble carbon sources such as vinasse and waste frying oil (Oliveira & Garcia-Cruz, 2013), used vegetable oil (Saisa-ard *et al.*, 2013) or crude glycerol from biodiesel industry (Sousa *et al.*, 2014) for biosurfactant production by different *Bacillus* species.

Nitrogen source: The production of biosurfactants is also affected by other medium constituents such as nitrogen. Makkar and Cameotra (1997) evaluated the effect of different organic and inorganic nitrogen sources on biosurfactant production by *Bacillus subtilis*. They reported that even in nitrogen-free medium *B. subtilis* showed a reduction in surface tension. However, the highest yields of surfactin production were obtained when using sodium nitrate and potassium nitrate as a nitrogen source, compared to organic compounds such as peptone, yeast and beef extract. Likewise, Abushady *et al.* (2005) also found that inorganic nitrogen sources were superior to organic nitrogen sources for the production of biosurfactants.

Growth conditions: Environmental factors such as pH, temperature, agitation speed and oxygen also influence biosurfactant production through their effect on cellular growth and cell activity. It has been reported that the production of surfactin by *B. subtilis* is depended on the strain and so are the optimum environmental conditions (Abushady *et al.*, 2005). However, Makkar and Cameotra (1998) studied the production of biosurfactant at mesophilic and thermophilic conditions by a strain of *B. subtilis*. They found that the obtained biosurfactant was stable even at high temperatures above 100°C and within a wide pH range from 3.0 to 11.0.

1.4 Recovery of biosurfactants

The most widely used methods in biosurfactant recovery processes are extraction with solvents such as chloroform/methanol, butanol, ethyl acetate and acetone. The hydrophobic moieties of biosurfactants are soluble in these solvents and therefore help to separate the crude product. However, disadvantages of using organic solvents include the large amount of solvents required as well as the toxic and harmful character of most compounds (Satpute *et al.*, 2010). Surfactin becomes insoluble at a low pH-value therefore acidic precipitation is a common recovery method for surfactin producing *B. subtilis* strains. Moreover, acidic precipitation is efficient in crude biosurfactant recovery and an inexpensive alternative to organic solvent extraction (Soberón-Chávez, 2011).

1.5 Detection of biosurfactants

For small scale experiments, thin-layer chromatography (TLC) is a commonly used method for the determination of sample constituents. As other methods of chromatography, separation by TLC is based on the different migration of the sample components through the stationary phase with a mobile phase as carrier. The rate of migration depends on the different affinities of the sample for the mobile and stationary phase. The stationary phase in TLC is a layer of fine particles bound to a glass plate, aluminum foil or plastic sheet. The mobile phase is selected depending on the sample properties and consists of a single component or mixture of solvents (Sherma & Fried, 2003).

In some cases, the sample needs to be visualized upon identification. This can be done using different methods such as fluorescence labeling of the stationary phase or showing the spots up chemically. Common chemical compounds are ninhydrin, which reacts with amino acids, sulfuric acid and many organic compounds, or *p*-anisaldehyde, which forms colored adducts upon heating and therefore allows an easy distinction (Jork *et al.*, 1990). The spots can be further identified by comparing the distance they have migrated with those of known reference material. The distance travelled by the individual spot is divided by the total distance of the solvent moved. The resulting ratio is called retardation factor (R_f), which gives values ranging from 0.0 to 1.0 (Gruenwedel & Whitaker, 1984).

$$R_f = \frac{distance\ travelled\ by\ compound}{distance\ travelled\ by\ solvent}$$

1.6 Applications

The increasing interest in microbial biosurfactants derives from their potential for wide applicability and advantages over synthetic surfactants. Surfactants are widely used in a variety of industries that produce household and industrial cleaners, personal care products, and in various types of manufacturing including food processing and the production of plastics, paints and coatings, textiles, pulp and paper, and agricultural products. Additionally, these compounds are also used in the specialty chemical market as components of cosmetic products, pharmaceuticals, emulsifiers, wetting agents, and in the synthesis of fine chemicals (Myers, 2006). This broad spectrum of applications illustrates the importance of developing new compounds with specific properties. Presently, the majority of surfactants used are synthetic; however, biosurfactants with their unique chemical characteristics have been recognized for their utility in various fields of industrial as well as biotechnological applications (Vijayakumar & Saravanan, 2015).

1.6.1 Biomedical science

The biological features of biosurfactants have led to a great potential for applications in the medical field. They are useful as antimicrobial, antifungal and antiviral agents, and may be used as safe and effective alternative to synthetic medicines (Rodrigues *et al.*, 2006). For instance, the influence of biosurfactants from two probiotic bacteria, *Lactobacillus lactis* 53 and *Streptococcus thermophilus* A, on biofilm formation was evaluated by Rodrigues *et al.* (2004). They found that both biosurfactants greatly reduced the microbial number of a variety of bacterial and yeast strains isolated from voice prostheses. Fernandes *et al.* (2007) emphasized the possible antimicrobial role of biosurfactants produced by *Bacillus subtilis* R14 against twenty-six multidrug-resistant bacteria. They reported that all resistant strains were sensitive to the lipopeptide surfactant, in particular *Enterococcus faecalis*.

In addition to antimicrobial properties, surfactants have also been related to several biological activities caused by viral infections. Vollenbroich *et al.* (1997) showed that the biosurfactant surfactin from *Bacillus subtilis* is active against several viruses, including Semliki Forest virus, herpes simplex virus (HSV), suid herpes virus, vesicular stomatitis virus, simian immunodeficiency virus, feline calicivirus and murine encephalomyocarditis virus. The inactivation of enveloped viruses, especially herpes- and retroviruses, was much more efficient than that of non-enveloped viruses. Therefore, the authors suggest that the antiviral action seems to be due to a physicochemical interaction between the membrane-active surfactant and the virus lipid membrane.

1.6.2 Bioremediation

Major concerns to the environment are hydrocarbons such as oil spills, oily waste and oil leakage. Hydrophobic organic compound (HOC) contaminants have very low water solubility and bioavailability due to their highly hydrophobic nature (Liu *et al.*, 2015).

Biosurfactants have great potential in bioremediation because of their low toxicity and ability to solubilize, mobilize, emulsify and degrade insoluble contaminants (Singh *et al.*, 2011). Emulsification is improved by high-molar mass bioemulsifiers, compared to solubilization and mobilization which are promoted by low-molar mass (Figure 5). Mobilization occurs below the CMC, at such concentrations biosurfactants reduce the surface and interfacial tension between air/water and soil/oil system. The contact between the surfactant and contaminated soil increases, while the capillary force holding oil and soil together is reduces. In turn, above the CMC the solubilization takes place, where molecules associate to form micelles and further increase the solubility of oil (Ismail *et al.*, 2016).

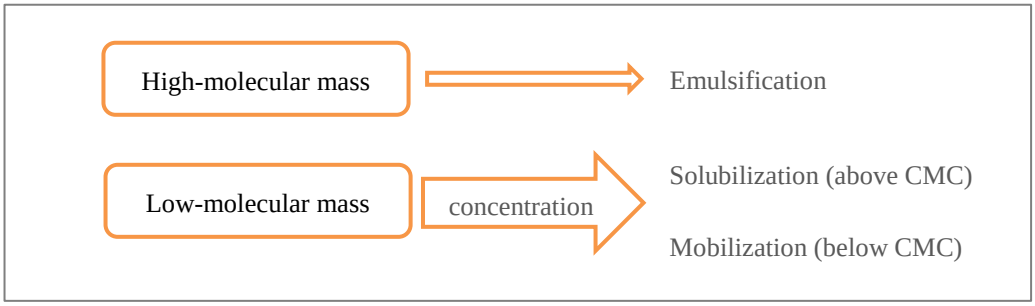

Figure 5: Mechanism of hydrocarbon removal by biosurfactant, modified from (Ismail *et al.*, 2016)

Lai *et al.* (2009) studied the ability of surfactin and rhamnolipid surfactant to remove oil-contaminated soil. The results show that biosurfactants exhibit much higher petroleum hydrocarbon removal efficiency than their synthetic counterparts and therefore provide a useful tool for bioremediation of oil-polluted environments. Liu *et al.* (2016) studied the application of *Bacillus licheniformis* in remediation of petroleum contaminated soil. They found that the biosurfactant from *B. licheniformis* strain Y-1 has an excellent emulsifying activity and showed a significant improvement in soil remediation.

Biosurfactants can also be useful in microbial enhanced oil recovery. This process involves microorganisms or their metabolites, including biosurfactants, to increase the recovery of remaining oil in reservoirs (Banat *et al.*, 2000). After primary (mechanical) and secondary (physical) recovery procedures, the residual oil is often located in regions that are difficult to access or even trapped in pores. By adding biosurfactants, the interfacial tension between oil/water and oil/rock is reduced. This reduces the capillary force and enhances mobilization of the oil through the rock pores (Figure 6) (Sen, 2008).

