



Study on a new low-cost process for producing edible oils derived from microorganisms using membrane separation technology and food residues

高城, 博也

(Degree)

博士 (工学)

(Date of Degree)

2025-09-25

(Date of Publication)

2026-09-01

(Resource Type)

doctoral thesis

(Report Number)

甲第9325号

(URL)

<https://hdl.handle.net/20.500.14094/0100498365>

※ 当コンテンツは神戸大学の学術成果です。無断複製・不正使用等を禁じます。著作権法で認められている範囲内で、適切にご利用ください。



博士論文

Study on a new low-cost process for producing edible oils
derived from microorganisms using membrane separation
technology and food residues

(膜分離技術と食品残渣を利用した低コストな微生物由来
食用油脂の新規生産プロセスに関する研究)

2025年7月

神戸大学大学院工学研究科

(氏名) 高 城 博 也
HIROYA TAKI

Acknowledgment

My time spent on my doctoral studies at Kobe University was challenging yet deeply rewarding. As I come to this milestone, I would like to take this opportunity to express my heartfelt thanks to everyone who has supported me.

Above all, I would like to express my heartfelt gratitude to Prof. Matsuyama, my supervisor. His appropriate guidance, insightful advice, and unwavering support have illuminated my academic journey. I consider it a great fortune and honor to have had the opportunity to learn from him.

I am also deeply grateful to Institute Prof. Kumagai for his tremendous support. It would have been impossible for me to complete my doctoral research while working at the company without his help. He not only guided me in how to conduct my research and write my thesis, but also provided direct guidance on experimental techniques. Although opportunities for face-to-face discussion were limited due to work in company, he provided heartfelt guidance and suggestions through close communication by email.

I would also like to express my gratitude to Dr. Takagi. He provided me with a great deal of advice and reviews in the process of completing this doctoral thesis. His kindness contributed greatly to improving my thesis.

I would also like to thank Prof. Funahashi, Prof. Ogino, Associate Prof. Kamio, and Associate Prof. Nakagawa for their careful review of this thesis. Furthermore, I would like to express my sincere gratitude to Prof. Yoshioka, Assistant Prof. Guan, Assistant Prof. Okamoto, Assistant Prof. Matsuoka, and the members of the Membrane Engineering Group, including Ms. Miyake, Ms. Kamio, and Ms. Ono, for their dedicated support, which significantly contributed to the smooth progress of my research.

Finally, I would like to express my deepest gratitude to my parents for their unwavering love and support. Thanks to their encouragement and support, I was able to gain the strength to pursue my study while working in the company.

Hiroya Taki
Kobe University, Japan, July, 2025

Table of contents

Acknowledgment.....	I
Table of contents	II
Chapter 1.....	1
1.1. Research background.....	1
1.2. Structure and characteristics of oil and fat.....	2
1.3. Oil and fat application and challenges	3
1.4. Oleaginous microorganisms	4
1.4.1. Various types of oleaginous microorganisms	5
1.4.2. Oleaginous yeasts	6
1.4.2.1. Characteristics and types of oleaginous yeasts	6
1.4.2.2. <i>Lipomyces starkeyi</i>	7
1.5. Process of oil production by <i>L. starkeyi</i>	10
1.5.1. Process flow and oil extraction.....	10
1.5.2. Cost for oil production.....	11
1.5.2.1. Upstream process	12
1.5.2.2. Downstream process.....	13
1.6. TAG synthesis mechanism in <i>L. starkeyi</i>	13
1.7. Membrane and membrane separation technology	15
1.7.1. Types and characteristics of membranes	16
1.7.2. Application of membrane technology	18
1.8. Current challenges in oil production by <i>L. starkeyi</i>	18
1.9. Purpose and overview of this dissertation	19
Chapter 2.....	23
2.1. Introduction	23
2.2. Experimental.....	24
2.2.1. Strain and growth conditions	24
2.2.2. Crushing treatment and preparation of emulsifying solution	25
2.2.3. Filtration separation and oil extraction	25
2.2.4. Analysis of fatty acid composition and calculation of TAG equivalent for extracted oil.....	27
2.2.5. Estimation for oil extraction performances	28
2.2.6. Required energy and cost estimation	29
2.2.7. Verification of extraction mechanism by tracking material balance	29
2.2.8. Verification of extraction mechanism by using model solution	30
2.2.9. Statistical analysis	30
2.3. Results and discussion	30
2.3.1. Oil extraction with hexane from crushed <i>L. starkeyi</i> culture.....	30
2.3.2. Oil extraction with pure solvent after nonwoven filtration.....	31
2.3.3. Oil extraction with solvent mixture after nonwoven filtration	32
2.3.4. TAG extraction efficiency and TAG selectivity.....	33
2.3.5. Estimation of the required energy and cost	35
2.3.6. Verification of extraction mechanism by tracking material balance	36
2.3.7. Verification of extraction mechanism by using model solution	37
2.4. Conclusions	38
2.5. Supplemental information	39
Chapter 3.....	43
3.1. Introduction	43
3.2. Experimental.....	45
3.2.1. Strain and growth conditions	45
3.2.2. Preparation of okara extract solution	45
3.2.3. Membrane concentration of okara extract solution	46
3.2.4. Analysis	46

3.2.4.1. Viscosity.....	46
3.2.4.2. Total organic carbon (TOC).....	47
3.2.4.3. Total nitrogen (T-N).....	47
3.2.4.4. Ion concentration.....	47
3.2.5. Evaluation of TAG production	48
3.2.5.1. Measurement of cell concentration	48
3.2.5.2. Measurement of TAG	48
3.2.5.3. Microscope observation	48
3.2.6. Statistical Analysis	48
3.3. Results and discussion	49
3.3.1. TAG production using okara as a nitrogen source	49
3.3.2. Preparation of okara extract and evaluation as nitrogen source for culture media	51
3.3.3. Membrane concentration of nitrogenous components in the okara extract solution	54
3.3.4. Culture evaluation of okara membrane concentrate as a raw material for culture media.....	57
3.4. Conclusions	61
Chapter 4.....	63
4.1. Introduction	63
4.2. Experimental.....	64
4.2.1. Yeast strain, growth conditions, and chemicals.....	64
4.2.2. Analysis for NH_4^+ , glucose, cell concentration, and TAG	65
4.2.3. Metabolome analysis	65
4.2.4. Data analysis.....	66
4.3. Results and discussion	67
4.3.1. Quantitative evaluation of nitrogen components in okara and yeast extract media.....	67
4.3.2. Effect of ammonium salt addition	68
4.3.3. Effects of ammonium sulfate addition based on a metabolome analysis	73
4.3.4. Estimated mechanism of enhanced GSH metabolism and improved oil productivity mediated by ammonium sulfate addition.....	81
4.4. Conclusions	85
Chapter 5.....	87
5.1. Simple and economical downstream process development for edible oil production from oleaginous yeast <i>Lipomyces starkeyi</i>	87
5.2. Utilization of okara as a culture medium by membrane concentration process for high oil production by oleaginous yeast, <i>Lipomyces starkeyi</i>	88
5.3. Effects of inorganic nitrogen addition to okara-utilized medium on oleaginous yeast, <i>Lipomyces starkeyi</i> and estimation of metabolism involved in the high oil production	89
5.4. Summary of cost reduction effects on total oil production process of <i>L. starkeyi</i>	89
5.5. Overall summary of the study.....	92
References.....	95
Publication list.....	111

Chapter 1

General introduction

1.1. Research background

Oils and fats play an important role in the biological functions of plants and animals [1]. Oils and fats are best known for their role as a source of energy, providing 9 kcal of energy per gram, which is more than twice as much as other nutrients such as carbohydrates and proteins [2]. Also, linoleic acid, alpha-linolenic acid, and arachidonic acid in oils and fats are essential for humans, but cannot be synthesized in their body. Thus, they must be taken in through diet [3]. In addition to the foods mentioned above, oils and fats are widely used around the world in chemical products, industrial applications, and fuel, therefore, oils and fats are essential resources for human life.

The production of vegetable oils reached over 200 million metric tons (mt) in 2024 [4]. Among them, palm oil is currently the most produced and used for various applications because its cultivation is characterized by lower prices and higher productivity per unit area than other vegetable oils [4, 5]. However, the palm oil industry faces various issues such as environmental and human rights concerns [5, 6]. Additionally, oil palm trees can only be cultivated fundamentally in tropical regions such as Malaysia and Indonesia. Thus, there is a risk that climate change caused by global warming may further reduce cultivation areas in the future [7]. For these reasons, considering a sustainable and stable supply of oil and fat, there is a need for alternatives that can replace vegetable oils such as palm oil.

In this thesis, oleaginous microorganisms that accumulate oil and fat in cells [8] were focused on. Some of them can produce oil and fat from low-cost food residues, enabling an economical

and sustainable process. Therefore, the development of a low-cost and stable method for supplying alternative palm oil by using the microorganisms and food residues was considered.

1.2. Structure and characteristics of oil and fat

Oils and fats are organic compounds formed by the ester bonds between fatty acids and glycerin (glycerol), mainly referring to triglycerides (triacylglycerols, TAG) (**Fig. 1.1**). The properties of TAG depend on the types of fatty acids in their composition and their configuration. Considering the number of possible combinations, there are n^3 (where n is the number of fatty acids) combinations, which means that there are a wide variety of TAG with diverse properties. In particular, fatty acids are classified into unsaturated fatty acids, which contain double bonds in their structure, and saturated fatty acids, which do not. Oils and fats with high contents of unsaturated fatty acids are often liquid at room temperature (normally called “oils”, e.g., olive oil and rapeseed oil), while oils and fats with rich saturated fatty acids are solid (normally called “fats”, e.g., butter and lard) [9]. In addition, the length of the carbon chains of the constituent fatty acids and their stereochemical positions affect crystallization, which in turn affects the rheological properties, hardness, melting point, and other properties of oils and fats [10].

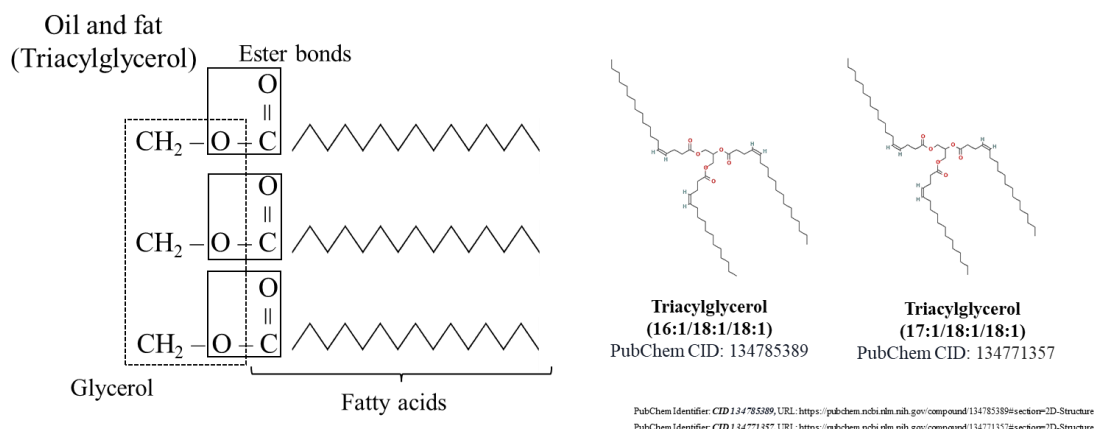


Fig. 1.1 Structure and examples of triacylglycerol (TAG).

1.3. Oil and fat application and challenges

Oils and fats are widely used around the fields in food, chemical products, and industrial applications due to their diverse chemical and functional properties. In food applications, oils and fats contribute to flavor and physical properties. For example, butter enhances flavor with its aroma and acts as an emulsifier to make foods soft and creamy [11]. Also, as oils and fats used for frying (e.g., for fried noodles and snack foods), they must be chemically stable, mainly for oxidation, at high temperatures. Therefore, oils and fats with a high proportion of saturated fatty acids are used, which are highly stable due to no double bonds in their structure [12]. Additionally, to prevent oil leakage from the fried products during transportation and storage, oils with solid properties at room temperature are preferred, such as palm oil, which is commonly used as frying oil, has a melting point of about 35°C and is solid at room temperature, making it suitable for the manufacturing of them [4].

As chemical products, fatty acids such as lauric acid mainly derived from palm oil are chemically converted through esterification and alkylation to produce surfactants, which are then processed into the products such as shampoos, conditioners, and detergents [13].

In industrial applications, oils and fats are sometimes used as fuels and lubricants. Also, fatty acid methyl esters obtained by reaction of TAG with methanol are used as biodiesel fuels [14]. In recent years, with the global demand for carbon neutrality, biodiesel fuel has been attracting attention due to its carbon neutrality [15]. Therefore, the demand for oil and fat as a substitute for fossil fuels is expected to continue to increase year by year.

In fact, the total production of vegetable oils, including coconut oil, cottonseed oil, olive oil, palm kernel oil, peanut oil, rapeseed oil, sunflower oil, soybean oil, and palm oil, was approximately 80 million metric tons (mt) in 2000, and then reach 223.8 million metric tons (mt) in 2024 [4]. Furthermore, global demand for vegetable oils is predicted to increase by 46% by 2050 compared to 2020 levels [16]. Among them, palm oil is the most produced [4], with its production increasing by 229% from 2000 to the present. And it is expected that global palm oil production will exceed 80 million tons over the two-year period of 2024/2025 [4]. This significant increase in production highlights the importance of palm oil, which is now the most important edible oil and industrial oil in the world, accounting for more than 35% of global vegetable oil production. The high cultivation efficiency and low cost of palm oil cultivation are the main factors behind the increase in production [4, 5]. However, the palm oil industry faces various issues such as environmental destruction, climate change risk and human rights concerns [5, 6, 7]. Therefore, from the point of view of a sustainable and stable supply of oil and fat resources, vegetable oils alternatives such as palm oil are urgently needed.

1.4. Oleaginous microorganisms

Table 1.1 summarizes various oleaginous microorganisms and their oil and fat contents reported, including bacteria, fungi, microalgae and yeast. They are defined as the microorganisms that accumulate oil and fat as TAG equivalent to more than 20% of their cell weight [8]. In addition, they

have shorter life cycles and higher production efficiency than animals and plants, making them attractive as oil and fat alternatives.

Table 1.1 Types of oleaginous microorganisms and their oil and fat content.

Species	Organisms	Oil and Fat Contents %(w/w)	References
Bacteria	<i>Rhodococcus</i> sp.	33	[17]
	<i>Streptomyces</i> sp.	50	[17]
	<i>Bacillus subtilis</i>	40	[18]
Fungi	<i>Mortierella alpina</i>	52	[20]
	<i>Mortierella alliacea</i>	42	[21]
	<i>Mucor moelleri</i>	24	[22]
Microalgae	<i>Chlorella</i> sp.	54	[23]
	<i>Botryococcus braunii</i>	32	[24]
Yeast	<i>Yarrowia lipolytica</i>	89	[26]
	<i>Rhodospiridium toruloides</i>	77	[26]
	<i>Lipomyces starkeyi</i>	87	[26]

1.4.1. Various types of oleaginous microorganisms

Among bacteria, the genera *Rhodococcus* [17], *Streptomyces* [17], and *Bacillus* [18] are known as oleaginous microorganisms. For example, *Streptomyces* sp. strain G25 showed 50% lipid (TAG and fatty acids) accumulation by dry cell weight in a medium using cellobiose as a carbon source [17]. However, in general, the lipid productivity of bacteria is not as high as that of other oleaginous microorganisms, so their suitability as alternative producers of inexpensive oil and fat is not high.

In filamentous fungi, oils and fats produced by *Mortierella alpina* are characterized by their high content of arachidonic acid, an essential fatty acid. Furthermore, they have been certified as GRAS (Generally Recognized as Safe) by the US Food and Drug Administration (FDA) and are being

investigated for commercialization [19]. However, their oils differ from vegetable oils and animal fats in their fatty acid composition, and their TAG production is not as high as that of other oleaginous microorganisms [20-22].

Microalgae are actively being studied because they can contribute to the prevention of global warming, and to the establishment of a recycling-oriented society. They have the ability to grow and produce organic matter through photosynthesis using carbon dioxide as a carbon source, and to accumulate part of this organic matter as oil and fat [23, 24]. However, they also have some issues such as low productivity, the need for vast cultivation areas for photosynthesis, and the risk of contamination due to their open cultivation environment [25].

1.4.2. Oleaginous yeasts

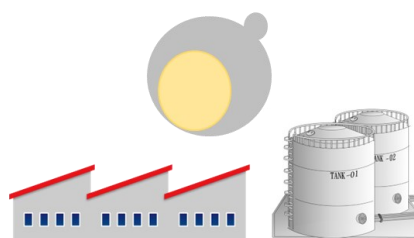
1.4.2.1. Characteristics and types of oleaginous yeasts

A yeast species called oleaginous yeasts is thought to have the potential to solve the problems of oleaginous microorganisms described above. Oleaginous yeast can accumulate lipids up to 70% of their dry weight, which have a fatty acid profile similar to vegetable and fish oils, making it suitable for a variety of applications, including biofuels and foods [26, 27]. Furthermore, they are known for their broad sugar utilization and can produce oils using sugars derived from inedible biomass as a carbon source [27, 28]. **Fig. 1.2** shows a comparison between oil production from conventional oil plants and oleaginous yeast. Oil plants have advantages in terms of price and usage experience, while the oils from oleaginous yeast can be produced in culture tanks, so it does not require large areas of land or specific climatic condition. This offers significant advantages in terms of environmental destruction, climate change risk, and stable supply, resulting in active research in recent years [29, 30].



Oil plants

- Lower price
- Traditional industrial use experience
- × Environmental impact
- × Climate change risk
- × Stable supply risk
- × Cultivation restrictions



Oleaginous yeast

- × Higher cost
- × Industrial application examples
- Potential for reducing environmental impact
- No climate change risk
- Stable supply possible
- No production location restrictions

Fig. 1.2 Comparison of oil plants and oleaginous yeast.

Yarrowia lipolytica [31], *Rhodospiridium toruloides* [32] and *Lipomyces starkeyi* [33] are well known as oleaginous yeasts. *Y. lipolytica*, the most representative oleaginous yeast, has long been studied as a model organism, which can accumulate more than 20% of oil by dry cell weight. However, in terms of oil productivity, it is generally considered to be less advantageous than *R. toruloides* and *L. starkeyi* [26, 31]. *R. toruloides* is an oleaginous yeast known for its high oil productivity, characterized by the accumulation of red oil containing carotenoids in the cells [26, 32]. It has also been shown to exhibit high cell density and high oil-to-sugar conversion rate, making it a yeast with growing potential as a producer of alternative oils and fats [26, 29].

1.4.2.2. Lipomyces starkeyi

L. starkeyi has been reported to have the ability to accumulate intracellular oil by a dry cell weight ratio of more than 85% [26, 34]. The fatty acid composition of its oil is mainly oleic acid and palmitic acid, which is highly similar to that of palm oil (**Table 1.2**) [35, 36].

Table 1.2 Fatty acid composition (%) of various vegetable oils.

	Palmitic acid (C16:0)	Palmitoleic acid (C16:1)	Stearic acid (C18:0)	Oleic acid (C18:1)	Linoleic acid (C18:2)	Others
<i>L. starkeyi</i>	35.7	3.1	5.5	46.5	3.7	5.5
Palm	39.6	5.4	-	42.9	8.9	3.2
Canola	4.5	3.3	-	65.7	15.1	11.4
Corn	12.9	2.2	-	19.9	68.1	-
Grapeseed	7.2	4.8	-	19.9	68.1	-
Olive	11.0	4.3	0.1	75.7	4.8	4.1
Soybean	12.2	6.5	0.1	29.1	44.1	8.0
Sunflower	5.5	4.2	-	26.2	64.1	-

The fatty acid composition of *L. starkeyi* was referenced from the report by Yamazaki *et al.* [35]. Other than those described below was based on the report by Alves *et al.* [36]. Example: “C16:1” means that carbon chain length is 16 and there is one unsaturated bond.

Additionally, it is known to have the ability to utilize a wide range of sugars, including glucose, xylose, arabinose, galactose, mannose, and cellobiose [37], making it a subject of active studies on the effective utilization of wastes (**Table 1.3**) [38-45].

Table 1.3 Examples of oil production using *L. starkeyi* from waste materials.

Material	Pretreatment	Products	Reference
Potato starch wastewater	-	Biomass yield 2.59 Oil content 8.88	[39]
Soluble starch	-	Oil yield 6	[40]
Sugarcane bagasse	Hydrolysis	Biomass yield 9.6 Oil yield 2.51 Oil content 26.1	[41]
Soybean hulls	Hydrolysis	Biomass yield 26.5 Oil content 42.5	[42]
Rice straw	Hydrolysis	Biomass yield 12.76 Oil yield 4.55 Oil content 35.65	[43]
Volatile fatty acids	-	Biomass yield 30.0 Oil yield 5 Oil content 16.8	[44]
Olive oil mill wastewater	-	Biomass yield 10.4 Oil yield 0.23 Oil content 22.4	[45]

Biomass yield (g/L) = g dry cell weight/ L culture medium, Oil yield (g/L) = g oils produced/ L culture medium, Oil content (%) = g oils produced/ g dry cell weight

It has a particularly high starch utilization capacity, and has been reported to produce oil from starch and potato starch wastewater [39, 40, 46]. Also, *L. starkeyi* has an excellent ability to utilize non-edible biomass, proposed by several studies on oil production from hemicellulose degradation products (sugarcane bagasse, soybean hulls, rice straw, etc.), which is plant cell wall components [41-43]. As described above, *L. starkeyi* can produce oil and fat efficiently not only from glucose, which is commonly used, but also from wastes containing multiple types of sugars and

inedible biomass. These advantages allow for reduced culture costs and make it a suitable alternative oil producer, as an alternative for palm oil with high demand worldwide.

1.5. Process of oil production by *L. starkeyi*

1.5.1. Process flow and oil extraction

Since the *L. starkeyi* accumulates oil within its cells, extraction and purification processes are necessary for production and practical application of the oil. In previous studies, the following processes have been implemented to obtain oil as shown in **Fig. 1.3** [35, 47-49]: (1) culture, (2) cell collection, (3) freeze-drying, (4) cell crushing, (5) extraction, and (6) purification.

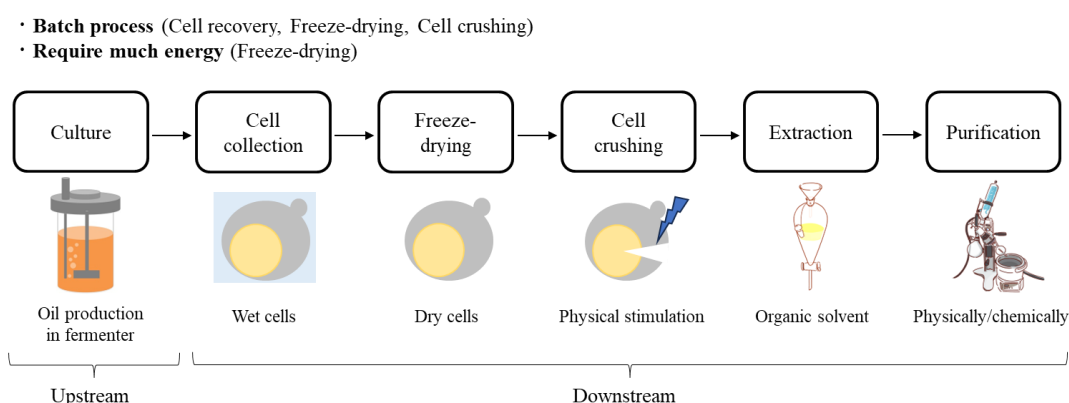


Fig. 1.3 Process flow and issues of oil production from oleaginous yeast.

Freeze-drying is performed to improve the efficiency of physical cell crushing, but it requires a significant amount of power. The disruption process of dried cells typically employs batch-type methods such as bead shockers and ball mills. Organic solvent extraction was performed by chloroform and methanol due to high efficiency of oil extraction and recovery. However, they are not suitable for the food industry because of its harmfulness to the human body.

Therefore, it is necessary to consider continuously processing without a drying step by crushing cells in liquid and supplying the treated culture liquid to the oil extraction process. However,

the treated culture is strongly emulsified by water, yeast oil, and yeast-derived components. Thus, extraction using hexane, which is a conventional extraction method in the food industry, should be difficult [50].

1.5.2. Cost for oil production

As mentioned above, palm oil is cheaper than other vegetable oils, with the price of crude palm oil in 2022 was 5,087 RYM per ton (approximately US\$1,094 at the time) [51]. The conversion rate of sugar to oil by oleaginous yeast is about 20-30% [52]. Therefore, the price of sugar as raw material must be less expensive than US\$200 to US\$300 per ton for the oil production to be profitable. This simple calculation does not account for other process costs such as medium components, labor, and energy costs. On the other hand, the price of glucose was approximately US\$900 per ton in 2025 [53], and it is clear that the use of glucose for oil production by yeast is not feasible from a cost perspective. Therefore, the actual price of raw sugar used in oil production by oleaginous yeast must be further reduced to ensure economic viability, indicating that research and development to reduce culture costs and process costs is critically important.

In this study, the concept of cost reduction for oil production will be evaluated based on the cost per unit weight of oil. For example, if the costs of medium raw materials are reduced by half, the cost per unit weight of oil will be half. On the other hand, if the amount of oil obtained per liter of culture medium (oil yield) is doubled, the cost per unit weight would also be half. Based on the above, strategies for cost reduction can be broadly categorized into two main approaches: (1) reducing process costs and (2) increasing oil yield per unit of culture medium.

1.5.2.1. Upstream process

The upstream process of oil production by the yeast is a culture process, and the main nutrients in the culture medium are nitrogen and carbon. A previous report showed that culture media cost accounts for 60-75% (average: 67.5%) of the total cost, and carbon sources account for 80% of culture media cost [27]. Therefore, the cost of nitrogen sources accounts for about 13.5% of the total ($67.5 - 67.5 \times 80/100$). In addition, stirring and air supply are necessary to operate the culture tank and perform fermentation. The cost of the power required for this is estimated to be about 17.6% [54]. Based on the above, the total cost in the upstream process is estimated to be roughly 85%.

In the medium composition, yeast extract is commonly used as the nitrogen source, and glucose as the carbon source [30, 33] (**Table 1.4**). The research on these raw material substitutes is highly effective for reducing medium costs, there are many reports on the utilization of wastes as a carbon source [26, 38]. On the other hand, organic nitrogen sources such as yeast extract, peptone, and urea show high oil and fat productivity, but are considered economically unfeasible [38]. Nevertheless, there are no reports on the replacement of nitrogen sources.

Table 1.4 Culture medium components in oleaginous yeast and their roles.

