Screening of Oleaginous Yeasts and Optimization for Lipid Production Using Crude Glycerol as a Carbon Source

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ชื่อวิทยานิพนธ์ การคัดแยกยีสต์ไขมันสูงและสภาวะที่เหมาะสมในการผลิตลิปิดโดยใช้กลีเซอรอลดิบเป็นแหล่งคาร์บอน

ผู้เขียน นางสาวศุลีพร กิจจะ

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ปีการศึกษา 2554

บทคัดย่อ

งานวิจัยนี้ศึกษาการคัดแยกยีสต์จากดินและของเสียของโรงงานสกัดน้ำมันปาล์มและโรงงานผลิตไบโอดีเซลในภาคใต้ของประเทศไทยโดยใช้กลูโคสหรือกลีเซลรอลดิบเป็นแหล่งคาร์บอนในสภาวะที่เป็นกรด (พีเอช 4.0) และสภาวะที่เป็นกรดเล็กน้อย (พีเอช 6.0) ที่เติมสารคลอแรมฟินิคอลร้อยละ 0.0001 พบว่าสามารถคัดแยกเชื้อยีสต์ได้ทั้งหมด 889 ไอโซเลท เมื่อทดสอบการสะสมลิปิดโดยการย้อมด้วยซูดานแบล็คบี พบเชื้อยีสต์ที่ย้อมติดสีซูดานแบล็คบีทั้งหมด 23 ไอโซเลท เมื่อนำเชื้อที่คัดเลือกได้มาเลี้ยงในอาหารที่ใช้กลีเซอรอลดิบเป็นแหล่งคาร์บอน พบว่าเชื้อไอโซเลท BY4-523 มีปริมาณลิปิดต่อเซลล์สูงสุดถึงร้อยละ 53.28 ในขณะที่เชื้อไอโซเลท JU4-57 เจริญเร็วที่สุดและให้ปริมาณชีวมวลสูงสุด โดยให้ปริมาณลิปิดต่อเซลล์ร้อยละ 41.5 จากการจำแนกสายพันธุ์ด้วยการวิเคราะห์ลำดับเบส 26 เอสอาร์ดีเอ็นเอ ยีสต์สายพันธุ์ JU4-57 และ BY4-523 คือ สายพันธุ์ *Trichosporonoides spathulata* (GenBank accession number AF335526.1) และ *Kodamaea ohmeri* (GenBank accession number FM180533.1) ตามลำดับ มีความเหมือนร้อยละ 99 (497/502 bp) และ 99 (457/460 bp) ตามลำดับ

ในการทดลองหาสภาวะที่เหมาะสมในการเลี้ยงเชื้อทั้งสองสายพันธุ์โดยใช้กลีเซอรอลดิบเป็นแหล่งคาร์บอน พบว่าแหล่งไนโตรเจนอินทรีย์ที่เหมาะสม คือ ยีสต์สกัดผสมเปปโตน (1:1) ซึ่งให้ปริมาณชีวมวลสูงสุดเท่ากับ 17.05 กรัมต่อลิตร สำหรับเชื้อ *T. spathulata* JU4-57 และ 11.1 กรัมต่อลิตร สำหรับเชื้อ *K. ohmeri* BU4-523 และทำให้เชื้อผลิตลิปิดสูงสุดเท่ากับ 10.23 กรัมต่อลิตร สำหรับเชื้อ *T. spathulata* JU4-57 และ 4.53 กรัมต่อลิตร สำหรับเชื้อ *K. ohmeri* BU4-523 อย่างไรก็ตามเนื่องจากแหล่งไนโตรเจนอินทรีย์มีราคาสูง เพื่อเป็นการลดต้นทุนของแหล่งไนโตรเจน ในงานวิจัยนี้ได้ศึกษาผลของการใช้แหล่งอนินทรีย์ไนโตรเจนที่มีราคาถูกกว่ามาก ผลการทดลองพบว่าแอมโมเนียมซัลเฟทเป็นแหล่งอนินทรีย์ไนโตรเจนที่เหมาะสมที่ให้ชีวมวลสูงกว่าแหล่งอนินทรีย์ไนโตรเจนอื่น องค์ประกอบอาหารที่เหมาะสมทั้งสอง

สายพันธุ์ คือ แอมโมเนียมซัลเฟทร้อยละ 0.5 และกลีเซอรอลดิบร้อยละ 10 (คิดเป็นสัดส่วนคาร์บอนต่อไนโตรเจนเท่ากับ 17.2) ภายใต้สภาวะนี้เชื้อ *T. spathulata* JU4-57 ให้ชีวมวลสูงสุดเท่ากับ 10.23 กรัมต่อลิตร และผลิตลิปิดเท่ากับ 3.87 กรัมต่อลิตร เช่นเดียวกับ *K. ohmeri* BU4-523 ให้ชีวมวลสูงสุดเท่ากับ 10.5 กรัมต่อลิตร และผลิตลิปิดเท่ากับ 3.22 กรัมต่อลิตร ผลการทดลองนี้แสดงให้เห็นว่ายีสต์ที่แยกได้ใหม่นี้สามารถเจริญและสะสมลิปิดต่อเซลล์สูงในอาหารที่ใช้กลีเซอรอลดิบเป็นหลักและเติมเพียงแอมโมเนียมซัลเฟท เมื่อทดสอบชนิดของลิปิดที่ผลิตพบว่า *T. spathulata* JU4-57 ผลิตลิปิดที่มีคุณสมบัติเป็นกลางในรูปของโมโนเอซิลกลีเซอไรด์ถึงร้อยละ 68.7 และไตรกลีเซอไรด์ร้อยละ 29.3 ขณะที่เชื้อ *K. ohmeri* ผลิตโมโนกลีเซอไรด์เป็นหลักถึงร้อยละ 94.5 และไตรกลีเซอไรด์เพียงเล็กน้อย

งานวิจัยนี้ทำการเลี้ยงสาหร่ายขนาดเล็กร่วมกับยีสต์เพื่อเพิ่มชีวมวลและการผลิตลิปิด เนื่องจากสาหร่ายขนาดเล็กมีความสามารถในการดูดซับคาร์บอนไดออกไซด์และให้ออกซิเจน พบว่าการเลี้ยงเชื้อผสมของยีสต์ *T. spathulata* JU4-57 กับ *Chlorella vulgaris* var. *vulgaris* TISTR 8261 ให้ชีวมวลดีที่สุด คือ 11.13 กรัมต่อลิตร และผลิตลิปิดสูงสุดเท่ากับ 4.55 กรัมต่อลิตร ในขณะที่การเลี้ยงเชื้อร่วมกันของ *T. spathulata* JU4-57 กับสาหร่ายน้ำจืดขนาดเล็ก *Chlorella* sp. และสาหร่ายทะเลขนาดเล็ก *Chlorella* sp. ให้ชีวมวลเท่ากับ 10.6 กรัมต่อลิตร และ 10.18 กรัมต่อลิตร ตามลำดับ จากการศึกษาสภาวะที่เหมาะสมสำหรับการเลี้ยงเชื้อผสม พบว่ามีการผลิตลิปิดเพิ่มขึ้นเท่ากับ 4.93 กรัมต่อลิตร เมื่อใช้สัดส่วนยีสต์และสาหร่ายขนาดเล็กที่ 3.4×10^6 และ 3.9×10^5 เซลล์ต่อมิลลิลิตร ตามลำดับ และให้แสงเท่ากับ 4,000 ลักซ์ ซึ่งสูงกว่าการผลิตลิปิดของยีสต์และสาหร่ายขนาดเล็กที่เลี้ยงแบบเชื้อเดี่ยวรวมกันที่มีค่าเท่ากับ 4.14 กรัมต่อลิตร นอกจากนี้งานวิจัยนี้ยังศึกษาการเลี้ยงยีสต์ไขมันสูงสายพันธุ์ *Rhodotorula glutinis* TISTR 5159 ร่วมกับสาหร่ายขนาดเล็ก *C. vulgaris* var. *vulgaris* TISTR 8261 พบว่าการเลี้ยงเชื้อผสมให้ชีวมวลและลิปิดสูงกว่าการเลี้ยงเชื้อเดี่ยวเช่นเดียวกับการทดลองข้างต้น และจากการศึกษาสภาวะที่เหมาะสม พบว่ามีการผลิตชีวมวลและลิปิดเพิ่มขึ้นเป็น 5.7 และ 3.8 เท่า ของสภาวะเริ่มต้น ตามลำดับ เมื่อใช้ยูเรียเป็นแหล่งไนโตรเจนเติมในกลีเซอรอลบริสุทธิ์ที่สัดส่วนคาร์บอนต่อไนโตรเจนเท่ากับ 32

T. spathulata JU4-57 ให้ชีวมวลและการผลิตลิปิดสูงสุด คือ 11.33 และ 5.03 กรัมต่อลิตร ตามลำดับ เมื่อควบคุมพีเอชที่ 6.0 และอัตราการให้อากาศที่ 1 ปริมาตรอากาศต่อปริมาตรอาหารต่อนาที ในการผลิตลิปิดแบบกะในถังปฏิกรณ์ชีวภาพขนาด 5 ลิตร ซึ่งใช้เพียงกลีเซอรอลดิบที่เติมเพียงแอมโมเนียมซัลเฟท นอกจากนี้การเลี้ยงเชื้อผสมของ *T. spathulata* JU4-57 และสาหร่าย *C. vulgaris* var. *vulgaris* TISTR 8261 ในถังปฏิกรณ์ชีวภาพขนาด 5

ลิตร ที่มีการควบคุมพีเอชที่ 6.0 สามารถผลิตชีวมวลและลิปิดได้เร็วกว่าและสูงกว่าการเลี้ยงเชื้อเดี่ยว

ในการผลิตลิปิดแบบกึ่งกะที่มีการป้อนกลีเซอรอลดิบและแอมโมเนียมซัลเฟทที่สัดส่วนคาร์บอนต่อไนโตรเจนเท่ากับ 17.2 พบว่าเมื่อเพิ่มความเข้มข้นของกลีเซอรอลในอาหารที่เติมจากร้อยละ 4 เป็นร้อยละ 12 ทำให้ชีวมวลเพิ่มขึ้นจาก 12.98 กรัมต่อลิตร เป็น 17.3 กรัมต่อลิตร และผลิตลิปิดเพิ่มขึ้นจาก 6.55 กรัมต่อลิตร เป็น 7.25 กรัมต่อลิตร แต่มีการสะสมน้ำมันลดลงจากร้อยละ 50 เป็นร้อยละ 41.9 นอกจากนี้ยังพบว่ากระบวนการหมักแบบสองขั้นตอนสำหรับการผลิตลิปิดจุลินทรีย์โดยการเติมกลีเซอรอลดิบอย่างเดียวในขั้นตอนที่สองทำให้มีชีวมวลน้อยลงเท่ากับ 13.8 กรัมต่อลิตร แต่มีเชื้อมีการสะสมน้ำมันเพิ่มขึ้นเป็นร้อยละ 55 และทำให้ผลิตลิปิดเพิ่มขึ้นเป็น 7.78 กรัมต่อลิตร

ลิปิดที่ผลิตโดยเชื้อ *T. spathulata* JU4-57 มีองค์ประกอบเป็นกรดไขมันสายยาวคาร์บอน 16 และ 18 อะตอมเป็นส่วนใหญ่ ซึ่งมีความคล้ายกับองค์ประกอบของน้ำมันพืช จึงมีศักยภาพในการใช้เป็นวัตถุดิบเพื่อผลิตไบโอดีเซล

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ABSTRACT

Eight hundreds and eighty nine yeast strains were isolated from soils and wastes from palm oil mill and the biodiesel plant of Prince of Songkla University in southern region of Thailand using glucose or glycerol as carbon source and at acidic condition (pH 4.0) or neutral condition (pH 6.0) with 0.0001% chloramphenicol. According to Sudan Black B test, 23 strains were screened as potential lipid producers or oleaginous yeasts. Strain BY4-523 accumulated highest lipid content up to 53.28% while JU4-57 grew fastest and gave comparable high lipid content 41.50% in crude glycerol based medium containing 4% of crude glycerol, 1% of yeast extract and 1% of peptone. The strains JU4-57 and BY4-523 were identified base on their 26S rDNA sequence as *Trichosporonoides spathulata* (99% identical over 497/502 bp; GenBank accession number AF335526.1) and *Kodamaea ohmeri* (99% identical over 457/460 bp; GenBank accession number FM180533.1), respectively.

Among organic nitrogen sources tested, a mixture of yeast extract and peptone (1:1) gave the best biomass (17.05 g/L for *T. spathulata* JU4-57 and 11.1 g/L for *K. ohmeri* BU4-523) and the maximum lipid production (10.43 g/L for *T. spathulata* JU4-57 and 4.53 g/L for *K. ohmeri* BU4-523). In the view point of economic strategy, the cheaper inorganic nitrogen sources were also tested. Among inorganic nitrogen sources tested, ammonium sulfate was selected as a suitable nitrogen source. The optimal medium composition for both strains was 0.5% ammonium sulfate and 10% crude glycerol (C/N ratio of 17.2). Under this condition, the maximum biomass of 10.23 g/L and lipid production of 3.87 g/L were achieved for *T. spathulata* JU4-57. Similarly, *K. ohmeri* BU4-523 also reached the maximum biomass of 10.50 g/L and lipid production of 3.22 g/L. The results showed that the newly isolated yeasts could grow and accumulate high lipid content in crude glycerol

based medium supplemented with only ammonium sulfate. *T. spathulata* JU4-57 could produce 68.7% of the neutral lipid, which was monoglycerides, followed by 29.3% of triacylglycerides. While *K. ohmeri* BY4-523 produced mainly monoglycerides up to 94.5% and only small amount of triacylglycerides.

In this study, microalga was used to mix with yeast for enhancing biomass and lipid production because it has a great ability to consume CO_2 and provide extra oxygen. The mixed cultivation of *T. spathulata* JU4-57 with *C. vulgaris* var. *vulgaris* TISTR 8261 gave the best biomass of 11.13 g/L and the maximum lipid production of 4.55 g/L, followed by the mixed culture of *T. spathulata* JU4-57 with a fresh water microalga *Chlorella* sp. (10.6 g/L of biomass) and a marine microalga *Chlorella* sp. (10.16 g/L of biomass). The optimal condition for the mixed cultivation was the ratio of yeast and microalga at 3.4×10^6 cells/mL: 3.9×10^5 cells/mL and providing light intensity at 4,000 lux. Under this condition, the lipid production was enhanced up to 4.93 g/L which was higher than the sum of lipid from yeast and microalga of 4.14 g/L. The mixed culture of an oleaginous yeast *Rhodotorula glutinis* TISTR 5159 with *Chlorella vulgaris* var. *vulgaris* TISTR 8261 also gave higher biomass and lipid than the pure culture. The biomass and lipid increased 5.7 and 3.8 fold, respectively in the presence of pure glycerol and urea at C/N ratio of 32.

T. spathulata JU4-57 gave the highest biomass (11.33 g/L) and lipid production (5.03 g/L) in batch fermentation using a 5 L bioreactor with pH control at 6.0 and aeration rate controlled at 1 vvm. The biomass and lipid production in the mixed culture of *T. spathulata* JU4-57 and *C. vulgaris* var. *vulgaris* TISTR 8261 increased faster and higher than that of the pure culture without and with pH control in a 5 L bioreactor. The biomass and lipid production was further enhanced up to 17.3 g/L and 7.25 g/L, respectively. However, the lipid content decreased from 50% by batch fermentation to 41.9% by fed-batch fermentation feeding with crude glycerol and ammonium sulfate. Two-stage fed-batch fermentation feeding with only crude glycerol in the second stage led to a lower biomass of 13.8 g/L but a higher lipid content of 55% and consequently higher lipid production of 7.78 g/L.

The fatty acid composition of *T. spathulata* JU4-57 lipid was mainly C16 and C18 which is similar to that of plant oil. This indicates that it has potential to be used as feedstocks for biodiesel production.

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CHAPTER 1

INTRODUCTION

Biodiesel is prepared through transesterification of oils with short chain alcohols. It has become more attractive recently because of its environmental benefits, and the fact that it is made from renewable resources. It contributes no net carbon dioxide or sulfur to the atmosphere and emits less gaseous pollutants than normal diesel. In the production of worldwide biodiesel, various plant oils have been chosen. However, all these plant oils require energy and acreage for sufficient production of oilseed crops. In addition, if plant oils are used for biodiesel production, the cost of source has accounted to 70–85% of the whole production cost. Therefore, exploring ways to reduce the high cost of biodiesel is of much interest in recent research, especially for those methods concentrating on lowering the cost of oil raw material. Moreover, the lack of oil feedstocks also limits the large-scale development of biodiesel to some extent. Recently, much attention has been paid to the development of microbial oils and it has been found that many microorganisms, such as microalgae, yeast, bacteria, and fungi, have the ability to accumulate oils under some special cultivation conditions. Microorganisms that can accumulate oils in lipid form more than 20% of their biomass are defined as oleaginous species. Some yeast strains, such as *Rhodosporidium* sp., *Rhodotorula* sp. and *Lipomyces* sp. can accumulate intracellular lipids as high as 70% of their biomass dry weight. The yeast oils are now believed as a promising potential feedstock for biodiesel production due to their similar composition of fatty acids to that of vegetable oils (Li *et al*. 2007). It has been reported that such yeast oils can be used as oil feedstocks for biodiesel production with the catalysis either by lipase or chemical catalyst (Li *et al*., 2008). Compared to other plant oils, microbial oils have many advantages, such as short life cycle, less labor required, less affection by venue, season and climate, and easier to scale up. In addition, oleaginous yeasts can not only accumulate lipids within a short period of time but grow well on a variety of substrates, even inexpensive materials such as

nutritional residues from agriculture and industry, thus lowering the cost of oils (Zhu *et al*., 2008). With the rapid expansion of biodiesel, microbial oils might become one of potential oil feedstocks for biodiesel production in the future, though there are many works associated with microorganisms producing oils need to be carried out further.

Biodiesel production is a process in which 10% crude glycerol is separated from the triacylglycerides. The future supply of crude glycerol is expected to increase as biodiesel plants increase production, and the output will greatly outpace demand. Some alternative use for this crude glycerol is a substrate for fermentation process because it contains metal ions such as calcium, potassium, magnesium, sulfur and sodium in addition to glycerol (Morita *et al*., 2007). The previous works also reported that some oleaginous yeasts have ability to grow and accumulate lipids on crude glycerol such as *R. glutinis* and *M. isabellina* (Saenge *et al*., 2011; Papanikolaou *et al*., 2008) and have short generation times, and very minimal nutrient requirements (Meesters *et al*., 1996). Thus, using the crude glycerol to produce yeast oils to be used as biodiesel feedstock would provide an added bonus of offsetting costs of production.

The objective of this study was to produce yeast oils from crude glycerol, a by-product of biodiesel plant. The oleaginous yeast was screened using glucose and glycerol as carbon sources. The optimal concentrations of crude glycerol and suitable nitrogen source were determined. In addition, the possibility of enhancing lipid production by mixed culture of oleaginous yeast and oxygen producer microalgae was also investigated. Fed-batch fermentation and various feeding strategies were attempted to enhance lipid production.

Objectives of the study

1. To screen high lipid accumulating oleaginous yeasts using glucose and glycerol as carbon sources.
2. To optimize medium components for cell growth and lipid production of a selected yeast using crude glycerol from biodiesel plant as a sole carbon source.
3. To enhance lipid production by mixed culture of the isolated oleaginous yeast with oxygen producer microalga and compare to mixed culture of a reference oleaginous *Rhodotorula glutinis* with microalga.
4. To enhance lipid production by fed-batch fermentation and various feeding strategies.

CHAPTER 2

LITERATURE REVIEWS

1. Microorganisms available for biodiesel production

To be a viable substitute for a fossil fuel, an alternative fuel should not only have superior environmental benefits and be economically competitive over the fossil fuel and be producible in sufficient quantities to make a meaningful impact on energy demands, but also provide a net energy gain over the energy sources used to produce it. One of the most prominent renewable energy resources is biodiesel, which is produced from renewable biomass by transesterification of triacylglycerols, yielding monoalkyl esters of long-chain fatty acids with short-chain alcohols, for example, fatty acid methyl esters (FAMEs) and fatty acid ethyl esters (FAEEs). It contributes no net carbon dioxide or sulfur to the atmosphere and emits less gaseous pollutants than normal diesel (Antolin *et al*., 2002; Lang *et al*., 2001; Vicente *et al*., 2004). Biodiesel has been widely used in many countries and regions such as US, Europe, Japan and Thailand. Raw material for biodiesel production in Thailand mainly come from vegetable oil such as palm oil, coconut oil, soybean oil, ground nut oil, castor, sesame oil, sunflower oil and jatropha oil. Besides, the use of vegetable oils as raw material for biodiesel production would compete with edible oils, thus leading to the soar of food price. Using recovered animal fats and used frying oils as feedstock can efficiently reduce the price of biodiesel, however, the amount of waste oils is limited and cannot meet the increasing needs for clean renewable fuels. Microbial oils, namely single cell oils, produced by oleaginous microorganisms involving bacteria, yeasts, moulds and microalgae, are now believed as a promising potential feedstock for biodiesel production due to their similar composition of fatty acids to that of vegetable oils (Li *et al*., 2007).

Oleaginous microorganisms are defined as microorganisms with the content of microbial lipid excess of 20% (Meng *et al*., 2009). Although there are all kinds of microorganisms storaging oils, such as microalgae, bacilli, fungi and yeasts,

not all of them are available for biodiesel production (Table 1). In microorganisms, the extent of lipid accumulation is determined by the genetic constitution, as maximum attainable lipid contents can vary enormously among species and even among individual strains (Meng *et al*., 2009). According to different microorganisms and different culture conditions (such as temperature, pH, culture time, etc.), oil content and composition are different (Table 2). The accumulated oil is almost invariably in the form of triacylglycerols, which is exactly the same form as plant oils. The fatty acyl groups of both neutral and polar lipids are usually conventional plant-like entities such as *Yarrowia lipolytica*, containing stearic, oleic, linoleic and palmitic acid (Papanikolaou *et al.*, 2002).

Table 1. Oil content of some microorganisms

Microorganisms	Oil content (% dry wt)	Microorganisms	Oil content (% dry wt)
Microalgae		Yeasts	
Botryococcus braunii	25–75	*Candida curvata*	58
Cylindrotheca sp.	16–37	*Cryptococcus albidus*	65
Nitzschia sp.	45–47	*Lipomyces starkeyi*	64
Chlorella sp.	28-32	*Rhodotorula glutinis*	72
Bacteria		Fungi	
Arthrobacter sp.	>40	*Aspergillus oryzae*	57
Acinetobacter	27–38	*Mortierella isabellina*	86
calcoaceticus	24–25	*Humicola lanuginosa*	75
Rhodococcus opacus	18–24	*Mortierella vinacea*	66
Bacillus alcalophilus			

Source: Meng *et al*. (2009)

Bacteria, in general, do not produce triacylglycerols but, instead, produce poly-β-hydroxy-butyrates and -alkanaoates as storage polymers (Ratledge, 2004). Oil accumulation is therefore found only in some yeasts, fungi and microalgae. Numerous oleaginous yeasts and microalgae have been reported to grow and accumulate lipid in significant amounts similar to vegetable oils. In some microalgae, they are not

considered for commercial production of polyunsaturated fatty acids-rich oils, because triacylglycerols are not the major lipid component and, instead, there are numerous lipid types present that are involved in the make-up of the photosynthesizing membrane systems. This complexity of microalgal lipids is disadvantageous to them being used commercially. Also microalgae, being obligate phototrophs, pose severe difficulties for their large-scale cultivation which have not yet been overcome. Furthermore, some types of fungi have the ability to produce oils, most fungi are explored mainly for the production of some special lipids, such as docosahexaenoic acid, gamma linolenic acid, eicosapentaenoic acid and arachidonic acid, and there are few reports on the utilization of fungal oils for biodiesel production (Li *et al*., 2008). The main difficulty is represented by the slow growth of fungi in bioreactors compared with other microorganisms and the occurrence of frequent contamination. Contamination is further favoured by mycelium washing and encapsulating (Rossi *et al*., 2002). Moreover, there seems to be a limitation related to insufficient knowledge of transport phenomena involved in fungal growth in bioreactors. In conventional bioreactor such as continuous stirred-tank reactor, a higher shear rate would damage mycelium, where lipid is accumulated, because the impellers can produce an intense fragmentation of the mycelium, affecting its viability. If the agitation is kept at a low level, the viscosity will increase with fungal growth and a consequent reduction of nutrient transport and oxygen- and heat-transfer coefficients will take place. On the other hand, some yeast does not have mycelia. Since yeasts contain no unusual fatty acids, they contain no deleterious fatty acids. The oleaginous yeast can accumulate lipids within a short period of time and it also grow well on a variety of substrates, even inexpensive material, such as nutritional residues from agriculture and industry (Zhu *et al*., 2008), thus lowering the cost of oils.

Table 2. Lipid composition of some microorganisms.

Microorganisms	Lipid composition (w/total lipid)					
	C16:0	C16:1	C18:0	C18:1	C18:2	C18:3
Microalgae	12–21	55–57	1–2	58–60	4–20	14–30
Yeasts	11–37	1–6	1–10	28–66	3–24	1–3
Fungi	7–23	1–6	2–6	19–81	8–40	4–42
Bacteria	8–10	10–11	11–12	25–28	14–17	–

Source: Meng *et al*. (2009)

2. The biochemistry of oil-accumulation

Of crucial importance in the further development of the single cell oils process is the understanding of how microorganisms synthesize their fatty acids and how they are able to accumulate so much oil. In order to achieve lipid accumulation in a microorganism, it needs to be grown in a medium with an excess of carbon substrate and a limiting amount of nitrogen. Then, when the organism grows, it exhausts the supply of nitrogen quickly but it continues to assimilate the carbon source (usually glucose or an alternative carbohydrate) (Meng *et al*., 2009). This is channeled directly into lipid synthesis with the resulting build of triacylglycerols within the cell as discrete oil droplets. Oil accumulation may reach over 70% of the cell biomass but not in every oleaginous specy. Non-oleaginous microorganisms, by definition, do not accumulate lipid. When non-oleaginous microorganisms were placed in the same nitrogen-limiting growth medium, they either tend to cease further cell proliferation or, if they continue to assimilate available carbohydrate, then this is diverted into various polysaccharides, including glycogen and various glucans, mannans, etc. Oil accumulation, beyond a very small level (usually less than 10% of the biomass), does not occur.

Therefore, the ability of an organism to accumulate large quantities of oil must lie outside the immediate area of fatty acid biosynthesis, as this biosynthetic machinery is common to all microorganisms. The reasons for oleaginicity would appear to be two-fold:

• The ability to produce a continuous supply of acetyl-CoA directly in the cytosol of the cell as a necessary precursor for fatty acid synthetase (FAS), and,

• The ability to produce a sufficient supply of NADPH as the essential reductant used in fatty acid biosynthesis.

Figure 1 shows a scheme of lipid synthesis in oleaginous microorganisms. The formation of acetyl-CoA in oleaginous microorganisms has been attributed to the presence of ATP:citrate lyase (ACL, reaction no. 1) which does not appear to occur in the majority of non-oleaginous specy:

$$\text{Citrate} + \text{CoA} + \text{ATP} \rightarrow \text{acetylCoA} + \text{oxaloacetate} + \text{ADP} + P_i \qquad (1)$$

to operate efficiently, its substrate, namely citric acid must itself be made readily available and, moreover, to be available in the cytosol of the cell where fatty acid sythesis occurs. Citric acid is, of course, synthesised as part of the tricarboxylic acid (TCA) cycle within the mitochondrion of the cell (All oleaginous microorganisms are eukaryotes and so have mitochondria). The feature that is unique to the oleaginous microorganisms, and which allows citric acid accumulation, is that the activity of isocitrate dehydrogenase as a component of the TCA cycle is dependent on the presence of AMP; no such dependency occurs with the enzyme from non-oleaginous microorganisms. The concentration of AMP itself is regulated by the activity of AMP deaminase (reaction no. 2):

$$\text{AMP} \rightarrow \text{inosine } 5' \text{ monophosphate} + NH_3 \qquad (2)$$

It is this enzyme whose activity is up-regulated at the onset of nitrogen limitation in the growth medium of the oleaginous microorganism possibly as a means of trying to scavenge additional ammonium ions from intracellular materials. Nitrogen limitation in the cultivation of an oleaginous microorganism induces a cascade of reactions leading to the formation of acetyl-CoA:

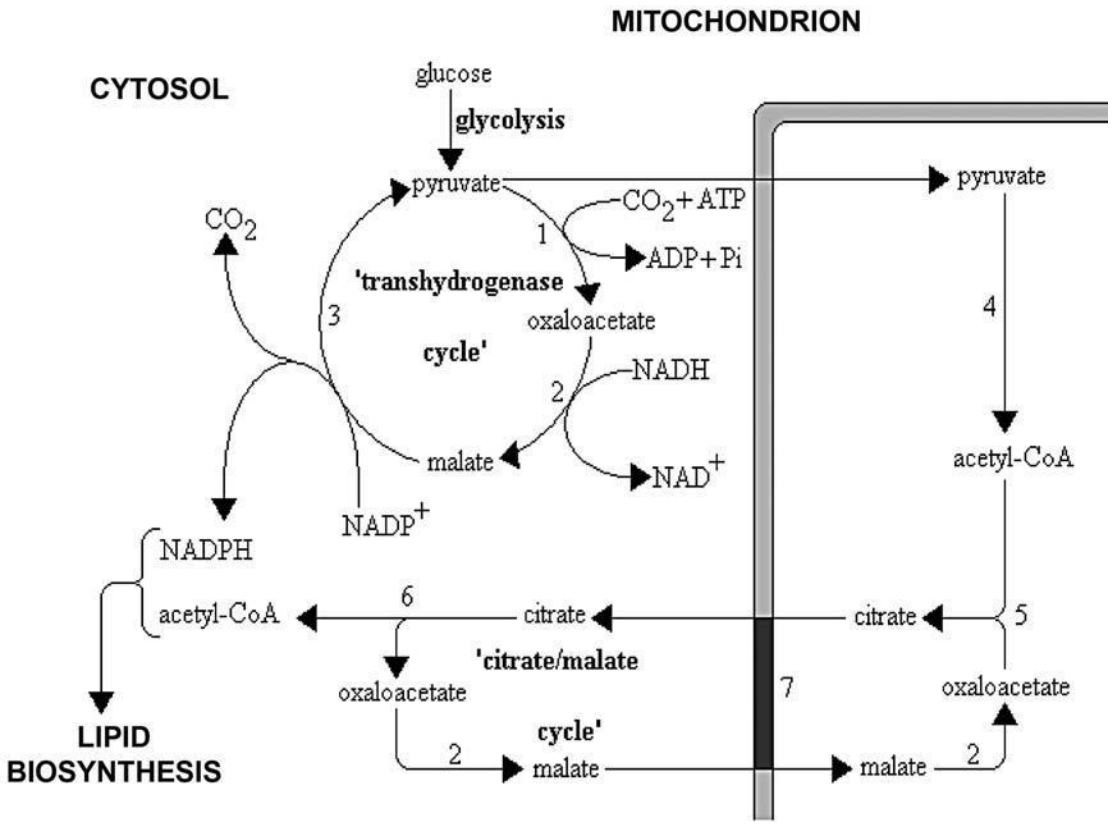


Figure 1. A scheme to show how the citrate/malate cycle and the cytosolic 'transhydrogenase' cycle could provide sufficient precursors of acetyl-CoA and NADPH for lipogenesis in oleaginous microorganisms. Enzymes: 1, pyruvate decarboxylase; 2, malate dehydrogenase; 3, malic enzyme; 4, pyruvate dehydrogenase; 5, citrate synthase; 6, ATP:citrate lyase; 7, citrate/malate translocase. Net carbon balance: pyruvate $\rightarrow$ acetyl-CoA + CO_2. Net reaction for NADPH production: NADH + $NADP^+$ +ATP $\rightarrow$ NAD^+ + NADPH + P_i. The transhydrogenase cycle can operate independently of the carbon flux from citrate in the mitochondrion to acetyl-CoA in the cytosol and consequently can provide all the NADPH needed for both fatty acid biosynthesis and fatty acid desaturation and elongation reactions.

Source: Ratledge (2004)

• At the onset of nitrogen exhaustion, oleaginous cells show an increased activity of AMP deaminase, which is up to five-fold greater than in cells before N limitation.

• The increased activity of AMP deaminase decreases the cellular content of AMP, including its content in the mitochondrion

• The diminished content of AMP in the mitochondrion stops isocitrate dehydrogenase from working as in oleaginous cells, this enzyme is strictly dependent on AMP for activity.

• As a result, isocitrate cannot be metabolised; it thus accumulates and is then readily equilibrated with citric acid (via aconitase).

• Citrate therefore accumulates in the mitochondrion.

• An efficient citrate efflux system exists in the mitochondrial membrane for the export of citrate (in exchange for malate, see in Figure 1).

• Citrate enters the cytosol and is cleaved by ACL to give acetyl-CoA and oxaloacetate.

• The acetyl-CoA is used for fatty acid biosynthesis.

• The oxaloacetate is converted via malate dehydrogenase to malate, which is then used as the counterion in the citrate efflux system.

This sequence of events is shown diagrammatically in Figure 1. Although this metabolism of glucose to acetyl-CoA is able to account for the flux of the carbon substrate into fatty acid biosynthesis under nitrogen-limited conditions, it is not the complete story. Some microorganisms have been found in which ACL activity is present though without the cells being able to accumulate lipid; however, the corollary is not true: no oil-accumulating microorganism has yet been reported that does not have ACL activity. Some other enzyme must be needed, therefore, to ensure lipid accumulation.

Fatty acids, it must be remembered, are highly reduced materials and to achieve their synthesis as a ready supply of reductant as NADPH is essential. The synthesis of 1 mol of a C18 fatty acid requires 16 mols NADPH to be provided as 2 mols NADPH are needed to reduce each 3-keto-fattyacyl group arising after every condensation reaction of acetyl-CoA with malonyl-CoA as part of the standard fatty acid synthetase complex into the saturated fatty acyl chain, which then undergoes a

further cycle of chain lengthening. The major supplier of NADPH for fatty acid biosynthesis is now considered to be malic enzyme (reaction no. 3):

$$\text{Malate} + \text{NADP}^{+} \rightarrow \text{pyruvate} + \text{CO}_2 + \text{NADPH} \quad (3)$$

Malic enzyme activity has been found in most oleaginous microorganisms where it is proposed to form an integrated metabolon complex that combines with ACL and fatty acid synthase (FAS) to ensure a direct channelling of acetyl-CoA into fatty acids, which are finally esterified with glycerol into triacylglycerols and incorporated via the endoplasmic recticulum into fatty acid droplets (Figure 2).

Malic enzyme activity does not, however, appear to be ubiquitous amongst oleaginous microorganisms and may be absent in some oleaginous yeasts, including *Lipomyces* sp. and some *Candida* sp. Here, it is probable that an alternative NADPH-generating enzyme, such as a cytosolic NADPH-dependent isocitrate dehydrogenase, though other enzymes are possible, will be found that will be dedicated to fatty acid biosynthesis much in the same way as malic enzyme is considered to be functionally associated with the fatty acid metabolon (Figure 2) (Ratledge, 2004).

Storage molecules such as triglycerides (TAG) and steryl esters (SE) are not suitable for integration into phospholipid bilayers. Therefore, they cluster to form the hydrophobic core of so-called lipid bodies (LB). Originally, LBs were considered only as a depot of neutral lipids which can be mobilized under starving conditions. However, the view of the LB as a simple storage compartment, excluded from any metabolic process, had to be revised since many of its proteins were identified as enzymes involved in lipid metabolism, especially in TAG synthesis and degradation. In yeasts, TAG synthesis follows the Kennedy pathway free fatty acids (FFA) are activated to coenzyme A (CoA) and used for the acylation of the glycerol backbone to synthesize TAG. Figure 3 shows different pathways involved in FA synthesis and storage and degradation of neutral lipid. In the first step of TAG assembly, glycerol-3-phosphate (G3P) is acylated by G3P acyltransferase to lysophosphatidic acid (LPA), which is then further acylated by LPA acyltransferase to

phosphatidic acid (PA). This is followed by dephosphorylation of PA by PA phosphohydrolase (PAP) to release diacylglycerol (DAG). In the final step, DAG is acylated either by DAG acyltransferase or by phospholipid DAG acyltransferase to produce TAG (Beopoulos *et al*., 2008).

