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Glycoside Hydrolase Family 43 from *Paenibacillus curdlanolyticus* Strain B-6; An Accessory Enzyme to Enhance the Hydrolysis of Pretreated Rice Straw

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Abstract Glycoside hydrolase family 43 (GH43) from *Paenibacillus curdlanolyticus* strain B-6 (Xyl43B6) was preformed heterologous expression in *Escherichia coli*. Enzymatic function determination revealed that Xyl43B6 exhibited trifunctional properties of α-L-arabinofuranosidase, β-xylosidase, and endo-xylanase. Xyl43B6 showed broad specificity of substrates on aryl-arabinoside, aryl-xylopyranoside, xylooligosaccharides, xylobiose, low branching xylan, and highly substituted arabinoxylan. Xylose liberation rate from xylooligosaccharides was higher than from xylobiose. However, trace amount of xylose was detected from highly substistuted rye flour arabinoxylan, indicating influence of chain length and substitution groups on xylose liberation efficiency of Xyl43B6. Moreover, co-hydrolysis of Xyl43B6 with GH10 endo-xylanase (Xyn10E) on pretreated rice straw revealed the boosting effect and increase in liberated reducing sugar as a result of Xyl43B6/endo-xylanase synergy. This report proposed new enzymatic properties of GH43 which showed the ability to enhance biomass biorefinery efficiency.

Keywords α-L-Arabinofuranosidase/β-xylosidase/xylanase activities, Glycoside hydrolase family 43, Multifunctional enzyme, *Paenibacillus curdlanolyticus*, Rice straw biorefinery

1. Introduction

Lignocellulosic biomass is the most abundant renewable resource obtained from agricultural residues, herbaceous grasses and forest products. Generally, lignocellulosic biomass is composed of approximately 15-40% cellulose, 10-30% hemicelluloses and pectin, and 5-20% lignin. [1] Xylan is the major component of hemicelluloses encompassing a linear β-(1,4)-D-xylopyranose backbone decorated with different groups of side chain. Common substituents found on the backbone of xylan are acetyl, arabinosyl, and glucuronosyl residues. Furthermore, type and degree of the substituted side chains vary among botanical origins. [2] Due to the recalcitrant architecture of xylans, hydrolysis of xylans requires the action of a multiple-enzyme system. The group of xylanolytic enzymes works synergistically to depolymerize xylan to its sugar constituents. Xylanolytic enzymes are usually composed of non-debranching enzymes (endo-xylanase and β-xylosidase) and debranching enzymes (α-arabinofuranosidase, α-glucuronidase, acetyl xylan esterase and phenolic acid esterase). [2]

A concept of xylan bioconversion has been emphasized in the last few decades. Thus, effective utilization of xylan would play a significant role in economical production of highly valued substances. Xylan bioconversion required a combination of debranching and non-debranching enzymes. Releasing the substituted side groups from xylan allows endo-xylanase to act efficiently for complete hydrolysis. [2] The glycoside hydrolase family 43 plays a role as an accessory enzyme that boosts the hydrolysis efficiency of endo-xylanases. GH43 is a group of enzymes, encompassing monofunctional enzymes such as α-L-arabinofuranosidase (EC 3.2.1.55), β-xylosidase (EC 3.2.1.37), arabinanase (EC 3.2.1.99), endo-xylanase (EC 3.2.1.8), galactan 1,3-β-galactosidase (EC 3.2.1.145), α-1,2-L-arabinofuranosidase

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(EC 3.2.1.-), exo-α-1,5-L-arabinofuranosidase (EC 3.2.1.-), exo-α-1,5-L-arabinanase (EC 3.2.1.-), and β-1,3-xylosidase (EC 3.2.1-). Furthermore, multifunctional enzymes, such as β-xylosidase/α-L-arabinofuranosidase [3], β-xylosidase/ exo-xylanase (GenBank ID: ABD48561.1), and β-1,4-xylosidase/α-1,5-arabinofur(pyr)anosidase/β-1,4-lactase/α-1,6-raffinase/α-1,6-stachyase/β-galactosidase/α-1,4-glucosidase [4], have been reported.

