JMBFS

Journal of Microbiology, Biotechnology and Food Sciences

International peer-reviewed scientific online journal

Published by Faculty of Biotechnology and Food Sciences

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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https://doi.org/10.55251/jmbfs.9945

ARTICLE INFO

Received 3. 3. 2023
Revised 10. 9. 2023
Accepted 25. 9. 2023
Published 1. 12. 2023

Regular article

OPEN ACCESS

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 ºC, respectively. Its operational pH and temperature ranged between 10.2 - 11.0 (optimum, 10.5) and 45 - 57 ºC (optimum 50 ºC), respectively. Metal ions e.g., K^+, Ca^{2+}, Mg^{2+}, Fe^{3+}, Cu^{2+}, Na^+, Mn^{2+}, and Zn^{2+} 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 ºC, but this improved to 200 % in the presence of 1.5 mM Ca^{2+} 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 process(es).

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^+, Mg^{2+}, or Ca^{2+}**(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- galacturonates 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 cleave 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 ºC) 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.

MATERIALS AND METHODS

Materials

Alkaliphilic *Bacillus halodurans* LBW 5117 used in this study was isolated in our laboratory from a sample collected from Lake Bogoria, Kenya **(Oluoch *et al*., 2018)**. Citrus pectins containing various DEs i.e., DE ≤ 25, 20-34, 50 - 75, and ≥ 85 %), peptone, yeast extract, ruthenium red dye, potassium sodium tartrate tetrahydrate, Tween 20, 3,5-Dinitrosalicylic acid, carboxy methyl cellulose (CMC) - sodium salt, avicel, and cellobiose were purchased from Sigma-Aldrich (St. Louis, MO, USA). De-ionized water was bought from a local supermarket (Carrefour). Rivatex (EA) Ltd (Eldoret, Kenya), donated the 100 % industrially woven plain grey cotton (contained corn-starch sizing material).

Bacterial cell culture for pectinase production

A stock of the bacterium, in 40 % (v/v) glycerol, was inoculated on a solid agar medium prepared according to **Horikoshi (1972)**, but with a slight modification. The medium contained (g L^{-1}): Citrus pectin (galacturonic acid ≥ 74 %), 10.0; Peptone 3.0; Yeast extract, 3.0; K_2HPO_4, 0.1, $Mg_2SO_4 \cdot 7H_2O$, 0.02; $MnCl_2$, 0.004 and NaCl, 0.15 [The pH of the medium was adjusted to 10.5 with 20% (w/v) Na_2CO_3]. The plate was incubated at 37 ºC for 12 h. A single bacterial colony from the plate was then used to inoculate a 20 ml seed-culture medium (prepared as described above, but without agar) and the flask incubated in a thermoshaker incubator (Gallenkamp, London, UK) at 37 ºC and 100 rpm for 12 h. This seed culture was used to inoculate 80 ml main broth culture medium (prepared as described above) and the bacterium was grown for 48 h using the same culture conditions. The cells were centrifuged at 5,000-x g for 30 min at 4ºC. The appearance and odor of the crude enzymatic solution were noted down, after which it was stored at -20 ºC until use.

Detection and assay for pectinase

The pectinase in the crude enzymatic solution was detected using the cup-plate assay method previously described by **Jurick et al. (2010),** and identified and quantified using the liquid assay method **(Taylor &Secor, 1988)**. For the detection of the enzyme, a gel medium containing 1.0 % (w/v) agarose prepared in 50 mM glycine-NaOH buffer (pH 10.5) with 0.1 % (w/v) polygalacturonic acid sodium salt as the substrate was prepared. The medium was heated to dissolve the polygalacturonic acid and agarose and then transferred (50 ml) to a petri dish. A cork borer was used to punch two depressions (0.5 cm diameter) in the solidified gel. One depression was filled with 35 µl enzyme and the other with a similar volume of the heat-inactivated enzyme (95 ºC for 30 min). The assay plate was incubated at 50 ºC for 48 h, after which the gel was developed by flooding the plate with 30 ml 0.05 % (w/v) ruthenium red dye solution for 30 min at 20ºC. Excess dye was drained from the plate and the gel was rinsed with several changes of deionized water and then examined for enzymatic activity. The presence of a colorless or pale zone around the depression on the gel is indicative of polygalacturonase or pectate lyase **(Cruickshank & Wade, 1980)**.

