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Microbial Lipid Production from Corn Stover by the Oleaginous Yeast *Rhodosporidium toruloides* Using the PreSSLP Process

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Abstract: Dry acid pretreatment and biodetoxification (DryPB) has been considered as an advanced technology to treat lignocellulosic materials for improved downstream bioconversion. In this study, the lipid production from DryPB corn stover was investigated by the oleaginous yeast *Rhodosporidium toruloides* using a new process designated prehydrolysis followed by simultaneous saccharification and lipid production (PreSSLP). The results found that prehydrolysis at 50 °C and then lipid production at 30 °C improved lipid yield by more than 17.0% compared with those without a prehydrolysis step. The highest lipid yield of 0.080 g/g DryPB corn stover was achieved at a solid loading of 12.5%. The fatty acid distribution of lipid products was similar to those of conventional vegetable oils that are used for biodiesel production. Our results suggested that the integration of DryPB process and PreSSLP process can be explored as an improved technology for microbial lipid production from lignocellulosic materials.

Keywords: corn stover; dry acid pretreatment and biodetoxification; microbial lipids; prehydrolysis; *Rhodosporidium toruloides*

1. Introduction

Our growing concerns on fossil fuels depletion and environmental deterioration have prompted rapid development and application of biofuels especially biodiesel in recent years [1]. While biodiesel has been considered as safer, greener and renewable biofuel, however, the primary disadvantage connected with biodiesel application is the consumption of a large volume of conventional lipid resources [2,3]. Microbial lipids produced by oleaginous microorganisms were considered as alternative feedstocks for biodiesel production [4]. Since the expense of single cell oil come largely from raw materials and production processes [5], it is key to use cheap feedstocks and develop a more efficient process.

Lignocellulosic materials consisting of lignin and two major polysaccharides cellulose and hemicellulose are renewable and abundant in nature. Cellulose is composed of glucose unit and hemicellulose is composed of pentoses and hexoses such as xylose, arabinose and galactose units. However, cellulose, hemicellulose and lignin form very complex and recalcitrant structures that

present a major challenge for fractionation and utilization [6]. A pretreatment step is usually required to breakdown interactions among cellulose, hemicellulose and lignin to increase the enzymatic digestibility [7,8]. Lignocellulosic biomass, like rice straw [9], corn stover [10,11], corn cob [3], sugarcane bagasse [12], wheat straw [13] and waste office paper [8] have been explored for lipid production. However, pretreatment methods in those studies suffered from problems such as high costs, high energy consumption, toxic wastes or generation of more inhibitory compounds. Therefore, a better process remains to be introduced for the conversion of biomass into lipids.

Previously we developed the dry acid pretreatment and biodetoxification (DryPB) technology which featured low water input, no wastewater discharge and biodetoxification using the ascomycete fungus *Amorphotheca resinae* ZN1 [14–17]. The biodetoxification process effectively removes inhibitors like furfural, formic acid, 5-hydroxymethylfuurfural [18,19], and thus has been proved to be beneficial to the subsequent fermentation process [19,20]. So far, DryPB has been adopted to pretreat biomass such as corn stover, wheat straw and rice straw [21]. Recently, ethanol production from DryPB biomass through a simultaneous saccharification and co-fermentation process achieved an ethanol titer of 12.8% (v/v) with only 2.241 ton wastewater per ton ethanol [21]. However, lipid production remained less effective with hydrolysates of DryPB corn stover [19,22]. In parallel, we reported the simultaneous saccharification and lipid production (SSLP) process for effective and direct produce single cell oil from cellulosic materials [11,23]. For the SSLP process, oleaginous microbes readily assimilate the sugars released by enzymatic hydrolysis [11].

In this study, we explored microbial lipid production from DryPB corn stover by the oleaginous yeast *Rhodosporidium toruloides*. We designed a new process designated prehydrolysis and SSLP (PreSSLP) of which corn stover was prehydrolyzed at 50 °C and then lipid production at 30 °C. The PreSSLP process improved lipid yield by more than 17.0% compared with those without the prehydrolysis step. Our results suggest the integration of DryPB process and PreSSLP process can be explored as a prospective approach for microbial lipid production from lignocellulosic biomass.

2. Materials and Methods

2.1. Materials

The corn stover was gathered from Bayan Nur, Inner Mongolia, China in 2015. The preliminary treatment of corn stover includes four steps: milling, washing, drying and screening using a mesh (10 mm) and then was stored in a plastic bag. The content of cellulose, hemicellulose, lignin and ash in raw corn stover were 35.4%, 24.6%, 16.1% and 3.5%, respectively [21].

The cellulase used in this study was the product Cellic CTec2 from Novozymes (China) Investment Co. Ltd. (Tianjin, China). The filter paper activity and cellulase activity were determined as 252.0 FPU/mL and 7081.6 CBU/mL following a published procedure [24]. The total protein concentration was 96.4 mg/mL and quantified using the BSA methodology [25]. All the other analytical chemicals were purchased from the local company.

