Promotive Effects of Cellulolytic Enzymes Produced by Biomass-Degrading Bacteria on Saccharification of Different Pretreated Corn Stovers

Yanwen Wu
Nanjing Forestry University
Sarita Shrestha
Lakehead University - Thunder Bay Campus: Lakehead University
Haipeng Guo
Ningbo University
Jinchi Zhang
Nanjing Forestry University
Haisong Wang
Dalian Polytechnic University
Wensheng Qin (wqin@lakeheadu.ca)
Lakehead University https://orcid.org/0000-0003-2178-4003

Research Article

Keywords: enzymes, cellulase, saccharification, synergistic effect

Posted Date: May 25th, 2021

DOI: https://doi.org/10.21203/rs.3.rs-481742/v1

Abstract

Enzymatic saccharification of corn stover can be enhanced by partially replacing commercial enzymes with bacterial crude enzyme extracts. Three bacteria, *Bacillus* sp. A0, *Bacillus* sp. CH20S1, and *Exiguobacterium* sp. AS2B, were cultured in a media with corn stover as the substrate. The cultural conditions were monitored and optimized to maximize CMCase and xylanase activity in the crude enzyme extracts. After 72 h of hydrolysis of corn stover with diluted crude enzymes (DCE) from the three strains, reducing sugars were released from non-pretreated and pretreated corn stovers. Values of the released sugars ranged from 48.23–71.69 mg g^{-1}, which were lower than those released by commercial cellulase (100–400 mg g^{-1}). The synergistic effects were observed when 12 FPU g^{-1} and 4 FPU g^{-1} of commercial cellulase were added to the DCE of the CH20S1 strain producing 315.90 mg g^{-1} and 320.65 mg g^{-1} reducing sugars, respectively. It was shown that an effective combination of bacterial DCE with commercial enzymes could achieve more cost-efficient saccharification of lignocellulosic biomass compared to either of the two enzymes used alone.

Statement Of Novelty

The novelty of this study entails enzyme production from bacteria for saccharification of corn stovers. The study also focuses on the effects of bacterial and commercial enzyme combination on saccharification. The synergistic effect was observed significant for non-pretreated corn stover than pretreated one. This study further illustrates the potential of total cost reduction in pretreatment and saccharification of biomass by supplementing commercial enzyme with crude enzyme.

Introduction

The massive consumption of crude oil for producing transportation fuels and industrial chemicals increases crude oil prices, greenhouse gas emissions, and social tensions among oil-producing nations. To mitigate these problems, several studies have focused on the development of alternative and sustainable energy sources [1]. Biofuels show significant advantages in reducing greenhouse gas emissions compared with fossil fuels, especially bioethanol, a common renewable fuel [2]. Lignocellulosic biomasses are currently considered as promising feedstocks for bioethanol production because of their extensive distribution and abundant reserves [3].

A considerable number of studies have been conducted to explore efficient technologies for lignocellulosic ethanol conversion [4]. In general, lignocellulosic ethanol production involves pretreatments (chemical or physical) to break down the plant cell wall, enzymatic saccharification to release fermentable sugars, and yeast or other microbial fermentation to produce ethanol [5, 6]. So far, numerous pretreatment technologies, such as alkaline, ammonia, dilute acid, and steam explosion pretreatments, have been developed to solve the biomass recalcitrance problem for efficient enzymatic saccharification [7, 8]. However, harsh pretreatments can lead to the degradation of free sugars, while too weak pretreatments result in low enzyme accessibility [9]. Pretreatments via alkaline hydrogen peroxide

(AHP) and dilute acid have been evaluated jointly with enzymatic saccharification and showed a remarkable effect on bioethanol production [10, 11].

Saccharification of the lignocellulosic material was developed to determine the yield of fermentable sugars for producing bioethanol [12]. Hence, the process of saccharification is one of the main constraints in converting lignocellulosic materials to ethanol [13]. In general, biomass materials are saccharified by using acids, alkalis, or enzymes [14]. Enzymatic hydrolysis is considered the best method for subsequent fermentation because of its high specificity, high yield, and lower formation of undesirable products [15, 16]. However, these strategies have a costly production process with the use of commercial enzymes. Cellulase enzymes contributed 10–20% to the overall costs of ethanol production from biomass, according to recent techno-economic evaluations [17–19], a cost considered a significant part of the overall production costs [20]. Thus, the improvement of cellulolytic microorganisms and enhancement of the hydrolytic capacity of cellulases are essential to reduce the costs for biomass-to-bioethanol production. Although microbial cellulolytic enzymes were studied here to carry out the enzymatic saccharification for cost reduction, the majority of studies focus on the cellulolytic enzymes produced by fungi, such as *Penicillium roqueforti* and *Trichoderma reesei* [14, 21]. We suggest that bacteria are considered more appropriate to produce the hydrolytic enzymes for saccharification on an industrial scale due to the shorter generation time [22], ability to be artificially cultivated [23], and excellent tolerance to environmental stresses [24].

As such, three bacterial strains (A0, AS2B, and CH20S1) were studied to produce cellulolytic enzymes using corn stover as the substrate and to examine the possibility of bacterial enzymes in the saccharification process. The hydrolytic efficiency of the crude enzyme extracts from the strains examined was evaluated from saccharification assays measuring the reducing sugars released from AHP/ H_2SO_4 in pretreated and non-pretreated corn stovers. Further, the potential of bacterial cellulolytic enzymes in industrial bioethanol production was investigated. Finally, the crude enzyme extract from the best-performing strain in the saccharification assays was selected and combined with commercial enzymes in different concentrations.

Materials And Methods

Biomass material and bacterial strains

The bacterial isolates, *Bacillus* sp. A0 (Accession no. KP974676), Bacillus sp. CH20S1 (Accession no. HQ331531), and *Exiguobacterium* sp. AS2B (Accession no. HM134063), and their enzymatic characteristics have been previously reported by our lab [25-27]. All the strains stored at -80°C in our lab were activated with Luria-Bertani (LB) medium (37°C, 200 rpm) for 12 h before the experiments. The corn stover containing high contents of glucan and xylan was chosen to induce cellulolytic enzymes from the three strains. The untreated corn stover provided by Agriculture and Agri-Food Canada was dried at 50ºC until a constant weight was achieved, then cut into 2–5 cm pieces, and then ground through a 50 mesh sieve for further study.

Pretreatment

AHP and H_2SO_4 have been proven as effective methods to pretreat corn stover [28, 29], so they were used in this study.

AHP pretreatment

Milled corn stover samples were pretreated with alkaline peroxide. The pretreatment was carried out by slurring the samples 5% (w/v) with 30% (w/v) H_2O_2 (pH to 11.5 adjusted with 2.0 M NaOH). The mixture was shaken at 150 rpm at 50°C for 1 h then the solid and liquid fractions were obtained by filtration. The solid fractions were washed with deionized water until pH 7, dried in an oven at 80°C overnight, and used for composition analysis.

