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Utilization of Okara as a Culture Medium by Membrane Concentration Process for High Oil Production by Oleaginous Yeast, *Lipomyces starkeyi*

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Abstract: Palm oil, widely used in various products, poses environmental and climate change risks. "Yeast oil" produced by *Lipomyces starkeyi*, an oil-producing yeast, is one of the sustainable alternatives for palm oil and was successfully produced as an edible substitute for palm oil. However, the high cost of the culture medium for oil production remains a challenge for practical applications. Okara is a by-product of tofu and soymilk production. Because yeast extract contributes to the high cost of the culture medium, we considered using okara, a cheap and nitrogen-rich substitute, to reduce costs. In the initial study with okara, the production of yeast oil was confirmed, but its productivity was low due to the high viscosity caused by its insoluble solids. To overcome this, we extracted and concentrated nitrogen components in okara using the membrane concentration process. Using NF (nanofiltration) membrane concentration, oil production increased 1.69 and 1.44 times compared to the unconcentrated extract solution (added 90% (v/v) in the culture medium) and yeast extract (added 5% (w/v) in the culture medium), respectively. These findings indicate the potential for a significant cost reduction in the culture medium and high oil yield in yeast oil production.

Keywords: oil-producing microorganism; *Lipomyces starkeyi*; lipid; environmentally friendly; bio-oil; alternative palm oil; upcycling food residue; membrane concentration process

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1. Introduction

Palm oil is used worldwide in a variety of commercial products, including cosmetics, chemicals, and foods (snacks, chocolate, instant fried noodles, etc.), and the global demand for palm oil is increasing [1,2]. However, the palm oil industry is facing global environmental issues, including climate change and the risk of environmental destruction associated with the development of oil palm plantations [3,4]. Rapidly increasing demand for palm oil, poor working conditions, and conflicts between local residents and land developers are also serious problems [1,5].

In recent years, many palm oil-related companies have been promoting the procurement of palm oil that is environmentally friendly and takes labor and human rights issues into consideration and are increasingly procuring Roundtable on Sustainable Palm Oil (RSPO) certified palm oil that can be traced throughout the entire supply chain from oil

palm plantation to end products [6]. However, these procurement efforts alone do not provide fundamental solutions to environmental impacts and climate change risks, and alternatives to palm oil are being sought to address these issues.

In nature, there exist oil-producing microorganisms (bacteria, yeasts, molds, algae, etc.) that accumulate intracellular oils and fats that are more than 20% of their cell weight [7]. The first studies on oleaginous microorganisms date back to the 1870s [8,9], but the economic competitiveness of microbial-derived oils over vegetable oils was considered doubtful at the time [10]. However, in recent years, the concepts of bioeconomy and circular economy have emerged rapidly, leading to an increasing number of papers reexamining the subject. Microalgae typically have a lipid content of 20–50%; promising and widely studied species that can accumulate large amounts of lipids include *Chlorella*, *Monoraphidium*, and *Botryococcus* [11–13]. However, while they have large storage capacities, they require larger areas and longer incubation times than bacteria and yeasts. Some bacteria are capable of accumulating oils under certain environmental conditions, and triacylglycerol (TAG) accumulation by the genera *Bacillus*, *Streptomyces*, and *Rhodococcus* has been reported. Bacterial species are characterized by high growth rates but low lipid content [14–16].

Oil-producing yeasts, such as the genera *Yarrowia*, *Rhodosporidium*, and *Lipomyces*, are attractive as microbial factories. Their ability to accumulate large amounts of lipids, grow rapidly, and utilize a wide variety of feedstocks, coupled with their ease of cultivation in large fermenters, make them the best candidates for biorefinery processes compared to other oleaginous microorganisms [17–20].

Lipomyces starkeyi is known as an oleaginous yeast that produces oils and can accumulate TAG in the form of fat globules in its cells at more than 65% of its dry cell weight [21,22]. Previous studies have shown that *Lipomyces starkeyi* can produce oil from a variety of sugars (e.g., starch, glucose, xylose, and mannose) and assimilate a variety of nitrogen sources [23]. Furthermore, it is characterized by being tolerant of a low pH and fermentation inhibitors such as acetic acid and 5-hydroxymethylfurfural (HMF) [24]. Moreover, as a suitable host for genetic engineering to improve lipid production, it has great industrial potential as an excellent oil producer [25,26]. The oil produced by *Lipomyces starkeyi* (hereafter referred to as "yeast oil") is mainly composed of the fatty acids (palmitic and oleic), which are very similar to those of palm oil compared to other vegetable oils (i.e., similar in melting point and other physical properties), and its expected use as an alternative palm oil has been studied [27,28].

