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Cryptococcus terricola is a promising oleaginous yeast for biodiesel production from starch through consolidated bioprocessing

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Starch is considered a potential feedstock for biofuel production, particularly in light of the large-scale landfilling of food waste and other starchy materials worldwide. Lipid accumulation by oleaginous yeast is a promising method for biodiesel production from starch. However, most oleaginous yeasts are grown on monosaccharides or oligosaccharides because they cannot directly utilize starch. We therefore investigated the starch-assimilation ability of 1,200 yeasts. We found that *Cryptococcus terricola* could be used for fuel production through consolidated bioprocessing. *C. terricola* JCM 24523 exhibited the highest lipid content of 61.96% on medium with 5% starch at 10 days. Fatty acid methyl ester analysis showed that this strain produced high proportions of C16:0 and C18 fatty acids when grown on starch, which are ideal for use in biodiesel. Considering the yield and cost, lipids derived from starch using *C. terricola* would be a promising alternative source for biodiesel production.

Biodiesel has received increasing attention as a result of globally rising crude oil prices, increasing carbon dioxide emissions, and growing expectations that biomass will be utilized instead of wasted. Biolipids, including triacylglycerol, produced by oleaginous yeasts have been confirmed to be among the most effective raw materials for biodiesel production^{1,2}. The advantages of lipid production by oleaginous yeast include the potential to produce lipids from organic waste matter containing various types of polysaccharides, such as starch and cellulose³. Because, in general, food wastes contain large amounts of starch, we are focusing on biolipid production from starch through consolidated bioprocessing (CBP) using oleaginous yeasts. A CBP protocol for producing lipids in a single step without the use of amylolytic enzymes from starchy materials would have the advantage of being very cost-effective, since it would eliminate the requirements for enzyme addition, long reaction time, and electricity for heating⁴⁻⁶.

Various starchy wastes, such as cassava pulp and wastewater from food plants, are discarded. Cassava pulp, which contains 50–60% residual starch on a dry weight basis, is a byproduct of starch production in tropical regions, and much of it (approximately 552,000 tons per year) is wasted⁷. Starchy wastewater contains high concentrations of chemical oxygen demand (COD) and biochemical oxygen demand (BOD), and causes serious environmental threats⁸. It is reported that at least 0.60 m³ of wastewater is formed in the processing of one ton of cassava⁹. However, this wastewater can be a major resource for biolipid production.

In addition to yeasts, there are kinds of microorganisms that accumulate lipids, such as microalgae, bacteria, and fungi. Although microalgae accumulate lipids at high levels (60–70% of dry cell weight)¹⁰ by using sunlight energy, carbon dioxide, and nitrogen, they cannot convert starch or other feedstocks to lipids. Bacteria accumulate lipids in their outer membrane, from which they are difficult to extract¹¹. The filamentous fungus *Mortierella alpina* can assimilate starch and produce lipid¹², but challenges remain with respect to the accumulation speed and quantity. Therefore, oleaginous yeast is a potential alternative oil resource for biodiesel production from starch.



It is known that oleaginous yeasts, such as *Rhodospiridium toruloides*, *Cryptococcus curvatus*, *Lipomyces starkeyi*, and *Yarrowia lipolytica*, accumulate lipids in more than 20% of their dry cells^{13–17}. From a taxonomical viewpoint, it has been shown that several oleaginous yeasts can grow on medium containing soluble starch as a sole carbon source¹⁸. To the best of our knowledge, however, there has been no comprehensive screening for oleaginous yeasts that directly produce lipids from soluble starch.

Recently, Takashima et al. reported on the great taxonomic diversity of the yeasts within subtropical and cool temperate areas of Japan¹⁹. We consider that the yeast strains collected in Japan, the majority of which have been classified as basidiomycetes, would also demonstrate great functional diversity, including lipid-production ability. Because the yeast strains classified into the basidiomycetes family have broad assimilation spectra, we considered that these yeasts would be suitable for screening a useful strain for lipid production from soluble starch. We therefore performed a comprehensive screening of the collected basidiomycete yeast strains in order to identify a strain that produces large amounts of lipid from soluble starch. Starch contains two types of polysaccharides: amylose and amylopectin²⁰. Amylose forms a colloidal dispersion in hot water, whereas amylopectin is completely water-insoluble. The proportions of amylose to amylopectin can vary considerably between plants. We employed hot-water-soluble starch to enable comprehensive screening of lipid production strains as a model of food waste.

