CHARACTERIZATION OF AN ALKALINE AMYLASE AND A PECTINASE FROM ALKALIPHILIC *BACILLUS HALODURANS* LBW 5117 ISOLATED FROM LAKE BOGORIA IN KENYA AND DEMONSTRATION OF THEIR APPLICATIONS IN BIO-PROCESSING WOVEN COTTON

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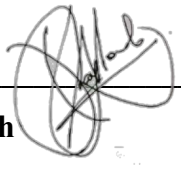
A THESIS SUBMITTED IN FULFILMENT FOR THE DEGREE OF DOCTOR OF PHILOSOPHY IN BIOCHEMISTRY IN THE DEPARTMENT OF BIOCHEMISTRY, FACULTY OF SCIENCE AND TECHNOLOGY, UNIVERSITY OF NAIROBI

OCTOBER, 2023

DECLARATION

This thesis is my original work and has not been presented for a degree in any other University.

Signature  **Date:** 16.10.2023

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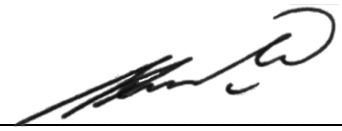
Signature  **Date:** 16/10/2023

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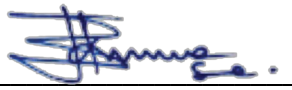
Signature  **Date:** 16.10.2023

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DEDICATION

This thesis is dedicated to my wife Maureen Mideva Imbayi and daughter Natasha Romina Oluoch, who have been a constant source of inspiration and encouragement during graduate school and life challenges. I am truly thankful for having you in my life. This work is also dedicated to my parents, the late Philip Nabboth Clement Oluoch and Celestine Rita Oluoch, for inspiring me to work hard for the things I aspire to achieve. Your constant prayers and encouragement throughout this challenging academic journey are cherished. Likewise, I dedicate this work to my brothers the late Thomas Everest Oluoch and Allan Douglas Oluoch, and sisters Mildred Rose Oluoch, Evelyn Dorothy Oluoch, the late Angela Oluoch, Carolyn Judith Oluoch, and the late Adah Mgele Oluoch, for their solid support throughout my academic journey and life in general.

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LIST OF ABBREVIATIONS AND ACRONYMS

A	Absorbance
Amy	Amylase
AATCC	American Association of Textile Chemists and Colorists
BLAST	Basic Local Alignment Search Tool
Bt	*Bacillus Thuringiensis*
CD	Cyclodextrin
CGTase	Cyclodextrin glycosyltransferase
CTA	Cotton Textile and Apparel
Dc	Diameter of colony
DE	Degree of esterification with methyl esters
Dh	Diameter of halo
dNTPs	Deoxyribonucleotide triphosphates
DSMZ	Deutsche Sammlung von Mikroorganismen und Zellkulturen German Collection of Microorganisms and Cell Cultures)
EA	East Africa
EPZ	Export Processing Zone
GoK	Government of Kenya
IPR	Institute of Primate Research
Kbp	Kilobase pair
KES	Kenya Shillings
LBK	Lake Bogoria Kenya
LBS	Lake Bogoria soil
LBW	Lake Bogoria water
Ltd	Limited
MUSCLE	MUltiple Sequence Comparison by Log- Expectation
NCBI	National Center for Biotechnology Information
NEMA	National Environmental Management Authority of Kenya
PCR	Polymerase chain reaction
PE	Pectinesterase
PecL	Pectate lyase
PGase	Polygalacturonase
PMGase	Polymethylgalacturonase
PNL	Pectin lyase
REAL	Rivatex East Africa Limited
SI	Solubilization index
TCA	Trichloroacetic acid
TEGEWA	TExtilhilfsmittel (textile auxiliaries), GErbstoffe (tanning agents) and WAschrohstoffe

LIST OF APPENDICES

LIST OF PAPERS

This thesis is based on the following three (3) papers:

Paper I **Oluoch K R,** Okanya P W, Hatti-Kaul R, Mattiasson B and Mulaa F J. Protease-, pectinase- and amylase- producing bacteria from a Kenyan soda lake. *The Open Biotechnology Journal* (2018) 12: 33-45 https://doi:10.2174/1874070701812010033.

Paper II **Oluoch K R,** Muge E K, Mwangi Y W and Mulaa F J. Characterization of an alkaline active endo-α-1-4-amylase from alkaliphilic *Bacillus halodurans* LBW 5117 and demonstration of its desizing potential. *Journal of Applied Biology and* Biotechnology (2023) (*In press*).

Paper III **Oluoch K R,** Muge E K, Mwangi Y W and Mulaa F J. Characterization of an alkaline active endo-polygalacturonase from alkaliphilic *Bacillus halodurans* LBW 5117 and demonstration of its bioscouring potential. *Journal of Microbiology, Biotechnology and Food Sciences* (2023) (*https://office2.jmbfs.org/index.php/JMBFS/article/view/9945*).

ABSTRACT

De-sizing is a key textile wet processing step that is carried out on sized woven cotton to remove the starch impurity from cloth. On the other hand, scouring is done on de-sized fabrics to remove the natural pectins and other non-cellulosic impurities from the cotton fibers of the cloth. Both processes are done to obtain sufficiently hydrophilic cellulosic fibers that will allow the cloth to be evenly bleached, mercerized, and dyed/printed in the subsequent processes. Both desizing and scouring are commercially carried out using conventional chemicals or enzymes. Although the chemicals are cheap and effective in removing impurities, they are environmentally unfriendly, and can also damage the fabrics. On the other hand, using enzymes offers better advantages e.g., they produce higher-quality fabrics and generate fewer pollution loads. However, most of the enzymes operate at or near neutral pH, making their use risky due to possible damage to the fabrics by cellulases from neutrophilic contaminants. In addition, the requisite enzymes are expensive because they are often high-temperature enzymes that have to be imported from China, Denmark, and the US. To address these challenges, low-temperature alkaline enzymes from indigenous alkaliphilic microorganisms should be sought and utilized as an alternative to both the costly commercial enzymes and the fabric-damaging and environmentally unfriendly chemicals. The study aimed to characterize an alkaline protease, pectinase, and amylase obtained from an alkaliphilic bacterium that was isolated from Lake Bogoria (soda lake), Kenya, and to demonstrate their potential to desize (amylase) and scour (pectinase or protease) woven cotton. The study began with the isolation and identification of alkaliphilic microbial soda lake isolates that exhibit protease-, pectinase- and amylase- activities. 16 isolates were identified as *B. halodurans* and 2 as *B. pseudofirmus*. All the *B. halodurans* and *B. pseudofirmus* isolates produced amylases while pectinases were produced by all the *B. halodurans* isolates. On the other hand, proteases were produced by 12 *B. halodurans* and all the *B. pseudofirmus* isolates. Among the isolates, *Bacillus halodurans* LBW 5117 was ranked as one of the most potent producers of both amylases (0.32 U/ml) and pectinases (0.09 U/ml). This bacterium was

therefore cultured for the individual production and characterization of both enzymes (amylase = Amy LBW 5117 and pectinase = PGase LBW 5117). Amy LBW 5117 was an endo-α-1-4-amylase while PGase LBW 5117 was an endo-polygalacturonase.The optimum storage condition for Amy LBW 4117 was in liquid form at 4 ^{o}C for six weeks while that for PGase LBW 5117 was at 4 - 30 ^{o}C for one year. On the other hand, the optimum operating conditions for Amy LBW 5117 was pH 10.0 at 60 ^{o}C in the presence of 1.0 mM Ca^{2} and 0.05 mM Tween 20 while that for PGase LBW 5117 was pH 10.5 at 50 ^{o}C in the presence of 1.5 mM Ca^{2+} and 0.05 mM Tween 20. In addition, these additives also enhanced the thermo-stability property of both enzymes, with Amy LBW 5117 retaining 96 % of its original activity after 3 h of incubation at 60 C and PGase LBW 5117 retaining 200 % of its activity after 8 h of incubation.at 50 ^{o}C. Furthermore, metal ions that are commonly found in cotton fibers and tap water either stimulated the catalytic activities of both enzymes or had no significant effect on them. Moreover, Amy LBW 5117 was cellulase-free and could hydrolyze the different types of starch products that are used to make textile sizing agents. Similarly, PGase LBW 5117 was cellulase-free and could hydrolyze different pectins with various degrees of methyl esters that are found in cotton fibers. Indeed, a preliminary desizing application study of Amy LBW 5117 revealed that it could desize woven cotton that contained starch as the size and yield a fabric with a commercially acceptable amount of residual starch (0.0725%) (TEGEWA rating 7.5). Similarly, PGase LBW 5117 could remove pectin and other non-cellulosic impurities from an Amy LBW 5117 desized fabric and yield a cloth with improved wettability (drop test = 10 sec) and dye-ability (capillary rise test = 28 mm after 30 min) properties. This study demonstrates that Amy LBW 5117 and PGase LBW 5117 have great potential to be effective textile desizing and scouring agents due to their good operational properties.

CHAPTER ONE

1.0 INTRODUCTION

1.1 Background of the study

Most lives on earth are sustained under moderate environmental conditions i.e., near neutral pH, temperatures of 20 - 40 ^{o}C, air pressure of 1 atm and sufficient nutrient and salt concentrations. It has however become increasingly clear that life also thrives in extreme environments. For example, microbes have been found in extremes of: a) temperature [high temperature (thermophiles) and low temperature (psychrophiles)] b) high pressure (barophiles/piezophiles) c) salinity (halophiles) d) pH [acidic (acidophiles) and alkaline (alkaliphiles) f) low water vailability (xerophiles) and g) high concentrations of metal/heavy metals (metallophiles) (Merino *et al*., 2019). These microorganisms are therefore called extremophiles.

The extracellular proteins produced by extremophiles have developed molecular mechanisms of adaptation to the extreme physicochemical conditions in these microorganisms thrive in (Hough and Danson, 1999). The unusual properties of these proteins, particularly enzymes (extremozymes) have attracted a lot of attention in research in the past few decades and consequently, they have found applications in various industrial processes where similar harsh conditions are required (Table 1.1). Other advantages of using these enzymes in industrial processes include high selectivity and catalysis, easy disposal and environmental compatibility.

In the present study, special attention has been given to alkaliphiles, which comprise microorganisms that grow optimally at pH > 9.0 (Merino *et al*., 2019). Alkaliphiles have been isolated from a variety of habitats, including soils, ground and seawater, and alkaline industrial effluents from industries such as cement production, leather tanning, and paper and board production (Zeynep and Metin, 2002). However, a more diversified community of alkaliphiles can be found in soda lakes, which are the most stable naturally occurring

highly alkaline environments on earth and whose pH values can go beyond 10.0 (Jones *et al*., 1998). The best studied soda lakes are those found in the Kenyan - Tanzanian Rift valley (Grant and Sorokin, 2011).

Alkaliphilic microorganisms, particularly those that belong to the genus *Bacillus* have attracted a lot of attention in the last couple of decades due to their ability to produce extracellular polymer degrading enzymes that are active and stable in alkaline environments (alkaline enzymes) (Martins, 2003). Among these enzymes are cellulases, xylanase, proteases pectinases and amylases, which have found applications in the food, chemical, pharmaceutical, pulp and paper, leather and textile industries (Table1). In the textile industry, commercial alkaline amylases and pectinases are increasingly finding applications, to remove the added starch and natural pectin (alongside other non-cellulosic substances) impurities, from woven cotton (Sultana *et al.*, 2014; Ansell and Mwaikambo, 2009). These pretreatments are done in order to obtain sufficiently hydrophilic cellulosic structures of the cloth and, thus improve its absorbency for bleach, dye and/or print agents in further processing steps (Chinnammal and Arunkumar, 2013; Thiagarajan and Selvakumar, 2008). It is also important to note that both of these enzymatic pretreatment steps do not affect the cellulosic structures of the cloth and thus, fiber damage is avoided. This, in turn, helps to reduce the pollution loads generated in the effluents. Moreover, the enzymes used are safe and easy to handle, hence pose no serious threat to the personnel; operate under mild conditions, implying low cost of fabric production; efficient in a wide range of temperature; degradable and require the use of less chemicals (eco-friendly).

Table 1.1 Extremophiles and their biotechnological applications (Coker, 2016; Preiss *et al*., 2015; Sarethy *et al*., 2011).

Extremophiles	Optimal growth	Enzyme and other biomolecules	Industrial application
Thermophiles	55 – 113 ºC	Amylases	Starch hydrolysis
		Cellulase	Cellulose hydrolysis
		Xylanase	Paper bleaching
		Proteases	Food processing, baking and detergents
		DNA polymerases	PCR technology/Genetic engineering
		Pullulanases	High glucose syrups
Psychrophiles	-2 to 20 ºC	Pectinases	Clarification of fruit juices
		Proteases, amylases and lipases	Energy saving detergents
		β-galactosidases	Lactose hydrolysis in milk
		Proteases	Cheese manufacturing
Acidophiles	pH < 3	Cellulases and proteases	Animal feed component
		Oxidases	Desulfurization of coal
			Metal extraction e.g. Fe
Halophiles	>0.5 M NaCl	Bioplastics	Packaging material
		Glycerol	Pharmaceutical, food and chemical
		Carotenoids	
		β-carotene	Food coloring
		Cathaxanthn	Food dye and feed additive, cosmetics
		Bacteriorhodopsin	Artificial retinas
		Trehalose	Antibody and vaccine stabilizer
		Ectoin	Skin moisturizer
		Halocins (peptides)	Antimicrobial, antifungal, antiviral and antitumor properties
Alkaliphiles	pH > 9.0	Lipases and proteases	Leather tanning
		Cellulases, amylases, proteases, lipases and xylanases	Laundry detergent additives bleaching agent
		Cellulases	Bio-polishing, bio-stoning, de-inking
		Lipases	Organic synthesis, food and detergent industries
		Amylase, pectinases and xylanase	Plant fiber processing for use in textile production and paper making
		CGTase	Production of cyclodextrins
		Catalase	Degradation of H_2O_2 in textile bleach wastewaters

1.2 Problem statement

One of the biggest challenges facing Kenya's textile industry is the high cost of importing commercial semi-purified amylases and pectinases that can be used for desizing and scouring of woven cotton, respetcivley. In addition, these enzymes often operate optimally at or near neutral pH, implying that both bio-processes are susceptible to contamination from neutrophilic microorganisms, which may in turn produce cellulases that end up attacking the cellulosic structures of the cloth and lead to the production of poor quality fabrics and generation of higher pollution loads in their respective effluents. Furthermore, they often operate optimally at high temperatures, meaning high cost of energy and purchase of specialized equipment that are resistant to heat [http://www.sunsonenzyme.com/Products/Textile/Desizing/ (cited date 05 December 2022)], [https://biosolutions.novozymes.com/en/textiles/products/desizing/aquazym (cited date 05 December 2022). To circumvent these problems, the Kenyan textile industry relies on the use of chemical methods of desizing and scouring. Both of these pretreatments require the use of harsh chemicals e.g., sodium hydroxide/hydrogen peroxide (oxidative cdesizing) and caustic soda (caustic scouring).

Although these chemicals are cheap, effective in removing the undesirable impurities from woven cotton and have a supplementary cleaning effect, they present other problems. For example, peroxide is risky to handle because it can; a) irritate and burn the eyes and skin, b) irritate the nose, throat and lungs causing coughing and c) cause shortness of breath and pulmonary edema (Ellis, 2008). On the other hand, caustic soda can cause thermal and chemical burns on the tissues it comes into contact with, and irritate the mucous membranes of the nose, throat and respiratory tract if inhaled [(https://wwwn.cdc.gov/TSP/MMG/MMGDetails.aspx?mmgid=246&toxid=45) cited date 05 Dec 2022]. Furthermore, its use in the scouring process is carried out at high temperatures thus, contributing to the high cost of energy and treatment of affected personnel (in case of an accident). This has a negative impact on the profits earned.

Besides, both chemicals can attack the cloth (air-induce cellulose degradation) and cause high fabric weight losses (weak fabric) associated with low tensile strengths and rough 'feel' [https://www.fibre2fashion.com/industry-article/2813/common-problems-in-desizing-their-countermeasures (cited date 05 Dec 2022)] (Singh *et al*., 2018).

Such poor-quality fabrics also affect the company's profit margins. Moreover, the effluents generated from both pretreatments are often high in pollution loads [e.g., Biological Oxygen Demand (BOD), Chemical Oxygen Demand (COD) and Total Dissolved Solids (TDS)] and are therefore a potential threat to the environment (Singh *et al*., 2018; Moksov, 2012; Wambaire, 2017). In addition, the CO_2 that is emitted during oxidative desizing is a greenhouse gas that contributes to global warming, which is a threat to biodiversity (Ul-Haq and Nasir, 2012; United Nations, 2020). Furthermore, both pretreatments are expensive, since large volumes of increasingly hot (50 - 95 ^{o}C) and cold waters (room temperature) are required to wash the pretreated fabrics [personal communication from Hosea Too, Rivatex (EA) Ltd, Kenya]. This in turn, generates large volumes of chemically contaminated wastewaters, which are not only harmful to the flora and fauna in potential receiving water bodies, but can also affect the underground drinking water supplies.

Finally, both processes take long to complete i.e., 8 - 12 h (oxidative desizing) and 6 - 8 h (caustic scouring) [personal communication from Hosea Too, Rivatex (EA) Ltd (REAL), Kenya]. This means that the turnover rate of the pretreated fabrics is low, and this has a negative impact on the company's profits. In conclusion, the local textile industry is facing serious challenges and efforts to develop cleaner production technologies are crucial for its survival. To address these challenges, there is great interest in the search for indigenous microorganisms as biotechnological sources for enzymes with improved catalytic and stability properties for more efficient bioprocesses in the local textile industry as an alternative to the fabric-damaging and eco-unfriendly conventional chemicals.

1.3 Justification and significance of the study

Kenya's Cotton, Textile and Apparel (CTA) sector is the second largest manufacturing industry after food processing and has the potential to anchor the country deeper into middle-income status through massive employment, growth and export. The sector earned KES 33.7 billion in nine months to September 2021 from both the local and international markets and has potential to generate more [(https://kenyanwallstreet.com/earnings-from-textile-exports-hit-33-7-b/#:~:text=7%20Billion%20in%202021,-byEunniah%20Mbabazi) cited date 20 Dec 2022], [(https://rivatex.co.ke/about/) cited date 20 Dec 2022].

Despite the huge potential that the local textile industry has on the Kenyan economy, the industry is still not associated with the use of fabric- and eco-friendly neutral desizing and scouring enzymes due to their high cost of importatition. Instead, it has embrased the use of conventional fabric damaging and environmental unfriendly desizing and scouring chemicals. Therefore, adopting the use of alkaline desizing and scouring enzymes obtained from indigenous alkaliphilic microorganisms, in the textile industry, appear to be an ideal solution to the existing problems. This will lead to the production of high quality fabrics in a more cost-effective way while minimizing or eliminating the pollution loads.

Alkaliphilic bacteria, particularly those of the genus *Bacillus*, are a rich source of industrial enzymes that are active and stable in alkaline environments. The Kenyan African Rift valley region has a number of alkaline (soda) lakes and hot springs, and these are two environments that are rich in alkaliphilic microorganisms (Kambura *et al*., 2016; Okanya, 2006). In order to avail some of the alkaline enzymes produced by these microorganisms to the biotechnology community in the region, this research initially focused on: a) establishing a collection of well-characterized alkaliphilic microorganisms that produce alkaline amylases and pectinases, b) determining the optimum operating and

storage conditions of an amylase and pectinase from the most potent producer(s) of these enzymes and c) carrying out a preliminary investigation to assess the desizing and bioscouring potential of the amylase and pecinase, respectively.

The research work is important because it will provide a platform for further experiments to be carried out to establish the optimum bio-preparation conditions for woven cotton with a view to improving the quality of the processed fabric while reducing both the: a) pollution loads generated and b) operational costs in terms of process times, and water- and energy consumption. Thus, the effort will establish the groundwork for the development of powerful tools for the local textile industry, while also giving an incentive to use eco-friendly and sustainable technologies.

1.4 General objective

To characterize an alkaline protease, pectinase and amylase obtained from alkaliphilic bacteria isolated from Lake Bogoria (a soda lake found in the Kenyan Rift valley region), and to demonstrate their potential to desize (amylase) and scour (pectinase or protease) industrially woven cotton.

1.4.1 Specific objectives

1. To isolate and identify soda lake alkaliphilic bacteria that produce alkaline proteases, amylases and pectinases.
2. To determine the operating and storage conditions of an alkaline amylase from the most potent producer of the enzyme and to demonstrate its desizing potential.
3. To determine the optimum operating and storage conditions of an alkaline pectinase from the most potent producer of the enzyme and to demonstrate its bio-scouring potential.

1.5 Research questions

The study was based on the following research questions:

1. What is the identity of the amylase- and pectinase- producing soda lake alkaliphilic bacteria?
2. What are the optimum operating and storage conditions of the amylase from the most potent producer of the enzyme? Can the enzyme desize woven cotton under the established optimum operating conditions?
3. What are the optimum operating and storage conditions of the pectinase from the most potent producer of the enzyme? Can the enzyme scour woven cotton under the established optimum operating conditions?

1.6 Hypotheses

1.6.1 Alternate hypothesis

The amylase and pectinase from the most potent producer of these enzymes possess good operational and storage properties that make them good candidates for desizing (amylase) and scouring (pectinase) woven cotton.

1.6.2 Null hypothesis

The amylase and pectinase from the most potent producer of these enzymes do not possess good operational and storage properties hence, making them unsuitable candidaesfor desizing (amylase) and scouring (pectinase) woven cotton.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Alkaliphiles

The term alkaliphile is derived from the words '*al-qualiy*' - (Arabic for soda ash) and '*phile*' – (loving) (Uilukanli and Digrak, 2002). An alkaliphile is defined as a microorganism that grows optimally at pH > 9.0 (often between 10 and 12), but cannot grow or grow slowly at near neutral pH. On the other hand, an alkali-tolerant is a microorganism that is capable of growing slowly at pH values > 9.0, but grow optimally around neutrality (Kroll, 1990). This definition may however not be accurate because microorganisms exhibit more than one optimum pH of growth, depending on the growth conditions e.g., nutrients and temperature among others (Horikoshi, 1999).

2.1.1 Habitats for alkaliphiles

Alkaliphiles inhabit both aquatic and terrestrial alkaline environments e.g., those generated by: a) human industrial activities, b) microbial activities/processes and, c) geochemical activities. Industrial processes such as cement manufacture, leather tanning, food processing, mining, indigo dye preparation, electroplating, paper and pulp production, and rayon manufacture generate alkaline environments that are home to alkaliphiles (Grant and Tindall, 1986).

Alkaliphiles also inhabit localized alkaline environments that arise from microbial activities e.g., ammonification, oxygenic photosynthesis and sulfate reduction. These environments may however be short lived because they are often unstable at pH > 10.0 (Grant and Tindall, 1986; Jones *et al*., 1998).

Yet another habitat that alkaliphiles can be found is alkaline ground water generated from the natural geochemical process of weathering of silicate minerals containing Ca^{2+} and Mg^{2+} (Uilukanli and Digrak, 2002). These environments are characterized by high pH

(about 11.0), high Ca^{2+} ions and low buffering capacity due to low solubility of $Ca(OH)_2$ and exposure to atmospheric CO_2 (Jones *et al*., 1998). They are also referred to as Ca^{2+} environments and are found in Turkey, Cyprus, California, Jordan, Oman and former Yugoslavia (Uilukanli and Digrak, 2002).

A significant number of alkaliphiles are found in alkaline soils (Kristjánsson & Hreggvidsson, 1995), and also in near neutral and acidic soils (Horikoshi, 1991). The number of alkaliphilic microorganisms found in ordinary neutral soils is about 1/10 to 1/100 that of the population of neutrophilic microorganisms (Horikoshi, 1996). Alkaliphiles have also been isolated from termite gut (Thongaram *et al*., 2005), fecal matter (Horikoshi, 1999) and in deep-sea sediments obtained at depths of 10,898 m of the Mariana Trench (Takami *et al*., 1997).

Alkaliphiles are also found in soda lakes and soda deserts, which represent some of the most stable naturally occurring alkaline environments found worldwide. These environments arise due to a combination of geographical, topographical and climatic conditions, which result in the generation of high concentrations of mineral carbonates (usually Na_2CO_3, which is responsible for alkalinity and provision of buffering capacity to the lake waters) and low concentrations of Mg^{2+} and Ca^{2+} (Uilukanli and Digrak, 2002). Although soda lakes are widely distributed worldwide, those found in the East African Rift valley and that of Wadi Natrum in Egypt are among the best-studied in terms of geochemical, limnological, biological and microbiological investigations (Duckworth *et al*., 1996; Grant *et al*., 1990; Jones *et al*., 1998; Rees *et al*., 2004). The alkalinity of these lakes evolves with simultaneous precipitation of other ions, particularly Na^+ and Cl^-, leading to the formation of saline alkaline lakes. In lakes with low salinity, the concentration of CO_3^{2-} ions exceed that of Na^+ ions and vice versa for highly saline lakes.

Alkaliphiles are also domiciled in carbonated hot springs, which may/may not be associated with soda lakes. These hot springs are characterized by low salinity and low pH values of ≤ 9.0 (Kristjánsson & Hreggvidsson, 1995).