Thus far, the authors of this study have succeeded in producing yeast oil as an "edible alternative palm oil" [29]. In the next step, cost reduction is necessary for its practical application. In the production of oils and fats from microorganisms, it is reported that the cost of the culture medium accounts for a large portion of the production cost; accordingly, it is important to reduce the cost of the culture medium [30]. The commonly used medium nutrient sources for yeast oil production are yeast extract and glucose as the nitrogen and sugar sources, respectively [25,26,29,31]. However, while there have been many reports on feedstock substitution studies to reduce the cost of the yeast oil production medium, mainly by examining the use of woody biomass and food residues as sugar sources, there have been few reports on nitrogen source feedstock substitution [32].

Okara is a by-product of the tofu and soymilk production process from soybeans. Approximately 3.9 million tons are generated annually worldwide, most of which are landfilled or incinerated due to their low shelf life [33,34]. However, okara remains highly nutritious, containing about 40–60% carbohydrates (mainly insoluble fiber), 20–30% protein, and 10–20% fat (all on a dry basis) [35]. Owing to its high nutritional value, the fermentation of okara by fungi and bacteria has been well studied; in contrast, there are a few studies on the fermentation of okara by yeast [36].

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 [37]. Therefore, okara may be a suitable growth medium for oleaginous yeast. In the example of *Saccharomyces cerevisiae*, glutaminase (YbaS) from *E. coli* was expressed on the cell surface via genetic recombination, and ammonia was successfully fermented and produced from okara outside the yeast cell [38]. As shown above, there are a few fermentation studies exploring yeast using okara, but none on *Lipomyces starkeyi* and no examples of oil production.

In this study, we directly replaced the yeast extract in a glucose/yeast extract medium with okara. Next, the nitrogen component of okara was extracted, and the insoluble solids were separated using a simple solid–liquid separation process. Moreover, we attempted a membrane concentration process in order to increase the concentration of the extracted nitrogen component and achieve a higher oil yield. The main advantages of employing the membrane concentration process for liquids containing useful components are high throughput, continuous processing, low energy consumption, and the use of moderate temperatures [39,40]. The process has been widely studied in the concentration of liquid foods, such as the concentration of fruit juices [41,42] and extracts from foods that should not be heated [43,44]. 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 study aims to establish a foundation for the commercial production of yeast oil by maximizing the use of a sustainable biomass resource (i.e., okara) and improving the efficiency of bio-oil production using yeast. Moreover, it contributes to the important environmental policy goals of upcycling food industry by-products and waste reduction.

2. Materials and Methods

2.1. Strain and Growth Conditions

Pre-cultures of *Lipomyces starkeyi* CBS1807 were conducted in 500 mL baffled flasks (Shibata Kagaku, Tokyo, Japan) containing 100 mL of a YG (yeast extract/glucose) medium composed of 5% (w/v) yeast extract BSP-570 (Oriental Yeast Industry, Tokyo, Japan) and 10% (w/v) D (+)-glucose (Fujifilm Wako Pure Chemical, Tokyo, Japan) with shaking at 150 rpm in an orbital incubator shaker (Innova S44i, Eppendorf, Hamburg, Germany) for 3 days 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 5 days 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.

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.

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 (Ultra Filtration) 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% (The membrane information is from the manufacturers). For membrane concentration experiments, a CF042D-FO cell (Sterlitech, Auburn, AL, USA, filtration area: 42 cm^2) 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 (1):

$$J = \frac{V_p}{A \times t} \tag{1}$$

where V_p is the permeate volume (L), A is the filtration area (m^2), and t is the time (h).

The rejection ratio (R) (%) of the components in solution was calculated using the following Equation (2):

$$R = \left(1 - \frac{C_p}{C_f}\right) \times 100 \tag{2}$$

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

2.4. Analysis

2.4.1. Viscosity

The 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.

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.

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 N_2 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.

2.4.4. Ion Concentration

Samples were filtered through a 0.45 µm PTFE filter (Advantec) and diluted with 0.1 M HNO_3 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).

