Potential Use of Oleaginous *Rhodotorula glutinis* for Bioconversion of Palm Oil Mill Effluent and Crude Glycerol to Oil Feedstock for Biodiesel Production

Chanika Saenge

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Author Miss Chanika Saenge

Major Program Environmental Management

Major Advisor :

.....Benjamas Cheirsilp.....

(Asst. Prof. Dr. Benjamas Cheirsilp)

Co-advisor :

.....Thunwadee Suksaroj.....

(Dr. Thunwadee Suksaroj)

Examining Committee :

.....Chairperson

(Asst. Prof. Dr. Chonlatee Cheewasedtham)

.....Benjamas Cheirsilp.....

(Asst. Prof. Dr. Benjamas Cheirsilp)

.....Duangporn Kantachote.....

(Assoc. Prof. Dr. Duangporn Kantachote)

.....

(Asst. Prof. Dr. Sumate Chaiprapat)

.....

(Assoc. Prof. Dr. Galaya Srisuwan)

The Graduate School, Prince of Songkla University, has approved this thesis as partial fulfillment of the requirements for the Doctor of Philosophy Degree in Environmental Management

.....

(Assoc. Prof. Dr. Krerkchai Thongnoo)

Dean of Graduate School

ชื่อวิทยานิพนธ์ ศักยภาพการใช้ยีสต์ไขมันสูง *Rhodotorula glutinis* ในกระบวนการแปรรูปน้ำทิ้งโรงงานสกัดน้ำมันปาล์มและกลีเซอรอลดิบให้เป็นวัตถุดิบน้ำมันเพื่อใช้ในการผลิตไบโอดีเซล

ผู้เขียน นางสาวฌานิกา แซ่แง่

สาขา การจัดการสิ่งแวดล้อม

ปีการศึกษา 2552

บทคัดย่อ

งานวิจัยนี้ศึกษาศักยภาพของยีสต์ไขมันสูง *Rhodotorula glutinis* เพื่อใช้ในการแปรรูปของเสียทางชีวภาพ ได้แก่ น้ำทิ้งจากโรงงานสกัดน้ำมันปาล์มและกลีเซอรอลดิบ เพื่อผลิตน้ำมันในรูปของลิปิดและแคโรทีนอยด์ที่มีมูลค่า โดย *Rhodotorula glutinis* TISTR 5159 เป็นยีสต์ที่สามารถสะสมได้ทั้งลิปิดและแคโรทีนอยด์ จากการศึกษาการเลี้ยงเชื้อยีสต์ในน้ำทิ้งจากโรงงานสกัดน้ำมันปาล์มและกลีเซอรอลดิบจากกระบวนการผลิตไบโอดีเซล พบว่าการเติม แอมโมเนียมซัลเฟตเพื่อเป็นแหล่งไนโตรเจน และ Tween 20 เพื่อเป็นตัวกระตุ้นทำให้เชื้อมีการเติบโตเพิ่มขึ้นและสามารถผลิตลิปิดและแคโรทีนอยด์ได้มากขึ้น และยังช่วยส่งเสริมการลดซีโอดีในน้ำทิ้งจากโรงงานสกัดน้ำมันปาล์มและการใช้กลีเซอรอล จากการศึกษาหาสภาวะที่เหมาะสมด้วยวิธีพื้นผิวตอบสนอง Response Surface Methodology (RSM) เมื่อเลี้ยงเชื้อในน้ำทิ้งจากโรงงานสกัดน้ำมันปาล์มโดยกำหนดตัวแปรที่ศึกษา คือ ซีโอดี อัตราส่วนคาร์บอนต่อไนโตรเจน และความเข้มข้นของ Tween 20 และกำหนดค่าการตอบสนอง คือ ปริมาณเซลล์ ปริมาณลิปิด และ ปริมาณแคโรทีนอยด์ พบว่าอัตราส่วนคาร์บอนต่อไนโตรเจนเป็นปัจจัยที่มีผลต่อการผลิตเซลล์ ลิปิด และแคโรทีนอยด์อย่างมีนัยสำคัญ และพบว่าที่อัตราส่วนคาร์บอนต่อไนโตรเจนเท่ากับ 140 เป็นสภาวะที่เหมาะสมในการผลิตเซลล์ ในขณะที่อัตราส่วนคาร์บอนต่อไนโตรเจนเท่ากับ 180 และ 170 เป็นสภาวะที่เหมาะสมสำหรับการผลิตลิปิดและแคโรทีนอยด์ ตามลำดับ แสดงว่าอัตราส่วนคาร์บอนต่อไนโตรเจนต่ำหรือปริมาณไนโตรเจนที่สูงนั้นสามารถเพิ่มการผลิตเซลล์แต่ให้การผลิตลิปิดและแคโรทีนอยด์น้อย ดังนั้นจึงต้องศึกษาการเลี้ยงเชื้อแบบสองขั้นตอนโดยในขั้นตอนแรกสำหรับการเติบโตและขั้นที่สองสำหรับการผลิตผลิตภัณฑ์ จากผลการทดลองพบว่าการเลี้ยงแบบสองขั้นตอนสามารถผลิตลิปิดและแคโรทีนอยด์ได้เพิ่มขึ้นจากการเลี้ยงแบบขั้นตอนเดียวร้อยละ 50 และร้อยละ 20 ตามลำดับ เมื่อเลี้ยงในถังหมักที่มีการควบคุมพีเอชที่ 6.0 และให้อากาศที่ 2 ปริมาตรอากาศต่อปริมาตรอาหารต่อนาที ทำให้การผลิตลิปิดและแคโรทีนอยด์เพิ่มขึ้น และจากการเลี้ยงแบบกึ่ง

ต่อเนื่อง พบว่าเชื้อสามารถสะสมลิปิดและแคโรทีนอยด์ในปริมาณสูงได้เป็นระยะเวลานาน นอกจากนี้ยังสามารถลดค่าซีโอดีในน้ำทิ้งได้ดีอีกด้วย

ในการหาสภาวะที่เหมาะสมด้วยวิธีพื้นผิวตอบสนอง สำหรับการเลี้ยงเชื้อ *R. glutinis* TISTR 5159 โดยใช้กลีเซอรอลดิบเป็นสับสเตรท ผลการทดลองพบว่าอัตราส่วนคาร์บอนต่อไนโตรเจน เป็นปัจจัยที่มีผลต่อการเพิ่มปริมาณเซลล์ ลิปิด และแคโรทีนอยด์ อย่างมีนัยสำคัญ และปัจจัยร่วมที่มีผลต่อการผลิตลิปิดคือ อัตราส่วนคาร์บอนต่อไนโตรเจนร่วมกับความเข้มข้นของกลีเซอรอล และอัตราส่วนคาร์บอนต่อไนโตรเจนร่วมกับความเข้มข้นของ Tween 20 ซึ่งเมื่อวิเคราะห์จากพื้นผิวการตอบสนองโดยกำหนดให้ความเข้มข้นของ Tween 20 คงที่ที่ 1.5 กรัมต่อลิตร พบว่าสภาวะที่เหมาะสมสำหรับการเพิ่มปริมาณเซลล์คือ ความเข้มข้นของกลีเซอรอลร้อยละ 8.5 และอัตราส่วนคาร์บอนต่อไนโตรเจนเท่ากับ 60 ในขณะที่สภาวะที่เหมาะสมในการผลิตลิปิดและแคโรทีนอยด์คือ ความเข้มข้นของกลีเซอรอลร้อยละ 9.5 และอัตราส่วนคาร์บอนต่อไนโตรเจนเท่ากับ 85 เมื่อเลี้ยงในถังหมักที่มีการควบคุมพีเอชที่ 6.0 และให้อากาศที่ 2 ปริมาตรอากาศต่อปริมาตรอาหารต่อนาทีทำให้ปริมาณลิปิดและแคโรทีนอยด์เพิ่มขึ้น และจากการเลี้ยงแบบกึ่งกะที่ควบคุมให้ความเข้มข้นของกลีเซอรอลคงที่พบว่าสามารถผลิตลิปิดได้ 6.05 กรัมต่อลิตร และผลิตแคโรทีนอยด์ได้ 135.25 มิลลิกรัมต่อลิตร

ในการผลิตไบโอดีเซลจากลิปิดจากยีสต์ทำการผลิตแบบสองขั้นตอน โดยทำปฏิกิริยาเอสเทอริฟิเคชั่นกับเมทานอลโดยใช้กรดเป็นตัวเร่งปฏิกิริยาแล้วตามด้วยปฏิกิริยาทรานเอสเทอริฟิเคชั่นกับเมทานอลโดยใช้ด่างเป็นตัวเร่งปฏิกิริยา พบว่าไบโอดีเซลที่ได้นั้นมีองค์ประกอบส่วนใหญ่เป็นกรดไขมันโอเลอิกและกรดไขมันปาล์มเมติก ซึ่งคล้ายกับไบโอดีเซลที่ผลิตจากน้ำมันพืชและมีสมบัติใกล้เคียงกับมาตรฐานไบโอดีเซล

การศึกษาการขยายขนาดเป็นการผลิต 10 ลิตร โดยใช้น้ำทิ้งจากโรงงานสกัดน้ำมันปาล์มเป็นสับสเตรท พบว่าสามารถผลิตลิปิดและแคโรทีนอยด์ได้สูงสุดที่ 7.64 กรัมต่อลิตร และ 189.32 มิลลิกรัมต่อลิตร ตามลำดับ ในขณะที่การใช้กลีเซอรอลดิบเป็นสับสเตรท พบว่าผลิตลิปิดและแคโรทีนอยด์ได้สูงสุดที่ 6.76 กรัมต่อลิตร และ 162.31 มิลลิกรัมต่อลิตร ตามลำดับ

เนื่องจากการเลี้ยง *R. glutinis* TISTR 5159 สามารถประยุกต์เลี้ยงในระบบบำบัดน้ำเสียได้ ทำให้สามารถลดต้นทุนในส่วนของถังหมักและค่าไฟฟ้าในการเลี้ยงเชื้อได้ จากการศึกษาความเป็นไปได้ทางเศรษฐศาสตร์ พบว่าต้นทุนสารเคมีสำหรับการผลิตลิปิดจากน้ำทิ้งโรงงานสกัดน้ำมันปาล์มคือ 0.036 บาทต่อกิโลกรัม และต้นทุนสารเคมีสำหรับการผลิตลิปิดจากกลีเซอรอลดิบคือ 8.5 บาทต่อกิโลกรัม เมื่อรวมค่าไฟฟ้าสำหรับการสกัดน้ำมัน (2.50 บาทต่อกิโลวัตต์ชั่วโมง)

0.006 บาทต่อกิโลกรัม ทำให้ต้นทุนรวมในการผลิตลิปิดจากน้ำทิ้งจากโรงงานสกัดน้ำมันปาล์มและกลีเซอรอลดิบคือ 0.042 บาทต่อกิโลกรัม และ 8.566 บาทต่อกิโลกรัม ตามลำดับ

Thesis Title	Potential Use of Oleaginous *Rhodotorula glutinis* for Bioconversion of Palm Oil Mill Effluent and Crude Glycerol to Oil Feedstock for Biodiesel Production
Author	Miss Chanika Saenge
Major Program	Environmental Management
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ABSTRACT

This study has shown the potential use of oleaginous red yeast *Rhodotorula glutinis* for bioconversion of wastes including palm oil mill effluent (POME) and crude glycerol to value-added oil in lipid form and carotenoids. An oleaginous red yeast *Rhodotorula glutinis* TISTR 5159 that accumulates both lipids and carotenoids was cultured in POME and crude glycerol. The addition of ammonium sulfate and Tween 20 improved biomass, lipids and carotenoids production as well as the removal of chemical oxygen demand (COD) in POME and glycerol consumption.

Response surface methodology (RSM) was applied to optimize the medium composition for bioconversion of POME including COD in POME, C/N ratio and Tween 20 concentration. Among three investigated factors, C/N ratio contributed a significant effect on lipids and carotenoids production. The analysis of response surface plots revealed that the optimum C/N ratio for biomass was 140, while that for lipid content and carotenoids were higher at 180 and 170, respectively. The high level of nitrogen source (with a low C/N ratio) enhanced the biomass, making the accumulation of lipids and carotenoids less preferable. Hence, the two-stage process was attempted as an optimal way for cell growth in the first stage and product accumulation in the second stage. The lipid yield and carotenoid production obtained in the two-stage process were increased 50% and 20%, respectively, from those obtained from the one-stage process. The production of lipid and carotenoids were further improved in a stirred tank bioreactor with pH controlled at 6.0 and aeration

rate at 2 vvm. In the semi-continuous fermentation, *R. glutinis* TISTR 5159 was found successfully accumulated a high lipid content and produced considerable high concentration of carotenoids during long-term cultivation. Moreover, efficient COD removal by *R. glutinis* TISTR 5159 was also observed.

Crude glycerol, a by–product of biodiesel plants, was used as the sole carbon source for *R. glutinis* TISTR 5159. Among the factors investigated using response surface methodology, the C/N ratio contributed a significant effect on biomass, lipid content and production of carotenoids. The synergic effects of the C/N ratio and glycerol concentration as well as the C/N ratio and Tween 20 concentration were observed in the accumulation of lipids. The analysis of the response surface plots revealed that when Tween 20 was fixed at maximum level of 1.5 g/L the optimum condition for biomass was glycerol concentration of 8.5% and C/N ratio of 60, while that for lipid content and carotenoids production was glycerol concentration of 9.5% and C/N ratio of 85. The production of lipids and carotenoids were further improved in a stirred tank bioreactor with pH controlled at 6.0 and aeration rate at 2 vvm. In fed–batch fermentation, the highest lipid production of 6.05 g/L with a cellular lipid content of 60.7 % and carotenoids production of 135.25 mg/L were obtained when the glycerol concentration was maintained at the optimum level throughout the fermentation process.

The production of biodiesel from yeast lipids was carried out in two steps. After hydrolysis reaction, esterification with methanol was carried out using acid catalysis. The biodiesel derived from yeast lipids was mainly composed of oleic and palmitic acids, which were similar to those from plants oil, and it also showed the favorable biodiesel properties.

The scale up of lipids production from POME and crude glycerol to a 10 L production scale was performed. The maximum lipids yield and carotenoids concentration obtained from POME were 7.64 g/L and 189.32 mg/L, respectively. While the use of crude glycerol provided lower lipids yield and carotenoids concentration of 6.76 g/L and 162.31 mg/L, respectively.

Since the cultivation of *R. glutinis* TISTR 5159 could be applied in conventional wastewater treatment, the costs for bioreactor and electricity for cultivation could be reduced. The study of economic feasibility found that the costs of

chemical for lipids production were 0.036 baht/kg from POME and 8.5 baht/kg from crude glycerol. The costs of electricity for oil recovery (based on 2.50 baht/kw.h) were 0.006 baht/kg. Hence, the sum of costs for lipids production by *R. glutinis* TISTR 5159 cultivated in POME and crude glycerol were 0.042 baht/kg and 8.566 baht/kg, respectively.

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CHAPTER 1

INTRODUCTION

Biodiesel is well known as an alternative, biodegradable, nontoxic, and clean renewable fuel with properties similar to conventional diesel. It is produced from renewable resources, and has low emission profiles. So it is environmentally beneficial. The raw materials for biodiesel production now mainly include biological sources such as vegetable seed oil, soybean oil and some recovered animal fats (Ana and Enoch, 2003; Han *et al.*, 2005). However the cost of biodiesel is high due to the high cost of raw material (about 70–75% of the total cost). So, biodiesel is still not commonly used in daily life mostly due to the high production cost involved, though this fuel has been developed for about three decades (Ma and Hanna, 1999; Leunc, 2001). A cheaper raw material for biodiesel production could be a solution. Microbial oils, namely single cell oils, produced by oleaginous microorganisms including bacteria, yeasts, moulds and algae, are now interested as a promising potential feedstock for biodiesel production due to their similar fatty acid compositions to that of vegetable oil (Li *et al.*, 2007). Compared with the production of vegetable oils, the culture of oleaginous microorganisms is more advantage because it is affected neither by seasons nor by climates.

Among oleaginous microorganisms, yeast has an advantage over algae, fungi and bacteria due to its unicellular relatively high growth rate and rapid lipids accumulating ability with utilizing low cost fermentation media such as nutritional residues from agriculture and industry. *Rhodotorula glutinis* is one of most interesting oleaginous yeasts because it can accumulate both lipids and carotenoids (Somashekar and Joseph, 2000). In addition to lipid, carotenoids are gaining importance as natural food colorants in view of possible safety hazards of chemical colorants. Therefore, there is a great potential for using *Rhodotorula* carotenoids in foods and feeds.

Palm oil process can be categorized into a dry and a wet milling standard processes. The wet process of palm oil milling is the most common and typical way of extracting palm oil, especially in Thailand. It is estimated that for each

ton of crude palm oil that is produced, 5–7.5 ton of water are required, and more than 50% of this water ends up as palm oil mill effluent (POME) (Ahmad *et al.*, 2003). The high chemical oxygen demand (COD) of POME makes its disposal a pollution problem. Therefore, use of POME as an inexpensive medium for fermentation process has long been of industrial interest. COD in POME is a suitable carbon source for many microorganisms. In addition, POME is rich in minerals and contains vitamins which may provide valuable nutrients to stimulate cell growth and product formation. The possibility of reusing POME as fermentation media is largely due to the fact that POME contains high concentrations of carbohydrate, protein, nitrogenous compounds, lipids and minerals (Habib *et al.*, 1997).

Glycerol is the main by-product of the conversion of oils into biodiesel, comprising approximately 10% by mass of the oils fed to the system (Dasari *et al.*, 2005). The increased production of biodiesel has caused a sudden increase in production of glycerol creating a glut in the glycerol market (Johnson and Taconi, 2007). It is believed that refining crude glycerol to high purity is too costly and energy-intensive. Therefore, it is urgent to discover innovative utilizations for crude glycerol that will make biodiesel production more profitable and sustainable. Researchers around the world are currently looking at the thermal, chemical, and biological conversion of crude glycerol to a variety of value-added products. One application that has been evaluated is the potential of using crude glycerol from biodiesel in animal feeds (Cerrate *et al.*, 2006). Another application for crude glycerol that is currently being investigated is the fermentation of glycerol to 1,3-propanediol, an intermediate compound for the synthesis of polymers used in cosmetics, foods, lubricants, and medicines (Mu *et al.*, 2006). Less expensive carbon sources, such as glucose, have limited the use of glycerol in fermentations. Now that glycerol has decreased in price, as a result of a dramatic increase in biodiesel production, its use as a carbon source needs to be re-evaluated. If feasible, large-scale cultivation in industry will create a substantial demand for glycerol. The alternative ways of glycerol valorization leading to its biotransformation to either single cell oil or citric acid with the aid of the non-conventional polymorphic yeast *Yarrowia lipolytica* have been developed (Rymowicz *et al.*, 2006).

Statistical experimental design techniques especially response surface methodology (RSM), are very useful tools for the optimizing the process parameters as they can provide statistical models which help understand the interactions among the parameters at varying levels and calculation of the optimal level of each parameter for a given target (Guo *et al.*, 2008). The application of RSM in medium optimization can improve growth and the production and also reduce process variability, development time, and overall costs. However, the use of palm oil mill effluent and crude glycerol for concomitant production of lipids and carotenoids have not been studied.

In this study, lipids and carotenoids production by cultivating *R. glutinis* TISTR 5159 in POME and crude glycerol were attempted. The suitable nitrogen source and surfactant were first screened. The effects of substrate concentration, C/N ratio and surfactant concentration on both lipids and carotenoids production were then simultaneously investigated using RSM. The process parameters including pH control and aeration rate were also optimized. The long-term cultivation of *R. glutinis* TISTR 5159 using POME and crude glycerol were performed in semicontinuous and fed-batch fermentation, respectively. Finally, the scale-up to 10 L production in 15 L bioreactor was attempted and the economic feasibility was studied. In addition, the biodiesel properties of lipids obtained was also evaluated.

Objectives of the study

1. To produce lipids and carotenoids using *Rhodotorula glutinis* TISTR 5159 cultured in palm oil mill effluent and crude glycerol.
2. To optimize growth, lipids and carotenoids production of *R. glutinis* TISTR 5159 using response surface methodology.
3. To perform lipids and carotenoids production in semi-continuous fermentation using POME and in fed-batch using crude glycerol.
4. To scale-up lipids and carotenoids production in a 15 L bioreactor.
5. To convert lipids to biodiesel and evaluate its biodiesel properties.

CHAPTER 2

LITERATURE REVIEWS

2.1 Microorganisms available for biodiesel production

To be a viable substitute for a fossil fuel, an alternative fuel should not only have superior environmental benefits over the fossil fuel it displaces, be economically competitive with it, 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. Oleaginous microorganisms are defined as microbial with the content of microbial lipids excess of 20%. Biodiesel production using microbial lipids, which is named as single cell oils (SCO), has attracted great attention in the whole world. Although there are all kinds of microorganism storaging oils, such as microalgae, bacteria, fungi and yeast, not all of them are available for biodiesel production (Table 1). The first commercial production of an SCO did not begin until 1995 and this only lasted for 6 years before it was closed down as no longer being cost effective (Ratledge, 2004). In recent years, a large number of hydrophobic lipids accumulating microorganisms have been studied as substrates for single cell oil production, especially used in the production of biodiesel. In microorgamisms, the extent of lipids accumulation is determined by the genetic constitution, as maximum attainable lipids contents can vary enormously among species and even among individual strains. According to different microorganisms and different culture conditions (such as temperature, pH, culture time, etc.), oil content and composition are different, see Table 2 (Alvarez and Steinbuchel , 2002; Papanikolaou ***et al***., 2002).

Among oleaginous microorganism, microalgae are sunlight-driven cell factories that convert carbon dioxide to potential biofuels including methane, biohydrogen and oils (Metting and Pyne, 1996). Although microalgae are high lipids microbial, they need a larger acreages to culture algaes and long fermentation period than bacteria. Table 1 shows that nearly all bacteria accumulate lower lipids than microalgaes, the average oil content is about 20–40% of dry biomass, such as

Arthrobacter sp. and *Acinetobacter calcoaceticus*, the oil content is 40% and 38%, respectively.

Table 1. Oil content of some microorganisms

Microoganisms	Oil content (%)	Microorganisms	Oil content (%)
Microalgae		Yeast	
Botryococcus braunii	25–75	*Candida curvata*	58
Cylindrothca sp.	16–37	*Cryptococcus albidus*	65
Nitzschia sp.	45-47	*Lipomyces starkeyi*	64
Schizochytrium sp.	50-77	*Rhodotorula glutinis*	72
Bacteria		Fungi	
Arthrobacter sp.	>40	*Aspergillus oryzae*	57
Acinetobacter calcoaceticus	27–38	*Mortierella isabellina*	86
Rhodococcus opacus	24–25	*Humicola lanuginosa*	75
Bacillus alcalophilus	18–24	*Mortierella vinacea*	66

Source : Meng *et al.* (2009)

Table 2. Lipids composition of some microorganisms

Microorganisms	Lipids composition (w/total lipid)					
	C16:0	C16:1	C18:0	C18:1	C18:2	C18:3
Microalga	12–21	55–57	1–2	58–60	4–20	14–30
Yeast	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
Bacterium	8–10	10–11	11–12	25–28	14–17	–

Source : Meng *et al.* (2009)

Bacteria have a superiority in the production of biodiesel with highest growth rate (reach huge biomass only need 12–24 h) and easy culture method. As an alternative, storage-lipid-accumulating bacteria, in particular those of the

actinomycete group may be used, and these bacteria are capable of synthesizing remarkably high amounts of fatty acids (up to 70% of the cellular dry weight) from simple carbon sources like glucose under growth-restricted conditions and accumulate them intracellularly as triacylglycerol (Alvarez and Steinbuchel, 2002). However, most bacteria are generally not oil producer, only a few bacteria accumulate complicated lipoid (i.e., polyhydroxyalkanoates) (Steinbuchel *et al*.,1998). It is difficult to extract because these lipoid are generated in the outer membrane, so there is no industrial significance in the actual production of biodiesel by using oleaginous bacteria as raw material. Yeasts and fungi (especially molds) are considered as favourable oleaginous microorganisms since 1980s (Abraham and Srinivasan, 1984; Ratledge, 1993). 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 most efficient oleaginous yeast *Crptococcus curvatus* can accumulate storage lipids up to >60% on a dry weight basis, while when it grows under N-limiting conditions, these lipids usually consist of single cell oil 90% w/w triacylglycerol with a percentage of saturated fatty acids (% SFA) of about 44% which is similar to many plant seed oils (Ratledge, 1982).

Oleaginous yeasts and molds accumulate triacylglycerols rich in polyunsaturated fatty acids or having specific structure (Kavadia *et al.*, 2001), somewhat limited: oleic (18:1) and linoleic (18:2) acids together with palmitic (16:0) or palmitoleic acids (C16:1) are the most frequently found fatty acids (See Table 2), and nearly all of them are unsaturated fatty acid. For example, *Rhodosporidium toruloides* Y4 contained mainly long-chain fatty acids with 16 and 18 carbon atoms, which is a well-known microbial lipids producer and extensive characterization of the oleaginous profile of *R. toruloides* CBS 14 has been reported (Evans and Ratledge, 1984). Recently, report showed that it can reach a dry biomass and cellular lipids content of 151.5 g/L and 48.0% (w/w), respectively, in flask fed-batch cultures run for 25 days (Li *et al*., 2007). A filamentous fungus – *Mortierella alliacea* strain YN-15, accumulated arachidonic acid (AA, C20:4 n-6) mainly in the form of triglyceride in its mycelia, which yielded 46.1 g/L dry cell weight, 19.5 g/L total fatty acid, and 7.1 g/L AA by 7-d cultivation in a 50-L jar fermenter (Takeno *et al*., 2005). Based on these data, oleaginous yeasts and mold are all potential alternative oil resources for

biodiesel production. However, the mold have a lower growth rate compared with yeast. They also form mycelia in the culture that makes the culture has a high viscosity causing problems in oxygen transfer and culture dispersal (Certik *et al.*, 1996). On the other hand, yeast can produce high amount of lipids contents which has the characteristics similar to vegetable oil. And it also has high growth rate and can be cultured in a single media with low cost substrate. In addition, they have high capacity to resist to virus and bacteria because most yeast species are acidolic. Moreover, the yeast cell are big in size which makes the saparation of cells from culture become easy to handle (Zhu *et al.*, 2008)

2.2 Lipids synthesis and accumulation in oleaginous microorganisms

2.2.1 Patterns of accumulation

Not all microorganisms can be considered as abundant sources of oils and fats, though, like all living cells, microorganisms always contain lipids for the essential functioning of membranes and membranous structures. Those microorganisms that do produce a high content of lipids may be termed “oleaginous” in parallel with the designation given to oil bearing plant seeds. Of the some 600 different yeast species, only 25 or so are able to accumulate more than 20% lipid; of the 60,000 fungal species fewer than 50 accumulate more than 25% lipids (Ratledge, 2002). The lipids which accumulates in oleaginous microorganisms is mainly triacylglycerol. If lipids other than this type are required then considerations other than those expressed here might have to be taken into account to optimize their production. With few exceptions, oleaginous microorganisms are eukaryotes and thus representative species include algae, yeasts, and molds. Bacteria do not usually accumulate significant amounts of triacylglycerol but many do accumulate waxes and polyesters which are now of commercial interest. The essential mechanism which operates is that the organism is unable to synthesize essential cell materials – protein, nucleic acids, etc. – because of nutrient deprivation and thus cannot continue to produce new cells. Because of the continued uptake of carbon and its conversion to lipid, the cells can then be seen to become engorged with lipids droplets (Figure. 1)

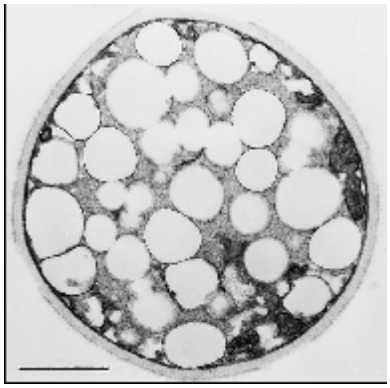

Figure 1. Electron micrograph of *Cryptococcus curvatus* strain D grown for 2 days on nitrogen-limiting medium.
Source: Holdsworth *et al*. (1988)

Few microorganisms are known to accumulate lipids to a significant level. Those species able to do so to a level corresponding to more than 20% of their biomass are described as oleaginous. Fewer than 30 of the 600 species of microorganisms investigated in one study were found to be oleaginous (Ratledge, 1994). The best known oleaginous yeasts include genus of *Candida, Cryptococcus, Rhodotorula, Rhizopus, Trichosporon* and *Yarrowia*. On average, these yeasts accumulate lipids to a level corresponding to 40% of their biomass. However, in conditions of nutrient limitation, they may accumulate lipids to levels exceeding 70 % of their biomass. Nevertheless, lipids content and profile differ between species. For instance, *Cryptococcus curvatus* and *Cryptococcus albidus* accumulate lipids to equivalent levels (58% and 65%, respectively), but their fatty acid profiles differ significantly. *C. curvatus* accumulates large amounts of palmitic acid, whereas oleic acid is the principal fatty acid accumulating in *C. albidus*. By contrast, in *Rhodotorula* species, lipids content diverges significantly (*Rhodotorula glutinis* and *R. graminis* accumulate lipids at levels corresponding to 72% and 36% of their biomass, respectively), but fatty acid composition remains similar. *Y. lipolytica* accumulates lipids to lower levels than some other oleaginous species, but it is the only yeast known to be able to accumulate such a high proportion of linoleic acid (more than 50% of the fatty acid residues present).

