DOKUZ EYLÜL UNIVERSITY
GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES

PRODUCTION AND PARTIAL CHARACTERIZATION OF BIOSURFACTANT FROM LIGNOSELLULOSIC WASTES

by
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December, 2014
İZMİR

PRODUCTION AND PARTIAL CHARACTERIZATION OF BIOSURFACTANT FROM LIGNOSELLULOSIC WASTES

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We have read the thesis entitled **"PRODUCTION AND PARTIAL CHARACTERIZATION OF BIOSURFACTANT FROM LIGNOSELLULOSIC WASTES"** completed by **ZÜLFİYE VELİOĞLU** under supervision of **PROF. DR. RAZİYE ÖZTÜRK ÜREK** and we certify that in our opinion it is fully adequate, in scope and in quality, as a thesis for the degree of Master of Science.

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Zülfiye VELİOĞLU

PRODUCTION AND PARTIAL CHARACTERIZATION OF BIOSURFACTANT FROM LIGNOSELLULOSIC WASTES

ABSTRACT

Biosurfactants produced by microorganisms have emulsification, wetting, solubilization and phase-dispersion properties. As their biodegradation ability, low toxicity, ecological acceptability and ability to be produced from renewable and cheaper substrates, biosurfactants are used in a wide variety of fields such as chemistry, food, cosmetic, pharmaceutics industries, biomedical and agriculture. The usage areas of biosurfactants depend on their type and physicochemical properties. The producer microorganism, production conditions and used substrates are important parameters that influence biosurfactant type and productivity.

In this study, economical production of biosurfactant was performed by white rot fungi *Pleurotus* spp. using sunflower seed shell, potato peel or grape waste on solid state fermentation. After determination of biosurfactant produced by *P. djamor* had the maximum activity, optimization studies were carried out. Optimum production conditions were determined as 5 g sunflower seed shell with 1 centimeter square particle surface area and sunflower oil, 10 mL liquid medium, at 29 degree Celsius incubation temperature. The produced biosurfactant at optimal condition amount of 10.21 g in per liter has high surface activity with 28.82 mN in per meter surface tension, 44.44 percents emulsification index and 3.9 centimeter oil spreading activity. Regarding the results of the chemical contents analysis, HPLC, FT-IR and 1H-NMR, it can be concluded that the produced and isolated biosurfactant has complex structure. Besides, resistance of its activity to environmental factors, critical micelle concentration and thermal stability were investigated. Also ligninolytic and lipase enzyme activities during biosurfactant production were searched.

Consequently, economical biosurfactant production was enabled using wastes which cause environmental pollution. The determination of activity and chemical structure of produced biosurfactant from a new species (*P. djamor)* provided a basis

to form different usage areas. Also, simultaneous enzymes production is important in terms of being sample for multiple productions in a single process.

Keywords: Biosurfactant, *Pleurotus* spp., solid state fermentation, ligninolytic enzymes, lipase, partial characterization.

LİGNOSELLÜLOZİK ATIKLARDAN BİYOSÜRFAKTAN ÜRETİMİ VE KISMİ KARAKTERİZASYONU

ÖZ

Biyosürfaktanlar, mikroorganizmalar tarafından üretilmekte olup; emülsifiye etme, ıslatma, çözünürleştirme ve faz-dağıtma özelliklerine sahiptirler. Biyodegradasyon yetenekleri, düşük toksisiteleri, ekolojik uyumlulukları ve yenilenebilir ucuz substratlardan üretilebilmeleri nedeniyle biyosürfaktanlar; kimya, gıda, kozmetik, farmasötik sanayi, biyomedikal ve tarımda kullanılmaktadırlar. Biyosürfaktanların kullanım alanları, türüne ve fizikokimyasal özelliklerine göre değişiklik göstermektedir. Üretici mikroorganizma, üretim koşulları, kullanılan substrat, üretilen biyosürfaktanın türünü ve verimini etkileyen önemli parametrelerdir.

Çalışmada, *Pleurotus* spp. beyaz çürükçül funguslarla ayçiçeği çekirdeği kabukları, patates kabukları veya üzüm atıkları kullanılarak katı hal fermentasyonu ile ekonomik biyosürfaktan üretimi gerçekleştirilmiştir. Maksimum aktivitede biyosürfaktan üretiminin, *P. djamor* ile gerçekleştiği belirlendikten sonra optimizasyon çalışmaları yapılmıştır. Optimum üretim koşulları; partikül yüzey alanı 1 santimetre kare olan 5 g ayçiçeği çekirdek kabuğu ve ayçiçeği yağı varlığında, 10 mL sıvı besin ortamı, 29 derece Santigrat inkübasyon sıcaklığı olarak belirlenmiştir. Optimum koşullarda litrede 10,21 g üretilen biyosürfaktan metrede 28,82 mN yüzey gerilimi, yüzde 44,44 emülsifikasyon indeksi ve 3,9 santimetre yağ yayma etkinliği ile yüksek yüzey aktivitesine sahiptir. Üretilen ve izole edilen biyosürfaktanın kimyasal içerik analizleri, HPLC, FT-IR ve 1H-NMR sonuçlarına göre kompleks yapıya sahip olduğu belirlenmiştir. Ayrıca biyosürfaktan aktivitesinin çevresel etkenlere dayanıklılığı, kritik misel konsantrasyonu ve termal stabilitesi araştırılmıştır. Biyosürfaktan üretimi esnasında ligninolitik ve lipaz enzim aktiviteleri de incelenmiştir.

Sonuç olarak; çevre kirliliği oluşturan atıklar kullanılarak, ekonomik biyosürfaktan üretimi sağlanmıştır. Yeni bir türden (*P. djamor*) üretilen biyosürfaktanın aktivitesinin ve kimyasal yapısının belirlenmesi, farklı kullanım alanlarının oluşmasına zemin hazırlamıştır. Ayrıca, eş zamanlı gerçekleştirilen enzimlerin üretimi tek proseste çoklu üretime örnek oluşturması açısından önemlidir.

Anahtar kelimeler: Biyosürfaktan, *Pleurotus* spp., katı hal fermentasyonu, ligninolitik enzimler, lipaz, kısmi karakterizasyon.

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CHAPTER ONE
INTRODUCTION

1.1 Surface Active Agents

A surface active agent (= surfactant) is a substance which lowers the surface tension of the medium in which it is dissolved, and/or the interfacial tension with other phases. Surfactants achieve this effect by acting as a bridge between the two materials meeting at the interface (Marchant, & Banat, 2012). Surface tension is the energy or work, required to increase the surface area of a liquid due to intermolecular forces (Figure 1.1 (a)). At room temperature, the surface tension of water is approximately 72 mN/m and this is the highest surface tension of any biologically relevant liquid which is resulted from the effects of hydrogen bonding (Gerson, 1993). A good surfactant can lower the surface tension of water to 35 mN/m (Mulligan, 2005). Interfacial tension is defined as an intermolecular attractive force held within the molecules in a liquid (Figure 1.1 (a)). Surfactants are amphiphilic molecules that contain both hydrophobic groups and hydrophilic groups (Figure 1.2). In general, surfactants are classified on the basis of the charge of polar head group and the common practice is to divide the surfactants into anionic, cationic, non-ionic and zwitterions.

Surfactants are commonly produced using a variety of organic chemistry methods, depending on the type and structure of the molecule desired. The non-polar, hydrophobic, water insoluble tail is frequently a hydrocarbon chain while the polar, hydrophilic, water soluble head is carboxylate, sulphate, sulphonate, phosphates or an amine product. In each case, the hydrophilic head of the surfactant is strongly attracted to the water molecules. As a result, the surfactant molecules align themselves as the hydrophilic heads are toward the water and the hydrophobic tails assemble away from the water. This formation of surfactant molecules is defined as a micelle (Figure 1.2).

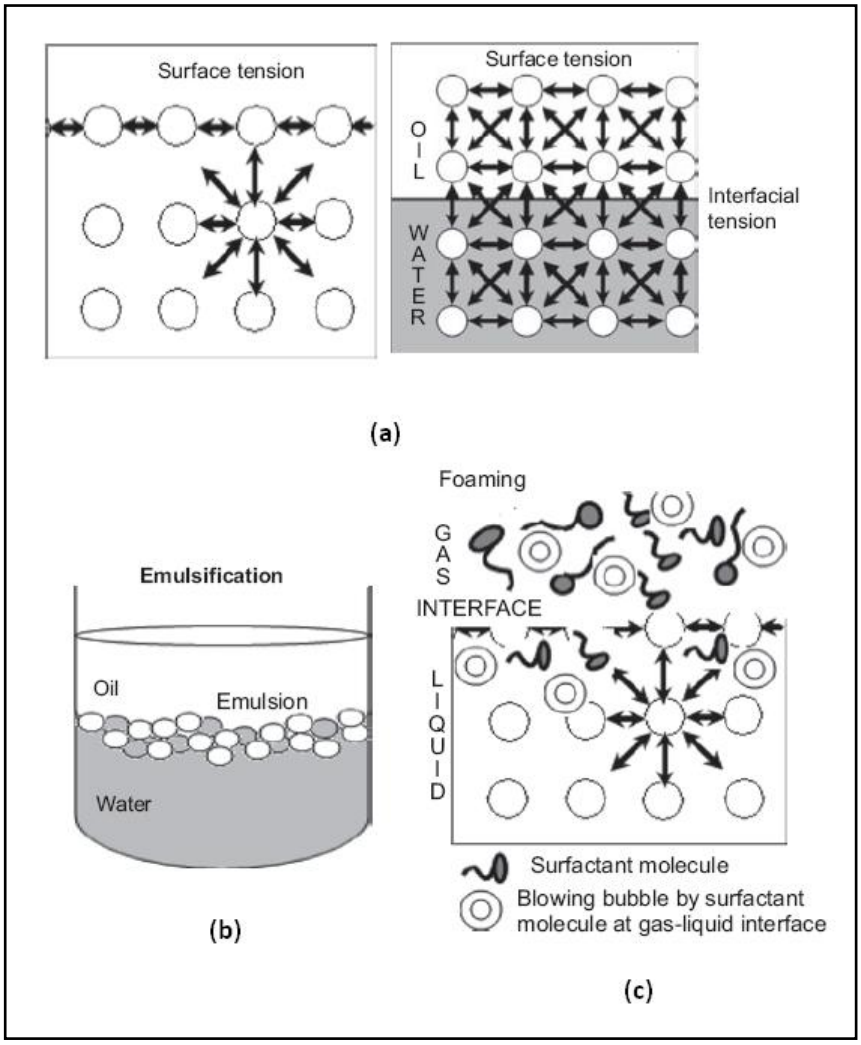

Figure 1.1 Different functional properties of surfactants (a) Surface and interfacial tension, (b) Emulsification, (c) Foaming (Satpute, Banpurkar, Dhakephalkar, Banat, & Chopade, 2010)

The capacity of aggregation in a solution is one of the characteristics of surfactants (Mohajeri, & Noudeh, 2012). As the aggregation formed, within a narrow concentration range, some physical properties of the surfactant solutions change abruptly. Micelles are one type of aggregation, and the narrow concentration range is called the critical micelle concentration (CMC), above which micelles are formed in the solutions (Figure 1.2). The shape and size of a micelle depends on molecular geometry of surfactant molecules, surfactant concentration, temperature, pH and ionic strength. At low surfactant concentration the surfactant molecules arrange on the surface. When more surfactant is added, the surface tension of the solution starts to rapidly decrease since more and more surfactant molecules will be on the surface.

When the surface becomes saturated, the addition of the surfactant molecules will lead to formation of micelles at CMC (Román–Guerrero, Vernon–Carter, & Demarse, 2010). CMC causes rapid changes in the physical properties of a solution such as surface tension, conductivity, viscosity, density, osmotic pressure, turbidity and chemical shifts (Satpute et al., 2010). Below the CMC, surface tension in aqueous systems falls to a minimum value approximately 29 mN/m (Desai, & Banat, 1997). CMC is used evaluate surfactant efficiency and an efficient surfactant has very low CMC value.

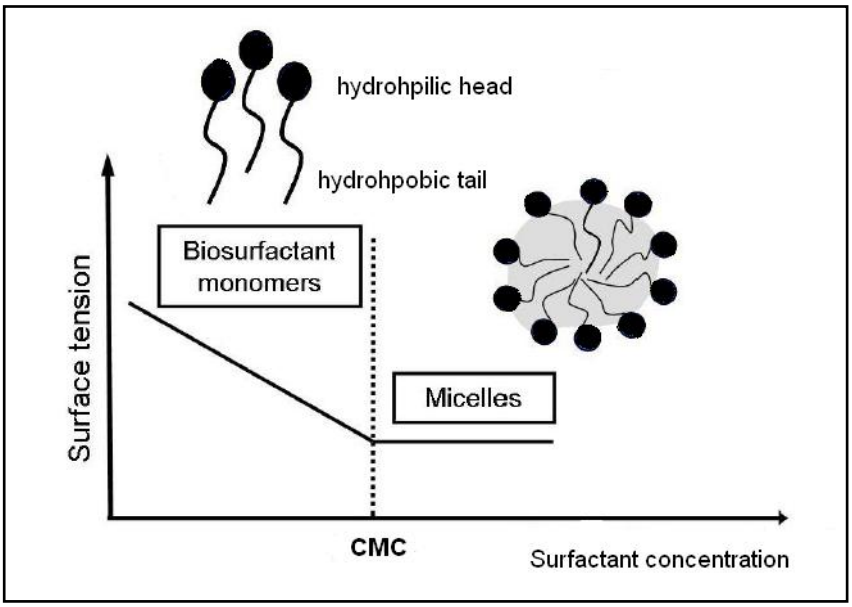

Figure 1.2 Monomeric surfactant molecules, micelle structure and the relationship between surfactant concentration, surface tension and CMC (Whang, Liu, Ma, & Cheng, 2008)

Their amphiphilic nature gives surfactant molecules unique properties such as to act as emulsifiers, wetting, dispersants, foaming and detergents agents (Marchant, & Banat, 2012). Although there are similarities in these functions, in practice the surfactants required to perform these functions widely different. Emulsification is dispersion of one liquid into another leading to the mixing of two immiscible liquids (Figure 1.1 (b)). Emulsions are generally unstable and they are stabilized with surfactants. Also, surfactants act as wetting agents with spreading and penetrating power. In addition surfactants get concentrated at a gas-liquid interface generating foam formation (Figure 1.1 (c)). The dispersant property of surfactants keeps

insoluble particles in suspension by preventing them from aggregations with each other. The function of detergency or cleaning is a complex combination of all the previous functions.

Due to their attractive properties, surfactants form main ingredients of many product formulations ranging from household detergents, shampoos, personal care products, pharmaceutical products and paints. According to the report "Surfactants Market by Product type, Substrates and Applications - Global Trends & Forecast to 2019" surfactant consumption is reach 22,802.1 kilotons, and the surfactant market value reach $40.286.3 million, by 2019. The choice of surfactant is firstly based on product cost, and secondly charge-type, physicochemical behavior, solubility and adsorption behavior are some of the selection criteria. In this competitive market, more economical an effective new product will replace the older ones.

Most of surfactants are petro-chemical origin and they are generally toxic to environment and non-biodegradable (Banat, Makkar, & Cameotra, 2000). After use, residual surfactants and their by-products are discharged to sewage treatment plants or directly to surface waters (Ying, 2006). Long-term use of surfactants has adverse effects on environment and human. Environmental concerns and the biodegradation rate have become one of the main driving forces for the development of new surfactants (Negm, & Tavvfik, 2013). New environmental regulations and increasing awareness to protect the ecosystem have effectively resulted in an increasing interest in alternatives to surfactants (Banat et al., 2000).

1.2 Biological Surface Active Agents

The biological surface active agents, biosurfactants, are structurally diverse groups of surfactants synthesized by wide variety microorganisms. The chemical structure of biosurfactants is formed from a hydrophilic tail and a hydrophobic head. The synthesis of both hydrophobic and hydrophilic moieties is dependent on the substrate used for biosurfactant production. Many microorganisms appear to produce

a complex mixture of biosurfactants, particularly during their growth on water-immiscible substrate (J. D., Desai, & A. J., Desai, 1993).

The natural roles of biosurfactants are current research topics. The cell membrane or cell wall of microorganisms represents the cell's primary interface with the environment. The diversity of microbial species and their unique physiologically interaction with different environmental interfaces is often mediated by cell-associated or secreted extracellular biosurfactants (Ward, 2010). In this regard the most fundamental requirement for cell survival and proliferation of microbial species relates to nutrient supply from the cell's external environment. Therefore, biosurfactants play various roles in facilitating that supply by mediating solubilisation, mobilization, accession and biodegradation of synthetic organic molecules. The best-known function of biosurfactants is increasing the surface area and bioavalibilty of hydrophobic, water-insoluble substrates (Van Hamme, Singh, & Ward, 2006). Biosurfactants improves the availability of the substrate to the microorganisms by allowing emulsion formation (Gerson, 1993). This expands the interfacial area at the aqueous-substrate interface, increasing the rate of substrate dissolutions. In addition, biosurfactants increases wetting of the insoluble substrates. Microorganisms, that degrade solid insoluble substrates, also produce biosurfactants to facilitate substrate break down and dissolution (Gerson, 1993). Also microorganisms produce biosurfactants to regulate the attachment and detachment of cells to and from surfaces (Rosenberg, & Ron, 2013). Biosurfactants play an important role in cell signaling, biofilm formation and cellular differentiation (Van Hamme et al., 2006). Additionally, biosurfactants increase bioavailability of heavy metals and lower toxicity levels of toxic materials. It is also possible that some biosurfactants are produced as external carbon storage. Another potential role of biosurfactants is enhancing gene uptake via transfection.

