CHARACTERIZATION OF AN ALKALINE ENDO-POLYGALACTURONASE (PGase LBW 5117) FROM ALKALIPHILIC BACILLUS HALODURANS LBW 5117 AND DEMONSTRATION OF ITS BIO-SCOURING POTENTIAL

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ABSTRACT

The demand for pectinases in the global market has been on the rise in recent years due to their significant biotechnological uses. The present study involves the characterization of a crude alkaline pectinase from Bacillus halodurans LBW 5117 and the demonstration of its bioscouring potential. The enzyme was an endo polygalacturonase (PGase LBW 5117) that was brown and had a slight fermentation odor. It exhibited an activity of 0.21 U/ml and retained 67, 90.5, 95.2, and 95.2 % of this activity after 1 year of storage at 20, 4, 20, and 30 °C, respectively. Its operational pH and temperature ranged between 10.2 - 11.0 (optimum, 10.5) and 45 - 57 °C (optimum 50 °C), respectively. Metal ions, e.g., K+, Ca2+, Mg2+, Fe3+, Cu2+, Na+, Mn2+, and Zn2+ either stimulated or did not significantly affect its activity at 1 mM. The enzyme retained only 15 % of its activity after 8 h of incubation at 50 °C, but this improved to 200 % in the presence of 1.5 mM Ca2+ and 0.05 mM Tween 20. The enzyme was cellulase-free and hydrolyzed pectin in an endo-manner. Moreover, when used under the established optimized operating conditions, it degraded and eliminated pectin (and other non-cellulosic hydrophobic impurities that adhere to it) from woven cotton (weight loss = 0.68 %) and produced a more hydrophilic fabric with improved wettability for water (drop test = 10 sec) and dye (capillary rise test = 28 mm after 30 min). This result shows that PGase LBW 5117 possesses good operational properties, potentially making it a good bioscouring agent. It is, however, recommended that the bioscouring process be optimized, and its effectiveness be compared with that of standard bio- and/or chemical-scouring processes.

Keywords: Pectinase; Characterization; Woven cotton; Bioscouring; Absorbency test

INTRODUCTION

Pectic substances are complex, heterogeneous, and acidic glycosidic polysaccharides predominantly found in plants’ middle lamella and primary cell walls (Jayani et al., 2005). They are predominant in vegetables, fruits, cereals, and fibers and, are characterized by a backbone of D-galacturonic acid residues linked by α-(1,4) linkages with a few residues of rhamnose in the main chain and lactose, xylose arabinose, and galactose on the side chains (Kohli & Gupta, 2015). The C-6 of galacturonic acid residues contain carboxyl groups, which are partially esterified by methyl groups, resulting in: a) low degrees of esterification (DE) (DE ≤ 25), b) medium DE (50-75 %) and c) high DE (≥ 85 %). Alternatively, they may be partially or completely neutralized by metal ions such as K+, Na+, Mg2+, or Ca2+(Jayani et al., 2005).

Enzymes that hydrolyze pectic substances are broadly referred to as pectic enzymes or more, commonly pectinases. Micheli (2001), and Oumer and Abate (2017) classified pectinases into three major groups, depending on their mode of action: a) protopectinases (no EC number assigned), b) esterases (also called pectinesterase (PE) or pectin methylesterase (PME) (EC 3.1.1.11), and c) depolymerases. Depolymerases are further divided into four different groups, depending on the preference of enzyme for the substrate, the mechanism of cleavage, and the splitting of the glycosidic bonds. They include pectin lyases (PNLs), pectate lyase (PLs) polymethylgalacturonases (PMGases), and polygalacturonases (PGases). PGases depolymerize polygalacturonic acid by catalyzing the hydrolytic cleavage of the α-(1,4) glycoside bonds in the chain. They include a) endo-PG (EC 3.2.1.15), which randomly cleaves the internal α-(1,4) glycoside bonds in the galacturonan chain to liberate oligo-galacturonates, and b) exo-PG I (EC 3.2.1.67) and exo-PG II (EC 3.2.1.82), which cleave the glycosidic bonds at the non-reducing ends of the galacturonan chains to liberate mono- and di-galacturonate respectively. On the other hand, PLs depolymerize polygalacturonic acid via β-elimination of the α-(1,4) glycoside bonds. They include a) endo-pectate lyase (endo-PEcL), which cleaves the internal α-(1,4) glycoside bonds in the galacturonan chain to liberate 4,5 unsaturated oligo-galacturonates and b) exo-PEcL, which cleaves the linkages at the non-reducing end of the chain to liberate 4,5 unsaturated mono- galacturonates.

