

Combined Fungal and Mild Acid Pretreatment of *Glycyrrhiza uralensis* Residue for Enhancing Enzymatic Hydrolysis and Oil Production

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The feasibility of the combination of fungal with mild acid pretreatments of *Glycyrrhiza uralensis* residue (GUR) for enzymatic hydrolysis and oil production was studied. Combined pretreatment with *Phanerochaete chrysosporium* and 2.5% sulfuric acid was shown to be more effective than the acid-only pretreatment. Subsequently, the residue obtained from acid hydrolysis was subjected to enzymatic hydrolysis to generate fermentable sugars for oil production by *Chlorella protothecoides*. The reducing sugar yield of enzyme hydrolysate from co-treated GUR was 1.08- to 1.71-fold higher than that obtained from acid-treated GUR under the same conditions. The highest cell dry and oil yield from co-treated GUR reached 4.16 and 1.66 g/L dry weight, respectively, values which were 2.1- and 3.32-fold higher than those using glucose as a carbon source. This study suggested that combined pretreatment with *P. chrysosporium* and 2.5% sulfuric acid is an effective strategy for enhancing enzymatic hydrolysis and microalgal oil production from GUR.

Keywords: *Glycyrrhiza uralensis*; Fungal pretreatment; Acid pretreatment; Enzymatic hydrolysis; Oil production

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INTRODUCTION

Glycyrrhiza uralensis Fisch. ex DC, a species of licorice native to Asia, is extensively used in traditional Chinese medicine (Liu *et al.* 2007). The rhizomes of *G. uralensis* are used to isolate bioactive compounds, such as glycyrrhizin and flavonoids, and they also contain high amounts of starch, cellulose, and hemicelluloses (Obolentseva *et al.* 1999). In the traditional glycyrrhizin manufacturing process, cellulose and hemicelluloses in the rhizomes are neglected and discarded or directly burnt in the field, leading to a serious waste of natural resources and further aggravation of environmental pollution. However, some studies have indicated that *G. uralensis* residue (GUR) is a potential resource for production of microbial oil, ethanol fuel, biogas, and other valuable products because vast quantities of *G. uralensis* are produced annually in China and the residues contain large amounts of cellulose and hemicelluloses (Liu *et al.* 2007; Sdykov and Abduazimov 2002). Microbial oil, produced by various oleaginous microorganisms (such as yeast, fungi, bacteria, and microalga), can be used as a promising feedstock for biodiesel production to alleviate the energy crisis and reduce environmental pollution (Liang and Jiang 2013).

Microbial oil production from GUR involves enzymatic hydrolysis and fermentation. Pretreatment prior to enzymatic hydrolysis is essential to break down lignin and allow cellulases easier access to cellulose (Janga *et al.* 2012). Acid pretreatments are

effective methods for removing lignin and hemicelluloses and retaining most of the cellulose in lignocellulosic biomass (Baboukani *et al.* 2011; Kupiainen *et al.* 2012; Ratsamee *et al.* 2012). The conventional processes are energy-intensive, environmentally unfriendly, and produce a number of inhibitory substances to restrain the growth of some microorganisms during the downstream fermentation process. Despite these issues, acid pretreatments are usually employed to disrupt the lignin of lignocellulosic biomass at high temperature and pressure (Yu *et al.* 2011). In addition, a preliminary study has indicated that acid pretreatment of GUR followed by enzymatic hydrolysis could significantly increase the enzymatic hydrolysis ratio of GUR (data not shown).

Biological pretreatment is based on the capacity of certain microorganisms (fungi and bacteria) to perform delignification and enhance the accessibility of cellulases to cellulose (Salvachúa *et al.* 2011; Sánchez 2009). Fungi, such as white-, brown- and soft-rot fungi, are the best-known microorganisms capable of degrading lignin; among these, white-rot fungi can most actively degrade the lignin component (Zheng *et al.* 2009). The white-rot fungus *Phanerochaete chrysosporium* has received considerable attention in recent years because it grows fast and secretes an abundance of extracellular lignin peroxidase and manganese peroxidase to selectively degrade lignin, azo dye, and organopollutants more rapidly than other microbes (Liong *et al.* 2012; Shi *et al.* 2012; Singh *et al.* 2011). Therefore, this organism can be used to develop environmentally friendly technologies for wastewater purification and municipal solid waste management (Raut *et al.* 2008; Yildirim *et al.* 2011). However, previous studies have resulted in controversy concerning bio-pretreatment because the mass loss of holocellulose during fungal cultivation and a long incubation period reduced overall productivity (Salvachúa *et al.* 2011). To solve the above-mentioned problems, Salvachúa *et al.* (2011) suggested that biological pretreatment combined with other pretreatments could be more effective at enhancing the digestibility of cellulosic materials. Unfortunately, there are few reports on lignocellulosic biomass co-treated with fungus and mild acid.

