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Full Length Research Paper

Degrading capability and activity of extracellular xylanase secreted by a composite microbial system XDC-2

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The natural lignocellulose degrading capabilities of extracellular enzyme secreted by a composite microbial system XDC-2 were studied. Peptone cellulose solution (PCS) medium was beneficial to the degradation of lignocellulosic materials and ATCC 1053 medium promoted enzyme production of XDC-2. The exocellular xylanase activities of the crude enzymes were stable below 40°C. The crude enzyme has an effective capability of degrading natural lignocellulose, especially natural hemicellulose. The corn stalk core and rice straw lost 21.1 and 11.9% of its weight, respectively, after 48 h hydrolysis by the crude enzyme, and the weight loss of hemicellulose of corn stalk core and rice straw was 84.7 and 27.8%, respectively. Qualitative scanning electron microscopes (SEM) images indicated that after 48 h crude enzymes hydrolysis at 35°C, the material structure was modified. The production of the **soluble carbohydrates was up to 2, 400 mg·L^{-1} for corn straw and 1, 300 mg·L^{-1} for rice straw. It would hold the potential of further development and application of XDC-2 with the ability to hydrolyze natural lignocelluloses and release soluble carbohydrates.**

Key words: Composite microbial system, lignocellulose degradation, exocellular xylanase, hydrolysis ability.

INTRODUCTION

Lignocelluloses are the most abundant and renewable biomass resources in the world (Wong et al., 1988). The crucial step of converting lignocellulose into bioenergy is the soluble sugar release. Microbial degradation and enzymatic hydrolysis of lignocellulosic materials to produce fermentable reducing sugars have been the focus of extensive research (Gan et al., 2002). However, most studies focused on a single isolated microorganism (Chen et al., 2010). Regularly, the pure cultures are characterized by unsatisfactory lignocellulolytic activities and cannot break down the complex natural lingocelluloses (Chang and Holtzapple, 2000; McMillan, 1994; Puri, 1984; Gregg and Saddler, 1996; Mansfiled et al., 1999).

To date, the degradation of natural lignocellulose is the bottleneck of research and application. The rate-limiting step in biomass degradation is the conversion of cellulose and hemicellulose polymers to sugars. In recent years, more attention has been paid to natural lignocellulose (rice straw, corn stalk and wheat straw) degradation by mixed microorganisms which are more enzymatically effective than any single isolate (Schwarz, 2001). Cui et al. (2002) constructed a microbial community (MC1) under artificial conditions based on the natural microflora, which could effectively degrade natural lignocellulose (Cui et al., 2002; Haruta et al., 2002). However, MC1 does not secrete extracellular enzymes. Degradation products such as reducing sugar were quickly utilized by the living microorganisms, resulting in inefficient accumulation of catabolism (Niu et al., 2005). Recent work showed that a mixture of several enzymes could improve the lignocellulolytic degradation capabilities (Seling et al., 2008), but there are only a few reports on enzymes that could effectively hydrolyze natural lignocellulose that were not treated under rigorous conditions. Therefore, the absence of enzymes with efficient degrading capacities is still the major limitation

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on the conversion of lignocellulosic materials into energy.

Recently, a lignocellulose degrading composite microbial system (XDC-2), which could secrete extracellular xylanase effectively, was developed in our laboratory (Guo et al., 2010). Xylanase treatment removes re-precipitated xylan on the surface of the fiber thereby making the fiber more permeable to lignin removal (Subramaniyan and Prema, 2002), hence leading to significant enhancement of the hydrolysis of lingocellulose substrates. In the present work, we focused on degrading efficiency and accumulation of hydrolysates of the crude enzymes of XDC-2, described the characterization and degradation capabilities of the crude enzymes of XDC-2 and provided the experimental basis for preparing lignocellulose degrading composite enzymes from a lignocellulose degrading composite microbial system, which could have a significant impact on conversion of natural lignocellulose.

