Screening for *Lipomyces* strains with high ability to accumulate lipids from renewable resources

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The yeast *Lipomyces* accumulates triacylglycerols (TAGs) as intracellular fat globules, and these TAGs can be used as source materials for biodiesel production. In this study, we aimed to use this yeast to produce lipids from renewable resources. Using plate culture and micrograph methods, strains with a high lipid-accumulation ability were screened from 15,408 types of systems combining renewable resources, strains, and culture temperatures. The lipid-accumulation ability of the strains was estimated from the fat globule volume, which was calculated using a micrograph. The reliability of this method was examined, and strains with a high lipid-accumulation ability were identified for each renewable resource. Seventy-seven *Lipomyces* strains (7 deposit, 68 wild-type, 2 mutants) with a high lipid-accumulation ability were selected. A few strains possessed the ability to accumulate large amounts of TAGs from more than four different renewable resources. We found that strains with a high lipid-accumulation ability could efficiently convert consumed carbon sources into TAGs, which could be easily recovered from the fat globules of these strains through physical disruption.

Key Words: biodiesel; fat globule; lipid-accumulation ability; *Lipomyces* yeast; renewable resource; screening; triacylglycerol conversion ratio; triacylglycerol recovery

Introduction

Light oil purified from low-cost fossil fuels can be used efficiently for diesel engines with a high heat conversion efficiency; however, this method results in the accumulation of carbon dioxide. Triacylglycerols (TAGs) obtained from oil crops can be used in biodiesel as substitutes for light oil and can reduce carbon dioxide accumulation to help slow global warming.

Lipid production with microorganisms was studied to ameliorate lipid shortages during the First and Second World Wars (Hongou, 1956; Iwamoto, 1958). Since then, lipid production with microorganisms has been intermittently studied. Recent papers have concerned, for example, oleaginous yeasts for biodiesel (Sitepu et al., 2014); oil production by *Lipomyces* deposit strains grown in glucose, xylose, glycerol, and wood saccharification solution (Wang et al., 2014); lipid production by oleaginous yeasts from crude glycerol (Spier et al., 2015); and the identification of superior lipid-producing strains from 18 members of deposit oleaginous yeasts (Dien et al., 2016).

Our laboratory has been carrying out research on the physiology, biochemistry and ecology of yeast *Lipomyces* (Naganuma et al., 1985, 1986, 1989, 1999; Watanabe et al., 1997). In these studies, we used deposit strains and strains isolated from Japanese soils. These *Lipomyces* strains were used for the screening of strains with high lipid-accumulation ability from renewable resources (Fig. 1).

A small-scale, simple method is needed to screen strains with a high lipid-accumulation ability under conditions of combining 12 renewable resources, 428 strains, and three culture temperatures.
There are three experiments necessary to achieve the research. The first step is the screening of strains with a high lipid-accumulation ability corresponding to the renewable resources. The second step is the development of the culture medium and the conditions for efficiently producing TAGs. The third step is the development of methods for efficiently recovering TAGs from fat globules in the cell.

*Lipomyces* cells typically have a single fat globule in each cell, and this fat globule can be observed clearly under a microscope. The fat globule is filled with TAGs, among which the major fatty acids are oleic acid and palmitic acid (Uzuka et al., 1975).

In this paper, we describe a small-scale, simple method for measuring the size of the fat globule in many samples on micrographs and for calculating the fat globule volume. We then compare the fat globule volume measured by the simple method and the amount of TAGs per cell to select strains with a large fat globule volume adapted to each renewable resource. Our results demonstrate that strains with a high lipid-accumulation ability (high fat globule volume) are advantageous for the efficient conversion of TAGs and the recovery of intracellular TAGs.

**Materials and Methods**

**Strain, culture, and observation.** Isolation of genus *Lipomyces* from field soil and primary identification: The features of genus *Lipomyces* yeast are the formation of a fat globule in the cell (Slooff, 1970), resistance to cycloheximide (Barnett et al., 2000) and the ability to grow on nitrogen-free medium (Robert, 1945).

The isolation medium was a glucose-mineral medium (pH 10) and shaken with a mixer (Model CM-1000; Eyela, Hitachi Living Systems, Ltd.) and then added to the medium. Yacon was crushed (MM1; TANINAKA O&K Co., Ltd.) and then squeezed (squeezer: HC-JH type; SUN FOOD Machinery Co., Ltd.). Powder materials were used. Unshipped peaches were crushed (crusher: 5SW type; TANINAKA O&K Co., Ltd.) and then squeezed (squeezer: HC-JH type; SUN FOOD MACHINERY Co., Ltd.). Yacon was crushed (MM1; Hitachi Living Systems, Ltd.) and then added to the medium.

