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Growth of oleaginous *Rhodotorula glutinis* in an internal-loop airlift bioreactor by using lignocellulosic biomass hydrolysate as the carbon source

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The conversion of abundant lignocellulosic biomass (LCB) to valuable compounds has become a very attractive idea recently. This study successfully used LCB (rice straw) hydrolysate as a carbon source for the cultivation of oleaginous yeast-*Rhodotorula glutinis* in an airlift bioreactor. The lipid content of 34.3 ± 0.6% was obtained in an airlift batch with 60 g reducing sugars/L of LCB hydrolysate at a 2 vvm aeration rate. While using LCB hydrolysate as the carbon source, oleic acid (C18:1) and linoleic acid (C18:2) were the predominant fatty acids of the microbial lipids. Using LCB hydrolysate in the airlift bioreactor at 2 vvm achieved the highest cell mass growth as compared to the agitation tank. Despite the low lipid content of the batch using LCB hydrolysate, this low cost feedstock has the potential of being adopted for the production of β-carotene instead of lipid accumulation in the airlift bioreactor for the cultivation of *R. glutinis*.

[**Key words:** Hydrolysate; *Rhodotorula*; Oleaginous; Rice straw; Airlift bioreactor]

Various renewable lipids have been explored for the production of biodiesel to replace fossil-fuel derived diesel, including vegetable oils, animal fats, and kitchen waste oils. However, the major obstacle to commercialization of biodiesel production is its high price compared to the low market price of conventional petroleum-based diesel. It has been estimated that over 70% of the market price of biodiesel lies in the feedstock cost (1). Consequently, much effort has been made toward finding cheap and renewable oil sources for biodiesel production. To this end, the conversion of abundant lignocellulosic biomass (LCB) into biofuels for use as transportation fuels has been presented as a viable option for improving energy security and reducing greenhouse gas emissions (2).

LCB is the world's most abundant and attractive biomass resource that can be used as a raw material for the economic production of microbial oils. The total worldwide production of cellulose and hemicellulose is about 85×10^9 tons/annum, with cereal straw estimated to exceed 2.9×10^9 tons/annum (3). Although the bioconversion of lignocellulosic residues into ethanol or butanol has been performed successfully, it has been both technically and economically challenging to produce biodiesel from LCB, and the technology has yet to progress from the laboratory stage to the demonstration stage. The major LCB resources come from residues generated by agricultural, forestry, and industrial sources. The difficulty of converting lignocellulosic material to useful biofuel products at economic competitive cost is the major obstacle to widespread utilization of this important resource (4). Hence, there is considerable economic interest in the development of processes that can pretreat and convert inexpensive cellulosic wastes into valuable products to be used as fuel. Some such processes already have been developed, but, so far, the hydrolysis process is still the most challenging step from an economic perspective (5,6). A technology called the simultaneous saccharification and enhanced lipid production (SSELP) process that is highly advantageous in terms of converting cellulosic materials into lipids had been examined by using *Cryptococcus curvatus* for the lipids accumulation (7).

The biological production of single cell oils (SCO) from oleaginous microorganisms as the oil feedstock is considered one of the feasible routes to biodiesel production in lieu of using vegetable oils. Since microbial lipids have many advantages over vegetable oils, such as a short life cycle and no need for agricultural land, they have attracted much interest as a potential non-food feedstock for biodiesel production. Oleaginous microorganisms are classified as strains that have a microbial lipid content in excess of 20% (g/g) (8). Numerous oleaginous yeasts and microalgae have been reported to grow and accumulate significant amounts of lipids. More specifically, the characteristics of rapid growth and the ability to utilize a range of carbon sources (e.g., glucose, glycerol) makes *Rhodotorula glutinis* an attractive candidate for microbial oil production (9–12). Further, an oil content as high as 72% obtained in *R. glutinis* has been reported (9). Pan and his colleagues obtained a cell density of 185 g/l in an 84-h fed-batch culture of *R. glutinis* aerated with oxygen-enriched air (13). In general, the components of fatty acids extracted from *R. glutinis* are mainly palmitic acid (C16:0), palmitoleic acid (C16:1), stearic acid (C18:0), oleic acid (C18:1) and linolenic acid (C18:2), where palmitic acid and oleic acid often account for over 80% of total fatty acids (14). To further convert the microbial oils to biodiesel, a direct methanolysis process by the assisting of acids had been explored, which reached up to 97% of

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yield under several specific conditions (15). Besides the accumulation of high lipids, *R. glutinis* is also well-known in the industry as a β-carotene producer (16), which β-carotene is regarded as a valuable compound in the health industry.