Therefore, the objectives of this study were as follows: (1) to compare the effect of acid pretreatment alone, relative to the combination of biological pretreatment (*P. chrysosporium*) with dilute sulfuric acid pretreatment on the reducing sugar yield; and (2) to explore the use of the hydrolysate from enzymatic hydrolysis of co-treated GUR as a substrate for oil production using *Chlorella protothecoides*.

EXPERIMENTAL

Microorganisms and Cultivation

P. chrysosporium was purchased from the Guangdong Culture Collection Center (GIM3.383), Guangzhou, P. R. China. The strain was maintained on a potato-dextrose agar (PDA) slant at 4 °C. A plug of the strain activated for 5 to 6 days on a PDA slant at 28 °C was inoculated onto a PDA plate and incubated at 28 °C for 7 days. The actively growing mycelium from the PDA plate was inoculated into a 250-mL Erlenmeyer flask with 100 mL of potato dextrose broth (PDB) medium (pH=6.0) and incubated at 28 °C and 180 rpm for 7 days on a reciprocal shaker. The culture can be used as inocula for biological pretreatment.

C. protothecoides was purchased from the Freshwater Alga Culture Collection of the Institute of Hydrobiology (FACHB-2), Wuhan, P. R. China. The organism was maintained in selenite enrichment (SE) media at 25 °C. The strain was inoculated into a

250-mL Erlenmeyer flask with 100 mL of fresh SE media and incubated in an illumination incubator with illumination intensities of 3000 lux at 25 °C under static conditions for 7 days. The culture can be used as inocula for heterotrophic cultivation.

Preparation of GUR

G. uralensis roots, collected from Xinjiang Autonomous Region of P. R. China, were washed to remove dirt, air-dried, and chopped into small pieces (about 0.5 cm in length). Then, the pieces were further ground using a grinder, and the granules (0.5 to 1.0 mm) were extracted with methanol at 25 °C for 2 days. The ratio of liquor to GUR was 20:1. The extracted granules were then dried in a vacuum-drying oven at 60 °C for 3 days and ground using a grinder. The treated granules were prepared for the following experiments.

Biological Pretreatment of GUR

Biological pretreatment with *P. chrysosporium* was carried out in a 250-mL Erlenmeyer flask with 5 g of treated GUR granules and 15 mL of distilled water. The samples were autoclaved at 121 °C for 30 min and aseptically inoculated with 20 mL of activated seed culture. All flasks were incubated statically at 28 °C for 21 days. Non-inoculated samples were treated under the same conditions and used as controls. All experiments were performed in triplicate.

Acid Pretreatment of GUR

The un-pretreated and pretreated GUR samples were hydrolyzed with 2.5% (v/v) sulfuric acid at various temperatures (25, 50, 75, and 100 °C) for 0.5 to 3 h and then filtered. The reducing sugar in the filtrate was measured after neutralization with lime (Mohagheghi *et al.* 2006). The residues of acid hydrolysis were washed with double distilled water (DD water) to neutralize them, dried in a vacuum drying oven at 60 °C for 3 days, and ground using a grinder. The granules were prepared for enzymatic hydrolysis.

Enzymatic Hydrolysis

Commercial cellulase produced by *Trichoderma longibrachiatum* was purchased from Shanxi Imperial Jade Biotechnology Co., Ltd (Yinchuan, P. R. China). Enzymatic hydrolysis was conducted using the method described by Ma *et al.* (2010). After filtration, the enzymatic hydrolysate was used for reducing sugar measurement and heterotrophic cultivation of *C. protothecoides*.

Heterotrophic Cultivation

As a culture medium for *C. protothecoides*, concentrated enzymatic hydrolysate of GUR that contained 10 g/L reducing sugars was supplemented with nutrients following the method of Ji *et al.* (2011). Heterotrophic cultivation of *C. protothecoides* was carried out in a 500-mL Erlenmeyer flask with 180 mL of fermentation medium (pH=7) and 20 mL of activated homogenized seed culture (OD₅₄₀=1) at 28 °C and 200 rpm for 7 days. The SE medium with 10 g/L glucose was used as the control. All experiments were performed in triplicate.