2.2.2 Biochemistry and regulation of lipids accumulation

Oleaginous microorganisms begin to accumulate lipids when an element in the medium becomes limiting and the carbon source (such as glucose) is present in excess. Many elements can induce lipids accumulation. Nitrogen limitation is generally used in lipids accumulation studies in microorganisms. Nitrogen limitation is the easiest condition to control and is generally the most efficient type of limitation for inducing lipids accumulation. During the growth phase, the carbon flux is distributed between the four macromolecular pools (carbohydrate, lipid, nucleic acid, protein). Nitrogen is essential for the protein and nucleic acid synthesis required for cellular proliferation. This process is therefore slowed by nitrogen limitation. However, in conditions of nitrogen limitation, the catalytic growth rate slows down rapidly, whereas the rate of carbon assimilation slows more gradually. This results in the preferential channeling of carbon flux toward lipids synthesis, leading to an accumulation of triacylglycerols within discrete lipids bodies in the cells. If non-oleaginous microorganisms are placed in the same nutrient-limiting medium, further cell proliferation tends to cease, with carbon flux into the cell maintained but, in this case, the carbon is converted into various polysaccharides, including glycogen and various glucans and mannans.

During the transition between the growth phase (growth with the production of catalytic biomass) and the lipids accumulation phase (decrease in growth rate due to nutrient limitation and the diversion of excess carbon to lipids production), some pathways are repressed (nucleic acid and protein synthesis), whereas others are induced (fatty acid and triacylglycerol synthesis). This transition is induced by the establishment of nitrogen limitation (see below). In addition, during the accumulation phase, precursors (acetyl-CoA, malonyl-CoA and glycerol) and energy (ATP, NADPH) are required for lipids synthesis. The role of the key enzymes involved in regulating lipids accumulation potential was described as follows: AMP deaminase is activated by the exhaustion of nitrogen in the medium during the growth of an oleaginous microorganism. AMP deaminase catalyzes the following reaction (Ratledge, 2002):

$$AMP \longrightarrow IMP + NH^{+}_{4}$$

The activation of AMP deaminase decreases mitochondrial AMP concentration and increases cellular ammonium concentration. The decrease in AMP concentration inhibits isocitrate dehydrogenase, blocking the citric acid cycle at the isocitrate level. Aconitase mediates the accumulation of citrate in mitrochondria, with exit from the mitochondria mediated by the citrate/malate cycle (Ratledge, 2002). This reaction provides large amounts of acetyl-CoA for fatty acid synthesis. Acetyl-CoA is provided by the cleavage of citrate coming from the mitochondria by ATP-citrate lyase (ACL) in the cytosol. ACL cleaves the citrate to give oxaloacetate and acetyl-CoA.

$$\text{Citrate} + \text{HS-CoA} + \text{ATP} \longrightarrow \text{acetyl} - \text{CoA} + \text{oxaloacetate} + \text{ADP} + \text{Pi}$$

This enzyme is absent from non-oleaginous yeasts, such as *S. cerevisiae*, but has been shown to be present in *Y. lipolytica*. Whereas the human ACL consists of a single protein encoded by a single gene, the ACL enzymes of both *Y. lipolytica* and *Neurospora crassa* consist of two subunits, Aclap and Aclbp, encoded by ACL1 (YALI0E34793g) and ACL2 (YALI0D24431g), respectively. This enzyme requires an ammonium ion for activation and is dependent on adenosine mono- and diphosphate (Ratledge, 2002). However, ammonium ions are scarce in the absence of nitrogen, due to the induction of AMP deaminase (Ratledge, 2002). In addition to acetyl-CoA, fatty acid synthesis requires a continuous supply of malonyl-CoA and NADPH. Malonyl-CoA can also be generated from acetyl-CoA, in a reaction catalyzed by acetyl-CoA carboxylase (Acc1p) (Al-Feel *et al.*, 1992).

$$\text{Acetyl-CoA} + HCO^{-}_{3} + \text{ATP} \longrightarrow \text{malonyl} - \text{CoA} + \text{ADP} + \textit{P}\text{i}$$

In mammalian cells, this enzyme is activated in the presence of tricarboxylic acid intermediates, such as citrate (Goodridge *et al.*, 1991). In yeast, however, Acc1p undergoes allosteric activation as a function of citrate concentration (Gill and Ratledge, 1972). NADPH is required for the function of the fatty acid

synthase (FAS). It is thought that NADPH concentration is controlled by the activity of malic enzyme (ME). This enzyme catalyzes the following reaction:

$$\text{Malate} + \text{NADP}^{+} \longrightarrow \text{pyruvate} + \text{NADPH}$$

The first evidence of ME involvement in lipids accumulation was provided by the inhibition of this enzyme by sesamol in Mucor circinelloides, resulting in a decrease in lipids accumulation from 25% to 2% of cell biomass. Ratledge *et al*. subsequently demonstrated a direct correlation between decreasing ME activity during the lipids accumulation phase and the extent of lipids accumulation. ME overproduction in *M. circinelloides*, through the expression of the gene under the control of the strong constitutive promoter of the glyceraldehyde-3-phosphate dehydrogenase gene (gpd1), was recently shown to increase lipids accumulation by a factor of 2.5 (Zhang *et al.*, 2007).

2.3 Yeasts as potential lipids producers

Oleaginous yeasts are single celled fungi defined as having at least 20% of their dry weight made up of lipids. Not only do these yeasts contain membrane lipids, but they accumulate lipids in the from 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 lipids accumulation.

Non-oleaginous yeasts, when placed in the same environment, tend to accumulate a little extra lipids 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 oil were myristic acid, palmitic acid, stearic acid, oleic acid, linoleic acid, and linilenic acid. It has been reported that such yeast oil 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 lipids accumulation in oleaginous yeasts may reach, and on occasions even exceed, 70% of biomass weight. The lowest amount of lipids required to be accumulated by yeast for inclusion in this list is somewhat arbitrary, and the limit usually chosen in about 20%, since this excludes many non-oleaginous species that can be pushed into accumulating lipids to about this level.

The evidence for true oleaginicity among some of the yeasts 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 lipids accumulation, and indeed its absence in species such as *S. cerevisiae* and *Candida utilis* can explain why these yeasts are unable to accumulate lipids beyond a normal limit of about 10%. However, lipids accumulation is a complex biochemical process and does not rely on the presence of single enzyme. The prospects of genetically engineering a non-oleaginous yeast into a lipids producer must therefore be regarded as remote, at least for the time being. The extraction of yeast lipids usually coextracts other lipids 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 lipids 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)

Table 3. Lipids production by different yeasts

Species	Maximum lipids 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. albidus	63
Cryptococcus laurentii	32
Endomycopsis vernalis	65
Hansenula ciferri	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	28
Trichosporon cuteneum	45
Trichosporon fermentans	62
Trichosporon pullulans	33
Trigonopsis variablis	40
Yarrowia lipolytica	36

Source : Verachtert and Mot (1989)

Table 4. Lipids composition of selected oleaginous yeast

Yeast	Relative % (w/w) of component[a]							
	TAG	DAG	MAG	FFA	S	SE	PL	GL
Cryptococcus 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	82	1	-	-	10	1	4	-

[a]TAG = triacylglycerol, DAG = diacylglycerol, MAG = monoacylglycerols, FFA = free fatty acid, S = sterol; SE : sterol ester; PL : phospholipid; G: glycolipid
Source : Ratledge and Tan (1990)

2.4 Factors affecting lipids accumulation

Lipids accumulation depends primarily on microorganism physiology, nutrient limitation and environmental conditions, such as temperature and pH. It is also affected by the production of secondary metabolites.

2.4.1 Carbon source

Yeasts are chemoorganotrophic organism. This means that they obtain carbon and energy from compounds in fixed, organic linkage. These compounds are most common sugars of which glucose is the most widely utilized by yeast. The costs of microbial oil production are currently higher than those of vegetable oil, but there are many methods to drastically improve the techno-economics of microbial oil production processes. So far, the most commonly used carbon source for single cell oil production is glucose. Therefore, it is very important to use a low cost raw material instead of glucose in order to reduce the cost of single cell oil production. It has been confirmed that ***R. mucilaginosa*** can assimilate xylose. So in the research of Mei ***et al***. (2010) sucrose, glucose, xylose and hydrolysate of cassava starch were used as the carbon sources for oil production by ***R. mucilaginosa*** TJY15a. The results in Figure 2 show that when the carbon source in the production medium was hydrolysate of cassava starch, the yeast could accumulate more lipids in its cells than in the production media containing other carbon sources. The optimal concentration of hydrolysate of cassava starch was 2.0% (w/v).

Under this condition, the yeast could accumulate 45.9% (w/w) of oil in its cells and dry cell mass reached 10.9 g/L (Figure. 2).

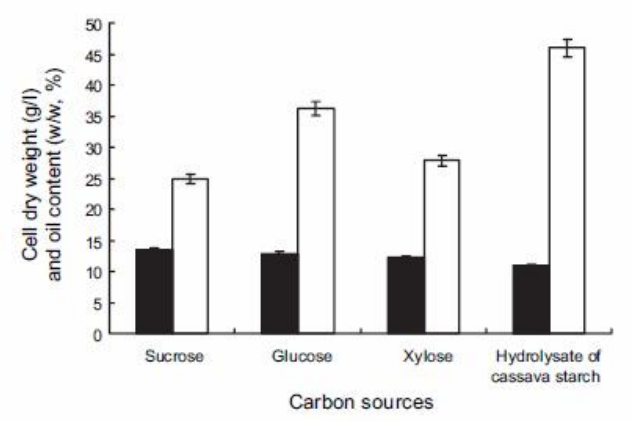

Figure 2. Effects of different carbon sources on cell growth and lipids accumulation of *Rhodotorula mucilaginosa* TJY15a. The concentrations of the carbon sources were 2.0% (w/v) and the concentration of yeast extract was 0.05% (w/v),■ cell dry weight (g/L); □ oil content (w/w, %). Data are given as means ± SD, n=3.
Source : Mei *et al*. (2010)

Saxena *et al*. (1998) studied the effect of carbon source including glucose, fructose, galactose, and lactose on growth rate and lipids yield of *Rhodotorula minuta*. The specific growth rates of an oleaginous strain on different substrates indicate the affinity of the strain toward the substrate used as a carbon source. 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). The specific growth rate (0.34 h^{-1}) of *R. minuta* IIP33 on glucose was comparatively higher than that of other oleaginous yeasts, e.g., *R. glutinis* CFR-1 (0.16 h^{-1}) and *Lipomyces starkeyi* (0.12 h^{-1}) as reported by Jacob and Krishnamurthy (1990). However, the specific growth rate of *R. minuta* IIP-33 on glucose was the same as that of *R. glutinis* IIP-30 on glucose (0.34 h^{-1}) but comparatively slower (0.22 h^{-1}) on sucrose compared to that of *R. glutinis* IIP-30 (Johnson *et al*., 1995). Lipids accumulation of *R. minuta* IIP-33 was also maximal on glucose (0.48) compared to other carbohydrates, e.g., sucrose (0.36), fructose (0.30) and galactose (0.11).

Table 5. Growth and lipids accumulation by *Rhodotorula minuta* IIP-33

Carbon substrate	Specific growth rate (h^{-1})	Lipids yield (w/w%)
Glucose	0.34	0.48
Fructose	0.30	0.30
Sucrose	0.22	0.36
Galactose	0.15	0.11

Source : Johnson *et al.* (1995)

Zhu *et al.* (2008) reported that an excess carbon is diverted to lipids biosynthesis in many oleaginous microorganisms. Table 6 shows the effect of carbon source on cell growth and lipids accumulation of *Trichosporon fermentans*. The maximum biomass was obtained when glucose was used as a carbon source, followed by fructose, sucrose, xylose and lactose. All the carbon sources tested gave relatively high lipids content. It is obvious that *T. fermentans* has a broad spectrum of carbon sources. Similar lipids production from *Rhodotorula glutinis* was obtained when used glucose as a 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 lipids accumulation of *T. fermentans*

Carbon source	Biomass (g/L)	Lipids content (%)	Lipids 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

Source : Zhu *et al.* (2008)

Eastering *et al.* (2009) reported the differences in oil accumulation by *Rhodotorula glutinis* between those cultures provided single carbon source and those provided mixtures of carbons source. Yeast cultured for 24 h on a medium containing dextrose, xylose, glycerol, dextrose and xylose, xylose and glycerol, or dextrose and

glycerol accumulated 16, 12, 25, 10, 21, and 34% triacylglycerol on a dry weight basis, respectively. When comparing the 24 and 48 h data for all experiments, lipids content of the glycerol grown *R. glutinis* increased on average 12.97% while dextrose grown 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. The data suggests that using glycerol as a sole carbon source may result in greater lipids production by the oleaginous yeast *R. glutinis*. There was 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 lipids than when the carbon source are used individually. It can be submitted, however, that glycerol grown *R. glutinis* accumulates more lipids under these experimental conditions than dextrose grown and xylose grown cultures.

2.4.2 Nitrogen source

Yeast cells normally have a nitrogen content of around 10% of their dry weight. 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 lipids synthesis independent of the nitrogen concentration in culture medium. Nutrient imbalance in the culture medium has long been known to trigger lipids accumulation by oleaginous microorganisms. When cell 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 lipids fermentation, different substrates and cultivation modes have been used (Li *et al.*, 2007). It has been reported that 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 (Papanikolaou *et al.*, 2004). Figure 3 shows the effect of different nitrogen sources on oil accumulation in the cells of *R. mucilaginosa* TJY15a. The results indicate that yeast extract was the most suitable nitrogen source for lipids accumulation by *R. mucilaginosa* TJY15a. In the medium containing 2.0% (w/v) hydrolysate of cassava

starch and 0.33% (w/v) yeast extract, the yeast stored 47.9% of oil in its cells and dry cell mass reached 20.2 g/L. Huang *et al.* (1998) reported that inorganic nitrogen sources were good for cell growth, but not suitable for oil production, while organic nitrogen sources were good for oil production, but not suitable for cell growth. *R. toruloides* has been found to accumulate more lipids when an organic nitrogen source was employed (Evans and Ratledge, 1984). The nitrogen sources for lipids accumulation of *Mortierella isabellina* ATHUM 2935 were ammonium sulfate and yeast extract at 0.5 g/L of each (Papanikolaou *et al.*, 2004).

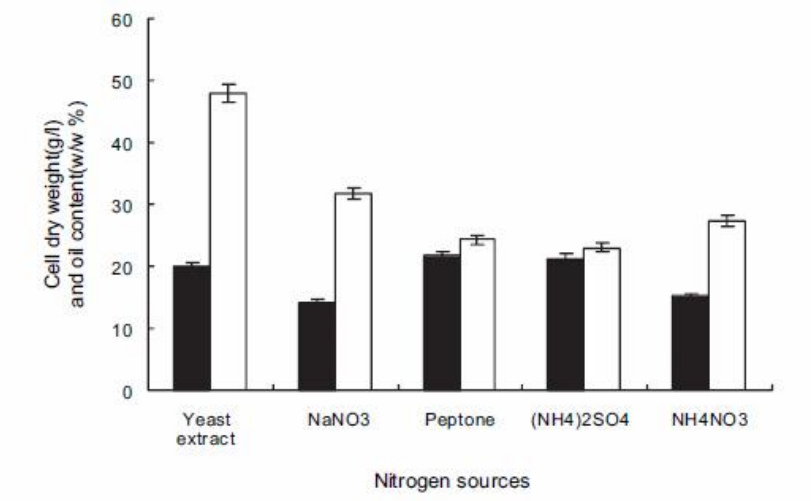

Figure 3. Effects of different nitrogen sources on cell growth and lipids accumulation. The concentrations of the nitrogen sources were 0.33% (w/w). **n** = cell dry weight (g/L); □ = oil content (w/w, %). Data are given as means ± SD, n=3.
Source : Mei *et al.* (2010)

2.4.3 C/N ratio

Indeed, C/N ratio has been found to be the major impact factor for oil accumulation by the oleaginous microorganisms (Papanikolaou *et al.*, 2004). When oleaginous organisms are grown with an excess of carbon and limited quantity of nitrogen (high C/N), they may accumulate high concentration of intracellular lipid. The exact ratio of C to N chosen for the medium was originally considered to be of little consequence provided N as the limiting nutrient and sufficient carbon remained

to ensure good lipids accumulation. A C/N ratio between 20 and 50 was optimal for intracellular lipids accumulation in the culture of *Candida curvatus* (Hassan *et al.*, 1996). After nitrogen depletion, significant fat quantities were accumulated inside the fungal mycelia of *M. isabellina* grown on high-sugar content media (50–55%, wt/wt oil in dry biomass), resulting in a notable single cell oil production of 18.1 g/L of culture medium (Papanikolaou *et al.*, 2004). However, Ykema *et al*. (1988) showed that a range of lipids yields in an oleaginous yeast, *Apiotrichum curvatum* (originally *Candida curvata* but now *Cryptococcus curvatus*), were traversed in continuous culture by varying the C/N ratio of the growth medium.

There was a hyperbolic relationship between the C/N ratio and the maximum growth (dilution) rate that the organism could attain: the lowest growth rate was at the highest C/N ratio of 50 and this, in turn, controlled the amount of lipids produced and the efficiency of yield (g lipids per g glucose used) with which it was produced. Although the highest lipids contents of the cell (50% w/w) were obtained with a C/N ratio of 50 or over, the optimum ratio for maximum productivity was at a ratio of 25 with glucose (Ykema *et al*., 1988) and at 30–35 when whey permeates were used with the same yeast. Similar results for describing the optimum C/N ratio for lipids accumulation have been developed by Granger *et al.* (1993) using *Rhodotorula glutinis*. Zhu *et al*. (2008) studied on the effect of C/N ratio on cell growth and lipids 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. Lipids 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 7). Further rise in C/N ratio beyond 140 resulted in a slight drop in lipids content but a continuous increase in biomass up to 163 and the highest lipids 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 7. Effects of C/N ratio on cell growth and lipids accumulation of *T. fermentans*

C/N ratio	Biomass (g/L)	Lipids content (%)	Lipids 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

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. Lipids 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 lipids production in continuous cultures demonstrated that cellular lipids content in whey permeat 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. At C/N ratios higher than 25-30 lipids content gradually increased to nearly 50% at C/N ratio 70 and the maximal obtainable dilution rate decreased to 0.02/h at C/N ratio 70. From these studies it could be derived that maximal lipids 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 lipids production rate (Ykema *et al.*, 1988).

2.4.4 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 acids) are more inhibitor to yeast growth compared with those acidified with mineral acids (e.g. hydrocholoric, phosphoric acids). This is because undissociated organic acid can lower intracellular pH following translocation across the yeast plasma membrane. This form 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 lipids accumulation of *T. fermentans*, comparatively high biomass and lipids content could be achieved at the pHs ranging from 4.0 to 10.0 The maximum biomass (28.1 g/L) and lipids 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 lipids production from *R. glutinis* was obtained when cultivatied at initial pH 5.0 (Dai *et al.*, 2007). The pH-value on lipids accumulation was studied using a C/N-ratio of 100. 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 lipids content 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 (Angerbauer *et al.*, 2008). In the literature different pH-values are reported for optimal lipids accumulation which seemed to depend on the carbon sources used. An optimum pH of 4.9 for lipids accumulation was found when glucose was used as a carbon source (Naganuma *et al.*, 1985), while pH 4.0 was the optimum when ethanol was used as a carbon-source (Holdsworth and Ratledge, 1988). However the pH-range from 5.5 to 7.0 is not reported. *R. glutinis* was found to be not suitable to grow in an acid condition. Cell growth and the COD degradation ratio were both very low at pH 2.5, however, the two parameters was relatively stable between pH 4.5 and 7.0. And the three target parameters, COD degradation ratio, biomass (dry cell weight/culture broth volume) and lipids content peak at pH 5.5. So

pH 5.5 was chosen as the optimal value (Xue *et al., 2006*). Husain *et al.*(2009) studied the effect of initial pH value of the cultivation medium, seven levels of different pH were performed. Little change in the final pH value was found between the different treatments. At pH 5.5 treatment, the dry cell weight of *Cryptococcus curvatus* NRRLY-1511 reached to 2.4 g/L with single cell oil weight of 1.7 g/L. On the other hand, pH 6.5 recorded the highest sugar consumption to be 56.2 g/L. It was found that microbial oil production was maximum when the mold was cultivated at pH 6.5. They found that total lipids drastically decreased at pH 8.0 and at pH 4.0. They also reported that there was an increase in total lipids concentration in the pH range of 3.0 to 6.0.

2.4.5 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.

Zhu *et al.* (2008) reported on the effects of temperature on cell growth and lipids accumulation (Table 8). Both biomass and lipids 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 lipids 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 lipids decreased from 71.8% to 52.0%, indicating that a low temperature was favorable for the formation of unsaturated fatty acids.

Table 8. Effects of temperature on cell growth and lipids accumulation of *T. fermentans*

Temperature (°C)	Biomass (g/L)	Lipids content (%)	Lipids 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

Source: Zhu *et al.* (2008)

Temperature induced changes in lipids biosynthesis are significant to know whether the organism is capable of adapting itself to wide ranges of temperature and how it affects its lipids yield and fat coefficient besides its fatty acid composition. Lipids yield and the fat coefficient profile of ***R. minuta*** IIP-33, cultured at different growth phase temperature, i.e., 30 to 38 °C. A maximal lipids yield of 0.25 and a fat coefficient of 14% in the growth phase were observed at a temperature of 32°C. The overall lipids 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 lipids biosynthesis, and the lipids yield and fat coefficient decreased to 0.12 and 3%, respectively, at 38°C. 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). The general fatty acid profile of ***R. minuta*** grown at different temperatures shows a wide range of fatty acids (C7 to C18), presumably owing to temperature-sensitive acyyl-carrier protein, 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 predorminant 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 (Sexena *et al*., 1998).

2.4.6 Aeration rate

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 from lanosterol and for the synthesis of unsaturated fatty acyl coenzyme-A esters. Oxygen should therefore be regarded as an important yeast growth factor (Walker, 1998). *R. glutinis*, is an aerobic microorganism and therefore requires the provision of oxygen. Aerating and agitating the fermentation broth normally satisfy the oxygen demand of a fermentation process. The effect of agitation and aeration on lipids production is extremely important for the successful progress of the fermentation. Agitation is important for adequate mixing, mass transfer and heat transfer. It not only assists mass transfer between the different phases present in the culture, but also maintains homogeneous chemical and physical conditions in the culture by continuous mixing. Agitation creates shear forces, which affect microorganisms in several ways, causing morphological changes, variation in their growth and product formation and also damaging the cell structure. Aeration could be beneficial to the growth and performance of microbial cells by improving the mass transfer characteristics with respect to substrate, product/by-product and oxygen. The morphology of the microorganism can strongly influence the product formation, since it affects broth rheology and consequently the mass and heat transfer capabilities of the fermentation broth (Atkinson and Mavituna, 1985).

2.4.7 Activator

Some agents, such as detergent additives, oils and surfactants have been known to increase lipids and carotenoids production (Kim *et al*. 1997). Supplementation of fermentation medium with surface-active substances can alter the physiological properties of microorganisms, improve metabolite production, stimulate

growth and respiration, and change the organization and permeability of cell membranes *(*Benchekroun and Bonaly, 1992*)*. The fatty acyl chains of phospholipids and the ratio of saturated to unsaturated fatty acids are among the most important factors modulating the fluidity and integrity of the membrane (Panchal and Stewart, 1980). Sterols are also involved in maintaining the dynamic state of the membrane, and altered levels lead to a change in membrane permeability and membrane-associated functions such as the activities of membrane-bound enzymes *(*Grunze and Deuticke, 1974*)*. Nemec and Jernejc. (2002) studied the addition of 0.1% of nonionic surface-active Tween 80 to a medium for optimization of pectolytic enzyme production of *Aspergillus niger*. The addition of Tween 80 increased the amount of enzymes excreted by 70% and also the amount of sterol esters and triacylglycerols was increased.

During the course of cultivation the amounts of precursors for ergosterol biosynthesis diminished with an increase of ergosterol. *A. niger* incorporated cholesterol from the medium, partly converting it to cholesterol esters. Sterol esters were formed only with selected fatty acids. Oleic acid, the hydrophobic part of Tween 80, was mainly incorporated in phospholipids and glycolipids. Nikkila *et al*. (1995) reported that oleic acid was incorporated into fungal cells after enzyme liberation, most probably by esterases and thus increased the amount of unsaturated fatty acids. In glycolipids and phospholipids as well as sterol esters oleic acid was substantially increased by Tween 80 supplementation. The nature of the fatty acyl chain of phospholipids is one of the most important factors modulating the fluidity and integrity of the membrane. The ratio of saturated to unsaturated fatty acids may also greatly influence membrane properties. A lower ratio can give rise to better permeability. Different lipids contents might effect the metabolism in a number of ways and perhaps changed lipids composition of membranes can increase protein transportation as well.

2.5 Carotenoids production by yeast

Naturally occurring carotenoids are tetraterpenoids consisting of highly unsaturated isoprene derivatives. These compounds are the most widely distributed class of pigments in nature, displaying yellow, orange, and red colour. Industrially

carotenoid pigments, such as β-carotene and astaxanthin are of increasing demand and a wide variety of market applications: as food colouring agents, e.g.margarine, soft drinks, and baked goods; as precursors of Vitamin A (pro-Vitamin A) in food and animal feed; as additives to cosmetics, multivitamin preparations; and in the last decade as antioxidants to reduce cellular or tissue damage. Carotenoids are produced primarily by filamentous fungi and yeasts and by some species of bacteria, algae and lichens. Among microbial sources of carotenoids, besides algae like *Dunaliella* species, yeasts such as *Phafia rhodozyma* and *Rhodotorula glutinis* are of commercial interest (Aksu and Eran, 2005). Although yeast is a nonphotosynthetic microorganism, there are yeasts that can biosynthesize carotenoids in the cell so there has been considerable interest in the commercial use of the yeast.

Rhodotorula yeasts, distributed widely in nature, can also biosynthesize characteristic carotenoids, such as β-carotene, torulene and torularhodin, in various proportions. *R. glutinis* is also a carotenoid-producing yeast and synthesizes β-carotene as the major carotenoid (Perrier *et al.*, 1995). The pH value of growth medium affects not only biosynthesis activity of culture, but also culture growth rate. Temperature is an another important parameter affecting the performance of cells and product formation. The temperature of the growth medium had also a considerable effect on both the growth and carotenoid production of *R. glutinis.* If the microorganism requires oxygen, aerating the growth medium is very important for the successful progress of the fermentation. As *R. glutinis* is an aerobic microorganism (Aksu and Eran, 2007). Some agents, such as detergent additives, oils, surfactants have been known to increase carotenoids productivity (Kim *et al.*, 1997).

