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THESIS

OPTIMIZATION OF LIPID PRODUCTION BY OLEAGINOUS YEAST

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A Thesis Submitted in Partial Fulfillment of
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Pakawat Kraisintu 2009: Optimization of Lipid Production by Oleaginous Yeast. Master of Science (Microbiology), Major Field: Microbiology, Department of Microbiology. Thesis Advisor: Professor Savitree Limtong, Dr.Eng. 95 pages.

Newly isolated oleaginous yeast *Rhodosporidium toruloides* DMKU3-TK16 was obtained from three steps screening from 63 strains of known species in the genus *Rhodotorula* and 104 newly isolated strains of unidentified species. Lipid production by this strain was highest in shaking flask cultivation at 150 rpm and 28 ºC in nitrogen-limited medium II containing per liter 70 g glucose, 0.75 g yeast extract, 0.55 g $(NH_4)_2SO_4$, 0.4 g KH_2PO_4, 2.0 g $MgSO_4 \cdot 7H_2O$ and pH 5.5. Under these optimal conditions, when the C/N ratio of the medium was 140, *R. toruloides* DMKU3-TK16 produced lipid of 9.26 g/l, which was 71.30 % of dry biomass (13.33 g/l) after 168 h of cultivation. Lipid production in a 5 l jar fermenter with agitation speed of 300 rpm, aeration rate of 3.0 vvm and 30 ºC throughout the cultivation, *R. toruloides* DMKU3-TK16 yielded a final lipid of 15.12 g/l, which was 67.23 % of dry biomass (22.49 g/l) after 156 h of cultivation. The major fatty acids of the cellular lipid were oleic acid (40.93%), palmitic acid (20.42%), linoleic acid (18.04%) and steric acid (11.65%).

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Student's signature Thesis Advisor's signature

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Pakawat Kraisintu

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TABLE OF CONTENTS

Page

LIST OF TABLES

LIST OF FIGURES

LIST OF FIGURES (Continued)

LIST OF FIGURES (Continued)

LIST OF FIGURES (Continued)

LIST OF FIGURES (Continued)

LIST OF FIGURES (Continued)

LIST OF FIGURES (Continued)

OPTIMIZATION OF LIPID PRODUCTION BY OLEAGINOUS YEAST

INTRODUCTION

Over the last 50 years, the world demand for fats and oils has increased so the production of microbial oil by oleaginous microorganism known as single cell oil (SCO) has been introduced to be alternative fats and oils source (Ratledge 1982; Davies, 1988). Among microorganisms, oil accumulation is only found in some yeast, fungi and small number of algae (Ratledge, 2001). Yeasts that can accumulate lipid higher than 20% of their biomass are defined as oleaginous yeast such as species of *Rhodotorula* and *Lipomyces* (Thorpe and Ratledge, 1972). Recent developments in yeast lipid biotechnology are the results of the search for beneficial lipid compounds using as biodiesel production and dietary supplement (Li *et al*., 2007). The advantages of using yeasts as potential lipid producers are that yeast lipid compositions are similar to vegetable oils and fats, yeast can be grown well on cheap raw material such as industrial waste, their lipid can be produced using short time in large scale and most of yeast for lipid production seem to be nontoxic to human (Jacob, 1993)

In order to obtain yeasts that confer high lipid content to be used as potential lipid producers isolation and screening such yeasts from various habitats are appreciated. The selected yeast strain with high lipid content obtained will be optimized for high lipid production which may further utilize for production of biodiesel or dietary supplement.

OBJECTIVES

1. To isolate and screen oleaginous yeast strains with high lipid content from various habitats.

2. To optimize cultivation conditions for lipid production of the selected oleaginous yeast strain.

LITERATURE REVIEW

1. Lipids

In biological systems, fatty acids are mostly classified as components of lipids. Lipids are organic compounds that are insoluble in water but soluble in organic solvents. There are two general types of lipids including complex and simple lipids. The complex lipids such as the triacylglycerols, can be hydrolyzed to smaller molecules but the simple lipids such as the steroids cannot be hydrolyzed into smaller ones (McMurry, 1988). The Acyl- and phosphor-glycerols, the most common lipids are based on structure of glycerol and fatty acids. Acylglycerols have a glycerol backbone linked to one, two and three fatty acids with ester bonding to provide mono-, di- and tri-acylglycerols, respectively (Dyal and Narine, 2005). Phosphoglycerols are composed of a glycerol backbone esterified to two fatty acids and a phosphate group (Gurr and Harwood 1991).

Fat is lipid material that is solid at room temperature whereas oil is liquid at similar temperature. The major components in natural oils and fats are triacylglycerols (Figure 1). Thus, the defined terms of oil and fat are often used to mean triacylglycerols. However, the other components still exist in small amounts within natural fats and oils including mono- and di-acylglycerols, phospholipids, waxes, steroids and carotenoids (Stauffer, 1996).

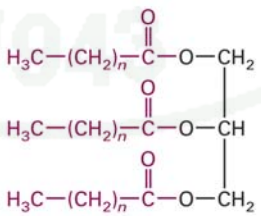

Figure 1 Chemical structure of triacylglycerol.

Source: Schweizer (1990)

2. Biological functions of lipids

Lipids are absolutely necessary for growth and survival of all organisms. They are considerable structural components of membranes (Lehninger, 1993). In many organisms, they play an important role as carbon and free-energy storage. In general, triacylglycerols are stored in organisms but when starvation occurring this storage triacylglycerols can be used (Swaff, 2003). Yeast can store triacylglycerol as intracellular oil droplets or lipid bodies under stress conditions (Murphy *et al*, 1991; Czabany *et al*., 2007). The lipids that provide to the structure and function of biological membranes are called structural lipids which contain mostly phospholipids, glycolipids (lipids including a sugar constituent), sphingolipids and steroids (Gurr and Harwood, 1991).

3. Molecular structure of fatty acids

Fatty acids are composed of a long hydrocarbon chain and a terminal carboxylate group. Over 1000 fatty acids are known with different types, positions, configurations and chain lengths of unsaturation, and the addition of substituent along the chain. However, only around 20 fatty acids can be found widely in nature (Schweizer, 1989). Most fatty acids are unbranched and consist of an even number of carbon atoms (10-24) as shown in Table 1. They may have various degrees of unsaturation from 0 to 6 double bonds (Swaff, 2003). Unbranched fatty acids can be described in symbol as x:y, where x represents the number of carbon atoms and y as the number of double bonds. Carbon atoms are counted from the carboxyl terminus. The positions of the double bonds are represented by Δ and a number. For example, Δ9 18:1 denotes a fatty acid with 18 carbon atoms and with one double bound at position of 9 that counted from the carboxyl terminus (Ratledge, 1988).

Table 1 Some common, systematic and short name of fatty acids.

Common name	Systematic name*	Short name
Saturated fatty acids		
Lauric acid	Dodecanoic acid	12:0
Myristic acid	Tetradecanoic acid	14:0
Palmitic acid	Hexadecanoic acid	16:0
Stearic acid	Octadecanoic acid	18:0
Monounsaturated fatty acids		
Palmitoleic acid	Δ9-Hexadecenoic acid	Δ9 16:1
Oleic acid	Δ9-Octadecenoic acid	Δ9 18:1
ω-6 Polyunsaturated fatty acids		
Linoleic acid (LA)	Δ9, Δ12-Octadecadienoic acid	ω-6 18:2
γ-Linolenic acid (GLA)	Δ6, Δ9, Δ12-Octadecatrienoic acid	ω-6 18:3
Arachidonic acid (ARA)	Δ5, Δ8, Δ11, Δ14-Eicosatetraenoic acid	ω-6 20:4
ω-3 Polyunsaturated fatty acids		
α-Linolenic acid (LNA)	Δ9, Δ12, Δ15-Octadecatrienoic acid	ω-3 18:3
Eicosapentaenoic acid(EPA)	Δ5, Δ8, Δ11, Δ14, Δ17-Eicosapentaenoic acid	ω-3 20:5
Docosahexaenoic acid (DHA)	Δ4, Δ7, Δ10, Δ13, Δ16, Δ19-Docosahexaenoic acid	ω-3 22:6

*All double bonds are in cis-configuration

Source: Swaff (2003)

Long-chain polyunsaturated fatty acids (PUFAs) have more than one double carbon bond and 18 or more carbon atoms in their structure. They are classified with the position of the first double bond where counted from the methyl end (Swaff, 2003). An ω-3 PUFA has first double bound at position 3 where counted from the methyl end. Either symbol ω or n is often written for classification of PUFAs. For example, α-Linolenic acid (18:3, n-3) donated 18 carbon atoms with three double bonds at positions 9, 12, 15 (counting the carboxy group as the beginning) so that the final bond (between C15 and C16) is n-3 from the end (Ratleadge and Wynn, 2002).

4. Fatty acid biosynthesis

4.1 Chain elongation

For fatty acid synthesis process in yeast and other eukaryotes, acetyl-CoA is converted to the fatty acid palmitic acid (16:0) by enzymatic activities in the fatty acid synthase system (FAS) (Stryer, 1988). The first step, malonyl-CoA is formed by carboxylation of acetyl-CoA and catalytic enzyme is acetyl-CoA carboxylase (Table 2). The CO_2 involved in this reaction that is again released in a subsequent reaction (step 4). In fact, additional carbon atoms of the fatty acids (with an even number of carbon atoms) are derived from acetyl-CoA. Acetyl transacylase catalyzes the conversion of acetyl-CoA and acyl carrier protein (ACP) to acetyl-ACP and CoA as products (step 2). Malonyl transacylase catalyzes the conversion of malonyl-CoA and ACP to malonyl-ACP and CoA (step 3). In four subsequent reactions (steps 4-7) butyryl-ACP, CO_2, ACP, H_2O and 2 $NADP^+$ are formed that require acetyl-ACP, malonyl-ACP and 2 ($NADPH + H^+$) for catalytic reactions. There are many catalytic enzymes to be used in these conversions including acyl-malonyl-ACP condensing enzyme, β-ketoacyl-ACP-reductase, 3-hydroxyacyl-ACP-dehydratase and enoyl-ACP reductase, respectively (Koolman and Roehm, 2005).

Table 2 Principle reactions in fatty acid synthesis.

Step	Reaction	Enzyme
1	Acetyl-CoA + HCO^-_3+ ATP → malonyl-CoA + ADP + Pi + H^+	Acetyl-CoA carboxylase
2	Acetyl-CoA + ACP ↔ acetyl-ACP + CoA	Acetyl transacylase
3	Malonyl-CoA + ACP ↔ malonyl-ACP + CoA	Malonyl transacylase
4	Acetyl-ACP + malonyl-ACP → acetoacetyl-ACP + ACP + CO_2	Acyl-malonyl-ACP condensing enzyme
5	Acetoacetyl-ACP + NADPH + H+ ↔ D-3-hydroxybutyryl-ACP + $NADP^+$	β-Ketoacyl-ACP-reductase
6	D-3-hydroxybutyryl-ACP ↔ crotonyl-ACP + H_2O	3-hydroxyacyl-ACP-dehydratase
7	Crotonyl-ACP + NADPH + H^+ → butyryl-ACP + $NADP^+$	Enoyl-ACP reductase

Source: Stryer (1988); Koolman and Roehm (2005)

In each next cycle of elongation the fatty acyl chain increases with two carbon atoms (Figure 2). The overall stoichiometry of the synthesis of palmitate (16:0) is as follow (Stryer, 1988)

8 Acetyl-CoA + 7 ATP + 14 ($NADPH+H^+$) → palmitate + 14 $NADP^+$+ 8 CoA + $6H_2O$ + 7 ADP + 7 Pi

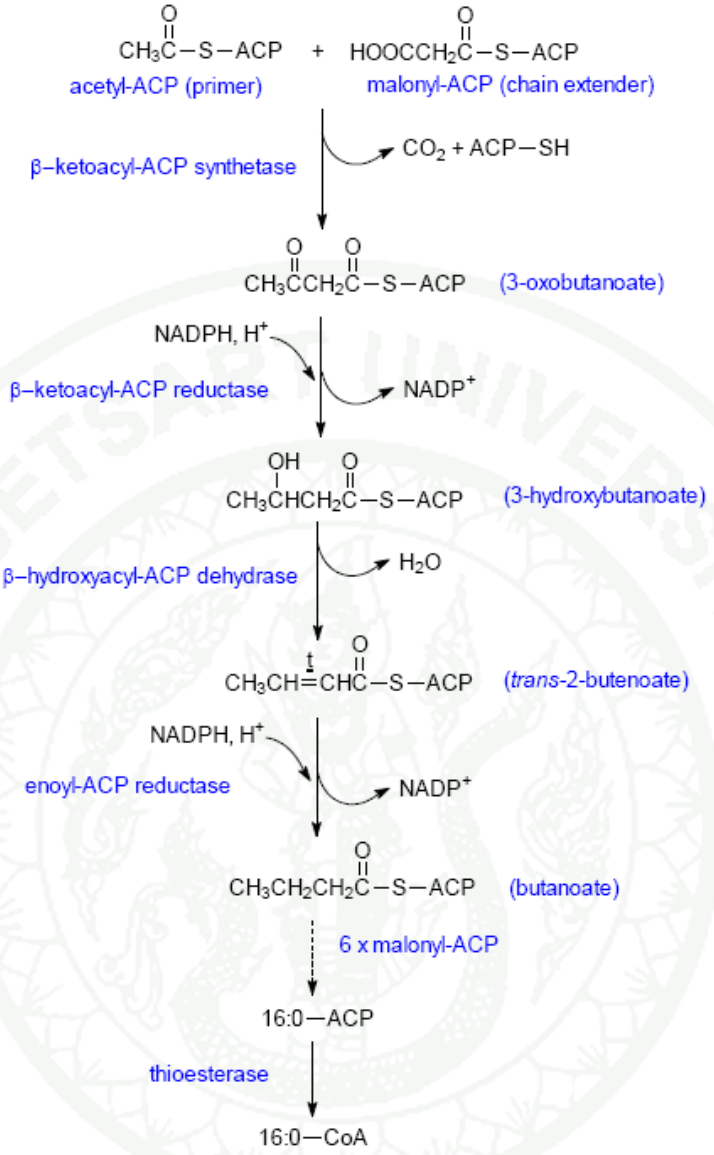

Figure 2 Reaction schemes of fatty acid synthesis and elongation.

Source: Tehlivets *et al.* (2007)

4.2 Desaturation

Fatty acids with double bonds are synthesized by anaerobic condition in many prokaryotes such as *Escherichia coli* (Bloch, 1969). These double bonds are the effect of a lack of enoyl reduction after the dehydratation (Hopwood and Sherman, 1990) and thus the double bond remains to inserts within fatty acid chain. Eukaryotes, cyanobacteria and some bacilli can insert double bonds into fatty acids by using activity of oxygen-dependent desaturases (Bloomfield and Bloch, 1960; Shanklin and Cahoon, 1998). The various desaturases are called according to the position of the double bond they insert in the fatty acid chain. For example, a Δ9 desaturase inserts a double bond at position Δ9. (Bloch, 1969; Shanklin and Cahoon, 1998). In yeasts, desaturases are three-component enzyme systems containing of cytochrome b_5 reductase, cytochrome b_5 and the desaturase itself as shown in Figure 3 (Ratledge, 2001; Stryer, 1988).

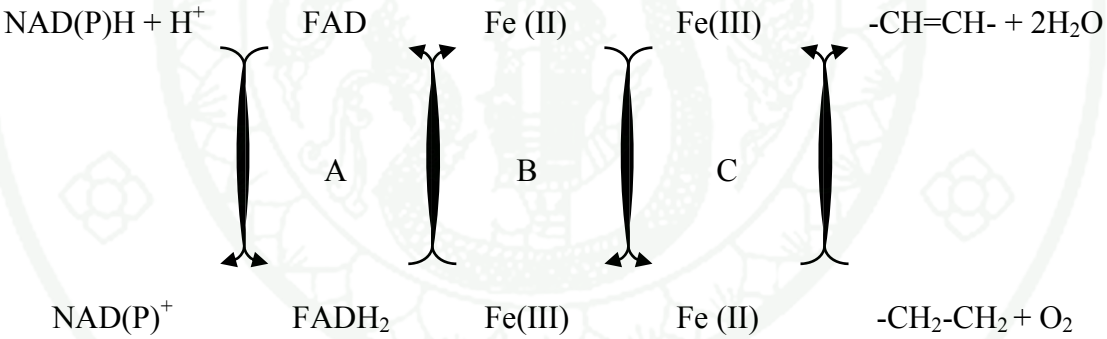

Figure 3 Diagram of the desaturases. A= cytochrome b_5 reductase; B= cytochrome b_5; C= fatty acid desaturase. Either NADPH or NADH is used.

Source: Ratledge (2001); Stryer (1988)

The best well known desaturases are the Δ9 desaturases as enzyme for the conversion of 16:0 to Δ9 16:1 and of 18:0 to Δ9 18:1. In *Saccharomyces cerevisiae,* the *OLE1* gene encoding the Δ9 desaturase is neccessary for the production of

monounsaturated fatty acids (Stukey *et al*., 1989; Stukey *et al*., 1990). Furthermore**,** the regulation of its expression still needs to be studied in detail (Ratledge and Wynn, 2002).

5. Oleaginous yeasts

Although all microorganisms have to synthesize minimum amount of lipid content for their membranes and other structures, only small number of microorganisms can accumulate lipid in lipid bodies higher than 20% of their biomass. In general, lipid accumulation is found in some yeast, fungi and small number of algae. They are named as oleaginous microorganisms (Ratleadge, 2002). Therefore, some yeast that accumulate high concentration of lipid is defined as "oleaginous yeast" (Thorp and Ratledge, 1972). It was reported that only 25 species in 700 yeast species can accumulate lipid more than 20% of biomass such as *Rhodotorula* spp., *Yarrowia lipolitica*, *Lipomyces starkeyi* and *Cryptococcus curvatus* (Ratledge, 2004). The lipid accumulated in oleaginous yeasts is mainly in triacylglycerol form and equivalent in chemical composition of the vegetable oils and fats (Rattray *et al*., 1975, Ratledge and Evan, 1989). The relative amount of fatty acid compositions in oleaginous yeasts are oleic acid (18:1) > palmitic acid (16:0) > linolenic acid (18:2) = stearic acid (18:0), respectively as shown in Table 3 (Ratledge and Tan, 1990). The key of lipid accumulation occurs in culture with limited nitrogen and excess carbon source. The high C/N ratio culture condition has lower effect on growth rate than on lipid biosynthesis and then yeasts allowed excess carbon to be converted into lipid (Ratledge and Wynn, 2002).

Table 3 Profile of fatty acid compositions of oleaginous yeasts used as single cell oil and compared to vegetable oil.

	Major fatty acid residues (relative % w/w)						
	14:0	16:0	16:1	18:0	18:1	18:2	18:3
Yeasts							
Cryptococcus curvatus	-	32	-	15	44	8	-
Lipomyces starkeyi	-	34	6	5	51	3	-
Rhodosporidium toruloides	-	18	3	3	66	-	-
Rhodotorula glutinis	-	37	1	3	47	8	-
Rhodotorula graminis	-	30	2	12	36	15	4
Yarrowia lipolytica	-	11	6	1	28	51	1
Vegetable oil							
Soybean	-	12	-	3	23	56	6
Palm	-	43	-	5	41	10	-
Cotton seed	-	20	-	3	19	55	-
Corn	-	12	-	2	25	61	-
Sun flower	-	6	-	3	17	74	-

- : no data

Source: Ratledge and Wynn (2002); Ma and Hanna (1999)

6. Lipid bodies

Triacylglycerol (TAG) and steryl esters are the main storage lipids in eukaryotic cells. In *Saccharomyces cerevisiae*, these storage lipids were accumulated during stationary growth phase within cellular lipid bodies (Sandager *et al*, 2002). These lipid compounds of yeast are inserted into lipid droplets or lipid bodies which are organelles consisting of neutral lipid surrounded by a phospholipid monolayer as shown in Figure 4 (Murphy and Vance, 1999). The yeast lipid droplets are believed to

form by budding from the endoplasmic reticulum, which also keep most of the enzymes for sterol synthesis and esterification, and triacylglycerol formation (Kurat *et al*., 2006).

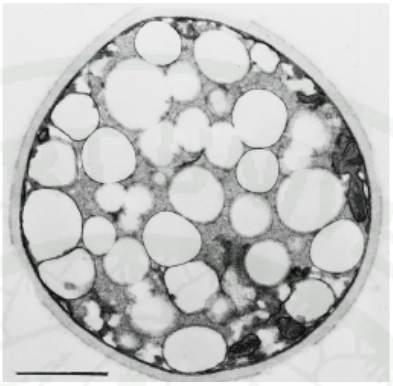

Figure 4 Electron micrograph of *Cryptococcus curvatus* strain D grown for 2 days on nitrogen-limiting medium showing presence of multiple lipid droplets. Total lipid content approximately 40%. Marker bar: 1 mm.

Source: Ratledge (1989)

7. The patterns of yeast lipid accumulation

Lipid accumulation in oleaginous yeast has been known to be occurred by stress conditions in the medium (Ratledge and Wynn, 2002). When cells start depletion of the nutrients such as nitrogen, the excess carbon in culture medium and is converted to cellular lipid (Aggelis and Sourdis, 1997). This pattern is observed in the lipid-accumulating yeast and filamentous fungi (Figure 5).

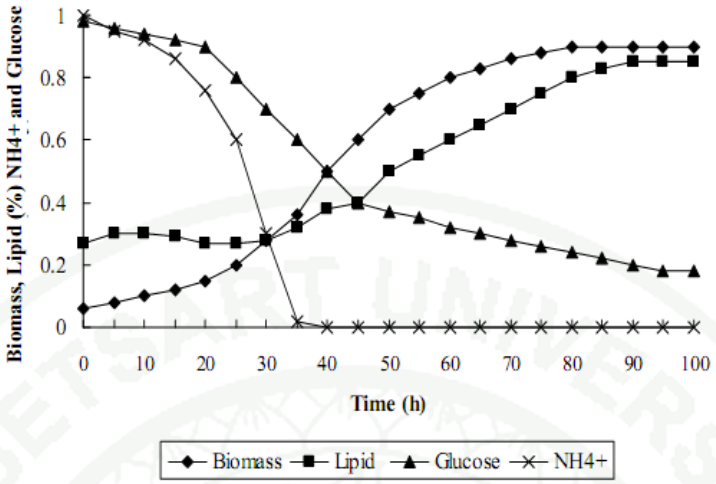

Figure 5 Lipid accumulation in batch cultivation that is pattern of oleaginous yeasts and filamentous fungi showing that lipid accumulation does not start until nitrogen is exhausted from the medium.

Source: Ratledge and Wynn (2002)

8. The biochemistry of yeast lipid accumulation

For the further development of the microbial oil production from yeast it is necessary to understand how oleaginous yeasts synthesize fatty acid and accumulate lipid in their cells. In some oleaginous yeasts, the content of storage lipid may reach 70% of their biomass when culture in optimized medium. Furthermore, the accumulated lipid is in the triacylglycerol form, as in commercial vegetable oils (Ratleadge, 2004).

In order to promote lipid accumulation in oleaginous yeasts, cultivation in a medium with an excess of carbon source and a limiting amount of nitrogen source is neccessary. The excess carbon is converted to lipid by lipid biosynthesis which is accumulated within lipid bodies (Ratleadge, 2004). Non-oleaginous yeasts such as *Saccharomyces cerevisiae* (baker's yeast) and *Candida ultilis* (food yeast) never

accumulate lipid higher than 10% of their biomass (Rattray, 1989). When cultivation of non-oleaginous yeasts in nitrogen-limiting medium they convert excess carbon source into various polysaccharides for example glucans and mannans (Ratleadge and Wynn, 2002).

The first biochemical reaction after nitrogen exhaust from the medium is an activation of adenosine monophosphate deaminase which catalyzes the conversion of AMP to inosine 5'-monophosphate and ammonium (Evan and Ratledge, 1985).

$$\text{AMP} \xrightarrow{\text{deaminase}} \text{inosine 5'-monophosphate} + NH_4^+$$

This reaction is ammonia-scavenging mechanism. The decrase in AMP concentration results in changing of oxygen consumption by the cells and releasing of CO_2 (Wynn *et al*., 2001). This is attributable to reduce or stop isocitrate dehydrogenase activity. The adenosine monophosphate deaminase enzyme in oleaginous microorganism has absolutely requirement for AMP, but in the non-oleaginous cell, the enzyme has no such absolute dependency (Botham and Ratledge, 1979). An inactivation of isocitrate dehydrogenase is resulted from the decrease in AMP concentration. The isocitrate, is not metabolized via tricarboxylic acid cycle because the depletion of isocitrate dehydrogenase activity, would then equilibrate to citrate via activity of aconitase (Evan and Ratledge, 1983).

$$\text{Isocitrate} \leftrightarrow \text{Aconitate} \leftrightarrow \text{Citrate}$$

Citrate is transported out of mitochondria to cytosol by malate/citrate translocase system (Evan and Ratledge, 1985). The citrate within cytosol is cleaved by ATP:citrate lyase, the enzyme that is not found in non-oleaginous microorganism, as shown in the following equation (Botham and Ratledge, 1979).

$$\text{Citrate} + \text{CoA} + \text{ATP} \rightarrow \text{acetyl-CoA} + \text{oxaloacetate} + \text{ADP} + \textit{Pi}$$

ATP: citrate lyase is reported as one of the key enzymes that must be present in eukaryotic microbial cell which can accumulate triacylglycerol lipids. Figure 6 shows the information that describes sequence of lipid accumulation in oleaginous yeasts and molds (Ratledge and Wynn, 2002).

