Screening of Biosurfactant-Producing Yeasts, Purification, Characterization and Application

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A Thesis Submitted in Partial Fulfillment of the Requirements

for the Degree of Doctor of Philosophy in Biotechnology

Prince of Songkla University

2008

Thesis Title	Screening of Biosurfactant-Producing Yeasts, Purification, Characterization and Application
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ชื่อวิทยานิพนธ์ การคัดเลือกยีสต์ที่ผลิตสารลดแรงตึงผิวชีวภาพ การทำบริสุทธิ์ คุณลักษณะ และการประยุกต์ใช้

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ปีการศึกษา 2550

บทคัดย่อ

การแยกยีสต์จากดินที่มีการปนเปื้อนน้ำมันบริเวณภาคใต้ของประเทศไทย สามารถแยกยีสต์ได้ 81 ไอโซเลท ซึ่งเจริญได้ในอาหารที่มี weathered crude oil เป็นแหล่งคาร์บอน โดย 5 ไอโซเลทสามารถผลิตสารลดแรงตึงผิวชีวภาพที่ลดแรงตึงผิวของอาหารเลี้ยงเชื้อได้ต่ำกว่า 60 มิลลิ-นิวตันต่อเมตร เมื่อใช้กลูโคส weathered crude oil เฮกซะดีเคน หรือไซลีน เป็นแหล่งคาร์บอน ยีสต์ไอโซเลท SR4 ให้ค่ากิจกรรมของสารลดแรงตึงผิวชีวภาพสูงสุด เมื่อเทียบเคียงยีสต์ SR4 ด้วยการทดสอบทางชีวเคมี และการหาลำดับเบส 26S rDNA ได้เป็น *Issatchenkia orientalis* SR4 ซึ่งสามารถลดแรงตึงผิวของอาหารเลี้ยงเชื้อจาก 61.5 ลงเหลือ 49.0 มิลลินิวตันต่อเมตร เมื่อใช้ไซลีนเป็นแหล่งคาร์บอน นอกจากนี้ยังพบว่า *I. orientalis* SR4 เมื่อเจริญในอาหารที่มีกลูโคส 10 กรัมต่อลิตร เป็นแหล่งคาร์บอน ให้ค่า emulsification activity ต่อไซลีน เฮกซะดีเคนและน้ำมันก๊าดเท่ากับ 84.62 47.62 และ 68.43 % ตามลำดับ ในขณะที่โซเดียมโดเดซิลซัลเฟต 10 กรัมต่อลิตรให้ค่า emulsification activity 76.92% จากการศึกษาสภาวะที่เหมาะสมต่อการผลิตสารลดแรงตึงผิวชีวภาพจากยีสต์ที่มีไซลีน 1 g/L เป็นแหล่งคาร์บอน ยูเรีย 3 g/L เป็นแหล่งไนโตรเจน ที่พีเอชเริ่มต้น 5.0 พบว่าเมื่อเลี้ยงยีสต์ SR4 ในระดับฟลาสก์เขย่าโดยใช้อาหารเลี้ยงเชื้อ 50 มิลลิลิตรในฟลาสก์ขนาด 250 มิลลิลิตร ด้วยอัตราการเขย่า 200 รอบต่อนาที ที่อุณหภูมิ 30 องศาเซลเซียส เชื้อมีการเจริญสูงสุด 3.0×10^{7} เซลล์ต่อมิลลิลิตร ในชั่วโมงที่ 48 และค่าแรงตึงผิวของอาหารเลี้ยงเชื้อมีค่าต่ำสุดเท่ากับ 49.0 มิลลินิวตันต่อเมตร และมีค่า emulsification activity 41.0% และเลี้ยงเชื้อในถังหมัก 2 ลิตร ซึ่งบรรจุน้ำหมัก 1 ลิตร อัตราการให้อากาศ 0.5 ปริมาตรอากาศต่อปริมาตรอาหารต่อนาที ด้วยอัตราการกวน 200 รอบต่อนาที ที่อุณหภูมิ 30 องศาเซลเซียส และไม่ควบคุมการเป็นกรด-ด่าง พบว่าการเจริญและการผลิตสารลดแรงตึงผิวชีวภาพสอดคล้องกับผลการทดลองในระดับ
ฟลาสก์เขย่า เมื่อนำส่วนใสมาสกัดด้วยตัวทำละลายผสมระหว่างคลอโรฟอร์มและเมทานอลในอัตราส่วน 2 ต่อ 1 (ปริมาตรต่อปริมาตร) ส่วนสกัดหยาบที่ได้นำมาทำการแยกโดยคอลัมน์โครมา-โตกราฟฟีชนิดซิลิกาเจล จากนั้นนำส่วนย่อยที่มีกิจกรรมของสารลดแรงผิวชีวภาพสูงสุดไปทำ

บริสุทธิ์ต่อด้วยวิธี preparative HPLC ด้วยคอลัมน์โครมาโตกราฟีชนิด Inertsil ODS-3โดยมี acetonitrile 95% ซึ่งเติม trifluoroacetic acid (TFA) 0.05% เป็นสารละลายเคลื่อนที่ และวิเคราะห์หาโครงสร้างทางเคมีของสารลดแรงตึงผิวชีวภาพที่แยกได้โดยวิธี GC-MS พบว่า fragmentation pattern และ mass spectrum จากฐานข้อมูล เป็น เมทิลโอเลเอท (methyl oleate) การจำแนกสารเบื้องต้นโดยการยืนยันด้วยการวิเคราะห์ร่วม (co-injection) กับเมทิลโอเลเอททางการค้า พบว่าสารลดแรงตึงผิวที่บริสุทธิ์แล้วเป็นเมทิลโอเลเอท นอกจากนี้ยังวิเคราะห์หาโครงสร้างสารลดแรงตึงผิวชีวภาพที่แยกได้ต่อไปด้วยวิธี HPLC โดยเปรียบเทียบกับกรดโอเลอิก (authentic oleic acid) พบว่าสารลดแรงตึงผิวที่บริสุทธิ์แล้วมีค่า retention time ตรงกับกรดโอเลอิก และนี้เป็นฉบับแรกที่รายงานว่าเชื้อยีสต์ *I. orientalis* SR4 สามารถผลิตสารลดแรงตึงผิวชีวภาพชนิดกรดโอเลอิก เมื่อใช้ไซลีนเป็นแหล่งคาร์บอน

การศึกษาคุณลักษณะของสารลดแรงตึงผิวชีวภาพ พบว่าส่วนสกัดหยาบไม่มีความคงตัวต่อโซเดียมคลอไรด์ 2% ขึ้นไป แต่มีความคงตัวต่อแคลเซียมคลอไรด์และแมกนีเซียมคลอไรด์ ในช่วง 0-0.02 และ 0-0.05% ตามลำดับ นอกจากนี้ส่วนสกัดหยาบมีความคงตัวที่พีเอชในช่วง 4 ถึง 10 และความคงตัวต่ออุณหภูมิในช่วง 4 ถึง 80 องศาเซลเซียส เป็นเวลา 24 ชั่วโมง ในขณะที่กิจกรรมของสารลดแรงตึงผิวจะลดลงที่อุณหภูมิ 100 องศาเซลเซียส เมื่อเวลาผ่านไป 3 ชั่วโมง การนำสารลดแรงตึงผิวชีวภาพที่ผลิตจากเชื้อยีสต์ *I. orientalis* SR4 ไปประยุกต์ใช้ พบว่าสารสกัดหยาบสารลดแรงตึงผิวช่วยเพิ่มการละลายของสาร polycyclic aromatic hydrocarbons ได้แก่ naphthalene และ phenanthrene ได้ดีกว่าสารลดแรงตึงผิวสังเคราะห์คือโซเดียมโดดีซิลซัลเฟต ในการย่อยสลายน้ำมันหล่อลื่นเครื่องยนต์ที่ใช้แล้ว เมื่อนำสารสกัดหยาบสารลดแรงตึงผิวเติมลงในดินที่มีการปนเปื้อนน้ำมันหล่อลื่นเครื่องยนต์ที่ใช้แล้ว พบว่า สารสกัดหยาบสารลดแรงตึงผิวส่งเสริมการย่อยสลายน้ำมันหล่อลื่นเครื่องยนต์ที่ใช้แล้ว โดยทำให้องค์ประกอบของน้ำมันหล่อลื่นเครื่องยนต์ที่ใช้แล้ว ได้แก่ saturate, aromatic และ resin ลดลง 67.80, 20.00 และ 51.86% ตามลำดับ ในเวลา 30 วัน ในขณะที่ชุดการทดลองที่ไม่มีการฆ่าเชื้อและไม่เติมสารสกัดหยาบสารลดแรงตึงผิวนั้น องค์ประกอบ saturate, aromatic และ resin ลดลงเพียง 23.00, 11.52 และ 23.13% ตามลำดับ

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Major Program	Biotechnology
Academic Year	2007

ABSTRACT

Eighty-one yeasts were isolated from oil contaminated soils in Southern Thailand to test for the ability to grow on weathered crude oil as a sole carbon source. Five isolated produced high biosurfactant activity which reduced surface tension of the culture broth to less than 60 mN/m when used glucose, weathered crude oil, *n*-hexadecane or xylene as a carbon source. The isolated yeast strain SR4 showed the highest biosurfactant activity and was identified as *Issatchenkia orientalis* SR4 by biochemical tests and based on 26S rDNA sequencing. It reduced the surface tension of a culture broth from 61.5 to 49 mN/m when cultivated in the medium with xylene as a carbon source. When *I. orientalis* SR4 was grown in the medium with 10 g/L glucose as a carbon source it showed emulsification activity (%EA) with xylene (84.62%), *n*-hexadecane (47.62%) and kerosene (68.43%). While 10 g/L sodium dodecyl sulfate (SDS) could emulsify these hydrocarbons with 76.92% EA. The optimized medium for the production of the biosurfactant by *I. orientalis* SR4 contained 1 g/L xylene as a carbon source, 3 g/L urea as a nitrogen source with an initial pH 5.0. When it was cultivated in this medium in shake-flask (50 ml medium in 250 ml flask) at 200 rpm and 30°C, it showed maximum growth of 3.0×10^7 cells/ml at 48 h and the surface tension of the culture broth was at the minimum of 49 mN/m and showed 41.0% EA. Growth and biosurfactant production in 2L fermentor containing 1L optimal medium, with the aeration rate 0.5 vvm, agitation speed at 200 rpm and incubation temperature 30°C without controlled pH showed the same result as the shake-flask cultivation.

The culture supernatant obtained was extracted twice with an equal volume of chloroform/methanol (2:1). The highest biosurfactant activity fraction was purified by using preparative HPLC on Inertsil ODS-3 eluting with 95% aqueous acetonitrile containing 0.05%

trifluoroacetic acid (TFA). The fragmentation pattern and a mass spectral library-search by GC-MS analysis suggested the purified compound was methyl oleate. This preliminary identification was confirmed by co-injection of authentic methyl oleate. When the purified compound was analyzed by HPLC it showed the same retention time as authentic oleic acid and the identity was confirmed by co-injection. This is the first report on a biosurfactant, oleic acid produced by yeast, *I. orientalis* SR4 when xylene was used as a carbon source.

The crude biosurfactant was not stable in NaCl up to 2% while it was stable in 0-0.02% $CaCl_2$ and 0-0.05% $MgCl_2$. In addition, the crude biosurfactant was stable at pH range 4.0 to 10.0. It was stable at 4 to 80°C for 24 h and still retained activity at 100°C for 3 h. The crude biosurfactant produced by *I. orientalis* SR4 enhanced the solubility of polycyclic aromatic hydrocarbons such as naphthalene (49.0 mg/L) and phenanthrene (4.2 mg/L) more than synthetic surfactant (SDS). It also promoted the degradation of waste lubricating oil and resulted in a decrease in saturate, aromatic and resin fractions 67.80, 20.00 and 51.86%, respectively at 30 days while the biotic treatment without crude biosurfactant degraded 23.27, 11.52 and 23.13% of saturate, aromatic and resin fractions of waste lubricating oil, respectively.

Acknowledgements

I would like to express my deep appreciation and sincere gratitude to my advisors, Assoc. Prof. Dr. Aran H-Kittikun of the Department of Industrial Biotechnology, Faculty of Agro-Industry, Prince of Songkla University, for his kindness, guidance and assistance in reading, correcting and criticizing this thesis.

I also would like to express my profound gratitude to my co-advisor, Prof. Dr. Fusako Kawai of Laboratory of Applied Microbiology, Research Institute for Bioresources, Okayama University, Japan, for her helpful suggestion, comment and correcting of this thesis.

I am grateful to my co-advisor, Dr. Suppasil Maneerat Department of Industrial Biotechnology, Faculty of Agro-Industry, Prince of Songkla University, for his guidance, comment and correcting of this thesis.

Special thanks to Assoc. Prof. Dr. Duangporn Kantachote, Department of Microbiology, Faculty of Science, Prince of Songkla University and Assoc. Prof. Dr. Benjamas Thanomsub, Department of Microbiology, Faculty of Medicine, Srinakharinwirot University for their helpful suggestion and dedicating valuable time for thesis examination.

Special thanks for Prof. Dr. Hiroshi Kanzaki and Assoc. Prof. Dr. Teruhiko Nitoda, The Graduate School of Natural Science and Technology, Okayama University, Japan for their kindness, guidance and suggestion of this thesis.

I am appreciative to Mrs. Junya Intamanee, Department of Chemical Engineering, Faculty of Engineering, Prince of Songkla University for her helpful and support about Tensiometer.

I would like to appreciate the Graduate School, Prince of Songkla University and the Scholarship under the Commission on Higher Education Staff Development Project, Commission on Higher Education, for provide the budget for thesis exeperiment.

Many thanks to my dear friends and technicians at Department of Industrial Biotechnology, Faculty of Agro-Industry, Prince of Songkla University

Finally, I would like to express my deepest appreciation to my family for great understanding, encouragement and support throughout my study.

Wichuda Katemai

CONTENTS

CONTENTS (CONTINUED)

LIST OF TABLES

LIST OF TABLES (CONTINUED)

LIST OF FIGURES

LIST OF FIGURES (CONTINUED)

LIST OF FIGURES (CONTINUED)

LIST OF FIGURES (CONTINUED)

LIST OF ABBREVIATION

BSM	Basal Salt Medium
CMC	Critical Micelle Concentration
EA	Emulsification Activity
EI	Emulsification Index
EI-MS	Electron-Ionization Mass Spectrometry
GC-MS	Gas Chromatography-Mass Spectrometry
^{1}H-NMR	Proton Nuclear Magnetic Resonance
HPLC	High Performance Liquid Chromatography
m/z	mass per charge
mN/m	millinewton per meter
ODA	Oil Displacement Area
PAHs	Polycyclic Aromatic Hydrocarbons
PSI	pound per square inch
rpm	round per minute
SDS	Sodium Dodecyl Sulphate
TFA	Trifluoroacetic acid
TLC	Thin Layer Chromatography
TLC-FID	Thin Layer Chromatography-Flame Ionization Detector
vvm	volume per volume per minute
WCO	Weathered Crude Oil

CHAPTER 1

INTRODUCTION

Introduction

Biosurfactants are surface active compounds derived from living organisms, mainly microorganisms which now bring much attention (Kim *et al*., 1999). These molecules reduce surface and interfacial tensions in both aqueous solutions and hydrocarbon mixtures (Khire and Khan, 1994). Microbial surface active agents are important biotechnological products with a wide range of applications in many industries such as in food, cosmetic and pharmaceutical industries (Kitamoto *et al*., 2002). Almost all surfactants currently in use are chemically derived from petroleum. However, in recent years, the interest in chemical surfactants has been substituted by the increase of microbial surfactants (Desai and Banat, 1997; Kim *et al*., 2000). Comparing with chemical surfactants, biosurfactants have more advantages, for example, their lower toxicity, higher biodegradability, better environmental compatability, higher foaming, higher selectibility and specific activity at extreme temperature, pH level and salinity and ability to be synthesized from renewable feed-stocks (Kim *et al*., 2000). In the petroleum industry, there are several applications of biosurfactants such as microbial enhancement of oil recovery, cleaning of oil tankers, pumping of crude oils by use of bioemusifier, demulsification of crude oils and viscosity reduction of heavy crude oils (Zhou and Kosaric, 1995).

Recently, a considerable number of studies have been published on surfactants produced by a wide variety of microorganisms such as bacteria, yeasts and filamentous fungi (Mercade and Manresa, 1994). Most known biosurfactants are of bacterial origin, and only a few biosurfactants come from yeasts and molds. Except for mannosylerythritol lipids (MEL) and sophorolipids, there has been no reported on the production of yeast biosurfactant in large quantities (Rosenberg and Ron, 1999). Biosurfactants have not yet been employed extensively in industry because of the relatively high production and recovery costs involved. To reduce the costs of biosurfactant production, it is necessary to select microorganisms capable of high-yield biosurfactant production. In recent years, there has been a growing isolation and identification of new microbial surfactants

(Cirigliano and Carman, 1985) that may have applications in environmental protection, petrochemical production and enhancement of oil recovery processes (Lang and Wullbrandt, 1999).

This study aimed for screening and identification of isolated yeasts from oil contaminated soil with potential to produce surface-active compound and optimize conditions for growth and biosurfactant production. The biosurfactant was purified and its structure was elucidated. The purified compound was characterized and applied for bioremediation of polycyclic aromatic hydrocarbons (PAHs) and waste lubricating oil contaminated soil.

Literature Review

1. Synthetic surfactants and biosurfactants

Surfactants are amphiphilic molecules consisting of hydrophilic and hydrophobic moieties in one molecule. The non-polar, hydrophobic part is frequently a hydrocarbon chain. The polar component or polar group appears in many variations such as amino acid or peptide, carbohydrate, phosphate, alcohol and carboxylic acid. The most common non-ionic surfactants are ethoxylates, ethylene and propylene oxide co-polymers and sorbitan esters. Examples of commercially available ionic surfactants include fatty acids, ester sulphonates or sulphates (anionic) and quartenary ammonium salts (cationic) (Finnerty, 1994). The rapid development of the chemical industry that began in the 1960s led to the development of a wide variety of petroleum-based chemical surfactants. The surface tension correlates with the concentration of the surface-active compound until the critical micelle concentration (CMC) is reached (Fig. 1). Efficient surfactants have a low critical micelle concentration (i.e. less surfactant is necessary to decrease the surface tension). The CMC is defined as the minimum concentration necessary to initiate micelle formation (Mulligan, 2005). In practice, the CMC is also the maximum concentration of surfactant monomers in water and is influenced by pH, temperature and ionic strength. Figure 1 shows how other parameters vary as a function of surfactant concentration. Concentration of surfactant increased as the solubility increased was investigated. On the other hand, natural surfactants produced from animal or plant materials, such as soap, lecithin and saponin, have long been consumed for home and industrial use before chemical surfactants were produced and became widespread. With increasing environmental awareness and emphasis on a sustainable society in harmony with the global environment, natural surfactants (Holmberg, 2001) have been becoming much more important. Microbial compounds which exhibit particularly high surface activity and emulsifying activity are classified as biosurfactants (Makkar and Cameotra, 1999). Biosurfactants produced by various microorganisms. Their properties of interest are: in changing surface active phenomena, such as lowing of surface and interfacial tensions, wetting and penetrating actions, spreading, hydrophilicity and hydrophobicity actions. Biosurfactants can be produced from cheap raw materials which are available in large quantities; the carbon source may come from hydrocarbons, carbohydrates and/or lipids, which may be used seperately or in combination with each other. Biosurfactants can also be produced from industrial wastes and by-products and this is of particular interest for bulk production (Kosaric, 2001).

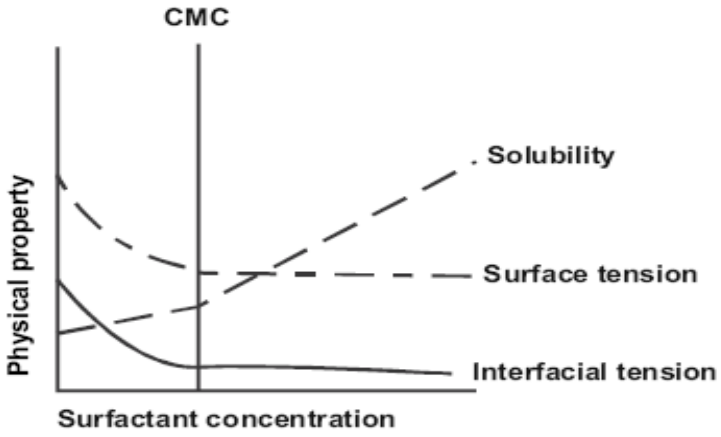


Figure 1. Surface tension, interfacial tension and solubilization as a function of surfactant concentration (CMC represents critical micelle concentration).

Source : Mulligan (2005)

2. Nature of biosurfactants

Many amphiphillic compounds, which consist of a hydrophobic and a hydrophilic group in the moeity, are known in microorganisms, plants and animals. These compounds play important roles in exchanging of energy, substances and signals on various interfaces, and in creating ordered biological systems. The structures of surfactants are largely reflected in that of interfacial characteristics. The combinations of these hydrophobic and hydrophilic groups are very elegant in biosurfactants. The whole structures of biosurfactants have been refined and optimized during the integration process of microbial evolution (Kitamoto *et al*., 2002).

2.1 Biosurfactant classification

Biosurfactants are produced mainly by aerobically growing microorganisms in aqueous media from a carbon source feedstock, e.g. carbohydrates, hydrocarbons, oils and fats or mixtures thereof. The emulsifiers are secreted into the culture medium during the growth of microorganisms and assist in the transport and translocation of the insoluble substrates across cell membranes. All biosurfactants are of the nonionic or anionic type. There are no literature reports of cationic structures however in some instances the presence of nitrogen-containing groups imparts a certain degree of cationic character to parts of the molecule thereby affecting, for example, adsorption on dispersed solids and particle flocculation. The lipophilic moiety of biosurfactant can

be a protein or a peptide with a high proportion of hydrophobic side chains, but is usually the hydrocarbon chain of a fatty acid with 10–18 carbon atoms, although higher molecular weight fatty acids have been reported. The hydrophilic moiety of biosurfactant is an ester, a hydroxy, a phosphate or carboxylate group or a carbohydrate (Bognolo, 1999).

The nature of their polar grouping, biosurfactants are categorized mainly by their structure (Desai and Banat, 1997). Table 1 summarizes recent studied on type and microbial origin of biosurfactants. In general, their structure includes a hydrophilic moiety consisting of amino acids or peptides anions or cations; mono-, di-, or polysaccharides; and a hydrophobic moiety consisting of unsaturated, saturated fatty acids. The major classes of biosurfactants include glycolipids, lipopeptides and lipoproteins, phospholipids and fatty acids and polymeric surfactants.

Table 1. Type and microbial origin of biosurfactants

Type of surfactant	Microorganism
Glycolipids	*Alcanivorax borkumensis*,
	Arthrobacter sp.,
	Corynebacterium sp.,
	Tsukamurella sp.,
	Rhodococus erythropolis,
	Serratia marcescens,
	Candida apicola,
	Candida bombicola
Trehalose lipids	*Arthrobacter paraffineus,*
	Corynebacterium spp*.,*
	Mycobacterium spp*.,*
	Rhodococcus erythropolis,
	Nocardia sp.
Rhamnolipids	*Pseudomonas aeruginosa,*
	Pseudomomas sp*.,*
	Serratia rubidea
Sophorose lipids	*Candida apicola,*
	Candida bombicola,
	Candida lipolytica,
	Candida bogoriensis
Cellobiose lipids	*Ustilago maydis*
Lipopolysaccharides	*Acinetobacter calcoaceticus RAG1,*
	Pseudomonas sp*.,*
	Candida lipolytica
Lichenysin A and B	*Bacillus licheniformis*
Viscosin	*Pseudomonas fluorescens*

Table 1. (Continue)

Type of surfactant	Microorganism
Surfactin	*Bacillus subtilis,*
	Bacillus pumilus
Phospholipids	*Acinetobacter* sp.
Neutral lipids	*Nocardia erythropolis*
Fatty acids (corynomycolic acids,	*Capnocytophaga* sp.,
spiculisporic acids, etc.)	*Penicillium spiculisporum*,
	Corynebacterium lepus,
	Arthrobacter paraffineus,
	Nocardia erythropolis
Alasan	*Acinetobacter radioresistens*
Liposan	*Candida lipolytica*
Polysaccharide-fatty acid complex	*Candida tropicalis*

Source: modified from Mulligan (2005)

2.1.1 Glycolipids

Most known biosurfactants are glycolipids. They are carbohydrates in combination with long-chain aliphatic acids or hydroxyaliphatic acids. Among the glycolipids, the best known are mannosylerythritol lipids, rhamnolipids, trehalolipids, sophorolipids and cellobiose lipids.

Mannosylerythritol lipids (MELs) are microbial extracellular glycolipids composed of mannosylerythritol and fatty acids as hydrophilic and lipophilic moieties, respectively (Fig. 2). They act as effective surfactants. They are produced by *Candida antarctica* T-34 (Kitamoto *et al.*, 1990) and *Ustilago maydis* (Spoeckner *et al.,* 1999).

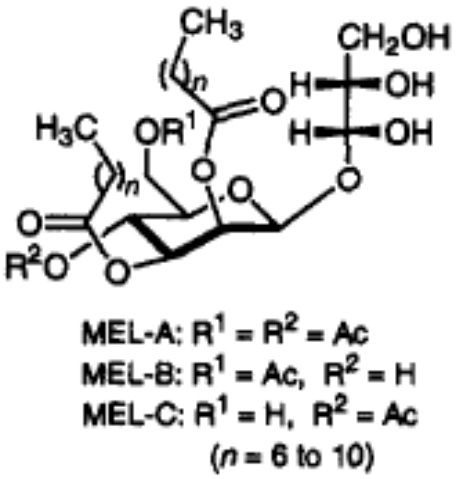


Figure 2. Structure of mannosylerythritol lipids (MELs).

Source : Kitamoto *et al*. (2002)

Rhamnolipids: which one or two molecules of rhamnose are linked to one or two molecules of β-hydroxydecanoic acid. They are the best-studied glycolipids such as L-rhamnosyl-L-rhamnosyl-β-hydroxydecanoyl-β-hydroxydecanoate (Fig. 3).

Trehalolipids: several structural types of microbial trehalolipid biosurfactants (Fig. 4) have been reported (Li *et al*., 1984). Disaccharide trehalose linked at C-6 and C-6′ to mycolic acids is associated with most species of *Mycobacterium, Nocardia* and *Corynebacterium*. Mycolic acids are long-chain, α-branched-β-hydroxy fatty acids. Trehalolipids from different organisms differ in the size and structure of mycolic acid, the number of carbon atoms, and the degree of unsaturation. Trehalose dimycolate produced by *Rhodococcus erythropolis* (Kurashige *et al*., 1989).

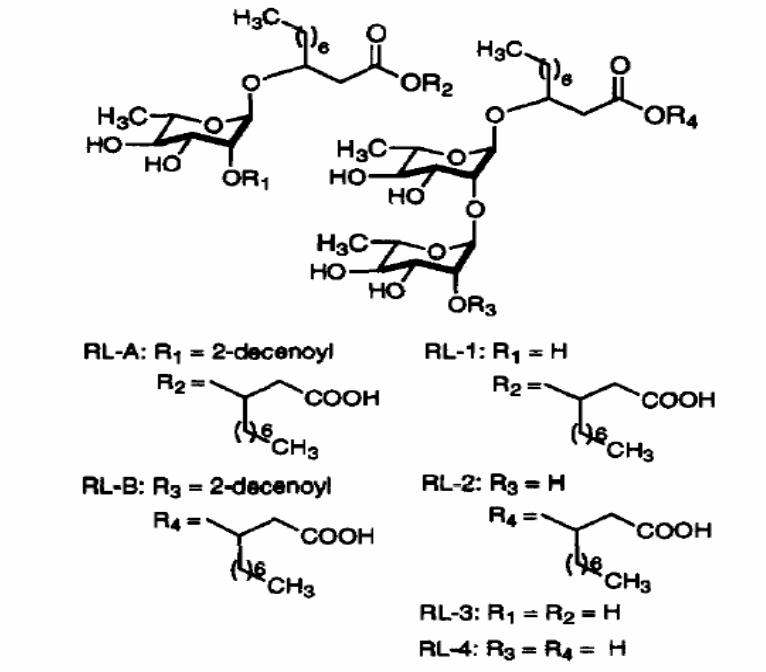


Figure 3. Structure of rhamnolipids.

Source : Kitamoto *et al*. (2002)

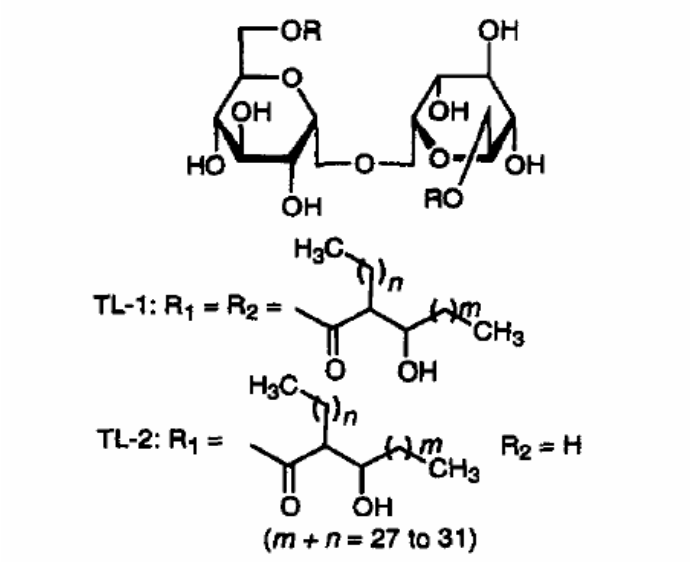


Figure 4. Structure of trehalolipids.

Source : Kitamoto *et al*. (2002)

Sophorolipids: which are produced mainly by yeasts such as *Torulopsis bombicola* (Cooper and Paddock, 1984) and *T. petrophilum* (Cooper and Paddock, 1983), consist of a dimeric carbohydrate sophorose linked to a long-chain hydroxyl fatty acid. These biosurfactants are a mixture of at least six to nine different hydrophobic sophorosides (Fig. 5).

The lipid moieties of the cellobiose lipids (CL) (Fig. 6) are considered to be acids with C2:0, C6:0, 3-OH-C6:0, 3-OH-C8:0, 15, 16-dihydroxy-C16:0 and 2, 15, 16-trihydroxy-C16:0. Moreover, the production of the more polar cellobiose lipids produced by the smut fungus *Ustilago maydis* was enhanced when glucose was used as a carbon source (Spoeckner *et al.,* 1999).

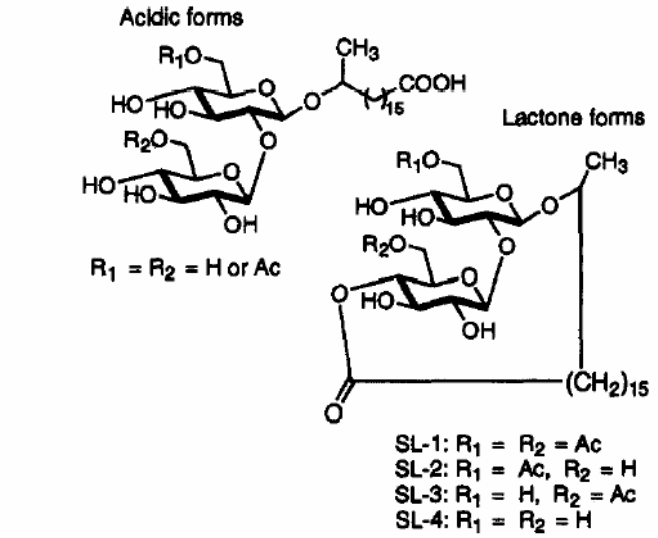


Figure 5. Structure of sophorolipids.

Source : Kitamoto *et al*. (2002)

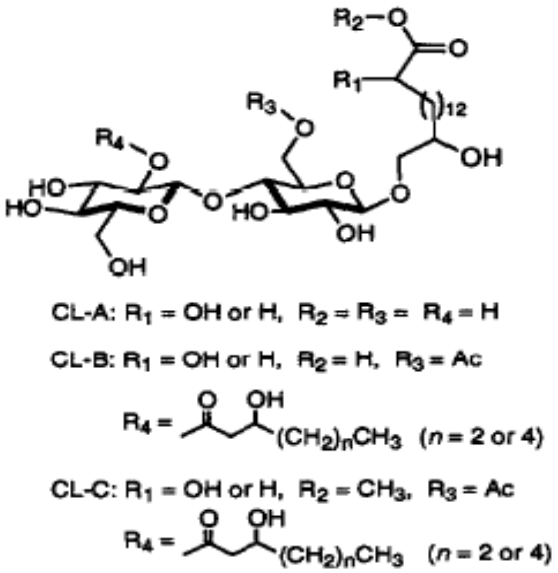


Figure 6. Structure of cellobiose lipids.

Source : Kitamoto *et al*. (2002)

2.1.2 Lipopeptides and lipoproteins

A number of cyclic lipopeptides including decapeptide antibiotics (gramicidins) and lipopeptide antibiotics (polymyxins), produced by *Bacillus* sp., possess remarkable surface-active properties. The cyclic lipopeptide surfactin (Fig. 7), produced by *B. subtilis* ATCC 21332, is one of the most powerful biosurfactants. It lowers the surface tension of water from 72.0 to 27.9 mN/m at concentration as low as 0.005% (Desai and Banat, 1997).

Yakimov *et al*. (1996) showed the production of a new lipopeptide surfactant, lichenysin A, by *B. licheniformis* BAS50 which reduced the surface tension of water from 72 to 28 mN/m with critical micelle concentration (CMC) of as little as 12 μM, comparing favorably with surfactin (24 μM).

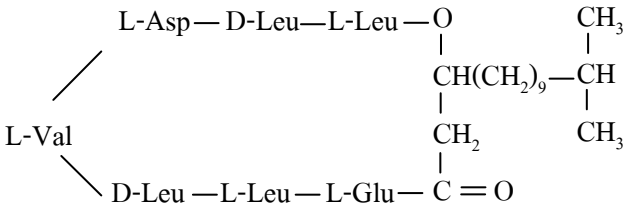


Figure 7. Structure of surfactin produced by *Bacillus subtilis*.

Source : Desai and Banat (1997)

2.1.3 Fatty acids, phospholipids, and neutral lipids

Several bacteria and yeasts produce large quantities of fatty acids and phospholipids surfactants. Phosphatidylethanolamine (Fig. 8) produced by *Rhodococcus erythropolis* growth on *n*-alkane caused a lowing of interfacial tension between water and hexadecane to less than 1 mN/m (Desai and Banat, 1997). Phospholipids (Fig. 9) are present in every microorganism, there are very few examples of extracellular production, the most notable one being the biosurfactants produced by *Corynebacterium lepus*. Fatty acids and neutral lipids, e.g. ustilagic acid, the corynomycolic acids (Fig.10), the lipotheichoic acids (sometimes classified as glycolipids) and the hydrophobic proteins were reported by Bognolo (1999).