Aksu and Eran (2007) studied the production of carotenoids by *Rhodotorula glutinis* in a batch system as a function of initial pH, temperature, aeration rate and activator (cotton seed oil and Tween 80) addition. It was found that the specific growth and total carotenoids production rates increased with raising the pH and reached to a maximum level at pH 6. On the other hand, a further increase of the pH over 6.0 resulted in a reduction of both the rates. For the effect of temperature, it was found that the highest specific growth rate of yeast was 0.238 h^{-1} at 30 $^{\circ}$C. The total carotenoids formation rate was also affected by the variation of fermentation temperature; enhanced strictly up to 30 $^{\circ}$C and increased slightly with further increase

in temperature. The effect of the aeration rate on the growth and total carotenoids formation of the yeast was also examined. The results obtained showed that the specific growth and total carotenoids formation rates changed significantly with varying the aeration rate from 0 to 2.4 vvm. In the growth medium aerated at 2.4 vvm, the total carotenoids per gram of glucose consumed were 7.1 mg/g. In order to enhance the production of carotenoids the medium was supplemented with the activators of 1% (v/v) of cotton seed oil and 1% (v/v) of Tween 80. It was found that cotton seed oil increased the total carotenoids per gram of glucose consumed up to 10.4 mg/g.

Mantzouridou *et al.* (2002) study the effect of the aeration rate and agitation speed on β-carotene production of *Blakeslea trispora*. In cultures grown at agitation speed of 150 and 325 rpm, the concentration of β-carotene increased significantly when the aeration rate was increased from 0.5 to 1.5 vvm. Aeration results in better mixing of the fermentation broth, thus helping to maintain a concentration gradient between the interior and the exterior of the cells. This concentration gradient works in both directions; through better diffusion it helps maintain a satisfactory supply of sugars and other nutrients to the cells, while it facilitates the removal of gases and other by-products of catabolism from the microenvironment of the cells. On the other hand, a further increase of the agitation speed over 325 rpm resulted in a decrease of β-carotene concentration when the aeration rate was increased from 0.5 to 1.5 vvm. This may be explained by the fact that a high agitation speed and aeration rate causes oxidation of the pigment.

2.6 Modes of culture for ensuring high levels of lipids accumulation

2.6.1 Batch culture

Zhu *et al.* (2008) reported that the optimal nitrogen source, carbon source and C/N molar ratio for production of lipids by *Trichosporon fermentans* in batch culture were peptone, glucose and 163, respectively. The favorable initial pH of the medium and temperature were 6.5 and 25 ◦C. Under the optimized conditions, a biomass of 28.1 g/L and a lipids content of 62.4% could be achieved after culture for 7 days. Hwang *et al.* (2005) studied the production of arachidic acid (ARA) by

Marsupella alpina DSA-12 in batch cultures. The glucose (50 g/L) was completely consumed after 6 days. The cell concentration was increased rapidly for the first 4 days and then more slowly to reach 22 g/L after 6 days. The production of total lipids was increased in parallel with the cell mass to achieve the final intracellular content of 52% (w/w) and concentration of 11.4 g/L after 6 days. Dai *et al*. (2007) studied the production of lipids by *Rhodotorula glutinis* in batch culture. The optimal fermentation conditions were obtained as follows: glucose as a carbon source 100 g/L; yeast extract and peptone as nitrogen sources at, respectively, 8 and 3 g/L; initial pH of 5.0; inoculation volume of 5%; temperature at 28^{o}C, shaking speed of 180 rpm, cultivated for 96 h. Under these conditions, *R. glutinis* accumulated lipids up to 49.25% on a cellular biomass basis and the corresponding lipids productivity reached 14.66 g/L. Batch fermentation with a 5-L bioreactor under the optimal culture conditions showed that *R. glutinis* accumulated lipids up to 60.69%, resulting in 23.41 g/L of lipids productivity. More encouraging results were observed for the lipids production with alternative carbon sources. Corn stalk and *Populus euramevicana* leaves hydrolysate could be used to substitute glucose. Chemical analysis indicated that biodiesel obtained by transesterification possessed similar composition to that from vegetable oil, one of the widely used feedstock for biodiesel.

An oleaginous yeast strain, *Cryptococcus curvatus* NRRLY-1511 was used for the production of single cell oil using a low cost cultivation medium containing beet molasses and corn gluten meal as carbon and nitrogen sources (Husain *et al*., 2009). Obtained results showed that the optimum concentrations for carbon and nitrogen were 125 and 0.130 g/L, respectively. In addition, the optimal temperature, incubation period, pH value of cultivation medium and agitation speed were 28°C, 72 h, pH 5.5 and 200 rpm, respectively. The extracted lipids were mainly 30.68% linoleic acid (C18:2), 22.66% oleic acid (C18:1) and 16.74% palmitic acid (C16:0). Furthermore, the gas chromatography analysis also showed that the total saturated fatty acids (n = 9) represented 41.96% while the value of the total unsaturated fatty acids (n = 6) was 58.04%.

2.6.2 Fed-batch

Fed-batch fermentation is the technique that industrially employs to produce chemicals and biochemicals. In fed-batch mode, the reactor is started with a small volume of fermentation medium and when the sugar is utilized a new amount of concentrated sugar will be added. The basic advantage of fed-batch processes is that the concentrations of the feed nutrients in the culture liquid in the bioreactor can be controlled voluntarily by changing the feed rate. Therefore, the fed-batch mode of operation is usually superior to conventional batch operation when changing concentrations of a nutrient affect the yield or productivity of the desired metabolite. Fed-batch cultivation modes have been widely applied for microbial lipids production.

Pan *et al*. (1986) reported on the fed-batch cultures of *Rhodotorula glutinis* with feeding medium containing 600 g/L glucose, 20 g/L yeast extract and 9 g/L $MgSO_4 \cdot 7H_2O$, and the final cellular lipids content was 40% (w/w). Yamauchi *et al.* (1983) obtained a high cell density of 153 g/L and lipids content of 54% (w/w) with *Lipomyces starkeyi* using a complicated feeding medium and a high seed inoculum of 50% (v/v) was also employed in this experiment. Ykema *et al*. (1988) examined lipids production in various culture modes with whey permeate using *Candida curvatus.* A cell density of 91.4 g/L and lipids content of 33% (w/w) were observed using a partial recycling method, while both nitrogen and carbon sources were supplied. Meesters *et al*. (1996) used glycerol as a carbon source in fed-batch fermentation with *Candida curvatus* and realized a cell density of 118 g/L in 50 h with a lipids production rate of 0.59 g/L/h. However, the final cellular lipids content was only 25% (w/w). It should be mentioned that these previous fed-batch lipids fermentation processes simultaneously introduced a carbon and nitrogen sources, which might produce a disfavored C/N ratio for lipids accumulation. In addition, this feeding operation may also be complicated.

2.6.3 Semicontinuous mode

In semi-continuous fermentation, a portion of the culture is withdrawn at intervals and fresh medium is added to the system. This method has the advantages of the continuous and batch operations. There is no need for a separate inoculum

vessel, except at the initial startup. Time is also not wasted in non-productive idle time for cleaning and resterilization. Another advantage of this operation is that not much control is required (Caylak, 1998)**.** Semi-continuous fermentation is easy to set up with low cost its also can prevent culture from having high viscosity that makes substrate utilization become more effective. Until now, the semi-continuous fermentation of lipids production has not been attempted.

2.7 Exploration of cheap carbon sources for yeast oil accumulation

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. Recently, *R. glutinis* was used for wastewater treatment. Xue *et al*. (2006) used the monosodium glutamate wastewater after diluted and well treated as a cheap fermentation broth for *Rhodotorula glutinis* to biosynthesize lipids as the raw material for the production of biodiesel. The optimum conditions were: the initial COD of inlet water was 10,000 mg/L, pH was 5.5, and the inoculum concentration of 12%. The flask treatment results indicated that the COD degradation ratio and the lipids content could reach 85.51% and 9.04 %, respectively. And the transesterification of the crude lipids obtained indicated that methyl ester yield was 92.54 %.

To reduce the cost of microbial oils, exploring other carbon sources instead of glucose is very important especially for such oils applied to biodiesel production. It was reported that xylose, arabinose, mannose, glycerol, and other agricultural and industrial waste could be used as the carbon sources for yeast oil accumulation, and the related information was listed in Table 9. It was reported that *C. potothecoides* could accumulate oils with starch hydrolysate as the carbon sources, and cell growth in the starch hydrolysate was even much better than that with glucose as the carbon sources (Han *et al*. 2006). Du *et al* (2007) found that sweet potato starch processing waste also could be used as the carbon source for yeast oil production, and such industrial waste was always rich in simple sugar, which could be utilized effectively by many microorganisms.

Table 9. Different carbon source used for yeast oil production

Species	Carbon source	Lipids content% (w/w)	Reference
L. starkeyi	Xylose	52.6	Kong *et al*. (2007)
C. potothecoides	Starch hydrolysate	46.13	Han *et al*. (2006)
T. cutaneum	Spartina anglica hydrolysate	46.3	Shen *et al.* (2007)
C. curvatus	Glycerol	25	Meesters *et al*. (1996)
C. echinula	Sweet potato starch processing waste	37.6	Du *et al*. (2007)
L. starkeyi	Sewage sludge	50.8	Angerbauer *et al*. (2008)
T. fermentans	Molasses	35.3	Zhu *et al*., 2008
C. lipolytica	Methanol	4.9	Rupcˇic´ *et al.* (1996)
C. echinulata	Orange peel	1.7	Gema *et al*. (2002)
R. glutinis	Monosodium glutamate	9.0	Xue *et al*. (2006)
M. isabellina	Starch	36.0	Papanikolaou *et al*. (2007)
C. echinulata	Starch	28.0	Papanikolaou *et al*. (2007)
M. isabellina	Pectin	24.0	Papanikolaou *et al*. (2007)
C. echinulata	Pectin	10.0	Papanikolaou *et al*. (2007)

Source: Papanikolaou *et al.* (2004)

During the process of biodiesel production, byproduct glycerol will be produced. It was reported that some microorganism even had the ability to use glycerol as the carbon sources for oil accumulation (Meesters *et al.*, 1996). In the future, with the large-scale development of biodiesel, more and more byproduct glycerol will be produced, and the utilization of crude glycerol for yeast oil production might be another interesting research filed with much prospect. Apart from glycerol, recently, much attention has been paid to the utilization of cellulose hydrolysate as the carbon sources for microbial oil production, but such cellulose hydrolysate usually contains some toxic components, such as acetic acid, formic acid, and furfural, which might have some negative effect on cell growth (Shen *et al*., 2007). Therefore, before using such cheap carbon sources for oil production, detoxication

is very necessary. 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.

2.8 A brief glance at palm oil mill effluent (POME)

In general, the palm oil milling process can be categorized into a dry and a wet (standard) process. The wet process of palm oil milling is the most common and typical way of extracting palm oil, especially in Thailand. It is estimated that for each ton of crude palm oil that is produced, 5–7.5 tons of water are required, and more than 50% of this water ends up as palm oil mill effluent (POME) (Ahmad *et al.*, 2003). Raw POME is a colloidal suspension containing 95–96% water, 0.6–0.7% oil and 4–5% total solids. Included in the total solids are 2–4% suspended solids,which are mainly constituted of debris from palm fruit mesocarp generated from three main sources, i.e. sterilizer condensate, separator sludge and hydrocyclone wastewater (Ma, 2000).

If the untreated effluent is discharged into water sourses, it is certain to cause considerable environmental problems (Davis and Reilly, 1980) due to its high biochemical oxygen demand (25,000 mg/L), chemical oxygen demand (53,630 mg/L), oil and grease (8370 mg/L), total solids (43,635 mg/L) and suspended solids (19,020 mg/L) (Ma, 1995).

The palm oil mill industry has thus been identified as the one discharging the largest pollution load into the rivers throughout the country (Hwang *et al.*, 1978). Ponding system is the most conventional method for treating POME (Khalid and Mustafa, 1992) but other processes such as aerobic and anaerobic digestions, physicochemical treatments and membrane filtration may also provide the palm oil industries with a possible insight into the improvement of current POME treatment process. However, the treatment that is based mainly on biological treatments of anaerobic and aerobic systems, is quite inefficient to treat POME, which unfortunately leads to environmental pollution issues (Ahmad *et al.*, 2005). This is because the high BOD loading and low pH of POME, together with the colloidal nature of the suspended solids, render treatments by conventional methods difficult

(Stanton, 1974). A detailed cost calculation for Indonesia has also shown that the conventional system of POME treatment, such as the ponding system, is not only the system with the highest environmental pollution and the lowest utilization of renewable resources, but also the system giving rise to the lowest profit (Schuchardt *et al.*, 2005).

The high compositions and concentrations of carbohydrate, protein, nitrogenous compounds, lipids and minerals in POME (Habib *et al.*, 1997). The presence of pentose in POME has been reported previously (Hwang *et al.*, 1978) and its most likely sources are the cell walls. Water-soluble carbohydrates, in terms of glucose, reducing sugars and pectin, are also found to be present in the soluble fraction of POME. However, the low concentrations of total soluble carbohydrate (0.390 g/100 ml POME) may restrict the usefulness of the soluble fraction of POME as a possible feedstock for substrate conversion via direct single-cell protein production (Ho *et al.*, 1984). Preliminary investigations on enzymatically hydrolyzed substrates from POME have indeed demonstrated the possibility of such substrates supporting the growth of *Candida tropicalis* (Wang *et al.*, 1981). On the other hand, Barker and Worgan (1981) noted that unhydrolyzed POME could support good growth of *Aspergillus oryzae* in the presence of an added inorganic nitrogen source. Their results also revealed that celluloses, polyphenols and nitrogenous compounds were the least biodegradable of the substrate constituents. This lends further support to the view that a proper hydrolysis step is essential in obtaining an optimal level of readily biodegradable sugars from the plant cell materials for a meaningful microbial bioconversion.

2.9 A brief glance at crude glycerol

Glycerol is present in the form of its esters (glycerides) in all animal and vegetable fats and oils. For every 9 kg of biodiesel produced, about 1 kg of a crude glycerol by-product is formed (Dasari *et al.*, 2005). For a current biodiesel production of 150 million gallons/year, the glycerol amount is 50 million kg. High purity glycerol is a very important industrial feedstock. Its applications are found in food, drug, cosmetic and tobacco industries. In the past decade, industrial glycerol price was in the range of $1.28 to $1.65 (Kirk-Othmer Encyclopedia of Chemical

Technology, 2004). However, crude glycerol derived from biodiesel production possesses very low value because of the impurities. Further refining of the crude glycerol will depend on the economy of production scale and/or the availability of a glycerol purification facility. Larger scale biodiesel producers refine their crude glycerol and move it to markets in other industries. It is generally treated and refined through filtration, chemical additions, and fractional vacuum distillation to yield various commercial grades. If it used in food, cosmetics, and drugs, further purifications are needed such as bleaching, deodoring, and ion exchange to remove trace properties. Purifying it to that stage, however, is costly and generally out of the range of economic feasibility for the small to medium sized plants. As more and more crude glycerol is continuously generated from the biodiesel industry, it is very important that economical ways of the low-grade glycerol utilization be explored to further defray the cost of biodiesel production in the growing global market.

The usage of low-grade quality of glycerol obtained from biodiesel production is a big challenge as this glycerol cannot be used for direct food and cosmetic uses. An effective usage or conversion of crude glycerol to specific products will cut down the biodiesel production costs. This paper aims to cover possible conversion of glycerol into useful products. The products are 1,3-propanediol, 1,2-propanediol, dihydroxyacetones, hydrogen, polyglycerols, succinic acid, and polyesters. Glycerol, when used in combination with other compounds yields other useful products. For example glycerol and ethylene glycol together can be used as a solvent for alkaline treatment of poly fabrics (Yang and Tsai, 1997). Glycerol reductions with magnesium synthesize the carbon onions (Du *et al*., 2005). Glycerol can be used as dielectric medium for compact pulse power systems (Brown *et al.*, 1999). Glycerol acts as a medium in electrodeposition of Indium-Antimony alloys from chloride tartrate solutions (Kochegarov and Belitskaya, 1971). Biomass is converted to liquid fuel using glycerol that can be blended with gasoline as an alternative fuel (Demirbas, 2000). Mixed culture fermentation of glycerol synthesizes short and medium chain polyhydroxyalkanoate blends (Koller *et al*., 2005).

2.10 Quality of biodiesel from oleaginous microorganism lipids

To assess the potential of biodiesel as a substitute of diesel fuel, the properties of biodiesel such as density, viscosity, flash point, cold filter plugging point, solidifying point and heating value were determined (Miao and Wu, 2006). An important fuel criterium for biodiesel is bound glycerol, which functions the residual amount of triglycerides and partial glycerides in the biodiesel. Biodiesel fuel, in the form of fatty acid methyl ester is now manufactured in many countries. In the United States, the relevant standard is the ASTM Biodiesel Standard D 6751 (ASTM. 2006), while in Europe Union, separate standard exists for biodiesel intended for vehicle use (Standard EN 14214) and for use as heating oil (Standard EN 14213). The ASTM D 6751 biodiesel acidnumber limitwas harmonized with the European biodiesel value of 0.5. Microorganism oils quite differ from most vegetable oils in being quite rich in polyunsaturated fatty acids (Belarbi *et al.,* 2000). No such limitation exists for biodiesel intended for use as heating oil, but acceptable ones must meet other criteria relating to the extent of total unsaturation of the oil, which is indicated by its iodine value. Standard EN 14214 and EN 14213 require the iodine value of biodiesel to exceed 120 and 130 g iodine/100 g biodiesel, respectively.

Zhu *et al*. (2008) showed that the lipids extracted from *T. fermentans* mainly contained palmitic acid, stearic acid, oleic acid and linoleic acid, and the unsaturated fatty acids amount to about 64%, which is similar to that of vegetable oils. The acid value of the microbial oil is quite high (5.6 mg KOH/g). For the purpose of reducing the acid value to about 1 mg KOH/g, the microbial oil was firstly pretreated by the reported method (Sreenivasan and Viswanath, 1973). The methanolysis of the pretreated microbial oil was subsequently carried out at 65 ^{o}C in the presence of KOH (1% based on oil weight), with the molar ratio of methanol to oil being 6:1, and a methyl esters yield of 92% was obtained after reaction for 1 h.

Angerbauer *et al.* (2007) show the fatty acid composition, the content of free fatty acids, phosphorus and sulphur of *Lipomyces starkeyi*. For the production of biodiesel the preparation of fatty acid methyl esters has to be carried out in two steps due to the high content of free fatty acids. After pre-esterification with methanol transesterification can be carried out under alkaline catalysis. The fatty acid composition is quite similar to palm oil with a very high content of palmitic acid.

Compared to palm oil also the content of stearic acid is higher. Because of the total content of saturated fatty acids of about 70% excellent burning characteristics like very high cetane number of the fatty acid methyl esters can be estimated (Mittelbach and Remschmied, 2004). However, the high content of saturated fatty acid acids also leads to poor low temperature behaviour of the resulting biodiesel, however, blending with mineral diesel could overcome this problem. The sulphur and phosphorus content is very low, so the limits for biodiesel according to EN 14214 with 10 mg/kg each can be met easily. After fatty acids in the extracted lipids were transmethylated and analyzed by GC, 85.8% of the fatty acids from the yeast strain TJY15a was C16:0 and C18:1, especially C18:1 (63.5%). This means that the lipids from *R. mucilaginosa* TJY15a are a good oil feedstock for biodiesel production.

The lipids from *R. toruloides* Y4 also contain mainly long-chain fatty acids with 16 and 18 carbon atoms. The fatty acids of lipids from oleaginous yeast *C. curvatus* were mainly oleic (C18:1), palmitic (C16:0) and stearic (C18:0). This means that fatty acid composition of *R. mucilaginosa* TJY15a was similar to that of other oleaginous yeast (Li *et al*., 2010). Dai *et al*. (2007) studied the biodiesel generation from *Rhodotorula glutinis* cultured with xylose. The biodiesel from yeast lipids was produced by transesterification with the yield of 81.7% and the fatty acid esters were analyzed by GC-MS. The results revealed that the composition of biodiesel was as follows: myristic acid (14:0) methyl ester 1.29%, palmitic acid (16:0) methyl ester 18.74%, stearic acid (18:0) methyl ester 1.16%, oleic acid (18:1) methyl ester 66.96%, linoleic acid (18:2) methyl ester 4.57% and low concentration of other methyl esters. The composition feature was quite similar to biodiesel from vegetable oil. Therefore, *R. glutinis* could be considered as a potential strain to convert lignocellulosic hydrolyzates into a raw material for biodiesel production.

Lipids samples produced by the fermenter fed-batch culture system were analyzed by gas chromatography. It is clear that *R. toruloides* Y4 is composed mainly of long-chain fatty acids with 16 and 18 carbon atoms. These data show that the distribution of some fatty acids, namely, C14:0 (myristic acid), C16:0 (palmitic acid), C16:1 (palmitoleic acid) C18:1 (oleic acid) and C18:3 (linolenic acid), were almost constant during the whole process. A clear decrease in the relative content of was found, while an increase for C18:0 (stearic acid) was observed over time. It is

also interesting to note that C18:2 (linoleic acid) showed an increased presence during the early stage, namely from 8.2% in the 6-h sample to 11.9% in the 18-h sample. However, linoleic acid content drastically decreased when lipids accumulation initiated and turned out to be 4.7 and 8.8%, in the 38 and 78-h samples, respectively. It is known that fatty acid distribution impacts on the saponification number (SN) and iodine value (IV) of the particular lipids (Kalayasiri *et al.*, 1996), which can also determine the cetane number (CN) of the corresponding biodiesel product (Li *et al.*, 2007).

CHAPTER 3

METERIALS AND METHODS

3.1 Materials

3.1.1 Microorganism

Rhodotorula glutinis TISTR 5159 obtained from the Thailand Institute of Scientific and Technological Research was used for the production of lipids and carotenoids.

3.1.2 Palm oil mill effluent (POME) and Crude glycerol

Palm oil mill effluent (POME) was obtained from Srijareon Palm oil mill (Krabi, Thailand). The original characteristics of POME after filtation by filter paper are listed as: pH 4.5, COD 48 g/L, total nitrogen 262 mg/L, reducing sugar 12 g/L, oil and grease 280 mg/L.

Crude glycerol was obtained from Prince of Songkla University biodiesel plant (Songkhla, Thailand) and kept at 4°C until used and adjusted pH to 6 prior to use. The COD in crude glycerol was 325 g/L. It contained 50% glycerol, 10% sodium chloride, 5% methanol, 5% organic matters, 25% water, and 5% other components (Sutharuk Bunchod, 2004). This indicated that the main carbon source in crude glycerol was glycerol.

3.2 Analytical method

3.2.1 Biomass concentration

The sample broth was centrifuged at 7500 rpm for 15 min to remove the cells. The cells precipitates were washed with acetone and resuspended in distilled water and centrifuged again to wash the cells. The washed cells were dried at 105 °C for 5 h in hot air oven and then weighed to constant weight after cooling in a desiccator (Kavadia *et al*., 2001).

3.2.2 Lipid extraction

The dry biomass was ground into a fine powder. One gram powder was blended with 3 mL chloroform/methanol (2:1) and the mixture was agitated during 20 min in an orbital shaker at room temperature. Solvent phase was recovered by centrifugation 5000 rpm 15 min. The same process was repeated three times. The whole solvent was evaporated and dried under vacuum (Bligh and Dyer, 1959).

$$\text{Lipid content (\%)} = \frac{\text{Oil weight (g)}}{\text{Cell dry weight (g)}} \times 100$$

3.2.3 Carotenoids determination

The washed cells were suspended in 10 ml of acetone and methanol (3:1) and disrupted with sonication at 70 Hz for 30 min. Solid material was removed by centrifugation 7500 rpm 15 min. The color of the supernatant was determined at 450 nm. The concentration of carotenoids present in the sample was determined by comparison with a standard calibration curve prepared from pure beta-carotene dissolved in acetone and methanol (3:1) (Aksu and Eren, 2005) (see Appendix 1).

3.2.4 Glycerol determination

The glycerol concentration was determined in the water phase of the reactor with KIO_4. One mL of sample and 0.4 mL of 0.0025 M KIO_4 were reacted for 5 min. Then, 0.1 mL of 0.5 M sodium arsenate was added. After 10 min, 9 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 placed in boiling water bath for 30 min. The cooled tubes were adjusted to a volume of 25 mL with water, and the absorbance at 570 nm was measured (Kosugi *et al.*, 1994) (see Appendix 2).

3.2.5 Determination of fatty acid composition by GC analysis

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 2x3 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. The fatty acids were identified by comparison of their retention times with those of standard ones, quantification being based on their respective peak areas and normalized (Jham *et al.*,1982) (see Appendix 4).

3.2.6 COD and TN determination

COD, total nitrogen (TN), oil and grease were determined according to the procedures used in standard methods (APHA, 2005) (see Appendix 6-10).

3.2.7 C/N ratio calculation

The nitrogen content in organic nitrogen sources was calculated by assuming that the yeast extract (Labscan, Spain), urea (Ajax Finechem Pty, Australia), and peptone (Ajax Finechem Pty, Australia) contained 11% (w/w), 46.65% (w/w), and 36.6% (w/w) of nitrogen, respectively.

The C/N ratio in POME was calculated from COD/TN. The C/N ratio in crude glycerol was calculated based on the molar basis of the carbon content from glycerol and the nitrogen content from nitrogen source. The formula used for the conversion of glycerol concentration from % (w/v) to mol carbon/L was [glycerol] (%) × 0.333 = mol carbon/L.

3.2.8 Statistical analysis

The statistical significance of the results was evaluated by one way ANOVA and Duncan's multiple range tests ($P<0.05$) using the SPSS version 10 software.

3.3 Experimental method

3.3.1 Inoculum preparation

A plate culture was incubated at 30°C for 24 h. The cells were transferred to 250 ml Erlenmeyer flasks containing 50 mL of culture medium (g/L, glucose 10, peptone 5, yeast extract 3, malt extract 3). The flasks were incubated at 30°C and 200 rpm for 24 h for seed culture.

3.3.2 Screening of nitrogen source and surfactant in shake-flask cultivation

The starter culture (10%) was transferred into the medium contained 90 mL diluted POME (COD = 10 g/L), which was sterilized at 121°C for 15 min. To study the effect of the nitrogen source, various organic and inorganic nitrogen sources, including yeast extract, peptone, urea, ammonium sulfate, ammonium chloride, and ammonium nitrate were each added to the diluted POME to obtain a COD/TN ratio of 160. Three surfactants at 1% concentration, Tween 20, Tween 80, and gum arabic, were screened for enhancing lipids and carotenoids production. All cultures were grown at 30℃ and 200 rpm for 72 h at least in duplicate. The samples were taken to determine biomass, lipids content, carotenoids production and COD removal at 0, 6, 12, 24, 36, 48, 60, 72 h of cultivation.

The starter culture (10%) was transferred into the medium contained 90 mL diluted crude glycerol (10% of glycerol concentration) The suitable nitrogen source from the previous experiment was added to obtain the C/N ratio of 60. The effect of addition of nitrogen source was compared with the control (without adding nitrogen source). Three surfactants at 1% concentration, Tween 20, Tween 80, and gum arabic, were screened for enhancing lipids and carotenoids production. All cultures were grown at 30℃ and 200 rpm for 72 h at least in duplicate. The samples

were taken to determine biomass, lipids content, carotenoids production and glycerol consumption at 0, 6, 12, 24, 36, 48, 60, 72 h of cultivation.

3.3.3 Medium optimization through RSM

A Box-Behnken design with three variables at three levels was followed to determine the response pattern and also to determine the synergy of the variables (Table 10 for POME based medium and Table 11 for crude glycerol based medium). According to this design, 15 runs were conducted containing three replications at the central point for estimating the purely experimental uncertainty variance. The relationship of the variables was determined by fitting a second order polynomial equation to data obtained from the 15 runs. A uniform design and analysis of data were done. The response surface analysis was based on the multiple linear regressions taking into account the main, quadratic and interaction effects, in accord with the following equation:

$$Y = \beta_0 + \sum \beta_i x_i + \sum \beta_{ii} x_i^2 + \sum \beta_{ij} x_i x_j \quad (1)$$

where Y is the predicted response, x_i and x_j represent the variables or parameters, β_0 is the offset term, β_i is the linear effect, β_{ij} is the first order interaction effect and β_{ii} is the squared effect. The goodness of fit of the model was evaluated by the coefficient of determination (R^2) and the analysis of variance (ANOVA). Response surface plots were developed to indicate an optimum condition. This was done by using the fitted quadratic polynomial equations obtained by holding one of the independent variables at a constant value and changing the levels of the other two variables.