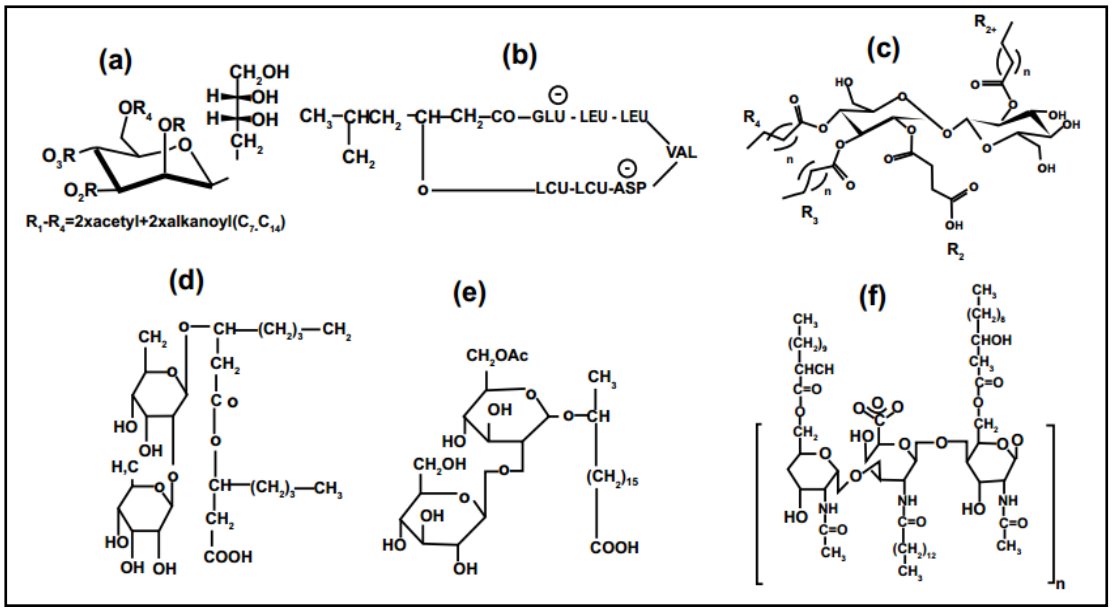

Figure 1.3 Chemical structures of some common biosurfactants (a) Mannosylerythritol lipid, (b) Surfactin, (c) Trehalose lipid, (d) Sophorolipid, (e) Rhamnolipid, (f) Emulsan (Fakruddin, 2012)

Biosurfactants have very different structures and surface properties. Physical and chemical properties, surface tension reduction and stability of the emulsion formed are very important in the search for a potential biosurfactant. These properties are used in evaluating biosurfactants and in screening potential microorganism for biosurfactant production. While synthetic surfactants are usually classified according to the nature of their polar group, biosurfactants are commonly differentiated on the basis of their biochemical nature and the microbial species producing them (J. D., Desai, & A. J., Desai, 1993). The major classes of biosurfactants (Figure 1.3; Table 1.1) are glycolipids, lipopeptides, phospholipids, polymeric and particulate surfactants (Nitschke, & Costa, 2007). The hydrophobic parts of molecule based on long-chain fatty acid, hydroxyl fatty acids or α-alkyl-β-hydroxy-fatty acids. The hydrophilic parts can be a carbohydrate, amino acid, cyclic peptide, phosphate, carboxylic acid or alcohol. Most biosurfactants are either anionic or neutral, only those containing amine groups are cationic.

Table 1.1 Major types of biosurfactants and producer microorganisms (Nitschke, & Costa, 2007)

Biosurfactant class	**Microorganism**
Glycolipids	
Rhamnolipids	*Pseudomonas aeruginosa*
Trehalose lipids	*Rhodococcus erithropolis, Arthobacter sp.*
Sophorolipids	*Candida bombicola, C. apicola*
Mannosylerythritol lipids	*C. antartica*
Lipopeptides	
Surfactin/iturin/fengycin	*Bacillus subtilis*
Viscosin	*P. fluorescens*
Lichenysin	*B. licheniformis*
Serrawettin	*Serratia marcescens*
Phospholipids	*Acinetobacter sp., Corynebacterium lepus*
Fatty acids/ Neutral lipids	
Corynomicolic acids	*C. insidibasseosum*
Polymeric biosurfactants	
Emulsan	*A. calcoaceticus*
Alasan	*A. radioresistens*
Liposan	*C. lipolytica*
Lipomanan	*C. tropicalis*
Particulate biosurfactants	
Vesicles	*A. calcoaceticus*
Whole microbial cells	Cyanobacteria

Glycolipids are carbohydrates like mono-, di-, tri- and tetrasaccharides that include glucose, mannose, galactose, glucuronic acid, rhamnose and galactose sulphate combined with long chain aliphatic acids or hydroxyl aliphatic acids (Mukherjee, & Das, 2010). These biosurfactants have low molecular weight. The most commonly studied glycolipids are rhamnolipids, sophorolipids, trehalose lipids and mannosylerythritol lipids. Lipopeptides, another low molecular weight biosurfactant type, have a hydrophilic peptide head group and hydrophobic lipid tail.

Surfactin, iturin, fengycin, viscosin, lichenysin and serrawettin are best known lipopeptide type biosurfactants. Such bioactive peptides usually occur as mixtures of closely similar compounds which show insignificant differences in their amino acid composition and/or their lipid portion. The structural analysis revealed that it is a mixture of lipopeptides with major components ranging in size from 979 to 1091 Da (Desai, & Banat, 1997). Certain hydrocarbon degrading microorganisms produce extracellular free fatty acids when grown on alkanes and exhibit good surfactant activity. The fatty acids as biosurfactants are saturated fatty acids in the range of C_{12} to C_{14} and complex fatty acids containing hydroxyl groups and alkyl branches (Mukherjee, & Das, 2010). Polymeric biosurfactants are high molecular weight biopolymers, which exhibit properties like high viscosity, tensile strength and resistance to shear. Emulsan, alasan and biodispersan are best studied ones. Emulsan has the heteropolysaccharide backbone contains a repeating trisaccharide of *N*-acetyl-D-galactosamine, *N*-acetylgalactosamine uronic acid and an unidenfied *N*-acetyl amino sugar (Desai, & Banat, 1997). Some of polymeric surfactants are composed of 50% carbohydrate, 19% protein and 10% lipid. Extracellular membrane vesicles partition hydrocarbons to form a microemulsion which plays an important role in alkane uptake by microbial cells. Some examples of particulate biosurfactants are extracellular membranes vesicles of microbial cells, which help in emulsification of hydrocarbons.

Biosurfactants have numerous advantages compared to their chemically synthesized counterparts; their surface activities are generally higher than synthetic surfactants. Many of the structures and properties of biosurfactants differ from synthetic surfactants, providing new possibilities for industrial applications. Many biosurfactants can conserve their surface activities and stabilities at changing environmental conditions such as temperature, pH and salinity. Probably, the most important advantage of biosurfactants is their ecological acceptability. Many synthetic surfactants cause ecological problems due to their resistance to biodegradation, toxicity and accumulation in natural ecosystems. Unlike synthetic surfactants, biosurfactants are easily degradable (Mohan, Nakhla, & Yanful, 2006). Biosurfactants are generally low or non-toxic products and therefore, convenient for

several application areas such as pharmaceutical, cosmetic and food industry. Due to antimicrobial activity of biosurfactants against bacteria, fungi, algae and viruses, they have various application fields.

1.3 Applications of Biosurfactants

With increasing environmental awareness and emphasis on sustainable society in conformity with the global environment, during the recent years, biosurfactants are getting much more attention as compared to the synthetic surfactants. In comparison to chemically synthesized surfactants, biosurfactants have many advantages such as environmentally friendly and biodegradable structure, less toxicity, higher selectivity and stability at extreme temperatures, pH and salinity (Pacwa-Płociniczak, Płaza, Piotrowska-Seget, & Cameotra, 2011). They can be used as emulsifiers, de-emulsifiers, wetting, detergency and foaming agents and functional food ingredients in petroleum, environmental management, agrochemicals, foods and beverages, cosmetics and pharmaceuticals, commercial laundry detergents as bio-detergent and in mining and metallurgical industries (Mukherjee, & Das, 2010).

Biosurfactants are generally less toxic and more biodegradable than synthetic surfactants, they commonly used in environmental applications. As they increase the oil-water surface area, they are used in oil degradation process from water or soil (Mullingan, 2005). This property of biosurfactants enables oil recovery in petroleum industry. Microbial enhanced oil recovery (MEOR) is an important application, in which microorganisms and/or their metabolic by products such as biosurfactants are utilized for the mobilization of crude oil from the oil reservoirs (Singh, Van Hamme, & Ward, 2007). Use of biosurfactants in MEOR reduce the hazardous chemicals during oil drilling and has several environmental advantages. Due to excellent emulsifying properties of biosurfactants, they are used as detergents in cleaning up hydrocarbon/crude oil storage tanks (Mukherjee, & Das, 2010). Such clean-up process is highly desirable as it is eco- friendly application. Also, biosurfactants are used to clean up soils contaminated with heavy metals (Whang et al., 2008). Biosurfactants can be applied to a small part of contaminated soil, the bond formed

between the positively charged metal and the negatively charged surfactant is so strong and the complex treated to precipitate out biosurfactant, leaving behind the metal. Moreover some biosurfactants are sufficient to increase the bioavailability of poorly soluble organic compounds such as polycyclic aromatic hydrocarbons (Rosenberg, & Ron, 2013).

Additionally in detergent and cleaning industries, biosurfactants are used in detergent formulation due to their high detergency activity and eco-friendly nature. The produced cyclic lipopeptide biosurfactant by *B. subtilis* showed good emulsion formation capability with vegetable oils and demonstrated excellent compatibility and stability with commercial laundry detergents (Mukherjee, 2007).

Some of biosurfactants are capable of forming very stabile emulsions, which are especially beneficial in cosmetic and food industries (Rosenberg, & Ron, 2013). As some biosurfactants concentrate the oil by adhering to the oil, these biosurfactants are valuable materials in low-fat product processes. In bakery and ice cream formulations biosurfactants control rheological properties and solubilizing flavor oils, improve shelf-life (Nitschke, & Costa, 2007). Moreover several applications such as control the agglomeration of fat globules, stabilize aerated system, improve texture and shelf-life, modify rheological properties and antimicrobial property of biosurfactants is an important specialty for convenience food industry (Mukherjee, & Das, 2010). In food industry, biosurfactants are also used in bakery and meat products as they influence the rheological characteristics of flour or fat tissue.

Many biosurfactant features such as emulsification and de-emulsification, foaming, water binding capacity, spreading and wetting properties are important for many food products as well as cosmetic applications (Williams, 2009). Some of biosurfactants are used in skin smoothing, anti- wrinkle and anti- ageing products due to their anti-microbial, dispersant and emulsification properties. Also several body and hair wash products contain biosurfactant which has high wetting and foaming activities. Sophorolipids are commercially used by Kao. Co. Ltd. (Japan) in a cosmetic product which contains 1 mol of sophorolipid and 12 mol of propylene

glycol has excellent skin compatibility (Desai, & Banat, 1997). Additionally their biocompatible and digestible features allow them to use in several cosmetic and pharmaceutical products.

Biosurfactants are useful as antibacterial, antifungal, antiviral, anticancer and adhesive agents, and as major immunomodulatory molecules in medical applications (Rodrigues, Banat, Teixeira, & Oliveira, 2006). They are used in gene transfection, in vaccines, as ligands in immunology and as adjuvant for antigens. Furthermore, biosurfactants have the potential to be used as anti-adhesive biological coatings for biomaterials, thus reducing hospital infections and use of synthetic drugs and chemicals. Recently, very few studies have been directed towards the possible wound healing properties of biosurfactants (Marchant, & Banat, 2012).

In addition biosurfactants are useful in agriculture as an eco-friendly bio-control agent against herb or insects (Rosenberg, & Ron, 2013). Studies have shown that the lipopeptide biosurfactant produced by *B. subtilis* exhibit insecticide activity against fruit fly (Assie et al., 2001). Also biosurfactants could be used biodegradation of chlorinated pesticides (Mukherjee, & Das, 2010).

Several companies in different countries are now manufacturing biosurfactants on various scales (Marchant & Banat, 2012). Highly purified rhamnolipids are produced by AGEA Technologies (USA) in small quantities. Larger production is being carried by Jeneil Biotech (USA) which is a general food additive company. Saraya Co. Ltd. (Japan) manufactures sophorolipids using *Pseudozyma* sp. with palm oil as substrate. Ecover (USA) also markets some products that contain sophorolipid and MG Intobio (Korea) markets soaps containing sophorolipids specifically for acne treatment. Soliance (France) also produce sophorolipids for cosmetic applications in skin care through antimicrobial activity.

1.4 Factors Affecting Biosurfactant Production

The nature, chemical structure, activity and amount of produced biosurfactant depend on the type of microorganism, the carbon and nitrogen source and C:N ratio, chemical and physical parameters such as temperature, aeration, pH and metal ions (Saharan, Sahu, & Sharma, 2011).

Biosurfactants produced by a variety of microorganisms mainly bacteria, fungi and yeasts (Table 1.1). They have diverse chemical composition and their nature, activity and the production yield depend on the type of microorganism. While the biosurfactants produced by bacterial species are well examined, fungi and yeast are less known as producers of biosurfactants. A white rot fungus *Pleurotus ostreatus* was declared as emulsifying agent producer which was correlated to the ligninolytic enzyme production (Nikiforova, Pozdnyakova, & Turkovskaya, 2009). As far as we know, this is one of the reports that indicate biosurfactant production potential of *Pleurotus* sp. which is a ligninolytic enzyme producer.

As the carbon source is necessary for energy and structural molecules, the amount and the type of carbon source are important for all bioprocesses. The type and physicochemical properties of biosurfactant are affected and influenced by the nature of carbon source (Rahman, & Gakpe, 2008). Diesel, crude oil, glucose, sucrose, glycerol are good sources of carbon substrate for biosurfactant production (Saharan et al., 2011). Little biosurfactant production is observed when microorganisms are growing on a readily available carbon source, when all the soluble carbon is consumed and when water-immiscible hydrocarbon is available biosurfactant production increase (Desai, & Banat, 1997). Nitrogen source is another important factor in the biosurfactant production because it is an essential component of the proteins and enzymes. Several sources of nitrogen have been used for the production of biosurfactants, such as urea, peptone, ammonium sulphate, ammonium nitrate, sodium nitrate, yeast extract, meat extract and malt extract (Fakruddin, 2012). In addition C:N ratio is an important factor that influence biosurfactant production. The production of biosurfactants often occurs when the nitrogen source is depleted in the

medium (Saharan et al., 2011). Maximum rhamnolipid production is occurred at C:N ratio of 16:1 to 18:1 and no surfactin production below a C:N ratio 11:1 (Desai, & Banat, 1997).

The producer microorganism, the type of the biosurfactant and the cost are the most important parameters in the choice of C and N sources. The producing from raw materials is an important advantage of biosurfactants. Using agro-industrial wastes such as cassava peel, soybean hull, sugar beet waste, potato peel, corn bagasse, or residue from different applications such as oil wastes, dairy industry wastes, molasses, soap stock is an economic alternative for C and N sources in biosurfactant production media (Saharan et al., 2011).

Environmental factors such as temperature, pH and salinity are quite important in the yield and characteristics of the biosurfactant produced. Temperature effects biochemical reactions in microorganism cells such as biosurfactant production. Most of the biosurfactant productions reported suitable temperature at 25-30°C (Saharan et al., 2011). In reference to Zinjarde and Pant (2002), the best production of biosurfactant occurred at pH 8.0 while in another study the production of biosurfactant reached maximum at pH 5.5 (Bednarski, Adamczak, Tomasik, & Płaszczyk, 2004). The optimum pH value shows changes according to the biosurfactant type, production condition and producer microorganism. Also salt concentration is an important effect on the biosurfactant production as the cellular activities of microorganisms are affected by salt concentration (Fakruddin, 2012). Metal ions play an important role in biosurfactant production since they form cofactors of some enzymes. In addition, aeration and agitation are important factors as simplify the oxygen transfer and facilitate nutrient transport to microorganisms.

1.5 Biosurfactant Production

Biosurfactants are potentially useful in every industry due to high activity and specificity, effective physicochemical properties, biodegradability, reduced toxicity and broad range of structure. The main limiting factor to the commercialization of

biosurfactants is the economics of production. The surfactant market is intensely competitive and only in low-volume high-applications such as cosmetic and pharmaceutical industries could tolerate high costs. Biosurfactants must be produced less expensively or with distinct advantages.

Economic strategies such as choice of inexpensive raw materials as substrate, increase of biosurfactant yield and production rate, optimization of production conditions, reduction of product recovery costs and production of biosurfactants suitable for specified applications must be developed to enable competition with synthetic surfactants. According to numerous studies, the fermentation type, submerged fermentation (SmF) and solid state fermentation (SSF), is an effective parameter on biosurfactant production (Colla et al., 2010; Neto, Meira, Araújo, Mitchell, & Krieger, 2008).

1.5.1 Biosurfactant Production in Submerged Fermentation

SmF is a cultivation method of microorganisms in liquid nutrient broth (Colla et al., 2010). This involves growing carefully selected microorganisms (bacteria, fungi or yeast) in closed vessels containing a rich broth of nutrients medium and a high concentration of oxygen. In SmF, measure of process parameters such as pH, temperature, oxygen concentration is easier. This fermentation type is especially suitable for bacterial productions. On the other hand, the disadvantages of SmF are large reactor need, high cost and contamination risk.