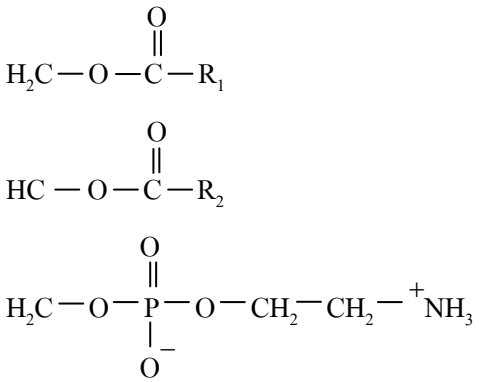


Figure 8. Structure of phosphatidylethanolamine, a potent biosurfactant produced by *Acinetobacter* sp. R_1 and R_2 are hydrocarbon chains of fatty acids.

Source : Desai and Banat (1997)

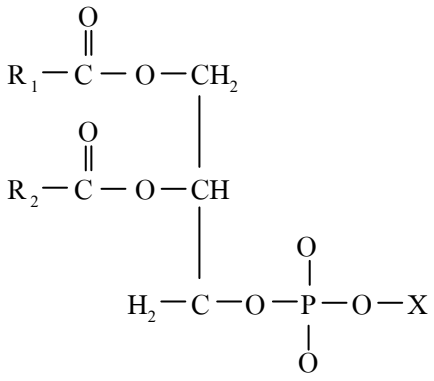


Figure 9. Structure of phospholipid biosurfactants.

R_1 and R_2 are alkyl

X is hydrogen, ethylamine, inositol

Source: Bognolo (1999)

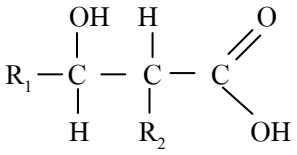


Figure 10. Structure of corynomycolic acid.

R_1 and R_2 are alkyl

Source: Bognolo (1999)

2.1.4 Polymeric biosurfactants

The best studied polymeric biosurfactants are emulsan, liposan, mannoprotein, and other polysaccharide-protein complexes. *Acinetobacter calcoaceticus* RAG-1 produces a potent polyanionic amphipathic heteropolysaccharide bioemulsifier (Fig. 11) called emulsan (Rosenberg *et al*., 1979). Liposan is an extracellular water-soluble emulsifier synthesized by *Candida lipolytica* and is composed of 83% carbohydrate and 17% protein (Cirigliano and Carman, 1984).

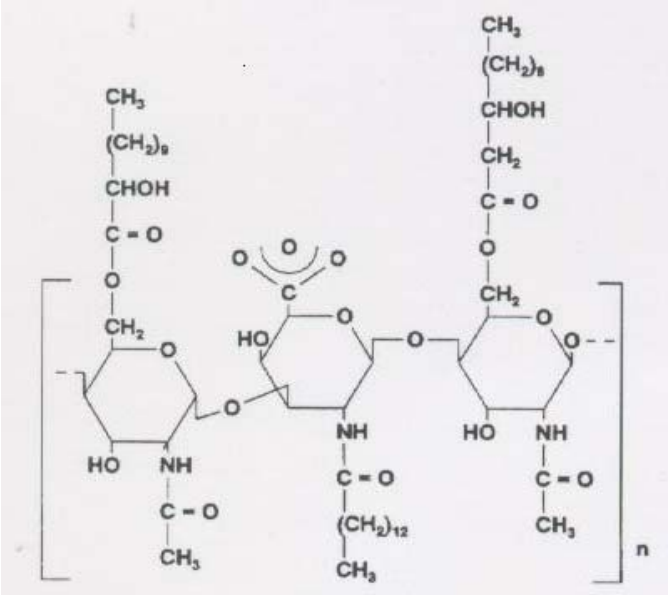


Figure11. Structure of emulsan, produced by *Acinetobacter calcoaceticus*, in which fatty acids are linked to a heteropolysaccharide backbone.

Source: Desai and Banat (1997)

Rosenberg and Ron (1999) suggested that biosurfactants can be divided by molecular weight into low-molecular-mass molecules, which efficiently lower surface and interfacial tensions, whereas the higher-molecular-mass molecules are more effective at stabilizing oil-in-water or water-in-oil emulsions.

The low-molecular-weight biosurfactants are generally glycolipids or lipopeptide. The best studied glycolipid bioemulsifiers, rhamnolipids, trehalolipids and sophorolipids are disaccharides that are acylated with long-chain fatty acids or hydroxyl fatty acids. Several lipopeptide antibiotics show potent surface-active properties. *Bacillus subtilis* produces a cyclic lipopeptide called surfactin, which has been reported to be the most active biosurfactant that has been discovered to date. The amphipathic nature of surfactin may contribute to some of its interesting biological properties, such as the formation of ion-conducting pores in membranes. *Pseudomonas* strains produce viscosin, a peptidolipid biosurfactant that lowers surface tension to 27 mN m^{-1} (Ron and Rosenberg, 2001).

A large number of bacterial species from different genera produce exocellular polymeric surfactants composed of polysaccharides, proteins, lipopolysaccharides, lipoproteins or

complex mixtures of these biopolymers. The protein component appears to play an important role in both the structure and the activity of the complex. The biopolymer, referred to as biodispersan, binds to powdered calcium carbonate and changes its surface properties in a way that allows for better dispersion in water. Yeasts produce a number of emulsifiers, which is particularly interesting because of the food-grade status of several yeasts. Liposan is an extracellular emulsifier produced by *C. antarctica* (Cirigliano and Carman, 1985). It is composed of 83% carbohydrate and 17% protein. Mannanprotein emulsifiers are produced by *Saccharomyces cerevisiae* (Ron and Rosenberg, 2001).

3. Estimation of biosurfactant activity

The fields of microbial surfactants are largely attributed to the development of quick, reliable methods for screening biosurfactant-producing microbes and assessing their potential. The measurement of surface tension has traditionally been used to detect biosurfactant production and most of the other methods that measure the surface properties of biosurfactant use surface tension reduction as the standard (Makkar and Cameotra, 1997).

Oil spread technique was carried out according to Morikowa *et al*. (2000). Fifty ml of distilled water was added to Petri dishes followed by addition of 100 µl of crude oil to the surface of the water. Then, 10 µl of the culture filtrates was put on the crude oil surface. The diameter of the clear zone on the oil surface was observed.

The surface tension is a property of the surface of a liquid that causes it to behave as an elastic sheet. Moreover, it's mean the maximum force needed to detach the ring from the liquid surface. Surface tension was measured with a ring-tensiometer (Kruss Digital-Tensiometer 10, Hamburg, Germany) at room temperature (McInerney *et al*., 1990). The ring hanging from the balance hook is immersed into the liquid. Then, the ring is slowly pulled up, by lowering the sample cup. Finally, the force applied on the ring while pulling through the surface is recorded. The surface tension of distilled water is 72.0 mN/m.

Emulsification is the process by which emulsions are prepared. An emulsion is a mixture of two immiscible (unblendable) substances. One substance (the dispersed phase) is dispersed in the other (the continuous phase). The emulsifying activity (%EA) was determined using a modification of the method described by Cooper and Goldenberg, (1987). To measure emulsification activity, 1 ml of xylene was added to 1 ml of supernatant in test tube. The mixture

was mixed by vortex mixer at high speed for 2 min and allowed to stand for 10 min and 24 h (emulsification index). The emulsion stability was determined after 10 min (%EA) and 24 h (the emulsification index (EI-24)) was calculated by dividing of measured height of the emulsion layer by the height of the oil or hydrocarbons phase and multiplying by 100.

Furthermore, the emulsifying activity was determined using a modified turbidometric method (Kim *et al*. 1997) and expressed as the turbidity at 620 nm. The surfactants (1 mg) were introduced into a 50 ml flask with distilled water to make 10 ml, then 0.1 ml of each hydrophobic liquid was added. The mixtures were incubated at 30°C with reciprocal shaking (160 strokes per min) for 1 h and allowed to stand for 10 min. The lower phase was taken and its turbidity measured at 620 nm. Soybean oil (Sigma Co.), *n*-hexadecane/2-methylnaphthalene (1:1, v/v), and crude oil (Arabian light, 1% sulfur content) were used as the hydrophobic liquids. A non-surfactant reaction was used as the control.

However, the measurement of surface tension is time-consuming, which makes it inconvenient to use for screening of a large number of isolates. The drop collapse technique depends on the principle that a drop of a liquid containing a biosurfactant will collapse and spread completely over the surface of oil (Bodour and Miller-Maier, 1998). This method is easy and can be used to screen large number of samples (Bodour *et al*., 2003), but it has not been correlated to surface tension reduction to confirm its reliability. The oil spreading technique measures the diameter of clear zones caused when a drop of a biosurfactant-containing solution is placed on an oil–water surface (Morikawa *et al*., 2000).

Youssef *et al*. (2004) reported three methods to detect biosurfactant production, drop collapse, oil spreading, and blood agar lysis and were compared for their ease of use and reliability in relation to the ability of the cultures to reduce surface tension. The oil spreading technique better predicted biosurfactant production than the drop collapse method. The use of the drop collapse method as a primary method to detect biosurfactant producers, followed by the determination of the biosurfactant concentration using the oil spreading technique, constitutes a quick and easy protocol to screen and quantify biosurfactant production. Hemolytic activity was carried out as described by Carrillo *et al*. (1996). Isolated strains were screened on blood agar plates containing 5% (v/v) blood and incubated at 45°C for 24–48 h. Hemolytic activity was detected as the presence of a clear zone around a colony. The large number of false negatives and positives obtained with the blood agar

lysis method and its poor correlation to surface tension demonstrated that it is not a reliable method to detect biosurfactant production.

The drop-collapse technique was carried out in the polystyrene lid of a 96-microwell plate as described by Bodour and Miller-Maier (1998). One-hundred µl culture supernatant was added to wells of a 96-well microliter plate lid, then 5 µl of crude oil was added to the surface of the culture supernatant. Biosurfactant-producing culture gave flat drops. Aliquots from a culture of each strain were analyzed on two separate microliter plates.

Blood agar lysis, drop-collapse method, oil spreading and surface tension (ST) measurements were used to detect biosurfactant production. Emulsification activity for culture broth was also tested using xylene, toluene, petroleum and diesel oils. Although surface tension reduction was a good measure of biosurfactant production, it did not correlate well with emulsion ability. Several bacteria isolates had good emulsifying abilities with all hydrocarbons tested. The simplicity of the above techniques allows effective screening of biosurfactant-producing microorganisms. Although hemolytic activity has been reported as an initial selection criterion for biosurfactant producers, other more conclusive tests such as surface tension measurements should be carried out for confirmation of the obtained results (Pyaza *et al*., 2006).

4. Screening of potential biosurfactant-producing microorganisms

Biosurfactant production is a desirable property of hydrocarbon-degrading microorganisms. Bento *et al*. (2005) characterized biosurfactant producing microbial populations from a Long Beach soil, California (USA) and a Hong Kong soil (China), contaminated with diesel oil. A total of 33 hydrocarbon-utilizing microorganisms were isolated from the soils. Twelve isolates and three defined consortia were tested for biosurfactant production and emulsification activity. The high reduction of surface tension was achieved with a consortium of L1, L2 and L3 isolates from a Long Beach soil (41.4 mN/m). Isolate L1 (*Acinetobacter junii*) displayed the highest reduction of surface tension (46.5 mN/m). The emulsifying capacity evaluated by the emulsification index (EI24) was highest in the culture of the isolate L5 (*Bacillus pumilus*) (74%). No substantial emulsification was achieved with the cell-free extracts, indicating that the emulsifying activity was not extracellular.

Twenty colonies of yeast-forming halos on agar plates covered with crude oil were isolated from soil samples. Isolated colonies were cultured in the isolation liquid medium containing *n*-hexadecane, soybean oil, or glucose as a carbon source, and then, by measuring the oil film-

collapsing activity of the culture broth by drop collapse technique, the three strains showing a large halo (more than 2 cm diameter) were selected. These samples were assessed for surface tension and emulsification activity. The yeast strain, *Candida* sp. SY16 which was capable of effectively emulsifying crude oil, vegetable oil, and hydrocarbons, and reduced the surface tension of the culture broth to 30 dyne/cm (Kim *et al*., 1999).

Among 200 yeast strains isolated from plants in Thailand, 50 strains produced relatively high lipase and 30 of them were biosurfactant producers. Eight strains exhibited thermotolerancy at 45°C. Strain Y12, *Candida ishiwadae* was selected as the highest biosurfactant producer (Thanomsub *et al*., 2004)

More than 200 strains were isolated from oil and wastewater samples. These yeast strains were cultured in fermentation media, 11 strains showing large halos (more than 3 cm diameter) were isolated by measuring the oil film-collapsing activity of the culture broth by drop collapse technique. A yeast strain Y2A producing a large amount of biosurfactants was isolated from oil-containing wastewater and identified as *Wickerhamiella domercqiae* (Jing *et al*., 2006).

5. Medium components affecting on growth and biosurfactant production by microorganisms

5.1 Carbon sources

The carbon source generally used in biosurfactant production could be divided into three categories, carbohydrates, hydrocarbons and vegetable oils. The highest production of sophorolipids produced by *Candida bombicola* was obtained when both vegetable oil (safflower oil) and sugar (glucose) were used as substrates (Zhou and Kosaric 1995).

C. antarctica SY 16 required a vegetable oil as the carbon source to produce a biosurfactant, mannosylerythritol lipid (MEL-SY16). Biosurfactant production was 31 g/l after 7 days in a batch culture and was not growth associated. In a two-stage culture, glycerol and oleic acid were used as an initial and a feeding carbon source, respectively and 41 g/l^{-1} biosurfactant was produced after 8 days (Kim *et al*., 2002b). In addition, in fed-batch fermentation, glucose and soybean oil (1:1, w/w) were used in combination as the initial carbon sources for cell growth, and soybean oil was used as the feeding carbon source during the MEL production phase (Kim *et al*., 2006).

Daniel *et al*. (1998) have shown production of sophorolipid from *C. bombicola* ATCC 22214 required whey and rapeseed oil as the carbon source to produce a biosurfactant. Biosurfactant production was 42 g/l.

C. antarctica T-34 produced mannosylerythritol lipid to 14 g/l in the culture containing *n*-octadecane as a carbon source (Kitamoto *et al*., 2001).

Davila *et al*. (1992) reported a high yield of sophorolipids by overcoming product inhibition in *C. bombicola* CBS 6009 through the addition of ethyl esters of rapeseed oil fatty acid in D-glucose medium.

T. apicola IMET 43747 produced glycolipid as high as 90 g/l with a medium containing D-glucose and sunflower oil (Stuwer *et al*., 1987).

In addition, Lee and Kim (1993) demonstrated that in batch culture, 37% of carbon input was channeled to produce 80 g/l of sophorolipid by *T. bombicola*. However, in fed-batch cultures, about 60% of the carbon input was incorporated into biosurfactant, increasing the yield to 120 g/l.

Candida ishiwadae produced relatively large amounts of biosurfactants when soyabean cooking oil was used as a carbon source (Thanomsub *et al*., 2004).

Jing *et al*. (2006) reported the sophorolipid production from *Wickerhamiella domercqiae* when rapeseed oil was used as a carbon source.

Summary recent studied on microbial production of biosurfactants when various carbon sources were used (Table 2).

Table 2. Production of biosurfactants by microorganisms when various carbon sources were used

Biosurfactant	Microorganism	Yield (g/l^{-1})	Carbon source	Reference
Mannosylerythritol lipid				
MEL-A, -B and –C	*Candida antarctica* T-34	14	*n*-Octadecane	Kitamoto *et al.* (2001)
MEL-SY16	*C. antarctica* KCTC 7804	47	Soybean oil	Kitamoto *et al.* (1992)
	C. antarctica KCTC 7804	41	Glycerol, oleic acid	Kim *et al.* (1999)
MEL-A, -AB and –B	*Ustilago maydis* DSM 4500	30	Sunflower oil	Spoeckner *et al.* (1999)
Sophorolipid				
SL mixture	*Candida bombicola* ATCC 22214	42	Whey, rapeseed oil	Daniel *et al.* (1998)
SL mixture	*C. bombicola* CBS6009	32	Glucose, rapeseed esters	Davila *et al.* (1992)
(SL-1: 73%)	*C. bombicola* ATCC 22214	16	Glucose, canola oil	Zhou and Kosaric (1995)
SL (lactone)	*C. apicola* IMET 43147	90	Glucose, sunflower oil	Hommel *et al.* (1994)
Rhamnolipid				
RL-1 and –2	*Pseudomonas aeruginosa* DSM 7107	11	Soybean oil	Lang and Wullbrandt (1999)

Table 2. (Continue)

Biosurfactant	Microorganism	Yield (g/l^{-1})	Carbon source	Reference
Trehalose lipid				
Trehalose-tetraester	*Rhodococcus erythropolis* DSM 43215	32	*n*-Decane	Lang and Philp (1998)
TL-1 and –2	*R. erythropolis* SD-74	40	*n*-Hexadecane	Uchida *et al.* (1989)
Oligosaccharide lipid				
GL-1, -2 and –3	*Tsukumurella* sp. DSM 44370	30	Sunflower oil	Vollbrecht *et al.* (1999)
Cellobiose lipid				
CL-A, -B and –C	*Ustilago maydis* ATCC 14826	16	Coconut oil	Fautz *et al.* (1986)

Source: modified from Kitamoto *et al*. (2002)

5.2 Nitrogen sources

Medium constituents other than carbon source also affect the production of biosurfactants. Among the inorganic salts tested, ammonium salts and urea were prefer as nitrogen sources for biosurfactant production by *Arthobacter paraffineus*, whereas nitrate supported maximum surfactant production in *Pseudomonas aeruginosa* (MacElwee *et al*., 1990).

Lichenysin-A production was enhanced two- and four-fold in *Bacillus licheniformis* BAS50 by addition of L-glutamic acid and L-asparagine, to the medium (Yakimov *et al*., 1996).

Makkar and Cameotra (1997) have studied the effect of nitrogen source on biosurfactant production of *Bacillus subtilis*. They found that in nitrogen-free medium, *B. subtilis* grew and produced small amount of biosurfactant whereas sodium nitrate, potassium nitrate and

urea were the best sources of nitrogen. Ammonium sulfate was not good for growth and biosurfactant production but *B. subtilis* was able to utilize ammonium nitrate for growth.

Robert *et al*. (1989) observed nitrate was the best source of nitrogen for biosurfactant production by *Pseudomonas* strain 44T1 growing on olive oil. The production started after 30 h of growth, when the culture reached nitrogen limitation, and continued to increase up to 58 h of fermentation. Similarly, nitrogen limitation caused biosurfactant production increase in *Candida tropicalis* IIP-4 (Singh *et al*., 1990) and *Nocardia* strain SFC-D (Kosaric *et al*., 1990).

Nitrogen exhaustion was thought to switch on biosurfactant production, and the results demonstrated that sophorolipids were secondary metabolites. The effect of yeast extract content on the production of sophorolipids in the cultivation of *C. bombicola* in a 1-L fermenter was studied. In a medium containing 10.5% glucose and 10.5% canola oil, best yields of the sophorolipids were obtained at 0.4% yeast extract (YE). Below 0.2% YE and above 0.6 % YE, sophorolipid biosynthesis diminished dramatically. These results indicated that nitrogen concentration strongly controls sophorolipid biosynthesis. Limitation of nitrogen leads to increased activity of the enzymes involved in sophorolipid biosynthesis, a maximum of about 160 g/l sophorolipids was obtained at 0.4% YE (Zhou and Kosaric 1995).

Candida sp. SY16 produced larger amounts of MEL from NH^+_4 salts than $NaNO_3$ or $NaNO_2$, and the highest yield of MEL was with NH_4NO_3, rather than $(NH_4)_2SO_4$ or NH_4Cl, which may have been attributed to the smaller decrease in the pH during cultivation; the final pH of the NH_4NO_3-culture was 4.5, whereas the other ammonium salts dropped the culture pH to below 2.5 after 7 days. The maximum MEL-SY16 production was attained at 1 g NH_4NO_3 per liters (Kim *et al*., 2002b).

When cultivated in vegetable oils and their derivatives, the smut fungus *Ustilago maydis* DSM 4500 and ATCC 14826 produces several glycolipids under nitrogen-limiting conditions a yield of 30 g/l glycolipid was achieved (Spoeckner *et al*., 1999).

5.3 Salt and minerals

Salt concentration also affected biosurfactant production depending on its effect on cellular activity. Some biosurfactants produced by *Rhodococcus* bacterium however, were not affected by salt concentrations up to 10% (w/v), although slight reductions in the CMCs were

detected (Abu-Ruwaida *et al*., 1991). Sutthivanitchakul *et al*. (1999) showed that addition of 5% NaCl in the culture broth improved the surface activity of the biosurfactant from *B. licheniformis* F 2.2 however, the activity was reduced when NaCl concentration higher than 10%.

5.4 Environmental factors

Growth conditions and environmental factors such as pH, temperature, agitation and oxygen availability also affect biosurfactant production through their effect on cellular growth or activity.

5.4.1 pH

The pH of medium plays an important role in sophorolipid production by *T. bombicola* (Gobbert *et al*., 1984). Robert *et al*. (1989) reported the production of rhamnolipid when cultivated *Pseudomonas* spp. in batch cultivation. Maximum yield of rhamnolipid showed at a pH range from 6 to 6.5 and decreased sharply above pH 7.

The pH range 7.0-9.0 was appropriate for growth and biosurfactant production by *Bacillus licheniformis* F2.2. This bacterium was unable to reduce the surface tension of culture medium when pH was 4.0 and 4.5 (Sutthivanitchakul *et al*., 1999).

The highest sophorose lipid production by *Candida bombicola* was obtained when the whole fermentation was carried out at pH 3.5 (Davila *et al*., 1997).

The effect of the initial pH on MEL-SY16 production was investigated. The highest production was at an initial pH of 7.9, which resulted in a final pH of 4.5 after 7 days. The MEL-SY16 production was significantly inhibited below pH 3 based on observing the final pH. From these results, it would appear that the MEL production was sensitive to the culture pH. Nitrogen and phosphate concentrations were also optimized to prevent a decrease of the pH to below 3 during the culture (Kim *et al*., 2002b).

The surface properties of the sophorolipids (SLs) were produced from *Candida bombicola* obtained from the soy molasses/oleic acid fermentation had minimum surface-tension values of 37 mN/m (pH 6) and 38 mN/m (pH 9) (Solaiman *et al*., 2004).

The effect of pH control on the production of MEL-SY16 produced by *Candida* sp. strain SY16 was also examined in batch fermentation. The highest production yield of MELSY16 was obtained when pH was controlled at 4.0, and the production was significantly improved compared to batch fermentation without pH control (Kim *et al*., 2006).

5.4.2 Temperature

The effects of temperature on MEL production by resting cells and by growing cells, *Candida antarctica* strain T-34 were studied. In both cultures, the highest yield was observed at 25°C and the difference in MEL yield was prominent at 20°C. The resting cells were able to produce MEL over a wide range of temperature (Kitamoto *et al*., 1992).

Thanomsub *et al*. (2004) described glycolipid biosurfactants produced by a thermotolernt yeast, *Candida ishiwadae* survived at 45°C and produced relatively large amount of biosurfactants.

The highest sophorose lipid production by *Candida bombicola* was obtained when the whole fermentation was carried out at incubation temperature 25°C (Davila *et al*., 1997).

5.4.3 Agitation and aeration

The effect of aeration on MEL production was examined. Different volumes (20-60 ml) of the medium were put into 300 ml Erlenmeyer flasks, because the rate of aeration is a function of the volume of the medium. The highest yield was observed at a volume of 30 ml (Kitamoto *et al*., 1992).

6. Recovery of biosurfactants

Biosurfactants are unlikely to be produced at low cost if extensive refining is required. Process development must be therefore focused around biosurfactants which can be recovered by simple and inexpensive techniques such as gravity separation. The most common biosurfactant recovery methods are either extraction with solvents (e.g. chloroform-methanol, dichloromethane-methanol, butanol, ethyl acetate) or acid precipitation. However, there are reports of the use of ammonium sulfate precipitation, crystallization centrifugation, adsorption, foam fractionation etc. (Makkar and Cameotra, 2002).

Glycolipid produced by *T. bombicola* was extracted by chilled ethyl acetate after adsorption on charcoal (Cooper and Paddock, 1984).

Both the glycolipid produced by *Ustilago zeae* and the mannosylerythritol lipid produced by *Candida* spp. are sedimented by centrifugation and then extracted in either ethanol or methanol (Kim *et al*., 1999).

Bryant (1990) improved the method for isolating the biosurfactant glycolipids by using diafiltration and isopropanol precipitation. This method removed protein and isolated the glycolipids by using organic extraction and salicylic acid chromatography.

Nocardia sp. L-417 strain grown in *n*-hexadecane as a carbon source produced two types of biosurfactant that had different characteristics. These biosurfactants were purified by procedures including ammonium sulphate fractionation, chilled acetone and hexane treatments, silica-gel column chromatography and Sephadex LH-20 gel filtration consecutively. The biosurfactant type I had strong properties as an emulsifying agent and as an emulsion-stabilizing agent, whereas type II showed a strong ability reduce surface tension (Kim *et al*., 2000).

Mannoprotein in the cell wall of *Saccharomyces cerevisiae* was extracted by autoclaving in citrate buffer (pH 7), and purified. The yield of emulsifier extracted from commercial strain of *S. cerevisiae* was 80–100 g/kg. The mannoprotein consisted of approximately 380–410 g/kg protein and 210 g/kg carbohydrate (Torabizadeh *et al*., 1996). Various processes employed for recovery of biosurfactants are shown in Table 3.

Table 3. Downstream process for recovery of biosurfactants

Process	Biosurfactant recovered
Ammonium sulfate precipitation	Emulsan
	Biodispersion
	Bioemulsifier
Acetone precipitation	Bioemulsifier
	Glycolipid
Solvent extraction	Trehaloselipids
	Sophorolipid
	Liposan
Acid precipitation	Surfactin
Crystallization	Cellobiolipids
	Glycolipids
Centrifugation	Glycolipids
Adsorption	Rhamnolipids
	Lipopeptide
	Glycolipids
Foam fractionation	Surfactin
Tangential flow filtration	Mixed biosurfactant
Diafiltration and precipitation	Glycolipids

Source: modified from Desai and Banat (1997)

7. Purification of biosurfactants

Another technique frequently employed for the characterization and quantification of biosurfactants has been thin-layer chromatography (TLC). Although TLC analysis can provide qualitative and quantitative information about biosurfactants, time consuming pre-purification procedures, such as precipitation and organic extraction, are generally required. Compared to tension measurement and TLC analysis, high-performance liquid chromatography (HPLC) represents an effective alternative for biosurfactant analysis with the desired sensitivity and selectivity. Using HPLC for quantitative analysis and/or for the purification of some lipopeptides have been reported. However, the development of these HPLC methods generally required relatively pure biosurfactant samples, which cannot be obtained without tedious isolation and purification operations including HPLC. It is, therefore, necessary to explore a general approach for the development of efficient HPLC methods for biosurfactant analysis and purification (Yakimov *et al*., 1995). From the culture filtrate of a thermotolerant yeast, *C. ishiwadae*, two glycolipid biosurfactants, a and b, were purified by solvent fractionation, silica gel and ODS column chromatographies (Thanomsub *et al*., 2004).

The development of HPLC analysis for surfactin, a lipopeptide biosurfactant produced by *Bacillus subtilis*, was reported to demonstrate the feasibility of this approach. Nevertheless, the proposed approach can be used for the development of HPLC analysis for practically any microbial surfactants. The techniques used are also useful for the preparation of homogeneous biosurfactant samples required for determining the CMC and for performing chemical analysis, such as Fourier infrared (FT-IR) analysis and nuclear magnetic resonance (NMR) spectroscopy, for the elucidation of chemical structures of biosurfactants (Lin *et al*., 1998).

8. Characterization of biosurfactants

Kim *et al*. (2002a) described the molecular weight of MEL-SY16 from *Candida* sp. SY16 was 634 Da and its hydrophobic lipophilic balance (HLB) was 8.8. It decreased the surface tension to 30 dyne cm^{-1} at a critical micelle concentration of 15.8 μM, and the minimum interfacial tension against kerosene was 0.1 dyne cm^{-1}. MEL-SY16 was stable from pH 4 to pH 10, up to 90°C for 1 h, and againt NaCl up to 100 mM.

The molecular weight of the bioemulsifier mannoprotein from *Saccharomyces cerevisiae* was estimated to be 14,000–15,800 by SDS-PAGE method. Physical and chemical stability

analysis showed that emulsions of 60% oil in water, 8 g/L bioemulsifier and 5–50 g/L sodium chloride were stable for 3 months at 4 °C at pH 3–11 (Torabizadeh *et al*., 1996).

Nocardia sp. L-417 produced biosurfactants when *n*-hexadecane was used as a carbon source. The biosurfactants were very stable over a board range of pH 2-12 and temperature at 100° C for 3 h (Kim *et al*., 2000).

Monoacrylglycerols; l-linoleylglycerol and l-oleyglycerol prouced by *Candida ishiwadae* exhibited higher surfactant activities tested by the drop collapse test than several artificial surfactants such as sodium dodecyl sulphate (Thanomsub *et al*., 2004).

9. Applications of biosurfactants

Surfactants and emulsifiers are indispensable components of daily life. They are widely used in the pharmaceutical, cosmetic, petroleum, and food industries. Many different types of surfactants are already being used in industry, but it is important to develop even more new compounds to broaden the spectrum of specific properties and applications (Cameotra and Makkar, 1998).

Worldwide production of surfactants amounted to 17-19 million tons in 2000 (including soap for less than 50%). The expected further growth rates is 3-4% per year globally and 1.5-2.0% in the European Union. The growth rate is closely related to the world demand in detergents, since this sector uses over 50% of surfactant production (Deleu and Paquot, 2004). Biosurfactants have been tested in environmental applications such as bioremediation and dispersion of oil spills, enhanced oil recovery and transfer of crude oil, are thought to be potential candidates to replace chemical surfactants in the future, especially in the food, cosmetic, and health care industries, industrial cleaning of products and in agricultural chemicals (Banat *et al*., 2000).

9.1 Food industry

Biosurfactants also have several applications in the food industry as food additives. Lecithin and its derivatives, fatty acid esters containing glycerol, sorbitol or ethylene glycol and ethyloxylated derivatives of monoglycerides including a recently synthesized oligopeptide are in use as emulsifiers in the food industry worldwide. Other applications of biosurfactants are in bakery and meat products where they influence the rheological characteristics of flour or the emulsification of partially broken fat tissue (Fiechter, 1992).

In dairy products (soft cheese and ice creams) the addition of emulsifiers improves the texture and creaminess. This quality is of special value for low-fat products (Rosenberg and Ron, 1999).

Evaluation of emulsifying ability of biosurfactants is in general related to hydrocarbons such as kerosene because of their potential in environmental applications. Few attempts have been made to evaluate emulsion forming by biosurfactants with oils and fats used in food industry. A lipopeptide obtained from *B. subtilis* was able to form stable emulsions with soybean oil and coconut fat, suggesting its has potential use as emulsifying agent in foods (Nitschke and Pastore, 2006).

A mannoprotein from *Kluyveromyces marxianus* was able to form emulsions with corn oil and were stable for 3 months; suggesting potential application as food bioemulsifier (Lukondeh *et al*., 2003).

Shepherd *et al*. (1995) tested a range of microorganisms for production of extracellular, high molecular weight emulsifiers for potential use in foods. The good producer organisms included the yeasts *Candida utilis, Candida ualida* and *Hansenula anomal.* Of these, *C. utilis* was selected for further study due to the excellent emulsification properties of its extracellular products and the food-grade status of the organism. Crude preparations of the bioemulsifier from *C. utilis* exhibited low viscosity and had a carbohydrate content of over 80%. Preliminary trials showed that the bioemulsifier from this organism had potential for use in salad cream.

9.2 Oil industry

Biosurfactants are used for enhance oil recovery. Due to the potential use in the oil industry with minimum purity specification so that whole-cell broth could be used. Compared with chemical surfactants, they are very selective and required in small quantities and are effective under broad ranges of oil and reservoir conditions.

B. licheniformis JF-2 was isolated from oil field injection water. In addition to producing most effective biosurfactants (CMC, 10 mg/l; interfacial tension of saline against decane lowered to 10^{-3} dynes/cm), *B. licheniformis* JF-2 had other properties such as being anaerobic, halotolerant and thermotolerant (Desai and Banat, 1997).

Biosurfactants have been tested in enhanced oil recovery and the transportation of crude oils. They were demonstrated to be effective in the reduction of the interfacial tension of oil

and water in situ, the viscosity of the oil, the removal of water from the emulsions prior to processing, and in the release of bitumen from tar sands. The high molecular weight Emulsan® has been commercialized for this purpose. It contains a polysaccharide with fatty acids and proteins attached. Other high molecular weight biosurfactants are reviewed by Ron and Rosenberg (2002).

9.3 Cosmetic industry

A broad potential application area is the cosmetic industry where surface-active substances are found in shampoo and many skin-care products (Fiechter, 1992). Biosurfactants have found a niche in the personal care market because of their moisturizing properties and skin compatability (Desai and Banat, 1997). Many biosurfactant properties such as emulsification and de-emulsification, foaming, water binding capacity, spreading and wetting properties effect on viscosity and on product consistency, can efficiently be utilized by the cosmetic industries. Emulsifiers are important additives to many cosmetic preparations such as lipsticks, eye shades, mascaras, soap, tooth pastes and polishes, health and beauty products and moisturizers (Kosaric, 1992).

9.4 Biomedical sciences

One useful property of many biosurfactants that has not been reviewed extensively is their antimicrobial activity. Several biosurfactants have strong antibacterial, antifungal and antiviral activity. Other medically relevant uses of biosurfactants include their role as anti-adhesive agents to pathogens, making them useful for treating many diseases and as therapeutic and probiotic agents. (Singh and Cameotra, 2004).

Kulakovskaya *et al*. (2004) purified glycolipid from the culture liquid of the ustilaginomycetous yeast *Sympodiomycopsis paphiopedili*. The minimal effective concentrations leading to ATP leakage and growth inhibition were 45 and 160 μg/ ml for *Cryptococcus terreus* and *Candida albicans*, respectively.

Rhamnolipid A produced by *Pseudomonas aeruginosa* B189 isolated from a milk factory waste showed significant antiproliferative activity against human breast cancer cell line (MCF-7) at minimum inhibitory concentration (MIC) at 6.25 μg/mL while rhamnolipid B showed MIC against insect cell line C6/36 at 50 μg/mL (Thanomsub *et al*., 2007).

Jing *et al*. (2006) reported the sophorolipid produced by *Wickerhamiella*

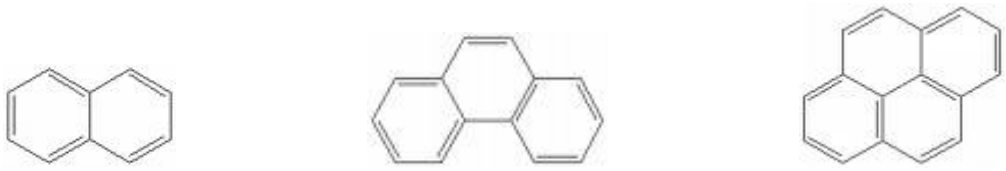
domercqiae have anticancer activity. The cytotoxic effects of the sophorolipid on cancer cells of H7402, A549, HL60 and K562 were investigated by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay. The results showed a dose-dependent inhibition ratio on cell viability according to the drug concentrations $\leq$ 62.5 μg/ml.