MATERIALS AND METHODS

Bacterial strains, media and growth conditions

For obtaining bacterial strains, lignocellulose degradation composite microbial system XDC-2 was screened from composted agricultural and animal waste amended soil following a long-term directed acclimation (Guo et al., 2010). The XDC-2 was cultured in the modified peptone cellulose solution (M-PCS) (peptone 5 g, yeast extract 1 g, NaCl 5 g, K_2HPO_4 1 g, $MgSO_4 \cdot 7H_2O$ 0.35 g, $CaCO_3$ 3 g, and 1 L H_2O (pH 7.2)) which contained 1% (w/v) carbon source (corn stalk or rice straw). After inoculation (seed volume of 5%), the medium was cultured under static dark conditions at 35°C. Except for M-PCS used in the original screening of composite microbial system XDC-2, the following four media were used: ATCC 1053 medium (Bhat and Barker 1947), Mandels' medium (Mandels and Reese 1960), Czapek's medium (Maghraby et al., 1991) and Cellulose-yeast extract medium (Cellulose-YE) (Mohagheghi et al., 1988).

For all treatments, inoculation volume was 5% (v/v) and 1% (w/v) corn stalk was added. All systems were cultured at 35°C under static conditions. The samples were obtained at the same time for the further determination of relevant indicators. Each medium was tested in triplicate.

Pretreatment of lignocellulosic materials and culture for enzyme production

The air-dried lignocellulosic materials (rice straw and corn stalk) were obtained locally (Beijing, China). The materials were submerged in 1% (w/v) sodium hydroxide at 25°C for 48 h, washed with tap water to neutral pH, then oven-dried at 80°C. In some cases, after delignification, the dried pretreated materials were milled to 1 mm length. Subsequently, 20 ml XDC-2 after five-day cultivation was inoculated into 500 ml flask (containing 400 ml ATCC medium plus 4 g corn stalks). The system was cultured for 48 h at 35°C under static conditions for determination of xylanase and relevant indicators.

Preparation of crude enzyme and hydrolytic treatment and extracellular xylanase assay

Culture samples were centrifuged at 8000 × *g* for 10 min at 4°C and the supernatants were filtered through a sterile 0.22 μM filter. One hundred milliliters of filtrate was placed in a 150-ml sterile flask (containing 1% milled corn stalk or rice straw). Each treatment was performed in quadruplicate. Xylanase activities were assayed according to Bailey et al. (1992). The substrate solution containing 1% (w/v) oat spelt xylan (Sigma) was dissolved in phosphate butter (pH 6.0). The reaction mixture consisted of 2.0 ml substrate solution and 0.5 ml of appropriately diluted enzyme. The reaction mixture was incubated at 40°C for 30 min prior to reducing sugar estimation.

Enzyme and reagent blank were also simultaneously incubated with the test samples. Colour development was measured at 520 nm using the 3,5-dinitrosalicylic acid (DNS) method (Miller 1959). β-glucosidase activities were assayed according to Shoemaker et al. (1978). The substrate solution contained 1% (w/v) D- salicin (Alfa Aesar) was dissolved in phosphate butter (pH 6.0). Similarly, the activity of carboxymethyl cellulose (CMCase) was assayed using 1% (w/v) CMC (Sigma) as substrate solution in the same conditions. The enzyme activities were expressed per milliliter of original volume of fermentation broth. One unit (U) of enzymatic activity was defined as the amount of enzyme required to liberate 1 μg of xylose or glucose in 1 min from xylan or carboxymethyl cellulose respectively.

Degradation of lignocellulosic materials by extracellular xylanase hydrolysis

Degradation of lignocellulosic materials by extracellular xylanase hydrolysis were measured after 48 h, all enzymatic hydrolysis materials (including fermentation broth and residual lignocellulosic materials) were centrifuged at 5000 × *g* for 10 min. The precipitates were washed with deionized water, centrifuged at 5000 × *g* for 10 min, and then the supernatant was discarded. After repeating this process twice, the precipitates were dried at 80°C to constant mass and weighed. Afterward, 0.5 g sample was transferred into a special pocket (Model F57, USA). Components of residual lignocellulosic materials were analyzed using fiber analysator (Model ANKOM^{220}, USA) as previously described (Guo et al., 2008).

Determination of soluble carbohydrates production

The water soluble carbohydrates produced during the hydrolytic process were determined according to the Anthrone colorimetry (Tomasm, 1977) method.