Table 10. Experimental range and levels of the three independent variables used in RSM using POME based medium

Trial	Independent variables		
	COD (g/L)	C/N ratio (-)	Surfactant (g/L)
	x_1	x_2	x_3
1	1(40)	1(180)	0(1.0)
2	1(40)	-1(140)	0(1.0)
3	-1(10)	1(180)	0(1.0)
4	-1(10)	-1(140)	0(1.0)
5	1(40)	0(160)	1(1.5)
6	1(40)	0(160)	-1(0.5)
7	-1(10)	0(160)	1(1.5)
8	-1(10)	0(160)	-1(0.5)
9	0(25)	1(180)	1(1.5)
10	0(25)	1(180)	-1(0.5)
11	0(25)	-1(140)	1(1.5)
12	0(25)	-1(140)	-1(0.5)
13	0(25)	0(160)	0(1.0)
14	0(25)	0(160)	0(1.0)
15	0(25)	0(160)	0(1.0)

Table 11. Experimental range and levels of the three independent variables used in RSM using crude glycerol based medium

Trial	Independent variables		
	Glycerol (%)	C/N ratio (-)	Surfactant (g/L)
	x_1	x_2	x_3
1	1(14)	1(85)	0(1.0)
2	1(14)	-1(35)	0(1.0)
3	-1(5)	1(85)	0(1.0)
4	-1(5)	-1(35)	0(1.0)
5	1(5)	0(60)	1(1.5)
6	1(14)	0(60)	-1(0.5)
7	-1(5)	0(60)	1(1.5)
8	-1(5)	0(60)	-1(0.5)
9	0(9.5)	1(85)	1(1.5)
10	0(9.5)	1(85)	-1(0.5)
11	0(9.5)	-1(35)	1(1.5)
12	0(9.5)	-1(35)	-1(0.5)
13	0(9.5)	0(60)	0(1.0)
14	0(9.5)	0(60)	0(1.0)
15	0(9.5)	0(60)	0(1.0)

3.3.4 Batch fermentation in a 2L bioreactor

The batch fermentation was carried out in a 2L bioreactor (B. Braun Biotech International, Germany). The bioreactor contained 1L of the optimized medium from RSM. The culture conditions were 10% (v/v) seed culture and a temperature of 30°C. Agitation was provided by two turbine impellers located at 5 and 10 cm above the bottom of the vessel (Figure 4). The impeller speed was 100 rpm. The aeration rate was varied 0-3 vvm. The pH was monitored and maintained at 6.0 with 2.5 N NaOH.

Figure 4. 2L bioreactor

3.3.5 Semi-continuous using POME based medium

Since the concentration of organic matters in POME was not high enough to perform fed-batch, the semi-continuous mode was applied when using POME based medium. The start-up of the process was the same as for the batch operation. Following a growth period of 48 h, half of the content of the medium (500 mL) was removed and 500 mL of fresh medium was added. During the 336 h of total cultivation. The samples were taken at 0, 24, 48, 96, 144, 192, 240, 288, 336 h of cultivation. The time courses of biomass, lipids content, production of carotenoids, and COD removal were determined.

3.3.6 Fed-batch using crude glycerol based medium

Since the high concentration of glycerol would inhibit biomass and product formation, the fed-batch fermentation was attempted when using crude glycerol based medium. The aim of the fed–batch process was to avoid the substrate inhibition and enhance the production of lipids and carotenoids by feeding additional substrate. The culture with an initial working volume of 800 mL was first operated at batch mode. The start–up of the process was the same as with the batch operation. The feeding started in the middle of the exponential phase and every 12 h to maintain the concentration of glycerol at a optimal level obtained from RSM. The samples were taken at 0, 6, 12, 24, 36, 48, 60, 72 h of cultivation. The time courses of biomass, lipids yield per liter, carotenoids production and glycerol concentration were determined.

3.3.7 Scale up in 15 L bioreactor

The batch fermentation was carried out in a 15 L bioreactor. Agitation was provided by two turbine impellers located at 5 and 15 cm above the bottom of the vessel (Figure 5). The impeller speed was 100 rpm. The aeration rate was varied 0-3 vvm. The pH was monitored and maintained at 6.0 with 2.5 N NaOH. The aeration was controlled at optimum level obtained from section 3.3.4. The bioreactor contained 10 L optimized medium from RSM. The cultivation condition were 10% (v/v) seed culture. The temperature was 30°C. The samples were taken to determine biomass, lipids content, carotenoids production and COD removal for POME based medium

and glycerol consumption for crude glycerol based medium at 0, 6, 12, 24, 36, 48, 60, 72 h of cultivation.

Figure 5. 15 L bioreactor

3.3.8 Biodiesel production

The tranesterification process was performed on the product from the esterification process molar ratios of oil to methanol at 1 : 12 with 1 wt% of H_2SO_4 and a reaction time of 12 h) using KOH (1.5 wt% of KOH/oil) dissolved in methanol. The reactants were mixed in a 250 mL boiling flask equipped with a reflux condenser that was heated in heating system (Ma and Hanna, 1999).

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Production of lipids and carotenoids by oleaginous red yeast *Rhodotorula glutinis* cultured in palm oil mill effluent

4.1.1 Effect of nitrogen source

The low nitrogen level in the POME (262 mg/L) and the resultant high C/N ratio prompted the need for supplementation with an external source of nitrogen. Nitrogen sources have long been known to affect the growth and suppress the biosynthesis of lipids and other secondary metabolites. A number of researchers have tested the effect of a nitrogen source on biomass and the lipids content of various microorganisms (Li *et al*., 2008; Hansson and Dostalek, 1998). In this study, the POME was supplemented with several organic and inorganic nitrogen sources. The effect of the nitrogen source on biomass, lipids content and the production of carotenoids of *R. glutinis* TISTR 5159 are shown in Table 12. The addition of either organic or inorganic nitrogen sources was found to increase the biomass, lipids content and production of carotenoids. The highest values of biomass, lipids content and carotenoid concentration were obtained with yeast extract (6.33 g/L, 32.63 % and 129.94 mg/L, respectively) followed by ammonium sulfate (6.29 g/L, 29.15 % and 115.76 mg/L, respectively). While peptone, ammonium nitrate, and ammonium chloride, gave poor growth and lower lipids content and carotenoid production. The addition of yeast extract increased the production of carotenoids was also previously reported for *Rhodotorula* sp. cultured in sugarcane molasses (Bhosale and Gadre, 2001). The reason that yeast extract is an excellent nitrogen source for microorganism growth and production could be because it contains metal ions and other required micronutrients.

The biomass and lipids content obtained with ammonium sulfate were comparable to those with yeast extract. This suggested that certain essential amino acids could be synthesized from inorganic nitrogen sources by *R. glutinis* TISTR

5159. Although yeasts cannot fix molecular nitrogen, simple inorganic nitrogen sources are wildly utilized. Ammonium sulfate is a commonly used nitrogen source in yeast growth media since it also provides a source of assimilable sulphur. Yeasts require sulphur principally for the biosynthesis of sulphur-containing amono acids. Sulphur sources can be in the form of a variety of sulphur compounds including: sulphate, sulphite, thiosulphate, methionine and glutathione. However, inorganic sulphate and the sulphur amino acid methionine are the two compounds central to the sulphur metabolism of yeast (Ratledge, 2002).

The COD removal when using ammonium sulfate as a nitrogen source was also found comparable with yeast extract (Table 12). The possible conversion of organic compounds in POME to lipids and carotenoids by ***R. glutinis*** has importance for waste treatment and valorization. It was reported that the POME corresponds to carbohydrates in nature indicating the possible presence of pentose. The presence of pentose in POME has been reported previously and its most likely source is the palm cell wall (Habib ***et al.,*** 1997). Water-soluble carbohydrates, in terms of glucose, reducing sugars and pectin, are also found to be present in the soluble fractions of POME. Although yeast extract gave the highest biomass, lipids content and carotenoids production, its high cost was considered to have a negative impact on its economic use in industrial-scale processes. Therefore, in order to improve the economic parameters of lipids and carotenoids, cheaper ammonium sulfate was selected as a suitable nitrogen source for further study.

Table 12. Effects of nitrogen source on biomass, lipids content, lipids yield, carotenoids production and COD removal of *R. glutinis* TISTR 5159 cultured in POME

Nitrogen source at C/N ratio of 160	Biomass (g/L)	Lipids content (%)	Lipids yield (g/L)	Carotenoids production (mg/L)	COD removal (%)
Control	4.15±0.09^{e}	20.97±1.65^{c}	0.87±0.09^{c}	85.26±0.01^{f}	40.50±1.41^{f}
Yeast extract	6.33±0.01^{a}	32.63±0.08^{a}	2.07±0.01^{a}	129.94±1.68^{a}	65.13±1.20^{a}
Urea	5.44±0.01^{b}	29.11±1.41^{b}	1.58±1.10^{b}	109.11±1.55^{c}	53.92±1.40^{c}
Peptone	4.46±0.03^{d}	26.89±2.04^{b}	1.20±0.06^{b}	99.55±1.09^{d}	43.48±1.27^{e}
Ammonium sulfate	6.29±0.16^{a}	29.15±1.41^{b}	1.83±0.08^{b}	115.76±2.49^{b}	59.29±1.40^{b}
Ammonium nitrate	4.80±0.11^{c}	27.77±0.01^{b}	1.12±0.04^{b}	105.85±1.57^{c}	46.99±1.59^{d}
Ammonium chloride	4.19±0.04^{e}	26.82±1.27^{b}	1.33±0.03^{b}	92.74±1.24^{e}	43.00±1.41^{e}

Values are means ± SD (n=2). Control: the culture without addition of nitrogen source. Different letters in the same column indicate significant treatments difference (P<0.05).

4.1.2 Effect of surfactant

Some surfactants have been known to increase the productivity of lipids and carotenoids (Kim, 2006). The mechanism of this stimulation is not completely clear, but the agents appear to cause different alterations in membrane fluidity (Kruszewska *et al.*, 1990). The enhancement of cell permeability by surfactants depends on the chemical composition of the cell, such as the membrane sterol content (Dalmau *et al.*, 2000). In this study a number of surfactants, including Tween 20, Tween 80, and gum arabic at 1% concentration, were tested for the enhancement of lipids and carotenoid production of *R. glutinis* TISTR 5159. The yeast was cultivated in POME with the addition of ammonium sulfate as the nitrogen source. The results are shown in Table 13. There were significant increases in the amounts of biomass and lipids content when surfactant was added, compared to the control to which no surfactant was added. Of the three surfactants, only Tween 20 effectively increased both lipids content and carotenoid production. The COD removal was also improved by the addition of all surfactants. This could be due to the emulsifying action of surfactant on hydrocarbon based compounds, which altered

them down into more manageable molecules so that the microbes digest more efficiently (Barker and Worgan, 1981).

The highest amounts of biomass, lipids content and production of carotenoids (7.07 g/L, 38.15 %, 125.94 mg/L, respectively) as well as the highest COD removal (66.85 %) were obtained when Tween 20 was added to the POME. This result was in accord with reports for the performance of other microorganisms. It was reported that Tween 20, when added into a medium, stimulated the growth, improved the growth rate and hence enabled excretion of the product from out of cells (Stredanska and Sajbidor, 1993). Previous research has found that the use of Tween 20 as a carbon source increased lipids accumulation but it was not suitable for cell growth (Dalmau *et al.*,2000). In contrast, the addition of Tween 20 at 1% concentration in POME enhanced both cell growth and the production of lipids and carotenoids of *R. glutinis* TISTR 5159.

Table 13. Effects of surfactant on biomass, lipids content, lipids yield, carotenoid production and COD removal of *R. glutinis* TISTR 5159 cultured in POME added with ammonium sulfate as nitrogen source (ammonium sulfate 37.38 mg/L and surfactant 1%)

Culture condition	Biomass (g/L)	Lipids content (%)	Lipids yield (g/L)	Carotenoids production (mg/L)	COD removal (%)
Control	6.29 ± 0.16^{b}	29.15 ± 1.41^{c}	1.83 ± 0.04^{c}	115.76 ± 2.49^{b}	59.29 ± 1.40^{b}
$(NH_4)_2SO_4$+Tween 20	7.07 ± 0.23^{a}	38.15 ± 1.46^{a}	2.70 ± 0.11^{a}	125.94 ± 1.95^{a}	66.85 ± 1.57^{a}
$(NH_4)_2SO_4$+Tween 80	6.64 ± 0.28^{ab}	34.15 ± 1.37^{b}	2.27 ± 0.01^{b}	118.92 ± 1.33^{b}	65.12 ± 1.54^{a}
$(NH_4)_2SO_4$+Gum arabic	6.62 ± 0.42^{ab}	33.45 ± 1.41^{b}	2.21 ± 0.05^{b}	115.32 ± 1.09^{b}	64.43 ± 1.20^{a}

Values are means ± SD (n=2). Control: the culture without addition of surfactant. Different letters in the same column indicate significant treatments difference (P<0.05).

4.1.3 Medium optimization through RSM

Since most industrial experiments usually involve many variables, a full factorial design entails a large number of experiments. To reduce the number of experiments to a practical level, only a small set from all the possibilities is normally selected. The significance of the variables can be explained as follows. The basic physiology of lipids accumulation in microorganisms has been well studied. A nutrient imbalance in the culture medium is known to trigger lipids accumulation in oleaginous microorganisms. Lipids production requires a medium with an excess of sugar or similar components and limited other nutrients, usually nitrogen. Thus oleaginous potential is critically affected by the carbon-to-nitrogen (C/N) ratio of the culture. 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 lipids. In this study, POME was thought to be suitable for lipids production since it contains high COD and macro elements such as calcium, potassium, magnesium, sulfur and sodium (Ahmad *et al*., 2003). In addition to C/N ratio, the levels of initial COD and Tween 20 may also affect the growth and production of lipids and carotenoids of ***R. glutinis*** TISTR 5159.

Consequently, three main fermentation factors, COD (x_1), C/N ratio (x_2) and Tween 20 (x_3), were selected for the optimization of biomass, lipids content and carotenoid production of ***R. glutinis*** TISTR 5159 using RSM. The Box-Behnken design leading to a total 15 sets of experiments was carried out in this study (Table 14). The results obtained by the Box-Behnken design were analyzed by ANOVA (Table 15). The second order regression equations for biomass (Y_1), lipids content (Y_2) carotenoid production (Y_3) and lipids yield (Y_4) as a function of COD (x_1), C/N ratio (x_2) and Tween 20 (x_3) are given as follows:

$$\text{Biomass } (Y_1) = 6.8465 - 0.0614\,x_1 - 0.00979\,x_2 + 4.1166\,x_3 + 0.000861\,x_1^2 + 0.0000469\,x_2^2 - 0.7250\,x_3^2 + 0.000292\,x_1x_2 - 0.01167\,x_1x_3 - 0.01375\,x_2x_3 \quad (2)$$

$$\text{Lipids content } (Y_2) = -54.6700 + 0.8510\,x_1 + 1.0230\,x_2 - 20.5300\,x_3 - 0.00869\,x_1^2 - 0.00292\,x_2^2 + 1.9520\,x_3^2 - 0.00313\,x_1x_2 + 0.04067\,x_1x_3 + 0.1090\,x_2x_3 \quad (3)$$

$$\text{Carotenoid production } (Y_3) = -182.6500 - 0.05528\,x_1 + 4.3270\,x_2 - 110.8700\,x_3 - 0.03069\,x_1^2 - 0.01367\,x_2^2 + 48.3900\,x_3^2 + 0.00939\,x_1x_2 + 0.1200\,x_1x_3 + 0.09575\,x_2x_3 \quad (4)$$

$$\text{Lipids yield } (Y_4) = -2.959 + 0.04606x_1 + 0.05396x_2 + 0.06083x_3 - 0.0000963x_1^2 - 0.000135x_2^2 - 0.227x_3^2 - 0.000225x_1x_2 + 0.00375x_2x_3 + 0.000894\,x_1x_3 \quad (5)$$

The models fitted satisfactorily to the experimental data as indicated by their goodness of fit expressed by R^2 and P values. The R^2 values of the models for Y_1, Y_2 Y_3 and Y_4 were 0.93, 0.97 0.93, and 0.92 respectively. This indicated that up to 93–97% of the variations in biomass, lipids content carotenoid production and lipids yield can be explained by these equations. The coefficients of variance (CV) indicate the degree of precision with which the experiments are compared. The lower reliability of the experiment is usually indicated by high value of CV (> 15). In the present case, acceptable CV values were observed for the model of biomass, lipids content, carotenoid production and lipids yield (3.61, 4.89, 5.75 and 5.67 respectively). This denotes that the experiments performed were reliable. The P values of the models for biomass, lipids content production of carotenoids and lipids yield were 0.020, 0.024, 0.003 and 0.024 respectively. The P value ≤ 0.05 indicates the significance of the coefficients. The statistical significance of the model equation

was also confirmed by *F* values of the model, which were 49.00, 40.53 269.4 and 67.20 for biomass, lipids content, carotenoid production and lipids yield, respectively.

Further statistical analysis showed that the C/N ratio (x_2) had a significant effect on lipids carotenoid production and lipid yield, while COD (x_1) had a significant effect on biomass ($P<0.05$) (Table 15). The terms of x_1x_2 and x_2x_3 indicating the interactions of C/N ratio with COD and Tween 20, respectively, were also found significant for lipids, carotenoid production and lipid yield. On the other hand, the effect of Tween 20 concentration (x_3) was found insignificant on all responses in the range of examined intervals. Thus, the mathematical model was simplified by using Tween 20 concentration (x_2) terms at optimum value (1.5 g/L). Then, in further regression analysis, the main, quadratic and interaction effects of x_1 and x_2 were maintained. The second order regression equation for biomass (Y_1), lipids content (Y_2) carotenoid production (Y_3) and lipids yield (Y_4) as a function of COD (x_1) and C/N ratio (x_2) generated a regression relationship as given in Eqs. (6-9).

$$Y_1 = 11.39028 - 0.0788\,x_1 - 0.0304\,x_2 + 0.000861\,x_1^2 + 0.0000468\,x_2^2 + 0.000292\,x_1x_2 \quad (6)$$

$$Y_2 = -81.073 + 0.912\,x_1 + 1.186\,x_2 - 0.00869\,x_1^2 - 0.00292x_2^2 - 0.00313\,x_1x_2 \quad (7)$$

$$Y_3 = -204.077 + 0.1247\,x_1 + 4.470\,x_2 - 0.03069\,x_1^2 - 0.01367\,x_2^2 + 0.00939\,x_1x_2 \quad (8)$$

$$Y_4 = -3.3777 + 0.04606\,x_1 + 0.0595\,x_2 - 0.0000963\,x_1^2 - 0.000135\,x_2^2 - 0.000225\,x_1x_2 \quad (9)$$

Regression models were employed to develop response surface plots as shown in Figure 6. The response surface and contour plots of biomass, lipids content carotenoid production and lipid yield illustrated the effects of COD and C/N ratio. Based on response surface plots, the interaction between two variables and their optimum levels can be easily understood and located. Figure 6A shows that biomass increased with increasing COD up to 40 g/L and decreasing the C/N ratio to 140. On

the contrast, the maximum lipids content was obtained at moderate COD of 25 g/L and when increasing C/N ratio up to 180 (Figure 6B).

Table 14. Experimental range and levels of the three independent variables used in RSM in terms of actual and coded factors and experimental data for the three-factor with three-level response surface analysis

Trial	Independent variables			Dependent variables			
	COD (g/L)	C/N ratio (-)	Tween 20 (g/L)	Biomass (g/L)	Lipids content (%)	Carotenoids production (mg/L)	Lipids yield (g/L)
	x_1	x_2	x_3	Y_1	Y_2	Y_3	Y_4
1	1(40)	1(180)	0(1.0)	7.54	37.50	124.26	2.83
2	1(40)	-1(140)	0(1.0)	8.05	35.13	116.56	2.83
3	-1(10)	1(180)	0(1.0)	7.33	38.48	118.58	2.82
4	-1(10)	-1(140)	0(1.0)	7.65	33.35	122.15	2.55
5	1(40)	0(160)	1(1.5)	7.85	36.92	139.56	2.90
6	1(40)	0(160)	-1(0.5)	7.82	35.55	138.28	2.78
7	-1(10)	0(160)	1(1.5)	7.60	35.88	135.82	2.73
8	-1(10)	0(160)	-1(0.5)	7.30	35.73	138.15	2.61
9	0(25)	1(180)	1(1.5)	7.50	42.56	155.23	3.19
10	0(25)	1(180)	-1(0.5)	7.55	37.40	144.29	2.82
11	0(25)	-1(140)	1(1.5)	7.50	34.03	132.58	2.55
12	0(25)	-1(140)	-1(0.5)	7.00	33.24	125.47	2.33
13	0(25)	0(160)	0(1.0)	7.50	37.16	132.57	2.79
14	0(25)	0(160)	0(1.0)	7.60	37.86	132.32	2.88
15	0(25)	0(160)	0(1.0)	7.55	37.44	133.39	2.83

Note: values in parentheses are the actual independent variables.

Table 15. Regression of coefficients and analysis of variance of the second order polynomial for response variables

Coefficient	Biomass (g/L) Y_1	Lipids content (%) Y_2	Carotenoids (mg/L) Y_3	Lipids yield (g/L) Y_4
β_0	6.8465*	-54.6700*	-182.6500*	-2.959*
Linear				
x_1	-0.0614*	0.8510	-0.05528	0.0460
x_2	-0.00979	1.0230*	4.3270 *	0.0539*
x_3	4.1166	-20.5300	-110.8700	0.0608
Interaction				
x_1x_2	0.000292	-0.00313*	0.00939*	-0.000225*
x_1x_3	-0.01167	0.04067	0.1200	0.00008
x_2x_3	-0.01375	0.1090*	0.09575*	0.0037*
Quadratic				
x_1^2	0.000861*	-0.0869	-0.03069	0.000096
x_2^2	0.0000469	-0.00292*	-0.01367*	-0.000135*
x_3^2	-0.7250	1.9520	48.3900	-0.227
Variability				
R^2 of model	0.93	0. 97	0.93	0.92
F value of model	49.00	40.53	269.4	67.20
P >F	0.020	0.024	0.003	0.024
CV of model	3.61	4.89	5.75	5.67

* means significant at 5% level. x_1, x_2 and x_3 are COD, C/N ratio and Tween 20 concentration, respectively

The maximum production of carotenoids was obtained at COD of 30 g/L and relatively high C/N ratio of 170 (Figure 6C). When the C/N ratio remained constant the production of lipids and carotenoids decreased with an increase in the COD above the optimum levels. Since the optimum condition for biomass was different from that for lipids content, the lipids yield per liter (biomass × lipids content) from each optimal condition was compared (Table 16). The optimum condition for lipids content (COD of 25 g/L and C/N ratio 180) gave the highest lipids yield of 3.00 g/L. Although the optimum condition for biomass (COD of 40 g/L and C/N ratio 140) gave the highest biomass of 7.91 g/L, only 32.78% of lipids content was accumulated and resulted in a lower lipids yield of 2.59 g/L. In addition, since the optimum condition for carotenoid production (COD of 30 g/L and C/N ratio of 170) was similar to that for lipids content, a comparable lipids yield of 2.96 g/L was obtained. The lipids content of *R. glutinis* TISTR 5159 using POME in this study was higher than that reported by Xue *et al*. (2007). In their study, only 20% of lipids was accumulated by *R. glutinis* when using monosodium glutamate wastewater as the culture medium. This might be due to the higher carbon content in POME compared to that in monosodium glutamate wastewater.

The level of lipids synthesis in oleaginous microorganisms depends mostly on a high C/N ratio (Ratledge, 1982; Sattur and Karanth, 1989). This is attributed to the induction of nitrogen-scavenging reactions, the effect of which is lowered levels of Adenosine monophosphate (AMP). This consequently disrupts the citric acid cycle due to dependence of the isocitrate dehydrogenase reaction on AMP. The influence of the C/N ratio on carotenoid production has, however, remained unevaluated until now. A study by Somashekar and Joseph (2000), found that a medium with a high C/N ratio tended to produce lipids rather than carotenoids. As the culture growth progresses, a change in the C/N ratio is expected with lower levels of nitrogen. The conditions for lipids and carotenoid production might become available at the later stage of culture growth.

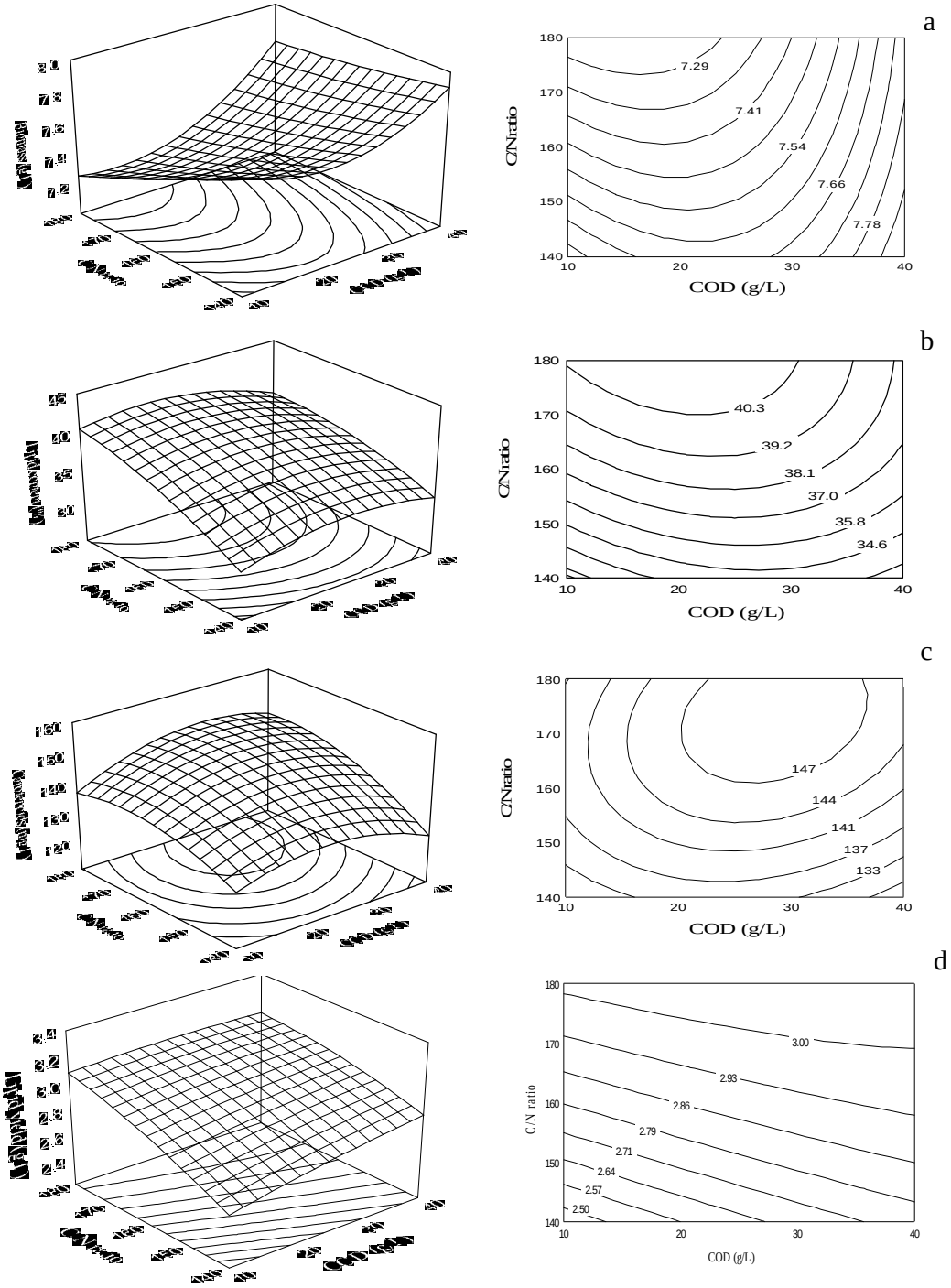

Figure 6. Response surface plots and contour plots for the effect of COD (x_1) and C/N ratio (x_2) on biomass (a), lipids (b), carotenoids (c) and lipid yield (d) when Tween 20 concentration was fixed at 1.5 g/L.