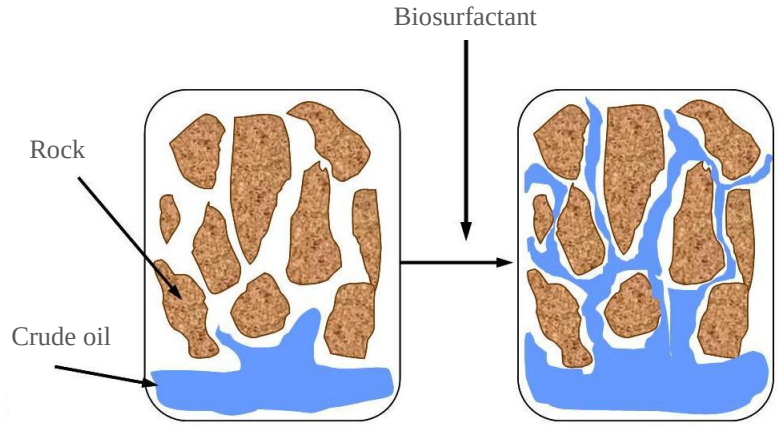

Figure 6: Mechanism of enhanced oil recovery by biosurfactants, modified from (Pacwa-Plociniczak *et al.*, 2011)

Pornsunthorntawee *et al.* (2008), studied the oil recovery activities of two types of biosurfactant-producing bacteria, *B. subtilis* and *P. aeruginosa*, compared to synthetic surfactants. For this purpose, a sand-packed column inoculated with motor oil was prepared. The results showed, that both biosurfactants could recover oil more effective than the three

synthetic counterparts, including polyoxyethylene sorbitan monooleate (Tween 80), sodium dodecyl benzene sulfonate (SDBS) and sodium alkyl polypropylene oxidebsulfate (Alfoterra).

1.6.3 Removal of heavy metals

Heavy metals are persistent soil contaminants and are not biodegradable (Singh & Cameotra, 2004). However, several metals can be transformed from one chemical state to another or be influenced by microorganisms and their products, e.g. biosurfactants. Some microorganisms can accumulate metals and influence their mobility or toxicity by adjusting the pH or stimulating other substances involved in the process (Miller, 1995). The mechanism of heavy metal removal by biosurfactants consists of three steps (Figure 7): firstly, sorption and binding of the biosurfactant to the soil surface and metal contaminant; secondly, separation of the heavy metal from the soil to the solution and lastly, association of the metal with micelles. When the heavy metals are trapped within the micelles, they can be easily recovered through precipitation or membrane separation techniques (Santos *et al.*, 2016)

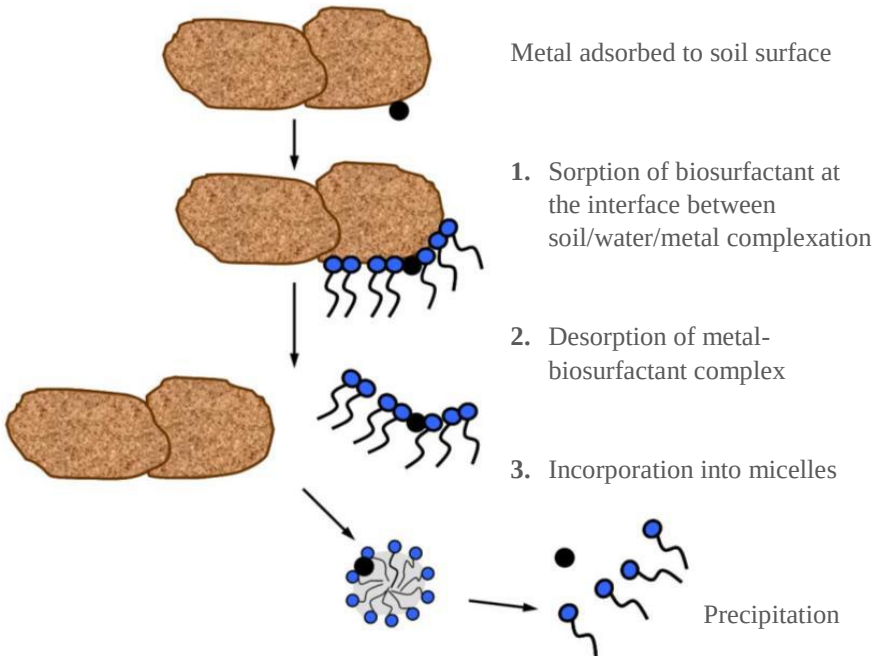

Figure 7: Mechanism of biosurfactant activity in metal-contaminated soil (Pacwa-Plociniczak *et al.*, 2011)

Mulligan *et al.* (2001) evaluated the feasibility of using surfactin, rhamnolipid and sophorolipid for the removal of Cu and Zn metals from sediments. Sequential extraction of the sediment after washing with the various surfactants indicated that the biosurfactants, rhamnolipid and surfactin, could remove the organically-bound copper and that the sophorolipid could remove the carbonate and oxide-bound zinc. They also postulated that metal removal by biosurfactants occurs through sorption of the biosurfactant into the surface, followed by complexation and detachment of the metal and hence association with the surfactant micelles.

Besides heavy metal removal from contaminated soil, another way to apply biosurfactants is in the treatment of metal-containing wastewater. Das *et al.* (2009) investigated the role of biosurfactants in removing heavy metal-containing solutions in their study. The surfactant derived from a marine bacterium, later identified as *Bacillus circulans* (Das *et al.*, 2008), showed almost complete removal of 100 ppm lead and cadmium at a concentration five times the CMC. They stated that not only micelles are involved in metal removal but also the formation of insoluble precipitates by microbial products, e.g. biosurfactants.

Chapter 2 Aim of the thesis

This work aims to study the effect of carbon source, nitrogen source, pH-value and shaking speed on the production of biosurfactant by a *Bacillus subtilis* B37 isolated from palm oil-contaminated soil. Furthermore, general characterization and recovery methods of the surface-active product were evaluated.

1. Optimization studies

   Optimization of the medium composition (C- and N-source) as well as environmental conditions (pH and agitation) for growth and biosurfactant production by the selected strain

2. Recovery method

   Extraction of biosurfactant from *B. subtilis* B37 with different solvents and acid precipitation to determine the most suitable recovery method

3. Characterization

   Examination of the optimum pH-value as well as study the effect of pH and temperature on the stability of the obtained biosurfactant

   Primary structure elucidation by using thin-layer chromatography

Chapter 3 Materials and Methods

3.1 Bacterial strain and cultivation conditions

The examined bacterial strain *Bacillus subtilis* B37 was kindly supplied from the Faculty of Agro-Industry, Department of Industrial Biotechnology, Prince of Songkla University. The strain was isolated from soil contaminated with palm oil from a palm oil refinery factory in Songkhla, Thailand. The biosurfactant-producing strains were isolated and screened according to Saisa-ard *et al.* (2014). The pure cultures were stored at -20°C in nutrient broth (HiMedia, India) mixed with sterile glycerol at a final concentration of 30 %.

For biosurfactant production a minimal salt medium (MSM) with the following composition (g/L) was used: K_2HPO (0.8), KH_2PO_4 (0.2), $CaCl_2$ (0.05), $MgCl_2$ (0.5), $FeCl_2$ (0.01), $NaCl_2$ (5.0) and distilled H_2O filled up to 1,000 mL (Saimmai *et al.*, 2012). Carbon and nitrogen sources were added separately. Cultivation was performed in 250 mL flasks containing 50 mL MSM broth at room temperature and stirred in a rotary shaker (Vision Scientific Co., South Korea) at 150 rpm, 30°C for 48 h.

3.2 Chemicals

All chemicals and solvents used were purchased from various suppliers as listed in Table 2.