In this study, the hydrolysate of LCB (rice straw) was explored for the cultivation of oleaginous yeast- *R. glutinis* to produce SCO and β-carotene. Due to the potential inhibitors existing in the hydrolysate solution, the suitability of hydrolysate as a carbon source for the growth of *R. glutinis* is firstly examined. The C/N ratio effects by varying the adding amount of yeast extract on total lipid production and β-carotene accumulation are also evaluated. To achieve the scaled-up production, the performance of *R. glutinis* in the internal-loop airlift bioreactor and in the conventional agitation tank using LCB hydrolysate is also compared in this study.

MATERIALS AND METHODS

Microorganism and medium Freeze-dried *R. glutinis* BCRC 22360 was obtained from the Bioresource Collection and Research Center, Taiwan (BCRC). The seed medium composition and the cultivation methods followed suggestions provided by the BCRC. The fermentation medium (per liter) comprised defined amounts of different carbon sources (including glucose, glycerol, crude glycerol and LCB hydrolysate), 2 g of yeast extract, 2 g of $(NH_4)_2SO_4$, 1 g of KH_2PO_4, 0.5 g of $MgSO_4 \cdot 7H_2O$, 0.1 g of $CaCl_2$ and 0.1 g of NaCl (17). Sodium hydroxide at 1.0 N or hydrogen chloride at 1.0 N was used to adjust the pH. LCB hydrolysate was kindly provided by the Institute of Energy Research, Taiwan, which was prepared through 2 stages of LCB hydrolysis of agriculture wastes and vegetable residues (pretreatment by dilute acid to remove hemicellulose and using cellulase for the following cellulose hydrolysis). The detail hydrolysis procedure was still the confidential process to the Institute of Energy Research, Taiwan, which could not be revealed here. The components of LCB hydrolysate is shown in Table 1. Before the use of LCB hydrolysate, it would be diluted properly to the designed reducing sugar concentration. The crude glycerol was purchased from a local biodiesel manufacture company (Yu-Hwa biodiesel company, Taiwan), with crude glycerol being the by-product of the conventional base catalyst transesterification process. No any extra purification process was performed before crude glycerol was used as one of the medium ingredients.

Fermentation in 5-L conventional agitation fermentor The batch fermentation was operated in a conventional 5-L stirred desk-top fermentor (model BTF-A, Biotop Ltd., Taiwan) with a 2-L working volume, the fermentation medium of which was described in the above section. The pH level was maintained at 5.5 by automatically feeding NaOH solution (1.0 N). The fermentor was operated at 24°C with dissolved oxygen controlled at over 30% by adjusting the agitation rate in the range of 200–500 rpm with a 1 vvm aeration rate.

Fermentation in 5-L internal-loop airlift bioreactor Batch fermentation was respectively carried out in a in a 5-L internal-loop glass airlift bioreactor (30 cm in height, with a 10 cm outer diameter and 7.7 cm inner tube diameter) with a working volume of 3 L. All experiments were controlled at 24°C and the pH was controlled at 5.5 by using 1 N NaOH solution. The aeration rate was performed at 1.5 vvm to explore the effects of using LCB hydrolysate on cell growth and on the accumulation of total lipids.

Analytical methods Infrared balance was adopted to rapidly measure the cell mass concentration. Five ml of broth was centrifuged at 7000 rpm for 10 min. After removing the supernatant, about an equal volume of distilled water was added to eliminate the impurities. The washing procedure was performed several times, and the final liquor was dried by using infrared balance at 150°C to evaporate the water content.

The total lipid analysis was based on a modification of the procedure used by Bligh and Dyer (18). The dry cell mass was ground into a fine powder; then, 0.05 g of the powder was blended with 5 ml chloroform/methanol (2:1), and the mixture was agitated for 20 min at room temperature in an orbital shaker. The solvent phase was recovered by centrifugation. The same process was repeated twice, and the whole solvent was evaporated and dried under vacuum conditions. The dinitrosalicylic acid (DNS) analysis was adopted for the measurement of reducing sugars of LCB hydrolysate.