**Screening of *Lipomyces*, a lipid yeast**

Fig. 1. Biodiesel production using TAGs (triacylglycerols) obtained from the *Lipomyces* yeast cultured from a renewable resource.

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After incubation at 25°C, the yeast colonies were picked and placed on the same nitrogen-depleted plate medium. The yeast colonies were purified on a nitrogen-depleted plate medium without cycloheximide, and single cells were picked using a Joy- stick Micromanipulator (MN-151; Narishige, Tokyo, Japan) attached to an inverted microscope (IMT-2; Olympus, Tokyo, Japan).

Method for obtaining mutants with repression of extracellular polysaccharide synthesis (Iefuji et al., 2012): The yeast was cultured in YM liquid medium (10 g of glucose, 5 g of peptone, 3 g of yeast extract, and 3 g of malt extract in 1 L of distilled water) using L-shaped test tubes at 25°C. After 3 days of cultivation, the cells were harvested and washed with distilled water. Cells suspended in distilled water were agitated for 90 s at 50 cm below a UV germicidal lamp until a survival rate of approximately 10% on a clean bench was reached (MCV-710ATS; Sanyo [Panasonic], Tokyo, Japan). The cell suspension (20 μL) was transferred to a YM plate medium (10 g of glucose, 5 g of peptone, 3 g of yeast extract, 3 g of malt extract, and 20 g of agar in 1 L of distilled water) and spread. The YM plate medium was incubated for approximately 7 days at 25°C, and white colonies (fewer extracellular polysaccharides) were picked.

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Medium: The optimum concentrations of the renewable resources and the optimum media were determined by observing the formation of clear colonies (>0.5 mm). S agar plate medium was used for strains obtained from the BioResource Center Collection and UV treatment. The S medium comprised 3.5 g of (NH₄)₂SO₄, 1.0 g of KH₂PO₄, 0.5 g of MgSO₄·7H₂O, 0.1 g of NaCl, 0.1 g of CaCl₂·2H₂O, 1 g of yeast extract, the appropriate amount of renewable resource, and 20 g of agar in 1 L of distilled water. In the case of a 1/10 YM medium agar plate, the YM medium was diluted 10 fold and added to 20 g/L agar. This medium was used for strains isolated from the field.
Preparation of agar plates: After autoclaving, 20 mL of medium was immediately poured into sterilized plastic dishes (90 × 15 mm; IWAKI & Co., Ltd.). Agar plates were allowed to stand in a clean bench until inoculation. The inoculation method is described in the Results and Discussion section.

Culture and measurement of fat globules: The relevant details are described in the Results and Discussion section.

**Verification of the reliability of the apparent lipid-accumulation ability based on the volume of the fat globule.**

Strains: *Lipomyces* wild-type strain (No. 67, 320, 335, 347, 350), *Lipomyces kononenkoeae* CBS 8113 and CBS 8114, the mutant of *Lipomyces* wild-type No. 8.

Medium: The [1/10]N-S medium contained 0.035% of the ammonium sulfate concentration of S medium. Waste peach juice and leftover boiled rice were used as carbon sources.

Culture: Quantitative analysis of TAGs in the intracellular fat globule requires a high number of yeast cells. Therefore, the yeast cells in 100 mL of liquid medium in a 500-ml shaking flask were cultivated with a shaker. The culture conditions were 28 or 33°C and 120 strokes/min.

Measurement of the fat globule volume: The method for measuring the fat globule volume as a measure of apparent lipid-accumulation ability is described in Section “Results and Discussion”.

**Quantitative estimation of lipid-accumulation ability.**

Cell disruption for analysis of TAGs in intracellular fat globules: A test tube containing 2.5 g of glass beads (ø, 1.00–1.05 mm) and 1 mL of cell suspension with 10^8–10^9 cells was shaken with a mixer (Model CM-1000; EYELA) at 2,500 rpm until complete cell disruption (approximately 60 min) (Naganuma et al., 1984).

