Characterization of a Novel Thermophilic Endopolygalacturonase Produced by *Bacillus licheniformis* IEB-8

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Endopolygalacturonases characterized until now have either low working temperatures, working pH in acidic range, high Michaelis–Menten constant (K_m), or a high production cost. These characteristics are a hurdle in the industrial applications of these endopolygalacturonases. The purpose of this work was to characterize a novel endopolygalacturonase produced by *Bacillus licheniformis* IEB-8. Phylogenetic analysis of *Bacillus licheniformis* IEB-8 showed that the isolate was unique. Citrus peels were used as the only nutrient source for the growth of *Bacillus licheniformis* IEB-8, allowing a cheap production of endopolygalacturonase. All the synthetic carbon sources showed a negative impact on the production of endopolygalacturonase, while ammonium sulfate enhanced its production. Among different metal ions, Zn^{+2} showed a negative effect while Mg^{+2} and Ca^{+2} did not have any significant effect on the endopolygalacturonase activity. A Lineweaver-Burk plot was prepared for the characterization of the kinetic parameters including K_m and V_{max}, which were 0.45 mg/mL and 285.7 µM/min, respectively. A comprehensive comparison of the endopolygalacturonase from this study with the available literature indicated that it is better than the reported and commercially available endopolygalacturonases in having the optimum working temperature of 55 °C, a low K_m of 0.57 mg/mL, and pH of 7 to 8, which indicated its novelty.

Keywords: Endopolygalacturonase; Bacillus licheniformis; Thermophilic; Phylogenetic tree; Kinetics

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INTRODUCTION

Pectinolytic enzymes are polysaccharidases that degrade pectins in the middle lamella and the primary cell walls of plants. This process is widely used in winemaking because pectinases improve liquefaction, juice yield, clarification, filterability, and can increase the release of color and flavor compounds entrapped in grape skins (Merín *et al.* 2015; Sandri and da Silveira 2018). Pectic enzymes may be alkaline or acidic in nature depending on their source of production. Alkaline pectinases are synthesized by prokaryotic microorganisms, and acidic pectinases are produced from eukaryotic microorganisms (Kubra *et al.* 2018). Among the various pectinases, bacterial pectinases

have advantages over other pectinases (Roy *et al.* 2018). The *Bacillus* genus is the predominant bacterial workhorse in microbial fermentation. They produce more than two dozen biologically active molecules, providing a high potential for biotechnological and biopharmaceutical applications. *Bacillus subtilis* and *Bacillus licheniformis* have been given the US Food and Drug Administration's Generally Regarded as Safe (GRAS) status. In addition, many researchers use *Bacillus* strains (*e.g.*, *B. subtilis*) that grow on cheap substrates such as agro-wastes (Kavuthodi and Sebastian 2018). Alkaline pectinases are used in the degumming and retting of fiber crops and in the pretreatment of pectic wastewater from fruit juice industries. These enzymes come mostly from bacterial sources (Kashyap *et al.* 2001; Roy *et al.* 2018).

The discovery of novel enzymes for their commercial applications in the food industry is a challenge for food scientists and biotechnologists (Abbasi *et al.* 2011; Kubra *et al.* 2018). The current study characterized a novel endopolygalacturonase (endo-PG) produced by *B. licheniformis* IEB-8 and tested different factors to enhance the production of endo-PG. Endopolygalacturonase produced by *B. licheniformis* IEB-8 was compared with the commercially available pectinases and endo-PGs to check the novelty.

EXPERIMENTAL

Maintenance of *Bacillus licheniformis* IEB-8

Hadri *et al.* (2018) isolated *B. licheniformis* IEB-8 from rotten fruits and vegetables. *Bacillus licheniformis* IEB-8 was cultured on YEP medium (Munir *et al.* 2015). Different growth parameters to produce endo-PG were optimized using Response Surface Methodology (RSM) under central composite design as outlined by Hadri *et al.* (2018). Pure slants of *B. licheniformis* IEB-8 were preserved in the form of stab cultures at 4 °C and 50% glycerol stock solutions at -80 °C (Garcia-Maceira *et al.* 2001; Ngo *et al.* 2008; Handa *et al.* 2016; Khan and Latif 2016).