Pectinases are also classified into acidic and alkaline pectinases, depending on their pH requirement for optimum activity (Li et al., 2008). Alkaline pectinases, particularly those that degrade polygalacturonic acid, e.g., endo-PEcLs, have been shown to exhibit both environmentally friendly and economic applications in industrial biotechnological processes such as pulp and paper making, pretreatment of pectic wastewaters, coffee bean- and tea leave-fermentation, animal feed production, and processing of textile fibers (Kohli & Gupta, 2015). Of importance to the textile industry, is their application in the scouring (bioscouring) of woven cotton, where they are used to specifically degrade and eliminate the undesirable high content of polygalacturonic acid found in both the outer cuticular layer of cotton fibers and the adjacent primary cell walls in woven cotton (Ansell & Mwaikambo, 2009). This also results in the removal of the loosened hydrophobic waxes and other non-cellulosic substances (e.g., hemicellulose, proteins, natural colorants, etc.) that adhere to it, leading to the exposure of the desired intact soft and smooth cellulosic structures of the cloth which, due to their good hydrophilicity; exhibit excellent absorbency properties (Colombi et al., 2021). Other advantages of using endo-PEcLs as bioscouring agents include; a) they do not require the use of harsh chemicals and b) they are biodegradable, implying that they are both fabric- and eco-friendly. Commercial bioscouring pectinases are however not used to scour woven cotton in developing countries like Kenya. This is because they are often costly high-temperature enzymes that have to be imported. As an alternative, the scouring process is carried out using strong caustic soda (pH 12 - 14) at high temperatures (95 °C) and pressure (25 - 30 psi) for 12 - 14 h. Under these conditions, the alkali may attack the cloth indiscriminately and lead to the production of damaged or poor-quality fabrics while generating high pollution loads in a time-consuming process [personal communication from Hose Too of Rivatex (EA) Limited]. Therefore, there is a need to replace this chemical scouring method with a more cost-effective fabric- and eco-friendly bioscouring process that utilizes a locally produced alkaline pectinase. However, for such an enzyme to be effective, it must perform optimally with respect to pH, temperature, metal ions, surfactant requirements, etc. Therefore, an understanding of the enzyme’s properties is also paramount. It is important to note that alkaline endo-polygalacturonases also have the potential to scour woven cotton, although there are no reports available in the literature regarding their use in such an application.

The objective of our study was to: a) determine the operational and storage properties of an alkaline endo-polygalacturonase from a locally isolated alkaliphilic bacterial strain (Bacillus halodurans LBW 5117), and b) demonstrate its potential to scour woven cotton under the established operating conditions.
Effect of pH on the activity of the enzyme

This was studied by carrying out the enzyme assays using 0.5 % (w/v) polygalacturonic acid sodium salt (substrate) prepared in different buffers (pH 3.0 - 13.0). The 50 mM buffers used in the study were prepared according to Gomori (1955) and the website http://delloyd.50megs.com/moreinfo/buffers2.html. They included citrate/diphosphate (pH 3.0, 4.0, 5.0), monophosphate/diphosphate (pH 7.0, 8.0), Tris-HCl (pH 8.0, 9.0), Glycine/sodium hydroxide (pH 9.0, 10.0, 10.5), sodium hydrogen carbonate/sodium hydroxide (pH 10.5, 11.0), diphosphate/sodium hydroxide (pH 11.0, 12.0) and potassium chloride/sodium hydroxide (pH 12.0, 13.0).

Effect of temperature on the activity of the enzyme

This was investigated by performing the enzyme assays at different temperatures ranging from 30 - 90 °C using Glycine/sodium hydroxide buffer (pH 10.5).

Effect of metal ions on the activity of the enzyme

The enzyme was incubated in the presence of K+, Ca2+, Mg2+, Fe2+, Cu2+, Na+, Mn2+ and Zn2+ (final concentrations ranging from 1, 5 and 10 mM, respectively) at 29 ± 3 °C. After 30 min, the residual activity of the enzyme was determined under the standard assay conditions. The activity of the enzyme was further determined in the presence of 1.0, 1.5, 2.0, 3.0, 5.0, and 10.0 mM Ca2+ ions in a similar fashion.

Effect of surfactants on the activity of the enzyme

The enzyme was incubated in the presence of Tween 20 (final concentrations = 0.25, 0.05, 0.1, and 0.15 mM) at 29 ± 3 °C. After 30 min, the residual activity of the enzyme was determined under the standard assay conditions.

Effect of optimum temperature on the stability of the enzyme

The enzyme was incubated in the absence and presence of Ca2+ and Tween 20 (final concentrations = 1.5 and 0.05 mM, respectively), individually, and in combination, at 50 °C, for 8 h. The samples were withdrawn after 0.5, 1, 2, 3, 4, 6, and 8 h for the determination of the residual activity of the enzyme under the standard assay conditions.

Effect of different substrates on the activity of the enzyme

The influence of pectin methylation on the activity of the enzyme was studied both qualitatively and quantitatively at pH 10.5. For the qualitative assay, a modified version of the method described by Heidari-Zadeh et al. (2018) was used. Ten (10) μl enzyme containing CaCl2 and Tween 20 (final concentrations = 1.5 mM and 0.1 mM, respectively) was aliquoted in small depressions (diameter = 0.5 cm) on the surfaces of agar media containing 0.5 % (w/v) a low degree of esterified pectin (DE ≤ 25 DE 20 - 34 %) (polygalacturonic acid), b) medium degree of esterified pectin (DE 25 - 75 %) and c) high degree of esterified pectin (DE ≥ 85 %), respectively. Similarly, the enzyme [containing CaCl2 and Tween 20 (final concentrations = 1.5 mM and 0.1 mM, respectively)] was inoculated in another set of wells on solid media containing 1 % (w/v) CMC sodium salt, 1 % (w/v) avicel and 1 % (w/v) cellobiose, respectively. The depressions were made using a sterile cork borer. The plates were incubated at 50 °C for 6 h, after which the pectin and cellulose plates were flooded with Lugol’s iodine (Hitha & Girijsa, 2014) and Gram’s iodine (Kasana et al., 2008) dye solutions, respectively. The activities of the enzyme and those of endoglucanase, exo- glucanase, and cellulase were calculated from the size of the hydrolyzation halos formed around the wells (if any) in the respective plates and expressed as solubilization index (SI) values (Hitha & Girijsa, 2014). For the controls, heat-denatured enzyme (95 °C for 30 min) was used.

