ความหลากหลายของยีสต์ที่ผลิตสารลดแรงตึงผิวชีวภาพที่มีประสิทธิภาพและ

การผลิตสารลดแรงตึงผิวชีวภาพจาก *Cyberlindnera samutprakarnensis* sp. nov. JP52$^{\text{T}}$

นางสาวจำรูญศรี   พุ่มเทียน

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต

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คณะวิทยาศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย

ปีการศึกษา 2555

ลิขสิทธิ์ของจุฬาลงกรณ์มหาวิทยาลัย

DIVERSITY OF EFFICIENT BIOSURFACTANT-PRODUCING
YEASTS AND BIOSURFACTANT PRODUCTION
BY *Cyberlindnera samutprakarnensis* sp. nov. JP52[T]

Miss Jamroonsri Poomtien

A Dissertation Submitted in Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy Program in Microbiology
Department of Microbiology
Faculty of Science
Chulalongkorn University
Academic Year 2012
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Thesis Title   DIVERSITY OF EFFICIENT BIOSURFACTANT-PRODUCING YEASTS AND BIOSURFACTANT PRODUCTION BY *Cyberlindnera samutprakarnensis* sp. nov. JP52$^{\text{T}}$

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จำรูญศรี พุ่มเทียน : ความหลากหลายของยีสต์ที่ผลิตสารลดแรงตึงผิวชีวภาพที่มีประสิทธิภาพและการผลิต
สารลดแรงตึงผิวชีวภาพจาก *Cyberlindnera samutprakarnensis* sp. nov. JP52^T (DIVERSITY OF EFFICIENT
BIOSURFACTANT-PRODUCING YEASTS AND BIOSURFACTANT PRODUCTION BY
Cyberlindnera samutprakarnensis sp. nov. JP52^T) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: รศ.จิราภรณ์ ธนียวัน, อ.ที่ปรึกษา
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จากการแยกเชื้อจำนวน 368 ไอโซเลตจากตัวอย่างดิน วัสดุทางการเกษตรที่พบการปนเปื้อนน้ำมันพืชหรือน้ำมันปาล์มและของ
เสียจากโรงงานอุตสาหกรรมในประเทศไทยและนำมาคัดเลือกยีสต์ที่ผลิตสารลดแรงตึงผิวชีวภาพที่มีประสิทธิภาพให้ค่าแรงตึงผิวได้ต่ำกว่า 40
mN m^{-1} พบว่ามีอยู่จำนวน 94 ไอโซเลต จากการจัดจำแนกยีสต์โดยอาศัยอนุกรมวิธานระดับโมเลกุลด้วยการเปรียบเทียบลำดับนิวคลีโอไทด์
บริเวณ D1/D2 ของ large subunit (LSU) rRNA gene พบว่าสามารถจัดเป็นสปีชีส์ยีสต์ที่มีการอธิบายแล้ว 93.6 % สปีชีส์ที่ยังไม่มีการอธิบาย แต่มี
ความใกล้เคียงกับยีสต์ที่มีการอธิบายแล้ว 2.1 % และสปีชีส์ใหม่ 4.3 % งานวิจัยนี้ได้จัดจำแนกยีสต์ที่เป็นสปีชีส์ใหม่โดยการวิเคราะห์ดีเอ็นเอ
บริเวณ D1/D2 ของ 26S rDNA LSU rRNA gene และบริเวณ ITS gene (ITS1-5.8S rRNA gene-ITS2; ITS1-2) การสร้างแผนภูมิความสัมพันธ์
ทางวิวัฒนาการ (phylogenetic analysis) และการศึกษาลักษณะฟีโนไทป์ของยีสต์สปีชีส์ใหม่ พบว่ายีสต์ในกลุ่มแอสโคมัยซีตัส จำนวน 3 ไอโซ
เลต (JP52, JP59 และ JP60) ไม่พบการสร้างสปอร์แบบมีเพศ โดยสายพันธุ์ JP52 มีความใกล้ชิดกับ *Cyberlindnera mengyuniae* CBS 10845^T โดย
มีความแตกต่างของลำดับนิวคลีโอไทด์แทนที่ในบริเวณ D1/D2 และยีน ITS1-2 เท่ากับ 2.9 และ 4.4 % ตามลำดับ จึงเสนอเป็นยีสต์สปีชีส์ใหม่ให้
ชื่อว่า *Cyberlindnera samutprakarnensis* sp. nov. (Type strain JP52^T; = BCC 46825^T = JCM 17816^T = CBS 12528^T, MycoBank no. MB800879)
สำหรับสายพันธุ์ JP59 และ JP60 มีลำดับนิวคลีโอไทด์ในบริเวณ D1/D2 และบริเวณ ITS ที่เหมือนกันทุกประการและต่างมีความใกล้ชิดกันมาก
กับ *Scheffersomyces spartinae* CBS 6059^T โดยมีความแตกต่างของลำดับนิวคลีโอไทด์แทนที่ในบริเวณ D1/D2 และยีน ITS1-2 เท่ากับ 0.9 และ
1.3 % ตามลำดับและในบริเวณ actin gene และ translational elongation factor gene ที่ต่างกัน 6.5 และ 4.8% ตามลำดับ ซึ่งนำมาใช้เป็นข้อมูล
สนับสนุน นอกจากนี้ความแตกต่างกันทางฟีโนไทป์ด้านชีวเคมีและสรีรวิทยาของสปีชีส์ใหม่ทั้งสองชนิดกับยีสต์ที่มีการอธิบายแล้วยังเป็นข้อมูล
บ่งชี้ชัดว่าสายพันธุ์ JP59 และ JP60 เป็นยีสต์สปีชีส์เดียวกันและเป็นสปีชีส์ใหม่ที่ให้ชื่อว่า *Candida thasaenensis* sp. nov. (Type strain JP59^T =
BCC 46828^T = JCM 17817^T = CBS 12529^T, MycoBank no. MB800880).

งานวิจัยนี้ได้คัดเลือกยีสต์ *Cyberlindnera samutprakarnensis* JP52^T ซึ่งมีความสามารถในการผลิตสารลดแรงตึงผิวชีวภาพที่มี
ประสิทธิภาพสูงสุดเมื่อเลี้ยงในอาหารเหลวปรับปรุงสูตรกลูโคสและน้ำมันปาล์มที่ประกอบด้วย 0.02% KH$_2$PO$_4$ 0.02% MgSO$_4$.7H$_2$O 0.4%
NaNO$_3$, 0.1% สารสกัดยีสต์ 2% กลูโคสและ 2% น้ำมันปาล์ม ความเป็นกรดด่างในอาหารเริ่มต้นเท่ากับ 5.5 ภาวะการเลี้ยงเชื้อที่อุณหภูมิ 30 °ซ
ในระดับขวดเขย่าด้วยอัตราเร็ว 200 รอบต่อนาที เป็นเวลา 7 วัน สารลดแรงตึงผิวชีวภาพที่ผลิตได้มีค่าแรงตึงผิวต่ำสุด 30.9 mN m^{-1} ค่าจุดวิกฤต
ของการเกิดไมเซลล์ (CMC) เท่ากับ 457.1 mg L^{-1} และให้ผลผลิต 1.89 g L^{-1} และเมื่อศึกษาลักษณะสมบัติทางชีวเคมีพบว่าสามารถทำงานได้ดีและ
มีความเสถียรที่ความเป็นกรดด่างเท่ากับ 8 มีความเสถียรต่ออุณหภูมิต่างๆ ได้ถึงอุณหภูมิ 121 °ซ และยังคงความเสถียรได้ดีในภาวะที่มี
โซเดียมคลอไรด์เข้มข้น 1.0-10.0% นอกจากนี้ยังสามารถก่ออิมัลชันต่อน้ำมันต่างๆ เช่น น้ำมันงา น้ำมันปาล์ม น้ำมันมะกอก น้ำมันดอกคำฝอย
น้ำมันเมล็ดฝ้ายและน้ำมันสลัด แสดงค่า E$_{24}$ > 80% จากการวิเคราะห์สารลดแรงตึงผิวชีวภาพที่ผลิตได้โดยใช้ preparative TLC พบว่ามี
ส่วนประกอบ 3 ส่วน (F1GP F2GP และ F3GP) ที่มีค่า Rf เท่ากับ 0.89, 0.77 และ 0.69 ตามลำดับ ซึ่ง F3GP ให้ค่าการกระจายน้ำมันสูงสุดและให้
ผลบวกกับการทดสอบ Molish จากนั้น F3GP ถูกนำไปวิเคราะห์มวลโมเลกุลและโครงสร้างทางเคมีต่อไปด้วย MALDI-TOF MS และ NMR ซึ่ง
แสดงค่ามวลโมเลกุลของสารส่วนใหญ่มีค่าเท่ากับ 574 และ 662 ซึ่งเทียบเคียงได้กับสารโซโฟโรลิพิดที่มีโครงสร้างเป็น non acetylated lactonic
sophorolipids (SL-C18) และ monoacetylated acidic sophorolipid (SL-Ac-C19:1) หรือ diacetylated diacetylated lactonic sophorolipids (SL-
Ac-Ac-C16) ตามลำดับ ดังนั้น *Cyberlindnera samutprakarnensis* JP52^T เป็นยีสต์สปีชีส์ใหม่ที่ผลิตสารลดแรงตึงผิวชีวภาพชนิดโซโฟโรลิพิดที่
อาจนำไปใช้ประโยชน์ได้หลากหลาย

ภาควิชา ....จุลชีววิทยา...................... ลายมือชื่อนิสิต ....

สาขาวิชา ....จุลชีววิทยา...................... ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก..

ปีการศึกษา ....2555............................. ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์ร่วม..

ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์ร่วม..

5173892023: MAJOR MICROBIOLOGY
KEYWORDS: BIOSURFACTANTS-PRODUCING YEAST / *Cyberlindnera samutprakarnensis* sp. nov. JP52^T / PALM OILS / SOPHOROLIPIDS

JAMROONSRI POOMTIEN: DIVERSITY OF EFFICIENT BIOSURFACTANT-PRODUCING YEASTS AND BIOSURFACTANT PRODUCTION BY *Cyberlindnera samutprakarnensis* sp. nov. JP52^T.
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SASITORN JINDAMORAKOT, Ph.D., 186 pp.

In the course of a survey on the biosurfactants-producing yeasts, 368 strains were isolated from soil, agricultural wastes and samples contaminated with vegetable or palm oils in Thailand. The efficient biosurfactant producing strains were selected by their activities of lowering surface tension to below 40 mN m^{-1}. As a result, 94 strains were selected and further identified by molecular DNA analysis using the D1/D2 sequencing method. Among them, 93.6% were known species, 2.1% were undescribed species and 4.3% were novel yeast strains. Based on the phylogenetic sequence analysis of the D1/D2 region of the large subunit (LSU) rRNA gene, the internal transcribed spacer region (ITS1-5.8S rRNA gene-ITS2; ITS1-2) and their physiological characteristics, the three strains (JP52, JP59 and JP60) were found to represent two novel species of the anamorphic ascomycetous yeast. Strain JP52 represent a novel species which was named *Cyberlindnera samutprakarnensis* sp. nov. (Type strain JP52^T; = BCC46825^T = JCM17816^T = CBS12528^T, MycoBank no. MB800879), it is classified as a members of the *Cyberlindnera* clade which was different from the closely related species, *Cyberlindnera mengyuniae* CBS10845^T by 2.9% and 4.4% sequence divergence in the D1/D2 region and ITS1-2, respectively. Strain JP59 and JP60 were identical in the D1/D2 region and ITS1-2 which were closely related to *Scheffersomyces spartinae* CBS6059^T by 0.9 and 1.3% sequence divergence respectively. Gene analysis of 6.5 and 4.8% of sequence divergence in the actin and translational elongation factor gene and their differences from the closely related species in some biochemical and physiological characteristics were also supported a distinct species. These two strains were assigned as a single novel species which was named *Candida thasaenensis* sp. nov. (Type strain JP59^T = BCC46828^T = JCM17817^T = CBS12529^T, MycoBank no. MB800880).

In this work, *Cyberlindnera samutprakarnensis* JP52^T was found to be an efficient biosurfactant-producing yeast that showed strong activity of surface tension reduction and oil displacement test when cultured in glucose and palm oil medium (medium broth consisted of 0.02% KH$_2$PO$_4$, 0.02% MgSO$_4$.7H$_2$O, 0.4% NaNO$_3$, 0.1% yeast extract, 2% (w/v) glucose and 2% (v/v) palm oil), initial pH 5.5. The optimum cultivation was performed at 30 ^{o}C in shake flask at 200 rpm. After 7 days of cultivation the biosurfactant produced possessed minimum surface tension of 30.9 mN m^{-1}, the critical micelle concentration (CMC) of 457.1 mg L^{-1} and yield of 1.89 g L^{-1}. The physicochemical studies of the product obtained revealed that this biosurfactant has optimum pH as well as pH stability at pH 8.0. It was stable at high temperature up to 121 ^{o}C and could tolerate to NaCl at 1.0-10.0%. In addition, it could form emulsion with various oils including sesame oil, palm oil, olive oil, safflower oil, cotton seed oil and salad oil by showing E$_{24}$ value over 80%. For purification, thin layer chromatography revealed that strain JP52^T produced three major biosurfactants in GP medium, namely F1GP, F2GP and F3GP with Rf values of 0.89, 0.77 and 0.69, respectively. F3GP gave the highest oil displacement activity and was glycolipid positive by the Molish test. Structural analyses by MALDI-TOF MS and NMR suggested that F3GP contained sophorolipid-type biosurfactants with molecular weights of 574 and 662 that are likely to be non acetylated lactonic sophorolipids (SL-C18) and either a monoacetylated acidic sophorolipid (SL-Ac-C19:1) or diacetylated lactonic sophorolipids (SL-Ac-Ac-C16), respectively. We propose *Cyberlindnera samutprakarnensis* JP52^T as new sophorolipid biosurfactant-producing yeast that produce attractive biosurfactants for diverse applications.

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Field of Study …Microbiology…………… Advisor's Signature…………………......................................
Academic Year…2012 …………………… Co-advisor's Signature……………………...............…….....
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ACKNOWLEDGEMENTS

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LIST OF ABBREVIATIONS

mN m^{-1}	-	Milinewton per meter
mg L^{-1}	-	Milligram per liter
g L^{-1}	-	Gram per liter
v/v	-	Volume per volume
w/v	-	Weight per volume
h	-	Hour
d	-	Day
rpm	-	Revolutions per minute
ca.	-	About
BS		Biosurfactant
DCW		Dry cell weight
cmc	-	Critical micelle concentration
ODA	-	Oil displacement activity
DNA	-	Deoxyribonucleic acids
PCR	-	Polymerase chain reaction
nt.	-	Nucleotide
IT		Interfacial tension
ST		Surface tension
ΔST, delta ST	-	Surface tension reduction
TLC	-	Thin Layer Chromatography
MALDI-TOF MS	-	Matrix Assisted Laser Desorption/Ionization - Time of Flight Mass Spectrometry
NMR	-	Nuclear Magnetic Resonance

CHAPTER I

INTRODUCTION

1.1 Background and rationale

The focus on potential of microbial-derived surface active compounds or being called as biosurfactants to substitute chemical surfactants has been increased due to high biodegradable properties, low toxicity, environment compatibility and specific activity in extreme conditions of these compounds. The significant volume of surfactant utilization in the worldwide market has been risen up to 17 -19 metric tons in 2000, with expected future globally growth rate 3-4% per year (Deleu and Paquot, 2004). By environmental awareness of healthy biosphere in reduction greenhouse gas emission, the biosurfactants have intensive role in sharing stakeholder of surfactant consumption. Biosurfactants have gained importance in the fields of enhanced oil recovery, environmental bioremediation, food processing and pharmaceuticals (Muthusamy et al., 2008).

Biosurfactants are surface active compounds, synthesized by microorganisms such as bacteria, yeast and filamentous fungi (Mulligan, 2005). They are amphiphilic compounds containing both hydrophilic and hydrophobic moieties thus are soluble in both water and organic solvents. Such molecules are accumulated at the phase boundary between the organic and aqueous phases, thereby capable of reducing surface and interfacial tensions, facilitating hydrocarbon uptake and forming emulsions (Thavasi et al., 2009). Typical, a good surfactant is capable of reducing the surface tension (ST) of water from 72 to 35 mN m^{-1} and the interfacial tension (IT) of a water/hexadecane interphase from 40 to 1 mN m^{-1} (Mulligan, 2005). Biosurfactants exhibit great structural diversity and group in 5 classes including glycolipids, lipopeptides, phospholipids, polymeric surfactants and particulate biosurfactants (Rosenberg and Ron, 1999; Deleu and Paquot, 2004). They have an amazingly wide

range of chemical structures depending on substrates used during cultivation and each appears to play different roles in the life cycle of the producing microorganism (Ron and Rosenberg, 2001). For biosurfactants from yeast are mainly possessed in glycolipids (sophorolipids, trehalose lipids, mannosylerythritol lipids) and polymeric surfactants (mannan-lipid-protein, glucolipids). With diverse properties, new value adding opportunities will result from the identification of new biosurfactants with different specific activities, especially in relation to their biological effects where they have potential application as surface active agents, bioemulsifiers, solubilizers, cleansing agents, antiadhesives and antimicrobial agents (Deleu and Paquot, 2004; Mulligan, 2005). Among the previously reported of biosurfactants, 46% of patented-biosurfactants are produced by bacteria, such as *Acinetobacter* spp., *Bacillus* spp. and *Pseudomonas* spp., whilst 24% are from sophorolipid producing yeasts, like *Candida* spp. (Shete et al., 2006). However, few reports are available with the biosurfactants producing yeasts that production and characterization of biosurfactants by yeasts are minor and less diverse as compared to that obtained from bacterial strains.

Sophorolipids are glycolipid-type biosurfactants, formerly known as sophorosides, which are found and excreted into the culture medium by *Candida* and related yeast species. There are reports regarding the production of glycolipid biosurfactants from different substrates such as glucose and other sugars, vegetable oil, molasses, whey or waste from refinery oil processing (Muthusamy, 2008). However, sophorolipids are now known to be synthesized by a phylogenetically diverse group of yeasts and have diverse chemical structures (Van Bogaert, 2007). *Starmerella* (*Candida*) *bombicola* was first reported by Spencer et al. (1970) and is well-known sophorolipid-producing yeast. Interest in its products is revealed by the highest number of patents being attributed to its products compared to other yeasts (Shete et al., 2006). Mannosylerythritol lipids, MEL glycolipid biosurfactant is well known for its utilization in external pharmaceutics and cosmetics. Konishi et al. (2007b) and Morita et al (2008b) used thin-layer chromatography, high-performance liquid chromatography and nuclear magnetic resonance spectroscopy to analyze MEL from *Pseudozyma*. Moreover, the molecular phylogenetic analysis was used to

categorize MEL producer into group of MEL-A, MEL-B and MEL-C which were differed in their chemical structures.

Biosurfactant-producing organisms have been isolated from several environments. Most of such bacteria used are isolated from contaminated sites usually containing petroleum hydrocarbon by products and/or industrial wastes (Carrillo et al., 1996; Rahman et al., 2002). For isolation and screening of the biosurfactant producing yeast had been reported from Thai fermented foods from Chonburi province (Chianguthai et al., 2006), oil contaminated sites such as soil from hot spring and garages (Techaoei et al., 2007), petroleum contaminated lagoon water (Ilori et al., 2008) and including studies in diversity of bioemulsifier producing yeasts isolated from wastewater treatment plant of a dairy industry (Monteiro et al., 2010). Literature review had shown the biosurfactant production from yeast when grown on varieties of vegetable oils such as soybean oil, rapeseed oil, olive oil or palm oil and agro industrial wastes (Amaral et al., 2008). The majority of the carbon sources in biosurfactant production are carbohydrate and lipid or hydrocarbon.

In this study, biodiversity of the effective biosurfactant producing yeasts were investigated from soil and agricultural waste and samples contaminated with oil. The efficient biosurfactant producers of the isolated yeast strains were selected based on their activities that showed lowering surface tension values to below 40 mN m^{-1}. These findings will bring to the discovery of strains capable of producing biosurfactants and further the molecular phylogenetic analysis may indicate novel yeast strains. Then an effective biosurfactant-producing yeast strains including the optimum conditions for their cultivation and biosurfactant production may be obtained. The properties of biosurfactant regarding physical and chemical characteristics were carried out. This research will provide biological databases as a resource for biosurfactant production from yeast for the application in various environment and food industries related to tensioactive compounds.

1.2   Objectives of the dissertation

The aims of the present study are:

1. To investigate the biodiversity of efficient biosurfactant-producing yeast isolated from samples contaminated oil.

2. To screen for novel yeast or efficient yeast strains with high potential to produce biosurfactant.

3. To optimize for cultivation and biosurfactant production process by the selected isolate.

4. To characterize the chemical structures and properties of biosurfactants produced by the selected yeast.

1.3   Experimental procedures

Part 1 Diversity of the efficient biosurfactant-producing yeasts

1.1 Isolation and screening of biosurfactant producing yeasts.

1.2 Identification of the isolated yeast strains by molecular technique and phylogenetic analysis.

1.3 Phenotypic characterization of interesting novel biosurfactant-producing yeasts

Part 2 Optimization for cultivation and biosurfactant production conditions

2.1 Selection of a strain of yeast having high biosurfactant producing ability.

2.2 Optimization of physical factors affecting cultivation and biosurfactant production.

2.3 Optimization of nutritional requirements affecting cultivation and biosurfactant production.

Part 3 Purification and characterization of biosurfactant produced by the selected strain

3.1 Isolation and determination of biosurfactant composition.

3.2 Purification and chemical structure characterization of biosurfactant by thin layer chromatography (TLC), mass spectrometry (MS) and nuclear magnetic resonances (NMR)

3.3 Physicochemical properties analysis of the biosurfactant.

CHAPTER II

LITERATURE REVIEWS

2.1   Introduction to biosurfactants

2.1.1 Definition and properties of biosurfactants

Biosurfactants are surface active compounds, synthesized by microorganisms, such as bacteria, yeast and filamentous fungi. They are totally or partially extracellular substances with amphiphillic compounds containing both polar and nonpolar moieties. The hydrophobic (non-polar) portion of the biosurfactant is insoluble in water and may have a long-chain of fatty acids, hydroxyl fatty acids or α-alkyl-β-hydroxy fatty acids. The hydrophilic (polar) portion can be a carbohydrate, amino acid, cyclic peptide, phosphate, carboxylic acid or alcohol (Van Ginkel, 1989). Due to the biosurfactants like to be accumulate at the interfaces (air-water and oil-water) as well as at air-solid and liquid-solid surfaces, the repulsive forces between two different phases were also reduced which two phases can easily interact and mix together very well. Regarding, the efficiency of a surfactant is determined by the capacity to reduce the surface or interfacial tensions and forming emulsions (Desai and Banat, 1997).

When surfactant monomers are added into the solution, the surface or interfacial tension will decrease until the surfactant concentration reaches at the critical micelle concentration (cmc). At this cmc or above, the surface or interfacial tension values are kept up constantly at the lowest although the surfactant concentrations gradually are increased (Figure 2.1A). At the cmc, monomer surfactants are able to aggregates in various forms, such as micelles, spherical or cylindrical micelles or lamellae (continuous bilayer) (Figure 2.1B). Micelles are the structure having the hydrophobic tails form the core of the aggregate and the

hydrophilic heads are in contact with the surrounding liquid. The shape of the aggregates depends on the chemical structure of the surfactants, subject to the balance of the sizes of the hydrophobic tail and hydrophilic head. However, the cmc for any surfactant is relied on the surfactant structure as well as the pH, ionic strength and the temperature of the solution.

2.1.2 Classification of biosurfactants

Biosurfactants are classified by their chemical compositions and microbial origin into the two categories of (i) low-molecular-mass molecules, which efficiently reduce the surface tension (ST) and (ii) high-molecular-mass molecules, which are effective as stabilizing emulsions (Rosenberg and Ron, 1999; Deleu and Paquot, 2004). The major classes of low-mass surfactant include glycolipids, lipopeptides and phospholipids whereas high-mass includes polymeric and particulate surfactants. Overall data in the major types of surfactants produced from microorganisms and the involved microorganisms were shown in Table 2.1. (Chavez and Maier, 2011). As noted that bacteria are the predominant group of surfactant-producing organisms however some yeast species are also involved. Bodour et al., (2004) have previously described a new class of biosurfactants named flavolipids produced by *Flavobacterium* sp isolated from a soil. Their biosurfactant molecule structures consisted a hydrophobic moiety containing saturated, unsaturated and/or hydroxylated fatty acids or fatty alcohols which is usually the hydrocarbon chain of a fatty acid with 10-18 carbon atoms and a hydrophilic moiety consisting of an ester, an hydroxy, a phosphate or mono-, oligo- or polysaccharides, peptides or proteins (Bognolo, 1999).

(A)

(B)
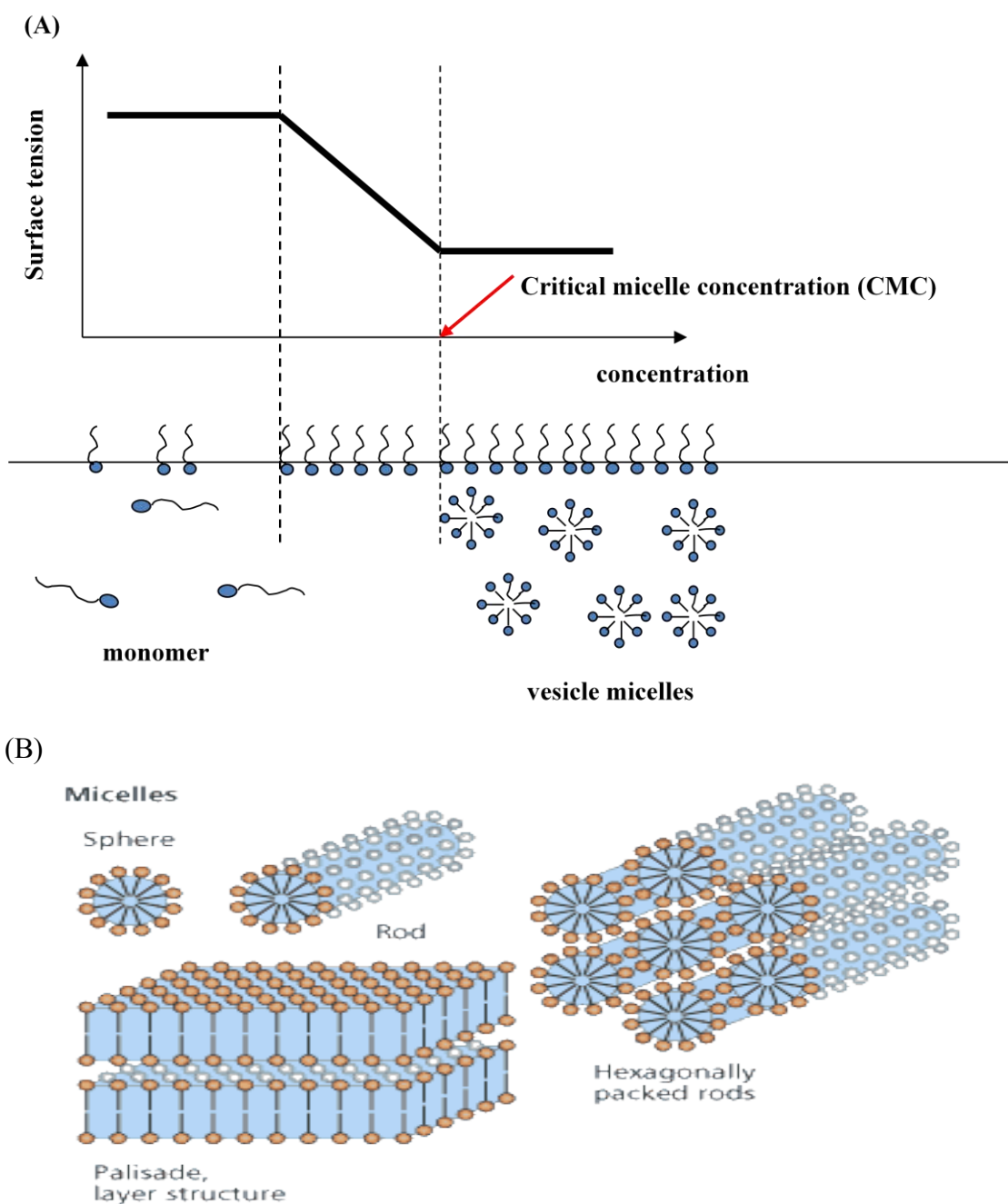
Figure 2.1 Formation of micelles at critical micelle concentration (A) and variation structures of aggregated micelles in sphere or vesicle, rod, hexagonally packed rods and layer structure (B)

(Online: www.rsc.org/chemistryworld/Issues/2003/July/amphiphiles.asp., 14th February, 2013)

Table 2.1 Examples of the more common biosurfactants and their origin

Class	Biosurfactant	Microorganisms	References
Glycolipids	Mannosylerythritol lipids	*Pseudozyma* (yeast), *Candida antartica, Ustilago maydis*	Kitamoto et at., 2002
	Sophorolipids	*Candida batistae, Torulopsis bombicola, Candida lypolytica, Candida. bombicola, Torulopsis apicola, Torulopsis petrophilum. Candida bogoriensis*	Van Bogaert et al., 2007
	Rhamnolipids	*Pseudomonas* sp. *Pseudomonas aeroginosa*	Reiling et al., 1986
	Trehalose lipids	*Rhodococcus* sp. *Arthrohacter* sp. *Rhodococcus erythropolis, Norcadia erytbropolis*	Lang and Philp, 1998
	Cellobiolipids	*Ustilago zeae Ustilago maydis*	Hewald et al., 2005
Lipopeptides	Surfactin	*Bacillus subtilis. Bacillus pumilus* A	Seydlova and Svobodova, 2008
	Viscosin	*Pseudomonas fluorescens, Pseudomonas libanensis*	Laycock et al., 1991
	Serrawettin	*Serratia marcescens*	Matsuyama et al., 1992
Fatty acid Neutral lipids Phospholipid	Fatty acid Neutral lipids and Phospholipid	*Corynlebacterium lepus* *Nocardia erythropolis* *Thiobacillus thiooxidans*	Cooper et al., 1981 Kretschmer et al., 1982 Knickerbocker et al., 2000
Polymeric	Emulsan Biodispersan Mannan-lipid-Protein Alasan	*Acinetobacter calcoaceticus* *Acinetobacter calcoaceticus* *Candida tropicalis* *Acinetobacter radioresistens*	Rosenberg and Ron, 1999 Rosenberg and Ron, 1997 Rosenberg and Ron, 1999 Navonvenezia et al., 1995
Siderophore	Flavolipids	*Flavobacterium* sp.	Bodour et al., 2004

Source: Chavez and Maier, 2011

2.1.2.1 Glycolipids

Glycolipids are the most well-known biosurfactants that compose of carbohydrates and long-chain aliphatic acids or hydroxyaliphatic acids. Among the glycolipids, the best known are rhamnolipids, trehalolipids, mannosylerythritol lipids and sophorolipids (Figure 2.2A-D).

Rhamnolipids are surface-active glycolipids. They contain one or two molecules of rhamnose linked to one or two molecules of β-hydroxydecanoic acid. The OH group of one of the acids is involved in glycosidic linkage with the reducing end of the rhamnose disaccharide, whereas the OH group of the second acid is involved in ester formation. Rhamnolipids were firstly found as exoproducts of the opportunistic pathogen *Pseudomonas aeruginosa* and described as a mixture of four congeners: α-L-rhamno pyranosyl-α-L-rhamnopyranosyl-β-hydroxydecanoyl-β-hydroxydecanoate (Rha-Rha-C10-C10), α-L-rhamnopyranosyl-α-L-rhamnopyranosyl-β-hydroxydecanoate (Rha-Rha-C10), as well as their mono-rhamnolipid congeners Rha-C10-C10 and Rha-C10. The development of more sensitive analytical techniques has lead to the further discovery of a wide diversity of rhamnolipid congeners and homologues (about 60) that are produced at different concentrations by various *Pseudomonas* species and by bacteria belonging to other families, classes, or even phyla like *Burkholderia* species (Mohammand et al., 2010).

Trehalose lipids, or Trehalolipids, are glycolipids containing trehalose as hydrophilic moiety and complex fatty acids varying from short simple to long chains as hydrophobic moiety. Disaccharide trehalose linked at C-6 and C-6′ to mycolic acids that are long chain, α-branched-β-hydroxy fatty acids. Trehalose lipids from different organisms differ in the size and structure of mycolic acid, the number of carbon atoms and the degree of unsaturation (Asselineau and Asselineau, 1978). They are mainly produced by Gram positive, high GC content bacteria of Actinomycetales including the genera of *Rhodococcus*, *Mycobacterium*, *Nocardia*, *Gordonia*, *Corynebacteria*, *Brevibacteria*, *Arthrobacter*, etc. They are active to reduce water surface and interfacial tension for example trehalose lipids from *Rhodococcus erythropolis* and *Arthrobacter* sp. reduced the surface and interfacial tension in culture broth from 40 to 25 and 5 to 1 mN m^{-1} respectively (Kretschmer et al., 1982). *Arthrobacter paraffineus* could produce emulsion layers as trehalose dimycolates in culture broths when the cells were grown on hydrocarbon substrates (Suzuki et al., 1969). With these properties, they were performed as possible application in enhanced oil recovery. Moreover, there are seldom reported the trehalose lipids production by yeast and other fungus (Shao, 2011).

Mannosylerythritol lipids (MEL) are belonged to glycolipid containing 4-O-β-D-mannopyranosyl-*meso*-erythritol as the hydrophilic group and a fatty acid and/or an acetyl group as the hydrophobic moiety (Kitamoto, 2008). Structural diversity of MEL based upon the number and position of the acetyl group on mannose or erythritol or both, number of acylation in mannose including fatty acid chain length and their saturation (Arutchelvi et al., 2008) They are produced by *Ustilago zeae, U. maydis* (as a minor component along with cellobiose lipid (CL) (Haskins et al., 1955; Spoeckner et al., 1999), *Schizonella melanogramma* (Deml et al., 1980), and yeast strains of the genus *Pseudozyma* sp. (as a major component) such as *P. aphidis,P. antarctica* and *P. rugulosa* (Kitamoto et al.,1990; Konishi et al., 2007a, b). The synthesis of MEL is not growth associated; it can also be produced by using resting (stationary phase) cells of yeast. MEL acts as an energy storage material in the yeast cells similar to triacylglycerols. MELs are shown to reduce the surface tension of water to less than 30 mN m^{-1} (Kim et al., 2006, Kitamoto et al., 2002).

Sophorolipids (SLs) consist of a dimeric sugar (sophorose) and a hydroxyl fatty acids, linked by a β-glycosidic bond (Asmer *et al.*, 1988). According to Hu and Ju, (2001) there are two types of SLs namely, the acidic (non-lactonic) SLs and the lactonic SLs. The hydroxyl fatty acids moiety (mostly C16-C18) of the acidic SLs has a free carboxylic acid functional group whilst that of the lactonic SLs forms a macrocyclic lactone ring with the 4′′-hydroxyl group of the sophorose by intramolecular esterificaion. As previously reported, sophorolipids were produced by yeast strain such as *Starmerella* (*Candida*) *bombicola* , *Candida* (*Torulopsis*) *apicola*, and *Rhodotorula* (*Candida*) *bogoriensis*, *Wickerhamiella domerquiae*, *Candida batistae* (Spencer, 1970; Hommel et al., 1994; Tulloch et al., 1968; Chen et al., 2006b; Konishi et al., 2008). Sophorolipids has been reported capable of lowering both surface and interfacial tension, though they are not effective emulsifying agents (Cooper and Paddock, 1984; Kitamoto et al., 2002). Both lactonic and acidic sophorolipids lowered the surface tension to 33 mN m^{-1} and the interfacial tension against *n*-hexadecane and water from 40 to 5 mN m^{-1} with 10 mg L^{-1} of pure sophorolipid. It is also showed a remarkable stability toward pH and temperature changes. The surface active properties were consistent between pH values of 6-9 and

temperature ranging from 20-90 ℃. Sophorolipids are the most important biosurfactants used in cosmetics and therapeutics (Maingault, 1999). In addition, lactonic SLs have been reported to have attracted more commercial and scientific attention than their acidic counterparts. They have measurable biocide activity (Lang et al., 1989), whilst the acetylated lactonic SLs have been applied in cosmetics as antidandruff, bacteriostatic agents and deodorants (Mager et al., 1987).

(A) Rhamnolipids
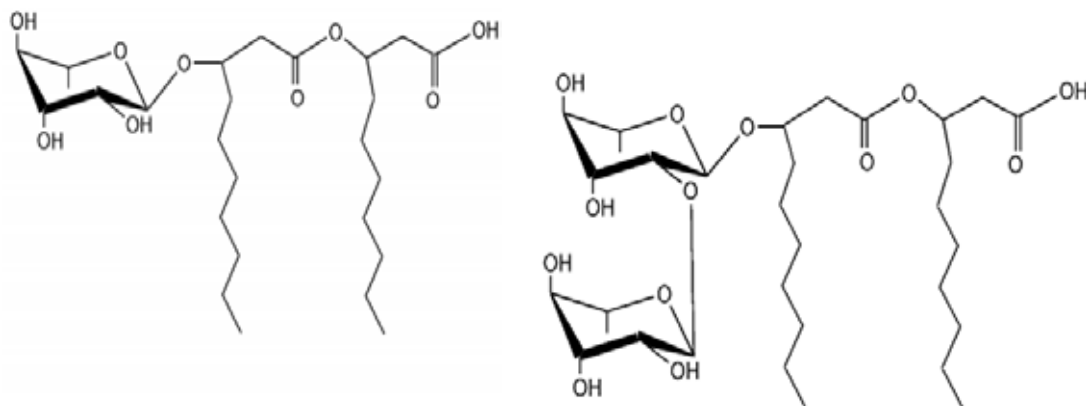
Mono-rhamnolipids   Di-rhamnolipids

(B) Trehalose lipids
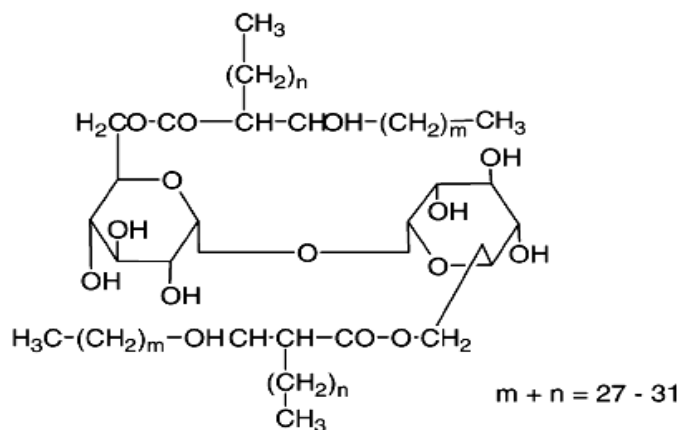
(C) Mannosylerythritol lipids (MEL)
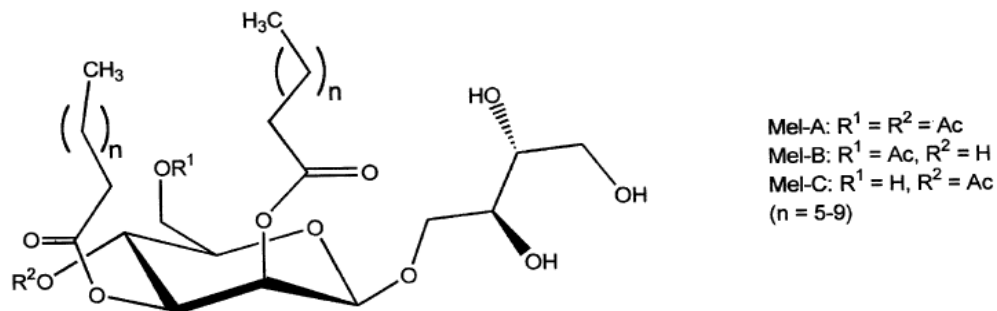
(D) Sophorolipids

$$R = -COCH_3 \text{ and/or } -H$$

Acidic sophorolipid   Lactonic sophorolipid
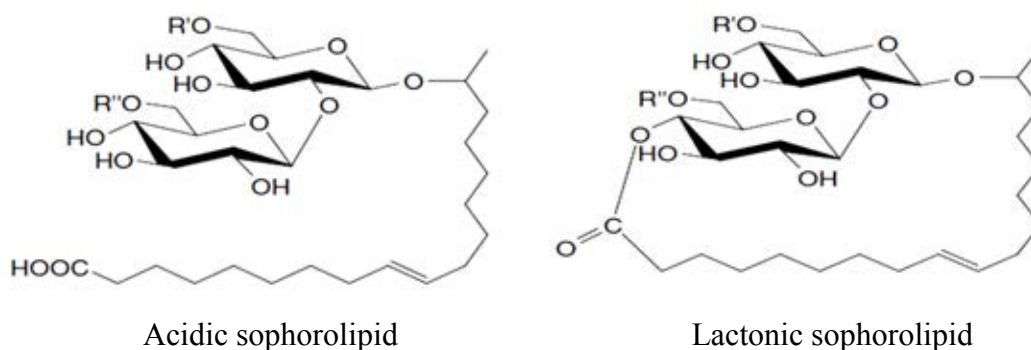
Figure 2.2 Structure of glycolipid biosurfactants. (A) Rhamnolipids from
Pseudomonas aeruginosa, (B) Trehalose lipids from *Rhodococcus erythropolis*, (C)
Mannosylerythritol lipids from *Pseudozyma antarctica* and (D) Sophorolipids from
Starmerella bombicola.
Source: Lang, 2002; Muthusamy et al., 2008

2.1.2.2 Lipoproteins and Lipopeptides

Lipopeptides consist of both hydrophilic of amino acid composition
and lipid portion which is mostly a hydroxy fatty acid. A family of cyclic lipopeptides
consists of 8 to 17 amino acids and a lipid portion which is composed of 8 to 9
methylene groups and a mixture of linear and branched tails (Desai and Banat, 1997).
Cyclic lipopeptides are produced by a number of *Bacillus* species as antibiotic
molecules. Among these, the most important compound is surfactin produced by

Bacillus subtilis because of its very high activity (Desai and Banat 1997; Rosenberg and Ron 1999) and lichenysin by *Bacillus licheniformis* (Yakimov et al., 1995) (Figure 2.3A, B).

Surfactin The cyclic lipopeptide surfactin, produced by *Bacillus subtilis* ATCC21332, is one of the most powerful biosurfactants. It is composed of a seven amino-acid ring structure linked to a fatty-acid chain via lactone linkage. It could reduce the surface tension from 72 to 27.9 mN m^{-1} at concentrations as low as 0.005% (Arima et al., 1968).

Lichenysin Lichenysin are also lipoheptapeptide, similar in structural and physio-chemical properties to the surfactin (McInerney et al., 1990). *Bacillus licheniformis* produces several biosurfacants which act synergistically and exhibit excellent temperature, pH and salt stability. The surfactants produced by *B. licheniformis* are capable of lowering the surface tension of water to 27 mN m^{-1} and the interfacial tension between water and *n*-hexadecane to 0.36 mN m^{-1}.

2.1.2.3 Fatty Acids, Phospholipids and Neutral Lipids

Fatty acid and phospholipid derived from alkanes have received considerable attention as surfactants. They produced by several bacteria during growth on *n*-alkanes (Rosenberg and Ron, 1999). The hydrophilic and lipophilic balance (HLB) is directly related to the length of the hydrocarbon chain in their structures. Microorganisms synthesized complex fatty acids containing hydroxyl groups and alkyl branches (mostly found C12 - C14 of saturated fatty acids), for example the corynomycolic acids produced by *Nocardia erythropolis*, *Corynebacterium lepus* (MacDonald et al. 1981) and phospholipids produced by *Acinetobacter* spp. (Kaeppeli and Finnerty, 1979) and *Thiobacillus thiooxidans* (Beebe and Umbreit, 1971).

In phosphatidylethanolamine-rich vesicles were produced by *Acinetobacter* sp. HO1-N (Kaeppeli and Finnerty, 1980) which formed optically clear microemulsions of alkanes in water (Figure 2.3C). Phosphatidyl-ethanolamine produced by *Rhodococcus erythropolis* grown on *n*-alkane causes a lowering of

interfacial tension between water and hexadecane to less than 1 mN/m and a critical micelle concentration (CMC) of 30 mg L^{-1} (Kretschmer et al., 1982).

2.1.2.4 Polymeric biosurfactants

Polymeric biosurfactants are emulsan, liposan, alasan, lipomanan and other polysaccharide–protein complexes were produced by either bacterial species or yeast. Bioemulsans produced by different species of *Acinetobacter* (Rosenberg and Ron 1998). *Arthrobacter calcoaceticus* RAG-1 produces an extracellular potent polyanionic amphipathics heteropolysaccharide bioemulsifier (Rosenberg et al., 1979) (Figure 2.3D). Emulsan is an effective emisifying agent for hydrocarbons in water (Zosim et al., 1982), even at a concentration as low as 0.001 to 0.01%. Liposan is an extracellular water-souble emulsifier synthesized by *Candida lipolytica* and is composed of 83% carbohydrate and 17% protein with the carbohydrate portion being a heteropolysaccharide consisting of glucose, galactose, galactosamine and galactoronic acid. (Cirigliano and Carman, 1985). Cameron et al. (1988) demonstrated the production of large amounts of mannoprotein by *Saccharomyces cerevisiae*. When purified, the emulsifier contains 44% mannose and 17% protein. The mannoprotein exhibited excellent emulsifying activity toward several oils, alkanes and organic solvents. Other polymeric biosurfactants such as biodispersan, alasan, food emulsifiers, protein complexes and insectides emulsifiers have also been reported.

2.1.2.5 Particulate biosurfactants

This type of biosurfactant includes vesicles and fimbriae produced by *Acinetobacter* sp. The purified vesicles are composed of protein, phospholipid and lipopolisaccharide. This extracellular membrane vesicles partition hydrocarbons form a microemulsion, which plays an important role in alkane uptake by microbial cells (Kappeli and Finnerty, 1979).

(A) Surfactin	(B) Lichenysin
(C) Phosphatidylethanolamine	(D) Emulsan
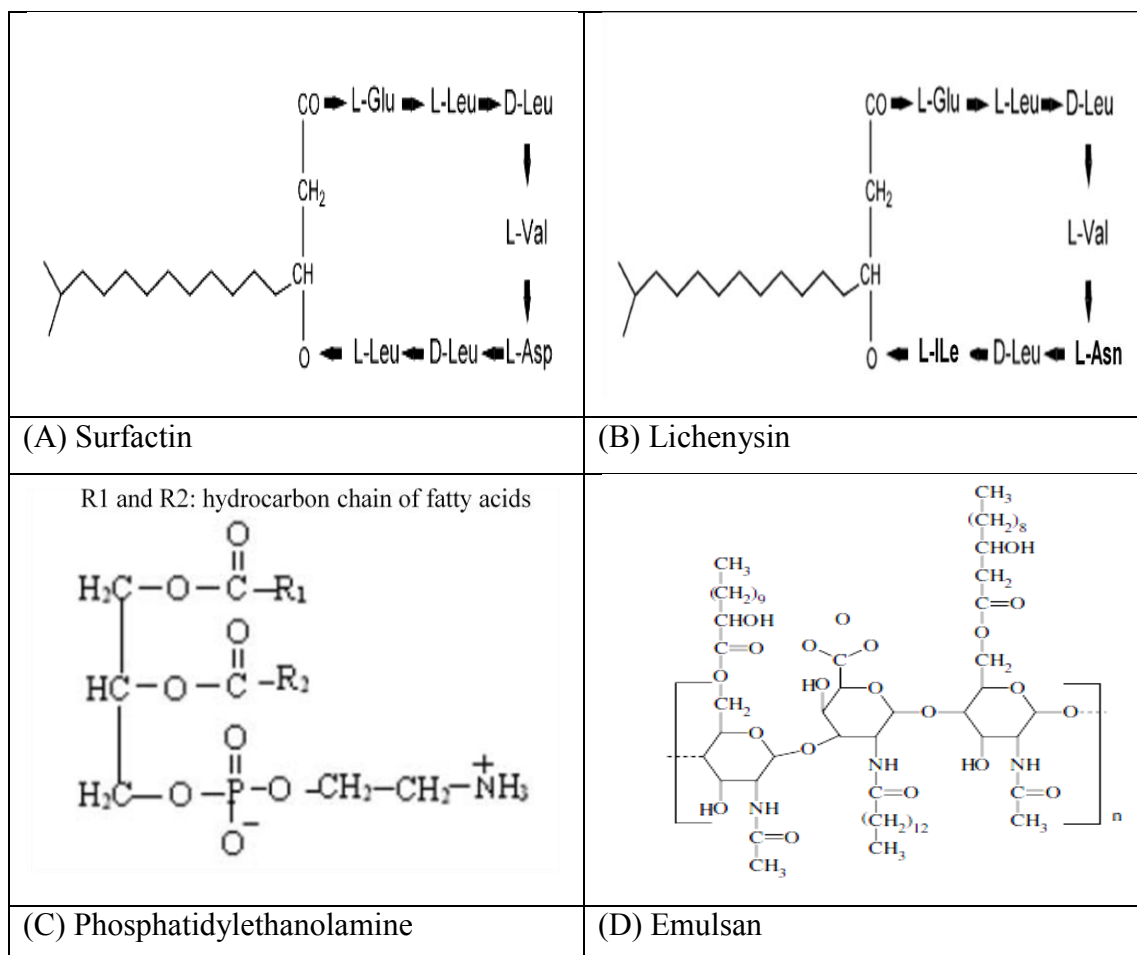
Figure 2.3 Structure of other biosurfactants. (A) surfactin produced by *Bacillus subtilis* (B) lichenysin produced by *Bacillus licheniformis*, (C) phosphatidyl-ethanolamine from *Acinetobacter* sp. (Desai and Banat, 1997) and (D) Emulsan from *Arthrobacter calcoaceticus* RAG-1(Banat et al., 2010).