Paenibacillus curdlanolyticus strain B-6 is a cellulolytic/ xylanolytic bacterium that produces a unique extracellular xylanolytic-cellulolytic multienzyme complex-like structure capable of degrading insoluble substrates. [5] We found one open reading frame (ORF) from the B-6 strain encoding a protein belonging to GH family 43 which comprises a broad variety of enzyme specificities and similarities. [6] We speculated that this GH43 may be a good accessory enzyme in xylanolytic enzymes cocktail that boosts the hydrolysis efficiency for the bioconversion of biomass. Therefore, synergy analysis was elucidated in this study.

2. Materials and Methods

2.1. Strains and Plasmid

P. curdlanolyticus B-6 was isolated from an anaerobic digester and fed with pineapple waste. [5] All cloning strategies were performed in *Escherichia coli* DH5α (New England Biolabs, Ipswich, *MA, USA*). *E. coli* BL21 (DE3) (Novagen, Darmstadt, Germany) was used as the host for the derivative of pET28b(+) expression system (Novagen).

2.2. Gene Cloning and Expression

The p*Xyl43B6* plasmid construction and *Xyl43B6* expression were conducted as described by Wongratpanya *et al*. [6]. Protein purification by affinity chromatography method was achieved via HisTrap™ FF columns (GE healthcare, Little Chalfont, UK). Protein concentration was determined as described by Lowry *et al*. [7].

2.3. Enzyme Assays

α-L-Arabinofuranosidase and β-xylosidase activities were assayed on *p*-nitrophenyl-α-L-arabinofuranoside (*p*NPA) and *p*-nitrophenyl-β-D-xylopyranoside (*p*NPX), respectively (Sigma- Aldrich, St. Louis, MO, USA), as described previously. [6] One unit (U) of enzyme activity was defined as the amount of enzyme required for liberating 1 µmol of *p*-nitrophenol per min. Xylanase activity was assayed on 1% xylan from birchwood (Sigma-Aldrich) in 50 mM sodium phosphate buffer (SPB) (pH 7.0) at 50°C for 10 min. Liberated reducing sugars were analysed by the Nelson-Somogyi method [8]. One unit (U) of xylanase activity was defined as the amount of enzyme used for liberating 1 µmol of xylose per minute.

2.4. Substrate Specificity

Hydrolysis of Xyl43B6 was determined on various structural types of substrates which were: 0.025% (w/v) xyloligosaccharides (X3-X6) and xylobiose (Megazyme International, Wicklow, Ireland), 1% (w/v) xylans from birchwood and oat spelt (Sigma-Aldrich), wheat flour arabinoxylan and rye flour arabinoxylan (Megazyme). The hydrolysis products were separated by thin layer chromatography (TLC), as described by Sornyotha *et al*. [9] and high performance liquid chromatography (HPLC) (Shimadzu, Kyoto, Japan) with a reflective index detector (Shimadzu RID-10A) on BP-100 Pb^{++} carbohydrate columns (Benson Polymeric, Sparks, NV, USA) operated at 85°C with deionised water as a mobile phase at a flow rate of 0.6 mL/min.

2.5. Biomass Hydrolysis

Enzymatic hydrolysis of biomass was performed using 1% grinded rice straw at pH 7.0 using 50 mM SPB at 50°C. One nmol of enzymes (each): Xyl43B6, Xyn10A [10], Xyn10B [11], Xyn10C [12], Xyn10D [13], and Xyn10E (unpublished data) was mixed and incubated for 16 h. The liberated reducing sugars were quantified by the Nelson-Somogyi method.

2.6. Synergistic Analysis on Rice Straw Hydrolysis

Pretreatment of rice straw was carried out by soaking in 27% ammonium hydroxide at a solid:liquid ratio of 1:10 at 60°C for one week. The sample was neutralized by 1 N HCl and the pretreated solid was retrieved, dried, milled by a blender and used as a substrate for enzymatic hydrolysis. The synergistic effect of Xyl43B6 and GH10 endo-xylanase was studied by using 1% (w/v) of pretreated rice straw in 50 mM SPB (pH 7.0). One nmol of each enzyme was mixed with the substrate and incubated at 50°C. Liberated reducing sugars were quantified by the Nelson-Somogyi method.