H_2SO_4 pretreatment

Acid pretreatment was conducted as described by Xu *et al*. [30] with some minor modifications. In brief, the biomass samples (0.5 g) were added into 10 mL H_2SO_4 (1%, v/v) in 15 ml plastic centrifuge tubes, mixed well, and autoclaved at 121°C for 20 min. After cooling down, the tubes were shaken at 150 rpm and 50°C for 2 h, then centrifuged at 3,000 g for 5 min. The residues were washed with deionized water until the pH of the washing fluid was approximately 7.0 and finally dried at 80°C overnight for plant cell wall composition analysis. All samples were prepared in triplicate.

Biomass composition analysis

The cellulose and hemicellulose analyses were conducted by measuring the content of glucan and xylan according to the methods described by Shrestha *et al*. [31]. The anthrone-sulfuric acid method [32] and orcinol-hydrochloric acid method [33] were used for hexose and pentose content determination, respectively. The Klason lignin analysis was performed by the method reported by Ibanez and Bauer [34].

Obtaining crude enzymes and enzyme assay

Each strain was inoculated (2%, v/v) in 50 ml of mineral salt medium (1.0 g L^{-1} $NaNO_3$, 1.0 g L^{-1} K_2HPO_4, 1.0 g L^{-1} KCl, 0.5 g L^{-1} $MgSO_4$, 0.5g L^{-1} yeast extract, and 3.0 g L^{-1} peptone) using 0.5% (w/v) corn stover as the sole carbon source. The samples were incubated at 37°C, 200 rpm, and for 6 days. After harvesting the culture, the samples were centrifuged at 12,000 g for 3 min. The supernatants were then taken as crude enzymes for the analysis of CMCase and xylanase activity after sterile filtration using a 0.22-μm Millipore filter. The crude enzymes, including maximum CMCase and xylanase, were used for the hydrolyzing experiments. The CMCase and xylanase activities were determined as described by Guo *et al*.

[27], detecting the release of reducing sugars from CMC and xylan, respectively. The reducing sugar content was measured by DNS methods [35].

Influences of temperature and pH on enzyme activities

The effect of temperature (40°C to 80°C with 5°C intervals) and the influence of pH (4 to 8 with 0.5 intervals) were studied. The buffer solutions used were pH 4–7 in a 0.05 M citrate buffer and pH 7.8–9.0 in a 0.05 M Tris-HCl buffer.

Enzymatic saccharification

Enzymatic saccharification of corn stover using different strains

Crude enzyme extracts obtained from strains A0, CH20S1, and AS2B after 12 h of incubation were used to saccharify pretreated corn stover. For each sample, 3.5 ml of crude enzyme extract was taken, adjusted to a pH of 5, and diluted to 5 ml with 0.05 M sodium citrate buffer (pH 5). Enzymatic saccharification was conducted by DCE containing 1% (w/v) pretreated corn stover and 0.005% sodium azide [14]. The reaction mixture was incubated at 55°C with an agitation of 200 rpm for 72 h. The supernatants were collected every 12 h to determine the amount of reducing sugars. In addition, 5.0 ml of DCE solution was set as the control.

Enzymatic saccharification of corn stover using commercial cellulase with crude enzymes

A commercial cellulase from *Trichoderma reesei* ATCC 26921 (Celluclast 1.5 L, Novozymes, Franklinton, NC, USA) and the crude enzymes produced from strain CH20S1 were combined in three sets to study enzymatic saccharification: 5.0 ml of DCE were added to 4, 8, and 12 FPU g^{-1} dry biomass of commercial cellulase. Also, 20 FPU g^{-1} of commercial cellulase without the crude enzyme extract was set as the control group and the same amount of commercial cellulase for the hydrolysis was set at the maximum as a reference to the previous report [36]. The measuring method was the same as described in section 2.6.1.

Statistical analysis

The statistical analysis was carried out by one-way analysis of variance (one-way ANOVA) using SPSS (SPSS Inc., USA, version 13.0). The data are taken in triplicate and the values are mentioned in mean ±

standard deviation.

Results And Discussion

Characterization of corn stover before and after pretreatments

The effects of different pretreatments on the glucan, xylan, and lignin content of the pretreated corn stover are shown in Table 1 and suggest that both pretreatments can drastically change the cell wall composition of corn stover.

Table 1

Composition of corn stover expressed as percentage of dry matter. Different letters indicate significant differences (P < 0.05) among different pretreatments. Different letters indicate significant differences (P < 0.05) among different pretreatments.

Pretreatment	Component (%)		
	Glucan	Xylan	Acid insoluble lignin
Non-pretreated	27.43±0.50^{c}	18.91±1.13^{a}	21.13±1.45^{b}
AHP	41.03±4.87^{b}	20.67±0.33^{a}	15.47±0.92^{c}
H□SO□	53.67±3.26^{a}	16.69±0.76^{b}	27.93±0.61^{a}

The raw material used in this investigation contained 46.34% total carbohydrate (27.43% glucan and 18.91% xylan) and 21.13% acid-insoluble lignin, which is consistent with that of the corn stover analyzed by Liu et al., 2013 [37]. After the treatment of AHP, the content of lignin was 15.47%, which showed a significant reduction compared to the raw material (P < 0.05), while the content of glucan significantly increased by 49.58% with a slight rise of xylan (Table 1). In this study, 1% H_2SO_4 pretreatment resulted in a significant increase of glucan content (53.67%, P < 0.05) compared to that of the raw material, while the xylan content significantly decreased to 16.69% (P < 0.05) (Table 1). In a previous study, the alkali pretreatment removed most of the lignin by breaking hydrogen and other covalent bonds, leaving behind a highly porous cell wall for better penetration of enzymes [38]. In contrast, acid pretreatment such as H_2SO_4 mainly induces the chemical conversion of xylan to xylose [39] which is soluble in acidic liquor and hence removed from the solids after pretreatment. Thus, the acid-pretreated corn stover had a reduced xylan content compared to non-pretreated corn stover (Table 1).

Production of CMCase and xylanase by A0, AS2B, and CH20S1

All three bacterial strains, A0, AS2B, and CH20S1, showed excellent CMCase and xylanase activity when corn stover was used as the sole carbon source, and with a similar variation tendency (Fig. 1). After 12 h of incubation, all tested strains secreted the majority of CMCase and xylanase into the medium. The maximum CMCase activities for A0, AS2B, and CH20S1 were 2.11, 2.58, and 1.96 U ml^{-1}, respectively (Fig. 1A), while the highest corresponding xylanase activities were 20.26, 26.65, and 29.44 U ml^{-1}, respectively (Fig. 1B).

Both CMCase and xylanase activity decreased rapidly after reaching peak values, possibly because the CMCase and xylanase were degraded by proteolytic enzymes [40]. Previous studies have proven that several *Bacillus* sp. strains can produce CMCase and xylanase and degrade cellulose and hemicellulose [41, 42]. In addition, the maximum CMCase activity of the three strains studied here (1.96-2.58 U ml^{-1}) were all remarkably higher than the typical cellulase-producing *Bacillus* sp. strain (0.81 U ml^{-1}) previously reported [43]. The highest increase in xylanase activity (29.44 U ml^{-1}) detected in this study was significantly more than other *Bacillus* sp. strains (1.8-4.03 U ml^{-1}) and the fungal strain *Aspergillus wentii* (8.1 U ml^{-1}) [44, 45]. The results indicate that corn stover containing rich cellulose and hemicellulose [46] could effectively induce the production of cellulolytic enzymes in bacteria.