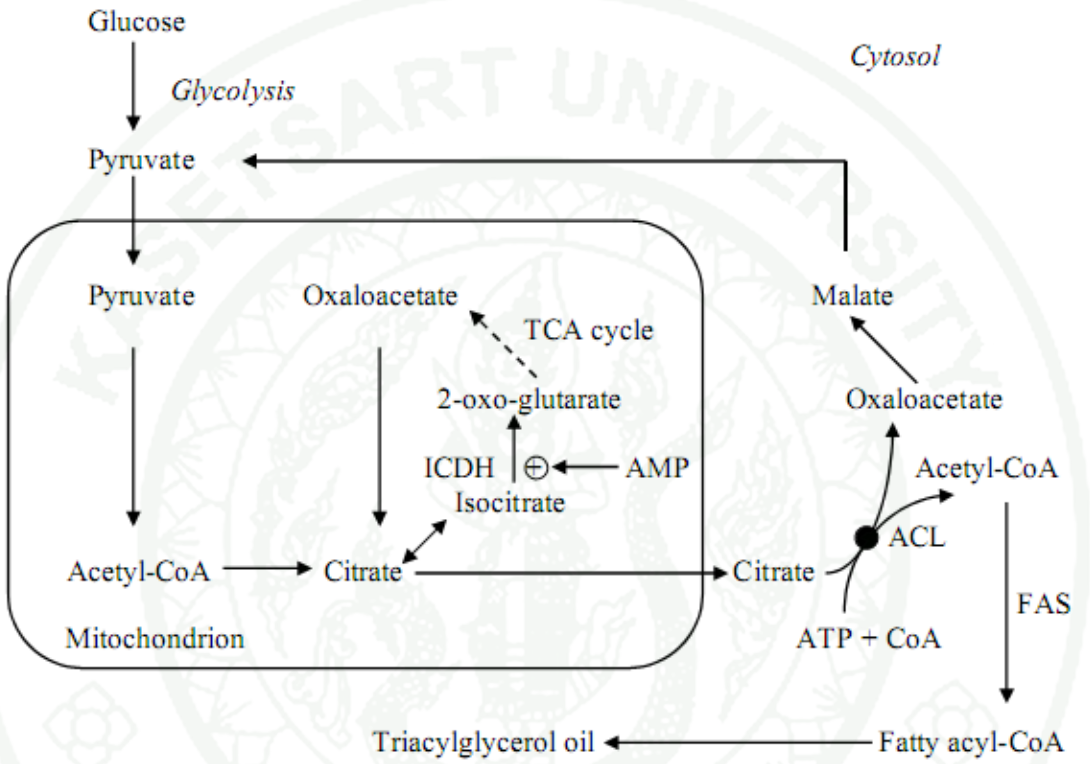

Figure 6 Outline of the main event leading to accumulation in oleaginous yeasts and molds. Lipid accumulation is triggered by a sequence of events described in the text. ICDH: isocitrate dehydrogenase (AMP depent); TCA cycle: tricarboxylic acid cycle; ACL: ATP:citrate lyase; FAS fatty acid synthase.

Source: Ratledge and Wynn (2002)

There is a direct correlation between ATP:citrate lyase activity and the ability of lipid accumulation in yeasts, filamentous fungi and other oleaginous microorganism (Ratledge and Wynn, 2002). No organism has been found to accumulate lipid higher than 20% of their biomass without ATP:citrate lyase activity

as shown in Table 4. However, there are a small number of yeasts that have been found this activity but do not accumulate lipid. There is no correlation between the absolute activity of ATP:citrate lyase and the concentration of lipid acumulation in various yeasts and fungi. Thus, it cannot be the sole explanation for accumulation of lipid. Other enzymes have to be responsible for controlling the lipid biosynthesis in each organism (Ratledge, 2002).

Table 4 Possible correlation of ATP:citrate lyase activity with high lipid content in oleaginous yeasts.

Yeast	ATP:citrate lyase activity (nmol min^{-1}mg $protein^{-1}$)	Lipid content (% dry weight)
Cryptococcus curvatus	7	34
Candida tropicalis	0	4
Candida utilis	0	4
Hansenula saturnus	11	25
Lipomyces lipofer NCYC 944	50	36
Lipomyces lipofer NCYC 692	0	2
Rhodosporidium toruloides CBS 6016	0	3
Rhodosporidium toruloides CBS 5490	42	26
Rhodosporidium toruloides CBS 2590	45	4
Rhodosporidium toruloides CBS 2921	52	5
Rhodotorula graminis	42	24
Saccharomyces cerevisiae	0	6

Source: Ratledge and Wynn (2002)

9. Triacylglycerol biosynthesis in yeasts

For mammalian cells, triacylglycerol (TAG) synthesis can be described in two different phases. The first phase contains different reactions leading to diacylglycerol (DAG) formation. In the second phase, another acylation reaction involves the the conversion of DAG to TAG (Czabany *et al*., 2007). In yeast, *de novo* synthesis of DAG can be divided into two pathways, namely the glycerol-3-phosphate (G-3-P) pathway and the dihydroxyacetone phosphate (DHAP) pathway (Rajakumari *et al*., 2008). An important precursor for TAG biosynthesis is phosphatidic acid (PtdOH) shown in Figure 7. The route of PtdOH synthesis in yeast involves two reactions with either G-3-P or DHAP as an alternative starting precursor (Sorger and Daum, 2003). The first route, G-3-P is acylated by G-3-P acyltransferase (GAT) at the *sn-1* position to form 1-acyl-G-3-P (lyso-phospatidic acid, LPA), and then by 1-acyl- G-3-P acyltransferase (AGAT) in the *sn-2* position, providing phosphatidic acid as product. The second route, DHAP is acylated at the *sn-1* position by DHAP acyltransferase (DHAPAT), and the formation of 1-acyl- DHAP is reduced by 1-acyl-DHAP reductase (ADR) to LPA, which is further acylated to PA by AGAT. phosphatidic acid can also be synthesized from phospholipids by the enzymatic activity of phospholipase D, or by phosphorylation of DAG through DAG kinase (Figure 7). Dephosphorylation of PA by a phosphatidate phosphatase (PAP) provides DAG (Figure 7), which is also synthesized from TAG by TAG lipase or from phospholipids with phospholipase reaction (Czabany, 2007; Rajakumari, 2008; Sorger and Daum, 2003).

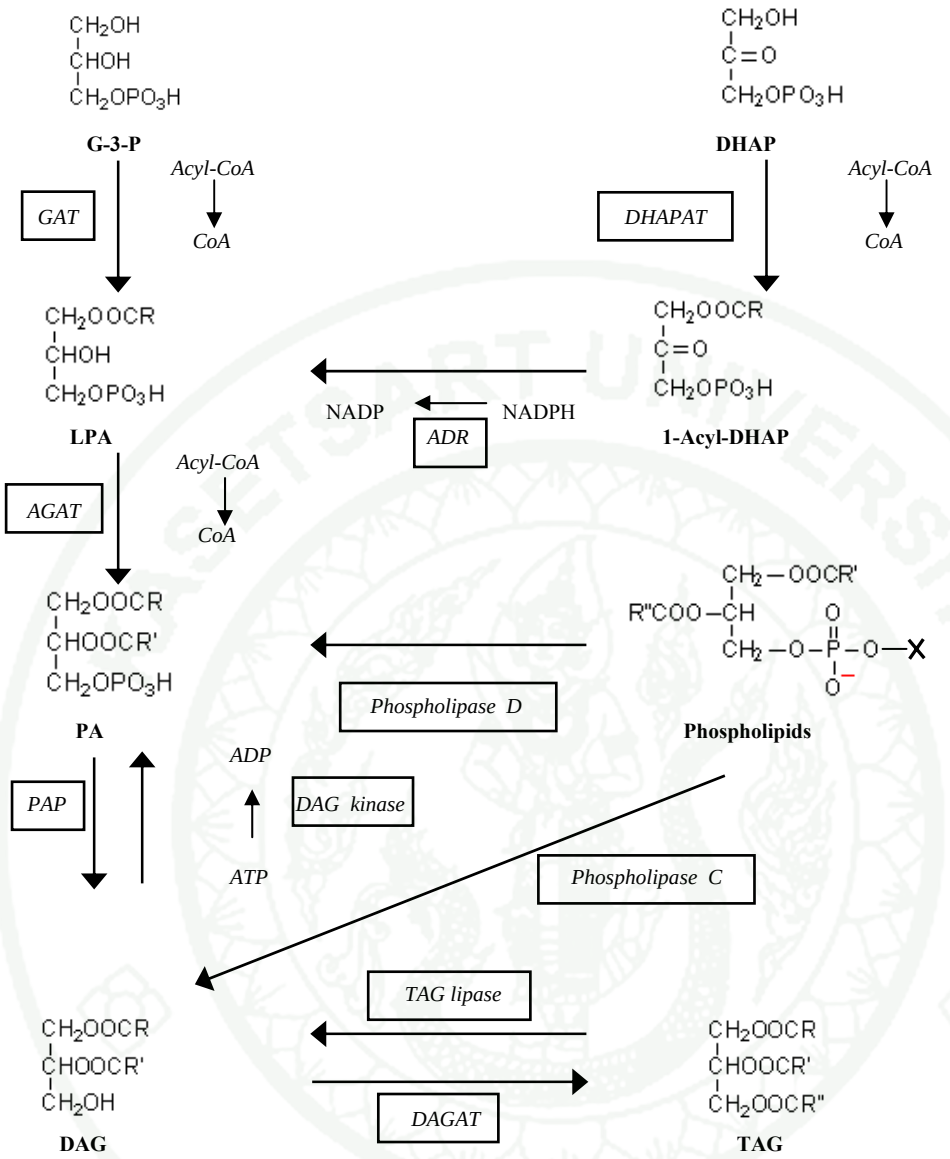

Figure 7 Pathways for triacylglycerol (TAG) synthesis in *Saccharomyces cerevisiae* *AGAT*: 1-acyl-glycerol-3-phosphate acyltransferase, *ADR*: 1-acyl-dihydroxyacetone-phosphate reductase, *DAG*: diacylglycerol, *DAG*: kinase diacylglycerol kinase, *DAGAT*: diacylglycerol acyltransferase, *DHAPAT*: dihydroxyacetone-phosphate-acyltransferase, *DHAP*: dihydroxyacetone phosphate, *1-acyl DHAP*: 1-acyl dihydroxyacetone phosphate, GAT: glycerol-3-phosphate acyltransferase, G-3-P: glycerol-3-phosphate, *LPA*: lyso phosphatidic acid, *PA*: phosphatidic acid, *PAP*: phosphatidate phosphatase, *TAG*: triacylglycerol, *TAG lipase*: triacylglycerol lipase.

Source: Sorger and Daum (2003)

10. Efficiency of yeasts lipid accumulation

The fatty acid profiles depend on oleaginous yeast types and growth conditions. Environmental conditions such as temperature, pH, substrate, C/N ratio and oxygen have influence on efficiency of accumulating lipid (Jacob, 1993). In principle, a good range of productivity of lipid product can be achieved in batch, fed-batch and continuous processes.

The process of yeast lipid production was improved in both batch and single stage continuous fermentation (Evans and Ratleadge, 1984; Gill *et al*., 1977). In continuous cultivation, the medium must be adjusted with high C/N ratio at approximately 50:1 (Ykema *et al*., 1986). Similar result of high C/N ratio on high lipid content (50% of cells dry weight) of *Apiotrichum curvatum* (*Cryptococcus curvatus*) growing with whey permeates in continuous fermentation was reported by Ykema *et al*. (1988). Gill *et al*. (1977) used single stage continuous process for culturing oleaginous yeast, *Candida* 107, and found that cell of *Candida* 107 accumulated lipid of 35% of dry biomass when glucose was used as a substrate. Lipid production by *Candida curvatas* (*Cryptococcus curvatus*) was investigated both in batch and continuous fermentation using media containing glucose, sucrose, lactose, xylose or ethanol (Evans and Ratledge, 1983). Results showed that when xylose was used, lipid production of 49 % of dry biomass was obtained in batch fermentation while 37% of dry biomass was attended with chemostat fermentation. It is also showed that in continuous fermentation, lipid accumulation profiles depended on the dilution rate (0.05/h) Papanikolaou *et al*. (2002) investigated lipid production by *Yarrowia lipolytica* growing on industrial fat composted of saturated free fatty acid in batch fermentation and lipid of 44-54 % of biomass was obtained. Lipid production by *Rhodosporidium toruloides* Y4 in batch fermentation resulted in accumulating of lipids up to 76% of dry biomass when glucose was used as a substrate (Li *et al*., 2006).

Although most studies on microbial lipid accumulation have been carried out by using batch and continuous fermentation, the other cultivation systems have also been investigated. In particular, fed-batch cultivation system has been applied to increase both cell growth and microbial lipid production (Yamauchi *et al*., 1983). Pan and Rhee (1986) reported the lipid production as 40% of dry biomass by *Rhodotorula glutinis* using fed-batch cultivation with feeding medium containing 600 g/l glucose. Ykema et al. (1989) investigated lipid production by *Candida curvatus* using various culture systems in whey permeate. A cell density of 91.4 g/l and lipid content of 33% of dry biomass were obtained when a partial recycling method was used. Meesters *et al*. (1996) used glycerol as a carbon source in fed-batch fermentation with *Candida curvatus* and obtained a cell density of 118 g/l with a lipid production rate of 0.59 g/ l/h; however, the lipid content was only 25% dry biomass. An oleaginous yeast, *Rhodosporidium toruloides* Y4, was reported to produce lipid as 67.5% of dry biomass by using fed-batch mode (Li *et al*., 2007).

11. Yeasts as cocoa butter equivalents (CBEs) producers

Oleaginous yeasts are used to produce cocoa butter equivalents (CBEs). Cocoa butter has high saturated fatty acid content (60% of total fatty acid) that consists of stearic acid (35% of total fatty acid) and palmitic acid (25% of total fatty acid) (Dyal and Narine, 2005). One alternative CBEs producer has been reported in Δ9-desaturated (Ufa) yeast mutants belonging to the yeast *Cryptococcus curvatus* (formerly, *Apiotrichum curvatum*; originally, *Candida curvata* D) (Ykema *et al*., 1990). The Δ9-desaturase is an enzyme that catalyzed conversion of stearic acid to oleic acid and palmitic acid to palmitoleic acid. The lack of Δ9-desaturase activity in these mutants resulted in higher stearic acid content which is similar to the fatty acid composition of cacoa butter (Table 5).

Table 5 Profile of fatty acid composition of yeast used as source of CBEs in comparison with cocoa butter.

Source	Relative fatty acid composition (%w/w)				
	16:0	18:0	18:1	18:2	18:3
Yeast isolate K7-4	20	24	40	7	2
Rhodosporidium toruloides	20	47	22	5	2
Cryptococcus curvatus F33.10	24	31	30	6	-
Cacoa butter	28	35	35	2	-

Source: Ratledge and Wynn (2002)

12. Yeast lipid as a potential source of an alternative biodiesel production

Biodiesel, one of the alternative fuels, has received considerable attention in recent years as it is renewable, biodegradable and nontoxic. Currently, biodiesel is produced from vegetable oils and animal fats. Although it has been used in many countries such as Germany, Italy, France, USA, Australia, Japan, China, Brazil, Argentina, Indonesia and Malaysia (Du *et al*., 2008; Fukuda *et al*., 2001; Zhu *et al*., 2008), manufacturing fuel from vegetable oils is undesirable because this competes with the use of these oils for human consumption and prevents the long-term development and large scale-use of biodiesel (Miao and Wu, 2006; Zhu *et al*., 2008). To minimize this, other renewable sources of oils should be considered. Recently microbial oils have been getting more attention because they, like conventional vegetable oils, are basically triacylglycerols that require a tranesterification process to convert them to biodiesel. From this viewpoint, microbial oils are potential sources for biodiesel production in the future (Li *et al*., 2008). Interest in lipid production by oleaginous yeasts has increased significantly because its recognition as potential source for biodiesel production. A red yeast, *Rhodosporidium toruloides* Y4, was examined both in batch and fed-batch fermentation for enhancing cell density and lipid content for lipid production as biodiesel raw material (Li *et al*, 2007). The novel method has been developed by Xue *et al*. (2006) for the biodiesel production from

monosodium wastewater containing COD of 10,000 mg/l. After treatment, the COD removal was 85% with 10% production of crude lipid. A total of 92% of lipid produced by *Rhodotorula glutinis* was converted to biodiesel with tranesterification (Xue *et al*, 2006). Xue *et al*. (2008) reported the lipid production by an oleaginous yeast, *Rhodotorula glutinis*, in medium containing monosodium glutamate wastewater using batch fermentation. Li *et al*. (2007) reported the conversion yeast lipid into 90 % biodiesel yield with lipase-catalyzed methanolysis.

13. Staining of lipids by Nile red

13.1 Chemical properties of Nile red

Nile red (9-diethylamino-5H-benzo[α]phenoxazine-5-one) is a benzophenoxazone dye (Figure 8). In older chemical literature it is referred as Nile blue A-oxazone. It is produced by boiling a solution of nile blue with sulfuric acid which amino groups are replaced by a carboxyl groups. Nile red is poorly soluble in water but is dissolved in various organic solvents (Greenspan *et al*., 1985). Nile red is intensely fluorescent in all organic solvents depending on the relative hydrophobic properties of solvent. The fluorescence colors ranged from golden yellow to deep red. Golden yellow fluorescent (excitation, 450–500 nm; emission, >528 nm) emission appeared when it is dissolved in neutral lipids such as cellular lipid droplets and red fluorescent (excitation, 515–560 nm; emission, >590 nm) emission appeared when dissolved in more polar solvents or phospholipids **(**Diaz *et al*., 2008**).**

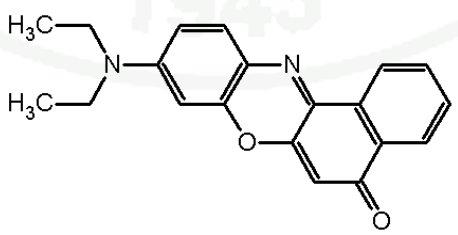

Figure 8 Chemical structure of Nile red.

Source: Mayer (1985)

METERIALS AND METHODS

1. Isolation of yeasts for lipid production

Two grams of soil sample was added into 50 ml of acidified (pH 3.7-3.8) nitrogen-limited medium I (30 g/l glucose, 1.5 g/l yeast extract, 0.5 g/l NH_4Cl, 7.0 g/l KH_2PO_4, 5.0 g/l $Na_2HPO_4 \cdot 12H_2O$, 1.5 g/l $MgSO_4 \cdot 7H_2O$, 0.08 g/l $FeCl_3 \cdot 6H_2O$, 0.01 g/l $ZnSO_4 \cdot 7H_2O$, 0.1 g/l $CaCl_2 \cdot 2H_2O$, 0.1 mg/l $MnSO_4 \cdot 5H_2O$, and 0.1 mg/l $CuSO_4 \cdot 5H_2O$ (Suuatari *et al*., 1993) supplemented with 0.2 mg/ml chroramphenical and 1 mg/ml sodium propionate, adjusted to pH 3.7-3.8 in a 250 ml Erlenmeyer flask and incubated on a rotary shaker at 150 rpm and 28 ºC for 72h. Culture broth was streaked onto nitrogen-limited agar plate and plate was incubated for 4 days at 28 ºC. Yeast colonies appear on agar plate were picked and purified by cross streaking on YM agar (10 g/l glucose, 5 g/l peptone, 3 g/l yeast extract, 3 g/l malt extract, 20 g/l agar). The pure culture was maintained in a deep freezer (-80 ºC) after suspended in YM broth supplemented with 10% glycerol as a cryoprotectant.

2. Screening for oleaginous yeast strain

Yeast strains obtained by isolation procedure described in 1 and the strains of known yeast species were subjected to screening of oleaginous yeast strains.

2.1 Primary screening

To preliminary determination cellular lipid content of yeast, Nile red staining was used. Inoculum were prepared by transferring one loop full of 24 h yeast culture grown on YM agar slant to a test tube (16 X 150 mm) containing 5 ml nitrogen-limited medium I and incubated on a rotary shaker at 150 rpm and 28 ºC for 24 h and then transferred as the amount of 5% into 5 ml of nitrogen-limited medium I (pH 5.5) contained in a test tube (16 X 150 mm). The test tube was incubated on a rotary shaker at 150 rpm at 28 ºC for 72 h. After 72 h, culture was taken and diluted with sterile water to appropriate cell concentration and stained by Nile red (Fluka,

Germany). Nile red stain was prepared by dissolving 10 µg of Nile red in 1 l of absolute ethanol (99.8% w/v). A 40 µl of cell suspension was mixed with 10 µg/l Nile red stain. After 5 minutes, lipid body of yeast cells was observed by fluorescence microscopy (Olympus BX51, Japan) using a 450-490 nm excitation filter, a 505-nm diachronic mirror X40 objective lens. Nile red shows strong yellow-gold emission when stain on a lipid body. Microscopic photographs were taken with a digital camera (Olympus DP11, Japan). Yeast strain which contain many and large lipid body was selected for further screening.

2.2 Secondary screening

To secondary screen yeast strains with high cellular lipid content, yeast was cultivated in nitrogen-limited medium I and lipid was determined by the method described in 6.1.

2.2.1 Inoculum preparation

One loop of yeast cells growing on YM agar slant at 28 ºC for 24 h was inoculated into 5 ml of nitrogen-limited medium I which pH was adjusted to 5.5 in a test tube (16 X 150 mm). The test tube was incubated on a rotary shaker at 150 rpm at 28 ºC for 24 h.

2.2.2 Cultivation for lipid production

Two and a half milliliters of 24-h preculture of yeast was transferred to 50 ml of nitrogen-limited medium I in 250 ml Erlenmeyer flask and incubated on a rotary shaker at 180 rpm and 28 ºC for 72 h. Cells were harvested for lipid analysis by the method described in 6.1 and dry weight determination by the method described in 6.3. Yeast strain which produce high cellular lipid content was selected for further screening.

2.3 Tertiary screening

Tertiary screening for yeast strains with high cellular lipid content, was carried out by cultivation of the selected yeast strains obtained from 2.2 in nitrogen-limited broth II (30 g/l glucose, 0.1 g/l $(NH_4)_2SO_4$, 0.75 g/l yeast extract, 1.5 g/l $MgSO_4 \cdot 7H_2O$, 0.4 g/l KH_2PO_4, 0.22 g/l $CaCl_2 \cdot 2H_2O$, 0.55 µg/l $ZnSO_4 \cdot 7H_2O$, 24.2 µg/l $MnCl_2 \cdot 4H_2O$ and 25 µg/l $CuSO_4 \cdot 5H_2O$) which pH was adjusted to 6.0 (Li *et al*., 2007) and determination of their cellular lipid content.

2.3.1 Inoculum preparation

One loop of yeast cells growing on YM agar slant at 28 ºC for 24 h was inoculated into 5 ml of nitrogen-limited broth II in a test tube (16 X 150 mm). The test tube was incubated on a rotary shaker at 150 rpm 28 ºC for 24 h. Two and half milliliters of 24-h preculture was transferred to 50 ml of nitrogen-limited medium II in 250 ml Erlenmeyer flask and incubated on a rotary shaker at 150 rpm at 28 ºC for 24 h.

2.3.2 Cultivation for lipid production

Five milliliters of 24-h preculture was transferred to 100 ml of nitrogen-limited medium II in 500 ml Erlenmeyer flask and incubated on a rotary shaker at 150 rpm and 28 ºC for 120 h. Cells were harvested every 12 h of 120 h cultivation for lipid analysis by the method of 6.1 and dry weight determination by the method of 6.3. Yeast strain which produce the highest lipid content was selected for optimization.

3 Identification of the selected oleaginous yeast strain

The sequence of the D1/D2 domains of the large subunit (LSU) rRNA gene was determined from PCR products from genomic DNA extracted from yeast cells using the slightly modified method described by Lachance *et al.* (1999). The D1/D2

domain of the LSU rRNA gene was amplified by a PCR with the forward primer NL1 and the reverse primer NL4 (O'Donnell, 1993). The PCR product was checked by agarose gel electrophoresis purified using the QIAquick purification kit (Qiagen, Ontario, USA) and cycle-sequenced using and ABI BigDye terminator cycle sequencing Kit Version 3.1 (Applied Biosystems, California, USA) according to the manufacturer's instruction. The sequences were compared pairwise using BLASTN homology search program (Altschul *et al*., 1997). Identification was based on the Kurtzman and Robnett (1998) statement that yeast strains showing nucleotide substitutions greater than 1% in the D1/D2 domain of the LSU rRNA gene is usually different species.

4. Optimization for lipid production of the selected yeast strain

The selected yeast strains from tertiary screening which accumulated the highest cellular lipid content was determined for optimal nutrient compositions of the medium and environmental conditions for the highest lipid production. The experiment was carried out in a 500 ml Erlenmeyer flask containing 100 ml of nitrogen limited medium II.

4.1 Inoculum preparation

Inoculum was prepared as explained in 2.3.1

4.2 Cultivation for lipid production

Five milliliters of inoculum was transferred to 100 ml of nitrogen-limited medium II in 500 ml Erlenmeyer flask. After inoculation, the flask was shaken on a rotary shaker at 150 rpm and 28 ºC for optimization of nutrient composition and pH. During cultivation, two milliliters of cultured sample were harvested at time intervals of 0 h, 12h, 24h, 36h, 48h, 60h, 72h, 84h, 108 h, 120 h, 132 h, 144 h, 156 h, 168 h and 180 h. All samples were determined for dry biomass, lipid production, cellular lipid content and glucose concentration.

4.3 Nutrient compositions

4.3.1 Effect of nitrogenous compounds

Different nitrogen sources both organic and inorganic compounds were individually added into the nitrogen-limited broth II. The combined nitrogen sources as yeast extract-ammonium sulfate, yeast extract-ammonium chloride, peptone-ammonium sulfate and peptone-ammonium chloride were also applied. They were selected based on their abilities to achieve the highest lipid production.

4.3.2 Effect of C/N-ratio

Optimum carbon and nitrogen sources from previous experiments were used for determining carbon/nitrogen ratio. The C/N-ratio was adjusted to 65:1, 90:1, 115:1 and 140:1.

4.3.3 Effect of carbon source

The nitrogen-limited broth containing different concentrations of glucose was used in this experiment. The glucose concentrations were adjusted to 50, 70 and 90 g/l. The concentration that provides the highest lipid concentration was selected for further study.

4.3.4 Effect of phosphate concentration

The potassium dihydrogen phosphate concentration was adjusted to 0.4, 1.6, 2.8 and 4.0 g/l in nitrogen-limited medium II containing glucose and nitrogen source at optimal C/N ration obtained from 4.3.2.

4.3.5 Effect of magnesium concentration

The magnesium sulfate concentration was adjusted to 0.5, 1, 1.5, 2.0, 2.5 and 3.0 g/l in nitrogen-limited II.

4.4 Environmental conditions

4.4.1 Effect of initial pH

The selected yeast strain was inoculated into 100 ml nitrogen-limited broth II with optimal nutrient compositions in 500 Erlenmeyer flask and adjusted pH to 5, 5.5, 6 and 6.5.