Type	Roles	Examples
Carbon sources	Materials for lipid conversion	Glucose, Xylose, Cellobiose, Starch, Oligosaccharide
	Energy production	
	Cell proliferation	
Nitrogen sources	Materials for nucleic acids and proteins	<u>Organic</u>
	Cell proliferation	- Yeast extract, Peptone, Urea
		<u>Inorganic</u> - NH_4^+ , NO_3^-
Mineral salts	Auxiliary role in proliferation and lipid production	MgSO_4 , CuSO_4 , MnSO_4 , ZnSO_4

In yeast, nitrogen components are essential for the synthesis of proteins and nucleic acids and are particularly important for cell proliferation. They are classified into organic nitrogen (such as proteins, peptides, and amino acids) and inorganic nitrogen (such as ammonia and nitrate). In the report on budding yeast, *Saccharomyces cerevisiae*, glutamic acid is said to supply 85% of total cellular nitrogen, with glutamine supplying the remaining 15% [55]. This report is consistent with reports that organic nitrogen contributes greater oil productivity than inorganic nitrogen in the oleaginous yeast.

1.5.2.2. Downstream process

The downstream processes is the process after oil and fat production by yeast cells in the culture process. They involve cell collection and freeze-drying in conventional methods, resulting in high energy cost. According to the results of cost estimates for upstream processes, the proportion of total downstream costs is approximately 15%. The cell collection process is considered to account for 25-60% of the downstream process cost [56]. Referring to a report evaluating the economic efficiency of oil production using oleaginous yeast, the crushing process is estimated to account for 7%, and the oil extraction process is expected to account for 33% of the downstream process [54]. Therefore, there is a need to develop a new production process that are energy-saving and cost-effective focusing on cell collection and oil extraction.

1.6. TAG synthesis mechanism in *L. starkeyi*

The basic mechanism of TAG synthesis by *L. starkeyi* has been clarified, and an example using glucose as a sugar source is shown in **Fig. 1.4**. Glucose taken up from the extracellular is converted into glycerol-3-phosphate, one of the components of TAG, by the glycolysis pathway or the pentose phosphate pathway. Fatty acids, another component, are synthesized from acetyl-CoA and malonyl-CoA produced when glucose is taken up from the glycolysis pathway into the tricarboxylic

acid (TCA) cycle. Then, glycerol-3-phosphate and fatty acids combine, and TAG is synthesized in the endoplasmic reticulum [38, 57].

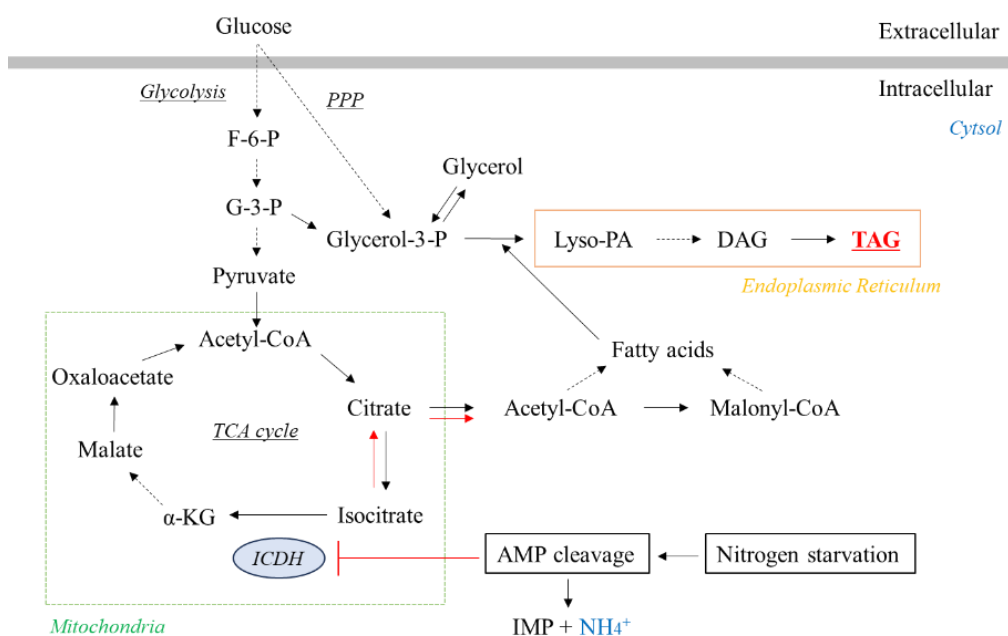


Fig. 1.4 TAG synthesis pathway in *L. starkeyi*.

Abbreviation in **Fig. 1.4**: F-6-P, Fructose-6-phosphate; G-3-P, Glyceraldehyde-3-phosphate; PEP, Phosphoenolpyruvate; Glycero-3-P, Glycerol-3-phosphate; Lyso-PA, Lysophosphatidic acid; DAG, Diacylglycerol; TAG, Triacylglycerol; PPP, Pentose Phosphate Pathway; α-KG, α-Ketoglutaric acid; ICDH, Isocitrate dehydrogenase; AMP, Adenosine monophosphate; IMP, Inosine monophosphate.

Also, nitrogen source limitation is considered to be one of the main factors affecting oil accumulation in oleaginous microorganisms, and the following mechanisms have been reported [33]: When nitrogen sources are limited, intracellular adenosine monophosphate (AMP) is cleaved to provide ammonium ions (NH₄⁺). The decrease of AMP concentration reduces the activity of isocitrate dehydrogenase (ICDH) in the TCA cycle in mitochondria, which causes an accumulation of isocitrate. This leads to negative feedback in the TCA cycle, resulting in citric acid accumulation and release into the cytosol. This citric acid activates the fatty acid synthesis and the oil/lipid (mainly triacylglycerol,

TAG) synthesis pathways. TCA cycle is a metabolic system for cell proliferation associated with nucleic acid synthesis. Therefore, to increase the oil yield per culture, TCA cycle should predominate in the presence of nitrogen components in the first half of the culture. Then, the metabolic system should be shifted to fatty acids and TAG synthesis in the absence of nitrogen components in the second half of the culture process. As a result, more yeast cells with high oil accumulation were obtained, and the oil yield per culture increased.

1.7. Membrane and membrane separation technology

There are various technologies for separating substances, such as distillation [58], crystallization [59], extraction [60], and membrane separation [61]. Membrane separation utilizes a sieving effect by pore on the membrane surface and interactions between the membrane surface and substances to perform various separation operations. The operation include removing particles contained in liquids or gases, filtering substances dissolved in solutions, and concentrating and purifying substances. In membrane filtration, the membrane behaves as a semi-permeable material and controls the transport rate of various molecules between two liquid phases. The most commonly used process is a pressure-driven membrane process, which utilizes applied pressure to push water molecules through the membrane [61]. In this process, larger particles are retained and concentrated in a feed, called retentate, and the water that passes through the membrane is called permeate. Membrane filtration is energy efficient, easy to operate, and low maintenance costs, however, membrane uncleanliness (called fouling) is a significant issue [62]. Membrane fouling causes a decrease in permeate flux due to substances (called foulant) on the membrane surface or within the pores, requiring expensive chemical cleaning to reduce the effects of fouling [63].

Membrane performance is evaluated based on two factors, permeate flux (treatment speed) and rejection rate (selectivity). Fouling affects both factors, so the ease of recovery from fouling

through cleaning are also important factors. They affect the frequency of membrane replacement in processes and are therefore considered important aspects of membrane functionality from an economic perspective [64, 65]. Regarding the evaluation criteria for these membrane properties, active research is being conducted to improve performance through the examination of membrane materials, surface patterning, and pretreatment methods [64]. On the other hand, it is also important to select membranes and operating conditions that are suitable for each specific application process [66]. As noted above, membrane processes involve numerous factors, and detailed examination of each factor contributes to the advancement of membrane technology.

1.7.1. Types and characteristics of membranes

Membranes are generally classified depending on a material into polymer-based organic membranes or inorganic ceramic membranes [67]. Furthermore, they are classified into microfiltration (MF) membranes, nanofiltration (NF) membranes, ultrafiltration (UF) membranes, and reverse osmosis (RO) membranes based on molecular weight cut-off (MWCO) [68]. **Fig. 1.5** shows the performance of membrane processes for various sizes of target components in water.

Particle size (μm)	0.0001	0.001	0.01	0.1	1.0	10	100
Molecular weight (Da)	< 100	200	20,000	> 500,000			
Membrane type	Reverse osmosis (RO)		Ultrafiltration (UF)		Conventional filtration		
		Nanofiltration (NF)			Microfiltration (MF)		
Separation target	Ions		Dissolved organics e.g., Peptides, amino acids, sugars, acids, etc.		Other particles		
			Protein				
				Bacteria, yeast			

Fig. 1.5 Types of membranes and separation targets.

MF is suitable for removing microorganisms such as bacteria and yeast, as well as particles, and is therefore used in sterilization filters and liquid clarification [69-71]. UF is suitable for separating high-molecular-weight substances such as proteins and is used for protein removal in research applications and as a pretreatment for RO membrane processing [72, 73]. The studies of UF are being conducted on improving fouling resistance by coating the surface as a membrane modification [73, 74]. NF has features between UF and RO, and in addition to separation by pores, it has properties such as high permeability and fouling resistance through modification such as membrane surface coating and graft polymerization [75, 76]. RO is suitable for treating seawater and wastewater because they block almost all substances other than water. In wastewater treatment, chemical fouling and biofouling caused by various organic substances and microorganisms often reduce permeate flux. To solve this problem, many technologies have been developed to suppress the adhesion of microorganisms and organic matter by controlling the surface charge or modifying the surface with hydrophilic polymers [77-80].

1.7.2. Application of membrane technology

Recently, membrane technologies have been widely applied in various fields, such as the food industry [70, 71, 81], drinking water production [82-84], biotechnology [85, 86], and wastewater treatment [66, 87, 88], taking advantage of their respective characteristics. Also, membrane separation technologies are promising for the separation, concentration, and purification of useful substances particularly because the properties of them can be maintained by using moderate temperatures during the processes [89]. For example, there are examples of recovering high value-added compounds, such as research on separating and recovering phenols from food processing waste using membranes [90] and recovering ammonia from anaerobic digested materials [91]. In addition, in the biomass enzymatic hydrolysis process, enzymes are recovered from the post-reaction solution and reused to improve cost efficiency [92]. Enzymes are particularly sensitive to heat, so membrane filtration systems are attractive because they can be used under mild conditions that do not affect enzyme activity. In the field of material production using microorganisms, there are examples of membrane use bioethanol production process using yeast. Membrane distillation processes have been investigated for ethanol recovery and concentration of sugars used in culture media, contributing to the development of simple and energy-efficient processes [93, 94].

1.8. Current challenges in oil production by *L. starkeyi*

Oils produced using the oleaginous yeast *L. starkeyi* is highly expected to be a substitute for palm oil worldwide, but it faces various challenges including production costs and application for food industry. However, the research related to these issues is insufficient to make its production economically viable.

First, regarding the upstream process, the use of low-cost food residues as culture media is very effective in reducing costs, but there are no reports on alternatives to nitrogen sources. Therefore,

research is needed on nitrogen substitutes that can achieve high oil production while maintaining productivity.

Next, in the downstream process, namely oil extraction and purification, most reports indicate that batch-type processes with high energy consumption and organic solvents unsuitable for food processing are used. Membrane separation technology, which is used in bio production, is often used as a low-energy and suitable for continuous processes. However, there are no report of the introduction of membrane processes in the oil production process of oleaginous yeasts, including *L. starkeyi*.

1.9. Purpose and overview of this dissertation

Therefore, as mentioned above, this research aims to solve the two challenges, (1) expensive nitrogen nutrient for oil production by yeast (2) batch-type processes with high energy consumption and organic solvents unsuitable for food processing. Finally, this research aims to establish a novel oil production process of *L. starkeyi*, which is low-cost and food-grade process.

To this end, the introduction of membrane separation technology into the upstream and downstream processes was investigated. In addition, research was conducted on alternative nitrogen source in the culture process with the aim of achieving higher oil productivity.

This dissertation includes five chapters as shown below.

Chapter 1: The research background and objectives of this research are generally introduced.

Chapter 2: In this chapter, a food-grade and cost-effective process for oil from oleaginous yeast *L. starkeyi* was investigated, proposing a novel process that eliminates freeze-drying step. The conventional crushing process without drying faces challenges due to oil emulsification, which hinders oil extraction. A new separation method, nonwoven filtration separation, was applied to the emulsified solution, and a 3:1 hexane/ethanol mixture was effective for oil extraction. The new process reduced

energy consumption and processing costs to 26% and 34% of the conventional method in oil extraction, respectively. Additionally, achieving high-efficiency oil extraction and recovery while using solvents suitable for food processing is noteworthy. This efficient process has the potential to support the practical application of edible palm oil substitutes.

Chapter 3: Chapter 2 focuses on the downstream process of oil production derived from the yeast, however, it is also necessary to reduce the cost in upstream process for reducing the cost of the oil. Therefore, in this chapter, the utilization of okara, a byproduct of tofu and soymilk, for nitrogen source of culture medium was examined. The okara medium showed lower oil yield than conventional medium probably due to high viscosity caused by high content of insoluble solids in okara. Therefore, oil yield was greatly improved by using an extract of nitrogen components from okara. As further optimization of culture condition, okara extract solution concentrated with NF, UF, and RO membranes were utilized in the culture medium. Culture medium using 5-fold NF membrane-enriched resulted in better oil yields than yeast extract or okara extract. There was a positive correlation between total nitrogen concentration and oil yield up to a certain threshold, after which yields leveled off or decreased. This result suggested that the NF membrane concentration process was optimal at a cutoff of about 500 Da, balancing the concentration of growth-promoting components with the permeation of inhibitory substances. These findings have achieved a significant cost reduction of the culture medium and high oil yield in yeast oil production.

Chapter 4: In Chapter 3, extraction of okara component and membrane concentration process was investigated to solve the problem of using okara solid medium for oil production. In contrast, in this chapter, the possible deficiency of nitrogen nutrient components in the initial environment in okara medium was focused on. Therefore, the effect of inorganic nitrogen addition was examined. Among them, ammonium sulfate addition significantly increased oil yield in okara medium, exceeding that of conventional medium. And the culture experiments results indicated that

the combination of SO_4^{2-} and NH_4^+ ions was important for this effect. Furthermore, metabolomic analysis of the culture supernatant strongly suggested that the addition of ammonium sulfate activated glutathione-related metabolic pathways and ammonia assimilation pathways. These pathways may contribute to maintaining cellular functions and improving oil productivity. In addition, the cost of nitrogen source materials for the oil production medium was significantly reduced compared to the conventional. This study is the first report showing that oil yield can be improved in *L. starkeyi* by adding inorganic nitrogen to okara medium, and that ammonium sulfate affects GSH metabolism and oil yield.

Chapter 5: A summary of this dissertation is given.

Chapter 2

*Simple and economical downstream process development for edible oil production from oleaginous yeast *Lipomyces starkeyi**

2.1. Introduction

The oil produced by *Lipomyces starkeyi* (hereinafter referred to as yeast oil), is expected to be a sustainable alternative because its fatty acid composition and characteristics are similar to that of palm oil [35, 36]. However, the production process of yeast oil remains at the research level, using reagents in media and organic solvents that are not recognized as edible. In the proposed yeast oil production process, the cell recovery and freeze-drying (freezing and vacuum drying) methods enable efficient cell crushing and allow for high-yield oil recovery using organic solvents due to their low moisture content [35, 95]. However, these methods are particularly time-consuming and require large amounts of energy because of the freezing and heating processes involved. In addition, batch crushing, for example, using a ball mill, is commonly used in the cell crushing process; however, it is unsuitable for practical processes because it requires considerable labor for the production of a large amount of oil. Instead, crushing cells in the liquid culture and feeding the crushed culture for the oil extraction step should be considered for continuous processing. Regarding the extraction of oil using solvents, the Bligh and Dyer method or Folch method, which uses chloroform and methanol to extract lipids, is a well-known procedure and have been applied to oil extraction from oleaginous yeast [96–98]. However, because chloroform and methanol are among hazardous solvents, alternative and safe solvents are needed. Extraction using safer solvents such as hexane and cyclopentyl methyl ether have

been studied, but they require complex pre-treatment procedures to obtain high recovery ratios [99-103].

Because palm oil is especially cheap among edible oils, it is difficult to industrialize yeast oil production using the conventional process from the viewpoint of throughput and cost. Therefore, a new process must be developed. To omit the batch-type processes of cell recovery and freeze-drying, a method involving the disruption of cells in the culture medium and the recovery of yeast oil has been proposed. However, the resulting cell disruption solution strongly emulsifies due to the presence of water, yeast oil, and disrupted yeast, making hexane-added extraction, a common extraction method in the food industry, still challenging [50].

This chapter aimed to establish a food-grade and cost-effective process for oil production from *L. starkeyi*. Filtration separation and various combinations of organic solvents were examined in order to overcome the difficult oil extraction from emulsified solutions generated by crushing yeast culture liquid. As organic solvents, hexane, ethanol, and 2-propanol, which are applicable to food processing, were used. Oil recovery rate, extraction efficiency, and TAG selectivity were analyzed and evaluated. Further, the energy consumption of the proposed process and its required costs were also estimated.

2.2. Experimental

2.2.1. Strain and growth conditions

Lipomyces starkeyi CBS1807 was cultured in a yeast extract/glucose (YG) medium containing 5% (w/v) yeast extract (Oriental Yeast Industry, Tokyo, Japan) and 10% (w/v) D (+)—glucose (Fujifilm Wako Pure Chemical, Tokyo, Japan). Preculturing was performed using a 500 mL baffle-flask, including a 100 mL YG medium at 30 °C and 150 rpm. The strain was aerobically cultured at 30 °C for 7 days using a microbial 5 L jar fermenter (Able, Tokyo, Japan). The culture conditions were as follows: stirring at 600 rpm and an aeration rate of 3 L/min. The D (+)—glucose concentration

in the medium was measured four times per day, and D (+)—glucose was added to maintain a concentration of 10% (w/v) every time a glucose concentration was measured. Foaming was constantly detected using a heating-type antifoaming sensor FOH (Able, Tokyo, Japan) and controlled using the silicone antifoaming agent KM-72 (Shin-Etsu Chemical Industry, Tokyo, Japan). All of these ingredients have been approved for use in food processing by the Ministry of Health, Labour and Welfare of Japan.

2.2.2. Crushing treatment and preparation of emulsifying solution

Using 1 L of *L. starkeyi* culture and high-pressure homogenizer (Jyoko, NAGS20), 10 passes (pulses equal to 10 times the sample volume) of yeast cell crushing was performed at 200 MPa at room temperature for 250 min. To confirm the degree of crushing and emulsification, a BX53 microscope (Olympus, Tokyo, Japan) was used to verify that the particle size was less than 3 μm .

2.2.3. Filtration separation and oil extraction

Vacuum freeze-drying was performed for 72 hours with a vacuum dryer (Tokyo Rika, freeze dryer FD-550P) after pre-freezing ($-80\text{ }^{\circ}\text{C}$) overnight. Centrifugation was performed at 5000 rpm for 15 min (CR-22N; Eppendorf Himac Technologies, Ibaraki, Japan). The filtration and extraction processes are illustrated in **Fig. 2.1**. Filtration was performed using a nonwoven fabric and pressurization using nitrogen gas at 30 kPa. A nonwoven filter (SYNTEX nano 6, Mitsui Chemicals, Tokyo, Japan, average pore size: 5.6 μm , frazier ventilation degree: $10\text{ cm}^3 / (\text{cm}^2 \cdot \text{s})$) was installed in a stirred cell with a filtration portion diameter of 47 mm, and 15 mL of the crushed culture was used.

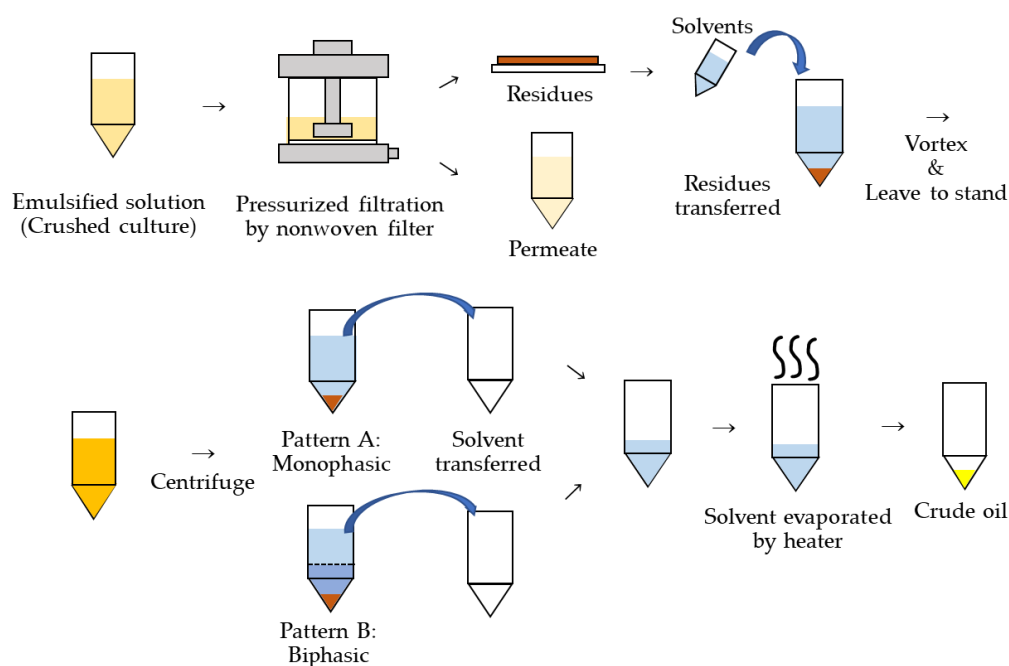


Fig. 2.1 Schematic of the oil extraction processes for the emulsified solutions (crushed *L. starkeyi* culture). Pattern A: monophasic extraction; Pattern B: biphasic extraction (Bligh and Dyer and hexane/ethanol methods).

After filtration, the cell residue containing oil on the nonwoven filter was collected, and the liquid was obtained as the permeate. The cell residue was then raked using a medicinal spoon and transferred into a conical tube. Ten milliliters of pure or mixed combinations of solvents (hexane, ethanol, and 2-propanol, Nacalai Tesque, Kyoto, Japan) were added to the conical tube and stirred for 1 min. After 15 min of standing, centrifugation was performed at 5000 rpm for 15 min. The solvent layer was recovered using a pipette (pattern A in **Fig. 2.1**; monophasic). For the Bligh and Dyer method, chloroform and methanol (Nacalai Tesque, Kyoto, Japan) were prepared and extracted as previously reported [96, 98]. In the cases of the Bligh and Dyer and hexane/ethanol methods, the layer became two phases, and only the upper layer was recovered because it may contain the target yeast oil owing to its greater lipophilicity (pattern B, Biphasic). Meanwhile, in both patterns A and B, the recovered

solution was collected in a conical tube and heated at 90 °C for 60 min using a dry-thermo-unit (DTU-1CN, TITEC, Saitama, Japan) to volatilize the solvent. The weight of oil remaining in the conical tubes was measured.

2.2.4. Analysis of fatty acid composition and calculation of TAG equivalent for extracted oil

The fatty acid composition of the extracted oils was analyzed using the gas chromatography-hydrogen flame ionization detector (GC-FID). A mixture of chloroform and methanol (2:1) was added to the extracted oil sample, heated to reflux, inverted into chloroform, and purified by using column chromatography on a silica gel support after the addition of triheptadecanoin as an internal standard. A mixture of diethyl ether and hexane (2:98) was used for washing, whereas a mixture of diethyl ether and hexane (8:92) was used for elution. After solvent removal, a sufficient amount of hexane and sodium methylate (final concentration 0.2 mol/L) was added for methyl esterification. Finally, water and acetic acid were added, and the resulting hexane layer was subjected to GC-FID analysis.

The TAG equivalent (TAG amount calculated from the fatty acid content) was calculated from the results of fatty acid composition analysis and saturated and unsaturated fatty acid quantitative analyses. The quantitative analyses of the saturated and unsaturated fatty acids were performed simultaneously, and the amount of each fatty acid per 100 g was determined by multiplying the sum of saturated and unsaturated fatty acids with the fatty acid composition ratio (%). The TAG equivalent was calculated using Equation (2.1) and used as the quantitative value for TAG [104].

$$\begin{aligned} &\text{TAG (g)} \\ &= \sum \{(\text{amount of each fatty acid per 100 g}) \\ &\times (\text{molecular weight of that fatty acid} + 12.6826)/(\text{molecular weight of that fatty acid})\} \end{aligned} \quad (2.1)$$

2.2.5. Estimation for oil extraction performances

Firstly, in order to determine the theoretical oil recovery weight of the emulsified solution samples, calculations were performed using conventional methods that can extract TAGs almost completely. Dried yeast cells were crushed using a multi-bead shocker (Yasui Instruments, Osaka, Japan), the oil was extracted with hexane, and the TAG portion in extracted oils was quantified using the method described previously. The TAG amounts in the emulsified solutions were analyzed in the same manner. In addition to TAG, diglycerides, monoglycerides, and free fatty acids may be present in the oil. The theoretical oil recovery weight in the solution was calculated using Equation (2.2).

$$\text{Theoretical oil recovery weight (g)} = \frac{\text{TAG amount in the emulsified solutions (g)}}{\text{TAG portion in the extracted oils (-)}} \quad (2.2)$$

Then, the experimental oil recovery ratio (%) was calculated using Equation (3).

$$\text{Oil recovery ratio (\%)} = \frac{\text{measured extracted oil weight (g)}}{\text{theoretical oil recovery weight (g)}} \times 100 \quad (2.3)$$

Because TAG is the major lipid species of interest, the TAG extraction efficiency was calculated using Equation (4).

$$\text{TAG extraction efficiency (mg/mL)} = \frac{\text{TAG (mg)}}{\text{Solvent volume (mL)}} \quad (2.4)$$

TAG selectivity was used to represent the effectiveness of a solvent system in selecting TAG from other undesirable constituents present in the cell lysate, and it was calculated using Equation (2.5).

$$\text{TAG selectivity (mg/mg \%)} = \frac{\text{TAG (mg)}}{\text{Total extracted oil (mg)}} \quad (2.5)$$

These calculations were based on a previous study by Probst *et al.* [98].

2.2.6. Required energy and cost estimation

The required processing energy (RPE) and required processing cost (RPC) were evaluated in terms of kilowatt hours (kWh) and yen required to treat 1 kg of culture, and they were calculated using the following Equations (2.6)–(2.9):

$$RPE = E_f + E_{ex}, \quad (2.6)$$

$$RPE' = E_{pf} + E_d + E_{ex}', \quad (2.7)$$

$$RPC = C_m + E_f \alpha + C_o + C_{ex}, \quad (2.8)$$

$$RPC' = (E_{pf} + E_d) \cdot \alpha + C_o' + C_{ex}', \quad (2.9)$$

where E_f is the energy required for filtration with a separator machine, kWh; E_{ex} is the energy required for extraction, kWh; E_{pf} is the energy required for freezing before vacuum drying, kWh; E_d is the required energy for vacuum drying, kWh; C_m is the required cost of material for filtration, yen; α is the electric cost–power coefficient, yen/kWh; C_o is the cost of organic solvents required for extraction, yen; C_{ex} is the required cost for extraction, yen. The symbols with apostrophes represent the values of the conventional method with hexane extraction after vacuum freeze-drying. The detailed calculations are provided in the supplemental information at the end of this chapter.

