Production and Characterization of Partially Purified Thermostable Endoxylanase and Endoglucanase from Novel *Actinomadura geliboluensis* and the Biotechnological Applications in the Saccharification of Lignocellulosic Biomass

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Extracellular endoxylanase and endoglucanase from halo- and thermo-tolerant *Actinomadura geliboluensis* were produced, purified, characterized, and used in the saccharification of native and pretreated lignocellulosic biomasses. The molecular mass of endoxylanase and endoglucanase were 30 and 38 kDa, respectively. The optimum pH and temperature values for both endoxylanase and endoglucanase activities were pH 6.0 and 60 °C, respectively. They were both stable within a pH range of 4.0 to 8.0 and up to 70 °C. The half-lives of endoxylanase and endoglucanase at 70 °C were calculated as 180 min and 60 min, while their half-lives at 80 °C were detected as 60 min and 50 min, respectively. Both the endoxylanase and endoglucanase obeyed Michaelis-Menten kinetics. The endoxylanase and endoglucanase from *A. geliboluensis* were strongly inhibited by Hg^{2+}. Endoxylanase was activated by Mg^{2+} and Ca^{2+} and endoglucanase was activated by Fe^{2+} and Ca^{2+}. The potential application of endoxylanase and endoglucanase in saccharification of lignocellulosic biomass was further evaluated. The reduced sugar was 265.12 mg/g biomass after both endoxylanase and endoglucanase were incubated with wheat straw, which was pretreated by 1 % NaOH at 121 °C for 15 min. Endoxylanase and endoglucanase were produced from novel *A. geliboluensis*, which could potentially be used in biotechnological applications.

Keywords: Actinomadura; Endoglucanase; Endoxylanase; Reducing Sugar; Thermostable

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INTRODUCTION

Lignocellulose, which is the most abundant biopolymer on earth, consists of three major components: lignin (10% to 25%), hemicellulose (20% to 40%), and cellulose (35% to 55%) (Nanda *et al.* 2013). Composition of lignocellulose depends on biomass source (Beg *et al.* 2001; Ang *et al.* 2015; Pellegrini *et al.* 2015). Cellulose is a major structural component of lignocellulosic biomass and is composed of repeating (1,4)-D-glucopyranose units numbering from 100 to 20,000. Unlike cellulose, hemicellulose is a branched heteropolysaccharide. The main components of hemicellulose are xylan, xyloglucan, glucomannan, mannan, and galactomannan (Pérez *et al.* 2002). Agricultural, forestry, and municipal wastes comprise most of the abundant and cheap lignocellulosic biomass sources (Chandra *et al.* 2012). The production of microbial enzymes by using wastes, which contains lignocellulose, as carbon sources reduces the fermentation cost

(Ravindran and Jaiswal 2013).

Endoglucanases (EC 3.2.1.4; 1,4-β-D-glucan 4-glucanohydrolase), which hydrolyze β-1,4-glucosidic bonds of cellulose chains and create reducing and non-reducing ends, are used in some biotechnological applications such as bioethanol production, pulp and paper biobleaching, fruit juice clarification, food processing, and animal feed improvement (Pellegrini *et al.* 2015). Endoxylanases (EC 3.2.1.8; 1,4-β-D-xylan xylanohydrolase) that hydrolyze xylan chains in hemicellulose have biotechnological potential in various industries, including bioethanol, food, animal feed, textile, and paper (Beg *et al.* 2001).

The market value of strong, thermostable, and specific industrial enzymes was 3.6 billion U.S. dollars in 2010. Approximately 75% of these enzymes are hydrolases such as cellulases, xylanases, amylases, and pectinases (Ang *et al.* 2015). The cost of carbon sources plays a main role in the economics of endoglucanase and endoxylanase production. In this study, the production of endoglucanase and endoxylanase from halo- and thermo-tolerant *Actinomadura geliboluensis* was investigated. The enzymes were partially purified, characterized, and used in the saccharification of raw and pretreated wheat straw, banana leaves, and *R.communis* stalks from agricultural waste.

EXPERIMENTAL

Chemicals and Raw Materials

The chemicals used in this study were purchased from Merck (Darmstadt, Germany), Sigma (Missouri, USA), and Alfa Easer (Karlsruhe, Germany). Lentil straw, banana leaves, barley straw, corn stover, and sawdust were obtained from a local market in Mersin, Turkey. Pine needle, pine wood, and *R. communis* stalk were collected from a local forest in Mersin, Turkey. These lignocellulosic biomasses were chopped, milled, washed twice with distilled water, and dried at 80 °C before use.

Microorganism and Culture Conditions

The microorganism used in this study was a novel actinobacterium *Actinomadura geliboluensis*. The strain was isolated and identified by Sazak *et al.* (2012). The strain was grown on a yeast extract-malt extract agar (ISP2) medium that contained 4 g/L yeast extract, 10 g/L malt extract, 4 g/L glucose, and 20 g/L agar. The final pH of the medium was adjusted to 7.2 ± 0.2. One loop of *A. geliboluensis* colonies was inoculated to a minimal salts-yeast extract nutrient medium (MS-YEM) (pH 7.0 ± 0.2) that contained 6 g/L yeast extract, 0.1 g/L ammonium sulphate, 0.3 g/L sodium chloride, 0.1 g/L magnesium sulphate, 0.02 g/L calcium carbonate, and 1 mL of trace-elements solution. The culture was incubated at 30 °C and 150 rpm for 7 days. One mL of culture was used as a preculture. All fermentation experiments were performed in 50 mL of the MS-YEM in 250-mL flasks.

Measurement of Enzyme Activity and Protein Content

The culture was centrifugated at 10,000 x g for 5 min at 4 °C at the end of the incubation and then 100 µL of the supernatant and 100 µL of 1% substrate were incubated in 50 mM phosphate buffer (pH 7.0) at 37 °C for 10 min. Birchwood xylan and carboxymethylcellulose (CMC) were used as substrates for determining the endoxylanase and endoglucanase activities, respectively. The amount of reducing sugar was calculated

using the 3,5-dinitrosalysilic acid (DNS) method (Miller 1959). One unit of enzyme activity was defined as the amount of enzyme required to liberate 1 µmol xylose or glucose equivalent per minute under the conditions described above. The protein content was estimated according to the Bradford method (Bradford 1976). The standard curve was plotted by using bovine serum albumin.

Determining the Lignocellulosic Structure of Biomass

The hemicellulose, lignin, and cellulose contents of wheat straw, banana leaves, and *R. communis* stalks were estimated according to Ali *et al.* (2012).