9.5 Bioremediation

Biosurfactants have been shown to promote biodegradation of hydrocarbons. Original data for biosurfactant assisted biodegradation of a selected herbicide methochlor, chlorinated aromatics and naphthalene. Furthermore, pilot plant and large scale bioremediations of soil contaminated with polycyclic aromatic hydrocarbons (PAHs) and heavy oil were performed. In the presence of selected biosurfactants, a preferential and significant (or complete) removal of PAHs was observed after only 22 days of bioremediation (Kosaric, 2001). Chemical characteristics and structure of PAHs are shown in Table 4 and Figure 12, respectively.

Table 4. Chemical characteristics of polycyclic aromatic hydrocarbons (PAHs)

PAHs (mg/L)	Chemical formula	Molecular weight	Water solubility (mg/L) at 30 °C
Naphthalene	$C_{10}H_8$	128.00	31.690
Phenanthrene	$C_{14}H_{10}$	178.20	1-1.6
Pyrene	$C_{16}H_{10}$	202.25	0.135



Naphthalene Phenanthrene Pyrene

Figure 12. Structure of polycyclic aromatic hydrocarbons (PAHs).

Another application of biosurfactants is oil storage tank cleaning. In a pilot field investigation, an evaluation of the ability of aqueous biosurfactant solutions (aescin, lecithin, rhamnolipid, saponin and tannin) for possible applications in washing crude oil contaminated soil was carried out. Results showed that the biosurfactants were able to remove significant amount of crude oil from the contaminated soil at different solution concentrations for instance rhamnolipid and SDS removed up to 80% oil and lecithin about 42%. The performance of water alone in crude oil removal was equally as good as those of the other biosurfactants. Oil removal was due to mobilization, caused by the reduction of surface and interfacial tensions. Solubilization and emulsification effects in oil removal were negligible due to the low crude oil solubilization of 0.11% (Urum and Pekdemir, 2004).

Tan *et al*. (1994) investigated the potential of rhamnolipid biosurfactants produced by *P. aeruginosa* ATCC 9027 in the removal of metals from soils contaminated with cadmium and reported 92% complexation of Cd^{2+} in a 0.5 mM solution of $Cd(NO_3)_2$ using a 5 mM solution of rhamnolipid (22 $\mu g\ mg^{-1}$ rhamnolipid).

The biosurfactant BS-UC was produced by *Candida antarctica* from *n*-undecane as the substrate. It was found that the addition of BS-UC influenced positively the emulsification and the biodegradation of a variety of *n*-alkanes substrates. Hence it will make BS-UC a promising choice for bioremediation of petroleum contamination (Hua *et al*., 2003).

Under optimum conditions, the rhamnolipid produced by *P. aeruginosa* removed as much as 70% of the phenanthrene and 60% of the diesel in the sand. These results indicated that using of biosurfactants in the flushing process is favorable, not only with respect to the environment, but also on removal efficiencies (Shin and Kim 2004).

Phenanthrene solubilization and biodegradation with a rhamnolipid solution were investigated as a function of pH. Batch phenanthrene solubilization experiments were performed in the pH range 4–8 and the highest solubilities with the biosurfactant were detected around a pH of 4.5–5.5. The apparent solubility at pH 5.5 was 3.8 times greater than at pH 7 in the presence of 240 ppm rhamnolipid (Shin *et al*., 2004).

Alasan, a high-molecular-weight bioemulsifier complex of an anionic polysaccharide and proteins that is produced by *Acinetobacter radioresistens* KA53, enhanced the aqueous solubility and biodegradation rates of polyaromatic hydrocarbons (Barkay *et al*., 1999).

Batch washing experiments were used to evaluate the feasibility of using biosurfactants for the removal of heavy metals from sediments. Surfactin from *Bacillus subtilis*, rhamnolipids from *Pseudomonas aeruginosa* and sophorolipid from *Torulopsis bombicola* were evaluated using metal-contaminated sediment (110 mg/kg copper and 3300 mg/kg zinc). A single washing with 0.5% rhamnolipid removed 65% of the copper and 18% of the zinc, whereas 4% sophorolipid removed 25% of the copper and 60% of the zinc. Surfactin was less effective, removing 15% of the copper and 6% of the zinc (Mulligan *et al*., 2001).

Rahman *et al*. (2003) examined the bioremediation of *n*-alkanes in petroleum sludge. The sludge contained an oil and grease content of 87.4%. C_8–C_{11} alkanes in 10% sludge were degraded 100%, while C_{12}–C_{21}, 83–98%, C_{22}–C_{31}, 80–85% and C_{32}–C_{40}, 57–73% after 56 days with addition of a bacterial consortium, nutrients, and rhamnolipids. Lower rates of biodegradation occurred as the chain length increased. However, the rates were still significant even for C_{32}–C_{40} compounds, indicating the benefit of rhamnolipid addition for assisting the biodegradation of these low solubility compounds.

The products of hydrocarbon degradation, introduced to the central tricarboxylic acid cycle, have a dual function. They are substrates of the energy metabolism and building blocks for biosynthesis of cell biomass and growth (Fig. 13). Two mechanisms are involved in the uptake of these lipophilic substrates: the attachment of microbial cells at oil droplets and the production of biosurfactants. The uptake mechanism linked to attachment of the cells is still unknown, whereas the effect of biosurfactants has been studied well (Fig. 14). Biosurfactants are molecules consisting of a hydrophilic and a lipophilic moiety. They act as emulsifying agents, by decreasing the surface tension and by forming micelles. The microdroplets may be encapsulated in the hydrophobic microbial cell surface. *n*-Alkanes are the main constituents of mineral oil contaminations. Long-chain *n*-alkanes (C_{10}-C_{24}) are degraded most rapidly by mechanisms demonstrated in Figure 15. Short-chain alkanes (less than C_9) are toxic to many microorganisms, but they evaporate rapidly from petroleum contaminated sites. Oxidation of alkanes is classified as being terminal or diterminal. The monoterminal oxidation is the main pathway. It proceeds via the formation of the corresponding alcohol, aldehyde, and fatty acid. β-Oxidation of the fatty acids results in the formation of acetyl-CoA (Fritsche and Hofrichter, 2000).

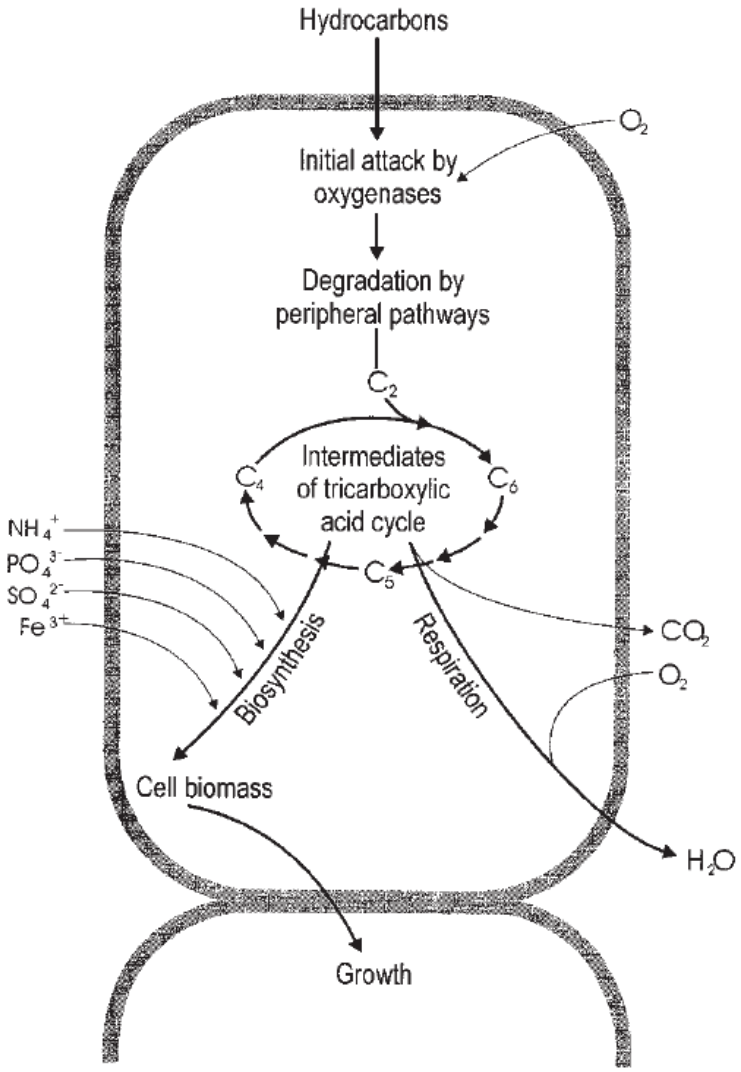


Figure 13. Main principle of aerobic degradation of hydrocarbons: growth associated processes.

Source : Fritsche and Hofrichter (2000)

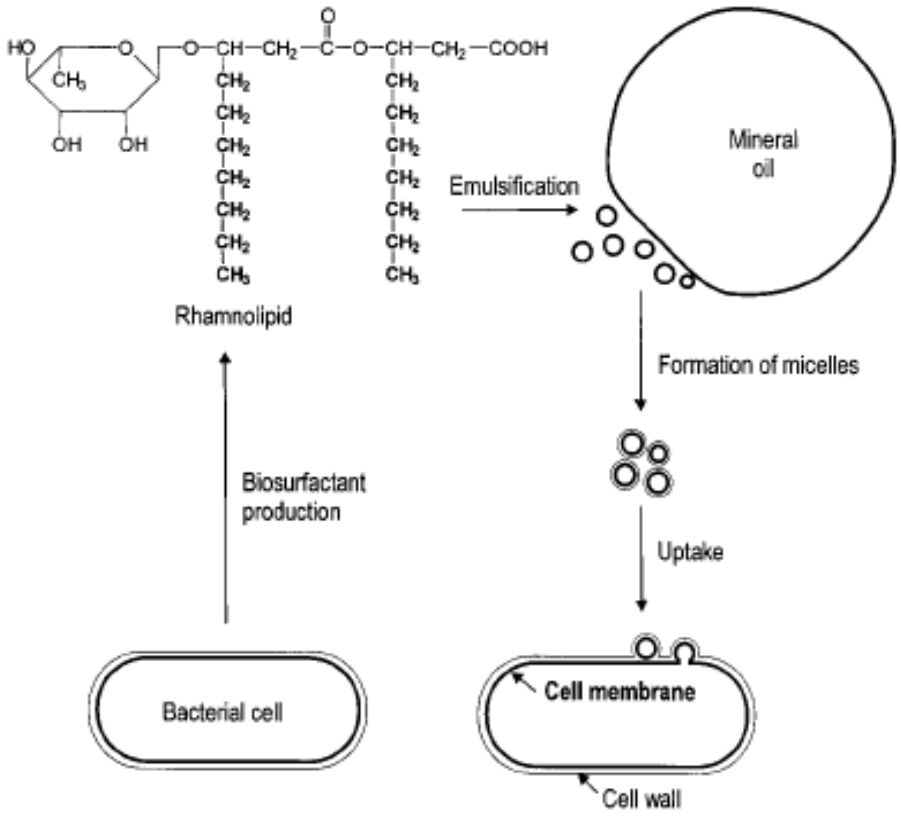


Figure 14. Involvement of biosurfactants in the uptake of hydrocarbons.

The figure demonstrated the emulsifying effect of a rhamnolipid produced by *Pseudomonas* spp. within the oil-water interphase and the formation of micelles. Lipid phases were printed in bold.

Source : Fritsche and Hofrichter (2000)

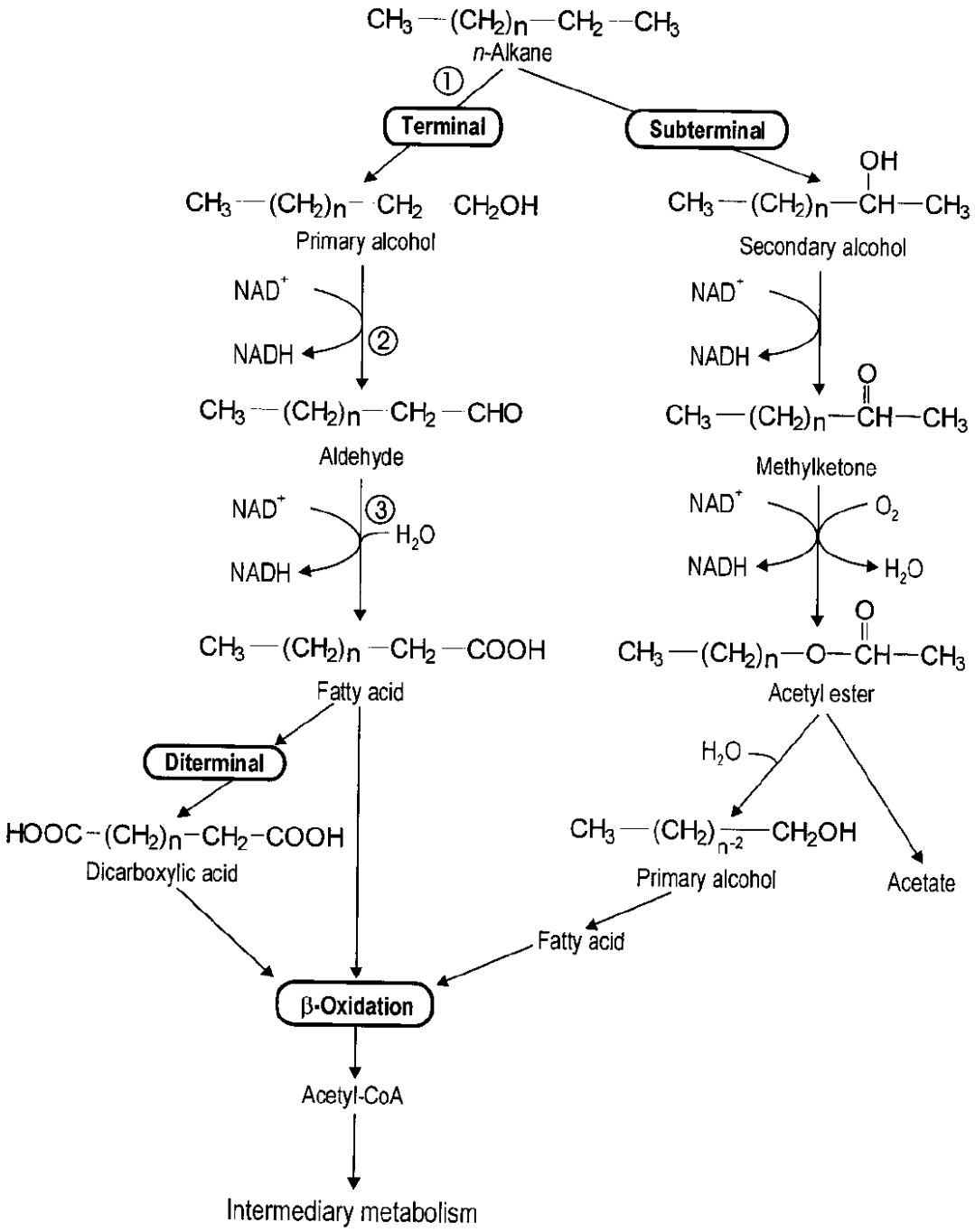


Figure 15. Peripheral pathways of alkane degradation.

The main pathway is the terminal oxidation to fatty acids catalyzed by ① *n*-alkane monoxygenase, ② alcohol dehydrogenase and ③ aldehyde dehydrogenase.

Source : Fritsche and Hofrichter (2000)

The applications of biosurfactants are very wide in a variety of industries as shown in Table 5.

Table 5. Applications of biosurfactants

Use	Effect of surfactant
Food and beverages	
Bakery and ice cream	Solubilize flavor oils, control consistency, retard staling
Cooking fat and oils	Prevent spattering due to super heat and water
Fruits and vegetables	Improve removal of pesticides, and in wax coating
Petroleum production/products	
Drilling fluids	Emulsify oil, disperse solids, modify rheological properties of drilling fluids for oil and gas wells
Textiles	
Dyeing and printing	Wetting, penetration, solubilization, emulsification dye leveling, detergency and dispersion
Elastomers and plastics	
Foamed polymers	Introduction of air, control of cell size
Latex adhesive	Promote wetting, improve bond strength
Industrial cleaning	
Soft goods	Detergents for laundry and dry cleaning
Leather	
Skins	Detergent and emulsifier in degreasing
Tanning and dyeing	Promote wetting and penetration
Waxes and polishes	Emulsify waxes, stabilize emulsions, antistat

Table 5. (Continue)

Use	Effect of surfactant
Agriculture	
Phosphate fertilizers	Prevent caking during storage
Spray application	Wetting, dispersing, suspending of powdered pesticides and emulsification of pesticide solutions
Paper	
Pulp treatment	Deresinification, washing
Paper machine	Defoaming, color leveling and dispersing
Calendar	Wetting and leveling, coating and coloring
Metals	
Concentration of ores	Wetting and foaming, collectors and frothers
Cutting and forming	Wetting, emulsification, lubrication and corrosion inhibition in rolling oils, cutting oils, lubricants, etc.
Casting	
Rust and scale removal	Mold release additives
Plating	In pickling and electrolytic cleaning
	Wetting and foaming in electrolytic plating

Source : modified from Kosaric (2001)

Objectives

1. To isolate and screen the biosurfactant producing-yeasts
2. To optimize conditions for growth and biosurfactant production by the selected yeast
3. To purify the biosurfactant produced by the selected yeast
4. To elucidate the chemical structure of the biosurfactant produced by the selected yeast
5. To apply the biosurfactant for bioremediation of hydrocarbons

Scope of Study

Biosurfactant producing-yeasts will be isolated from oil contaminated soils in the Southern Thailand. Then, the production of biosurfactant by the selected yeast will be optimized. The biosurfactant obtained will be purified, elucidated for chemical structures and applied to hydrocarbons bioremediation.

CHAPTER 2

MATERIALS AND METHODS

Materials

1. Sample of palm oil and hydrocarbon contaminated soils

1.) Pure Palm Oil Co., Ltd., Hat Yai, Songkhla : PO

PO1: soil near storage oil tank site

PO2: soil from walk street

PO3: Pond 1

PO4: Pond 2

PO5: Pond 3

2.) Garage 1 (Saun Praw restaurant, Hat Yai, Songkhla): GR1

3.) Garage 2 (Noodle restaurant, Hat Yai, Songkhla): GR2

4.) Garage 3 (Thung Lung, Hat Yai, Songkhla): GR3

5.) Garage 4 (near 109 PSU, Hat Yai, Songkhla): GR4

6.) Thai Tallow and Oil Co., Ltd., Surathani: SR

SR1: Pond 1

SR2: Pond 2

SR3: Pond 3

SR4: Pond 4

SR5: Pond 5

7.) Biodiesel Plant, Department of Chemical Engineering, PSU, Hat Yai, Songkhla: BP

8.) Asian Palm Oil Co., Ltd., Krabi: KB

KB1: Pond 1

KB2: Pond 2

KB3: Pond 3

2. Chemicals

Weathered crude oil (WCO) is by product obtained after Oman crude oil distillation at the temperature of 350°C. It was obtained from the Department of Chemical Engineering, Faculty of Engineering, Prince of Songkla University. All chemicals and solvents used were reagent grade and purchased from various suppliers as follows:

Chemical	Supplier
Acetonitrile (HPLC grade)	Nacalai Tesque, Kyoto, Japan
Ammonium nitrate	Merck, Germany
Ammonium sulphate	Merck, Germany
Anhydrous methanolic 5% HCl	Tokyo Kasei Kogyo Co., Tokyo, Japan
p-Anisaldehyde	Nacalai Tesque, Kyoto, Japan
Bromocresol green	Nacalai Tesque, Kyoto, Japan
Chloramphenicol	Sigma-Aldrich, Germany
Chroloform	Nacalai Tesque, Kyoto, Japan
Dichloromethane	Lab Scan, Lab Scan Asia Co., Ltd., Thailand
Ethyl acetate (HPLC grade)	Nacalai Tesque, Kyoto, Japan
n-Hexadecane	Fluka, Switzerland
Hexane (HPLC grade)	Nacalai Tesque, Kyoto, Japan
Oleic acid (GC grade)	Wako Pure Chemical Industries, Osaka, Japan
Magnesium sulphate	Merck, Germany
Methyl oleate (GC grade)	Nacalai Tesque, Kyoto, Japan
Methanol	Lab Scan, Lab Scan Asia Co., Ltd., Thailand
Methanol (HPLC grade)	Nacalai Tesque, Kyoto, Japan
Naphthalene	Fluka, Switzerland
Phenanthrene	Fluka, Switzerland
Potassium dihydrogen phosphate	Merck, Germany
Pyrene	Fluka, Switzerland
Sodium dodecyl sulphate (SDS)	Bio-Rad, US
Toluene	Lab Scan, Lab Scan Asia Co., Ltd., Thailand

Chemical	Supplier
Trifluoroacetic acid	Nacalai Tesque, Kyoto, Japan
Triton X-100	Nacalai Tesque, Kyoto, Japan
Triton X-114	Nacalai Tesque, Kyoto, Japan
Tween 20	Labchem, Australia
Tween 80	Labchem, Australia
Urea	Merck, Germany
Wako gel C-100, 200 (silica gel)	Wako Pure Chem. Ind., Ltd., Osaka, Japan
Xylene (mixed isomer)	Lab Scan, Lab Scan Asia Co., Ltd., Thailand

Instruments

Equipments	Series	Supplier
Autoclave	SS-325	Tomy Seiko Co., Ltd
Centrifugation	5403	Eppendorf
Chromarods	SM- III	Iatron Laboratories, Japan
Chromarods holder	SD-5	Iatron Laboratories, Japan
Developing tank	TD-150	Iatron Laboratories, Japan
Rotary evaporator	SB-XL 651	Tokyo Rikakikai, Japan
Gas chromatography	HP5890 Series II	JEOL, Tokyo, Japan
GC column	DB-1 (30 m x 0.25 mm, 0.25 μm film thickness)	J&W Scientific, Folsom, CA, USA
Haemacytometer	BOE14	Boeco, Germany
HPLC	HP	Waters Associates, Milford, MA, USA
HPLC column	Inertsil ODS-3 (4.6. x 250 mm)	GL Sciences Inc., Tokyo, Japan
Hot air oven	UM 200	Memmert, Germany
Incubator	MIR-153	Sanyo, Japan

Equipments	Series	Supplier
Laminar air flow	Hotpack	Scientific promotion Co., Ltd., Thailand
Light Microscopy	YS100	Nikon Corporation, Japan
pH meter	420A	Orion Research Inc., USA
Spectrophotometer	GENESYS^{TM}10	Thermo Electron Corporation, USA
TLC-FID (Iatroscan)	MK 5	Iatron Laboratories, Japan
Tensiometer	OS	Torsion balance supplier
Incubator shaker	M.3525	LAB-Line Instruments, Inc
Silica gel 60 plate	F_{254}	Merck, Germany
Vortex mixer	MS 1	IKA-Works, Inc., USA

3. Media

Samples were grown on YM agar (peptone, 5g; yeast extract, 3g; malt extract, 3g; glucose, 10g; agar 20g per liter of distilled water, pH 5.0) supplemented with 12.5 μg/ml chloramphenicol. The basal salt medium (BSM) for production of the biosurfactant contains 3g NH_4NO_3, 0.2g KH_2PO_4, 0.2g $MgSO_4 \cdot 7H_2O$, 0.5g yeast extract, supplemented with 1g WCO per liter of distilled water, pH 5.0 (modified from Thanomsub *et al*., 2004).

4. Statistical analysis

The data were calculated with mean values, and standard deviations (mean±SD) were determined from triplicate determinations. Statistical significance of the results were evaluated by one way ANOVA (analytical of variance) and Duncan's multiple range tests ($P < 0.05$) using SPSS 10 software.

5. Analytical methods

5.1 TLC analysis

Thin-layer chromatography of the sample was performed on silica gel 60 F_{254}-coated aluminium sheets (Merck, Darmstadt, Germany) with the solvent system hexane/ethyl

acetate (7: 3, v/v). Chromatograms were viewed in visible and UV light (254 nm and 365 nm) prior to sprayed with either anisaldehyde-sulfuric acid reagent (mixture of 0.5 ml of *p*-anisaldehyde, 0.5 ml of concentrated sulfuric acid, and 9 ml of ethanol) or bromocresol green reagent (0.04 % bromocresol green in methanol adjusted to a blue color (pH 7.5) with 0.1 M NaOH).

5.2 GC-MS analysis for structure elucidation

The purified compound was dissolved in anhydrous methanolic 5% HCl and heated at 100°C for 2 h. The reaction mixture was neutralized with 0.5 M $NaHCO_3$ and extracted with $CHCl_3$. The $CHCl_3$ layer was analysed with a GC-MS system (Automass 20, JEOL, Tokyo, Japan). GC-MS analysis was carried out under the following conditions: GC, Hewlett Packard HP5890 Series II; column, DB-1 (30 m x 0.25 mm, 0.25 μm film thickness, J&W Scientific, Folsom, CA, USA); carrier gas, He; injection temperature, 250°C; column temperature program, 150°C (1 min), 150-280°C at 10°C /min, 280°C (20 min); MS, electron ionization positive mode; ionization voltage, 70 eV; ion source temperature, 250°C. The compounds corresponding to the peaks in total ion chromatogram were tentatively identified by library search of the JEOL data system (Lucy version 5.0 software, JEOL).

5.3 HPLC analysis

HPLC analysis of the purified compound was performed on an Inertsil ODS-3 HPLC column (4.6. x 250 mm, GL Sciences Inc., Tokyo, Japan). The isocratic mobile phase was 95% aqueous acetonitrile with 0.05% TFA employed at a flow rate of 0.53 ml/min at 30°C and the effluent was monitored at 210 nm.

5.4 Biosurfactant activity assay

5.4.1 Surface tension measurement

The surface tension of the cell-free supernatant was measured by the ring method using a tensiometer (OS, Torsion balance supplier, Germany) (Sarubbo *et al*., 2007). A 30-mL volume was put into a clean glass vessel (50 mL) that was placed on the tensiometer platform. A platinum wire ring was submerged into the solution and then slowly pulled through the liquid–air

interface. Between each measurement, the platinum wire ring was rinsed three times with water and three times with acetone and allowed to dry.

The critical micelle concentration (CMC) was determined by measuring the surface tensions of dilutions of the isolated biosurfactant in distilled water up to a constant value of surface tension.

5.4.2 Emulsification activity (%EA) and emulsification index (EI24)

To measure emulsification activity, 1 ml of xylene was added to 1 ml of supernatant in a test tube. The mixture was mixed by vortex mixer at high speed for 2 min and allowed to stand for 10 min and 24 h (emulsification index). The emulsification activity was calculated by using the following equation (modified from Cooper and Goldenberg, 1987).

$$\text{EA (\%)} = \frac{\text{height of emulsion formed}}{\text{total height of solution}} \times 100$$

5.4.3 Oil displacement area (ODA)

Fifty ml of distilled water was added to a large Petri dish (15 cm diameter) followed by an addition of 20 µl of WCO to the surface of water to make an oil layer. Ten µl of sample solution was then added to the surface of oil, resulting in a clear zone according to its oil-displacing ability (surface activity) (Youssef *et al*., 2004). Each test was performed in triplicate. Methanol was used as negative control (no clear zone was found). The area of this circle was measured and calculated for oil displacement area (ODA) using the following equation

$$\text{ODA (cm}^2\text{)} = 22/7\ (\text{radius})^2$$

The minimum active dose (MAD) of the biosurfactant was defined as the amount of the biosurfactant necessary for giving a detectable clear zone by naked eye on an oil layer (by the ODA test).

5.5 TLC-FID analysis for waste lubricating oil composition

Waste lubricating oil contaminated soil was collected from the motorcycle garage at Prince of Songkla University (PSU). Waste lubricating oil was extracted twice by *n*-hexane. *n*-Hexane phase was separated and dried by rotary evaporator. Samples were diluted in hexane. One µl of prepared sample solution was spotted on each chromarod and developed according to increasing order of polarity using *n*-hexane, toluene and dichloromethane/methanol (95:5 v/v) as eluent. The development heights were 10 cm for the first development (by 100% *n*-hexane) and 5.5 cm for the second (by 20% *n*-hexane and 80% toluene). The rods were developed in the third TLC tank with dichloromethane/methanol (95:5 v/v). The rods were removed when the solvent front has traveled 2 cm and allowed to dry for 5 minutes in an oven at 105°C. The chromarods were placed in the TLC/FID analyzer. A pure hydrogen flow of 160 ml/min and air flow of 2 L/min were used in this study. The scanning speed was 30s/scan (Goto *et al*., 1994). The remaining oils from each isolate were extracted using *n*-hexane, then dry weight was determined and the oil degradability was calculated based on (%) weight loss and (**%**) oil degradation, respectively (Shirai *et al*., 1995) as follows.

$$(\%)\ \text{weight loss} = \frac{(\text{weight of oil (negative control)} - \text{weight of oil (degraded)})}{\text{weight of oil (negative control)}} \times 100$$

$$(\mathbf{\%})\ \text{oil degradation} = \frac{(\text{component in negative control} - \text{component in sample})}{\text{component in negative control}} \times 100$$

5.6 GC-MS analysis for waste lubricating oil composition

Extracted waste lubricating oil was analyzed by a GC-MS. GC-MS analysis was carried out under the following conditions: GC, Hewlett Packard HP5890 Series II; column, Rtx-5MS (30 m x 0.25 mm, 0.25 µm film thickness); carrier gas, He carrier flow 1.0 ml/min, splitless 0.9 min; injection temperature, 240°C; column temperature program, 50°C (1.5 min), 180°C at 15°C /min, 275°C (15 min); MS, electron ionization positive mode; ionization voltage, 70 eV; ion source temperature, 275°C, solvent delay time 3.0 min (Dabrowska *et al*., 2003).

Methods

1. Isolation and screening of biosurfactant producing-yeasts from palm oil and hydrocarbon contaminated soil

Five grams of soil samples were added in a 45 ml of 0.85% NaCl, samples were serially diluted and spread on YM agar plates supplemented with 12.5 μg/ml chloramphenicol. The plates were incubated at 30°C for 24 h. The purified isolates were inoculated into 10 ml BSM containing 1g/L WCO and incubated at 30°C for 24 h with 200 rpm agitation. Culture broth was centrifuged at 9,000 rpm, 4 °C for 15 min. The cell-free supernatant was used to determine biosurfactant activity by oil displacement area (ODA) (Youssef *et al*. 2004), surface tension by the ring method using a tensiometer (Kim *et al*., 2002b) and emulsification activity (%EA) (modified from Cooper and Goldenberg, 1987).

2. Identification of the isolated yeast strain

The selected yeast strain which exhibited the highest biosurfactant activity was selected and identified based on the physiological and biochemical properties (Barnett *et al*., 2000). In addition, the 18S and 26S rDNA fragments of the selected strain were amplified using universal oligonucleotide primers described by Vasdinyei and Deak (2003). Sequences of the amplified fragments were analyzed with an ABI PRISM™ 3100 DNA Sequencer (PE Applied Biosystems, Foster City, CA, USA) and a BigDye Terminator Cycle Sequencing kit (Applied Biosystems) according to the manufacturer's instruction manuals. The BLAST program (http://www.ncbi.nlm.nih.gov/BALST) was used to analyze sequence homology (Thompson *et al*., 1994).

3. Optimization of biosurfactant production in shake-flask cultivation

The optimization conditions were studied in order to obtain the maximum biosurfactant production from the selected yeast strain. The selected yeast was grown into 50 ml of YM medium at initial pH 5.0 in 250-ml flask and incubated with shaking at 200 rpm and 30°C for 24 h. The culture was diluted with the medium to obtain yeast cell of 10^6 cells/ml and used as the starter culture. Ten percent of starter culture was transferred to 50 ml BSM in 250 ml flask. The culture was cultivated on a rotary shaker with 200 rpm at 30°C. Triplicate samples were taken at 48 h and

growth was measured by direct microscopic count, pH, %EA and surface tension were also measured.

3.1 Effect of carbon sources

3.1.1 Type of carbon sources

Cultivation was performed in the BSM with various carbon sources: glucose and sucrose at 10 g/L and *n*-hexadecane, xylene and naphthalene at 1 g/L. Carbon source rendering the highest biosurfactant production was chosen for the next study.

3.1.2 Concentration of carbon sources

Cultivation was performed in the BSM containing the selected carbon source from section 3.1.1 with varying concentrations 0, 1, 2, 3, 4 and 5 g/L. Concentration of carbon source which gives the highest biosurfactant production was chosen for further study.

3.2 Effect of nitrogen sources

3.2.1 Type of nitrogen sources

Cultivation was performed in the BSM containing the selected carbon source with proper concentration from section 3.1.2 with varying 1 g/L of nitrogen sources ($(NH_4)_2SO_4$, NH_4NO_3, urea and yeast extract). Nitrogen source showing the highest biosurfactant production was chosen for further study.

3.2.2 Concentration of nitrogen sources

Cultivation was performed in the BSM containing the selected carbon source with proper concentration from section 3.1.2 with varying concentration 0, 1, 2, 3, 4 and 5 g/L of the selected nitrogen source from section 3.2.1. Concentration of nitrogen source which gives the highest biosurfactant production was chosen for further study.

3.3 Effect of initial pH

Cultivation was performed in the BSM containing the selected carbon and nitrogen sources with their proper concentrations from section 3.2.2 varying initial pH of culture

medium at 4.0, 4.5, 5.0, 5.5 and 6.0 by 0.5 N HCl and 0.5 N NaOH. The initial pH of BSM showing the highest biosurfactant production was chosen for further study.

3.4 Effect of temperature

Cultivation was performed in the BSM containing the selected carbon and nitrogen sources with the proper concentrations and initial pH from section 3.3 incubating at different temperatures at 30, 37 and 45 °C. The suitable incubation temperature of BSM which gives the highest biosurfactant production was chosen for further study.

3.5 Effect of agitation

Cultivation was performed in the BSM containing the selected carbon and nitrogen sources with the proper concentrations, initial pH and incubation temperature from section 3.4 varying speed of rotary shaker at 150, 200 and 250 rpm. The suitable agitation speed showing the highest biosurfactant production was chosen for further study.

3.6 Time course of growth and biosurfactant production in shake-flask cultivation

The BSM consisting of carbon and nitrogen sources with the proper concentrations, initial pH, incubation temperature and agitation speed were chosen from section 3.5. Triplicate samples were taken at 0, 12, 24, 36, 48, 60, 72, 84 and 96 h. The cultivation, growth was measured by direct microscopic count, pH, %EA and surface tension were also measured.

3.7 Optimization for biosurfactant production in fermentor

Cultivation was performed in a 2 liters fermentor (working volume 1 liter) of optimal condition (resulted from section 3.5). Then the samples were taken at 0, 12, 24, 36, 48, 60, 72, 84, 96 and 120 h and determined as described in section 3.6. All parameters were analyzed as mentioned in the shake-flask cultivation.

3.7.1 Effect of pH

Cultivation under uncontrolled and controlled pH (pH 5.0) was compared. The agitation speed was 200 rpm with aeration rate 0.5 vvm. The suitable condition showing the highest biosurfactant production was chosen for further study.

3.7.2 Effect of aeration rate

Cultivation under the selected condition from section 3.7.1 was prepared. The aeration rate was varied from 0, 0.5 and 1.0 vvm. The suitable aeration rate which giving the highest biosurfactant production was chosen for further study.