Synthesis of FA (Acyl-CoA) is catalyzed by the FA synthesis complex from the basic blocks acetyl-CoA and malonyl-CoA. Acyl-CoA can be stored either as SE or as TAG. The synthesis of steryl esters (SE) is catalyzed by SE synthases homologous to the human acyl-CoA:cholesterol acyltransferase and their mobilization by the SE hydrolases, releasing sterol and FFA. The synthesis of TAG require acyl-CoA and G3P. G3P could be produced from glycerol or from dihydroxyacetone (DHAP). *GUT1* encodes a glycerol kinase that converts glycerol to G3P in the cytosol. The product of this reaction can be oxidized to DHAP by the G3P dehydrogenase (G3PDH) encoded by the *GUT2* gene. DHAP can enter either glycolysis or gluconeogenesis. G3P could also be used as a skeleton for TAG synthesis. Three acyls are added to the G3P backbone to give TAG, and this process requires four enzymatic steps. First, an acyl is added at the sn-1 position of G3P by a G3P acyltransferase to produce LPA, and then a second acyl is added at the sn-2 position by a 1-acyl G3P acyltransferase to produce PA, which is then dephosphorylated by PAP, yielding DAG. Finally, the third acyl can be added at the sn-3 position either by the acetyl-CoA dependent pathway (directly from acyl-CoA) or by the acetyl-CoA-independent pathway (from a glycerophospholipid). TAG can be mobilized by the conversion to FFA and DAG upon hydrolysis by TAG lipase. The FFA can then be degraded in the β-oxidation pathway. This pathway requires four enzymatic steps. In *Yarrowia lipolytica*, six genes (*POX1* to *POX6*) that code for acyl-CoA oxidases are involved in the second step of β-oxidation. Proteins encoded by the genes in parentheses were found to be associated with lipid particles (LB) in *Y. lipolytica* (Beopoulos *et al*., 2008).

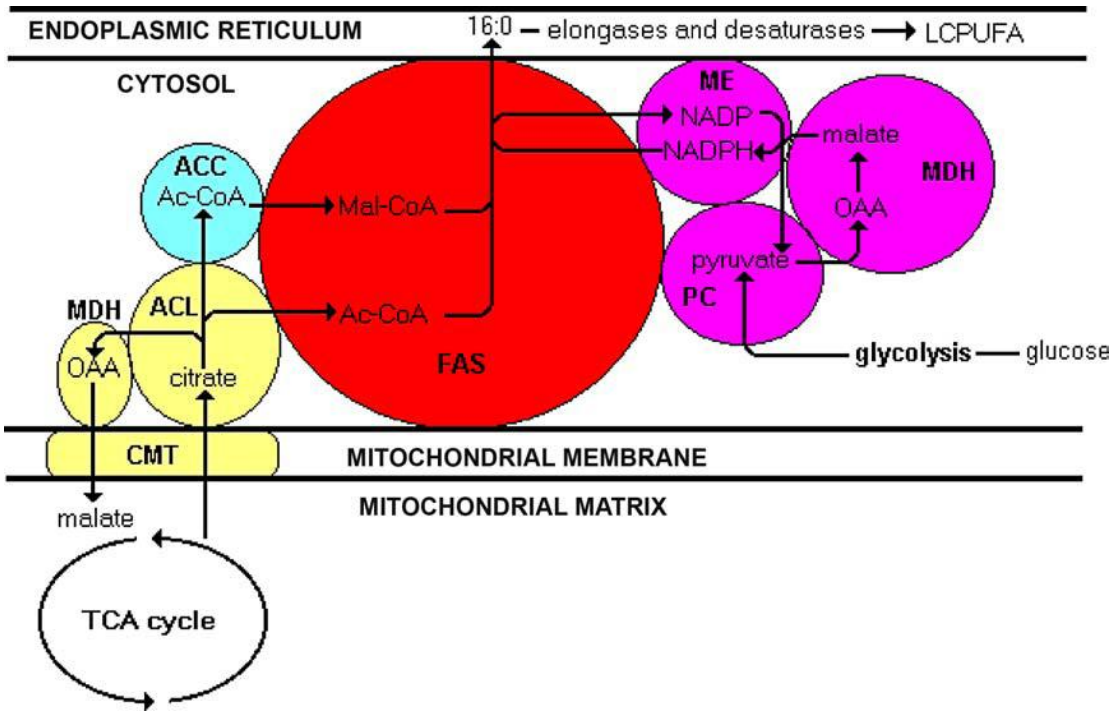


Figure 2. A diagram to show the organisation of a hypothesised lipogenic metabolon. The flux of carbon from the mitochondrion, via citrate efflux and acetyl-CoA formation in the cytosol, hence into fatty acids and finally into long chain PUFAs (LCPUFA) occurring in the membranes of the endoplasmic reticulum, is shown by the continuous lines. The system uses pyruvate (from glycolysis) as the provider of intramitochondrial acetyl-CoA and for citric acid production as is shown in Figure 1 but which is not repeated here for clarity. OAA: oxaloacetate;AC-CoA: acetyl-CoA; Mal-CoA: malonyl-CoA; FAS: fatty acid synthase;ACL: ATP:citrate lyase; ACC: acetyl-CoA carboxylase; CMT: citrate/malate translocase; ME: malic enzyme; PC: pyruvate carboxylase; MDH: malate dehydrogenase. The 'yellow' enzymes form the citrate/malate cycle while the 'purple' enzymes represent the cytosolic transhydrogenase cycle.

Source: Ratledge (2004)

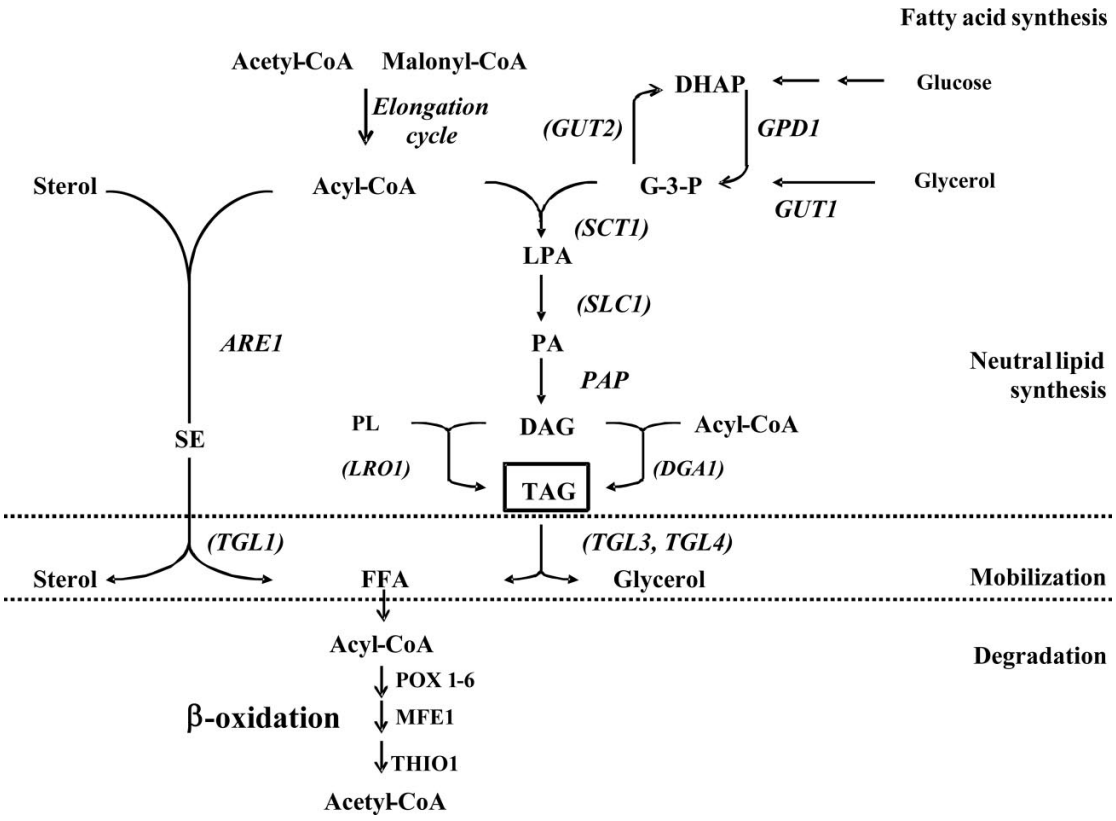


Figure 3 Overview of different pathways involved in fatty acids synthesis and storage and degradation of neutral lipids.

Sourece: Beopoulos *et al*. (2008)

3. Yeasts as potential lipid producers

Oleaginous yeasts are single-celled fungi defined as having at least 20% of their dry weight made up of lipids (Easterling *et al*., 2009). Not only do these yeasts contain membrane lipids, but they also accumulate lipid in the form of triacylglycerol (TAG) (Davoli *et al*., 2004). *Rhodotorula glutinis* is oleaginous yeast which is able to activate non-esterified fatty acids for the synthesis of triacylglycerol (Gangar *et al*., 2001). In *R. glutinis*, fatty acids are activated in an ATP dependent manner prior to being used. Gangar *et al*. (2002) have demonstrated that an enzyme, acyl–acyl carrier protein (ACP) plays a role in activating fatty acids for triacylglycerol biosynthesis. There is plenty evidence to suggest that organism has the potential to be a source of fatty acids for the production of biodiesel. There are a small number of yeasts, which have the propensity to accumulate large amounts of intracellular lipid.

They do so by being grown in a medium with a high C/N ratio (usually about 30:1) so that, in either batch or continuous culture they have an excess of carbon over the supply of nitrogen. The carbon then continues to be assimilated by the yeasts and, in the absence of protein and nucleic acid synthesis due to the lack of nitrogen, the carbon is then channeled into lipid accumulation. Nonoleaginous yeasts, when placed in the same environment, tend to accumulate a little extra lipid-up to 15% or even 20% in some cases-but the majority of the excess carbon is converted into polysaccharide materials.

Many yeast species, such as *Cryptococcus albidus*, *Lipomyces lipofera*, *Lipomyces starkeyi*, *Rhodosporidium toruloides*, *Rhodotorula glutinis*, *Trichosporon pullulan*, and *Yarrowia lipolytica*, were found to be able to accumulate oils under some cultivation conditions, and it was reported that different yeast species led to different oil accumulation (Table 3). The main fatty acids in yeast oils were myristic acid, palmitic acid, stearic acid, oleic acid, linoleic acid, and linolenic acid. It has been reported that such yeast oils can be used as oil feedstocks for biodiesel production with the catalysis either by lipase or chemical catalyst (Li *et al*., 2008). The extent of lipid accumulation in oleaginous yeasts may reach, and on occasions even exceed, 70% of biomass weight. Table 3 lists the yeasts that are currently considered to be "oleaginous." The lowest amount of lipid required to be accumulated by yeast for inclusion in this list is somewhat arbitrary, and the limit usually chosen is about 20%, since this excludes many "non-oleaginous" species that can be pushed into accumulating lipid to about this level. The evidence for true oleaginicity among some yeast listed in Table 3 still needs to be evaluated. It is possible that oleaginicity could be defined biochemically by examining yeasts for the presence of the enzyme ATP: citrate lyase, which is considered to play a key role in ensuring lipid accumulation, and indeed its absence in species such as *Saccharomyces cerevisiae* and *Candida utilis* can explain why these yeasts are unable to accumulate lipid beyond a normal limit of about 10%. However, lipid accumulation is a complex biochemical process and does not rely on the presence of single enzyme. The prospects of genetically engineering a nonoleaginous yeast into a lipid producer must therefore be regarded as remote, at least for the time being.

The extraction of yeast lipid usually coextracts other lipid fractions (phospholipids, sterols, sterol esters, etc.) associated with the cell membrane. Reports often show the presence of free fatty acids in the extracted lipid, but these arise by uncontrolled lipolysis occurring during the extraction. Representative analyses of the lipid constituents of oleaginous yeasts are given in Table 4. The triacylglycerols themselves show a distribution of the fatty acyl constituents similar to that found in plant oils; that is, the central position of the glycerol is occupied almost exclusively by an unsaturated acyl group (Verachtert and Mot, 1989).

The time course of cell growth, glucose exhaustion and lipid production of *Trichosporon fermentans* are shown in Figure 4 (Zhu *et al*., 2008). It is apparent that glucose was used mainly for cell growth at the beginning of the fermentation. Biomass, lipid content and utilized glucose gradually increased with time after inoculation. On the 7^{th} day, lipid content and lipid yield reached the maximum of 57.0% and 12.3 g/L. A slight decrease was found in biomass on the day 8 while utilized glucose increased. The possible reason may be that nitrogen source was exhausted and a great deal of glucose consumption led to a decrease of pH, thus inhibiting cell growth. During the period between days 9 and 10, there was a clear increase in biomass. However, lipid content showed an apparent decrease. While *R. glutinis* accumulated lipids up to 60.69% on a cellular biomass basis with biomass yield of 38.6 g/L for 72 h, which corresponding to 23.41 g/L lipid productivity (Dai *et al*., 2007).

Table 3. Lipid production by different yeasts.

Species	Maximum lipid content (%)
Candida sp. 107	45
Candida curvata D	58
Candida curvata R	51
Candida diddensiae	37
Cryptococcus (*terricolus*) *albidus* var. *albidus*	65
Cryptococcus albidus var. *aerius*	63
Cryptococcus laurentii	32
Endomycopsis vernalis[a]	65
Hansenula ciferri[b]	22
Hansenula saturnus	28
Lipomyces lipofer	64
Lipomyces starkeyi	63
Lipomyces tetrasporus	67
Rhodosporidium toruloides	51
Rhodotorula glutinis (*gracilis*)	72
Rhodotorula glaminis	41
Rhodotorula mucilaginosa[b]	28
Trichosporon cuteneum	45
Trichosporon fermentans	62
Trichosporon pullulans[a]	33
Trigonopsis variablis	40
Yarrowia (*Candida, Saccharomycopsis*) *lipolytica* (*paralipolytica*)[b]	36

[a]Probably equivalent species.

[b]Uncertain whether true oleaginous species; more information needed.

Source: Verachtert and Mot (1989)

Table 4. Lipid composition of some oleaginous yeast

Yeast	Relative % (w/w) of components[a]							
	TAG	DAG	MAG	FFA	S	SE	PL	GL
Cryptococcus (*terricolus*) *albidus* var. *albidus*	92	2.5	1	3	1	1	2	
Lipomyces starkeyi	95	1		<1	1		3	
Rhodotorula glutinis	67			4	2	7	11	6
Trichosporon pullulans (= *Endomycopsis vernalis*)	82	1			10	1	4	

[a]TAG = triacylglycerol; DAG = diacylglycerol; MAG = monoacylglycerol; FFA = free fatty acid; S = sterol; SE = sterol ester; PL = phospholipid; GL = glycolipid

Source: Verachtert and Mot (1989)

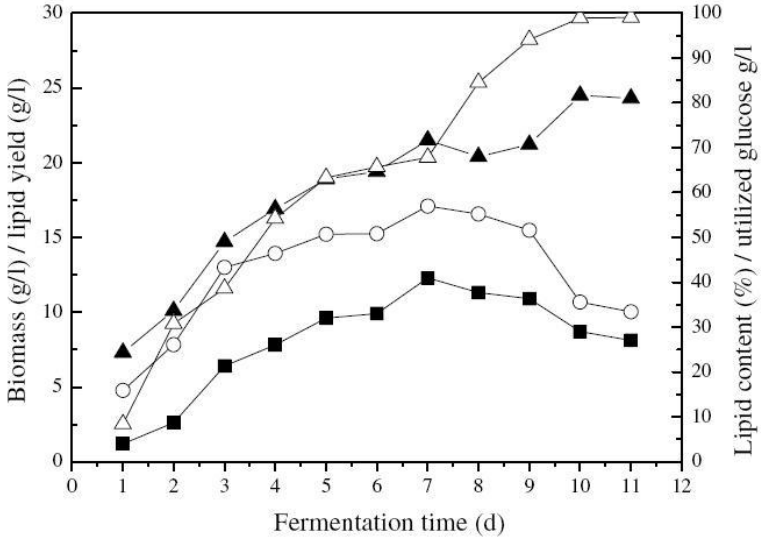


Figure 4. Time course of cell growth and lipid accumulation with *T. fermentans*. Culture was performed in the original nitrogen-limited medium. (▲) Biomass; (■) lipid yield; (o) lipid content; and (Δ) utilized glucose.

Source: Zhu *et al*. (2008)

4. Microalgae oil

Microalgae are currently being considered as feedstocks for biodiesel production because of their rapid growth rates and high yield of lipids (Huang *et al*., 2009). Microalgae-based technologies could provide a key tool for reducing greenhouse gas emissions from coal-fired power plants and other carbon intensive industrial processes. Because microalgae are rich in oil and can grow in a wide range of conditions, many companies are betting that it can create fuels or other chemicals cheaper than existing feedstocks. The aim of microalgae biofixation of CO_2 is to operate large-scale systems that are able to convert a significant fraction of the CO_2 outputs from a power plant into biofuels (Demirbas, 2011). Thus microalgae are superior to oil crops for the sustainable production of biomass in an environmental-friendly operation mode. When a microalga is considered for mass biodiesel production, two key factors, namely cell biomass and lipid content are essential for the initial assessment.

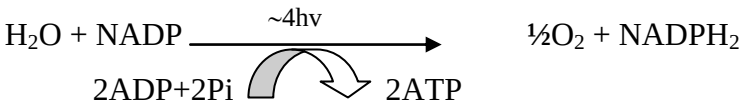
Photosynthesis in microalgae

The term photosynthesis means "synthesis with the help of light". This is generally ascribed to the synthesis of organic matter by plants, although it implies a variety of organic as well as inorganic and physical reactions. Photosynthesis is the basic process of life on earth.

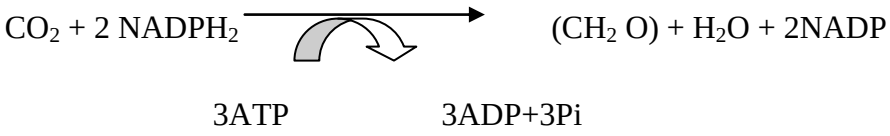
Photosynthesis in microalga is superimposed on the reverse process, respiration, i.e. slow combustion of organic matter to form water and CO_2 with release of chemical energy in the form of heat and "energy-rich" phosphate bands. An excess of photosynthesis over respiration is what permits the growth of photosynthetic organisms and storage of food reserves such as starch, oil and fat.

The photosynthetic process can be divided into two parts, the light reaction and the dark reactions. In the light reactions, which take place in the thylakoid membrane system, hydrogen is withdrawn from water and passed along a series of hydrogen carriers to NADP, so that $NADPH_2$ is formed and oxygen is liberated. Associated with this hydrogen (or electron) transport there is a conversion of ADP and inorganic phosphate to ATP, probably two (or, on average, some fractional number between one and two) ATP molecules being formed for every two

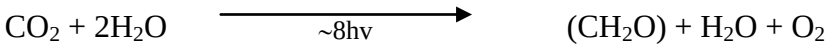
electrons transferred or molecule of NADP reduced. These chemical changes are associated with a considerable increase in free energy: this is made possible by the light energy absorbed by the chloroplast pigments. Thus we may summarize the light reactions by the equation



In the dark reactions, which take place in the stroma of the chloroplast, the $NADPH_2$ produced in the light reactions is used to reduce CO_2 to the level of carbohydrate. This too is associated with an increase in free energy, the energy being supplied by the concomitant breakdown of ATP produced in the light reactions. The dark reactions can be summarized by the equation.



Thus, the overall photosynthetic process can be represented by



The green microalgae in the genus *Chlorella* consist of about 10 species that can grow photoautotrophically, mixotrophically and heterotrophically with high biomass concentration (Miao and Wu, 2004). Considering their lipid contents, *C. vulgaris* and *C. protothecoides* have been reported to be the candidates for biodiesel production under photoautotrophic or heterotrophic culture conditions (Miao and Wu, 2004). Certain *Chlorella* species was reported to attain very high biomass concentration in heterotrophic culture (Sun *et al*., 2008). In addition, lipids extracted from heterotrophically grown cells had similar properties to fuel oil in terms of oxygen content, heating value, density and viscosity (Miao and Wu, 2004). Moreover, cultivating *Chlorella* heterotrophically could eliminate the light requirement that could definitely reduce the cost of the final product (Chen, 1996).

Many microalgae accumulate lipids as storage materials and their accumulation is stimulated under environment stress, such as nutrient deficiency

(Dunahay *et al*., 1996) or salt stress (Takagi *et al*., 2006). Widjaja *et al*. (2009) reported that maximum lipid content of *Chlorella vulgaris* was only 26% under normal nutrition medium with nitrogen ($NaNO_3$). Considering their lipid contents, *C. vulgaris* and *C. protothecoides* have been reported to be the candidates for biodiesel production under photoautotrophic or heterotrophic culture conditions (Miao and Wu, 2004).

Usually the temperature drops during cultivation, which lowers the cell density in the system. For example, the most suitable temperature range of *Chlorella vulgaris* for maintaining both proper biomass and lipid production was between 25 and 30 °C (Lee *et al*., 1996). In another study, *Chlorella sorokiniana* was able to grow up to a maximum biomass of 7 g/L at the optimum temperature of 35 °C (Lee *et al*., 1996). Mayo (1997) reported that *C. vulgaris* survived and even grew under very acidic conditions. This is probably because the photosynthetic system for this microalga, which is in the chloroplasts, is surrounded by cytoplasm of neutral pH. However, when the temperature was increased to 40°C, the cells became less resistant to acidic pH, than they did at 35°C and below. It is possible that cytoplasm was damaged at higher temperatures, allowing hydrogen ion to penetrate easily to the chloroplasts.

5. Factors affecting growth and lipid production of oleaginous yeast

It has been reported extensively that cultivation conditions such as C/N ratio, carbon source, nitrogen source, temperature, pH and oxygen would have various influences on oil accumulation.

5.1 Nutrients

5.1.1 Carbon sources

Yeasts are chemoorganotrophic organisms. This means that they obtain carbon and energy from compounds in fixed, organic linkage. These compounds are most commonly sugars of which glucose is the most widely utilized by yeast. *Yarrowia lipolytica* is the yeast that is able to utilize hydrophobic substrates (e.g., alkanes, FA, and oils) efficiently as a sole carbon source (Fickers *et al*., 2005). Its superior capacity to accumulate lipids when grown on these substrates is probably

related to protrusions formed on cell surfaces, facilitating the uptake of hydrophobic substrates from the medium. Internalized aliphatic chains can be either degraded for growth requirements or accumulated in an unchanged or a modified form (Fickers *et al*., 2005).

Saxena *et al*. (1998) reported on the specific growth rate of an oleaginous *Rhodotorula minuta* IIP-33 on different substrates indicates the affinity of the strain toward the substrate used as a carbon source. The specific growth rate and lipid yield of the strain were determined by using carbohydrates, e.g., glucose, fructose, galactose, and lactose. The strain grew at a maximal specific growth rate of 0.34 h^{-1} on glucose. Growth on other substrates was comparatively slow, and no growth was observed on lactose (Table 5). Lipid accumulation of *R. minuta* IIP-33 was maximal on glucose (0.48) at the end of fed-batch fermentation, compared to other carbohydrates, e.g., sucrose (0.36), fructose (0.30) and galactose (0.11), as carbon substrate. However, the strain was unable to grow on lactose. Lipid-synthesizing characteristics of the strain on different carbohydrates have indicated that whey would be an unsuitable feedstock for lipid synthesis because lactose was not assimilated by the strain.

Table 5. Growth and Lipid Accumulation by *Rhodotorula minuta* IIP-33

Carbon substrate	Sp. growth rate (μ, h^{-1})	Lipid yield $Y_{p/x}$ (w/w)
Glucose	0.34	0.48
Fructose	0.30	0.30
Sucrose	0.22	0.36
Galactose	0.15	0.11

Source: Saxena *et al*. (1998)

While Zhu *et al*. (2008) reported on excess carbon is diverted to lipid biosynthesis in many oleaginous microorganisms. As depicted in Table 6, the maximum biomass was obtained when glucose was used as the carbon source, followed by fructose, sucrose, xylose and lactose. All the carbon sources tested gave relatively high lipid content. It is obvious that *T. fermentans* has a broad spectrum of

carbon sources. Similar lipid production from *Rhodotorula glutinis* was obtained when used glucose as carbon source at 100 g/L. *R. glutinis* accumulated lipids up to 49.25% based on biomass (Dai *et al*., 2007).

Table 6. Effect of carbon source on cell growth and lipid accumulation of *T. fermentans.*

Carbon source	Biomass (g/L)	Lipid content (%)	Lipid yield (g/L)
Glucose	24.1	56.6	13.6
Sucrose	19.5	62.6	12.2
Xylose	17.1	57.8	9.9
Lactose	16.9	49.6	8.4
Fructose	21.5	40.7	8.8

All cultures were performed at 25°C, pH 6.0 and 160 rpm for 7 days.
Source: Zhu *et al*. (2008)

Easterling *et al*. (2009) reported on the data demonstrates differences in oil accumulation by *Rhodotorula glutinis* between those cultures provided single carbon sources and those provided mixtures of carbons sources (Figure 5). Yeast cultured 24 h on medium containing dextrose, xylose, glycerol, dextrose and xylose, xylose and glycerol, or dextrose and glycerol accumulated 16, 12, 25, 10, 21, and 34% TAG on a dry weight basis, respectively. When comparing the 24 and 48 h data for all experiments, lipid content of the glycerol grown *R. glutinis* increased on average 12.97% while dextrose and xylose grown *R. glutinis* decreased 8.56% and 9.08%, respectively. The dextrose plus xylose grown culture increased 1.11% from 24 to 48 h. While the data suggests that using glycerol as a sole carbon source may result in greater lipid production by the oleaginous yeast *R. glutinis* the standard error shows there is not sufficient evidence to determine whether using glycerol in conjunction with a six or five carbon sugar will cause the oleaginous yeast *R. glutinis* to produce more lipid than when the carbon sources are used individually. It can be submitted, however, that glycerol grown *R. glutinis* accumulates more lipid under these experimental conditions than dextrose grown and xylose grown cultures.

Xylan, the principal component of hemicellulose, is a polymeric form of xylose and, like other polysaccharides, is not readily utilized by many yeast. However, Fall and coworkers (Verachtert and Mot, 1989) examined a number of oleaginous yeasts for the direct utilization of xylan itself and found that both *Cryptococcus albidus* and *Trichosporon pullulans* could produce good yields of intracellular triacylglycerols from the xylan taken from the wood of larch, birch and oats.

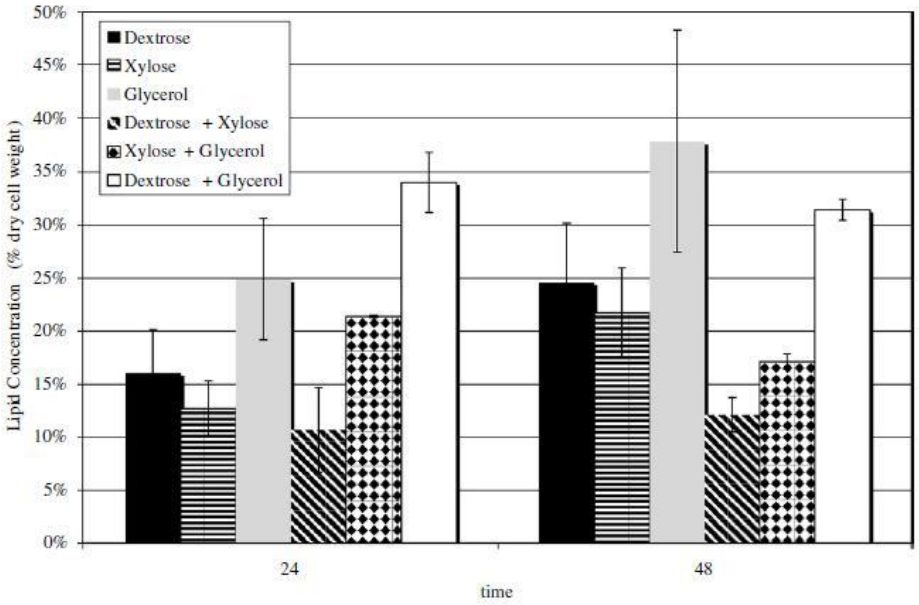


Figure 5. The effect of glycerol and culture duration on the total lipid production of *Rhodotorula glutinis* when grown at 35°C with agitation (112 rpm).

Source: Easterling *et al*. (2009)

Microbial lipid production by the oleaginous yeast *Rhorosporidium toruloides* Y4 was studied using glucose as a carbon source. Batch cultures demonstrated that there was little inhibitory effect with a substrate concentration of up to 150 g/L (Figure 6). When the initial glucose concentration increased from 10 to 90 g/L, the biomass, lipid yields and cellular lipid content increased from 4.7, 1.13 g/L and 24.0% to 18.6, 8.63 g/L and 46.4%, respectively. However, the biomass and lipid accumulation slightly dropped to 18.0 and 8.60 g/L for cultures with an initial glucose concentration of 120 g/L. These data were further reduced to 17.7 and 8.30 g/L, respectively for cultures with 150 g/L glucose. When the substrate concentration

reached 200 g/L, biomass and lipid production were greatly decreased, suggesting that a considerable inhibitory effect had occurred (Li *et al*., 2007).

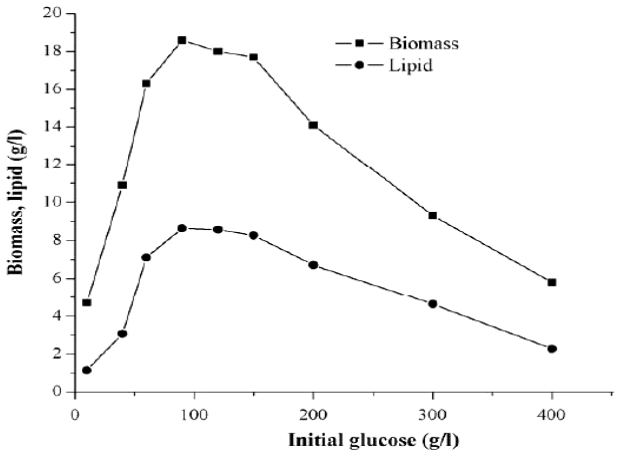


Figure 6. Biomass and lipids accumulated by *R. toruloides* Y4 at 120 h vs. initial glucose concentration.

Source: Li *et al*. (2007)

5.1.2 Nitrogen source and C/N ratio

Yeast cells have a nitrogen content of around 10% of their dry weight (Walker, 1998). Yeasts are capable of utilizing a range of different inorganic and organic sources of nitrogen for incorporation into the structural and functional nitrogenous component of the cell. Nitrogen will boost cell growth and lipid synthesis independent of the nitrogen concentration in the culture medium. Nutrient imbalance in the culture medium has long been known to trigger lipid accumulation by oleaginous microorganisms. When cells run out of key nutrients, usually nitrogen, excess substrate continues to be assimilated by the cells and converted into fat for storage. However, under nitrogen-limited conditions, cell propagation is drastically depressed, which in many cases restricts cell density. To achieve a high-density cell culture for microbial lipid fermentation, different substrates and cultivation modes have been used (Li *et al*., 2007). Although iron, zinc, phosphorus, nitrogen, and concomitant limitations of nitrogen and phosphorus or magnesium have resulted in lipid accumulation (Gill *et al.*, 1997; Ykema *et al.*, 1988), nitrogen limitation has been used more extensively to create an environment conducive to lipid hoarding.

Different nitrogen sources also had varied influence on oil production. Both inorganic nitrogen sources and organic nitrogen sources can be used for yeast cultivation with varied influence on oil accumulation (Liu *et al*. 2000). Huang *et al*. (1998) reported that inorganic nitrogen sources were good for cell growth but not suitable for oil production, while organic nitrogen sources such as peptone was good for oil production but not suitable for cell growth. Zhu *et al*. (2008) studied the effects of culture medium components on biomass and lipid production of *Trichosporon fermentans* (Table 7). Among the nitrogen sources tested, urea supported the maximum biomass (23.1 g/L), followed by peptone (19.7 g/L). With respect to lipid content, peptone was the best (54.9%) and the maximum lipid yield (10.8 g/L) was achieved with peptone as the nitrogen source. Disappointingly, $(NH_4)_2SO_4$, NH_4Cl and NH_4NO_3 gave both poor biomass and lipid content, indicating that organic nitrogen sources are more beneficial to lipid production of *T. fermentans* than inorganic nitrogen sources. While the maximum lipid production from *R. glutinis* was obtained when used yeast extract and peptone as nitrogen sources (Dai *et al*., 2007).

Carbon has been determined to be limiting at C/N of 4:1 and surfeit for oleaginous yeast C*ryptococcus curvatus* at C/N of 25:1 (Gill *et al.*, 1997). A C/N ratio of 10:1 was provided in the test media used in these *R. glutinis* batch culture experiments, therefore, rather than optimizing lipid production, the goal was to observe the basic lipid producing capability of the yeast when given different carbon sources (Easterling *et al*., 2009).

Table 7. Effect of nitrogen source on cell growth and lipid accumulation of *T. fermentans.*

Nitrogen source	Biomass (g/L)	Lipid content (%)	Lipid yield (g/L)
Peptone	19.7	54.9	10.8
Urea	23.1	23.0	5.3
$(NH_4)_2SO_4$	3.5	12.4	0.4
NH_4Cl	4.5	15.7	0.7
NH_4NO_3	3.7	14.8	0.5

All cultures were performed at 25°C, pH 6.0 and 160 rpm for 7 days.

Source: Zhu *et al*. (2008)

An optimum initial C/N molar ratio of about 77 was found for maximum lipid production from glucose or fructose with a nitrogen source from either $(NH_4)_2SO_4$, NH_4NO_3 or urea. The fatty acid composition of the lipids was affected by the initial C/N ratio and by the type of sugar but not significantly by the type of nitrogen (Turcotte and Kosaric, 1989).

Zhu *et al*. (2008) studied on the effect of C/N ratio on cell growth and lipid accumulation of *T. fermentans*. The biomass increased gradually with the increase of C/N molar ratio and reached the maximum of 24.0 g/L at 163. Lipid content was quite low at the C/N molar ratio of 108, then showed a sharp increase when C/N molar ratio increased from 108 to 140, and reached the maximum of 63.1% at 140 (Table 8). Further rise in C/N molar ratio beyond 140 resulted in a slight drop in lipid content but a continuous increase in biomass up to 163 and the highest lipid yield of 14.8 g/L was achieved at 163. There are two possible reasons for this phenomenon. One is that a high concentration of glucose could result in a high osmotic pressure and the other may be the excessive glucose consumption led to a sharp decrease in pH, which has been confirmed by examining the medium pH after fermentation (pH 3.5).

Table 8 Effect of C/N ratio on cell growth and lipid accumulation of *T. fermentans.*

C/N ratio	Biomass (g/L)	Lipid content (%)	Lipid yield (g/L)
108	19.6	56.8	11.1
130	20.8	59.9	12.5
140	21.8	63.2	13.8
158	22.4	61.7	13.8
163	24.0	61.6	14.8
186	20.9	61.3	12.8
238	18.7	60.8	11.4
248	14.8	59.3	8.8

All cultures were performed by using glucose as carbon source at 25°C, pH 6.0 and 160 rpm for 7 days.

Source: Zhu *et al*. (2008)

The potential of accumulation of lipids by *Lipomyces starkeyi* when grown on sewage sludge was studied by Angerbauer *et al*. (2008). On a synthetic medium, accumulation of lipids strongly depended on the C/N ratio. The highest content of lipids was measured at a C/N ratio of 150 with 68% lipids of the dry matter while at a C/N ratio of 60 only 40% were accumulated.

Lipid production of the oleaginous yeast *Apiotrichum curvatum* was studied in whey permeate to determine optimum operation conditions in this medium. Studies on the influence of the carbon to nitrogen ratio (C/N ratio) of the growth medium on lipid production in continuous cultures demonstrated that cellular lipid content in whey permeate remained constant at 22% of the cell dry weight up to a C/N ratio of about 25. The maximal dilution rate at which all lactose was consumed in whey permeate with excess nitrogen was found to be 0.073 h^{-1}. At C/N ratios higher than 25-30 lipid content gradually increased to nearly 50% at C/N = 70 and the maximal obtainable dilution rate decreased to 0.02 h^{-1} at C/N = 70. From these studies it could be derived that maximal lipid production rates can be obtained at C/N ratios of 30-35 in whey permeate. Since the C/N ratio of whey permeate normally has a value between 70 and 101, some additional nitrogen is required to optimize the lipid production rate (Ykema *et al*., 1988).

Growth and lipid accumulation profiles of *Rhodotorula minuta* IIP-33 on glucose in batch and fed-batch fermentation are displayed a growth-associated lipid yield of 0.25 under growth-phase batch fermentation, where the initial C/N ratio of the batch was maintained at 17, and the temperature at 32°C. The lipid yield of the cells increased to 0.48 at the end of fed-batch mode, where the initial C/N was maintained at 30. On further increasing the C/N to 40 in the fed-batch mode, lipid yield was decreased to 0.33. Thus, severe nitrogen limitation is not favorable for lipid accumulation by *R. minuta* IIP-33 (Saxena *et al*., 1998).