2.1.1.1 Soda lakes found in the East African Rift Valley

The Afro-Arabian Rift valley is a faulted trough that stretches from Jordan through the Red sea to the Gulf of Aden and further southwards to East Africa and down to Mozambique. It covers a distance of about 5000 - 6000 km and varies in width and depth from about 30 - 50 km and a few hundred to several thousand meters, respectively. In East Africa, the valley splits into two: the east and west African Rift valleys. These regions are volcanically active (Hashim, 2004; Mamo, 2006).

The East African Rift valley has a number of soda lakes in Kenya (e.g., Bogoria, Elementaita, Magadi and Nakuru) and in Tanzania (Eyasi, Makati, Manyara and Natron). Their formation requires a combination of geological, topographical and climatic conditions. For instance: a) formation of alkaline environments (pH 10 - 11.5) containing high CO_3^{2-}, Na^+ and silicates and low Ca^{2+} and Mg^{2+}, b) restriction of the water to shallow basins (no outflow) and, c) evaporation concentration and sufficient rainfall to sustain a standing water body (Uilukanli and Digrak 2002; Grant, *et al*., 1990). The high concentration of CO_3^{2-} and Na^+ gives rise to saline alkaline environments whose salinity ranges from approximately 5% (w/v) NaCl in the more dilute northern lakes (e.g., Nakuru, Elementaita and Bogoria) to 30 % (w/v) NaCl (or higher) in the more saturated southern lakes (e.g., Magadi and Natron (Grant and Tindall 1986; Grant *et al*., 1990).

Also found within the volcanic region of the east African Rift valley are hot springs on the shores of the lakes. These springs are more dilute and less alkaline than the lakes they feed, but where mixing occurs, complex pH, temperature and salinity gradients occur, affording a range of different soda lake habitats, from where novel extremophiles such as

alkaliphiles, thermophiles, alkalithermophiles and haloalkaliphiles can be found (Duckworth *et al*. 1996).

The Kenyan section of the East African and the location of Lake Bogoria (00º 15’ N and 36º 06’ E), from where the samples used in this study were collected, is shown in Figure 2.1.

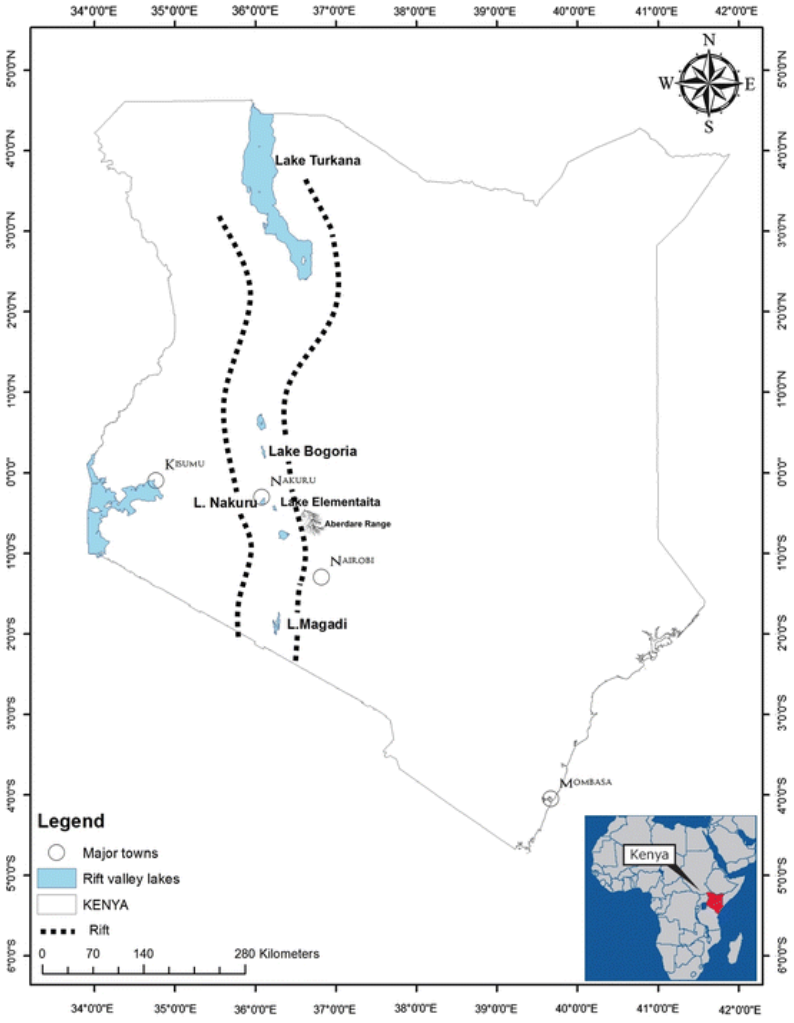

Figure 2.1: Section of the Rift Valley region and the position of Lake Bogoria on the Kenyan map. Inset is the position of Kenya on the African continent (Agembe *et al*., 2016).

2.1.2 Diversity of alkaliphiles in soda lakes

Despite the high alkalinity and saline conditions, soda lakes are considered to be among the world's most productive aquatic environments, with productivity rates reaching 10 g cells/m^2/d, or more, presumably because of the relatively high day light intensities, ambient temperatures and unlimited access to CO_2, in these carbonate-rich waters (Melack and Kilham, 1974; Grant *et al*., 1990).

Primary productivity in dilute soda lakes is largely attributed to the presence of dense populations of alkaliphilic cyanobacteria, namely *Spirulina* spp., (Grant *et al*., 1990) and to some extent eukaryotic algae of the genera *Nitzschia* and *Navicula* (Jones *et al*., 1994). On the contrary, primary productivity in the saturated soda lakes is largely attributed to alkaliphilic microorganisms belonging to the genera *Ectothiorhodnspira* and *Halorhodospira* (Grant and Tindall, 1986; Tindall, 1980). However, after extensive rainfall has caused dilution of the brine in these lakes, cyanobacteria may colonize them and contribute to the productivity therein, alongside the anoxygenic phototrophic bacteria.

A dense population of diverse non-phototrophic aerobic bacteria are found in the dilute soda lakes (Duckworth *et al*., 1996). They include Gram-negative bacteria belonging to the genera *Halomonas* (Duckworth *et al*., 2000), *Aeromonas*, *Vibrio, Pseudomonas*, and *Alteromonas* (Duckworth *et al*., 1996; Jones *et al*., 1998) and Gram-positive bacteria with low and high G + C contents. Gram positive bacteria with low G + C contents include those from the genus *Bacillus* e.g., *Bacillus clarkii*, *Bacillus alcalophilus*, *Bacillus halodurans*, *Bacillus pseudofirmus* while those with high G + C contents include those that belong to the genera *Dietzia*, *Arthrobacter* and *Terrabacter* (Duckworth *et al*., 1996). On the contrary, the concentrated brines of hypersaline soda lakes e.g., Magadi and Natron support the growth of alkaliphilic Archaea of the family *Halobacteriaceae* (halobacteria) and genera *Natronobacterium, Natronococcus, Natronomonas, Natrialba, Natronorubrum and Halorubrum* (Tindal, 1988). Members of this genera are the most

halophilic organisms known and they typically color brines red due to C50 carotenoids. (Tindal *et al*., 1988).

Soda lakes found in East Africa also support anaerobic microbial populations such as sulfate reducing bacteria (*Desulfonatronovibrio hydrogenovorans*) (Zilina *et al*., 1997), methanogenic bacteria (*Methanohalophilus zhilinae* strain Z-7936) (Kevbrin *et al*., 1997) and acetogenic ammonifiers (*Natronincola histidinovorans* and *Tindallia magadiensis*) (Zhilina *et al*., 1998; Kevbrin *et al*., 1998). In addition, the hot springs around these lakes harbor thermophilic anaerobes of the genera *Anaerobranca* and *Thermotogales* (Zhilina , 1994; Duckworth *et al.*, 1996; Jones *et al.*, 1998).

Although alkaliphiles are represented by several genera, the majority are Gram-positive bacteria that belong to the *Bacillus* sp. This is attributed to the manner in which sampling is carried out, culture conditions employed and the choice of isolates for study (Jones *et al*., 1998).

2.1.3 Isolation of alkaliphiles

Horikoshi and his colleagues have previously isolated a large number of alkaliphilic microorganisms using a recommended isolation protocol that they developed (Horikoshi, 1999). The isolation is carried out using 0.5 - 2.0 % (w/v) Na_2CO_3 (autoclaved separately) to adjust the pH of the medium to around 8.5 - 11.0. For cultivation periods longer than 48 h, close monitoring of the pH is highly recommended because high pH medium tends to absorb CO_2 from the environment thereby reducing the pH values markedly (Grant and Tindall, 1980). In addition to providing the alkaline environment, Na_2CO_3 provides Na^+ ions required for growth, germination and sporulation of most alkaliphilic *Bacillus* isolates (Horikoshi, 1996). Table 2.1 shows the composition of the alkaline isolation medium developed by Horikoshi and his colleagues (Horikoshi I), and a modified (enriched) version of the medium used in our laboratory to isolate alkaliphiles used in this study**.**

Table 2.1 Basal medium for isolating alkaliphiles (Horikoshi, 1999).

Ingredients	Horikoshi I (g/L)	Modified Horikoshi I (g/L)
Glucose	10	1.0
Polypeptone	5.0	-
Yeast extract	5.0	5.0
KH_2PO_4	1.0	-
$Mg_2SO_4 \cdot 7H_20$	0.2	-
Na_2CO_3	10.0	10.0
Agar	20.0	20.0
Bactopeptone	-	10.0
Malt extract	-	5.0

2.1.4 Adaptation of alkaliphiles to alkaline environment

Most alkaliphiles grow optimally at around pH 10 while their intracellular enzymes (e.g., α-galactosidase from alkaliphile *Micrococcus* sp. strain 31-2) operate optimally at pH 7.5 thus, suggesting that their cytoplasmic pH is around neutral (Horikoshi, 2004). Furthermore, their cell-free protein synthesizing machinery has been established to optimally assemble amino acids into protein at pH 8.2 - 8.5, which is just 0.5 pH units higher than that of neutrophilic *B. subtilis* (Horikoshi. and Akiba, 1982).

Several physiological studies have been carried out to find out how these alkaliphiles are able to grow under such an extreme environment while maintaining their internal cytoplasmic pH values near neutral. In this regard, mechanisms involving their cell wall components and high cytoplasmic buffering capacity have been suggested (Krulwich *et al*., 1997, 1998).

2.1.4.1 Cell wall composition

One such study reported that the cell wall of Gram-positive alkaliphiles consists of peptidoglycan and anionic polymers [e.g., galacturonic acid, gluconic acid, glutamic acid, aspartic acid, phosphoric acid, teichoic acid, teichuronic acid and teichuronopeptides (contains uronic acids)] whose concentrations tend to increase when these bacteria grow under alkaline conditions (Aono and Horikoshi, 1983; Aono *et al*., 1995). These

negatively charged acidic polymers repel the OH^- ions thus, lowering the local pH within vicinity of the outer membrane surface, and this helps the cells to grow in the alkaline environments (Yumoto, 2002). In addition, the cell membrane components found in alkaliphiles have been reported to vary from those in non-alkaliphiles e.g., the phospholipid heads in the former are highly negatively charged thus, suggesting that they could be involved in pH homeostasis (Hicks and Krulwich, 1995).

2.1.4.2 Cytoplasmic buffering capacity

In addition to being an essential mineral that contributes to the growth, germination and sporulation in most alkaliphilic *Bacillus* isolates (Horikoshi, 1996), Na+ ions have also been shown to play a key role in regulating cytoplasmic pH in alkaliphilic *Bacillus pseudofirmus* and *Bacillus halodurans* C-125) (Krulwich *et al*., 2001).

This pH regulatory system is an intricate Na^+ cycle that couples Na^+ expulsion to electron transport for pH homeostasis and energy transduction (Figure 2.2). The cycle begins with a net influx of H^+ with a concomitant efflux of Na^+ via the Na^+/H+ antiporters followed up by a second phase that involves mechanisms that facilitate the re-entry of Na^+ via the; a) Na^+/amino acid or oligosaccharide symporters and, b) possibly the channel associated with Na^+ - dependent flagella motion (Krulwich *et al*., 2001).

Whereas the pH homeostasis mechanisms discussed above provide an insight into the regulation of cytoplasmic of alkaliphilic *Bacillus* species, further studies are needed to clarify the mechanism of adaptation of phylogenetically different alkaliphiles to high pH. In addition, it is clear that although the cytoplasmic pH of alkaliphiles is at near neutral pH, their extracellular enzymes are active and stable under alkaline conditions.

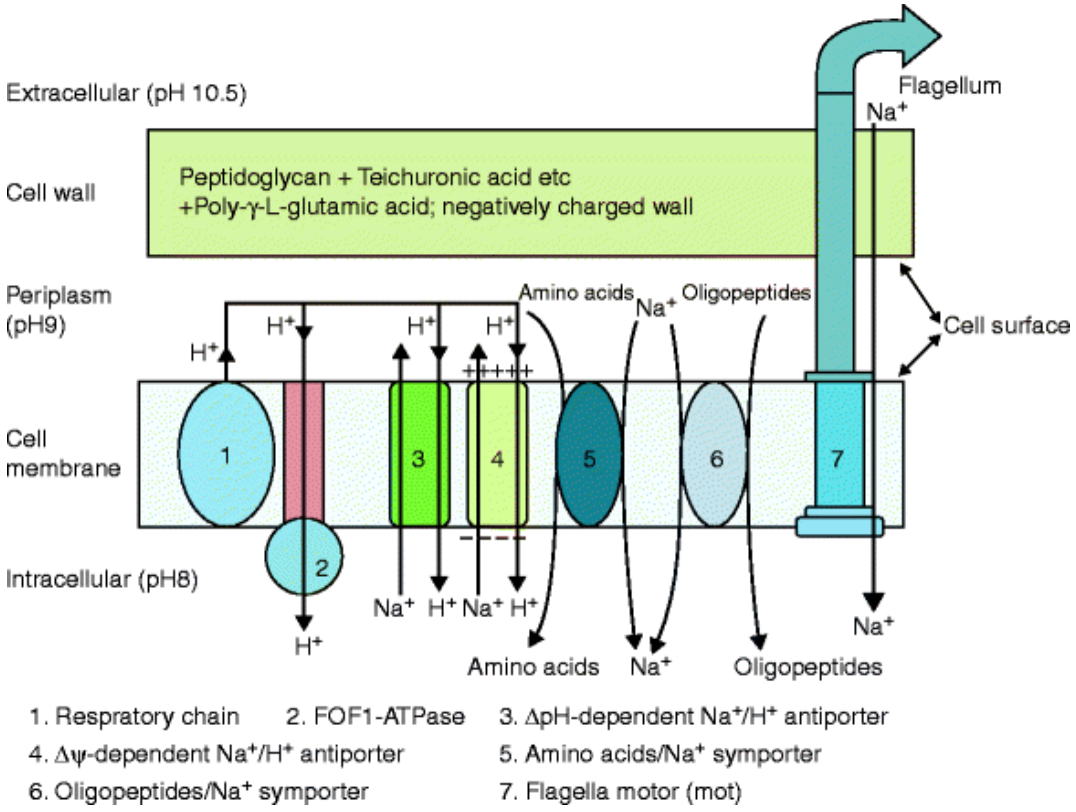

Figure 2.2: Schematic illustration of cytoplasmic pH regulation in alkaliphilic *B. halodurans* C-125 (Horikoshi, 2011).

2.1.5 Biotechnological application of enzymes produced by alkaliphiles

Alkaliphiles, particularly those of the genus *Bacillus* sp., have piqued the interest of researchers in recent decades due to their ability to produce extracellular enzymes that are active and stable under alkaline environments (alkaline enzymes). This unusual property makes them suitable for use in industrial processes that demand similar conditions. Many alkaline enzymes of alkaliphilic origin are available in the world market for various industrial applications, including:

a) Additives in laundry and automatic/hand dishwasher detergents

Alkaline enzymes from *Bacillus* sp. e.g., proteases, amylase, lipases, pectinases and phosphodiesterases and mannanase are often added to laundry and automatic/hand dishwasher detergents [https://biosolutions.novozymes.com/en/laundry (cited date 2023 Jan 16)]. The enzymes' role is to hydrolyze the protein-, starchy-, fatty-, pectin-, body grime (dead cells, sweat and sebum) and mannan containing gummy (guar/locust bean)-stains, respectively, on the surface of soiled garments, china or cutlery into smaller molecules and thus, make it easier for the detergent's surface-active agents to gain access and effectively remove the partially hydrolyzed stains.

Some commercially available laundry detergent alkaline enzymes include: *Progress® Uno* 100L (protease), *Amplify® Prime* 100 L (amylase), *Lipex® Evity® 200 L* and Xpect® 1000 T (pectinase) and *Pristine® 100 L* (phosphodiesterase) from Novozymes A/S Bagsvaerd, Denmark. Others include; *Intensa® Evity® 145 T* and *Intensa Core 220 L*, which are suitable for automatic- and hand- dish washing, respectively [https://biosolutions.novozymes.com/en/laundry (cited date 2023 Jan 16)].

(b) Leather dehairing and degreasing

Hides and skin contain a lot of proteins and fats in between the collagen fibers, which eventually forms the leather. In several processes such as soaking, bating and degreasing, the raw material is gradually transformed to leather. Several of these steps require harsh conditions e.g., high pH, and in the process, generate high pollution loads in the process streams.

NUE™ 6.0T (protease) and *Greasex® Ultra* (lipase) are among the commercially available alkaline enzymes that are increasingly used to dehair and degrease hides and

skins, respectively, thereby reducing the use of traditional chemicals e.g., sodium sulphide [(https://biosolutions.novozymes.com/en/leather) cited date 2023 Jan 16].

(c) Recovery of silver from used photographic and x-ray films

Photographic and x-ray films contain about 2 % silver on gelatin layers. This silver is recovered from waste photographic and x-ray films, and is conventionally retrieved by burning the films, a process that contributes to environmental hazards. Alkaline proteases from alkaliphilic *Bacillus* sp. can carry out the hydrolysis of the gelatin layer in an eco-friendly way, allowing the polyester base of the film to be recycled as well as assisting the retrieval of silver (Al-abdalall & Al-khaldi, 2016).

(d) Management of industrial and household proteinaceous wastes

Waste feathers make up about 5 % of the body weight of poultry and are considered a high protein source for food and feed, provided their rigid keratin structures are destroyed. The use of keratinolytic proteases to manage waste keratinous material from poultry refuse and as depilatory agent to remove hair from house-hold drains have been reported (Ichida *et al*., 2001).

In addition, the treatment of pectic wastewaters from both citrus processing and vegetable food processing industries with alkaline pectinases or alkaliphilic pectinolytic microbes have been reported to facilitate the removal of pectinaceous material thus, rendering the treated wastewaters suitable for decomposition by activated sludge treatment (Tanabe *et al*., 1987; Thakur & Kumar, 2019).

(e) Production of poultry and animal feed

When poultry and animals digest their feed, their stomachs and small intestines secrete a wide range of enzymes that break down starch, protein, pectins and fats into their

respective smaller components, which are then absorbed as vital nutrition. This however makes up about 30 - 90 % of the nutrients in the feed, implying that the rest pass out of the animal as waste in the faeces [(https://biosolutions.novozymes.com/en/animal-nutrition) cited date 2023 Jan 28], (Jayani *et al*., 2005). To improve the net benefit that the poultry and animals can derive from the feeds, the feed digestibility is improved using alkaline enzymes e.g., proteases, amylases, xylanases, amylases, β-glucanases, cellulases, xyloglucanase and phytases. Addition of the enzyme: a) reduces the viscosity of the feed, which in turn increases absorption of nutrients and, b) liberates nutrients by hydrolysis of nondegradable fibers or by liberating nutrients blocked by these fibers. This translates to a reduction in the amount of faeces and increase in both body weight gain and health.

Some of the commercially available enzymes that are used in the preparation of animal and poultry feeds include: a) *Ronozyme® Pro* Act (protease) b) *Ronozyme®* (amylases) c) *Ronozyme® MultiGrain* (multi-enzyme - xylanases, β-glucanases, cellulases and xyloglucanase) and d) *HiPhorius™* (phytases) [(https://biosolutions.novozymes.com/en/animal-nutrition) cited date 2023 Jan 28].

(f) Production of cyclodextrins

Cyclodextrins (CDs) are a family of non-reducing cyclic oligosaccharides composed of 6, 7 and 8 D-glucose repeating units linked by α-(1,4) glycosidic linkages. Their interior and exterior surfaces are hydrophobic and hydrophilic, respectively - a property that enables them accommodate various organic hydrophobic ‘guest’ compounds within their cavities and in the process, form inclusion complexes that alter or improve the physical and chemical properties of the compound e.g., solubility, chemical reactivity and sensitivity to heat, oxygen and light. The altered characteristics of the encapsulated compounds have led to the application of CDs in various industries e.g., pharmaceutical, cosmetic, analytical chemistry, food, textile, plastic and agricultural industries, among others (Yousaf *et al*., 2022; Saini *et al*., 2022). Commercial production of CDs starts with the

gelatinization of starch followed by enzymatic liquefaction (thermostable α-amylase) of the viscous gel formed and finally, cyclization of the dextrins formed by CGTase from *Bacillus macerans*. The disadvantage of this process is that the cyclization reaction is carried out at a temperature that is much lower (60 ^{o}C) than that of liquefaction (90 - 105 ^{o}C) because of the low thermostability property of *Bacillus* CGTase. This can however be resolved using thermostable CGTases from thermophiles.

(g) Coffee and tea fermentation

Tea prepared using instant tea granules/powder often generate foam or froth due to the high concentration of pectins in the granules or powder. To avoid this, alkaline pectinases e.g., polygalacturonases are added during tea fermentation to degrade the pectins, which are associated with the foam-forming property of the granules. As a result, the tea's quality improves, its color changes, and it becomes more valuable in the market. (Haile & Ayele, 2022).

Alkaline pectinases are also used to remove mucilage (outer coat) from raw coffee beans during coffee fermentation (Haile & Ayele, 2022). During this process, raw coffee beans are fermented with pectinolytic bacteria, which degrade and remove the pectin-rich mucilagenous layer from the beans. Oumer and Abate, 2017 observed that complete de-mucilisation of pectinase treated coffee beans occurred within 24 h of incubation, but in the case of natural fermentation, this process was incomplete even after 36 h.

(h) Textile processing

(i) Silk-degumming

Raw silk consists of a gummy soluble non-filamentous protein called sericin that encloses a filamentous insoluble fibroin protein. The process of eliminating sericin (gum) to obtain the desired fibroin is referred to as degumming. It involves the selective cleavage of sericin peptide bonds followed by the removal of sericin hydrolysate from the fibroin. This is

typically carried out in 0.1 - 0.5 % (w/v) Na_2CO_3 containing soap at 92 - 98 ^oC for 2 - 4 h. Although this treatment is fast, inexpensive and effective in removing the gum, it produces poor quality fibroin fibers (low tensile strength) and high pollution loads (BOD and COD) in the generated effluents. Furthermore, it is a high water- and energy- consuming process (More, 2015). To circumvent this problem, a number of alkaline proteases have been reported to remove the sericin protein layer of the silk and maintain the original fibroin physical (natural color, softness and smoothness, luster or shine and tensile strength] and chemical (good moisture absorbancy and dye ability) - properties (Rajasekhar *et al*., 2011). However, the use of enzymes in degumming silk is yet to be performed in large-scale because: a) it consumes large volumes of enzymes in a market with an insufficient supply and, b) the price of enzymes is higher than that of chemical reagents.

(ii) Desizing and bioscouring of woven cotton

In the textile industry, an aqueous solution of starch (sizing material) is applied on warp yarns (sizing) to facilitate a fast and secure weaving process, after which it is removed in a safe and eco-friendly process using amylases (e.g., Aquazym ® Prime 12000L under alkaline conditions) in a process called enzymatic desizing (Sultana *et al*., 2014) [(https://biosolutions.novozymes.com/en/textiles/products/desizing/aquazym) cited date 25 July 2023]. This is done to prevent the starch from reacting with alkali and thus, bring about color changes in the textile product (Chinnammal, & Arunkumar, 2013), and also to allow for easier penetration of dyes in the subsequent pretreatment steps.

(iii) Bioscouring of woven cotton

Once the fabric has been desized, it is subjected to the process of scouring. This involves the use of pectinases e.g., BioPrep 3000L under alkaline conditions) to remove 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 water and dye absorbency properties (Colombi *et al*., 2021). Pectinase may be used in combination with proteasesand lipases to improve the efficiency of the bioscouring process.