3.7.3 Time course of growth and biosurfactant production under optimal condition

Cultivation under optimal condition from section 3.7.2 was prepared. Triplicate samples were taken at 0, 12, 24, 36, 48, 60, 72, 84, 96 and 120 h. The parameters were determined and compared to those from the shake-flask cultivation.

4. Recovery of biosurfactant produced by the selected yeast strain

The selected yeast strain was cultivated in BSM under the optimized conditions (section 3.7.3). The culture broth was separated by centrifugation at 9,000 rpm at 4°C for 20 min. The extraction methods of culture supernatant were compared as follows:

4.1 The culture supernatant was extracted twice with 2 volume of ethyl acetate, and the ethyl acetate phase was treated with anhydrous Na_2SO_4 to remove residual water. After evaporation of ethyl acetate, the remaining material was dissolved in 1 volume of methanol, and the oily residue in the methanol was discarded (Kim *et al*. 1999).

4.2 The culture supernatant was acidified with 5 N HCl to pH 2 and subsequently extracted two times with an equal volume of ethyl acetate (Rau *et al*., 2005). The mixture was vortexed for 1 min and centrifuged for 5 min at 6000 rpm. The ethyl acetate phase was treated with anhydrous Na_2SO_4. The combined extracts were concentrated to dryness with a vacuum rotary evaporator.

4.3 The culture supernatant was extracted with an equal volume of mixtures of chloroform/methanol (2:1) (modified from Mercade *et al*. 1996). The solvent phase was treated with anhydrous Na_2SO_4 to remove residual water. The combined extracts were concentrated to dryness with a vacuum rotary evaporator.

The weight of the crude biosurfactant from 3 methods was measured and biosurfactant activity was monitored by measuring critical micelle concentration (CMC).

5. Purification of biosurfactant produced by the selected yeast strain

The culture supernatant (10L) of the selected yeast strain was extracted with mixtures of chloroform/methanol (2:1). The combined extracts were concentrated to dryness *in vacuo*. The crude extract was separated by chromatography on a silica gel column (1.0×30 cm, Wako gel C-100, Wako Pure Chem. Ind., Ltd., Osaka, Japan) by sequential elution with hexane, ethyl acetate and methanol. All fractions were collected, dried and tested for the biosurfactant activity by ODA test. The pooled highest active fraction was fractionated by chromatography on a silica gel column (1.0×30 cm, Wako gel C-200, Wako Pure Chem. Ind., Ltd., Osaka, Japan) by sequential elution with hexane, ethyl acetate and methanol, which was confirmed by oil displacement area test and *p*-anisaldehyde-positive spots on TLC. The fraction with the highest surfactant activity was purified further using a preparative HPLC on Inertsil ODS-3 (20×250 mm, GL Sciences Inc. Japan) eluting with 95% aqueous acetonitrile containing 0.05% trifluoroacetic acid (TFA). The preparative HPLC was performed with pressure 500 pound per square inch (PSI) at flow rate 10 ml/min. The surface-active compound as an oily material was finally recovered, which determined by analytical Inertsil ODS-3 HPLC column (4.6. x 250 mm, GL Sciences Inc., Tokyo, Japan) when eluting with 95% acetonitrile supplemented with 0.05%TFA and on TLC analyses.

6. Structure elucidation of the surface-active compound

The purified compound from the section 5 was elucidated the chemical structure by ^{1}H-NMR spectra and recorded in CD_3OD with Varian VXR 500 instrument. Mass spectra were obtained with SX-102A equipment (JEOL, Tokyo, Japan). GC-MS analysis was performed by Automass 20 (JEOL) using DB-1 (ϕ 0.25 mm × 30 m, 0.25 μm thickness) column.

7. Characteristics of crude biosurfactant

The obtained crude biosurfactant (0.00625 mg/ml) from the section 4 was used for characterization the crude biosurfactant as follows:

7.1 Effect of salts on the stability

The resistance of the crude biosurfactant to 0-5% of NaCl and 0-0.1% of $MgCl_2$ and $CaCl_2$ were investigated. The samples were allowed to stand for 24 h at 30°C before determining ODA test.

7.2 Effect of pH on the stability

The pH stability of the crude biosurfactant was determined in the pH range 2-10 which adjusted by 0.5 N HCl or NaOH for 24 h at 4°C. Then the samples were determined as described in section 7.1 at 30°C.

7.3 Effect of temperature on the stability

The stability of the crude biosurfactant was tested over a wide range of temperatures at 4-80°C for 48 h and 100°C for 24 h. Then the samples were determined as described in section 7.1 at 30°C.

7.4 Comparison with synthetic surfactants

The purified biosurfactant (6.25 μg/ml) from the section 5 was compared with commercial surfactants such as Triton X-100, Triton X-114 and sodium dodecyl sulfate (SDS). The biosurfactant activity was assayed by oil displacement area (ODA) test and %EA.

8. Application of biosurfactant for bioremediation

8.1 Solubility of polycyclic aromatic hydrocarbons (PAHs)

Stock solutions of PAHs (naphthalene, phenanthrene and pyrene), 1000 μg/ml were prepared in methanol and distributed into screw cap test tubes to a final concentration of 100 μg of PAHs per tube. Tubes were left open inside an operating chemical fume hood to remove solvent. The crude biosurfactant obtained from the section 4 was dissolved in 5 ml distilled water to

obtain the concentration of 10, 50 and 100 mg/L compared with SDS and distilled water and was added into each PAHs test tube. All experiments were done in triplicate. Tubes were incubated at 30°C and shaked at 200 rpm for 48 h. The samples were subsequently centrifuges at the speed of 8,000 rpm at 4 °C for 30 min to separate the undissolved PAHs. An appropriate aliquot of the supernatant was carefully withdrawn and analyzed by the spectrophotometer at 205 nm (naphthalene) and 254 nm (phenanthrene and pyrene) (modified from Barkay *et al*., 1999). The concentration of naphthalene, phenanthrene and pyrene were determined from the standard curves of naphthalene, phenanthrene and pyrene (Appendix 3).

8.2 Bioremediation of waste lubricating oil contaminated soil

This experiment was performed in 500 ml erlenmeyer flasks. 25 g of waste lubricating oil contaminated soil which contained 12.5 mg waste lubricating oil at the beginning was suspended in 225 ml sterile deionized water. Soil slurry was mixed with 35 mg of the crude biosurfactant. The soil samples were shaked at 200 rpm and 30°C and the samples were taken at 0, 15 and 30 days (modified from Kosaric, 2001). The culture flasks of each treatment were follows:

Treatment 1 Abiotic treatment (sterile soil) as the negative control

Treatment 2 Abiotic with crude biosurfactant

Treatment 3 Biotic treatment (non sterile soil)

Treatment 4 Biotic with crude biosurfactant

The remaining oils from each treatment were extracted two times by *n*-hexane. The amount of microorganisms was determined on plate count agar (PCA) (Appendix 1). The oil degradability was calculated based on weight loss (%) (Shirai *et al*., 1995) and the composition of the oil was analyzed by TLC-FID (Goto *et al*., 1994) and GC-MS (Dabrowska *et al*., 2003), respectively.

CHAPTER 3

RESULTS AND DISCUSSION

1. Isolation and screening of biosurfactant-producing yeasts from oil contaminated soils

A total of 237 yeasts were isolated from oil contaminated soils from Southern region of Thailand by cultivation in the BSM supplemented with 1g/L weathered crude oil (WCO) as a sole carbon source at 30°C. Among 81 yeast isolates showing growth on this medium, only 7 yeast isolates, PO1.2, PO3.6, PO3.13, PO4.2, GR2.4, GR4.2 and SR4 showed high biosurfactant activities as indicate in term of surface tension reduction and emulsification activity of the supernatant when compared with sodium dodecyl sulphate (SDS). These yeast isolates were further screened for biosurfactant production in BSM supplemented with different carbon sources i.e., glucose, weathered crude oil and xylene.

Table 6 showed the biosurfacant activities of these isolated yeast which could reduce the surface tension of the supernatant to less than 60 mN/m. When these yeast isolates were growing in the BSM medium using glucose and xylene as a carbon source, the supernatant of the isolate SR4 showed the best surface tension reduction from 66.00 to 52.00 and 49.5 mN/m, respectively. When WCO was used as a carbon source, the supernatant of the isolate PO3.6 showed the best surface tension reduction from 66.00 to 54.5 mN/m but the supernatant of the isolate SR4 showed little surface tension reduction (from 66.0 to 60.5 mN/m).

The characteristic of good emulsifier should show the emulsification activity and emulsion stability with various hydrocarbons. Kerosene, xylene and *n*-hexadecane were used for this study. The structure of kerosene is consisted of the mixture of paraffin, naphthalene and aromatic hydrocarbon. So, it was the most difficult to emulsify than xylene and *n*-hexadecane which are consisted of 1 benzene ring and long chain hydrocarbon, respectively. Emulsification activity (%EA) and emulsification index (E_{24}) of the supernatants which produced by 5 yeast isolates when grow in the BSM medium contained glucose, WCO and xylene as a carbon source were shown in Table 7. The results indicated that the types of yeasts, carbon sources and hydrocarbons have effect on %EA and EI_{24}. When the yeast isolates PO1.2 and PO4.2 were grew in BSM with glucose or xylene as a carbon source, the organisms did not produce a biosurfactant which showed emulsification activity. However, when they are grown in the BSM medium with

WCO as a carbon source, the biosurfactants were produced and showed the emulsification activity with kerosene but not with xylene and *n*-hexadecane. When the yeast isolates GR2.4 and GR4.2 were grown in the BSM medium with glucose as a carbon source, the produced biosurfactant could emulsify only kerosene. They did not produce the biosurfactant with emulsification activity in the medium with WCO or xylene as a carbon source. However, the biosurfactant produced by the yeast isolate SR4 grown in BSM medium with glucose as a carbon source could emulsify kerosene, xylene and *n*-hexadecane. In addition, it could emulsify xylene when xylene was used as a carbon source but when WCO was used as a carbon source no emulsification activity was observed. The WCO is the by product obtained after distillation of Oman crude oil at 350°C and is a mixture of a large number of different hydrocarbons; the most commonly found molecules are alkanes with approximately up to 20 carbon atoms, cycloalkanes, aromatic hydrocarbons, or more complicated chemical s like asphaltenes (Meng *et al*., 2006). From above information, the WCO was not suitable to be a carbon source for cell growth and biosurfactant production.

The synthetic surfactant, 1%SDS showed emulsification activity with all tested hydrocarbons. However, it was noted that the isolate SR4 showed better emulsification activity with xylene than the SDS. Furthermore, isolated colonies from soil sample were cultured in the isolation liquid medium containing *n*-hexadecane, soybean oil, or glucose as a carbon source, and then, by measuring the oil film-collapsing activity of the culture broth by drop collapse technique. These samples were assessed for surface tension and emulsification activity. *Candida* sp. SY16 was capable of effectively emulsifying crude oil, vegetable oil, and hydrocarbons, and reduced the surface tension of the culture broth to 30 dyne/cm (Kim *et al*., 1999). The surface tensions of the culture broth produced by *Wickerhamiella domercqiae* Y2A was isolated from an oil-contaminated wastewater sample was 37.3 mN/m when supplemented with rapeseed oil as a carbon source (Jing *et al*., 2006) while the supernatant produced by yeast isolate SR4 can reduce the surface tension of BSM from 65.0 to 49.5 mN/m when xylene was used as a carbon source. Besides, xylene is a hydrocarbon which consisting of 1 benzene ring may be more difficult to use for carbon source than sugar or vegetable oil. However, this study was the first report of biosurfactant-producing yeast when xylene was used as a carbon source.

From the result of isolation and screening of biosurfactant-producing yeasts, the yeast strain SR4 showed the highest biosurfactant production. It was performed not only the lowest surface tension but also the emulsification activity. Thus, this yeast strain SR4 was selected for further study.

Table 6. Effect of carbon sources on the surface tension reduction by biosurfactant producing-yeasts

		Surface tension reduction (mN/m)		
		Carbon sources		
Isolate	Source	1.0%Glucose	0.1%WCO	0.1%Xylene
PO1.2	Palm oil Factory	54.0±0.35(66.0) *	59.0±0.00(67.0)	55.0±0.00(65.0)
PO3.6	Palm oil Factory	63.0±0.00(66.0)	54.5±0.00(67.0)	61.5±0.00(65.0)
PO3.13	Palm oil Factory	64.0±0.00(66.0)	58.5±0.35(67.0)	63.5±0.35(65.0)
PO4.2	Palm oil Factory	66.0±0.00(66.0)	67.0±0.00(67.0)	66.0±0.00(66.0)
GR2.4	Motor Oil, Garage	62.5±0.35(66.0)	56.5±0.00(67.0)	60.0±0.00(65.0)
GR4.2	Motor Oil, Garage	66.0±0.00(66.0)	67.0±0.00(67.0)	66.0±0.00(66.0)
SR4	Palm oil Factory	52.0±0.00(66.0)	60.5±0.00(67.0)	49.5±0.35(65.0)

Cultivation in BSM with 200 rpm at 30°C for 48 h.

* Surface tension of BSM supplemented with each carbon source without inoculated culture.

Table 7. Effect of yeast isolates and carbon source on emulsification activity (%EA) and emulsification index (EI_{24}) of their supernatants

Isolate	Carbon source	Emulsify with	%EA*	EI_{24} (%)†
PO1.2	Weathered crude oil	kerosene	50.00	38.10
		xylene	0	0
		n-hexadecane	0	0
PO3.6	Weathered crude oil	kerosene	0	0
		xylene	0	0
		n-hexadecane	0	0
PO3.13	Weathered crude oil	kerosene	0	0
		xylene	0	0
		n-hexadecane	0	0
PO4.2	Weathered crude oil	kerosene	65.00	55.00
		xylene	0	0
		n-hexadecane	0	0
GR2.4	glucose	kerosene	39.17	0
		xylene	0	0
		n-hexadecane	0	0
	xylene	kerosene	0	0
		xylene	54.55	0
		n-hexadecane	36.36	0
GR4.2	glucose	kerosene	39.17	0
		xylene	0	0
		n-hexadecane	0	0
SR4	glucose	kerosene	63.43	63.43
		xylene	84.62	84.62
		n-hexadecane	47.62	47.62

Table 7. Continue

Strains	Carbon source	Emulsify with	%EA*	EI_{24} (%)†
SR4	Weathered crude oil	kerosene	0	0
		xylene	0	0
		n-hexadecane	0	0
	xylene	kerosene	0	0
		xylene	41.67	41.67
		n-hexadecane	0	0
1%SDS		kerosene	76.92	76.92
		xylene	76.92	76.92
		n-hexadecane	76.92	76.92

*%EA: Emulsion at 10 min

†E_{24}: Emulsion at 24 h

2. Identification of biosurfactant-producing yeast

Conventional identification methods such as culture characteristics and physiological properties of yeast isolate SR4 were examined because of the yeast isolate SR4 showed the highest biosurfactant production. It was performed not only the lowest surface tension (49.5 mN/m) of BSM but also the emulsification activity thus, this yeast isolate SR4 was selected for identification. The yeast isolate SR4 was isolated as a crude oil-utilizing, reduce surface tension and emulsifying yeast and characterized on YM agar plate as followed: growth at 30°C, off-white color, oval shape, dull, and smooth with margins ranging from smooth to lobed. Morphology of the yeast isolate SR4 under microscopy showed oval shape and budding cells (Fig. 16). The results of characteristics of the selected yeast isolate SR4 (Table 8) suggested the yeast isolate SR4 was *Candida krusei*. While when the microscopical examination for ascospores was studied in acetate agar (McClary *et al*., 1959) the result showed that the isolate SR4 had ascus with 1 to 4 round ascospores. So, this yeast isolate SR4 had sexual reproduction (teleomorph) but *Candida krusei* has asexual reproduction (Barnett *et al*., 2000). Then, the isolate SR4 was further identified

by DNA sequencing analysis. The 26S rDNA sequences of isolate SR4 were amplified by universal oligonucleotide primers, GGAAGGGRTGTATTTATTAG (EF4) and TCCGTCAATTCCTTTAAG (NS4). The 26S rDNA sequences obtained (600 base pairs) was aligned with sequences available from GenBank, DDBJ, and EMBL. Identification of the strain SR4 was performed on the basis of nucleotide sequence of yeast 26S rDNA gene. The DNA sequence of this strain showed 99% similarity with *I. orientalis* WL2002 26S ribosomal RNA (Appendix 4). The phylogenetic tree was constructed by the neighbor-joining method, using the bootstrap resampling method with 1000 replicates. Phylogenetic and molecular evolutionary analyses were performed using MEGA version 3.1. Isolate SR4 was clustered into genus *Issatchenkia orientalis* by 98% bootstrap confidence based on 26S rDNA (Figure 17). Therefore this yeast isolate was identified as *Issatchenkia orientalis* strain SR4. This is the first report to show that the yeast *Issatchenkia orientalis* strain SR4 produced biosurfactant which isolated from palm oil contaminated soil.

In addition, Kim *et al*. (1999) examined the morphological properties of the strain SY16 after 3 days cultivation on YM agar at 25°C. Assimilation tests and growth characteristics suggested that it belong to the genus *Candida*. Although some differences were found in the assimilation tests, the morphological, physiological, and other taxonomical characteristics of SY16 were very similar to those of *Candida antarctica*. Moreover, results of biochemical and physiological studies of the yeast strain Y12 indicated no ascospore could be detected by spore staining. The assimilation of nitrate, nitrite, lysine and ethylamine were positive. Urea hydrolysis and diazonium blue B reaction was negative. These results suggested that strain Y12 belongs to *Candida ishiwadae* (Thanomsub *et al*., 2004). By using BIOLOG analysis and conventional methods for yeast identification, strain Y2A was identified as *Wickerhamiella domercqiae*, the probability and similarity values for Y2A and *W. domercqiae* were 100% and 0.63, respectively. Cell morphology, growth properties and colony appearance of strain Y2A were identical to those of *W. domercqiae* (Jing *et al*., 2006).

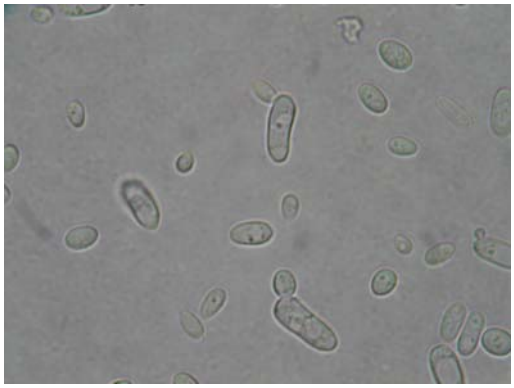


Figure 16. Morphology of *Issatchenkia orientalis* SR4 under microscopy (400x)

Table 8. Characteristics of the selected yeast isolate SR4

Assimilation of carbon compounds	Isolate SR4	*Candida krusei*[*]
1 Dextrose	Positive	Positive
2 Maltose	Negative	Negative
3 Sucrose	Negative	Negative
4 Lactose	Negative	Negative
5 Galactose	Negative	Negative
6 Melibiose	Negative	Negative
7 Cellobiose	Negative	Negative
8 Inositol	Negative	Negative
9 Xylose	Negative	Negative
10 Raffinose	Negative	Negative
11 Trehalose	Negative	Negative
12 Dulcitol	Negative	Negative
Fermentation of Glucose	Positive	Positive
Germ tube	Negative	Negative
Urease test	Negative	Negative
Phenoloxidase	Negative	Negative
Color on CHROMagar Candida	-	Pink
Ascospore	Positive	Negative

[*] code from Barnett *et al*. (2000).

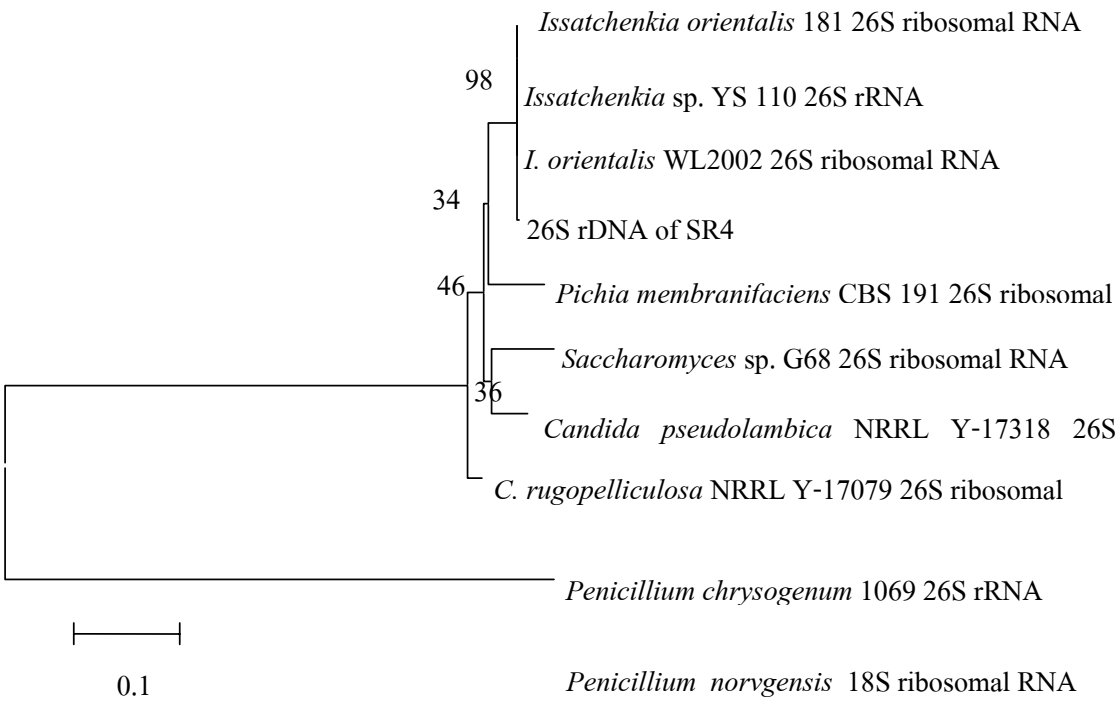


Figure 17. Phylogenetic tree of 26S rDNA of the selected yeast isolate SR4.

3. Optimization of biosurfactant production in shake-flask cultivation

3.1 Effect of medium compositions on growth and biosurfactant activity in shake-flask cultivation

3.1.1 Carbon sources

The effect of various carbon sources on growth and biosurfactant production by *Issatchenkia orientalis* SR4 was shown in Table 9. Cultivation was performed in the BSM with initial pH 5.0 and 200 rpm at 30°C for 48 h. The *I. orientalis* SR4 could grow in the BSM medium containing all of the carbon sources tested (glucose, sucrose, xylene *n*-hexadecane and naphthalene). It had the highest cell numbers of 4.9×10^8 cells/ml when glucose was used as a carbon source. Whereas insoluble carbon source such as xylene, *n*-hexadecane and naphthalene was used as a carbon source this yeast strain grew slowly. It might due to the insoluble carbon source is difficult to use or toxic to yeast cell. When this yeast was cultivated in BSM without carbon source, it also grew because there was 0.5 g/L yeast extract in BSM medium.

When the yeast strain SR4 was grown in BSM medium with glucose as a carbon source it showed the highest emulsification activity (84.62%) but the surface tension of the culture broth was slightly reduced (53.0 mN/m). The yeast strain SR4 did not show emulsification activity when *n*-hexadecane and naphthalene were used as a carbon source. On the other hand, the yeast strain SR4 showed the highest surface tension reduction of BSM from 63 to 49.5 mN/m and could emulsify xylene with 41.67%EA when xylene was used as a carbon source. Glucose is the best carbon source for growth and emulsification activity. Although xylene was not a good carbon source for growth but it produced the best surface tension reduction and also produced moderately emulsification activity. Therefore, xylene was selected as a carbon source for further study. In addition, this is the first report to show that xylene was used as a carbon source for biosurfactant production by the yeast *I. orientalis* SR4.

The results indicated that the types of carbon source have effect on growth and biosurfactant activity of *I. orientalis* SR4 because of the structure of biosurfactant depend on carbon source. In addition to the carbon substrates examined for biosurfactant production by *Candida* sp. SY16, neither glucose nor *n*-hexadecane stimulated any significant biosurfactant production (Kim *et al*., 2002b). Moreover, *n*-alkanes ranging from C_{12} to C_{18} were converted into glycolipid biosurfactants, mannosylerythritol lipids (MEL), by resting cells of *Pseudozyma* (*Candida*) *antarctica* T-34 (Kitamoto *et al*., 2001). Furthermore, these authors suggested that the water-soluble substrate is used primarily for cellular metabolism and for synthesis of the hydrophilic sophorolipid moiety, while the lipophilic substrate is used exclusively for production of the hydroxy-carboxylic acid moiety. This is related to the observation that *Candida* yeasts are capable of directly incorporating fatty acids into the sophorolipid product (Weber *et al*., 1992). *Candida bombicola* could produce sophorolipid molecules with surfactant properties when grown in a medium composed of two different carbon sources, 100 g/L glucose and 100 g/L sunflower oil (Casas and Ochoa, 1999).

3.1.2 Concentration of carbon source

Table 10 showed the effect of xylene on growth and biosurfactant activity of *I. orientalis* SR4. Cultivation was performed in the BSM with xylene as a carbon source with an initial pH 5.0 and 200 rpm at 30°C for 48 h. The result showed that xylene

concentration more than 1 g/L could inhibit or was toxic on yeast cell because cell growth of the yeast strain SR4 was decreased, thus it also had effect on biosurfactant production. In addition, the high concentration of xylene could inhibit or cause delayed growth and development of yeast cell. When cultivation in BSM without xylene, it could grow by using yeast extract in BSM medium as a carbon source.

I. orientalis SR4 could produce the highest biosurfactant activity when 1 g/L of xylene was used as a carbon source in the BSM medium. The supernatant of this cultivation could reduce the surface tension of BSM medium from 65.0 to 49.5 mN/m and emulsify xylene with 41.70%EA. When 2 g/L of xylene was added in the BSM medium the *I. orientalis* SR4 also grow moderately and produced 20%EA with xylene. From the results, 1 g/L xylene was selected as a carbon source for further study.

Table 9. Effect of carbon sources on growth and biosurfactant activity produced by *Issatchenkia orientalis* SR4

Carbon sources	Cells/ml	Final pH	Surface tension (mN/m)	%EA
Glucose	$4.90 \times 10^{8} \pm 0.29^{a‡}$	5.60 ± 0.29	$53.0 \pm 0.50^{b}(66)^{†}$	84.62 ± 0.00
Sucrose	$3.85 \times 10^{8} \pm 0.00^{b}$	5.65 ± 0.29	59.0 ± 0.50^{e} (68)	0
Xylene	$3.08 \times 10^{7} \pm 0.00^{c}$	6.18 ± 0.50	49.5 ± 0.29^{a} (65)	41.67 ± 0.00
n-Hexadecane	$7.5 \times 10^{6} \pm 0.29^{e}$	5.82 ± 0.00	57.0 ± 0.58^{d} (60)	0
Naphthalene	$8.6 \times 10^{6} \pm 0.00^{f}$	5.55 ± 0.29	62.0 ± 1.25^{f} (71)	0
Control*	$1.62 \times 10^{7} \pm 0.00^{d}$	6.00 ± 0.00	55.0 ± 0.29^{c} (69)	0

Cultivation in BSM with NH_4NO_3 and yeast extract as a nitrogen source, initial pH 5.0 under shaking at 200 rpm and 30°C for 48 h.

*BSM without carbon source.

†Surface tension of BSM supplemented with each carbon source without inoculated culture.

‡Different letters in the same column indicate the significant differences ($p<0.05$).

Table 10. Effect of xylene on growth and biosurfactant activity produced by *Issatchenkia orientalis* SR4

Concentration of xylene (g/L)	Cells/ml	Final pH	Surface tension (mN/m)	%EA
0	$1.73 \times 10^7 \pm 0.50^{c‡}$	5.98±0.29	56.0 ± 0.00^{b} (69.0) †	0
1	$3.10 \times 10^7 \pm 0.00^{a}$	6.19±0.50	49.5 ± 0.75^{a} (65.0)	41.70±0.00
2	$2.24 \times 10^7 \pm 0.29^{b}$	5.33±0.50	65.0 ± 0.76^{c} (60.5)	20.00±0.00
3	$1.29 \times 10^7 \pm 0.00^{d}$	5.18±0.29	65.5 ± 0.50^{c} (58.5)	0
4	$8.7 \times 10^6 \pm 0.00^{e}$	5.15±0.00	64.0 ± 0.58^{c} (57.0)	0
5	$3.9 \times 10^6 \pm 0.50^{f}$	5.20±0.29	65.0 ± 0.76^{c} (54.5)	0

Cultivation in BSM with NH_4NO_3 and yeast extract as a nitrogen source under shaking at 200 rpm and 30°C for 48 h.

†Surface tension of BSM supplemented with xylene without inoculated culture.

‡Different letters in the same column indicate the significant differences ($p<0.05$).

3.1.3 Nitrogen sources

The effect of nitrogen sources on growth and biosurfactant production by *I. orientalis* SR4 in the BSM contained 1 g/L xylene as a carbon source was shown in Table 11. Growth of the yeast strain SR4 in the BSM with inorganic nitrogen sources such as NH_4NO_3, $(NH_4)_2SO_4$ and organic nitrogen sources such as yeast extract, peptone, urea at 1 g/L concentration were compared with control (without nitrogen source) and the original BSM which added nitrogen sources as 3 g/L NH_4NO_3 and 0.5 g/L yeast extract. The result showed the highest cell growth of the yeast strain SR4 was obtained in the medium with peptone a nitrogen source. Meanwhile when cultivated in the medium without nitrogen source, it exhibited the lowest cell growth.

The surface tension reduction of the medium by this yeast strain SR4 showed the same activity when $(NH_4)_2SO_4$, yeast extract and peptone were used as a nitrogen source while the highest biosurfactant activity was obtained when urea was used as a nitrogen source. The supernatant produced by *I. orientalis* SR4 in the BSM medium with xylene as a

carbon source and urea as a nitrogen source could reduce the surface tension of the medium from 61.5 to 49.0 mN/m and emulsify xylene with 40%EA. So, urea was selected as a nitrogen source for further study.

The type of nitrogens presented in the medium (whether NH_4^+, NO_3^-, urea or amino acid) influenced on the biosurfactant production (Haba *et al*., 2000). *Candida bombicola* produced the highest sophorolipid when grown in a medium composed of yeast extract (Casas and Ochoa, 1999) while Kim *et al*. (2002b) reported the maximum MEL-SY16 production by *Candida* sp. SY16 when NH_4NO_3 was used as a nitrogen source. Furthermore, the smut fungus *Ustilago maydis* (DSM 4500 and ATCC 14826) produced several glycolipids under nitrogen-limiting conditions (Spoeckner *et al*., 1999) similarly, when the culture reached nitrogen limitation caused biosurfactant production increase in *Candida tropicalis* IIP-4 (Singh *et al*., 1990).

3.1.4 Concentration of nitrogen source

The effect of urea concentration on growth and biosurfactant production by *I. orientalis* SR4 in the BSM contained 1 g/L xylene as a carbon source was shown in Table 12. Growth of the yeast strain SR4 was increased when supplemented with urea from 1 to 5 g/L. The highest cell growth was obtained in the medium with 4 and 5 g/L urea as a nitrogen source. The increasing of nitrogen concentrations could improve cell growth and biosurfactant production (Kim *et al*., 2006).

The surface tension reduction and emulsification activity were not different when 1 and 2 g/L urea were used as a nitrogen source. The supernatant produced by *I. orientalis* SR4 showed the highest biosurfactant activity when 3 g/L urea was used as a nitrogen source. Thus, 3g/L urea was selected as a nitrogen source for further study.

In addition, *Candida bombicola* could be able to produce the highest sophorolipid molecules with surfactant properties when grown in a medium composed of 1g/L yeast extract (Casas and Ochoa, 1999). Kim *et al*. (2002b) reported the maximum MEL-SY16 production by *Candida* sp. SY16 was attained at 1 g/L NH_4NO_3.

Table 11. Effect of nitrogen sources on growth and biosurfactant activity produced by *Issatchenkia orientalis* SR4

Nitrogen source	Cells/ml	Final pH	Surface tension (mN/m)	%EA
NH_4NO_3	$4.03 \times 10^7 \pm 0.00^{c‡}$	5.98 ± 0.00	61.0 ± 0.35^{d} (61.5) †	35 ± 0.35^{d}
$(NH_4)_2SO_4$	$3.90 \times 10^7 \pm 0.29^{d}$	5.90 ± 0.29	60.0 ± 0.00^{c} (68.0)	32 ± 0.00^{e}
Yeast extract	$4.24 \times 10^7 \pm 0.50^{b}$	6.00 ± 0.29	60.0 ± 1.06^{c} (59.0)	38 ± 0.00^{c}
Peptone	$4.49 \times 10^7 \pm 0.00^{a}$	5.81 ± 0.50	60.0 ± 0.00^{c} (60.0)	35 ± 0.00^{d}
Urea	$3.20 \times 10^7 \pm 0.00^{e}$	6.58 ± 0.29	49.0 ± 0.71^{a} (61.5)	40 ± 0.00^{a}
NH_4NO_3 + Yeast extract	$3.05 \times 10^7 \pm 0.29^{f}$	6.12 ± 0.00	50.0 ± 0.35^{b} (63.0)	39 ± 0.35^{b}
Control*	$2.02 \times 10^6 \pm 0.00^{g}$	5.76 ± 0.00	61.5 ± 0.71^{d} (65.0)	30 ± 0.35^{f}

Cultivation in BSM (1g/L xylene) under shaking at 200 rpm and 30°C for 48 h.

*BSM without nitrogen source.

†Surface tension of BSM supplemented with each nitrogen source without inoculated culture.

‡Different letters in the same column indicate the significant differences ($p<0.05$).

Table 12. Effect of urea on growth and biosurfactant activity produced by *Issatchenkia orientalis* SR4

Concentration of urea (g/L)	Cells/ml	Final pH	Surface tension (mN/m)	%EA
0	$2.05 \times 10^7 \pm 0.50^{d‡}$	5.40 ± 0.00	61.0 ± 0.35^{b} (65.0)†	37 ± 0.35^{c}
1	$2.50 \times 10^7 \pm 0.00^{d}$	5.40 ± 0.29	51.5 ± 0.00^{b} (62.0)	37 ± 0.35^{c}
2	$2.98 \times 10^7 \pm 0.29^{c}$	5.42 ± 0.50	52.0 ± 0.35^{b} (61.5)	37 ± 0.00^{c}
3	$3.34 \times 10^7 \pm 0.00^{b}$	6.43 ± 0.00	49.0 ± 0.00^{a} (61.5)	40 ± 0.35^{a}
4	$3.49 \times 10^7 \pm 0.00^{a}$	6.80 ± 0.50	54.0 ± 0.35^{c} (60.0)	38 ± 0.00^{b}
5	$3.42 \times 10^7 \pm 0.50^{a}$	7.54 ± 0.29	55.0 ± 0.35^{d} (59.0)	38 ± 0.00^{b}

Cultivation in BSM (1g/L xylene) under shaking at 200 rpm and 30°C for 48 h.

†Surface tension of BSM supplemented with urea without inoculated culture.

‡Different letters in the same column indicate the significant differences ($p<0.05$).