Quantitative estimation of TAGs: The homogenate (20 μL) was transferred into a test tube containing 3.0 mL of Cleantech TG assay kit reagents (Triglyceride E-test Wako; Wako Pure Chemical Industries, Ltd.). The solution was incubated at 37°C for 10 min and centrifuged at 2,500 × g for 5 min. The supernatants were filtered using a 0.45-μm membrane filter (DISMIC-25CS; ADVANTEC). The absorbance of the filtrates was then measured at 600 nm (Naganuma et al., 1982).

Measurement of cell number: The cell number was measured using a counting chamber (Thoma deep 0.1 mm; Erma) (Naganuma et al., 1975).

Calculation of the concentration (quantitative) of TAGs per 10^8 cells: The amount of TAGs accumulated in the intracellular fat globules (TAGs [mg]/10^8 cells) was estimated as follows: (TAGs [mg]/culture medium [mL])/(cell number/culture medium [mL]) × 10^8.

The amount of TAGs in 10^8 cells was defined as the quantitative lipid-accumulation ability.

**Association between quantitative lipid-accumulation ability and TAG conversion ratio.**

Strains and medium: These are each as described in the section “Verification of the reliability of the apparent lipid-accumulation ability based on the volume of the fat globule”.

Quantitative lipid-accumulation ability: The quantitative lipid-accumulation ability was measured as described previously.

Sugar consumption: The amount of sugar in the peach juice medium was measured using a pocket refraction meter (PAL-1; Atago). The starch concentration in the medium was analyzed by measuring the glucose concentration after starch hydrolysis, as follows: 250 μL of the medium was added to 7 mL of 2.5% HCl, and the starch was hydrolyzed at 121°C for 60 min (specified analysis method of National Tax Agency (2007)). The glucose concentration was estimated using a diagnostic glucose assay kit (Glucose C-test Wako; Wako Pure Chemical Industries, Ltd.).

The TAG conversion ratio (%) was expressed in terms of grams of TAGs produced per 100 g of sugar consumed (Park et al., 1990).

**Relationship between quantitative lipid-accumulation ability and the recovery ratio of TAGs in fat globules.**

Strains: *Lipomyces* wild-type strain (No. 67, 320, 335, 350), *Lipomyces kononenkoeae* CBS 8113, the mutant of *Lipomyces* wild-type No. 8. To investigate the strain character of leaking TAGs from fat globules, two *Lipomyces* wild-type strains (No. 35 and 357) with approximately the same volume of fat globules were used.

Medium: Media are the same as described in “Verification of the reliability of the apparent lipid-accumulation ability based on the volume of the fat globule”.

Quantitative lipid-accumulation ability: The quantitative lipid-accumulation ability was measured as described above.

Cell disruption: Complete cell disruption was performed at 2,500 rpm for 60 min. The conditions used for weak cell disruption were as follows: 1,000 rpm for 10 min.

The recovery ratio of the TAGs in the fat globule was calculated as follows: recovery (%) = (amount of TAGs leaked by weak disruption)/(amount of TAGs leaked by complete disruption) × 100.

**Results and Discussion**

**Screening for Lipomyces strains with a high lipid-accumulation ability.**

Development of small-scale, simple methods for the screening of strains with a high lipid accumulation. The combination of 12 types of renewable resources, 428 strains, and three culture temperatures required 15,408 experiments. Thus, we attempted to develop a small-scale, simple method for the screening of strains with a high lipid-accumulation ability.

*Lipomyces* yeasts can accumulate TAGs in intracellular fat globules, which can be observed using a microscope. The lipid-accumulation ability can therefore be estimated by microscopically measuring the sizes of the fat globules in the cells.

Six strains were inoculated on agar plate medium divided into six fractions. Spot inoculation was performed because the surface of the plate medium with a natural substance was uneven. The plastic dishes were placed into incubators at 20, 28, and 35°C (Fig. 2). Many strains were
Yeasts were stored in 15% glycerol in a deep freezer (−80°C).

Yeasts were cultured for 3-7 days on 5 or 1/10 YM liquid medium at 28°C with a shaker (120 rpm).

Ten microliters of culture medium were inoculated in an agar plate medium.

Two spots / each block

Agar plate medium with renewable resources divided into 6 blocks.

The plastic dishes were stacked into incubators at temperatures of 20, 28, and 35°C.