The considerable majority of biosurfactant production studies use SmF (Krieger, Neto, & Mitchell, 2010). The microorganisms generally used for biosurfactant production are aerobic organisms and they need aeration and agitation during fermentation. The essential problem in biosurfactant production by SmF is large quantities of foam formation due to aeration and agitation. The foam reduces the efficiency of gas transfer between the gas and liquid phases in the bioreactor. Also there is a tendency for the microorganism to accumulate within the foam and remove from the culture medium. In addition, the foam formation increases the risk of

contamination of the bioreactor. The addition of antifoaming agents decrease the efficiency of oxygen and carbon dioxide transfer between the gas and liquid phases and effective antifoam agents are expensive. Also they may have toxic effects to microorganism and they cause chemical contamination. Mechanical foam breaking devices are expensive and not effective when large quantities of foam are produced (Krieger et al., 2010).

Generally, SmF is a high- priced fermentation type. Due to complex and high cost nutrients are required, large scale production by SmF is much more expensive. Additionally, costs and huge dimensions of large bioreactors, energy for aeration, agitation and sterilization and product isolation increase operation expenses. Also large amounts of composed waste and waste water are discharged after production process. Disposal of composed waste and purification of waste water after fermentation is a serious problem.

1.5.2 Biosurfactant Production in Solid State Fermentation

SSF is defined as the fermentation type in which microorganisms grow on solid materials without the presence of free liquid (Bhargav, Panda, Ali, & Javedb, 2008). The majority of the water in the system is absorbed within the solid particles (Figure 1.4). Although droplets of water may be present between the particles and there may be thin water film at the particle surface, the inter-particle spaces are filled by the gas phase (Mitchell, Berovič, & Krieger, 2006).

SSF may be appropriate when the product is only produced under SSF conditions is produced in much levels and the yield is much higher than SmF. Also SSF is preferable when using solid waste is necessary in order to avoid the environmental impacts that would caused by its direct disposal. Recently, various biotechnological products are produced by SSF such as enzymes (amylases, proteases, lipases, pectinases, cellulases), pigments, antibiotics, ethanol, oxalic acid, lactic acid and biological control agents (Bhargav et al., 2008; Pandey, 2003). There are also studies

into the use of microorganisms growing in SSF conditions to mediate processes such as decolorization of dyes, bioleaching, biopulping and bioremediation.

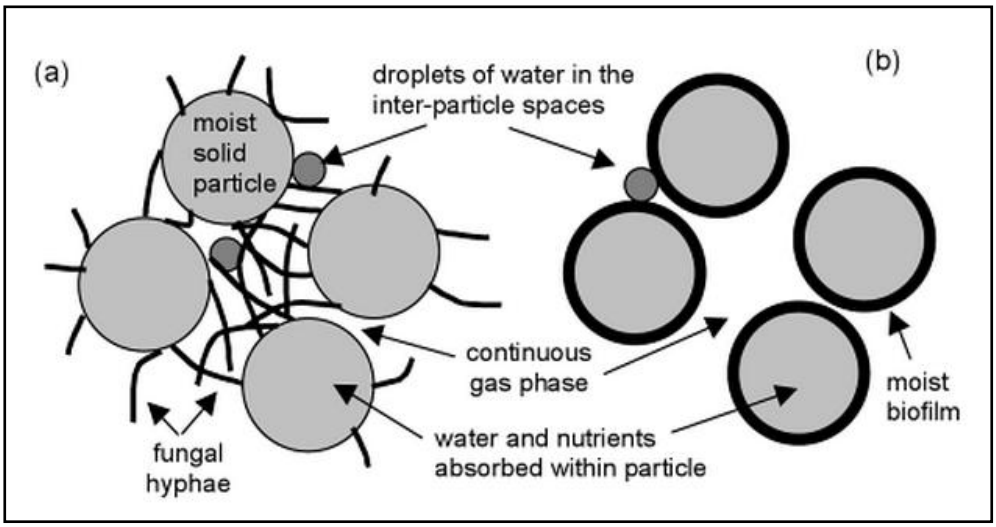

Figure 1.4 The arrangement of moist solid particles and gas phase in SSF (a) involving a filamentous fungus and (b) a unicellular organism

SSF has some advantages such as use of low-cost substrates and simple equipments, low volumes of water, low energy demand and higher concentration of products obtained in comparison to SmF (Akpinar, & Ozturk Urek, 2012; Akpinar, & Ozturk Urek, 2014). The composed waste after fermentation process is lower than SmF systems. Additionally, the structure of the SSF system prohibits the foaming problem that affects SmF for biosurfactant production (Krieger et al., 2010). Although there are problems about homogeneity and scaling up, SSF has gained importance with being an economical and ecological fermentation type.

The most essential challenges in the biosurfactant production in SSF are suitable solid substrate and microorganism selection, bioreactor selection and downstream processing (Martins et al., 2011). The substrate used in SSF processes are generally by-products or wastes of agriculture, forestry or food processing (Mitchell et al., 2006). When using residual substrates in biosurfactant production processes, production costs can be greatly reduced, and the volume of waste is also decrease (Accorsini, Mutton, Lemos, & Benincasa, 2012). Unlike other microorganisms, fungi grow in nature on solid substrates such as pieces of wood, seeds, stems and roots.

SSF is a productive fermentation when fungi are used although some SSF processes include bacteria or yeast (Bhargav et al., 2008). Also desired end product is an important factor in selection of solid substrate and microorganism. Generally, in biosurfactant production studies potatoes and cassava, sugar cane molasses, banana and orange peels, grape pomace have been used in SSF (Table 1.2).

Table 1.2 Production of various biosrufactant in SSF

Microorganism	**Solid Substrate**	**Produced Biosurfactant**	**Reference**
B. subtilis	Okara	Iturin and surfactin	Ohno, Ano, & Shoda, 1996
B. subtilis	Wheat bran	Surfactin	Veenanading, Gowthaman, & Karanth, 2000
Aspergillus fumigatus	Rice husk and rice bran	Biosurfactant	Martins, Kalil, Bertolin, & Costa, 2006
B. subtilis	Potato peels	Lipopeptide	Das, & Mukherjee, 2007
P. aeruginosa	Sugarcane bagasse and sunflower seed meal	Rhamnolipid	Neto et al., 2008
Aspergillus sp.	Soybean meal and rice husk	Biosurfactant	Colla et al., 2010
B. pumilus	Okara and sugarcane bagasse	Surfactin	Slivinski, Mallmann, Araújo, Mitchell, & Krieger, 2012
B. subtilis	Millet	Biosurfactant	Ghribi et al., 2012

The major considerations in choosing a bioreactor for biosurfactant production in SSF are the capital and operation costs and the effectiveness of heat removal and

moisture. Besides that, the process variables such as pretreatment, particle size of substrate, medium ingredients, moisture, temperature, pH, agitation and aeration, have a considerable affect on process efficiency.

For downstream processing in which it is desired to extract the biosurfactant from solids, it will be necessary to determine the most efficient extraction method. SSF is an interesting technique for the production of biosurfactant and might make commercial production processes economically viable.

1.6 Recovery, Purification and Chemical Characterization of Biosurfactans

Downstream processing cost generally account for approximately 60% of the total production costs (Satpute et al., 2010). Therefore, it is important to designate the most efficient and economic procedure when choosing recovery and/or purification methods. To choose most suitable methods, biosurfactant properties and yield of procedure are important.

Generally, to precipitate of high molecular weight biosurfactants acetone, ethanol or ammonium sulphate is used as precipitant (Satpute et al., 2010). After precipitation, dialysis is used to remove any small molecules. Depending upon the type and amount of biosurfactant, concentration and volume of precipitant chemical can be changed. For low molecular weight biosurfactant precipitation, acidification is mostly used method. In addition, adsorption-desorption ability of biosurfactants is used for their purification process. Also ion exchange chromatography, isoelectric focusing, crystallization, foam fractionation, solvent extraction and ultra-filtration methods are used for various biosurfactants purification.

The most commonly used techniques to chemical analysis of biosurfactant are colorimetric assays. The protein content of biosurfactant is often analyzed with Folin phenol method (Lowry, Rosebrough, Farr, & Randal, 1951) or according to Bradford (1976) method. For further analysis, amino acid sequence is determined by Edman degradation (Zachara, & Gooley, 2000). Also sodium dodecyl sulphate-

polyacrylamide gel electrophoresis is used for protein separation and determination of molecular mass of biosurfactants. Additionally carbohydrate and fatty acid moieties of biosurfactant can be observed by gas chromatography mass spectroscopy (GC-MS). There are simpler methods for estimation of carbohydrate and lipid concentration. For example phenol- sulfuric acid reaction (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956) is very sensitive to determine total carbohydrate content. Reducing sugars are determined by dinitrosalicylic acid (DNS) (Miller, 1959). Also there are some colorimetric methods for detection and quantification of only gylcolipid type biosurfactants such as anthrone and orcinol assays (Bailey, 1958; Heyd et al., 2008). In addition gravimetric methods are used to determine lipid content. For further characterization, various chromatographic and spectroscopic analyses are used. Thin layer chromatography is generally used for determination of different functional groups from biosurfactant. High pressure liquid chromatography (HPLC) is commonly used for separation of lipopeptide type biosurfactants or free rhamnose from rhamnolipid (Satpute et al., 2010). GC-MS is used for chemical analysis especially for glycolipid type biosurfactants. Another spectroscopic method is infra red (IR) spectroscopy which determines the functional groups of gases, liquids and solid samples. Additionally nuclear magnetic resonance (NMR), is based on transitions in atoms with a magnetic moment when an external magnetic field is applied, is also suitable for structure determination of all kinds of biosurfactants.

1.7 *Pleurotus* spp.

Pleurotus spp. belong to the genus *Pleurotus* (Quel.) Fr., tribe Lentineae Fayod, family *Polyporaceae* (Fr.) Fr., and they are widely distributed throughout the Northern Hemisphere, such as Europe, North Africa, Asia and North America (Singer, 1986). This fungus has spores as 6.5- 9 × 2.8- 3.5 μm size, cylindrical or cylindrical-ellipsoidal, smooth and hyaline, with vacuoles. The color of spores is dingy grey or pale lilac grey. The geographic distribution of the fungi varies according to its species. For example, *P. pulmonarius* and *P. cystidiosus* are distributed in the tropical and subtropical region, while *P. eryngii* are found in southern Europe, North Africa and central Asia.

Pleurotus spp. are saprophytic, white rot fungi that are cultivated on substrates containing lignin or cellulose such as agro-industrial wastes. Usage of these materials is dependent on secreted enzymes by *Pleurotus* spp. including peroxidases, laccases, cellulases, hemicellulases and xylanases (Cohen, Persky, & Hadar, 2002). The enzymes responsible for lignin degradation are mainly laccase (Lac), lignin peroxidase (LiP) and manganese peroxidase (MnP). Other biological functions are that Lac and LiP defend the fungi against pathogens. Also Lacs have roles such as spore resistance, rhizomorph and fruit bodies formation and pigment synthesis.

Lacs belong to multicopper oxidase family and catalyze the oxidation of various substrates with the simultaneous reduction of molecular oxygen to water (Maciel, Costa e Silva, & Ribeiro, 2010). Most Lacs are reported from fungal organisms and most biotechnologically useful Lacs are also of fungi origin. Fungal Lacs are mostly inducible, extracellular, monomeric glycoproteins with carbohydrate contents of 10-20% which may contribute to the high stability of Lacs (Mayer, & Staples, 2002). Lacs are multinuclear enzymes and the active site of Lac comprises four copper atoms in three groups. The T1 copper is responsible for the blue color of the enzyme and has a characteristic absorbance around 610 nm. The T2 copper is colorless and cannot be detected spectrophotometrically. The T3 copper displays a spectral absorbance shoulder in the region of 330 nm and also displays a characteristic fluorescence spectrum. Lacs from different sources exhibit a wide range of redox potentials. Lacs exhibit low substrate specificity and can oxidize a range of compounds, such asdiphenols, aryl diamines, and aminophenols (Wong, 2009).

LiPs belong to the family of oxidoreductases (Hammel, & Cullen, 2008). This enzyme has been recorded for several species of white-rot basidiomycetes and in actinomycetes. LiP is an extracellular hemeprotein, dependent of H_2O_2, with an unusually high redox potential and low optimum pH. It is capable of oxidizing a variety of reducing substrates including polymeric substrates. LiP shows little substrate specificity, reacting with a wide variety of lignin model compounds and even unrelated molecules. It has the distinction of being able to oxidise methoxylated

aromatic rings without a free phenolic group, generating cation radicals that can react further by a variety of pathways, including ring opening, demethylation, and phenol dimerisation. LiP in contrast with Lac does not require mediators to degrade high redox potential compounds but it needs H_2O_2 to initiate the catalysis. Due to their enlarged substrate range and high redox potentials LiPs have great potential for application in various industrial processes.

MnP belong to the family of oxidoreductases (Wong, 2009). Subsequent investigations have shown that MnP is distributed in almost all white-rot fungi and it seems to be more widespread among white rot fungi than LiP. MnP oxides Mn^{2+} to Mn^{3+}, and phenolic compounds to phenoxyl radicals. The product Mn^{3+} is highly reactive and complex with chelating organic acid, as oxalate or malate, which are produced by the fungus. The redox potential of the MnP system is lower than LiP system and it has shown capacity for preferable oxidize *in vitro* phenolic substrates. MnP may oxidize Mn(II) without H_2O_2 with decomposition of acids, and concomitant production of peroxyl radicals that may affect lignin structure.

Ligninolytic enzymes are highly versatile in nature and can be used in a range of industrial processes. These enzymes have established their applications in bio-remediation, pollution control and in the treatment of industrial effluents containing recalcitrant and hazardous chemicals such as textile dyes, phenols and other xenobiotics (Nigam, 2013). The paper and pulp industry requires a step of separation and degradation of lignin from plant material, where the ligninolytic enzymes are used. The ligninolytic enzyme system is used in bio-bleaching of craft pulp and in other industries such as for the stabilization of wine and fruit juices, denim washing, cosmetic industry and biosensors.

The aim of this study is economical and high productive biosurfactant production by white rot fungus *Pleurotus* sp. on sunflower seed shell, potato peel or grape waste in SSF. The first step of this study was determination of the best producer strain and the most suitable solid substrate. In the second step, effects of size and amount of solid substrate, volume of medium, temperature, pH and Fe^{2+} ion concentrations

were investigated to optimize biosurfactant production conditions. The biosurfactant production was screened by oil spreading technique, emulsification index and surface tension assay. In the last step of the study, produced biosurfactant in optimum production conditions was further characterized by chromatographic (HPLC) and spectroscopic (FT-IR and NMR) analyses. Also physicochemical properties, such as CMC value, thermal stability and resistance to changes of environmental factors, of produced biosurfactant were investigated. Additionally, suitability of produced biosurfactant for oil recovery was investigated.

CHAPTER TWO
MATERIALS AND METHODS

2.1 Materials

2.1.1 Microorganism

Four different *Pleurotus* strains were used in the study: *P. djamor* (Rumph. Ex Fr.) Boedijn (MCC15), *P. ostreatus* (Jacq.) Pleurotus Kumm. (MCC16) and *P. sajor caju* (Fr.) Singer (MCC29) and *P. eryngii* (DC.) Gillet (MCC58).

2.2 Methods

2.2.1 Maintenance of Microorganisms

P. djamor, P. ostreatus and *P. sajor caju* were grown on potato dextrose agar (39 g/L, pH 5.6) at 25^{o}C for 7 days, *P. eryngii* was grown on malt extract: peptone: agar (30: 3: 15 g/L, pH 5.6) at 25^{o}C for 14 days.

To prepare the media, the contents of medium were mixed and sterilized at 121°C in autoclave for 20 min. After the medium was cooled, it was transferred to Petri dishes. After solidification of the medium, inoculation was performed.

2.2.2 Solid State Fermentation Media and Production

For biosurfactant production in SSF, three different solid substrate and two different extra carbon source were used (Table 2.1). To prepare production medium, 5 g solid substrate was weighed in 250 mL Erlenmeyer flasks. Basal medium (Table 2.1) was the same for all the solid substrates and pH was adjusted to 6.0 (Bazalel, Hadar, & Cerniglia, 1997). Three different liquid media were used. The first one included basal medium and D-glucose (10 g/L), the second one included basal

medium and sunflower seed oil (10 mL/L) and the last one included only basal medium. Potato peels and a part of grape wastes were dried at 60°C for 24 h.

Table 2.1 Basal medium components, solid substrates and extra carbon sources used in SSF

Basal Medium (g/L)	**Solid Substrate (5 g)**	**Extra Carbon Source**
NH_4NO_3, 0.724	Sunflower seed shell	D-glucose (10 g/L)
KH_2PO_4, 1.0	Potato peel	Sunflower seed oil (10 mL/L)
$MgSO_4.7H_2O$, 1.0	Grape waste (wet or dried)	
KCl, 0.5		
yeast extract, 0.5		
$FeSO_4.7H_2O$, 0.001		
$ZnSO_4.7H_2O$, 0.0028		
$CaCl_2.2H_2O$, 0.033		
peptone, 10.0		

Solid substrates and liquid medium were sterilized at 121°C in autoclave for 20 min. After sterilization, 10 mL liquid medium was added into solid substrate (humidity 70%) and 7 agar plugs (1 cm^2) were inoculated. Flasks were incubated at 29°C and samples from flasks were harvested on 5, 9, 13, 16 and 20 days of incubation period.

