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Biological co-production of biodiesel and bioethanol from wheat straw

Wheat straw

Dilute acid prehydrolysis

Hemicellulosic sugars

Fermentation by oleaginous yeast

Microbial lipid

Enzymatic transesterification

Biodiesel

Pretreated slurry

Cellulase enzymes

Simultaneous saccharification and fermentation

Saccharomyces cerevisiae

Bioethanol
Biological co-production of ethanol and biodiesel from wheat straw: A case of dilute acid pretreatment

Yuichi Morikawa 
Xuebing Zhao 
Dehua Liu

A process for co-production of ethanol and biodiesel from wheat straw was proposed. Dilute acid pre-hydrolysis of hemicellulose followed by enzymatic hydrolysis of cellulose were optimized to maximize recovery of total sugars. It was found that xylose yield obtained by super-dilute acid (0-1%) pretreatment under the experimental conditions was too low. By using moderate condition (140-160°C) with higher sulfuric acid concentration (0.3-0.6%), xylose recovery could be greatly increased to 60-70%. The relatively optimum condition for dilute acid pretreatment was 0.5% H2SO4 at 140°C for 1 h. 15.1 g/L ethanol with approximate 58% of theoretic yield was obtained by SSF of the pretreated solid. The hydrolyzate was directly converted to microbial lipid using a mutagenized Rhodospirillum toruloides. The extracted lipid was well converted to biodiesel with 90% conversion ratio under the catalysis of immobilized lipase. Mass balance showed that 0.80 g biodiesel and 10.1 g ethanol were produced from 100 g wheat straw. This work thus can provide a novel idea for biological production of biofuels from lignocellulosic biomass.

Introduction

Lignocellulosic biomass is one of the most abundant natural organic materials on our planet. Production of biofuels from biomass has attracted a great deal of attention in recent years due to the escalating energy depletion and environmental pollutions caused by combustion of fossil fuels. Among the biomass-derived biofuels, bioethanol and biodiesel, which are promising and renewable alternatives for the fossil gasoline and diesel respectively, have been well developed in commercial scales in many countries. As a renewable material, lignocellulosic biomass is the most important feedstock for producing the second-generation bioethanol. Lignocellulose needs saccharification prior to fermentation. However, it has to undergo pretreatment to increase the enzymatic digestibility because of the biomass recalcitrance constructed by its chemical compositions and physical structures. Various pretreatment techniques have been developed during last several decades, among which dilute acid pretreatment has been considered as one of the leading pretreatments with a great commercialization potential. It has the advantage of not only solubilizing hemicelluloses but also converting the solubilized hemicelluloses to fermentable sugars. Generally, in the reported works for an effective pretreatment of biomass using dilute acid hydrolysis, the acid concentration is usually higher than 0.5% (typically 1%) at temperatures of higher than 160°C. However, there are some problems by using relatively “high” acid concentration, e.g. more serious corrosion to the facilities, more significant degradation of xylose and more generation of inhibitors to microbes, and more loss of fermentable sugars in the neutralization process. One of the solutions for these problems is to use relatively lower acid concentration to release xylose from lignocellulosic biomass and minimize sugar degradation. Another important problem associated with bioethanol production from lignocellulosic biomass refers to the utilization of pentose (mainly xylose). In agricultural residual biomass, hemicellulose which mainly consists of xylan can account for as high as 30% of the biomass. This part of polysaccharide should be utilized in order to reduce production cost. However, conventional Saccharomyces cerevisiae cannot convert xylose into ethanol, unless that a genetic-modified strain is used.

Biodiesel is derived from transesterification of triglyceride or esterification of free fatty acids with short-chain alcohols. Conventional oil feedstocks for biodiesel production are vegetable oils such as soybean oil, rapeseed oil, palm oil etc. However, the utilization of edible oils as feedstock is not sustainable. Thus exploitation of non-edible oil feedstock is important to the sustainable development of biodiesel industries. Regarding to the issue of xylose utilization involved in lignocellulosic ethanol production, an alternative pathway is to convert the xylose produced during the pretreatment into microbial lipid that can be used as feedstock for biodiesel production, because that some microorganisms e.g. fungi, yeast and algae, can easily utilize cheap and non-edible carbon sources such as xylose for accumulating intracellular lipids. Compared with vegetable oils, production of microbial lipids has many advantages, i.e. short life cycle, less labor required, less affection by venue, season and climate, and easiness to scale up. Particularly, many oleaginous microorganisms can use detoxified lignocellulosic biomass hydrolyzate for lipid accumulation with lipid content of 20-60%, which shows a great sustainability for oil feedstock supply. However, when using the dilute acid hydrolyzate for microbial oil production, the presence of inhibitors such as organic acids, phenol compounds and furfural usually show inhibitions to both growth and lipid accumulation of the microorganisms. Therefore, the conditions for acid hydrolysis of biomass should be re-optimized to maximize sugar recovery while minimize the production of inhibitors.

