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Short Communication

Oil production on wastewaters after butanol fermentation by oleaginous yeast *Trichosporon coremiiforme*

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HIGHLIGHTS

- Oleaginous yeast *Trichosporon coremiiforme* was used for microbial oil production.
- Wastewaters after butanol fermentation were used for microbial oil production.
- COD degradation of the wastewaters by the treatment of *T. coremiiforme* was attractive.
- Biomass and lipid content of *T. coremiiforme* showed its potential for oil production.
- Lipid composition of *T. coremiiforme* showed its feasibility for biodiesel production.

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ABSTRACT

From the distillation process after butanol fermentation, wastewaters mainly consisted of organic acids and residual sugars and with high COD (usually >20,000 mg/L) are generated. Without any pretreatment and adding other nutrients (nitrogen sources and trace elements), these wastewaters were used as substrate for microbial oil production by oleaginous yeast *Trichosporon coremiiforme*. After 5 days' lipid fermentation, all the sugars and organic acids measured were totally utilized by *T. coremiiforme* and a 68% of COD degradation could be obtained. The highest biomass and lipid content of *T. coremiiforme* on the wastewaters were 5.8 g/L and 19.1%, respectively. This work shows that *T. coremiiforme* is a promising strain for microbial oil production on the wastewaters after butanol fermentation.

1. Introduction

Microbial oil, namely single cell oils (SCO), was focused in recent years not only for its important function as the supplier of valuable lipids but also its great potential as the feedstock for biodiesel production (Papanikolaou and Aggelis, 2011). However, the high cost of fermentation substrates limits its further application and industrialization. Thus, various low-cost raw materials such as industrial oils and fats (Papanikolaou and Aggelis, 2001), glycerol (Easterling et al., 2009), and lignocellulosic biomass (Huang et al., 2012) were used for microbial oil production. Besides, many works showed that oleaginous yeasts could accumulate lipid on various wastewaters such as corncob waste liquor (Venkata Subhash and Venkata Mohan, 2011), monosodium glutamate wastewaters (Xue et al., 2006), and olive oil mill wastewaters (Yousuf et al., 2010). This bioconversion process could not only solve the environmental problems of wastewaters but also reduce the cost of microbial oil production significantly.

Nowadays, liquid fuels produced by fermentation have been focused by many researchers in order to solve the problem caused by the shortage of petroleum sources. Among them, bio-butanol was considered as an attractive one since that it could be used as a gasoline additive, or even as a complete gasoline replacement. Also, it has higher energy content, lower volatility and less corrosiveness when compared with ethanol (Li et al., 2011). After butanol fermentation by different microorganisms, various products (butanol, ethanol, and acetone) in the fermentation broth could be easily recovered by distillation process. However, the remaining fermentation broth whose COD was usually higher than 20,000 mg/L needs further treatment before drainage. Different from other wastewaters, besides residual sugars, these wastewaters mainly contain different organic acids (mainly butyric acid and acetic acid).

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To date, the oleaginous yeasts used for wastewaters treatment mainly included *Lipomyces starkeyi* (Angerbauer et al., 2008), *Cryptococcus curvatus* (Chi et al., 2011), *Rhodotorula glutinis* (Xue et al., 2006), and etc. Interestingly, volatile fatty acids were proven to be a promising carbon source for microbial oil production by the oleaginous yeast *Cryptococcus albidus* recently (Fei et al., 2010). Base on this phenomenon, oleaginous yeasts might have potential to use wastewaters after butanol fermentation which contained lots of organic acids for microbial oil production. In the present study, *Trichosporon coremiiforme*, an oleaginous yeast was investigated for its capability to produce oil when grown on wastewaters from the distillation process after butanol fermentation.

2. Methods

2.1. Wastewaters preparation

The original wastewaters after butanol fermentation were obtained from ZHONGKE New Energy Co., Ltd. (Ying-kou, China). According to ZHONGKE New Energy Co., Ltd., their butanol fermentation was carried out on the medium containing cellobiose, xylose, glucose, and arabinose. After fermentation, more than 99% of butanol, ethanol, and acetone in the fermentation broth were recovered by distillation. The remaining fermentation broth which contained the residual sugars and organic acids were the wastewaters used in this work. The initial COD of the wastewaters was about 23,560 mg/L.

2.2. Microorganism and microbial oil production on wastewaters

Oleaginous yeast *Trichosporon coremiiforme* CH005 (stored by Laboratory of Energy and Biochemical Engineering, Guangzhou Institute of Energy Conversion, Chinese Academy of Sciences) was used for microbial oil production. It is an adapted strain isolated after cultivation of *T. coremiiforme* CICC 1256 (obtained from China Center of Industrial Culture Collection) on the wastewaters after butanol fermentation for more than half a year.