2.2. Corn Stover Pretreatment Procedure

The corn stover was pretreated using the DryPB method [16,17]. In short, 5% (w/w) sulfuric acid and corn stover were mixed at a solid loading of 50% (2.5 g H_2SO_4 per 100 g dry corn sotver) and put it in the reactor and heated to 175 °C for 5 min followed by biological detoxification process with *A. resinae* ZN1. The content of cellulose, hemicellulose, lignin and ash in the resulting DryPB corn stover on the dry weight basis were 47.6%, 4.5%, 30.3% and 3.4%, respectively. The elemental composition of DryPB corn stover was 41.9% carbon, 5.2% hydrogen and 0.8% nitrogen.

2.3. Strain and Inoculum Culture Condition

The yeast strain *R. toruloides* AS 2.1389 was acquired from China General Microbiological Culture Collection Centre (CGMCC). The strain was stored at 4 °C on YEPD agar plate supplemented with

20 g/L glucose, 10 g/L peptone, 10 g/L yeast extract, 15 g/L agar, pH 6.0 and sub-cultured once a month. The strain *A. resinae* ZN1 acquired from CGMCC and was plated on PDA. The YEPD medium contains 20 g/L glucose, 10 g/L peptone, 10 g/L yeast extract, pH 6.0. The media were sterilized at 121 °C for 20 min. The inoculum culture was carried out in a 250-mL flask with 50 mL YEPD medium at 30 °C 200 rpm for 36 h. Cells were collected by centrifugation at 8000 rpm for 5 min, washed twice with distilled water and used as inoculum.

2.4. Lipid Production from DryPB Corn Stover

The SSLP process was conducted in 250-mL flasks with an initial volume of 50 mL [11,23]. To potassium phosphate buffer (50 mM, pH 5.2) was added DryPB corn stover at a solid loading of 10.0%, cellulase, 50 mg/L ampicillin (used to avoid bacterial contamination) and fresh *R. toruloides* cells at an inoculum size of 3.0 g dry cell weight per liter. The flasks were placed in a shaker at 37 °C, 200 rpm. The culture broth was withdrawn to analyze glucose and total reducing sugars (TRS) concentration.

For the PreSSLP process, the mixture containing DryPB corn stover, cellulase and 50 mg/L ampicillin in potassium phosphate buffer (50 mM, pH 5.2) was treated in a water-bath shaker at 50 °C, 200 rpm for 10 h. The mixture was cooled to 30 °C and adjusted solid loading to 10.0%, 12.5%, 15.0%, 17.5% and 20.0%, and inoculated with fresh *R. toruloides* cells at inoculum size of 3.0 g/L, 3.8 g/L, 4.5 g/L, 5.3 g/L and 6.0 g dry cell weight per liter, respectively, and held at 30 °C, 200 rpm. The culture broth was sampled every 12 h to analyze glucose and TRS concentrations.

2.5. Analytical Methods

The composition of corn stover samples was detected followed the protocol from National Renewable Energy Laboratory [26]. Corn stover residues and cells mixture were gathered by centrifugation at 8000 rpm for 5 min, washed two times with distilled water and desiccated at 105 °C to constant weight. Residual glucose was analyzed by an SBA-40D biosensor (Shandong Academy of Sciences, Jinan, China). TRS were measured following the DNS method as described by Miller [27].

Total lipids were extracted according to the methanol and chloroform method. In short, cells and corn stover residue mixture were gathered by centrifugation at 8000 rpm for 5 min, dried and treated with 4 M HCl at 78 °C for 1 h and then extracted two times with CH_3OH and $CHCl_3$ (1:1, *v*/*v*). The extract was washed with an equal volume of 0.1% sodium chloride and passed through anhydrous Na_2SO_4. The chloroform was removed by rotary evaporation at 34 °C. Crude lipids were re-dissolved in petroleum ether and the mixture was passed through a filter membrane to eliminate the insoluble substance, and the petroleum ether was wiped off by rotary evaporation at 34 °C. The total lipids were dried to constant weight at 105 °C [22,28]. The lipid yield was defined as 1 g lipid per 1 g DryPB corn stover.

The lipid products were transmethylated as described previously [4]. Then, compositional profiling of fatty acid was measured by a 7890F GC (Techcomp Scientific Instrument Co. Ltd., Shanghai, China), which was equipped with a cross-linked capillary FFAP column (30 m × 0.32 mm × 0.4 mm) and a flame ionization detector. The flow rates for N_2, H_2 and air were 720 mL/min, 30 mL/min and 100 mL/min, respectively. The injection port, oven and detector temperature was set at 250 °C, 190 °C and 280 °C, respectively. The injection volume was 0.2 µL. Fatty acids were identified and quantified by comparing with the retention time of those of standards and the respective peak areas and area normalization.

3. Results and Discussion

3.1. Lipid Production Using SSLP Process

When the SSLP process was applied for lipid production, enzyme dosage affected the rate and degree of corn stover hydrolysis, and consequently, cell growth and lipid synthesis. Additionally, higher culture temperature was found beneficial to enzymatic hydrolysis and led to more lipids [23].