In our previous work, a mutant of Rhodospirillum toruloides was isolated by atmospheric room-temperature plasma mutagenesis. The mutant showed a high tolerance to some inhibitors and thus can produce lipid by directly using sugarcane bagasse hydrolyzate. Therefore, the objective of this work is to investigate the feasibility of co-production of ethanol and biodiesel from wheat straw based on dilute acid pretreatment. This work includes the following three parts: 1) the pretreatment...
condition with relatively low sulfuric acid concentration (≤0.5%) at mild temperature (≤160°C) were optimized for maximum production of fermentable sugars from wheat straw; 2) enzymatic hydrolysis of pretreated solid was studied under various conditions, and ethanol was produced by simultaneous saccharification and fermentation (SSF); and 3) the hemicellulosic hydrolyzate was used as a carbon source for microbial lipid production, and the lipid is further converted to biodiesel by immobilized-lipase catalyzed transesterification.

Finally a general mass balance is obtained for co-production of biodiesel and ethanol from wheat straw.

Experimental

Materials

Wheat straw used in the experiments was collected from Shandong province, China. It contained 33.1 ± 0.5% glucan, 22.4 ± 0.3% xylan, 3.1 ± 0.1% arabinan, 2.0 ± 0.1% acetyl group, 1.9 ± 0.1% acid soluble lignin and 10.6 ± 0.7% acid-insoluble lignin. Standard compounds used for HPLC calibration, including glucose, xylose, arabinose and cellobiose were purchased from Sigma–Aldrich (Shanghai branch). Other chemicals were purchased locally. The cellulase used in experiments was Cellic CTec2 which was kindly donated by Novozymes.

Experimental scheme

The experimental scheme is shown in Figure 1. Wheat straw was pretreated by dilute acid hydrolysis to maximize the cellulose accessibility. The hemicellulosic hydrolyzate was used as a carbon source for production of microbial lipid, which was further converted to biodiesel under the catalysis of immobilized lipase. The pretreated cellulose solid was then used for ethanol production by SSF. One of the objectives of the present work is to optimize the pretreatment condition to maximize sugar recovery for ethanol and microbial lipid (biodiesel) productions.

![Fig. 1 Flow chart of the process to convert wheat straw to bioethanol and biodiesel](image)

Strain and media

Oleaginous yeast *Rhodosporidium toruloides* AACC 20341 was purchased from Agricultural Culture Collection of China. It was mutagenized with Atmosphere Room Temperature Plasma (ARTP) and screened in our previous work. The mutant strain was named as *R. toruloides* M18. The culture of inoculums and fermentation were conducted under the condition described in our previous work. For ethanol production, the yeast used was *Saccharomyces cerevisiae* CICC 31014. The medium contained 2 g/L (NH4)2SO4, 5 g/L KH2PO4, 5 g/L yeast extracts, 1 g/L MgSO4 and 0.2 g/L CaCl2.

Pretreatment

All pretreatments were carried out in a stainless steel reactor equipped with a temperature controller and an impeller-type mixer (TFCF5-15, Tianzhou Haidai Science and Technology Co., Ltd, China). For super-dilute acid pretreatment, 100 g 2-cm long wheat straw and 1 L super-dilute acid solution (0, 0.05, 0.1%/w/v H2SO4) were loaded in the reactor and heated to the desired temperature (160, 180 or 200°C). The pretreatment was then kept at 300 rpm for 20 min. It took 1-1.5 h to increase the temperature to 160-200°C. After pretreatment, the slurry was filtered to obtain solid fraction and liquid hydrolyzate (WSH). The solid fraction was washed with adequate water. The chemical compositions of wheat straw and pretreated solid were carried out according to the NREL laboratory analytical procedure. For dilute acid pretreatment, 100 g wheat straw and 1 L dilute acid solution (0.3-0.6%/w/v H2SO4) were mixed and heated to the desired temperature (140, 150 or 160°C) at 300 rpm for 1 h. After filtration, the solid fraction was washed with 200 mL water, and the filtrate was combined with the liquid fraction.

Preparation of the neutralized liquid hydrolyzate

The obtained hydrolyzate was first concentrated by evaporation under reduced pressure. Calcium hydroxide was then added to adjust the pH to 10.0 in a process called overliming. The mixture was maintained at 60°C in a water bath with stirring for 1 h, and cooled to room temperature. It was then re-adjusted to pH 6.0 with H2SO4 and filtered to remove the settled gypsum. Thereafter, the hydrolyzate was autoclaved for 20 min at 115°C.

Culture condition of *R. toruloides* M18 and oil extraction

The growth test of the oleaginous yeast *R. toruloides* M18 in WSH medium was performed in test tubes. 500 µL yeast preculture was inoculated in 5 mL nitrogen-abundant WSH medium, and cultivated at 30°C, 200 rpm. For liquid fermentation, 10 mL yeast preculture was inoculated in 100 mL prepared hydrolyzate medium in 500 mL flask, followed by cultivation at 30°C, 200 rpm for 9 days. The crude lipid was extracted by acid-heating procedure as described in our previous work.

