Pretreatment of *Dioscorea zingiberensis* for Microbial Transformation

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Abstract: *The influences of five pretreatments on fungal growth and enzyme production during microbial transformation of Dioscorea zingiberensis (DZW) were studied. The biomass, α-rhamnase and β-glucosidase activities in the fermentation system were employed in the study to determine how each method affected the efficiency of microbial transformation. The fungal strain grew better on the substrate which contained easily utilized carbon source. While lack of carbon source induced the strain produce more glucosidase. Among five pretreatment methods, complex enzymatic hydrolyzation can remove 84.3% starch and 76.5% fibre from DZW in form of sugar, which resulted in high α-rhamnase activity of 2.89 IU/mL and β-glucosidase activity of 8.17 IU/mL in fermentation broth.*

Keywords: *Microbial transformation, Pretreatment, α-rhamnase, β-glucosidase.*

Introduction

Dioscorea zingiberensis (DZW), commonly called yellow ginger, is a special plant cultivated for production of diosgenin in China. Diosgenin, an important precursor used in the synthesis of human steroidal drugs, exists in form of saponins in plant tissue [1, 3, 13]. To produce diosgenin from DZW, acid hydrolysis is usually applied. However, this method causes serious water pollution.

Nowadays, microbial transformation of saponins to obtain diosgenin was attracted attention because of its environment-friendly and low cost [4, 9, 11, 19]. Saponins in the plant cells are wrapped by cellulose and starch. The existence of cellulose and starch hinders the attachment of enzymes produced by microorganism and saponins [18]. Thus, to improve the efficiency of microbial transformation, in one hand, separation cellulose and starch from the plant tubers is important. While, in the other hand, an amount of cellulose and starch in the substance is essential to support fungal growth and enzyme production, which is also important for transformation efficiency. Prior studies showed that pretreatment of DZW using enzymatic saccharification [18] or solvent extraction [12] before biotransformation could increase diosgenin accumulation. While some other attempts, such as ultrasonic technology [14], complex enzymatic hydrolysis [5] and physical separation [8], can also release saponins from the network structure of starch. Their effects on biotransformation efficiency have not been demonstrated.

Up to now, most studies on the pretreatment effects have focused on recovery of starch and fibre, and improvement of diosgenin yield. In the microbial transformation process, the fungal growth, enzyme production and saponins change responsible for diosgenin yield and product

quality after different pretreatment have not been examined yet. Accordingly, the objective of this paper was to investigate the effects of different pretreatment methods on the fungal growth, enzyme production in microbial transformation procedure. Five pretreatments including physical (physical separation), chemical (catalytic solvent extraction) and biological (ultrasonic fermentation, complex enzymatic hydrolyzation, and enzymatic saccharification) methods were systematically studied to establish the best pretreatment method to maximize the diosgenin production of biotransformation.

Materials and methods

Materials

DZW tubers were supplied by ShiYan City, Hubei Province in China. α-amylase (2000 IU/g), saccharifying enzyme (10.000 IU/g) and cellulase (10.000 IU/g) were taken from Shandong Longda Bio-product Co., Ltd., Shangdong, China. ADH yeast was purchased from Angel Yeast Company, Yichang, Hubei, China.

The standard of diosgenin, p-nitrophenyl-α-L-rhamnopyranoside (pNPR) and p-nitrophenyl-β-D-glucopyranoside (pNPG) were purchased from Sigma Company, Shanghai, China.

Microorganism

The fungal strain *Trichoderma reesei* (ACCC 30597) used was prepared and subcultured as we described previously.

Pretreatment methods

DZW tubers were washed and then dried. The raw tubers were subjected to different pretreatment methods to remove starch and fibre. PDZWs were obtained. The contents of starch, reducing sugar, fibre and saponins [8] in DZW and PDZWs were determined.

Physical separation (P1)

100 g of dried DZW tubers were immerged in 3 L water for 24 h, and then cut into slices, grounded for 40 s. The wet powder was suspended and partitioned in a large amount of water to form three layers. The top layer is fibre, the middle and bottom lays were combined, grounded and suspended again to remove fibre. The residue was suspended and partitioned. The bottom layer was starch, the top and middle layers were combined, centrifuged and dried at 60°C and grounded to pass through a 60-mesh screen. PDZW1 was obtained.