3. Results and Discussion

According to Fosmid genomic library of strain B-6 constructed previously [11], an ORF *Xyl43B6* encoding a protein was confidently predicted by SMART software [14] to belong to a glycoside hydrolase family 43 catalytic domain. Heterologous expression of *Xyl43B6* was accomplished in *E. coli.* Protein expression and affinity purification was achieved via either C and N terminals histidyl tag, originated by *BamH*I/*Xho*I insertion site on pET28b(+) expression vector. A single protein band of approximately 39 kDa was observed on SDS-PAGE analysis.

Purified Xyl43B6 was determined the enzymatic hydrolysis function in various kinds of substrates. Xyl43B6

showed hydrolysis capability on *p*NPA and *p*NPX with the same activity (0.30 U/mg). Furthermore, determination of xylanase activity showed that Xyl43B6 exhibited xylanase activity versus various structural types of xylan. The highest xylanase activity (2.86 U/mg) was achieved on birchwood xylan, and 1.46 U/mg was determined on oat spelt xylan. Moreover, weak xylanase activity of Xyl43B6 on recalcitrant highly substituted xylan, wheat flour arabinoxylan and rye flour arabinoxylan was detected. As showed in Fig. 1, xylose was found as a sole hydrolysis product from xylooligosaccharides X3-X6 and xylobiose. Those results confirmed that Xyl43B6 exhibited multiple functions of hydrolysis activity including α-L-arabinofuranosidase, β-xylosidase and xylanase with broad substrate preference. Xyl43B6 could hydrolyze xylobiose, xylooligosaccharides, low branching xylan, and highly substited xylan. However, hydrolysis efficiency on highly substituted xylan was low. It might be postulated that increasing structural complexity of xylan had an effect on hydrolysis efficiency of Xyl43B6. On the other hand, β-xylosidse function of Xyl43B6 was different from true β-xylosidases which prefer xylobiose only [15]; the hydrolysis efficiency decreases with increasing degrees of polymerisation. [16]

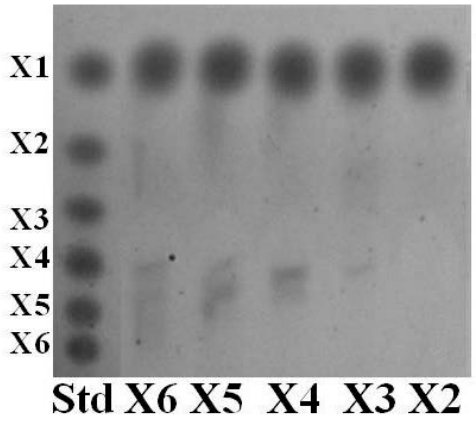


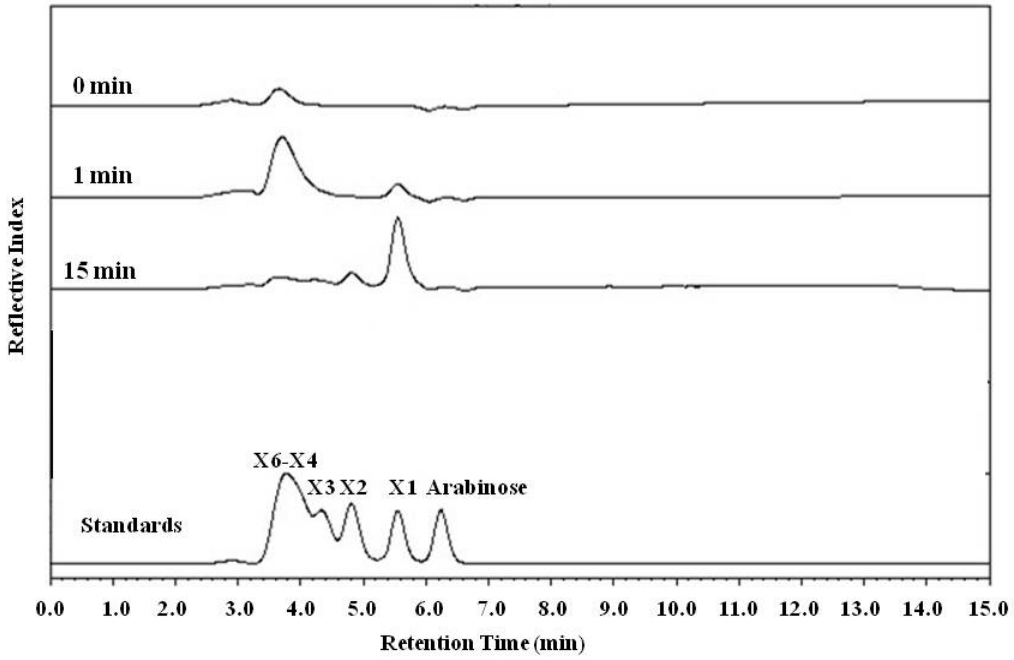
Figure 1. TLC chromatogram represents the hydrolysis products of xylooligosaccharides with degree of polymerization 3 to 6 and xylobiose hydrolyzed by Xyl43B6. Standard xylose (X1) and xylooligosaccharides, X2 to X6, were marked by Std. Hydrolysis was conducted in 50 mM SPB (pH 7.0) at 50°C for 16 h