Enzymatic hydrolysis and simultaneous saccharification and fermentation (SSF) of pretreated cellulosic solid

Enzymatic hydrolysis of the pretreated cellulosic solid was performed according to our previous work, namely at 50°C, pH 4.8 acetate buffer (0.05 M) in a 130 rpm air-bath shaker for 5 days with dry solid loading of 5% (w/v) and cellulase loading of 15 FPU/g dry solid (pretreated biomass solid). Samples were taken at 12, 24, 48, 72 and 96 h. For analysis of sugar concentration, the slurry was first centrifuged at 14000 rpm for 5 min, and the liquid supernatant was diluted with deionized water for 50 folds. For SSF, pretreated slurry containing 0.3 g dry mass and 10 mL deionized water (including water contained in the solid) were put into the flask and autoclaved at 115°C for 20 min. After cooled down to room temperature, Tween 20 and Tween 80 (0.05, 5 g/L) was added respectively, followed by adjusting the pH to 5.0 with 1 M NaOH before adding cellulase enzymes. After enzymatic pre-hydrolysis for 24 h, 1 mL preculture of *S. cerevisiae* was inoculated into the flask. The SSF was then...
performed for 72 h. The sampling procedure was the same as that of enzymatic hydrolysis.

**Analytical methods**

Quantitative analyses of glucose, xylose, arabinose, acetic acid, and furfural were performed with a Shimadzu10AVP HPLC system (Shimadzu Corp., Japan) equipped with a RID-R1A refractive index detector and Aminex HPX-87H column (300 × 7.8 mm, Bio-Rad, USA) at 65°C with 5 mM H₂SO₄ as the eluent at a flow rate of 0.8 mL/min⁻¹. The phenolic compounds, regarded as acid-soluble lignin in this research, were determined by measuring the absorbance at 205 nm with UV1800 spectrophotometer (Shimadzu Corp., Japan). For determination of fatty acid compositions, the crude lipid was first converted to fatty acid methyl ester (FAME) with excess methanol in a tert-butanol system under the catalysis of excess loading of immobilized lipase (NS 435 and NS 40044, Novozymes). The analysis of obtained FAME was performed with an Agilent 7890A GC (Agilent, USA) equipped with a CP-FAP CB capillary column (25 m × 0.32 m × 0.30 μm, Agilent, USA) and a flame ionization detector. Heptadecanoic acid methyl ester was used as internal standard. The column temperature was kept at 180°C for 0.5 min and heated to 250°C at 10°C/min, and then kept for 6 min. The temperatures of the injector and detector were set at 245 and 260°C, respectively.

**Results**

**Super-dilute acid pretreatment**

As aforementioned description, higher acid concentration may lead to more loss of C5 sugar with generation of more inhibitors. Therefore, we firstly attempted super-dilute acid pretreatment expecting to enhance sugar release by lowering acid concentration. The H₂SO₄ concentration and temperature were in the ranges of 0-0.1% and 160-200°C. The experimental results (Table 1) show that xylose concentration in hydrolyzate was in the range of 0.34-3.24 g/L, corresponding to 0.3-3.2 g xylose/100g wheat straw. This yield was unexpectedly low. Further analysis of the main chemical compositions of the remaining solid (see Table 1) indicated that the low xylose concentration by super-dilute pretreatment was mainly ascribed to two reasons: first, at low temperature (160°C) H₂SO₄ concentration was not high enough to hydrolyze hemicellulose, so that the percentage of xylan hydrolyzed was low (<35% depending on H₂SO₄ concentration); second, hemicellulose was well hydrolyzed at high temperature such as 200°C, but serious degradation of the produced xylose took place due to the severe condition. This fact can be proved by the high furfural concentration obtained at 200°C. According to experimental results, temperature showed more significant influence on polysaccharide hydrolysis during pretreatment. The percentage of xylan hydrolyzed increased from <35% at 160°C to >90% at 200°C. It should be noted that the degradation of formed xylose was significant even at 160°C. More than 80% of xylose degraded for all of experimental runs. Furfural concentration was less than 0.01 g/L at 160°C but increased to 2.43-3.74 g/L at 180°C depending on H₂SO₄ concentration. The concentrations of other inhibitors such as FA, AA, 5-HMF and phenols increased with temperature and H₂SO₄ concentration. Temperature was found to be more important than H₂SO₄ concentration for inhibitor generation.

Glucan hydrolysis was in the range of 3-20% as shown in Table 1. However, glucan content after pretreatment increased with temperature and acid concentration, which was mainly attributed to the hydrolysis of xylan. Similar phenomenon was observed for lignin. Acetyl group was removed during pretreatment. Particularly, the acetyl group content can be reduced to less than 1% at 180°C, while no acetyl group in pretreated solid was detected at 200°C.

The solids obtained by super-dilute acid pretreatment were further converted to glucose by enzymatic hydrolysis using cellulase. The solid pretreated with 0.1% H₂SO₄ at 200°C gave the maximum glucan conversion (96.4%) to glucose after 96 h hydrolysis. For comparison, the results of enzymatic hydrolysis courses at each temperature and H₂SO₄ concentration were averaged (Figure 2 A column). Glucan conversion increased with temperature significantly. The average glucan conversion was lower than 50% when pretreatment was conducted at 160°C, while higher than 80% at 200°C. However, H₂SO₄ concentration in the experiment range (0-0.1%) showed no significant effect on glucan conversion. It indicated that temperature was the most important variable to increase cellulose digestibility for super-dilute acid pretreatment. The experimental data illustrate that super-dilute acid pretreatment is not so satisfying as expected to obtain a high xylose yield, though good enzymatic digestibility could be achieved at 200°C. Significant degradation of xylose took place during pretreatment, which lowered the total sugar yield. Thus, a compromise should be made by properly decreasing pretreatment temperature and increasing H₂SO₄ concentration; however, cellulose enzymatic digestibility might be lowered accordingly.