Analytical Methods

Chemical component analysis

The carbohydrates, lignin, and ash contents of raw material and treated GUR were determined according to the procedures established by the National Renewable Energy Laboratory (NREL) (Sluiter *et al.* 2005, 2012). Total reducing sugars in the hydrolysate produced by each process were estimated by the 3,5-dinitrosalicylic acid method (Ma *et al.* 2010). The monosaccharides in the hydrolysate were analyzed by high-performance liquid chromatography (HPLC) (Agilent 1100, Agilent Technologies, Waldbronn, Germany) with a chromatograph equipped with a refractive index detector (Agilent Technologies) and a Shodex SP-0810 sugar column (Pb²⁺ form; Showa Denko, Tokyo, Japan).

*Analysis of cell growth and oil yield of *C. protothecoides**

Algal biomass was monitored using a UV/Visible spectrophotometer (UV-Vis spectrometer 752, Shenyang, China) at 540 nm. At the end of the cultivation period, the algal cells were harvested through centrifugation, freeze-dried to constant weight under vacuum at -50 °C, and then weighed. Oil was extracted from the microalga powder according to the procedure of Galloway *et al.* (2004). The amount of oil production was calculated as follows,

$$\text{Oil production yield (g/L)} = M_1/V_0 \quad (1)$$

where M_1 is the amount of oil production from dry microalga powder (g) and V_0 is the total volume of culture medium (L).

Analysis of fatty acid composition of the microalgal oil

Fatty acid methyl esters (FAMES) were prepared according to a method reported in the literature (Damiani *et al.* 2010) and analyzed by gas chromatography–mass spectrometry (GC-MS) (7890A/5975C, Agilent, USA) on an apparatus equipped with a flame-hydrogen detector and a capillary column (30 m × 0.25 mm × 0.25 μm, J&W Scientific, Folsom, CA, USA). FAMES were identified by comparison with standards (Sigma). The quantities of individual FAMES were estimated from the peak areas on the chromatogram using tridecanoic acid (C13:0) as the internal standard (Sigma Chemical Co. St. Louis, MO, USA).

RESULTS AND DISCUSSION

Main Chemical Components of Raw GUR

The main chemical components of raw GUR are listed in Table 1. The hemicellulose and lignin contents of the raw material were lower than those previously reported in the literature, but the cellulose and ash contents were in accordance with the reported data (Sdykov and Abduazimov 2002). These differences might originate from different habitats and different harvest times for *G. uralensis* (Stepanova and Sampiev 1997).

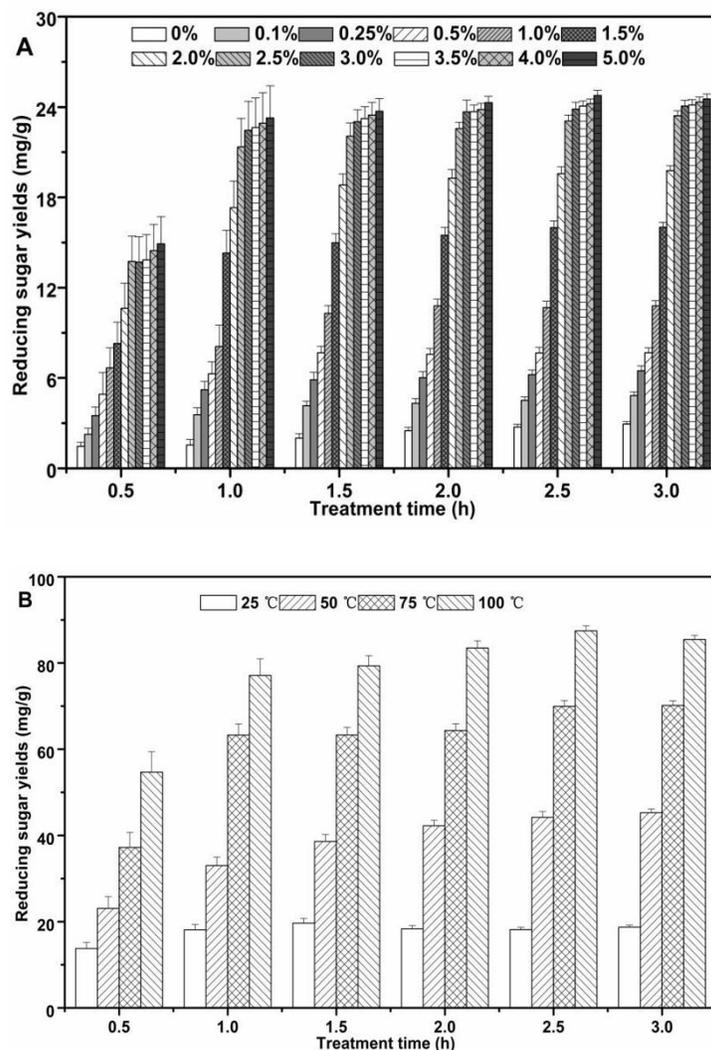
Table 1. Chemical Composition of Raw GUR (Percentage of Dry Weight)

Samples	Cellulose (%)	Hemicellulose (%)	Lignin (%)*	Ash (%)
Raw GUR	29.2±1.0	14.9±1.0	19.9±0.5	4.0±0.5
Licorice grass	30.7	18	25.1	4.1

*Lignin (%) = acid soluble lignin (%) + acid insoluble lignin (%)

The Reducing Sugar Yield of Acid Hydrolysate

Results are shown in Fig. 1.