2.2.3 Chemical Analyses of Solid Substrates

First of all, solid substrates were dried at 65°C for 24 h and they were crushed in the powder. The powder of solids was homogenized with potassium phosphate buffer (10 mM, pH 6). After centrifugation at 12000 rpm for 15 min, the supernatant was used for analyses of total carbohydrate and nitrogen content as explained below. Lignin concentrations of solid substrates used were determined by thioglycolic acid (TGA) lignin analysis method (Campbel, & Ellis, 1992). After incubation of 1 mg dried sample, 1.5 mL HCl (2 N) and 0.3 mL TGA at 95°C for 4 h, the sample was cooled to 0°C, centrifuged at 10000 rpm for 12 min, and the supernatant is discarded. After washing the pellet thoroughly by re-suspending in water three times, the

lignothioglycolate was extracted by 150 rpm shaking for 18 h in 1 mL 0.5 M NaOH. After centrifugation, the supernatant was reserved, and combined with the supernatant from washing the pellet with 0.5 mL 0.5 M NaOH. The combined alkali extracts were acidified with 0.3 mL concentrated HCl, and the precipitate formed after 4 h at 4°C was recovered by centrifugation. After drying, the pellet was res-uspended in 1 mL 0.5 M NaOH, and diluted with 0.5 M NaOH to yield an appropriate absorbance at 280 nm. Commercial lignin was used as standard in the range of 0.5-2.5 mg and the standard function was $y = 0.3604x$, $R^2 = 0.9987$.

Analysis of cellulose content was performed by Kürschner-Hanack method (Kulić, & Radojičić, 2011). The sample was degraded with a mixture of nitric acid and acetic acid and boiled. The solution was then filtered through a Büchner funnel. Then the filter paper containing an insoluble residue was dried in oven and measured gravimetrically.

2.2.4 Determination of Biosurfactant Activity

After incubation period, for extraction each flask was received 25 mL of distilled water and was agitated for 1 h at 150 rpm, 29^{o}C. The suspension was centrifuged at 5000 rpm and 4^{o}C for 15 min and supernatant was used for determining analysis below.

2.2.4.1 Emulsification Index

Emulsification index was measured by adding 3.5 mL of extract and 2 mL of sunflower seed oil (Pinto, Martins, & Costa, 2009). The mixture was agitated in a vortex agitator at high speed for 1 min. Non-fermented culture medium was used as blank. The emulsion index was calculated after 24 (E_{24}) and 48 (E_{48}) hours by the height of the emulsion layer divided by the total height (Figure 2.1), and multiplied by 100 (Equation 2.1) and emulsifying activity determined according to Equation 2.2.

$$E(sample) = \frac{H(emulsion\ layer)}{H(total)} * 100 \quad (2.1)$$

$$EI = \big(E(sample) - E(blank)\big) * D \quad (2.2)$$

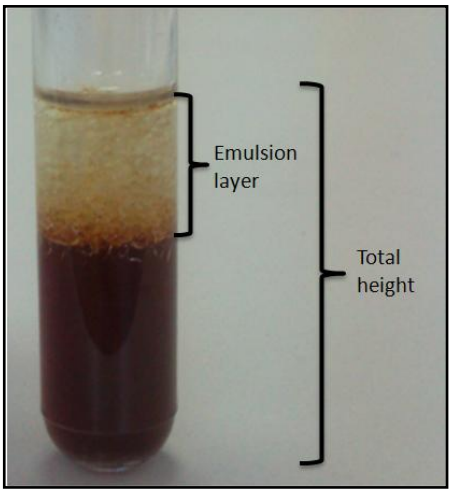

Figure 2.1 Emulsion layer and total height on a biosurfactant oil emulsification

2.2.4.2 Oil Spreading Test

200 µL sunflower seed oil was added onto the surface of distilled water filled in a 50 mL petri dish to form a layer on the surface. 20 µL cell free supernatant was gradually added to the centre of oil layer. The diameter of the clear zone on the oil surface was measured related to the concentration of biosurfactant. Distilled water was used as negative control and standard surfactants such as Tween-80 and Triton X-100 were used as positive controls (Youssef et al., 2004).

2.2.4.3 Measurement of Surface Tension

The cell free supernatant was used for the determination of the surface tension with Sigma 701 digital surface tensiometer (KSV Instruments LTD– Finland) at room temperature. This tensiometer works on the principle of the Du Nuoy ring method.

2.2.5 Optimization of Biosurfactant Production Conditions

After determination of the best strain, solid substrate and extra carbon source for biosurfactant production according to biosurfactant activity results, these parameters were used in optimization steps.

First of all the amounts of solid substrate (with 1 cm^2 surface area) were changed as 1, 3, 5, 7 and 10 g. Also solid substrate which had 0.25 cm^2 surface area was used in amounts of 1, 3, 5, 7 and 10 g. After determination of optimum surface area and amount of solid substrate, medium volume changed as 10, 25 and 50 mL. Lastly three different fermentation temperatures (25, 29 and 35°C), four different pH levels (5.5, 6, 7 and 8), different concentrations of Fe^{2+} ion (0, 3.5, 18 and 35 μM) were investigated.

Optimum condition parameters were determined according to biosurfactant activity results as explained above.

2.2.6 Determination of Chemical Composition of Production Medium

After production period, cell free supernatant was prepared as above and chemical composition analyses were performed.

2.2.6.1 Determination of Protein Content

Protein content of cell free supernatant of production media was determined by Bradford (1976) method using bovine serum albumin as standard in the range of 0-250 ppm. To prepare Bradford reagent, 100 mg of Coomassie Brillant Blue G-250 was dissolved in 50 mL 95% ethanol. The solution was added to 100 mL 85% H_3PO_4, and diluted to 1000 mL total volume with water. 100 μL sample (100 μL distilled water for reference) was added to 900 μL reagents and mixed. This solution was waited for 2 min at room temperature and the absorbance was measured at 595 nm against a reference sample. The standard function was $y = 0.0064x$, $R^2 = 0.9913$.

2.2.6.2 Determination of Reducing Sugar Content

For determination of reducing sugar content, DNS method was used (Miller, 1959). To prepare DNS reagent, solution B (30 g of sodium-potassium tartarate/ 50 mL distilled water) and solution A (1 g DNS/ 20 mL, 2N NaOH) were mixed with 100 mL total volume. To determine reducing sugar content, after mixing 500 µL supernatant (500 µL distilled water for reference) and 500 µL DNS reagent the mixture were boiled for 10 min and cooled to room temperature. After cooling, 5 mL distilled water was added and mixed. The absorbance was measured at 546 nm against a reference sample. D-glucose was used as standard in the range of 0-165.1 µg/mL, the standard function was $y = 0.007x$, $R^2 = 0.9988$.

2.2.6.3 Determination of Nitrogen Content

Nitrogen content of cell free supernatant was determined by phenol-hypochlorite method (Weatherburn, 1967). To prepare phenol reagent, solution A (5 g phenol/ 50 mL distilled water) and solution B (25 mg sodium-nitroprusside/ 50 mL distilled water) were mixed in equal volumes. Alkaline hypochlorite solution was prepared by mixing equal volume of solution C (5 g sodium hydroxide/ 100 mL distilled water) and solution D (26 g/L NaOCl). 2 mL supernatant (2 mL distilled water for reference), 500 µL phenol solution and 500 µL alkaline hypochlorite solutions were mixed and incubated for 5 min at 60°C. The absorbance was measured at 630 nm against a reference sample. Standard was prepared in the range of 0-500 µg/mL of ammonium sulfate and the standard function was $y = 0.1299x$, $R^2 = 0.9917$.

2.2.6.4 Determination of Total Carbohydrate Content

Total carbohydrate content was determined according to phenol-sulfuric acid method (Dubois et al., 1956). To determine total carbohydrate content, 1 mL cell free supernatant (1 mL distilled water for reference) was mixed with 1 mL 5% phenol solution and 5 mL concentrated H_2SO_4. After incubation for 20 min at room temperature the absorbance was measured at 470 nm against a reference sample.

Glucose was used as standard in the range of 0- 250 µg/mL and the standard function was y = 0.0042x, R^2 = 0.09982.

2.2.6.5 Determination of Uronic Acid Content

Uronic acid content of cell free supernatant was determined with borate-sulfuric acid- carbazole assay (Healy, Devine, & Murphy, 1996). 0.5 mL cell free supernatant (0.5 mL distilled water for reference) and 3 mL cold borate-sulphuric acid reagent (3.82 g sodium borate was dissolved in 10 mL hot water and 390 mL well-cooled concentrated sulphuric acid) were mixed and heated in boiling water bath for 20 min. after cooling to 0°C, 0.1 mL 0.2% carbazole solution were added. The solution was shaken well and heated again in the boiling water bath for 10 min. After cooling at room temperature for 15 min the absorbance was measured at 530 nm against a reference sample. Glucuronolactone was used as standard in the range of 0- 1 µmol/ mL, the standard function was y = 0.0012x, R^2 = 0.9956.

2.2.7 Determination of Enzyme Activities

2.2.7.1 Ligninolytic Enzyme Activities

Lac activity was determined by oxidation of 2,2- Azino-bis-3-ethyl-benzthiozoline-6-sulphonic acid (ABTS). Increase in absorbance for 2 min was measured by spectrophotometer at 420 nm and 25°C (ε = 36000 cm^{-1} M^{-1}). The reaction mixture contained 700 µL 100 mM sodium acetate buffer (pH 4.5), 100 µL 5 mM ABTS and 200 µL sample (Niku-Paavola, & Raaska, 1990).

MnP activity was determined by monitoring the oxidation of 2,6-dimethoxyphenol for 2 min at 469 nm and 25°C (ε = 27500 cm^{-1} M^{-1}). The reaction mixture contained 600 µL 250 mM Na-citrate buffer (pH 4.5), 50 µL 20 mM 2,6-dimethoxyphenol, 50 µL 30 mM $MnSO_4.H_2O$, 100 µL 4 mM H_2O_2 and 200 µL of sample (Kuwahara, Glenn, Morgan, & Gold, 1984).

LiP activity was determined by oxidation of veratryl alcohol for 2 min at 310 nm and 30°C (ε = 9300 cm^{-1} M^{-1}). The reaction mixture contained 850 µL 125 mM Na-tartarate buffer (pH 2.5), 100 µL 2 mM veratryl alcohol, 50 µL 4 mM hydrogen peroxide solution and 200 µL sample (Tien, & Kirk, 1988).

For all assays one unit of enzyme activity was defined as the amount of enzymes that oxidizes 1 µmol of substrate.

2.2.7.2 Lipase Enzyme Activity

Lipase activity was determined by using p-nitrophenyl palmitate (pNPP) as the substrate at 410 nm (ε = 1400 cm^{-1} M^{-1}) (Silva, Mitidieri, Schrank, & Vainstein, 2003). 0.1 mL sample was mixed with 0.9 mL substrate solution containing 3 mg pNPP dissolved in 1 mL propanol-2-ol diluted in 9 mL 50 mM Tris–HCl (pH 8.0) containing 40 mg of Triton X-100 and 10 mg of gum arabic. After 30 min of incubation at 37°C the absorbance was measured at 410 nm against an enzyme-free control.

One unit (U) was defined as the amount of enzyme that liberated 1 µmol pNPP per min.

2.2.8 Isolation and Purification of Produced Biosurfactant

After biosurfactant production at optimum condition, cells were removed from cultivation medium by centrifugation. The concentrated HCl was added with 20 mL supernatant to bring final pH of 2.0 and kept for overnight at 4°C (Yin et al., 2009). Resulted precipitation was collected by centrifugation at 5000 rpm and 4°C for 30 min. The supernatant was removed and 10 mL of chloroform: methanol (2:1 v/v) was added to precipitated pellet and incubated in a rotary shaker at 30°C and 200 rpm for 20 min. The content was centrifuged at 5000 rpm, 4°C for 30 min and the supernatant was evaporated by air drying (Chander, Lohitnath, Mukesh Kumar, & Kalaichelvan, 2012).

2.2.9 Partial Characterization of Produced Biosurfactant

2.2.9.1 Determination of Critical Micelle Concentration

The CMC was determined by measuring the surface tension of produced biosurfactant at different concentrations. The value of CMC was obtained from the plot of surface tension versus the biosurfactant concentration and determined as mg/mL of biosurfactant.

2.2.9.2 Chemical Composition of Produced Biosurfactant

Chemical structure of produced biosurfactant at optimum condition was determined by protein, total carbohydrate, reducing sugar, nitrogen and uronic acid assays as explained above. Additionally, lipid content of biosurfactant was determined by Mishra et al. (2014) method. To prepare reagent 0.6 g vanillin was dissolved in 10 mL ethanol and mixed 90 mL distilled water and 400 mL concentrated phosphoric acid. 2 mL concentrated sulfuric acid was added to 100 µL sample (100 µL distilled water for reference) and was heated for 10 min at 100°C, and was cooled for 5 min in ice bath. 5 mL of freshly prepared phospho-vanillin reagent was then added and the sample was incubated for 15 min at 37°C incubator shaker at 150 rpm. The absorbance was measured at 530 nm against a reference sample. Sunflower seed oil was used as standard in the range of 0- 100 µg/mL and the function was $y = 0.0044x$, $R^2 = 0.9927$.

Also lipase activity of produced biosurfactant was investigated as explained above.

2.2.9.3 Chromatographic and Spectroscopic Analyses of Produced Biosurfactant

To determine type of the produced biosurfactant at optimum condition and standard biosurfactants (surfactin and rhamnolipid) were analyzed by high

performance liquid chromatography (HPLC) (Agilent Technologies 1100) with ACE-221-2546 C18 column (250 x 4.6 mm).

The surfactin standard was solved in acetonitrile/ methanol (1:1, v/v). The mobile phase was 3.8 mM triflouroacetic acid/ acetonitrile (1:4, v/v) at 1 mL/ min. The absorbace of the eluent was monitored at 205 nm for 45 min (Wei, & Chu, 2002). The produced biosurfactant sample was prepared as surfactin standard.

The rhamnolipid standard was solved in mobile phase, acetonitrile/ water (3:2, v/v). The flow rate was kept at 1 mL/ min and the eluent was monitored at 280 nm for 80 min (Abouseoud, Maachi, Amrane, Boudergua, & Nabi, 2008). The produced biosurfactant sample was prepared as rhamnolipid standard.

For spectroscopic analyses, Fourier transform infrared spectroscopy (FT-IR) and nuclear magnetic resonance (NMR) were used. The FT-IR spectra were recorded on the Perkin Elmer Spectrum BX, in the 4000- 400 cm^{-1} spectral region. All samples were dried at 70°C overnight before analysis. KBr pellet was used as a back ground reference. Approximately 1 mg of the sample was milled with approximately 100 mg of dried KBr and then pressed to form a pellet for measurement. Rhamnolipid and surfactin were used as standard.

For NMR spectroscopy, the sample was recorded as solution in dimethyl sulfoxide. ^{1}H-NMR spectrum was recorded on a MERCURY Plus-AS 400 spectrometer at 400 MHz and 30°C. The chemical shifts were expressed in ppm.

2.2.9.4 Thermal Gravimetric and Differential Scanning Calorimetric Analyses (TGA and DSC)

TGA and DSC analyses of produced biosurfactant were carried out with Perkin Elmer- Diamond TG/DTA. About 3-5 mg of dry produced biosurfactant sample was loaded on a platinum pan and its energy level was scanned in the ranges of 30-500°C under a nitrogen atmosphere with a temperature gradient of 10°C/min. The

analyses were performed under gradual increase in temperature by plotting the weight loss percentage and differential weight percentage against temperature.

2.2.10 Effects of Environmental Factors on Biosurfactant Activity

The effects of temperature, NaCl concentration and pH on the activity of produced biosurfactant in optimal SSF condition were investigated in the cell free broth.

To study the effect of temperature on biosurfactant activity, the cell free broth was maintained at a constant temperature (4, 25, 40, 70 and 100°C) for 60 min and used for surface tension and emulsification index measurements as described above. Specific concentrations of NaCl (2, 5, 10 and 30%, w/v) were added to the cell free broth and surface tension and emulsification index was determined to investigate salt concentration effect. The effect of pH on surface tension and emulsification index was evaluated after adjustment of the cell free broth pH to 2, 5, 7, 9 and 12 with concentrated NaOH or HCl.

Also emulsification indexes with different oils (Sunflower seed oil, waste frying oil, lubricating oil) were investigated in the cell free broth.

2.2.11 Removal of Waste Frying Oil from Contaminated Sandy Soil Application

Suitability of produced biosurfactant for oil recovery was carried using 100 g of beach sand impregnated with 5 mL of waste frying oil (Jain, Mody, Mishra, & Jha, 2012). 25 g of the contaminated sand were transferred to 250 mL Erlenmeyer flasks and 25 mL distilled water was added to one of them as control, 25 mL (1000 ppm) biosurfactant solution was added to other one. The samples were incubated on a rotary shaker at 150 rpm, 25°C for 24 h. After incubation, the mixture was centrifuged at 5000 rpm for 20 min for separation of aqueous phase from sand containing oil which was further extracted with hexane in soxhlet. The hexane was

recovered using a rotary evaporator and the residual lubricant oil was measured gravimetrically (Latha, & Kalaivani, 2012).

2.2.12 Statistical Analysis

The SPSS 13 statistical program was used for statistical significance analyses. The values were the mean of three separate experiments. Comparisons were also made with Pearson's correlation.