2.5. Evaluation of TAG Production

2.5.1. Measurement of Cell Concentration

The cell concentration of the main culture was measured after 5 days using a Q500 sonicator (QSonica, Newrown, 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 [45].

2.5.2. Measurement of TAG

The extraction and measurement of TAG from L. starkeyi were conducted according to a previously reported study [45]. 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, Osaka, Japan) 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.

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, Tokyo, Japan) with differential interference contrast (DIC) images.

2.6. Statistical Analysis

Statistical analysis was performed using Dunnett's test with GraphPad Prism software, version 10.2.1 (GraphPad Software, San Diego, CA, USA). Differences were considered significant at $p < 0.05$. The values are given as the mean ± standard deviation calculated in triplicate experiments except for Table 1 (Yeast extract: $n = 2$).

Table 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/100 g	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 ± S.D. with $n = 3$ except for yeast extract (measured with $n = 2$).

3. Results and Discussion

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 (Figure 1a). Microscopic observations showed no difference in oil accumulation or cell density between the 2% (w/v) and 3% (w/v) addition groups (Figure 1b). 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 [46]. 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 (Figure 2). As oil-producing yeasts are aerobic microbes and their growth and production of TAG are promoted by the increased dissolved oxygen, we expected that if the viscosity of the medium is reduced, the yeast would be able to produce more TAG, comparable to or more than the YG medium.

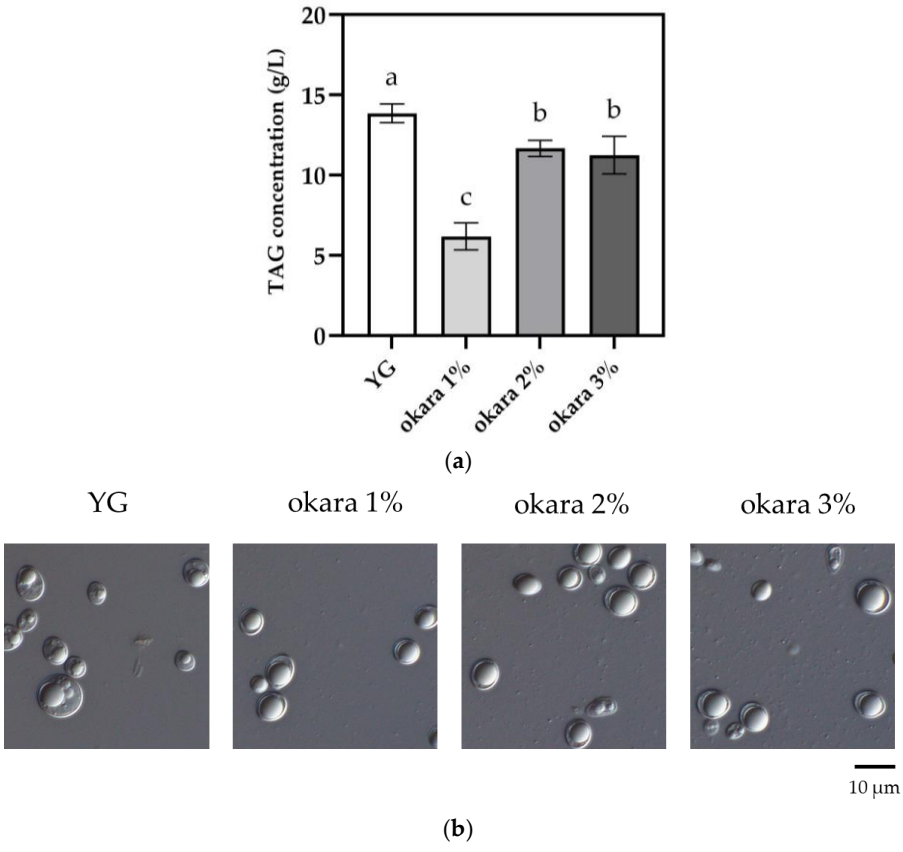

Figure 1. Replacement of yeast extract with okara in the *L. starkeyi* culture medium. (**a**) Comparison of TAG productivity in the YG medium and the okara-added medium. (**b**) Differential interference contrast (DIC) images of the yeast cells. Mean ± S.D., $n = 3$, Tukey's test, different letters above the columns for each experimental group indicate significant differences between groups ($p < 0.05$).

