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Bioconversion and saccharification of some lignocellulosic wastes by *Aspergillus oryzae* ITCC-4857.01 for fermentable sugar production

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Abstract The recent interest in bioconversion of agricultural and industrial wastes to chemical feedstock has led to extensive studies on cellulolytic enzymes produced by microorganisms. In the present study three lignocellulosic substrates viz. sugarcane bagasse, sawdust and water hyacinth were pre-treated with alkali and enzyme and their effect on bioconversion has been investigated. The ability of selected substrates for induction of cellulase enzyme by *A. oryzae* ITCC 4857.01 and for the potentiality of the induced enzyme to saccharify the substrates were also assessed. The maximum degree of conversion of substrate (0.415%) and improved specific substrate consumption (0.99 g substrate/g dry biomass) was exhibited in sugarcane bagasse after alkali treatment at 96 hrs. Both alkali-treatment and enzyme-treatment, water hyacinth was the best for cellulase induction and showed maximum endoglucanase activity of 11.42 U/ml. Reducing sugar yield ranged from 1.12 mg/ml for enzyme treated sawdust at 48 hrs to 7.53 mg/ml for alkali treated sugarcane bagasse at 96 hrs. Alkali-treated sugarcane bagasse gave the highest saccharification rate of 9.03% after 96 hrs. The most resistant substrate was sawdust which produced 5.92% saccharification by alkaline treatment. The saccharification of lignocellulosic substrates by enzyme produced by *A. oryzae* ITCC 4857.01 indicates the enzymes specificity towards the substrates. The use of such enzyme in lingo-cellulose hydrolysis will lead to efficient conversion of cellulose materials to other important products.

Keywords: *Aspergillus oryzae*, biomass, cellulase, lignocellulosic substrates, pre-treatment, saccharification

INTRODUCTION

Lignocellulosic biomass represents the largest renewable reservoir of potentially fermentable carbohydrates on earth (Mtui and Nakamura, 2005; Ahmadi et al. 2010). These materials generally contain up to 75% of cellulose and hemicelluloses which cannot be easily converted to simple monomeric sugars due to their recalcitrant nature. The utilization of cellulosic biomass for ethanol production continues to be a subject of worldwide interest in view of fast depletion of our oil reserves and food shortages (Singh et al. 2009). A lot of emphasis had been given to screening of the agricultural wastes for release of sugars by hydrolysis of lignocellulosics. The hydrolysis of cellulose to soluble sugars makes it available as feedstock in alcoholic fermentation, single cell protein production and other industrial processes (Rao et al. 1985; Biag et al. 2004; Louime and Uckelmann, 2008). Pre-treatment of cellulose opens up the structure and removes secondary bonds between glucose chains. Treatment with alkali can removes lignin, thus promoting hydrolysis and improving the glucose recovery from cellulose. Enzymatic hydrolysis of such cellulosic material by cellulase enzymes is the most promising approach to get high product yields. Fungi produce extracellular cellulase enzymes that break down cellulose into two or three glucose units which are readily degraded and assimilated as glucose monomers (Chinedu et al. 2010). *Aspergillus spp*. is a filamentous fungus which produces a

wide range of enzymes for the degradation of plant cell wall polysaccharides which are important to the food and feed industry (de Vries and Visser, 2001; Helal, 2006; Jahromi et al. 2010). Therefore, the present study was conducted to evaluate the potential of *Aspergillus oryzae* ITCC-4857.01 on bioconversion of some lignocellulosic substrates, and to examine the ability of this organism to induce cellulase and sacchrification on alkali and enzyme treated substrates.

MATERIALS AND METHODS

Microorganism

Aspergillus oryzae ITCC-4857.01 was used in the present study which was obtained from Plant Pathology, Mycology and Microbiology Laboratory, Department of Botany, Rajshahi University, Bangladesh. This strain was isolated from rice bran and confirmed by Indian Type Culture Collection, IARI. The strain was maintained in glycerol at 4ºC.

Substrates

Sugarcane bagasse, wood sawdust and water hyacinth were used as test substrates. Sample substrates (except sawdust) were cut into very small pieces and dried in an oven at 80ºC for 24 hrs. The dried samples were then milled in an electric grinder and sieved to a particle size of less than 0.5 mm. These substrates were used in the experiments conducted.