In this study, we show that *Cryptococcus terricola*, which was reported as an oleaginous yeast in the 1960s, has suitable characteristics for lipid production from soluble starch through CBP with high lipid content.

Results

Screening of yeast strains that grow on starch-containing agar medium. Among the 1,200 strains studied, 85% of the strains grew and 43% of the strains accumulated detectable amount of lipids in the primary screening (Fig. 1a) on medium containing soluble starch as a sole carbon source. Almost all of the yeast strains that could grow in the medium were basidiomycetes. All of the yeast strains that accumulated lipids at a high level belonged to *Cryptococcus terricola*.

Lipid-accumulating ability in liquid medium containing soluble starch. Nine starch-assimilating *C. terricola* strains (Table 1) were cultivated in SS5 liquid medium containing 5% soluble starch for 6 days, and intracellular lipid was estimated as the lipid content of fatty acids (secondary screening) (Fig. 1b). JCM 24523 attained the highest lipid content, at 36.10%. JCM 24518 exhibited a fairly high lipid content of 26.78%. The other strains also grew directly on starch substrates and accumulated lipids. The results were strain-specific.

Kinetic analysis of lipid accumulation of *C. terricola*. To gain further insight into lipid accumulation by *C. terricola*, kinetic analysis of JCM 24518 and JCM 24523 using SS5 or SS10 liquid medium containing 10% soluble starch was performed, and the results were compared with those for the control strain *L. starkeyi* NBRC 10381 (Fig. 2a,b). The culture broths were collected at 1, 2, 4, 7, and 10 days. In SS5 liquid medium, JCM 24518 and JCM 24523 showed the highest lipid contents: 57.76% and 61.96% at 10 days, respectively (Fig. 2a). These values were 18% higher than that of *L. starkeyi* NBRC 10381. For all strains, the lipid content observed using SS10 liquid medium was lower than that using SS5 medium (Fig. 2b).

Simultaneously, we measured the time courses of the starch concentrations of the strains *C. terricola* and *L. starkeyi* NBRC 10381 using SS5 or SS10 liquid medium at 1, 2, 4, 7, and 10 days (Fig. 2c,d). In both cases, the consumed starch of *L. starkeyi* NBRC 10381 was higher than that of *C. terricola*.

Fatty acid profiles. The fatty acid compositions of the lipids produced by the yeast strains using SS5 or SS10 liquid medium after 10 days

were examined (Table 2), as fatty acid composition is known to influence the quality of biodiesel produced from biolipids. No major differences in these compounds of three biolipids were found; the dominant fatty acid in these biolipids was oleic acid (18:1), which ranged from 72.01% to 81.46%, at each starch concentration. Suitable fatty acid compositions for biodiesel production are palmitic (16:0), stearic (18:0), oleic (18:1), linoleic (18:2), and linolenic (18:3) acids²¹. The two selected *C. terricola* strains contained these fatty acids in high proportions, ranging from 94.56% to 95.46%.

Discussion

C. terricola belongs to the basidiomycete family, the filobasidiales lineage, and the *aerius* clade¹⁸. This species is frequently isolated from soils²². In the 1960s, Petersen and co-workers published several reports on lipid production by *C. terricola*^{23–25}. To the best of our knowledge, these were the last reports published on the lipid production of these species. In the present work, therefore, we examined *C. terricola* because of its high lipid-accumulating ability, and focused further on nine strains of *C. terricola* (Table 1) that have a high lipid-accumulating ability. All the strains examined in this study were isolated from Rishiri Island, which is in a cool temperate climate zone, since *C. terricola* is known to thrive in colder climates¹⁸.