![Fig. 2 Enzymatic hydrolysis of wheat straw pretreated by super-dilute acid (A column) and dilute acid (B column): (a) Effect of H₂SO₄ concentration (each datum is the average result of those at 160,180 and 200°C, respectively). (b) Effect of temperature (each datum is the average result of those at H₂SO₄ concentration of 0, 0.05 and 0.1%) (Enzymatic condition: 5% solid consistence, 15 FPU/g solid cellulase loading)](image-url)
relatively high H$_2$SO$_4$ concentrations (0.3-0.6%) at relatively low temperatures (140-160 °C). In the hydrolyzate, glucose, xylose, arabinose, formic acid, acetic acid and furfural were detected by HPLC but 5-HMF was not detectable as shown Table 2. These hydrolyzates contained xylose of 13.26-16.66 g/L, which was much higher than those of super-dilute hydrolysates. The hydrolyzate obtained with 0.6% H$_2$SO$_4$ at 140 °C had the highest xylose yield (67.63%); however, no much difference was observed for the hydrolyzate obtained with 0.5% H$_2$SO$_4$ at 140°C (66.14%). Therefore, if the subsequent neutralization process is taken into account, the later pretreatment condition was preferred. 5-HFM was not detected at <160 °C, while furfural and phenol concentrations increased with pretreatment severity. The chemical compositions of the remaining solid after dilute acid pretreatment are shown in Table 2. A similar percentage of xylan hydrolyzed was obtained for pretreatments with 0.5% H$_2$SO$_4$ at 140 °C and 0.3% H$_2$SO$_4$ at 160 °C; however, the xylose degradation was more serious at 160 °C. Glucan and lignin contents increased after dilute acid pretreatment compared with those of raw material due to the removal of hemicelluloses.

### Table 1 Sugar and inhibitor concentrations in hydrolyzate and chemical compositions of pretreated solid after super-dilute acid pretreatment

<table>
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<tr>
<th>Run</th>
<th>H$_2$SO$_4$ (%)</th>
<th>Temp. (°C)</th>
<th>Glucose (g/L)</th>
<th>Xylose (g/L)</th>
<th>Arabinose (g/L)</th>
<th>FA (g/L)</th>
<th>AA (g/L)</th>
<th>5-HMF (g/L)</th>
<th>Furfural (g/L)</th>
<th>Phenols (g/L)</th>
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### Table 2 Sugar and inhibitor concentrations in hydrolyzate and chemical compositions of pretreated solid after dilute acid pretreatment

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<th>Run</th>
<th>H$_2$SO$_4$ (%)</th>
<th>Temp. (°C)</th>
<th>Glucan (%)</th>
<th>Xylan (%)</th>
<th>Arabinan (%)</th>
<th>Acetyl group (%)</th>
<th>Total lignin (%)</th>
<th>Glucan hydrolyzed (%)</th>
<th>Xylan hydrolyzed (%)</th>
<th>Xylose degradation (%)</th>
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<td>17.4</td>
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* based on initial xylan in wheat straw.

The pretreated solid was further converted to glucose by enzymatic hydrolysis (Figure 2 B column). The final glucan conversions of these samples at 96 h were in the range of 75-85%, but glucan conversions of solids pretreated at 150 °C and 160 °C were relatively higher (79.3% and 83.3%, respectively) than those pretreated at 140°C (75.8%-76.4%). For further
Enzymatic hydrolysis of pretreated cellulosic solid

The enzymatic glucan conversion is affected by not only pretreatment but also enzymatic hydrolysis conditions. Optimizations of some variables in the enzymatic hydrolysis process were thus further performed. The enzymatic glucan conversions under different hydrolysis conditions are shown in Figure 3.

Effect of buffer pH

It has been known that the optimum pH for cellulose hydrolysis by commercial cellulase is 4.8. However, some recent researches demonstrated that enzymatic saccharification of sulfite-pretreated substrates should be conducted at elevated pH 5.2–6.2. We thus investigated the effect of pH on the enzymatic hydrolysis of dilute acid-pretreated WS as shown in Figure 3(a). The maximum glucan conversion (76.4%) was obtained at pH 4.8, whereas only 33.4% of glucan conversion was obtained at pH 4.0. It indicated that the optimum pH for enzymatic hydrolysis of pretreated substrates might be relevant to lignin structure such as molecular weight, functional groups as well as surface charges. More details will be investigated in our future work.

