SIMULTANEOUS MICROBIAL OIL SYNTHESIS AND RECOVERY FROM OLEAGINOUS YEAST CULTIVATION FOR BIOLUBRICANT CONVERSION

KESSUDA NGERNSOMBAT

A THESIS SUBMITTED IN PARTIAL FULLFILLMENT OF THE REQUIREMENT FOR THE DEGREE OF MASTER OF SCIENCE PROGRAM IN APPLIED BIOLOGY
FACULTY OF SCIENCE AND TECHNOLOGY
RAJAMANGALA UNIVERSITY OF TECHNOLOGY THANYABURI
ACADEMIC YEAR 2023

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Thesis Title	Simultaneous Microbial Oil Synthesis and Recovery from Oleaginous Yeast Cultivation for Biolubricant Conversion
Name – Surname	Miss Kessuda Ngernsombat
Program	Applied Biology
Thesis Advisor	Assistant Professor Atsadawut Areesirisuk, Ph.D.
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Abstract

This study aimed to: 1) select the effective oleaginous yeast strain for enhancing yeast oil (YO) production and secretion, 2) monitor the expression of key lipogenic genes, 3) investigate *in-situ* and *ex-situ* oil recovery using oil capturing agents (OCAs), and 4) study the feasibility of biolubricant production using YO as the primary feedstock.

Seven yeast candidates for YO production were evaluated. NIS was added during cultivation to enhance intracellular oil secretion. Oil recovery was conducted through both *in-situ* and *ex-situ* methods using different OCAs. YO was utilized to synthesize biolubricants via trimethylolpropane (TMP) esterification.

Rhodosporidium toruloides TISTR 5186 exhibited the highest efficacy in YO production in a glycerol-based medium. The highest total YO (TO) formation and oil content were at 4.0 g/L and 78.38% (w/w), respectively, with 0.7% (w/v) Tween 20. Under this condition, the expression of *ACC* and *DGA* genes significantly increased by 3.4 and 3.6-fold, respectively. SP70 OCA effectively adsorbed extracellular oil (EO), providing 90.49% (w/w) *ex-situ* EO recovery within 1 day. A TO quantity of 53.37 g was achieved through online *ex-situ* oil recovery and fed-batch fermentation in a 5-L bioreactor. The conversion of YO into TMP-fatty acid esters as biolubricants on a laboratory scale was successful. Simultaneous YO production and recovery present a promising process to enhance TO production without cell disruption.

Keywords: non-ionic surfactants, oil capturing agent, oil recovery, batch fermentation, fatty acid

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Kessuda Ngernsombat

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List of Abbreviations

AO : Adsorb Oil

AMP : Adenosine monophosphate deaminase

AMPD : Adenosine monophosphate deaminase

Ct : Threshold cycles

DAG : Diacylglycerol

DHAP : Dihydroxyacetone phosphate

EO : Extracellular oil

FA : Fatty acid

FAME : Fatty acid methyl ester

FAS : Fatty-acyl-CoA synthase

FFAs : Free Fatty acids

G-OPM : Glycerol-based oil production medium

G-3-P : Glycerol-3-phosphate

GA-3-P : Glyceraldehyde 3-phosphate

GC-MS : Gas chromatography with mass spectrometry

IO : Intracellular oil

α-KG : Alpha-Ketoglutarate

LPA : Lysophosphatidic acid

m_S : Biomass maintenance coefficient (g/g h)

MUFA : Monounsaturated fatty acid

n : Power constant of product inhibition (dimensionless)

N : Number of measurements

NPG : Neopentyl glycol

NRMS : non-normalized root mean square

OAA : Oxaloacetate

OCA : Oil capturing agent

PA : Phosphatidic acid

P_i : Intracellular product concentration (g/L)

P_i* : Critical intracellular product-inhibition concentration (g/L)

List of Abbreviations (Cont.)

Q_p : Quality procedure

q_{cal} : Calculated data

q_{exp} : Experimental data

$\bar{q}_{exp}$: Average of all the experimental data

RT-qPCR : Reverse transcription-quantitative polymerase chain reaction

R^2 : Coefficient of determination (dimensionless)

ΔS : Consumed substrate (g/L)

SD : Standard deviation

t : Cultivation time (h)

TAG : Triacylglycerol

TCA : Tricarboxylic acid

TO : Total oil

SCOs : Single cell oils

X : Biomass concentration (g/L)

X_{max} : Maximum biomass concentration (g/L)

X_r : Residual biomass (g/L)

YM : Yeast malt medium

YO : Yeast oil

$Y_{Pi/S}$: Intracellular oil yield (g intracellular oil /g substrate)

$Y_{Pt/S}$: Total oil yield (g total oil /g substrate)

$Y_{X/S}$: Biomass yield (g cell/g substrate)

Greek letters

μ_{max} : Maximum specific growth rate (h^{-1})

α : Growth-associated production coefficient (g oil/g cell)

β : Non-growth associated production coefficient (g/g h)

CHAPTER 1
INTRODUCTION

1.1 Background and statement of problems

In the past, there have been many environmental concerns about using petroleum to produce various conventional products in the forestry, plantation, drilling, and marine industries [1]. Due to about 10-15 million tons of petroleum-producing oil spilling into the biosphere yearly, 40% is contaminated by water and soil [2]. Lubricants are oil-based chemicals, predominantly produced from mineral oil, which are widely used to minimize damage due to friction, wear, and overheating in machine parts [3]. In addition, most traditional lubricant products are poorly biodegradable and cause serious environmental pollution [4,5]. For these reasons, there is a growing impetus in the demand for and use of plants to produce bio-lubricants in various fields rather than petroleum-based lubricants [6,7]. However, the raw material cost of biolubricants produced using vegetable oil accounts for 75-80% of the total production cost [8]. In addition, it also requires an area and nutrients suitable for large-scale planting and a long growth cycle. Therefore, it is a long-term disadvantage to use plants for biolubricant production [9].

Microbial oil is a secondary metabolite produced by yeast, algae, and fungi. It can be used as an alternative feedstock for the production of oleochemicals and sustainable fuels such as wax esters and biodiesel [10-12]. The microbial oil produced through fermentation of raw resources has been studied for bio-lubricant production [13]. It has an excellent fatty acid (FA) composition, physicochemical, and tribophysical properties, making it suitable for high-quality lubricant formulations [14]. Oleaginous yeast can produce and accumulate oils above 20% of its cells [15]. Yeast has a high growth rate and oil content and can be grown in agricultural waste such as rice straw, corn cob, and glycerol [16]. It can produce the oil quickly and is unaffected by environmental conditions. Yeast oil also contains FAs similar to the composition of vegetable oils [17]. As a result, it has been used in various applications, including fuels, chemicals, and polymers [18].

Glycerol is a chemical compound used in various industries, including pharmaceuticals, cosmetics, chemicals, and food. Glycerol is a significant by-product of biodiesel manufacturing. This process generates a large amount of raw glycerol, with approximately 100 kg of crude glycerol produced per ton of biodiesel [19]. Previously, glycerol was used as a carbon source for microbial oil production under suitable cultivation conditions [20].

The microbial oil extraction process begins with the chemical or physical digestion of the cells, followed by the separation of the oil using organic solvents. This procedure will increase production costs and require more steps. Previous studies investigated using surfactants to release yeast oil from oleaginous cells into a culture medium in the form of extracellular oil. In addition, yeast oil removal from the medium during cultivation has been investigated. It has been reported that the oil capturing agent (OCA) can adsorb the yeast oil from fermentation broth. However, the integration of yeast oil released into cultivation broth and oil absorption procedure from broth has not been reported.

This research aims to develop yeast oil production integrated with oil recovery using non-ionic surfactants and oil capturing agents by *in situ* and *ex situ* batch modes, to develop the online oil capturing process in a 5-L bioreactor, and to evaluate the feasibility of bio-lubricant production using yeast oil as a raw material.

1.2 Purposes of the study

1.2.1 To select the highest potential oil-producing yeast from glycerol.

1.2.2 To study the simultaneous oil biosynthesis and recovery of oil-producing yeast with non-ionic surfactants and OCA in batch mode.

1.2.3 To study the online capture of yeast oil in 5-L bioreactor by batch and fed-batch modes.

1.2.4 To study the feasibility of bio-lubricants production using yeast oil as a raw material.

1.3 Scope of thesis

The scope of this research is the selection of the highest potential oil-producing yeast strains using glycerol as the main carbon source. Seven yeast strains used in this research are *Cryptococcus albidus* TISTR 5103, *Pseudozyma parantarctica* CHC28, *Rhodotorula glutinis* DB-RMUTT01, *R. glutinis* DB-RMUTT02, *R. glutinis* TISTR 5159, *Rhodosporidium toruloides* TISTR 5186 and *Yarrowia lipolytica* TISTR 5212. The effect of non-ionic surfactants on releasing of extracellular oil is investigated. *In situ* and *Ex situ* simultaneous yeast oil production and recovery are performed in batch modes. Then, the online yeast oil capture is operated in 5-L bioreactor by batch and fed-batch modes. The expression of the key gene associated with oil production is evaluated by real-time PCR (RT-PCR). The FA profiles of yeast oil produced from glycerol are defined by gas chromatography with mass spectrometry (GC-MS). The kinetic parameters of yeast growth, substrate consumption, and oil formation are described by mathematical modeling. Finally, the feasibility of biolubricant production from yeast oil is investigated on a laboratory scale.

1.4 Expectations of thesis

This thesis, I have the scope and limitations of studying which are concerned to the previous works which are:

1.4.1 Obtained the highest potential oil-producing yeast from glycerol.

1.4.2 Known the simultaneous oil biosynthesis and recovery of oil-producing yeast with non-ionic surfactants and OCA in batch mode.

1.4.3 Known the online capture of yeast oil in 5-L bioreactor by batch and fed-batch modes.

1.4.4 Known the feasibility of biolubricants production using yeast oil as a raw material.

CHAPTER 2
REVIEWS OF THE LITERATURE

2.1 Fossil resources for oil-based production

Over the past century, fossil oil has served as the most economical raw material for energy and chemical production, forming the foundation for much of modern society's materials and infrastructure. However, this scenario is undergoing rapid transformation. Decreasing oil reserves, coupled with escalating demand, have led to a drastic shift, with oil prices now soaring to levels ten times higher than those of 15 years ago [21]. Globally, fossil fuels are widely used in several industries. However, it has been a concern because it raises CO_2 levels and accumulates greenhouse gases, causing environmental problems and harming human and animal health. Fossil oils are a non-renewable energy source that takes millions of years to form. Rising oil prices often lead to recessions like the world and international conflict, especially in some developing countries [22-25]. Therefore, much research is being done to find and expand renewable energy sources from other sources such as solar power, waves, wind power, geothermal energy, and biomass such as algae, yeast, and fungi instead of fossil fuels [26,27]. Lubricant products are produced from fossil oil and are generally used in machine engines. Conventional petroleum-based lubricants are required for 7 billion people worldwide to operate in 600 million vehicles [28]. In 2019, the demand for lubricants was increasing at a rate of 2% per year and up to 45.4 million metric tons. Additionally, approximately 40-50% of used lubricant volume needs to be disposed of properly and has a negative impact on terrestrial and aquatic ecosystems [29].

2.2 Oleaginous yeast

Generally, oil is contained at 6-8% (w/w) of the dry cell weight in all microorganisms [15]. A number of microbes, both prokaryotic (like bacteria) and eukaryotic (like fungi, yeast, and algae), are able to synthesize large amounts of lipids, mostly as particles that store energy and carbon [30]. These microbial lipids, also known as single-celled oils (SCOs) [31], are used as a source of oil for the synthesis of biofuel as well as for the nourishment of humans and other animals [32]. The term oleaginous

describes a microbe that can accumulate at least 20% of its intracellular oil by dry weight [33,34]. The most promising microorganisms for biofuel production are oleaginous yeast, Triglycerides and steryl esters (SEs), which are commonly present in the form of fatty acids from C13 to C21, make up the majority of the accumulated oils, which are mainly nonpolar [35]. Triacylglycerols (TAGs) in oleaginous yeast usually have a lipid content ranging from 80% (w/w) to 90% (w/w) [36-40]. Both lipids from the yeast cell membrane and oil droplets are frequently included in the oils examined in investigations. Oleic acid (C18:1), palmitic acid (C16:0), linoleic acid (C18:2), and stearic acid (C18:0) are the main fatty acids found in yeast fat [41]. Yeast oil has been studied as raw material for biodiesel and biolubricant production, replacing fossil oils. The FA compositions of yeast oil are similar to vegetable and animal oil. In addition, yeast oil production presents several advantages over vegetable oils and animal fats. It is not affected by area or weather, requires less planting labor, and is easier to expand [42]. Oleaginous yeast cultivation can use different carbon sources as raw materials and grow in general operating conditions [43]. Various carbon sources are employed for yeast lipid production, with monosaccharides, glycerol and hydrolysates (pure and crude) being the most prevalent, collectively constituting approximately 71% of the raw materials utilized. Conversely, fatty acids, wastewater, oils and molasses are less common, occasionally supplemented by aromatics, aqueous extracts, alcohols, and other waste. The versatility in substrate utilization is a significant advantage of yeast oil, consistent with specific policies such as the European Union Renewable Energy Directive. Carbon sources notably influence lipid synthesis, with feedstock selection often based on organism suitability, nutrient content, simplicity, cost, and availability [44-49]. Nutrient restriction typically stimulates lipid accumulation, with excess citrate from the citric acid cycle being channeled towards fatty acid synthesis [50]. Nitrogen, phosphorus, iron, sulphur, zinc, and oxygen are among the nutrients that are frequently depleted to promote fat storage. When extracellular carbon sources are not enough for metabolic demands, storage lipids can go through beta-oxidation to liberate stored energy [51]. On the other hand, intracellular lipids may be released to promote cell division when nutrients are sufficient [52]. *De novo* oil synthesis is mainly divided between cell growth and lipid accumulation, with the carbon/nitrogen (C/N) or carbon/phosphorus (C/P) ratios being widely utilized to identify nutrient-

limiting circumstances [44- 46]. Citrate and other byproducts can build up as a result of ongoing nutritional shortages. Although it was once thought that oleaginous yeasts simultaneously accumulated intracellular polysaccharides and lipids through a *de novo* pathway, new findings suggest that certain species accumulate polysaccharides in nitrogen-rich environments prior to transitioning to oil production because of nitrogen limitations [53,54]. *Yarrowia lipolytica*, *Cutaneotrichosporon oleaginosus*, *Rhodosporidium toruloides*, *Lipomyces starkeyi*, and *Rhodotorular glutinis* are notable oleaginous yeast species that, when combined, account for over 50% of cultured oleaginous yeasts because of their high oil content, efficient growth, and genetic potential [32]. Among them, *R. toruloides* also referred to as *R. rubescens* or *R. gracile* stands out for having a high oil output, producing carotenoid and synthesizing enzymes, and having industrial potential as an oil producer [55,56]. *Rhodotorula* species produce a lot of carotenoid pigments, which gives them their characteristic red hue [56]. Among the native species used in lipid synthesis, native *R. toruloides* strains DSM 4444 (German Microbial and Cell Culture Group) and AS.2.1389 (China General Microbiological Culture Collection Centre) are particularly often used [32]. At least 25% of these strains are local to the world.

2.3 Microbial oil accumulation

The primary feature of oleaginous yeasts in biotechnological applications is their capacity for high lipid production when grown on volatile fatty acids (VFAs) rich digestate. Lipid quantification typically occurs at the end of fermentation, once the carbon source has been fully utilized and maximum yeast biomass has been achieved [57]. Oleaginous microorganisms are capable of accumulating lipids at levels exceeding 20% of dry cell weight, reaching up to 60% under certain conditions [58]. The lipid content of yeast can vary even within the same species. Apart from physiological factors influencing lipid accumulation, other elements such as substrate and environmental conditions also play a significant role [59].

The type of substrate utilized in culture affects lipid storage, with synthesis and accumulation occurring through two distinct pathways depending on whether glucose or other substrates are metabolized. When glucose is the carbon source, lipid synthesis

follows the de novo pathway. This process involves lipid accumulation during secondary metabolic growth, which is disrupted by nutrient (typically nitrogen) limitation. In nutrient-limited conditions, excess carbon sources in the growth medium are converted into storage lipids, primarily triacylglycerols. The importance of the carbon-to-nitrogen (C/N) ratio in de novo lipid synthesis is widely acknowledged [58].

On the other hand, the *ex-novo* oil manufacturing pathway starts when carbon sources other than hydrophobic substrates are used. Regardless of nutritional constraints, lipid formation in this pathway happens simultaneously with main metabolic development [60]. Complexities exist, nevertheless, in the metabolic pathways linked to oily yeasts' absorption of VFAs and in the critical elements that lead to the buildup of unknown lipids resulting from large amounts of ammonium produced during microalgae anaerobic fermentation. The present study's low C/N ratio (3.8 ± 0.2) implies that lipid accumulation happens during initial metabolic development, especially when the growth medium contains long-chain fatty acids as substrates [61].

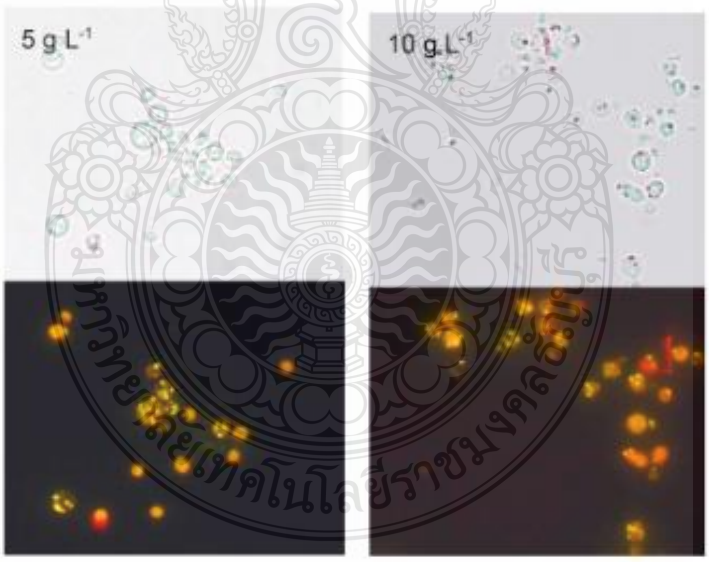


Figure 2.1 Oil droplets morphology in *R. toruloides* cultured on VFAs 5 and 10 g/L.

Source: Llamas *et al.* 2020 [62].

2.4 Carbon and nitrogen ratio in the growth medium for oil accumulation

Effective lipogenesis induction in oleaginous microorganisms depends on optimizing the carbon-to-nitrogen (C/N) ratio. Maintaining the appropriate C/N ratio in the fermentation medium is crucial for maximizing lipid production. In batch processes, the duration of the growth phase determines lipid conversion, which is influenced by the C/N ratio. Some oleaginous fungi exhibit an excess of carbon sources in the medium induces the overproduction of organic acids such as pyruvic acid, which can increase lipid accumulation [63]. For instance, in *Y. lipolytic*a, high initial C/N ratios (80–120 mol C/mol N) result in cell growth followed by citric acid production, thereby limiting lipid accumulation. In *Y. lipolytica* fermentations using glucose and glycerol with a C/N ratio of 62 (80 g/L of C and 3 g/L of N) after 48 h, the carbon-to-lipid conversion yield is approximately 20%, yielding about 16 g/L of lipids.

2.5 Triacylglycerol synthesis pathway in the oleaginous yeast

The synthesis of triacylglycerol (TAG) is initiated when yeast oil develops in a solution that is restricted in nitrogen but has an abundance of carbon. Carbon sources are used by oleaginous yeasts for growth, oil production, and TAG buildup. The accumulated oils provide survival energy, stress response, and cell development [64]. Two routes are involved in the synthesis of TAG: the Kennedy pathway, which leads from glycerol-3-phosphate to TAG, and the production of acyl-CoA (Figure 2.2). The glycolysis process in the cytosol transforms glucose into pyruvate. The pyruvate dehydrogenase complex converts pyruvate into acetyl-CoA once it has been transported to mitochondria. In order to feed the cell with nitrogen when nitrogen supplies are limited, adenosine monophosphate deaminase (AMPD) activity rises, AMP deteriorates, and cell levels of AMP fall [65]. Isocitrate dehydrogenase (*Idh*), the TCA cycle, and citrate retention in mitochondria are all inhibited by low AMP concentrations [66]. The malate/citrate translocase system transfers a significant amount of citrate from the mitochondria into the cytosol. Next, the ATP-citrate lyase converts the cytosolic citrate to oxaloacetate and acetyl-CoA [67]. Since pyruvate is converted into acetyl-CoA via pyruvate decarboxylase, acetyl-CoA synthetase, and acetaldehyde dehydrogenase, the synthesis mechanism of cytosolic acetyl-CoA differs from that of pyruvate dehydrogenase [68].

Acetyl-CoA carboxylase (*Acc1*) converts acetyl-CoA to malonyl-CoA in the cytosol [67] The FA synthase (*Fas1*, *Fas2*) complex then uses the acyl-CoA and malonyl-CoA molecules to synthesis FAs [69]. The Kennedy pathway is used to integrate C16 and C18 acyl-CoAs into lipids once they are produced in the endoplasmic reticulum [70].

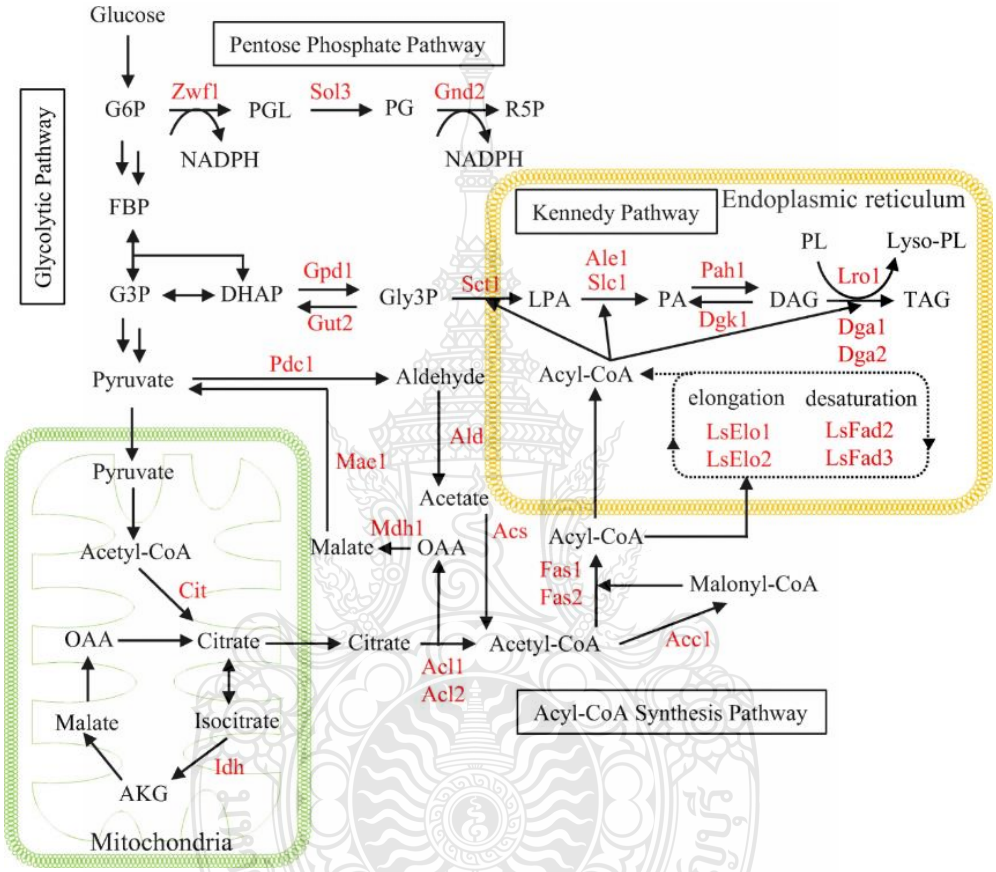


Figure 2.2 Acyl-CoA and TAG synthesis pathway in the oleaginous yeast.

Source: Takaku *et al.* 2020 [70].

2.6 Glycerol for microbial cultivation

Glycerol, a versatile chemical compound, finds applications in the pharmaceutical, biotechnology, cosmetics, and food industries. As a significant renewable feedstock, it serves as the primary by-product of the biodiesel industry, necessitating valorization to enhance the economic viability of biodiesel production.