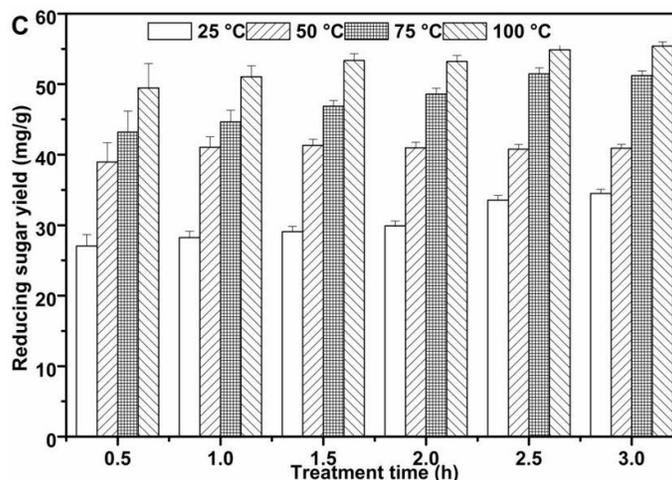


Fig. 1. The reducing sugar yield of GUR treated with 2.5% (v/v) sulfuric acid at different temperatures and different treatment times after 15 days of bio-pretreatment with *P. chrysosporium*. (A) Acid pretreatment with different concentrations of sulfuric acid; (B) acid-only pretreatment with 2.5% (v/v) sulfuric acid; (C) combined pretreatment with *P. chrysosporium* and 2.5% (v/v) sulfuric acid

Most of the hemicelluloses could be effectively dissolved and recovered as dissolved sugars, but the mass of cellulose remained in the solid state during dilute sulfuric acid pretreatment (Haghighi Mood *et al.* 2013; Hong *et al.* 2012). At 25 °C, the reducing sugar yield of the control was significantly increased by increasing the sulfuric acid concentration (from 0.1% to 2.5% (v/v)), but the amount of reducing sugar increased only slightly at an acid concentration beyond 2.5% (v/v) (Fig. 1A). These results might be explained by furfural formation from xylose at high concentrations of sulfuric acid solution (Lenihan *et al.* 2010). Therefore, 2.5% (v/v) sulfuric acid was selected to treat GUR in the rest of the experiments. At high temperatures (75 and 100 °C), the reducing sugar yield slightly increased as pretreatment time increased (Fig. 1B). The amount of reducing sugar of acid hydrolysate at mild conditions (from 4.86 to 24.76 mg/g dry weight) was relatively low, compared with the yield with high acid concentrations and/or high temperatures (Lavarack *et al.* 2002). These results might be caused by partial hydrolysis of hemicelluloses under mild conditions (Ma *et al.* 2010). In fact, high temperatures (120 to 200 °C) and/or high acid concentrations (2.5 to 10% (w/w)) are usually employed in dilute sulfuric acid pretreatment to enhance the hydrolysis ratio of hemicelluloses (Baboukani *et al.* 2011; Lenihan *et al.* 2010).

The bio-pretreated samples with *P. chrysosporium* were further hydrolyzed using 2.5% (v/v) sulfuric acid at 25, 50, 75, and 100 °C for 0.5 to 3 h. The reducing sugar yield of bio-pretreated samples (from 27.04 to 55.42 mg/g dry weight) was relatively lower than that obtained with acid-only pretreatment under the same conditions (Fig. 1C). This difference might originate from the degradation of hemicelluloses by *P. chrysosporium*. The xylose content of insoluble residues from acid hydrolysis of co-treated GUR was relatively lower than that from acid-treated samples (Table 2).