CHAPTER THREE
RESULTS AND DISCUSSION

3.1 Determination of the Best Biosurfactant Producer Strain and the Most Suitable Production Medium

In biosurfactant production by four different *Pleurotus* spp. on SSF sunflower seed shell, potato peel or grape waste were used as solid substrate. Additionally three different liquid media were used, which included extra carbon source [glucose (10%, w/v) or sunflower seed oil (10%, v/v)] or no extra carbon source. Emulsification indexes (E_{24} and E_{48}), oil spreading activities and surface tension values (Table 3.1) were considered to determination of maximum biosurfactant production.

In production media that included sunflower seed shell as solid substrate, all *Pleurotus* spp. had the maximum biosurfactant activities with sunflower seed oil as second carbon source. *P. djamor* and *P. eryngii* reached the maximum activity on 13^{th} day while *P. ostreatus* reached on 5^{th} day and *P. sajor-caju* reached on 16^{th} day of incubation period.

When grape waste was used as solid substrate, *P. djamor* and *P. sajor-caju* had the maximum biosurfactant activity on 9^{th} day while *P. eryngii* and *P. ostreatus* reached the maximum activity on 16^{th} day of incubation period. *P. ostreatus* was the only strain that had maximum biosurfactant activity on dry grape waste, other three strains indicated maximum biosurfactant activities on wet grape waste. While *P. djamor* and *P. ostreatus* demonstrated maximum biosurfactant activities with sunflower seed oil as second carbon source, *P. eryngii* and *P. sajor-caju* reached maximum activities with medium included no extra carbon source.

P. djamor, P. eryngii, *P. ostreatus* and *P. sajor-caju* had maximum biosurfactant activities on potato peels as solid substrate and sunflower seed oil as carbon source on 9, 5, 9 and 20^{th} day of incubation period, respectively.

Table 3.1 Maximum activity values of biosurfactant that produced by four different *Pleurotus* strains on three different solid substrates

		Sunflower seed shell	**Grape waste**	**Potato peel**
P. djamor	E_{24} (%)	45.71 ± 4.0	40 ± 4.0	42.86 ± 4.0
	E_{48} (%)	41.17 ± 4.0	38.24	41.23 ± 4.0
	Oil spreading (cm)	3.5 ± 0.2	0.1 ± 0.01	5 ± 0.4
	Surface tension (mN/m)	29.79 ± 0.3	43.63 ± 0.4 (wet waste)	30.9 ± 0.3
P. eryngii	E_{24} (%)	52.94 ± 5.0	41.18 ± 4.0	33.33 ± 3.2
	E_{48} (%)	52.94 ± 5.0	38.24 ± 3.2	28.21 ± 3.0
	Oil spreading (cm)	3.1 ± 0.2	0.6 ± 0.05	4 ± 0.3
	Surface tension (mN/m)	40.36 ± 0.4	59.3 ± 0.5 (wet waste)	39.65 ± 0.4
P. ostreatus	E_{24} (%)	42.4 ± 4.0	45.45 ± 4.0	28.57 ± 3.0
	E_{48} (%)	39 ± 3.5	31.25 ± 3.2	28 ± 3.0
	Oil spreading (cm)	4 ± 0.3	0.8 ± 0.05	3 ± 0.1
	Surface tension (mN/m)	30.6 ± 0.3	46.46 ± 0.4 (dry waste)	34.05 ± 0.3
P. sajor-caju	E_{24} (%)	54.55 ± 5.0	36.36 ± 3.2	38.24 ± 3.4
	E_{48} (%)	52.94 ± 5.0	35.29 ± 3.2	34.29 ± 3.2
	Oil spreading (cm)	1.9 ± 0.09	0.5 ± 0.05	1.2 ± 0.09
	Surface tension (mN/m)	41.93 ± 0.4	49.05 ± 0.5 (wet waste)	47.26 ± 0.2

Biosurfactant activity changes according to microorganism as well as type of substrate. As it is seen in Table 3.1, *P. eryngii* had maximum biosurfactant activity on potato peel while other three strains had maximum activity on sunflower seed shell. Additionally, all four strains reached maximum biosurfactant activity with sunflower seed oil as extra carbon source. Little biosurfactant production was observed when microorganisms grow on readily available carbon sources such as glucose, glycerol, mannitol etc. when all the soluble carbon was consumed and

water-immiscible haydrocarbon was viable, biosurfactant production was triggered (Desai, & Banat, 1997). Due to high carbohydrate content, potato peels are suitable sources for biosurfactant production (Saharan et al., 2011). In addition, it is known that oily materials such as sunflower seed shell much stimulate biosurfactant production (Makkar, Cameotra, & Banat, 2011). Although in several studies there were high biosurfactant activities that produced on grape waste which has suitable C:N ratio for biosurfactant production, in this study the lowest biosurfactant activities were determined in production on grape waste. Such waste materials are mostly burned in order to dispose and release CO_2 that advances to greenhouse effect. This study demonstrated the potential of bioconversion of these waste materials to value-added biotechnological products. Total carbohydrate, nitrogen, lignin and cellulose percentages of solid substrates used in the present study were shown in Table 3.2.

Table 3.2 Chemical composition of solid substrates (dry weight %)

Solid Substrate	**Total Carbohydrate**	**Nitrogen**	**Lignin**	**Cellulose**
Sunflower seed shell	3.72 ± 0.2	1.26 ± 0.1	18.65 ± 1.1	45 ± 3.8
Potato peels	45.97 ± 5.2	2.33 ± 0.2	0.47 ± 0.02	28.8 ± 1.9
Grape waste	52.2 ± 4.7	2.11 ± 0.2	20.4 ± 1.5	17.2 ± 1.1

In some studies glucose was used as carbon source which was an expensive substrate especially in large scale production (Prieto, Michelon, Burkert, Kalil, & Burkert, 2008; Rau, Hammen, Heckmann, Wray, & Lang, 2001; Reis, Servulo, & De Franca, 2004). In this present study sunflower seed oil was also used as second carbon source. According to Makkar et al., (2011) producing biosurfactant from oily substrates is an advisable strategy of waste management and low cost production. It is important to note that the selection of suitable waste with the right balance of nutrients that permits cell growth and product accumulation.

According to these results, the highest biosurfactant activity was determined in *P. djamor* on SSF with sunflower seed shell and sunflower seed oil. Figure 3.1 shows oil spreading activity, surface tension and E_{24} and E_{48} values of biosurfactant produced by *P. djamor* during incubation period. The highest emulsification indexes and oil spreading activity and the lowest surface tension value was detected on 13th day of incubation.

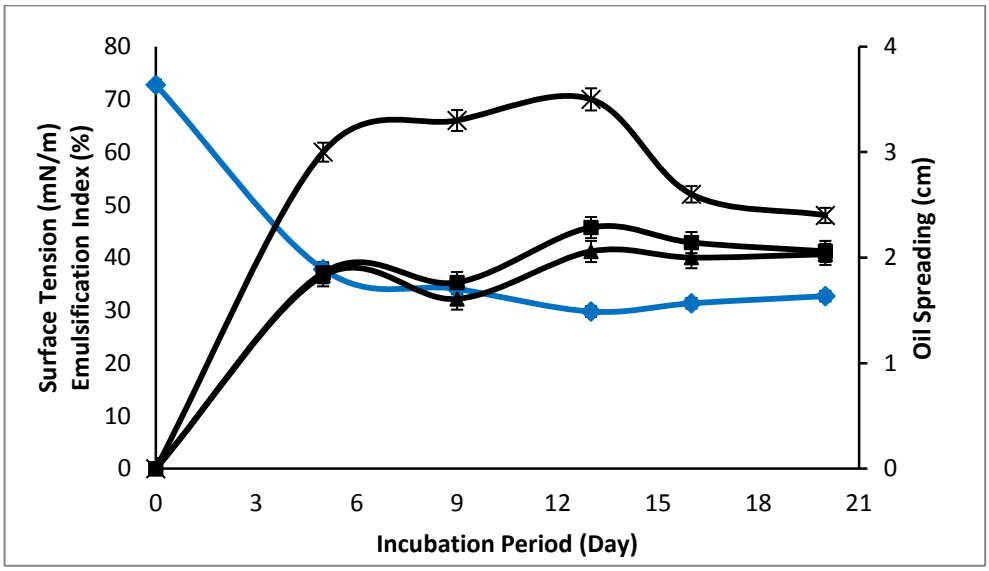

Figure 3.1 Surface tension (♦), emulsification indexes [E_{24} (■) and E_{48} (▲)] and oil spreading activity (×) of biosurfactant during the incubation period that produced by *P. djamor*

Chemical composition analyses of medium were carried out during incubation period (Figure 3.2). Maximum protein concentration was detected as 6260 ± 60 ppm on 9th day of incubation while protein concentration was 5320 ± 85 ppm on 13th day. Similarly, the maximum nitrogen concentration was recorded on 9th day of incubation as 21601.23 ± 260 ppm. The nitrogen concentration was determined as 18691.3 ± 225 ppm on 13th day of incubation on which the maximum biosurfactant activity was detected. Although concentrations of total carbohydrate and reducing sugar on 13th day of incubation were determined as 7151 ± 187 ppm and 183.2 ± 8.9 mM, respectively, their maximum concentrations were detected on 9th day of incubation as 10303 ± 100 ppm and 308.9 ± 15 mM, respectively. Unlike total carbohydrate concentration, maximum uronic acid concentration was detected on 5th

day of incubation as 54.58 ± 2.5 M. On 13th day its concentration was determined as 31.83 ± 1.1 M.

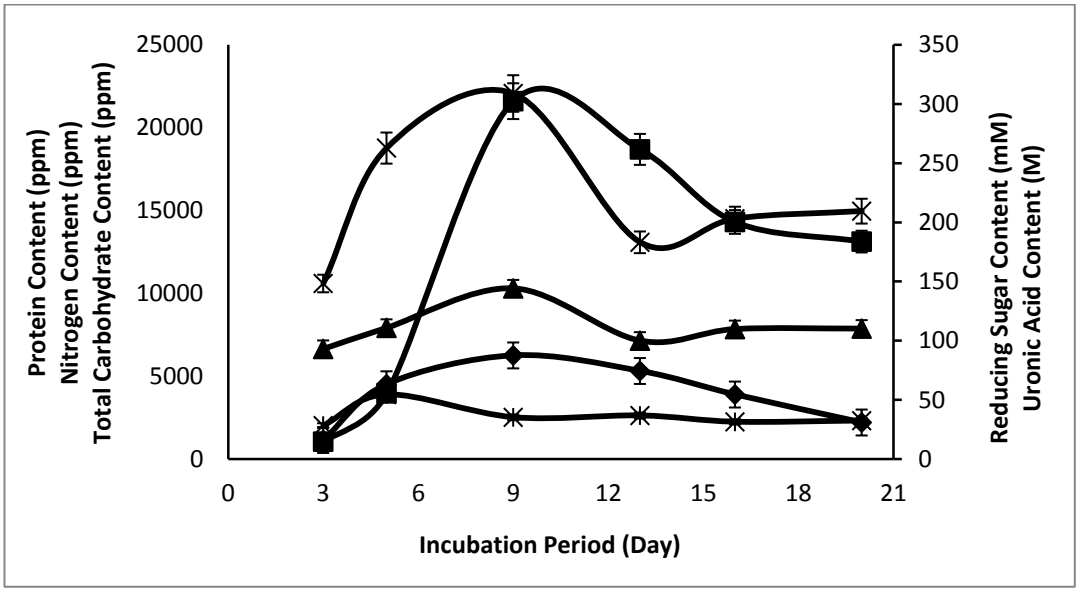

Figure 3.2 Protein (♦), nitrogen (■), total carbohydrate (▲), reducing sugar (×) and uronic acid (•) content of medium during incubation period

Carbon (C) and nitrogen (N) concentrations in production medium remained at high levels until 9th day of incubation and they reached maximum levels on 9th day. Monomeric components that occurred by degradation of solid substrate were caused high C and N concentrations. However, decreasing of C and N concentrations in the medium from the 9th day of incubation was indicator of consuming of nutrients by microorganisms. Decreasing of nutrients is beginning of the stress condition for microorganisms and they need extra metabolite to reach more nutrients. Before reaching the maximum biosurfactant activity, C and N concentrations were at maximum levels. It was concluded as, when C and N levels in the production medium reached certain levels biosurfactant production was triggered. Additionally, it is known that C:N ratio is one of the parameters that effects biosurfactant production (Saharan et al., 2011). In this present study suitable C:N ratio was occurred for biosurfactant production by *P djamor* after 9th day of incubation.

3.2 Ligninolytic and Lipase Enzyme Activities

Pleurotus sp. is known as good sources for ligninolytic enzyme producer (Akpinar, & Ozturk Urek, 2012; Akpinar, & Ozturk Urek, 2014). Due to lignocellulosic character of solid substrate used in this present study, ligninolytic enzyme production was searched during the biosurfactant production (Figure 3.2). The highest Lac and MnP activities were determined on 16th day of incubation as 2446.67 ± 58 and 850.91 ± 25 U/L, respectively. Additionally, the highest LiP activity was detected as 5832.26 ± 102 U/L on 9th day of incubation. While the highest Lac and MnP activities were detected after reaching the maximum biosurfactant activity, the highest LiP activity was determined before reaching the maximum biosurfactant activity.

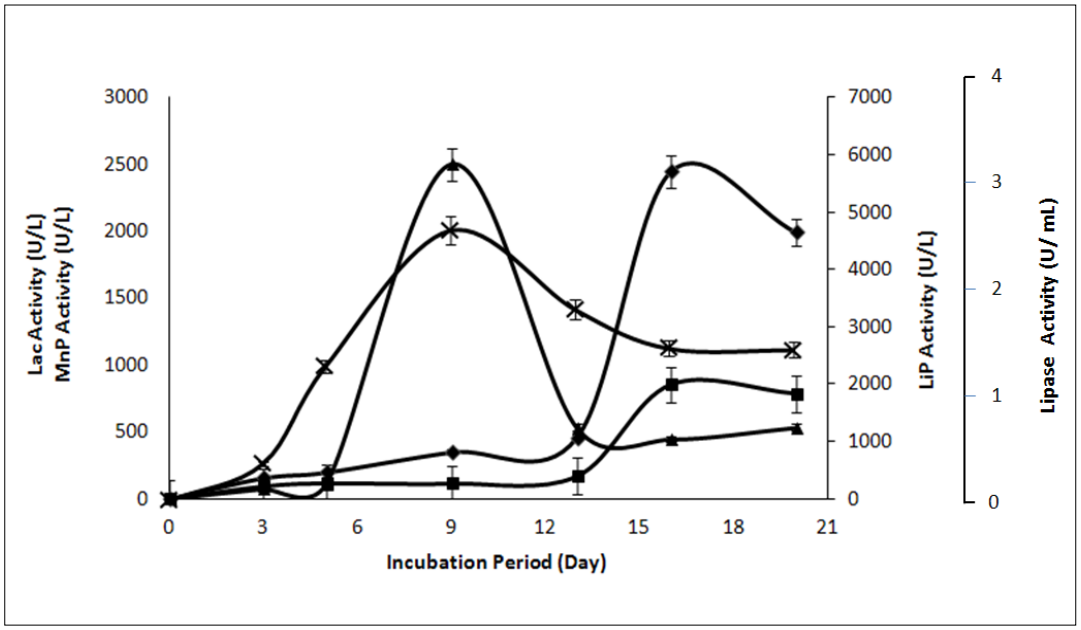

Figure 3.3 Variation of Lac (♦), MnP (■), LiP (▲) and lipase (×) activities during incubation period that produced by *P. djamor*

The interactions between ligninolytic enzyme production and biosurfactant production in SSF were investigated. The correlations between Lac and MnP activity and surface tension reducing ability of biosurfactant were meaningful (r_s = 0.763 and r_s = 0.840, respectively) between 13th and 20th days of cultivation in *P. djamor* culture. Also, there was a meaningful correlation (r_s = 0.758) between LiP activity

and surface tension reducing ability of biosurfactant in *P. djamor* culture between 9th and 20th days of cultivation.

Although the main aim of the study was not ligninolytic enzyme production, produced enzymes had high ligninolytic activities. In a study of Dritsa and Rigas (2013), maximum Lac and MnP activities produced by *Pleurotus sp.* were detected as 27.81 and 25.96 U/L, respectively. In this present study obtained Lac and MnP enzyme activities were approximately 90 and 35 fold higher than study of Dritsa and Rigas (2013), respectively. These results were important to demonstrate the correlation between biosurfactant and ligninolytic enzyme production. According to several studies *Pleurotus sp.* cannot produce LiP with high activity or no production is occurred (Dritsa, & Rigas, 2013; Peláez, Martinez, & Martinez, 1995). However, the highest activity (5832.26 ± 102 U/L) of LiP enzyme produced by *P. djamor* in this present study was much higher than several studies therefore it was concluded that there was a correlation between LiP and biosurfactant production. LiPs (~ - 1.35V) have high redox potentials than several peroxidases (~ - 0.8V) (Hammel, & Cullen, 2008). They are capable of oxidizing a variety of reducing substrates (Maciel et al., 2010). Due to lignocellulosic properties of solid substrates used in production medium, they stimulated production of ligninolytic enzyme production. Additionally, hydrocarbon component of solid substrate promoted biosurfactant production. Ligninolytic enzymes and biosurfactant produced by microorganisms in SSF facilitated degradation of solid substrate in a synergetic effect (Velioglu, & Ozturk Urek, 2014a).

As the hydrophobic character of the solid substrate and extra carbon source used in production medium lipase enzyme production was stimulated as well as biosurfactant production. Since microorganisms need to metabolize insoluble compounds, they synthesize lipase and biosurfactant (Desai, & Banat, 1997). Lipase activities during incubation period are shown in Figure 3.2. The highest lipase activity (2.34 ± 0.02 U/mL) was detected on 9th day of incubation. On 13th day of incubation, that the maximum biosurfactant activity was determined, lipase activity was detected as 1.65 ± 0.01 U/mL.

