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Taki, Hiroya
Mine, Kentaro
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Simple and Economical Downstream Process Development for Edible Oil Production from Oleaginous Yeast *Lipomyces starkeyi*

Hiroya Taki [1,2], Kentaro Mine [2], Shinji Matsuo [2], Kazuo Kumagai [3] and Hideto Matsuyama [1,3,*]

[1] Department of Chemical Science and Engineering, Kobe University, Kobe 657-8501, Japan; hiroya.taki@nissin.com
[2] Nissin Global Innovation Center, Nissin Foods Holdings Co., Ltd., Hachioji 192-0001, Japan; kentaro.mine@nissin.com (K.M.); shinji.matsuo@nissin.com (S.M.)
[3] Research Center for Membrane and Film Technology, Kobe University, Kobe 657-8501, Japan; k.kumagai@port.kobe-u.ac.jp
* Correspondence: matuyama@kobe-u.ac.jp

Abstract: The production of palm oil, which is used in various foods, is associated with environmental destruction and climate change risks; therefore, there is an urgent need for sustainable alternatives. "Yeast oil" produced by *Lipomyces starkeyi*, an oil-producing yeast, is expected to solve these problems because its fatty acid composition is similar to that of palm oil. To date, we have successfully developed yeast oil as an edible alternative to palm oil. However, conventional processes, including cell collection and lyophilization, are difficult to industrialize in terms of equipment and cost. Therefore, a method for extracting yeast oil from the emulsified liquid generated by crushing the culture was investigated. It is presumed that the emulsified state is stable owing to the components derived from yeast cells and metabolites; thus, solid–liquid filtration separation was attempted before extraction. The extraction recovery ratio of yeast oil was 98.2% when a hexane/ethanol mixture (3:1) was added to the residue after filtration. Furthermore, the energy consumption and processing cost of this new process were estimated to be 26% and 34%, respectively, of that of conventional methods, suggesting that the new process has potential for practical applications.

Keywords: oil-producing microorganism; *Lipomyces starkeyi*; lipid; environmentally friendly; bio-oil; alternative palm oil; economical process; oil extraction; oil–water separation

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1. Introduction

Palm oil is used in a wide variety of products worldwide, such as cosmetics, chemicals, and foods (e.g., snacks, chocolate, and instant fried noodles). However, the palm oil industry faces global environmental problems such as climate change and the risk of environmental destruction associated with the development of oil palm plantations. The rapid increase in demand for palm oil, poor working environments, and conflicts between local residents and land developers are also serious problems [1–3].

Recently, many palm oil-related companies have begun promoting sustainable palm oil sourcing, which is environmentally friendly, considers labor and human rights issues, and procures Roundtable on Sustainable Palm Oil (RSPO)-certified palm oil that can be traced throughout the entire supply chain from oil palm farms to products [4]. However, the risks associated with environmental destruction and climate change have not been fundamentally solved; therefore, alternative oils that can replace palm oil are required. Oleaginous yeasts are advantageous producers that can accumulate oil, whose major component is triacylglycerides (TAG), up to approximately 60% of their dry cell mass [5,6]. The oils produced by *Lipomyces starkeyi* (hereafter referred to as yeast oil) are similar in fatty acid composition (rich in palmitic acid and oleic acid) and physical properties (solid

at room temperature) to palm oil; therefore, it is expected to be an alternative palm oil from the viewpoint of flexible manufacturing locations and sustainability because there will be no need to harvest forests [7–10]. In addition, *Lipomyces starkeyi* has great industrial potential as an excellent lipid producer because it is a suitable host for genetic engineering to improve lipid production [11]. However, the preparation of yeast oil remains at the research level, using reagents in media and organic solvents that are not recognized as edible. We tried to utilize edible materials and processes, and we succeeded in producing yeast oil as an edible alternative to palm oil [12]. The proposed yeast oil production process includes (1) culturing, (2) cell recovery, (3) freezing, (4) vacuum drying, (5) cell crushing, (6) organic solvent extraction, and (7) oil purification [12,13]. In the cell recovery and lyophilization (freezing and vacuum drying) methods, the yeast cells can be crushed efficiently, and the oil can be recovered with organic solvents in high yields due to the low water content, though they 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. The Bligh and Dyer or Folch methods, which use chloroform and methanol for the extraction of lipids, are well-known procedures and have been applied for extracting oil from oleaginous yeasts [14–16]. 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 [17–21].