Table 2. Mass Balance of GUR

Carbohydrates	Control (mg/g ^a)	Acid Hydrolysis		Enzymatic Hydrolysis	
		Control (mg/g ^a)	Bio- pretreated GUR (mg/g ^a)	Acid treated Control (mg/g ^a)	Co-treated GUR (mg/g ^a)
Arabinose	20.8±2.3	14.7±0.9	9.4±1.0	10.1±0.3	7.3±0.5
Xylose	95.5±3.6	42.3±2.1	25.2±2.0	20.0±0.2	17.2±0.1
Mannose	18.5±1.5	12.3±1.3	10.5±1.6	8.8±0.5	7.8±0.7
Glucose	266.4±6.6	234.6±5.3	228.4±6.7	80.6±4.8	40.2±4.1
Galactose	13.7±2.3	10.5±1.3	7.9±0.8	9.4±0.6	5.3±0.3
Sugars in residues	414.9	314.4	281.4	128.9	77.8
Sugars in hydrolysates	-	87.46	55.42	175.47	192.07
Total Sugars	414.9	401.86	336.42	304.37	269.87
Sugars loss	-	15.04	78.48 ^b	10.03	11.53

^a Based on original GUR weight

^b Part of polysaccharides utilized by *P. chrysosporium* as carbon source for cell growth

The loss of hemicellulose reached 18% after bio-pretreatment, which was slightly higher than the reported data (Zhao *et al.* 2012). The difference might originate from different sources. These results further indicated that some of the hemicelluloses of GUR had been degraded and utilized by *P. chrysosporium* as carbon and energy sources for cell growth (Hori *et al.* 2012). Potumarthi *et al.* (2013) reported that the xylanase activity could reach its maximum value of 100.27 U/mL at the 20th day of bio-pretreatment with *P. chrysosporium*. In another study by Bak *et al.* (2009), hemicelluloses in corn stalk were degraded by xylanase from *P. chrysosporium*, similar to the results of this study.

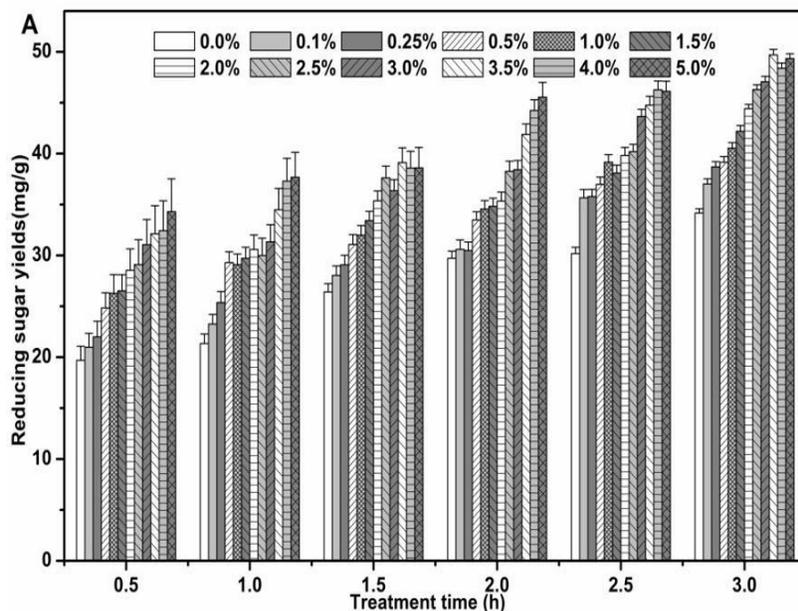
Enzymatic Hydrolysis of GUR

The solid residues of acid hydrolysis were subjected to enzymatic hydrolysis. The reducing sugar yield of enzymatic hydrolysis from the control treated with different concentrations of sulfuric acid at room temperature (from 20.98 to 49.34 mg/g dry weight) were relatively low compared with that of GURs treated with 2.5% (v/v) sulfuric acid at high temperatures (75 and 100 °C) with the rest of the conditions unchanged (Fig. 2A and 2B). This indicates that the physical protection of cellulose provided by lignin and hemicelluloses might not be effectively removed by dilute sulfuric acid pretreatment at 25 °C, leading to a decrease in the enzymatic hydrolysis of GUR (Öhgren *et al.* 2007). Furthermore, the strong binding affinity between cellulase components and the remainder of lignin might also decrease the saccharification ratio of GUR (Govumoni *et al.* 2013). The reducing sugar yield of GUR co-treated with *P. chrysosporium* and mild acid (from 50 to 192.07 mg/g) were 1.08- to 1.71-fold higher than that of acid-only treatment under the same conditions (Fig. 2C). These results may be due to the reduction of lignin and hemicelluloses, which can increase the pore size in GUR and make cellulose more susceptible to enzymatic hydrolysis after combined pretreatment (Zhao *et al.* 2012). The

reducing sugar yield from enzymatic hydrolysis of co-treated GUR (192.07 mg/g) was similar to that of the reported data (208 mg/g) (Zhao *et al.* 2012). The tiny difference might be attributed to the higher cellulose content of cornstalk. The glucose yields in this study (82.4%) are obviously higher than the previously reported one (55%) (Bak *et al.* 2009). This suggests that the combined pretreatment with *P. chrysosporium* and mild acid is a promising method to improve the enzymatic digestibility of GUR.