The catalytic activity of PGase in the crude enzymatic solution was determined by adding 0.2 ml enzyme to 0.8 ml (w/v) 0.5 % polygalacturonic acid sodium salt (DE ≤ 25 %) prepared in 50 mM glycine-NaOH buffer, pH 10.5. The reaction mixture was incubated at 50 ºC for 10 min and the amount of reducing sugars formed was determined according to **Wang *et al*. (1997).** One unit of enzyme activity was defined as the amount of enzyme liberating 1 µmol of mono galacturonic acid per min under the standard assay conditions. On the other hand, the catalytic activity of PecL was determined using the method described by **Soriano *et al*. (2006)**, but with a modification. 0.1 ml crude enzyme was added to 1.9 ml 0.2% (w/v) polygalacturonic acid sodium salt in 50 mM glycine NaOH buffer pH 10.5, which also contained 0.5 mM $CaCl_2$. The assay mixture was incubated at 50 ºC for 2.5 min and increase in A 232 nm measured at intervals of 0.5 min after terminating the reaction with 2 ml 50 mM HCl and centrifuging the tube at 14,000 rpm (5 min). One unit of PecL activity was defined as the amount of enzyme that liberated 1 µmole unsaturated galacturonates under the standard assay conditions. The molar extinction coefficient of the unsaturated product was assumed to be 4,600 M-1cm-1. For the control in both assays, a heat-inactivated enzyme (95 ºC, 30 min) was used instead of the active one.

Characterization of polygalacturonase activity

The crude enzyme designated PGase LBW 5117, was subjected to both physical and chemical characterization of its catalytic activity to establish its optimum operating and storage conditions. These are described below:

Effect of storage temperature on the stability of the enzyme

One (1 ml) of the enzyme was stored in a freezer (-20 ºC), refrigerator (4º C), and in two separate incubators (20 ºC and 30 ºC), respectively. The samples were retrieved after one year and the residual activities of the enzyme determination under the standard assay 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 ºC 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^+, Ca^{2+},Mg^{2+}, Fe^{3+}, Cu^{2+}, Na^+, Mn^{2+} and Zn^{2+}(final concentrations ranging from 1, 5 and 10 mM, respectively) at 29 ± 3 ºC. 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 Ca^{2+} 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 = 0.0.25, 0.05, 0.1, and 0.15 mM, respectively) at 29 ± 3 ºC. After 30 min, the residual activity of the enzyme was determined under the standard assay conditions. This procedure was repeated with a) Triton X-100 (final concentration = 0.1, 0.2, 0.25, and 0.3 mM, respectively) and b) sodium dodecyl sulfate (SDS) (final concentration = 0.1, 0.2, 0.25, and 0.3 mM, respectively).

Effect of optimum temperature on the stability of the enzyme

The enzyme was incubated in the absence and presence of Ca^{2+} and Tween 20 (final concentrations = 1.5 and 0.05 mM, respectively), individually, and in combination, at 50 ºC, 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 **Heidarizadeh *et al*. (2018)** was used. Ten (10) µl enzyme containing $CaCl_2$ 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 50 - 75 %) and c) high degree of esterified pectin (DE ≥ 85 %), respectively. Similarly, the enzyme [containing $CaCl_2$ 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 ºC for 6 h, after which the pectin and cellulosic plates were flooded with Lugol's iodine **(Hitha & Girija, 2014)** and Gram's iodine **(Kasana *et al*., 2008)** dye solutions, respectively. The activities of the enzyme and those of endoglucanase, exoglucanase, and cellobiose 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 & Girija, 2014)**. For the controls, heat-denatured enzyme (95 ºC for 30 min) was used.