Hence, the experiment was done with 10.0% solid loading at 37 °C in the presence of various cellulase dosage. The evolution profiles of glucose and TRS are shown in Figure 1. It was remarkable that TRS and glucose concentrations were low during the whole process with cellulase at a low loading of 1.0 mg/g or 2.5 mg/g, and that lipid titers were only 4.3 g/L and 5.5 g/L, respectively (Table 1, entries 1 and 2), indicating that corn stover hydrolysis was limiting. When cellulase dosage reached 5.0 mg/g, glucose and TRS concentrations were 10.8 g/L and 15.7 g/L, respectively, at 24 h, suggesting that the rate of sugar release was faster than assimilation by yeast cells. After 72 h, lipid titer was 6.2 g/L (Table 1, entry 3), which was increased by 12.7% and 44.2% compared with those using cellulase dosage of 1.0 mg/g and 2.5 mg/g, respectively. Interestingly, those sugar evolution profiles also indicated that *R. toruloides* cells assimilated both glucose as there was little TRS left in the media (vide infra). When more cellulase was loaded, lipid titer remained at the level of 6.2 g/L (Table 1, entries 4 and 5), albeit noticeably more TRS and glucose were observed at the early stage of the culture process. This was probably due that lipid production was inhibited at 37 °C. In the previous studies, higher product titers were obtained at temperatures higher than the optima for cell growth [23,29,30]. For example, our previous study achieved the highest lipid titer at 37 °C from cellulose by *Cryptococcus curvatus* ATCC 20509 [23], while Zhu et al. obtained the highest ethanol yield at 34 °C from DryPB corn stover by *Saccharomyces cerevisiae* SyBE005 [30]. However, these studies also pointed out that higher temperatures significantly affected cell metabolism. Hence, it was important to produce lipids at the optimal growth temperature, albeit enzymatic hydrolysis rates dropped substantially [31].

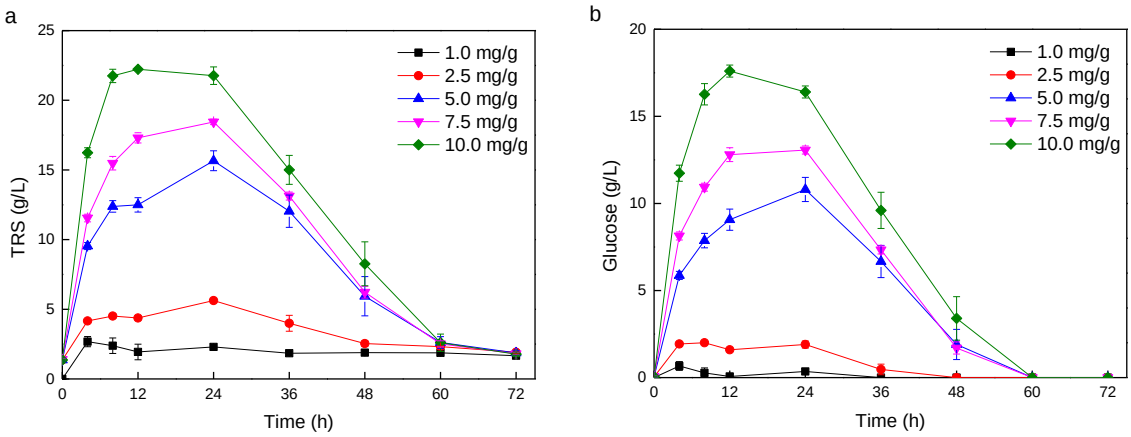

Figure 1. Total reducing sugar (TRS) (**a**) and glucose (**b**) evolution profiles of lipid production at 37 °C with various amounts of cellulase.

Table 1. The results of lipid production from DryPB corn stover at 37 °C with various amounts of cellulase.

Entry	Cellulase Dosage (mg/g) [a]	Total Solid Mass (g/L)	Residual TRS (g/L)	Lipid (g/L)	Lipid Yield (g/g)
1	1.0	40.6 ± 1.4	1.7 ± 0.2	4.3 ± 0.1	0.043 ± 0.001
2	2.5	40.5 ± 1.2	1.9 ± 0.1	5.5 ± 0.5	0.055 ± 0.004
3	5.0	41.2 ± 1.1	1.9 ± 0.2	6.2 ± 0.1	0.062 ± 0.001
4	7.5	43.0 ± 0.2	1.8 ± 0.1	6.2 ± 0.3	0.061 ± 0.003
5	10.0	39.9 ± 1.1	1.8 ± 0.1	6.2 ± 0.1	0.062 ± 0.001

[a] mg total protein per g DryPB corn stover.

3.2. Lipid Production Using PreSSLP Process

To balance different temperature requirements for enzymatic hydrolysis and lipid production, we added a prehydrolysis step before SSLP process, and the overall process is termed PreSSLP process. The results of lipid production from DryPB corn stover using PreSSLP process are shown in Figure 2 and Table 2. It was found that the prehydrolysis step at 50 °C for 10 h led to release substantial