The pre-culture was performed on pre-cultivation medium (g/L, glucose 20, peptone 10, yeast extract 10, and with initial pH of 6.0) at 28 °C and 150 rpm for 24 h. The wastewaters after butanol fermentation without adding other nutrients (nitrogen sources or trace elements) were used as the medium (initial pH 7.0) for microbial oil production by *T. coremiiforme*. 10% seed culture was inoculated to culture medium. Cultivation was performed in a 250 mL conical flask containing 50 mL fermentation broth in a rotary shaker at 28 °C and 150 rpm.

2.3. Analytical methods

Biomass was harvested by centrifugation and determined by its dry cell weight. After centrifugation, the supernatant of fermentation broth (wastewaters) was used for the detection of sugars and organic acids concentration. Extraction of lipid from dry biomass was used a mixture of chloroform: methanol (2:1, v/v). The extracted lipid was centrifuged to obtain a clear supernatant and the solvent was removed by vacuum evaporation. Lipid yield is expressed as the amount of lipid extracted from the cells in per liter fermentation broth (g/L) and lipid content is defined as the percentage of lipid to dry biomass (%, w/w).

The fatty acid composition was measured by converting fatty acids into fatty acid methyl esters and the fatty acid methyl esters were determined by GC (GC-2010, Shimadzu Corporation, Japan) with ionization detector and an Rtx®-Wax capillary column (Restek Corporation, USA). The column temperature was maintained at 195 °C for 12 min and upgraded from 195 to 230 °C at a rate of 10 °C min^{-1} and kept for 15 min. Nitrogen was used as the carrier gas at 1.0 mL min^{-1}. Split ratio was 1:30 (v/v). The injector and the detector temperatures were set at 250 °C and 280 °C, respectively.

The acetic acid and butyric acid in wastewaters were also analyzed by GC (GC-HP6820, Hewlett Packard Corporation, USA) with FID detector and a DB-FFAP capillary column (Agilent Technologies Co. Ltd., USA). The column temperature was maintained at 40 °C for 5 min and upgraded to 140 °C at a rate of 20 °C min^{-1} and kept for 3 min, then upgraded to 250 °C at a rate of 40 °C min^{-1} and kept for 3 min. Nitrogen was used as the carrier gas at 1.0 mL min^{-1}. Split ratio was 1:30 (v/v). The injector and the detector temperature were set at 250 and 300 °C, respectively.

Sugars (D-xylose, and L-arabinose and etc.) concentrations in the wastewaters were analyzed by HPLC (Waters 2685 systems, Waters Corp., USA), with a RI detector (Waters 2414), and on Aminex HPX-87H column (300 mm × 7.8 mm, Bio Rad Corp., USA) using 5 mM H_2SO_4 solution as mobile phase at 0.6 mL min^{-1}, and the HPLC was operated at 65 °C. COD and ammonium nitrogen in the wastewaters was evaluated by Hach DR2700 Water Quality Analyzer (Hach Company, USA).

3. Results and discussion

3.1. Lipid production on wastewaters

In the wastewaters generated after butanol fermentation, both glucose and cellobiose have been exhausted and the residual sugars were mainly xylose and arabinose. After distillation, butanol, ethanol, and acetone in the fermentation broth were almost totally recovered, and the organic acids remaining in the wastewaters were mainly acetic acid and butyric acid. Overall, the COD of the wastewaters used in this work was about 23,560 mg/L. Without any pretreatment or adding any nutrients, it was used as substrate for microbial oil production by *T. coremiiforme*.

The time courses of growth and lipid accumulation of *T. coremiiforme* were depicted in Fig 1A. During the first day of lipid fermentation, the biomass of *T. coremiiforme* increased slowly possibly due to its adaption to the environment of the wastewaters. Then, the growth of *T. coremiiforme* became faster until the 60th hour. After 3 days' fermentation, the biomass of *T. coremiiforme* maintained from 5 to 6 g/L. On the other hand, the lipid content of *T. coremiiforme* increased quickly during the first 36 h of fermentation. The highest lipid content of *T. coremiiforme* (19.1%) was achieved at the 36th hour. And then, the lipid content of *T. coremiiforme* decreased fast possibly due to using cellular lipid as carbon source to maintain its growth. This phenomenon so-called "lipid turnover" was also observed in other researches (Huang et al., 2009; Papanikolaou et al., 2001). Interestingly, after 72 h of fermentation, the lipid content of *T. coremiiforme* varied a little as the fermentation went on. This suggests that the process of lipid accumulation and lipid degradation might exist simultaneously when the carbon sources were limited for lipid fermentation on the wastewaters.

