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Enhancement of lipid productivity of *Rhodosporidium toruloides* in distillery wastewater by increasing cell density

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HIGHLIGHTS

- New inoculation method in the process of lipid production from wastewater by yeast.
- Applicable to raw distillery wastewater without addition of external nutrients.
- Applicable to distillery wastewater without sterilization and pH adjustment.
- High lipid production and high COD, TN, and TP removal.
- Relatively short cultivation time.

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ABSTRACT

This study is to improve the process of producing lipid convertible to biodiesel, from distillery wastewater while simultaneously removing organics and nutrients efficiently by inoculating oleaginous yeast *Rhodosporidium toruloides* in the presence of indigenous microorganisms. The lipid productivity of *R. toruloides* was studied using real wastewater obtained from distillery and local municipal wastewater treatment plants. Under the conditions of mix rate of 1:1 with domestic wastewater, initial soluble chemical oxygen demand (SCOD) over 20,000 mg/L and initial cell density of 2×10^7 cells/mL at 30 °C, lipid content and lipid yield achieved were 43.65 ± 1.74% and 3.54 ± 0.04 g/L, with the associated removal efficiencies for COD, total nitrogen (TN), and total phosphorus (TP), 86.11 ± 0.41%, 57.81 ± 0.29%, and 67.69 ± 0.73%, respectively, after three days of cultivation in real distillery wastewater without pH adjustment. The pH of wastewater increased from 3.71 to over 8 in 7 days of cultivation.

1. Introduction

The rapid shrink of fossil fuel global reserves, high energy prices, and environmental security has led to the increasing demand of substitute fuels. As a renewable and alternative source of energy, biodiesel has increasingly attracted attention worldwide. Biodiesel refers to a fuel consisting of mono-alkyl esters of long chain fatty acids derived from vegetable oils and animal fats, and alcohols of lower molecular weights in the presence of catalysts. The reaction adopted for the production of biodiesel is transesterification which could be catalyzed by homogeneous or heterogeneous catalysts (Atadashi et al., 2010; Mondala et al., 2009).

Biodiesel is an attractive new energy resource not only because it is renewable but also because it has some favorable environmental benefits in reducing generation of carbon dioxide and air pollutions related to sulfur. The carbon dioxide emission could be reduced by 10% when using biodiesel as a substitute of diesel (Atadashi et al., 2010). Furthermore, the biodegradability of biodiesel was recorded to be 84% after 30 days though still with some negative impact on indigenous microbial communities, while that of normal diesel was just 13.6% (Silva et al., 2012). Less environmental contamination in soil and water would follow if biodiesel used when leaking or spilling accidents happen (Wu et al., 2010).

Wastewater generated from food industries contains plenty of organic matters which render it more difficult to treat than the municipal one. If organic matters in wastewater could be recovered and converted into biodiesel by microorganisms, not only could it reduce the cost of wastewater treatment but also the waste could be turned into resource. There have been many studies about the production of biodiesel by microalgae, mainly cultured in sea water, normal fresh water, and municipal wastewater (Lardon et al., 2009; Mutanda et al., 2011), and oleaginous yeasts were shown to be slightly more widely used feedstock in the process of producing microbial lipid from wastewater with high amount of organic matter like food industry wastewater. Similar to microalgae, oleaginous yeasts show a great potential as

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Table 1
Composition of wastewater samples.

	SCOD (mg/L)	TN (mg/L)	TP (mg/L)	SS (mg/L)	pH	C/N	C/P
Distillery wastewater	52,900	2540	360	–	3.70	21:1	147:1
Domestic wastewater (TAI-I)	197	45	6.5	320	7.31	4:1	30:1
Mixture (1:1, v/v) wastewater	23,975	1227	195	–	3.71	20:1	123:1

SCOD, soluble chemical oxygen demand; TN, total nitrogen; TP, total phosphorus; SS, suspended solid.

biodiesel feedstock among microbial sources, with a relatively short doubling time of about 90 min (Sherman, 2002) and a cell cycle time of around 140–160 min (Abe et al., 1984; Lovrics et al., 2006) compared to microalgae (3.5–72 h) (Chisti, 2007; Sheehan et al., 1998). The doubling time of a newly isolated oleaginous microalgal strain *Chlorella* sp. was reported about 15 h during the exponential phase (Rasoul-Amini et al., 2011). In addition, oleaginous yeasts are less affected by the climate and seasonal prerequisites than plants and their cultures are more easily expandable than microalgae. Oleaginous yeast 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 can produce different lipids from different carbon sources in the culture medium. Oleaginous yeasts have been reported a good candidate in the production of polyunsaturated fatty acids (Ageitos et al., 2011; Subramaniam et al., 2010).