2.1.6 Alkaliphilic *Bacillus halodurans* and *Bacillus pseudofirmus*: characteristics and their enzymes

a) Bacillus halodurans

The word *'halodurans'* can be traced from the Greek word *'hals'* (salt) and the Latin word *'durans'* (enduring). Hence, *Bacillus halodurans* is a salt enduring microorganism (Nielsen *et al*., 1995). Strains of this microorganism were first classified as *Bacillus alcalophilus* subsp. *halodurans* because of their close taxonomic relationship with *Bacillus* alcalophilus (Boyer *et al*., 1973). However, further studies involving their physiological and genetic characterization revealed that they were new species and were therefore designated *Bacillus halodurans* (Fritze, *et al*., 1990). The type strain of this species is *Bacillus halodurans* DSM 497. Morphologically, its colonies are white and may or may not have filamentous margins while its cells are rod-shaped and produces sub-terminal ellipsoidal spores. Moreover, most can grow at pH 7.0 although they grow optimally at pH 9.0 - 10.0. Their growth temperature and salt tolerance range from 15 - 55 ^{o}C and > 0 - 12 % (w/v) NaCl, respectively**.** Other characteristics exhibited by this bacterial species include; hydrolysis of starch, casein, gelatin, and pullulan. It utilizes galactose, inositol, mannose, L-arabinose, xylitol, and N-acetylglucoseamine or 2-ketogluconate. It does not reduce nitrate and its G+C content is 42.1 and 43.9 % mol % (Nielsen, *et al*., 1995). Among the alkaline enzymes produced by alkaliphilic *Bacillus halodurans* are xylanases, amylases, proteases, pullulanases and pectinases. Some properties of these extracellular enzymes are shown in Table 2.2.

b) Bacillus pseudofirmus

The term *pseudofirmus* is derived from the Greek word pseudes, which means "false," and the particular epithet "*firmus*." Hence, *Bacillus pseudofirmus* is the false firmus; referring to the physiological similarities to *Bacillus firmus.* The type strain of this species is *Bacillus pseudofirmus* DSM 8715. Its colonies are yellow and round with regular or irregular margins while its cells are rod-shaped, producing spores. They hydrolyse Tween 40 and 60, casein, gelatin and starch**.** The strains are obligate alkaliphiles with optimum growth at around pH 9.0. Their growth temperature and salt tolerance range from 10 - 45 ^{o}C and > 0 – 16 or 17 % (w/v) NaCl, respectively**.** They can utilize ribose or D-xylose, but no growth on galactose, sorbitol, rhamnose, L-arabinose or lactose. The chromosomal DNA composition is between 39.0 and 40.8 mol % mol G + C (Nielsen *et al*., 1995). Alkaline enzymes produced by alkaliphilic *Bacillus pseudofirmus* include amylases, proteases, pullulanases and lipases, among others. Some properties of these extracellular enzymes are shown in Table 2.2.

Table 2.2 Properties of some extracellular alkaline enzymes produced by different strains of alkaliphilic *Bacillus halodurans* (Hashim *et al*., 2005; Mamo *et al*., 2006; Asha *et al*., 2013; Mei *et al*., 2013; Daoud *et al*., 2018) and *Bacillus pseudofirmus* (Dhundale *et al*., 2014; Sen *et al*., 2011; Lu *et al*., 2018; Tambekar *et al*., 2014).

Alkaliphilic *Bacillus* species	**Strain**	**Enzyme**	**Optimum temperature of activity (^{o}C)**	**Optimum pH of activity**
B. halodurans	S7	Xylanase	75 at pH 9, 70 at pH 10	9.0 - 9.5
	LBK 34	Amylase	60	10.5 - 11.5
	US193	Protease	70	10.0
	-	Pullulanase	50	10.0
	M29	Pectinase	80	10
	LBW 5117	Amylase	60	10
	LBW 5117	Pectinase	50	10.5
B. pseudofirmus	DW4 (1)	Amylase	40	10.0
	SVB1	Protease	40	10.0
	703	Pullulanase	45	7.0 - 8.0
	CS4	Lipase	60	9.0

2.2 Starch and starch converting enzymes

2.2.1 Occurrence, structure and applications of starch

Besides cellulose, starch ranks among the most abundant carbohydrate polymers on earth. It occurs in plants in the form of granules and is particularly abundant in cereal grains e.g., wheat (*Triticum aestivum*), rice (*Oryza sativa*) and corn (*Zea mays*), where it constitutes 40 - 90 of the dry weights. The polymer is also found in tubers (e.g., Irish potato (*Solanum tuberosum*), sweet potato (*Ipomoea batatas*) and tapioca (*Manihot esculenta*), where they constitute 15 - 25 % of the dry weights.

Starch is a complex carbohydrate polymer composed of α-glucose units linked by glycosidic bonds between the O-atom at C-1 on one glucose unit and the O-atom at C-4 (α-1,4) or C-6 (α-1,6) on the next glucose unit (Antranikian, 1990; Van Der Maarel *et al*., 2002). These bonds are stable under alkaline conditions, but hydrolyze under acidic conditions. This polysaccharide is made up of two kinds of glucose polymers;

a) Amylose

Amylose accounts for 15-25% of the starch content. It is a linear polymer composed of up to 6000 α-glucose units that are solely linked by α -1,4 glycosidic linkages [Figure 2.3a) (i)]. Experimental evidence however shows that due to hydrogen bonding, the polymer is not a straight chain, but is instead coiled to form a spiral structure that contains six glucose units per turn [Figure 2.3a (ii)].

b) Amylopectin

Amylopectin makes up 75 - 85 % of starch composition. It is a branched polymer consisting of short α-1,4 linked linear chains containing 10 - 60 glucose units and α-1,6 linked side chains with 45 - 60 glucose units (Figure 2.3b). In total, an amylopectin molecule may contain up to 2 million glucose units, thus making it one of nature's largest molecule (Antranikian, 1990; Van Der Maarel *et al*., 2002).

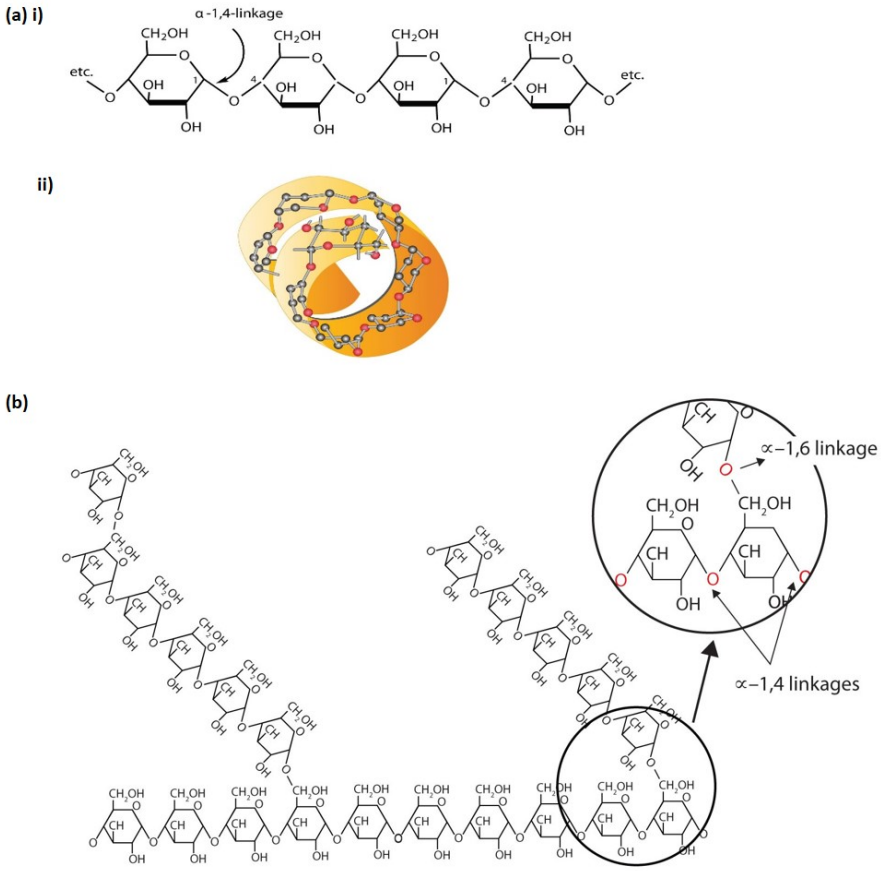

Figure 2.3: Structure of: (a) amylose [(i) and (ii)] and (b) amylopectin. Adapted from [https://saylordotorg.github.io/text_the-basics-of-general-organic-and-biological-chemistry/s19-07-polysaccharides.html (cited date 2022 Dec 23)].

The starch polymers serves as: a) storage carbohydrate in the plants b) food and c) source of industrial starch for conversion into malto-dextrins, cyclodextrins, maltose, high fructose and maltose syrups and crystalline sugar, which have various uses (Guzmàn-Maldonado and Paredes-Lòpez , 1995). Starch is also used in the textile industry to size warp yarns in order to facilitate a fast and secure weaving process (Sultana *et al*., 2014).

2.2.2 Enzymes involved in the degradation of starch

Due to the complex structure of starch, a combination of enzymes (starch degrading enzymes), are needed to coordinately degrade it to its final product (glucose), which can then be absorbed and used by organism' cells. These extracellular enzymes are called amylolytic enzymes or amylases and are produced by microorganisms (e.g., yeast, fungi and bacteria), plants and animals (Aiyer, 2005).

Starch degrading enzymes are classified into four groups: (a) endoamylases; (b) exoamylases; (c) debranching enzymes; and (d) transferases (Van Der Maarel *et al*., 2002):

a) Endoamylases

These enzymes exclusively cleave the α-1,4 glycosidic linkages present in the inner part of starch in a random fashion to form linear malto-oligosaccharides of varying lengths, with an α-configuration, and branched α-limit dextrins (Figure 2.4). Alpha amylase (EC 3.2.1.1) is a well-known endoamylase.

b) Exoamylases

Exoamylases either exclusively cleave the α-1,4 glycosidic linkages e.g. **β-amylase** (EC 3.2.1.2) or on both α-1,4 and α-1,6 glycosidic linkages e.g., **amyloglucosidase or glucoamylase** (EC 3.2.1.3) and **α-glucosidase** (EC 3.2.1.20). Exoamylases act on the external glucose residues from the non-reducing end of starch and thus produces only glucose (glucoamylase or glucosidase), or maltose and β-limit dextrin (β-amylase) (Figure 2.4). β-amylase and glucoamylase also convert the anomeric configuration of the liberated maltose from α to β. Furthermore, glucoamylase and α-glucosidase differ in their substrate preference where glucoamylase hydrolyzes long-chain polysaccharides best while α-glucosidase acts best on short maltooligosaccharides and liberates glucose with α-configuration.

c) Debranching enzymes

Debranching enzymes exclusively cleave the α-1,6 glycosidic linkages in both starch and limit dextrins to release long linear polysaccharides as end products (Figure 2.4). They include **isoamylase** (EC 3.2.1.68) and **pullulanase type I** (EC 3.2.1.41). The major difference between these two enzymes is that the latter is also able to cleave the α-1,6 glycosidic linkages in pullulan, a polysaccharide with a repeating unit of maltotriose that is α-1,6 linked. On the contrary, pullulanase type II enzymes (also known as **α-amylase-pullulanase or amylopullulanase pullulanase)** are able to cleave both α-1,4 and α-1,6 glycosidic linkages. The main degradation products are maltose and maltotriose. A special enzyme belonging to this group of pullulanases is **neopullulanase**, which can also perform transglycosylation, resulting in the formation of a new α-1,4 or α-1,6 glycosidic linkages (Van Der Maarel *et al*., 2002)

d) Transferases

This group of enzymes cleave the α-1,4 linkage of a donor sugar molecule and transfer part of it to another sugar (acceptor), resulting in the formation of a new glycosidic bond (transglycosylation (Figure 2.4). Examples of these enzymes include **amylomaltase** (EC 2.4.1.25) and **CGTases** (EC 2.4.1.19), which forms new α-1,4 glycosidic linkages and **branching enzyme** (EC 2.4.1.18), which form new α-1,6 glycosidic linkages. Both intramolecular and intermolecular transglycosylation have been demonstrated in these enzymes, depending on whether the fragment is transferred between two sugar molecules (amylomaltose) or within the same polysaccharide chain (CGTase and branching enzymes). In the former case, a linear polysaccharide is formed while in the latter case, a cyclic oligosaccharide product (CD) (usually with 6, 7, or 8 glucose residues) and highly branched high molecular weight dextrins are formed.

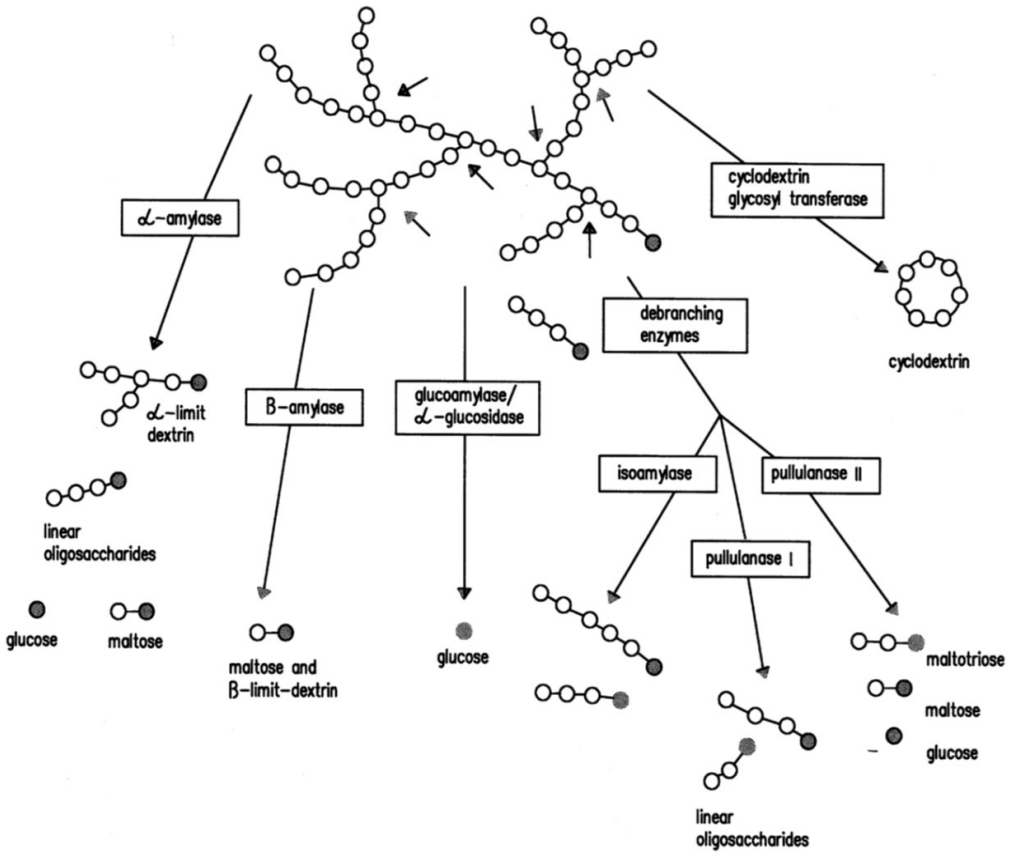

Figure 2.4: Action of starch degrading enzymes. (○) glucose unit, (●) glucose unit with a reducing end [Adapted from (Anranikian, 1991)].

2.3 Glycoside hydrolase family 13 (GH 13) amylases

Glycoside hydrolases (EC 3.2.1) are enzymes that cleave the glycosidic linkages in polysaccharides (Ly and Withers, 1999). Based on their amino acid sequence similarities, they are currently classified into more than 100 different families (GH families), with members of each family exhibiting a common 3-D structure and stereochemical mechanism of action [http://www.cazy.org/ (cited date 2022 Dec 25) (Drula *et al*., 2022)].

Glycoside hydrolase family 13, also known as the α-amylase family, consists of a big group of starch degrading amylases (e.g., α-amylase, pullulanase, CGTases, α-

glucosidase, maltooligosaccharide-producing amylases and glucoamylase) that exhibit more than 30 reaction specificities involving the α-1,4 and/or α-1,6 glycosidic linkages (MacGregor, 2001). All members of the α-amylase family share the following characteristics (Takata *et al*., 1992):

1) Act on α-glycosidic linkages to produce α-anomeric mono/oligosaccharides (hydrolysis), form α-1,4 or α-1,6 glycosidic linkages (transglycosylation), or show both activities.
2) Possess a $(\beta/\alpha)_8$ -barrel structure that is made up of eight parallel β-strands surrounded by eight α-helices and contains the catalytic residues (Figure 2.5a).
3) Have four highly conserved regions within the primary structure of their $(\beta/\alpha)_8$ – barrel. These regions contain amino acid residues that are essential for the stability of the conserved $(\beta/\alpha)_8$ - barrel, substrate-binding and catalysis. Among the residues that are important for catalysis are two Asp and one Glu.

Furthermore, most α-amylases are metalloenzymes and therefore require at least one or more Ca^{2+} ions which is/are essential for activity and stability (Sudha, 2012).

2.3.1 Domain organization within GH13 amylases

Members of GH13 amylases are composed of multi-domain proteins, whose type and organization determines the specificity of the enzyme (Van Der Maarel *et al*., 2002) (Jespersen *et al*., 1991). **Domain A** (catalytic domain) is the most conserved domain and consists of about 300 - 400 amino acid residues that are folded to form the characteristic $(\beta/\alpha)_8$ barrel (Figures 2.5a and b). This structure was first identified in triose phosphate isomerase (TIM) obtained from chicken muscle, and is therefore commonly referred to as the TIM - barrel structure. The loops between the β-strands and adjacent α-helices form the catalytic groove that accommodates conserved amino acid residues involved in substrate binding and catalysis (Figures 2.5a and b).

Protruding between β3-strand and α3-helix of domain A is a long loop (44 - 133 residues), which forms a separate domain i.e., **domain B (**Figure 2.5b**).** The length and sequence of this domain varies among GH13 members. This domain is responsible for the differences in substrate specificities within the α-amylase family. In addition, it contains an invariant Asp residue, which is involved in the binding of Ca^{2+} ions that helps to maintain the structural stability of the enzyme during catalysis (Boel *et al*., 1990).

In addition to domains A and B, present in all the α-amylase family members is **domain C** (Figure 2.5b). This is a Greek key motif, which occurs at the C-terminal part of the sequence and is responsible from protecting the hydrophobic residues of the TIM barrel from solvents (Janeček, 1997; Ramasubbu *et al*., 1996).

2.3.2 Conserved regions within the primary sequences of GH13 amylases

Although the amino acid sequence similarity among members of the α-amylase family is low (< 30 %), seven highly conserved sequence regions have been identified in the primary sequences of their TIM- barrel structure (I-VII). The first four conserved regions I, II, III and IV are located at or near the active site on β-strands 3, 4, 5 and in the loop connecting β7-strand to α7- helix within domain A of the TIM-barrel (Figure 2.5b). They contain amino acid residues that play a role in catalysis i.e., substrate binding, stabilization of the transition state, binding of calcium ligands and cleavage of the glycosidic bond (Table 2.3).

In addition to the four conserved regions, three more conserved regions in the GH13 amylase sequences have been proposed (Horváthová *et al*., 2001; Janecek, 1992). These are believed to play a role in the binding of calcium and enzyme specificity (Janeček, 1997).

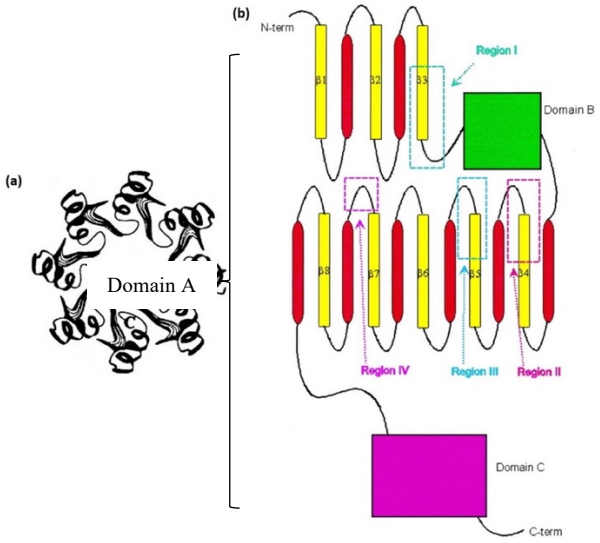

Figure 2.5: Schematic illustration of a GH13 amylase showing the (β/α)8 (TIM) – barrel (a) and the organization of its domains and conserved regions(b) (Nielsen and Borchert, 2000; Mehta and Satyanarayana, 2016).

Table 2.3 Conserved regions I to IV and their respective conserved catalytic residues in GH 13 amylases (Nielsen and. Borchert, 2000).

Region	Location	Residue	Function
I	β3-strand	Asp Asn His	Maintenance of active site integrity Coordinates the conserved Ca^{2+} between A and B domains Stabilizes the interaction between C-terminal of β-3 and the rest of the TIM barrel
II*	β4-strand	Asp, Arg Lys, His	Catalysis Bind to reducing end of glucose chain
III*	β5-stand	Glu	Catalysis (proton donor)
IV	Loop between β7-strand and α7-helix	Residues Asp His, Phe, Val, Asn	Shield the active site from solvents Substrate binding, substrate distortion and elevation of pKa value of Glu

*Believed to contain residues that confer bond type specificity e.g., α(1,4) or α(1,6)

2.3.3 Catalytic mechanism of GH 13 amylases

The widely accepted catalytic mechanism of the GH13 amylases is that of the α-retaining double displacement (Kuriki *et al*., 2005). In this mechanism, two catalytic residues in the active site are involved: a glutamate as acid/base catalyst and an aspartate as the nucleophile. It involves five steps as shown in Figure 2.6:

a) Prior to the binding of the substrate, un-ionized Glu230 shares a hydrogen bond with the proton of Asp297 and with other water molecules which are also present around the catalytic triad (Figure 2.6a)

b) The substrate binds to the enzyme, resulting in the exclusion of water from the cleft. This leads to the disruption of the hydrogen-bonding network, including that between Glu230 and Asp297. This results in the release of a proton by Glu230 (Figure 2.6b).

c) The proton from Glu230 attacks the glyosidic oxygen (close to Asp230) between two glucose residues within the substrate, resulting in the cleavage of the glycosidic linkage and the release of a reducing sugar from the cleft (Figure 2.6c). Meanwhile, Asp208 located at the bottom of the cleft promotes the formation of a carbonium ion intermediate, which it also stabilizes. In addition, the ionized Glu230 generates a repulsive force between itself and Asp297.

d) To neutralize the repulsive force generated between Glu230 and Asp297, a water molecule approaches from the outer side of the cleft between these two residues and weakens the repulsive forces by forming a hydrogen bond between the two catalytic residues (Figure 2.6d).

e) Glu230 accepts a proton from the water molecule, and this generates a OH^- ion of the water molecule which attacks the carbonium ion intermediate to form a new OH group at the C1 position of the intermediate (Figure 2.6e).

It is important to note that since the water molecule approaches the catalytic site from the position of Glu230 and Asp297, which is the α-anomeric side, and not from the bottom of

the cleft, it results in the retention of the α-anomeric configuration in the released products. In the case of transglycosylation, a glucose molecule moves into the active site in step IV, donates a H^+ to Glu230 and result in the formation of a new glycosidic bond between the carbonium intermediate and itself (Van Der Maarel *et al*., 2002).

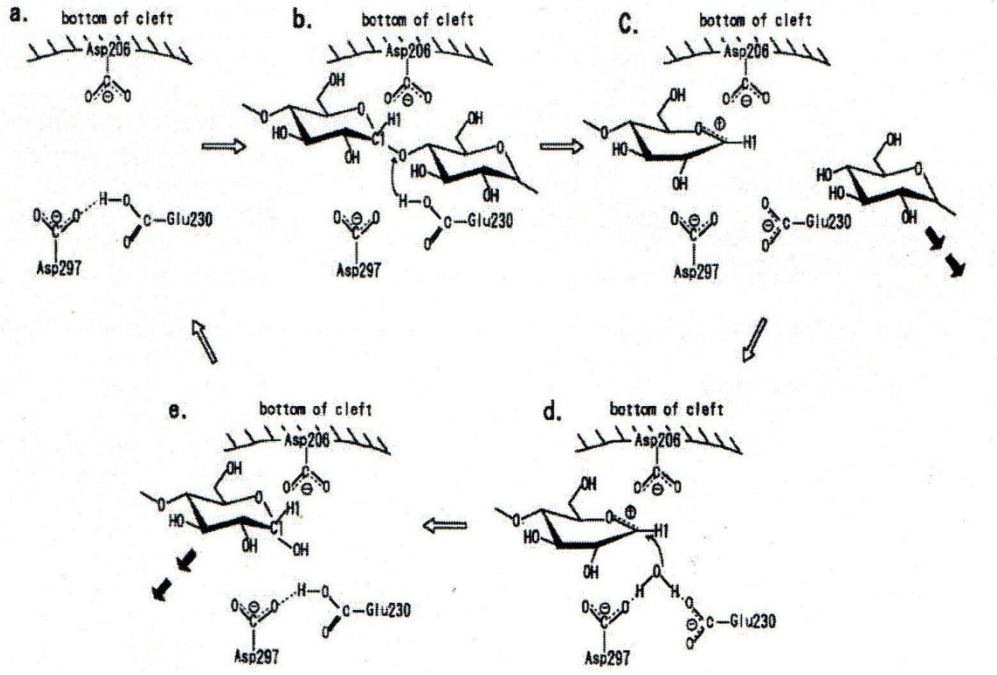

Figure 2.6: Catalytic mechanism of α-amylases (Kuriki *et al*., 2005).

2.4 Biotechnological application of α-amylases in the textile industry

Modern production of textiles include a process called weaving that introduces a lot of strain on warp yarns. To prevent these yarns from breaking, a detachable thin layer (size) is applied to them in a process called sizing. Among the materials used for this size layer is starch because it is inexpensive, easily available in most regions of the world, and is easily removable once the weaving is completed. The process starts with steaming the warp yarns with a hot (100 - 110 ^oC) homogenous solution that contains starch (100 kg) and beef tallow (8 kg) in a total volume of 1000 L H_2O. This is followed by drying them at 125 - 130 ^oC and then interlacing them at right angles with the weft yarn (weaving).