2.2 Diversity of biosurfactant producing microorganisms

Biosurfactants are a group of natural products of interest for biotechnological and industrial applications (Desai and Banat, 1997; Bodour and Maier, 2002). Interest in research concerning biosurfactant publication has increased considerably in the last decade (Figure 2.4) as well as other newly discovered biosurfactants or novel biosurfactant producers as evidenced by the rapidly growing number of publication on the topic of biosurfactants (Chavez and Maier, 2011).
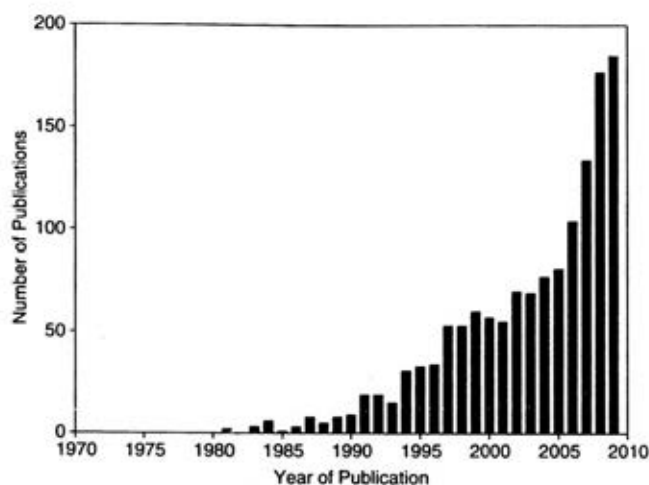
Figure 2.4 The number of publication obtained from a year by year search for the term "biosurfactant" on the Thomson Reuters ISI Web of Knowledge search platform (2009). (Source: Chavez and Maier, 2011)

Biosurfactant-producing microorganisms have been isolated from a wide diversity of environments including soil, sea water, marine sediments oil fields (Yamikov et al., 1998) and even extreme environments (Cameotra and Makkar., 1998). A large body of research has been focused to isolate and produce biosurfactant by selected bacteria that they have been described as biosurfactant producer in bacterial genera such as *Bacillus*, *Pseudomonas*, *Rhodococcus*, *Mycobacterium*, *Nocardia*, *Flavobacterium*, *Clostridium*, *Corynebacterium*, *Acinetobacter*, *Thiobacillus*, *Serratia*, *Arthrobacter* and *Alcanivorax* (Bodour et al., 2003). There is limitation on studies of the frequency and distribution of biosurfactant producers in the environment. Bodour et al. (2003) have studied the distribution of culturable bacteria that produced biosurfactants from undisturbed and contaminated sites, and then reported that 45 of 1,305 isolates from 21 sites or 3.4 % of the total isolates were found to be biosurfactant producers belonged to be *Bacillus subtilis*, *Pseudomonas aeruginosa*, *Pseudomonas* sp. and *Flavobacterium* sp. Furthermore, they suggested some environments having selective pressure may have greater for biosurfactant productions than others. Later, biosurfactant produced by *Flavobacterium* isolates was identified the chemical structures that found to be the flavolipids, an novel class of

biosurfactants. (Bodour et al., 2004). In recent study, Jaysree et al., 2011 isolated biosurfactant producing bacteria from environmental samples (contaminated soil and uncontaminated water) and screen bacteria as the biosurfactant producers by oil displacement and emulsification index methods. Consequently, *Bacillus cereus* obtained from contaminated soil are found to produce stronger biosurfactant activity than that of *Bacillus subtilis* obtained from uncontaminated water, therefore they suggested that the biosurfactant producing activity of *Bacillus subtilis* may be induced in the presence of pollutant.

A growing number of aspects related to the production of biosurfactants from yeasts have been the topic of research during the last decade (Amaral et al., 2008). From previous studies, isolation and screening of the biosurfactant producing microbes had been reported from Thai fermented foods from Chonburi province (Chianguthai et al., 2006), oil contaminated sites such as soil from hot spring and garages (Techaoei et al., 2007), petroleum contaminated lagoon water (Ilori et al., 2008) and including studied in diversity of bioemulsifier producing yeasts isolated from waste water treatment plant of a diary industry (Monteiro, et al., 2010). Chianguthai et al. (2006) and Thaniyavarn et al. (2008) demonstrated the efficient biosurfactant-producing yeast, *Pichia anomala* PY1, which was isolated from Thai fermented food and could produce sophorolipid which could significantly lower the surface tension to 28 mN m^{-1}.

Techaoei et al. (2007) preliminary studied biosurfactant-producing organisms and investigated diversity of 197 bacterial strains isolated from hot spring and garages in northern Thailand. They were isolated by using enrichment cultures containing 0.1% soybean oil as a carbon source and revealed that 25 strains of bacteria from garage site showed high biosurfactant activity in oil displacement area test and E_{24} emulsifying capacity.

Ilori et al. (2008) isolated 2 yeast strains from petroleum contaminated lagoon water in Nigeria and which were identified to be *Saccharomyces cerevisiae* and *Candida albicans*. These strains had long lag phase in mineral salt medium supplemented with 1% (v/v) diesel oil and reached exponential phase after 6 days. The

biosurfactant activities were initially detected on day 2 and reached maximum at stationary phase.

Recently Monteiro et al. (2010) reported 132 yeast isolates of which 31, were found to be bioemulsifier-producing strains isolated from dairy waste in Brazil and three effective bioemulsifier producing yeasts, *Trichosporon loubieri*, *T. montevideense* and *Geotrichum* sp. could produce biosurfactants consisting of lipid-polysaccharide complexes and showed high emulsion-stabilizing capacity. Likewise the discovery of new biosurfactant producing yeast strains, *Trichosporon asahii*, isolated from petroleum-contaminated soil in India was found to be the efficient producer of biosurfactant in mineral salt media containing diesel oil as the carbon source and found to be an great degrader of diesel oil (95%) over a period of 10 days (Chandran and Das, 2010). However, there are only few reports of diversity of biosurfactant-producing yeast from samples contaminated oil such as agricultural wastes contaminated with palm oil and waste from biodiesel plants; therefore it is interesting to search for new strain of biosurfactant producing yeasts.

2.3 Yeast diversity and molecular identification by D1/D2 analysis

Yeast is one of the important industrial microorganisms, because of its essential role in brewing, wine making, baking, and fuel alcohol production. They are also important infectious agents (Lachance and Starmer, 1998). In addition, yeast has been growing in importance in biosurfactant production, with production being reported mainly by the genders *Candida* sp., *Pseudozyma* sp. and *Yarrowia* sp. (Amaral et al., 2008). There were many reports of yeast diversity associated the fermentation process of food and beverages (Strauss et al., 2001; Pretorius et al., 1999) including in some natural habitats from individual area (Hong et al., 2002; Jindamorakot et al., 2006). Their work demonstrated that molecular identification methods based on the partial sequences of 26S rDNA are rapid and useful tools for yeast diversity studies.

The assignment of yeast and fungi strains to the family, and especially to the genera and species level has previously been primarily based upon morphology and

then physiological/biochemical characters. However, this has become apparent that this is somewhat problematic. Comparative molecular analyses of sequence variation in regions that show relatively similar mutation rates across species, such as the D1/D2 variable regions of the large subunit (LSU) RNA gene, the complete small subunit (SSU) RNA gene or the non-coding internal transcribed spacers (ITS1 - 5.8S rRNA- ITS2; ITS1-2) region are increasingly used for the rapid and development stage independent approach for identification of species. Consequently DNA sequencing of 26S rDNA of D1/D2 region has been widely adopted to understand species relationships because of its rapidness and resolution of both close and distant relationships (Kurtzman and Robnett, 1998; Fell et al., 2000). In addition it has already been demonstrated that some species have significant variability in the D1/D2 domain, sequencing of this region is generally accepted as a means for species delimitation, especially for yeasts in *Candida* species (Ramos et al., 2006). Several different species had proven to discriminate with greater than 1% nucleotide substitution from comparison of nuclear relatedness (Peterson and Kurtzman, 1991; Kurtzman and Robnett, 1998). From the nucleotide comparison, it was predicted that strains showing greater than 1% substitutions in the ca.600-nucleotide D1/D2 domain are likely to be different species and that strains with 0-3 nucleotide differences are either conspecific or sister species (Kurtzman and Robnett, 1998). Identification of yeasts based on the 1% substitution criterion of D1/D2 sequence is very rapid and reliable in contrast to the identification based on morphological or physiological data.

2.3.1 Classification of yeast

Classification of yeast is regularly performed according to guideline in yeast taxonomy, described in The Yeasts, a Taxonomic Study. The Yeasts, a Taxonomic Study 5th edition (Kurtzman, Fell and Boekhout) published in 2011, the lastest edition, collected 149 genera and nearly 1,500 species that are classified into the Phylum Ascomycota and Phylum Basidiomycota. The application of genes sequences analysis is largely responsible for the increase in the number of taxa presented in this edition. This book contains 3 volumes that have been described all currently known yeast species including characterization of species from morphology, growth tests and gene sequences. Species descriptions also include information on ecology, systematics,

phylogeny, biotechnological uses, importance to food and agriculture and clinical significance are detailed in volume 1. The ascomycetous yeasts (Phylum ascomycota) in volume 2 are distributed in 2 subphylum, 5 classes; Taphricomycotina (Neolectomycetes, Pneumocystidomycetes, Schizosaccharomycetes, Taphrinomycetes) and Saccharomycotina (Saccharomycetes). The classification system of ascomycetous yeasts is shown in Figure 2.5. The basidiomycetous yeasts (Phylum basidiomycota) in volume 3 are distributed in three subphylums: Pucciniomycotina (Pucciniomycetes, Mixiomycetes, Agaricostilbomycetes, Cystobasidiomycetes, Microbotryomycetes), Agaricomycotina (Tremellomycetes) and Ustilaginomycotina (Ustilaginomycetes, Exobasidiomycetes). The classification system of basidiomycetous yeasts is shown in Figure 2.6.

The criteria used for yeast identification are conventional (Yarrow, 1998), chemotaxonomic (Phaff, 1998; Yamazaki et al., 1998) and molecular taxonomic characteristics (Kurtzman, 1998; Kurtzman and Blanz, 1998).

2.3.1.1 Phenotypic characteristics

Yeast systematic is characterized by a thorough study of morphological characteristics including vegetative reproduction and characteristics of sexual reproduction such as ascospore formation and basidiospore formation.

Physiological and biochemical characteristics were based on the fermentation of carbohydrates, assimilation of carbon compounds, assimilation of nitrogen compounds, growth in vitamin-free medium and vitamin requirements, growth in media of high osmotic pressure, growth at 37°C and at other temperatures, acid formation from glucose, formation of extracellular amyloid compounds (starch formation), hydrolysis of urea and cycloheximide resistance (Wickerham, 1951).

2.3.1.2 Chemotaxonomic characteristics

Chemotaxonomic characteristics, such as carbohydrate composition of cell walls and capsules (Suzuki and Nakase, 1998; Prillinger et al., 1993), ubiquinone

system (Yamada and Kondo, 1973; Yamada et al., 1976, 1977), fatty acid composition (Cottrel et al.,1986; Viljoen et al., 1986) were extensively used for taxonomic distinctions and DNA base composition (mol% G+C) can differentiate phenotypically similar strains of different species (Kurtzman and Phaff, 1987).The range of nucleic acid base compositions differs for ascomycetous and basidiomycetous yeasts. Most ascomycetous yeasts have a mol% G+C about 27-50%, while that of basidiomycetous yeasts is approximately 50-70% (Kurtzman, 1998) except for the narrow range of 48-52%, where some overlap occurs.

2.3.1.3 Molecular characteristics

Molecular techniques that made a great impact on the taxonomy of yeasts is D1/D2 of the 26S rDNA sequencing. D1/D2 variable domains of the 26S large subunit ribosomal RNA are near the 5' end of large subunit (26S) rRNA (Peterson & Kurtzman, 1991). These regions are considered to be chronometers because of their universal occurrence, functional constraints and presences of both variable and conserved regions (Valente et al., 1999). The length of the sequenced of D1/D2 region was about 600 nucleotides (63-642 bp of *Saccharomyces cerevisiae*). This region is useful for analysis at the species level (Kurtzman and Robnett, 1998) and a large database is now available. However, the D1/D2 sequence might not distinguish varieties species or sister species so a phylogenetic analysis of the dataset provides an overview of close species relationships. The most frequently used numerical methods for sequence comparison are parsimony, neighbor-joining (Saitou and Nei**,** 1987) and maximum likelihood methods (Felsenstein, 1985). The phylogenetic trees need to be statistically tested to set confidence limits for branching order by bootstrap analysis (Felsenstein, 1985).

Figure 2.5 Phylogenetic relationship among ascomycetous yeast genera, as represented by type species, which were determined from neighbor-joining analysis of a concatenated dataset of gene sequences from LSU rRNA, SSU rRNA and translation elongation factor. Published family designations are listed. Where known, coenzyme Q determinations are given in brackets. Bootstrap values were determinate from 1,000 replicates. Y and YB prefixes are NRRL strain numbers. T=type strain, NT=neotype strain, A=authentic strain, Type species of genera *Ascobotryozyma* (anamorph, *Botryozyma*), *Coccidiascus*, *Endomyces*, *Helicogonium*, *Phialoascus*, *Saitoella*, *Macrorhabdus*, *Schizoblastosporon* (teleomorph, *Nadsonia*), some of which are not known from culture, were not included in the analysis (Kurtzman and Robnett, unpublished data).
Source: Kurtzman et al., 2011.
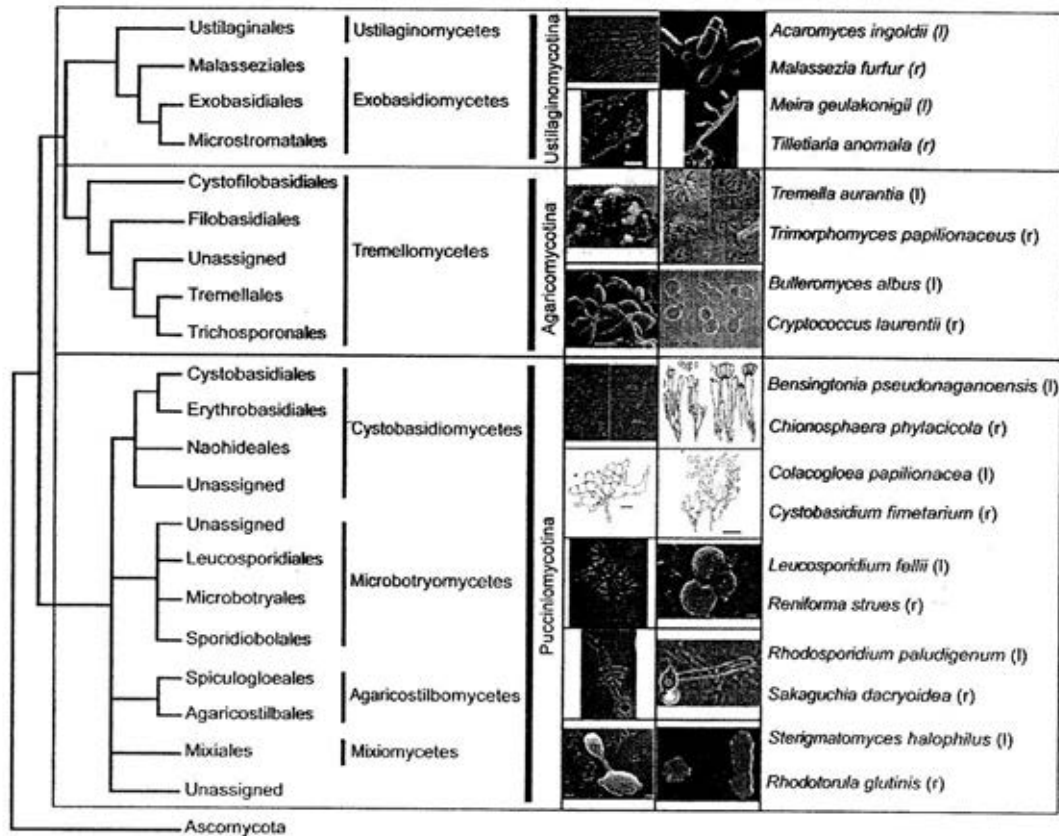
Figure 2.6 Phylogenetic relationships between three subphyla of the Basidiomycota that contain yeast states. r, right picture; l, left picture.

Source: Kurtzman et al., 2011.

At present, most molecular comparisons have been made from single genes and it is unclear whether the relationships depicted are representative of the evolutionary history of the organisms themselves. Additionally, basal branches in phylogenetic trees derived from single genes are often only weakly supported, resulting in uncertain relationships among more divergent species (Kurtzman and Robnett, 2003). They studied phylogenetically relationships among yeasts of the 'Saccharomyces complex' by using multigene sequence analyses. Analysis of combined gene sequences resolved the 75 species compared into 14 clades, many of which differ from currently circumscribed genera.

Concerning novel yeast in this research, some closely known yeast was later mentioned. As an example of the use of molecular approaches in ascomycetous yeasts, the yeast genera *Williopsis* and *Pichia* were originally classified as individual species in several diverse clades of ascomycetous yeasts. The classification of *Williopsis saturnus* species complex was designated as 5 varieties of var. *saturnus,* var. *mrakii,* var. *sargentensis,* var. *suaveolens* and var. *subsufficiens* by nuclear DNA reassociation (Kurtzman, 1991). Therefore, to clarify the taxonomic status of *Williopsis saturnus*, pairwise nucleotide differences among type strains of five varieties of *W. saturnus* for D1/D2 region of LSU rRNA gene, ITS1-2 rRNA and EF-1α genes was performed and showed that the five varieties of *W. saturnus* represent closely related, but genetically distinct taxa that can be regard as species of *Lindnera* (Kurtzman et al., 2008). The genus *Cyberlindnera* was introduced as a replacement name for *Lindnera*, with 21 new combinations (Minter, 2009). *Cyberlindnera mengyuniae* and *Cyberlindnera rhizosphaerae* were later described to be related to this clade (Chen et al., 2009, Mestre et al., 2011). Five novel species of genus *Candida* in the *Cyberlindnera* clade were proposed, hence the *Cyberlindnera* clade contains twenty-three recognized teleomorphic species and 12 *Candida* species are also related to this clade (Chang et al., 2012).

For resolving the clade containing the coenzyme Q-9 producing species of *Pichia, P. spartinae, P. acaciae* and *P. guilliermondii* were assigned to *Yamadazyma* and clustered among several clades (Billon-Grand 1989; Kurtzman and Robnett 1998). Kurtzman and Suzuki (2010) further classified the species of these genera into 12 well-supported clades, based on the sequence analysis of the D1/D2 region of the LSU and the SSU rRNA genes. In this analysis, *P. spartinae* was proposed to belong to the genus *Scheffersomyces. S. spartinae*, which does not ferment D-xylose is the basal species in the genus *Scheffersomyces,* and its placement is weakly supported. The addition of new species to the clade may strengthen placement of *S. spartinae* in the genus or provide evidence that it is a member of an undescribed sister genus. The new taxon *Candida gosingica* was proposed by Chang et al. (2011) as a novel species that was quite closely related to *S. spartinae*, based on sequence analysis of the SSU, the D1/D2 domain of the LSU and the ITS1-2 region of the rDNAs.

2.4   Important roles of biosurfactant in microbial physiology and behaviour

Microorganisms may be commonly found at interfaces as seen by biofilms, surface films and aggregates. They are impacted by interfacial phenomena, as its phospholipid bilayer of cell membrane or cell wall always interact with their environments; this is the natural roles and impacts of biosurfactants with respect to microbial behavior. (Rosenberg and Ron, 1999; Van Hamme et al., 2006). Biosurfactants play an essential natural role whenever a microbe encounters an interface. Biosurfactants are important for motility (gliding and swarming motility as well as de-adhesion from surfaces), cell–cell interactions (biofilm formation, maintenance and maturation, quorum sensing, amensalism and pathogenicity) and cellular differentiation, substrate accession (via direct interfacial contact and pseudosolubilization of substrates), as well as avoidance of toxic elements and compounds (Van Hamme et al., 2006). They may also be used as carbon and energy storage molecules, as a protective mechanism against high ionic strength, and may simply be by-products released in response to environmental change (e.g. extracellular coverings), all above as shown in Figure 2.7. However, several studies have been investigated on many roles of microbial biosurfactants, these included emulsification of water-insoluble substrates (Oberbremer et al., 1990; Zhang and Miller., 1992), direct contact of cells with hydrocarbon droplets and their interaction with emulsified droplets (Singh and Desai., 1986; Francy et al., 1991), cell adherence which imparts greater stability under hostile environmental conditions and virulence (Rosenberg, 1986), cell desorption to find new habitats for survival (Rosenberg and Rosenberg., 1981), antagonistic effects toward other microbes in the environment (Kitamoto et al., 1993; Lang et al., 1989; Uchida et al., 1989) as briefly shown in Figure 2.7.

Intracellular

Nutrient Storage
Intracellular bioavailability & efflux
    Nutrients
    Toxic components
    Regulatory molecules
Gene regulation

Bioavailability
    Gene uptake
    Nutrient uptake
    Sequestration of toxic components

Extracellular

Cellular differentiation
    Cell hydrophobicity change
    Mycelium formation
    LPS removal

Motility
Quorum sensing

Intercellular

Antagonism
Pathogenesis
Biofilm formation maintenance & maturation

Microbial community structure

Figure 2.7 Important roles of biosurfactants in microbial physiology and ecology.
Source: Van Hamme et al., 2006

2.5   Kinetics of biosurfactants

Most of the biosurfactants are secondary metabolites, which are released into the culture medium at the stationary phase. Some of them are also produced throughout the exponential phase (Cameotra and Makkar, 1998). The production of biosurfactant has been carried out in batch or continuous fermentation at low dilution rates. The kinetics of biosurfactant production exhibit many variations among various systems: (i) growth-associated production, (ii) production under growth-limiting conditions, (iii) production by resting or immobilized cells, and (iv) production with precursor supplementation (Desai and Banat, 1997). Schematic illustration as indicated in Figure 2.8 showing different types of fermentation kinetics for biosurfactant production. (A) Growth-associated production in which a parallel

relationship is obtained between growth, substrate utilization, and biosurfactant production as observed in *Acinetobacter calcoaceticus*. (B) Production under growth-limiting condition in which biosurfactant production is observed only when growth limitation occurs by exhaustion of nutrients such as nitrogen as in the case of *Pseudomonas* spp. (C) Production of biosurfactant by resting or immobilized cells where cells continue to utilize substrate and produce biosurfactant without cell multiplication as observed for some *Torulopsis* spp.
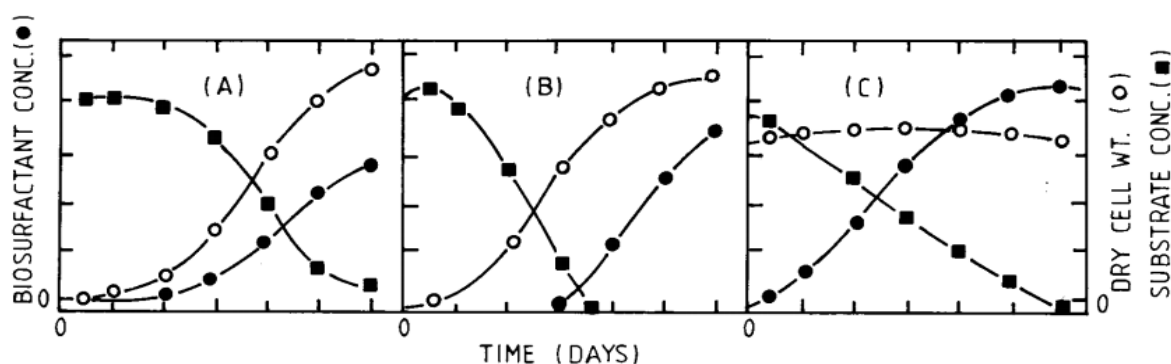
Figure 2.8 Schematic illustration showing different types of fermentation kinetics of biosurfactant production. (Biomass, glucose concentration and biosurfactant production).
Source: Desai and Banat, 1997

2.6   Biosynthesis of biosurfactant

With respect to the amphiphillic structures of biosurfactant, two primary metabolic pathways belong to hydrocarbons and carbohydrates were concerned in the synthesis of their hydrophobic and hydrophilic moieties, respectively. The pathways for the synthesis of these two groups of precursors are diverse and utilize specific sets of enzymes depend on genotype of individual microbial origin, types of biosurfactants and substrate used especially carbon substrates (Hommel and Ratledge, 1993). There are 4 possible evidences for biosurfactant synthesis of different moieties and their linkage according to Syldatk and Wagner (1987); Desai and Desai, 1993 as follows:

(i)   The hydrophilic and hydrophobic moieties are synthesized de novo by two independent pathways.

(ii)   The hydrophilic moiety is synthesized de novo while the synthesis of the hydrophobic moiety is induced by substrate.

(iii)   The hydrophobic moiety is synthesized de novo, while the synthesis of the hydrophilic moiety is substrate dependent.

(iv)   The synthesis of both the hydrophobic and hydrophilic moieties is substrate dependent.

For example, sophorolipids (SL), a kind of extracellular glycolipids biosurfactants, consist of a hydrophobic fatty acid tail of 16 or 18 carbon atoms and a hydrophilic carbohydrate head, sophorose. Sophorose is a glucose disaccharide with an unusual β-1, 2 bonds and can be acetylated on the 6′- and/or 6″-positions. One terminal or subterminal hydroxylated fatty acid is β-glycosidically linked to the sophorose molecule. The carboxylic end of this fatty acid is either free (acidic or open form) or internally esterified at the 4″ or in some rare cases at the 6′- or 6″-position (lactonic form). The hydroxy fatty acid itself counts in general 16 or 18 carbon atoms and can have one or more unsaturated bonds (Asmer et al., 1988; Davila et al., 1993). However, Asmer and co-worker (1988) reported that *C. bombicola* produced mixtures of sophorolipids containing varieties of structures differed in the fatty acid part (chain length, saturation, and position of hydroxylation) and the lactonization and acetylation pattern. Davila et al. (1993) analyzed the mixture of sophorolipids by HPLC method and used an evaporative light scattering to detect individual sophorolipids and found over 20 components of the fatty acid chains.

According to a schematic overview of the biochemical pathways of sophorolipid synthesis (Van Bogaert et al., 2007), glucose and a fatty acid are necessity of building blocks prior to synthesize sophorolipid. Both can be simply derived from medium consisted of carbohydrate and immiscible carbon. The biosynthesis of biosurfactant in glucose-grown cells involves glycolytic (glucose is degraded) and lipogenic (accumulation of synthesized fatty acids) metabolisms. In all cases, fatty acid biosynthesis begins with acetyl-CoA as the key intermediate (Figure 2.9). The differences of the synthesized unsaturated fatty acids occur mainly because

of the organization of the enzymes making up the individual fatty acid synthetase complexes (Hommel and Ratledge, 1993).
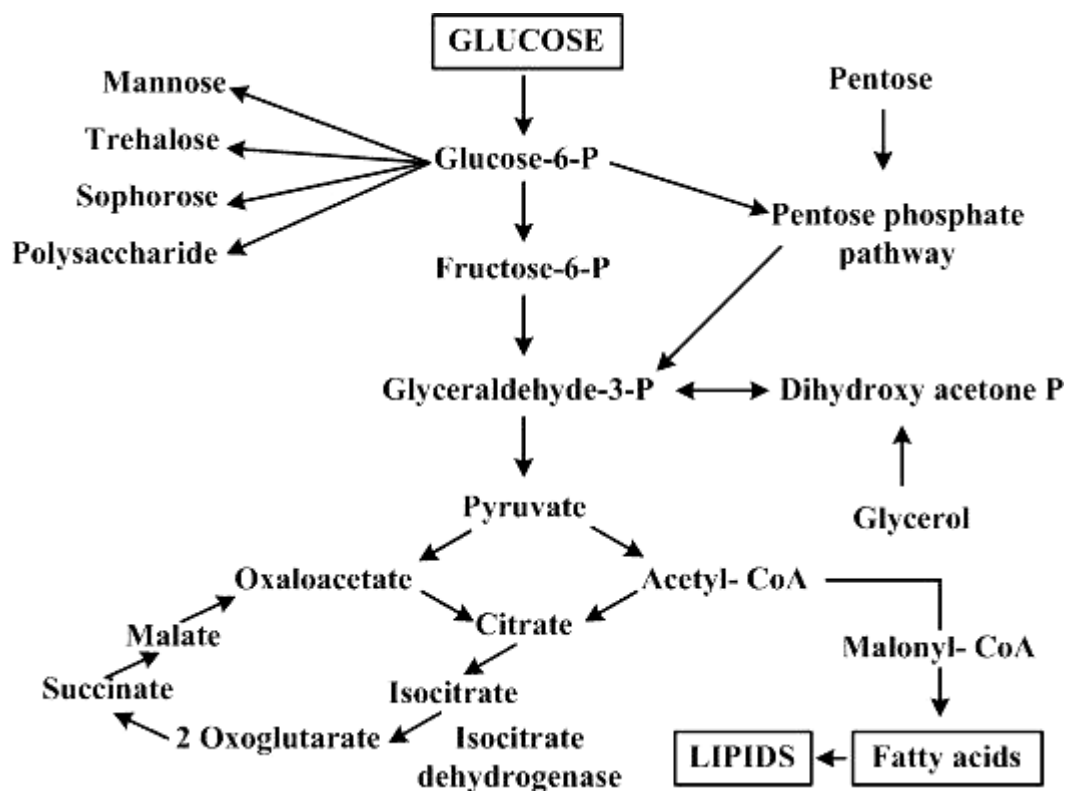
Figure 2.9 Metabolic pathway of glucose utilization during biosurfactant production. Source: Boulton and Ratledge, 1987; Mulligan and Gibbs, 1993

In case of only hydrophilic substrate present, fatty acids are formed by de novo pathway converting pyruvic acid to acetyl-CoA derived from glycolysis. As if the glucose concentration is low, fatty acids can be conducted toward the β-oxidation for cell maintenance whilst inductions of sophorolipid biosynthesis seem to be low.

Sophorolipid synthesis pathways are described in review of Van Bogeart et al. (2007) (Figure 2.10) as step1: the fatty acids are converted to a terminal (ω) or subterminal (ω-1) hydroxy fatty acid by NADP dependent monooxygenase enzyme, cytochrome P450.; Step 2: glucose is glycosidically coupled (position C1′) to the

hydroxyl group of the fatty acid through the action of a specific glycosyltransferase I The transferase reaction requires nucleotide-activated glucose [uridine diphosphate (UDP)-glucose] as glucosyl donor.; Step 3: a second glucose is glycosidically coupled to the C2′ position of the first glucose moiety by glycosyltranferase II. The majority of the sophorolipids are however further modified by both internal esterification (lactonization) and acetylation of the carbohydrate head. Lactonic sophorolipids are formed by an esterifaction reaction of the carboxyl group of the hydroxy fatty acid with a hydroxyl group of sophorose. The vast majority of lactones are esterified at the 4″-position, whereas a small percentage is esterified at either the 6′- or 6″-position (Asmer et al., 1988). The acetylation at the 6′- and/or 6″-position is carried out by an acetyl-coenzyme A (CoA) dependent acetyl transferase.
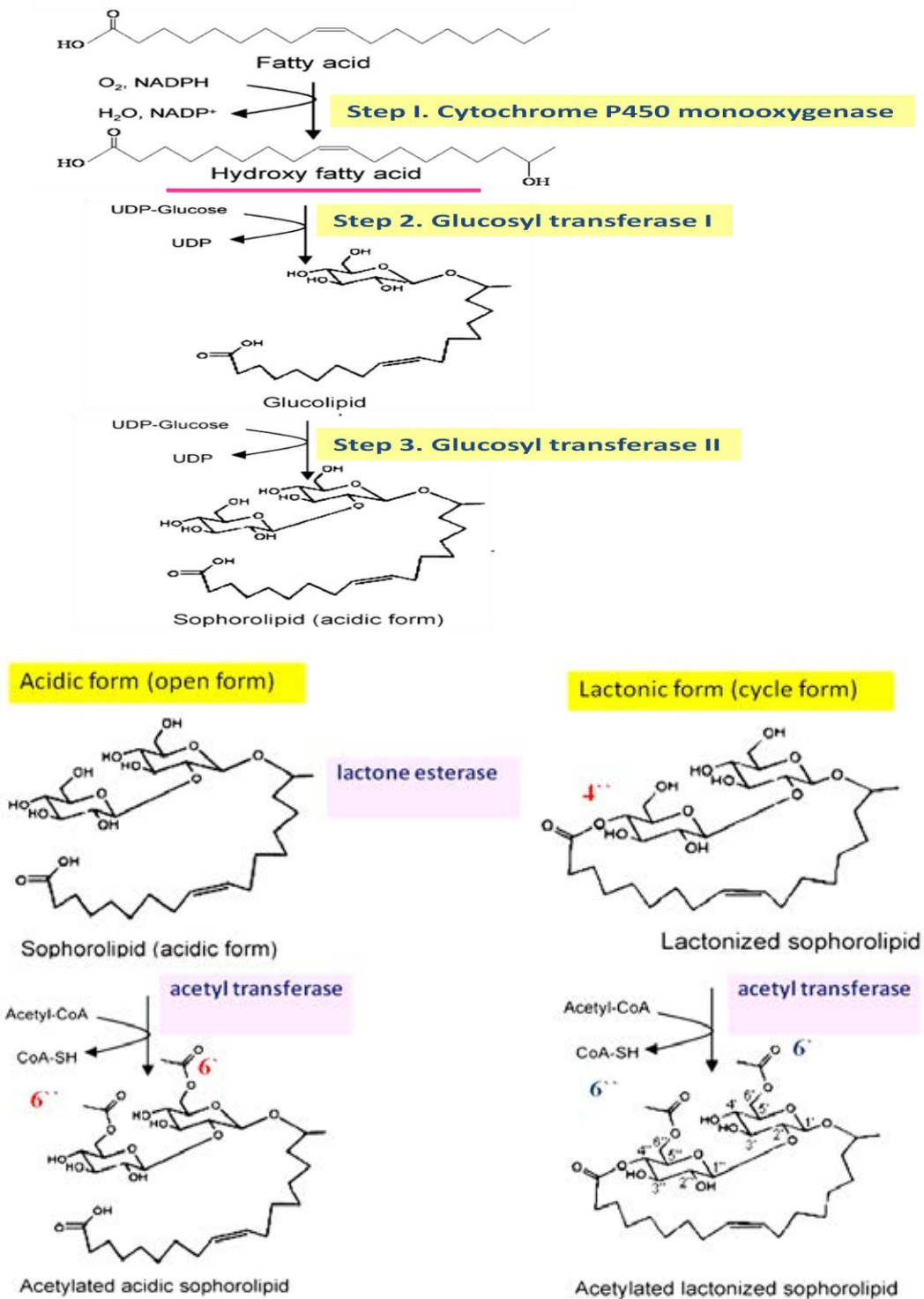
Figure 2.10 Proposed sophorolipid biosynthetic pathways.

Source: Van Bogaert et al., 2007

2.7   Factors affecting biosurfactant production by yeast

2.7.1   Effect of carbon sources

For biosurfactant production, various types of carbon substrates and concentrations have been investigated. Truly for the type, quality and quantity of biosurfactant production are affected and influenced by the nature of the carbon substrate used (Singer, 1985; Raza et al., 2007). The use of different carbon compounds may be desired when some properties are sought for a particular application (Cooper, 1986) Carbohydrates, hydrocarbons and vegetable oils had been previously used as the carbon sources.

With glucose as carbon source, the biosurfactant production by *Yarrowia lipolytica* IA1055 (Sarubbo et al., 2001), and another strain of IMUFRJ 50682 (Amaral et al., 2006) demonstrated high emulsification activity for oil-in-water emulsions and the authors demonstrated that the induction of biosurfactant production was not dependent on the presence of hydrocarbons. Lactose has also been used as soluble substrate for the production of compounds with emulsifying activity, such as the production of mannan-proteins by *Kluyveromyces marxianus* (Lukondeh et al., 2003). MEL and SL production obtained from glucose or sucrose as sole carbon substrates gave lower yield of biosurfactant than that of *n*-alkane or vegetable oils (Morita et al., 2009; Gobbert et al., 1984), however water-soluble carbon sources like sugars simplify the separation steps when compared to that of vegetative oils.

Candida lipolytica could produce surface-active compounds like emulsifying agents when grown in *n*-alkane as carbon source, but not in the glucose medium (Pareilleux, 1979). In a similar study, biosurfactant production by *Yarrowia lipolytica* NCIM3589, a marine yeast, was achieved in cultural media containing crude oil and alkanes (C10-C18), but not with soluble substrates such as glucose, glycerol, sodium acetate or alcohol (Zinjarde and Pant, 2002). Diesel and crude oil were identified to be good carbon sources for biosurfactant production by isolated organisms of *S.cerevisiae* and *C.albicans* (Ilori et al., 2008).

Vegetable oils (corn, soybean, sunflower and safflower oils) have been used as substrate for biosurfactant production by *T. bombicola*. The biosurfactants yields about 70 g L^{-1} were similar for all the oils investigated (Cooper and Paddock, 1984). Soybean oil, olive oil and safflower oil are the best carbon sources for MEL production by various *Pseudozyma* sp. (Morita et al., 2008a and b).

As previous studies, it is possible to produce biosurfactants in the presence of either hydrophillic or hydrophobic carbon sources as well. A great number of works demonstrating the combination of the hydrophillic and hydrophobic carbon compounds are the important nutritional carbon sources in biosurfactant production by yeast. Several studies showed that higher production yields are often obtained when hydrophobic substrates are added (Zhou and Kosaric, 1995; Solaiman et al., 2004; Rau et al., 1996). Casas and Garcia-Ochoa (1999) studied the medium composition of sophorolipids production by *Candida bombicola*. The carbon sources promoting the best biosurfactant production were a mixture of glucose (100 g L^{-1}) and sunflower oil (100 g L^{-1}), resulting in a biosurfactant concentration of 120 g L^{-1} after 144 hours of fermentation. Sarrubo et al., 2007 investigated the production of a biosurfactant by *C. lipolytica* in a medium containing canola oil (100 g L^{-1}) and glucose (100 g L^{-1}). The surface-active compound produced was constituted by a protein-lipid-polysaccharide complex and was able to reduce the surface tension of water from 71 mN m^{-1} to 30 mN m^{-1}.

2.7.2   Effect of nitrogen sources

The nitrogen source in the medium also has a great effect in the production of biosurfactants due to it is essential for cell growth including protein and enzyme synthesis (Amaral et al., 2008). It may also contribute to pH control. Organic nitrogen such as urea (Vance-Harrop et al., 2003), peptone (Kim et al., 2006), yeast extract (Adamczak, Bednarski., 2000; Chen et al., 2006a), and inorganic nitrogen such as ammonium sulphate (Zinjarde et al., 1997), ammonium nitrate (Thanomsub et al., 2004), sodium nitrate (Bednarski et al., 2004) have been used for biosurfactant production.

Several literatures focused on the influence of these nitrogen sources on biosurfactant production and yeast extract is the most frequently used, but its optimal concentration could not specified which might depend on microorganisms and culture medium. Casas and Garcia-Ochoa (1999) showed that among the tested yeast extract concentrations between 1-20 g L^{-1}, *C. bombicola* gave highest biosurfactant production at the lowest yeast extract concentration, 1 g L^{-1}. They pointed out that when nitrogen source is excess, biosurfactant production is decreased due to the carbon source is efficiently used for cell growth. However for biosurfactant production by *C. bombicola* in agitated flasks, 5 g L^{-1} of yeast extract gave higher biosurfactant production than those of ammonium nitrate, sodium nitrate, ammonium chloride or urea (Cooper and Paddock, 1984).

Zinjarde et al.(1997) studied the production of a bioemulsifier by *Yarrowia lipolytica* and found that ammonium sulphate and ammonium chloride were the best nitrogen sources while emulsifying activity was reduced to half when ammonium nitrate and urea were used and no emulsifying activity was detected with sodium nitrate. Fontes et al. (2010) investigated the effects of nitrogen sources (urea, ammonium sulfate, yeast extract, and peptone) on maximum reduction of surface tension (ΔST) and emulsification index (EI) by using full factorial design method. They found that the best results (67.7% of EI and 20.9mN m^{-1} of ΔST) were obtained in a medium composed of 10 g L^{-1} of ammonium sulfate and 0.5 g L^{-1} of yeast extract.

Kim et al. (2006) compared organic- and inorganic nitrogen sources for biosurfactant production by *Candida antarctica*. The biosurfactant synthesis was repressed, in spite of abundant cells and high cell growth rate, when organic nitrogen was used instead of inorganic nitrogen this might be due to it was preferentially utilized for cell growth rather than biosurfatant production.

Davila et al. (1992) reported that synthesis of sophorolipid started when the yeast cells entering stationary phase and under conditions of nitrogen limitation, indicating its synthesis was not growth-associated. This work was in agreement with that reported by Albrecht et al. (1996) who suggested that biosurfactant synthesis occurred in the limiting nitrogen conditions. Low nitrogen condition lead to the

decline of the specific activities of NAD- and NADP-dependent isocitrate dehydrogenase, which catalyses the oxidation of isocitrate to 2-oxoglutarate in the citric acid cycle. With the reduction of the activity of this enzyme, isocitrate accumulates lead to the accumulation of citrate in mitochondria. Citrate and isocitrate are, then, transported to the cytosol, where it is first cleaved by citrate synthase, forming acetyl-CoA, which is the precursor of fatty acids synthesis and, therefore, biosurfactant production is increased. Nitrogen limitation not only causes over production of biosurfactants, but also changes the composition of biosurfactants produced (Syldatk et al., 1985; Desai and Desai, 1993).

An important parameter studied by several researchers is the quantitative ratio between carbon and nitrogen sources (C/N) used in biosurfactant production. Different C/N ratios and hydrocarbons were used in biosurfactant production by *Candida tropicalis*. The emulsifying activity rose with the increase in C/N ratio for almost all cases, when nitrogen was the limiting factor (Singh et al., 1990). Similar results were obtained by Johnson et al. 1992 for the production of a biosurfactant from *R. glutinis*. The best C/N ratio for biosurfactant production by *Yarrowia lipolytica* IMUFRJ 50682 (Fontes et al.,2010) was 11.9/1 when using optimal concentrations of glucose (4% w/v), glycerol (2% w/v), ammonium sulfate (10 g L^{-1}) and yeast extract (0.5 g L^{-1}).

2.7.3 Effect of environmental factors

Growth conditions and environmental factors such as temperature, pH, agitation and oxygen availability also affect the production of biosurfactant. The production of a bioemulsifier by *Rhodotorula glutinis* during fed batch fermentation was significantly influenced by both pH and temperature, with the optimum conditions at 30 °C and pH 4.0 (Johnson et al., 1992). The acidity of the production medium was the parameter studied in the synthesis of glicolipids by *Candida antarctica* and *Candida apicola*. When pH was maintained at 5.5, the production of glicolipids reached a maximum. Without pH control, the synthesis of the biosurfactant decreased (Bernardski et al., 2004). Kim et al. (2006) studied extracellular production of a glycolipid biosurfactant, mannosylerythritol lipid, by *Candida* sp. SY16 and found

that the production of MEL-SY16 significantly improved when the pH was controlled at 4.0, but was repressed at 5.0. C*andida tropicalis* MTCC 230 showed the best result of emulsification activity when petrol and NH_4Cl was used as carbon and nitrogen source, respectively and optimum pH was 6.8 and temperature was 34 °C (Ashish et al., 2011). The pH of the medium plays an important role in sophorolipid production by *Candida bambicola* (Gobbert et al., 1984).