Pretreatment of substrates

Alkaline pretreatment: Twenty five gram of each substrate and 500 ml of NaOH (1%) solution were mixed in a flask and kept for duration of either 3, 6, 12 or 24 hrs. The alkali treated substrates were filtered through a nylon cloth, and washed in running tap water until neutralized. Excess water was removed by squeezing the substrate in the nylon cloth, after which the treated substrates was allowed to dry overnight in an oven at 80ºC.

Preparation of crude enzyme solution: *A. oryzae* ITCC-4857.01 was cultivated in 100 ml Czapek's broth medium which consists (in g/L) of sucrose (20.0 g), $NaNO_3$ (2.0 g), KH_2PO_4 (1.0 g), KCl (0.5 g), $MgSO_4.7H_2O$ (0.5 g) $FeSO_4.7H_2O$ (0.01 g) and $CuSO_4.5H_2O$ (0.05 g) with 1% of carboxylmethyl cellulose powder (CMC) and pH 5.6. The medium was inoculated with 1 x 10^6 spores/cells and allowed to grow at 28ºC on a Gallenkamp (England) rotary shaker at 100 rpm for 7 days. Fungal mat was removed by filtering and the filtrate was centrifuged at 10,000 rpm for 10 min at 4ºC using refrigerated ultracentrifuge (REMI, k-70) and stored at 4ºC with few drops of toluene.

Table 1. Bioconversion of untreated and enzyme treated cellulosic substrates by *A. oryzae* ITCC-4857.01 after 7 days of incubation at 28ºC.

Treatment/ substrate	Reducing sugar (mg/ml)	Conversion (%)	Protein (mg/ml)	Biomass (gm)	Substrate consumption (g/ g biomass)
Untreated					
Bagasse	0.629^{d}	0.06	481^{d}	0.42^{d}	2.38
Sawdust	0.361^{f}	0.04	227^{e}	0.27^{e}	3.70
Water hyacinth	0.514^{e}	0.05	343^{f}	0.38^{d}	2.63
Enzyme-treated					
Bagasse	3.24^{a}	0.32	815^{a}	0.97^{a}	1.03
Sawdust	1.80 ^{c}	0.18	612^{c}	0.67^{c}	1.49
Water hyacinth	2.62^{b}	0.26	787^{b}	0.85^{b}	1.17

Means with different letters within same column differ significantly at $p < 0.05$.

Enzymatic pre-treatment: The prepared crude enzyme induced by *A. oryzae* ITCC-4857.01 was used in the pre-treatment of substrates. One gram of substrate and 20 ml of crude enzyme solution was mixed in 250 ml flask and kept for 24 hrs at 28ºC. Then excess water was removed by squeezing the substrate with nylon cloth and then it was spread over an aluminum foil and allowed to dry overnight in an oven at 80ºC.

Bioconversion of cellulosic substrates

Pre-treated substrates (1%) were added with Czapek's broth medium and pH of the medium was adjusted to 5.6 and autoclaved. The medium was inoculated with 1 x 10^6 spores/cells and allowed to grow at 28ºC with shaking for 7 days. The fungal mat was removed from the solution by filtering and dried in oven at 80ºC until constant weight and amount of biomass was calculated by subtracting the weight of filter paper from the total mass. The yield was expressed as g/g substrate. Specific substrate consumption was calculated by weight of substrate used (g) /g dry biomass. The supernatant was centrifuged at 10,000 rpm for 10 min. The amount of reducing sugar in culture filtrate was measured by DNS method using a spectrophotometer (Spectronic 21) with the absorbance set at 550 nm. The protein content was determined by the method of Lowry et al. (1951) using bovine serum albumin (BSA) as protein standard.

Table 2. Bioconversion of alkali-treated cellulosic substrates by *A. oryzae* ITCC-4857.01 after 7 days of incubation at 28ºC.