The size formulation encloses the warp yarns and, eventually, the fabric. This makes the sized fabric less absorbent to chemicals e.g., dyes in subsequent processing steps; hence may lead to uneven dyeing, printing, or finishing if not completely removed after weaving. The process of removing size from the warp yarns in woven cotton is called desizing. The desizing methods include acid desizing, oxidative desizing, and enzymatic desizing (α-amylase) (Agrawal, 2016). Out of these methods, enzymatic desizing is the commercially preferred method for removing the starch sizes from woven cotton. This is because enzymes are safe, biodegradable, require fewer chemical (eco-friendly) and do not attack the cellulosic structures of the cloth compared to chemical methods, which require the use of chemicals that are not only risky to handle, but can also damage the cloth and generate high pollution loads in time consuming processes.

2.4.1 Principle of the enzymatic desizing process

Among the GH 13 amylases, α-amylase is the enzyme of choice for desizing woven cotton. This is because it randomly hydrolyzes the α-(1,4) glycosidic bonds in starch to generate soluble malto-oligo saccharides that can be washed off the surface of the fabric easily, implying a faster desizing process (more profits). This pretreatment is carried out in the following three steps after washing the fabric in hot water (80 - 95°C) to gelatinize the starch (Agrawal, 2016; Ul-Haq & Nasir, 2012):

i. *Impregnation:* The fabric is wetted with enzymatic solution, which also contains a wetting agent whose role is to enhance the absorption of the enzyme by the fabric.
ii. *Incubation:* The temperature of the desizing bath is raised to match that of the enzyme's optimum operating temperature. This allows the enzyme to randomly hydrolyze the α-(1,4) glycosidic linkages in the starch and in the process, form

water-soluble dextrins. For low enzyme activities, longer incubation times are recommended and vice versa.

iii. *Washing:* This involves washing the desized fabric to remove the dextrins. Other ingredients e.g., tallow are easily removed when the hydrolyzed starch is washed away. To achieve the best desizing results, the fabric is washed in hot water (93 - 85 ^{o}C) with a detergent and then dried.

Alpha amylases that are today available for commercial desizing include *LTAA31, HTAA25L, CoenzymeLTAA3P* and *Coenzyme DD990*L (Sunsonzymes, Beijing, China) and *Aquazyme® Prime 12000L* and *Aquazyme® Ultra 1200N* (Novozymes A/S, Copenhagen, Denmark), among others [http://www.sunsonenzyme.com/Products/Textile/Desizing/ (cited date 16 Feb 2023)], [https://biosolutions.novozymes.com/en/textiles/products/desizing/aquazym (cited date 16 Feb 2023)].

2.4.2 Enzymatic desizing technologies

Several technologies are available for carrying out the desizing of woven cotton using α-amylases. They include:

2.4.2.1 Jigger desizing

This method of desizing is carried out in a batch process using a jigger machine (Figure 2.7). The sized fabric is first washed with hot water (80 - 95 °C) to gelatinize the starch and then rolled in a desizing bath (contains wetting agent, enzyme and emulsifier in in appropriate buffer) using the let in roller (A) and allowed to impregnate. The desizing liquor's temperature is then adjusted to 55 – 95^{o}C (depending on the optimum temperature of activity of the enzyme) to allow the enzyme degade the starch into soluble dextrins. Finally, the fabric is rolled out of the machine using the let out roller (B) and then subjected to washing at (90 - 95°C) to remove the dextrins (Agrawal, 2016).

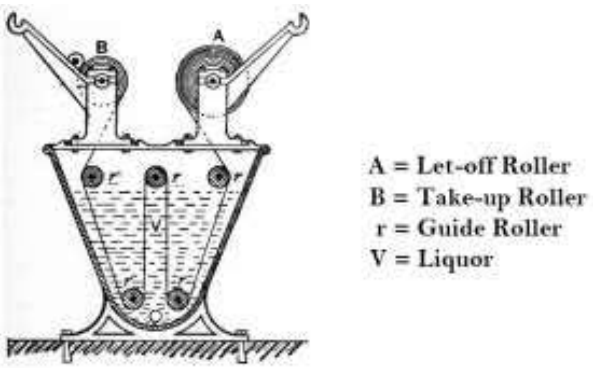

Figure 2.7: Process flow for jigger desizing method. Adapted from Agrawal, 2016.

2.4.2.2 Winch desizing

This method of desizing occurs in a continuous process. The grey fabric is passed through hot water to gelatinize the starch (Figure 2.8A) and then through the enzymatic solution (contains enzyme, salt, wetting agent and buffer to maintain the pH) to impregnate it. The fabric then passes into the winch through an entry pothole (E), which converts it from open-width form to rope form. This results in the fabric assuming a wavy structure as it accumulates in the pit of the winch (Figure 2.8B). The slope in the pit of the winch gives the required dwell time for desizing, after which the fabric is converted back to its open-width form by a second pothole (E) and then taken up for washing (Figure 2.8C). The desized fabric is then dried in preparation for the next step of processing i.e., scouring (Figure 2.8D). The advantages of this method of desizing include a) Shorter desizing times, b) higher production rates, since it is a continuous process and c) high quality fabrics, since cellulosic structures of the cloth are intact (enzymes act specifically towards their targets). A disadvantage associated with this method of desizing is decrease in desizing efficiency if the conditions e.g., temperature and pH are not maintained.

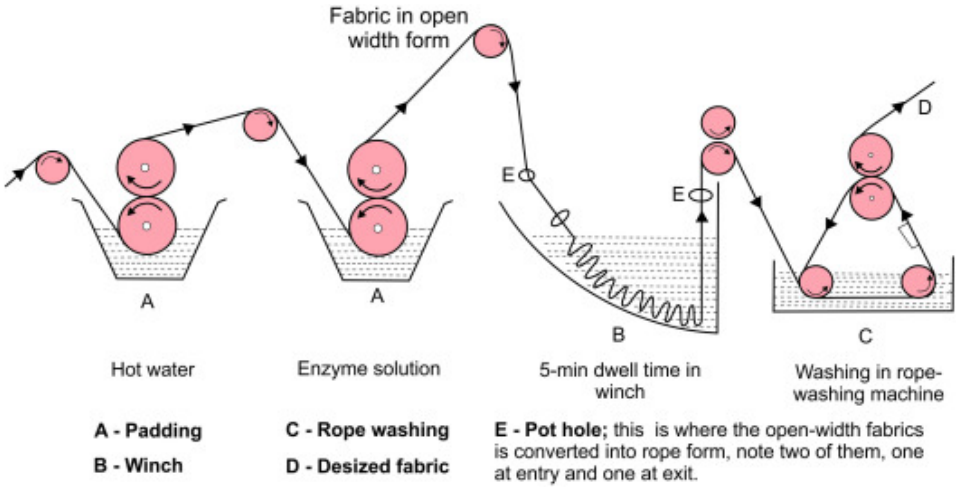

Figure 2.8: Process flow for winch desizing method. Adapted from Landage, 2022.

2.4.2.3 Pad-roll desizing

In terms of grey fabric movement, this is a semi-continuous process. [https://www.dspattextile.com/2022/05/padroll-and-padsteam-for-desizing.html (cited date 2023 Jan 21)], (Agrawal, 2016). The cloth, in open-width form, is impregnated/saturated with at least four dips in the desizing liquor containing a suitable α-amylase and other ingredients (Figure 2.9). This is quickly followed by passing it through a steam chamber (or by infrared radiations), where it is preheated before passing it to the batching chamber, where it is batched on a roll. The batched fabric is then passed to the incubation/storage chamber where it is left for 8 - 12 h to allow the enzyme digest the starch to water-soluble dextrins. During this period, it is important that the: a) fabric is gently rotated on the roller to prevent uneven desizing due to drainage and b) temperature is maintained by slowly feeding steam into the chamber. Once desizing is complete, the fabric is unwound from the roll and washed in open-width form using hot and cold water in that order, to remove the soluble starch hydrolysate products.

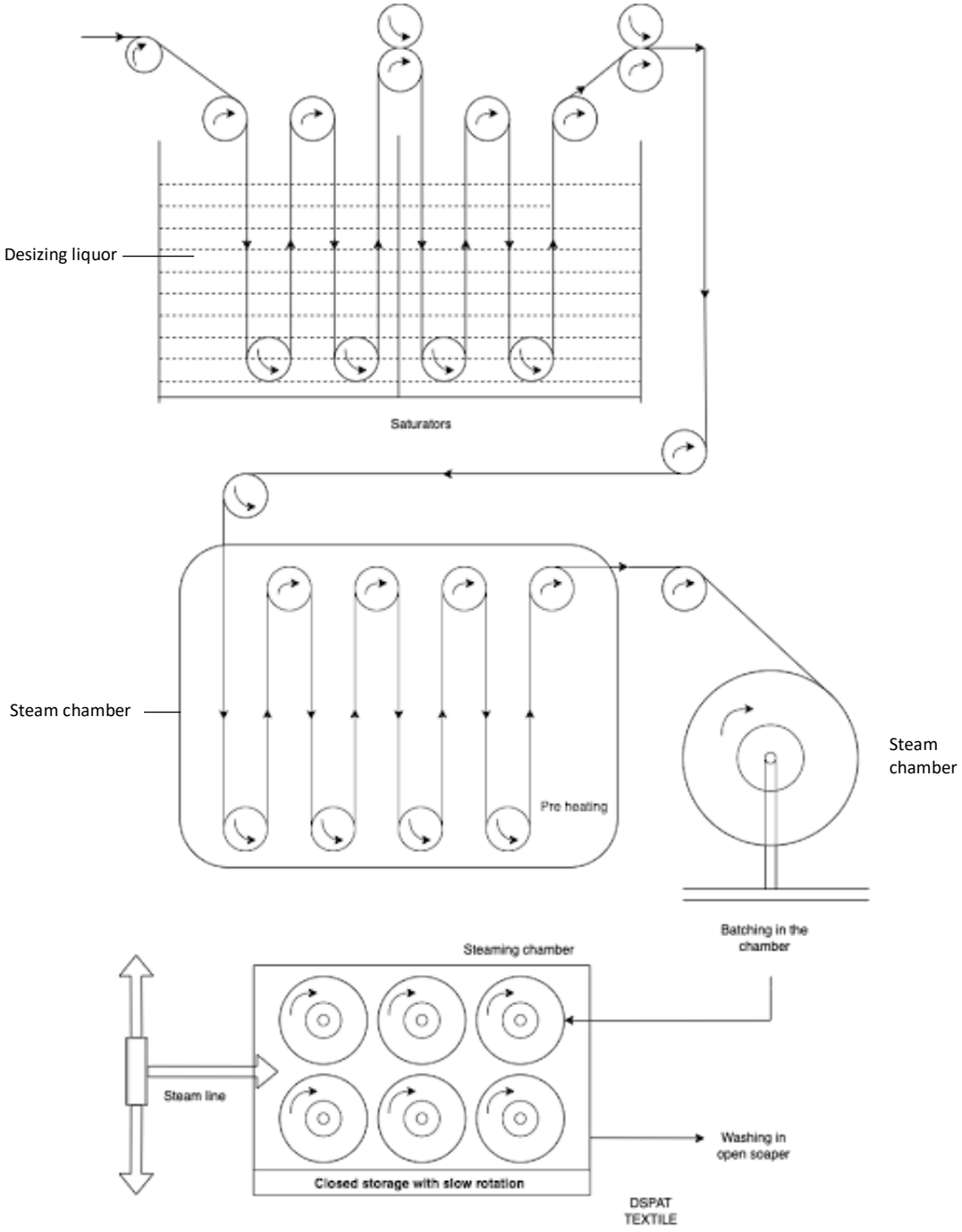

Figure 2.9: Process flow for pad-roll desizing method. Adapted from [https://www.dspattextile.com/2022/05/padroll-and-padsteam-for-desizing.html (cited date 21 Jan 2023)].

2.4.2.4 Pad-steam desizing

Pad-steam desizing is a totally continuous process performed in a J-box machine at extreme temperatures (e.g., 95 - 100 ºC or higher), necessitating the use of thermostable α-amylases. (Figure 2.10) [https://www.dspattextile.com/2022/05/padroll-and-padsteam-for-desizing.html (cited date 2023 Jan 21)]. The cloth, in rope form, is first impregnated/saturated in desizing liquor containing an α-amylase, wetting agent and salt then fed into a preheating chamber in open-width form and finally into the J-box, in rope-form, where it is steamed for 20 - 60 sec to allow the enzyme hydrolyze the starch into soluble dextrins. On completing the operation, the desized fabric is washed in open width-form to remove the water-soluble, dextrins after which it is dried.

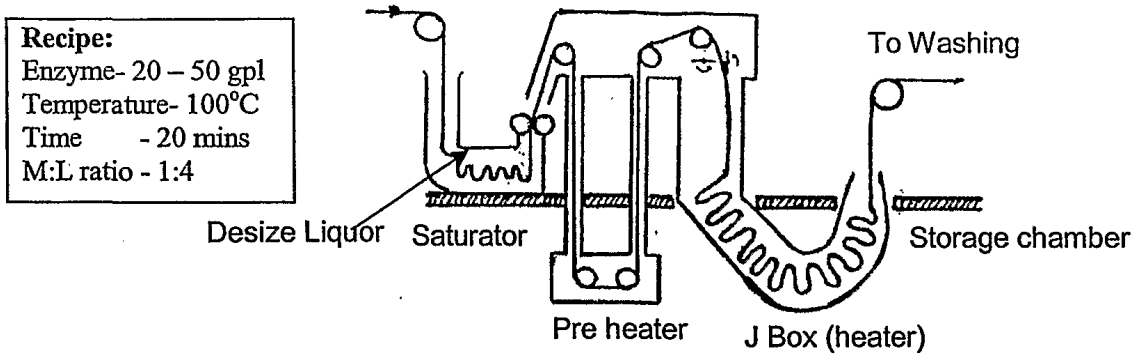

Figure 2.10: Process flow for pad-steam desizing method. Adapted from (Landage, 2022)

2.4.3 Evaluation of the desizing efficiency

The removal of a considerable amount of starch size from woven cotton gives an indication of the desizing efficiency. Among the preliminary tests that are carried out on desized fabrics to establish the efficiency of the process include the following;

2.4.3.1 Surface property determination

In this method, the fabric is 'handled' or 'felt' for softness/firmness, roughness/smoothness, and flexibility/stiffness, and the result compared with that from an un-desized fabric. A softer and smoother 'feel' with greater flexibility is an indication that starch has been removed to a greater extent, implying an efficient desizing process (Grinevičiūtė and Gutauskas, 2004). On the other hand, a firm and rough 'feel' with great stiffness is indicative of an undesized fabric and hence, an inefficient desizing process.

2.4.3.2 Weight loss (%) determination

In this method, the bone-dried weight loss (%) of the fabric sample is calculated before and after desizing. The fabric is first weighed accurately and its weight recorded as W_1, then it is desized, washed, dried and re-weighed. W_2 represents the final weight. The fabric's weight loss (%) after the treatment is calculated using the equation below (Au & Holme, 1999):

$$Fabric\ weight\ loss\ (\%) = \frac{W1 - W2}{W1} x\ 100\ \%$$

Where: W_1 and W_2 are the bone-dried weights of fabric sample before and after desizing The weight loss (%) incurred by the fabric is then compared with that of the control fabric and the net weight loss obtained is attributed to the removal of starch it by the enzyme.

2.4.3.3 Iodine-stain test

To perform the iodine-stain test, a specimen is cut from the fabric and immersed in a beaker containing Gram's iodine solution (1.27g iodine in 10 ml distilled water containing 2 g potassium iodide, and diluted to 300ml with distilled water) for 1 min (Dhawale *et al*., 1982). The stained fabric is then washed with cold water, mangled, dried and observed for color change. A brownish/beige color signifies the absence or near absence of starch to

signify its efficient removal by the enzyme while a deep bluish/purple/black color signifies the presence of a significant amount of starch and hence, an inefficient desizing process (Halim & Zhou, 2018).

2.4.3.4 TEGEWA rating and residual starch content (%) determination

The TEGEWA violet scale is a scale consisting of colors ranging from violet/blue/black to light brown/beige with corresponding numerical values ranging 1 to 9 and >1 to 0.04, respectively (Figure 2.11). A rating of 9 (light brown/beige) signifies complete or near complete removal of starch (residual starch content = 0.04 %) (efficient desizing process) while a rating of 1 (violet/blue/black) signifies the presence of a significant amount of starch (residual starch = > 1 %). The different shades of color between these two extreme colors indicate different desizing efficiencies. The recommended commercially acceptable amount of residual starch on desized fabrics is 0.125 – 0.085 % (rating of 6 - 7) (Harane & Adivarekar, 2017). This is important because improperly desized fabrics can develop different shades of color when subjected to subsequent processing steps e.g., dyeing (Agrawal, 2016).

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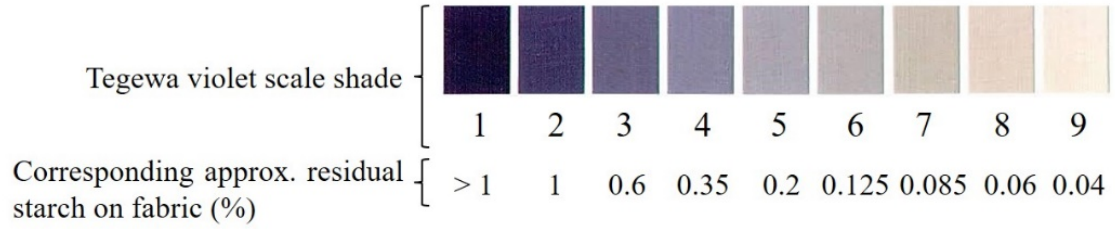


Figure 2.11: TEGEWA violet scale showing the range of violet colors and their corresponding numerical values and expected starch estimates (Au and Holme, 1999; Halim and Zhou, 2018).

2.5 Pectic substances and pectic-degrading enzymes

2.5.1 Occurrence, structure and applications of pectic substances

Pectic substances are a complex group of heterogeneous of high molecular weight polysaccharides found virtually in all plants, where they play the role of conferring rigidity to cells thereby contributing to their structures. They make up to about 30 % of the cell wall composition. They are also found in the middle lamella between plant cells walls, where they help to bind cells together. (Zdunek *et al*., 2021). In mature cotton fibers, they are found in the primary wall which is located between the outer protective cuticular layer and the desired inner secondary wall (Fig 2.12a) where it acts like biological glue that holds the non-cellulosic materials found there-in (hemicellulose, proteins and natural colorants) together (Fig 2.12b). It is also found in the cuticular layer of the fiber.

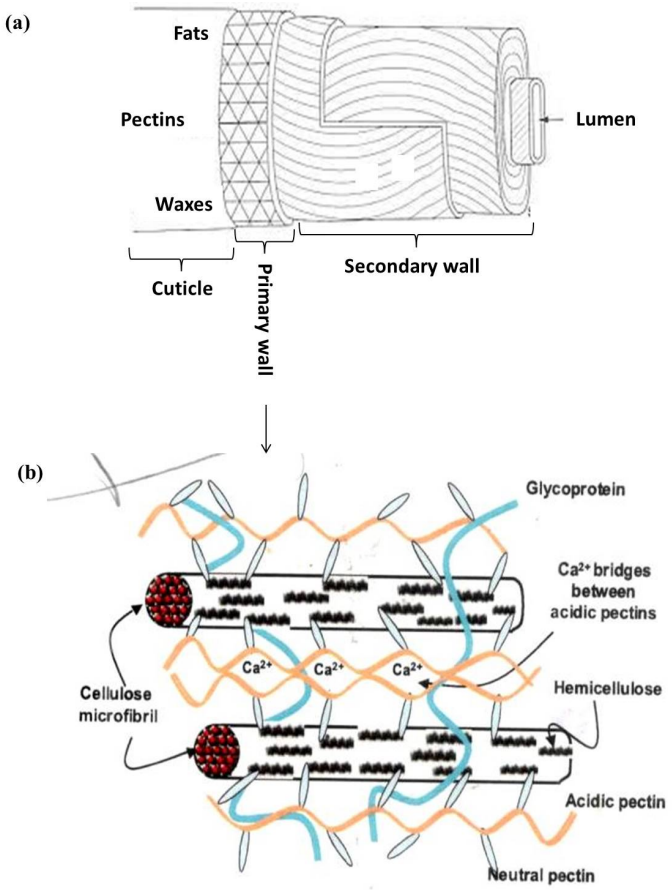


Figure 2.12: Schematic illustration of longitudinal section of: (a) a mature cotton fiber showing its various layers and (b) the chemical constituents of its primary wall bound by pectin.

Commercial pectins are derived from citrus fruits such as oranges, lemons, grapes and apples, as well as the by-products generated after their processing such as citrus and lemon peels and apple pulp. Cocoa husks, sunflower heads, sugar beet, pumpkin, watermelon, pears, and potato pulp are among more sources (Chandel *et al*., 2022). On dry weight basis, citrus peels contain about 20 - 30 % pectin and apple pulp contains about 10 - 15 % pectin while sugar beet and sunflower head residues contain 10 - 15 % pectin (Gawkowska, *et al*., 2018). Pectin levels in fruits, such as apricots, cherries, oranges, and carrots, are approximately 1.0, 0.4, 0.5 - 3.5, and 1.4%, respectively, based on fresh

weight. (Lara-Espinoza *et al*., 2018). Yet another source of pectins is the by-products generated after processing fruits. Sugar beet pulp, for example, produces yields of up to 23% pectin depending on the extraction conditions. (Li *et al*., 2015).

The American Chemical Society classified pectic substances are structurally into four groups (Jayani *et al*., 2005), (Atta and Ruiz-Larrea, 2022), namely:

- **Protopectin:** This is the parent form pectic substances. It is insoluble.
- **Pectin:** Its backbone is composed of repeating units α-(1,4) D-galacturonic acid residues esterified at their carboxylic groups with methanol. The extent of methoxylation [degree of esterification (DE)] ranges from 75 - 90 %. (Figure 2.13 a) They may also be partially esterified with acetyl groups at C-2 and C-3 (Figure 2.13a).
- **Pectic acid:** Its backbone is similar to that of pectin, but with a negligible amount of methoxyl groups (Figure 2.13b).
- **Pectinic acid:** Its backbone is also similar to that of pectin, but with a methyl ester content that is intermediate in quantity between that of pectic acid and pectin. Researchers however, quite often .refer to pectinic acid as pectins.

In addition, the galacturonic acid backbone of pectic substances is interrupted by rhamnose-rich regions and for this reason, pectic substances are considered to have at least three polysaccharide domains described below (Steigerwald *et al*., 2022).

a) Homogalacturonan (HG)

This is the primary constituent of pectic substances. It is entirely composed of D-galacturonic acid residues (GalA) residues that range in number from 72 - 100 in citrus fruits, apple and sugar beet. It may have a negligible number of methyl or acetyl groups (Thibault *et al*., 1993), but totally lacks side chains, for which reason it is referred to as a smooth region (Figure 2.13c).

b) Rhamnogalacturonan I (RGI)

Rhamnogalacturonan I is made up of repeated α-1,2-L-rhamnose and GalA residues (Figure 2.13c). Its side chains are made up of single sugar residues or mixed chains of arabinogalactans, arabinans or galactans that are attached at C-4 of the rhamnose residues, giving it a hairy appearance. The length of the RGI backbone differs from species to species e.g., in sugar beet the disaccharide is about 20 residues (Renard *et al*., 1995) while in sycamore cell walls it is 100 - 300 residues (Talmadge *et al*., 1973).

c) Rhamnogalacturonan II (RGII)

RGII has a backbone made up of GalA residues (similar to HG), but it is heavily branched with side chains at C-2 and C-3, making it a hairy region (Fig 2.13c). Arabinose, apiose, fucose, galactose, rhamnose, aceric acid, glucuronic acid, galacturonic acid, xylose, and fucose are examples of side chains.

In addition to these major domains (HG, RGI and RGII), others e.g., apiogalacturonan (AGA) and xylogalacturonan (XGA) are also present on galacturonic acid residues of HG (Figure 2.13c).