Table 16. Comparison of predicted responses from each optimum condition with different criteria

Criteria	Biomass (g/L)	Lipids content (%)	Lipids yield (g/L)	Carotenoids (mg/L)
Maximum biomass[a]	7.91	32.78	2.59	126.26
Maximum lipids content[b]	7.32	41.08	3.00	147.81
Maximum carotenoids[c]	7.47	39.74	2.96	148.77

[a]COD of 40 g/L and C/N ratio of 140; [b]COD of 25 g/L and C/N ratio of 180; [c]COD of 30 g/L and C/N ratio of 170.

The increased growth, lipids content and carotenoid production of ***R. glutinis*** TISTR 5159 cultured in POME were obtained when sufficient Tween 20 as an activator was supplied with a suitable COD and C/N ratio. This suggests that Tween 20 might influence the enzyme activities in the lipids and carotenoid biosynthetic pathway of ***R. glutinis*** TISTR 5159. It was reported that several enzymes involved in lipids and carotenoid biosynthesis in prokaryotic and eukaryotic cells are stimulated by Tween 20 (Dalmau, 2000). These include, for example, phytoene desaturase, β-carotene hydroxylase and lycopene cyclase.

Bioprocesses for lipids production may be designed on the ability for ***R. glutinis*** to grow and accumulate large amounts of lipids. In this study, the predicted condition for maximizing lipids content from RSM was experimentally tested in a one-stage process. A two-stage process was also attempted and compared to the one-stage process, in which cell growth was promoted in the first stage and the lipids production was then enhanced in the second stage. The experimental results are shown in Table 17. The first stage corresponded to the maximum biomass production obtained with high COD of 40 g/L and low C/N ratio of 140. At this stage, the highest biomass of 8.58 g/L was obtained while the lipids content and carotenoid production were 34.99% and 126.51 mg/L, respectively. The second stage corresponded to the establishment of lipids accumulation with COD of 25 g/L and high C/N ratio of 180. At this stage, the lipids content and carotenoid were enhanced up to 51.85 % and 176.45 mg/L, respectively. Hence, the overall lipids yield and carotenoids were increased from 3.10 g/L and 153.73 mg/L in the one-stage process up to 4.58 g/L and

176.45 mg/L or 1.5 fold and 1.2 fold, respectively, in the two-stage process. The two-stage system was also found suitable for the production of astaxanthin by *Haematococcus pluvialis* (Aflalo *et al.*, 2007).

Table 17. Experimental results of biomass, lipids content, lipids yield and carotenoids production by *R. glutinis* TISTR 5159 in one and two-stage processes

Strategy/Criteria	Biomass (g/L)	Lipids content (%)	Lipids yield (g/L)	Carotenoids (mg/L)
One-stage process[a]				
Maximum lipids content	7.59±0.37	40.85±1.83	3.10±0.14	153.73±12.01
Two-stage process[b]				
First stage	8.58±0.25	34.99±0.19	3.00±0.05	126.51±2.13
Second stage	8.82±0.18	51.85±0.64	4.58±0.11	176.45±3.19

[a] The cell was grown under condition for maximum lipids content for 72 h; [b]the cell was grown under condition for maximum biomass for 48 h in the first stage and then transferred to be cultured under condition for maximum lipids content for 24 h in the second stage.

4.1.4 Effect of pH control and aeration rate

To further increase the growth and production of lipids and carotenoids, batch fermentation was carried out in a 2L bioreactor equipped with pH control and aeration systems (Figure 7). Since the two-stage process was complicated and not practical in large scale production, the conditions for maximizing lipids production in one-stage process were applied.

The pH level is one of the most important environment parameters affecting cell growth and product formation. In general, the effects of pH on cell growth and product accumulation vary with different microorganisms, medium composition, and operational conditions. Some literatures dealing with the condition of *R. glutinis* reported the optimal pH for cell growth and accumulation of lipids and carotenoids in a shake flask (Meyerand Preez, 1994; Chan and Ho, 1999). To date, there have no been reports about the effects of pH control on cell growth, lipids content, and carotenoids production in a lab–scale fermentor. Figure 7 shows the time

courses of pH, cell growth, lipids yield, carotenoids production, and glycerol consumption, in a 2L stirred tank bioreactor with and without pH control at 6.0. The agitation speed was controlled at 100 rpm without aeration. Without pH control, the pH in the medium declined from 6.0 to 4.53 which may indicate the production of acids. When the pH was controlled by adding sodium hydroxide, there was a slight increase in lipids yield, carotenoids production and glycerol consumption. This could be because the inhibitory effect of low pH was alleviated. The lipids content was also enhanced from 48.41 % up to 54.18 % when the pH was kept constant.

As *R. glutinis* is an aerobic microorganism, aeration plays a major role in the growth rate, lipids content and carotenoids production since the oxygen transfer becomes a limiting factor when the cell grow 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 (Wu *et al.*, 2005; Mantzouridou *et al.*, 2002). The effects of aeration rate on cell growth, lipids yield, carotenoids production and glycerol consumption were examined (Figure 8). The aeration rate had a profound effect on biomass, consequently lipids production and glycerol consumption. Biomass and lipids yield significantly increased when increasing aeration rate from 0 to 2 vvm. At an aeration rate of 2 vvm, the biomass and lipids content reached 9.15 g/L and 60.62%, respectively, at 48 h of fermentation. This thus led to a lipids yield of 5.55 g/L. The carotenoid concentration was also increased up to 188.31 mg/L. The COD removal in batch fermentation was also evaluated and it was found that 69.60 % of the COD was reduced.

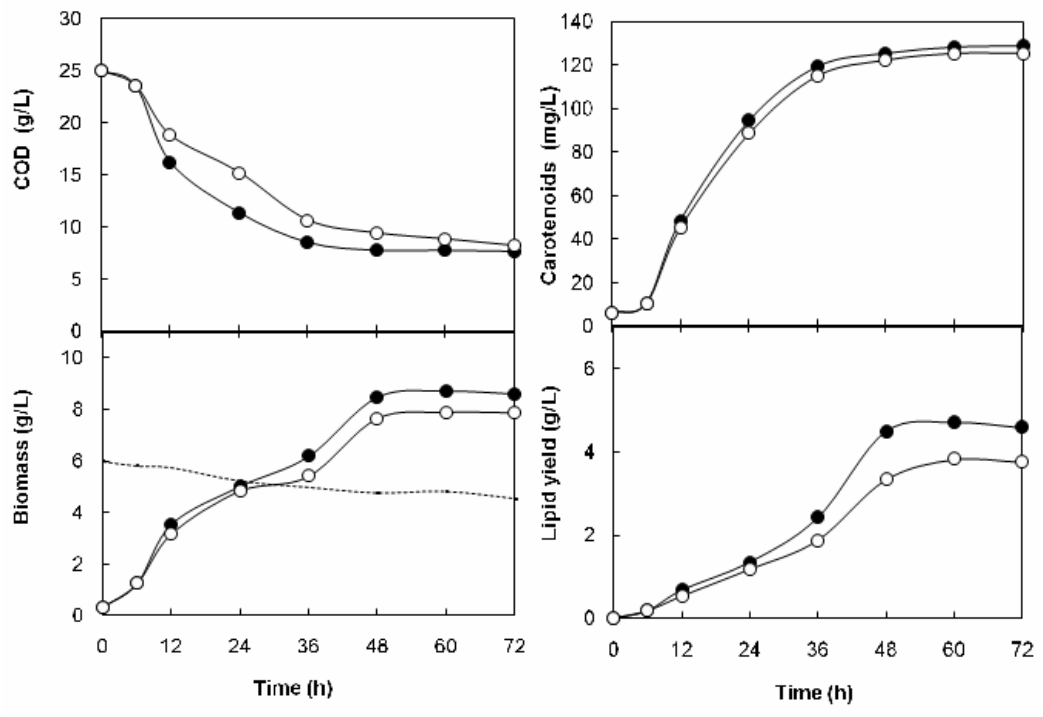

Figure 7. Effect of pH control on biomass, lipids yield, carotenoids production and COD removal by *R. glutinis* TISTR 5159 in a stirred tank bioreactor. Without pH control (open circle) and pH control (filled circle). The dashed line shows the profile of pH under the condition without pH control.

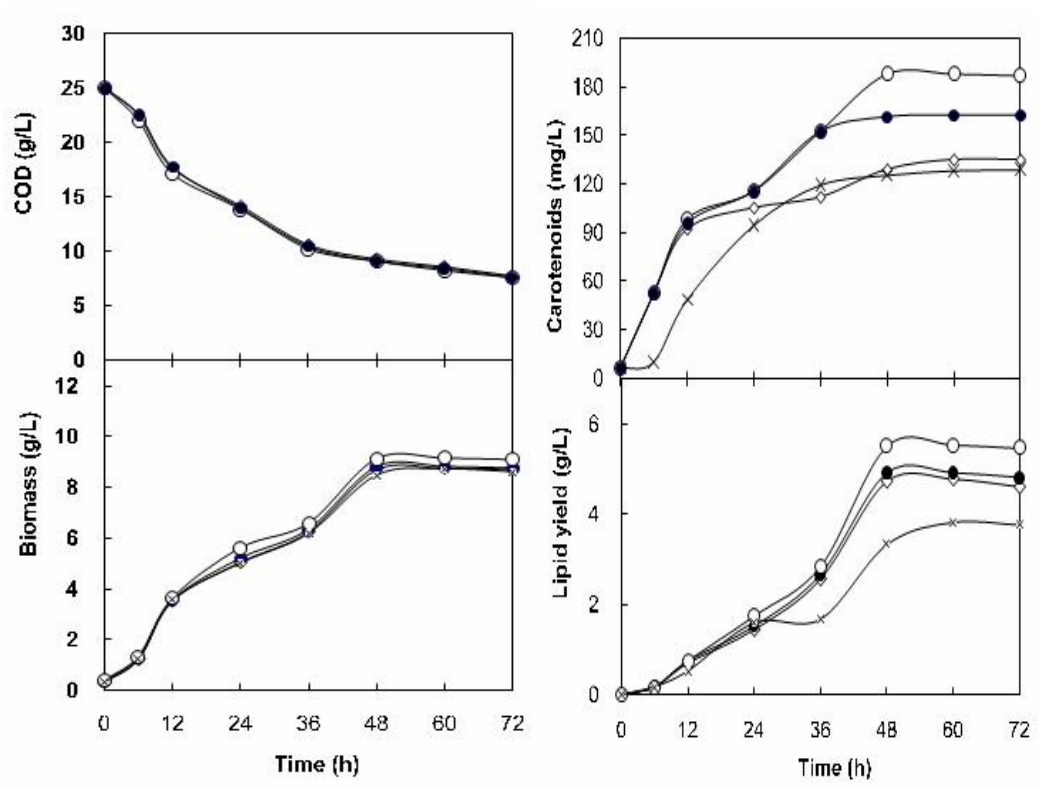

Figure 8. Effect of aeration rate on biomass, lipids yield, carotenoids production and COD removal by *R. glutinis* TISTR 5159 in a stirred tank bioreactor. The aeration rate was varied at 0 vvm (cross), 1vvm (open diamond), 1.5 vvm (filled circle) and 2 vvm (open circle).

4.1.5 Batch and semi-continuous fermentation

As the low value of COD in POME (48 g/L), the semi-continuous mode was suitable for using POME based medium. In semi-continuous fermentation, a portion of the culture is withdrawn at intervals and fresh medium is added to the system. This method has the advantages of the continuous and batch operations. There is no need for a separate inoculum vessel, except at the initial startup. Furthermore, time is not wasted in through non-productive activities such as cleaning and resterilization. Another advantage of this operation is that little control is required (Li *et al.*, 2010)**.** In the semi-continuous fermentation of ***R. glutinis*** TISTR 5159 cultured in POME, the first fill-and-withdraw operation was introduced at 48 h and repeated five times during the 336 h of fermentation. Time courses of biomass, COD, carotenoid, lipids yield and lipids content are shown in Figure 9. The specific rates include the specific growth rate, specific COD removal rate, specific production rates of carotenoids and lipids are shown in Figure 10. It was obvious that cells grew fast during the initial stage. The biomass increased rapidly from 0.37 to 9.3 g/L within 48 h. In this period, cell growth was linear and biomass increased with the highest specific growth rate of 0.024 h^{-1}. In the periods 48-96 h and 96-144 h the specific growth rate decreased to 0.016 h^{-1}, and after 144 h it further decreased and fluctuated at the levels of 0.009-0.013 h^{-1}. Therefore, the increase in biomass after 144 h was smaller than that before 144 h.

The maximum lipids yield of 7.4 g/L and maximum carotenoid concentration of 188.55 mg/L were achieved at 144 h. This was due to the maximum biomass of 10.9 g/L and the high lipids content of 67.27% obtained at 144 h. The lipids content was maintained above 60% until 204 h and it gradually decreased and reached 30% at the end of cultivation. Final biomass, lipids yield and carotenoid production were 7.5 g/L, 2.25 g/L and 77.81 mg/L, respectively. The effective COD removal was continued throughout the 336 h of cultivation with the overall specific COD removal rate of 0.06 g/g-cell/h. The specific lipids production rate was highest at 0.021 g/g-cell/h in the initial period of 0-48 h and reduced to 0.011 g/g-cell/h after 48 h and to 0.004 g/g-cell/h after 144 h. The specific carotenoid production rate was highest at 0.269 mg/g-cell/h in the initial period of 0-48 h and reduced to 0.110 mg/g-cell/h after 96 h and to 0.062 mg/g-cell/h after 144 h. Although, the effective COD

removal and moderate cell growth were observed during 144-336 h, the lipids and carotenoid producing ability drastically decreased. In the total volume of fermentation 4 L, biomass was 34.2 g, lipids yield was 19 g and carotenoids was 590 mg. This phenomenon could be explained that the cells might use most of the consumed substrate to maintain their activities in a long-term cultivation rather than accumulate as lipids and carotenoids.

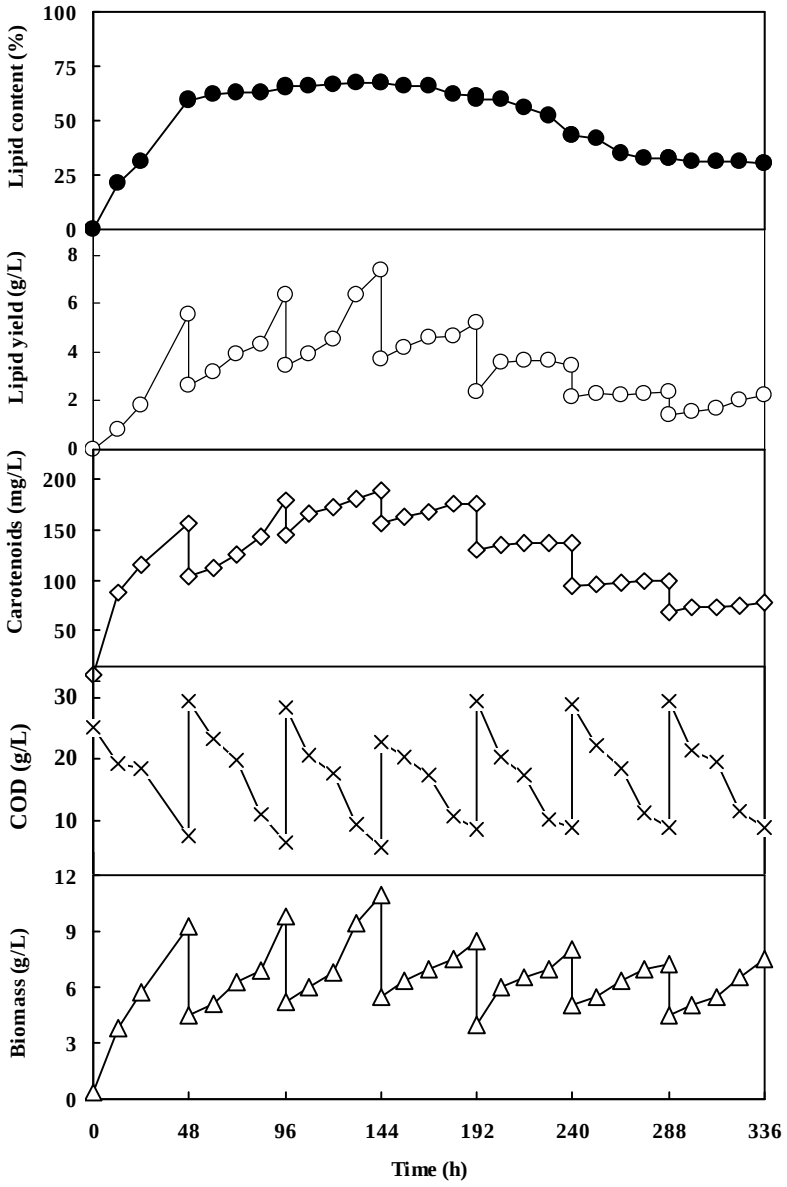

Figure 9. Semi-continuous fermentation of *R. glutinis* TISTR 5159 with the optimized medium in a stirred bioreactor. The pH was controlled at 6.0 and the aeration rate was 2.0 vvm. The fill-and-draw operation started at 48 h and the interval of reset was 48 h. Biomass (open triangle), lipids yield per liter (open circle), lipids content (close circle), carotenoids (open diamond), COD (cross).

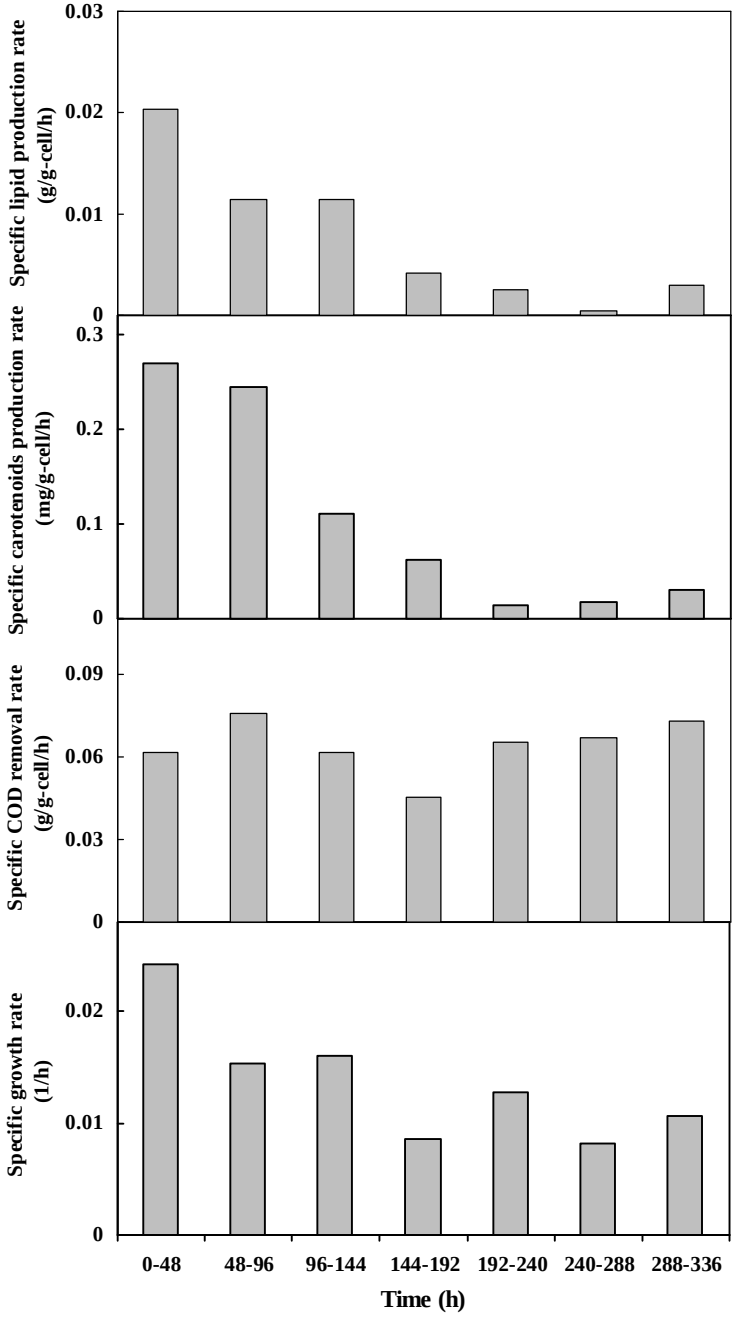

Figure 10. Time courses on specific growth rate, specific COD removal rate, specific carotenoid production rate and specific lipids production rate of *R. glutinis* TISTR 5159 in semi-continuous fermentation.

4.2 Production of lipids and carotenoids by oleaginous red yeast *Rhodotorula*

***glutinis* cultured in crude glycerol from biodiesel plant**

4.2.1 Screening of surfactant

Some agents, such as detergent additives, oil and surfactants have been known to increase lipids productivity (Kim *et al.*, 2006). The mechanism of this stimulation is not completely clear, but the agents appear to cause different alterations in membrane fluidity (Kruszewska *et al.*, 1990). In this study, the crude glycerol was diluted to obtain 10% glycerol concentration. Ammonium sulfate was then added as a low cost nitrogen source to obtain a C/N ratio of 60. A number of surfactants including Tween 80, Tween 20 and gum arabic at 1% concentration were tested for the enhancement of lipids and carotenoids production by *R. glutinis* TISTR 5159. The results are shown in Table 18. It was found that the crude glycerol could serve as a sole carbon source for *R. glutinis* TISTR 5159 without the addition of either nitrogen source or surfactant. The reason could be because it contained macro elements such as calcium, potassium, magnesium, sulfur and sodium (Bodour *et al*., 2003).

When ammonium sulfate was added as a nitrogen source to crude glycerol, the biomass, lipids content and carotenoids production were about increased 1.4-1.5 folds. The addition of nitrogen source was also required for the production of carotenoids by *Dietzia natronolimnaea* HS-1 (Khodaiyan *et al.*, 2008). Further increases in biomass, lipids content and carotenoids production were observed when surfactant was added. Among the three investigated surfactants, Tween 20 was found to be the most effective in increasing both lipids content and carotenoids production. It gave the highest amounts of biomass, lipids content and carotenoids production (5.47 g/L, 35.22 % and 101.94 mg/L, respectively). The enhancement of cell permeability by surfactants was dependent on the chemical composition of cell like membrane sterol content, the effect of the surfactants can be different according to the cells used (Lee *et al*., 1996). Moreover, the supply of Tween 20 may influence the enzyme activities in the lipids and carotenoids biosynthetic pathway of the yeast. Several enzymes involved in lipids and carotenoids biosynthesis in prokaryotic and eukaryotic cells, for examples, phytoene desaturase, β–carotene hydroxylase and lycopene cyclase are stimulated by activator (Nemec and Jernejc, 2002). Table 18 also shows that the glycerol consumption was improved by the addition of ammonium

sulfate and surfactant. The highest glycerol consumption of 47.15% was obtained with the addition of Tween 20. This could be because the surfactant emulsified hydrocarbon-based compounds, breaking them down into more manageable molecules, so that the microbes can then more efficiently digest (Bodour *et al*., 2003). This result was in accordance with the reports for the performance of other microorganisms. It was reported that Tween 20 added into the medium stimulated the growth, improved the growth rate and hence enabled the excretion of the product out of cells (Nemec and Jernejc, 2002). Previous research has found that Tween 20 as a carbon source was suitable for lipids production but it was not good for cell growth (Dalmau *et al*., 2000). In this study, the addition of Tween 20 enhanced both cell growth and product accumulation.

Table 18. Effect of surfactant on biomass, lipids content, lipids yield, carotenoid production, and glycerol consumption of *R. glutinis* TISTR 5159 cultured in crude glycerol added with ammonium sulfate (C/N ratio of 60) as a nitrogen source and various surfactants

Culture condition	Biomass (g/L)	Lipids content (%)	Lipids yield (g/L)	Carotenoids production (mg/L)	Glycerol consumption (%)
Control	3.06±0.50^{d}	16.12±1.28^{d}	0.49±0.03^{d}	65.86±2.23^{c}	29.65±1.55^{d}
$(NH_4)_2SO_4$	4.53±0.31^{c}	23.05±0.99^{c}	1.04±0.04^{c}	97.76±1.25^{b}	39.48±2.36^{c}
$(NH_4)_2SO_4$ +Tween 20	5.47±0.25^{a}	35.22±0.64^{a}	1.93±0.12^{a}	108.94±2.14^{a}	47.15±1.22^{a}
$(NH_4)_2SO_4$ +Tween 80	5.26±0.34^{b}	33.33±1.25^{b}	1.75±0.06^{b}	99.12±3.21^{b}	45.60±2.21^{b}
$(NH_4)_2SO_4$ +Gum arabic	5.22±0.21^{b}	31.18±2.44^{b}	1.63±0.20^{b}	98.77±1.14^{b}	43.14±2.32^{b}

Values are means ± SD (n=2). Control: crude glycerol without addition of either ammonium sulfate or surfactant. Different letters in the same column indicate significant treatments difference ($P< 0.05$).

4.2.2 Medium optimization through RSM

The basic physiology of lipids accumulation in microorganisms has been well studied. A nutrient imbalance in the culture medium is known to trigger lipids accumulation in oleaginous microorganisms. It is known that lipids production requires a medium with an excess of the carbon source and limited other nutrients, usually nitrogen. Thus the oleaginous potential is critically affected by the ratio of carbon and nitrogen sources (C/N ratio) of the culture. At a low C/N ratio, the carbon flux is distributed to allow cellular proliferation. Hence, a high number of cells could be obtained but with a low lipids content. While at a high C/N ratio, poor growth is observed and when the cells run out of nitrogen, they cannot multiply. The excess carbon substrate is then assimilated continuously to produce storage lipids (Li *et al.*, 2006). Although a high content of lipids is obtained, the lipids yield per liter might be low due to the low numbers of the cells. Therefore, it is important to determine the optimal condition for both biomass and lipids accumulation.

In this study, three main factors, namely glycerol concentration, C/N ratio and Tween 20 concentration, were selected for the optimization of biomass, lipids content and carotenoids production. The Box–Behnken experiment design led to a total 15 sets of experiments. The low, middle, and high levels of each variable and the experimental design and respective experimental results are given in Table 19. The results obtained by the Box–Behnken design were analyzed by ANOVA (Table 20).

The models fitted satisfactorily with the experimental data as indicated by their goodness of fit expressed by R^2 and P values (Table 20). The R^2 values of the models for Y_1, Y_2, Y_3, Y_4 were 0.98, 0.96, 0.95 and 0.95, respectively. This indicated that up to 95–98% of the variations in biomass, lipids content and carotenoids concentration can be explained by these equations. The coefficient of variation (CV) value indicates the degree of precision with which the experiments are compared. The lower reliability of the experiment is usually indicated by a high value of CV (> 15).

