Enhancement of Saccharification of Corn Stover by Cellulolytic Enzyme Produced from Biomass-degrading Bacteria

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Enzymatic saccharification of corn stover can be enhanced by partially replacing commercial enzymes with bacterial crude enzyme extracts. Thus, in this study, three bacteria (*Bacillus* sp. A0, *Bacillus* sp. CH20S1, and *Exiguobacterium* sp. AS2B) were cultured in a media with corn stover as the substrate to produce crude enzyme extract and saccharify corn stover. 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 ranging from 48.2 to 71.7 mg g^{-1} were released from non-pretreated and pretreated corn stover. Furthermore, the maximum reducing sugars of 316 and 321 mg g^{-1} were observed when 12 and 4 FPU g^{-1} of commercial cellulase were added to the DCE of the CH20S1 strain, respectively. It was shown that an effective combination of bacterial DCE with commercial enzymes could achieve higher saccharification of lignocellulosic biomass, which might be cost-efficient compared to their single-use. Overall, this study aims to show the enhanced enzymatic saccharification of corn stover.

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INTRODUCTION

Lignocellulosic biomass is used as an inexpensive green renewable resource for producing different value-added products. However, the different components present in lignocellulosic biomasses contribute to the recalcitrant properties of biomasses and are resistant to enzymatic and chemical degradation (Isikgor and Becer 2015; Sun *et al.* 2016; Shrestha *et al.* 2020). Thus, pretreatment is preferred as an essential step in breaking down cell walls and causing the efficient release of fermentable sugars from various lignocellulosic biomass that further can be used for producing other valuable products such as bioethanol (Rajendran *et al.* 2018). Numerous pretreatment technologies such as alkaline, ammonia, dilute acid, and steam explosion pretreatment have been developed to solve the biomass recalcitrance problem for efficient enzymatic saccharification. However, harsh pretreatment can lead to the degradation of sugars, while too weak pretreatment results in low enzyme accessibility (Chaturvedi and Verma 2013; Rajendran *et al.* 2018). Although the biomass composition plays a crucial role in the outcome of pretreatment, the

enzymatic hydrolysis after different physical or chemical pretreatment may be beneficial economically (Hendriks and Zeeman 2009; Chapla *et al.* 2010). In addition, the pretreatment facilitates conversion efficiency by destroying or breaking down the initial structure of the biomass (Sun *et al.* 2016; Zhang *et al.* 2021a). Thus, in this study, corn stover was used as biomass and pretreatment *via* alkaline hydrogen peroxide (AHP) and dilute acid was evaluated, followed by enzymatic saccharification.

Corn stover was used in this study as it is one of the most widely used renewable energy sources in bioconversion. AHP and dilute acid hydrolysis have been widely used to enhance the conversion of corn stover because the component fractions of corn stover can be efficiently hydrolyzed into monomeric components by using those two methods. Therefore, the conversion and enzyme accessibility can be highly improved (Akhlisah *et al.* 2021). However, due to problems such as high equipment requirements, different degrees of corrosion, and environmental pollution, there is still room for improvement in these two methods.

Enzymatic hydrolysis is the best method for subsequent fermentation because of its high specificity, high yield, and lower formation of undesirable products (Ostadjoo *et al.* 2019; Shrestha *et al.* 2020). Cellulases and xylanases are the important enzymes for the hydrolysis of cellulose and hemicellulose contained in the various biomasses (Prajapati *et al.* 2018; Shrestha *et al.* 2020). Hence, the improvement of cellulolytic microorganisms and enhancement of the hydrolytic capacity of cellulases are essential. This study aimed to study the saccharification of corn stover by bacterial cellulolytic enzymes for cost reduction because the majority of studies have focused on fungal cellulolytic enzymes (Zhang *et al.* 2014; Ferraz *et al.* 2018). Bacteria are considered more appropriate to produce the hydrolytic enzymes for saccharification on an industrial scale due to the shorter generation time, ability to be artificially cultivated (Sadhu and Maiti 2013), and excellent tolerance to environmental stresses (Vilanova *et al.* 2012; Thatoi *et al.* 2014).

The present research concentrated on three bacterial strains (A0, AS2B, and CH20S1) 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 studied strains was evaluated from saccharification assays measuring the reducing sugars released from pretreated and non-pretreated corn stover. Finally, the crude enzyme extract from the best saccharifying strain was selected and combined with commercial enzymes in different concentrations to study the saccharification efficiencies.

EXPERIMENTAL

Biomass Material and Bacterial Strains

The bacteria *Bacillus* sp. A0 (Accession no. KP974676), *Bacillus* sp. CH20S1 (Accession no. HQ331531), and *Exiguobacterium* sp. AS2B (Accession no. HM134063) used in this study were isolated from rotting wood samples, municipal waste, and peat. They were stored at -80 °C in the authors' lab. Their enzymatic characteristics have already been reported (Maki *et al.* 2012; Paudel *et al.* 2015; Guo *et al.* 2017). All the strains stored at -80 °C were activated by culturing in 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 was dried at 50 °C until a constant weight was attained, cut into small (2 to 5 cm) pieces,

and then ground through a 50-mesh sieve. The corn stover was provided by Agriculture and Agri-Food Canada (Ottawa, Canada).

Pretreatment

Prior to the enzymatic hydrolysis of corn stover, it was subjected for pretreatments (AHP pretreatment and H_2SO_4 pretreatment) to enhance the enzymatic hydrolysis efficiency by changing the structure of corn stover.