amounts of TRS and glucose and that the initial TRS and glucose concentrations correlated positively with enzyme dosage (Figure 2). Both TRS and glucose concentrations dropped over time after cells were added for lipid production at 30 °C. When 1.0 mg/g cellulase was used, TRS and glucose concentrations were 14.0 g/L and 9.2 g/L initially and 2.8 g/L and 0.0 g/L, respectively, at 24 h. The final total solid mass of 51.4 g/L and lipid yield of 0.037 g/g suggested that corn stover was not fully hydrolyzed (Table 2, entry 1). In contrast, when the SSLP process was applied at a cellulase dosage of 2.5 mg/g, very little TRS and glucose were detected at 12 h; while for the experiment with 5.0 mg/g cellulase, TRS and glucose concentrations were 8.2 g/L and 4.2 g/L, respectively, at 12 h. Thus, sugars were more readily available by PreSSLP process. For comparison, when 2.5 mg/g cellulase was applied, PreSSLP process afforded a lipid yield of 0.068 g/g than that (0.046 g/g) of SSLP process (Table 2, entry 2 vs. 3). When cellulase loading increased to 5.0 mg/g, the initial TRS and glucose concentrations were 28.9 g/L and 21.0 g/L, respectively, and lipid yield reached a high level of 0.073 g/g (Table 2, entry 4). Similarly, SSLP process with 5.0 mg/g cellulase gave a lipid yield of 0.059 g/g, which was 17% less than that of PreSSLP process (Table 2, entry 5). While even more cellulase was used for prehydrolysis, more initial TRS and glucose were observed, but no further improvement in terms of lipid yield. Noting that TRS dropped apparently slower at the early stage for those cultures with cellulase dosage more than 5.0 mg/g, indicating that cellulase-mediated hydrolysis at 30 °C had a large contribution to balance cell growth-associated substrate consumption.

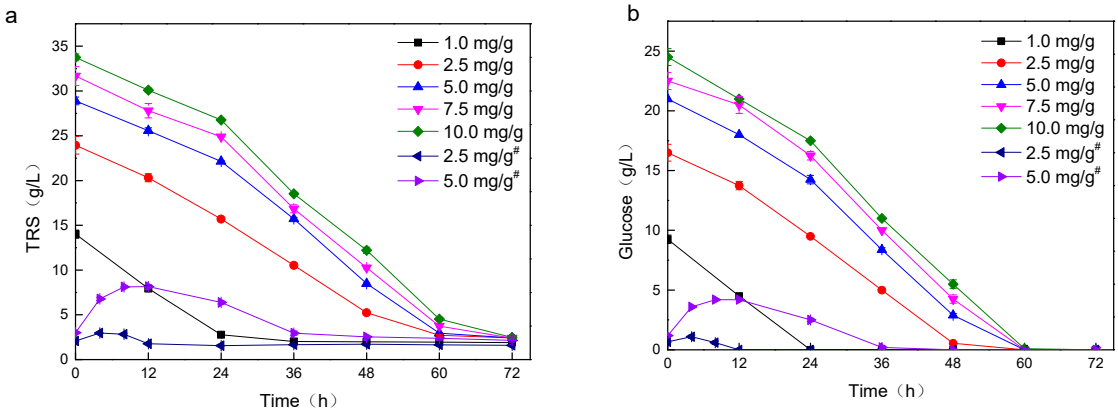

Figure 2. TRS (**a**) and glucose (**b**) evolution profiles of lipid production at 30 °C with various amounts of cellulase. #: without prehydrolysis.

Table 2. The results of lipid production at 30 °C with various amounts of cellulase.

Entry	Cellulase Dosage (mg/g) [a]	Total Solid Mass (g/L)	Residual TRS (g/L)	Lipid (g/L)	Lipid Yield (g/g)
1	1.0	51.4 ± 1.4	1.9 ± 0.1	3.7 ± 0.2	0.037 ± 0.002
2	2.5	41.9 ± 2.2	2.4 ± 0.0	6.8 ± 0.3	0.068 ± 0.003
3	2.5 [b]	41.0 ± 0.2	1.6 ± 0.0	4.6 ± 0.0	0.046 ± 0.000
4	5.0	40.4 ± 0.4	2.4 ± 0.0	7.3 ± 0.0	0.073 ± 0.000
5	5.0 [b]	41.3 ± 0.3	2.1 ± 0.0	5.9 ± 0.0	0.059 ± 0.000
6	7.5	40.0 ± 0.4	2.4 ± 0.1	7.3 ± 0.1	0.073 ± 0.001
7	10.0	40.9 ± 0.5	2.4 ± 0.0	6.9 ± 0.8	0.069 ± 0.008

[a] mg total protein per g DryPB corn stover. [b] without prehydrolysis.

It was clear that lipid yields of PreSSLP process at 30 °C were generally higher than those of the SSLP process at 37 °C excepting the experiment with 1.0 mg/g cellulase. When cellulase was applied at very low loading, the enzymatic hydrolysis of DryPB corn stover was limited, but lipid production at 37 °C could benefit from higher hydrolytic activity of cellulase. Similar results have been observed for cellulosic ethanol fermentation [32]. At a high cellulase loading, enzymatic hydrolysis secured sugar release such that the lipid production process was depending on yeast cell activity. It is known

that cellulases have an optimal temperature around 50 °C [33], whereas the optimal temperature for *R. toruloides* is 30 °C. To attain more hydrolytic activity of cellulase, SSLP process cultivated cells at a higher temperature, but cellular metabolism might be inhibited for lipid production [34,35]. In contrast, PreSSLP process assured optimal temperatures for enzymatic prehydrolysis and cell growth, yet collected residual hydrolytic activity of cellulase in the culture broth. Overall, PreSSLP process is more beneficial to convert lignocellulosic materials into lipids.