The second order regression equations for biomass (Y_1), lipids content (Y_2) carotenoids production (Y_3) and lipids yield (Y_4) as a function of glycerol concentration (x_1), C/N ratio (x_2) and Tween 20 concentration (x_3) are given as follows:

$$\text{Biomass } (Y_1) = 3.952 + 0.3480x_1 + 0.0382x_2 - 2.3300x_3 - 0.0209x_1^2 - 0.00035x_2^2 + 1.0530x_3^2 + 0.000044x_1x_2 + 0.0046\,x_2x_3 + 0.0056\,x_1x_3 \quad (10)$$

$$\text{Lipids content } (Y_2) = 20.2600 + 3.3100x_1 + 0.1270x_2 - 6.1550x_3 - 0.1790x_1^2 - 0.000709x_2^2 + 2.5780x_3^2 + 0.00131\,x_1x_2 + 0.02240\,x_2x_3 + 0.00889x_1x_3 \quad (11)$$

$$\text{Carotenoids } (Y_3) = 103.6100 + 4.7290x_1 - 0.7080x_2 - 37.0900x_3 - 0.2060x_1^2 + 0.00937x_2^2 + 16.0700x_3^2 - 0.01813x_1x_2 + 0.0960\,x_2x_3 + 0.1260\,x_1x_3 \quad (12)$$

$$\text{Lipids yield } (Y_4) = 0.570 + 0.307x_1 + 0.02026x_2 - 1.168x_3 - 0.01716x_1^2 - 0.000164x_2^2 + 0.510x_3^2 + 0.000044x_1x_2 + 0.0032x_2x_3 + 0.00222\,x_1x_3 \quad (13)$$

In the present case, acceptable CV values were observed for the model of biomass, lipids content, carotenoids production and lipids yield (7.14, 3.48, 5.92, and 7.55, respectively). This denotes that the experiments performed were reliable. The *P* values of models for biomass, lipids content, carotenoids production and lipids yield were 0.002, 0.013 0.028 and 0.024 respectively. The *P* values of the models ($\leq$ 0.05) indicate the significance of the coefficients.

Further statistical analysis in Table 20 showed that only the C/N ratio (x_2) had a significant effect on all the responses ($P<0.05$). The interaction terms of x_1x_2 and x_2x_3 were found significant for lipids content and lipid yield but not for carotenoids production. Among three variables, the effect of Tween 20 concentration in the range of examined intervals was found insignificant for all the responses. Thus,

the mathematical model was simplified by using term of Tween 20 concentration (x_2) at optimum value of 1.5 g/L.

Table 19. Experimental range and levels of the three independent variables used in RSM in terms of coded and actual factors and experimental data for the three-factor with three-level response surface analysis

Trial	Independent variables			Dependent variables			
	Glycerol (%)	C/N ratio (-)	Tween 20 (g/L)	Biomass (g/L)	Lipids content (%)	Carotenoids production (mg/L)	Lipids yield (g/L)
	x_1	x_2	x_3	Y_1	Y_2	Y_3	Y_4
1	1(14)	1(85)	0(1.0)	4.30	35.79	99.36	1.54
2	1(14)	-1(35)	0(1.0)	4.35	33.34	95.63	1.45
3	-1(5)	1(85)	0(1.0)	5.25	36.65	105.48	1.92
4	-1(5)	-1(35)	0(1.0)	5.32	34.79	93.59	1.85
5	1(5)	0(60)	1(1.5)	5.46	36.83	97.47	2.01
6	1(14)	0(60)	-1(0.5)	5.34	36.67	95.28	1.96
7	-1(5)	0(60)	1(1.5)	5.22	35.75	97.51	1.87
8	-1(5)	0(60)	-1(0.5)	5.15	35.67	96.45	1.84
9	0(9.5)	1(85)	1(1.5)	5.65	42.84	122.23	2.42
10	0(9.5)	1(85)	-1(0.5)	5.42	41.54	117.43	2.25
11	0(9.5)	-1(35)	1(1.5)	5.45	36.73	93.58	2.00
12	0(9.5)	-1(35)	-1(0.5)	5.45	36.55	93.58	1.99
13	0(9.5)	0(60)	0(1.0)	5.48	39.12	95.33	2.14
14	0(9.5)	0(60)	0(1.0)	5.44	39.08	97.58	2.13
15	0(9.5)	0(60)	0(1.0)	5.44	39.44	97.58	2.15

Note: values in parentheses are the un–coded independent variables.

Table 20. Regression of coefficients and analysis of variance of the second order polynomial for response variables

Coefficient	Biomass Y_1	Lipids content Y_2	Carotenoids Y_3	Lipids yield Y_4
β_o	3.952*	20.2600*	103.6100*	0.570*
Linear				
x_1	0.3480	3.3100	4.7290	0.307
x_2	0.0382*	0.1270*	–0.7080 *	0.0202*
x_3	–2.3300	–6.1550	–37.0900	-1.168
Interaction				
x_1x_2	0.000044	0.00131*	–0.01813	0.000044*
x_1x_3	0.0056	0.00889	0.1260	0.0022
x_2x_3	0.0046	0.02240*	0.0960	0.0032*
Quadratic				
x_1^2	–0.0209	–0.1790	–0.2060	–0.0171
x_2^2	–0.00035*	–0.000709*	0.00937*	–0.000164*
x_3^2	1.0530	2.5780	16.0700	0.510
Variability				
R^2 of model	0.98	0. 96	0.95	0.95
F value of model	438.14	71.61	33.85	55.61
$P > F$	0.002	0.013	0.028	0.024
CV of model	7.14	3.48	5.92	7.55

x_1, x_2, x_3 are glycerol concentration, C/N ratio and Tween 20 concentration, respectively. * means significant at 5% level.

Then, in further regression analysis, the main, quadratic, and interaction effects of x_1 and x_2 were maintained. The second order regression equation for biomass (Y_1), lipids content (Y_2), carotenoids production (Y_3) and lipids yield (Y_4) as a function of glycerol concentration (x_1) and C/N ratio (x_2) generated regression relationship as given in Eqs. (12–15).

$$Y_1 = 2.8260 + 0.3560\, x_1 + 0.0440\, x_2 - 0.02095\, x_1^2 - 0.000359\, x_2^2 + 0.000044\, x_1 x_2 \quad (12)$$

$$Y_2 = 16.8280 + 3.3230\, x_1 + 0.1600\, x_2 - 0.1790\, x_1^2 - 0.000709\, x_2^2 + 0.00131\, x_1 x_2 \quad (13)$$

$$Y_3 = 84.1330 + 4.9180\, x_1 - 0.5640\, x_2 - 0.2060\, x_1^2 + 0.00937\, x_2^2 - 0.01813\, x_1 x_2 \quad (14)$$

$$Y_4 = -0.0345 + 0.3103\, x_1 + 0.0250\, x_2 - 0.01716\, x_1^2 - 0.000164\, x_2^2 + 0.000044\, x_1 x_2 \quad (15)$$

The regression models were employed to develop response surface plots. The plots of biomass, lipids content and carotenoids production illustrated the effects of glycerol concentration and C/N ratio. The relationship that transpired between glycerol concentration and C/N ratio were illustrated as three-dimensional response surfaces and two–dimensional contour plots generated by the models as shown in Figure 11. Based on the response surface plots, the interaction between two variables and their optimum levels can be easily understood and located. The optimum values of these factors were obtained by solving the regression Eq. (12-15). The optimal levels of glycerol concentration and C/N ratios for biomass were 8.5 % and 60, respectively, at which the maximum biomass of 5.70 g/L would be obtained (Figure 11A). Under this condition, the lipids content and carotenoids production were 40.27 % (Figure 11B) and 103.96 mg/L (Figure 11C), respectively. At a glycerol concentration higher than 8.5% cell growth was inhibited. The possible reason for this

could be that a high concentration of glycerol could result in a high osmotic pressure and consequently inhibit the metabolic activity of the cell (Zhu *et al.*, 2008). Figure 11B and 11C. show that lipids content and carotenoids production increased with increasing glycerol concentration up to moderate concentration of 9.5% and a C/N ratio up to the maximum investigated level of 85. Under this condition, the maximum lipids content of 41.78% and carotenoids production of 117.38 mg/L were obtained. The amount of biomass was 5.49 g/L which was lower than that of the optimal condition for biomass.

The glycerol concentration and C/N ratio, as well as their balance in the culture medium are very important for metabolite biosynthesis. The interaction term of x_1x_2 with a positive regression coefficient in Eq.(6) and Figure 11B proved the synergic effect of glycerol concentration and C/N ratio on lipids accumulation. A low C/N ratio (increase in nitrogen content) gave higher biomass but lower lipids accumulation and carotenoids production. On the other hand, a high C/N ratio (decrease in nitrogen content) was favorable for high accumulation of the lipids.

It was reported that under the condition with limited nitrogen and an excess of the presence of the carbon source, the organisms started to store lipids (Angerbauer *et al.*, 2008). This is attributed to the induction of nitrogen–scavenging reactions, an effect of which is lowered level of adenosine monophosphate (AMP) with consequent disruption of the citric acid cycle due to dependence of the isocitrate dehydrogenase reaction on AMP (Ratledge, 1982). Patnayak and Sree (2005) found that a high C/N ratio significantly improved lipids production in *Bacillus subtilis* and *Pseudomonas* spp. This indicated an increment in lipids production with a depletion of the nitrogen source in the medium. Xue *et al.* (2007) found that the lipids content and lipids yield of *R. glutinis* using monosodium glutamate wastewater as culture medium were 20% and 5 g/L, respectively. Dai *et al.* (2007) studied on lipids production by *R. glutinis* using tree leaves hydrolytes as substitutes for the costly glucose. In their study, the lipids content of 28.59% and lipids yield of 4.73 g/L were obtained.

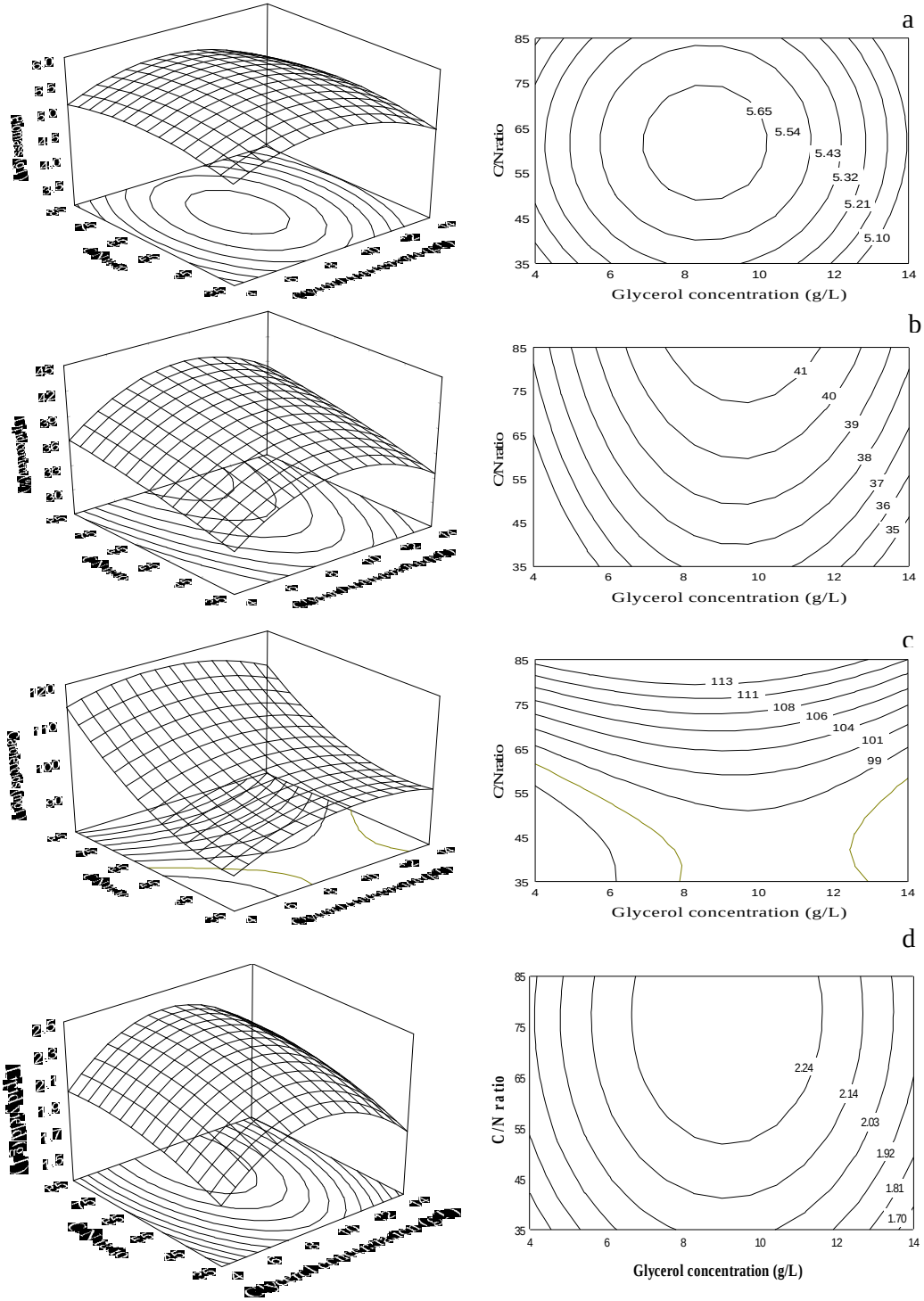

Figure 11. Response surface plots and contour plots for the effect of glycerol concentration (x_1) and C/N ratio (x_2) on biomass (a), lipids content (b), carotenoids (c) and lipids yield (d) when Tween 20 concentration was fixed at 1.5 g/L.

From Figure 11C it is obvious that the glycerol concentration affected the carotenoids production less than that did the C/N ratio. When the C/N ratio was kept constant, with an increase in the glycerol concentration beyond 9.5%, the carotenoids production was decreased. Glycerol may provide both the major energy source for cell metabolism and the carbon element for biosynthesis of biomolecules. However, excessive glycerol was found to repress the synthesis of the carotenoids. This was also observed in the carotenoids production of *Phaffia rhodozyma* due to the so–called Crabtree effect (Reynders, 1997). Numerous previous studies have shown that the C/N ratio has a significant influence on cell growth and carotenoid biosynthesis in some microorganisms including yeasts. Most studies have suggested that a high C/N ratio is more favorable for the biosynthesis of carotenoids in these organisms. Vustin *et al*. (2004) reported that as the C/N ratio of a medium decreased, the increase in biomass was coupled with a decrease in the carotenoids (astaxanthin) production by *Phaffia rhodozyma*.

They then suggested that abundant nitrogen in the medium (low C/N ratio) may enhance cell growth but suppress the enzymes for carotenoids production and its conversion to astaxanthin. Yamane *et al*. (1997) proposed that a high initial C/N ratio may decrease the consumption of NADPH for primary metabolism such as protein synthesis, so as to leave more NADPH available for carotenoids (astaxanthin) biosynthesis. In mycelia cultures of *Gibberella fujikuroi* fungus, while nitrogen feeding increased the cell growth, nitrogen limitation stimulated carotenoid biosynthesis, perhaps by imposing C/N imbalance and driving most of assimilated carbon to the secondary metabolism pathways (Garbayo *et al*., 2003). Carotenoids in yeasts have also been regarded as a secondary metabolite in several previous studies (Vustin *et al*., 2004; Johnson, 2003). Carotenoid is a secondary metabolite in *Rhodotorula* sp. and it begins to be formed during the very late stationary phase, while lipids accumulation always takes place during the early stationary phase.

All data support that maximum biomass, lipids and carotenoid production occurred at the optimum condition mentioned earlier. Since the optimum condition for biomass was different from that for lipids content and carotenoids production, the lipids yield per liter (biomass × lipids content) from each optimal

condition were calculated and compared. It was found that both conditions gave a comparable lipids yield of 2.30 g/L. In this study, the optimal condition for lipids content was chosen because of the lower requirement of the nitrogen source. This condition was then experimentally tested. The responses obtained from the predicted conditions compared with the actual values are shown in Table 21. The actual values of the biomass, lipids content and carotenoid production obtained using the above optimum condition were 5.98 g/L, 42.12% and 123.85 mg/L, respectively. The low value of CV indicated a close correlation between the experimental and predicted values. To further increase the biomass and production of lipids and carotenoids, batch fermentation was carried out in a 2L stirred tank bioreactor. The effects of the culture conditions including pH control and aeration rate were determined.

Table 21. Predicted and observed values for the dependent variables at optimum condition for lipids content

Response	Predicted value	Actual values (CV)
Biomass (g/L)	5.49	5.98 (13.24)
Lipids content (%)	41.78	42.12 (1.89)
Carotenoids (mg/L)	117.38	123.85 (1.95)

Note: the data in the bracket is the coefficients of variances (CV).

4.2.3 Effect of pH control and aeration rate

Figure 12 shows the time courses of pH, cell growth, lipids yield, carotenoids production, and glycerol consumption, in a 2L stirred tank bioreactor with and without pH control at 6.0. The agitation speed was controlled at 100 rpm with no aeration. 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 lipids yield, carotenoids production and glycerol consumption. This could be because the inhibitory effect of low pH was

alleviated. The lipids content was also enhanced from 42.12 % up to 48.21 % when the pH was kept constant.

The effects of aeration rate on cell growth, lipids yield, carotenoids production and glycerol consumption were examined (Figure 13). The aeration rate had a profound effect on biomass, consequently lipids production and glycerol consumption. Biomass and lipids yield significantly increased when increasing aeration rate from 0 to 2 vvm. At an aeration rate of 2 vvm, the biomass and lipids yield were highest at 8.17 g/L and 4.32 g/L, respectively. The further increase in aeration rate from 2 vvm to 3 vvm did not increase the biomass and the production of lipids. In addition, the effect of the aeration rate on carotenoids production was insignificant. One possible explanation might be because a high aeration rate causes oxidation of the pigment.

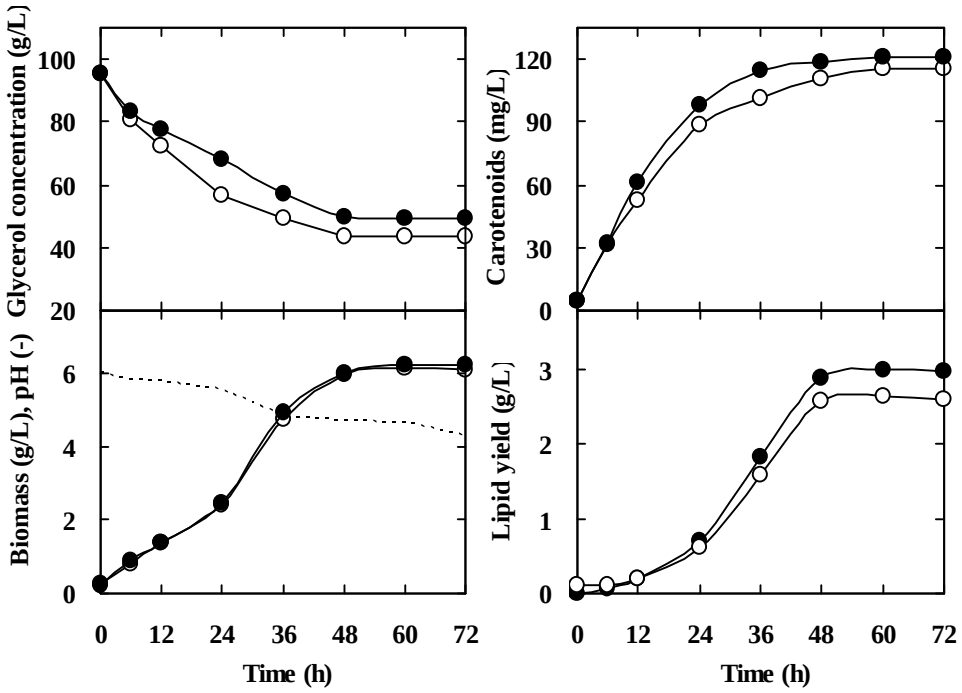

Figure 12. Effect of pH control on biomass, lipids yield, carotenoids production and glycerol consumption by *R. glutinis* TISTR 5159 in a stirred tank bioreactor. Without pH control (open circle) and pH control (filled circle). The dashed line shows the profile of pH under the condition without pH control.

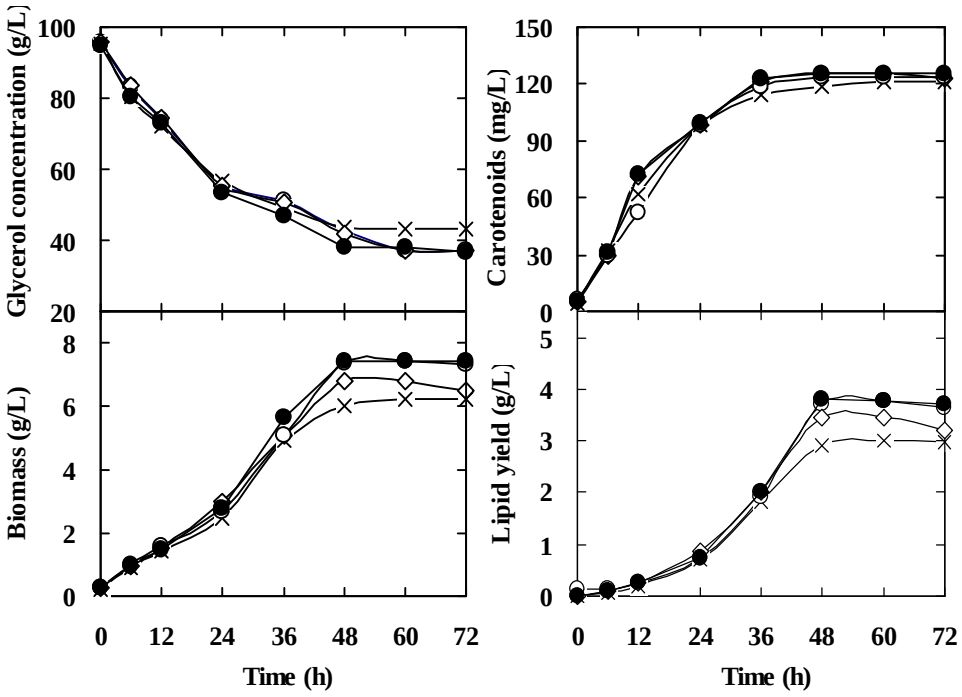

Figure 13. Effect of aeration rate on biomass, lipids yield, carotenoids production and glycerol consumption by *R. glutinis* TISTR 5159 in a stirred tank bioreactor. The aeration rate was varied at 0 vvm (cross), 1.5 vvm (open diamond), 2 vvm (filled circle) and 3 vvm (open circle).

4.2.4 Fed-batch fermentation

Since the results of the response surface plots (Figure 11) show that the high glycerol concentration would inhibit biomass and product formation, fed-batch fermentation was attempted. The aim of the fed–batch process was to avoid the substrate inhibition and enhance the production of lipids and carotenoids by feeding additional substrate. The culture with an initial working volume of 800 mL was first operated at batch mode. Then crude glycerol with the optimal C/N ratio for lipids content was added every 12 h up to 48 h of fermentation time to maintain the glycerol concentration at the optimum level of 9.5%. The culture pH was controlled at 6.0

throughout the fermentation process. The agitation speed and aeration rate were controlled at 100 rpm and 2 vvm, respectively. The profiles of cell growth, lipids yield, carotenoids production and glycerol consumption, are shown in Figure 14. The feeding of the substrate increased biomass, lipids content, lipids yield and carotenoids production up to 10.05 g/L, 60.70%, 6.10 g/L and 135.25 mg/L , respectively. The final total lipids yield in the fed-batch was 8.36 g which was about twice of the batch culture (4.33 g). This thus increased the productivity of lipids from 0.058 g/h in the batch culture to 0.116 g/h in the fed-batch culture. The final amount and productivity of carotenoids were also increased from 125.75 mg and 1.747 mg/h in batch culture to 180.20 mg and 2.504 mg/h in fed-batch culture. In the study of Li *et al*. (2007), there was little inhibitory effect on the culture of ***Rhorosporidium toruloides*** Y4 with a substrate concentration up to 150 g/L. When fed–batch cultures were applied the biomass was enhanced and the lipids content was increased up to 48.0%. Zhu *et al*. (2006) also found that the fed–batch culture of ***Mortierella alpine*** using glucose as a carbon source enhanced biomass and lipids yield up to 1.65–fold and 1.51–fold, respectively, compared with those of the batch culture. In the total volume of fermentation 1.31 L, biomass was 13.2 g, lipids yield was 7.99 g and carotenoids was 177.18 mg.

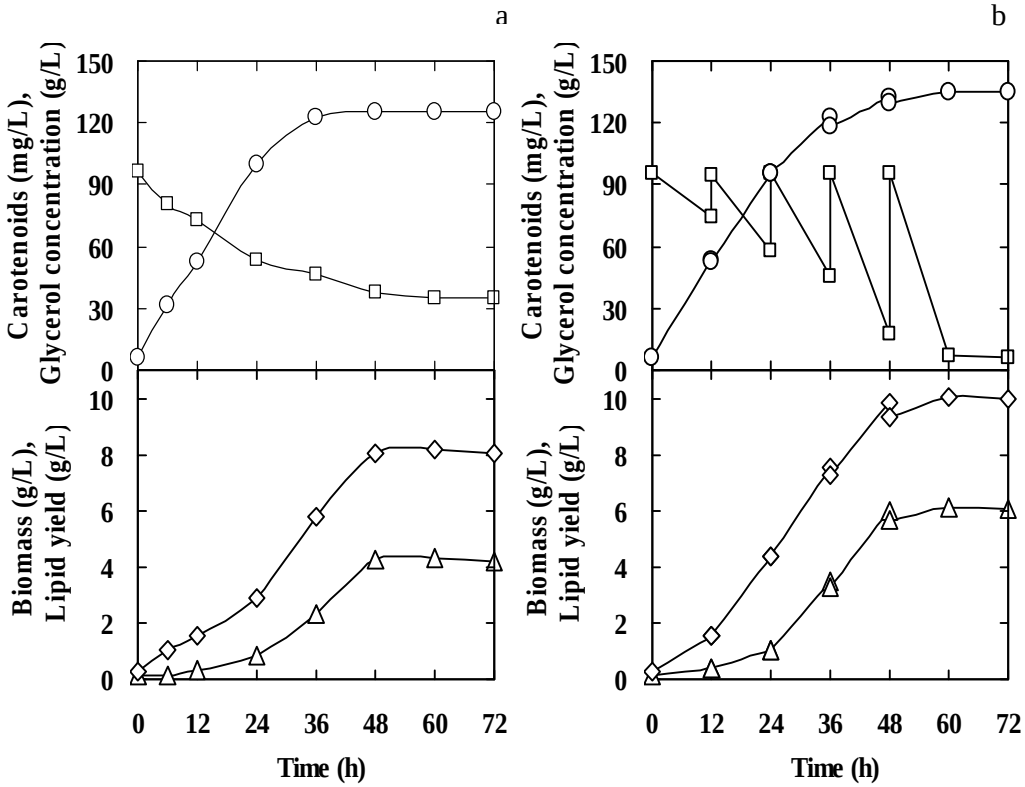

Figure 14. Comparison of batch fermentation (a) and fed-batch fermentation (b) in a stirred tank bioreactor. Biomass (open diamond), lipids yield (open triangle), carotenoids (open circle) and glycerol concentration (open square).

4.3 Scale-up of *R. glutinis* TISTR 5159 cultivation in a 15 L bioreactor

Scale-up is the study of the problem associated with transferring data obtained in laboratory and pilot plant equipment to industrial production. Main idea of scaling-up is to produce the large amount yield of interested product with enduring the results obtained in a small scale or improvement something on accepted principle basis. It will be good in operation with less possible step from the beginning of shaken flask to laboratory fermentation, pilot-scale fermenter and industrial or production fermenter, respectively. The values of parameter in larger scale must be set and stand as same or close as the variable of operation in smaller scale to expectably obtain the same results or product yield. Then, the results from each step are compared together to prove the problems or errors of operation. Scale-up should be carried out by using the same geometrically larger fermenter if possible. Working volume of each larger scaleis should be 10 time of smaller scale, in order to easily operate, control and prove of problems and errors. Operation in large scale about the large volume is deal with transferring of momentum, mass and energy that affects on processes and high volume, such as pressure and force of massive liquid and gasses, heat exchange, thermal capacity, controlling of mixing and foam formation, etc. Consequently, the performance of equipment and instrument should be appropriated and readily (Aiba *et al.*, 1973).

In this study, *R. glutinis* was cultivated in a large scale 15L bioreactor. The main reason for this was the economic of scale as a large scale system often requires a lower investment when compared to small system with the same volume. This section examined the performance of such system compared with that of smaller system. This is to investigate whether the large scale cultivation was, in fact, suitable to the growth and lipids production of *R. glutinis*. The lipids production was scaled up to a 10 L production scale in 15L bioreactor. The results from the cultivation of *R. glutinis* in the large scale system was illustrated in Figures 15 and 16. It was found that the maximum biomass and lipids content using POME were 10.98 g/L and 69.57%, respectively. The lipids yield, carotenoids concentration and COD removal were 7.64 g/L, 189.32 mg/L and 69.6% respectively. For the cultivation using crude glycerol as a substrate, the biomass and lipids content were 10.27 g/L and 65.78%,

respectively. The lipids yield, carotenoids concentration and glycerol consumption were 6.76 g/L, 162.31 mg/L and 81.61% respectively.