2.2.7. Verification of extraction mechanism by tracking material balance

To verify the mechanism of hexane/ethanol extraction, the amount of oil leaked from the residue by ethanol and the amount remaining in the residue were measured. First, 2.5 mL of ethanol was added to the residue obtained by nonwoven filtration, mixed and allowed to stand for 15 min, and then separated into the ethanol/water fraction and residue with a 0.22 μ m filter. 7.5 mL of hexane were added to each fraction and mixed, and the mixture was centrifuged at 5000 rpm for 15 min. Then, the

hexane layer was collected with a pipette, volatilized using a heater, and the weight of the collected oil was measured.

2.2.8. Verification of extraction mechanism by using model solution

In order to verify the effect of medium components on oil extraction recovery, oil extraction was attempted on model emulsion solutions in which culture medium had been removed via centrifugation. First, the dry cells were prepared by centrifuging and vacuum freeze-drying them from 500 mL of *L. starkeyi* culture by using the method described in Section 2.2.3. Then, 50 g of dry cells were suspended in 500 mL of water and crushed using a high-pressure homogenizer according to the method described in Section 2.2.2. 15 mL of this crushed solution were subjected to two pretreatments: (1) vacuum freeze-drying (2) nonwoven filtration for collecting the residue using the same method as described in Section 2.2.3. After these pretreatments, oil extraction was performed using 10 mL of hexane. The weight of extracted oil was determined as described in Section 2.2.3. Oil recovery ratio was calculated by comparison with the theoretical oil recovery weight determined as described in Section 2.2.5.

2.2.9. Statistical analysis

The values are given as the mean $\pm$ standard deviation calculated in triplicate experiments. In **Fig. 2.5**, the bar graph is presented with averages of triplicate analyses.

2.3. Results and discussion

2.3.1. Oil extraction with hexane from crushed *L. starkeyi* culture

When yeast oil was extracted from the emulsified solution of the crushed *L. starkeyi* culture using hexane, the oil recovery ratio was 0% (**Table 2.1**). This can be attributed to the stable emulsification state owing to the components derived from yeast cells and metabolites. Therefore, centrifugation, which is often used for de-emulsification, was performed before extraction. However,

the oil recovery ratio was still 0%. Even when the vacuum freeze-drying method, which omits water and crushes the emulsified state, was conducted, the oil recovery ratio remained low (44.4%). These results can be attributed to the strong emulsification of crushed *L. starkeyi* cultures with some yeast components such as cell wall compounds and metabolites.

Table 2.1 Oil recovery ratios using several extraction methods from *L. starkeyi* cell culture solution using hexane.

Sample	Oil Recovery Ratio (%)		
	Non-treated	Centrifugation	Vacuum Freeze-Drying
Crushed <i>L. starkeyi</i> culture	0	0	46.6 ± 10.6

2.3.2. Oil extraction with pure solvent after nonwoven filtration

To remove moisture and the water-soluble components from the crushed *L. starkeyi* culture, pressurized filtration was performed using a polyolefin-based nonwoven filter. A clear permeate was obtained via filtration. The oil recovery ratio of the residue that accumulated on the nonwoven filter was $7.28 \pm 3.09\%$ (**Table 2.2**). Although this value was lower than that of the vacuum freeze-drying sample (**Table 2.1**), the oil recovery ratio was higher compared with the direct hexane extraction from crushed *L. starkeyi* culture (0%). When ethanol and 2-propanol were used for extraction, the oil recovery ratio increased to $67.8 \pm 14.3\%$ and $67.5 \pm 7.7\%$, respectively. However, as shown in **Fig. 2.2**, the recovered oil was brownish, had a strange odor, and contained solid content even after heating at 60 °C or higher. This is because many non-target fat-soluble components in the culture solution were mixed with the extracted oil. Thus, the recovered oil is not a suitable alternative to palm oil.

Table 2.2 Oil recovery ratios by various organic solvents after nonwoven filtration.

Extraction Solvent	Oil Recovery Ratio (%)	
	With Filtration	Without Filtration
Hexane	7.3 ± 3.1	0
Ethanol	67.8 ± 14.3	0
2-Propanol	67.5 ± 7.7	0

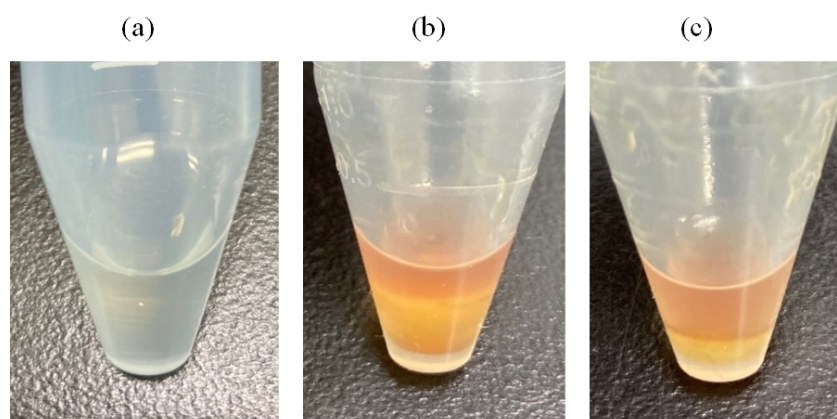


Fig. 2.2 Pictures of oils extracted by each pure organic solvent after nonwoven filtration. **(a)** Hexane, **(b)** Ethanol, **(c)** 2-Propanol. The extracted oils were heated at approximately 60 °C.

2.3.3. Oil extraction with solvent mixture after nonwoven filtration

Fig. 2.3 shows the relationship between the oil recovery ratio and solvent mixture composition. When a 3:1 (v/v) hexane/ethanol mixture was used as the extraction solvent, a recovery ratio of 98.2% was obtained, which was the highest in this study (**Fig. 2.3a**). The recovered oil is transparent, as shown in **Fig. 2.4a**. Even in the cases of the hexane/ethanol mixtures with ratios of 2:1 and 5:1, the oil recovery ratios were 80% or higher. As shown in **Fig. 2.3b,c**, when hexane/2-propanol or 2-propanol/ethanol mixtures were used, there were conditions for achieving the oil recovery ratios of 80% or higher with a 1:1 (v/v) hexane/2-propanol mixture, a 1:1 (v/v) 2-propanol/ethanol mixture, and a 3:1 (v/v) 2-propanol/ethanol mixture. However, the recovered oils under these conditions were

brownish, suggesting that many fat-soluble components other than the target yeast oil were extracted (Fig. 2.4b,c).

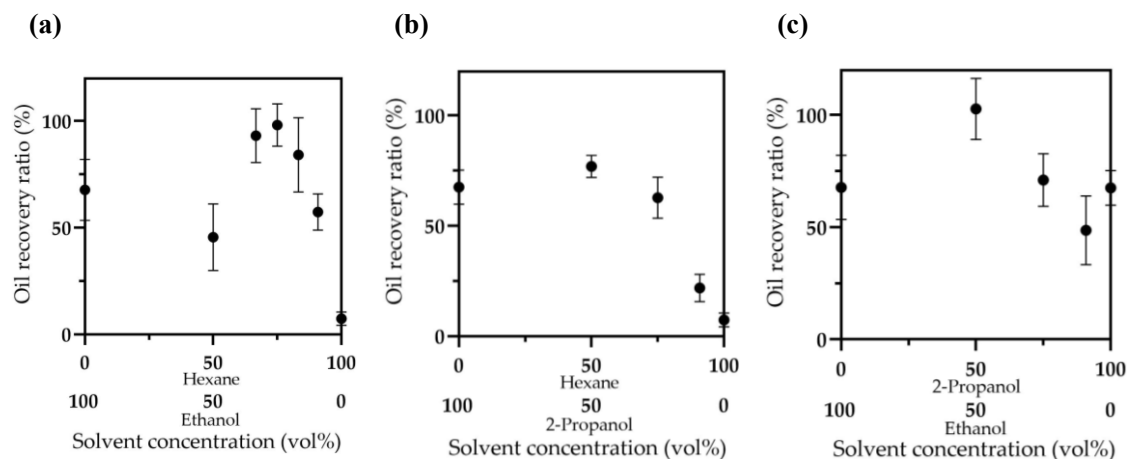


Fig. 2.3 Effect of solvent mixture composition on the oil recovery ratio. **(a)** Hexane/ethanol mixture, **(b)** hexane/2-propanol mixture, and **(c)** 2-propanol/ethanol mixture.

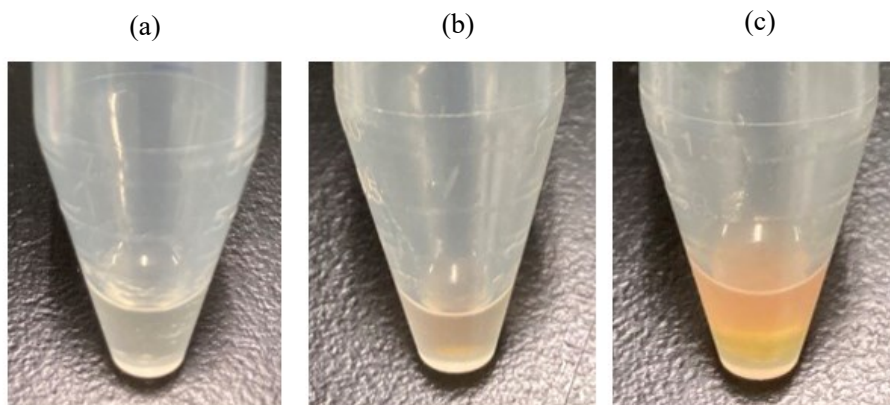


Fig. 2.4 Pictures of oils extracted from each solvent mixture. **(a)** 3:1 (v/v) hexane/ethanol mixture, **(b)** 3:1 (v/v) hexane/2-propanol mixture, and **(c)** 3:1 (v/v) 2-propanol/ethanol mixture. Extracted oils were heated at approximately 60 °C.

2.3.4. TAG extraction efficiency and TAG selectivity

The TAG extraction efficiency and TAG selectivity of various extraction solvents are summarized in **Table 2.3**. High TAG extraction efficiency and selectivity were obtained for the 3:1

(v/v) hexane/ethanol mixture, which were comparable to those of the Bligh and Dyer method, which uses a non-edible organic solvent. The results shown in **Table 2.3** indicate that the hexane/ethanol mixture is a useful extraction solvent in terms of TAG extraction efficiency and TAG selectivity.

Table 2.3 TAG extraction efficiency and TAG selectivity.

Extraction Solvent	Total Amount of Extracted Oil (mg)	TAG (mg)	TAG Extraction Efficiency (mg TAG/mL Solvent)	TAG Selectivity (%mg TAG/mg Oil)
Chloroform/methanol/ water (1:2:0.8)	257 ± 12	213 ± 14	21.3 ± 1.4	82.8 ± 2.9
Hexane	60.0 ± 10	52.7 ± 9.4	5.27 ± 0.94	87.7 ± 1.8
Ethanol	197 ± 42	27.6 ± 3.3	2.75 ± 0.33	14.3 ± 2.4
2-Propanol	307 ± 87	98.5 ± 7.8	9.85 ± 0.78	33.8 ± 9.1
Hexane/ethanol (3:1)	233 ± 58	174 ± 19	17.4 ± 1.9	74.6 ± 9.6

All data were measured in triplicate and are noted as mean ± standard deviation.

The fatty acid composition (change in physical properties) is affected the extraction method [105, 106]. Therefore, the fatty acid composition and amount of TAG by the sum of TAG equivalents from fatty acids in oils extracted using various solvents are plotted in **Fig. 2.5**. The fatty acid compositions of the oils extracted using pure hexane and a 3:1 (v/v) hexane/ethanol mixture were similar to those of palm oil, although the compositions in the cases of pure ethanol and 2-propanol were different. Therefore, it can be deduced that many non-target fat-soluble components were extracted using ethanol and 2-propanol. These results also indicate the usefulness of the 3:1 (v/v) hexane/ethanol mixture as an extraction solvent. The Bligh and Dyer system consisted of a chloroform:methanol:water (1:2:0.8) mixture.

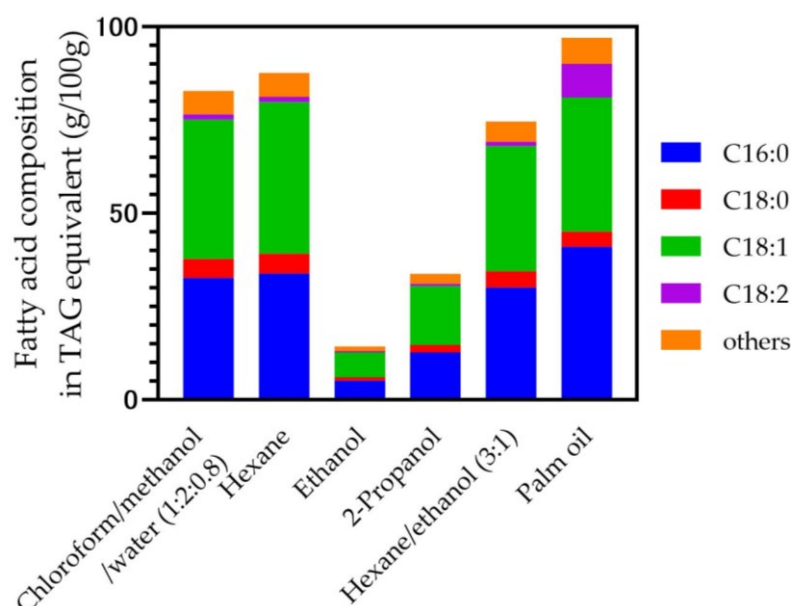


Fig. 2.5 Fatty acid composition in TAG equivalents of oils extracted with various solvents.

2.3.5. Estimation of the required energy and cost

The new yeast oil production process proposed in this chapter was compared with the conventional process in **Fig. 2.6**. Efficient oil recovery from the resulting emulsified solution have succeeded, which has been difficult previously; thus, the freeze-drying processes can be omitted in the new process. Hence, the new process is relatively simple, and as described previously, the obtained oil quality was sufficiently good. The required energy and processing costs of the new method (filtration and extraction) were calculated and compared with those of the conventional process (freezing and vacuum dry).

The required energies of new and conventional processes (RPE , RPE') are 0.24 kWh and 0.92 kWh per kg culture, respectively. The required processing costs (RPC , RPC') were estimated to be 5.8 yen and 17 yen per kg of culture for the new and conventional processes, respectively (see Supplementary Materials for more details). The required energy and cost of the new process were only 26% and 34% of those of the conventional process, respectively, indicating the superiority of the new

process. Thus, construction of a new filtration and extraction process for yeast oil without cell collection and vacuum freeze-drying was succeeded, which have previously restricted mass production. In addition, a new process, including cell crushing in the liquid state, would help to construct a continuous production process.

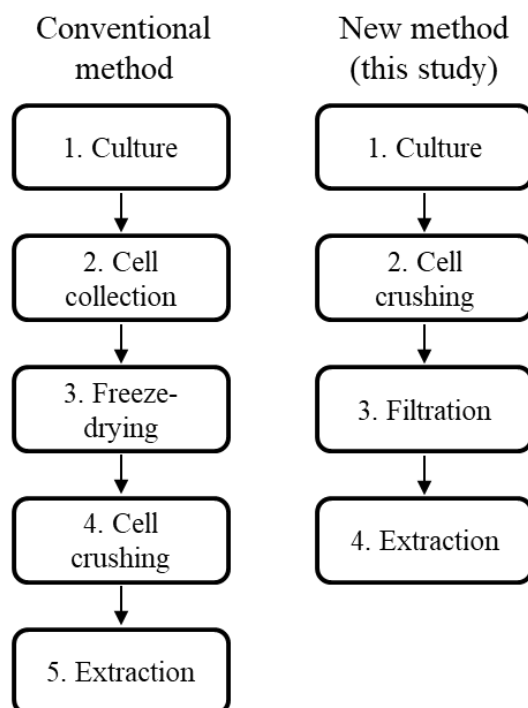


Fig. 2.6 Comparison of new and conventional processes for yeast oil production from *L. starkeyi*.

2.3.6. Verification of extraction mechanism by tracking material balance

The oil recovery ratio increased drastically with the addition of ethanol to hexane. Therefore, the following two hypotheses were considered. First, the fat-soluble components in the residues leak well into ethanol, which has a high affinity for water; the target oils are then extracted efficiently from the ethanol layer using hexane. This may be supported by the fact that oils containing not only TAG but also other fat-soluble components were extracted using ethanol alone, as shown in **Table 2.2** and **Fig. 2.3b**. Another hypothesis is that the affinity between the extraction solvent and the residue has increased.

The affinity between pure hexane and the residue is very low, however, it is thought that mixing ethanol with hexane increases this affinity. As a result, the contact efficiency between the oil in the residue and the hexane has improved, leading to improved extraction efficiency with hexane. Alternatively, it is possible that both contributed to the improvement in the oil recovery ratio.

To measure the amount of oil leaked from the residue via ethanol and that which remained in the residue, ethanol was added to the residue and mixed, the ethanol/water fraction and residue were separated via filtration, and hexane was added to each sample's extract oil. It was found that $78.1 \pm 9.1\%$ of the oil remained in the residue and only $21.9 \pm 9.1\%$ of the oil was leaked by ethanol, suggesting that the first hypothesis (leakage by ethanol) is not the main reason and that the second hypothesis (ease of contact of hexane with residue by ethanol) plays an important role in increasing the oil recovery ratio.

2.3.7. Verification of extraction mechanism by using model solution

Emulsification is considered to be the main factor inhibiting hexane extraction. The components of the yeast medium and metabolites may have contributed to this emulsification. Therefore, a model solution containing 10% (w/v) crushed dry cells was prepared and subjected to hexane extraction with two pretreatments: vacuum freeze-drying and nonwoven filtration. As shown in **Table 2.4**, the oil recovery ratios from the model solution after vacuum freeze-drying and filtration were $79.2 \pm 6.0\%$ and $67.2 \pm 20.5\%$, respectively. In contrast, in the case of the culture solution, these values were lower, at $46.6 \pm 10.6\%$ and $7.28 \pm 3.09\%$, respectively. In the model solution, the concentration of the water-soluble components was low because they were de-liquidated via centrifugation. Therefore, water-soluble components in the culture solution may inhibit hexane extraction. Specifically, a component derived from a medium or a metabolite of yeast is considered a candidate inhibitor.

Table 2.4 Oil recovery ratios from model solution and culture solution.

Sample	Oil Recovery Ratio (%)	
	Freeze-Drying	Filtration
Model solution (10% dry cells)	79.2 ± 6.0	67.2 ± 20.5
<i>L. starkeyi</i> culture solution	46.6 ± 10.6	7.28 ± 3.09

Although the addition of ethanol to hexane was effective, ethanol is expensive; therefore, the reduction of the ethanol amount or the use of only hexane should be considered. If the inhibitory component is identified and reduced or removed, less ethanol will be required during the extraction process, reducing the cost of oil production. The identification of the inhibitory component also contributes to the elucidation of the detailed extraction mechanism. This will be our next goal in the near future.

2.4. Conclusions

In this chapter, a food-grade process for the mass production of yeast oil using *L. starkeyi* was investigated. In contrast to a previously studied process that collected and crushed yeast cells after vacuum freeze-drying, a novel process that can eliminate these steps was proposed. The graphical abstract of this chapter is shown in **Fig. 2.7**. The conventional process is limited by the emulsification of the oil-containing liquid resulting from crushing, which interferes with oil extraction from the emulsified liquid. In this chapter, a new pretreatment method, nonwoven filtration, was applied to an emulsified solution. The 3:1 (v/v) hexane/ethanol mixture was found to be suitable for efficient oil extraction from residues. The energy consumption and processing cost of the new process were

calculated to be 26% and 34% of the conventional method, respectively, suggesting that the new process shows promise for industrial use.

This new process, which is capable of continuous operation and is efficient in terms of energy consumption and oil production costs, will greatly contribute to the practical use of edible palm oil substitutes for the realization of an environmentally friendly bioeconomy.

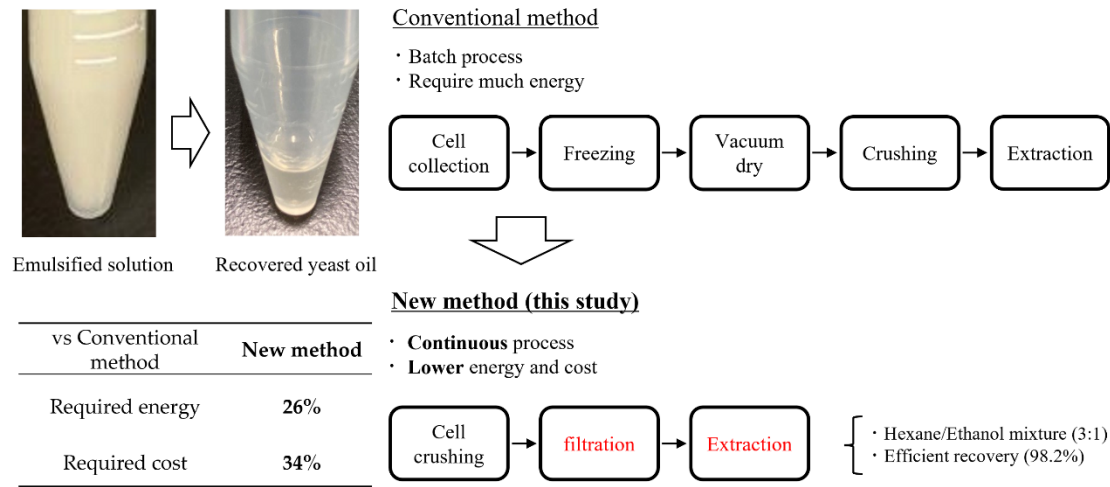


Fig. 2.7 Graphical abstract of this chapter.

2.5. Supplemental information

The details of the required energy and cost calculations are presented below. The values of the coefficients used in the calculations are listed in **Table 2.S1**.

E_f was calculated as follows, assuming a power of 9.4 kWh for the nonwoven filter separator and a throughput of 100 kg/h.

$$E_f = 9.4/100 = 0.094 \text{ (kWh)}$$

E_{ex} was calculated as follows, under conditions in which 0.500 L of hexane and 0.167 L of ethanol (mixing ratio 3:1) per kg of emulsified solution were used for extraction.

$$= 1.5 \times 0.01 + 12/15 = 0.82 \text{ (kWh)}$$

C_m was calculated using a nonwoven cost per area of 370 yen/m² and a throughput of 500 kg/m², as follows:

$$C_m = 370/500 = 0.74 \text{ (yen)}$$

C_o and C_o' were calculated as follows, under the condition where the conventional method requires 0.667 L of hexane per 1 kg of culture, and the new method requires 0.5 L of hexane and 0.167 L of ethanol per 1 kg of culture, and the solvent recovery ratio is 98%.

$$\begin{aligned} C_o &= \sum (\text{solvent usage}) \times (\text{solvent cost}) \times \{100 - (\text{solvent recovery rate})\}/100 \\ &= 0.5 \times 33.1 \times (100 - 98)/100 + 0.167 \times 84 \times (100 - 98)/100 \\ &= 0.61 \text{ (yen)} \end{aligned}$$

$$\begin{aligned} C_o' &= (\text{solvent usage}) \times (\text{solvent cost}) \times \{100 - (\text{solvent recovery rate})\}/100 \\ &= 0.667 \times 33.1 \times (100 - 98)/100 = 0.44 \text{ (yen)} \end{aligned}$$

C_{ex} and C_{ex}' was calculated using the following equations:

$$C_{ex} = E_{ex} \times \alpha = 0.154 \times 18 \approx 2.8 \text{ (yen)}$$

$$C_{ex}' = E_{ex}' \times \alpha = 0.103 \times 18 \approx 1.9 \text{ (yen)}$$

Thus, the required processing energy (RPE , RPE') and cost (RPC , RPC') were calculated as follows:

$$RPE = E_f + E_{ex} = 0.094 + 0.154 \approx 0.24 \text{ (kWh)}$$

$$RPE' = E_{pf} + E_d + E_{ex}' = 0.82 + 0.103 = 0.92 \text{ (kWh)}$$

$$RPC = C_m + E_f \cdot \alpha + C_o + C_{ex} = 0.74 + 0.094 \times 18 + 0.61 + 2.8 \approx 5.8 \text{ (yen)}$$

$$RPC' = (E_{pf} + E_d) \cdot \alpha + C_o' + C_{ex}' = 0.82 \times 18 + 0.44 + 1.9 \approx 17 \text{ (yen)}$$

Table 2.S1. The coefficients and input values.

Coefficient used for calculation	Value	Unit
power-calorific coefficient	0.000278	kJ/kWh
electric cost-power coefficient (α)	18	yen/kWh
solvent recovery rate	98	- (%)
power of extractor	0.001	kWh/L
room temperature	20	°C
hexane boiling point	68.74	°C
hexane evaporation latent heat	366.45	J/g
hexane specific gravity	0.654	-
hexane specific heat	2.304	J/g°C
hexane cost	50.6	yen/L
ethanol boiling point	78.34	°C
ethanol evaporation latent heat	948.24	J/g
ethanol specific gravity	0.789	-
ethanol specific heat	3.38	J/g°C
ethanol cost	84	yen/L

Chapter 3

*Utilization of okara as a culture medium by membrane concentration process for high oil production by oleaginous yeast, *Lipomyces starkeyi**

3.1. Introduction

Chapter 2 focuses on the downstream process of oil production derived from the yeast, however, it is also necessary to reduce the cost in upstream process for reducing the cost of yeast oil.

Okara is a by-product of the tofu and soymilk production process from soybeans. Approximately 14 million tons are generated annually worldwide, most of which are dumped and burned as waste [107]. Nevertheless, okara remains highly nutritious, containing about 40–60% carbohydrates (mainly insoluble fiber), 20–30% protein, and 10–20% fat (all on a dry basis) [108]. Therefore, if okara can be converted into something with added value, such as oil and fat, through fermentation by microorganisms, it will have a significant impact by reducing waste and securing resources. In fact, the fermentation of okara by fungi and bacteria has been well studied, in contrast, there are a few studies on yeast [109].

A report on *Yarrowia* genus yeast, a type of oleaginous yeast, examined biovalorization using okara as a raw material, focusing on the flavor and nutritional value of okara rather than on the growth value [110]. Therefore, okara may be a suitable growth medium for oleaginous yeast. In the example of *Saccharomyces cerevisiae*, glutaminase (YbaS) from *Escherichia coli* was expressed on the cell surface by genetic recombination, and ammonia was produced from okara outside the yeast

cell [111]. As shown above, there are a few fermentation studies exploring yeast using okara, but none on *Lipomyces starkeyi* and no examples of oil production.