Multiple Sequence Alignment and Phylogenetic Tree Analysis

The 16S rRNA gene sequence of *B. licheniformis* IEB-8 was blasted in the NCBI nucleotide database. The 16S rRNA gene sequence of the first ten strains showed resemblance to the isolate used for the multiple sequence alignment (ClustalW; https://www.genome.jp/tools-bin/clustalw) and for the construction of the phylogenetic tree using Multiple Evolutionary Genetic Analysis (MEGA) (Version 6, https://www.megasoftware.net/download_form) (Baroncelli *et al.* 2016; Khan and Latif 2016).

Use of Agriculture Wastes for the Cheap Production of Endo-PG

Different agriculture wastes including citrus peels, white radish peels, and apple peels were used as the only nutrient source for *B. licheniformis* IEB-8. The crude extracts of the three agriculture wastes were compared for endo-PG production, and the one with the highest endo-PG yield was used in subsequent experiments (Munir *et al.* 2015).

Effect of Carbon Sources on the Endo-PG Production

Glucose, fructose, galactose, and lactose were used as carbon sources to check for their effect on endo-PG production (Munir *et al.* 2015). Endo-PG produced in the current work was purified by ammonium sulfate precipitation and size exclusion chromatography

as mentioned by Hadri *et al.* (2018) and was used to check the endo-PG activity.

Effect of Nitrogen Sources on the Endo-PG Production

Ammonium sulfate, ammonium carbonate, ammonium phosphate, and ammonium nitrate were used as nitrogen sources to check for their effect on endo-PG production (Darah *et al.* 2013; Embaby *et al.* 2014; Kaur *et al.* 2016; Sethi *et al.* 2016).

Effect of Different Metal Ions on the Endo-PG Activity

Different metal ions including Mg^{+2}, Ca^{+2}, and Zn^{+2} were tested for their effect on endo-PG activity (Gupta and Kalpana 2011; Paudel *et al.* 2015; Sethi *et al.* 2016).

Kinetic Parameters Characterization of Endo-PG

An endo-PG assay was carried-out with different polygalacturonic acid concentrations (0.2, 0.4, 0.6, 0.8, and 1 mg/mL) to check for the effect of the substrate concentration on the enzyme activity and to find the values of the Michaelis–Menten constant (K_m) and V_{max} (Paudel *et al.* 2015). K_m and V_{max} were determined by preparing the Lineweaver–Burk plot (a double reciprocal plot) of $1/(V_o)$ *vs.* $1/(S)$.

Comparison with the Commercially Available Endo-PGs

The optimum temperature, pH, and K_m of the endo-PG produced by *B. licheniformis* IEB-8 were compared with those of the endo-PGs available from different commercial suppliers.

RESULTS AND DISCUSSION

Molecular Characterization of *B. licheniformis* IEB-8

The 16S rRNA gene sequence of *B. licheniformis* IEB-8 was submitted to GenBank with the accession number KY816931 (Hadri *et al.* 2018). The phylogenetic tree constructed after multiple sequence alignment consisted of three clades. Clade 1 consisted of seven *B. licheniformis* strains, clade 2 consisted of only one *B. licheniformis* strain, and clade 3 consisted of three *B. licheniformis* strains, including *B. licheniformis* IEB-8. *Bacillus licheniformis* IEB-8 is unique and is a single strain under the branch-tip of its node. *Bacillus licheniformis* IEB-8 showed some similarity with *Bacillus licheniformis* strain GY-43 and *Bacillus licheniformis* strain LZLJ005 because it was in the same clade. However, it was different when compared to the other known species of *B. licheniformis*, which indicated its uniqueness (Fig. 1).