A comparative study of structural complexity level versus hydrolysis efficiency was further elucidated. Hydrolysis of xylohexaose (X6) by Xyl43B6 at 1 min (Fig. 2a) revealed the sudden conversion of X6 to a mixture of X5-X4 xylooligosaccharides and xylose. Xylose was continuously increasing, and major xylose peak was observed after hydrolysis action proceeded for 15 min, while small amount of xylooligosaccharides and xylobiose still remained stable. In comparison with xylobiose hydrolysis (Fig. 2b), enzymatic conversion rate of xylobiose was slow. Xylobiose remained at 30 min. The major peak of xylose was found at 16 h of hydrolysis. These results indicated that degree of polymerization of β-(1,4)-D-xylopyranose had influenced hydrolysis efficiency of Xyl43B6. Meanwhile, overnight hydrolysis of rye flour arabinoxylan released major product of xylooligosaccharides and trace amount of xylose (Fig. 2c). The structural architecture of rye flour arabinoxylan is a linear chain of β-(1,4)-D-xylopyranose with high degree of substitution of both α-1,3 and α-1,2 monosubstitutions and O2, O3 disubstituted α-L-arabinofuranoside. [17] According to arabinoxylan structure, presence of substitution groups on main chain had an effect on xylose liberation by Xyl43B6. However, hydrolysis mechanism of Xyl43B6 was not elucidated in this study. The multifunctional actions of Xyl43B6 suggest that Xyl43B6 might be a good accessory enzyme for boosting xylanolytic enzyme cocktail and improving biomass biorefinery efficiency.

