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Using crude glycerol and thin stillage for the production of microbial lipids through the cultivation of *Rhodotorula glutinis*

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Single cell oils (SCO) produced from oleaginous microorganisms are a potential alternative oil feedstock for biodiesel production. The worldwide production of glycerol, a 10% (w/w) byproduct produced in the transesterfication process of oils converted to biodiesel, is increasing as more biodiesel is being produced. For the purposes of cost reduction, crude glycerol was regarded as a suitable carbon source for the cultivation of *Rhodotorula glutinis*. In addition to using renewable crude glycerol, waste solution collected from the brewing company (called thin stillage) was adopted as a substitute to replace a costly nitrogen source used in the medium. The results of using mixture of crude glycerol and thin stillage indicated about a 27% increase in total biomass as compared to that of using crude glycerol with a standard medium. Using glycerol instead of glucose as the carbon source could also alter the lipid profile, resulting in an increase in linolenic acid (C18:2) to comprise over 20% of the total lipid. Successfully using renewable crude glycerol and thin stillage for the cultivation of oleaginous microorganisms could greatly enhance the economic competition of biodiesel produced from SCO.

[**Key words:** Crude glycerol; Lipid profile; Stillage; Biodiesel; Single cell oils (SCO)]

It is estimated that the cost of lipid feedstock for biodiesel production would be in the range of 70–85% of the total production cost (1). Based on annual biodiesel consumption, this cost would be prohibitive as compared to having biodiesel produced from conventional oilseeds or animal fat. However, even where the availability of crop oils is high enough; this can lead to disputes concerning the best use of arable land. Therefore, lipid production from oleaginous microorganisms is regarded as a substitute for conventional oil crops in biodiesel production. Biodiesel production using microbial lipids, named single cell oils (SCO), has attracted considerable attention in recent years. Normally, microorganisms containing over 20% lipids are classified as potential oleaginous microorganisms for lipid production (2).

The yeast *Rhodotorula* is known for its high lipid content (as high as 70% or more), which can potentially provide large amounts of lipids for biodiesel production (2,3). Pan and his colleagues adopted oxygen-enriched air to obtain the highest cell density on record (185 g/L) in an 84-h fed-batch culture of *Rhodotorula glutinis* (4). Many factors affecting the growth of *R. glutinis* had been previously explored in the literature. It was first reported by Yen and his colleagues that irradiation could obviously increase the growth rate of *R. glutinis* as compared to a batch grown without irradiation (5), which resulted in a 79% increase in lipid productivity in a batch grown using three LED lights. The following study by the same authors indicated that the accumulation of potential inhibitive metabolic products probably impedes growth, thereby restricting accumulation of more biomass in the fed-batch operation with irradiation. Combining the fed-batch operation with irradiation and microfiltration can successfully improve the growth of *R. glutinis* to the maximum of 72.4 ± 0.6 g/L with 51.2 ± 4.9% of lipid content obtained (6).

Besides traditional carbon sources, crude glycerol produced from biodiesel has been evaluated as a substrate converted to lipid by *R. glutinis*. Glycerol is the main byproduct derived from the conversion of oils into biodiesel, comprising approximately 10% by mass of the oils fed to the process (7). The future supply and use of glycerol are expected to increase as the global production of biodiesel increases; this could in turn result in a surplus in the glycerol market (8). The process of refining crude glycerol into high purity is too costly and energy-intensive. Therefore, the discovery of a novel application for crude glycerol could make biodiesel production more profitable and sustainable. In the fed-batch operation with crude glycerol attained through cultivation of *R. glutinis*, 10.05 g/L of the highest biomass, 60.70% of the lipid content and a 6.1 g/L lipid yield were obtained (9). In addition to crude glycerol, certain other renewable industrial wastes were prospectively applied in the fermentation process. Stillage, also termed as distillery wastewater, distillery pot ale, distillery slops, distillery spent wash, vinasse, and thin stillage, is the aqueous byproduct attained during the distillation of ethanol following fermentation of carbohydrates (10). In this process, after removing ethanol by distillation, the residual ethanol-free slurry (whole stillage) is separated into a solid fraction

