Physico-chemical characterization of a recombinant endoglucanase from a clone derived from a mixed microbial culture from mudspring water of Mt. Makiling, Laguna, Philippines

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The endoglucanase gene harbored in *Escherichia coli* BL21 clone pEngl-24-21-63 developed in another study expressed the endoglucanase protein, MM-Engl in an autoinduction medium. The expressed endoglucanase protein, MM-Engl was obtained from a mixed culture resulting from cellulose enrichment of mudspring water from Mt. Makiling, Laguna, Philippines, through culture-independent molecular based approach. The protein was partially purified by ammonium sulfate precipitation at 60-80% saturation. The partially purified endoglucanase (PPEndo) was shown to be both acid and thermostable having an optimum temperature of 80 °C, thermal stability from 30 to 80 °C, optimum pH of 3.0 and pH stability from 2.0 to 6.0 based on carboxymethylcellulase (CMCase) activities. The enzymatic activities of the partially purified endoglucanase was greatly enhanced by $Mg^{2+} > K^{+} > Cu^{2+}$ ions, while negatively affected by compounds and ions, EDTA > Mn^{2+} > SDS > Zn^{2+}. Highest amount of reducing sugars was released using CMC as substrate as compared against cotton, filter paper and xylan.. The high resistance of MM-Engl to changes in pH and temperature makes it well suited for application in lignocellulose bioprocessing for bioethanol production and as an enzyme component in the feed industry. The stability of the enzymes can reduce the need of cooling the reaction mixture from the previous high temperature step which could hasten the reaction process and reduce production costs.

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INTRODUCTION

Volcanic vents and mudspring systems located on the mountainside of Mt. Makiling, Los Baños, Laguna are potential sources of thermophilic and hyperthermophilic microorganisms. These microorganisms are excellent sources of genomic DNA materials for the expression of enzymes and other genes encoding biomolecules with novel properties having potential or established industrial importance (Lantican et al. 2011; Montecillo et al. 2019; Tambalo et al. 2020).

Agricultural by-products present a promising alternative source of sugars for ethanol production. Dilute acid treatment is considered as a cheap and effective pre-treatment method due to low cost and easy availability of acids (Kim et al. 2005). Acid treatment is carried out in the presence of high and low concentrations of acids, and at high and low temperatures. At high temperatures, pretreatment of biomass is performed at temperature greater than 160 °C in continuous process containing a low concentration of biomass (5–10% substrate concentration) (Chaturvedi and Verma 2013). This is conducted to break the lignin seal, loosen up the lignocellulose structure and expose the recalcitrant crystalline cellulose. Enzymatic conversion of cellulose to glucose, cellobiose, cello-oligosaccharides using cellulase enzymes that break the β-1-4 glycosidic bonds of cellulose can be done (Linares-Pasten et al. 2014; Bernardi et al. 2019).

Due to the pH and temperature extremes involved in the biomass-to-biofuel conversion, stable endoglucanases and its synergistic enzymes, such as cellobiohydrolase and β-glucosidase, are sought for the enzymatic conversion of biomass. Enzymatic hydrolysis of cellulose to glucose is predominantly carried out by fungi, *e.g. Trichoderma, Penicillium* and *Aspergillus* at mesophilic (typically between 20 and 45 °C) temperature and slightly acid to neutral (4-7) pH conditions (Karnchanatat et al. 2008; Liu et al. 2006). Thermostable endoglucanases from extremophiles are considered promising because they typically exhibit valuable characteristics in biofuel production including optimal functionality at higher temperatures and the ability to withstand extreme pH changes (Yennamalli et al. 2013).

The use of thermostable and thermoactive enzymes during lignocellulose hydrolysis will allow enhanced cell-wall disorganization and degradability of these materials, improve the penetration by enzymes for lignocellulose conversions, allow easy mixing and improve substrate solubility, allow high mass transfer rate and lower the risk of contamination (Pfeifer et al. 1984; Tran and Chambers 1986). Efficient hydrolysis is expected to achieve high sugar yields, lower the formation of inhibitory by-products with concomitant decrease in the use of chemicals and water used for detoxification and neutralization. Cellulase, the enzyme that catalyzes cellulose degradation to glucose, is actually a complex mixture of several enzymes including endoglucanase, cellobiohydrolases (CBH, also known as exoglucanases), and β-glucosidase. The concerted action of these three groups of enzymes ensures an efficient hydrolysis of cellulosic materials and diminished product inhibition (Zhu 2005; Enze 2006).

In a previous study, a putative thermostable endoglucanase gene was identified from a mixed culture resulting from the inoculation of Brock-CMC (1%) broth with mudspring water from Mt. Makiling, Laguna, Philippines that had been incubated at 90 °C (Tambalo et al. 2020). Genomic DNA was extracted from the cellulose-enriched mixed culture and endo1949 forward and reverse primers (Brouns et al. 2005) were used to amplify the endoglucanase gene, which was cloned into pCR-script. Blastn alignment of the sequenced insert revealed 99.69% similarity to the glycosyl hydrolase, sso1354 (CelA1; Q97YG7) from *Saccharolobus solfataricus.* The endoglucanase gene (GenBank accession number MK984682) was inserted into a pET21 vector and transformed in *E. coli* BL21 for expression. Initial test on the expressed recombinant endoglucanase (MM-Engl) demonstrated heat stability up to 80 °C.

This study was conducted to characterize the expressed recombinant endoglucanase, MM-Engl, and to determine its potential application in the hydrolysis of complex bio-materials/agricultural by-products at high temperature and dilute acid conditions.

MATERIALS AND METHODS

Microbial culture

The transformant *Escherichia coli* BL21 clone pEngl-24-21-63 containing a pET21 plasmid with an endoglucanase gene insert developed by Tambalo et al. (2020) was used in this study.

Growth Conditions of *Escherichia coli* BL21 clone pEngl-24-21-63

The seed culture of the transformant *E. coli* BL21 clone pEngl-24-21-63 was made by inoculating a loopful of an overnight culture to 5 ml Luria Bertani (LB) broth medium (HiMedia, Mumbai, India) containing ampicillin (100 µg/ml). The culture was incubated at 37 °C for 18-20 hours with shaking (1.50 x*g*). The seed culture was inoculated to 200 ml of autoinduction medium (AIM) (Studier 2005) containing ampicillin (100 ug/ml). The inoculated flask was incubated at 37 °C with shaking (1.5 x*g*) for 16 h. A 100 ml aliquot of the 16 h culture broth was inoculated to 2.0 liter AIM medium in a 4.0 liter Erlenmeyer flask and incubated at 37 °C with shaking (1.5 x*g*) for 24 h. The resulting culture broth was harvested by centrifugation at 1,000 x*g* for 15 min at 4 °C. The protocol of the Grunden laboratory for the preparation of recombinant proteins for expression studies was followed in this study (Grunden 2011). The supernatant was collected in a sterile container while the biomass pellets were placed inside an autoclavable plastic bag for decontamination. The supernatant was heat treated at 85 °C for 30 min in a hot water bath and centrifuged at 1,000 x*g* for 15 min at 4 °C to precipitate the denatured proteins. The supernatant was collected in a sterile container.