Scanning electron microscopy (SEM)

Structures of the pretreated lignocellulosic materials (rice straw and corn stalk) and these materials after enzymatic hydrolysis were examined by SEM (HITACHI-S3400) as previously described (Seling, 2007). All the experimental results are the average of three replicates, unless specified otherwise.

RESULTS

Determination of media for producing enzyme

To determine a suitable culture medium for lignocellulose degradation by the composite microbial system XDC-2, we selected five types of media commonly used for lignocellulose degradation microbe culture. The results

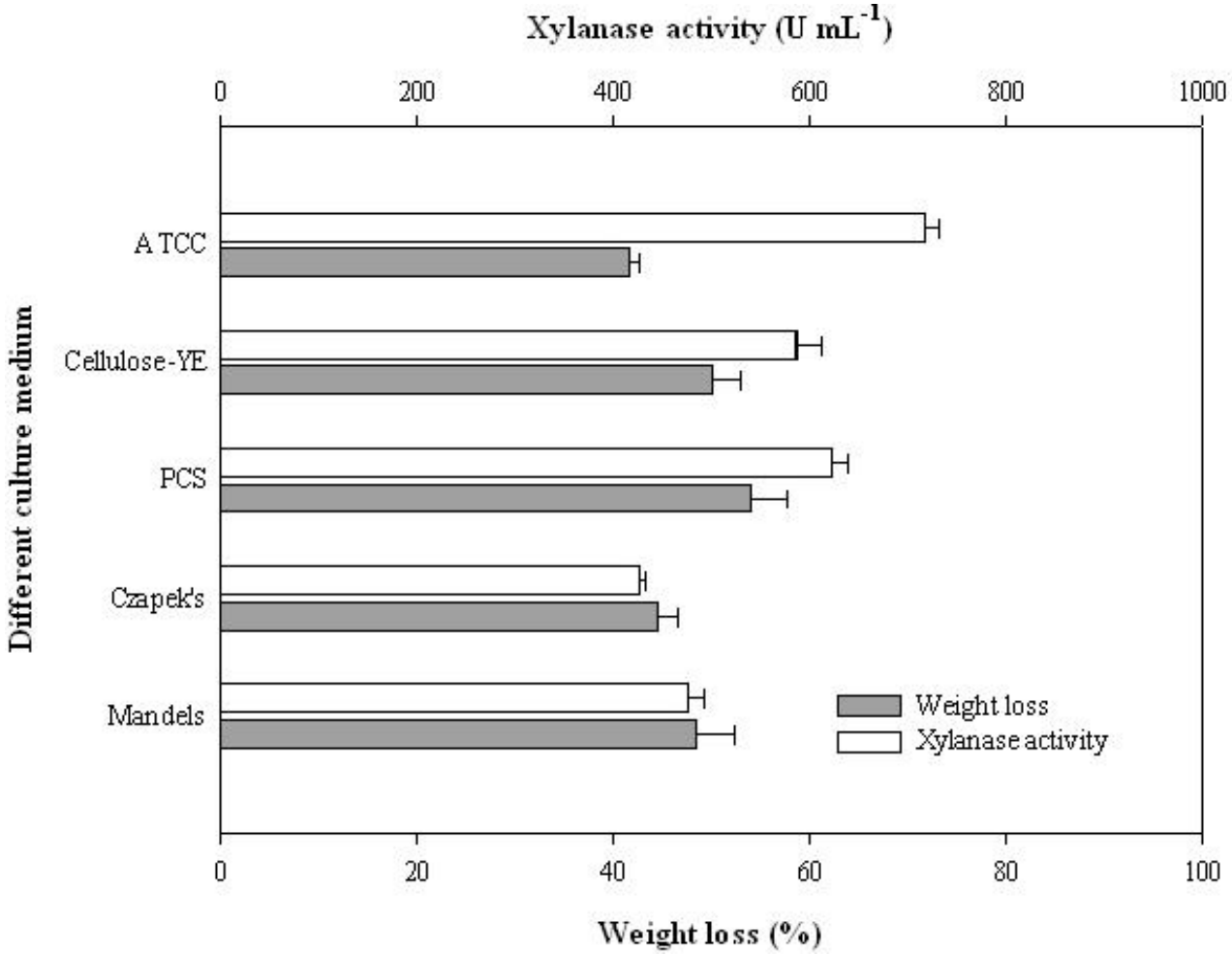

Figure 1. The effects of different media on xylanase activities and weight loss of corn stalk during degradation process.