**Fig. 2.** Inoculation into agar plate medium containing the renewable resource.

A medium consisting of 2% agar and the renewable resources was autoclaved at 121°C. The medium was poured into sterilized plastic dishes (90 x 15 mm; IWAKI & Co., Ltd.). Six strains were inoculated into a plate medium.

A digital camera (NIKON COOLPIX P5100) was attached to a microscope (OLYMPUS BX51) through an attachment (MICRONET NY-P5000 90455). The objective lens was a UPlanFI 40x (OLYMPUS).

**Fig. 3.** Measurement of fat the globule volume.

A digital camera (NIKON COOLPIX P5100) was attached to a microscope using an attachment. Micrographs of cells within clear single colonies (> 0.5 mm) were acquired with a digital camera. The diameters of six large globules and six small globules were measured using a ruler. The fat globule volume (μm³) was calculated.

obtained from different isolation sources, and many types of renewable resources as carbon sources were used; therefore, we assumed that the optimum culture temperature for TAG accumulation may also vary. Accordingly, we tested culture temperatures of 20, 28, and 35°C.

Small amounts of cells were collected from each colony to measure the fat globule diameter. A digital camera (NIKON COOLPIX P5100) was then used to obtain micrographs. After printing the micrographs, the sizes of the fat globules were measured with a ruler. Each large and small fat globules of three, six, fifteen, thirty, fifty, seventy, and one hundred were measured. In over six of the fat globules, the standard deviation was almost same. For reducing the experimental period, the number of measurement fat globules was set to six large and small, the diameters of large fat globules and small fat globules were measured, and the average fat globule volume was calculated (Fig. 3).

Natural substances in the renewable resources negatively affected the quantitative analysis in some cases. However, the intracellular fat globules in medium containing these materials are easily visible with a microscope, and the lipid-accumulation ability is therefore measured easily.

The calculated fat globule volume was defined as the apparent lipid-accumulation ability.

**Verification of the reliability of the apparent lipid-accumulation ability.** By measuring the volume of the fat globules (μm³) with a small-scale, simple method, the apparent lipid-accumulation ability was estimated. In contrast, the calculation of the quantitative lipid-accumulation ability (TAGs [mg]/10⁸ cells) requires a complicated analytical process, large amounts of cells, and expensive reagents. To investigate the quantitative relationship between the sizes of fat globules and the amounts of TAGs in the globules, globules of different sizes were used.

The reliability of the apparent lipid-accumulation ability based on the volume of the fat globule.

Three shaking flasks were used per single strain, the shaking flasks contained waste peach juice or leftover boiled rice. The apparent lipid accumulation ability is the fat globule volume determined by the micrograph measurement method. The quantitative lipid accumulation ability is the amount of TAGs per cell. Error bars are the standard deviation from the mean. For selecting strains to experiment in Fig. 4, several pre-experiments were performed using many strains, and the strains which had reliable values for the relationship between the fat globule volume and the quantitative lipid-accumulation ability were used. The strain designation on each circle are as follows: (1) the mutant of *Lipomyces* wild-type No. 8, (2) *Lipomyces kononenkoe CBS 8113*, (3) No. 67 of *Lipomyces* wild-type strain, (4) *Lipomyces kononenkoe CBS 8114*, (5) No. 320 of *Lipomyces* wild-type strain, (6) No. 335 of *Lipomyces* wild-type strain, (7) No. 350 of *Lipomyces* wild-type strain, and (8) No. 347 of *Lipomyces* wild-type strain.

**Fig. 4.** Reliability of the apparent lipid accumulation ability based on the volume of the fat globule.
**Yeast strains corresponding to renewable resources as carbon sources.** Fat globule volumes of >46 μm³ were needed for selecting more one strain with a high lipid-accumulation ability from one renewable resource.

Table 1 shows the number of strains that had fat globules with volumes >46 μm³ (apparent lipid-accumulation ability) from each renewable resource.

In 15,408 types of systems combining renewable resources, strains with a high lipid-accumulation ability, and culture temperatures, 117 types were selected. Seventy-seven *Lipomyces* strains (7 deposit, 68 wild-type, 2 mutants) were obtained from 428 strains which were the subject of screening. Crude glycerol, waste peach juice, yacon, and rice bran all had impurities, and few yeasts were selected from these substances. Cellobiose, xylose, and glycerol had no impurities, and many yeasts were selected from these substances.

