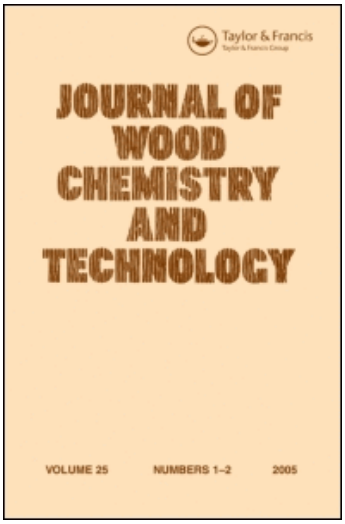

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Pretreatment of Rice Hulls for Cellulase Production by Solid Substrate Fermentation

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Abstract: Rice hulls via pretreatment served as a solid substrate for cellulase production. Different pretreatment methods, including microwave irradiation, organosolv process catalyzed by acid or alkali were compared. With rice hulls pretreated by microwave irradiation coupled with alkaline pretreatment and rice hulls pretreated with organosolv process catalyzed with acid or alkali, the maxima of filter paper activity (FPA) obtained during the fermentation were 18%, 21%, 31% higher, and maxima of the carboxy-methyl cellulase (CMCase) were 7.7%, 14%, 16% higher than those obtained with the rice hulls pretreated only with alkali, respectively. The ratios of cellulose degradation were also estimated and they showed similar tendency with the maxima of FPA detected in the fermentation processes.

Keywords: Microwave irradiation, organosolv process, pretreatment, cellulase, lignocellulose, solid substrate fermentation

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INTRODUCTION

Rice hulls are one of the most abundant renewable resources in the world. China alone generates about 32 million tons of rice hulls every year, the vast majority of which are not well-utilized. For example, rice hulls have not been used extensively in papermaking because of their low α-cellulose content. The generally short cellulose chains found in rice hulls are not appropriate for manufacturing high-quality paper.

However, new opportunities for the use of rice hulls do exist. For example, cellulase production is one of the most important steps in the economically important production of ethanol, single cell protein, and other chemicals from renewable cellulosic materials. The use of rice hulls for cellulase production may represent an effective and economically attractive use of rice hulls.

Unfortunately, natural lignocellulosic materials as rice hulls, mainly composed of cellulose, hemicellulose, and lignin, are difficult to break down with cellulase or microorganisms because of their special crystal structure and the protection of the cellulose provided by the lignin and hemicellulose.[1] So, the materials are usually pretreated before being degraded with cellulase or microorganisms. Many pretreatment processes, such as acidic hydrolysis, alkaline hydrolysis, steam explosion and wet oxidation, have been studied in connection with cellulase production and enzymatic hydrolysis on the cellulosic materials. [2] An investigation using rice hulls specifically in cellulase production also showed that the FPA and the CMCase obtained with the rice hulls pretreated by a solution of 4% NaOH increased by 27.5% and 25.1% over those without pretreatment.[3] Obviously the pretreated materials are degraded more easily.

Microwave heating technology has now been fruitfully applied to treatment of materials containing cellulose. For example, it has been shown that microwave irradiation can accelerate the esterification of cellulose and pyrolysis of wood blocks. [4,5] Moreover, this pretreatment can enhance the susceptibility of cellulose in lignocellulosic materials to enzymatic attack.[6]

In this article, microwave was applied for pretreating rice hulls for improving cellulase production, and the comparison was also made with other common pretreatment methods.

EXPERIMENTAL

Microorganism and Seed Culture

Trichoderma viride 3.2942 used for fermentation was from Chinese Academy of Science (Beijing, China). The spores of *T. viride* 3.2942 were inoculated to a 500 ml shaking flask with 50 ml wheat bran juice, and cultivated at 30°C and 130 rpm for 24 h. The wheat bran juice was prepared as follows: 10 g wheat

bran was submerged in 100 ml distilled water, heated to boiling, and kept boiling for 20 min. After cooling, the juice was filtered with pledget and diluted to 100 ml with distilled water. Then, equal portions of the solution were poured into two shake flasks and autoclaved at 115°C for 20 min.

Substrate Pretreatment

Pretreatment with Alkali and Microwave Irradiation

Rice hulls were milled to 1–2 mm particle size. The milled hulls were then immersed in a solution of 4% NaOH for 24 h at 40°C. The proportion of rice hulls to NaOH solution was 1:8 (w/w). After that, the treated rice hulls were washed with water until neutrality, and then dried at 80°C.