The graphical abstract is shown in **Fig. 3.1**. In this chapter, the yeast extract in a YG medium was directly replaced with okara. Next, the nitrogen component of okara was extracted, and the insoluble solids were separated using a simple solid–liquid separation process. Moreover, a membrane concentration process was attempted in order to increase the concentration of the extracted nitrogen component and achieve a higher oil yield. In this oil-producing yeast fermentation production process, the adoption of the membrane concentration process has the following two advantages: (1) a high oil yield can be achieved because nitrogenous components extracted were concentrated, and (2) the volume of the extract can be reduced, which, in turn, lowers storage and transportation costs.

This chapter aims to maximize the utilization of okara, a sustainable biomass resource, and establish a foundation for commercial production of yeast oil by improving oil yield using yeast, thereby achieving cost reduction.

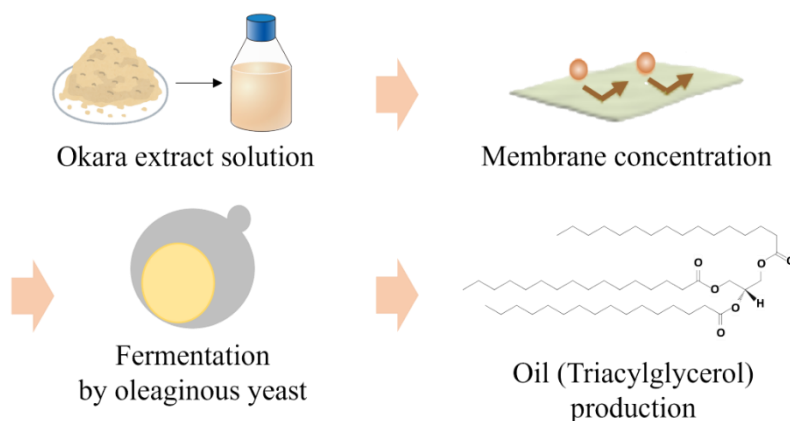


Fig. 3.1 Graphical abstract of this chapter.

3.2. Experimental

3.2.1. Strain and growth conditions

Pre-cultures of *L. starkeyi* CBS1807 were conducted in 500 mL baffled flasks containing 100 mL of a YG (yeast extract/glucose) medium composed of 5% (w/v) yeast extract BSP-570 (Oriental Yeast Industry) and 10% (w/v) D (+)-glucose (Fujifilm Wako Pure Chemical) with shaking at 150 rpm in an orbital incubator shaker (Innova S44i, Eppendorf, Hamburg, Germany) for 72 hours at 30 °C. The main culture was then conducted in a 200 mL baffled flask containing 50 mL of a production medium. The YG medium, the okara medium composed of 1%, 2%, and 3% (w/v) okara (Nissin Foods Co., Ltd., Hachioji, Japan), the okara extract solution medium containing a 30%, 60%, and 90% (v/v) okara extract solution, or the okara concentration medium composed of a 60% (v/v) okara extract concentrate (3×, 5× and 7× concentration ratio) for 96 hours at 30 °C was shaken at 150 rpm. All media other than the YG medium were similarly supplemented with 10% D (+)-glucose as a carbon source. Media were sterilized by autoclaving at 121 °C for 20 min.

3.2.2. Preparation of okara extract solution

Okara obtained from a certain factory, which contains a soymilk process, was added with 20, 30, or 40 times as much water as the weight of the okara. Then, papain (Mitsubishi Chemical, Tokyo, Japan) was added with 1% (w/w) according to the weight of okara and incubated at 55 °C for 2 h to extract nitrogenous components. The solution was heated at 90 °C for 10 min to inactivate the enzyme. Thereafter, solid–liquid separation was performed using 5A filter paper (Toyo Roshi Kaisha, Tokyo, Japan) to remove solids from the okara solution. The filtrate was further filtered using a 0.45 µm membrane filter (Toyo Roshi Kaisha) to remove suspended solids. The permeate (okara extract solution) was kept at 4 °C until use in the membrane concentration step.

3.2.3. Membrane concentration of okara extract solution

To concentrate the okara extract solution, the membrane process was performed using three types of membranes: UA60 (TriSep, Gaviota, CA, USA), a UF (Ultrafiltration) membrane with a molecular weight cut-off of 1000 Da; XN45 (TriSep), an NF membrane with a molecular weight cut-off of 500 Da; and UTC-82V (Toray, Tokyo, Japan), an RO (Reverse Osmosis) membrane with a NaCl rejection rate of 99.7% (according to the manufacturers). For membrane concentration experiments, a CF042D-FO cell (Sterlitech, Auburn, AL, USA, filtration area: 42 cm²) was used at 25 °C or 50 °C and a back pressure of 0.3 (UF), 0.5 (NF) and 1.0 (RO) MPa. The permeate flux (J) (LMH, Lm⁻² h⁻¹) was obtained every 1 min with Equation (3.1):

$$J = \frac{V_p}{A \times t} \quad (3.1)$$

where V_p is the permeate volume (L), A is the filtration area (m²), and t is the time (h).

The rejection rate (R) (%) of the components in the solution was calculated using the following Equation (3.2):

$$R = \left(1 - \frac{C_p}{C_f}\right) \times 100 \quad (3.2)$$

where C_p is the concentration of permeate (ppm) and C_f is the concentration of feed solution (ppm).

3.2.4. Analysis

3.2.4.1. Viscosity

Viscosity was measured using a Rapid Visco Analyzer 4500 (PerkinElmer, Waltham, MA, USA). The temperature was set at 30 °C, and the agitation was 150 rpm. The cP (mPa·s) values were recorded 5 min after measurement commenced.

3.2.4.2. Total organic carbon (TOC)

Prior to the TOC measurements, samples were filtered through a 0.45 µm hydrophilic polytetrafluoroethylene (PTFE) membrane (DISMIC 13HP045AN, Advantec, Tokyo, Japan). The TOC of the samples was measured using a TOC analyzer (TOC-VCSH, Shimadzu, Kyoto, Japan). First, inorganic carbon was removed from the samples as carbon dioxide by adjusting the pH below 3 via the addition of 1 N HCl. Next, the samples were combusted at 680 °C over a platinum catalyst. The combustion gas was cooled and dehumidified, and the carbon dioxide in the gas was detected using a non-dispersive infrared gas detector. As a standard for calibration, a potassium hydrogen phthalate solution was used.

3.2.4.3. Total nitrogen (T-N)

Samples were filtered through a 0.45 µm PTFE filter (Advantec) and diluted with MilliQ water to an appropriate concentration for analysis. The total nitrogen concentration was determined by two methods: (1) The Dumas method to measure solid samples (okara) involves combusting them in a 900° chamber in the presence of oxygen, and then measuring the generated N₂ gas. (2) TOC-TN analyzer (TNM-L ROHS, Shimadzu) at 720 °C by the contact pyrolysis/chemiluminescence method. Potassium nitrate was used for the calibration curve.

3.2.4.4. Ion concentration

Samples were filtered through a 0.45 µm PTFE filter (Advantec) and diluted with 0.1 M HNO₃ to enter the quantification range. Multi-element mixed standard solutions for ICP analysis (Fujifilm Wako Pure Chemical) were used as a standard. The concentration of Na, K, Ca, Mg, and P in the solution was measured by using inductively-coupled plasma–atomic emission spectrometry (ICP-AES, ICPE-9800, Shimadzu, Japan).

3.2.5. Evaluation of TAG production

3.2.5.1. Measurement of cell concentration

The cell concentration of the main culture was measured after 96 hours using a Q500 sonicator (QSonica, Newtown, CT, USA) to ensure the dispersion of yeast cells in the solution and a CDA-1000 electronic particle counter (Sysmex, Kobe, Japan), as previously reported [35].

3.2.5.2. Measurement of TAG

The extraction and measurement of TAG from *L. starkeyi* were conducted according to a previously reported study [35]. Briefly, cells were collected from 1 mL of culture via filtration using a 0.45 µm cellulose mixed ester filter (Advantec), suspended in 500 µL of phosphate-buffered saline (PBS) (Fujifilm Wako Pure Chemical), and 1 g of 0.5 mm diameter glass beads (As One, Osaka, Japan) were added. Next, cells were disrupted using a Multi-Beads Shocker (Yasui Kikai) for 15 min at 2500 rpm at 25 °C. After cell disruption, 500 µL of PBS was added to the homogenates, which were then incubated with shaking using the Micro Mixer E-36 (Taitec, Saitama, Japan) at 37 °C for 10 min. The TAG concentration in the cell homogenates supernatant was determined via enzymatic analysis using LabAssay Triglyceride (Fujifilm Wako Pure Chemical), according to the manufacturer's instructions.

3.2.5.3. Microscope observation

Microscopic observations were performed to confirm cell growth and oil accumulation and to check the contamination during membrane concentration experiments using a BX53 microscope (Olympus) with differential interference contrast (DIC) images.

3.2.6. Statistical Analysis

Statistical analysis was performed using Tukey's test with GraphPad Prism software, version 10.2.1 (GraphPad Software). Differences were considered significant at $p < 0.05$. The values are given

as the mean $\pm$ standard deviation calculated in triplicate experiments except for **Table 3.1** (Yeast extract: $n = 2$).

3.3. Results and discussion

3.3.1. TAG production using okara as a nitrogen source

In this study, the evaluation of okara as a nitrogen source for TAG production was first conducted by replacing yeast extract in the YG medium with okara. The results showed that when okara was added to the culture medium at concentrations of 1–3% (w/v), TAG was produced, but its yield was lower than that of the YG medium (44.6%, 84.1%, and 81.1% for the addition of 1%, 2%, and 3% (w/v) okara, respectively). In particular, there was no increase in the TAG yield in the 3% (w/v) addition group compared to the 2% (w/v) addition group, despite the increase in okara concentration (**Fig. 3.2a**). Microscopic observations showed no difference in oil accumulation or cell density between the 2% (w/v) and 3% (w/v) addition groups (**Fig. 3.2b**). These low TAG yields may be due to the high insoluble solids content of okara, which might cause the increased viscosity of the medium and reduce the dissolved oxygen in the medium [112]. Therefore, the viscosity of the YG medium and the okara-added medium (1%, 2%, and 3%) were measured. The viscosity of the YG medium was 8.3 ± 1.2 mPa·s, while those of the okara-added medium group with 1%, 2%, and 3% (w/v) additions were 10.0 ± 2.2 , 21.0 ± 4.2 , and 47.3 ± 2.9 mPa·s, respectively, indicating that the viscosity of the 3% (w/v) okara addition group was five times that of the YG medium (**Fig. 3.3**). As oleaginous yeasts are aerobic microbes and their growth and production of TAG are promoted by the increased dissolved oxygen, it was expected that if the viscosity of the medium decreased, yeast would be able to produce TAG equivalent to or greater than that produced in YG medium.

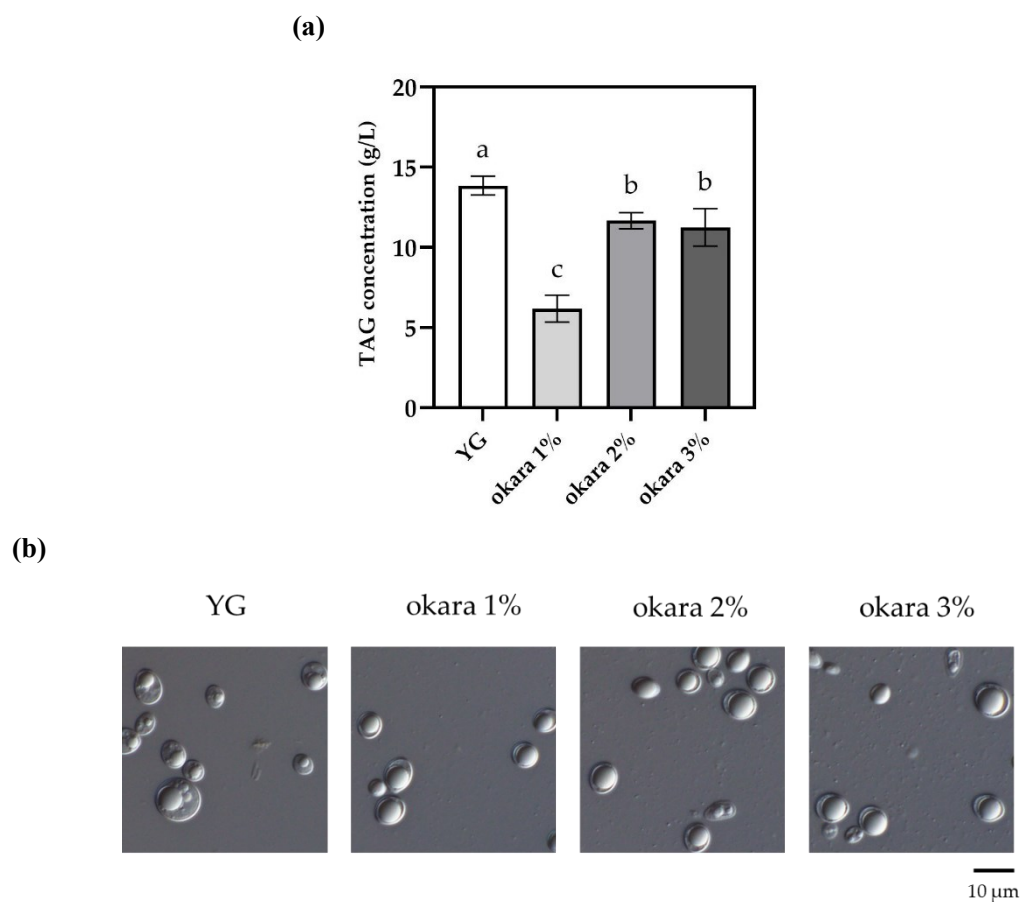


Fig. 3.2 Replacement of yeast extract with okara in the *L. starkeyi* culture medium. **(a)** Comparison of TAG productivity in the YG medium and the okara-added medium. **(b)** Differential interference contrast (DIC) images of the yeast cells. Mean $\pm$ S.D., $n = 3$, Tukey's test, different letters above the columns for each experimental group indicate significant differences between groups ($p < 0.05$).

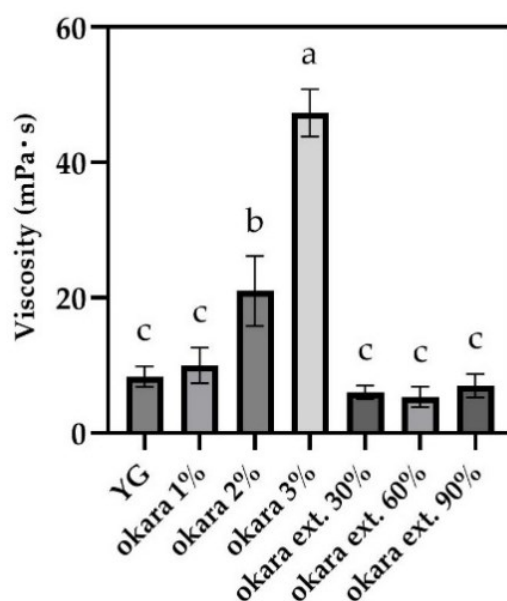


Fig. 3.3 Viscosity of the mediums from different nitrogen sources. Okara was added with 1%, 2%, and 3% (w/v) per medium, and the okara extract solution (extracted with 30 times water from okara) was added with 30%, 60%, and 90% (v/v) per medium. The values are mean $\pm$ S.D., $n = 3$, Tukey's test, different letters above the columns for each experimental group indicate significant differences between groups ($p < 0.05$).

3.3.2. Preparation of okara extract and evaluation as nitrogen source for culture media

To reduce the viscosity of the okara-containing medium, extraction of nitrogenous components from okara was attempted. The amount of water used for extraction was 20, 30, and 40 times the weight of okara, and there was no difference in the T-N recovery ratio in the extracts (**Table 3.1**). To make a highly concentrated extract, the amount of water should be low as possible, but 30 times the amount of water was chosen because, in the extraction with 20 times the amount of water, the liquid was still so viscous that it required significant power for stirring in the enzymatic reaction. Next, the okara ex-tract solution extracted with 30 times water from the okara was evaluated as a nitrogen source in the culture medium. The viscosity of the medium was greatly reduced by using the okara extract solution instead of the okara itself and was even lower than that of the YG medium (**Fig.**

3.3). Next, TAG production was evaluated using the medium with the okara extract solution. TAG productivity was found to be dependent on the added amount of okara extract solution, and a high TAG yield (13.8 ± 0.39 [g/L]) was obtained for the 90% (v/v) addition of the okara extract solution (**Fig. 3.4a**). A concentration-dependent increase in the accumulation of oil per cell and the cell concentration with the addition of the okara extract solution was also noted in the microscopic observations (**Fig. 3.4b**). These results suggest that if the extract could be concentrated and added to the culture medium in larger amounts, the TAG productivity might be even higher.

Table 3.1 T-N content of nitrogen sources used in the medium and nitrogen extraction rate of okara extract solution.

	Unit	Yeast extract	Okara	Okara extract solution		
				extracted with 20 times the volume of water	with 30 times	with 40 times
T-N	g/100g	10.0	3.7 ± 0.2	0.17 ± 0.02	0.11 ± 0.01	0.08 ± 0.02
Nitrogen extraction ratio	%	-	-	92 ± 9	92 ± 6	90 ± 8

Results presented as mean $\pm$ S.D. with $n = 3$ except for yeast extract (measured with $n = 2$).

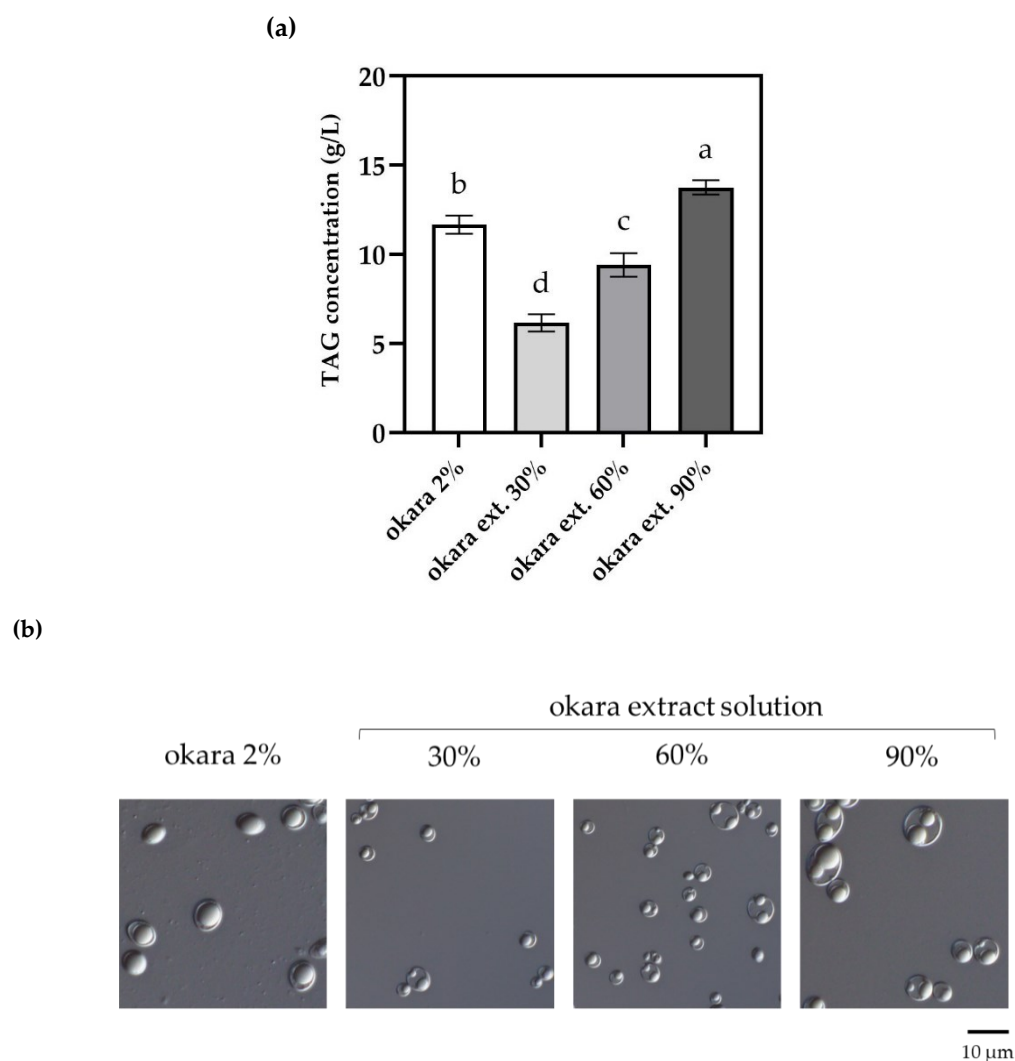


Fig. 3.4 Replacement of okara with the okara extract solution in the *L. starkeyi* culture medium as a nitrogen source. **(a)** TAG productivity in various concentrations of okara extract solution. **(b)** DIC images. Okara ext.: okara extract solution. Mean $\pm$ S.D., $n = 3$, Tukey's test, and different letters above the columns for each experimental group indicate significant differences between groups ($p < 0.05$).

3.3.3. Membrane concentration of nitrogenous components in the okara extract solution

From the above results, the membrane concentration of the okara extract solution was attempted. As proteins and peptides were considered the main nitrogenous components in the okara extract solution, an NF membrane with a molecular weight cut-off of approximately 500 Da, which was considered suitable to reject and concentrate these components, was first selected. The liquid temperature was controlled at two temperatures (25 °C and 50 °C) during the membrane concentration process to evaluate the effect of membrane fouling, which would be more severe at 25 °C due to germ growth, while at 50 °C, germ growth would be suppressed. The results indicated that the average flux was 30 LMH at 50 °C compared with 20 LMH at 25 °C during 320 g permeation (**Fig. 3.5**). As high flux is preferable for concentration, subsequent membrane concentration experiments determined that 50°C should be used.

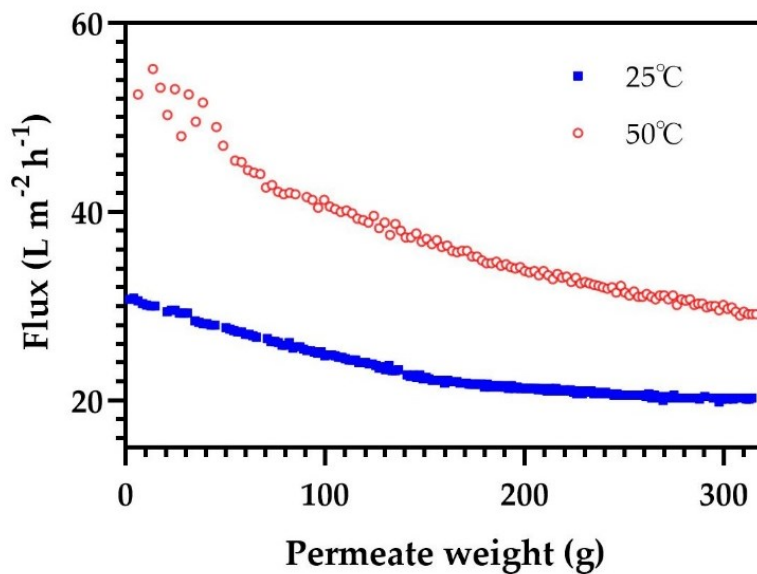


Fig. 3.5 Flux of concentration of okara extract solution for the NF membrane in different temperature conditions (25 °C and 50 °C).

Next, the membrane concentration of the okara extract solution was performed using UF and RO membranes in order to determine which is the optimal type of membrane for the concentration among the NF, UF, and RO membranes. In this concentration experiment, the total nitrogen (T-N), total organic carbon (TOC), ion concentration (Na, K, Mg, Ca, and P) in the feed, and permeate solutions were measured and compared their rejection rates (**Table 3.2**). The T-N rejection rates were 76.0%, 84.5%, and 98.7% for the UF, NF, and RO membranes, respectively, and corresponded to the pore size of the membranes. TOC rejection showed a similar tendency. With regard to ion rejection, the RO membrane had a high rejection rate of more than 88–98% for all components, while UF and NF membranes had lower rejection rates than the RO membranes. For the NF membrane, multivalent ions (Ca, Mg, and P) were rejected at high rejection rates (86–98%), while monovalent ions (Na and K) were rejected at lower rates (75–78%). The K^+ ion is the most abundant ion in the extract solution and accounts for more than 70% of the total ions by weight. These rejection results show that in concentration by the RO membrane, all components are highly rejected, indicating that both the T-N and all ions are accumulated in the concentrates and causing high osmotic pressure. On the contrary, in the case of NF and UF membrane, K was rejected less than T-N, indicating that T-N was more concentrated than K^+ , which denotes less osmotic pressure. The concentrates were obtained as 3 $\times$, 5 $\times$, and 7 $\times$ concentrates at 50 °C.

Table 3.2 Characteristics of rejection by different types of membranes for concentration of the okara extract solution.

Membrane type	UF			NF			RO		
MWCO (Da)	~1,000			~500			N/A		
	Concentration (ppm)		Rejection rate (%)	Concentration (ppm)		Rejection rate (%)	Concentration (ppm)		Rejection rate (%)
	Feed solution	Permeate		Feed solution	Permeate		Feed solution	Permeate	
T-N	946.5 ± 42.1	201.4 ± 13.8	78.7 ± 2.3 ^c	903.6 ± 31.1	135.0 ± 6.5	85.1 ± 0.8 ^b	954.0 ± 22.6	13.0 ± 0.5	98.6 ± 0.1 ^a
TOC	4543.7 ± 239.4	962.0 ± 4.6	78.8 ± 1.1 ^c	4320 ± 22.6	518.8 ± 13.4	88.0 ± 0.4 ^b	4493.7 ± 59.1	54.6 ± 0.6	98.8 ± 0.0 ^a
Na	11.6 ± 1.8	4.2 ± 0.7	63.7 ± 9.3 ^c	10.3 ± 1.3	2.1 ± 0.1	79.4 ± 2.6 ^b	12.5 ± 2.5	0.3 ± 0.0	97.3 ± 1.0 ^a
K	264.0 ± 33.0	62.6 ± 5.9	76.3 ± 3.4 ^b	250.0 ± 32.2	54.6 ± 2.6	78.2 ± 3.3 ^b	260.3 ± 9.7	4.0 ± 0.3	98.5 ± 0.1 ^a
Ca	9.4 ± 0.5	1.5 ± 0.0	83.9 ± 1.0 ^c	9.6 ± 0.3	0.3 ± 0.0	96.7 ± 0.3 ^b	9.5 ± 0.7	0.01 ± 0.0	99.8 ± 0.1 ^a
Mg	10.7 ± 0.6	1.5 ± 0.2	86.3 ± 1.9 ^b	11.3 ± 0.2	0.2 ± 0.0	98.0 ± 0.2 ^a	11.2 ± 0.7	0.01 ± 0.0	99.9 ± 0.0 ^a
P	59.8 ± 14.1	12.3 ± 0.8	79.4 ± 4.0 ^c	61.9 ± 2.4	7.9 ± 0.5	87.2 ± 1.3 ^b	66.4 ± 3.4	0.2 ± 0.1	99.8 ± 0.2 ^a

The values are mean ± S.D., $n = 3$, Tukey's test, and different letters on right shoulder of rejection rate for each experimental group indicate significant differences between groups ($p < 0.05$). MWCO: molecular weight cut-off. N/A: not available.