The influence 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 50 - 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 **(Kiio *et al*., 2016)**. Briefly, 500 ul 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 ºC 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 º 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 800 µl 1 % (w/v) polygalacturonic acid was prepared in 50 mM glycine/sodium hydroxide buffer (pH 10.5) and pre-incubated in a water bath at 50 ºC. 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 ºC for 5 min (to denature the enzyme), centrifuged (5,000 rpm, 5 min) and the supernatants stored at 4 ºC 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) H_2SO_4 (5 sec), air-dried again, and finally developed by heating at 80 ºC until spots appeared. The sheet was then photographed.

Bioscouring of woven cotton using PGase LBW 5117

Desizing: An industrially woven piece of plain grey cotton, containing corn starch as the sizing material, was first washed with distilled water (15 min at room temperature), dried (105 ºC), 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 $Ca^{2+,}$ and 40.5 U of PGase LBW 5117 (pH 10.5)] at room temperature (29 ± 3 ºC) (Material liquor ratio was maintained at 1:20). After 30 min, the reaction mixture was transferred to a thermoshaker incubator (Gallenkamp, London, UK) set at 50 ºC 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 ºC, for 10 min) followed by several washes with cold water before drying (105 ºC 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 ºC for 30 min) instead of the active one. Finally, the fabric was re-weighed. The preparedness of the bioscoured 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 (%) determination

The weight loss of both the bioscoured and control fabrics was determined using the equation stated below:

$$\text{Weight loss (\%)} = \frac{\text{Initial weight} - \text{Final weight}}{\text{Initial weight}} \times 100$$ **(Halim & Zhou, 2018)**

Absorbency test

The bioscoured- 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 bioscoured 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 using a stopwatch, and recorded 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 bio-scoured 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 noncircular and small, the fabric is scoured unevenly and the process is incomplete.
- If the drops are circular and small, the fabric is evenly scoured, but the process is incomplete.
- If the drops are circular and big, the fabric is evenly scoured and the process is complete.
- If the drops do not get absorbed into the fabric, the process does not take place.

ii) Capillary rise (wicking) test
This was carried out according to **Bristi *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 down-stream processing steps (e.g., centrifugation and concentration), the enzyme was observed to be a brown liquid with a slight fermentation odor **(Headon & Walsh, 1994) (https://biosolutions.novozymes.com/en/leather-textiles/textiles/biopreparation), (http://www.sunsonenzyme.com/Products/Textile/Bio_scouring), (https://biosolutions.novozymes.com/en/leather-textiles/textiles/biopreparation), (https://microbiosci.creative-biogene.com/enzyme-application/textile-industry-1283.html).** 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 hydrolyzation 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 PecL 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).** The low activity can be attributed to the unoptimized culture conditions that were used to grow the microorganism **(Haile & Ayele, 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 (-20ºC) and in liquid forms (4, 20, and 30 ºC) 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 ºC, 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).

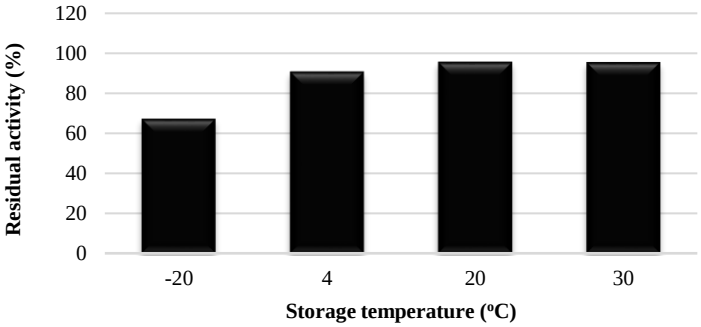

Figure 2 Effect of storage temperature on the activity of the enzyme. Enzyme activity corresponding to 100 % was 0.21 U/ml at pH 10.5.