Catalytic solvent extraction (P2)

100 g of dried DZW tubers were grounded. The dried powder was mixed with 1.2 L of 60% EtOH, 0.15 g of $NaHCO_3$ and 0.3 g of NaOH was then added. The mixture was stirred at 70°C for 2 h. The slurry was then filtrated. The residue was extracted with 50% EtOH again. The two filtrates were combined and concentrated. PDZW2 was obtained.

Ultrasonic fermentation (P3)

100 g of dried DZW tubers were grounded to pass 60-mesh screen. The overflow was fibre. The underflow was mixed with 350 mL water and ultrasonic pre-irradiated at 50 KHZ, 100 w for 20 min. The slurry was holed for 1h. The supernant was fibre. The residue was fermented with 0.8 g yeast dosage at 30°C for 3 days. The hydrolysate was centrifuged and washed with water. The residue was dried at 60°C and grounded to pass through a 60-mesh screen. PDZW3 was obtained.

Complex enzymatic hydrolyzation (P4)

100 g of dried DZW tubers were grounded for 2 min. The dried powder was mixed with 600 mL water, and then incubated with 1.5 g cellulose and 1.5 g amylase at 55°C and pH 4.0 for 6 h. The enzymatic hydrolysate was centrifuged and washed with water. The residue was dried at 60°C and grounded to pass through a 60-mesh screen. PDZW4 was obtained.

Enzymatic sacchrification (P5)

100 g of dried DZW tubers were grounded for 2 min. The dried powder was mixed with 600 mL water and boiled for 1h. After cooling, the slurry was incubated with 2 g amylase at 70°C and pH 6.5 for 1 h. The hydrolysate was then transformed with 3 g saccharifying enzyme at 60°C and pH 4.0 for 8 h. The hydrolysate was then centrifuged and washed with water. The residue was dried at 60°C and grounded to pass through a 60-mesh screen. PDZW5 was obtained.

Microbial transformation

Microbial transformation experiment was taken out with DZW or PDZW as substrate as we reported previously [18]. The fermentation medium containing 2.67% peptone, 0.27% K_2HPO_4, 0.73% Tween 80 and 10% substrate. Every 12 h, 30 mL of the sample were taken out. Reducing sugar [8], biomass, *α*-rhamnase, *β*-glucosidase, amylase [15] in the fermentation broth were detected to evaluation of the growth of *T. reesei* in different substrates

Analysis

Biomass

Because of the presence of substrate, the cell dry weight concentration of *T. reesei* could not be analyzed directly. Intracellular protein concentration was detected instead with the method described by Zhang et al. [16]. The broth sample (10 mL) was centrifuged, collected and washed with distilled water twice. The solid was dissolved in 20 mL of 0.2 mol/L NaOH at 100°C for 2 minutes, and then filtrated. The protein concentration in the filtrate was detected with the standard Lowry method using UV/Vis spectrophotometer at 595 nm.

α-Rhamnase and β-glucosidase activities

The fermentation broth (5 mL) was centrifuged at 4°C and 10000 rpm for 15 min. The suspension was obtained for enzyme activity analysis. *α*-rhamnase or *β*-glucosidase activity was determined using pNPR or pNPG as substrate, respectively. 1 mL of 5 mM pNPR or pNPG was maintained at 50°C for 10 min, and then 1 mL of appropriately diluted enzyme was added. The enzyme reaction was stopped by adding 1 mL of 1 M Na_2CO_3 after 10 min. The mixture was then measured at 420 nm. One unit of *α*-rhamnase (or *β*-glucosidase) activity was defined as the amount of enzyme that released 1 μmol p-nitrophenol under the above conditions in one minute.

Results and discussion

Compositions of different PDZW

The compositions of different PDZW are demonstrated in Fig. 1. It can be seen that P3 and P5 were two most effective methods for removing starch from DZW. While, P2 and P4 could recover fibre in form of sugar from DZW, effectively. About 2.78% total saponins were detected in raw material. P1 resulted in lowest saponins concentration of 2.13%, because some saponins were wrapped in recovered starch and lost in the grinding and screening

process. The contents of saponins in DZW, PDZW1, PDZW2 and PDZW3, PDZW4 and PDZW5 were 2.78%, 2.13%, 2.65%, 2.96%, 2.94% and 2.97%, respectively.