3.3.4. Culture evaluation of okara membrane concentrate as a raw material for culture media

TAG production was evaluated using culture media prepared with a membrane-concentrated extract solution. Oil yield (TAG concentration, g/L), cell growth (cell concentration, cells/mL), and oil accumulation (amount of TAG per cell, mg/10⁸ cells) were determined (**Fig. 3.6a-i**). The results showed that the highest TAG production was obtained with the NF membrane concentrate, and the maximum oil yield (20.0 g/L) for the 5× NF membrane concentrate was 1.71 times higher than 2% okara (**Fig. 3.6d**), which is equivalent to 1.44 times higher than that of the yeast extract and 1.69 times higher than that of the 90% okara extract solution. On the other hand, in the medium using the 3× UF membrane concentrate, the oil yield (17.1 g/L) was 1.47 times higher than that of the 2% okara (**Fig. 3.6a**), and equivalent to 1.23 and 1.44 times higher than that of the YG and 90% okara extract solutions, respectively. On the contrary, the oil yield was relatively low for the RO membrane concentrates (**Fig. 3.6g**). The oil yield of 5× RO membrane concentrate (14.0 g/L) was 1.20 times higher than 2% okara, which is equivalent to 1.01 times higher than that of the yeast extract and 1.18 times higher than that of the 90% okara extract solution. In regard to cell growth, all of the extract concentrates showed higher cell growth than the okara 2% extract (**Fig. 3.6b,e,h**), and in regard to TAG amount per cell, it has been confirmed that membrane concentration is effective in increasing TAG production per cell. However, the effect turned to a decline at a higher addition concentration for all types of membranes, especially for the NF and RO membranes (**Fig. 3.6c,f,i**), suggesting that there may be a component that is concentrated in the membrane and re-tards TAG production.

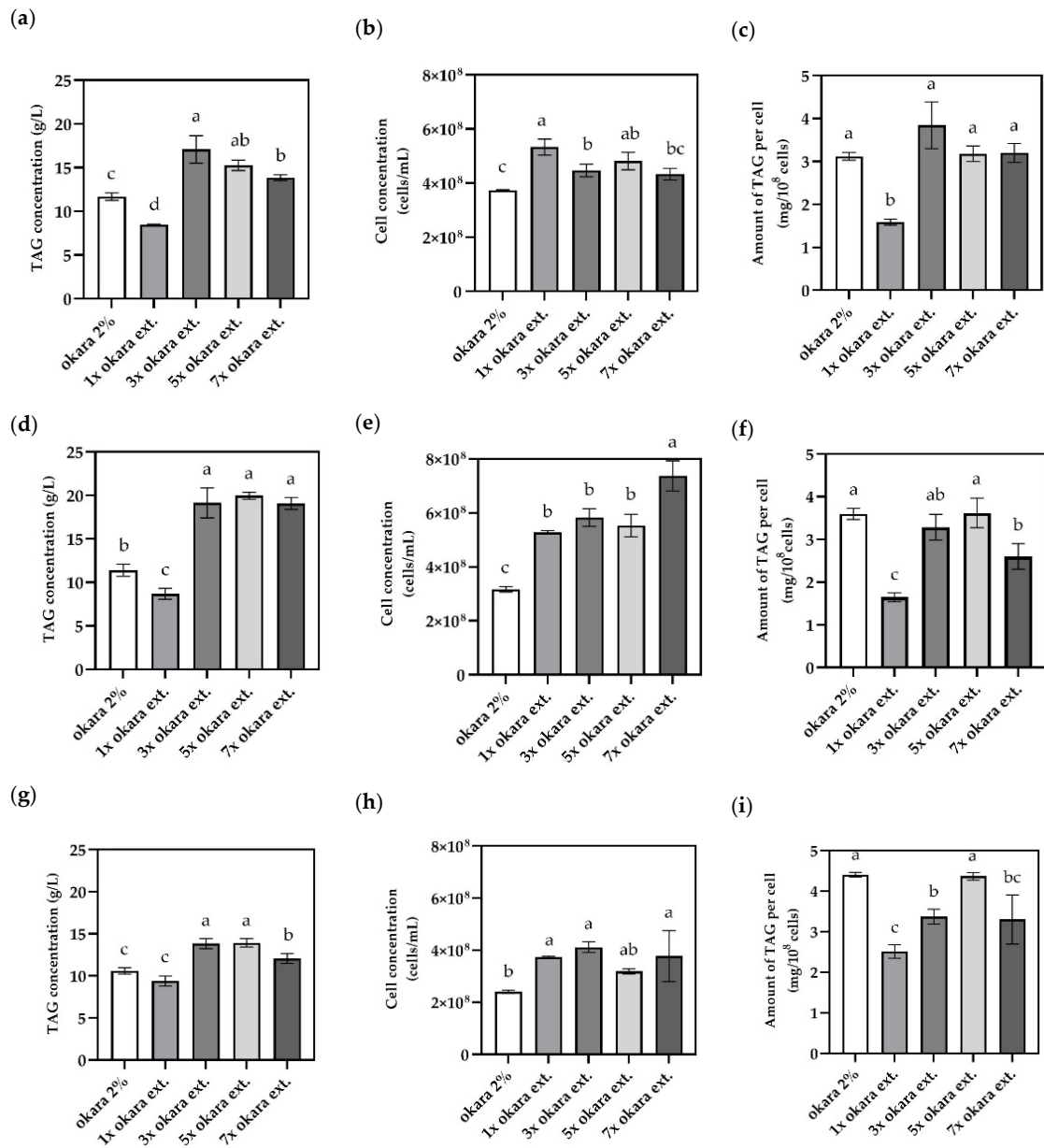


Fig. 3.6 TAG production using membrane-concentrated okara extract solutions as a nitrogen source of the culture medium. **(a–c)**: TAG concentration (g/L), cell concentration (cells/mL), amount of TAG per cell (mg/10⁸ cells) using UF membrane-concentrated extract solutions. **(d–f)**: Those using NF membrane concentrates. **(g–i)**: Those using RO membrane concentrates. Data are given in mean ± S.D., $n = 3$, Tukey's test, different letters above the columns for each experimental group indicate significant differences between groups ($p < 0.05$).

To analyze the effect of okara and its extract solution and membrane concentration on TAG production in more detail, the T-N concentration in the medium and the TAG concentration obtained using cultivation were taken as the horizontal and vertical axes, respectively, and their relationship was plotted, as shown in **Fig. 3.7a**.

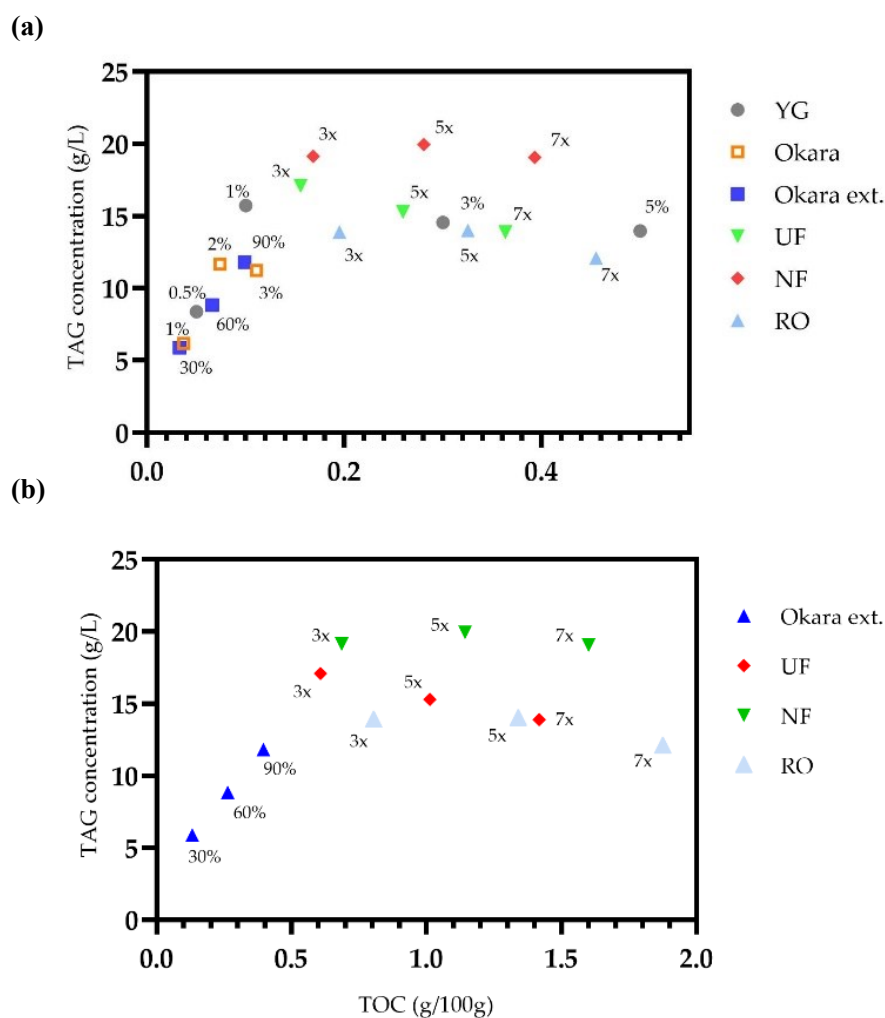


Fig. 3.7 (a) Relationship between T-N concentration and TAG productivity. **(b)** Relationship between TOC concentration and TAG productivity. 3×, 5×, 7×: Concentration magnification for each membrane concentration, UF, NF, and RO: Type of membrane used to prepare the membrane concentrate. The data are presented with averages of triplicate analyses.

In the low T-N concentration range up to 0.2 g/100 g, the TAG concentration increased in proportion to the T-N content (correlation coefficient (r) = 0.85), but the TAG concentration leveled off or fell above 0.2 g/100 g of the T-N concentration (r = -0.40). Especially, for the okara extract solution group (blue square), the TAG concentration increased linearly with the T-N concentration (r > 0.99). In the okara-added group (white squares with orange frame), the dependence of TAG concentration on the T-N concentration was lower (r = 0.88), which could be due to the increase in medium viscosity caused by the insoluble solids in the okara, as observed in **Fig 3.2**. To examine the effect of carbon source concentration on oil production, the relationship between TOC concentration in the medium and TAG yield was plotted (**Fig. 3.7b**). The results were similar to the relationship between T-N and TAG concentrations, indicating that higher TOC did not increase TAG yield.

On the other hand, there was almost no relationship between the T-N concentration and the TAG concentration for the 3×, 5×, and 7× membrane concentrates, which does not necessarily mean an increase in TAG concentrations in case of high T-N concentrations. TAG productivity was found to be NF > UF > RO because in the UF membrane concentration, the membrane pore size was so large that some components necessary for yeast growth were permeated, resulting in a lack of them in the culture medium. Conversely, in the RO membrane concentration, where the membrane pore size was small and almost all substances were concentrated, it is thought that some components that inhibit the growth of yeast were also concentrated and negatively affected TAG production. However, in the NF membrane concentration, which had a pore size between the UF and RO membranes, some of the necessary components that were permeated by the UF membrane were rejected, and some of the inhibitory components that were concentrated by the RO membrane were permeated through the NF membrane, resulting in a high oil yield. Chemical and physiological stresses (e.g., osmotic pressure and inhibitory compounds in the culture medium) are known to cause intracellular water loss, cell shrinkage, cytoskeletal collapse, and even mitochondrial dysfunction in yeasts [113, 114]. Additionally,

acetic acid, furfural, phenolic compounds, and some sugars are known to be inhibitory components of yeast culture [115]. Regarding osmotic pressure, considering the high salt rejection by the RO membrane compared to the UF and NF membranes, it is possible that the concentrated ions from the okara ex-tract solution caused high osmotic stress, adversely affecting oil yield.

Based on the above results, the components necessary for growth and oil production may include compounds with a molecular weight of 500–1000, while the growth-inhibitory components that have been concentrated by the RO membrane are thought to have a molecular weight of less than 500. Therefore, sugar-like compounds, organic acids, aromatic compounds, and salts were considered. In the future, identification of the necessary and inhibitory components in okara extract solution will lead to the construction of a more optimized membrane concentration process and subsequent further cost reduction and industrialization of yeast oil production.

3.4. Conclusions

This study explores the potential of using a soybean by-product, okara, as a culture medium for oleaginous yeast culture to improve oil yields and reduce costs for industrial applications. Initially, direct substitution of yeast extract with okara in the YG medium resulted in a low oil yield, which was attributed to increased medium viscosity and reduced dissolved oxygen due to the high insoluble solids content of okara. To address this problem, nitrogenous components were extracted from okara and used in the culture medium, resulting in a significant improvement in the oil yield. As a further optimization, membrane concentration processes using NF, UF, and RO membranes were attempted to concentrate nitrogenous components from the okara extract solution. The results demonstrated that oil yield was significantly enhanced when the 5× NF membrane concentrate was used, exceeding yields from the yeast extract and the okara extract solution. The relationship between T-N concentration and oil yield (TAG concentration) was also investigated, revealing a positive correlation up to a certain T-N threshold, beyond which the yield plateaued or decreased. The NF membrane

process, with a molecular weight cut-off of ~500 Da, was found to be optimal, potentially due to balancing the rejection of growth-promoting components and permeation of inhibitory substances for yeast growth. This study suggests that the identification of the necessary growth components and inhibitory substances could lead to further optimization of the membrane concentration process, thereby reducing costs and advancing the industrialization of yeast oil production.

Chapter 4

*Effects of inorganic nitrogen addition to okara-utilized medium on the oleaginous yeast *Lipomyces starkeyi* and assessment of metabolism involved in increased oil production*

4.1. Introduction

In Chapter 3, *L. starkeyi* showed growth on a medium based on solid okara as the single nitrogen source; however, its oil yield was not comparable to that obtained when using yeast extract medium (**Fig. 3.2**). Then, when the okara extract solution was prepared using protease and thermal treatment, and then concentrated through membrane separation, the oil yield was higher than that obtained using the yeast extract medium (**Fig. 3.4**). In solid okara-containing media, the solubilization of nitrogen components by yeast proteases is inadequate in the early stages of culture, suggesting insufficient nitrogen sources. However, the effect of adding inorganic nitrogen, which is often used in combination with organic nitrogen source [116, 117], has not been examined.

Nitrogen components are essential for protein and nucleic acid synthesis and are important for cell proliferation, and the limitation of them lead to improvement of oil accumulation in oleaginous microorganisms [27, 38]. Therefore, to increase the oil yield per culture, cell proliferation should predominate in the presence of nitrogen components in the first half of the culture. Then, the metabolic system should be shifted to fatty acids and TAG synthesis in the absence of nitrogen components in the second half of the culture process.

Sulfate ion (SO_4^{2-}) is often used as a counterion to ammonia and magnesium, zinc, and copper ions when they are added to oleaginous yeast media [34, 37, 118]. Sulfate ions are taken up by

cells and utilized in the cysteine and glutathione (GSH) biosynthetic pathway as sulfur ions [119]. GSH is an intracellularly synthesized tripeptide composed of glutamic acid, cysteine, and glycine, and is present in various organisms. Moreover, it reacts directly with free radicals and protects cells against reactive oxygen species (ROS) by acting as a cofactor for oxidoreductases, such as glutathione peroxidase and glutathione reductase [120]. In budding yeast, GSH is an essential metabolite necessary for resistance to oxidative stress [121], and thus, it is also thought to play an important role in oleaginous yeasts, including *L. starkeyi*. Regarding the relationship between oil synthesis and GSH, the enhanced expression of antioxidant-related genes, including GSH-related genes, increases oil accumulation in *Y. lipolytica* [122], however, there are no similar reports pertaining to *L. starkeyi*.

In this chapter, the possibility of deficiency of nitrogen in initial environment of okara medium was focused on. The effect of adding inorganic nitrogen to a medium okara, an organic nitrogen source, was investigated by using a combination of culture experiments and metabolome analysis. The aim was to improve the culture process using okara, which has the potential for use in oil production at a low cost. Furthermore, the synergistic effects of ammonium and sulfate ions to the *L. starkeyi* culture medium and the mechanisms involved in the improved oil productivity are also presented.

4.2. Experimental

4.2.1. Yeast strain, growth conditions, and chemicals

Lipomyces starkeyi CBS1807 was cultured as described in Chapter 3. All reagents were purchased from Fujifilm Wako Pure Chemical (Osaka, Japan), unless otherwise noted. As the main culture method, the yeast was grown in yeast extract medium and okara medium composed of 1%, 2%, and 3% (w/v) okara (Nissin Foods Co., Ltd., Hachioji, Japan) for 4 or 7 days at 30 °C, with shaking at 150 rpm. All media were supplemented with 10% D(+)-glucose as the carbon source. The medium was sterilized by autoclaving at 121 °C for 20 min.

4.2.2. Analysis for NH_4^+ , glucose, cell concentration, and TAG

Samples for analysis were filtered through a centrifugal-type 0.45 μm polyolefin filter (Kurabo Industries, Osaka, Japan) and diluted with distilled water to enter the quantification range. The NH_4^+ concentration in the culture supernatant was determined via enzymatic analysis using an Ammonia Assay Kit (Cell Biolabs, San Diego, USA), according to the manufacturer's instructions. The glucose concentration in the culture supernatants was measured using the Wako Glucose C-II Test, following the manufacturer's instructions. The cell concentration and TAG concentration were measured the same as in Chapter 3.

4.2.3. Metabolome analysis

Metabolite extraction was conducted as follows: the cultured medium was filtered through centrifugal- type 0.45 μm polyolefin filter (Kurabo) at $9200 \times g$ at 4 °C to remove cells and other debris, and then, 80 μL of the supernatant was mixed with 20 μL of Milli-Q water containing internal standards (H3304-1002, Human Metabolome Technologies, Inc. (HMT), Tsuruoka, Yamagata, Japan). The mixture was centrifugally filtered through a 5 kDa cutoff filter (ULTRAFREE MC PLHCC, HMT) at $9100 \times g$ at 4 °C to remove macromolecules. The filtered culture supernatant was then used for the metabolomic analysis at HMT. Metabolome analysis was conducted according to HMT's Basic Scan package using capillary electrophoresis time-of-flight mass spectrometry (CE-TOFMS), based on previously described methods [123, 124].

Briefly, CE-TOFMS analysis was performed using an Agilent CE capillary electrophoresis system equipped with an Agilent 6230 time-of-flight mass spectrometer (Agilent Technologies, Santa Clara, CA, USA). The systems were controlled using Agilent MassHunter Workstation Data Acquisition (Agilent Technologies) and connected by a fused silica capillary (50 μm i.d. $\times$ 80 cm) with a commercial electrophoresis buffer (H3301-1001 and I3302-1023 for cation and anion analyses,

respectively; HMT) as the electrolytes. The mass spectrometer was scanned from m/z 50 to 1000, and the peaks were extracted using the MasterHands automatic integration software (Keio University, Tsuruoka, Japan) to obtain peak information, including m/z , peak area, and migration time (MT) [125]. Signal peaks corresponding to isotopomers, adduct ions, and other product ions of known metabolites were excluded, and the remaining peaks were annotated according to the HMT metabolite database based on their m/z values and MTs. The areas of the annotated peaks were normalized to those of the internal standards. Furthermore, 110 primary metabolites, containing amino acids and GSH, were quantified based on one-point calibrations using their respective standard compounds.

4.2.4. Data analysis

For culture evaluations, statistical analysis was performed using Student's t-test or Tukey's test with GraphPad Prism (version 10.2.1; GraphPad Software, San Diego, USA). Differences were considered statistically significant at $p < 0.05$. Values are presented as mean $\pm$ standard deviation, calculated from triplicate experiments. The oil conversion rate (% [w/w]) from glucose by yeast was calculated using Equation (4.1).

$$\text{Oil conversion rate (\%)} = \frac{\Delta(P)}{\Delta(S)} \quad (4.3)$$

where $\Delta(P)$ and $\Delta(S)$ indicate differences in the produced oil and consumed glucose, respectively, at the sampling and starting points of cultivation. For the metabolomic analysis, hierarchical cluster analysis (HCA) and principal component analysis (PCA) were performed using HMT's proprietary MATLAB and R programs [126], respectively. The detected metabolites were linked to a metabolic pathway index based on the HMT database constructed from public databases.

4.3. Results and discussion

4.3.1. Quantitative evaluation of nitrogen components in okara and yeast extract media

The total nitrogen content of okara, a residue from tofu and soymilk production, has been reported to be $3.7 \pm 0.2\%$ (w/w) in **Table 3.1**, with the free amino acid content being only 0.1% (w/w) [110]. Therefore, to confirm the nitrogen source components in the okara medium used in this study (composed of okara and glucose), free amino acid and NH_4^+ concentrations were measured and compared with those in the yeast extract medium. The concentration of total free amino acids was 80.6 ± 8.5 mM in the 5% (w/v) yeast extract medium, whereas it was 1.9 ± 0.02 mM in the 2% (w/v) okara medium, which is less than 1/42 of that in the yeast extract medium (**Fig. 4.1a**). The NH_4^+ concentration was 57.6 ± 2.97 mM in the yeast extract medium and 3.5 ± 0.09 mM in the okara medium, which was less than 1/16 of that in the yeast extract medium (**Fig. 4.1b**). These results suggest that the low cell growth and low TAG yields associated with cultures using only okara as the nitrogen source (**Fig. 3.2**) could be due to a lack of nitrogen, especially in the early stages (cell proliferation phase) of culture. Insufficient nitrogen sources not only hinder cell proliferation but also cause metabolic fluctuations in which AMP is consumed to compensate for the lack of nitrogen sources in yeast cells, which may induce a metabolic shift from cell proliferation to TAG synthesis, even early in culture [27, 38]. Thus, supplementing the okara medium with a nitrogen source may promote cell growth in the early stages of culture and eventually lead to increased oil yields.

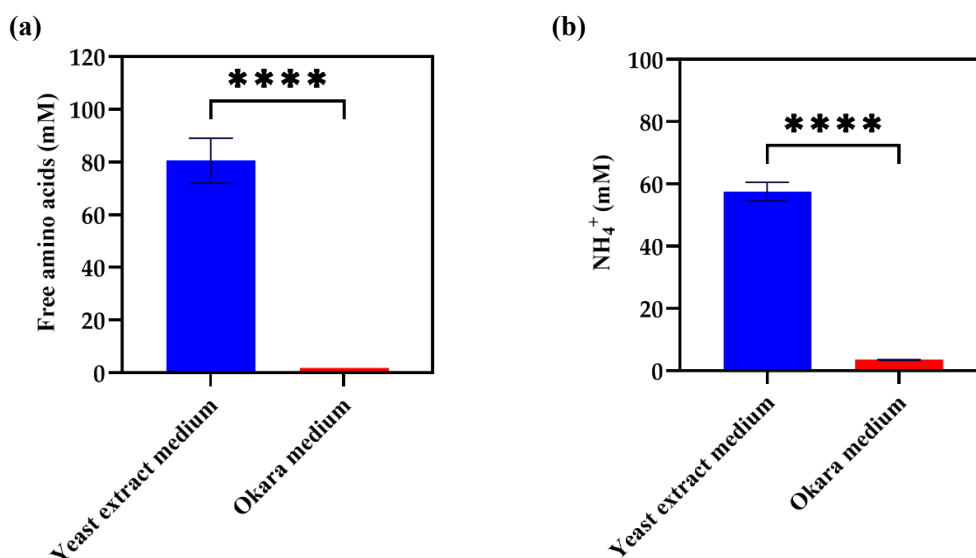


Fig. 4.1 Quantitative analysis of nitrogen sources in the culture media. **(a)** Concentration of free amino acids and **(b)** NH_4^+ in the culture medium. The yeast extract medium contained 5% (w/v) yeast extract and 10% (w/v) glucose. The okara medium contained 2% (w/v) okara and 10% (w/v) glucose. Mean $\pm$ S.D., **** $p < 0.0001$, $n = 3$, Student's t-test.

4.3.2. Effect of ammonium salt addition

Ammonium sulfate ($[\text{NH}_4]_2\text{SO}_4$), a commonly used inorganic nitrogen source in culture media, was added to the okara medium (1, 2, and 3% [w/v] okara with 10% [w/v] glucose) to increase cell proliferation by resolving the lack of nitrogen components in the early stages of culture. Its addition significantly increased not only the cell concentration but also TAG production in the okara medium after 4 days of culture (**Fig. 4.2a,b**). The addition of 1% (w/v) ammonium sulfate to 2% (w/v) and 3% (w/v) okara media resulted in higher oil yields than those in the yeast extract medium, by 1.29 ± 0.04 (fold) and 1.33 ± 0.15 (fold), respectively (**Fig. 4.2a**). Without the addition of ammonium sulfate, the cell concentrations were as low as 0.31 ± 0.02 (fold), 0.45 ± 0.03 (fold), and 0.47 ± 0.03 (fold) in 1% (w/v), 2% (w/v), and 3% (w/v) okara media, respectively, compared to those in the yeast

extract medium. The addition of 1% (w/v) ammonium sulfate also significantly increased the cell concentration to 0.82 ± 0.12 (fold), 0.86 ± 0.09 (fold), and 1.01 ± 0.03 (fold), respectively, which was comparable to that achieved with the yeast extract medium (**Fig. 4.2b**). The total nitrogen concentration in 3% (w/v) okara medium supplemented with 1% (w/v) ammonium sulfate was calculated as 0.32% (w/w), lower than the 0.5% (w/w) in the yeast extract medium. Therefore, the improvement in cell concentration and oil yield may be due more to the effect of adding ammonium sulfate itself than to the effect of increased total nitrogen in the medium.