3.2 Effect of environmental conditions on growth and biosurfactant activity in shake-flask cultivation

3.2.1 Initial pH

Issatchenkia orientalis SR4 was cultivated in the optimal medium containing 1 g/L of xylene as a carbon source and 3 g/L urea as a nitrogen source with shaking at 200 rpm and incubation temperature at 30°C. Initial pH of the medium was adjusted to 4.0, 4.5, 5.0, 5.5 and 6.0 (Table 13). The highest growth of *I. orientalis* SR4 was obtained in the medium with an initial pH of 5.0.

The maximum biosurfactant production was also obtained in the medium with an initial pH of 5.0. The supernatant produced by *I. orientalis* SR4 had the surface tension 49 mN/m and could emulsify xylene with 41.5%EA (Table 13).

Most of yeasts grow very well between pH 4.0-6.5 (Walker, 1998) but production of a biosurfactant by microorganisms is depended on the type of microorganisms, type of a medium as well as pH of a medium. The sophorose lipid production by *Candida bombicola* showed the highest performance when the fermentation was carried out at pH 3.5 (Davila *et al*., 1997). Furthermore, the biosurfactants activity produced by *Candida bombicola* using soy molasses and oleic acid as co-substrates exhibited minimum surface tension values of 37 and 38 mN/m when adjusted initial pH at 6.0 and 9.0, respectively (Solaiman *et al*., 2004).

Table 13. Effect of initial pH on growth and biosurfactant activity produced by *Issatchenkia orientalis* SR4

Initial pH	Cells/ml	Final pH	Surface tension (mN/m)	%EA
4.0	$2.78x10^7 \pm 0.00^{b‡}$	5.64±0.50	55.0 ± 0.35^{e} (60.0) †	35.0 ± 0.35^{e}
4.5	$2.48x10^7 \pm 0.29^{c}$	6.00±0.35	52.5 ± 0.70^{c}(60.0)	38.0 ± 0.00^{c}
5.0	$2.85x10^7 \pm 0.00^{a}$	6.37±0.00	49.0 ± 0.00^{a} (61.0)	41.5 ± 0.35^{a}
5.5	$2.74x10^7 \pm 0.50^{b}$	6.59±0.29	50.0 ± 0.35^{b} (62.0)	40.0 ± 0.35^{b}
6.0	$2.47x10^7 \pm 0.29^{c}$	6.89±0.50	54.0 ± 0.35^{d} (61.5)	37.0 ± 0.00^{d}

Cultivation in BSM (1 g/L xylene, 3 g/L urea) under shaking at 200 rpm and 30°C for 48 h.

†Surface tension of BSM at each initial pH without inoculated culture.

‡Different letters in the same column indicate the significant differences ($p<0.05$).

3.2.2 Incubation temperature

The effect of incubation temperature on growth and biosurfactant production by *I. orientalis* SR4 cultivated in optimal medium (1 g/L of xylene, 3 g/L urea and initial pH at 5.0 with the agitation speed at 200 rpm) was shown in Table 14. Growth of the yeast strain SR4 decreased when the temperature was higher than 30°C. In addition, most laboratory and industrial yeasts are mesophilic and generally grow best between 20-30°C (Walker, 1998).

Incubation temperature had effect not only on cell growth but also biosurfactant production. When temperature was increased to 37 and 45°C, the supernatant of *I. orientalis* SR4 could not reduce the surface tension of BSM medium. Furthermore, incubation temperature at 45°C did not show emulsification activity. The highest biosurfactant production was occurred at the incubation temperature 30°C. Thus incubation temperature at 30°C was selected for further study.

Growth and metabolic activity of yeasts at various temperatures are functions not only of the genetic background of the cells but also of the growth medium composition and other physical growth parameters. Yeasts have the cellular damage, meaning that yeast cell viability will rapidly decline when temperatures are increased (Walker, 1998).The effects of temperature on mannosylerythritol lipids (MEL) production by resting cells and growing cells of *Candida antarctica* were examined. The highest yield was observed at 25°C (Kitamoto *et al*., 1992). Sophorose lipid production by *Candida bombicola* exhibited the highest performance when the whole fermentation was carried out at temperature 25°C (Davila *et al*., 1997).

Table 14**.** Effect of incubation temperature on growth and biosurfactant activity produced by *Issatchenkia orientalis* SR4

Temperature (°C)	Cells/ml	Final pH	Surface tension (mN/m)	%EA
30	$2.95x10^{7}\pm0.00^{a‡}$	6.30±0.29	49.0 ± 0.35^{a} (65) †	41.5±0.00
37	$1.28x10^{7}\pm0.00^{b}$	5.74±0.50	65.0 ± 0.00^{b} (65)	20.0±0.00
45	$8.4x10^{6}\pm0.00^{c}$	5.56±0.00	66.0 ± 0.00^{c} (65)	0

Cultivation in BSM under shaking at 200 rpm for 48 h.

†Surface tension of BSM at each temperature without inoculated culture.

‡Different letters in the same column indicate the significant differences ($p<0.05$).

3.2.3 Shaking speed

The effect of shaking speed on growth and biosurfactant production by *I. orientalis* SR4 which cultivated in optimal medium (1 g/L of xylene, 3 g/L urea) and condition (initial pH at 5.0 and the incubation temperature 30°C) was shown in Table 15. The cell growth of the yeast strain SR4 increased when the agitation speed increased. The maximum cell growth was exhibited when the agitation speed was at 250 rpm.

The highest biosurfactant production was obtained when the agitation speed was at 200 rpm. The supernatant produced by *I. orientalis* SR4 could reduce the surface tension of BSM from 65.0 to 49.0 mN/m and emulsify xylene with 41.5%EA . Thus, the agitation speed with 200 rpm was selected for further study.

Table 15. Effect of shaking speed on growth and biosurfactant activity produced by *Issatchenkia orientalis* SR4

Agitation speed (rpm)	Cells/ml	Final pH	Surface tension (mN/m)	%EA
150	$2.87 \times 10^7 \pm 0.00^{c‡}$	6.20±0.50	$53.5 \pm 0.00^{c}(65)^{†}$	30.0 ± 0.00^{c}
200	$2.99 \times 10^7 \pm 0.00^{b}$	6.41±0.35	$49.0 \pm 0.35^{a}(65)$	41.5 ± 0.00^{a}
250	$3.10 \times 10^7 \pm 0.00^{a}$	6.16±0.00	$52.5 \pm 0.71^{b}(65)$	32.0 ± 0.00^{b}

Cultivation in BSM with an initial pH 5.0 and 30°C for 48 h.

†Surface tension of BSM at each speed of agitation without inoculated culture.

‡Different letters in the same column indicate the significant differences ($p<0.05$).

3.3 Time course of growth and biosurfactant production under optimal medium in shake-flask cultivation

Cultivation of *I. orientalis* SR4 in the 50 ml optimal medium (contained 1g/L xylene, 3g/L urea) with an initial pH 5.0 in 250 ml flask at 200 rpm and 30°C was shown in Figure 18. *I. orientalis* SR4 grew rapidly during 24 h of cultivation and slightly increased to the

maximum growth (3.0×10^7 cells/ml) at 48 h. The biosurfactant was produced after 12 h of cultivation and the maximum biosurfactant activity was obtained at 48 h of cultivation. It could reduce the surface tension of the supernatant from 63.0 to 49.5 mN/m and emulsify xylene with 41.0%EA. In addition, the yield of crude biosurfactant produced by *I. orientalis* SR4 was 200 mg/L. The result showed that the biosurfactant production by *I. orientalis* SR4 was growth associated. While *Candida antarctica* (sp. SY16) required a vegetable oil as the carbon source to produce mannosylerythritol lipid (MEL-SY16). Biosurfactant production was 31 g/L in a batch culture for MEL-SY16 production was carried out for 7 days in 500 ml Erlenmeyer flasks containing 50 ml of the defined medium as the basal medium and was not growth associated (Kim *et al*., 2002b).

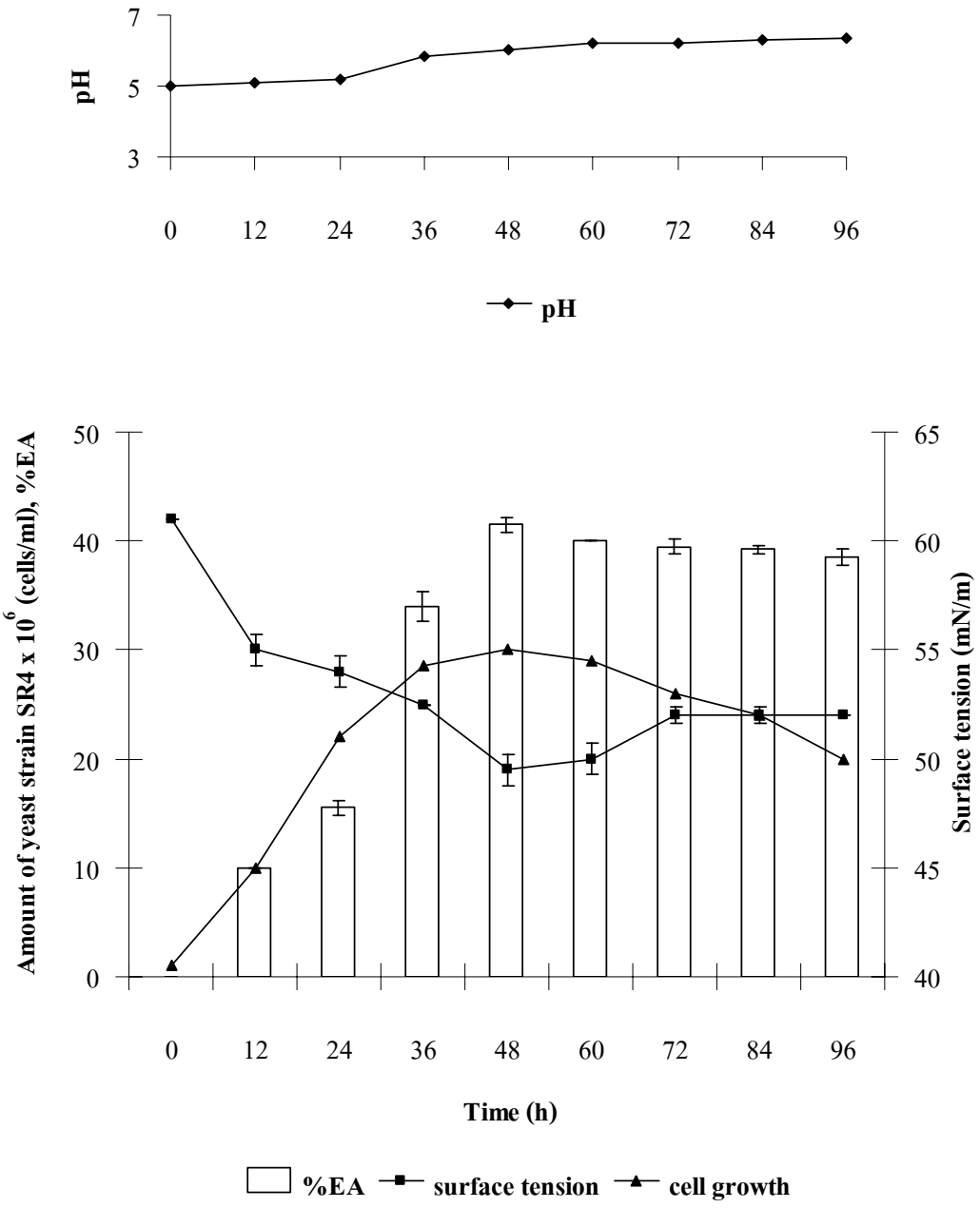


Figure 18. Time course of growth and biosurfactant production by *Issachenkia orientalis* SR4 in optimal medium (1 g/L of xylene, 3 g/L of urea, initial pH 5.0) at 200 rpm and 30°C.

3.4 Effect of environmental conditions on growth and biosurfactant production in fermentor

3.4.1 Effect of pH

According to the effect of pH on cell growth and biosurfactant production by *I. orientalis* SR4 in shake-flask cultivation (section 3.3), the initial pH at 5.0 exhibited the maximum cell growth and biosurfactant activity. Thus, pH 5.0 was selected to study the effect of controlled and uncontrolled pH in a fermentor.

I. orientalis SR4 was cultivated in the 2L fermentor containing 1L optimal medium (1 g/L of xylene as a carbon source, 3 g/L of urea as a nitrogen source) with the aeration rate 0.5 vvm and agitation speed of 200 rpm at 30°C, uncontrolled and controlled pH (pH 5.0). The result showed that the yeast strain SR4 grew well at both conditions while the biosurfactant production was higher under uncontrolled pH than controlled pH condition but not significant difference ($p>0.05$) (Fig. 19). So, uncontrolled pH was selected for further studied.

This result showed different with the effect of pH control on the production of MEL-SY16 by *Candida* sp. strain SY16 was also examined in batch fermentation. The highest production yield of MELSY16 was when the pH was controlled at 4.0, and the production was significantly improved compared to batch fermentation without pH control (Kim *et al*., 2006).

3.4.2 Effect of aeration rates

The effect of aeration rates on growth and biosurfactant production by *I. orientalis* SR4 was studied and showed in Figure 20. In the medium with no aeration the cell growth was still observed (2.15×10^7 cells/ml) may be due to the effect of shaking speed with 200 rpm. While the cell growth of *I. orientalis* SR4 increased when the aeration rates was raised. The maximum growth (3.1×10^7 cells/ml) and biosurfactant production were occurred at the aeration rate 0.5 vvm and 1.0 vvm with no significantly difference ($p>0.05$). The supernatant produced by *I. orientalis* SR4 could reduce the surface tension of culture broth from 60.25 to 49.50 mN/m and emulsify xylene with 41%EA. The aeration rate 0.5 vvm was selected for biosurfactant production as the reason of energy saving when comparison with aeration rate 1.0 vvm.

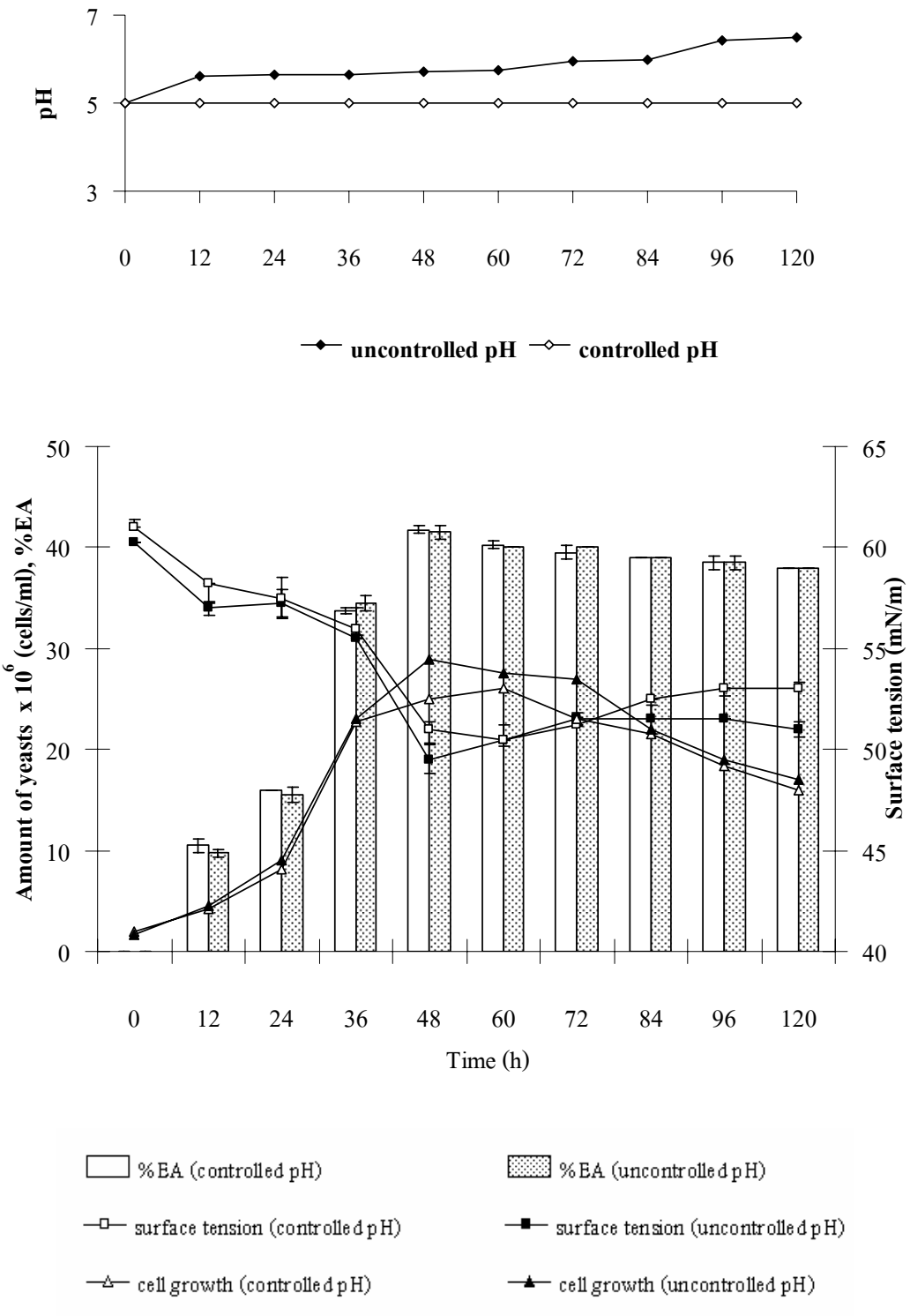


Figure 19. Growth and biosurfactant production by *Issachenkia orientalis* SR4 during cultivation under uncontrolled and controlled pH (in 2L fermentor with 1L working volume at 30°C, 200 rpm and 0.5 vvm).

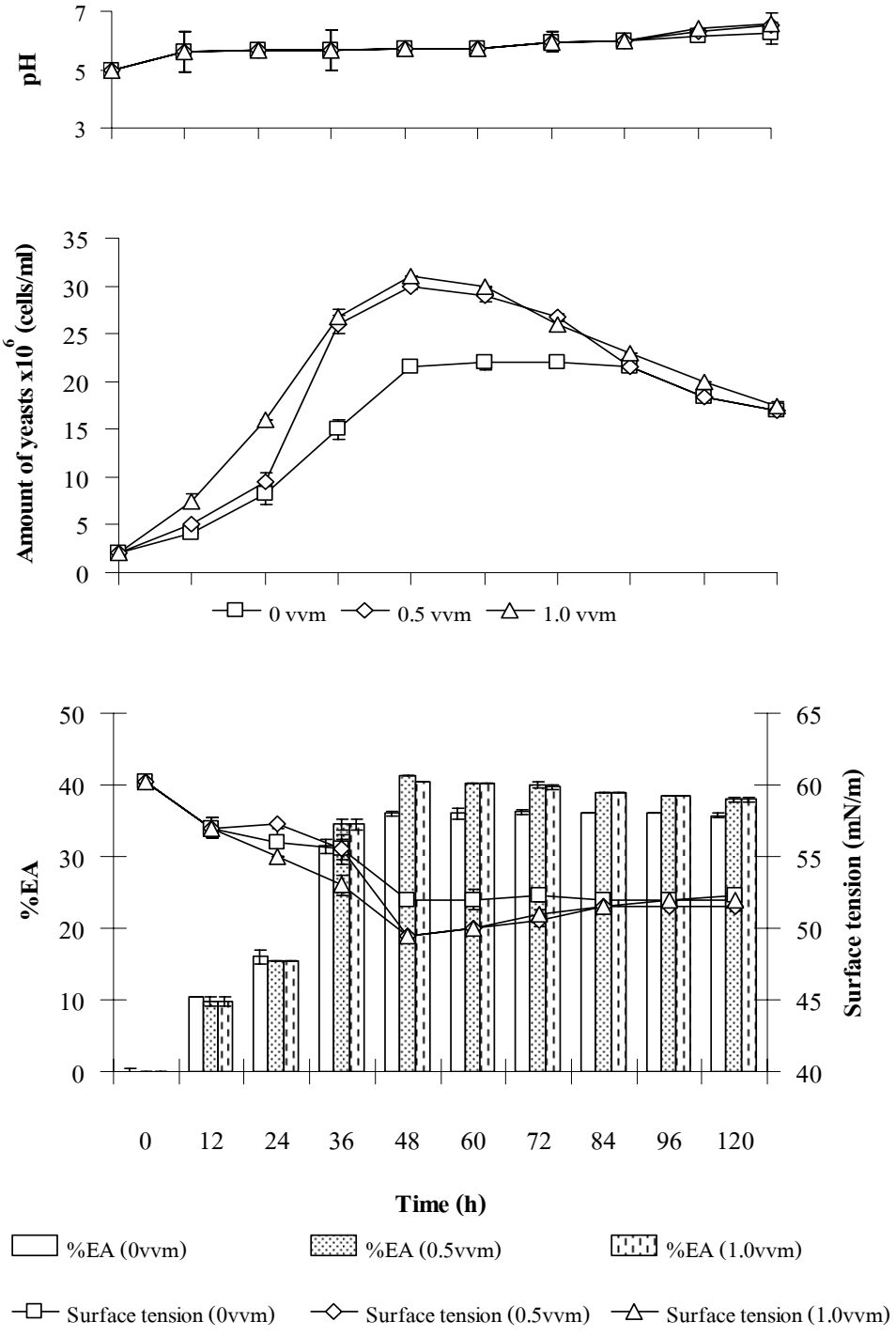


Figure 20. Effect of aeration rate on growth and biosurfactant production by *Issachenkia orientalis* SR4 (in 2L fermentor with 1L working volume in optimal medium, uncontrolled pH, at 30°C and 200 rpm).

3.4.3 Time course of growth and biosurfactant production in fermentor cultivation

I. orientalis SR4 was cultivation in 2L fermentor containing 1L optimal medium, which contained 1 g/L of xylene as a carbon source, 3 g/L of urea as a nitrogen source with the aeration rate 0.5 vvm, agitation speed at 200 rpm and incubation temperature 30°C, without controlled pH. Figure 21 exhibited maximum cell growth of the yeast strain SR4 3.0×10^{7} cells/ml at 48 h of cultivation. In addition, the specific growth rate (μ) was 0.05.

The results of biosurfactant activity in the fermentor, yeast, *I. orientalis* SR4 could reduce surface tension of culture medium from 61.5 to 49.0 mN/m and showed 41.25%EA. *I. orientalis* SR4 showed the same results of the growth and biosurfactant production as in the shake-flask cultivation. In addition, the yield of crude biosurfactant produced by *I. orientalis* SR4 was 250 mg/L.

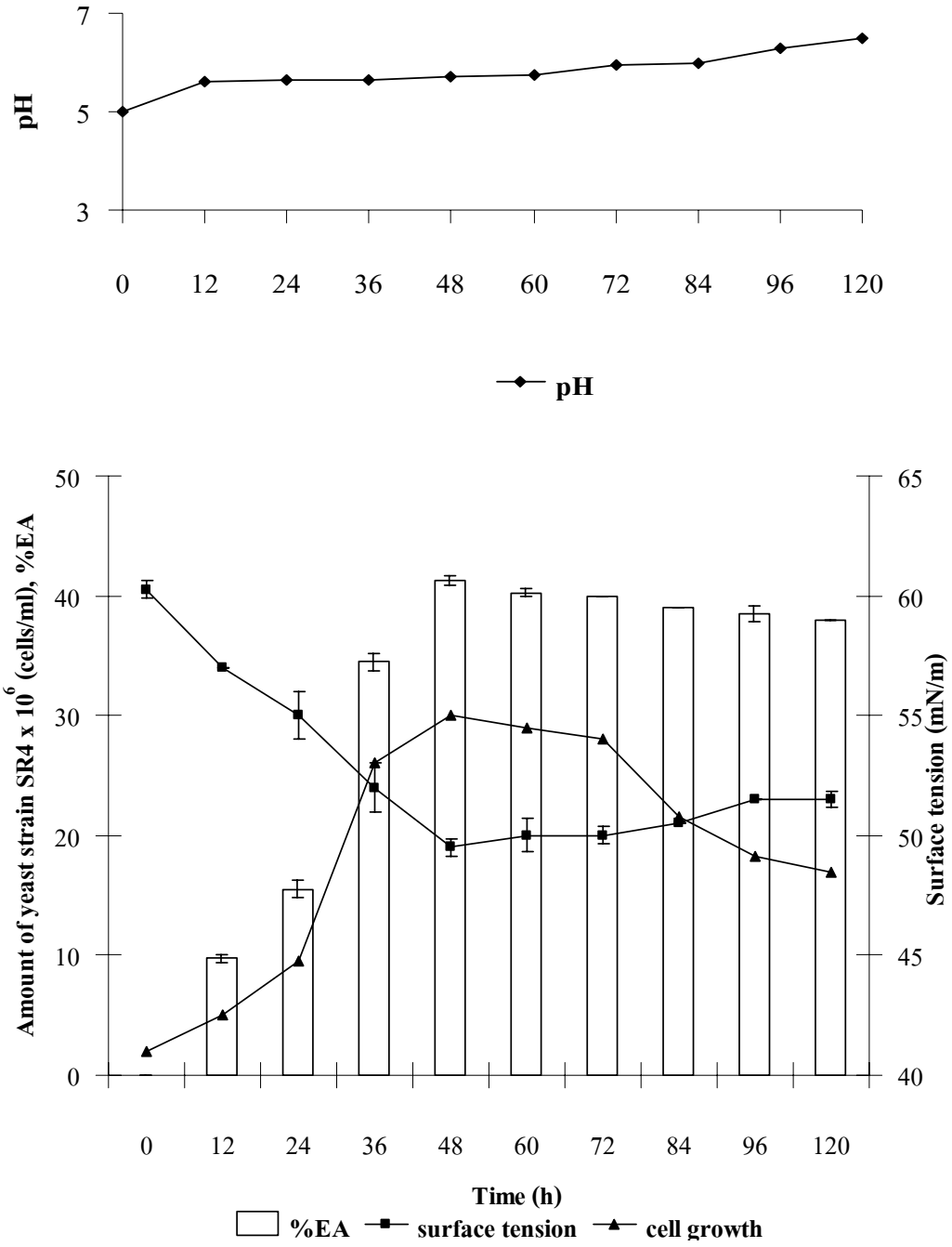


Figure 21. Time course of growth and biosurfactant production by *Issachenkia orientalis* SR4 in a 2L fermentor contained optimal medium (1 g/L of xylene, 3 g/L of urea, uncontrolled pH, shaking speed 200 rpm, at 30°C and aeration rate 0.5 vvm).

4. Recovery of biosurfactant produced by *Issatchenkia orientalis* SR4

The culture broth was obtained from biosurfactant production of *Issatchenkia orientalis* SR4 in a 2L fermentor when cultivated in 1L optimal medium (1 g/L of xylene, 3 g/L of urea, uncontrolled pH, agitation speed with 200 rpm, incubation at 30°C and aeration at 0.5 vvm) and separated by centrifugation at 9,000 rpm for 20 min. The supernatant was extracted with three solvents, ethyl acetate, ethyl acetate (acid condition (pH2.0)) and mixture of chloroform/methanol (2:1) based on the critical micelle concentration (CMC) of crude biosurfactants. The results are showed in Figure 22. The crude biosurfactant extracted by ethyl acetate could reduce the surface tension of water from 72 to 44 mN/m at CMC 0.025 mg/ml. The same result was obtained when extracted by ethyl acetate (acid condition) but at less CMC (0.008 mg/ml). The crude biosurfactant extracted by the mixture of chloroform/methanol could reduce the surface tension of water from 72 to 30 mN/m at CMC 0.00625 mg/ml. The mixture of chloroform/methanol was the best solvent system for crude biosurfactant extraction in this study because it contained both non polar (chloroform) and quite polar (methanol) compounds. They had effect on the structure of the biosurfactant which was composed of hydrophobic and hydrophilic moieties. Thus, the mixture of chloroform/methanol system could extract crude biosurfactant better than single solvent such as ethyl acetate. The mixture of chloroform/methanol (2:1) system was selected for further study.

Extractions of glycolipids and cerebrosides from *Absidia corymbifera* F-295 and *Mortierella alpina* strain KG-1/95 by chloroform/methanol system were also reported (Batrakov *et al*., 2002, 2003).

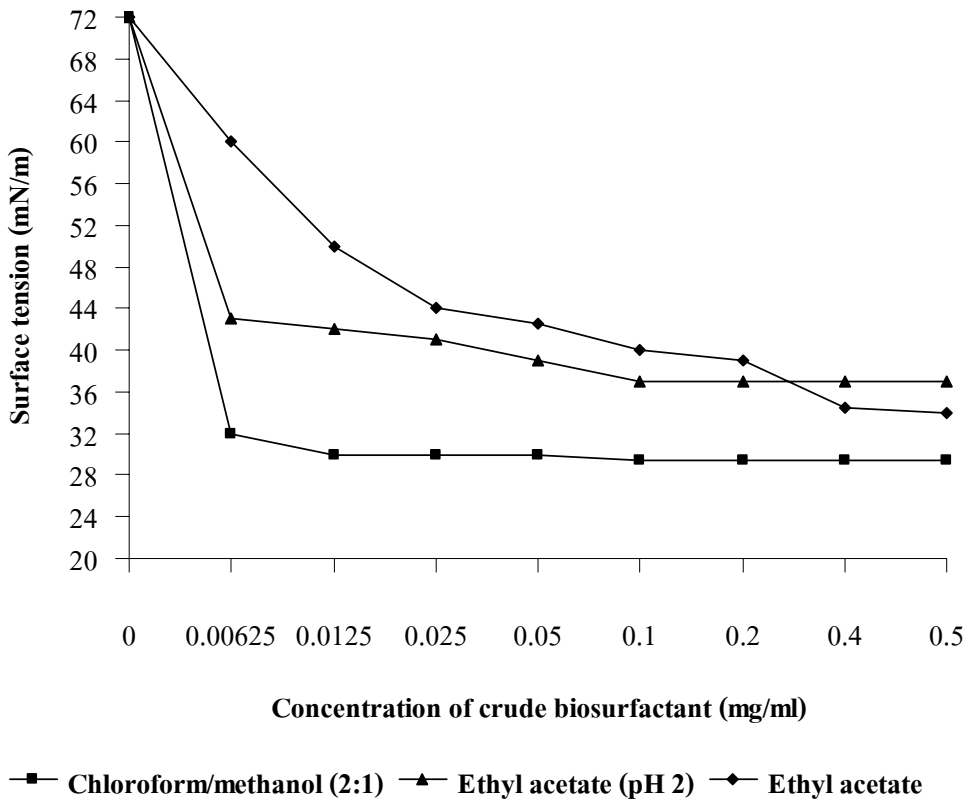


Figure 22. Critical micelle concentration of crude biosufactant of *Issatchenkia orientalis* SR4 extracted by ethyl acetate, ethyl acetate (acid condition, pH 2) and chloroform/methanol (2:1) (v/v).

5. Purification of crude biosurfactant produced by *Issatchenkia orientalis* SR4

Crude biosurfactant was extracted from the culture supernatant two times by chloroform/methanol (2:1) (v/v). Then, the obtained crude biosurfactant was separated by thin layer chromatography (TLC) on silica gel plate using hexane/ethyl acetate (7:3) (v/v) as a developing solvent system. The crude biosurfactant was separated into 7 spots by *p*-anisaldehyde spray reagent (Fig. 23). When the biosurfactant activity was analyzed by ODA test, 5 spots showed biosurfactant activity. The violet spot was observed by *p*-anisaldehyde reagent on TLC plate at Rf 0.34 exhibited the highest biosurfactant activity. The obtained crude extract was extracted two times by chloroform/methanol (2:1) (v/v) and dried by a vacuum rotary evaporator. The dried crude extract was purified by silica gel C-100 column chromatography using

sequencing washes of hexane/ethyl acetate (50:50), (40:60), (30:70) and (20:80), ethyl acetate/methanol (99:1) and 100% methanol. All of the eluted fractions were collected, dried, tested for the biosurfactant activitiy by ODA test and checked pattern of each spot on TLC plate using hexane/ethyl acetate (7:3) (v/v) as a developing system. Two active fractions were eluted with hexane/ethyl acetate in the ratio of 1:9 and 100% ethyl acetate, respectively in the silica gel column chromatography showed 2 spots with Rf value at 0.50 and 0.34, respectively when analyzed by TLC plate using hexane/ethyl acetate (7:3) (v/v) as a developing solvent. The compound from the spot at Rf 0.34 exhibited higher biosurfactant activity than the compound from the spot at Rf 0.50. So, the compound from the spot at Rf 0.34 was chosen for further purification. This active fraction was purified by silica gel (C-200) column chromatography using sequencing washes of hexane/ethyl acetate (20:80), (10:90) and 100% ethyl acetate and ethyl acetate/methanol (99:1) and 100% methanol. All eluted fractions were collected, dried, tested for the biosurfactant activity and checked pattern of spot on TLC plate. The pooled highest active fraction showed the highest biosurfactant activity at Rf 0.34 (violet spot) using hexane/ethyl acetate (7:3) (v/v) as a developing solvent system on TLC plate. The pooled highest active fraction was then applied to Inertsil ODS-3 column preparative HPLC with pressure 500 pound per square inch (PSI) and at flow rate of 10 ml/min. The preparative HPLC chromatogram of crude biosurfactant produced by *I. orientalis* SR4, the results are shown in Figure 24. It could be seen that the crude biosurfactant are mixture containing many compounds. The main product was occurred at retention time 32.88 min because of it was the highest amount and biosurfactant activity. The purified compound was finally recovered, which exhibited single peak at a retention time 8.59 min by analytical HPLC (Fig. 25) when eluting with 95% acetonitrile supplemented with 0.05%TFA and gave a single spot of Rf, 0.34 on TLC plate (Fig. 26). The purified biosurfactant was used for further structure elucidation.

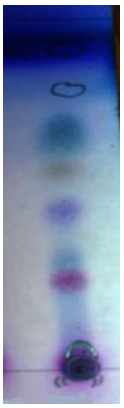
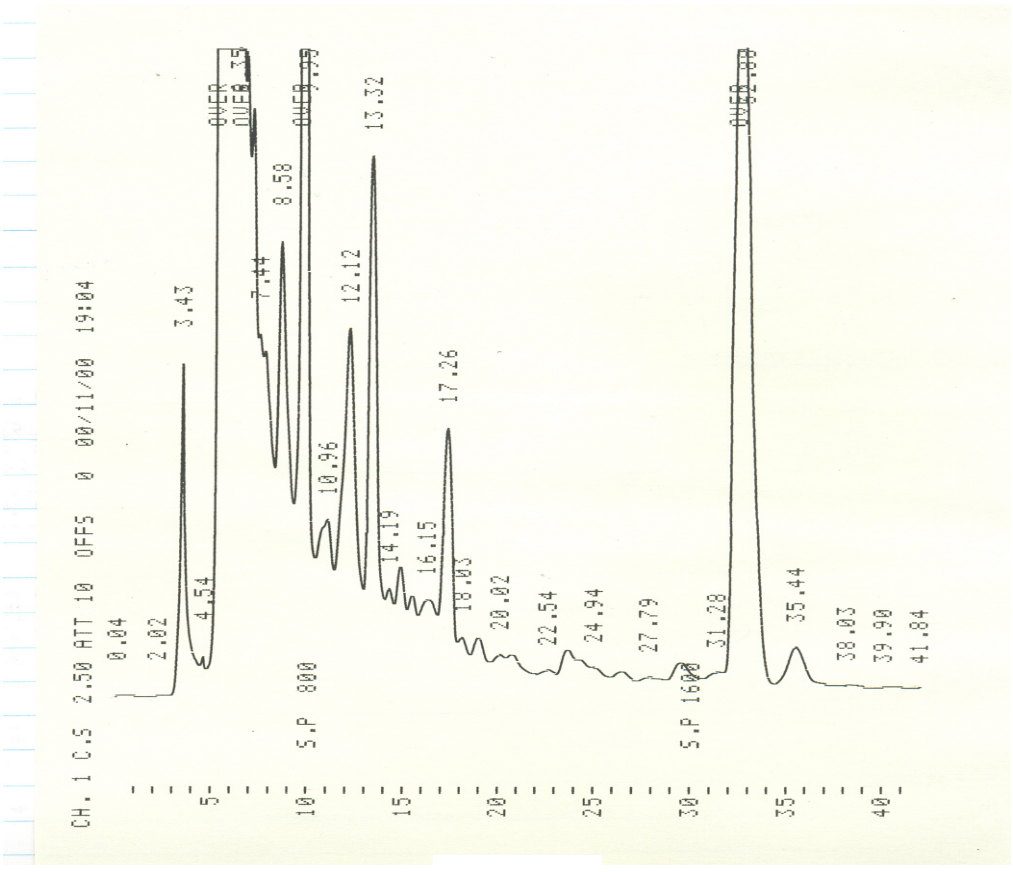


Figure 23. Thin layer chromatography analysis of the crude biosurfactant using hexane/ethyl acetate (7:3) (v/v) as a developing solvent system, sprayed with *p*-anisaldehyde reagent.