Effect of solid loading

Solid loading is another important parameter affecting enzymatic glucan conversion and glucose concentration in the hydrolyzate. As shown in Figure 3(b), glucan conversion did not change with the increase of solid loading from 3% to 7.5%, but a little bit decreased at solid loading of 10%. Therefore, it is preferred that enzymatic hydrolysis is performed at solid loading of less than 7.5% for obtaining a high glucan conversion. However, glucose concentration increased with solid loading. For example, after hydrolysis for 96 h, the glucose concentrations in the enzymatic hydrolyzate were 12.11, 19.49, 30.07 and 37.89 g/L for solid loadings of 3.0%, 5.0%, 7.0% and 10.0%, respectively. Therefore, solid loading should be selected by comprehensively considering both glucan conversion and glucose concentration.

Effect of enzyme loading

The pretreated WS was hydrolyzed with 10, 15 and 20 FPU/g solid of cellulase loadings, respectively, as shown in Figure 3(c). The conversions before 48 h increased with cellulase loading dramatically, but there was no notable difference for the conversions at 72 h. When hydrolysis was performed for 24 h, the glucan conversions were 50.4%, 54.0% and 62.9% at cellulase loadings of 10, 15 and 20 FPU/g solid, respectively. The glucan conversions at 96 h were found to be 67.3% (10 FPU/g), 68.5% (15 FPU/g) and 75.4% (20 FPU/g), respectively. Therefore, cellulase loading should be selected with consideration of hydrolysis time.

Effect of surfactant loading

Nonionic surfactants have been found to effectively increase enzymatic hydrolysis of pretreated biomass by reducing the nonproductive adsorption of cellulase onto lignin matrix. In this work, Tween 20 and 80 were used to enhance the enzymatic glucan conversion. As shown in Figure 3(d) and (e), the addition of 5.0 g/L Tween 20 increased the enzymatic glucan conversion of the pretreated solid from 64.3% (control) to 70.4%. The addition of 5.0 g/L Tween 80 increased the glucan conversion from 64.3% (control) to 72.3%.

Additional comments

In order to test whether oleaginous yeast can grow in dilute acid hydrolyzate without detoxification by activated charcoal adsorption, R. toruloides M18 was cultured in WSH obtained by pretreatment with 0.3-0.5% H$_2$SO$_4$ at 140-160°C (initial xylose: 37.7 ± 2.5 g/L) with presence of abundant nitrogen source (10 g/L yeast extract and 20g/L peptone). As shown in Figure 4(a), the OD660 of cell growth in WSH obtained with 0.4% and 0.5% H$_2$SO$_4$ at 140 °C reached 17.1 and 14.4, respectively, which were much higher than those in WSH obtained with 0.4% H$_2$SO$_4$ at 140 °C.
150 °C and 0.3% H₂SO₄ at 160 °C (10.9 and 8.6 respectively). This was because that the WSH obtained at 150 °C and 160 °C contained more inhibitors such as acetic acid, furfural and phenols as shown in Table 2. It further indicated that 140 °C was preferred for wheat straw pretreatment, at which the acid hydrolyzate showed a high fermentability.

**Lipid accumulation in hydrolyzate medium with limited nitrogen source**

WSHs obtained with 0.4% and 0.5% H₂SO₄ at 140 °C were further used as carbon source for microbial lipid production by R. toruloides M18 with limited nitrogen source. The cell growth and xylose consumption during fermentation are shown in Figure 4(b) and (c). For comparison, pure xylose and glucose were used in control experiments at different initial concentrations. Cell growth steadily increased as xylose gradually consumed. Higher cell growth rates were observed for control experiments; however, more sugars were consumed for WSH media as shown in Table 4. Xylose concentration decreased to less than 10 g/L at 192 h for all of WSH samples, while the residual sugar concentration were in the range of 3.5-21.0g/L for controls depending on the initial sugar concentration. WSH could obtain dry cell weight of 5.94-8.52 g/L with sugar consumption of 32.4-35.0 g/L. The lipids productivity was 46.2-31.5 g/L and lipid contents in dry cell were 25-27%. Corresponding sugars-to-lipid conversions were 4.5-6.6%. For the control samples, the dry cell weights were 6.90-8.65 g/L and sugar consumptions were 23.5-34.5 g/L. Corresponding lipid productivities were 2.50-3.55 g/L and lipid contents in dry cell reached 36.2-41.3%. The sugar-to-lipid conversions of controls were much higher than those of WSH. This was probably due to the presence of inhibitors in WSH, so that the yeast had to consume more carbon source for energy production and synthesize corresponding enzymes to tolerate the inhibitors.