Moreover, harnessing glycerol for value-added product formation can mitigate environmental challenges [71]. In recent years, the utilization of glycerol and its conversion to microbial biomass and products has been studied [72]. Serving as the structural backbone of TAGs, glycerol is chiefly derived from biodiesel production, making it a prime candidate for yeast lipid production. The integration of microbial lipid processes into biodiesel production plants, with surplus lipids converted into biodiesel, has been widely proposed [66-68]. However, the impurities contained in crude glycerol inhibit growth and product formation. The crude glycerol requires purification before being used as a carbon source in microbial cultivation. The bioconversion of glycerol to microbial or single cell oils (SCOs) has attracted much interest [73]. SCOs represent a significant alternative biofuel source. Traditional biofuels often face challenges such as a low benefit-to-cost ratio and scalability issues. However, microbial biofuels, including SCOs, offer a more environmentally and economically viable solution. They play a crucial role in addressing waste management issues across various industries. In particular, SCO production has the potential to enhance energy yield per hectare of land by an order of magnitude compared to traditional oil crop production [74]. in addition, there are also different strains of oleaginous yeast using glycerol as carbon source shown in Table 2.1 Notably, several strains of oleaginous yeast, including *Rhodotorula* species and *Yarrowia lipolytica*, efficiently utilize glycerol [90, 128, 133], making it a favored carbon source among researchers in the field (Figure 2.3). Past studies have demonstrated increased production of conjugated linoleic acid (CLA) and achieved lipid yields of approximately 0.20 g/g on crude glycerol [66,73] and 0.27 g/g on pure glycerol [47].

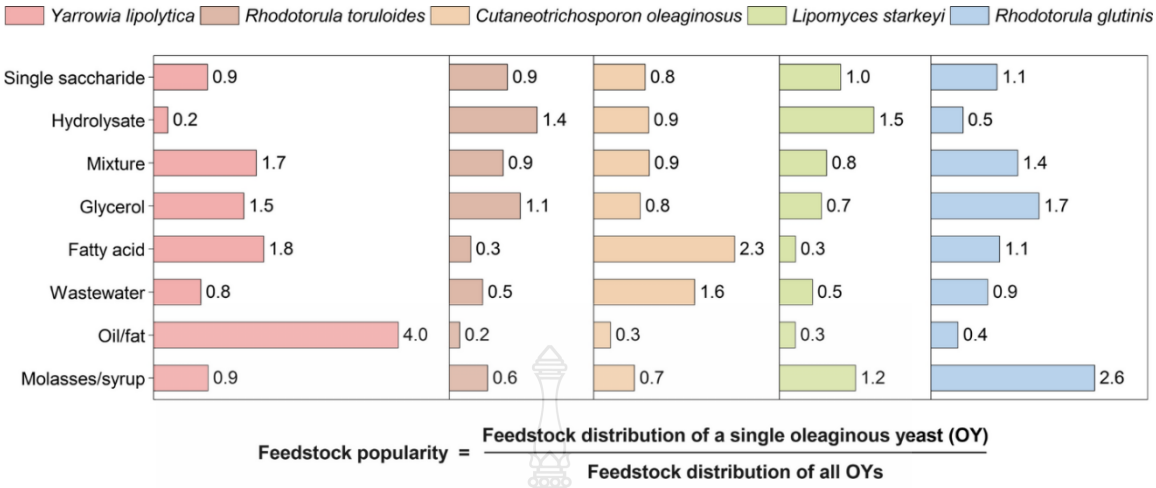


Figure 2.3 Feedstock popularity of the most prominent for oleaginous yeasts.

Source: Abeln *et al*. 2021 [32].

Table 2.1 The Different oleaginous yeasts using glycerol as carbon source as reported in literature.

Yeast strain	Carbon source	Total oil concentration (g/L)	References
R. toruloides 32489	Pure glycerol	5.18	(Gao *et al*., 2016) [77]
	Crude glycerol	6.20	
	Glucose	5.58	
R. toruloides ATCC10788	Crude glycerol	11.27	(Uprety *et al*., 2017) [67]
Rhodotorula minuta LOCKR19	Pure glycerol	1.82	(Gientka *et al*., 2017) [49]
Candida silvae DMKU-CPC19	Pure glycerol	1.2	(Polburee *et al*., 2015) [79]
Pichia manshurica DMKU-UbC9	Pure glycerol	0.5	(Polburee *et al*., 2015) [79]

2.7 Yeast oil production process

2.7.1 Oleaginous yeast cultivation

Oleaginous yeast has a remarkable ability to cultivate with abundant renewable or waste material in various fermentation forms. (e.g., glycerol, hemi-cellulose hydrolysates, whey permeate, sewage sludge, xylose-based materials, etc.) [81-83]. Cultivating oleaginous yeast during malnutrition can stimulate TAG accumulation in yeast cells [83]. Compared to fungi and algae, oleaginous yeasts have significantly higher specific growth rates [51]. As a result, oleaginous yeast can be regarded as a potential oil-producing strain for producing the second generation of oleochemical products [51].

2.7.2 Conventional yeast oil extraction

The extraction of yeast oil is an important step that depends on various factors. Conventional microbial oil recovery is carried out with cell hydrolyzation and solvent extraction. The oleaginous biomass is hydrolyzed by mechanical and chemical procedures. The released intracellular oils are extracted by organic solvent using chloroform and methanol [84]. The oil-dissolved solvent is evaporated, and yeast oils are collected. Even though this method is simple, reproducible, and broadly achievable [29,79]. However, it requires numerous physical and chemical steps and is impractical for large-scale production. Numerous methods for oil extraction have been documented, and the processes for various biological materials are applicable to microbial lipids with minor modifications. There are three main types of oil extraction procedures: Direct solvent extraction, solvent extraction and a wide range of solvent extraction techniques. The choice of extraction method significantly impacts oil recovery. Table 2.2 illustrates that yeast oil comprises both polar and non-polar components. Thus, suitable solvent systems (either individually or in combination) are used to ensure effective extraction. The diverse composition of yeast oil requires specific steps for total or specific lipid extraction [86].

Table 2.2 The facilitate extraction of yeast oil from various methods and solvent systems.

Method	Effect
Physical Methods	
Dry (freeze or thermal)	Inhibition of lipase activity, degradation of bonds connecting lipids to other polymers increases and sensitivity to lysis
Pressure extrusion	Cell liquid oscillation, impingement, cell wall disruption
Sonication	cell wall disruption
Solvent Systems	
Polar solvents (water)	Swelling of carbohydrate molecules, polar oil extraction
Polar solvents (alcohol)	Dehydration, hydrolysis of proteins, etc. Biopolymer Extraction of oils polar.
Nonpolar solvents	Non-polar oil extraction, hydrolysis, protein denaturation, Lipase stimulation

Source: Kyle *et al*. 2016 [86].

2.7.3 Releasing of intracellular oil

Surfactants are commonly categorized as ionic and non-ionic surfactants. Ionic surfactants are further classified as cationic, anionic, and zwitterionic types, while non-ionic surfactants are further classified as ether and ester types. Non-ionic surfactants consist of a hydrophilic head group and a hydrophobic tail. It is a non-ionic and relatively non-toxic used to prepare niosomes [87]. The hydrophobic tail of surfactants can be alkyl (T), fluoroalkyl, or natural steroids. In comparison, the hydrophilic head groups have a wide variety of available vesicle-forming surfactants, such as, glucosyl dialkyl ethers, polyglycerol alkyl ethers, ester-linked surfactants, crown ethers, Brij, polyoxyethylene alkyl ethers, Spans (sorbitan esters), and Tweens (polysorbates). They are used to prepare the generally regarded as safe (GRAS) category niosomes and are safe to use [88].

Various non-ionic surfactants are amphibious molecules adsorbed in the interface to reduce skin tension. Surfactant molecules have a hydrophobic part and a hydrophobic region. This property allows them to adsorb across the interface formed when mixing two phases of different polarity [89]. They especially permeabilize cell membranes when included in microbial culture media, which increases the secretion of intracellular metabolites and enzymes outside of cells [90]. Surfactants with an amphipathic molecular structure have been applied in biochemical product synthesis by enhancing electron transfer. The permeability of cell membranes has been modulated by the activity of key metabolic enzymes and other effects [43]. The non-ionic surfactants have higher surface activity, water resistance, and low foaming ability. It can increase the production rate of microbial metabolites and improve the product secretion of microorganisms [91].

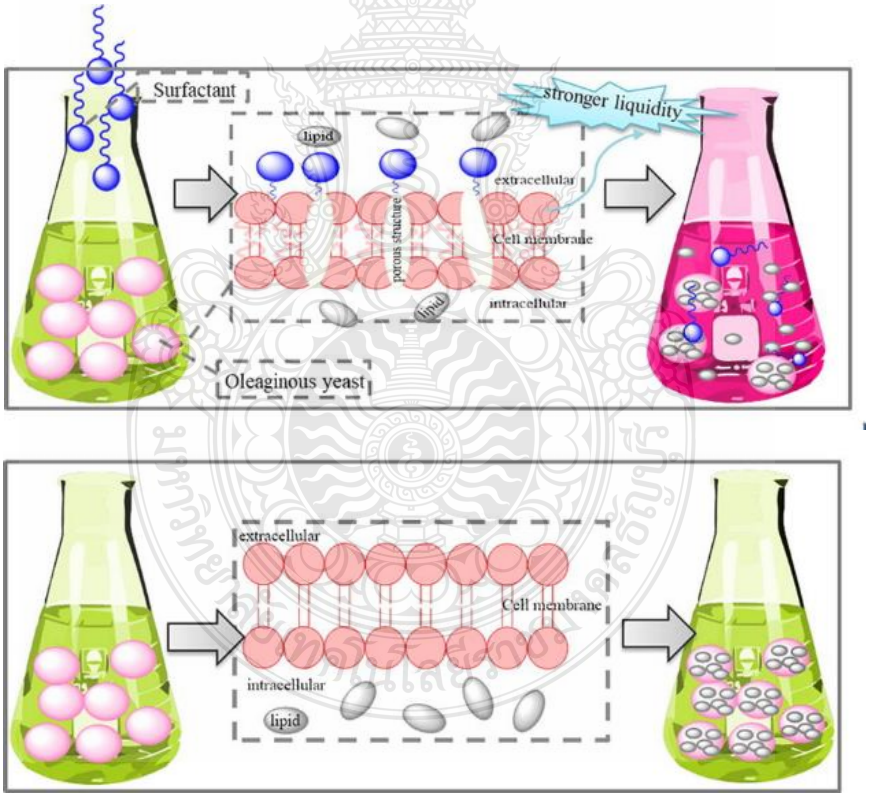


Figure 2.4 Mechanism of non-ionic surfactants.

Source: Huang *et al.* 2019 [91].

2.7.4 Oil capturing agents

The effect of OCAs on oil absorption has been reported in a previous study. The presence of OCA indicates the removal of attached oil droplets from the cell surface [91]. This evidence allows glucose uptake and ultimately redirects the flux to TAG synthesis. The OCA traps oils from the cell surface and cultivation medium. It is assumed that TAGs can be moved out of the cell. This mechanism facilitates higher and longer-lasting oil production in the stagnant cell phase. The addition of OCA into the fermentation cycle has demonstrated that continuous cell extraction positively influences oil yield by preventing and reducing fat re-utilization [92]. The main characteristic of oil capturing resin adsorption is the binding force between the adsorbent and the adsorbate, which is usually weaker than that found in activated carbon adsorption. In addition, the OCA can be regenerated with solvent to reuse in the next operation, causing it to have potential agents for recovering intracellular oils [93].

Table 2.3 Physical properties of XAD-16 and SP70

Properties	XAD-16	SP70
Matrix	Macroreticular polymer (Styrenic-DVB)	Ethylvinylbenzene-DV
Specific gravity	1.015 a 1.025	-
Harmonic mean size	0.56–0.71 mm.	-
Surface area (m^2/g)	≥800	870
Porosity	≥0.55 mL/mL	1.5 ml/g
Particle size (um)	560–710	850
Pore size (A°)	150	70
Pore volume (mL/g)	-	1.5
References	Dávila-Guzman *et al*. [94], Phillips *et al.* [95]	Pawar *et al*. [96]

Most widely used nonionic adsorbent resins are inherently hydrophobic due to their polystyrenic backbone crosslinked with divinylbenzene. They exhibit a strong affinity for aromatic and hydrophobic substances, binding these metabolites through π-π

bonding or hydrophobic interaction [96]. This polystyrenic scaffold has been modified over time to include different functions including hydroxyl, amino, and cyano groups, which have changed the binding selectivity. But most of these functionalized polystyrenic adsorbents are meant for chromatographic applications; their tiny particle sizes and high cost make it difficult to recover them from fermentation broth. Non-aromatic acrylic ester-based polymeric adsorbents such as XAD-7 (Rohm and Haas—Dow Chemical) and HP2MG (Mitsubishi) are frequently used as substitutes for attaching less hydrophilic metabolites. Adsorbent resins fluctuate in bead size, pore size, surface area, and density in addition to variations in polymer chemistry. The benefit of elution in organic solvents over aqueous elution buffers, which are employed with ion exchange resins, is another benefit of nonionic polymeric adsorbents. This facilitates easy evaporation of solvents to minimize processing volumes and seamless integration with subsequent isolation steps [95].

2.8 Petroleum-based lubricants and biolubricant

Petroleum-based lubricants are currently highly required. It is estimated that 30 to 40 million tonnes of lubricants are used annually, of which 20 million tonnes of used lubricants will be returned to the environment [97]. Petroleum-based lubricants are toxic and non-renewable. The continuous combustion and improper disposal of these products can impact the environment in the long term [98]. As a result, environmentally friendly alternative lubricants must be developed to meet future demands. The biolubricant made from vegetable oil is an innovative product with environmentally friendly and sustainable chemistry. Most vegetable oils are mono-, di, or triglycerides, composed of free FAs (long chains) and hydroxyl groups enamored by an ester bound [100,101]. This product is more valuable than petroleum-derived lubricants because it can be biodegradable and environmentally friendly, and it has a high viscosity index, excellent lubricity, and a high flash point [102-104]. As esters are present, biolubricants have a 30% to 80% viscosity higher than mineral oil. Biolubricants degrade 25–35% more rapidly than petroleum-based lubricants, which results in lower disposal costs and an effective economy [64]. Lubricants are classified as biodegradable if their degradation percentage in standard tests exceeds specified values. Vegetable oils are generally more biodegradable than mineral

oil and other products [104]. Base oil has a major impact on lubricant biodegradability [100,101], which is dependent on the molecular structure of organic components. When lubricants are applied, base oils undergo chemical composition changes due to exposure to air, temperature, metals, humidity, and pressure. Biodegradability is impacted by these modifications to chemical structures throughout service [104]. Vegetable oil compositions, however, continue to provide challenges for the creation of biolubricants, including low thermal stability, low oxidation stability, and limited performance at low temperatures [63,64]. Because of this, the manufacture of biolubricants necessitates a suitable chemical modification in order to suggest several industrial uses [64].

2.9 The standard of lubricants

Many standards, including those from the European Committee for Standardisation (CEN), the American Society for Testing and Materials (ASTM International), and others, control the qualities of lubricants. In order to monitor the chemical and physical characteristics of lubricants and assist establish biolubricants as a superior substitute, the properties of lubricants have been tested using the necessary instrumental procedures [109].

2.10 Compositions and properties of oils

The principle of lubricants is mineral substances. They have many different types of hydrocarbons with a typical average molecular weight between 300 and 600. Molecular structures of the lubricant are shown in Figure 2.3. The predominant structures are long-chain saturated hydrocarbons (paraffins) with straight or branch chains containing 15–30 carbon atoms and chained 5 or 6-membered saturated hydrocarbons (naphtenases). Aromatic components are also present in small proportions of lubricants. Additionally, it consists of one or more benzene rings with saturated side chains. Other large components may be present in smaller quantities [111].

The primary objectives of lubrication utilization include: (1) reducing wear and heat loss resulting from contact between moving surfaces, thereby minimizing the friction coefficient between two contact surfaces; (2) preventing rust and oxidation; (3) acting as insulation in transformer applications; and (4) serving as a seal to protect against dirt,

dust, and water. Lubricants function by reducing friction and wear through the provision of a protective film between two moving surfaces. This lubrication occurs when two surfaces are separated by a film of lubricant, which can be in liquid, solid, or gas form. High-quality lubricants exhibit specific characteristics such as a high viscosity index (VI), high boiling point, low thermal stability, freezing point, corrosion prevention ability, and high oxidation resistance [112]. Additionally, biolubricants offer several valuable physicochemical properties and technical advantages not commonly found in petroleum-based lubricants. These advantages include high flash point, high VI, a high lubricity and low evaporation losses[108-112].The production of biolubricants involves various routes, with the choice of method depending on factors e.g. hydrolytic and oxidative instability, lubricating. limitations at compatibility with paints and sealants and low temperatures [113-115]The most commonly employed processes in the literature involve use the catalysts is lipases , including esterification, transesterification, hydroesterification, ring-opening processes and sequential epoxidation [93,108-111]. The chemical process known as transesterification involves switching out the alkyl group of alcohol from one ester for another. Different alcohols, mostly polyols like TMP, PE, and NPG, replace the glycerol molecule in the structure of TAG during this process [116-121]. A single reaction step involving TAG and alcohol [123-125], or two consecutive transesterification processes can be used to produce biolubricants. The first step involves the conversion of TAG to methyl or ethyl ester, and the second step involves the interaction of these esters with various alcohols, including TMP [126,108,120,122,125,127]. Transesterification is a common method for generating biolubricants, as seen in Figure 2.4. This is mainly because TAG has a high viscosity, which might impede mass transfer during the reaction and cause problems during the purification stage [125].

Table 2.4 Specification of standard lubricating oil

Types of lubricating oil	Physical properties				
	Kinematic viscosity 40°C	(cSt) 100°C	Viscosity index	Pour point	Flash point
Engine oil	-	4.1-6.6	>90	<-5	>190
2 stoke oil	-	5.6-7.8	>95	<-5	>70
Automatic gear oil	-	13.5-15.5	>85	<-5	>200
Industrial gear oil	28.8-35.2	-	>90	<-10	>200
Hydraulic oil	9.0-11.0	2.5 Typical	>75	<12	>125
Turbine oil	28.8-35.2	5.0 Typical	>90	<-6	>160
Refrigerator compressor oil	28.8-35.2	5.7 Typical	>90	<-20	>200
Air compressor oil	28.8-35.2	5.6 Typical	>90	<-10	>200
Mineral base oil	29.0-31.0	-	>100	<-9	>204

Source: Inkerd *et al*. 2015 [110].

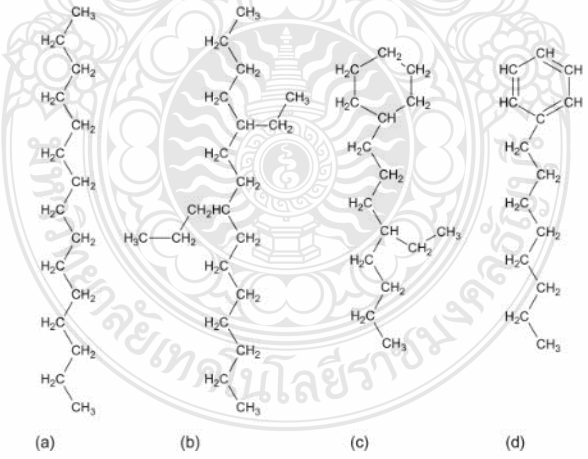


Figure 2.5 Examples of structures of some hydrocarbon moleules present in oils: (a) paraffin (alkane); (b) branched paraffin (alkane); (c) naphthenic; (d) aromatic.

Source: Hutchings *et al*. 2017 [111]

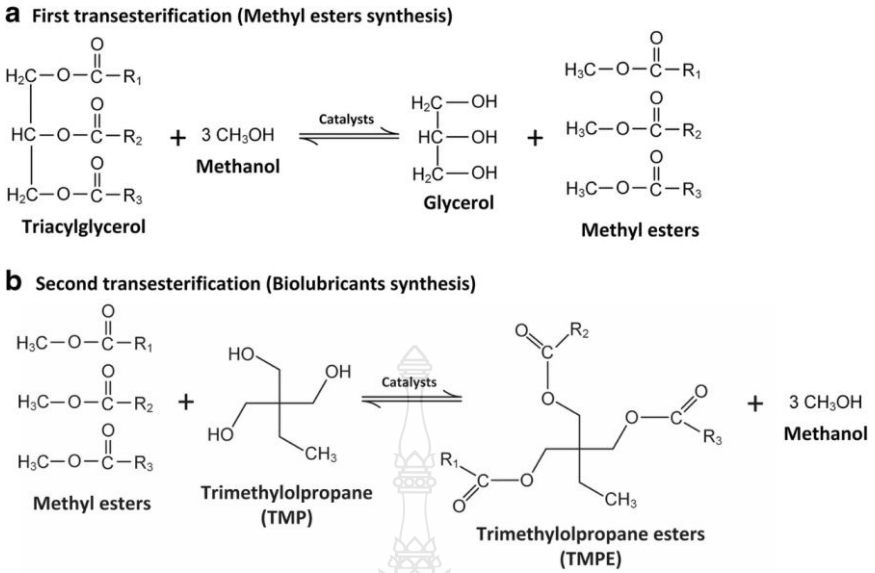


Figure 4.6 Representative scheme of biolubricant synthesis via a two-step transesterification reaction.
Source: Bolina *et al*. 2021 [112].

2.11 Reviews of the literature

Burgstaller *et al.* have studied the influence of different carbon sources on growth and SCO production in oleaginous yeasts *Apiotrichum brassicae* and *Pichia kudriavzevii.* The result found that both species can utilize all carbon sources for oil production. Moreover, the high volumetric lipid production capacity (0.4 g/Lh) and lipid content (68%) are particularly accessible with acetic acid as a carbon source [126].

Chebbi *et al*. investigated biodiesel production from microbial oil derived from yeast cultivated on industrial glycerol and wastewater from olive oil factories. The results demonstrated that oleaginous yeast can effectively utilize carbon sources for oil production. Specifically, *M. caribbica* MH267795 achieved a total dry weight of 42.12 g/L, with an oil content of 48.14% (w/w). Furthermore, the biodiesel derived from this oil exhibited a high fatty acid methyl ester content of 90.2% (w/w) [15].

Saran *et al*. examined the optimization of the culture process and oil accumulation in *R. toruloides* A29, an oleaginous yeast. This study recognized the importance of microbial lipids as a source of biodiesel and sought to assess the potential of yeast strains,

namely *R. toruloides* A29, for biodiesel generation. Utilizing statistical modelling techniques improved biomass yield, resulting in a lipid yield of 0.436 g/g of dry cell weight. Additionally, a higher lipid yield of 0.535 g/g CDW was obtained from the effective culture of *R. toruloides* A29 in a scaled-up 30-liter bioreactor. FAME (biodiesel) with a composition like that of biodiesel made from vegetable oil was synthesized by the transesterification of yeast oil. In order to address the increasing need for energy, the microbial lipid from *R. toruloides* A29 presents a viable alternative for sustainable biodiesel production, since the physical and chemical characteristics of the SCO transesterified fulfilled conventional biodiesel standards [30].

Pawar *et al*. investigated the simultaneous lipid production and recovery of *Y. lipolytica* oleaginous yeast. In this work, *Y. lipolytica* was cultured on a glucose-based medium for 380 h, and oil was simultaneously recovered *in-situ* from the fermentative production broths using OCA. Through the fluidization of the fermentation broth, the OCA made it easier to collect extracellular oil from both the medium and the cell surface. This method produced an oil yield equal to the expected oil yield over glucose, or 0.33 g/g of glucose eaten. Furthermore, the addition of OCA resulted in an 89% oil content and total substrate utilization. But it also resulted in an overall procedure utilizing OCA [92].

Patel *et al*. investigated the tribological performance of single-cell oils as green bio-lubricants. Analysis of oils produced by different oleaginous microorganisms, such as yeasts and microalgae, was part of the research. They were analyzed for wear, thermal stability, friction coefficient, viscosity, and fatty acid composition. They contain a large amount of monounsaturated fatty acids, specifically oleic acid (C18:1), yeast strains like *R. toruloides* and *C. curvatus* synthesized lipid contents of 56.42% and 52.66%, respectively. These strains proved to have outstanding physicochemical and tribological qualities. The oil obtained from these microorganisms showed promising anti-wear and friction-reducing capabilities that outperformed those of oil generated from thrastochytride. Because the balanced ratio of saturated and unsaturated fatty acids in microalgal and yeast oils reduced friction and improved protection against surface wear. On metallic surfaces, microalgal and yeast oils showed noticeably lower friction coefficients and less wear than conventional mineral-based lubricants like PEG 200 [127].

Papadaki *et al*. investigated developing bioprocesses to produce biolubricants using microbial oil that is produced by fermentation from waste materials in the confectionery business. The research found that two fungal strains and five yeast strains produced microbial oil when grown on crude hydrolysate from wheat milling and confectionary waste streams. In this two-step bioprocess, waste from the confectionery industry was first hydrolyzed enzymatically, and then crude enzymes were produced via solid-state fermentation. Based on the fatty acid content and fermentation efficiency for the manufacture of biolubricants, *R. toruloides* and *C. curvatus* were chosen for additional assessment. Trimethylolpropane (TMP) and neopentyl glycol (NPG) were used in a solvent-free method to esterify the free fatty acids obtained from the enzymatic hydrolysis of the oil recovered from the yeast. For *R. toruloides* and *C. curvatus* NPG esters, the maximum conversion yields were 88% and 82.7%, respectively [13].

Huang *et al.* reported using a non-ionic surfactant as an accelerator to increase extracellular lipid production by oleaginous yeast *Cryptococcus curvatus* MUCL 29819 with acetic acid as a carbon source. In a comparison of Brij 58 and Triton X-100, the result showed that Brij 58 could increase the total lipids, with a yield of up to 2.84 g/L (extracellular lipid up to 47%), and also increased the metabolic flow of acetic acid to lipid accumulation (maximum conversion of 0.54 g/g at 1.0 g/L) [91].