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 cell recovery and lyophilization steps, various methods for crushing in a culture medium state without performing cell collection and extraction to recover the yeast oil were examined. Yeast oil cells are dispersed in a high-viscosity liquid and have a cell structure that are stronger than other microorganisms; thus, a high-pressure homogenizer (approximately 200 MPa) was used to uniformly crush the yeast cells. Compared to the batch-type ball mill method, high-pressure homogenizers are considered useful because of the possibility of continuous operation [22]. However, the prepared cell-crushing liquid is strongly emulsified by water, yeast oil, and crushed yeast. Therefore, extraction via hexane addition, which is a conventional extraction method for the food industry, remains challenging [23].

In this study, a solid–liquid filtration separation for de-emulsification and extraction using a combination of various organic solvents against a crushed emulsified solution was investigated. Hexane, ethanol, and 2-propanol were used as organic solvents for food processing. The oil recovery ratio, 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. Materials and Methods

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. 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.

2.3. Solid-Liquid Filtration and Oil Extraction

Vacuum freeze-drying was performed for 72 h with a vacuum dryer (Tokyo Rika, freeze dryer FD-550P) after pre-freezing (−80 °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 Figure 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 cm^3 /(cm^2 · s)) was installed in a stirred cell with a filtration portion diameter of 47 mm, and 15 mL of the crushed culture was used. After filtration, the cell residue 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 Figure 1; monophasic). For the Bligh and Dyer method, chloroform and methanol (Nacalai Tesque, Kyoto, Japan) were prepared and extracted as previously reported [14,16]. 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.

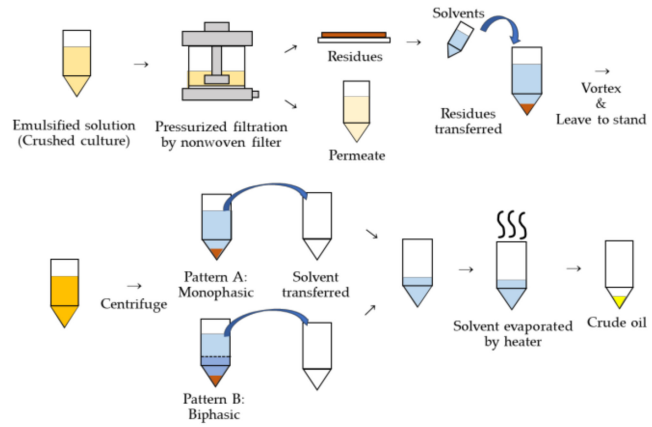

Figure 1. Schematic of the oil extraction processes for the emulsified solutions (crushed *L. starkeyi* culture). Pattern A: monophasic extraction; Pattern B: biphasic extraction (Bligh and Dyer and hexane/ethanol methods).

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 (1) and used as the quantitative value for TAG [24].

$$\begin{aligned}\text{TAG (g)} = \sum\{&(\text{amount of each fatty acid per 100 g}) \\ \times &(\text{molecular weight of that fatty acid} + 12.6826)/(\text{molecular weight of that fatty acid}\})\end{aligned} \quad (1)$$

2.5. Estimation for Oil Extraction Performances

To determine the theoretical oil recovery weight of the emulsified solution samples, calculations were performed using conventional methods that can extract TAGs almost completely. First, dry 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).

$$\text{Theoretical oil recovery weight (g)} = \frac{\text{TAG amount in the emulsified solutions (g)}}{\text{TAG portion in the extracted oils (}-\text{)}} \quad (2)$$

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

$$\text{Oil recovery ratio (\%)} = \frac{\text{measured extracted oil weight (g)}}{\text{theoretical oil recovery weight (g)}} \times 100 \quad (3)$$

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

$$\text{TAG extraction efficiency (mg/mL)} = \frac{\text{TAG (mg)}}{\text{Solvent volume (mL)}} \quad (4)$$

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 (5).

$$\text{TAG selectivity (mg/mg \%)} = \frac{\text{TAG (mg)}}{\text{Total extracted oil (mg)}} \quad (5)$$

These calculations were based on a previous study by Probst et al. [16].

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 (6)–(9):

$$RPE = E_f + E_{ex}, \tag{6}$$

$$RPE' = E_{pf} + E_d + E'_{ex}, \tag{7}$$

$$RPC = C_m + E_f \cdot \alpha + C_o + C_{ex}, \tag{8}$$

$$RPC' = \left(E_{pf} + E_d\right) \cdot \alpha + C'_o + C'_{ex}, \tag{9}$$

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 Materials.