5.1.3 Metal ions

Trace metal ions also affect oil accumulation to a varied extent. Biomass and oil content could be improved significantly by the optimization of Mg^{2+}, Zn^{2+}, Mn^{2+}, Cu^{2+}, and Ca^{2+} concentrations (Li *et al*., 2006). Dyal *et al*. (2005) studied the effect of metal ion on growth and lipid accumulation in *Mortierella ramanniana*

var. *ramanniana*. The biomass yields increased with increasing Fe^{2+}concentration and decreased when Cu^{2+}, Mg^{2+}, or Zn^{2+} concentrations increased. In the case of Ca^{2+}, biomass yields appeared to be the same for 5 and 50 mg/L and increased for 500 mg/L. Mn^{2+} ions induced highest yield for metal ion concentration of 5 mg/L and lowest for 500 mg/L. The lipid yields per gram biomass of the media supplemented with metal ions were higher than those obtained with the unsupplemented media except for Zn^{2+} at 5 mg/L and Fe^{2+} at 50 and 500 mg/L. Lipid yields for Mg^{2+} decreased slightly with increasing metal ion concentration while those for Cu^{2+} and Fe^{2+} were highest at 5 mg/L then leveled up for 50 and 500 mg/L. The lipid yields of Zn^{2+}, and Mn^{2+} supplemented media were optimum at 50 mL/g with slightly higher values than those recorded for 50 and 500 mg/L while the yields of Ca^{2+} supplemented media increased and leveled up for the last two concentrations.

Granger *et al*. (1993) studied the effects of nitrogen (N), phosphorus (P), zinc (Zn) or iron (Fe) limitations on lipid and especially α-linolenic acid (ALA), which is an organic compound found in many common vegetable oils and an essential dietary requirement for all mammals, synthesis in the oleaginous yeast *Rhodotorula glutinis*. Exhaustion of the limiting element in the medium resulted in an enhancement of both the fatty acid cell content and the corresponding productivity. Except for Fe-limited media, lipid accumulation was always coupled with an increase in the yield of ALA synthesis, whereas in all the limiting conditions the productivity of ALA declined.

5.2 Culture conditions

5.2.1 Temperature

Temperature is one of the most important physical parameters which influence yeast growth. Most laboratory and industrial yeasts generally grow best between 20-30°C. Notable exceptions to this range are found when studying yeasts in natural habitats. Growth and metabolic activity of yeasts at various temperatures are functions not only of genetic background of the cell but also of the growth medium composition and other physical growth parameters. The accumulation of yeast metabolites, both extracellularly and intracellularly, may also influence the temperature profiles of yeast.

Table 9 Effect of temperature on cell growth and lipid accumulation of *T. fermentans.*

Temperature (°C)	Biomass (g/L)	Lipid content (%)	Lipid yield (g/L)
20	20.7	55.2	11.4
25	27.5	60.6	16.7
28	20.9	54.9	11.5
30	18.7	53.8	10.1
35	13.5	36.5	4.8

All cultures were performed at initial pH 6.0 and 160 rpm for 7 days.
Source: Zhu *et al*. (2008)

Zhu *et al*. (2008) reported on the effects of temperature on cell growth and lipid accumulation (Table 9). Both biomass and lipid content reached the maximum of 27.5 g/L and 60.6% at 25°C and lower or higher temperature was not suitable for cell growth and lipid accumulation of *T. fermentans*. It is worth noting that in the range from 20°C to 35°C, the total amount of unsaturated fatty acids of the lipid decreased from 71.8% to 52.0%, indicating that a low temperature was favorable for the formation of unsaturated fatty acids. While optimal temperature of *R. glutinis* was temperature at room temperature (Dai *et al*., 2007).

Temperature-induced changes in lipid biosynthesis are significant to know whether the organism is capable of adapting itself to wide ranges of temperature and how it affects its lipid yield and fat coefficient besides its fatty acid composition. Lipid yield and the fat coefficient profile of *R. minuta* IIP-33, cultured at different growth phase temperatures, i.e., 30 to 38°C, are illustrated in Figure 7. A maximal lipid yield of 0.25 and a fat coefficient of 14% in the growth phase were observed at a temperature of 32°C. The overall lipid yield and fat coefficient at the end of the accumulation phase were also maximal, i.e., 0.48 and 20.2%, respectively, at the same temperature. However, a temperature above 34°C was unfavorable for overall lipid biosynthesis, and the lipid yield and fat coefficient decreased to 0.12 and 3%, respectively, at 38°C (Sexena *et al*., 1998).

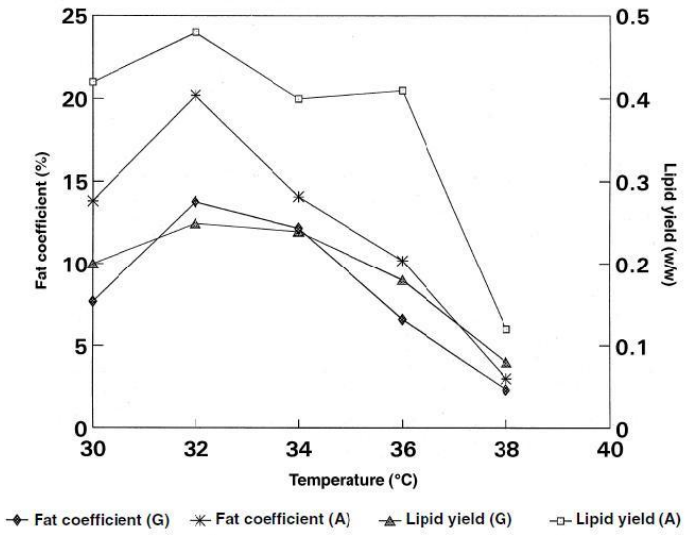


Figure 7. Effect of temperature on fat coefficient and lipid yield of *R. minuta* in growth (G) and accumulation (A) phases.

Source: Saxena *et al*. (1998)

Temperature also plays an important role in regulation of fatty acid composition of membrane lipids of a microorganism. The variation of the fatty acid composition of lipids accumulated in the growth phase of *R. minuta* cultured at different temperatures (30 to 38°C) (Saxena *et al*., 1998). The fatty acid profile of *R. minuta* grown at different temperatures showed a wide range of fatty acids (C7 to C18), presumably owing to temperature-sensitive acyl-carrier proteins, part of a key enzyme associated with chain elongation of fatty acids. Synthesis of long-chain fatty acids, e.g., C16, C18, C18:1, and C18:2, was predominant at 30–32°C, i.e., near the optimal growth temperature, whereas short-chain acids, e.g., C7, C8, C9, were predominantly synthesized at 38°C. However, a negligible variation in composition of middle-ranged (C10–C14) fatty acids was observed as a function of temperature. The degree of unsaturation (DUS mole^{-1}) was maximal (0.57) at 32°C and decreased to 0.02 at 38°C. That meant *R. minuta* could produce unsaturated fatty acids higher than saturated fatty acid at 32°C. The decrease in degree of unsaturation is probably due to a decrease in activity of desaturase enzymes at 38°C, which results in an increase of saturated short-chain (C7–C9) fatty acids in the lipids. The strain *R. minuta* IIP-33 exhibited biphasic behavior of the temperature-dependent degree of unsaturation.

Papanikolaou *et al*. (2002) studied the effect of the incubation temperature on cell growth of *Yarrowia lipolytica* at 19, 24, 28, 33, and 39°C. Temperatures of 19 and 39°C did not allow high growth, whilst significant growth was observed at temperatures of 24–33°C (x_{max}= 7.5–8.7 g/L). The specific growth rate was 0.26 ± 0.1 h^{-1} in the exponential phase. The highest lipid production was obtained at room temperature (L_{max}=3.8 g/L, $Y_{L/x}$ = 0.44 g/g), whereas at 24 and 33°C, the amounts of lipid were lower ($Y_{L/x}$ = 0.22–0.35 g/g).

5.2.2 Initial pH

Other physical growth requirements for yeasts relate to media pH. Most yeast grow very well between pH 4.5 and 6.5, but nearly all species are able to grow, albeit to a lesser extent, in more acidic or alkaline media (around pH 3 and pH 8, respectively). Media acidified with organic acids (e.g. acetic, lactic acid) are more inhibitory to yeast growth compared with those acidified with mineral acids (e.g. hydrocholoric, phosphoric acids). This is because undissociated organic acids can lower intracellular pH following translocation across the yeast plasma membrane. It is the basis of action of weak acid preservatives in inhibiting food spoilage yeast growth (Walker, 1998).

Zhu *et al*. (2008) reported on the effect of initial pH on cell growth and lipid accumulation of *T. fermentans* (Table 10), comparatively high biomass and lipid content could be achieved at the pHs ranging from 4.0 to 10.0. The maximum biomass (28.1 g/L) and lipid content (62.4%) were achieved at pH 6.5 which were much higher than the original values (19.4 g/L and 50.8%). While maximum lipid production from *R. glutinis* was obtained when cultivated at initial pH 5.0 (Dai *et al*., 2007).

Table 10. Effect of initial pH on cell growth and lipid accumulation of *T. fermentans.*

initial pH	Biomass (g/L)	Lipid content (%)	Lipid yield (g/L)
4.0	17.8	52.4	9.3
4.5	18.1	56.9	10.0
5.0	18.4	57.5	10.6
5.5	19.7	58.7	11.6
6.0	23.1	60.9	14.1
6.5	28.1	62.4	17.5
7.0	27.9	61.9	17.3
7.5	26.0	61.8	16.1
8.0	24.7	61.3	15.1
8.5	22.8	59.5	13.6
9.0	21.2	58.8	12.5
9.5	19.9	58.8	11.7
10.0	18.7	58.6	11.0

All cultures were performed at 25°C and 160 rpm for 7 days.

Source: Zhu *et al*. (2008)

The influence of initial pH on growth and lipid accumulation of *Yarrowia lipolytica* was studied in media with stearin, $(NH_4)_2SO_4$, and temperature 28°C. Kinetics were measured at pH 5, 5.5, 6, 6.5, and 7. Substantial growth was observed at pH 6 and 6.5 (x_{max} 9.5 and 8 g L^{-1}, respectively; x = total biomass), whereas lipid (L) accumulation was favored at pH 6 [L_{max}=2.7 g L^{-1}, $Y_{L/x}$ (lipid produced per total biomass) =0.28 g g^{-1}]. At pH 5 and 7, restricted microbial growth was observed. The pH changed slightly during growth, as low amounts of organic acids (mainly citrate at 0.2–0.8 g L^{-1}) were produced. An initial pH of 6 was chosen for all following experiments (Papanikolaou *et al*., 2002).

Johnson *et al*. (1992) reported that the maximum lipid production (66% w/w dry wt) in *Rhodotorula glutinis* IIP-30 utilizing glucose in a fed-batch fermentation under N-limiting was obtained at pH 4. At pH 3, 5 and 6, the lipid contents were 12%, 48% and 44%, respectively. There was only a small change in the

fatty acid profile over the pH range examined, although the ergosterol content decreased by a third as the pH increased.

Angerbauer *et al*. (2008) studied the influence of pH on lipid accumulation of *Lipomyces starkeyi*. Raw sewage sludge from a treatment plant varies quite strongly with regard to the pH-value (from 5 to 7.5) and thus a pH range from 5.0 to 7.5 was chosen for these experiments. The highest lipid content of *L. starkeyi* was found at a pH 5.0 while the yield per litre was highest at a pH 6.5. Thus, there was not a big influence on the accumulation of lipids in the pH-range 5.5 to 6.5. However, at pH 7.0 the accumulation of lipids decreased dramatically. In the literature different pH-values are reported for optimal lipid accumulation which seemed to depend on the carbon sources used.

5.2.3 Aeration

Yeasts are unable to grow well in the complete absence of oxygen. This is because, as well as providing a substrate for respiratory enzymes during aerobic growth, oxygen is required for certain growth-maintaining hydroxylations such as those involving the biosynthesis of sterols and unsaturated fatty acids. Specifically, yeasts need molecular oxygen for the mixed-function oxidase mediated cyclization of squalene 2, 3-epoxide to form lanosterol and for the synthesis of unsaturated fatty acyl coenzyme-A esters. Oxygen should therefore be regarded as an important yeast growth factor. Different yeasts possess different requirements for molecular O_2 base on agitation rate (Table 11) and pure oxygen can even be strongly inhibitory to yeast cells. The toxicity of hyperbaric oxygen towards yeasts may be cell cycle-dependent (Walker, 1998).

Table 11. Effect of agitation rate on cell growth and lipid accumulation from yeasts species.

Species	Aeration (rpm)	Biomass (g/L)	Lipid content (% w/w)	Reference
Lipomyces starkeyi	120	9.4	68	Angerbauer *et al*., 2008
Rhodotorura glutinis	140	29.77	49.25	Dai *et al*., 2007
Trichosporon fermantans	160	28.1	62.4	Zhu *et al*., 2008

5.2.4 Fed-batch fermentation

Fed-batch culture has been used successfully to improve the productivity of both homologous and heterologous proteins in high cell density cultures. It is an effective technique for overcoming cellular regulatory mechanisms such as the Crabtree effect (Siso, 1994), catabolite repression (Hendy *et al*., 1984) and product inhibition (Konstantinov *et al*., 1990). Moreover, several recent studies have demonstrated the ability of fed-batch culture to enhance plasmid stability in recombinant cell fermentations (Cheng *et al*., 1997). Fed-batch cultures are frequently operated in feed-forward modes with feedback controls to provide a corrective scheme to compensate for process disturbances and model inaccuracies. The effectiveness of feedback controls depends on the choice of control variables and their ability to reflect culture performance**.**

Meesters *et al*. (1996) studied growth and lipid production of *Cryptococcus curvatus* on glycerol in fed-batch fermentation mode. They performed a fermentation consisting of two phases. The first phase is the biomass production phase in which there is no nutrient limitation except for short periods of glycerol exhaustion. The substrate feed was controlled by the dissolved oxygen tension. In the second phase nitrogen limitation was introduced, which causes lipid accumulation. This way very high cell densities of 118 g/L in 50-h fermentation could be reached. With a lipid production rate of 0.59 g lipid $L^{-1}h^{-1}$, a cellular lipid content of 25% was obtained. The growth and lipid accumulation phase are characterized by different cellular fatty acid compositions. In the growth phase, a relatively high amount of C18:2 (linoleic acid) is present, which is a major component of membrane lipids.

C18:0 (stearic acid) and C18:1 (oleic acid) are major constituents of the accumulated triglycerides and therefore the relative amount of C18:2 decreases during the lipid accumulation phase.

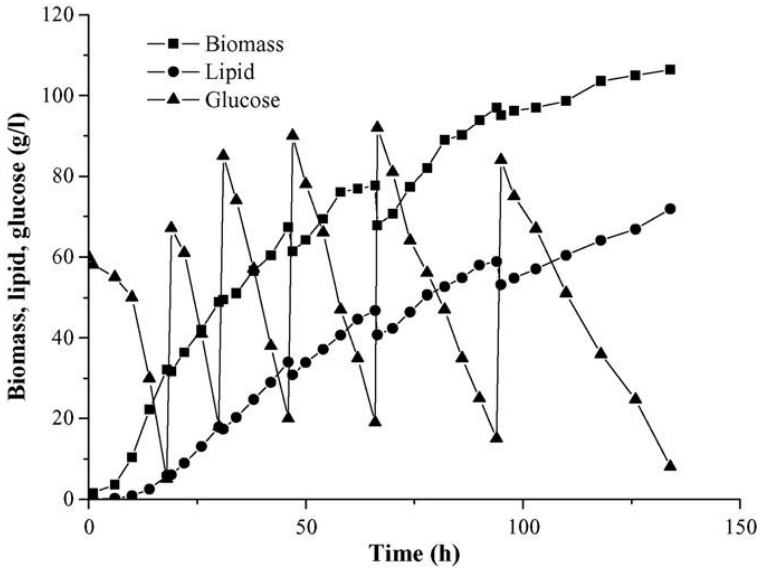


Figure 8. Pilot-scale fed-batch fermentation profile for *R. toruloides* Y4 in a 15-L fermenter.

Source: Li *et al*., 2007

Li *et al*. (2007) conducted a pilot-scale fed-batch culture using a 15-L stirred-tank fermenter. The typical time-course of glucose concentration, biomass and lipid production for this experiment are shown in Figure 8. It was obvious that cell growth was fast during the initial stage. Biomass increased rapidly from 0.3 to 32.2 g/L within18 h, and biomass yield was 0.59 g/g glucose. In the period of 10–18 h, growth was linear and biomass increased with a high specific growth rate of about 0.13 h^{-1}. The first batch of feeding medium was introduced at 18 h and repeated four times during the 134-h-long fermentation. The overall biomass yield, lipid yield and cellular lipid content were 0.35 g/g, 0.23 g/g and 67.5% (w/w), respectively. The overall lipid production rate was 0.54 g/L.h. Within the 134-h-long fermentation, 2500 g of glucose was fed into the reactor, which corresponds to a final substrate concentration of 307 g/L.

Xue *et al*. (2008b) studied on lipid production by *Rhodotorula glutinis* fermentation using monosodium glutamate wastewater (MSG) wastewater and glucose added MSG wastewater as a culture medium. Three different strategies, including initial addition, fed-batch addition and glucose feedback addition were attempted. The results found that the addition of glucose was favorable not only for cell growth but also for lipid synthesis. Of the three adding methods glucose feedback addition was the most effective one: about 25 g/L of biomass, 20% of lipid content and 45% of COD degradation were obtained, respectively.

5.2.5 Mixed culture

Mixed culture of microalgae (*Spirulina platensis*) and yeast (*Rhodotorula glutinis*) for lipid production was studied by Xue *et al*. (2008a). Mixing cultivation of the two microorganisms significantly increased the accumulation of total biomass and total lipid yield. The result of mix cultivation in the mixed medium was compared with *R. glutinis* cultivated in yeast medium or *S. platensis* cultivated in Zarrouk medium. During mix cultivation, it was observed that the color of *S. platensis* was yellow rather than green, which meant that the synthesis of chlorophyll *a* was restrained and mixotrophic process was conducted instead because of the presence of glucose. As shown in Table 12, the highest total biomass concentration in the *R. glutinis* culture was 4,784 mg/L and the highest lipid content was 16.02% in the *S. platensis* culture. The highest total lipid yield was 467 mg/L obtained from the mix cultivation, which was 3.18 times of *R. glutinis* culture and 3.92 times of *S. platensis* culture.

Table 12. Comparison of mix cultivation and single microorganism cultivation.

Medium	Microorganisms	Residual glucose (g/L)	Lipid yield (mg/L)	Biomass (mg/L)	Lipid content (%)
Mixed	*Rhodotorula glutinis*	0.05	135±2	1,702±12	7.91±0.07
	Spirulina platensiss	28.5	13±1	203±10	6.39±0.01
	Rhodotorula glutinis and				
	Spirulina platensis	0.01	467±15	3,673±18	12.71±0.40
Yeast	*Rhodotorula glutinis*	0.03	147±2	4,784±19	3.13±0.04
Zarrouk	*Spirulina platensis*	–	119±3	743±8	16.02±0.26

Cultivation condition: 30 °C, 140 rpm, cultivated for 5 days.

Source: Xue *et al*. (2008a)

6. Lipid production using waste as substrates

Although there are many works such as process optimization and scaling up that need to be carried out further, utilizing cheap carbon sources for yeast oil production opens a new way for oil cost reduction, which is very important for such oils used for biodiesel production in the future. Microbial oils, which are renewable and potentially inexhaustible source of energy as potent as diesel fuel, have attracted much attention in recent years. Due to recent increment in petroleum prices, more concerns as shown (Papanikolaou *et al*., 2004) in microbial oil fuels arise nowadays. Recently, *Rhodotorula glutinis* was used for wastewater treatment (Xue *et al*., 2006). Monosodium glutamate (MSG) wastewater was used as a cheap fermentation broth, which served as the raw material for the production of biodiesel using lipid from *R. glutinis*. However, both the biomass and lipid content were very low. Yang *et al*. (2005) have recently shown the ratio of C:N:P (1:2.4:0.005) in MSG wastewater is not appropriate for microbial growth and lipid synthesis: phosphorus and carbon are obviously not sufficient while the concentration of NH_4^+ -N is too high, which will boost cell growth but inhibit the lipid synthesis. It has been stated that glucose could enhance lipid production with some microorganism species, so the effect of glucose addition in the MSG wastewater on lipid production was studied

(Feng *et al*., 2005). It was found that addition of glucose was favorable not only for cell growth but also for lipid synthesis.

Easterling *et al*. (2009) studied on the effects of different growth substrates on TAG accumulation and fatty acids produced by *R. glutinis*. Yeast cultured 24 h on medium containing dextrose, xylose, glycerol, dextrose and xylose, xylose and glycerol, or dextrose and glycerol accumulated 16, 12, 25, 10, 21, and 34% TAG on a dry weight basis, respectively. Lipids were extracted from *R. glutinis* culture and transesterified to form fatty acid methyl esters. The results show a difference in the degree of saturation for the carbon sources tested. Cells cultivated on glycerol alone had the highest degree of unsaturated fatty acids at 53% while xylose had the lowest at 25%. *R. glutinis* can be cultivated on all sugars tested as single carbon substrates or in mixtures. Glycerol may be used as a secondary or a primary carbon substrate.

Trichosporon fermentans could grow well in pretreated waste molasses and a lipid yield of 12.8 g/L could be achieved with waste molasses of 15% total sugar concentration (w/v) at pH 6.0, representing the best result with oleaginous microorganisms on agro-industrial residues. Addition of various sugars to the pretreated molasses could efficiently enhance the accumulation of lipid and the lipid content reached as high as above 50%. Similar to vegetable oils, the lipid mainly contains palmitic acid, stearic acid, oleic acid and linoleic acid and the unsaturated fatty acids amount to about 64% of the total fatty acids. The microbial oil with an acid value of 5.6 mg KOH/g was transesterified to biodiesel by base catalysis after removal of free fatty acids and a high methyl ester yield of 92% was obtained (Zhu *et al*., 2008).

7. Biodiesel production from microbial oils

Lewis *et al*. (2000) studied effect of different extraction techniques on the recovery of fatty acids from freeze-dried biomass of two lipid-producing microheterotrophs. Two procedures were used: the extraction of lipids from biomass followed by transesterification of the fatty acids (extraction-transesterification); and the direct transesterification of biomass to produce fatty acid methyl esters (i.e. without the initial extraction step). Variable factors in the extraction-

transesterification experiment were the sequence in which solvents were added to the samples, the relative amount of methanol in the solvent mix, and sonication of biomass while in the solvent mix. Variable factors in the direct transesterification experiment were sample size, and reaction duration. Statistical analysis of data showed that: (1) extraction of total fatty acids prior to transesterification was significantly more efficient when solvents were added in the order of increasing polarity; (2) neither sonication nor increasing the proportion of methanol in the extraction solvent significantly affected extraction of fatty acids prior to transesterification; (3) efficiency of direct transesterification of fatty acids increased significantly with reaction time; (4) efficiency of direct transesterification of fatty acids was not significantly affected by sample size; (5) the most efficient method for extraction of fatty acids prior to transesterification yielded significantly less fatty acids than the most effective direct transesterification method.

CHAPTER 3

MATERIALS AND METHODS

Materials

1. Microorganisms and crude glycerol

The yeast strains were isolated from soil and wastes of palm oil mill and biodiesel plant in southern region of Thailand.

Rhodotolura glutinis TISTR 5159 as the reference strain and *Chlorella vulgaris* TISTR 8261 was purchased from a stock culture of Thailand Institute of Scientific and Technological Research (TISTR), Bangkok, Thailand.

A freshwater microalga *Chlorella* sp. and a marine microalga *Chlorella* sp. was purchased from National Institute of Coastal Aquaculture in southern region of Thailand.

Crude glycerol, waste discharged from biodiesel manufacturing process was received from a biodiesel plant located at the Faculty of Engineering, Prince of Songkla University, Songkhla, Thailand. Crude glycerol was filtered through mesh to separate suspension solid. It was then characterized based on pH, glycerol concentration, total nitrogen (Koroleff's method, Merck, 2009), Chemical Oxygen Demand (COD, Open Reflux method) and oil and grease (Partition-Gravimetric method) according to the standard method (APHA, AWWA and WPCF, 1998; Appendix 1-3). It contained glycerol 39.3% (w/v), methanol 2% (w/v), total nitrogen 0.11% (w/v), COD 189% (w/v), oil and grease 0.001% (w/v) and water 58% with pH 10.27.

2. Media preparation and cultivation

2.1 Medium for yeast

2.1.1 Enrichment and isolation medium

Waste samples from palm oil mill and biodiesel plant were enriched in yeast extract-peptone-dextrose medium (YPD) (yeast extract 1% and peptone 1%) by

using 4% glucose or glycerol as a carbon source at acid pH (4.0) or slightly acidic pH (6.0) with 0.0001% chloramphenicol. Then, 0.1 mL diluted culture was transferred on YPD agar medium (yeast extract 1%, peptone 1%, glucose or glycerol and 1.2% agar) using spread-plate technique and was incubated at room temperature for 72 h.

2.1.2 Inoculum medium

Oleaginous yeast strains were precultured in inoculum medium (glucose 4%, yeast extract 1% and peptone 1%, pH 6.0) at room temperature with shaking at 140 rpm for 24 h.

2.1.3 Crude glycerol based medium

Seed culture (10%) of 24 h old preculture was inoculated in crude glycerol based medium (crude glycerol 10%, yeast extract 1% and peptone 1 %, pH 6.0) at the C/N molar ratio of 9.8 (Appendix 4) and incubated at room temperature with shaking at 140 rpm or 72 h (Li *et al*., 2007).

2.2 Chu 13 medium for microalgae

Microalgae were precultured in Chu13 medium (g/L: 0.2 g KNO_3, 0.04 g K_2HPO_4, 0.1 g $MgSO_4 \cdot 7H_2O$, 0.054 g $CaCl_2 \cdot 2H_2O$, 0.01 g Fe citrate, 0.1 g citric acid, 0.036 g $NaHCO_3$ and 1 mL of micro element with consist of 2.85 g H_3BO_3, 1.8 g $MnCl_2 \cdot 4H_2O$, 0.02 g $ZnSO_4 \cdot 7H_2O$, 0.08 g $CuSO_4 \cdot 5H_2O$, 0.08 g $CoCl_2 \cdot 6H_2O$ and 0.05 g $Na_2MoO_4 \cdot 2H_2O$, pH was adjust to 6.7 with 1 M KOH (Largeau *et al*., 1980)) at room temperature and 140 rpm for 7 days with continuous cool-white fluorescence illumination (2,000 lux).

3. Instruments

All instruments used in this work listed and shown in Table 13, were in Faculty of Agro-Industry, Prince of Songkla University, Thailand.

Table 13. List of instruments.

Instruments	Series	Supppliers
Autoclave	SS-325	Tommy, USA
Balance	PA214	OHAUS, USA
Centrifuge	CF-10	Wise Spin, Korea
Chromarods	SM-III	Iatron Laboratories, Japan
Chromarods holder	SD-5	Iatron Laboratories, Japan
Developing tank	TD-150	Iatron Laboratories, Japan
Digital lux meter	Lx 801	Nicety, USA
DO meter	DO200	Clean, USA
Fluorescence	Cool-white, 36W	SYLVANIA, Thailand
Gas Chromatography	6890	Hewlett Packard, USA
GC column 30 m×0.32 mm	FFAP column	Agilent, USA
Heamacytometer	-	Diamond, Taiwan
Hot air oven	ED115	BINDER, USA
Laminar flow	-	Clean, USA
Phase contrast microscope	ECLIPSE E100	Nikon, Japan
pH controller	4801	DIN, Taiwan
pH meter	EF-201	Mettler Toledo, China
Shaker	VRN-480	GEMMY, Taiwan
Sonicator	E30 H	Elma, Germany
Spectrophotometer	LIBRA-S22	Biochrom, England
Stirrer	MS115	ML, Thailand
Stirrer tank bioreactor, 5 L	-	S.T.S., Taiwan
TLC-FID (Iatroscan)	MK5	Iatron Laboratories, Japan
Vortex	VM-10	WiseMix, Korea

Analytical Methods

1. Staining with Sudan black B technique

Total yeast strains were stained with Sudan black B technique, which was a lysochrome (fat-soluble dye) diazo dye used for staining of neutral triglycerides and lipids, by preparing the smear on a slide, let it dry thoroughly in the air, and heat fix. Flood the entire slide with Sudan black B solution (0.3 g of Sudan black B powder in 100 ml of 60% ethanol), and allow the slide to remain undisturbed at room temperature for 5 minutes and drain off excess stain, wash with water and air dry. The stained yeasts were observed under a phase contrast microscope on oil immersion for the presence of blue or greyish colored fat globules within the cell (Patnayak and Sree, 2005).

2. Determination of biomass concentration and total lipid

2.1 Biomass

Biomass concentration was determined gravimetrically and expressed as dry cell weight (gram dry weight per liter). Samples containing 2 mL fermentation broth withdrawn from the flasks would be centrifuged at 4,000 rpm for 10 min, the cell pellet was collected and washed twice with distilled water, and then was dried at 60°C to constant weight for 2 days (Xue *et al*., 2008a).

2.2 Lipid extraction

The dry biomass was ground into a fine powder; 0.1 g powder was blended with 1 mL chloroform: methanol (2: 1) and glass bead (ca. 0.5 g × 0.5 mm diameter). The mixture was sonicated for 30 min at 70 Hz at room temperature. Solvent phase was recovered by centrifugation. The extraction process was repeated two more times. The combined solvent was removed by drying at 60°C to constant weight. Lipid content was expressed as gram lipid per gram dry biomass (Xue *et al*., 2008a).

2.3 Cell counting of yeast and microalga by using a heamacytometer

The viable yeast cell and microalga numbers were stained with 0.02 % (w/v) methylene blue and counted directly using heamacytometer. The culture broth was mixed well and used a Pasteur pipette to transfer a small amount of the cell suspension to the counting chamber. Cells were counted in the four corner squares. Each square of the heamacytometer (with cover slip in place) represented a volume of 0.1 mm^3 or 10^{-4} cm^3. Since 1 cm^3 was equivalent to 1 mL, the subsequent cell concentration per mL (and the total number of cells) was determined using the following calculation (Frei, 2011).

$$\text{Cells per mL} = \text{the average number of cells per square} \times 10^4 \times \text{dilution}$$

3. Determination the compositions of glycerides by Thin Layer Chromatography analysis (TLC-FID)

The compositions of extracted lipid from oleaginous yeasts were determined using a thin layer chromatography equipped with flame ionization detector or TLC-FID (Rosu *et al*., 1998). One µL of lipid solution (diluted in chloroform at appropriate dilution) was spotted on to the Chromarods, which was then developed in a solvent mixture of benzene/chloroform/acitic acid (50:30:0.5 v/v/v) until the solvent front reached 10 cm (approximately 35 min). The Chromarods were dried at 105°C for 5 min and scanned process was performed using hydrogen flow rate of 160 mL/min, an air flow rate 2.0 L/min and scanning speed was 30 sec/rod to produce a chromatogram. The glyceride compositions were calculated as percentage based on the peak area of each component (Appendix 6).

4. Determination of fatty acid composition by Gas Chromatography (GC)

The method for converting extracted lipids to fatty acid methyl esters (FAME) involved hydrolysis of the lipids followed by esterification. Hydrolysis of the lipids (50 µL) was done with 1 mL of KOH/MeOH (0.5 M) at 100 °C for 5 min. Esterification, the hydrolysis mixture was added 400 µL of aq. HCl/MeOH (4:1, v/v) and the mixture was heated in an oil bath for 15 min at 100 °C. The tube was cooled

and 2 mL of water was added and then extracted with 2×3 mL of petroleum ether. The organic layer was dried quickly over anhydrous Na_2SO_4, evaporated and redissolved in 500 µL of $CHCl_3$, and 0.5 µL was used for gas chromatography (GC). The fatty acid composition in FAME was analyzed using a HP6850 Gas Chromatograph equipped with a cross-linked capillary FFAP column (length 30 m, 0.32 mm I.D, 0.25 µm film thickness) and a flame ionization detector. The operating conditions were as follows: inlet temperature 290 °C, oven temperature initial 210°C hold 12 min ramp to 250 °C at 20°C/min hold 8 min and detector temperature 300°C. Fatty acids were identified by comparison of their retention times with those of standard ones and calculated as percentage based on their respective peak areas using a standard mixture of FAME (Appendix 9) (Jham *et al*.,1982).

5. Determination of glycerol concentration

The glycerol concentration was determined in the water phase of the reactor and centrifuged at 4,000 rpm for 10 min. The supernatant solution was mixed with KIO_4. 0.5 mL of sample and 0.2 mL of 0.0025 M KIO_4 were reacted for 5 min. Then, 0.05 mL of 0.5 M sodium arsenate was added. After 10 min, 2 mL of chromotropic acid reagent was added (110 mg of chromotropic acid disodium salt in 10 mL water with 120 mL of 50% H_2SO_4 added). The tubes were adjusted to a volume of 5 mL with water. The absorbance at 570 nm was measured and the glycerol concentration was calculated using a standard curve of glycerol (Appendix 5) (Kosugi *et al*., 1995).

6. Kinetic of growth and lipid production from microbial oil

6.1 Specific growth rate of single cultivation of yeast:

$$\text{Specific growth rate, } \mu \ (h^{-1}) = \frac{\ln\frac{x}{x_0}}{\Delta t}$$

x total biomass (g/L)

x_0 initial total biomass (g/L)

t time (h)

6.2 Lipid productivity

$$\text{Lipid productivity, } Q_p \text{ (g/L.h)} = \frac{\text{Maximun lipid production (g/L)}}{\text{Cultivation tiime (h)}}$$

7. Statistical analysis

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

Experimental Methods

1. Screening and optimization of medium components

1.1 Quantitative lipid production from crude glycerol by oleaginous yeasts

1.1.1 Screening of oleaginous yeasts

The samples were collected from soil and waste of palm oil mill and biodiesel plant in southern region of Thailand. Ten percentages of samples were enriched in 25 mL YPD medium for 48 h at room temperature. The culture was diluted in 0.85% normal saline. Then, 0.1 mL diluted culture was inoculated onto YPD agar medium using spread-plate technique and incubated at room temperature for 72 h. Total yeast strains were stained with Sudan black B technique and observed under a phase contrast microscope on oil immersion for the presence of blue or greyish colored fat globules within the cell (Patnayak and Sree, 2005). The yeast strains showing fat globules within the cell by using *R. glutinis* TISTR 5159 as the reference strain were selected for further quantitative analysis. The yeast strains were precultured in inoculum medium, and then 24 h old preculture was inoculated in 25 mL crude glycerol based medium and incubated at room temperature with shaking speed at 140 rpm for 72 h. The yeast strains which gave the highest lipid production (biomass × lipid content) were selected for further step (section 1.1.2).

1.1.2 Identification of the selected oleaginous yeasts using 26S rDNA sequence

The selected oleaginous yeast strains were identified based on its 26S rDNA sequence. The selected oleaginous yeast strains were inoculated and grown in 50 mL of YPD broth (yeast extract 1%, peptone 1% and dextrose 4%) at 28°C on a reciprocal shaker at 140 rpm. The culture was centrifuged at 14,000 rpm for 10 min. The cells were washed twice with 2 mL of distilled water, resuspended in 2 mL of distilled water and 1 mL of the suspension was pipetted into each of two 1.5 mL microcentrifuge tubes. DNA isolation for PCR was performed by a modified version of the sodium dodecyl sulfate protocol of Raeder and Broda (1985). The microcentrifuge tube was added with ca. 0.5 mL of glass beads (0.5 mm diameter). The tube was shaken for 20 min on a wrist action shaker at maximum speed. This treatment visibly fractured about 25% of the cells. The cell were suspended in 1 mL of extraction buffer (200 mM Tris-HCl at pH 8.0, 250 mM NaCl, 25 mM EDTA at pH 8.0 and 0.5% sodium dodecyl sulfate) and extracted with phenol and chloroform. DNA was precipitated from the aqueous phase by adding 0.54 volume of isopropanol and pelleted for 3 min at 14,000 rpm. The pellet was washed gently with 70% ethanol, resuspended in 100 µL of TE buffer (10 mM Tris-HCl, 1 mM EDTA at pH 8.0) and dissolved by incubation at 55°C for 1 h.

The eukaryotic 26S rDNA gene was amplified by PCR with thermal cycler (Kurtzman and Robnett, 1998). The PCR was performed in a final volume of 100 µL containing DNA sample 0.5 µL, 10×PCR buffer 10 µL, deoxynucleoside triphosphate (dNTPs) 4 µL, 25 mM $MgCl_2$ 4 µL, primer 4 µL, *Taq* DNA polymerase 1 µL and water 76.5 µL. The oligonucleotide primers used for the yeast 26S rRNA were various universal primer sets of F63-Forward; 5´-GCA TAT CAA GCG GAG GAA AAG-3´. The reactions were run for 35 cycles: denaturation was at 94°C for 60 s, annealing at 52°C for 30 s and extension at 72°C for 60 s. Amplification products were detected by electrophoresis on a 0.8% agarose gel, staining with ethidium bromide. Reaction products which displayed bands corresponding to the correctly size product (about 500 base pair) were purified using QIAquick PCR purification kit (QIAGEN, German). For sequencing of PCR products used automated DNA sequencer (Biosystems GeneScan 3700, Foster City, CA). The obtained sequences

were BLAST searched against National Center for Biotechnology Information (NCBI) database (http://www.ncbi.nlm.nim.gov).