Tamano *et al.* have studied the high-efficiency extracellular release of free FAs from *Aspergillus oryzae* using non-ionic surfactants. The results showed that the free FA is more released at 80% when cultured in 1% (w/v) of Trion X-100. The other non-ionic surfactants in the same ether series as Brij 58, IEPAL CA-630, and Tergitol NP-40 stimulated a similar release of the FFA [90].

Lamas *et al.* studied the screening of five oleaginous yeast strains for lipid production using volatile FAs as the substrate. The result showed that the difference between the five strains could grow and provide biomass yields from VFAs between 0.22 and 0.37 g/g. The highest oil content in dry biomass was observed in *Cutaneotrichosporon curvatum* and *Cyberlindnera saturnus* (36.9 and 33.9% on dry biomass, respectively), corresponding to lipid yields from VFAs 0.11 and 0.13 g/g , respectively. The oleic, palmitic, and linoleic acids were the major FAs that account for

more than 70% of the FAs in all yeast fats. This composition was similar to general vegetable oil [62].

Maina *et al.* examined the microbial synthesis of oil from several carbon sources by recently discovered fruit-derived oleaginous yeasts. Their capacity to acquire a high intracellular microbial oil content (20–48% w/w of total dry weight) led to their selection. Isolate VV D4 had the greatest concentration of oleic acid at 62.7% (w/w) and the highest level of saturated FAs at 68.7% (w/w). Using fed-batch bioreactor cultures, the VV D4 generated a total dry weight of 40 g/L and a microbial oil content of 39% (w/w). International criteria are met by the essential characteristics of biodiesel derived from microbial oil, which are evaluated based on their FA content. The novel isolate VV D4's microbial oil may be utilized to make biodiesel [128].

Chmielarz *et al.* have demonstrated the microbial synthesis of lipids using oleaginous yeasts with crude glycerol and hemicellulosic hydrolysate. Twenty-seven examined strains with crude glycerol as a carbon source were found to be able to develop on the plating test. Specifically, strains of *Rhodotorula* could be grown in hemicellulosic and mixer crude glycerol. With *R. tuberculoides* in 10% on hemicellulosic medium mixed with 55g/L crude glycerol, the greatest biomass and lipid content were obtained at 19.4 g/L and 10.6 g/L, respectively. Furthermore, the FA content was akin to that of vegetable oils and other *R. toruloides* strains [72].

Louhasakul *et al.* studied on the direct transesterification of oleaginous yeast lipids into biodiesel. The study revealed that *Y. lipolytica*, an oleaginous yeast with an oil content exceeding 30%, was directly utilized in the transesterification process. A 2 L vigorously stirred tank reactor equipped with a balloon-whisk impeller was designed to simultaneously disrupt yeast cells and convert yeast lipids into biodiesel in a single reactor. The highest biodiesel yields of 94.99% and 80.91% were achieved using dried and wet yeast cells, respectively. The cell fraction remaining after the reaction contained 63.41% carbohydrates and 15.76% protein, indicating its potential as a nutrient source for further fermentation processes [12].

Ramadan *et al.* investigated the biodegradation of used lubricating and diesel oils by a novel yeast strain, *Candida viswanathii* KA-2011. The study aimed to isolate microorganisms capable of effectively decomposing lubricating oil. *C.viswanathii* KA-

2011 was isolated from used lubricating oil and demonstrated efficient degradation of lubricating oil and diesel fuel (58.6% and 93.9%, respectively) at a salt concentration of 6% within just four days. This strain holds promise for the removal of lubricants, diesel, or vegetable oil pollution from soil, wastewater, and seawater. Thus, *C. viswanathii* KA-2011 could be utilized in the biological treatment of gasoline-contaminated lubricating oil or seawater, mitigating pollution in aquaculture. This presents new possibilities for utilizing contaminated soil and wastewater in agriculture [129].

Ferreira-Dias *et al.* examined the removal of certain chemicals from olive residual oil miscella using different adsorbents. All resins tested (Amberlite XAD-4, XAD-7, and XAD-16) produced consistent findings. With each sorbent, the removal of carotenoids and green pigments like pheophytin and chlorophyll was successful. Contrary to the 20% removal of conjugated hydroperoxide (HP) obtained with resin, activated carbon and soil showed a 50–60% reduction of HP. However, a 60% removal rate was also noted for the final oxidation product (FOP) using 20% resin, 20% carbon powder, and 30% clay. Furthermore, carbon powder or activated carbon eliminated 20% of the free fatty acids (FFA), whereas resin eliminated 40%. Subsequent application of the corresponding sorbents (clay or carbon, then XAD-7 resin) allowed the leftover FFA, HP, and FOP [130].

CHAPTER 3
MATERIALS AND METHODS

3.1 Chemicals, Apparatus and Equipments

	Company / Brand
3.1.1 2-Mercaptoethanol	Himedia
3.1.2 96-well microplate	THERMO SCIENTIFIC
3.1.3 Acetylacetone	SIGMA ALDRICH
3.1.4 Agar	Himedia
3.1.5 Aluminium foil	Renolds metal
3.1.6 Amberlite™ XAD16	SIGMA ALDRICH
3.1.7 Ammonium sulfate ($(NH_4)_2SO_4$)	UNIVAR
3.1.8 Analytical balance	OHAUS
3.1.9 Autoclave	N-BIOTEC/NB-1080
3.1.10 Beaker	SCHOOT
3.1.11 Bioreactor	MS Major Science
3.1.12 Biosafety cabinet (BSC)	ESCO
3.1.13 Brij 58	SIGMA ALDRICH
3.1.14 Calcium chloride dehydrate ($CaCl_2 \cdot 2H_2O$)	UNIVAR
3.1.15 Centrifuge	SIGMA/2-16PK
3.1.16 entrifuge tube	Nalgene
3.1.17 Chloroform	RCI Labscan
3.1.18 Cylinder	Isolab
3.1.19 Disposable syringe	Nipro
3.1.20 Duran bottle	SCHOOT
3.1.21 Erlenmeyer flask	PYREX
3.1.22 Ethanol	KEMAUS
3.1.23 Filter tip	SARSTEDT
3.1.24 Gas chromatography (GC-MS-3.1.1.18QP2010 SE)	Bara scientific
3.1.25 Glass column	

	Company / Brand
3.1.26 Glycerol	SRL
3.1.27 Glucose ($C_6H_{12}O_6$)	SRL
3.1.28 Heptadecanoic acid (C17:0)	MERCK
3.1.29 Hot air oven	Binder
3.1.30 Hot plate stirrers	THERMO SCIENTIFIC
3.1.31 Hydrochloric acid (HCl)	QRëC
3.1.32 Incubator and shaker	N-BIOTEK/NB-205V
3.1.33 Iron (III) chloride RPE ($FeCl_3$)	CARLO ERBA Reagents
3.1.34 Light microscope	Olympus
3.1.35 Magnesium sulfate heptahydrate ($MgSO_4 \cdot 7H_2O$)	UNIVAR
3.1.36 Malt extract powder	SRL
3.1.37 Methanol (CH_3OH) AR Grade	KEMAUS
3.1.38 Methanol (CH_3OH) HPLC grade	RCI Labscan
3.1.39 Microcentrifuge	JSR
3.1.40 Micropipette	Gilson
3.1.41 Microplate reader	BMG LABTECH
3.1.42 Microscope slide and cover glass	
3.1.43 Microtube	Nalgene
3.1.44 nanodrop spectrophotometer	Eppendorf
3.1.45 Peptone	SRL
3.1.46 Plastic plate	Hycon
3.1.47 Potassium carbonate (K_2CO_3)	Ajax Finechem
3.1.48 Potassium dihydrogen phosphate (KH_2PO_4)	SRL
3.1.49 Potassium hydroxide (KOH)	MERCK
3.1.50 PrimeScript RT reagent Kit	PCR Biosystems
3.1.51 propan-2-ol	KEMAUS
3.1.52 qPCRBIO SyGreen Mix Lo-ROX	PCR Biosystems
3.1.53 RNA purification kit	OMNI International

	Company / Brand
3.1.54 Vacuum rotary evaporator	BUCHI
3.1.55 RT-PCR machine	PCR max
3.1.56 Silicone oil	KEMAUS
3.1.57 Silicone tube	Dura
3.1.58 Septa,Red PTFE/Wgite Silicone	National scientific
3.1.59 di-Sodium hydrogen phosphate dodecahydrate ($Na_2HPO_4 \cdot 12H_2O$)	KEMAUS
3.1.60 Spectrophotometer	SHIMADZU/UV-1800
3.1.61 SEPABEADS™ SP70	THERMO SCIENTIFIC
3.1.62 sodium chloride	KEMAUS
3.1.63 sodium periodate	Ajax Finechem
3.1.64 sulfuric acid (H_2SO_4)	QRëC
3.1.65 Syringe Filters PVDF	nationalscientific
3.1.66 Test tube	PYREX
3.1.67 Triton X-100	ALFA AESAR
3.1.68 Trimethylolpropane (TMP)	TCI
3.1.69 Tween 20	THERMO SCIENTIFIC
3.1.70 Ultrapure distilled water	Invitrogen
3.1.71 Volumetric flask	PYREX
3.1.72 Vortex mixture	Scientific Industries/G560E
3.1.73 Water bath	FALC
3.1.74 Yeast extract	IYEAST
3.1.75 Zinc sulfate heptahydrate ($ZnSO_4 \cdot 7H_2O$)	Ajax Finechem

3.2 Methods

3.2.1 Yeast strains and cultivation

The following seven oleaginous yeast strains were cultured on YM agar: 20 g/L agar, 5 g/L peptone, 10 g/L glucose, 3 g/L malt extract, and 3 g/L yeast extract. The strains were *C. albidus* TISTR 5103, *P. parantarctica* CHC28, *R. toruloides* TISTR 5186, *Y. lipolytica* TISTR 5212, *R. glutinis* DB-RMUTT01, *R. glutinis* TISTR 5159, and *R. glutinis* DB-RMUTT02 were grown on YM agar. Then, 30 mL of YM broth were added to one loopful of yeast from each strain, and the mixture was shaken at 200 rpm for an additional 24 h at 30 °C in a 250 mL Erlenmeyer flask. Next, 0.8 mL of the culture and 0.2 mL of 75% v/v sterile glycerol were aseptically combined in a 2 mL microtube. This mixture was used as the master stock culture for the remaining tests, and it was kept at -80 °C.

In order to prepare the starter, 1 mL of the master yeast stock was added to 30 mL of new YM broth, and the mixture was aerobically incubated for 24 h at 30 °C at 200 rpm. Subsequently, the same conditions were used for the re-inoculation of 0.5 mL of active yeast into 30 mL of new YM broth. This culture served as the experimental starter for the study.

3.2.2 Growth and YO production by glycerol-based medium

Fifteen milliliters of yeast starter were transferred to 135 mL of glycerol-based oil production medium (G-OPM) (composition per liter: 0.15 g $CaCl_2 \cdot 2H_2O$, 1 g $(NH_4)_2SO_4$, 50 g glycerol, 6.3 g $Na_2HPO_4 \cdot 12H_2O$, 1.5 g $MgSO_4 \cdot 7H_2O$, 0.09 g $FeCl_3$, 7 g KH_2PO_4, 0.02 g $ZnSO_4 \cdot 7H_2O$, and 0.5 g yeast extract) and cultured under the previous condition [29]. The concentrations of IO, glycerol consumption, and biomass were measured by periodically withdrawing samples. Triple testing was used for every experiment. The yield of biomass ($Y_{X/S}$) and IO ($Y_{Pi/S}$) as well as the oil content were calculated to determine the YO production efficiency. The strain of yeast that produced the highest amount of oil was selected to be used in the subsequent experiments.

The efficiency of YO production was described by the fermentation parameters, namely $Y_{X/S}$, $Y_{Pi/S}$, and oil content as Eqs. (1) to (3), respectively:

$$Y_{X/S} = \frac{X}{\Delta S} \quad (1)$$

$$Y_{Pi/S} = \frac{P\mathrm{i}}{\Delta S} \quad (2)$$

$$\text{Oil content (\% w/w)} = \frac{P}{X} \times 100 \quad (3)$$

where X, Pi, ΔS, and P are biomass (g/L), intracellular YO (g/L), consumed substrate (g/L), and YO (g/L), respectively.

3.2.3 Effect of non-ionic surfactant on the release of extracellular yeast oil

The effect of non-ionic surfactants on extracellular YO release was examined. Following the above-described inoculation procedure, a 500 mL flask holding 135 mL of G-OPM media was incubated with a 15 mL aliquot of *R. toruloides* TISTR 5186 starter culture. Non-ionic surfactants (Triton X-100 (Alfa Aesar, USA), Tween 20 (Thermo Scientific, USA), and Brij 58 (Sigma-Aldrich, USA)) were added to the culture at different doses ranging from 0.1 to 1% w/v during the middle-exponential growth phase (at 72 h). Samples were obtained at various points during the culture to quantify biomass, intracellular and extracellular oils, and residual glycerol. Every experiment was carried out three times. The results of earlier studies were used to assess YO production [46]. The performance of non-ionic surfactants was expressed as the yield of total YO (TO) as Eq. (4):

$$Y_{Pt/S} = \frac{P\mathrm{t}}{\Delta S} \quad (4)$$

where $Y_{Pt/S}$ and P_t are the yield of TO and TO concentration (g/L), respectively.

3.2.4 Simultaneous oil biosynthesis and recovery of *R. toruloides* TISTR 5186 with oil capturing agent (OCA)

3.2.4.1 *In-situ* oil capturing mode

The 15 mL of *R. toruloides* TISTR 5186 starter was inoculated into 135 mL of G-OPM in 500 mL flask. The fermentation medium was incubated under the same conditions as seed cultivation. The Tween 20 was then added to the culture for 0.7% v/v

at 72 h (mid-exponential growth phase). The OCA was regenerated and sterilized by propanol-2-ol. The regenerated OCA Amberlite™ XAD-16 (Sigma Aldrich, USA) and SEPABEADS™ SP70 (Thermo Scientific, USA) were added together with Tween 20 by varying the concentrations (0.5 to 2.0% w/v) [92]. The sample was collected throughout cultivation, and fermented parameters were measured following the above experiment.

3.2.4.2 *Ex-situ* oil capturing mode

The 15 mL of *R. toruloides* TISTR 5186 starter was inoculated into 135 mL of G-OPM in 500 mL flask. The culture was incubated under the same conditions as seed cultivation. Tween 20 was added to the culture for 0.7% v/v at 72 h (mid-exponential growth phase). At the end of fermentation, SP70 was added to the cultured medium and then shaken at 200 rpm for 1 to 3 days. Then, the cultured medium was separated from OCA by filtration with clean gauze. The OCA-free cultured medium determined the biomass, IO, and EO according to the analytical method. The oil adsorbed by OCA was extracted with a methanol-chloroform mixture and expressed as adsorbed oil (AO) [92].

3.2.4.3 Scaling up of simultaneous oil biosynthesis and *in-situ* recovery by batch fermentation in 5-L bioreactor

Scaling up simultaneous oil biosynthesis and in-situ recovery was operated using a batch process. The 1.8 L of oil production medium contained in a 5-L bioreactor was inoculated with 10% v/v of yeast starter. The cultivation was carried out at 30ºC, 200 rpm agitation rate, and 1.0 vvm of aeration. At 72 h of cultivation, Tween 20 (0.7% v/v) and SP70 (2% w/v) were added to the cultured medium. The sample was withdrawn throughout fermentation to determine the growth of oleaginous yeast, glycerol consumption, and oil production performance.

3.2.5 Simultaneous oil biosynthesis and recovery by online oil capturing in 5-L bioreactor

3.2.5.1 Batch fermentation with online oil capturing mode

The yeast oil production was operated by batch process in 5-L bioreactor following the previous experiment. The glass column (20 cm height × 2.5 cm internal diameter) was packed with 40 g of activated and sterilized SP70 and connected to the bioreactor (Figure 4.14). The SP70 was activated by propan-2-ol and sterilized with

sterile water at least three times [92]. The cultured medium was continuously fluidized through the bottom of the SP70 column and recirculated to bioreactor by a peristaltic pump at 10 mL/min. The SP70 column was removed and replaced with a new column every 3 days. The sample was collected to determine the yeast growth, glycerol consumption, and oil production performance.

3.2.5.2 Fed-batch fermentation with online oil capturing mode

The YO production and *in-situ* oil recovery were initiated by batch mode following the abovementioned process. The G-OPM mixed with Tween 20 (0.7% v/v) was added to the bioreactor every 6 days, and The SP70 column was replaced every 3 days until yeast growth and oil production were diminished.

3.2.6 *ACC* and *DGA* gene expression

This study examined the expression of the *ACC* and *DGA* genes linked to oleaginous yeast oil production using the reverse transcription-quantitative polymerase chain reaction (RT-qPCR) technique with relative quantification. The testing conditions involved centrifuging the yeast cultures for 10 minutes at 4 °C and 10,000 rpm (9,168 × *g*). A nanodrop spectrophotometer (Kinetic, BioSpectrometer, Eppendorf, USA) was used to measure the quantity and quality of the total RNA extracted from yeast cells using the bead mill yeast RNA purification kit (26-012B Yeast RNA Purification Kit, OMNI International, USA) in compliance with the manufacturer's instructions. From the total RNA, the complementary DNA was synthesized using the PrimeScript RT reagent kit (qPCRBIO cDNA Synthesis Kit, PCR Biosystems, USA). Table 3.1 displays the primers that were utilized in the real-time PCR. Using an RT-PCR equipment (Eco 48 Real-time PCR, PCR max, USA) and qPCRBIO SyGreen Mix Lo-ROX (2XqPCRBIO SyGreen Mix Lo-ROX, PCR Biosystems, USA), the expression of the *ACC* and *DGA* genes was measured. The sample was first denaturated for two minutes at 95 °C in order to achieve the ideal temperature profile for the real-time PCR experiment. 45 cycles of 15 s at 95 °C and 30 s at 60 °C came next. Version 5.2.12 of the Eco programme was utilized to determine the threshold cycles (Ct). The transcription levels of the *ACC* and *DGA* genes in *R. toruloides* TISTR 5186 treated with non-ionic surfactant were measured using the comparative computerized tomography technique [55]. The internal housekeeping gene,

18s rRNA, functioned as the internal control for each treatment [56]. Each data point represents the average of three trials, and the Ct values from triplicate reactions were averaged for each set. The $2^{(-\Delta\Delta Ct)}$ method was utilized to calculate the normalized transcription levels [55].

Table 3.1 Primer sequences used for real-time PCR.

Gene	Primer	Sequence
ACC	ACC_FP	5'–CGG GTC GTA AAG TAC CTC GG–3'
	ACC_RP	5'–CTG CGG CGA TAC CAT TGT TG–3'
DGA	DGA_FP	5'–GCC TGA CCG GAA GTA CGT CTT–3'
	DGA_RP	5'–TAG AGC GGG AGC TTG AAG TTG–3'
18s rRNA	18s rRNA_FP	5'–GAG CCT TAC CTC CTG GTG AAC A–3'
	18s rRNA_RP	5'–CGG CGA TCT CAG AAA CCA AC–3'

3.2.7 Preliminary bio-lubricant production from yeast oil

The preliminary biolubricant production was investigated by modifying the method of Mehdi *et al.* [48] consists of two main steps. Firstly, esterification synthesizes fatty acid methyl esters (FAMEs) from free fatty acids (FFAs). The FFAs were reacted with methanol using 5% w/w H_2SO_4 as an acid catalyst until the FFAs were decreased to less than 1%. The FFA to methanol molar ratio of 1:3. The esterification was conducted at 65°C for 1 h. The residual H_2O was then evaporated from the mixture to avoid adverse effects. Subsequently, the triglyceride in yeast oils was transferred to FAMEs using 2% w/w KOH as an alkaline catalyst. The obtained FAMEs were cleaned with H_2O. Secondly, the FAMEs were reacted with trimethylolpropane (TMP) to generate trimethylolpropane fatty acid triester (TFATE). Fifty grams of FAMEs were transferred to a round bottle connected with a condenser and thermometer. The FAMEs were warmed at 60°C, and TMP (molar ratio of 5:3) was added and gently mixed until completely dissolved. Potassium carbonate (2% by weight of FAMEs) was then added to the mixture, heated to 130°C with a vacuum pressure of 425 mmHg, and cooled. The chemical structure of biolubricant was analyzed by nuclear magnetic resonance (NMR) spectrometry at 500 MHz frequency [49].

3.2.8 Mathematical modeling

The microbial cells changed throughout the biological fermentation process. Yeast growth rate ($\frac{dX}{dt}$) was described according to a dynamic logistic growth model, while product inhibitory effect was associated with the growth rate model [59-61] as Eq. (5):

$$\frac{dX}{dt} = \mu_{max} X\left(1 - \frac{X}{X_{max}}\right)\left(1 - \frac{P_i}{P_i^*}\right)^n \quad (5)$$

where μ_{max} is the maximum specific growth rate (h^{-1}), X and X_{max} are the biomass and the maximum biomass concentration (g/L), P_i and P_i* are the intracellular product concentration (g/L) and critical intracellular product-inhibition concentration (g/L), n is a power constant of product inhibition (dimensionless), and t is the cultivation time (h). In Eq. (5), μ_{max}, X_{max}, P_i*, and n are model constants.

The substrate consumption rate ($\frac{dS}{dt}$) is related to cell growth and oil formation, with the kinetic model for glycerol consumption established [62] as Eq (6):

$$-\frac{dS}{dt} = \frac{1}{Y_{X/S}}\left(\frac{dX}{dt}\right) + m_S X \quad (6)$$

where $Y_{X/S}$ is the biomass yield on the consumed substrate and m_S is the biomass maintenance coefficient (g/g h).

The IO production rate ($\frac{dP_i}{dt}$) depends on the yeast growth and can be described by the Luedeking-Piret equation [59], as Eq. (7):

$$\frac{dP_i}{dt} = \alpha\frac{dX}{dt} + \beta X \quad (7)$$

where P_i is the IO yield on the consumed substrate, and α and β are growth-associated (g/g) and non-growth-associated production coefficients (g/g h).

The kinetic parameters of the mathematical models (i.e., μ_{max}, X_{max}, P_i*, $Y_{X/S}$, m_S, α, and β) were defined as the required values to best fit the model (Eqs. (5) – (7)) to the experimental data by Berkeley Madonna™ software version 8.3.18 [63].

The proportion of the dependent variable estimated from the independent variable is commonly determined using the coefficient of determination (R^2) [131], [132], and defined as Eq. (8):

$$R^2 = \frac{\sum_{i=1}^{N} (q_{cal} - \bar{q}_{exp})^2}{\sum_{i=1}^{N} (q_{cal} - \bar{q}_{exp})^2 + \sum_{i=1}^{N} (q_{cal} - q_{exp})^2} \quad (8)$$

where q_{cal} is the calculated data of an examined variable achieved from the kinetic model, and q_{exp} and $\bar{q}_{exp}$ are the experimental data and the average of all the experimental data, respectively. The non-normalized root mean square ($NRMS$) was employed to evaluate the actual error between the calculated experimental data at all points [64] as Eq. (9):

$$NRMS = \sqrt{\frac{\sum_{i=1}^{N} \left(q_{cal} - q_{exp}\right)^2}{N}} \quad (9)$$

where N is the number of paired data.

3.2.9 Kinetic modeling of microbial oil production

3.2.9.1 Yeast growth

The yeast growth rate ($\frac{dX}{dt}$) is described according to the logistic growth model [133] in Eq. (10):

$$\frac{dX}{dt} = \mu_{max} X \left(\frac{X - X_{max}}{X_{max}}\right) \quad (10)$$

where μ_{max} is the maximum specific growth rate (h^{-1}); X and X_{max} are the biomass and the maximum biomass concentration (g/L); S is the substrate concentration (g/L), and t is the cultivation time (h). In Eq. (10), μ_{max} and X_{max} are the model constants.

3.2.9.2 Substrate consumption

The substrate consumption rate ($\frac{dS}{dt}$) is importantly related to cell growth and product formation. The kinetic model for glycerol consumption is established [134] as Eq (11):

$$\frac{dS}{dt} = \frac{1}{Y_{x/s}}\left(\frac{dX}{dt}\right) + m_s X \qquad (11)$$

where $Y_{x/s}$ is the biomass yield on the consumed substrate, and m_s is the biomass maintenance coefficient (g/g h).

3.2.9.3 Product formation

The yeast oil production rate ($\frac{dP}{dt}$) which depends on the yeast growth is described by Luedeking–Piret equation [133], as Eqs. (12) and (13):

$$\frac{dP}{dt} = \alpha\frac{dX}{dt} + \beta X \qquad (12)$$

$$Y_{p/s} = \alpha Y_{x/s} \qquad (13)$$

In Eq. (12), a growth-related oil formation coefficient is denoted by α, whereas β is not connected with growth. Oil generation in yeast is directly correlated with yeast growth if $\alpha \neq 0$ and $\beta = 0$; correlated with yeast growth only if $\alpha \neq 0$ and $\beta \neq 0$; and uncorrelated with yeast growth if $\alpha = 0$ and $\beta \neq 0$. $Y_{p/s}$ is the yeast oil yield on the consumed substrate in Eq. (13).