The target endoglucanase protein in the supernatant was precipitated by 60-80% saturation of ammonium sulfate (Yin et al. 2010) and kept at 4 °C overnight. The mixture was then centrifuged (1,000 x*g* for 15 min at 4 °C) to obtain the precipitated proteins and dialyzed against 20 mM phosphate buffer (pH 8.0) overnight at 10 °C. The suspended partially purified endoglucanase (PPEndo) was collected in a sterile glass vial and used for subsequent characterization studies, endoglucanase activity assay and protein content determination. The samples were subjected to SDS-PAGE using a Mini-Protein II system (BIORAD, California, USA). Electrophoresis was performed at room temperature for ~45 min at 200 volts according to the method described by Laemmli (1970).

Endo-β-1,4-glucanase Assay

Endo-β-1,4-glucanase activity expressed as CMCase activity of PPEndo was determined by measuring the amount of reducing sugar released from carboxymethylcellulose (CMC) (SIGMA, Missouri, USA) after enzyme treatment using dinitrosalicylic (DNS) acid reagent (Ghose 1987; Miller 1959). The standard assay mixture contained 100 ul of the enzyme and 900 µl of 1% CMC in 20 mM Na_2HPO_4/citric acid buffer (pH 3.0). The mixture was incubated at 50 or 80 °C. After 30 min reaction, 1 ml of DNS was added and boiled in a water bath for 5 min to stop the reaction. The resulting samples were cooled to room temperature and absorbance was measured at 540 nm (A_{540}). Reducing sugar content was calculated using a standard curve with 0–1.0 mM monosaccharide (glucose) solution.

One unit (umol/min) of endo-β-1,4-glucanase activity per ml solution is defined as the amount of enzyme that could hydrolyze CMC and release 1 µg of glucose within 1 min reaction at 50 or

80 °C (Miller 1959). Enzyme assays were carried out in duplicate for each treatment.
Protein concentration, expressed as milligram per ml solution (mg/ml) was determined by Lowry (1951) method using bovine serum albumin (BSA) as standard. Specific activity was calculated by dividing the number of units/ml by the protein concentration in mg/ml to get µmol/min/mg.

Effects of temperature and pH on endoglucanase activity
The residual activity after subjecting PPEndo to various conditions was determined by measuring the amount of reducing sugar released from 1% CMC (SIGMA, Missouri, USA) using the DNS colorimetric method (Ghose 1987; Miller 1959).

Temperature optimum
To 900 µl of 1% CMC in 20 mM Na_2HPO_4/citric acid buffer (pH 3.0), 100 µl of appropriate concentrations of the PPEndo were added and incubated at various temperatures (5, 15, 32, 40, 50, 60, 70, 80, 90 and 100 °C) for 30 min. After incubation, the reducing sugars released was determined and CMCase activity was then measured.

Temperature stability
PPEndo (100 µl) in 20 mM Na_2HPO_4/citric acid buffer (pH 3.0) was incubated at various temperatures (5, 15, 32, 40, 50, 60, 70, 80, 90 and 100 °C). After 1 h incubation, the enzyme was used for activity assay and the residual CMCase activity was measured.

pH optimum
PPEndo (100 µl) was added to 900 µl of 1.0% CMC in buffers adjusted to various pH levels (50 mM glycine/HCl buffer, pH 2.0; 50 mM Na_2HPO_4/citric acid buffer, pH 3.0, 4.0, 5.0. 6.0; 50 mM phosphate buffer, pH 7.0, 8.0; 50 mM carbonate buffer, pH 9.0, 10.0) and incubated at 80 °C for 30 min after which the reducing sugars released was determined and CMCase enzyme activity was measured.

pH stability
PPEndo (100 µl) was incubated in buffers adjusted to various pH levels (50 mM glycine/HCl buffer, pH 2.0; 50 mM Na_2HPO_4/citric acid buffer, pH 3.0, 4.0, 5.0; 50 mM phosphate buffer, pH 6.0, 7.0, 8.0; 50 mM carbonate buffer, pH 9.0, 10.0) at 80 °C for 1 h. After the 1 h pre-incubation period, the enzyme was used for activity assay and residual CMCase activity was measured.

Effects of metal ions and other compounds on CMCase activity
CMCase activity of PPEndo (100 µl) was examined at 5 mM concentrations of metal ions: Na^+, Mg^{2+}, Ca^{2+}, Mn^{2+}, Fe^{2+}, Zn^{2+}, Cu^{2+}, K^+ and Co^{2+}, in chloride form and/or sulfate. Ions were added to the reaction mixture containing 2% CMC in 20 mM Na_2HPO_4/citric acid buffer (pH 3.0). The mixture was incubated at 80 °C for 30 min. The CMCase activity of the treated PPEndo was determined by measuring the amount of reducing sugar released from CMC as stated above. The effects of ethylene diamine tetraacetic acid (EDTA) and sodium dodecyl sulfate (SDS) were examined under the same reaction conditions. The relative activity is equal to the CMCase activity observed after incubation with the metal ions and chemical reagents over that in the absence of metal ions and chemical reagents multiplied by 100.

Substrate specificity
The hydrolytic ability of the PPEndo (100 µl) against 1% each of CMC, cotton, filter paper and xylan in 20 mM Na_2HPO_4/citric acid buffer (pH 3.0) incubated at 80 °C for 30 min was determined to evaluate substrate specificity. The CMCase activity of the PPEndo was determined by measuring the amount of reducing sugar released from the different substrates using DNS acid reagent (Ghose 1987; Miller 1959).
All experiments were conducted for two (2) trials using duplicate set-ups per trial. From each set-up, collection of enzyme reaction mixtures were done twice with each sample assayed in duplicate. Presented data are the averages of the two (2) experiment trials.