Most biosurfactant productions reported were performed in a temperature ranging from 25 to 30 °C. Casas and Garcia-Ochoa (1999) showed that the amount of sophorolipids obtained in the culture medium of *Candida bombicola* at temperature of 25 °C or 30 °C was similar. However, cell biomass was lower and glucose consumption rate was higher when performed at 25 °C. In a similar study, Desphande and Daniels (1995) observed that the growth of *Candida bombicola* reached a maximum at a temperature of 30 °C while 27 °C was the best temperature for the production of sophorolipids. In the culture of *Candida antarctica*, temperature causes variations in the biosurfactant production. The highest mannosylerythritol lipids production was observed at 25 °C for the production by both growing and resting cells (Kitamoto et al., 1992; Kitamoto et al., 2001)

Table 2.2 Summerization of glycolipid production by yeasts when grown in various carbon and nitrogen sources

Yeasts strains	C sources (g L^{-1})	N sources[a] (g L^{-1})	Types of biosurfactant[b]	Production level	Yields (g L^{-1})	Cultivation time (h)	References
Torulopsis bombicola ATCC22214	Glucose (500) + Safflower oil (1000)	YE (5)	sophorolipids	Batch	18	48	Cooper and Paddock, 1984
Torulopsis apicola IMET43747	Glucose + vegetable oils	YE (1) (NH$_4$)$_2$SO$_4$	sophorolipids	Batch	5-90	450	Stüwer et al.,1987
Candida tropicalis ATCC20336	n-hexadecane (10)	YE (0.3) Peptone (0.5)	Liposan lipid 40%, protein 22 %, CHO 17.5%	Batch	0.81 U	72	Singh and Desai, 1989
Debaryomyces polymorphus ATCC20449	n-hexadecane (10)	YE (0.3) Peptone (0.5)	Liposan lipid 40%, protein 22 %, CHO 17.5%	Batch	0.11 U	72	Singh and Desai, 1989
Candida bombicola CBS6009 (ATCC22214)	Glucose (100) + Ethyl ester rapseed oil (100)	Dry corn steep liquor (5) (NH$_4$)$_2$SO$_4$ (4)	sophorolipids	fermentor	320	200	Davila et al., 1992
Candida antractica T-34	Nut oil (80)	YE (1)	MEL	Batch Resting cell	47	144	Kitamoto et al., 1992
Candida bombicola ATCC22214	Glucose (100) Animal fat (100)	corn steep liquor (4), Urea (1.5)	sophorolipids	Batch	120	68	Deshpande and Daniels,1995
Torulopsis bombicola ATCC22214	Glucose (100) + soybean oil (100)	YE (5) (NH$_4$)$_2$ SO$_4$(3.3)	sophorolipids	fermentor	40-80	168	Kim et al., 1997
Candida bombicola ATCC22214	Rapseed oil (400)	Whey (1.5 L)	sophorolipids	Fed batch	422	25 d	Daniel et al., 1998
Candida bombicola NRRL Y-17069	Glucose (100) + Sunflower oil (100)	YE (1)	sophorolipids	Batch-Resting cell	120	192	Casas and Ochoa, 1999
Candida antractica ATCC20509	Soybean oil (80)	YE (1)	MEL	Batch	46	144	Adamczak & Bednarski., 2000
Candida antractica T-34	n-octadecane (60)	YE (1) NaNO$_3$(2)	MEL	Batch-Resting cell	40.5	144	Kitamoto et al., 2001
Candida lipolytica IA1055	Babassu oil (50)	Urea (0.25)	Liposan lipid 20% protein 50%, CHO (8%)	Fed-batch	0.66 U/ml	60	Vance-Harrop et al., 2003
Kluyveromyces marxianus FII510700	Lactose (40)	YE (2) (NH$_4$)$_2$ SO$_4$ (5)	Poly. BS (CHO 90% + protein 4-6%	Batch	ND	ND	Lukondeh et al., 2003
Candida antractica ATCC20509	Glucose (40) + Soapstock (100)	YE (1) NaNO$_3$(2)	Glycolipid	Feed-batch	13.2	144	Bednarski et al., 2004
Candida apicola ATCC96134	Glucose (40) + Soapstock (100)	YE (1) NaNO$_3$ (2)	Glycolipid	Batch	10.3	144	Bednarski et al., 2004

Yeasts strains	C sources (g L^{-1})	N sources[a] (g L^{-1})	Types of biosurfactant[b]	Production level	Yields (g L^{-1})	Cultivation time (h)	References
Candida ishiwadae	Soybean frying oil(40)	YE (0.5) NH$_4$NO$_3$ (3)	Monoacylglycerols	Batch	0.25 ug	168	Thanomsub et al., 2004
Yarrowia lipolytica IMUFRJ 50682	Glucose (20)	Peptone (6.4) YE (10)	Liposan	Batch	2.0 U/ml	170	Amaral et al., 2006
Wickerhamiella domercqiae	Glucose (50) + rapeseed oil (20)	YE (2)	sophorolipids	Shake flasks	ND	96	Chen et al., 2006a
Candida sp. SY16	Glucose (15) + Soybean oil (15)	Peptone (1) NH$_4$NO$_3$ (3)	MEL	Fed-batch	95	200	Kim et al., 2006
Candida glabrata UCP1002	Glucose (50) + cotton seed oil (75)	YE (3) NH$_4$NO$_3$ (1)	Poly. BS (CHO 10% + protein 45% + lipid 20%)	Shake flasks	10	144	Sarubbo et al., 2006
Candida lipolytica UCP0988	Glucose (100) + canola oil (100)	YE (2)	sophorolipids	Shake flasks	8	48	Sarubbo et al., 2006
Candida ingens CB-216	corn oil (20)	YE (2)	ND	Batch	0.048	168	Amezcua et al., 2007
Candida bombicola ATCC22214	Glucose (100) + canola oil (2)	YE (1) (NH$_4$)$_2$ SO$_4$ (1)	sophorolipids	Shake flasks	1.44	96	Shah and Prabhune (2007)
Candida bombicola ATCC22214	Waste oil from restaurants Oleic acid	YE (1) (NH$_4$)$_2$ SO$_4$ (1)	sophorolipids	Shake flask Batch fermentor Shake flask Batch fermentor	30 34 38 42	168	Shah et al., 2007
Candida lipolytica UCP0988	Glucose (100) + Canola oil (100)	YE (2) NH$_4$NO$_3$ (1)	Poly. BS (CHO 24% + protein 40.2%+ lipid 19.5%)	Shake flask	8	144	Sarubbo et al., 2007
Candida bombicola ATCC22214	Soy molasses (100)+ Oleic acid (90 ml) Glucose (100) + Oleic acid (90 ml)	YE (10) urea (2)	sophorolipids	Fermentor fermentor	53 100	168	Solaiman et al., 2007
Candida bombicola ATCC22214	Glucose (100) + tallow fatty acids (40) Glucose (100) + coconut fatty acids (40)	YE (10) urea (1)	sophorolipids	Fed-batch Fed-batch	120 40	168	Felse et al., 2007
Candida batistae CBS8550	Glucose (50) + olive oil (50)	YE (5) NaNO$_3$ (3)	sophorolipids	Shake flask	6	72	Konishi et al., 2008
Candida sphearica UCP0995	DW+ ground-nut oil refinery residue (50)	corn steep liquor (25)	Glycolipids (CHO 25% + lipid 75%)	Shake flask	4.5	144	Sobrinho et al., 2008

Yeasts strains	C sources (g L^{-1})	N sources[a] (g L^{-1})	Types of biosurfactant[b]	Production level	Yields (g L^{-1})	Cultivation time (h)	References
Pichia anomala PY1	Soybean oil (40)	YE (1) NaNO$_3$ (4)	sophorolipids	Shake flask	0.2	168	Thaniyavarn et al., 2008
	Glucose (40)	YE (1) NaNO$_3$ (4)	sophorolipids	Shake flask	0.06	168	Thaniyavarn et al., 2008
Starmerella bombicola NRRL Y-17069	Soybean oil + sugarcane molasses	YE (10) Urea (1)	sophorolipids	fermentor	63.7	192	Daverey and Pakshirajan, 2010a
Trichosporon asahii	Diesel oil (20)	NH$_4$NO$_3$ (1)	Glycolipids (CHO 31.9% + lipid 68.2%)	Shake flask	0.9	240	Chandran and Das, 2010
Candida glabrata UCP1002	vegetable fat waste (50)	YE (2)	CHO–protein–lipid complex (CHO 48% + Protein 34%+ lipids 18%)	Shake flask	7.16	144	Gusma˜o et al., 2010
Candida sphaerica UCP 0995	ground–nut oil refinery residue (%)	corn steep liquor (9%)	Glycolipids (CHO 15.0% + lipid 70%)	Shake flask	9	144	Luna et al., 2013

a: YE is yeast extract used as a N source.

b: CHO is carbohydrate

Source: these data were modified fromAmaral et al's review in 2008.

2.8   Potential commercial applications

Biosurfactants have vastly known and prominently interested for applications in a wide variety of commercial areas and industrial processes such as oil recovery enhancement (Lin, 1996), bioremediation of oil-polluted soil and water (Volkering et al., 1997), replacement of chlorinated solvents used in the cleaning up of oil-contaminated pipes (Robinson et al., 1996), use in the detergent industry (Rosenberg and Ron, 1998) and in the formulation of oil-in-water emulsions in food, biotechnological, pharmaceutical and cosmetic industries.

For bioremediation, the effects of biosurfactants on the biodegradation of petroleum compounds were investigated by Hua et al. (2004). They reported that addition of a biosurfactant produced by *Candida antarctica* T-34 could improve the biodegradation rate of some n-alkanes (90.2% for n-decane, 90.2% for n-undecane, 89.0% for dodecane), a mixture of n-alkanes (82.3%) and kerosene (72.5%) and showed that this biosurfactant could substitute synthetic surfactants.

Biosurfactants have also showed several promising applications in the food industry as food additives. Lecithin and its derivatives, fatty acid esters containing glycerol, sorbitan, or ethylene glycol and ethyoxylated derivatives of monoglycerides are currently in use as emulsifiers in the food industries worldwide. A bioemulsifier from *Candida utilis* has shown potential use in salad dressing (Sheperd et al., 1995) and good results were obtained with mannoproteins extracted from *S. cerevisiae* cell wall in mayonnaise formulation (Torabizadeh et al., 1996).

Biosurfactants can also have a very important position in the cosmetic industries. A product containing 1 mol of sophorolipid and 12 mol of propylene glycol has excellent skin compatibility and is used commercially as a skin moisturizer (Yamane et al., 1987). Sophorolipids are being produced and used by Kao Co. Ltd. as a humectant in cosmetics (Banat et al., 2000).

Some other potential commercial applications of biosurfactants currently under study were shown in Table 2.3

Table 2.3 Biosurfactant-producing yeasts and their applications in recent years.

Yeast	Type of biosurfactants	Potential applications	Reference
Candida bombicola	Sophorolipids	Antiaging, use in cosmetic (skin lotion) US patent, 2003	Borzeix and Frederique, 2003
Candida bombicola	Modified sophorolipids (Lactonic SL-leucine)	Antibacterial, Anti HIV, Spermicidal activity	Shah et al., 2005; Azim et al., 2006
Wickerhamiella domercqiae	Sophorolipids	Anticancer, Induce apoptosis	Chen et al., 2006a,b
Pseudozyma siamensis	Mannosyl erythritol lipids	Promising yeast BS	Morita et al. 2008a
Pseudozyma graminicola	Mannosyl erythritol lipids	Washing detergents	Morita et al. 2008b
Pseudozyma hubeiensis	Glycolipid	Bioremediation of marine oil pollution	Fukuoka et al. 2008
Pseudozyma parantarctica	Mannosyl erythritol lipids	Emulsifiers and/or washing detergents	Morita et al. 2009
Candida bombicola	Sophorolipids	Environmental applications.	Daverey and Pakshirajan 2010a, b)
Trichosporon asahii	Glycolipids	Biodegradation of diesel oil	Chandran and Das, 2010
Candida sphaerica	Glycolipids	Oil recovery, petroleum industry	Sobrinho et al., 2008 Luna et al., 2013

Source: Makkar et al., 2011.

CHAPTER III

MATERIALS AND METHODS

3.1   Microorganisms

The biosurfactant-producing yeasts involved in this study were isolated from the samples contaminated oils in Thailand (Table 4.1 in Chapter IV). Those isolates included possible yeast like colonies obtained by dilution plating and enrichment culture techniques. Details of isolation for these yeasts were shown in Section 3.3. They were screened for the efficient biosurfactant-producing ability from the capability of reducing ST value to below 40 mN m^{-1} via the surface tension value measurement. (Section 3.5). The selected yeast of total isolates was used for further study in molecular analysis.

For screening the efficient yeast, six strains were used in this study as shown in Table 3.1. *Pichia anomala* PY1 was a biosurfactant-producing yeasts as a referenced strain. It was a thermotolerant strain isolated from fermented food, produced sophorolipid as glycolipid biosurfactant (Thaniyavarn et al., 2008).

Table 3.1 Six yeast strains used for screening the efficient biosurfactant-producing yeast

Yeast species	strain	ST values[a] (mN m^{-1})	Reference
Trichosporon asahii	JP11	39.67	This work
Trichosporon faecale	JP30	31.33	This work
Trichosporon asahii	JP70	29.17	This work
Meyerozyma guilliermondii	JP39	26.17	This work
Cyberlindnera samutprakarnensis	JP52	34.83	This work
Candida thasaenensis	JP59	40.33	This work
Pichia anomala	PY1	ND[b]	Thaniyavarn et al., 2008

a: surface tension values of supernatant when cultured in YM broth supplemented with 1% palm oil

b: Not determined the surface tension values of supernatant when cultured in YM broth supplemented with 1% palm oil

Yeast strain JP11, JP30 and JP70 were belonged to yeast species in genus *Trichosporon* that were reported to be biosurfactant producing yeast (Monteiro et al., 2010; Chandran and Das, 2010). Yeast strain JP39 as *Meyerozyma guilliermondii* showed the lowest surface tension values of supernatant when cultured in YM broth supplemented with 1% palm oil. Yeast strain JP52 and JP59 as new yeast species were obtained from this study to screen the efficient biosurfactant- producing yeast.

3.2   Sample collection

3.2.1   Location of sample collection

The samples contaminated with oil were interesting sources to screen for biosurfactant-producing microorganisms. Therefore, the collected samples in this study were obtained from soils contaminated with oil especially the surrounding soil of palm oil trees and heaped soil with palm oil tree, agricultural wastes as rotten oil palm, biodiesel wastes, and wastes from palm oil and cosmetics factories from Surattani, Chumphon, Ranong, Prachuabkirikhan, Rayong, Samutsakhon and Samutprakan Province, Thailand.

3.2.2   Sampling methods

The collected samples in this study were used either solid or aqueous types. Most samples were kept as solid-type from those such as various agricultural soils in the palm oil gardened-land sources, heaped soils near the pooled palm oil fruit bunches area, agricultural wastes as rotten oil palm fruitlet and soil sediment palm biodiesel pond. Soil samples were collected from the surface soil at 3-5 cm in depth of contaminated soil and kept in zipped plastic bags in ice-cooled box. Aqueous samples such as wastes from cosmetic factories, water contaminated motor oils near the fishery pier were collected at the surface area, put into sterile bottle and placed in ice-cooled box. The samples were transported and stored at 4 °C until used.

3.3 Isolation of biosurfactant producing yeasts

Each collected sample was analyzed for pH value and moisture content. Moisture content analysis is used to determine the percentage of water in a sample by drying the sample to a constant weight. Yeast strains were isolated by two isolation methods including standard plate culture technique and enrichment technique. For the standard plate culture technique, ten grams of each sample were suspended in 90 ml of sterile saline solution (0.85% (w/v) NaCl). The serials dilution were spreaded onto acidified YM agar (0.3% (w/v) yeast extract, 0.3% (w/v) malt extract, 0.5% (w/v) peptone and 1.0% (w/v) glucose), pH4.5 supplemented with 1% (v/v) palm oil, 100 μg ml^{-1} chloramphenicol, and 0.2% (w/v) sodium propionate incubated at 30 °C for 3 days (Appendix 1.3) to obtain yeast colonies and inhibit bacterial and mold growth. Then the numbers of colonies were counted and calculated in CFU. g^{-1}. For enrichment isolation, ten grams of each sample were inoculated in 90 ml of YM broth containing 100 μg L^{-1} chloramphenicol and 1% (v/v) palm oil and incubated at room temperature on a rotary shaker at 200 rpm. At interval period during cultivation, the enriched culture was streaked onto YM agar plate and incubated for 3, 7, 14 and 28 days. The isolated strains were maintained in YM broth containing 20% (w/v) glycerol and stored at -20 °C.

3.4 Growth and maintenance of yeast

3.4.1 Inoculum preparation

A fresh single pure colony of each yeast isolates was transferred aseptically from yeast extract malt extract (YM agar) plate into yeast extract malt extract (YM) broth, pH 4.5 using a sterile wire loop. The culture was grown at 30 °C for 24 h with shaking at 200 rpm. The culture was diluted to an optical density (OD600) of 1.0 and used as inoculum. Ten percent of each inoculum was transferred to an experimental medium in a 250 ml.

3.4.2 Culture maintenance and storage

The purified isolates were maintained in YM broth supplemented with 20% (w/v) glycerol as a cryoprotectant (Appendix 1.4) and stored in deep freezer (-20

°C). They were regularly subcultured into fresh medium of YM agar for short-term storage.

3.5   Screening of biosurfactant producing yeasts

For biosurfactant-producing yeast screening, the isolated yeasts were cultivated in YM broth containing 1% (v/v) palm oil and analyzed for biosurfactant activity during cultivation period of 7 days. Their abilities to reduce surface tension and oil displacement capacity were determined in cell-free broth. After centrifugation of the culture broth at 8,000 rpm and 4 °C for 20 min, the supernatant was collected and examined for 3 parameters; (1) pH measurement (2) oil displacement capacity (Morikawa et al., 2000) and (3) the surface tension value measurement by the Du Nouy Ring method using a K6 Kruss tensiometer (section 3.6.3.2). In this study, the efficient biosurfactant producing yeast strains were selected based on their activities that showed surface tension values lower than 40 mN m^{-1}. All efficient strains were further identified by molecular genetic analysis of D1/D2 sequencing.

3.6   Analytical methods

3.6.1   Determination of cell biomass

Yeast cells were sedimented by centrifugation at 8,000 rpm for 20 min and supernatant was kept. Cell sediments were washed twice with normal saline and washed with hexane to get rid of vegetable oil which was present in the medium, after that baked in hot air oven (105 ºC) for 24 hrs and weighed the dried cells.

3.6.2   pH measurement

The supernatant was determined for pH value by using pH meter (Metler toledo model S20) at room temperature.and the measurement was repeated two times.

3.6.3   Analysis of biosurfactant activity

Biosurfactant activities in the cell free broth were determined by measuring the surface tension value by Du Nouy Ring method using a K7 Kruss tensiometer

and the oil displacement capacity (Morikawa et al., 2000). Δ Surface tension (ΔST) was derived from the surface tension of the initial medium subtracted by the surface tension of the supernatant. Δ Surface tension was used as a criterion to select the optimum experimental condition to be used or the maximal biosurfactant activity of strains to be selected.

3.6.3.1 Oil displacement test

The oil displacement test is a rapid method for screening capacity of surfactant. If biosurfactant is present in the cell free culture broth, oil will be displaced with an oil free clearing zone and diameter of this clearing zone indicates the surfactant activity, also called oil displacement activity. It was performed by measuring the diameter of the clear zone formed on the surface of an oil-water phase which occurs after dropping a surfactant solution on an oil-water interphase (Morikawa et al., 2000). The oil displacement test was done by adding 50 ml of distilled water to a glass petri dish with a diameter of 15 cm. After that 15 μl of synthetic oil was dropped onto the surface of the water (surface of an oil-water phase 153.9 cm^2 surface area), followed by the addition of 10 μl of supernatant. The diameter of the clear halo zone was obviously measured after 30 s. The measurement was repeated three times and performed at room temperature. Calculation of oil displacement area derived from area of clear zone as πr^2; the radius (r) of a normal circle is always one-half of the diameters and π is 3.14. A negative control was maintained with uninoculated medium (without surfactant), in which no or a few of oil displacement area or clear zone was observed.

3.6.3.2 The surface tension value measurement

The surface tension value measurement used in this study was to determine the surface force between a liquid and air in a liquid medium sample. Surface tension of the supernatant or surfactant solution was measured by the Du Nouy Ring method using a K6 Kruss tensiometer. It was rapid, simple, user-friendly operation at measuring range 1-90 mN m^{-1}.

The Du Nouy Ring method operated with a platinum-iridium ring and sample glass-container. The instrument was set on the equilibrium platform and all glass vessels were surely cleaned to get an accurate measurement. A

platinum-iridium ring was flamed with the alcohol lamp to completely remove all residues. The sample was determined by the following method:

- The platinum-iridium ring was hold in directional rectangular to precision balance and firstly setting the force scale to zero.

- A sample of volume 30 ml was poured into cleaned glass vessel and placed onto the tensiometer platform. A vessel of sample was raised by adjusting the tensiometer sample carrier platform until the ring was fully wetted and sunk in the liquid.

- A platinum-iridium ring is suspended from a torsion balance, and the force (in mN m^{-1}) necessary to pull the ring free from the surface film is measured. The platform was then lowered while the ring was raised by increasing the tension in the balance. This was operated simultaneously manual to get the precision force of surface tension.

For calibration of the instrument, the surface tension of the pure water was measured before each set of experiment and belonged generally known at 72 mN m^{-1}. After that, the surface tension of each sample was determined at least repeated three times and an average value was obtained. The surface tension of uninoculated cultural medium performed at room temperature was used as control.

3.7 Identification of the isolated yeast strains by molecular DNA analysis

Molecular genetics identification of yeast was done by comparing the D1/D2 region of the large subunit (LSU) rRNA gene with other sequences retrieved from the database Genbank. The molecular technique was based on extraction of genomic DNA, followed by DNA amplification of D1/D2 region by using PCR method and sequence analysis of the D1/D2 region further including constructed phylogenetic tree from the evolutionary distance data. Furthermore, this work also revealed three novel yeast strains discovery then focused to study new yeast identification according to yeast taxonomy and systematic (Kurtzman and Robnett, 1998).

3.7.1 Extraction of genomic DNA

Genomic DNA extraction was performed according to Jindamorakot (2006). Pure culture on YM agar plate (2 day old) was transferred to 1.5 ml eppendorf tube and suspended in 200 µl of lysis buffer (100 mM Tris, 30 mM EDTA, 0.5% SDS) and then boiled in water bath for 15 min. The suspension was added with 200 ul of 2.5 M potassium acetate and placed in ice for 60 min then centrifugation at 14,000 rpm for 5 min. Supernatant was collected and extracted twice by adding equal volumes of chloroform-isoamyl alcohol, followed by isopropanol precipitation. Subsequently the DNA pellet was washed with 70% and 90% ethanol respectively, dried up and dissolved in 20-30 µl of sterilized nano pure water. (The volume of purified water was adjusted upon the amount of DNA precipitation.)

3.7.2 DNA amplification by using PCR method

The D1/D2 domain of LSU rDNA gene was amplified using the primers NL1 and NL4, according to the methods of Kurtzman and Robnett (1998). PCR mixture reaction preparation was shown in Appendix 4. A polymerase chain reaction was performed by pre-denaturation at 94 °C for 5 min; 30 cycles with denaturation at 94 °C for 1 min, annealing at 52 °C for 1.5 min, and extension at 72 °C for 2.5 min and a final extension at 72 °C for 10 min. Additionally, among the novel species and interested strains had been further analyzed for the ITS regions flanking 5.8s rDNA using the primer pairs of ITS1/ITS4, primers ITS1 and ITS4. Amplification of ITS gene was performed using an initial denaturation during 5 min at 95 °C, followed by 40 cycles consisting of 1 min at 95 °C, 2 min at 58 °C and 3 min at 72 °C. A final step of 10 min at 72 °C was carried out (White et al., 1990). The PCR products were purified by using the PCR DNA fragment extraction kit (Geneaid) as shown in Appendix1.4. Amplification products were verify detected and separated in 0.8% agarose gel electrophoresis and detected by staining with 0.005% ethydium bromide dye. Their purities of amplified DNA were determinated by Nano drop meter to evaluate optical density ratio at OD260/280.

For using multigene analysis to support correctly of two novel strains, JP59 and JP60, the actin gene *ACT1* and the elongation factor 2 genes *EF2* were also

examined. The gene *ACT1* and *EF2* were amplified by using the primers ACT1 and ACT2 for *ACT1* and EFIIF1 and EFIIR2 for *EF2*. PCR was performed according to the method described by Diezmann et al. (2004). All PCR products were delivered to Macrogen Company in Korea to analyze their nucleotides sequences.

Table 3.2   Primer used in DNA amplification by using PCR method

DNA region	primer	Nucleotide sequences	Reference
D1/D2	NL1	5′-GCATATCAATAAGCGGAGGAAAAG-3′	Kurtzman and Robnett , 1998
	NL4	5′-GGTCCGTGTTTCAAGACG-3′	
ITS1-2	ITS1	5′-TCCGTAGGTGAACCTGCGG-3′	White et al., 1990
	ITS4	5′-TCCTCCGCTTATTGATATGC-3′	
Actin gene (*ACT1*)	ACT1	5′ 59-TACCCAATTGAACACGGTAT-39 3′	Diezmann et al., 2004
	ACT2	5′ 59-TCTGAAT CTTTCGTTACCAAT-39 3′	
Elongation factor gene (*EF2*)	EFIIF1	5′ 59-AGTCTCCAAACAAAGCATAAC-39 3′	Diezmann et al., 2004
	EFIIR2	5′ 59-GGGAAAGCTTGACCACCAGTAGC-39 3′	

3.7.3   Yeast identification by comparing DNA sequence divergence

All studied DNA sequences of the D1/D2 sequences, ITS regions, actin gene and elongation factor gene were compared with nucleotide databases by nucleotide BLAST analysis tool as BLASTn Homology Search (http:// www.ncbi. nlm.nih.gov /blast) as previously reported in Altschul et al., 1997. Several different species had proven to discriminate with greater than 1% nucleotide substitution from comparison of nuclear relatedness (Peterson and Kurtzman, 1991; Kurtzman and Robnett, 1998). According to Kurtzman and Robnett (1998) used the number of nucleotide substitution of D1/D2 sequences as criterion to resolve for yeast species identification. They suggested D1/D2 sequences of that strain pairs showing with 0-3 nucleotide differences are either conspecific or sister species and six nucleotides (1%) or greater differences in that sequence are likely to be separate species. Therefore D1/D2 sequences of studied strains were indicated to answer either known, new species or sister species (undescribed yeast strains). The nucleotide sequences of novel yeast strain determined in this study have been deposited with GenBank under the accession numbers as later described.

3.7.4 Phylogenetic tree construction for novel yeast species

Phylogenetic tree construction by retrieving DNA sequencing analysis has clearly provided genetic relationships among species. DNA sequences of new species and related yeasts as type strain were aligned using the CLUSTAL X ver. 2.0 software (Larkin et al., 2007) and phylogenetic trees were constructed by using Neighbor-Joining analysis (Saitou and Nei, 1987). Sites where gaps existed in any sequences were excluded. Bootstrap analyses (Felsenstein, 1985) were performed 1,000 random resamplings.

3.8 Identification of novel yeast strains by conventional and chemotaxonomic studies

Not only classification based on molecular genetic analyses that have considered reliable genetic similarities and difference for yeast identification, but also conventional and chemotaxonomic studies necessity examined for the scheme of polyphasic taxonomy. They were prescribed in *The fifth edition of The Yeasts, a Taxonomic Study* that was edited by Kurtzman et al. (2011). The isolates were characterized morphologically, physiologically and biochemically following standard yeast identification methods for conventional studies (Yarrow, 1998). The nitrogen assimilation was examined on solid media with starved inoculum (Nakase and Suzuki 1986a), while the ability to grow at various temperatures was determined in YM broth using metal block baths. Vitamin requirements were determined by the method of Komagata and Nakase (1967).

3.8.1 Morphological characteristics examination

3.8.1.1 The morphology of vegetative cells in liquid media and on solid media

Actively growing yeast cells were inoculated to yeast extract-malt extract (YM) in broth and on agar plates further incubated at 25 ºC for 3 days. With observation of yeast cells characterization, cultural broth and single colony on agar plate were undertaken by wet mount slides technique. It was used to examine microscopically to observe the cell shape, cell arrangement, and the formation of hypha and modes of reproduction. The length and breadth of at least

20 cells were measured and the extreme values noted. This examination were done and again after 4 wks.

3.8.1.2 Yeast growth characteristics on liquid and solid media

Actively growing yeast cells were cultivated in three types of YM including broth, agar plates and agar slant then later incubated at 25 °C for 4 weeks. Yeast growth characteristics in liquid media, solid media both agar plate and slant were noted at interval cultivation time 3 day and every weeks. The growth of yeasts in liquid media were observed both change on surface and sediment at the bottom that result in the formation of compact, coherent, flocculent or mucoid sediment, a ring, floating islets or a pellicle. The features of the appearance colonies were noted as item of form, texture, color, surface, elevation and margin. Growths on agar slant were observed the apparently features of filiform, arborescent, beaded, effuse, rhizoid and echinulate.

3.8.1.3 Filamentation formation

Formation of pseudohyphae and true hyphae was examined on corn meal agar (CMA), potato dextrose agar (PDA) and YM using the Dalmau plate technique (Kurtzman & Fell, 1998; Barnett et al., 2000). A petridish containing a V-shaped glass rod supporting a glass microscopic slide is sterilized by using autoclave and dried prior to use in this experiment. A sterile slide was covered with melted agar on in order to form a thin-coated layer and replaced on glass rod. After the surface of agar dried, the actively growing yeast were strike slightly and streaked a straight line on the agar surface and steriled cover slide was placed on line of inoculation. To drying protection, a little sterile water was poured in to the petridish. Yeast was later incubated at 25 °C or properly temperature for up to 21 days. It was examined microscopically at interval of 3 days for pseudohyphae formation from yeast growth both under and around the cover slip.

3.8.1.4 Ascospore formation

Ascospore formation was examined on YM agar, 5% Malt extract agar, Fowell's acetate agar. Yeast was streaked onto plates and then incubated at 25 °C for 4 weeks. Ascospores were observed microscopically after 2-4 days and

every week. Ascospore was examined in formation, variation in shape, color, surface and the number formed per ascus.

3.8.2 Physiological and biochemical characteristics examination

3.8.2.1 Fermentation of sugars

The fermentation of D-glucose, D-galactose, sucrose, maltose, lactose, melibiose, raffinose and fructose was tested. Durham tubes containing 2% solutions of sugar (except in the case of raffinose having 4%) have been used for routine test. A yeast suspension was used to inoculate the fermentation medium in sterile test tubes containing Durham tubes. The tubes were incubated at 25 °C for up to 4 weeks and examined everyday (1-7 days) and read the results later in every week. A positive result was indicated by accumulation of gas in the Durham tubes. The results were recorded and scored as follows, upon the change of color in the indicator, the time and the amount of the gas accumulation in that Durham tubes:

+, strongly positive, insert filled within 7 days;

l, delayed positive (latent), insert rapidly filled, but only after more than 7 days;

s, slowly positive, insert slowly filled after more than 7 days;

w, weakly positive, the insert is not fully filled with gas (e.g., less than 1/3 full is often considered weak)

-, negative, no accumulation of gas in the insert;

v, variable, some strains are positive, others are negative.

3.8.2.2 Assimilation of carbon compounds

This experiment examined assimilation of carbon compounds for routine identification from various 44 types, namely: D-glucose, D-galactose, L-sorbose, sucrose, maltose, cellobiose, trehalose, lactose, melibiose, raffinose, melezitose, inulin, soluble starch, D-xylose, L-arabinose, D-arabinose, D-ribose, L-rhamnose, ethanol, glycerol, erythritol, ribitol, galactitol, D-mannitol, D-glucitol, α-methyl-D-glucoside, salicin, glucono-δ-lactone, 2-keto-D-gluconate, 5-keto-D-gluconate, DL-lactic acid, succinate, citrate, inositol, D-glucoronic acid, D-galacturonic acid, xylitol, L-arabinitol, D-gluconate, D-glucosamine, N-acetyl-D-

glucosamine, propane-1, 2 diol, butane-2, 3 diol, and methanol. Negative control used basal medium without carbon compound. Each tube contains a standard 2 ml amount of liquid yeast nitrogen base medium with one test substrate, except those for the negative controls with absence carbon source. An aliquot (0.2 ml) of filter-sterilized yeast nitrogen base containing 10X strength of the carbon compound under test was aseptically added to sterile distilled water (1.8 ml) in test tubes. The tubes were inoculated by aseptically adding 2 drops (approximately 0.1 ml) of a visible suspension of an actively growing culture in sterile distilled water. The tubes were incubated at 25 ºC and examined after 2-4 days, every week until at the end of 4 weeks. Results were read after the tests have been mixed well to thoroughly disperse the yeast. A positive reaction was assessed by visual inspection for an increase in the turbidity of the solution on examination at the different intervals. Each test was scored against 5 black lines with thickness 0.75 mm and width distance 5 mm on a white card. The result is scored as 3+ if the lines are completely obscured; as 2+ if the lines appear as diffuse bands; as 1+ if the lines are distinguishable as such but have blurred edges; as negative (-) if the lines are distinct and sharp edged.

The results were summarized from cultivation for 4 weeks and recorded as follows, upon the time and degree of growth rate that assessed from the turbidity of the solution on examination at the different intervals:

+, positive, either a 2+ or a 3+ reading after 1 week, or 2 weeks in some laboratories;

l, delayed positive (latent), either a 2+ or a 3+ reading develops rapidly, but after 2 weeks or longer;

s, slowly positive, a 2+ or a 3+ reading develops slowly over a period exceeding 2 weeks;

w, weakly positive, a 1+ reading

-, negative;

v, variable, some strains are positive, others are negative.

3.8.2.3 Assimilation of nitrogen compounds

The nitrogen assimilation was examined on solid media with starved inoculum (Nakase and Suzuki 1986a). Active cultural yeast on YM agar

was solely inoculated in yeast carbon base (YCB) broth by using needle to avoid residual media. Yeast grew at 25 ºC for a week to prepare starved inoculums prior nitrogen compound assimilation test. The nitrogen compounds tested were nitrate, nitrite, ethylamine, L-lysine and cadaverine. Ammonium sulphate was used as a positive control. MoltenYCB agar contained individual nitrogen compound were prepared as agar plate (Appendix 1.14). After solidification, the plates were kept, lid side up, at 25 ºC for a few hours to obtain a dry surface agar. Starved inoculums of each strain was dropped on agar plate with sterile Pasteur pipette and incubated at 25 ºC. Results were observed yeast growth and recorded every 2-4 days up to 16 days.

3.8.2.4 Growth in vitamin-free medium

The use of the ability or inability to grow in a vitamin requirement test medium devoid of all vitamins was used as a diagnostic property. Actively growing yeast cells from a slant were inoculated by needle into tubes containing mineral medium devoid of vitamins and incubated at 25 ºC for 7 days to obtain vitamins carried over from inoculum. Growth was observed after 7 days after that one drop of inoculums from the first tube inoculated into newly vitamin-free tube. The cultures were incubated and inspected after 3 and 7 days for evidence of growth.

3.8.2.5 Growth in media of high osmotic pressure

The ability of yeasts to grow on high concentrations of sugar was usually tested against high osmotic pressure resistance. These experiments were used agar media containing 50% (w/w) and 60% (w/w) glucose. Actively growing cultures were lightly inoculated as a streak on the glucose agar slant in tubes. Tubes were incubated at 25 ºC and growth was examined after 3 and 7 days for evidence of growth.

3.8.2.6 Growth at various temperatures

To determine yeast growth at various temperatures, actively growing yeasts were inoculated into tubes containing YM broth and incubated via using metal block bath at various temperatures; 25, 30, 35, 37, 40, 42 and 45 ºC.

Growth was observed after 3 and 7 days up to 21 days. The results were read and scored as well as for the carbon assimilation test.

3.8.2.7 Acid production from glucose

The ability of yeasts to produce traces of volatile and non- volatile acids in culture were determined when calcium carbonates in the Custer's chalk medium was dissolving by releasing some acid. Actively growing cultures were lightly streaked onto the acid chalk agar plate and incubated at 25 ºC. The growth of tested yeast was inspected after 3 and 7 days for up to 21 days for evidence of clear zone around the cultural growth.

3.8.2.8 Formation of extracellular amyloid compounds (starch formation)

Extracellular amyloid compounds or starch formation was simultaneously carried out by adding 2 drops of Lugol's iodine reagent to the cultural tube for the carbon assimilation test or nitrogen assimilation test agar containing ammonium sulphate after 28 days of incubation. A positive result is indicated by the development in the culture of a color varying from dark blue to green, and the result were noted the color change.

3.8.2.9 Hydrolysis of urea

The urease test was done by using commercial Christensen's urease agar medium. Actively growing cultures were consistently streaked onto the urea agar slant and incubated at 25 ºC. The culture was inspected daily for up to 4 days. The test was taken to be positive when there was development of a deep pink color in the agar but not change in the control.

3.8.2.10 Cycloheximide resistance

Antibiotic cycloheximide (Acti-dione) test was the first report and used as criterion for dividing yeast categories of antibiotic sensitivity; markedly sensitive, moderately sensitive and tolerant (Whiffen, 1948). Actively growing yeasts were inoculated in the same manner as for the carbon (glucose) assimilation tests, into tubes containing filter sterilized 0.01% (100 ppm) and 0.1% (1000 ppm) solutions of cycloheximide. Tubes were incubated at 25 ºC and examined 3 and 7

days for up to 21 days. The results were read and scored as well as for the carbon assimilation test.

3.8.2.11 Gelatin liquefaction

Growth characteristics in gelatin medium were used as a diagnostic property to investigate the ability of few types of yeast is strongly proteolysis of gelatin. Actively growing yeasts were inoculated with a needle as stab into gelatin agar deep tube. Tubes were incubated at 20 ºC and examined 3 and 7 days for up to 21 days. The results were observed for the signs of liquefaction and growth characteristics in gelatin medium.

3.8.3 Chemotaxonomic study: ubiquinone analysis

Classifications of ubiquinone (coenzyme Q) system are well established as a chemotaxonomic criterion at the generic level of yeast (Yamada et al, 1989). Extraction and purification of ubiquinone were carried out according to Nakase and Suzuki (1986b).

3.8.3.1 Cultivation

Yeast cells grew in YPD medium 500 ml, on a rotary shaker at 150 rpm at 25 °C were harvested in 2 days old via centrifugation at 8,000 rpm for 15 min. Cells were washed with distilled water and freeze-dried.

3.8.3.2 Extraction of ubiquinone

Freeze–dried cells were grinded in sterile mortar and pestles then suspended in 50 ml chloroform/methanol (2:1) and kept at RT overnight. Cell pellets were removed by filtration (pore size 0.45 μm) and the filtrates were evaporated to dryness at 40 °C. Crude extracts dissolve with 0.5 ml of acetone.

3.8.3.3 Purification and identification of ubiquinone

Purification of Co-Q was performed by preparative Thin Layer Chromatography (0.5 mm silica gel 60, F254 layers on 20x20 cm glass TLC plate, Merck Germany) using hexane/diethyl ether (85:15) as developer. TLC system took about 30 min. A yellow band (Rf 0.3-0.5), corresponding to a yellow spot of the reference standard and also visualized as a dark band under short wave UV

light (254 nm), was scrapped off. The silica gel powder is transferred to a tube and extracted with 1 ml of acetone then kept overnight in dark condition at RT. The solution was filtered (pore size 0.22 μm) and concentrated by N_2 gas. The solution of ubiquinone isoprenologues stored in the freezer (-20 °C). Ubiquinone isoprenologues were identified by HPLC using a Cosmosil (Waters C18) and methanol: isopropanol (2:1) at 1 ml min^{-1} as the elution system with UV spectrophotometry at wavelength at UV 275 nm. Known Co-Q was used as standards.

3.9 Optimization conditions for growth and biosurfactant production

Among 94 biosurfactant-producing yeast strains which could reduce surface tension to below 40 mN m^{-1} have been screened (section 3.5) and selected for further study. In this section, growths of six selected strains were optimized and their biosurfactant activities were observed under optimum growth conditions. A powerful biosurfactant producing yeast was selected by cultivation in flask containing medium supplemented by either glucose or palm oil or both glucose and palm oil after that cell free broth was measured for biosurfactant activity. For optimization conditions for cultivation and biosurfactant production of the best efficient yeast, the major nutritional requirements for growth and biosurfactant production such as carbon source of glucose and/or vegetable oil and nitrogen source were studied, respectively. The effects of physical factors in each variation of initial pH and temperature during cultivation periods were also investigated. All experiments were carried out in shake flasks.

3.9.1   Selection of a yeast strain having high biosurfactant producing ability.

Yeast strains were cultivated in YM broth, modified Hua's medium supplemented 10% palm oil and modified Hua's medium supplemented 2% glucose and 2% palm oil. The modified Hua's medium used in this study was a medium containing 0.4% (w/v) $NaNO_3$, 0.02% (w/v) $MgSO_4.7H_2O$, 0.02% (w/v) KH_2PO_4, 0.1% (w/v) yeast extract (Hua et al., 2003). Test cultures were carried out in duplicate with each of culture medium. The inoculums having OD 1.0 at wavelength 600 nm (10% v/v) was introduced to an experimental medium. All

cultivation medium were set at initial pH 5.5, with shaking 200 rpm at 30 °C for 7 days. After the end of cultivation period, cell biomass and biosurfactant activities of each studied strains were analyzed to compare biosurfactant capability in reducing surface tension. Cell biomass was measured in terms of cell dry weight. Biosurfactant activities in the cell free broth were determined by measuring the surface tension value by Du Nouy Ring method and the oil displacement capacity (Morikawa et al., 2000). Culture supernatants were tested in triplicate to analyze for biosurfactant activity. Calculation of Δ surface tension defines as reducing value of surface tension was used as a criterion to select the strongest efficient biosurfactant producing yeast strain among the six interested yeast strains then further studies the optimum cultivation conditions used.

3.9.2 Optimization of nutritional requirements affected on cultivation and biosurfactant production

3.9.2.1 Carbon sources

For the effect of carbon sources, the biosurfactant was produced by the selected yeast in 50 ml of modified Hua's medium at an initial pH 5.5 as described in Appendix 1.21 in the presence of various carbon sources including glucose, glycerol, palm oil or soybean oil as sole carbon source. Additionally cultivation in the mixture of two carbon sources was used such as glucose and another hydrophobic carbon source. For the optimum concentration of the carbon source, the concentration of the selected carbon source was varied. All experimental conditions were investigated under constant cultivation temperature at 30 °C with shaking at 200 rpm. The inoculums having OD 1.0 at wavelength 600 nm (10% v/v) was introduced to a tested medium. Each experiment having three sets of flasks was determined at interval cultivation period of 1, 3, 5 and 7 days for cell biomass and biosurfactant activities. The tested carbon source contained in medium that gave the high cell biomass and its supernatant having excellent biosurfactant activity of surface tension reduction and oil displacement test were selected for further studies.

3.9.2.2 Nitrogen sources

For the effect of nitrogen sources, the biosurfactant was produced by the selected yeast in 50 ml of modified Hua's medium supplemented with optimum carbon source obtained from section 3.9.2.1 and 0.4% of various inorganic nitrogen compound, such as $NaNO_3$, NH_4NO_3, $(NH_4)_2SO_4$ and NH_4Cl in a 250 ml Erlenmeyer flask with shaking at 200 rpm at 30 °C. Cell biomass and biosurfactant activity were determined. To determine the optimum concentration of a nitrogen source, 0.2-0.6% of suitable inorganic nitrogen compound were used. The tested nitrogen source contained in medium that gave the high cell biomass and its supernatant having excellent biosurfactant activity of surface tension reduction and oil displacement test were selected for further studies.

3.9.3 Optimization of physical factors affected on cultivation and biosurfactant production

3.9.3.1 Initial pH

The effect of initial pH on cell growth and biosurfactant activity of yeast strain JP52 in a flask containing 50 ml, modified Hua's medium supplementing with optimum carbon and nitrogen source obtained from section 3.9.2.1and 3.9.2.2 were investigated at 30 °C, shaking 200 rpm. Various pH (4.5, 5.0, 5.5, and 6.0) were studied. The cultural sample was collected intervally during cultivation period and determined for cell dry weight, pH and biosurfactant activity. The tested initial pH of medium that gave the high cell biomass and its supernatant having excellent biosurfactant activity of surface tension reduction and oil displacement test was selected for further studies.

3.9.3.2 Temperature

The effect of different temperatures on the growth and biosurfactant activity by yeast strain JP52 in modified Hua's medium with optimum carbon and nitrogen source obtained from section 3.9.2.1and 3.9.2.2 were examined at 30, 35 and 40 °C under optimal initial pH value. The sample was collected intervals during cultivation period and determined for cell dry weight, pH and biosurfactant activity. The tested temperature during cultivation that gave the high cell biomass

and its supernatant having excellent biosurfactant activity of surface tension reduction and oil displacement test was selected for further studies.

3.10 Growth profile, evaluation of substrate consumption and time course of biosurfactant production

The selected yeast strain was grown in a 250 ml- Erlenmeyer flask containing 50 ml of modified Hua's medium with optimum carbon and nitrogen source including optimum initial pH and temperature obtained from section 3.9.2and 3.9.3 with shaking at 200 rpm. Time course of biosurfactant production was performed by periodically determining yeast cell dry weight (DCW), pH, biosurfactant activity as surface tension (ST) values (mN m^{-1}) and oil displacement areas (ODA) including substrate consumption during cultivation. Two percent each of glucose (w/v) and palm oil (v/v) were selected as substrates for this study as they showed excellent growth and biosurfactant activity from the early studies. Biomass as cell dry weight was determined after centrifugation. The cell pellet was washed twice with distilled water, placed on a pre-weighed foil-plate and dried at 105 ºC to obtain constant weight. The supernatant was examined for biosurfactant activity by oil displacement test and ST value measurement. For substrate analysis, total reducing sugars were determined from the supernatant in the absence of oil by dinitrosalicylic acid (DNS) method (Miller, 1959). The residual vegetable oil was determined from the hexane extracts from the cell pellet combined with that of the supernatant as reported by Kawashima et al. (1983). The nitrate concentration was determined colorimetrically by the brucine sulfate method (Ciapina et al., 2006). All data were reported as the mean values of triplicate samples.

3.11 Study of the nitrogen limitation affected on cultivation and biosurfactant production

3.11.1 Microscopic observation of intracellular lipid accumulation of yeast cell

Yeast were cultured in YM, medium supplementing 2% glucose and 2% palm oil (GP) and nitrogen limited medium (Easterling et al., 2009) with initial pH 5.5 at 30°C on a rotary shaker (200 rpm) for interval cultivation time. After that

day of incubation with the above medium, the culture medium was centrifuged at 8,000 rpm for 20 min and the supernatant was removed. The precipitated cells were washed at least three times with saline water (0.8% NaCl), and then suspended in the saline water. The stock solution of Nile red was added to the cell suspension at a final concentration of 0.1 μg ml^{-1}. After 5 min of incubation at 37 °C, the cells were rinsed with the saline water. A stock solution containing 500 μg ml^{-1} Nile red (Wako, Japan) in acetone was stored at 4°C. Fluorescence microscopy was carried out using a BIOREVO Keyence microscope BZ-9000 equipped with a fluorescence filter cube (480–510 nm red excitation filters), using a 100 × objective lens.

3.11.2 Optimization the amount of C: N (w/w) affected on growth and biosurfactant production

For the effect of the amount of C: N (w/w), the biosurfactant was produced by selected yeast strain in 50 ml of medium limited nitrogen containing 0.2 % (w/v) NaNO$_3$ and 0.05 % (w/v) yeast extracts (YE) and varied an equal amount of carbon source of glucose and palm oil at an initial pH 5.5. Their experiments were cultivated at 30°C under shaking 200 rpm. Cell biomass and biosurfactant activity were determined. To determine the optimum amount of C: N (w/w) in GP medium limited nitrogen for biosurfactant production, 16:1 to 100:1 of the amount of C: N (w/w) were studied. The medium limited nitrogen with optimal the amount of C:N (w/w) gave the high cell biomass and its supernatant having excellent biosurfactant activity of surface tension reduction and oil displacement test were compared with GP medium containing 2% glucose and 2% palm oil.

Time-course study of the cell growth, biosurfactant production including following periodically substrate consumption during cultivation were performed as described in section 2.11 when yeast strain was cultivated in the selected optimal medium limited nitrogen and GP medium containing 2% glucose and 2% palm oil. All data were reported as the mean value of duplicate samples.

Table 3.3 The concentration of carbon and nitrogen sources in medium limited nitrogen and GP medium.

Medium		Carbon sources	Nitrogen sources
amount of C: N (w/w)	C: N ratio		
60:1 medium limit N	103.4/1	7.5% (w/v) glucose, 7.5% (v/v) palm oil	0.2 % (w/v) NaNO$_3$ and 0.05 % (w/v) YE
8:1 (GP medium)	25.95/1	2% (w/v) glucose, 2% (v/v) palm oil	0.4 % (w/v) NaNO$_3$ and 0.1 % (w/v) YE

Biosurfactant production was analyzed in 400 ml of production medium obtained from medium limited nitrogen with the amount of C: N at 60:1 (ratio of C/N 103.4/1) for day 7 and 9 and GP medium containing 2% glucose and 2% palm oil (ratio of C/N 25.95/1) at day 7 of cultivation periods. Yeast were grown in 200 ml of medium with initial pH 5.50 contained in 500 ml Erlenmeyer flask at 30 ºC with shaking at 200 rpm. Isolation of biosurfactant and partial purification of crude extracts by TLC analysis were done according to method in section 3.12 and 3.13.1.

3.12 Biosurfactant production by the selected yeast strain

The selected strain was grown in modified Hua's medium supplemented with optimum carbon and nitrogen source including optimum initial pH and temperature obtained from section 3.9.2 and 3.9.3 at 30 °C with shaking at 200 rpm for 7 days. *Pichia anomala* PY1 cultivated in medium containing 2% glucose and 2% palm oil at the same condition. The supernatant was harvested by centrifugation (8,000 rpm, 4 °C, 20 min), and then extracted with hexane at the raitio of 1:3 (v/v) hexane: supernatant. The aqueous phase was harvested and extracted three times with ethyl acetate at the ratio of 1:1 (v/v) ethyl acetate: aqueous phase. The ethyl acetate extracts were pooled, evaporated to dryness (yellowish residue) in a vacuum at 40 °C, and weighed to determine the yield. The production medium that gave the maximal yields and its supernatant having excellent biosurfactant producing capability was selected for further studies.

3.13   Purification and characterization of biosurfactant produced by selected efficient yeast

3.13.1 Isolation and determination of biosurfactant composition by TLC analysis

Crude biosurfactant productions from a selected efficient yeast strain cultivated in optimum medium obtain from section 3.11. Each crude biosurfactant were resolved and then compared their mobility properties of resolution pattern undertaken on preparative thin layer chromatography (TLC) plates at same condition. The crude extracts of biosurfactant by selected efficient yeast from medium GP and G were then suspended in ethyl acetate to 20 mg mL^{-1} and were resolved on silica gel (TLC) plates with thickness 2.0 mm (G60, Merck, and Darmstadt, Germany) using a 65:25:4 (v/v/v) ratio chloroform:methanol:water mixture as the mobile phase. Positive areas of glycolipid on the resolved TLC plate were visualized by iodine vaporing for fatty acid detection, seen as dark yellowish spots. The relative mobility (Rf) value of the positive fractions were recorded and then they were scraped, solvated in ethyl acetate and assayed for carbohydrate components by the Molish test and oil displacement activity, as previously reported.