It was found that the higher starch concentration does not promote lipid accumulation (Fig. 2a,b). It can be surmised that high starch concentration led to a highly gelatinized solution, and then the diffusion of amylase was limited and its accessibility to glycosidic linkages was restricted^{26,27}. It should be noted that the lipid contents of the strains increased rapidly between culture days 4 and 10 (Fig. 2a,b). It is known that the lipid accumulation in oleaginous yeast occurs under stress conditions in the medium²⁸. When cells begin the depletion of nutrients, such as nitrogen, the excess carbon in the culture medium is converted into cellular lipids²⁹. Nitrogen is one of the main components of media for yeast growth and metabolism. As previously reported, the role of nitrogen is to promote the growth of cells in order to prepare for the accumulation of lipids³⁰. Unlike monosaccharides or oligosaccharides, starch is not assimilated directly by most yeasts³¹. To grow on starchy substrates, yeasts need to degrade starch to oligosaccharides enzymatically by using their own extracellular amylases. We consider that the CBP is divided into three parts: a growth phase (from days 0 to 2), a degradation phase (days 2 to 4), and a lipid accumulation phase (days 4 to 10) (Fig. 2a,b). During the growth phase, yeasts hydrolyze starch and assimilate the released glucose simultaneously. Lipid accumulates gradually, and yeast cells continue to proliferate throughout this period. During the degradation phase, starch hydrolysis becomes predominant and yeast cells enter a stationary phase. This pattern corresponded with the starch consumption profiles; the starch concentrations decreased until day 4 (Fig. 2c,d). The lipid-accumulation phase corresponds to the lipid body enlargement phase under nitrogen limitation conditions^{32–34}.

Assimilated excess starch was used for cell growth; the cell masses of JCM 24523 at 10 days of culture were 4.88 g/L and 6.01 g/L for the SS5 and SS10 liquid media, respectively. However, just because a good deal of starch was assimilated, it does not necessarily follow that the lipid content was higher. Rather, the differences in starch assimilation may have been related to specific characteristics of the metabolic systems of these species. Further investigation is needed to clarify the difference between *C. terricola* and *L. starkeyi* with respect to the enzymatic degradation mechanism of starch, as well as to the relationship between nitrogen concentration and lipid content in these species.

The fatty acid profile of rapeseed oil includes a high quantity of oleic acid, very similar to the profile obtained for *C. terricola* cultivated in starch medium in the present study. Rapeseed oil can be used for the production of biodiesel³⁵. For example, according to the Japan