The result of lipids yield in large scale (10 L) is higher than that in small scale (1L). The lipids yield observed in 1L using POME and crude glycerol were 5.5 g/L and 4.32 g/L, respectively. While the lipids yield in 10 L using POME and crude glycerol were 7.6 g/L and 6.7 g/L, respectively. The cultivation of ***R. glutinis*** in the large scale system was demonstrated to increase the production. This was mainly due to the increase in the harvested volume when compared to the small system. In order to produce commercially lipids by microorganism, scale-up process is essential from lab scale to industry scale. Scale-up is usually based on various criteria such as geometrical similarity, power input, volumetric oxygen transfer coefficient, bioreactor fluid dynamics and experiences, and so on. Although the scale-up process in this study was based on the experiences, the better results in the 10L scale were obtained. It was demonstrated that scale-up was effective.

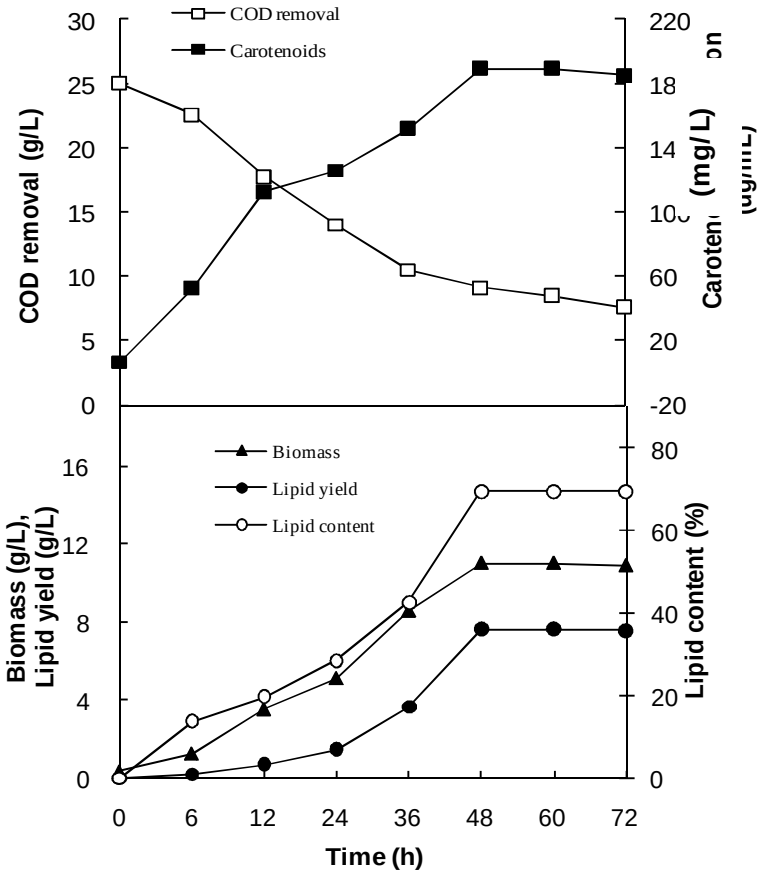

Figure 15. Time courses of cultivation of *R. glutinis* TISTR 5159 using POME in 10L production scale.

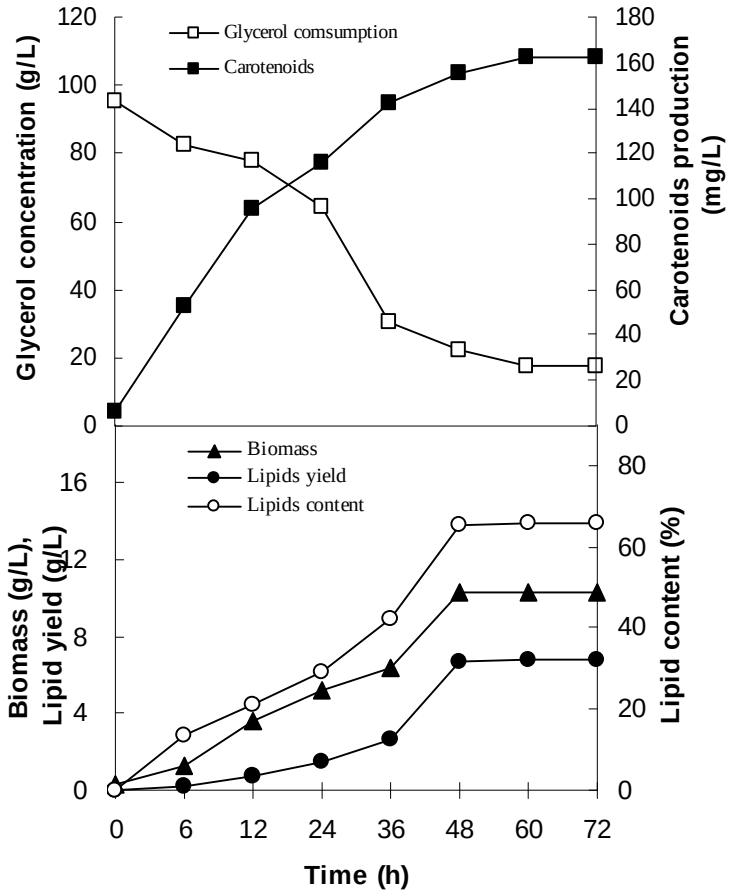

Figure 16. Time courses of cultivation of *R. glutinis* TISTR 5159 using crude glycerol in 10 L production scale.

4.4 Biodiesel production from yeast oil

For the production of biodiesel, the preparation of fatty acid methyl esters (FAME) was carried out in two steps. The tranesterification process was performed on the product from the esterification process (molar ratios of oil to methanol, 1 : 12) with 1 wt% of H_2SO_4 and a reaction time of 12 h) using KOH (1.5 wt% of KOH/oil) dissolved in methanol. The reactants were mixed in a 250 mL boiling flask equipped with a reflux condenser that was heated in heating system (Ma and Hanna, 1999). Table 22 and 23 show the fatty acid composition of biodiesel produced from yeast lipids using POME and crude glycerol, respectively. The data show that the main fatty acids in biodiesel produced from yeast lipids using POME were long-chain fatty acids with 16 and 18 carbon atoms including oleic acid (C18:1) 47.88%, palmitic acid (C16:0) 20.37%, stearic acid (C18:0) 10.33% and linoleic acid (C18:2) 7.31%. The composition of fatty acids in biodiesel produced from yeast lipids using crude glycerol was also mainly long-chain fatty acids including oleic acid (C18:1) 45.75%, linoleic acid (C18:2) 17.92% and palmitic acid (C16:0) 16.80%.

The similar fatty acid compositional profile to that of plant oil indicates that lipids from *R. glutinis* TISTR 5159 is of great potential as biodiesel feedstock. The lipids from other oleaginous yeasts also contain mainly long-chain fatty acids with 16 and 18 carbon atoms. *Rhodosporidium toruloides* Y4 contained four main fatty acids including oleic acid 46.9%, palmitic acid 20%, stearic acid 14.6% and linoleic acid 13.1% when it was grown on glucose (Li *et al*., 2007). While when *R. toruloides* Y4 was grown on lignocellulose biomass hydrolyzate, it contained mainly oleic acid 50% and palmitic acid 33% (Hu *et al*., 2009). *Rhodotorula mucilaginosa* TJY15a also contains mainly oleic acid (63.5%) and palmitic acid 22.3% when using cassava starch hydrolysate as a substrate (Li *et al*., 2010).

Compared to the commonly used soybean oil and rapeseed oil as feedstocks for biodiesel production in the US and the EU, respectively, the biodiesel derived from yeast lipids were more saturated. Soybean oil contains mostly linoleic acid and oleic acid at 53.7% and 23.3%, respectively, while rapeseed oil also contains similar fatty acids at 23.3% and 64.4%, respectively (O'Brien, 1988).

Table 22. Fatty acids composition of biodiesel derived from lipids of *R. glutinis* TISTR 5159 cultured in POME

Distribution of fatty acids	%
Lauric acid C12:0	0.18
Myristic acid C14:0	1.04
Palmitic acid C16:0	20.37
Palmitoleic acid C16:1	0.83
Heptadecanoic acid C17:0	1.38
Stearic acid C18:0	10.33
Oleic acid C18:1	47.88
Linoleic acid C18:2	7.31
Linolenic acid C18:3	0.85
Behenic acid C22:0	1.50
Lignoceric acid C24:0	1.03
Not identified	7.30
Total	100

Therefore, the yeast lipids tend to provide favorable properties to the biodiesel derived from soybean oil and rapeseed oil such as increased cetane number (CN), decreased NOx emissions, shorter ignition delay time, and oxidative stability. To ensure the quality of the FAME from yeast lipids, some fuel properties were determined with standard methods and compared with the biodiesel standards of Thailand's regulations and ASTM D6751-09 (US). Test methods and obtained results are shown in Table 20.

Most properties of FAME met Thailand's regulation except viscosity. However, the viscosity of FAME met ASTM D6751-09. Two important parameters for low temperature applications of a fuel are cloud point and pour point. The cloud point is the temperature at which wax first becomes visible when the fuel is cooled. The pour point is the lowest temperature at which the oil specimen can still be moved (Canan *et al.*, 2009). The FAME from yeast lipids has cloud point and pour point of 7 and 4 $^{\circ}$C (POME) 8 and 5 $^{\circ}$C (glycerol), respectively.

Table 23. Fatty acids composition of FAME derived from lipids of *R. glutinis* TISTR 5159 using crude glycerol as a sole carbon source

Distribution of fatty acids	%
Capric acid C10:0	0.06
Lauric acid C12:0	0.10
Myristic acid C14:0	0.87
Palmitic acid C16:0	16.80
Palmitoleic acid C16:1	0.81
Heptadecanoic acid C17:0	1.24
Stearic acid C18:0	3.68
Oleic acid C18:1	45.75
Linoleic acid C18:2	17.92
Linolenic acid C18:3	4.33
Behenic acid C22:0	0.70
Erucic acid C22:1	0.36
Lignoceric acid C24:0	0.91
Not identified	6.47
Total	100

These values are close to those specified in ASTM D6751-09. Among the fuel properties, the CN is one of the important parameter, which is considered during the selection of methyl esters for being used as biodiesel. The CN is the ability of fuel to ignite quickly after being injected. Better ignition quality of the fuel is always associated with higher CN value.

The CN also affected a number of engine performance parameters like combustion, stability, drivability, white smoke, noise and emissions of carbon monoxide (CO) and hydrocarbons (HC) (Antolin *et al*., 2002). Since, the FAME from yeast lipids in this study shows high value of CN and meets the biodiesel standards, it has a great potential for its use as biodiesel.

Therefore, the lipids from *R. glutinis* TISTR 5159 tends to give favorable properties to the biodiesel derived from soybean oil and rapeseed oil. These

include an increased cetane number (CN), decreased NOx emissions, shorter ignition delay time, and oxidative stability. It is known that fatty acid distribution impacts on the CN values. Considering the percentage distribution of fatty acids shown in Table 24 and the empirical equation (Xue *et al.*, 2007), the yeast lipids could produce biodiesel with CN values higher than 54. The minimal requirement for CN values have been set at 47, 49 and 51 by biodiesel standards ASTMD 6751 (USA), DIN 51606 (Germany) and EN 14214 (European Organization), respectively. Since the biodiesel produced from lipids of ***R. glutinis*** TISTR 5159 meet these standards, it has a great potential for biodiesel production.

Table 24. Some important fuel properties of FAME in this study compared with the biodiesel standards of Thailand's regulations and ASTM D6751-09

Property	Test method	FAME in this study (POME)	FAME in this study (Glycerol)	Thailand's regulations[a]	ASTM D6751-09
ME content (%)	TLC-FID[b]	99.8	99.7	≥ 96.5	-[c]
Diacylglycerol content (%)	TLC-FID[b]	0	0.20	≤ 0.20	-[c]
Monoacylglycerol content (%)	TLC-FID[b]	0.23	0.13	≤ 0.80	-[c]
Cetane number	ASTM D613	58.44	54.11	≥ 51	≥ 47
Viscosity at 40°C (mm^2/s)	ASTM D445	5.78	5.92	3.5–5.0	1.9-6.0
Water and sediment (wt%)	ASTM D2709	0.03	0.03	≤ 0.05	≤ 0.05
Pour point (°C)	ASTM D97	4	5	-[c]	-15 to 10
Cloud point (°C)	ASTM D2500	7	8	-[c]	-3 to 12

[a] Standard specification for biodiesel fuel 2009 from Department of Energy Business, Ministry of Energy, Thailand.

[b] Thin-layer chromatography with flame ionization detection (Cheirsilp *et al.*, 2008)

[c] Not defined

4.5 Economic feasibility

The aim of this economic assessment was to investigate the feasibility of lipids production by yeast using POME and crude glycerol. For lipids to be commercially successful, they must compete with refined oil and crude palm oil, which typically sell for 31 and 28 baht/kg, respectively. However, the scope of this study did not include the retail cost of lipids production and other measures of process profitability such as rate of return, pay back period and net present value. Therefore, it might not be able to directly compare with the cost of refine oil and crude palm oil but it will give the valuable information to be further investigated before the investment.

Since, *R. glutinis* TISTR 5159 is a potential yeast strain for treatment of wastes due to its high endurance to low pH and high salinity, the cultivation of it could be applied in conventional wastewater treatment. This system could save a lot of labour and costs. From experimental data, a total cycle time of 2 days is reasonable. Detailed cost analyses have not been reported on the cost of yeast oil production. The study of economic feasibility for biodiesel production from yeast lipids was calculated in 1000 L production scale using the experimental data from 10 L production scale. The costs of biodiesel production included only raw material and electricity.

From the diagrams in Figure 17, it is required only 11.5 g ammonium sulfate when cultivated in 1000 L POME based medium. This was due to the presence of nutrients in POME that can be used as a nitrogen source. While 1000 L crude glycerol based medium required much higher of nitrogen source (2.39 kg ammonium sulfate) (Figure 18). Mulligan and Gibbs (1992) commented that economic strategies might include choice of inexpensive raw materials, increase of product yields and reduction of total batch time, which includes media preparation, fermentation, harvesting and cleaning. This translates into reduced materials, labor and capital costs and hence reduced production costs. The inexpensive raw materials including POME, crude glycerol and ammonium sulfate we chose were industrially attractive. Consequently, the production cost of lipids by *R. glutinis* TISTR 5159 cultivated in POME and crude glycerol were estimated to be 0.036 baht per kg and 8.56 baht per kg, respectively. These values were 86,027 and 361 times lower than that cultivated in the complex medium (Table 25).

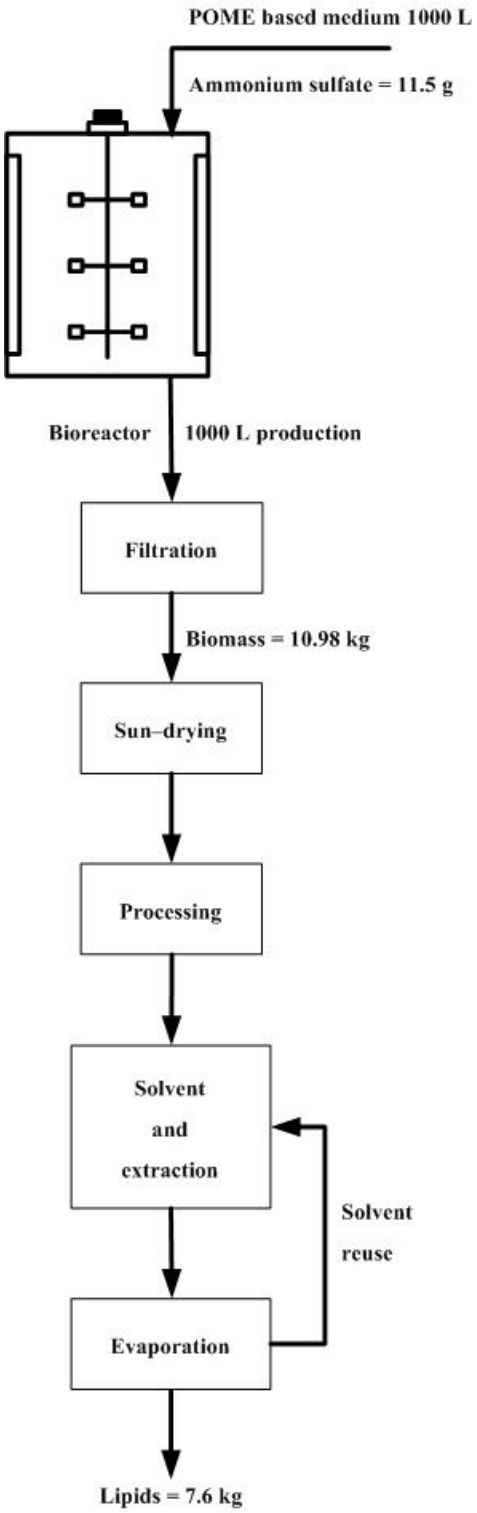

Figure 17. Show the fermentation diagram of lipids production from POME for economic analysis.

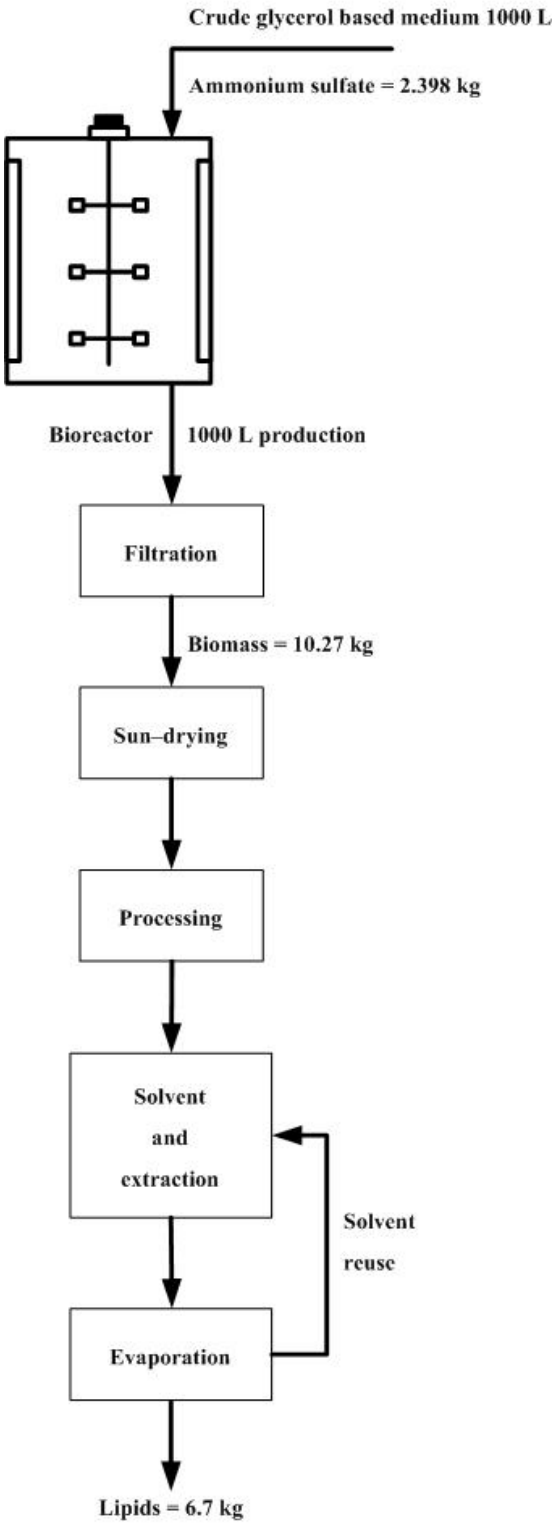

Figure 18. Show the fermentation diagram of lipids production from crude glycerol for economic analysis.

Table 25. Production costs per kg lipids of *Rhodotorula glutinis* using wastes compared with complex media

Medium	Component	Amount required for cultivation (g/L)	Cost (Baht/kg)	Yield (g/L)	Production cost (Baht/kg)
POME	Ammonium sulfate	0.0115	24	7.6	0.036
Total costs per kg lipids					0.036
Crude glycerol	Ammonium sulfate	2.39	24	6.7	8.56
Total costs per kg lipids					8.56
Complex[a]	Glucose	100	32	14.66	218.24
	Yeast extract	8	3960		2162.16
	Peptone	3	3500		717.5
Total costs per kg lipids					3097.9

[a]Data from Dai et al. (2007)

Table 26 show the composition and costs of a proposed production. The cost of electricity for oil recovery including sonication and evaporation was 0.045 baht (based on 2.50 baht/kW.h). The total costs of lipids production were 0.042 and 8.566 baht per kg lipids for POME and crude glycerol, respectively. The treatment of such a high concentration of organic pollutants in POME and crude glycerol by conventional activated sludge processes consumes a lot of energy, resulting in high treatment costs. Therefore, the efficient and economical treatment of such fermentative POME is becoming a big challenge. A favourable process is the use of POME to produce the valuable yeast lipids as oil feedstock for biodiesel production. In addition to the lipids, the by-products such as carotenoids and single cell protien are also the valuable products. Single cell protein from yeast is normally used in animal food additive as high protein sources. There are many amino acids contained in the biomass. The excess biomass from the yeast system was, therefore, a kind of good single cell protein with a high protein and low fat (SCP: 15$: kg =525 baht/kg). Carotenoids is an important compound because of its role as a precursor of vitamin A and it has also been used in food and feed products (Caratenoids 1000 $/kg = 35000 baht/kg). Therefore, the recovery of this part of carotenoids and single cell protein could counteract some part of the cost of wastewater treatment.

Table 26. Economic analysis of lipids production in 1000 L

Category	POME		Crude glycerol	
	Amount	Cost (baht)	Amount	Cost (baht)
Raw material				
Ammonium sulfate (kg)	0.0115	0.276	2.398	57
Electricity (2.5 baht/kwh)				
Recovery(Sonicator+Evaperator)(kW)	0.11	0.046	0.11	0.046
Lipids (kg)	7.6		6.7	
Total costs per kg lipids		0.042		8.566
By product				
Single cell protein (kg)	3.38	1775	3.57	1874
Carotenoids (kg)	1.90	66500	1.60	56000

CHAPTER 5

CONCLUSION

This study has shown the potential use of oleaginous red yeast *Rhodotorula glutinis* for bioconversion of wastes including POME and crude glycerol to value-added lipids and carotenoids. Improved biomass, lipids content and carotenoid production of *R. glutinis* TISTR 5159 cultured in POME have been achieved by medium optimization using RSM. The proposed model equation illustrated the quantitative effect of variables and also the interactions among the variables on biomass, lipids content, and carotenoids production. The optimized condition was COD of 40 g/L and C/N ratio of 140 for biomass production. While COD of 25 g/L and C/N ratio of 180 were optimum for lipids production and COD of 30 g/L and C/N ratio of 170 were optimum for carotenoids. Under the optimum condition for lipids content the highest lipids yield of 3.00 g/L and carotenoids production of 183.81 mg/L were obtained.

The two-stage process was demonstrated as an optimal way which cell growth (1^{st} stage) and accumulate lipids (2^{nd} stage). In the first stage using the condition of COD 40, C/N ratio 140, the highest biomass, lipids content and carotenoids production were 8.58 g/L, 34.99% and 126.51 mg/L, respectively, and COD removal for this condition was 73.27%. In the second stage using condition of COD 25, C/N ratio 180, the highest lipids content and carotenoids production were 51.85 % and 176.45 mg/L, respectively and COD removal for this condition was 73.58%. Further process optimization by pH control at 6.0 and aeration rate at 2 vvm improved the lipids and carotenoids production up to 60.62% and 188.31 mg/L, respectively. In semi-continuous process using POME, *R. glutinis* TISTR 5159 was found successfully able to accumulate high lipids content and produce considerable high carotenoids during long-term cultivation. Moreover, the efficient COD removal by *R. glutinis* TISTR 5159 was also observed in semicontinuous mode.

The bioconversion of crude glycerol using *R. glutinis* was preformed. The enhancement of biomass, lipids content and carotenoid production of *R. glutinis* using crude glycerol as a sole carbon source have been achieved by medium optimization using RSM. The proposed model equation illustrated the quantitative effect of variables and also the interactions among the variables on biomass, lipids content and carotenoids production. The optimal medium for maximum lipids accumulation and carotenoids production were glycerol concentration of 9.5%, C/N ratio of 85, and Tween 20 of 1.5 g/L. Under this condition, the biomass, lipids content and carotenoid production obtained were 5.98 g/L, 42.12% and 123.85 mg/L, respectively. The process optimization by pH control at 6.0 and aeration at 2 vvm enhanced both lipids content and carotenoids production. When fed-batch fermentation was applied, the highest lipids content and carotenoids production of 60.70% and 135.25 ug/mL , respectively, were achieved.

The scale up of lipids production to a 10 L production using POME and glycerol were performed. The use of POME provided the maximum biomass and lipids content of 10.98 g/L and 69.57%, respectively. The lipids yield, carotenoids concentration and COD removal were 7.64 g/L, 189.32 mg/L and 69.6%, respectively. While the use of crude glycerol provided the maximum biomass and lipids content of 10.27 g/L and 65.78%, respectively. The lipids yield, carotenoids concentration and glycerol consumption were 6.76 g/L, 162.31 mg/L and 81.61 %, respectively.

The fatty acid composition of biodiesel produced from yeast lipids using POME were long-chain fatty acids with 16 and 18 carbon atoms including oleic acid (C18:1) 47.88%, palmitic acid (C16:0) 20.37%, stearic acid (C18:0) 10.33% and linoleic acid (C18:2) 7.31%. The composition of fatty acids in biodiesel produced from yeast lipids using crude glycerol were also mainly long-chain fatty acids including oleic acid (C18:1) 45.75%, linoleic acid (C18:2) 17.92% and palmitic acid (C16:0) 16.80%. The similar fatty acid compositional profile to that of plant oil indicates that lipids from *R. glutinis* TISTR 5159 is of great potential as biodiesel feedstock.

The study of economic feasibility found that the costs of chemical for lipids production were 0.036 baht/kg from POME and 8.5 baht/kg from crude

glycerol. The costs of electricity for oil recovery (based on 2.50 baht/kw.h) were 0.006 baht/kg. Hence, the sum of costs for lipids production by *R. glutinis* TISTR 5159 cultivated in POME and crude glycerol were 0.042 baht/kg and 8.566 baht/kg, respectively.

Recommendations

1. For the future work, the non-diluted wastewater and without addition of nitrogen source should be used for lipids production because the high cost of nitrogen source was considered to have a negative impact on its economic use in industrial-scale processes.

2. Although, glycerol was the main component in crude glycerol, other components such as fatty acid might be additional carbon source for the yeast. Therefore, the C/N ratio in crude glycerol should be calculated based on COD/TN.

3. The effective method for simultaneous recovery of lipids and carotenoids should be investigated.

4. Various methods have been investigated to recover lipids and carotenoids from microorganism. These include adsorption, solvent extraction and molecular distillation. However, these processes involve the usage of organic solvents, chemical modifications and generate waste that has to be treated before it is allowed to be discharged to watercourse. Over the last two decades, supercritical fluid extraction (SFE) has been well received as green and promising technology for extraction of natural products. Therefore, the extraction of carotenoids and lipids by supercritical CO_2 will be free of potential harmful organic solvents.

5. Since the pH in the fermentation without pH control declined from 6.0 to 4.3, this indicated the production of acids. Therefore, the determination of this acids should be done.

6. To study the economic feasibility in detail, the retail cost of lipids production and other measures of process profitability such as rate of return, pay back period and net present value should be evaluated.

7. Since the addition of nitrogen source is costly, the wastewater that contain high concentration of nitrogen such as wastewater from seafood processing plant should be used to mix with POME or crude glycerol.