Optimization of Endoglucanase and Endoxylanase Production

The effect of different nutritional, physical, and environmental parameters such as incubation time (0 to 120 h), carbon source (lentil straw, banana leaves, barley straw, corn stover, sawdust, pine needle, pine wood, and *R. communis* stalk), particle size of the carbon source (0.25 to 1.00 mm), nitrogen source (asparagine, glycine, casein, peptone, yeast extract, soybean meal, urea, and gelatin), incubation temperature (25 to 40 °C), the initial pH of the medium (4.0 to 9.0), and agitation rate (50 to 250 rpm) on endoxylanase and endoglucanase production were studied. Each parameter studied was incorporated into subsequent experiments.

Partial Purification

The crude extract was concentrated 20-fold by a 100,000 molecular weight cut-off ultra-filtration membrane before purification. The concentrated sample was loaded on to the gel filtration column that was packed with Sephadex G-100 column material according to the manufacturer's instructions. The sample was eluted with 50 mM potassium phosphate buffer (pH 7.0). Fractions (1.5 mL) were collected at a flow rate of 30 mL/h. Endoxylanase and endoglucanase activities and absorbance at 280 nm of each fraction were determined.

Native- and SDS-PAGE

Native polyacrylamide gel electrophoresis (Native PAGE) and SDS-PAGE was performed using an OmniPAGE mini vertical electrophoresis unit (Cleaver Scientific LTD). The resolving gel was composed of 0.375 M Tris HCl buffer (pH 8.8), acrylamide (10%), bis-acrylamide (0.27%), SDS (10%), ammonium persulfate (0.006%), and tetramethylethylenediamine (TEMED) (0.15%). The stacking gel consisted of 0.125 M Tris HCl buffer (pH 6.8), acrylamide (4%), bis-acrylamide (0.105%), SDS (10%), ammonium persulfate (0.012%), and TEMED (0.30%). The reservoir buffer consisted of 0.25 M Trisma base, and 1.92 M glycine and SDS (10%). The sample buffers were prepared with 0.625 M Tris-HCl (pH 6.8), glycerol (50 %), SDS (10%), 2-mercaptoethanol (25%), and bromophenol blue (0.05%) (Laemmli 1970). The protein bands were displayed with rapid silver staining protocol (Nesterenko *et al.* 1994). Native PAGE was performed with SDS-free resolving gel, stacking gel, reservoir buffer, and sample buffer, similarly. The gel was incubated into a 3% substrate solution that was prepared with 50 mM phosphate buffer (pH 6.0) at 60 °C for 30 min, Congo red solution at room temperature for 15 min, and 1 M NaCl solution at room temperature for 30 min for zymography.

Characterization of Partially Purified Endoxylanase and Endoglucanase

The effect of temperature on endoxylanase and endoglucanase activities was determined at 30 to 90 °C (pH 7.0). The thermostability was assessed at 30 to 90 °C for 0 to 6 h in a 50 mM phosphate buffer (pH 7.0). The optimum pH of the enzyme was determined by carrying out the reaction in the pH range of 3.0 to 9.0 using different assay buffers for 10 min each: 50 mM sodium acetate buffer (pH 4.0 to 6.0), phosphate buffer (pH 6.0 to 7.0), and Tris-HCl (pH 7.0 to 9.0) at 60 °C.

The effects of various metal ions and inhibitors, including Mn^{+2}, Ca^{+2}, Mg^{+2}, Cu^{+2}, Zn^{+2}, Fe^{+2}, Hg^{+2}, Ag^{+}, NaCl, EDTA, DTT, and SDS, on endoxylanase and endoglucanase activities were also examined. Each enzyme with substance (1 and 10 mM) was incubated for 1 h in a phosphate buffer at optimum pH before performing the standard enzyme assay. The relative activity was calculated by comparing with a control without metal ions and inhibitors. The enzyme was pre-incubated in the presence of 20% (v/v) of different substances including formaldehyde, methanol, acetone, ethanol, isopropanol, glycerol, tween 80, isoamyl alcohol, butanol, triton X-100, toluene, and benzene at 60 °C for 1 h to determine the effects of organic solvents and chemical reagents on activity. The relative activity was calculated by comparison on the control without substance.

Application of Endoxylanase and Endoglucanase in Biomass Hydrolysis

Crude and pretreated wheat straw, banana leaves, and *R. communis* stalk was used in the hydrolysis experiment. Pretreatment was carried out with 1% NaOH (solid to liquid ratio of 1/10) at 121 °C for 15 min using an autoclave. Pretreated biomass was washed by distilled water four times and dried overnight at 80 °C before hydrolysis. Hydrolysis was performed in 100 mM phosphate buffer (pH 6.0) containing 2.5% biomass, 0.005% sodium azide, 20 U/mL (800 U/g biomass) endoxylanase, and 10 U/mL (400 U/g biomass) endoglucanase. During hydrolysis (0 to 6 h), sufficient hydrolysis liquid was taken at regular intervals and then reducing sugar was estimated by the DNS method (Miller 1959). The saccharification percentage was calculated as the amount of sugar after enzymatic hydrolysis divided by the amount of carbohydrate in the substrate.

RESULTS AND DISCUSSION

Optimization of Endoglucanase and Endoxylanase Production

The use of pure xylan and cellulose as carbon sources is not economical for production of endoxylanase and endoglucanase. Therefore, cost-effective and easily available lignocellulosic wastes as primary the carbon source were used for endoxylanase and endoglucanase production. Enzyme production was performed in MS-YEM supplemented with different carbon sources (5 g/L) at 30 °C and 150 rpm for 3 days. *Actinomadura geliboluensis* showed the highest endoxylanase (170.91 U/mL) and endoglucanase (6.20 U/mL) production when wheat straw was used as the primary carbon source (Fig. 1). *Actinomadura geliboluensis* produced 164.98, 122.37, and 100.47 U/mL endoxylanase in MS-YEM supplemented with corn stover, *R. communis* stalk, and *Lolium* sp., respectively. Endoglucanase activities were 5.47 and 5.41 U/mL in MS-YEM supplemented with barley straw and corn stover, respectively. Similarly, wheat straw has been reported as a good substrate for enzyme production from some actinomycetes (Kohli *et al*. 2001; Nadia *et al*. 2010; Kumar *et al*. 2012).

The particle size of the substrate is another important parameter for endoxylanase and endoglucanase production. In this study, the highest endoxylanase (192.76 U/mL) and endoglucanase (7.27 U/mL) production were obtained with the smallest particle size of wheat straw (≤ 0.25 mm) (Fig. 2).