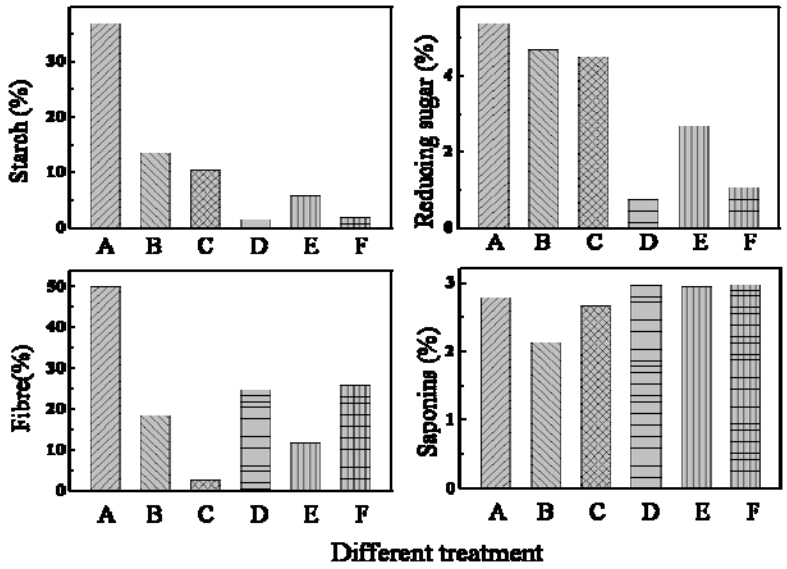

Fig. 1 Composition of different pretreated DZW
A: DZW; B: PDZW1; C: PDZW2; D: PDZW3; E: PDZW4; F: PDZW5

Effects of pretreatments on T. reesei growth

DZW and PDZW were then subjected to microbial transformation with *T. reesei*, respectively. The fungal growths on different pretreated DZW were shown in Fig. 2a. The biomass of *T. reesei* with DZW as the substrate was significantly higher than those obtained with other five PDZW. Fungal growth on PDZW1 or PDZW2 was better than that on PDZW3, PDZW4 or PDZW5. The maximum fungal biomass of 5.43 and 5.17 g/L were obtained with PDZW1 and PDZW2 as substrate, while the values were 2.17, 2.33 and 2.11 g/L using PDZW3, PDZW4 and PDZW5 as substrate, respectively.

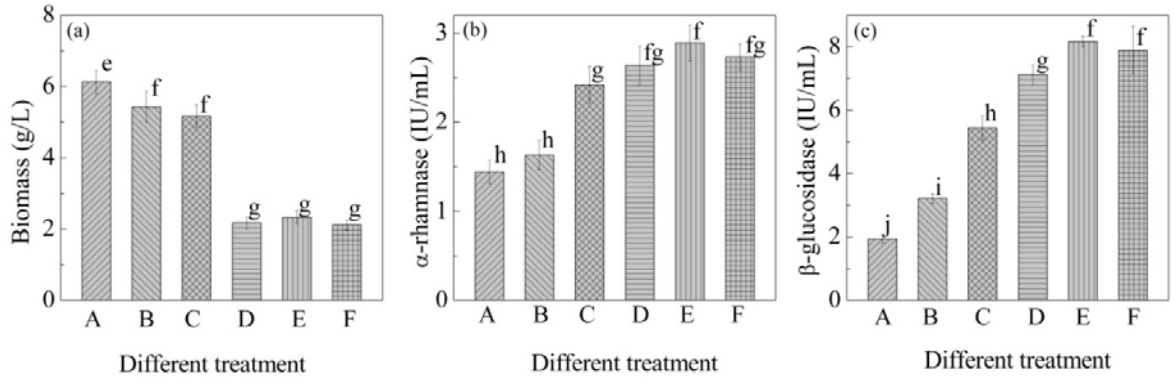

Fig. 2 Highest biomass (a), *α*-rhamnase (b), and *β*-glucosidase (c)
in bioreactor cultivation of DZW and PDZW by *T. reesei*.
Values not sharing same letters are significantly different
(one-way ANOVA with SNK method, $p < 0.05$).
A: DZW; B: PDZW1; C: PDZW2; D: PDZW3; E: PDZW4; F: PDZW5.