Next, to determine whether NH_4^+ ions or SO_4^{2-} ions are important for TAG production, various ammonium or sulfate salts were added to the culture medium (**Fig. 4.3a**). Diammonium hydrogen phosphate ($[\text{NH}_4]_2\text{HPO}_4$, 0.9% [w/v]), ammonium chloride (NH_4Cl , 0.7% [w/v]), and sodium sulfate (Na_2SO_4 , 1.0% [w/v]) were added to a 2% (w/v) okara medium. Here, the TAG concentration was significantly increased only in the ammonium sulfate-added group (13.4 ± 0.52 [g/L]) compared to that in the okara medium group (8.7 ± 0.69 [g/L]). However, no significant increase in the oil yield was observed in the ammonium chloride-added group (9.2 ± 0.23 [g/L]), and the oil yield was significantly decreased in the diammonium hydrogen phosphate-added group (5.9 ± 0.70 [g/L]). In the case of sodium sulfate addition, the oil yield was 9.4 ± 0.59 (g/L), showing no significant increase. These results indicate that adding NH_4^+ alone does not increase the oil yield and that the combination of NH_4^+ and SO_4^{2-} is important for increasing oil production. Additionally, regarding the significant decrease in oil yields observed with the addition of ammonium dihydrogen phosphate, the excessive addition of phosphorus may have negatively affected oil accumulation, in accordance with a previous report showing that phosphorus limitations can induce oil accumulation [52]. Regarding the cell concentration, all groups, except the ammonium chloride-supplemented group, showed a significant increase compared to that with the okara medium (**Fig. 4.3b**). The effect of diammonium hydrogen phosphate addition is thought to be due to a change in metabolism toward an increase in the

cell concentration caused by excessive phosphorus addition. For sodium sulfate, the positive effect was on cell proliferation but not on the TAG yield, indicating that the oil accumulation per cell decreased, which is also thought to be due to the change in metabolism with increasing cell concentration. With ammonium chloride addition, a significant decrease in the cell concentration was observed, suggesting that NH_4^+ addition alone does not lead to an increase in cell proliferation, whereas phosphate ions and SO_4^{2-} may contribute to the cell concentration increase.

Furthermore, ammonium sulfate was tested as the sole nitrogen source without the addition of okara. The oil yields for 1% (w/v) and 2% (w/v) ammonium sulfate media were 2.2 ± 0.44 (g/L) and 2.1 ± 0.20 (g/L), respectively, which were significantly lower than those in the yeast extract group (12.9 ± 0.24 [g/L]) (**Fig. 4.3c**). Similarly, cell concentrations were also significantly lower, at $1.02 \pm 0.16 \times 10^8$ (cells/mL) and $1.11 \pm 0.19 \times 10^8$ (cells/mL), respectively, compared to those in the yeast extract group ($7.57 \pm 0.25 \times 10^8$ [cells/mL]) (**Fig. 4.3d**). This indicates that ammonium sulfate alone does not improve cell growth or TAG production. The previously described results (**Fig. 4.2** and **4.3**) indicate that okara, NH_4^+ , and SO_4^{2-} are the three elements necessary for high oil yields from *L. starkeyi*. Additionally, NH_4^+ and SO_4^{2-} had supplementary roles in promoting the growth of *L. starkeyi*, whereas organic nitrogen (proteins and amino acids) derived from okara was primarily utilized during cultivation.

Therefore, ammonium sulfate can compensate for nitrogen deficiency in the early stages of cultivation; however, this alone was not sufficient to fully elucidate the mechanism underlying the increased oil yield and cell concentration. And it was suggested that NH_4^+ and SO_4^{2-} may complement the rate-limiting metabolic processes and components.

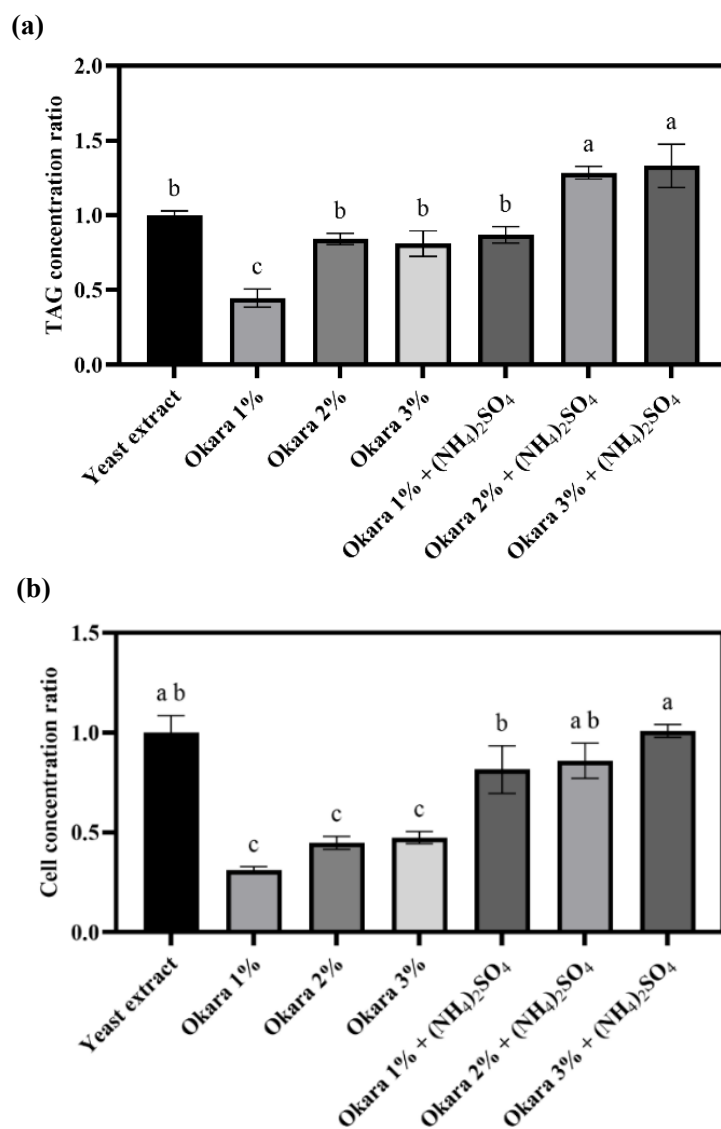


Fig. 4.2 Effect of (NH₄)₂SO₄ addition (1% [w/v]) to the okara medium on the **(a)** TAG productivity and **(b)** cell concentration. The data were obtained after 96 h of incubation. Mean ± S.D., $n = 3$, Tukey's test, different letters above the columns for each experimental group indicate significant differences between groups ($p < 0.05$).

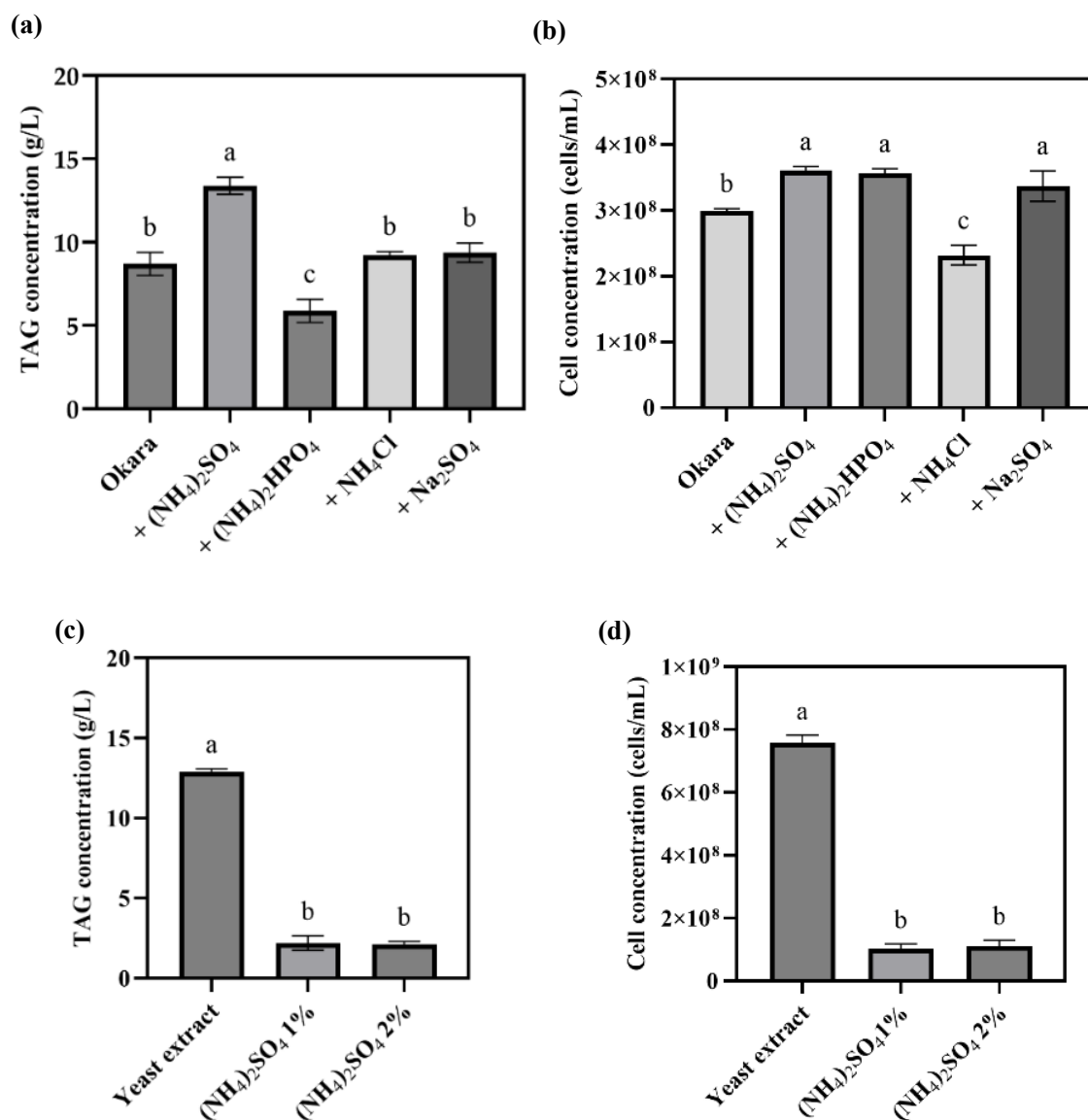


Fig. 4.3 Effect of the addition of various salts on the **(a)** TAG productivity and **(b)** cell concentration. Each salt was added such that the concentrations of NH_4^+ ions among ammonium salts or SO_4^{2-} ions among sulphate salts were the same. Comparison of **(c)** TAG productivity and **(d)** cell concentrations between the yeast extract medium and media using $(\text{NH}_4)_2\text{SO}_4$ as a single nitrogen source. The data were obtained after 96 hour of incubation. Mean $\pm$ S.D., $n = 3$, Tukey's test, different letters above the columns for each experimental group indicate significant differences between groups ($p < 0.05$).

4.3.3. Effects of ammonium sulfate addition based on a metabolome analysis

To investigate the mechanism underlying the increased oil production induced by ammonium sulfate addition, a metabolome analysis of the culture supernatants was performed. **Table 4.1** shows the culture samples analyzed by metabolome analysis and the culture experiment data. Similar to the results shown in **Fig. 4.2**, the group with 2% (w/v) okara supplemented with 1% (w/v) ammonium sulfate (hereafter referred to as the okara-ammonia group) showed higher oil yields than the group with 5% (w/v) yeast extract (hereafter referred to as the yeast extract group). The cell concentration was also increased in the okara-ammonia group compared to that in the 2% (w/v) okara group (hereafter referred to as the okara group). The oil accumulation per cell (amount of TAG per cell) was similar between the okara and okara-ammonia groups on day 4 and was higher than that in the yeast extract group. On day 7, the accumulation in the okara-ammonia group was higher than that in the okara group. The okara medium group had higher residual glucose content than the okara-ammonia and yeast extract groups, suggesting that the low oil yield in the okara medium was due to low cell concentration and slow sugar consumption. On day 7, the amount of TAG per cell in the okara-ammonia group was almost the same as on day 4, but those in the yeast extract and okara medium groups decreased, suggesting that the presence of ammonium sulfate may suppress TAG degradation. The glucose-to-oil conversion rate per cell was highest in the okara group ($28.6 \pm 3.78\%$) on day 4, but on day 7, the okara-ammonia group exhibited the highest rate ($19.8 \pm 0.45\%$). Considering that the oil yield and cell concentration in the okara group on day 4 were the lowest among the three groups, it is thought that the oil conversion rate increased because glucose was used for oil production and not for cell proliferation. These results suggest that the high oil yield of the okara-ammonia group was mainly due to an increase in cell concentration and that the oil produced was maintained within the cells.

Table 4.1 Comparison of oil productivity and culture medium conditions used for metabolome analysis.

Culture medium condition	A: Yeast Extract		B: Okara	C: Okara-ammonia
	Carbon source	Glucose, 10% (w/v)		
	Nitrogen source	Yeast extract, 5% (w/v)	Okara, 2% (w/v)	Okara, 2% (w/v) + (NH ₄) ₂ SO ₄ , 1% (w/v)
Cell concentration (10 ⁸ cells/mL)	day4	5.8 ± 0.31	3.7 ± 0.12	4.7 ± 0.01
	day7	6.3 ± 0.09	3.8 ± 0.33	4.9 ± 0.43
TAG concentration (g/L)	day4	14.3 ± 0.40	13.8 ± 1.81	18.8 ± 1.18
	day7	12.2 ± 0.59	9.4 ± 0.10	19.7 ± 0.44
Amount of TAG per cell (mg/10 ⁸ cells)	day4	2.5 ± 0.13	3.8 ± 0.13	4.0 ± 0.10
	day7	1.9 ± 0.03	2.5 ± 0.21	4.0 ± 0.35
Oil conversion rate (% [w/w])	day4	19.4 ± 0.56	28.6 ± 3.78	25.2 ± 1.80
	day7	12.3 ± 0.60	11.3 ± 0.40	19.8 ± 0.45
Residual glucose content (%)	day4	2.6 ± 0.04	5.2 ± 0.10	2.5 ± 0.26
	day7	0.10 ± 0.05	1.6 ± 0.29	0.09 ± 0.02

TAG, triacylglycerol

Next, a metabolome analysis based on CE-TOF-MS was performed, and 400 metabolites were detected in the three culture groups. The metabolites detected in each culture medium were classified using a metabolic index based on the HMT database. The number of metabolites detected in each sample was expressed as a percentage of the total number of metabolites (400 compounds) detected in the whole group as detection ratio (**Fig. 4.4**). The metabolic index associated with each metabolite was also indicated by color coding in the figure. Metabolites were linked to various metabolic indices, such as energy and amino acid metabolism. The detection ratio was the lowest and decreased with culture time in the okara group. In contrast, in the okara-ammonia group, the detection ratio increased with the culture time and reached a level similar to that in the yeast extract group, indicating that the addition of ammonium sulfate activated yeast metabolism.

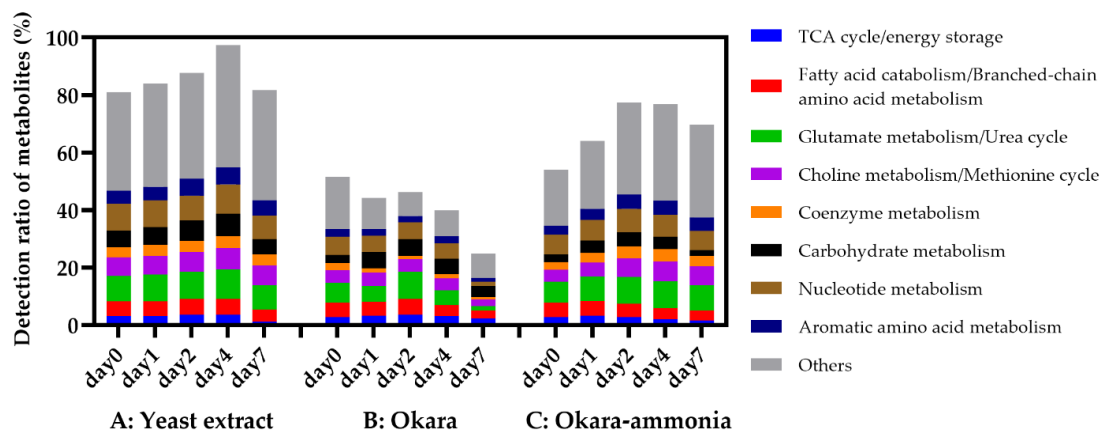


Fig. 4.4 Detection ratio of metabolites in each medium group and metabolic symbols. The metabolites detected in each culture supernatant were classified based on their metabolic symbols and the number of them was presented as a percentage of the total 400 metabolites detected. Values represent the averages of each sampling point ($n = 3$). When multiple metabolic symbols are associated with a metabolite, they overlap.

In addition, PCA was performed on all metabolites, resulting in three different medium groups classified into three distinct clusters (**Fig. 4.5**). PC1 (horizontal axis) primarily reflected the differences between the yeast extract group (A, green) and the two okara-based groups, the okara group (B, blue) and the okara-ammonia group (C, red), whereas PC2 (vertical axis) primarily reflected the differences between the groups over time. The okara group (B) showed less metabolic variation over time than the other two groups, whereas the okara-ammonia group (C) exhibited obvious metabolic variation with the culture duration, with performance similar to that of the yeast extract group (A). These results support our hypothesis that metabolic activation induced by ammonium sulfate addition contributes to increased cell concentration and oil yields.

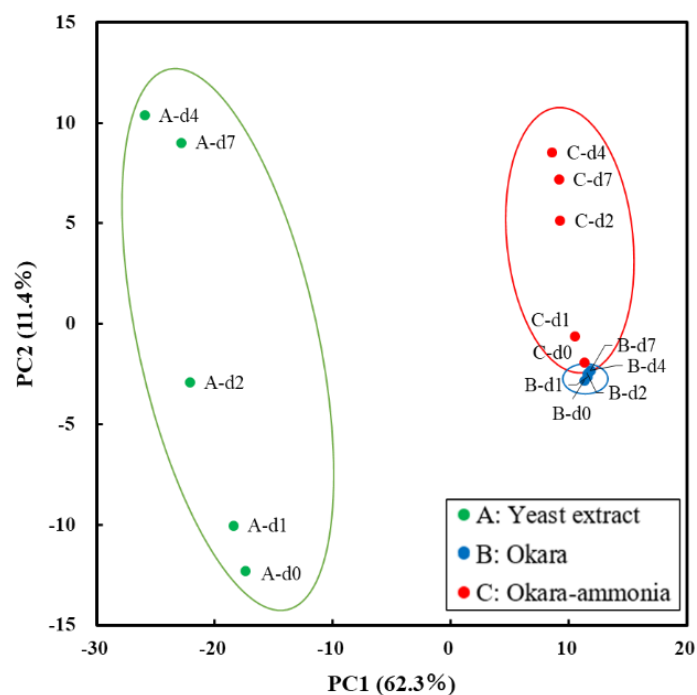


Fig. 4.5 Principal component analysis (PCA) of the metabolome. The graph was drawn using the first and second principal components (PC1 and PC2). Group A (green): yeast extract. Group B (blue): okara. Group C (red): okara-ammonia.

To identify the activated metabolic pathways, heatmap generation and HCA were performed to compare the okara and okara-ammonia groups. As shown in **Fig. 4.6** and **Table 4.2**, GSH, γ -Glu-Cys, Cys (cysteine), and Gln (glutamine) were included in the metabolic clusters with the largest variations. This indicated that metabolic clusters related to the GSH metabolic pathway were largely increased from day 2 to 4. Therefore, it is strongly suggested that the GSH metabolic pathway is enhanced in the okara medium supplemented with ammonium sulfate.

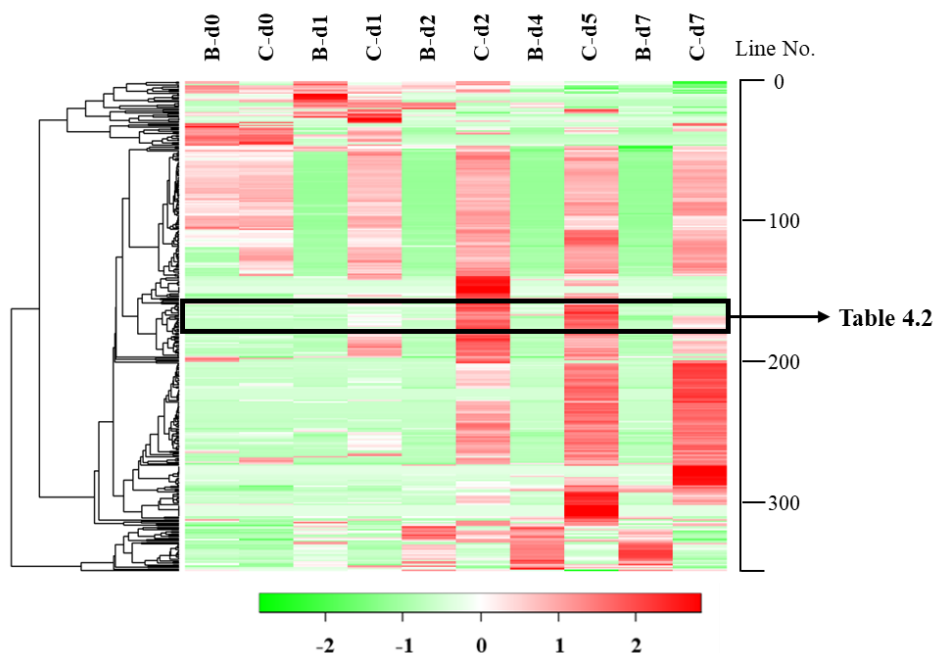


Fig. 4.6 Heatmap and hierarchical clustering analysis of metabolome of *Lipomyces Starkeyi*

in okara or okara-ammonium medium. Overall view of the heatmap of the metabolome profiles ordered based on the average slope of the fold-change and results of the HCA. All metabolic substance values were standardized (average: $\mu = 0$, dispersion: $\sigma = 1$) to align the data scales and are presented as the standardized relative area. The correlation coefficient was used as an indicator of the distance between peaks, whereas the unweighted pair group method with arithmetic mean (UPGMA method) was used to calculate the distance between clusters and create a tree diagram.

Table 4.2 Metabolite clusters with large variation and their standardized relative area.

Compounds in metabolite clusters shown in the black square frame in **Fig. 4.6** were listed.

Line No.	Compound name	Standardized Relative Area									
		B-d0	C-d0	B-d1	C-d1	B-d2	C-d2	B-d4	C-d4	B-d7	C-d7
160	Glutathione (GSH)	-0.730	-0.723	-0.470	-0.053	-0.170	1.952	-0.359	1.735	-0.738	-0.443
161	Butyric acid	-0.695	-0.695	-0.354	-0.199	-0.170	1.627	-0.172	2.049	-0.695	-0.695
162	Thiamine phosphate	-0.471	-0.471	-0.471	-0.471	-0.471	2.143	-0.471	1.623	-0.471	-0.471
163	Quinolinic acid	-0.474	-0.474	-0.474	-0.474	-0.474	1.924	-0.474	1.870	-0.474	-0.474
164	AMP	-0.606	-0.587	-0.502	-0.256	-0.553	1.914	-0.212	1.844	-0.598	-0.445
165	Noradrenaline	-0.461	-0.461	-0.461	-0.461	-0.461	1.350	-0.461	2.340	-0.461	-0.461
166	DOPA	-0.454	-0.454	-0.454	-0.454	-0.454	1.193	-0.454	2.435	-0.454	-0.454
167	UMP	-0.679	-0.679	-0.679	-0.032	-0.679	1.592	0.742	1.774	-0.679	-0.679
168	<i>S</i> -Adenosylmethionine	-0.271	-0.195	-0.786	0.220	-0.458	1.175	-0.884	2.018	-1.259	0.441
169	5-Oxo-2-tetrahydrofurancarboxylic acid	-0.326	-0.315	-0.281	-0.130	-0.589	1.280	-1.105	1.981	-1.105	0.589
170	Succinyladenosine	-0.682	-0.660	-0.823	-0.131	0.176	1.366	-0.618	1.804	-1.159	0.727
171	GABA	-0.659	-0.622	-0.688	0.045	-0.655	1.930	-0.646	1.483	-0.770	0.582
172	Cys	-0.636	-0.636	-0.636	-0.256	-0.636	1.833	-0.636	1.683	-0.636	0.560
173	Pyridoxamine 5'-phosphate	-0.671	-0.671	-0.671	0.073	-0.671	1.699	-0.671	1.785	-0.671	0.470
174	Guanidinosuccinic acid	-0.628	-0.628	-0.628	-0.026	-0.628	1.708	-0.628	1.920	-0.628	0.169
175	γ-Glu-Cys	-0.635	-0.635	-0.635	0.257	-0.635	1.897	-0.635	1.705	-0.635	-0.047
176	NMN	-0.619	-0.619	-0.573	-0.548	-0.544	2.018	-0.498	1.612	-0.541	0.312
177	Hypotaaurine	-0.746	-0.746	-0.524	0.099	-0.462	2.112	-0.746	1.249	-0.746	0.508
178	GMP	-0.663	-0.663	-0.663	0.295	-0.663	2.135	-0.663	1.284	-0.663	0.263
179	CMP	-0.642	-0.642	-0.642	-0.221	-0.642	2.124	-0.642	1.164	-0.642	0.783
180	γ-Glu-Asn	-0.681	-0.681	-0.681	0.325	-0.681	1.357	-0.681	2.034	-0.681	0.370
181	<i>N</i> -Acetylalanine	-0.730	-0.730	-0.730	0.432	-0.730	1.585	-0.730	1.577	-0.730	0.784
182	Saccharopine	-0.282	-0.304	-0.956	0.135	-0.539	2.106	-0.614	1.180	-1.117	0.392
183	Arg	-0.466	-0.246	-0.872	0.767	-0.867	1.920	-0.870	1.120	-0.878	0.390
184	Uridine	-0.460	-0.458	-0.581	0.550	-0.633	2.332	-0.644	0.718	-1.055	0.232
185	Gln	-0.588	-0.574	-0.611	0.798	-0.612	2.325	-0.616	0.818	-0.623	-0.316

Table 4.3 shows the changes in the concentrations of the 20 natural amino acids and GSH in all three groups. In the okara group, the amino acid content was low; most amino acids were consumed after 24 h of culture, and all were below the detection limit by day 7. In the yeast extract group, amino acids, except Cys and Gln, were present at high levels (several hundred to 10,000 ppm, even on day 7), indicating an excess amount of amino acids in the culture medium. In the okara-ammonia group, arginine (Arg), glutamine (Gln), and glutamic acid (Glu) levels increased during the

early to-mid culture period and were consumed during the mid-to-late culture period. In particular, Gln increased more than 50-fold from day 0 to day 3 (23 to 1361 ppm) and disappeared in the latter half of the culture (1361 to 142 ppm), suggesting that Gln- or glutamine-related metabolism has a major effect on oil productivity. In contrast, Cys was detected only on day 4 in the yeast extract group, but low levels of Cys were detected in the okara-ammonia group on days 2, 4, 5, and 7, suggesting that Cys-related metabolism was maintained in the okara-ammonia group. Gln is converted intracellularly to Glu, which combines with Cys, mediated by γ -glutamylcysteine synthetase, to form γ -Glu-Cys, which further combines with Gly, mediated by glutathione synthetase, to form γ -Glu-Cys-Gly (glutathione, GSH). However, GSH is degraded to form Gly, Cys, and γ -Glu and is metabolized again [127]. Therefore, the Cys, Gln, and Glu concentration variations were consistent with the GSH-related metabolic variations revealed through HCA, suggesting that ammonium sulfate affects GSH-related metabolic pathways.