Some studies have been done for the dual purpose of treating wastewater and producing microbial lipid. The lipid content of *Rhodotorula glutinis* and *Cryptococcus curvatus* grown in distillery wastewater generated from the tequila production process was reported 27.02 ± 2.36% and 25.2 ± 1.98% of cell dry weight, respectively, with the COD removal efficiency of 78.98% and 84.44%, respectively, in pure cultures (Gonzalez-Garcia et al., 2013). *Rhodosporidium toruloides* Y2 was reported able to produce 3.8 g/L of lipid with the lipid content of 34.9% of cell yield and the COD removal efficiency of 72.3% for the bioethanol wastewater, and the lipid yield and content could be further increased by 39.5% and 53.8%, respectively, with the addition of 1.2 g/L/d of glucose under the sterile condition (Zhou et al., 2013). However, very little study has been done using real non-sterile wastewater without the addition of other nutrients like glucose, while the process of sterilization would consume a lot of energy. This study, therefore, is first to improve the lipid productivity of oleaginous yeast in real wastewater obtained from distillery and local domestic wastewater treatment plants and to further investigate the associated removal efficiencies for organic matters (COD) as well as nutrients (nitrogen and phosphorus containing compounds) in wastewater.

2. Methods

2.1. Strain, medium, and wastewater

Yeast strain *R. toruloides* AS 2.1389 used in this study was purchased from the China General Microbiological Culture Collection Center, sub-cultured on distillery wastewater agar medium plates, and maintained on the distillery wastewater agar medium 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 (Li et al., 2005, 2010; Wu et al., 2010) and (Wu et al., 2011; Zhao et al., 2011). The distillery wastewater agar medium was made of rice wine distillery wastewater (vinasses) 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 (Li et al., 2005). Both media were sterilized at 121 °C for 20 min before used. The rice wine distillery wastewater (vinasses) was obtained from the S1 distillery in Foshan city, China, and the domestic wastewater was from the Taipa municipal wastewater treatment plant in Macau SAR, China. The 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.

2.2. Experimental setup

The oleaginous yeast *R. toruloides* strain grown from the distillery wastewater medium slant or plate was transferred to 150-mL flask containing 50 mL YPD medium, cultivated at 30 °C and 200 rpm for 24 h, and used as seed culture for the orthogonal experiment I. Cells from another seed culture cultivated for 36 h were centrifuged at 4000 rpm for 10 min to obtain the high cell density of 2.5×10^9 cells/mL (Lin et al., 2010), and used for the orthogonal experiment II using the mixture of distillery wastewater and domestic wastewater. Cell density was measured and calculated using cell chamber.

Thirty millilitres of distillery wastewater diluted with distilled water was added to 150-mL flasks and sterilized at 121 °C for 20 min before used as culture medium in orthogonal experiment I and the respective distillery wastewater without sterilization was used in orthogonal experiment II. The seed culture was inoculated to wastewater under conditions of 200 rpm, different temperatures (25, 30, and 35 °C), different inoculum sizes (5%, 10%, and 15%; v/v) or different initial cell densities in culture medium (0.5×10^8, 1.0×10^8, and 2.0×10^8 cells/mL), and different incubation periods (3–7 days). The orthogonal experimental design table L_9 (3^4) was used in this experiment (Lazic and Mastorakis, 2008). After incubation, samples were centrifuged at 4000 rpm for 10 min (Li et al., 2005), and COD and pH of supernatant and cell yield and lipid yield of pellet were analyzed.

For the mixture of distillery and domestic wastewater (1:1 ratio; Table 1), used as culture medium, 30 mL non-sterile wastewater mixture was first added to 150-mL flasks. Then, the seed culture with different cell densities (0.2×10^8, 0.3×10^8, 0.4×10^8, and 0.5×10^8 cells/mL) was inoculated into the mixture culture medium and cultivated at 30 °C and 200 rpm for 7 days. Samples were taken in every 24 h for the analyses of COD, TN, TP, NH_3-N, and pH in supernatants and cell yield and lipid yield in pellets.

The yeast seed culture was prepared in 150-mL flasks containing 30 and 50 mL of sterilized YPD medium and cultivated at 30 °C and 200 rpm for 24 and 36 h. All the cell densities were measured using cell chamber, and all the experiments were conducted in replicates.

2.3. Analytical methods

Chemical oxygen demand (COD), total nitrogen (TN), and total phosphorus (TP) were measured using Hach's reagents with Hach reactor DRB200 and spectrophotometer DR2800, following the HACH methods (HACH DR/2800, Hach Company, Loveland, Colorado) and the Standard Methods APHA (American Public Health Administration)/AWWA (American Water Works Association)/