It was shown that lipid composition of oleaginous microorganism could change significantly throughout the fermentation process (Fakas et al., 2009). Thus, in this work, the lipid composition of *T. coremiiforme* during the lipid fermentation was analyzed. As shown in Table 1, the lipid composition of *T. coremiiforme* varied significantly during the fermentation process. During the first 2 days' fermentation, interestingly, the ratio of linoleic acid decreased firstly and then increased again. Surprisingly, the ratio of linoleic acid decreased significantly after 2 days' fermentation, and linoleic acid even disappeared. However, after 4 days' fermentation, linoleic acid was generated by *T. coremiiforme* again. When

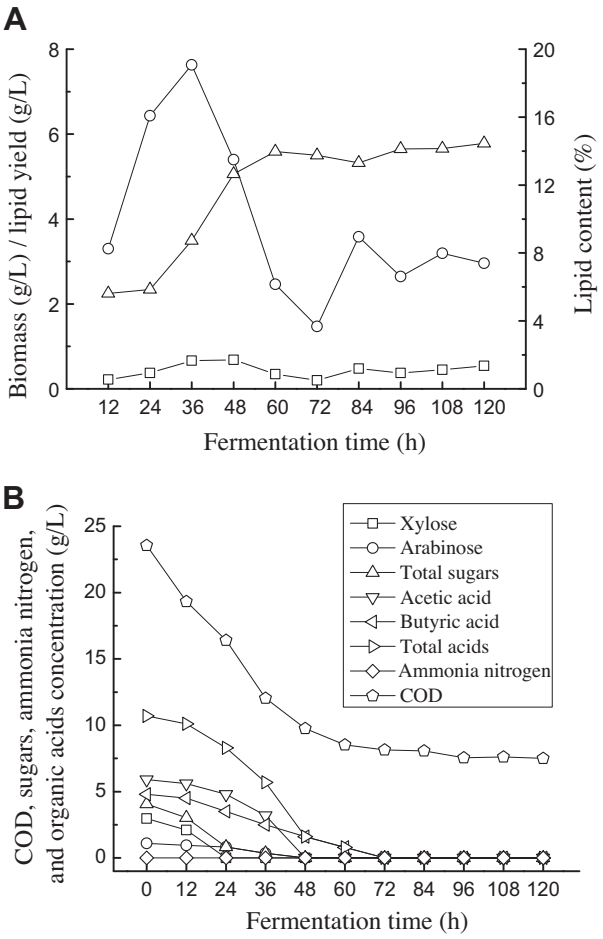

Fig. 1. Production of microbial oil on the wastewaters after butanol fermentation by *T. coremiiforme* (A): Time course of cell growth and lipid accumulation. (△) Biomass; (□) lipid yield; (○) lipid content; (B): Change of sugar and organic acid concentration, COD value, and ammonium nitrogen concentration.

Table 1
lipid composition of *T. coremiiforme* during the lipid fermentation process on the wastewaters after butanol fermentation.

Fermentation time (h)	Lipid composition of *T. coremiiforme* (%)				
	C16:0	C18:0	C18:1	C18:2	Others[a]
12	21.5	29.8	26.6	13.5	8.6
24	32.2	24.9	29.6	8.8	4.5
36	36.3	26.3	25.7	7.1	4.5
48	26.2	19.1	35.1	15.2	4.3
60	37.8	23.6	29.2	2.9	6.5
72	44.3	30.9	14.6	ND[b]	10.3
84	41.4	27.7	23.3	ND	7.6
96	41.6	29.0	21.7	ND	7.7
108	30.8	19.9	33.4	9.5	6.4
120	29.3	22.3	36.4	7.0	5.1

[a] Others were C8:0, C10:0, C12:0, C14:0, C16:1, C18:3, C20:0 C20:1, C20:2, C22:0, and C24:0.
[b] ND means not detected.

most assimilable carbon sources were depleted (Fig 1B), *T. coremiiforme* might firstly prefer using unsaturated fatty acids to maintain its growth. However, when the linoleic acid was used up, it might stimulate the desaturase activity of *T. coremiiforme* and thus linoleic acid was accumulated again. Finally, the fatty acids ratio of *T. coremiiforme* became stable after 4 days' fermentation. Overall, the lipid extracted from *T. coremiiforme* mainly contained palmitic acid, stearic acid, oleic acid and linoleic acid and this lipid composition is similar to that of vegetable oils. Thus, it is a promising feedstock for biodiesel production.