AHP pretreatment

This pretreatment method followed the method reported by Banerjee *et al.* (2011) with slight modification. In brief, 30% (w/v) H_2O_2 (pH to 11.5 adjusted with 2.0 M NaOH) was mixed with 5% (w/v) of milled corn stover samples and was shaken at 150 rpm at 50 °C for 1 h. After 1 h, the mixture was centrifuged to obtain the solid and liquid fractions. The solid fraction was 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*. (2012) with some minor modifications. In brief, 0.5 g of biomass samples were mixed with 10 mL H_2SO_4 (1%, v/v) in 15 mL plastic centrifuge tubes, 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 neutral pH of the washing fluid and finally dried at 80 °C overnight for plant cell wall composition analysis.

Biomass Composition Analysis

The biomass composition analysis was performed as mentioned in Wu *et al.* (2021), in which cellulose and hemicellulose were analyzed by measuring the glucan and xylan contents. The Klason lignin analysis was performed by anthrone-sulfuric acid and orcinol-hydrochloric acid methods.

Obtaining Crude Enzymes and Enzyme Assay

Each strain was inoculated (2%, v/v) in 50 mL of mineral salt medium (consisting of 1.0 g L^{-1} $NaNO_3$, 1.0 g L^{-1} K_2HPO_4, 1.0 g L^{-1} KCl, 3.0 g L^{-1} peptone, 0.5 g L^{-1} $MgSO_4$, and 0.5 g L^{-1} yeast extract) containing 0.5% (w/v) non-pretreated corn stover. The media contained only 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. Then the supernatants as crude enzymes were processed for sterile filtration using a 0.22-μm Millipore filter (Billerica, MA, USA). In addition, the supernatants were used for the analysis of xylanase and CMCase activity. The supernatant solutions with maximum CMCase and xylanase were later used for the hydrolyzing experiments. The CMCase and xylanase activities were determined by DNS methods (Miller 1959), where the reducing sugars released from CMC and xylan were calculated as described by Guo *et al*. (2017).

Influences of Temperature and pH on Enzyme Activities

The effects of temperature (40 to 80 °C with 5 °C intervals) and the influence of pH (4 to 8 with 0.5 intervals) on enzyme activities were studied. The buffer solutions, 0.05 M citrate buffer for pH 4 to 7 and 0.05 M Tris-HCl buffer for pH 7.8 to 8.0, were used in

maintaining different pH for enzyme activities. The enzyme activities were calculated as relative activity. The relative activity is the ratio of activity at a certain time by activity at an initial time multiplied by 100, which is as,

$$\text{Relative activity (\%)} = \frac{A_t}{A_o} \times 100\% \quad (1)$$

where A_t is the enzyme activity at time equal to t and A_o is the initial enzyme activity.

Enzymatic Saccharification

Enzymatic saccharification of corn stover using different strains

For enzymatic saccharification of corn stover, crude enzyme extracts obtained from three bacterial strains A0, CH20S1, and AS2B were used. For each pretreated corn stover, 3.5 mL of crude enzyme extract was added after adjusting pH of 5 with 0.05 M sodium citrate buffer (pH 5). 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. Also, 5.0 mL of diluted crude enzymes (DCE) solution without any treatment was set as the control.

Combined effect of commercial cellulase and crude enzyme for saccharification of corn stover

A commercial cellulase (Celluclast 1.5 L, Novozymes, Franklinton, NC, USA) from *Trichoderma reesei* ATCC 26921 was used in this study. The crude enzyme produced from strain CH20S1 was combined with commercial cellulase in three sets: 5.0 mL of DCE were added to 4, 8, and 12 FPU g^{-1} dry biomass of commercial cellulase to study enzymatic saccharification of corn stover. Also, the control group contained 20 FPU g^{-1} of commercial cellulase without the crude enzyme extract, with reference to the previous report by Singh *et al.* (2009).

Statistical Analysis

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

RESULTS AND DISCUSSION

Characterization of Corn Stover Before and After Pretreatments

The effects of pretreatments including AHP and H_2SO_4 on the corn stover and the differences in the content's compositions (glucan, xylan, and lignin) of the pretreated corn stover are shown in Table 1. The result illustrated that both pretreatments were able to change the cell wall composition of corn stover. It is known that different pretreatment methods improve the hydrolysis of biomass and change the chemical and physical structures of biomass resulting in enzymes accessibility to corn stover and yielding of fermentable sugars (Sun *et al.* 2016; Avci *et al.* 2019).

Table 1. Composition of Corn Stover Expressed as Percentage of Dry Matter

Pretreatment	Component (%)		
	Glucan	Xylan	Acid insoluble lignin
Non-pretreated	27.4 ± 0.5[c]	18.9 ± 1.1[a]	21.1 ± 1.5[b]
AHP	41.0 ± 4.9[b]	20.7 ± 0.3[a]	15.5 ± 0.9[c]
H_2SO_4	53.7 ± 3.3[a]	16.7 ± 0.8[b]	27.9 ± 0.6[a]

Different letters indicate significant differences ($P < 0.05$) among different pretreatments

The corn stover used as a raw material revealed acid-insoluble lignin 21.1% and glucan and xylan of 27.4% and 18.9% respectively. Besides, there are other carbohydrates and components present in corn stover that are not described in Table 1. This result was found to be consistent with that of the corn stover analyzed in another study (Liu *et al.* 2013). Also, the contents of corn stover were observed to be almost in the range mentioned; cellulose 30 to 60%, hemicellulose 20 to 40%, and lignin 15 to 25% (Dahadha *et al.* 2017; Avci *et al.* 2019). After the AHP treatment, the content of lignin was 15.5%, which showed a significant reduction compared to the raw material ($P < 0.05$), while the glucan content increased significantly by 49.6% with a minor increase of xylan. However, 1% H_2SO_4 pretreatment resulted in a significant increase of glucan content (53.7%, $P < 0.05$) compared to that of the raw material, and the xylan content significantly decreased to 16.7% ($P < 0.05$) (Table 1). The alkali assisted acidic pretreatment in another study has determined 59.5%, 5.2%, and 8.3% of cellulose, lignin, and hemicellulose, respectively (Goyal *et al.* 2014). The alkali pretreatment removes most of the lignin by dissolving lignin as a result of breaking ether linkages, hydrogen bonds, and other covalent bonds, leaving behind a highly porous cell wall for better penetration of enzymes (Chaturvedi and Verma 2013; Goyal *et al.* 2014; Sun *et al.* 2016). In contrast, acid (H_2SO_4) pretreatment mainly induces the hydrolysis of cellulose and hemicellulose, produces monomeric sugars (Chaturvedi and Verma 2013), and hence the monomeric sugars are removed from the biomasses after pretreatment. Thus, the non-pretreated corn stover had higher xylan content compared to acid-pretreated corn stover. Furthermore, the concentrations of chemicals used and time of treatment also play a major role in determining the biomass contents (Mensah *et al.* 2021).