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

Enzyme	Appearance		Odor	Storage temp (ºC)	pH of activity		Temp* of activity (ºC)		Ref#
	Color	State			Operational	Optimum	Operational	Optimum	
Coenzyme® SDT-1	White	Powder	Slight fermentation odor	< 30, for 3 - 6 months	-	-	-	80, 1 h	[a]
Coenzyme SPA-5 (PG, PE, and PL)	Brown	Liquid	Normal fermentation odor	Dry & and cool place, for 9 months	7.0 - 10.0	9.8	45 - 65	58	[a]
BioPrep 3000L (PecL)	Brown	Liquid	Slight fermentation odor	-	-	8.5	-	55	[b]
Scourzyme L (PecL)				-	7.5 - 8.5	7.5 - 8.5	25 - 60	55	[c]
Alkaline pectinase (MBS-SE22)	-	-	Slight fermentation odor	5 - 35, for 9 - 12 months	-	-	-	-	[d]
Alkaline pectinase (MBS-1851)	-	L/P**	-	-20 ºC	-	-	-	-	[d]
Alkaline pectinase (MBS- SE23)	-	Liquid	Slight fermentation odor	5 - 35, for 6 - 9 months	-	-	-	-	[d]
PGase LBW 5117	Brown	Liquid	Slight fermentation odor	4 - 30 ºC for 1 year	10.2 - 11.0	10.5	45 - 57	50	This study

Temp ***** = Temperature
Ref# = Reference
L/P** = Liquid/powder
[a]: **http://www.sunsonenzyme.com/Products/Textile/Bio_scouring**
[b]:**https://biosolutions.novozymes.com/en/leather-textiles/textiles/biopreparation**
[c]:**https://www.textiletoday.com.bd/introducing-bio-scouring-a-cost-effective-and-eco-friendly-process-for-the-generation-next/ (Nikolov, 2005)**
[d]: **https://microbiosci.creative-biogene.com/enzyme-application/textile-industry-1283.html**

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 ºC. 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.

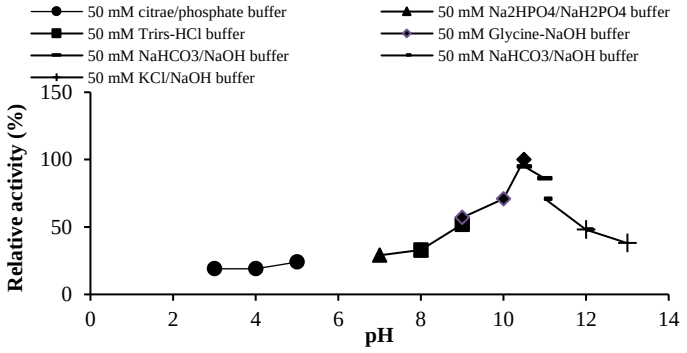

Figure 3 Effect of pH on the activity of the enzyme at 50 ºC. The enzyme activity corresponding to 100 % was 0.21 U/ml.

Optimum temperature of enzyme activity

The activity of the enzyme was measured at different temperatures ranging from 30 - 90 ºC. As seen in Figure 4, there was a rapid increase in the activity of the enzyme from 57 % at 30 ºC to 100 % at 50 ºC. This was followed by a rapid decline in the activity, reaching 11 % at 90 ºC. The enzyme exhibited optimum pH at 50 ºC, with over 80 % of its original activity falling within the temperature range of 45 - 57 ºC (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.

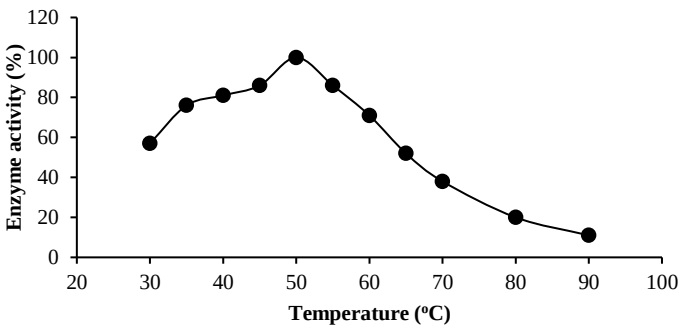

Figure 4 Effect of temperature on the activity of the enzyme at pH 10.5. Enzyme activity corresponding to 100 % was 0.21 U/ml.

