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Enhancement of extracellular lipid production by oleaginous yeast through preculture and sequencing batch culture strategy with acetic acid

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ABSTRACT

Oleaginous yeast *Cryptococcus curvatus* MUCL 29819, an acid-tolerant lipid producer, was tested to spill lipids extracellularly using different concentrations of acetic acid as carbon source. Extracellular lipids were released when the yeast was cultured with acetic acid exceeding 20 g/L. The highest production of lipid (5.01 g/L) was obtained when the yeast was cultured with 40 g/L acetic acid. When the yeast was cultivated with moderate concentration (20 g/L) of acetic acid, lipid production was further increased by 49.6% through preculture with 40 g/L acetic acid as stimulant. When applying high concentration (40 g/L) of acetic acid as carbon source in sequencing batch cultivation, extracellular lipids accounted up to 50.5% in the last cycle and the extracellular lipids reached 5.43 g/L through the whole process. This study provides an effective strategy to enhance extracellular lipid production and facilitate the recovery of microbial lipids.

1. Introduction

With the emerging of worldwide energy crisis, biodiesel has become a popular alternative to fossil fuel due to its sustainability and renewability (Sajid et al., 2016). Microbial lipids produced by different microorganisms are promising feedstock for biodiesel production. Microbes, such as oleaginous yeasts, can accumulate high amounts of intracellular lipids by utilizing organic carbon in a short duration, regardless of seasonal variation (Li et al., 2008). However, the high cost of biodiesel production is the main obstacle for its broader commercialization. Those costs mainly stem from the fermentation processes, where expensive fermentation substrates like edible oils are usually used (Huang et al., 2013). Besides, for the extraction of lipids, the conventional oil extraction processes that involve mechanical cell rupture, extraction and phase separation require large amounts of energy, which is also highly cost-dependent (Martin, 2016).

Recently, many studies have focused on seeking for low-cost fermentation substrates for synthesis of microbial lipids to make biodiesel production competitive in the energy market. By-products from food manufacturing industries can be used as cheap fermentation substrates including sugar-contained substrates and lignocellulosic biomass (Chatzifragkou et al., 2010; Wu et al., 2010; Yu et al., 2011). However, transportation and continuous supply of these raw materials should be considered (Huang et al., 2013). Recently, volatile fatty acids (VFAs) generated from anaerobic fermentation of various organic wastes, turn out to be effective carbon source for lipid production in certain culture conditions (Liu et al., 2016, 2017a). Scholars have explored different culture modes, including two-stage cultivation (Christophe et al., 2012; Fontanille et al., 2012), sequencing batch cultivation (Liu et al., 2016) to enhance the production of microbial lipids from VFAs. Among all VFAs derived from wastes, acetic acid is generally the major component, accounting for 43%–69% (Yuan et al., 2011), followed by propionic and butyric acids. Acetic acid is a superior candidate for lipid accumulation over other acids because of its relatively shorter conversion pathway to acetyl-CoA, an important precursor in lipid biosynthesis (Zheng et al., 2012). Huang et al. (2016) found that the oleaginous yeast *Rhodosporidium toruloides* AS 2.1389 accumulated higher content of lipid when acetic acid was used as sole carbon source compared with glucose under the same culture conditions. Gong et. al. (2015) also found that the yeast *Cryptococcus curvatus* accumulated the highest lipid content up to 73.4% using acetate as the carbon source. However, recent study has shown that high concentration of acetic acid exhibited adverse effect on cell growth of the yeast, sharply decreasing the utilization ratio of acetic acid and cell mass (Li et al., 2015). Liu et al. (2017b) also reported *Cryptococcus curvatus* ATCC 20509 could only use 22.8% of the 40 g/L acetic acid (pH 7) as sole carbon source. The adverse effect of high concentration of acetate was resulted from the low external pH of the media which was close to the pKa (4.76). Under this condition, the lipophilic undissociated form of acetic acid predominates, permeates into the plasma membrane, dissociates

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intracellularly, and leads to cytosolic acidification, which may impose stress on cell metabolism (Mira et al., 2010). To alleviate the adverse effect of the acid on cell growth and increase lipid production, some strategies such as pH regulation (Beligon et al., 2015) and increase in inoculation concentration were explored (Guo and Olsson, 2016).

Another barrier for the commercialization of biodiesel production is the high energy consumption of downstream processes for oil extraction. To date, organic solvent extraction coupled with mechanical disruption remains the most efficient and economical technology for oil extraction from microorganisms (Armenta and Valentine, 2013). Solvent extraction for intracellular lipids involves cell disruption, solvent extraction, and transesterification, which occupy more than 40% of the total biodiesel production cost due to energy-intensive steps (Ledesma-Amaro et al., 2016). Thus, energy balance should be achieved through a correctly designed extraction process (Martin, 2016). Among the extraction steps, cell rupture is energy-consuming but vital to remove physical barriers and facilitate the contact of intracellular TAG (Tricarboxylic acid glyceride) with the solvent. Several pretreatment methods, such as homogenization, bead milling, and microwave, have been performed to increase the recovery yields of lipids (Meullemiestre et al., 2016). What's more, several chemical surfactants, such as Triton-100, sodium dodecyl sulfate (SDS), and certain biodegradable anionic detergents, have been used to disrupt the cell wall, instead of performing mechanical disruption that requires high energy input (Yellapu et al., 2016). In addition, fatty acid secretion by genetically modified strains is a promising strategy to reduce the extraction cost and enhance the lipid production. For example, the engineered strains of *Yarrowia lipolytica* can secrete lipids to the culture broth with glucose as carbon source and the lipid content reached 120% of the dry cell weight (DCW) (Ledesma-Amaro et al., 2016). Liu et al. (2011) also genetically modified cyanobacteria to secrete fatty acids for lipid production. The yield of fatty acids secreted increased to 197 mg/L when the yeast was cultured at a cell density of 1.0×10^9 cells/mL by adding codon-optimized thioesterase genes.

