International Conference on Renewable Energies and Power Quality (ICREPQ'14)
Cordoba (Spain), 8th to 10th April, 2014
Renewable Energy and Power Quality Journal (RE&PQJ)
ISSN 2172-038 X, No.12, April 2014

Optimization of Initial Cell Density and Usage Efficiency of Seed Culture Medium During Lipid Production from Distillery Wastewater by *Rhodosporidium toruloides*

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Abstract. For the environmentally sustainable development, the recovery of resource or energy from wastes has attracted worldwide attention in recent years. The wastewater produced from distillery is more difficult to treat than the domestic wastewater due to the high organic loads. This study explores the possibility of developing a process, which could produce microbial lipid convertible to biodiesel by recovering nutrients from non-sterile wastewater while simultaneously removing part of organic matters through cultivating oleaginous microorganisms. Especially, it is intended to further optimize the initial cell density and the usage efficiency of seed culture medium. The lipid productivity by oleaginous yeast *Rhodosporidium toruloides* was studied using wastewater from rice wine distillery, at 30ºC under different initial cell densities (2 x 10^7, 1 x 10^7, 0.8 x 10^7, and 0.5 x 10^7 cells/mL) and incubation periods (2-5 days). Chemical oxygen demand (COD) removal efficiency, cell yield, and lipid yield were analyzed and the potential for bioenergy recovery from wastewater was assessed. The seed culture medium was found reusable for the second time without the addition of extra nutrient and possible for the third time with the addition of glucose.

Key words

Initial cell density, lipid, non-sterile distillery wastewater, oleaginous yeast, seed culture medium.

1. Introduction

As a renewable and alternative source of energy, biodiesel has been receiving an increasing attention all over the world. It is produced from biomass by the catalytic transesterification of triglycerides, yielding mono-alkyl esters of long chain fatty acids derived from vegetable oils and animal fats, and short-chain alcohols. Biodiesel is an attractive energy resource option for any eco-friendly energy strategy and offers some advantages over standard fossil fuels due to such characteristics as biodegradability, renewable source of energy, and reduction of carbon dioxide generation and air pollution relative to sulfur [1].

Despite its environmental benefits, the large-scale development of biodiesel is limited by oil feed stocks. Much attention has been paid to the development of microbial biodiesel from algae, yeast, bacteria, and fungi. Compared to plant oils, microbial oils have many advantages, such as short life cycle, less labor required, less effect by season and climate, and easier to scale up [2]. Among the microorganisms that have been commonly used for the biodiesel production, oleaginous yeast can be a promising candidate in the production of polyunsaturated fatty acids [3]. In general, oleaginous yeast cultures are more easily expandable than microalgae and can produce lipid from a variety of low cost carbon sources. Some oleaginous yeasts can accumulate oil up to 80% of their dry weights and produce different lipids from different carbon sources in the culture medium.

From our previous work, when the initial cell density was higher than 2 x 10^7 cells/mL, *Rhodosporidium toruloides* was not significantly affected by indigenous microorganisms and was able to survive and produce relatively high amount of lipid from non-sterile distillery wastewater [4]. It was also observed that large amount of seed culture medium was remained during the high cell density seed culture preparation, which encourages us to explore the possibility of reutilizing the seed culture medium to be more cost effective and to evaluate whether it can still produce the same amount of cells in seed culture.

2. Materials and Methods

A. Microorganism, Medium, and Wastewater

Yeast strain *Rhodosporidium toruloides* AS 2.1389 was purchased from the China General Microbiological Culture Collection Center, Beijing. Yeast stock culture was sub-cultured on distillery wastewater agar plates and stored in distillery wastewater agar slants at 4°C. The composition of lipid produced by *R. toruloides* AS 2.1389 from the medium containing glucose as single carbon source and distillery wastewater was reported to be mainly palmitic acid, palmitoleic acid, stearic acid, oleic acid, and linoleic acid, which are suitable for the biodiesel production [5-9]. The distillery wastewater agar medium was made of rice wine distillery wastewater and

20 g/L of agar with pH adjusted to 5.5. The YPD medium used for seed culture contained (per liter) glucose 20 g, yeast extract 10 g, and peptone 20 g at pH 6.0 [5]. Both media were sterilized at 121°C for 20 min before use. The rice wine distillery wastewater was obtained from the 'S1' distillery in Foshan city, China. The domestic wastewater was obtained from a local wastewater treatment plant. Both wastewater samples were filtered through the filter paper (47 mm diameter, 0.7 μm pore size glass-fiber) and then stored at 4°C before use.