Table 2: List of chemicals

Chemical	Supplier
Acetic acid	Sigma-Aldrich Co. LLC, Germany
Acetone	RCI Labscan Ltd., Thailand
p-Anisaldehyde	n/a
Butanol	J.T. Baker, Avantor Performance Materials TCC, USA
Calcium chloride	Ajax Finechem Pty. Ltd., Australia
Chloroform	RCI Labscan Ltd., Thailand
Copper (II) sulphate	n/a
Dipotassium hydrogen phosphate	Ajax Finechem Pty. Ltd., Australia
Ethanol	J.T. Baker, Avantor Performance Materials TCC, USA
Ethyl acetate	J.T. Baker, Avantor Performance Materials TCC, USA
Glucose	Utopia Co. Ltd., Thailand
Iron (III) chloride hexahydrate	Loba Chemie Pvt. Ltd., India
Kerosene	Sigma-Aldrich Co. LLC, Germany
Magnesium chloride hexahydrate	Ajax Finechem Pty. Ltd., Australia
Methanol	J.T. Baker, Avantor Performance Materials TCC, USA
Molasses[i]	Songkhla Province, Thailand
Ninhydrin	n/a
Nutrient Broth	HiMedia Laboratories Pvt. Ltd., India
Peptone	HiMedia Laboratories Pvt. Ltd., India
Phosphoric acid	Ajax Finechem Pty. Ltd., Australia
Potassium dihydrogen phosphate	Ajax Finechem Pty. Ltd., Australia
Soapstock[ii]	Narathiwat Province, Thailand
Sodium chloride	Ajax Finechem Pty. Ltd., Australia
Sodium dodecyl sulphate	HiMedia Laboratories Pvt. Ltd., India
Sodium nitrate	Merck, Germany
Sulfuric acid	RCI Labscan Ltd., Thailand
Urea	Riedel-de Haen, Honeywell, USA
Used palm oil[iii]	Hat Yai, Songkhla Province, Thailand
Yeast	HiMedia Laboratories Pvt. Ltd., India

[i]Molasses was obtained from a sugar refinery in Songkhla, Thailand.
[ii]Soapstock was generated as a by-product during the refining process of palm oil in Narathiwat, Thailand.
[iii]Used palm oil was provided by a restaurant in Hat Yai, Thailand from frying processes.

3.3 Instruments

The instruments used during the optimization, recovery and characterization studies are listed in Table 3.

Table 3: List of instruments

Equipment	Model	Supplier
Autoclave	SS-325	Tomy Seiko Co. Ltd., Japan
Centrifuge	Sorvall Legend XTR	Thermo Fisher Scientific Inc., USA
Hot air oven	UM 200	Memmert, Germany
Laminar air flow	Hotpack 527044	Scientific Promotion Co. Ltd., Thailand
pH meter	Starter 2100	OHAUS, USA
Rotary evaporator	Eyela N-1000	Tokyo Rikakikai Co. Ltd., Japan
Shaking incubator	853-041/1-1/46	Vision Scientific Co. Ltd., South Korea
Spectrophotometer	Genesys 10 uv	Thermo Electron Corporation, USA
Table Centrifuge	5424 R	Eppendorf, Germany
Tensiometer	OS	Torsion Balance Supplies, UK
Water bath		Memmert, Germany
Weighing balance	FX-2000i	A&D Company Ltd., Japan
Weighing balance	Pioneer PA214	OHAUS, USA

3.4 Statistical Analysis

The obtained data were calculated with mean values and standard deviations (mean±SD) from all experiments performed with three determinations. Statistical significance of the results was evaluated by one-way analysis of variance (ANOVA) and Waller-Duncan's multiple range test ($p< 0.05$) using SPSS Statistics 17.0 software.

3.5 Analytical Methods

3.5.1 Bacterial growth

- Biomass estimation by cell dry weight (CDW)

The biomass was determined by measuring the cell dry weight (CDW). A 12-mL aliquot of the culture broth was transferred to pre-weighted 15 mL tubes and centrifuged at 8,500 rpm, 4°C for 15 min (Thermo Fisher Scientific Inc., USA). The cell pellet was further dried in an oven (Memmert, Germany) at 105°C for 24 h. After drying, the tubes were weighted as before and the difference in weight gives the cell dry weight (CDW) in g/L.

- Biomass estimation by spectrophotometer (OD_{600})

The growth of bacteria was monitored by measuring the optical density of the culture sample at a wavelength of 600 nm at time intervals. The 1 mL sample was subjected to centrifugation for 5 min at 10,000 and the supernatant was then decanted. The precipitate was washed once with a 0.85 % NaCl solution and the pellet further dissolved in distilled water. The optical density of the culture sample at 600 nm was measured using a UV and visible light spectrophotometer (Genesys series 10). Distilled water was used as a blank.

3.5.2 Biosurfactant activity assay

- Surface tension measurement

The surface tension was measured by the du Noüy ring method using a tensiometer (OS, Torsion balance supplies, UK) at room temperature (du Noüy, 1919). Roughly 12 mL volume of the cell-free supernatant was put into a clean glass vessel that was placed on the tensiometer platform. A platinum wire ring was submerged into the solution and then slowly pulled up through the liquid-air surface. The validity of these measuring was confirmed by taking surface tension measurements of distilled water (72±0.5 mN/m) before sample reading. Before conducting the experiment and between each pair of measurements, the sample cup and platinum wire ring was washed with methanol and distilled water and then allowed to dry.

- Critical micelle concentration (CMC)

The measurement of the surface tension after sequential dilution of the solution provides the concentration at which the surface tension starts to increase, the so-called critical micelle concentration (CMC). The CMC was determined by plotting the surface tension versus concentration of biosurfactant in the solution (Dominguez *et al.*, 1997). In order to obtain this, different concentrations ranging from 0 to 2 g/L of crude biosurfactant in distilled water were prepared.

- Emulsification index (EI_{24})

Evaluation of the emulsification activity of the biosurfactant was conducted by measuring the emulsification index after 24 hours (EI_{24}). The emulsification index was investigated by adding 2 mL kerosene (Sigma-Aldrich Co., Germany) to the same amount of the cell-free supernatant (ratio 1:1) (Cooper & Goldenberg, 1987). The emerging liquid interface and liquid-air surface was labeled and then heavily mixed by vortex for 1 min. Afterwards, the mixture was allowed to stand for 24 hours at room temperature. The EI_{24} is given as percentage of height of emulsified layer (mm) divided by total height of the liquid column (mm) (Patel & Desai, 1997).

3.5.3 Thin-layer chromatography (TLC) analysis

Thin-layer chromatography was performed on a silica gel 60 F_{254}-coated aluminum sheet (Merck, Germany) with a solvent system of chloroform/methanol/water (1:2:0.1). For visualizing the sample spots, the chromatogram was treated with an *p*-anisaldehyde-sulfuric acid reagent (1 mL anisaldehyde and 1 mL 97 % sulfuric acid in 18 mL ethanol) to detect sugars (Stahl & Glatz, 1982), a mixture of ninhydrin-butanol (0.2 g ninhydrin solved in 95 mL butanol and 5 mL 10 % acetic acid) to visualize amino acids (Sherma & Fried, 2003) and a copper (II) sulphate solution (10 % copper (II) sulphate with 8 % phosphoric acid) to identify fatty acids (Jork *et al.*,1990).

3.6 Optimization of biosurfactant production in shake-flask cultivation

The optimization was conducted in a series of experiments changing one variable at a time. Four factors were chosen to obtain higher biosurfactant production of the selected bacterial strain: carbon source, nitrogen source, pH-value and agitation speed.

A 200 µL stock-solution of the *Bacillus subtilis* B37 was transferred to test tubes containing 5 mL nutrient broth (HiMedia, India) and shaken at 200 rpm (BBI, Germany), 30°C overnight. For the starter inoculum, 1 mL of the culture broth was inoculated into 50 mL nutrient broth in a 250-mL flask to obtain a cell density of about 10^6 cells/mL. The culture was cultivated on a rotary shaker at 30°C and 150 rpm for 24 h. Then, 1 mL starter culture was added to several 250 mL flasks containing 50 mL MSM, each supplemented with different parameters tested as descripted below.

- Effect of carbon source

Cultivation was performed in 50 mL MSM with four different carbon sources (10 g/L): glucose (Utopia Co., Thailand), used palm oil (used frying oil), soapstock (by-product of refinery oil) and molasses (by-product of refinery cane sugar), with sodium nitrate serving as nitrogen source. The carbon source which gives the highest biosurfactant production was chosen for further study.

- Effect of nitrogen source

For evaluation effect of the nitrogen source on biosurfactant production, $NaNO_3$ (Merck, Germany), peptone (HiMedia, India), urea (Riedel-de Haen, USA) and yeast extract (HiMedia, India) were added to 50 mL MSM in a concentration of 1 g/L and supplemented with the optimal carbon source. The most appropriate nitrogen source to produce biosurfactant was selected for further studies.

- Effect of initial pH

To determine the optimal pH for biosurfactant production, various pH-values were tested: 4.0, 5.0, 7.0 and 8.0. The pH of the culture medium containing the selected carbon and nitrogen source, were adjusted with 1 M NaOH or 1 M HCl solution using a pH-meter. The initial pH of MSM showing the highest surfactant production was chosen for subsequent studies.

- Effect of agitation

The effect of agitation was examined in 50 mL MSM consisting of the optimized carbon and nitrogen sources with the proper initial pH-value. The rotary shaker speed varied from 100, 150, 200 and 250 rpm to specify the most suitable agitation for biosurfactant production.