Effects of pretreatments on enzyme activity

Previously studies demonstrated that *α*-rhamnase and *β*-glucosidase play key roles in biotransformation saponins to diosgenin [10, 17, 18]. The activities of the two enzymes involved in microbial transformation DZW or PDZW with *T. reesei* were shown in Fig. 2b and Fig. 2c. When PDZW1 was used as the substrate, *α*-rhamnase activity of 1.63 IU/mL was obtained.

Highest *α*-rhamnase activity of 2.89 IU/mL and 2.73 IU/mL were achieved after incubating *T. reesei* with PDZW4 and PDZW5 for 84 h, respectively. The two values were at the same level. The activity of *α*-rhamnase produced by *T. reesei* was 2.42 IU/mL or 2.64 IU/mL with PDZW2 or PDZW3 as substrate, respectively. With respect to *β*-glucosidase, when incubated with different substrates, the changes of enzyme activity during the microbial transformation were similar. Fig. 2c demonstrated that the activity of *β*-glucosidase produced by *T. reesei* with DZW was 1.94 IU/mL. Fermented with PDZW1-5 respectively, the activity of *β*-glucosidase inceased more significantly. The maximum enzyme activities of 3.21, 5.44 and 7.12 IU/mL were obtained for PDZW1, PDZW2 and PDZW3. From the results of PDZW4 and PDZW5, the activities of *β*-glucosidase in the two fermentation broth were at the same level, reached maximum values of 8.17 and 7.89 IU/mL.

The result of Fig. 2 showed that when DZW, PDZW1, PDZW2 were used as the substrates, low glucosidase activities and high biomass were achieved, when PDZW3, PDZW4, PDZW5 were used as the substrate, high glucosidase activities and low biomass were obtained. During biotransformation, reducing sugar was the most easily utilized carbon source for *T. reesei*. *T. reesei* utilizes the reducing sugar from substrate to build up its own biomass.

Table 1. Calculated initial contents of reducing sugar, starch, hemicellulose and saponins in the fermentation broth of different pretreated DZW

	Untreated	**P1**	**P2**	**P3**	**P4**	**P5**
Reducing sugar (g/L)	5.36 ± 0.32^{c}	10.7 ± 1.45^{b}	22.0 ± 1.11^{a}	2.26 ± 0.74^{d}	10.6 ± 1.11^{b}	3.02 ± 0.14^{d}
Starch (g/L)	36.8 ± 1.59^{b}	30.9 ± 4.15^{b}	50.8 ± 7.04^{a}	4.26 ± 0.28^{d}	23.3 ± 4.01^{c}	5.21 ± 0.61^{d}
Hemicellulose (g/L)	40.0 ± 1.83^{b}	23.3 ± 2.46^{c}	12.2 ± 1.09^{d}	55.4 ± 1.38^{a}	22.5 ± 3.23^{c}	50.9 ± 4.28^{a}
Saponins (g/L)	2.78 ± 0.18^{d}	4.84 ± 0.33^{c}	13.04 ± 0.35^{a}	8.97 ± 1.01^{b}	11.9 ± 1.88^{ab}	8.74 ± 1.38^{b}

$^{a-d}$ - indication letters. Values in the same raw not sharing same letters are significantly different (one-way ANOVA with SNK method, $p < 0.05$)

As shown in Table 1, the initial concentrations of reducing sugar in the six fermentation broth were 5.36, 10.7, 22.0, 2.26, 10.6, 3.02 g/L, respectively. The result did not show direct relationship between initial concentration of reducing sugar and fungal growth. Besides reducing sugar, starch, fibre and saponins in the substrates are potential carbon sources for *T. reesei*. They can be hydrolyzed to reducing sugar by the enzymes from fungal strain. It was assumed that the fungal growth and glucosidase activity were determined by the composition of starch, fibre and saponins in the substrates.

In order to verify our assumptions, the changes of the reducing sugar concentration and amylase activity in each fermentation broth were assessed and demonstrated in Fig. 3 and Fig. 4.

Taking PDZW1 and PDZW4 for example, the initial reducing sugar concentrations in the two substrates were at the same level. When *T. reesei* was grown on PDZW1 (Fig. 3e-h), reducing sugar in the fermentation broth decreased sharply in the first 32 h. The fungal utilized the carbon source to build up its own biomass. While from 32 to 48 h, reducing sugar increased from 1.43 g/L to 4.54 g/L. At 48 h, amylase of 10.94 IU/mL and *β*-glucosidase of 0.35 IU/mL were detected in the fermentation suspension. Most of the reducing sugar was produced from starch.