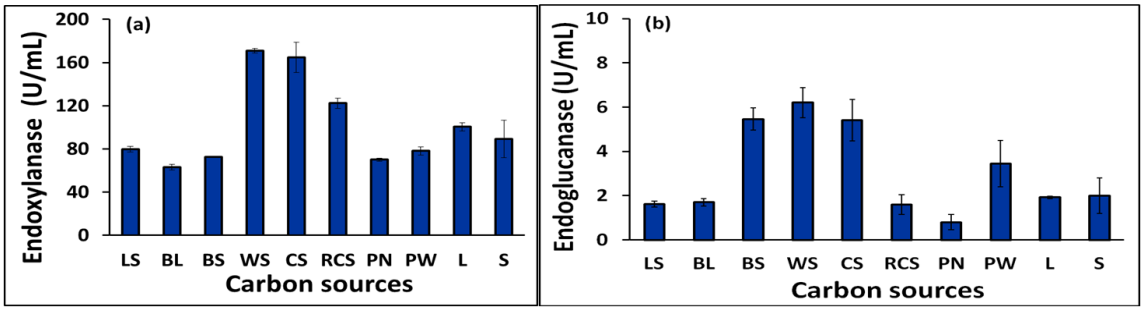

Fig. 1. Effect of different lignocellulosic carbon sources on (a) endoxylanase and (b) endoglucanase production. Incubation was performed in MS-YEM supplemented with different lignocellulosic substances such as lentil straw (LS), banana leaves (BL), barley straw (BS), wheat straw (WS), corn stover (CS), *Ricinus communis* stalk (RCS), pine needle (PN), pine wood (PW), *Lolium* sp. (L), and sawdust (S) as primary carbon sources at 30 °C and 150 rpm for 3 days. The data are presented as means of three replicates with SE.

The lowest endoxylanase (77.15 U/mL) and endoglucanase (0.77 U/mL) production were recorded when wheat straw that was passed through the 1-mm IS sieve was used in a medium as the primary carbon source. Consequently, the enzyme production decreased when the particle size of wheat straw was increased due to the decrease in the surface area of the straw for microbial attack.

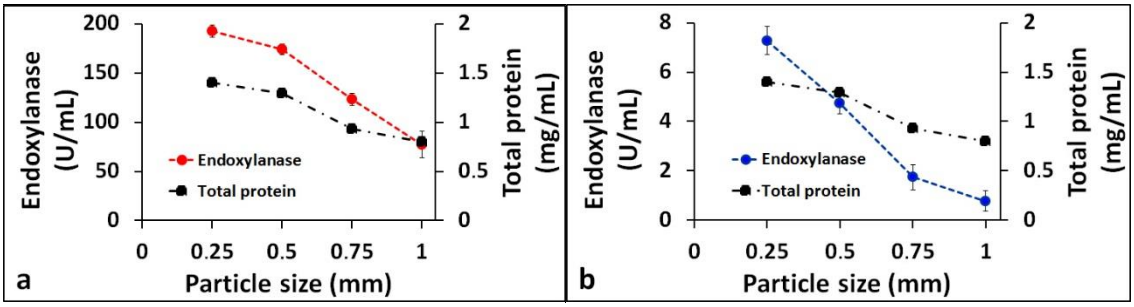

Fig. 2. Effect of the particle size of wheat straw on endoxylanase (a) and endoglucanase (b) production. Incubation was carried out in MS-YEM supplemented with wheat straw of different particle sizes as the primary carbon source at 30 °C and 150 rpm for 3 days. Data are presented as means of three replicates with SE.

The effect of nitrogen sources on endoxylanase and endoglucanase production was evaluated. The highest endoxylanase (193.27 U/mL) and endoglucanase (8.04 U/mL) activities were determined in a medium containing yeast extract as a nitrogen source. Also, noticiable endoxylanase (144.55 U/mL) and endoglucanase (6.98 U/mL) production were obtained when the medium was supplemented with peptone as a nitrogen source. In contrast, relatively low endoxylanase activity was determined when glycine (80.94 U/mL), casein (76.25 U/mL), asparagine (62.64 U/mL), or gelatine (46.27 U/mL) was used in the medium. The lowest endoglucanase production occurred when casein (2.47 U/mL) and gelatine (2.66 U/mL) were used in the medium (Fig. 3).

Similarly, *Streptomyces thermovulgaris* TISTR1948 (Chaiyaso *et al*. 2011) and *Streptomyces* sp. B-PNG23 (Bettache *et al*. 2013) secreted high amounts of enzyme when the yeast extract was used in the medium.

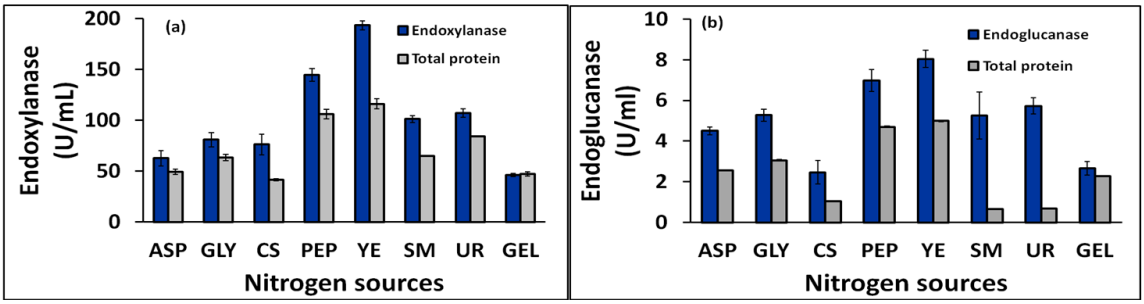

Fig. 3. The effect of different nitrogen sources on (a) endoxylanase and (b) endoglucanase production; incubation was carried out in MS-YEM supplemented with wheat straw (≤0.25 mm) as the primary carbon source and with different nitrogen sources (Asparagine: ASP; Glycine: GLY; Casein: CS; Peptone: PEP; Yeast extract: YE; Soybean meal: SM; Urea: UR; Gelatine: GEL) at 30 °C and 150 rpm for 3 days; data are presented as means of three replicates with SEs

The suitable incubation temperature for endoxylanase and endoglucanase production from *A. geliboluensis* was determined. *Actinomadura geliboluensis* showed the maximum production of endoxylanase (189.26 U/mL) and endoglucanase (7.76 U/mL) at 30 °C. The enzyme production increased with the increase in incubation temperature from 25 to 30 °C (Fig. 4). *Actinomadura geliboluensis* produced 127.58 and 32.21 U/mL endoxylanase when incubation was carried out at 35 and 40 °C, respectively. Similarly, the endoglucanase production decreased drastically when the incubation temperature was increased to 40 °C (0.64 U/mL). Generally, microorganisms produce high amounts of enzyme at their optimum growth temperature (Nagar *et al*. 2010).