In this study, *Cryptococcus curvatus* MUCL 29819, an acid-tolerant oleaginous strain, exhibited excellent ability to accumulate lipids when cultured with high concentration of acetic acid. The integrity of the cell membrane during cultivation was assessed by flow cytometry. The spilled extracellular lipids were analyzed and the explanation for spilling was explored. Two strategies were applied to increase the proportion of extracellular lipids produced by the yeast cultured with moderate and high concentrations of acetic acid. These strategies are possible routes for decreasing the cost of lipid recovery.

2. Materials and methods

2.1. Strain and media

C. curvatus MUCL 29819 was obtained from the Biological Resource Center, National Institute of Technology and Evaluation (Tokyo, Japan). The yeast was maintained at 4 °C on YM agar slants and activated at 30 °C on YM agar plates for 5 days prior to cultivation in liquid media. A loop full of cells was inoculated into 50 mL of YPD medium in 250 mL flasks and incubated for 36 h. The YPD medium contained 20 g/L glucose, 10 g/L fish peptone, and 10 g/L yeast extract, and the initial pH was adjusted to 6.0. The fermentation medium contained 1.5 g/L of $MgSO_4{\cdot}7H_2O$, 0.4 g/L of KH_2PO_4, and different concentrations of acetic acid or glucose. The yeast extract was added with $(NH_4)_2SO_4$ to obtain a ratio of 7.5:1 (w:w) due to the different concentrations of the carbon source for maintaining the set C/N ratio in the experiment. The nitrogen source in the yeast extract (OXOID, LP0021) accounted for 11% of the total weight. Both the YPD and fermentation media were autoclaved at 115 °C for 30 min before use. All cultivations were performed in a gyratory shaker under 200 rpm and 30 °C.

2.2. Feeding strategies

2.2.1. Batch cultivation

Briefly, 5 mL of the seed culture was inoculated into 45 mL of fermentation medium in 250 mL flasks. The seed cells were collected via centrifugation (3600 × *g* at 4 °C for 5 min), washed once with normal saline for preventing the effects of nutrients on the YPD media, and resuspended in sterile distilled water to generate cell suspension solution. The fermentation medium was incubated under the same conditions as seed cultivation. The initial cell concentration of the fermentation culture medium was controlled at approximately 0.8–1.2 (optical density at 600 nm, OD_{600}) unless otherwise stated. *C. curvatus* MUCL 29819 was cultivated in the fermentation media containing different concentrations of acetic acid (5, 10, 20, 30, and 40 g/L) or glucose (40 g/L) as carbon source. The initial pH was 7.0.

2.2.2. Preculture cultivation strategy

The YPD medium of *C. curvatus* MUCL 29819 was replaced by nitrogen-rich medium (C/N = 10) containing different concentrations of acetic acid (15, 30, and 40 g/L) and cultured for 30 h to determine the effect of short acid shock cultivation on lipid production. Subsequently, 5 mL of the culture was inoculated into 45 mL of the fermentation medium in 250 mL flasks. Seed cells were collected via centrifugation (3600 × *g* at 4 °C for 5 min), washed once with normal saline, and resuspended in sterile distilled water to generate cell suspension and avoid the effects of nutrients in the precultured media. The fermentation medium was nitrogen-limited medium (C/N = 100) containing 20 g/L acetic acid to produce excess microbial oils. The initial pH was 7.0.

2.2.3. Sequencing batch cultivation strategy

C. curvatus MUCL 29819 was cultivated in 50 mL of fermentation medium in 250 mL flasks. During cultivation, OD_{600} and acetic acid concentration were monitored. When the yeast reached the stationary growth phase, the medium was replaced with fresh medium for the next growth cycle. Four growth cycles were run in this study. The carbon source in each cycle was 40 g/L acetic acid, with a C/N ratio of 100. The initial pH was 7.0.

2.3. Analytical methods

2.3.1. Determination of cell mass

During the growth period, cell mass concentration was determined using OD_{600} to describe the growth curve. At the end of the cultivation, the samples were centrifuged (3600 × *g*, 4 °C) for 5 min. The pellets were rinsed using a normal saline solution and dried by vacuum lyophilization at −50 °C for 24 h. The amount of cell mass accumulated was determined by weighing.

2.3.2. Determination of lipid and fatty acid methyl ester (FAME)

Dried yeast cells were weighed, suspended in 1 mL of methanol containing 0.5 mm-diameter glass beads, and disrupted by the MonoLyser™ lysing system (RotaPrep Inc., Tustin, CA, USA). For lipids released outside the cells, 30 mL of the well-mixed supernatant was obtained. After their respective pretreatments, lipids in dried or liquid samples were extracted twice with chloroform/methanol (1:1, v/v). The extracts were washed with the same volume of 0.1% NaCl solution and dried by nitrogen blowing at 50 °C (Gong et al., 2012). The lipid content is defined by two different equations as follows due to the existence of extracellular lipids:

$$\text{Cell mass (g/L)} = \text{Intracellular lipid (g/L)} + \text{Nonlipid (g/L)} \quad (1)$$

$$\text{Intracellular lipid content (\%)} = \frac{\text{Intracellular lipid (g/L)}}{\text{Cell mass (g/L)}} \quad (2)$$