Effect of pectin methylation on the activity of the enzyme was also studied qualitatively. The assays were carried out in liquid media, each containing 0.5 (w/v) pectin with a different degree of methylation (DE < 25 % DE 20 - 34 %; DE 25 - 75 %) and DE ≥ 85 %), under the standard assay conditions. Similarly, the effect of 1 % (w/v) CMC, 1 % (w/v) avicel, and 1 % (w/v) cellobiose (substrates) on the activity of the enzyme was studied (Klio et al., 2016). Briefly, 500 μl enzyme was incubated with a similar volume of 1 % (w/v) CMC sodium salt, avicel, and cellobiose, respectively, prepared in 50 mM glycine NaOH buffer (pH 10.5). The mixtures were incubated at 50 °C for 1 h, and the amount of reducing sugars formed in each assay was determined using the 3, 5-Dinitrosalicylic acid (DNS) method with glucose as the standard (Wang et al., 1997). In all the assays, heat-inactivated enzymes (95 °C, 30 min) were used as controls.
Mode of action of the enzyme
A modified version of the Thin layer chromatography (TLC) method described by Anand et al. (2016) was used to determine whether the enzyme hydrolyzes polygalacturonic acid in an exo- or an endo-fashion. A volume of 500 µl 1 % (w/v) polygalacturonic acid was prepared in 50 mM sodium/sodium hydroxide buffer (pH 10.5) and pre-incubated in a water bath at 50 °C. After 5 min, 0.05 U PGase LBW 5117 was added to the substrate. Aliquots (8.0 µl) of the reaction mixture were withdrawn after 15, 30, and 45 min, and 1, 2, 4, 8, and 18 h, heated at 95 °C for 5 min (to denature the enzyme), centrifuged (5,000 rpm, 5 min) and the supernatants stored at 4 °C until use. Five (5 µl) samples were spotted on a precoated Kieselgel 60 F254 silica gel aluminum sheet. A similar volume of the enzyme, and 0.1 % (w/v) each of mono galacturonic acid and polygalacturonic acid sodium salt were also spotted, as control and standards, respectively. The sheet was air-dried and then developed in a jar containing a mobile phase of 1-butanol, water, and acetic acid in the volume ratio 5:3:2, with two ascents. The sheet was air-dried, immersed in 15 % (v/v) H2SO4 (5 sec), air-dried again, and finally developed by heating at 80 °C until spots appeared. The sheet was then photographed.

Bioscouring of woven cotton using PGase LBW 5117

Bioscouring: An industrially woven piece of plain grey cotton, containing cotton starch as the sizing material, was first washed with distilled water (15 min at room temperature), dried (105 °C), and then cut to obtain a small piece measuring 5 cm x 5 cm. The latter was subjected to enzymatic desizing in a batch process using the method described by Oluoch et al. (unpublished data). The desized fabric was trimmed along its edges to obtain fringe-free cotton and then weighed.

Bioscouring: The desized fabric was subjected to bioscouring (batch process) using the method described by Rajendran et al. (2011). The fabric was initially wetted to approximately 100 % pick up using bioscouring liquor [0.05 mM Tween 20, 1.5 mM CaCl2 and 40.5 U of PGase LBW 5117 (pH 10.5)] at room temperature (29 ± 3 °C) (Material liquor ratio was maintained at 1:20). After 30 min, the reaction mixture was transferred to a thermostaker incubator (Gallenkamp, London, UK) set at 50 °C and 100 rpm, and the bioscouring process allowed to proceed for 4 h. The fabric was then removed from the bath, washed with hot water (95 °C, for 10 min) followed by several washes with cold water before drying (105 °C for 1 h), cooling (in a desiccator), and reweighing. For the control, a second piece of enzymatically desized fabric (5 cm x 5 cm) was weighed and treated similarly, but with heat-inactivated PGase LBW 5117 (95 °C for 30 min) instead of the active one. Finally, the fabric was re-weighed. The preparedness of the bioscouring fabric towards the absorption of water and dye was assessed by determining its: a) weight loss (%) and b) absorbency rate, and then comparing the results obtained with those from the control fabric. This was done as follows:

Weight loss (%) = \frac{Initial weight − Final weight}{Initial weight} \times 100

Absorbency test

The bioscouring- and control-fabrics were each cut into two equal parts to obtain two sets of samples, each measuring 2.5 cm x 5 cm. One of the bioscouring fabric samples and its control counterpart were subjected to the drop penetration test, and the other two to the capillary rise test. These were carried out as described below:

i) Drop penetration test

This was carried out using the AATCC test method 39 - 1980 (evaluation of wettability; AATCC Technical Manual 1980). The fabric sample was placed over the top of a beaker so that the center was unsupported. A drop of water (100 µl) was placed on its surface by dropping it from a height of 1 cm. The time taken (sec) for the water drop to make contact with the fabric and its complete absorption into the sample was taken as the wetting time; as the wetting time; the shorter the time, the more efficient the process. This procedure was repeated at a different point on the fabric and the average wetting time was calculated from the two readings and compared with that from the control fabric.

In addition to the time taken for the water drop to be absorbed into the fabric, the efficiency of the process was assessed by determining if the fabric was evenly bioscouring or not (Halim & Zhou, 2018). This was carried out using 5 mg. L⁻¹ malachite green dye (for better visibility). Briefly, a drop of the dye solution was placed at a different point on the surface of the fabric by dropping it from a height of 1 cm, and the size and shape of the absorbed area were determined and compared with that of the control fabric. The assessment was done as follows:

- If the drops are circular and small, the fabric is bioscouring unevenly and the process is incomplete.
- If the drops are circular and large, the fabric is evenly bioscouring and the process is complete.

ii) Capillary rise (wicking) test

This was carried out according to Briti et al. (2019), but with a slight modification in that malachite green dye was used instead of Reactive red dye. In this experiment, the fabric was hung (using support) over a 50 ml beaker with its bottom edge immersed 1 cm in malachite green dye solution (5 mg. L⁻¹) contained in the beaker. Once immersed, the dye was allowed to rise up the fabric by capillary force for 30 min, after which the distance moved by the colored solution (wicking height) was recorded and compared with that of the control fabric.

