Journal of Bioscience and Bioengineering
VOL. 121 No. 2, 209–212, 2016
www.elsevier.com/locate/jbiosc

Cultivation of oleaginous *Rhodotorula mucilaginosa* in airlift bioreactor by using seawater

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Received 29 April 2015; accepted 16 June 2015
Available online 28 August 2015

The enormous water resource consumption is a concern to the scale-up fermentation process, especially for those cheap fermentation commodities, such as microbial oils as the feedstock for biodiesel production. The direct cultivation of oleaginous *Rhodotorula mucilaginosa* in a 5-L airlift bioreactor using seawater instead of pure water led to a slightly lower biomass being achieved, at 17.2 compared to 18.1 g/L, respectively. Nevertheless, a higher lipid content of 65 ± 5% was measured in the batch using seawater as compared to the pure water batch. Both the salinity and osmotic pressure decreased as the cultivation time increased in the seawater batch, and these effects may contribute to the high tolerance for salinity. No effects were observed for the seawater on the fatty acid profiles. The major components for both batches using seawater and pure water were C16:0 (palmitic acid), C18:1 (oleic acid) and C18:2 (linoleic acid), which together accounted for over 85% of total lipids. The results of this study indicated that seawater could be a suitable option for scaling up the growth of oleaginous *R. mucilaginosa*, especially from the perspective of water resource utilization.

[**Key words:** Microbial oils; Oleaginous; Airlift bioreactors; Seawater; Salinity]

The rapid deterioration of the environment resulting from the over-consumption of fossil fuels makes the use of bioenergy increasingly attractive. Biodiesel is one such option, and can be produced through the transesterification of several oil-containing feedstocks, such as conventional oil crops, waste kitchen oils and microbial oils. Among all potential feedstocks, microbial oils from oleaginous microorganisms are especially attractive, as they can avoid the problem of competition with arable land use while using non-food carbon sources for the accumulation of microbial oils. Oleaginous microorganisms are defined as those species containing more than 20% of lipid content per dry biomass (1). Numerous oleaginous yeasts and microalgae have been reported to be capable of accumulating large amounts of lipids, with some studies reporting a lipid content of more than 70% (2). The majority of these lipids are triacylglycerol (TAG) containing long-chain fatty acids, and the fatty acid profiles of most oleaginous microorganisms are comparable to those of conventional oil crops, and are suitable for use as the feedstock for biodiesel production. More specifically, *Rhodotorula mucilaginosa* is especially suitable for microbial oil production, due to its characteristics of rapid growth and high lipid content by using various carbon sources (3,4).

R. mucilaginosa can accumulate a large amount of lipids from the hydrolysate of cassava starch, with a lipid content of about 48% (w/w) obtained during batch cultivation, and 53% in the fed-batch cultivation (3). The resulting fatty acids are mainly composed of palmitic acid (C16:0), palmitoleic acid (C16:1), stearic acid (C18:0), oleic acid (C18:1) and linolenic acid (C18:2), suggesting that the fatty acids of *R. mucilaginosa* could be a good feedstock for biodiesel production (3). When the hydrolysate of inulin is used, a lipid content of about 48.8% of can be accumulated in *R. mucilaginosa*, reaching 14.8 g/L of biomass during batch cultivation. Lipid contents of 48.6% and 52.2% have been reported in the batch and fed-batch operations using the hydrolysate extracted from Jerusalem artichoke tubers, respectively, with a biomass of 14.4 g/L and 19.5 g/L. Over 87.6% of the fatty acids from *R. mucilaginosa* cultivated in the hydrolysate extracted from Jerusalem artichoke tubers is C16:0, C18:1 and C18:2, with the major component being C18:1, accounting for 54.7% of the total fatty acids (4). In addition to the high accumulated lipid content, *R. mucilaginosa* is also known to be a good carotene producer. As *R. mucilaginosa* is an obligate aerobe, it is necessary to provide enough dissolved oxygen for its growth. In addition to the biomass, the total carotenoids concentration and production yield are significantly enhanced when the aeration rate is increased to 2.4 vvm (5). Moreover, an initial ammonium sulphate concentration of 2 g/L gives the maximum carotenoids production, with the highest concentration of 89.0 mg total carotenoids per liter of fermentation broth (5).