Although any extra optimization study wasn't carried out for lipase production, the detected enzyme activities were comparable level with several studies in literature. In a study of Açıkel, Erşan and Açıkel (2011) the activity of lipase produced in optimum conditions was 0.964 U/mL. The detected lipase activity in the present study was noted as 2.43 times higher than this value. By optimization of lipase production conditions, it is also possible to achieve higher lipase activity in a biosurfactant production process.

Lipase and LiP activities reached highest levels before the detection of maximum biosurfactant activity. By reducing the amount of nutrients in the production medium an increasing in enzyme activities was occurred, followed by increased activity of biosurfactant. Lastly Lac and MnP activities were reached to their highest levels. Microorganism used the substrate in production medium at maximum efficiency by synergistically using biosurfactant and enzyme activities. Simultaneous and economic production of biosurfactant and industrial enzymes is important for economical applications like bioremediation of oil residues, waste management and cosmetic industry.

3.3 Optimization of Biosurfactant Production Conditions

After determination of the best biosurfactant producer strain and the most suitable solid substrate and extra carbon source, optimization studies were carried out. First of all, optimum amount and surface area of sunflower seed shell were determined (Table 3.3) in optimization steps. As mentioned above, the maximum biosurfactant activity was determined on 13^{th} day of incubation so in this part of the study, samples of 13^{th} day were analyzed. The minimum surface tension value (29.79 ± 0.3 mN/m) and the maximum E_{24} value (45.71 ± 4.0%) were determined in control condition. Also, diameter of clear zone according to oil spreading was 3.5 ± 0.2 cm in this condition. The emulsification index stability designates the strength of a surfactant and the produced biosurfactant in control condition conserved the emulsification index by 90.07 ± 8.7% for 48 hours.

Table 3.3 Emulsification indexes (E_{24} and E_{48}) and surface tensions of produced biosurfactants by *P. djamor* with different surface area and amount of solid substrate

Surface area of solid substrate (cm^2)	Amount of solid substrate (g)	E_{24} (%)	E_{48} (%)	Surface tension (mN/m)
1	1	36.84 ± 3.2	35.14 ± 3.2	37.03 ± 0.4
	3	35.14 ± 3.2	37.84 ± 3.2	33.067 ± 0.3
	5	45.71 ± 4.0	41.17 ± 4.0	29.79 ± 0.3
	7	37.5 ± 3.2	36.84 ± 3.2	38.61 ± 0.4
	10	13.16 ± 1.1	13.89 ± 1.1	39.88 ± 0.4
0.25	1	35.16 ± 3.2	34.21 ± 3.2	36.6 ± 0.3
	3	36.11 ± 3.2	35.14 ± 3.2	32.88 ± 0.3
	5	31.58 ± 3.1	28.95 ± 2.5	33.45 ± 0.3
	7	37.84 ± 3.2	36.12 ± 3.2	35.19 ± 0.3
	10	34.21 ± 3.2	28.21 ± 2.5	42.36 ± 0.4

Surface tension values didn't show alterations except control condition. With regard to surface tension and emulsification index values, the optimum amount and surface area of solid particles were in control condition. Also, results of oil spreading test supported these findings. Biosurfactants exhibit important roles in increasing bioavailability of substrates (Van Hamme et al., 2006). Increment of surface area expedited getting substrates, thus microorganism didn't need large amounts of biosurfactant production. Similarly production with large amount of solid substrate made easier to reach substrate although microorganisms produced biosurfactant to get more nutrients in control condition.

In the next optimization step it was demonstrated the volume of liquid medium (Table 3.4) after determination of optimum surface area and amount of solid substrate. According to surface tension results, optimum volume of medium was 10

mL (control condition). Also the maximum emulsification index and oil spreading activity values were determined in control condition while no growth was observed in 50 mL medium. Additionally, there was a strong correlation between surface tension and medium volume ($r_s = 0.946$, $p < 0.05$).

Table 3.4 Emulsification indexes (E_{24} and E_{48}), diameter of clear zones and surface tension values of produced biosurfactant by *P. djamor* with different volume of medium

Volume of medium (mL)	**E_{24} (%)**	**E_{48} (%)**	**Diameter of clear zones (cm)**	**Surface tension (mN/m)**
10	45.71 ± 4.0	41.17 ± 4.0	3.5 ± 0.2	29.79 ± 0.3
25	37.84 ± 3.2	35.89 ± 3.2	2.6 ± 0.15	32.1 ± 0.3
50	0	0	0	Not detected

As humidity exceeded 70%, no growth was observed in 50 mL medium. In this present study, we also aimed economic production, so the volume of liquid medium is an important factor that effects production cost. The optimum medium volume was determined as 10 mL which was suitable for an economic and higher production.

Table 3.5 Emulsification indexes (E_{24} and E_{48}) and surface tensions of produced biosurfactant by *P. djamor* at different temperature

Temperature (°C)	**E_{24} (%)**	**E_{48} (%)**	**Surface tension (mN/m)**
25	28.95 ± 2.5	27.03 ± 2.5	34.14 ± 0.3
29	45.71 ± 4.0	41.17 ± 4.0	29.79 ± 0.3
35	38.46 ± 3.2	37.84 ± 3.2	45.29 ± 0.5

After determination of optimum surface area, amount of solid substrate and volume of liquid medium, optimum process temperature was investigated (Table 3.5). As maximum emulsification index values and minimum surface tension value were observed in control condition, optimum temperature for biosurfactant production process was 29°C. A meaningful correlation was determined between temperature and surface tension ($r_s = 0.775$, $p < 0.05$).

In order to get large amounts of biosurfactant, it is necessary to optimize the environmental conditions (Saharan et al., 2011). Temperature is one of the important factors that affect type and amount of produced biosurfactant. Surface tension values of biosurfactant produced at different temperatures showed considerable changes. Temperature effects biochemical reactions in microorganism cells, hence changes in biosurfactant production were expected situation.

pH is another environmental factor that affects yield and characteristics of the produced biosurfactant. The next step was optimization of pH value of liquid medium. As the minimum surface tension value (28.82 ± 0.3 mN/m) was detected at pH 5.5, optimum pH level was determined as 5.5 (Table 3.6). In this condition, biosurfactant conserved emulsification index by 90 ± 8.7% ($p < 0.05$). As a result of this finding, pH value of control condition was changed as 5.5.

Table 3.6 Emulsification indexes (E_{24} and E_{48}), diameter of clear zones and surface tensions of produced biosurfactant by *P. djamor* at different pH values

pH value of medium	**E_{24} (%)**	**E_{48} (%)**	**Diameter of clear zones (cm)**	**Surface tension (mN/m)**
5.5	44.44 ± 4.0	40 ± 4.0	3.9 ± 0.2	28.82 ± 0.3
6	45.71 ± 4.0	41.17 ± 4.0	3.5 ± 0.2	29.79 ± 0.3
7	37.84 ± 3.2	36.84 ± 3.2	1.5 ± 0.05	29.45 ± 0.3
8	36.84 ± 3.2	36.84 ± 3.2	4 ± 0.3	29.02 ± 0.3

According to Zinjarde and Pant (2002), the best production of biosurfactant occurred at pH 8.0 while in another study the production of biosurfactant reached maximum at pH 5.5 (Bednarski et al., 2004). The optimum pH value shows changes according to the biosurfactant type, production condition and producer microorganism. In this present study significant changes wasn't determined between surface tensions of biosurfactant produced at different pH values.

In the last optimization step, effects of different Fe^{2+} ion concentrations on biosurfactant production were investigated (Table 3.7). There was no significant

effect of Fe^{2+} ion concentration on biosurfactant production in investigated culture condition and concentrations (r_s = -0.157, p > 0.05). According to surface tension values the optimum Fe^{2+} ion concentration was 3.5 µM. Also maximum emulsification index and diameter of clear zone were determined in control condition.

Metal ions play an important role in biosurfactant production since they form cofactors of some enzymes (Saharan et al., 2011). Fe is an important activator of isocitrate lyase enzyme which is involved in cell growth on hydrophobic substrates (Hommel, & Ratledge, 1993). In this present study, excessive concentration or deficiency of Fe^{2+} ion didn't considerably effect biosurfactant production. In a study, biosurfactant production by *B. subtilis* was optimal with 4 mM Fe^{2+} (Wei, Wang, & Chang, 2004). The authors supposed that poor growth was observed at low iron concentration due to the chelating effects of biosurfactant resulting in less iron for cell metabolism. At high iron concentration, growth was reduced due to the acidification of media.

Table 3.7 Emulsification indexes (E_{24} and E_{48}), diameter of clear zones and surface tensions of produced biosurfactant by *P. djamor* with different Fe^{2+} ion concentrations

Fe^{2+} ion concentration (µM)	**E_{24} (%)**	**E_{48} (%)**	**Diameter of clear zones (cm)**	**Surface tension (mN/m)**
Deficiency	33.33 ± 3.2	30.23 ± 3.1	1.5 ± 0.1	29.52 ± 0.3
3.5	44.44 ± 4.0	40 ± 3.7	3.9 ± 0.3	28.82 ± 0.3
18	32.43 ± 3.1	27.03 ± 2.5	2.2 ± 0.2	29.37 ± 0.3
35	38.89 ± 3.3	35.14 ± 3.2	3.0 ± 0.3	29.08 ± 0.3

According to results optimum conditions were determined as surface area and amount of solid substrate 1 cm^2 and 5 g, volume of medium 10 mL, temperature 29°C, pH level 5.5 and Fe^{2+} ion concentration 3.5 µM. In the optimized conditions levels of protein, total carbohydrate, reducing sugar, lipid, nitrogen, and uronic acid of medium were 3214.29 ± 98 ppm, 8969.69 ± 102 ppm, 205.3 ± 9.2 mM, 1395.5 ±

42 ppm, 12933.03 ± 162 ppm and 32750 ± 471 mM, respectively. Also lipase activity was detected as 1.467 ± 0.01 U/mL. In these conditions, levels of protein and lipid reduced while the level of total carbohydrate increased in reference to control condition. The increasing level of carbohydrate was supposed by the increase in levels of reducing sugar and uronic acid. Due to the reduced level of lipid, decrease of lipase activity in optimum conditions was an unexpected result. In addition, the produced biosurfactant in optimum conditions was isolated and purified and the amount was detected as 10.21 ± 0.5 g/L.

In the study of Kalyani, Naga Sireesha, Aditya, Girija Sankar and Prabhakar (2014) olive oil was used as carbon source to produce rhamnolipid by *Streptomyces coelicoflavus* and they produced 0.475 g/L biosurfactant in optimum conditions. Rufino, Sarubbo and Campos-Takaki (2007) studied the cultivation of *C. lipolytica* grown on ground nut oil and produced 4.5 g/L biosurfactant. Similarly a low-cost agricultural byproduct palm oil was used as carbon source and achieved biosurfactant amount was 2.3 g/L (Oliveira, Vazquez, De Campos, & De Franca, 2009). In another study, sunflower and olive oil were used as carbon sources and 2.7 g/L biosurfactant produced with 32-36 mN/m surface tension (Haba, Espuny, Busquets, & Manresa, 2000). As distinct from these studies, in this present study two low cost substrates were used, as sunflower seed shell and oil and the amount of produced biosurfactant was 10.21 ± 0.5 g/L with high surface tension reducing activity (28.82 ± 0.3 mN/m).

3.4 Partial Characterization of Produced Biosurfactant

The CMC value was investigated which is an important characteristic for surfactants. CMC is a measure of surfactant efficiency, the lower CMC indicates less surfactant is needed to saturate interfaces and form micelles. As shown in Figure 3.4, before reaching the CMC value, the surface tension changed strongly with the concentration of the produced biosurfactant. However when it reached the CMC value, the surface tension changed with a lower slope and remained relatively constant at high concentrations. The CMC of produced biosurfactant was determined as 0.964 ± 0.09 mg/mL and the surface tension was 30.98 ± 0.3 mN/m at this point.

In a study that used canola oil and glucose as carbon source, CMC value of produced biosurfactant by *C. lipolytica* was 25 mg/mL and surface tension was 30 mN/m at this point (Sarubbo, Farias, & Campos-Takaki, 2007). Similarly, CMC value of produced biosurfactant by *P. aeruginosa* LB1 growing on oil refinery waste was detected as 120 mg/mL (Benincasa, Abalos, Oliveira, & Manresa, 2004). Rhamnolipid type biosurfactant produced by *P. aeruginosa* UG2 with corn oil had CMC value as 230 mg/mL (Abalos et al., 2001). Low CMC value (0.964 ± 0.09 mg/mL) of economically produced biosurfactant in this present study, allow economically use of the biosurfactant in various applications.

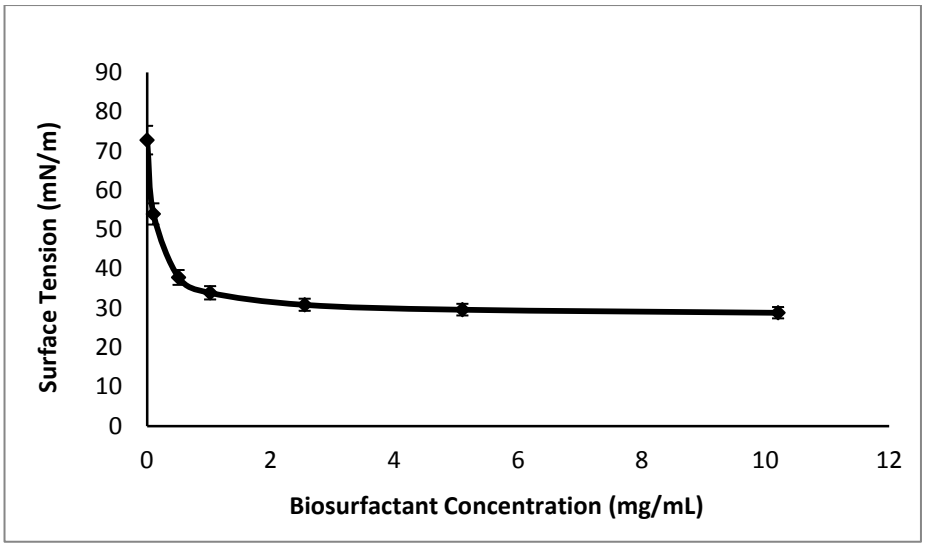

Figure 3.4 Surface tension versus concentration of the produced biosurfactant by *P. djamor*

After optimization steps the produced biosurfactant in optimum conditions was extracted, purified and its chemical characterization was analyzed. The amount of purified biosurfactant was 10.21 ± 0.5 g/L. The chemical composition of 1 mg extracted biosurfactant was formed 92.38 ± 5.2 µg protein, 15.8 ± 0.5 µg total carbohydrate, 13 ± 0.5 µg reducing sugar, 13.27 ± 0.8 µg lipid, 56.2 ± 2.4 µg nitrogen, and 14.97 ± 0.5 µg uronic acid. Results of chemical composition analyses demonstrated complex structure of produced and purified biosurfactant. Also, purified biosurfactant demonstrated considerable lipase activity (0.695 ± 0.01 U/mg).

After determination of chemical composition of produced biosurfactant, the investigation of biosurfactant type was performed by chromatographic and spectroscopic methods.