No. 296 of *Lipomyces* wild-type strain accumulated a large amount of TAGs from six types of renewable resources (cellobiose, xylose, xylooligosaccharide, potato, waste peach juice, and rice bran). No. 260 of *Lipomyces* wild-type strain accumulated TAGs from four types of renewable resources (cellobiose, xylitol, crude glycerol•maker B, and tapioca). *Lipomyces kononenkoae* CBS 7681 accumulated TAGs from four types of renewable resources (xylose, glycerol, glycerol•maker B, and yacon).

Even at 35°C, many strains accumulated large amounts of TAGs from the renewable resources. In particular, *Lipomyces kononenkoae* CBS 7681 accumulated TAGs at 20°C (glycerol), 28°C (yacon), and 35°C (xylose and glycerol•maker B). *Lipomyces kononenkoae* CBS 7682 accumulated TAGs at 20°C (glycerol), 28°C (glycerol•maker A), and 35°C (glycerol•maker B).

**Characteristics of strains with a high lipid accumulation**

**TAG conversion ratio.** The relationship between quantitative lipid-accumulation ability and the TAG conversion ratio (TAGs [g]/100 g sugar) is shown in Fig. 5. Yeasts with a high lipid accumulation tended to have a high TAG conversion ratio as well.

**Recovery ratio of TAGs in fat globules.** Microscopic observations revealed that cells with larger fat globules leaked TAGs in the fat globule upon exertion of a slight force. We investigated the effects of lipid-accumulation ability (size of fat globule volume) on TAG recovery; the results are shown in Fig. 6. High lipid accumulation caused a high recovery ratio; however, in some cases, the size of the fat globule was not correlated with the recovery ratio. This result suggests that both the lipid-accumulation ability and the strength of the fat globule membrane could affect TAG recovery.

The glycolytic pathway, pentose phosphate cycle, glycerol 3-phosphate pathway, tricarboxylic acid cycle, fatty-acid biosynthesis pathway, and carbon uptake are involved in TAG biosynthesis in cells, and different enzymes regulate these pathways (Hübscher, 1970; Naganuma et al., 1987; Wakil, 1970). It is suggested that the high lipid accumulation is induced by the activation of these pathways.
### Table 1. Strains with a high lipid-accumulation ability obtained on renewable resources.