It is assumed that these pectin domains are covalently linked (Ishii and Matsunaga., 2001; Coenen *et al.*, 2007) since they cannot be easily separated without chain-cleavage agents such as pectic enzymes (Zhan *et al*., 1998)

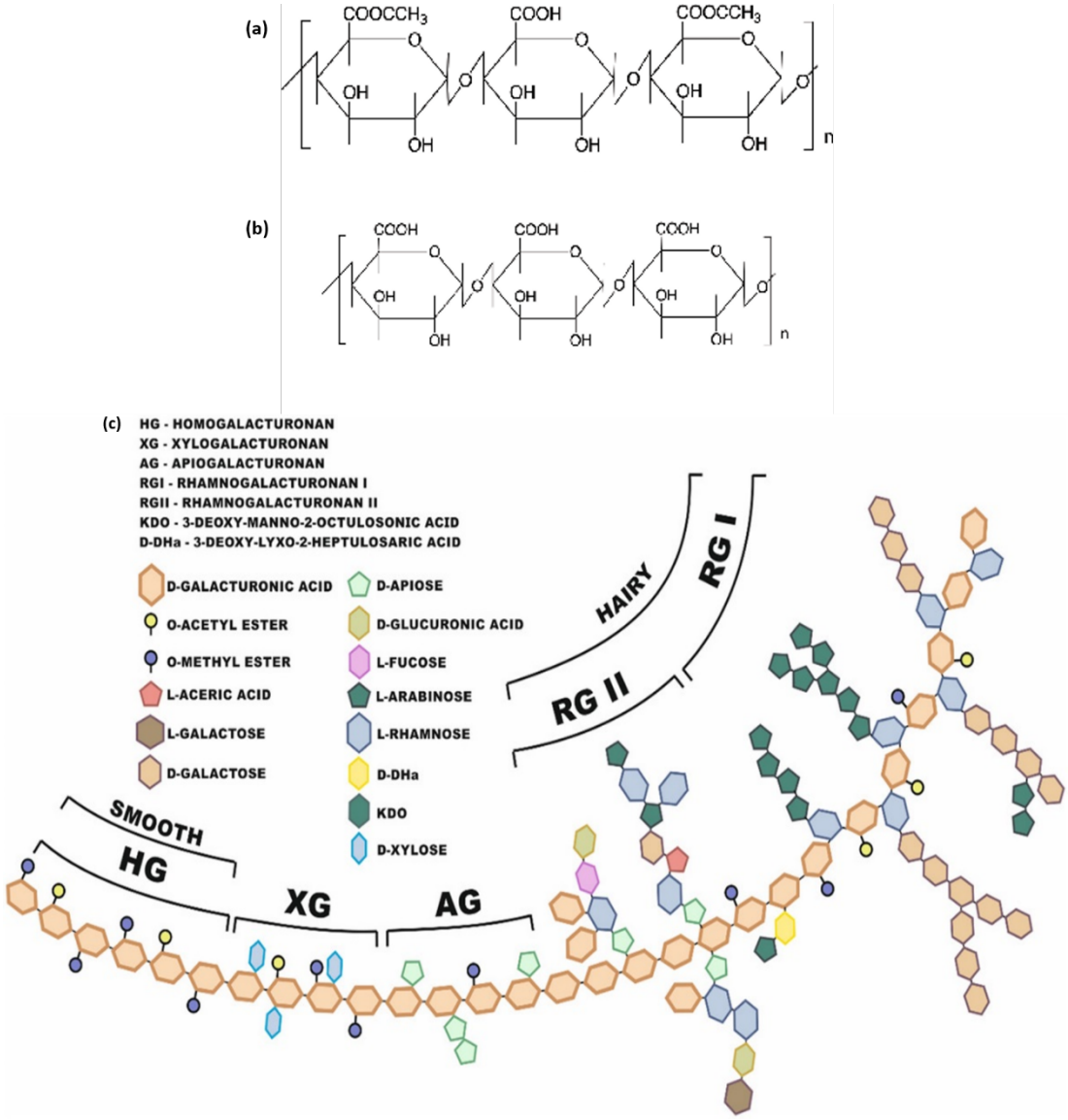


Figure 2.13: Model structure of pectic substance showing: (a) esterified pectin, (b) pectic acid and (c) domains (HG), XG, AG, RGI and RG II) present there-in (Schmidt *et al*., 2015; Steigerwald *et al.*, 2022).

Pectins are used in the food and beverage industries as emulsifiers, stabilizers, and gelling agent, thickening agent in the production of jam, yoghurt drinks, fruity milk drinks, and ice cream. Pectin is also an important source of dietary fiber with therapeutic properties e.g., easier bowel function (Steigerwald *et al*., 2022).

2.5.2 Enzymes involved in the degradation of pectic substances

Enzymes that are active on pectic substances are known as pectic enzymes, and more commonly as pectinases. They are widespread in nature and are produced by plants, where they play the role of breaking down pectic substances in cell walls and thus, promote cell elongation and growth (Jayani *et al*., 2005). Bacteria, yeast, fungi, insects, nematodes and protozoa are also major producers of pectinases (Jayani *et al.*, 2005). Pectinases are classified into three broad groups: a) protopectinases b) esterases and c) depolymerases (Jayani *et al.*, 2005):

a) Protopectinase

Protopectinases (PPases) degrade insoluble protopectin aggregates, resulting in the formation of highly polymerized soluble pectins (see reaction below).

$$\text{Protopectin (insoluble)} + H_2O \xrightarrow{\text{Protopectinase}} \text{Pectin (soluble)}$$

There are two types of PPases: a) A-type PPases, which react with the inner polygalacturonic acid region of proto-pectin, and b) B-type PPases, which react on the outer side of polysaccharide chains that may connect the polygalacturonic acid chain and cell wall constituents.

b) Esterases

Pectinesterase (PE) (also called pectin methylesterase) PME (EC 3.1.1.11), catalyzes the de-esterification of pectin by removing the methoxyl esters in the presence of water thereby, converting it into pectic acid (polygalacturonic acid) and methanol. (Figure 2.14) (Micheli, 2001), (Oumer & Abate, 2017).

(c) Depolymerases

They act on pectic substances by two different mechanisms: a) hydrolysis, where they catalyze the cleavage of α-(1,4) glycosidic bonds linking the galacturonic acid residues with the introduction of water across the oxygen bridge and b) trans-elimination lysis, where they break the glycosidic bonds via trans-elimination reaction without any participation of water molecule. They are 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.

i) Polygalacturonase and polymethylgalacturonase

Polygalacturonases (PG) and polymethylgalacturonase (PMG) depolymerize polygalacturonic acid and pectin chains, respectively, by catalyzing the hydrolytic cleavage of the α-(1,4) glycoside bonds in their respective chains (Figure 2.14). They are divided into two groups based on their modes of action:

- Endo-PG (EC 3.2.1.15) and endo-PMG (EC number not defined), randomly cleave the internal α-(1,4) glycoside bonds in their respective galacturonan chains to liberate oligo- and methyl oligo- galacturonates, respectively.
- Exo- PG I (EC 3.2.1.67), exo-PG II (EC 3.2.1.82) and exo- PMG (EC number not defined) cleave the glycosidic bonds at the non-reducing ends of their respective galacturonan chains to liberate mono-, di- and methyl mono- galacturonates respectively.

ii) Pectate lyases and pectin lyases

Polygalacturonate lyase (PGL) [or pectate lyase (PL)] and polymethylgalacturonate lyase (PMGL) [(or pectin lyases PNL)] depolymerize polygalacturonic acid and pectin chains, respectively, but via β-elimination of the α-(1,4) glycoside bonds in their respective chains (Figure 2.14). They are divided into two groups based on their modes of action:

- Endo-PGL and endo PMGL randomly cleavage the internal α-(1,4) linkages of their respective chains to liberate 4,5 unsaturated oligo- and 4,5 unsaturated methyl oligo- galacturonates, respectively.
- Exo-PGL and exo-PMGL, cleave the linkages at the non-reducing end of their respective chains to liberate 4,5 unsaturated mono- and 4,5 unsaturated methyl mono- galacturonates respectively.

Other enzymes involved in the depolymerization ofpectin include;

- Rhamnogalacturonase (RG) (EC 3.2.1.171), which depolymerize pectin by catalyzing the hydrolytic cleavage of α-(1,2) glycosidic bonds between D-galacturonic acid and L-rhamnose.
- Xylogalacturonan hydrolase (XGH) (EC not defined), which depolymerize pectin by catalysing random hydrolytic cleavage of the glycosidic bond between D-galacturonic acid and L-xylose.

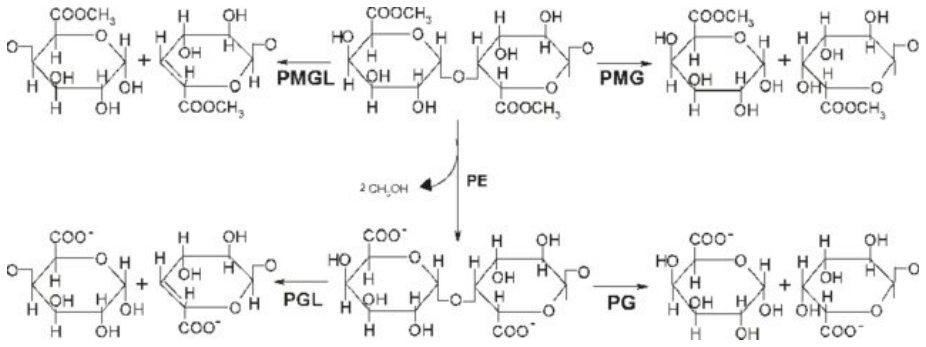


Figure 2.14: Mode of action of pectic enzymes: a) PMGL (polymethylgalacturonate lyase), b) PMG (polymethylgalacturonase), c) PE (pectinesterase), d) PGL (polygalacturonate lyase and e) PG (polygalacturonase)

2.6 Glycoside hydrolase family 28 pectinases

Based on amino acid sequence similarities, glycoside hydrolases that degrade pectins are classified under family 28 in the CAZy database [http://www.cazy.org/GH28.html (cited

date 21 Feb 2022)]. Members of the family are structurally related and exhibit a common catalytic mechanism, but with divergent specificities. They are involved in the hydrolysis of the: a) smooth homogalacturonan (endo-PG (EC 3.2.1.15), exo-PG I (EC 3.2.1.67) and exo-PG II (EC 3.2.1.82) (Henrissat, 1991) and b) hairy rhamnogalacturonan (rhamnogalacturonases (EC 3.2.1.171) and xylogalacturonan (xylogalacturonan hydrolase (EC not identified) regions of pectin.

All members of GH family 28 pectinases exhibit the following characteristics:

1) Act on α-(1,4) or α-(1,2) .glycosidic linkages in pectin to produce α-anomeric mono/oligogalacturonates (hydrolysis) or mono/oligo.
2) Possess ten (10) complete coils of right-handed parallel β-sheets conformation. Each coil is formed by three or four β-strands (Figure 2.15)
3) Have highly four conserved regions located on or before β-strands 5, 6, 7 and 8. These regions contain amino acid residues that are essential for the stability of the active site, substrate binding and catalysis.

2.6.1 Domain organization within GH28 pectinases

Members of GH28 pectinases have a single domain composed of ten (10)-turn right-handed parallel beta-helix with an additional α-helix coil at the N-terminal end and an incomplete coil at the C-terminal end of the protein (no C-terminal extension) (Figure 2.15. The last strand of β-strand two is responsible for secretion of the enzyme. Flexibility is lowest in the central turns, but increases towards the N- and C- termini of the parallel β-strands. The active site is on the surfaces of β-sheets 1, 4, 5, 6, 7, and 8, as well as on the loops preceding these strands. It has two disulfide bonds: one between Cys15 residue prior to the first β-strand of PB2 and cys35 after the C-terminal end of the first α-helix. The second disulfide, Cys^{89}- Cys^{99} is in the long loop that precedes strand two of PB1.

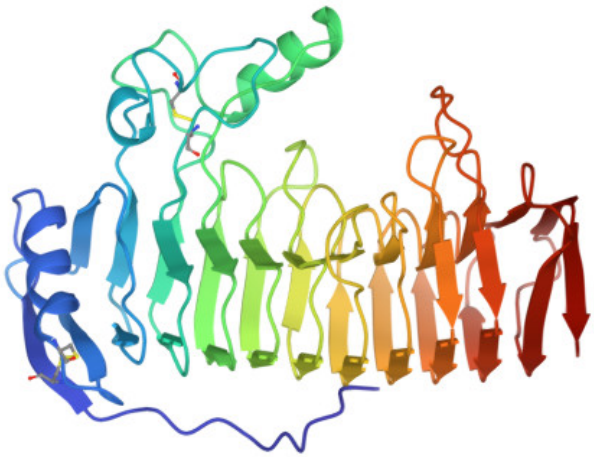


Figure 2.15: Schematic representation of a polygalacturonase showing the single domain ten complete coils of right-handed parallel β-sheets.

2.6.2 Conserved regions within the primary sequence of GH 28 pectinases

There are four highly conserved regions in the primary sequences of PGs located on PB1. These regions in increasing numerical order are shown in Table 2.4, along with the conserved residues they contain. The clustering of these residues on or before β-strands 5, 6, 7, and 8 is an indication that the surfaces of β-strands 5 - 8 of PB1 and the adjacent loops form the catalytic site. This site is located in a pronounced cleft, which supports this proposal (Figure 2.15).

The substrate-binding cleft is a tunnel-like structure formed by two long loops that precede β-strands 2 and 3 of PB1 and three loops that follow β-strands 7, 8 and 9. Ser 257 residue found in the loop preceding β-strand 7 in PB1 is believed to maintain the geometry of this cleft. It is further characterized by the presence of conserved aspartates and Lys229 and 282, Arginines and lysines also line its sides thus, making the overall electrostatic potential in the substrate-binding cleft positive.

Table 2.4 Conserved regions I to IV and their respective conserved catalytic residues in GH 28 pectinases.

Region	Location	Residue
I	Within β-strand 5	Asn201, Thr202, Asp203
II	Turn before β-strand 6	Gly222, Asp223, Asp224
III	Turn before β-strand 7	Gly250, His251, Gly252
IV	Within β-strand 9	Arg280, ile281, Lys282

2.6.3 Catalytic mechanism of GH 28 pectinass

2.7 Biotechnological application of endo polygalacturonases or pectale lyases in the textile industry

Once woven cotton has been properly desized (see section 2.4), it is subjected to a second wet pretreatment step called scouring. Herein, the undesirable polygalacturonic acid found in both the outer cuticular layer of cotton fibers and the adjacent primary cell walls in woven cotton (Figure 2.12) is removed alongside 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 hydrophilic cellulosic structures of the cloth (exhibit excellent water and dye absorbency properties (Colombi *et al*., 2021).

There are two methods that are used for commercial scouring of woven cotton: a) caustic-scouring and bio-scouring. Out of these methods, the latter is preferred because it requires the use of enzymes, which offer numerous advantages e.g., enzymes are safe, increase the rate of catalysis (more profits), biodegradable and require use of less aggressive chemicals (eco-friendly), and do not attack the cellulosic structures of the cloth (high quality fabrics) compared to the former method, which requires the use of hot caustic soda that is not only risky to handle, but can also damage the cloth and result in the generation of high pollution loads in a time-, water- and energy- consuming process. Besides endo-polygalacturonases (EC 3.2.1.15), pectate lyases (EC 4.2.2.2) have been a favorite bioscouring agent.

2.7.1 Principle of the bioscouring process

Like in the desizing process, bioscouring of woven cotton is carried out in three steps;

i. *Impregnation:* The fabric is wetted with scouring liquor containing endo-PecL, wetting agent, salt and enzyme stabilizer. This is carried out at room temperature.
ii. *Incubation:* The temperature of the scouring liquor is raised to match that of the enzyme's optimum operating temperature to allow the latter (enzyme) to randomly cleave the α-(1,4) glycosidic linkages in the polygalacturonic acid chains via β-elimination and liberate water-soluble 4,5 unsaturated oligo-galacturonates, and the loosened hydrophobic waxes and other non-cellulosic substances that adhere to it and thus, lead to the exposure of the desired hydrophilic cellulosic structures of the cloth.
iii. *Washing:* This involves washing the bioscoured fabric to remove the unsaturated oligo-galacturonates and other undesired non-cellulosic substances from its surface followed by drying. To get the best desizing result, the fabric is washed with a detergent using hot water (93 - 95 ºC) and then dried.

2.7.2 Bioscouring technologies

2.7.2.1 Jigger bioscouring

Bioscouring of desized woven cotton is carried out in a batch process using a Jigger machine similar to that used for desizing (see section 2.4.2) [https://www.textiletoday.com.bd/introducing-bio-scouring-a-cost-effective-and-eco-friendly-process-for-the-generation-next (cited date 31 July 2023)]. The fabric is rolled into the souring bath (contains a wetting agent, enzyme, and emulsifier in the appropriate buffer) using the let-off roller (A) where it is allowed to undergo impregnation (Figure 2.16) . The temperature of the bioscouring liquor is then adjusted to 55 - 95 °C (depending on the optimum temperature of activity of the enzyme) to allow the enzyme to degrade the polygalacturonic into soluble oligogalacturonates. The fabric is then rolled out of the machine using the take-up roller and subjected to washing with hot water (90 - 95°C) to

remove the oligogalacturonates and other non-cellulosic substances (e.g., hemicellulose, proteins, natural colorants, etc) that adhere to them.

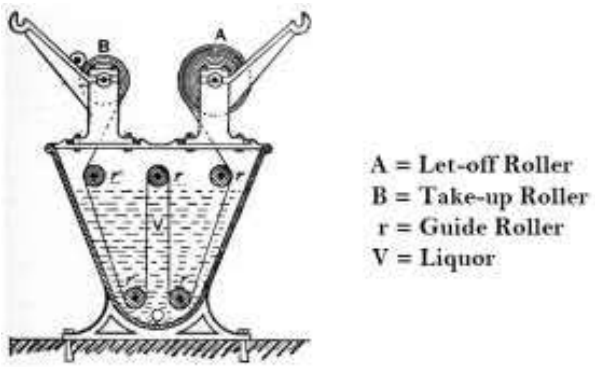


Figure 2.16: Process flow for jigger bioscouring method. Adapted from [https://www.textiletoday.com.bd/introducing-bio-scouring-a-cost-effective-and-eco-friendly-process-for-the-generation-next (cited date 31 July 2023)].

2.7.2.2 J-box bioscouring

Bioscouring of desized woven cotton can also be carried out in a continuous process using a J-box machine similar to that used for desizing woven cotton using the pad-steam desizing method (see section 2.4.2.4). The fabric is first passed through the guide roller into the bioscouring liquor (pectinase, wetting agent and salt) where it is saturated with this solution, in rope form. It is then fed into the pre-heating chamber in open-width form and finally into the J-box, in rope form where it is steamed up to allow the enzyme hydrolyze and eliminate the pectin, along with and other non-cellulosic components that adhere to it on the cotton fibers that make up the cloth. This exposes the desired hydrophilic cellulosic structures of the cloth, and the latter can now be washed to remove the pectin hydrolysate from its surface.

2.7.3 Evaluation of the scouring efficiency

The extent to which the pectin and other non-cellulosic impurities are enzymatically removed from the cuticular and primary layer of cotton fibers on woven cotton gives an

indication of the bioscouring efficiency. Among the preliminary tests that are carried out to determine the efficiency of the process include assessing the properties of the pretreated fabric e.g., a) weight loss (%) determination, b) surface-handle or 'feel' and c) absorbency tests, and then comparing the results with those from the control fabric (treated with denatured enzyme). This is done as follows;

2.7.3.1 Surface property determination

The bioscouring efficiency can be evaluated by 'handling' or 'feeling' the fabric for softness/firmness, roughness/smoothness, and flexibility, and then comparing the result with that of an un-scoured fabric. A much softer and smoother 'feel' with greater flexibility is an indication that the hydrophilic cellulosic structures of the cotton fibers are exposed and that the process is therefore efficient or tends towards being efficient. Exposure of the cellulosic structures of the cloth is attributed to the enzymatic hydrolysis and elimination of pectin alongside other non-cellulosic impurities that adhere to it in the cuticular and primary walls of cotton fibers that make up the cloth.

2.7.3.2 Weight loss (%) determination

The bio-scouring effieincy can also be evaluated by determining the bone-dried weight loss (%) that the pretreated fabric incurs after being subjected to the process of bioscouring, and can be carried out using the method described inection 2.4.3.2. The weight loss (%) that the fabric incurs as a result of the treatment is then computed using the equation below:

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

Where: W_1 and W_2 are the bone-dried weights of fabric sample before and after desizing

The weight loss (%) incurred by the fabric is then compared with that of the un-scoured fabric and the net weight loss obtained is attributed to the enzymatic removal of pectin and other non-cellulosic substances from the cotton fibers in the fabric.

2.7.3.3 Absorbency test

The efficiency of the bioscouring process can also be assessed performing water and dye absorbency tests on the pretreated fabric, as follows;

2.7.3.3.1 Drop penetration test

Water absorbency is evaluated using AATCC technical manual 39 - 1980 edition (wettability, evaluation of) (Tzanov *et al*., 2001). The fabric is placed over the top of a beaker so that the centre is unsupported. A drop of water (100 µl) is placed on its surface by dropping it from a height of 1 cm, and the time taken (sec) for it to make contact with the fabric and its complete absorption into the sample is taken using a stopwatch, and recorded as the wetting time. This procedure is repeated at different spots on the ame fabric and the average wetting time is calculated from the readings and compared with that from the control fabric. The shorter the time, the more efficient is the bioscouring processs (a standard absorbency time of < 5 sec is recommended (Teli & Adere, 2016).

The efficiency of the bioscouring process can also be determinedby the method descrbed by Halim & Zhou (2018). A drop of dye solution is placed on the surface of a bioscoured fabric by dropping it from a height of 1 cm, and the size and shape of the absorbed area is determined and compared with that from the control fabric. The assessment is done as follows:

- If the drop is noncircular and small, the fabric is scoured unevenly and the process is incomplete.
- If the drop is circular and small, the fabric is evenly scoured, but the process is incomplete.

- If the drop is circular and big, the fabric is evenly scoured and the process is complete.
- If the drop does not get absorbed into the fabric, the process did not take place.

2.7.3.3.2 Capillary rise (wicking) test

Anotther method that can be used to assess the efficiency of the bioscouring process is the capillary rise test (Bristi *et al*., 2019), A piece of the pretreated fabric is hung (using a support) over a beaker with its bottom edge immersed 1 cm in dye solution (5 mg l^{-1}) contained in the beaker. Once immersed, the dye ias allowed to rise up the fabric by capillary action for 30 min, after which the distance moved by the colored solution (wicking height) is recorded and compared with that from the un-bioscoured fabric. The bioscoured fabric should exhibit a greater wicking height compared to that of the un-bioscoured fabric. This is because a bioscoured fabric is more absorbent than the an un-bioscoured one due to the exposure of the hydrophilic celluloisic structures of the cotton fibers following the degradation and elimination of pectin and other non-cellulosic substances from it by the enzyme.

CHAPTER THREE

3.0 PROTEASE-, PECTINASE- AND AMYLASE-PRODUCING BACTERIA FROM A KENYAN SODA LAKE

The work described in this chapter was published in the a) *Open Biotechnology Journal* in 2018, Volume 12, pp 33 - 45 (https://doi:10.2174/1874070701812010033) and appears as **paper I** in the 'list of papers' in this thesis (page xxi) and b) local Standard newspaper dated July 28, 2018 [https://www.standardmedia.co.ke/health/health-science/article/2001289771/scientists-discover-treasure-trove-at-lake-bogoria (Cited date 03 Oct 2023)].

The work was also presented at the a) 11th International Congress on Extremophiles, September 12 - 16, 2016, Kyoto University, Kyoto, JAPAN, and b) 5th Annual Conference of the Faculty of Science and Technology, University of Nairobi, October 26 -28, 2022, KENYA.

3.1 Introduction

Soda lakes are some of the main types of alkaline environments that occur naturally on Earth. They are characterized by high alkalinity (pH 8 > 12), large amounts of Na_2CO_3 and NaCl [5 % (w/v) NaCl to saturation], and high and low concentrations of Na^+ and Mg^{2+}/Ca^{2+} ions, respectively, due to evaporative concentration (Grant and Tindall, 1986; Grant and Horikoshi, 1992). Although soda lakes have a wide geographical distribution on Earth, their hostile nature and inaccessibility has contributed to only a few of them being explored from the limnological and microbiological point of view. Among the most studied soda lakes are those fed by carbonated hot springs in the Kenyan Rift Valley area. These alkaline water bodies also support a considerably diverse population of alkaliphiles i.e. microorganisms that are able to grow at optimal pH values > 9.0, but fail to grow/grow

slowly at around pH 7 (Horikoshi, 1999). They include archaea, Gram-negative protobacteria (e.g. *Pseudomonas*, *Halomonas* and *Deleya*) and Gram-positive bacteria/eubacteria (e.g. *Bacillus* and *Clostridium*) (Grant and Jones, 2000).

Alkaliphilic bacteria, especially those that belong to the genus *Bacillus* have generated a lot of interest because of their ability to produce alkaline and stable enzymes (Fujinami and Fujisawa, 2010). This unique characteristic of the enzymes present an opening for their utilization in industrial technologies where similar conditions are used. For example, alkaline proteases, pectinases, amylases and lipases have been shown to have a great impact in laundry and dish/handwashing detergents (Sarethy *et al*., 2011). Other areas of application of alkaline enzymes are in the: a) leather industry (proteases and lipases), b) pharmaceutical, chemical, food and feed industries (proteases, amylases), c) pulp and paper industry (pectinases and amylases) and d) textile industry (amylases, proteases and pectinases). In addition, they are used to treat food wastewater (proteases and pectinases), recover silver from both x-ray and photographic films (proteases) and cyclodextrin production (CGTases) (Starnes, 1990; Ellaiah *et al*., 2002; Sarethy *et al.*, 2011; Sundarram and Murthy, 2014; Kohli & Gupta, 2015).

Despite the fact that alkaline enzymes are currently used commercially in many industrial processes, there has been renewed interest in the search for more enzymes with better catalytic and stability properties for more efficient bioprocesses. In this article, we report on the identification of alkaliphilic bacteria that produce alkaline proteases, pectinases and amylases. The bacteria were bacteria were isolated from Lake Bogoria, a soda lake found in the Kenyan Rift Valley area.

3.2 Materials and Methods

3.2.1 Materials

Glucose, yeast extract, nutrient agar, peptone, citrus pectin (DE ≥ 74 %), polygalacturonic acid sodium salt (DE ≤ 25 %), potassium sodium tartrate tetrahydrate and 3.5-Dinitrosalicylic acid (DNS), Gram staining kit and Gordon-Mcleod reagent and Folin-Ciocalteau reagent were purchased from Sigma-Aldrich (St. Louis, MO, USA). Soluble starch was purchased from Merck (Darmstadt, Germany). DNA extraction- and PCR purification- kits were bought from Invitrogen Life Technologies™, CA, USA) and Qiagen (Limburg, Netherlands) respectively. Hydrogen peroxide [30 % v/v] was purchased from a local chemist while chicken quill feathers were donated from a local chicken slaughterhouse. Reference strains *Bacillus halodurans* DSM 497 and *Bacillus pseudofirmus* DSM 8715 were purchased from Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ), Braunschweig, Germany.