Substrates	Time (hrs)	Reducing sugar (mg/ml)	Conversion (%)	Protein (mg/ml)	Biomass (gm)	Substrate consumption (g/ g biomass)
Bagasse	3	3.15[d]	0.32	517[j]	0.65de	1.53
	6	3.47[c]	0.35	640[e]	0.82c	1.21
	12	3.82[b]	0.39	812[c]	0.99b	1.01
	24	4.15[a]	0.42	985[a]	1.01a	0.99
Sawdust	3	1.06[j]	0.11	355[l]	0.48f	2.08
	6	1.59[i]	0.16	528[i]	0.59e	1.69
	12	1.98[h]	0.20	583[g]	0.64de	1.56
	24	2.40[g]	0.24	622[f]	0.78c	1.28
Water hyacinth	3	2.15[f]	0.22	463[k]	0.51f	1.96
	6	2.82[e]	0.28	552[h]	0.68d	1.47
	12	3.10[e]	0.31	697[d]	0.76c	1.66
	24	3.75[b]	0.38	863[b]	0.91b	1.09

Means with different letters within same column differ significantly at $p < 0.05$.

Cellulase induction for saccharification

For cellulase induction three pre-treated natural lignocellulosic substrates were used and beside these CMC (semi synthetic cellulosic substrate) was used as control for comparison of enzyme activity. Two gram of each substrate was placed separately in 100 ml Czapek's broth medium and autoclaved. The medium was inoculated with standard inoculums of test organism and incubated at 37ºC for 7 days. Then the supernatant was harvested as crude enzyme solution and endoglucanase activity (C_x) was measured. Enzyme activity was measured as the amount µmol glucose released min/mlof culture filtrate and expressed as U/ml. These enzyme solutions were used for saccharification studies.

Saccharificaiton

To determine the percentage of saccharification, 100 mg dry weight equivalent of cellulosic material were mixed with 1 ml of 0.1 M citrate buffer (pH 5.5) and incubated with 1 ml crude culture filtrate at 50ºC for 48, 72, 96 hrs. The amount of reducing sugar released was measured by the DNS method. Saccharification was calculated by applying the equation of Spano et al. (1976).

$$\text{Saccharification (\%)} = \frac{\text{Reducing sugar (mg/ml) x 0.9}}{\text{Substrate (mg/ml)}} \times 100$$

Table 3. Production of reducing sugar by *A. oryzae* ITCC- 4857.01 culture filtrates on untreated and alkali-treated different cellulosic substrates.

Substrate and induced enzyme	Substrate for hydrolysis	Reducing sugar (mg/ml)		
		48 hrs	72 hrs	96 hrs
CMC (8.23 U/ml)	Untreated bagasse	1.10	2.57	3.82
	Treated bagasse	2.91	4.05	5.85
	Untreated sawdust	0.97	1.75	2.12
	Treated sawdust	1.49	2.78	3.94
	Untreated WH	1.05	2.15	3.55
	Treated WH	2.64	3.92	5.15
Sawdust (6.68 U/ml)	Untreated bagasse	0.95	1.22	1.65
	Treated bagasse	1.52	2.15	2.72
	Untreated sawdust	0.86	0.92	1.25
	Treated sawdust	1.18	1.38	2.52
	Untreated WH	0.90	1.18	1.47
	Treated WH	1.36	1.77	2.62
Bagasse (9.16 U/ml)	Untreated bagasse	1.89	2.72	4.98
	Treated bagasse	3.53	4.86	6.41
	Untreated sawdust	1.12	1.96	2.80
	Treated sawdust	2.05	3.13	4.35
	Untreated WH	1.24	2.54	3.92
	Treated WH	2.78	4.16	5.88
Water hyacinth (11.42 U/ml)	Untreated bagasse	2.72	3.76	5.25
	Treated bagasse	4.72	5.57	7.53
	Untreated sawdust	1.73	2.10	2.92
	Treated sawdust	2.45	3.78	4.94
	Untreated WH	2.18	3.56	4.24
	Treated WH	3.95	5.38	6.91

CMC= Carboxyl methyl cellulose, WH= water hyacinth.

RESULTS AND DISCUSSION

Bioconversion of lignocellulosic substrates

In the present investigation untreated, alkali (1% NaOH) and enzyme (2.4 U/ml endoglucanase of *A. oryzae* ITCC 4857.01) treated substrates *i.e*. sugar cane bagasse, sawdust and water hyacinth were used for bioconversion and the results are summarized in Table 1 and Table 2. The significant ($p < 0.05$) amount of protein and reducing sugar was obtained in all treated substrates compare to untreated. The maximum amount of reducing sugar (4.15 mg/gm) and protein (985 µg/ml) was measured in sugarcane bagasse after alkali-treatment at 24 hrs and lowest in sawdust for all conditions. The present results support the work of Ja'afaru and Fagade (2007) who recorded the highest reducing sugar 5.55 mg/ml for alkali-treated corn cob at 48 hrs. The increase in glucose production depends on availability of cellulose in the medium and also due to the specific binding of the enzymes with substrates (Omojasola et al. 2008).