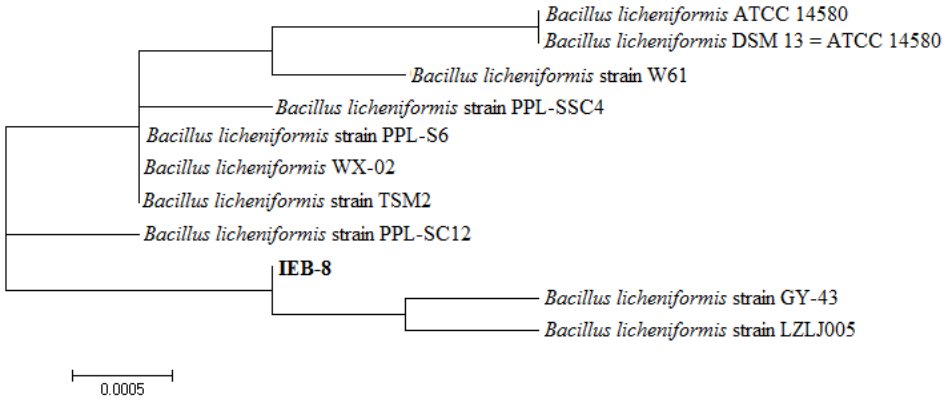

Fig. 1. Phylogenetic analysis of 16S rRNA gene sequence of *B. licheniformis* IEB-8

Use of Agriculture Wastes for the Cheap Production of Endo-PG

B. licheniformis IEB-8 showed maximum endo-PG production with citrus peels. There was a highly significant difference (indicated by $p < 0.01$) when producing the endo-PG by *B. licheniformis* IEB-8 with different peels (Fig. 2). The citrus peels were used in subsequent experiments.

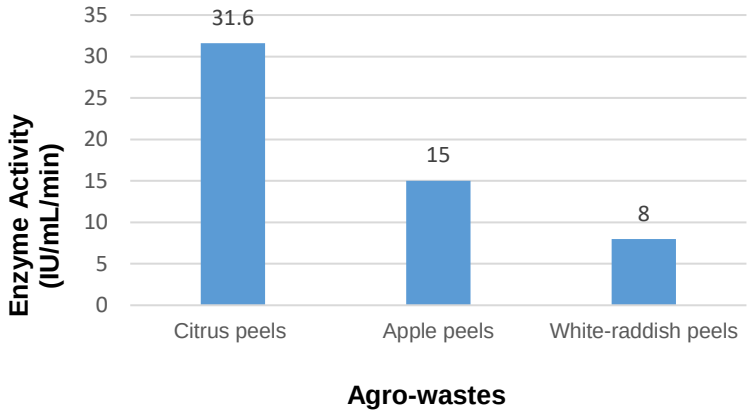

Fig. 2. Comparison of the endo-PG produced by *B. licheniformis* IEB-8 using three agro-wastes (citrus peels, apple peels, and white radish peels)

Use of Carbon Sources to Produce Endo-PG

The carbon sources at all the concentrations had a negative impact on the production of endo-PG by *B. licheniformis* IEB-8. There was a decrease in endo-PG production compared with the control (only citrus peels), which produced the maximum endo-PG with an activity of 48 IU/mL/min (Fig. 3). Galactose with a concentration of 2.5% produced the least amount of endo-PG with an activity of 0.0065 IU/mL/min. Lactose with a concentration of 0.5% gave the maximum production, after the control, which was 35 IU/mL/min.

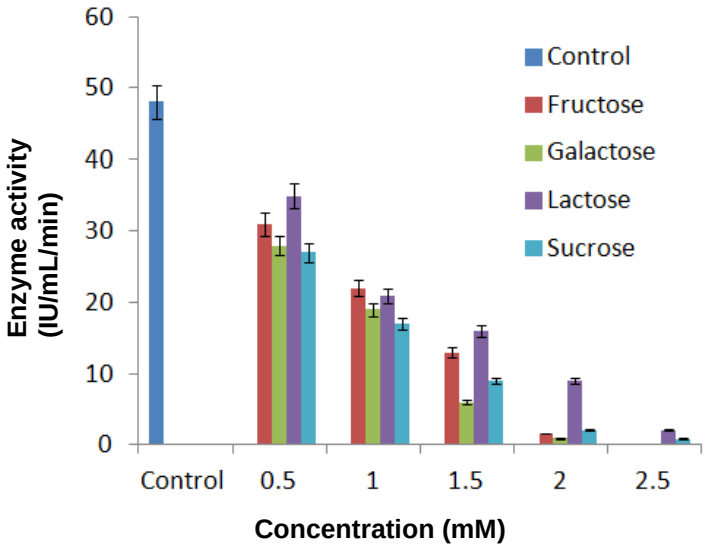

Fig. 3. Effect of carbon sources on the production of endo-PG by *B. licheniformis* IEB-8. (Error bars indicate standard error)

