Bioresource Technology 153 (2014) 230–235

Contents lists available at ScienceDirect

Bioresource Technology

journal homepage: www.elsevier.com/locate/biortech

# Selection of oleaginous yeasts with high lipid productivity for practical biodiesel production

Ayumi Tanimura [a], Masako Takashima [b], Takashi Sugita [c], Rikiya Endoh [b], Minako Kikukawa [a], Shino Yamaguchi [a], Eiji Sakuradani [d], Jun Ogawa [d], Jun Shima [a,*]

[a] Research Division of Microbial Sciences, Kyoto University, Kitashirakawa Oiwake-Cho, Sakyo-ku, Kyoto 606-8502, Japan
[b] Japan Collection of Microorganisms, RIKEN BioResource Center, Koyadai, Tuskuba, Ibaraki 305-0074, Japan
[c] Department of Microbiology, Meiji Pharmaceutical University, Tokyo 204-8588, Japan
[d] Division of Applied Life Sciences, Graduate School of Agriculture, Kyoto University, Kitashirakawa Oiwake-Cho, Sakyo-ku, Kyoto 606-8502, Japan

## HIGHLIGHTS

- The lipid-producing ability of 500 newly isolated yeast strains was evaluated.
- Lipid content, fatty acid composition, lipid productivity, and biomass were examined.
- The yeast *Cryptococcus musci* was capable of the highest lipid productivity.

## ARTICLE INFO

*Article history:*
Received 24 September 2013
Received in revised form 25 November 2013
Accepted 30 November 2013
Available online 8 December 2013

*Keywords:*
Oleaginous yeast
Fatty acids
Lipid productivity
*Cryptococcus musci*
*Cryptococcus podzolicus*

## ABSTRACT

The lipid-accumulating ability of 500 yeast strains isolated in Japan was evaluated. Primary screening revealed that 31 strains were identified as potential lipid producers, from which 12 strains were cultivated in a medium containing 3% glucose. It was found that JCM 24511 accumulated the highest lipid content, up to 61.53%, while JCM 24512 grew the fastest. They were tentatively identified as *Cryptococcus* sp. and *Cryptococcus musci*, respectively. The maximum lipid concentration of 1.49 g/L was achieved by JCM 24512. Similarly, JCM 24511 also achieved a high lipid production of 1.37 g/L. High lipid productivity is the most important characteristic of oleaginous yeasts from the viewpoint of practical production. Among the strains tested here, JCM 24512 had the best lipid productivity, 0.37 g/L/day. The results show that the isolated yeasts could be promising candidates for biodiesel production.



## 1. Introduction

Biolipids, including triacylglycerol produced by oleaginous yeast, have been confirmed to be one of the most important raw materials for biodiesel production (Meng et al., 2009). The quality of biodiesel depends upon the fatty acid composition of the biolipids (Knothe, 2011). In general, biolipids produced by oleaginous yeast are suitable feedstock for biodiesel, because the fatty acid composition satisfies important criteria i.e., chain length and saturation degree. However, the fatty acid composition of biolipids is strain specific, it is therefore important to select oleaginous yeast strains to ascertain their suitability for biodiesel production.

The other advantage of oleaginous yeast is their ability to produce lipids from non-utilized biomass, including lignocellulosic biomass (Gong et al., 2013; Liang et al., 2012). It is known that many oleaginous yeasts such as *Rhodosporidium toruloides*, *Cryptococcus curvatus*, *Lipomyces starkeyi* and *Yarrowia lipolytica* accumulate lipids to more than 20% of the dry yeast cells (Feofilova et al., 2010; Papanikolaou and Aggelis, 2010; Thiru et al., 2011; Wild et al., 2010; Wu et al., 2011) and that several oleaginous yeast, including *R. toruloides* and *L. starkeyi*, can assimilate xylose (Gong et al., 2012; Oguri et al., 2012; Zhao et al., 2012).

One of the difficulties in the commercialization of biolipid production by oleaginous yeasts may be due to low productivity (Feofilova et al., 2010). In general, the growth of oleaginous yeasts is much slower, compared with that of ascomycetes such as *Saccharomyces cerevisiae*, and biolipid production by oleaginous yeasts requires a prolonged period to produce maximum yields (Ageitos et al., 2011).

To evaluate the lipid-accumulating ability of oleaginous microorganisms, lipid content (% of dry cell weight) is the most commonly used parameter (Meng et al., 2009; Song et al., 2013). There are other derived values, for example, lipid concentration

* Corresponding author. Tel.: +81 75 753 9545; fax: +81 75 753 9544.
*E-mail address:* shimaj@kais.kyoto-u.ac.jp (J. Shima).

0960-8524/$ - see front matter © 2013 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.biortech.2013.11.086

(g-lipid/L), lipid coefficient (lipid yield on glucose consumption; g-lipid/g-glucose), biomass concentration (g-cell/L) and lipid productivity (specific rate of lipid production; g-lipid/L/h or g-lipid/L/day). However, lipid productivity is currently receiving a lot of attention as the selection of rapid-growing and accumulative strains is fundamental to the success of practical biodiesel production (Griffiths and Harrison, 2009; Ordog et al., 2013). Based on this background, the selection of oleaginous yeasts which can rapidly accumulate lipid, that is, high lipid productivity with a suitable fatty acid composition in typical culture media was undertaken.