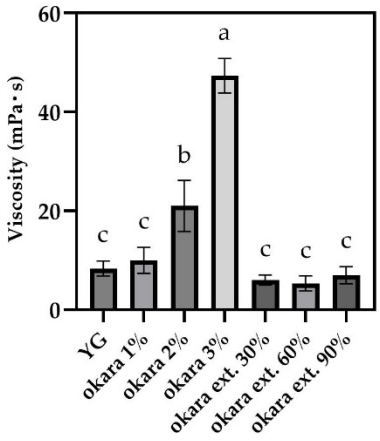

Figure 2. Viscosity of the mediums from different nitrogen sources. Okara was added with 1%, 2%, and 3% (w/v) per medium, and the okara extract solution (extracted with 30 times water from okara) was added with 30%, 60%, and 90% (v/v) per medium. The values are mean ± S.D., $n = 3$, Tukey's test, different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

3.2. Preparation of Okara Extract and Evaluation as a Nitrogen Source for Culture Media

To reduce the viscosity of the okara-containing medium, we attempted to extract nitrogenous components from okara. 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 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 extract 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 (Figure 2). 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 (Figure 3a). 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 (Figure 3b). 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.

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 (Figure 4). As high flux is preferable for concentration, we decided to use 50 °C for subsequent membrane concentration experiments.

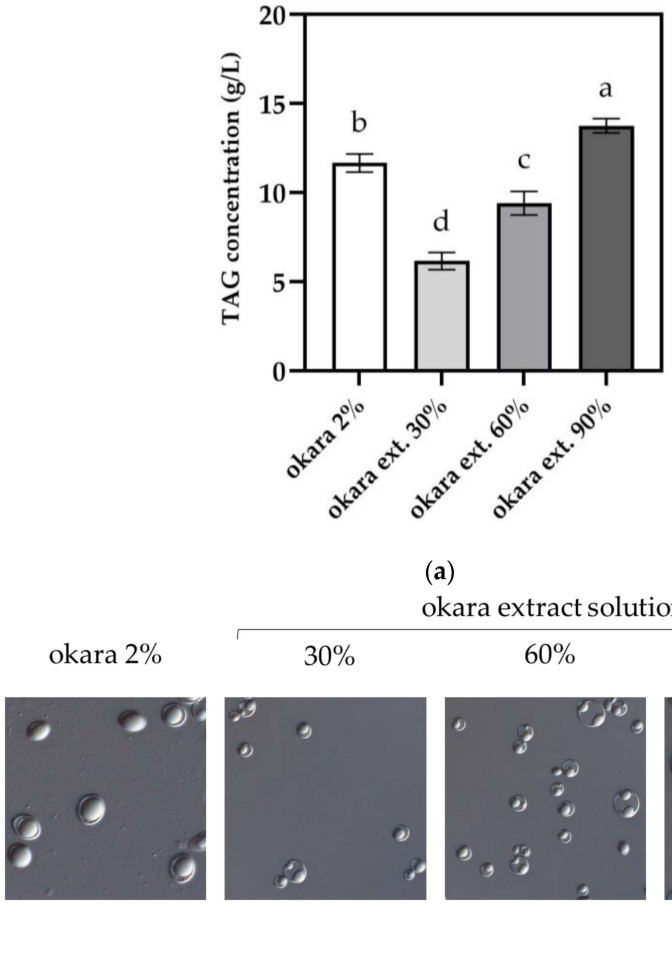

Figure 3. Replacement of okara with the okara extract solution in the *L. starkeyi* culture medium as a nitrogen source. (**a**) TAG productivity in various concentrations of okara extract solution. (**b**) DIC images. Okara ext.: okara extract solution. Mean ± S.D., $n = 3$, Tukey's test, and different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