Different studies show the positive and negative effects of using different bacteria and fungi to produce endo-PG. Phutela *et al.* (2005) mention the positive effect of sucrose on the production of pectinolytic enzymes, produced by *Aspergillus fumigatus*, which leads to the enhanced production of the enzymes. They further reported a decrease in the production of the pectinolytic enzymes in the presence of glucose. Rehman *et al.* (2015) reported a decrease in the production of pectinase in the presence of the carbon sources lactose, glucose, sucrose, maltose, and galacturonic acid. In addition, Embaby *et al.* (2014) report the negative effects of glucose, xylose, maltose, and sucrose. Sethi *et al.* (2016) additionally mention the negative effects of the carbon sources they use, excluding the mannitol because it has a positive effect when producing endo-PG. According to Ahlawat *et al.* (2009), catabolite repression decreases pectinase production in the presence of the carbon sources. Similar reasons are reported by others (Solis-Pereira *et al.* 1993; Darah *et al.* 2013).

Use of Nitrogen Sources to Produce Endo-PG

Different nitrogen sources impacted differently on endo-PG production by *B. licheniformis* IEB-8 (Fig. 4). Ammonium sulfate, with a concentration of 1%, produced the most endo-PG with an activity of 78 IU/mL/min. Calcium nitrate, with a concentration of 2.5%, produced the least endo-PG with an activity of 1.2 IU/mL/min. Ammonium sulfate and ammonium chloride produced more endo-PG than the control (only citrus peels). Ammonium nitrate and calcium nitrate negatively impacted endo-PG production.

Nitrogen sources have variable effects on the production of pectinolytic enzymes. Darah *et al.* (2013) reported that the best nitrogen source was yeast extract followed by ammonium sulfate, peptone, and ammonium hydrogen phosphate. In contrast, urea, ammonium nitrate, and sodium nitrate inhibited the production of polygalacturonases. Embaby *et al.* (2014) reported a decrease in pectinase production when in the presence of nitrogen sources. Rehman *et al.* (2015) reported the positive effects of using yeast extract and peptone compared with inorganic nitrogen sources.

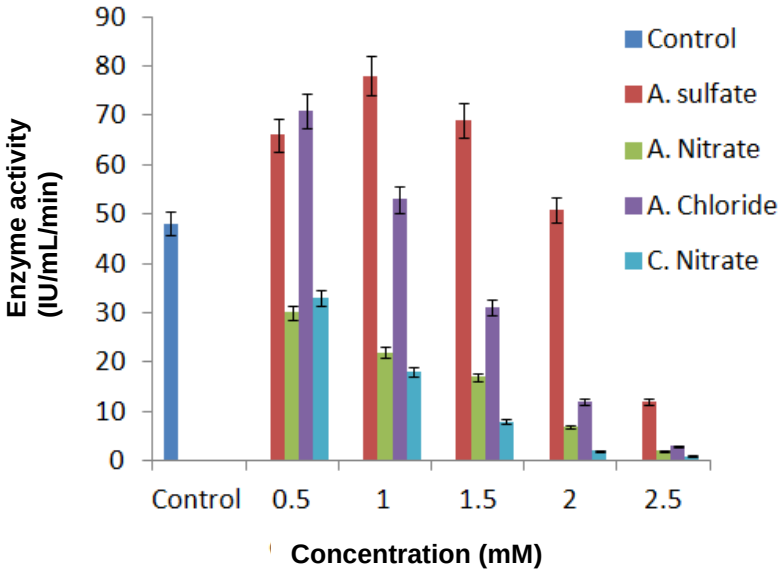

Fig. 4. Effect of different nitrogen sources on the production of endo-PG by *B. licheniformis* IEB-8. (Error bars indicate standard error)

Effect of Metal Ions on the Endo-PG Activity

None of the metal ions tested during the endo-PG assay impacted the endo-PG activity when tested at lower concentrations, excluding Zn^{2+}, which negatively impacted the endo-PG activity (Paudel *et al.* 2015). This result suggests that Zn^{2+} acts as an inhibitor of endo-PG. Additionally, Mg^{2+} and the control showed similar activity (47 IU/mL/min and 44 IU/mL/min, respectively), while Ca^{2+} had less activity than Mg^{2+} and the control (Fig. 5). Gupta and Kalpana (2011) reported that polygalacturonase does not require metal ions for its activity, and some metal ions, such as Al^{+3}, Zn^{+2}, and Ca^{+2}, have a negative effect on its activity.