**Table 3** Sugar productions via dilute pretreatment of wheat straw followed by enzymatic hydrolysis under different pretreatment conditions

<table>
<thead>
<tr>
<th>Temp. °C</th>
<th>H₂SO₄%</th>
<th>GₚH</th>
<th>g/L</th>
<th>XₚH</th>
<th>g/L</th>
<th>GₐH</th>
<th>g/L</th>
<th>XₐH</th>
<th>g/L</th>
<th>TSR</th>
<th>g</th>
<th>TSY</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>140</td>
<td>0.4</td>
<td>1.2</td>
<td>13.3</td>
<td>25.0</td>
<td>2.2</td>
<td>42.2</td>
<td>69.1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>140</td>
<td>0.5</td>
<td>1.6</td>
<td>16.3</td>
<td>26.9</td>
<td>2.4</td>
<td>47.2</td>
<td>77.3</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>140</td>
<td>0.6</td>
<td>2.4</td>
<td>16.7</td>
<td>25.3</td>
<td>2.9</td>
<td>47.3</td>
<td>77.4</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>150</td>
<td>0.4</td>
<td>1.9</td>
<td>15.4</td>
<td>24.1</td>
<td>1.9</td>
<td>43.4</td>
<td>71.0</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>160</td>
<td>0.3</td>
<td>1.5</td>
<td>13.9</td>
<td>26.8</td>
<td>2.1</td>
<td>44.4</td>
<td>72.7</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

G: Glucose concentration; X: Xylose concentration; subcript: PH-prentreatment hydrolyzate; EH-Enzymatic hydrolyzate; TSR: Total sugar recovery from 100 g WS, TSY: Total sugar yield based on theoretic yield of glucose plus xylose

After fermentation, the intracellular lipid was extracted. The fatty acid compositions of the lipid were analyzed as shown in Table 4. The major fatty acid compositions of the lipids were found to be C16:0 (palmitic acid), C18:0 (stearic acid), C18:1 (oleic acid) and C18:2 (linoleic acid), which accounted for more than 85% of the total fatty acids. Oleic acid was the most abundant fatty acid in the lipid, which accounts for 55.0% of the lipids obtained by WSH and 32.3% of the lipid obtained by control (pure sugar) medium. Compared with those of control lipid, the lipid obtained by WSH contained much higher C18:1 but much less C18:2. This result indicated that inhibitors present in WSH might cause the change of metabolic pathway of R. toruloides M18, which resulted in alteration of the main fatty acid composition. However, the fatty acid compositional profile of the yeast lipid was in the range of various plant oil and animal tallow, indicating that the lipid could be used as biodiesel feedstock.

**Conversion of microbial lipid to biodiesel**

The extracted lipid was further converted to biodiesel by enzymatic transesterification as shown in Figure 4(d). For comparison, refined soybean oil was also used as feedstock for biodiesel production. The lipid could be well converted to biodiesel, though the conversion rate was lower than that of soybean oil. The lipid obtained from WSH showed the lowest reaction rate. This was probably because the extracted crude lipid contained some impurities such as phospholipid that might reduce transesterification rate. However, after reaction for 24 h, the lipid-to-biodiesel conversion could reach 90%, similarly to that of soybean oil. It indicated that the yeast lipid indeed could be used as feedstock for biodiesel production.

**Fig. 4** Cell growth and xylose consumption during conversion of dilute acid hydrolyzate to microbial lipid by R. toruloides M18, and enzymatic conversion of obtained lipid to biodiesel. (a) Cell growth in different hydrolysates with abundant nitrogen source; (b) Cell growth in different hydrolysates and control media with limited nitrogen source; (c) Xylose concentration in different hydrolysates and control media with limited nitrogen source; (d) Lipid conversion could reach 90%, similarly to that of soybean oil. It indicated that the yeast lipid indeed could be used as feedstock for biodiesel production.

**Mass balance for co-production of biodiesel and bioethanol from wheat straw**

According to experimental data, a general mass balance for co-production of ethanol and biodiesel from wheat straw via dilute acid pretreatment with 0.5% H₂SO₄ at 140°C was obtained as shown in Figure 5. 0.90 g microbial lipid was produced from 100 g wheat straw, from which 0.8 g biodiesel was obtained. 10.1 g ethanol was produced from 100 g wheat straw. Theoretically, if xylan and cellulose of wheat straw are completely converted to microbial lipid and ethanol respectively (without formation of biomass and other products), corresponding yields are 7.3 g lipid/100 g WS and 18.6 g ethanol/100g WS. However, the experimental data demonstrated that co-production of biodiesel and ethanol from wheat straw is thermodynamically feasible but economically unfeasible.
and ethanol from wheat straw is technically feasible, though more optimization should be performed.

Table 4 Lipid accumulation of *R. toruloides* M18 using WSH liquid medium and nitrogen-limited medium (control), and the fatty acid composition of extracted lipid

<table>
<thead>
<tr>
<th>Lipid accumulation</th>
<th>Medium No.</th>
<th>Initial glucose &amp; xylose DCW</th>
<th>Lipid in medium</th>
<th>Lipid content</th>
<th>Glucose &amp; xylose consumption</th>
<th>C-source consumption</th>
<th>Sugars to lipid conversion</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A</td>
<td>34.3 &amp; 5.98</td>
<td>1.49</td>
<td>25.06</td>
<td>32.4</td>
<td>39.8</td>
<td>4.6</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>38.2 &amp; 5.94</td>
<td>1.46</td>
<td>24.68</td>
<td>32.6</td>
<td>36.9</td>
<td>4.5</td>
</tr>
<tr>
<td>C (control)</td>
<td></td>
<td>45.6</td>
<td>3.55</td>
<td>41.31</td>
<td>30.5</td>
<td>30.5</td>
<td>12.0</td>
</tr>
<tr>
<td>D (control)</td>
<td></td>
<td>38.0 &amp; 6.65</td>
<td>3.20</td>
<td>37.07</td>
<td>34.5</td>
<td>34.5</td>
<td>9.3</td>
</tr>
<tr>
<td>E</td>
<td></td>
<td>52.4</td>
<td>2.31</td>
<td>27.09</td>
<td>35.0</td>
<td>40.9</td>
<td>6.6</td>
</tr>
<tr>
<td>F (control)</td>
<td></td>
<td>52.0</td>
<td>2.50</td>
<td>36.23</td>
<td>23.5</td>
<td>23.5</td>
<td>10.6</td>
</tr>
</tbody>
</table>