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^+, Ca^{2+}, Mg^{2+}, Fe^{3+}, Cu^{2+}, Na^+, Mn^{2+}, and Zn^{2+}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 Mn^{2+}, which partially inhibited the activity (Table 2). On increasing the concentration of the metal ions to 5 and 10 mM, only K^+ and Ca^{2+} 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 Ca^{2+}. A similar observation was reported for an alkaline polygalacturonase from *Bacillus* No. P-4-N **(Horikoshi, 1972)**. Reports indicate that Ca^{2+}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) (Chrome- extension: //efaidnbmnnnibpcajpcglclefindmkaj/https://wasreb.go.ke/downloads/Water_Quality_&_Effluent_Monitoring_Guidelines.pdf, 2008).**

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

Metal ion	Residual activity (%)		
	1 mM	5mM	10 mM
Control	100	100	100
K^+	124	126	116
Ca^{2+}	167	138	110
Mg^{2+}	106	36	34
Fe^{3+}	103	38	16
Cu^{2+}	121	69	68
Na^+	118	106	79
Mn^{2+}	78	34	12
Zn^{2+}	112	101	70

The activity of the enzyme was significantly stimulated in the presence of 1 mM Ca^{2+} (Table 2), which suggests that the metal ion plays a vital role in maintaining its active conformation. For this reason, the influence of Ca^{2+} 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 Ca^{2+} and declined thereafter at higher concentrations of the metal ion. It has been reported that low concentrations of Ca^{2+} preferentially enhance the activity of PGases by binding 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)**.

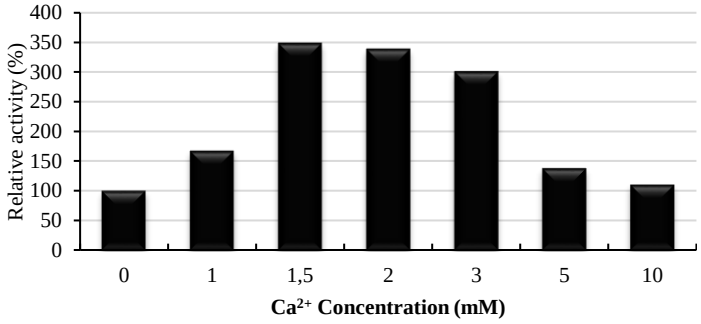

Figure 5 Effect of Ca^{2+} ion concentration on the activity of the enzyme at pH 10.5 and 50 ºC. Enzyme activity corresponding to 100 % was 0.21 U/ml (Control).

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 138 % 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 71 % 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 (CMCs) (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.