During the next 24 hours, the reducing sugar decreased from 4.54 g/L to 1.02 g/L. The biomass increased sharply, from 3.25 g/L to5.43 g/L. At 72h, the activities of amylase, *α*-rhamnase and *β*-glucosidase in the fermentation broth were 7.33, 0.48 and 0.96 IU/mL. In this period, starch was the main source of reducing sugar. From 72 to 168 h, reducing sugar concentration changed slightly, and the biomass decreased slightly. The activities of *α*-rhamnase and *β*-glucosidase reached highest values of 1.63 and 3.21 IU/mL at 96 h and 108 h, respectively. In this phase, the amylase activity was relatively low; most of the sugars were hydrolyzed from saponins.

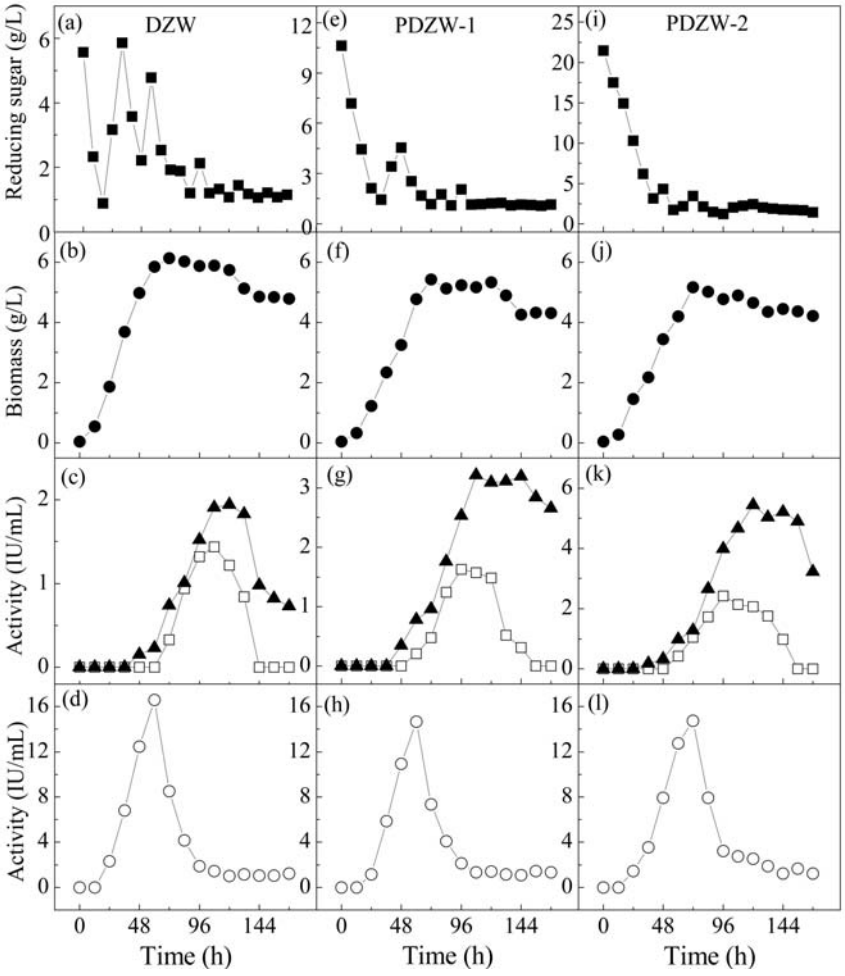

Fig. 3 Time course of reducing sugar (■), biomass (●), *α*-rhamnase (□), *β*-glucosidase (▲) and amylase (○) in bioreactor cultivation of DZW and PDZW by *T. reesei*
(a-d: DZW; e-h: PDZW1; i-l: PDZW2)

When PDZW4 was incubated with *T. reesei* (Fig. 4e-h), reducing sugar in the fermentation suspension decreased from 10.15 to 1.22 g/L in the first 32 h. While from 32 to 40 h, reducing sugar increased from 1.22 g/L to 2.11 g/L. At 36 h, *α*-rhamnase of 0.36 IU/mL, *β*-glucosidase of 0.83 IU/mL and amylase of 1.93 IU/mL were detected in the suspension. In this phase, reducing sugar was from starch and saponins.