The kinetic parameters of the mathematical models (i.e., μ_{max}, X_{max}, $Y_{x/s}$, m_s, α, and β) are defined as the required values to fit the model best (Eqs. (10)–(13)) to the experimental data. The data fitting is achieved using the Berkeley Madonna™ software version 8.3.18 (Berkeley Madonna Inc., USA).

3.2.10 Analysis

After harvesting wet yeast biomass from the culture solution, the sample was centrifuged twice with sterile deionized water (Sigma 3-18KS, Sartorius, Germany) at 4

°C and 10,000 rpm (9,168 × *g*) for 10 minutes. After being cleaned, wet cell pellets were dried at 80 °C to a consistent weight. Using gravimetric techniques, the biomass was quantified and represented as grammes of biomass per litre of sample.

Glycerol contained in cell-free suspension was analyzed by using colorific spectrophotometry, following the modified method of Bondioli and Della Bella [57], as described by Rakkitkanphun *et al.* [29]. A 0.5 mL portion of the cell-free supernatant was mixed with 0.6 mL of sodium periodate solution (10 mM) and 0.5 mL of 95% ethanol. After that, the mixture was heated in a water bath for one minute at 70°C while being manually stirred and treated with 0.6 mL of a 0.2 M acetylacetone solution. After heating, the solution was rapidly cooled, and a microplate reader (SPECTROstar Nano, BMG LABTECH, Germany) was used to measure the absorbance at 410 nm. The glycerol standard curve was used to calculate the glycerol concentration.

YO was found to be IO and EO. Yeast biomass was separated from the G-OPM medium by centrifugation at 4 °C and 10,000 rpm for 10 min. The material was then twice cleaned with sterile deionized water for the IO measurement. After cleaning, the yeast biomass was digested for 2 h at 60°C using 4 N HCl. A combination of methanol and chloroform was used to extract the cell-released IO, use an altered version of Bligh and Dyer's technique [58]. After being collected, the IO-rich organic solvent fraction was evaporated until all of the solvent was gone. After 24 h, the solvent-free IO was dried at 70 °C to eliminate moisture, and its weight remained constant. The amount of IO present in the medium was measured in grams per liter.

Liquid-liquid extraction was used to quantify the EO liberated from the yeast biomass and accumulated in the production medium. The cell-free supernatant was supplemented with an equal volume of methanol and chloroform solution in a 1:1 ratio. The dissolved substance in the top aqueous phase was separated and the oil-rich solvent in the bottom phase was transferred to a fresh test tube. To extract the remaining oil, chloroform was added again to the cell-free supernatant. The test tube containing all of the oil-rich aliquots was combined. The gravimetric measurement was finished after the solvent had evaporated and the oil-filled test tube had dried to a constant weight. The amount of EO in each litre of the culture medium was measured in grammes. The level

of IO and EO helped to clarify how non-ionic surfactants affected the yeast strain's overall oil-producing potential.

The AO on SP70 was recovered by elution of methanol and chloroform mix solution followed by pure chloroform. The oil-contained organic solvent phase was evaporated to obtain AO. The AO was dried and quantified gravimetrically [135].

The procedure detailed by Rakkitkanphun *et al.* [29] was used to convert the FAs given in both IO and EO to fatty acid methyl ester (FAME). The temperature programme was run using an InertCap Wax capillary column (0.25 mm I.D. × 30 m; GL Science Inc., Japan). The split ratio employed was 1:80, and the injection temperature was set at 250 °C. Helium was used as the carrier gas, and its flow rate was 1 mL/min.

3.2.11 Statistical analysis

One-way ANOVA with Duncan's multiple range test was used to investigate significant differences between the experimental values using SPSS 15.0 software (SPSS Inc., USA) with a confidence interval of 95% (p-value ≤ .05).

3.3 Experimental place

This study was performed at Biotechnology Laboratory, Division of Biology, Faculty of Science and Technology, Rajamangala University of Technology Thanyaburi, Thanyaburi, Pathum Thani, Thailand.

CHAPTER 4
RESULTS AND DISCUSSION

4.1 Performance of yeast strains on oil production in glycerol-based medium

The IO production performance of yeast strains in G-OPM was investigated in an orbital shaking flask, with results illustrated in Figure 4.1. From the start of cultivation in G-OPM, all strains of yeast exhibited rapidly propagation without any observable lag phase (Figure 4.1A). After 96 h of incubation, the biomass of *R. toruloides* TISTR 5186, *R. glutinis* TISTR 5159, *R. glutinis* DB-RMUTT01, and *R. glutinis* DB-RMUTT02 reached the stationary phase. On the other hand, at 120 h, the growth of *Y. lipolytica* TISTR 5212, *C. albidus* TISTR 5103, and *P. parantarctica* CHC28 entered the stationary phase. Table 4.1 shows that of these strains, *Y. lipolytica* TISTR 5212 had the lowest biomass at 8.94 ± 0.22 g/L, and *R. glutinis* TISTR 5159 had the highest biomass at 15.87 ± 0.15 g/L. Different yeast strains had different profiles of IO generation during cultivation (Figure 4.1 B). While *Y. lipolytica* TISTR 5212 and *C. albidus* TISTR 5103 produced less oil, *R. toruloides* TISTR 5186 produced the most at 4.00 ± 0.07 g/L at 144 h.

Signori *et al.* examined into the synthesis of oil using three different oleaginous yeasts (*R. toruloides*, *Lipomyces starkeyi*, and *C. curvatus*) and glycerol as a carbon source. The strains under investigation were shown to have the highest oil content, the fastest rate of glycerol consumption in *L. starkeyi*, and the maximum oil productivity in *R. toruloides* [136]. Under optimal conditions, oil yield and content at 7.5 g/L and 35.7% w/w were achieved using crude glycerol at 70 g/L as a carbon source for oil production by *L. starkeyi* AS 2.1560 [43]. Furthermore, 4.07 g/L of YO with an oil concentration of 45% w/w was produced by growing *P. parantarctica* in batch fermentation using purified crude glycerol generated from biodiesel [137]. Poli *et al.* studied utilizing crude glycerol as a substrate for oil production and brewery waste to enhance *Y. lipolytica* QU21's production of linolenic acid helped to alleviate the environmental effects of waste disposal [138]. Crude glycerol generated from biodiesel has also been studied as a potential renewable fuel for *Candida viswanathii* Y-E4 to produce microbial oil. At 51.9% w/w, this strain produced more oil than other strains [139]. Similarly, Pulburee et

al. used a two-stage cultivation method to create microbial oil by *Rhodosporidiobolus fluvialis* DMKU-RK253 using inexpensive raw materials such as monosodium glutamate and crude glycerol. High oil output and content were produced under ideal circumstances, indicating the possibility of growing yeast to make inexpensive oils [140]. The results suggested that lipogenic yeast could successfully utilize glycerol as its main carbon source for producing YO. However, the examined species and growing method affect how well YO formation performs.

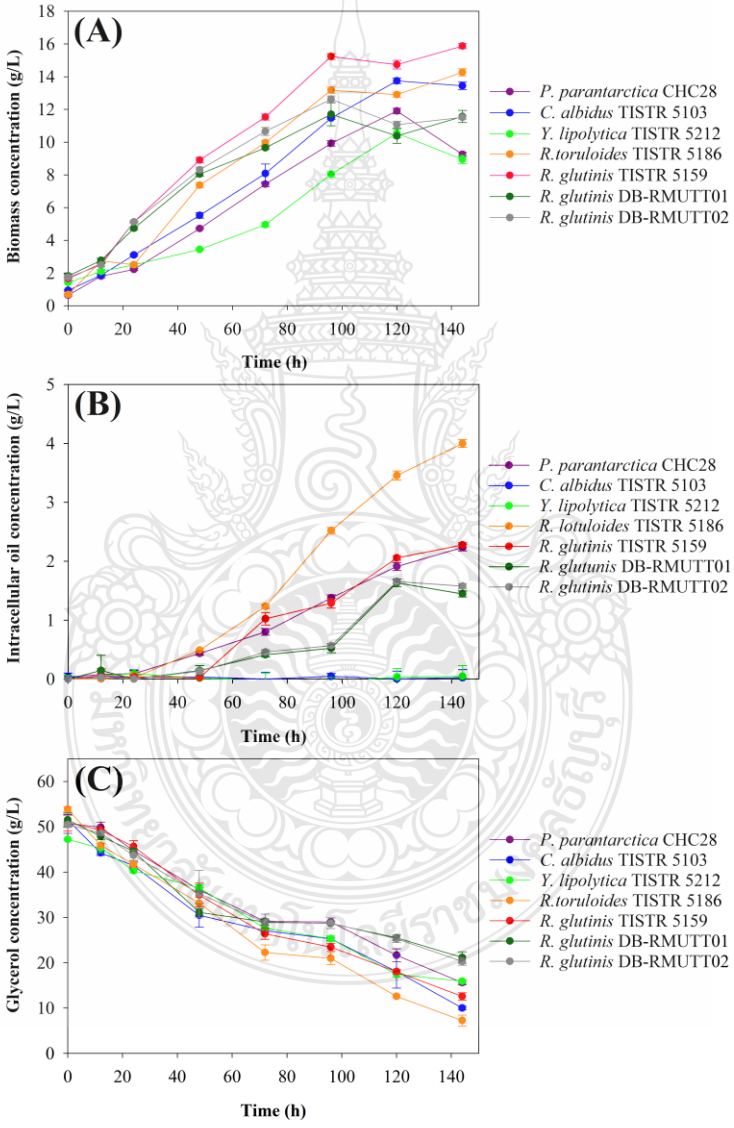


Figure 4.1 Schematics of growth and IO production of different yeast strains cultured in G-OPM. (A) biomass, (B) Intracellular oil, and (C) glycerol concentration. Data are represented as mean with ± SD bar.

Table 4.1 Fermentation parameters for yeast oil production by various yeast strains at 144 h incubation.

Strain	X (g/L)	X_r (g/L)	P_i (g/L)	ΔS (g/L)	$Y_{X/S}$	$Y_{Pi/S}$	Oil content (%)
P. parantarctica CHC28	9.26 ± 0.14^{e}	7.02 ± 0.10^{d}	2.23 ± 0.06^{b}	35.18 ± 2.14^{d}	0.26 ± 0.01^{e}	0.06 ± 0.0^{b}	24.13 ± 0.0^{b}
C. albidus TISTR 5103	13.44 ± 0.23^{c}	13.42 ± 0.36^{a}	0.10 ± 0.05^{d}	41.50 ± 0.77^{b}	0.32 ± 0.0^{c}	NC	0.75 ± 0.0^{e}
Y. lipolytica TISTR 5212	8.94 ± 0.22^{e}	8.90 ± 0.43^{c}	0.13 ± 0.0^{d}	31.30 ± 0.38^{e}	0.28 ± 0.0^{d}	NC	1.45 ± 0.22^{e}
R. toruloides TISTR 5186	14.27 ± 0.38^{b}	10.27 ± 0.28^{b}	4.00 ± 0.07^{a}	46.63 ± 1.41^{a}	0.31 ± 0.01^{c}	0.09 ± 0.0^{a}	28.04 ± 0.01^{a}
R. glutinis TISTR 5159	15.87 ± 0.15^{a}	13.59 ± 0.15^{a}	2.28 ± 0.04^{b}	38.33 ± 1.09^{c}	0.41 ± 0.01^{a}	0.06 ± 0.0^{b}	14.37 ± 0.0^{c}
R. glutinis RMUTT01	11.57 ± 0.38^{d}	10.12 ± 0.34^{b}	1.44 ± 0.05^{c}	30.37 ± 2.31^{e}	0.38 ± 0.01^{b}	0.05 ± 0.0^{c}	12.49 ± 0.0^{d}
R. glutinis RMUTT02	11.51 ± 0.10^{d}	9.93 ± 0.13^{b}	1.58 ± 0.04^{c}	30.15 ± 0.29^{e}	0.38 ± 0.01^{b}	0.05 ± 0.0^{c}	13.71 ± 0.0^{cd}

Different superscripts in the same column indicate significant differences at p-value $\leq .05$. NC, not calculated.

4.2 Effect of non-ionic surfactants on extracellular yeast oil release

Figure 4.2 shows the growth, IO generation, and glycerol consumption of *R. toruloides* TISTR 5186 under various non-ionic surfactant treatments. In the first stage (Figure 4.2 A), the yeast expanded without a lag phase; however, during the middle exponential growth phase (72 h), the addition of non-ionic surfactants had an impact on the development of *R. toruloides* TISTR 5186. Compared to Tween 20, Triton X-100 and Brij 58 demonstrated greater inhibitory effects on yeast proliferation. When Triton X-100 was added at concentrations between 0.1 and 1 g/L, the most inhibitory effect was seen, and biomass dropped between 22.1 and 37.3% in comparison to the control (data not shown). However, according to other studies, Triton X-100 had no effect at 1 g/L on the growth of *Y. lipolytica*, *Aspergillus oryzae*, and *C. curvatus* [90,91,141]. Biomass production of *R. toruloides* TISTR 5186 was inhibited by Brij 58 from 22.4 to 28.6%, similar to the effect of Triton X-100. This result concurred with Huang *et al.* [91] They found that whereas biomass decreased by 53 and 43.5%, respectively, Brij 58 at 0.1 to 1 g/L had no discernible effect on the growth of *C. curvatus* MUCL 29819. Furthermore, Wei *et al.* [142] discovered that, only at 0.5 g/L, Brij 30 had a detrimental effect on Saccharomyces cerevisiae growth during glutathione synthesis.

The use of Tween 20 during the culture process did not affect the growth rate of *R. toruloides* TISTR 5186, according to the results. The concentration of Tween 20 was raised from 0.1 to 1 g/L, resulting in the highest biomass of 12.91 ± 0.02 to 14.79 ± 0.04 g/L (Figure 4.2 A and Table 4.2). At 0.1 g/L, Tween 20 had a 9.3% negative effect on *R. toruloides* TISTR 5186 growth in comparison to the control treatment. However, when 1 g/L Tween 20 was added, the biomass that was collected was comparable to the control condition [143] The effect of surfactants was examined, and it was discovered that *R. glutinis* TISTR 5159's biomass increased by 17.2 and 13.9%, respectively, when 1% w/v Tween 20 and Tween 80 were added. Their findings concurred with Taoka *et al.* [144] who reported that adding Tween 80 greatly enhanced the biomass of *Thraustochytrium aureum* ATCC 34304. By contrast, Xu *et al.* [145] showed that the addition of Tween 20 and Tween 80 had no effect on *R. toruloides* cell growth, indicating that the impact of non-ionic surfactants varied based on the particular microbial species and their structure.

Different patterns in IO accumulation of *R. toruloides* TISTR 5186 are displayed in Figure 4.2 B. The addition of Triton X-100 caused the IO levels to rise progressively for 96 h before abruptly declining. Additionally, the effects of Brij 58 and Tween 20 on IO buildup were noted. IO accumulation increased with the addition of Tween 20 and Brij 58, with Tween 20 recording higher IO accumulation than Brij 58. The effects of each non-ionic surfactant on the yeast TISTR 5186's glycerol intake varied (Figure 4.2 C). Notably, when Triton X-100 was introduced, glycerol consumption was reduced in comparison to Tween 20 and Brij 58. In yeast TISTR 5186, the addition of non-ionic surfactants had an impact on IO buildup and glycerol consumption (Figure 4.2 Band C). Surfactants are substances with amphiphilia [142].

Figure 4.3 displays the IO buildup, EO, and TO production results at 144 h. When using 0.7% w/v Triton X-100 and 1% w/v Tween 20, respectively, the IO accumulation in yeast increased to 1.80 ± 0.0 and 2.51 ± 0.04 g/L (Figure 4.3 A). In contrast, when the concentration of non-ionic surfactants increased, EO steadily increased but IO buildup decreased in response to an increase in Brij 58. The highest amount of EO was 8.21 ± 0.01 g/L at 0.7% w/v Tween 20 (p-value ≤ .05) (Figure 4.3 B), indicating that the IO that was kept in the yeast cytoplasm was liberated to produce EO due to an increase in non-ionic surfactant. Figure 3C illustrates TO production. The results showed that raising non-ionic surfactant significantly increased TO production. These three non-ionic surfactants contributed significantly to the oil content and TO generation in *R. toruloides* TISTR 5186, accounting for a large percentage of the cell weight. Tween 20 exhibited the most prominent promoting effect, providing TO and oil contents of 10.62 ± 0.06 g/L and 78.4% w/w, respectively at 0.7% w/v Tween 20 (Table 4.2).

The role of surfactants in promoting extracellular metabolites has been shown before. It is commonly recognized that the amphiphilic characteristic of the surfactant increases membrane penetration by attenuating the formation of hydrogen and van der Waals bonds in the membrane [91,146,147,148,p.]. Due to its combination with the lipid membrane, the membrane-adsorbed surfactant uses hydrophobic contact to permeate the cell membrane. This molecular activity modifies the original structure of membrane, resulting in the formation of several areas inside the membrane [91,146,149]. Higher concentrations of surfactant cause the surfactant-lipid clusters to become more soluble or

dispersible in the aqueous phase. When the surfactant separates from the membrane area, an increasing rise in membrane permeability, the formation of membrane holes, and the interchange of internal components with the external environment are frequent characteristics. At sufficiently high surfactant concentrations, these effects facilitate cell lysis and death [9]. Surfactants also affect the lysis and permeability of membranes. Cytoplasmic metabolites are released when the cytoplasmic membranes of bacteria burst due to high surfactant concentrations. The cytoplasmic membrane of the cell underwent structural changes due to permeabilization processes triggered by surfactants, whereas the outer membrane was left intact [141].

Based on these findings, non-ionic surfactant supplementation increased the release of yeast EO, resulting in a larger generation of TO compared to a culture without such supplementation. Table 4.2 lists the fermentation parameters for various non-ionic surfactants. All three non-ionic surfactants inhibited the accumulation of non-oil biomass (also known as residual biomass, or X_r), with Tween 20 and Brij 58 having the strongest effects (Table 4.2). This result concurred with Huang *et al.* (2019) and Mirbagheri *et al.* (2011) who found that Triton X-100 and Brij 58 promoted the synthesis of microbial products [91,141] Furthermore, when Tween 20 was introduced at 0.7% w/v, the X_r of *R. toruloides* TISTR 5186 was at its lowest, 2.93 ± 0.05 g/L. Under these conditions, the highest glycerol utilization was measured at 39.43 ± 0.18 g/L, leading to 0.34 for biomass yield ($Y_{X/S}$) and 0.27 for TO yield ($Y_{Pt/S}$). The experiment's findings indicate that glycerol was mostly transformed into YO rather than other non-oil biomass, leading to a maximum oil concentration of 78.38 ± 0.37% w/w.

These findings suggested that at certain concentrations, Triton X-100, Tween 20, and Brij 58 could enhance the oleaginous yeast *R. toruloides* TISTR 5186's growth, YO production performance, and oil content. The properties of non-ionic surfactants were found to be responsible for variations in growth and YO production, potentially due to distinct modes of action. Triton X-100 and Brij 58 are categorized as ether-type chemicals [91], while Tween 20 is a type of polyoxyethylene compound [143,145]. Although Brij 58 has higher surface activity, it interacts with cell membranes more strongly and has a lower critical lysis concentration when it comes to cells. [91]. These characteristics implied the depletion of microbial metabolism in *C. curvatus* similar to carbon source

transformation into YO by *R. toruloides* TISTR 5186 in this study. The surfactant qualities of Brij 58 cause yeast cells to lose their ability to maintain their structural integrity at high doses, which causes cell breakdown and the subsequent release of EO [91]. Tween 20 or polyoxyethylene sorbitan monolaurate is a non-ionic surfactant and contains a mono-unsaturated FA. The monopalmitate, monostealate, and monooleate found in the tween aqueous series, which includes tween 40, tween 60, and tween 80, are accordingly. These compounds accelerate the transfer of nutrients from the environment into the cytoplasm and interfere with the permeability of cell membranes [143,150]. On the other hand, Triton X-100 lowers the carbon source's transformation efficiency into YO and moderates surface activity. Then, by increasing the fluidity of the triton X-100 cell membrane, the IO is transferred to the external environment outside the cell [91].

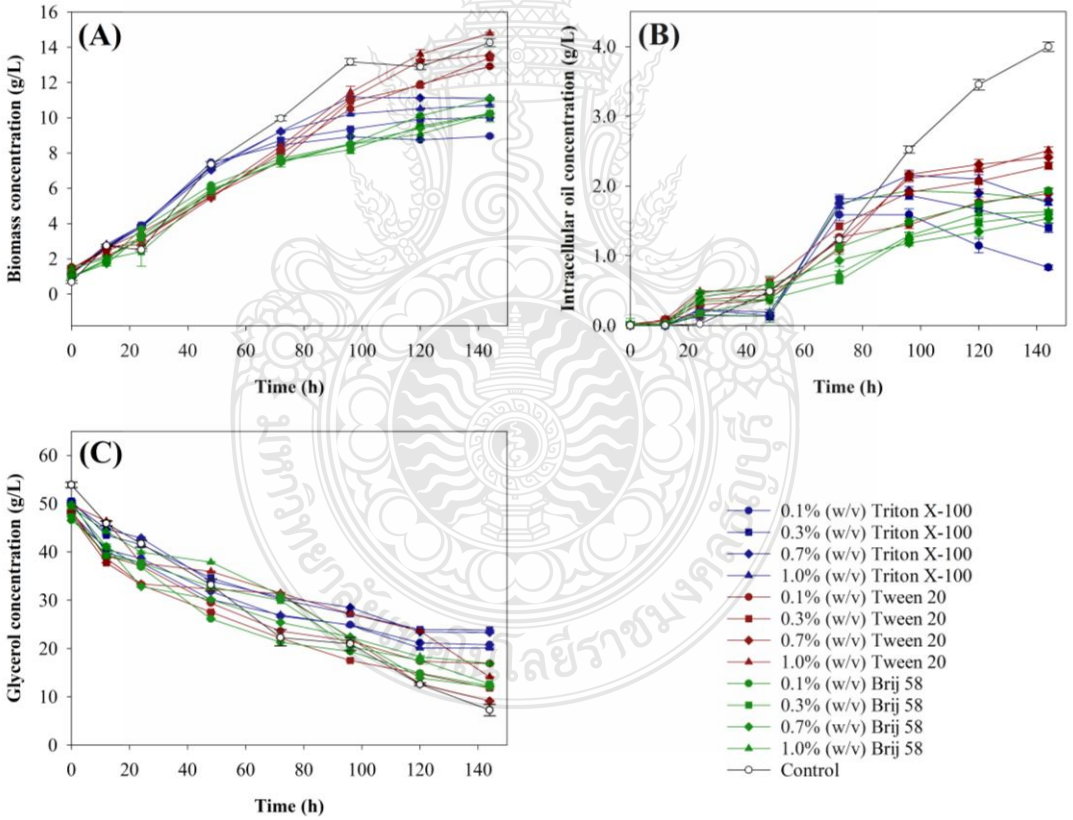


Figure 4.2 Schematics of growth and IO production of *R. toruloides* TISTR 5186 cultured in G-OPM containing different non-ionic surfactants. (A) biomass, (B) Intracellular oil, and (C) glycerol concentration. Data are presented as mean values ± SD bar.