- Effect of carbon concentration

The carbon concentration was tested at 0, 5, 10 and 20 g/L with the examined optimal carbon source. All other conditions such as nitrogen source, pH of the medium as well as agitation speed were adjusted to the determined values. Concentration of the carbon source which gives the highest biosurfactant production and biomass was chosen for the following evaluation studies.

- Effect of nitrogen concentration

The most suitable nitrogen concentration for the selected nitrogen source was evaluated using four different concentrations (g/L) ranging from 0 to 3. The MSM was prepared using the optimized carbon source with proper concentration as well as the optimal initial pH-value and agitation speed. Nitrogen concentration showing the highest reduction in surface tension was selected for further studies.

The samples containing the different medium composition or concentration were incubated for 48 hours at 30°C with a speed of 150 rpm (Vision Scientific Co. Ltd., South Korea). After incubation, the cell pellet was separated from the supernatant containing the excreted biosurfactant by centrifugation. Supernatant was further used to determine the biosurfactant activity by measuring the surface tension and emulsification index (EI_{24}). All assays were carried out in triplicate.

3.6.1 Time course study of production and growth under optimized conditions

Biosurfactant production by the examined *Bacillus subtilis* B37 was carried out in 250 mL Erlenmeyer flasks containing 150 mL MSM consisting of the selected carbon and nitrogen source with proper concentrations as well as optimized initial pH and agitation speed. Cultivation was performed at room temperature for two days in an orbital shaker. At time intervals, samples were withdrawn for growth and biosurfactant activity as previously described. All analyses were performed in triplicate.

3.7 Recovery of biosurfactant produced by *Bacillus subtilis* B37

The cultivation of *B. subtilis* B37 was performed in a shake-flask as described in section 3.6. The culture broth at time which gives the highest biosurfactant production was separated by centrifugation at 8,500 rpm for 15 min at 4°C. For subsequent experiments, 150 mL aliquots of the resulting supernatant were transferred to glass vessels. Four different recovery procedures were used: a mixture of chloroform and methanol (2:1), precipitation with chilled acetone, precipitation with methanol and acid precipitation.

a) Acid precipitation

A 150-mL sample of the culture supernatant was acidified with 6 M HCl to a pH-value of 2.0. The solution was further placed at 6°C overnight. After that, the samples were centrifuged at 8,500 rpm for 15 min at 4°C (Singh & Tiwary, 2016). The supernatant was removed and the remaining pellet was dried at room temperature for two days.

b) Acetone precipitation

The biosurfactant was recovered from the culture supernatant by cold acetone precipitation as described by Ilori & Amund (2001) with minor modifications. The cell-free supernatant was precipitated with an equal volume of chilled acetone (RCI Labscan, Thailand) and incubated at 6°C overnight. This was followed by centrifugation at 8,500 rpm for 15 min at 4°C. The supernatant was discarded and the pellet was dried in a desiccator for 48 h.

c) Methanol precipitation

One volume of ice-cold methanol (J.T. Baker, USA) was mixed with one volume of culture supernatant. After 24 h of incubation at 6°C, the samples were centrifuged at 8,500 rpm for 15 min at 4°C. Finally, the supernatant was removed and the pellet was air dried for two days (Cooper & Goldenberg, 1987).

d) Chloroform/methanol extraction

The culture supernatant was added to the same volume of a chloroform/methanol (RCI Labscan, Thailand) mixture (2:1) in a separating funnel and mixed several times. After phase separation, the solvent phase was collected and the supernatant again extracted with a fresh chloroform/methanol mixture. The solvent phase was further removed by air drying.

After drying, the crude biosurfactant from all four recovery methods was weighted. Subsequently, sample concentrations ranging from 0.0125, 0.025, 0.05, 0.1, 0.2, 0.4, 0.5, 1, 1.5 to 2 g/L with distilled H_2O were prepared to determine the critical micelle concentration (CMC). The biosurfactant activity at different concentrations of all four recovery methods was measured using a ring-tensiometer. The CMC was obtained from a plot of surface tension

versus biosurfactant concentration. The recovery method which gives the lowest CMC value was chosen for further studies.

3.7.1 Determination of biosurfactant yield

A 150-mL shake-flask culture was prepared and cultivation performed as previously described, with optimized medium composition and cultivation conditions. The sample was transferred to pre-weighted 50 mL tubes and centrifuged at 8,500 rpm for 15 min and 4°C. The bacterial cell pellet was dried in an oven at 105°C overnight to determine the cell dry weight. Supernatant was subjected to chloroform/methanol extraction (2:1) as described in section 3.7. The organic phase was transferred to a round-bottom flask connected to a rotary evaporator under vacuum to remove the solvent. The concentrated liquid obtained was used to calculate the yield of biosurfactant produced by *Bacillus subtilis* B37.

$$Yield\ (\%) = \frac{crude\ biosurfactant\ (\frac{g}{L})}{cell\ dry\ weight\ (\frac{g}{L})}$$

3.8 Characterization of crude biosurfactant produced by *B. subtilis* B37

3.8.1 Study of biosurfactant stability

The obtained biosurfactant from the most suitable recovery method was used to characterize the crude biosurfactant. The optimum pH-value as well as the effect of pH and temperature on the stability was determined at the examined CMC in a total volume of 10 mL for each tube. Sodium dodecyl sulphate (SDS, CMC at 1.12 g/L) was used to compare as a commercial.

- Optimum pH and pH stability of biosurfactant

The crude biosurfactant was adjusted to pH-values ranging from 3.0 to 11.0 with 0.1 M HCl or NaOH solutions. The surface tension was measured in order to determine the optimal pH-value. After that, the samples were allowed to stand for 24 h at room temperature. Then, all samples were re-adjusted to the optimal pH and biosurfactant activity was measured using surface tension measurement by the ring method.

- Thermal stability of biosurfactant

Thermal stability of the biosurfactant was studied by incubating the biosurfactant at a broad range of temperatures (30-100°C) for 1 hour and at 121 °C for 15 min. The samples were allowed to stand at room temperature for 24 h before measuring the surface tension.

3.8.2 Structural characterization of biosurfactant

The obtained biosurfactant from the most suitable recovery method was used to characterize the composition of biosurfactant. The characterization of the target compound was performed by thin-layer chromatography (TLC) analysis. Thus, a small amount of the extract was dissolved in methanol and spotted on a silica gel 60 F_{254} plate coated with aluminum. The TLC plate was put into a covered vessel containing a mixture of chloroform/methanol/water (1:2:0.1) as a mobile phase. The resulted spots on the TLC plate were visualized by dipping them into different color developing reagents: *p*-anisaldehyde-sulfuric acid, ninhydrin-butanol or copper (II) sulphate to detect sugars, amino acids and fatty acids, respectively. After drying, the TLC plates were heated in an oven to develop the spots.

Chapter 4 Results and Discussion

4.1 Optimization of biosurfactant production in shake-flask cultivation

The production of biosurfactants is dependent not only on the type of microorganism, but also on the composition of the medium and condition of the cultivation. In this study, the effect of various carbon and nitrogen sources as well as initial pH and agitation speed on growth and biosurfactant production by *Bacillus subtilis* B37 was examined. Cultivation was performed in MSM with 150 rpm at 30°C for 48 h.

4.1.1 Effect of medium composition on growth and biosurfactant activity

Biosurfactant-producing microorganisms produce and secrete their product into culture medium. Accordingly, the production of biosurfactants can be monitored by measuring the reduction in surface tension of the culture broth (Makkar & Cameotra, 1997).

First, effect of carbon sources on cell growth and surface tension reduction were tested and the results are shown in Table 4. The strain could grow in MSM containing all different carbon sources tested but showed highest surface tension reduction of MSM supplemented with glucose and molasses as a carbon source. Whereas with soapstock and used palm oil the surface tension was only slightly reduced, which is most likely due to the difficulty of using such insoluble carbon sources. This was also demonstrated by Abushady *et al.* (2005), who showed that soluble carbon sources such as glucose, sucrose and mannose enhance the production of surfactant when added to the production medium of *Bacillus subtilis*. They found that the addition of hydrocarbons or vegetable oils to the production medium resulted in a low level of surfactant production. Another study by Kim *et al.* (1997) also reported that *B. subtilis* C9 is produced in a higher yield using easily available carbon sources like glucose or sucrose, compared to insoluble hydrocarbons. Unlike other biosurfactant-producing microorganisms such as *Pseudomonas aeruginosa* (Robert *et al.*, 1989) or *Candida* sp. (Kitamoto *et al.*, 2002), *B. subtilis* require only carbohydrates to produce lipopeptide type biosurfactants like most *Bacillus* species (Banat, 1993; Horowitz *et al.*, 1990).