Production of CMC and Xylan Hydrolysing Enzymes

When three bacterial strains (A0, CH20S1, and AS2B) were cultured in the media containing corn stover as only carbon source, they exhibited an excellent CMCase and xylanase. Also, the enzyme activities by these strains were produced in a similar variation tendency (Fig. 1). The majority of CMCase and xylanase were secreted into the medium in 12 h of incubation period for all tested strains. The highest CMCase activities for A0, CH20S1, and AS2B were 2.1, 1.9, and 2.6 U mL^{-1}, respectively (Fig. 1a). Similarly, the maximum corresponding xylanase activities were 20.3, 29.4, and 26.7 U mL^{-1}, respectively (Fig. 1b). In another study, different biomasses including algae, wheat bran, agave, corn stover, wood dust, CMC, and pine chip were used for enzymes production. Moreover, the study revealed *Bacillus* sp. produced maximum CMCase (17.8 U g^{-1}) and xylanase (195.8 U g^{-1}) from corn stover (0.5% w/v) among the various biomasses and they used corn stover for further study (Wu *et al.* 2021).

Both CMCase and xylanase activity increased continuously until 12 h of incubation because the strains in this study had suitable cultural conditions and nutrients for their enzymatic activities. However, activities decreased rapidly after reaching peak values at

12 h, possibly because the substrate may have been used up, or maybe there was change in pH making unfavorable conditions, or some inhibiting products may have been produced in the media. The maximum CMCase activity (1.9 to 2.6 U mL^{-1}) and xylanase activity (29.4 U mL^{-1}) of these three strains studied here were all remarkably higher than the previously reported typical cellulase-producing *Bacillus* sp. strain (Kim *et al.* 2012). The maximum xylanase (7.9 U mL^{-1}) was produced from *Bacillus pumilus* under optimized condition. Xylanase in that manner has the potential to be useful in paper and pulp industries (Lawrence *et al.* 2015). The results indicate that corn stover containing rich cellulose and hemicellulose could effectively induce the production of cellulolytic and hemicellulolytic enzymes in bacteria. In addition, the combined effect of xylanase and cellulase are more effective in the degradation of lignocellulosic biomass (Prajapati *et al.* 2018).

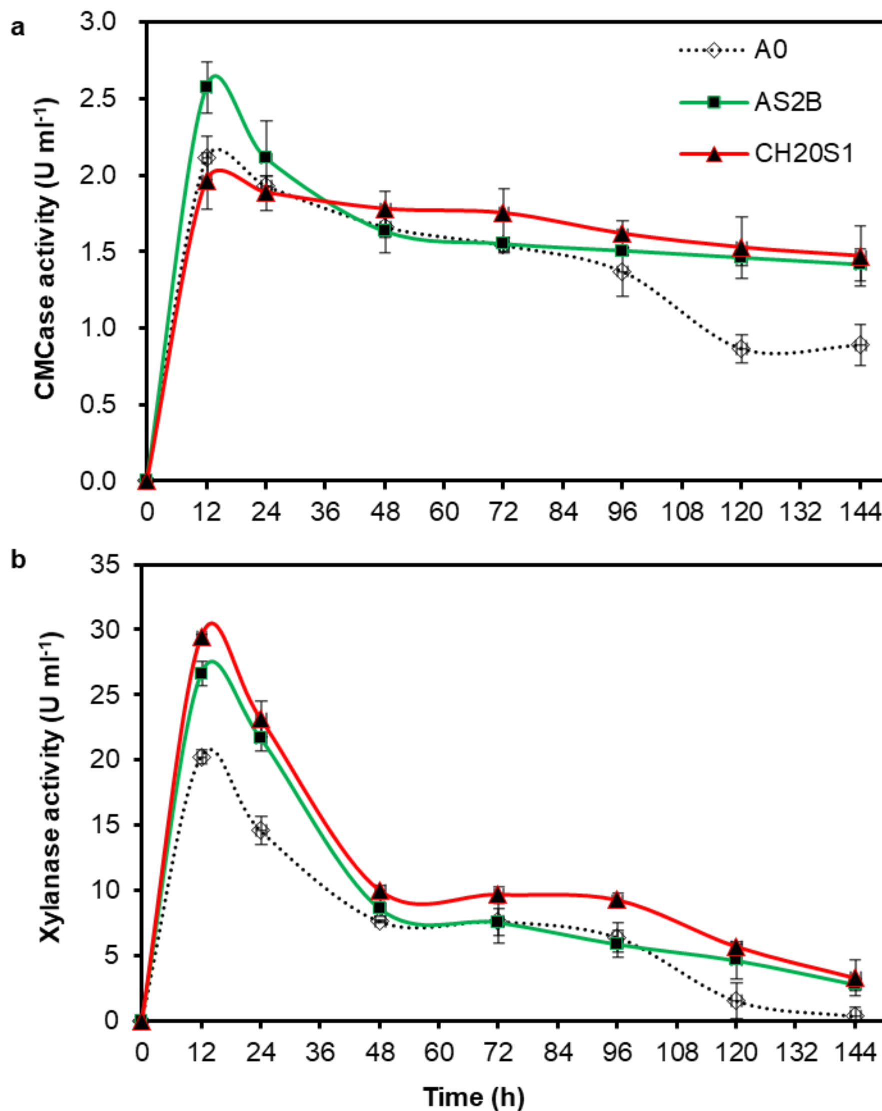

Fig. 1. CMCase (a) and xylanase (b) activity in strains A0, AS2B, and CH20S1 using non pretreated corn stover as the substrate in submerged fermentation with shaking for 144 h at 37 °C. Bars indicate the standard deviation (n = 3). The symbol * indicates a significant difference at $P < 0.05$.