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APPENDIX

1. Carotenoids determination (Aksu and Eren, 2005**)**

The washed cells were suspended in 10 ml of acetone and methanol (3:1) and disrupted with sonication at 70 Hz for 30 min. Solid material was removed by centrifugation 7500 rpm 15 min. The color of the supernatant was determined at 450 nm. The concentration of carotenoids present in the sample was determined by comparison with a standard calibration curve prepared from standard beta-carotene dissolved in acetone and methanol (3:1)

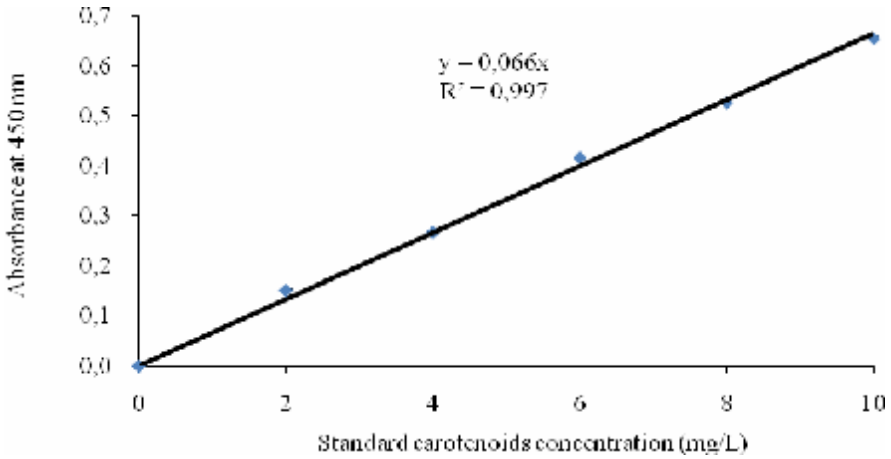


Figure 16. Carotenoids standard curve.

2. Glycerol determination (Kosugi *et al.*, 1994)

The glycerol concentration was determined in the water phase of the reactor with KIO_4. One mL of sample and 0.4 mL of 0.0025 M KIO_4 were reacted for 5 min. Then, 0.1 mL of 0.5 M sodium arsenate was added. After 10 min, 9 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 placed in boiling water bath for 30 min. The cooled tubes were adjusted to a volume of 25 mL with water, and the absorbance at 570 nm was measured.

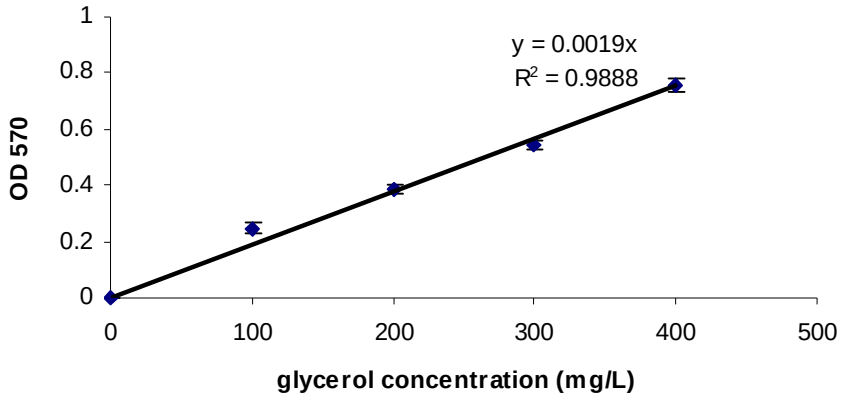


Figure 17. Glycerol standard curve.

3. Lipid extraction (Bligh and Dyer, 1959)

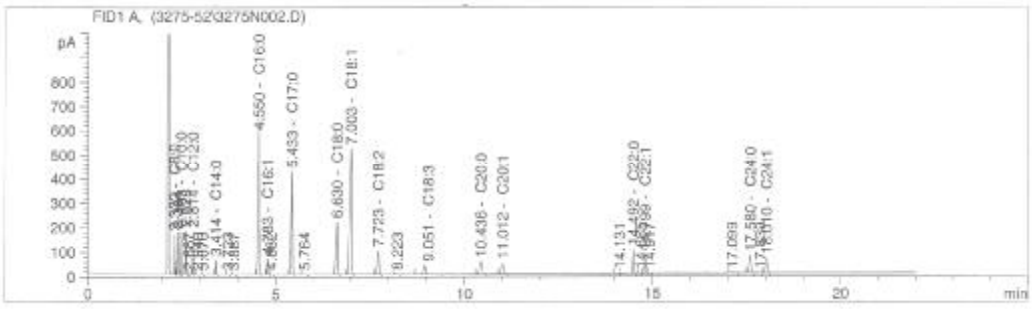
The dry biomass was ground into a fine powder. One gram powder was blended with 3 mL chloroform/methanol (2:1) and the mixture was agitated during 20 min in an orbital shaker at room temperature. Solvent phase was recovered by centrifugation 5000 rpm 15 min. The same process was repeated three times. The whole solvent was evaporated and dried under vacuum.

$$\text{Lipid content (\%)} = \frac{\text{Oil weight (g)}}{\text{Cell dry weight (g)}} \times 100$$

4. Determination of fatty acid composition by GC analysis (Jham *et al.*,1982)

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 2x3 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

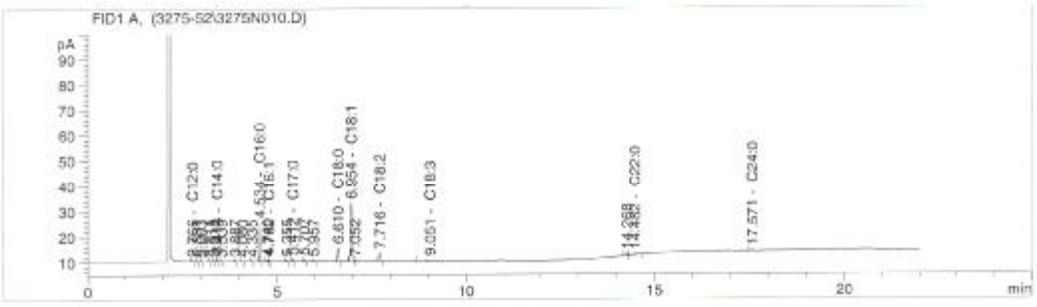
equipped with a cross-linked capillary FFAP column (length 30 m, 0.32 mm I.D, 0.25 µm film thickness) and a flame ionization detector. The operating conditions were as follows: inlet temperature 290 °C, oven temperature initial 210°C hold 12 min ramp to 250 °C at 20°C/min hold 8 min and detector temperature 300°C. The fatty acids were identified by comparison of their retention times with those of standard ones, quantification being based on their respective peak areas and normalized.



Signal 1: FID1 A,

Peak #	RetTime [min]	Type	Width [min]	Area [pA*s]	Area %	Name
1	2.322	BV	0.0192	194.21054	2.32951	C8:0
2	2.493	VB	0.0194	214.20706	2.56936	C10:0
3	2.814	PP	0.0205	229.87621	2.75731	C12:0
4	3.414	BB	0.0241	89.35952	1.07185	C14:0
5	4.550	BP	0.0313	1400.72424	16.80136	C16:0
6	4.783	BP	0.0328	136.12474	1.63278	C16:1
7	5.433	BP	0.0383	1088.58887	13.05737	C17:0
8	6.630	BP	0.0469	682.72614	8.18914	C18:0
9	7.003	BB	0.0514	1956.39124	23.46646	C18:1
10	7.723	BB	0.0537	330.60779	3.96556	C18:2
11	9.051	PBA++	0.0697	147.34647	1.76739	C18:3
12	10.436	BP	0.0692	247.00786	2.96280	C20:0
13	11.012	BB	0.0726	216.83221	2.60085	C20:1
14	14.492	VB	0.0415	256.17569	3.07277	C22:0
15	14.799	VV	0.0408	208.56201	2.50165	C22:1
16	17.580	BP	0.0523	261.30869	3.13434	C24:0
17	18.010	VB	0.0579	228.92313	2.74588	C24:1
Totals :				7888.97239	94.6264	

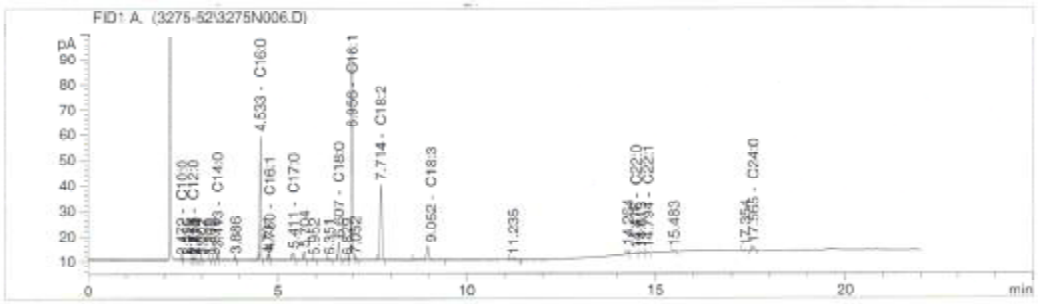
Figure 18. GC chromatogram of standard fatty acid mixture.



Signal 1: FID1 A,

Peak #	RetTime [min]	Type		Width [min]	Area [pA*s]	Area %	Name
1	2.322		+	0.0000	0.00000	0.00000	C8:0
2	2.493		+	0.0000	0.00000	0.00000	C10:0
3	2.766	PB	+	0.0168	2.68492e-1	0.18332	C12:0
4	2.881	PB		0.0212	4.26744e-1	0.29137	?
5	3.003	BB		0.0146	1.51770e-1	0.10362	?
6	3.211	BP		0.0196	2.17351e-1	0.14840	?
7	3.318	BP		0.0186	3.41780e-1	0.23336	?
8	3.414	PB	+	0.0238	1.52239	1.03944	C14:0
9	3.539	PP		0.0220	3.85597e-1	0.26327	?
10	3.887	BB		0.0212	5.63901e-1	0.38501	?
11	4.080	BP		0.0179	2.20933e-1	0.15085	?
12	4.335	BP		0.0193	2.15842e-1	0.14737	?
13	4.534	BB	+	0.0306	29.83689	20.37171	C16:0
14	4.740	BV		0.0244	7.67551e-1	0.52406	?
15	4.782	VP	+	0.0295	1.21268	0.82798	C16:1
16	5.255	PP		0.0344	1.08652	0.74184	?
17	5.412	BB	+	0.0354	2.02620	1.38343	C17:0
18	5.707	BP		0.0391	2.18814	1.49399	?
19	5.957	BP		0.0310	9.32594e-1	0.63675	?
20	6.610	BB	+	0.0432	15.13028	10.33049	C18:0
21	6.954	BV	+	0.0464	70.12226	47.87732	C18:1
22	7.052	VP		0.0312	9.92686e-1	0.67778	?
23	7.716	BB	+	0.0492	10.70948	7.31210	C18:2
24	9.051	PVA	++	0.0579	1.24594	0.85069	C18:3
25	10.436		+	0.0000	0.00000	0.00000	C20:0
26	11.012		+	0.0000	0.00000	0.00000	C20:1
27	14.268	BV		0.0403	2.19517	1.49879	?
28	14.482	VB	+	0.0841	2.19461	1.49841	C22:0
29	14.799		+	0.0000	0.00000	0.00000	C22:1
30	17.571	BP	+	0.0453	1.50658	1.02865	C24:0
31	18.010		+	0.0000	0.00000	0.00000	C24:1
Totals :					146.46239		

Figure 19. GC chromatogram of fatty acid composition of lipids from *R. glutinis* TISTR 5159 cultured in POME.



Signal 1: FID1 A,

Peak #	RetTime [min]	Type		Width [min]	Area [pA*s]	Area %	Name
1	2.322		+	0.0000	0.00000	0.00000	C8:0
2	2.472	PP	+	0.0180	3.56094e-1	0.06256	C10:0
3	2.596	VB		0.0299	3.68982e-1	0.06482	?
4	2.766	PV	+	0.0200	5.90606e-1	0.10376	C12:0
5	2.813	VV		0.0209	4.57933e-1	0.08045	?
6	2.874	VP		0.0290	3.89977e-1	0.06851	?
7	3.002	BP		0.0184	1.74718e-1	0.03069	?
8	3.209	BB		0.0197	2.59000e-1	0.04550	?
9	3.316	PP		0.0215	3.79664e-1	0.06670	?
10	3.413	BP	+	0.0240	4.94552	0.86883	C14:0
11	3.886	BP		0.0272	1.55772	0.27366	?
12	4.533	BP	+	0.0309	95.63665	16.80153	C16:0
13	4.731	BV		0.0280	1.81663	0.31915	?
14	4.780	VP	+	0.0328	4.58818	0.80606	C16:1
15	5.411	BP	+	0.0379	7.05842	1.24003	C17:0
16	5.704	BB		0.0408	9.15232	1.60789	?
17	5.952	PB		0.0330	8.16707e-1	0.14348	?
18	6.351	PP		0.0327	8.83281e-1	0.15518	?
19	6.607	BB	+	0.0422	20.93875	3.67854	C18:0
20	6.829	BV		0.0285	5.04922e-1	0.08871	?
21	6.956	VV	+	0.0482	260.40018	45.74733	C18:1
22	7.052	VP		0.0428	2.61686	0.45973	?
23	7.714	BB	+	0.0527	102.02194	17.92330	C18:2
24	9.052	VVA++		0.0742	24.65895	4.33211	C18:3
25	10.436		+	0.0000	0.00000	0.00000	C20:0
26	11.012		+	0.0000	0.00000	0.00000	C20:1
27	11.235	PP		0.0745	3.43267	0.60305	?
28	14.264	BV		0.0447	6.64869	1.16805	?
29	14.476	VV	+	0.0763	4.00307	0.70326	C22:0
30	14.613	VB		0.0539	3.82112	0.67130	?
31	14.794	BV	+	0.0469	2.04784	0.35977	C22:1
32	15.483	PB		0.0493	1.71382	0.30108	?
33	17.354	PB		0.0599	1.77893	0.31252	?
34	17.565	BP	+	0.0478	5.19378	0.91245	C24:0
35	18.010		+	0.0000	0.00000	0.00000	C24:1
Totals :					569.21391		

Figure 20. GC chromatogram of fatty acid compositionof lipids from *R. glutinis* TISTR 5159 cultured in crude glycerol.

5. Biodiesel production (Dai *et al.*, 2007)

The condition for biodiesel production using acid catalyst was molar ratio (methanol to oil) 12:1 mol/mol, 1% v/wt sulfuric acid as catalyst, reaction temperature at 98 °C at reaction time at 12 hours.

6. Chemical oxygen demand (COD) (APHA, 2005)

Wash culture tube and caps with 20% H_2SO_4 before first use to prevent contamination. Place sample in culture tube or ampule and add digestion solution. Carefully run sulfuric acid reagent down inside of vessel so an acid layer is formed under the sample-digestion solution layer. Tightly cap tubes or seal ampoules, and invert each several times to mix completely. Place tubes or ampoules in block digester or oven preheated to 150 ºC and reflux for 2 h. Cool to room temperature and place vessels in a test tube rack. Remove culture tube caps and add small TFE-covered magnetic stirring bar. If ampoules are used, transfer contents to a larger container for titrating. Add 0.05 to 0.10 mL (1-2 drop) ferroin indicator and stir rapidly on magnetic stirrer while titrating with 0.10 M ferrous ammonium sulfate (FAS). The end point is a sharp color change from blue-green to reddish brown, although the blue-green may reappear within minutes. In the same manner reflux and titrate a blank containing the reagents and a volume of distilled water equal to that of the sample

$$\text{COD} = \frac{(A-B) \times M \times 8000}{\text{mL sample}}$$

where :

A = mL FAS used for blank,

B= mL FAS used for sample

M = molarity of FAS

7. Nitrogen (Ammonia) (APHA, 2005)

Add 500 mL water and 20 mL borate buffer to a distillation flask and adjust pH to 9.5 with 6 N NaOH solution. Add a few glass beads or boiling chips and use this mixture to steam out the distillation apparatus until distillate shows no traces of ammonia. Use 500 mL dechlorinated sample or a portion diluted to 500 mL with water. When NH_3-N concentration is less than 100 µg/L, use a sample volume of 1000 mL. Remove residual chlorine by adding, at the time of collection, dechlorinating agent equivalent to the chlorine residual. If necessary, neutralize to approximately pH 7 with dilute acid or base, using a pH meter. Add 25 mL borate buffer solution and adjust to pH 9.5 with 6 N NaOH using a pH meter. To minimize contamination, leave distillation apparatus assembled after steaming out and until just before starting sample distillation. Disconnect steaming-out flask and immediately transfer sample flask to distillation apparatus. Distill at a rate of 6 to 10 mL/min with the tip of the delivery tube below the surface of acid receiving solution. Use 50 mL in dicating boric acid solution for titrimetric method. Collect at least 200 mL distillate. Lower collected distillate free of contact with delivery tube and continue distillation during the last minute or two to cleanse condenser and delivery tube. Dilute to 500 mL with water. Titrate ammonia in distillate with standard 0.02 N H_2SO_4 titrant until indicator turns a pale lavender.

$$NH_3\text{-N (mg/L)} = \frac{(A-B) \times 280}{\text{mL sample}}$$

where :

A = volume of H_2SO_4 titrated for sample, mL

B = volume of H_2SO_4 titrated for blank, mL

8. Nitrogen (Nitrite) (APHA, 2005)

If sample contains suspended solids, filter through a 0.45 µm pore size membrane filter. If sample pH is not between 5 and 9, adjust to that range with 1 N HCl or NH_4OH as required. To 50 mL sample, or to a portion diluted to 50 mL, add 2 mL color reagent and mix. Between 10 min and 2 h after adding color reagent to

samples and standard, measure absorbance at 543 nm. As a guide use the following light paths for the indicated NO_2^- -N concentration:

Light Path Length (Cm)	NO_2^- -N µg/L
1	2-25
5	2-6
10	<2

9. Nitrogen (Nitrate) (APHA, 2005)

Insert a glass wool plug into bottom of reduction column and fill with water. Add sufficient Cu-Cd granules to produce a column 18.5 cm long. Maintain water level above Cu-Cd granules to prevent entrapment of air. Wash column with 200 mL dilute NH_4Cl-EDTA solution. Activate column by passing through it, at 7 to 10 mL/min, at least 100 mL of a solution composed of 25% 1.0 mg NO_3^--N/L standard and 75% NH_4Cl-EDTA solution. Using the intermediate NO_3^-- N solution, prepare standards in the range 0.05 to 1.0 mg NO_3^--N/L by diluting the following volumes to 100 mL in volumetric flasks: 0.5, 1.0, 2.0, 5.0, and 10.0 mL. Carry out reduction of standards exactly as described for samples. Compare at least one NO_2^- standard to a reduced NO_3^- standard at the same concentration to verify reduction column efficiency. Reactivate Cu-Cd granules.

10. Nitrogen (organic) (APHA, 2005)

Place a measured volume of sample in an 800 mL kjeldahl flask. Select a sample size from the following tabulation:

Organic nitrogen in sample (mg/L)	Sample size (mL)
0-1	500
1-10	250
10-20	100
20-50	50
50-100	25

Add 25 mL borate buffer and then 6N NaOH until pH 9.5 is reached. Add a few glass beads or boiling chips and boil off 300 mL. If desired, distill this fraction and determine ammonia nitrogen. Alternately, if ammonia has been determined by the distillation method, use residue in distilling flask for organic nitrogen determination. Cool and add carefully 50 mL digestion reagent to distillation flask. Add a few glass beads and, after mixing, heat under a hood or with suitable ejection equipment to remove acid fumes. Boil briskly until the volume is greatly reduced and copious white fumes are observed. Then continue to digest for an additional 30 min.

11. Determination of oil composition by TLC/FID analyzer

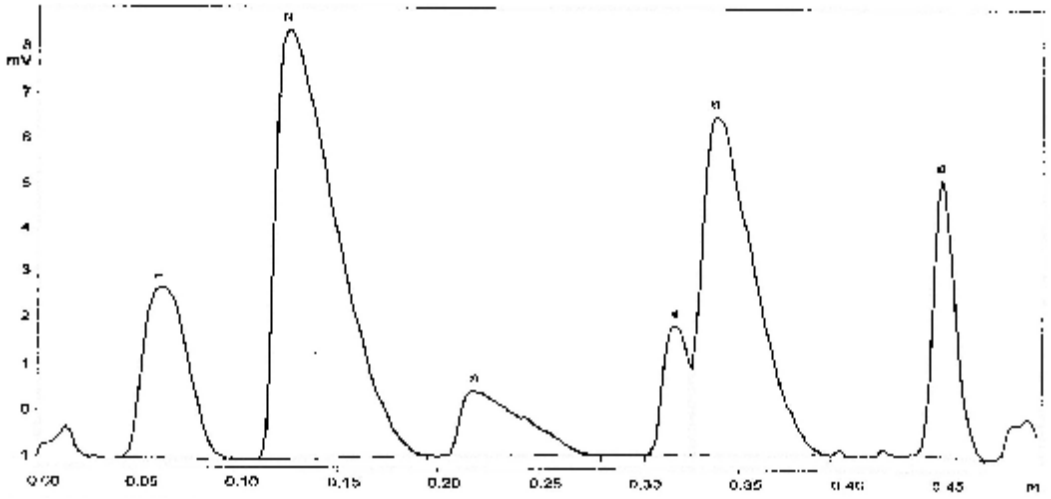
Condition

Stationary phase : CHROMAROD-SIII

Mobile phase : benzene: chloroform: acetic acid (50:20:0.7)

Gas flow : H_2 160 mL/min, Air 2.0 L/min

Scanning speed : 30 s/scan

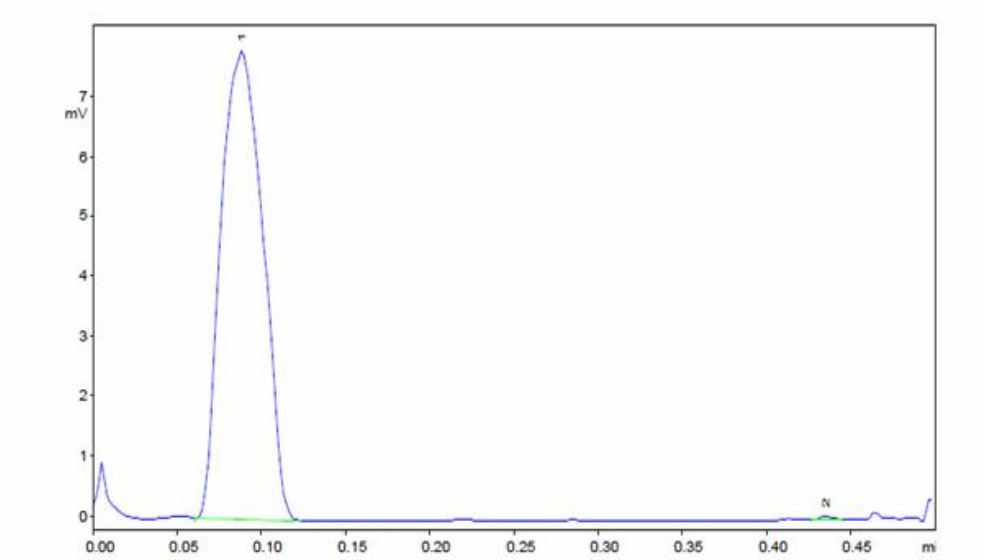


Peak no	Ret. Time (min)	Pk.Start (min)	Pk.End (min)	Area	Height (mV)	Area %
1	0.062	0.042	0.100	2952	3.68	11.670
2	0.125	0.112	0.195	10147	9.38	40.118
3	0.218	0.205	0.282	1615	1.40	6.383
4	0.328	0.303	0.327	1284	2.80	5.078
5	0.338	0.327	0.395	6877	7.43	27.189
6	0.450	0.435	0.472	2418	6.11	9.561
				25294	30.79	100.000

Figure 21. TLC/FID chromatogram of oil composition.

Table 25. TLC/FID report.

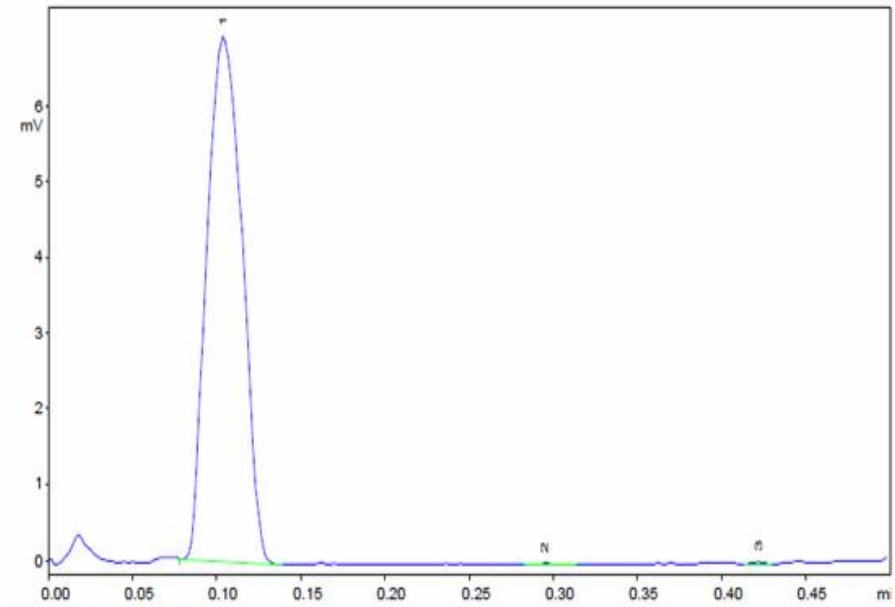
Peak no	Compound name	% of compound
1	Fatty acid ethyl ester	11.67
2	Triglyceride	40.11
3	Free fatty acid	6.38
4	1,3 Diglyceride	5.08
5	1,2 Diglyceride	27.19
6	Monoglyceride	9.56



Calculation Method : Percent

Peak-No	Ret.Time (min)	Pk.Start (min)	Pk.End (min)	Area	Height (mV)	Area%
1	0.088	0.060	0.123	7068	7.80	99.755
2	0.435	0.427	0.445	17	0.06	0.245
				7086	7.86	100.000

Figure 22. TLC/FID chromatogram of oil composition of *R. glutinis* TISTR 5159 cultured in POME.



Calculation Method : Percent

Peak-No	Ret.Time (min)	Pk.Start (min)	Pk.End (min)	Area	Height (mV)	Area%
1	0.103	0.078	0.138	5258	6.93	99.663
2	0.295	0.283	0.313	11	0.02	0.204
3	0.422	0.413	0.430	7	0.03	0.133
				5276	6.97	100.000

Figure 23. TLC/FID chromatogram of oil compositions of *R. glutinis* TISTR 5159 cultured in crude glycerol.

VITAE

Name Miss Chanika Saenge

Student ID 4910930005

Educational Attainment

Degree	Name of Institution	Year of Graduation
B.Sc. in Food Science	Songkhla Rajabhat University	2002
M.Sc in Environmental Management	Prince of Songkha University	2005

List of Publication and Proceedings

Publications

Saenge, C., Cheirsilp, B., Tachapattawearakul, T. and Bourtoom, T. Concomitant Production of Carotenoids and Single Cell Oil by *Rhodotorula glutinis* TISTR 5159 Growning on Crude Glycerol from Biodiesel Plant. Process Biochem. (Submitted)

Saenge, C., Cheirsilp, B., Tachapattawearakul, T. and Bourtoom, T. Simultaneous Optimization of Single Cell Oil and Carotenoids Production by Oleaginous Yeast *Rhodotorula glutinis* TISTR 5159 Cultured in Palm Oil Mill Effluent. Biotech. Bioprocess Eng. (Submitted)

Presentations

Saenge, C., Cheirsilp, B., Tachapattawearakul, T. and Bourtoom, T. 2009. Single Cell Oil and Carotenoids Production by Oleaginous Yeast *Rhodotorula glutinis* TISTR 5159 Cultured in Palm Oil Mill Effluent. International Conference of the Thai Society for Biotechnology, 24-25 September, 2009, Queen Sirikit National Convention Center, Bangkok, Thailand.