3.13.2 Chemical structure characterization of biosurfactants

Positive fraction(s) with a high oil displacement activity were further analyzed using a matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF-MS) to identify the glycolipid type. The result of the m/z for peaks of molecular mass analysis was proposed and assigned the chemical structural of biosurfactants by NMR method.

3.13.2.1 Molecular mass analysis by MALDI-TOF-MS

MALDI-TOF-MS analysis was performed as previously described (Kurtzman et al., 2010 and Price et al., 2012) on a Voyager instrument (Bruker Daltonics, Billerica, MA) operating in linear mode with positive ion detection. Samples were suspended in acetonitrile to 100 μg/μL and then 0.5 μL was mixed with 5 μL of the matrix (2, 5-dihydroxybenzoic acid at 10 mg/mL in 50%

Acetonitrile) and allowed to dry in a 100 well plate at RT prior to analysis. An acceleration voltage of 20 kV and time delay of 110 ns was used, with a total of 100 laser shots being summed for each spectrum.

3.13.2.2 Structure elucidation of the glycolipids by nuclear magnetic resonance (NMR) analysis

^{1}H and ^{13}C NMR spectra of the partially purified glycolipids were recorded in deuterated methanol (CD$_3$OD) on a Bruker Avance spectrometer (Bruker Biospin Corp., Billerica, MA) operating at 500.11 MHz using a standard 5 mm z-gradient BBI probe at 27 ℃. Two-dimensional NMR spectra, ^{1}H- ^{1}H- correlation spectroscopy (COSY), the ^{1}H- ^{1}H- total correlation spectroscopy (TOCSY), heteronuclear single quantum correlation (HSQC) and heteronuclear multiple bond correlation (HMBC) were performed at 600 MHz using standard procedures. The chemical shifts were expressed in parts per million (ppm) downfield from an internal standard of tetramethylsilane.

3.14 Determination of physicochemical properties of crude biosurfactants

The crude extracts (acquired from section 3.11) were analyzed for the critical micelle concentration as CMC value according to Sheppard and Mulligan (1987), emulsification index of E24 value and emulsion stability followed by Cooper and Goldenberg (1987) compared with the synthetic surfactants. For physical properties analysis, the crude biosurfactants were subjected to biosurfactant efficiency test under various extreme conditions of pH, temperature and salinity.

3.14.1 Measurement of CMC value of crude biosurfactant

In this experiment, the yellow oily extracted biosurfactant from medium supplementing glucose and palm oil (GP) was studied and dissolved in 50 mM Tris-HCl, pH 8.0 and serially diluted to allow the concentration at 10^{-2}-10^{5} and 5 x 10^{5} mg L^{-1} prior to the measurement of surface tension. The CMC was obtained from a plot of the surface tension as a function of the biosurfactant concentrations. The concentration at which micelles began to form was represented as the CMC.

Above this concentration, there would be no increment could be detected for the reduction of surface tension.

3.14.2 Measurement of emulsification activity

Emulsification ability of the selected crude biosurfactant as the extracted biosurfactant from GP medium towards various vegetable oils and hydrocarbons was studied. A mixture of 6 ml of studied vegetable oils or hydrocarbons and 4 ml of 1 mg ml^{-1} of the crude biosurfactant solution was vortexed at a high speed for 2 min. The emulsion activity was investigated after 24 h and the emulsification index (E24) was calculated by dividing the measured height of the emulsion layer by the total height of the mixture and multiplying by 100 (Cooper and Goldenberg, 1987). Furthermore those emulsions were left standing to reexamine on day 7 and evaluated their emulsion stabilization.

3.14.3 Stability characterization of biosurfactant

Stability studies were carried out using 1 mg ml^{-1} of the extracted biosurfactant from GP medium suspended in 50 mM Tris-HCl, pH 8.0 and investigated the effect of several environmental parameters (pH, salt and temperature variation) on the surface activity of the biosurfactant. For the effect of pH, the biosurfactant solution was adjusted with 0.1 M NaOH or HCl in the pH range 2-10 and for salt tolerance test; it was prepared at 0-10% w/v NaCl concentration. All tested tubes was analyzed the activity of surface tension reduction, measured their ST value when left standing for 3 hours (day 1) and 72 hours (day 3). For heat stability of the crude biosurfactant solution, it incubated at tested temperatures (4, 30, 60 or 100 ºC) or sterilization process via autoclave then monitored the biosurfactant activity at interval incubation time up to 9 hours or past sterilization 5 times.

CHAPTER IV

RESULTS

4.1   Diversity of the efficient biosurfactant producing yeasts

4.1.1   Isolation of biosurfactant producing yeasts

This research studied the biodiversity of the efficient biosurfactant producing yeasts isolated from soil, agricultural waste contaminated with oil, biodiesel wastes and industrial wastes. The samples were collected upon the features of sample sources contaminated with oil from Suratthani, Chumphon, Ranong, Prachuabkirikhan, Rayong, Samutsakhon and Samutprakan Province, Thailand. All samples were determined for pH value and the total number of colonies. There were total of 38 samples obtained from 19 locations and from 7 provinces as shown in Table 4.1. The moisture content determinations were also undertaken only with solid samples such as soil, rotten oil palm fruitlet or bunches and sediment, which showed the percentage of moisture in the range 1.95-62.64%. The pH values were in the range of 4.26-7.24, but most of the samples were found to be below pH 7.00. Each sample was analyzed for enumeration of microbial population by spread plate technique on acidified YM agar supplemented with 1% (v/v) palm oil, 100 µg ml^{-1} chloramphenicol, and 0.2% (w/v) sodium propionate. The total numbers of aerobic cultures per gram of samples were found to be 3.3E+02 to 2.61E+06 CFU g^{-1}. No yeast colonies were formed in two samples from sea water collected from fishery pier.

Thirty-eight samples contaminated with oil were classified into six types including agricultural waste (AW), waste from biodiesel plant (BP), liquid of effective microorganisms (EM), industrial wastes (IW), sea water (SW) and soil (S). Isolation methods were taken by dilution plating and enrichment cultures to obtain as many kinds of yeast as possible which resulted in 368 isolates as shown in Table 4.2. The

results showed that besides yeast strains, some other microorganisms were also isolated which were 17 strains of bacterial and 37 strains of filamentous fungi.

Table 4.1 Results of moisture content, pH value and total number of colonies of collected samples with contaminated oil

source	location	Collected Date	Sample types	code	Moisture content (%)	pH value	Total number of colonies (CFU g^{-1})
1	Palm oil tree garden in Krang District, Rayong	24-1-2009	Wetted-soil[a]	RK1	12.32	5.68	4.80E+03
		24-1-2009	Dried-soil[a]	RK2	1.95	5.45	1.78E+05
		24-1-2009	rotten oil palm bunches	RK3	7.18	6.53	1.25E+05
2	Biodiesel plant in Prachuabkirikhan	6-2-2009	Soil sediment	PB	9.96	4.97	8.50E+05
3	Cosmetic factory in Bangplee District, Samutprakarn	6-2-2009	Waste water	SM	ND	5.34	3.86E+04
4	Palm oil tree of Orchid village in Bangsaotong District, Samutprakarn	16-3-2009	Topsoil[a]	OR	12.44	5.78	2.18E+05
5	Palm oil tree garden of Bangsapannoi District,Prachuabkirikhan at KM.428	13-5-2009	Soil at depth 5 cm	PC1	15.51	5.17	1.30E+03
		13-5-2009	Topsoil[a]	PC2	25.7	6.15	9.150E+03
		13-5-2009	rotten oil palm bunches	PC3	12.63	6.25	5.55E+03
6	Fishery pier at Paknum Chumporn District, Chumporn	13-5-2009	Sea water that contaminated oil	CH1	ND	6.84	No growth
		13-5-2009	Sea water that contaminated oil	CH2	ND	6.89	No growth
7	Fishery pier at Tayang District, Chumporn	13-5-2009	Surface water that contaminated oil	CH3	ND	6.99	No growth
		13-5-2009	Surface soil that contaminated oil	CH4	48.05	7.24	No growth

Sample collection					Moisture content (%)	pH value	Total number of colonies (CFU g^{-1})
source	location	Collected Date	Sample types	code			
8	Chumporn cabana resort at Chumporn	14-5-2009	EM broth	CH5	ND	4.26	6.95E+03
		14-5-2009	Topsoil[a]	CH6	45.03	6.86	4.15E+03
		14-5-2009	rotten oil palm bunches	CH7	62.64	4.68	8.00E+03
		14-5-2009	Topsoil[b]	CH8	3.12	5.4	No growth
9	Palm oil biodiesel production plant (Chumporn Palm Oil industry) at Thasae District, Prachuabkirikhan	15-5-2009	Soil sediment pond	CH9	25.51	5.45	6.40E+04
		15-5-2009	wastewater	CH10	ND	4.93	1.89E+04
10	Pooled palm oil fruit bunches shop in Pakjan District, Ranong	14-5-2009	rotten oil palm fruitlet	RN1	44.65	5.69	2.61E+06
		14-5-2009	rotten oil palm bunches	RN2	58.53	5.62	2.25E+05
11	Palm oil tree garden in Pakjan District, Ranong	14-5-2009	Soil at depth 5 cm	RN3	34.96	5.96	1.87E+03
		14-5-2009	Topsoil[a]	RN4	39.52	5.95	1.76E+03
		14-5-2009	Topsoil[a]	RN5	54.01	6.13	6.65E+03
		14-5-2009	rotten oil palm bunches	RN6	54.16	6.96	9.35E+03
12	Palm oil tree garden in Kraburi District, Ranong	14-5-2009	rotten oil palm fruitlet	RN7	49.22	5.37	1.36E+06
		14-5-2009	Soil at depth 5 cm	RN8	23.73	4.41	1.78E+03
		14-5-2009	Topsoil[a]	RN9	26.82	5.26	7.00E+04
13	Mangroove forest at Laoon District, Ranong	14-5-2009	Wetted-soil	RN10	61.23	6.13	3.80E+05
14	Biodiesel plant in Samutsakorn	19-5-2009	wastewater	SK1	ND	6.38	2.92E+03
		19-5-2009	Topsoil[b]	SK3	19.44	5.32	3.30E+02
		19-5-2009	Topsoil[b]	SK4	34.39	4.55	5.15E+03
15	Palm oil tree garden in Suratthani	12-5-2009	Topsoil[a]	SOM1	45.14	5.75	3.00E+05
		12-5-2009	Topsoil[a]	SOM2	29.36	5.80	1.17E+05
16	Palm oil tree garden in Wangjan District, Rayong	12-5-2009	Topsoil[a]	PS1	11.12	6.29	4.30E+04

Sample collection					Moisture content (%)	pH value	Total number of colonies (CFU g^{-1})
source	location	Collection date	Sample types	code			
17	Palm oil tree garden in Thasae District, Chumporn	14-4-2009	Topsoil^a	POM1	6.06	7.02	1.82E+04
18	Palm oil tree garden in Bangsapannoi District,Prachuabkirikan	14-4-2009	Topsoil^a	POM2	10.26	6.69	6.75E+04
19	Palm oil tree garden in Suradthani	13-4-2009	Topsoil^a	POM3	12.75	5.95	2.18E+03

ND: Not determined due to these were water typed-samples.
No growth: Total aerobic plate counts were not found when using dilution plating on YM + 1% palm oil + antibacterial
a: soils surrounding of palm oil trees
b: soils surrounding area of biodiesel plant

Table 4.2 Six types of samples with contaminated oil and diversity of total cultural isolates and efficient biosurfactant producing yeast strains having surface tension ≤ 40 mN m^{-1}

Type of sample	Numbers of sample analysis	Code of samples	Total isolates (strains)	Efficient BS yeast (strains)	Proportion of efficient BS yeasts^a (%)
Agricultural waste (AW)	7	CH7, PC3, RK3, RN1, RN2, RN6, RN7	52	12	23.1
Biodiesel plant waste (BP)	4	PB, SK1, SK3, SK4	53	16	30.2
EM liquids (EM)	1	CH5	4	4	100.0
Industrial waste (IW)	3	CH9, CH10, SM1	49	15	30.6
Sea water (SW)	3	CH1, CH2, CH3	19	0	0.00
Soil (S)	20	CH4, CH6, CH8, OR, PC1, PC2, POM1, POM2, POM3, PS1, RK1, RK2, RN3, RN4, RN5, RN8, RN9, RN10, SOM1, SOM2	191	47	24.6
	38		368	94	

a: Proportion of efficient biosurfactant-producing yeasts (%) derived from dividing the number of efficient yeast with the number of total isolates then multiplying with 100.

4.1.2   Screening of biosurfactant producing yeasts

All isolates were screened for efficient biosurfactant producing yeasts by cultivating in acidified YM broth containing 1% (v/v) palm oil for 7 days then they were determined for pH value, oil displacement activity and the surface tension of cell free broth. These isolated strains were grouped according to surface tension reduction ability as shown in Figure 4.1. A good surfactant is capable of reducing the surface tension (ST) of water from 72 to 35 mN m^{-1} and the interfacial tension (IT) of a water/hexadecane interphase from 40 to 1 mN m^{-1} (Mulligan, 2005). This work aimed to screen and select the yeast strains isolated from various oil-contaminated samples for efficient biosurfactant production that could reduce the ST below to 40 mN m^{-1}. The population diversity of the effective biosurfactant producers having ST value below to 40 mN m^{-1} were 122 strains (33.15%) out of the total numbers of isolated strains. They could reduce cell free broth surface tension from 50 mN m^{-1} to lower than 40 mN m^{-1}. Out of 122 strains, 94 strains were found to be highly effective biosurfactant producing yeasts when grown in growth medium containing glucose plus palm oil. The results of measurable biosurfactant activity and pH value analysis of individual isolated strains were shown in the Appendix 9. The distribution of isolated strains and efficient biosurfactant producing yeast strains among six types of samples were shown in Table 4.2 and Figure 4.2. From this experiment, probability of screening efficient biosurfactant producing yeast strains from biodiesel wastes and industrial wastes were 30.19 and 30.61%, respectively which were higher than those from agricultural wastes (23.08%) and soils (24.61%). From screening results, effective 94 yeast strains were then selected for further studies including DNA sequencing analysis for strains identification.
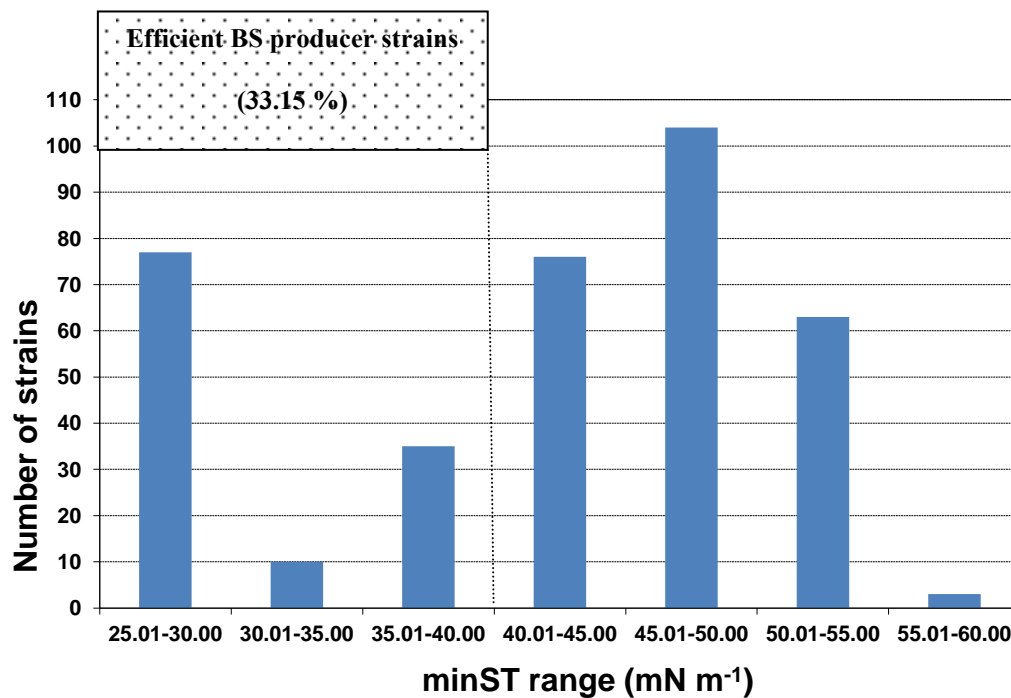
Figure 4.1 Grouping of yeast and fungal strains according to surface tension reduction activity.
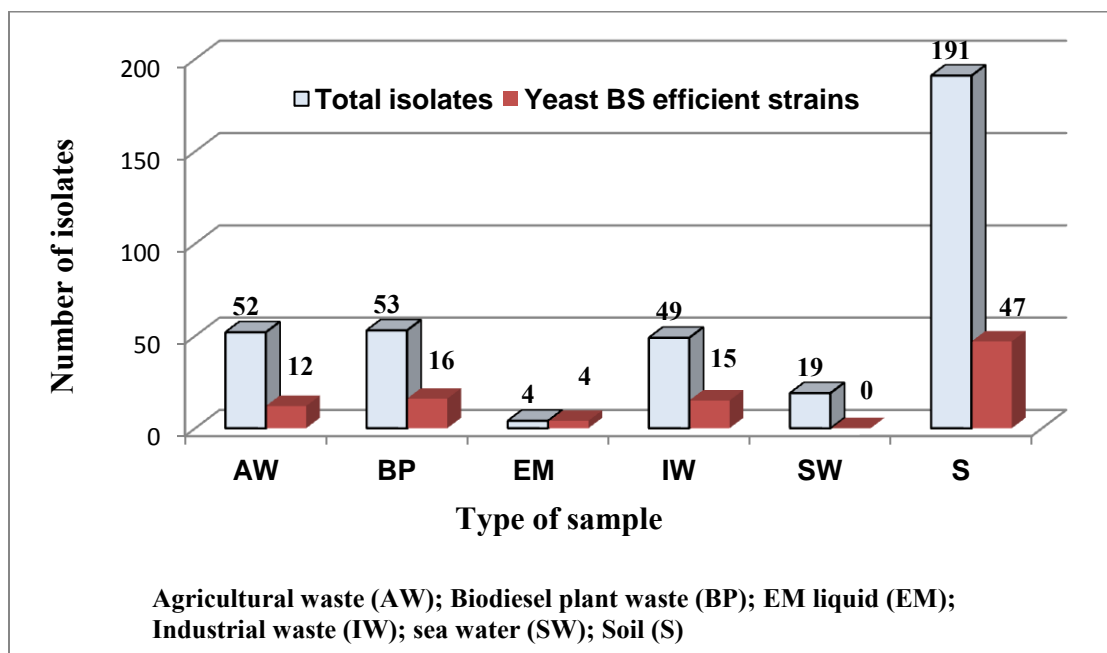
Figure 4.2 The distribution of total isolated cultures and efficient biosurfactant producing yeast strains having surface tension ≤ 40 mN m^{-1} in different types of sample contaminated oils.

4.1.3 Identification of the isolated yeast strains by molecular technique analysis

For molecular genetic analysis of yeasts for strain identification, the 94 yeast strains were identified based on D1/D2 sequences of LSU rDNA gene. D1/D2 region located on the sequenced portion of region 25S–635 was extended to ~600 nucleotides sequence in length (Kurtzman and Robnett, 1997). Intended DNA fragments of all efficient yeast strains were successfully amplified with primers NL-1 and NL-4. The PCR products were determined by Nano drop meter and gel electrophoresis for the quantity and purity. PCR products from all tested strains were obviously seen as a single fragment each with a molecular size of approximately 550-600 bp when analyzed on 0.8% agarose gel (Figure 4.3).
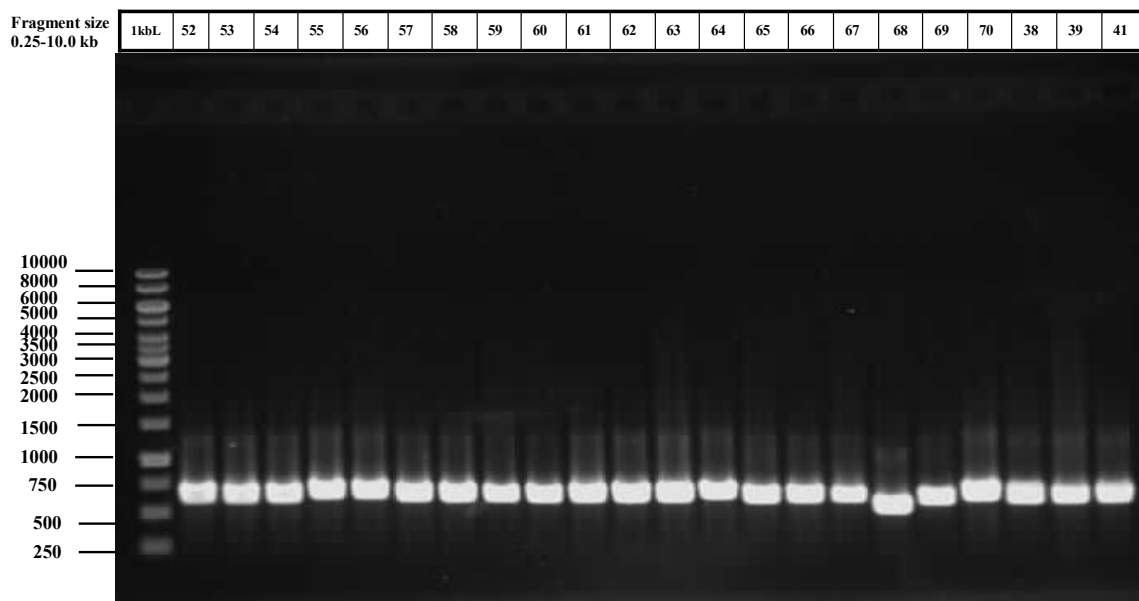
Figure 4.3 The PCR products of D1/D2 region of efficient yeast strain (JP52-JP70, JP38, JP39 and JP41) comparing with DNA marker in fragment size 0.25-10.0 kb (left lane).

The guideline of yeast identification suggested that yeast strains showing nucleotide substitutions at 1% in the D1/D2 region of the LSU rRNA gene are usually different species (Kurtzman and Robnett, 1998). Base pair variation of the D1/D2

region with greater 1 % substitutions in the ca. 600-nucleotides are likely to be different species, whilst that with 0-3 are either conspecific species or sister species. The results in Table 4.3 showed identification of 94 yeast strains comparing to available sequences data retrieved from Genbank. They were consisted of query length 537-625 nucleotides as likewise other entries of yeast species. Nucleotide sequences of each strain were aligned to the closest described species showing the highest matched sequence identity. With respect to the calculated percentage of nucleotide substitution, each strain was acquired the answer whether known or novel yeast species.

Table 4.3 Identification of efficient biosurfactant producing yeast by comparing to sequences data available from Genbank

No.of seq. JP	Closet species (Accession number)	Query length	Nucleotide identity in D1/D2 region		No. of nt. diferrence	No. of gap	No. of nt. substitutions	% substitution	Results
			no. of nt. identity / total nt.	% Identity					
1	*Meyerozyma guilliermondii* (U45709)	577	569/570	99.82	1	0	1	0.18	Known species
2	*Meyerozyma guilliermondii* (U45709)	625	568/570	99.82	2	1	1	0.18	Known species
3	*Candida mucifera* (AJ508572)	584	575/577	99.65	2	0	2	0.35	Known species
4	*Candida tropicalis* (U45749)	578	570/570	100.00	0	0	0	0.00	Known species
5	*Candida viswanathii* (U45755)	575	569/570	100.00	1	1	0	0.00	Known species
6	*Candida mucifera* (AJ508572)	584	575/577	99.65	2	0	2	0.35	Known species
7	*Meyerozyma guilliermondii* (U45709)	577	577/577	100.00	0	0	0	0.00	Known species
8	*Candida mucifera* (AJ508572)	585	574/577	99.65	3	1	2	0.35	Known species
9	*Candida mucifera* (AJ508572)	579	575/577	99.65	2	0	2	0.35	Known species
10	*Candida tropicalis* (U45749)	577	577/577	100.00	0	0	0	0.00	Known species
11	*Trichosporon asahii* (EF559350)	606	602/606	99.83	4	3	1	0.17	Known species
12	*Candida tropicalis* (U45749)	576	576/576	100.00	0	0	0	0.00	Known species
13	*Trichosporon asahii* (EF559350)	601	601/601	100.00	0	0	0	0.00	Known species
14	*Trichosporon asahii* (EF559350)	605	605/605	100.00	0	0	0	0.00	Known species
15	*Meyerozyma guilliermondii* (U45709)	537	536/537	99.81	1	0	1	0.19	Known species
16	*Meyerozyma guilliermondii* (U45709)	579	579/579	100.00	0	0	0	0.00	Known species
17	*Meyerozyma guilliermondii* (U45709)	582	569/571	99.82	2	1	1	0.18	Known species
18	*Meyerozyma guilliermondii* (U45709)	579	569/571	99.82	2	1	1	0.18	Known species
19	*Meyerozyma guilliermondii* (U45709)	580	569/570	99.82	1	0	1	0.18	Known species
20	*Candida mucifera* (AJ508572)	582	574/577	99.65	3	1	2	0.35	Known species
21	*Meyerozyma guilliermondii* (U45709)	578	569/570	99.82	1	0	1	0.18	Known species
22	*Meyerozyma guilliermondii* (U45709)	580	569/571	99.82	2	1	1	0.18	Known species
23	*Candida mucifera* (AJ508572)	586	575/577	99.65	2	0	2	0.35	Known species
24	*Meyerozyma guilliermondii* (U45709)	577	569/571	99.82	2	1	1	0.18	Known species
25	*Candida mucifera* (AJ508572)	583	574/577	99.65	3	1	2	0.35	Known species
26	*Meyerozyma guilliermondii* (U45709)	577	569/570	99.82	1	0	1	0.18	Known species
27	*Meyerozyma guilliermondii* (U45709)	578	569/570	99.82	1	0	1	0.18	Known species
28	*Meyerozyma guilliermondii* (U45709)	577	569/570	99.82	1	0	1	0.18	Known species
29	*Candida palmioleophila* (U45758)	577	567/570	100.00	3	3	0	0.00	Known species
30	*Trichosporon faecale* (AF105395)	602	599/601	99.67	2	0	2	0.33	Known species

No.of seq. JP	Closet species (Accession number)	Query length	Nucleotide identity in D1/D2 region		No. of nt. diferrence	No. of gap	No. of nt. substitutions	% substitution	Results
			no. of nt. identity / total nt.	% Identity					
31	*Trichosporon asahii* (EF559350)	602	602/602	100.00	0	0	0	0.00	Known species
32	*Candida mucifera* (AJ508572)	585	575/578	99.65	3	1	2	0.35	Known species
33	*Candida mucifera* (AJ508572)	582	574/578	99.65	4	2	2	0.35	Known species
34	*Candida mucifera* (AJ508572)	583	574/577	99.65	3	1	2	0.35	Known species
35	*Meyerozyma guilliermondii* (U45709)	579	569/572	99.83	3	2	1	0.17	Known species
36	*Meyerozyma guilliermondii* (U45709)	578	569/570	99.82	1	0	1	0.18	Known species
37	*Candida mucifera* (AJ508572)	581	574/577	99.65	3	1	2	0.35	Known species
38	*Candida mucifera* (AJ508572)	583	575/577	99.65	2	0	2	0.35	Known species
39	*Meyerozyma guilliermondii* (U45709)	575	569/570	99.82	1	0	1	0.18	Known species
40	*Candida mucifera* (AJ508572)	583	575/577	99.65	2	0	2	0.35	Known species
41	*Candida mucifera* (AJ508572)	584	574/577	99.65	3	1	2	0.35	Known species
42	*Meyerozyma guilliermondii* (U45709)	577	569/571	99.82	2	1	1	0.18	Known species
43	*Candida mucifera* (AJ508572)	584	574/577	99.48	3	0	3	0.52	Sister species
44	*Candida mucifera* (AJ508572)	581	575/577	99.65	2	0	2	0.35	Known species
45	*Candida mucifera* (AJ508572)	585	575/577	99.65	2	0	2	0.35	Known species
46	*Candida mucifera* (AJ508572)	587	575/577	99.65	2	0	2	0.35	Known species
47	*Candida mucifera* (AJ508572)	583	575/577	99.65	2	0	2	0.35	Known species
48	*Candida mucifera* (AJ508572)	586	575/577	99.65	2	0	2	0.35	Known species
49	*Meyerozyma guilliermondii* (U45709)	576	569/570	99.82	1	0	1	0.18	Known species
50	*Meyerozyma guilliermondii* (U45709)	575	569/570	99.82	1	0	1	0.18	Known species
51	*Candida mucifera* (AJ508572)	582	573/577	99.65	4	2	2	0.35	Known species
52	*Candida mengyuniae* (AB741072)	591	564/587	97.27	23	7	16	2.73	New species
53	*Candida tropicalis* (U45749)	573	570/570	100.00	0	0	0	0.00	Known species
54	*Candida tropicalis* (U45749)	577	570/570	100.00	0	0	0	0.00	Known species
55	*Trichosporon asahii* (EF559350)	604	604/604	100.00	0	0	0	0.00	Known species
56	*Trichosporon asahii* (EF559350)	600	599/599	100.00	0	0	0	0.00	Known species
57	*Candida tropicalis* (U45749)	579	570/571	100.00	1	1	0	0.00	Known species
58	*Candida tropicalis* (U45749)	577	570/570	100.00	0	0	0	0.00	Known species
59	*Scheffersomyces spartinae* (JQ689045)	576	569/577	98.96	8	2	6	1.04	New species
60	*Scheffersomyces spartinae* (JQ689045)	573	566/575	98.96	9	3	6	1.04	New species

No.of seq. JP	Closet species (Accession number)	Query length	Nucleotide identity in D1/D2 region		No. of nt. diferrence	No. of gap	No. of nt. substitutions	% substitution	Results
			no. of nt. identity / total nt.	% Identity					
61	*Candida tropicalis* (U45749)	578	570/570	100.00	0	0	0	0.00	Known species
62	*Candida tropicalis* (U45749)	576	570/570	100.00	0	0	0	0.00	Known species
63	*Meyerozyma guilliermondii* (U45709)	579	568/571	99.65	3	1	2	0.35	Known species
64	*Trichosporon asahii* (EF559350)	607	607/607	100.00	0	0	0	0.00	Known species
65	*Candida palmioleophila* (U45758)	576	567/568	100.00	1	1	0	0.00	Known species
66	*Candida tropicalis* (U45749)	577	570/570	100.00	0	0	0	0.00	Known species
67	*Meyerozyma guilliermondii* (U45709)	575	568/570	99.65	2	0	2	0.35	Known species
68	*Candida rugosa* (U45727)	622	484/484	100.00	0	0	0	0.00	Known species
69	*Pichia kudriavzevii* (U76347)	571	564/564	100.00	0	0	0	0.00	Known species
70	*Trichosporon asahii* (EF559350)	603	602/603	99.83	1	0	1	0.17	Known species
71	*Candida palmioleophila* (U45758)	575	566/567	99.82	1	0	1	0.18	Known species
72	*Candida mucifera* (AJ508572)	584	574/577	99.65	3	1	2	0.35	Known species
73	*Meyerozyma guilliermondii* (U45709)	578	568/570	99.65	2	0	2	0.35	Known species
74	*Meyerozyma guilliermondii* (U45709)	582	569/570	99.82	1	0	1	0.18	Known species
75	*Candida mucifera* (AJ508572)	586	574/578	99.65	4	2	2	0.35	Known species
76	*Meyerozyma guilliermondii* (U45709)	578	569/570	99.82	1	0	1	0.18	Known species
77	*Meyerozyma guilliermondii* (U45709)	577	569/570	99.82	1	0	1	0.18	Known species
78	*Candida mucifera* (AJ508572)	584	571/577	99.13	6	1	5	0.87	Sister species
79	*Meyerozyma guilliermondii* (U45709)	579	569/570	99.82	1	0	1	0.18	Known species
80	*Meyerozyma guilliermondii* (U45709)	577	569/570	99.82	1	0	1	0.18	Known species
81	*Candida tropicalis* (U45749)	578	570/570	100.00	0	0	0	0.00	Known species
82	*Trichosporon asahii* (EF559350)	606	606/606	100.00	0	0	0	0.00	Known species
83	*Candida tropicalis* (U45749)	579	570/570	100.00	0	0	0	0.00	Known species
84	*Prototheca zopfii* var. zopfii (AB183201)	590	570/583	98.11	13	2	11	1.89	New species
85	*Meyerozyma guilliermondii* (U45709)	578	569/570	99.82	1	0	1	0.18	Known species
86	*Meyerozyma guilliermondii* (U45709)	579	569/570	99.82	1	0	1	0.18	Known species
87	*Meyerozyma guilliermondii* (U45709)	578	569/570	99.82	1	0	1	0.18	Known species
88	*Meyerozyma guilliermondii* (U45709)	578	569/570	99.82	1	0	1	0.18	Known species
89	*Meyerozyma guilliermondii* (U45709)	578	569/570	99.82	1	0	1	0.18	Known species
90	*Meyerozyma guilliermondii* (U45709)	579	569/570	99.82	1	0	1	0.18	Known species

No.of seq. JP	Closet species (Accession number)	Query length	Nucleotide identity in D1/D2 region		No. of nt. diferrence	No. of gap	No. of nt. substitutions	% substitution	Results
			no. of nt. identity / total nt.	% Identity					
91	*Meyerozyma guilliermondii* (U45709)	578	568/570	99.65	2	0	2	0.35	Known species
92	*Candida palmioleophila* (U45758)	576	567/567	100.00	0	0	0	0.00	Known species
93	*Meyerozyma guilliermondii* (U45709)	578	569/570	99.82	1	0	1	0.18	Known species
94	*Meyerozyma guilliermondii* (U45709)	577	569/570	99.82	1	0	1	0.18	Known species

BLASTn analysis of the 94 yeast strains based on sequences of D1/D2 analysis indicated that 93.62% (88 strains) were known species, 2.13% (2 strains) were undescribed species and 4.26% (4 strains) were novel species. The diversity among these 94 efficient biosurfactant producing yeast strains was shown in Figure 4.4. Among these known yeasts, ascomycetous yeasts were found diversely species with number higher than basidiomycetous yeasts when investigated from 5 groups of contaminated oil samples. *Meyerozyma guilliermondii* were the most frequent yeast species and *Candida mucifera* as the second of ascomycetous, which were found 39.36% and 23.40% respectively. Both species were obtained abundantly from soils as the prominent sources. Basidiomycetes were minor in number of isolates and a few of species e.g. *Trichosporon asahii* and *Trichosporon faecale* were observed with 9.57% and 1.06% of analyzed strains. There were 2 undescribed strains or sister species (2.13%) that differed in 3 and 5 nucleotide substitutions with *Candida mucifera* as the closest known yeast. This output of surveying biosurfactant producing yeasts also generated four novel strains which one strain might be a microalga of *Chlorellales* (not described) and three strains which had been later described in this study to be two new yeast species.

4.1.3.1 Described known yeast

The results of efficient biosurfactant producing yeast identification showed 93.62% (88 isolates) of described yeast or known species which were classified as Ascomycota at 88.63% (78 isolates) and Basidiomycota at 11.36% (10 isolates) of total described yeasts. With respect to reveal in Ascomycota, these isolates were grouped into 4 genus and 7 species, they were placed similarly in order Saccharomycetales class Saccharomycetes but distinguished 4 families as described below.

Family Debaryomycetaceae Genus *Meyerozyma*: *Meyerozyma guilliermondii* was found the most frequent at 37 isolates and distributed vastly in various contaminated oil samples such as agricultural wastes, biodiesel plant wastes, industrial wastes and especially a lot from soils.

Family Trichomonascaceae Genus *Trichomonascus*: *Trichomonascus* (*Candida*) *mucifera* was placed in *Trichomonascus ciferrii* complex. There were 22 isolates obtained from agricultural wastes, biodiesel plant

wastes, EM liquid and soils.

Family Pichiaceae Genus *Pichia*: *Pichia kudriavzevii* had one isolate from biodiesel plant wastes.

Family Saccharomycetales (mitosporic) Genus *Candida*; 4 species e.g. *C. tropicalis* 12 isolates, *C. palmiophila* 4 isolates, *C. rugosa* 1 isolate, *C. viswanathii* 1 isolate. *C. tropicalis* was found rather than others in this genus *Candida* obtained from agricultural wastes, industrial wastes and soils.
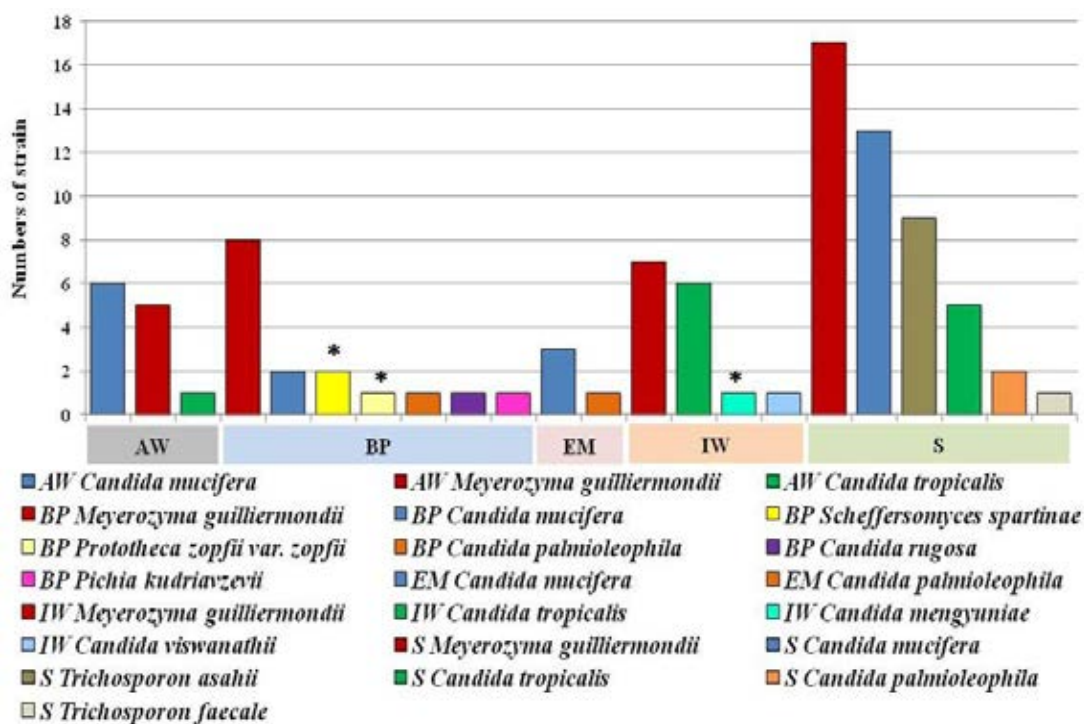
Figure 4.4 The diversity of efficient biosurfactant producing yeast strains as known yeast and the closest related species (*) in different types of sample contaminated oils.

About the 10% as rest of known species were identified to be yeast in phylum Basidiomycota obtain from soil only. They were in sub phylum Agaricomycotina, class Tremellomycetes, order Tremellales, family mitosporic Tremellales and genus *Trichosporon*. It was belonged to 9 isolates of *Trichosporon asahii* and one isolate of *Trichosporon faecale*.

4.1.3.2 Novel species and undescribed yeast

As a result of D1/D2 domain sequences of 26S LSU rRNA gene when compared with GenBank database, if strains with 0-3 nucleotide differences are considered either conspecific or sister species and with greater than 1 % substitutions in the ca. 600-nucleotides are certainly determined as novel species (Kurtzman and Robnett, 1998). The results found two isolates with 3 and 5 nucleotide substitutions then they were sister species or undescribed yeast. Both were closely related with known yeast *Candida mucifera*. Novel yeast strains having potential in biosurfactant production were 4 strains including 1 strain (JP52) belong to the genus *Cyberlindera*, 2 strains (JP59 and JP60) belong to the genus *Scheffersomyces*, and 1 strains (JP84) belong to the genus *Prototheca* in family Chlorellaceae. Strain JP52 differed remarkably with the closest known yeast at 16 nucleotide substitutions whilst JP59 and JP60 had each difference with 6 nucleotide substitutions. This study concerned three strains of yeast (JP52, JP59 and JP60) that have been later described and proposed to be two novel yeast species.

4.1.4 Identification of novel yeast species followed by polyphasic taxonomy studies

Polyphasic approach was undertaken to be the efficient strategy of microbial identification (Arias et al., 2002). So, novel yeast identification in our study for molecular biological analysis was accuracy performed by using multi-gene analysis at least two regions of D1/D2 region of LSU rRNA gene sequences and ITS1-2 region. In addition to conventional study, the novel yeast strains were characterized morphologically, physiologically and biochemically following standard yeast identification methods (Yarrow, 1998) including classification of ubiquinone system according to Nakase & Suzuki (1986b).

4.1.4.1 *Cyberlindnera samutprakarnensis* sp. nov. JP52[T]

Strain JP52 was isolated from the waste-water of a cosmetic factory (Milott laboratory Ltd.) in Bangplee, Samutprakarn province, Thailand, using an enrichment technique in acidified YM broth containing 1% (v/v) palm oil. The discrimination of novel yeast strain JP52 was determined from the rRNA gene

sequences from the D1/D2 region of the LSU rRNA gene and the ITS1-2 region. The sequences data of JP52[T] revealed D1/D2 and ITS1-2 region have been deposited in the GenBank database under the following accession numbers AB598079 and AB695388 respectively. Strain JP52[T] was closely related to *Cyber. mengyuniae* CBS 10845[T], by 2.9% (16 substitutions, 7 indels) and 4.4% (28 substitutions, 49 indels) in the D1/D2 region and ITS1-2 region, respectively then closed to *Cyberlindnera saturnus* varieties, *Cyber. saturnus*, *Cyber. suaveolens*, *Cyber. subsufficiens*, *Cyber. mrakii* and *Cyber. sargentensis* by 4.6-5.3% sequence divergence. Table 4.4 showed the sequence divergence of the D1/D2 LSU rDNA (upper triangle) and ITS1-2 region (lower triangle) of strain JP52[T] and their related species with *Cyberlindnera saturnus* varieties, *Cyber. meyerae*, *Cyber. vartiovaarae* and *Cyber. jadinii*. Nucleotide variation of strain JP52[T] discriminated against among those closely related yeasts with 16-32 base substitutions of D1/D2 region and increasingly reached 28-57 nucleotides in region of ITS1-2 gene. Strains with more than 6 nucleotide substitutions or 1% sequence divergence in these region suggested that they belong to different species (Kurtzman and Robbnett, 1998). In addition to the phylogenetic tree based on the concatenated ITS1-2 and D1/D2 region of LSU rRNA gene sequences, strain JP52 placed in the *Cyberlindnera* clade and distinct from the described species of *Cyberlindnera*. This phylogram of JP52 showed JP52 connected to *Cyberlindnera mengyuniae* with high bootstrap confidence then connect with a branch of *C. mrakii*, *C. saturnus*, *C. suaveolens*, *C. subsufficiens* and *C. sargentensis* (Figure 4.5).

Table 4.4 The sequence divergence of the D1/D2 LSU rDNA (upper triangle) and ITS1-2 region (lower triangle) of strain JP52[T] and their related species, *Cyber. mengyuniae, Cyber. mrakii, Cyber. saturnus, Cyber. sargentensis, Cyber. suaveolens, Cyber. subsufficiens, Cyber. meyerae, Cyber. vartiovaarae* and *Cyber. jadinii*. The numbers in front of the parenthesis are indicating substitutions and numbers in parenthesis are the number of indels.

	JP52	Cyber .men	Cyber. mra	Cyber. sat	Cyber. sar	Cyber. sua	Cyber. sub	Cyber. mey	Cyber. var	Cyber. jad
JP52	0	16 (7)	25 (7)	27 (12)	25 (7)	27 (12)	27 (7)	29 (14)	31 (13)	32 (4)
Cyber. mengyuniae	28 (49)	0	36 (74)	36 (69)	38 (69)	36 (69)	32 (74)	52 (69)	40 (38)	43 (80)
Cyber. mrakii	36 (127)	14 (5)	0	1 (0)	0	1(0)	4 (0)	19 (2)	12 (1)	48 (5)
Cyber. saturnus	40 (114)	13 (5)	2 (1)	0	0	0	4 (0)	20 (2)	13 (1)	48 (5)
Cyber. sargentensis	37 (122)	13 (5)	7 (1)	5 (2)	0	0	4 (0)	20 (2)	13 (1)	48 (5)
Cyber. suaveolens	40 (114)	13 (5)	3 (1)	1 (0)	4 (2)	0	4 (0)	20 (2)	13 (1)	48 (5)
Cyber. subsufficiens	38 (117)	16 (5)	6 (4)	4 (3)	5 (5)	5 (3)	0	21 (2)	11 (1)	50 (5)
Cyber. meyerae	57 (94)	18 (5)	53 (75)	53 (74)	51 (70)	54 (74)	51 (67)	0	77 (5)	132 (26)
Cyber.vartiovaarae	44 (69)	20 (6)	23 (17)	21 (22)	20 (26)	21 (22)	19 (22)	46 (47)	0	133 (15)
Cyber.jadinii	42 (92)	33 (8)	48 (114)	50 (113)	49 (111)	50 (111)	49 (116)	55 (88)	47 (85)	0
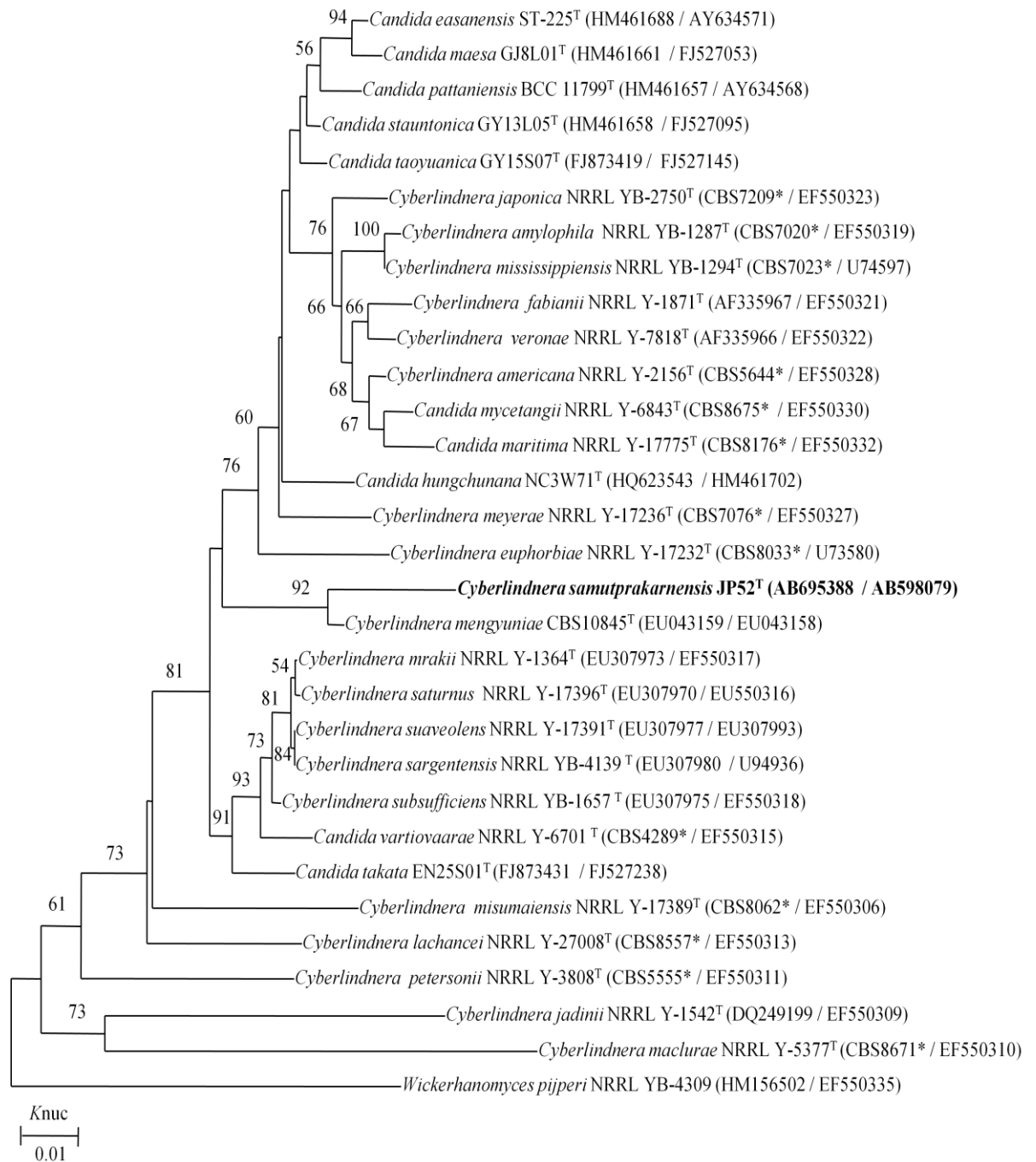
Figure 4.5 NJ-based phylogenetic tree for *Cyberlindnera samutprakarnensis* nov. (JP52^T) based on the concatenated ITS and D1/D2 region of the LSU rRNA gene sequence. *Wickerhanomyces pijperi* NRRL YB-4309^T is used as the outgroup. The numerals at each node represent the percentages from 1,000 replicate bootstrap resamplings (excluded when < 50%). Sequences were retrieved from the NCBI Genbank databases and CBS database (*). *Bar* 0.01 substitutions per nucleotide position.

