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Converting Chemical Oxygen Demand (COD) of Cellulosic Ethanol Fermentation Wastewater into Microbial Lipid by Oleaginous Yeast *Trichosporon cutaneum*

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Abstract Cellulosic ethanol fermentation wastewater is the stillage stream of distillation column of cellulosic ethanol fermentation broth with high chemical oxygen demand (COD). The COD is required to reduce before the wastewater is released or recycled. Without any pretreatment nor external nutrients, the cellulosic ethanol fermentation wastewater bioconversion by *Trichosporon cutaneum* ACCC 20271 was carried out for the first time. The major components of the wastewater including glucose, xylose, acetic acid, ethanol, and partial of phenolic compounds could be utilized by *T. cutaneum* ACCC 20271. In a 3-L bioreactor, 2.16 g/L of microbial lipid accumulated with 55.05% of COD reduced after a 5-day culture of *T. cutaneum* ACCC 20271 in the wastewater. The fatty acid composition of the derived microbial lipid was similar with vegetable oil, in which it could be used as biodiesel production feedstock. This study will both solve the environmental problem and offer low-cost lipid feedstock for biodiesel production.

Keywords *Trichosporon cutaneum* · Cellulosic ethanol fermentation wastewater · Chemical oxygen demand (COD) · Microbial lipid

Introduction

Biorefining process of lignocellulose biomass for production of fuel ethanol includes pretreatment to disrupt the rigorous structure, detoxification to remove inhibitor compounds, enzymatic hydrolysis

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to yield fermentable sugars, fermentation to produce ethanol, and distillation to anhydrous ethanol up to the purity of 99.5% (*v*/*v*) [1, 2]. In the final distillation step, ethanol fermentation broth is stripped to 30–40% (*v*/*v*) of ethanol as the distillate stream and the water-solid slurry as the stillage stream. After the solids in the stillage are removed by solid/liquid separation operation, the stillage liquid is sent to the wastewater treatment unit [3]. The wastewater generated is approximately 6–80 t per ton of fuel ethanol produced according to the specific process options, which are much more than the wastewater generation of corn-based ethanol [4]. The wastewater contains high chemical oxygen demand (COD, approximately 125 g/L) [5] and various residual sugars and inhibitor compounds from pretreatment step. A practical solution of wastewater treatment should be found before the commercial production of fuel ethanol from lignocellulose [5].

Several methods had been used to remove the COD from fermentation wastewater. In the cellulosic ethanol process design by the National Renewable Energy Laboratory (NREL), USA, the wastewater is treated by a series of steps including anaerobic treatment (biogas generation), activated sludge system ammonia-nitrogen removal, membrane filtration, evaporation, and centrifugation (biological sludge dewatering) [6]. Wang and Zheng [7] used anaerobic sludge blanket (UASB) and membrane bioreactor (MBR) to treat the cellulose ethanol fermentation wastewater to lead to 99% of COD reduction [8]. Microbial lipid fermentation is one of the options to reduce the COD by converting the residual sugars and organic compounds in fermentation wastewater into lipid [9]. Wastewater streams from different fermentation processes had been tried, including glutamic acid fermentation wastewater [10], municipal wastewater [11], and distillery wastewater [12]. Of the COD in ethanol fermentation wastewater from sweet potato, 72.3% was reduced by oleaginous yeast *Rhodosporidium toruloides* Y2 with 1.33 g/L of lipid formation [13]. The COD degradation of the acetone-butanol-ethanol (ABE) fermentation wastewater by *Trichosporon cutaneum* CH002 and *Trichosporon dermatis* CH007 reached to 68% with 1.00 g/L of lipid [14, 15]. At present, biodiesel was mainly produced from vegetable oils that would result in a shortage of edible oils and would increase the price of food [16]. Microbial lipid is an important feedstock for biodiesel production [17], and the accumulation of microbial lipid from cellulosic ethanol wastewater treatment provides an alternative.

Lipid fermentation using lignocellulose feedstock by various oleaginous yeast strains had been studied in details [15, 18–20]. However, lipid fermentation using cellulosic ethanol fermentation wastewater as feedstock had not yet been reported up to now. In this study, an oleaginous yeast *T. cutaneum* ACCC 20271 was used as the fermenting strain using cellulosic ethanol fermentation wastewater for COD reduction and lipid accumulation. An obvious COD reduction and a lipid accumulation were observed using the cellulosic fermentation wastewater feedstock. This study provided a practical solution on cellulosic fermentation wastewater treatment by lipid accumulation.