R. mucilaginosa was reported to have a salinity tolerance as high as 7% of NaCl, although several biological activities (such as the bioreduction function of some chemicals) and the growth rate are reduced at this level (6,7). While the reasons for this tolerance are not clearly understood, a common explanation is the formation of intracellular glycerol under high osmotic conditions, which can be helpful to the process of osmoregulation (8,9). Successful biotechnological processes for producing biodiesel feedstock should be supported by the utilization of cheap substrates, as this would

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http://dx.doi.org/10.1016/j.jbiosc.2015.06.007

make the commercialization of single cell oil (SCO) possible; and for this reason, crude glycerol is a particularly appealing material. About 10% (w/w) of crude glycerol is produced in the biodiesel manufacturing process as the main by-product (10). Since the global production of biodiesel is increasing, the amount of crude glycerol production has also risen significantly, and consequently its market price has fallen, leading to crude glycerol being a very promising potential substrate for the cultivation of oleaginous microorganisms (11, 12).

The large amount of water needed in the fermentation process remains a problem with regard to scaling up operations, as this also yields the argument of water resource arrangement. One way to avoid using water from aquifers is to directly use seawater for the fermentation (13), and this could also reduce the related process costs. Consequently, the present study investigated the effects of salinity on the growth of oleaginous *R. mucilaginosa* by adding various amounts of NaCl and using combinations of seawater and pure water at different ratios. Furthermore, the growth of *R. mucilaginosa* in a 5-L airlift bioreactor using crude glycerol as the sole carbon source will be carried out using seawater and pure water, respectively, and the results then compared.

MATERIALS AND METHODS

Microorganism and medium Freeze-dried *R. mucilaginosa* was provided by Professor Nelson Chang's lab (National Formosa University, Taiwan), and this had been mutated for high lipid content strain screening using NTG mutation method. The seed medium composition was consisting of (per liter); 60 g of crude 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 (14). Solutions of 1.0 N NaOH or 1.0 N HCl were used to adjust the initial pH to 5.5. The crude glycerol was provided by a local biodiesel manufacturing company of Taichung, Taiwan (Yeow-Hwa Biodiesel Company), as it is the by-product of the conventional base catalyst transesterification process. The composition of the crude glycerol was 47 ± 8% (w/w) of glycerol, 16 ± 8% methanol and 29 ± 6% of ash. The actual content of crude glycerol greatly depends on the biodiesel production batch. Seawater was collected from the seashore close to Taichung, Taiwan. Salinity and osmolality of pure seawater are about 31 ± 0.8 g/L and 885 ± 37 mmol/kg. No any pretreatments of the seawater were performed before it was adopted for the medium preparation.

Fermentation in 5-L airlift bioreactor Batch fermentation was carried out 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 set at 1.5 vvm to enhance cell growth (15). No agitation device is mounted for this airlift bioreactor. The mixing effects was completely provided by the air flow at the aeration rate of 1.5 vvm.

Analytical methods An infrared balance (IR 35, Denver Instrument) was used to rapidly measure the biomass concentration. Broth (5 ml) was centrifuged at 7000 rpm for 10 min. After removing the supernatant, about an equal volume of distilled water was added to eliminate impurities. This washing procedure was performed several times, and the final liquor was dried using the 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 (16). The dry biomass was first ground into a fine powder, and then 0.05 g of the powder was blended with 5 ml chloroform/methanol (2:1), and subsequently agitated for 20 min at room temperature in an orbital shaker. The solvent phase was recovered by centrifugation at 7000 rpm for 10 min. The same process was repeated twice, and the whole solvent was evaporated and dried under vacuum conditions.

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 N5ODS, 250 mm × 4.6 mm, Vercotech, Taiwan). The mobile phase was composed of 0.01 N H_2SO_4 with a flow rate of 0.4 ml/min (17).