4.4.2 Effect of incubation temperature

Study of the effect of temperature on lipid production was carried out at the range from 16 to 34 ºC in L-tubes containing 10 ml nitrogen-limited medium II and incubated on a temperature gradient incubator model TN-3 (Toyo Kagaku Sangyo Co. Ltd., Japan).

5. Batch fermentation in fermentor

Batch fermentation was carried out in a 5 l stirred tank fermentor (Microferment NewBrunwick, USA) with 3 l working volume. Nutrient compositions and environmental conditions obtained from optimization in shaking flask were used for determination of lipid production in fermentor. The effect of aeration at 1.0, 2.0 and 3.0 vvm was studied. The effect of agitations was determined at 200 and 300 rpm for the highest lipid production.

6. Analytical methods

6.1 Lipid analysis

6.1.1 Lipid extraction

Cellular lipid content was reported on the basis of the total fatty acid. To determine total fatty acid, lipid components were extracted according to a modified method of Bligh and Dyer (1959). Five milliliters of culture broth was transferred to screw cap test tube (16 X 100 mm), centrifuged at 3,000 rpm for 15 minutes and removed supernatant. To add 0.5 ml distilled water was added to yeast cells sediment and mixed vigorously for 15 seconds. Cell suspension was subjected to lipid extraction with 3.75 ml chloroform-methanol (1:2) mixture added to the suspension and vortexed for 1 minute. Chloroform of 1.25 ml,was added to the mixture, followed by vortexing for 1 minute. Fatty acid internal standard (Pentadecanoic acid) of 0.1 ml was added this step, if the sample were to be analyzed by GC. To the suspension 1.25 ml of distilled water was added, respectively. The mixture was vortexed for 15 seconds and centrifuged at 3,000 rpm for 15 minutes to facilitate phase separation. The bottom chloroform layer was transferred to a new screw cap test tube for further analysis.

6.1.2 Tranmethylation of fatty acid

Derrivation of fatty acids into methyl ester was prepared according to method of Holub and Skeaff (1987). Lipid in the chloroform layer, obtained from previous extraction in Teflon lined screw cap test tube, were dried at 38 ºC under nitrogen atmosphere. Tranesterification was carried out by adding 2 ml of methanol containing 6%H_2SO_4. After mixing for 30 seconds, the screw cap was tightened to avoid leakage. The test tubes were tranmethylated for 14-18 h at 80 ºC and then cooled at room temperature. Two milliliters of petroleum ether were added to the aqueous methanol mixtures and vortexed for 30 seconds. The mixture was washed with 1.25 ml distilled water and mixed for 15 seconds. The upper petroleum ether

layer was then transferred to 2 ml screw cap vial. Petroleum ether was evaporated in vacuum rotary evaporator at 40 ºC under nitrogen stream. The fatty acid methyl ester was recovered in 100 µl hexane layer and then applied to the capillary gas chromatography.

6.1.3 Determination of fatty acid methyl ester

Fatty acid methyl ester was analyzed using gas chromatography (GC14-A, Shimazu, Japan) with capillary column containing silica megabore column (30 mX 0.52 mm X 1 µm, Durabond 225, J and W Scientific, USA) and flame ionized detector. Operating conditions are as follows: helium carrier gas 10 ml/min, nitrogen carrier gas 40 ml/min, column temperature is 210 ºC, injection and detection temperatures are 250 ºC (Holub and Skeaff, 1987). Fatty acids are identified by comparison of their retention times with standard mixtures.

6.2 Determination of dry biomass

A 20 ml of culture broth was harvested and washed with distilled water and then dried at 110 ºC to constant weight. The weight of biomass was determined gravimetrically.

6.3 Determination of glucose concentration

Glucose concentration was measured by HPLC Differential Refractometor R401 (Millipore, Water). ULTRON PS-80C (Shinwa Chemical Industries LTD., Japan) column was used for glucose analysis. The mobile phase was deionized water with the flow rate of 1 ml min^{-1}. The solution containing 1% analytical grade glucose was used as a standard solution of analysis.

RESULTS AND DISCUSSIONS

1. Isolation of yeasts from soil

The newly isolated 104 yeast strains was isolated from various collected soil samples in the natural environment of Thailand by an enrichment technique in nitrogen-limited medium I (Table 6). These strains were purified by conventional streaking technique and maintained at -80 ºC.

Table 6 Number of yeasts isolates from various parts of Thailand.

Year	Parts					Total
	Northern	North-Eastern	Eastern	Central	Southern	
2006	17	20	19	16	11	**83**
2007	12	0	0	0	9	**21**
Total	**29**	**20**	**19**	**16**	**20**	**104**

2. Primary screening

A total of 104 yeast isolates were obtained from soil samples collected from various locations in Thailand. Hence, 170 strains, including 61 strains of *Rhodotorula mucilaginosa*, one strain of *Rhodotorula glutinis*, one strain of an unknown *Rhodotorula* species, and three reference oleaginous yeast strains (*Cryptococcus curvatus* CBS 570, *Lipomyces starkeyi* CBS 1807 and *Rhodosporidium toruloides* CBS 14) from Centraalbureau voor Schimmelcultures (CBS), Utrecht, the Netherlands, were subjected to Nile red staining (Kimura *et al*., 2004). Kimura *et al*. (2004) indicated that lipid bodies in oleaginous microorganisms have different shapes depending on species and culture conditions. The yeast lipid bodies were believed to form by budding from the endoplasmic reticulum, and contained most of the enzymes for sterol synthesis and esterification (Kurat *et al*., 2006). The result of lipid body staining revealed that 48 strains contained many and large lipid bodies comparable to

the reference yeast strains. These strains were then selected for further screening. Photomicrographs of all selected yeast strains obtained with the method described above are shown in Figure 9-25. The lipid bodies of selected strains were spherical in shape with the range of less a 0.5-1.0 µm in diameter.

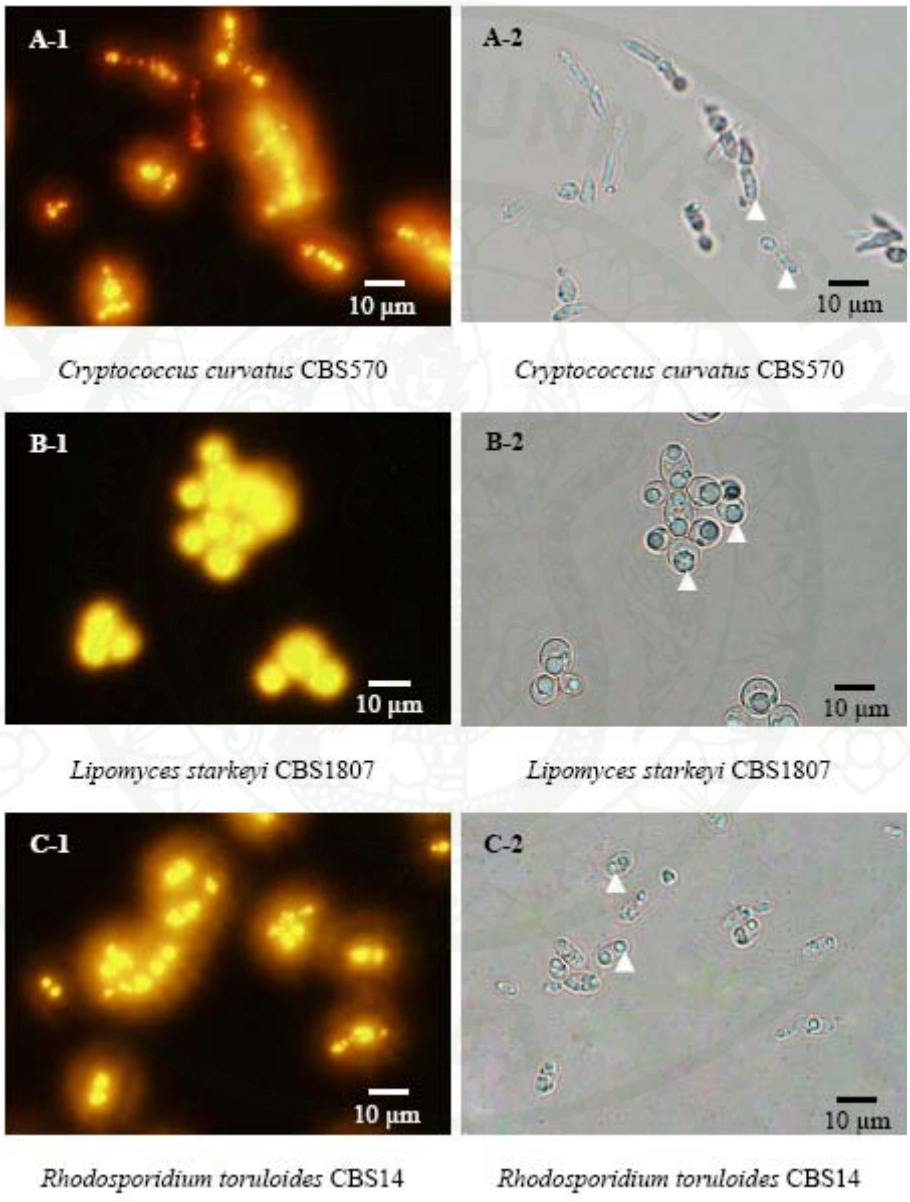

Figure 9 Photomicrographs of Nile red stained cells of *C. curvatus* CBS 570, *L. starkeyi* CBS 1807 and *R. toruloides* CBS 14 observed under fluorescence microscope showed yellow gold emission of lipid bodies (A-1), (B-1) and (C-1) and light microscope (A-2), (B-2) and (C-2) respectively. Arrows indicate lipid bodies.

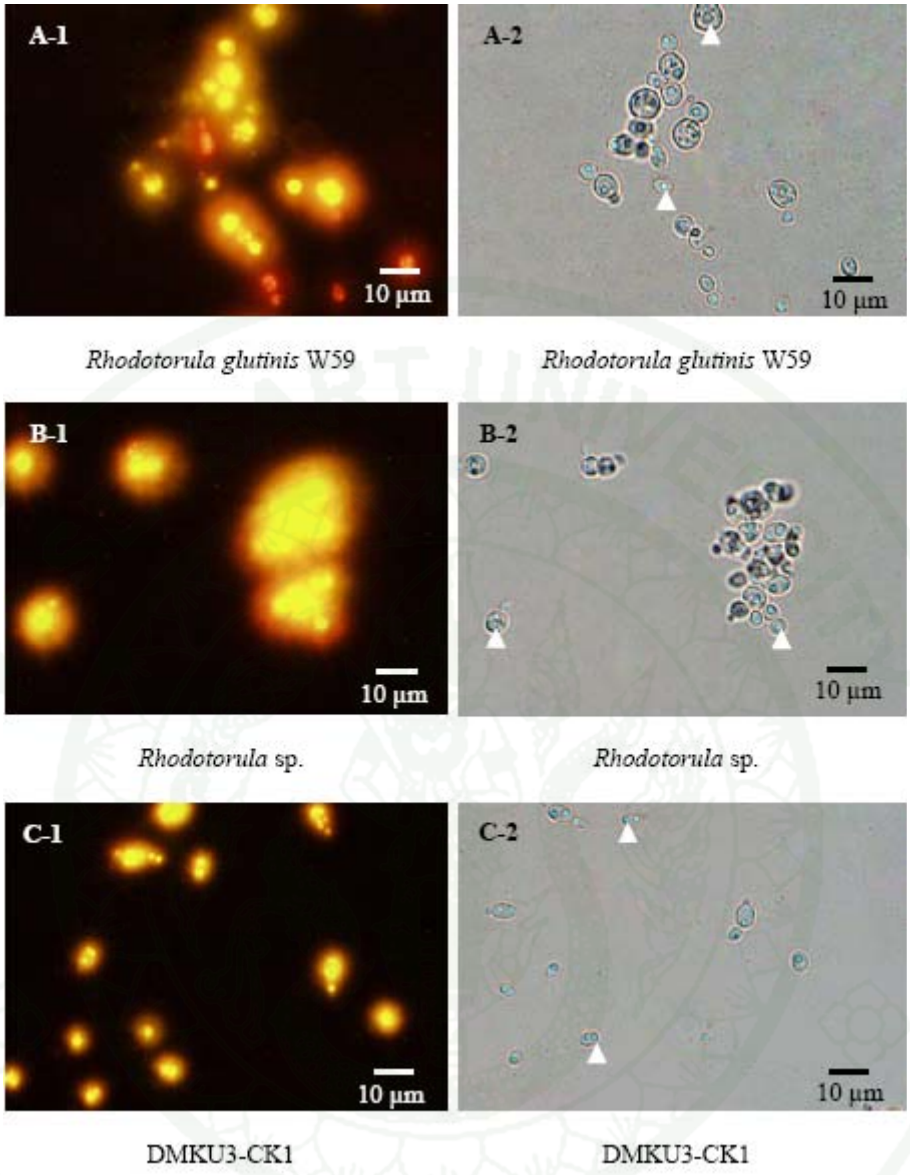

Figure 10 Photomicrographs of Nile red stained cells of *R. glutinis* W59, *Rhodotorula* sp. and DMKU3-CK1 observed under fluorescence microscope showed yellow-gold emission of lipid bodies (A-1), (B-1) and (C-1) and light microscope (A-2), (B-2) and (C-2). Arrows indicate lipid bodies.

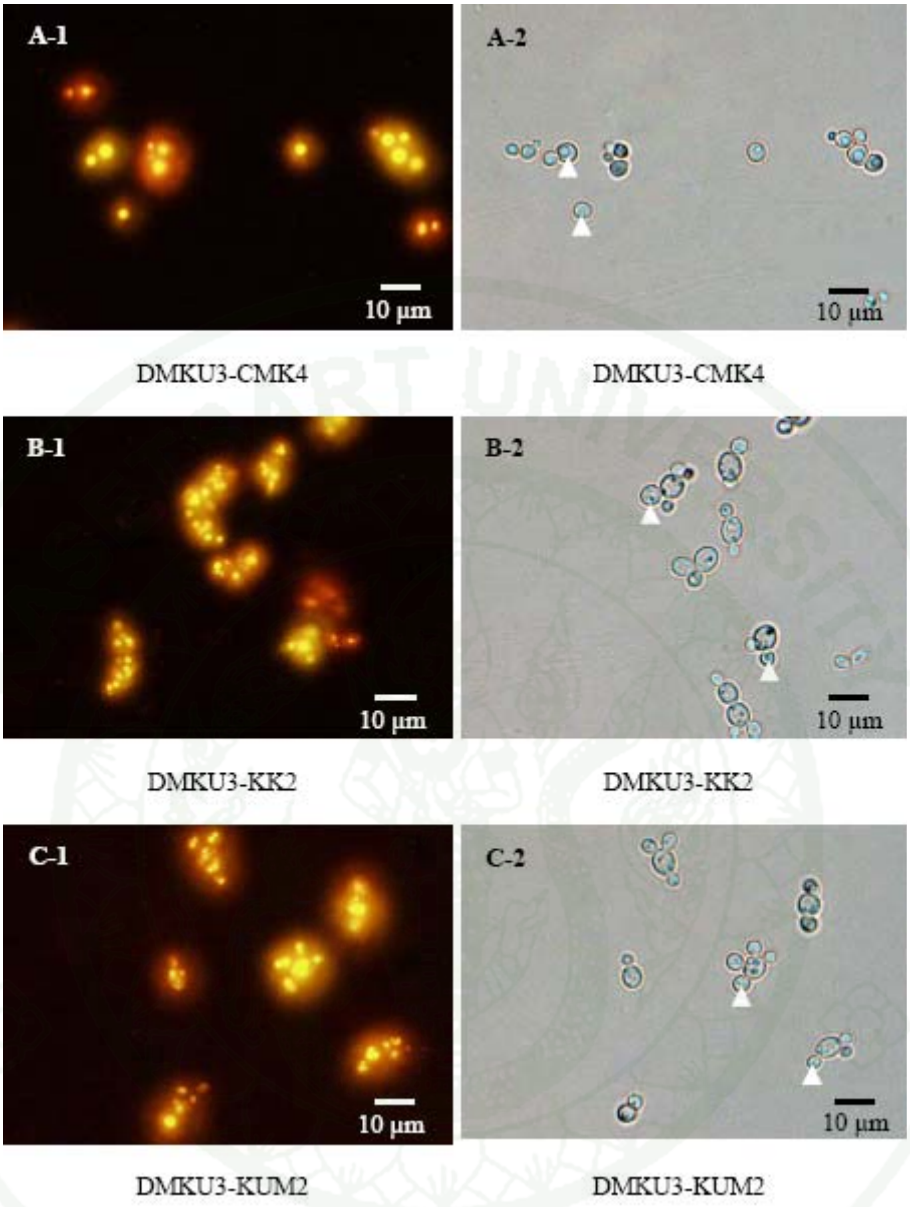

Figure 11 Photomicrographs of Nile red stained cells of DMKU3-CMK4, DMKU3-KK2 and DMKU3-KMU2 observed under fluorescence microscope showed yellow-gold emission of lipid bodies (A-1), (B-1) and (C-1) and light microscope (A-2), (B-2) and (C-2) , respectively. Arrows indicate lipid bodies.

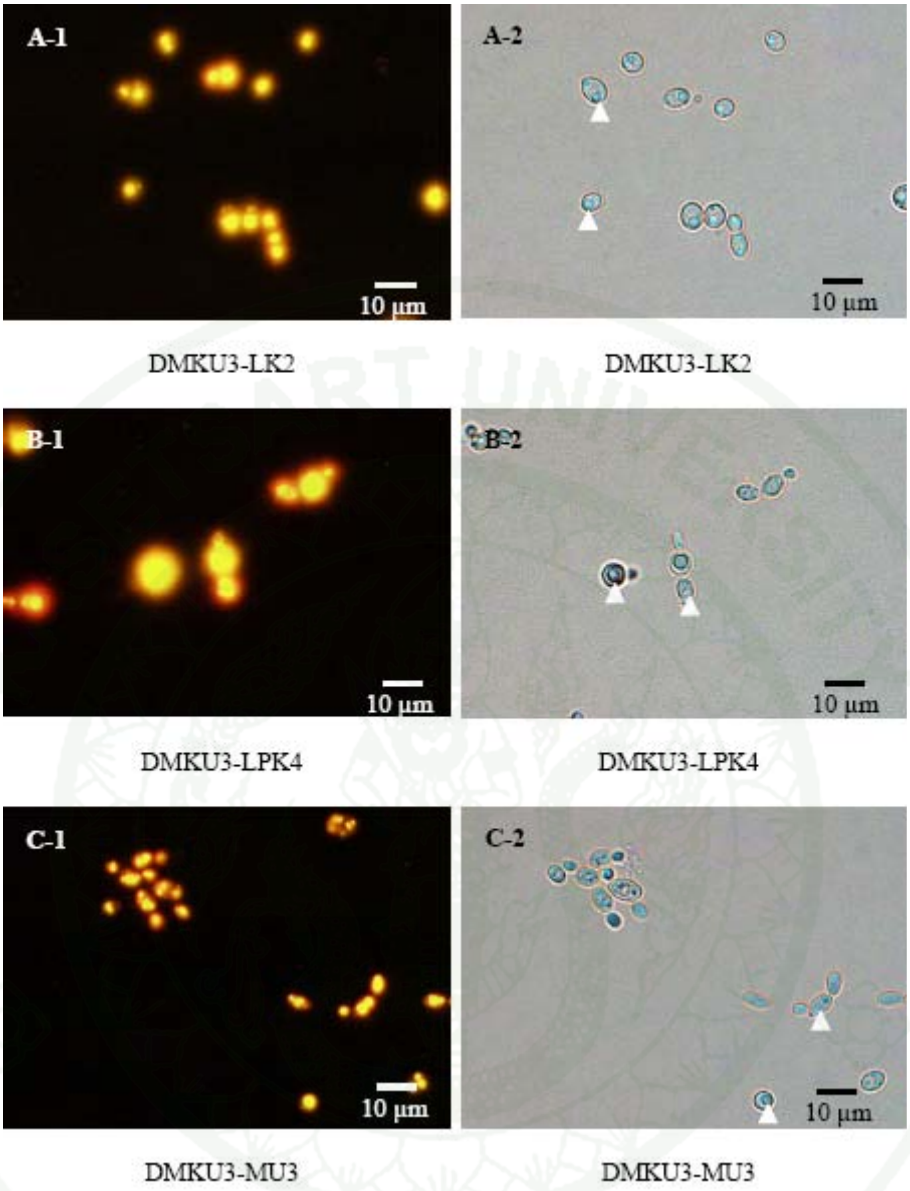

Figure 12 Photomicrographs of Nile red stained cells of DMKU3-LK2, DMKU3-LPK4 and DMKU3-MU3 observed under fluorescence microscope showed yellow-gold mission of lipid bodies (A-1), (B-1) and (C-1) and light microscope (A-2), (B-2) and (C-2), respectively. Arrows indicate lipid bodies.

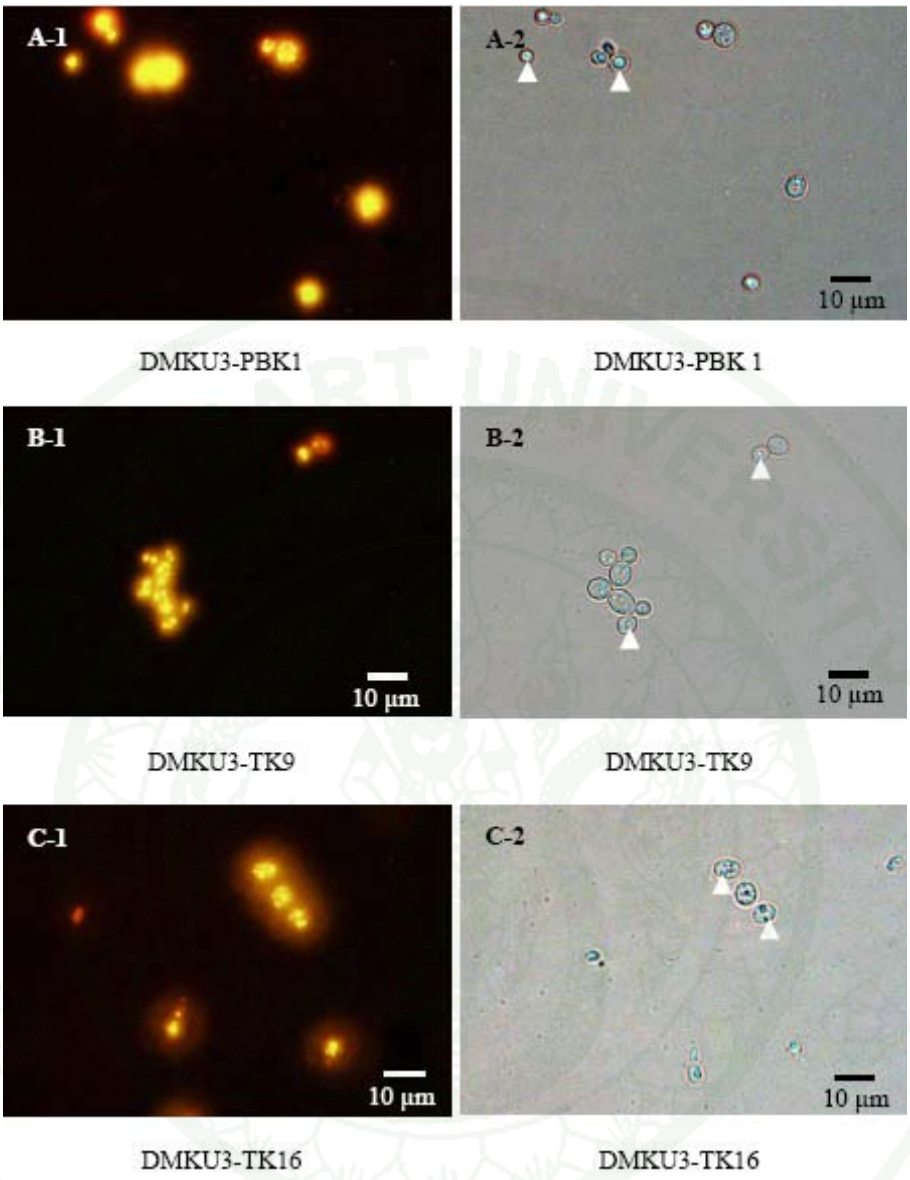

Figure 13 Photomicrographs of Nile red stained cells of DMKU3-PBK1, DMKU3-TK9 and DMKU3-TK16 observed under fluorescence microscope showed yellow-gold emission of lipid bodies (A-1), (B-1) and (C-1) and light microscope (A-2), (B-2) and (C-2), respectively. Arrows indicate lipid bodies.