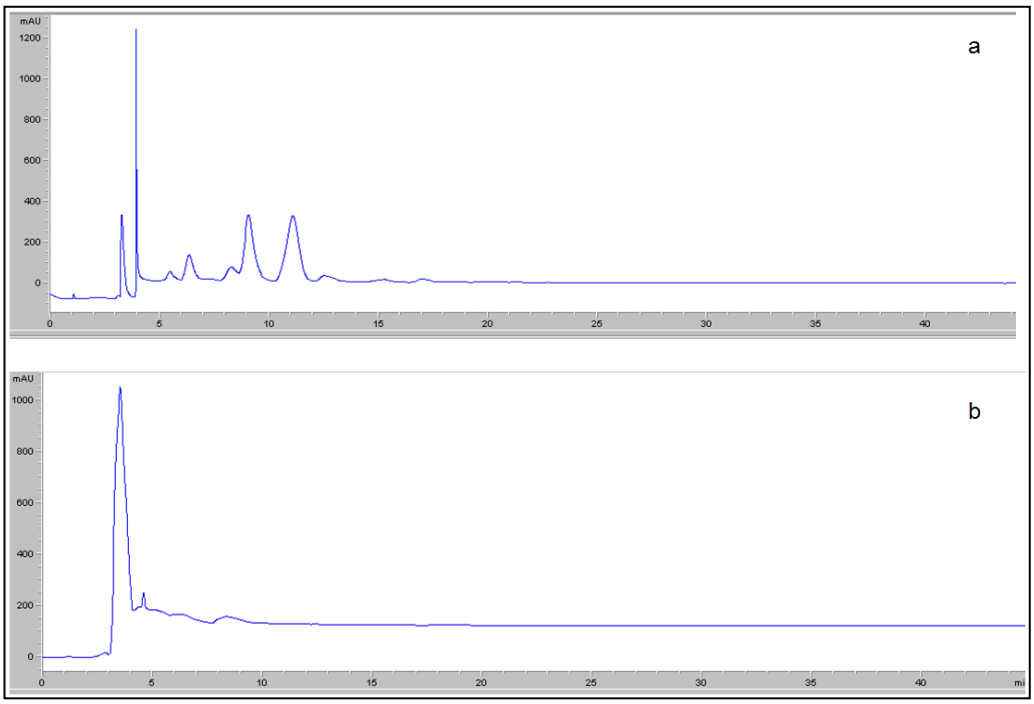

Figure 3.5 HPLC chromatogram of (a) standard surfactin, (b) produced biosurfactant by *P. djamor*

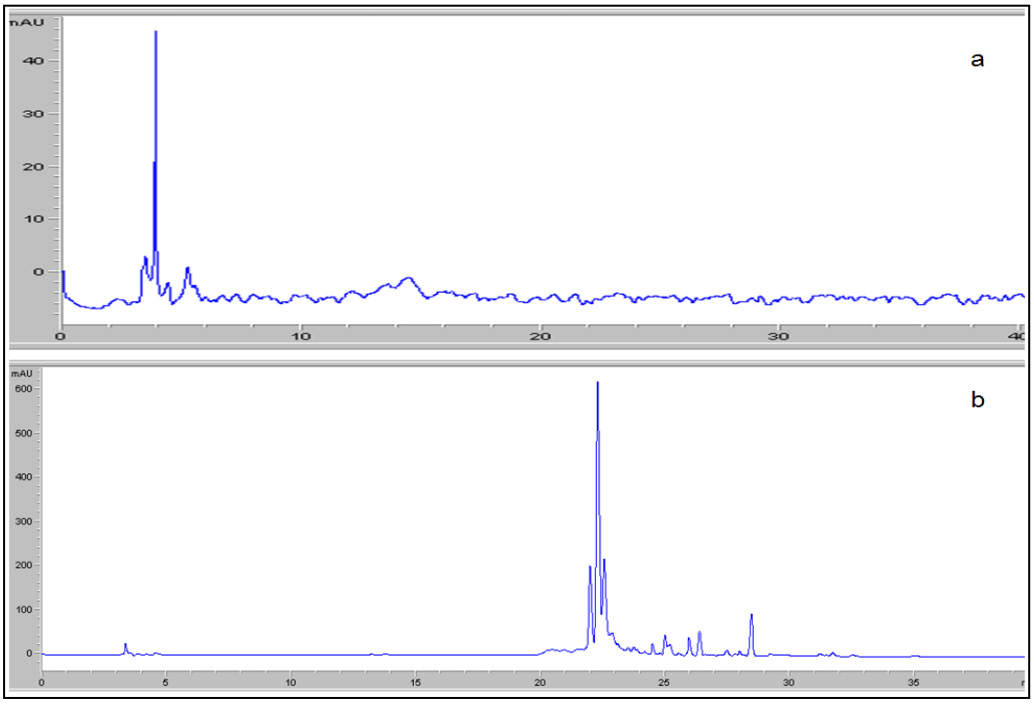

Figure 3.6 HPLC chromatogram of (a) standard rhamnolipid, (b) produced biosurfactant by *P. djamor*

Surfactin and rhamnolipid were used as standard in HPLC and chromatograms of standard biosurfactants and produced biosurfactant were compared (Figure 3.5 and Figure 3.6).

When compared HPLC chromatograms of produced biosurfactant (Figure 3.5 (b)) and standard surfactin (Figure 3.5 (a)), it was clear that they didn't have same structure. In the standard surfactin chromatogram there were six major peaks (Wei, & Chu, 2002). Although these peaks didn't appear in chromatogram of the produced biosurfactant, the resulted peaks were related to complex structure of produced biosurfactant. In a similar way it was definite that the structures of rhamnolipid and produced biosurfactant were different (Figure 3.6 (a-b)). The occurred peaks in chromatogram of produced biosurfactant may be due to the complex structure.

As well as chromatographic analysis spectroscopic analysis was applied to the produced biosurfactant. Figure 3.7 illustrated FT-IR spectra of standard and produced biosurfactants. Although the produced biosurfactant (Figure 3.7 (a)) had common peaks with rhamnolipid and surfactin (Figure 3.7 (b-c)), it was neither gylcolipid nor lipopeptide type biosurfactant.

In FT-IR spectra of rhamnolipid and produced biosurfactant, the peak at 3490 cm^{-1} was associated with O-H stretching in polysaccharide structure and also it was indicator of intermolecular H bonds. In FT-IR spectra of surfactin and produced biosurfactant, the peak located next to 3300 cm^{-1} was related to N-H stretching. The peak at 2991-2924 cm^{-1}, which was figured in all three spectrums, showed CH_3 asymmetric stretching which was associated with lipid, carbohdyrate or protein structure. The peak at 2856 cm^{-1} was formed as a result of CH_2 symmetric resonance in lipid and carbohydrate structure (Figure 3.7 (a) and 3.7 (c)). The peaks next to 1650 cm^{-1} were relevant with C=O stretching on protein structure and the peak belongs to N-H bending and C-N stretching was next to1542 cm^{-1} (Figure 3.7 (a) and 3.7 (b)). The peak at 1468 cm^{-1} was associated with CH_2 bending mostly on lipid slightly on protein structure (Figure 3.7 (a) and 3.7 (b)). The peaks next to 1404 and

1209 cm^{-1} were related to asymmetric stretching of hydrocarbon chain and phospholipid structures, which were figured in all three spectra.

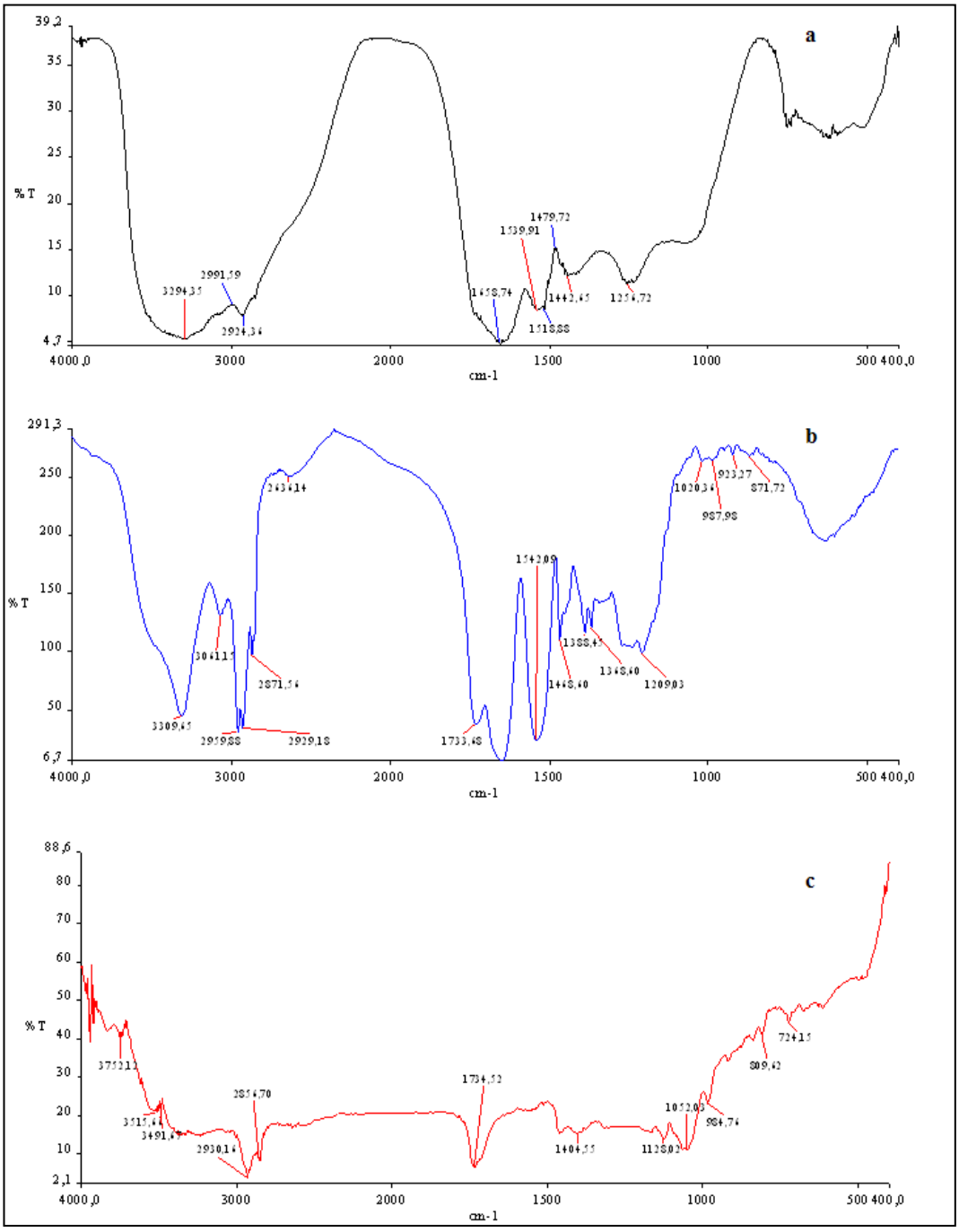

Figure 3.7 FT-IR spectra of (a) produced biosurfactant by *P. djamor*, (b) standard surfactin, (c) standard rhamnolipid

The most important bands of produced biosurfactant were located at 3294 cm^{-1} (O-H stretching) and at 2991-2924 cm^{-1} (C-H bands: CH_2-CH_3 stretching) was associated with the stretching vibration of C–H bond of constituent sugar residues. The peak at 1658 cm^{-1} (C=O stretching) suggested the presence of carbonyl functionality present in carboxylate or amide moieties of protein and peptide amines and also the peaks at 1539 cm^{-1} (N-H bending) were indication of proteins. The peaks at 1200- 1400 cm^{-1} were indication of hydrocarbon chain (Velioglu, & Ozturk Urek, 2014a-b).

Lastly, according to ^{1}H-NMR spectrum (Figure 3.8) of produced biosurfactant in optimum conditions, presence of peaks at 0.797- 1.912 ppm indicated the methyl group (-CH_3) corresponding to straight chain fatty acids. Additionally, CH signals at 2.130-2.738 ppm and NH signals at 6.948-7.419 ppm indicated amino acid and peptide structure. The peaks at 3.075-4.498 ppm belonged to sugar ring.

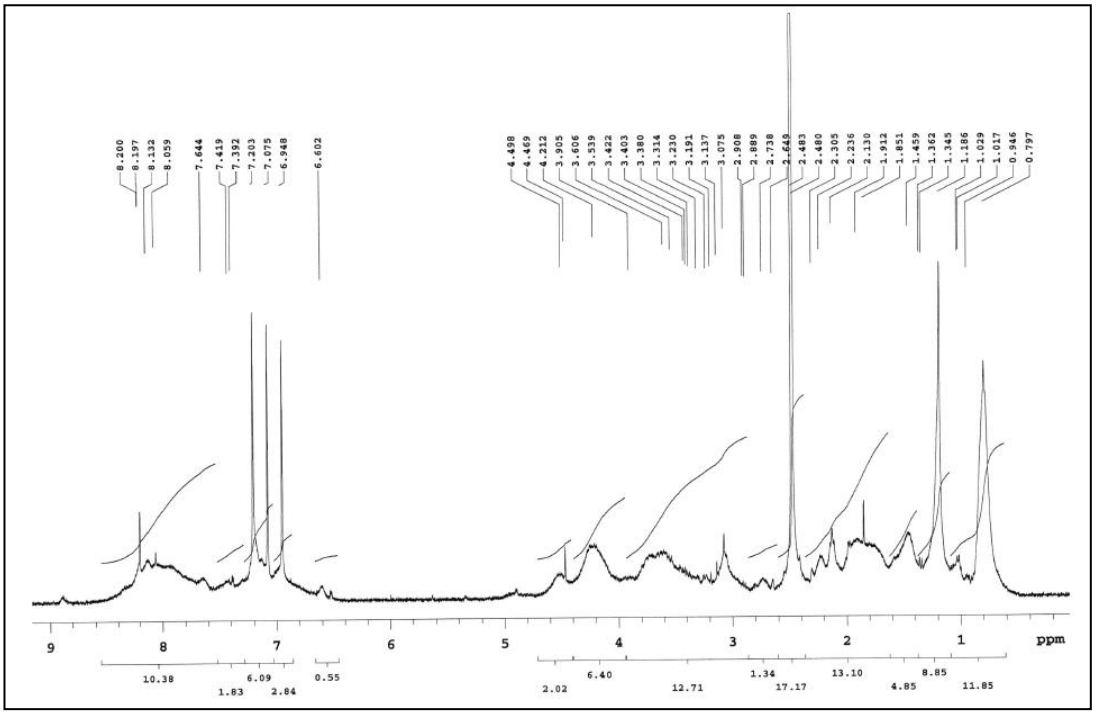

Figure 3.8 ^{1}H-NMR spectrum of produced biosurfactant by *P. djamor*

The obtained results demonstrated that produced biosurfactant in optimum conditions by *P. djamor* had functional groups, bonds and structures which are

presence in glycolipid or lipopeptide type biosurfactants. It was concluded that produced biosurfactant had a complex structure by having carbohydrate, protein and lipid contents. Also, chemical composition analyses of produced biosurfactant supported this result.

The thermal stability of biosurfactant is an important characteristic with respect to its applications. Degradation of biosurfactant occurred by three steps as observed in TG analysis (Figure 3.9). In the first step 6.72 ± 0.4% of weight loss was recorded from 126 to 169°C due to loss of alcohol molecules and moisture. In the second phase degradation 6.43 ± 0.4% weight loss was observed between 183-225°C. Lastly maximum degradation was determined from 260 to 462°C where a weight loss 43.9 ± 4% was observed. The maximum degradation temperature was 302°C. In a similar study, the maximum degradation temperature of produced biosurfactant by *Cronobacter sakazakii* was recorded as 300°C (Jain et al., 2012). The produced rhamnolipid by *P. aeruginosa* with soybean oil as carbon source had maximum degradation temperature at 250°C (Abbasi et al., 2012).

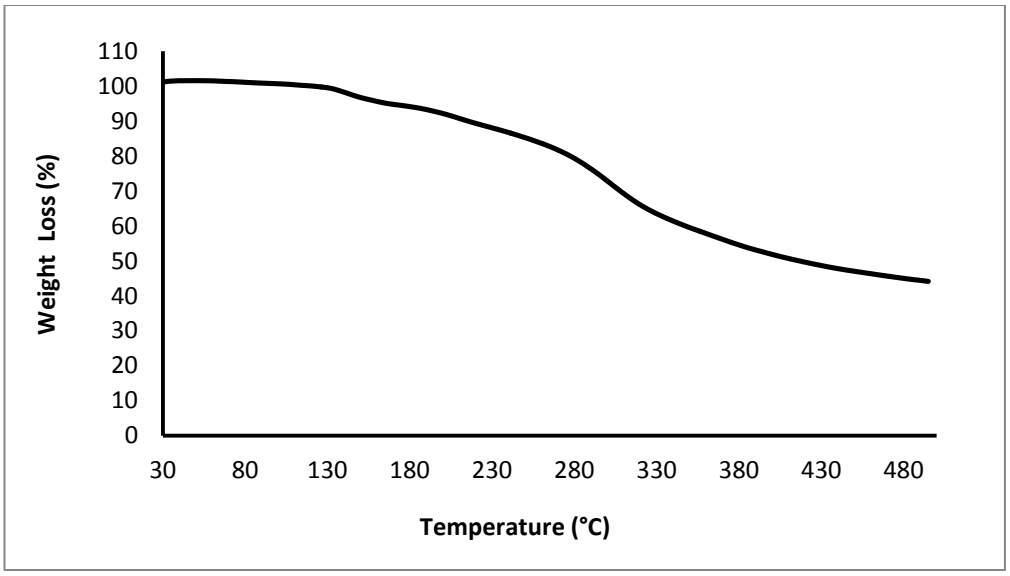

Figure 3.9 TGA diagram of the produced biosurfactant by *P. djamor*

The first weight loss was occurred at high temperatures than 100°C and low weight loss was detected in first two steps. These indicated high thermal stability of

produced biosurfactant by *P. djamor*. Also in last step occurred weight loss was not high, it supported these results. This situation was interpreted contributing to the thermal stability of the complex structure of produced biosurfactant.

The DSC thermogram of produced biosurfactant illustrated endothermic peak (Figure 3.10) with crystallization temperature (T_c = 141.81°C) and melting points (T_{m1} = 206.55°C, T_{m2} = 302.48°C).

The obtained results indicated the possibility of biosurfactant usage in widespread processes carried out at high temperatures such as food industry and some waste removal, due to thermal stability of produced biosurfactant.