B. Experimental Setup

The oleaginous yeast *R. toruloides* strain grown in the distillery wastewater agar slant or plate was transferred to 150-mL flask containing 30 mL YPD medium, cultivated at 30°C and 200 rpm for 36 h, and used as seed culture. The seed culture was centrifuged at 4,000 rpm for 10 min to obtain the high cell density of 1.5×10^9 cells/mL. The cell density was measured and calculated using cell chamber.

Distillery wastewater was mixed with domestic wastewater at 1:1 ratio (Table I) and was used as culture medium. Thirty mL non-sterile diluted wastewater was first added to 150-mL flasks. Then, the seed culture with different cell densities (2×10^7, 1×10^7, 0.8×10^7, and 0.5×10^7 cells/mL) was inoculated into the culture medium and cultivated at 30°C and 200 rpm for 5 days. Samples were taken in every 24 h from the 2nd day for the analyses of chemical oxygen demand (COD), total nitrogen (TN), total phosphorus (TP), ammonia nitrogen (NH_3-N), pH in supernatants, and cell yield and lipid yield in pellets.

To evaluate the possibility of reutilizing the remained seed culture medium, 300 mL YPD medium used once and twice with or without the addition of glucose (0, 5, and 10 g/L) and sterilized at 121°C for 20 min was used to make the seed culture for the second time. The number of cells produced by the remained seed culture was compared with the one produced by fresh YPD medium.

C. Analytical Methods

COD, TN, TP, and NH_3-N were measured using Hach reactor DRB200 and spectrophotometer DR2800, following the HACH methods (HACH DR/2800, Hach company, Loveland, Colorado) and the Standard Methods [10]. The pH was measured using HACH ONE laboratory pH meter.

After the culture was centrifuged at 4,000 rpm for 10 min at ambient temperature, cell pellets were washed with distilled water twice and then dried at 80°C until the weight remained constant, and the cell dry weight was measured as cell yield. The total lipids were analyzed according to Bligh and Dyer [11] with modifications described by Li [12] and Zhou et al. [13].

3. Results and Discussion

A. Optimization of Initial Cell Density

The optimization of initial cell density of yeast *R. toruloides* was done by checking lipid production and lipid content of dry biomass while organics and nutrients from distillery wastewater were simultaneously removed. As shown in Fig. 1, the COD removal as well as the removal of TN and TP did not show much difference with the change of initial cell density. The highest removal of COD, TN, and TP were obtained on the 4th day, which reached about 85%, 55%, and 95%, respectively. These results suggest that the indigenous microorganisms present in the distillery wastewater would be able to degrade organic matter and nutrients in case yeast did not grow well in wastewater. It was also shown that slightly more NH_3-N was generated in wastewater when the initial cell density was 2×10^7 cells/mL compared to other lower initial cell concentrations (Fig. 2). The growth of yeast seemed to contribute to the rise of NH_3-N and consequently it could be one reason for the increased pH.

As shown in Table II and Fig. 2, the change of initial cell density (from 0.5×10^7 to 2×10^7 cells/mL) did not show that much influence on biomass production due to the contribution of the growth of indigenous microorganisms in wastewater. However, in case of the lipid production, the same behavior was not observed. The lipid production in samples with the initial cell density of 2×10^7 cells/mL was higher than in samples with lower initial cell densities (0.5×10^7, 0.8×10^7, and 1×10^7 cells/mL), as shown in Fig. 2 and Tables III-IV. When the initial cell density was 2×10^7 cells/mL, relatively high lipid content and lipid yield were obtained on the 3rd day, 56.35±14.97% and 2.89±0.12 g/L, respectively, while those for other initial cell densities were not higher than 45% and 2.30 g/L. The associated removal of COD, TN, and TP under the condition of initial cell density 2×10^7 cells/mL and cultivation time of three days was 82.31±0.70%, 52.64±0.46%, and 88.46±2.08%, respectively. Previous results also indicated that the lipid production of samples with the initial cell density of 2×10^7 cells/mL was higher or similar to samples with the initial cell densities of 20×10^7, 10×10^7, 5×10^7, 4×10^7, and 3×10^7 cells/mL, suggesting that the initial cell density of 2×10^7 cells/mL was the optimal value for lipid production from distillery wastewater by yeast *R. toruloides* [4].

B. Biomass in Reused Seed Culture Medium

For the 300 mL fresh YPD medium, which contained 20 g/L glucose, the cell amount produced in seed culture was 6.85×10^{10} cells. In comparison, for the YPD medium used for the second time, 6.34×10^{10} cells were produced in seed culture without the addition of glucose, while the number of cells produced after the addition of 5 and 10 g/L glucose was 9×10^{10} and 7.38×10^{10} cells, respectively. This result suggests that the seed culture medium has a possibility to be used for the second time or even for the third time with the addition of glucose. Further studies are warranted to evaluate whether the cells produced in this reused seed culture medium could produce similar amount of lipid from non-sterile distillery wastewater.