Optimal temperature and pH for enzymatic activities

The best hydrolytic performance of enzymes produced by the three strains was determined by the influence of temperature and pH on enzyme activity. The optimal temperature for CMCase activity was 55°C for A0, AS2B, and CH20S1, however, the optimal temperatures for xylanase activity were 60°C, 50°C, and 60°C, respectively (Fig. 2).

The optimal pH of CMCase and xylanase activity was 5.5 in A0, while for both AS2B and CH20S1 the optimal pH was 5.0 (Fig. 3). These optimal temperature and pH conditions were consistent with the CMCase and xylanase produced by some fungi and bacteria, with the optimal values ranging from 40°C to 70°C and pH 4.0 to 6.5, respectively [47-49].

Saccharification of corn stover using bacterial crude enzyme extracts

The crude enzymes extracted from the three strains in the current study exhibited different abilities to release reducing sugars from pretreated and non-pretreated corn stover in the saccharification process (Fig. 4). After 72 h of incubation, the DCE from the A0 strains hydrolyzed AHP and H_2SO_4 pretreated and non-pretreated corn stovers, and produced 63.27, 53.16, and 48.23 mg g^{-1} reducing sugars, respectively. Similarly, strain AS2B released 70.36, 53.89, and 52.27 mg g^{-1} reducing sugars, respectively, and strain CH20S1 produced 71.69, 63.89, and 55.24 mg $g^{-1}$of reducing sugars, respectively (Fig. 4). When

comparing these three strains, CH20S1 demonstrated an advantage of hydrolytic ability on AHP and H_2SO_4 pretreated and non-pretreated corn stovers and released the highest amount of reducing sugars.

The hydrolysis effects on pretreated materials were superior to non-pretreated materials, mainly because of the fiber exposure of the materials after pretreatments [50]. Moreover, the yields of reducing sugars released by DCE were 3.3–7.0 fold higher than that of the buffer solution alone (Fig. 4), demonstrating that the addition of bacterial enzymes can significantly improve the hydrolysis of corn stover. However, according to previous research [51], the contents of reducing sugars released from non-pretreated/pretreated corn stover by commercial cellulase ranged from 100 to 400 mg g^{-1}, which is higher than the results of this study.

Several enzymes, such as endoxylanases, β-xylosidases, and various accessory enzymes, are required in abscission of bonded side groups to the main chain of substituted xylan [52]. Cellulolytic enzymes from bacteria can hydrolyze CMC and xylan into reducing sugars, and the hydrolysis efficiency is limited to the various lignocellulosic biomasses used as substrates [12, 53]. Furthermore, the types and activities of enzymes induced by different biomasses differ greatly [53]. So, we can perhaps conclude that the remarkably different hydrolysis effects between the crude bacterial enzymes and commercial cellulase studied here could be due to the absence of some necessary hydrolytic enzymes for efficient hydrolysis of lignocellulosic biomass in the bacterial crude enzyme extracts.

Comparison of effects of crude enzymes and commercial cellulase on saccharification

To further explore the possibility of the practical application of bacterial enzymes in the industrial production of bioethanol, more enzymatic saccharification experiments were performed by partially replacing commercial cellulase with bacterial enzyme extracts. Strain CH20S1 was selected from the three strains previously studied for its ability to hydrolyze corn stover, and 5.0 ml of DCE was added to each treatment. The reducing sugar yields of each treatment group were markedly different (Fig. 5).

After 72 h of incubation, the contents of reducing sugars released from non-pretreated, AHP pretreated, and H_2SO_4 pretreated corn stovers by using 20 FPU g^{-1} of commercial cellulase were 162.25, 260.08, and 317.60 mg g^{-1}, respectively. The reducing sugar yield from other treatments are depicted in Fig. 5. The maximum amount of reducing sugars obtained from non-pretreated raw materials was almost the same as that of the pretreated corn stovers, which demonstrates these treatments were efficient in the hydrolysis of corn stovers with or without pretreatments. Furthermore, these amounts were significantly higher than the reducing sugar produced by using the crude enzymes from bacteria (Fig. 4) and confirms our previous hypothesis that the crude enzyme extracts lack the necessary hydrolytic enzymes, which might be supplemented by commercial cellulase.

More importantly, for AHP pretreated corn stover, the treatments using commercial cellulase with crude enzymes (260.08–320.65 mg g^{-1}) generally exhibited superior hydrolysis effects compared to the treatment using 20 FPU g^{-1} of commercial cellulase only. In addition, the content of reducing sugar released by 4, 8, and 12 FPU g^{-1} of commercial cellulase added to DCE declined with values of 320.65, 302.57, and 260.08 mg g^{-1}, respectively (Fig. 5B). These results illuminate a synergistic effect in the enzymatic hydrolysis process of AHP pretreated corn stover when bacterial enzyme extracts were used to partially replace the commercial cellulase. Furthermore, the synergistic effect increased with the proportion of crude enzymes used in this study. According to previous research, the non-hydrolytic protein isolated from fresh corn stover substantially increased both the hydrolysis rate of cellulose and the yield of reducing sugar [54]. The addition of non-hydrolytic proteins was considered not only for enzyme deactivation but also to loosen the tightly packed and highly ordered regions of the cellulose resulting in more access for the cellulolytic enzymes to the cellulose [55, 56]. The water-soluble extracts of wheat straw were recently proven to contain non-hydrolytic proteins and a promotional effect on the enzymatic hydrolysis of pretreated wheat straw. The synergistic effect found in this study could therefore possibly have been caused by the non-hydrolytic proteins contained in the crude enzyme extracts produced by strain CH20S1 when corn stover was used as a carbon source. A similar synergistic effect appeared in the enzymatic hydrolysis of non-pretreated corn stover, while the synergistic effect increased with the rise in the proportion of commercial cellulase (Fig. 5A). Specifically, after 72 h of incubation, the yields of reducing sugar from non-pretreated corn stover using 4, 8, and 12 FPU g^{-1} of commercial cellulase with DCE, were 181.73, 183.58, and 315.90 mg g^{-1}, respectively (Fig. 5A). The main reason for this increase could be that the cell wall structure of non-pretreated corn stover was not damaged, thus requiring more hydrolytic enzymes than pretreated corn stover [29]. In addition, the bacterial enzymes showed no synergistic effect when commercial cellulase and H_2SO_4 pretreated corn stover was used as the substrate. The H_2SO_4 pretreatment might generate inhibitors of the non-hydrolytic proteins in the reaction mixture, while partial by-products of acidic pretreatment have been reported to promote deactivation of proteins [57, 58]. Despite all this, our results suggest that partially replacing costly commercial enzymes has the potential to be an economically friendly and efficient saccharification process in biofuel production.