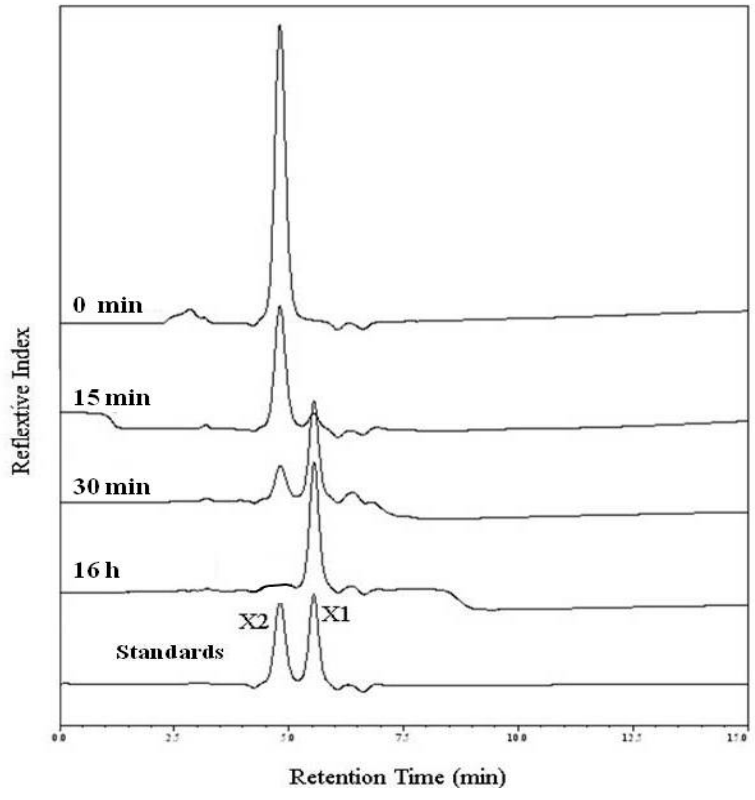
Synergy analysis of endo-xylanase and Xyl43B6 was studied on rice straw. Rice straw is a non-food renewable resource found abundantly in agricultural waste. Rice straw has a recalcitrant structure encompassing 41% of cellulose, 21.5% of hemicelluloses, and 9.9% of lignin [18]. Hence, over 60% of hexose and pentose content in rice straw is persuaded into the bioconversion to valued products. In this study, endo-xylanase GH10s and Xyl43B6 were applied for biorefining rice straw. The GH10 endo-xylanases collection in our laboratory including Xyn10A [10], Xyn10B [11], Xyn10C [12], Xyn10D [13], and Xyn10E (unpublished data) was used. At 16 h of hydrolysis hydrolysis, we revealed that the maximum reducing sugar liberation from rice straw was achieved by Xyn10E. Nevertheless, Xyl43B6 showed higher reducing sugar liberation than other GH10s from strain B-6 (Fig. 3a). Hence, we selected Xyn10E and Xyl43B6 to study synergism of rice straw biorefinery. Synergy analysis on 1% grinded rice straw showed that adding Xyl43B6 boosted the hydrolysis efficiency of Xyn10E, and liberation of reducing sugar increased with 1.77 degree of synergy (48 μg/mL) at 4 h (Fig. 3b). Nevertheless, the amount of liberated reducing sugar from Xyn10E/Xyl43B6 synergy was quite low; thereby, pretreatment process of rice straw was applied. In this study liquid-ammonia was selected to pretreat rice straw. Ammonia was used as a delignifying reagent which reacts selectively with lignin over carbohydrates. [19] Liquid-ammonia cleaves C–O–C bonds in lignin, as well as ether and ester bonds in the lignin–carbohydrate complex resulting in desirable characteristics including swollen structure of biomass. [19] Using liquid-ammonia soaked rice straw showed 4-fold dramatic increase in the amount of liberated reducing sugar (205.9 μg/mL) (Fig. 3c). Pretreatment of plant biomass is an important tool for achieving effective enzymatic hydrolysis. It should be focused on the removal or relocalization of lignin [20] and reducing structural complexity of the biomass [21]. Presence of lignin could limit accessibility of biomass-degrading enzymes [22] and may also inhibit the hydrolysis performed by an enzyme [23]. Hence, removing or redistributing lignin allowed the enzyme to hydrolyze the biomass efficiently.

4. Conclusions

In summary, an ORF Xyl43B6 from the strain B-6 genomic fosmid library was heterologously expressed in *E. coli*. Xyl43B6 exhibited multifunctional enzymatic activities with broad substrate specificity. Xyl43B6 plays a role as an accessory enzyme that boosts liberation of reducing sugar of GH10 endo-xylanase on rice straw hydrolysis. Hence, co-hydrolysis of Xyl43B6 with endo-xylanase could improve biomass biorefinery efficiency.



(a)