show that after four days inoculation, the extracellular xylanase activities of ATCC medium (717.7 U·ml^{-1}) were significantly higher than the others (476.2 U·ml^{-1} of Mandels medium, 426.9 U·ml^{-1} of Czapek's medium, 622.8 U·ml^{-1} of PCS medium and 587.2 U·ml^{-1} of cellulose-yeast extract medium) (Figure 1). The total degradation ratio varied: ATCC medium was 41.6%, and the highest was 54.0% of PCS medium. There were no significant differences in degradation ratio among other three media.

The extracellular enzyme activities of composite microbial system

The extracellular activities (avicelase activity, CMCase activity and xylanase activity) of the XDC-2 were determined on day 0 (immediately after inoculation), three, six, nine, 12 and 15. The xylanase activities amounted to the highest value (738.3 U·ml^{-1}), while avicelase activity and CMCase activity and were only 27.7 and 19.7 U·ml^{-1} and 3.11 U·ml^{-1} respectively (Table. 1). On the basis of this experiment result, we preliminarily infer that this composite microbial system has a high capability of degrading natural hemicellulose.

Effect of temperature on extracellular xylanase activity stability

To investigate the temperature stability of the enzymes, the crude enzyme was incubated at 35, 40 and 45°C, and extracellular xylanase activities were determined at 2, 6, 12, 24, 36 and 48 h. Results indicate that the dynamics of the crude extracellular xylanase activities were similar at 35 and 40°C. The relative extracellular xylanase activities amounted to 87.3 and 73.5% after 6 h. Following a slight decrease from 6 to 12 h, the relative extracellular xylanase activities were 63.1 and 55.1%, respectively. No significant change occurred from 12 to 36 h. After 48 h, the extracellular xylanase activities were closed to deactivation at 35 and 40°C (Figure 2). At 45°C, the

Table 1. Enzyme activity over time in rice straw degradation.

Time (day)	Enzymes activity (U·ml^{-1})			
	Avicelase	CMCase	β-glucosidase	Xylanase
0	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
3	6.90±0.72	14.90±1.08	10.20±0.60	583.20±12.22
6	27.7±0.62	19.70±0.85	3.11±0.21	738.27±6.42
9	7.20±0.44	5.40±0.36	1.27±0.11	645.07±4.92
12	5.93±0.38	0.61±0.29	0.00±0.00	351.87±4.54
15	3.30±0.20	0.34±0.12	0.00±0.00	308.53±11.81