Data presentation and analysis

The data for characterization of PGase LBW 5117 concerning its activity, stability, substrate specificity, and mode of action are presented in graphical or tabular forms and were conducted in duplicate with calculated mean. On the other hand, the data for the bio-scoured fabric is presented in both tabular and pictorial forms and was conducted once.

RESULTS AND DISCUSSION

Appearance, detection, identification, and activity of pectinase

The culture of B. halodurans LBW 5117 was subjected to a single downstream processing step (i.e., clarification) to obtain crude pectinase. Like bulk, technical, or industrial enzymes, whose preparations also require few downstream processing steps (e.g., centrifugation and concentration), the enzyme was observed to be a brown liquid with a slight fermentation odor (Headon & Walsh, 1994). The detection of the pectinase was carried out on a polygalacturonic acid-gel detection medium (pH 10.5) using the cup-plate assay method. As seen in Figure 1, a pale hydrolysis zone was formed around the depression on the gel where the enzyme was aliquoted into (white arrow) while the area around the depression that contained the heat-inactivated enzyme (control) remained pink in color, similar to that of the background of the gel. This suggests that the pectinase could be polygalacturonase, pectate lyase, or a combination of both enzymes (Cruickshank and Wade, 1980).

To identify the enzyme, specific quantitative assays were performed for polygalacturonase and pectate lyase in liquid media that contained polygalacturonic acid (pH 10.5) as the substrate. The catalytic activity of polygalacturonase was found to be 0.21 U/ml while that of Pcel, was undetected under the standard assay conditions. This, therefore, confirms that the enzyme under investigation is alkaline polygalacturonase (PGase LBW 5117). The catalytic activity of this enzyme was however observed to be much lower than that of a commercial bioscouring pectinase i.e., COENZYME SPA-5 (10,000 U/ml) (http://www.sunsonenzyme.com/Products/Textile/Bio_scouring), (https://microbiosochi.creative-biogene.com/enzyme/application/textile-industry-1283.html). The low activity can be attributed to the unoptimized culture conditions that were used to grow the microorganism (Halle & Aylee, 2022) and/or the fact that the enzyme was not concentrated.

Figure 1 Detection of pectinase using the cup-plate assay method. The pale zone around the depression on the gel (indicated by arrow) depicts polygalacturonase- or pectate lyase-activity, or both.

Physico-chemical properties of PGase LBW 5117

Storage stability

The stability of the alkaline PGase LBW 5117 was studied by storing it frozen (-20°C) and in liquid forms (4, 20, and 30 °C) for 1 year. The frozen enzyme retained
67% of the original activity while those stored in a liquid state (had a foul smell) retained over 90% of the activity (Figure 2). The relatively high loss of activity exhibited by the frozen enzyme could be due to the high freezing rate that it might have encountered at the onset of the storage period. This can cause an increase in the dehydration rate at the interphase between its surface and ice/unfrozen solution and thus, lead to denaturation, unfolding, and aggregation (Park et al., 2021). On the other hand, the relatively high activity it retained after storage at higher temperatures can be attributed to the good thermostability property that alkaline enzymes exhibit at high pH. This can be attributed to several factors e.g., a decrease in the content of alkali-sensitive amino acids e.g., Asn (Gülich et al., 2000), and an increase in the relative content of His, Gln, and Arg in the protein sequence (Dubnovitsky et al., 2005; Shirai et al., 1997, 2001), due to presence of acidic residues on the protein surface (Dubnovitsky et al., 2005; Mamo et al., 2009), and an increase in the number of salt bridges (De Lemos Esteves, 2005; Hakulinen et al., 2003; Shirai et al., 1997). This result shows that although PGase LBW 5117 can be produced, stored in a liquid state at 4-30 °C, and used within a year (shelf life = 1 year) in an application that involves the degradation of pectin, it would be desirable to concentrate and add preservatives to it to reduce large volumes of the enzyme and the foul smell, respectively. This can help reduce its storage space and also save on the cost of its production. Most of the commercially available alkaline bioscouring pectinases are also stored in a liquid state and have similar shelf lives (Table 1).

### Table 1
Comparison of some physical properties of PGase LBW 5117 with those of pectinases that are used for commercial bioscouring.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Appearance</th>
<th>Odor</th>
<th>Storage temp (°C)</th>
<th>pH of activity</th>
<th>Temp of activity (°C)</th>
<th>Ref*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Color</td>
<td>State</td>
<td></td>
<td>Operational Optimum</td>
<td>Operational Optimum</td>
<td></td>
</tr>
<tr>
<td>Coenzyme® SDT-1</td>
<td>White</td>
<td>Powder</td>
<td>Slight fermentation odor</td>
<td>&lt; 30, for 3 - 6 months</td>
<td>-</td>
<td>80.1 h a</td>
</tr>
<tr>
<td>Coenzyme SPA-5 (PG, PE, and PL)</td>
<td>Brown</td>
<td>Liquid</td>
<td>Normal fermentation odor</td>
<td>Dry &amp; cool place, for 9 months</td>
<td>7.0 - 10.0</td>
<td>45 - 65</td>
</tr>
<tr>
<td>BioPrep 3000L (PecL)</td>
<td>Brown</td>
<td>Liquid</td>
<td>Slight fermentation odor</td>
<td>-</td>
<td>8.5</td>
<td>55 b</td>
</tr>
<tr>
<td>Scourzyme L (PecL)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Alkaline pectinase (MBS-SE22)</td>
<td>-</td>
<td>-</td>
<td>Slight fermentation odor</td>
<td>5 - 35, for 9 - 12 months</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Alkaline pectinase (MBS-1851)</td>
<td>-</td>
<td>L/P***</td>
<td>-</td>
<td>-20 °C</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Alkaline pectinase (MBS-SE23)</td>
<td>-</td>
<td>Liquid</td>
<td>Slight fermentation odor</td>
<td>5 - 35, for 6 - 9 months</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>PGase LBW 5117</td>
<td>Brown</td>
<td>Liquid</td>
<td>Slight fermentation odor</td>
<td>4 - 30 °C for 1 year</td>
<td>10.2 - 11.0</td>
<td>45 - 57</td>
</tr>
</tbody>
</table>