The osmolality was measured using a vapour osmometer (Vapro 5600, Wescor Company). This instrument measures the dew point temperature depression of a solution in vapour equilibrium in a closed chamber for the calculation of osmolality. The salinity measurement was performed using a handheld refractometer (Master-BX/S28M, Atago).

All shaker conditions were performed in triplicate to have the expression of mean ± standard deviation. The data of 5-L airlift bioreactor operation will be acquired on three separate time points after the steady-state condition achieved, and the values would be expressed as the mean ± standard deviation.

RESULTS AND DISCUSSION

Effects of NaCl concentrations in the shaker trials The purpose of this study aimed to explore the feasibility of directly using seawater for the growth of *R. mucilaginosa*. It is well known that the salinity of seawater is about 3.5% of NaCl. Therefore, the effects of initial NaCl concentration (0.1–40 g/L) adding in the pure water on the growth of *R. mucilaginous* were examined in the shaker flasks using 60 g/L of crude glycerol as the carbon source. The results are shown in Fig. 1, which indicates that high salinity will lead to a decrease in biomass from 8.7 ± 0.6 to 6.9 ± 0.1 g/L for the trials containing 0.1 to 40 g/L of NaCl. Besides the inhibition of cell growth, the lipid content and β-carotene content were also observed to be slightly reduced with the increase of salinity, and these levelled off when the NaCl concentration was over 30 g/L. This is in contrast to an earlier report (18) which indicated that an increase in the osmotic pressure in the medium will lead to an increase in the lipid content and a fall in the polysaccharide content, while the relative proportion of polyols increased, although the qualitative composition of intracellular lipids did not change. In the cultivation of *Rhodotorula rubra*, an increase in the salt concentration to high levels of 4%, 8% and 12% resulted in a decrease in the specific cell growth rate from 0.27 to 0.05 (1/h). Nevertheless, the total lipid content of the cells increased along the salinity, which was not observed in the current study (9). Although the reasons for the tolerance of *Rhodotorula* under conditions of high salinity remain unclear, a close correlation between intracellular glycerol concentration and medium salinity has been observed, suggesting that the formation of glycerol pool concentrations inside the cells may balance the osmotic pressure in highly saline media (9). The intracellular glycerol concentration was not measured in this study. Nevertheless, a high NaCl tolerance of *R. mucilaginous* growth was concluded in this study. The results obtained by Zheng and his colleagues (7) also showed that *R. mucilaginosa* could tolerate very high salinity, of up to 7% NaCl. Although an increase in the NaCl concentration did not increase the lipid content in the current study, in contrast to the findings in the literature (9), relatively good growth of *R. mucilaginosa* was still obtained at a high NaCl concentration of 40 g/L. Therefore, the next section will examine the effects of adding seawater at various ratios on the growth of *R. mucilaginosa*.

The effects of seawater/pure water ratios on the growth of *R. mucilaginosa* As shown in the previous section, *R. mucilaginosa* has the potential to be cultured in a medium with salinity as high as 40 g/L of NaCl. Nevertheless, the growth rate of biomass was slightly impeding by the increase of NaCl

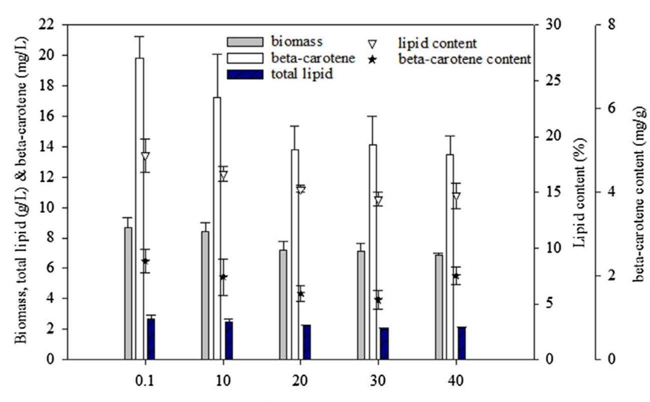

FIG. 1. Effects of adding NaCl on the growth of *R. mucilaginosa* in the shaker trials.