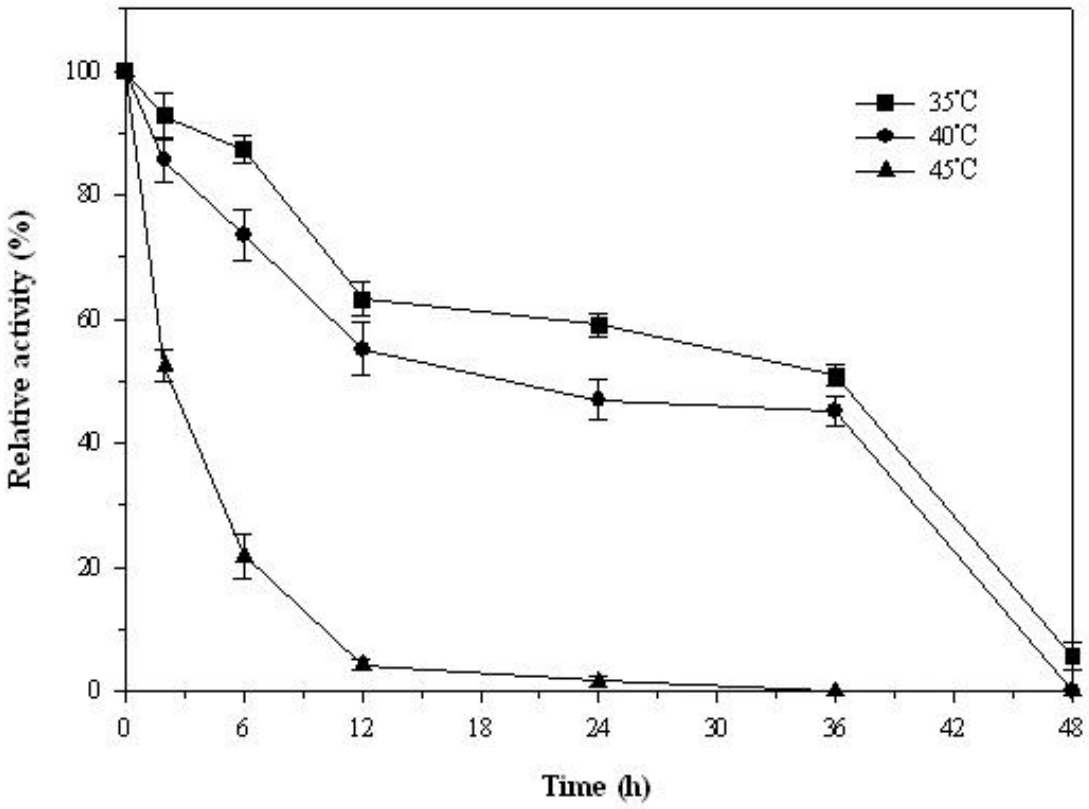

Figure 2. Effect of temperature on xylanase activities.

extracellular xylanase activities ceased after 12 h.

Soluble carbohydrates production by enzymatic hydrolysis

To determine the production of the soluble carbohydrates, milled corn stalk core or rice straw was used as the sole carbon source. During 48 h hydrolysis, the production of the water soluble carbohydrates were measured every 12 h. The results show that the soluble carbohydrates were increased to 2, 400 mg·L^{-1} (Figure 3). With rice straw as the substrate, the content of soluble carbohydrates increased to 1, 300 mg·L^{-1}, while with corn stalk core as the substrate, the content of soluble carbohydrates increased slowly from 0 to 12 h. There was a significant increase from 24 to 36 h. The production of soluble carbohydrates reached 2, 400 mg·L^{-1} at 36 h, but did not increase after this time-point. In contrast, when the corn stalk core was degraded by composite microbial system XDC-2, the content of soluble carbohydrates in fermentation broth was decreased to 300 mg·L^{-1}.