3.2. Carbon and nitrogen metabolism of *T. coremiiforme*

To get a deeper insight on the metabolism of oleaginous yeast *T. coremiiforme* on the wastewaters, the evolution of sugar and organic acid, COD value, and ammonium nitrogen during lipid fermentation were further measured (Fig 1B). After butanol fermentation, most sugars have been exhausted and 4.1 g/L residual sugars which were mainly xylose and arabinose still remained in the wastewaters. As shown in Fig 1B, the utilization of xylose by *T. coremiiforme* was much faster than that of arabinose. After 24 h of fermentation, all the xylose has been exhausted by *T. coremiiforme* and the utilization of arabinose became faster. Finally, all the sugars were used up by *T. coremiiforme* after 48 h of fermentation. The utilization of organic acids by *T. coremiiforme* was different from that of sugars utilization. Obviously, *T. coremiiforme* could assimilate acetic acid and butyric acid simultaneously. However, as shown in Fig 1B, acetic acid was a better carbon source for *T. coremiiforme* than butyric acid in the wastewaters that acetic acid was totally consumed by *T. coremiiforme* after 2 days' fermentation but butyric acids was exhausted by *T. coremiiforme* after 3 days' fermentation. All the sugars and organic acids measured were totally utilized by *T. coremiiforme*, indicating that it had a broad spectrum of carbon source and therefore is a potential oleaginous microorganism for microbial oil production from various low-cost substrates.

Obviously, the high initial COD value of the wastewaters generated from the distillation process after butanol fermentation was partly due to the relatively high concentration of residual organic acids and sugars present in it. As shown in Fig. 1B, the COD value decreased fast during the first 2 days' fermentation. This was because of the fast utilization of sugars and organic acids for the growth and lipid accumulation of *T. coremiiforme*. However, after the depletion of fermentable sugars and organic acids in the wastewaters, the COD value decreased much slower. Overall, the highest COD degradation was about 68% after 5 days' fermentation. Some reasons were possible to explain the incomplete COD degradation. On the one hand, the wastewaters used in this work were generated from butanol fermentation. Besides acetic acid and butyric acid, other complex organic acids or chemical compounds might also exist in the wastewaters. It is possible that these chemical compounds were difficult to be degraded by this oleaginous yeast. On the other hand, it is also possible that the metabolic activity of *T. coremiiforme* might be weaker during the lipid fermentation, which also influenced its performance on the degradation of COD.

Generally speaking, there are two types of lipid accumulation process in oleaginous microorganisms, namely *de novo* lipid accumulation and *ex novo* lipid accumulation (Papanikolaou and Aggelis, 2011). Different from the lipid fermentation on hydrophobic substrates (*ex novo* lipid accumulation), *de novo* lipid accumulation (on the sugars or other hydrophilic substrates) usually requires limited nitrogen sources. In this work, the ammonium nitrogen concentration was extremely low (less than 6 mg/L) throughout the lipid fermentation process. However, it is worth noting that the lipid content (less than 20%) of *T. coremiiforme* was not high during the lipid fermentation process. It is possible that the concentration of carbon sources in the wastewaters were too low and insufficient for lipid synthesis of *T. coremiiforme*, thus resulted in its low lipid content.

3.3. Comparison of lipid production on various wastewaters

To date, many kinds of wastewaters have been used as the substrates for microbial oil production. Thus, the microbial oil produc-

Table 2
Lipid production on different wastewaters by various microorganisms.

Strains	Wastewaters	Biomass (g/L)	Lipid content (%)	Initial COD (mg/L)	COD degradation (%)	References
Oleaginous consortium	Municipal wastewater	0.6	20	2450	81.0	Hall et al. (2011)
Aspergillus sp.	Corncob waste liquor	2.0	22.1	5000	60.0	Venkata Subhash and Venkata Mohan (2011)
R. glutinis	Monosodium glutamate wastewater	1.5	9.1	20,000	43.8	Xue et al. (2006)
L. starkey	Olive oil mill wastewaters	11.0	22.4	43,000	ND[a]	Yousuf et al. (2010)
T. coremiiforme	Wastewater after butanol fermentation	5.8	19.1	23,560	68.0	This work

[a] No definite COD degradation in this work, the removal rate of sugars, proteins, and phenols were 48%, 82%, and 43%, respectively.

tion by *T. coremiiforme* on the wastewaters after butanol fermentation was compared with other SCO production on various wastewaters. As shown in Table 2, the COD degradation of wastewaters in this work was higher than other wastewaters except the municipal wastewaters. However, it is worth noting that the initial COD value of the wastewaters in this work was higher than that of the municipal wastewaters. The lipid content of *T. coremiiforme* was close to that of other oleaginous microorganisms in Table 2. On the other hand, the biomass of *T. coremiiforme* was higher than other microorganism except *L. starkey*. The high biomass value of *L. starkey* on the olive oil mill wastewaters was possibly due to its high initial COD value. Overall, the lipid yield of *T. coremiiforme* (depend on its biomass and lipid content, see Fig 1A) on the wastewaters in this work showed its potential to be further scaled up in future.

4. Conclusions

Without any pretreatment and adding other nitrogen sources or trace elements, the wastewaters after butanol fermentation could be potential substrates for microbial oil production by *T. coremiiforme*. This bioconversion could both solve the environmental problem and offer low-cost lipid feedstock for biodiesel production.

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