9 g samples of the alkali-treated rice hulls (ATRH) were mixed in 500 ml beakers with 9 g of a mineral solution containing 1% $(NH_4)_2SO_4$, 0.3% KH_2PO_4, 0.05% $CaCl_2$, and 0.05% $MgSO_4$. The samples were irradiated with microwaves at 300 watts for 0.5, 1, 2, or 3 min, respectively. After microwave irradiation, water was added to the beakers to return the weight to its original 18 g, thereby replacing water that may have been lost during the process of microwave irradiation. These ATRH samples irradiated with microwave were designated as AMRH.

NaOH-Ethylene Glycol Organosolv Process

The milled rice hulls were submerged in ethylene glycol solution containing 1.5% sodium hydroxide as a catalyst, heated to 150°C and kept at 150°C for 1 h. The ratio of rice hulls to liquor was 1:4 (w/w). After cooling down, the treated rice hulls were washed with water until neutrality, and then washed with acetone two times, and at last dried at 80°C. The rice hulls pretreated by NaOH-ethylene glycol organosolv process were coded as NGRH.

H_2SO_4-Ethylene Glycol Organosolv Process

The procedure was same as that used in the previous case of NaOH-ethylene glycol organosolv process, except that the catalyst was sulfuric acid. The rice hulls from H_2SO_4-ethylene glycol organosolv process were coded as HGRH.

Solid Substrate Fermentation

The solid-state medium consists of pretreated rice hulls/wheat bran (3/7, w/w) and mineral solution (see earlier). The ratio of dried solid substrate to mineral solution was 1:1(w/w). The medium was added into 500 ml beakers, each of which contained 30 g solid-state substrate and 30 g mineral

solution. The inoculation size was at ratio of 20%(v/w), and the fermentation was conducted at 30°C for 120 h in a 95% relative humidity chamber.

Analysis Methods

FPA and CMCase Determination

After sampling, 1 g moldy medium was soaked in 10 ml acetate buffer of pH 4.8 at 30°C for 3 h to extract cellulase. The mixture was filtered and the filtrate was centrifuged at 4000 rpm for 15 min to remove spores. The resulting supernate solution in the centrifuge tube was analyzed for its cellulase activity. The final cellulase concentration was estimated for carboxy-methyl cellulase (CMCase), as well as filter paper activity (FPA) according to IUPAC recommendations.[7] The enzyme concentration was expressed as international unit (IU), which denotes the micromoles of glucose released per min of the reaction, whereas the enzyme activities were calculated for 1 g substrate dry matter.

RCD Determination

RCD is the ratio of cellulose degradation and it can be calculated by the following correlation.

$$\mathrm{RCD} = \frac{M_1 - M_2}{M_1} \times 100\%$$

where M_1 = mass of cellulose in medium before fermentation; M_2 = mass of cellulose in medium after fermentation.

The contents of cellulose in medium were all determined by HNO_3-C_2H_5OH Method,[8] while the bran should be firstly treated with hot distilled water, and then benzene-ethanol solution and the medium after fermentation should be washed and dried, prior to the determination of the cellulose content.

RESULTS AND DISCUSSION

Cellulase production with microwave irradiation pretreatment was greater than that obtained without the pretreatment (see Figure 1), especially for 1 min irradiation. The maximum FPA (when culture time = 48 h) increased by 18% with AMRH irradiated for 1 min, compared with ATRH. At the same time, there was also an increase of 7.7% in CMCase. In addition, AMTH was degraded more easily than ATRH and the values of RCD paralleled those of maximum cellulase production with different microwave

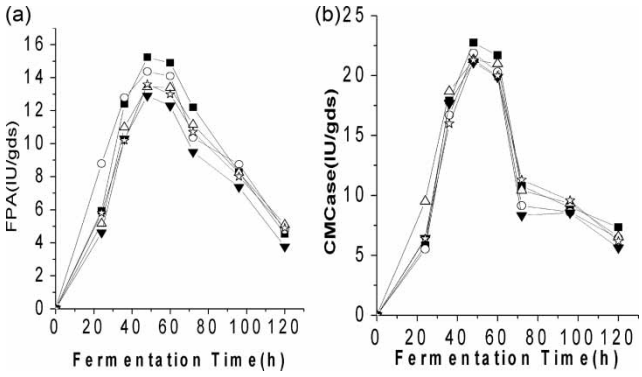

Figure 1. Effects of microwave irradiation time on celluase production. Symbols: (☆) 0.5 min; (■) 1 min; (○) 2 min; (△) 3 min; (▼) 0 min.

irradiation time from Table 1. RCD with AMRH irradiated for 1 min increased by about 20% ((30.1 − 25.1)/25.1 × 100%), as compared with that with ATRH. Hence, microwave irradiation is good for both enzyme production and the degradation of cellulose.