Recently, Takashima et al. (2012) reported the taxonomic richness of yeasts in Japan within subtropical and cool temperature areas. It can be considered that the yeast strains collected in Japan also exhibit functional diversity, including lipid-producing ability. Therefore, a comprehensive evaluation of the lipid-accumulating ability of yeast strains in the collection was performed.

In this study, it was found that several yeast strains classified as basidiomycete, including *Cryptococcus* sp. and unclassified strains, accumulated biolipids with high productivity, compared with previously reported oleaginous yeasts.

## 2. Methods

### 2.1. Yeast strains

Yeast strains collected and taxonomically identified by Takashima et al. were used as the main screening resource (Takashima et al., 2012). The yeast strains isolated from the campus of Kyoto University (Kyoto, Japan) were also assessed. As control strains, *L. starkeyi* NBRC 10381 and *R. toruloides* NBRC 0559 were obtained from the National Institute of Technology and Evaluation (NITE) Biological Resource Center.

### 2.2. Media

YM agar medium (Difco, Detroit, MI, USA) was used for maintenance of yeast strains. YPD medium (1% yeast extract [Difco], 2% peptone [Difco] and 2% glucose) was used for the liquid cultivation of yeast cells, unless otherwise indicated. Synthetic defined (SD) medium (0.17% yeast nitrogen w/o ammonium sulphate and amino acids [Difco], 0.5% ammonium sulphate and 3% glucose) was used for the primary comprehensive screening of biolipid-accumulating ability. SS2 medium (3% glucose, 0.5% ammonium sulphate, 0.05% magnesium sulphate, 0.01% sodium chloride, 0.01% calcium chloride and 0.01% yeast extract [Difco]) was used for the secondary screening of oleaginous yeasts with high lipid productivity.

### 2.3. 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 using a hydrochloric acid-catalysed direct methylation (Ichihara and Fukubayashi, 2010). In brief, after cultivation, the yeast cells were harvested by centrifugation and lyophilization. The lyophilized cells were dissolved in toluene and methanol, then directly transmethylated with 8% methanolic HCl at 45 °C overnight. The resultant fatty acid methyl esters (FAMEs) were extracted with *n*-hexane and analysed using gas chromatography (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, California, 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, it was then increased at a rate of 5 °C/min to 240 °C, which was held for 3 min. Helium was the carrier gas, which was pumped at 1.0 mL/min, and nitrogen was used as the make-up gas. The injector temperature was 250 °C, the detector temperature was 300 °C, with a split ratio of 50:1. The identification of major peaks was performed based on 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.

### 2.4. Comprehensive evaluation of biolipid-accumulating ability of strains in the yeast collection

Yeast strains were inoculated into 3 mL of YPD medium in test tubes and incubated overnight at 30 °C, with reciprocal shaking at 150 opm (preculture). The preculture was then suspended in 6 mL of SD medium in a test tube, to a cell optical density at 600 nm ($OD_{600}$) of 0.2, then cultured at 30 °C with reciprocal shaking for 3 days at 150 opm. Cells from 3 mL of culture broth were harvested by centrifugation (15,000 rpm for 10 min) and washed twice with distilled water. Intracellular total lipids were determined after lyophilizing the wet cells (primary screening).

### 2.5. Selection of oleaginous yeasts which accumulate intracellular lipids at a high level

The yeast strains screened by the primary screening were cultivated in Erlenmeyer flasks, containing 100 mL of SS2 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, 3, and 4 days of cultivation, by centrifugation (15,000 rpm for 10 min), and washed twice with distilled water. Intracellular total lipids, fatty acid composition and cell mass were determined after lyophilizing the wet cells (secondary screening). All experiments were performed in triplicate.

### 2.6. Calculation of biodiesel properties

Based on the equations of Hoekman et al. (2012), the biodiesel properties, namely viscosity, specific gravity, cloud point, cetane number, iodine number and higher heating value (HHV), were estimated.

Average unsaturation (AU) was calculated from the compositional profiles in Table 2 as

$$\mathrm{AU} = \sum N \times C_i \tag{1}$$

where $N$ is the number of carbon–carbon double bonds of unsaturated fatty acids and $C_i$ is the concentration (mass fraction) of the component.

Each property was calculated using Eqs. (2)–(7) (Hoekman et al., 2012).

$$\text{Viscosity} = -0.6316\mathrm{AU} + 5.2065 \tag{2}$$

$$\text{Specific gravity} = 0.0055\mathrm{AU} + 0.8726 \tag{3}$$

$$\text{Cloud point} = -13.356\mathrm{AU} + 19.994 \tag{4}$$

$$\text{Cetane number} = -6.6684\mathrm{AU} + 62.876 \tag{5}$$

$$\text{Iodine number} = 74.373\mathrm{AU} + 12.71 \tag{6}$$

$$\text{HHV} = 1.7601\mathrm{AU} + 38.534 \tag{7}$$