Description of *Cyberlindnera samutprakarnensis* sp. nov. JP52[T]

After growth in YM broth for three days at 25 °C, cells in the sediment are ovoid to ellipsoidal, 2-4 x 4-8 μm, and occur singly, in pairs and sometimes arranged in chain, budding or in pairs (Figure 4.6A and 6B). Flocculent and sediment were formed after 3 days. Growth on YM agar after one month at 25 °C, the streak culture is whitish to cream-colored, butyrous, circular, smooth, glistening and convex with entire margins. A pellicle is not present during growth on the surface of assimilation medium. Pseudomycelium and true mycelium are not formed on Dalmau plates on corn meal agar (CMA) after 14 days at 25 °C. Ascospores did not produce on 5% malt extract agar, Fowell's acetate agar and YM agar after 28 days at 25 °C. The major ubiquinone is Q-7.
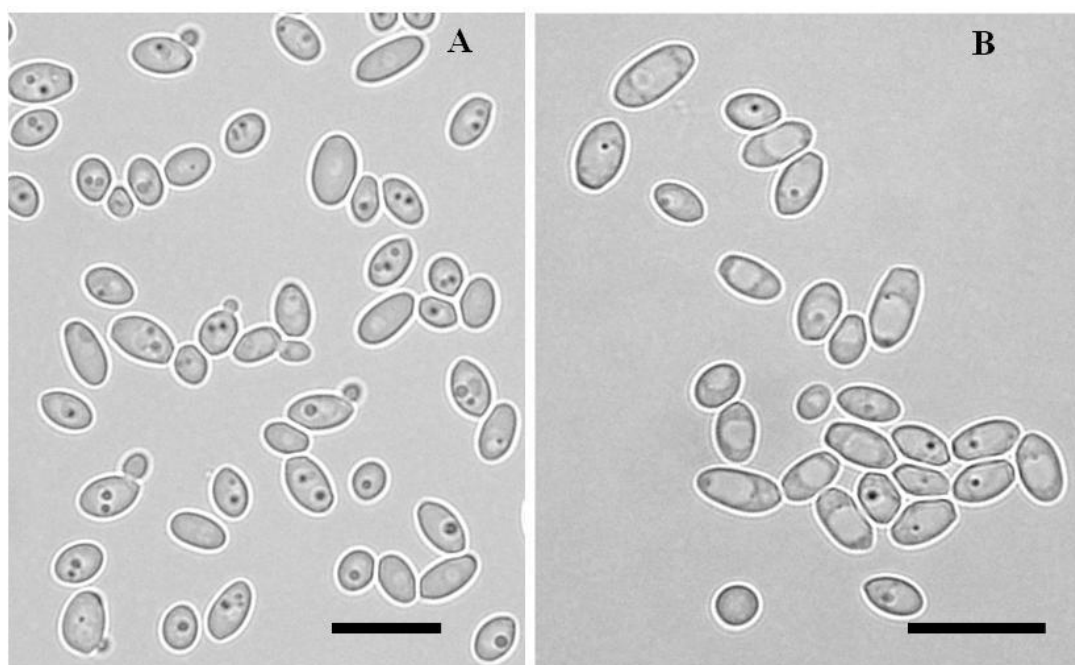
Figure 4.6 Morphology of vegetative cells of *Cyberlindnera samutprakarnensis* JP52[T]

A: in sediment grown in YM broth for 3 days at 25 °C.

B: grown in YM agar for 28 days at 25 °C.

Scale bars=10μm

Physiological characteristics of *Cyberlindnera samutprakarnensis* sp. nov. JP52[T]

Carbohydrate fermentation:

Glucose	+	Lactose	-
Galactose	-	Melibiose	-
Maltose	-	Raffinose	+ (weak)
Sucrose	+	Fructose	+

Carbon compounds assimilation:

Glucose	+	Glycerol	+
Galactose	-	Erythritol	-
L-Sorbose	-	Ribitol	-
Sucrose	+	Galactitol	-
Maltose	+	D-Mannitol	+
Cellobiose	+	D-Glucitol	+
Trehalose	+	Xylitol	-
Lactose	+ (slow)	L-Arabinitol	-
Melibiose	+ (slow)	Inositol	-
Raffinose	+	Methyl-α-D-glucoside	+ (slow)
Melezitose	+	Salicin	+
Inulin	+	Glucono-δ-lactone	+
Soluble starch	+	D-Gluconic acid	-
D-Xylose	+	2-Ketogluconic acid	+ (slow)
L-Arabinose	-	5-Ketogluconic acid	-
D-Arabinose	-	DL-Lactic acid	-
D-Ribose	+ (slow)	Succinic acid	+
L-Rhamnose	+	Citric acid	+
D-Glucosamine	-	D-Glucuronic acid	-
N-Acetyl-D-glucosamine	-	D-Galacturonic acid	-

Methanol	-	Propane-1,2-diol	+
Ethanol	+	Butane-2,3-diol	+

Nitrogen compounds assimilation:

Potassium nitrate	+	L-Lysine	+
Sodium nitrite	+	Cadaverine	+
Ethylamine	+		

-, Negative; +, positive; d, delayed positive (after 14 d or more); w, weak; -, +, variable.

Other characteristics:

Vitamins required:	Biotin & thiamine
Production of starch-like substances:	Negative
Growth on 50% glucose agar slant:	Negative
0.1% cycloheximide resistance:	Negative
Maximum growth temperature:	42 °C
Liquefaction of gelatin:	Negative
Acid production on chalk agar:	Positive
Urease:	Negative

Phenotypic comparison of strain JP52[T] with closely related species demonstrated that it differed from *Cyberlindnera mengyuniae* in their ability to assimilate maltose, cellobiose, melezitose, soluble starch, rhamnose, salicin and succinic acid and from *Cyberlindnera mrakii* as shown in Table 4.5. It could grow at 40 and 42 °C but not at 45 °C whilst *Cyberlindnera mrakii* probably grew at 37 °C.

Table 4.5 Differential phenotypic characteristics of *Cyberlindnera samutprakarnensis* JP52^T and their closely related species.

Characteristic	*Cyber. samutprakarnensis*	*Cyber mengyuniae**	*Cyber. mrakii**
Assimilation of:			
galactose	-	+	-
sucrose	+	+	-
maltose	+	-	V
cellobiose	+	-	+
trehalose	+	+	-
melezitose	+	-	V
inulin	+	+	-
soluble starch	+	-	-
L-rhamnose	+	-	V
salicin	+	-	+
succinic acid	+	-	+
citric acid	+	+	-

+, Positive; V, variable; -, negative.

* Data for *Cyberlindnera mengyuniae* were taken from Chen et al. (2009). *Cyberlindnera mrakii* were taken from Kurtzman et al (2011).

Based on the results mentioned above, they supported doubtlessly that JP52 was a new species in genus *Cyberlindnera*. It is described as *Cyberlindnera samutprakarnensis* sp. nov. JP52^T. The MycoBank number is MB800879.

Cyberlindnera samutprakarnensis (sa.mut.pra.karn.en´ sis. N.L. fem.adj. *samutprakarnensis* referring to Samutprakarn, Thailand, where this strain was isolated). Holotype is strain JP52^T, isolated from the wastewater of a cosmetic factory (Milott laboratory Company Ltd.), collected in Bangplee, Samutprakarn Prov., Thailand, in Feb. 2009, by Jamroonsri Poomtien. It was deposited at (i) the BIOTEC Culture Collection (BCC), National Center for Genetic Engineering and Biotechnology (BIOTEC), Pathumthani, Thailand, as BCC 46825^T; (ii) the Japan Collection of Microorganisms (JCM), RIKEN BioResource Center, Hirosawa,

Wako, Saitama, Japan, as JCM 17816[T], and (iii) Centraalbureau voor Schimmelcutures (CBS), Utrecht, The Netherlands, as CBS 12528[T]. The strain is maintained by freezing and/or lyophilization.

Etymology: The specific epithet "*samutprakarnensis*" was derived from "Samutprakarn Province", where a sample of wastewater containing *C. samutprakarnensis* was collected.

4.1.4.2 *Candida thasaenensis* sp. nov. JP59[T] and JP60

Yeast strain JP59[T] and JP60 isolated from a soil sediment pond of a palm oil biodiesel production plant (Chumporn Palm Oil industry), were collected in Thasae District, Chumporn Province, Thailand. Each discrete colony of both isolates grew on acidified YM broth containing 1% (v/v) palm oil when cultivated by using dilution plating technique. In accordance with sequence comparison results of D1/D2 region and ITS1-2 region, strains JP59[T] and JP60 were conspecific species that had identical sequences of D1/D2 region and ITS1-2 region. Two strains were closely related with *Scheffersomyces spartinae* as the closet related species, so they differed from 0.9% sequence divergence (5 substitutions, 2 indels) of D1/D2 region and 1.02% sequence divergence (6 substitutions, 0 indels) of ITS1-2 region. The nucleotide substitutions of D1/D2 domain include the ITS region of JP59 and JP60 was slightly differed from closely related species thus the other conserved DNA region such as actin gene and EF gene was also further determined. The pairwise sequence analysis of actin gene *ACT1* and transitional elongation factor gene *EF2* of both *Scheffersomyces spartinae* and *Candida gosingica* with strains JP59[T] provided better supportive information to resolve the novel species and confirm distinct status. Strains JP59[T] and JP60 were differed from both *Scheffersomyces spartinae* and *Candida gosingica*, 6.5% sequence divergence (35 substitutions, 0 indels) and 4.8% sequence divergence (26 substitutions, 0 indels), respectively in the actin gene and translational elongation factor gene. The sequences data of JP59[T] and JP60 analyzed in this study were deposited in GenBank with the accession numbers shown in Table 4.6.

Table 4.6 The accession numbers of DNA region from strain JP59[T] and JP60

DNA region	Strain NO. (accession numbers)	Strain NO. (accession numbers)
D1/D2 domain	JP59[T] (AB598080)	JP60 (AB686643)
ITS region	JP59[T] (AB686644)	JP60 (AB686645)
actin gene	JP59[T] (AB742430)	JP60 (AB742431)
EF gene	JP59[T] (AB742433)	JP60 (AB742434)

Their phylogenetic position was supported by the phylogenetic trees construction based on the internal transcribed spacer (ITS1/ITS2) region and D1/D2 region gene sequences. Phylogenetic analysis based on the concatenated sequences of ITS and D1/D2 region showed that strains JP59[T] and JP60 were single species which phylogenetically related to *Scheffersomyces spartinae* and *Candida gosingica* with a high bootstrap confidence (Figure 4.7). Overall results of multiple gene analysis among four DNA regions provided confidentially to suggest a new species of *Candida thasaenensis* nov. for JP59[T] and JP60.
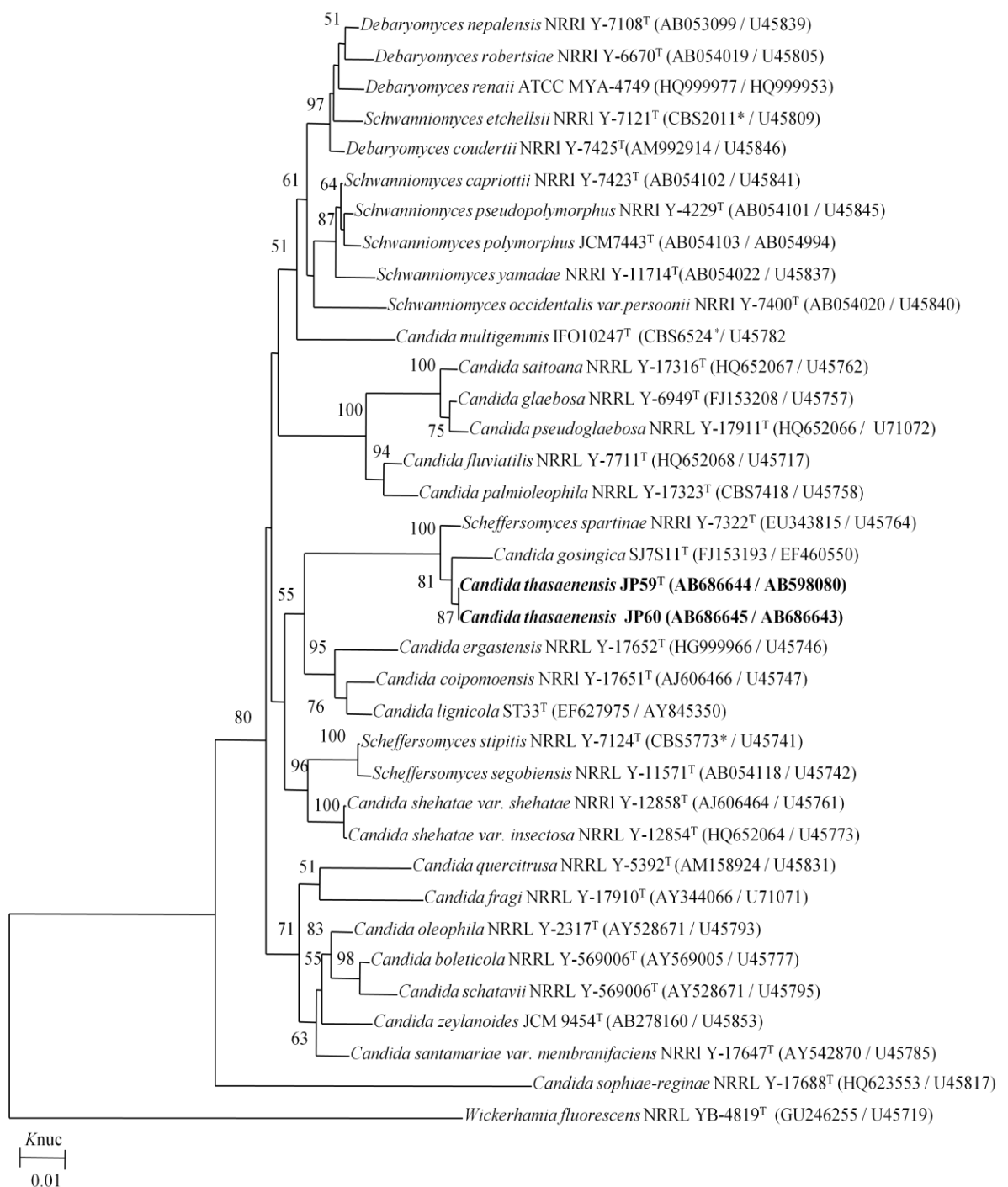
Figure 4.7 NJ-based phylogenetic tree for *Candida thasaenensis* sp. nov. (JP59^T, JP60) based on the concatenated ITS and D1/D2 region of the LSU rRNA gene sequence. *Wickerhamia fluorescens* NRRL YB-4819^T is used as the outgroup. The numerals at each node represent the percentages from 1,000 replicate bootstrap resamplings (excluded when < 50%). Sequences were retrieved from the NCBI Genbank databases and CBS database (*). *Bar* 0.01 substitutions per nucleotide position.

Description of *Candida thasaenensis* sp. nov. (JP59[T], JP60)

After growth in YM broth for three days at 25 °C, cells in sediment are spheroidal, ovoidal shape (2.7-4.7 x 3.3-6.0 μm), or ellipsoidal and elongate (2.7-3.3 x 6.7-21.3 μm) in shape, occur singly, in pair or form in chains (Figure 4.8A and B). Pseudomycelium is well developed. Growth on YM agar after one month at 25 °C, the streak culture is pale yellowish. Membranous growth generally results from profuse formation of filaments. Yeast colonies are raised, moderately rough, dull, and fringed with pseudomycelium. A pellicle is not present during growth on the surface of assimilation medium. In Dalmau plates after seven days on slide culture on CMA, potato dextrose agar (PDA) and YM agar at 25 °C, pseudomycelium is abundant on these three media but true hypha do not develop. The extensive pseudomycelium as Candida-type consists of spheriodal blastospores singly and in short chains are formed on media CMA and YM, and form clusters like mycotoruloides-type on PDA (Fig. 4.8 C-E). Ascospores are not produced on 5% malt extract agar, Fowell's acetate agar and YM agar after 28 days at 25 °C. The major ubiquinone is Q-9.
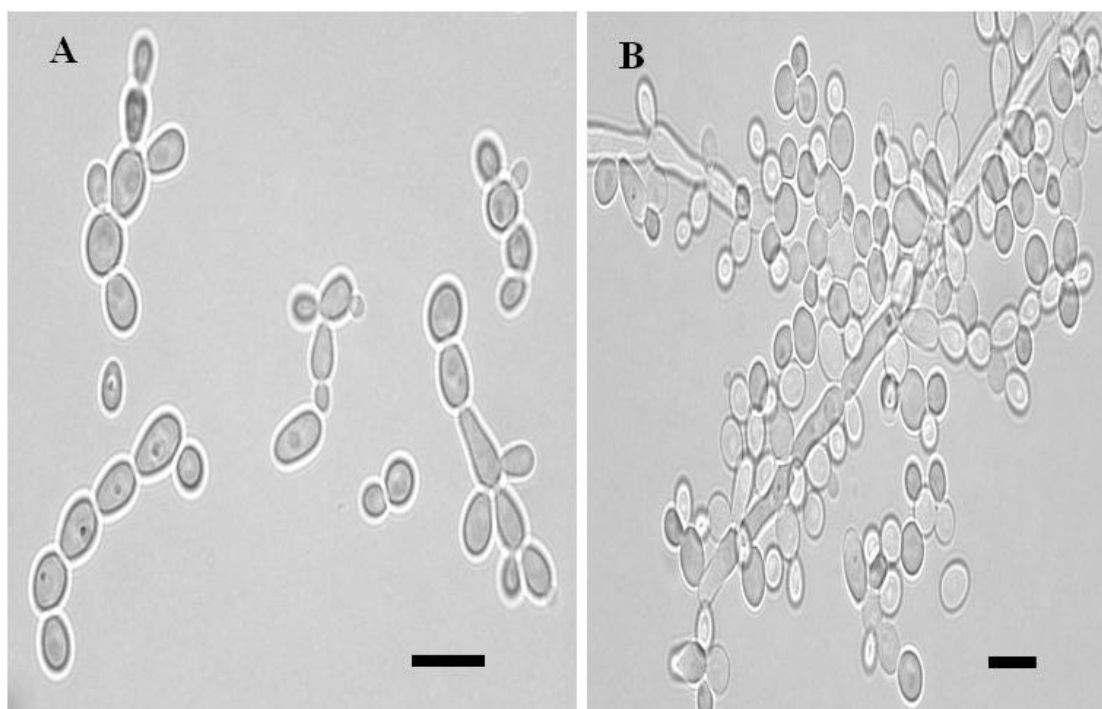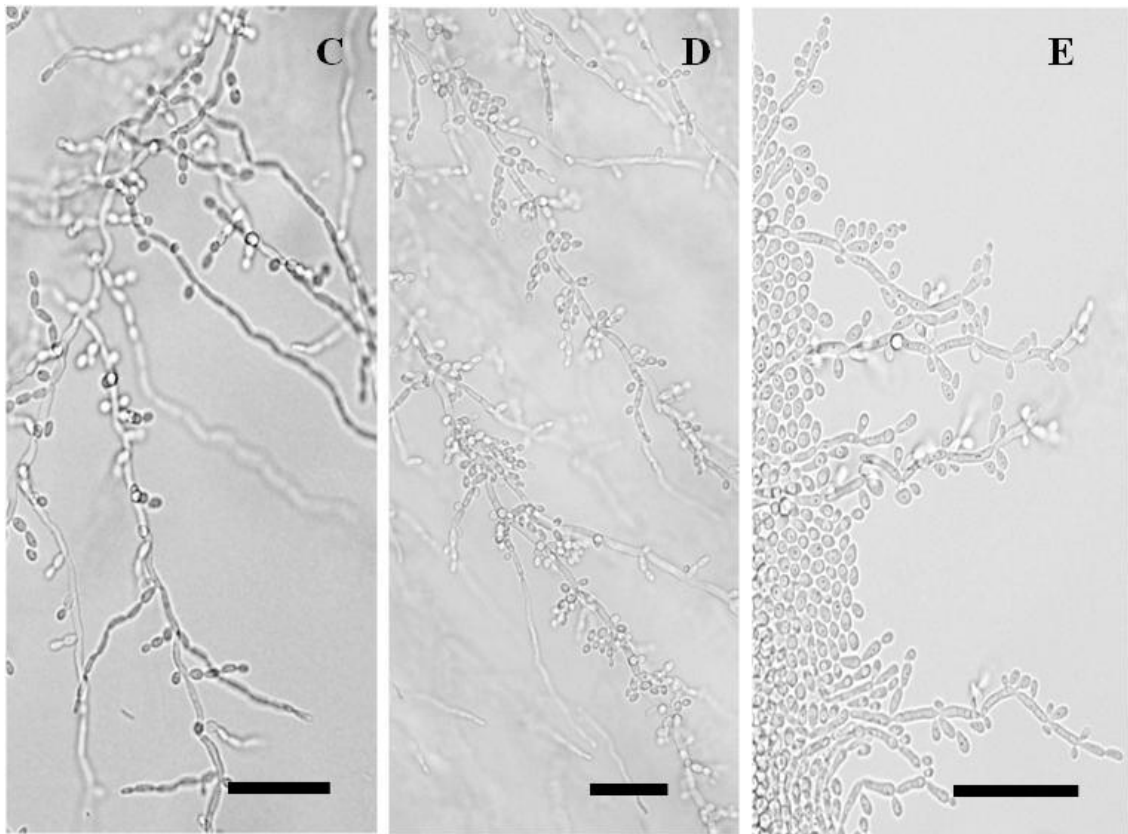
Figure 4.8 Morphology of *Candida thasaenensis*

A: Vegetative cells of *C. thasaenensis* JP59^T grown in YM broth for 3 days at 25 °C.

B: Vegetative cells of *C. thasaenensis* JP60 grown in YM broth for 3 days at 25 °C.

Scale bars=10μm

C-E: Pseudomycelia of *C. thasaenensis* JP59^T developed on slide culture with CMA, PDA and YM agar, respectively after 7 days at 25 °C.

Scale bars=30μm

Physiological characteristics of *Candida thasaenensis* sp. nov. JP59^T, JP60

Carbohydrate fermentation:

Glucose	+	Lactose	-
Galactose	-	Melibiose	-
Maltose	-	Raffinose	-
Sucrose	+(weak)	Fructose	+

Carbon compounds assimilation:

Glucose	+	Glycerol	+
Galactose	-	Erythritol	-
L-Sorbose	-	Ribitol	+
Sucrose	+	Galactitol	-
Maltose	+	D-Mannitol	+
Cellobiose	+	D-Glucitol	+ (slow)
Trehalose	+	Xylitol	+
Lactose	-	L-Arabinitol	-
Melibiose	-	Inositol	-
Raffinose	-	Methyl-α-D-glucoside	+
Melezitose	+	Salicin	+
Inulin	-	Glucono-δ-lactone	+ (slow)
Soluble starch	+ (latent)	D-Gluconic acid	-
D-Xylose	-	2-Ketogluconic acid	+
L-Arabinose	-	5-Ketogluconic acid	-
D-Arabinose	-	DL-Lactic acid	+ (slow)
D-Ribose	-	Succinic acid	+
L-Rhamnose	+ (slow)	Citric acid	+
D-Glucosamine	-	D-Glucuronic acid	-
N-Acetyl-D-glucosamine	-	D-Galacturonic acid	-

Methanol	-	Propane-1,2-diol	+
Ethanol	+	Butane-2,3-diol	-

Nitrogen compounds assimilation:

Potassium nitrate	-	L-Lysine	+
Sodium nitrite	-	Cadaverine	+
Ethylamine	+		

-, Negative; +, positive; d, delayed positive (after 14 d or more); w, weak; -, +, variable.

Other characteristics:

Vitamins required:	Biotin & thiamine
Production of starch-like substances:	Negative
Growth on 50% glucose agar slant:	Positive
0.1% cycloheximide resistance:	Negative
Maximum growth temperature:	37 °C
Liquefaction of gelatin:	Negative
Acid production on chalk agar:	Positive
Urease:	Negative

Phenotypic characteristics of strain JP59[T] compared with closely related known yeast as *Scheffersomyces spartinae* and *Candida gosingica* resulted that it was distinguished from *Scheffersomyces spartinae,* the most closely related species, by its ability to assimilate L-rhamnose and that it could grow on 50% (w/v) glucose and from *Candida gosingica,* by its ability to assimilate L-rhamnose, glycerol and citrate, its ability to grow at 37 °C but not at 40 °C and its inability to assimilate L-sorbose, 0.1% cycloheximide and could not ferment maltose. Both species were well separated from other species on the basis of these and other growth characteristics (Table 4.7).

Table 4.7 Differential phenotypic characteristics of *Candida thasaenensis sp*. nov., and their closely related species.

Characteristics	*Candida thasaenensis*	*Scheffersomyces spartinae**	*Candida gosingica**
Fermentation of maltose	-	w/-	+
Assimilation of:			
L-sorbose	-	+	+
soluble starch	D	-	-
L-rhamnose	+	-	-
glycerol	+	S	-
citric acid	+	+	-
Growth on 50% glucose	+	-	+
Resistance of 0.1% cycloheximide	-	-	+
Growth at/with 37 °C	+	-	-

+, Positive; D, delayed; S, slow; -, negative; ND, not determined.

* Data for *Candida gosingica* were taken from Chang et al. (2011).

Scheffersomyces spartinae were taken from Kurtzman et al (2011).

With respect to the molecular and physiological characteristics described above, the results demonstrated that strain JP59[T] and JP60 represent novel species, then both strains were proposed as *Candida thasaenensis* sp. nov. (MycoBank No. MB800880) with JP59[T] as the type strain.

Candida thasaenensis (tha.sae.nen.sis. N.L. fem. adj. *thasaenensis* referring to Thasae District, Chumporn Province in Thailand, where two strain were isolated). Holotype is strain JP59[T], isolated from a soil sediment pond of a palm oil biodiesel production plant (Chumporn Palm Oil industry), collected in Thasae, Chumporn Prov., Thailand, in May 2009, by Jamroonsri Poomtien. It was deposited at (i) the BIOTEC Culture Collection (BCC), National Center for Genetic Engineering and Biotechnology (BIOTEC), Pathumthani, Thailand, as BCC 46828[T], (ii) Japan Collection of Microorganisms (JCM), RIKEN BioResource Center, Hirosawa, Wako, Saitama, Japan, as JCM 17817[T], and (iii)

Centraalbureau voor Schimmelcutures (CBS), Utrecht, The Netherlands, as CBS 12529^{T}. The strain is maintained by freezing and/or lyophilization.

Etymology: The specific epithet "*thasaenensis*" was chosen because this species was isolated from Thasae District, Chumporn Province.

4.2   Optimization conditions for growth and biosurfactant production

4.2.1   Selection for yeast strains having high biosurfactant producing ability

Selections of yeast strains were obtained from their ability to produce biosurfactant with stable activity. For selection of an efficient biosurfactant producing yeast, strain JP11, JP30, JP39, JP52, JP59, and JP70 from this study and *Pichia anomala* PY1 (Thaniyavarn et al., 2008) were selected for further optimization condition for cultivation and biosurfactant production as described in section 3.1. These six strains including *Pichia anomala* PY1, a reference strain, were tested by cultivating in a flask containing medium supplemented with either glucose or palm oil or both compounds as carbon sources. Palm oil is vegetable oil and supply abundantly in Thailand including the South East Asia and has previously reported for biosurfactant production; therefore it is interesting to be a carbon substrate. After 7 days of cultivation, cell biomass was determined from dry cell weight and the cell free culture broth was analyzed for pH value and biosurfactant activity by surface tension reduction and oil displacement tests. The results were shown in Table 4.8 with three different media including YM broth, modified Hua's medium supplemented with 10% palm oil (10% PO) and modified Hua's medium supplemented with 2% glucose plus 2% palm oil (2% GP) were used.

Table 4.8 Growth and biosurfactant activity of six yeast strains and PY1, a reference strain, cultivated for 7 days in 3 different media: (A) YM broth, (B) modified Hua's medium supplemented with 10% palm oil and (C) modified Hua's medium supplemented with 2% glucose and 2% palm oil (2% GP).

(A) YM broth

Yeast strain	Cell dry weight (g L^{-1})	Surface tension (mN m^{-1})	Δ Surface tension[a] (mN m^{-1})	ODA[e] of supernatant (cm^2)	pH value
JP11	6.98 ± 0.01	47.3 ± 0.6	5	0.50 ± 0.05	6.98 ± 0.07
JP 30	7.52 ± 0.03	43.0 ± 0.5	9	0.39 ± 0.03	6.93 ± 0.10
JP 39	4.54 ± 0.01	48.0 ± 0.8	4	0.50 ± 0.05	7.20 ± 0.05
JP 52	5.14 ± 0.01	45.3 ± 0.6	7	0.59 ± 0.05	6.12 ± 0.03
JP 59	5.3 ± 0.01	45.0 ± 0.5	7	0.59 ± 0.03	4.84 ± 0.06
JP 70	7.46 ± 0.03	46.0 ± 0.4	6	0.64 ± 0.10	6.74 ± 0.11
PY1[d]	5.00 ± 0.01	41.3 ± 0.5	11	0.59 ± 0.03	6.80 ± 0.05

[a] Surface tension value of YM broth at initial was 52.0 mN m^{-1}

(B) Modified Hua's medium supplemented with 10% palm oil (10%PO)

Yeast strain	Cell dry weight (g L^{-1})	Surface tension (mN m^{-1})	Δ Surface tension[b] (mN m^{-1})	ODA[e] of supernatant (cm^2)	pH value
JP 11	7.6 ± 0.03	46.6 ± 2.3	10	0.17 ± 0.05	4.46 ± 0.09
JP 30	7.0 ± 0.02	52.2 ± 0.4	4	0.15 ± 0.05	4.39 ± 0.07
JP 39	4.9 ± 0.01	45.8 ± 2.2	11	1.61 ± 0.21	3.51 ± 0.03
JP 52	4.6 ± 0.01	40.6 ± 0.6	16	2.41 ± 0.42	6.39 ± 0.06
JP 59	4.7 ± 0.01	43.8 ± 1.7	13	0.52 ± 0.13	4.13 ± 0.14
JP 70	7.4 ± 0.01	52.2 ± 0.4	4	0.55 ± 0.05	4.57 ± 0.06
PY1[d]	7.6 ± 0.02	30.5 ± 0.6	26	2.74 ± 0.42	6.66 ± 0.05

[b] Surface tension value of medium supplemented with 10 % palm oil at initial was 56.3 mN m^{-1}

(C) Modified Hua's medium supplemented with 2% glucose and 2% palm oil (2%GP)

Yeast strain	Cell dry weight (g L^{-1})	Surface tension (mN m^{-1})	Δ Surface tension[c] (mN m^{-1})	ODA[e] of supernatant (cm^2)	pH value
JP 11	2.95 ± 0.01	36.3 ± 0.8	17	0.48 ± 0.04	3.06 ± 0.06
JP 30	3.42 ± 0.01	44.7 ± 1.1	9	0.24 ± 0.05	3.71 ± 0.06
JP 39	5.12 ± 0.02	38.8 ± 2.6	15	0.27 ± 0.17	3.46 ± 0.06
JP 52	5.3 ± 0.12	33.3 ± 1.3	20	0.46 ± 0.05	7.72 ± 0.02
JP 59	7.11 ± 0.01	38.5 ± 0.5	15	0.32 ± 0.03	3.62 ± 0.03
JP 70	3.07 ± 0.00	36.5 ± 2.4	17	0.35 ± 0.05	2.85 ± 0.03
PY1[d]	11.18 ± 0.01	34.2 ± 0.4	19	0.52 ± 0.08	8.00 ± 0.11

[c] Surface tension value of medium supplemented with GP at initial was 53.3 mN m^{-1}

[d] *Pichia anomala* PY1 was used as reference strain.

[e] Oil displacement area of supernatant when cultured in each medium at 7 days.

Error values indicated the standard deviation from triplicate determinations.

Biosurfactant activities in term of the surface tension reduction (ΔST) of the six strains grown in different media were compared while *Pichia anomala* PY1, the efficient biosurfactant producing yeast reported earlier (Thaniyavarn et al., 2008), was used as a reference strain (Figure 4.9). It was found that most of the new isolated yeast strains except for PY1 showed higher ΔST values when grown in 2% GP than those of the other media. Among the six tested strains, JP52 showed the highest ΔST values of 20.0 mN m^{-1} and 16.0 mN m^{-1} when grown in modified Hua's medium supplemented with 2% GP and medium supplemented with 10% PO, respectively. Whereas *Pichia anomala* PY1 showed strongest biosurfactant activity with ΔST values of 25.8 mN m^{-1} when grown in the medium supplemented with 10% PO and 19.1 mN m^{-1} when grown in the medium supplemented with 2% GP. Therefore among the six new isolates, JP52 was selected for further optimization for cultivation and biosurfactant production conditions. JP52 was reported in the early part of the present study as a novel species of yeast and it was named as *Cyberlindnera samutprakarnensis* JP52[T].
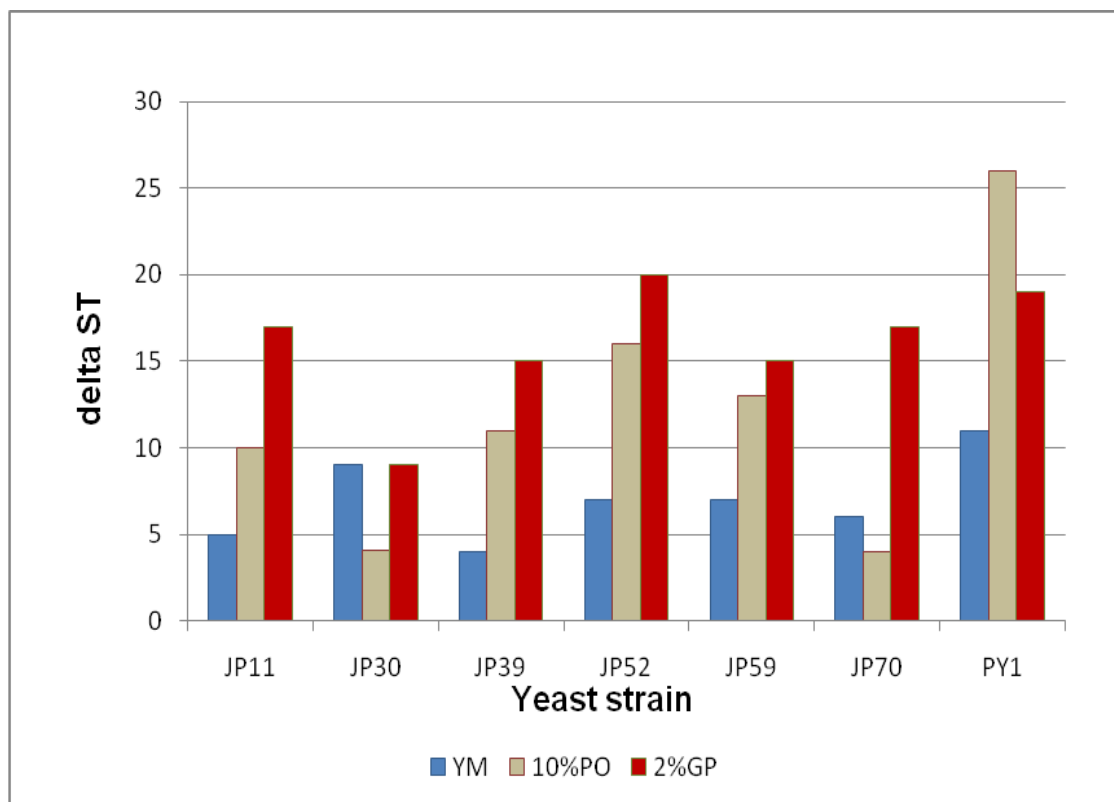
Figure 4.9 Comparison of biosurfactant activity of six isolates (JP11, JP 30, JP 39, JP 52, JP 59 and JP 70) including a reference yeast strain (PY1), when cultivated for 7 days in YM or modified Hua's medium with 10% palm oil (10%PO) or modified Hua's medium with 2% glucose and 2% palm oil (2% GP).

4.2.2 Nutritional requirements for optimum condition for growth and biosurfactant production by *Cyberlindnera samutprakarnensis* JP52

4.2.2.1 Effect of various carbon sources on growth and biosurfactant production

There were many evidences on the importance of carbon source and its concentration in the production of surface-active compounds by microbes (Desai and Banat, 1997). Therefore, the present work determined the effect of various carbon sources on biosurfactant production by *Cyberlindnera samutprakarnensis*. It was cultured in the presence of 2% (w/v) glucose or 2% (v/v) of either glycerol, palm oil or soybean oil as the sole carbon source or in the presence of a mixture of 2% (w/v) glucose with either 2% (v/v) of glycerol, palm oil or soybean oil supplemented in modified Hua's medium at initial pH of 5.5. Growth was carried out in shake flask as batch culture at 30 ºC. Table 4.9 showed

the incubation periods giving the maximum growth and the maximum biosurfactant activity.

Table 4.9 The effect of various carbon sources on the maximum growth and biosurfactant activity determined as min ST, ΔST and oil displacement area of *Cyberlindnera samutprakarnensis*.

Carbon type	Max. growth (at day)	DCW[a] (g l^{-1})	Min. ST (at day)	Min. ST (mN m^{-1})	Δ ST[b]	ODA[c] (cm^2)	pH	ST at initial (mN m^{-1})
Single carbon source at 2% (w/v) of:								
Glucose	3	7.15 ± 0.01	7	34.5 ± 0.7	25.8	0.27 ± 0.05	8.04 ± 0.02	60.3
Glycerol	3	8.44 ± 0.03	7	35.8 ± 0.7	17.5	0.39 ± 0.05	7.59 ± 0.08	53.3
Palm oil	3	0.49 ± 0.00	7	38.5 ± 2.1	6.50	0.18 ± 0.07	6.89 ± 0.1	45.0
Soybean oil	3	0.43 ± 0.00	7	39.9 ± 0.6	6.80	0.27 ± 0.02	6.87 ± 0.0	46.7
Mixed carbon source of 2% (w/v) glucose plus 2% (v/v) of:								
Glycerol	3	8.68 ± 0.01	7	37.8 ± 0.5	12.4	0.18 ± 0.02	7.99 ± 0.1	50.2
Soybean oil	3	6.44 ± 0.00	7	41.8 ± 1.2	12.9	0.28 ± 0.02	7.27 ± 0.1	54.7
Palm oil	3	7.01 ± 0.02	7	33.2 ± 1.7	20.0	0.46 ± 0.01	7.89 ± 0.0	53.3

[a] Dry cell weights at maximum growth.

[b] The difference of surface tension value of medium at initial and supernatant at day 7.

[c] Oil displacement area of supernatant when cultured in each medium at day 7.

Cyberlindnera samutprakarnensis grew well after 24 h of incubation in almost all media except for the medium with either palm oil or soybean oil as a sole carbon, then reached the maximum growth on day 3. Cell growth was highest in medium containing (2% (w/v) glucose plus 2% (v/v) glycerol, it grew fairly well with 2% (w/v) glucose or 2% (v/v) glycerol as sole carbon source (Table 4.9). But it had poor growth with vegetable oils such as palm oil or soybean oil as a carbon source (Figure 4.10).
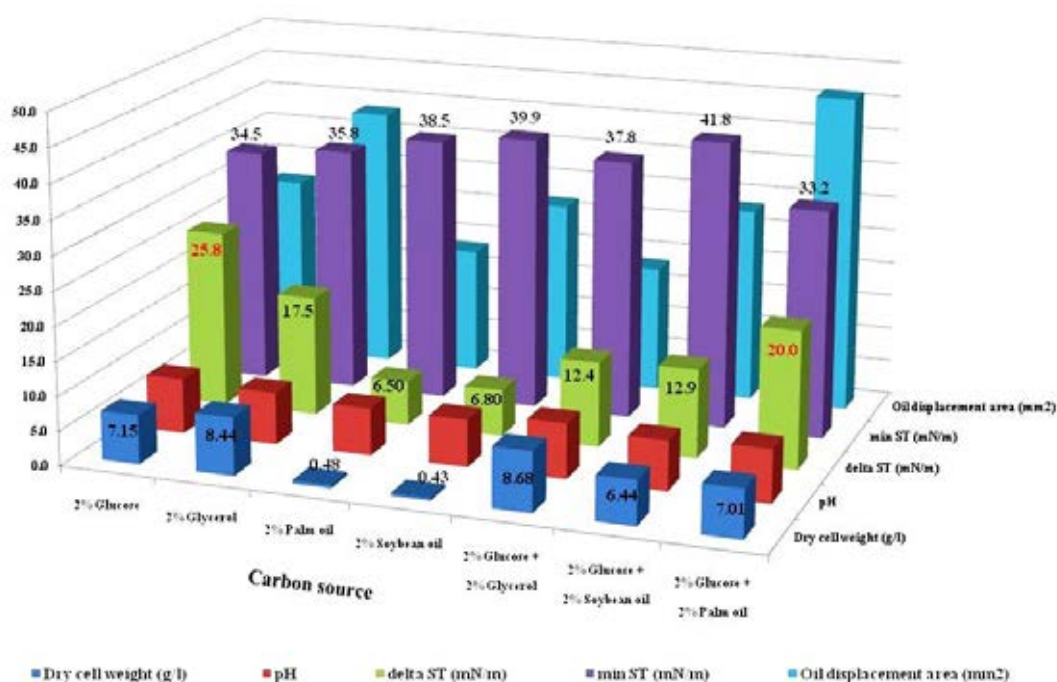
Figure 4.10 Maximum cell growth at day 3, maximum biosurfactant activity and pH of the cell free broth of *Cyberlindnera samutprakarnensis* grown in the presence of various carbon sources at day 7

For biosurfactant activity, *Cyberlindnera samutprakarnensis* cultivated in the presence of 2% glucose gave the highest ΔST value of 25.8 but oil displacement activity (ODA) was only 0.27 cm^2. Whereas cultivation in the presence of 2% glucose and 2% palm oil gave fairy high ΔST value of 20.0 and quite high ODA of 0.46 cm^2. The final pH values close to 8.0 were observed with the culture broths having high biosurfactant activities. From these results, the medium containing glucose and palm oil was then chosen for further study on optimization of the concentration of the carbon sources.

4.2.2.2 Effect of concentration of carbon source on growth and biosurfactant production

Production of biosurfactants by co-utilization of carbohydrates, especially glucose and vegetable oils has been found to be more effective on both their production and properties (Muthusamy et al., 2008). This study found that *Cyberlindnera samutprakarnensis* grown in the mixture of carbon sources (2% glucose and 2% palm oil) gave fairly good activity of biosurfactant. In this part,

further study on the effect of various glucose concentrations with the fixed amount of palm oil (2%) on biosurfactant production was investigated. The results were shown in Table 4.10. *Cyberlindnera samutprakarnensis* exhibited a relatively good growth in the presence of glucose concentrations between 2-20% (w/v) with 2% (v/v) palm oil. However, the maximum growth was found at day 9 in the presence of 10% (w/v) glucose which was higher than that of 15 and 20% (w/v) glucose (Figure 4.11A). It also showed that when *Cyberlindnera samutprakarnensis* was grown in the medium with 10% glucose or higher concentrations it could utilize the second carbon source to boost cell biomass as indicated by diauxic growth pattern. Whereas at low glucose concentrations, maximum growth was found on day 3 and day 5 when cultivated in the medium having 2% and 5% of glucose, respectively. It was observed that the pH values of the cultures (Figure 4.11B) were in near basic range (pH 7-8) when grown in the medium without glucose added or with 2%, 5% and 10% of glucose at late log phase while those in the medium with 15% and 20% of glucose were maintained in acidic pH throughout the cultivation period.

For biosurfactant activities, maximum activity of surface tension reduction with ΔST value of 21.0 mN m^{-1} (from 53.3 mN m^{-1} to 32.3 mN m^{-1}) was obtained from the cell free broth of 2% (w/v) glucose and 2% (v/v) palm oils at day 7 of cultivation (Figure 4.11C). The best oil displacement capacity value of 0.61 cm^2 was from cell free culture broth of 10% glucose plus 2% palm oil at day 3 of cultivation and the value of 0.46 cm^2 was obtained from the cell free broth of 2% (w/v) glucose and 2% (v/v) palm oils at day 7 (Figure 4.11D).

Table 4.10 The effect of different glucose concentrations and 2% (v/v) palm oil on the maximum growth and biosurfactant activity determined as min ST, ΔST and oil displacement area of *Cyberlindnera samutprakarnensis*.

Glucose conc. % (w/v)	Max. growth (at day)	DCW[a] (g l⁻¹)	min. ST (at day)	min ST (mN m⁻¹)	Δ ST[b]	ODA[c] (cm²)	pH	ST at initial (mN m⁻¹)
0	3	0.48 ± 0.00	7	38.5 ± 2.1	6.50	0.18 ± 0.07	6.89 ± 0.10	45.0
2	3	6.63 ± 0.02	7	32.3 ± 1.5	21.0	0.46 ± 0.01	7.72 ± 0.02	53.3
5	5	10.89 ± 0.03	3	39.6 ± 0.12	11.7	0.39 ± 0.05	6.13 ± 0.06	51.3
10	9	12.52 ± 0.04	3	39.2 ± 2.59	13.3	0.61 ± 0.07	4.47 ± 0.06	52.5
15	9	7.42 ± 0.01	3	38.9 ± 0.35	13.1	0.48 ± 0.16	4.53 ± 0.00	52.0
20	9	7.90 ± 0.04	3	38.1 ± 2.47	14.4	0.22 ± 0.05	4.61 ± 0.09	52.5

[a] Dry cell weights at maximum growth.

[b] The difference of surface tension value of medium at initial and supernatant at the maximum activity.