In the present study, the surface tension reduction of molasses (25.5 mN/m) is slightly lower than the surface tension reduction of glucose (27.5 mN/m). Nevertheless, molasses is a cheap by-product of the sugar refinery and therefore preferable as carbon source, especially considering large scale production. Furthermore, the highest cell growth of *B. subtilis* B37 was obtained in the minimal salt medium containing molasses. Thus, molasses was selected as carbon source for further studies.

Table 4: Effect of carbon source on biosurfactant production by *Bacillus subtilis* B37, which was cultivated in 250 mL flask containing 50 mL MSM medium at 30°C in a shaking incubator at 150 rpm for 48 h (N-source: 1 g/L of sodium nitrate).

C-source	CDW (g/L)	Final pH*	Surface tension (mN/m)	ST reduction (mN/m)	EI_{24} (%)
Glucose	0.33±0.05[b**]	5.91±0.03	33.0±1.0 (61)[†]	27.5[a]	0
Soapstock	0.02±0.15[b]	4.50±0.05	41.8±0.29 (43)	0.7[c]	0
Used palm oil	0.26±0.66[b]	5.75±0.03	49.0±0.50 (50)	1.0[c]	0
Molasses	1.08±0.09[a]	8.40±0.02	28.5±0.50 (54)	25.5[b]	0

*Values are given as mean±SD from triplicate determinations.
**Different superscript letters in the same column indicate significant differences ($p < 0.05$).
†Surface tension of MSM supplemented with each carbon source without inoculated culture.

The characteristic of emulsifiers such as biosurfactants implies emulsification ability with hydrocarbons, which can be measured using the so-called emulsification index (EI_{24}). However, the supernatants produced by *B. subtilis* strain B37 mixed with kerosene did not show any bubbles at the medium/kerosene interface after 24 h. According to Desai & Banat (1997), low-molecular weight biosurfactants are usually less effective in stabilizing emulsions of oil-in-water than high-molecular weight bioemulsifiers. Most *Bacillus* sp. produce low-molecular weight biosurfactants such as lipopeptides and glycolipids, therefore not all strains show the ability to emulsify hydrocarbons (Oliveira & Garcia-Cruz, 2013). Previous studies have been reported EI_{24} of 33 % with motor oil (Makkar & Cameotra, 1998) and EI_{24} of 40 % with n-hexadecane (Pereira *et al.*, 2013) of the biosurfactant produced by a thermophilic *B. subtilis* strain and three *B. subtilis* isolates from crude oils, respectively. Sousa *et al.* (2014) obtained the best emulsifying index for *B. subtilis* ATCC 6633 by using n-hexadecane (53 %), followed by soybean oil (43 %). They also reported no measurable emulsifying index when kerosene was used.

Second, the ability of the strain B37 to reduce surface tension of MSM containing four different nitrogen sources was tested (Table 5). All organic nitrogen sources such as yeast extract, peptone and urea as well as inorganic $NaNO_3$ could reach a high reduction of the surface tension. The slightly greatest reduction in surface tension from 55.0 to 30.3 mN/m was yet obtained by $NaNO_3$. Additionally, sodium nitrate had the highest biomass output of 1.4 g/L CDW and was therefore chosen for the following optimization trials.

Kim *et al.* (1997) showed that inorganic nitrogen sources such as ammonium bicarbonate (NH_4HCO_3) resulted in a greater yield of biosurfactant production by *B. subtilis* C9. They also found that *B. subtilis* strain C9 requires a supplementary organic nitrogen source in the media for the production of larger biosurfactant amounts. Likewise, Makkar & Cameotra (1997) also reported that the best biosurfactant production by *B. subtilis* was obtained when using sodium nitrate or potassium nitrate, compared to organic nitrogen sources such as peptone or yeast extract.

Table 5: Effect of nitrogen source on biosurfactant production by *Bacillus subtilis* B37, which was cultivated in 250 mL flask containing 50 mL MSM medium at 30°C in a shaking incubator at 150 rpm for 48 h (C-source: 10 g/L of molasses).

N-source	CDW (g/L)	Final pH*	Surface tension (mN/m)	ST reduction (mN/m)	EI_{24} (%)
$NaNO_3$	1.36±0.16[a**]	8.68±0.08	30.3±0.58 (55)†	24.7[a]	0
Peptone	0.62±0.01[c]	6.77±0.02	29.7±0.29 (53)	23.3[b]	0
Urea	0.75±0.00[c]	8.41±0.11	30.2±0.29 (51)	20.8[c]	0
Yeast	0.93±0.06[b]	6.87±0.01	29.0±0.00 (50)	21.0[c]	0

*Values are given as mean±SD from triplicate determinations.
**Different superscript letters in the same column indicate significant differences ($p < 0.05$).
†Surface tension of MSM supplemented with each nitrogen source without inoculated culture.

The selected strain could not emulsify kerosene after 24 h; therefore, no emulsification index was calculated.

4.1.2 Effect of environmental factors on growth and biosurfactant activity

Besides medium composition, pH and agitation speed also affected the growth and biosurfactant production by the selected strain. *Bacillus subtilis* B37 was cultivated in the optimal medium containing 10 g/L molasses as a carbon source and 1 g/L $NaNO_3$ as a nitrogen source. The initial pH of the MSM was adjusted to 4.0, 5.0, 7.0 and 8.0 (Table 6).

Table 6: Effect of pH values on biosurfactant production by *Bacillus subtilis* B37, which was cultivated in 250 mL flask containing 50 mL MSM medium at 30°C in a shaking incubator at 150 rpm for 48 h (C-source: 10 g/L of molasses, N-source: 1 g/L of sodium nitrate).

Initial pH	CDW (g/L)	Final pH*	Surface tension (mN/m)	ST reduction (mN/m)	EI_{24} (%)
4.0	0.00±0.02[b**]	4.33±0.05	57.2±0.29 (58)†	0.8[c]	0
5.0	1.19±0.03[a]	8.42±0.10	31.0±0.00 (54)	23.0[b]	0
7.0	1.19±0.07[a]	8.97±0.04	31.3±0.58 (57)	25.7[a]	0
8.0	1.39±0.27[a]	9.09±0.07	30.5±0.50 (54)	23.5[b]	0

*Values are given as mean±SD from triplicate determinations.
**Different superscript letters in the same column indicate significant differences ($p < 0.05$).
†Surface tension of MSM supplemented with each pH-value without inoculated culture.

Whereas *B. subtilis* B37 could not growth in a pH as low as 4.0, all other examined pH-values reached a reasonable bacterial growth and reduction in surface tension. The cell growth of the selected strain increases with increasing pH and reached a maximum at pH 8.0 with a value of 1.4 g/L CDW. The lowest surface tension was obtained in the medium with an initial pH of 7.0. The supernatant produced by *B. subtilis* B37 could reduce the surface tension of the pH neutral medium from 57.0 to 31.3 mN/m and was chosen for the upcoming experiments. Many authors reported that the optimum pH-range for biosurfactant-producing *B. subtilis* strains is between pH 6.5 to 7.0 (Abushady *et al.*, 2005; Jacques *et al.*, 1999; Joshi *et al.*, 2008; Kim *et al.*, 1997). Compared to this, rhamnolipid production by *Pseudomonas* sp. (Guerra-Santos *et*

al., 1984) was at its maximum in a pH-range of 6.0 to 6.5 and decreased sharply above pH 7.0.

The effect of agitation on growth and biosurfactant activity by *B. subtilis* B37 in optimized medium with 10 g/L molasses, 1 g/L $NaNO_3$ and a pH-value of 7.0, is shown in Table 7. The different shaking speeds ranging from 100, 150, 200 to 250 rpm could all reach a high biosurfactant production. The slightly highest surface tension reduction of MSM from 57.0 to 31.5 mN/m was performed at 200 rpm. Thus, the agitation speed at 200 rpm was selected for further studies.

The effects of biosurfactant on oxygen transfer have been investigated by Sheppard & Cooper (1990). It was observed that biosurfactant production in yeast and bacteria was increased with increasing the agitation speed. They concluded that oxygen transfer is one of the key parameters for the process optimization and scale-up of biosurfactant production in *B. subtilis*.

Table 7: Effect of agitation speed on biosurfactant production by *Bacillus subtilis* B37, which was cultivated in 250 mL flask containing 50 mL MSM medium at 30°C in a shaking incubator at 150 rpm for 48 h (C-source: 10 g/L of molasses, N-source: 1 g/L of sodium nitrate, pH 7.0).

Agitation (rpm)	CDW (g/L)	Final pH*	Surface tension (mN/m)	ST reduction (mN/m)	EI_{24} (%)
100	1.13±0.24	8.70±0.04	31.0±0.00 (49)†	18.0b**	0
150	1.13±0.05	8.68±0.07	31.8±0.76 (50)	17.7b	0
200	0.99±0.13	8.45±0.34	31.5±1.50 (57)	25.5a	0
250	1.15±0.00	8.62±0.06	32.3±0.58 (57)	24.7a	0

*Values are given as mean±SD from triplicate determinations.
**Different superscript letters in the same column indicate significant differences ($p < 0.05$).
†Surface tension of MSM supplemented with each agitation speed without inoculated culture.