Conclusions

Significant synergies existed between the crude enzyme extracts from cellulolytic enzyme-producing bacterial strain and commercial cellulase in the enzymatic hydrolysis of pretreated and non-pretreated corn stover. Compared to using commercial cellulase only, the mixed enzymes used for saccharification of AHP pretreated and non-pretreated corn stovers increased the reducing sugar content at most by 23.29% and 94.70%, respectively. Moreover, the highest amount of reducing sugars obtained from non-pretreated corn stovers by mixed enzymes (315.90 mg g^{-1}) was almost as much from pretreated corn stovers (317.60-320.65 mg g^{-1}). Therefore, commercial enzymes and pretreatment requirements could be reduced to allow for more cost-effective production of bioethanol.

Declarations

Acknowledgements

This work was supported by a Natural Sciences and Engineering Research Council of Canada Grant [RGPIN-2017-05366] to W. Q. and a Priority Academic Program Development of Jiangsu Higher Education Institutions (PAPD) scholarship to Y. W.

Ethic declaration:

Not applicable

Conflict of interest:

There is no conflict of interest among the authors.

References

1. Huber, G.W., Dale, B.E.: Grassoline at the pump. Sci. Am. **301**, 52–59 (2009)
2. Farrell, A.E., Plevin, R.J., Turner, B.T., Jones, A.D., O'hare, M., Kammen, D.M.: Ethanol can contribute to energy and environmental goals. Science **311**, 506–508 (2006)
3. Limayem, A., Ricke, S.C.: Lignocellulosic biomass for bioethanol production: current perspectives, potential issues and future prospects. Prog. Energy Combust. Sci. **38**, 449–467 (2012)
4. Chundawat, S.P., Beckham, G.T., Himmel, M.E., Dale, B.E.: Deconstruction of lignocellulosic biomass to fuels and chemicals. Annual Review of Chemical and Biomolecular Engineering **2**, 121–145 (2011)
5. Himmel, M.E., Ding, S.-Y., Johnson, D.K., Adney, W.S., Nimlos, M.R., Brady, J.W., Foust, T.D.: Biomass recalcitrance: engineering plants and enzymes for biofuels production. Science **315**, 804–807 (2007)
6. Rubin, E.M.: Genomics of cellulosic biofuels. Nature **454**, 841–845 (2008)
7. da Costa Sousa, L., Chundawat, S.P., Balan, V., Dale, B.E.: 'Cradle-to-grave'assessment of existing lignocellulose pretreatment technologies. Curr. Opin. Biotechnol. **20**, 339–347 (2009)
8. Garlock, R.J., Balan, V., Dale, B.E., Pallapolu, V.R., Lee, Y., Kim, Y., Mosier, N.S., Ladisch, M.R., Holtzapple, M.T., Falls, M.: Comparative material balances around pretreatment technologies for the conversion of switchgrass to soluble sugars. Biores. Technol. **102**, 11063–11071 (2011)
9. Koshy, J., Nambisan, P.: Ethanol production from spent substrate of pleurotus eous. International Journal of Applied Biology and Pharmaceutical Technology **3**, 280–285 (2012)
10. Hernández-Salas, J., Villa-Ramírez, M., Veloz-Rendón, J., Rivera-Hernández, K., González-César, R., Plascencia-Espinosa, M., Trejo-Estrada, S.: Comparative hydrolysis and fermentation of sugarcane

and agave bagasse. Biores. Technol. **100**, 1238–1245 (2009)

11. Sendelius, J.: Steam pretreatment optimisation for sugarcane bagasse in bioethanol production. Master of Science Thesis Department of Chemical Engineering, Lund University, Sweden 2005
12. Sun, Y., Cheng, J.: Hydrolysis of lignocellulosic materials for ethanol production: a review. Biores. Technol. **83**, 1–11 (2002)
13. Chen, F., Dixon, R.A.: Lignin modification improves fermentable sugar yields for biofuel production. Nat. Biotechnol. **25**, 759–762 (2007)
14. Ferraz, J.LdA.A., Souza, L.O., Soares, G.A., Coutinho, J.P., de Oliveira, J.R., Aguiar-Oliveira, E., Franco, M.: Enzymatic saccharification of lignocellulosic residues using cellulolytic enzyme extract produced by *Penicillium roqueforti* ATCC 10110 cultivated on residue of yellow mombin fruit. Bioresource Technology 2017
15. Sadaf, A., Khare, S.: Production of *Sporotrichum thermophile* xylanase by solid state fermentation utilizing deoiled *Jatropha curcas* seed cake and its application in xylooligosachharide synthesis. Biores. Technol. **153**, 126–130 (2014)
16. Chapla, D., Divecha, J., Madamwar, D., Shah, A.: Utilization of agro-industrial waste for xylanase production by *Aspergillus foetidus* MTCC 4898 under solid state fermentation and its application in saccharification. Biochem. Eng. J. **49**, 361–369 (2010)
17. Sassner, P., Galbe, M., Zacchi, G.: Techno-economic evaluation of bioethanol production from three different lignocellulosic materials. Biomass Bioenerg. **32**, 422–430 (2008)
18. Wingren, A., Galbe, M., Roslander, C., Rudolf, A., Zacchi, G.: Effect of reduction in yeast and enzyme concentrations in a simultaneous-saccharification-and-fermentation-based bioethanol process. Appl. Biochem. Biotechnol. **122**, 485–499 (2005)
19. Sassner, P., Zacchi, G.: Integration options for high energy efficiency and improved economics in a wood-to-ethanol process. Biotechnol. Biofuels **1**, 4 (2008)
20. Galbe, M., Sassner, P., Wingren, A., Zacchi, G.: Process engineering economics of bioethanol production. Biofuels 2007:303–327
21. Zhang, Z., Liu, J.-L., Lan, J.-Y., Duan, C.-J., Ma, Q.-S., Feng, J.-X.: Predominance of *Trichoderma* and *Penicillium* in cellulolytic aerobic filamentous fungi from subtropical and tropical forests in China, and their use in finding highly efficient β-glucosidase. Biotechnol. Biofuels **7**, 107 (2014)
22. Sangrila, S., Maiti, T.: Cellulase production by bacteria: a review. British Microbiology Research Journal **3**, 235–258 (2013)
23. Otajevwo, F., Aluyi, H.: Cultural conditions necessary for optimal cellulase yield by cellulolytic bacterial organisms as they relate to residual sugars released in broth medium. Modern Applied Science **5**, 141–151 (2011)
24. Daniel, G., Nilsson, T.: Developments in the study of soft rot and bacterial decay. Forest products biotechnology 1998:37–62