During the next 24 h, the reducing sugar decreased from 2.11 g/L to 0.82 g/L and then increased to 1.35 g/L. Similar change was found from 56 h to 168 h. At 60 h, the activities of *α*-rhamnase, *β*-glucosidase in the fermentation broth were 1.04, 3.05 IU/mL, and increased to 2.89 and 8.17 IU/mL at 84 and 96 h. While, the activity of amylase was 2.16 IU/mL at 60 h, and then decreased sharply. In this period, saponins were the main source of reducing sugar. This growth trend of *T. reesei* was in accordance with the results obtained by Hasegawa et al. and Bae et al. [2, 6-7], which suggested that saponins can be the only carbon source for microorganisms.

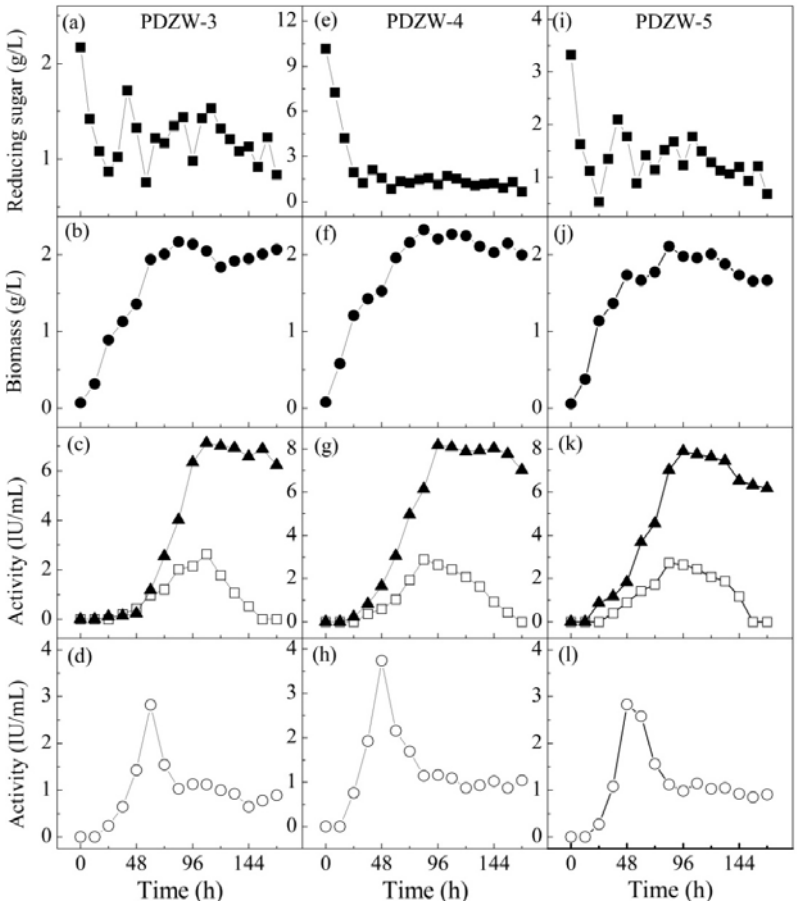

Fig. 4 Time course of reducing sugar (■), biomass (●), *α*-rhamnase (□), *β*-glucosidase (▲) and amylase (○) in bioreactor cultivation of PDZW by *T. reesei*
(a-d: PDZW3; e-h:PDZW4; i-l: PDZW5)

Conclusion

It can be seen that, with phsical separation, the easily utilized starch was still in substrate, and was the main carbon source for *T. reesei*. In this system, high biomass and low *α*-rhamnase and *β*-glucosidase activities were detected. While in PDZW4, saponins were the main carbon source for *T. reesei*. Because easily utilized starch and hemi-cellulose were excluded from DZW by amylase and cellulase, the reduce starch in PDZW4 was not an attractive carbon source for *T. reesei*. Lack of carbon source induced *T. reesei* to produce *α*-rhamnase and *β*-glucosidase to hydrolyze sugar chains from saponins, resulted in high enzyme activities in this system. So, complex enzymatic hydrolyzation was the best pretreatment method for microbial transformation.

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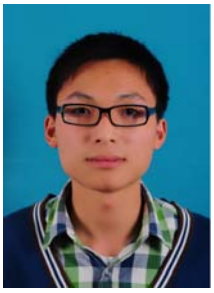

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