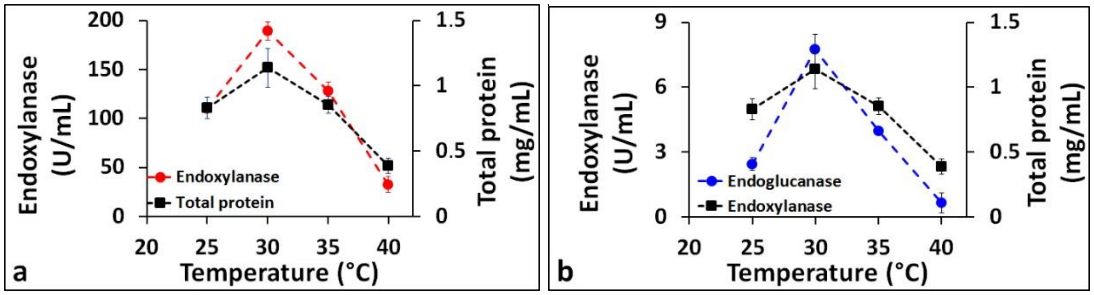

Fig. 4. Effect of incubation temperature on endoxylanase (a) and endoglucanase (b) production. Incubation was carried out in MS-YEM supplemented with wheat straw (≤ 0.25 mm) as the primary carbon source at different temperatures and 150 rpm for 3 days. Data are presented as means of three replicates with SE.

The effect of the culture medium initial pH on endoxylanase and endoglucanase production was investigated. The highest endoxylanase production (200.71 U/mL) was observed when the initial pH of culture medium was adjusted to 6.0. Endoxylanase production was 195.31 and 155.58 U/mL at pH 7.0 and 8.0, respectively. However, endoxylanase production decreased drastically at pH 5.0 (55.35 U/mL) and 4.0 (5.11 U/mL) (Fig. 5). The culture medium of *Streptomyces olivaceoviridis* E-86 demonstrated a similar optimum initial pH for endoxylanase production (Ding *et al*. 2004). There are,

however, also some reports of endoxylanase production at neutral and alkali conditions (Nawel *et al*. 2011). The best initial pH of the culture medium for endoglucanase production was 7.0 (9.33 U/mL). *A. geliboluensis* produced 4.40, 7.25, and 7.48 U/mL endoglucanase when the initial pH of the culture was adjusted to 5.0, 6.0, and 8.0, respectively.

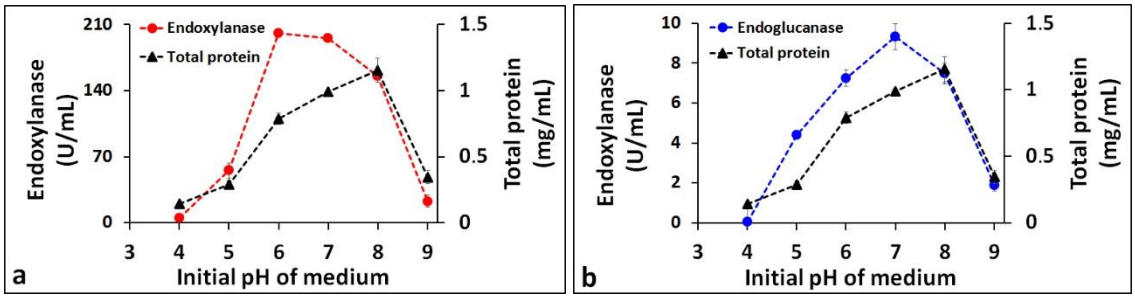

Fig. 5. Effect of the incubation medium initial pH on endoxylanase (a) and endoglucanase (b) production. Incubation was carried out in MS-YEM supplemented with wheat straw (≤ 0.25 mm) as the primary carbon source at 30 °C and 150 rpm for 3 days. Data are presented as means of three replicates with SE.

Agitation affected the endoxylanase and endoglucanase production notably. The highest endoxylanase (199.63 U/mL) and endoglucanase (11.98 U/mL) production were obtained at an agitation speed of 200 rpm. Endoxylanase (150.56 U/mL) and endoglucanase (7.24 U/mL) production decreased as the agitation speed increased from 200 to 250 rpm (Fig. 6). However, when the agitation speed was less than 200 rpm, noticeable reduction in both endoxylanase and endoglucanase production occurred. Observations showed that agitation speed was one of the remarkable physicochemical parameters for enzyme production. The results of this study showed that suitable agitation speed might promote enzyme production by increasing the availbility of nutrients and soluble oxygen into the medium.

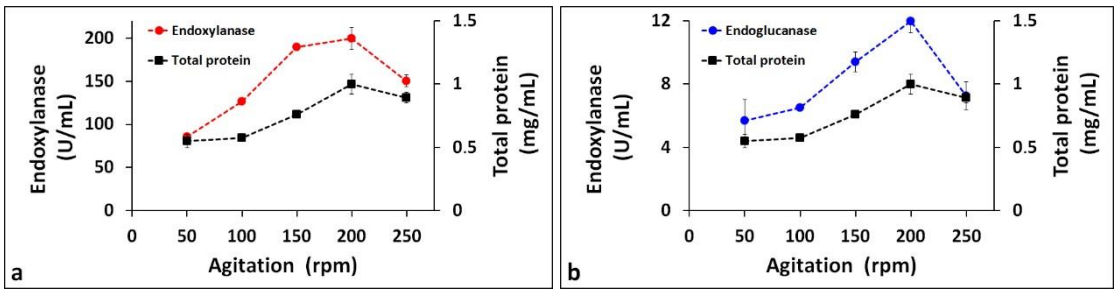

Fig. 6. Effect of the agitation rate on the endoxylanase (a) and endoglucanase (b) production. *A. geliboluensis* incubated in MS-YEM supplemented with wheat straw (≤ 0.25 mm) as the primary carbon source at different agitation rates at 30 °C for 3 days

Purification of Endoxylanase and Endoglucanase

For the purification experiment, *A. geliboluensis* was incubated in MS-YEM medium supplemented with 5 g/L wheat straw (0.25 mm particle size) at 30 °C and 200 rpm for 3 days. After centrifugation of culture liquid at 10,000 x g for 5 min, supernatant was used for crude enzyme preparation. Endoxylanase and endoglucanase activities of the supernatant were 232.55 U/mL and 17.76 U/mL, respectively, and the protein content of the supernatant was 2.39 mg/mL. The supernatant was concentrated 20-fold by an

Amicon ultrafiltration chamber using 10 kDa cut-off ultrafiltration membrane, and 1 mL of concentrated sample was loaded onto the gel filtration column that was packed with Sephadex G-100. Phosphate buffer (pH 7.0 and 50 mM) was used to elute the proteins at 30 mL/h. Fractions containing endoxylanase (fraction numbers 130 to 135) and endoglucanase (fraction numbers 125 to 129) were pooled and concentrated (Fig. 7). Specific activities of purified endoxylanase and endoglucanase were 182.23 U/mg and 17.65 U/mg, respectively. The purities of the endoxylanase and endoglucanase were calculated as approximately 1.87- and 2.37-fold greater than that of the crude sample. Molecular masses of enzymes were estimated as 30 kDa for endoxylanase and 38 kDa for endoglucanase by SDS-PAGE (Fig. 8).