1.2 Optimization of medium components

A loop of oleaginous yeast from a stock culture was inoculated into 5 mL medium in a test tube and cultivated at room temperature and 140 rpm. Then 10% of 24-h-old precultured inoculum was transferred into 50 mL of sterilized crude glycerol based medium in a 250-mL Erlenmeyer flask and incubated at room temperature on rotary shaker at 140 rpm for 3 days.

1.2.1 Nitrogen sources

The initial amount of crude glycerol was fixed at 10% (0.13 M of carbon). The nitrogen sources tested were organic nitrogen sources (yeast extract 3.00 %, peptone 1.50 %, urea 0.46 %) and inorganic nitrogen sources ($(NH_4)_2SO_4$ 0.99 %, NH_4Cl 0.80 %, NH_4NO_3 1.20 %). The initial amount of nitrogen source was fixed at the C/N molar ratio of 8.6 (Appendix 4). The medium containing no nitrogen source was used as a control. The seed culture was inoculated and grown at room temperature on a reciprocal shaker at 140 rpm. The samples were taken every 12 h until 72 h and determined for pH, cell dry weight, lipid content, lipid production, specific growth rate and lipid productivity (Analytical method 2 and 6). The nitrogen source which gave the highest lipid production was selected for further step (section 1.2.2).

1.2.2 Nitrogen concentration

The effects of nitrogen concentrations were studied at 0, 0.5, 1.0, 1.5 and 2.0%. Ten percentage of the seed culture were inoculated and grown at room temperature on a shaker at 140 rpm. The samples were taken every 12 h until 72 h to determine for pH, cell dry weight, lipid content, lipid production, specific growth rate and lipid productivity (Analytical method 2 and 6). The nitrogen concentration which gave the highest lipid production was selected for further step (section 1.2.3).

1.2.3 Glycerol concentration

Crude glycerol was used as a carbon source at concentrations of 5%, 10%, 15% and 20% (w/v). The optimal nitrogen concentration was selected from 1.2.2. The seed culture was inoculated and grown at room temperature on a shaker at 140 rpm. The samples were taken every 12 h until 72 h and determined pH, cell dry weight, lipid content, lipid production, specific growth rate and lipid productivity (Analytical method 2 and 6). Crude glycerol concentration which gave the highest lipid production was selected for further step (section 2.1).

1.2.4 Glycerides compositions analysis

The dry biomass was ground into a fine powder; 0.1 g powder was blended with 1 mL chloroform: methanol (2: 1) and glass bead (ca. 0.5 g × 0.5 mm diameter). The mixture was sonicated for 30 min at 70 Hz at room temperature. Solvent phase was recovered by centrifugation. The extraction process was repeated two more times. The combined solvent was removed by drying at 60°C to constant weight. The compositions of glycerides of oleaginous yeasts were determined using TLC (Analytical method 3).

2. Enhancing lipid production by mixed culture of oleaginous yeast and microalga

A seed culture of cells (oleaginous yeast and microalga) was incubated at room temperature and 140 rpm. Microalgae were incubated for 7 days with 16:8 light:dark cycle (2,000 lux), while oleaginous yeast was incubated for 24 h without special light illumination (Xue *et al*., 2008).

2.1 Mixed culture of oleaginous yeast *T. spathulata* and microalga

Seed cultures of oleaginous yeast (1×10^6cells/mL) and microalga (1×10^5 cells/mL) were transferred into 250 mL Erlenmeyer flasks containing 50 mL of crude glycerol based medium. The flasks were incubated at room temperature and 140 rpm. For microalgae, the culture was cultivated under 16:8 light:dark cycle (2,000 lux). The samples were taken every 12 h and determined for pH, cell dry weight, lipid

content, lipid production, specific growth rates of yeast and microalga and lipid productivity (Analytical method 2 and 6).

Seed cultures of each oleaginous yeast and microalgae (yeast and microalga ratio at 1×10^6:1×10^5 cells/mL) were transferred into 250 mL Erlenmeyer flasks containing 50 mL of crude glycerol based medium (from section 2). The flasks were incubated at room temperature and 140 rpm for 24 h for oleaginous yeast and 5 days for microalga under 16:8 light:dark cycle (2,000 lux) for microalga. The samples were taken every 12 h and determined for cell counting, pH, cell dry weight, lipid content, lipid production, specific growth rates of yeast and microalga and lipid productivity (Analytical method 2 and 6).

2.1.1 Selection of microalgae for enhancing biomass and lipid production

Seed cultures of each oleaginous yeast and three microalgae including *Chlorella vulgaris* var. *vulgaris* TISTR 8261, a fresh water microalga *Chlorella* sp. and a marine microalga *Chlorella* sp., were transferred into 250 mL Erlenmeyer flasks containing 50 mL of crude glycerol based medium. The samples were taken every 12 h and determined for cell counting, pH, cell dry weight, lipid content, lipid production, specific growth rates of yeast and microalga and lipid productivity (Analytical method 2 and 6). The microalga specie which gave the highest lipid production was selected for further step (section 2.1.2).

2.1.2 Inoculum ratio

The initial oleaginous yeast : microalgae ratio on cell growth and lipid production of oleaginous yeast and microalgae were varied at 1×10^6:1×10^4, 1×10^6:1×10^5, 1×10^6:1×10^6 and 1×10^6:1×10^7 cells/mL and incubated at room temperature and 140 rpm under 16:8 light:dark cycle (2,000 lux). The samples were taken every 12 h and determined for pH, cell count, cell dry weight, lipid content, lipid production, specific growth rates of yeast and microalga and lipid productivity (Analytical method 2 and 6). The inoculum ratio which gave the highest lipid production was selected for further step (section 2.1.3).

2.1.3 Light intensity

The light intensity on cell growth and lipid production of oleaginous yeast and microalgae were varied at 2,000 4,000 and 8,000 lux. The samples were taken every 12 h and determined for pH, cell count, cell dry weight, lipid content, lipid production, specific growth rates of yeast and microalga and lipid productivity (Analytical method 2 and 6).

2.2 Mixed culture of oleaginous yeast *Rhodotorula glutinis* and microalga

2.2.1 Biomass and lipid production from pure glycerol by single culture and mixed culture of yeast and microalga

Seed cultures of *R. glutinis* (1×10^6cells/mL) and microalgae (1×10^5 cells/mL) were transferred into 250 mL Erlenmeyer flasks containing 50 mL using 1% pure glycerol as a carbon source and ammonium sulfate as a nitrogen source with the C/N molar ratio of 16. The flasks were incubated at room temperature and 140 rpm. For microalgae, the culture was cultivated under 16:8 light:dark cycle (2,000 lux). The samples were taken every 12 h and determined for pH, cell dry weight, lipid content, lipid production, specific growth rates of yeast and microalga and lipid productivity (Analytical method 2 and 6).

2.2.2 Optimizing the medium for the co-culture of yeast and microalga

2.2.2.1 Nitrogen source

The initial amount of pure glycerol was fixed at 1%. The nitrogen sources tested were ammonium chloride, ammonium nitrate, urea and ammonium sulfate.The seed culture was inoculated and grown at room temperature on reciprocal shaker at 140 rpm. The flasks were incubated at room temperature and 140 rpm. The culture was cultivated under 16:8 light:dark cycle (2,000 lux). The samples were taken every 12 h and determined for pH, cell dry weight, lipid content, lipid production, specific growth rates of yeast and microalga and lipid productivity (Analytical method 2 and 6).

2.2.2.2 C/N ratio

To determine the effect of C/N ratio, 1% pure glycerol was used as a carbon source and the suitable nitrogen source were used as a nitrogen source with various C/N ratios of 16, 24 and 32. The flasks were incubated at room temperature and 140 rpm. The culture was cultivated under 16:8 light:dark cycle (2,000 lux). The samples were taken every 12 h and determined for pH, cell dry weight, lipid content, lipid production, specific growth rates of yeast and microalga and lipid productivity (Analytical method 2 and 6).

2.2.3 Pure and crude glycerol concentration

The concentrations of pure and crude glycerol were increased from 1% to 2%, 3% and 4%. The flasks were incubated at room temperature and 140 rpm. The culture was cultivated under 16:8 light:dark cycle (2,000 lux). The samples were taken every 12 h and determined for pH, cell dry weight, lipid content, lipid production, specific growth rates of yeast and microalga and lipid productivity (Analytical method 2 and 6).

2.2.4 Fatty acid composition analysis

The dry biomass of the yeast, microalga and the mixed culture under optimal condition was ground into a fine powder; 0.1 g powder was blended with 1 mL chloroform: methanol (2: 1) and glass bead (ca. 0.5 g × 0.5 mm diameter). The mixture was sonicated for 30 min at 70 Hz at room temperature. Solvent phase was recovered by centrifugation. The extraction process was repeated two more times. The combined solvent was removed by drying at 60°C to constant weight. The compositions of extracted lipid were determined using GC (Analytical method 4).

3. Process optimization in a 5-L bioreactor

3.1 Effect of pH control

Ten percentage of 24-h-old precultured inoculum was transferred into a 5-L bioreactor containing 2 L sterilized crude glycerol based medium and incubated at room temperature for 120 h. For microalgae, the culture was cultivated under 16:8 light:dark cycle (4,000 lux). The cell growth and lipid production of oleaginous yeast

and microalga were performed. The pH was controlled at 6.0 using automatic control with 5.0 M NaOH. Aliquots of 10 mL were taken at 12-h time intervals to estimate DO, glycerol concentration, cell dry weight, lipid production, lipid content, specific growth rates of yeast and microalga and lipid productivity (Analytical method 2 and 6). The pH which gave the highest cell growth and lipid production was chosen for further step (section 3.2).

3.2 Effect of aeration rate

Ten percentage of 24-h-old precultured inoculum was transferred into a 5-L bioreactor containing 2 L sterilized crude glycerol based medium, incubated at room temperature and controlled pH at optimal by automatic control with 5.0 M NaOH for 120 h. The aeration rate on cell growth and lipid production of oleaginous yeast were varied at 1, 2, 3 and 4 vvm. Aliquots of 10 mL were taken at 12-h time intervals to estimate DO, glycerol concentration, cell dry weight, lipid production, lipid content, specific growth rate of yeast and lipid productivity (Analytical method 2 and 6). The aeration rate which gave the highest cell growth and lipid production was chosen for further step (section 3.3).

3.3 Fed-batch fermentation

Ten percentages of 24-h-old precultured inoculum was transferred into 2 L sterilized crude glycerol based medium in a 5-L bioreactor for 5 days with crude glycerol based medium. The different feeding strategies were attempted. Aliquots of 10 mL were taken at 12-h time intervals to estimate glycerol concentration, cell dry weight, lipid production, lipid content, specific growth rate of yeast and lipid productivity (Analytical method 2 and 6).

4. Fatty acid composition analysis

The dry biomass of oleaginous yeast was ground into a fine powder; 0.1 g powder was blended with 1 mL chloroform: methanol (2: 1) and glass bead (ca. 0.5 g × 0.5 mm diameter). The mixture was sonicated for 30 min at 70 Hz at room temperature. Solvent phase was recovered by centrifugation. The extraction process was repeated two more times. The combined solvent was removed by drying at 60°C

to constant weight. The composition of extracted lipid from oleaginous yeast was determined using GC (Analytical method 4).

CHAPTER 4

RESULTS AND DISCUSSION

1. Screening and optimization of medium components

1.1 Quantitative lipid production from crude glycerol by oleaginous yeasts

1.1.1 Screening of oleaginous yeasts

Eight hundred and eighty nine yeast strains were isolated from soil and waste of palm oil mills and a biodiesel plant in southern region of Thailand in YPD medium using glucose or glycerol as a carbon source at acid pH (4.0) or slightly acidic pH (6.0) with 0.0001% chloramphenicol as shown in Table 14. In Table 15, 226 and 176 yeast isolates were obtained using glycerol as a carbon source at initial pH 4 and 6, respectively. Two hundred and sixty seven and two hundred and twenty yeast isolates were obtained using glucose as a carbon source at initial pH 4 and 6, respectively. They were stained with Sudan black B technique and observed under a phase contrast microscope on oil immersion (1000×) for the presence of blue or greyish fat globules within the cell as shown in Figure 9. Eighty six and sixty three yeasts isolates showed fat globules within the cell (Figure 9b) but only 5 and 3 yeast isolates showed big fat globules indicating high production of oil (Figure 9c) in the medium containing glycerol as a carbon source at pH 4 and pH 6, respectively (Table15). When glucose was used as a carbon source, 107 and 81 yeasts isolates showed fat globules within the cell but only 13 and 2 yeast isolates showed big fat globules in the medium with pH 4 and pH 6, respectively. These 23 yeast isolates which showed big globules were selected as the potential lipid biomass producers (Table 16-17)**.**

Table 14 Sources and isolated yeast from soil and waste of palm oil mill and biodiesel plant in southern region of Thailand.

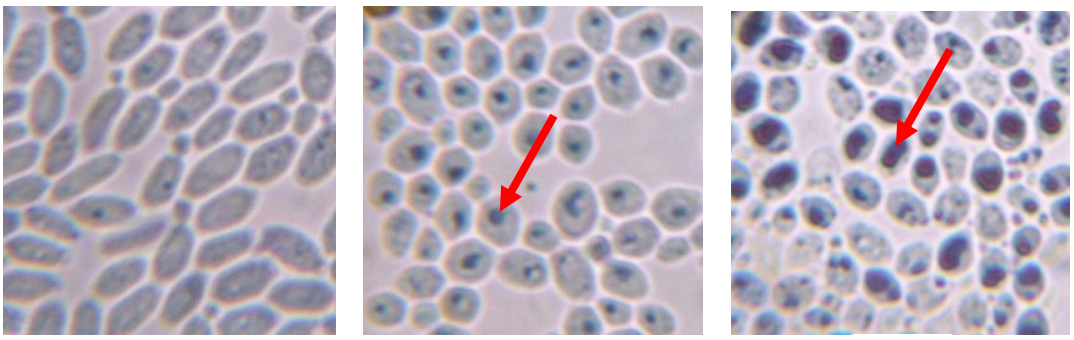
Sources	Picked up colony						After staining											
	Glycerol			Glucose			Glycerol						Glucose					
							+			++			+			++		
	Total	pH 4	pH 6	Total	pH 4	pH 6	Total	pH 4	pH 6	Total	pH 4	pH 6	Total	pH 4	pH 6	Total	pH 4	pH 6
Thai Tallow And Oil Co., Ltd.																		
1. Wastewater	2	1	1	4	2	2	2	1	1	-	-	-	-	-	-	-	-	-
2. Palm seed	5	2	3	5	2	3	4	2	2	-	-	-	-	-	-	-	-	-
3. Decanter cake	16	8	8	15	6	9	15	8	7	-	-	-	1	1	-	-	-	-
Unipalm Industry Co., Ltd.																		
1. Decanter cake	4	2	2	8	4	4	-	-	-	-	-	-	1	-	1	-	-	-
2. Palm seed	4	2	2	10	6	4	2	2	-	-	-	-	3	3	-	-	-	-
3. Palm fiber	4	2	2	4	2	2	2	2	-	-	-	-	1	-	1	-	-	-
4. Decanter cake	8	4	4	14	6	8	5	3	2	-	-	-	2	1	1	-	-	-
Sang Siri Palm Oil Co., Ltd.																		
1. Palm fiber	11	6	5	24	12	12	9	5	4	-	-	-	11	5	6	-	-	-
2. Palm seed	5	2	3	12	6	6	4	1	3	-	-	-	7	6	1	-	-	-
3. Decanter cake	8	4	4	17	11	6	7	4	3	-	-	-	15	9	6	-	-	-

- : Negative; +: Positive; ++: Highly Positive

Table 14. (Continous).

Sources	Picked up colony						After staining											
	Glycerol			Glucose			Glycerol						Glucose					
							+			++			+			++		
	Total	pH 4	pH 6	Total	pH 4	pH 6	Total	pH 4	pH 6	Total	pH 4	pH 6	Total	pH 4	pH 6	Total	pH 4	pH 6
Natural Palm Oil Co., Ltd.																		
1. Decanter cake	11	5	6	17	5	12	7	2	5	-	-	-	5	3	2	-	-	-
2. Palm fiber	13	5	8	19	6	13	11	5	6	-	-	-	5	3	2	-	-	-
3. Palm seed	6	3	3	5	1	4	2	-	2	-	-	-	3	-	3	-	-	-
J-K Industry Co., Ltd.																		
1. Decanter cake	8	-	8	20	11	9	1	-	1	1	-	1	11	7	4	6	4	2
2. Oil	2	2	-	3	3	-	2	2	-	-	-	-	2	2	-	1	1	-
3. Palm fiber	7	4	3	15	7	8	1	1	-	-	-	-	7	4	3	-	-	-
5. Soil	7	7	-	19	17	2	6	6	-	1	1	-	2	-	2	3	3	-
Biodiesel plant of Prince of Songkla University																		
1. Soil	257	149	111	241	121	70	95	40	27	10	3	3	50	69	52	5	5	-
2. Wastewater	-	-	-		-	-	-	-	-	-	-	-	-	-	-	-	-	-
3. Waste oil	24	21	3	35	29	6	10	8	2	2	2	-	15	14	1	-	-	-
Total	402	226	176	487	267	220	149	86	63	8	5	3	185	107	81	15	13	2

- : Negative; +: Positive; ++: Highly Positive



a b c

Figure 9. Phase contrast microscope photographs of the isolated yeasts stained with Sudan black B on oil immersion (1000×). They were cultivated on YPD agar using glycerol as the carbon source at 28°C for 72 h. (a) Negative; (b) Positive and (c) *R. glutinis* stained positive with Sudan Black B as a reference oleaginous yeast strain.

Table 15. Number of yeast strains were isolated by using glucose and glycerol as a carbon source

Initial pH	Carbon sources					
	Glycerol			Glucose		
	Total isolates	+	++	Total isolates	+	++
pH 4	226	86	5	267	107	13
pH 6	176	63	3	220	81	2
Total	402	149	8	487	185	15

+: showing fat globules; ++: showing big fat globules

Table 16. Codes of isolated yeast when used glycerol and glucose as the carbon sources.

Medium	Isolated yeasts
Glycerol as a carbon source	
Initial pH 4	BY4-197, BY4-199, BY4-523, BY4-571, JY4-42
Initial pH 6	BY35, BY37, JY7
Glucose as a carbon source	
Initial pH 4	BU4-230, BU4-598, BY4-600, BY4-606, BU4-622, JU4-48, JU4-52, JU4-53, JU4-57, JU4-59, JU4-74, JU4-75, JU4-76,
Initial pH 6	JU16, JU22

Table 17. Codes of isolated yeast from different sources

Sources	Isolated yeasts
Thai Tallow And Oil Co., Ltd.	-
Unipalm Industry Co., Ltd.	-
Sang Siri Palm Oil Co., Ltd.	-
Natural Palm Oil Co., Ltd.	-
J-K Industry Co., Ltd.	JU4-48, JU4-52, JU4-53, JU4-57, JU4-59, JU4-74, JU4-75, JU4-76, JY4-42, JU16, JU22
Biodiesel plant of Prince of Songkla University	BU4-230, BU4-598, BY4-600, BY4-606, BU4-622, BY4-197, BY4-199, BY4-523, BY4-571, BY35, BY37, JY7

The isolated 23 yeast strains, which showed highly positive fat globules in the cell, were precultured in inoculum medium, and then 24 h old preculture were inoculated in 125 mL flasks containing 25 mL crude glycerol based medium and incubated for 72 h at room temperature with shaking at 140 rpm. Among 23 isolates, there were only 12 isolates accumulating lipid within the cell more than 20% as shown in Table 18. Four of them (BY4-523, BY4-571, JU4-57 and BU4-598) gave high lipid production with lipid content higher than 40% when grown on crude glycerol based medium. BY4-523 and BY4-571 were isolated from soil of the

biodiesel plant using glycerol as a carbon source at pH 4.0 while BU4-598 was isolated using glucose at pH 4.0. JU4-57 was isolated from soil of a palm oil mill using glucose as a carbon source at pH 4.0. These four isolates were cultured using crude glycerol as a carbon source. It was found that BY4-523 gave highly lipid content up to 53.28% on a dry weight basis (10.28 g/L of biomass and 5.48 g/L of lipid production), followed by JU4-57 accumulated 51.13% of lipid content (14.48 g/L of biomass and 7.40 g/L of lipid production), BY4-571 gave 49.34% of lipid content (9.58 g/L of biomass and 4.73 g/L of lipid production), BU4-598 gave 41.19% of lipid content (16.33 g/L of biomass and 6.73 g/L of lipid production). Although BU4-606 accumulated the highest lipid content up to 63.62% but it showed low biomass and lipid production (6.73 g/L of biomass and 4.83 g/L of lipid production). Therefore, JU4-57, BY4-523, BY4-571 and BU4-598 strains were selected as lipid producers.

Figure 10 shows time courses of cell growth and lipid production of four strains. It was found that BY4-523 accumulated highest lipid content up to 53.28% (10.28 g/L of biomass and 5.48 g/L of lipid production) while JU4-57 grew fastest and gave comparable high lipid content 41.50% (17.05 g/L of biomass and 7.48 g/L of lipid production). The specific growth rate and lipid productivity were 0.061 h^{-1} and 129 mg/L.h, respectively. It should be noted that the isolated yeast strains were not inhibited by 10% crude glycerol. The previous works also reported that some oleaginous yeast has ability to grow and accumulate lipids on crude glycerol. For example, *R. glutinis* gave biomass of 4.53 g/L and lipid production of 1.04 g/L (Saenge *et al*., 2011) and *M. isabellina* gave biomass of 8.1 g/L and lipid production of 4.4 g/L (Papanikolaou *et al*., 2008). It was also reported that the crude glycerol from biodiesel manufacturing contains some element such as calcium, potassium, magnesium, sulfur and sodium (Meesters *et al*., 1996). Thus, using the crude glycerol to produce yeast oils as biodiesel feedstock would provide an added bonus of offsetting costs of production. The isolate yeasts JU4-57 and BY4-523 which gave highest lipid production and highest lipid content, respectively, were selected for the next study.

Table 18**.** The lipid content of oleaginous yeast strains.

Isolate number	Biomass (g/L)	Lipid production (g/L)	Lipid content (%)
R. glutinis	10.13 ± 0.04^{h}	4.05 ± 0.14^{f}	40.00 ± 1.26^{d}
BY35	12.08 ± 0.12^{g}	4.01 ± 0.21^{f}	33.13 ± 1.37^{e}
BY37	12.40 ± 0.12^{f}	3.17 ± 0.04^{h}	25.58 ± 0.59^{gh}
BY4-197	8.22 ± 0.25^{l}	1.30 ± 0.21^{lm}	15.85 ± 3.06^{ij}
BY4-199	15.34 ± 0.15^{c}	2.57 ± 0.08^{i}	16.76 ± 0.72^{ij}
BY4-523	10.28 ± 0.11^{h}	5.48 ± 0.18^{c}	53.28 ± 1.17^{b}
BY4-571	9.58 ± 0.04^{j}	4.73 ± 0.18^{d}	49.34 ± 1.66^{c}
BU4-230	9.45 ± 0.21^{jk}	1.72 ± 0.11^{k}	18.25 ± 0.71^{i}
BU4-598	16.33 ± 0.04^{a}	6.73 ± 0.18^{b}	41.19 ± 0.99^{d}
BU4-600	9.23 ± 0.18^{k}	1.05 ± 0.14^{mn}	11.37 ± 1.32^{l}
BU4-606	6.73 ± 0.01^{n}	4.28 ± 0.07^{e}	63.60 ± 1.18^{a}
BU4-622	15.98 ± 0.11^{b}	4.38 ± 0.25^{e}	27.39 ± 1.73^{fgh}
JY7	8.08 ± 0.04^{l}	1.12 ± 0.04^{mn}	13.93 ± 0.38^{jkl}
JY4-42	7.30 ± 0.17^{m}	2.13 ± 0.07^{j}	29.20 ± 1.65^{f}
JU16	4.07 ± 0.09^{p}	1.14 ± 0.06^{mn}	28.04 ± 0.76^{fg}
JU22	5.98 ± 0.25^{o}	0.67 ± 0.04^{o}	11.29 ± 0.12^{l}
JU4-48	9.96 ± 0.08^{i}	1.48 ± 0.02^{kl}	14.81 ± 0.34^{jk}
JU4-52	5.88 ± 0.04^{o}	1.55 ± 0.14^{kl}	26.39 ± 2.57^{fgh}
JU4-53	7.05 ± 0.14^{m}	0.98 ± 0.04^{n}	13.83 ± 0.22^{jkl}
JU4-57	14.48 ± 0.11^{d}	7.40 ± 0.14^{a}	51.13 ± 1.35^{bc}
JU4-59	6.15 ± 0.07^{o}	0.98 ± 0.11^{n}	15.86 ± 1.91^{ij}
JU4-74	14.61 ± 0.18^{d}	3.58 ± 0.16^{g}	24.47 ± 1.41^{h}
JU4-75	13.74 ± 0.14^{e}	2.19 ± 0.16^{j}	15.90 ± 1.02^{ij}
JU4-76	13.79 ± 0.13^{e}	1.75 ± 0.03^{k}	12.69 ± 0.32^{kl}

Crude glycerol was used as the carbon (10%) and the initial amount of nitrogen source was yeast extract 1% and peptone 1%. They were incubated at room temperature under shaking speed of 140 rpm for 72 h. Different small letters in the table indicate significant difference between strains (P<0.05).

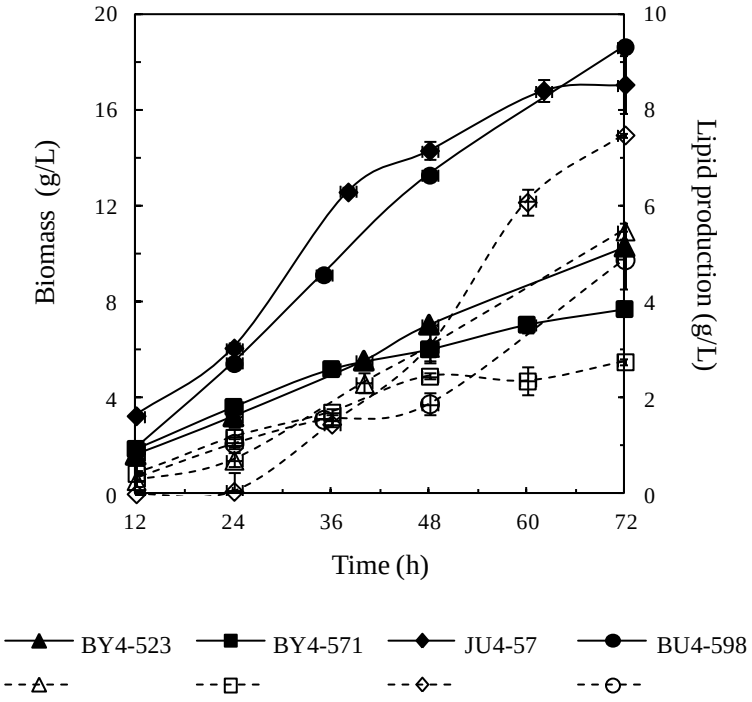
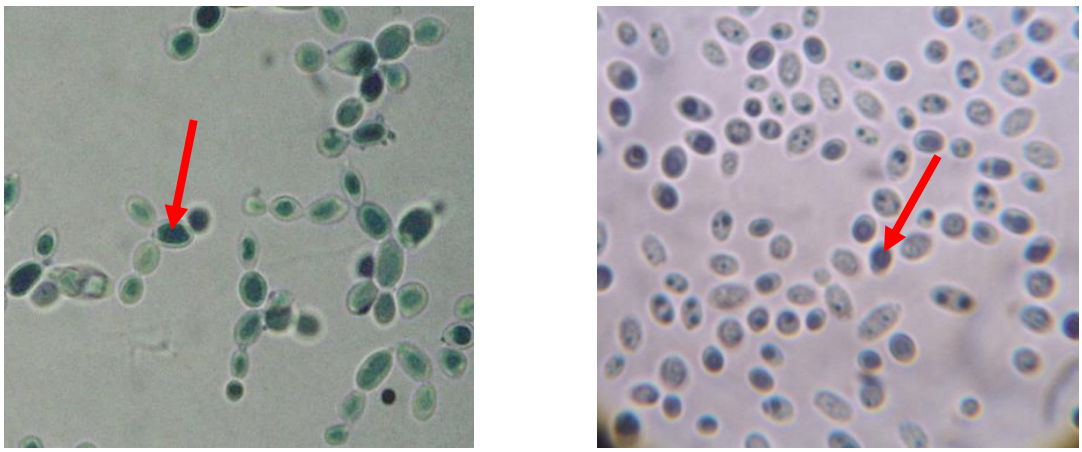


Figure 10. Time course of cell growth (close symbol) and lipid accumulation (open symbol) of oleaginous yeasts. Ten percentage of crude glycerol (0.13 M of carbon) was used as a carbon source and 1% yeast extract and 1% peptone were used as nitrogen sources with the C/N molar ratio of 9.8.

1.1.2 Identification of oleaginous yeast strain

Strains JU4-57 and BY4-523 were grown on YPD agar with staining and observed under the phase contrast microscope on oil immersion (1000×) as shown in Figure 11. These oleaginous yeast strains were identified based on its 26S rDNA sequence. The obtained sequences were BLAST searched against those available in the GenBank of National Center for Biotechnology Information (NCBI) database (http://www.ncbi.nlm.nim.gov). The results showed high sequence similarity up to 99% (497/502 bp) with the type strain of *Trichosporonoides spathulata* (GenBank accession number AF335526.1) for JU4-57 and 99% (457/460 bp) with the type strain of *Kodamaea ohmeri* (GenBank accession number FM180533.1) for BY4-523 as shown in the Figure 12 and 13. Therefore, JU4-57 and BY4-523 isolates were called as *T. spathulata* JU4-57 and *K. ohmeri* BY4-523, respectively.



(a) (b)

Figure 11. Phase contrast microscope photographs of the isolated oleaginous yeasts *T. spathulata* JU4-57 (a) and *K. ohmeri* BY4-523 (b) stained with Sudan black B on oil immersion (1000×). Yeast cells were cultured on YPD agar medium using glycerol as a carbon source at 28°C for 72 h.

Score = 898 bits (486), Expect = 0.0

Identities = 497/502 (99%), Gaps = 1/502 (0%)

Strand=Plus/Minus

```
Query  1    CAGATTTGAAAGCTCCCGCCAGGGCGCATTGTAAGCTGGAGACGTGCCTCGAGCGGCGCG  60
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Sbjct  512  CAGATTTGAAAGCTCCCGCCAGGGCGCATTGTAAGCTGGAGACGTGCCTCGAGCGGCGCG  453

Query  61   GCTGGACGCAAGTCTGCTGGAAAGCAGCATCAAAGAGGGTGAGAATCCCGTGCTTGGTCT  120
            |||||||||||||||||||||||||||||||| ||||||||||||||||||||||||||||
Sbjct  452  GCTGGACGCAAGTCTGCTGGAAAGCAGCATCAGAGAGGGTGAGAATCCCGTGCTTGGTCT  393

Query  121  GGCTGTGCGCCGTGTTGTGGGGTGCGCTCGACGAGTCGCGTTGTTTGGGAATGCAGCGCA  180
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Sbjct  392  GGCTGTGCGCCGTGTTGTGGGGTGCGCTCGACGAGTCGCGTTGTTTGGGAATGCAGCGCA  333

Query  181  AAGAGGGGTGGTAAACGCCATCCAAGGCTAAATACCGGGGAGAGACCGATAGCGAACAAG  240
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Sbjct  332  AAGAGGGGTGGTAAACGCCATCCAAGGCTAAATACCGGGGAGAGACCGATAGCGAACAAG  273

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Sbjct  272  TACCGTGAGGGAAAGATGAAAAGCACTTTGGAAAGAGAGTTAAAGAGTACGTGAAATTGC  213

Query  301  CAAGAGGGAAGCGCTGGCAGTCAGTGCCGTAGCGCTGCTGGTCCCGCCtttttttGGGAG  360
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Sbjct  212  CAAGAGGGAAGCGCTGGCAGTCAGTGCCGTAGCGCTGCTGGTCCCGCCTTTTTTTGGGAG  153

Query  361  GTTGATGCCGGCAGTGTGGGGCCCGCGTCGGTTGCTGTTTTGGTGGGGGAGAAGGCAGAG  420
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Sbjct  152  GCTGATGCCGGCAGTGTGGGGCCCGCGTCGGTTGCTGTTTTGGTGGGGGAGAAGGCAGAG  93

Query  421  CGCCAAGGTGGCTTCCCtttttttGGGGGGAGTGTTATAGCCGCTTTGTGGATGCCCTGC  480
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Sbjct  92   CGG-AAGGTGGCTTCCCTTTTTTTGGGGGGAGTGTTATAGCCGCTTTGTGGATGCCCTGC  34

Query  481  TAGCGACCGATGACCGCTTTTT  502
            |||||||||| ||||||||||
Sbjct  33   TAGCGACCGAGGACCGCTTTTT  12
```

Figure 12. Comparison of 26S rDNA nucleotide sequences of strain JU4-57 with *Trichosporonoides spathulata* JU4-57.

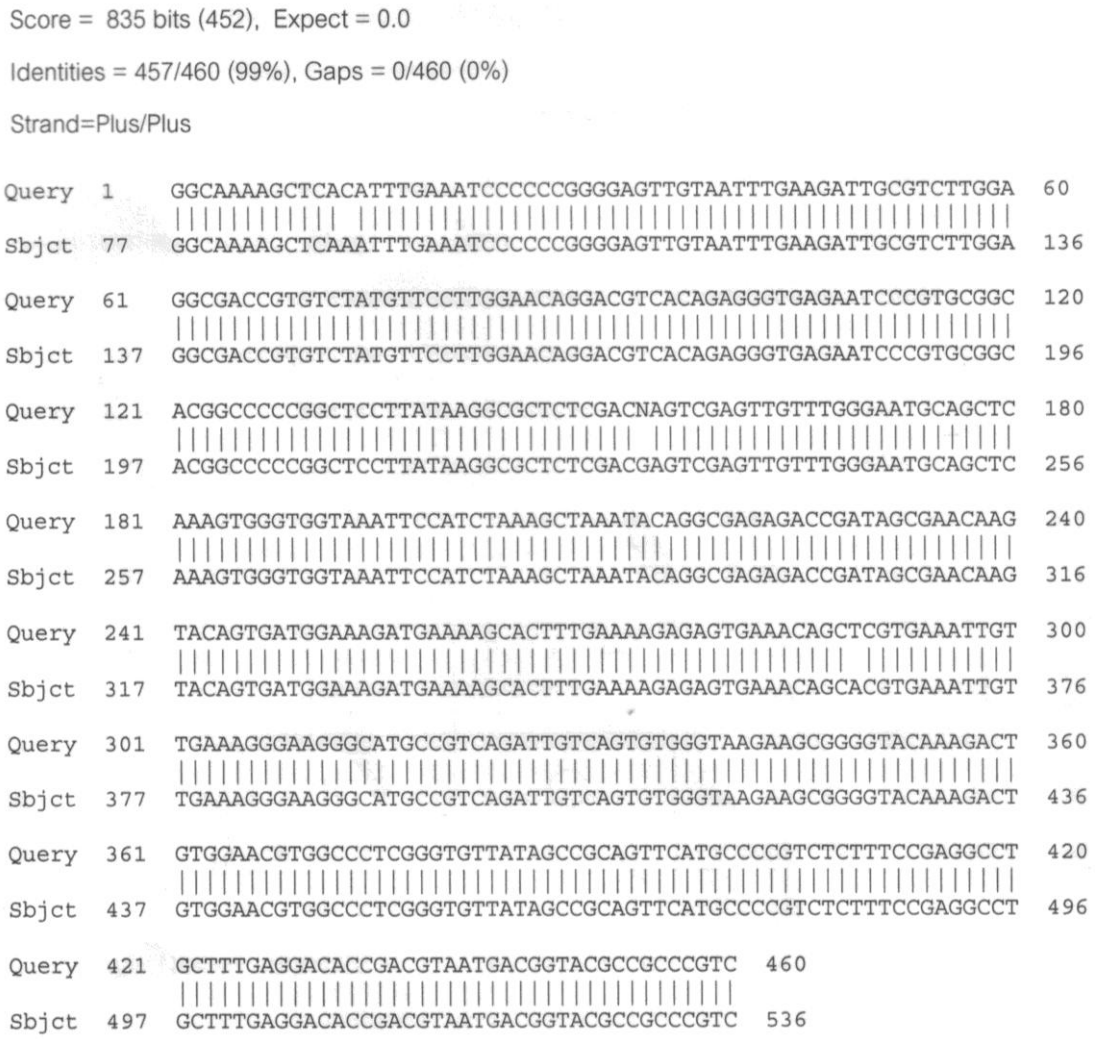

Figure 13. Comparison of 26S rDNA nucleotide sequences of strain BY4-523 with *Kodamaea ohmeri*.