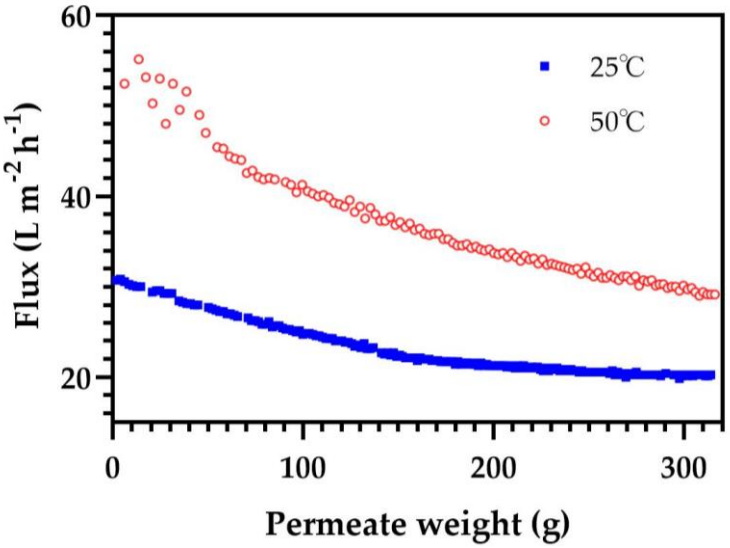

Figure 4. Flux of concentration of okara extract solution for the NF membrane in different temperature conditions (25 °C and 50 °C).

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, we measured the total nitrogen (T-N), total organic carbon (TOC), ion concentration (Na, K, Mg, Ca, and P) in the feed, and permeate solutions and compared their rejection rates (Table 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 a 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×, 5×, and 7× concentrates at 50 °C.

Table 2. Characteristics of rejection by different types of membranes for concentration of the okara extract solution.

Membrane Type	UF			NF			RO		
MWCO (Da)	~1000			~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.

3.4. 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/10^8 cells) were determined for the comparison (Figure 5). 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 1.7 times higher than 2% okara (Figure 5d), which is equivalent to 1.4 times higher than that of the yeast extract and 1.7 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 1.4 times higher than that of the 2% okara (Figure 5a), and equivalent to 1.2 and 1.4 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 (Figure 5g). In regard to cell growth, all of the extract concentrates showed higher cell growth than the okara 2% extract (Figure 5b,e,h), and in regard to TAG amount per cell, we confirmed that the membrane concentration was 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 (Figure 5c,f,i), suggesting that there may be a component that is concentrated in the membrane and retards TAG production.