[c] Oil displacement area of supernatant when cultured in each medium at the maximum activity.
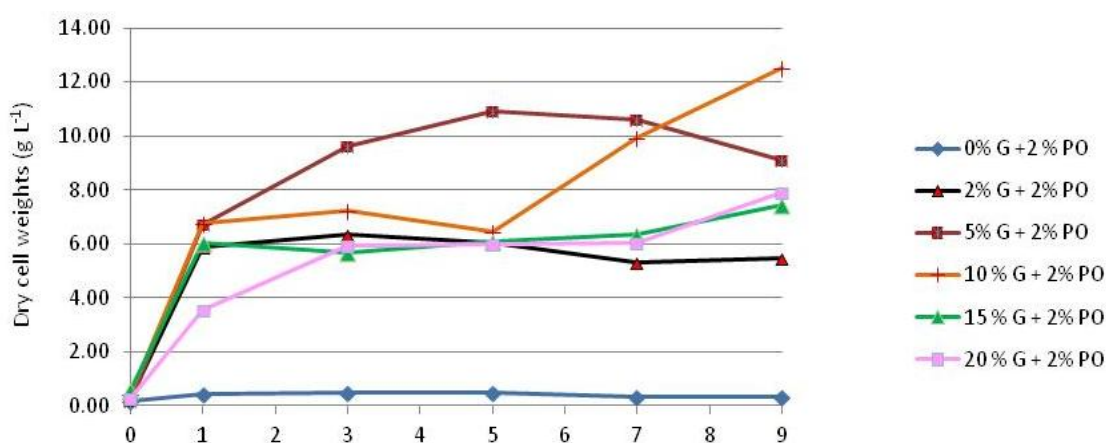
(A) Cell biomass
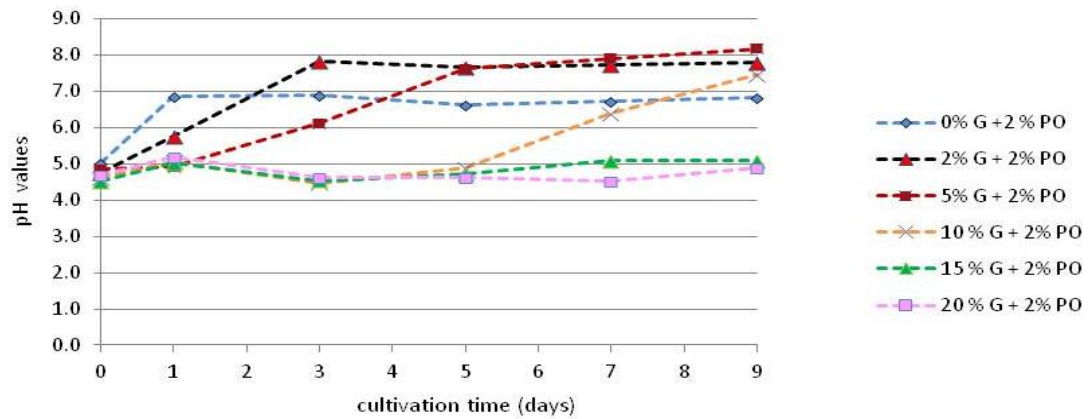
(B) pH values
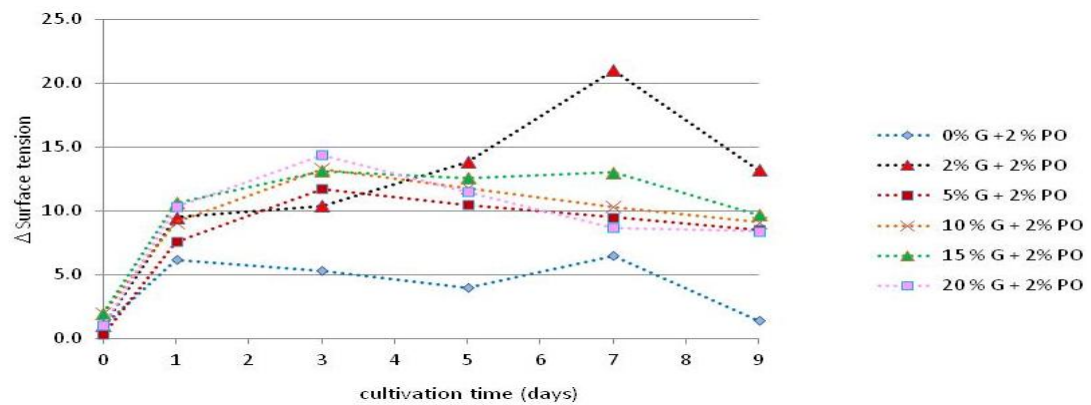
(C) Surface tension reduction (ΔST) values
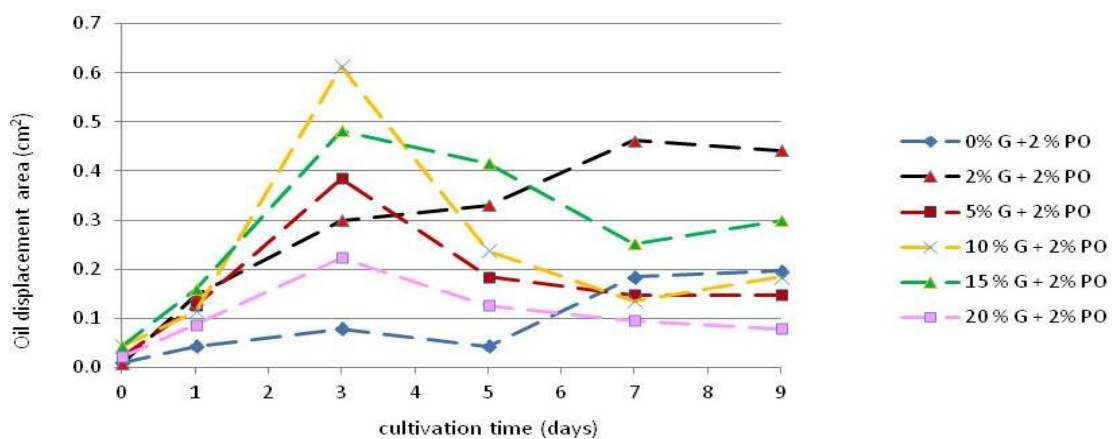
 (D) The oil displacement areas

Figure 4.11 The effect of different glucose concentration (G) and fixed 2 % (v/v) of palm oil (PO) of *Cyberlindnera samutprakarnensis* that presented of: cell biomass (A); pH values (B); surface tension reduction (ΔST) values (C); the oil displacement areas (D)

Furthermore the various concentration both glucose and palm oil were studied. The results were shown in Table 4.11 and Figure 4.12, *Cyber. samutprakarnensis* grew very well in the presence of glucose and palm oil concentrations at each 12.5% and showed diauxic growth after day 7 to boost cell growth at maximum on day 9. However, the ΔST value of biosurfactant obtained from medium containing 2% glucose and 2% palm oil gave the highest values among other concentrations.

Table 4.11 The effect of different glucose and palm oil concentrations on the maximum growth and biosurfactant activity determined as min ST, ΔST and oil displacement area of *Cyberlindnera samutprakarnensis*.

Glucose % (w/v)	Palm oil % (v/v)	Max. growth (at day)	DCW[a] (g l^{-1})	min. ST (at day)	min ST (mN m^{-1})	ΔST[b]	ODA[c] (cm^2)	pH	ST at initial (mN m^{-1})
1.25	1.25	1	4.93 ± 0.04	3	37.3 ± 1.0	9.7	0.41 ± 0.07	7.74 ± 0.10	47.0
2	2	3	6.63 ± 0.02	7	32.3 ± 1.5	21.0	0.46 ± 0.01	7.72 ± 0.02	53.3
2.5	2.5	3	7.03 ± 0.01	7	38.8 ± 0.35	11.2	0.47 ± 0.01	7.83 ± 0.04	50.0
12.5	12.5	9	11.03 ± 0.03	9	35.6 ± 0.12	13.2	0.46 ± 0.05	7.4 ± 0.06	48.8
25	25	3	8.14 ± 0.04	7	39.5 ± 2.59	8.7	0.35 ± 0.07	4.54 ± 0.06	48.2

[a] Dry cell weights at maximum growth.

[b] The difference of surface tension value of medium at initial and supernatant at the maximum activity.

[c] Oil displacement area of supernatant when cultured in each medium at the maximum activity.

Figure 4.12 Maximal cell growth, maximal biosurfactant activity and pH of cell free broth of *Cyberlindnera samutprakarnensis* grown in medium supplementing various glucose (G) and palm oil (PO) concentration.

From the above results, it was concluded that the medium with suitable carbon sources and concentrations was the medium supplemented with 2% (w/v) glucose and 2% (v/v) palm oil which will be named as GP medium. This medium showed maximum biosurfactant activity of the newly biosurfactant producing yeast *Cyber. samutprakarnensis* JP52[T].

4.2.2.3 Effect of various nitrogen sources on growth and biosurfactant activity

Various inorganic nitrogen sources at 0.4% (w/v) including $NaNO_3$, NH_4NO_3, $(NH_4)_2SO_4$ and NH_4Cl were added into GP medium to test their effects on biosurfactant production by *Cyberlindnera samutprakarnensis*. It was observed that cultivation in the presence of $NaNO_3$ showed highest surface tension reduction (Table 4.12, Figure 4.13) while similar oil displacement activities were obtained with those of $NaNO_3$ and NH_4Cl. However, $NaNO_3$ was selected as a nitrogen source for further concentration optimization as it gave highest ΔST value.

Table 4.12 The effect of different inorganic nitrogen sources on growth and biosurfactant activity determined as min ST, ΔST and oil displacement area of *Cyberlindnera samutprakarnensis* when cultured in GP medium

Nitrogen source	Max. growth (at day)	DCW[a] (g l^{-1})	min. ST (at day)	min ST (mN m^{-1})	ΔST[b]	ODA[c] (cm^2)	pH	ST at initial (mN m^{-1})
NH$_4$Cl	3	5.83 ± 0.01	7	35.0 ± 1.9	12.0	0.48 ± 0.02	2.0 ± 0.04	47.0
NH$_4$NO$_3$	7	6.88 ± 0.02	7	36.6 ± 1.3	5.60	0.40 ± 0.02	5.35 ± 0.06	42.2
(NH$_4$)$_2$SO$_4$	9	5.68 ± 0.01	7	38.3 ± 1.1	8.70	0.25 ± 0.05	2.5 ± 0.02	47.0
NaNO$_3$	3	7.01 ± 0.02	7	33.2 ± 1.7	20.0	0.46 ± 0.01	7.72 ± 0.02	53.3

[a] Dry cell weights at maximum growth.

[b] The difference of surface tension value of medium at initial and supernatant at the maximum activity.

[c] Oil displacement area of supernatant when cultured in each medium at the maximum activity.

Figure 4.13 Maximal cell growth, maximal biosurfactant activity and pH of cell free broth of *Cyberlindnera samutprakarnensis* grown in GP medium with various inorganic nitrogen sources.

4.2.2.4 Effect of NaNO₃ concentration on growth and biosurfactant activity

Different concentrations of NaNO₃ (0.2 – 0.6% w/v) were tested for their effect on growth and biosurfactant activity. The results in Figure 4.14 showed that NaNO₃ at the concentration higher than 0.4% (w/v) resulted in the decrease in biosurfactant activity in both the ΔST value and oil displacement area. Therefore, NaNO₃ at 0.4% (w/v) was the optimal concentration for cultivation of *Cyberlindnera samutprakarnensis* to give highest ΔST value and oil displacement area.

Figure 4.14 Maximal cell growth, maximal biosurfactant activity and pH obtained from cell free broth of *Cyberlindnera samutprakarnensis* grown in GP medium with various concentrations of NaNO₃.

4.2.3 Optimization condition of other factors affected on growth and biosurfactant production.

4.2.3.1 Effect of initial pH

The influence of initial pH of medium on growth and biosurfactant production of *Cyberlindnera samutprakarnensis* was investigated. For this experiment, GP medium with initial pH of 4.5, 5.0, 5.5 and 6.0 were used. After the first day of cultivation, pH values of all above had increased to 5.9-6.1

during exponential growth phase then gradually climbed up to 8.1-8.2 until late log phase after day 3 and further slightly decreased in stationary phase at day 7. Maximal cell growth and biosurfactant activity of *Cyberlindnera samutprakarnensis* grown in GP medium at various initial pH were shown in Table 4.13, Figure 4.15. The highest dry cell mass of 7.32 g l^{-1} was observed on day 3 when grown in GP medium at the initial pH of 5.5. It was also found that the maximum biosurfactant activity was found on day 7 with ΔST value of 21.9 mN m^{-1}, which reduced the medium-surface tension from 52.8 mN m^{-1} to 30.9 mN m^{-1}, when grown in GP medium at the same initial pH of 5.5.

Table 4.13   The effect of initial pH on growth and biosurfactant activity determined as min ST, ΔST and oil displacement area of *Cyberlindnera samutprakarnensis* when grown in GP medium

Initial pH	Max. growth (at day)	DCW[a] (g l^{-1})	min. ST (at day)	min ST (mN m^{-1})	ΔST[b]	ODA[c] (cm^2)	pH	ST at initial (mN m^{-1})
pH4.5	3	6.99 ± 0.02	7	44.0 ± 1.9	10.0	0.18 ± 0.07	7.82 ± 0.08	54.0
pH5.0	3	7.15± 0.00	7	42,0 ± 0.71	16.0	0.18 ± 0.02	7.92± 0.00	58.0
pH5.5	3	7.32 ± 0.05	7	30.9 ± 0.6	21.9	0.42 ± 0.05	7.61 ± 0.05	52.8
pH6.0	3	7.14 ± 0.01	7	44.5 ± 1.7	12.2	0.22 ± 0.01	7.78 ± 0.14	56.7

[a] Dry cell weights at maximum growth.

[b] The difference of surface tension value of medium at initial and supernatant at the maximum activity.

[c] Oil displacement area of supernatant when cultured in each medium at the maximum activity.
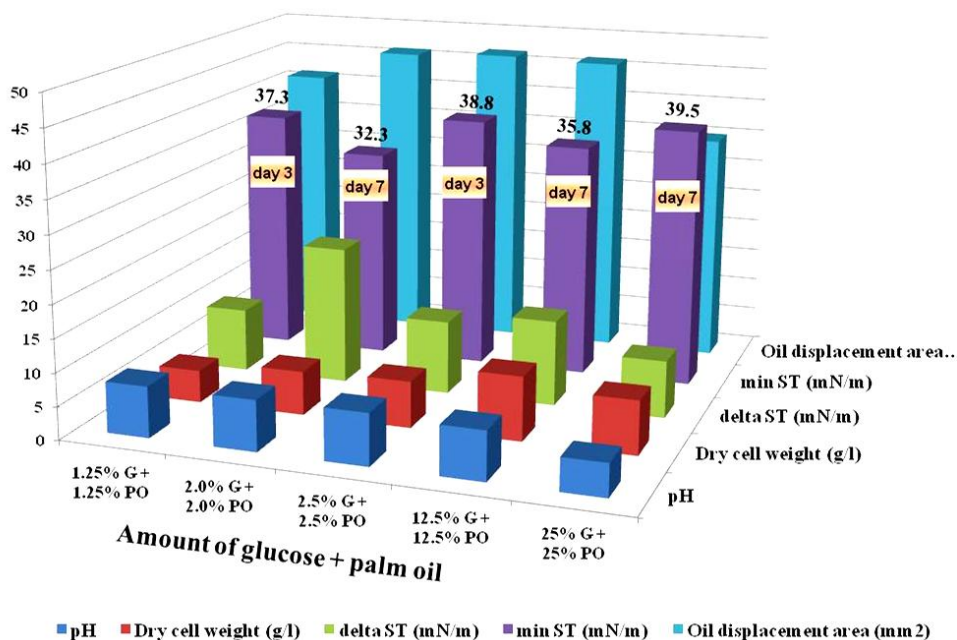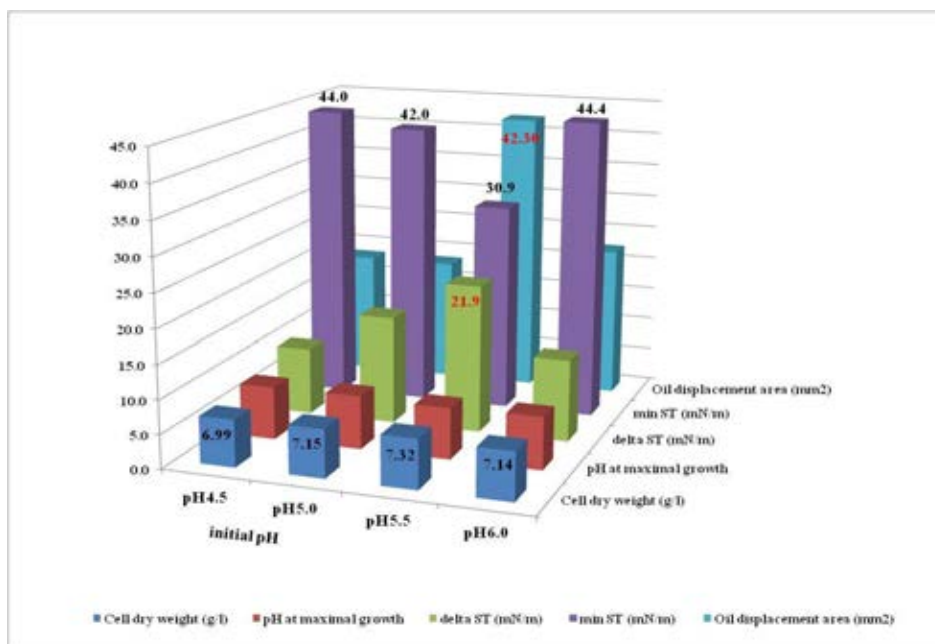
Figure 4.15 Maximal cell growth, maximal biosurfactant activity and pH obtained from cell free broth of *Cyberlindnera samutprakarnensis* when grown in GP medium at various initial pH.

4.2.3.2 Effect of temperature

The effect of temperature on growth and biosurfactant activity was studied by cultivating *Cyberlindnera samutprakarnensis* in GP medium at initial pH 5.5 but at various temperatures including 30, 35 and 40 ºC. Maximum growth was observed on day 3 at 30 ºC (Table 4.14, Figure 4.16) but low growth was found at 40 ºC. Good biosurfactant activities determined from surface tension reduction and oil displacement were also observed when grown at 30 ºC on day 7. At higher temperatures of 35 and 40 ºC, biosurfactant activities were lower and maximum activity was found on day 9 which was slower than that of at 30 ºC.

Table 4.14 The effect of various incubation temperatures on growth and biosurfactant activity determined as min ST, ΔST and oil displacement area of *Cyberlindnera samutprakarnensis*.

Temperature (°C)	Max. growth (at day)	DCW[a] (g l⁻¹)	min. ST (at day)	min ST (mN m⁻¹)	ΔST[b]	ODA[c] (cm²)	pH	ST at initial (mN m⁻¹)
30	3	7.32 ± 0.05	7	30.8 ± 0.6	20.1	0.42 ± 0.05	7.61 ± 0.05	50.7
35	3	6.62± 0.02	9	37.5 ± 1.75	13.2	0.30 ± 0.02	7.84± 0.02	50.7
40	3	4.63 ± 0.01	9	36.8 ± 1.7	13.9	0.24 ± 0.01	6.91 ± 0.10	50.7

[a] Dry cell weights at maximum growth.

[b] The difference of surface tension value of medium at initial and supernatant at the maximum activity.

[c] Oil displacement area of supernatant when cultured in each medium at the maximum activity.

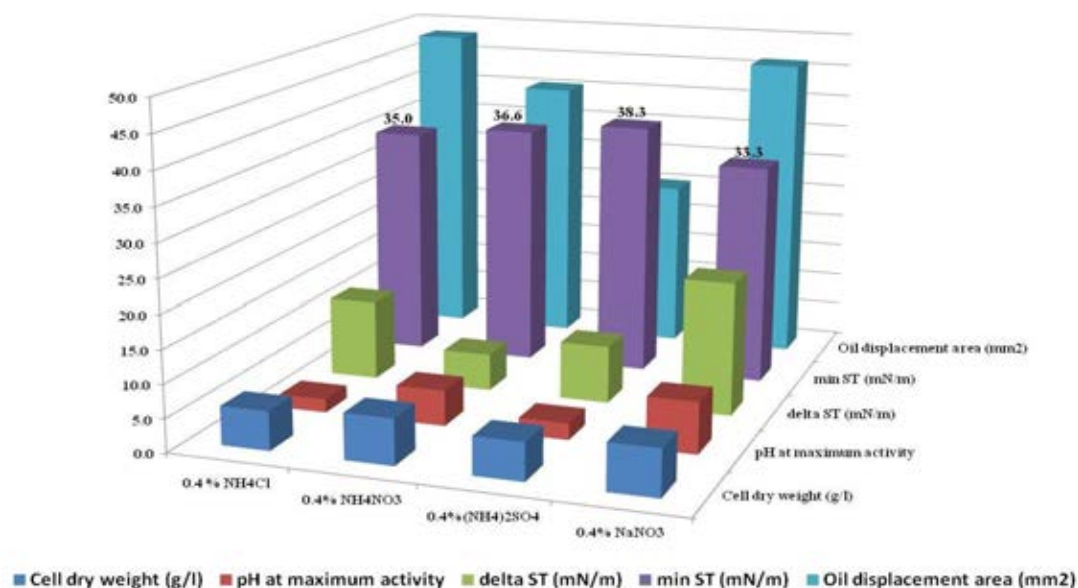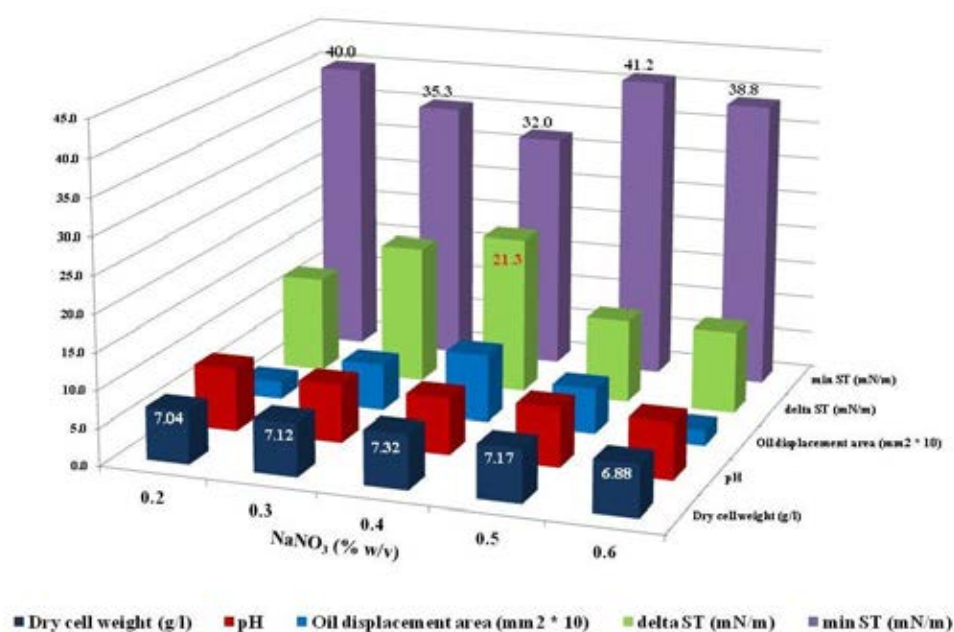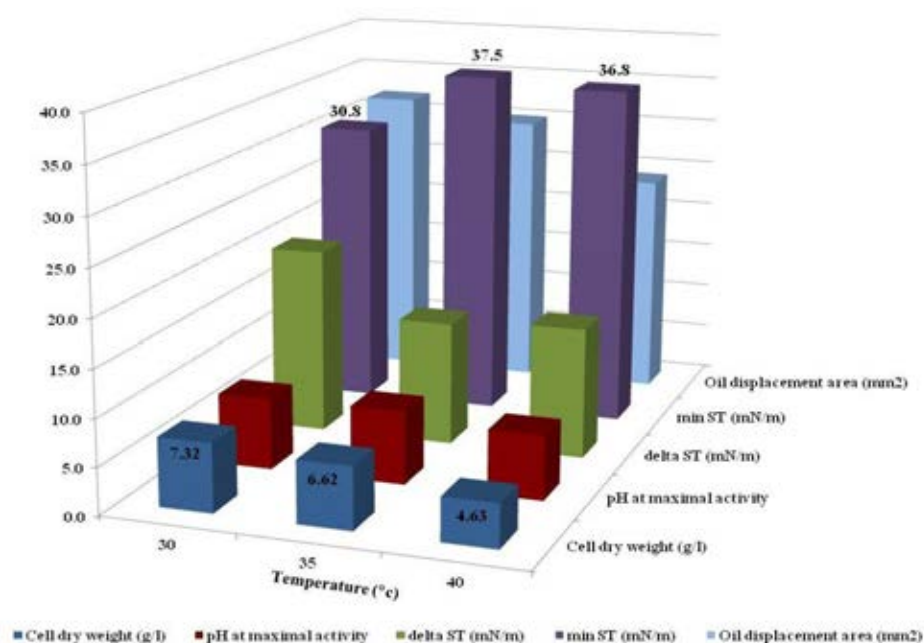**Figure 4.16** Maximal cell growth, maximal biosurfactant activity and pH of cell free broth of *Cyberlindnera samutprakarnensis* when cultivated in GP medium at various temperatures.

From this study, the highest efficiency for biosurfactant production by *Cyberlindnera samutprakarnensis* sp. nov. JP52[T] was obtained when it was grown

in modified medium consisting of 0.02% KH_2PO_4, 0.02% $MgSO_4.7H_2O$, 0.4% $NaNO_3$, 0.1% yeast extract, 2% (w/v) glucose and 2% (v/v) palm oil, initial pH 5.5. The optimum cultivation was performed at 30 ^oC in shake flask at 200 rpm.

4.3 Kinetics studies of cell growth and evaluation of substrate utilization when grown in medium supplemented with glucose and palm oil (GP)

Growth of *Cyberlindnera samutprakarnensis*, substrate consumption and production of biosurfactant determined by its activity were shown in Figure 4.17A and B. Maximum biomass determined as cell dry weight of 7.01 g L^{-1} was achieved on day 3 of cultivation and cell growth was slightly decreased until constant in the stationary phase (Figure 4.17A). The pH of the medium was increased from 5.0 to 7.9 in the exponential phase and remained constant until the end of cultivation. Biosurfactant ability shown by reducing surface tension from the initial value of 52 to 43 mN m^{-1} and by increasing oil displaced area was found during exponential growth (day 3). However, biosurfactant activity was highly effective after late exponential growth which gave the minimum surface tension of 33 mN m^{-1} and highest oil displacement area on day 7 (Figure 4.17A). Biosurfactant activity was gradually increased in the exponential phase but it was significantly increased in the stationary phase indicating the biosurfactant production is non-growth associated.

Kinetic behaviors of *Cyberlindnera samutprakarnensis* growth and biosurfactant production in medium supplemented with 2% (w/v) glucose plus 2% (v/v) palm oil, GP, were investigated. Cell biomass, biosurfactant activity determined by oil displacement test and surface tension reduction, substrate consumption determined as relative amounts of total reducing sugar, palm oil and nitrate compound in culture broth were shown in Figure 4.17B. At the beginning of exponential growth in the first 24 h glucose was almost exhausted with rapid increase in cell biomass to the maximum level, after that the remaining hydrophilic carbon compound in the culture was completely depleted after day 3 of cultivation. However, palm oil which is more hydrophobicity comparing to the other carbon sources in the medium was slowly utilized by *Cyberlindnera samutprakarnensis*, in which only 20 % consumption rate was observed at the end of the cultivation period when compared to that of the un-cultured broth. When the total amounts of

nitrate supplied from yeast extract and sodium nitrate were determined, it was found that the amounts of both nitrogen sources were reduced to about 50 % within 24 h and significantly decreased when yeast cell biomass reached the maximum level and they were completely exhausted on day 7. The above results indicated that glucose, a more hydrophilicity carbon source, and nitrogen source were essential for cell maximum growth, while palm oil, a more hydrophobicity carbon source, and enhanced biosurfactant production as *Cyberlindnera samutprakarnensis* showed strong biosurfactant activity at the end of the exponential phase.

(A) Profiles of cell growth, pH and biosurfactant activities
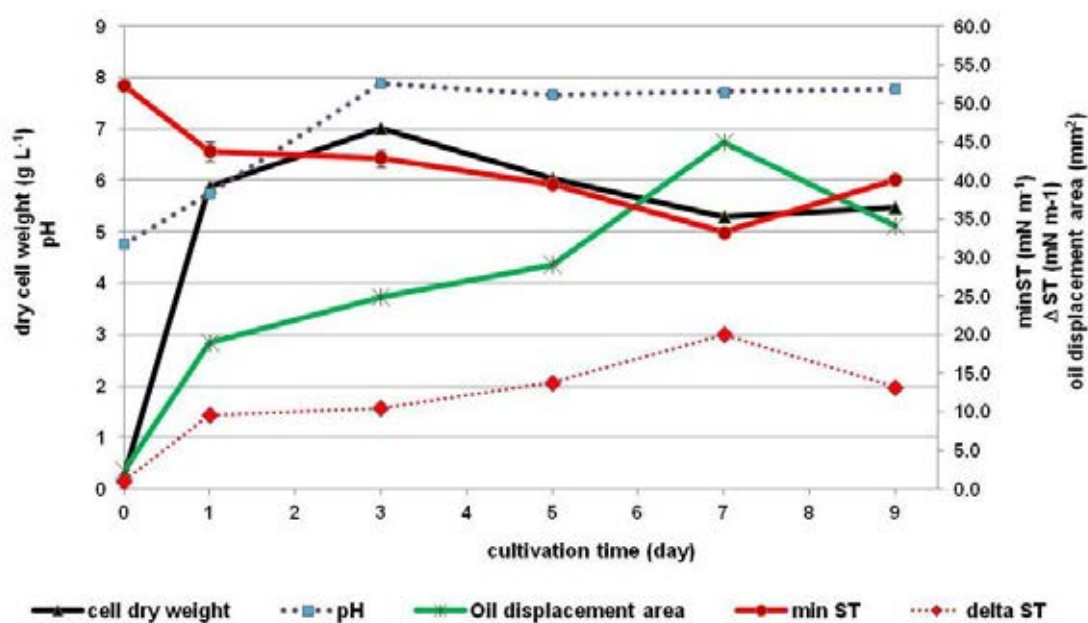
(B) Kinetic studies of cell growth and substrates consumption
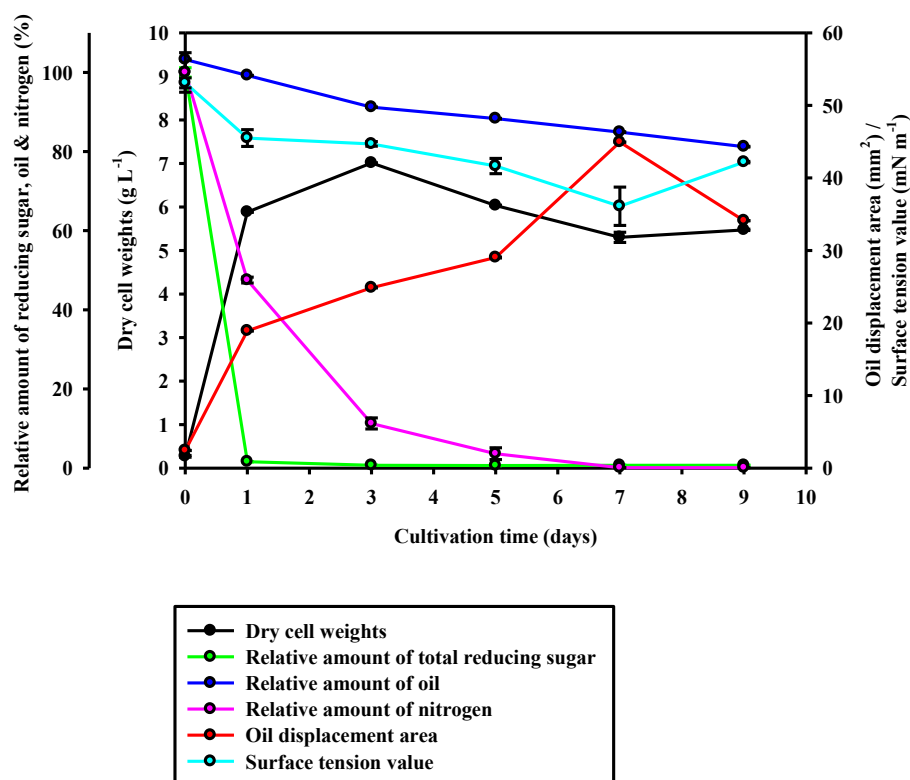
Figure 4.17 Profiles of cell growth, pH and biosurfactant activity (**A**). Kinetic studies of cell growth, substrates consumption (**B**) determined as relative amounts of total reducing sugars, oil and nitrate of *Cyberlindnera samutprakarnensis* when cultured in medium supplemented with 2% (w/v) glucose plus 2% (v/v) palm oil.

4.4 Study of the nitrogen limitation affected on cultivation and biosurfactant production

4.4.1 Microscopic observation of intracellular lipid accumulation of *Cyberlindnera samutprakarnensis*

The dye nile red, 9-diethylamino-5H-benzo[a]phenoxazine-5-one,is an excellent vital stain for the detection of intracellular lipid droplets by fluorescence microscopy and flow cytofluorometry (Greenspan et al., 1985). Nile reds were used to observe intracellular accumulation of polar lipids such as glycolipids in the *Pseudozyma* yeasts (Morita et al., 2006; 2007). These observation results showed the intracellular accumulation of polar lipids by *Cyberlindnera samutprakarnensis*, fluorescence microscopy with nile red were observed the orange-red florescent

colors inside of cell yeast when obtained at 5 and 9 days from nitrogen limited medium (Figure 4.18A) and GP medium (Figure 4.18B). Yeast cells obtained from YM were not shown the orange-red florescent colors. However, the orange-red fluorescences were much stronger in *Cyberlindnera samutprakarnensis* yeast cells grown with nitrogen limited medium than in GP medium.

(**A**) Yeast cells in a nitrogen limited medium for 5 and 9 days.
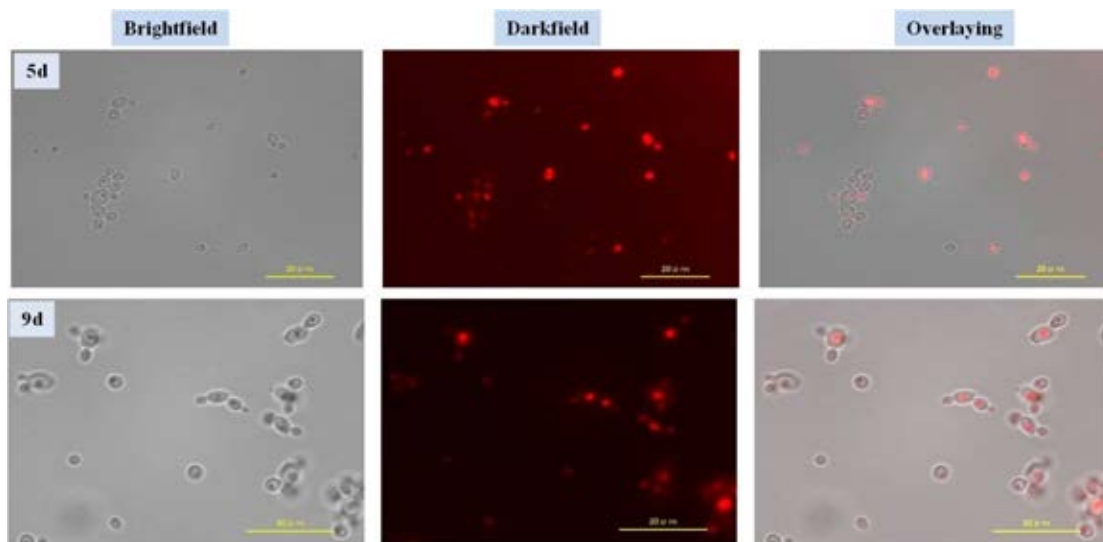
(**B**) Yeast cells in GP medium for 5 and 9 days.
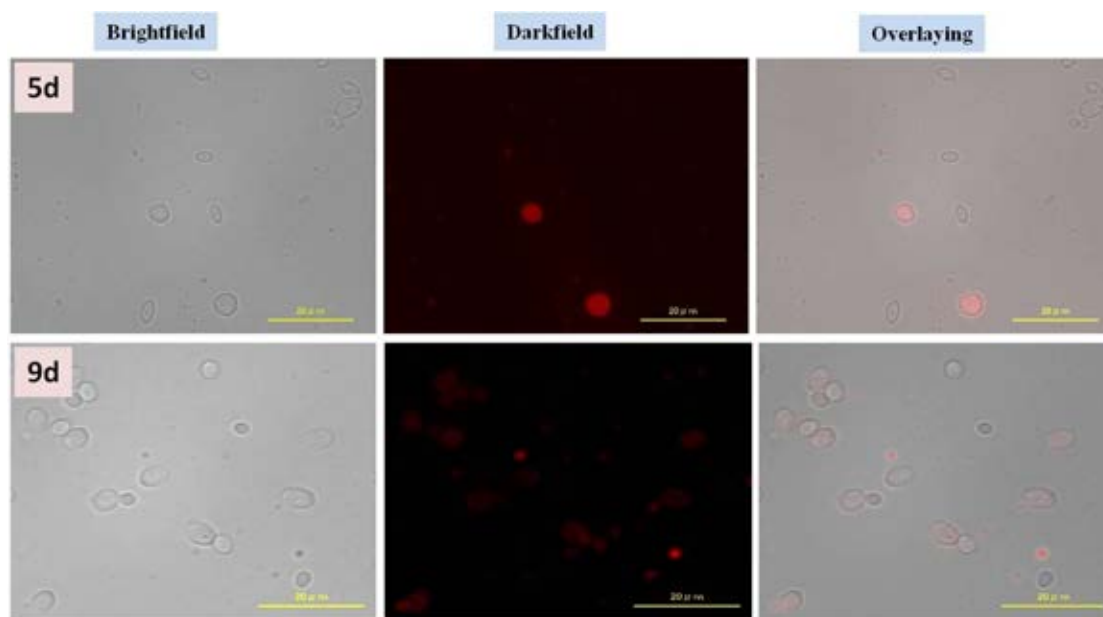
Figure 4.18 Fluorescence microscopy observation of glycolipids accumulated in the cells of *Cyberlindnera samutprakarnensis*. These yeast cells were cultured in a nitrogen limited medium (**A**) and GP medium (**B**) at 30 °C for 5 and 9 days. Nile red staining was clearly observed on dark field of fluorescent mode (middle panels). Brightfield (left panels) and Overlaying (right panels).

4.4.2 Optimization the amount of C: N (w/w) affected on growth and biosurfactant production

The accumulation of lipids produced from carbon substrates is activated by nutrient limitation. Nitrogen limitation is the principal type of limitation governing lipid accumulation (Ratledge and Wynn, 2002). Factors such as carbon and nitrogen sources have significant influences on cell growth and lipid accumulation (Wu et al., 2011). To study the amount of C: N in medium limited nitrogen on growth and biosurfactant production, the amount of C: N in media with the same 0.2 % (w/v) NaNO$_3$ concentration and the same 0.05 % (w/v) yeast extracts were prepared. As shown in Figure 4.19, yeast growth was poor (maximum cell biomass ~ 3.6 g L^{-1} at 9th day) in the medium limited nitrogen supplementing the amount of C: N at 16:1 to 40:1. Remarkably increases in biomass concentrations were observed on day 7 when *Cyberlindnera samutprakarnensis* grew in medium limited nitrogen supplementing the amount of C: N at 60:1 and 80:1, which gave maximum cell biomass at 9.41 and 9.00 g L^{-1} in respectively. However cell growth of *Cyberlindnera samutprakarnensis* was level off at 7.79 g L^{-1} when cultured in medium limited nitrogen supplementing the amount of C: N at 100:1 for day 9. The maximum biosurfactant activities by *Cyberlindnera samutprakarnensis* was found on day 9 which demonstrated ΔST 13.3 mN m^{-1} (reduced the surface tension of medium from 48.7 mN m^{-1} to 35.4 mN m^{-1}) and oil displacement area 1.93 cm^2 in medium limited nitrogen supplementing the amount of C: N 60:1.
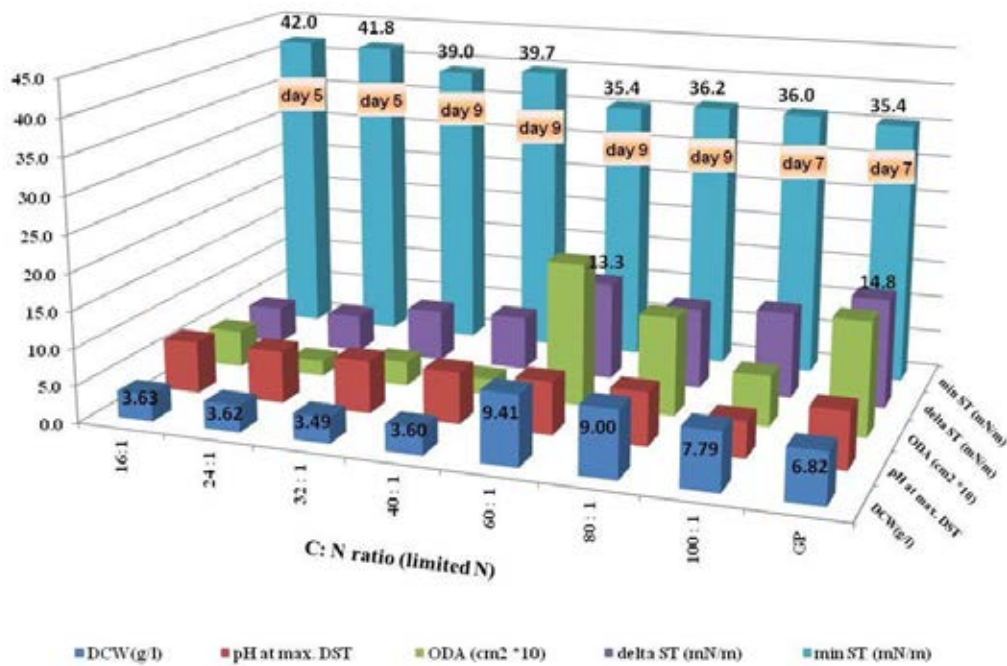
Figure 4.19 Maximal cell growth, maximal biosurfactant activity and pH obtained from cell free broth by *Cyberlindnera samutprakarnensis* in the amount of C/N variation of medium limited nitrogen.

However comparing with GP medium, actually consisted of 2% (w/v) glucose, 2% (v/v) palm oil, 0.4 % (w/v) NaNO$_3$ and 0.1 % (w/v) yeast extracts, yeast growth reached at stationary phase and observed at maximum growth on day 3 and maximum biosurfactant activities, ΔST 14.8 mN m^{-1} on day 7, while their growth and ΔST from all medium limited nitrogen having higher amount of carbon were observed at maximum on cultivation times of day 3 above (Figure 4.20A and B). Overall results of optimization study of the amount of C: N affected on growth and biosurfactant production, medium limited nitrogen with the amount of C: N at 60:1 supported yeast growth and showed the highest ΔST that it was equal to ΔST from GP medium.
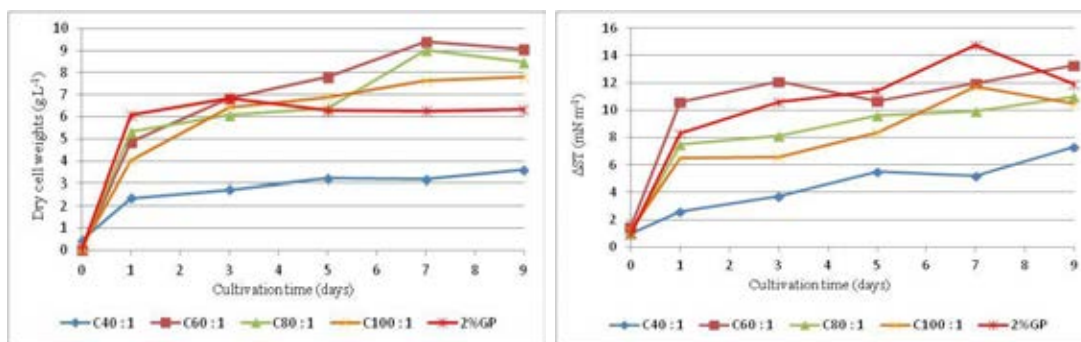
(A) Cell biomass   (B) Surface tension reduction values

Figure 4.20 The effect of the different amount of C: N of medium limited nitrogen (40:1, 60:1, 80:1, 100:1) and 2% GP medium of *Cyberlindnera samutprakarnensis* that presented of cell biomass (**A**); surface tension reduction values (**B**).

Growth profiles of *Cyberlindnera samutprakarnensis* and biosurfactant production in medium limited nitrogen with the amount of C: N at 60:1 and 2% GP were investigated. Cell biomass, biosurfactant activity determined surface tension reduction, substrate consumption determined as relative amounts of total reducing sugar, palm oil and nitrate compound in culture broth of each medium were shown in Figure 4.21A and B. The produced biosurfactant from cell free broth of both medium showed high ability to reduce surface tension from ST at initial during exponential growth (day 3). However, biosurfactant activity showed significantly great effective after late log phase that gave the highest ΔST occurred on day 7 for GP medium and on day 9 for medium limited nitrogen with the amount of C: N at 60:1.

As it can be seen in Figure 4.21A, growth in exponential phase were investigated that glucose was almost exhausted rapidly in the first 24 h for GP medium, and 3d for C/N 60:1with N limitation. The same trend was observed about palm oil consumption when cultivated in medium C/N 60:1with N limitation and GP medium, they were slowly utilized by *Cyberlindnera samutprakarnensis*, decreased only 20 % consumption rate at the end of the cultivation period. When the total amounts of nitrate supplied from yeast extract and sodium nitrate were determined, it was found that the amounts of both nitrogen sources were reduced to about 30% and 50 % within 24 h when cultivated in medium limited nitrogen with the amount of C: N at 60:1, and GP in respectively. Nitrate contents significantly

decreased when yeast cell biomass reached the maximum level and they were completely exhausted on day 5 for medium limited nitrogen and on day 7 for GP medium (Figure 4.21B).

(**A**) Medium
C: N at 60:1
(N limitation)

(**B**) GP medium
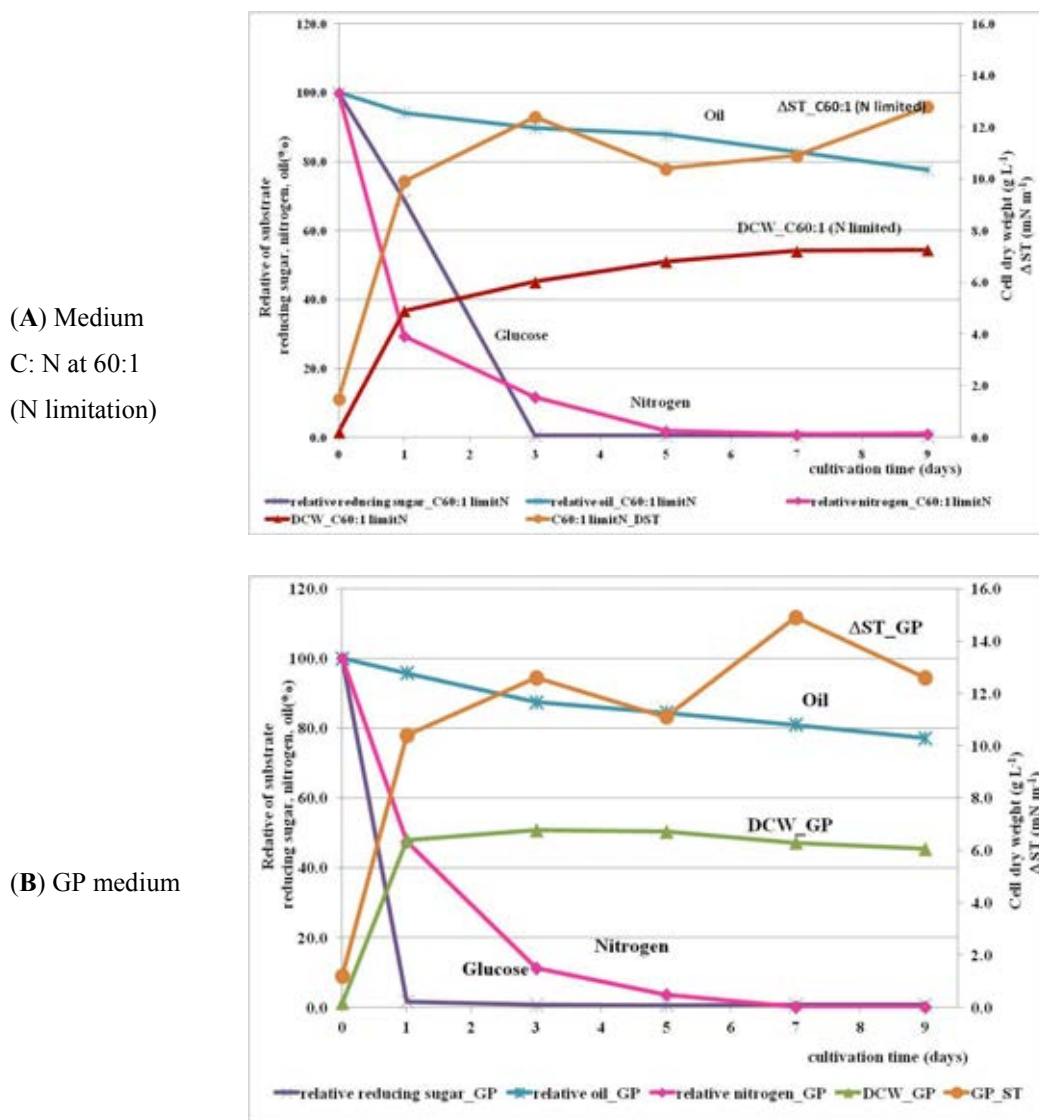
Figure 4.21 Kinetic studies of cell growth, substrate consumption determined as relative amounts of total reducing sugars, oil and nitrogen, and biosurfactant activity (ΔST) of *Cyberlindnera samutprakarnensis* when cultured in medium limited nitrogen with C: N at 60:1 (**A**) and GP medium (**B**).