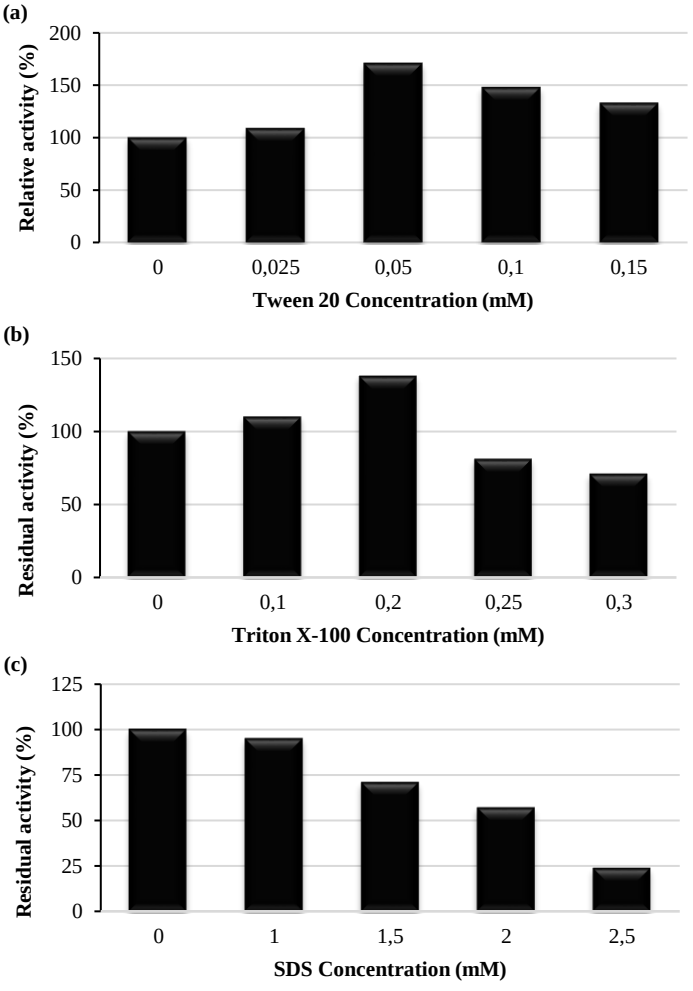

Figure 6 Effect of different surfactant concentrations on the activity of the enzyme at pH 10.5 and 50 ºC. Enzyme activity corresponding to 100 % was 0.21 U/ml (control).

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 Ca^{2+} 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, Ca^{2+}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.

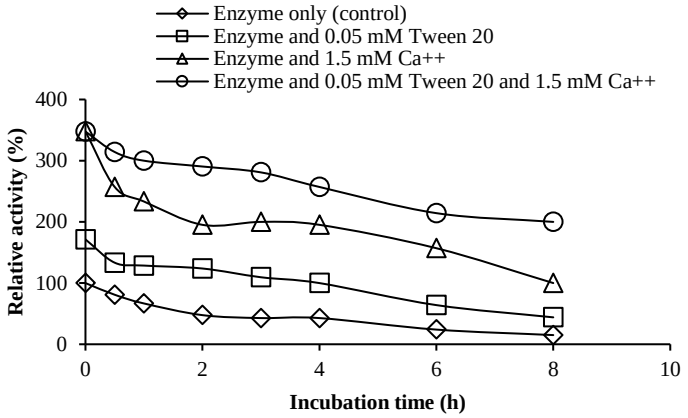

Figure 7 Effect of temperature on the stability of the enzyme at pH 10.5 and 50 ºC in the absence (control) and presence of Tween 20, Ca^{2+}, and Ca^{2+} and Tween 20 additives together. Enzyme activity corresponding to 100 % was 0.21 U/ml (control), 0.36 U/ml in the presence of 0.05 mM Tween20, and 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 cellulosic 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 cellobiose 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 cellulosic substrates in different liquid media.