The highest degree of conversion (0.42%) was exhibited in sugarcane bagasse while that for water hyacinth and sawdust were 0.38% and 0.24%, respectively. Degree of conversion rate was increased

with increase in duration of treatment. The lowest degree of conversion and poor specific substrate consumption was recorded in untreated sawdust. Improved specific substrate consumption (0.99 g substrate/g dry biomass) was shown when sugarcane bagasse was used as substrate. Comparatively sugarcane bagasse contains higher amount of available carbohydrates from other substrates, therefore, the organism can take it easily and followed by the results of biomass production. Analysis of the results using Duncan's multiple range test (DMRT) showed that there was significant ($P < 0.05$) difference in reducing sugar production among the treatments, time and using substrates. However, alkali-treated sugarcane bagasse is the best for microbial conversion.

Table 4. Production of reducing sugar by *A. oryzae* ITCC- 4857.01 culture filtrate on untreated and enzyme treated different cellulosic substrates.

Substrate and induced enzyme	Substrate for hydrolysis	Reducing sugar (mg/ml)		
		48 hrs	72 hrs	96 hrs
CMC (7.43 U/ml)	Untreated bagasse	1.24	2.24	3.75
	Treated bagasse	2.52	3.32	5.73
	Untreated sawdust	0.92	1.67	2.04
	Treated sawdust	1.79	2.50	4.29
	Untreated WH	1.12	2.00	3.26
	Treated WH	2.32	3.08	5.17
Sawdust (5.53 U/ml)	Untreated bagasse	0.95	1.12	1.53
	Treated bagasse	1.37	1.57	2.05
	Untreated sawdust	0.75	0.90	1.12
	Treated sawdust	1.12	1.31	1.75
	Untreated WH	0.84	1.00	1.35
	Treated WH	1.26	1.42	1.91
Bagasse (9.10 U/ml)	Untreated bagasse	1.62	2.52	4.48
	Treated bagasse	2.98	4.72	6.17
	Untreated sawdust	1.27	1.76	2.65
	Treated sawdust	2.05	2.75	4.54
	Untreated WH	1.25	2.30	3.71
	Treated WH	2.75	4.13	5.58
Water hyacinth (10.42 U/ml)	Untreated bagasse	2.54	3.62	5.00
	Treated bagasse	3.30	5.00	6.60
	Untreated sawdust	1.62	2.00	2.65
	Treated sawdust	2.70	3.76	4.74
	Untreated WH	1.95	3.12	4.16
	Treated WH	2.97	4.62	6.23

CMC= Carboxyl methyl cellulose, WH= water hyacinth.

Cellulase induction by *A. oryzae* ITCC 4857.01

The production of cellulases is inducible and is affected by the nature of the substrates. Therefore, the choice of an appropriate inducing substrate is important (Kang et al. 2004). In the present study, using different cellulosic substrates for production of cellulase showed that alkali treated water hyacinth was the most susceptible substrate which produced the highest activity for endoglucanase (C_x 11.42 U/ml) while semi-synthetic substrate CMC showed moderate enzyme activity (Table 3 and Table 4). Sawdust was found to be most resistant. Earlier, Kocher et al. (2008) reported exoglucanase (C_1), C_x and C_B activities of 0.09, 0.12 and 1.12 IU ml^{-1}, respectively using rice straw inoculated with *Trichoderma harzianum* Rut-8230. Wen et al. (2005) reported C_1, C_x and C_B activities of 1.74, 12.22, and 0.097 IU $ml^{-1,}$ respectively by *Trichoderma reesei* using dairy manure as substrate. Ja'afaru and Fagade (2007) reported newspaper, groundnut shell and sawdust are poor inducers of the enzymes probably due to the high lignin content in these substrates. In nature, it is known that lignin physically encrusts cellulose that makes it resistant to enzymatic degradation. This leads to shortage of utilizable carbohydrates in lignin-rich substrates and consequently poor growth of organism and low enzyme yield.