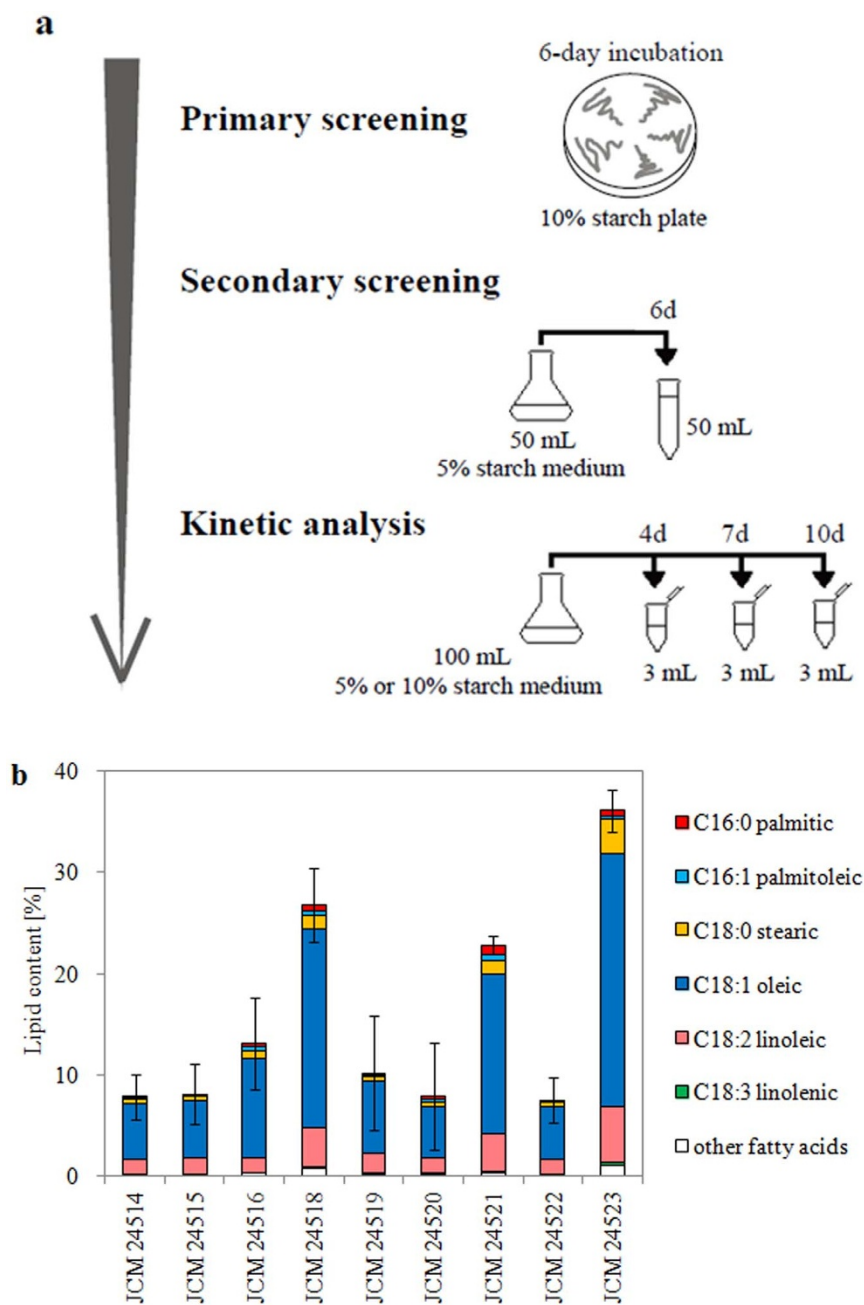


Figure 1 | (a) Screening flow scheme. (b) Comparison of the lipid contents of nine selected *C. terricola* after a 6-day culture. The sole carbon source was 5% starch. Data are the means \pm standard deviation (error bars) of three assays.

Table 1 | The nine strains of *Cryptococcus terricola* selected for the second screening

JCM number	Species	Higher taxa	DDBJ accession no.	Source
JCM 24514	<i>Cryptococcus terricola</i>	Filobasidiales, Agaricomycotina	AB726925	Soil, Rishiri Island
JCM 24515	<i>Cryptococcus terricola</i>	Filobasidiales, Agaricomycotina	AB726924	Soil, Rishiri Island
JCM 24516	<i>Cryptococcus terricola</i>	Filobasidiales, Agaricomycotina	AB726973	Soil, Rishiri Island
JCM 24518	<i>Cryptococcus terricola</i>	Filobasidiales, Agaricomycotina	AB726896	Soil, Rishiri Island
JCM 24519	<i>Cryptococcus terricola</i>	Filobasidiales, Agaricomycotina	AB726906	Soil, Rishiri Island
JCM 24520	<i>Cryptococcus terricola</i>	Filobasidiales, Agaricomycotina	AB726970	Soil, Rishiri Island
JCM 24521	<i>Cryptococcus terricola</i>	Filobasidiales, Agaricomycotina	AB727265	Soil, Rishiri Island
JCM 24522	<i>Cryptococcus terricola</i>	Filobasidiales, Agaricomycotina	AB726890	Soil, Rishiri Island
JCM 24523	<i>Cryptococcus terricola</i>	Filobasidiales, Agaricomycotina	AB726902	Soil, Rishiri Island