25. Maki, M.L., Idrees, A., Leung, K.T., Qin, W.: Newly isolated and characterized bacteria with great application potential for decomposition of lignocellulosic biomass. J. Mol. Microbiol. Biotechnol. **22**, 156–166 (2012)
26. Paudel, Y.P., Qin, W.: Characterization of novel cellulase-producing bacteria isolated from rotting wood samples. Appl. Biochem. Biotechnol. **177**, 1186–1198 (2015)
27. Guo, H., Chen, H., Fan, L., Linklater, A., Zheng, B., Jiang, D., Qin, W.: Enzymes produced by biomass-degrading bacteria can efficiently hydrolyze algal cell walls and facilitate lipid extraction. Renewable Energy **109**, 195–201 (2017)
28. Uppugundla, N., da Costa Sousa, L., Chundawat, S.P., Yu, X., Simmons, B., Singh, S., Gao, X., Kumar, R., Wyman, C.E., Dale, B.E.: A comparative study of ethanol production using dilute acid, ionic liquid and AFEX™ pretreated corn stover. Biotechnol. Biofuels **7**, 72 (2014)
29. Banerjee, G., Car, S., Scott-Craig, J.S., Hodge, D.B., Walton, J.D.: Alkaline peroxide pretreatment of corn stover: effects of biomass, peroxide, and enzyme loading and composition on yields of glucose and xylose. Biotechnol. Biofuels **4**, 16 (2011)
30. Xu, N., Zhang, W., Ren, S., Liu, F., Zhao, C., Liao, H., Xu, Z., Huang, J., Li, Q., Tu, Y.: Hemicelluloses negatively affect lignocellulose crystallinity for high biomass digestibility under NaOH and H_2SO_4 pretreatments in *Miscanthus*. Biotechnol. Biofuels **5**, 58 (2012)
31. Shrestha, P., Ibáñez, A.B., Bauer, S., Glassman, S.I., Szaro, T.M., Bruns, T.D., Taylor, J.W.: Fungi isolated from *Miscanthus* and sugarcane: biomass conversion, fungal enzymes, and hydrolysis of plant cell wall polymers. Biotechnol. Biofuels **8**, 38 (2015)
32. Leyva, A., Quintana, A., Sánchez, M., Rodríguez, E.N., Cremata, J., Sánchez, J.C.: Rapid and sensitive anthrone-sulfuric acid assay in microplate format to quantify carbohydrate in biopharmaceutical products: Method development and validation. Biologicals **36**, 134–141 (2008)
33. Tomoda, M.: Colorimetric determination of pentoses. IV. determination with orcinol reagent. Chem. Pharm. Bull. **11**, 809–812 (1963)
34. Ibáñez, A.B., Bauer, S.: Downscaled method using glass microfiber filters for the determination of *Klason* lignin and structural carbohydrates. Biomass Bioenerg. **68**, 75–81 (2014)
35. Miller, G.L.: Use of dinitrosalicylic acid reagent for determination of reducing sugar. Anal. Chem. **31**, 426–428 (1959)
36. Singh, R., Varma, A., Laxman, R.S., Rao, M.: Hydrolysis of cellulose derived from steam exploded bagasse by *Penicillium* cellulases: comparison with commercial cellulase. Biores. Technol. **100**, 6679–6681 (2009)
37. Liu, C., van der Heide, E., Wang, H., Li, B., Yu, G., Mu, X.: Alkaline twin-screw extrusion pretreatment for fermentable sugar production. Biotechnol. Biofuels **6**, 1–11 (2013)
38. Mosier, N., Wyman, C., Dale, B., Elander, R., Lee, Y., Holtzapple, M., Ladisch, M.: Features of promising technologies for pretreatment of lignocellulosic biomass. Biores. Technol. **96**, 673–686 (2005)

39. Zhang, W., Yi, Z., Huang, J., Li, F., Hao, B., Li, M., Hong, S., Lv, Y., Sun, W., Ragauskas, A.: Three lignocellulose features that distinctively affect biomass enzymatic digestibility under NaOH and H_2SO_4 pretreatments in *Miscanthus*. Biores. Technol. **130**, 30–37 (2013)
40. Lin, C., Shen, Z., Qin, W.: Characterization of xylanase and cellulase produced by a newly isolated *Aspergillus fumigatus* N2 and its efficient saccharification of barley straw. Appl. Biochem. Biotechnol. **182**, 559–569 (2017)
41. Nagar, S., Mittal, A., Kumar, D., Gupta, V.K.: Production of alkali tolerant cellulase free xylanase in high levels by *Bacillus pumilus* SV-205. Int. J. Biol. Macromol. **50**, 414–420 (2012)
42. Shrinivas, D., Savitha, G., Raviranjan, K., Naik, G.R.: A highly thermostable alkaline cellulase-free xylanase from thermoalkalophilic *Bacillus* sp. JB 99 suitable for paper and pulp industry: purification and characterization. Appl. Biochem. Biotechnol. **162**, 2049–2057 (2010)
43. Horikoshi, K., Nakao, M., Kurono, Y., Sashihara, N.: Cellulases of an alkalophilic *Bacillus* strain isolated from soil. Can. J. Microbiol. **30**, 774–779 (1984)
44. Subramaniyan, S., Prema, P.: Cellulase-free xylanases from Bacillus and other microorganisms. FEMS Microbiol. Lett. **183**, 1–7 (2000)
45. de Castro, A.M., de Andréa, T.V., Carvalho, D.F., Teixeira, M.M.P., dos Reis Castilho, L., Freire, D.M.G.: Valorization of residual agroindustrial cakes by fungal production of multienzyme complexes and their use in cold hydrolysis of raw starch. Waste Biomass Valoriz. **2**, 291–302 (2011)
46. Öhgren, K., Bura, R., Saddler, J., Zacchi, G.: Effect of hemicellulose and lignin removal on enzymatic hydrolysis of steam pretreated corn stover. Biores. Technol. **98**, 2503–2510 (2007)
47. Schinner, F., Von Mersi, W., Xylanase-: CM-cellulase-and invertase activity in soil: an improved method. Soil Biol. Biochem. **22**, 511–515 (1990)
48. Qinnghe, C., Xiaoyu, Y., Tiangui, N., Cheng, J., Qiugang, M.: The screening of culture condition and properties of xylanase by white-rot fungus *Pleurotus ostreatus*. Process Biochem. **39**, 1561–1566 (2004)
49. Aa, K., Flengsrud, R., Lindahl, V., Tronsmo, A.: Characterization of production and enzyme properties of an endo-β-1, 4-glucanase from *Bacillus subtilis* CK-2 isolated from compost soil. Antonie Van Leeuwenhoek **66**, 319–326 (1994)
50. Karagöz, P., Rocha, I.V., Özkan, M., Angelidaki, I.: Alkaline peroxide pretreatment of rapeseed straw for enhancing bioethanol production by same vessel saccharification and co-fermentation. Biores. Technol. **104**, 349–357 (2012)
51. Li, C., Cheng, G., Balan, V., Kent, M.S., Ong, M., Chundawat, S.P., daCosta Sousa, L., Melnichenko, Y.B., Dale, B.E., Simmons, B.A.: Influence of physico-chemical changes on enzymatic digestibility of ionic liquid and AFEX pretreated corn stover. Biores. Technol. **102**, 6928–6936 (2011)
52. Kocabaş, D.S., Güder, S., Özben, N.: Purification strategies and properties of a low-molecular weight xylanase and its application in agricultural waste biomass hydrolysis. Journal of Molecular Catalysis B: Enzymatic **115**, 66–75 (2015)