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 µm filter. Hexane (7.5 mL) was added to each fraction and mixed, and the mixture was centrifuged at 5000 rpm for 15 min. The hexane layer was collected with a pipette, volatilized using a heater, and the weight of the collected oil was measured.

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 emersion 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 2.3. 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. Two 15 mL portions of this crushed solution were subjected to two pretreatments: vacuum freeze-drying and nonwoven filtration for collecting the residue using the same method as described in Section 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 2.3. Oil recovery ratio was calculated by comparison with the theoretical oil recovery weight determined as described in Section 2.5.

2.9. Statistical Analysis

The values are given as the mean ± standard deviation calculated in triplicate experiments. For Figure 5, the bar graph is presented with averages of triplicate analyses.

3. Results and Discussion

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

Sample	Oil Recovery Ratio (%)		
	Original Solution	**Centrifugation**	**Vacuum Freeze-Drying**
Crushed *L. starkeyi* culture	0	0	46.6 ± 10.6

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 ± 3.09% (Table 2). Although this value was lower than that of the vacuum freeze-drying sample (Table 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 ± 14.3% and 67.5 ± 7.7%, respectively. However, as shown in Figure 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. Oil recovery ratios after nonwoven filtration.

Extraction	Oil Recovery Ratio (%)	
Solvent	**With Filtration**	**Without Filtration**
Hexane	7.28 ± 3.09	0
Ethanol	67.8 ± 14.3	0
2-Propanol	67.5 ± 7.7	0

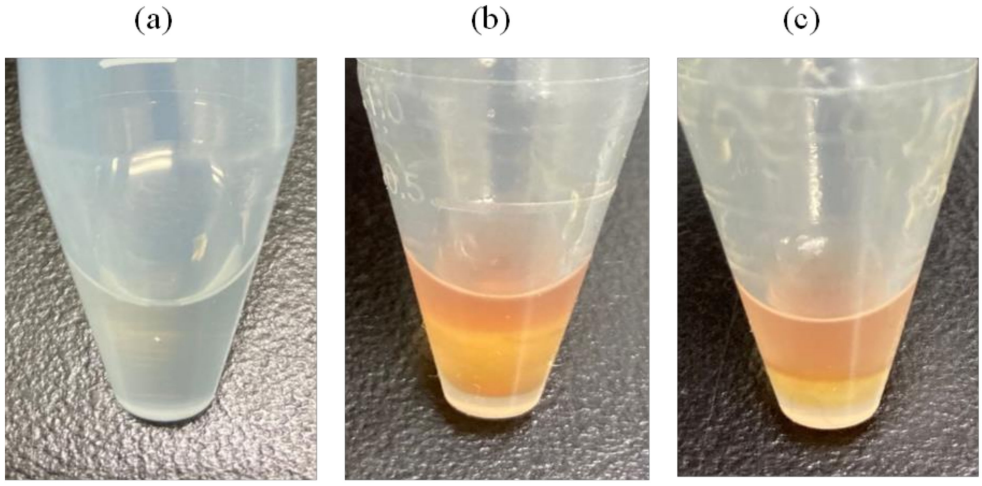

Figure 2. Pictures of oils extracted by each pure organic solvent after nonwoven filtration. (**a**) Hexane, (**b**) ethanol, (**c**) 2-propanol. The extracted oils were heated at approximately 60 °C.

3.3. Oil Extraction with Solvent Mixture after Nonwoven Filtration

Figure 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 (Figure 3a). The recovered oil is transparent, as shown in Figure 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 Figure 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 (Figure 4b,c).