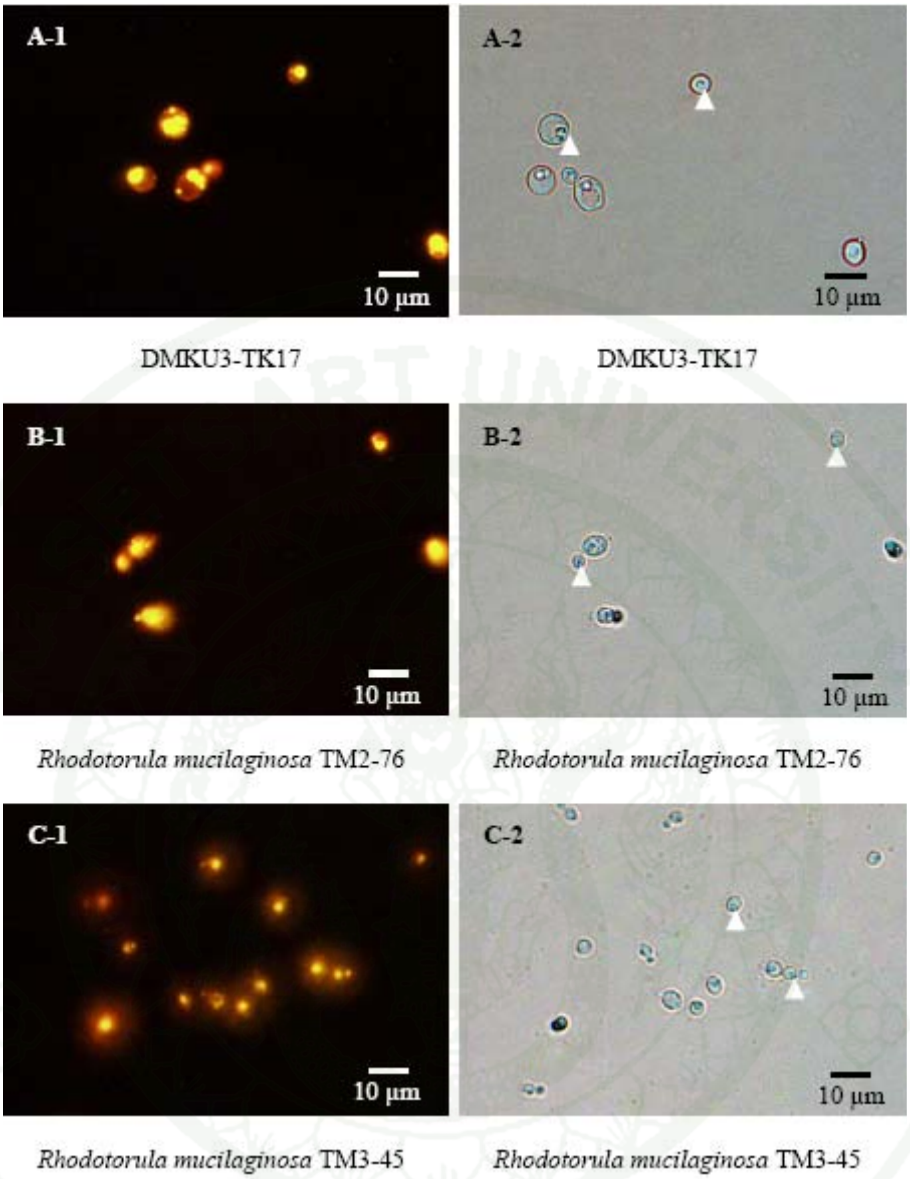

Figure 14 Photomicrographs of Nile red stained cells of DMKU3-TK17, *R. mucilaginosa* TM2-76 and *R. mucilaginosa* TM3-45 observed under fluorescence microscope showed yellow-gold emission of lipid bodies (A-1), (B-1) and (C-1) and light microscope (A-2), (B-2) and (C-2), respectively. Arrows indicate lipid bodies.

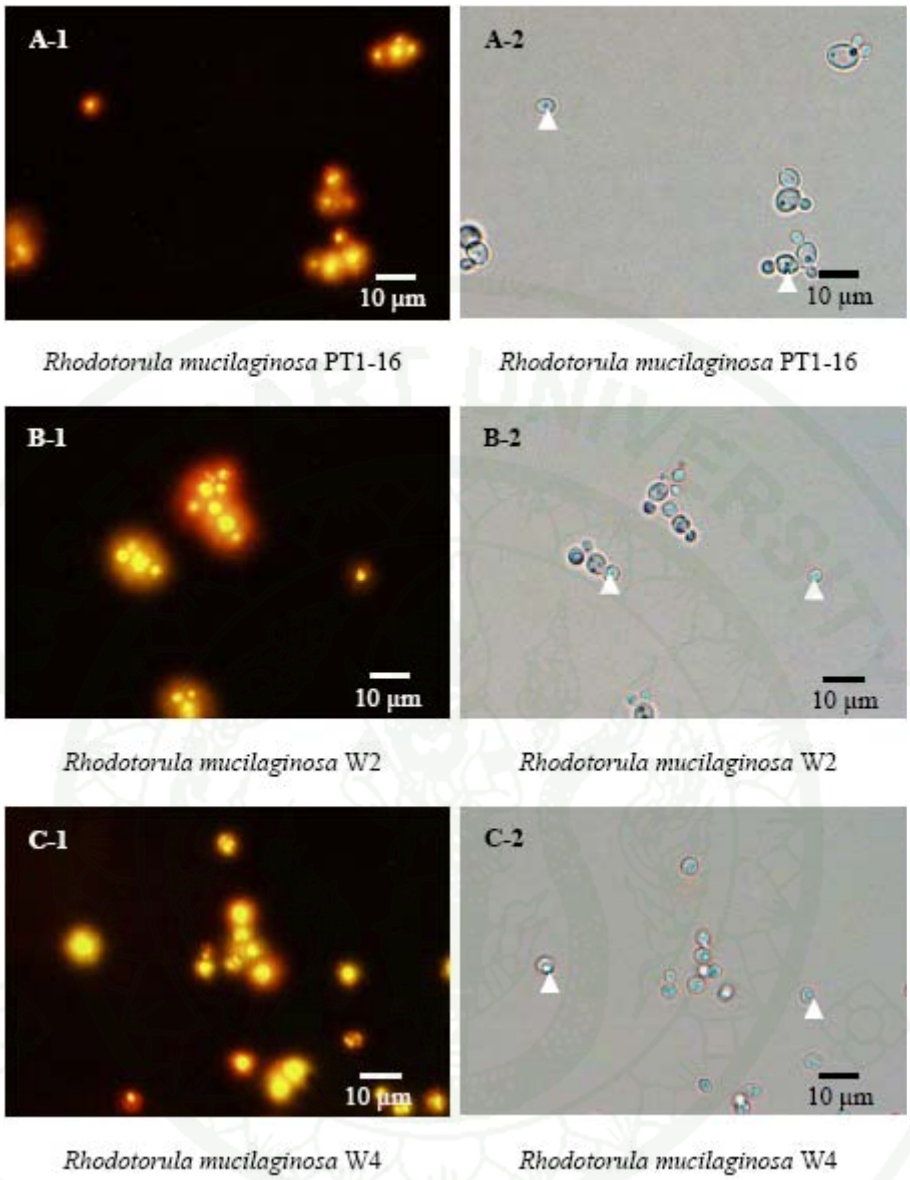

Figure 15 Photomicrographs of Nile red stained cells of *R. mucilaginosa* PT1-16, *R. mucilaginosa* W2 and *R. mucilaginosa* W4 observed under fluorescence microscope showed yellow-gold emission of lipid bodies (A-1), (B-1) and (C-1) and light microscope (A-2), (B-2) and (C-2), respectively. Arrows indicate lipid bodies.

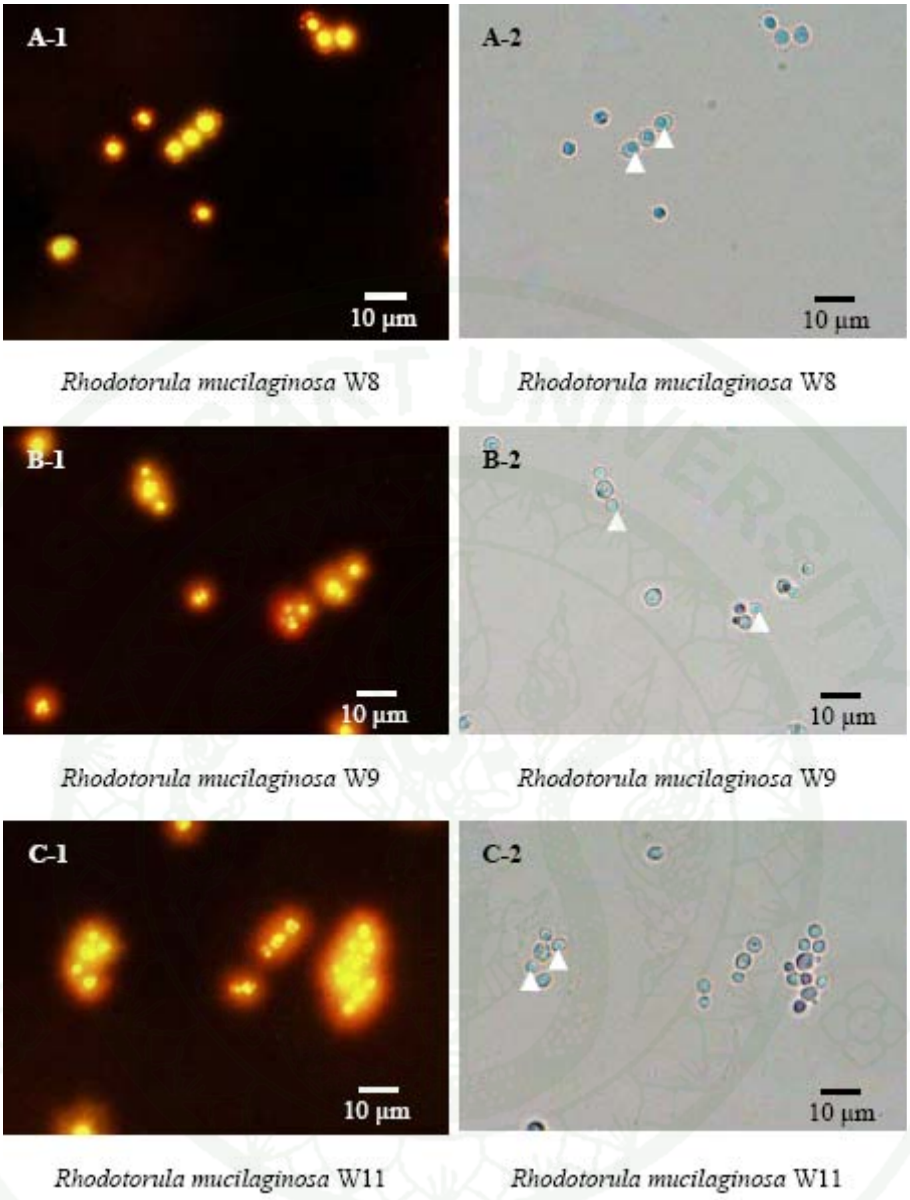

Figure 16 Photomicrographs of Nile red stained cells of *R. mucilaginosa* W8, *R. mucilaginosa* W9 and *R. mucilaginosa* W11 observed under fluorescence microscope showed yellow-gold emission of lipid bodies (A-1), (B-1) and (C-1) and light microscope (A-2), (B-2) and (C-2), respectively. Arrows indicate lipid bodies.

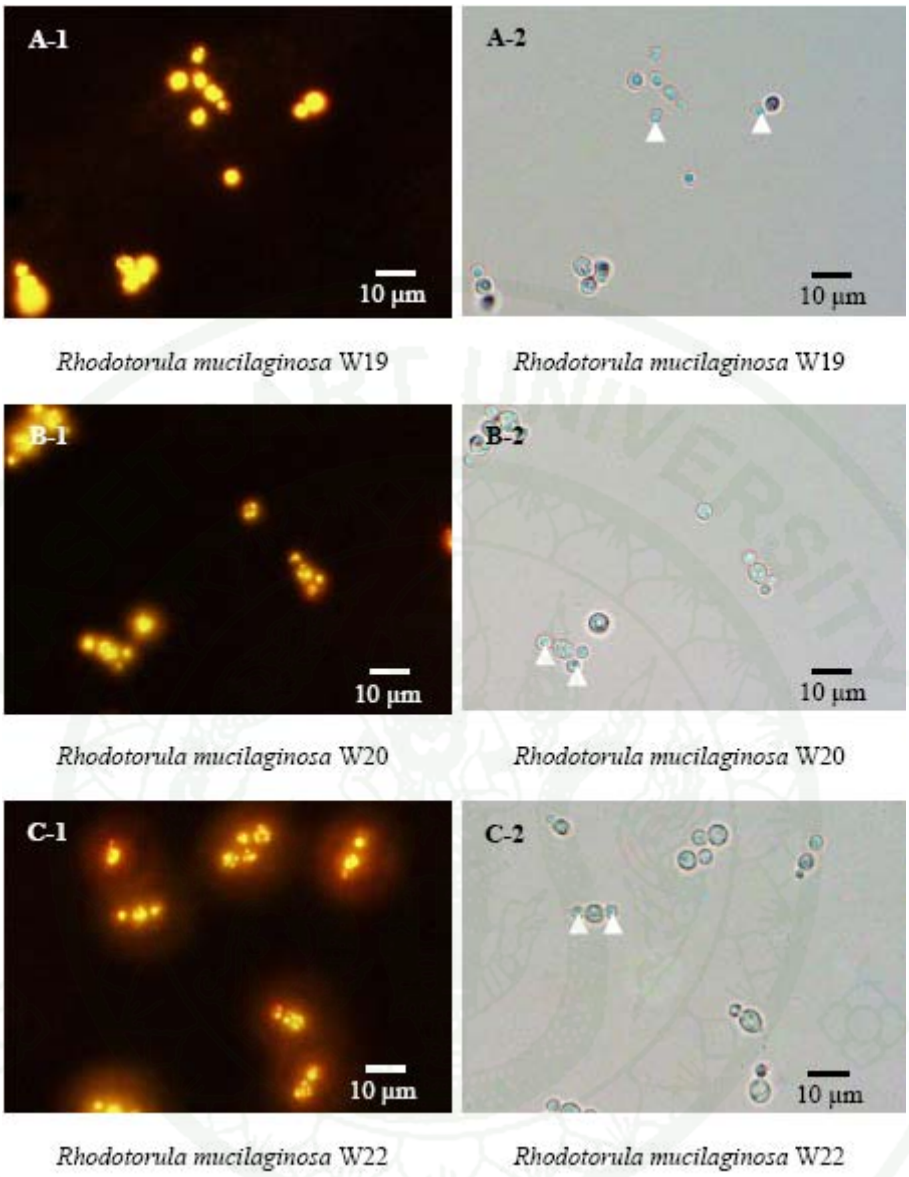

Figure 17 Photomicrographs of Nile red stained cells of *R. mucilaginosa* W19, *R. mucilaginosa* W20 and *R. mucilaginosa* W22 observed under fluorescence microscope showed yellow-gold emission of lipid bodies (A-1), (B-1) and (C-1) and light microscope (A-2), (B-2) and (C-2), respectively. Arrows indicate lipid bodies.

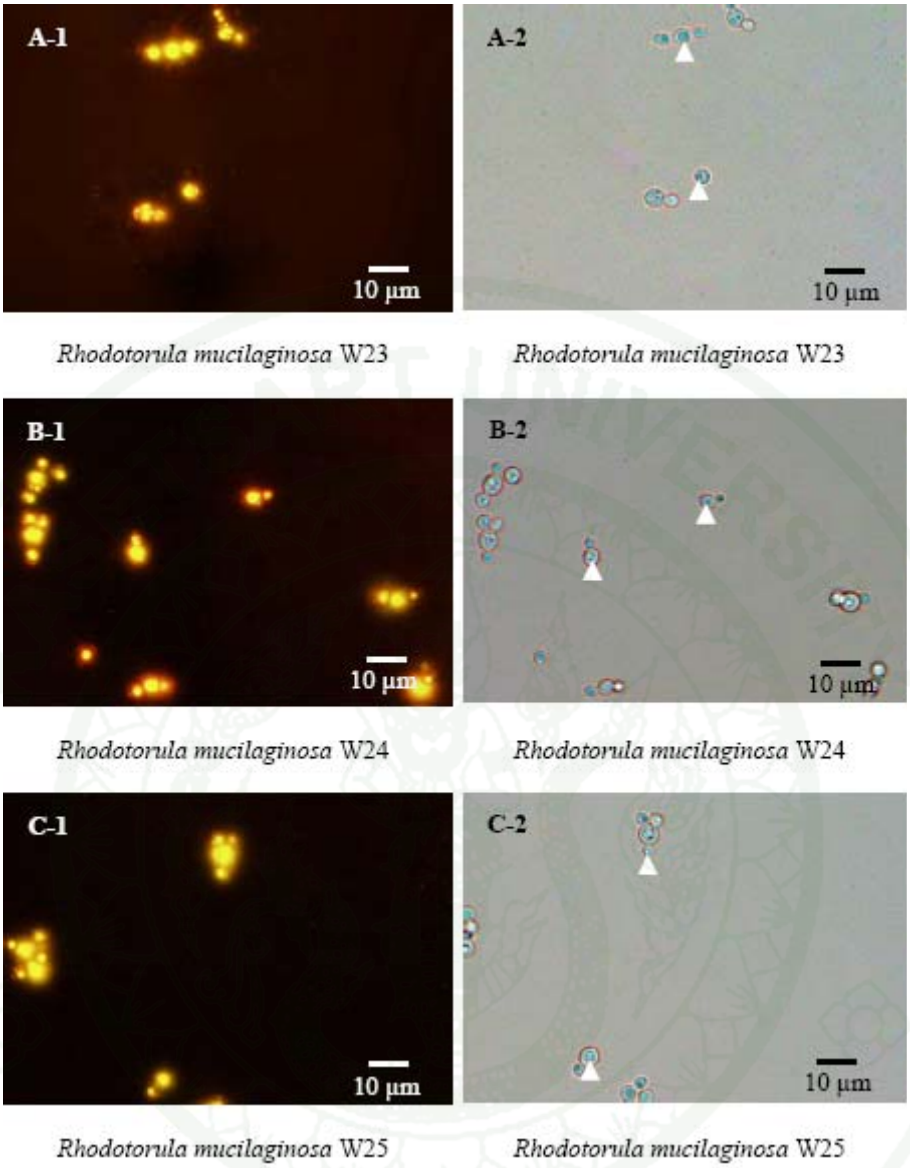

Figure 18 Photomicrographs of Nile red stained cells of *R. mucilaginosa* W23, *R. mucilaginosa* W24 and *R. mucilaginosa* W25 observed under fluorescence microscope showed yellow- gold emission of lipid bodies (A-1) and (B-1) and (C-1) and light microscope (A-2), (B-2) and (C-2) respectively. Arrows indicate lipid bodies.

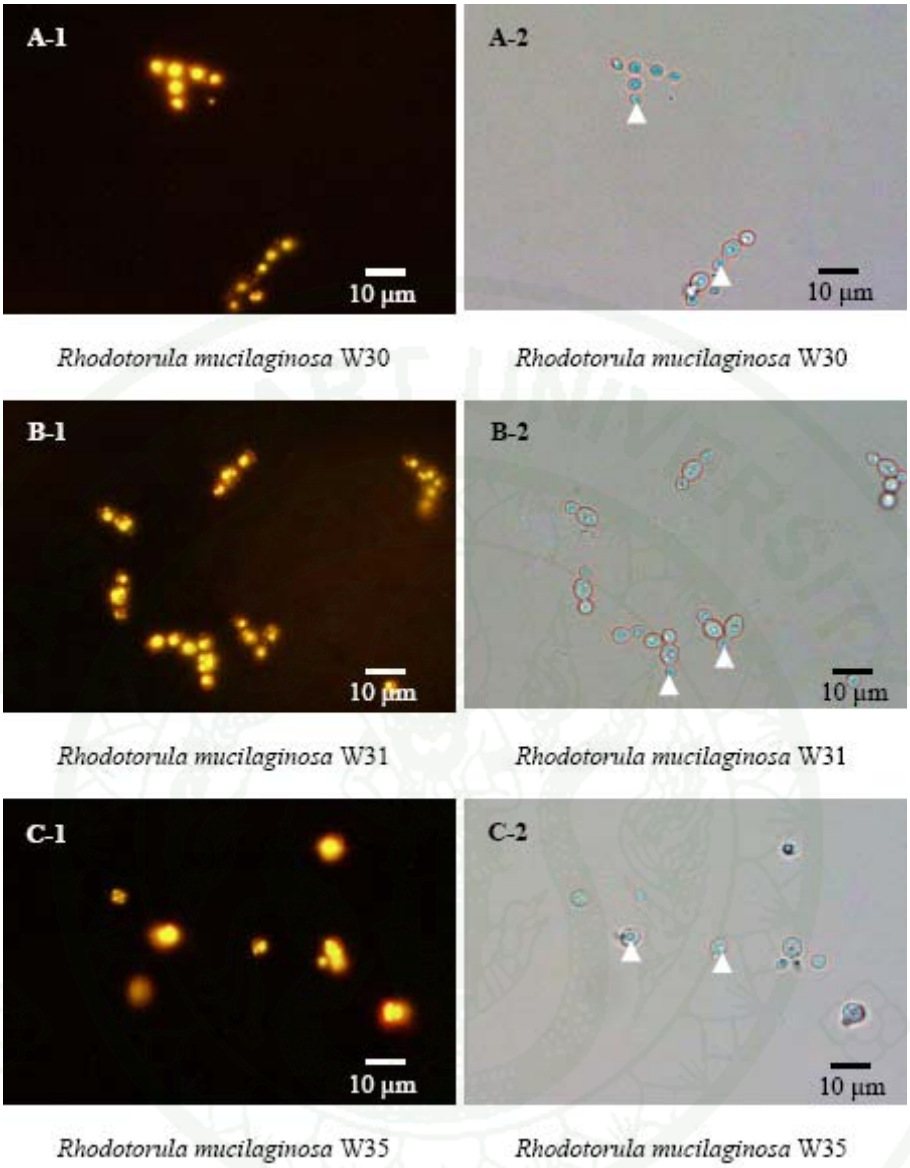

Figure 19 Photomicrographs of Nile red stained cells of *R. mucilaginosa* W30, *R. mucilaginosa* W31 and *R. mucilaginosa* W35 observed under fluorescence microscope showed yellow-gold emission of lipid bodies (A-1), (B-1) and (C-1) and light microscope (A-2), (B-2) and (C-2), respectively. Arrows indicate lipid bodies.

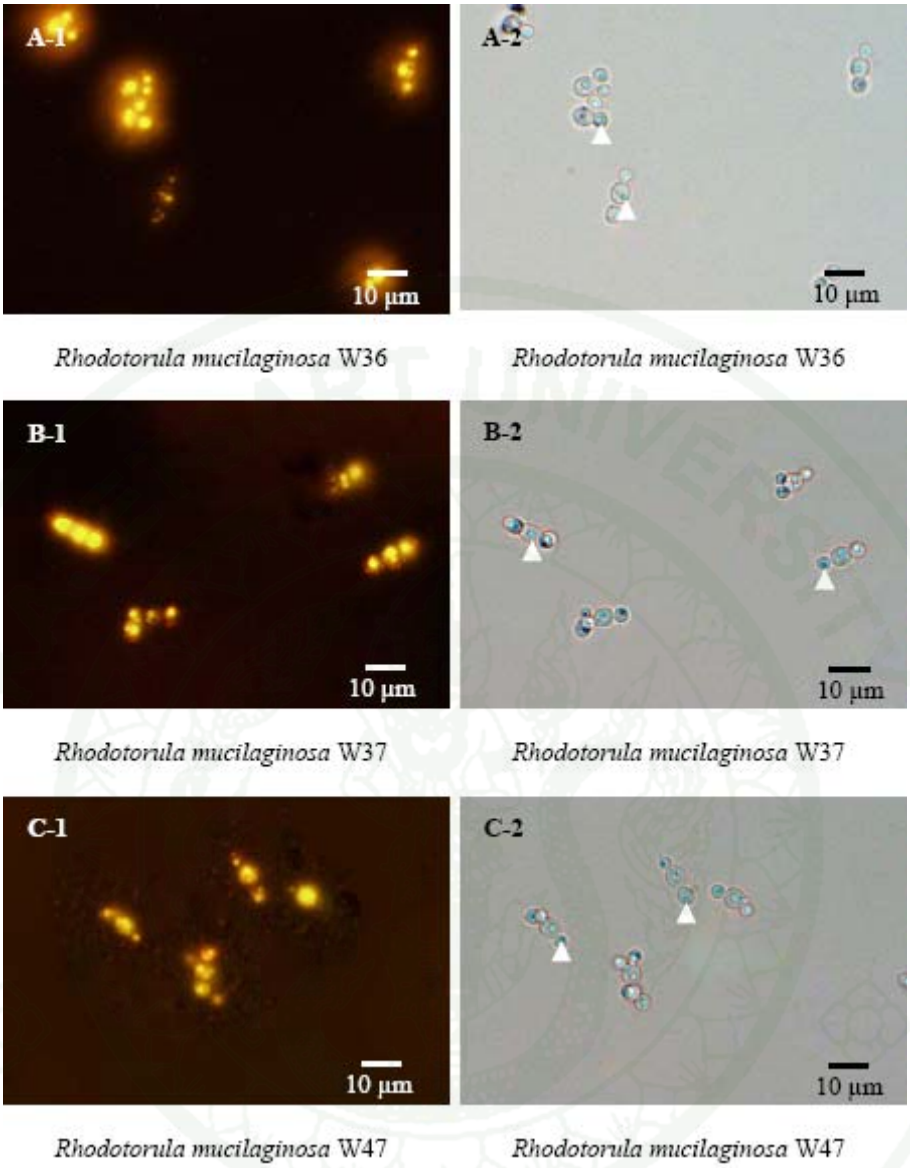

Figure 20 Photomicrographs of Nile red stained cells of *R. mucilaginosa* W36, *R. mucilaginosa* W37 and *R. mucilaginosa* W47 observed under fluorescence microscope showed yellow-gold emission of lipid bodies (A-1), (B-1) and (C-1) and light microscope (A-2), (B-2) and (C-2), respectively. Arrows indicate lipid bodies.