Optimal Temperature and pH for Enzymatic Activities

The best hydrolytic performance of enzymes is affected by the optimal temperature and pH. Thus, the enzymes produced by these three strains were employed in further tests to determine the influence of temperature and pH on enzyme activity. The optimal temperature for maximum CMCase activities was 55 °C exhibited by AS2B, A0, and CH20S1 strains. However, the optimal temperatures for xylanase activity were 50, 60, and 60 °C for the mentioned strains, respectively (Fig. 2).

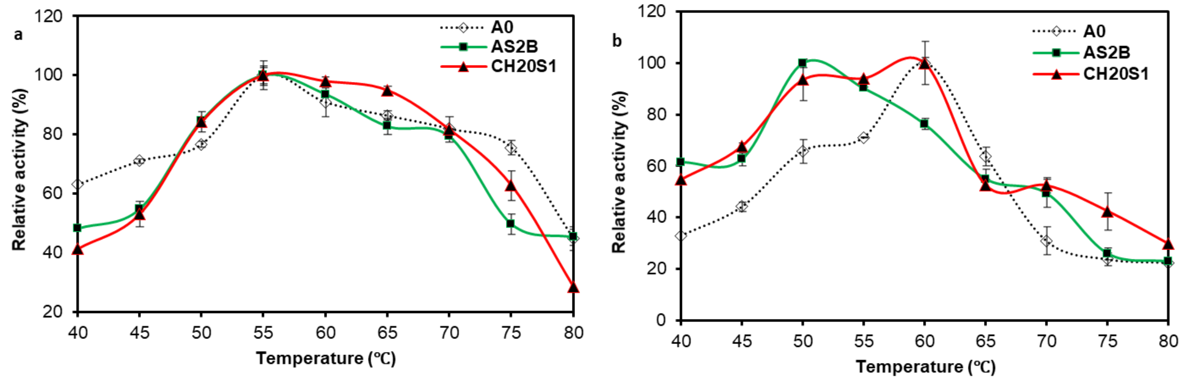

Fig. 2. Effects of different temperatures on CMCase (a) and xylanase (b) activity. The temperature for enzymatic activities ranges from 40 °C to 80 °C, and bars indicate the standard deviation (n = 3).

The CMCase and xylanase activities were observed to be maximum at pH 5.5 for A0 strain, pH 5.0 for AS2B, while pH 5 to pH 6 was optimal pH for CH20S1 strain (Fig. 3). The pH and temperature play an important role, affecting the metabolic rate and growth of bacteria. The optimal pH and temperature differ with different fermentation conditions and strains. Lawrence *et al.* (2015) demonstrated pH 7 and 50 °C as the optimum pH and temperature for *Bacillus pumilus* to produce xylanase. Similarly, another study revealed pH 7 and 37 °C were the optimum pH and temperature for *Bacillus pumilus* (Kaur *et al.* 2017).

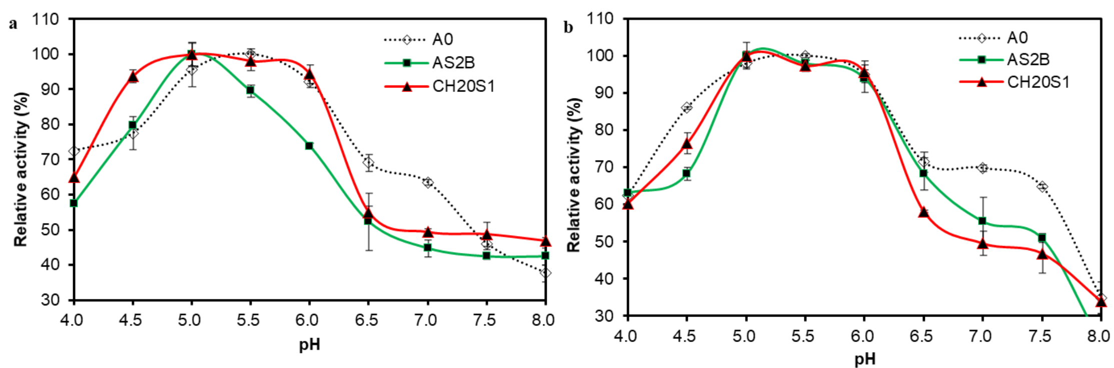

Fig. 3. Effects of pH on CMCase (a) and xylanase (b) activity. The pH for enzymatic activities ranges from 4 to 8, and bars indicate the standard deviation (n = 3).

Bacterial Crude Enzyme Extracts and Saccharification of Corn Stover

The crude enzymes extracted from the three strains exhibited different yields of reducing sugars from pretreated and non-pretreated corn stover (Fig. 4). After 72 h of incubation, the DCE from the A0 strain hydrolyzed AHP and H_2SO_4 pretreated and non-pretreated corn stover, and produced 63.3, 53.2, and 48.2 mg g^{-1} reducing sugars, respectively. Similarly, strain AS2B released 70.4, 53.9, and 52.3 mg g^{-1} reducing sugars, respectively, and another strain CH20S1 produced reducing sugars of 71.7, 63.9, and 55.2 mg g^{-1}, respectively (Fig. 4). When comparing these three strains, CH20S1 demonstrated an advantage of hydrolytic ability relative to AHP and H_2SO_4 pretreated and non-pretreated corn stover, releasing the highest amount of reducing sugars.