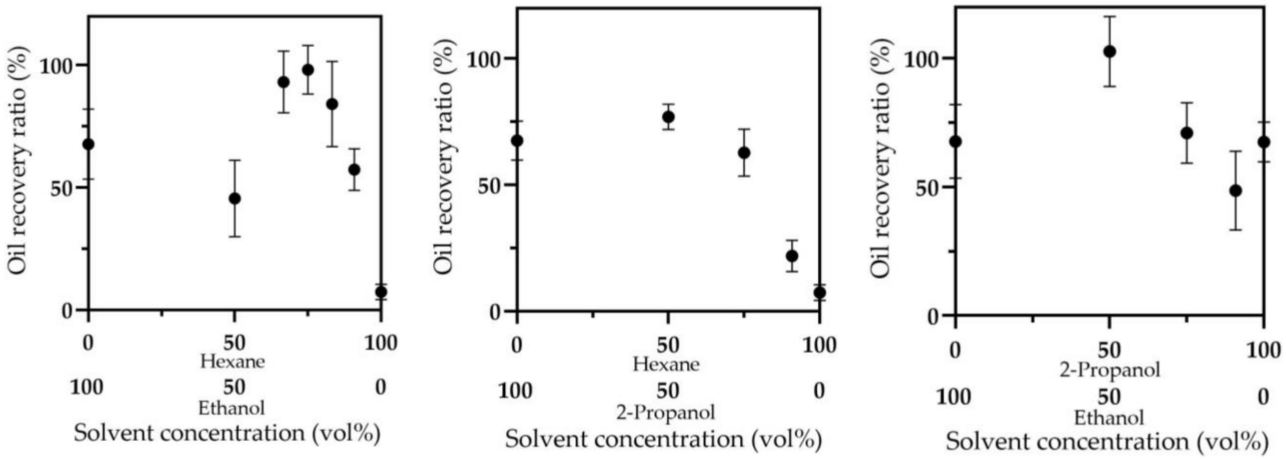

Figure 3. Effect of solvent mixture composition on the oil recovery ratio. (**a**) Hexane/ethanol mixture, (**b**) hexane/2-propanol mixture, and (**c**) 2-propanol/ethanol mixture.

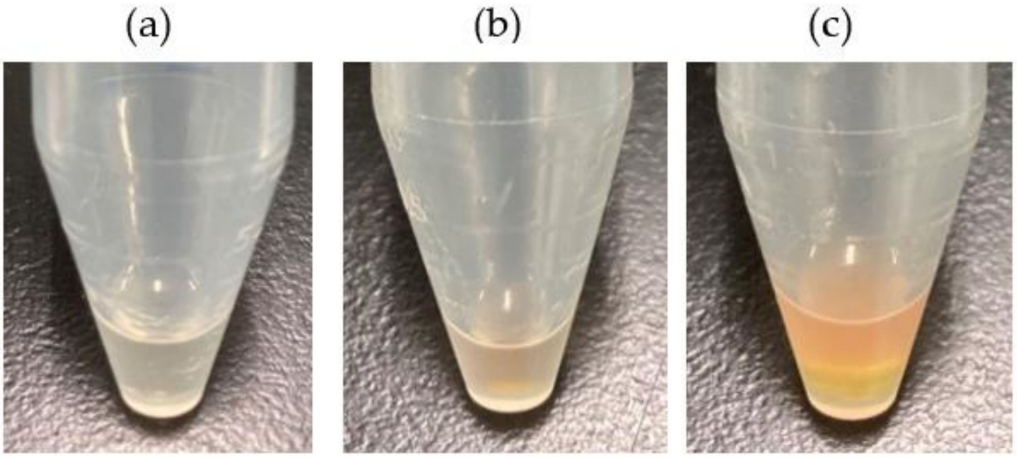

Figure 4. Pictures of oils extracted from each solvent mixture. (**a**) 3:1 (v/v) hexane/ethanol mixture, (**b**) 3:1 (v/v) hexane/2-propanol mixture, and (**c**) 3:1 (v/v) 2-propanol/ethanol mixture. Extracted oils were heated at approximately 60 °C.

3.4. TAG Extraction Efficiency and TAG Selectivity

The TAG extraction efficiency and TAG selectivity of various extraction solvents are summarized in Table 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 3 indicate that the hexane/ethanol mixture is a useful extraction solvent in terms of TAG extraction efficiency and TAG selectivity. All data were measured in triplicate and are noted as mean ± standard deviation.

Table 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

The fatty acid composition (change in physical properties) is affected the extraction method [10,25]. 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 Figure 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.