The emulsification index could not be determined once again, which leads to the assumption that the examined *B. subtilis* strain B37 produces a biosurfactant which shows no emulsification activity with kerosene. Considering that low-molecular weight surfactants are less effective for emulsifying, the results confirmed that the biosurfactant tested belongs to this class of surface-active agents.

Smyth *et al.* (2010) reported on the isolation and analysis of lipopeptides compared to high-molecular weight biosurfactants. They found that low-molecular weight surfactants efficiently reduce surface and interfacial tension, whereas high-molecular weight bioemulsifiers are usually more effective in stabilizing emulsions but do not lower the surface tension as much. The most efficient biosurfactants that form stable emulsions are emulsan and liposan, produced by *Acinetobacter* sp. and *Candida lipolytica*, respectively. The former is regarded as one of the most powerful emulsion stabilizer with concentrations as low as 0.01-0.001 % and emulsan-to-hydrocarbon ratios of 1:100 to 1:1000 (Ron & Rosenberg, 2001).

4.1.3 Effect of medium concentrations on growth and biosurfactant activity

The effect of carbon source concentrations on growth and biosurfactant production is shown in Table 8. Cultivation was performed in MSM containing molasses as a carbon source with sodium nitrate as a nitrogen source (1 g/L), an initial pH of 7.0 at room temperature and shaking at 200 rpm for two days. Growth of the bacterial strain B37 was enhanced with increasing molasses concentration. The highest cell growth of 1.7 g/L was obtained when using the maximal examined molasses concentration of 20 g/L. The supernatant of this cultivation could also reduce the surface tension of the medium from 54.0 to a value as low as 30.7 mN/m. From these results, the highest examined concentration of molasses (20 g/L) was selected for following tests.

A previous study by Abushady *et al.* (2005) reported the effect of carbon concentrations ranging from 5 to 60 g/L on the biosurfactant production by *B. subtilis*. The results obtained elucidated that there was a significant increase in surfactin production upon addition of glucose up to 30 g/L. They further found that there was an almost linear increase in surfactin concentration with increasing the initial glucose concentration up to 30 g/L. Kim *et al.* (1997) reported similar findings, and found that with increasing carbon concentration the production yield increases linear with a little change over 40 g/L glucose.

Table 8: Effect of molasses concentration on biosurfactant production by *Bacillus subtilis* B37, which was cultivated in 250 mL flask containing 50 mL MSM medium at 30°C in a shaking incubator at 200 rpm for 48 h (N-source: 1 g/L of sodium nitrate, pH 7.0).

Molasses (g/L)	CDW (g/L)	Final pH*	Surface tension (mN/m)	ST reduction (mN/m)	EI_{24} (%)
0	0.15±0.09^{c**}	7.37±0.01	51.3±1.53 (67)†	15.7^{c}	0
5	0.43±0.19^{c}	8.13±0.06	31.0±0.00 (55)	24.0^{a}	0
10	1.19±0.06^{b}	8.24±0.08	30.3±0.29 (50)	19.2^{b}	0
20	1.70±0.20^{a}	7.73±0.04	30.7±0.29 (54)	23.3^{a}	0

*Values are given as mean±SD from triplicate determinations.
**Different superscript letters in the same column indicate significant differences ($p< 0.05$).
†Surface tension of MSM supplemented with molasses without inoculated culture.

Table 9 shows the effect of nitrogen concentrations on the biosurfactant production and growth of the examined *Bacillus subtilis* B37. The reduction in surface tension was not significant different when using concentrations of 0, 2 or 3 g/L sodium nitrate. However, the slightly highest biosurfactant activity was reached when using 1 g/L sodium nitrate as nitrogen source. Thus, 1 g/L sodium nitrate was chosen for further studies.

Abushady *et al.* (2005) examined the surfactin production after addition of different concentrations of ammonium nitrate $(NH_4)_2NO_3$ to the medium. They observed that the surfactin concentration increased with an increasing ammonium nitrate concentration up to 4.6 g/L.

Table 9: Effect of sodium nitrate concentration on biosurfactant production by *Bacillus subtilis* B37, which was cultivated in 250 mL flask containing 50 mL MSM medium at 30°C in a shaking incubator at 200 rpm for 48 h (C-source: 20 g/L of molasses, pH 7.0).

$NaNO_3$ (g/L)	CDW (g/L)	Final pH*	Surface tension (mN/m)	ST reduction (mN/m)	EI_{24} (%)
0	0.23±0.09^{c**}	7.15±0.01	33.3±0.58 (54)†	20.7^{b}	0
1	1.83±0.05^{b}	7.75±0.01	31.3±0.58 (55)	23.7^{a}	0
2	2.14±0.05^{a}	8.50±0.02	31.8±0.76^{b} (53)	21.2^{b}	0
3	2.24±0.08^{a}	8.46±0.08	31.7±0.58 (52.5)	20.8^{b}	0

*Values are given as mean±SD from triplicate determinations.
**Different superscript letters in the same column indicate significant differences ($p< 0.05$).
†Surface tension of MSM supplemented with sodium nitrate without inoculated culture.

4.1.4 Time course study of production and growth under optimized conditions

Bacillus subtilis B37 was cultivated in 250 mL Erlenmeyer flasks containing 150 mL optimized medium (20 g/L molasses, 1 g/L sodium nitrate) with an initial pH of 7.0 at 200 rpm and 30°C. During culture growth, samples were collected at 0, 6, 12, 24, 36 and 48 hours. Figure 8 shows the time course profile of the growth kinetics and biosurfactant production by *B. subtilis* B37. Cell growth rapidly increased during 24 hours of cultivation and slightly increased to the maximum growth (OD_{600}= 1.5) after 36 hours. Cell dry weight reached its maximum (3.2 g/L) at the end of the exponential growth phase after 24 hours of cultivation. A significant reduction in the surface tension of *B. subtilis* B37 supernatant (35 mN/m) was obtained after 12 hours of incubation, then reaching its minimal value (30 mN/m) after 24 hours of growth by the end of the exponential phase. Thereafter, a slight reduction in the surface tension was up to the end of cultivation. That may be attributed to the critical micelle concentration (CMC) value, in which the surface tension stayed stable (30 mN/m). The results revealed that the production of biosurfactant from palm oil-contaminated soil occurred predominately throughout the exponential phase indicating that the biosurfactant is a growth-associated primary metabolite (Elazzazy *et al.*, 2015).

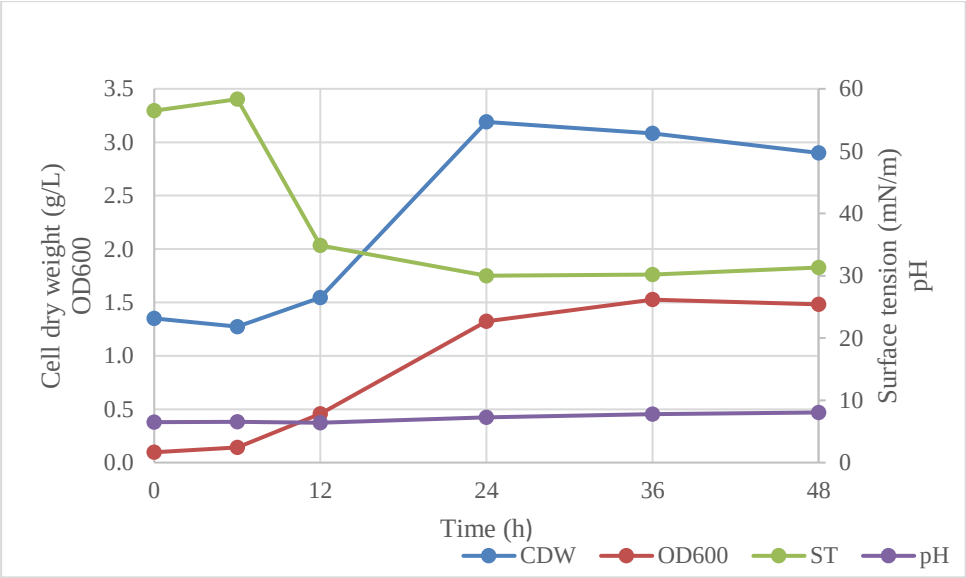

Figure 8: Time course of growth and biosurfactant production by *B. subtilis* B37 in optimized medium (20 g/L molasses, 1 g/L sodium nitrate, initial pH 7.0) at 200 rpm and 30°C.