Degradation of rice straw and corn stalk core by crude enzymatic hydrolysis

After 48 h enzymatic hydrolysis, extracellular xylanase

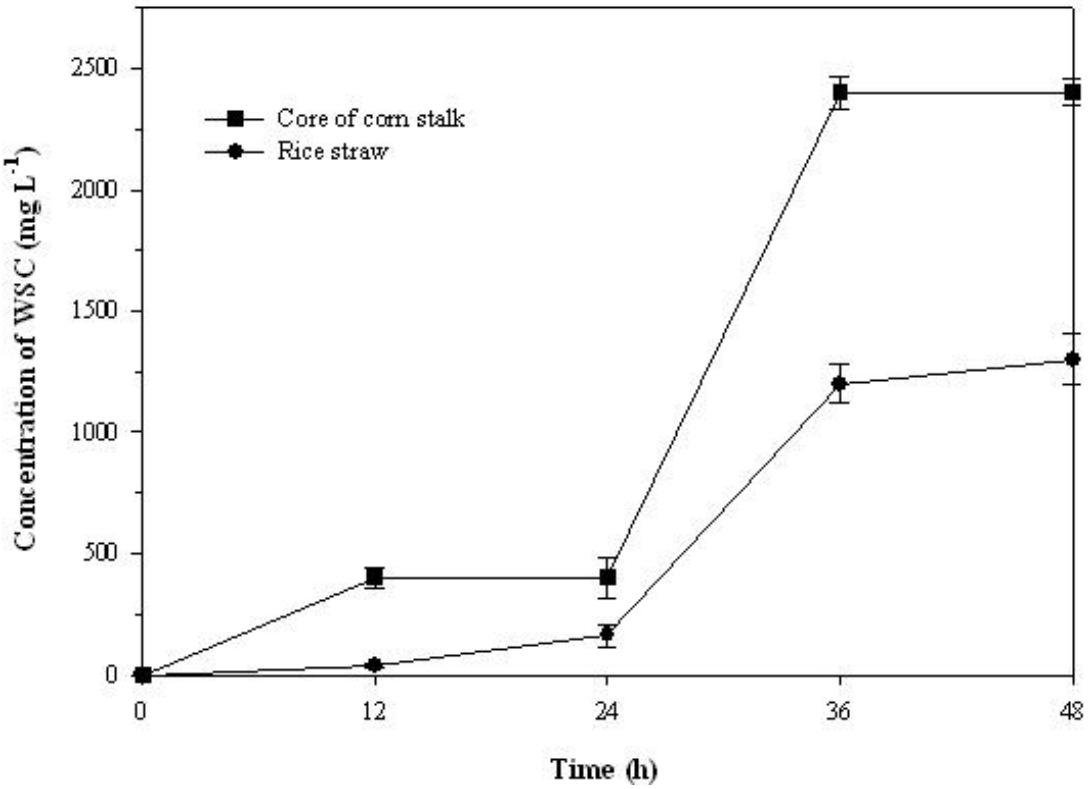

Figure 3. Levels of soluble carbohydrates during enzymatic hydrolysis using corn stalk core and rice stalk as substrates.

activities decreased, while soluble carbohydrates content did not increase. At this time, we terminated the enzymatic hydrolysis process and determined total degradation ratio, weight loss of soluble substance, cellulose, hemicellulose, lignin and ash content of the solid residues. The total degradation ratio of corn stalk core was 21.1%, and for rice straw was 11.9% (Figure 4). The weight loss of hemicellulose of corn stalk core was 84.7%, whereas rice straw weight loss was only 27.8%. Cellulose weight loss was only 2.1 and 1.9%, respectively.

Structural changes as determined by SEM

The structure changes in rice straw and corn stalk before and after the enzymatic hydrolysis were shown by using SEM (Figure 5). The plant cell wall structure of rice straw and corn stalk core showed evidence of plant cell wall vascular bundles and a highly fibrillar structure(Figures 5A and C). Enzymatic hydrolysis disrupts the lingocellulosic structure by mainly dissolving hemicellulose. As a result, major microfibrous cellulose structures remain (Figures 5B and D) and some lignin or lignin-carbohydrate complexes may be condensed on the surface of the cellulose fibers. Hydrolysis with crude enzymes significantly alters the fibrillar structure.

DISCUSSION

XDC-2 is a novel lignocellulose-degrading composite microbial system. The composite microbial system could degrade lignocellulose such as corn stalk and rice straw as well as hemicelluloses; it also secreted extracellular xylanase effectively. The extracellular xylanase of the XDC-2 system was stable over a wide range of pH (3.0 to 10.0) at temperatures of 25 to 35°C. The extracellular xylanase of XDC-2 could maintain high activity at pH 5.0 to 9.0 and temperature of 25 to 35°C over three days (Guo et al., 2010).

ATCC medium includes nutrients which accelerate the rapid growth of XDC-2 microbes. Therefore, extracellular xylanase activity after four-days cultivation was significantly higher than others. However, XDC-2 might give priority to utilize beef extract which was contained in ATCC medium, and resulted in lower corn stalk total degradation ratio. These results show that nutritive elements in the culture medium have significant influence on enzyme activities of XDC-2. The specific influences of different nutritive elements require further study. In addition, from the enzyme activities assays, we suggested that XDC-2 has a high capability to degrade natural hemicellulose. However, enzyme activity does not necessarily reflect the efficient degradation of the natural