| Renewable resource | Species | Strain | Sampling date | Culture temperature (°C) | Quantitative lipid-accumulation ability (TAGS (mg/10^6 cells)) |
|--------------------|---------|--------|---------------|---------------------------|---------------------------------------------------------------|
|                    |         |        | Vegetation    | Locality                  |                                                               |
| Cellulbiose (Reagent) | Wild type | No. 1  | Cherry tree   | Kofu city                 | 35                                                           | 5.0 ± 0.1                                                   |
|                     | Wild type | No. 68 | Green field   | Fujisohida city           | 20                                                           | 7.2 ± 0.0                                                   |
|                     | Wild type | No. 163| Mt. Maruyama  | Narusawa mura             | 28                                                           | 4.0 ± 0.0                                                   |
|                     | Wild type | No. 172| Taro field    | Tokorozawa city           | 28                                                           | 4.4 ± 0.0                                                   |
|                     | Wild type | No. 241| Needle leaf   | Chino city                | 28                                                           | 4.9 ± 0.0                                                   |
|                     | Wild type | No. 248| Grape orchard | Fuefuki city              | 28                                                           | 8.5 ± 0.2                                                   |
|                     | Wild type | No. 259| Peach orchard | Koshu city                | 28                                                           | 3.7 ± 0.0                                                   |
|                     | Wild type | No. 260| Peach orchard | Koshu city                | 35                                                           | 5.5 ± 0.0                                                   |
|                     | Wild type | No. 262| Grape orchard | Koshu city                | 35                                                           | 4.2 ± 0.0                                                   |
|                     | Wild type | No. 265| Peach orchard | Fuefuki city              | 35                                                           | 3.7 ± 0.0                                                   |
|                     | Wild type | No. 266| Grape orchard | Minami-alps city          | 28                                                           | 4.8 ± 0.1                                                   |
|                     | Wild type | No. 296| Rice field    | Yokote city               | 28                                                           | 3.6 ± 0.0                                                   |
|                     | Wild type | No. 305| Pine tree     | Omiya city                | 28                                                           | 4.6 ± 0.0                                                   |
|                     | Wild type | No. 314| Broad leaf    | Kofu city                 | 28                                                           | 3.9 ± 0.0                                                   |
|                     | Wild type | No. 320| Hinoki cypress| Kanazawa city             | 28                                                           | 4.7 ± 0.0                                                   |
|                     | Wild type | No. 323| Pine tree     | Sado city                 | 28                                                           | 4.2 ± 0.0                                                   |
|                     | Wild type | No. 335| Orange grove  | Yatsushiro city           | 28                                                           | 9.9 ± 0.1                                                   |
| Xylose (Reagent)    | Wild type | No. 1  | Cherry tree   | Kofu city                 | 35                                                           | 4.5 ± 0.0                                                   |
|                     | Wild type | No. 23 | Strawberry field | Kamagai city             | 28                                                           | 4.6 ± 0.1                                                   |
|                     | Wild type | No. 35 | Cuppice      | Fuefuki city              | 35                                                           | 3.7 ± 0.0                                                   |
|                     | Wild type | No. 44 | Cuppice      | Hokote city               | 28                                                           | 4.2 ± 0.0                                                   |
|                     | Wild type | No. 55 | Grassland    | Kawakami-mura             | 28                                                           | 4.1 ± 0.0                                                   |
|                     | Wild type | No. 57 | Cuppice      | Minami-alps city          | 28                                                           | 4.4 ± 0.0                                                   |
|                     | Wild type | No. 63 | Lava area    | Fujisohida city           | 28                                                           | 5.1 ± 0.0                                                   |
|                     | Wild type | No. 67 | Pine tree    | Oshinomura               | 28                                                           | 5.3 ± 0.0                                                   |
|                     | Wild type | No. 68 | Green field  | Fujisohida city           | 28                                                           | 4.6 ± 0.0                                                   |
|                     | Wild type | No. 76 | Garden       | Yokoha city               | 28                                                           | 7.2 ± 0.0                                                   |
|                     | Wild type | No. 225| Shore of Lake Motoko | Minobu-cho | 28                           | 4.5 ± 0.0                                                   |
|                     | Wild type | No. 260| Peach orchard | Koshu city                | 35                                                           | 3.9 ± 0.0                                                   |
|                     | Wild type | No. 261| Grape orchard | Koshu city                | 35                                                           | 3.6 ± 0.0                                                   |
|                     | Wild type | No. 263| Grape orchard | Fuefuki city              | 28                                                           | 7.3 ± 0.0                                                   |
|                     | Wild type | No. 295| Apple orchard | Yokote city               | 28                                                           | 4.5 ± 0.0                                                   |
|                     | Wild type | No. 296| Rice field   | Yokote city               | 35                                                           | 4.8 ± 0.0                                                   |
|                     | Wild type | No. 332| Sugarcane field | Kishima city             | 35                                                           | 3.6 ± 0.0                                                   |
|                     | Wild type | No. 381| Pine tree    | Minami-alps city          | 28                                                           | 5.0 ± 0.0                                                   |
|                     | Wild type | No. 862-B | Vineyard | Yamanashi city           | 28                                                           | 4.2 ± 0.0                                                   |
| konnenkoae          | Wild type | No. 24 | Pine tree    | Yoro cho                  | 28                                                           | 3.9 ± 0.0                                                   |
|                     | Wild type | No. 57 | Cuppice      | Minami-alps city          | 35                                                           | 5.0 ± 0.0                                                   |
|                     | Wild type | No. 175| Wilderness   | Nara city                 | 35                                                           | 9.9 ± 0.1                                                   |
|                     | Wild type | No. 262| Grape orchard | Koshu city                | 35                                                           | 4.3 ± 0.0                                                   |
|                     | Wild type | No. 331| Rice field   | Kishima city              | 35                                                           | 4.5 ± 0.0                                                   |
|                     | Wild type | No. 334| Cuppice      | Taishinomi city           | 35                                                           | 3.6 ± 0.0                                                   |
|                     | Wild type | No. 4a | Primeval forest with tree fern | Nago city | 35 | 6.9 ± 0.1 |
| Glycerol (Reagent)  | Wild type | No. 89-3| Cryptomeria forest | Koshu city | 28 | 3.9 ± 0.0 |
| konnenkoae          | konnenkoae | CBS 7535|              |                           | 35                                                           | 4.2 ± 0.1                                                   |
|                     | konnenkoae | CBS 7683|              |                           | 35                                                           | 3.8 ± 0.0                                                   |
|                     | konnenkoae | CBS 7682|              |                           | 35                                                           | 6.9 ± 0.1                                                   |
|                     | konnenkoae | CBS 8114|              |                           | 35                                                           | 4.9 ± 0.0                                                   |
|                     | konnenkoae | KW-3    |              |                           | 35                                                           | 4.9 ± 0.0                                                   |
| starkeyi            | CBS 1807 |        |              |                           | 35                                                           | 6.9 ± 0.1                                                   |
### Table 1. (continued).