**Fatty acid composition (%) of extracted lipid**

<table>
<thead>
<tr>
<th>Media</th>
<th>C14:0</th>
<th>C16:0</th>
<th>C16:1</th>
<th>C18:0</th>
<th>C18:1</th>
<th>C18:2</th>
<th>C18:3</th>
<th>Others</th>
</tr>
</thead>
<tbody>
<tr>
<td>B</td>
<td>1.3</td>
<td>24.7</td>
<td>1.2</td>
<td>9.1</td>
<td>55.0</td>
<td>5.8</td>
<td>0.4</td>
<td>2.4</td>
</tr>
<tr>
<td>D</td>
<td>1.4</td>
<td>29.1</td>
<td>1.2</td>
<td>8.1</td>
<td>32.3</td>
<td>20.7</td>
<td>4.8</td>
<td>2.4</td>
</tr>
</tbody>
</table>

Note: A: WSH obtained with 0.4% H$_2$SO$_4$ at 140 °C; B: WSH obtained with 0.5% H$_2$SO$_4$ at 140 °C; C: Control 1#; D: Control 2#; E: WSH obtained with 0.6% H$_2$SO$_4$ at 140 °C; F: Control 3#; DCM: Dry cell weight; C-source consumption was calculated as the sum of consumption of glucose, xylose and acetic acid.

Discussions

Bioethanol and biodiesel are two important liquid biofuels. However, currently both of them are mainly produced from food feedstocks such as edible vegetable oils and grains. Therefore, production of biofuels from non-edible feedstock such as lignocellulosic biomass is necessary for a sustainability of biofuel industries. The structural carbohydrates of agricultural residues mainly consist of glucan and xylan. However, conventional *Saccharomyces cerevisiae* cannot efficiently convert xylose into ethanol, unless a genetic-modified strain is used. Therefore, in the present work, we have proposed to convert glucose to ethanol by *Saccharomyces cerevisiae* and xylose to microbial lipid by *Rhodosporidium toruloides* M18. The lipid was further chemically extracted and converted to biodiesel enzymatically. By this process, biological co-production of ethanol and biodiesel can be achieved using lignocellulosic biomass as a single feedstock.

Release of fermentable sugar is the prerequisite for biologically converting lignocellulosic biomass to ethanol and biodiesel. Dilute acid pre-hydrolysis of hemicellulose followed by enzymatic hydrolysis of pretreated cellululosic solid has been found to be a promising method for fermentable sugar production. In order to reduce acid dosage, we first performed super-dilute acid pretreatment expecting to reduce sugar degradation. Nevertheless, the experiment results showed that xylan could not be well hydrolyzed at low temperature, while most of the formed xylose was degraded at high temperature. The relatively long retention time was also a reason for the serious degradation of xylose. Probably, controlling hydrolysis time according to the reaction kinetics may guide the maximum recovery of xylose, but such kinetic modeling works on super-dilute acid hydrolysis of wheat straw were not found in literatures. According to the kinetics of xylan hydrolysis by dilute acid, retention time was only seconds to minutes in order to obtain the maximum xylose yield at high temperature. However, because of reactor limitation, fast increase of temperature to 140°C or higher cannot be fulfilled in the present work. On the other hand, the experimental data demonstrated that high temperature was preferred for increasing the enzymatic digestibility. This is mainly because that higher temperature leads to more destruction of wheat straw cell wall thus more efficiently exposing cellulose. The lignin structure changes caused by high temperature might also contribute to the increase of cellulose digestibility.

Fig. 5 Mass balance for biodiesel and ethanol production from wheat straw via dilute acid pretreatment

To decrease the degradation of xylose, moderate condition should be employed. Based on a kinetic model prediction, several acid concentrations and temperature were selected for...
pretreatment. Xylose concentration could be greatly enhanced at these moderate conditions. With consideration of sugar yield as well as inhibitor concentrations, 0.5% H$_2$SO$_4$ and 140°C were found to be the “optimum” condition. 77.3% of theoretic total sugar (glucose and xylose) yield was obtained, which was comparative with reported results $^{26,32}$. However, the acid dosage used in the present work was much lower.