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(wet distillers grains) and a liquid fraction (thin stillage) through centrifugation (11). The stillage of alcoholic fermentation derived from raw carbohydrate materials often presents a considerable disposal or treatment problem. The stillage is difficult to treat because of its high biological oxygen demand (BOD), high organic content and low pH. With the global increase in ethanol production for use as biofuel, it is essential to determine how to deal with the enormous quantity of stillage produced.

Rice spirit is a distilled alcoholic beverage used mainly for Chinese cooking in Taiwan. Considerable volumes of wastewater are produced by rice distilleries with the quantity of thin stillage from rice-spirit distilleries in Taiwan estimated to be as high as 2106 L/day in 1998 (12). The production of acid protease by *Aspergillus niger* with thin stillage from a rice-spirit distillery had been successfully performed by Yang and his colleagues (13). Appropriate additions of C-source, N-source and soybean oil into the stillage medium increase the activity of protease (13). The production of butanol from thin stillage by *Clostridium pasteurianum* DSM 525 was also evaluated; 6.2–7.2 g/L of butanol can be produced utilizing the glycerol in thin stillage as the main carbon source, with yields of 0.32–0.44 g of butanol produced per gram of glycerol consumed (14).

Since the substrate cost is detrimental to the economical competition of microbial lipids converted to biodiesel, this study focused on the effects of the use of crude glycerol and thin stillage on the growth of the oleaginous yeast *R. glutinis*, and on its production of lipids.

MATERIALS AND METHODS

Microorganism and medium The freeze-dried *R. glutinis* BCRC 22360 (Bioresource Collection and Research Center) was kindly provided by Dr. Yaw-Nan Chang (Department of Biotechnology, National Formosa University, Taiwan). The fermentation medium (per liter) was comprised of defined amounts of glucose (or glycerol), 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, which was expressed as the standard medium in this text (15). Two different glycerol sources were adopted in this study, including pure glycerol (purchased from Sigma) and crude glycerol. The crude glycerol obtained directly from a local industrial biodiesel manufacturing plant, which did not have any pretreatment or purification process performed in the lab before being used as the substrate. The crude glycerol appeared as a slightly viscous solution, and the color presented slightly dark brown to almost black. In the trials using thin stillage as the nitrogen source, only defined amounts of crude glycerol and thin stillage were used as the carbon and nitrogen source without the addition of any other ingredients. The thin stillage was provided by the Taichung winery and the Taiwan Tobacco and Wine Board. This organization provided the residual waste products derived during the rice wine manufacturing process. In the process, the substrate of rice was fermented using yeast to produce alcohol. After the distillation procedure to remove ethanol from the broth, the residual supernatant (without solid matters) became known as thin stillage. The ingredients of thin stillage, as seen in Table 1, are shown to contain many nutrients. Sodium hydroxide at 1.0 N was used as the pH neutralizer to adjust the initial pH level at 5.5.

Batch cultivation of *R. glutinis* in shaker One ml of prepared cell suspension was inoculated into a 250-ml flask containing 50 ml of BCRC suggested medium (per liter), comprising 3 g of yeast extract, 3 g of malt extract, 5 g of peptone and 10 g of dextrose as the seed medium; it was shaken at 150 rpm at 24°C for 24 h under aerobic conditions. Next, 5 ml of seed medium (10%) was transferred into 250-ml flasks containing designated amounts of fermentation medium, as described previously, and was cultured at 24°C and shaken at 150 rpm under aerobic conditions.