The sugar composition of the solid residue produced by each process was analyzed and quantified using HPLC and shown in Table 2. As can be seen from Table 2, the reducing sugars in raw GUR totaled 414.9 mg/g dry weight. After acid hydrolysis, the reducing sugar content (281.4 mg/g) of the acid-insoluble residue of bio-pretreated GUR was less than that of the control. These results can be attributed to the utilization of hemicelluloses and cellulose by *P. chrysosporium* as a carbon source for cell growth during biological pretreatment (Bak *et al.* 2009). The small sugar loss might also be caused by washing the bio-pretreated GUR with DD water before acid hydrolysis.

The maximum efficiency of enzymatic hydrolysis reached 68.3% of the theoretical maximum yield, which was higher than that of the bio-pretreatment only (Zhao *et al.* 2012). This also was lower than that of physical and chemical methods (Lu *et al.* 2007). Nevertheless, in comparison with physical and chemical methods, fungus-assisted mild acid pretreatment has its own advantages, such as less fermentation inhibition, milder conditions, and safety. These results further demonstrated that GUR co-treated with *P. chrysosporium* and mild acid can be efficiently hydrolyzed using a commercial cellulase.



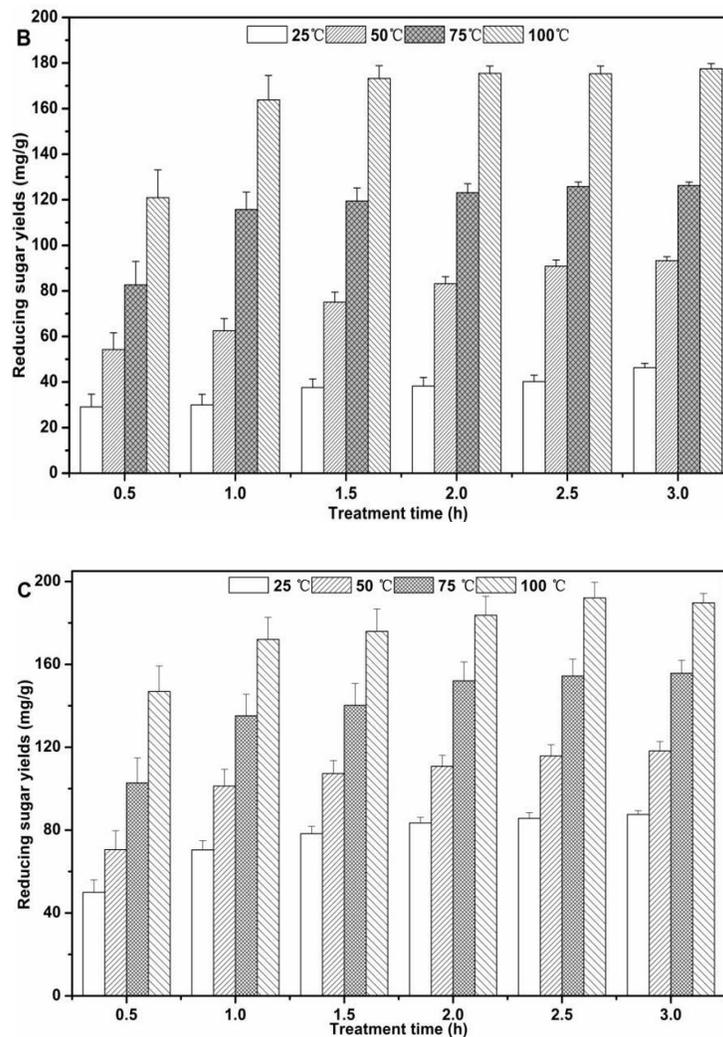


Fig. 2. The reducing sugar yield of enzymatic hydrolysis from GUR treated with 2.5% (v/v) sulfuric acid at different temperatures and different treatment times after 15 days of bio-pretreatment with *P. chrysosporium*. (A) Acid pretreatment with different concentrations of sulfuric acid; (B) acid-only pretreatment with 2.5% (w/w) dilute sulfuric acid; (C) combined pretreatment with *P. chrysosporium* and 2.5% (v/v) sulfuric acid

To further demonstrate the potential viability of the combined pretreatment, a mass balance was roughly determined (Table 2) on a weight basis. The HPLC analysis showed that the reducing sugar yields from acid hydrolysate of the bio-pretreated sample was about 13.4% and the reducing sugar yields from the enzymatic hydrolysate of the co-treated sample was about 68.3%. As compared with the contents of chemical components of the insoluble residue, the mass balance of the treatment process was satisfactory.