Table I. Wastewater Characteristics Used for the Initial Cell Density Optimization Experiment

	SCOD (mg/L)	TN (mg/L)	TP (mg/L)	NH_3-N (mg/L)	pH
Distillery Wastewater	42,000	2,220	275	226.5	3.77
Domestic Wastewater	80	42	4	19.1	3.79
Mixed Wastewater	15,827	825	110	51.3	8.15

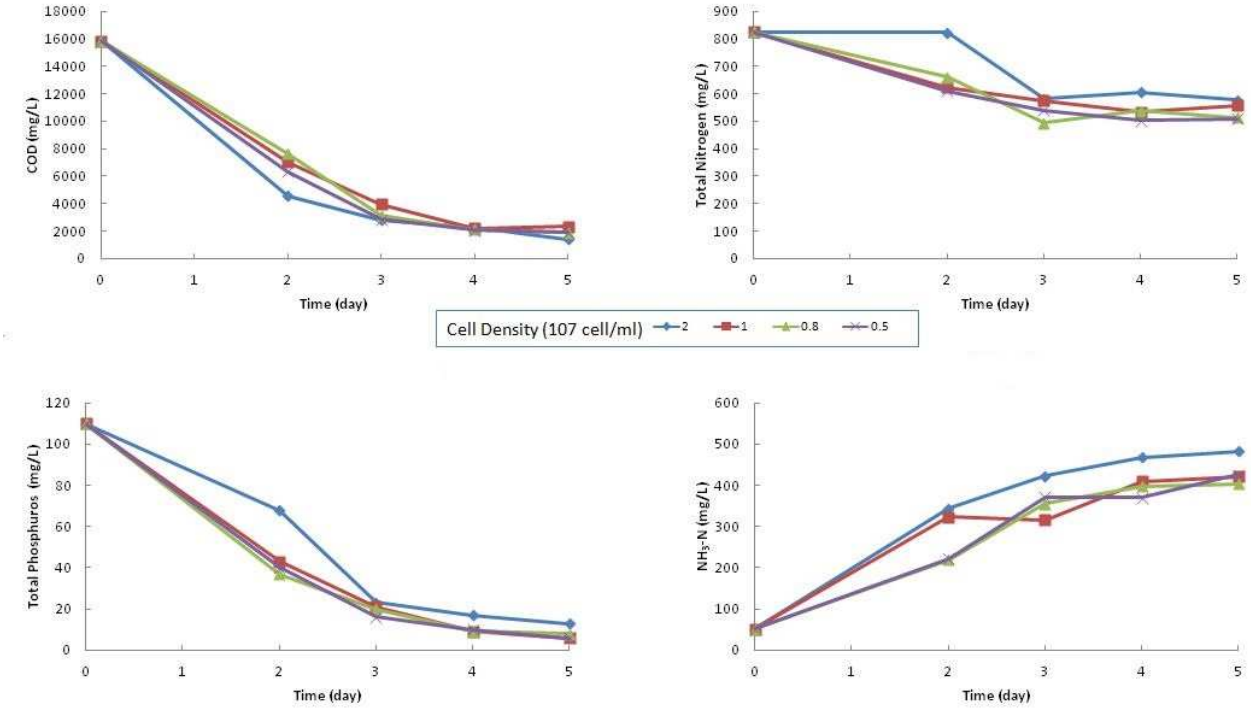

Fig. 1. Changes in COD, TN, TP, and NH_3-N levels in non-sterile mixed (distillery and domestic) wastewater at different initial cell densities