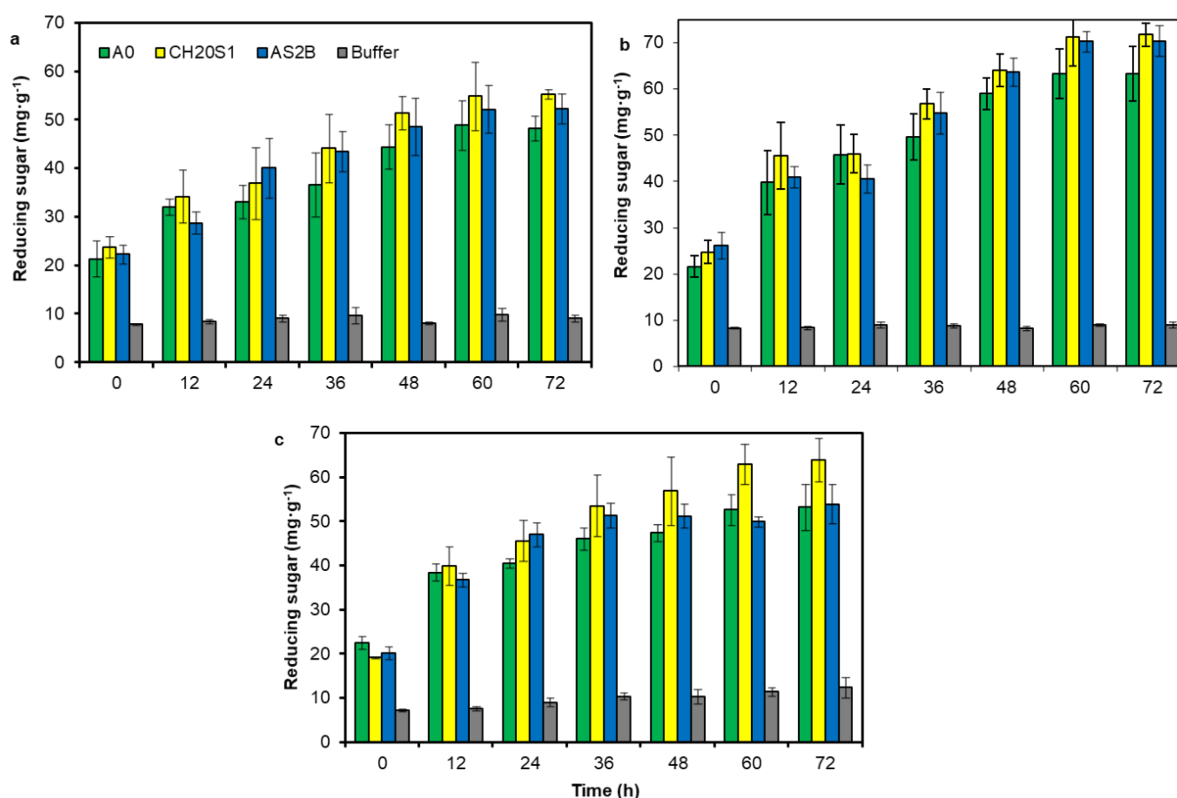

Fig. 4. Saccharification of non-pretreated (a), AHP pretreated (b), and H_2SO_4 pretreated (c) corn stover using crude enzymes produced by strains A0, AS2B, and CH20S1. Saccharification was performed at 55 °C, pH 5.0, with 200 rpm for 72 h, using a substrate concentration of 1%. Bars indicate the standard deviation (n = 3).

The hydrolysis effects on pretreated materials were superior to non-pretreated materials, mainly because of the fiber exposure of the materials after pretreatments (Dutra *et al.* 2018). Moreover, the yields of reducing sugars released by DCE were 3.3 to 7.0 fold higher than that of the blank (Fig. 4), showing that the bacterial enzymes supplementation can significantly improve the hydrolysis of corn stover. However, according to previous research, the contents of reducing sugars released from non-pretreated/pretreated corn stover by commercial cellulase ranged from 100 to 400 mg g^{-1} (Li *et al.* 2011), which is higher than the results of this study. Similarly, enzymatic hydrolysis and alkali

pretreatment of corn stover produced in Ghana yielded 158 mg mL^{-1} reducing sugar in 24 hours (Mensah *et al.* 2021).

Several enzymes and other accessory enzymes cut off the bonded side groups from the main chain, for example β-xylosidases, and endoxylanases hydrolyse the side group from the primary main structure of substituted xylan (Kocabaş *et al.* 2015). Further cellulolytic enzymes from bacteria can hydrolyze CMC into reducing sugars, and the hydrolytic efficiency is dependent on types of lignocellulosic biomasses (substrates) used (Sadhu and Maiti 2013; Prajapati *et al.* 2018; Shrestha *et al.* 2020). Furthermore, the types and activities of enzymes induced by different biomasses differ greatly (Maki *et al.* 2012). So, it can perhaps be concluded that the remarkable different hydrolysis effects between the crude bacterial enzymes and commercial cellulase studied here could be due to the lack of some essential hydrolytic enzymes in bacterial enzyme extract for lignocellulosic biomass hydrolysis effectively.