Time (min)

Figure 24. Preparative HPLC chromatogram of crude biosurfactant produced by *Issatchenkia orientalis* SR4 using 95% acetonitrile supplemented with 0.05% trifluoroacetic acid as an eluent.

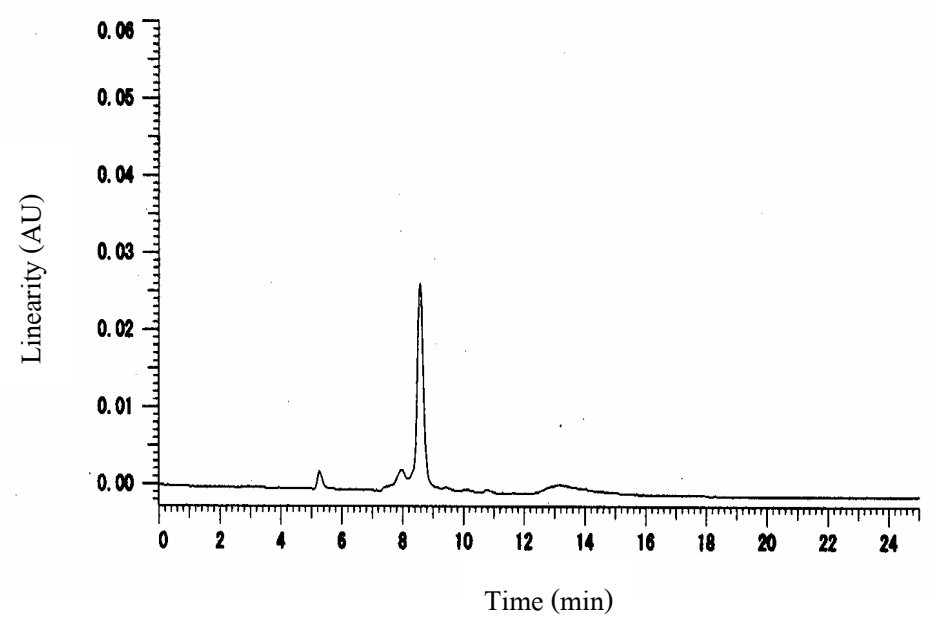


Figure 25. Analytical HPLC chromatogram of purified compound by preparative HPLC using 95% acetonitrile supplemented with 0.05% trifluoroacetic acid as an eluent.

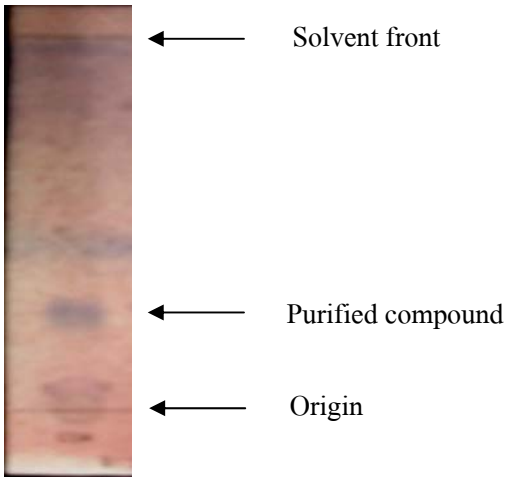


Figure 26. Thin layer chromatography analysis of the purified biosurfactant using hexane/ethyl acetate (7:3) (v/v) as a developing solvent system, sprayed with *p*-anisaldehyde reagent.

6. Structure elucidation of the purified biosurfactant

The purified biosurfactant obtained from a preparative HPLC when eluting with 95% acetonitrile supplemented with 0.05%TFA was elucidated chemical structure by ^{1}H-NMR spectrum (Fig. 27). The ^{1}H-NMR spectrum indicated olefinic protons at the chemical shift 5.4, suggested the presence of a monounsaturated fatty acid moiety in this compound. According to the result of ^{1}H-NMR spectrum, this purified biosurfactant could not be identified because of a few impurity was contaminated in the compound at the chemical shift 2.07 (-CH_2-CH=CH-) and 2.30(-CH_2-CH_2-COOR). Thus, this compound should be purified and then further elucidated chemical structure. The purified biosurfactant obtained from a preparative HPLC was analyzed by GC-MS. The purified compound was a mixture of compounds differing in carbon chain lengths namely, 9-octadecanoic acid (Z) at a retention time 10:13 min was the major, pentadecanoic acid at a retention time 8:32 min was the second major and octadecanoic acid at a retention time 10:29 min was the last by GC-MS analysis (Fig. 28). When these compounds were separated by methanolysis with HCl-methanol, the result after methanolysis showed GC-MS total ion chromatogram of the derivative obtained and exhibited a single peak with a retention time 10:11 min (Fig. 29A). It showed the same retention time with commercial methyl oleate (Fig. 29B). Furthermore, the purified compound was analyzed by co-injection with commercial methyl oleate at retension time 10.10 min (Fig 29C). Thus, the fatty acid moiety was determined to be oleic acid methyl ester (methyl oleate) by GC-MS analysis.

The mass spectrum of this peak showed a weak molecular ion at m/z 296 and prominent ions at m/z 265, 223 and 181 (Fig. 30A) was compared with commercial methyl oleate (Fig. 30B). The fragmentation pattern and a mass spectral library-search suggested that the compound corresponding to this peak was methyl oleate. This preliminary identification was confirmed by co-injection of authentic methyl oleate (Fig. 30C).

Structure elucidation of purified compound after methanolysis with HCl-methanol was confirmed by analytical ODS-HPLC (Fig. 31A) comparison with oleic acid (Fig. 31B). The result of co-injection of purified compound and oleic acid showed that there was the presence of oleic acid at a retention time 8.29 min (Fig. 31C). Purified biosurfactant could be detected and observed by sprayed reagent. The result showed yellow spot (organic acid) after sprayed with bromocresol green and violet spot with *p*-anisaldehyde on TLC plate. Thin layer chromatography

of the purified compound after ODS-HPLC was shown in Figure 32A comparison with authentic oleic acid (Fig. 32B).

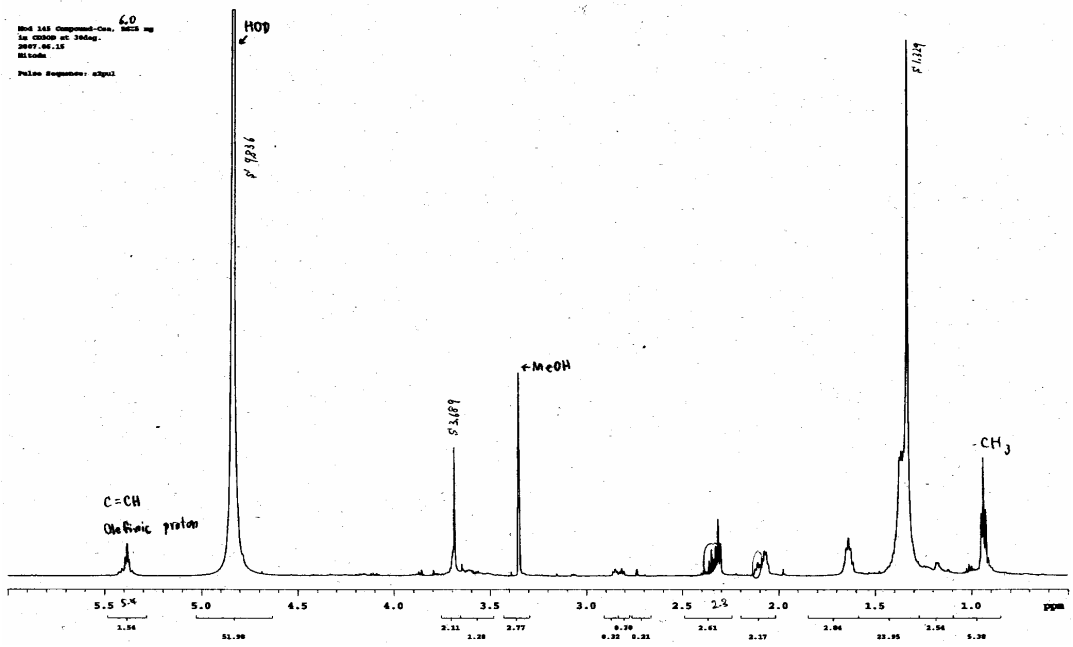


Figure 27. ^{1}H-NMR spectrum of purified compound.

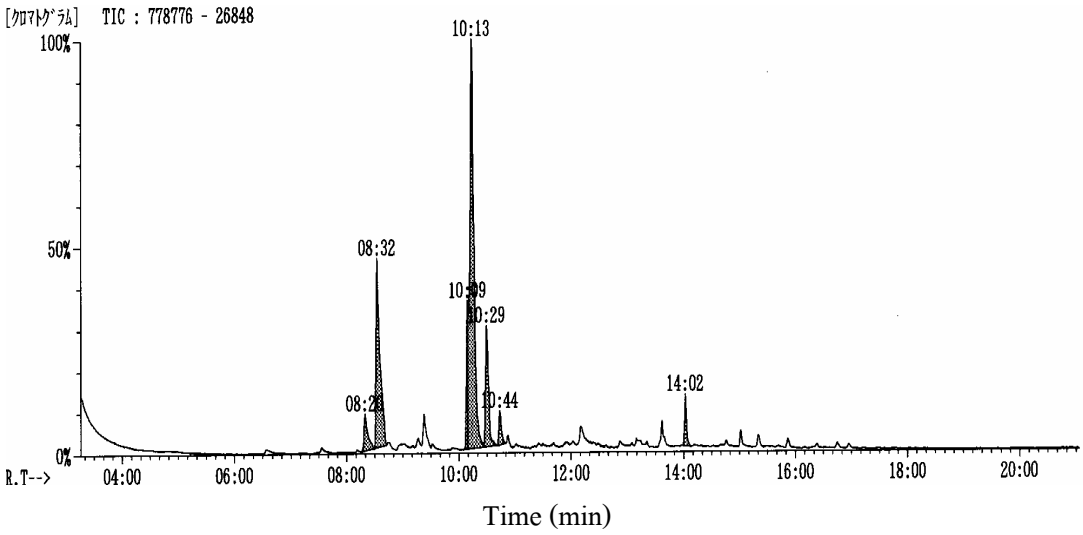


Figure 28. Chromatogram of purified compound analyzed by GC-MS.

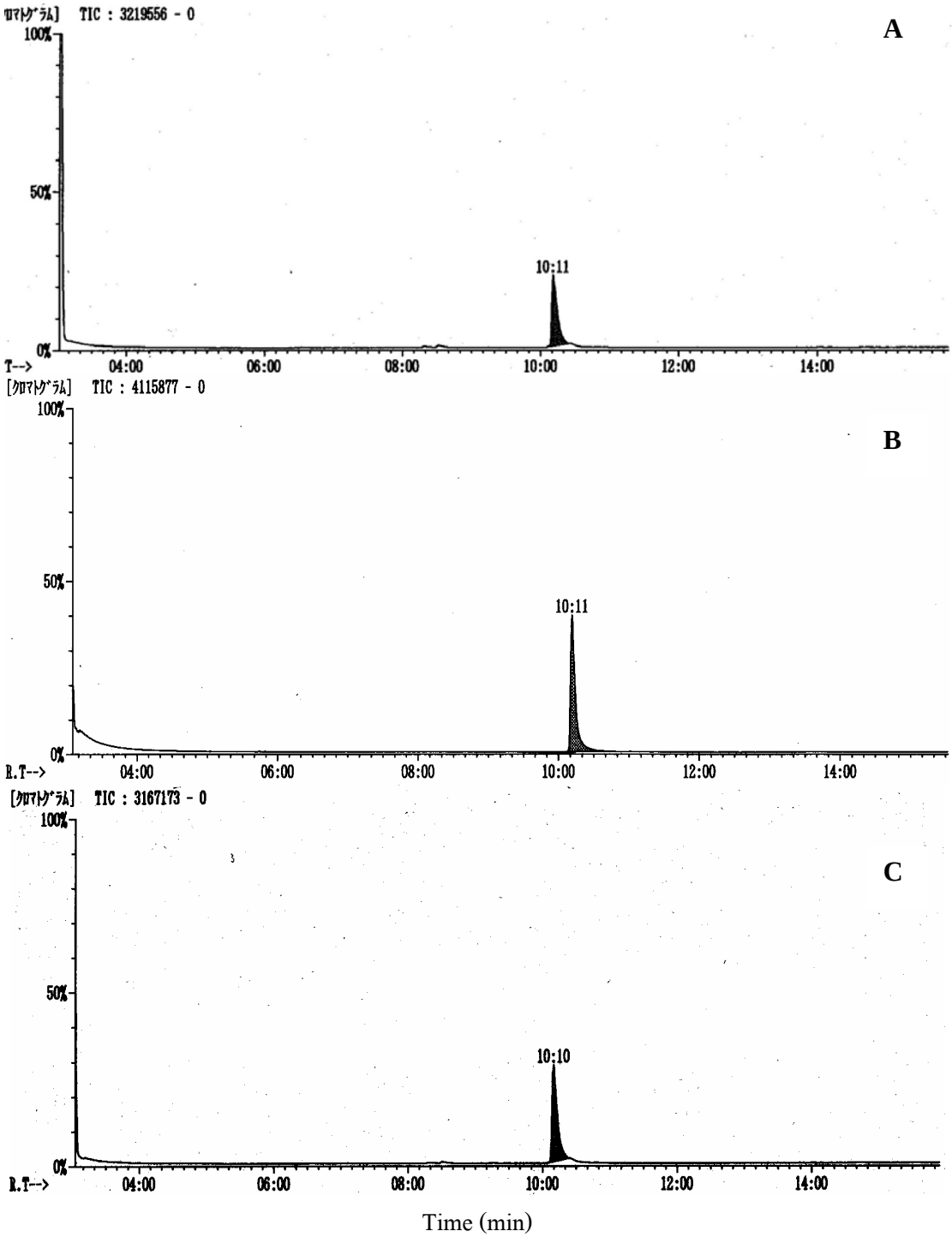


Figure 29. Chromatogram of purified compound by a methanolysis and GC-MS analysis, purified compound (A), Methyl oleate (B) and Co-injection of purified compound and methyl oleate (C).

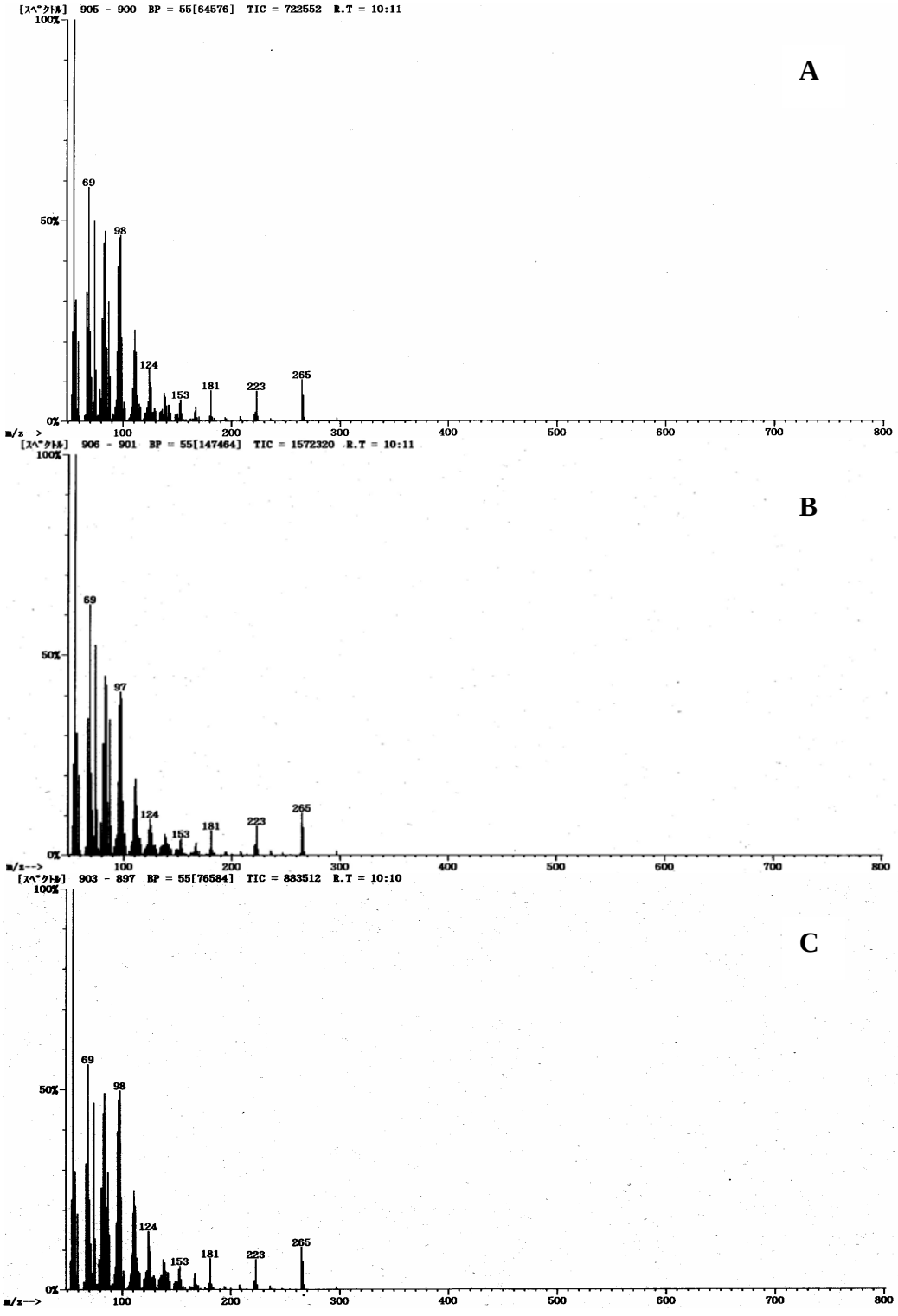


Figure 30. EI-MS spectrum of purified compound methyl ester (A), methyl oleate (B) and co-injection of purified compound methyl ester and methyl oleate (C).

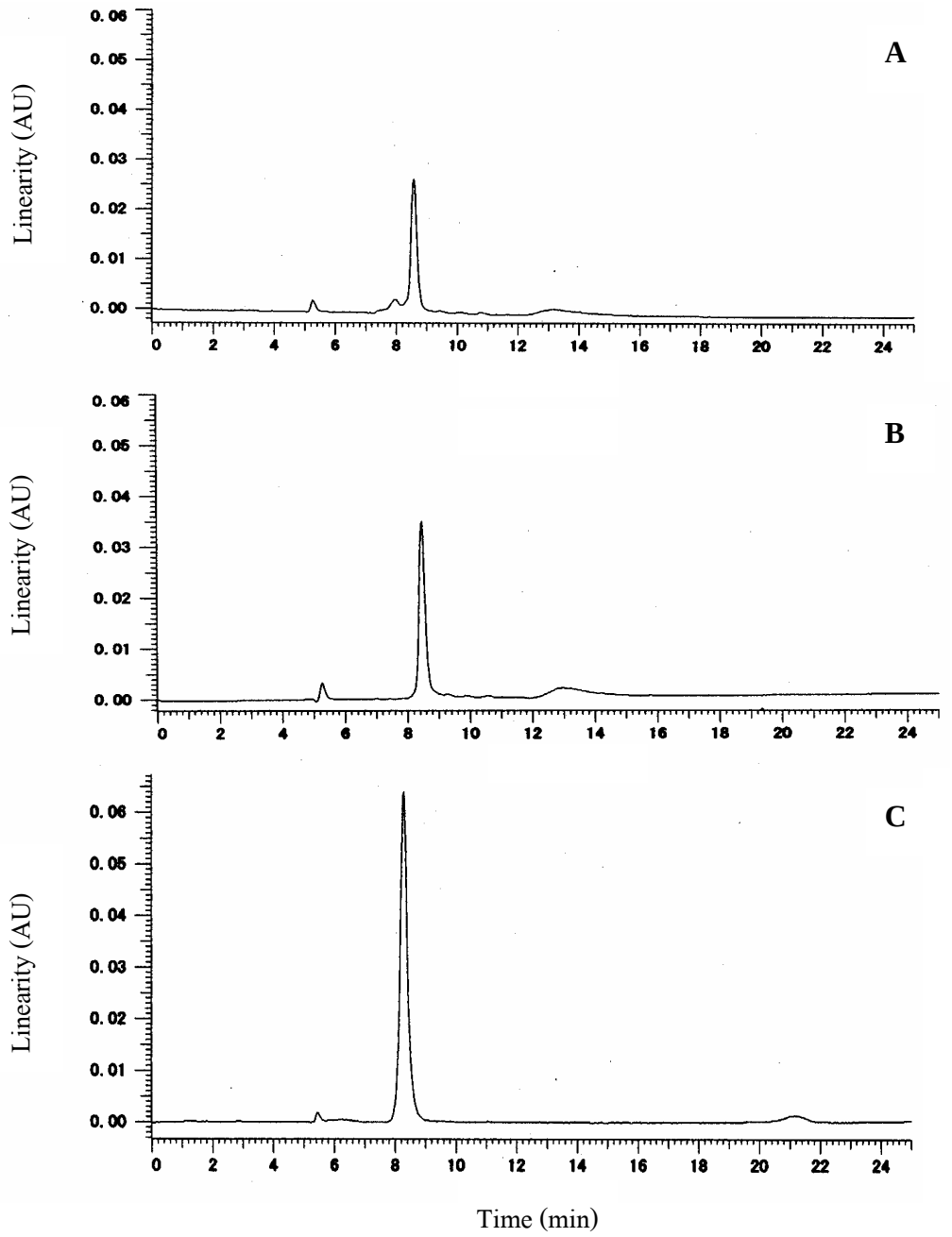


Figure 31. Chromatogram of purified compound (A), oleic acid (B) and co-injection of purified compound and oleic acid (C) using 95% acetonitrile supplemented with 0.05% trifluoroacetic acid as an eluent by analytical HPLC (detected at wavelength 210 nm).

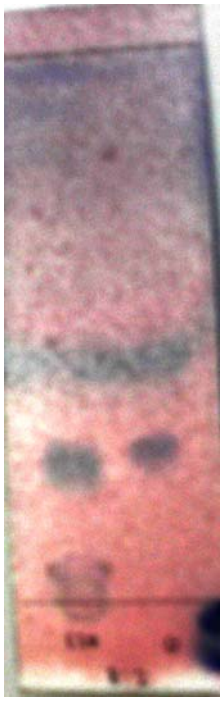


Figure 32. Thin layer chromatography analysis of the purified compound (A) after ODS-HPLC was compared with oleic acid (B) using hexane/ethyl acetate (7:3) (v/v) as a developing solvent system, sprayed with *p*-anisaldehyde reagent.

6.1 Origin of biosurfactant

The origin of the biosurfactant was examined from the BSM supplemented with 1 g/L of xylene without inoculated culture, BSM supplemented with 1 g/L of xylene with inoculated yeast strain SR4 and the purified compound were extracted using the same protocol as the biosurfactant extraction. No spot was detected in the BSM without inoculated culture while one spot of BSM with inoculated yeast strain SR4 was detected as a blue-green spot with *R*f value of 0.34 on TLC by *p*-anisaldehyde sulfuric acid reagent hexane/ethyl acetate (7:3) as a developing solvent system (Fig. 33). In addition, a target peak at retention time 8.29 min of biosurfactant was detected only in the crude extract of BSM with inoculated yeast strain SR4 by analytical HPLC (Fig. 34). It was agreeable with the result of co-injection of purified compound and oleic acid by analytical ODS-HPLC (Fig. 34C) which showed that there was the presence of oleic acid at a retention time 8.29 min in the compound. This result proved that the biosurfactants was produced by the yeast, *I. orientalis* SR4.

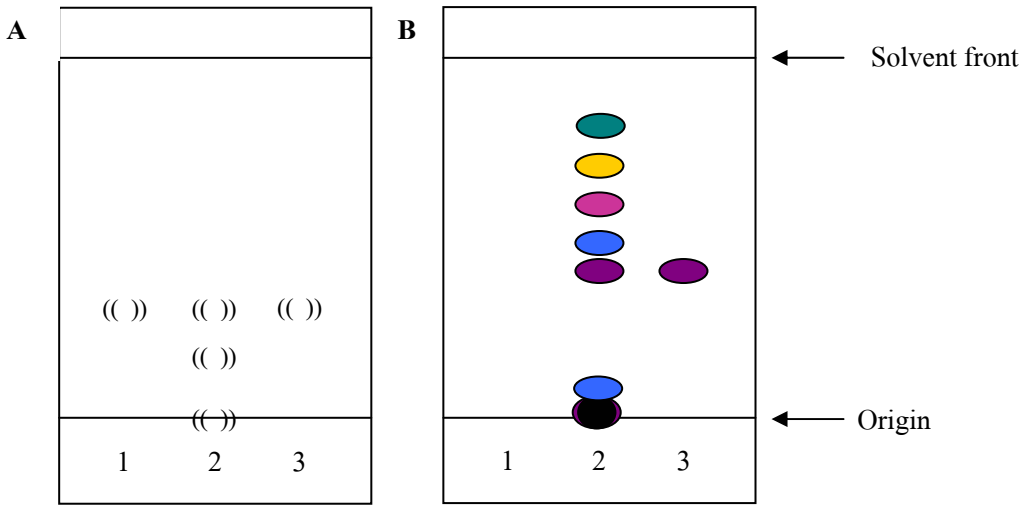


Figure 33. Thin layer chromatography analysis of crude extract of BSM (without inoculated culture) (1), crude extract of BSM (with inoculated yeast strain SR4) (2) and the purified compound (3) using hexane/ethyl acetate (7:3) as a developing solvent system.

Note: (( )) mean wavelength 365 nm under UV (A) and by *p*-anisaldehyde spray reagent (B) observation.

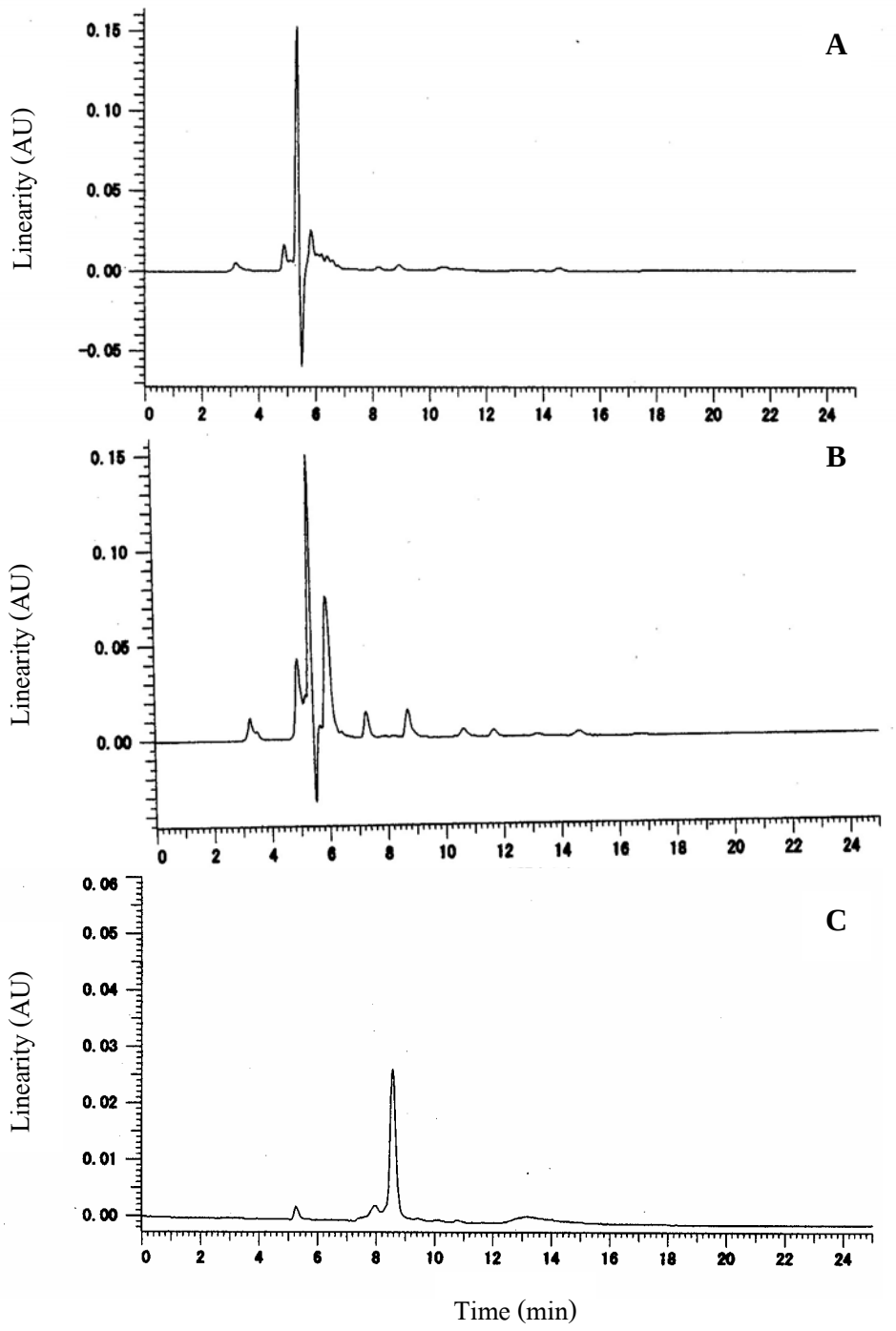


Figure 34. Chromatogram of crude extract when extracted from BSM without inoculated yeast strain SR4 (A) BSM with inoculated culture (B) and co-injection of purified compound and oleic acid (C) using 95% acetonitrile supplemented with 0.05% trifluoroacetic acid as an eluent analyzed by HPLC (detect at wavelength 210 nm).

7. Characteristic of crude biosurfactant

7.1. Effect of salts on the stability

An average, seawater in the world's oceans has a salinity of about 3.5%. The dissolved salts of sea water mostly, but not entirely, the ions of NaCl (3.02%) and small amount of $CaCl_2$ (0.04%) and $MgCl_2$ (0.13%). The biosurfactant might have applications in environmental protection for example, oil contaminated in sea water. The effect of salts on stability of biosurfactant activity was studied.

The effect of NaCl on the stability of the crude biosurfactant which produced by *I. orientalis* SR4 was shown in Figure 35. The activity of the biosurfactant was decreased when increased the concentration of NaCl to 2%. According to this result the crude biosurfactant was not suitable for application in sea water which contains NaCl 3.02%.

This result was better than a glycolipid-type biosurfactant, mannosylerythritol lipid (MEL-SY16) produced by *Candida* sp. SY16 against NaCl to 0.58% (Kim *et al*., 2002a) but more inferior to a biosurfactant produced by the newly isolated *Candida glabrata* which was stable against NaCl concentrations up to 10% (Sarubbo *et al*., 2006).

Thimon *et al*. (1992) reported the ion of salts had effect on the structure of biosurfactant. Salt ions will bind the carboxylic group of biosurfactant and then the surface tension reduction area between water/air was lost. In addition, Figure 36 showed the resistance of crude biosurfactant to $CaCl_2$ and $MgCl_2$. The activity of the crude biosurfactant was stable in 0-0.02% and 0-0.05% of $CaCl_2$ and $MgCl_2$, respectively.

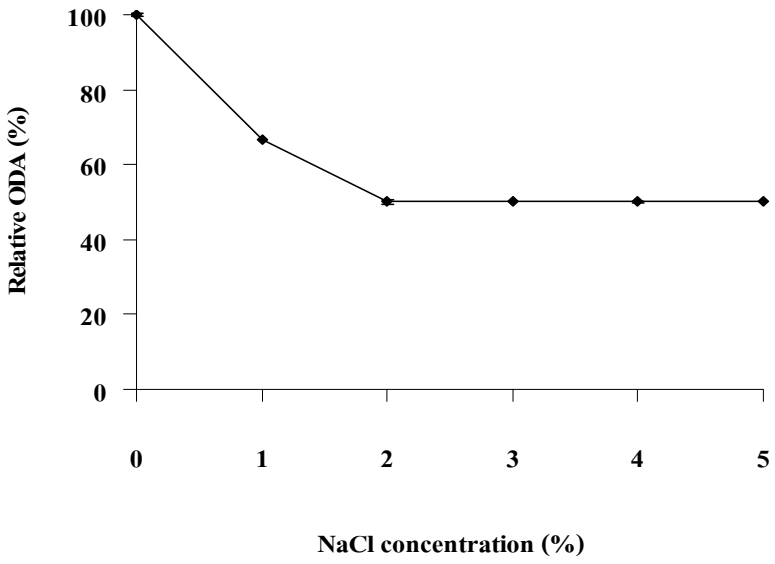


Figure 35. Effect of NaCl on the activity of crude biosurfactant produced by *Issatchenkia orientalis* SR4.

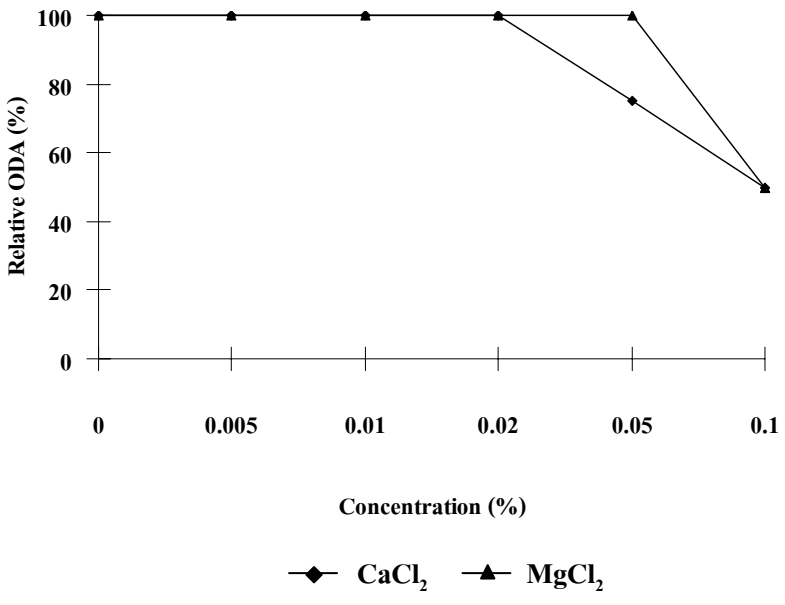


Figure 36. Effect of $CaCl_2$ and $MgCl_2$ on the activity of crude biosurfactant produced by *Issatchenkia orientalis* SR4**.**

7.2 Effect of pH on the stability

The effect of pH on the stability of crude biosurfactant by the selected yeast strain, SR4 was shown in Figure 37. The solution of the crude extract was adjusted to various pH ranging from 2.0 to 10.0 by 0.5 N HCl or 0.5 N NaOH and kept for 24 h at 4°C. The crude extract could retain the activity (100%) at the pH range 4.0 to 10.0.