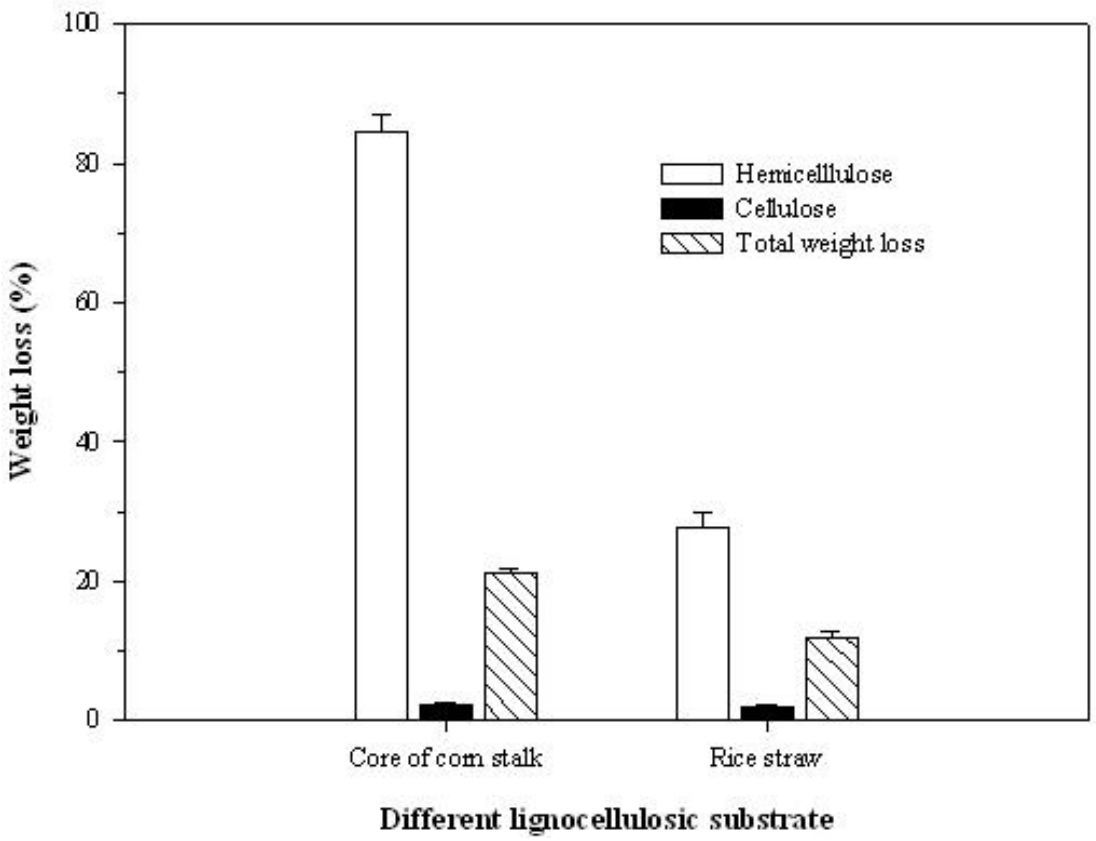

Figure 4. Degradation of lignocellulosic components of different substrates in enzymatic hydrolysis process.