RESULTS AND DISCUSSION

Protein expression
Growing the transformant *E. coli* BL21 (Tambalo et al. 2020) in auto-induction medium allowed the endoglucanase gene contained in pEngl-21-63 to be expressed and secreted into the medium. An intense band of ~37 kDa molecular size was present in the samples: whole cell extract (WCE), cell free extract (CFE), heat treated CFE and supernatant (lanes 1, 2, 3 and 5 Figure 1). The ~37 kDa band was not present in the CFE sample of the *E. coli* BL21 containing the pET 21 plasmid without the inserted gene even after induction. The approximate molecular mass of ~37 kDa was in good accordance with the molecular mass of ~37 kDa or 37386 Da calculated from the deduced amino acid count of 333 using pepstart-EMBOSS program (Tambalo et al. 2020; http://www.bioinformatics.nl/emboss-explorer).

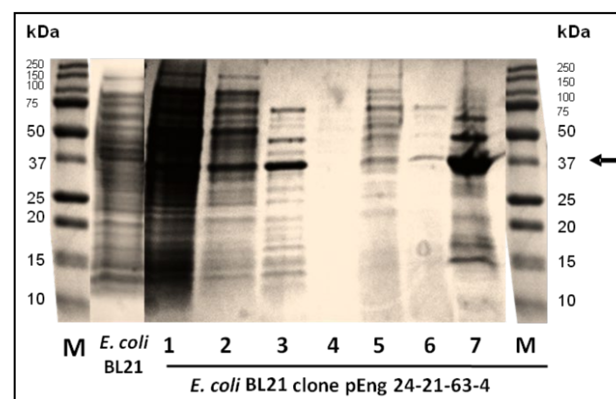

Figure 1: SDS-PAGE chromatogram of protein fractions from small scale expression of *Escherichia coli* BL21 clone pEngl-24-21-63 grown in auto-induction medium. *E. coli* BL21 contains pET21 without the gene insert; 1 = whole cell extract (WCE); 2 = cell free extract (CFE); 3 = CFE heat treated (HT) at 85 °C for 30 min; 4 = periplasmic extract; 5 = supernatant (S) from culture broth; 6 = supernatant heat treated (HT) at 85 °C for 30 min; and 7 = protein suspension after ammonium sulfate precipitation of heat treated supernatant; M = molecular weight marker

Although the intensity of the expressed recombinant endoglucanase, MM-Engl protein band (~37 kDa) was much more intense in the cell free extract (CFE) (lane 2, Figure 1) consisting of cytoplasmic proteins, it was also present in the supernatant albeit at a lower concentration (lane 5, Figure 1). This signifies that the expressed protein was being secreted to the medium by the transformant host *E. coli* BL21 during its growth. The target protein band was not present in the periplasmic extract (lane 4, Figure 1) or if present in the periplasmic space, was in very low quantity such that the resulting band was not discernible. After heat treatment at 85 °C for 30 min, the target protein band was still present in the banding pattern of the heat treated CFE (lane 3, Figure 1) and heat treated supernatant (lane 6, Figure 1) pointing to its heat stable characteristic. Heat treatment as a rapid and partial purifications step denatures and precipitates non heat-stable proteins thereby reducing the number of proteins in the mixture resulting to a thermostable protein suspension without contaminating components (Patchett et al. 1989; Phillips 2020).

The protein suspension was attained after a series of centrifugation, ammonium sulphate precipitation, centrifugation to obtain the precipitated protein and suspension in phosphate buffer (6.0). Concentration of the target protein in the suspension was attained after ammonium sulfate precipitation as shown by the increased band intensity (lane 7, Figure 1). Other protein bands were visible in lane 7 which could be heat stable proteins precipitated and concentrated in the enzyme suspension. The study of Kwon et al. (2008) was able to identify 17 heat-stable proteins from *E. coli* after treatment for 10 minutes at 85 °C. The chaperonin (GroEL and GroES), molecular chaperones (DnaK and FkpA) and peptidyl prolyl isomerases (trigger factor and FkpA) that function in the protection of other proteins against denaturation such as pyrophosphatase (Ppa), histidine binding protein and enolase were included but no cellulase nor endoglucanase enzyme was identified. Also, none of the identified proteins had the molecular mass of ~37 kDa.

As shown in a previous study by Tambalo et al. (2020), the amino acid count determined from the recombinant endoglucanase MM-Engl was close to the hypothetic endo-β-glucanase, SSO1949 encoded by 334 amino acids and SSO1354 encoded by 335 amino acids, which were cloned from the thermoacidophilic archaeon, *Sulfolobus solfataricus* P2 by Huang et al. (2005) and (Girfoglio et al. 2012), respectively. The molecular size of the recombinant endoglucanase (~37 kDa) that was secreted extracellularly was also close to the molecular mass of a cellulase from *Thermoascus aurantiacus* RBB-1 at 35 kDa (Dave et al. 2015), a thermostable endoglucanase isolated from the wood-decaying fungus *Daldinia eschscholzii* of 46.4 kDa (Karnchanatat et al. 2008) and a monomeric endoglucanase (43 kDa) from culture filtrates of *Aspergillus awamori* strain F18 (Singh et al. 2011).

Some other endo-glucanases in the 25-45 kDa molecular mass range were also reported such as 27 kDa from *Mucor circinelloides* (Saha 2004), 35 kDa from *Chalara paradoxa* (Lucas et al. 2001), 40 kDa from *Bacillus* strains (Mawadza et al. 2000), 40 kDa from *Aspergillus niger* (Akiba et al. 1995) and thermostable endocellulases CelA (29 kDa) and CelB (30 kDa) from *Thermotoga neapolitana* (Bok et al. 1998).

The Gram-negative bacterium *Escherichia coli* is commonly used as host for the industrial production of various chemical products with a variety of sugars including xylose, as carbon sources. However, this strain neither produces endogenous cellulose degradation enzymes nor secrets heterologous cellulases for its poor secretory capacity (Gao et al. 2015; Salamanca-Cardona et al. 2014). An earlier study by Wulff et al. (2003) also studied and reported that *E. coli* does not have endoglucanase proteins thus, all endoglucanase activity observed in this study is derived from the cloned gene.

Effects of Temperature and pH on Activity of the partially purified endoglucanase

Temperature optimum and stability

The PPEndo showed its highest activity at 80 °C incubation temperature (Figure 2). After 1 h of incubation at different temperatures and subsequent assay at 80 °C, pH 3.0, the PPEndo had no enzymatic activity at temperatures 5 and 15 °C with increasing activity recorded from 30 to 70 °C which peaked at 80 °C. Slight decrease in residual activity at 90 °C with a complete loss of activity at 100 °C was observed.

The optimum activity at 80 °C of the PPEndo observed in this study corresponds to the optimum temperature (80 °C) of the endoglucanase SSO1949 from *Sulfolobus solfataricus* reported by Huang et al. (2005). Cellulases from thermophilic microorganisms have commonly been described as having a high thermostability (Wang et al. 2017; Patel et al. 2019). Since the endoglucanase gene possibly originated from the hyperthermophilic *Saccharolobus solfataricus* (Tambalo et al.