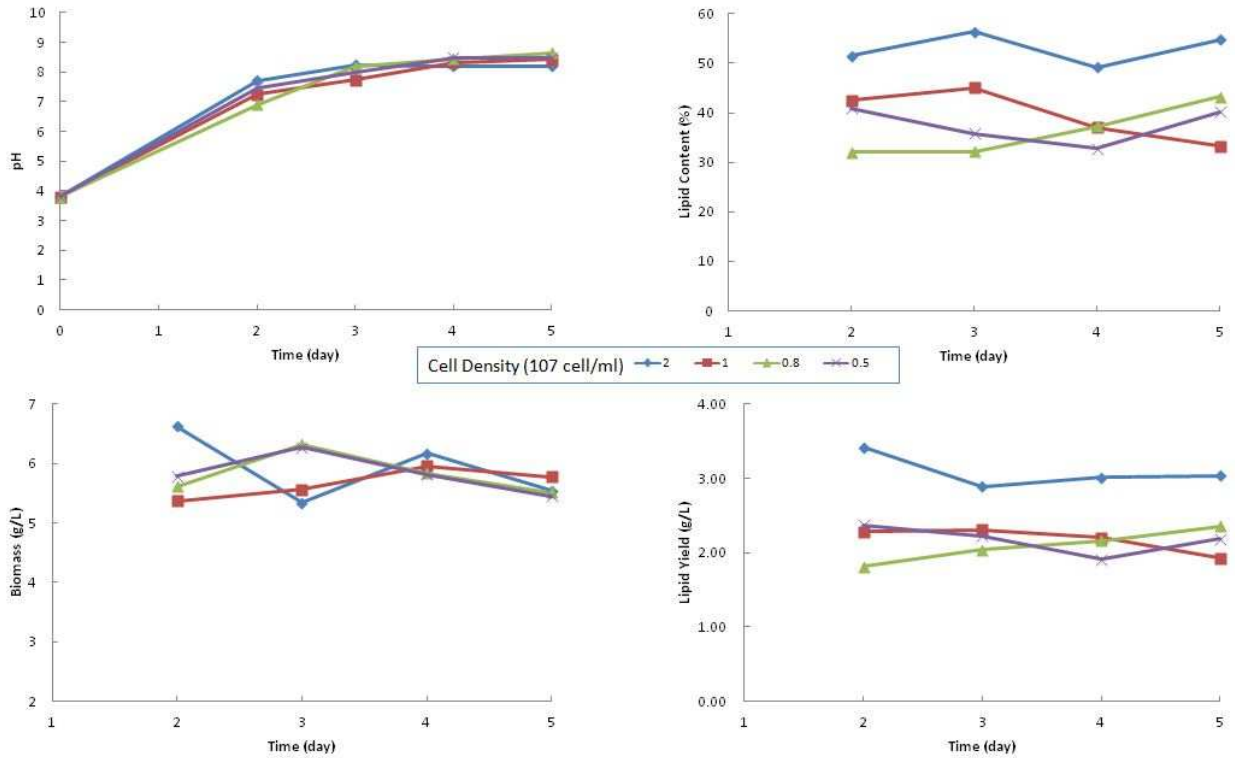

Fig. 2. Changes in pH, lipid content, biomass, and lipid yield in non-sterile mixed (distillery and domestic) wastewater at different initial cell densities

Table II. Biomass (g/L) of Yeast at Different Initial Cell Densities

Time (day) / Cell Density (cells/mL)	2	3	4	5
2 x 10^7	6.63±0.12	5.34±1.64	6.17±0.39	5.55±0.05
1 x 10^7	5.37±0.55	5.56±1.56	5.96±0.31	5.77±0.27
0.8 x 10^7	5.61±0.44	6.32±0.59	5.83±0.20	5.51±0.42
0.5 x 10^7	5.78±0.14	6.27±0.62	5.82±0.29	5.45±0.17

Table III. Lipid Content (%) of Yeast at Different Initial Cell Densities

Time (day) / Cell Density (cells/mL)	2	3	4	5
2 x 10^7	51.52±4.97	56.35±14.97	49.17±12.69	54.73±9.39
1 x 10^7	42.48±3.15	45.00±19.45	37.06±3.14	33.32±1.39
0.8 x 10^7	32.09±10.77	32.15±6.74	37.24±10.48	43.21±10.08
0.5 x 10^7	40.96±6.99	35.87±5.27	32.76±1.43	40.21±10.80

Table IV. Lipid Yield (g/L) of Yeast at Different Initial Cell Densities

Time (day) / Cell Density (cells/mL)	2	3	4	5
2×10^7	3.42±0.39	2.89±0.12	3.01±0.59	3.04±0.55
1×10^7	2.28±0.26	2.30±0.30	2.20±0.13	1.92±0.14
0.8×10^7	1.81±0.66	2.04±0.47	2.16±0.54	2.35±0.37
0.5×10^7	2.37±0.43	2.23±0.13	1.91±0.18	2.18±0.53

4. Conclusion

The optimal initial cell density for the lipid production from distillery wastewater by the oleaginous yeast strain *Rhodosporidium toruloides* AS 2.1389 was shown 2×10^7 cells/mL. The removal of organic matter and nutrients was similar when the initial cell density changed from 0.5×10^7 to 2×10^7 cells/mL, while the higher lipid production was obtained when the initial cell density was 2×10^7 cells/mL. The remained seed culture medium after the first time use was still able to produce nearly the same amount of cells in seed culture as the fresh seed culture medium without the addition of any extra nutrient. This further suggests the possibility for the seed culture medium to be reused for the second time or even for the third time with the addition of glucose, resulting in cost savings.

Acknowledgement

This research was supported by a grant from the University of Macau Research and Development Administration Office (RDAO) through the Multi-Year Research Grant (MYRG).

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