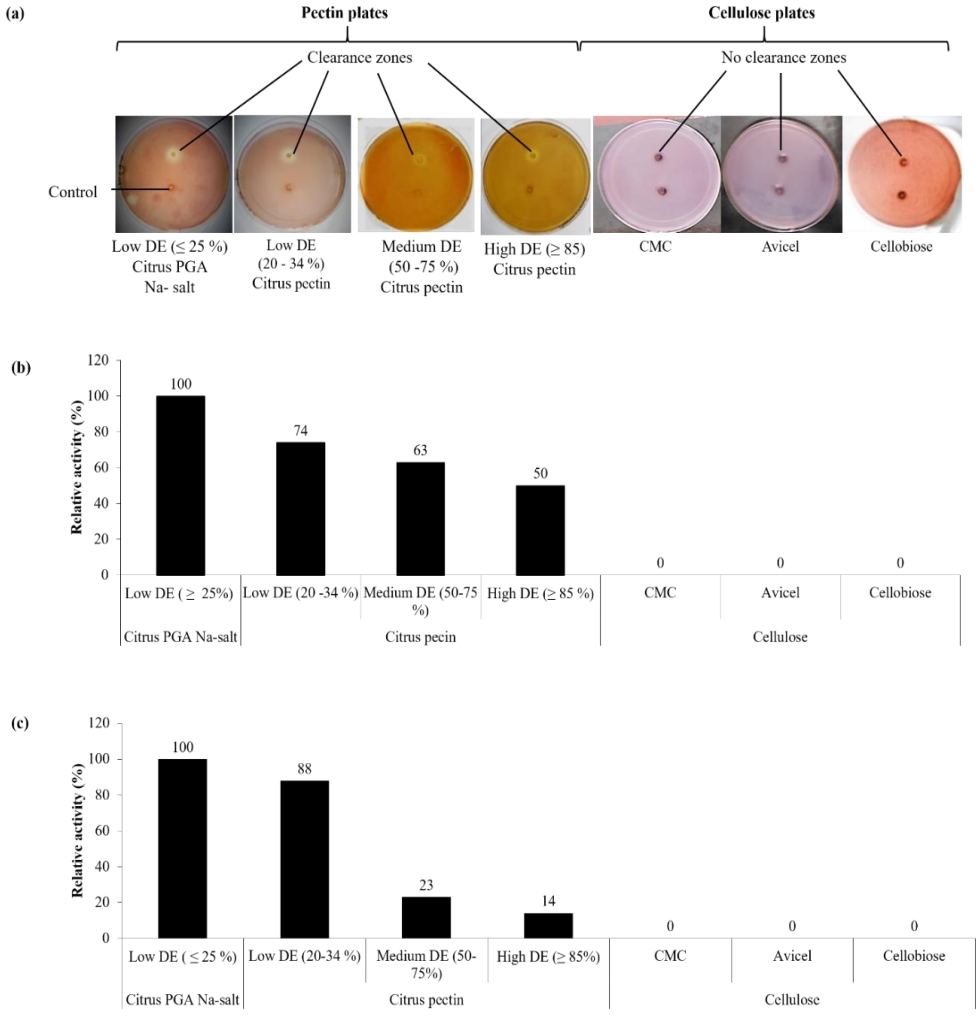

Figure 8 Effect of pectins with various DEs and celluloses (substrates) on the activity of the enzyme on solid (a) and liquid (c) media at pH 10.5 and 50 ºC. The activities are seen as clear hydrolyzation zones around the depressions on all the pectin plates, but not on the cellulosic plates (a), and 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 celluloses, 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/leather-textiles/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 makeup 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 lyases (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 lyases, which can be expensive since they have to be imported.

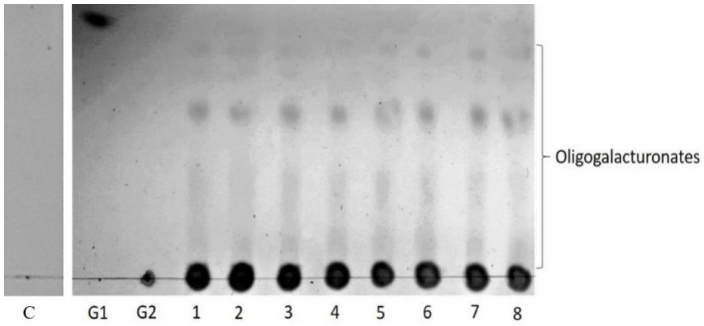

Figure 9 TLC profile of the hydrolytic action of PGase LBW 5117 on polygalacturonic acid sodium salt: C is enzyme only (control); G1 and G2 are mono galacturonic acid and polygalacturonic acid standards; lanes 1 - 3 reaction products after 15, 30, and 45 min and lanes 4 - 8 reaction products after 1,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

Assessment of the bioscoured fabric

The enzyme PGase LBW 5117 was used to scour a 5 cm x 5 cm piece of enzymatically desized 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) and 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-scoured with pectinases, **Rajendran *et al.* (2011)** have reported a higher weight loss (0.89 %) from a piece of fabric that they bioscoured 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 3 Analysis of the bioscoured fabric by weight loss (%) determination.

Weight	Fabric treated with	
	Active enzyme	Denatured enzyme (control)
Initial (g)	0.3515	0.3593
Final (g)	0.3489	0.3591
Weight loss (%) incurred	0.74 (0.68)	0.06

***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 **(Teli & 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 bioscoured 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)**.