| Renewable resource | Species | Strain | Sampling date | Culture temperature (°C) | Quantitative lipid-accumulation ability (TAGs (mg)/10^6 cells) |
|--------------------|---------|--------|---------------|---------------------------|-------------------------------------------------------------|
|                    |         |        | Vegetation    | Localidad                |                                                             |
| Crude glycerol     | Maker A | Wild type | No. 15 | Cherry tree | Kofu city | 28 | 4.2 ± 0.1 |
|                    |         | Wild type | No. 50 | Cryptomeria forest | Kawakami-mura | 20 | 4.0 ± 0.1 |
|                    |         | Wild type | No. 58 | Pumpkin field | Minami-ku city | 28 | 3.9 ± 0.1 |
|                    |         | Wild type | No. 310 | Broad leaf tree | Minami-ku city | 28 | 7.7 ± 0.0 |
|                    |         | Wild type | No. 354 | Rice field | Yatsushiro city | 28 | 4.5 ± 0.0 |
|                    |         | Wild type | No. 89-1 | Cryptomeria forest | Koshu city | 28 | 15.9 ± 0.2 |
|                    |         | Wild type | No. 89-4 | Cryptomeria forest | Koshu city | 28 | 7.5 ± 0.1 |
| Potato             | Wild type | No. 91-2 | Green field | Yamanashi city | 28 | 5.5 ± 0.1 |
| (Edible powder material) | Wild type | No. 91-2 | Green field | Yamanashi city | 28 | 6.6 ± 0.0 |
|                    |         | Mutant | CBS 1807 |         | 35 | 4.8 ± 0.1 |
|                    | No. 271 | Grape orchard | Kofu city | 28 | 4.6 ± 0.0 |
|                    | No. 295 | Apple orchard | Yokote city | 35 | 3.8 ± 0.0 |
|                    | No. 296 | Rice field | Yokote city | 35 | 8.6 ± 0.1 |
|                    | No. 311 | Lotus garden | Choe city | 28 | 7.6 ± 0.0 |
|                    | No. 327 | Cabbage | Suzuka city | 28 | 5.0 ± 0.0 |
|                    | No. 330 | Rice field | Kumaoka city | 28 | 3.6 ± 0.0 |
|                    | No. 346 | Cabbage | Nikko city | 28 | 4.0 ± 0.0 |
|                    | No. 347 | Tree | Sendai city | 28 | 9.3 ± 0.2 |
|                    | No. 347 | Pineapple field | Nago city | 35 | 3.7 ± 0.0 |
|                    |         | Mutant | CBS 1807 |         | 35 | 5.8 ± 0.0 |
| Xylooligosaccharides | (Reagent)| Wild type | No. 35 | Cabbage | Fukuoka city | 35 | 4.3 ± 0.1 |
|                    |         | Wild type | No. 98 | Cherry tree | Kofu city | 28 | 3.7 ± 0.0 |
|                    |         | Wild type | No. 296 | Rice field | Yokote city | 35 | 6.3 ± 0.0 |
|                    |         | Wild type | No. 320 | Hizoki cypress | Kumaoka city | 35 | 4.2 ± 0.0 |
|                    |         | Wild type | No. 324 | Cabbage | Fukuoka city | 35 | 4.0 ± 0.0 |
|                    |         | Wild type | No. 355 | Orange grove | Yatsushiro city | 35 | 3.6 ± 0.2 |
|                    |         | Wild type | No. 347 | Tree | Sendai city | 35 | 8.0 ± 0.1 |
|                    |         | Wild type | No. 350 | Tree | Iwakuni city | 35 | 6.3 ± 0.1 |
|                    |         | Mutant | CBS 1807 |         | 35 | 4.1 ± 0.0 |
| Crude glycerol     | Maker B | Wild type | No. 1 | Cherry tree | Kofu city | 35 | 4.0 ± 0.0 |
|                    |         | Wild type | No. 67 | Pine tree | Oshinomura | 28 | 4.5 ± 0.0 |
|                    |         | Wild type | No. 259 | Peach orchard | Koshu city | 35 | 5.3 ± 0.0 |
|                    |         | Wild type | No. 263 | Grape orchard | Fukuoka city | 35 | 5.6 ± 0.0 |
|                    |         | Wild type | No. 295 | Apple orchard | Yokote city | 35 | 6.5 ± 0.1 |
|                    |         | Wild type | No. 296 | Rice field | Yokote city | 35 | 8.4 ± 0.0 |
|                    |         | Wild type | No. 86R-1 | Vineyard | Yamanashi city | 20 | 4.3 ± 0.1 |
|                    |         | Mutant | CBS 7543 | Peach orchard | Kofu city | 35 | 5.9 ± 0.0 |
|                    |         | Mutant | CBS 7581 | Peach orchard | Kofu city | 35 | 3.6 ± 0.0 |
|                    |         | Mutant | CBS 8114 | Peach orchard | Kofu city | 35 | 4.0 ± 0.0 |
| Tapioca (Edible powder material) | Wild type | No. 193 | Fendob in Hino-o-dam | Yamanashi city | 28 | 4.6 ± 0.0 |
|                    |         | Wild type | No. 260 | Peach orchard | Kofu city | 35 | 5.0 ± 0.0 |
|                    |         | Mutant | CBS 7543 | Peach orchard | Kofu city | 35 | 3.6 ± 0.0 |
|                    |         | Mutant | CBS 7581 | Peach orchard | Kofu city | 35 | 5.0 ± 0.0 |
|                    |         | Mutant | CBS 8114 | Peach orchard | Kofu city | 35 | 4.0 ± 0.0 |
| Yacon              | Wild type | No. 25 | Bamboo forest | Sagamihara city | 28 | 5.1 ± 0.0 |
|                    | Wild type | No. 4-1 | Bamboo forest | Sagamihara city | 28 | 5.1 ± 0.0 |
|                    | Wild type | No. 2-1A | Prunus | Naha city | 28 | 4.0 ± 0.0 |
|                    | Mutant | CBS 7618 |         | Nago city | 28 | 3.9 ± 0.0 |
|                    | Mutant | CBS 8114 |         | Nago city | 20 | 4.2 ± 0.0 |
| Rice bran          | Wild type | No. 186 | Swamp | Kofu city | 28 | 6.0 ± 0.0 |
|                    | Wild type | No. 296 | Rice field | Yokote city | 35 | 5.1 ± 0.0 |
|                    | Wild type | No. 313 | Needle leaf tree | Kofu city | 35 | 5.1 ± 0.0 |
|                    | Mutant | No. 8 | Swamp | Kofu city | 28 | 7.0 ± 0.3 |
| Crude glycerol     | Maker C | Wild type | No. 44 | Cabbage | Kofu city | 28 | 3.7 ± 0.0 |