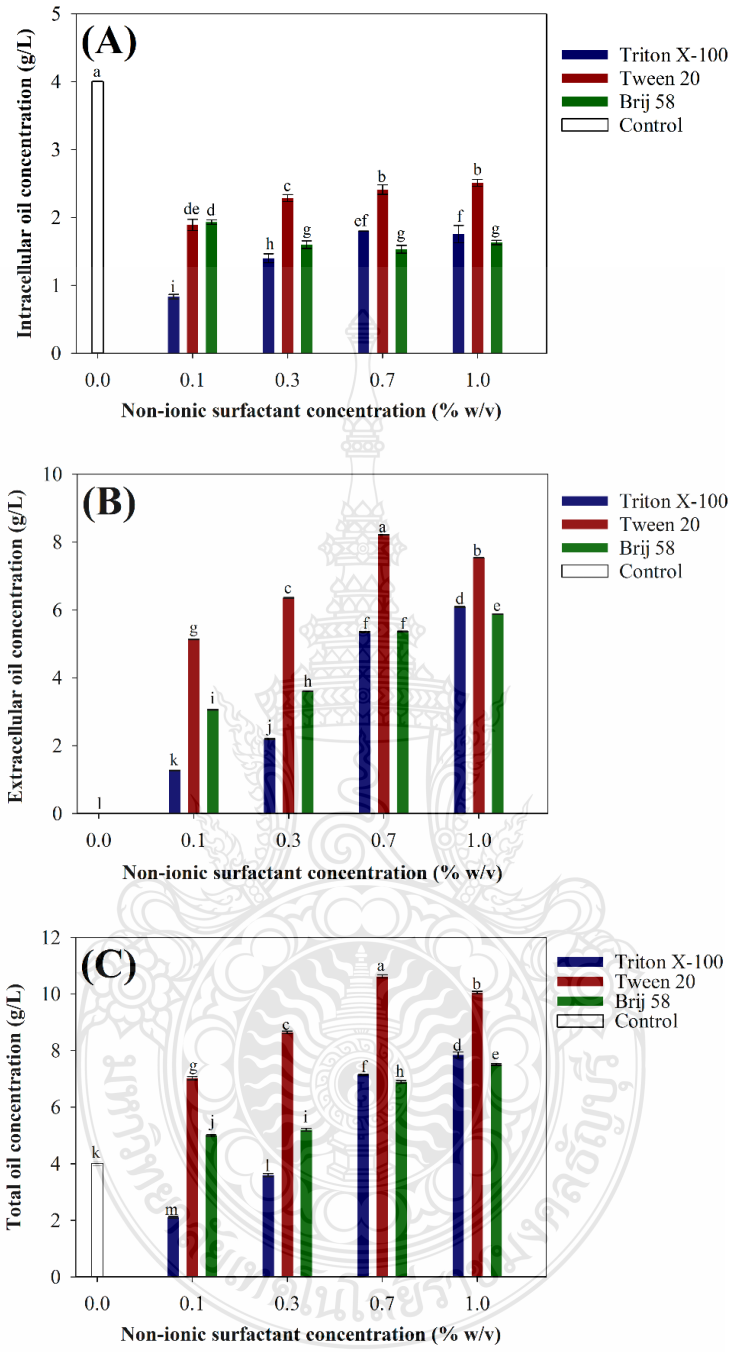


Figure 4.3 Oil compositions of *R. toruloides* TISTR 5186 cultured in G-OPM containing different non-ionic surfactants. (A) intracellular oil, (B) extracellular oil, and (C) total oil. Data are presented as mean values ± SD bar. Different lowercases indicated significant differences at *p-value* ≤ .05.

Table 4.2 Fermentation parameters of yeast oil production under different non-ionic-surfactants.

Condition	Fermentation parameters					
	X (g/L)	Xr (g/L)	ΔS (g/L)	$Y_{X/S}$	$Y_{Pt/S}$	Oil content (%)
Triton X-100 (% w/v)						
0.1	8.95 ± 0.06^{g}	8.12 ± 0.09^{a}	27.37 ± 0.21^{i}	0.33 ± 0.0^{e}	0.08 ± 0.0^{i}	23.54 ± 0.49^{i}
0.3	10.01 ± 0.02^{f}	8.61 ± 0.07^{b}	26.72 ± 0.19^{j}	0.37 ± 0.0^{b}	0.14 ± 0.0^{g}	35.93 ± 0.58^{h}
0.7	11.11 ± 0.04^{d}	9.31 ± 0.03^{e}	26.70 ± 0.31^{j}	0.41 ± 0.0^{a}	0.27 ± 0.0^{b}	64.31 ± 0.14^{d}
1.0	10.71 ± 0.04^{e}	8.96 ± 0.08^{g}	30.35 ± 0.11^{h}	0.35 ± 0.0^{c}	0.26 ± 0.0^{c}	73.21 ± 0.82^{b}
Tween 20 (% w/v)						
0.1	12.91 ± 0.02^{c}	11.02 ± 0.08^{c}	31.01 ± 0.24^{g}	0.42 ± 0.0^{a}	0.23 ± 0.0^{e}	54.39 ± 0.54^{e}
0.3	13.40 ± 0.05^{b}	11.11 ± 0.09^{d}	35.74 ± 0.07^{c}	0.37 ± 0.0^{b}	0.24 ± 0.0^{d}	64.50 ± 0.57^{d}
0.7	13.55 ± 0.06^{b}	11.14 ± 0.05^{g}	39.43 ± 0.18^{a}	0.34 ± 0.0^{d}	0.27 ± 0.0^{b}	78.38 ± 0.37^{a}
1.0	14.79 ± 0.04^{a}	12.28 ± 0.02^{d}	35.60 ± 0.17^{c}	0.42 ± 0.0^{a}	0.28 ± 0.0^{a}	67.94 ± 0.16^{c}
Brij 58 (% w/v)						
0.1	11.08 ± 0.07^{d}	9.14 ± 0.01^{c}	34.29 ± 0.09^{e}	0.32 ± 0.0^{ef}	0.15 ± 0.0^{f}	45.03 ± 0.40^{g}
0.3	10.19 ± 0.11^{f}	8.59 ± 0.11^{d}	34.67 ± 0.03^{d}	0.30 ± 0.0^{g}	0.15 ± 0.0^{f}	51.04 ± 0.61^{f}
0.7	10.22 ± 0.03^{f}	8.69 ± 0.06^{f}	37.79 ± 0.12^{b}	0.27 ± 0.0^{h}	0.18 ± 0.0^{f}	67.47 ± 0.56^{c}
1.0	10.19 ± 0.36^{f}	8.56 ± 0.34^{g}	32.30 ± 0.24^{f}	0.31 ± 0.01^{f}	0.23 ± 0.0^{e}	73.79 ± 2.46^{b}

Different superscripts in the same column indicate significant differences at p-value $\leq$ .05.

4.3 *ACC* and *DGA* gene expression in *R. toruloides* cultivation with non-ionic surfactant addition

FA and TAG are typical products of acyl-CoA synthesis and Kennedy pathways (Figure 4.4) as the relevant mechanisms in oleaginous yeast [70]. generally, the mitochondrial TCA cycle produces citrate, which is then transported to the cytosol via the acyl-CoA production pathway. After acyl-CoA production, ATP-citrate lyase activity converts the citrate to acetyl-CoA. Certain acyl-CoA molecules can also be produced directly from acetyl-CoA in the cytosol via pyruvate formation from glycolysis and

acetate synthesis from aldehyde. The *ACC* gene controls the cytosolic acetyl-CoA carboxylase activity, which further converts acetyl-CoA to malonyl-CoA. Through acyl-CoA synthesis, the FAS complex initiates a series of reactions that convert malonyl-CoA to increased long-chain acyl-CoA. The Kennedy routes are then used to synthesize TAG by sequentially acylating G-3-P with acyl-CoA (Figure 4.4). The *DGA* gene regulates the conversion of diacylglycerol (DAG) to triacylglycerol, and genes encoding enzymes linked to Kennedy pathways and acyl-CoA production are crucial for stimulating TAG synthesis (Figure 4.4).

After the yeast was cultivated and treated with non-ionic surfactants for 72 h, the gene expression linked to YO production of *R. toruloides* TISTR 5186 was confirmed. The investigation of the *ACC* and *DGA* gene expressions occurred 12 h following the administration of non-ionic surfactant (Figure 4.5). Except Brij 58 (1.4-fold), which did not differ significantly at the 95% confidence interval, all non-ionic surfactants had higher *ACC* gene expression than the control condition. In Triton X-100 and Tween 20, the *ACC* gene expression was significantly higher than the housekeeping gene expression by 2.9 and 3.4-fold, respectively (Figure 4.5). The *DGA* gene was expressed at 3.6, 3.6, and 1.4-fold under Triton X-100, Tween 20, and Brij 58, respectively. In addition, it was significantly expressed in all non-ionic surfactant additions compared to the control treatment.

One of the essential genes for the synthesis of FA is the *ACC* gene. Acetyl-CoA carboxylase converts acetyl-CoA to malonyl-CoA during the first stage of FA production [151]. By combining two carbon acetyl units of acetyl-CoA by numerous rounds of concurrent decarboxylation and condensation of malonyl-CoA, the carbon chain of FA is expanded [152]. The expression of the *ACC* gene controls the amount of cytosolic pooled acetyl-CoA that is directly used in FA production [153]. The final stage of the Kennedy pathways is the conversion of DAG into TAG by the diacylglycerol acyltransferase, which is encoded by the *DGA* gene.

Xue *et al.* [154] and Qiao *et al.* [74] revealed that total oil content of *Y. lipolytica* rose when the *DGA* gene was overexpressed. Thus, the *ACC* and *DGA* genes are necessary to regulate the generation of YO. Simultaneous overexpression of *ACC* and *DGA* greatly boosted YO production in *Y. lipolytica* and *R. toruloides* compared to the

solo effects of *ACC* or *DGA* overexpression [65,155]. Moreover, Takaku *et al.* [156] revealed that the synthesis of FA and TAG in *L. starkeyi* was significantly influenced by elevated *ACC* and *DGA* expression. The overexpression of *ACC* and *DGA*, respectively, increased the production of acetyl-CoA carboxylase and diacylglycerol acyltransferase, which was a critical mechanism for improving the efficiency of oil generation in oleaginous yeasts [156].

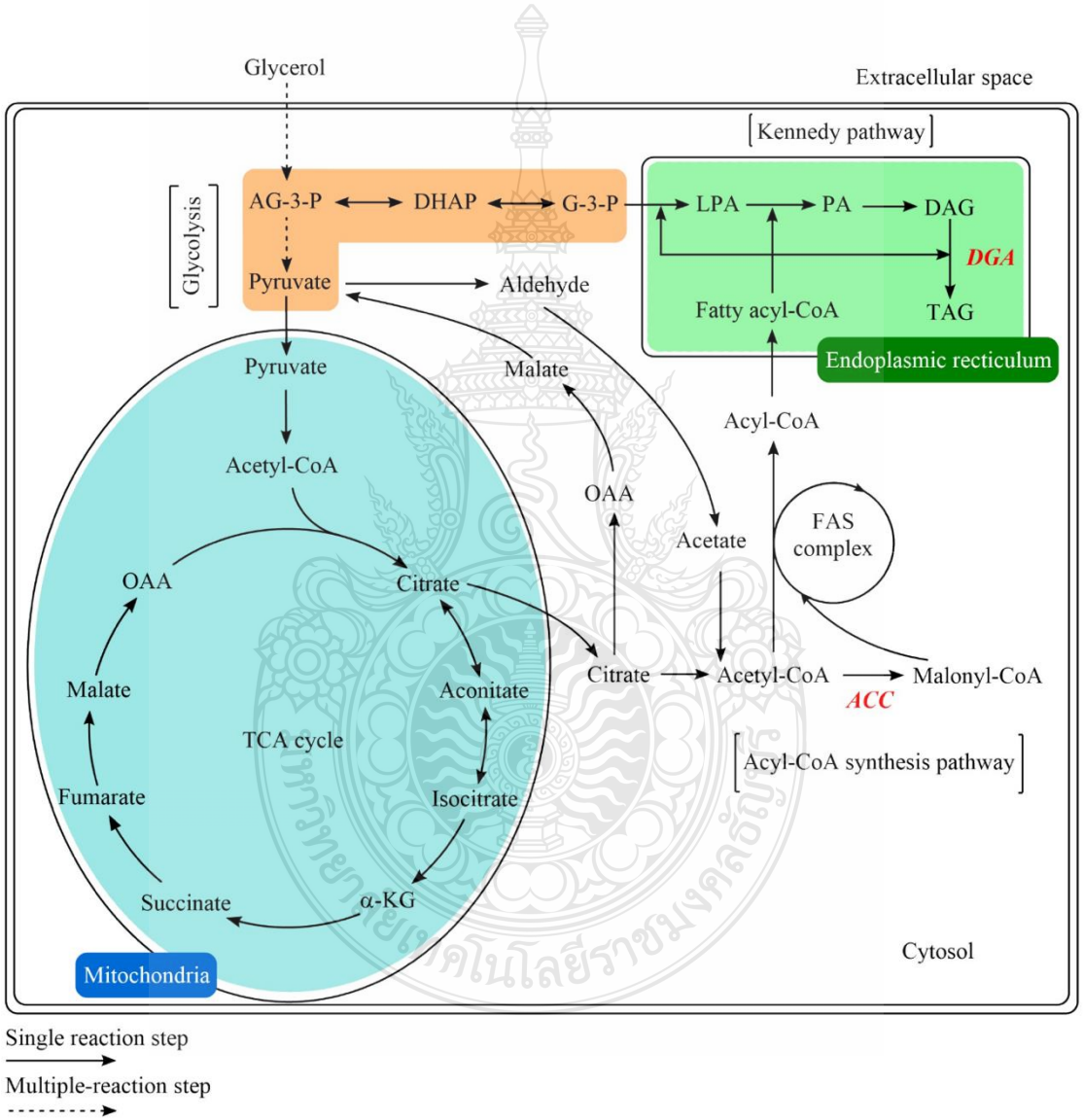


Figure 4.4 Metabolic pathways of *R. toruloides* using glycerol as a carbon source. The ACC and DGA genes are involved in the biosynthesis of FAs and TAG pathways, respectively. The pathway map was modified from Wen *et al.* and Zhao *et al.* [157,158].

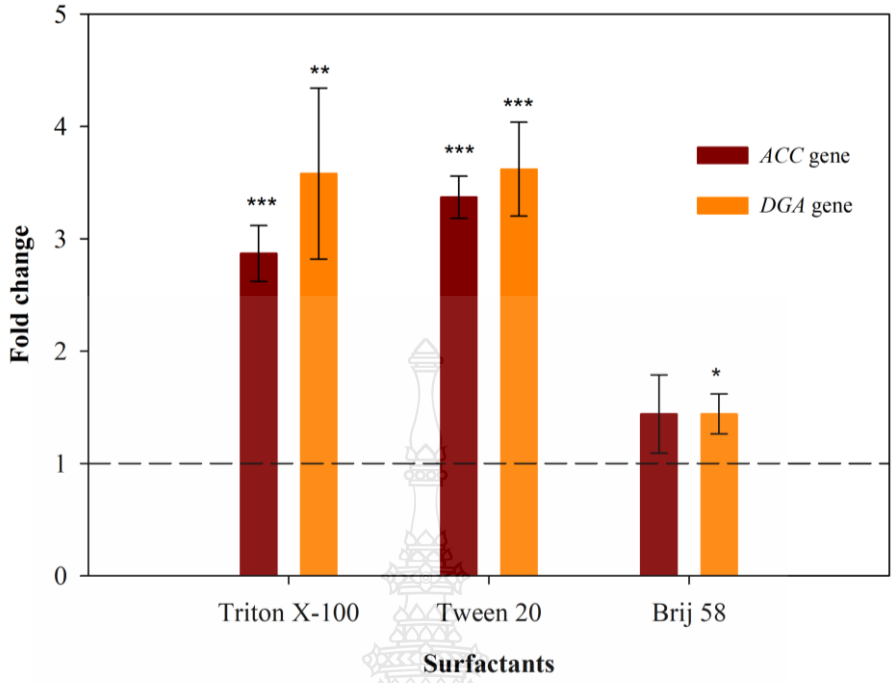


Figure 4.5 Fold change levels of the *ACC* and *DGA* genes in *R. toruloides* TISTR 5186 cultivation with non-ionic surfactant addition. Significant differences were calculated using the Student's t-test compared to the expression of *18s rRNA* (**p*-value < .05, ***p*-value < .01, and ****p*-value < .001). Data are presented as mean values of three independent experiments ± SD bar.

4.4 Kinetic modeling of YO production under non-ionic surfactant addition

Equations (5) to (7) were utilized to fit the experimental data on yeast growth, glycerol consumption, and IO production rate to characterize the YO production of *R. toruloides* TISTR 5186 in the presence of non-ionic surfactant (Figure 4.6). The logistic equation is one of the most popular unstructured mathematical models used to explain how microbes multiply. This model, which is not dependent on a substrate, accurately explains how biomass inhibits cell growth in traditional batch fermentations [43,159]. Increased IO accumulation during culturing impeded yeast growth. To simultaneously represent the non-linear growth profile driven by YO accumulation, the logistic equation and the product inhibition equation were merged (Eq. (5)). Figure 4.6 shows a simulation of this yeast growth schematic under the addition of non-ionic

surfactant. With the addition of Triton X-100, Tween 20, and Brij 58, the R^2 value of the logistic model (Eq. (5)) effectively suited the development of TISTR 5186. The values ranged from 0.817 to 0.994 (Figure 4.6A-1 to A-4), 0.831 to 0.929 (Figure 4.6B-1 to B-4), and 0.974 to 0.955 (Figure 4.6C-1 to C-4), respectively. For every treatment, there was satisfactory agreement with the substrate consumption model (Eq. (6)) throughout the glycerol consumption time course ($R^2 \geq 0.920$) (Figure 4.6).

The R^2 of Tween 20 and Brij 58 supplementation varied from 0.730 to 0.936 (Figure 4.6B-1 to B-4) and 0.905 to 0.972 (Figure 4.6 C-1 to C-4), respectively, according to curve fitting of IO production using the Luedeking-Piret equation (Eq. (7)). On the other hand, when Triton X-100 was used, the obtained R^2 varied from 0.557 to 0.777 (Figure 4.6A-1 to A-4). As time passed, the IO concentration decreased (especially after 72 h). A nonlinear regression model that demonstrates the relationship between microbial growth and product generation is the Luedeking-Piret equation [159, 160] and, therefore, did not accurately describe this situation. Except for product loss or degradation, the Luedeking-Piret equation is typically employed to simulate time-dependent product production during the experimental period. The measured product in this investigation represented the difference between the synthesized and released YO and showed how much IO was still present. To enhance the performance of the Luedeking-Piret equation in this specific scenario, a new term representing the product release schematic must be constructed and incorporated into the model.

Table 4.3 presents the fermentation results together with the kinetic parameters derived via nonlinear fitting. Similar to the control treatment (14.25 g/L), the X_{max} values of *R. toruloides* TISTR 5186 obtained from Tween 20 inclusion ranged from 12.03 to 14.73 g/L. These values were higher than the inclusions of Triton X-100 and Brij 58. According to Taoka *et al.*, Tween 20 functions as a carbon source, improving the permeability of the cell membrane and promoting the uptake of nutrients into the cell bodies [144]. On the other hand, Triton X-100 and Brij 58 reduced the formation of non-oil biomass, resulting in a lesser buildup of biomass than Tween 20. After adding Triton X-100 to the TISTR 5186 culture, the μ_{max} remained lower than the control condition, but it was higher than the other non-ionic surfactants. The critical IO concentration for non-ionic surfactant treatment, or P_i*, was calculated to be 4 g/L for the control treatment.

The release of IO from *R. toruloides* TISTR 5186 happened outside the yeast cells with the addition of non-ionic surfactants, resulting in a drop to below 4 g/L (Figure 4.3A). Therefore, non-ionic surfactants could be added to yeast TISTR 5186 culture to increase TO formation and lessen the effects of product inhibition (Figure 4.3C).

Compared to Brij 58 and the control circumstances, the estimated values of $Y_{X/S}$ for Triton X-100 and Tween 20 were higher (Table 4.3). This indicated that Triton X-100 and Tween 20 were more effective at promoting the conversion of glycerol into oleaginous biomass in *R. toruloides* TISTR 5186 than Brij 58 supplementation. Brij 58 had a notably more potent inhibitory effect; in the presence of 0.1 and 1 g/L Brij 58, respectively, biomass decreased by 53 and 43.5% It is possible that different non-ionic surfactant types and concentrations had distinct effects on yeast growth, which resulted in different amounts of inhibition [91]. In every instance, the cell maintenance coefficient, or m_S, was equal to zero. This discovery demonstrated that the production of biomass and YO without cell maintenance was the principal role of glycerol. When $\alpha \neq 0$ and $\beta = 0$, product generation is directly correlated with microbial growth as per the criteria of Eq. (7) [161]. Since the values of β were close to zero and the α values were larger than those of any other treatment, it can be concluded that IO production in this study was growth-associated (Table 4.3).

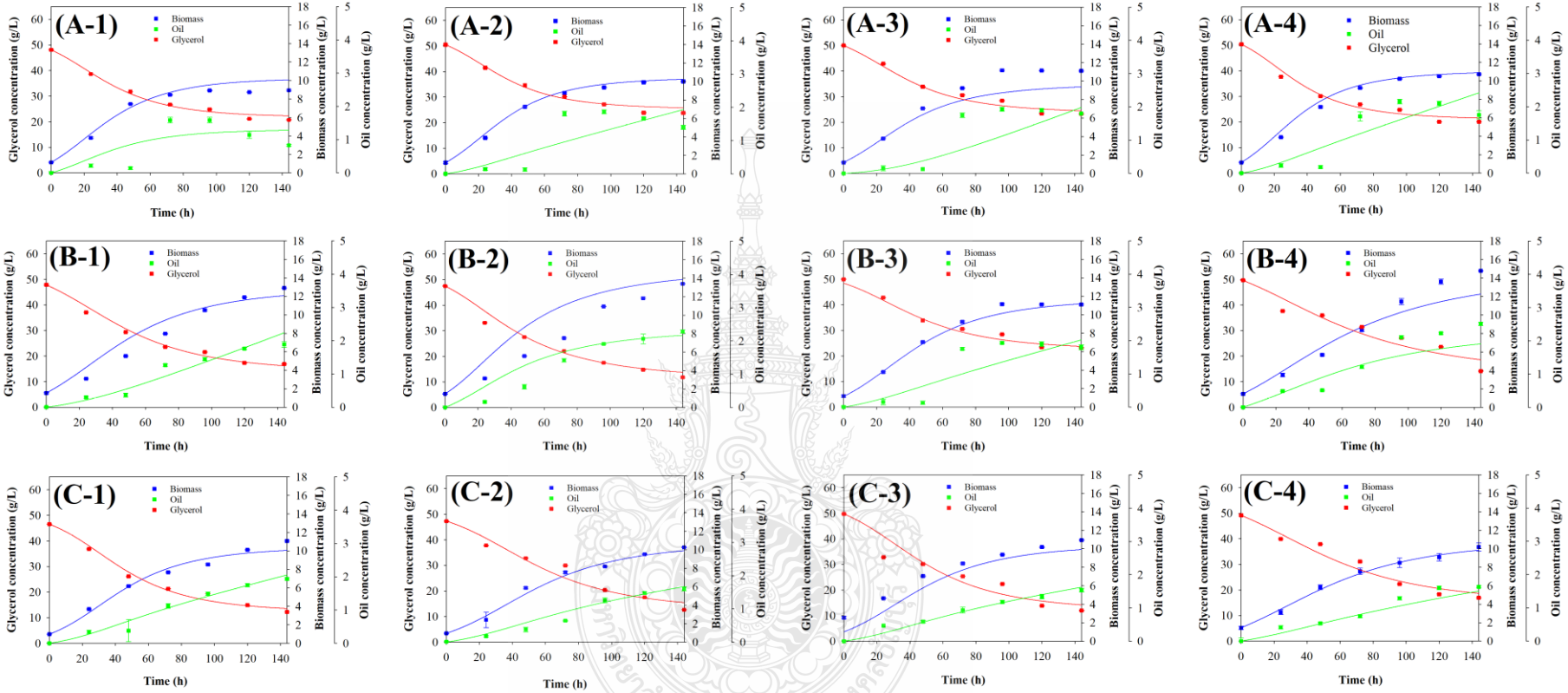


Figure 4.6 Experimental results (symbols) and model predictions (lines) of glycerol, biomass, and oil concentration during *R. toruloides* TISTR 5186 cultivation under addition of non-ionic surfactants. Triton X-100 (A-1) 0.1% w/v; (A-2) 0.3% w/v; (A-3) 0.7%; w/v (A-4) 1% w/v, Tween 20 (B-1) 0.1% w/v; (B-2) 0.3% w/v; (B-3) 0.7% w/v; (B-4) 1%, w/v and Brij 58 (C-1) 0.1% w/v; (C-2) 0.3% w/v; (C-3) 0.7% w/v; (C-4) 1% w/v. Data are presented as mean values ± SD bar.

Table 4.3 Estimated kinetic parameters of intracellular oil production under non-ionic surfactant addition.