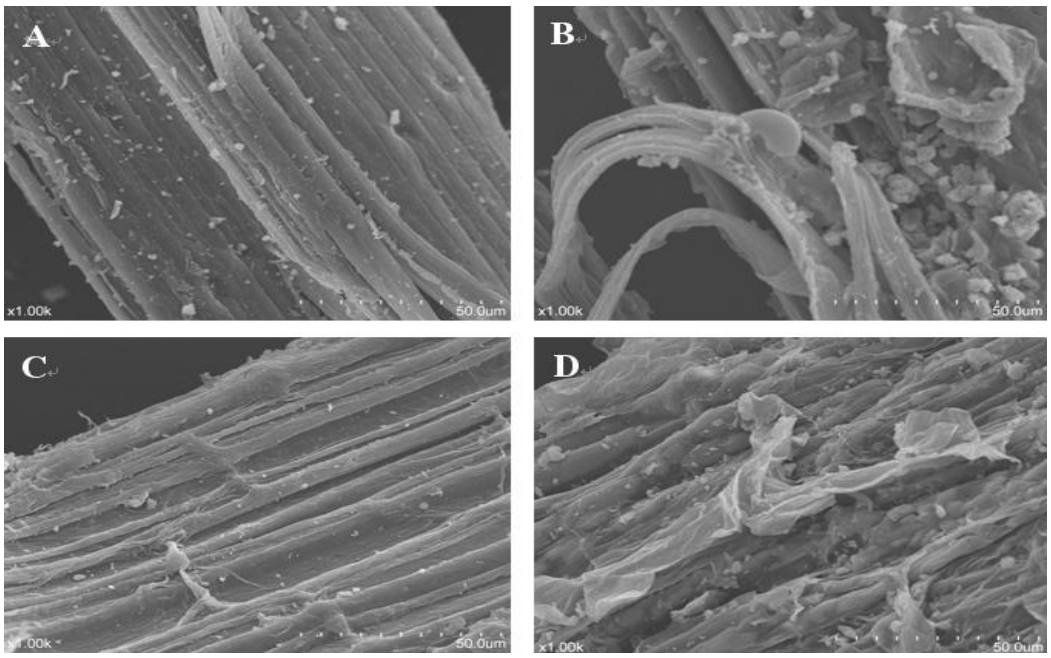

Figure 5. SEM of rice straw and corn stalk samples. Rice straw before enzymatic hydrolysis (A), corn stalk before enzymatic hydrolysis (B); rice straw after enzymatic hydrolysis (C) and corn stalk (D). SignalName=SE, accelerating voltage=20000 volt, Deceleration voltage=0 v, Magnification=1000, working reistance=9400 um, Emission current=50000 nA.

lignocellulosic substrates (Yawada 1988; Johnson et al., 1982; Schwarz 2001). Therefore, the degrading capacity of the composite enzyme system of the XDC-2 was investigated in the degradation experiment, which suggested that the composite enzyme system has a high capability of degrading natural hemicellulose. The weight loss of corn stalk core was higher than rice straw (Figure 4). The loss of hemicellulose was the primary component of lost mass during the degradation of corn stalk and rice straw. Cellulose is a sturdy material, ideally suited to insure the structural stability of land plants, where it is a main component of the primary cell wall (Schwarz, 2001). The presence of hemicellulose and lignin is reported to make the access of cellulase enzymes to cellulose difficult, thus reducing the efficiency of hydrolysis (Solange et al., 2008). The composite enzyme system of the XDC-2 solubilized the hemicellulose significantly, and would result in the access of cellulase to cellulose easily, thus enhancing the cellulose enzymatic hydrolysis.

Pure cultures microorganism has limited ability, while a composite microbial system can exhibit a synergistic effect where the degradation ability of the composite microbial system is greater than the sum of the degradation ability of the individual microorganism (Haruta et al., 2002). Much attention has been devoted to research on mixing different microorganisms to improve the capability to degrade natural lignocelluloses. Lewis et al. (1988) mixed rumen microorganisms to degrade rice straw with a weight loss of 55.8%, but the extracellular xylanase activity of this mixture was not described. The xylanase activities of XDC-2 were much higher than those previously reported. Temperature stability of the crude enzyme showed that this composite enzyme system were stable between 35 to 40°C. The result shows the importance of utilizing this composite enzyme system for enzymatic hydrolysis of lignocellulosic substrates to obtain good process economy than thermophilic enzymes. This characteristic could provide a convenient option to explore effective xylanase preparations.

Corn stalk core is softer than rice straw and the content of hemicellulose is apparently higher (Guo et al., 2010). These differences lead to different enzymatic hydrolysis exhibition. XDC-2 was able to adhere to corn stalk core more easily than rice straw. As a result, the extracellular xylanase activities were high, and more soluble carbohydrates accumulated. However, increased levels of soluble carbohydrates may be utilized by micro-organisms quickly, resulting in inefficient accumulation (Niu et al. 2005). Saving products by using enzymes instead of microorganisms in lignocelluloses utilization must not be made at the expense of the biomass convert, which is still the most important parameter in achieving lower utilization costs.

Before enzymatic hydrolysis, the substrates exhibited rigid and highly ordered fibrils (Figures 5A and C). Qualitative SEM images indicate that after crude enzymes hydrolysis at 35°C, the hemicellulose removal modified the material structure, but the main structures were not broken down (Figures 5B and D), and these substrates exhibited significant differences in their supramolecular structures. Hydrolyzed substrates show that a major destruction of the fibers is hemicellulose. These findings combined with the results earlier mentioned suggest that the effects of composite enzyme system are mainly attributed to the solubilization of the hemicellulose fractions, and to the beneficial effects that the enzymatic hydrolysis has on changing the morphology and fine structure of the cellulosic residue. This will be helpful for cellulosic residue conversion and utilization.

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