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1.2 Optimization of medium components and culture condition

1.2.1 Effect of nitrogen sources on growth and lipid accumulation

Many factors including medium components, such as carbon source, nitrogen source and C/N molar ratio etc. as well as culture conditions (temperature and pH) have significant influences on cell growth and lipid accumulation of oleaginous microorganism (Papanikolaou *et al*., 2007). Effects of nitrogen source on biomass and lipid production of *T. spathulata* JU4-57 and *K. ohmeri* BY4-523 strains were presented in Figure 14. Among the nitrogen sources tested for *T. spathulata* JU4-57 strain (Figure 14a), a mixture of yeast extract and peptone (1:1) gave the maximum biomass (17.05 g/L) and lipid production (10.43 g/L), followed by yeast extract (16.02 g/L biomass and 7.12 g/L lipid production), peptone (10.88 g/L biomass and 5.43 g/L lipid production) and ammonium sulfate (9.17 g/L biomass and 3.85 g/L lipid production). As shown in Table 19, the mixture of yeast extract and peptone (1:1) gave the highest specific growth rate for *T. spathulata* JU4-57 up to 0.069 h^{-1} and lipid productivity up to 144.8 mg/L.h. Among inorganic nitrogen sources, ammonium sulfate gave the highest specific growth rate and lipid productivity (0.05 h^{-1} and 53.5 mg/L.h, respectively)**.**

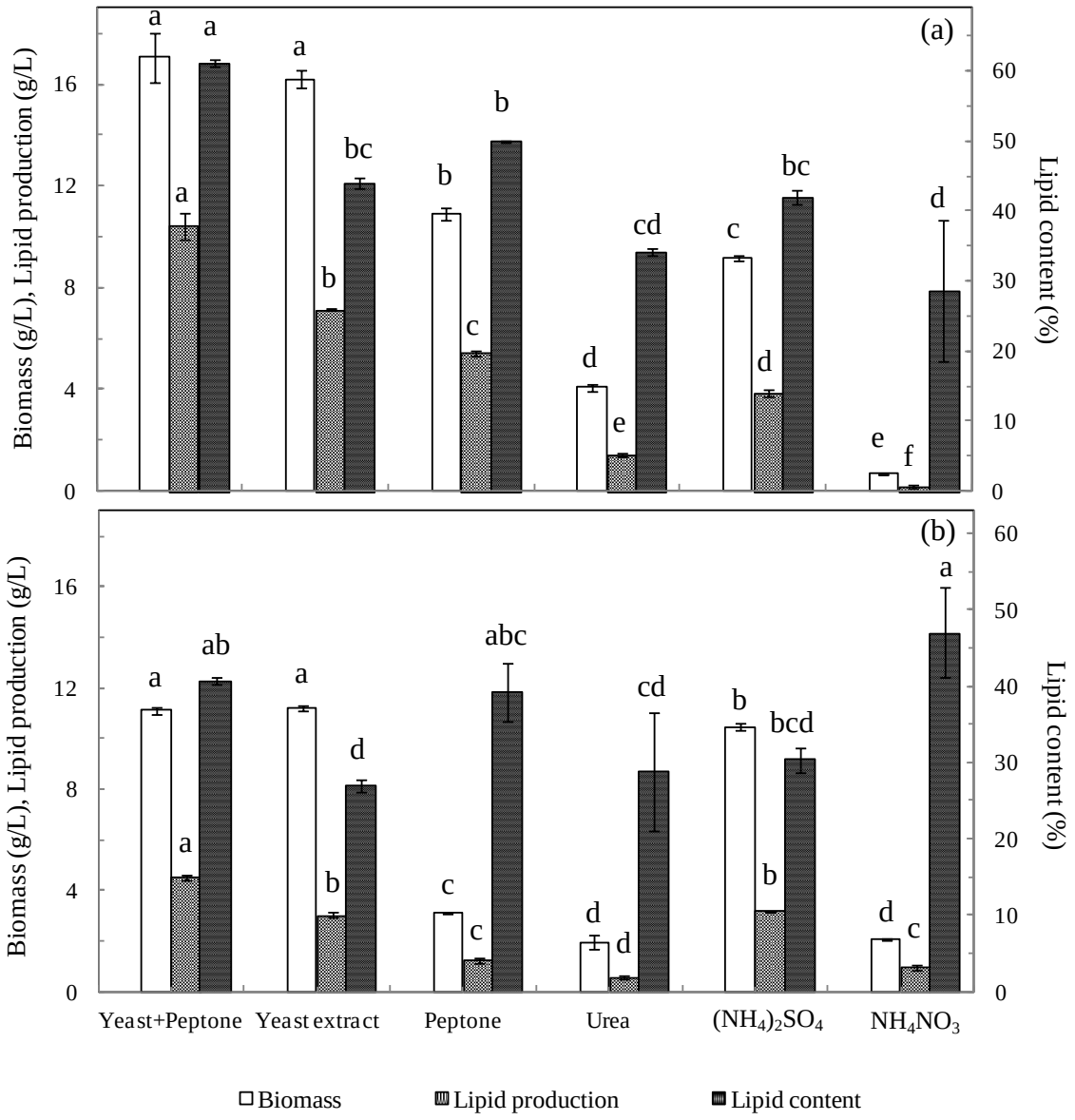

Figure 14. Effect of nitrogen sources on cell growth, lipid production and lipid content of *T. spathulata* JU4-57 (a) and *K. ohmeri* BY4-523 (b). Ten percentage of crude glycerol (0.13 M of carbon) was used as a sole carbon source and the nitrogen source was added to obtain the C/N molar ratio of 8.6. Different small letters on the bar indicate significant difference between treatments ($P<0.05$).

Among the nitrogen sources tested for *K. ohmeri* BY4-523 strain (Figure14b), there was no significant difference in biomass between the mixture of yeast extract and peptone (1:1) (11.1 g/L) and yeast extract alone (11.2 g/L), followed by $(NH_4)_2SO_4$ (10.45 g/L). For the lipid production, the mixture of yeast extract and peptone gave the maximum lipid production (4.53 g/L), followed by yeast extract and $(NH_4)_2SO_4$ (3.17 g/L and 3.03 g/L, respectively). The mixture of yeast extract and peptone (1:1) also gave the highest specific growth rates for *K. ohmeri* BY4-523 up to 0.0527 h^{-1} and lipid productivity up to 62.8 mg/L.h. Among inorganic nitrogen sources, ammonium sulfate gave the highest specific growth rate and lipid productivity (0.051 h^{-1} and 44.1 mg/L.h, respectively) as shown in Table 19**.** Disappointingly, urea and NH_4NO_3 gave both poor biomass and lipid production by *T.spathulata* JU4-57 and *K. ohmeri* BY4-523 strains, indicating that organic nitrogen sources are more beneficial to lipid production of oleaginous yeast than inorganic nitrogen sources. On the contrast, Zhu *et al*. (2008) reported that urea supported the maximum biomass of *Trichosporon fermentans* (23.1 g/L), followed by peptone (19.7 g/L) when using glucose as a carbon source. While peptone gave the best lipid content (54.9%) and the maximum lipid production (10.8 g/L).

Yeast extract was the best organic nitrogen source for biomass and lipid production, followed by peptone but they were expensive organic nitrogen sources. If yeast extract and peptone were used as a nitrogen source, the cost of yeast extract (3.95 Baht/g) and peptone (3.9 Baht/g) were more expensive than $(NH_4)_2SO_4$ (0.02 Baht/g). Although $(NH_4)_2SO_4$ was selected as a suitable nitrogen source because of its cheaper cost and inorganic nitrogen is more economical choice and can efficiently reduce the cost of lipid production.

1.2.2 Effect of ammonium sulfate concentration on growth and lipid accumulation

As shown in Figure 15a, the biomass of *T. spathulata* JU4-57 increased when ammonium sulfate was added. It reached the maximum value of 10.15 g/L of biomass and the specific growth rates of 0.514 h^{-1} at 0.5% ammonium sulfate as shown in Table 19. At this concentration, the maximum lipid production was also obtained (3.85 g/L) and gave the highest lipid productivity up to 53.8 mg/L.h (Table

19). Further rise in ammonium sulfate concentration beyond 0.5% resulted in a drop of biomass, lipid production and lipid content. In Figure 15b, biomass of *K. ohmeri* BY4-523 increased gradually with the increase of ammonium sulfate concentration and reached the maximum biomass of 10.45 g/L and the specific growth rate of 0.0594 h^{-1} at 0.5% ammonium sulfate. Lipid production showed a sharp increase when ammonium sulfate concentration increased from 0.25% to 0.5%, and reached the maximum lipid production of 3.17 g/L and lipid productivity of 44.8 mg/L.h at 0.5%. It can be seen that at lower concentration of nitrogen source, the lipid content was higher. This result indicated that the exhaustion of ammonium-N apparently greatly promoted lipid accumulation. The similar results were also observed in lipid content of *Rhodosporidium toruloides* Y4, *Rhodotorula glutinis* and *Trichosporon fermentans* (Li *et al*., 2007).

1.2.3 Effect of glycerol concentration on growth and lipid accumulation

The biomass of *T. spathulata* JU4-57 increased gradually with an increase of glycerol concentration and reached the maximum value of 10.23 g/L at 4% glycerol concentration (10% crude glycerol) (Figure 16a) in 72 h. Lipid production also increased when glycerol concentration increased to 10%, and reached the maximum lipid of 3.87 g/L for 72 h. As shown in Table 19, the specific growth rate and lipid productivity of *T. spathulata* JU4-57 were 0.0570 h^{-1} and 53.8 mg/L.h, respectively. The biomass and lipid production of *K. ohmeri* BY4-523 also increased gradually with an increase of glycerol concentration and reached the maximum values at 4% glycerol (Figure 16b). *K. ohmeri* BY4-523 also reached the highest biomass of 10.5 g/L and the maximum lipid production of 3.22 g/L at 72 h. The specific growth rate and lipid productivity of *K. ohmeri* BY4-523 were 0.0594 h^{-1} and 44.8 mg/L.h, respectively (Table 19).

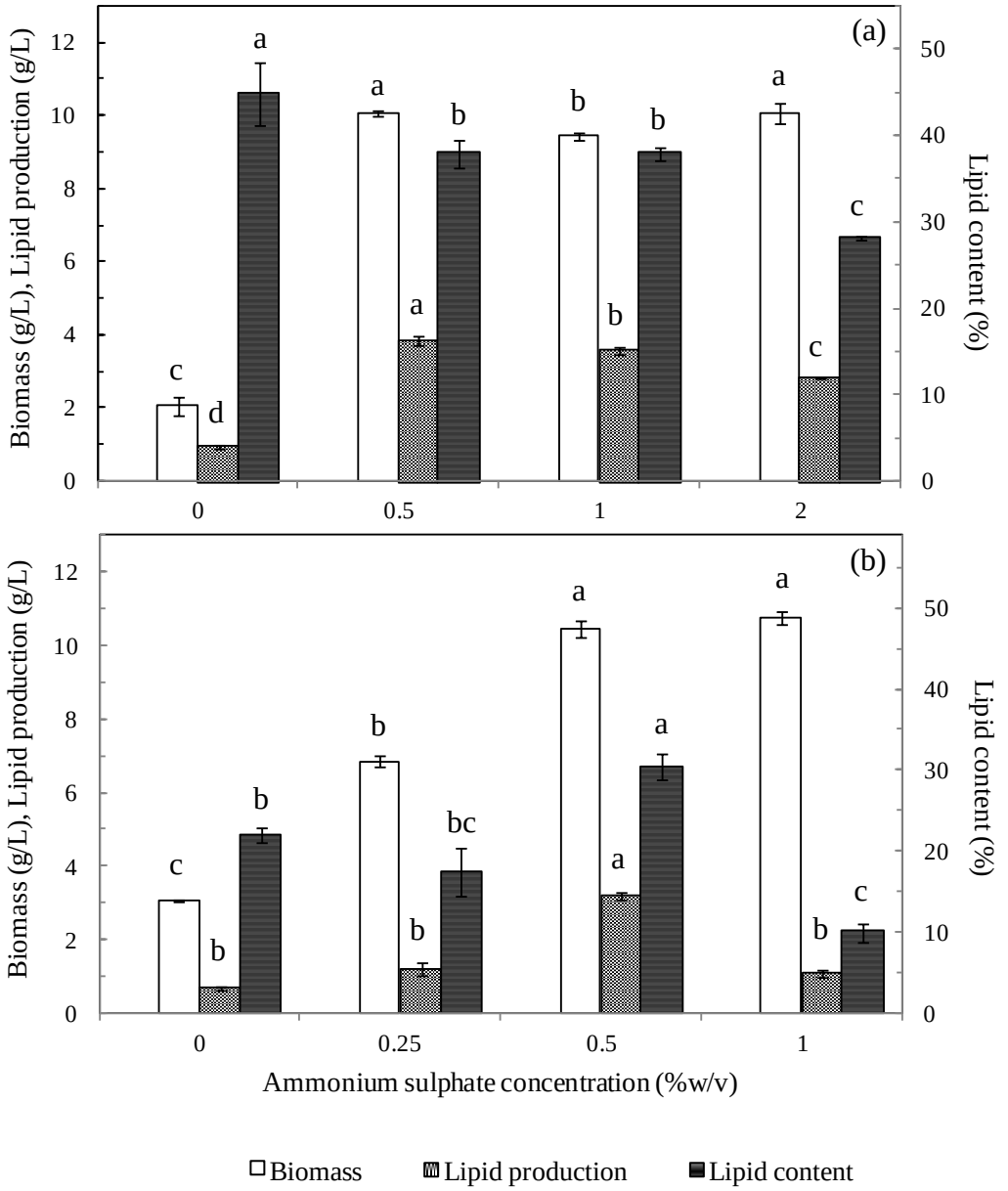

Figure 15. Effect of ammonium concentration on cell growth and lipid accumulation of *T. spathulata* JU4-57 (a) and *K. ohmeri* BY4-523 (b). Ten percentage of crude glycerol (0.13 M of carbon) was used as a sole carbon source. Different small letters on the bar indicate significant difference between treatments ($P<0.05$).

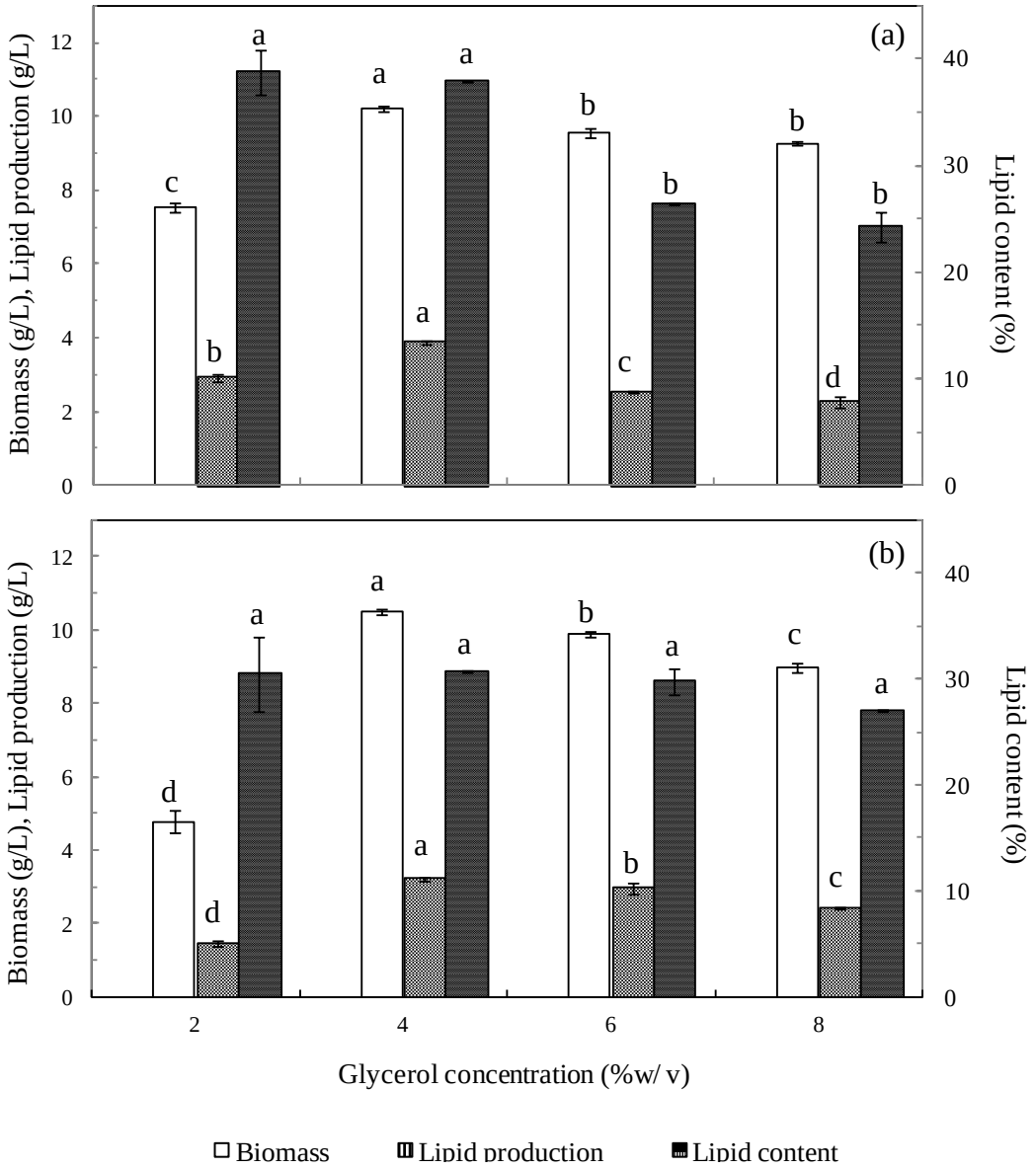

Figure 16. Effect of glycerol concentration on cell growth and lipid accumulation of *T. spathulata* JU4-57 (a) and *K. ohmeri* BY4-523 (b). The ammonium sulfate concentration was fixed at 0.5% (w/v). Different small letters on the bar indicate significant difference between treatments ($P<0.05$).

Table 19. Growth and lipid productivity of *T. spathulata* JU4-57 and *K. ohmeri* BY4-523.

Parameter	Specific growth rate (h^{-1})		Lipid productivity (mg/L.h)	
	T. spathulata	*K. ohmeri*	*T. spathulata*	*K. ohmeri*
Nitrogen source[a]				
Yeast extract and peptone (1:1)	0.0690	0.0527	144.8	62.8
Yeast extract	0.0579	0.0528	98.9	42.0
Peptone	0.0524	0.0351	75.4	17.0
Urea	0.0389	0.0285	19.4	7.6
$(NH_4)_2SO_4$	0.0500	0.0518	53.5	44.1
NH_4NO_3	0.0143	0.0294	2.8	13.5
Nitrogen concentration (% w/v)[b]				
0.0	0.0298	0.0417	12.8	9.4
0.5	0.0514	0.0594	53.8	44.8
1.0	0.0520	0.0518	49.7	44.1
2.0	0.0466	0.0542[*]	39.2	16.3[*]
Crude glycerol concentration (% w/v)[c]				
2	0.0574	0.0396	40.6	20.1
4	0.0570	0.0594	53.8	44.8
6	0.0588	0.0498	35.1	41.0
8	0.0561	0.0484	31.2	33.7

All cultures were performed at the initial pH 6.0 and 140 rpm for 72 h.

[a] The carbon source was crude glycerol (4% w/v) and nitrogen source was fixed at the C/N molar ratio of 8.6.

[b] Ammonium sulfate was selected as the nitrogen source.

[c] Ammonium sulfate was fixed at 0.5% (w/v).

[*] The concentration of ammonium sulfate was 0.25% (w/v).

The time courses of biomass and lipid accumulation of *T. spathulata* JU4-57 and *K. ohmeri* BY4-523 using 10% crude glycerol and 0.5% ammonium sulfate as a carbon source and a nitrogen source, respectively, are shown in Figure 15. *T. spathulata* JU4-57 gave the highest biomass of 10.23 g/L and the maximum lipid production of 3.87 g/L at 72 h (Figure 17a). *K. ohmeri* BY4-523 also gave the highest biomass of 10.5 g/L and the maximum lipid production of 3.22 g/L at 72 h (Figure 17b). It should be noted that the lipid content of *T. spathulata* JU4-57 reached the maximum value at 24 h and gradually decreased. The similar changes were also observed in lipid content of *Yarrowia lipolytica, Cunninghamella echinulata* and *Mortierella isabellina* after exhaustion of the carbon source in the growth environment. The use of lipid for cell proliferation owing to the lack of glycerol could account for this phenomenon. It has been reported that the breakdown of lipid could be repressed in multiple-limited media (Fakas *et al*., 2007; Papanikolaou *et al*., 2004).

1.2.4 Glycerides compositions analysis

A thin-layer chromatography equipped with flame ionization detector or TLC-FID was also used for measuring stored triglycerides from the extracted lipid of oleaginous yeasts. It was found that the composition of *T. spathulata* JU4-57 lipid was 68.7% monoglycerides (MAG), 29.3% triacylglycerides (TAG), 1.2% free fatty acid (FFA) and 0.45% diglycerides (DAG) (Appendix 7). These compositions can be used as feedstock for biodiesel production. *K. ohmeri* BY4-523 lipid was also mainly MAG up to 94.5% and only small amount of TAG and DAG at 3.93% and 1.57%, respectively (Appendix 8). Since *T. spathulata* JU4-57 gave higher lipid production than *K. ohmeri* BY4-523, it was then used for further studies.

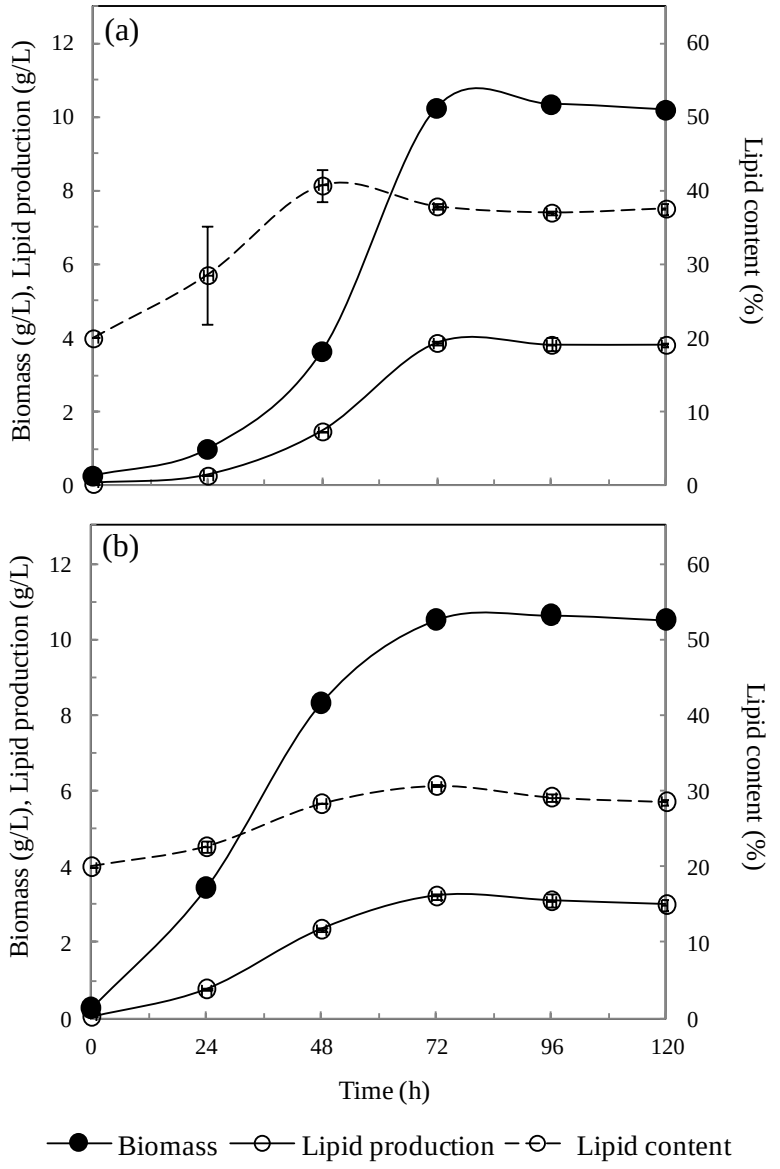

Figure 17. Time courses of biomass (close symbol), lipid production (open symbol) and lipid content (open symbol and dash line) of *T. spathulata* JU4-57 (a) and *K. ohmeri* BY4-523 (b). Ten percentage of crude glycerol was used as a sole carbon and 0.5% (w/v) ammonium sulfate was used as a nitrogen source.

2. Enhancing lipid production by mixed culture of oleaginous yeast and microalgae

2.1 Mixed cultures of oleaginous yeast *T. spathulata* JU4-57 and microalgae

2.1.1 Selection of microalgae for enhancing biomass and lipid production

Selection of microalgae for enhancing biomass and lipid production was shown in Figure 18. The result of pure and mixed cultures of *T. spathulata* JU4-57 with three microalgae including *Chlorella vulgaris* var. *vulgaris* TISTR 8261, a fresh water microalga *Chlorella* sp. and a marine microalga *Chlorella* sp. were attempted in crude glycerol based medium. It was found that the mixed culture of *T. spathulata* JU4-57 with *C. vulgaris* var. *vulgaris* TISTR 8261 gave the best biomass of 11.13 g/L, the maximum lipid production of 4.55 g/L and lipid content of 40.81%, followed by *T. spathulata* JU4-57 with fresh water microalga *Chlorella* sp.**,** *T. spathulata* JU4-57 with a marine microalga *Chlorella* sp. and pure culture of *T. spathulata* JU4-57. As shown in Table 20, the mixed culture of *T. spathulata* JU4-57 with *C. vulgaris* var. *vulgaris* TISTR 8261 enhanced the specific growth rates for yeast and microalga and lipid productivity up to 0.0681 h^{-1}, 0.0014 h^{-1} and 63.2 mg/L.h, respectively, followed by mixed culture with fresh water microalga *Chlorella* sp. (0.0609 h^{-1}, 0.0012 h^{-1} and 57.6 mg/L.h, respectively) and mixed culture with a marine microalga *Chlorella* sp. (0.0583 h^{-1}, 0.0012 h^{-1} and 56.6 mg/L.h, respectively). Xue *et al*. (2008) also studied pure and mixed culture of *Spirulina platensis* and *Rhodotorula glutinis*. It was found that the highest total lipid production was 467 mg/L obtained from the mixed culture, which was 3.18 times of *R. glutinis* pure culture and 3.92 times of *S. platensis* pure culture.

2.1.2 Inoculum ratio

Because the mixed culture of yeast and microalga gave greater biomass and lipid production. As the microalga grew slower than the yeast, an increase in the initial amount of microalga was attempted. The effect of the amount of microalga was investigated by increasing the amount of microalga 4.0×10^4 cells/mL to 1.0×10^7 cells/mL and the amount of yeast was fixed at 3.4×10^6 cells/mL, the results are shown in Figure 19. There was no significant increase in biomass and lipid production

when the amount of microalga was increased from 3.9×10^5 cells/mL to 1.0×10^7 cells/mL. However, lipid productivity increased slightly from 55 mg/L.h to 66 mg/L.h with increasing the amount of microalga from 4.0×10^4 cells/mL to 1.6×10^6 cells/mL and slightly dropped when increasing the amount of microalga up to 1.0×10^7 cells/mL as shown in Table 20. This would be because light intensity was shaded from high cell density of microalga and yeast. The specific growth rate of yeast increased slightly from 0.0615 h^{-1} to 0.0715 h^{-1} with increasing the amount of microalga from 4.0×10^4 cells/mL to 1.0×10^7 cells/mL. While the specific growth rate of microalga decreased when its initial amount was higher or lower than 3.9×10^5 cells/mL. A low inoculum size would result in a long lag phase. Cheirsilp *et al.* (2011) also studied on an increase in the initial amount of microalga. The effect of the amount of microalga was investigated by increasing the amount of microalga to two and three folds. There was no significant increase in biomass and lipid production when amount of microalga was increased.

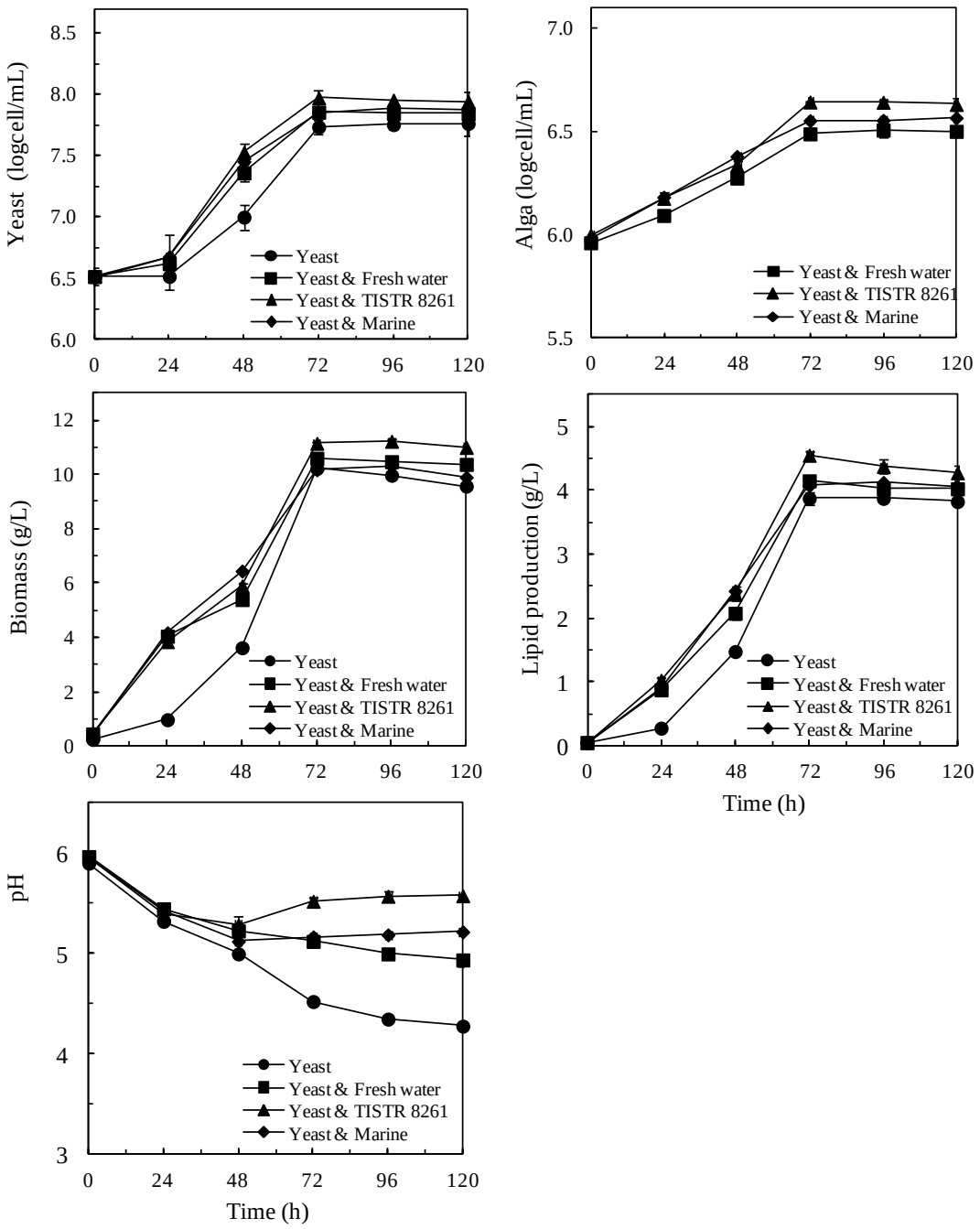

Figure 18. Comparison of cell number of yeast, cell number of microalgae, biomass and lipid production in the mixed culture of *T. spathulata* JU4-57 with a marine microalga *Chlorella* sp., *T. spathulata* JU4-57 with *Chlorella vulgaris* var. *vulgaris* TISTR 826, *T. spathulata* JU4-57 with Fresh water microalga *Chlorella* sp. and the pure culture of *T. spathulata* JU4-57.

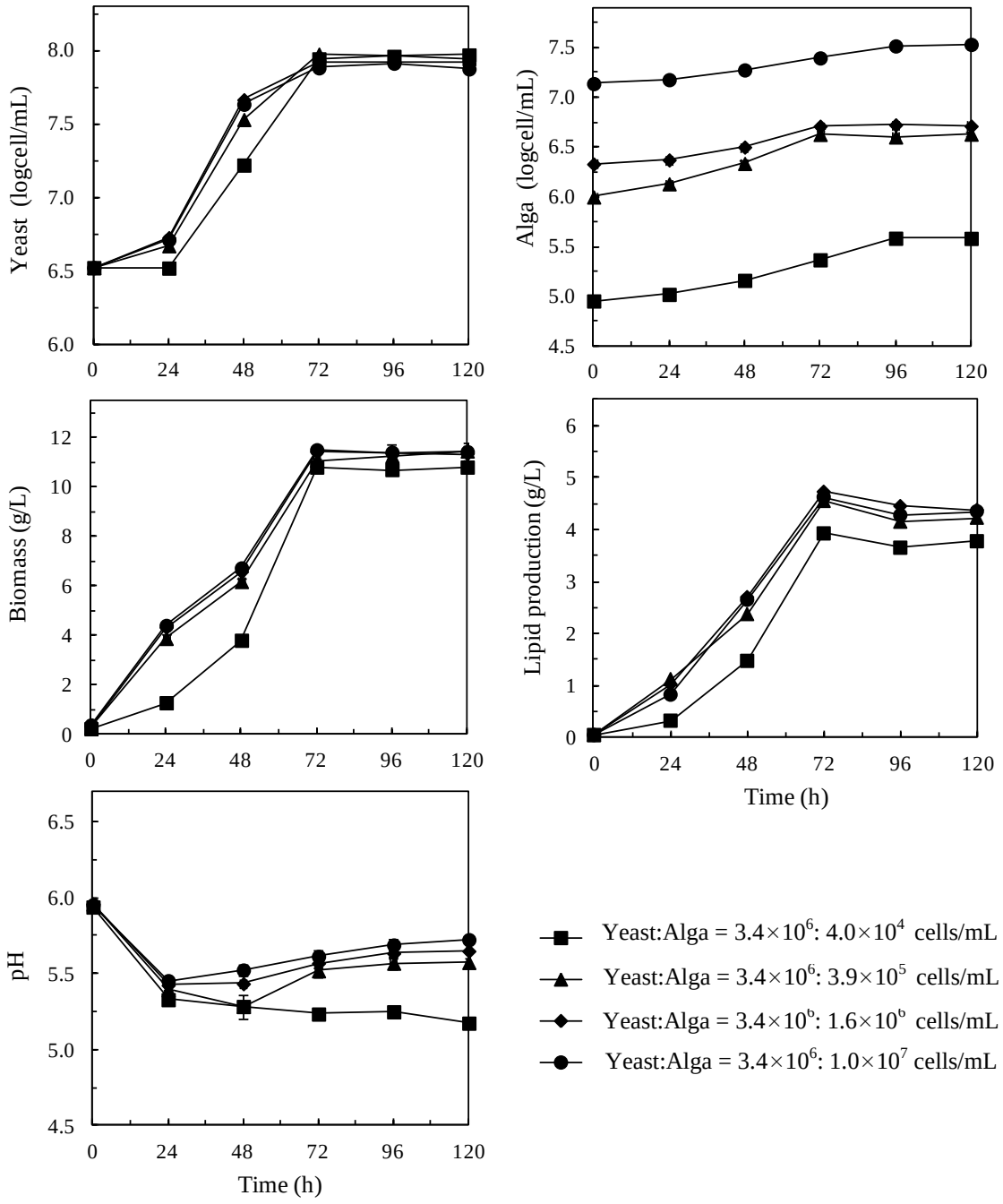

Figure 19. Effect of yeast and microalga inoculum size ratio on cell number of yeast, cell number of microalgae, biomass, and lipid production in the mixed culture.

2.1.3 Light intensity

Light is the source of energy which drives this reaction and in this regard intensity, spectral quality and photoperiod need to be considered. Photosynthesis in microalga is superimposed on the reverse process, respiration, i.e. slow combustion of organic matter to form water and CO_2 with release of chemical energy in the form of heat and "energy-rich" phosphate bands. An excess of photosynthesis over respiration is what permits the growth of photosynthetic organisms and storage of food reserves such as starch, oil and fat. Light intensity plays an important role, but the requirements vary greatly with the culture depth and the density of the microalgal culture: at higher depths and cell concentrations the light intensity must be increased to penetrate through the culture. Light may be natural or supplied by fluorescent tubes. Too high light intensity (e.g. direct sun light, small container close to artificial light) may result in photo inhibition. Also, overheating due to both natural and artificial illumination should be avoided. The effect of light intensity of mixed culture on cell growth and lipid accumulation were shown in Figure 20. It was found that the mixed culture could grow faster with increasing light intensity from 2,000 lux to 8,000 lux. It used high light intensity because light intensity was shaded by crude glycerol based medium. The specific growth rate of yeast increased from 0.0681 h^{-1} to 0.0835 h^{-1} with increasing light intensity from 2,000 lux to 8,000 lux as shown in Table 20. The specific growth rate of microalga also increased from 0.0014 to 0.0019 h^{-1} with increasing light intensity from 2,000 lux to 4,000 lux and slightly dropped when light intensity was increased up to 8,000 lux (Table 20) because the effect of photoinhibition.