Batch operation in a lab-top fermentor First, 200 ml of seed medium was transferred into a 5-L stirred desk-top fermentor (model BTF-A, Biotop Ltd., Taiwan) of a 2-L working volume. The pH level was automatically maintained at 5.1 by automatically feeding NaOH solution (1.0 N) into the medium. The fermentor was operated at 24°C with dissolved oxygen controlled at a 25 ± 10% saturation level. The agitation during the process was limited to a range of 200–400 rpm to avoid potential damage resulting from the high shear force.

TABLE 1. Properties of thin stillage (adapted from the values provided by the alcohol manufacture company).

pH	3.18–3.28
Residual starch (w/v)	0.1%
Total sugars (w/v)	0.26%
Reducing sugars (w/v)	0.112%
Total phosphorus (mg/L)	128.65
Total nitrogen (g/L)	1.97
COD (g/L)	51
BOD (g/L)	24

Glucose analysis and biomass measurement Glucose concentration was detected using a glucose biosensor (YSI 2300 STAT Glucose Analyzer). For the measurement of yeast growth, dry biomass concentration was determined by measuring the turbidity of the diluted sample at 660 nm, using a standard curve of absorbance against dry cell mass concentration. Absorbance measurements were carried out using a Genesys™10 Series spectrometer.

Total lipid analysis Extraction of lipids from wet biomass was based on a modification of the procedure used by Bligh and Dyer (16). The dry biomass was ground into a fine powder: 0.05 g of 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 carried out two times. The whole solvent was evaporated and dried under vacuum conditions.

Analysis of the 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 broth were treated in a flask with 4 ml of a 0.5 N KOH/methanol solution at 100°C for 15 min, following which 5 ml of BF3 diethyl etherate and 5 ml of methanol were added. The mixture was refluxed for 15 min, cooled, diluted with distilled water and 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 40 ml/min, injection port temperature 230°C, oven temperature 200°C and detector temperature 230°C. Fatty acids were identified by a comparison of their retention time to those of standard ones, quantified based on their respective peak areas and normalized (5).

RESULTS AND DISCUSSION

Glycerol as the carbon source Due to the rapid increase of global biodiesel production, there has been an immense increase in the global supply of glycerol. The use of glycerol has attracted much attention recently (17). Two different sources of glycerol (pure glycerol and crude glycerol) were examined for use in the production of microbial lipids as compared to using glucose as the carbon source through the cultivation of *R. glutinis*. The results of using pure glycerol, crude glycerol and glucose each at 30 g/L are shown in Fig. 1. As seen in Fig. 1, the growth of *R. glutinis* in the batch with glucose as the carbon source had the highest biomass, which is about 1.5 times greater than that of the batch produced using crude glycerol. However, the batch produced using crude glycerol had the highest lipid content of all batches, which led to the total lipid production about 30% less than that of glucose. Saenge et al. (9) obtained a maximum biomass of 8.71 g/L and a lipid content of 52.91% in the batch using crude glycerol for the

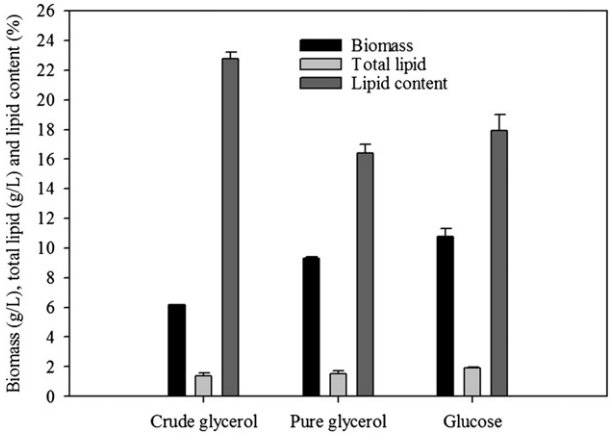

FIG. 1. The effects of different carbon sources (crude glycerol, pure glycerol and glucose each at 30 g/L) on biomass and total lipid production after 48 h cultivation.