Materials and Methods

Strains and Seed Culture

T. cutaneum ACCC 20271, which was purchased from Agricultural Culture Collection of China (ACCC), Beijing, China (http://www.accc.org.cn/), was selected as the microbial lipid fermentation strain. The xylose-utilizing ethanol fermentation strain *Saccharomyces cerevisiae* XH7 was granted from Professor Xiaoming Bao's group at School of Life Science, Shandong University, China. *Amorphotheca resinae* ZN1 (CGMCC 7452) was isolated in our previous

work [21] and used as a biodetoxification strain for inhibitor degradation. The seed culture of yeast strains and *A. resinae* ZN1 was carried out according to Wang et al. [18].

Raw Material and Enzyme

Corn stover was obtained from Bayan Nur, Inner Mongolia, China, in fall 2015. The raw corn stover contained 35.38% of cellulose, 24.62% of hemicellulose, 16.05% of lignin, and 3.47% of ash on dry weight base (*w*/*w*) determined by a two-step sulfuric acid hydrolysis according to the NREL laboratory analytical procedure (LAP) protocols [22, 23].

Cellulase enzyme CTec2 was provided by Novozymes, Beijing, China. According to the methods of NREL LAP-006 [24] and Ghose [25], its filter paper activity and cellobiase activity were 203.20 FPU/g and 4900 CBU/mL, respectively.

Biorefining of Corn Stover for Ethanol Production

The dry dilute acid pretreatment of corn stover was carried out according to Zhang et al. [21] and He et al. [26]. The pretreated corn stover contained 37.64% of cellulose and 4.40% of hemicellulose according to NREL LAP protocols [22, 23], along with 15.29 mg of furfural, 3.28 mg of HMF, and 2.77 mg of acetic acid per gram of dry solid matter.

Inhibitors that existed in the pretreated corn stover were biodegraded by *A. resinae* ZN1 according to Zhang et al. [21] and He et al. [27]. The inhibitors were all completely degraded by *A. resinae* ZN1 except of acetic acid with the concentration of 1.27 mg/g dry solid matter.

The simultaneous saccharification and cofermentation (SSCF) operation was performed in a 5-L bioreactor equipped with a helical ribbon impeller as described by Zhang et al. [28]. The prehydrolysis stage was started when cellulase enzyme was fed into the bioreactor and lasted for 12 h at 50 °C and pH 4.8, at 30% (*w*/*w*) solid loading. Then, SSCF stage started after inoculating an engineered *S. cerevisiae* XH7 strain (with xylose utilization function) and lasted for 96 h at 30 °C, pH 5.5.

Lipid Fermentation Using Cellulosic Fermentation Wastewater

Lipid fermentation in flasks by *T. cutaneum* ACCC 20271 was carried out in a 500-mL conical flask containing 50 mL of cellulosic ethanol fermentation wastewater at 30 °C and different nutrient additions (0.22 g/L of $(NH_4)_2SO_4$, 0.5 g/L of $MgSO_4{\cdot}7H_2O$, 1.0 g/L of KH_2PO_4, 0.5 g/L of YE), pH values, or rotation rates for 120 h.

Lipid fermentation in a 3-L bioreactor (Baoxing Biotech Co., Shanghai, China) by *T. cutaneum* ACCC 20271 was performed at 30 °C, aeration rate of 1.67 vvm, and the stirring rate at 600 rpm, and pH was maintained at 5.0 automatically. Ten percent (*v*/*v*) of seed culture broth was inoculated into the 1 L cellulosic ethanol fermentation wastewater without any other nutrient additions. All experiments were performed twice, and the derivation was calculated.

Determination of COD in Wastewater

The COD concentration of wastewater was assayed according to the China Standard for water quality [29]. Wastewater was diluted to the COD at the range of 0.05 to 0.7 g/L. Twenty milliliters of the dilute wastewater and 30 mL of sulfate silver solution (10 g/L) with zeolite particles were mixed in a 250-mL flask and boiled for 2 h. Then, 90 mL of distilled water was added after the

temperature was reduced to room temperature. It was titrated with a 0.1-M ferrous ammonium sulfate solution till the solution changed to red-brown from yellow and blue-green with ferrous spirit as the indicator, with equal volume of pure water as the blank. Calculate the concentration of the ferrous ammonium sulfate solution according to the following equation:

$$\mathrm{COD}\ (\mathrm{mg/L}) = \frac{(V_0 - V_1) \times C \times 8 \times 1000}{V}$$

where C, ferrous ammonium sulfate solution concentration, 0.1 M; V_0, the volume of ferrous ammonium sulfate solution in titration of blank (mL); V_1, the volume of ferrous ammonium sulfate solution in titration of wastewater (mL); V, the volume of wastewater (mL); and 8, molar mass of one-half oxygen.