3.3. Lipid Production with Higher Solid Loadings

Increasing solid loading is more favorable because of its potential to achieve a higher product titer. Ye et al. found that ethanol was 82.8 g/L at high solid loading of 34.0% from mixtures of sugarcane bagasse and Dioscorea composita extracted residue using SSF process [36]. To demonstrate the effectiveness of PreSSLP process and improve lipid titer, we performed more experiments with 5.0 mg/g cellulase and solid loading up to 20.0%. It was found that more TRS and glucose were obtained at higher solid loading (Figure 3). At a solid loading of 12.5%, TRS and glucose concentrations were 40.4 g/L and 27.5 g/L, respectively. The lipid yield was 0.080 g/g (Table 3, entry 2), which was slightly higher than the results obtained with 10.0% solid loading. When the solid loading was 15.0% or higher, lipid titer increased proportionally to solid loading (Table 3, entries 3-5); but lipid yield dropped and longer culture time was required. When comparing the results obtained at a solid loading of 20.0% with those of 17.5%, it was clear that lipid titer was almost identical but lipid yield dropped (Table 3, entry 4 vs. 5). Albeit lipid production at higher solid loading may encounter difficulties including higher concentrations of inhibitory components [19,22], high viscosity and reduced oxygen transfer [37], it remains attractive due to reduced consumption of water, energy, as well as resources used for lipid recovery [2,10,38]. The mass balance of microbial lipid production from DryPB corn stover is summarized in Figure 4. Lipid yield was only 6.2 g per 100 g DryPB corn stover using SSLP process, while it produced 7.3 g lipid using PreSSLP process. However, PreSSLP process gave better results than those of SSLP process in terms of lipid yield at a wide range of solid loading, indicating that PreSSLP process was robust to convert DryPB corn stover into lipids. The highest lipid of 8.0 g from 100 g DryPB corn stover was achieved at solid loading of 12.5%. Further, it also suggested that DryPB process afforded raw materials suitable for enzymatic hydrolysis and microbial lipid production.

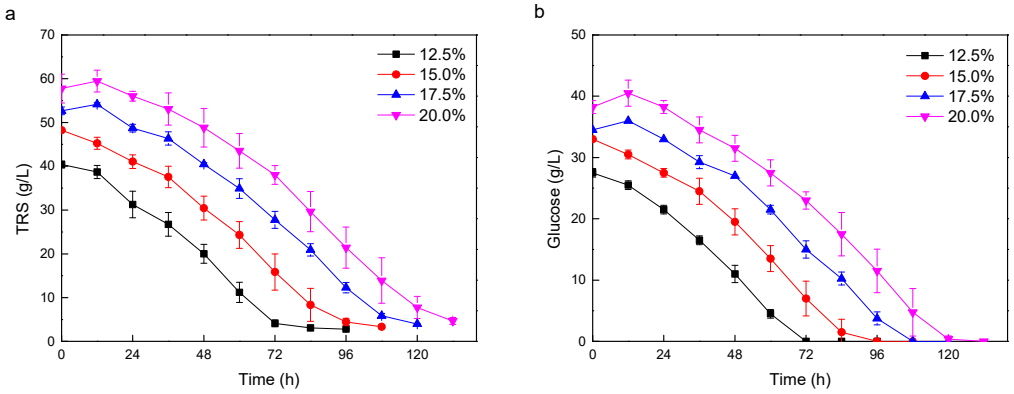

Figure 3. TRS (**a**) and glucose (**b**) evolution profiles of PreSSLP process with various solid loading.

Summarized in Table 4 are results of lipid production from corn stover being pretreated with various methods by oleaginous yeasts. A similar study was done by *Trichosporon cutaneum* CX1, but lipid yields were substantially lower [22]. In another study, hydrolysates of DryPB corn stover were first prepared with high initial solid loading for subsequent lipid production by *T. cutaneum* ACCC 20271, and lipid titers were relatively low [19]. In addition, hydrolysates of corn stover being pretreated by the AFEX process were used for lipid production by *Lipomyces tetrasporus* NRRL Y-11562, and lipid yield was only 0.037 g/g [10]. It should be noted that, for lipid production, SSLP process was superior

to SHF process due to low cost, low energy consumption and high productivity [11]. Further, PreSSLP process produced even more lipids than those previous results at identical solid loading.

Table 3. The results of lipid production at 30 °C under various solid loadings.

Entry	Solid Loading (%)	Total Solid Mass (g/L)	Residual TRS (g/L)	Lipid (g/L)	Lipid Yield (g/g)
1	10.0	40.4 ± 0.4	2.4 ± 0.0	7.3 ± 0.0	0.073 ± 0.000
2	12.5	52.2 ± 0.7	2.8 ± 0.1	10.1 ± 0.4	0.080 ± 0.003
3	15.0	60.3 ± 2.3	3.3 ± 0.5	11.6 ± 0.7	0.077 ± 0.005
4	17.5	73.9 ± 0.3	4.0 ± 0.1	12.4 ± 0.6	0.071 ± 0.004
5	20.0	82.9 ± 2.6	4.7 ± 0.8	12.5 ± 0.1	0.062 ± 0.000

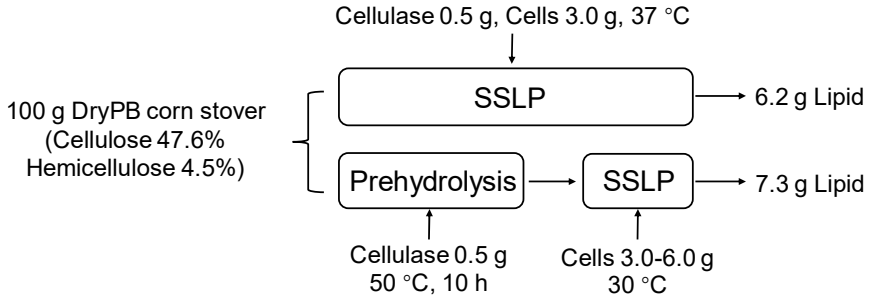

Figure 4. The mass balance of microbial lipid production from DryPB corn stover using SSLP process and PreSSLP process.