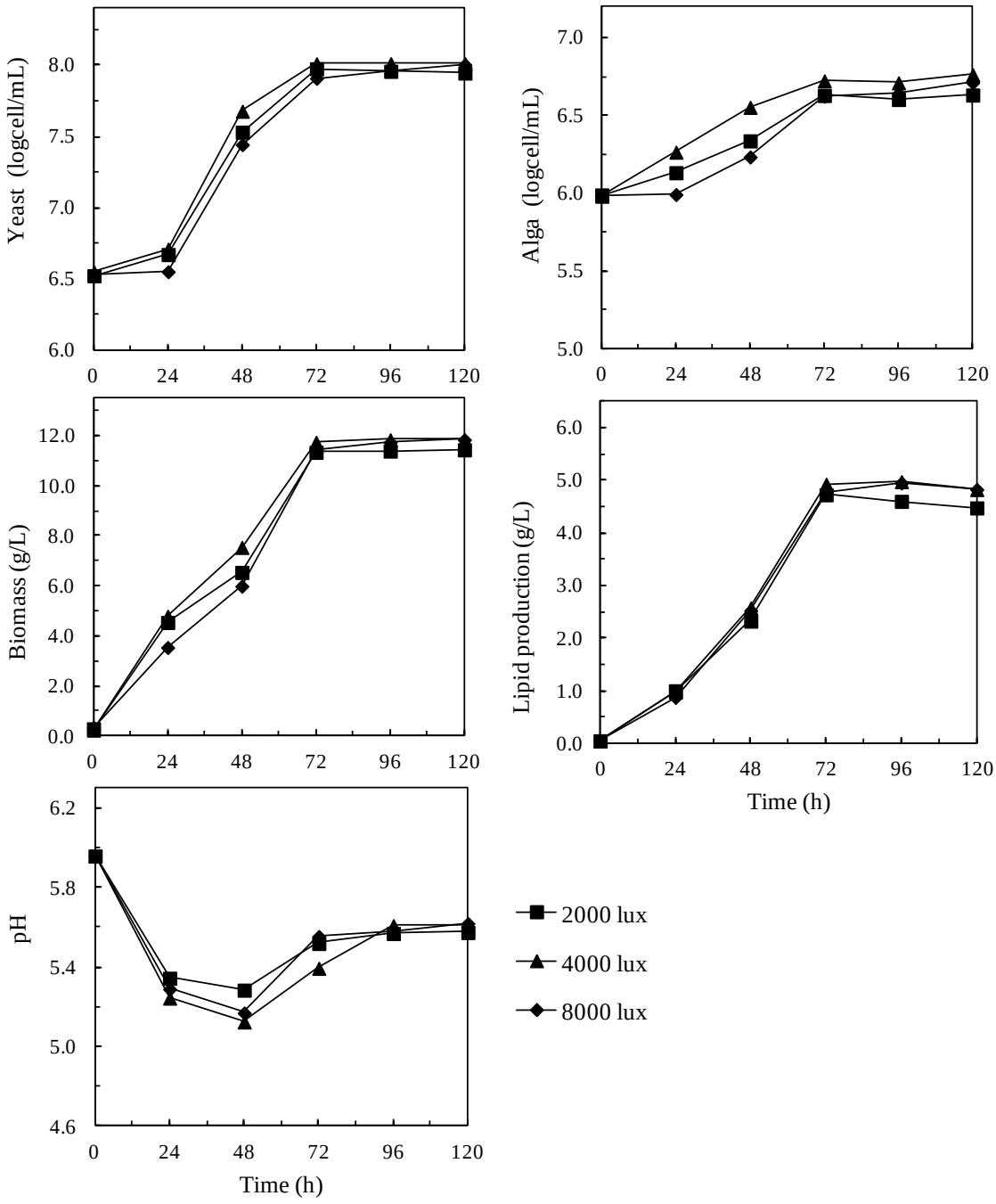

Figure 20. Effect of light intensity on cell number of yeast, cell number of microalgae, biomass and lipid production in the mixed culture.

Table 20. Growth and productivity of oleaginous yeast *T. spathulata* JU4-57 and *C. vulgaris* var. *vulgaris* TISTR 826.

Parameter	Specific growth rate (h^{-1})		Lipid productivity (mg/L.h)
	Yeast	Alga	
Selection of microalgae			
Fresh water microalga *Chlorella* sp.	0.0609	0.0012	57.6
C. vulgaris var. *vulgaris* TISTR 826	0.0681	0.0014	63.2
Marine microalga *Chlorella* sp.	0.0583	0.0012	56.6
Yeast and microalga inoculum ratio			
Yeast:Alga = 3.4×10^6: 4.0×10^4 cells/mL	0.0615	0.0006	54.5
Yeast:Alga = 3.4×10^6: 3.9×10^5 cells/mL	0.0681	0.0014	63.2
Yeast:Alga = 3.4×10^6: 1.6×10^6 cells/mL	0.0704	0.0008	65.6
Yeast:Alga = 3.4×10^6: 1.0×10^7 cells/mL	0.0715	0.0005	64.2
Light intensity			
2000 lux	0.0681	0.0014	65.6
4000 lux	0.0774	0.0019	68.4
8000 lux	0.0835	0.0014	66.3

The cell counts of yeast and microalga and lipid production in the single culture and mixed culture using 10% crude glycerol as a carbon source and ammonium sulfate as a nitrogen source with the C/N molar ratio of 17.2.

During mixed cultures, it was observed that the color of microalgae were yellow rather than green, which meant that the synthesis of chlorophyll *a* was restrained and mixotrophic process was conducted instead because of the presence of glycerol. According to Marquez *et al*. (1993), mixotrophic culture is much better than phototrophic culture, for the growth rate of mixotrophic culture is the sum of the phototrophic culture and the heterotrophic culture. Crude glycerol based medium was used to cultivate *T. spathulata* JU4-57 and *C. vulgaris* var. *vulgaris* TISTR 8261 separately. In the Figure 21 shows the time courses of biomass, lipid production and lipid content of *T. spathulata* JU4-57, *C. vulgaris* var. *vulgaris* TISTR 8261 and mixed culture of *T. spathulata* JU4-57 and *C. vulgaris* var. *vulgaris* TISTR 8261 in

10% crude glycerol (0.13 M of carbon) was used as a sole carbon and 0.5% (w/v) ammonium sulfate was used as a nitrogen source. As shown in Table 21, the *T. spathulata* JU4-57 biomass concentration was 10.23 g/L and the *C. vulgaris* var. *vulgaris* TISTR 8261 biomass concentration was 0.75 g/L. The sum of biomass concentration of the two microorganisms was 10.98 g/L, which was lower than that of the mixed culture (11.75 g/L). The result of total lipid of mixed culture (4.93 g/L) was also higher than the sum of *T. spathulata* JU4-57 lipid (3.87 g/L) and *C. vulgaris* var. *vulgaris* TISTR 8261 lipid (0.27 g/L). The specific growth rate and lipid productivity of the mixed culture were also higher that the pure culture of yeast and microalga (0.0774 h^{-1} and 68.4 mg/L.h). Xue *et al*. (2008) also studied mixed culture of microalgae (*S. platensis*) and yeast (*R. glutinis*) for lipid production. It was found that mixing cultivation of the two microorganisms significantly increased the accumulation of total biomass and total lipid production. This suggested that, the interaction between the two microorganisms played an important role on the cell growth and lipid synthesis of the microorganisms. At the same time, glycerol was consumed 99%. Furthermore, *C. vulgaris* var. *vulgaris* TISTR 8261 cultivation also used glycerol as a carbon source, which meant that biomass of *C. vulgaris* var. *vulgaris* TISTR 8261 could be also produced through heterotrophic cultivation under dark condition, release carbon dioxide and use oxygen. Alternatively they are cultivated heterotrophically or mixotrophically using organic compounds as energy and carbon sources. For the heterotrophic or mixotrophic cultivation, organic carbon compounds such as glucose are responsible for higher production costs. Glucose used in this process comprises about 80% of the total costs (Li *et al*., 2007). Pyle *et al*. (2008) reported that the glycerol derived biomass of microalga *Schizochytrium limacinum* contained 45-50% lipid, 14-20% protein, and 25% carbohydrate, with 8-13% ash content. Palmitic acid (C16:0) and DHA were the two major fatty acids in the microalgal lipid. The microalgal biomass was rich in lysine and cysteine, relative to many common feedstuffs.

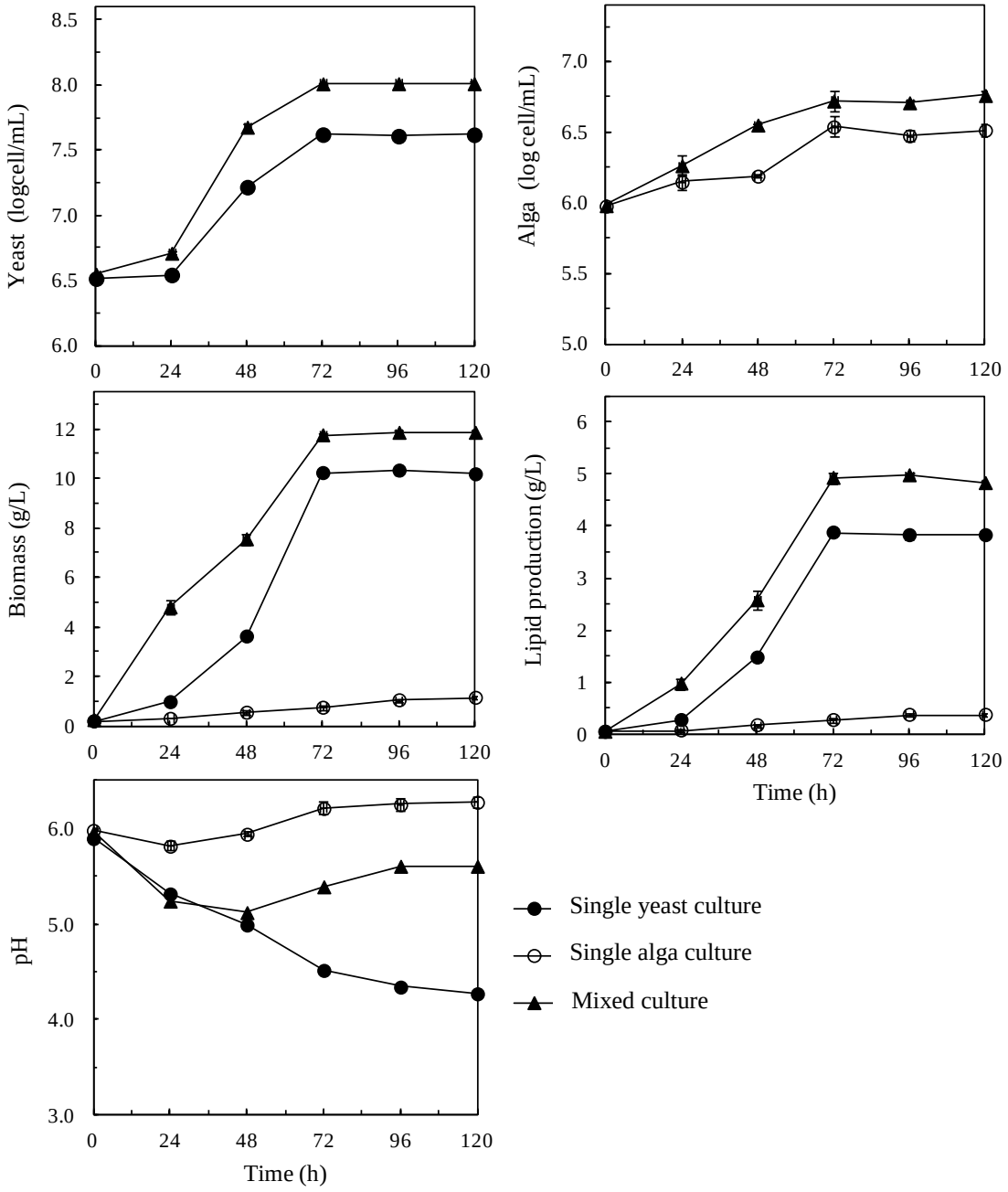

Figure 21. Time courses of cell number of yeast, cell number of microalgae, biomass and lipid production of *T. spathulata* JU4-57, *C. vulgaris* var. *vulgaris* TISTR 8261 and mixed culture of *T. spathulata* JU4-57 and *C. vulgaris* var. *vulgaris* TISTR 8261 in 10% crude glycerol (0.13 M of carbon) was used as a sole carbon and 0.5% (w/v) ammonium sulfate was used as a nitrogen source.

Overall, the results show that crude glycerol was a suitable carbon source for microalgal fermentation. The crude glycerol-derived microalgal biomass had a high level of DHA and a nutritional profile similar to that of commercial microalgal biomass, suggesting a great potential for using crude glycerol-derived microalgae in omega-3-fortified food or feed.

Xue *et al*. (2008) reported that the dissolved oxygen (DO) in the *Rhodotorula glutinis* culture was monitored using an autoclavable O_2 sensor (Mettler Toledo, Greifensee, Switzerland). In the early phase of yeast cultivation, the DO decreased rapidly because of continuous consumption of oxygen. When *Spirulina platensis* was added into the culture, DO increased rapidly from 7.45% to 120.5% in 5 h, which meant that the addition of *S. platensis* could provide extra oxygen for yeast to enhance aerobic metabolism. Thus, the yeast could grow better. During yeast metabolism, organic acids were synthesized and the pH of the culture decreased then carbon dioxide was released in acidified environment containing $NaHCO_3$. On one hand, the culture was adjusted to a less acidic environment so that the inhibition of yeast growth was alleviated. On the other hand, in the photosynthesis process of *S. platensis*, carbon dioxide, from yeast metabolism of mixed medium, was utilized as carbon source by *S. platensis* and simultaneously, oxygen was released leading to DO increasing.

Table 21. Comparison of single microorganism cultivations and mixed cultivation.

Microorganisms	Residual glycerol	Total biomass		Total lipid		Yields		Specific growth rate	Lipid productivity
	% w/v	g/L	CV	(g/L)	CV	$Y_{x/s}$	$Y_{p/x}$	(h^{-1})	(mg/L.h)
T. spathulata JU4-57	0.05± 0.001	3.87±0.06^{b}	0.91	10.23±0.04^{b}	1.04	0.267	0.381	0.0620	53.8
C. vulgaris var. *vulgaris* TISTR 8261	2.35± 0.013	0.27±0.04^{c}	12.8	0.75±0.07^{c}	9.43	0.021	0.367	0.0019	3.8
spathulata JU4-57 and *C. vulgaris* var. *vulgaris* TISTR 8261	0.03± 0.002	4.93±0.11^{a}	2.15	11.75±0.07^{a}	0.60	0.308	0.419	0.0774	68.4

- Cultivations were compared in crude glycerol (glycerol 4% (w/v)) based medium supplemented with only ammonium sulfate.
- Cultivation condition: 28±2 °C, 140 rpm, cultivated for 3 days.
- Different small letters in the table indicate significant difference between biomass, lipid production and lipid content (P<0.05).

2.2 Mixed culture of oleaginous yeast *Rhodotorula glutinis* and microalga

2.2.1 Biomass and lipid production from pure glycerol by single culture and mixed culture of yeast and microalga

The previous works also reported that *R. glutinis* has the ability to grow and accumulate lipids on glycerol (Saenge *et al*., 2011). Therefore, it was interesting to enhance lipid production by mixed culture of *R. glutinis* with oxygen producer microalga. The single culture and mixed culture of yeast *R. glutinis* and microalga *C. vulgaris* using 1% pure glycerol as a carbon source and ammonium sulfate as a nitrogen source with the C/N molar ratio of 16 are compared in Figure 22. The single culture of yeast grew faster than the single culture of microalga. The specific growth rate of yeast and lipid productivity in the mixed culture were 0.0079 h^{-1} and 9.87 mg/L.h, respectively, which were higher than those in the single culture as shown in Table 22. While the specific growth rate of microalga in the mixed culture was lower than that in the single culture of microalga. The biomass of yeast increased continuously until the end of cultivation while the numbers of yeast cell increased until day 2 of cultivation. In the co-culture, during 2 days of cultivation the numbers of yeast cell as well as its biomass increased faster than those in the single culture. The microalga grew slowly either in the single culture or in the co-culture. It should be noted that the numbers of microalga cell in the mixed culture was less than that in the single culture. This could be because the light may have hardly penetrated through the high concentration of yeast cell in the co-culture.

At day 5 of cultivation, the biomass of yeast in the single culture became little higher than that in the co-culture. This may have resulted from the faster depletion of the nutrients in the mixed culture than that in the single culture. It should be noted that the glycerol did not deplete during the cultivation. The glycerol consumption by the mixed culture was 76.7% which was higher than that of the single cultures of yeast (64.8%) and microalga (72.1%). It has been reported that glycerol could be used as a carbon source for *C. vulgaris* under mixotrophic condition (Liang *et al*. 2009). In this study, the light and dark period was set at 16:8 hours considering the further application in outdoor photobioreactors with the natural light and dark cycles.

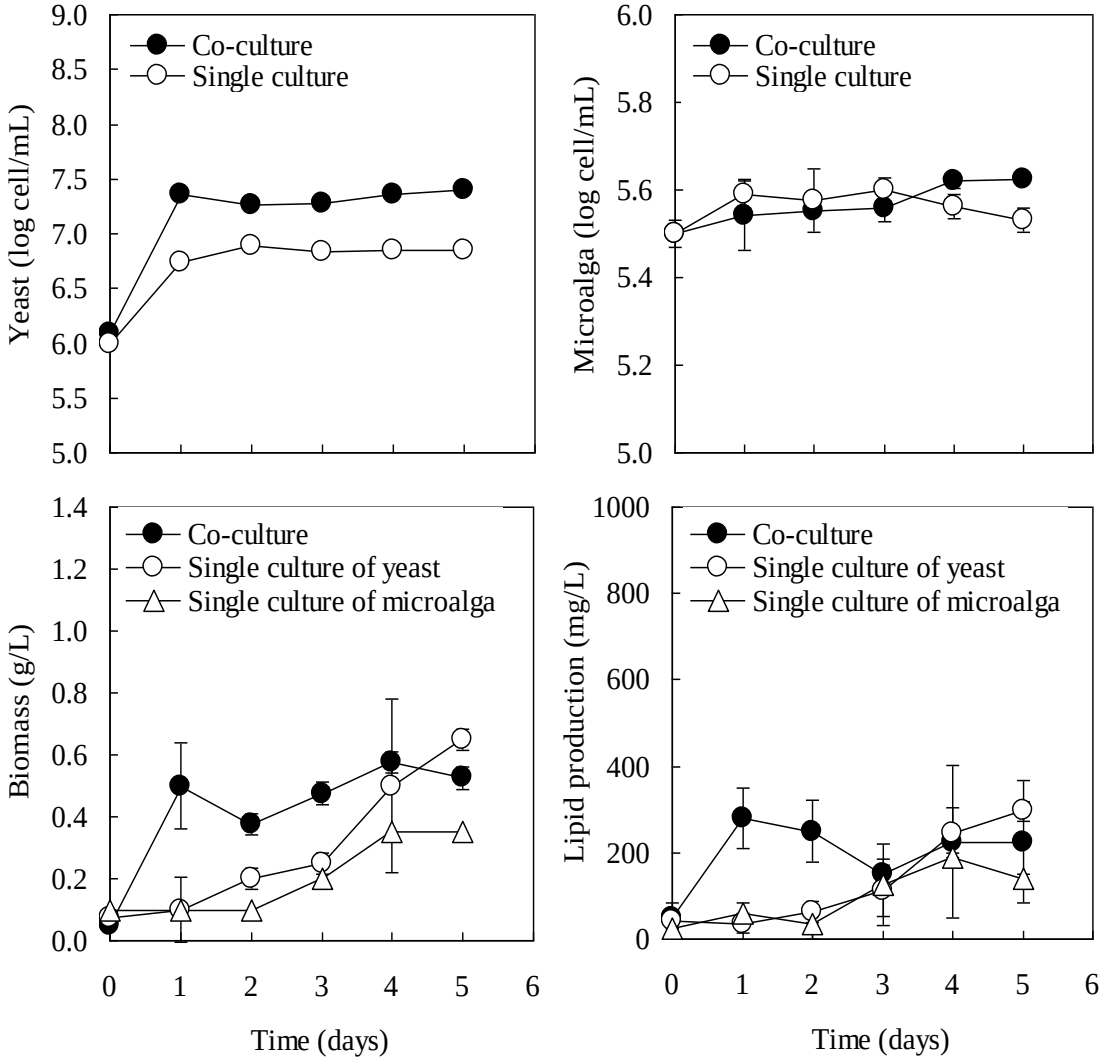

Figure 22. The cell counts of yeast and microalga, biomass and lipid production in the single culture and mixed culture using 1% pure glycerol as a carbon source and ammonium sulfate as a nitrogen source with the C/N molar ratio of 16.

During 4 days of cultivation, as the biomass in the mixed culture was higher than that in the single culture of each, so did the lipid production. The slight decrease in lipid production after 3 days of cultivation by the mixed culture could be due to the internal degradation of storage lipids by microorganisms. It has been reported that it is common for oleaginous microorganisms to reserve lipid during the growth especially in any nitrogen starvation phase then degrade it under carbon starvation conditions (Papanikolaou *et al*. 2004; Fakas *et al*. 2007). However, since the remarkable amount of glycerol remained in the medium after 3 days of cultivation (3.43 g/L), the lipid turnover in this yeast might not be due to the depletion of glycerol. It is possible that the decrease in glycerol concentration might reduce its uptake rate and when the uptake rate of the extra-cellular carbon source is reduced the lipid turnover occurs. The lipid turnover of yeast *Rhodotorula* sp. was also observed in the study of Chatzifragkou *et al*. (2011) when the glycerol concentration reduced to the level lower than that would be as "threshold" for glycerol conversion to lipid.

The contributions to the higher productivity in the mixed culture were obviously different between the two species. Since the yeast dominated in the mixed culture in terms of number of cells, therefore, it is reasonable to postulate that the yeast benefited more from the mutualism. Thus, the improvement in the biomass in the mixed culture mainly resulted from the promoted growth of the yeast. It is possible that the microalga may function as an oxygen producer in the mixed culture and enhance the growth of yeast. Xue *et al*. (2010) found that the dissolved oxygen in the culture of yeast *R. glutinis* drastically increased when microalga *Spirulina platensis* was added into the culture. In addition, the relationship would be symbiotic, as the yeast produced CO_2 that could be used by the microalga. In the co-culture, both the two metabolic reactions of CO_2 release and uptake were combined and were complementary. Since the production of lipid from microorganisms is subjected to the constraints of high operation costs, even a small improvement in the culture techniques could result in substantial savings in their biomass production.

2.2.2 Optimizing the medium for the mixed culture of yeast and microalga

2.2.2.1 Nitrogen source

Since the productivity of biomass and lipid in the mixed culture of yeast and microalga was higher than that in the single culture of each, further experiments to optimize the medium composition were carried out. The co-cultures of yeast and microalga using 1% pure glycerol as a carbon source and various nitrogen sources with the C/N molar ratio of 16 are shown in Figure 23. To economically produce lipid, several cheap inorganic nitrogen sources such as ammonium sulfate, ammonium chloride, ammonium nitrate and a relatively cheap organic nitrogen source, urea, were tested. The addition of ammonium chloride, ammonium nitrate and urea increased the numbers of yeast cell compared to the use of ammonium sulfate. The highest value of yeast cell was obtained when using urea as a nitrogen source. The specific growth rates of yeast and microalga and lipid productivity were 0.0102 h^{-1}, 0.0013 h^{-1} and 17.29 mg/L.h, respectively (Table 22). It was found that ammonium nitrate and urea could enhance the growth of microalga. However, the numbers of microalga cell decreased after 2 days of cultivation when using ammonium nitrate as a nitrogen source. Therefore, urea was considered to be the most suitable nitrogen source because it enhanced the growth of both strains and gave the highest values of biomass and lipid. In the study of Shia *et al*. (2000), they also found that urea was the most suitable nitrogen source for the heterotrophic growth of *Chlorella protothecoides*.

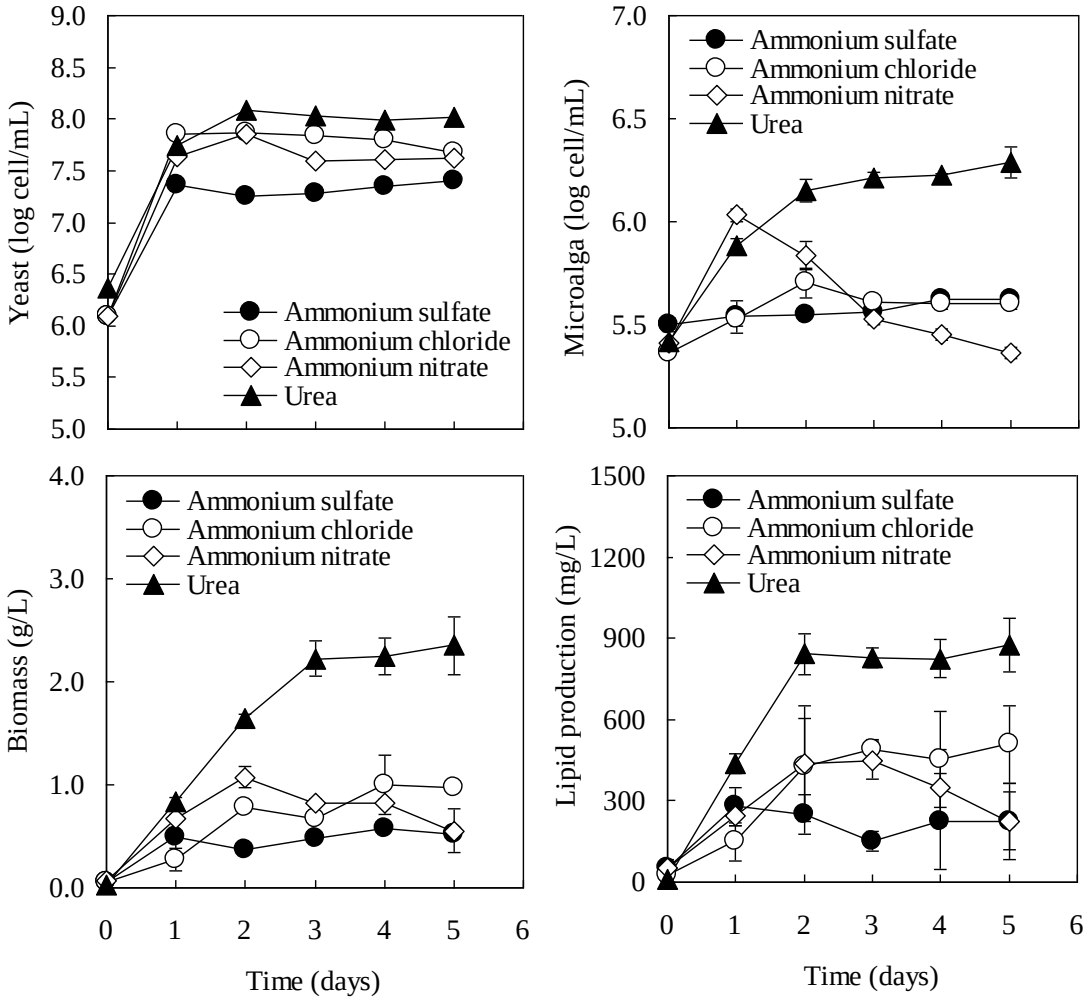

Figure 23. Effect of nitrogen source on the cell counts of yeast and microalga, biomass and lipid production in the co-cultures using 1% pure glycerol as a carbon source and various nitrogen sources at the C/N molar ratio of 16. The nitrogen source was ammonium sulfate, ammonium chloride, ammonium nitrate and urea.

2.2.2.2 C/N ratio

A nutrient imbalance in the culture medium is known to trigger lipid accumulation in oleaginous microorganisms. Lipid production requires a medium with an excess of carbon source and limited other nutrients, usually nitrogen. Thus, oleaginous potential is critically affected by the C/N ratio of the culture. At a low C/N, the carbon flux is distributed to allow cellular proliferation. At a high C/N ratio, when cells run out of nitrogen, they cannot multiply and excess carbon substrate is assimilated continuously to produce storage lipid. To determine the effect of C/N ratio, 1% pure glycerol was used as a carbon source and urea were used as a nitrogen source with various C/N ratios of 16, 24 and 32 (Figure 24). The numbers of yeast cell increased significantly when the C/N ratio increased from 16 to 24. There was no significant difference in the numbers of microalga cell with various C/N ratios. The highest biomass and lipid production was obtained at a C/N molar ratio of 32, which gave the highest lipid productivity of 17.92 mg/L.h. It should be noted that the biomass was maximum at day 2 of cultivation while that for lipid production was at day 3. As the culture growth progresses, a change in the C/N ratio is expected with relatively lower levels of nitrogen. The conditions for lipid production might therefore be more favourable during the later stage of culture growth. According to Angerbauer *et al*. (2008), the lipid production by *Lipomyces starkeyi* was enhanced when the C/N ratio was increased. A high C/N ratio at 25 was also used for the lipid production from glycerol by *C. curvatus* (Meester *et al*. 1996). In the study of Illman *et al*. (2000), they also reported that the lipid production by microalga *C. vulgaris* increased significantly when it was grown in low nitrogen medium, while the difference in final dry weights was small.

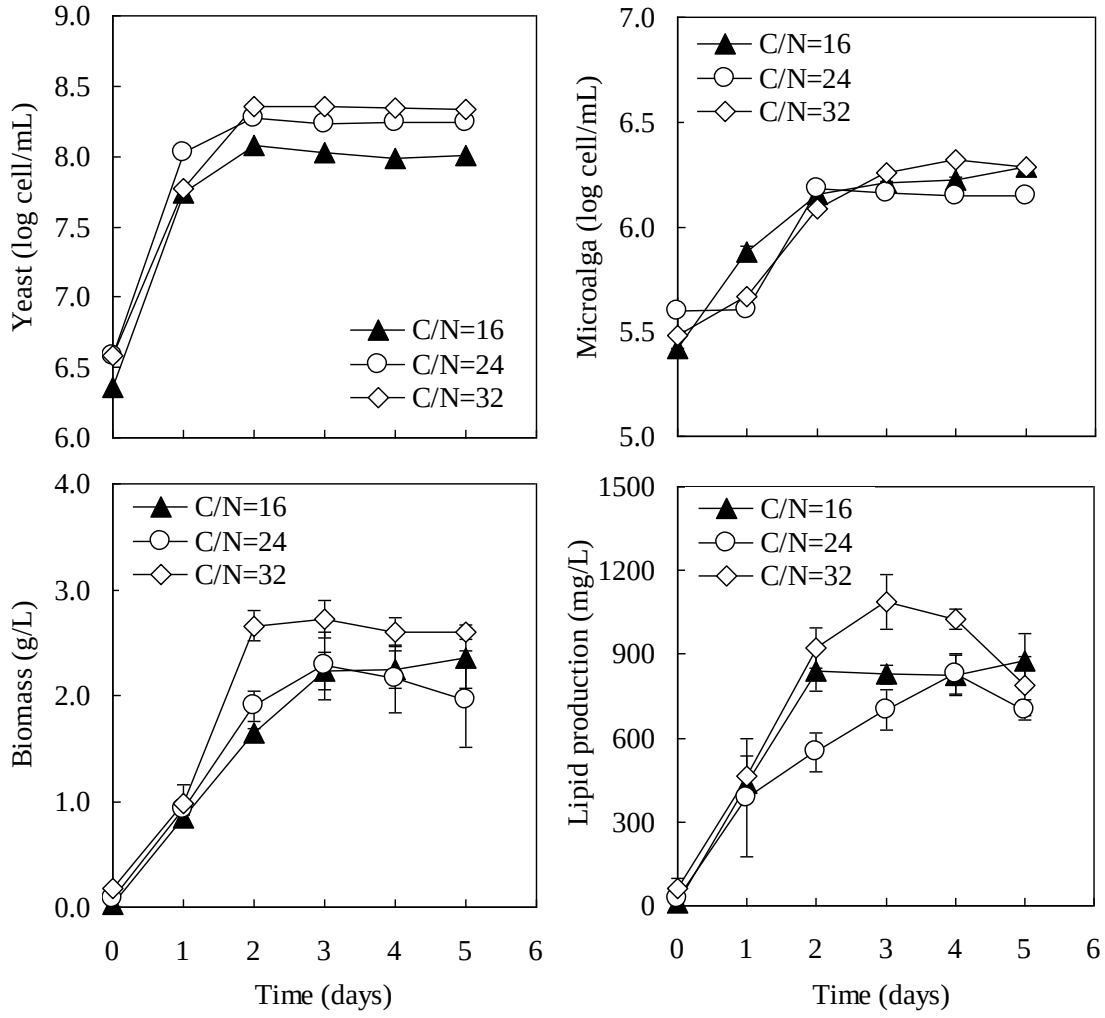

Figure 24. Effect of C/N molar ratio on the cell counts of yeast and microalga, biomass and lipid production in the co-cultures using 1% pure glycerol as a carbon source and urea as a nitrogen source. The C/N molar ratio was varied from 16 to 24 and 32.

Table 22. Growth and lipid productivity of oleaginous yeast *R. glutinis* and *C. vulgaris* var. *vulgaris* TISTR 826.

Parameter	Specific growth rate (h^{-1})		Lipid productivity (mg/L.h)
	Yeast	Microalga	
Single culture and mixed culture			
Single culture of yeast	0.0048	-	2.10
Single culture of microalga	-	0.0007	1.69
Mixed culture	0.0079	0.0003	9.87
Nitrogen source			
Ammonium sulfate	0.0079	0.0003	9.58
Ammonium chloride	0.0105	0.0013	8.77
Ammonium nitrate	0.0094	0.0045	8.08
Urea	0.0102	0.0013	17.29
C/N ratio			
16	0.0102	0.0013	17.29
24	0.0083	0.0021	10.42
32	0.0069	0.0014	17.92

The results of the single and mixed cultures using 1% pure glycerol as a carbon source and ammonium sulfate as a nitrogen source with the C/N molar ratio of 16.

2.2.3 Glycerol concentration

In order to further increase the biomass and lipid production, the concentration of pure glycerol was increased from 1% to 2%, 3% and 4% while using urea as the nitrogen source and maintaining the C/N molar ratio at 32. In addition, to reduce the cost of lipid production, pure glycerol was replaced with crude glycerol, a by-product from a biodiesel plant, at the same glycerol content. The results of biomass, lipid production and lipid production are shown in Figure 25. The biomass and, consequently, lipid production increased with increasing concentration of glycerol. However, there was little increase of biomass when the pure glycerol was increased from 3% to 4% and this led to a lower lipid production. In the study of Meester *et al*. (1996), when the concentration of pure glycerol was increased higher

than 32 g/L (3.2%) there was no further increase in the growth of *C. curvatus* and its growth was inhibited at glycerol concentration of 64 g/L (6.4%). In this study, the biomass and lipid production using crude glycerol were lower than those using pure glycerol. This could be due to the presence of impurities in the crude glycerol. Liang et al. (2010) also reported that *C. curvatus* was inhibited when grown with crude glycerol compared with pure glycerol. However, it was reported that *Y. lipolytica* could grow with crude glycerol just as well as with pure glycerol (Papanikolaou and Aggelis, 2002). This could be due the higher purity of glycerol (65-85%) used in their study compared to that used in this study (50%).

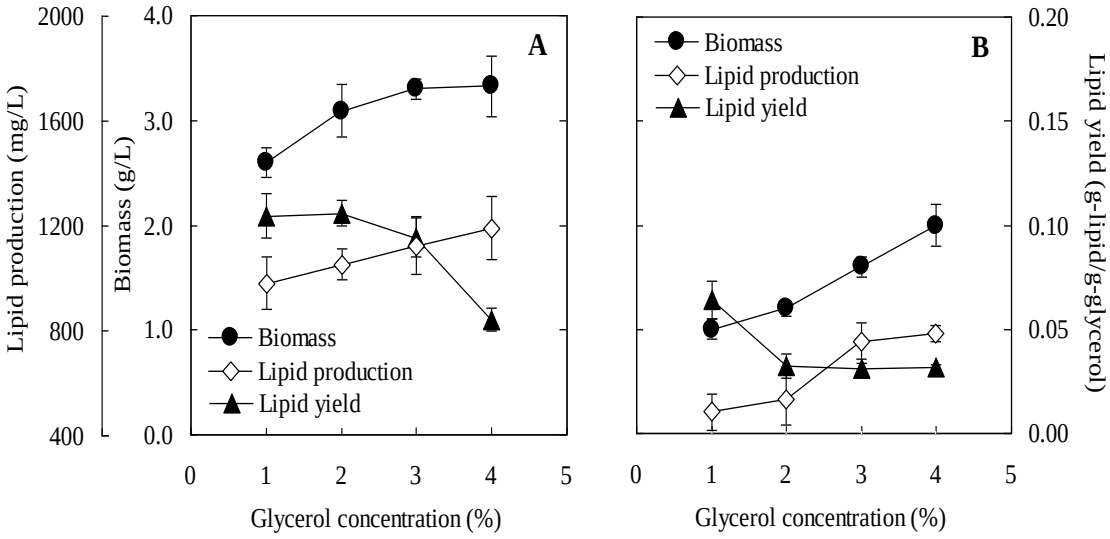

Figure 25. Effect of glycerol concentration on biomass, lipid production and lipid production in the co-cultures using pure glycerol (A) and crude glycerol (B) as a carbon source and urea as a nitrogen source with the C/N molar ratio of 32.