53. Maki, M., Leung, K.T., Qin, W.: The prospects of cellulase-producing bacteria for the bioconversion of lignocellulosic biomass. International Journal of Biological Sciences **5**, 500 (2009)
54. Han, Y., Chen, H.: Synergism between corn stover protein and cellulase. Enzyme and Microbial Technology **41**, 638–645 (2007)
55. Arantes, V., Saddler, J.N.: Access to cellulose limits the efficiency of enzymatic hydrolysis: the role of amorphogenesis. Biotechnol. Biofuels **3**, 4 (2010)
56. Coughlan, M.P.: The properties of fungal and bacterial cellulases with comment on their production and application. Biotechnol. Genet. Eng. Rev. **3**, 39–110 (1985)
57. Cabiscol Català, E., Tamarit Sumalla, J., Ros Salvador, J.: Oxidative stress in bacteria and protein damage by reactive oxygen species. Int. Microbiol. **3**, 3–8 (2000)
58. Jönsson, L.J., Martín, C.: Pretreatment of lignocellulose: formation of inhibitory by-products and strategies for minimizing their effects. Biores. Technol. **199**, 103–112 (2016)

Figures

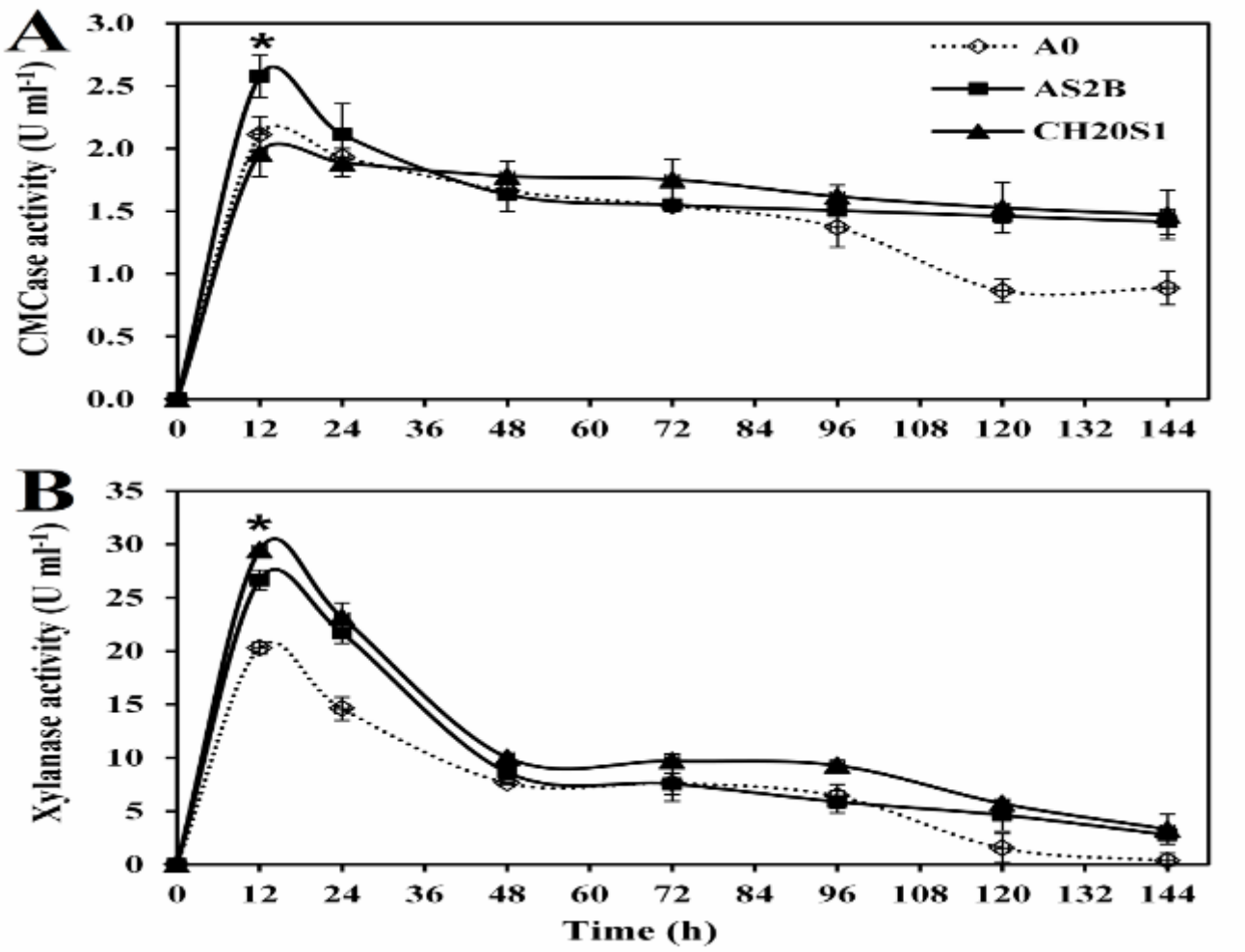

Figure 1

CMCase (A) and xylanase (B) activity in strains A0, AS2B, and CH20S1 using corn stover as the substrate in submerged fermentation with shaking for 144 h at 37°C. Bars indicate the standard deviation (n = 3). The symbol * indicates a significant difference at P < 0.05.