Analysis Method

The concentrations of glucose, xylose, acetic acid, and ethanol were analyzed by HPLC (LC-20AD, Shimadzu, Japan) equipped a Bio-Rad Aminex HPX-87H column at 65 °C and a refractive index RID-10A detector. The mobile phase was 5 mM sulfuric acid with a flow rate of 0.6 mL/min.

The total sugars were measured by the dinitrosalicylic acid (DNS) method using glucose as the standard [30]. Three-milliliter dilute wastewater and 0.2 mL 6 M HCl to a calibration tube were mixed and incubated at 121 °C for 30 min. Adjust the pH to 6.0 with 6 M NaOH and constant volume to 10 mL. The sum of reducing sugars was equal to total sugar content.

The total phenolic content of wastewater was determined using the Folin-Ciocalteu reagent [31]. Of the dilute (50 to 100 times) wastewater, 0.5 mL was mixed with 1.0 mL 15% (*v*/*v*) Folin-phenol reagent, and then, 4.0 mL of a 1-M $NaCO_3$ solution was added into the mixture. The solutions were mixed and allowed to stand at room temperature for 2 h. The total phenolic content was determined by the absorbance at 765 nm. The calibration curve was drawn using the standard solutions of 0.100 mg/mL of gallic acid at five dilute concentrations.

Dry cell weight (DCW) was evaluated gravimetrically after being dried at 80 °C overnight. The lipid in yeast cells was extracted according to the chloroform-methanol method [32]. The fatty acid compositional profile of the lipid derived from cellulosic ethanol wastewater was performed according to Zhang et al. [33].

Results and Discussion

Distillation Stillage Liquid Preparation from Cellulosic Ethanol Fermentation

Corn stover was pretreated in a dry dilute acid way, followed by biodetoxification and fermentation using a xylose-utilizing yeast with only 3.07 g/L of glucose and 6.91 g/L of xylose left in the final fermentation broth. The ethanol fermentation broth was directly distillated into approximately 70% (*v*/*v*) crude ethanol solution as distillate and the slurry containing water-insoluble solids (WIS) as the stillage. The stillage liquid contained 7.25 g/L of glucose, 11.52 g/L of xylose, 38.56 g/L of the total reducing sugars, 3.43 g/L of ethanol, 5.01 g/L of acetic acid, and 5.21 g/L of the total phenolic compounds after the solids were removed in the solid/liquid separation. The COD concentration of the stillage liquid from cellulosic ethanol fermentation wastewater was 112.41 g/L.

Lipid Fermentation in Flasks Using Cellulosic Ethanol Fermentation Wastewater

The feasibility of COD reduction of the stillage liquid by lipid fermentation of *T. cutaneum* ACCC 20271 was investigated in flasks. Various fermentation parameters affecting the COD reduction and lipid accumulation were tested (Fig. 1). The detailed data of the flask fermentation is shown in Supplemental Material Table S1.

Nutrient addition included 0.5 g/L of $MgSO_4 \cdot 7H_2O$, 1.0 g/L of KH_2PO_4, 0.22 g/L of $(NH_4)_2SO_4$, and 0.5 g/L of YE. The results show that the cell growth, lipid accumulation, and COD reduction were not affected by single or multiple nutrient additions (Fig. 1a). The effect of pH was found to have a turning point at 5 (Fig. 1b). Below pH 5, the cell growth, lipid accumulation, and COD reduction were negligible, but a significant improvement was observed when the pH was above 5. Oxygen requirement was also tested by changing the shaking rate under the constant liquid filling volume. The cell growth, lipid accumulation, and COD reduction maintained almost constant when the minimum oxygen was supplied (Fig. 1c). The COD degradation ratio was only 10.15% when *T. cutaneum* ACCC 20271 was cultured in a non-agitated flask, while it increased obviously with mixing strength, and the dry cell weight, lipid titer, and COD reduction increased and maintained constant.