The lipid productions of the mixed culture with pure glycerol in this study were 0.05-0.12 g-lipid/g-glycerol, while those with crude glycerol were only 0.03-0.06 g-lipid/g-glycerol. In recent investigation, the maximum lipid production from glycerol by yeast that could be met in optimized culture conditions was 0.20-0.22 g/g (Papanikolaou and Aggelis, 2009). The lower lipid production in this study presumably is due to the relative poor regulation of the enzymes involved in the primary metabolic steps of glycerol assimilation (Fakas *et al*. 2009). In the study of Chatzifragkou *et al*. (2011), a number of yeast strains were screened in relation to their ability to convert raw glycerol into lipid. Although, strains belonging to the genus *Rhodotorula* have been reported to accumulate significant lipid quantities, in their study noticeable differences related with glycerol uptake, biomass production and lipid accumulation were observed between the two *Rhodotorula* strains studied. One *Rhodotorula* strain accumulated lipid up to 22% based on its biomass while the other accumulated only 3.4%. Therefore, the oleaginous character is strongly strain-dependent capability. Apart from using glycerol as a substrate, *Rhodotorula* spp. have been revealed capable of producing high lipid during growth on various natural substrates (Li *et al*., 2010; Zhao *et al*., 2010) and industrial effluent (Saenge *et al*. 2011).

After optimization of the co-culture, the biomass and lipid production were enhanced by 5.7-fold and 3.8-fold, respectively compared to unoptimized condition. In addition, since higher glycerol concentration gave a lower lipid production, perhaps the use of the fed-batch fermentation technique would further improve the biomass and lipid production by these two microorganisms. Moreover, since it was reported that the lipid production by *Y. lipolytica* was enhanced in highly aerated cultures (Papanikolaou and Aggelis, 2002), the mixed culture system would be an alternative method to further improve the lipid production by this strain.

2.2.4 Fatty acid composition of microbial lipid produced from crude glycerol

Table 23 shows the fatty acid composition of lipid extracted from yeast, microalga and mixed culture growing with crude glycerol. The fatty acid chain lengths were in the range of C12:0 to C24:0. The lipid from *R. glutinis* is composed mainly of long-chain fatty acid with 16 and 18 carbon atoms including oleic acid as the predominant fatty acid (48.91%) followed by linoleic acid (19.16%) and palmitic acid (17.96%). The predominant fatty acid in lipid from microalga was oleic (37.49%) followed by palmitic acid (32.32%) and linoleic acid (15.69%). The main fatty acids were long-chain fatty acids with 16 and 18 carbon atoms including palmitic acid as the predominant fatty acid (40.52%) followed by oleic acid (21.30%) and stearic acid (17.15%) from mixed culture. In the mixed culture of yeast and microalga, the content of palmitic acid was higher than that of the single cultures of both while the content of oleic acid was lower than that of the single cultures. Since the lipid from the single culture of *R. glutinis* grown with crude glycerol (Saenge *et al*., 2010) and other oleaginous yeasts such as *Rhodosporidium toruloides* Y4 (Li *et al*., 2007) and *Rhodotorula mucilaginosa* TJY15a (Li *et al*., 2010) contained oleic acid as the predominant fatty acid, the high content of palmitic acid could be derived from the presence of microalgal lipid. It has been reported that the fatty acids found in *Chlorella vulgaris* were mainly palmitic acid (47-63%) and oleic acid (10-37%), depending on the culture conditions (Converti *et al*., 2009). Petkov and Garcia (2007) also found that palmitic acid was the main fatty acid in *C. vulgaris* (26%) followed by linoleic acid (24%), linolenic acid (20%) and oleic acid (16%). It was also possible that the extra oxygen provided by microalga might stimulate the production of saturated fatty acid (palmitic acid) rather than unsaturated fatty acid (oleic acid). Cai *et al*. (2007) also reported that the mixed culture of the microalga *Isochrysis galbana* 8701 and the yeast *Ambrosiozyma cicatricose* was richer in saturated fatty acids than the two single cultures. The fatty acid compositional profile in this study is similar to that of plant oil which contains mainly palmitic and oleic acids. This indicates that the microbial lipid from this mixed culture have potential as a biodiesel feedstock. Although, the high content of saturated fatty acid (palmitic acid) would evidence

lower fuel properties at low temperatures, but it would provide better oxidative stability.

Table 23. Fatty acid composition of yeast and microalgal lipid produced from crude glycerol.

Fatty acids	Fatty acid relative content (%)		
	Yeast	Microalga	Mixed
Capric acid (C10:0)	0.06	0.00	0.00
Lauric acid (C12:0)	0.11	0.00	0.98
Myristic acid (C14:0)	0.93	0.97	2.98
Palmitic acid (C16:0)	17.96	32.32	40.52
Palmitoleic acid (C16:1)	0.87	2.31	1.15
Heptadecoic acid (C17:0)	1.33	0.00	1.10
Stearic acid (C18:0)	3.93	8.27	17.15
Oleic acid (C18:1)	48.91	37.49	21.30
Linoleic acid (C18:2)	19.16	15.69	1.41
Linolenic acid (C18:3)	4.63	2.94	0.00
Behenic acid (C22:0)	0.75	0.00	1.44
Erucic acid (C22:1)	0.38	0.00	8.89
Lignoceric acid (C24:0)	0.97	0.00	3.08

3. Process optimization in a 5-L bioreactor

3.1 Effect of pH control

3.1.1 Culture of yeast, microalga and mixed without pH control

The pH level is one of the most important environment parameters affecting cell growth and production. Some literatures dealing with the oleaginous yeasts reported the optimal pH for cell growth and accumulation of lipids in a shake flask. In this study, the effect of pH control and without out pH control were investigated in a 5 L stirrer tank bioreactor working volume 2 L under batch mode as shown in Figure 26.

Figure 26. The set apparatus for *T. spathulata* JU4-57 in a stirred tank bioreactor under batch and fed-batch fermentation.

Figure 27 shows the time courses of cell growth, lipid production, lipid content and DO without pH control. The agitation speed was controlled at 100 rpm without aeration in a 5-L bioreactor and under light intensity of 4,000 luk for microalgal and mixed culture. The pH in the medium declined from 6.0 to 4.31 (Figure 27a) for the single culture of yeast and the pH in the medium of the mixed culture slightly decreased from 6.0 to 5.56 (Figure 27b). By contrast, the pH of cultivation of microalga slightly increased from 6.0 to 6.43 (Figure 27c). The mixed culture showed a short lag phase and gave higher biomass than that of the pure culture. The specific growth rate of the mixed culture was 0.0586 h^{-1} and it was also

higher than the pure culture of yeast (0.0499 h^{-1}) and microalga (0.0012 h^{-1}). This would be because in the mixed culture yeast might produce some acids that can be used by microalga. It has been reported that microalgae are usually directly involved in the treatment by the assimilation of not only nitrogen and phosphorus, but also organic matter (Pearson *et al*., 1987). Laliberte and de la Noue (1993) also reported the ability of phytoplankton to use different organic compounds, such as acetate and glucose, as a carbon source in the dark (heterotrophic activity) or in the light (mixotrophic activity). In the culture of microalga, the DO decreased under dark condition and increased again under continuous light illumination because microalgae released O_2 under light condition and fixed CO_2 (photoautotrophic mode).

3.1.2 Pure culture of yeast with pH control

Figure 28 shows the time courses of cell growth, lipid production and lipid content with and without pH control at 6.0. The agitation speed was controlled at 100 rpm with aeration at 1 vvm. Without pH control, the pH in the medium declined from 6.0 to 4.2 which may indicate that the production of acids (Figure 28a). When the pH was controlled by adding sodium hydroxide, the cell grew faster and gave slightly higher amount of biomass (Figure 28b). The specific growth rate and lipid productivity of yeast with pH control were 0.064 h^{-1} and 69.8 mg/L.h, respectively, which were higher than yeast culture without pH control (0.0527 h^{-1} and 67 mg/L.h, respectively). This could be because the inhibitory effect of low pH was alleviated. With pH control, the cell could also accumulate the lipid faster but there was no significant difference in the lipid production with and without pH control at 72 h. Saenge *et al.* (2011) found that the yeast *R. glutinis* accumulated a stable amount of lipids during the exponential phase. Without pH control, the pH in the medium declined from 6.0 to 4.3 which may indicate the production of acids. When the pH was controlled by adding sodium hydroxide, there was a slight increase in lipid production. The lipid content was also enhanced from 42.12% up to 48.21% when the pH was kept constant.

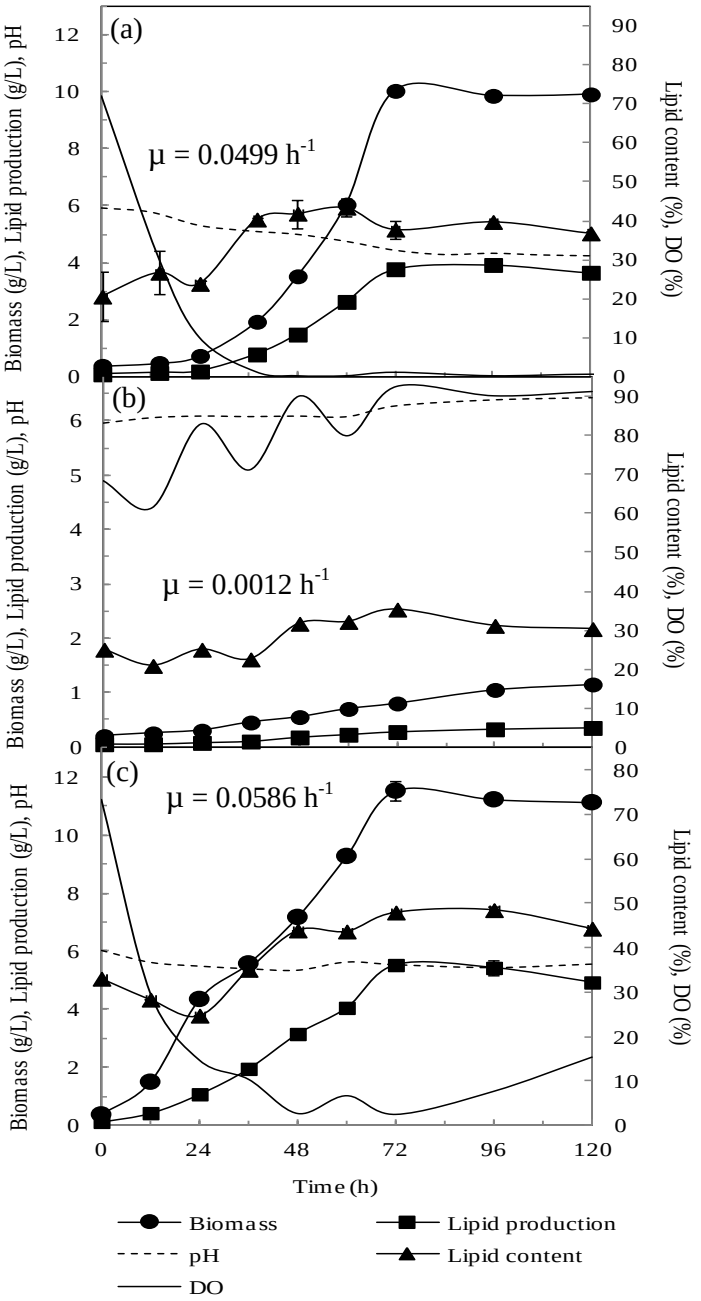

Figure 27. Effect of without pH control on biomass, lipid production, lipid content and DO by *T. spathulata* JU4-57 (a), *C. vulgaris* var. *vulgaris* TISTR 8261 (b) and mixed culture of *T. spathulata* JU4-57 and *C. vulgaris* var. *vulgaris* TISTR 8261 (c) in a 5-L bioreactor under light intensity of 4,000 luk.

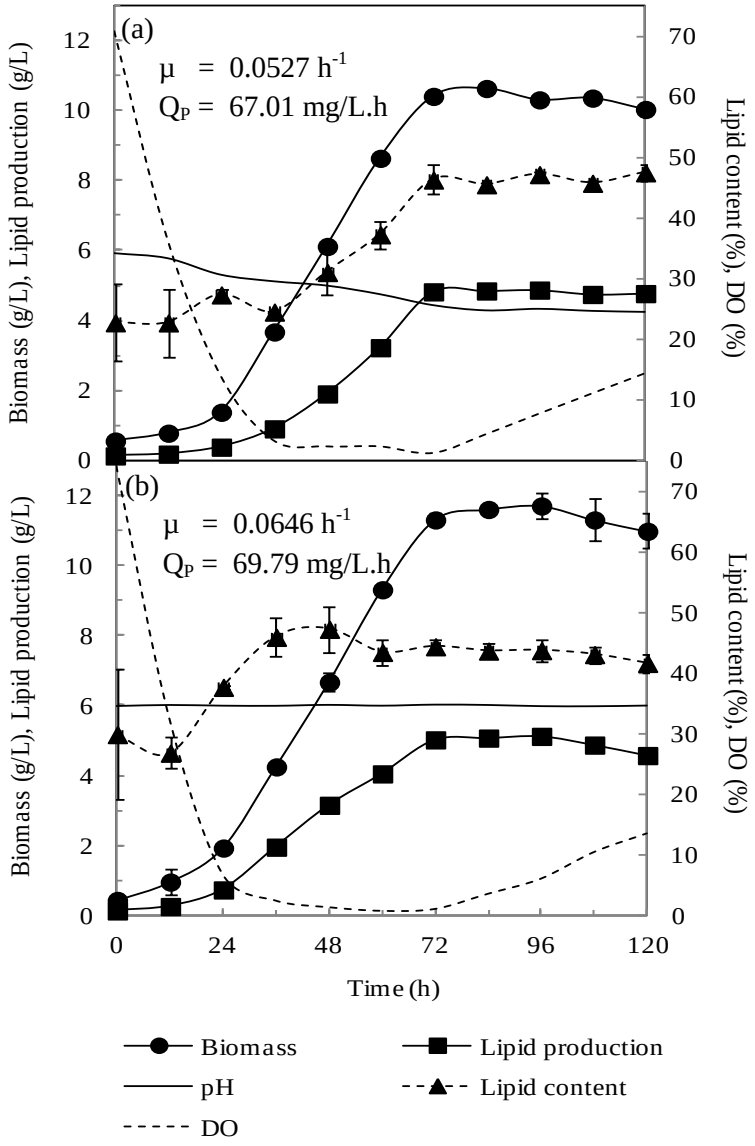

Figure 28. Effect of pH control on biomass, lipid production and lipid content by *T. spathulata* JU4-57 in a stirred tank bioreactor at aeration rate of 1 vvm. Without pH control (a) and pH control (b).

3.1.3 Mixed culture of oleaginous yeast and microalga

The mixed culture of yeast *T. spathulata* JU4-57 and microalgal *C. vulgaris* var. *vulgaris* TISTR 8261 was also cultivated in a 5 L stirrer bioreactor tank working volume 2 L under batch mode to investigate the effect of pH control. The growth, lipid production, lipid content and DO were investigated as shown in Figure 29. The biomass concentration and lipid production in the mixed culture increased faster and higher compared with that in the pure culture for both cases without (Figure 27a and Figure 28a) and with pH control (Figure 28b). At 72 h, there was no significant difference in the biomass concentration of the mixed culture and that of the pure culture of yeast. This may have resulted from the depletion of the nutrients in the culture medium used. It is possible that the microalga may function as an oxygen producer in the mixed culture and enhance the growth of yeast. Xue *et al*. (2010) found that the dissolved oxygen in the culture of yeast *R. glutinis* drastically increased when microalga *Spirulina platensis* was added into the culture. In addition, the relationship would be symbiotic, as the yeast produced CO_2 that could be used by the microalga. The higher DO concentration in the mixed culture (Figure 27a and 27b) compared with the pure culture (Figure 28) was also observed in this study.

During the yeast cultivation, organic acids were synthesized and the pH of the culture propped from 6.0 to 4.3. Bhosale and Gadre (2001) reported that the optimum initial pH for the growth rate of *R. glutinis* was 6.0. In addition, the media acidified with organic acids (e.g. acetic, lactic acids) are more inhibitor to yeast growth compare with those acidified with mineral acids (e.g. hydrochloric, phosephoric acids). This is because undissociated organic acid can lower intracellular pH following translocation across the yeast plasma membrane (Walker, 1998). In the mixed culture, there was a slight drop from 6.0 to 5.56 at 72 h. When CO_2 dissolves in water at neutral pH, bicarbonate (HCO_3^-) is formed. During microalgal photosynthesis activity HCO_3^- is converted to CO_2 and hydroxide ion (OH^-). Hence, when CO_2 is consumed by microalgae, the OH^- is formed, and the pH becomes more alkaline (Richmond, 1986). It was reported that some of yeast metabolites such as pyruvic and acetic acids might be utilized by microalga (Xue *et al*., 2010). Hence, the inhibition of yeast growth by those organic acids could be alleviated.

pH control also was investigated using sodium hydroxide and ammonia in the mixed culture (Figure 29b and 29c, respectively). When the pH was controlled using sodium hydroxide and ammonia, the cell could grow and accumulate lipid faster than without pH control. The pH control using ammonia gave the highest specific growth rate 0.0648 h^{-1}. However, there was no significant difference in the final biomass and lipid production between the mixed cultures without and with pH control using either sodium hydroxide or ammonia. The cell number of yeast and microalga were also monitored as shown in Table 24. With pH control adding sodium hydroxide, the cell number of yeast and microalga increased from 5.95×10^6 cells/mL and 2.12×10^6 cells/mL to 4.24×10^7 cells/mL and 4.94×10^6 cells/mL, respectively. The pH control using ammonia gave the highest cell number of yeast and microalga. The cell number of yeast and microalga could increase from 6.51×10^6 cells/mL and 2.20×10^6 cells/mL to 5.55×10^7 cells/mL and 5.76×10^6 cells/mL, respectively. This could be because the inhibitory effect of low pH was alleviated, and yeast and microalga could use ammonia as nitrogen source for their growth.

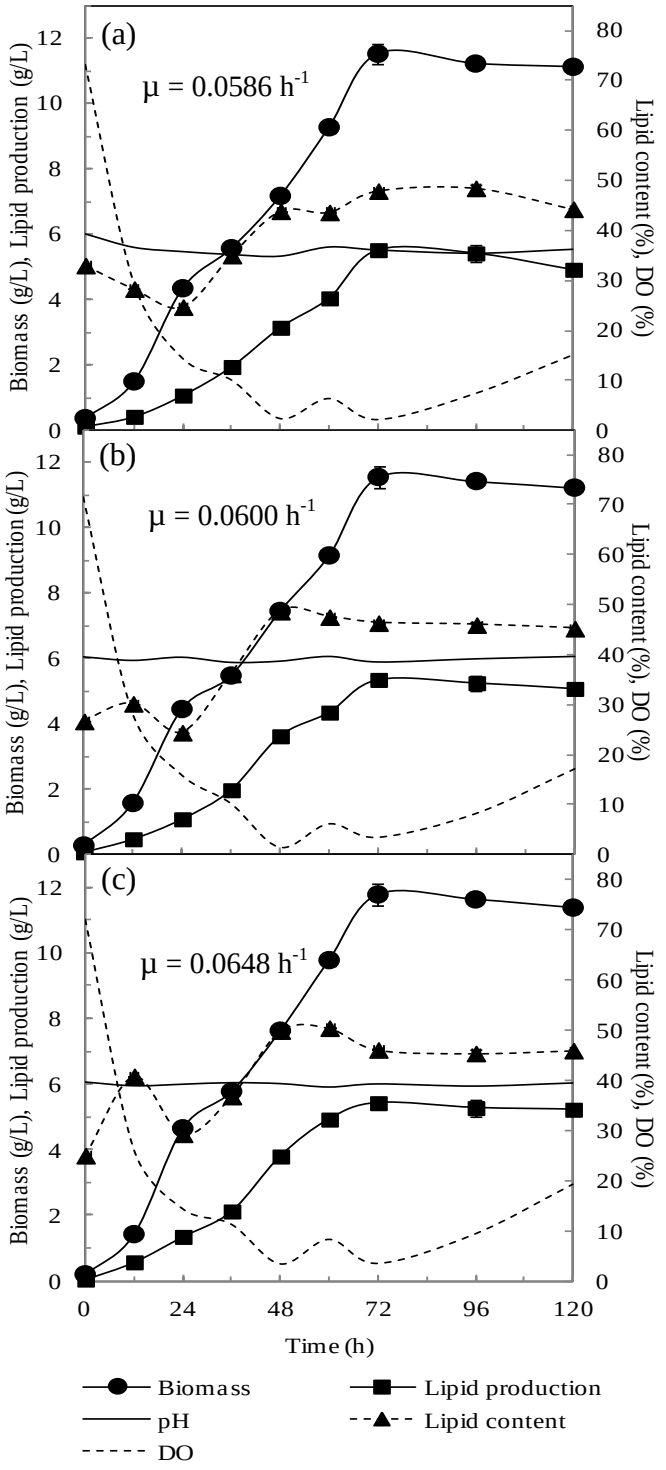

Figure 29. Effect of without (a) and with pH control using sodium hydroxide (b) and ammonia (c) on biomass, lipid production, lipid content and DO by mixed culture of *T. spathulata* JU4-57 and *C. vulgaris* var. *vulgaris* TISTR 8261 in a 5-L bioreactor under light intensity of 4,000 luk.

Table 24. Effects of culture conditions on the growth of the yeast *T. spathulata* JU4-57 and the microalga *C. vulgaris* var. *vulgaris* TISTR 8261 in the mixed cultures.

Culture conditions	Cell counts at each cultivation (cell/mL)					
	12 h		72 h		120 h	
	Yeast	Microalga	Yeast	Microalga	Yeast	Microalga
Without pH control	5.30×10^{6}	1.91×10^{6}	3.38×10^{7}	4.33×10^{6}	3.48×10^{7}	4.38×10^{6}
Control pH at 6.0 by NaOH	5.95×10^{6}	2.12×10^{6}	4.26×10^{7}	4.66×10^{6}	4.24×10^{7}	4.94×10^{6}
Control pH at 6.0 by ammonia	6.51×10^{6}	2.20×10^{6}	5.56×10^{7}	5.31×10^{6}	5.55×10^{7}	5.76×10^{6}

3.2 Effect of aeration rate of *T. spathulata* JU4-57

Aeration plays a major role in the growth rate and the lipid production since the oxygen transfer becomes a limiting factor when the cell grows and the viscosity of the broth increases. In the aerobic culture, the rate of the dissolved oxygen (DO) supply must at least equal the rate of oxygen demand (Mantzouridou *et al*., 2002; Meesters *et al*., 1996). Figure 30 shows the effect of aeration rate on cell growth, lipid production, glycerol consumption and dissolved oxygen. The aeration rate had a profound effect on biomass, consequently lipid production and glycerol consumption. The cell slightly grew faster and the specific growth rate slightly increased from 0.0640 h^{-1} to 0.0646 h^{-1} when increasing aeration rate from 1 to 3 vvm but the final cell concentration was not significantly different. On the other hand, increasing the aeration rate the lipid content gradually decreased. The maximum biomass (11.33 g/L) and lipid production (5.03 g/L) were obtained at the aeration rate of 1 vvm. It is possible that when the aeration was increased the energy was used for cell proliferation rather than accumulated as lipid content. In the early phase of yeast cultivation, glycerol concentration decreased rapidly and nearly to 0 g/L after 72 h. There was also a rapid decrease in the DO, from over 70% nearly to 0% at 60 h. The DO became increased again after 72 h when glycerol was exhausted. The cascade control of DO was also attempted to increase DO by increasing agitation speed and

aeration rate (Figure 30d). However, this strategy could not recover the DO level even using the maximum agitation speed at 200 rpm and aeration rate at 3 vvm.

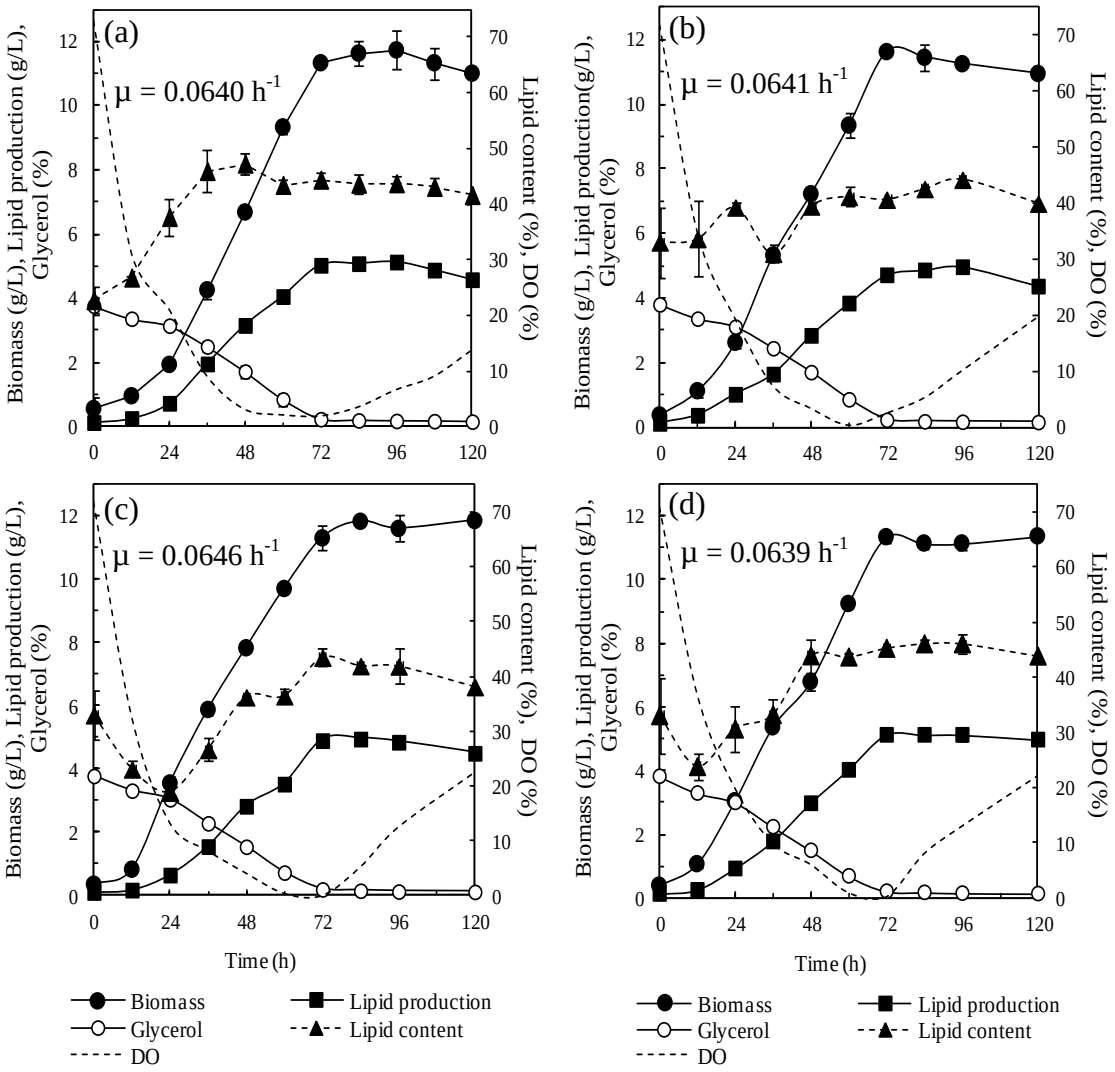

Figure 30. Effect of aeration rate on cell growth, lipid production, lipid content and dissolved oxygen (DO). The aeration rate was varied at 1 vvm (a), 2 vvm (b), 3 vvm (c) with the constant agitation rate at 100 rpm (d) was the result of cascade control of DO by agitation and aeration.

3.3 Fed-batch fermentation

From the results described above, it is likely that the lack of the glycerol prevents biomass and lipid production reaching their maximum at the end of the fermentation. The additional glycerol is presumably helpful in enhancing the cell biomass and lipid production and productivity. However, the high concentration of glycerol required for high final biomass and lipid inhibit growth when it was added in total at the start of the fermentation. Thus, the fed-batch strategy in that feeding glycerol during fermentation was adopted to avoid the inhibitory effects of nutrient on the biosynthesis and to increase the final cell biomass and lipid accumulation.

3.3.1 Feeding with crude glycerol and ammonium sulfate

To enhance both biomass and lipid production the fed with crude glycerol and ammonium sulfate was attempted. The feeding solution containing glycerol and ammonium sulfate at the C/N ratio of 17.2 was added into the bioreactor after 60 h of fermentation and every 12 h thereafter until 120 h. Four fed-batch fermentations were performed in which feeding solution contained different glycerol concentrations of 4, 8, 12 and 16%. The profiles of biomass and lipid production of *T. spathulata* JU4-57 in four fed-batch fermentations are shown in Figure 31. The residual glycerol concentration and DO were also monitored. Each feeding led to different residual glycerol concentrations around 0.3, 0.5, 1 and 2%, respectively. The feeding solution with 4% glycerol could maintain the glycerol concentration at lowest level at 0.3%. In this fed-batch, the concentration of biomass and lipid production reached 12.98 g/L and 6.55 g/L, respectively (Figure 31a). Increasing the concentration of crude glycerol in the feeding solution gave higher biomass and lipid production. However, the residual glycerol concentration also increased. This indicated that the nutrient supply rate was higher than the consumption rate by the yeast. When 12% of glycerol was fed, the concentration of biomass and lipid increased up to 17.30 g/L and 7.25 g/L, respectively, where the residual glycerol concentration was about 1% (Figure 31c). However, when 16% of glycerol was fed, the concentration of biomass and lipid slightly dropped to 16.23 g/L, and 7.22 g/L, respectively. This could be due to a considerable inhibitory effect since the residual glycerol concentration remarkably increased up to 2%.

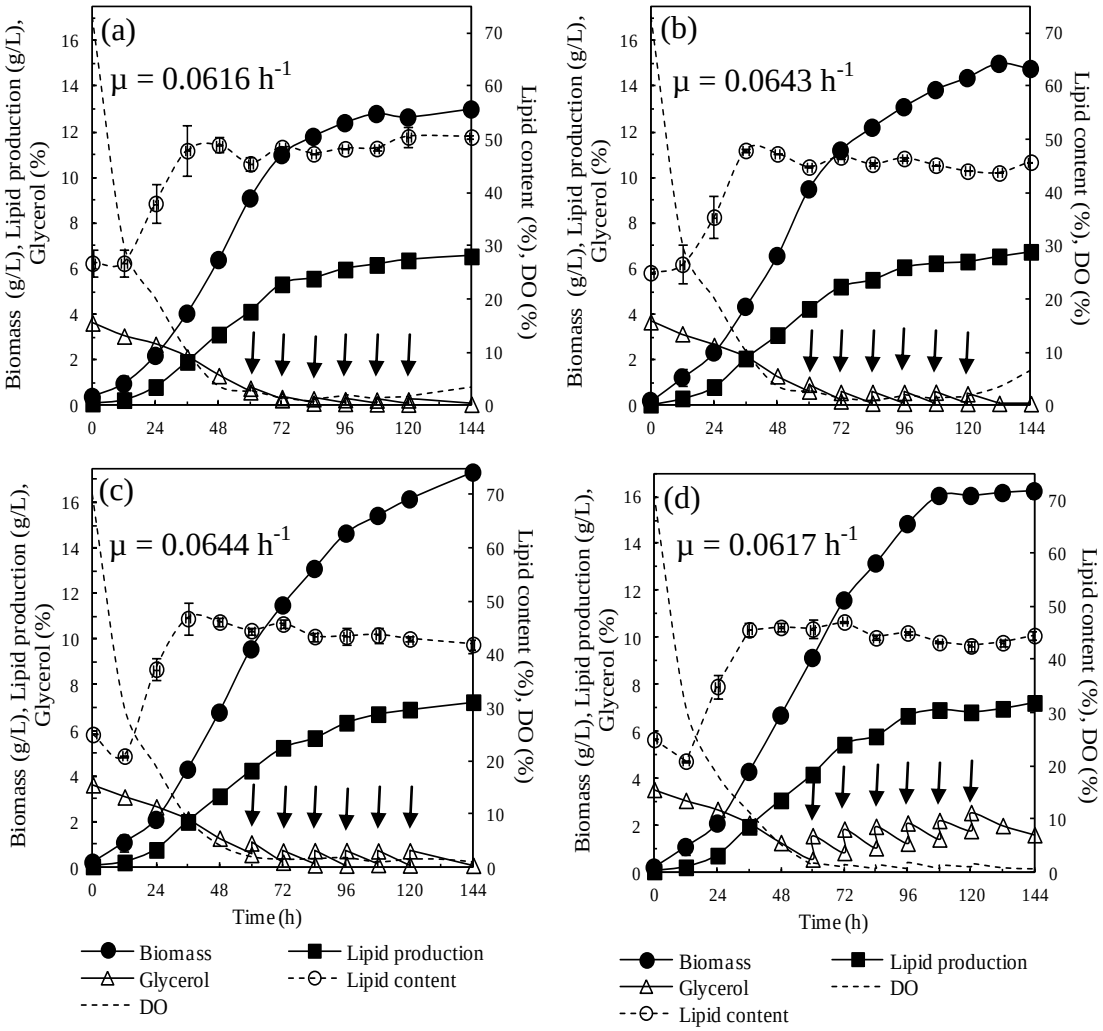

Figure 31. Fed-batch fermentation of *T. spathulata* JU4-57 in a 5-L bioreactor. Glycerol concentration in feeding solution was 4% (a), 8% (b), 12% (c) and 16% (d). The ammonium sulfate was added in the feeding solution at C/N ratio of 17.2. The feeding solution was fed at 60, 72, 84, 96, 108 and 120 h.

The lipid content slightly decreased from 45% to 41.9% when higher concentration of crude glycerol was fed. This suggested that the fed with higher concentration of nutrient led to higher biomass production but the yeast accumulated lower amount of lipid. There was no significant difference in DO between four fed-batch fermentations. The low level of DO indicated the high activity of the yeast and the sufficient supply of nutrient. The results presented here have demonstrated that fed-batch culture was useful for enhancing biomass and lipid production of *T. spathulata* JU4-57. In the study of Saenge *et al*. (2011), the feeding of the substrate increased biomass, lipid content, and lipid production of *Rhodotorula glutinis* up to 10.05 g/L, 60.70%, and 6.10 g/L, respectively. The glycerol consumption was also improved in the fed-batch fermentation. Zhu *et al*. (2008) also found that the fed-batch culture of *Mortierella alpine* using glucose as a carbon source enhanced biomass and lipid yield up to 1.65-fold and 1.51-fold, respectively, compared with those of the batch culture.

3.3.2 Feeding with only crude glycerol

It has been reported that the high C/N ratio, namely high concentration of carbon source with low concentration of nitrogen source, enhanced the lipid production. Some oleaginous microorganisms begin to accumulate lipid when a nutrient is exhausted in the medium whereas a surfeit of carbon was supplied (Lin *et al*., 2011). In this study, a two-stage fermentation process for microbial lipid production was investigated (Figure 32). In the fist stage, the oleaginous yeast *T. spathulata* JU4-57 cells were cultivated in crude glycerol based medium which contained both crude glycerol and ammonium sulfate as a carbon source and nitrogen source to support cell propagation. Then, after 60 h of fermentation the crude glycerol without the addition of ammonium sulfate was fed to start the second stage and enhance lipid accumulation. The crude glycerol concentrations in the feeding solution were the same as those using the section 3.3.1. It should be noted that some growth was observed even feeding with only crude glycerol. This could be due to the crude glycerol also contained some nutrient that could be used as a nitrogen source. However, as expected the feeding with only crude glycerol gave lower amount of biomass when compared with those feeding with both crude glycerol and ammonium

sulfate.

Interestingly, the lipid content increased substantially when only crude glycerol was fed. The maximal lipid content of 55.04% was obtained when feeding with 12% glycerol. The highest biomass and lipid production were 13.83 g/L and 7.78 g/L, respectively. Noticeably, each feeding with only crude glycerol with glycerol concentration of 4, 8, 12 and 16% led to the same residual glycerol concentration as those feeding with both crude glycerol and ammonium sulfate. Although the biomass with feeding only crude glycerol was lower than that feeding with crude glycerol and ammonium sulfate, the lipid production by this strategy was higher due to high lipid content. From the economic point of view, the high lipid content is much attractive because this could thus reduce the extraction cost of lipid.