Comparison of Commercial Cellulase and Crude Enzymes Effects on Saccharification

To further explore the possibility of the practical application of bacterial enzymes in the industries, more enzymatic saccharification experiments were performed by partially replacing commercial cellulase with bacterial enzyme extracts. Strain CH20S1 showed the highest hydrolysis of corn stover, so this strain was selected among the three strains studied. For each treatment, 5.0 mL of DCE was added, but the reducing sugar yields for each group were noticeably different (Fig. 5). Since DCE was obtained from the supernatant of bacterial culture, in which corn stover was used as the sole carbon source for the growth of bacteria, there was small amount of reducing sugar existing in DCE on day 0 (Fig. 5).

After 72 h of incubation, the contents of reducing sugars released from non-pretreated, AHP pretreated, and H_2SO_4 pretreated corn stover by using 20 FPU g^{-1} of commercial cellulase were 162.2, 260.1, and 317.6 mg g^{-1}, respectively. The other treatments such as the addition of different concentrations of commercial cellulase and reducing sugar yields 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 stover, which demonstrated that these treatments were efficient in the hydrolysis of corn stover with or without pretreatment. Furthermore, the reducing sugars produced by the addition of commercial cellulase were significantly higher than the reducing sugar produced by using the crude enzymes from bacteria. This fact confirmed the current authors' previous hypothesis that the crude enzyme extracts lack the necessary hydrolytic enzymes, which might be supplemented by commercial cellulase. In addition, the enzymes effectively need to bind with the substrates for their hydrolysis. Besides, there might be other essential proteins or other compounds that help to loosen the structures of substrates and enhance the enzymes efficiency. For example, expansin has been found to enhance the enzyme adsorption for cellulose hydrolysis (Zhang *et al.* 2021b).

More importantly, for AHP-pretreated corn stover, treatments using commercial cellulase with crude enzymes 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 commercial cellulase added to DCE declined, showing the negative relationship between the concentration of commercial cellulase addition and the released reducing sugar for AHP treated corn stover (Fig. 5b). The study described alkaline pretreatment removed lignin and increased cellulose hydrolysis (Sun *et al.* 2016), but

higher loading of commercial enzyme did not increase the reducing sugars. The excess enzyme might restrict the increase of initial rate and diffusion because the excess enzyme gets adsorbed in the substrate surface (Martín *et al.* 2012). These results illustrate a cooperative effect of bacterial enzyme extract addition to replace the commercial cellulase partially for enzymatic hydrolysis of pretreated (AHP) corn stover.

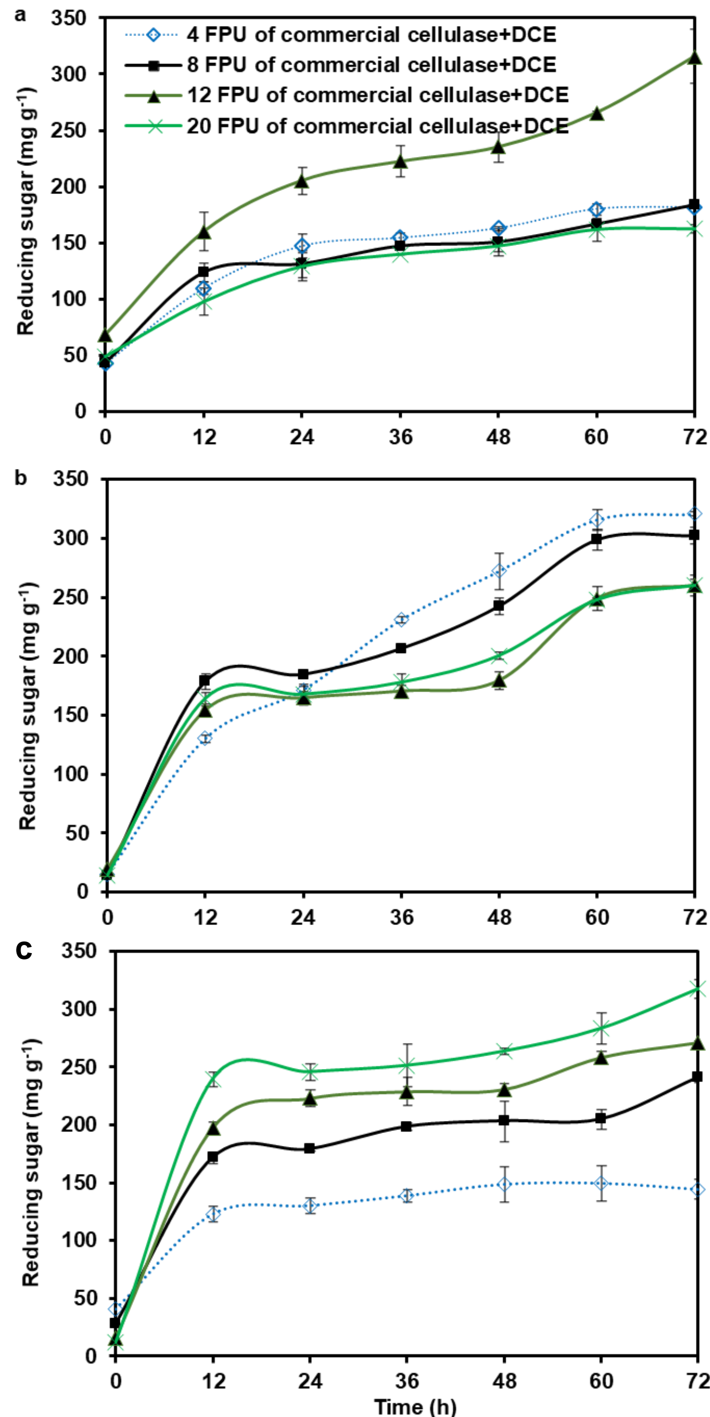

Fig. 5. Saccharification of non-pretreated (a), AHP pretreated (b), and H_2SO_4 pretreated (c) corn stover using bacterial enzyme extracts with different additions of commercial cellulases. Saccharification was performed at 55 °C, pH 5.0, with 200 rpm for 72 h, using a substrate concentration of 1%. Bars indicate the standard deviation (n = 3).