3.2.2 Sample collection

The non-probability (purposive/selective) sampling method was used to collect soil, water and sediment samples from Lake Bogoria (00º 15'N and 36º 06' E), its surrounding hot springs (wells) and streams that channel the hot spring waters into the lake, (Figure 3.1). The samples were placed separately in sterile bottles and labelled. Samples from the lake were labelled using the acronym LB (Lake Bogoria) while those from the hot springs (wells) and streams as LBW (Lake Bogoria Well) and LBS (Lake Bogoria Stream), respectively. The samples were transported to the Department of Biochemistry, University of Nairobi, Kenya, where they were stored frozen at -20 ºC until use.

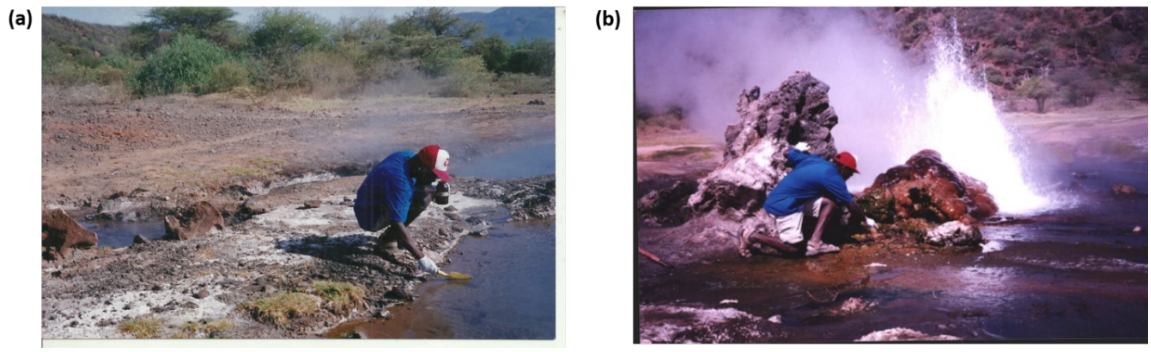


Figure 3.1: Photograph showing the collection of samples from: (a) the shores of Lake Bogoria and (b) a drainage channel from a hot spring leading into the lake.

3.2.3 Media preparation

3.2.3.1 Isolation medium (Horikoshi I)

The medium for isolating alkaliphiles was prepared according to Horikoshi, (1999), but with a slight modification. The modification included the addition of: a) 2 g glucose instead of 10 g, b) 10 g bactopeptone instead of 5 g, c) 15 g agar instead of 18 g and d) 5 g malt extract. These ingredients were added to 950 ml distilled water, and the medium was sterilized in a 3870 MLV autoclave (Tauttnauer Innovation Legacy Partnership, Hoeksteen, Netherlands) at 15 psi and 121 ^{o}C for 30 min, cooled to about 60 ^{o}C and 20 % (w/v) $NaCO_3$ and trace elements solution (see composition and preparation method in appendix 1a) added to it in the proportion of 50 ml- and 300 µl- per liter, respectively (pH 10.5). Both the Na_2CO_3 and stock trace elements solution were autoclaved separately before adding them to the mdium. Finally, 20 - 25 ml of the sterilized alkaline nutrient medium was poured in sterile petri dishes in a Heraeus HS12 class II biological safety cabinet (Richmond Scientific, Ore, East Sussex, UK) and allowed to solidify at room temperature.

3.2.3.2 Screening media

3.2.3.2.1 Gelatin medium

The protocol described by Juwon and Emanuel, (2012) was used to prepare the medium for screening for protease producing microbial isolates. A stock solution of 8 % (w/v) gelatin was prepared by dissolving 4 g gelatin in 50 ml distilled water and sterilizing it in a 3870 MLV autoclave (Tauttnauer Innovation Legacy Partnership, Hoeksteen, Netherlands) at 15 psi and 121 ^{o}C for 30 min. The sterile medium was then cooled to about 60 ^{o}C before adding sterile 1.5 % (w/v) nutrient agar and 20 % (w/v) Na_2CO_3 in the ratio of 5 ml 8 % gelatin: 50 ml nutrient agar and 5 ml Na_2CO_3 (pH 10.5). Finally, 20 - 25 ml of the sterilized alkaline gelatin medium was poured in a sterile petri dish in a Heraeus HS12 Class II Biological safety cabinet (Richmond Scientific, Ore, East Sussex, UK) and allowed to solidify at room temperature.

3.2.3.2.2 Pectin medium

The screening for pectinase producing microbial isolates was done using medium prepared according to Horikoshi (1972): 10 g citrus pectin (DE ≥ 74 %), 3 g peptone, 3 g yeast extract, 0.04 g $MnCl_2 \cdot 2H_2O$, 0.2 g $MgSO_4 \cdot 7H_2O$, 1 g K_2HPO_4, 1.5 g NaCl, 10 g and 18 g agar were dissolved in 950 ml distilled water. The medium was sterilized in a 3870 MLV autoclave (Tauttnauer Innovation Legacy Partnership, Hoeksteen, Netherlands) at 15 psi and 121 ^{o}C for 30 min, allowed to cool to about 60 ^{o}C and then 50 ml 20 % (w/v) sterilized Na_2CO_3 added to it to raise the pH to 10.5. Finally, 20 - 25 ml of the sterilized alkaline pectin medium was poured in a sterile petri dish in a Heraeus HS12 Class II Biological safety cabinet (Richmond Scientific, Ore, East Sussex, UK) and allowed to solidify at room temperature.

3.2.3.2.3 Starch (Horikoshi II) medium

The medium for screening for amylase producing microbial isolates was prepared according to Horikoshi, (1999): 10 g soluble starch, 5 g yeast extract, 5 g polypeptone, 1 g K_2HPO_4, 0.2 g $MgSO_4 \cdot 7H_2O$ and 18 g agar were dissolved in 950 ml distilled water. The medium was sterilized in a 3870 MLV autoclave (Tauttnauer Innovation Legacy Partnership, Hoeksteen, Netherlands) at 15 psi and 121 ^oC for 30 min, allowed to cool to about 60 ^oC and 50 ml 20 % (w/v) sterilized Na_2CO_3 added to it to raise the pH to 10.5. Finally, 20 - 25 ml of the sterilized alkaline starch medium was poured in a sterile petri dish in a Heraeus HS12 Class II Biological safety cabinet (Richmond Scientific, Ore, East Sussex, UK) and allowed to solidify at room temperature.

3.2.3.3 Nutrient medium

The medium for growing the microbial isolates for their subsequent characterization was nutrient agar, prepared according to the manufacturer's instructions [https://www.sigmaaldrich.com/KE/en/product/sial/n9405 (cited date 09 Feb 2023)]. Briefly, 28 g of nutrient agar was dissolved in 950 ml distilled water. The mixture was sterilized in a 3870 MLV autoclave (Tauttnauer Innovation Legacy Partnership, Hoeksteen, Netherlands) at 15 psi and 121 ^oC for 30 min, allowed to cool to about 60 ^oC and 50 ml 20 % (w/v) sterile Na_2CO_3 added to it to raise the pH to 10.5. About 20 - 25 ml of the sterilized medium was poured in sterile petri dishes in a Heraeus HS12 Class II Biological safety cabinet (Richmond Scientific, Ore, East Sussex, UK) and allowed to solidify at room temperature. Nutrient agar medium pH 7.0 was also prepared in a similar manner, but using NaOH to adjust its pH to neutral.

3.2.3.4 Enzyme production media

Eighteen 50- ml protease production media (pH 10.5) were prepared by adding 0.5 g chicken quill feathers in 250 ml shake flasks, each containing 47.5 ml distilled water. The media were autoclaved in a 3870 MLV autoclave (Tauttnauer Innovation Legacy

Partnership, Hoeksteen, Netherlands) at 15 psi and 121 ºC for 30 min and then allowed to cool to about 60 ºC before adding 2.5 ml 20 % (w/v) Na_2CO_3 and 15 ul trace elements solution (see composition in Appendix 2a) to them.

Eighteen 50- ml pectinase production media (pH 10.5) were prepared in 250 ml conical flasks, each containing the nutrients described in section 3.2.3.2.2, but without agar. The media were autoclaved in a 3870 MLV autoclave (Tauttnauer Innovation Legacy Partnership, Hoeksteen, Netherlands) at 15 psi and 121 ºC for 30 min and then allowed to cool to about 60 ºC before adding 2.5 ml 20 % (w/v) Na_2CO_3 to them.

Fifty (50)- ml amylase production (Horikoshi II) medium (pH 10.5) was prepared in 250 ml conical flasks, each containing the nutrients described in section 3.2.3.2.3, but without agar. The media were autoclaved in a 3870 MLV autoclave (Tauttnauer Innovation Legacy Partnership, Hoeksteen, Netherlands) at 15 psi and 121 ºC for 30 min and then allowed to cool to about 60 ºC before adding 2.5 ml 20 % (w/v) Na_2CO_3 to them.

3.2.4 Isolation of alkaliphilic microbial isolates

This was carried out by the spread-plate method (Sanders, 2012). Briefly, soil, polymer and maize-cob samples (0.1 g) were suspended in separate 15 ml falcon tubes, each containing 9.9 ml saline solution [0.9 % (w/v) NaCl], and serially diluted. One hundred (100) µl inoculums from the desired dilutions (10^{-3} to 10^{-7}) were then aliquoted on the surface of isolation media (pH 10.5); spread evenly on the agar surface using a sterile bent glass rod and the inoculated plates incubated in a LEEC Research GA2000 incubator (Akribis Scientific Limited, Cheshire, UK) at 45 ºC for 12 - 24. Individual microbial colonies that formed on the surface of the plates were isolated and re-streaked on new agar media until single, uniform colonies were seen (Figure 3.2). One hundred (100) - µl water samples were similarly diluted and the desired dilutions (10^{-3} to 10^{-7}) used to inoculate isolation medium. The plates were incubated at 45 ºC for 12 – 24h.

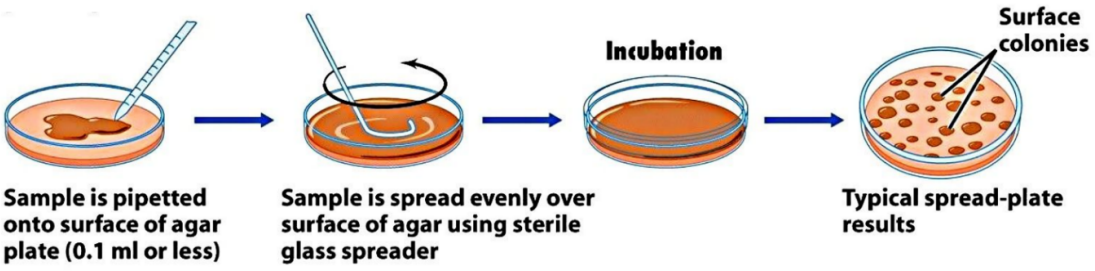


Figure 3.2: Schematic illustration of the spread-plate method that was used to isolate pure microbial isolates from the soda lake samples. Adapted from: https://biologyideas.com/spread-plate-technique/ (cited date 09 Feb 2023).

One hundred ninety (190) pure microbial colonies (alkaliphiles) grew on the isolation alkaline medium (pH 10.5). They were retrieved from the plates under sterile conditions and sub-cultured individually in 50 ml isolation broth (without agar) medium in a Gallenkamp thermoshaker incubator (London, UK) at 45 ^{o}C and 100 rpm for 24 h.

Forty (40) % (v/v) glycerol stocks of the cell cultures were prepared by mixing three (3) volumes of each culture with two (2) volumes of sterile 87 % glycerol in separate eppendorf tubes. The tubes were labelled according to: a) a number assigned to the microbial isolates and b) the origin of the sample from which the isolate was obtained e.g., isolate LBW 2719 was the 19th isolate obtained from a sample collected from site no. 7 in well no 2. All the glycerol stocks of alkaliphilic isolates were stored frozen at -80 ^{o}C.

3.2.5 Screening the isolates for the production of alkaline proteases, pectinases and amylases

Eighteen (18) out of the one hundred and ninety (190) isolates were chosen for this study based on their dominance in growth during the isolation process. The designations given to these isolates and a brief description of the sites where they were sampled from is shown in Table 3.1.

Table 3.1 Designations given to the isolates used in this study and some physical parameters recorded at their sampling sites.

Serial no.	Isolate designation	Sampling site	Sample type	pH	Temp* (°C)
1.	LBW 2719	Hot spring well no. 2	Soil	9.0	65
2.	LBW226	Hot spring well no. 2	Polymer	9.0	65
3.	LBW 318	Hot spring well no. 3	Maize-cob	9.0	63
4.	LBS 77	Upstream drainage channel of hot spring well no. 7	Water	9.0	60
5.	LBK 261	Periphery of hot spring well no. 2	Water	9.0	54
6.	LBW 4512	Periphery of hot spring well no. 4	Polymer	9.0	50
7.	LBW 327	Periphery of hot spring well no. 3	Polymer	9.0	57
8.	LBW 434	Periphery of hot spring well no. 4	Polymer	9.0	52
9.	LBW 7526a	Periphery of hot spring well no. 7	Polymer	9.5	55
10.	LBW 39	Periphery of hot spring well no. 3	Polymer	9.0	58
11.	LBW 7526b	Periphery of hot spring well no. 7	Polymer	9.5	56
12.	LBW 328	Periphery of hot spring well no. 3	Polymer	9.0	57
13.	LBS 16	Downstream drainage channel of hot spring well no. 1	Soil	9.0	45
14.	LBW 625	Periphery of hot spring well no. 6	Soil	9.0	55
15.	LBW 446	Periphery of hot spring well no. 4	Polymer	9.0	50
16.	LBW 5117	Periphery of hot spring well no. 5	Soil	8.0	51
17.	LBW 313	Periphery of hot spring well no. 3	Maize-cob	9.0	50
18.	LBW 317	Periphery of hot spring well no. 3	Maize-cob	9.0	48

* Temperature

The isolates were subjected to plate screening tests for the production of alkaline proteases, pectinases, and amylases (Dhawale *et al.*, 1982; Soares *et al*, 1999; Juwon and Emanuel, 2012). One (1) µl glycerol stock of each isolate was inoculated on the screening plates containing gelatin-, pectin- and starch- agar media (pH 10.5), respectively (see section 3.2.3.2 on how the media were prepared), and the plates incubated in a LEEC Research GA2000 incubator (Akribis Scientific Limited, Cheshire, UK) at 37 °C for 72 h. The complete degradation of gelatin, pectin and starch by the isolates is manifested by the formation of halos around protease-, pectinase- and amylase -producing colonies after flooding the plates with saturated $(NH_4)_2SO_4$ (769 g^{-1}), Lugol's iodine (5.0 g KI, 1.0 g I and 330 ml distilled water) and Gram's iodine (1.27 g iodine in 10 ml distilled water containing 2 g KI, and dilute to 300 ml with distilled water) dye solutions, respectively.

The activity of the enzymes produced by the colony of each isolate was determined semi-quantitatively in terms of solubilization index (SI) values calculated using the equation below. The SI values obtained were then used to rank the isolates using the formulae shown in Table 3.2:

$$SI = Diameter\ of\ halo\ (mm) / Diameter\ colony(mm)$$ …….. (Hitha & Girija, 2014)

Table 3.2 Ranking of the isolates based on their SI values obtained during screening.

SI Value	Rank
>3.0	Excellent producer of the enzymes
2.0 < SI ≤3.0	Very good producer of the enzyme
1.0 < SI ≤ 2.0	Good producer of the enzyme
0 < SI ≤ 1.0 (not clear)	Weak producer of the enzyme
Size of halo cannot be determined	Minor producer of the enzyme
No halos	Poor producer of the enzyme

3.2.6 Characterization of the bacterial isolates

The isolates whose colonies exhibited protease, pectinase and/or amylase activities during the screening process were retrieved from the freezer (-20 ºC), inoculated on nutrient medium (pH 10.5) and the plates incubated in a LEEC Research GA2000 incubator (Akribis Scientific Limited, Cheshire, UK) at 37 ºC for various time intervals. The isolates were identified by subjecting them to morphological, biochemical, physiological and molecular characterization, as described below:

3.2.6.1 Morphological characterization

3.2.6.1.1 Colony and cell morphology

The colony morphology of the isolates were studied by examining their 48-h old cultures with the naked eye for characters such as color, opacity, shape, elevation, texture and margins while their cell morphology were studied by mixing a small amount of 12-h old colonies with a drop of saline (0.9 % w/v) solution on separate microscope slides. The mixtures were carefully stirred using a toothpick, covered with a cover slip and examined

under a phase contrast Leica ICC50 microscope (Leica Microsystems Ltd, Heerbrugg, Switzerland) (focused at x 100 objective). The shape, arrangement and motility of the cells was recorded.

3.2.6.1.2 Spore formation test

Colony (72-h old) of each isolate was mixed with a drop of saline [0.9% (w/v)] solution on separate microscope slides. The slides were covered with cover slips, observed under a phase contrast Leica ICC50 microscope (Leica Microsystems Ltd, Heerbrugg, Switzerland) and the presence/absence of spores recorded.

3.2.6.1.3 Gram-staining and KOH tests

Twelve hour (12 h) old colonies of isolates LBW 261, LBW 4512 , LBW 327, LBW 434, LBW 7526a, LBW 39, LBW 7526b, LBW 328, LBS 16, LBW 625, LBW 446, LBW 5117, LBW 313 and LBW 317 and 48 h old colonies of isolates LBW 2719, LBW 226, LBW 318 and LBS 77 were subjected to Gram-stain test (Gerhardt *et al*., 1994). A small amount of each colony was suspended in a small drop of saline [0.9 % (w/v)] solution on a microscope slide and left to dry. The cells were fixed by passing the slide (cell side up) over a flame. A drop of crystal violet dye was then added to stain the heat-fixed cells (1 min) before rinsing the slide with water. Gram's iodine solution [1 % (w/v) I and 2 % (w/v) KI in water) was added to the sample to fix the dye. The sample was then rinsed with water and 95 % ethanol applied to it (10 sec) to remove the unbound crystal violet dye, leaving Gram-positive bacteria stained purple and Gram-negative bacteria, if any, colorless. The samples was rinsed with water, counterstained with safranin (1 min), air-dried and a drop of immersion oil added to it. Finally, the slide was examined under a phase contrast Leica ICC50 microscope (Leica Microsystems Ltd, Heerbrugg, Switzerland) (magnification x 100) and the cells recorded as positive for Gram stain if they appeared purple and Gram negative if they appeared pink.

To complement the Gram-staining test, each colony was subjected to the KOH test (Gregersen, 1978). With the help of a toothpick, the colony was mixed with a drop of 3 % (w/v) KOH on a microscope slide. The dense suspension was stirred continuously for 60 sec and the toothpick gently pulled away and observed. A thick and stringy suspension indicates that the isolate is Gram-negative bacteria while its absence indicates that it is Gram-positive.

3.2.6.2 Biochemical characterization

3.2.6.2.1 Catalase test

The method described by Gerhardt *et al*., (1994) was used. A loop-full growth of 24 h old colonies of each isolate was mixed with a drop of 3 % (v/v) H_2O_2 on a microscope slide and the mixture observed against a dark background. Production of effervescence due to catalase-catalyzed breakdown of O_2 (equation below) indicates a positive reaction.

$$H_2O_2(aq) \rightarrow H_2O\ (aq) + O_2\ (g)$$

3.2.6.2.2 Oxidase test

Twelve-hour (12-h) old colonies of isolates LBW 261, LBW 4512 , LBW 327, LBW 434, LBW 7526a, LBW 39, LBW 7526b, LBW 328, LBS 16, LBW 625, LBW 446, LBW 5117, LBW 313 and LBW 317 and 48 h old colonies of isolates LBW 2719, LBW 226, LBW 318 and LBS 77 were subjected to the oxidase test. This was done using oxidase reagent according to Gordon-Mcleod [https://www.sigmaaldrich.com/KE/en/search/gordon-mcleod-reagent?focus=products&page=1&perpage=30&sort=relevance&term=gordon-mcleod%20reagent&type=product_name (cited date 2023 Feb 09)]. With the help of a toothpick, the isolates were transferred from the solid nutrient medium to a filter paper moistened with Gordon-Mcleod oxidase reagent and then observed for the appearance of a deep blue -purple color within 10 sec to signify a positive oxidase test.

3.2.6.3 Physiological characterization

3.2.6.3.1 Alkaliphily test

The isolates were grown on nutrient agar (pH 7) in a LEEC Research GA2000 incubator (Akribis Scientific Limited, Cheshire, UK) at 37 ^{o}C for 72 h. No growth at this pH indicated that the particular isolate is an obligate alkaliphile while better growth indicated that it is a facultative alkaliphile (alkalitolerant).

3.2.6.3.2 Oxygen requirement test

To determine whether the isolates require oxygen for growth, a small amount of each colony was inoculated deep into 0.6 % (w/v) solid nutrient medium (pH 10.5) contained in different test tubes. The tubes were covered with aliminium foil and incubated in a LEEC Research GA2000 incubator (Akribis Scientific Limited, Cheshire, UK) at 37 ^{o}C for 72 h. The tubes were observed to establish whether growth occurred inside the medium or on its surface.

3.2.6.3.3 *Effect of temperature on growth of the isolates*

The cells were grown on solid nutrient medium (pH 10.5) in a LEEC Research GA2000 incubator (Akribis Scientific Limited, Cheshire, UK) at 25 ^{o}C, 37 ^{o}C, 45^{o}C, 55^{o}C and 65 ^{o}C, respectively, for 72 h. The sizes of the colonies formed were the visually compared.

3.2.6.3.4 Effect of salinity on growth of the isolates

The effect of salinity on the growth of the isolates was studied by growing the cells on solid nutrient- medium (pH 10.5) containing 0, 2.5, 5.0, 7.5, 10, 12.5 and 15 % (w/v) NaCl, respectively, in a LEEC Research GA2000 incubator (Akribis Scientific Limited, Cheshire, UK) at 37 ^{o}C, for 72 h, and then comparing the size of the colonies formed visually.

3.2.6.3.5 Oxygen requirement test

To determine whether the isolates require oxygen for growth, a small amount of each colony was inoculated deep into 0.6 % (w/v) solid nutrient medium (pH 10.5) contained in different test tubes. The tubes were covered with aliminium foil and incubated in a LEEC Research GA2000 incubator (Akribis Scientific Limited, Cheshire, UK) at 37 ^{o}C for 72 h. The tubes were observed to establish whether growth occurred inside the medium or on its surface.

3.2.6.4 Molecular characterization

3.2.6.4.1 DNA extraction

Genomic DNA was extracted from 24 h old colonies grown on solid nutrient medium (pH 10.5). This was carried out using *PureLink*® Genomic DNA extraction kit (Invitrogen Life Technologies™, CA, USA) according to the manufacturer's instructions. Briefly, the cell mass of each colony was harvested from the plate using individual sterile toothpicks and suspended in separate tubes, each containing 180 µl lysozyme digestion buffer (20 mg/ml). The contents of each tube was mixed by vortexing and then incubating it at 37º C. After 30 min, 20 µl proteinase K was added to the cell lysates and the mixtures vortexed briefly. Two hundred (200) µl genomic lysis/binding Buffer was added to the mixtures and the tubes vortexed (to yield homogenous solutions) before incubating them at 55 °C. After 30 min, 200-µl 96 % ethanol was added to the cell lysates and the tube contents mixed by vortexing to yield homogenous solutions.

Genomic DNA from the cell lysates were purified using the spin column-based centrifugation procedure. Briefly, 640 µl of each cell lysate was added to a separate spin column with a collection tube attached at the bottom and spun in a Hettich Mikro 200R centrifuge (Merck KGaA, Darmstadt, Germany) at 10,000-x g for 1 min at room temperature. The collection tubes were discarded and the spin columns placed in another set of clean collection tubes. Five hundred-(500) µl wash buffer 1 was added to the

columns and the latter centrifuged at 10,000-x g for 1 min at room temperature. The collection tubes were again discarded, after which the spin columns were placed in a new set of clean collection tubes. Five hundred-(500) µl wash buffer 2 was added to the columns followed by centrifugation at 20,200 g for 3 min. at room temperature. The collection tubes were discarded and the spin columns placed in sterile 1.5-ml micro centrifuge tubes. One hundred (100) µl genomic elution buffer was added to the columns and left to stand at room temperature for 1 min before centrifuging at 20,200 g for 1 min at room temperature to elute the DNA from the spin columns. The columns were removed and discarded to obtain purified genomic DNA samples in the tubes.

3.2.6.4.2 16S rDNA amplification

Genomic DNA from the isolates were subjected to PCR amplification of their 16S rRNA genes using universal forward 8-27F (5-AGAGTTTGATCCTGGCTCAG-3') and reverse 1492R (5'-CTACGGCTACCTTGTTACGA-3') primers (Frank *et al*., 2008) in a Gene Amp PCR system 9700 Thermal Cycler (Applied Biosystems, CA, USA). PCR reactions were performed in 50 µl containing 50 ng genomic DNA template (5 µl), 10 pmoles each of the forward and reverse primers (1 µl, each), 2 x PCR master mix containing PCR buffer, dNTPs and *Taq* DNA polymerase (25 µl), and nuclease free water (18 µl). The PCR cycling conditions were an initial denaturation at 94 ^{o}C for 5 min followed by 35 cycles of 94 ^{o}C for 1 min (DNA denaturation), 57 ^{o}C for 1 min (primer annealing) and 72 ^{o}C for 2 min (primer extension) with a final extension at 72 ^{o}C for 7 min. The amplified 16S rDNA fragments were resolved in a 1 % (w/v) agarose gel in 1X TAE buffer containing 0.5 µg/ml ethidium bromide and visualized under a Vilber smart imaging UV trans-illuminator (Marne-la-Vallée, France). A negative control containing nuclease free water instead of DNA was included in the experiment.