Model and kinetic parameters	Control	Triton X-100 (%w/v)				Tween 20 (%w/v)				Brij 58 (%w/v)			
		0.1	0.3	0.7	1	0.1	0.3	0.7	1	0.1	0.3	0.7	1
Growth rate													
X_{max} (g/L)	14.25	10.23	10.55	9.94	11.26	13.73	14.73	12.03	14.48	10.57	10.87	10.55	11.06
μ_{max} (1/h)	0.133	0.130	0.128	0.120	0.129	0.102	0.100	0.118	0.100	0.084	0.080	0.094	0.080
P_i* (g/L)	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0
n	0.21	0.26	0.21	0.16	0.24	0.19	0.38	0.20	0.22	0.37	0.30	0.26	0.23
R^2	0.960	0.954	0.994	0.817	0.988	0.929	0.831	0.986	0.883	0.974	0.986	0.995	0.993
NRMS	0.927	0.695	0.252	1.466	0.384	1.030	2.098	0.424	1.380	0.527	0.379	0.226	0.244
Substrate consumption rate													
$Y_{X/S}$ (g/g)	0.32	0.35	0.37	0.32	0.34	0.33	0.37	0.41	0.35	0.28	0.28	0.25	0.28
m_S	0	0	0	0	0	0	0	0	0	0	0	0	0
R^2	0.934	0.987	0.979	0.979	0.976	0.988	0.975	0.979	0.923	0.986	0.957	0.920	0.955
NRMS	3.766	1.034	1.266	1.315	1.583	1.235	1.905	1.285	3.108	1.409	2.405	3.734	2.304
Oil production rate													
α (g/g)	0.007	0.143	0.058	0.004	0.064	0.039	0.174	0.072	0.176	0.092	0.114	0.094	0.097
β (g/gh)	0.002	0	0.001	0.001	0.001	0.001	0	0.001	0	0.001	0	0	0
R^2	0.856	0.557	0.655	0.662	0.777	0.936	0.858	0.730	0.757	0.968	0.948	0.972	0.905
NRMS	0.498	0.408	0.476	0.526	0.440	0.201	0.316	0.424	0.385	0.128	0.136	0.095	0.170

4.5 Simultaneous YO production by *R. toruloides* TISTR 5186 and *in-situ* YO recovery with oil capturing agent (OCA)

4.5.1 *In-situ* oil capturing procedure

The simultaneous YO production and in-situ YO recovery with different oil-capturing agents (OCA) were investigated in the bath process. The intracellular oil produced by *R. toruloides* TISTR 5186 was secreted to the cultural medium as extracellular oil (EO) by adding Tween 20 at 72 h. The EO was captured *in situ* by OCA, which was contained in the medium. The cultural profile is illustrated in Figure 4.7A to C. The biomass of TISTR 5186 cultured was increased time dependently under simultaneous YO production and recovery condition (Figure 4.7 A). When cultivated for 144 h with SP70, the biomass ranged 10.33 to 11.43 g/L (Table 4.4). Meanwhile, the biomass was produced for 10.22 to 1.43 g/L by using XAD-16. It was observed that the oleaginous and residual biomass were decreased with increasing SP70 and XAD-16. Under OCA addition, the IO was lower increased than control experiment (Figure 4.7 B). In addition, the IO was lower accumulated when using XAD-16 than SP70. During the exponential phase, the glycerol was more rapidly consumed than the control condition until 96 h of cultivation (Figure 4.7 C). However, the glycerol consumption was similar to XAD-16 and higher than SP70 adding condition. Pawar *et al*. reported that using OCA can impact the growth and non-oil biomass of oleaginous yeast. OCA aids in the removal of adsorbed oil droplets from the cell surface, thereby improving substrate assimilation and enhancing triacylglycerol (TAG) synthesis. The presence of OCA is associated with an increase in substrate consumption rate, leading to enhanced TAG production. Moreover, OCA is proposed to facilitate the removal of intracellular TAGs from the cells, resulting in prolonged oil production during the stationary phase. This mechanism allows for oil secretion into the culture medium and extends the duration of oil production [92]. The effect of YO recovery is presented in Table 4.4. The adsorbed oil (AO) gradually increased in raising both OCAs. It was demonstrated that the quantity captured oil was related to the increase of OCA utilizing weight. The EO was adsorbed by SP70 at a higher concentration than XAD-16 and maximized at 2% (w/v) SP70 for 3.28 g/L. As the AO increased, the TO also increased with OCA. The maximum TO was presented for 8.39 g/L at 2% (w/v) SP70 (Table 4.4). SP70 is a synthetic copolymer consisting of styrene

divinyl benzene (~ 850 micro-meter spherical particles), providing a highly hydrophobic surface area of 870 m^2/g for selective oil capture [95]. Similarly, XAD-16 is a crosslinked, nonionic, hydrophobic divinylbenzene matrix with a nonionic macroporous (or macroreticular) resin structure. It contains a continuous pore phase and a continuous polymer phase that adsorbs and releases ionic species through hydrophobic and polar interactions [94].

The simultaneous oil production integrated *in-situ* recovery by SP70 and XAD-16 provided $Y_{x/s}$ and $Y_{p/s}$ ranged from 0.26 to 0.33 and 0.24 to 0.26, respectively. The oil content expressed as the oil synthesized per biomass produced was maximized at 2% SP70 for 91.72% (w/w). These results demonstrated that using OCA could promote YO production efficiency in simultaneous processes.

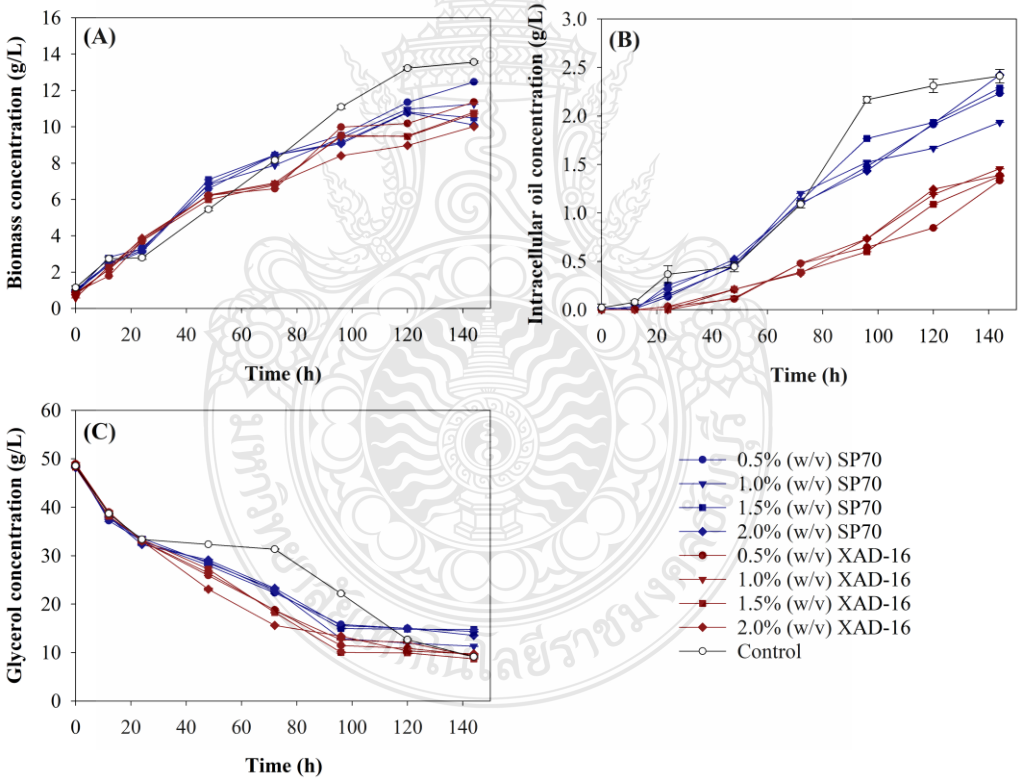


Figure 4.7 Time course of simultaneous YO production by *R. toruloides* TISTR 5186 and *in-situ* YO recovery with OCA.

Table 4.4 Kinetic parameters of simultaneous YO production by *R. toruloides* TISTR 5186 and in-situ YO recovery with OCA.

OCA	Conc. (%w/v)	X (g/L)	X_r (g/L)	ΔS (g/L)	$Y_{x/s}$	$Y_{p/s}$	Oil content (%)
Control	0	13.55±0.06^{a}	11.14±0.05^{a}	39.43±0.18^{a}	0.34±0.00^{a}	0.27±0.00^{a}	78.38 ± 0.37^{b}
SP70	0.5	11.43±0.12^{b}	9.20±0.14^{b}	34.56±0.06^{d}	0.33±0.00^{a}	0.20±0.00^{d}	60.17±0.82^{d}
	1.0	10.33±0.11^{c}	8.40±0.11^{c}	37.18±0.20^{c}	0.28±0.00^{b}	0.19±0.00^{e}	68.46±0.62^{c}
	1.5	9.62±0.04^{d}	7.33±0.03^{e}	34.02±0.13^{e}	0.28±0.00^{b}	0.22±0.00^{c}	77.37±0.36^{b}
	2.0	9.14±0.08^{d}	6.72±0.11^{f}	34.66±0.07^{d}	0.26±0.00^{c}	0.24±0.00^{b}	91.72±1.20^{a}
XAD-16	0.5	10.46±0.02^{c}	9.12±0.05^{bc}	39.51±0.22^{a}	0.26±0.00^{c}	0.08±0.00^{i}	32.05±0.45^{g}
	1.0	10.22±0.11^{c}	8.77±0.17^{cd}	39.65±0.17^{a}	0.26±0.00^{c}	0.12±0.00^{h}	46.23±1.04^{f}
	1.5	9.86±0.40^{d}	8.48±0.40^{c}	39.58±0.09^{a}	0.25±0.01^{d}	0.13±0.00^{g}	52.73±1.46^{e}
	2.0	9.26±0.17^{d}	7.87±0.16 d	38.98±0.00^{b}	0.24±0.00^{e}	0.14±0.00^{f}	59.63±0.60^{d}

Different superscripts in the same column indicate significant differences at p-value ≤ .05.

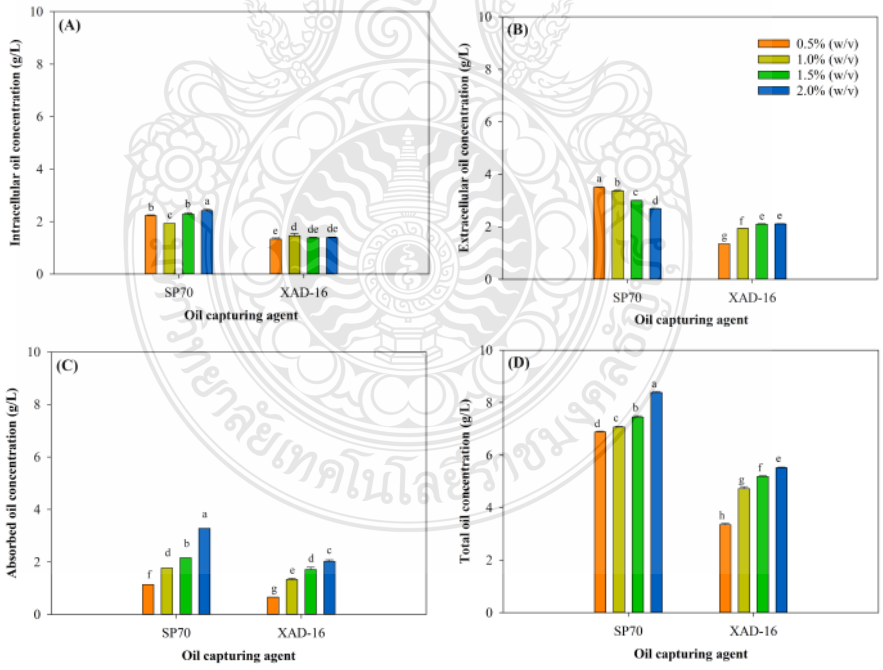


Figure 4.8 In-situ oil capturing process at 144 h. (A) IO, (B) EO, (C) CO, and (D) Total oil.

The ratio (%w/w) of IO, EO, and AO of *in-situ* recovery process by SP70 and XAD-16 was illustrated in Figure 4.9A and B. The result explored that the IO was released from yeast cell to be EO. Then EO was captured by OCA as AO. The ratio of AO was increased according to an increase of OCA. Moreover, the AO ratio was higher than that of IO and EO at 2% OCAs. Especially, the SP70 provided the AO ratio better than XAD-16 (Figure 4.9 A and B). The highest recovered AO (46.81 %w/w) was obtained with 2% SP70 (Figure 4.9A). It was suggested that OCA could remove the EO from the cultural medium of YO production.

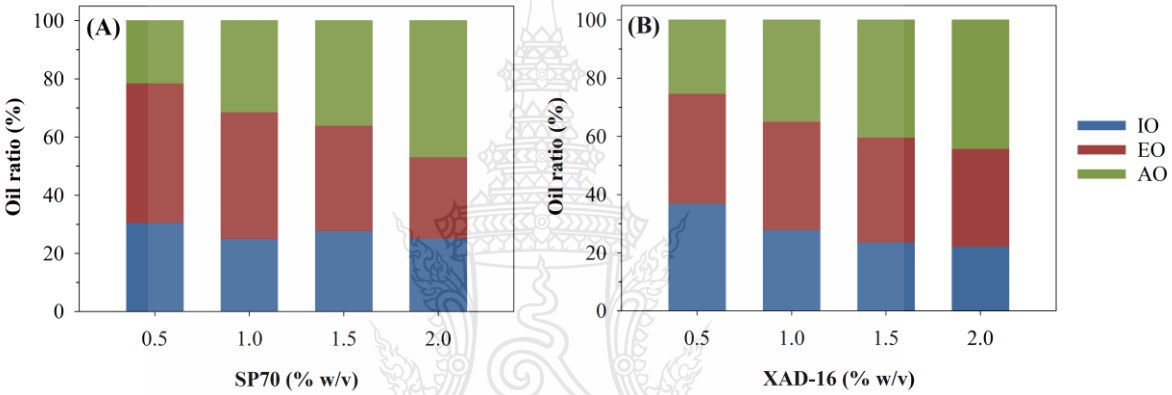


Figure 4.9 Oil ratio of *in-situ* oil recovery by (A) SP70 and (B) XAD-16.

4.5.2 *Ex-situ* oil capturing procedure

Ex-situ oil recovery mode was conducted by introducing SP70 to the culture medium at the conclusion of fermentation with varying amounts and absorption times. The oil concentrations observed under the ex-situ mode are depicted in Figure 4.10 (A) to (D). The IO ranged between 2 to 10 g/L (Figure 4.10 A). The EO decreased as the absorption time increased to the 3rd day and was lowest at 0.5 g/L (Figure 4.10 B). Conversely, the AO increased with the enhancement of absorption time (Figure 4.10 C). This suggests that EO was adsorbed by SP70, leading to a decrease in EO and an increase in AO. Moreover, the total oil (TO) experienced a slight decrease on the 3rd day of the *ex-situ* mode (Figure 4.10 D). Pawar *et al.* reported on the use of OCA for capturing the EO produced by *Y. lipolytica*. The physical consequence of directly adding the resin to the fermentation broth in a batch method is that complete adsorption on OCA occurs over a

period of 72 h [92]. Consequently, this has a negative effect on oil production within the cells, as it inhibits the biosynthesis of intracellular oils and disrupts cellular homeostasis [74]. Therefore, it becomes crucial to minimize EO saturation on both the cell surface and in the culture medium [162].

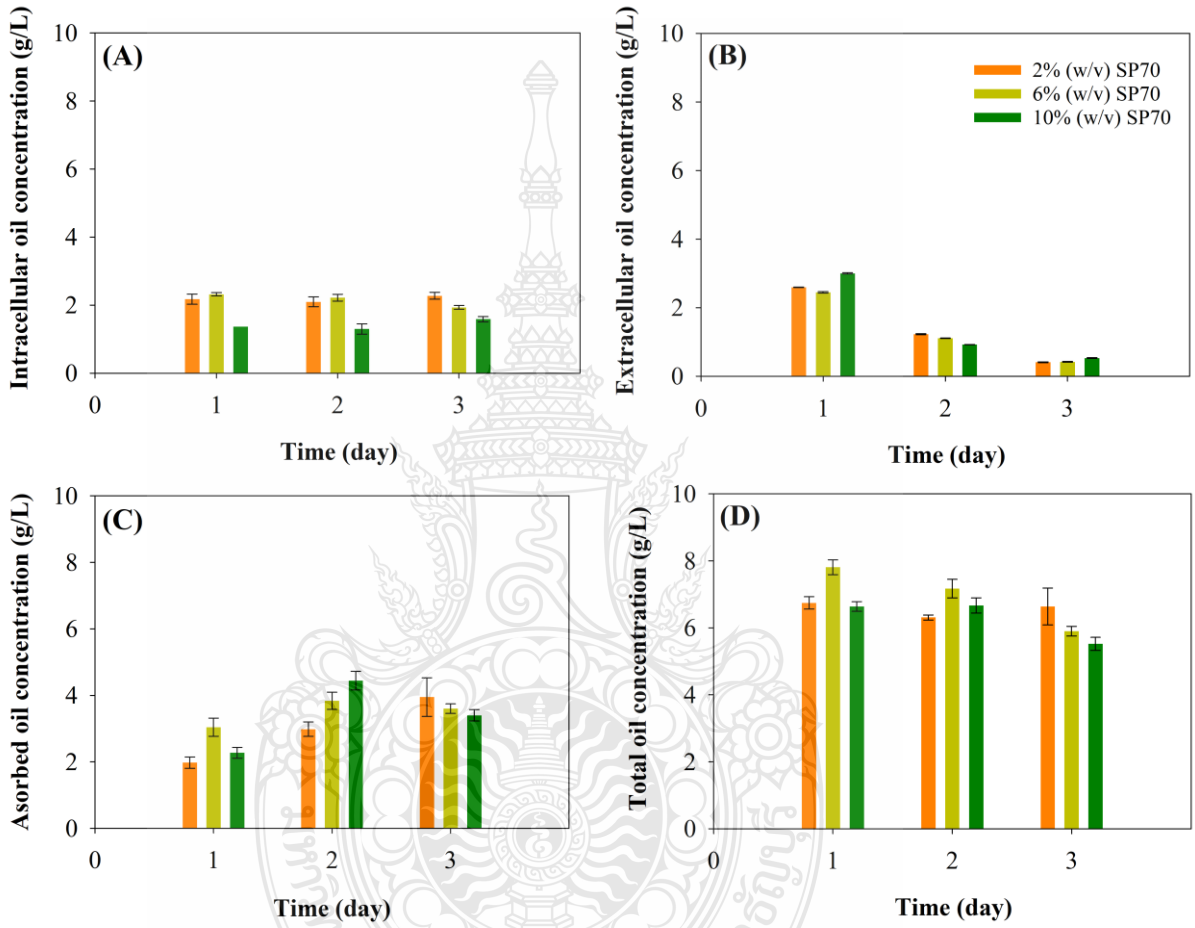


Figure 4.10 Efficiency of ex-situ oil recovery mode. (A) IO, (B) EO, (C) CO, and (D) TO.

In an above experiment on *in-situ* YO recovery, the quantity of SP70 was further investigated by varying from 2 to 10% (w/v) in the ex-situ YO recovery condition (Figure 4.11 A to C). The ratio of EO decreased as SP70 increased from 2 to 10% (w/v). Conversely, the ratio of AO increased with the enhancing of SP70. This demonstrates that an increase in the quantity of SP70 positively affects YO absorption and recovery.

Additionally, at 2 and 6% (w/v) SP70 (Figure 4.11 A to B), an increase in soaking time also led to enhanced AO. However, at 10% (w/v) SP70, the AO ratio remained similar under different soaking times (Figure 4.11 C). It was suggested that 10% (w/v) SP70 was sufficient for oil absorption. Consequently, AO could be completely adsorbed in a shorter time.

The SP70 has the capacity to adsorb yeast oil (YO) from the broth, accumulating it instead [92]. When OCA is introduced into the oil production broth based on its binding capacity, the dispersed OCA beads in the aqueous broth provide a large surface area for oil adsorption and cell contact [92]. These OCAs generally possess hydrophobic characteristics due to their polystyrenic backbone crosslinked with divinylbenzene. They exhibit a strong affinity for aromatic and hydrophobic substances, binding these metabolites through π-π bonding or hydrophobic interactions [96]. As demonstrated with XAD-16 resin, other functionalities, such as hydroxyl, amino, and cyano groups, have been added to this polystyrenic scaffold, changing the binding selectivity. It is challenging to recover most of these expensive, small-particle functionalized polystyrenic adsorbents from fermentation broth. Furthermore, surface area, density, pore size, and bead size are commonly used to differentiate adsorbent resins. The added benefit of nonionic polymeric adsorbents is that elution is typically performed in organic solvents that are easily evaporated to reduce processing volumes and are frequently simpler to integrate with subsequent separation procedures [95].

Microbial-based technologies face challenges in scaling up and still require significant technological advancements to be commercialized [163]. In terms of economics, downstream processing is the main cause of cost escalation [164]. The in-situ oil adsorptive fermentation system enables extended oil production periods and recovery by the cells, demonstrating effective integration of upstream and downstream processing that is industrially feasible [165].

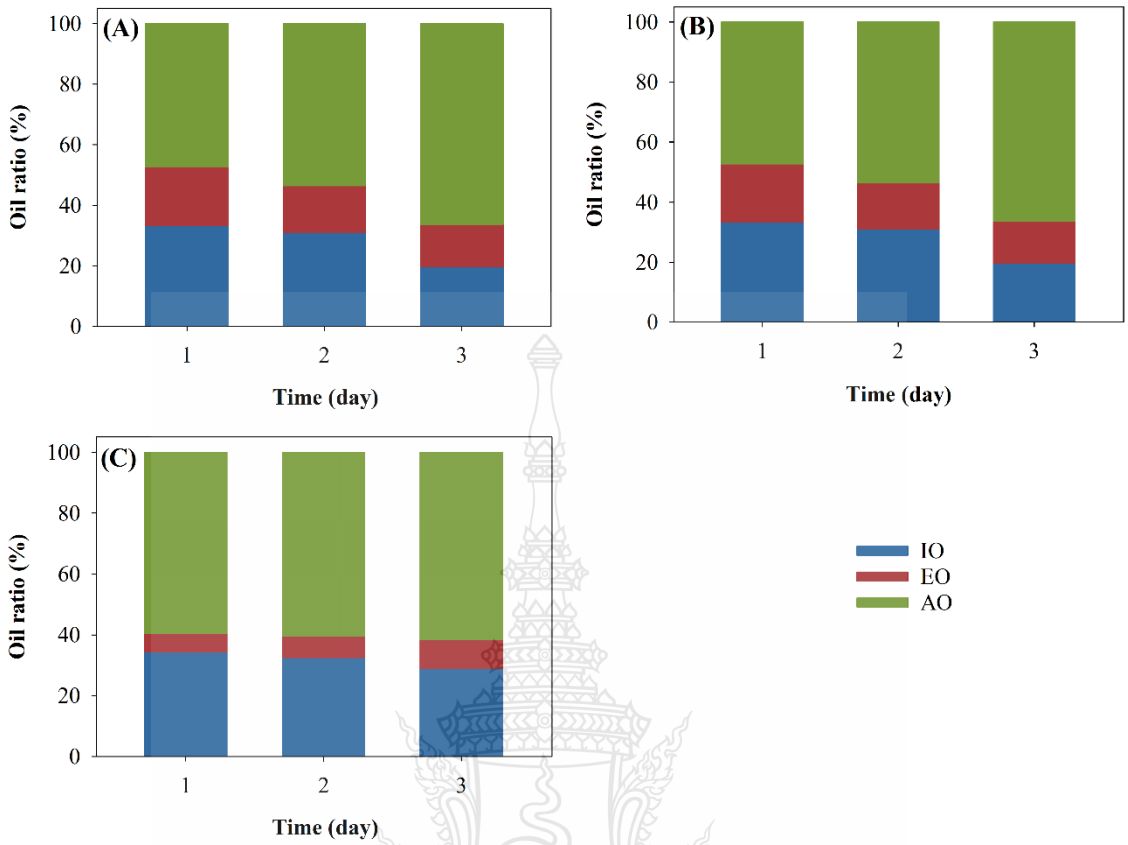


Figure 4.11 Ratio of ex-situ oil recovery by SP70 of (A) 2% (w/w), (B) 6% (w/w), and (C) 10% (w/w).

The cell released-oil recovery efficiency obtained from *in-situ* and *ex-situ* oil recovery mode is depicted in Figure 4.12 A and B. The EO recovery was significantly increased with increasing of SP70 and XAD-16 (Figure 4.12 A). The result observed that the maximum EO recovery was presented of 54.95% (w/w) using 2% (w/v) SP70 (Figure 4.12 A). The ex-situ EO recovery by SP70 was presented in Figure 4.12 B. The result shown that the amount of SP70 and absorption time significantly affected on EO recovery. The amount of SP70 added increases, the EO recovery increases. In addition, the absorption time also impacted on EO recovery. The highest EO recovery was achieved of 90.49% (w/w) at 1st day of adsorption.

Comparison of SP70 and XAD-16, synthesized by polymerization using synthetic crosslinking polymers, reveals a wide variety of precise pore sizes. The physical and adsorptive characteristics of these adsorbents have been impacted. Particularly, SP70 exhibits a greater specific surface area than XAD-16, resulting in higher oil absorption capabilities [96]. Pawar *et al.* have reported that the addition of hydrophobic OCA resins can enhance the efflux of oil across the semipermeable membrane into the external environment, thereby reducing intracellular oil (IO) levels in the presence of in situ adsorbents [96]. The use of different in situ adsorbent resins causes variations in oil dispersion, affecting each adsorbent's capacity to extract IO from the cells [96]. Additionally, Phillips *et al.* have discussed the principal mechanisms responsible for titer enhancement and identified three variables to consider when employing solid-phase adsorption during fermentation: (1) the choice of resin, (2) the quantity of resin to add, and (3) the timing of its addition [95]. However, oil production halts once the OCA becomes fully saturated, necessitating the termination of the batch and replacement of the OCA [92]. Therefore, the duration of OCA soaking is crucial for effective EO absorption. Incorporating the adsorptive system into the fermenter enhance the oil production periods and minimize the oil reuse of cell. This improvement enhances the process economics and environmental friendliness.