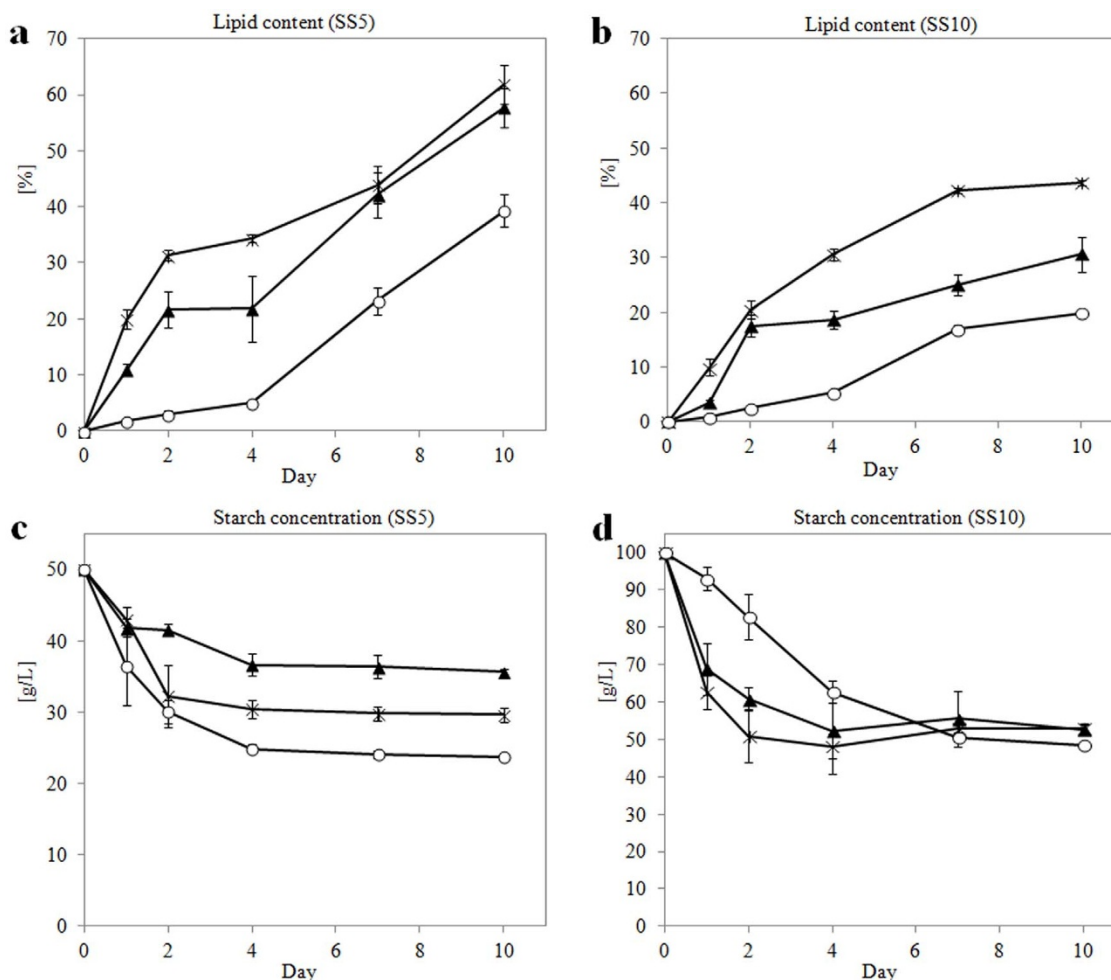


Figure 2 | Time courses of the lipid contents of JCM 24518 (filled triangle), JCM 24523 (asterisk), and *L. starkeyi* NBRC 10381 (open circle). The sole carbon source was 5% (a) or 10% (b) starch. Time courses of the starch consumption of these strains. The sole carbon source was 5% (c) or 10% (d) starch. Data are the means \pm standard deviation (error bars) of three assays.

Oilseed Processors Association, the 2011/2012 rapeseed oil harvest in Japan was 10.18 million tons per 1,000 hectares, for a yield of 1,018 kg/ha/year. Assuming a lipid production of *C. terricola* JCM 24523 of 3.02 g/L (calculated from 61.96% of lipid content and 4.88 g/L of cell mass) from SS5 liquid medium at 10 days, approximately 337 m³ of medium would be required for the production of the equivalent of 1 hectare's worth of rapeseed oil. The daily amount of starchy wastewater from Udon noodle restaurants in Japan's Kagawa Prefecture is almost the same (more than 300 m³) and causes

serious environmental pollution. *C. terricola*-based oil production requires only 10 days. Such production could be used to reduce food-industry waste in various countries, such as to reduce landfilling of cassava pulp in Thailand.