3.2.6.4.3 Purification of amplified 16S rDNA fragments and sequencing

The amplified 16S rDNA fragments were purified from the gel using Qiagen DNA purification kit (Limburg, Netherlands) according to the manufacturer's manual. Their absorbances were measured at A260 and A280 using a *NanoDrop*™ 2000/2000c spectrophotometer (Waltham, MA, USA), which also calculated their concentrations and degrees of purity (Johnson, 1994). Fifty (50) µl of the purified 16S rDNA fragments were sequenced on both strands using the dideoxy chain termination method and ABI Prism Big Dye Terminator Cycle Sequence Ready Reaction kit V2.1, in an ABI 377 DNA analyzer, at Macrogen Europe Laboratory (Amsterdam, Netherlands).

3.2.6.4.4 Phylogenetic Analysis and accession numbers

DNA Baser Sequence Assembler (version 4.20) was used to edit and assemble the contigs from chromatograms obtained from *Macrogen Europe Laboratory*. Assembled 16S rDNA sequences were compared with those of known 16S rDNA sequences at the GenBank of the NCBI database using *BLASTn* algorithm [https://blast.ncbi.nlm.nih.gov/Blast.cgi (cited date 10 February 2023)]. The identification of the isolates used in this study both at the genus and species levels was defined as a 16S rDNA sequence similarity of ≥ 99 % with that of the prototype strain sequence in the GenBank. The sequences, together with those of the reference strains derived from the GenBank, were aligned using MUSCLE (Edgar, 2004). A Phylogenetic tree was constructed from the aligned nucleotide sequences using the Bayesian phylogenetic method executed in MrBayes bioinformatics software version 3.1.2 (Huelsenbeck *et al*., 2002) and visualized using Fig Tree software version 1.2.2 [http://tree.bio.ed.ac.uk/ (cited date 2023 Feb 10)].

The 16S rDNA sequences were deposited in the GenBank at the NCBI database under accession numbers AY423275 and KU321024 to KU321040.

3.2.7 Cell culture and enzyme activity determination

One hundred (100)-µl glycerol stock of each isolate was aliquoted in separate 250 ml conical flasks containing 50 ml protease production medium, prepared as described in section 3.2.3.4. This procedure was repeated with pectinase and amylase production media (also prepared as described in section 3.2.3.4). The flasks were incubated in a Gallenkamp thermoshaker incubator (London, UK) at 37 ^{o}C and 200 rpm. After 48 h, the cell cultures were spun in a Supra 22K centrifuge (Hanil Science Industrial Co. Ltd, South Korea) at 4,000 rpm and 4 ^{o}C for 30 min, and the activity of the crude enzymes in the cell-free culture supernatants determined as follows:

3.2.7.1 Protease activity determination

This was done on the cell-free culture supernatants obtained from the protease production medium (Bakhtiar *et al.*, (2003). An aliquot of 0.5 ml enzyme was added to 0.5 ml 1 % (w/v) casein prepared in 50 mM glycine-NaOH buffer pH 10.5. The reaction mixture was then incubated in an LKB 2209 Multitemp Heating/Cooling Circulating water bath (Colora Messtechnik GmbH, Lorch/Wűrtt, Germany) at 50 ^{o}C for 20 min, after which the reaction was stopped by the addition of 0.5 ml 10 % (w/v) ice-cold TCA and left to stand for 5 min at 4 ^{o}C. The tube was centrifuged at 6,600 g at the same temperature for 2 min. Supernatant (0.5 ml) was obtained and 2.5 ml 0.5 M Na_2CO_3 and 0.5 ml three-fold diluted Folin-Ciocalteau phenol reagent added, respectively. The reaction mixture was left undisturbed at room temperature for 30 min and the absorbance read at 600 nm. One unit of protease activity was defined as the amount of enzyme that liberated one µmol of amino acids, as tyrosine, per min under the standard assay conditions.

3.2.7.2 Pectinase activity determination

This was done on the cell-free culture supernatants obtained from the pectin production medium using a modified version of the method described by Wang *et al*.(1997). An aliquot (0.2 ml) of enzyme was added to 0.8 ml 0.5% (w/v) polygalacturonic acid sodium

salt prepared in 50 mM glycine-NaOH buffer pH 10.5. The reaction mixture was incubated in a LKB 2209 Multitemp Heating/Cooling Circulating water bath (Colora Messtechnik GmbH, Lorch/Wűrtt, Germany) at 55 ^{o}C for 10 min. The reaction was stopped by adding 1 ml Dinitrosalicylic acid (DNS) reagent (see composition and preparation method in appendix 1b) followed by boiling in a Brinkmann Instruments Buchi Type 311350 1200W water bath (Flawil, Switzerland) for 10 min. One (1) ml each of 50 % (w/v) sodium potassium tartrate tetrahydrate (Rochelle salt) and distilled water were added respectively and the mixture left to stand under tap water for 5 min. The amount of reducing sugars formed was then determined (Wang *et al*., 1997). One unit of pectinase activity was defined as the amount of enzyme that liberated one µmol of reducing sugars, as monogalacturonic acid, per min under the standard assay conditions.

3.2.7.3 Amylase activity determination

This was carried out using a modified version of the method described by The enzyme (0.1 ml) was added to 0.4 ml 0.3% (w/v) soluble starch prepared in 50 mM glycine-NaOH buffer pH 10.5. The reaction mixture was incubated in a LKB 2209 Multitemp Heating/Cooling Circulating water bath (Colora Messtechnik GmbH, Lorch/Wűrtt, Germany) water bath at 55 ^{o}C and after 10 min, the amount of reducing sugars formed was then determined (Wang *et al*., 1997). One unit of amylase activity was defined as the amount of enzyme that liberated one µmol of reducing sugars, as glucose, per min under the standard assay conditions.

3.2.8 Data presentation and analysis

Data from screening the isolates for production of the enzymes was obtained after performing the experiment once (qualitative analysis), and are presented herein in both pictorial and tabular forms. The data for the characterization and identification of the positive producers of the enzymes was also obtained after performing the experiments once (qualitative analysis), and are presented herein in both tabular and figure forms.

Lastly, data for the production of the enzymes in the different broth media, by the isolates, was obtained after conducting the enzyme assays in duplicate, with calculated means, and is presented in graphical form.

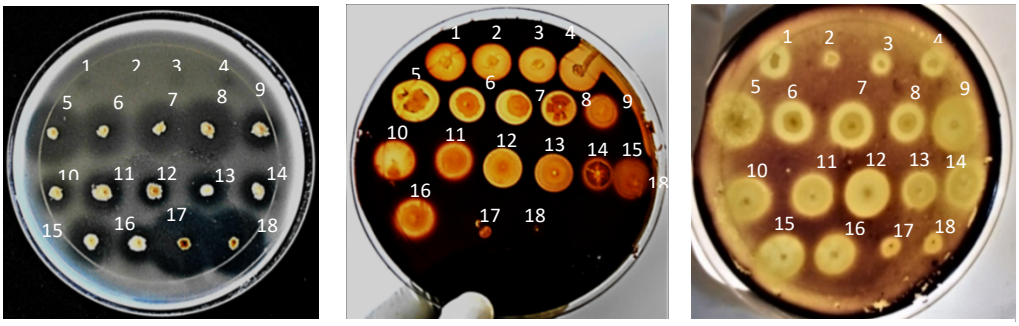
3.3 Results and Discussion

3.3.1 Detection of isolates that produce alkaline proteases, pectinases and amylases

Eighteen (18) Lake Bogoria isolates were plate-screened on solid gelatin, pectin and starch media (pH 10.5) for the production of alkaline proteases, pectinases and amylases, respectively. The screening data revealed that fourteen (14) isolates formed clear halos around visible colonies on the gelatin plate while four (4) isolates did so vaguely around invisible colonies on the same plate (Figure 3.3a). This is a confirmation that all the isolates produced extracellular alkaline proteases, which hydrolyzed or solubilized the substrate (gelatin) around their respective colonies thus, leaving no proteins for $(NH_4)_2SO_4$ solution to precipitate. In addition, sixteen (16) isolates formed clear halos around visible colonies after flooding the pectin plate with Lugol's iodine dye solution while two (2) did not (Figure 3.3b). This is a confirmation that the 16 isolates produced extracellular alkaline pectinases, which solubilized pectin around their respective colonies thus, leaving no pectin for Lugol's iodine dye solution to bind. Furthermore, all eighteen (18) isolates formed clear halos around visible colonies after flooding the starch plate with Gram's iodine dye solution (Figure 3.3c). This is a confirmation that the all the isolates produced amylases, which solubilized the starch around their respective colonies thus, leaving no starch for Gam's iodine dye solution to bind.

The activity of the enzymes produced by the colony of each isolate during the screening process was determined semi-quantitatively in terms of solubilization index (SI) values using equation 3.2 (section 3.2.5), and the values obtained used to rank the isolates as excellent-, very good-, good-, weak-, minor- and poor- producers of the enzymes. As seen in Table 3.3, seven (LBW 261, LBW 4512, LBW 327, LBW 434, LBW 446, LBW 313,

and LBW 317)- and four (LBW 2719, LBW 226, LBW 318, and LBS 77)- isolates exhibited SI values that were > 3.0 for protease and pectinase/amylase activities, respectively. For this reason, they were ranked as excellent producers of these enzymes. The rest of the isolates were ranked as either very good (2.0 < SI ≤ 3.0), good (1.0 < SI ≤ 2.0), or poor producers (no halos formed) of the enzymes. In addition, isolates LBW 2719, LBW 226, LBW 318 and LBS 77 were ranked as producers of minor and unquantifiable proteases as they presented with vague halos due to poor (invisible) growth of the isolates.



(a) **(b)** **(c)**

Figure 3.3: Gelatin (a), pectin (b) and starch (c) screening plates showing halos around the bacterial colonies that are positive for production of extracellular alkaline proteases, pectinases, and amylases, respectively. 1 = isolate LBW 2719; 2 = isolate LBW 226; 3 = isolate LBW 318; 4 = isolate LBS 77; 5 = isolate LBK 261; 6 = isolate LBW 4512; 7 = isolate LBW 327; 8 = isolate LBW 434; 9 = isolate LBW 7526a; 10 = isolate LBW 39; 11 = isolate LBW 7526b; 12 = isolate LBW 328; 13 = isolate LBS 16; 14 = isolate LBW 625; 15 = isolate LBW 446; 16 =isolate LBW 5117; 17 = isolate LBW 313 and 18 = isolate LBW 317.

Table 3.3 Enzymatic production profiles of the isolates based on their SI values.

	Isolate	Protease			Pectinase			Amylase		
		Dc (mm)	Dh (mm)	SI	Dc (mm)	Dh (mm)	SI	Dc (mm)	Dh (mm)	SI
1.	LBW 2719	[a]	2	[b]	5	20	4.0	4	15	3.8
2.	LBW226	[a]	2	[b]	5	20	4.0	3	10	3.3
3.	LBW 318	[a]	2	[b]	5	20	4.0	3	11	3.7
4.	LBS 77	[a]	2	[b]	5	22	4.4	4	14	3.5
5.	LBK 261	5	28	5.6	10	22	2.2	13	22	1.7
6.	LBW 4512	5	23	4.6	10	18	1.8	8	22	2.8
7.	LBW 327	5	23	4.6	10	18	1.8	9	21	2.3
8.	LBW 434	7	28	4.0	12	18	1.5	8	18	2.3
9.	LBW 7526a	8	19	2.4	10	18	1.8	13	23	1.8
10.	LBW 39	6	17	2.8	12	18	1.5	11	22	2.0
11.	LNW 7526b	8	17	2.1	10	18	1.8	11	20	1.8
12.	LBW 328	8	15	1.9	10	20	2.0	14	23	1.6
13.	LBS 16	5	15	3.0	10	20	2.0	11	20	1.8
14.	LBW 625	7	15	2.1	10	17	1.7	11	20	1.8
15.	LBW 446	6	19	3.2	15	22	1.5	13	28	2.2
16.	LBW 5117	7	17	2.4	10	18	1.8	11	24	2.2
17.	LBW 313	5	32	6.4	2	[c]	[d]	8	12	1.5
18.	LBW 317	5	30	6.0	1	[c]	[d]	8	12	1.5

Key**:**
[a] No visible growth
[b] Minor and unquantifiable enzyme
[c] No halo
[d] No enzyme produced

3.3.2 Identification of the isolates

Based on colony morphological tests, the majority of the isolates exhibiting enzymatic activities had white, translucent, and glistening pigmentations. In addition, they had circular shapes, flat surfaces, smooth textures, and entire margins **(**Figure 3.4 and Table 3.4). A few notable exceptions, however, were the colonies of isolates LBW 2719, LBW 226, LBW 318, and LBS 77, which presented with different morphological characteristics. Moreover, they initially grew and slowly into the agar, gradually becoming irregular and undulating in their later stages of growth. Another notable exception was the colonies of isolates LBW 313 and LBW 317, which were yellow in color.

Figure 3.4: Colony morphological characteristics of the isolates after 48 h of growth on a nutrient agar medium (pH 10.5). 1 = isolate LBW 2719; 2 = isolate LBW 226; 3 = isolate LBW 318; 4 = isolate LBS 77; 5 = isolate LBK 261; 6 = isolate LBW 4512; 7 = isolate LBW 327; 8 = isolate LBW 434; 9 = isolate LBW 7526a; 10 = isolate LBW 39; 11 = isolate LBW 7526b; 12 = isolate LBW 328; 13 = isolate LBS 16; 14 = isolate LBW 625; 15 = isolate LBW 446; 16 = isolate LBW 5117; 17 = isolate LBW 313 and 18 = isolate LBW 317.

Table 3.4 Colony morphological characteristics of the isolates after 48 h of growth on a nutrient agar medium.

Serial No.	Isolate	Pigment	Shape, height, texture, margin
1.	LBW 2719	White, opaque, and dull	Circular, raised, rough and dry, entire
2.	LBW226	White, opaque, and dull	Circular, raised, rough and dry, entire
3.	LBW 318	White, opaque, and dull	Circular, raised, rough and dry, entire
4.	LBS 77	White, opaque, and dull	Circular, raised, rough and dry, entire
5.	LBK 261	White, translucent, and glistening	Circular, flat, smooth, entire
6.	LBW 4512	White, translucent, and glistening	Circular, flat, smooth, entire
7.	LBW 327	White, translucent, and glistening	Circular, flat, smooth, entire
8.	LBW 434	White, translucent and glistening	Circular, flat, smooth, entire
9.	LBW 7526a	White, translucent and glistening	Circular, flat, smooth, entire
10.	LBW 39	White, translucent, and glistening	Circular, flat, smooth, entire
11.	LBW 7526b	White, translucent and glistening	Circular, flat, smooth, entire
12.	LBW 328	White, translucent, and glistening	Circular, flat, smooth, entire
13.	LBS 16	White, translucent, and glistening	Circular, flat, smooth, entire
14.	LBW 625	White, translucent, and glistening	Circular, flat, smooth, entire
15.	LBW 446	White, translucent, and glistening	Circular, flat, smooth, entire
16.	LBW 5117	White, translucent, and glistening	Circular, flat, smooth, entire
17.	LBW 313	Yellow, translucent, and glistening	Circular, flat, smooth, entire
18.	LBW 317	Yellow, translucent, and glistening	Circular, flat, smooth, entire

Based on cell morphological and biochemical tests, all isolates were motile, rod-shaped, Gram-positive, catalase- and oxidase- positive endospore-forming bacterial cells,

implying that they belonged to the genus *Bacillus* (Ash *et al*., 1991). Representative cells exhibiting some of these phenotypic characteristics are shown in Figure 3.5. It is worth noting that the colonies of all the isolates did not produce stringy suspensions when treated with KOH thus, confirming the Gram staining test (results not shown).

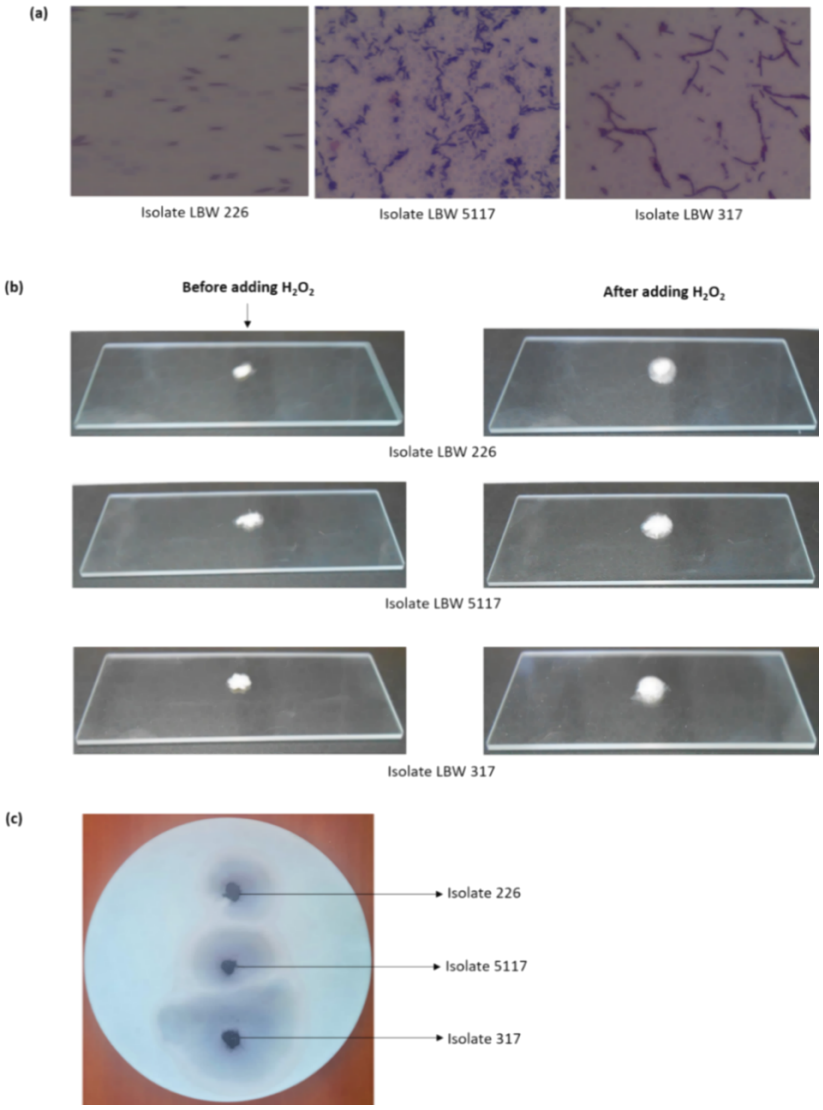


Figure 3.5: Some morphological and biochemical characteristics of the representative isolates: a) Gram staining -, b) Catalase - and c) Oxidase -tests.

Tests based on the physiological characterization of these *bacilli* isolates revealed that they all grew at pH > 9.0 (Figure 3.4 and Table 3.5), implying that they were alkaliphilic *bacilli* isolates (Kroll, 1990). In addition, the oxygen requirement test revealed that all the isolates migrated from inside the agar to its surface where they grew. This not only confirmed their motility status, but also showed that they were strict aerobes (Figure 3.6). Further physiological characterization of the isolates revealed that they were divided into two groups:

a) Group 1

Comprised sixteen (16) isolates that grew in the pH range 7.0 - 11.0 (optimum 10.5), implying that they were facultatively alkaliphilic *bacilli* isolates **(**Table 3.5**)**. Additional physiological tests revealed that they were divided further into two sub-groups. Sub-group I comprised four (4) isolates that grew at temperatures of up to 65 ^{o}C (optimum 37 ^{o}C) and in the presence of up to 5 % (w/v) NaCl [optimum, 0 - 4 % (w/v) NaCl] **(**Table 3.5**)** (Ghojavand *et al*., 2008). These growth characteristics are similar to those exhibited by *Bacillus* sp. C-125 (re-identified as *Bacillus halodurans* C-125) (Takami and Horikoshi, 1999); *Bacillus* sp. AH-101 (re-identified as *Bacillus halodurans*) (Takami *et al*., 1999) and *Bacillus halodurans* M29 (Mei *et al*., 2013). Sub-group II comprised twelve (12) isolates that grew at temperatures of up to 55 ^{o}C (optimum 37 - 45 ^{o}C), but in the presence of higher salt concentration [up to 10 % (w/v) NaCl (optimum 0 - 5 %)] **(**Table 3.5**)**. Ghojavand *et al.*, (2008) and Nielsen, *et al*, (1995) have shown that *Bacillus halodurans* DSM 497 type strain exhibits similar growth characteristics. These results suggest that the isolates classified under sub-group I are phenotypic variants of those in sub-group II or vice versa. This school of thought is further supported by the differences observed earlier on a) at their sampling sites (temperature) (Table 3.3), b) in their enzyme production profiles during screening (Table 3.3), c) in their colony and cell morphological characteristics (Table 3.4 and Figure 3.5a and c) in their physiological characteristics (Tables 3.5).

b) Group 2

Comprised two (2) isolates that grew in the pH range > 7.0 - 11.0 to suggest that they were obligate alkaliphilic *bacilli* isolates (Table 3.5)**.** They also grew at temperatures of up to 45 ºC (optimum 37 - 45 ºC) and in the presence of up to 15 % (w/v) NaCl [optimum 0 - 5 % NaCl (w/v)] (Figure 3.5). These growth characteristics are similar to those exhibited by *Bacillus pseudofirmus* DSM 8715 type strain (Nielsen *et al*., 1995)

Overall, the results suggest that the sixteen (16) isolates classified under Group 1 were *Bacillus halodurans* while those classified under Group 2 were *Bacillus pseudofirmus*. Both bacterial species are common residents of soda lakes and/or their surrounding hot springs (Grant and Tindall, 1986).

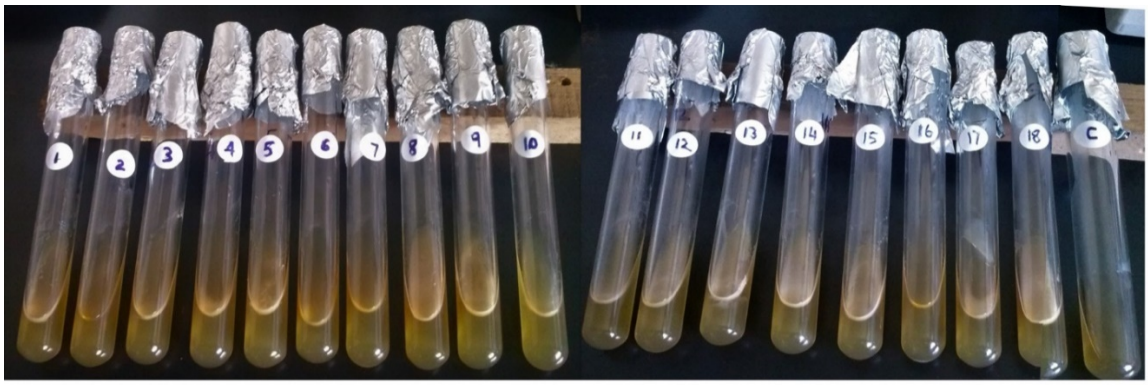


Figure 3. 6: Oxygen requirement test performed on the isolates. 1 = isolate LBW 2719; 2 = isolate LBW 226; 3 = isolate LBW 318; 4 = isolate LBS 77; 5 = isolate LBK 261; 6 = isolate LBW 4512; 7 = isolate LBW 327; 8 = isolate LBW 434; 9 = isolate LBW 7526a; 10 = isolate LBW 39; 11 = isolate LBW 7526b; 12 = isolate LBW 328; 13 = isolate LBS 16; 14 = isolate LBW 625; 15 = isolate LBW 446; 16 = isolate LBW 5117; 17 = isolate LBW 313 and 18 = isolate LBW 317.

Table 3.5 Effect of pH, temperature, and salinity on the growth of the isolates on nutrient agar medium.