The enzymatic hydrolysis of pretreated cellulosic solid is affected by not only pretreatment process but also the enzymatic conditions. The important parameters influencing enzymatic hydrolysis of cellulose mainly include pH, solid consistency and cellulase loading. After studying the enzymatic digestibility of pretreated wheat straw, a relatively optimum condition for enzymatic hydrolysis was obtained to guide the SSF process. All addition of non-ionic surfactant such as Tween-20 and Tween 80 was found to increase glucon conversion by 6-8% in pH-control system (buffer). This result was in accordance with those reported in literatures $^{26,32}$. Nevertheless, in SSF process, no improvement was observed when Tween 20 and 80 were added. It indicated that enzymatic hydrolysis condition might influence the improvement action of surfactants. The ethanol yield was only 58% of theoretic yield with ethanol concentration of 15.1 g/L. This was mainly ascribed to the un-controlled pH of the system, and thus glucose release became a limiting step to the final ethanol yield. However, this yield was comparatively similar to the reported results for dilute acid pretreated wheat straw $^{26,33}$. For example, Saha et al., reported an ethanol concentration of 13±2g/L with yield of 0.17g ethanol/g raw material after wheat straw was pretreated by 0.75% H$_2$SO$_4$ at 121°C for 1h $^{26}$. Karagöz and Özkan reported an ethanol yield of 0.2195 g ethanol/g sugar (43% of theoretic yield), corresponding to 0.1187g ethanol/g raw material after the biomass was pretreated with 1% H$_2$SO$_4$ at 140 °C for 90 min $^{33}$.

The neutralized acid hydrolyzate could be directly utilized by a mutagenized Rhodosporidium toruloides (M18) for lipid accumulation. Detoxification with activated charcoal thus can be eliminated, which shows promise to reduce production cost, because that detoxification with activated charcoal adsorption is usually expensive and may cause a significant loss of sugars. However, compared with pure sugar media, WSH media seemed to cause more consumption of carbon source to form other products rather than lipid. This was probably because that the strain needed to consume more carbon source for energy generation and synthesize more enzymes to tolerant the inhibitors present in the hydrolyzate.

Mass balance showed that both lipid and ethanol yields obtained in the present work were much lower than theoretic yields. This is mainly due to two reasons. First, the polysaccharide-to-sugar conversion is not high enough. In the present work, xylose yield by dilute acid hydrolysis was only approximate 66%, and glucose yield by enzymatic hydrolysis was approximate 74%. Second, the conversion of sugar to lipid is too low. As aforementioned description, this is mainly because that a great part of sugar flowed to other products rather than lipid. Therefore, much effort should be made to increase sugar yield. One possible improvement is to use two-step pretreatment. In the first step, higher xylose yield can be obtained under moderate condition with higher acid concentration, while in the second step, the pre-hydrolyzed solid is further treated at high temperature or with chemicals to achieve a high enzymatic conversion of cellulose. The sugar-to-lipid conversion also should be improved. For this purpose, genetic modification as well as metabolic engineering methodology might provide promising tools to enhance the flow of sugars to lipid synthesis. Anyway, this work has provided an idea to co-produce ethanol and biodiesel using lignocellulosic biomass as a single feedstock. However, a cost evaluation should be performed for estimating the economic feasibility of the process.

Conclusions

A biological co-production of bioethanol and biodiesel was proposed by using wheat straw as a feedstock. Super-dilute acid (0-0.1%) and dilute acid (0.3-0.6%) pretreatments were employed for release of sugars. Xylose recovery of super-dilute acid pretreatment under the experimental conditions was too low to be used for microbial lipid production. Temperature had a very significant effect on xylan hydrolysis, xylose degradation as well as enzymatic digestibility of cellulose. Xylan was not well hydrolyzed at a low temperature such as 160°C, while a good cellulose digestibility was obtained at a high temperature such as 200°C, but more than 80% of the formed xylose was degraded. By using moderate condition (140-160°C) with higher sulfuric acid concentration (0.3-0.6%), xylose recovery could be greatly increased to 60-70% with only 17-30% of the formed xylose degraded. The relatively optimum condition for dilute acid pretreatment of wheat straw was found to be 0.5% H$_2$SO$_4$, 140°C for 1 h. Under this condition, xylose yield of 66.14% in pretreatment and glucose yield of 76.4% in subsequent enzymatic hydrolysis were obtained. The pretreated solid showed an acceptable enzymatic digestibility. Ethanol concentration of 22.6% with approximate 58% of theoretic yield was obtained by SSF. The dilute acid hydrolyzate could be directly converted to microbial lipid using a mutagenized Rhodosporidium toruloides (M18) with lipid content of around 25%. However, the sugar-to-lipid conversion was relatively low due to the presence of multi-inhibitors. The extracted lipid was well converted to biodiesel with 90% conversion ratio under the catalysis of immobilized lipase. Mass balance showed that 0.80 g biodiesel and 10.1 g ethanol can be produced from 100 g wheat straw via dilute acid pretreatment. This work demonstrated that co-production of biodiesel and ethanol from wheat straw is technically feasible, though improvement should be made to increase sugar yield as well as microbial lipid and ethanol yields.

Acknowledgments

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† Electronic Supplementary Information (ESI) available for kinetic modeling of dilute acid hydrolysis for formation of xylose, and simultaneous saccharification and fermentation of dilute acid pretreated solid