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The effect of volatile fatty acids as a sole carbon source on lipid accumulation by *Cryptococcus albidus* for biodiesel production

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ABSTRACT

The use of volatile fatty acids (VFAs) for microbial lipid accumulation was investigated in flask cultures of *Cryptococcus albidus*. The optimum culture temperature and pH were 25 °C and pH 6.0, respectively, and the highest lipid content (27.8%) was obtained with ammonia chloride as a nitrogen source. The lipid yield coefficient on VFAs was 0.167 g/g of *C. albidus* with a VFAs (acetic, propionic, butyric acids) ratio of 8:1:1, which was in good agreement with a theoretically predicted lipid yield coefficient of the VFAs as a carbon source. The major fatty acids of the lipids accumulated by *C. albidus* were similar to those of soybean oil and jatropha oil. A preliminary cost analysis shows that VFAs-based biodiesel production is competitive with current palm and soybean based biodiesels. Further process development for lower aeration cost and higher lipid yield will make this process more economical.

1. Introduction

The current crisis of global warming is primarily attributed to CO_2 production from excessive use of fossil fuels during recent decades, and has increased demand for renewable biofuels tremendously. Lipids are drawing considerable attention in relation to the production potential of biodiesel on the basis of their non-toxic, sustainable, and energy efficient proprieties (Ratledge et al., 2008). Currently, edible and industrial lipids are obtained mostly from agricultural commodities. However, rising global population and concomitant competition with foodstuffs necessitates the identification of alternative lipid sources. Much attention has thus been focused on non-edible lipid sources such as jatropha and microorganisms. In particular oleaginous microorganisms carrying microbial lipid content in excess of 20% (Ratledge, 1991) have been considered as alternatives to agricultural commodities for the production of lipids and fats.

A number of studies on oleaginous microorganisms have been reported (Hansson et al., 1986; Morita et al., 2000; Papanikolaou et al., 2007; Liang et al., 2010). The amount of lipids produced and their composition vary depending on the strains, culture conditions, and carbon sources (Easterling et al., 2009). Oleaginous species have the capacity for continual intake of carbon sources from a medium, converting the carbon source into lipid storage materials. The microbial lipids accumulated in oleaginous cells can be easily converted to biodiesel through a transesterification process. The major fatty acids in the lipids produced by oleaginous microorganisms are myristic acid (C14:0), palmitic acid (C16:0), stearic acid (C18:0), oleic acid (C18:1), linoleic acid (C18:2), and linolenic acid (C18:3), which are the major compounds of biodiesel.

To date, most studies on lipid production on oleaginous microorganisms (microalgae, yeast, bacteria, etc.) have been carried out with glucose as the sole carbon source (Li et al., 2007; Papanikolaou et al., 2010; Steen et al., 2010). However, the high cost of biodiesel from oleaginous microorganisms mainly stems from the high cost of glucose, which is estimated to be about 80% of the total medium cost. Therefore, considerable efforts have been directed toward minimizing the carbon source cost and finding new alternative carbon sources, including starch and ethanol (Hansson et al., 1986), pectin and lactose (Papanikolaou et al., 2007), wastes (Xue et al., 2008; Fakas et al., 2008), and glycerol (Easterling et al., 2009; Fakas et al., 2009; Makri et al., 2010). However, the lipid yield coefficient on these carbon sources was too low to effectively reduce lipid production cost.

Oleaginous cells can directly convert some organic acids into acetyl-CoA, a central intermediate in lipid synthesis, by acetylcoenzyme A synthetase; this acetyl-CoA is then used for biosynthesis of polyunsaturated fatty acids and lipid accumulation in oleaginous yeast cells (Ratledge, 2004). Some pure or single organic acids were

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used for the production of polyunsaturated fatty acids and lipids (du Preez et al., 1995; Rodrigues and Pais, 2000; Yahara et al., 2007). du Preez et al., 1996 used a mixture of volatile fatty acids (VFAs) as a sole carbon source for gamma-linolenic acid production and obtained 0.3 g biomass/g VFAs and a lipid yield of less than 9% of the VFAs mixture. VFAs, which can be produced from foodwastes, sludge, and a variety of biodegradable organic wastes via a VFAs platform (Lim et al., 2008a, Chang et al., 2010), are promising alternative carbon sources for lipid accumulation by oleaginous microorganisms. With the aim of producing biodiesel more economically and effectively, in the present study VFAs are used for the first time as a sole carbon source for lipid accumulation by *Cryptococcus albidus*, which can accumulate high amounts of lipid, up to 65% of dry cell weight (Ratledge, 1991) and use various carbon sources for cell growth and lipid accumulation (Hansson et al., 1986).

The optimum culture conditions (temperature, initial pH, and nitrogen source) were investigated for lipid accumulation with VFAs. Several nitrogen sources were compared in order to obtain a higher lipid yield coefficient. Various VFAs ratios and concentrations were used to study their effects on lipid accumulation by *C. albidus*. Furthermore, the compositions of lipids produced by *C. albidus* with VFAs were analyzed and compared with the case of using glucose as a carbon source. A preliminary cost analysis was performed to evaluate whether VFAs-based biodiesel production would be feasible in comparison with traditional biodiesels. The overall aim of this study is to investigate the influence of culture conditions on microbial lipid production with VFAs as a carbon source, and to determine whether VFAs are a suitable carbon source for lipid and biodiesel production.