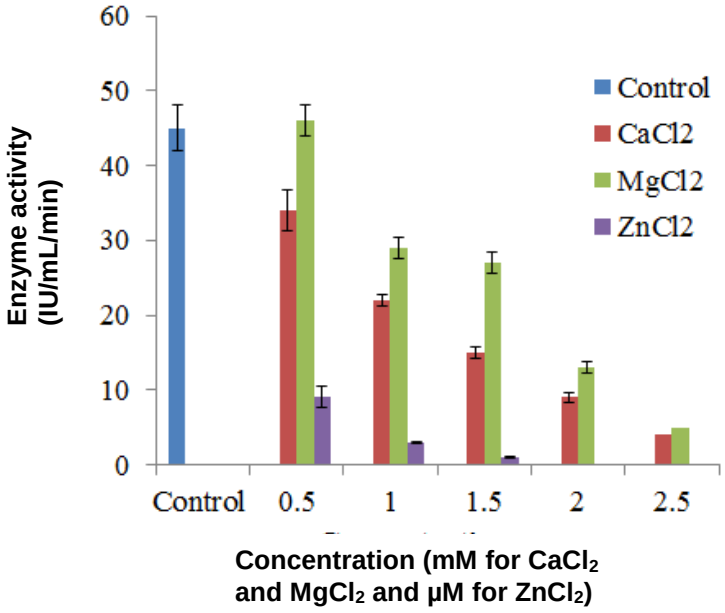

Fig. 5. Effect of different metal ions on the activity of endo-PG produced by *B. licheniformis* IEB-8. (Error bars indicate standard error)

Kinetic Parameters Characterization

The K_m and V_{max} values of the endo-PG were determined using a Lineweaver-Burk plot. For this purpose, 1/(*S)* (x-axis) was plotted against 1/(V_o) (y-axis). The K_m and V_{max} values of the endo-PG produced by *B. licheniformis* IEB-8 were 0.45 mg/mL and 285.7 µM/min, respectively (Fig. 6).

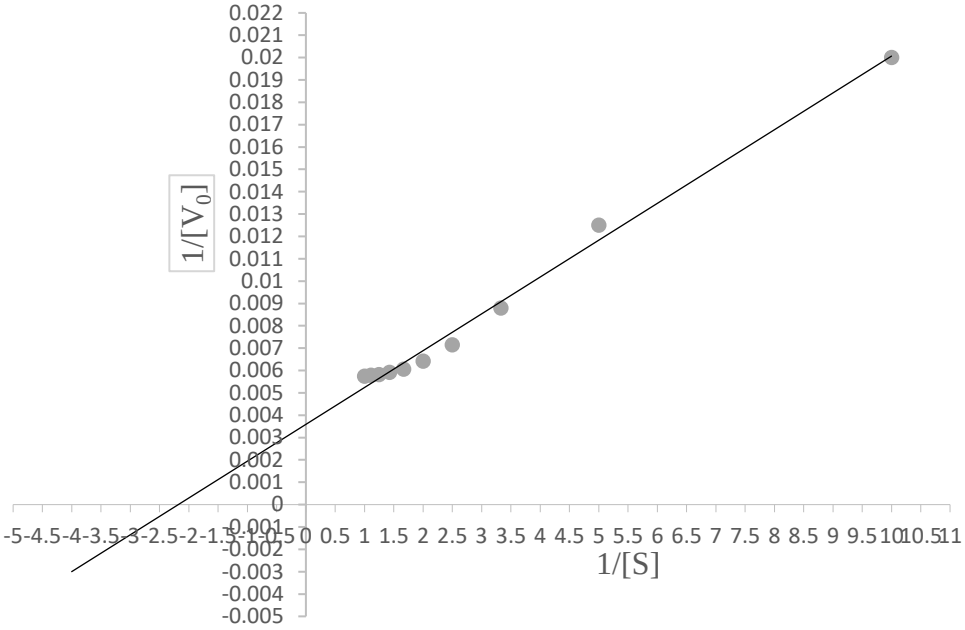

Fig. 6. Lineweaver-Burk plot of 1/(S) vs 1/(V_o) for the estimation of Km and Vmax of endo-PG produced by *B. licheniformis* IEB-8