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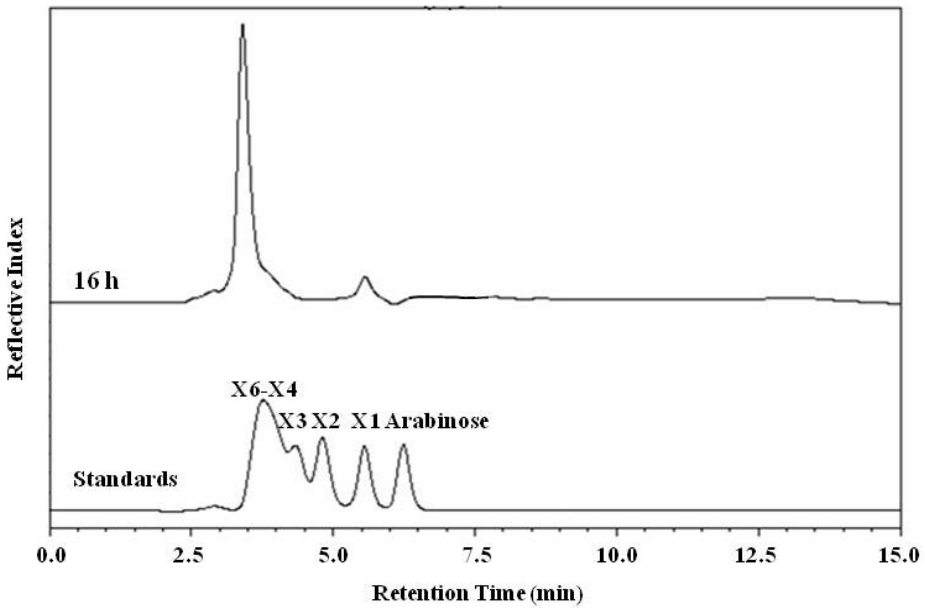
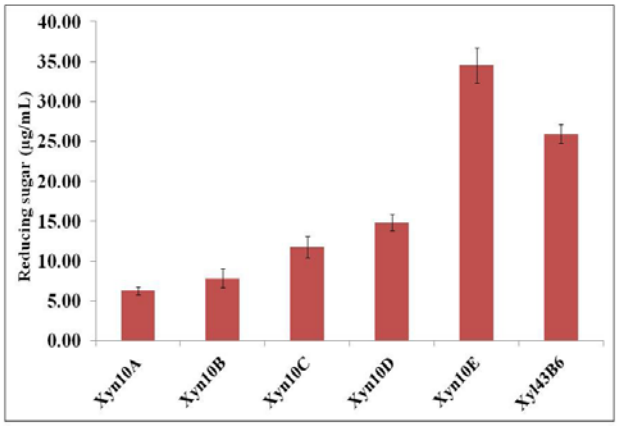
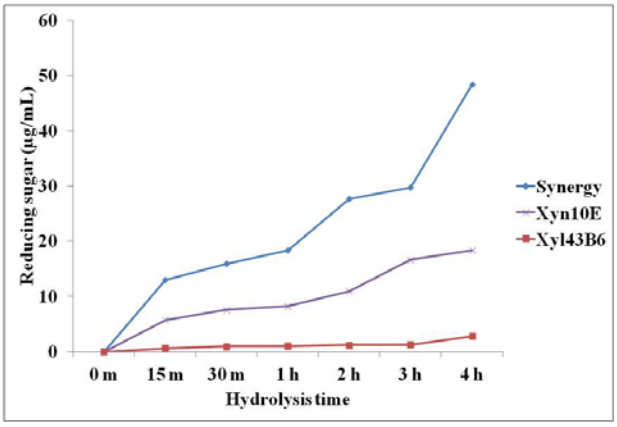


(c)

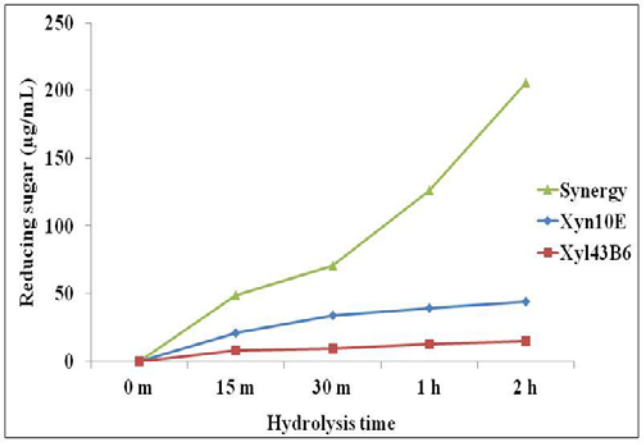
Figure 2. Chromatograms represent hydrolysis products of xylohexaose (a), xylobiose (b), and rye flour arabinoxylan (c) hydrolyzed by Xyl43B6. Hydrolysis products were analyzed via HPLC with reflective index detector



(a)



(b)



(c)

Figure 3. Reducing sugar liberation from 1% grinded rice straw hydrolysis by endo-xylanases GH10 B-6 and Xyl43B6 from strain (a) 1% w/v rice straw was hydrolyzed with 1 nmol of each Xyn10E and Xyl43B6 or a combination of Xyn10E and Xyl43B6 (1 nmol each) at 50°C pH 7.0 for 16 h. (b) Hydrolysis of pretreated rice straw (c) was conducted under the same conditions as described in (b). Reducing sugar was determined by Nelson-Somogyi method

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