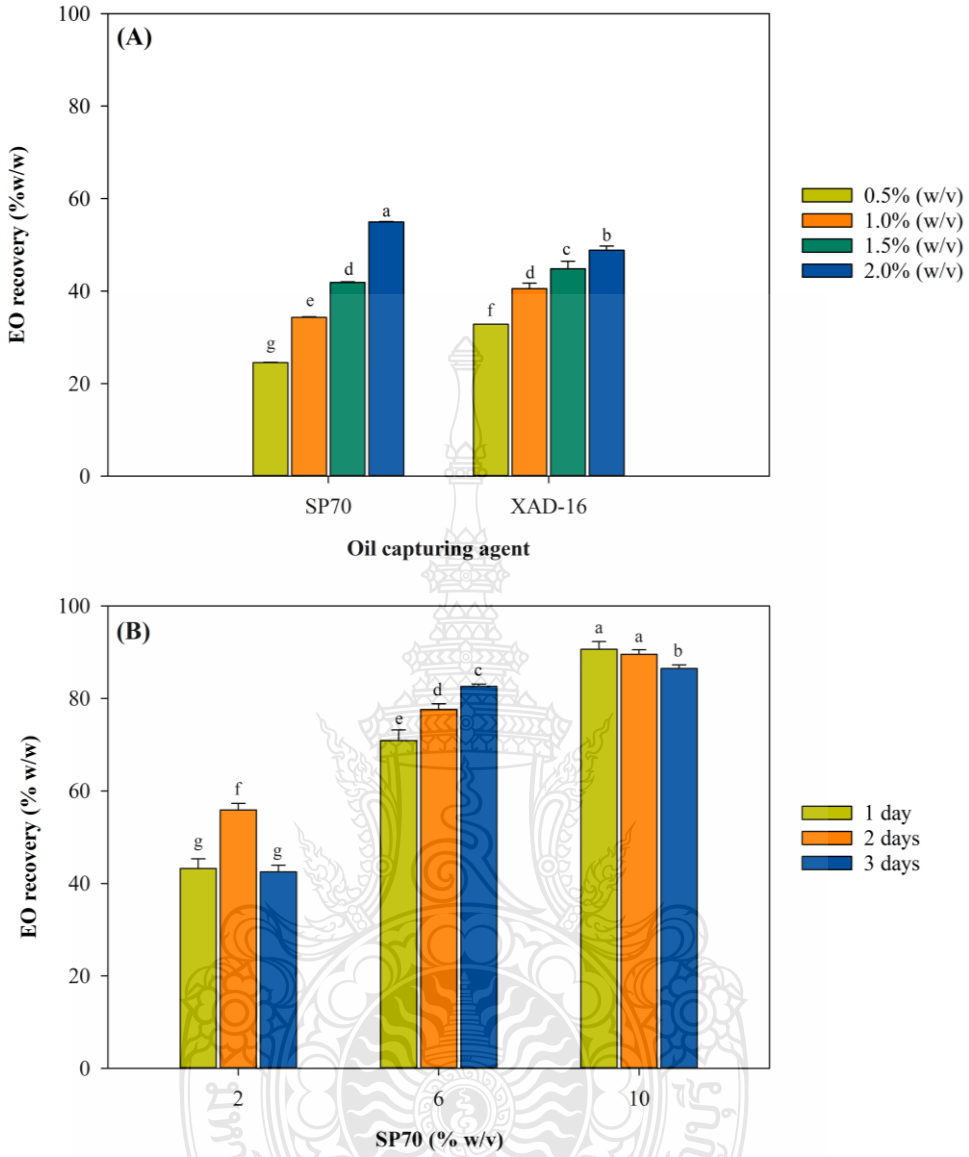


Figure 4.12 EO recovery by (A) in-situ and (B) ex-situ YO recovery process. Data present the mean ± SD bar. The different lowercase letter indicated significant different at p-value < .05.

4.6 Scaling up of simultaneous oil biosynthesis and *in-situ* recovery by batch fermentation in 5-L bioreactor

The scaling-up performance of *R. toruloides* in simultaneous oil production and offline *in-situ* recovery through a batch process is illustrated in Figure4.13. The results presented a more rapid glycerol consumption rate observed in the OCA-containing condition compared to the control during the exponential phase, influencing the growth rate and IO formation of *R. toruloides*. The yeast *R. toruloides* exhibited enhanced growth and IO production in the OCA-containing condition compared to the control. Under the SP70-containing condition. *R. toruloides* achieved a biomass and IO of 14.95 ± 0.17 g/L and 4.78 ± 0.06 g/L, respectively (Table 4.5). Furthermore, the TO, $Y_{X/S}$, and $Y_{P/S}$ were maximized in the aforementioned condition at 12.50 ± 0.09 g/L, 0.32, and 0.26, respectively (Table 4.5). The oil content (83.58 ± 0.72% (w/w)) was not significantly different with control treatment. The results suggest that the oil released from microbial cells has low solubility in water. This ensures that the oil adheres to the microbial membrane due to its hydrophobicity and remains close to the microbial cells, blocking transportation across the membrane. As a result, the assimilation of carbon sources and other nutrients is slowed down. Consequently, the metabolic flux associated with biochemical synthesis is performed slowly, leading to lower microbial oil production [92, 162]. During fermentation, the addition of an adsorbent-based OCA has performed selective isolation of organic compounds from the aqueous fermentation medium [92,166]. This employment can improve the efficiency of the downstream process in microbial oil production.

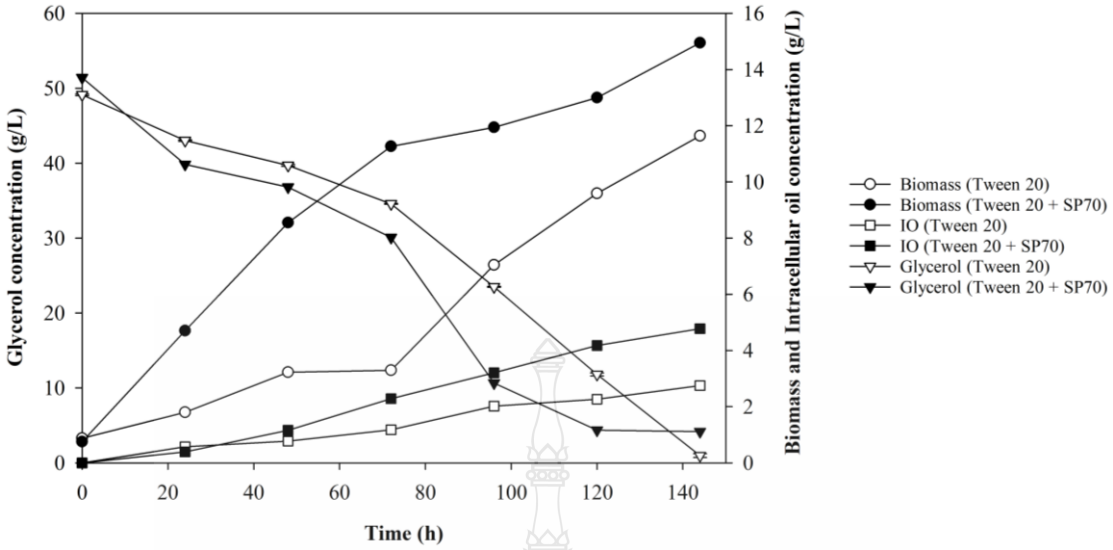


Figure 4.13 Time course of simultaneous YO production by *R. toruloides* TISTR 5186 and in-situ YO recovery with OCA in 5-L bioreactor. Data present the mean ± SD bar.

Table 4.5 Scale-up kinetic parameters of simultaneous YO production by *R. toruloides* TISTR 5186 and in-situ YO recovery with OCA.

Kinetic parameters	Tween 20 (0.7% w/v)	Tween 20 (0.7% w/v) and SP70 (2% w/v)
X (g/L)	11.64 ± 0.71	14.95 ± 0.17*
X_r (g/L)	8.89 ± 1.15	10.18 ± 0.17^{ns}
IO (g/L)	2.75 ± 0.51	4.78 ± 0.06**
EO (g/L)	7.78 ± 0.40	4.52 ± 0.07**
AO (g/L)	NA	3.20 ± 0.02
TO (g/L)	10.53 ± 0.60	12.50 ± 0.09*
ΔS (g/L)	48.17 ± 4.26	47.25 ± 0.57^{ns}
Yx/s	0.24 ± 0.04	0.32 ± 0.0*
Yp/s	0.22 ± 0.01	0.26 ± 0.0**
Oil content (%)	91.07 ± 10.66	83.58 ± 0.72^{ns}

NA is not available. * and ** present significant differences compared with non-OCA addition at t-value ≤ 0.05 and 0.01, respectively. ^{ns} presents not significant differences compared with non-OCA addition at t-value > .05.

Table 4.6 Total production in scale-up kinetic parameters of simultaneous YO production by *R. toruloides* TISTR 5186 and in-situ YO recovery with SP70.

Yeast oil	Tween 20 (0.7% w/v)	Tween 20 (0.7% w/v) and SP70 (2% w/v)
IO (g)	5.02 ± 1.08	8.71 ± 0.10
EO (g)	14.19 ± 0.85	8.26 ± 0.13
AO (g)	-	5.83 ± 0.03
TO (g)	19.21 ± 1.26	22.80 ± 0.16
EO recovery (% w/w)	-	41.39 ± 0.49

3.2 Online capture of yeast oil in 5-L bioreactor by batch and fed-batch modes

3.2.1 Batch mode

The batch cultivation in 5-L bioreactor integrated with SP70 OCA column was employed to simplify the YO recovery process. The SP70 was contained in a glass column and fluidized with the G-OPM (Figure 4.14). The SP70 column was replaced every 72 h with a new column throughout batch cultivation. Yeast *R. toruloides* could completely use the glycerol for growth and YO synthesis (Figure4.15 A). The biomass and IO were reached to 13.01 and 3.51 g/L, respectively. At 72 h, the biomass and their IO in the SP70 column were retained higher than in the bioreactor, resulting in the glycerol concentration being lower than the bioreactor (Figure4.15 B). While the biomass in SP70 column was higher but the IO and residue glycerol were similar to 5-L bioreactor at 144 h. In SP70 column, the total EO and AO were 0.02 ± 0.0 and 0.16 ± 0.04 g at 72 h (1^{st} SP70 column) (Table 4.7). Additionally, the total production of EO and AO reached 0.08 ± 0.0 and 2.03 ± 0.70 g, respectively, at 144 h (2^{nd} SP70 column) (Table 4.7). These findings indicate the absorption of EO by SP70 in the glass column. The TO obtained was 18.88 ± 1.24 g by the end of the process, resulting in biomass and TO yields of 0.258 ± 0.026 and 0.19 ± 0.01, respectively, presenting an oil content of 75% (w/w) (Table 4.7). SP70 can effectively remove oil droplets from the cell surface, thereby increasing substrate assimilation and directly regulating the metabolic flux of TAG synthesis [165]. Utilizing an SP70 OCA column could decrease the accumulation of YO produced in the bioreactor.

As a result, yeast growth and YO production in the bioreactor were promoted with the incorporation of the SP70 column.

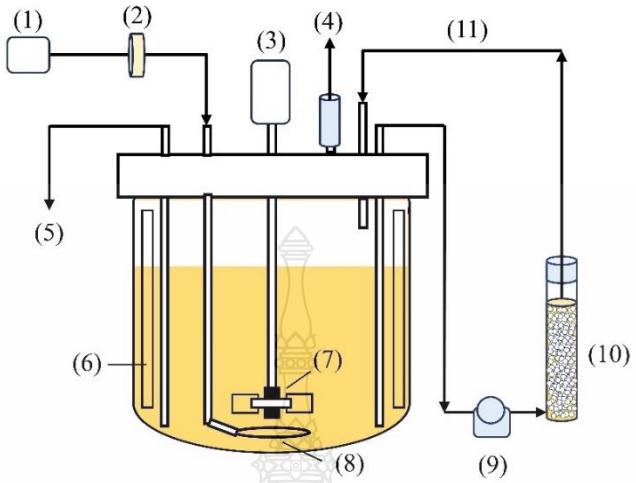


Figure 4.14 Process chart for simultaneous YO oil production and in-situ online SP70 OCA column. The G-OPM in 5-L bioreactor continuously flowed to fluidize an SP70 bed in the external OCA glass column and recirculate to the bioreactor; (1) Air compressor, (2) Air filter, (3) Motor, (4) Exhaust line and condenser, (5) Sampling line, (6) Baffle, (7) Disc-turbine, (8) Ring sparger, (9) Peristaltic pump, (10) SP70 bed, and (11) Recirculating line.

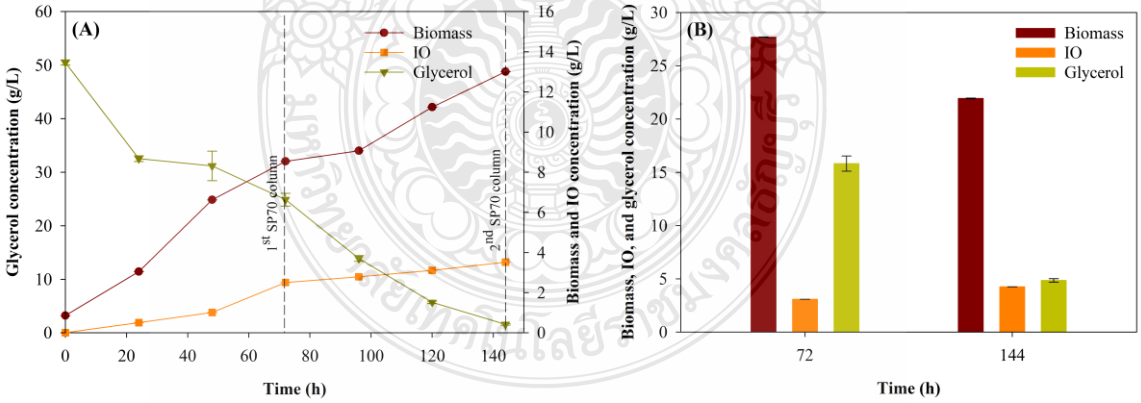


Figure 4.15 (A) Time course of simultaneous YO production with *R. toruloides* by batch mode in 5-L bioreactor and in-situ YO recovery using SP70 OCA column. The dash line presents the replacement time of the SP70 OCA column. (B) The biomass, IO, and glycerol concentration in SP70 OCA column. Data are presented as mean values ± SD bar.

3.2.2 Fed-batch mode

The fed-batch mode was implemented to prolong simultaneous YO production and *in-situ* recovery utilizing the SP70 OCA column, as depicted in Figure 4.15 A 5-L bioreactor containing 2 L of G-OPM was equipped with an EO recovery column containing 40 g of SP70 (2% w/w). The SP70 column was replaced with a fresh one every 3 days. The G-OPM was introduced into the bioreactor every 6 days of cultivation. A schematic outlining yeast growth and YO production is presented in Figure 4.16. In the initial batch, yeast actively utilized glycerol for both its growth and continuous IO production. The second batch, glycerol utilization was lower than in the first batch, resulting in the maximization of biomass at 13.82 ± 0.19 g/L. However, IO enhancement was not observed, and it remained within the range of 0.04 ± 0.02 to 0.06 ± 0.0 g/L. In the third batch, glycerol consumption and biomass were at 117.40 g/L and 18.45 g/L, lower than the previous batch, with IO production similar to that of the second batch. Notably, both biomass and IO production decreased in the fourth batch. This decline suggests a drop in yeast growth from glycerol, with its biomass retained in the SP70 column without recirculation to the bioreactor.

This study was observed that yeast could not continuously utilize glycerol for its growth, resulting in an increased accumulation of glycerol. Consequently, biomass enhancement was inhibited. Previous research by Li *et al.* has indicated that increasing substrate amounts decrease the biomass and oil production of *R. toruloides* Y4 [167]. Additionally, there may be an accumulation of yeast by-products, such as short-chain organic acids, including citric and succinic acid, which can inhibit growth during fed-batch fermentation operating over a prolonged period.

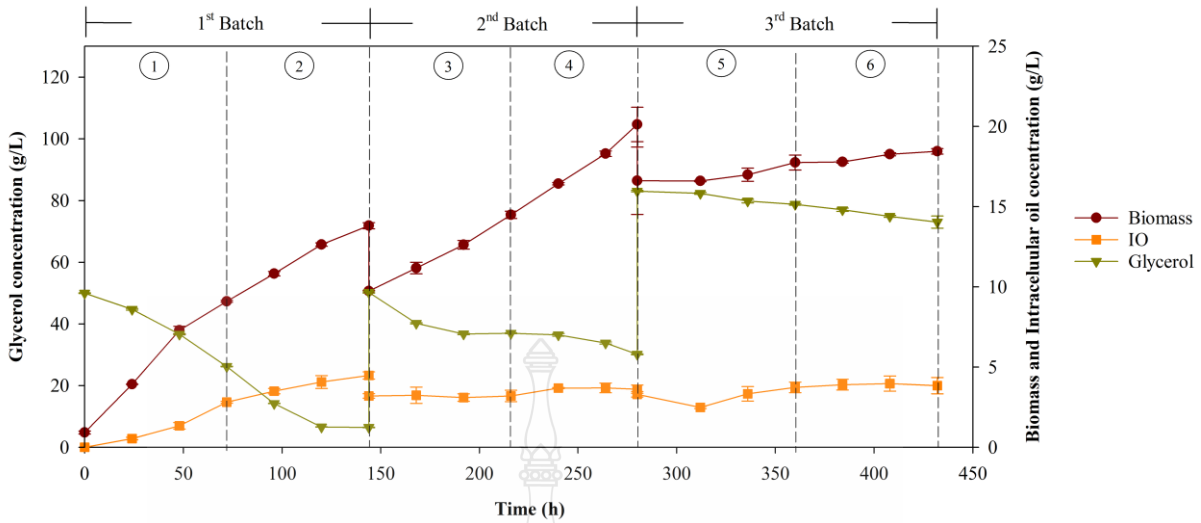


Figure 4.16 Time course of simultaneous YO production with *R. toruloides* by fed-batch mode in 5-L bioreactor and in-situ YO recovery using SP70 OCA column. The dashed line and number in the circle present the replacement time and sequencing SP70 column. Data are presented as mean values ± SD bar.

The efficiency of total YO production with online YO recovery-integrated fermentation in both batch and fed-batch modes is presented in Table 4.7. In the batch mode, the overall oleaginous biomass and TO produced by *R. toruloides* were 25.31 ± 2.30 g and 18.88 ± 1.24 g, respectively. Glycerol consumption was measured at 98.08 ± 1.05 g, converted to biomass and TO with yields of 0.258 ± 0.026 and 0.19 ± 0.01, respectively. In the fed-batch mode, the biomass produced in the bioreactor gradually decreased from the 1st batch to the 3rd batch (Table 4.7). Additionally, the profiles of glycerol consumption, intracellular oil (IO), and TO similar to the schematic of biomass production. Interestingly, while the biomass yield showed a decreasing trend, the TO yield increased by the 3rd batch. Overall production metrics indicated that the biomass and TO yields were achieved at 0.36 ± 0.02 and 0.37 ± 0.08, respectively, resulting in an oil content of 102% (Table 4.7).

Table 4.7 Efficiency of total YO production with online YO recovery-integrated fermentation by batch and fed-batch modes.

Production mode	X (g)	ΔS (g)	IO (g)	EO (g)	AO (g)	TO(g)	$Y_{x/s}$	$Y_{pTO/s}$	$Y_{pTO/x}$	Q_p (g/h)
Batch mode										
Bioreactor	23.68 ± 1.66	95.89 ± 1.03	6.84 ± 0.98	9.84 ± 1.24	ND	16.69 ± 0.59	0.25 ± 0.02	0.17 ± 0.01	0.71 ± 0.05	0.081
1st SP70 column	1.07 ± 0.04	1.39 ± 0.02	0.12 ± 0.0	0.02 ± 0.0	0.02 ± 0.0	0.16 ± 0.04	0.77 ± 0.20	0.11 ± 0.03	0.16 ± 0.08	0.001
2nd SP70 column	0.55 ± 0.40	0.80 ± 0.04	0.07 ± 0.02	0.08 ± 0.0	1.88 ± 0.72	2.03 ± 0.70	0.71 ± 0.54	2.54 ± 0.93	6.10 ± 4.48	0.011
Overall production	25.31 ± 2.30	98.08 ± 1.05	7.04 ± 1.00	9.94 ± 1.24	1.90 ± 0.72	18.88 ±1.24	0.26 ± 0.03	0.19 ± 0.01	0.75 ± 0.08	0.098
Fed-batch mode										
Bioreactor, 1st batch	25.28 ± 1.22	85.27 ± 1.05	8.77 ± 1.39	9.92 ± 1.93	ND	18.69 ± 3.27	0.30 ± 0.01	0.22 ± 0.04	0.74 ± 0.10	0.039
1st SP70 column	0.70 ± 0.34	1.22 ± 0.04	0.09 ± 0.02	0.05 ± 0.0	ND	0.14 ± 0.22	0.57 ± 0.26	0.11 ± 0.02	0.24 ± 0.14	0
2nd SP70 column	0.89 ± 0.54	0.85 ± 0.03	0.07 ± 0.05	0.16 ± 0.0	0.79 ± 0.43	1.02 ± 0.44	1.06 ± 0.67	1.21 ± 0.53	1.63 ± 1.10	0.002
Bioreactor, 2nd batch	20.32 ± 6.97	39.27 ± 5.65	3.23 ± 0.24	12.82 ± 0.71	ND	16.04 ± 0.74	0.51 ± 0.12	0.42 ± 0.08	0.89 ± 0.38	0.033
3rd SP70 column	0.62 ± 0.23	0.24 ± 0.01	0.06 ± 0.0	0.26 ± 0.0	1.48 ± 0.39	1.81 ± 0.39	2.55 ± 0.96	7.43 ± 1.39	3.47 ± 2.05	0.004
4th SP70 column	0.66 ± 0.25	0.14 ± 0.02	0.04 ± 0.02	0.18 ± 0.05	0.93 ± 0.06	1.16 ± 0.07	4.50 ± 1.25	8.15 ± 0.72	1.95 ± 0.68	0.002
Bioreactor, 3rd batch	1.82 ± 0.30	19.60 ± 4.35	1.29 ± 1.16	11.38 ± 6.05	ND	12.67 ± 5.14	0.10 ± 0.02	0.71 ± 0.44	7.46 ± 4.04	0.026
5th SP70 column	2.59 ± 0.11	0.17 ± 0.11	0.04 ± 0.01	0.16 ± 0.00	0.90 ± 0.31	1.09 ± 0.31	23.39 ± 17.17	9.21 ± 6.25	0.42 ± 0.12	0.002
6th SP70 column	0.27 ± 0.13	0.23 ± 0.11	0.03 ± 0.01	0.13 ± 0.03	0.60 ± 0.02	0.75 ± 0.04	1.30 ± 0.78	3.69 ± 1.24	3.85 ± 3.11	0.002
Overall production	53.15 ± 4.92	146.99 ± 7.51	13.61 ± 0.82	35.07 ± 8.76	4.70 ± 1.09	53.37 ± 9.20	0.36 ± 0.02	0.37 ± 0.08	1.02 ±0.27	0.111

ND is not detectable. Different superscripts in the same column indicate significant differences at p-value ≤ .05.

4.7 Fatty acid composition of intracellular and extracellular oils

FAME was produced from YO synthesized by several yeast strains that employed glycerol as their primary carbon source. GC-MS analysis of the FAME profile produced a heatmap that is shown in Figure 4.17. The findings showed that C16:0 (palmitic acid) and C18:1 (oleic acid) were the predominant FA components of YO, followed by C18:0 (stearic acid) and C18:2 (linoleic acid). Of them, C18:1 comprised 46%, with C16:0 being the main FA (given by *R. toruloides* TISTR 5186) at 46.7% and C18:1 at 29.3%. The sources included *R. glutinis* TISTR 5159, *R. glutinis* RMUTT01, *R. glutinis* RMUTT02, *Y. lipolytica* TISTR 5212, and *C. albidus* TISTR 5103. This result was in line with previous study findings.

Huang *et al.* [168] found that the majority of the FAs in the oil of *C. curvatus* MUCL 29819, or over 90% of the total, were C16:0, C18:0, C18:1, and C18:2. The predominant component, C18:1, exceeded 50%. According to Rakkitkanphun *et al.*, the primary FA in the oil generated by *P. parantarctica* CHC28 utilizing crude glycerol recovered from refined biodiesel was C16:0 [137]. Chang *et al.* [169] further found that the most common FA in the *Cryptococcus* sp. SM5S05 oils were C18:1, C16:0, C18:0, and C18:2, with percentages of 58.2, 22.2, 7, and 6.8%, respectively.