The mannosylerythritol lipid (MEL-SY16) produced by *Candida* sp. SY16 was also stable from pH 4 to10 (Kim *et al*., 2002a). However, the biosurfactant produced by *Candida glabrata* was stable at wider ranges (pH 2-12) (Sarubbo *et al*., 2006).

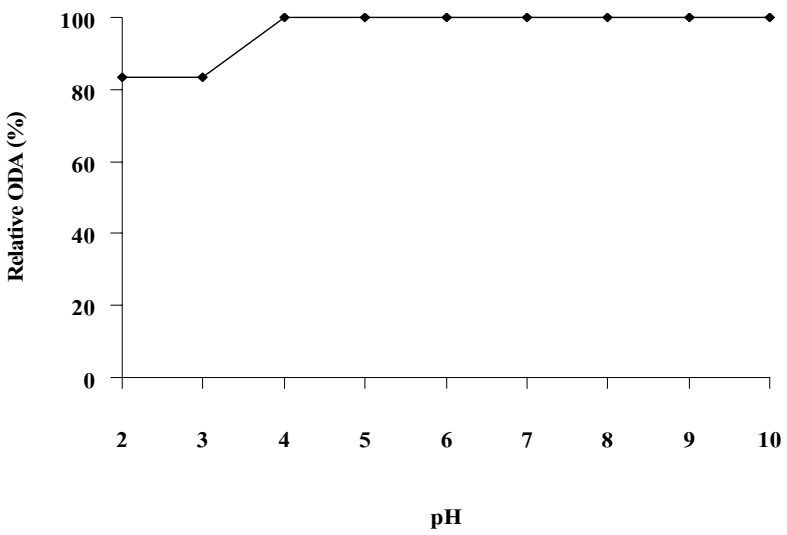


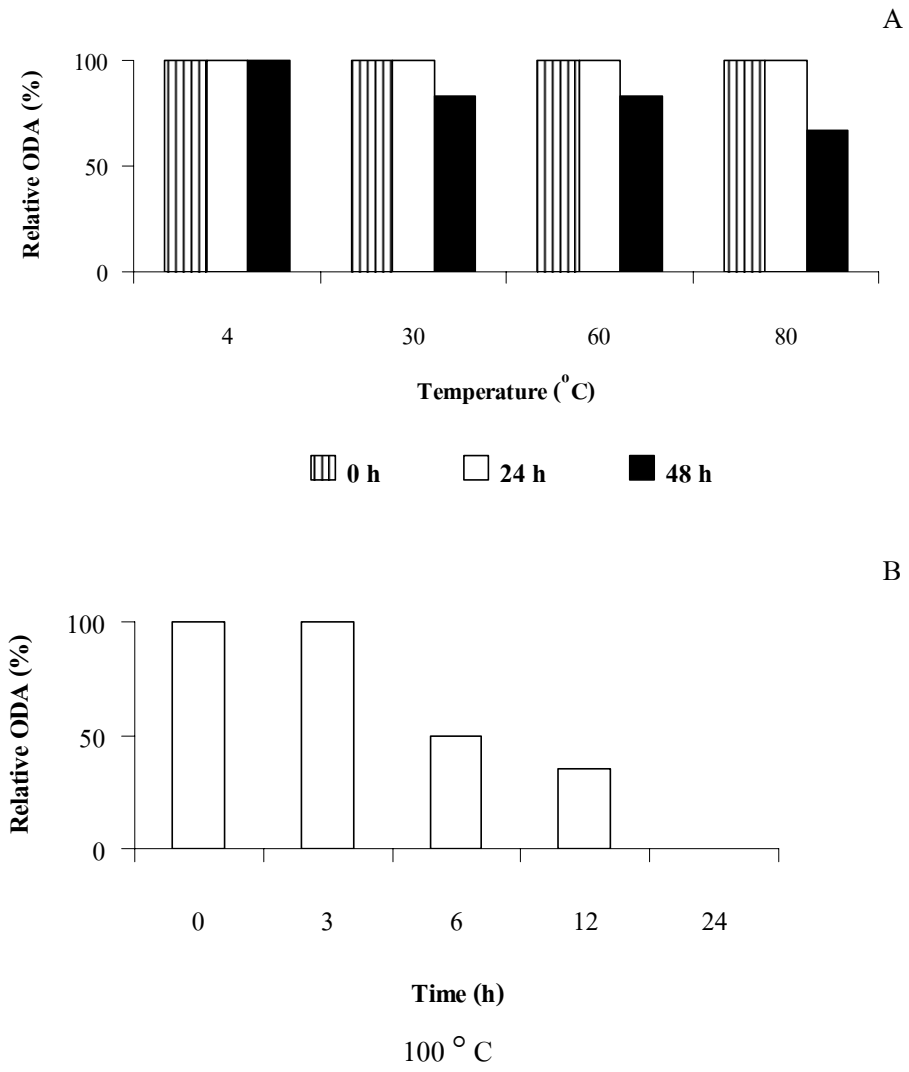
Figure 37. Effect of pH on the stability of crude biosurfactant produced by *Issatchenkia orientalis* SR4**.**

7.3 Effect of temperature on the stability

The temperature stability of the crude biosurfactant was examined by incubating the crude extract at various temperatures such as 4, 30, 60 and 80°C for 48 h and 100°C for 3, 6, 12 and 24 h. The samples were taken for measuring the ODA value. The result showed the stability of the crude biosurfactant at 4 to 80°C for 24 h (Fig. 38A). Moreover, the biosurfactant activity was stable at 4°C for 48 h might be due to this temperature was cool. While the temperature at 30 to 80°C after 24 h the biosurfactant activity was decreased. Furthermore, the

stability of the crude biosurfactant at 100°C, it retained 100% for 3 h after 3 h the biosurfactant activity was decreased (Fig. 38B). The increasing of temperature and incubation time had effect on the stability of biosurfactant activity. So when increasing temperature the stability of biosurfactant activity was decreased.

This result was the same as a biosurfactant was produced by the newly isolated and promising strain *Candida glabrata*. The stability of biosurfactant activity within a temperature (4-80°C) ranges (Sarubbo *et al*., 2006) whereas glycolipid-type biosurfactant, (MEL-SY16) produced by *Candida* sp. SY16 stable up to 90 °C for 1 h (Kim *et al*., 2002a).



100 ° C

Figure 38. Effect of temperature on the stability of crude biosurfactant produced by *Issatchenkia orientalis* SR4 at 4-80°C (A) and 100°C (B).

7.4 Comparison with synthetic surfactants

The purified biosurfactant of *I. orientalis* SR4 from a preparative HPLC when eluting with 95% acetonitrile supplemented with 0.05%TFA showed almost the same surface activity as an authentic oleic acid. The minimum active dose (MAD) of the purified biosurfactant was higher than that of synthetic surfactants such as SDS (anionic surfactant), triton X-100 and triton X-114 (non-ionic surfactant), as shown in Table 16. These synthetic surfactants were selected in this study because the biosurfactant usually were anionic and non-ionic surfactant. Table 17 showed emulsification activity of the purified compound when compared with authentic oleic and synthetic surfactants. The emulsification activity of the purified compound was equal to the emulsification activity of authentic oleic acid with xylene (25.0%EA) and *n*-hexadecane (30.0%EA). However, its emulsification activity was lower than those of the synthetic surfactants. The biosurfactant activity was not necessary to have both properties of surface tension reduction and emulsifing. Because the purified compound was the fatty acid biosurfactant, it was more eminent to reduce surface tension than to be emulsifier (Rosenberg and Ron, 1999).

With increasing environmental awareness and emphasis on a sustainable society in harmony with the global environment, biosurfactants have been becoming much more important than synthetic surfactants. The monoacylglycerols of the culture of a *Candida ishiwadae* which exhibited a relatively high surfactant activity. Comparative surfactant activity using a modified drop collapse method showed that the monoacylglycerols have higher activity than the synthetic surfactants (Thanomsub *et al*., 2004). Moreover, *Candida* sp. SY16 produces a glycolipid-type biosurfactant, MEL-SY16 could emulsify hydrocarbons, vegetable oil, and crude oil (Kim *et al*., 2002a).

Table 16. Minimum active dose (MAD) of purified biosurfactant, oleic acid and synthetic surfactants by oil displacement area test

Compound	MAD (μg)
Purified compound from the strain SR4 culture broth	0.625
Oleic acid (guaranteed reagent)	0.500
Sodium dodecyl sulfate (SDS)	6.250
Triton X-100	1.250
Triton X-114	1.250

Table 17. Emulsification activity of purified biosurfactant, oleic acid and synthetic surfactants

Compound	%EA	
	Xylene	*n*-Hexadecane
Oleic acid (purified from the strain SR4 culture broth)	25.0	30.0
Oleic acid (guaranteed reagent)	25.0	30.0
Sodium dodecyl sulfate (SDS)	76.9	76.9
Triton X-100	40.5	55.0
Triton X-114	40.5	55.0

8. Application of biosurfactant for bioremediation

8.1 Solubilization of polycyclic aromatic hydrocarbons (PAHs)

Polycyclic aromatic hydrocarbons (PAHs) are ubiquitous environmental pollutants. Excessive inputs from anthropogenic activities have caused serious contamination and adversely affect the health of aquatic life and human through bioaccumulation. PAHs are hydrophobic and readily adsorbed onto particulate matter; therefore, coastal and marine sediments

become the ultimate sinks and elevated concentrations have been recorded (Hughes *et al*., 1997). Solubilization of PAHs depends on the type and dose of the surfactant, the hydrophobicity, the surfactant- soil interactions, and the time that the contaminant has been in contact with the soil (Zhou and Rhue, 2000). The effect of crude biosurfactant produced by *I. orientalis* SR4 on solubility enhancement of PAHs such as naphthalene, phenanthrene and pyrene was investigated in comparison with synthetic surfactant (SDS) and distilled water. The obtained crude biosurfactant was extracted by a mixture of chloroform/methanol (2:1) and dissolved in 5 ml distilled water to obtain the concentration of 10, 50 and 100 mg/L. The crude biosurfactant enhance the solubility of naphthalene (49.0 mg/L) more than SDS (46.0 mg/L) and distilled water (31.0 mg/L) (Fig. 39A). In addition, the crude biosurfactant enhanced the solubility of phenanthrene (4.2 mg/L) more than SDS (4.0 mg/L) and distilled water (1.2 mg/L) (Fig. 39B). The crude biosurfactant enhanced the solubility of naphthalene and phenanthrene more than SDS and distilled water with significant difference ($p<0.05$). While the solubility of pyrene (0.9 mg/L) by this crude biosurfactant was lower than SDS (1.2 mg/L) but higher than distilled water (0.1 mg/L) (Fig. 39C) with significant difference ($p<0.05$). The structure of pyrene is consisting of 4 benzene rings so, it is more difficult to dissolve than naphthalene (2 benzene rings) and phenanthrene (3 benzene rings).

The results suggested that the crude biosurfactant produced by *I. orientalis* SR4 could enhance the solubility of hydrophobic compounds. So it has a potential application in bioremediation. In addition, alasan, a high-molecular-weight bioemulsifier complex of an anionic polysaccharide and proteins produced by *Acinetobacter radioresistens* KA53, enhanced the aqueous solubility and biodegradation rates of polycyclic aromatic hydrocarbons (PAHs). In the presence of 500 mg of alasan/ml, the apparent aqueous solubilities of phenanthrene, fluoranthene, and pyrene were increased 6.6-, 25.7-, and 19.8-fold, respectively. While the crude biosurfactant produced by by *I. orientalis* SR4 enhanced the solubility of naphthalene and phenanthrene from distilled water (negative control) which were increased 1.58-, 3.5-, and 9-fold, respectively. Physicochemical characterization of the solubilization activity suggested that alasan solubilizes PAHs by a physical interaction, most likely of a hydrophobic nature, and that this interaction is slowly reversible (Barkay *et al*., 1999).

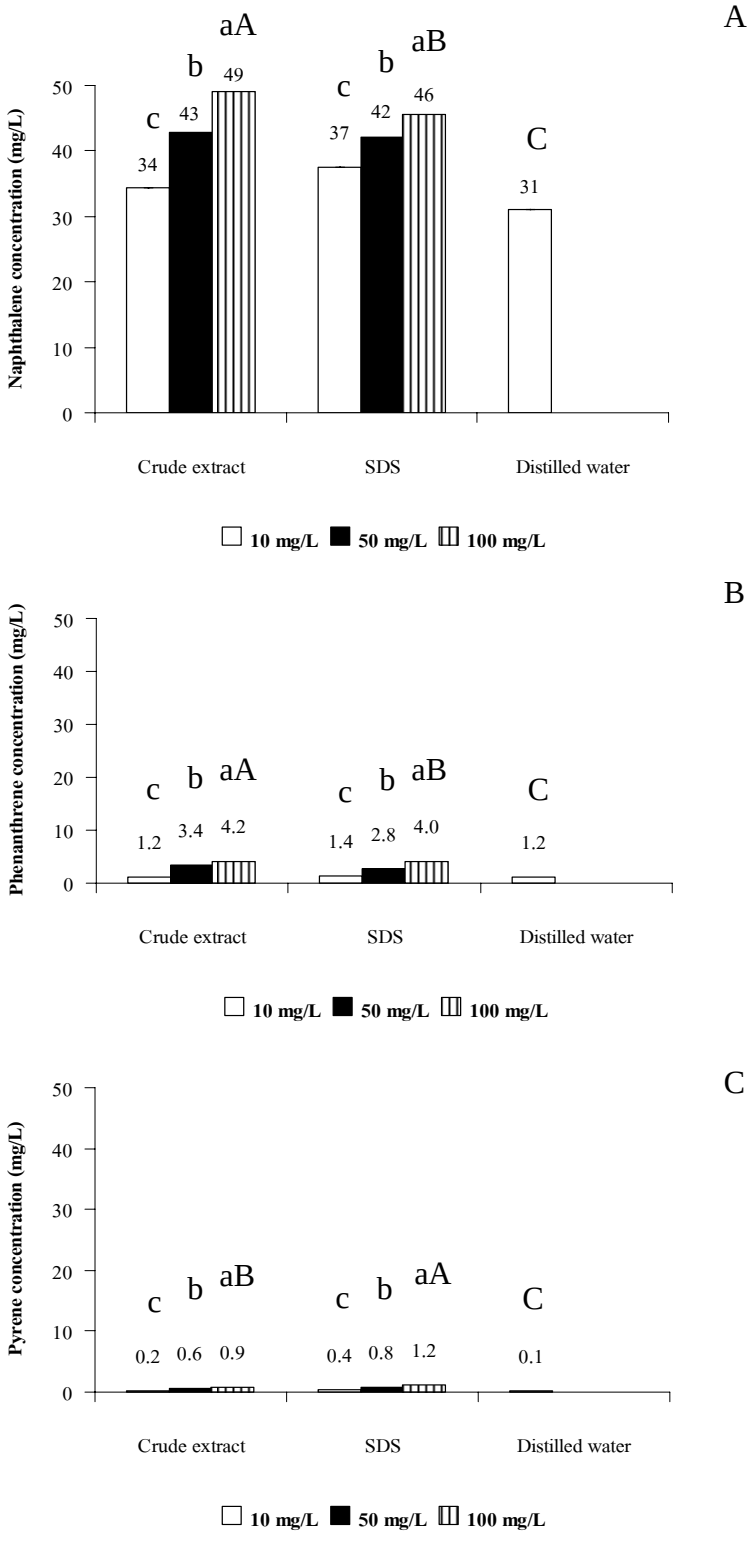


Figure 39. Effect of the crude biosurfactant concentration on naphthalene (A), phenanthrene (B) and pyrene (C) solubilization.

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8.2 Bioremediation of waste lubricating oil-contaminated soil

Waste or used lubricating oils have become a serious environmental problem. In the environment, the waste oil could bind to organic matter, mineral particles and organisms so waste lubricating oil was the target oil for biodegradation. Table 18 showed characteristics of waste lubricating oil contaminated soil. The soil sample was mainly sandy loam soil, consist of 73.94% sand. Numerous factors limit biodegradation of pollutants in the environment were investigated (Providenti *et al*., 1993). One factor was the limited bioavailability of contaminants to microorganisms. Contaminants may be unavailable because of their hydrophobic nature and sorption to soil. Polluted soils may be remediated by inoculating with microorganisms capable of degrading the target contaminants. Factors that could affect survival and degradation activity of microorganisms in soil include soil texture, environmental parameters (e.g. pH, temperature, redox potential, aeration rate), predation by protozoans, displacement by native microbial populations, and competitive fitness of introduced strains (Providenti *et al*., 1995).

The bioremediation of waste lubricating oil-contaminated soil had 4 treatments, abiotic treatment (sterile waste lubricating contaminated soil), abiotic treatment with crude biosurfactant, biotic treatment (non sterile waste lubricating contaminated soil) and biotic treatment with crude biosurfactant. The amount of microorganisms of each treatment was determined on plate count agar (PCA) (Appendix 1). The result of abiotic treatment (sterile waste lubricating contaminated soil) showed no microorganisms while another treatment, biotic treatment (non sterile waste lubricating oil contaminated soil) with crude biosurfactant exhibited the highest amount of microorganisms when determined from 0, 15 and 30 days of cultivation (Fig. 40).

Table 18. Characteristics of waste lubricating oil contaminated soil

	Soil property
Texture	Sandy loam
- clay (%)	10.44
- silt (%)	15.62
- sand (%)	73.94
pH	6.44
Moisture content (%)	5.23
Organic matter (%)	2.55
Total nitrogen (%)	0.25
Available phosphorus (%)	0.028

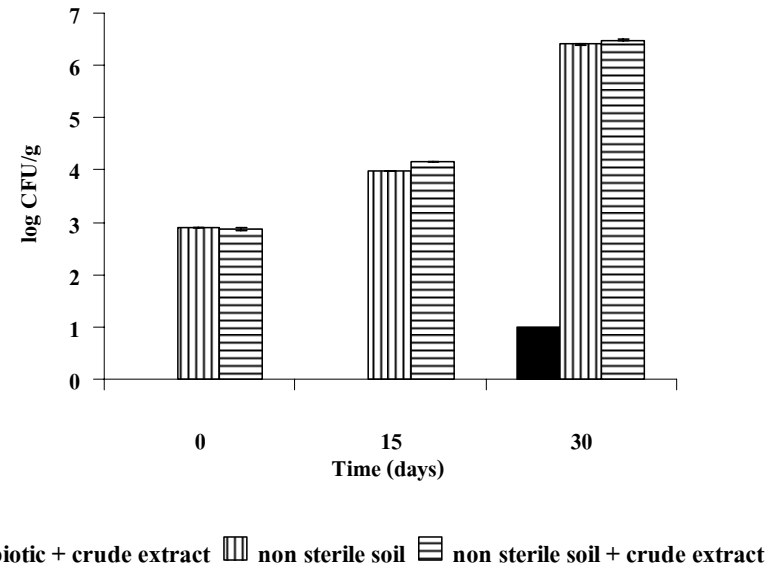


Figure 40. Number of microorganisms present in each treatment at 0, 15 and 30 days incubation of times.

The remaining waste lubricating oils from each treatment were extracted two times with *n*-hexane. Then, the degradability waste lubricating oil components was analyzed by TLC-FID. Chemical composition analyzed using TLC-FID of waste lubricating oil at the beginning contained saturate (retention time 0.100 min), aromatic (retention time 0.160 and 0.260 min), resin (retention time 0.343 min) and asphaltene (retention time 0.443 min) at 29.15, 17.66, 49.04 and 4.15%, respectively (Appendix 5). The TLC-FID chromatogram of abiotic treatment (sterile waste lubricating oil contaminated soil) showed in Figure 41. According to 30 days of cultivation the abiotic treatment showed 20.02% weight lost and degraded 13.27, 8.88 and 10.72% of saturate, aromatic and resin fractions of lubricating oil, respectively (Fig. 41C) may be because of some removal of hydrocarbons was also seen in the abiotic treatment. Abiotic weathering processes in polluted soils include evaporation, photochemical oxidation and adsorption onto particulate material (Ghazali *et al*., 2004).

The TLC-FID chromatogram of the abiotic treatment with crude biosurfactant was showed in Figure 42. This treatment exhibited 28.78% weight lost and degraded 13.09, 14.50 and 20.11% of saturate, aromatic and resin fractions of lubricating oil, respectively at 30 days of cultivation (Fig. 42C) might be due to the contamination of microorganisms from the crude biosurfactant which was not sterile. This result was correlated with the amount of microorganisms which was detected in abiotic treatment with crude biosurfactant at 30 days (Fig. 40).

The TLC-FID chromatogram of the biotic treatment (non sterile soil) was showed in Figure 43. This treatment showed 60.24% weight lost and degraded 23.00, 11.52 and 23.13% of saturate, aromatic and resin fractions of lubricating oil, respectively at 30 days of cultivation (Fig. 43C) because the native microorganisms in waste lubricating oil contaminated soil sample can degrade lubricating oil components.

The TLC-FID chromatogram of the biotic treatment with crude biosurfactant showed in Figure 44. The highest waste lubricating oil degradability was resulting in 66.94% weight lost and degraded 67.80, 20.00 and 51.86% of saturate, aromatic and resin fractions of lubricating oil, respectively at 30 days of cultivation (Fig. 44C) because the biosurfactant could promote the degradation of waste lubricating oil by native microorganisms presented in waste lubricating oil contaminated soil. The use of surfactants has been reported to increase the bioavailability of hydrophobic compounds in soil as well as in aqueous systems (Stelmack *et al*.,

1999). Used lubricating oil contaminated soil with surfactants could increase bioavailability to degrade by microorganisms (Providenti *et al*., 1995).

Table 19 showed the summarized of weight loss (%) and their degraded components in waste lubricating oil (%). In addition, pH of the abiotic treatment and abiotic treatment with the crude biosurfactant were relatively constant at around 6.45 throughout the experiment while pH of the biotic treatment and biotic treatment with the crude biosurfactant slightly dropped to around 6.32-6.21, significantly lower than that of the abiotic treatment, probably due to the release of proton ions formed during waste lubricating oil degradation (Yu *et al*., 2005). Thus, decreasing pH in the biotic treatment and biotic treatment with the crude biosurfactant may be increase the biodegradation of waste lubricating oil contaminated soil.

From the degradability of waste lubricating oil (Table 19), all of 4 treatments could not reduce the asphaltene component because of asphaltenes are not a specific family of chemicals with common functionality and varying molecular weight. Molecules in the asphaltene fraction can have many different sorts of polar functionality as well as varying molecular weight. Their only unifying property is insolubility in a specified *n*-alkane (Fan and Buckley, 2002).

The bioremediation waste lubricating oil contaminated soil when supplemented the crude biosurfactant produced by *I. orientalis* SR4 was more efficient than the degradability of used lubricating oil by the *Nocardia simplex* W9. Chemical composition of used lubricating oil was analyzed by TLC-FID demonstrated that *N. simplex* W9 degraded used lubricating oil and resulted in a decrease in saturate, aromatic and resin fractions to 52.46, 38.13 and 18.81%, respectively (Jirasripongpun, 2002). Moreover, the glycolipid biosurfactant produced by *P. aeruginosa* SB30 improved removal of weathered oil from gravel contaminated by the Exxon-Valdez spill (Harvey *et al*., 1990). In addition, the rhamnolipid biosurfactants produced by *P. aeruginosa* UG2 improved mobilization of several PAHs and two polychlorinated biphenyls (PCBs) from soil slurries (Scheibenbogen *et al*., 1994).

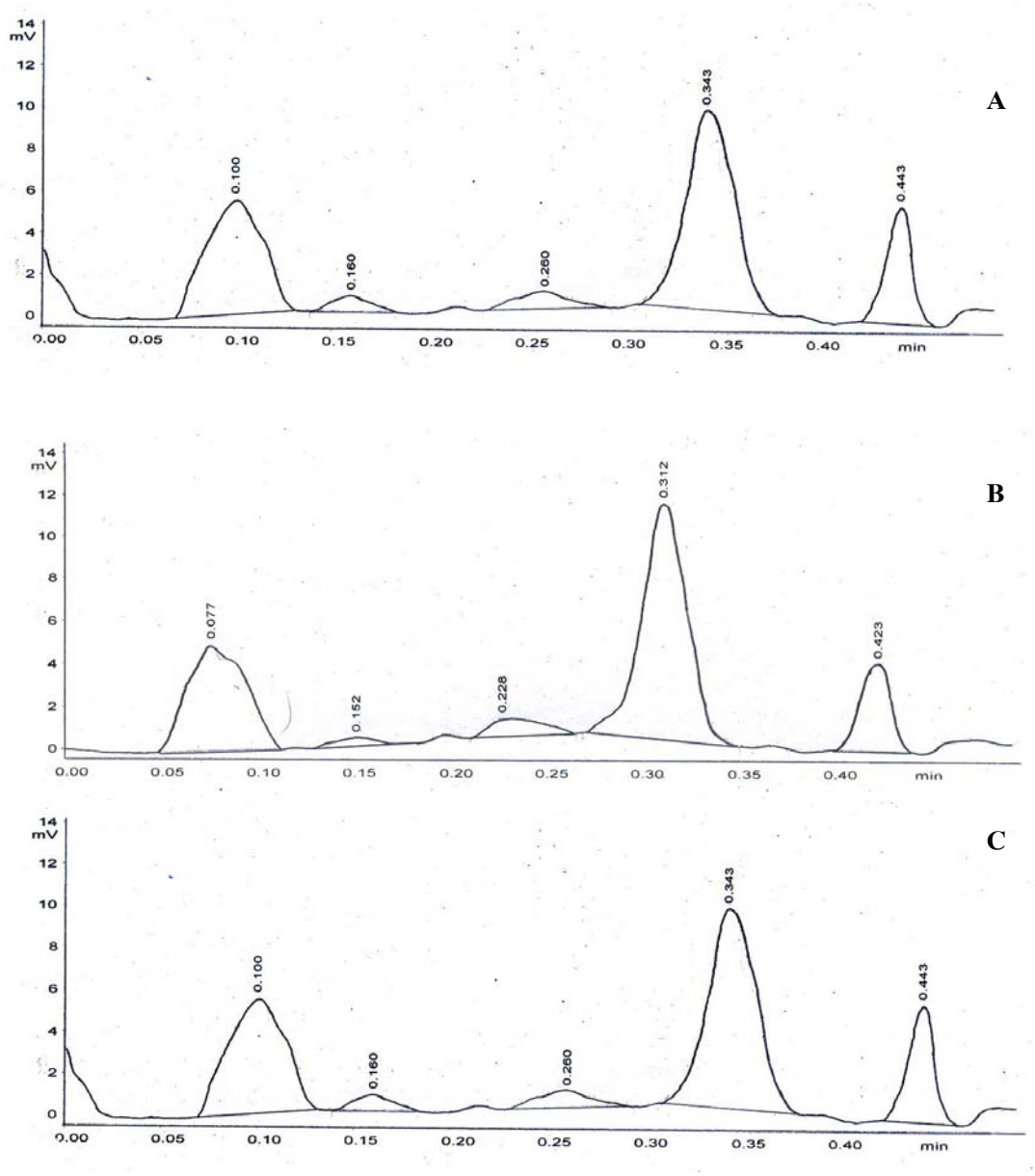


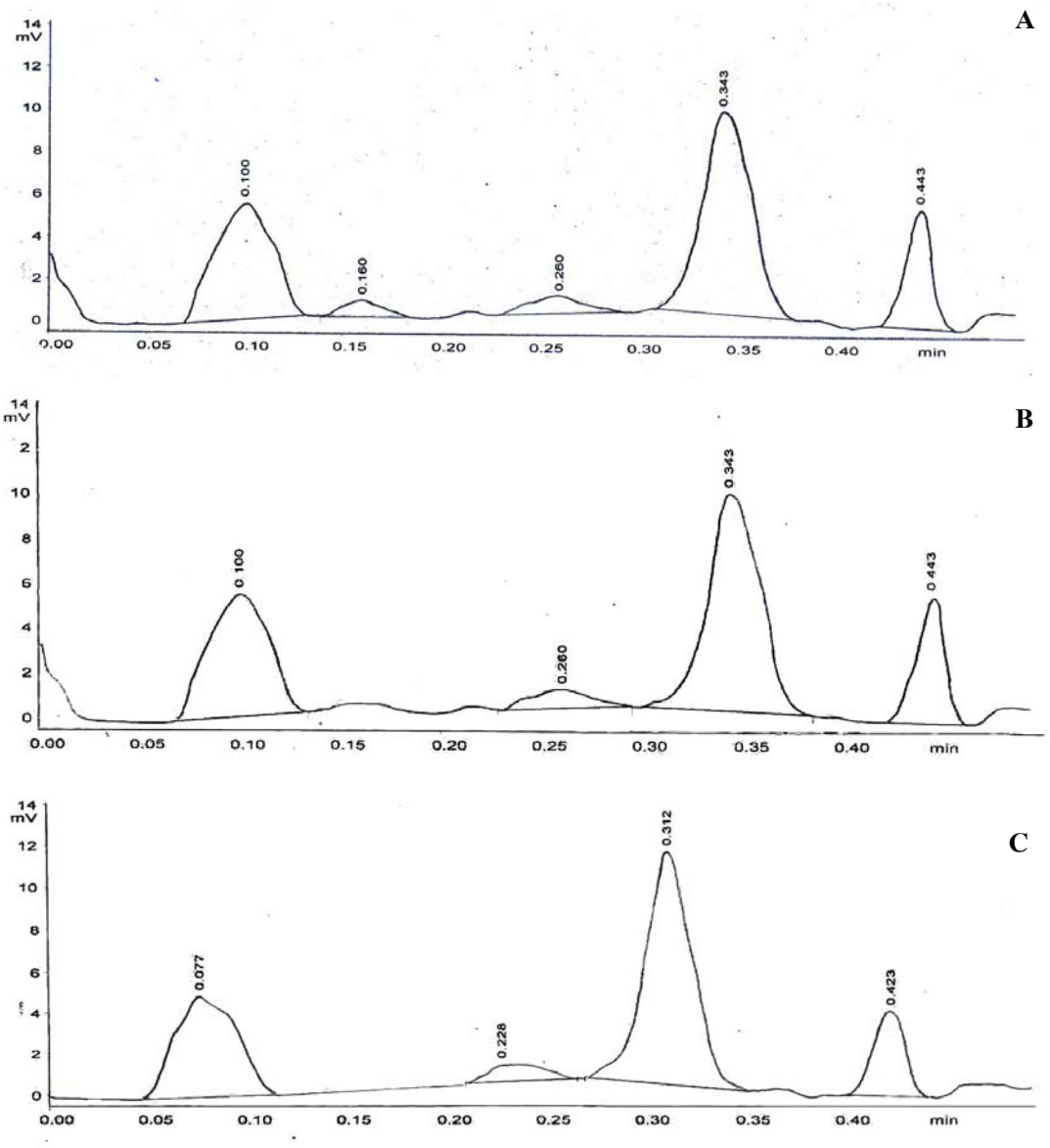
Figure 41. TLC-FID chromatogram of waste lubricating oil when extracted from lubricating oil contaminated soil (abiotic treatment) without biosurfactant.

A = 0 day of cultivation

B = 15 days of cultivation

C = 30 days of cultivation

Note: Saturated hydrocarbon (RT=0.089±0.01), Aromatic hydrocarbon (RT=0.246±0.02), Resin (RT=0.329±0.01) and Asphaltene (RT=0.434±0.01) analyzed by TLC-FID method.



Time (min)

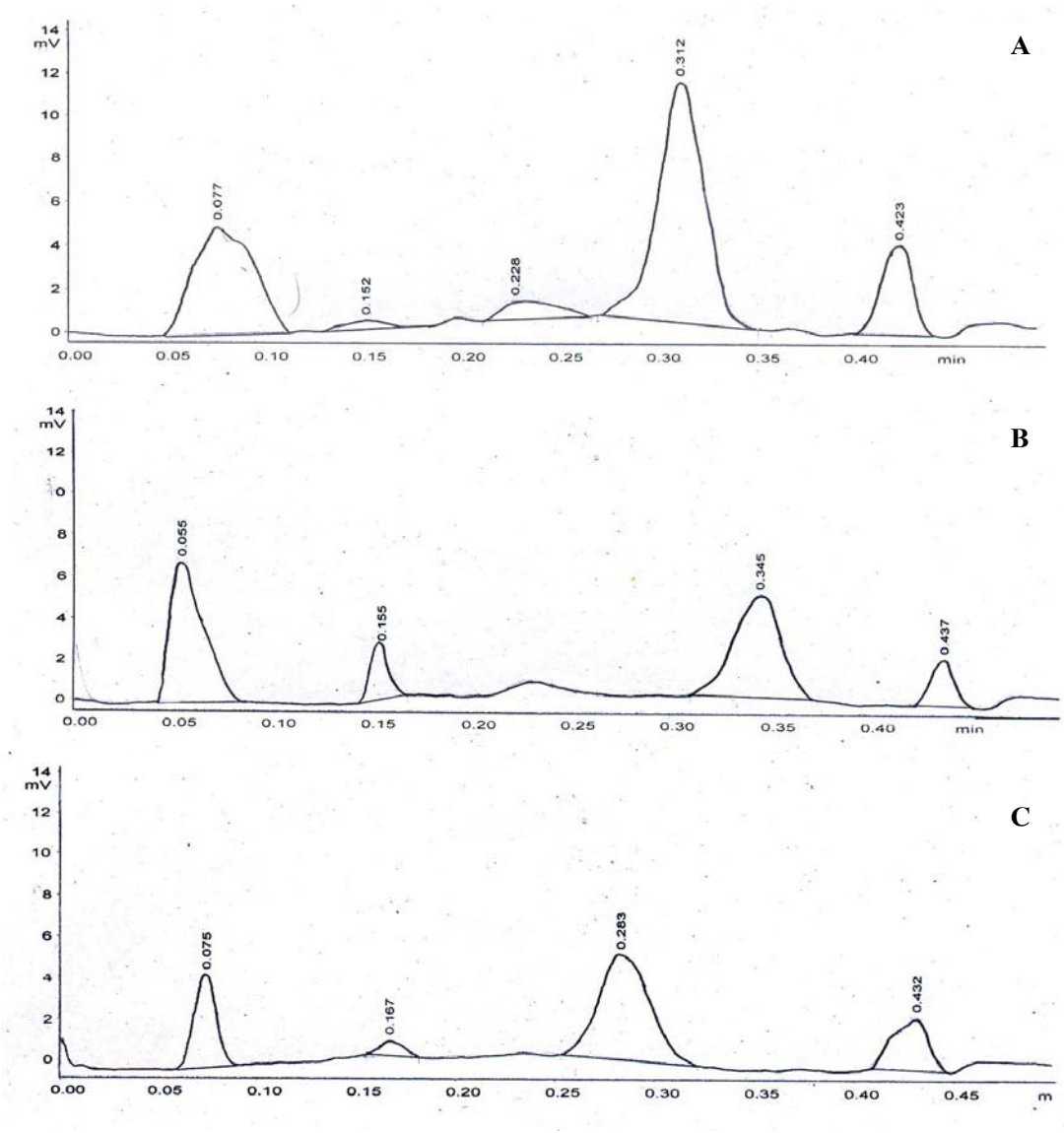
Figure 42. TLC-FID chromatogram of waste lubricating oil when extracted from lubricating oil contaminated soil (abiotic treatment) with biosurfactant.