The results indicate that *T. cutaneum* ACCC 20271 was able to utilize the organic compounds including glucose, xylose, acetic acid, ethanol, and partial phenolic compounds for lipid production and the COD was reduced simultaneously. The maximum COD reduction was from 114.2 to 53.96 g/L with the maximum lipid titer of 2.61 g/L at the conditions of pH 5.0 and 180 rpm and with all nutrient additions. The result was better than the lipid titer of *R. toruloides* Y2 (1.33 g/L) [12] and *T. cutaneum* CH002 (0.72 g/L) [34] using the ABE fermentation wastewater from sweet potato hydrolysate.

Lipid Fermentation in Fermentor Using Cellulosic Ethanol Fermentation Wastewater

Following the flask fermentation on COD reduction and lipid accumulation, the cellulosic ethanol fermentation wastewater was sent to a 3-L bioreactor for lipid fermentation under well control of temperature, pH, and oxygen input (Fig. 2). The DCW reached as high as 16.20 g/L, and the lipid titer was 2.16 g/L after the 120-h fermentation (Fig. 2a). The total reducing sugars were reduced from 47.89 to 21.65 g/L, while the total phenolic content was only reduced from 4.41 to 3.37 g/L (Fig. 2b). Glucose, acetic acid, and ethanol were completely utilized by *T. cutaneum* ACCC 20271 after 12, 12, and 72 h, while still 2.16 g/L of xylose was left in the wastewater (Fig. 2c). The COD was reduced gradually from 118.58 to 55.55 g/L within 96 h (Fig. 2d), while it almost did not reduce further (53.31 g/L at 120 h).

The fatty acid composition of the lipid accumulated in *T. cutaneum* ACCC 20271 cells was analyzed by gas chromatography-mass spectrometer (GC-MS) and compared with the lipid accumulated by the same strain (*T. cutaneum* ACCC 20271) in corn stover hydrolysate (Table 1). The results show that the lipid accumulated by *T. cutaneum* ACCC 20271 using cellulosic ethanol fermentation wastewater was more diverse than that from corn stover hydrolysate. The unsaturated fatty acid content was 64.96%, similar to the lipid derived from corn stover hydrolysate [16] as well as the vegetable oil used for biodiesel synthesis [35].

Due to the high initial glucose and xylose concentration, the DCW of *T. cutaneum* ACCC 20271 (16.20 g/L) was obviously higher than that of *Trichosporon coremiiforme* CH005 (5.80 g/L), *T. dermatis* CH007 (7.40 g/L), *T. cutaneum* CH002 (4.90 g/L), and *R. toruloides*