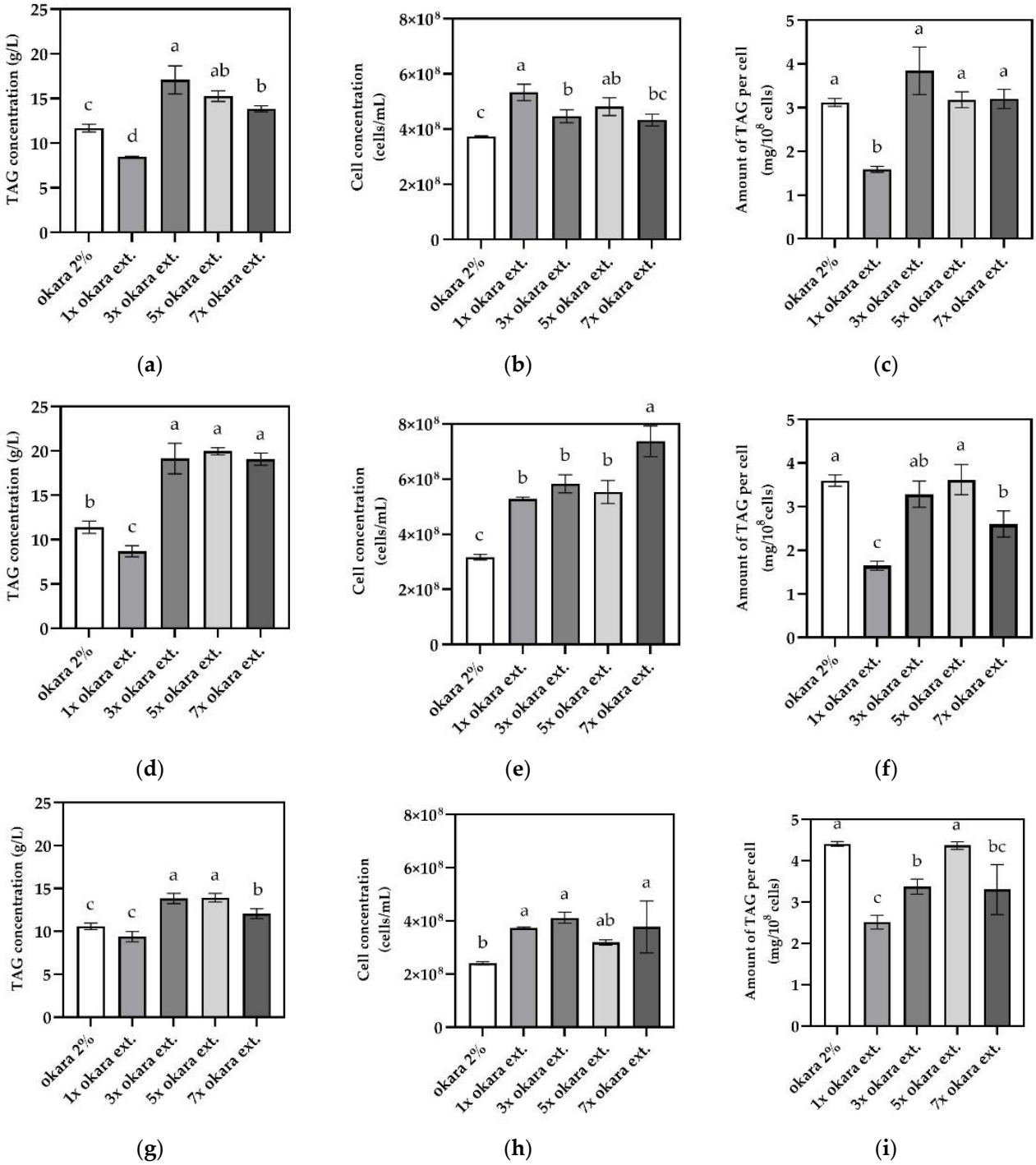

Figure 5. TAG production using membrane-concentrated okara extract solutions as a nitrogen source of the culture medium. (**a**–**c**): TAG concentration (g/L), cell concentration (cells/mL), amount of TAG per cell (mg/10^8 cells) using UF membrane-concentrated extract solutions. (**d**–**f**): Those 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 Figure 6. 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 Figure 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 (Figure S1). The results were similar to the relationship between T-N and TAG concentrations, indicating that higher TOC did not increase TAG yield.

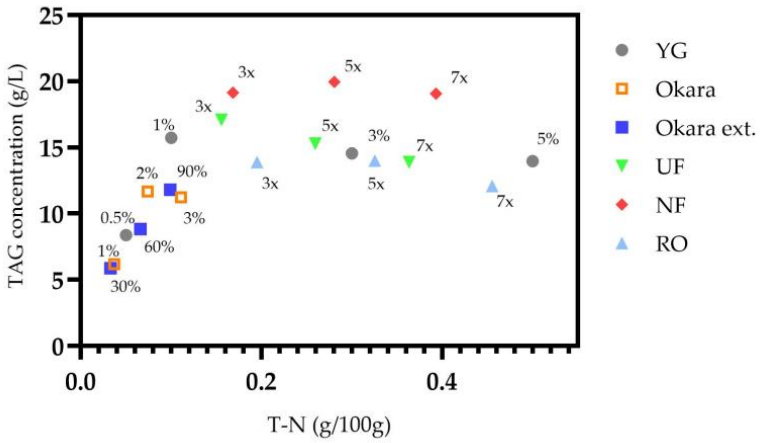

Figure 6. Relationship between T-N concentration and TAG productivity. 3×, 5×, 7×: Concentration magnification for each membrane concentration, UF, NF, and RO: Type of membrane used to prepare the membrane concentrate. The data are presented with averages of triplicate analyses.

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 [47,48]. Additionally, acetic acid, furfural, phenolic compounds, and some sugars are known to be inhibitory components of yeast culture [49]. 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 extract 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.

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.

5. Patents

A patent application claiming "Culture media for yeast culture and yeast culture methods" which is a part of this study is pending in Japan (Application Number: 2024-192907; Application Date: 1 November 2024; Inventor's Name: Hideto Matsuyama, Kazuo Kumagai, Hiroya Taki, Kentaro Mine).

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/fermentation11010007/s1, Figure S1: Relationship between TOC concentration and TAG productivity.

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Conflicts of Interest: Authors Hiroya Taki, Kentaro Mine, Mana Miyamoto, Jiro Seto and Shinji Matsuo were employed by the company Nissin Foods Holdings Co., Ltd. The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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