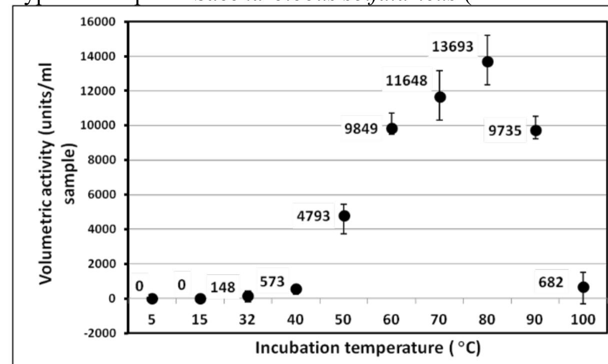

Figure 2: Effect of different temperature values on the CMCase activity of the partially purified recombinant endoglucanase from *Escherichia coli* BL21 clone pEngl-24-21-63 grown in auto-induction medium. Error bars indicate standard deviation with respect to the mean of 8 technical assay replicates.

2020), high thermostability was observed for PPEndo. The most thermophilic endoglucanase isolated to date was prepared from *Thermotoga* sp. strain FjSS3-B.1 (Bok et al. 1998) and has maximal activity at 106 °C. The endoglucanases of thermophilic fungi generally have optimal activity in the range 50–80 °C (Manheshwari et al. 2000). A thermostable cellulase from *Pyrococcus horikoshi* with an optimum temperature of 97 °C was reported by the group of Ando (2002), while the enzyme from *Pyrocuccus furiosus* showed optimal activity at 102–105 °C (Kengen et al. 1993). A cellulase from *Thermotoga maritima* MSB8 optimally active at 95 °C was reported by the group of Bronnenmeier (1995). CelA, with the ability to hydrolyze microcrystalline cellulose, was isolated from the extremely thermophilic bacterium *Anaerocellum thermophilum* (Zverlov et al. 1998) showed maximal activity at 85–95 °C.

After 1 h of incubation of PPEndo at different temperatures and subsequent assay at 80 °C, pH 3.0, the PPEndo retained its enzyme activity at the temperature range of 5 to 80 °C (Figure 3). A decrease in residual activity was obtained at 90 °C (40%) with a complete loss of activity at 100 °C (Figure 3). PPEndo maintained its high activity at high temperatures of 70-90 °C even afer 1 h of incubation. Similarly, Huang et al. (2005) reported that endoglucanase SSO1949 retained its activity on prolonged incubation at high temperature (80 °C for 2h), with significant decrease at higher than 85 °C and rapid inactivation at 95 °C.

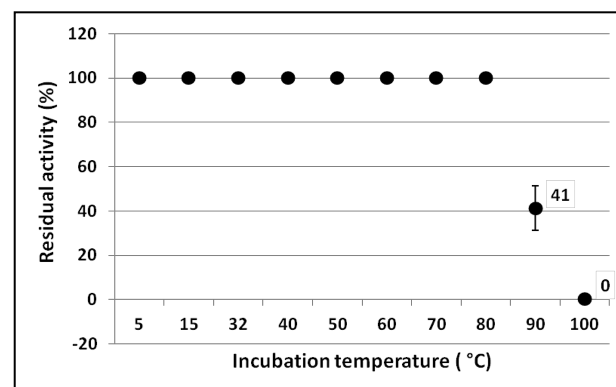

Figure 3: Thermal stability of the partially purified recombinant endoglucanase (PPEndo) from *Escherichia coli* BL21 clone pEngl-24-21-63 grown in auto-induction medium. The PPEndo was pre-incubated at different temperature (°C) values for 1 h prior to CMCase activity determination. Error bars indicate standard deviation with respect to the mean of 8 technical assay replicates.

Heat denaturation of proteins is usually irreversible at temperatures higher than 80 °C because under these conditions proteins generally aggregate after heat denaturation (Matsuura et al. 2015). Heat denaturation and loss of biological activity have been linked to the breakup of the 2-D-spanning water network around the protein (due to increased breakage of the hydrogen bonds with temperature), which otherwise acts restrictively on protein vibrational dynamics (Koizumi et al 2007; Brovchenko et al. 2005). As proteins denature, their structures initially become looser allowing them to take up more water with the water-exposed surface increasing by up to 50% as might be expected from reversing protein folding (Groot and Bakker 2016).

An endo-β-1,4-glucanase from *Thermoascus aurantiacus* RBB-1 studied by Dave et al. (2015) was stable up to 70 °C as it exhibited 100% relative activity. The enzyme was also observed to retain up to 90% activity even after 1 h of incubation at 80 °C; however, increasing the temperature to 90 °C led to a rapid decrease in the stability (70% activity). A novel thermostable endoglucanase from the wood-decaying fungus *Daldinia eschscholzii* had maximum activity at 70 °C and showed enzyme activities over the temperature range of 30–90 °C (Karnchanatat et al. 2008). The enzyme retained 80% of its maximum activity at 80 °C.

In terms of thermal stability, the temperature range of PPEndo (15-90 °C) was higher than cellulases obtained from *Bacillus* strains 0-50 °C (Mawadza et al. 2000) and from *Mucor circinelloides* (0-70 °C) (Saha 2004). These results are in complete agreement with other studies, which reported that majority of the thermophilic fungal cellulases are stable within the temperature range of 55–80 °C (Amouri and Gargouri 2006; Gao et al. 2008; Kaur et al. 2007). It has also been reported that endo-β-1,4-glucanase from *T. aurantiacus* was stable at 70 °C for 8 h (Khandke et al. 1989). Based on the thermal stability of the enzyme, pre-incubation temperatures higher than 85 °C are required to induce inactivation of the PPEndo enzyme.

The differences in thermal stability of the different cellulases indicated the different molecular properties of the enzymes, including the bonding that stabilizes the structures and the functional conformations among the various microorganisms of origin. Imanaka (2011) enumerated some molecular bases for thermophily, a) increase in internal hydrophobicity of an enzyme and stabilization of a secondary structure α-helix, b) ion pair network interaction between amino acid residues, and c) shift of hydrophobic residues to the interior of the molecule, and charged residues to the surface of the molecule that stabilize the internal packing of its tertiary structure. Vieille and Zeikus (2001) identified other possible contributors to protein stability at high temperatures, such as disulfide bridges, aromatic interactions or hydrogen bonds. The possibility that the specialized heat stable proteins known as chaperonins reported by Kwon et al. (2008) were produced by the *E. coli* host and were precipitated together with the target enzyme can also be considered. The study of Conway de Macario and Macario (2000) indicated that chaperonins may help thermostable proteins, in this case the PPEndo, to refold back after their denaturation to their native form and restored their functions.