*Temp *a = Temperature
*Ref* = Reference
*L/P** = Liquid/powder


### Optimum pH of enzyme activity

The activity of PGase LBW 5117 was studied at different pH values ranging from 3.0 - 13.0 at 50 °C. As seen in Figure 3, the activity of the enzyme remained constant at 19% between pH 3 - 4 and then increased gradually thereafter to 33% at pH 8. At higher pH values, the activity of the enzyme increased rapidly, reaching 100% at pH 10.5 and then declining to 38% at pH 13. The enzyme exhibited optimum pH at 10.5 in 50 mM glycine-NaOH buffer, with over 80% of its original activity falling within a narrow pH range of 10.2 - 11.0 (operational range). Both the optimum pH of the enzyme and its operational pH range are more alkaline than those reported for commercial pectinases that are used for scouring fabrics (Table 1). Thus, if the enzyme were to be used for such an operation, it would mean a reduction in incidents of contamination from neutrophilic microorganisms during its use.

### Optimum temperature of enzyme activity

The activity of the enzyme was measured at different temperatures ranging from 30 - 90 °C. As seen in Figure 4, there was a rapid increase in the activity of the enzyme from 57% at 30 °C to 100% at 50 °C. This was followed by a rapid decline in the activity, reaching 11% at 90 °C. The enzyme exhibited optimum pH at 50 °C, with over 80% of its original activity falling within the temperature range of 45 - 57 °C (operational range). The optimum temperature of the enzyme falls below those of known commercial pectinases that are used for bioscouring (Table 1). PGase LBW 5117 can therefore be considered as a low-temperature enzyme, implying that if it were to be used for bioscouring a) lower energy costs would be incurred and b) savings on the purchase of specialized equipment that is resistant to heat would be made.

Figure 3 Effect of pH on the activity of the enzyme at 50 °C. The enzyme activity corresponding to 100% was 0.21 U/ml.

Figure 4 Effect of temperature on the activity of the enzyme at pH 10.5. Enzyme activity corresponding to 100% was 0.21 U/ml.
Effect of metal ions on the activity of the enzyme

Metals ions play an important role in the functioning of enzymes in terms of taking part in their catalytic activities and/or altering their structures to improve their stability. However, the type of metal ion and its concentration must be carefully selected or else they can inhibit the enzyme. For this reason, the effect of K⁺, Ca²⁺, Mg²⁺, Fe³⁺, Cu²⁺, Na⁺, Mn²⁺, and Zn²⁺ ions on the activity of the enzyme was investigated at 1, 5, and 10 mM under optimized temperature and pH. All the metal ions tested either stimulated the activity of the enzyme at 1 mM or did not have a significant effect on it, except for Mn²⁺, which partially inhibited the activity (Table 2). On increasing the concentration of the metal ions to 5 and 10 mM, only K⁺ and Ca²⁺ stimulated the activity significantly. The rest of the metal ions either were partial/significant inhibitors of the enzyme or played no major role in altering the activity. This result suggests that PGase LBW 5117 requires metal ions at 1 mM, particularly Ca²⁺. A similar observation was reported for an alkaline polygalacturonase from Bacillus No. P-4-N (Horikoshi, 1972). Reports indicate that Ca²⁺ ions play the important roles of: a) linking the substrate to the active site of the enzyme to help stimulate its catalytic activity (Li et al., 2008) or b) acting directly on the pectin molecule, stabilizing the negatively charged carboxyl groups and indirectly stimulating the polygalacturonase activity (Vatanparast et al., 2014). The finding of a metal ion resistant PGase LBW 5117 is therefore attractive for the textile industry because metal ion impurities are found in cotton fibers and tap water, both of which the industry heavily relies on for scouring woven cotton (Brushwood & Perkins, 1994).

Table 2 Effect of metal ions on the activity of the enzyme at pH 10.5 and 50 °C. Enzyme activity corresponding to 100 % was 0.21 U/ml.