* With a lipid-globule volume of over 46 μm³.
* These values were obtained by transferring apparent lipid-accumulation ability (the globule volume [μm³]) into quantitative lipid-accumulation ability (TAGs (mg)/10^6 cells), using Fig. 4.
* Containing 0.05% xylose as inducer.
* Containing mainly fructooligosaccharides.
associated with TAG synthesis.

When reagents are used as carbon sources, there are no effects of impurities on the activity of enzymes and metabolic pathways (Taherzadeh et al., 2000). Therefore, in strains selected from reagents as carbon sources, rescreening of strains with a high lipid-accumulation ability under the presence of impurities is necessary.

Identifying strains with a high lipid-accumulation ability in the presence of impurities is an important part of achieving biodiesel production from renewable resources (Fig. 1). This issue was resolved by selecting several Lipomyces strains with a high lipid-accumulation ability in the presence of waste peach juice and rice bran.

Strains with a high lipid accumulation can also easily leak TAGs upon weak cell disruption; therefore, TAGs can be easily recovered from fat globules by physical methods at low cost.

By determining the optimal culture conditions and media for lipid production and obtaining a low-cost method for easy recovery of TAGs from fat globules, a practical production of source lipids for biodiesel production can be achieved. However, biodiesel production using Lipomyces yeast is more expensive than the production of fossil fuel diesel. Thus, for commercial purposes, a biodiesel production system with lower cost is required.

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