Given the numerous advantages of carrying out processes at temperatures above 50 °C, thermostability is one of the most desirable enzymatic properties in the industry (Moroz et al. 2015; Mohd Azhar et al. 2017; Azizi et al. 2015). Storage stability, as well as stability at the reaction conditions are very important since utilization of enzymes in industrial processes often encounters the problem of thermal inactivation of the enzyme (Shah and Madamwar 2005). The recombinant PPEndo in this study had a very wide range of temperature stability (15-90 °C), indicating a molecular structure that is resistant to changes in temperature. This provides the enzyme a potential application in the paper industry and in saccharification processes for the production of biofuel and other chemicals using cellulosic materials.

pH optimum and stability

The PPEndo achieved maximal enzymatic activity at pH 3.0 (Figure 4). The PPEndo was active at a pH range of 2.0 to 6.0 with maximal enzymatic activity observed at pH 3.0-4.0 while the group of Huang (2005) recorded an optimum pH of 1.8 for the endoglucanase SSO1949 isolated from *Sulfolobus solfataricus.*

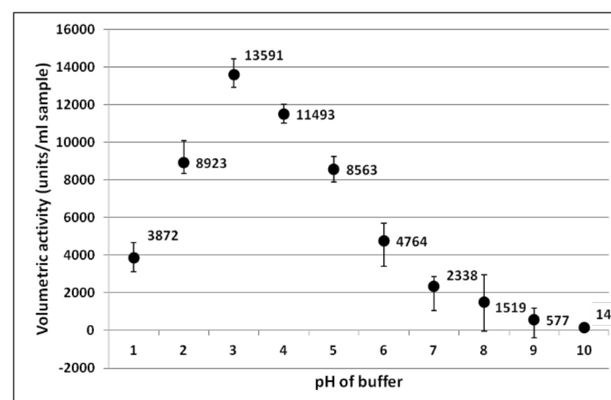

Figure 4: Effect of different pH values on the CMCase activity of the partially purified recombinant endoglucanase from *Escherichia coli* BL21 clone pEngl-24-21-63 grown in auto-induction medium. Error bars indicate standard deviation with respect to the mean of 8 technical assay replicates.

The endocellulases (CelA and CelB) from *Thermotoga neapolitana* obtained by Bok et al. (1998) had optimal pH at 6.0 and pH 6.0–6.6, respectively. The group of Zverlov (1998) was able to isolate an endoglucanase (CelA) from the extremely thermophilic bacterium *Anaerocellum thermophilum* with the ability to hydrolyze microcrystalline cellulose and maximal activity at pH 5.0–6.0. Highly thermostable endoglucanases with pH optimum at ranges at 6.8–7.8 and 6.0-7.0 were produced from *Thermotoga* sp. FjSS3-B1 (Ruttersmith and Daniel 1991) and *Thermotoga maritema* MSB8 (Bronnenmeier et al. 1995).

The stability of the PPEndo in this study had a wider pH stability range than those from *Mucor circinelloides*, 4.0-7.0 (Saha 2004), *Bacillus circulans*, 4.5-7.0 (Kim 1995), *Bacillus* strains, 5.0-6.5 (Mawadza et al. 2000), 6.0-7.0 from *Aspergillus niger* (Akiba et al. 1995), and 5.0-7.0 from *Lysobacter* sp. (Ogura et al. 2006). Endoglucanase activity between pH 5.0 and 9.0 with an optimum at pH 6 was reported for the thermophilic endoglucanase fungus, *Chaetomium thermophile* var. *coprophile* by the group of Ganju (1990).

The bell-shaped pH-activity profile of acidophilic glycosyl hydrolases (GHs) was observed for SSO1949 from hyperthermophilic *S. Solfataricus,* xylanase C from *A. kawachii* and xylanase I from *Trichoderma reesei* (Huang et al. 2005). Considering that the PPEndo sequences also possibly originated from hyperthermophilic *Saccharolobus solfataricus* (Tambalo et al. 2020), a similar bell shaped pH activity profile was obtained for the endoglucanase in the study. The pH-activity profile of PPEndo is likewise sharper and shifted to the acidic range of pH 3-4, whereas the SSO1949 of Huang et al. (2005) was lower at pH 1-2.

According to Huang et al. (2005), the bell-shaped pH–activity profile of GHs was caused by the ionization states of the two catalytic acidic residues. In the reaction mechanism of glycoside hydrolysis, two carboxylate side chains cooperate; one of them

acts as a nucleophile and is required to be in the de-protonated state, whereas the other donates a proton and therefore is required to be in the protonated state. In the pH–activity profile of SSO1949, two ionization steps of apparent p*K*a values of 1.7 and 2.3 are assumed to be involved in catalysis (Huang et al. 2005).

For the stability of the PPEndo after pre-incubation at different pH levels, low residual activity was recorded at pH 1.0, a range of 70 to 100% and down to 50% residual activities between pH levels of 2.0 to 6.0 and gradual decrease in residual activities from pH 7.0 to 10.0 (30-0%) (Figure 5). Highest stability value (100% residual activity) was measured in terms of CMCase activity at pH 3.0 which corresponds to the determined optimum temperature of PPEndo.

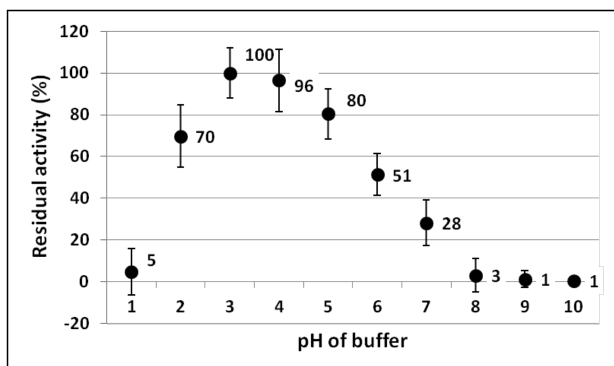

Figure 5: pH stability of the partially purified recombinant endoglucanase (PPEndo) from *Escherichia coli* BL21 clone pEngl-24-21-63 grown in auto-induction medium. The PPEndo was pre-incubated in buffers of different pH values at 85 °C for 1 h prior to CMCase activity determination. Error bars indicate standard deviation with respect to the mean of 8 technical assay replicates.