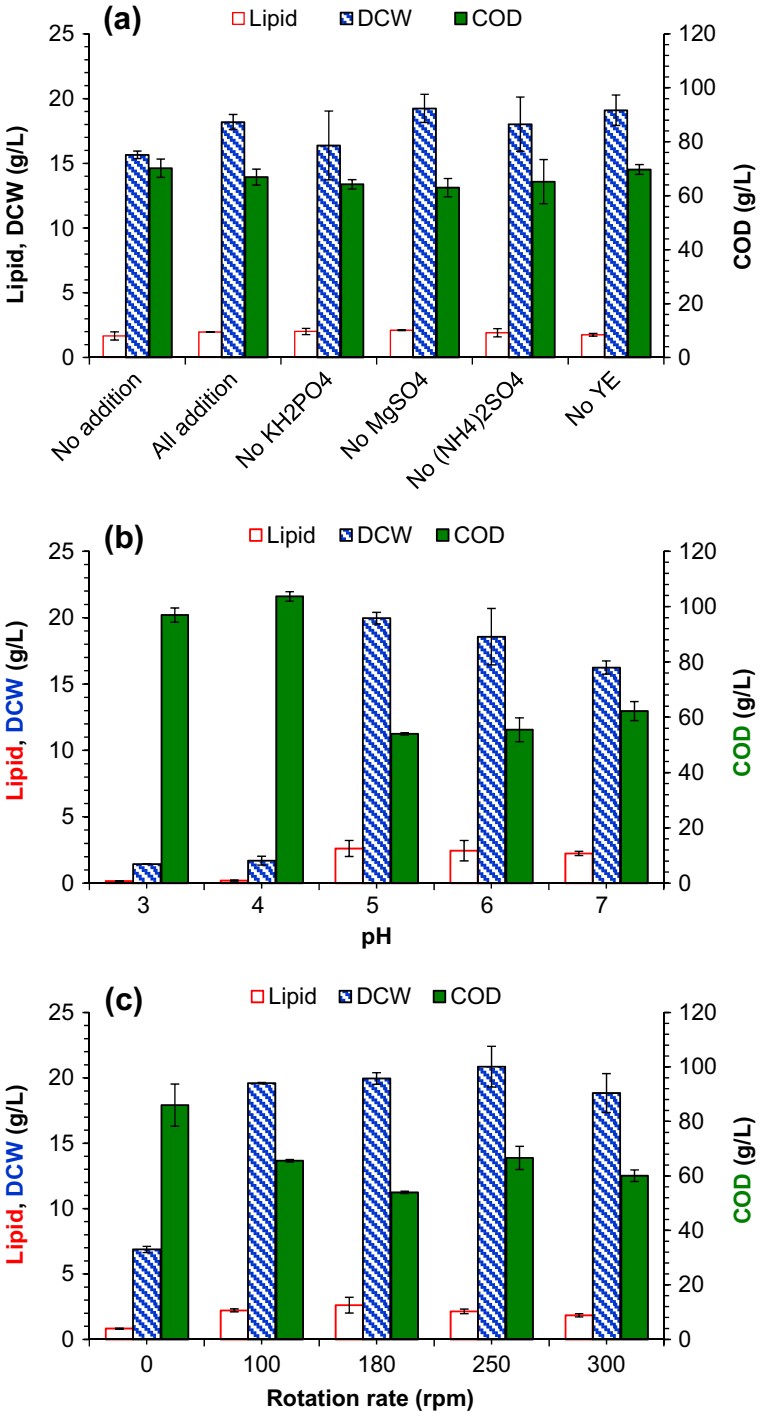

Fig. 1 Effects of nutrients, pH, and rotation rate on the COD reduction and lipid accumulation in cellulosic ethanol fermentation wastewater. **a** Nutrient addition. **b** pH. **c** Rotation rate. Culture conditions: 500-mL flask containing 50 mL of wastewater at 30 °C