Table 4.3 Amino acids and glutathione (GSH) concentration profile at each culture period.

Amino acids	A: Yeast extract							B: Okara							C: Okara-ammonia						
	Culture period (days)							Culture period (days)							Culture period (days)						
	d0	d1	d2	d4	d7	d0	d1	d2	d4	d7	d0	d1	d2	d4	d7	d0	d1	d2	d4	d7	
Ala	8,449	7,886	7,455	5,211	5,650	189	12	11	0	0	196	237	348	214	208	196	237	348	214	208	
Arg	4,052	3,933	3,309	2,360	2,377	79	1.2	2.2	1.5	0	121	314	534	381	242	121	314	534	381	242	
Asn	3,542	3,444	3,697	3,548	3,801	89	2.3	3.0	1.8	0	94	126	225	238	168	94	126	225	238	168	
Asp	4,435	4,348	4,587	4,504	4,816	100	6.7	2.4	2.1	0	102	130	164	139	126	102	130	164	139	126	
Cys	0	0	0	1.7	0	0	0	0	0	0	0	0.4	2.6	2.5	1.3	0	0.4	2.6	2.5	1.3	
Gln	42	106	108	202	67	16	5.9	5.5	3.3	0	23	656	1,361	666	142	23	656	1,361	666	142	
Glu	9,585	9,427	10,041	9,130	10,319	262	29	13	5.2	0	259	455	445	357	370	259	455	445	357	370	
Gly	5,300	5,088	5,471	5,344	5,732	141	9.3	9.6	4.9	0	149	157	175	153	151	149	157	175	153	151	
His	1,787	1,710	1,566	1,289	1,318	19	0.5	1.4	1.0	0	25	39	57	53	33	25	39	57	53	33	
Ile	3,619	3,406	3,427	3,114	3,381	99	2.6	3.2	2.3	0	107	100	115	83	78	107	100	115	83	78	
Leu	6,420	6,126	5,889	4,494	4,705	135	2.3	4.7	3.6	0	140	134	143	106	103	140	134	143	106	103	
Lys	4,032	3,912	3,989	3,841	4,265	67	2.2	2.8	3.1	0	85	121	192	204	141	85	121	192	204	141	
Met	1,905	1,924	1,572	1,114	1,186	20	0.2	0.7	0.5	0	18	20	20	12	14	18	20	20	12	14	
Phe	4,234	4,155	3,457	2,229	2,361	56	2.6	3.7	2.5	0	58	61	60	41	37	58	61	60	41	37	
Pro	4,157	3,949	4,351	4,351	4,701	133	3.9	4.7	3.6	0	136	121	127	131	133	136	121	127	131	133	
Ser	6,282	5,906	6,449	6,183	6,667	160	5.1	7.0	4.8	0	166	177	201	169	164	166	177	201	169	164	
Thr	3,390	3,335	3,595	3,522	3,789	90	3.9	4.5	2.8	0	94	111	129	112	113	94	111	129	112	113	
Trp	784	751	610	426	446	12	0.11	0.8	0.4	0	12	12	11	6.8	6.0	12	12	11	6.8	6.0	
Tyr	4,200	4,002	3,663	2,833	3,051	51	0.8	1.7	1.2	0	53	53	55	39	36	53	53	55	39	36	
Val	4,337	4,246	4,425	4,023	4,307	144	0	11	0	0	149	149	160	120	115	149	149	160	120	115	
GSH	0	4.9	42.5	127.4	1.1	0.4	14.7	31.2	20.8	0	0.8	37.6	147.6	135.6	16.2	0.8	37.6	147.6	135.6	16.2	

The data (in ppm) shown are averages. The data (ppm) are shown in a heatmap. The average of the maximum and minimum values of all data is colored white, while the closer to the maximum value, the darker red, and the closer to the minimum value, the darker green.

Furthermore, the quantitative results showed that the maximum amount of GSH (20–150 ppm) was detected in all groups during the mid-culture period (days 2 and 4), but most of it was lost by day 7 (1.1 μM for the yeast extract group, below the detection limit for the okara group, and 16 μM for the okara-ammonium group) (**Table 4.3**). The maximum concentration of GSH in the okara group was 31 μM at day 2, whereas it was 127 μM in the yeast extract group at day 4 and 148 μM in the okara-ammonium group at day 2, showing that GSH was detected in these two groups in amounts more than four times that in the okara group. This suggests that the addition of ammonium sulfate to the okara medium altered GSH-related metabolism to a level equal to or greater than that in the yeast extract group. On day 7, 16 μM GSH was detected in the okara-ammonium group, which was 10 times higher than that in the yeast extract group (1.1 μM), suggesting that GSH-related metabolism was enhanced for a longer period compared to that in the yeast extract group.

4.3.4. Estimated mechanism of enhanced GSH metabolism and improved oil productivity mediated by ammonium sulfate addition

The results of culture experiments and the metabolomic analysis revealed a relationship between enhanced GSH metabolism and improved oil productivity following the addition of ammonium sulfate to the okara medium. A previous study reported that budding yeast GSH synthase-mutants could not survive and exhibited apoptosis [128]. Another study showed that enhanced GSH synthase expression improves TAG synthesis in the oleaginous yeast *Y. lipolytica* [122]. Furthermore, a report of oil production and cellular physiological responses in budding yeast suggested that excessive fatty acids may induce endoplasmic reticulum stress and indirectly cause oxidative stress [129]. Combining these previous findings with the present results, GSH metabolism may be important for cell function maintenance and TAG synthesis in *L. starkeyi*, and the activation of GSH-related metabolism may contribute to the increased oil yield. In an activated state of metabolism, ROS are easily generated, in association with respiration for energy production [128], indicating that increased

GSH synthesis protects cells from oxidative damage. Furthermore, GSH serves as a sulfur reservoir [130], and the depletion of GSH observed in the late culture phase was presumed to be due to the reuse of reduced sulfur (Cys) accumulated in the mid-culture phase. This mechanism is thought to enable the maintenance of high metabolic levels.

Furthermore, culture experiments (**Fig. 4.2** and **4.3**) showed that both NH_4^+ and SO_4^{2-} are important for increasing the oil yield. Therefore, the following mechanism(s) is proposed for the activation of GSH-related metabolism by these ions (**Fig. 4.7**): SO_4^{2-} is taken up into cells, promoting the sulfur assimilation pathway (reduction pathway, from sulfate to Cys), and activating Cys synthesis. The increased Cys levels are then used for GSH synthesis [119]. However, it is also important to consider that the Gln and Glu pools expand owing to a sufficient NH_4^+ supply. The detection of large amounts of Gln during the middle stage of culture with the addition of ammonium sulfate suggests that the ammonium assimilation pathway [131], which synthesizes Gln from Glu and NH_4^+ , becomes active at high concentrations of NH_4^+ . This suggests that the synthesis pathways for nucleobases and nucleic acids are promoted by an abundant nitrogen supply, resulting in more efficient cell proliferation. In addition, an increase in Glu and its precursor Gln leads to an abundant supply of substrates for GSH synthesis, resulting in the activation of GSH-related metabolism.

In summary, when *L. starkeyi* was cultured in the okara medium, the cell concentration was low, but the oil accumulation per cell was higher than that in the yeast extract medium (**Table 4.1**). This was attributed to the initial nitrogen deficiency in the culture, which led to the degradation of AMP to supply NH_4^+ . This resulted in a decrease in ICDH activity and negative regulation of the TCA cycle, thereby activating the oil accumulation pathway before cell proliferation reaches a sufficient level. In contrast, the addition of ammonium sulfate to the okara medium increased the cell concentration, whereas the oil accumulation per cell was equal to that in the okara medium, resulting in a higher oil yield per culture compared to that in the okara and yeast extract media (**Fig. 4.2a**). This suggests that the addition of ammonium sulfate compensates for nitrogen source depletion in the early stages of culture, thereby contributing to improved cell proliferation through maintenance of the TCA cycle. However, the results of the culture experiment (**Fig. 4.3c,d**) showed that supplementation with NH_4^+ alone was insufficient to increase the oil yield, suggesting that the combination of NH_4^+ and SO_4^{2-} is important for activating metabolism and increasing oil yield. Metabolomic analysis results (**Fig. 4.6, Table 4.2 and Table 4.3**) suggest that SO_4^{2-} contributes to the supply of Cys mediated by sulfur assimilation pathway, whereas NH_4^+ may activate Gln and Glu metabolism through activation of the ammonia assimilation pathway, thereby inducing the activation of GSH-related metabolism. This suggests that abundant GSH may counteract the oxidative stress and apoptosis caused by ROS, thereby maintaining cell function and improving TAG synthesis. On the other hand, regarding culture medium costs, yeast extract costs from 20 to 60 dollars per kilogram, ammonium sulfate costs several dollars per kilogram, and okara costs tens of cents per kilogram. Therefore, the okara medium supplemented with ammonium sulfate can reduce the medium nitrogen source costs for *L. starkeyi* culture to a level of several % that of conventional yeast extract culture media.

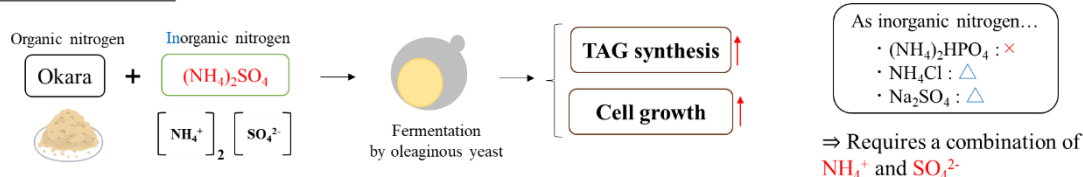
To our knowledge, this study is the first to demonstrate the relationship between the addition of ammonium sulfate, an inexpensive inorganic nitrogen source, and its effects on GSH metabolism

and oil yield in oleaginous yeast through culture experiments and metabolome analyses. These findings provide new insights into both the microbiology and practical applications of alternative oil production mediated by oil-producing microorganisms. In the future, more detailed mechanisms will be elucidated by measuring the intracellular metabolomes, gene expression changes, and enzyme activities.

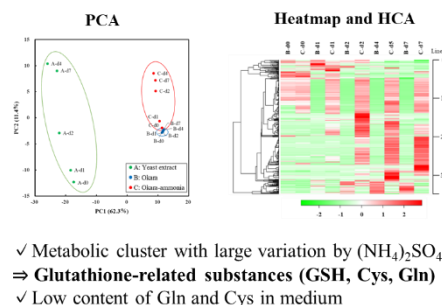
4.4. Conclusions

The graphical summary of this chapter was shown in **Fig. 4.8**.

Culture evaluation



Metabolome analysis for culture supernatant



Putative mechanism of oil yield increase by $(\text{NH}_4)_2\text{SO}_4$

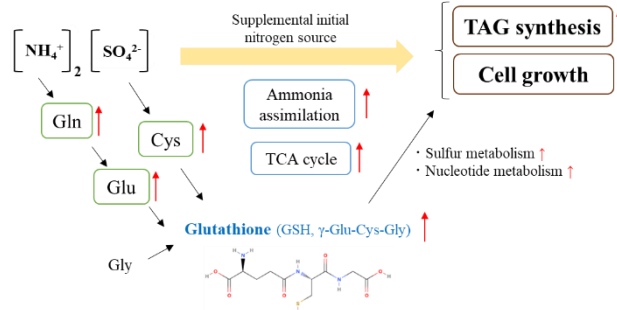


Fig. 4.8 Graphical summary of this chapter.

In this chapter, the effect of adding ammonium salts as a nitrogen supplement to an okara medium on oil production by *L. starkeyi* was investigated. Among the salts tested, ammonium sulfate was found to be the most effective in promoting cell growth and oil yield, indicating the importance of adding both NH_4^+ and SO_4^{2-} ions to the okara medium. Metabolome analysis of the culture medium revealed that ammonium sulfate addition activated GSH-related metabolic and ammonia assimilation pathways,

strongly suggesting that these pathways contribute to the maintenance of cellular functions and TAG synthesis. Based on these results, it was concluded that the addition of ammonium sulfate to the okara medium promoted cell proliferation by supplementing the initial nitrogen source depletion and, furthermore, increased oil production through the activation of GSH metabolism and ammonia assimilation. The cost of the okara medium with ammonium sulfate is as low as approximately several % of that of the yeast extract medium. These findings will provide new insights into yeast microbiology and industrial applications of oil production by oleaginous yeast.

Chapter 5

Conclusions

Oils and fats are widely used in foods, fuels, chemical products, detergents, ink solvents, etc. around the world. Among edible oils, palm oil is particularly popular and accounts for more than 35% of global vegetable oil production because it is cheaper than other vegetable oils and has high chemical stability. However, palm oil production faces various issues that threaten its stable supply such as environmental and human rights problems, as well as the risk of future climate change. Therefore, alternatives to palm oil are needed to ensure a sustainable and stable supply. For this reason, oleaginous yeast *Lipomyces starkeyi* is a promising candidate as an alternative oil producer due to its high productivity of oils similar to palm oil. However, oil production by *L. starkeyi* has not yet been industrialized because feasible and suitable production processes for the food industry have not been established.

This study focuses on the development of upstream and downstream processes for low-cost oil production by *L. starkeyi* applicable to the food industry by applying membrane separation technology and utilizing inexpensive food residues such as okara for culture medium. All detailed results are described in the Chapters 2 to 4, and are summarized below:

5.1. Simple and economical downstream process development for edible oil production from oleaginous yeast *Lipomyces starkeyi*

This chapter investigates a food-grade process for the low-cost and industrial production of oil from the oleaginous yeast *L. starkeyi*. Conventionally, the emulsification generated by crushing yeast cells in the presence of water inhibits oil extraction, so a drying process after cell recovery and

hazardous organic solvents were performed for efficient extraction. As a new method, filtration separation by nonwoven fabric was applied to an emulsified solution and oil extraction from the residue using a 3:1 hexane/ethanol mixture. As a result, the recovery rate of the oil was 98.2%, demonstrating its effectiveness. Based on the results of experiments using model liquids, the filtration separation method is considered to be a method that allows extraction-inhibiting substances to pass through and reduces their amount, in contrast to conventional drying methods. This new process eliminates the need for yeast cell recovery and vacuum freeze-drying, reducing energy consumption by 26% and processing costs by 34% compared to conventional oil extraction methods. Additionally, achieving high-efficiency oil extraction and recovery while using solvents suitable for food processing is noteworthy. This efficient process has the potential to support the practical application of edible palm oil substitutes.

5.2. Utilization of okara as a culture medium by membrane concentration process for high oil production by oleaginous yeast, *Lipomyces starkeyi*

In this chapter, okara, a food residue, was investigated as a nitrogen source for culture media in order to reduce upstream process costs. Okara medium showed a lower oil yield than conventional media, which was thought to be due to its high viscosity caused by the high content of insoluble solids in okara. Therefore, the oil yield was greatly improved by using okara extract solution from which insoluble solids had been removed by a filter paper. For further optimization, the concentrated extract solution using NF, UF, and RO membranes was applied for a culture medium. The culture medium concentrated 5-fold by NF membranes showed best oil yields (20 [g/L], 1.44 times than yeast extract, 1.69 times than okara extract solution). There was a positive correlation between total nitrogen concentration and oil yield up to a certain threshold, but beyond that, the yield remained flat or decreased. These results suggest that the NF membrane process is optimized at a cutoff of approximately 500 Da, achieving a balance between the concentration of growth-promoting

components and the permeability of inhibitory substances. These findings demonstrate the potential of okara for use in the upstream process of oil production by yeast, enabling significant cost reductions in culture media and high oil yields.

5.3. Effects of inorganic nitrogen addition to okara-utilized medium on oleaginous yeast, *Lipomyces starkeyi* and estimation of metabolism involved in the high oil production

In this chapter, focusing on the deficiency of nitrogen nutrients in the initial environment of okara medium, the effect of adding ammonium salt, which is an inorganic nitrogen, was investigated. Among ammonium salts, the addition of ammonium sulfate greatly improved not only cell concentration but also the oil yield of okara medium, exceeding that of conventional yeast extract medium. Furthermore, it was shown that the combination of SO_4^{2-} and NH_4^+ ions is important for this effect by culture experiments. On the other side, metabolomic analysis of the medium revealed that the addition of ammonium sulfate activates glutathione (GSH) -related metabolic pathways and ammonia assimilation pathways, possibly contributing to the maintenance of cellular function and enhanced oil productivity. This study is the first report showing that oil yield can be improved in *L. starkeyi* by adding inorganic nitrogen to okara medium, and that ammonium sulfate affects GSH metabolism and oil yield.

5.4. Summary of cost reduction effects on total oil production process of *L. starkeyi*

In the above three chapters, the upstream and downstream processes of oil production by *L. starkeyi* were examined using membrane separation technology and food residues. Based on these results, the cost reduction effects, which is one of the main objectives of this research are summarized in **Table 5.1**.

Table 5.1 Summary and comparison of cost reduction achievement in each chapter compared to conventional process.

	Improved point	Cost after reduction	Reference
Upstream	Okara extract concentration	60.2%	Chapter 3
	Okara + (NH ₄) ₂ SO ₄	66.2%	Chapter 4
Downstream	Filtration separation	90.7%	Chapter 2
Total	Okara extract concentration Filtration separation	53.7%*	-

*: The total cost after reduction was calculated based on the combination of okara extract concentration (upstream) and filtration separation (downstream), and the details are described in section 5.4..

As for the upstream process, oil production by culture of *L. starkeyi*, two approaches were conducted (Chapter 3 and 4). In Chapter 3, membrane concentration of nitrogen components in okara extract solution showed that it could be used as a substitute for conventional yeast extract, and the cost of nitrogen raw material for culture medium was estimated to be approximately 1/100. In addition, under the best conditions, the oil yield at day4 was 1.44 times higher compared to yeast extract medium (20.0 vs 13.9 [g/L], respectively). Then, the estimated cost reduction of this improvement in the overall process cost of oil production by *L. starkeyi* was calculated based on data from previous studies [58, 60]. As a result, it was calculated that the introduction of this process would reduce the total cost by 60.2%.

On the other hand, in Chapter 4, the addition of inorganic nitrogen (especially, (NH₄)₂SO₄) to a medium using okara was examined, and the cost of nitrogen material for the medium was estimated to be approximately 1/10 to 1/100 that of yeast extract. Furthermore, by adding ammonium

sulfate, the oil yield at day4 was 1.31 times higher than yeast extract medium (18.8 vs 14.3 [g/L], respectively). As a result of the estimated cost reduction in the total process, it was calculated that the introduction of this process would reduce by 66.2%. The above results demonstrate the advantages of the membrane concentration process for the okara extract solution in Chapter 3 when using okara in the upstream process.

Regarding downstream processes, oil extraction and recovery process, in Chapter 2, introduction of filtration separation process and oil extraction by mixed solvents was examined. As a result, the cost in the oil extraction process was reduced to 34% of the conventional. Then, the estimated cost in the overall process was calculated to be 90.7%.

Based on these results, the final total process cost after reduction achieved in this study was estimated. In the upstream process, a process using membrane concentration of okara extract as a culture medium (Chapter 3) was adopted, and in the downstream process, the process described in Chapter 2 was adopted. As a result, the estimated total cost after reduction was 53.7%. The detailed was shown following: About okara extract concentration, the use of okara reduced the cost of culture medium raw materials by 13.4% and further reduced the total cost by 30.5% by achieving an oil yield 1.44 times higher. About filtration separation, oil extraction costs were reduced by 66% ($100 - 34 = 66\%$) which accounted for 9.3% of total costs. Based on the above, the total cost after reduction was calculated by following Eq..

$$\begin{aligned}
 & [100 - (\text{raw material cost reduction}) + (\text{oil extraction cost reduction})] \\
 & \quad \div (\text{increase rate of oil yield}) \\
 & = [100 - (13.4 + 9.3)] \div 1.44 \\
 & = 53.7 (\%)
 \end{aligned}$$

As described above, the utilization of membrane separation technology and food residues resulted in significant cost reductions in the production of alternative oil using *L. starkeyi*.

5.5. Overall summary of the study

This study focused on membrane separation technology and the production of oil from unexpensive food residues, and examined upstream and downstream processes with the aim of establishing low-cost oil production process by *L. starkeyi* that also can be applied to the food industry.

Fig. 5.1 summarizes the overall outline of this research.

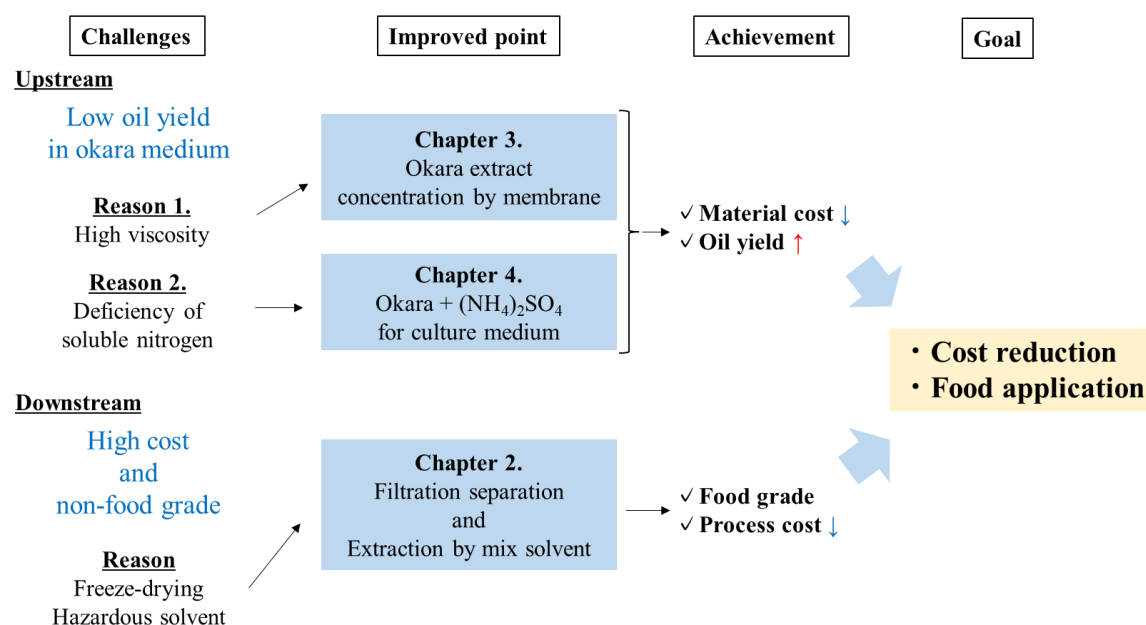


Fig. 5.1 Relationship between the chapters of this thesis and the aim of the research.

Through the development of an upstream process utilizing okara in Chapters 3 and 4, improvements in oil yield and reductions in material costs were achieved. Meanwhile, in Chapter 2, the development of a new oil extraction process as a downstream process resulted in reductions in process costs and enabled application to the food industry. Combining these results, the main objective of this study,

which was establishing a low-cost process applicable to the food industry, has been achieved. This established process could support the practical application of edible palm oil substitutes, which in turn helps ensure oil and fat, resources that are essential for humankind.

References

1. Ahmed S, Sha P, Ahmed O. Biochemistry, Lipids. [Updated 2023 May 1]. In: StatPearls [Internet]. Treasure Island (FL): StatPearls Publishing; 2025 Jan-. Available from: <https://www.ncbi.nlm.nih.gov/books/NBK525952/>
2. Akoh CC, Decker EA. Lipid-based fat substitutes. Crit Rev Food Sci Nutr. 1995;35(5):405–430.
3. Wolff J, Cober MP, Huff KA. Essential fatty acid deficiency in parenteral nutrition: historical perspective and modern solutions, a narrative review. Nutr Clin Pract. 2025;40(2):350-367.
4. Alhaji AM, Almeida ES, Carneiro CR, da Silva CAS, Monteiro S, Coimbra JSJR. Palm Oil (*Elaeis guineensis*): A journey through sustainability, processing, and utilization. Foods. 2024;13(17):2814.
5. Haryati Z, Subramaniam V, Noor ZZ, Hashim Z, Loh SK, Aziz AA. Social life cycle assessment of crude palm oil production in Malaysia. Sustain Prod Consum. 2022;29:90-99.
6. Wilcove DS, Koh LP. Addressing the threats to biodiversity from oil-palm agriculture. Biodivers Conserv. 2010;19:999–1007.
7. Murphy DJ, Goggin K, Paterson RRM. Oil palm in the 2020s and beyond: Challenges and solutions. CABI Agric Biosci. 2021;2(1):39.
8. Ratledge C, Wynn JP. The biochemistry and molecular biology of lipid accumulation in oleaginous microorganisms. Adv Appl Microbiol. 2002;51:1-51.
9. Sharma SK, Barthwal R, Saini D, Rawat N. Chemistry of food fats, oils, and other lipids. In: Chauhan OP, eds. Advances in Food Chemistry. Singapore: Springer; 2022.

10. Yang D, Lee YY, Lu Y, Wang Y, Zhang Z. Internal factors affecting the crystallization of the lipid System: Triacylglycerol structure, composition, and minor components. *Molecules*. 2024;29(8):1847.
11. Liu X, Zhu X, Han Z, Liu H. Recent advances in the mechanisms of quality degradation and control technologies for peanut butter: A literature review. *Foods*. 2025;14(1):105.
12. Qi J, Wang X, Wang X, Akoh CC, Jin Q. Effect of oil type and emulsifier on oil absorption of steam-and-fried instant noodles. *J Oleo Sci*. 2019;68(6):559-566.
13. Charoentanaworakun C, Assabumrungrat S, Sootitawatt A. Techno-economic-environmental analysis of sustainable anionic biosurfactant production from palm fatty acid distillate. *ACS Omega*. 2023;8(47):45045-45055.
14. Akinfalabi SI, Rashid U, Arbi Nehdi I, Yaw Choong TS, Sbihi HM, Gewik MM. Optimization and blends study of heterogeneous acid catalyst-assisted esterification of palm oil industry by-product for biodiesel production. *R Soc Open Sci*. 2020;7(1):191592.
15. Suhara A, Herawan SG, Tirta A, Idris M, Roslan MF, Putra NR, Hananto AL, Veza I. Biodiesel sustainability: Review of progress and challenges of biodiesel as sustainable biofuel. *Clean Technol*. 2024;6(3):886–906.
16. Meijaard E, Brooks TM, Carlson KM, et al. The environmental impacts of palm oil in context. *Nat Plants*. 2020;6:1418–1426.
17. Röttig A, Hauschild P, Madkour MH, Al-Ansari AM, Almakishah NH, Steinbüchel A. Analysis and optimization of triacylglycerol synthesis in novel oleaginous *Rhodococcus* and *Streptomyces* strains isolated from desert soil. *J Biotechnol*. 2016;225:48-56.
18. Zhang Q, Li Y, Xia L. An oleaginous endophyte *Bacillus subtilis* HB1310 isolated from thin-shelled walnut and its utilization of cotton stalk hydrolysate for lipid production. *Biotechnol Biofuels*. 2014;7(1):152.