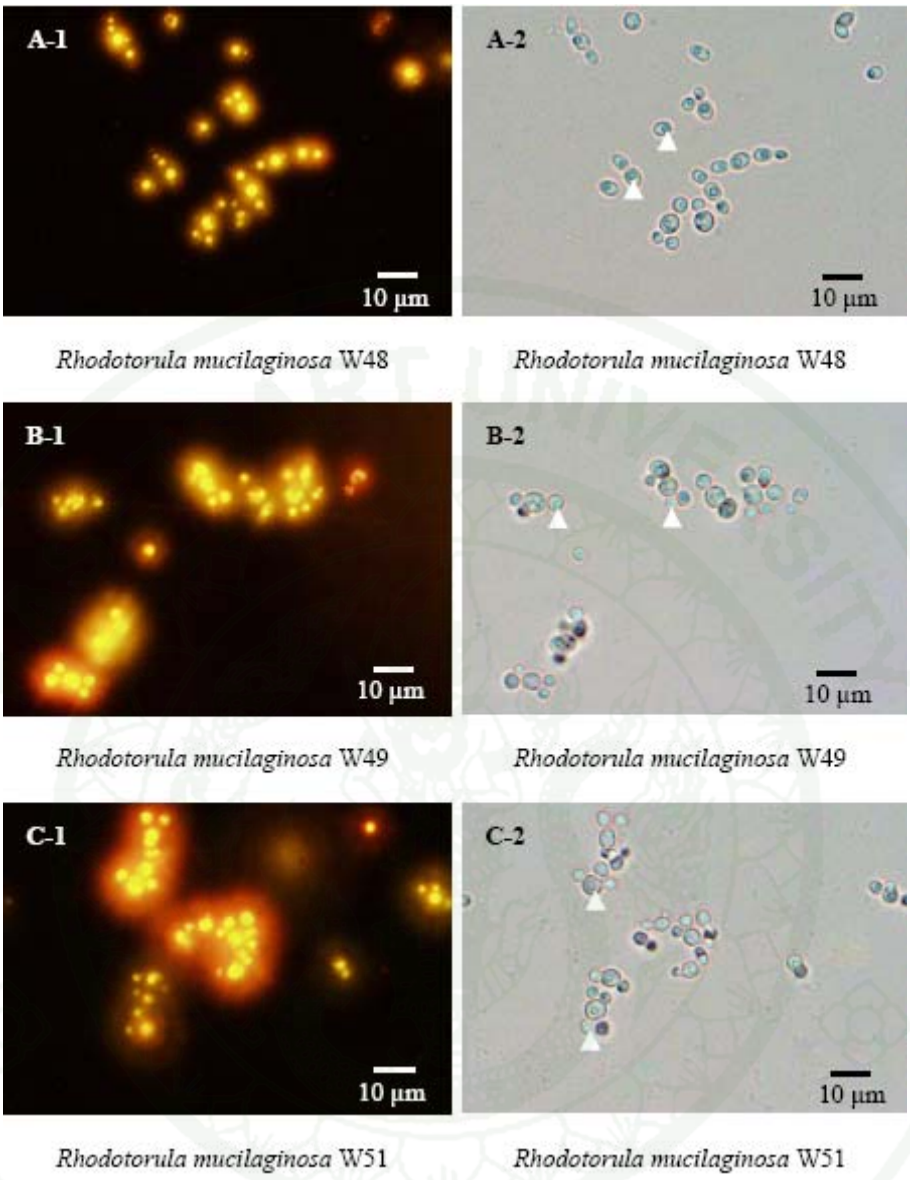

Figure 21 Photomicrographs of Nile red stained cells of *R. mucilaginosa* W48, *R. mucilaginosa* W49 and *R. mucilaginosa* W51 observed under fluorescence microscope showed yellow-gold emission of lipid bodies (A-1), (B-1) and (C-1) and light microscope (A-2), (B-2) and (C-2), respectively. Arrows indicate lipid bodies.

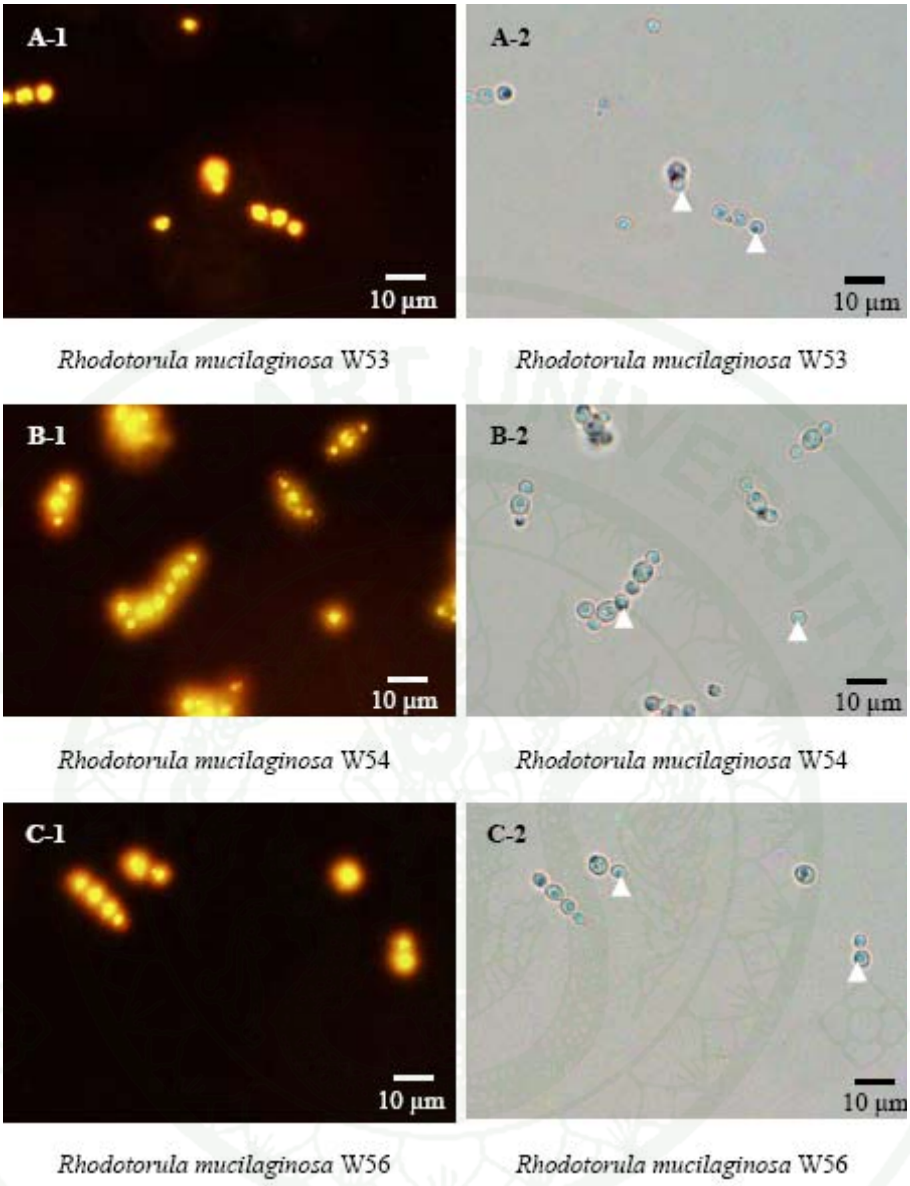

Figure 22 Photomicrographs of Nile red stained cells of *R. mucilaginosa* W53, *R. mucilaginosa* W54 and *R. mucilaginosa* W56 observed under fluorescence microscope showed yellow-gold emission of lipid bodies (A-1), (B-1) and (C-1) and light microscope (A-2), (B-2) and (C-2), respectively. Arrows indicate lipid bodies.

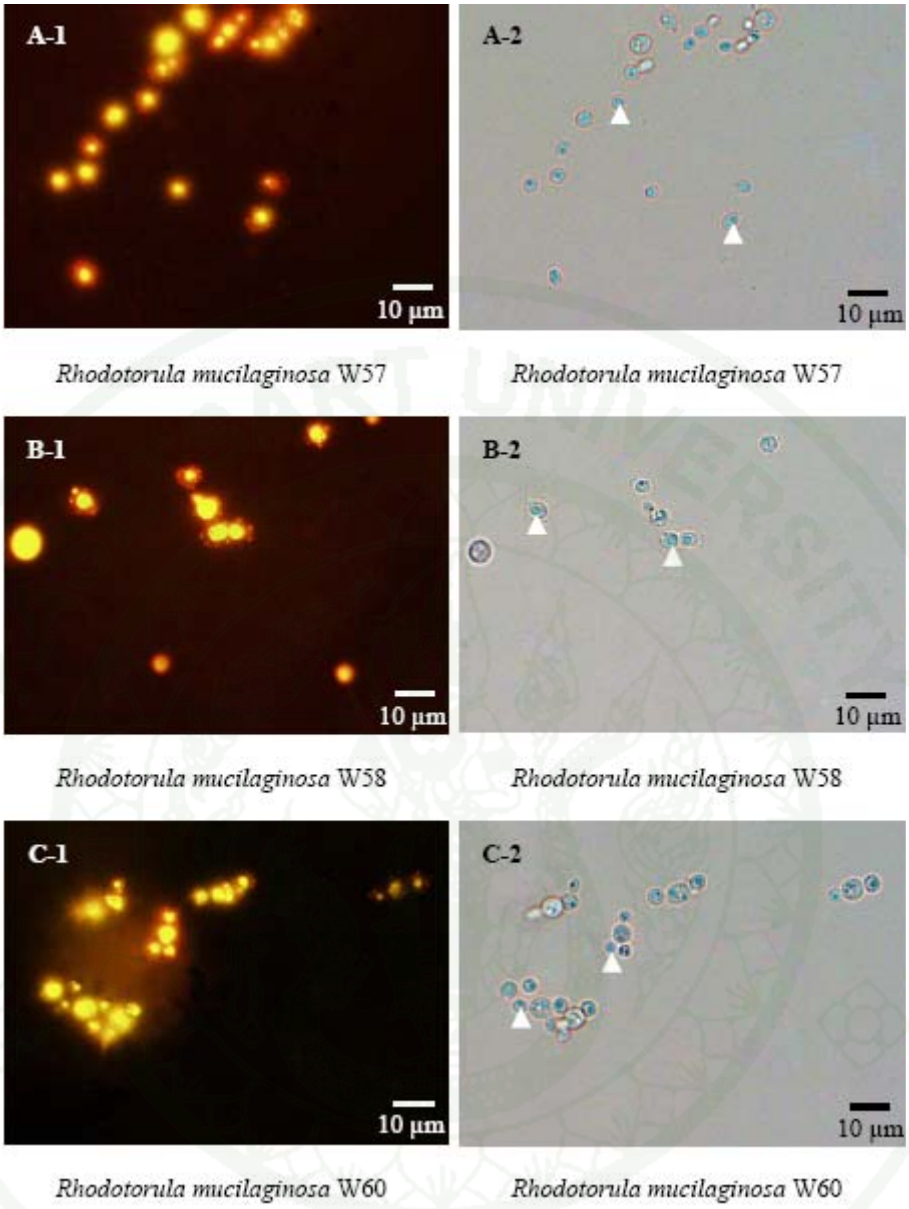

Figure 23 Photomicrographs of Nile red stained cells of *R. mucilaginosa* W57, *R. mucilaginosa* W58 and *R. mucilaginosa* W60 observed under fluorescence microscope showed yellow- gold emission of lipid bodies (A-1), (B-1) and (C-1) and light microscope (A-2), (B-2) and (C-2), respectively. Arrows indicate lipid bodies.

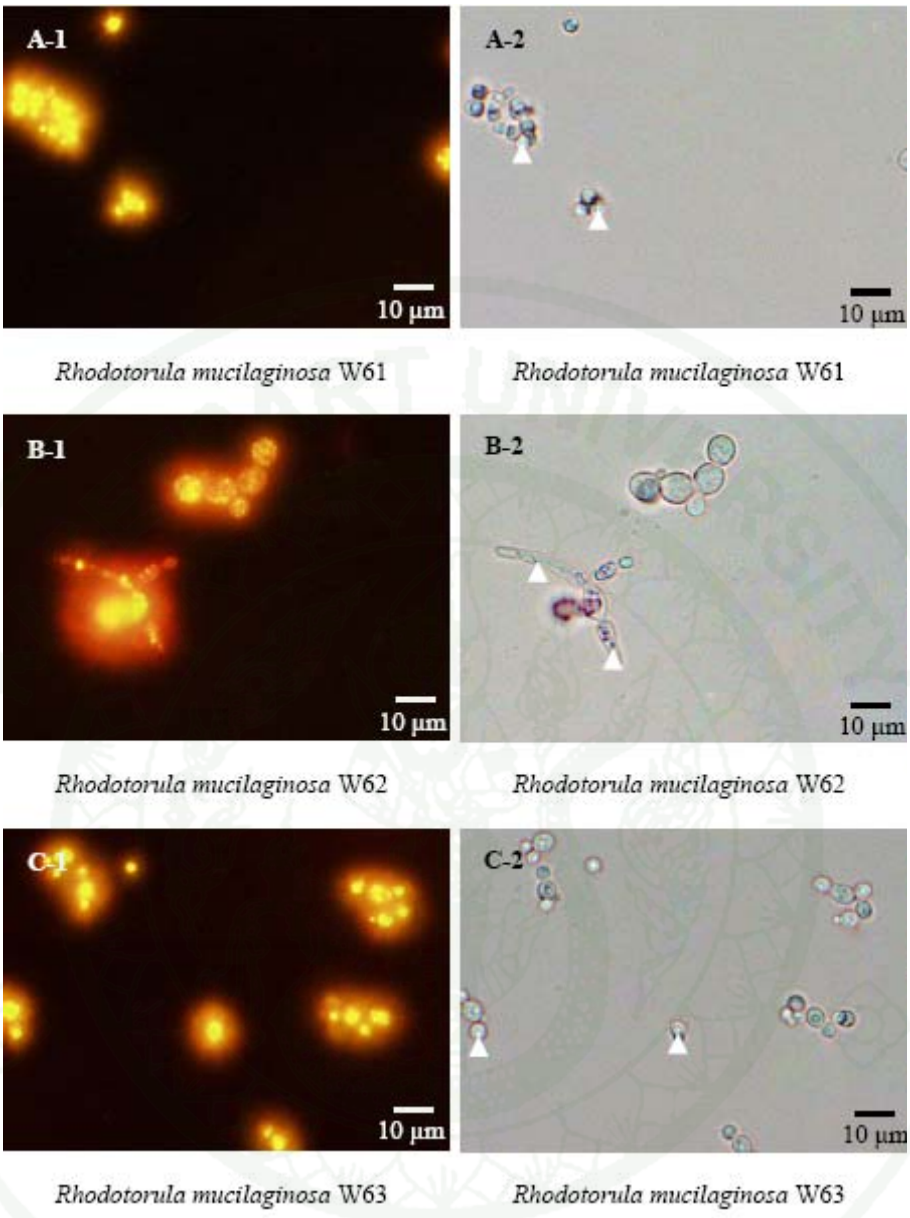

Figure 24 Photomicrographs of Nile red stained cells of *R. mucilaginosa* W61, *R. mucilaginosa* W62 and *R. mucilaginosa* W63 observed under fluorescence microscope showed yellow-gold emission of lipid bodies (A-1), (B-1) and (C-1) and light microscope (A-2), (B-2) and (C-2), respectively. Arrows indicate lipid bodies.

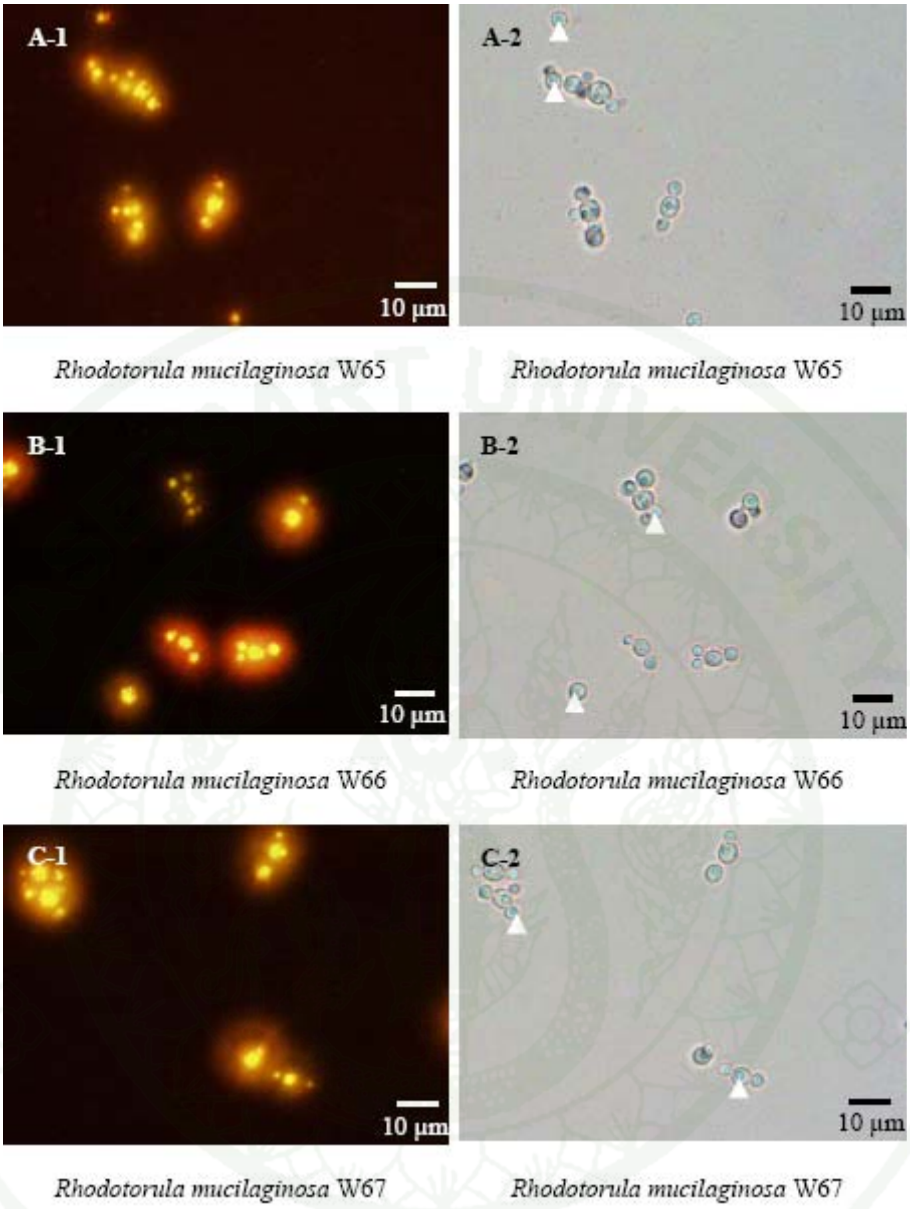

Figure 25 Photomicrographs of Nile red stained cells of *R. mucilaginosa* W65, *R. mucilaginosa* W66 and *R. mucilaginosa* W67 observed under fluorescence microscope showed yellow- gold emission of lipid bodies (A-1), (B-1) and (C-1) and light microscope (A-2), (B-2) and (C-2), respectively. Arrows indicate lipid bodies.

3. Secondary screening

Forty-eight yeast strains from the previous Nile red staining selection were screened for their lipid production. The selected yeast strains were cultivated in nitrogen-limited medium I in shaking flask at 30 ºC for 72 h of cultivation. The properties of oleaginous yeast can accumulate lipid greater than 20% of dry biomass under suitable culture conditions (Ratledge, 1991). The suitable culture conditions are different depending on oleaginous yeast species. The important factors in optimized culture conditions that have influences on lipid accumulation such as temperature, pH, substrate, C/N-ratio and oxygen (Jacob, 1993) Although there was no yeast strains including three reference oleaginous yeast strains accumulated lipid greater than 20% of dry biomass, yeast strains that accumulated high lipid when compared to the reference strains were selected for further screening. The results revealed that four stains, DMKU3-TK16, DMKU3-TK17, *Rhodotorula* sp. and *Rhodotorula mucilaginosa* W35 accumulated lipid greater than 5% of dry biomass as 12.43, 7.57, 9.94 and 5.49% of dry biomass, respectively. While three reference oleaginous yeast strains namely *L. starkeyi* CBS 1807, *C. curvatus* CBS 570 and *R. toruloides* CBS 14 produced lipid as 2.90, 6.22 and 6.69 % of dry biomass, respectively. The remaining 44 strains accumulated lipid at lower concentration ranged between 0.15-4.63 % of dry biomass (Table 7).

Table 7 Biomass, lipid production and cellular lipid content of selected yeasts compared with three reference oleaginous yeasts

No.	Name	Lipid production (g/l)	Biomass (g/l)	Lipid content (% of dry biomass)
1	*L. starkeyi* CBS 1807	0.24	8.37	2.90
2	*C. curvatus* CBS 570	0.35	5.55	6.22
3	*R.toruloides* CBS 14	0.47	7.00	6.69
4	*R.glutinis* W59	0.27	8.10	3.39
5	*Rhodotorula* sp.	0.86	8.63	9.94
6	DMKU3-KUM2	0.24	10.36	2.33
7	DMKU3-LK1	0.02	10.97	0.20
8	DMKU3-CK1	0.55	11.76	4.63
9	DMKU3-CMK4	0.03	7.08	0.40
10	DMKU3-KK2	0.02	10.42	0.15
11	DMKU3-LPK4	0.08	9.53	0.89
12	DMKU3-PBK2	0.20	8.37	2.35
13	DMKU3-MU3	0.02	10.97	0.15
14	DMKU3-TK9	0.02	9.50	0.16
15	DMKU3-TK16	1.24	10.00	12.43
16	DMKU3-TK17	0.67	8.82	7.57
17	*R. mucilaginosa* PT1-16	0.07	8.30	0.88
18	*R. mucilaginosa* TM2-76	0.08	8.41	0.90
19	*R. mucilaginosa* TM3-45	0.08	8.30	0.98
20	*R. mucilaginosa* W2	0.09	9.89	0.88
21	*R. mucilaginosa* W4	0.07	9.39	0.70
22	*R. mucilaginosa* W8	0.08	9.06	0.85
23	*R. mucilaginosa* W9	0.08	9.41	0.81
24	*R. mucilaginosa* W11	0.07	9.65	0.70
25	*R. mucilaginosa* W19	0.07	10.54	0.71
26	*R. mucilaginosa* W20	0.06	9.81	0.59

Table 7 (Continued)

No.	Name	Lipid production (g/l)	Biomass (g/l)	Lipid content (% of dry biomass)
27	*R. mucilaginosa* W22	0.06	10.24	0.62
28	*R. mucilaginosa* W23	0.06	10.05	0.64
29	*R. mucilaginosa* W24	0.08	9.86	0.82
30	*R. mucilaginosa* W25	0.19	10.93	1.77
31	*R. mucilaginosa* W30	0.07	9.35	0.79
32	*R. mucilaginosa* W31	0.08	9.23	0.87
33	*R. mucilaginosa* W35	0.48	8.80	5.49
34	*R. mucilaginosa* W36	0.06	9.64	0.67
35	*R. mucilaginosa* W37	0.08	8.67	0.97
36	*R. mucilaginosa* W47	0.08	9.32	0.86
37	*R. mucilaginosa* W48	0.09	9.24	0.95
38	*R. mucilaginosa* W49	0.06	8.62	0.73
39	*R. mucilaginosa* W51	0.08	8.67	0.88
40	*R. mucilaginosa* W53	0.06	8.70	0.72
41	*R. mucilaginosa* W54	0.08	9.45	0.83
42	*R. mucilaginosa* W56	0.07	8.71	0.83
43	*R. mucilaginosa* W57	0.07	9.38	0.75
44	*R. mucilaginosa* W58	0.06	9.07	0.71
45	*R. mucilaginosa* W60	0.08	9.11	0.86
46	*R. mucilaginosa* W61	0.07	8.63	0.78
47	*R. mucilaginosa* W62	0.16	4.10	3.88
48	*R. mucilaginosa* W63	0.11	9.22	1.22
49	*R. mucilaginosa* W65	0.07	9.15	0.73
50	*R. mucilaginosa* W66	0.15	8.95	1.63
51	*R. mucilaginosa* W67	0.05	8.61	0.63

4. Tertiary screening

Biomass, lipid production and cellular lipid content of the four strains, DMKU3-TK16, DMKU3-TK 17, *Rhodotorula* sp. and *R. mucilaginosa* W35 were determined by shaking cultivation in nitrogen-limited medium II at 28 ºC for 120 h. Biomass of strains DMKU3-TK16, *Rhodotorula* sp. and *R. mucilaginosa* W35 were comparable, i.e. 8.23, 8.49 and 8.25 g/l, respectively, while strain DMKU3-TK17 produced slightly less biomass as 7.38 g/l (Figure 26A). However, lipid production by DMKU3-TK16 was the highest at 4.56 g/l, while DMKU3-TK17 and *R. mucilaginosa* W35 produced extremely low lipid of 0.64 and 0.51 g/l, respectively (Figure 26B); these reflected to their cellular lipid content of 56.76, 8.99 and 6.52 %, respectively (Figure 26C). Therefore, the strain DMKU3-TK16 was selected for optimization of lipid production.

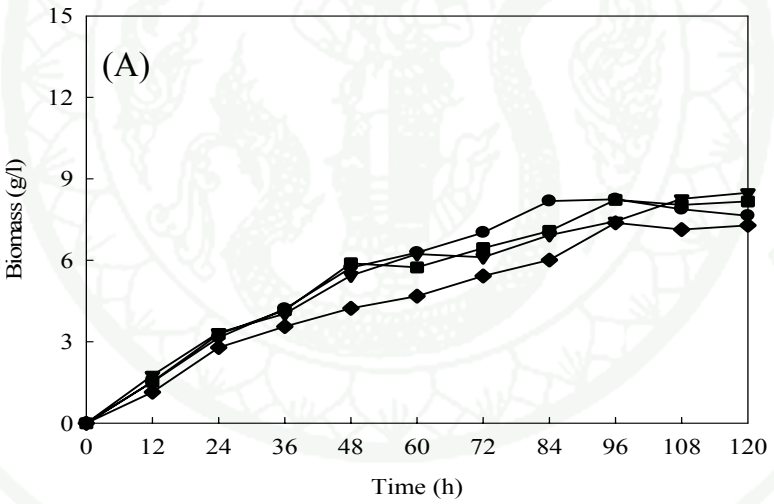

Figure 26 Time course of biomass (A), lipid production (B), and cellular lipid content (C) of *Rhodotorula mucilaginosa* W35 (●), *Rhodotorula* sp. (▼) DMKU3-TK16 (■) and DMKU3-TK17 (◆) in a nitrogen-limited medium II composed of 30 g/l glucose, 0.75 g/l yeast extract, 0.10 g/l $(NH_4)_2SO_4$, 0.4 g/l KH_2PO_4 1.5 g/l $MgSO_4 \cdot 7H_2O$ and pH of 6.0 by shaking flask cultivation at 150 rpm and 28 ºC.