According to the 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 (Hu *et al.* 2013). Also, the non-hydrolytic proteins deactivate the enzyme function and make accessibility easy for cellulolytic enzyme by loosening the highly ordered and tightly packed region of cellulose (Arantes and Saddler 2010). The non-hydrolytic proteins present in water-soluble extracts of wheat straw have 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 was observed for non-pretreated corn stover, as shown in (Fig. 5a), where the synergistic effect increased with the increase in commercial cellulase concentration. It was observed in 72 h of incubation that the yields of reducing sugars were 181.7, 183.6, and 315.9 mg g^{1} when DCE of non-pretreated corn stover contained 4, 8, and 12 FPU g^{-1} of commercial cellulase, respectively. The main reason for this increase could be due to the undamaged cell wall structure of non-pretreated corn stover requiring more hydrolytic enzymes than pretreated corn stover (Banerjee *et al.* 2011). In addition, the bacterial enzymes showed continuous increase in the release of reducing sugar when commercial cellulase was added to H_2SO_4 pretreated corn stover used as the substrate (Fig. 5c). The H_2SO_4 pretreatment might increase the susceptibility of substrate for enzymatic hydrolysis and promote deactivation of inhibitory proteins (Jönsson and Martín 2016). However, the pretreatment might interfere with the pH, resulting in the conformation change of corn stover and decreased in enzymatic activity. The combined pretreatment strategies could be considered as a means to achieve effective digestibility or hydrolysis of different lignocellulosic biomass and produce simple sugars that further can be used for various value-added products production (Sun *et al.* 2016). Despite all this, the present results suggest that one can partially replace costly commercial enzymes by crude enzyme extract. Also, the enzymes production in large scale by using this type of low-cost substrate can reduce the production cost. Furthermore, crude enzyme extract has the potential to be an environmentally friendly and helps in efficient saccharification process of biomass, which further can be used in different industries for various products production.

CONCLUSIONS

1. Significant synergies were found 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.
2. Compared to using commercial cellulase only, the mixed enzymes used for saccharification of AHP pretreated and non-pretreated corn stover increased the reducing sugar content at most by 23.3% and 94.7%, respectively. Moreover, the highest amount of reducing sugars obtained from non-pretreated corn stover by mixed enzymes (316 mg g^{-1}) was almost as much from pretreated corn stover (318 to 321 mg g^{-1}).
3. Therefore, commercial enzymes and pretreatment requirements could be reduced to allow for more cost-effective production of different products. Also, there might be benefits in technological development and in real applications.

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APPENDIX

Table S1. CMCase and Xylanase Activity in Strains A0, AS2B, and CH20S1 Using Non-pretreated Corn Stover as the Substrate in Submerged Fermentation with Shaking for 144 h at 37 °C

Time (h)	CMCase (U mL-1)			Xylanase (U mL-1)		
	A0	CH20S1	AS2B	A0	CH20S1	AS2B
0	0	0	0	0	0	0
12	2.11±0.14	1.96±0.19	2.57±0.17	20.26±0.56	29.44±0.18	26.65±0.94
24	1.93±0.07	1.89±0.11	2.11±0.25	14.59±1.09	23.15±1.36	21.68±0.97
48	1.66±0.07	1.78±0.11	1.63±0.13	7.63±0.11	10.02±0.40	8.63±0.63
72	1.54±0.04	1.75±0.16	1.55±0.04	7.61±1.69	9.69±0.64	7.56±1.01
96	1.37±0.16	1.62±0.09	1.50±0.02	6.39±1.12	9.27±0.49	5.87±1.05
120	0.86±0.09	1.53±0.20	1.46±0.05	1.53±1.36	5.70±0.18	4.63±1.44
144	0.89±0.13	1.47±0.19	1.42±0.10	0.34±0.70	3.29±1.40	2.79±0.49

Table S2. Optimal Temperature for CMCase and Xylanase Activities Produced by Strains A0, AS2B, and CH20S1

Temp (°C)	CMCase (Relative Activity %)			Xylanase (Relative Activity %)		
	A0	AS2B	CH20S1	A0	AS2B	CH20S1
40	63.07±0.80	48.27±1.39	41.33±3.61	32.97±1.19	61.41±4.46	54.74±5.11
45	71.02±0.80	54.85±0.94	53.06±4.33	44.20±1.71	62.61±2.42	67.71±1.23
50	76.70±0.99	84.73±0.33	84.18±3.43	65.82±4.61	100.00±1.69	93.52±8.11
55	100.00±4.82	100.00±3.32	100.00±2.72	71.14±0.17	90.24±0.48	94.14±0.53
60	90.91±4.82	93.59±0.00	97.96±1.61	100.00±8.29	76.27±2.12	100.00±2.11
65	86.36±1.61	82.76±2.79	94.89±1.44	63.82±3.68	54.95±3.82	52.62±2.12
70	81.95±0.19	79.31±0.69	81.63±4.33	30.92±5.46	49.39±5.31	52.49±2.99
75	75.57±2.41	49.75±3.48	62.76±5.05	23.79±2.56	25.97±2.33	42.52±7.23
80	44.88±4.02	45.32±2.79	28.57±1.27	22.46±0.56	22.82±1.27	29.80±0.17