Algal Biomass and Fatty Acid Profiles of the Algal Oil

To determine the feasibility of using enzymatic hydrolysate for microalgal oil production, enzymatic hydrolysate from the co-treated sample with the maximum reducing sugar concentration was used as the substrate for oil production by *C. protothecoides* (see Table 3). The highest biomass and oil contents reached 4.16 and 1.66 g/L, which were respectively 1.12- and 1.34-fold higher than those of acid-only treated

sample. Also, the biomass and oil contents were 2.1- and 3.32-fold higher than those obtained using glucose as the carbon source. These results implied that enzymatic hydrolysate of co-treated samples was the most suitable carbon source for oil production by *C. protothecoides*. This might be due to the decreased concentration of inhibitors or the production of some cultivation accelerants (such as proteins, amino acids, or other components) (Gao *et al.* 2010). Ma *et al.* (2010) previously reported that the combined pretreatment of water hyacinth with *Echinodontium taxodii* and dilute sulfuric acid significantly enhanced the ethanol yield because of the reduction of fermentation inhibitors or the increase in fermentation accelerants.

To further explore the feasibility of using enzymatic hydrolysate for microalgal oil production, a mass over the heterotrophic cultivation of *C. protothecoides* was roughly examined. The mass balance was on the basis of glucose weight. The biomass yields on glucose were respectively 0.4 and 0.416 g/g glucose in the media with enzymatic hydrolysates from acid-only treated and co-treated GUR, which were a little higher than that of the previously reported (Xiong *et al.* 2010). This might be due to the consumption of acetate as carbon source for cell growth in culture medium (Xu *et al.* 2006). Thus, compared with the contents of the remainder reducing sugar in culture medium (Table 3), together with the biomass yield of heterotrophic cultivation, the mass balance throughout the whole process was also very satisfactory.

Table 3. Effect of Glucose and Enzymatic Hydrolysate of Acid-treated and Co-treated GUR as Carbon Sources on the Biomass and Oil Yield of *C. protothecoides*

	Glucose	Enzymatic hydrolysate of acid-treated GUR	Enzymatic hydrolysate of co-treated GUR
Initial reducing sugar in medium (g/L)	10.1±0.3	10±0.5	10.2±0.4
Finally reducing sugar in medium (g/L)	4.2±0.25	0.72±0.21	0.2±0.13
Biomass (g/L)	2.02±0.15	3.71±0.23	4.16±0.3
Oil yield (g/L)	0.5 ±0.1	1.24±0.15	1.66±0.2

Microalgal oil from different carbon sources has been a popular research area recently, and many researchers have engaged in relevant work. Lu *et al.* (2010) investigated cassava starch with enzymatic hydrolysis and alga cultivation with *C. protothecoides*. In a shake flask, the biomass and oil yield reached 4.26 and 2.14 g/L, respectively, after 100 h of cultivation. Although the oil yield was higher, the biomass was in accordance with the data reported in this study. The low oil yield in this study might be due to the limited nutrients in the enzymatic hydrolysate medium from GUR. According to previous reports, the oil content of heterotrophic *C. protothecoides* can be significantly improved through nitrogen limitation (Xu *et al.* 2006). Chen and Walker (2011) used glycerol as a carbon substrate for oil accumulation *via* heterotrophic *C. protothecoides*. The highest biomass and lipid concentrations reached 23.5 and 14.6 g/L, respectively, after 6 days of cultivation in a 500-mL shake-flask using SE medium supplemented with glycerol and yeast extract; these values are respectively 5.65- and 8.8-fold higher than those obtained in this study. These differences further demonstrated that limited nutrition in the basal culture medium affects the biomass concentration and oil

content of heterotrophic *C. protothecoides*. However, the economic feasibility of heterotrophic *C. protothecoides* is poor because of the high cost of glycerol. It can be inferred that the performance of algal oil production using enzymatic hydrolysate of co-treated GUR as the substrate is comparable or even better than that with other carbon sources reported in previous studies.

After FAME synthesis, the FAMES were further analyzed by GC-MS (Fig. 3). The three most abundant components, including C16:0, C18:1, and C18:2, accounted for over 87.3% of total FAMES, which is different from those reported by Gao *et al.* (2010). Polyunsaturated fatty acids (such as C16:2, C16:3, and C18:3) were also detected in the experiments. However, some fatty acids, such as C17:1 and C19:1, that were found in previous studies were not detected (Table 4) (Gao *et al.* 2010). The differences might be caused by varied cultivation conditions and different nutrition in the culture medium. In addition, it might also be from different growth phases or other factors that have significant effects on the metabolism of *C. protothecoides*. Recent research has shown that biodiesel production from *C. protothecoides* as a feedstock represents a viable alternative to fossil fuels, with a high heating value and low viscosity (Miao and Wu 2004; Xu *et al.* 2006). This finding further indicated that biodiesel derived from the oil production of *C. protothecoides* is a promising alternative to conventional diesel fuel.