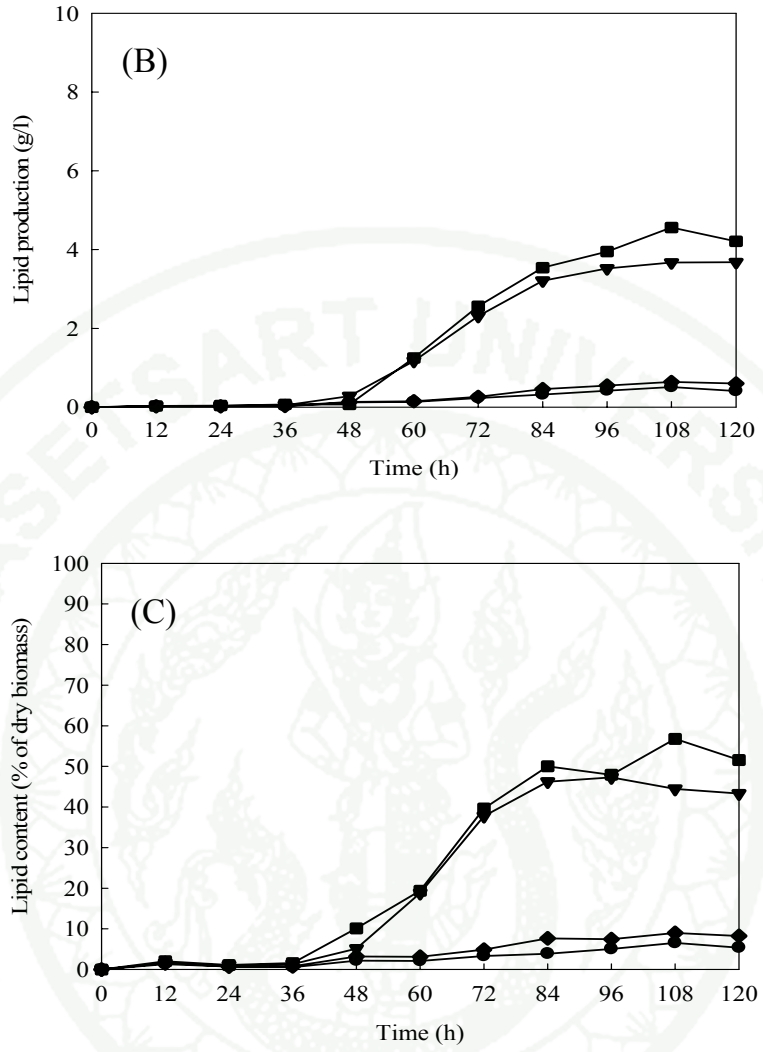

Figure 26 (Continued)

5. Identification of the selected oleaginous yeast strain

Identification of the strain DMKU3-TK16 was carried out using a molecular taxonomic approach. Molecular taxonomic studies based on analysis of nucleotide sequences of the D1/D2 domain of the LSU rRNA gene showed that the sequences of the strain DMKU3-TK16 (559 nucleotides) and the type strain of *Rhodosporidium toruloides*, *R. toruloides* CBS 14^T, were identical. Therefore, the strain DMKU3-TK16 was identified as *Rhodosporidium toruloides*. The D1/D2 domain of the LSU rRNA gene sequence of strain DMKU3-TK16 was deposited in GenBank under the accession number AB507798.

6. Optimization of lipid production by *Rhodosporidium toruloides* DMKU3-TK16 in Erlenmeyer flask

6.1 Effect of combined organic and inorganic nitrogen compounds on lipid production

The effect of combined organic (yeast extract or peptone at 0.75 g/l) and inorganic [$(NH_4)_2SO_4$ or NH_4Cl at 0.1 g/l] nitrogen compounds was determined in the nitrogen-limited medium II composed of 30 g/l glucose and adjusted to pH 6.0. Huang *et al*. (1998) reported that organic nitrogenous compound is good for lipid accumulation but not for cell growth, on the other hand inorganic nitrogenous compound is favorable for cell growth but not for lipid accumulation. The results showed that when using yeast extract with ammonium salt, higher biomass and lipid production were obtained while using peptone with ammonium salt resulted in higher cellular lipid content. The highest biomass (8.71 g/l) and lipid production (4.01 g/l) were obtained when yeast extract and $(NH_4)_2SO_4$ were used in combination (Figure 27A). The glucose was entirely depleted after 96 h. This resulted in a decrease of the lipid production and cellular lipid content in the following period of cultivation. The lipid production and biomass decreased markedly, for example, a lipid production of 3.03 g/l was reached, and whereas the biomass was 5.99 g/l when using peptone and NH_4Cl (Figure 27 C). With using peptone and $(NH_4)_2SO_4$, both the lipid production and biomass were 3.05 g/l and 5.91 g/l (Figure 27D). Because the biomass increased slowly that may be the reason why the yeast strain DMKU3-TK16 could not produce high lipid and the remaining glucose was observed (Figure 27C and D). However, higher cellular lipid content at 53.71% and 53.10% of dry biomass were yielded when using peptone together with NH_4Cl and peptone together with $(NH_4)_2SO_4$, respectively. Based on the highest biomass and lipid production, yeast extract and $(NH_4)_2SO_4$ were chosen for further study.

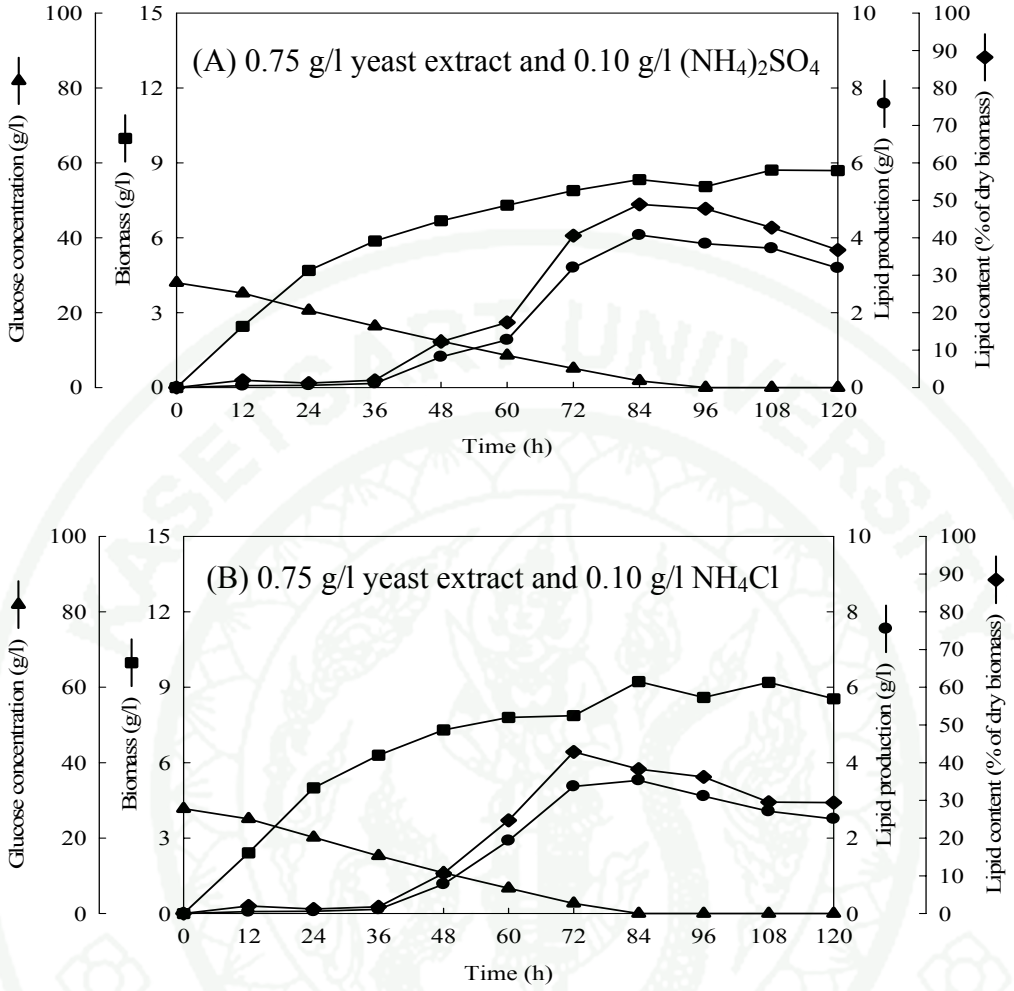

Figure 27 Time course of biomass (■), lipid production (●), cellular lipid content (◆) and glucose concentration (▲) by *R. toruloides* DMKU3-TK16 in a nitrogen-limited medium II composed of 30 g/l and various combined nitrogen compounds (A) 0.75 g/l yeast extract and 0.10 g/l $(NH_4)_2SO_4$, (B) 0.75 g/l yeast extract and 0.10 g/l NH_4Cl, (C) 0.75 g/l peptone and 0.10 g/l $(NH_4)_2SO_4$, (D) 0.75 g/l peptone and 0.10 g/l NH_4Cl with pH of 6.0 by shaking flask cultivation at 150 rpm and 28 ºC

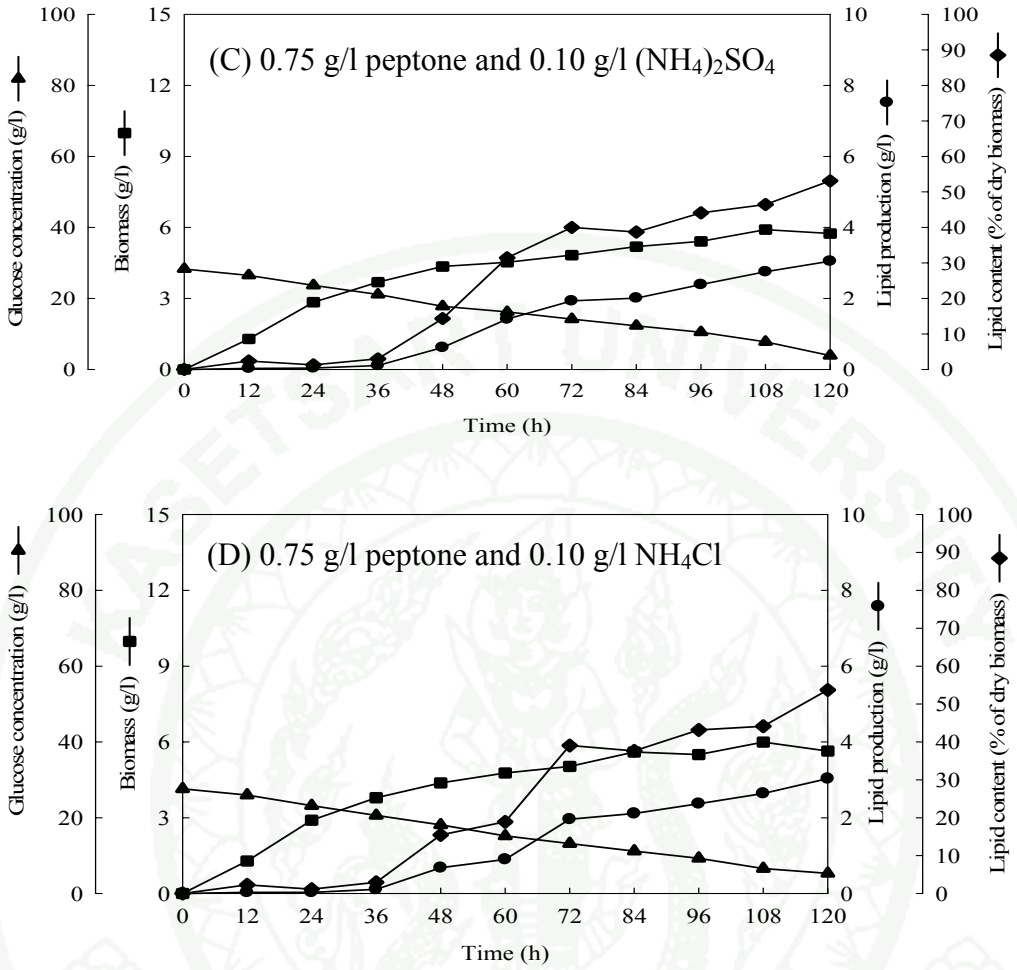

Figure 27 (Continued)

6.2 Effect of C/N-ratio on lipid production

The influence of C/N-ratio on biomass and lipid production was studied by varying C/N-ratios of 65, 90, 115 and 140 of the nitrogen-limited medium II composed of 30 g/l glucose, 0.75 g/l yeast extract with various $(NH_4)_2SO_4$ concentrations as shown in Table 8 and adjusted pH to 6.0.

Table 8 The C/N-ratio in nitrogen-limited medium II

C/N-ratio	Carbon source	Nitrogen sources
65	30 g/l glucose	0.75 g/l yeast extract and 0.48 g/l $(NH_4)_2SO_4$
90	30 g/l glucose	0.75 g/l yeast extract and 0.24 g/l $(NH_4)_2SO_4$
115	30 g/l glucose	0.75 g/l yeast extract and 0.10 g/l $(NH_4)_2SO_4$
140	30 g/l glucose	0.75 g/l yeast extract and 0.012 g/l $(NH_4)_2SO_4$

The C/N ratio was found to be very important factor for lipid accumulation (Angerbauer *et al*., 2008). The results revealed that the cellular lipid content was increased with an increasing of C/N-ratio. These results were in agreement with the literature as, in general, lipid accumulation in microorganisms is stimulated by an excess of a carbon source and a limitation in nitrogen (Ratledge and Wynn, 2002). The increase in cellular lipid content reflected the increase in lipid production though a decrease in biomass was obtained when the C/N-ratio was increased. The highest cellular lipid content, 62.30% of dry biomass, and lipid production, 4.23 g/l, were attained at the C/N ratio of 140 [30 g/l glucose, 0.75 g/l yeast extract and 0.014 g/l $(NH_4)_2SO_4$] but with the lowest biomass (6.79 g/l). The rapid growth was observed within the first 36 h of cultivation while the cellular lipid content increased from 23.10% at 60 h, to a maximum value of 69.61% at 120 h (Figure 28D). Although the biomass concentration did not further increase after 84 h, the glucose concentration continued to decrease, from 8.50 to 0 g/l in the final 36 h. At the C/N ratio of 115 (Figure 28C) high lipid production (4.13 g/l) and cellular lipid content (51.41 %) were observed. The lipid production and cellular lipid content decreased markedly at low C/N ratio of 90 (Figure 28B). The lipid production of 3.24 at 84 h was reached with the lipid content was only 33.18% of dry biomass at 84 h. At the lowest C/N-ratio of 65 the highest biomass (10.52 g/l) and the lowest lipid production (2.7 g/l) was found

(Figure 28A). It can then be concluded that the optimal C/N ratio for lipid production by the strain DMKU3-TK16 was 140 that was selected for further experiments.

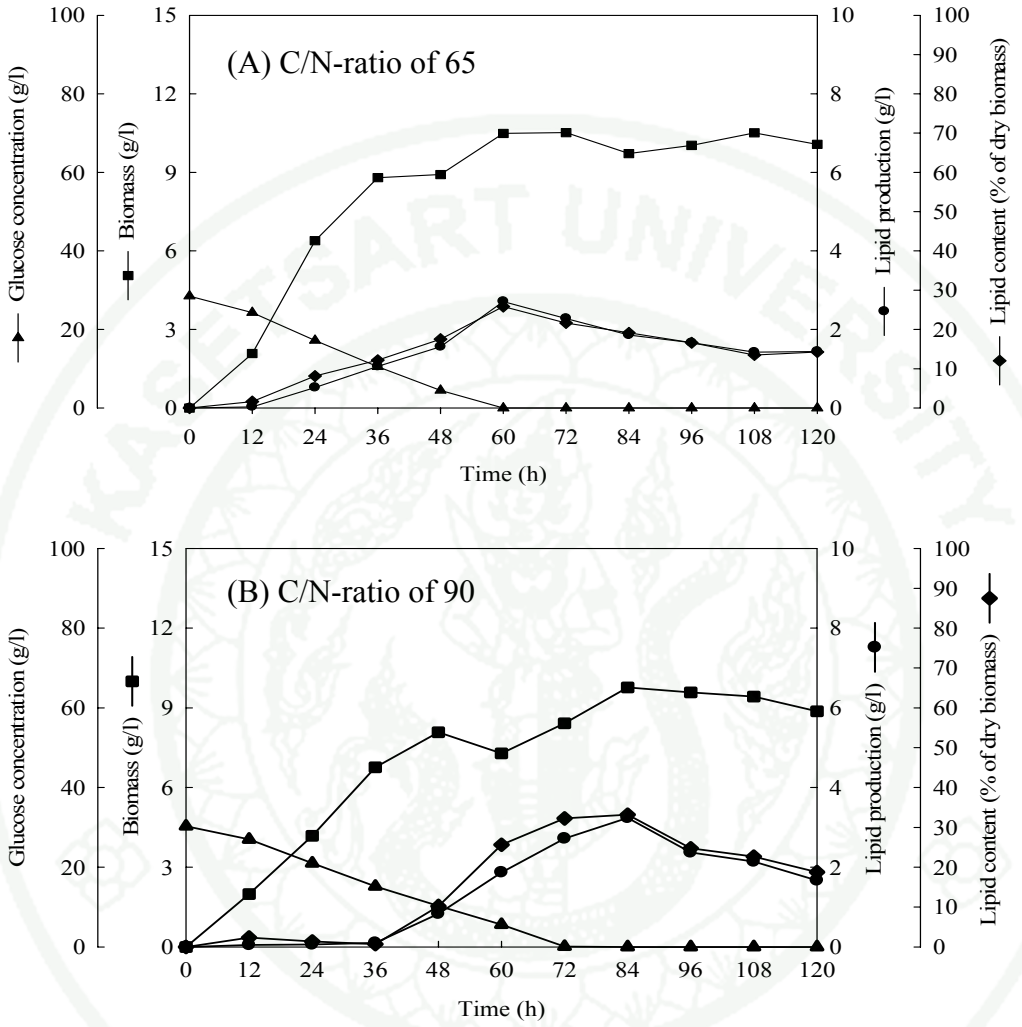

Figure 28 Time course of biomass (■), lipid production (●), cellular lipid content (◆) and glucose concentration (▲) by *R. toruloides* DMKU3-TK16 in a nitrogen-limited medium II composed of 30 g/l 0.75 g/l yeast extract, 0.10 g/l $(NH_4)_2SO_4$ and various C/N-ratios (A) C/N-ratio of 65, (B) C/N-ratio of 90, (C) C/N-ratio of 115, (D) C/N-ratio of 140 with pH of 6.0 by shaking flask cultivation at 150 rpm and 28 ºC.

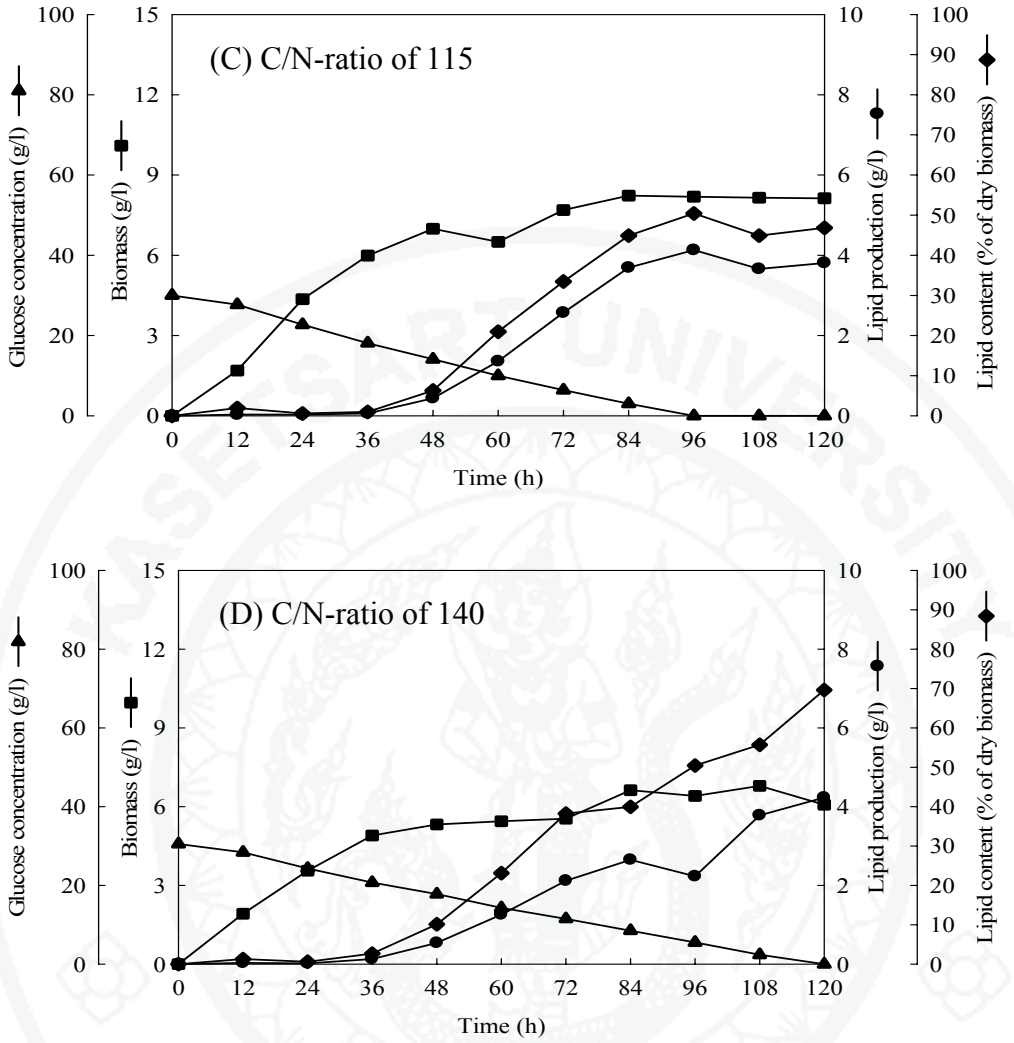

Figure 28 (Continued)

6.3 Effect of carbon source concentration on lipid production

The effect of carbon concentration was studied using nitrogen-limited medium II composed of glucose at 50, 70 and 90 g/l and yeast extract with $(NH_4)_2SO_4$ at the C/N-ratio of 140 and having pH 6.0 (Table 9).

Table 9 The C/N-ratio in nitrogen-limited medium I

C/N-ratio	Carbon source	Nitrogen sources
140	50 g/l glucose	0.75 g/l yeast extract and 0.28 g/l $(NH_4)_2SO_4$
	70 g/l glucose	0.75 g/l yeast extract and 0.55 g/l $(NH_4)_2SO_4$
	90 g/l glucose	0.75 g/l yeast extract and 0.82 g/l $(NH_4)_2SO_4$

The result of time course experiment revealed that at higher glucose concentration, the higher final biomass and lipid production were observed but only up to 70 g/l glucose (Figure 29). Further increase of glucose concentrations led to decrease in biomass and lipid production. The highest biomass (13.56 g/l), lipid production (8.11 g/l) and cellular lipid content of (64.43% of dry biomass) were achieved when 70 g/l glucose was used (Figure 29B). Moreover, glucose concentration during 0 to 180 h was slightly decreased until entirely exhausted at the end of the cultivation. The highest biomass was 13.56 g/l at 156 h. Exponential growth was observed within the first 36 h of cultivation. As observed in the growth curve, the increase of the biomass concentration became linear after this initial exponential growth phase (Figure 29A). After 120 h, growth remained constant throughout cultivation period. Increasing glucose concentration up to 90 g/l (Figure 29C) resulted in lipid production, cellular lipid content and biomass at 180 h as 7.95 g/l, 62.11 % of dry biomass and 12.96 g/l, respectively, which were lower that by using 70 g/l glucose. The possible reason is higher glucose concentration resulted in increasing osmotic pressure which inhibited growth and lipid production. At 180 h of cultivation the glucose was not entirely depleted. When *R. toruloides* DMKU3-TK16 was cultivated in medium composed of 50 g/l glucose, the lowest cellular lipid production (6.67 g/l) and biomass (10.48 g/l) were obtained. This may be due to the complete utilization of substrate for growth and lipid accumulation at 132 h (Figure 29C). Hence, glucose at 70 g/l was chosen for further study because it provided the highest lipid production (8.11 g/l) and cellular lipid content (64.43% of dry biomass).