The kind of substrate significantly influences lipid accumulation by yeasts. The fatty acid profiles produced by the selected strains from starch were suitable for biodiesel production. Our previous study revealed that oleaginous yeast achieved a lipid content of at most 61.53% from 3% glucose as a sole carbon source at day 4³⁶. In

Table 2 | Fatty acid composition of the two selected *C. terricola* strains and the control strain after a 10-day culture using SS5 or SS10 liquid medium

Medium	SS5			SS10		
	Strain:	JCM 24518	JCM 24523	<i>L. starkeyi</i> NBRC10381	JCM 24518	JCM 24523
C12:0 lauric	-	0.06	0.03	-	0.06	0.02
C14:0 myristic	0.47	0.63	0.51	0.42	0.57	0.59
C16:0 palmitic	2.86	2.55	4.11	2.53	2.58	4.20
C16:1 palmitoleic	3.06	3.09	7.44	2.84	3.29	8.00
C18:0 stearic	4.87	4.81	4.01	5.40	4.94	9.19
C18:1 oleic	73.16	73.17	81.46	72.42	72.01	76.54
C18:2 linoleic	13.91	13.77	1.85	14.33	14.45	0.85
C18:3 linolenic	0.65	0.55	0.10	0.72	0.59	0.08
C22:0 behenic	0.39	0.39	0.32	0.48	0.40	0.32
C24:0 lignoceric	0.61	0.99	0.18	0.86	1.11	0.19



the present study, on the other hand, the lipid content of JCM 24523 was 34.31% from 5% soluble starch at day 4 (Fig. 2a). This difference was not negligible. However, unlike monosaccharides and oligosaccharides, starch is considered the feedstock with the greatest potential for biolipid production, as it is cheap and does not compete with food resources. Considering the feedstock and enzyme costs, lipid production from soluble starch with *C. terricola* through CBP would compensate for the loss and therefore has great potential for cost-effective and time-efficient biodiesel production.

In the present study, we found that the oleaginous yeast *C. terricola* could directly assimilate soluble starch and thereby accumulate a considerable amount of lipids. Our results thus provide a novel process for lipid production from starch biomass. More importantly, a system that does not require enzymatic hydrolysis would encourage further development in practical biodiesel production.

Methods

Yeast strains. Yeast strains collected and taxonomically identified by Takashima et al. were used as the main resources¹⁹. Yeast strains isolated from the campus of Kyoto University (Kyoto, Japan) were also assessed. As a control strain, *L. starkeyi* NBRC 10381 was obtained from the National Institute of Technology and Evaluation (NITE) Biological Resource Center.

Media and cultivation conditions. YM agar medium (Difco, Detroit, MI, USA) was used for the maintenance of yeast strains. SS10 agar medium (0.17% yeast nitrogen w/o ammonium sulfate and amino acids [Difco], 0.5% ammonium sulfate, 10% soluble starch [Nacalai Tesque, Kyoto, Japan], and 2% agar) and SS5 liquid medium (0.5% ammonium sulfate, 0.05% magnesium sulfate, 0.01% sodium chloride, 0.01% calcium chloride and 0.01% yeast extract [Difco] and 5% soluble starch) were used for the primary and secondary screening of starch-assimilating ability, respectively. YPD medium (1% yeast extract [Difco], 2% peptone [Difco], and 2% glucose) was used for preculture. SS5 and SS10 (0.5% ammonium sulfate, 0.05% magnesium sulfate, 0.01% sodium chloride, 0.01% calcium chloride and 0.01% yeast extract [Difco], and 10% soluble starch) liquid medium were used for the kinetic analysis.

Screening of yeast strains with starch-assimilation ability. The strains were screened in three steps (Fig. 1a). They were cultivated on SS10 agar medium at 25°C for 6 days. The grown cells were scraped with a loop, suspended in distilled water, and then centrifuged at 15,000 rpm for 10 min. Intracellular total lipids were determined by gas chromatography after lyophilizing the harvested wet cells (primary screening).