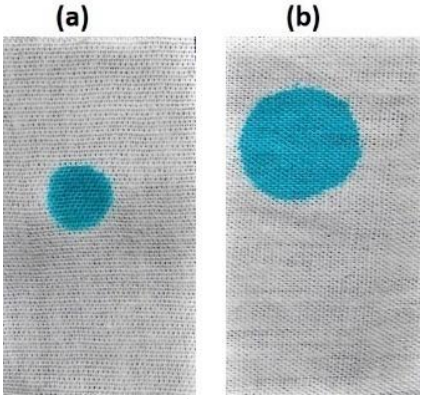

Figure 10 Drop penetration test performed on: (a) control and (b) bio-scoured fabrics using malachite green dye solution (5 mg. L^{-1}). The shape and size of the areas absorbed by the dye are shown on both fabrics as green circles.

ii) Capillary rise test

A photograph of the absorbency test showing the rise of malachite green dye up the control- and enzymatically treated (bioscoured) 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.

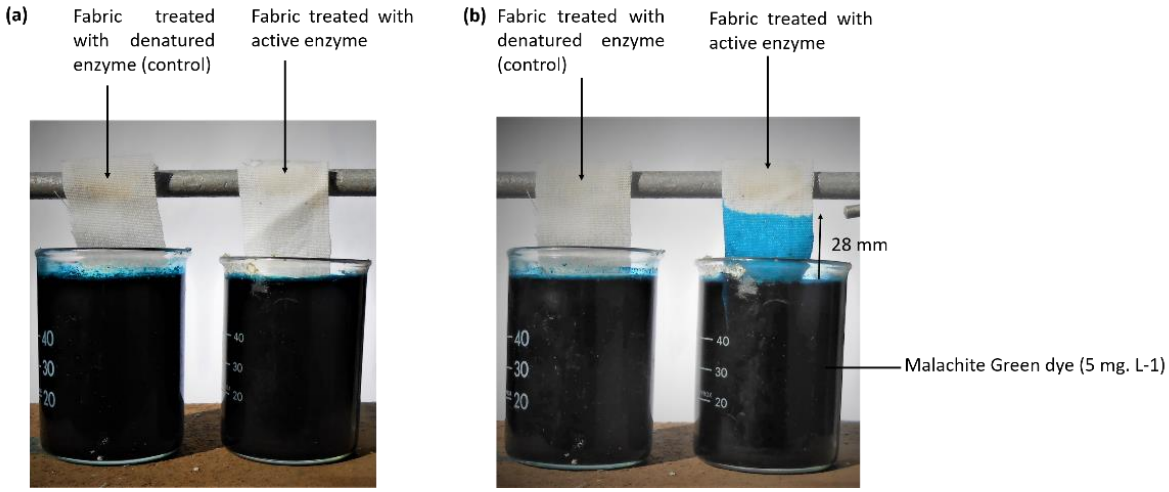

Figure 11 Dye absorbency test performed on the control and enzymatically treated (bioscoured) fabrics using the capillary rise (wicking) method: (a) before the experiment began (time t = 0) and (b) at the end of the experiment (time t = 30).

CONCLUSION

The study shows that the pectinase under investigation was a cellulase-free thermostable (albeit in the presence of Ca^{2+} and Tween 20) metallo endo-polygalacturonase that could be produced, stored (4 - 25 ºC) 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 ºC (optimum 50 ºC)] conditions. These properties make it suitable for the enzyme to be use 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 hydrophobic 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 process(es).

Acknowledgments: The authors would like to thank Mr. Hosea Too and his team at Rivatex (EA) Limited, for providing the fabric that was used in the study, and for the useful information regarding the setting-up of the bioscouring experiment. We are also grateful to Dr. Joseph M. Kamau of the Institute of Primate Research, Karen, Nairobi, for allowing us to use some of his laboratory facilities. Special thanks to Mr. Faustus Mmbaya of the Department of Biochemistry, University of Nairobi, for guidance with the TLC experiment.

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