The glycerol concentration was measured by HPLC (Agilent series 1100, Agilent Technologies, Santa Clara, CA, USA) with a refractive index detector, while the analysis was performed in a C-18 column (Vercopak N5 ODS, 250 mm × 4.6 mm, Taiwan). The mobile phase was composed of 0.01 N H_2SO_4 with a flow rate of 0.4 ml/min (19). The measurement of β-carotene was performed as following: 50 mg of cells, after freeze-drying, were mixed with a 2 ml mixture consisting of acetonitrile, isopropyl alcohol and ethyl acetate (40:40:20 v/v), followed by ultrasonication (power 5, reaction time 2 min) to crush the cells for pigment extraction. The extract was centrifuged and the supernatant filtered through a 0.45-μm membrane filter and subjected to HPLC analyses, which were performed on a reversed-phase C18 analytical column [N5 ODS (C-18) 4.6 mm i.d. × 250 mm]. The mobile phase was composed of acetonitrile, isopropanol and ethyl acetate (40:40:20 v/v) and had a flow rate of 0.7 ml/min. The column thermostat was set at 25°C, while the detector was operated at a wavelength of 457 nm.

Analysis of lipid composition To determine fatty acid composition, wet cells were directly transmethylated according to the following procedure. Wet cell pellets from 1 ml of culture were treated in a flask with 4 ml of a 0.5 N KOH/methanol solution at 100°C for 15 min, followed by the addition of 5 ml BF3 diethyl etherate and 5 ml methanol. The mixture was refluxed for 15 min, cooled, diluted with distilled water and then extracted with *n*-Hexane. The organic layer was washed with distilled water and subjected to fatty acid compositional analysis. Fatty acid methyl esters were analyzed using a gas chromatography instrument (Focus GC, Thermo, USA) equipped with a cross-linked capillary Column SEG BP20 (25 m × 0.22 mm × 0.25 μm) and flame ionization detector. Operating conditions were as follows: N_2 carrier gas at 40 ml/min; injection port temperature of 230°C; oven temperature of 200°C; and, a detector temperature of 230°C. Fatty acids were identified by a comparison of their retention times with those of standard ones, quantified based on their respective peak areas, and then normalized.

RESULTS AND DISCUSSION

Using hydrolysate from LCB as the carbon source Several studies have revealed results of conventional carbon sources being used as the carbon source for the growth of *R. glutinis* in the literature. In this study, glucose, glycerol, crude glycerol and LCB hydrolysate each at 30 g/L (hydrolysate solution was prepared at the equivalent of 30 g/L of reducing sugars) were compared for the growth of *R. glutinis*. As shown in Fig. 1, LCB hydrolysate solution can be a good carbon source for the growth of *R. glutinis*, which can produce a similar cell mass as compared to the batch using glucose. As seen in Table 1 of LCB hydrolysate components analysis, the glucose concentration was about 50% of reducing sugars (measured by the DNS method), which implied that there was still some other reducing sugars unclassified in the LCB hydrolysate. The concentration of glucose in the hydrolysate was even higher than that of xylose, which were 71.5 and 18.1 g/L, respectively. The high cell mass obtained in the batch using LCB hydrolysate suggests that the potential inhibitors (e.g., HMF, furfural) derived from the hydrolysis process would not inhibit the growth of *R. glutinis* under these cultivation conditions. Due to the recalcitrant nature of LCB products, different pretreatment process was often adopted for the destruction of complex cells was and exposed the cellulose for the further degradation of cellulolytic enzymes. On the other hand, lignocellulose pretreatment has the detrimental effect of also releasing a wide range of hydrolysis compounds, which are inhibitory to fermenting microorganisms and cellulolytic enzymes (20,21). Nevertheless, such kind of potential inhibitors seems not affecting the growth of *R. glutinis* in this study under the investigated conditions. It had been also reported that lipid production of *Rhodosporidium toruloides* afforded good results in the presence of six inhibitors at their respective concentrations usually found in biomass hydrolysates. Fatty acid compositional profile also indicated that those inhibitors had little effects on lipid biosynthesis (22).

The lipid composition analyses for each batch with different carbon source are shown in Table 2, which indicates that oleic acid (C18:1) dominated the fatty acid components among all batches.

TABLE 1. Components of LCB hydrolysate solution (g/L).

Reducing sugar	Glucose	Xylose	Arabinose	Formic acid	Acetic acid	HMF	Furfural
148.5 ± 4.6	71 ± 3.2	18.1 ± 1.5	0.5 ± 0.1	2.3 ± 0.2	4.9 ± 0.1	0.11 ± 0.1	0.2 ± 0