Comparison of the isolated crude extracts obtained from different concentration of carbon and nitrogen but similar with types, crude extracts of biosurfactants produced by *Cyberlindnera samutprakarnensis* from medium limited nitrogen with C: N at 60:1 and GP medium were determined volumetric productions (g L^{-1} of crude extracts in one liters of medium volume) and product

yields (g g^{-1} of crude extracts in one grams of carbon substrates). Furthermore, each crude extract was prepared at sample concentration of 20 mg mL^{-1} and analyzed oil displacement area. All above of results were shown in Table 4.15.

Table 4.15 Effect of the carbon concentration and nitrogen limitation in medium on the biosurfactant production levels and properties of *Cyber. samutprakarnensis*.

medium	% glucose (w/v)	% palm oil (v/v)	% NaNO$_3$ (w/v)	% YE (w/v)	Crude BS weight[a] (g L^{-1}), d	Crude BS yields[b] (g g^{-1})	ODA of crude BS[c] (cm^2)
C: N at 60:1 (N limitation)	7.5	7.5	0.2	0.05	4.6, 7d	0.31	31.9
					3.27, 9d	0.22	36.3
GP	2.0	2.0	0.4	0.1	1.93, 7d	0.48	64.7

[a] Weight of crude biosurfactant (BS) in one volume of production mediumwas analyzed at that day of cultivation time in term of g L^{-1}.

[b] Weight of crude biosurfactant (BS) in one unit of carbon substrate used was analyzed at that day of cultivation time in term of g g^{-1}.

[c] ODA: Oil displacement area was evaluated with 20 mg mL^{-1} of biosurfactant solution.

With respect to the biosurfactant production level from the different carbon concentration and nitrogen limitation, the crude biosurfactant level from the medium limited nitrogen with C: N at 60:1 was by far the highest at 4.6 g L^{-1} for 7 d of cultivation periods, including higher than that obtained from GP medium. However, product yields, derived from weight of crude extracts in one gram of carbon substrates, of GP medium showed the highest at 0.48 g g^{-1}. When tested at a concentration of 20 mg mL^{-1}, crude extracts from GP showed the highest biosurfactant activity in the oil displacement test at 64.7 cm^2, some 2-fold higher than that from medium limited nitrogen with C: N at 60:1.

In addition, by using analytical thin layer chromatography in this examination, crude extracts obtained from both medium were also analyzed and compared spots on TLC plate after visualization with iodine vapor as shown in Figure 4.22. The resolved biosurfactant bands obtained from medium limited

nitrogen with C: N at 60:1 of day 7 and day 9 showed also similar patterns that consisted of three major bands with each of its mobility at Rf value as well as from GP medium, day 7.
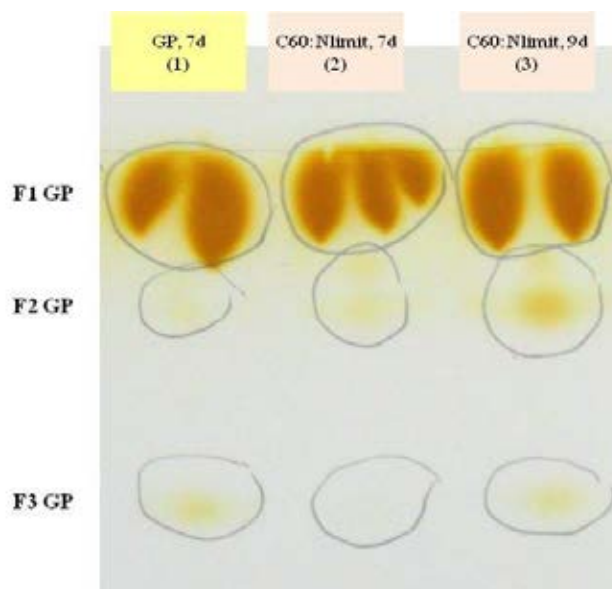
Figure 4.22 TLC analysis of the biosurfactant produced by *Cyberlindnera samutprakarnensis* when cultured in media supplemented with GP medium (**1**), GP medium limited nitrogen with C: N at 60:1 on day 7 (**2**) and day 9 (**3**). Spots were visualized by iodine vapor.

From overall study concerning the optimization condition for growth and biosurfactant activity, the highest efficiency for biosurfactant production by *Cyberlindnera samutprakarnensis* was obtained when it was grown in modified medium consisting of 0.02% KH_2PO_4, 0.02% $MgSO_4.7H_2O$, 0.4% $NaNO_3$, 0.1% yeast extract, 2% (w/v) glucose and 2% (v/v) palm oil, initial pH 5.5.

4.5 Biosurfactant production by *Cyberlindnera samutprakarnensis*

Several authors have reported that the efficiency of an individual biosurfactant-producing yeast strain depends upon the carbon source in the medium and the yeast growth phase (Kitamoto et al., 1992; Hommel et al., 1994).

This study determined the biosurfactant production when cultured in 2% (w/v) glucose and in presence of a mixture of 2% (w/v) glucose and 2% (v/v) of palm oil at optimum condition, 30 °C for 7 days. With respect to the biosurfactant production level from two carbon sources, the crude biosurfactant level from the GP medium was by far the highest at 1.89 g L^{-1}, some 63-fold higher than that obtained from 2% (w/v) glucose as the sole carbon source. In addition, when tested at a concentration of 20 mg mL^{-1}, it showed the oil displacement activity at 123.9 cm^2, some 1.84-fold higher than that from 2% (w/v) glucose as the sole carbon source. In the present study, cultivation medium supplemented with 2% (w/v) glucose and 2% (v/v) palm oil, GP medium, was selected as the substrate for biosurfactant production of the newly biosurfactant producing yeast *Cyberlindnera samutprakarnensis*.

Table 4.16 Crude biosurfactant production from medium 2% (w/v) glucose and medium supplemented with 2% (w/v) glucose and 2% (v/v) palm oil, GP medium of *Cyberlindnera samutprakarnensis*.

Yeast	Carbon sources	Crude BS weight[a] (g L^{-1})	ODA of crude extracts[b] (cm^2)
Cyber. samutprakarnensis JP52	2% glucose	0.030 ± 0.01	67.2 ± 0.06
	2% glucose + 2% Palm oil	1.89 ± 0.32	123.9 ± 0.21
P. anomala PY1	2% glucose + 2% Palm oil	0.22 ± 0.05	64.33 ± 0.1

Data are presented as the mean $\pm$ 1 SD and all experiments were conducted in triplicate.

[a] Weight of crude biosurfactant (BS) was analyzed at day 7 of culture (maximal biosurfactant activity).

[b] ODA: Oil displacement area was evaluated with 20 mg mL^{-1} of biosurfactant solution.

4.6   Purification and structural characterization of biosurfactant

4.6.1   TLC analysis of crude biosurfactants

Crude extracts of biosurfactants produced by *Cyber. samutprakarnensis* from medium with 2% of glucose and 2% palm oil (GP) and 2% glucose (G) as described in Table 4.16 were determined by using preparative thin layer chromatography. The extracts from the GP medium was an oily-yellowish color, whereas that from the 2% (w/v) glucose medium was a dark brownish color. These samples were then analyzed by preparative TLC and visualized with iodine vapor. The resolved biosurfactant fractions obtained from the GP medium revealed the presence of three major bands, F1GP, F2GP and F3GP with Rf values of 0.89, 0.77 and 0.69, respectively. There were also three major bands from the 2% (w/v) glucose medium, F1G, F2G and F3G, with Rf values of 0.90, 0.73 and 0.65, respectively which were slightly different from those of GP medium (Figure 4.23).

	Rf value	Oil dis. area (cm²) [a]			Rf value	Oil dis. area (cm²) [a]
F1GP	0.89	18.3		F1G	0.90	41.9
F2GP	0.77	18.3		F2G	0.73	11.6
F3GP	0.69	71.3		F3G	0.65	9.1
		(A)				(B)
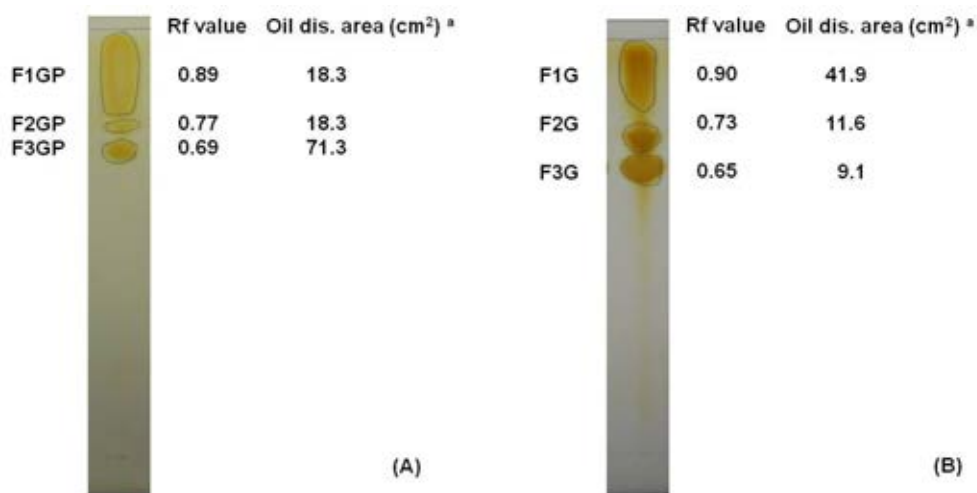
Figure 4.23 TLC analysis of the biosurfactant produced by *Cyberlindnera samutprakarnensis* when cultured in media supplemented with (**A**) 2% (w/v) glucose plus 2% (v/v) palm oil (GP medium) and (**B**) 2% (w/v) glucose, and the respective Rf and oil displacement activity values of each fraction. Spots were visualized by iodine vapor. Oil displacement area was measured at a sample concentration of 20 mg mL^{-1}. TLC plates shown and associated values given are representative of those seen from three independent samples.
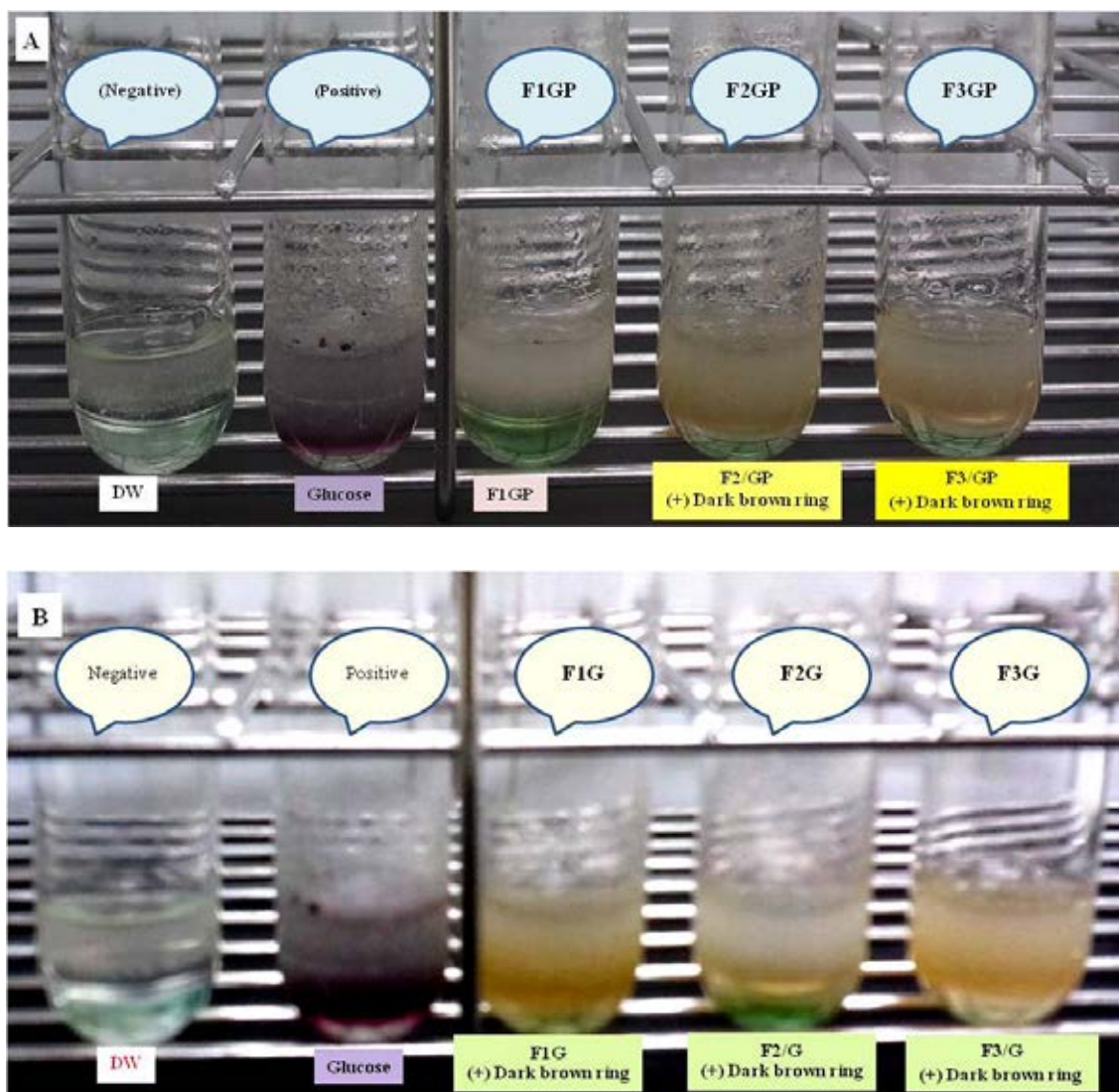
Figure 4.24 Carbohydrate components determination by molish test in each fractions of biosurfactant extracts at concentration 1 mg ml^{-1} from (A) GP and (B) 2% glucose medium produced by *Cyberlindnera samutprakarnensis*. Distilled water and 1 mg ml^{-1} of glucose was representative of negative and positive control.

Following the Molish test, F2GP and F3GP showed an obvious positive result after only a few minutes as did F1G and F3G, suggesting that these biosurfactants were glycolipids (Figure 4.24). However, in agreement with the slightly different Rf values, the oil displacement activity (as area) was not similar between the extracted samples from the corresponding spots from the GP and 2% (w/v) glucose media. These fractions of TLC and their oil displacement area (cm^2) gave results as F1GP/ 18.3 *vs*. F1G/ 41.9, F2GP/ 18.3 *vs*. F2G/ 11.6 and F3GP/ 71.3 *vs*. F3G/ 9.1 cm^2. The highest oil displacement activity was seen in F3GP

(some 7.8-fold higher than that in F3G), followed by that of F1G (2.3-fold higher than that in F1GP). Based on the efficiency of biosurfactant production by *Cyberlindnera samutprakarnensis* when cultured in GP medium and its biosurfactant activities, the F3GP fraction was selected for analysis of the structure of the glycolipid biosurfactant(s) present by MALDI-TOF-MS and NMR based analysis.

4.6.2 Purification and chemical characterization of biosurfactants

4.6.2.1 Proposed molecular weight and category identification of the main glycolipid biosurfactant components by MALDI-TOF-MS

The F3GP glycolipid extract was analyzed by MALDI-TOF-MS (Figure 4.25), which revealed that the major spectra were characterized by molecular ions at m/z 574.57 and 663.99. These values are either similar to the molecular weight of the compound or may be molecule adduct ions for the lactone and free acids form of sophorolipids, according to the nominal mass calculation. Sophorolipids are typically produced as complex mixtures in fermentation broths, with the composition depending on the carbon source (Hu and Ju, 2001; Asmer et al, 1988). In the case of F3GP, which was produced from *Cyber. samutprakarnensis* in GP medium, the sophorolipids produced were complex, with obtained molecular weights of 574 and 662 that correspond to non acetylated lactonic sophorolipids (SL-C18) and either a monoacetylated acidic sophorolipid (SL-Ac-C19:1) or diacetylated lactonic sophorolipids (SL-Ac-Ac-C16), respectively.
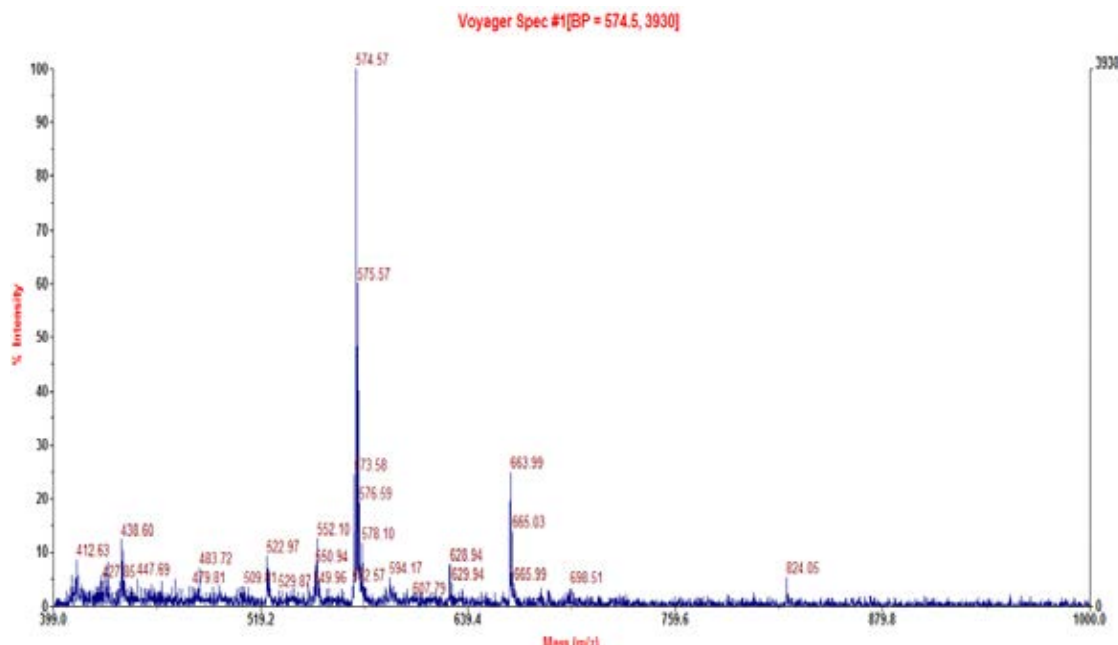
Figure 4.25 MALDI-TOF-MS spectra of the glycolipid F3GP from *Cyberlindnera samutprakarnensis*. Spectra shown is representative of those seen from two independent samples.

4.6.2.2 Structure elucidation of glycolipid biosurfactant by NMR analysis

Structure elucidation of the glycolipid F3GP was performed in CD_3OD. Although the sample was not pure, both the ^{1}H and ^{13}C NMR spectra could be assigned to be from a typical sophorolipid-type structure. Compared with previously known NMR assignments, F3GP presented the NMR signals of ^{1}H and ^{13}C nuclei derived from sophorose sugar (2-O-glucopyranosyl-D-glucopyranose) as indicated by the H1′/C1′ peak at 4.36 ppm /101.1 ppm and the H1′′/C1′′ peak at 4.57 ppm /106.3 ppm. They were similar to the evidence for Glc1 and Glc2 in *S. bombicola* and *W. domericqiae* (Price et al, 2012; Chen et al, 2006a), where the NMR signals of the glucosyl anomeric nuclei were established for the sophorolipids from *S. bombicola* (H1′/C1′ peak at 4.46 ppm /102.2 ppm and the H1′′/C1′′ peak at 4.63 ppm /104.0 ppm) and *W. domericqiae* (H1′/C1′ peak at 4.46 ppm /102.6 ppm and the H1′′/C1′′ peak at 4.56 ppm /104.0 ppm).

Sophorolipid structures are typically found as O-(1→2)-glycoside linkages between two glucose units (Glc1, C2′-O- Glc2, C1′′). In support of this,

the F3GP sophorolipid assigned H-1′′at 4.57 ppm shows a HMBC long range coupling across the 1′-anomeric oxygen to the C-2′ carbon (C2′ at 82.5 ppm) on the other glucosyl ring. In addition, the chemical shift at H2′/C2′ (3.55 ppm /82.5 ppm) and H2′′/C2′′ (3.31 ppm /75.1 ppm) support that C2′ resonated at higher values than C2′′ due to the deshielding effect of a higher electronegativity of C2′-O in Glc1 (Figure 4.26A-F).

With respect to the C6′ and C6′′ positions in glucose, normally located with an –O-(CH_2)- group, they differed in having –CH-, as revealed by the HSQC analysis where the two proton signals were noticeably shifted with C6′ at 4.40 and 4.25 ppm, C6′′ at 4.25 and 4.10 ppm. Noticeably, the R group (-OH or acetate) revealed two high chemical shifts in the ^{13}C –NMR spectrum at 174.7 and 174.3 ppm that are derived from the carbonyl groups (-CO-), indicating that these carbonyl groups were linked to the C6′ and C6′′ of glucose. This notion is supported by the HMBC analysis with peaks at 4.2-4.40 ppm (data not shown). Together, these data suggested that the major component of the glycolipid F3GP was an acetyl group containing sophorose. The lactonic sophorolipid was formed from the esterification of the end carboxylic acid of the fatty acid linked to the C4′′ of glucose, as evident from the H4′′ signals at 4.92 (Figure 4.26B).

With respect to the fatty acid moiety, the carbonyl groups at the terminal C1 carbon were detected with a high chemical shift of 177.6 ppm in the ^{13}C – NMR. The –CH=CH- group was represented with ^{1}H at 5.1-5.3 ppm and ^{13}C of two carbons in the range of 120.0 - 125.9 ppm. The multiple chemical signals of protons and ^{13}C in the long fatty acid chain were found at 1.2-2.25 ppm and 20.1-33.1 ppm, respectively (Figure 4.26D and E). The HSQC analysis revealed that the H/C NMR signal at the ω-1 position of the hydroxyl fatty acid were assigned at chemical shift [13]C 79.1 ppm and coupled to the adjacent ^{1}H proton at 3.87 ppm. This proton is remarkably shifted by the attached ω-1 position of the hydroxyl fatty acid, linked with the glucose ring. The terminal fatty acid was assigned by the H/C NMR signal at 1.17 ppm /20.05 ppm to be an acyl methyl group. The terminal proton showed a doublet spectra at 1.17 ppm (Figure 4.26C) that was influenced from the coupling with the environ nuclei proton at the ω-1 position of the hydroxyl fatty acid.
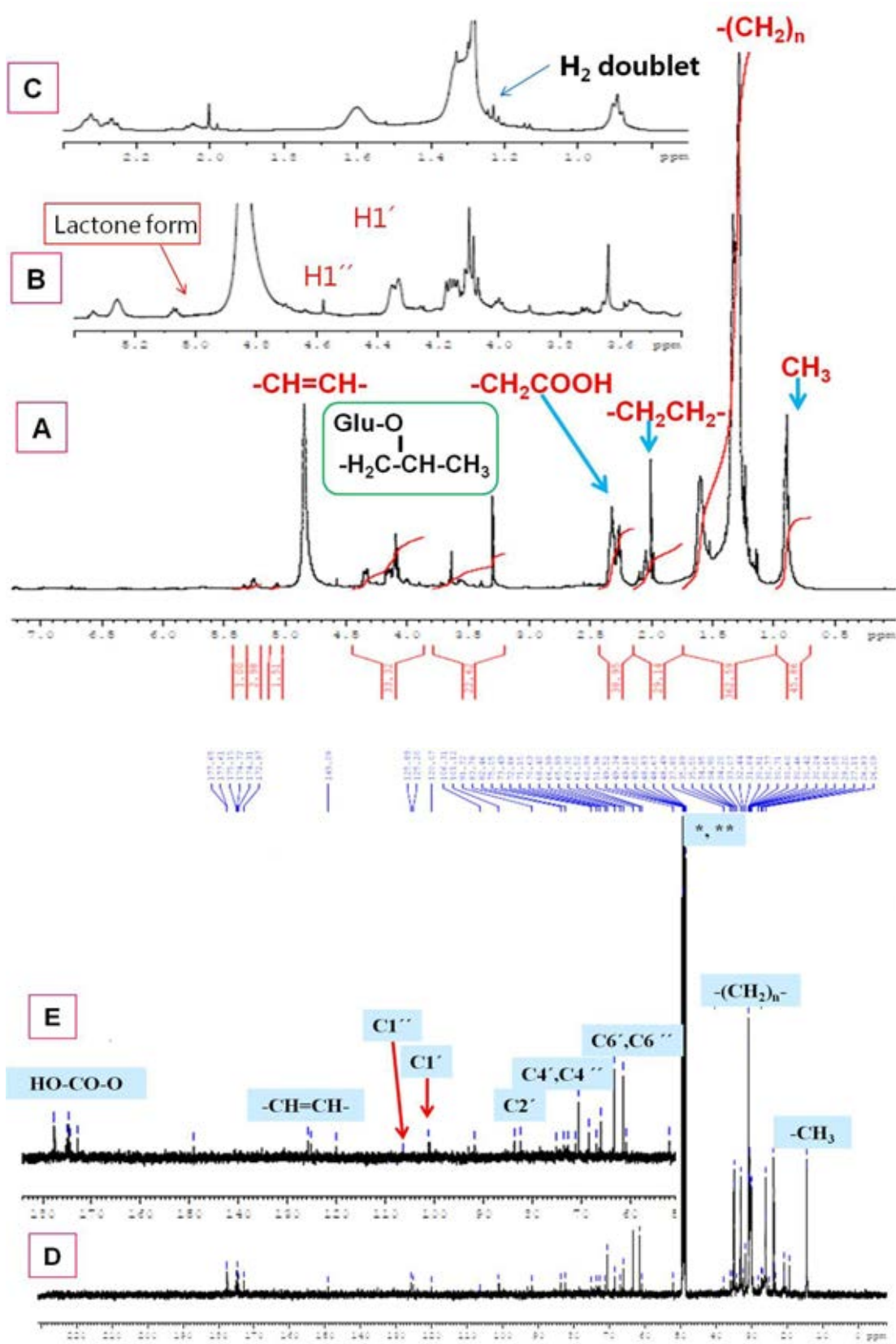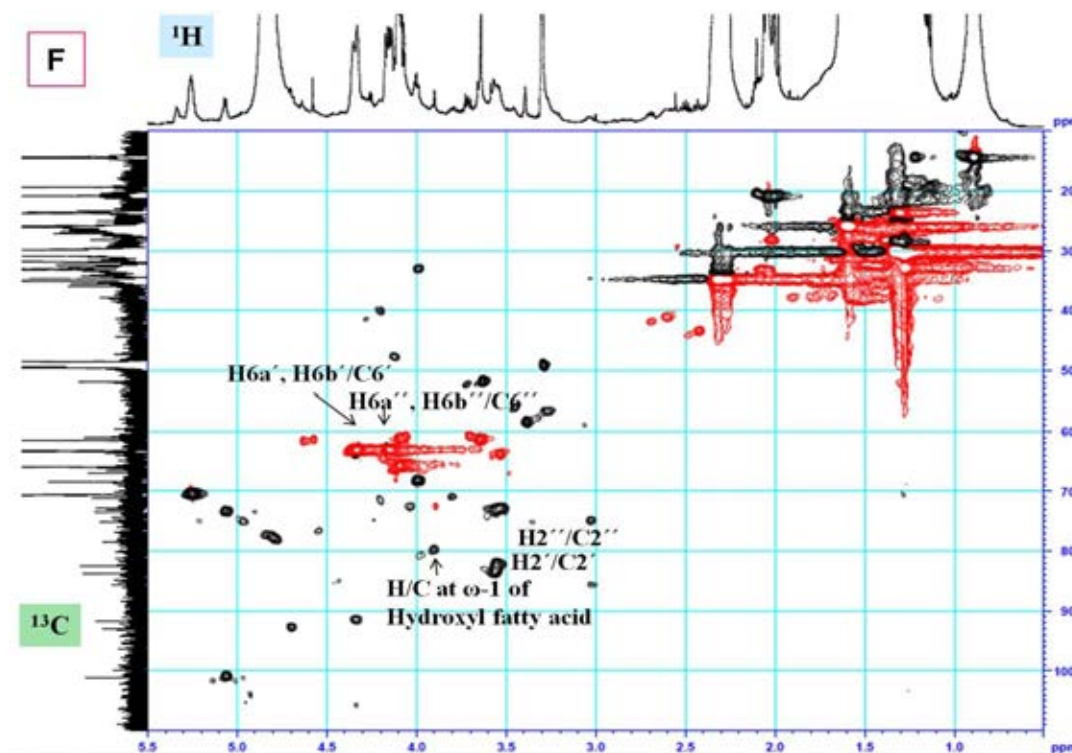
Figure 4.26 NMR data for the F3GP sophorolipids from *Cyber. samutprakarnensis*. Shown are the (**A-C**) ^{1}H NMR spectra, (**D & E**) ^{13}C NMR spectra and (**F**) heteronuclear single quantum correlation (HSQC) spectra. The solvents CD$_3$OH and CH$_3$OD are indicated by * and ** respectively. Spectra shown are representative of those seen from two independent samples.

4.7 Determination of physical and chemical properties of the crude biosurfactants

4.7.1 Evaluation critical micelle concentration (CMC) of biosurfactant

CMC value is defined as the minimum concentration of surfactant to initiate micelle structure. Whenever reaching the CMC, the surface tension was hardly able to decrease although the amount of surfactant continuously increased. Crude biosurfactant from *Cyberlindnera samutprakarnensis* showed high efficiency to reduce surface tension of Tris buffer, using as a control, from 55.7 to 30.9 mN m^{-1} when reached to CMC of 457.1 mg L^{-1} (0.046 %) as shown in Figure 4.27. Regardless of increasing biosurfactant concentration, reduction of the surface tension value is not further observed once the CMC has been reached at this point. Oil displacement area test was also used to evaluate for CMC value.
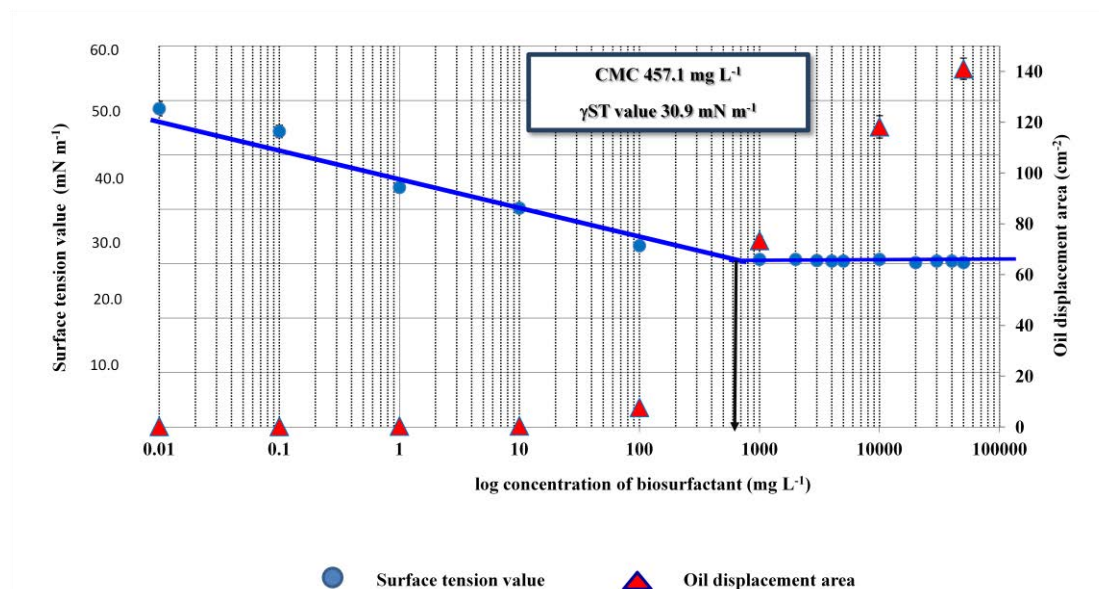
Figure 4.27 Relationship of biosurfactant capacity of surface tension reduction and oil displacement area enhancement of crude biosurfactant from *Cyberlindnera samutprakarnensis* when cultured in media supplemented with 2% (w/v) glucose plus 2% (v/v) palm oil. Arrow indicates γST value reached 30.9 mN m^{-1} at CMC 457.1 mg L^{-1} (0.046%).

4.7.2 Measurement of emulsification activity

The present work determined emulsification index (E24) and stability of emulsion of the crude biosurfactant at 1 mg ml^{-1}, from *Cyber. samutprakarnensis* JP52 tested against various vegetable oils and some hydrocarbon compounds (Figure 4.28). As shown in Figure 4.29, emulsion with 5 kinds of vegetable oils including olive oil, sesame oil, palm oil, cotton seed oil and safflower oil gave E24 values higher than 80% and the emulsions were stable for over a period of 7 days after that it was found that the emulsions formed with olive oil, cottonseed oil and safflower oil were collapsed from 90 to 50% after 1 month but not with palm oil and sesame oil. In contrast, this biosurfactant failed to emulsify any hydrocarbon tested including hexadecane, dodecane and toluene.
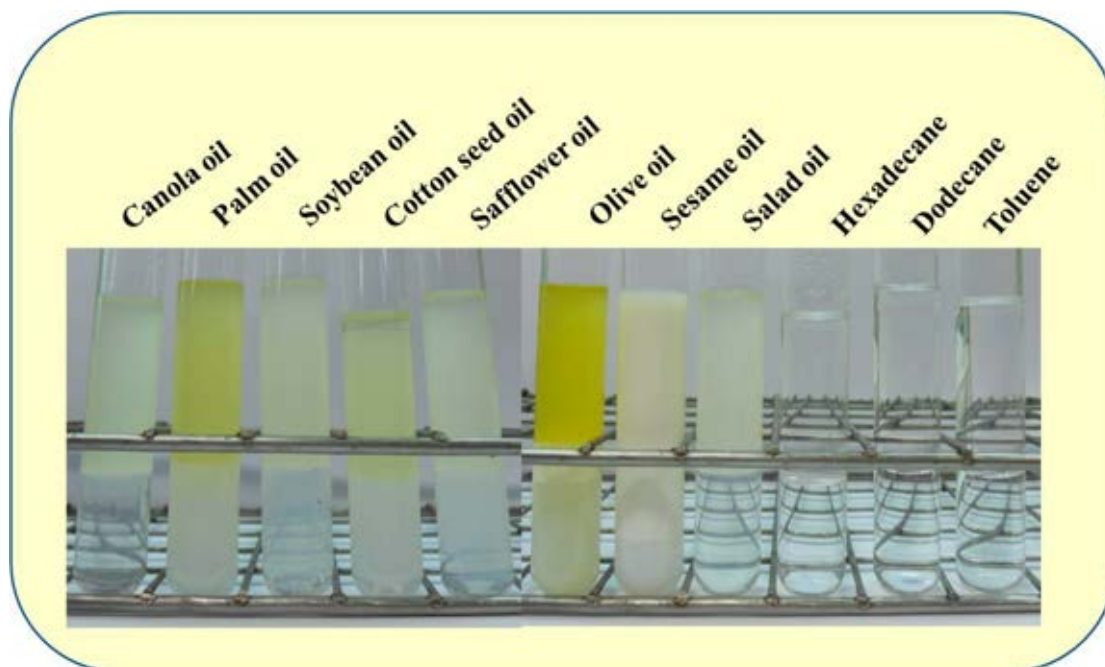
Figure 4.28 Emulsification formation in various vegetable oils and hydrocarbon when tested at 1-day-periods of crude biosurfactant from *Cyberlindnera samutprakarnensis* when cultured in medium supplemented with 2% (w/v) glucose plus 2% (v/v) palm oil.
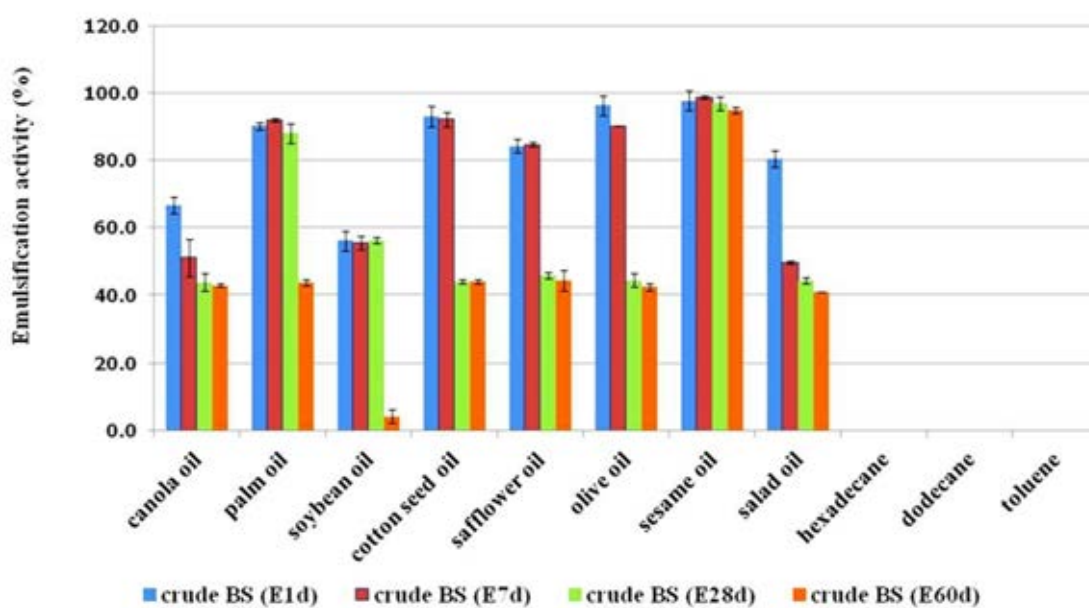
Figure 4.29 Emulsification stability tested at 1, 7, 28 and 60-day-periods (E1d or E24 index, E7d, E28d, E60d) of crude biosurfactant from *Cyber. samutprakarnensis* when cultured in medium supplemented with 2% (w/v) glucose plus 2% (v/v) palm oil.

4.7.3 Stability characterization of crude biosurfactant against pH, salt and temperature

The stability of crude biosurfactant from *Cyber. samutprakarnensis* was tested against pH, salt concentration and temperature . For pH, the samples of crude extract of biosurfactant at 1 mg ml^{-1} dissolved in 50 mM Tris-HCl at various pH from 5 to 9 showed high efficiency in reducing surface tension and retained strong activity although they were kept at the tested conditions for 3 days (Figure 4.30A). It was found that its optimal pHs activity was in neutral pH range however, it was still quite active in acidic and alkaline conditions. The salt tolerance of the biosurfactant was analyzed by determining surface tension-reduction activity of the biosurfactant in the presence of 0-10% (w/v) NaCl. As shown in Figure 4.30B, the biosurfactant was still active even at high salt concentrations and there were no differences when tested for 1- or 3-day-periods. For temperature stability, the biosurfactant was treated at 4, 28, 60 and 100°C for up to 9 h, after that the samples were left standing at ambient temperature and the ST values were determined. The samples treated at 4 up to 60°C still retained high activity throughout the treating periods, whereas the sample treated at 100°C could retain high activity up to 3 h of the treatment but after that it could retained moderate activity throughout the treating periods (Figure 4.30C). Moreover, we further investigated on its heat tolerance by heating the biosurfactant at 121°C under pressure via sterilization process up to 5 times; surprisingly it could still retain considerably high surface tension reduction activity and not much difference when compared to those of SDS (Figure 4.30D). From the stability properties of the biosurfactant from *Cyberlindnera samutprakarnensis* against various environmental effects such as pH, salinity and heat suggested that it is a good candidate for use in various environments and some industries related to tensioactive compounds.

A) pH variation on day 1 and 3

B) NaCl tolerance on day 1 and 3

C) Temperature variation

D) Heat tolerance via sterilization under pressure and comparing with SDS
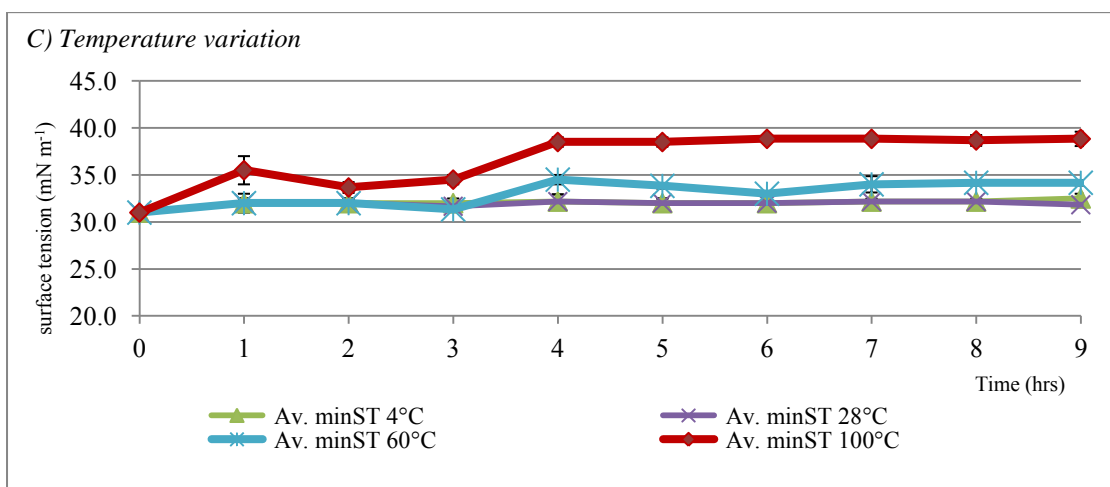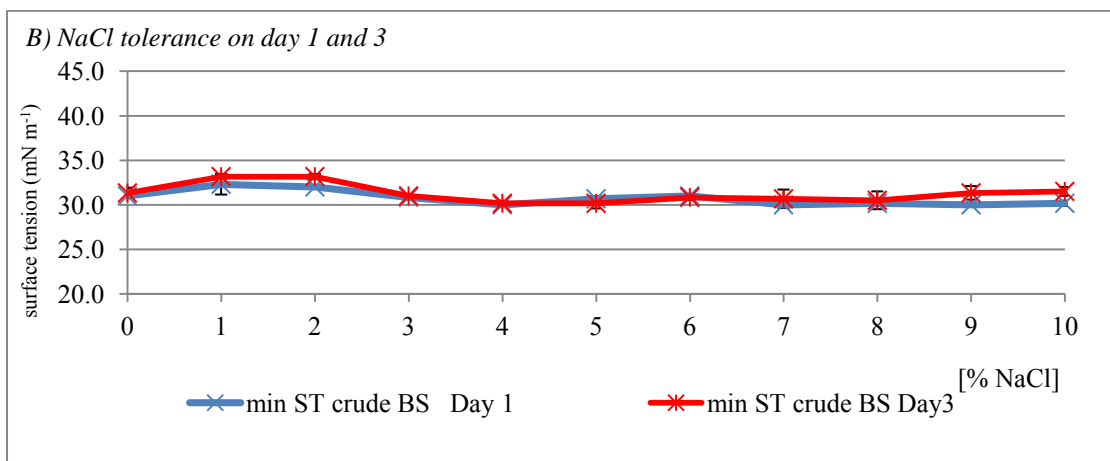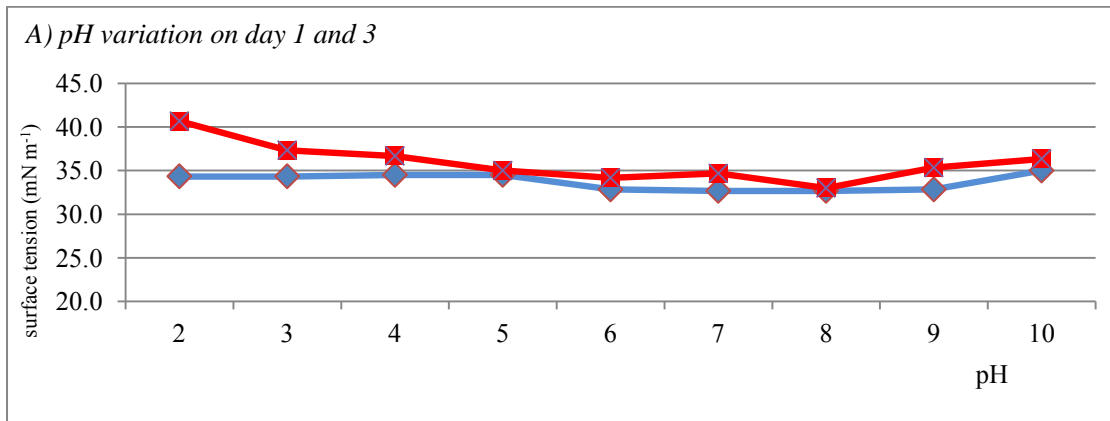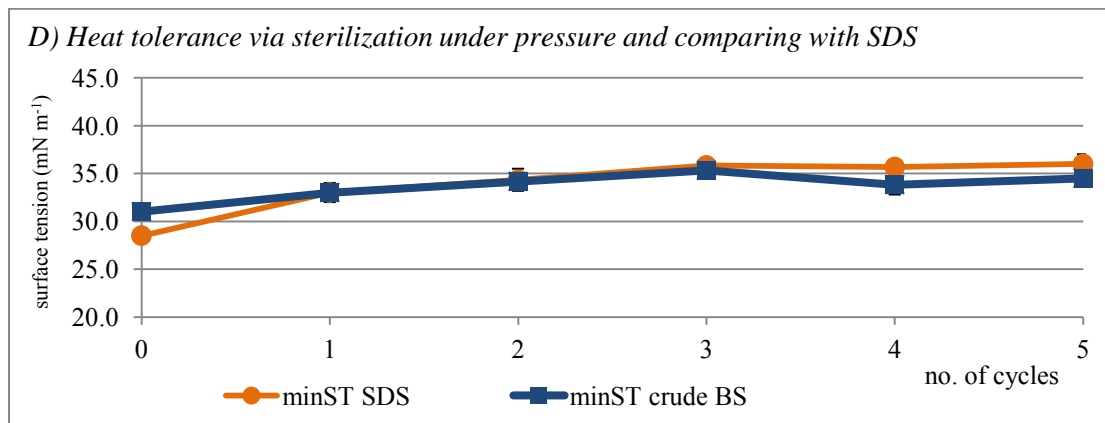
Figure 4.30 Effects of pH (**A**), NaCl concentrations (**B**), temperature (**C**) and sterilization (**D**) for tension-active capacity of crude biosurfactant from *Cyberlindnera samutprakarnensis* cultured in medium supplemented with 2% (w/v) glucose plus 2% (v/v) palm oil.

CHAPTER V

DISCUSSIONS AND CONCLUSIONS

This study revealed the diversity of the efficient biosurfactant producing yeasts at species level and demonstrated the probability to obtain efficient biosurfactant producers in various types of samples including agricultural wastes (23.08%), soil (24.61%) wastes from biodiesel plants (30.19%), and industrial wastes (30.61%). Of these samples, two samples enriched with oil contaminants had more number of efficient strains suggesting some environments or habitats having hydrophobic substrates may be a selective pressure for survival and adaptation of biosurfactant producers. This finding was similar to those reported by others such as Techaoei (2007) who suggested that soil sample from garage sites had positive surfactant activity and was a good source for screening of biosurfactant-producing bacteria than the hot spring samples. Gram-positive biosurfactant producing isolates were found in heavy metal contaminated or uncontaminated soils, while gram negative isolates were found from hydrocarbon contaminated or co-contaminated soils (Bodour et al., 2003). This study demonstrated that the efficient strains showed their activities of lowering the surface tension to below 40 mN m^{-1} were isolated from a wide diversity of environmental samples contaminated with oils. The samples enriched with oils or lipids such as wastes from biodiesel plant and industrial wastes were frequently found to have a high number of the isolates.