Table S3. Optimal pH for CMCase and Xylanase Activities Produced by Strains A0, AS2B, and CH20S1

pH	CMCase (Relative Activity %)			Xylanase (Relative Activity %)		
	A0	AS2B	CH20S1	A0	AS2B	CH20S1
4.0	72.47±3.97	57.56±0.82	65.00±1.77	62.49±2.75	63.05±1.26	60.34±0.76
4.5	77.53±4.77	79.65±0.82	93.75±1.77	85.99±0.33	68.30±1.77	76.49±2.85
5.0	95.51±4.77	100.00±3.29	100.00±3.53	97.89±1.10	100.00±1.26	100.00±3.52
5.5	100.00±1.58	89.53±1.64	98.13±2.65	100.00±0.55	97.89±1.01	97.37±1.52
6.0	92.13±1.58	73.83±0.82	94.37±2.65	94.86±1.43	93.96±3.67	95.18±2.95
6.5	69.10±2.38	52.32±8.22	55.00±1.76	71.44±2.64	68.31±4.30	58.18±0.38
7.0	63.48±0.79	44.77±2.46	49.37±0.88	69.81±0.77	55.43±6.45	49.56±3.23
7.5	46.07±1.58	42.44±0.82	48.75±3.53	64.82±0.55	50.87±1.13	46.86±5.33
8.0	37.64±2.38	42.44±2.47	46.87±0.88	34.86±4.40	22.50±0.13	34.01±3.14

Table S4. Saccharification of Non-pretreated (a), AHP Pretreated (b), and H_2SO_4 Pretreated (c) Corn Stover Using Crude Enzymes Produced by Strains A0, AS2B, and CH20S1

a)

	0 h	12 h	24 h	36 h	48 h	60 h
A0	21.57±2.32	39.73±6.91	45.79±6.35	49.56±4.93	58.97±3.45	63.28±5.39
CH20S1	24.73±2.48	45.57.166±	45.98±4.16	56.73±3.31	64.01±3.46	71.29±6.32
AS2B	26.07±2.86	40.94±2.25	40.52±3.04	54.78±4.53	63.65±3.06	70.20±2.23
Buffer	8.28±0.14	8.34±0.36	8.95±0.64	8.77±0.46	8.16±0.48	8.89±0.28

b)

	0 h	12 h	24 h	36 h	48 h	60 h
A0	22.44±1.40	38.43±1.98	40.46±1.07	46.04±2.56	47.38±1.93	52.59±3.51
CH20S1	19.12±0.18	39.889±4.29	45.59±4.58	53.53±6.94	56.85±7.73	62.88±4.51
AS2B	20.09±1.45	36.73±1.53	47.013±2.69	51.38±2.80	51.22±2.71	49.88±1.17
Buffer	7.18±0.37	7.552±0.50	9.01±1.011	10.34±0.78	10.34±0.78	11.39±1.01

c)

	0 h	12 h	24 h	36 h	48 h	60 h
A0	21.33±3.69	31.94±1.68	33.08±3.44	36.57±6.56	44.40±4.56	48.83±5.06
CH20S1	23.70±2.26	34.20±5.49	36.87±7.44	44.13±7.04	51.44±3.43	54.84±6.99
AS2B	22.24±1.89	28.74±2.26	40.03±6.14	43.50±4.17	48.59±5.86	52.11±4.95
Buffer	7.79±0.14	8.40±0.39	9.07±0.69	9.62±1.73	8.09±0.23	9.85±1.31

Table S5. Saccharification of Non-pretreated (a), AHP Pretreated (b), and H_2SO_4 Pretreated (c) Corn Stover using Bacterial Enzyme Extracts with Different Additions of Commercial Cellulases

a)

Time (h)	4 FPU of commercial cellulase+DCE	8 FPU of commercial cellulase+DCE	12 FPU of commercial cellulase+DCE	20 FPU of commercial cellulase+DCE
0	40.89±6.12	28.09±1.38	15.37±1.01	11.61±1.88
12	122.91±6.87	171.81±5.15	197.65±5.15	239.78±6.11
24	130.28±6.42	179.19±1.72	223.17±7.42	245.93±7.42
36	138.89±5.06	198.57±3.03	229.02±12.02	251.47±18.23
48	148.74±15.16	203.49±17.57	230.86±5.15	263.77±2.80
60	149.66±15.45	205.027±8.58	258.54±5.15	283.46±13.74
72	144.34±8.58	241.01±4.24	271.46±1.72	317.59±8.18

b)

Time (h)	4 FPU of commercial cellulase+DCE	8 FPU of commercial cellulase+DCE	12 FPU of commercial cellulase+DCE	20 FPU of commercial cellulase+DCE
0	14.96±0.87	14.15±1.01	19.89±0.37	13.99±0.84
12	130.16±3.43	178.73±6.87	154.44±3.43	164.16±4.86
24	171.44±3.43	184.79±1.71	164.97±5.05	167.79±8.58
36	230.94±2.53	206.65±1.72	170.63±5.06	177.92±7.37
48	272.22±15.45	242.67±7.01	179.54±7.42	200.58±3.43
60	315.93±8.58	298.93±8.58	249.15±10.30	247.94±1.72
72	320.65±1.72	302.57±6.87	260.08±5.15	260.08±8.58

c)

Time (h)	4 FPU of commercial cellulase+DCE	8 FPU of commercial cellulase+DCE	12 FPU of commercial cellulase+DCE	20 FPU of commercial cellulase+DCE
0	43.37±2.32	43.69±1.46	68.63±0.84	49.36±4.92
12	109.92±5.61	123.68±8.53	160.11±17.06	97.78±12.22
24	147.16±10.58	131.37±12.02	205.44±11.89	128.95±12.62
36	154.44±4.86	147.16±3.43	222.44±13.74	139.87±0.00
48	162.94±1.72	150.80±12.02	235.39±13.37	147.16±4.86
60	179.94±5.15	166.58±12.02	265.83±1.72	161.73±10.30
72	181.73±0.00	183.58±0.00	315.90±24.04	162.25±3.71

Note: Saccharification was performed at 55 °C, pH 5.0, with 200 rpm for 72 h, using a substrate concentration of 1% and reducing sugar is calculated in mg g^{-1}.