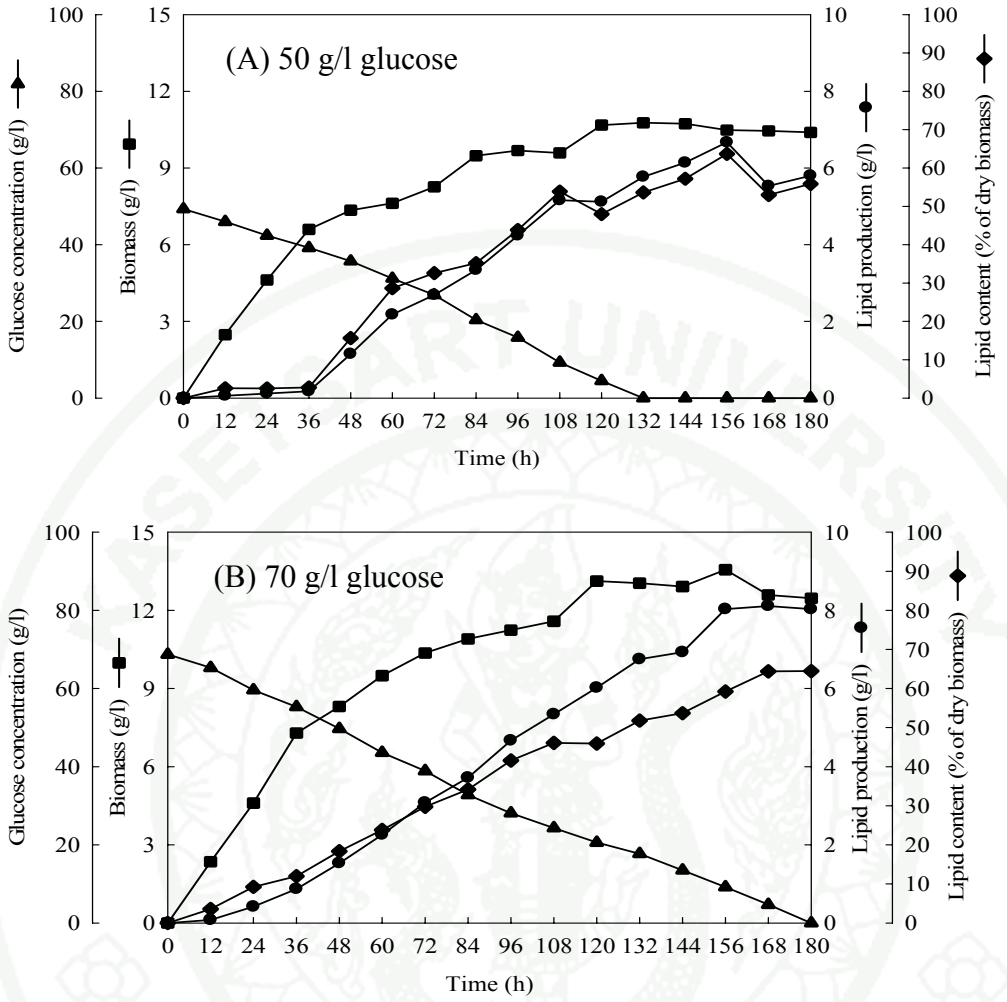

Figure 29 Time course of biomass (■), lipid production (●), cellular lipid content (◆) and glucose concentration (▲) by *R. toruloides* DMKU3-TK16 in a nitrogen-limited medium II composed of 0.75 g/l yeast extract, 0.55 g/l $(NH_4)_2SO_4$, C/N-ratio (140) and various (A) 50 g/l glucose, (B) 70 g/l glucose (C) 90 g/l glucose with pH of 6.0 by shaking flask cultivation at 150 rpm and 28 ºC

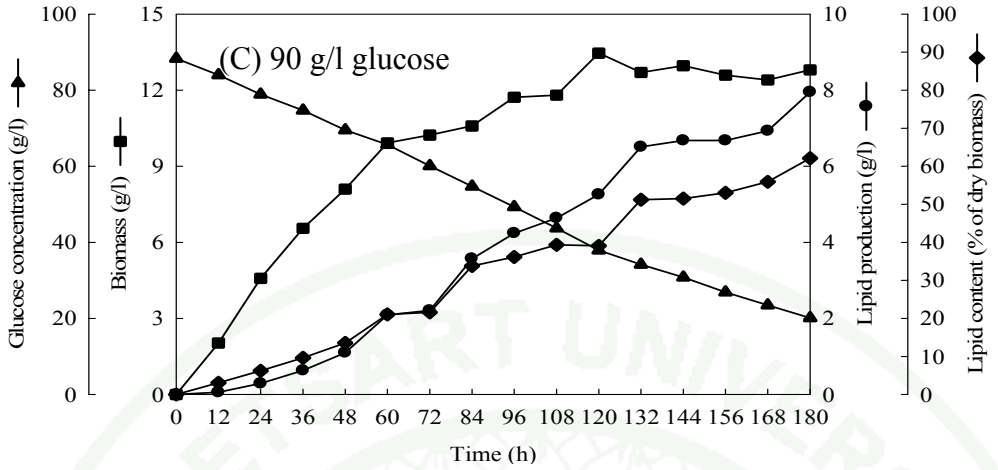

Figure 29 (Continued)

6.4 Effect of phosphate concentration on lipid production

The effect of phosphate concentration was studied by supplementation of KH_2PO_4 at 0.4-4 g/l to nitrogen-limited medium II composed of 70 g/l glucose, 0.75 g/l yeast extract, 0.55 g/l $(NH_4)_2SO_4$ and pH 6.0. The highest lipid production of 8.85 g/l was obtained when the medium was supplemented with 0.4 g/l KH_2PO_4 (Figure 30A). The decrease of glucose concentration was linear, indicating that substrate was slightly used for growth throughout cultivation period. While the lipid production and cellular lipid content obtained from various concentrations of KH_2PO_4 were only slightly different (Figure 30B-D). Thus, KH_2PO_4 at 0.4 g/l was chosen for further experiment.

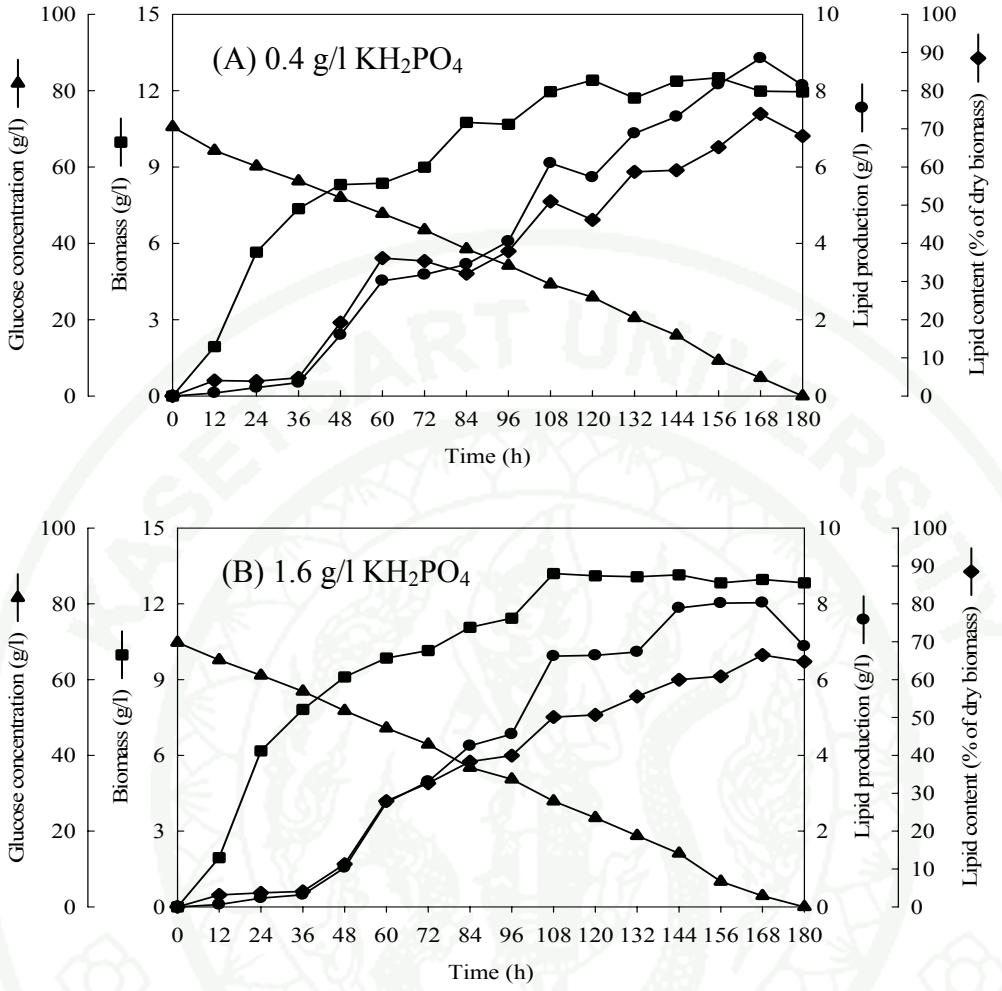

Figure 30 Time course of biomass (■), lipid production (●), cellular lipid content (◆) and glucose concentration (▲) by *R. toruloides* DMKU3-TK16 in a nitrogen-limited medium II composed of 70 g/l glucose, 0.75 g/l yeast extract, 0.55 g/l $(NH_4)_2SO_4$, C/N-ratio (140) and various phosphate concentrations (A) 0.4 g/l KH_2PO_4, (B) 1.6 g/l KH_2PO_4, (C) 2.8 g/l KH_2PO_4 and (D) 4.0 g/l KH_2PO_4 with pH of 6.0 by shaking flask cultivation at 150 rpm and 28 ºC

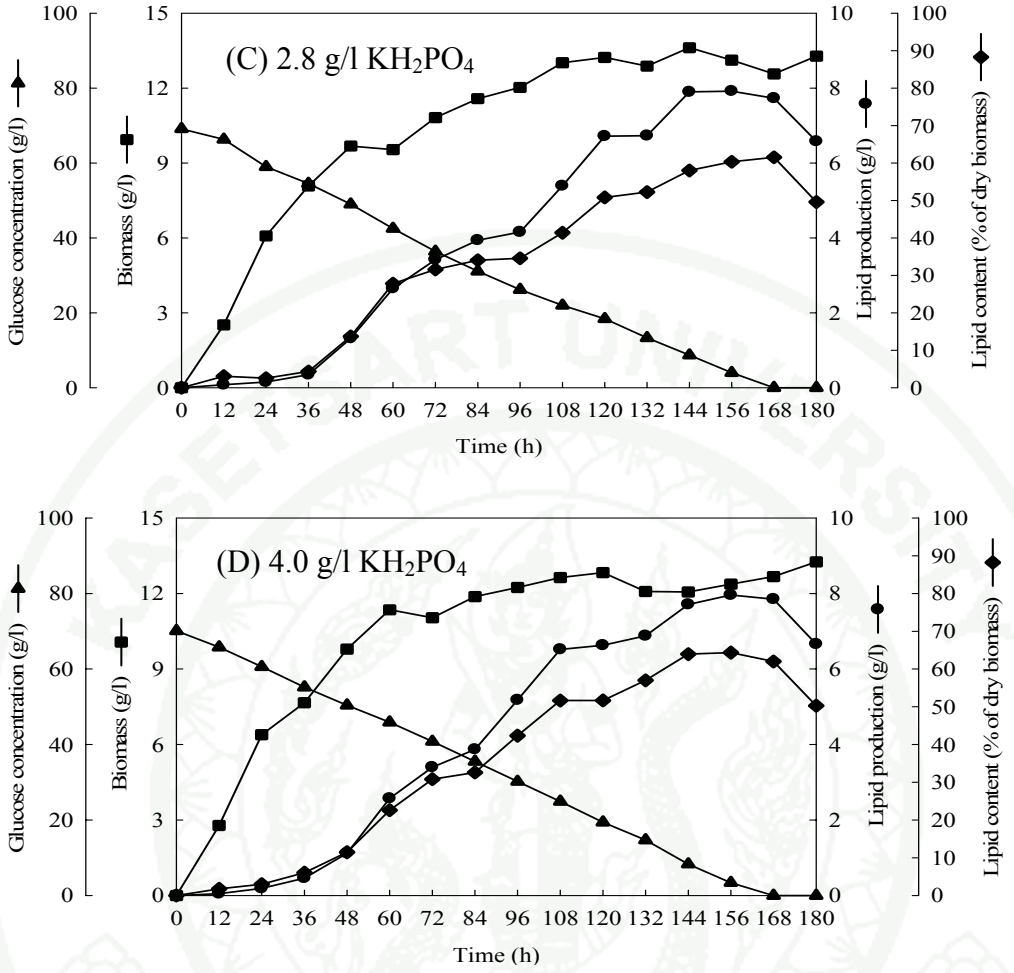

Figure 30 (Continued)

6.5 Effect of magnesium concentration on lipid production

The effect of magnesium was investigated using $MgSO_4 \cdot 7H_2O$ at concentrations ranged between 0.5-3.0 g/l in a nitrogen-limited medium II composed of 70 g/l glucose, 0.75 g/l yeast extract, 0.55 g/l $(NH_4)_2SO_4$, 0.4 g/l KH_2PO_4 and pH 6.0. The highest lipid production (8.79 g/l) and cellular lipid content (69.78% of dry biomass) were obtained when the medium was supplemented with 2.0 g/l $MgSO_4 \cdot 7H_2O$. The lipid production and cellular lipid content increased significantly from 36 h of cultivation and reached a maximum at 168 h (Figure 31D). Li *et al*. (2006) reported the effect of magnesium concentration on cellular lipid production of *R. toruloides*. They found that the optimal $MgSO_4 \cdot 7H_2O$ concentration was 1.5 g/l.

Further rise in the magnesium concentration beyond 2.0 g/l led to slightly decrease in lipid production. The lipid production and cellular lipid content were 8.58 g/l and 64.06 % of dry biomass when the medium was supplemented with 2.5 g/l $MgSO_4 \cdot 7H_2O$ (Figure 31E); and with 3.0 g/l $MgSO_4 \cdot 7H_2O$ supplementation lipid production of 8.08 g/l and cellular lipid content of 61.17% of dry biomass, were obtained (Figure 31F). Furthermore, the lipid production and cellular lipid content obtained from various concentrations of $MgSO_4 \cdot 7H_2O$ were only slightly different (Figure 31A-C). The glucose concentration of all treatments decreased linearly during cultivation period (Figure 31A-F). At the lowest $MgSO_4 \cdot 7H_2O$ concentration (0.5 g/l) tested, the lowest lipid production (7.19 g/l) and cellular lipid content (58.24% of dry biomass) were observed (Figure 31A).

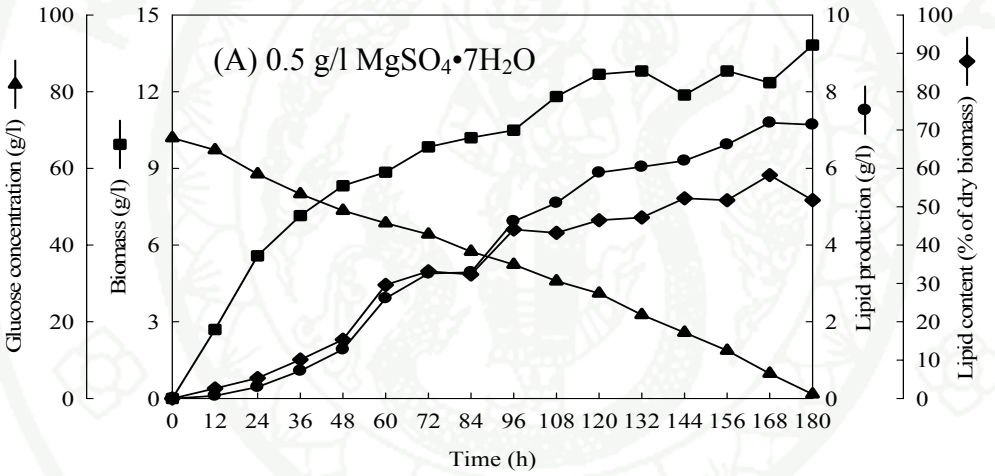

Figure 31 Time course of biomass (■), lipid production (●), cellular lipid content (◆) and glucose concentration (▲) by *R. toruloides* DMKU3-TK16 in a nitrogen-limited medium II composed of 70 g/l glucose, 0.75 g/l yeast extract, 0.55 g/l $(NH_4)_2SO_4$, C/N-ratio (140), 0.4 g/l KH_2PO_4 and various magnesium concentrations (A) 0.5 g/l $MgSO_4 \cdot 7H_2O$, (B) 1.0 g/l $MgSO_4 \cdot 7H_2O$, (C) 1.5 g/l $MgSO_4 \cdot 7H_2O$, (D) 2.0 g/l $MgSO_4 \cdot 7H_2O$, (E) 2.5 g/l $MgSO_4 \cdot 7H_2O$ and (F) 3.0 g/l $MgSO_4 \cdot 7H_2O$ with pH of 6.0 by shaking flask cultivation at 150 rpm and 28 ºC

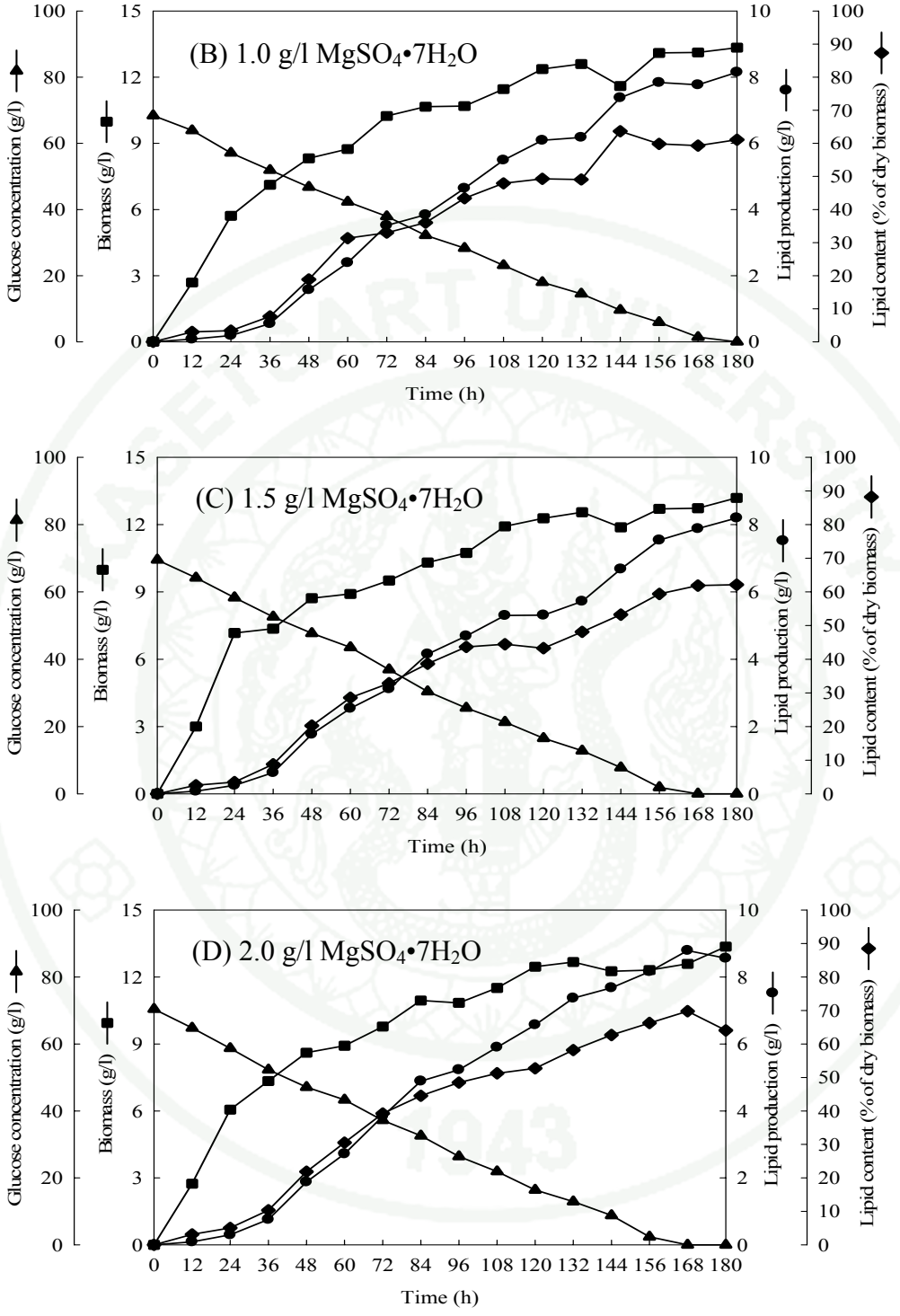

Figure 31 (Continued)

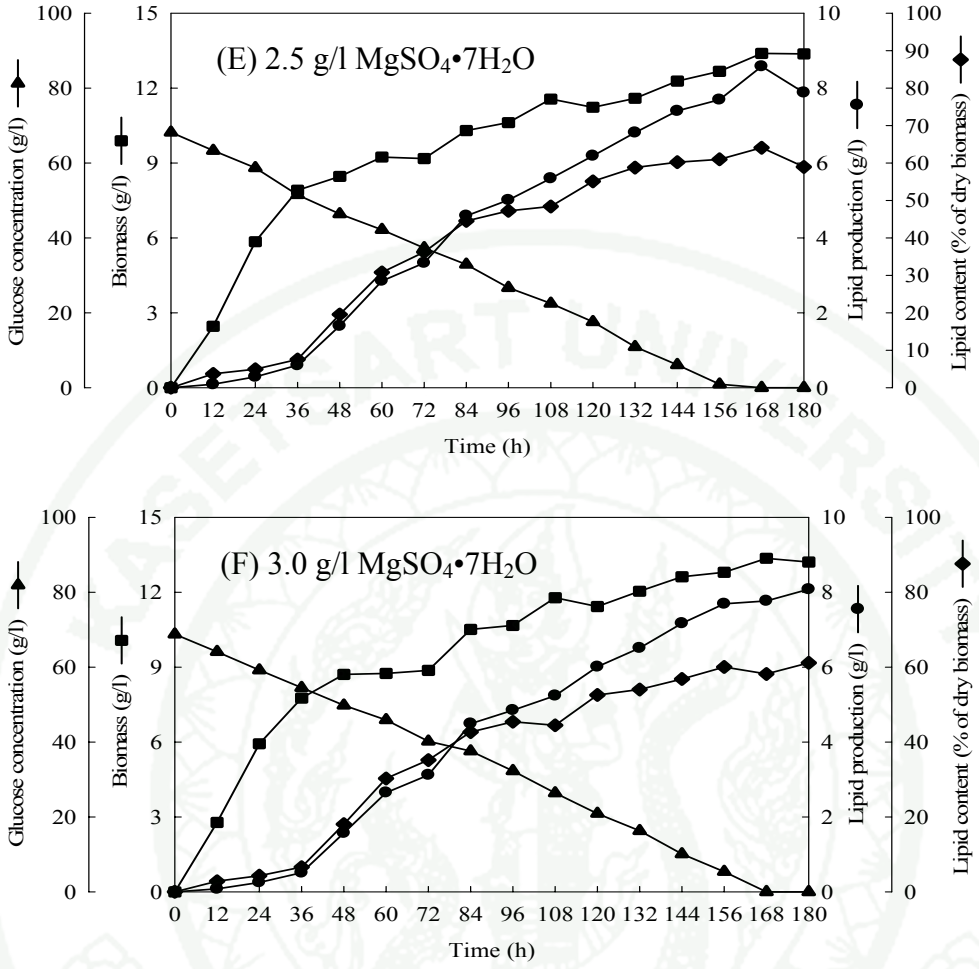

Figure 31 (Continued)

6.6 Effect of initial pH on lipid production

The influence of initial pH of the medium on lipid production was studied using pH ranged from 5.0 to 6.5. It was found that the highest cellular lipid content (71.30% of dry biomass) and lipid production (9.26 g/l) were obtained when pH of the medium was adjusted to 5.5 (Figure 32B). The biomass concentration increased gradually from 9.46 g/l (48 h) to maximum of 13.33 g/l (156 h) at pH of 5.5. Cultivation at pH 5.0, 6.0 and 6.5, resulted in slightly different of cellular lipid content and production (Figure 32A, C and D). At low pH (5.0) resulted in the lowest lipid production (8.34 g/l) and cellular lipid content (66.77% of dry biomass) (Figure 32A). From these results, the medium with low pH might not good for lipid

accumulation. Many investigators reported that pH of the medium had an effect on lipid production and seemed to depend on carbon sources used (Angerbauer *et al*., 2008). An optimal pH of 4.0 for lipid production by *Lipomyces starkeyi* was found when ethanol was used as a carbon source (Yamauchi *et al*., 1983). Holdsworth and Ratledge (1988) reported the optimum pH of *L. starkeyi* at 5.5 with glucose while Angerbauer *et al*. (2008) obtained the highest cellular lipid content when cultivated *L. starkeyi* at pH 5.0. Therefore, the pH of 5.5 was chosen for further study.

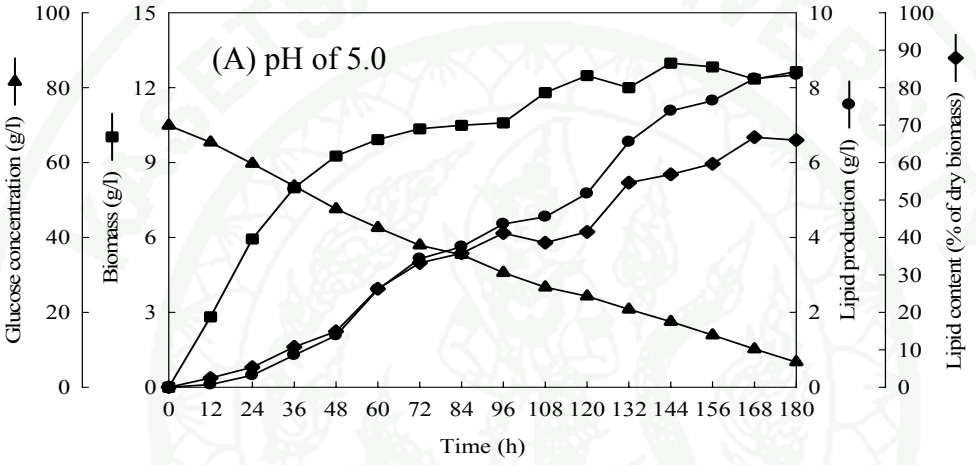

Figure 32 Time course of biomass (■), lipid production (●), cellular lipid content (◆) and glucose concentration (▲) by *R. toruloides* DMKU3-TK16 in a nitrogen-limited medium II composed of 70 g/l glucose, 0.75 g/l yeast extract, 0.55 g/l $(NH_4)_2SO_4$, C/N-ratios (140), 0.4 g/l KH_2PO_4, 2.0 g/l $MgSO_4 \cdot 7H_2O$ and various initial pH-values (A) pH of 5.0, (B) pH of 5.5, (C) pH of 6.0 and (D) pH of 6.5 by shaking flask cultivation at 150 rpm and 28 ºC

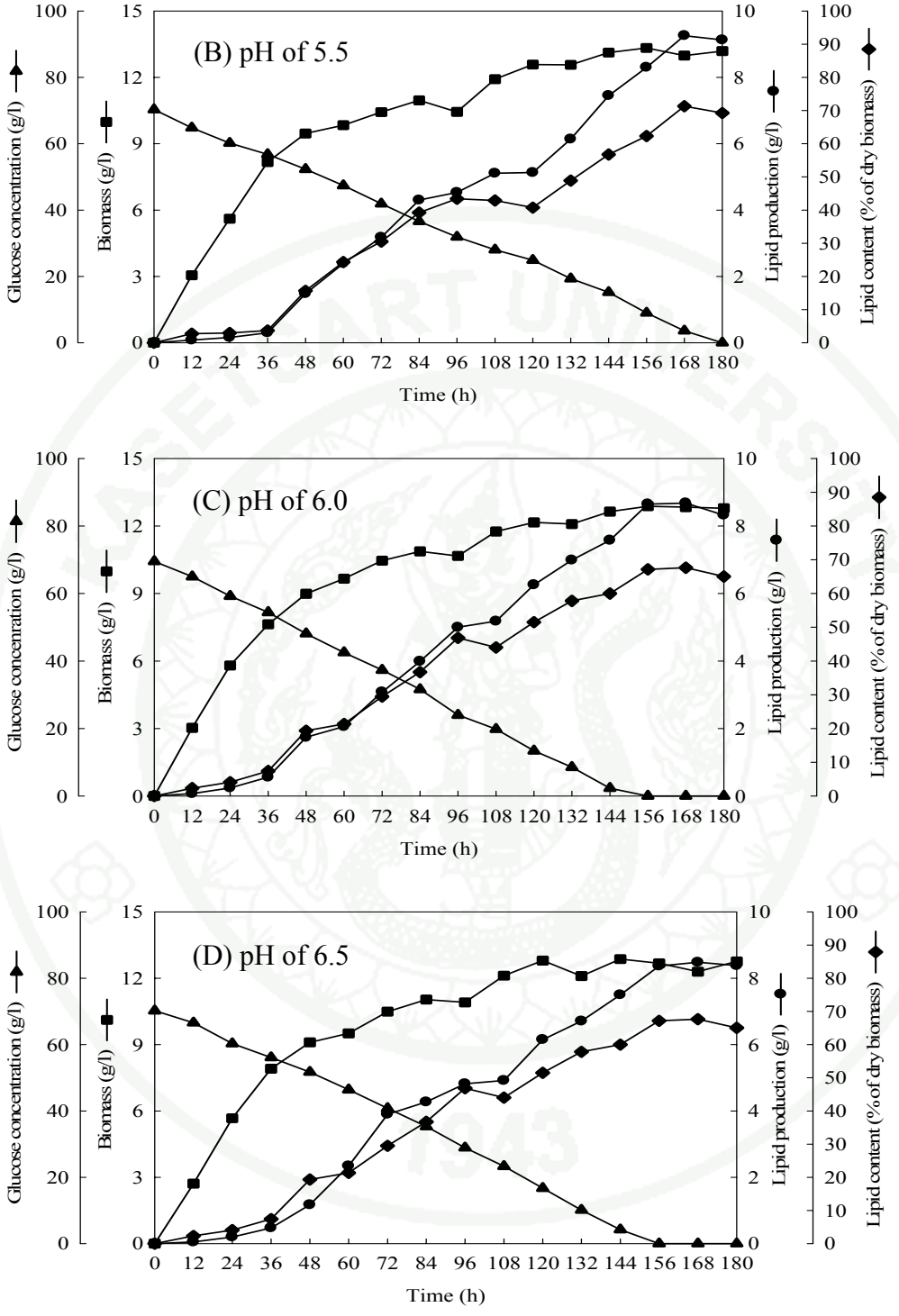

Figure 32 (Continued)

6.7 Effect of temperature on lipid production

The effect of temperature on lipid production by *R. toruloides* DMKU3-TK16 was investigated. The lipid accumulation was clearly effected by the incubation temperature. Papanikolaou *et al*. (2002) studied the influence of the incubation temperature and obtained high lipid content (44-55 % of dry biomass) at the temperature of 28-33 °C when cultivated Yarrowia lipolytica with animal fat.

