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Characterization of oleaginous yeasts accumulating high levels of lipid when cultivated in glycerol and their potential for lipid production from biodiesel-derived crude glycerol

Pirapan POLBUREE[a], *Wichien YONGMANITCHAI*[a], *Noppon LERTWATTANASAKUL*[a], *Takao OHASHI*[c], *Kazuhito FUJIYAMA*[c], *Savitree LIMTONG*[a,b,*]

[a]*Department of Microbiology, Faculty of Science, Kasetsart University, Chatuchak, Bangkok 10900, Thailand*
[b]*Center for Advanced Studied in Tropical Natural Resources, National Research University-Kasetsart University, Chatuchak, Bangkok 10900, Thailand*
[c]*International Center for Biotechnology, Osaka University, Suita-shi, Osaka 565-0871, Japan*

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ABSTRACT

This study attempted to identify oleaginous yeasts and selected the strain that accumulated the largest quantity of lipid for lipid production from glycerol. Two-step screening of 387 yeast strains revealed 23 oleaginous strains that accumulated quantities of lipid higher than 20 % of their biomass when cultivated in glycerol. These strains were identified to be four ascomycetous yeast species i.e. *Candida silvae*, *Kodamaea ohmeri*, *Meyerozyma caribbica*, and *Pichia manshurica*, and five basidiomycetous yeast species i.e. *Cryptococcus* cf. *podzolicus*, *Cryptococcus laurentii*, *Rhodosporidium fluviale*, *Rhodotorula taiwanensis*, and *Sporidiobolus ruineniae*. *Rhodosporidium fluviale* DMKU-RK253 accumulated the highest quantity of lipid equal to 65.2 % of its biomass (3.9 g L^{-1} lipid and 6.0 g L^{-1} biomass) by shaking flask cultivation in crude glycerol. The main fatty acids in the accumulated lipid of this strain consisted of oleic acid, linoleic acid, and palmitic acid. Therefore, *R. fluviale* DMKU-RK253 has potential for producing lipid for biodiesel manufacturing using crude glycerol as a feedstock.

Introduction

Concern for the increase in energy demand and the depletion of fossil fuel reserves has resulted in a rapid rise in crude oil prices, and therefore, securing alternative sources of energy is urgently required. One of the most promising renewable energy resources is biodiesel, which is produced from renewable biomasses by transesterification of triacylglycerols, yielding monoalkyl esters of long-chain fatty acids with short-chain alcohols, for example, fatty acid methyl esters and fatty acid ethyl esters (Meng *et al.* 2009). In different parts of the world, various renewable lipids have been chosen for production of biodiesel, including vegetable oils, animal fats, and waste oils (Aggelis *et al.* 1995). However, the use of

* *Corresponding author*. Tel.: +66 2 562 5444x4017; fax: +66 2 579 2081.
E-mail address: fscistl@ku.ac.th (S. Limtong).
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vegetable oils as raw material for biodiesel production would compete with their use as edible oils, thus leading to soaring food prices. Using recovered animal fats and used frying oils as feedstocks can efficiently reduce the price of biodiesel; however, the amount of these waste oils is limited and cannot meet the increasing needs for clean renewable fuels (Zhu *et al.* 2008). Thus, research for alternative sources for biodiesel production is currently an area of lively interest.

Oleaginous yeasts that are able to accumulate cellular lipid in quantities higher than 20 % of their biomass (Ratledge 1991) represent a potential feedstock for biodiesel production due to the fact that the composition of their fatty acids is similar to that of vegetable oils (Liu *et al.* 2011). Although currently, microbial lipids are not promising alternatives for biodiesel production due to their high processing cost, however, searching for high lipid producing microbe, using low cost raw materials and improvement of production process to reduce processing cost may result in reduction of overall production cost. In general, cells of oleaginous yeasts accumulate high quantities of lipid when cultivated in media with excess quantities of carbon such as glycerol, sugar, and polysaccharide but limited quantities of other nutrients, especially nitrogenous ones. In fact, only 3–10 % of the 1600 known yeast species have been reported as oleaginous yeast species (Sitepu *et al.* 2014). These included various species of the phylum Basidiomycota e.g. *Cryptococcus curvatus*, *Cr. laurentii*, *Cr. podzolicus*, *Cr. terricola*, *Rhodosporidium fluviale*, *R. rubra*, *R. toruloides*, *Rhodotorula glutinis*, and *Sporidiobolus ruineniae*, and the phylum Ascomycota e.g. *Candida tropicalis*, *C. utilis*, *Kodamaea ohmeri*, *Lipomyces starkeyi*, *L. lipofer*, and *Yarrowia lipolytica* (Meesters *et al.* 1996; Papanikolaou & Aggelis 2002, 2011; Ratledge & Cohen 2008; Pan *et al.* 2009; Johnson & Echavarri-Erasun 2011; Sitepu *et al.* 2012; Kanti *et al.* 2013; Kitcha & Cheirsilp 2013; Castanha *et al.* 2014; Schulze *et al.* 2014). Many oleaginous yeast species were isolated from plant surfaces (Clément-Mathieu *et al.* 2008) and soil (Kitcha & Cheirsilp 2011; Saenge *et al.* 2011). Yeasts can grow on a variety of substrates, even inexpensive materials such as wastes of agriculture and industry, and thus oleaginous yeast strains that can efficiently produce lipid from low-cost raw materials are of great interest.