In the study of Sielecki et al. (1990), the stability of the acidophilic porcine pepsin was attributed to the large excess of negatively charged groups over positively charged groups of the protein structure which also has an extremely low pI of 1. The rapid denaturation of the protein at pH >6.5 and resulting instability was explained by the repulsion of the excess negative charges at high pH. Similarly, the stability of the extremely acidophilic and acid-stable xylanase C has been interpreted in terms of an excess of acidic residues on coiled regions and has a negatively charged surface with a low pI (3.5) (Fushinobu et al. 1998).

The wide pH stability observed for the PPEndo is advantageous since in most industrial application cases, it is not economical or efficient to adjust the reaction conditions to neutral pH. Therefore, a broad range of pH stability is useful in an industrial setting (Turner et al. 2007).

Generally, alkaline-stable cellulases can be primarily applied as additives in washing powder and detergents. Cellulases that are stable under mild conditions (pH 5.0–6.0) can be used in brewing and biofuel production processes (Bernardi et al. 2018; Liu et al. 2015; Zhao et al. 2012). Acidophilic and acid-stable enzymes are favorable for application in the food and textile industries (Akbarzadeh et al. 2014; Zhao et al. 2012). According to Tavares et al. (2013), acidophilic enzymes are also suitable to industrial lignocellulose degradation, since most of the substrate is pretreated with inorganic acids.

Feed processing of lignocellulose in agricultural products is normally performed at high temperatures, therefore development and use of enzymes stable at extreme pH and conditions are imperative. High process temperatures are used during the production of pellets wherein the material is treated with moist heat (70-90 °C), followed by mechanical pressing. Different types of cellulases and hemicellulases are of interest to increase the fraction of digestible carbohydrates, which is an especially pronounced need in feeds for non-ruminant animals (e.g. poultry and pig) (Linares-Pasten et al. 2014).

Since enzymes need to be exposed to extreme conditions during several industrial processes, stability to high temperatures within a wide pH range, as well as tolerance to inhibition by reaction products, are the most desired enzymatic properties at the industrial level (Liu et al. 2015; Pellegrini et al. 2015). The PPEndo was observed to be both an acidophilic and thermophilic enzyme having broad ranges in temperature and pH stabilities required of enzymes for industrial application and consequently is well suited to the harsh process conditions used in lignocellulose bioprocessing, as well as during the incorporation of enzyme cocktails to the feed products.

Effect of metals ions and selected compounds on the CMCase activities of the partially purified endoglucanase

Among the different metal ions and selected compounds tested, manganese ion exerted the most enhancing effect on the enzyme activity of the PPEndo endoglucanase with about ~54% increase in activity (Figure 6). This was followed by potassium (~35%) and copper ions (7.5%). Other metal ions (Fe^{2+}, Ca^{2+}, Na^{+} and Co^{2+}) also enhanced the activity to a lesser degree. On the other hand, the enzyme activity was greatly affected negatively by ethylene diaminetetraacetic acid (EDTA), magnesium ion and SDS, and by Zn^{2+} to a lesser degree.

Metal ions can be associated with proteins and can also form complexes with other molecules linked to enzymes acting as electron donors or acceptors as Lewis´s acids, or as structural regulators (Riordan 1977). These ions can either activate or inhibit the enzymatic activity by interacting with amine or carboxylic acid group of the amino acids (Ishida et al. 1980).

The enhancing effect of Mn^{2+} ion on the endoglucanase activity was also shown in *Aspergillus niger* ANL301 and *Penicillium chrysogenum* PCL501, respectively (Azzeddine et al. 2013; Chinedu et al. 2011). It is assumed that this ion responds to certain amino acid residues in the active site of the protein, causing a conformational change in favor of higher activity (Tao et al. 2010). There is the possibility that the manganese ion may be a requirement for the enzyme activity and may even be an integral component of the enzyme complex (Chinedu et al. 2011). Similarly, studies on the kinetic enzyme properties of endoglucanases from *Aspergillus niger* AS101 by the group of Singh (1990) showed that the enzyme is a metallo-protein which requires certain metal ions for activation (Chinedu et al. 2011).

Activity of Cel5A, a metagenome-derived cellulase by Voget et al. (2006) was even enhanced in the presence of low concentrations of manganese, a phenomenon also reported for a cellulase from *Bacillus* (Sánchez-Torres et al. 1996). Other metal ions (Fe^{2+}, Ca^{2+}, Na^{+} and Co^{2+}) also enhanced the activity to a lesser degree. Co^{2+} and Mn^{2+} ions activated the cellulase from *Mucor circinelloides* (Saha 2004) and *Chalara paradoxa* (Lucas et al. 2001), respectively. Tavares et al. (2013) reported that for rEG A endoglucanases of *A. nidulans,* interaction with Ca^{2+}, Co^{2+} and Cu^{2+} resulted in increased enzyme activity, possibly because these ions exert a stabilizing effect on the enzyme structure without interfering in the catalytic site.

In contrast, Li et al. (2006) reported the inhibition of endoglucanase from *Bacillus* sp. AC-1 by Fe^{2+} and Cu^{2+}, while the enzyme from *Streptomyces* sp B-PNG23 was not affected by Fe^{2+} or Cu^{2+}. Fe^{2+} can complex with D/L-lysine and L-methionine (Vassilev et al. 2013) while Cu^{2+} can complex with