Table 10 Biomass, lipid production and cellular lipid content by *R. toruloides* DMKU3-TK16 in a nitrogen-limited medium composed of 70 g/l glucose, 0.75 g/l yeast extract, 0.55 g/l$(NH_4)_2SO_4$, C/N-ratios (140), 0.4 g/l KH_2PO_4, 2.0 g/l $MgSO_4 \cdot 7H_2O$ and pH 5.5 incubated on temperature gradient incubator for 180 h.

Temperature range (°C)	Biomass (g/l)	Lipid production (g/l)	Lipid content (% of dry biomass)
15-16	5.48	0.09	1.69
18-19	10.81	0.74	6.85
21-22	12.48	4.64	37.18
24-25	13.24	5.25	39.65
27-28	12.84	6.70	52.18
30-31	12.80	7.94	62.03
33-34	10.34	7.30	70.60

When cultivated at temperature of 30-31 °C, maximal lipid production (7.94 g/l) was obtained but with cellular lipid content of only 62.03% of dry biomass (Table 10). Increasing the cultivation temperature form 15-16 °C to 30-31 °C resulted in increasing of lipid production and cellular lipid content. Apparently, lipid production was more stimulated at the higher incubation temperature. However, when temperature raising up to 33-34 °C both biomass (10.34 g/l) and lipid production (7.30 g/l) were slightly decreased while cellular lipid content was increased to 70.60% of dry biomass. The effect of temperature for growth and lipid production of

R. toruloides was also investigated by Li *et al*. (2006). They found that the optimal temperature was 30 °C. On the basis of the highest lipid production, the temperature of 30-31 °C was used for further optimization.

7. Optimization of lipid production by *Rhodosporidium toruloides* DMKU3-TK16 in a jar fermenter

In order to study the possibility of high lipid production of *R. toruloides* DMKU3-TK16, batch cultivation in a 5 l jar fermenter with working volume of 3 l was performed.

7.1 Effect of aeration rate on lipid production

The influence of aerations at 1.0, 2.0 and 3.0 vvm on lipid production was investigated with agitation speed of 200 rpm throughout the cultivation period. The result revealed that the final lipid production increased with the increase of aeration. The highest lipid production (12.90 g/l), cellular lipid content (62.68 % of dry biomass) and biomass (20.58 g/l) were obtained at 156 h when aeration rate was adjusted to 3.0 vvm (Figure 33C). At this time the glucose was not entirely depleted. Although the lipid production, cellular lipid content and biomass did not further increase after 156 h, the glucose concentration continued to decrease, from 18.70 at 156 h to 11.10 g/l at 180 h. Generally, high biomass concentration contributed to the high cellular lipid accumulation. While the aeration rate at 2.0 vvm (Figure 33B), the cellular lipid and biomass concentration were declined to 8.13 g/l and 12.68 g/l, respectively. However, cellular lipid content (65.78 % of dry biomass) found at this aeration rate was higher than that obtained by using higher aeration rate (3.0 vvm). The glucose consumption decreased by decreasing in oxygen supplied rate. At the lowest aeration rate (1.0 vvm), *R. toruloides* DMKU3-TK16 revealed low growth (10.91 g/l) same as at 2.0 vvm aeration rate but produced extremely low lipid of 2.86 g/l (Figure 33A); these reflected to their cellular lipid content of 26.21 % of dry biomass. The result of low lipid accumulation supported by remaining high glucose concentration (50.80 g/l) in the medium.

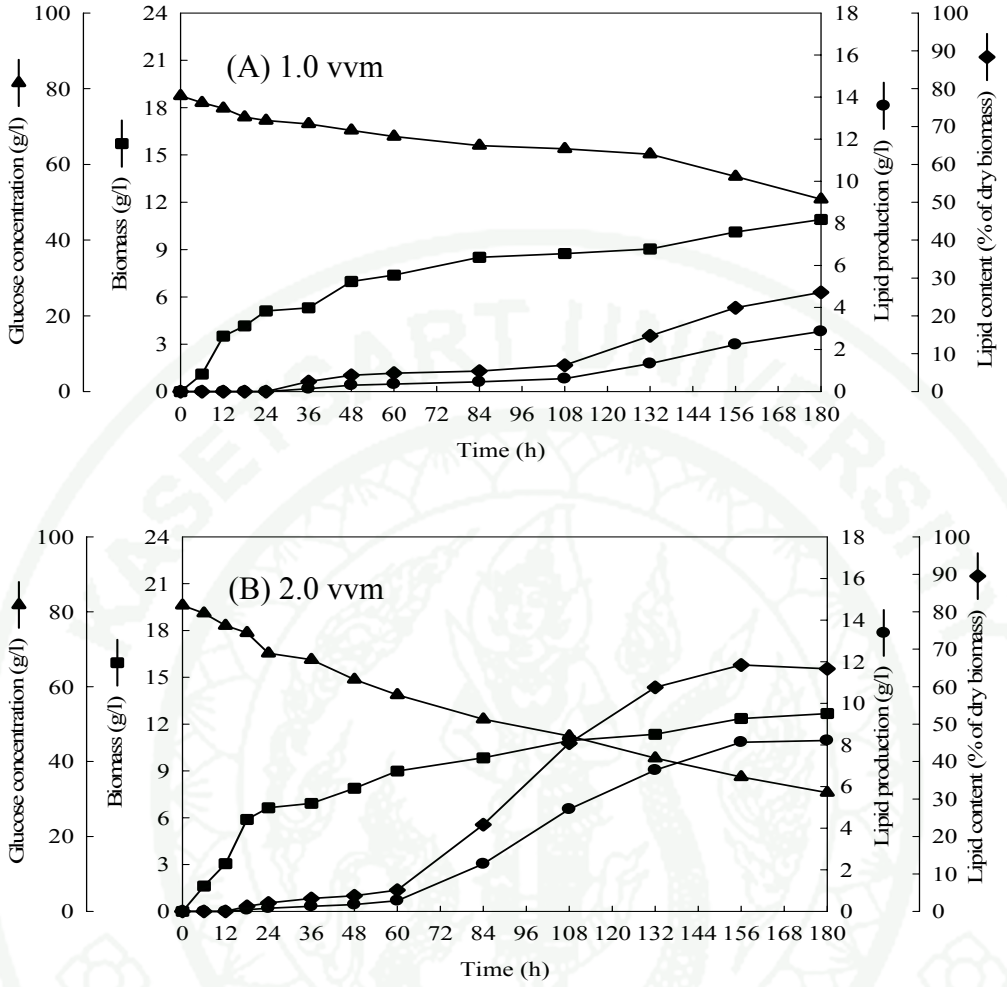

Figure 33 Time course of biomass (■), lipid production (●), cellular lipid content (◆) and glucose concentration (▲) by *R. toruloides* DMKU3-TK16 in a optimized medium composed of 70 g/l glucose, 0.75 g/l yeast extract, 0.10 g/l $(NH_4)_2SO_4$, C/N-ratios (140), 0.4 g/l KH_2PO_4, 2.0 g/l $MgSO_4 \cdot 7H_2O$, pH of 5.5 and various aerations (A) 1.0 vvm, (B) 2.0 vvm and (C) 3.0 vvm by jar fermenter cultivation at 200 rpm and 30 ºC.

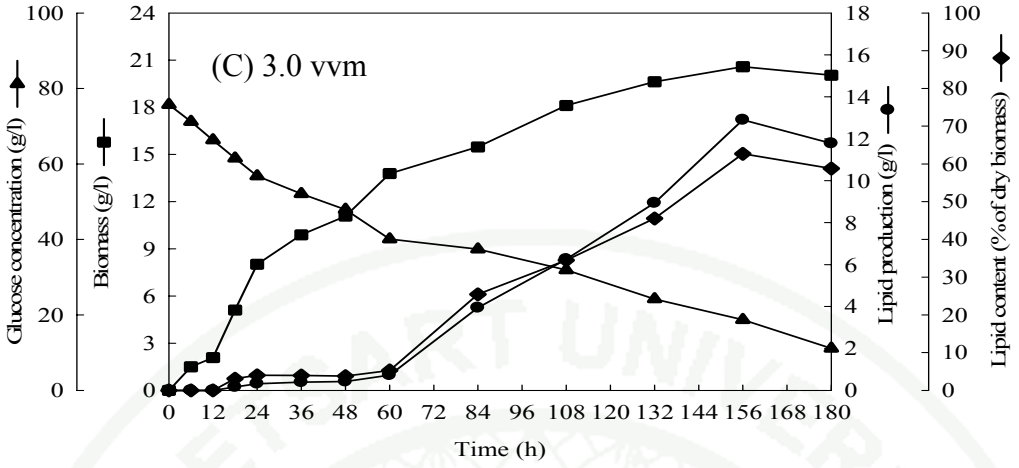

Figure 33 (Continued)

7.2 Effect of agitation on lipid production

Effect of agitation rate on lipid production by batch cultivation of *R. toruloides* DMKU3-TK16 was carried out in a 5 l jar fermenter with working volume of 3 l. The agitation speed was adjusted to 200 and 300 rpm with aeration at 3.0 vvm throughout the cultivation period. The lipid production, cellular lipid content and biomass concentration of *R. toruloides* DMKU3-TK16 were improved significantly by enhancing the agitation speed from 200 to 300 rpm, probably due to an enhanced oxygen supply. The lipid production and biomass concentration at 300 rpm had reached a maximum of 15.12 g/l and 22.49 g/l at 156 h, respectively (Figure 34B). These values were higher than that obtained at 200 rpm however; the maximal cellular lipid content (67.23% of dry biomass) obtained in this cultivation was lower than that found in shaking flask cultivation. The glucose was not entirely depleted at the end of cultivation period. At 200 rpm agitation rate lower biomass concentration (20.26 g/l), lipid production (11.98 g/l) and cellular lipid content (59.13% of dry biomass) were observed at 156 h (Figure 34A). It is clear that the lower agitation rate of batch cultivation resulted in slightly difference in both biomass and lipid production.

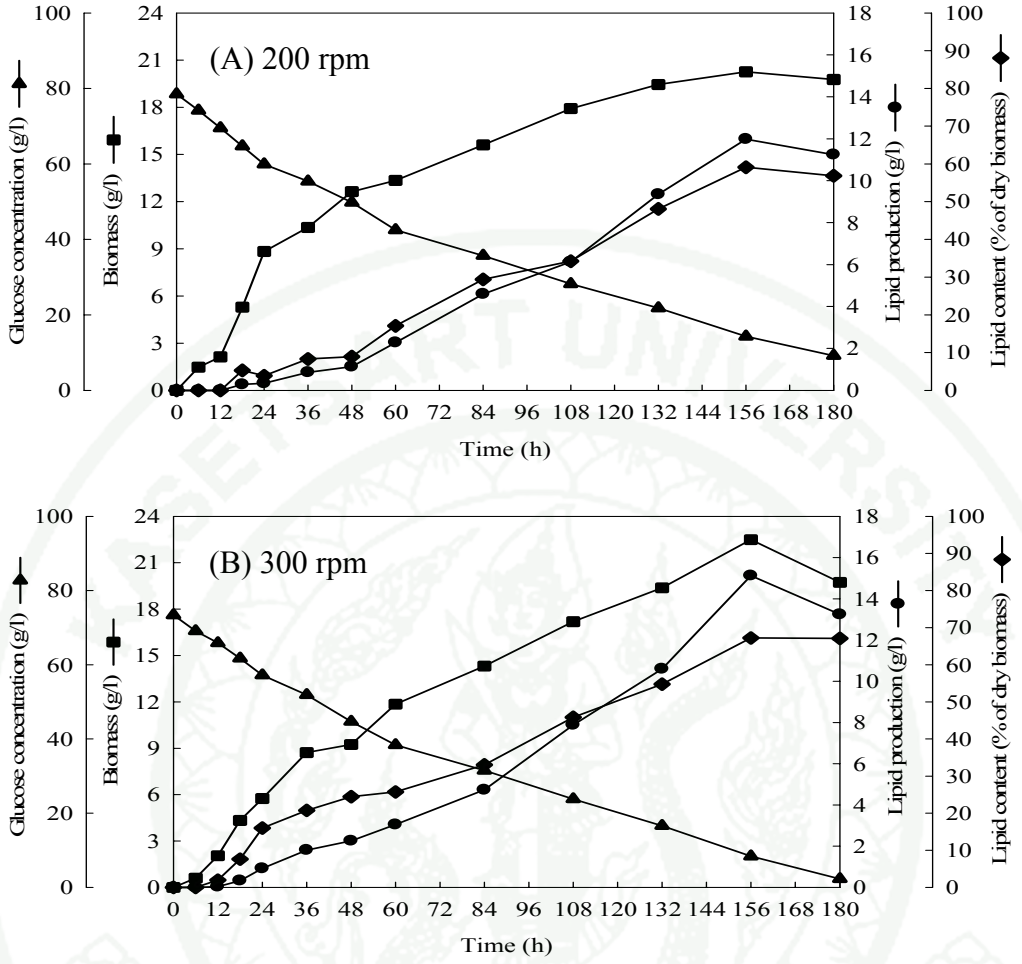

Figure 34 Time course of biomass (■), lipid production (●), cellular lipid content (◆) and glucose concentration (▲) by *R. toruloides* DMKU3-TK16 in a optimized medium composed of 70 g/l glucose, 0.75 g/l yeast extract, 0.10 g/l $(NH_4)_2SO_4$, C/N-ratios (140), 0.4 g/l KH_2PO_4, 2.0 g/l $MgSO_4 \cdot 7H_2O$, pH of 5.5, 3.0 vvm and various agitations (A) 200 rpm, (B) 300 rpm by jar fermenter cultivation at 30 ºC.

8 Fatty acid profiles

The fatty acid profiles of oleaginous yeast depend on the species and their growth conditions. Environmental conditions such as temperature, pH, substrate, C/N-ratio and oxygen have influence on efficiency of lipid accumulation (Jacob, 1993). During the final 96 h of cultivation, the fatty acid profile of *R. toruloides* DMKU3-TK16 under the optimal nutrient and environmental conditions in a nitrogen-limited medium II composed of 70 g/l glucose, 0.75 g/l yeast extract, 0.55 g/l $(NH_4)_2SO_4$, 0.4 g/l KH_2PO_4, 2.0 g/l $MgSO_4 \cdot 7H_2O$, with pH of 5.5 by jar fermenter cultivation at 300 rpm, 3.0 vvm and 30 ºC as percentage of total fatty acid was oleic acid (C18:1) (39.45-40.85 %), palmitic acid (C16:0) (20.42-25.60 %), linoleic acid (C18:2) (14.28-18.04 %), stearic acid (C18:0) (11.65-15.36 %), linolenic acid (C18:3) (4.25-6.97 %), myristic acid (C14:0) (0.80-1.32 %) and palmitoleic acid (C16:1) (0.96-1.49 %) as shown in Table 11 It is clearly seen that the major fatty acids were oleic, palmitic, linolenic and stearic acid (Table 11). The percentages of fatty acids remained at a constant level or decreased slightly but amounts of linolenic acid slightly increased during 84 and 180 h. Similar result was observed in the study of Li *et al.* (2007). They reported that oleic acid was the major fatty acid produced by *R. toruloides* while the other fatty acids were almost constant during the cultivation.

Table 11 Major fatty acid composition of the cellular lipid content in percentages of *R. toruloides* DMKU3-TK16 grown in a 5-l jar fermenter during cultivation

Time (h)	Major fatty acid residue (%w/w)						
	14:0	16:0	16:1	18:0	18:1	18:2	18:3
84	1.06	25.60	ndt	15.36	39.45	14.28	4.25
108	1.32	24.54	ndt	14.38	40.20	15.05	4.51
132	0.89	22.66	0.96	13.13	40.59	16.18	5.59
156	0.80	20.42	1.49	11.65	40.93	18.04	6.67
180	0.83	21.35	1.05	12.01	40.85	16.94	6.97

ndt = no detect

The fatty acid profile of *R. toruloides* DMKU3-TK16 under the optimal nutrient and environmental conditions in a 5 l jar fermenter at 156 h as percentage of total fatty acid was oleic acid (40.93 %), palmitic acid (20.42 %), linoleic acid (18.04 %), stearic acid (11.65 %), linolenic acid (6.67 %), myristic acid (0.80 %) and palmitoleic acid (1.49 %) are shown in Table 12. It is clearly seen that the fatty acid composition was similar to vegetable oils with four major fatty acids including oleic, palmitic, linolenic and stearic acids (Table 12).

Table 12 Comparison of fatty acid profiles of *R. toruloides* DMKU3-TK16 and the other vegetable oils

	Major fatty acid residue (%w/w)						
	14:0	16:0	16:1	18:0	18:1	18:2	18:3
R. toruloides DMKU3-TK16	0.80	20.42	1.49	11.65	40.93	18.04	6.67
Soybean [a]	nd	12	nd	3	23	56	6
Palm [a]	nd	43	nd	5	41	10	nd
Cotton seed [a]	nd	20	nd	3	19	55	nd
Corn [a]	nd	12	nd	2	25	61	nd
Sun flower [a]	nd	6	nd	3	17	74	nd

[a] Data from Ma and Hanna (1999); nd = no data

CONCLUSION AND RECOMMENDATION

In this study, newly isolated oleaginous yeast *Rhodosporidium toruloides* DMKU3-TK16 was obtained by three steps screening from 63 strains of known species in the genus *Rhodotorula* and 104 newly isolated strains of unidentified species. Optimization of lipid production by *R. toruloides* DMKU3-TK16 was carried out. The results revealed that the lipid production by this strain in shaking flask cultivation at 150 rpm and 28 ºC was highest in nitrogen-limited medium II containing per liter 70 g glucose, 0.75 g yeast extract, 0.55 g $(NH_4)_2SO_4$, 0.4 g KH_2PO_4, 2.0 g $MgSO_4 \bullet 7H_2O$ and pH 5.5. Under these optimal conditions, when the C/N ratio of the medium was 140, *R. toruloides* DMKU3-TK16 produced lipid of 9.26 g/l, which was 71.30 % of dry biomass (13.33 g/l) after 168 h of cultivation. The lipid production in a 5 l jar fermenter with an agitation speed of 300 rpm, an aeration rate of 3.0 vvm and 30 ºC throughout the cultivation, *R. toruloides* DMKU3-TK16 yielded a final lipid of 15.12 g/l, which was 67.23 % of dry biomass (22.49 g/l) after 156 h of cultivation. The major fatty acids of the cellular lipid were oleic acid (40.93%), palmitic acid (20.42%), linoleic acid (18.04%) and steric acid (11.65%). The fatty acid profiles of *R. toruloides* DMKU3-TK16 revealed that its lipid was similar to vegetable oil with four major fatty acids including oleic, palmitic, linolenic and stearic acid so that it can be used for biodiesel production.

In order to promote high biomass, lipid production and cellular lipid content of *R. toruloides* DMKU3-TK16 in a cost-effective process may require scaling up of the suitable cultivation mode with a cheap raw material in further development.

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APPENCICES

Appendix A

Medium

1. Yeast extract malt extract (YM) agar

Yeast extract	3.00	g
Malt extract	3.00	g
Peptone	5.00	g
Glucose	10.00	g
Agar	15.00	g
Distilled water	1,000.00	ml

Sterilized at 121 ºC for 15 min

2. Nitrogen-limited medium I agar

Glucose	30.0	g
Yeast extract	1.50	g
NH_4Cl	0.50	g
KH_2PO_4	7.00	g
$Na_2HPO_4 \cdot 12H_2O$	5.00	g
$MgSO_4 \cdot 7H_2O$	1.50	g
$FeCl_3 \cdot 6H_2O$	0.08	g
$ZnSO_4 \cdot 7H_2O$	0.01	g
$CaCl_2 \cdot 2H_2O$	0.10	g
$MnSO_4 \cdot 5H_2O$	0.10	mg
$CuSO_4 \cdot 5H_2O$	0.10	mg
Agar	20.00	g
Distilled water	1,000.00	ml

Sterilized at 121 ºC for 15 min

3. Nitrogen-limited medium I agar supplement with choramphinical (200 µg/l) and Sodium propionate (2.0 g/l)

Glucose	30.0	g
Yeast extract	1.50	g
NH_4Cl	0.50	g
KH_2PO_4	7.00	g
$Na_2HPO_4 \cdot 12H_2O$	5.00	g
$MgSO_4 \cdot 7H_2O$	1.50	g
$FeCl_3 \cdot 6H_2O$	0.08	g
$ZnSO_4 \cdot 7H_2O$	0.01	g
$CaCl_2 \cdot 2H_2O$	0.10	g
$MnSO_4 \cdot 5H_2O$	0.10	mg
$CuSO_4 \cdot 5H_2O$	0.10	mg
Choramphinical	200.00	µg
Sodium propionate	2.00	g
Agar	20.00	g
Distilled water	1,000.00	ml

Sterilized at 121 ºC for 15 min

4. Nitrogen-limited medium I broth (Suuatari *et al*., 1993)

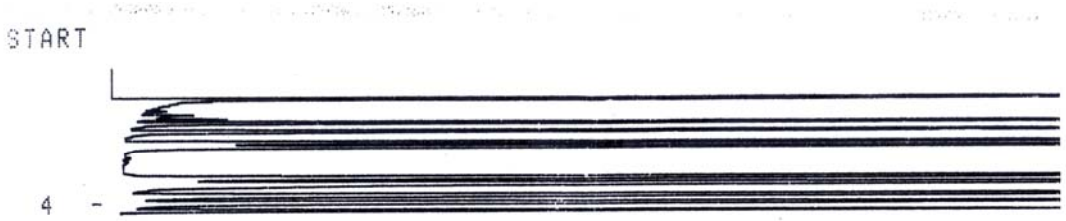
Glucose	30.00	g
Yeast extract	1.50	g
NH_4Cl	0.50	g
KH_2PO_4	7.00	g
$Na_2HPO_4•12H_2O$	5.00	g
$MgSO_4•7H_2O$	1.50	g
$FeCl_3•6H_2O$	0.08	g
$ZnSO_4•7H_2O$	0.01	g
$CaCl_2•2H_2O$	0.10	g
$MnSO_4•5H_2O$	0.10	mg
$CuSO_4•5H_2O$	0.10	mg
Distilled water	1,000.00	ml
Sterilized at 121 ºC for 15 min		

5. Nitrogen-limited medium II broth (Li *et al*., 2007)

Glucose	30.00	g
$(NH_4)_2SO_4$	0.10	g
Yeast extract	0.75	g
$MgSO_4 \cdot 7H_2O$	1.50	g
KH_2PO_4	0.40	g
$CaCl_2 \cdot 2H_2O$	0.22	g
$ZnSO_4 \cdot 7H_2O$	0.55	g
$FeCl_3 \cdot 6H_2O$	0.08	g
$ZnSO_4 \cdot 7H_2O$	0.01	µg
$MnCl_2 \cdot 4H_2O$	24.20	µg
$CuSO_4 \cdot 5H_2O$	25.00	µg
Distilled water	1,000.00	ml
Sterilized at 121 ºC for 15 min		

Appendix B

A typical gas chromatogram of fatty acid



CHROMATOPAC C-R6A FILE 9
SAMPLE NO 0 METHOD 843
REPORT NO 14129 SAMPLE WT 100
IS WT 1

PKNO	TIME	AREA	MK	IDNO	CONC	NAME
1	1.592	63560		2	0.9644	14:0
2	1.837	55341		1		IS
3	2.167	59892		3	1.0218	16:0
4	2.297	45217	V	4	0.9958	16:1
5	3.197	54134	V	5	1.1044	18:0
6	3.387	68127	V	6	1.0206	18:1
7	3.718	37956	V	7	1.0183	18:2
8	3.93	30112	V	8	1.0143	A18:3
9	4.193	29854	SV	9	1.0233	G18:3
	TOTAL	444192			8.1629	

Appendix Figure B1 A typical gas chromatogram of fatty acid

CIRRICULUM VITAE

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