The yeast cell count during the fed-batch fermentation feeding only crude glycerol increased less than 15% after the 72^{th} h when crude glycerol was fed while the glycerol concentration constantly decreased and the cellular lipid content continuously increased over time. This suggested that the lipid biosynthesis remained active throughout the culture even though the ammonium sulfate was not added during second stage. Lin *et al*. (2011) also studied the two-stage fermentation of *Lipomyces starkeyi* AS 2.1560 in glucose solution without auxiliary nutrients. In the first stage, cells were cultivated in a nutrient-rich medium for propagation. In the second stage, cells were resuspended in glucose solution to achieve high cellular lipid contents.

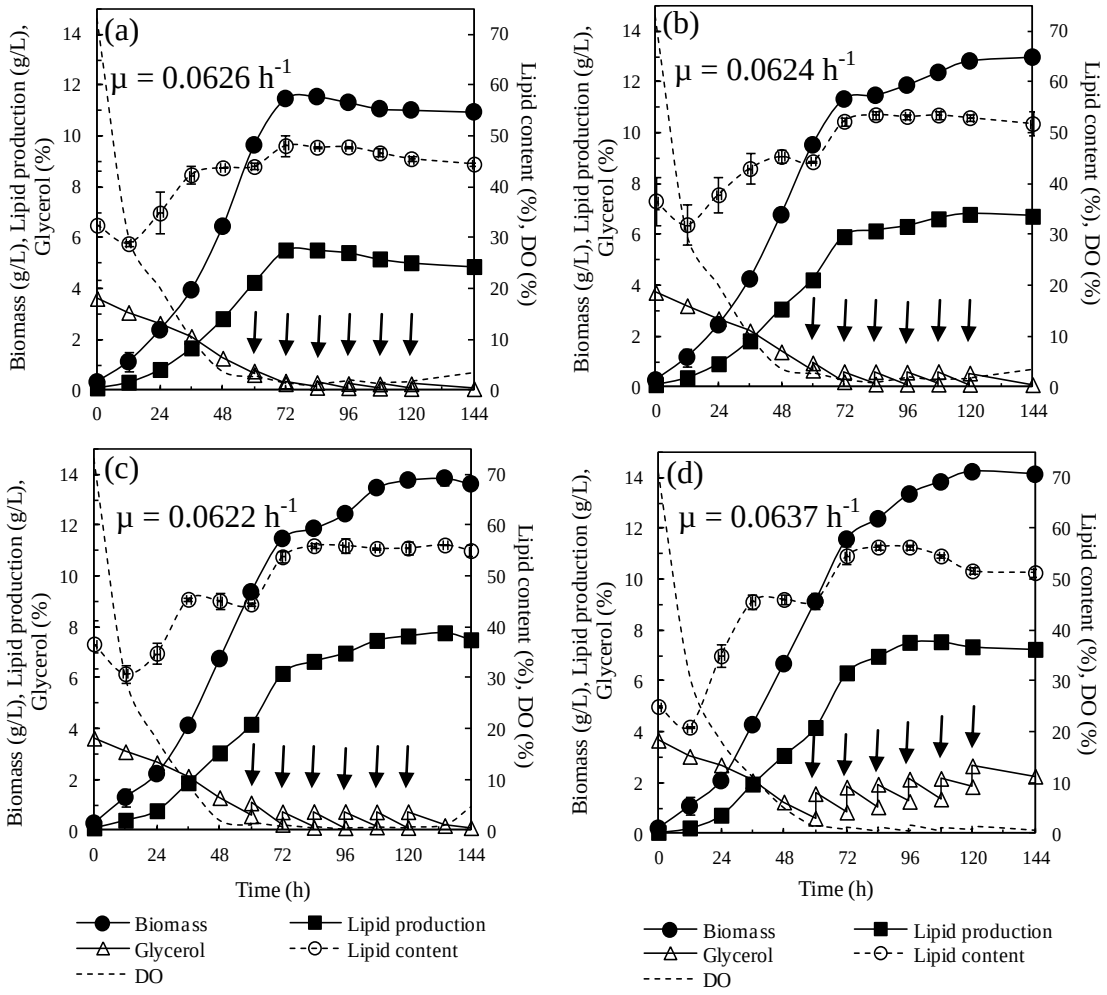

Figure 32. Two-stage fed-batch fermentation of *T. spathulata* JU4-57 in a 5-L bioreactor. The crude glycerol based medium with addition of ammonium sulfate was used in the first stage, feeding solution contained only crude glycerol was fed in the second stage. The glycerol concentration in feeding solution was 4% (a), 8% (b), 12% (c) and 16% (d). The feeding solution was fed at 60, 72, 84, 96, 108 and 120 h.

The biomass and lipid production of *T. spathulata* JU4-57 in this study was compared with those of several oleaginous microorganisms that were grown on glycerol, crude glycerol and industrial fats (Table 25). Apparently, the abilities of utilizing glycerol varied with microbial strains. The high lipid production of *T. spathulata* JU4-57 indicated its high potential to be used as a new source for biodiesel production. Interestingly, *Cryptococcus curvatus* gave the high cell density using glycerol as a carbon source because it consisted of several component containing Na_2HPO_4, KH_2PO_4, $MgSO_4 \cdot 7H_2O$, EDTA and NH_4OH (Meesters *et al*.,1996) as shown in Table 26, which led to increase the cost of lipid production.

Table 25. Lipid production on different carbon sources by various oleaginous microorganisms.

Strain	Carbon source	Culture mode	Biomass (g/L)	Lipid content (%)	Reference
T. spathulata	Crude glycerol	Batch	11.3 ± 0.18^{c}	44.3 ± 1.0^{b}	This study
T. spathulata	Crude glycerol	Fed-batch[1]	17.3 ± 0.07^{a}	41.9 ± 0.9^{c}	This study
T. spathulata	Crude glycerol	Fed-batch[2]	13.8 ± 0.07^{b}	55.0 ± 0.5^{a}	This study
C. curvatus	Glycerol	Fed-batch	118	25	Meesters *et al*.,1996
M. isabellina	Glycerol	Batch	7.8	25.6	Fakas *et al*.,2009
R. glutinis	Crude glycerol	Fed-batch	13.8	60.7	Saenge *et al*.,2011
Y. lipolytica	Glycerol	Batch	4.37	13.9	Makri *et al*.,2010
M. isabellina	Crude glycerol	Batch	8.6	51	Papanikolaou *et al*.,2008
Y. lipolytica	Industrial fats	Batch	8.7	44.0	Papanikolaou *et al*.,2001

[1]Feeding with crude glycerol and ammonium sulfate.

[2]Feeding with only crude glycerol in the second stage.

Different superscripts in the table indicate significant difference ($P<0.05$).

Table 26. Production costs per kg lipids.

Component	Amount (g/L)	Cost (Baht/kg)	Lipid (g/L)	Production cost (Baht/kg)	References
T. spathulata JU4-57			7.59	13.17	In this study
Crude glycerol	68.8	0			
$(NH_4)_2SO_4$	5	20			
C. curvatus			29.5	~2,425	Meesters *et al.*, 1996
Glycerol	120	37			
Yeast extract	7	3,958			
Na_2HPO_4	68.5	310			
KH_2PO_4	189	90			
$MgSO_4 \cdot 7H_2O$	14	20			
EDTA	7	125			

4. Fatty acid composition analysis

The fatty acid compositions of the lipid from *T. spathulata* JU4-57 when cultivated on glucose and crude glycerol are shown in Table 27. The lipid from *T. spathulata* JU4-57 when cultivated on glucose is composed mainly of long-chain fatty acid with 16 and 18 carbon atoms including oleic acid as the predominant fatty acid (40.2%) followed by palmitic acid (20.6%), linoleic acid (15.1%) and stearic acid (13.8%). Similarly, the predominant fatty acid in lipid from *T. spathulata* JU4-57 when cultivated on crude glycerol was oleic (41.1%) followed by palmitic acid (35.1%), linoleic acid (11.2%) and stearic acid (7.5%). The total unsaturated fatty acids amount was 54.4-56.1%, which is similar to that of plant oils. This indicates that lipid from oleaginous yeast *T. spathulata* JU4-57 has potential as a biodiesel feedstock. Interestingly, *T. spathulata* JU4-57 also accumulated heptadecoic acid (C17:0) up to 7% when cultivated on glucose. While when cultivated on crude glycerol, it accumulated heptadecoic acid only 0.3%. Devi and Mohan (2012) reported that haptadecanoic acid can be readily used in the diesel engines.

Table 27. Fatty acid compositions of lipid from *T. spathulata* JU4-57.

Fatty acids	Fatty acid relative content (%)	
	Glucose based medium	Glycerol based medium
Myristic acid (C14:0)	0.6	1.1
Pentacanoic acid (C15:0)	0.7	0.2
Palmitic acid (C16:0)	20.6	35.1
Palmitoleic acid (C16:1)	0.8	2.1
Heptadecoic acid (C17:0)	7.0	0.3
Stearic acid (C18:0)	13.8	7.5
Oleic acid (C18:1)	40.2	41.1
Linoleic acid (C18:2)	15.1	11.2
Arachidic acid (C20:0)	0.6	0.3
Linolenic acid (C24:0)	0.7	0.3

CHAPTER 5

CONCLUSIONS AND SUGGESTIONS

Conclusions

1. Twenty three isolates from soils and wastes of palm oil mill and biodiesel plant in southern region of Thailand were screened as oleaginous yeast. The isolate BY4-523 accumulated highest lipid content of 53.28% while JU4-57 grew fastest and gave comparable high lipid content of 41.50%. They were identified as *Kodamaea ohmeri* BY4-523 and *Trichosporonoides spathulata* JU4-57, respectively.

2. The newly isolates yeasts, *T. spathulata* JU4-57 and *K. ohmeri* BY4-523 could grow and accumulate lipid in the crude glycerol supplemented with ammonium sulfate only. The optimal medium composition for both strains was 0.5% ammonium sulfate and 10% crude glycerol (C/N ratio of 17.2).

3. Mixed culture of *T. spathulata* with *C. vulgaris* var. *vulgaris* TISTR 8261 gave the best biomass and the maximum lipid production. Under the optimal condition, the lipid production was higher than the sum of lipid from yeast and microalga. The mixed culture of an oleaginous yeast *R. glutinis* TISTR 5159 with *C. vulgaris* var. *vulgaris* TISTR 8261 also showed that the mixed culture gave higher biomass and lipid than did the pure culture.

4. The lipid production by *T. spathulata* using crude glycerol supplemented only ammonium sulfate was further enhanced by the process optimization in a 5 L bioreactor equipped with pH control and aeration system. The optimal conditions were pH control at 6.0 and aeration rate controlled at 1 vvm.

5. The biomass and lipid production was further enhanced up to 17.3 g/L and 7.25 g/L, respectively, with the lipid content of 45% by fed-batch fermentation feeding with crude glycerol and ammonium sulfate. Two-stage fed-batch fermentation feeding with only crude glycerol in the second stage led to lower biomass of 13.8 g/L but higher lipid content of 55% and consequently higher lipid production of 7.78 g/L.

6. The fatty acid composition of lipid was similar to that of plant oil. This indicates that the *T. spathulata* lipid is suitable to be used as feedstocks for biodiesel production.

Suggestions

1. Since *T. spathulata* is a new isolated oleaginous yeast strain and it can accumulate lipid in the form of triacylglycerols, the further scale up in pilot plant scale and continuous fermentation of this strain should be done.

2. The use of the industrial wastes which contain high nitrogen source such as rubber serum to replace ammonium sulfate should be attempted to reduce the production cost and make the lipid production by this strain more economical.

3. The biomass and lipid would be more improved if the oxygen could be supplied higher than the threshold level.

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APPENDIX

1. Chemical Oxygen Demand (COD) by Open Reflux Method (APHA, AWWA and WPCF, 1998)

Reagents

a. Standard potassium dichromate solution, 0.04167 M: Dissolve 12.259 g $K_2Cr_2O_7$, primary standard grade, previously dried at 150°C for 2 h, in distilled water and diluted to 1 L.

b. Sulfuric acid reagent: Added 5.5 g of Ag_2SO_4 to 1 kg of concentrated H_2SO_4. Let stand 2 days to dissolve.

c. Ferroin indicator solution: Dissolved 1.485 g of 1,10-phenanthroline monohydrate and 695 mg $FeSO_4 \cdot 7H_2O$ in distilled water and diluted to 100 mL.

d. Standard ferrous ammonium sulfate (FAS) titrant, approximately 0.25 M: Dissolved 98 g $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$ in distilled water. Added 20 mL concentrated H_2SO_4, cooled and diluted to 1 L. Standardize this solution daily against standard $K_2Cr_2O_7$ solution as follows:

Diluted 25 mL standard $K_2Cr_2O_7$ to 100 mL and added 30 mL concentrated H_2SO_4 and cooled. Titrated with FAS titrant using 0.1 to 0.15 mL (2 to 3 drops) ferroin indicator.

Molarity of FAS solution

$$= \frac{\text{Volume 0.04167 M } K_2Cr_2O_{27} \text{ solution titrated,mL}}{\text{Volume FAS used in titration,mL}} \times 0.25$$

e. Mercuric sulfate, $HgSO_4$.

f. Sulfamic acid: Required only if the interference of nitrites was to be eliminated.

g. Potassium hydrogen phathalate (KHP) standard, $HOOCC_6H_4COOK$: Lightly crushed and then dried KHP to constant weight at 110°C. Dissolved 425 mg in distilled water and diluted to 1 L. KHP had a theoretical COD[1] of 1.176 mg O_2/mg and this solution had a theoretical COD of 500 µg O_2/mg. This solution was stable when refrigerated, but not indefinitely.

Procedure

Samples (< 900 mg O_2/L COD > 50 mg O_2/L) 50 mL was added into a 500-mL refluxing flask. Added 1 g $HgSO_4$, several glass beads and very slowly added 5 mL sulfuric acid reagent, with mixing to dissolve $HgSO_4$. Cool while mixing to avoid possible loss of volatile material. Added 25 mL 0.04167 M $K_2Cr_2O_7$ solution and mixed. Attach flask to condenser and turned on cooling water. Added remaining sulfuric acid reagent (70 mL) through open end of condenser. Continued swirling and mixing while sulfuric acid reagent was added and refluxed for 2 h.

Cooled and washed down condenser with distilled water. Disconnected reflux condenser and diluted mixture to about twice its volume with distilled water. Cooled to room temperature and titrated excess $K_2Cr_2O_7$ with FAS using 0.10 to 0.15 mL ferroin indicator. Took as the end point of titration the first sharp color change from blue-green to reddish brown that persists for 1 min their average.

Caculation

$$\text{COD as mg } O_2/\text{L} = \frac{(A-B)\times M\times 8000}{\text{mL sample}}$$

where:

A = mL FAS used for blank,

B = mL FAS used for sample,

M = Molarity of FAS, and

8000 = milliequivalent weight of oxygen$\times$1000 mL/L.

2. Oil and grease by Partition-Gravimetric Method (APHA, AWWA and WPCF, 1998)

Reagents

a. Hydrochloric or sulfuric acid, 1:1 Mixed equal volumes of both acid and reagent water.

b. n-Hexane, boiling point 69°C. The solvent should leave no measurable residue on evaporation; distill if necessary.

c. Methyl-tert-butyl ether (MTBE), boiling point 55°C to 56°C. The solvent should leave no measurable residue on evaporation; distill if necessary.

d. Sodium sulfate, Na_2SO_4, anhydrous crystal.

e. Solvent mixture, 80% n-hexane/20% MTBE, v/v.

Procedure

Sample was acidified with either 1:1 HCl or 1:1 H_2SO_4 to pH 2. Carefully rinsed sample bottle with 30 mL extracting solvent and added solvent washings to separatory funnel. Shaked vigorously for 2 min. Let layers separate. Drain aqueous layer and small amount of organic layer into original sample container. Drain solvent layer through a funnel containing a filter paper and 10 g Na_2SO_4, both of which were solvent-rinsed, into a clean, tarred distilling flask and centrifuged for 5 min at 4,000 rpm. Recombine aqueous layers and any remaining emulsion. Extract twice more with 30 mL solvent each time. Distill solvent from flask in a water bath at 85°C for either solvent system. To maximize solvent recovery, fit distillation flask with a distillation adapter receiver. When visible solvent condensation stopped, removed flask from water bath and dried flasks on top of cover, with water bath still at 85°C for 15 min. Cooled in desiccators for at least 30 min and weighted. To determine initial sample volume, either fill sample bottle to mark with water and then poured water into a 1-L graduated cylinder, or weigh empty container and caped and calculated the sample volume by difference from the initial weight (assuming a sample density of 1.00).

Calculation

If the organic solvent was free of residue, the gain in weight of the tared distilling flask was due to oil and grease. Total gain in weight, A, of tared flask, less calculated residue from solvent blank, B, is the amount of oil and grease in the sample:

$$\text{mg oil and grease/L} = \frac{(\text{A} - \text{B}) \times 1000}{\text{mL sample}}$$

3. Total nitrogen cell test by Koroleff's Method (Merck, 2009)

Reagent

Package contents:

1 bottle of reagent N-1K

1 bottle of reagent N-2K

1 bottle of reagent N-3K

25 reaction cells and 1 cell with blank (white screw cap); required only when using the SQ 118

Photometer

1 sheet of round stickers for numbering the cells

Procedure

Sample 10 mL was pepetted into an empty cell and added 1 level blue microspoon of reagent N-1K and reagent N-2K. Heat the cell at 120°C in the preheated thermoreactor for 1 hour. Allow the closed cell to cool to room temperature in a test-tube rack. Reagent N-3K of 1 level yellow microspoon was placed into a reaction cell, immediately close the cell tightly and shaked vigorously for 1 min. The clear supernatant of 1.5 mL was very slowly and carefully allowed to run from the pipette down the inside of the tilted reaction cell onto the reagent. Immediately closed the cell tightly and mixed briefly. The cell must be held only by the screw cap and left the hot cell to stand for 10 min (reaction time). Measure the sample in the photometer.

4. C/N molar ratio

$$\mathrm{C/N\ molar\ ratio} = \frac{\text{moles C}}{\text{moles N}}$$

Glycerol, yeast extract and peptone were contained at 40 g/L, 10 g/L and 10 g/L, respectively. Peptone contains 14% N (w/w) and 8% C (w/w), and yeast extract includes 7% N (w/w) and 12% C (w/w).

Calculation;

$$\text{Total carbon of glycerol} = \frac{3\text{ C atoms} \times 12\text{ g} \times 40\text{ g/L}}{92.09\text{ g}} = 15.6\text{ g/L}$$

$$\text{Carbon of peptone} = 10\text{ g/L}_{\text{peptone}} \times \frac{8}{100}(\text{w/w})_{\text{N}} = 0.8\text{ g/L}$$

$$\text{Carbon of yeast extract} = 10\text{ g/L}_{\text{yeast ext}} \times \frac{12}{100}(\text{w/w})_{\text{N}} = 1.2\text{ g/L}$$

$$\text{Total carbon} = 2.0\text{ g/L}$$

$$\text{Total nitrogen of peptone} = 10\text{ g/L}_{\text{peptone}} \times \frac{14}{100}(\text{w/w})_{\text{N}} = 1.4\text{ g/L}$$

$$\text{Total nitrogen of yeast extract} = 10\text{ g/L}_{\text{yeast ext}} \times \frac{7}{100}(\text{w/w})_{\text{N}} = 0.7\text{ g/L}$$

$$\text{Total nitrogen} = 2.1\text{ g/L}$$

$$\text{C/N molar ratio} = \frac{15.6\text{ g/L} + 2.0\text{ g/L of C}}{2.1\text{ g/L of N}} \times \frac{14\text{ g}_{\text{N}}}{12\text{ g}_{\text{C}}} = 9.8$$

Glycerol concentration was fixed 40 g/L and ammonium sulfate was contained at 5 and 10 g/L.

Calculation;

Glycerol = $C_3H_8O_3$, C = 12 g

Ammonium sulfate = $(NH_4)_2SO_4$, N = 14 g

Calculate; ammonium sulfate 5 g/L

$$\text{C/N molar ratio} = \frac{\frac{3\text{ C atoms} \times 40\text{ g}}{92.09\text{ g}}}{\frac{2\text{ N atoms} \times 5\text{ g}}{132.1\text{ g}}} = 17.2$$

Calculation; ammonium sulfate 10 g/L

$$\text{C/N molar ratio} = \frac{\frac{3\ \text{C atoms} \times 40\ \text{g}}{92.09\ \text{g}}}{\frac{2\ \text{N atoms} \times 10\ \text{g}}{132.1\ \text{g}}} = 8.6$$

5. Standard curve of glycerol

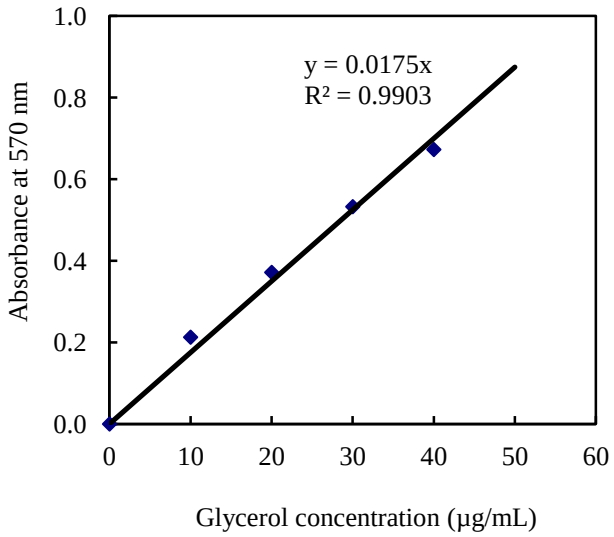
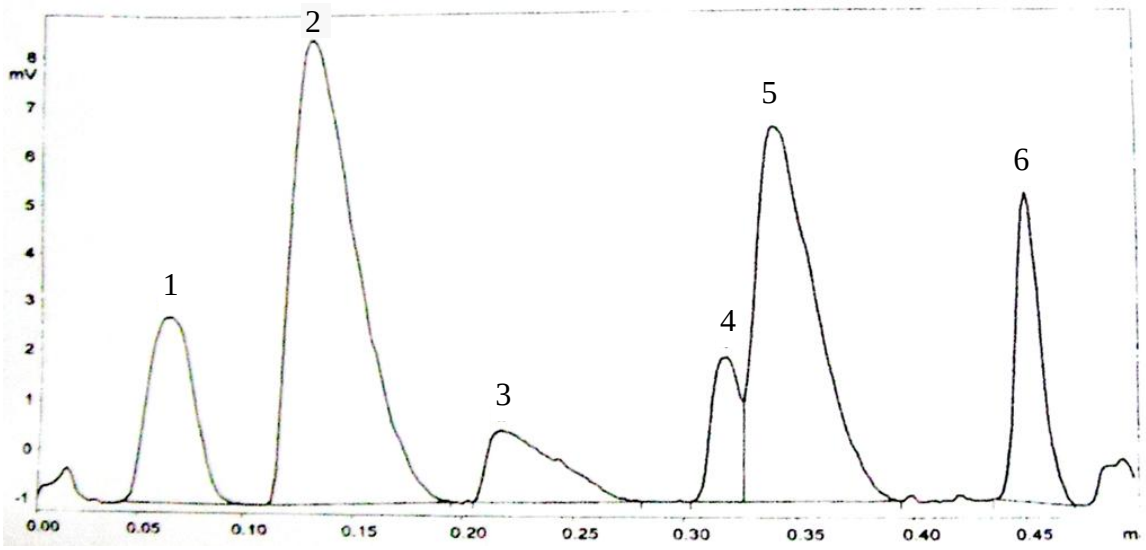


Figure 33. Standard curve of glycerol.

6. Standard TLC-FID chromatogram of oil composition



Peak No.	Name	Ret. Time (min)	Pk. Start (min)	Pk. End (min)	Area	Height (mV)	Area (%)
1	Fatty acid ethyl ester	0.062	0.042	0.100	2952	3.68	11.67
2	Triglyceride	0.125	0.112	0.195	10147	9.38	40.12
3	Free fatty acid	0.218	0.205	0.282	1615	1.40	6.38
4	1,3 Diglyceride	0.328	0.303	0.327	1284	2.80	5.08
5	1,2 Diglyceride	0.338	0.327	0.395	6877	7.43	27.19
6	Monoglyceride	0.450	0.435	0.472	2418	6.11	9.56
Total					25294	30.79	100.00

Condition:

Stationary phase : Chromarod-SIII

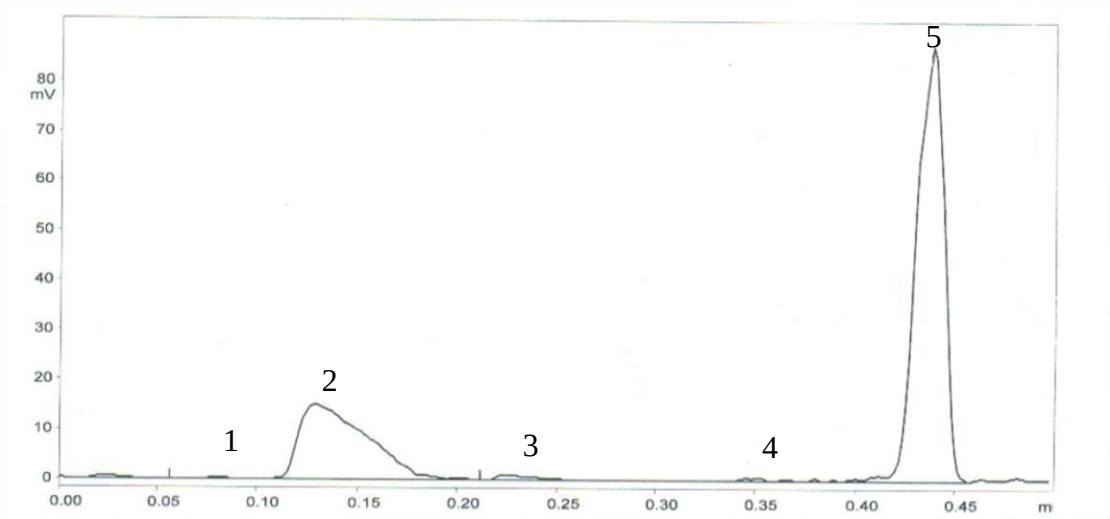
Mobile phase : H_2 160 mL/min, Air 2.0 L/min

Gas flow : H_2 160 mL/min, air flow rate 2.0 L/min

Scanning speed : 30 s/scan

Figure 34. TLC-FID chromatogram of standard oil.

7. TLC-FID chromatogram of extracted lipid from *T. spathulata* JU4-57



Peak No.	Name	Ret. Time (min)	Pk. Start (min)	Pk. End (min)	Area	Height (mV)	Area (%)
1	Fatty acid ethyl ester	0.080	0.057	0.097	201	0.38	0.314
2	Triglyceride	0.130	0.097	0.212	18761	15.06	29.263
3	Free fatty acid	0.227	0.212	0.267	793	1.15	1.237
4	1,2 Diglyceride	0.347	0.267	0.373	291	0.58	0.454
5	Monoglyceride	0.437	0.373	0.458	44067	82.64	68.733
Total					64113	99.359	100.00

Condition:

Stationary phase : Chromarod-SIII

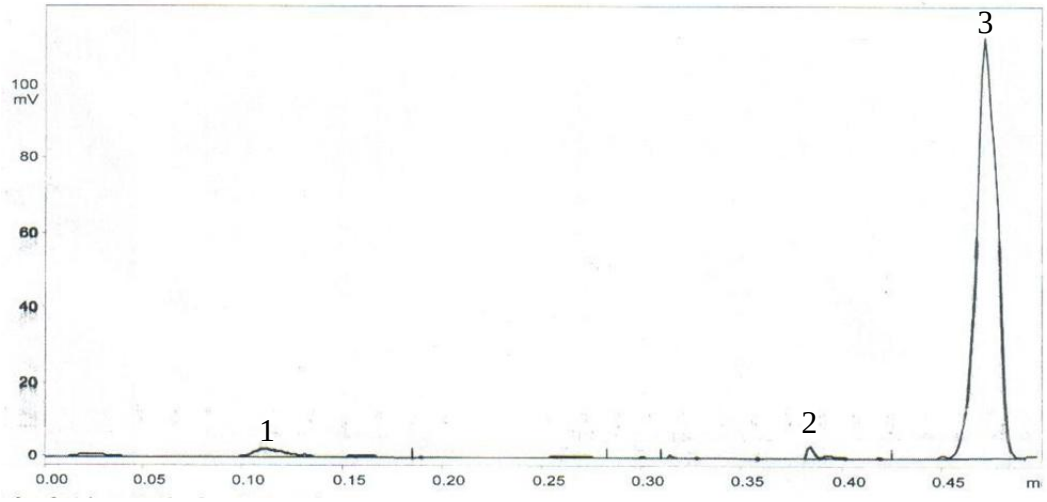
Mobile phase : H_2 160 mL/min, Air 2.0 L/min

Gas flow : H_2 160 mL/min, air flow rate 2.0 L/min

Scanning speed : 30 s/scan

Figure 35. TLC-FID chromatogram of extracted lipid from *T. spathulata* JU4-57.

8. TLC-FID chromatogram of extracted lipid from *K. ohmari* BU4-523



Peak No.	Name	Ret. Time (min)	Pk. Start (min)	Pk. End (min)	Area	Height (mV)	Area (%)
1	Triglyceride	0.112	0.043	0.142	1785	2.50	3.934
2	1,2 Diglyceride	0.393	0.347	0.425	714	0.94	1.575
3	Monoglyceride	0.472	0.425	0.492	42863	112.87	94.491
Total					45362	116.31	100.00

Condition:

Stationary phase : Chromarod-SIII

Mobile phase : H_2 160 mL/min, Air 2.0 L/min

Gas flow : H_2 160 mL/min, air flow rate 2.0 L/min

Scanning speed : 30 s/scan

Figure 36. TLC-FID chromatogram of extracted lipid from *K. ohmari* BU4-523.

9. Determination of fatty acid composition by GC analysis

Table 28. Retention time of standard fatty acid methyl esters.

Fatty acid	Retention time (min)
Caprylic acid (C8:0)	2.406
Capric acid (C10:0)	2.542
Lauric acid (C12:0)	2.800
Myristic acid (C14:0)	3.283
Palmitic acid (C16:0)	4.194
Palmitoleic acid (C16:1)	4.380
Heptadecoic acid (C17:0)	4.920
Stearic acid (C18:0)	5.886
Oleic acid (C18:1)	6.147
Linoleic acid (C18:2)	6.731
Linolenic acid (C18:3)	7.700
Arachidic acid (C20:0)	9.022
Eicosenoic acid (C20:1)	9.444
Behenic acid (C22:0)	13.626
Erucic acid (C22:1)	13.894
Lignoceric acid (C24:0)	16.513
Nervonic acid (C24:1)	16.841

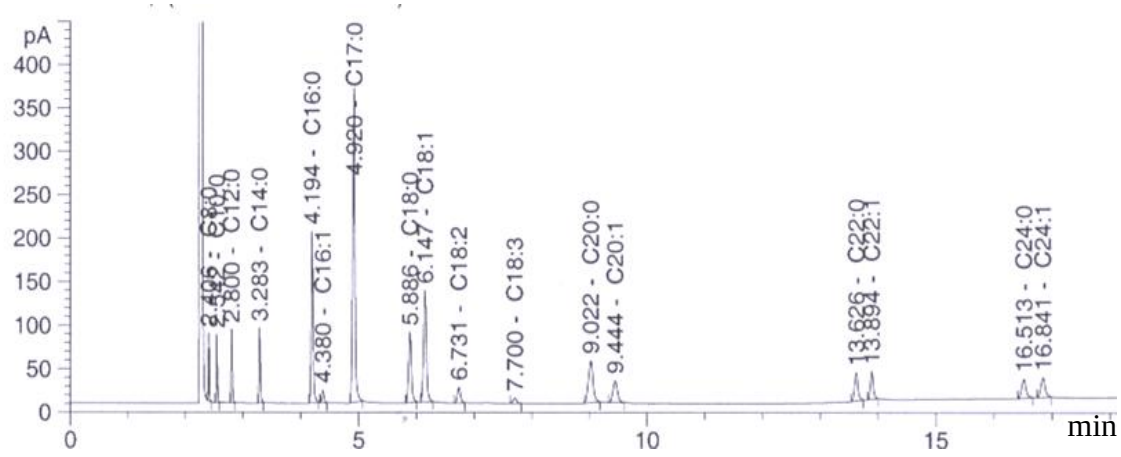


Figure 37. Fatty acid methyl ester chromatogram of GC analysis of standard quantitive.

10. Nucleotide sequence of 26S rDNA gene of *Trichosporonoides spathulata* JU4-57

Nucleotide sequence of 26S rDNA gene of *Trichosporonoides spathulata*

Sample name : JU4-57

Identify : *Trichosporonoides spathulata*

26S rDNA Sequence (502 bp)

GGGATGAGCCCAGATTTGAAAGCTCCCGCCAGGGCGCATTGTAAGCTGGA
GACGTGCCTCGAGCGGCGCGGCTGGACGCAAGTCTGCTGGAAAGCAGCAT
CAAAGAGGGTGAGAATCCCGTGCTTGGTCTGGCTGTGCGCCGTGTTGTGG
GGTGCGCTCGACNAGTCGCGTTGTTTGGGAATGCAGCGCAAAGAGGGGTG
GTAAACGCCATCCAAGGCTAAATACCGGGGAGAGACCGATAGCGAACAA
GTACCGTGAGGGAAAGATGAAAAGCACTTTGGAAAGAGAGTTAAAGAGT
ACGTGAAATTGCCAAGAGGGAAGCGCTGGCAGTCAGTGCCGTANCGCTGC
TGGTCCCGCCTTTTTTTGGGAGGTTGATGCCGGCAGTGTGGGGCCCGCGTC
NGTTGCTGTTTTGGTGGGNGAGAAGGCAGAGCGCCAANGTGNCTTCCCTT
TTTTTGGGGGGAGTGTTATAGCCGCTTTGTGGATGCCCTGCTAGCGACCNA
TGACCGCTTTTTTCCANANAAGANGNNGGCTTAATGGCTTTCNCGGCCCN
TCTGAAAAANACGGACCAA

11. Nucleotide sequence of 26S rDNA gene of *Kodamaea ohmeri* BU4-523

Nucleotide sequence of 26S rDNA gene of *Kodamaea ohmeri*

Sample name : BY4-523

Identify : *Kodamaea ohmeri*

26S rDNA Sequence (536 bp)

GANGCGGCAAAAGCTCANATTTGAAATCCCCCCGGGGAGTTGTAATTTGA
AGATTGCGTCTTGGAGGCGACCGTGTCTATGTTCCTTGGAACAGGACGTC
ACAGAGGGTGAGAATCCCGTGCGGCACGGCCCCCGGCTCCTTATAAGGCG
CTCTCGACNAGTCGAGTTGTTTGGGAATGCAGCTCAAAGTGGGTGGTAAA
TTCCATCTAAAGCTAAATACAGGCGAGAGACCGATAGCGAACAAGTACA
GTGATGGAAAGATGAAAAGCACTTTGAAAAGAGAGTGAAACAGCNCGTG
AAATTGTTGAAAGGGAAGGGCATGCCGTCAGATTGTCANTGTGGGTAAGA
AGCGGGGTACAAAGACTGTGGAACGTGGCCCTCGGGTGTTATAGCCGCAG
TTCATGCCCCGTCTCTTTCCGAGGCCTGCTTTGAGGACACCGACGTAATGA
CGGTACGCCGCCCGTCTGAAAAAACACGGACCA

VITAE

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M. Sc. Biotechnology	Prince of Songkla University	2007

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List of Publication and Proceedings

Publication

Cheirsilp, B., **Kitcha, S.** and Torpee, S. 2011. Co-culture of an oleaginous yeast *Rhodotorula glutinis* and a microalga *Chlorella vulgaris* for biomass and lipid production using pure and crude glycerol as a sole carbon source. Ann. Microbiol. 1-7.

Kitcha, S. and Cheirsilp, B. 2012. A newly isolated oleaginous yeast *Trichoaporonoides spathulata* as a new source for biodiesel production (Submitted).

Kitcha, S. and Cheirsilp, B. 2012. Mixed cultures of oleaginous yeast *Trichosporonoides spathulata* and microalgae *Chlorella* sp. for lipid production from crude glycerol (Submitted).

Proceedings

Kitcha, S., Cheirsilp, B. 2011. Screening of oleaginous yeasts and optimization for lipid production using crude glycerol as a carbon source. Energy Procedia 9: 274–282.

Kitcha, S. and Cheirsilp, B. 2011. Screening of Oleaginous Yeasts and Optimization for Lipid Production Using Crude Glycerol as a Carbon Source. 9th Eco-Energy and Materials Science and Engineering Symposium (EMSES), Wiang Inn Hotel, Chiang Rai, Thailand. 25-28 May 2011 (Oral Presentation).

Kitcha, S. and Cheirsilp, B. 2011. A Newly Isolated Oleaginous Yeast *Trichoaporonoides spathulata* as a New Source for Biodiesel Production. 11th International Conference on Clean Energy (ICCE-2011), Taichung, Taiwan, 2-5 November, 2011 (Poster Presentation).