Screening of lipid-accumulating strains in liquid medium. The yeast strains selected by the primary screening were inoculated into 3 mL of YPD medium in test tubes and incubated overnight at 27°C at 150 rpm (preculture). The preculture was then suspended in 50 mL of SS5 liquid medium in an Erlenmeyer flask, to a cell optical density at 600 nm (OD₆₀₀) of 0.2, then cultured at 27°C for 6 days at 150 rpm. Cells from 50 mL of culture broth were centrifuged (15,000 rpm for 10 min) then washed twice with distilled water. Intracellular total lipids, fatty acid composition, and cell mass were determined after the wet cells were lyophilized (secondary screening). In this assay, *L. starkeyi* was used as a control strain because it is known as an oleaginous yeast that can grow in starch¹⁶. All experiments were performed in triplicate.

Kinetic analysis of yeast strains with high lipid content. The yeast strains selected by the secondary screening were cultivated in Erlenmeyer flasks containing 100 mL of SS5 or SS10 liquid medium at 27°C, on a rotary shaker at 150 rpm. The preculture and cell dosage were the same as described above. Cells from 3 mL of culture broth were harvested after 1, 2, 4, 7, and 10 days of cultivation by centrifugation (15,000 rpm for 10 min), then washed twice with distilled water. Intracellular total lipids, fatty acid composition, and cell mass were determined after lyophilizing the wet cells. The starch concentrations of the culture supernatants were determined by following the procedures below. All experiments were performed in triplicate.

Measurement of intracellular fatty acids. Total intracellular lipids were estimated as total fatty acids. The fatty acids of the yeast strains were extracted from the lyophilized cells by a hydrochloric acid-catalyzed direct methylation method³⁷. In brief, after cultivation, the yeast cells were harvested by centrifugation and lyophilization. The lyophilized cells were dissolved in toluene and methanol, then directly methylated with 8% methanolic HCl at 45°C overnight. The resultant fatty acid methyl esters were extracted with n-hexane and analyzed using a gas chromatograph (GC-2010 Plus; Shimadzu, Kyoto, Japan) equipped with a flame ionization detector (FID) and an autosampler (AOC20; Shimadzu). A DB-23 capillary column (30 m × 0.25 mm ID and 0.25 μm film thickness) (Agilent Technologies, Palo Alto, CA, USA) was used. The column temperature was programmed to start at 50°C for 2 min and then increased by 10°C/min up to 180°C, where it remained for 5 min, before being increased at a rate of 5°C/min to 240°C and held at 240°C for 3 min. Helium, the carrier gas, was pumped at 1.0 mL/min, and nitrogen was used as the make-up gas. The injector temperature was 250°C and the detector temperature was 300°C, with a

split ratio of 50 : 1. The major peaks were identified based on the retention time using controls obtained from Sigma-Aldrich (Saint Louis, MO, USA). The fatty acid concentrations were determined using a standard curve generated by a series of external standards.

Measurement of starch in culture medium. Starch concentration was determined using a calibration curve obtained by measuring the enzymatic hydrolyzed glucose of the serial concentration of starch. A solution for determining starch concentration was prepared by adding 30 U/mg-solid of glucoamylase from *Rhizopus* sp. (Toyobo, Osaka, Japan) to 300 μL of supernatant of broth. After keeping the solution at 50°C for 15 min, the enzymatic reaction was stopped by boiling for 10 min. Glucose concentrations were determined using an HPLC system (Shimadzu) equipped with an Aminex Fermentation Monitoring Column (Bio-Rad Laboratories, Hercules, CA, USA) and Micro-Guard Cation H Refill Cartridges with a Standard Cartridge Holder (Bio-Rad Laboratories). Glucose was detected using a RID 10A refractive index detector (Shimadzu). The column was kept at 60°C using a CTO 20A column oven (Shimadzu). Sulfuric acid solution (5 mM) was used as the mobile phase at a constant flow rate of 0.6 mL/min. Portions (10 μL) were injected into the HPLC system with an SIL-20A auto sampler (Shimadzu).

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Author contributions

A.T. carried out the flask assays, gas chromatography analysis and data analysis, and drafted the manuscript. M.T., T.S., R.E. and M.O. isolated the tested yeast strains and identified them based on the sequence of the D1/D2 region of the LSU rRNA gene. M.K. and S.Y. performed the primary screening and HPLC analysis. E.S. and J.O. assisted with data analysis. J.S. managed the overall project and revised the manuscript.

Additional information

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