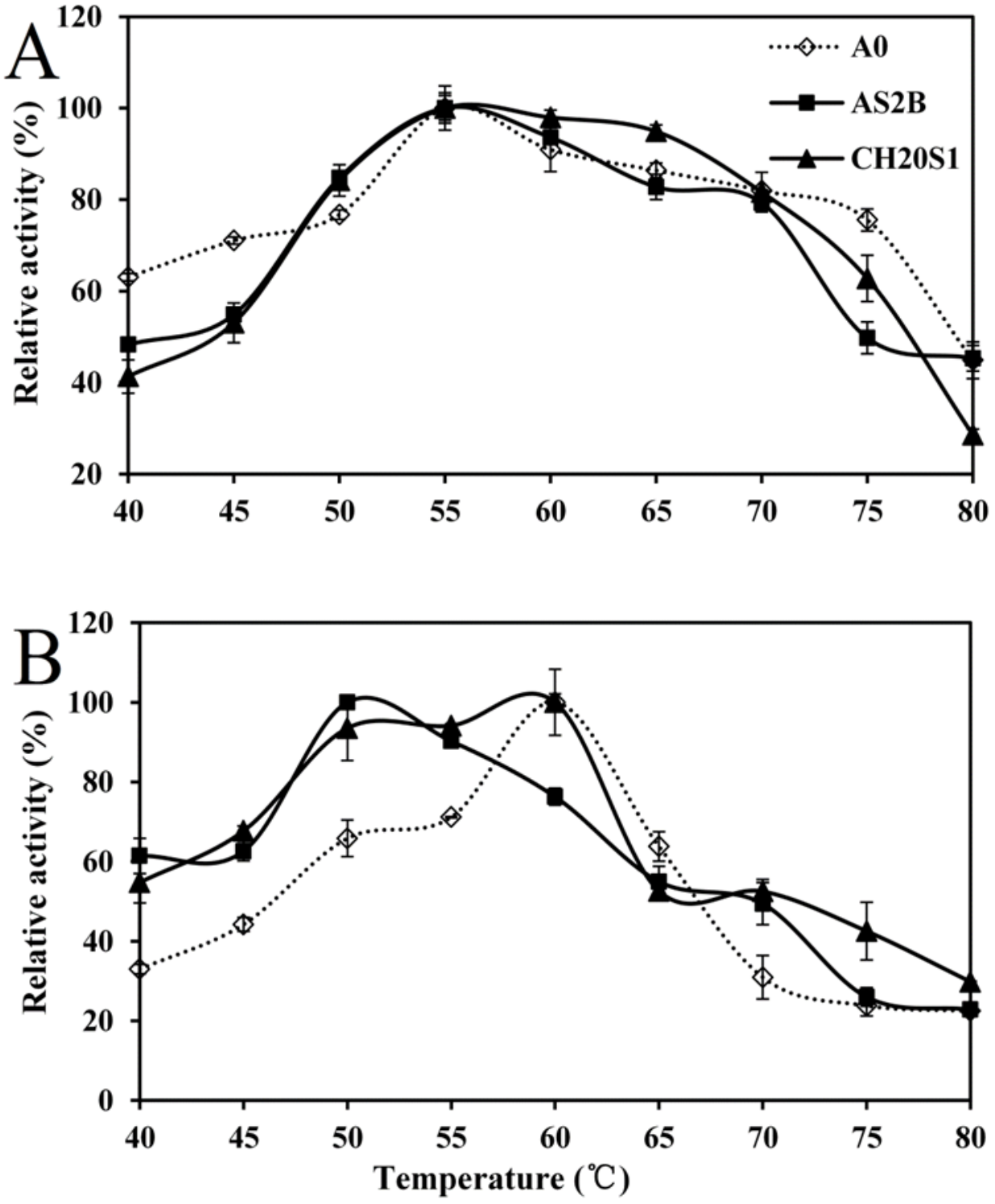

Figure 2

Effects of different temperatures on CMCase (A) and xylanase (B) activity. The temperature for enzymatic activities ranges from 40°C to 80°C, and bars indicate the standard deviation (n = 3).

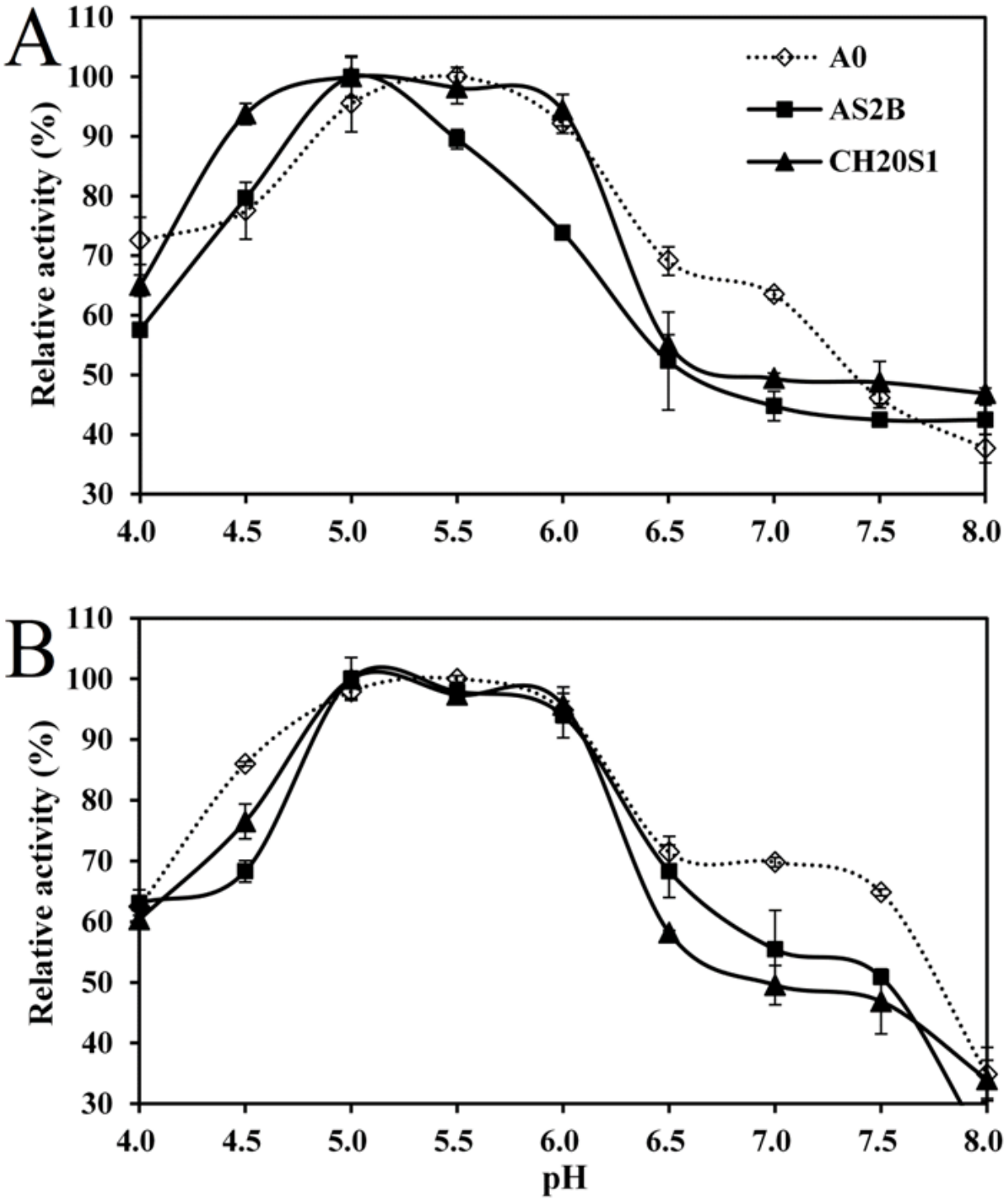

Figure 3

Effects of pH on CMCase (A) and xylanase (B) activity. The pH for enzymatic activities ranges from 4 to 8, and bars indicate the standard deviation (n = 3).