<table>
<thead>
<tr>
<th>Metal ion</th>
<th>Residual activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1 mM</td>
</tr>
<tr>
<td>Control</td>
<td>100</td>
</tr>
<tr>
<td>K⁺</td>
<td>124</td>
</tr>
<tr>
<td>Ca²⁺</td>
<td>167</td>
</tr>
<tr>
<td>Mg²⁺</td>
<td>106</td>
</tr>
<tr>
<td>Fe³⁺</td>
<td>103</td>
</tr>
<tr>
<td>Cu²⁺</td>
<td>121</td>
</tr>
<tr>
<td>Na⁺</td>
<td>118</td>
</tr>
<tr>
<td>Mn²⁺</td>
<td>78</td>
</tr>
<tr>
<td>Zn²⁺</td>
<td>112</td>
</tr>
</tbody>
</table>

The activity of the enzyme was significantly stimulated in the presence of 1 mM Ca²⁺ (Table 2), which suggests that the metal ion plays a vital role in maintaining its active conformation. For this reason, the influence of Ca²⁺ concentration on the activity of the enzyme was studied further. As seen in Figure 5, the activity of the enzyme increased from 100 % (control) to 348 % in the presence of 1.5 mM Ca²⁺ and declined thereafter at higher concentrations of the metal ion. It has been reported that low concentrations of Ca²⁺ preferentially enhance the activity of PGases by binding to the substrate to the enzyme (Li et al., 2008) while higher concentrations inhibit it by chelating the substrate through the formation of interlinkages that cause it to gel and therefore became inaccessible to the enzyme (Cabanne & Donche, 2002; Saeed et al., 2007).

![Figure 5](image)

Figure 5 Effect of Ca²⁺ ion concentration on the activity of the enzyme at pH 10.5 and 50 °C. Enzyme activity corresponding to 100 % was 0.21 U/ml (Control).

Effect of surfactants on the activity of the enzyme

The use of surfactants in industrial processes such as bioscouring is important because they help facilitate the wetting of fabrics and thus, make them more accessible to the scouring enzymes (Hamaki et al., 2019; Rocky, 2012). The type and concentration of the surfactant required must however be selected carefully otherwise the enzyme can become inactivated. In this context, the effect of different concentrations of Tween 20, Triton X-100, and SDS surfactants on the activity of the enzyme was investigated under optimized temperature and pH. As seen in Figures 6a and 6b, the activity of the enzyme increased from 100 % in the absence of surfactant (control) to 171 % and 73 % in the presence of 0.05- and 0.2-mM Tween 20 and Triton X-100, respectively. Any further increase in the concentrations of these surfactants resulted in a decline in the activity, culminating at 133 and 117 % for Tween 20 and Triton X-100, respectively. SDS on the other hand, gradually decreased the activity of the enzyme from the onset as its concentration was increased from 0 - 2.5 mM (Figure 6c).

TWEEN 20 and Triton X - 100 are nonionic surfactants (mild detergents) that do not interact extensively with protein surfaces when used at concentrations that are lower than that of their critical micellar concentrations (CMC) (CMC for Tween 20 is 0.06 mM and that for Triton X - 100 is 0.24). They instead lower the surface tension of aqueous solutions to facilitate contact frequencies between the active site of the enzyme and their substrates hence, speeding up the reactions (Doan et al., 2021; Kapoor et al., 2000). However, when used at higher concentrations than that of their respective CMCs, they form stable micelles that interact with enzymes to form colloidal detergent-protein suspensions that promote conformational changes in the active site of the enzyme, partial unfolding of enzyme or disruption of substrate binding. This lowers the activity of the enzyme (Doan et al., 2021; Kapoor et al., 2000). On the other hand, SDS is an ionic surfactant that binds non-specifically to protein surfaces and can therefore lead to protein unfolding, resulting in the loss of its conformation and activity (Doan et al., 2021). These results suggest that Tween 20 (final concentration = 0.05 mM) is the best wetting agent for use with PGase LBW 5117.

![Figure 6](image)

Figure 6 Effect of different surfactant concentrations on the activity of the enzyme at pH 10.5 and 50 °C. Enzyme activity corresponding to 100 % was 0.21 U/ml (control).

Thermostability of the enzyme

The effect of optimum temperature on the stability of the enzyme was investigated in the absence and presence of 1.5 mM Ca²⁺ and 0.05 mM Tween 20 (individually, and with both additives together) under optimized temperature and pH. As seen in Figure 7, both the activity and stability of the enzyme were enhanced in the presence of the additives. On the onset, the enzyme exhibited initial activities of 171.4, 348, and 348 % in the presence of Tween 20, Ca²⁺ions, and with both additives together, respectively, compared to its original activity of 100 % (control). After 8 h of incubation, the activities were reduced to 44, 100, and 200%, respectively, compared to the control’s 15 %. Upon extending the incubation
period to 20 h, the activities reduced drastically to 22, 33, and 102.3 %, respectively, compared to the control’s 0 % (data not shown). The enhanced stability of the enzyme in the presence of Ca\(^{2+}\) is attributed to the fact that the metal ion protects it from undergoing heat inactivation while the surfactant prevents: a) it from dissociating into its monomers and b) the removal of essential cofactors (or prosthetic group) from its active site (Bennamoun et al., 2016; Komori et al., 1993). A stable PGase LBW 5117 means that the enzyme can be reused and thus, help reduce its cost production.

![Figure 7](image-url) Effect of temperature on the stability of the enzyme at pH 10.5 and 50 °C in the absence (control) and presence of Tween 20, Ca\(^{2+}\), and Ca\(^{2+}\) and Tween 20 additives together. Enzyme activity corresponding to 100 % was 0.21 U/ml (control), 0.36 U/ml in the presence of 0.05 mM Tween20, and 0.73 U/ml in the presence of both Ca\(^{2+}\) and Ca\(^{2+}\)/Tween 20.