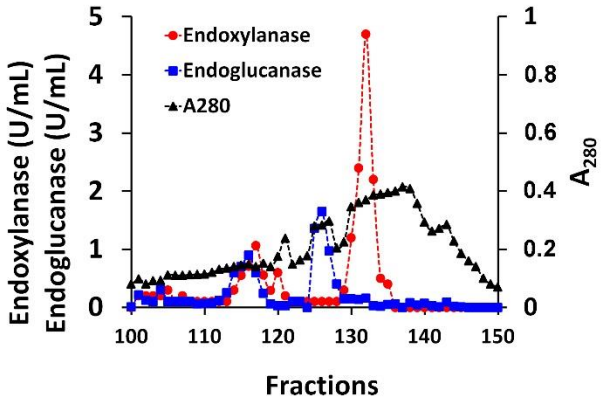

Fig. 7. Gel filtration chromatography with Sephadex G-100. The flow rate was 30 mL/h and the size of the fractions were 1.5 mL.

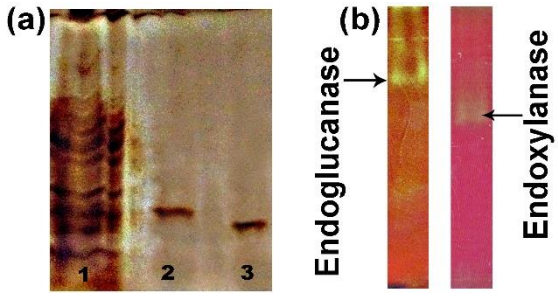

Fig. 8. Electrophoretic analyses of partially purified endoxylanase and endoglucanase produced by *A. geliboluensis*: (a) SDS-PAGE (Lane 1: crude enzyme; Lane 2: endoglucanase; Lane 3: endoxylanase). (b) Zymogram of endoxylanase and endoglucanase. The clear band that resulted from CMC and birchwood xylan hydrolysis from purified endoglucanase and endoxylanase is visible against a red background.

Characterization of Endoxylanase and Endoglucanase

The relative activities of the endoxylanase and endoglucanase at different temperatures were determined at pH 7.0 using a standard assay method. The relative endoxylanase activity at 30 °C, 40 °C, and 50 °C was 51.80 %, 62.60 %, and 97.15 %, respectively. The optimum temperature for endoxylanase was 60 °C. Endoxylanase retained up to 50% activity at temperatures ranging from 60 °C to 90 °C (Fig. 9). Xylanases derived from actinomycetes such as *Streptomyces* sp. RCK-2010 (Kumar *et al*. 2012), *Streptomyces* sp. SWU10 (Deesukon *et al*. 2011), *Streptomyces matensis* (Yan *et al*. 2009), *Streptomyces cyaneus* SN32 (Ninawe *et al*. 2008), *Streptomyces actuosus* A-

151 (Wang *et al*. 2003), and *Streptomyces althioticus* LMZM (Luo *et al*. 2016) have the same optimal temperature. However, the relative activity of xylanases from these microorganisms decreased rapidly when the temperature was increased from 60 °C to 90 °C. The optimum temperature of endoglucanase was 60 °C at pH 7.0, but it retained over 70% maximum activity in a broad temperature range (40 to 80 °C) (Fig. 9). The relative activity of endoglucanase decreased at 90 °C (56.28%). The optimum temperature of the *A. geliboluensis* endoglucanase was higher than some other endoglucanases obtained from *Streptomyces* sp. B-PNG23 (Bettache *et al*. 2013), *Bacillus subtilis* (Asha and Sakthivel 2014), and *Bacillus mycoides* S122C (Balasubramanian *et al*. 2012). Some researchers have also recently reported that endoglucanases showed maximum activity at 60 °C and above 60 °C (Saratale *et al*. 2012; Dipasquale *et al*. 2014).

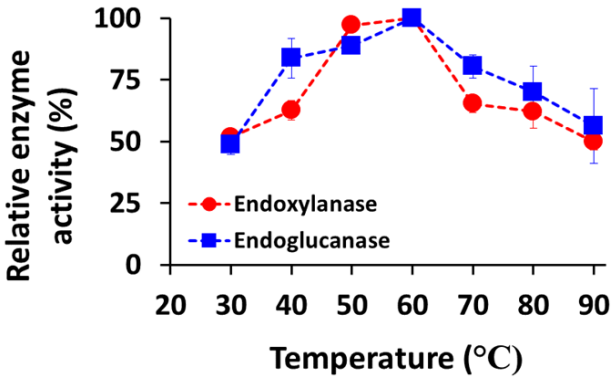

Fig. 9. Effect of the reaction temperature on endoxylanase and endoglucanase activity. After substrates (CMC and birchwood xylan) and enzymes incubated at specified temperatures for 10 min at pH 7.0, the DNS method was used to measure enzyme activity. Data are presented as means of three replicates with SE.

The effect of pH on endoxylanase and endoglucanase activities was determined at standard conditions at 60 °C. Endoxylanase exhibited its maximum activity at pH 6.0 (Fig. 10). Also, relative activities of endoxylanase at pH 4.0, 5.0, 7.0, and 8.0 were 70.98%, 81.31%, 77.50%, and 56.99%, respectively. Endoxylanase activity sharply decreased above pH 8.0. Similar pH optima have been previously reported for other endoxylanases (Nascimento *et al*. 2002; Bajaj and Singh 2010; Kim *et al*. 2010; Kumar *et al*. 2012). Endoglucanase also showed optimal activity at pH 6.0. Endoglucanase showed considerable activity in a pH range from 4.0 (73.93%) to 8.0 (86.74%) (Fig. 10). Similarly, endoglucanase that was obtained from *Streptomyces* sp. showed optimal activity at pH 6.0 to 8.0 (Jang and Chen 2003).