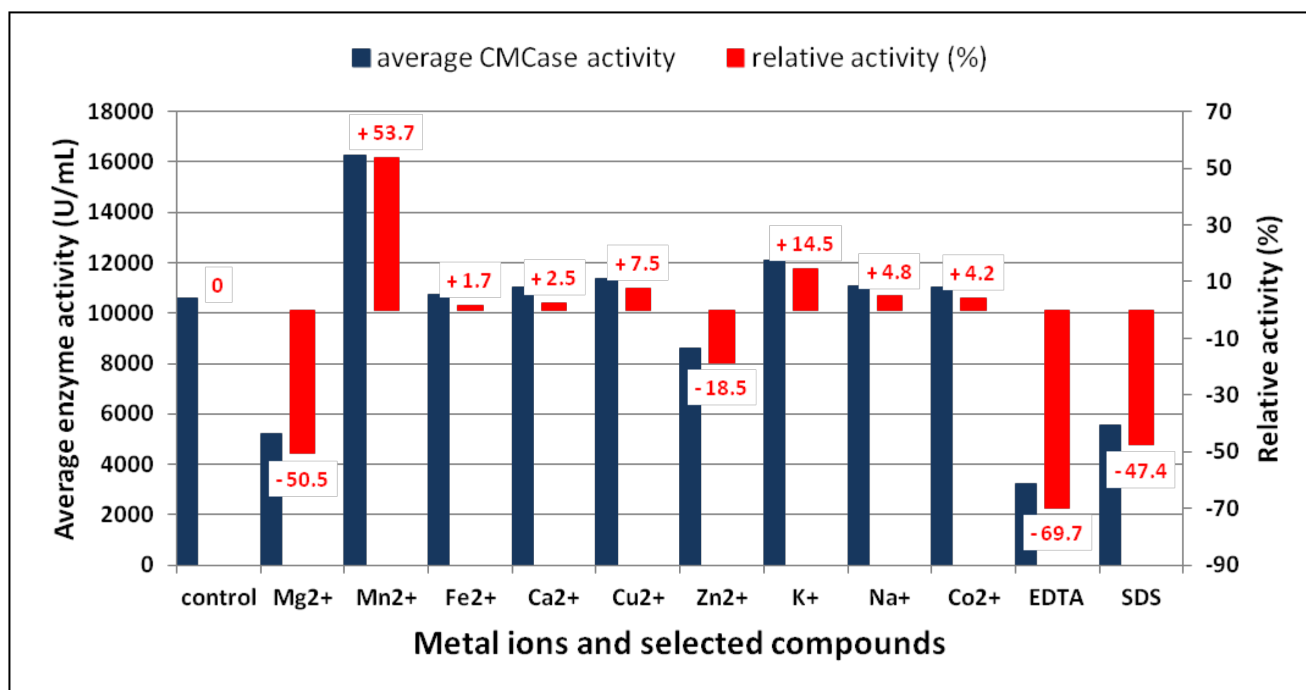

Figure 6: Effect of metal ions and selected compounds on the CMCase activity of the partially purified endoglucanase assayed at 80 °C for 30 min using carboxymethylcellulose (CMC) as substrate. The relative activity is equal to the CMCase activity observed after incubation with the metal ions and selected chemical compounds over that in the absence of metal ions and selected compounds multiplied by 100. The red colored bar represents the stimulatory (+ % relative activity) or inhibitory (- % relative activity) effect of the metal ions and selected compounds on the endoglucanase activity. Values denote the actual +/- % relative activity after treatment.

histidine (Kryukova et al. 2003). The negative effect of Cd^{2+}, Fe^{2+} and Hg^{2+} on the cellulase activity was related by Smriti and Sanwal (1999) to interference in the SH-groups present in the active site. Tao et al. (2010) observed that the endoglucanase from *Aspergillus glaucus* XC9 was inhibited by Cd^{2+}, Pb^{2+} and Cu^{2+}. It is not clear, but cations might have affected the amino acid structure of enzyme active site or other amino acid residues, though the impact is dependent on several characteristics such as the enzyme's amino-acid structure, buffer condition and other related characteristics (Ghose 1987).

Chelating agents such as EDTA, ethylene glycol (or β-mercaptoethanol), and 1,2-bis diphenylphosphino-ethylene (DPPE) may activate some enzyme activities, especially cellulases, by sequestering inhibitory metal ions from the aqueous system (Miyano et al. 1985). On the other hand, the negative effect of chelating agents on enzymatic activity suggests that enzyme activities depend on the inorganic ion that was sequestered (Pereira et al. 2016). Similarly, the strong chelating effect of EDTA for divalent metal ions (Chinedu et al. 2011) could have led to the removal of manganese ions from the enzyme mixture and subsequent decrease in enzyme activity of the PPEndo.

In contrast, the endoglucanases of *Streptomyces* sp. B-PNG23 seem resistant to SDS while dithiothreitol (DTT) and EDTA reduced its activity (Azzeddine et al. 2013). Magnesium ion and SDS, and by Zn^{2+} to a lesser degree also had a similar negative effect probably due to changes in electrostatic bonding, which would change the tertiary structure of the enzyme (Li et al. 2003). These non-ionic reagents could modify the cellulose surface's property, so this might affect the enzyme activity slightly (Han et al. 2005; Fu et al. 2010).

The knowledge about the activators and inhibitors of cellulase activity is very important in the context of industrial applications since ingredients/components in processing steps may contain inhibitory substances. Some metal ions may be present at significant levels in industrial enzymatic biomass converting systems, after being added as reagents, dissolved from a reactor/pipeline, or brought from water or other raw material sources (Tejirian and Xu 2010). Metal ions which are present in water and/or other reagents employed in industrial processes, or may come from equipment corrosion can influence the activity of the enzymes (Oviedo and Rodriguez 2003).

Lignocellulose is a highly complex and rigid substrate wherein crystallinity of cellulose, available surface area, and the distribution of lignin and hemicellulose therein are factors that limit the hydrolysis rate of plant cell walls. To enable the production of abundant amounts of hexose, as well as pentose, or monosaccharides for biofuel production, optimized thermostable multicomponent mixture of enzymes for both cellulose and hemicellulose hydrolysis will be essential to reduce the overall cost of production. Knowledge with regards to the effects of metal ions on each enzyme component will greatly help the stability and effectiveness of the whole enzyme mixture.

Effect of different substrates on the CMCase activities of the partially purified endoglucanase

The Volumetric CMCase activity of the PPEndo varied with the substrate used (Figure 7). Highest activity (9979 U/ml) was obtained using CMC followed by filter paper (958 U/ml). Low activity (521 U/ml) was obtained from xylan substrate with the lowest activity for cotton (110 U/ml).

Carboxymethylcellulose is a water-soluble long-chained cellulose with carboxymethyl substitutions commonly used as a model substrate for detecting β-1,4-endoglucanases. Similar to the results obtained in this study for PPEndo, the endo-1,4-glucanase purified from *D. eschscholzii* by Yin et al. (2010) and Karnchanatat et al. (2008) also showed highest activity against CMC with almost no hydrolysis ability against crystalline substrates of avicel, cotton fiber, filter paper, xylan or p-nitrophenyl-beta-D-glucoside (p-NPG). In the study of Yin et al. (2010) the *D. eschscholzii* endoglucanase degraded filter paper at approximately 25.3% of activity against carboxymethylcellulose but showed lower activity (wt %) against Avicel® PH-101, a brand of microcrystalline cellulose.