2. Methods

2.1. Microorganism and medium

C. albidus var. albidus (ATCC 10672) was obtained from the Korea Biological Resource Center (Republic of Korea). The strain was maintained by a monthly subculture on YM slants containing 2% agar. Flask culture experiments were performed using a minimal medium (pH 6.0) containing the following per liter of distilled water: NH_4Cl 1 g, KH_2PO_4 3 g, $MgSO_4 7H_2O$ 1 g, $FeCl_3 \cdot 6H_2O$ 15 mg, and $ZnSO_4 \cdot 7H_2O$ 7.5 mg. The seed culture contained 20 g/L glucose as a carbon source. VFAs were used as a sole carbon source in all experiments, and the concentration and ratio of VFAs were varied in different experiments. The pH of the medium was adjusted using 2 mol/L HCl and NaOH solutions.

2.2. Culture conditions

Flash culture experiments were performed in 250 ml Erlenmeyer flasks containing a 50 ml medium. All cultures were incubated in a rotary shaker at 150 rpm. The cultures were inoculated with a 10% (v/v) inoculum. The inoculum was grown under 22 °C and pH 6.0 at 200 rpm, and then used for cultures at 20, 25, 30, and 35 °C and pH ranging from 3.0 to 8.0 through careful adjustment with sterilized 1.0 mol/L HCl and NaOH. The optimum temperature and initial pH were identified for the inoculum and the cultures and employed in subsequent experiments. In all experiments, the ratio of VFAs (acetic acid: propionic acid: butyric acid) was 6:1:3, unless otherwise specified.

Ammonia sulfate, ammonia chloride, potassium nitrate, sodium nitrate, glycine, and urea were, respectively, used as a nitrogen source with an initial concentration of 0.02 mol N/L. Organic nitrogen sources (urea and glycine) were sterilized by membrane filtration (0.2 μm). NH_4^+-ions were typically exhausted after 20 h of cultivation. The cells were harvested in the stationary phase.

The influence of VFAs on lipid accumulation was studied by comparing their initial ratio and concentration. Four different VFAs ratios were tested for each volatile fatty acid effect on lipid accumulation and the initial concentration of VFAs was 2 g/L. The VFAs ratio was based upon the products produced from foodwastes by the VFAs platform (Chang et al., 2010). The effects of VFAs concentration were evaluated using concentrations of 2, 5, 8, 10 g/L with a ratio of 6:1:3.

2.3. Analyses

20 ml of sample was transferred to a pre-weighed centrifuge tube and centrifuged at 8000 rpm for 30 min. After rinsing the pellet twice with 0.9% NaCl, it was dried overnight under vacuum at 105 °C until no further decrease in weight was observed.

Culture broth samples were prepared for glucose and VFAs analyses. The culture broths were centrifuged, and the supernatants were filtered through a 0.2 μm pore-size, 13 mm diameter filter (Whatman and Pall). The concentrations of glucose and VFAs were determined by high performance liquid chromatography (Li et al., 2009).

Lipid extraction was performed according to a procedure reported by Xue et al. (2008) and subsequently by the "Folch wash" procedures (Fakas et al., 2008). The purified chloroform layer was carefully withdrawn and transferred to a glass vial and evaporated to dryness under a stream of N2 in order to avoid oxidation of unsaturated fatty acids. The residue was immediately weighed to give the total lipid content. The total lipids were transesterified using sodium methoxide (Sarina et al., 2009) and the fatty acid components were analyzed by a HP 5890 SERIES II plus a Gas Chromatograph coupled to a HP 5973 Mass Spectrometer Detector (Hewlett–Packard, Palo Alto, CA, USA). The MS scanned a range of m/z of 50–550 using the SCAN mode. The column used to separate each compound was an equity-1 (Supelco), with dimensions of 30 m (length) × 0.25 cm (inner diameter) × 0.1 μm (thickness). A flame ionization detector (FID) operated at 280 °C was also employed and the sample entrance was 45 cm/s. Nitrogen was used as a carrier gas. The initial temperature was 120 °C for 5 min. The temperature was then raised at 3 °C/min to 180 °C, where it was maintained for 2 min. The temperature was then increased at 10 °C/min to 220 °C, where it was then sustained for 30 min. HP 5972 MS and data processing software (HP G1034C Chemstation Software) were used for measuring and analyzing the data.

2.4. Statistical analysis

To evaluate the influence of culture conditions, nitrogen sources, and VFAs on the lipid accumulation by *C. albidus* in flask cultures, an ANOVA analysis was performed and the means of the significantly different main effects were compared at $p < 0.05$.

3. Results and discussion

3.1. The effect of culture temperature and initial pH on lipid accumulation with VFAs as a carbon source

The effect of temperature on lipid accumulation with VFAs as a carbon source was investigated at 20, 25, 30, and 35 °C, respectively. It was found that *C. albidus* grew well and a large amount of lipids was accumulated in a temperature range of 20–30 °C (Fig. 1). In contrast, lipids were not accumulated at 35 °C and cell growth lasted up to 48 h at this temperature. The maximum lipid concentrations were obtained at a temperature of 25 °C, and