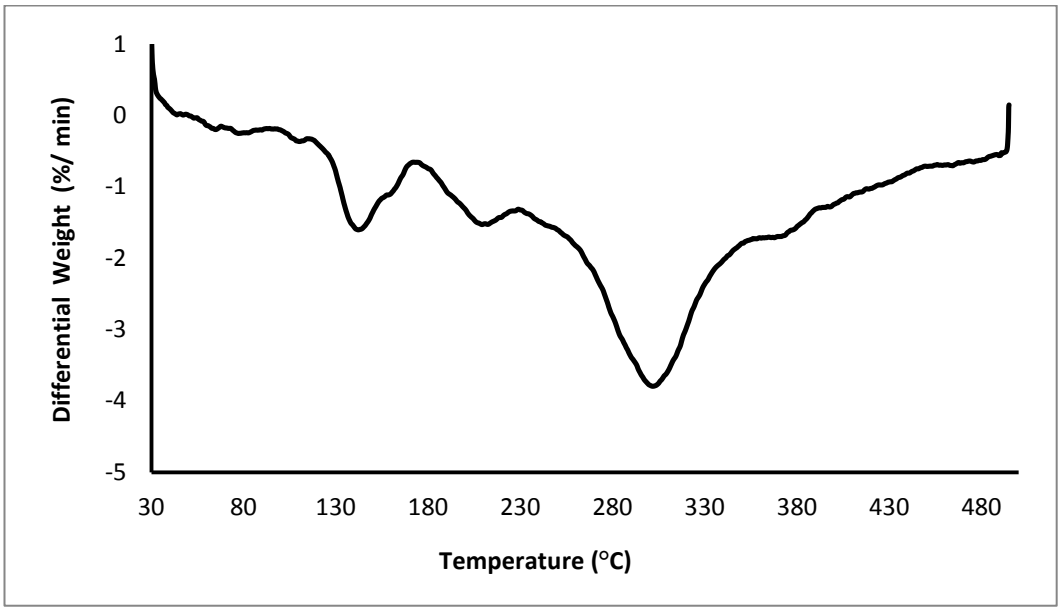

Figure 3.10 DSC analysis of the produced biosurfactant by *P. djamor*

3.5 Effects of Environmental Factors on Biosurfactant Activity

Environmental factors affect activity and stability of biosurfactants (Mulligan, 2005). The changes of surface activity of produced biosurfactant were investigated at different pH, temperature or NaCl concentrations (Figure 3.11). The surface activity of produced biosurfactant was stabile at investigated temperature values (4-100°C) and the maximum activity was detected at 70°C. The produced biosurfactant was

conserved emulsification index by 86 ± 8% for 96 h at 70°C which was an important indicator of thermo stability (Figure 3.11 (a)). The surface tension was almost stabile at different temperature and it reached minimum value at 70°C as similar to study of Luna, Sarubbo and Campos-Takaki (2009).

When the effect of the pH on the activity of biosurfactant was examined it was clear that at pH values 2 and 12 values the surface activities were not very high, but the produced biosurfactant showed high surface activity at pH values between 2 and 12. The produced biosurfactant indicated pH stability at wide pH range (Figure 3.11 (b)). At low pH values the surface tension was stabile while it reached minimum value at pH 9. These results had similarities with the study of Chooklin, Maneerat and Saimmai (2014).

In this present study variation in biosurfactant activity was also investigated at 2-30% concentration of NaCl. The surface activities of produced biosurfactant were high and stabile under 30% NaCl concentrations (Figure 3.11 (c)). The surface tension conserved stability between 2-10% NaCl concentrations as determined in the study of Rocha e Silva, Rufino, Luna, Santos and Sarubbo (2014).

These results demonstrated that produced biosurfactant in optimum conditions had high tolerance to temperature, pH and salinity. Multimeric association of the biosurfactant could be either due to hydrophobic interactions or due to ionic, hydrogen or covalent bonds (Markande, Acharya & Nerurkar, 2013). The high tolerance of produced biosurfactant against in changes of environmental factors will increase the possibility of use in various industrial applications.

Emulsification indexes and stability of produced biosurfactant were also investigated using different oils (Figure 3.12). The maximum emulsification index (E_{24} = 52.94 ± 5.0%) was determined with waste frying oil, and also it conserved stability about 92 ± 9% for 48 h. Although the emulsification index with lubricating oil was not very high (E_{24} = 34.29 ± 3.2%), it was stabile about 77 ± 7% for 48 h.

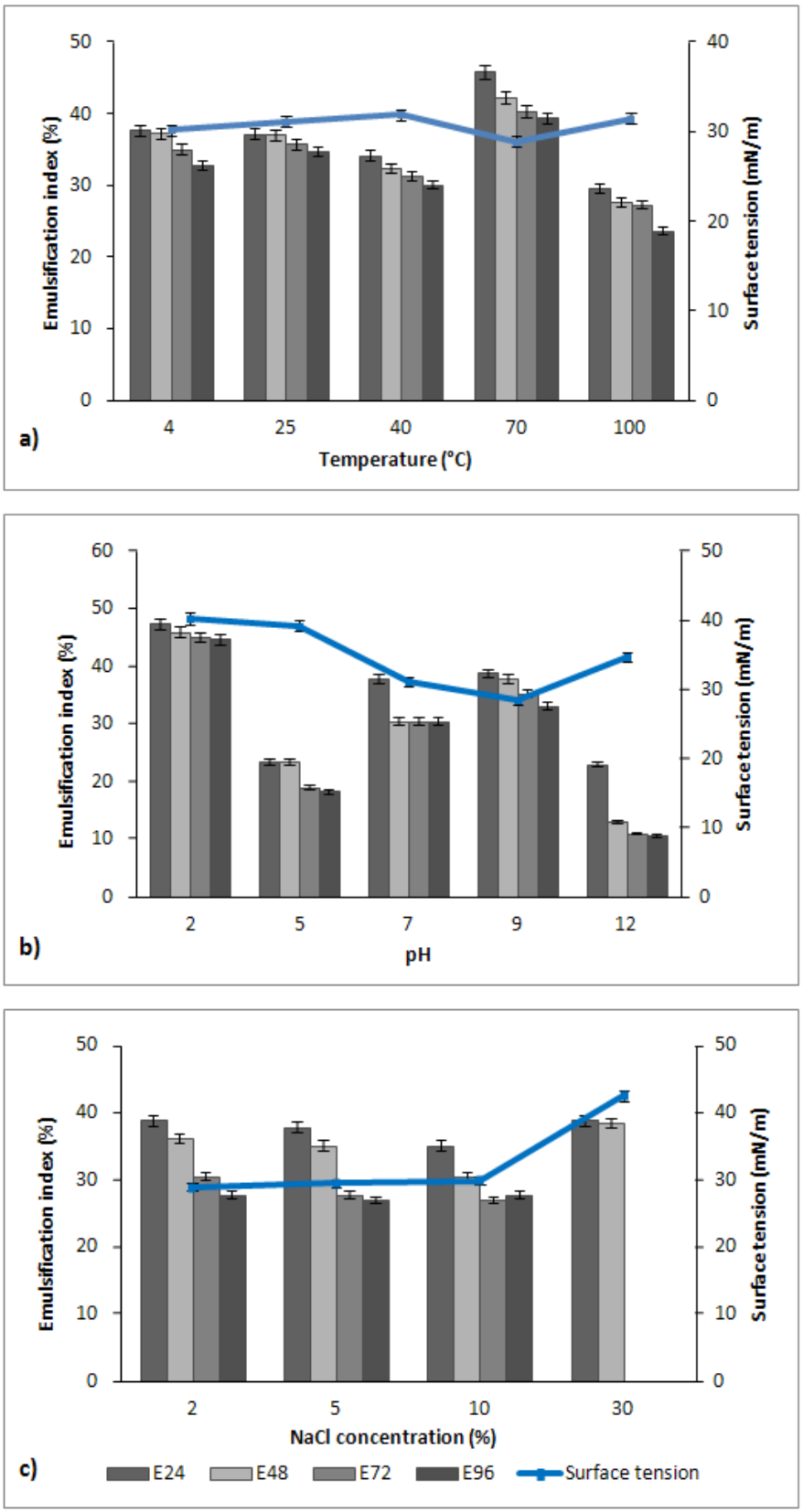

Figure 3.11 Effects of (a) temperature, (b) pH, (c) NaCl concentration on emulsification index and surface tension of produced biosurfactant by *P. djamor*

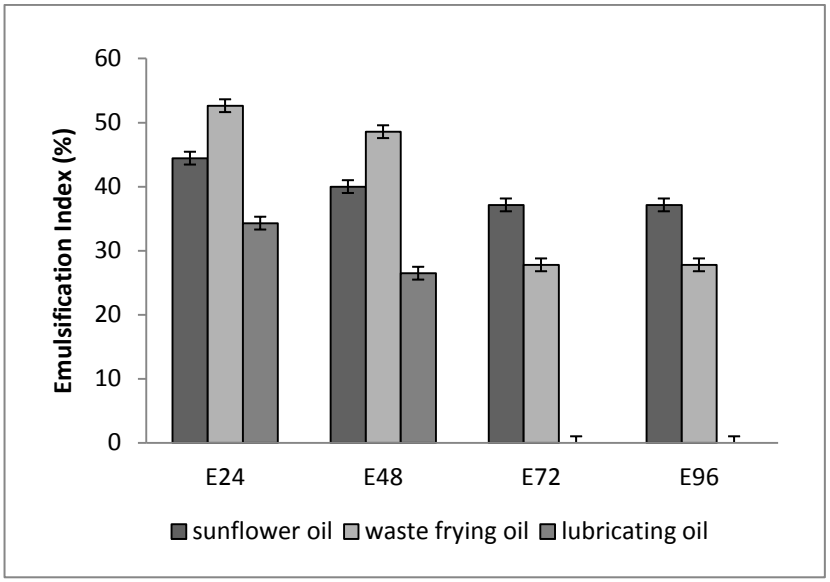

Figure 3.12 Emulsification indexes of produced biosurfactant with different oils

This result had importance for using in recovery of these types of waste oils. Additionally, occurred emulsification with lubricating oil had lower activity and it conserved its stability for 2 days. Due to different chemical structures of used oils, occurred emulsifications had different activities. Also as sunflower seed oil was used in production media, high emulsification activity may observed with this oil.

3.6 Removal of Waste Frying Oil from Contaminated Sandy Soil

The performance of produced biosurfactant in oil recovery was studied using waste frying oil contaminated sand. The results obtained demonstrated that the produced biosurfactant was capable to remove 76.57 ± 6% of waste frying oil absorbed in the sand while distilled water (control) removed 65 ± 6% of the oil. In the study of Pereira et al., (2013), the produced biosurfactant from different *Bacillus* sp. removed between 19-22% of oil. Thus, this characteristic indicated potential use of the produced biosurfactant in oil industry and environmental applications especially in microbial enhanced oil recovery. Also, biosurfactants have eco-friendly features over chemical surfactants as they remove oil contaminants without modifying the chemical nature of soil.

CHAPTER FOUR
CONCLUSION

Biologically produced biosurfactants by microorganisms have emulsification and de-emulsification, wetting, solubilization, foaming and phase-dispersion properties. Also they are as effective as synthetic surfactants in reducing surface and interfacial tension. Biosurfactants have several advantages over the synthetic surfactants such as high biodegradability, low toxicity, ecological features, high surface activity, high selectivity and specific activity at extreme conditions and ability to be produced from renewable and cheaper substrates. They are used in a wide variety of fields such as chemistry, food, cosmetic, pharmaceutics industries, biomedical and agriculture due to their excellent properties. The variety of chemical structures, physicochemical properties and types enable the formation of different usage areas. The producer microorganism, production conditions and used substrates are important parameters that influence biosurfactant type and productivity.

In this present study biosurfactant production by four different *Pleurotus* strains using sunflower seed shell, potato peel or grape waste on SSF was carried out. Nikiforova et al., (2009) have shown production of an emulsifying agent by *P. ostreatus* and as far as we know research on biosurfactant production by *Pleurotus* spp. is limited to this study. The production condition was optimized and chemical and physicochemical properties of produced biosurfactant in optimum conditions were investigated. In addition to solid substrates three different liquid media were used for production, which included extra carbon source (glucose or sunflower seed oil) or no extra carbon source. According to obtained results the most suitable solid substrates were detected as sunflower seed shell for *P. djamor, P. ostreatus* and *P. sajor-caju* strains and potato peels for *P. eryngii*. Also, all four strains reached maximum biosurfactant activity with sunflower seed oil as extra carbon source. According to obtained results, the highest biosurfactant activity (29.79 ± 0.3 mN/m surface tension, 3.5 ± 0.2 cm oil spreading activity and 45.71 ± 4% emulsification index) was determined in *P. djamor* on SSF with sunflower seed shell and sunflower seed oil on 13^{th} day of incubation.

During biosurfactant production period, ligninolytic enzyme activities produced by *P. djamor* were investigated, due to lignocellulosic character of solid substrate. The highest Lac and MnP activities were determined on 16^{th} day of incubation as 2446.67 ± 58 and 850.91 ± 25 U/L, respectively. Additionally, the highest LiP activity was detected as 5832.26 ± 102 U/L on 9^{th} day of incubation. Microorganisms that degrade solid insoluble substrates produce biosurfactants to facilitate substrate break down and dissolution (Gerson, 1993). The producer microorganism used in this study is known as a good ligninolytic enzyme producer. By concurrently producing biosurfactants and ligninolytic enzymes microorganisms altered the physical nature of the cell-substrate interaction, and significantly increased the bioavailability of the substrate and their growth potential.

As the hydrophobic character of the solid substrate and extra carbon source used in production medium lipase enzyme production was also investigated during incubation period. The highest lipase activity (2.34 ± 0.02 U/mL) was detected on 9^{th} day of incubation. Insoluble substrates can be rich sources for microbial growth; however the microbial utilization of insoluble substrates imposes a great restriction on the availability of substrate to the cell (Gerson, 1993). The main effect of the biosurfactants is to reduce the interfacial tension at the surface of insoluble substrate and to increase the availability of substrate to the microorganism. For liquid hydrocarbons, reduced interfacial tension facilitates emulsification, increasing the surface area available for dissolution, microbial attachment and substrate absorption. In this study, simultaneous production of lipase and biosurfactant facilitated utilization of oily substrates.

After determination of the best biosurfactant producer strain and the most suitable solid substrate and extra carbon source, optimization of size and amount of solid substrate, volume of medium, temperature, pH and Fe^{2+} ion concentrations were carried out. Optimal conditions were detected as surface area and amount of solid substrate 1 cm^2 and 5 g, volume of medium 10 ml, temperature 29°C, pH level 5.5 and Fe^{2+} ion concentration 3.5 μM. The produced biosurfactant in optimal condition

amount of 10.21 ± 0.5 g/L has high surface activity with 28.82 ± 0.3 mN/m surface tension, 44.44 ± 4% emulsification index and 3.9 ± 0.3 cm oil spreading activity.

The chemical and physicochemical properties of produced biosurfactant by *P. djamor* in optimum conditions were investigated after optimization studies. The CMC value was detected as 0.964 ± 0.09 mg/mL and the surface tension was 30.98 ± 0.3 mN/m at this point. CMC is a measure of surfactant efficiency, efficient surfactant has very low CMC value and the lower CMC indicates less surfactant is needed to saturate interfaces and form micelles. The partial characterization of the produced biosurfactant was studied by HPLC, FT-IR and ^{1}H-NMR. When compared HPLC chromatograms of produced biosurfactant and standard biosurfactants (surfactin and rhamnolipid), it was clear that they didn't have same structure. Similarly in FT-IR spectra, although the produced biosurfactant had common peaks with standard biosurfactants, it had a different structure than standards. Lastly, according to ^{1}H-NMR spectrum of produced biosurfactant in optimum conditions by *P. djamor* had functional groups, bonds and structures which are presence in glycolipid or lipopeptide type biosurfactants. It was demonstrated that produced biosurfactant had a complex structure and chemical composition analyses of produced biosurfactant also supported this result. In addition to chemical characterization thermal stability of produced biosurfactant was investigated by TGA and DSC analysis. The produced bisourfactant showed high thermal stability with the maximum degradation temperature at 302°C.

Additionally, the effects of environmental factors such as temperature, NaCl concentration and pH on the activity of biosurfactant produced in optimal SSF condition were investigated. The surface activity of produced biosurfactant was stabile at investigated temperature values (4-100°C) and the maximum activity was at 70°C. The thermal stability of produced biosurfactant was also supported by TGA and DSC analysis. Although at pH values 2 and 12 the surface activities were not very high, the produced biosurfactant showed high surface activity at pH values between 2 and 12. Also produced biosurfactant had high and stabile surface activity under 30% NaCl concentrations. The high tolerance to changes of environmental

factors may be due to complex structure of produced biosurfactant. Emulsification indexes and stability of produced biosurfactant were also investigated using different oils (sunflower seed oil, waste frying oil, lubricating oil) and high activity was detected with waste frying oil. Lastly, the performance of produced biosurfactant in oil recovery was studied and it was capable to remove 76.57 ± 6% of waste frying oil from contaminated sand. It was concluded that these properties will increase the possibility of use in various industrial applications.

In this present study, economical biosurfactant production was occurred by *Pleurotus* sp. in SSF using agro-industrial wastes which cause environmental pollution. By studying with a new biosurfactant producer fungus has contributed to the literature on this topic. The majority of biosurfactants described in literature is of bacterial origin. However, due to the pathogenic nature of such producing microorganisms, these products are not suitable for use in food, cosmetic or pharmaceutical industries. The studies of biosurfactant production by non toxic or pathogenic fungus like *Pleurotus* sp. has been growing in importance.

Additionally, the type of the producer microorganism is an important factor that affects yield, type and application activities of produced biosurfactant. Although literatures have several reports about any types of biosurfactants no single surfactant is suitable for all the potential applications such as solubility, surface tension reduction, wetting ability and foaming. This makes very important and urgent to develop even more multifunctional biosurfactants. The produced biosurfactant had high surface activity as well as high emulsification activity. It was demonstrated that the complex structure of produced biosurfactant enabled the high surface and emulsification activities. Additionally, concurrent production of biosurfactant and ligninolytic enzymes and lipase was a significant example to multiple product strategies. So, multifunctional biotechnological products were gained in a single process.

In addition to biosurfactant production with high activity, development of the cheaper process and use of low cost substrates are important for a successful

biosurfactant production process. In the present study used agro-industrial wastes as solid substrates facilitated an economical and ecological production. The use of industrial wastes achieved double benefit of reducing the pollutants and producing valuable biotechnological products. Also economical production of biosurfactants facilitates economical usage in several applications. In the present study economical and concurrent biosurfactant and enzyme production was carried out by using several agro-industrial wastes.

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