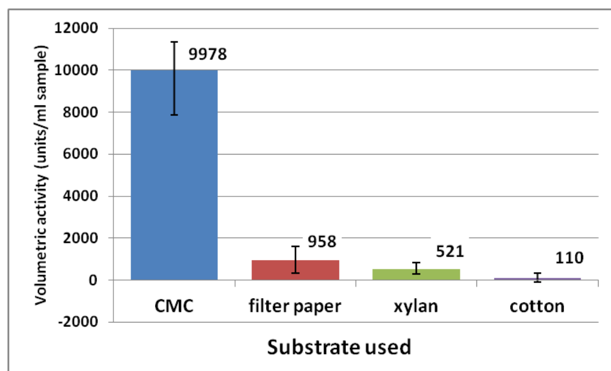

Figure 7: CMCase activity of the partially purified endoglucanase (PPEndo) from *Escherichia coli* BL21 clone pEngl-24-21-63 at 80 °C, pH 3.0 using different substrates. Error bars indicate standard deviation with respect to the mean of 8 technical assay replicates.

In the study of Huang et al. (2005), alternative substrates of β-glucanases, such as xylan, laminarin and lichenan, were not hydrolyzed by thermostable endoglucanase SSO1949 from *Sulfolobus solfataricus*. However, in their study, assay of endoglucanase SSO1949 for the aforementioned substrates was conducted at low temperatures and at neutral pH since at the optimal reaction condition of SSO1949 (high temperature and low pH), these polysaccharides show a high rate of spontaneous hydrolysis and the activity could not be measured reliably (Huang et al. 2005).

The PPEndo has low capability to cleave 1,4-β-D-xylosidic linkages in xylan similar to the endoglucanase from *Fusarium lini* reported by Rao et al. (1986), and the endoglucanase from *Trichoderma reesei* which exhibited hydrolytic activity towards a broad range of substrates including β-glucan, carboxymethylcellulose, hydroxyethyl-cellulose, RBB-xylan, methylumbelliferyl-β-d-cellobioside, and methylumbelliferyl-β-d-lactoside (Takashima et al. 1998). The low hydrolytic activity towards oat spelt xylan was also reported in fungal endoglucanases from *Aspergillus niger* (Akiba et al. 1995), *Macrophomina phaseolina* (Wang and Jones 1995), *Talaromyces emersonii* (Maloney et al. 1985), *Thermomonospora fusa* (Calza et al. 1985), and *Trichoderma koningii* (Wood and McCrae 1978). Low activities obtained from cotton and filter paper, even though they are cellulosic materials may probably be due to non-pretreatment of the substrates.

In the study of Huang et al. (2005), no hydrolytic activity was detected for the endoglucanase SSO1949 with microcrystalline cellulose as substrate which is characteristic of cellulases without cellulose binding domains (CBDs) in their genome. The hydrolytic activity of PPEndo against crystalline cellulose at both acidic and neutral pH values needs further investigation.

Pre-treatment of lignocellulosic residues is usually done under very high temperature (~200 °C) in combination with organic acids (pH 3-4) to break the lignin seal, loosen up the lignocellulose structure and expose the crystalline cellulose. This is followed by the use of cellulase enzymes that break the β-1-4 glycosidic bonds of cellulose to glucose, cellobiose, cello-oligosaccharides. The use of a thermo-acid stable enzyme like PPEndo during the enzyme hydrolysis step further hydrolyzes the cellulose and enhances the release of glucose molecules. The temperature of the system does not need to be cooled down to mesophilic (~30-40 °C) levels, thus savings in steam generation and shorter processing time can be achieved. In addition, a decrease in chemicals and water used for washings are achieved since the pH of the hydrolysis bath need not be adjusted to almost neutral (~pH 6-7) before adding the usually used mesophilic hydrolytic enzyme. The product of the hydrolysis is glucose which can be used in bioethanol production.

Furthermore, thermo-acid stable enzymes like PPEndo can be used as a component of feed formulations for non-ruminant animals (e.g. poultry and pig) to increase the fraction of digestible carbohydrates (Linares-Pasten et al. 2014). High process temperatures are used during the processing of lignocellulose in agricultural products and preparation of pellets wherein the material is treated with moist heat (70-90 °C), followed by mechanical pressing, conditions under which PPEndo can still be active. Throughout the pretreatment process, the recalcitrant structure of lignocellulose is disrupted resulting in breakage of lignin sheath, degradation of hemicellulose and reduction in crystallinity. The degree of polymerization of cellulose is greatly reduced producing simple components such as cellulose, hemicelluloses, and lignin (Baruah et al. 2018).

Enzyme supplementation is well documented as effective in breaking polymeric chains of non-starch polysaccharides (NSPs) (e.g. hemicellulose, cellulose and pectin) and hence improving the nutritive value of fibrous feedstuffs (Alemawor et al. 2009). Due to the chemical structure of plant cell wall matrix, a combination of various fibrolytic enzyme activities (including cellulases and hemicellulases) has been recommended to enhance saccharification of NSPs (Beldman et al. 1987). Commercially available enzyme "cocktails" contain different hemicellulases, e.g. xylanases and mannanases that act in synergy in the decomposition of the hemicellulose portion (Linares-Pasten et al. 2014) releasing xylan and mannans in the process. Considering that the concentration of the PPEndo used in the study was low, it was still able to hydrolyze non-crystalline CMC and xylan substrates albeit low enzyme activities were obtained. This ability together with its heat stability, point to its potential as a component of enzyme "cocktails" for feed product application.

CONCLUSION AND RECOMMENDATIONS

The recombinant endoglucanase, MM-Engl was characterized to be optimally active at high temperature and slightly acidic conditions with a broad range of temperature and pH stabilities. Its high resistance to changes in pH and temperature makes it well suited for application in lignocellulose bioprocessing for bioethanol production and as an enzyme component in the feed industry.

Cellulose is hydrolysed by organic acids at high temperatures and use of thermo-acid stable enzymes promote penetration of the enzymes in the lignocellulosic residue in the subsequent step of enzymatic hydrolysis. The stability of the enzymes can reduce the need of cooling the reaction mixture from the previous high temperature step which could hasten the reaction process and reduce production costs.

Utilization of the recombinant endoglucanase as an enzyme component in enzyme "cocktails" for saccharification of lignocellulosic hydrolysates from high temperature-dilute acid pre-treatment needs to be conducted. Likewise, incorporation of the recombinant endoglucanase in enzyme "cocktails" for feed formulations should be investigated and demonstrate the actual stability of the enzyme under pre-treatment conditions and efficacy for hydrolysis of NSPs in fibrous feedstuffs. The thermal stability of MM-Engl beyond 1 h needs to be investigated in order to characterize the half-life of the protein at specific conditions.

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CONFLICT OF INTEREST

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

CONTRIBUTIONS OF INDIVIDUAL AUTHORS

RDT was directly involved in the expression and characterization experiments, data analysis, and drafting the manuscript. AKR supervised the experiments and edited the manuscript. All authors read and approved the final manuscript.

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