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Cultivation and lipid production of yeast *Cryptococcus curvatus* using pretreated waste active sludge supernatant

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ABSTRACT

Oleaginous yeast *Cryptococcus curvatus* has become some of the most promising feedstock for biodiesel production due to their high production efficiency. However, high cost of cultivation, especially substrate cost, hinders rapid commercialization of yeast-based biodiesel. In this study, waste activated sludge (WAS), which is rich in nutrients and organic matters, was examined as an economic substitute for organic substances. To be efficiently bioavailable, WAS must be pretreated. Hydrodynamic cavitation reaction time and pH were identified as experimental factors, whereas growth rate was selected as response parameter. The experimental factors were optimized employing Response Surface Methodology (RSM). Growth rate of 1.13/h was optimized at reaction time 12.5 min and pH 8.65. When sludge pretreated under these optimal conditions was used as a substitute to yeast extract, 9.84 g/L of biomass was obtained in a day and lipid was accumulated up to 23% of dry weight.

1. Introduction

Biodiesel has drawn increasing attention as a renewable, biodegradable and environment friendly fuel. However, sources used for the production of commercial biodiesel are mainly food based such as soybean oil, rapeseed oil, palm oil, and corn oil. These food-based biofuels have raised intense public concerns. For example, if grain needed to fill a 25 gallon oil tank of sport utility vehicle was instead consumed for food, it would provide one person with enough calories for a year (Courtney, 2008).

Oil originated from microbes is considered as an ideal alternative for biodiesel production due to its high productivity (Chisti, 2007; Miao and Wu, 2006; Xu et al., 2006; Li et al., 2007). Ethical controversies are not of an issue here, since microbes are not normally used for food. These oil-rich microbes, collectively called oleaginous microbes, include microalgae, yeasts, fungi, and even bacteria. Among these microbes, oleaginous yeast *Cryptococcus curvatus* has drawn noticeable attention, as it accumulates oil up to 60% of cell dry weight (Ratledge, 1991). The composition of fatty acid in this microbe is quite similar to that of plant seed oils such as palm oil (Davies, 1988).

Oleaginous yeast utilizes organic carbon substances for their growth, unlike photoautotrophic microalgae. However, the cost of substrates accounts for up to 50% of the biodiesel production (Li et al., 2007). Even with this cons, oleaginous yeast presents distinct pros such as easy maintenance of growth conditions, incomparably high productivity and increased final cell density (Chen, 1999; Yee and Blanch, 1993; Shay et al., 1987; Chang et al., 1993). Therefore, if the cost of substrates can be reduced to economically sufficient level, the oleaginous yeast would become a competitive route for the biofuel production.

Waste activated sludge (WAS) is a promising feedstock due to high content of organic carbon and nutrients. However, WAS is limited because of its low biodegradability, and hence limited bioavailability. Nutrients in WAS are insoluble and are trapped in suspended solids such as aggregates of organic matter, extracellular polymers, cells and cellular debris or absorbed on their surfaces (Pham et al., 2007). The proper disintegration of WAS is required before its use as a substrate for the yeast growth.

Alkali treatment has been known to be relatively effective in sludge solubilization, in the efficiency order of NaOH > KOH > $Mg(OH)_2$ and $Ca(OH)_2$ (Kim et al., 2003). Physical disintegration with ultrasonic waves can synergistically boost up the efficiency of the alkaline pretreatment. However, issues of high energy consumption and scaling-up make impractical for the purpose of commercialization (Chakinala et al., 2008). Hydrodynamic cavitation (HC), which employs similar working principles to the sonication, is an excellent alternative, as it is much more energy-efficient and can be easily scaled-up (Wang et al., 2009; Braeutigamm et al., 2009). HC can simply generated by a constriction using an orifice plate. Fluid passing through an orifice will experience a drop in pressure. If a pressure falls below the threshold level for cavitation, micro bubbles are generated. Subsequently, as a liquid jet expands, pressure recovers and bubbles are collapsed. At the

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point of collapse, temperature within micro bubble may be several thousand kelvin and pressure is several atmospheres.

This paper focusses on finding the optimal condition for the mass cultivation of *C. curvatus* using WAS as a cheap substrate. To this end, sludge pretreatment was conducted, taking advantage of HC as a physical means and NaOH as a chemical catalyst. Lipids in *C. curvatus* can then be used for biodiesel production.

2. Methods

2.1. Yeast strain, media, and cultivation

C. curvatus was purchased from the Korean Collection for Type Culture (KCTC) with depository number KCTC 17162. YM Agar Medium (KCTC) was used for pre-culture and solid culture, and it contained the following ingredients in 1 L of deionized water: 3 g of yeast extract, 3 g of malt extract, 5 g of peptone, 10 g of dextrose, in 20 g/L of agar. For the cell growth, Cryptococcus Growth Medium (CGM) was mainly used, containing 40 g of glucose, 0.5 g of NH_4Cl, 2.7 g of KH_2PO_4, 0.95 g of Na_2HPO_4, 1 g of $MgSO_4{\cdot}7H_2O$, trace elements solution (DSMZ medium 320) 10 mL in 1 L of deionized water. To confirm its known effect on boosting-up the microbial growth, yeast extract was supplemented at different levels: 0, 0.1, 0.3, 0.5, 0.7, 1.0, and 1.2 g. Both YM and CGM media were autoclaved. CGM media was cultivated in a shaking incubator (200 rpm).