19. Chang L, Chen H, Tang X, Zhao J, Zhang H, Chen YQ, Chen W. Advances in improving the biotechnological application of oleaginous fungus *Mortierella alpina*. Appl Microbiol Biotechnol. 2021;105(16-17):6275-89.
20. Hao G, Chen H, Gu Z, Zhang W, Chen W, Chen YQ. Metabolic engineering of *Mortierella alpina* for arachidonic acid production with glycerol as carbon source. Microb Cell Fact. 2015;14:205.
21. Xin Meng, Jianming Yang, Xin Xu, Lei Zhang, Qingjuan Nie, Mo Xian. Biodiesel production from oleaginous microorganisms. Renew Energy. 2009;34(1):1-5.
22. Carota E, Crognale S, D'Annibale A, Petruccioli M. Bioconversion of agro-industrial waste into microbial oils by filamentous fungi. Process Saf Environ Prot. 2018;117.
23. Feng P, Xu Z, Qin L, Asraful Alam M, Wang Z, Zhu S. Effects of different nitrogen sources and light paths of flat plate photobioreactors on the growth and lipid accumulation of *Chlorella* Sp. GN1 outdoors. Bioresour Technol. 2020;301.
24. Nascimento IA, Cabanelas ITD, Santos JN dos, Nascimento MA, Sousa L, Sansone G. Biodiesel yields and fuel quality as criteria for algal-feedstock selection: Effects of CO₂-supplementation and nutrient levels in cultures. Algal Research. 2015;8:53-60.
25. Jorquera O, Kiperstok A, Sales EA, Embiruçu M, Ghirardi ML. Comparative energy life-cycle analyses of microalgal biomass production in open ponds and photobioreactors. Bioresour Technol. 2010;101(4):1406-13.
26. Abeln F, Chuck CJ. The history, state of the art and future prospects for oleaginous yeast research. Microb Cell Fact. 2021;20(1):221.
27. Poontawee R, Lorliam W, Polburee P, Limtong S. Oleaginous yeasts: Biodiversity and cultivation. Fungal Biol Rev. 2023;44:100295.

28. Poontawee R, Yongmanitchai W, Limtong S. Efficient oleaginous yeasts for lipid production from lignocellulosic sugars and effects of lignocellulose degradation compounds on growth and lipid production. *Process Biochem.* 2017;53:44-60.
29. Zhao Y, Song B, Li J, Zhang J. *Rhodotorula toruloides*: an ideal microbial cell factory to produce oleochemicals, carotenoids, and other products. *World J Microbiol Biotechnol.* 2021;38(1):13.
30. McNeil BA, Stuart DT. *Lipomyces starkeyi*: an emerging cell factory for production of lipids, oleochemicals and biotechnology applications. *World J Microbiol Biotechnol.* 2018;34(10):147. doi:10.1007/s11274-018-2532-6
31. Beopoulos A, Mrozova Z, Thevenieau F, Le Dall MT, Hapala I, Papanikolaou S, Chardot T, Nicaud JM. Control of lipid accumulation in the yeast *Yarrowia lipolytica*. *Appl Environ Microbiol.* 2008;74:7779–89.
32. Pham KD, Shida Y, Miyata A, et al. Effect of light on carotenoid and lipid production in the oleaginous yeast *Rhodospiridium toruloides*. *Biosci Biotechnol Biochem.* 2020;84(7):1501-12.
33. Lin J, Shen H, Tan H, Zhao X, Wu S, Hu C, Zhao ZK. Lipid production by *Lipomyces starkeyi* cells in glucose solution without auxiliary nutrients. *J Biotechnol.* 2011;152(4):184-8.
34. Juanssilfero AB, Kahar P, Amza RL, Miyamoto N, Otsuka H, Matsumoto H, Kihira C, Thontowi A, Yopi, Ogino C, Prasetya B, Kondo A. Effect of inoculum size on single-cell oil production from glucose and xylose using oleaginous yeast *Lipomyces starkeyi*. *J Biosci Bioeng.* 2018;125(6):695-702.

35. Yamazaki H, Kobayashi S, Ebina S, et al. Highly selective isolation and characterization of *Lipomyces starkeyi* mutants with increased production of triacylglycerol. *Appl Microbiol Biotechnol.* 2019;103(15):6297-6308.
36. Alves AQ, da Silva VA, Góes AJ, Silva MS, de Oliveira GD, Bastos IV, de Castro Neto AG, Alves AJ. The fatty acid composition of vegetable oils and their potential use in wound care. *Adv Skin Wound Care.* 2019.
37. Oguri E, Masaki K, Naganuma T, Iefuji H. Phylogenetic and biochemical characterization of the oil-producing yeast *Lipomyces starkeyi*. *Antonie van Leeuwenhoek.* 2012;101(2):359-68.
38. Caporusso A, Capece A, De Bari I. Oleaginous yeasts as cell factories for the sustainable production of microbial lipids by the valorization of agri-food wastes. *Fermentation.* 2021;7(2):50.
39. Mine K, Taki H, Kim J, Seto J, Matsuo S, Sato R, Takaku H. Transcriptomic analysis reveals 3 important carbohydrate-active enzymes contributing to starch degradation of the oleaginous yeast *Lipomyces starkeyi*. *Biosci Biotechnol Biochem.* 2025;89(3):446-58.
40. Liu JX, Yue QY, Gao BY, Wang Y, Li Q, Zhang PD. Research on microbial lipid production from potato starch wastewater as culture medium by *Lipomyces starkeyi*. *Water Sci Technol.* 2013;67(8):1802-8.
41. Xavier MCA, Coradini ALV, Deckmann AC, Franco TT. Lipid production from hemicellulose hydrolysate and acetic acid by *Lipomyces starkeyi* and the ability of yeast to metabolize inhibitors. *Biochem Eng J.* 2017;118:11-19.
42. Martinez-Burgos WJ, Porto De Souza Vandenberghe L, Karp SG, Murawski De Mello AF, Thomaz Soccol V, Soccol CR. Microbial lipid production from soybean hulls using *Lipomyces starkeyi* LPB53 in a circular economy. *Bioresour Technol.* 2023;372:128650.

43. Azad AK, Yousuf A, Ferdoush A, Hasan MM, Karim MR, Jahan A. Production of microbial lipids from rice straw hydrolysates by *Lipomyces starkeyi* for biodiesel synthesis. Microb Biochem Technol. 2014;1–6.
44. Llamas M, Dourou M, González-Fernández C, Aggelis G, Tomás-Pejó E. Screening of oleaginous yeasts for lipid production using volatile fatty acids as substrate. Biomass Bioenergy. 2020;138:105553.
45. Yousuf A, Sannino F, Addorisio V, Pirozzi D. Microbial conversion of olive oil mill wastewaters into lipids suitable for biodiesel production. J Agric Food Chem. 2010;58:8630–8635.
46. Punpeng B, Nakata Y, Hayashida S. A novel raw-starch-digesting yeast α -amylase from *Lipomyces starkeyi* HN-606. J Ferment Bioeng. 1992;73:108-11.
47. Suzuki T, Takigawa A, Hasegawa K. Lipid extraction methods for *Lipomyces starkeyi*. Agric Biol Chem. 1973;37:2653–2656.
48. Zain NA, Kahar P, Sudesh K, Ogino C, Kondo A. Production of single cell oil by *Lipomyces starkeyi* from waste plant oil generated by the palm oil mill industry. J Biosci Bioeng. 2024;138(2):153-162.
49. Takaku H, Ebina S, Kasuga K, et al. Isolation and characterization of *Lipomyces starkeyi* mutants with greatly increased lipid productivity following UV irradiation. J Biosci Bioeng. 2021;131(6):613-621.
50. Willis RM, McCurdy AT, Ogborn MK, Wahlen BD, Quinn JC, Pease LF, Seefeldt LC. Improving energetics of triacylglyceride extraction from wet oleaginous microbes. Bioresour Technol. 2014;167:416-424.

51. Mat Enh A, Mustafa H, Ahmed F, Wahab A. Impact of the Russia-Ukraine conflict on the quality and quantity of Malaysia's palm oil production: A time series analysis. PLoS One. 2024;19(5):e0302405.
52. Wu CC, Okano K, Religia P, et al. Combination of two-stage continuous feeding and optimized synthetic medium increases lipid production in *Lipomyces starkeyi*. Eng Life Sci. 2025;25(1):e70003.
53. United States Department of Agriculture. Economic Research Service. Sugar and sweeteners outlook: April 2025. SSS-M-440. April 16, 2025. Available from: https://ers.usda.gov/sites/default/files/_laserfiche/outlooks/111409/SSS-M-440.pdf?v=26003
54. Bonatsos N, Marazioti C, Moutousidi E, Anagnostou A, Koutinas A, Kookos IK. Techno-economic analysis and life cycle assessment of heterotrophic yeast-derived single cell oil production process. Fuel. 2020;264:116839.
55. Lampitt LH. Nitrogen Metabolism in *Saccharomyces cerevisiae*. Biochem J. 1919;13(4):459-86.
56. Tomás-Pejó E, Morales-Palomo S, González-Fernández C. Microbial lipids from organic wastes: Outlook and challenges. Bioresour Technol. 2021;323:124612.
57. Sutanto S, Zullaikah S, Tran-Nguyen PL, Ismadji S, Ju YH. *Lipomyces starkeyi*: Its current status as a potential oil producer. Fuel Process Technol. 2018;177:39–55.
58. Meng K, Zhang G, Ding C, Zhang T, Yan H, Zhang D, Fang T, Liu M, You Z, Yang C, Shen J, Jin X. Recent advances on purification of lactic acid. Chem Rec. 2020;20(11):1236-1256.
59. Lightfoot EN. Bioseparation. Biotechnol Bioeng. 2004;87(3):259-273.

60. Aziz ZAA, Ahmad A, Setapar SHM, Karakucuk A, Azim MM, Lokhat D, Rafatullah M, Ganash M, Kamal MA, Ashraf GM. Essential oils: Extraction techniques, pharmaceutical and therapeutic potential - A review. *Curr Drug Metab.* 2018;19(13):1100-1110.
61. Chiam CK, Sarbatly R. Purification of aquacultural water: Conventional and new membrane-based techniques. *Sep Purif Rev.* 2011;40(2):126–160.
62. Nunes SP. Can fouling in membranes be ever defeated? *Curr Opin Chem Eng.* 2020;28:90-95.
63. Zulkefli NF, Alias NH, Jamaluddin NS, et al. Recent mitigation strategies on membrane fouling for oily wastewater treatment. *Membranes (Basel).* 2021;12(1):26.
64. Barambu NU, Bilad MR, Wibisono Y, Jaafar J, Mahlia TMI, Khan AL. Membrane surface patterning as a fouling mitigation strategy in liquid filtration: A review. *Polymers (Basel).* 2019;11(10):1687.
65. Uragami T. *Science and technology of separation membranes.* Chichester, UK: John Wiley & Sons, Ltd.; 2017. Introduction to membrane science and technology; pp. 1–12.
66. Yang F, Huang Z, Huang J, Wu C, Zhou R, Jin Y. Tanning wastewater treatment by ultrafiltration: Process efficiency and fouling behavior. *Membranes (Basel).* 2021;11(7):461.
67. Zulkefli NF, Alias NH, Jamaluddin NS, et al. Recent mitigation strategies on membrane fouling for oily wastewater treatment. *Membranes (Basel).* 2021;12(1):26.
68. Alzahrani S, Mohammad AW. Challenges and trends in membrane technology implementation for produced water treatment: A review. *J Water Process Eng.* 2014;4:107-133.
69. Burke M, Wells E, Larison C, Rao G, Bentley MJ, Linden YS, Smeets P, DeFrance J, Brown J, Linden KG. Systematic review of microorganism removal performance by physiochemical water treatment technologies. *Environ Sci Technol.* 2025.

70. Watanabe AP, Ushikubo FY, Viotto LA. Evaluation of permeate flux in microfiltration of Tamarind (*Tamarindus indica* L.) juice using polypropylene membrane. Desalination. 2006;200:337–338.
71. Ushikubo FY, Watanabe AP, Viotto LA. Microfiltration of umbu (*Spondias tuberosa* Arr. Cam) juice. J Membr Sci. 2007;288:61–66.
72. Soto-Salcido LA, Anugwom I, Mänttari M, Kallioinen-Mänttari M. Cellulose nanofibers derived surface coating in enhancing the dye removal with cellulosic ultrafiltration membrane. Membranes (Basel). 2022;12(11):1082.
73. Bonnelye V, Guey L, Del Castillo J. UF/MF as RO pre-treatment: the real benefit. Desalination. 2008;222(1–3):59-65.
74. Yu W, Brown M, Graham NJ. Prevention of PVDF ultrafiltration membrane fouling by coating MnO₂ nanoparticles with ozonation. Sci Rep. 2016;6:30144.
75. Vrijenhoek EM, Hong S, Elimelech M. Influence of membrane surface properties on initial rate of colloidal fouling of reverse osmosis and nanofiltration membranes. J Membr Sci. 2001;188(1):115-128.
76. Mallya DS, Abdikheibari S, Dumée LF, Muthukumaran S, Lei W, Baskaran K. Removal of natural organic matter from surface water sources by nanofiltration and surface engineering membranes for fouling mitigation - A review. Chemosphere. 2023;321:138070.
77. Shafi HZ, Khan Z, Yang R, Gleason KK. Surface modification of reverse osmosis membranes with zwitterionic coating for improved resistance to fouling. Desalination. 2015;362:93-103.
78. Mahlangu OT, Nackaerts R, Thwala JM, Mamba BB, Verliefde ARD. Hydrophilic fouling-resistant GO-ZnO/PES membranes for wastewater reclamation. J Membr Sci. 2017;524:43-55.

79. Xiao N, Huang X, He W, Yao Y, Wu N, Xu M, Du H, Zhao Y, Tu Y. A review on recent advances of egg byproducts: Preparation, functional properties, biological activities and food applications. *Food Res Int.* 2021;147:110563.
80. Wen-Qiong W, Yun-Chao W, Xiao-Feng Z, Rui-Xia G, Mao-Lin L. Whey protein membrane processing methods and membrane fouling mechanism analysis. *Food Chem.* 2019;289:468-481.
81. Darnon E, Morin E, Belleville MP, Rios GM. Ultrafiltration within downstream processing: some process design considerations. *Chem Eng Process: Process Intensification.* 2003;42(4):299-309.
82. Bottino A, Capannelli C, Del Borghi A, Colombino M, Conio O. Water treatment for drinking purpose: ceramic microfiltration application. *Desalination.* 2001;141:75–79.
83. Takabatake H, Taniguchi M, Kurihara M. Advanced technologies for stabilization and high performance of seawater RO membrane desalination plants. *Membranes (Basel).* 2021;11(2):138.
84. Wu F, Li Q, Zhang Z, Zhou X, Pang R. A review on antifouling polyamide reverse osmosis membrane for seawater desalination. *Environ Res.* 2025;274:121305.
85. Bailey SM, Meagher MM. Separation of soluble protein from inclusion bodies in *Escherichia coli* lysate using crossflow microfiltration. *J Membr Sci.* 2000;166:137–146.
86. Carrère H, Blaszkow F, de Balmann HR. Modeling the clarification of lactic acid fermentation broths by cross-flow microfiltration. *J Membr Sci.* 2001;186:219–230.
87. Lin SH, Lan WJ. Waste oil/water emulsion treatment by membrane processes. *J Hazard Mater.* 1998;59:189–199.
88. Benito JM, Sánchez MJ, Pena P, Rodríguez MA. Development of a new high porosity ceramic membrane for the treatment of bilge water. *Desalination.* 2007;214:91–101.

89. Wei P, Cheng LH, Zhang L, Xu XH, Chen HL, Gao CJ. A review of membrane technology for bioethanol production. *Renew Sustain Energy Rev.* 2014;30:388–400.
90. Castro-Muñoz R, Yáñez-Fernández J, Fila V. Phenolic compounds recovered from agro-food by-products using membrane technologies: An overview. *Food Chem.* 2016;213:753–762.
91. Rivera F, Muñoz R, Prádanos P, Hernández A, Palacio L. A systematic study of ammonia recovery from anaerobic digestate using membrane-based separation. *Membranes (Basel).* 2021;12(1):19.
92. Hammed A, Polunin Y, Voronov A, Pryor SW. Glucan conversion and membrane recovery of biomimetic cellulosomes during lignocellulosic biomass hydrolysis. *Appl Biochem Biotechnol.* 2021;193(9):2830–2842.
93. Peng P, Lan Y, Liang L, Jia K. Membranes for bioethanol production by pervaporation. *Biotechnol Biofuels.* 2021;14(1):10.
94. Shirazi MM, Kargari A. Concentrating of sugar syrup in bioethanol production using sweeping gas membrane distillation. *Membranes (Basel).* 2019;9(5):59.
95. Suzuki T, Takigawa A, Hasegawa K. Lipid extraction methods for *Lipomyces starkeyi*. *Agric Biol Chem.* 1973;37:2653–2656.
96. Bligh EG, Dyer WJ. A rapid method of total lipid extraction and purification. *Can J Biochem Physiol.* 1959;37:911–917.
97. Folch J, Lees M, Stanley GHS. A simple method for the isolation and purification of total lipides from animal tissues. *J Biol Chem.* 1957;226:497–509.
98. Breil C, Abert Vian M, Zemb T, Kunz W, Chemat F. “Bligh and Dyer” and folch methods for solid–liquid–liquid extraction of lipids from microorganisms. *Int J Mol Sci.* 2017;18:708.

99. Probst KV, Wales MD, Rezac ME, Vadlani PV. Evaluation of green solvents: Oil extraction from oleaginous yeast *Lipomyces starkeyi* using cyclopentyl methyl ether (CPME). *Biotechnol Prog.* 2017;33:1096–1103.
100. Drévillon L, Koubaa M, Nicaud JM, Vorobiev E. Cell disruption pre-treatments towards an effective recovery of oil from *Yarrowia lipolytica* oleaginous yeast. *Biomass Bioenergy.* 2019;128:105320.
101. Kim J, Lee EJ, Lee KE, Nho YH, Ryu J, Kim SY, Yoo JK, Kang S, Seo SW. Lipid extract derived from newly isolated *Rhodotorula toruloides* LAB-07 for cosmetic applications. *Comput Struct Biotechnol J.* 2023;21:2009–2017.
102. Wu CY, Okuda T, Ando A, Hatano A, Kikukawa H, Ogawa J. Isolation and characterization of the Ω 3-docosapentaenoic acid-producing microorganism *Aurantiochytrium* sp. T7. *J Biosci Bioeng.* 2022;133:229–234.
103. Watsuntorn W, Chuengcharoenphanich N, Niltaya P, Pinyakong O, Pathom-Aree W, Lertcanawanichakul M. A novel oleaginous yeast *Saccharomyces cerevisiae* CU-TPD4 for lipid and biodiesel production. *Chemosphere.* 2021;280:130782.
104. Ministry of Education, Culture, Sports, Science and Technology. Standard Tables of Food Composition in Japan-2020-, 8th ed. Nagano, Japan: Tsutamoto Printing Co., Ltd.; 2020:1–660.
105. Tuhanioglu A, Alpas H, Cekmecelioglu D. High hydrostatic pressure-assisted extraction of lipids from *Lipomyces starkeyi* biomass. *J Food Sci.* 2022;87:5029–5041.
106. Bonturi N, Matsakas L, Nilsson R, Christakopoulos P, Miranda EA, Berglund KA, Rova U. Single cell oil producing yeasts *Lipomyces starkeyi* and *Rhodospiridium toruloides*: Selection of extraction strategies and biodiesel property prediction. *Energies.* 2015;8:5040–5052.

107. Nguyen TAH, Ngo HH, Gio WS, Kim SH, Guo WS, Chang SW, Nguyen DD. Feasibility of iron loaded 'okara' for biosorption of phosphorous in aqueous solutions. *Bioresour Technol.* 2013;150:42–49.
108. Li B, Qiao M, Lu F. Composition, nutrition, and utilization of okara (soybean residue). *Food Rev Int.* 2012;28:231–252.
109. Vong WC, Liu SQ. Biovalorisation of okara (soybean residue) for food and nutrition. *Trends Food Sci Technol.* 2016;52:139–147.
110. Vong WC, Au Yang KL, Liu SQ. Okara (soybean residue) biotransformation by yeast *Yarrowia lipolytica*. *Int J Food Microbiol.* 2016;235:1–9.
111. Watanabe Y, Kuroda K, Tatemichi Y. Construction of engineered yeast producing ammonia from glutamine and soybean residues (okara). *AMB Expr.* 2020;10:70.
112. Wang Z, Hao Q, An X, Zhang J, Li X, Chen Y, Liu Y. Optimization of mopan persimmon wine fermentation with pectinase and analysis of its mechanism of action. *Foods.* 2023;12:1246.
113. Wu CC, Ohashi T, Misaki R, Ogino C, Shimizu N. Ethanol and H₂O₂ stresses enhance lipid production in an oleaginous *Rhodotorula toruloides* thermotolerant mutant L1-1. *FEMS Yeast Res.* 2020;20:foaa030.
114. Hohmann S. Osmotic stress signaling and osmoadaptation in yeasts. *Microbiol Mol Biol Rev.* 2002;66:300–372.
115. Pomraning KR, Collett JR, Kim J, Panisko EA, Culley DE, Dai Z, Deng S, Hofstad BA, Kuo TM, Magnuson JK, Olstad J, Peterson EJ, Rodriguez A, Romero EJ, Schilling CH, Baker SE. Transcriptomic analysis of the oleaginous yeast *Lipomyces starkeyi* during lipid accumulation on enzymatically treated corn stover hydrolysate. *Biotechnol Biofuels.* 2019;12:162.

116. Liu L, Zong M, Hu Y, Li n, Lou W, Wu H. Efficient microbial oil production on crude glycerol by *Lipomyces starkeyi* AS 2.1560 and its kinetics. *Process Biochem.* 2017;58:230–8.
117. Matsakas L, Sterioti AA, Rova U, Christakopoulos P. Use of dried sweet sorghum for the efficient production of lipids by the yeast *Lipomyces starkeyi* CBS 1807. *Ind Crop Prod.* 2014;62:367–72.
118. Zhao X, Kong X, Hua Y, Feng B, Zhao Z (Kent). Medium optimization for lipid production through co-fermentation of glucose and xylose by the oleaginous yeast *Lipomyces starkeyi*. *Euro J Lipid Sci Technol.* 2008;110(5):405–12.
119. De Vero L, Bonciani T, Verspohl A, Mezzetti F, Giudici P. High-glutathione producing yeasts obtained by genetic improvement strategies: a focus on adaptive evolution approaches for novel wine strains. *AIMS Microbiol.* 2017;3(2):155–70.
120. Walker MC, van der Donk WA. The many roles of glutamate in metabolism. *J Ind Microbiol Biotechnol.* 2016;43(2-3):419–30.
121. Grant CM, MacIver FH, Dawes IW. Glutathione is an essential metabolite required for resistance to oxidative stress in the yeast *Saccharomyces cerevisiae*. *Curr Genet.* 1996;29(6):511–5.
122. Xu P, Qiao K, Stephanopoulos G. Engineering oxidative stress defense pathways to build a robust lipid production platform in *Yarrowia lipolytica*. *Biotechnol Bioeng.* 2017;114(7):1521–30.
123. Ohashi Y, Hirayama A, Ishikawa T, Nakamura S, Shimizu K, Ueno Y, et al. Depiction of metabolome changes in histidine-starved *Escherichia coli* by CE-TOFMS. *Mol Biosyst.* 2008;4(2):135–47.

124. Ooga T, Sato H, Nagashima A, Sasaki K, Tomita M, Soga T, et al. Metabolomic anatomy of an animal model revealing homeostatic imbalances in dyslipidaemia. *Mol Biosyst.* 2011;7(4):1217–23.
125. Sugimoto M, Wong DT, Hirayama A, Soga T, Tomita M. Capillary electrophoresis mass spectrometry-based saliva metabolomics identified oral, breast and pancreatic cancer-specific profiles. *Metabolomics.* 2010;6(1):78–95.
126. Yamamoto H, Fujimori T, Sato H, Ishikawa G, Kami K, Ohashi Y. Statistical hypothesis testing of factor loading in principal component analysis and its application to metabolite set enrichment analysis. *BMC Bioinformatics.* 2014;15:51.
127. Xiao W, Loscalzo J. Metabolic responses to reductive stress. *Antioxid Redox Signal.* 2020;32(18):1330–47.
128. Perrone GG, Tan SX, Dawes IW. Reactive oxygen species and yeast apoptosis. *Biochim Biophys Acta.* 2008;1783(7):1354–68.
129. Jiang W, Li C, Li Y, Peng H. Metabolic Engineering Strategies for Improved Lipid Production and Cellular Physiological Responses in Yeast *Saccharomyces cerevisiae*. *J Fungi.* 2022;8(5):427.
130. Elskens MT, Jaspers CJ, Penninckx MJ. Glutathione as an endogenous sulphur source in the yeast *Saccharomyces cerevisiae*. *J Gen Microbiol.* 1991;137(3):637–44.
131. Guillamón JM, Riel NAW, Giuseppin MLF, Verrips CT. The glutamate synthase (GOGAT) of *Saccharomyces cerevisiae* plays an important role in central nitrogen metabolism. *FEMS Yeast Res.* 2001;1(3):169–75.

Publication list

Chapter 2:

Taki H, Mine K, , Matsuo S, Kumagai K, and Matsuyama H*. Simple and economical downstream process development for edible oil production from oleaginous yeast *Lipomyces starkeyi*. Processes. 2023;11(5):1458.

Chapter 3:

Taki H, Mine K, Miyamoto M, Seto J, Matsuo S, Kumagai K, and Matsuyama H*. Utilization of okara as a culture medium by membrane concentration process for high oil production by oleaginous yeast, *Lipomyces starkeyi*. Fermentation. 2025;11(1):7.

Chapter 4:

Taki H, Mine K, Miyamoto M, Seto J, Kim J, Takaku H, Kumagai K, and Matsuyama H*. Effects of inorganic nitrogen addition to okara-utilized medium on the oleaginous yeast *Lipomyces starkeyi* and assessment of metabolism involved in increased oil production (Submitted).

神戸大学博士論文「膜分離技術と食品残渣を利用した低コストな微生物由来食用油脂の新規生産プロセスに関する研究」全111頁

提出日 2025年7月7日

本博士論文が神戸大学機関リポジトリ Kernel にて掲載される場合、掲載登録日（公開日）はリポジトリの該当ページ上に掲載されます。

©高城博也

本論文の内容の一部あるいは全部を無断で複製・転載・翻訳することを禁じます。