The therrmostability of endoxylanase and endoglucanase was studied at 40 to 80 °C for 6 h. The maximum stability for endoxylanase was observed at 40 °C. The relative activities of endoxylanase were 99.61%, 95.62%, 93.28%, 88.81%, 84.46%, and 74.46% after the preincubation at 40 °C for 1, 2, 3, 4, 5, and 6 h, respectively. However, endoxylanase was moderately stable when the enzyme was pre-incubated at 50 °C (63.01%) and 60 °C (51.11%) for 5 h (Fig. 11a). Remarkable loss in endoxylanase activity was observed at 70 °C and 80 °C. The half-lives of endoxylanase at 70 °C and 80 °C were approximately 3 h and 1 h, respectively. The thermostability exhibited by endoxylanase that was observed from *A. geliboluensis* was higher than endoxylanase from *Streptomyces cyaneus* SN32 (Ninawe *et al*. 2008), but it was lower than *Actinomadura* sp. Cpt20 (Taibi *et al*. 2012). The relative endoglucanase activities at 40,

50, and 60 °C for 3 h incubation were 77.24%, 73.87%, and 53.05%, respectively. The half-lives of endoglucanase at 70 °C and 80 °C were approximately 60 and 50 min, respectively (Fig. 11b). The thermostability of endoglucanase that was produced by *A. geliboluensis* was better than that of some actinomycetes such as *Streptomyces malaysiensis* (Nascimento *et al*. 2009), *Streptomyces viridobrunnes* SCPE-09 (Da Vinha *et al*. 2011), and *Streptomyces drozdowiczii* (De Lima *et al*. 2005). However, endoglucanase that was produced by *Thermomonospora* sp. was more stable between 50 and 70 °C (George *et al*. 2001). In another study, the half-life of endoglucanase was obtained from *Streptomyces* sp. SLBA-08 at 8 h and 50 °C (Macedo *et al*. 2013).

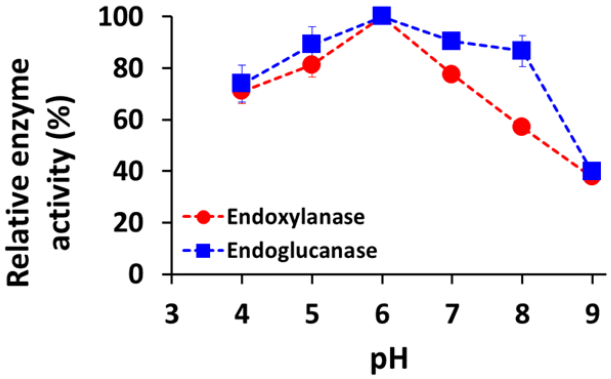

Fig. 10. Effect of incubation pH on endoxylanase and endoglucanase activity. Reactions were performed at specified pH and 60 °C for 10 min; enzyme activity was calculated using the DNS method. Data are presented as means of three replicates with SE.

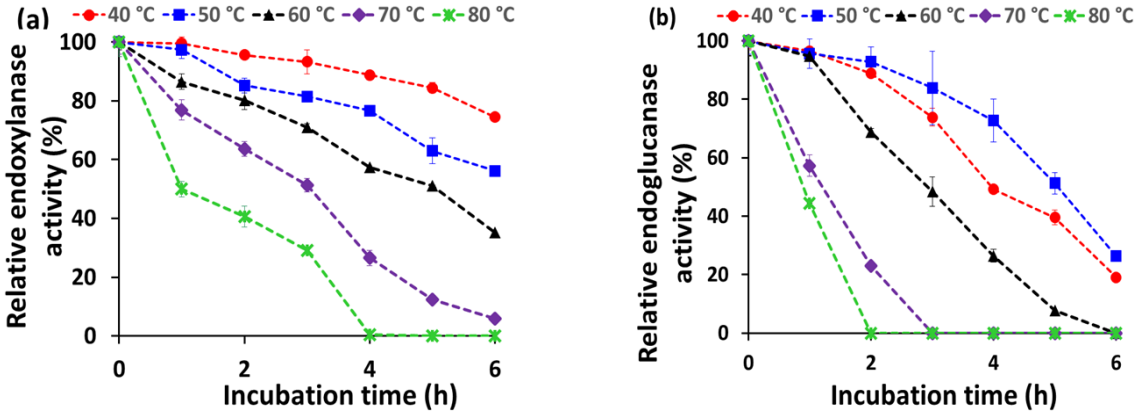

Fig. 11. The thermostability profiles of (a) endoxylanase and (b) endoglucanase. The enzymes were incubated at 40 °C (●), 50 °C (■), 60 °C (▲), 70 °C (◆), and 80 °C (x) for 6 h, and residual activities were detected at 1 h intervals. Data are presented as means of three replicates with SE.

The effect of certain metal ions and inhibitors at the concentration of 1 mM and 10 mM on the stability of endoxylanase and endoglucanase was studied at pH 6.0 and 60 °C. As shown in Table 1, some metal ions and inhibitors at 1 mM and 10 mM concentration, such as Mn^{+2}, Cu^{+2}, Zn^{+2}, Ag^{+}, EDTA, DTT, and SDS, did not affect endoxylanase activity (80% to 100% relative activity) notably. Endoxylanase activity was enhanced (100% to 110%) by Ca^{+2} and Mg^{+2} at 1 mM and 10 mM concentrations. On the other hand, Hg^{+2} (1 and 10 mM) showed inhibitor effects on endoxylanase that was obtained from *A. geliboluensis*. Similarly, some researchers reported that Hg^{+2} showed

inhibitor effects on endoxylanases (Sharma and Bajaj 2005; Taibi *et al*. 2012; Luo *et al*. 2016). Some metal ions and inhibitors at 1 mM and 10 mM concentrations, such as Mn^{+2}, Cu^{+2}, Mg^{+2}, Zn^{+2}, Ag^{+}, EDTA, DTT, and SDS, inhibited the endoglucanase moderately. The endoglucanase activity decreased after preincubation of enzyme with 1 mM and 10 mM Hg^{+2} while it was stimulated in the presence of Ca^{+2} and Fe^{+2} ions. Inhibition by Hg^{+2} and stimulation by Ca^{+2} and Fe^{+2} has also been indicated in previous reports (De Lima *et al*. 2005; Lee *et al*. 2008; Wang *et al*. 2008).