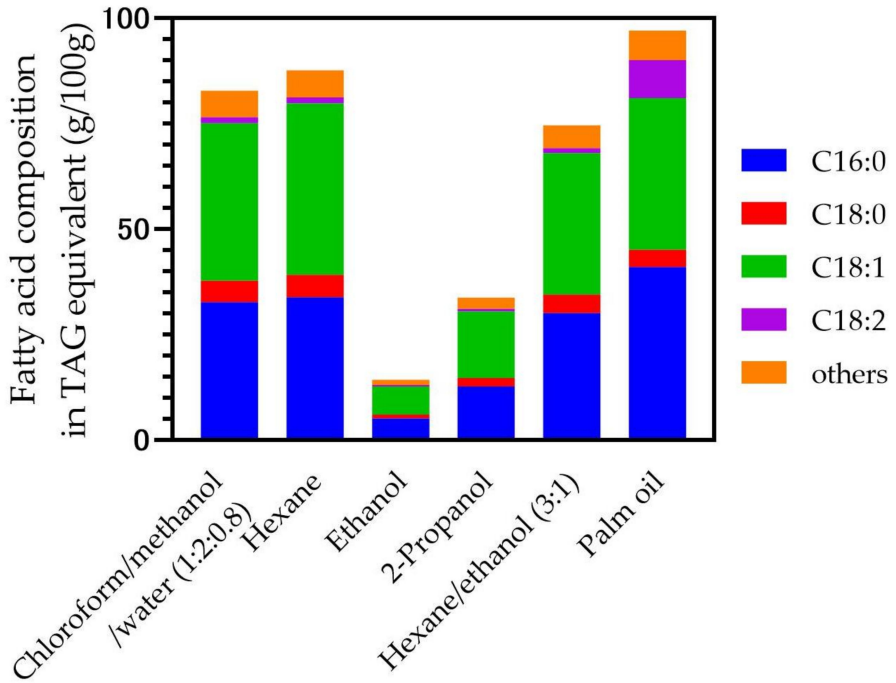

Figure 5. Fatty acid composition in TAG equivalents of oils extracted with various solvents.

3.5. Estimation of the Required Energy and Cost

Figure 6 compares the new yeast oil production process proposed in this study with the conventional process. We have succeeded in efficient oil recovery from the resulting emulsified solution, which has been difficult previously; thus, the lyophilization 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).

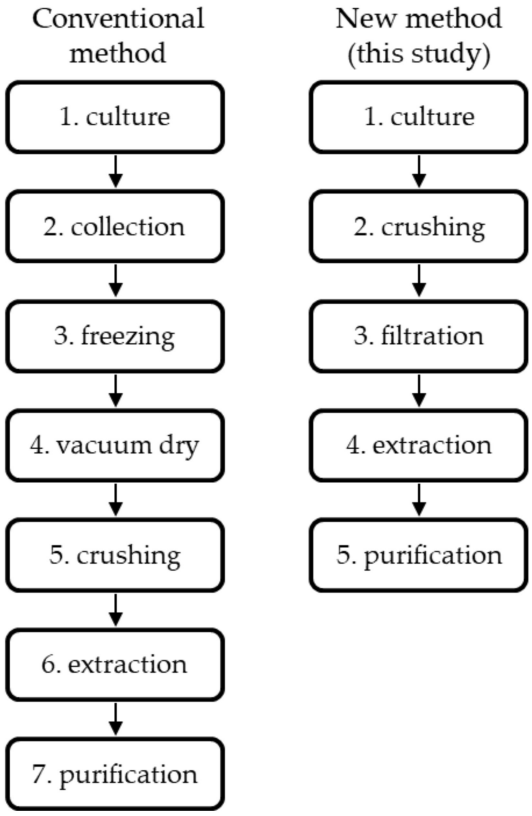

Figure 6. Comparison of new and conventional processes for yeast oil production from *L. starkeyi*.

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, we succeeded in constructing a new filtration and extraction process for yeast oil without cell collection and vacuum freeze-drying, 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.

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 and Figure 3b. Another hypothesis is that the affinity between the extraction solvent and the residue, which is quite low in the case of pure hexane, increased when hexane is mixed with ethanol, resulting in improved contact efficiency between hexane and the oil in the residue, resulting in improved extraction efficiency in 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.

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 4, the oil recovery ratios from the model solution after vacuum freeze-drying and filtration were 79.2 ± 6.0% and 67.2 ± 20.5%, respectively. In contrast, in the case of the culture solution, these values were lower, at 46.6 ± 10.6% and 7.28 ± 3.09%, respectively. In the model solution, the concentration of the water-soluble components was low because they were de-liquidated via centrifugation. Water-soluble components in the culture solution may inhibit hexane extraction. Specifically, a component derived from a medium or a metabolite of yeast cells is considered a candidate inhibitor.

Table 4. Oil recovery ratios from model solution and culture solution.

Sample	Oil Recovery Ratio (%)	
	Vacuum Freeze-Drying	Filtration
Model solution (10% dry cells)	79.2 ± 6.0	67.2 ± 20.5
L. starkeyi culture	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.

4. Conclusions

In this study, 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 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 study, 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.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/pr11051458/s1, Table S1: The coefficients and input values.

Author Contributions: Conceptualization, H.T. and K.M.; methodology, H.T., K.M., K.K., and H.M.; writing—original draft preparation, H.T.; writing—review and editing, S.M., K.K., and H.M. All authors have read and agreed to the published version of the manuscript.

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