Molecular genetics identification of yeast has vastly expanded the scope of classification especially using in yeast diversity studies by comparing the D1/D2 region of the large subunit (LSU) rRNA gene with other sequences retrieved from the Genbank database. Based on the sequences of D1/D2 (ca.600 nucleotides) domain of 26S LSU rRNA gene according to a guideline of Kurtzman and Robnett (1998), 94

yeast strains were identified that 93.62% (88 strains) were known species, 2.13% (2 strains) were sister species and 4.26% (4 strains) were novel species. From this survey, biosurfactant-producing yeasts, *Meyerozyma guilliermondii* and *Trichosporon asahii* were dominant species in the ascomycetous and basidiomycetous yeasts among the known yeasts. Species such as *Candida mucifera*, *Issatchenkia orientalis*, *C. tropicalis*, *C. palmiophila*, *C. rugosa*, *C. viswanathii*. *C. tropicalis* and *T. faecale* were also investigated and distributed in this survey unless having only a bias of selective medium containing glucose and palm oil as carbon sources. However, different yeasts are able to utilize different carbon sources and nutritional selectivity determines yeast species diversity in particular niches (Phaff et al., 1978).

There were many reports on screening of biosurfactant-producing bacteria from environmental samples while only limited studies were with yeasts. These limited studies included screening for biosurfactant-producing yeasts, *Saccharomyces cerevisiae* and *Candida albicans*, from a polluted lagoon water which the isolates grew effectively on diesel and crude oil as a sole carbon source to produce biosurfactants (Ilori et al., 2008), other study was on the hydrocarbon-uptake into cells of *Candida*, six *Candida* species (*C. tropicalis*, UCP0996; *C. lipolytica*, UCP0988; *C. sphaerica*, UCP0995; *C. glabrata*, UCP1002; *C. buinensis*, UCP0994; and *C. guilliermondii*, UCP0992) in which cell hydrophobicity, emulsifying activity, surface tension, and interfacial tension of the cell free culture medium were investigated (Coimbra et al., 2009). Furthermore, basidiomycetous yeasts such as *Trichosporon loubieri*, *Trichosporon montevideense* and *Geotrichum* sp. (Monteiro et al., 2010), *Trichosporon asahii* (Chandran and Das, 2010) were also reported on their capability of biosurfactant production. From this study, identification of yeasts with high surface tension reduction activity may lead to potential to obtain efficient biosurfactant producers and increase database resources for biosurfactant producers.

In the course of a survey on the biosurfactants-producing yeasts, three anamorphic yeast strains were found to represent two novel species which were closely related to *Cyberlindnera mengyuniae* and *Scheffersomyces spartinae*. They are described as *Cyberlindnera samutprakarnensis* sp. nov. for strain JP52[T] and *Candida thasaenensis* sp. nov. for strains JP59[T] and JP60. Among them, three strains were

differentiated from any known species by using multigene analysis of the D1/D2 region of the large subunit (LSU) rRNA gene, the internal transcribed spacer (ITS1–5.8S rRNA gene–ITS2; ITS1-2) region for generalized analysis, and in this addition, actin gene and translational elongation factor gene gave supportive information in the case of strains JP59[T] and JP60 and represent two novel species. Strain JP52[T] was obtained from the waste-water of a cosmetic factory and the other two strains, JP59[T] and JP60, were from the sediment ponds of a palm biodiesel production plant. With a strength suggestion from this research, two novel species of *Cyberlindnera samutprakarnensis* sp. nov. JP52[T] and *Candida thasaenensis* sp. nov. JP59[T] and JP60 performed as new yeasts capable to produce biosurfactant from their tensoactive reduction properties.

Cyberlindnera samutprakarnensis sp. nov. JP52[T] was selected among six of tested strains as it showed highest biosurfactant producing ability which was determined by surface tension reduction (ΔST value 20 mN m^{-1} of cell free broth in 2% glucose and 2% palm oil). As a result of optimal cultivation conditions, the highest efficiency for biosurfactant production was obtained when it was grown in modified medium (GP) consisting of 0.02% KH_2PO_4, 0.02% $MgSO_4.7H_2O$, 0.4% $NaNO_3$, 0.1% yeast extract, 2% palm oil and 2% glucose, pH 5.5. The cultivation was performed at 30°C in shake flask at 200 rpm. Cultivation in GP medium, biosurfactant from cell free broth of strain JP52 possessed strong activitities showing high ΔST value of 20 mN m^{-1} and the oil displacement activity of 0.46 cm^2. In addition, when tested at a concentration of 20 mg mL^{-1}, it showed the highest biosurfactant activity in the oil displacement test, 123.9 cm^2; this was 1.84-fold higher than that from 2% (w/v) glucose as the sole carbon source.

According to the literature, biosurfactants produced by culturing with co-utilization of carbohydrates especially glucose and vegetable oils have gained more effective of production and their properties. There have been many reports mentioned on biosurfactant production from the combination of substrates , glucose and vegetable oil, these included glucose and corn oil utilized by *Candida bombicola* (Pekin and Vardar-Sukan, 2006), glucose plus cotton seed oil by *Candida glabrata*

(Sarubbo et al., 2006) glucose and canola oil by *Candida lipolytica* (Sarubbo et al., 2007). However, only few reports on biosurfactants production from palm oil, such as sophorolipid was produced by *Candida bombicola* using palm oil and the methyl ester of palm oil (Davila et al., 1994). Palm oil is important domestic plant oil and a raw material for biodiesel precursor and diversely uses in many applications. It is abundantly available in Southeast Asia and cheaper price than many other vegetable oils. Thus, production of biosurfactants from renewable resources like palm oils or used palm oils or in combination with carbohydrates is one of strategies for the industries to use low cost substrates and may also reduce the generation of waste. In the present study, cultivation medium supplemented with 2% (w/v) glucose and 2% (v/v) palm oil, GP medium, was selected as the substrate for growth profile and biosurfactant production-study of the newly biosurfactant producing yeast *Cyberlindnera. samutprakarnensis* JP52^T.

Several authors have reported that the efficiency of an individual biosurfactant-producing yeast strain depends upon the carbon source in the medium and the yeast growth phase (Kitamoto et al. 1992; Hommel and Huse, 1993). According to kinetic behaviors of *Cyberlindnera samutprakarnensis* study of cell growth and biosurfactant activities, maximum biomass as cell dry weight (7.01 g L^{-1}) of yeast strain JP52 in media supplemented with 2% (w/v) glucose plus 2% (v/v) palm oil was achieved when cultivating on day 3 and the level of cell growth was slightly decreased until gradually constant during in the stationary phase. The produced biosurfactant showed ability to reduce surface tension from initial ST 52 to 43 mN m^{-1} and increase oil displaced area during exponential growth (day 3). However, biosurfactant activity demonstrated remarkably great effective after late exponential growth that gave the minimum ST (33 mN m^{-1}) and highest oil displacement area occurred on day 7. Biosurfactant production gradually rose in the exponential phase and significantly increased whilst yeast grew in stationary phase that indicated the biosurfactant production is non-growth associated. Similar observations have been made for other biosurfactant-producing yeast, growth and biosurfactant production by *Candida lipolytica* UCP0988 using 10% glucose plus 10% canola oil showed a non growth-associate production, while the maximum production and ability to reduce ST value at

the minimum (34 mN m^{-1}) occurred after the end of exponential growth phase (Sarubbo et al., 2007). For evaluation of substrate utilization by *Cyberlindnera samutprakarnensis*, glucose as hydrophilic carbon compound in the culture was completely depleted after day 3 of cultivation, palm oil as hydrophobicity compound in the medium was slowly utilized, used only 20 % consumption rate and the amounts of total nitrogen were reduced to about 50 % within 24 h and significantly decreased when yeast cell biomass reached the maximum level and they were completely exhausted on day 7. This indicated that glucose, a more hydrophilicity carbon source, and nitrogen source were essential for cell maximum growth, while palm oil enhanced biosurfactant production as *Cyberlindnera samutprakarnensis* showed strong biosurfactant activity at the end of the exponential phase. This finding is in agreement with other previous reports such as sophorolipid production by *Candida bombicola* which was found to place when the yeast entered stationary phase under condition of nitrogen limitation, indicating biosurfactant synthesis was not growth-associated (Davila et al., 1992; Hu and Ju, 2001).

Purification by preparative TLC analysis of this biosurfactant indicated the presence of 3 major bands, F1, F2 and F3 with Rf values of 0.89, 0.77 and 0.69, respectively. Among them, F3GP gave highest oil displacement activity and gave positive results with Molisch test indicating the presence of sugar moiety in the molecule. It was undergoing further characterize chemical structures of biosurfactants using MALDI-TOF-MS and NMR methods. The F3GP glycolipid extract was analyzed by MALDI-TOF-MS which revealed that the major spectra were characterized by molecular ions at m/z 574.57 and 663.99 including both elucidated the ^{1}H and ^{13}C NMR spectra could be assigned to be from a typical sophorolipid-type structure. Sophorolipids are typically produced as complex mixtures in fermentation broths, with the composition depending on the carbon source (Hu and Ju, 2001; Asmer et al., 1988). When *Candida bombicola* ATCC22214 was cultured with glucose plus soybean oil as the carbon source the sophorolipid complex produced contained both lactonic and acidic sophorolipids with saturated and unsaturated C16 and C18 fatty acid moieties (Hu and Ju, 2001). In the case of F3GP, this was produced from *Cyberlindnera samutprakarnensis* in GP medium, the sophorolipids produced were

complex, with obtained molecular weights of 574 and 662 that correspond to non acetylated lactonic sophorolipids (SL-C18) and either a monoacetylated acidic sophorolipid (SL-Ac-C19:1) or diacetylated lactonic sophorolipids (SL-Ac-Ac-C16), respectively.

Physicochemical characterization of crude extracts of biosurfactants produced by *Cyberlindnera samutprakarnensis* studied CMC values, emulsification index and stability including stability of the biosurfactant over a wide range of pH, temperature and NaCl. CMC values of crude biosurfactant from *Cyberlindnera samutprakarnensis* at 457.1 mg L^{-1} (0.046 %) showed the lowest surface tension (γST) at 30.9 mN m^{-1}. When compared to biosurfactants from other sources, it was found that the biosurfactant from *Cyberlindnera samutprakarnensis* had the minimum surface tension (γST) value less than those of from *C. bombicola* (39-43.5 mN m^{-1}) (Pekin and Vardar-Sukan, 2006) from *Candida antarctica* (35 mN m^{-1})(Adamczak and Bednarski, 2000) and from *Yarrowia lipotylica* (50 mN m^{-1}) (Amaral et al., 2006). Furthermore, it also had lower CMC value than those of other biosurfactansts from other yeasts previously reported including 0.6% for the biosurfactant from *Candida antractica* (Adamczak and Bednarski, 2000) and 2.5% for the biosurfactant from *Candida lipolytica* (Sarubbo et al., 2007). Moreover, it had greater CMC value than that of SDS, a chemical surfactant, with CMC value of 1280 mg L^{-1} (Pornsunthorntawee et al., 2008).

Crude biosurfactants from *Cyberlindnera samutprakarnensis* could form emulsion with 5 kinds of vegetable oils including olive oil, sesame oil, palm oil, cotton seed oil and safflower oil and gave E24 values higher than 80%. The emulsions were high stable (> 80 %) over a period of 7 days and after 1 month with palm oil and sesame oil. Other studies related with the emulsification activity and stability denoted that the SL produced by *Torulopsis bombicola* ATCC22214 was not able to stabilize emulsions containing water and either hydrocarbons or vegetable oils (Cooper and Paddock, 1984). Sophorolipids from *Torulopsis bombicola* had been shown to reduce surface and interfacial tension, but were not good as emulsifiers (Cavalero and Cooper, 2003). For biosurfactant from *Candida glabrata* UCP1002, it

was efficient in emulsification cotton seed oil and no significant difference in the emulsification index was observed for this substrate when compared to n-hexadecane (Sarubbo et al., 2006). However, the present study showed that crude biosurfacnt produced by *Cyberlindnera samutprakarnensis* when grown on glucose and palm oil had better emulsification activity and stability when compared to those reported by others in the literature (Sarubbo et al., 2006; Cooper and Paddock, 1984). From the stability properties of the biosurfactant from *Cyberlindnera samutprakarnensis* against various environmental effects, this biosurfactant extracts had optimum pH as well as pH stability at pH 8.0. It was stable at high temperature up to 121°C and could tolerate to NaCl at 1.0-10.0%. With physical and chemical properties of crude extracts, this suggested that biosurfacatant produced by *Cyberlindnera samutprakarnensis* has high potential performance to be a good candidate for use in various environments and some industries related to tensioactive compounds.

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Conclusions

A novel yeast namely *Cyberlindnera samutprakarnensis* JP52[T] was found to produce significant levels of glycolipid biosurfactants when cultured in Hua's medium supplemented with 2% (w/v) glucose plus 2% (v/v) palm oil (GP) as the carbon source and the biosurfactant activities were superior when compared with those of the other various tested carbon sources. Of the three main components found in the biosurfactant by TLC, F3GP gave highest oil displacement activity and was confirmed to be a glycolipid by the Molish test. The chemical characterization of its molecular weight and structure by MALDI-TOF-MS and NMR analysis revealed that the major components of the F3GP biosurfactant fraction had molecular weights of 574 and 662, which corresponds to non-acetylated lactonic sophorolipids (SL-C18) and either a monoacetylated acidic sophorolipid (SL-Ac-C19:1) or diacetylated lactonic sophorolipids (SL-Ac-Ac-C16), respectively. Thus, *Cyberlindnera samutprakarnensis* JP52 is a new species of sophorolipid biosurfactant-producing yeast that produces potentially attractive biosurfactants for diverse applications. This biosurfactant was tested to be a good emulsifier and had excellent tensio-active properties. Furthermore, it was produced by using palm oil and glucose as carbon sources, therefore, it could be

produced in large scale with low cost. Therefore from its good properties, this biosurfactant might be an attractive choice for food and oil industrial application.

Suggestions

This research has provided biological database as a resource for biosurfactant producing yeasts and increased two novel yeast species in the ascomycetous yeasts. The presence study has procured much important information on the course of surveying biosurfactant-producing yeast and biosurfactant production by the isolated new species and their physicochemical properties of the biosurfactant produced. However, the available information is still limited on their biosynthetic pathways and their genes involvement. Therefore, further studies from different fields are required in order to promote the research and development of biosurfactant (Van Bogaert et al., 2009).

Optimization condition for yeast growth and biosurfactant production by *Cyberlindnera samutprakarnensis* JP52^T should be additional studied the other on the hydrophilic substrates such as starch, molasses and the hydrophobic substrates such as palm oil fatty acid methyl ester (palm oil FAME) (Davila et al., 1994), waste frying oil (Fleurackers, 2006) including studied the effect of C/P, C/Fe, C/Mg ratios and yeast extract concentration (Amezcua-Vega et al., 2007). The production of biosurfactant can also be investigated in batch or fed-batch fermentation process to enhance the production of biosurfactant. The fed-batch culture could maintain the low level of the residual substrate concentration in the system and avoid the toxic effects. Fed batch process using two stages could supplement the second carbon source in late log phase to increase product yields (Daniel et al., 1998).

Molecular mass analysis by MALDI-TOF MS and the structural elucidation determination by high resolution 1H- and 13C-NMR spectroscopy of biosurfactant produced by *Cyberlindnera samutprakarnensis* JP52^T in medium with 2% glucose and 2% palm oil revealed the complex mixtures of sophorolipid with acidic and lactonic form. These finding may be use in various application in washing detergent, antimicrobial substances in cosmetics or further modify chemical derivatization of sophorolipids. In the study of Azim and coworker (2006) evaluated the derivative

sophorolipids to test antibacterial, anti-HIV and spermicidal activity. Especially the physicochemical properties of crude biosurfactant of GP medium demonstrated high activities and stabilization in tensio-active properties against to high salts, heat tolerance and active in broaden pH condition including the strong emulsification with various vegetable oils, consequently it should be further studied the emulsifying property used in food industry.

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APPENDIX

APPENDIX 1   **Culture media**

1.1   **Yeast extracts malt extract (YM) broth composition**

Yeast extract	3	g
Malt extract	3	g
Peptone	5	g
Glucose	10	g
Deionized water	1000	ml

Sterile at 121ºC for 15 min.

1.2   **Yeast extract malt extract (YM) agar**

Yeast extract	3	g
Malt extract	3	g
Peptone	5	g
Glucose	10	g
Agar	15	g
Deionized water	1000	ml

Sterile at 121ºC for 15 min.

1.3   **Acidified yeast extract malt extract agar**

Yeast extract	3	g
Malt extract	3	g
Peptone	5	g
Glucose	10	g
Chloramphenicol	0.1	g
Sodium propionate	0.20	g
Agar	20	g
Deionized water	1000	ml

Adjust pH 3.7-3.8 with 1 N HCl

Sterile at 121ºC for 15 min.

1.4   YM broth supplemented with 20% (w/v) glycerol: Media for yeast maintenance and storage

Yeast extract	3	g
Malt extract	3	g
Peptone	5	g
Glucose	10	g
Glycerol	100	ml
Deionized water	1000	ml

Sterile at 121ºC for 15 min.

1.5   Yeast extract peptone dextrose (YPD) agar

Yeast extract	10	g
Peptone	20	g
Glucose	20	g
Agar	15	g
Deionized water	1000	ml

Sterile at 121ºC for 15 min.

1.6   Potato dextrose agar (PDA)

Potato	200	g
Dextrose	20	g
Agar	15	g
Deionized water	1000	ml

Sterile at 121ºC for 15 min.

1.7   Corn meal agar (CMA)

Corn meal agar	1.7	g
Deionized water	1000	ml

Sterile at 121ºC for 15 min.

1.8   5% Malt extract agar (MEA)

Malt extract	50	g
Agar	15	g
Deionized water	1000	ml

Sterile at 115ºC for 15 min.

1.9   Fowell's acetate agar

Sodium acetate	5	g
Agar	20	g
Deionized water	1000	ml

Sterile at 121ºC for 15 min.

1.10   Sugar stock solutions for fermentation test

Sugar stock solutions: glucose (positive control), galactose, sucrose, maltose, lactose, melibiose and fructose were prepared at 6% except for raffinose was at 12% , they were sterilized by filtration (0.22 µm membrane filter) and kept in screw-capped sterile test tubes and stored at -20ºC.

1.10.1 Fermentation basal medium

Yeast extract	4.5	g
Peptone	7.5	g
Deionized water	1000	ml

Add 4 ml of bromothymol blue (stock: 50mg/75 ml) to give a

sufficiently dark green color.

Transfer 2 ml of fermentation basal medium into 13 x 100 mm cotton plugged test tubes containing Durham tubes. Sterile at 121ºC for 15 min and check for the air inside Durham tube. Add 1 ml of each stock solution of sugars aseptically to give a final concentration of 2% (w/v) except for raffinose was 4%.

1.10.2 Fermentation test of glucose

Yeast extract	3	g
Peptone	5	g
Glucose	20	g
Deionized water	1000	ml

Add a few drops of bromothymol blue to give a sufficiently dark green color.

Transfer 3 ml of glucose fermentation medium into 13 x 100 mm cotton plugged test tubes containing Durham tubes. Sterilize at 110ºC for 15 min.

1.11 Media for assimilation of carbon compounds

Carbon compounds: D-glucose, D-galactose, L-sorbose, sucrose, maltose, cellobiose, trehalose, lactose, melibiose, raffinose, melezitose, inulin, soluble starch, D-xylose, L-arabinose, D-arabinose, D-ribose, L-rhamnose, ethanol, glycerol, erythritol, ribitol, galactitol, D-mannitol, D-glucitol, α-methyl-D-glucoside, salicin, glucono-δ-lactone, 2-keto-D-gluconate, 5-keto-D-gluconate, DL-lactic acid, succinate, citrate, inositol, D-glucoronic acid , D-galacturonic acid, xylitol, L-arabinitol, D-gluconate, D-glucosamine, N-acetyl-D-glucosamine, propane-1,2 diol, butane-2,3 diol, and methanol were used. Negative control was a basal medium without carbon compound added.

Stock solution of carbon compound (10X)

Yeast nitrogen base	6.7	g
Carbon compound	5	g
Deionized water	100	ml

Filter sterilization using membrane filter(pore size 0.22 μm) and store in freezer

Add 0.2 ml of stock solution of each carbon compound (10X) into 13 x 100 mm cotton plugged test tubes containing 1.8 ml of sterile water and mix well. Place at ambient temperature for 3 day to check for contamination.

For assimilation test of 7 carbon compounds including inulin, ethanol, methanol, soluble starch, galactitol, 2-keto-D-gluconate and 5-keto-D-gluconate, the carbon compound solutions were freshly at 1x strength and sterilized by filtration.

1.12   Stock solution of yeast carbon base, YCB, (10X)

Yeast carbon base   11.7   g

Deionized water   100   ml

Sterilize at 121ºC for 15 min.

1.13   Yeast carbon base (1X)

Add 0.2 ml of yeast carbon base stock solution (10X) into 13 x 100 mm cotton plugged test tubes containing 1.8 ml of sterile water and mix well. Place at ambient temperature for 3 day to check for contamination.

1.14   Media for assimilation of nitrogen compounds

Stock nitrogen solution (10X)

Yeast carbon base   11.7   g

Nitrogen compound   X   g

Deionized water   100   ml

Filter sterilization using membrane filter (pore size 0.22 μm)

and store at -20ºC

Nitrogen compound: Ammonium sulphate [$(NH_4)_2SO_4$] 0.5 g, Potassium nitrate [KNO_3] 0.78 g, Sodium nitrite [$NaNO_2$] 0.26 g, Ethylamine hydrochloride 0.64 g, L-lysine-HCl 0.56 g and Cadaverinedihydrochloride 0.68 g, pH 5.5-6.5 were used.

Media for assimilation of nitrogen compounds were prepared by suspending 1.67 g of agar in 90 ml of water and sterilized at 121ºC for 15 min, after that 10 ml each of stock nitrogen solutions was add aseptically into molten agar, rapidly mix well and pour into sterilized petridish. YCB agar plus ammonium sulphate was used as a positive control whilst YCB agar without nitrogen compound was used as a negative control.

1.15 Vitamin free medium

Glucose	10	g
Vitamin assay cassamino acids	5	g
Potassium dihydrogenphosphate (KH_2PO_4)	1	g
Magnesium sulphate ($MgSO_4.2H_2O$)	0.5	g
Sodium chloride (NaCl)	0.1	g
Calcium chloride ($CaCl_2.2H_2O$)	0.1	g
Deionized water	1000	ml

Adjust to pH 5.5, distribute in test tubes (2ml/tube)

Sterilize at 121ºC for 15 min.

1.16 Acid chalk medium (Custer's chalk medium)

Yeast extract	5	g
Glucose	50	g
Calcium carbonate ($CaCO_3$)	5	g
agar	15	g
Deionized water	1000	ml

Sterilize at 121ºC for 15 min.

Molten medium at approximately 50ºC was agitated gently to resuspend $CaCO_3$ thoroughly and poured into petridish.

1.17 High osmotic pressure for growth test

Media supplementing with 50% (w/v) glucose

Yeast extract	1	g
Glucose	50	g
agar	1.3	g
Deionized water	100	ml

Distribute in test tubes and sterilized at 110ºC for 15 min and made into slants.

Media supplementing 60% (w/v) glucose

Yeast extract	1	g
Glucose	60	g
agar	1.3	g
Deionized water	100	ml

Distribute in test tubes and sterilize at 110ºC for 15 min and made into slants.

1.18   Urease test (Christensen's urea agar)

Peptone	1	g
Glucose	1	g
NaCl	5	g
KH_2PO_4	2	g
Phenol red	12	µg
agar	15	g
Deionized water	1000	ml

Adjusted pH to 6.8

Distribute 4.5 ml in test tubes and sterilized at 121ºC for 15 min. Then add 0.5 ml of filtered sterile 20% solution of urea, mix and made into slant.

1.19   Cycloheximide resistance test

Basal medium (10X)

Cycloheximide 1 g suspend in 2.5 ml of acetone

Yeast nitrogen base	6.7	g
Deionized water	100	ml

Filter sterilization using membrane filter (pore size 0.22 µm) and store in refrigerator.

An aliquot (0.2 ml) of antibiotic basal medium was aseptically added to sterile distilled water (1.8 ml) in test tubes.

(Liquid yeast nitrogen base with D-glucose, as for the assimilation test, with cycloheximide added to give a final concentration of either 0.1% or 0.01% (w/v). The results are read and scored as well as for the carbon assimilation test.

1.20 Gelatin liquefaction test

gelatin	100	g
Glucose	5	g
agar	6.7	g
Deionized water	1000	ml

Distribute 4.5 ml in test tubes and sterilize at 121ºC for 15 min and made into slants.

1.21 Hua's medium

KH_2PO_4	0.20	g
$MgSO_4.7H_2O$	0.20	g
$NaNO_3$	4.0	g
Yeast extracts	1.0	g
Deionized water	1000	ml

Sterile at 121ºC for 15 min.

APPENDIX 2   Chemicals

2.1 Bromothymol blue stock solution

Dissolve 50 mg of bromothymol blue in 75 ml of deionized water.
For instruction, 4 ml of bromothymol blue solution was added into 100 ml of fermentation basal medium and fermentation test of glucose.

2.2 Lugol's solution

iodine	1	g
Potassium iodide (KI)	2	g
Deionized water	300	ml

2.3   50X TAE buffer

Tris buffer	48.4	g
0.5 M EDTA (pH8.0)	20	ml
Glacial acetic acid,conc.	11.5	ml
Deionized water, adjust to	100	ml
Sterile at 121ºC for 15 min.		

2.4   10X TBE buffer

Tris base	10.8	g
Boric acid	5.5	g
0.5 M EDTA (pH8.0)	4	ml
Deionized water, adjust to	100	ml
Sterile at 121ºC for 15 min.		

2.5   Loading dye

Bromphenol blue	0.25	g
Glycerol	30	ml
Deionized water, adjust to	100	ml

2.6   Stock ethidium bromide

1 g of ethidium bromide was dissolved in 100 ml of purified water. The solution was kept in a dark bottle at room temperature.

APPENDIX 3 Preparation of visible suspension of an actively growing culture

A loop of active growing yeast of about 2-3 days old was suspended in 3 ml of sterile distilled water and then mixed well to disperse the cells thoroughly. A yeast suspension was assessed by visual inspection for an increase in the turbidity of the solution. Each tube was scored against 5 black lines with thickness 0.75 mm and width distance 5 mm on a white card. The result is scored as 3+ if the lines are completely obscured; as 2+ if the lines appear as diffuse bands; as 1+ if the lines are distinguishable as such but have blurred edges; as negative (-) if the lines are distinct and sharp edged.

For growth test evaluation in this experiment, yeast suspension was acquired if the turbidity result has read as +2. Each of test tubes containing the test media was inoculated with 2 dropped of the yeast suspension with sterile Pasteur pipette.

APPENDIX 4   **PCR mixture reaction**

4.1   **D1/D2 domain of LSU rDNA**

Component	Volume (µl)
Sterile nano pure water	57.5
10X Taq buffer	10.0
MgCl$_2$ (25 mM)	8.0
dNTP mixture (2.5 mM)	8.0
Primer NL1 (10pM)	3.0
Primer NL4 (10pM)	3.0
Taq polymerase (Fermentas; 5U/µl)	0.5
DNA template *	10.0
Total volume	100

PCR mixture without DNA template was mixed very well and then transfered to PCR tube (90 µl of reaction mixture/tube, total volume was equal to 100 µl/tube).

Add DNA template (< 1 µg) 1 µl/tube for optimization (total volume = 10 µl), 10 µl of optimum DNA template/tube for large scale (total volume = 100 µl). Mixed and short spin.

4.2   **ITS1-2 region**

This mixture reaction for ITS1-2 region was prepared similarly to that of D1/D2 domain as described above except the primers were ITS1 and ITS4.

4.3   ***EF2* and*ACT1* gene of LSU rDNA**

The resulting amplicons ranged in size from approximately 600 (*EF2* and*ACT1*) to 1,400 (26S rDNA) nucleotides. The PCR mixture consisted of 25 µl of 75 mM Tris-

HCl (pH 8.0), 20 mM $(NH_4)_2SO_4$, 1.5 mM $MgCl_2$, 0.01% Tween 20, 7.5 pmol of each primer, 100 μM deoxynucleoside triphosphates, 1.0 U of *Taq*polymerase (Marsh), and 20 ng of genomic DNA.

APPENDIX 5   **Purification of PCR product by using Gel/PCR DNA fragments extraction kit (Geneaid)**

5.1   Sample preparation

5.1.1 Transfer 100 μl of PCR-reaction product to a micro centrifuge tube.

5.1.2 Add 5 volumes of DF buffer to 1 volume of the sample and mix by vortex.

5.2   DNA binding

5.2.1 Place a DF column in a 2 ml collection tube.

5.2.2 Apply the sample mixture from the previous step into the DF column.

5.2.3 Centrifuge at 13,000 rpm for 30 seconds.

5.2.4 Discard the flow-through and place the DF column back into the collection tube.

5.3   Wash

5.3.1 Add 600 μl of wash buffer (absolute ethanol added) in the center of the DF column.

5.3.2 Centrifuge at 13,000 rpm for 30 seconds.

5.3.3 Discard the flow-through and place the DF column back into the collection tube.

5.3.4 Centrifuge again for 3 minutes at 13,000 rpm to dry the column matrix.

5.4   DNA elution

5.4.1 Transfer the dried column to a new microorganism tube.

5.4.2 Add 30 μl of nano pure water in the center of the column matrix.

5.4.3 Let stand for 5-10 minutes (ethanol is evaporated) until the water is absorbed by the matrix. Centrifuge for 2 minutes at full speed to elute the purified DNA.

APPENDIX 6   **Gel electrophoresis**

Agarose preparation

0.8% agarose in 1X TAE buffer (50 X TAE buffer: 242 g Tris base, 57.1 ml glacial acetic acid, 37.2 g Na_2EDTA)

1. Drop 1.0 µl of dye solution on paraffin paper.
2. Add 3 µl of PCR product and mix well.
3. Add DNA solution to agarose gel.
4. Run time approximately 35 minutes at 100 volts.

APPENDIX 7　　　　**Glucose standard curves**

y = 0.5916x
R² = 0.9883
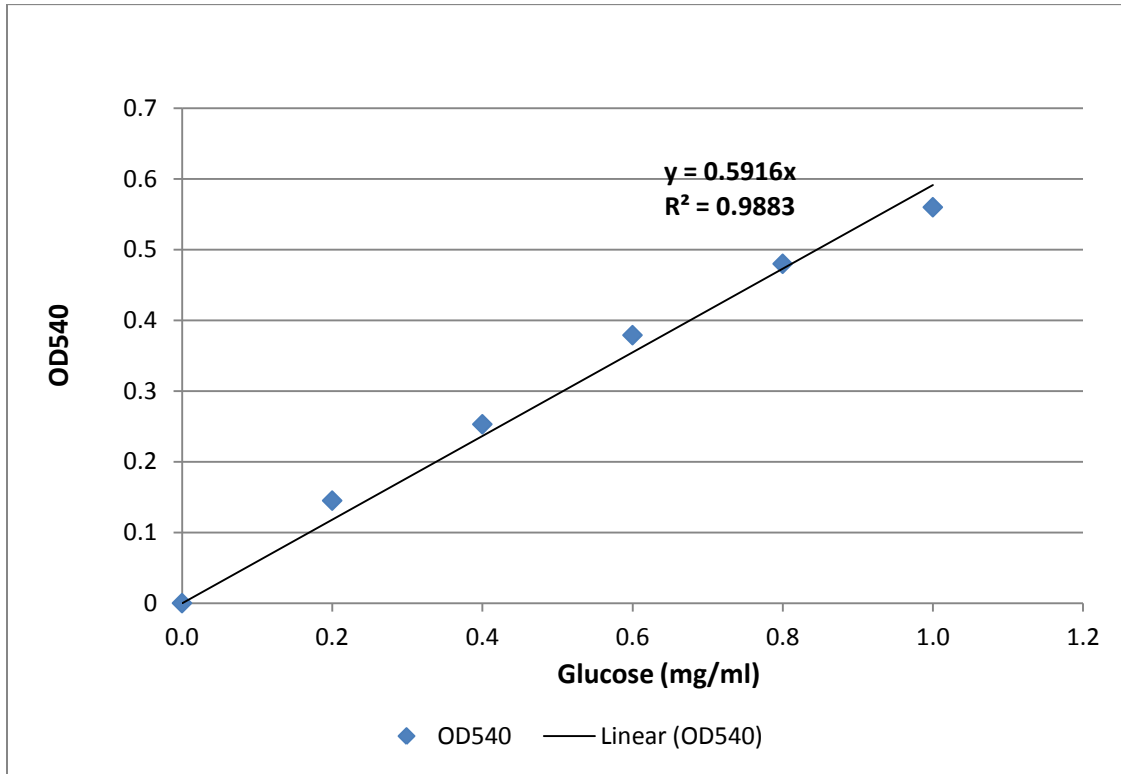
APPENDIX 8   **Nitrogen standard curves**

$y = 0.283x$

$R^2 = 0.9934$
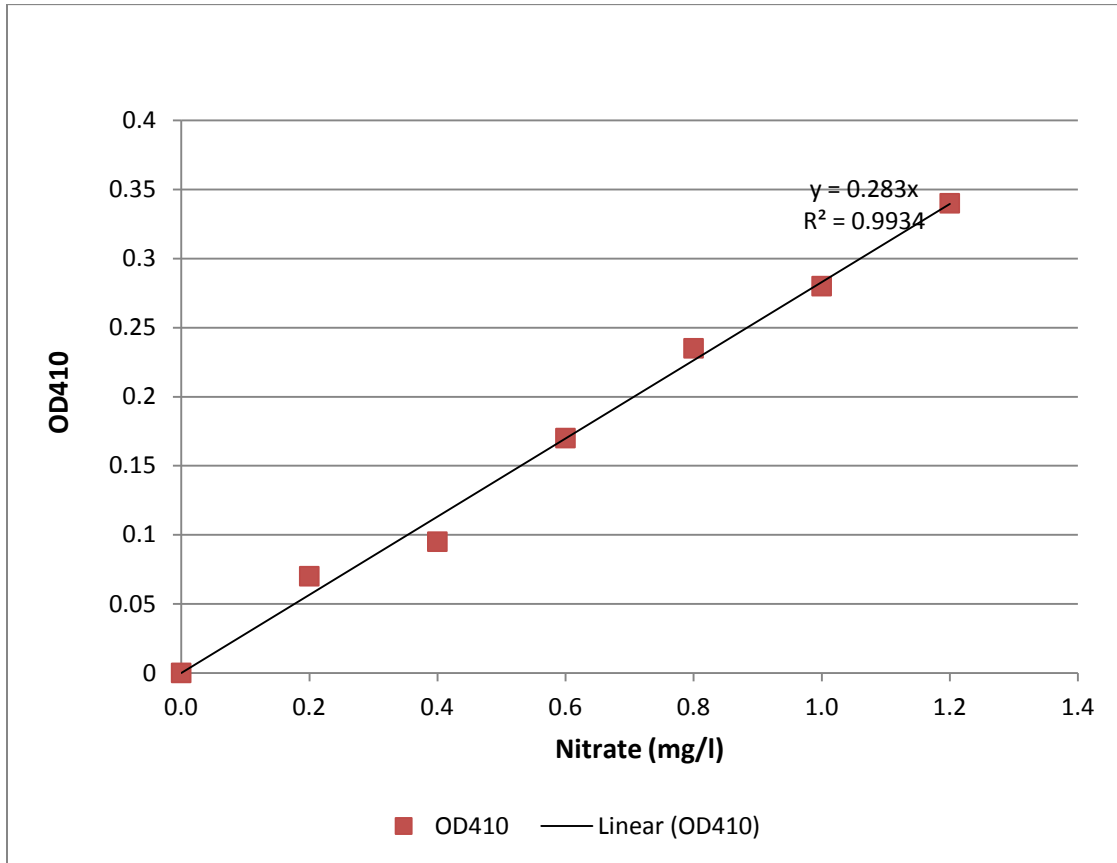
APPENDIX 9   Biosurfactant activities determining oil displacement areas (ODA) and surface tension (ST) of 94 isolates analyzed by DNA sequencing

Seq.isolates JP	Closet species (Accession number)	Results	Name	pH	ODA (cm²)	minST (mN/m)
1	*Meyerozyma guilliermondii* (U45709)	Known species	RN8-2A	8.16	26.13	29.50
2	*Meyerozyma guilliermondii* (U45709)	Known species	SOM2-B2-3A	7.82	22.35	29.00
3	*Candida mucifera* CBS7409^T (AJ508572)	Known species	PS1-5B	6.85	0.22	33.33
4	*Candida tropicalis* (U45749)	Known species	RK1-Y1-1	4.02	0.32	39.83
5	*C.viswanathii* NRRL Y-17317 (U45755)	Known species	SM1-Y4-1	4.52	4.78	36.50
6	*Candida mucifera* CBS7409^T (AJ508572)	Known species	SOM1-Y1-1A	8.27	22.63	29.00
7	*Meyerozyma guilliermondii* (U45709)	Known species	RN8-1A	7.86	28.60	29.67
8	*Candida mucifera* CBS7409^T (AJ508572)	Known species	SOM1-Y1-2B	8.14	20.17	29.33
9	*Candida mucifera* CBS7409^T (AJ508572)	Known species	SK1-3A	7.6	44.99	28.67
10	*Candida tropicalis* (U45749)	Known species	RK2-Y1-1	4.18	0.25	35.83
11	*Trichosporon asahii* CBS2479^T (EF559350)	Known species	RK1-Y1-2	7.08	0.89	39.67
12	*Candida tropicalis* (U45749)	Known species	SM1-Y3-2	4.34	0.32	37.83
13	*Trichosporon asahii* CBS2479^T (EF559350)	Known species	SOM2-B2-3A	7.82	22.35	29.00
14	*Trichosporon asahii* CBS2479^T (EF559350)	Known species	SOM2-B2-3B	7.82	22.35	29.00
15	*Meyerozyma guilliermondii* (U45709)	Known species	SOM2-1A	8.15	20.97	29.00
16	*Meyerozyma guilliermondii* (U45709)	Known species	SOM2-1B	8.15	20.97	29.00
17	*Meyerozyma guilliermondii* (U45709)	Known species	CH10-Y2-2A	8.18	43.80	29.17
18	*Meyerozyma guilliermondii* (U45709)	Known species	CH10-Y2-2B	8.18	43.80	29.17
19	*Meyerozyma guilliermondii* (U45709)	Known species	RN8-2B(A)	8.16	26.13	29.50
20	*Candida mucifera* CBS7409^T (AJ508572)	Known species	RN8-2B(B)	8.16	26.13	29.50
21	*Meyerozyma guilliermondii* (U45709)	Known species	RN8-1B	7.86	28.60	29.67
22	*Meyerozyma guilliermondii* (U45709)	Known species	SOM1-Y1-2A	8.14	20.17	29.33
23	*Candida mucifera* CBS7409^T (AJ508572)	Known species	SK4-2A	8.12	31.85	29.50
24	*Meyerozyma guilliermondii* (U45709)	Known species	SK4-2B	8.12	31.85	29.50
25	*Candida mucifera* CBS7409^T (AJ508572)	Known species	RN6-4A	7.07	35.62	27.33
26	*Meyerozyma guilliermondii* (U45709)	Known species	RN8-B3-1A	7.38	33.20	28.83
27	*Meyerozyma guilliermondii* (U45709)	Known species	RN8-5A	6.86	33.54	26.17
28	*Meyerozyma guilliermondii* (U45709)	Known species	RN8-5B	6.97	0.79	36.67
29	*Candida palmioleophila* (U45758)	Known species	OR-B1-1A	6.88	21.25	31.33
30	*T. faecale* CBS4828^T, (AF105395)	Known species	OR-B1-1B	6.88	21.25	31.33
31	*Trichosporon asahii* CBS2479^T (EF559350)	Known species	OR-Y1-1	7.03	39.24	27.00
32	*Candida mucifera* CBS7409^T (AJ508572)	Known species	PC1-B1-1	6.92	22.35	27.33
33	*Candida mucifera* CBS7409^T (AJ508572)	Known species	RN3-Y1-1	6.24	12.99	26.33
34	*Candida mucifera* CBS7409^T (AJ508572)	Known species	SOM2-3A	8	15.68	29.33
35	*Meyerozyma guilliermondii* (U45709)	Known species	RN8-1B	7.86	28.60	29.67
36	*Meyerozyma guilliermondii* (U45709)	Known species	SK1-3B	7.6	44.99	28.67

Seq.isolates JP	Closet species (Accession number)	Results	Name	pH	ODA (cm^2)	minST (mN/m)
37	*Candida mucifera* CBS7409^T (AJ508572)	Known species	OR-Y1-1A	7.03	39.24	27.00
38	*Candida mucifera* CBS7409^T (AJ508572)	Known species	RN8-B3-1B	7.38	33.20	28.83
39	*Meyerozyma guilliermondii* (U45709)	Known species	CH6-Y1-1	6.86	33.54	26.17
40	*Candida mucifera* CBS7409^T (AJ508572)	Known species	PC3-Y1-2	6.82	22.07	29.00
41	*Candida mucifera* CBS7409^T (AJ508572)	Known species	PC3-B2-1	7.4	41.49	26.50
42	*Meyerozyma guilliermondii* (U45709)	Known species	PC3-Y1-1	7.99	0.22	39.83
43	*Candida mucifera* CBS7409^T (AJ508572)	Sister species	CH5-B1-2	4	0.32	39.33
44	*Candida mucifera* CBS7409^T (AJ508572)	Known species	CH5-Y1-1	7.5	32.52	26.83
45	*Candida mucifera* CBS7409^T (AJ508572)	Known species	PC1-Y3-2B	6.92	22.35	27.33
46	*Candida mucifera* CBS7409^T (AJ508572)	Known species	RN1-B2-2	6.1	0.15	35.33
47	*Candida mucifera* CBS7409^T (AJ508572)	Known species	PC3-B1-1	5.71	16.15	28.67
48	*Candida mucifera* CBS7409^T (AJ508572)	Known species	PC3-2	6.92	20.70	27.67
49	*Meyerozyma guilliermondii* (U45709)	Known species	PC3-Y2-2	7.6	43.41	30.00
50	*Meyerozyma guilliermondii* (U45709)	Known species	CH6-Y2-1A	7.47	1.07	38.83
51	*Candida mucifera* CBS7409^T (AJ508572)	Known species	CH6-Y2-1B	6.92	27.04	28.00
52	*C. mengyuniae* strain Bu F3-16 (AB741072)	New species	SM1-Y1-1	6.79	0.42	34.83
53	*Candida tropicalis* (U45749)	Known species	RK8-Y1-2	4.16	0.50	39.67
54	*Candida tropicalis* (U45749)	Known species	SM1-Y3-3	5.95	0.22	37.83
55	*Trichosporon asahii* CBS2479^T (EF559350)	Known species	SOM2-Y3-1	8.26	14.75	30.00
56	*Trichosporon asahii* CBS2479^T (EF559350)	Known species	SOM2-Y3-2	7.9	16.63	31.33
57	*Candida tropicalis* (U45749)	Known species	SM1-B3-4	4.26	0.25	40.17
58	*Candida tropicalis* (U45749)	Known species	RN1-Y2-1	4.07	0.25	36.67
59	*Scheffersomyces spartinae* (JQ689045)	New species	PB4-7	3.92	0.50	40.33
60	*Scheffersomyces spartinae* (JQ689045)	New species	PB1-3-8	4.37	0.84	38.83
61	*Candida tropicalis* (U45749)	Known species	SM1-B2-1	3.97	0.28	39.67
62	*Candida tropicalis* (U45749)	Known species	SM1-B4-4	3.95	0.39	36.67
63	*Meyerozyma guilliermondii* (U45709)	Known species	PB1-3-2	3.84	0.68	36.50
64	*Trichosporon asahii* CBS2479^T (EF559350)	Known species	SOM1-Y3-1	8.01	30.53	29.17
65	*Candida palmioleophila* (U45758)	Known species	PC1-B2-3	7	38.13	27.00
66	*Candida tropicalis* (U45749)	Known species	PC1-B3-1	4.17	0.25	40.17
67	*Meyerozyma guilliermondii* (U45709)	Known species	SOM2-B1-1	8.13	30.86	30.33
68	*Candida rugosa* (U45727)	Known species	SK4-Y2-2	7.33	0.13	40.00
69	*Issatchenkia orientalis* (U76347)	Known species	SK3-Y1-2	4.95	0.15	36.83
70	*Trichosporon asahii* CBS2479^T (EF559350)	Known species	SOM1-B2-1	8.19	30.86	29.17
71	*Candida palmioleophila* (U45758)	Known species	CH5-B3-1	7.38	5.73	28.67
72	*Candida mucifera* CBS7409^T (AJ508572)	Known species	CH6-B3-1A	7.48	47.40	29.17
73	*Meyerozyma guilliermondii* (U45709)	Known species	PC2-Y3-2A	6.75	0.32	39.17
74	*Meyerozyma guilliermondii* (U45709)	Known species	RN1-4	6.41	2.94	40.33
75	*Candida mucifera* CBS7409^T (AJ508572)	Known species	CH5-B2-1	7.32	46.99	29.33
76	*Meyerozyma guilliermondii* (U45709)	Known species	SK4-B1-1	8.14	45.38	29.00

Seq.isolates JP	Closet species (Accession number)	Results	Name	pH	ODA (cm²)	minST (mN/m)
77	*Meyerozyma guilliermondii* (U45709)	Known species	SK4-B2-1	7.19	2.18	39.00
78	*Candida mucifera* CBS7409ᵀ (AJ508572)	Sister species	SOM1-B1-3	5.49	0.20	40.33
79	*Meyerozyma guilliermondii* (U45709)	Known species	CH9-2	7.15	21.79	28.17
80	*Meyerozyma guilliermondii* (U45709)	Known species	SOM1-1	7.84	0.09	40.33
81	*Candida tropicalis* (U45749)	Known species	SOM1-Y2-1	4.67	0.35	38.50
82	*Trichosporon asahii* CBS2479ᵀ (EF559350)	Known species	SOM1-Y3-2	7.75	12.16	29.33
83	*Candida tropicalis* (U45749)	Known species	SM1-B3-1	5.29	0.35	39.83
84	*Prototheca zopfii* var. zopfii ATCC16533 (AB183201)	New species	PB4-4	3.63	0.32	40.33
85	*Meyerozyma guilliermondii* (U45709)	Known species	CH9-5	4.68	9.44	28.67
86	*Meyerozyma guilliermondii* (U45709)	Known species	SK1-5B	7.14	0.35	38.33
87	*Meyerozyma guilliermondii* (U45709)	Known species	CH10-Y2-1	7.59	46.99	29.17
88	*Meyerozyma guilliermondii* (U45709)	Known species	SK4-Y1-1	7.55	0.15	36.00
89	*Meyerozyma guilliermondii* (U45709)	Known species	SK4-Y2-2	7.33	0.13	40.00
90	*Meyerozyma guilliermondii* (U45709)	Known species	CH7-Y1-1	4.07	0.25	37.83
91	*Meyerozyma guilliermondii* (U45709)	Known species	CH7-Y2-1	5.33	3.47	30.00
92	*Candida palmioleophila* (U45758)	Known species	SK3-Y3-1	5.4	0.17	36.00
93	*Meyerozyma guilliermondii* (U45709)	Known species	CH10-B2-1	8.18	43.80	29.17
94	*Meyerozyma guilliermondii* (U45709)	Known species	CH10-B1-2	6.62	4.40	32.17

BIOGRAPHY

Miss Jamroonsri Poomtien was born on January 14, 1970 in Bangkok, Thailand. She gradutated with the degree of Master of Science (Industrial Microbiology) from the Department of Microbiology, Faculty of Science, Chulalongkorn University in 1995. She has studied for the Doctor of Philosophy (Ph.D.) Degree in the Microbiology Program at Department of Microbiology, Faculty of Science, Chulalongkorn University since 2008.

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She had presented some parts of her works in:

Poomtien, J., Jindamorakot, S., Pinpanichakarn, P., and Thaniyavarn, J. (2010). Biodiversity of efficient biosurfactant-producing yeasts isolated from soil and agricultural materials. In Proceedings of International Conference on iotechnology for Healthy Living, The 22nd Annual Meeting of the Thai Society for Biotechnology, pp.707-714. 20-22 October 2010, Prince of Songkla University, Trang Campus, Thailand. (Poster Presentation).

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