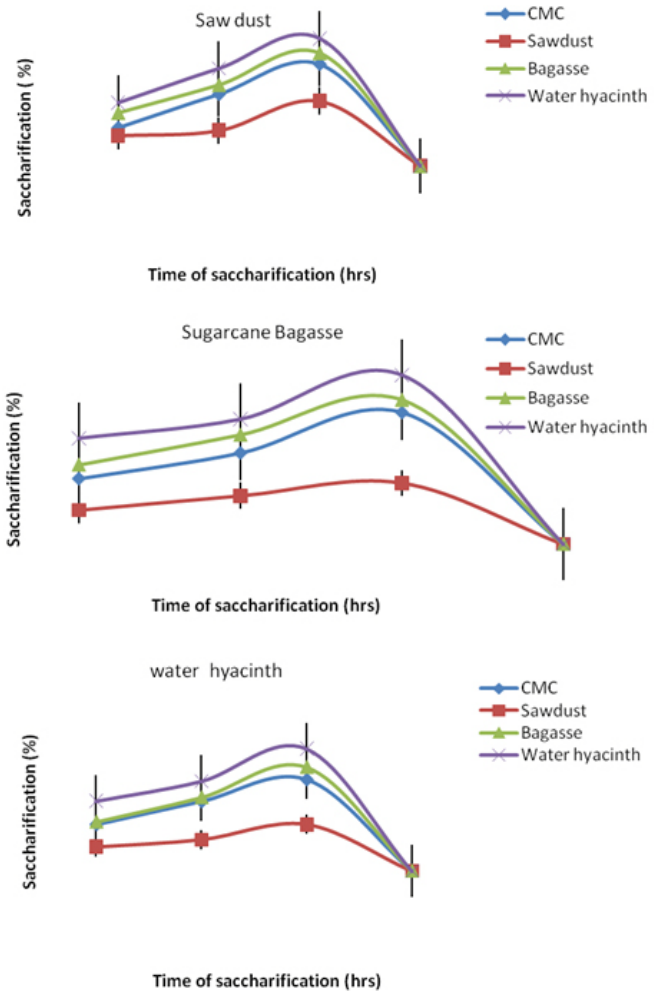

Fig. 1 Effect of time on saccharification of sawdust, sugarcane bagasse and water hyacinth by culture filtrate of *A. oryzae* ITCC-4857.01 grown on alkali-treated different cellulosic substrates.

Hydrolysis of substrates

The results of hydrolysis of untreated, alkali and enzyme treated cellulosic substrates with culture filtrate of *A. oryzae* ITCC-4857.01 grown in different cellulosic substrates are presented in Table 3 and Table 4. It was found that higher amounts of reducing sugar were produced when pre-treated substrates were used as compared to untreated substrate. Reducing sugar yield ranged from 1.18 mg/ml for alkali-treated sawdust at 48 hrs to 7.53 mgh/ml for alkali-treated sugarcane bagasse at 96 hrs while enzyme treated substrate ranged 1.12 to 6.60 mg/ml. The highest amount of reducing sugar was recorded in alkali-treated sugarcane bagasse at 96 hrs when alkali treated water hyacinth induced enzyme (11.42 U/ml) was used. From the reducing sugar releasing spectra it was exhibited that sugarcane bagasse was the best substrate used. A considerable amount of reducing sugar was liberated in enzyme treatment but the amount was comparatively lower than that of alkali treatment. This decrease may have been due to the removal of potentially usable sugar from the substrate by C_x component in the enzyme solution. The test organism *A. oryzae* ITCC-4857.01 has more C_x enzyme but low amount C_1 enzyme; therefore, some form of pre-treatment of the substrate, for example alkali treatment, is necessary in order to use cellulosic substrate hydrolysis effectively before using culture filtrate of *A. oryzae* ITCC 4857.01. The effect of the alkali treatment is, somewhat similar to that of C_1 activity in facilitating the degradation of cellulose by the C_x enzyme (Han and Callihan, 1974).