Microwave irradiation may enhance enzyme activities through its effect on ultrastructure of cellulose.[9] Water molecules, water-swelled chain segments of cellulose, and chain segments of cellulose of crystal regions differ in their ability to absorb microwave irradiation. Water molecules have the strongest absorbability of microwave irradiation because of their stronger polarity and will, therefore, be heated most quickly. This makes the rate of the thermal motion of water molecules more rapid than that of water-swelled chain segments of cellulose and chain segments of cellulose of crystal regions. Therefore, the water molecules moving rapidly collide with the chains of cellulose, creating damage in the crystal region; that is, the total crystal region of cellulose is reduced. This damage will increase the susceptibility of AMRH to enzymatic or microbial degradation.

However, in Figure 1, enzyme activity decreased when microwave irradiation time was more than 1 min, the reason for which might be that excessive microwave irradiation could result in some broken-down hydrogen bonds in water-swelled cellulose linking again to form more

Table 1. Effects of microwave irradiation time on ratio of cellulose degradation

Microwave irradiation time (min)	0	0.5	1	2	3
Ratio of cellulose degradation[a] (%)	25.1	26.5	30.1	27.9	26.4
The highest FPA[a] (IU/gds)	12.9	13.6	15.2	14.4	13.4

[a]Experiments were performed in triplicates and each error was less than 6.0%.

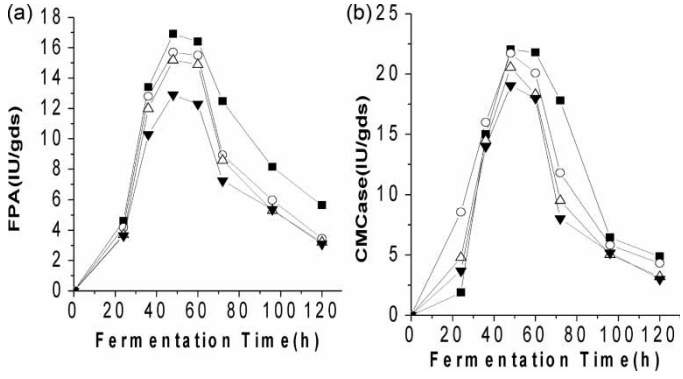

Figure 2. Effects of rice hull pretreatments on cellulase production. Symbols: (■) NaOH-catalyzed ethylene glycol-treated rice hull (NGRH); (○) H_2SO_4-catalyzed ethylene glycol-treated rice hull (HGRH); (△) alkali-treated rice hull irradiated with microwave (AMRH); (▼) alkali-treated rice hull (ATRH).

regular structure and micropores opened by water molecules being closed. Moreover, it could make oriented chain segments of cellulose in non-crystal regions form new crystal regions. This process is called keratinization.[9]

The results of several different pretreatments are shown in Figure 2. The FPA and CMCase obtained from the strain were higher with both NGTH and HGTH than with ATRH, and even higher than with AMRH. The best results were obtained with NGRH. The maximum FPA and CMCase with NGRH (when culture time was 48 h) increased by about 31% and 16%, respectively, compared with ATRH, and they also increased by about 11% and 7.3%, respectively, compared with AMRH.

The tendency of RCD for the different pretreatments also corresponds with that of cellulase production, which means that the higher FPA results in the higher RCD (see Table 2). The RCD with NGTH increased by about 64% $((41.0 - 25.1)/25.1 \times 100\%)$, as compared with that with ATRH.

Table 2. Effect of rice hull pretreatments on ratio of cellulose degradation

Pretreated substrates	NGRH[b]	HGRH[c]	AMRH[d]	ATRH[e]
Ratio of cellulose degradation[a] (%)	41.0	33.8	30.1	25.1
The highest FPA[a](IU/gds)	16.9	15.6	15.2	12.9

[a]Experiments were performed in triplicates and relative error was less than 7.0%.
[b]NGRH—NaOH-catalyzed ethylene glycol-treated rice hull.
[c]HGRH—H_2SO_4-catalyzed ethylene glycol-treated rice hull.
[d]AMRH—alkali-treated rice hull irradiated with microwave.
[e]ATRH—alkali-treated rice hull.

Therefore, ethylene glycol pretreatment of rice hulls also benefits both enzyme production and degradation of cellulose.

CONCLUSIONS

The present work indicates that microwave irradiation on alkali-treated rice hulls increases cellulase production. As the microwave irradiation pretreatment is environmentally benign and easy to implement and optimal irradiation time is not long, it is an advisable pretreatment method. The work also demonstrates that ethylene glycol treatment of rice hulls with either alkali or acid as catalyst could cause higher enzyme activity than alkali treatment alone.

Microwave irradiation pretreatment method was compared with another three by detecting their effects on cellulase production. The enzyme activity available by the different pretreatments of rice hulls decrease in the following order: NaOH-ethylene glycol organosolv process > H_2SO_4-ethylene glycol organosolv process > combination of alkali- and microwave-pretreatment > alkali treatment alone.

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