4.2 Recovery of biosurfactant produced by *Bacillus subtilis* B37

Bacillus subtilis B37 was cultivated in the optimized MSM with molasses (10 g/L) and $NaNO_3$ (1 g/L) as a carbon and nitrogen source, respectively, a pH-value of 7.0 and agitation speed of 200 rpm. The supernatant at 48 hours of cultivation was extracted with: chloroform/methanol mixture, precipitation with acetone, methanol, as well as precipitation in an acidic condition (pH 2.0). The latter three methods formed pellets, which leads to a low reduction in surface tension. Only the recovery with chloroform/methanol extraction could obtain a good surface tension reduction profile. The mixture consists of a polar as well as non-polar compound and could therefore extract the amphiphilic biosurfactant better than single solvents. Hence, a mixture of chloroform/methanol (2:1) was chosen for further studies.

The CMC of obtained crude biosurfactant from *B. subtilis* B37 was obtained by plotting a graph of surface tension versus the surfactant concentration as shown in Figure 9. The surface tension of water decreased gradually with increasing biosurfactant concentration from 72.0 to 30.5 mN/m and then remained constant. This change of slope marks the CMC, with a biosurfactant concentration of 1.5 g/L.

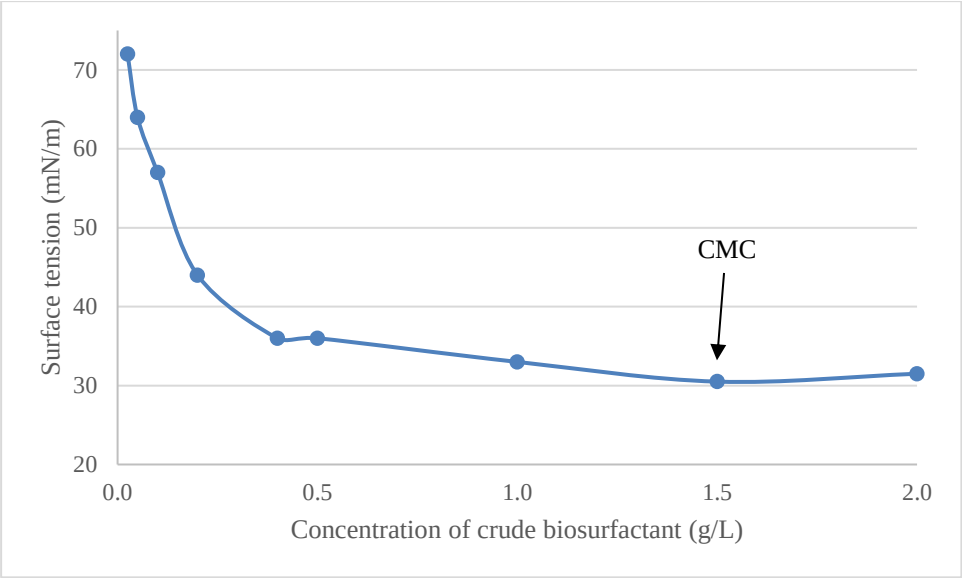

Figure 9: CMC of crude biosurfactant obtained from *Bacillus subtilis* B37 extracted with chloroform/methanol (2:1).

The critical micelle concentration is specific to each surfactant and low CMC values represent the effectiveness of a biosurfactant (Mulligan, 2005). The biosurfactant produced by *B. subtilis* B37 proved to be efficient as the result was lower when compared to SDS, which has a CMC of 2.1 g/L (Chen *et al.*, 2012). In general, biosurfactants have about a 10- to 40-fold-lower CMCs than chemical surfactants (Desai & Banat, 1997). For example, surfactin isolated from different *B. subtilis* strains shows surface CMC values as low as 10 mg/L (Soberón-Chávez, 2011). Compared to this, the CMC of rhamnolipids has been reported to be around 40 mg/L (Zhang & Miller, 1992).

4.2.1 Determination of biosurfactant yield

After determination of the most suitable recovery method as well as medium composition (20 g/L molasses, 1 g/L sodium nitrate) and cultivation conditions (200 rpm, pH= 7.0, 30°C), the biosurfactant yield was calculated. The biosurfactant yield is defined as the crude biosurfactant concentration divided by the cell dry weight.

$$Yield\ (\%) = \frac{0.52\ g/L}{1.98\ g/L} = 26.20\ \%$$

The final yield of the biosurfactant produced by the examined *B. subtilis* strain B37 after chloroform/methanol (2:1) extraction and optimized conditions was 26.20 %. This result indicates that the purity of the surfactant preparation obtained by the proposed process was relatively low and therefore an additional purification step, e.g. high-performance liquid chromatography (HPLC), is necessary.

4.3 Characterization of crude biosurfactants produced by *B. subtilis* B37

4.3.1 Biosurfactant stability studies

The effect of pH and temperature on the stability of the biosurfactant was investigated to characterize surface-active compounds by the selected *Bacillus subtilis* strain B37. Therefore, the crude biosurfactant obtained from chloroform/methanol extraction was diluted to its CMC of 1.5 g/L in a total volume of 10 mL. The effect of pH and temperature on the activity of sodium dodecyl sulphate at its CMC of 1.12 g/L was also studied.

- Effect of pH on the stability of biosurfactant

The surface tension was measured at pH-values ranging from 3.0 to 11.0. The surface tension of the biosurfactant remained stable at a wide range of pH (5.0-10.0) (Figure 10). The highest reduction in surface tension (31 mN/m) was obtained at neutral pH. Although a significant stable surface activity was also observed at acidic pH (pH= 3.0, ST= 35 mN/m) as well as alkaline pH (pH= 10.0, ST= 34 mN/m), a drop in the surface activity was observed at pH higher than 10. Extreme pH may cause partial precipitation of the biosurfactant (Abouseoud *et al.*, 2008). Compared to this, the surface tension of the synthetic surfactant SDS fluctuates from 44 to 48 mN/m.

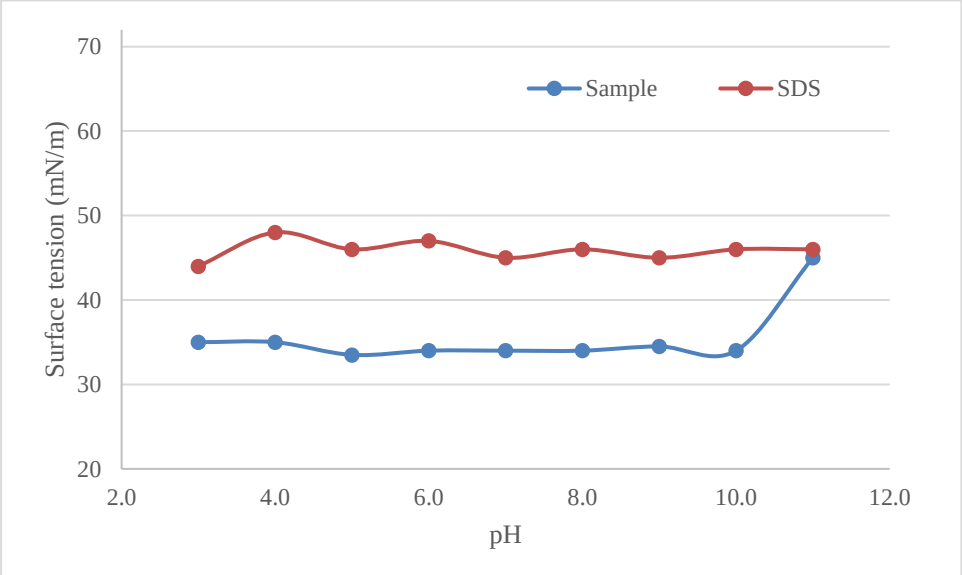

Figure 10: Effect of pH on the surface tension of the sample (*B. subtilis* B37) and sodium dodecyl sulphate (SDS).

- Effect of temperature on the stability of biosurfactant

The effect of temperature on the stability of crude biosurfactant obtained from *B. subtilis* B37 is shown in Figure 11. At different temperatures, no significant changes were observed in the surface tension after incubation period of 1 hour (30-100°C) or 15 min (121°C). The examined biosurfactant remained stable over the whole temperature scale tested and could retain its full activity. The biosurfactant produced by *B. subtilis* B37 was found to be thermostable because

heating at 80-121°C caused no significant effect on the ability to reduce surface tension. Temperature is one of the most important parameters that significantly influences the growth of microorganisms and thus biosurfactant production (Singh & Tiwary, 2016). Moreover, the results enable the potential application of the examined biosurfactant in various industries such as pharmaceutical, food, cosmetics as well as in microbial enhanced oil recovery (MEOR) where heating is very important (El-Sheshtawy *et al.*, 2015; Abouseoud *et al.*, 2008). In contrast, SDS could reduce the surface tension of water only from 72.0 to 44.0 mN/m at its maximum, but also remained stable over the examined temperature range.