**Substrate specificity**

Substrate specificity studies of the enzyme were carried out by performing semi-quantitative assays on solid agar media (pH 10.5) containing citrus pectin with low (DE ≤ 25 %, DE 20 - 34 %), medium (DE 50 - 57 %) and high (DE ≥ 85 %) degrees of methyl esterification, respectively. After staining the plates with Lugol’s iodine dye solution, clearance zones were observed around the depressions on all the plates (Figure 8a) thus, confirming the capability of the enzyme to degrade different types of pectins irrespective of their degrees of methylation. The largest clearance zone (SI = 5.4) was observed on the pectin plate containing the lowest DE (i.e., PGA-Na salt), and this was therefore considered to represent an activity of 100 %. Using this as a reference point, the activities of the enzyme in the remaining plates were observed to reduce with an increase in the DE content of pectin, reaching 50 % in the plate containing pectin with the highest DE (≥ 85 %) (Figure 8b). A similar pattern of enzymatic activities was observed when the assays were carried out using the more sensitive quantitative liquid assay method (Figure 8c). Overall, these results show that the amount of methyl-ester groups has a clear influence on the activity of the enzyme, as the rate of hydrolysis was maximum in the range of DE ≤ 25 % and decreased thereafter with an increase in the DE content. A similar observation was made for a polygalacturonase from *Fusarium moniliforme* (Bonnin et al., 2002). No information is however available in the literature regarding the degradation of different types of pectins by commercial pectinases. Substrate specificity studies were also carried out by performing semi-quantitative assays on cellulotic solid agar media (pH 10.5). After staining the plates with Gram’s iodine solution, no clearance zones were observed around the depressions on the CMC - sodium salt, avicel, and cellulbiose plates to indicate undetectable levels of endo-glucanase, exo-glucanase, and β-glucosidase, respectively, in the enzymatic solution (Figures 8a and 8b), presumably because 6 h of incubation may have been a very short time for weak enzymes, if any, to hydrolyze the substrates. Therefore, a more sensitive (quantitative) assay was performed using the three cellulotic substrates in different liquid media.

![Figure 8](image-url) Effect of pectins with various DEs and celluloses (substrates) on the activity of the enzyme on solid (a) and liquid (c) media at pH 10.5 and 50 °C. The activities are seen as clear hydrolyzation zones around the depressions on all the pectin plates, but not on the cellulotic plates (a), and are expressed as a percentage against the substrate responsible for producing the highest amount of the enzyme (b). The activities of the enzyme in liquid media containing the different pectins and cellulloses, as substrates, are also shown in (c). The activity corresponding to 100 % was 5.4 SI and 0.73 U/ml for the plate and liquid assays, respectively.
The results confirmed that none of the three cellulases were detected (Figure 8c). These results are similar to those of commercially available pectinases that are used for bioscouring woven cotton (http://www.sunsonenzyme.com/Products/Textile/Bio_scouring), (https://biosolutions.novozymes.com/en/fabrics/textiles/biopreparation).

The finding of a cellulase-free PGase LBW 5117 that can hydrolyze pectins with various DEs is important to the textile industry. This is because it has the potential to degrade and eliminate all types of pectins and the undesired hydrophobic non-cellulosic substances that adhere to it from cotton fibers without damaging the desired hydrophilic cellulosic structures that make cloth. This can lead to the production of high-quality cellulosic fabrics with better softness and good absorbency properties (Kohli & Gupta, 2015).

Enzyme’s mode of hydrolysis

The mode of action of PGase LBW 5117 was determined by the thin-layer chromatography method. The reaction spots appeared between the spots represented by mono galacturonic- (G1) and polygalacturonic-acids (G2) (Figure 9) thus, revealing an endo-type of reaction mechanism that produced oligogalacturonates of different molecular weight masses. This group of polygalacturonases randomly cleaves multiple internal α-1,4 glycosidic linkages in polygalacturonic acid substrate, resulting in the rapid production of oligogalacturonates bearing the α-conformation. This makes PGase suitable for many industrial processes where the rapid degradation of pectins, particularly polygalacturonic acid, is required e.g., processing of textile fibers (Kohli & Gupta, 2015). On the contrary, the known commercial bioscouring pectinases are endo-pectate lysases (Table 1). This is yet another group of pectinases that randomly cleave multiple internal 1,4 glycosidic bonds in the same substrate as that for PGase LBW 5117, but via β-elimination to rapidly produce saturated and unsaturated oligogalacturonates (Zhen et al., 2020). Based on this argument, the locally produced PGase LBW 5117 may provide an alternative bioscouring agent to the commercially available pectate lysases, which can be expensive since they have to be imported.

![TLC profile of the hydrolytic action of PGase LBW 5117 on polygalacturonic acid sodium salt.](image)

**Figure 9** TLC profile of the hydrolytic action of PGase LBW 5117 on polygalacturonic acid sodium salt: C is enzyme only (control); G1 and G2 are mono galacturonic acid and polygalacturonic acid standards; lanes 1 - 3 reaction products after 15, 30, and 45 min and lanes 4 - 8 reaction products after 1, 2, 4, 8 and 18 h.

Assessment of the bioscouring fabric

The enzyme PGase LBW 5117 was used to scour a 5 cm x 5 cm piece of enzymatically desired fabric, and the removal of the pectins and other non-cellulosic components of the cuticular and primary wall of the cotton fibers was assessed by determining the: a) weight loss (%) that the fabric incurred and b) new absorbency properties it acquired. The results obtained were as follows:

a) Weight loss (%) incurred by the fabric

The fabric lost 0.74 % of its original weight compared to the control (treated with denatured enzyme), which lost only 0.06 % (Table 3). Assuming that the weight loss incurred by the control fabric was due to the removal of small particles and loss of fringe yarns from its surface and edges, respectively, during the pre-treatment, then the same can be argued for the fabric that was pre-treated with the active enzyme. This implies that the latter incurred an additional weight loss of 0.68 %, which is attributed to the enzymatic degradation and elimination of pectin (and other natural hydrophobic impurities that adhered to it) from it. Although there are no reports available on the measurements of weight losses incurred by fabrics that have been commercially bio-scour with pectinases. Rajendran et al. (2011) have reported a higher weight loss (0.89 %) from a piece of fabric that they biosoured in their laboratory. The difference in the weight loss exhibited by the two fabrics may be attributed to the unoptimized bioscouring conditions used in our study.