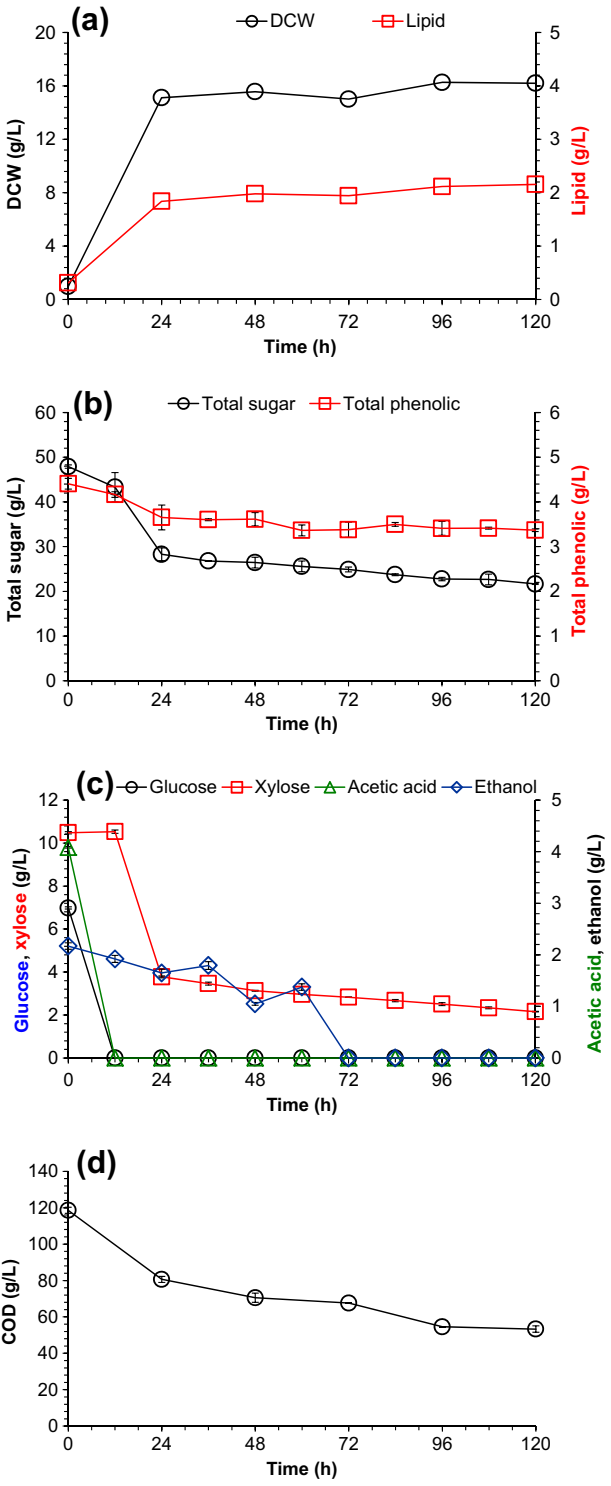

Fig. 2 Profiles of ethanol fermentation wastewater treatment and microbial lipid production by *T. cutaneum* ACCC 20271 in 3-L bioreactor. **a** DCW and lipid. **b** Total sugar and total phenolic. **c** Time course of glucose, xylose, acetic acid, and ethanol utilization by *T. cutaneum* ACCC 20271. **d** COD. Culture conditions: pH 5.0, 30 °C, 600 rpm, and aeration rate 1.67 vvm

Table 1 Fatty acid composition of microbial lipid from ethanol fermentation wastewater and the comparison with lipid from different corn stover feedstocks

Feedstocks		Hydrolysate of corn stover (*w/w*, %) [15]	Ethanol fermentation wastewater of corn stover (*w/w*, %) (this study)
Laurate	C12:0	–	–
Myristate	C14:0	0.23	0.31
Pentadecanoate	C15:0	0.67	1.09
Palmitoleate	C16:1	10.69	0.55
Palmitate	C16:0	19.41	14.21
Margaroleate	C17:1	7.90	–
Margarate	C17:0	3.56	1.88
Oleate	C18:1	42.11	67.81
Stearate	C18:0	5.98	10.12
Linoleate	C18:2	4.26	0.36
Linolenate	C18:3	–	–
Nonadecenoate	C19:1	–	0.20
Nonadecanoate	C19:0	0.22	0.16
Eicosenate	C20:1	–	0.48
Arachidate	C20:0	–	0.43
Erucate	C22:1	–	–
Behenate	C22:0	–	0.31
Lignocerate	C24:0	0.46	1.65
Pentacosanoate	C25:0	–	0.45
Hexacosanoate	C26:0	–	–

– Standing for trace or not detectable

Y2 (6.70 g/L). *T. cutaneum* ACCC 20271 utilizes acetic acid and ethanol as the carbon source for the cell growth, but the lipid accumulation of *T. cutaneum* ACCC 20271 (13.3%) was lower than that of *T. coremiiforme* CH005 (19.14%), *T. dermatis* CH007 (13.5%), *T. cutaneum* CH002 (14.7%), and *R. toruloides* Y2 (15.5%) because the existence of cellulase enzyme leads to low C/N ratio. Lipid fermentation using the wastewater from butanol fermentation by *T. coremiiforme* CH005 and *T. dermatis* CH007 both led to the COD degradation ratio of 68% [11, 36]. For bioethanol fermentation wastewater, the COD reduction by *R. toruloides* Y2 was 72.3% [13], while the COD of glutamic acid fermentation wastewater was reduced by 85.51% by *Rhodotorula glutinis* [10]. In this study, the COD reduction of cellulosic ethanol fermentation wastewater by *T. cutaneum* ACCC 20271 was 55.05%, which was obviously lower than that using different wastewaters, perhaps due to the existence of high inhibitors and low organic substance in cellulosic ethanol fermentation wastewater. Besides, the very high initial COD concentration (up to 112.41 g/L) is also one of the critical reasons resulting into the high final COD concentration. Moreover, the residual cellulase enzyme existing in the cellulose ethanol wastewater also provided as a carbon source that partly resulted into the high initial COD concentration. However, 2.16 g/L of lipid recovery would be a positive result balancing the high cost of cellulosic ethanol production.

Conclusion

The wastewater with high COD obtained after cellulosic ethanol fermentation broth was approved to be a potential feedstock for microbial lipid production by *T. cutaneum* ACCC

20271. The maximum reduction of COD of cellulosic ethanol fermentation wastewater was 55.05%, and the fatty acid composition of the derived microbial lipid was similar with vegetable oil, in which it could be used for biodiesel synthesis.

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