2.2. Sludge pretreatment

Sewage samples were obtained from the Sewage Treatment Plant in Daejeon, Republic of Korea. The properties of the activated sludge, whose approximate concentration was 1% (w/v), are listed in Table 1.

Hydrodynamic cavitation (HC) system was employed to mechanically disrupt cell structure and any extra materials limiting bioavailability. A reactor for HC reactor was made of stainless steel as Fig. 1. The reactor was connected to a water pump (TPH2TK6KS; Walrus Pump (Taiwan) Ltd.). The maximum capacity of the system was 1.5 L. The solution pH was adjusted between 8–10 using sodium hydroxide (5 M). After the HC-treated sludge was centrifuged at 12000 rpm for 10 min, the supernatant was neutralized to pH 5.5, filtered with 0.45 μm PES filter (Whatman), and thereafter used.

2.3. Sludge as substrates

Pretreated activated sludge supernatant was used for the growth of *C. curvatus*. Two different experiments were performed to see how the pretreated sludge affected the growth, i.e., either as a substitute for yeast extract or as a nearly complete nutrient source (i.e., carbon source). As a yeast extract substitute, the sludge supernatant was mixed with CGM which contains glucose but not yeast extract (1:4, v/v). As the complete nutrient source, the sludge supernatant was mixed with CGM without glucose and yeast extract (1:1, v/v). Cultivation was done at 200 rpm and 30 °C.

Table 1
Compositional change in activated sludge before and after being pretreated at pH 8.65 for 12.5 min using hydrodynamic cavitation.

	Values (mg/L)	
	Before pretreatment	After pretreatment
T-COD	10110	2143
S-COD	15	2143
T-Protein	93	21
S-Protein	11	21
T-N	650	135
S-N	5	135
T-P	287	61
S-P	6	61

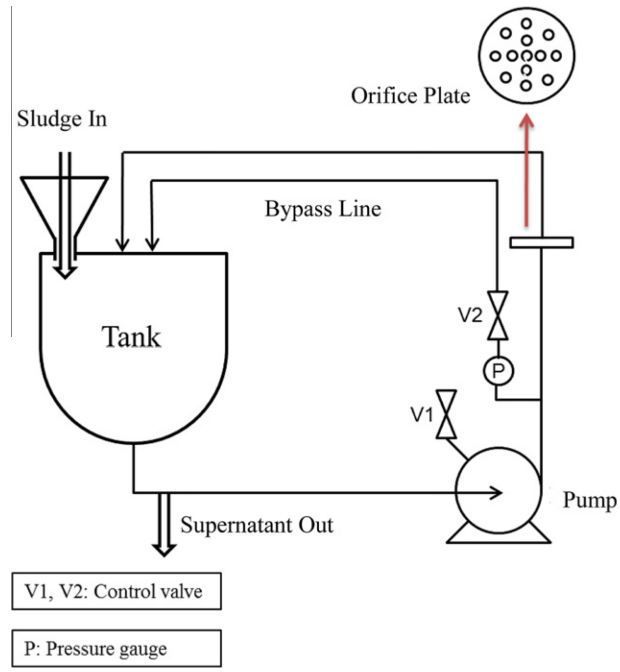

Fig. 1. Schematic diagram of hydrodynamic cavitation system.

2.4. Optimization of sludge disintegration

Response surface methodology (RSM) was employed to obtain the optimal pretreatment conditions that exhibited the shortest growth rate (Y). Two factors were used: pH (A), and hydrodynamic cavitation operating (HC) time (B). The pH level was varied between 8 and 10, whereas HC time was varied between 5 and 20 min. Total 13 experimental runs including five replicates of a center point were executed. Data analysis was carried out via Design Expert.

2.5. Measurement of disintegration degree of sludge and microbial growth rate

The pretreated sludge supernatant was analyzed for the composition of soluble substances such as chemical oxygen demand (COD), nitrogen (N), phosphorus (P), and protein. Protein concentration was measured according to Lowry et al. (1951). Concentrations of N, P, and COD were measured using a Hach DR 5000 UV–Vis Laboratory Spectrophotometer (Hach, USA).

The growth of yeast was monitored by measuring absorbance at 600 nm. A calibration curve was plotted with the absorbance versus cell dry density. Dry cell was obtained using the following process: (1) five milliliters of culture was centrifuged at 4000 rpm for 10 min. (2) The cells were washed twice with 5 mL of deionized water. (3) And then dried to constant weight at 60–80 °C.

2.6. Lipid extraction and analysis

Lipid contents and compositions were analyzed according to the procedure described by Liang et al. (2010) with slight modification. The cells were harvested by centrifugation (12000 rpm, 10 min), washed twice with deionized water, oven-dried at 50 °C for 1 day, and then disrupted in 15 mL falcon tubes with a bead-beater (BioSpec Products). Bead-beating was conducted for 2 min with 1 mm glass beads to approximately 5 mL and 2 mL of methanol. The entire content was then transferred to a 50 mL centrifuge tube and chloroform added to make a 2:1 (v/v) chloroform/methanol ratio. The tube was vortexed for 5 min and was allowed to stand for