Table 1. Comparison of Specific Activity, Optimum Temperature, and Optimum pH of the Commercial Pectinolytic Enzymes with Endo-PG Produced by *B. licheniformis* IEB-8

Enzyme (Code)	Company	Source	Specific Activity (U/mg)	Optimum Temperature	Optimum pH
Endo-PG by IEB-8	Hadri *et al.* (2018)	*Bacillus licheniformis* IEB-8	32	55	7
Endo-PG (P5079)	Sigma-Aldrich (Merck)	*Aspergillus japonicus*	200 to 600	30	5
PG (P3304)	Sigma-Aldrich (Merck)	*Aspergillus japonicus*	300 to 1500	30	5
Pectolyase (P3026)	Sigma-Aldrich (Merck)	*Aspergillus japonicus*	≥ 0.3	25	5.5
Pectinase (P2401)	Sigma-Aldrich (Merck)	*Rhizopus* sp.	0.4 to 0.8 (400 to 800 U/g)	25	4
Pectinase (17389)	Sigma-Aldrich (Merck)	*Aspergillus niger*	> 1	50	4
Pectinase (P-4716)	Sigma-Aldrich (Merck)	*Aspergillus niger*	≥ 5	25	4
Endo-PG (E-PGALUSP)	Megazyme	*Aspergillus aculeatus*	150	40	5.5
Exo-PG (E-EXPGA)	Megazyme	Recombinant (*Yersinia enterocolitica)*	220	60	6
Pectate Lyase (E-PCLYAN)	Megazyme	*Aspergillus* sp.	180	40	8

In enzymatic reactions, the kinetic parameter, which describes the enzyme efficiency, is important (Oumer and Abate 2017). There are different K_m and V_{max} values for the PGs produced by different organisms. Oumer and Abate (2017) reported the K_m and V_{max} values of pectinase produced by the strain *Bacillus subtilis* Btk27 as 1.879 mg/mL and 149.6 µM/min, respectively. Additionally, Fratebianchi *et al.* (2017) report the K_m and V_{max} values of endo-PG produced by *Aspergillus sojae* to be 0.134 mg/mL and 9.6 µM/mg/min. The endo-PG produced by *B. licheniformis* IEB-8 has affinity towards polygalacturonic acid because of its lower K_m value (0.57 mg/mL), which is better than the K_m value of the pectinase mentioned by Oumer and Abate (2017).

Comparison with the Commercially Available Endo-PGs

Commercial pectinolytic enzymes, including endo-PG, are available from companies such as Sigma-Aldrich (Merck) and Megazyme. Table 1 shows the source, optimum temperature, optimum pH, and the specific activity of the endo-PG produced by *B. licheniformis* IEB-8 and the commercial pectinolytic enzymes. Only the endo-PG produced by *B. licheniformis* IEB-8 has an optimum temperature above 50 °C, a pH in the neutral-alkaline region, and a low K_m value. The other enzymes either have a lower optimum temperature, an optimum pH in the acidic range, or have a high K_m value.

CONCLUSIONS

1. Based on the present study it is reported that endo-PG produced by *B. licheniformis* IEB-8 is the only reported endo-PG that can efficiently be applied in industries such as the textile industry. Additionally, it can be applied for the maceration of vegetables because it has an optimum working temperature (as reported in the current study) of 55 °C (being the suitable temperature for the industrial application), a pH of 7 to 8, and a low K_m value.
2. These properties of endo-PG by *B. licheniformis* IEB-8 were shown to be more effective than other available endo-PGs including the commercially available pectinolytic enzymes produced by Sigma-Aldrich (Merck) and Megazyme.
3. Ammonium sulfate and ammonium chloride enhanced the production of endo-PG as compared to the control (having only citrus peels as the nutrient source), while all of the synthetic carbon sources reduced the production of endo-PG.

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