Table 4. Lipid production from corn stover by different approaches.

Strain	Pretreatment Method	Detoxification Method	Culture Mode	Solid Loading (%)	Lipid (g/L)	Lipid Yield (g/g) [a]	Reference
T. cutaneum CX1	Dry acid pretreatment	Biodetoxification	SSF	10.0	3.0	0.030	[22]
				15.0	4.0	0.027	
T. cutaneum ACCC 20271	Dry acid pretreatment	Biodetoxification	SHF	15.0	5.9	-	[19]
				20.0	7.4	-	
				25.0	8.0	-	
L. tetrasporus NRRL Y-11562	AFEX	No	SHF	22.1	8.4	0.037	[10]
R. toruloides AS 2.1389	Dry acid pretreatment	Biodetoxification	SSLP	10.0	6.2	0.062	This study
R. toruloides AS 2.1389	Dry acid pretreatment	Biodetoxification	PreSSLP	10.0	7.3	0.073	This study
				12.5	10.1	0.080	
				15.0	11.6	0.077	
				17.5	12.4	0.071	

[a] base on pretreated corn stover.

3.4. Fatty Acid Composition

The total lipids produced by *R. toruloides* according to PreSSLP process under different solid loading were transmethylated and detected by GC. The results of the fatty acid composition analysis are presented in Table 5, the majority were saturated and unsaturated fatty acids including palmitic acid (C16:0), stearic acid (C18:0), and oleic acid (C18:1), which were similar to the fatty acid compositions of conventional vegetable oils. These samples also showed similar fatty acid compositions to those lipids produced by other oleaginous yeasts [3,39,40]. These data suggested that microbial lipids from corn stover may be used as a biodiesel feedstock.

Table 5. Fatty acid compositional profiles of lipids produced from DryPB corn stover by *R. toruloides* according to the PreSSLP process.

Entry	Solid Loading (%)	Lipid (g/L)	Relative Fatty Acid Contents (%)					
			Myristic Acid	Palmitic Acid	Palmitoleic Acid	Stearic Acid	Oleic Acid	Linoleic Acid
1	10.0	7.3 ± 0.0	2.5 ± 0.2	46.0 ± 1.0	0.6 ± 0.0	15.0 ± 0.4	35.0 ± 0.6	1.0 ± 0.2
2	12.5	10.1 ± 0.4	2.6 ± 0.2	44.6 ± 1.2	1.0 ± 0.0	15.5 ± 0.9	36.0 ± 0.5	0.7 ± 0.0
3	15.0	11.6 ± 0.7	2.5 ± 0.6	41.0 ± 5.4	1.1 ± 0.1	14.9 ± 1.5	38.6 ± 5.6	1.9 ± 1.7
4	17.5	12.4 ± 0.6	3.6 ± 0.6	55.0 ± 1.7	0.8 ± 0.3	21.3 ± 3.1	19.3 ± 4.7	0.0 ± 0.0

4. Conclusions

Here we showed that DryPB corn stover was effectively converted into lipids using *R. toruloides* as a lipid producer. PreSSLP process obtained higher titer and lipid yield of 7.3 g/L and 0.073 g/g, respectively, compared with those of SSLP process. When higher solid loading was used, lipid titer and lipid yield could reach 12.5 g/L and 0.080 g/g, respectively. Thus, the DryPB process and PreSSLP process can be fully integrated for microbial lipid production from corn stover to make it industrially feasible.

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Conflicts of Interest: The authors declare no conflict of interest.