Table 1. Effect of Metal Ions and Reagents on Enzyme Activity*

Metal Ions and Reagents	Concentration (mM)	Relative Endoxylanase Activity (%)	Relative Endoglucanase Activity (%)
Mn^{+2}	1	88.56 ± 1.62	90.36 ± 4.22
	10	89.65 ± 1.70	79.86 ± 8.35
Ca^{+2}	1	102.57 ± 2.67	102.37 ± 1.62
	10	109.01 ± 0.99	107.74 ± 2.06
Mg^{+2}	1	113.55 ± 0.44	91.24 ± 2.69
	10	123.15 ± 0.99	76.18 ± 7.45
Cu^{+2}	1	85.69 ± 0.14	83.93 ± 0.30
	10	85.09 ± 0.89	59.69 ± 1.88
Zn^{+2}	1	91.19 ± 0.31	83.67 ± 4.67
	10	84.41 ± 0.38	70.85 ± 0.09
Fe^{+2}	1	90.23 ± 0.37	102.71 ± 1.62
	10	78.07 ± 1.67	113.75 ± 1.62
Hg^{+2}	1	69.30 ± 0.84	56.35 ± 1.26
	10	53.85 ± 2.95	47.97 ± 0.36
Ag^{+}	1	88.15 ± 0.63	90.27 ± 7.63
	10	82.93 ± 0.13	74.79 ± 4.49
NaCl	1	91.31 ± 0.99	98.60 ± 5.65
	10	89.27 ± 0.59	87.44 ± 2.33
EDTA	1	90.79 ± 1.54	90.82 ± 0.72
	10	83.25 ± 0.61	84.56 ± 2.24
DTT	1	92.59 ± 0.70	85.62 ± 3.32
	10	81.92 ± 1.66	78.89 ± 1.62
SDS	1	90.09 ± 2.62	91.16 ± 6.55
	10	82.32 ± 0.43	82.06 ± 0.72

*The enzyme reaction mixture without metal ions and reagents was taken as the control (100%). Data are presented as means of three replicates with SD.

The effects of various organic solvents and chemical reagents (20%) on endoxylanase and endoglucanase activities are shown in Table 2. Formaldehyde, methanol, isopropanol, glycerol, and toluene stimulated endoxylanase at 60 °C. However, acetone, ethanol, tween 80, isoamyl alcohol, butanol, triton X-10, and benzene slightly inhibited the endoxylanase activity. The endoglucanase activity was enhanced by acetone, glycerol, and tween 80, and it was inhibited by formaldehyde, methanol, ethanol, isopropanol, isoamyl alcohol, and butanol.

The kinetic parameters K_m and V_{max} were determined from Lineweaver-Burk double reciprocal plots of endoxylanase and endoglucanase activities at 60 °C using various concentrations (0 to 10 mg/mL) of birchwood xylan and CMC as substrates. The V_{max} and K_m values of endoxylanase were 292.2 (± 5.72) U/min and 3.87 (± 0.16)

mg/mL, and the V_{max} and K_m values of endoglucanase were 18.03 (± 0.35) U/min and 3.57 (± 0.22) mg/mL, respectively. The K_m value of endoxylanase from *A. geliboluensis* was lower than endoxylanase obtained from *Streptomyces cyaneus* SN32 (11.1 mg/mL) (Ninawe *et al*. 2008) and *Streptomyces althioticus* LMZM (43.03 mg/mL) (Luo *et al*. 2016). The K_m value of endoglucanase has been reported in the range of 3.0 to 4.0 mg/mL for CMC (Wang *et al*. 2009; Rastogi *et al*. 2010). The kinetics of endoglucanase obtained from *Actinomadura* sp. have not been previously characterized. Lower K_m values were important for the industrial application of enzymes due to increasing substrate affinity.

Table 2. Effect of Organic Solvents and Chemical Reagents on Enzyme Activity*

Organic Solvents and Chemical Reagents (20%)	Relative Endoxylanase Activity (%)	Relative Endoglucanase Activity (%)
Formaldehyde	74.38 ± 1.60	59.01 ± 4.85
Methanol	63.65 ± 2.59	52.71 ± 0.36
Acetone	95.24 ± 0.88	104.99 ± 6.82
Ethanol	83.38 ± 5.40	76.10 ± 2.96
Isopropanol	79.42 ± 6.36	63.32 ± 1.70
Glycerol	77.04 ± 2.76	102.37 ± 2.42
Tween 80	98.27 ± 5.54	105.92 ± 4.22
Isoamyl Alcohol	95.59 ± 1.97	68.15 ± 2.51
Butanol	87.35 ± 3.20	58.97 ± 1.26
Triton X-100	87.90 ± 1.65	67.51 ± 4.04
Toluene	76.70 ± 0.63	63.20 ± 1.26
Benzene	80.51 ± 4.11	69.67 ± 1.17

*The enzyme reaction mixture without organic solvents and chemical reagents was taken as the control (100%). Data are presented as means of three replicates with SD.

Hydrolysis of Biomass

Second generation biomasses include agricultural and municipal lignocellulosic waste. They are preferable in the production of value-added products, such as bioethanol and some organic acids, because they are cheap and abundant. Also, the use of lignocellulosic waste can decrease environmental problems. Because wheat straw, banana leaves, and *R. communis* stalks are cheap and abundant, they are good candidates for the production of reducing sugar with hydrolysis. For this reason, this study evaluated milled (0.25 mm particle size) wheat straw, banana leaves, and *R. communis* stalks with endoxylanase and endoglucanase that were obtained from *A. geliboluensis*.

The maximum reducing sugar was obtained from the hydrolysis of pretreated wheat straw (265.1 mg/g biomass). Reducing sugars were obtained *via* the enzymatic hydrolysis of native wheat straw, pretreated banana leaves, pretreated *R. communis* stalk, native banana leaves, and native *R. communis* stalk, which after 6 h were 138.3, 120.0, 82.1, 57.5, and 51.4 mg/g biomass, respectively (Fig. 12a). The lignocellulosic content of biomass (Table 3) was taken into consideration when saccharification was calculated.

Table 3. Contents of Natural Wheat Straw (NWS), Pretreated Wheat Straw (PWS), Natural Banana Leaves (NBL), Pretreated Banana Leaves (PBL), Natural *R. communis* Stalk (NRC), and Pretreated *R. communis* Stalk (PRC)*

Biomass	Cellulose (%)	Hemicellulose (%)	Lignin (%)
NWS	35.93 ± 2.54	29.80 ± 3.35	18.63 ± 1.97
PWS	41.29 ± 3.39	33.13 ± 1.95	12.52 ± 1.29
NBL	28.63 ± 1,65	31.96 ± 1.30	18.91 ± 1.60
PBL	34.30 ± 1.24	40.04 ± 2.76	13.87 ±1.60
NRC	32.43 ± 3.87	40.36 ± 1.74	20.65 ± 2.45
PRC	36.40 ± 7.60	43.28 ± 1.05	14.09 ± 2.05

*Data are presented as means of three replicates with SD.