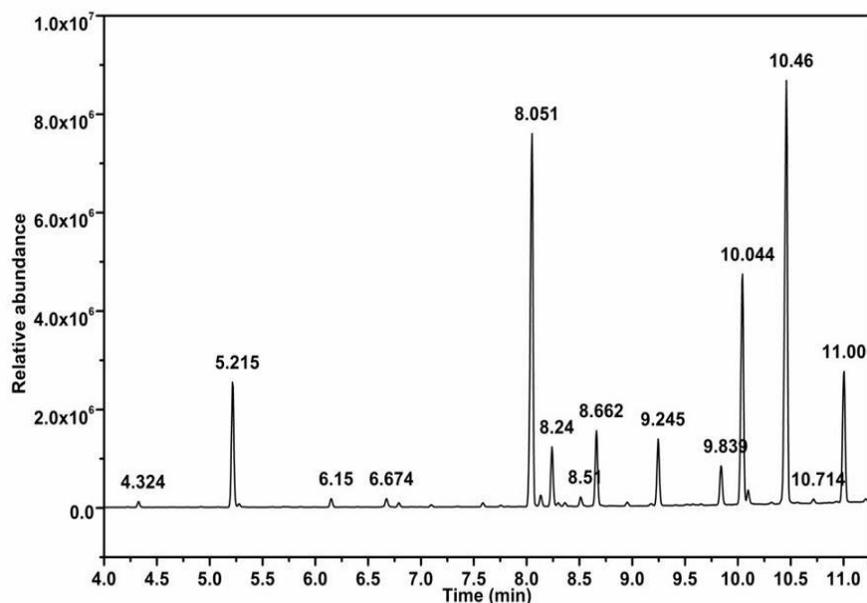


Fig. 3. Fatty acid compounds of microalgal oil analyzed by GC-MS

Table 4. Fatty Acid Profiles of Microalgal Oil Produced using Glucose and Enzymatic Hydrolysate of Acid-treated and Co-treated GUR as Substrates

Fatty Acid	Relative Content (%)		
	Glucose	Acid Hydrolysate	Enzymatic Hydrolysate
C14:0	0.20	0.31	0.40
C16:0	10.25	10.09	10.84
C16:1	0.24	1.26	1.60
C16:2	ND*	1.89	2.05
C16:3	ND*	1.97	2.00
C17:0	1.23	0.52	0.66
C18:0	1.38	1.24	1.49
C18:1	60.75	58.50	61.7
C18:2	17.48	12.02	11.72
C18:3	ND*	3.16	4.46
C19:1	0.55	ND*	ND*

*not detected

CONCLUSIONS

1. The present study showed the feasibility of using *Glycyrrhiza uralensis* residue (GUR) as a cheap and abundant source of biomass for oil production. GUR as a source of biomass for oil production can be cost-effective relative to other materials such as cassava because GUR is cheaper and easier to collect.
2. Combined pretreatment with *P. chrysosporium* and 2.5% sulfuric acid proved to be more effective than acid-only pretreatment under moderate conditions. After the combined pretreatment, the reducing sugar yield of enzymatic hydrolysate from co-treated GUR increased 1.08- to 1.71-fold more than that of acid-only treatment.
3. Enzymatic hydrolysate from co-treated samples was fit for *C. protothecoides* to accumulate intracellular oil. The highest biomass and oil yields of *C. protothecoides* using SE medium with enzymatic hydrolysate from co-treated samples as a culture medium were respectively 1.12- and 1.34-fold higher than that obtained from acid-only treated samples.

ACKNOWLEDGMENTS

The authors acknowledge the financial support of the National Natural Science Foundation of P. R. China (NSFC) (Nos. 31070089, 31170078 and J1103514), the National High Technology Research and Development Program of P. R. China (863 Program) (No. 2011AA02A204), and the Innovation Foundation of HUST (2011TS100). Many thanks are due to Mrs. Chen from the Centre of Analysis and Testing, Huazhong University of Science and Technology, for measuring the compositions of monosaccharides and biodiesel.

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Article submitted: July 27, 2013; Peer review completed: August 31, 2013; Revised version received and accepted: September 9, 2013; Published: September 12, 2013.