References

1. Kumar, D.; Singh, B.; Korstad, J. Utilization of lignocellulosic biomass by oleaginous yeast and bacteria for production of biodiesel and renewable diesel. *Renew. Sustain. Energy Rev.* **2017**, *73*, 654–671. [CrossRef]
2. Jin, M.J.; Slininger, P.J.; Dien, B.S.; Waghmode, S.; Moser, B.R.; Orjuela, A.; Sousal, L.D.; Balan, V. Microbial lipid-based lignocellulosic biorefinery: Feasibility and challenges. *Trends Biotechnol.* **2015**, *33*, 43–54. [CrossRef] [PubMed]
3. Liu, Y.T.; Wang, Y.P.; Liu, H.J.; Zhang, J.A. Enhanced lipid production with undetoxified corncob hydrolysate by *Rhodotorula glutinis* using a high cell density culture strategy. *Bioresour. Technol.* **2015**, *180*, 32–39. [CrossRef] [PubMed]
4. Li, Y.H.; Zhao, Z.B.; Bai, F.W. High-density cultivation of oleaginous yeast *Rhodosporidium toruloides* Y4 in fed-batch culture. *Enz. Microb. Technol.* **2007**, *41*, 312–317. [CrossRef]
5. Koutinas, A.A.; Chatzifragkou, A.; Kopsahelis, N.; Papanikolaou, S.; Kookos, I.K. Design and techno-economic evaluation of microbial oil production as a renewable resource for biodiesel and oleochemical production. *Fuel* **2014**, *116*, 566–577. [CrossRef]
6. Vanneste, J.; Ennaert, T.; Vanhulsel, A.; Sels, B. Unconventional pretreatment of lignocellulose with low-temperature plasma. *ChemSusChem* **2017**, *10*, 14–31. [CrossRef]
7. Sun, S.N.; Sun, S.L.; Cao, X.F.; Sun, R.C. The role of pretreatment in improving the enzymatic hydrolysis of lignocellulosic materials. *Bioresour. Technol.* **2016**, *199*, 49–58. [CrossRef] [PubMed]
8. Annamalai, N.; Sivakumar, N.; Oleskowicz-Popiel, P. Enhanced production of microbial lipids from waste office paper by the oleaginous yeast *Cryptococcus Curvatus*. *Fuel* **2018**, *217*, 420–426. [CrossRef]
9. Huang, C.; Zong, M.H.; Wu, H.; Liu, Q.P. Microbial oil production from rice straw hydrolysate by *Trichosporon fermentans*. *Bioresour. Technol.* **2009**, *100*, 4535–4538. [CrossRef]
10. Xue, Y.P.; Jin, M.J.; Orjuela, A.; Slininger, P.J.; Dien, B.S.; Dale, B.E.; Balan, V. Microbial lipid production from AFEX (TM) pretreated corn stover. *RSC Adv.* **2015**, *5*, 28725–28734. [CrossRef]
11. Gong, Z.W.; Shen, H.W.; Yang, X.B.; Wang, Q.; Xie, H.B.; Zhao, Z.B.K. Lipid production from corn stover by the oleaginous yeast *Cryptococcus curvatus*. *Biotechnol. Biofuels* **2014**, *7*, 158. [CrossRef]

12. Brar, K.K.; Sarma, A.K.; Aslam, M.; Polikarpov, I.; Chadha, B.S. Potential of oleaginous yeast *Trichosporon* sp., for conversion of sugarcane bagasse hydrolysate into biodiesel. *Bioresour. Technol.* **2017**, *242*, 161–168. [CrossRef]
13. Yu, X.C.; Zheng, Y.B.; Dorgan, K.M.; Chen, S.L. Oil production by oleaginous yeasts using the hydrolysate from pretreatment of wheat straw with dilute sulfuric acid. *Bioresour. Technol.* **2011**, *102*, 6134–6140. [CrossRef]
14. Zhang, J.A.; Zhu, Z.N.; Wang, X.F.; Wang, N.; Wang, W.; Bao, J. Biodetoxification of toxins generated from lignocellulose pretreatment using a newly isolated fungus, *Amorphotheca resinae* ZN1, and the consequent ethanol fermentation. *Biotechnol. Biofuels* **2010**, *3*, 26. [CrossRef]
15. Zhang, J.A.; Wang, X.S.; Chu, D.Q.; He, Y.Q.; Bao, J. Dry pretreatment of lignocellulose with extremely low steam and water usage for bioethanol production. *Bioresour. Technol.* **2011**, *102*, 4480–4488. [CrossRef]
16. He, Y.Q.; Zhang, J.; Bao, J. Dry dilute acid pretreatment by co-currently feeding of corn stover feedstock and dilute acid solution without impregnation. *Bioresour. Technol.* **2014**, *158*, 360–364. [CrossRef]
17. He, Y.Q.; Zhang, L.P.; Zhang, J.; Bao, J. Helically agitated mixing in dry dilute acid pretreatment enhances the bioconversion of corn stover into ethanol. *Biotechnol. Biofuels* **2014**, *7*, 1. [CrossRef]
18. He, Y.Q.; Zhang, J.; Bao, J. Acceleration of biodetoxification on dilute acid pretreated lignocellulose feedstock by aeration and the consequent ethanol fermentation evaluation. *Biotechnol. Biofuels* **2016**, *9*, 19. [CrossRef]
19. Wang, J.; Gao, Q.Q.; Zhang, H.Z.; Bao, J. Inhibitor degradation and lipid accumulation potentials of oleaginous yeast *Trichosporon cutaneum* using lignocellulose feedstock. *Bioresour. Technol.* **2016**, *218*, 892–901. [CrossRef]
20. Zhou, P.P.; Meng, J.; Bao, J. Fermentative production of high titer citric acid from corn stover feedstock after dry dilute acid pretreatment and biodetoxification. *Bioresour. Technol.* **2017**, *224*, 563–572. [CrossRef]
21. Liu, G.; Zhang, Q.; Li, H.X.; Qureshi, A.S.; Zhang, J.; Bao, X.M.; Bao, J. Dry biorefining maximizes the potentials of simultaneous saccharification and co-fermentation for cellulosic ethanol production. *Biotechnol. Bioeng.* **2018**, *115*, 60–69. [CrossRef] [PubMed]
22. Liu, W.; Wang, Y.M.; Yu, Z.C.; Bao, J. Simultaneous saccharification and microbial lipid fermentation of corn stover by oleaginous yeast *Trichosporon Cutaneum*. *Bioresour. Technol.* **2012**, *118*, 13–18. [CrossRef]
23. Gong, Z.W.; Shen, H.W.; Wang, Q.; Yang, X.B.; Xie, H.B.; Zhao, Z.B.K. Efficient conversion of biomass into lipids by using the simultaneous saccharification and enhanced lipid production process. *Biotechnol. Biofuels* **2013**, *6*, 36. [CrossRef] [PubMed]
24. Ghose, T.K. Measurement of cellulase activities. *Pure Appl. Chem.* **1987**, *59*, 257–268. [CrossRef]
25. Bradford, M.M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **1976**, *72*, 248–254. [CrossRef]
26. National Renewable Energy Laboratory (NREL). Determination of Structural Carbohydrates and Lignin in Biomass. Laboratory Analytical Procedure (LAP); NREL/TP-510-42618; 2012. Available online: https://www.nrel.gov/docs/gen/fy13/42618.pdf (accessed on 20 November 2017).
27. Miller, G.L. Use of dinitrosalicylic acid reagent for determination of reducing sugar. *Anal. Chem.* **1959**, *31*, 426–428. [CrossRef]
28. Wu, S.G.; Hu, C.M.; Jin, G.J.; Zhao, X.; Zhao, Z.K. Phosphate-limitation mediated lipid production by *Rhodosporidium toruloides*. *Bioresour. Technol.* **2010**, *101*, 6124–6129. [CrossRef]
29. Qin, L.; Zhao, X.; Li, W.C.; Zhu, J.Q.; Liu, L.; Li, B.Z.; Yuan, Y.J. Process analysis and optimization of simultaneous saccharification and co-fermentation of ethylenediamine-pretreated corn stover for ethanol production. *Biotechnol. Biofuels* **2018**, *11*, 118. [CrossRef]
30. Zhu, J.Q.; Qin, L.; Li, W.C.; Zhang, J.; Bao, J.; Huang, Y.D.; Li, B.Z.; Yuan, Y.J. Simultaneous saccharification and co-fermentation of dry diluted acid pretreated corn stover at high dry matter loading: Overcoming the inhibitors by non-tolerant yeast. *Bioresour. Technol.* **2015**, *198*, 39–46. [CrossRef]
31. Paulova, L.; Patakova, P.; Rychtera, M.; Melzoch, K. High solid fed-batch SSF with delayed inoculation for improved production of bioethanol from wheat straw. *Fuel* **2014**, *122*, 294–300. [CrossRef]
32. Qiu, J.W.; Tian, D.; Shen, F.; Hu, J.G.; Zeng, Y.M.; Yang, G.; Zhang, Y.Z.; Deng, S.H.; Zhang, J. Bioethanol production from wheat straw by phosphoric acid plus hydrogen peroxide (PHP) pretreatment via simultaneous saccharification and fermentation (SSF) at high solid loadings. *Bioresour. Technol.* **2018**, *268*, 355–362. [CrossRef] [PubMed]