Saccharification

The culture filtrate of *A. oryzae ITCC* 4857.01 showed comparatively low saccharification rates ranged 1.41 to 9.03% on natural cellulosic materials (Figure 1 and Figure 2). The saccharification rate was increased with duration of time. The highest saccharification was obtained for alkali-treated sugarcane bagasse at 96 hrs when water hyacinth induced enzyme was used. The most resistant substrate was sawdust which produced 5.64 and 5.92% saccharification by enzyme and alkaline treatment at 96 hrs, respectively. El-Naghy et al. (1991) reported 3.55% saccharification rate for saw dust, 1.5% for cotton and 3.05% for newspaper using *Sporotrichum thermophile* culture filtrate. Ja'afaru and Fagade (2007) also reported 3.9% saccharification rate for saw dust, 5.0% for corn cob after 48 hrs incubation with *A. niger* culture filtrate. In the present study, the saccharification rates of alkaline treated substrates were higher than those of enzymatic treated substrates. Perhaps the cellulases of *A. oryzae* ITCC4857.01 have less ability to absorb tightly onto the sawdust compared to that of bagasse and water hyacinth. Hence, sawdust consists high amount of recalcitrant lignin which are closely associated with cellulose and the used fungus was cellulolytic that are not able to dipolymerise lignin consequently low percentage of saccharification was obtained.

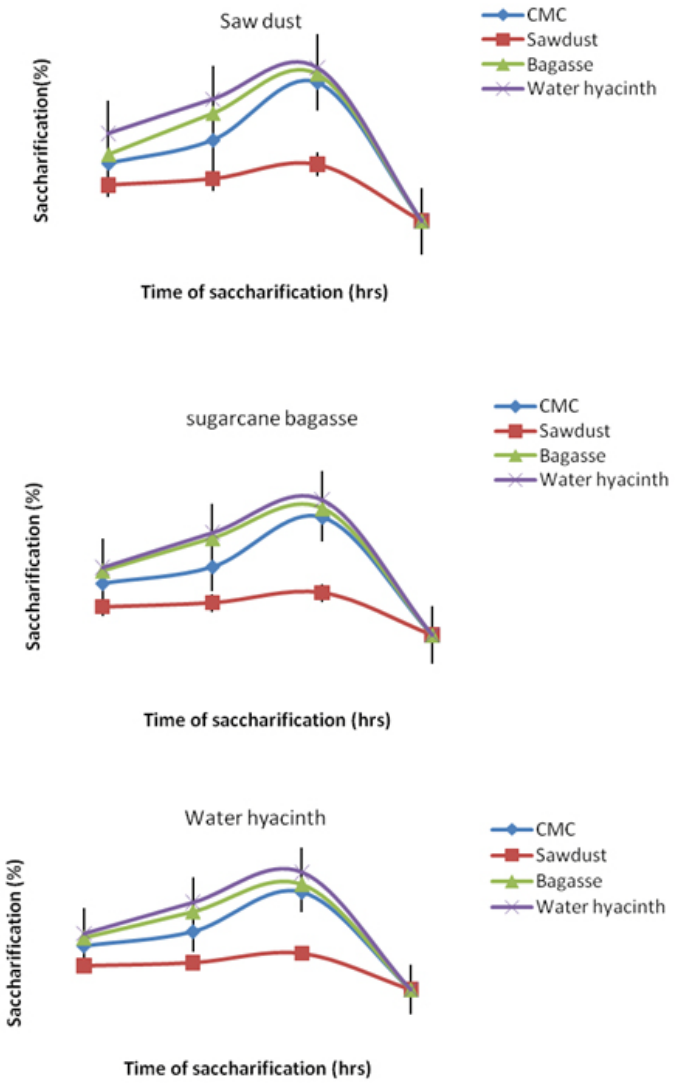

Fig. 2 Effect of time on saccharification of sawdust, sugarcane bagasse and water hyacinth by culture filtrate of *A. oryzae* ITCC-4857.01 grown on enzyme-treated different cellulosic substrates.

From above findings it may be concluded that strains *A. oryzae* ITCC 4857.01 showed good performance for the production of reducing sugar, protein and biomass and also for enzyme production. Among the tested substrates, water hyacinth was the best cellulase inducer and sugarcane bagasse was found to be the best for releasing maximum reducing sugar and saccharification. Further, water hyacinth induced enzyme was performed maximum percentage of saccharification that may be used in future to produce fermentable sugar for ethanol production or others. This research may be meaningful both in the conversion and utilization of renewable biomass, and in the reduction of environmental pollution.

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