<table>
<thead>
<tr>
<th>Weight</th>
<th>Fabrics treated with active enzyme</th>
<th>Denatured enzyme (control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial (g)</td>
<td>0.3515</td>
<td>0.3593</td>
</tr>
<tr>
<td>Final (g)</td>
<td>0.3489</td>
<td>0.3591</td>
</tr>
<tr>
<td>Weight loss (%) incurred</td>
<td>0.74 (0.68)</td>
<td>0.06</td>
</tr>
</tbody>
</table>

b) Absorbency of the fabric

The pre-treated fabric was subjected to absorbency tests using the drop penetration and capillary rise (wicking) tests, and the following results were obtained:

i) Drop penetration test

It took an average of 10 sec for the two water drops to be absorbed into the enzymatically treated fabric compared to the control fabric, which did not absorb the water drops even after extending the waiting period to 300 sec. This shows that the enzyme degraded and eliminated the pectin from both the outer cuticular layers and adjacent primary cell walls of cotton fibers in the woven cotton, and in the process, also removed the loosened hydrophobic waxes and other non-cellulosic substances (e.g., hemicellulose, proteins, and natural colorants, etc) that adhere to it (Ansell & Mwaikambo, 2009), resulting in the exposure of the desired hydrophilic cellulosic structures of the cloth, which easily absorbed the water drops. Although a standard absorbency time of < 5 sec is recommended (Teil & Adere, 2016), this result is acceptable since the study was carried out under unoptimized bioscouring conditions. Moreover, when a drop of malachite green dye was added on a separate point on both the control- and enzymatically treated-fabrics, it (dye) was not absorbed in the former to indicate that bioscouring did not take place (Figure 10a) but was quickly absorbed in the latter and the colored area appeared as a big green circle to indicate that the fabric was evenly bioscour and the process was complete (Figure 10b). This further confirms that the enzyme removed the pectin and other hydrophobic non-cellulosic structures that adhere to it, from the fibers in the cloth, leading to exposure of the desired hydrophilic cellulosic structures, which in turn exhibited excellent dye absorbency properties. Similar results were obtained by Halim & Zhou (2018).

![Drop penetration test performed on: (a) control and (b) bio-scoured fabrics using malachite green dye solution (5 mg. L⁻¹).](image)

**Figure 10** Drop penetration test performed on: (a) control and (b) bio-scoured fabrics using malachite green dye solution (5 mg. L⁻¹). The shape and size of the areas absorbed by the dye are shown on both fabrics as green circles.

ii) Capillary rise test

A photograph of the absorbency test showing the rise of malachite green dye up the control- and enzymatically treated (bioscour) fabrics, by capillary action, is shown in Figure 11. It is clear that the wicking height of the control fabric hardly left its baseline over the 30 min treatment time while that of the enzymatically treated fabric increased from 0 mm at time t = 0 (Figure 10a) to 28 mm at time t = 30 min (Figure 10b). This result further confirmed that the enzyme degraded the pectins from the cloth, and in the process, also removed the loosened hydrophobic waxes and other non-cellulosic impurities that adhere to it (Ansell & Mwaikambo, 2009). Consequently, this resulted in the exposure of the desired intact cellulosic structures of the cloth which, due to their good hydrophilicity, exhibited excellent dye absorbency properties (Colombi et al., 2021). Although Halim & Zhou (2018) obtained a better wicking height of 40 cm, this result is acceptable, given that the study was a preliminary investigation into the bioscouring potential of the enzyme and was therefore carried out under unoptimized process conditions.
CONCLUSION

The study shows that the pectinase under investigation was a cellulase-free thermostable (albeit in the presence of Ca²⁺ and Tween 20) metallo endo-polygalacturonase that could be produced, stored (4 - 25°C) and used within a year to degrade pectins with various degrees of methyl esters under alkaline (pH of 10.2 - 11.0 (optimum, 10.5)) and relatively low temperature (45 - 57°C (optimum 50°C)) conditions. These properties make it suitable for the enzyme to be used in applications that require the degradation of pectin at low operational costs (e.g., low energy consumption, no need to purchase heat-resistant equipment or metal ion chelators, reusability) without having to worry about contamination from neutrophilic microorganisms. A preliminary application study of the enzyme showed that it could degrade and eliminate the undesirable pectins from cotton fibers in cloth, and also remove the loosened hydrophilic waxes and other non-cellulosic substances (e.g., hemicellulose, proteins, and natural colorants) that adhere to it and thus, yield a more hydrophilic fabric with improved wettability (drop test = 10 sec) and dye-ability (capillary rise test = 28 mm after 30 min) properties. The overall result showed that the enzyme has good operational and storage properties that potentially make it a cost-effective bioscouring agent. It is however, important to emphasize that further experiments be carried out to optimize the bioscouring conditions and a comparison of its effectiveness be made with that of standard bio- and/or caustic scouring processes.

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REFERENCES


Figure 11 Dye absorbency test performed on the control and enzymatically treated (bioscouring) fabrics using the capillary rise (wicking) method: (a) before the experiment began (time t = 0) and (b) at the end of the experiment (time t = 30).


Cotton 


Cotton 

Stabilized Pectinolytic Enzymes