33. Kumar, R.; Singh, S.; Singh, O.V. Bioconversion of lignocellulosic biomass: Biochemical and molecular perspectives. *J. Ind. Microbiol. Biotechnol.* **2008**, *35*, 377–391. [CrossRef] [PubMed]
34. Dong, C.Y.; Wang, Y.; Chan, K.L.; Bhatia, A.; Leu, S.Y. Temperature profiling to maximize energy yield with reduced water input in a lignocellulosic ethanol biorefinery. *Appl. Energy* **2018**, *214*, 63–72. [CrossRef]
35. Rudolf, A.; Baudel, H.; Zacchi, G.; Hahn-Hagerdal, B.; Liden, G. Simultaneous saccharification and fermentation of steam-pretreated bagasse using *Saccharomyces cerevisiae* TMB3400 and *Pichia stipitis* CBS6054. *Biotechnol. Bioeng.* **2008**, *99*, 783–790. [CrossRef] [PubMed]
36. Ye, G.Y.; Zeng, D.F.; Zhang, S.S.; Fan, M.S.; Zhang, H.D.; Xie, J. Ethanol production from mixtures of sugarcane bagasse and *Dioscorea composita* extracted residue with high solid loading. *Bioresour. Technol.* **2018**, *257*, 23–29. [CrossRef] [PubMed]
37. Hodge, D.B.; Karim, M.N.; Schell, D.J.; McMillan, J.D. Soluble and insoluble solids contributions to high-solids enzymatic hydrolysis of lignocellulose. *Bioresour. Technol.* **2008**, *99*, 8940–8948. [CrossRef] [PubMed]
38. Kristensen, J.B.; Felby, C.; Jorgensen, H. Yield-determining factors in high-solids enzymatic hydrolysis of lignocellulose. *Biotechnol. Biofuels* **2009**, *2*, 11. [CrossRef]
39. Yang, X.B.; Jin, G.J.; Gong, Z.W.; Shen, H.W.; Bai, F.W.; Zhao, Z.K. Recycling biodiesel-derived glycerol by the oleaginous yeast *Rhodosporidium toruloides* Y4 through the two-stage lipid production process. *Biochem. Eng. J.* **2014**, *91*, 86–91. [CrossRef]
40. Yang, X.B.; Jin, G.J.; Wang, Y.D.; Shen, H.W.; Zhao, Z.K. Lipid production on free fatty acids by oleaginous yeasts under non-growth conditions. *Bioresour. Technol.* **2015**, *193*, 557–562. [CrossRef]