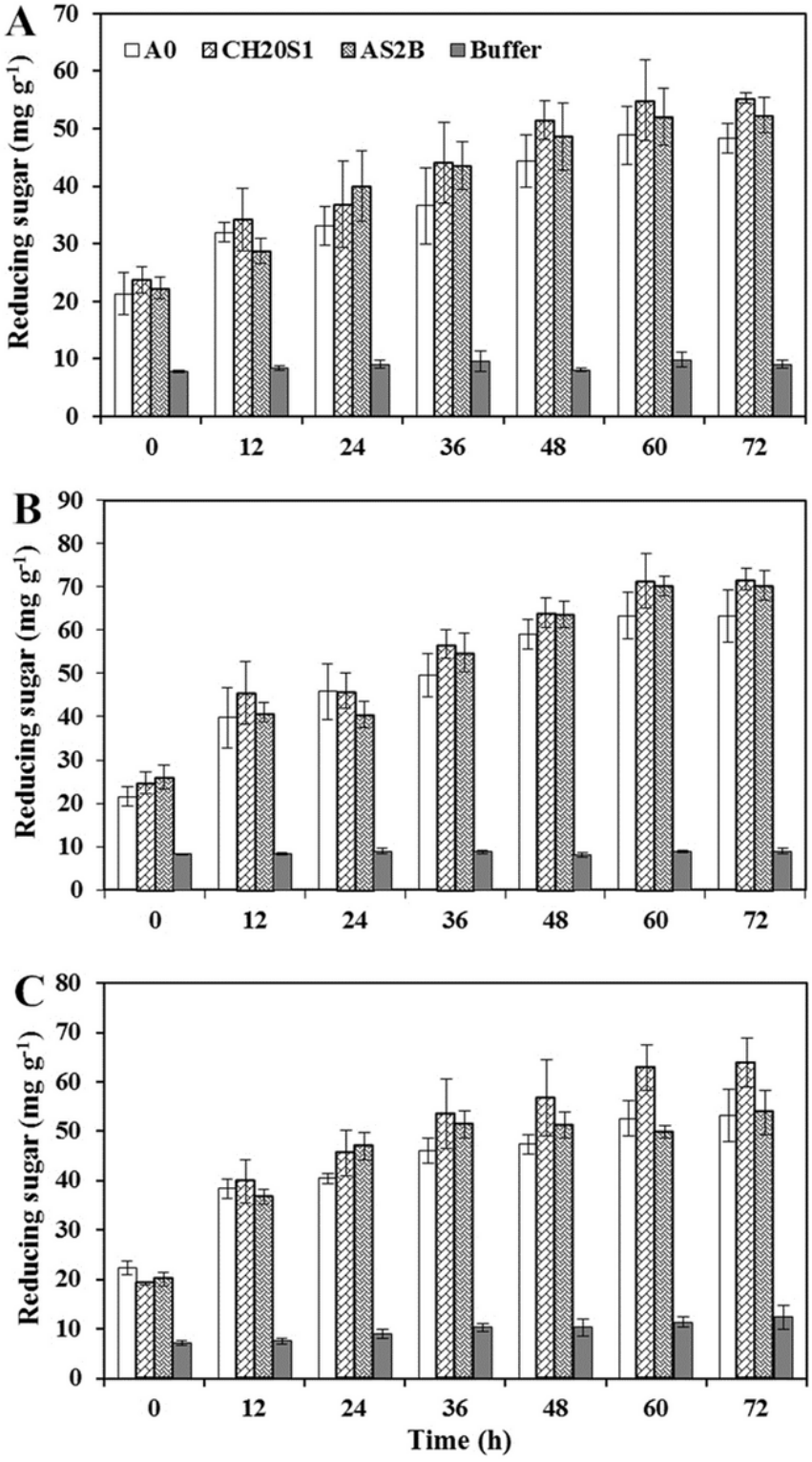

Figure 4

Saccharification of non-pretreated (A), AHP pretreated (B), and H2SO4 pretreated (C) corn stover using crude enzymes produced by strains A0, AS2B, and CH20S1. Saccharification was performed at 55°C, pH 5.0, with 200 rpm for 72 h, using a substrate concentration of 1%. Bars indicate the standard deviation (n = 3).

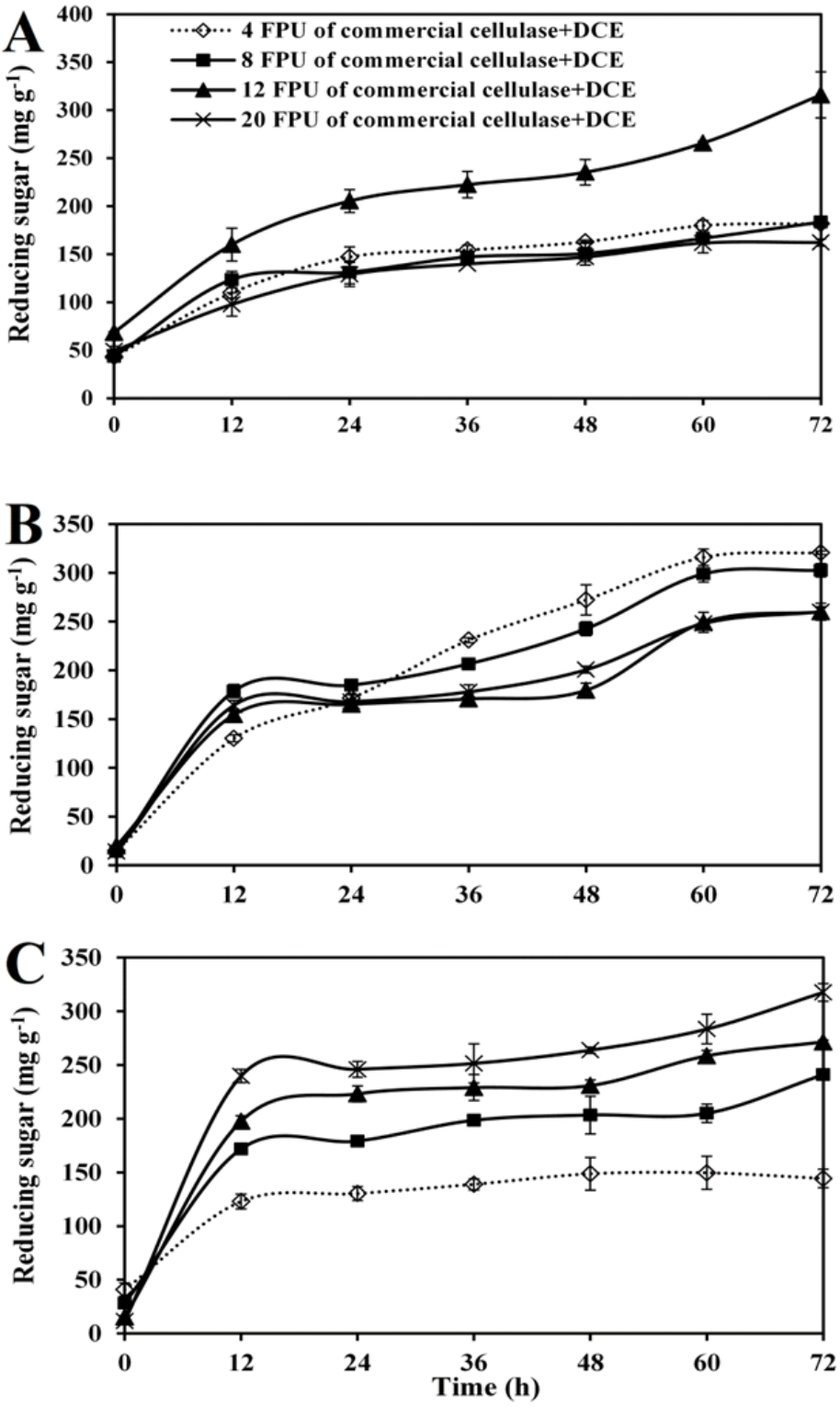

Figure 5

Saccharification of non-pretreated (A), AHP pretreated (B), and H2SO4 pretreated (C) corn stover using bacterial enzyme extracts with different additions of commercial cellulases. Saccharification was performed at 55°C, pH 5.0, with 200 rpm for 72 h, using a substrate concentration of 1%. Bars indicate the standard deviation (n = 3).

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- GraphicalAbstarct.png