A = 0 day of cultivation

B = 15 days of cultivation

C = 30 days of cultivation

Note: Saturated hydrocarbon (RT=0.096±0.02), Aromatic hydrocarbon (RT=0.236±0.02), Resin (RT=0.347±0.02) and Asphaltene (RT=0.447±0.01) analyzed by TLC-FID method.



Time (min)

Figure 43. TLC-FID chromatogram of waste lubricating oil when extracted from lubricating oil contaminated soil without biosurfactant.

A = 0 day of cultivation

B = 15 days of cultivation

C = 30 days of cultivation

Note: Saturated hydrocarbon (RT=0.069±0.01), Aromatic hydrocarbon (RT=0.228±0.00), Resin (RT=0.313±0.03) and Asphaltene (RT=0.431±0.01) analyzed by TLC-FID method.

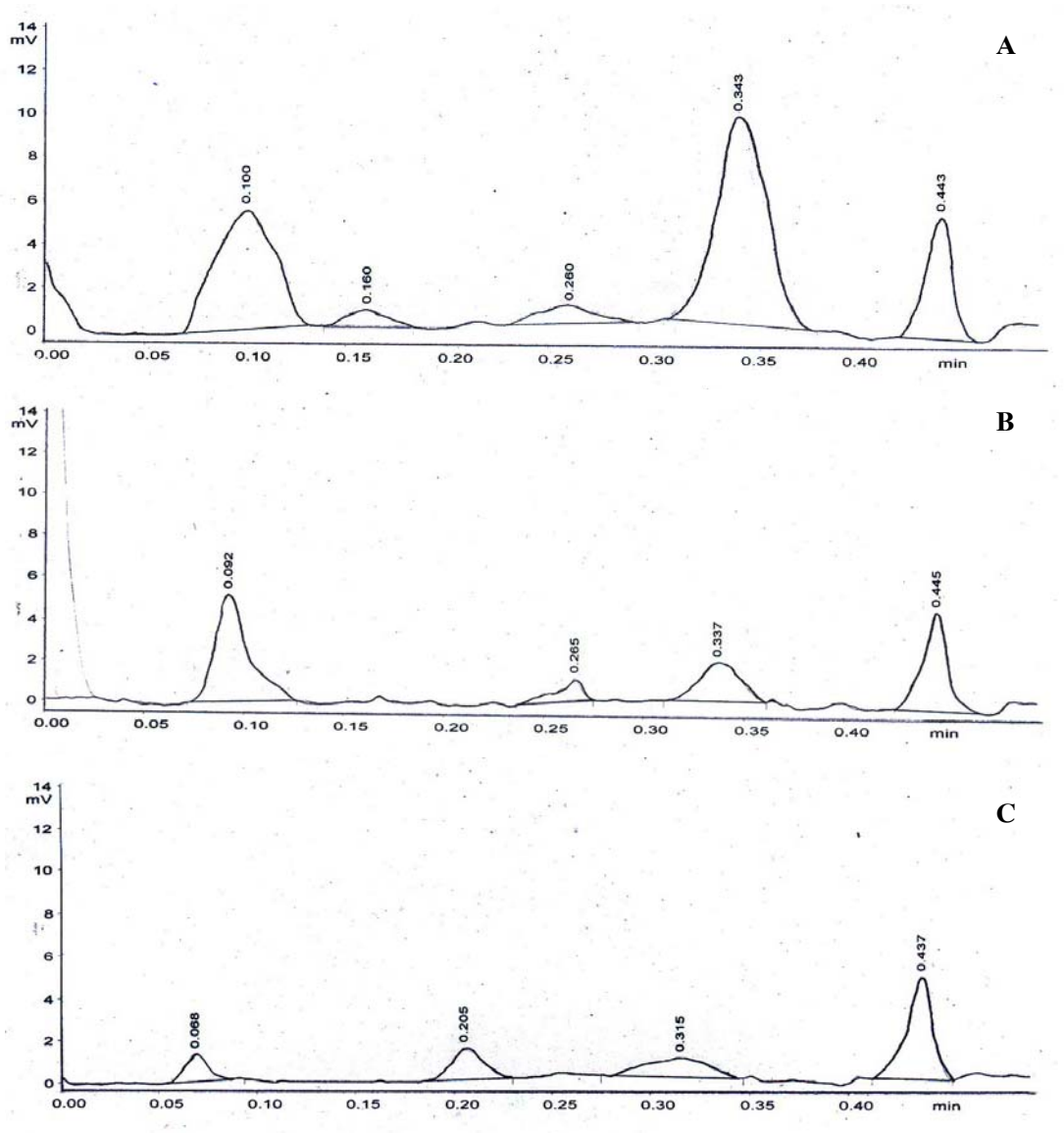


Figure 44. TLC-FID chromatogram of waste lubricating oil when extracted from lubricating oil contaminated soil with biosurfactant.

A = 0 day of cultivation

B = 15 days of cultivation

C = 30 days of cultivation

Note: Saturated hydrocarbon (RT=0.087±0.01), Aromatic hydrocarbon (RT=0.243±0.03), Resin (RT=0.332±0.01) and Asphaltene (RT=0.441±0.00) analyzed by TLC-FID method.

Table 19. Effect of crude biosurfactant on the degradation of waste lubricating oil

Experiments	Final pH[*]	Weight loss (%)[**]	Degradation of waste lubricating oil (%)[†]		
			Saturates	Aromatics	Resins
1. Abiotic (sterile soil)	6.45±0.00[c‡]	20.02±0.01[d]	13.27±0.00[c]	8.88±0.02[d]	10.72±0.00[d]
2. Abiotic + crude bisurfactant	6.40±0.01[c]	28.78±0.02[c]	13.09±0.01[c]	14.50±0.02[b]	20.11±0.02[c]
3. Biotic (non sterile soil)	6.32±0.02[b]	60.24±0.00[b]	23.00±0.02[b]	11.52±0.01[c]	23.13±0.01[b]
4. Biotic + crude biosurfactant	6.21±0.02[a]	66.94±0.02[a]	67.80±0.00[a]	20.00±0.00[a]	51.86±0.02[a]

[*]Final pH at 30 days.

[**](%) weight loss = weight of oil (0 day) minus weight of oil (degraded 30 days) divided by weight of oil (0 day) multiplied by 100

[†] (%) oil degradation = component in negative control (0 day) minus component in sample (30 days) divided by component in negative control (0 day) multiplied by 100.

[‡]Different letters in the same column indicated the significant differences ($p<0.05$).

The analysis of the biodegradation of waste lubricating oil contaminated soil by TLC-FID analysis could determined only the group of waste lubricating oil components for example, saturate, aromatic, resin and asphaltene fractions while GC-MS analysis could analyze the details of each component. The best treatment (biotic treatment with crude biosurfactant) from TLC-FID analysis was selected for GC-MS analysis. Analysis of the hydrocarbon extracts from the waste lubricating oil from waste lubricating oil contaminated soil at the beginning of this study detected aliphatic compounds C_{12}-C_{29} (Fig. 45). At the end of the 30 days study (Fig. 46), a level of biodegradation was seen when the waste lubricating oil contaminated soil was supplemented with the crude biosurfactant and detected the saturated hydrocarbon fractions as C_{12}, C_{16}, C_{17}, C_{20}, C_{22}, C_{24} and C_{26}. Oxidation of alkanes is classified as being terminal or diterminal. The monoterminal

oxidation is the main pathway. It proceeds via the formation of the corresponding alcohol, aldehyde, and fatty acid. β-Oxidation of the fatty acids results in the formation of acetyl-CoA. Fatty acids of a physiological chain length may be directly incorporated into membrane lipids, but the majority of degradation products are introduced into the tricarboxylic acid cycle (Fritsche and Hofrichter, 2000).

According to the result of GC-MS analysis was correlated with the result of TLC-FID analysis. So, the addition of the crude biosurfactant into the contaminated soil sample significantly promoted biodegrading microorganisms which could reduce the amounts of hydrocarbons in the waste lubricating oil contaminated soil after 30 days.

The analysis of alkanes in both unused and used lubricating oils using GC-MS revealed that used lubricating oil contained components of higher carbon (C > 29) in much higher quantities than components of the lower carbon (C_{14}- C_{28}) as detected in unused oil. The higher carbon (C > 29) alkane could result in a low solubility and less degradable than the lower carbon (C_{14}- C_{28}) alkane (Eastcott *et al*., 1988) as found on used lubricating oil.

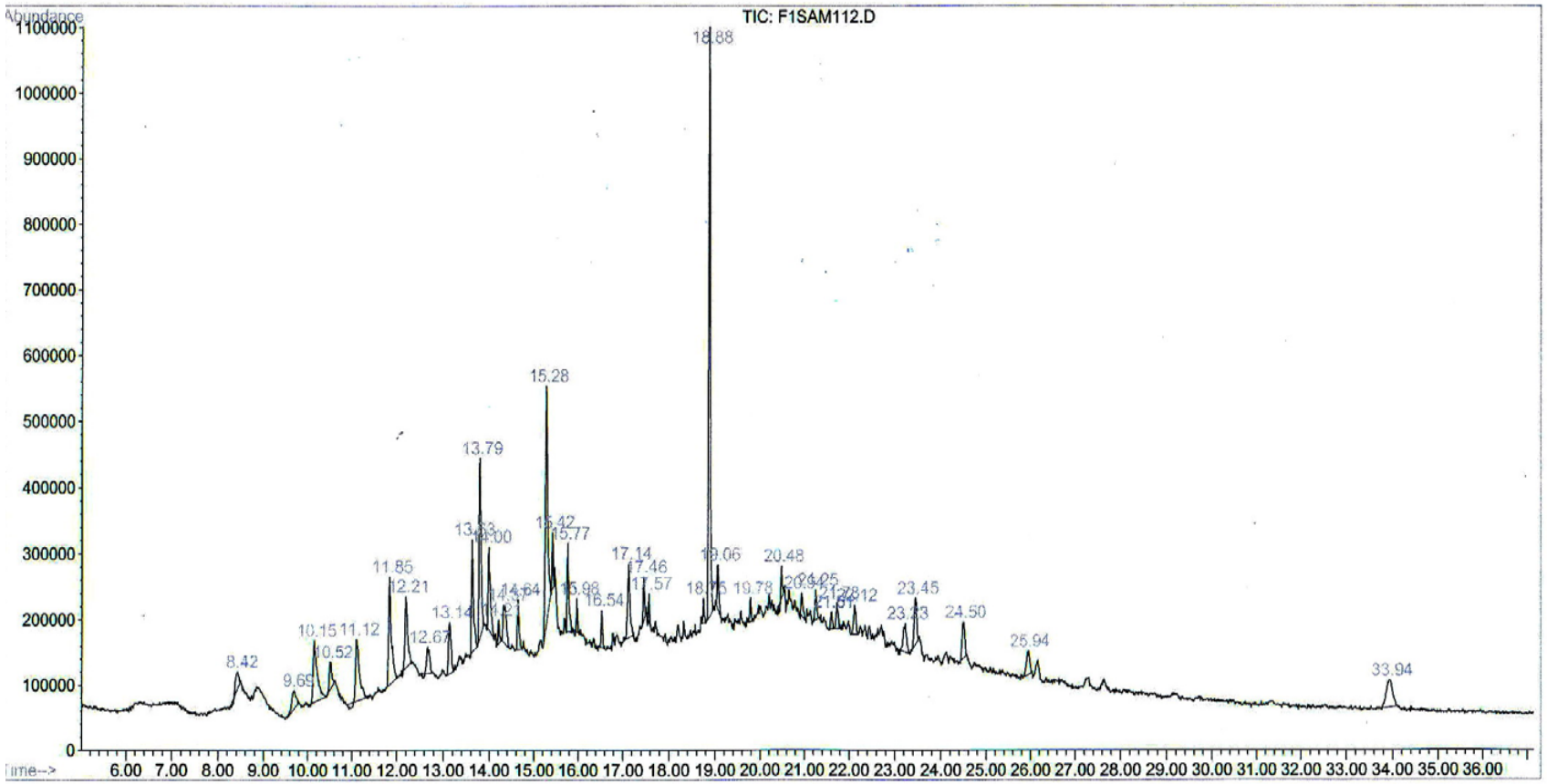


Figure 45. GC-MS chromatogram of saturated hydrocarbon fractions from waste lubricating oil contaminated soil with biosurfactant.

Numbers indicate *n*-alkane chain length, incubation time 0 day.

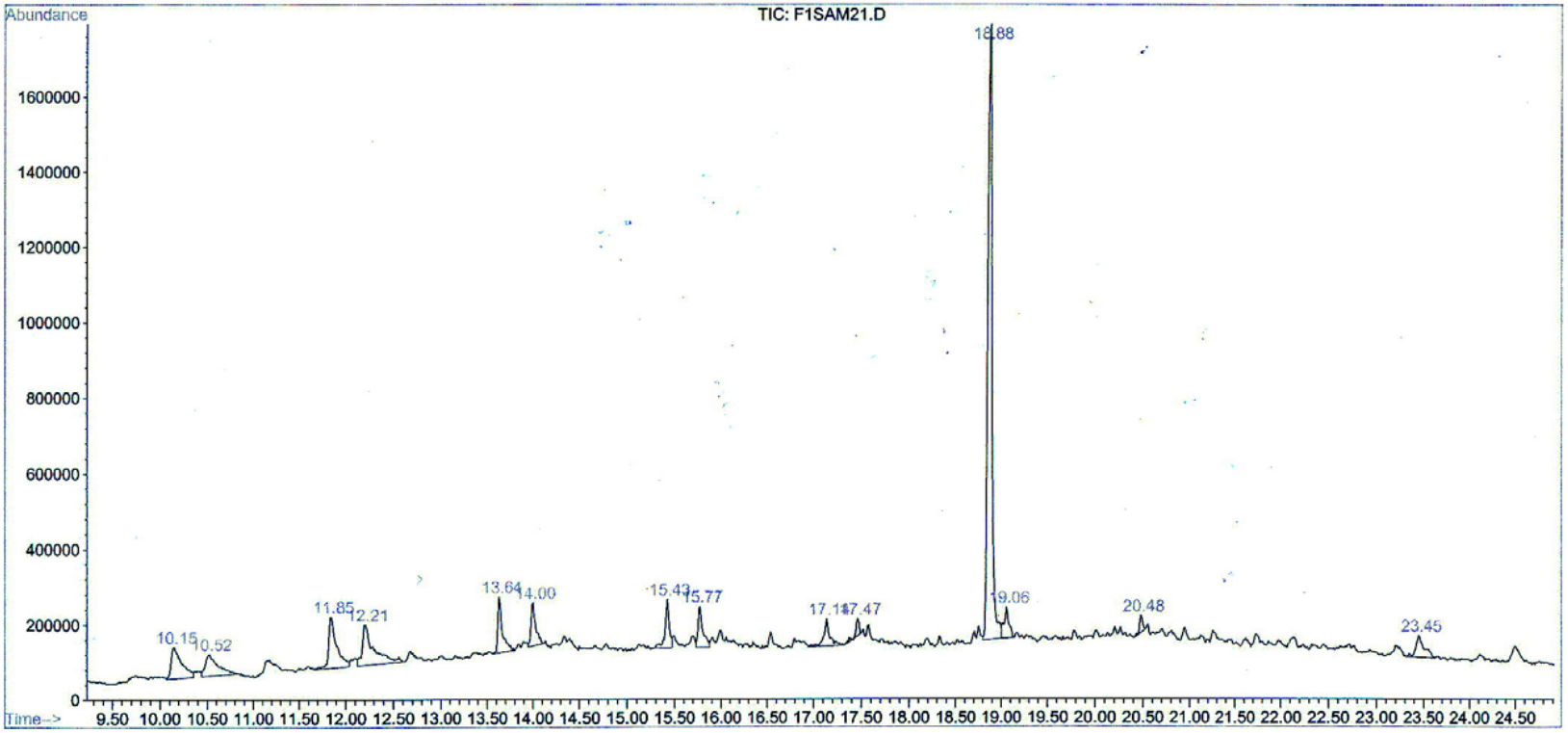


Figure 46. GC-MS chromatogram of saturated hydrocarbon fractions from waste lubricating oil contaminated soil with biosurfactant.

Numbers indicate *n*-alkane chain length, incubation time 30 days.

CHAPTER 4

CONCLUSION

Eighty-one yeast isolates were isolated from oil contaminated soils in Southern region of Thailand to test for the ability to grow on weathered crude oil as a sole carbon source. Seven isolates, PO1.2, PO3.6, PO3.13, PO4.2, GR2.4, GR4.2 and SR4 produced high biosurfactant activity which reduce surface tension of the culture broth to less than 60 mN/m when used glucose, weathered crude oil, *n*-hexadecane or xylene as a carbon source. Accordingly, the isolation and screening of biosurfactant-producing yeasts, the yeast strain SR4 showed the highest biosurfactant production. It reduced the surface tension of a culture broth from 61.5 to 49 mN/m when cultivated in a medium with xylene as a carbon source. When this yeast strain SR4 was grown in the medium with 10 g/L glucose as a carbon source it showed emulsification activity (%EA) with xylene (84.62%), *n*-hexadecane (47.62%) or kerosene (68.43%). While 10 g/L sodium dodecyl sulfate (SDS) could emulsify xylene, *n*-hexadecane or kerosene with emulsification activity only 76.92%. It was performed not only the lowest surface tension but also the emulsification activity. So this study was the first report of biosurfactant-producing yeast when xylene was used as a carbon source.

The yeast isolate SR4 was identified as *Issatchenkia orientalis* strain SR4 based on physiological, biochemical and DNA sequencing analysis. This is the first report to show that the yeast *Issatchenkia orientalis* strain SR4 produced biosurfactant which isolated from palm oil contaminated soil. The optimum medium for the production of the biosurfactant by *I. orientalis* SR4 contained 1 g/L of xylene as carbon source, 3 g/L of urea as nitrogen source with the aeration rate 0.5 vvm, agitation speed at 200 rpm and incubation temperature 30°C, without controlled pH when cultivation in 2L fermentor containing 1L optimal medium. It showed the maximum cell growth of yeast strain SR4 3.0×10^{7} cells/ml at 48 h of cultivation. The results of biosurfactant activity, *I. orientalis* SR4 could reduce the surface tension of culture medium from 61.5 to 49.0 mN/m and emulsify xylene with 41.25%EA. The obtained culture supernatant was extracted twice with an equal volume of mixtures of chloroform/methanol (2:1). The crude biosurfactant was separated by chromatography on a silica gel column and preparative HPLC. The purified biosurfactant was elucidated the chemical structure based on ^{1}H-NMR, GC-MS,

HPLC analysis and TLC plate and identified as oleic acid. This is the first report on a biosurfactant, oleic acid produced by yeast, *I. orientalis* SR4 when xylene was used as a carbon source. The characteristics of the crude biosurfactant were investigated. The crude biosurfactant was not stable in 1-5% NaCl while it was stable in 0-0.02% and 0-0.05% of $CaCl_2$ and $MgCl_2$, respectively. In addition, this crude biosurfactant was stable at pH 4-10 and thermostable at 4 to 80°C for 24 h. Moreover, the purified compound showed almost the same surface activity as an authentic oleic acid, and the activity was higher than that of synthetic surfactants such as SDS, triton X-100 and triton X-114. Application of the crude biosurfactant for bioremediation, the crude biosurfactant produced by *I. orientalis* SR4, enhanced the solubility rates of polycyclic aromatic hydrocarbons (PAHs) such as naphthalene (49.0 mg/L) and phenanthrene (4.2 mg/L) more than synthetic surfactant (SDS) and distilled water. Biodegradation of waste lubricating oil, chemical composition of waste lubricating oil analyzed by TLC-FID demonstrated that crude biosurfactant produced by *I. orientalis* SR4 could promote the degradation of waste lubricating oil and resulted in a decrease in saturate, aromatic and resin fractions to 67.80, 20.00 and 51.86%, respectively when cultivated for 30 days. So, the addition of the crude biosurfactant into the contaminated soil sample significantly promoted biodegrading microorganisms which could reduce the amounts of hydrocarbons in the waste lubricating oil contaminated soil.

Suggestions

The results of this work lead to the following suggestions:

1. Determination the biosurfactant activity of biosurfactant produced by *Isssatchenkia orientalis* SR4 with palm oil.
2. Study on the inoculum size of *I. orientalis* SR4.
3. Application of biosurfactant for palm oil wastewater treatment.
4. Study on the concentration of crude biosurfactant for bioremediation hydrocarbons.

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1. Culture media and preparation

1.1 Modified Basal Salt Medium

weathered crude oil	1.0	g
NH_4NO_3	3.0	g
yeast extract	0.5	g
KH_2PO_4	0.2	g
$MgSO_4 \cdot 7H_2O$	0.2	g

per liter of distilled water, pH 5.0. Autoclave at 121 °C for 15 min.

1.2 YM broth

yeast extract	3.0	g
malt extract	3.0	g
peptone	5.0	g
glucose	10.0	g

per liter of distilled water, pH 5.0. Autoclave at 121 °C for 15 min.

1.3 YM agar

yeast extract	3.0	g
malt extract	3.0	g
peptone	5.0	g
glucose	10.0	g
agar	20.0	g

per liter of distilled water, pH 5.0. Autoclave at 121 °C for 15 min.

1.4 Plate Count Agar (PCA)

tryptone	5.0	g
yeast extract	2.5	g
dextrose	1.0	g
agar	15.0	g

per liter of distilled water, pH 7.0. Autoclave at 121 °C for 15 min.

2. Spray reagent

2.1 Anisaldehyde-sulfuric acid reagent

Anisaldehyde-sulfuric acid reagent was prepared by slowly adding 0.5 mL of 98% H_2SO_4 to an ice cooled mixture of 9 mL of ethanol. To this solution added 0.5 mL of anisaldehyde and mixed well. The anisaldehyde-sulfuric acid reagent was colorless and should be stored in a refrigerator. If a color develops, the reagent must be discarded.

2.2 Bromocresol green reagent

0.04 % bromocresol green in methanol adjusted to a blue color (pH 7.5) with 0.1 M NaOH.

3. Solubility of polycyclic aromatic hydrocarbons (PAHs)

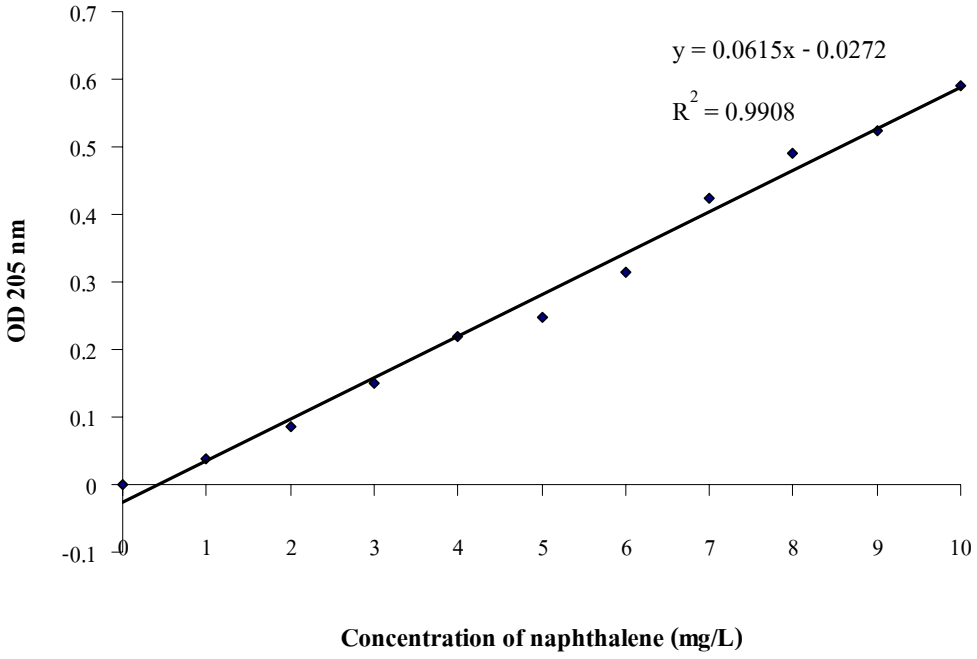

Figure 47. Standard curve of naphthalene.

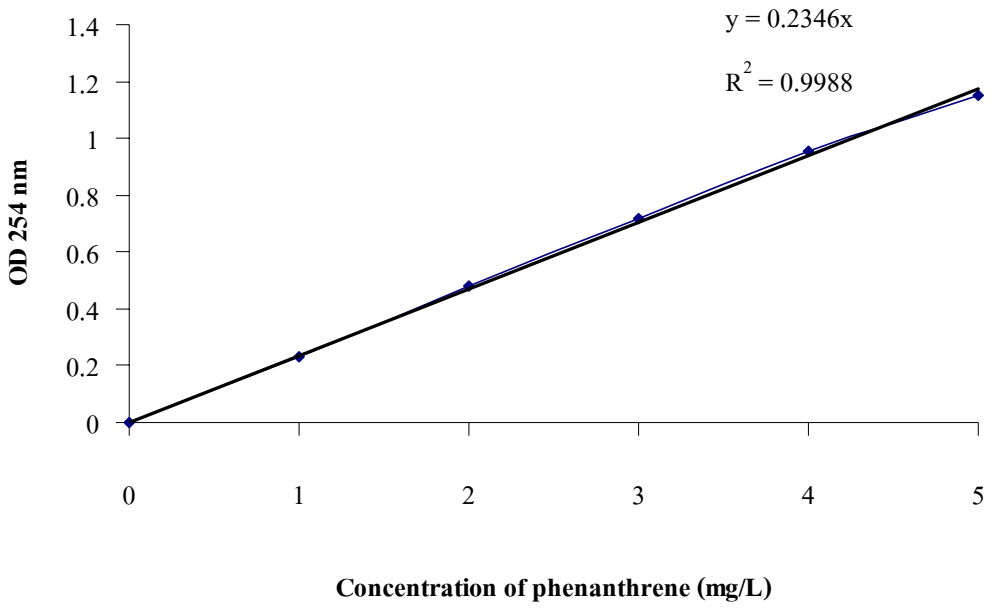

Figure 48. Standard curve of phenanthrene.

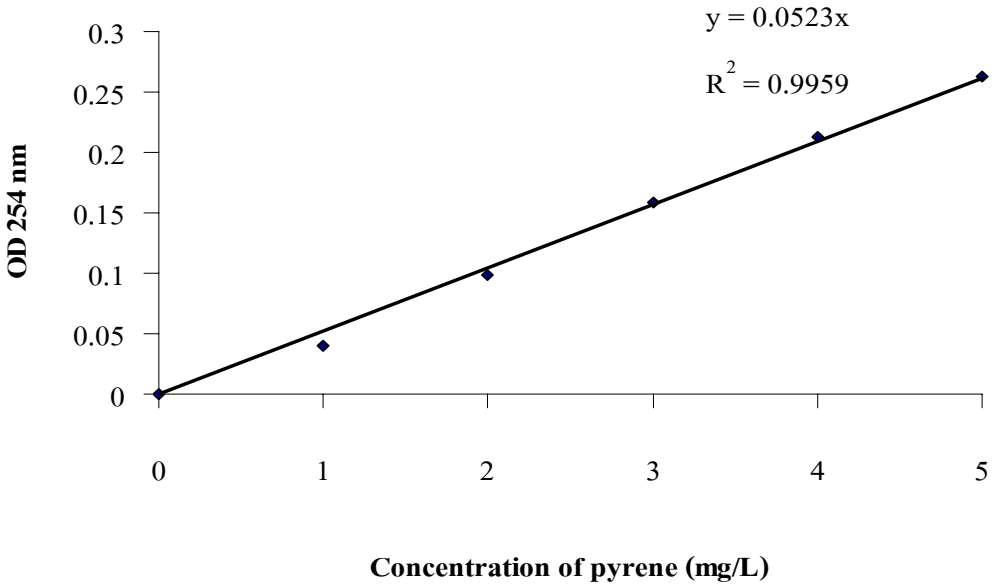

Figure 49. Standard curve of pyrene.

4. Nucleotide sequence of 26S rDNA gene of *Issatchenkia orientalis* SR4

Sample name : SR4

Identify : *Issatchenkia orientalis*

26S rDNA Sequence (600 bp)

GGATTGCCTCAGTAGCGGCGAGTGAAGCGGCAAGAGCTCAGATTTGAAATCGTGCTT
TGCGGCACGAGTTGTAGATTGCAGGTTGGAGTCTGTGTGGAAGGCGGTGTCCAAGTC
CCTTGGAACAGGGCGCCCAGGAGGGTGAGAGCCCCGTGGGATGCCGGCGGAAGCAG
TGAGGCCCTTCTGACGAGTCGAGTTGTTTGGGAATGCAGCTCCAAGCGGGTGGTAAA
TTCCATCTAAGGCTAAATACTGGCGAGAGACCGATAGCGAACAAGTACTGTGAAGGA
AAGATGAAAAGCACTTTGAAAAGAGAGTGAAACAGCACGTGAAATTGTTGAAAGGG
AAGGGTATTGCGCCCGACATGGGGATTGCGCACCGCTGCCTCTCGTGGGCGGCGCTC
TGGGCTTTCCCTGGGCCAGCATCGGTTCTTGCTGCAGGAGAAGGGGTTCTGGAACGT
GGCTCTTCGGAGTGTTATAGCCAGGGCCAGAATGCTGCGTGCGGGGACCGAGGACTG
CGGCCGTGTAGGTCACGGGTGCGGCAGAACG

Blast Result

RID: ZZ2VWJEH016

Database: All GenBank+EMBL+DDBJ+PDB sequences (but no EST, STS, GSS, environmental samples or phase 0, 1 or 2 HTGS sequences) 6,689,110 sequences; 23,477,585,937 total letters

> gb|AY707865.1| Issatchenkia orientalis WL2002 26S ribosomal RNA gene, partial sequence
Length=609

Score = 985 bits (533), Expect = 0.0
Identities = 540/543 (99%), Gaps = 2/543 (0%)
Strand=Plus/Plus

```
Query  1    GGATTGCCTCAGTAGCGGCGAGTGAAGCGGCAAGAGCTCAGATTTGAAATCGTGCTTTGC  60
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  36   GGATTGCCTCAGTAGCGGCGAGTGAAGCGGCAAGAGCTCAGATTTGAAATCGTGCTTTGC  95

Query  61   GGCACGAGTTGTAGATTGCAGGTTGGAGTCTGTGTGGAAGGCGGTGTCCAAGTCCCTTGG  120
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  96   GGCACGAGTTGTAGATTGCAGGTTGGAGTCTGTGTGGAAGGCGGTGTCCAAGTCCCTTGG  155

Query  121  AACAGGGCGCCCAGGAGGGTGAGAGCCCCGTGGGATGCCGGCGGAAGCAGTGAGGCCCTT  180
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  156  AACAGGGCGCCCAGGAGGGTGAGAGCCCCGTGGGATGCCGGCGGAAGCAGTGAGGCCCTT  215

Query  181  CTGACGAGTCGAGTTGTTTGGGAATGCAGCTCCAAGCGGGTGGTAAATTCCATCTAAGGC  240
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  216  CTGACGAGTCGAGTTGTTTGGGAATGCAGCTCCAAGCGGGTGGTAAATTCCATCTAAGGC  275

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            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
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Query  301  TGAAAAGAGAGTGAAACAGCACGTGAAATTGTTGAAAGGGAAGGGTATTGCGCCCGACAT  360
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Query  481  CCAGAATGCTGCGTGCGGGGACCGAGGACTGCGGCCGTGTAGGTCACGGGTGC-GGCAGA  539
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Sbjct  575  ACG  577
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5. TLC-FID chromatogram of waste lubricating oil

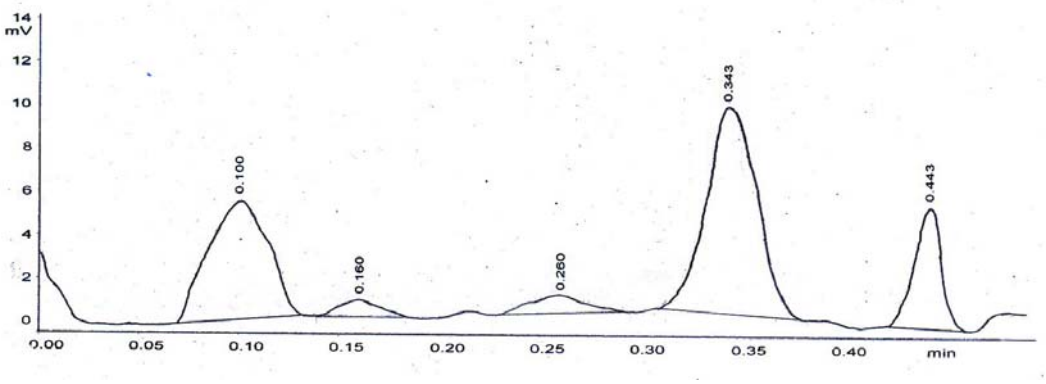

Figure 50. TLC-FID chromatogram of waste lubricating oil components**.**

Note: Saturated hydrocarbon (RT=0.089±0.01), Aromatic hydrocarbon (RT=0.246±0.02), Resin (RT=0.329±0.01) and Asphaltene (RT=0.434±0.01) analyzed by TLC-FID method.

VITAE

Name Miss Wichuda Katemai

Student ID 4783001

Educational Attainment

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Scholarship Award during Enrolment

Scholarship under the Commission on Higher Education Staff Development Project, Commission on Higher Education, Thailand

List of Publication and Proceedings

Publication

Katemai, W., Maneerat, S., Kawai, F., Kanzaki, H., Nitoda, T. and H-Kittikun, A. 2008. Purification and characterization of a biosurfactant produced by *Issatchenkia orientalis* SR4. Journal of General Applied Microbiology. 54: 71-74.

Proceedings

Katemai, W., Maneerat, S., Kawai, F. and H-Kittikun, A. Isolation and Screening of Biosurfactant-Producing Yeast from Oil Contaminated soils. The 5th JSPS-NRCT Joint Seminar on Development of Thermotolerant Microbial Resources and Their Applications. Aisawan Resort & Spa, Chonburi, Thailand. 7-10 November, 2006.

H-Kittikun, A., Maneerat, S., Katemai, W., Prommachan, O., Nitoda, T., Kanzaki, H. and Kawai, F. Isolation of biosurfactant producing microorganisms and their applications. JSPS-NRCT Concluding Joint Seminar on Development of Thermotolerant Microbial Resources and Their Applications. Walailak University, Nakhon Si Thammarat, Thailand. 18-20 October, 2007.