Glycerol is generated as a by-product of the biodiesel production process. The glycerol produced at the transesterification stage is crude glycerol that is obtained at high concentration in a weight ratio of 1/10 (glycerol/biodiesel) and that is a component of a mixture of glycerol (65–85 %, w/w), methanol, and soap (Rossi *et al.* 2011). With the ever-growing production of biodiesel in recent years, the annual amount of crude glycerol emerging from this biodiesel production presently amounts to some 1.9 Mton and this will continue to increase (Amaral *et al.* 2009). Therefore, various ways to use glycerol should be developed. Thus, the cultivation of oleaginous yeasts in glycerol-based media is attracting great interest and biodiversity is increasingly being explored to discover oleaginous yeast species that use glycerol for growth and lipid production. Hence, we carried out this study to gain a greater understanding about the diversity of oleaginous yeasts that accumulate high quantities of lipid when cultivated in glycerol and to find strains that accumulate high quantities of lipid when crude glycerol is used as the sole carbon source.

Materials and methods

Yeast isolation

Yeasts were isolated from soil and other materials, e.g. palm oil fruits and the female palm flowers collected from various habitats, by an enrichment technique using two enrichment media i.e. nitrogen-free medium (0.117 g L^{-1} Difco-yeast carbon base, 0.1 g L^{-1} sodium propionate, 0.2 g L^{-1} chloramphenicol and pH 3.3) and nitrogen-limited medium I, both containing 30 g L^{-1} pure glycerol as a sole source of carbon (30 g L^{-1} pure glycerol, 1.5 g L^{-1} yeast extract, 0.5 g L^{-1} NH_4Cl, 7.0 g L^{-1} KH_2PO_4, 5.0 g L^{-1} $Na_2HPO_4 \cdot 12H_2O$, 1.5 g L^{-1} $MgSO_4 \cdot 7H_2O$, 0.08 g L^{-1} $FeCl_3 \cdot 6H_2O$, 0.01 g L^{-1} $ZnSO_4 \cdot 7H_2O$, 0.1 g L^{-1} $CaCl_2 \cdot 2H_2O$, 0.1 mg L^{-1} $MnSO_4 \cdot 7H_2O$, 0.1 mg L^{-1} $CuSO_4 \cdot 5H_2O$, and pH 5.5) (Kraisintu *et al.* 2010) supplemented with 0.1 g L^{-1} sodium propionate and 0.2 g L^{-1} chloramphenicol. Fifty millilitres of either nitrogen-free medium or nitrogen-limited medium I in a 250 ml Erlenmeyer flask was incubated with 5 g of soil or the other materials by shaking at 150 rpm 25 °C for 3 d. The enrichment culture was spread on a solid medium of the same composition as the enrichment medium in a Petri dish and incubated at 25 °C until yeast colonies appeared. Yeast colonies of different morphologies were picked and purified by cross streaking on YM agar. Purified yeast cultures were maintained on YM agar at 8 °C.

In addition to the newly isolated strains, 64 yeast strains from the private culture collection were used in this study (Table 1). These strains were isolated from the surface of plant leaves by an enrichment technique using yeast extract-malt extract (YM) broth (10 g L^{-1} glucose, 5 g L^{-1} peptone, 3 g L^{-1} yeast extract, 3 g L^{-1} malt extract) supplemented with 0.25 g L^{-1} sodium propionate and 0.2 g L^{-1} chloramphenicol (Limtong & Koowadjanakul 2012; Limtong *et al.* 2014).

Cryptococcus curvatus CBS 570 and *Rhodosporidium toruloides* CBS 14, which were reported to be oleaginous yeast strains by Meesters & Eggink (1996) and Evans & Ratledge (1984), respectively, were obtained from the Centraalbureau voor Schimmelcultures (CBS), Utrecht, the Netherlands and used as the reference strains.

Screening for oleaginous yeasts

To obtain oleaginous yeast strains which accumulate high quantities of lipid when cultivated in pure glycerol, a two-step evaluation of lipid accumulation was performed. In the first step, yeast was cultivated in a test tube holding 5 ml nitrogen-limited medium I containing 30 g L^{-1} pure glycerol and incubated on a rotary shaker (Lab Companion IS-971R, Korea) at 300 rpm and room temperature (28 ± 3 °C) for 72 h. Cellular lipid bodies were qualitatively analyzed by staining cells with Nile red following the method of Kimura *et al.* (2004). The culture broth (15-μl) was mixed with 5 μl of Nile red solution (10 μg Nile red in 1 L absolute ethanol). Yeast cells were observed with a fluorescence microscope (Olympus BX51, Japan) using a 450–490 nm excitation filter, a 505-nm diachronic mirror ×40 objective lens, under which the Nile red-stained lipid bodies showed a yellow-gold emission. The