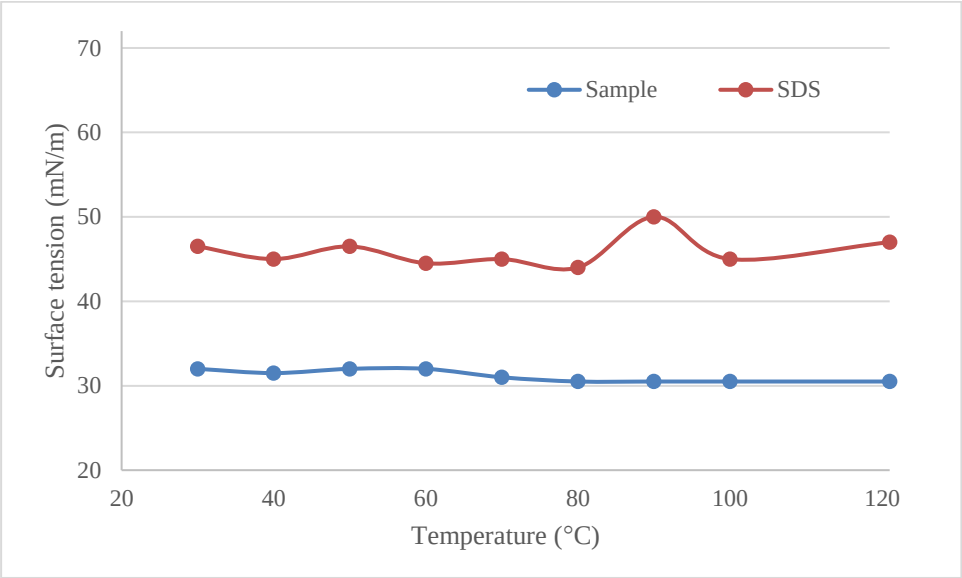

Figure 11: Effect of temperature on the surface tension of the sample (*B. subtilis* B37) and sodium dodecyl sulphate (SDS).

4.3.2 Structural characterization of biosurfactant

The examined biosurfactant was recovered from the culture broth by chloroform/methanol extraction. Separation of the sample compounds was completed by thin-layer chromatography (TLC). The developed silica gel plates were dipped into different stains to detect colorless organic compounds and observe TLC results. The chromatograms (Figure 12) showed positive reaction with *p*-anisaldehyde and copper (II) sulphate, indicating the presence of carbohydrates and lipid moieties respectively. When dipped into ninhydrin reagent, no spots were detected confirming the absence of free amino acids. The presence of glycosyl units and lipid moieties on the same spots but negative reactions for amino groups reveals that the sample is a glycolipid type biosurfactant.

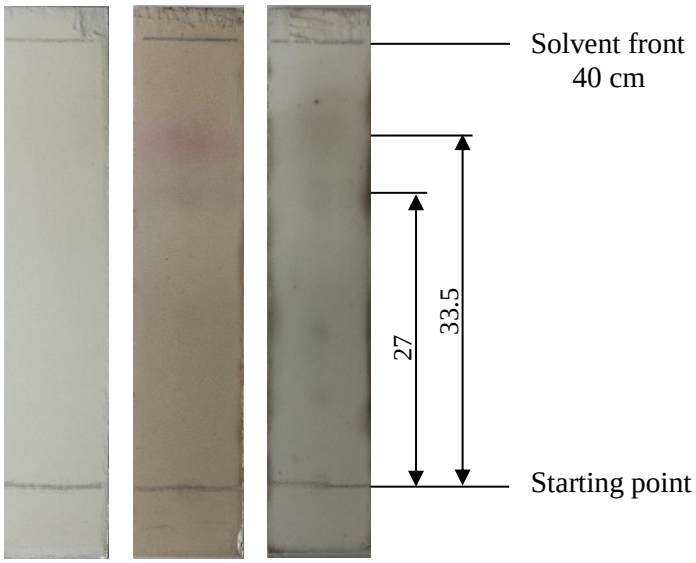

Figure 12: Thin-layer chromatography analysis of crude biosurfactant using chloroform/methanol/H_2O (1:2:0.1) (A) ninhydrin (B) p-anisaldehyde (C) copper (II) sulphate.

The ratio of the distance travelled by the compound in a particular solvent to the distance travelled by the solvent is known as retardation factor (R_f). The major component in the sample extract has an R_f-value of 0.84. Similar reports of the production of glycolipid biosurfactants by *Pseudomonas aeruginosa* (R_f= 0.85) and *P. cepacian* (R_f= 0.9) are in the literature (Silva *et al.*, 2010; Silva *et al.*, 2014).

$$R_f = \frac{33.5\ mm}{40\ mm} = 0.84$$

The results from the TLC analysis suggest the glycolipid nature of the biosurfactant by *Bacillus subtilis* B37 in the present study, consisting of a carbohydrate moiety linked to fatty acids. Even though most reported *Bacillus* spp. produce lipopeptides, the chemical nature of biosurfactants varies with both species and strains within the genus *Bacillus* (Thavasi *et al.*, 2008).

Conclusion

With increasing environmental awareness and emphasis on a sustainable society in accordance with the environment, natural surfactants produced by microorganisms have been becoming much more important. The key factor governing the success of biosurfactant production is the development of an economical process that uses low-cost materials and gives high productivity. The efficiency of biosurfactant production by microorganisms can be enhanced by using inexpensive medium components such as food industry by-products or waste, since they represent about 50 % of the total production costs. In the present thesis, a low-cost carbon source based on a by-product of cane sugar refining has been successfully evaluated for biosurfactant production by the bacteria *Bacillus subtilis* B37. The combination of the medium composition with optimized cultivation conditions and recovery method resulted in a relatively high biosurfactant production yield. Structural characterization by thin-layer chromatography confirmed that the biosurfactant produced *B. subtilis* B37 is a glycolipid in nature. The stability studies further showed that the biosurfactant retained its activity at extreme temperatures and pH-values. Furthermore, the excellent surface tension reducing property of the examined strain B37 and critical micelle concentration suggest the possible usage of this new biosurfactant in a wide variety of industrial applications such as food, pharmaceutical and cosmetic industries. This strain may also be used for bioremediation of hydrocarbon contaminated soils.

The future of biosurfactants will depend on their costs and applications. High production costs of biosurfactants is a limiting factor for their wide applicability, regarding this, microbial surfactants are not yet competitive with chemical surfactants. Hence, an effort should be made on different aspects of production to find a suitable and economically viable process. The results obtained from the present investigation indicated that a promising biosurfactant-producing *B. subtilis* B37 has been optimized and specified. Further research on structural characterization as well as purification studies, in addition to more knowledge on the genetics and metabolism of this strain will be required.

Appendix

4.4 References

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4.5 List of Figures

4.6 List of Tables

4.7 List of Abbreviation

Abbreviation	Full name
Abs	Absorbance units
ANOVA	Analysis of variance
B37	Strain of *Bacillus subtilis*
C	Carbon
$CaCl_2$	Calcium chloride
CDW	Cell dry weight
CMC	Critical Micelle Concentration
C/N	Carbon to nitrogen ratio
Cu	copper
EI_{24}	Emulsification index after 24 hours
F	Force
FDA	American Food and Drug Administration
$FeCl_2$	Iron (II) chloride
g	Gram
GRAS	Generally regarded as safe
h	Hour
HCl	Hydrogen chloride
H_2O	Water
HOC	Hydrophobic organic compounds
HPLC	High-performance liquid chromatography
HSV	Herpes simplex virus
K_2HPO	Dipotassium phosphate
KH_2PO_4	Monopotassium phosphate
L	Liter
MEOR	Microbial enhanced oil recovery
mg	Milligram
$MgCl_2$	Magnesium chloride
min	Minutes
mL	Milliliter
mm	Millimeter
mN/m	Millinewton per meter
MSM	Minimal salt medium
N	Nitrogen
n/a	Not available
$NaCl_2$	Sodium chloride

$NaNO_3$	Sodium nitrate
NaOH	Sodium hydroxide
NH_4HCO_3	Ammonium bicarbonate
$(NH_4)_2NO_3$	Ammonium nitrate
nm	Nanometer
OD_{600}	Optical density at a wavelength of 600 nm
o/w	Oil-in-water
ppm	Part per million
p-value	Probability
R_f	Retardation factor
RO	Reverse osmosis
rpm	Revolutions per minute
SD	standard deviations
SDBS	Sodium dodecyl benzene sulfonate
SDS	Sodium dodecyl sulphate
SPSS	Statistical Package for the Social Sciences
ST	Surface tension
TLC	Thin-layer chromatography
USDA	United States Department of Agriculture
USP	Used palm oil
UV	Ultraviolet
µL	Microliter
µM	Microgram
w/o	Water-in-oil
Zn	Zinc