The FA profile of IO and EO generated for different non-ionic surfactant additions by *R. toruloides* TISTR 5186 is displayed in Figure 4.18. As in the surfactant-free condition, C16:0 was the principal FA of IO and EO in every instance. The maximal C16:0 in IO values for Triton X-100, Tween 20, and Brij 58 were 69.3, 79.6, and 73.5%, respectively. For Triton X-100, Tween 20, and Brij 58, the EO, which is made up of C16:0, peaked at 61.7, 75.9, and 64.7%, respectively. Compared to IO, the overall C16:0 content of FA in EO was lower when utilizing a comparable non-ionic surfactant. On the other hand, EO outperformed IO (21.9%) in terms of total C18:1 (26.7%). Similar FA profiles of YO synthesized by *R. toruloides* TISTR 5186 were also reported. Furthermore, the FA profile of YO recovery using *in-situ* and *ex-situ* procedures is depicted in Figure 4.18A and B. In the case of in-situ recovery, C16:0 emerged as the predominant FA in both IO and EO for both SP70 and XAD-16 OCAs (Figure 4.18 A). Additionally, FA analysis revealed the presence of C14:0, C16:0, and C18:0 as primary FA of AO. Moreover, the FA composition of IO and EO predominantly consisted of C16:0 and

C18:1 as the main components (Figure 4.18 B). Interestingly, C16:0 was identified as the primary FA in AO during the *ex-situ* oil recovery procedure. According to Fontanille *et al.*, in all studies, including C16:0, C18:0, C18:1, and C18:2, the total levels of unsaturated FAs recovered from *Y. lipolytica* cultured using volatile FA were 80% [170]. This FA content was comparable to that of vegetable oils, which are employed in the manufacturing of goods derived from oil [46,47].

Microbial oils have been utilized to generate various oil-based products including biodiesel [15], [137], [165], bio-polyurethane foam [71], [172], [173], and bio-lubricasnt [13], [174]. Oil-based product qualities were influenced by the FA compositions present in the oil. According to Hoekman *et al.*, two important variables influencing the characteristics of biodiesel were the degree of unsaturation of FA (connected to the number of carbon–carbon double bonds) and chain length (linked to carbon number) [175]. The relationship between the unsaturation degree and biodiesel properties has been reported. Higher saturated FA percentage produced microbial oil-based biodiesel with a higher cetane number, viscosity, and cloud point. In contrast, the biodiesel's high heating value, iodine number, and specific gravity were all elevated by the presence of monounsaturated FA (MUFA) [175]. The oil composition of *R. pacifica* cultivated in molasse-integrated sugarcane bagasse hydrolysate with a high MUFA content was shown to be more suited for biodiesel synthesis than jatropha oil, as it offered improved oxidative stability and cold filter plug point [176]. This information was reported by Deeba *et al.* High MUFA content produced by *L. starkeyi* in a related investigation showed strong cold flow qualities and oxidative stability, which are legitimate traits of new-generation biodiesel [177]. This result showed that utilizing glycerol as the only carbon source, YO produced by *R. toruloides* TISTR 5186 under the effect of several non-ionic surfactants was appropriate for producing biodiesel.

YO has been applied to form bio-polyurethane foam at the laboratory scale. Uprety *et al.* shown that to use oleaginous yeast *R. toruloides* ATCC 10788 to convert crude glycerol to YO, which was subsequently chemically transformed to polyurethane foam. Results summarized that YO mainly contained C18:1 (47.2%) and C16:0 (24.4%), which can be used as a feedstock to produce polyols and subsequent conversion to polyurethane foams [71]. Samavi *et al.* have reported on the manufacture of polyol from

YO of *C. curvatus* by a more environmentally friendly enzymatic process utilizing a wood-based hemicellulose stream [172]. Results exhibited that YO was successfully converted to epoxidized oil, followed by a ring-opening reaction to produce polyol as a precursor of polyurethane foam formation. Samranrit *et al.* [173] explored the viability of converting *P. parantarctica* CHC28's YO synthesis into bio-polyurethane foam. Their findings showed that YO may be effectively transformed into bio-polyurethane foam as a backup technique for making BPU foam.

The YO of *R. toruloides* DSM 4444 and *C. curvatus* ATCC 20509, which were obtained from the side streams of wheat milling and confectionery, was assessed for use as a feedstock in the manufacturing of bio-lubricants. In both YOs, C18:1 and C16:0 FAs predominated. After the isolated YOs were hydrolyzed enzymatically, the free FAs were esterified in a solvent-free system using trimethylolpropane and neopentyl glycol (NPG) to create NPG esters using Lipomod 34-MDP. At more than 82%, YO can efficiently convert to an NPG ester. NPG esters show promise as physicochemical replacements for traditional lubricants in bio-based lubricant compositions [13]. Ma *et al.* [174] proved that the oil from *R. glutinis* CGMCC 2258 was appropriate for making bio-lubricants. The oil's unsaturated FA helped to enhance the tribological properties of bio-lubricants as well as their low-temperature lubrication. These bio-lubricants made synthetically have superior lubricating qualities than lubricants made from minerals of similar viscosity, suggesting that they could eventually replace conventional lubricants. These research' data demonstrated how different yeast strain species affect the compositions of FAs. Non-ionic surfactants were applied to *R. toruloides* TISTR 5186, which had a minor impact on the different FA compositions of both IO and EO. The results of this study indicated that YO obtained from *R. toruloides* TISTR 5186 was a suitable and feasible substitute feedstock for more research into the synthesis of other oil-based products.

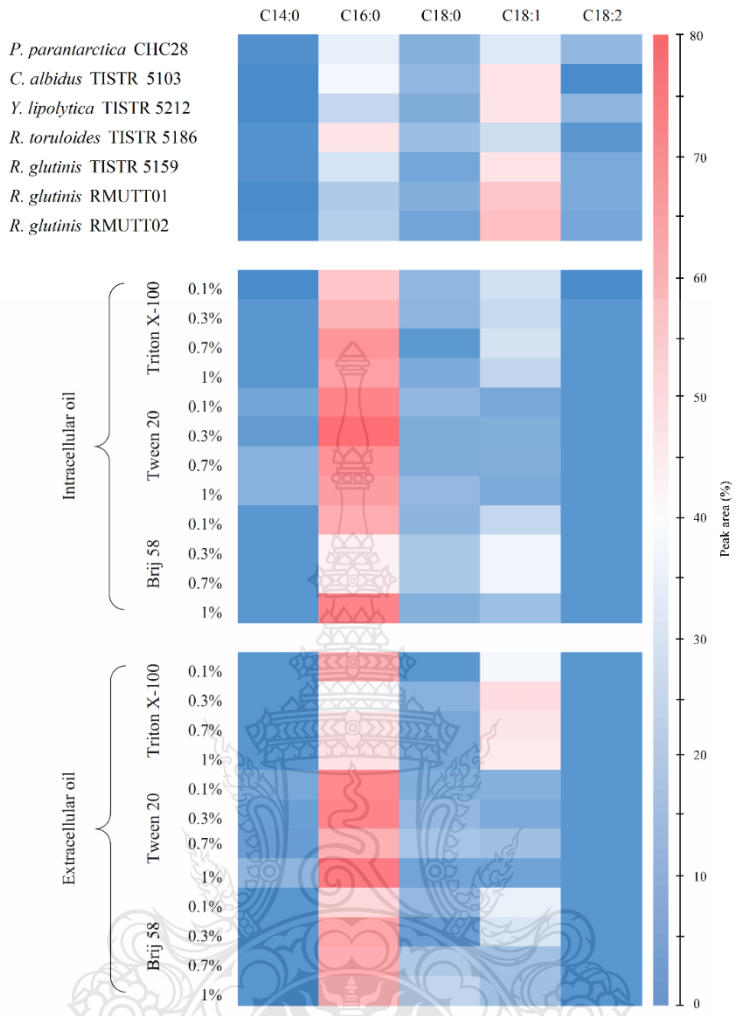


Figure 4.17 Heatmap of FA profiles in yeast oil.

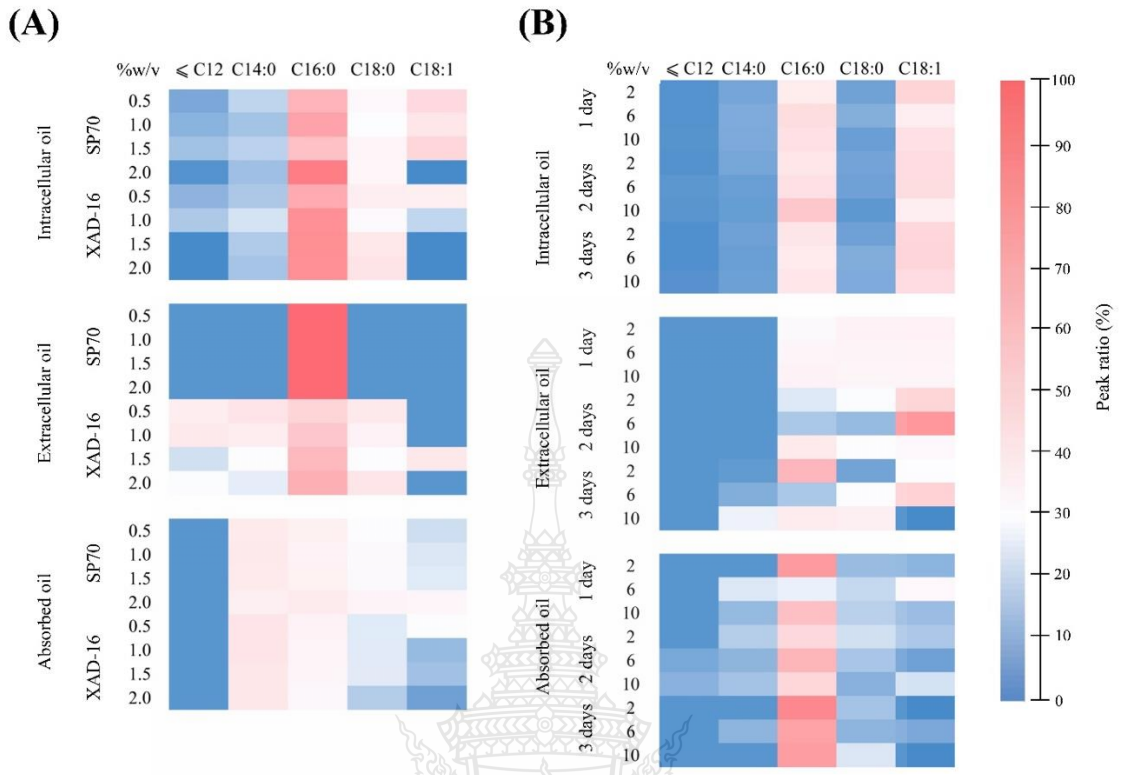


Figure 4.18 Heat map of FA compositions of YO achieved from in-situ and ex-situ oil recovery.

4.8 Preliminary biolubricant production

The preliminary biolubricant production from YO was evaluated. To avoid saponification, the free FA contained in YO was initially converted to FAME by esterification with methanol using HCl as a catalyst. Then, mono-, di-, and triglycerides remained in YO and were transformed to FAME by transesterification with methanol using NaOH as a catalyst. The FAMEs were reacted with TMP to be TMP ester as a biolubricant. The conversion profile of *R. toruloides* FAMEs to TMP ester is presented in Figure 4.19. The conversion yield was increased to 75.4% at 2 h and slightly enhanced to the maximum level of 85.90% at 24 h. The results showed that YO could successfully convert to biolubricant under this process. This finding is similar to the results reported by Mehdi *et al.*, who documented the conversion of FA to TMP triester, yielding a sustainable lubricant from *Pistacia atlantica* mutica (PAM) oil. The reaction of biolubricant from PAM oil is effectively conducted and optimized. The highest reaction

yield achieved was 86%, with optimal conditions including a vacuum pressure of 424.5 mmHg and a FAME to TMP molar ratio of 5:1[178].

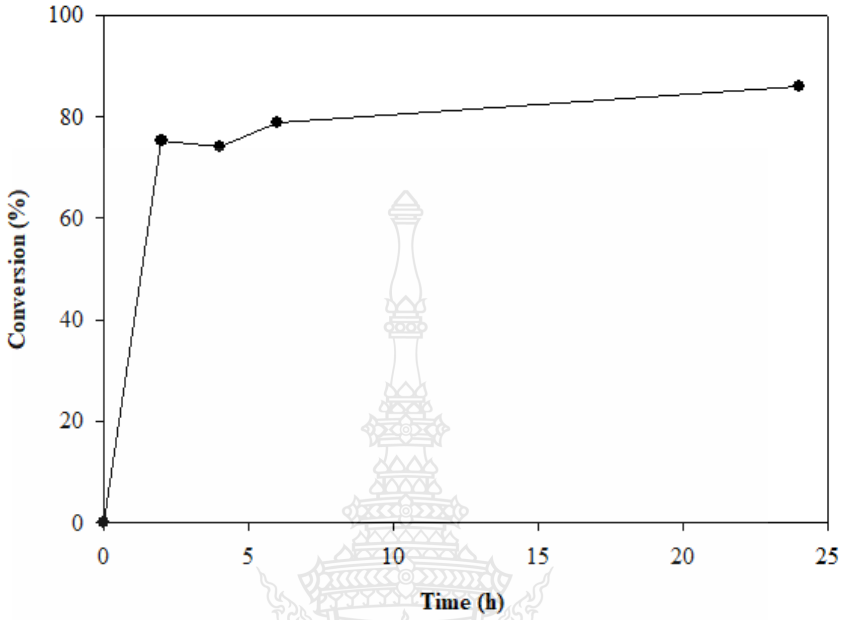
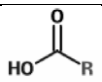
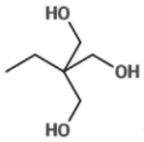
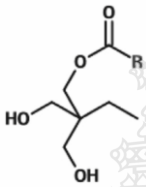
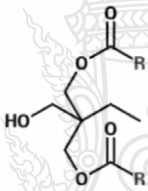
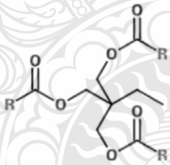


Figure 4.19 Time course of biolubricant production by esterification using the FAME of *R. toruloides* with TMP.

The composition of TMP esters according to the content of mono-, di-, and triesters was investigated by NMR analysis (Table 4.8). The esterification (2 h) employed using YO FAMEs with TMP synthesized mainly TMP monoesters (32.3%) and TMP diesters (16.9%). The non-esterified TMP was only 14.7%, corresponding to 24.6% free FAME (Table 4.8). At 24 h, the TMP monoesters (32.3%) and TMP diesters (16.9%) remained the major components. Additionally, free TMP and FAME decreased slightly to 9.8% and 14.2%, respectively. Similarly, Papadaki *et al.* reported the bioprocess development for biolubricant production utilizing *R. toruloides* oil and TMP. The quantity of TMP ester was determined by NMR analysis. The biolubricant product formed at 24 h consisted of triesters (47.8%), diesters (15.9%), and monoesters (13.8%), while the non-esterified TMP accounted for 7.4%, corresponding to 15.1% of free FAME [48].

Table 4.8 NMR analysis of biolubricant produced by reverse transesterification of FAMEs derived from *R. toruloides* YO with TMP.

Components	Chemical structure	% of each component			
		2 h	4 h	6 h	24 h
FAME		24.6	25.6	20.9	14.2
Free TMP		14.7	12.4	9.8	9.8
TMP monoesters		32.3	28.9	28.8	25.5
TMP diesters		16.9	17.5	19.2	21.2
TMP triesters		3.1	3.4	3.9	6.0

CHAPTER 5
CONCLUSIONS

This study showed how well yeast strains could produce YO from glycerol when non-ionic surfactants were applied. Compared to the other yeast strains under investigation, *R. toruloides* TIRTR 5186 produced YO with more efficiency. Non-ionic surfactant was added, and this caused YO to secrete, which increased EO and TO. The generation of TO was boosted by Tween 20 at 0.7% w/v, 2.7 times more than in the control treatment. The synthesis of YO was enhanced by non-ionic surfactants in response to the modification of *ACC* and *DGA* gene expression. The expression of the *ACC* and *DGA* genes increased by 3.4 times when Tween 20 was added. The unconstructed mathematical modeling provided a good description of the kinetic parameters. Non-ionic surfactants inhibited the specific growth rate, as shown by the modified logistic equation.

Consequently, the simultaneous YO production and recovery were investigated. The IO produced by *R. toruloides* TISTR 5186 was secreted to the cultural medium as extracellular oil (EO) by adding Tween 20 at 72 h. OCAs were then added to the cultivation medium to adsorb EO released from YO production. For in-situ oil recovery, SP70 provided higher oil recovery than XAD-16 by 32.09%. Using 2% (w/v) SP70, the maximum oil recovery and TO production were obtained at 91.72% and 8.39 g/L, respectively. In addition, the oil recovery was increased to 90.49% by ex-situ process with 10% SP70 for 1st day.

Subsequently, scaling up of simultaneous YO biosynthesis and *in-situ* recovery by batch fermentation in 5-L bioreactor. The results presented a more rapid glycerol consumption rate observed in the OCA-containing condition compared to the control during the exponential phase, influencing the growth rate and IO formation of *R. toruloides*. The yeast *R. toruloides* growth and IO production were enhanced in the OCA-containing condition compared to the control experiment. Under the SP70-containing condition, *R. toruloides* achieved a biomass and IO of 14.95 ± 0.17 g/L and 4.78 ± 0.06 g/L, respectively. Furthermore, the TO, $Y_{X/S}$, and $Y_{P/S}$ were maximized in the aforementioned

condition at 12.50 ± 0.09 g/L, 0.32, and 0.26, respectively. The oil content (83.58 ± 0.72% (w/w)) was not significantly different with control treatment. Online oil capturing integrated with YO production by batch and fed-batch modes in 5-L bioreactor was explored. In the batch mode, the overall oleaginous biomass and TO of *R. toruloides* were 25.31 ± 2.30 g and 18.88 ± 1.24 g, respectively. Glycerol consumption was measured at 98.08 ± 1.05 g, converted to biomass and TO with yields of 0.258 ± 0.026 and 0.19 ± 0.01, respectively. In the fed-batch mode, overall production indicated that the yield of biomass and TO were achieved at 0.36 ± 0.02 and 0.37 ± 0.08, respectively, resulting in an oil content of 102%.

The synthesis of YO from various strains and production methods using glycerol did not affect the FA compositions of *R. toruloides* significantly. The FA profiles were predominantly composed of C16:0 and C18:1. Additionally, YO was efficiently converted into biolubricant, achieving a conversion ratio of 85.90% within 24 h.

This study offers insights into moderating YO secretion and enhancing YO production efficiency in *R. toruloides*. Moreover, simultaneous YO biosynthesis and recovery can improve YO production performance. YO provides a promising alternative feedstock for biolubricant production.

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APPENDICES

APPENDIX A

Spectrum of FTIR

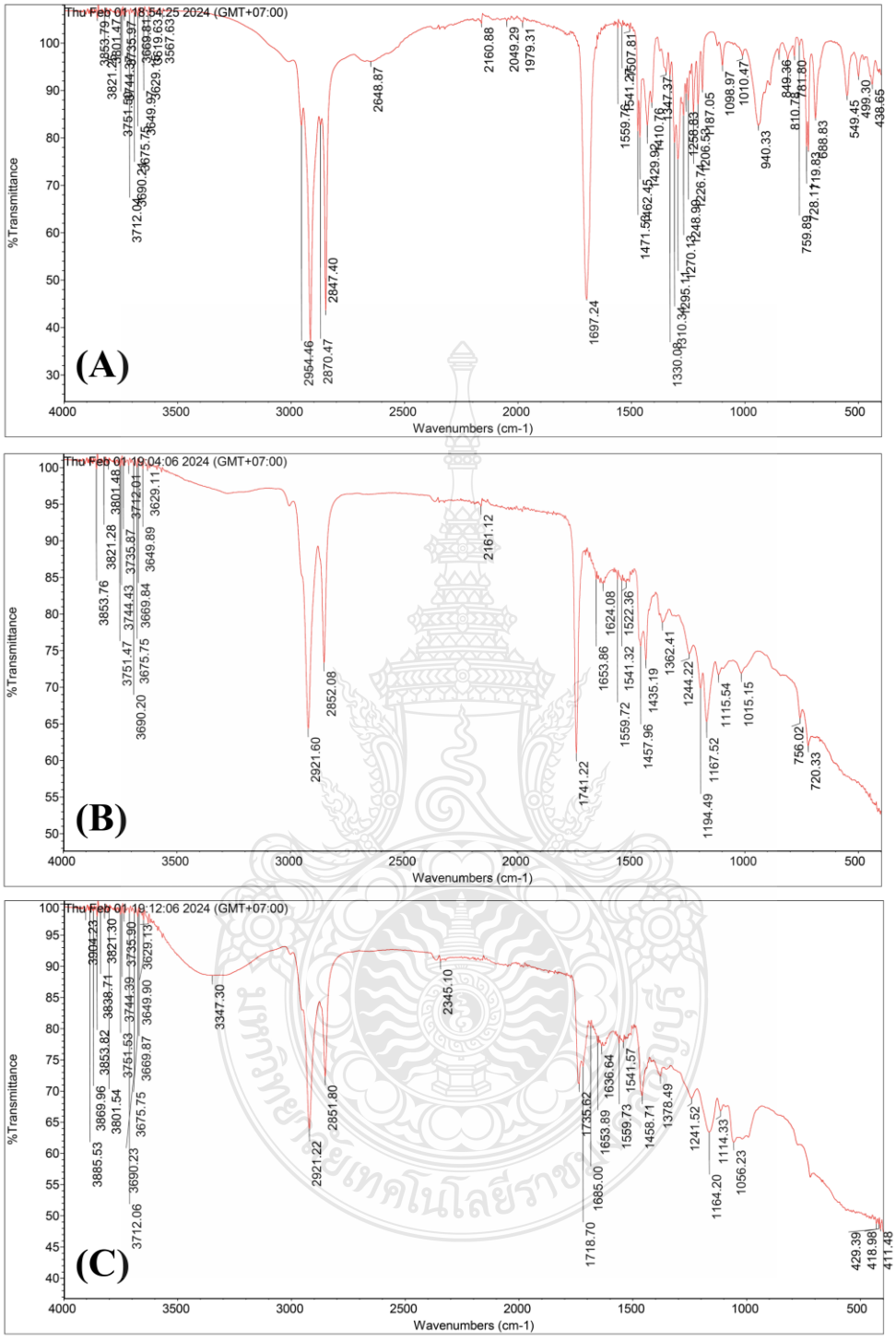

Figure A1 FTIR Spectrum of (A) YO, (B) FAMEs, and (C) biolubricant.

APPENDIX B

^{1}H proton NMR

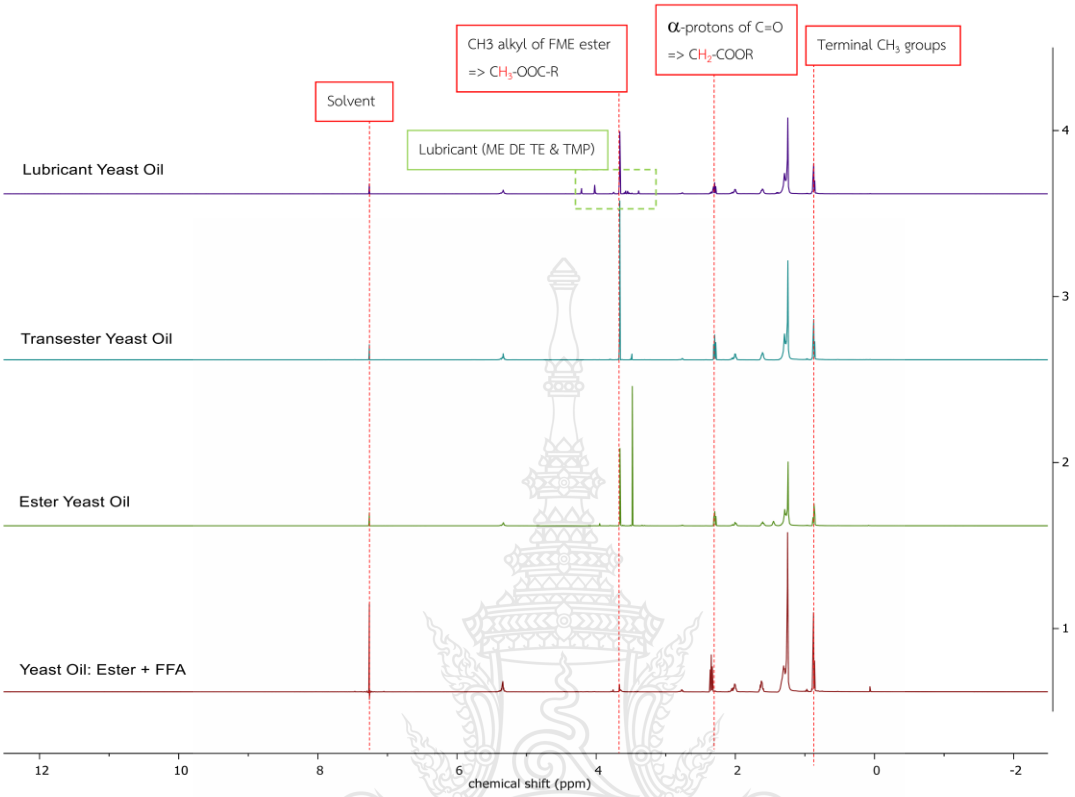

Figure B1 ^{1}H proton NMR of biolubricant, tranestered-YO, and estered-YO.

Biography

Name - Surname	Miss Kessuda Ngernsombat
Date of Birth	November 28, 1997
Address	111/1209, M. 1, Bueng Yeetao, Thanyaburi, Pathum Tani, 12130, Thailand
Education	Mater of Science (Applied Biology Program) (2021-2023)
Telephone Number	097-270-5450
Email address	K.ngernsombat@gmail.com