The saccharification yields of native wheat straw, pretreated wheat straw, native banana leaves, pretreated banana leaves, native *R. communis* stalk, and pretreated *R. communis* stalk were 21.0%, 35.6%, 9.5%, 16.1%, 7.1%, and 10.3%, respectively (Fig. 12b). After pretreatment, the hemicellulose and cellulose content increased, and lignin content decreased (Table 3). Therefore, the reducing sugar yield increased with pretreatment of the biomass after hydrolysis. Table 4 shows compares the reducing sugar yields that were determined previously.

Table 4. Comparison of Reducing Sugar Yields Obtained by Enzymatic Hydrolysis of Different Biomass Materials

Biomass	Enzymes	Enzyme Source	Pretreatment	Hydrolysis Yield	Hydrolysis Time	Ref.
Parthenium hysterophorus	CMCase (10 U/g biomass)	*Bacillus amyloliquefaciens* SS35	130 min autoclaving with 1 % (v/v) H_2SO_4	271.5 mg TRS/g biomass	120 h	(Singh *et al*. 2015)
Maize straw (80 g/L)	20 FPU/g substrate	*T. reesei* ZU-02	2% NaOH at 80 °C for 1 h	50 g/L (400 mg/g biomass) (approximately)	48 h	(Chen *et al.* 2008)
2.0%(w/v) wheat bran	0.5 U/mL xylanase	Recombinant xylanase from *Bacillus* sp. HJ14	None	19.0 µmol/mL reducing sugar	12 h	(Zhou *et al*. 2014)
Wheat straw	500 IU of xylanase, 80 FPU of FPase, 160 IU β-glucosidase	*B. pumilus* VLK-1	0.1 N NaOH for 2 h at room temperature	553 ± 12 mg sugars/g biomass	6 h	(Kumar *et al*. 2013)
Sorghum straw	Crude enzyme	*Bacillus altitudinis* DHN8	3% alkaline hydrogen per- oxide	34.94 mg/g biomass	36 h	(Adhyaru *et al*. 2014)
Water hyacinth biomass	Cellulase (103.75 U/g) and xylanase (650.18 U/g	Commercial	5 % NaOH for 1 h at room temperature and for 10 min at 150 °C	465 mg/g	36 h	(Ganguly *et al*. 2013)
Sugarcane bagasse	Cellulase(25 FPU/g)	*Penicillium oxalicum* EU2106	10 % NaOH + 10 % peracetic acid	30 g/L	96 h	(Huang *et al*. 2015)
Wheat straw	Celluclast 1.5 L (0.24 FPU/mL)	Commercial	Thermomechanical pretreatment	31 g reducing sugar/ 100 g straw	18h	(Pierre *et al*. 2011)
Wheat straw	2.5 U/mLCMCase	Commercial	Fungal pretreatment	225 mg/g straw (approximately)	35 days	(Dias *et al*. 2010)
Wheat straw	Endoxylanase (800 U/g biomass) and endoglucanase (400 U/g biomass)	*A. geliboluensis*	%1 NaOH for 15 min at 121 °C.	265.12 mg/g biomass	6 h	This study
Banana leaves	Endoxylanase (800 U/g biomass) and endoglucanase (400 U/g biomass)	*A. geliboluensis*	%1 NaOH for 15 min at 121 °C.	119.97 mg/g biomass	6 h	This study
R. communis stalk	Endoxylanase (800 U/g biomass) and endoglucanase (400 U/g biomass)	*A. geliboluensis*	%1 NaOH for 15 min at 121 °C.	82.14 mg/g biomass	6 h	This study

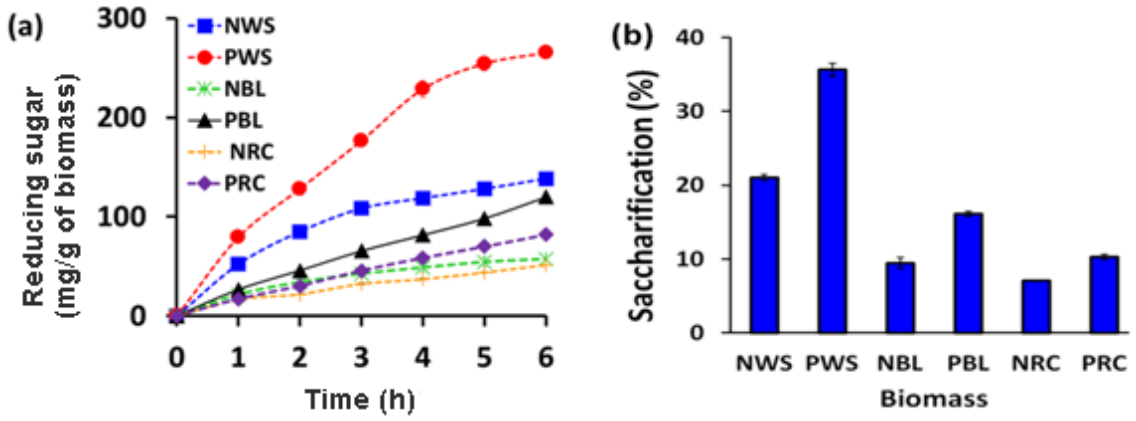

Fig. 12. (a) The reducing sugar and (b) saccharification rates, after hydrolysis of natural wheat straw (NWS), pretreated wheat straw (PWS), natural banana leaves (NBL), pretreated banana leaves (PBL), natural *R. communis* stalk (NRC), and pretreated *R. communis* stalk (PRC). Hydrolysis was performed at 60 °C for 6 h. Data are presented as means of three replicates with SE.

CONCLUSIONS

1. This study showed that novel *A. geliboluensis* can produce endoxylanase and endoglucanase using lignocellulosic wastes in submerged fermentation.
2. The molecular mass of endoxylanase and endoglucanase purified by gel filtration chromatography were found to be approximately 30 kDa and 38 kDa. Endoxylanase and endoglucanase display thermotolerant characteristics and promising activity in the broad range of pH. Kinetic analysis of endoxylanase showed that V_{max} and K_m values of endoxylanase were 292.2 (±5.72) U/min and 3.87 (±0.16) mg/mL. The endoglucanase exhibited maximum activity (V_{max}) of 18.03 (±0.35) U/min with its corresponding K_m value of 3.57 (±0.22).
3. Hydrolysis experiment in this study revealed that endoxylanase and endoglucanase obtained from *A. geliboluensis* may be useful in saccharification of lignocellulosic feedstocks.

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