Group			pH of growth (optimum growth)	Growth at (ºC)					Growth in the presence of NaCl (w/v)				
				25	37	45	55	65	0-4	5	10	15	18
Alkaliphilic *bacilli* isolates	**Group 1** Facultatively alkaliphilic *bacilli* isolates	Sub-group I	7.0 - 11.0 (pH 10.5)	+	++	+	+	+[a]	++	+[b]	-	-	-
			7.0 - 11.0 (pH 10.5)	+	++	+	+	+[a]	++	+[b]	-	-	-
			7.0 - 11.0 (pH 10.5)	+	++	+	+	+[a]	++	+[b]	-	-	-
			7.0 - 11.0 (pH 10.5)	+	++	+	+	+[a]	++	+[b]	-	-	-
		Sub-group II	7.0 - 11.0 (pH 10.5)	+	++	++	+	-	++	++	+	-	-
			7.0 - 11.0 (pH 10.5)	+	++	++	+	-	++	++	+	-	-
			7.0 - 11.0 (pH 10.5)	+	++	++	+	-	++	++	+	-	-
			7.0 - 11.0 (pH 10.5)	+	++	++	+	-	++	++	+	-	-
			7.0 - 11.0 (pH 10.5)	+	++	++	+	-	++	++	+	-	-
			7.0 - 11.0 (pH 10.5)	+	++	++	+	-	++	++	+	-	-
			7.0 - 11.0 (pH 10.5)	+	++	++	+	-	++	++	+	-	-
			7.0 - 11.0 (pH 10.5)	+	++	++	+	-	++	++	+	-	-
			7.0 - 11.0 (pH 10.5)	+	++	++	+	-	++	++	+	-	-
			7.0 - 11.0 (pH 10.5)	+	++	++	+	-	++	++	+	-	-
			7.0 - 11.0 (pH 10.5)	+	++	++	+	-	++	++	+	-	-
			7.0 - 11.0 (pH 10.5)	+	++	++	+	-	++	++	+	-	-
	Group 2: Obligately alkaliphilic *bacilli* isolates		> 7.0 - 11.0 (pH 10.5)	+	++	++	-	-	++	++	+	+	-
			> 7.0 - 11.0 (pH 10.5)	+	++	++	-	-	++	++	+	+	-

Key:
+, Growth; ++, Optimum growth; +[a], Poor growth after 72 h, better growth after 96 h; +[b], Weak growth; -, no growth

For molecular characterization, the PCR performed on the isolates' 16S rRNA genes using universal forward 8-27F- and reverse 1492R- primers amplified the expected 1.5 Kbp DNA fragments in all the DNA samples (Figure 3.7). The amplified PCR products were purified, quantified, (see their concentrations and degrees of purity in appendix 2), sequenced, and manually edited. A BLAST search of these sequences against those available in the geneBank of the NCBI database was carried out and the results are summarized in Table 3.6. All the sequences, including nine (9) retrieved from the NCBI database were aligned using MUCLE algorithm, and a phylogenetic tree was constructed (Figure 3.8). The tree divided the isolates into two distinct clusters. The largest cluster comprised the sixteen (16) isolates previously classified into group 1 phenotypically. Their sequences exhibited similarities ranging from 98.7 (isolate LBW 5117) -to- 99.8 (isolates LBW 4512, LBW 327, LBW 434, LBW 7526a, LBW 39, LBW 7526b, LBW 328, LBS 16, LBW 625 and LBW 446) -to- 99.9 (LBW 2719; 2 = LBW 226; 3 = LBW 318; 4 = LBS 77) -to- 100 % (LBK 261) with that of *Bacillus halodurans* DSM 497 type strain. On the other hand, the smallest cluster comprised of the two (2) isolates previously classified into group 2 phenotypically. Their sequences were 100 % similar to that of the *Bacillus pseudofirmus* DSM 8715 type strain. These results, therefore, confirmed the identity of the isolates as previously revealed by phenotypic analysis.

Both *Bacillus halodurans* and *Bacillus pseudofirmus* species are among the Gram-positive alkaliphiles commonly found in the Kenyan Rift Valley soda lakes (Duckworth *et al*., 1996). The limited number of alkaliphilic bacterial species in this study can be attributed to the sampling method and limited isolation conditions employed in the field and laboratory, respectively. It is interesting to note that although the isolates in sub-groups I and II shared high sequence identities with *Bacillus halodurans* DSM 497 type strain, they exhibited different: a) enzymatic production profiles (Table 3.3) and b) phenotypic characteristics (Table 3.4). This suggests that the four (4) sub-group I isolates may have evolved from those in sub-group II to enable them to adapt to their new ecological niches where the temperatures were relatively

high (Table 3.1) thus, giving rise to a variant sub-population with different gene expressions and new phenotypic characteristics (Rodríguez, 1995; Sousa *et al*., 2011).

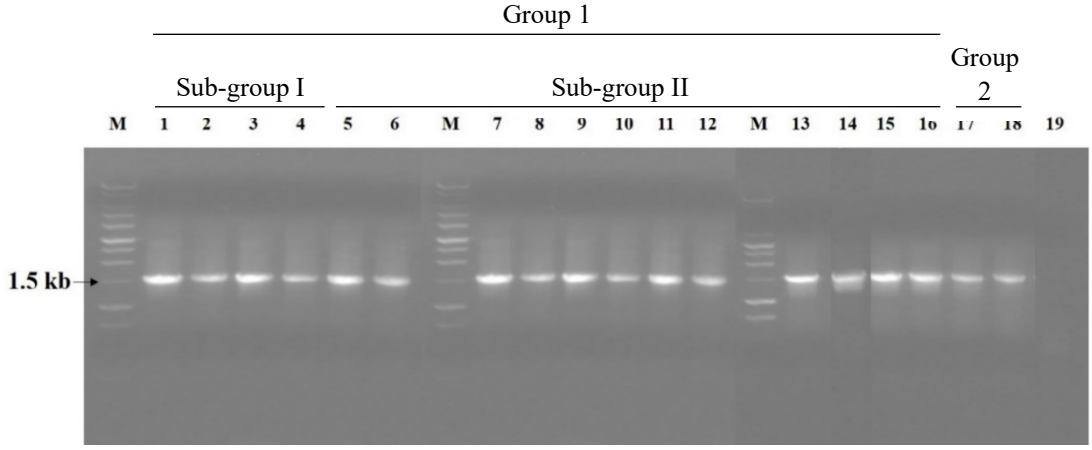


Figure 3.7: One (1) % (w/v) agarose gel showing PCR amplified 16S rDNA fragments of the isolates. M = 1 Kb Plus DNA ladder; 1 = LBW 2719; 2 = LBW 226; 3 = LBW 318; 4 = LBS 77; 5 = LBK 261; 6 = LBW 4512; 7 = LBW 327; 8 = LBW 434; 9 = LBW 7526a; 10 = LBW 39; 11 = LBW 7526b; 12 = LBW 328; 13 = LBS 16; 14 = LBW 625; 15 = LBW 446; 16 = LBW 5117; 17 = LBW 313 and 18 = LBW 317; 19 Negative control.

Table 3.6 Blast search results for the 16S rRNA gene sequences of the isolates.

Serial No.	Isolate	Query sequence length (bp)	GenBank accession number	Sequence similarity (%)	Closest species		
1.	LBW 2719	1383	KU321025.1	99.9	*Bacillus halodurans*	SG I	G 1
2.	LBW 226	1397	KU321024.1	99.9	*Bacillus halodurans*		
3.	LBW 318	1285	KU321028.1	99.9	*Bacillus halodurans*		
4.	LBS 77	1267	KU321036.1	99.9	*Bacillus halodurans*		
5.	LBK 261	1468	AY423275	100	*Bacillus halodurans*	SG II	
6.	LBW 4512	1312	KU321033.1	99.8	*Bacillus halodurans*		
7.	LBW 327	1340	KU321029.1	99.8	*Bacillus halodurans*		
8.	LBW 434	1315	KU321031.1	99.8	*Bacillus halodurans*		
9.	LBW 7526a	1298	KU321037.1	99.8	*Bacillus halodurans*		
10.	LBW 39	1320	KU321040.1	99.8	*Bacillus halodurans*		
11.	LBW 7526b	1305	KU321038.1	99.8	*Bacillus halodurans*		
12.	LBW 328	1308	KU321030.1	99.8	*Bacillus halodurans*		
13.	LBS 16	1267	KU321035.1	99.8	*Bacillus halodurans*		
14.	LBW 625	1282	KU321034.1	99.8	*Bacillus halodurans*		
15.	LBW 446	1283	KU321032.1	99.8	*Bacillus halodurans*		
16.	LBW 5117	1100	KU321039.1	98.7	*Bacillus halodurans*		
17.	LBW 313	1267	KU321026.1	100	*Bacillus pseudofirmus*	G 2	
18.	LBW 317	1289	KU321027.1	100	*Bacillus pseudofirmus*		

Key; G 1 = Group 1; SG I = Sub-group I; SG II = Sub-group II and G 2= Group 2

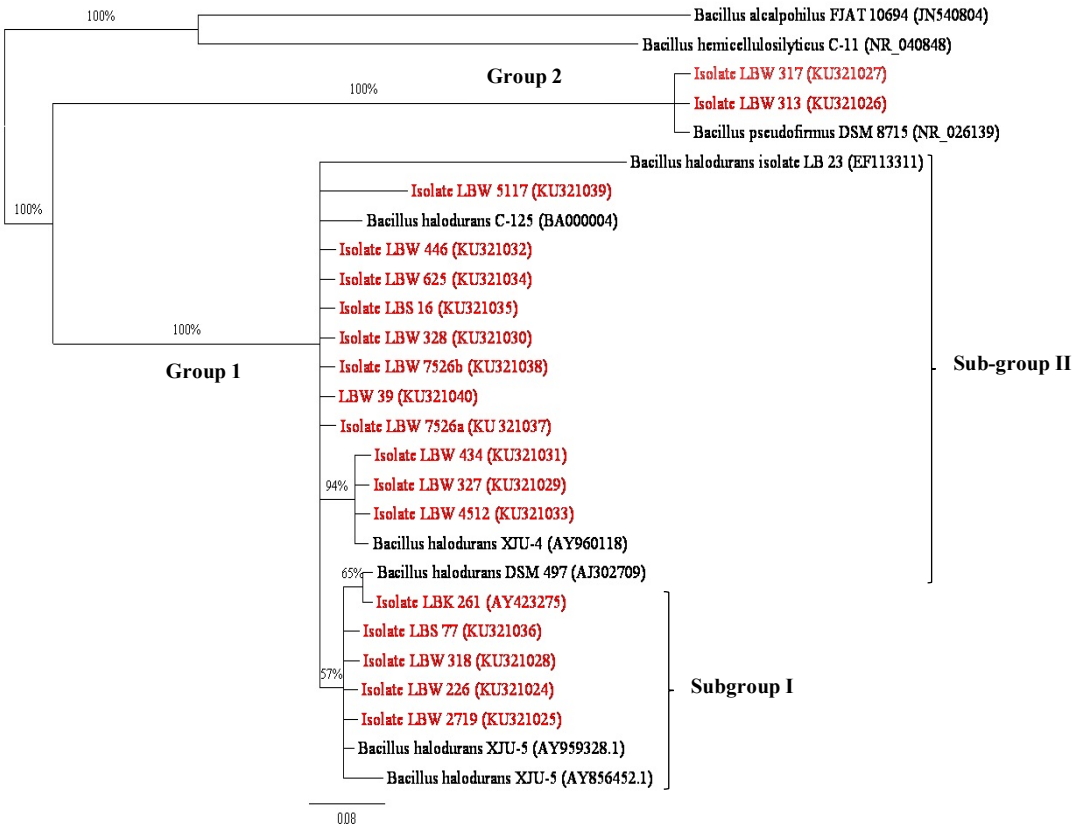


Figure 3.8: An unrooted phylogenetic tree constructed from the analysis of the 16S rRNA gene sequences of the alkaliphilic isolates and their closest relatives obtained from the geneBank in the NCBI database. The scale represents the average number of nucleotide substitutions per site. Bootstrap values for every 1000 trees generated are shown at the nodes. The accession numbers of the 16S rRNA sequences for each isolate are shown in brackets.

3.3.3 Enzyme activities of the isolates

3.3.3.1 Proteases

The majority of the isolates produced detectable extracellular alkaline proteases when cultured in liquid media containing chicken quill feathers (pH 10.5) as sole carbon and nitrogen sources, hence providing an economical and readily available substrate for cultivation of the isolates. Among the positive producers of the enzyme were *Bacillus halodurans* isolates classified under subgroup II (0.01 - 0.32 U/ml) and *Bacillus pseudofirmus* isolates in group 2 (0.38 - 0.40 U/ml), the majority of which produced titers that were within the range of those produced by their respective reference strains (Figure 3.9). On the contrary, the enzyme was not detected in the culture supernatants

of *Bacillus halodurans* isolates classified under sub-group I. This enzyme production profile further supports the theory that sub-group I isolates were a variant sub-population of those in sub-group II, as was also established based on their phenotypic characteristics (see section 3.3.2). The overall pattern of protease production by isolates from both bacterial species correlated well with those observed on the gelatine solid medium during the screening process (Table 3.3).

The highest protease producer was *Bacillus pseudofirmus* LBW 313, with a titer of 0.40 U/ml. Proteases hydrolyze proteins hence, this enzyme can find applications in several industries, including:

a) Detergent industry - as an additive in detergents for removal of protein-based stains in laundry and dishes during washing.
b) Leather industry - to dehair hide and produce high-quality leather
c) Recovery of silver from photographic and x-ray films - by hydrolyzing the gelatin layer in films and thus, allow the polyester base of the film to be recycled as well as assisting in the recovery of silver.
d) Food industry - to solubilize the proteins in protein-containing wastes in order to recover liquid concentrates or dry solids of nutritional values for incorporation in fish or livestock feeds (waste management).
e) Feed-manufacturing industry - to hydrolyze poultry and animal feed proteins to shorter peptides and amino acids. This helps to reduce the viscosity and digestibility of the feeds, resulting in increased absorption of nutrients, which translates to reduced fecal matter and increased weight and health of poultry/animals.
f) Textile industry - to degum silk during textile manufacture.

3.3.3.2 Pectinases

All *Bacillus halodurans* isolates classified under group 1 produced detectable extracellular alkaline pectinases, with those in sub-group I yielding relatively higher titres of the enzyme (0.12 - 0.46 U/ml) compared to their counterparts in sub-group II

(0.05 - 0.10 U/ml), whose production profile was within range of that produced by the reference strain, *Bacillus halodurans* DSM 497 (Figure 3.9). This altered enzymatic production profile between isolates in two sub-groups of the same bacterial species further supports the fact that sub-group I isolates were a variant sub-population of those in sub-group II, as was also established based on their phenotypic characteristics (see section 3.3.2) and in their protease production profiles (see section 3.3.3.1). On the other hand, no detectable pectinase activity was detected in the cell-free culture supernatants of *Bacillus pseudofirmus* isolates (group 2), and so did its reference strain, *Bacillus pseudofirmus* DSM 8715. The overall pattern of pectinase production by both *Bacillus halodurans* and *Bacillus pseudofirmus* isolates correlated well with those observed on solid pectin medium during the screening process (Table 3.3).

The highest pectinase producer was *Bacillus halodurans* 318, with a titer of 0.46 U/ml. Pectinase hydrolyze pectins hence, this enzyme can therefore find applications in the following industries:

a) Detergent industry - as an additive in detergents for removal of pectin-based stains in laundry and dishes during washing.
b) Food industry waste - to remove pectic waste waters from citrus and vegetable processing industries to facilitate the removal of pectinaceous material and thus, render the treated wastewaters suitable for decomposition by activated sludge treatment.
c) Coffee and tea industry - to remove mucilage from coffee beans and also break down the pectins in tea granules to prevent the formation of froth or form during the preparation of instant tea.
d) Textile industry - to remove pectins and other non-cellulosic materials that adhere to it from cotton fibers (scouring) during textile manufacture), among others.

3.3.3.3 Amylases

All *Bacillus halodurans* and *Bacillus pseudofirmus* isolates produced extracellular alkaline amylases whose titers ranged from 0.2 - 0.4 and 0.06 - 0.1 U/ml, respectively (Figure 3.9). These titers were within the range of those produced by their respective type strains. Interestingly, these enzymatic titers did not correlate with those produced by the same isolates on solid starch medium (Table 3.3). These can be attributed to differences in the; a) uptake of nutrients by the isolates in the two types of media and/or b) activity detection rule *e.g*. in the solid medium the disappearance of the substrate is measured while in the liquid medium the appearance of the product is measured, as was also reported by Castro *et al.* (1993).

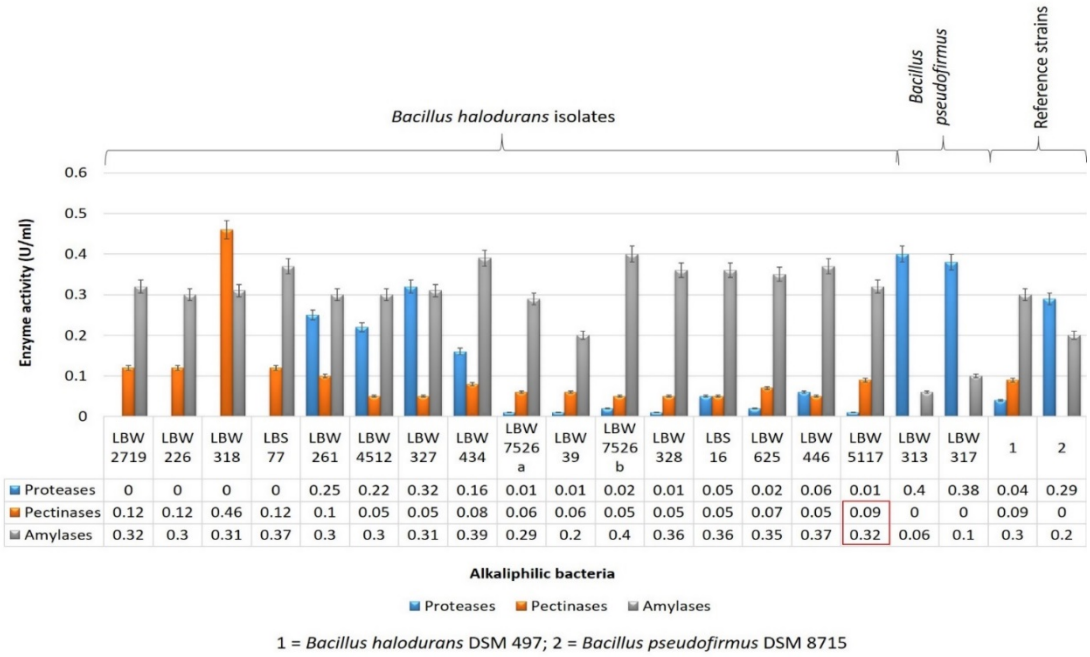
The highest amylase producer was *Bacillus halodurans* LBW 7526b (0.40 U/ml). Amylases hydrolyze starch hence, this enzyme can find applications in several industries, including:

a) Detergent industry - as an additive in detergents for removal of starchy-based stains in laundry and dishes during washing.
b) Feed-manufacturing industry - to hydrolyze poultry and animal feed starch to shorter oligosaccharides. This helps to reduce the viscosity and digestibility of the feeds, resulting in increased absorption of nutrients, which translates to reduced fecal matter and increased weight and health of poultry/animals.
c) Production of cyclodextrins whose hydrophobic cavities can form complexes with ‘guest molecules e.g., organic foods and, pharmaceutical- and cosmetic-products to improve their solubility and stability properties, for use in the pharmaceutical, cosmetic, analytical chemistry, food, plastic, and agricultural industries, etc.
d) Textile industry - to remove starch-based sizing material from woven cotton (desizing) during textile manufacture.

Among the most potent producer of both amylases and pectinases was alkaliphilic *Bacillus halodurans* LBW 5117, with yields of 0.32 and 0.09 U/ml, respectively. If

these enzymes are to find applications in the textile industry, as desizing and scouring agents respectively, then they must first be characterized to establish their optimum operating conditions, to ensure greater efficiency and hence production, and then used to demonstrate their potential in the intended industrial applications.

The characterization of an alkaline amylase and pectinase from alkaliphilic *Bacillus halodurans* LBW 5117 is described in chapters four and five, respectively. Their potential to desize (amylase) and scour (pectinase) industrially woven cotton are also described therein.



	LBW 2719	LBW 226	LBW 318	LBS 77	LBW 261	LBW 4512	LBW 327	LBW 434	LBW 7526 a	LBW 39	LBW 7526 b	LBW 328	LBS 16	LBW 625	LBW 446	LBW 5117	LBW 313	LBW 317	1	2
Proteases	0	0	0	0	0.25	0.22	0.32	0.16	0.01	0.01	0.02	0.01	0.05	0.02	0.06	0.01	0.4	0.38	0.04	0.29
Pectinases	0.12	0.12	0.46	0.12	0.1	0.05	0.05	0.08	0.06	0.06	0.05	0.05	0.05	0.07	0.05	0.09	0	0	0.09	0
Amylases	0.32	0.3	0.31	0.37	0.3	0.3	0.31	0.39	0.29	0.2	0.4	0.36	0.36	0.35	0.37	0.32	0.06	0.1	0.3	0.2

Figure 3.9: Enzymatic production profiles of the isolates: a) group 1 (composed of sub-groups I and II) *Bacillus halodurans*- and b) group 2 *Bacillus pseudofirmus*- isolates, and c) reference strains (1 = *Bacillus halodurans* DSM 497 and 2 = *Bacillus pseudofirmus* DSM 8715). Experimental details are in the text.

3.4 Conclusion and Recommendations

The present findings show that Lake Bogoria and its surrounding hot springs are inhabited by alkaliphilic microorganisms that can produce industrially important enzymes. Sixteen of these isolates were identified as *Bacillus halodurans* (group 1),

with 4 members of this group classified further into sub-group I and the remaining 12 into sub-group II. Two (2) other isolates were identified as *Bacillus pseudofirmus*. Isolates belonging to both bacterial species were amylase producers while pectinase was only produced by *Bacillus halodurans* isolates. Proteases were produced by *Bacillus halodurans* isolates belonging to sub-group II as well as their *Bacillus pseudofirmus* counterparts. These enzymes can find applications in various industries e.g., a) detergent (proteases, pectinases, and amylases)-, b) leather (proteases)-, c) poultry and animal feed (proteases, pectinases and amylases) - d) coffee and tea (pectinases)-, and e) textile (proteases, amylases, and pectinases) industries. However, they must first be characterized to establish their optimum operating conditions and then used, under optimized conditions, to evaluate their potential in the intended industrial applications. This is to guarantee maximum efficiency and therefore, greater production. In this regard, the characterization of amylase and pectinase from alkaliphilic *Bacillus halodurans* LBW 5117 is described in **chapters four** and **five**, respectively. Their ability to desize (amylase) and scour (pectinase) industrially woven cotton are also described therein.

CHAPTER FOUR

4.0 CHARACTERIZATION OF AN ALKALINE ENDO-α-1-4-AMYLASE FROM ALKALIPHILIC *BACILLUS HALODURANS* LBW 5117 AND DEMONSTRATION OF ITS DESIZING POTENTIAL

The work described in this chapter was accepted in the *Journal of Applied Biology and Biotechnology*, and appears as **paper II** in the 'list of papers' in this thesis (page xxi).

4.1 Introduction

The genus *Bacillus* is one of the major sources of industrial enzymes (Jan, 1983). The application of such enzymes in the detergent, pulp, and paper, and textile industries, has prompted the isolation of strains from a variety of alkaline environments as a source of stable enzymes with suitable activities (Horikoshi, 1996; Preiss *et al*., 2015). Many alkaliphilic *Bacillus* species have been identified and among them is *Bacillus halodurans*, which was first described by Nielsen *et al*. (1995). Reports available on this species have focused on polysaccharide degrading enzymes (e.g., pectinase, amylase, and xylanase, etc) with potential in different industrial processes (Kumar & Satyanaana, 2014; Mei *et al*., 2013; Murakami *et al*., 2007). Isolation of amylase-producing alkaliphilic *Bacillus halodurans* and *Bacillus pseudofirmus* isolates from samples collected from Lake Bogoria, a soda lake found in Kenya's Rift Valley area, has been reported in chapter three of this thesis (Oluoch *et al*., 2018). Screening of the microorganisms for starch-degrading enzymes revealed that *Bacillus halodurans* isolates were among the highest producers of amylases (chapter three) (Oluoch *et al*., 2018)

Amylases catalyze the hydrolysis of 1,4 -O- and 1,6-O- glycosidic bonds in starch or that of its degradation products (Yahya *et al*., 2021). Among these enzymes is endo-α-1-4- amylase (or α-amylase) [EC 3.2.1.1], which randomly cleaves multiple internal 1,4-O- glycosidic bonds in starch to rapidly produce water-soluble low molecular weight linear α-anomeric maltooligosaccharides and α-dextrins (Tiwari *et al*., 2015).

Endo-α-1-4- amylases are produced mainly by *Bacillus* species, although production by other bacterial (e.g., *Chromohalobacter*, *Halomonas*, *Rhodothermus*) and fungal (e.g., *Aspergillus, Thermomyces, Streptomyces* and *Penicillium*) species has also been reported (Hussain *et al*., 2013; Sundarram & Murthy, 2014). Their characterization is vital if they are to find applications in various industrial sectors. This is because enzymes possess unique properties that enable them to operate optimally under different conditions (Martin *et al*., 2019; Martins *et al*., 2001). For example, different enzymes require different operational conditions (e.g., pH, temperature, and metal ion and surfactant requirements, etc) to optimally hydrolyze substrates into their respective products. Therefore, an understanding of an enzyme's characteristics is paramount.

Endo-α-1-4- amylases have widespread applications in several industries e.g., detergent, brewing, baking, paper, and textile (Putri & Nakagawa, 2020). In the textile industry, an aqueous solution of starch (sizing material) is applied on warp yarns (sizing) in order to facilitate a fast and secure weaving process, after which it is removed using crude (or partially purified) endo-α-1-4-amylases (enzymatic desizing) to facilitate further processing of the fabric e.g., scouring, bleaching; dyeing, printing and finishing (Sultana *et al*., 2014).

Endo-α-1-4- amylases offer numerous advantages as desizing agents e.g., they are: a) safe and easy to handle b) efficient in a wide range of temperatures and c) degradable. Furthermore, they require the use of fewer chemicals (eco-friendly) and do not cause damage to cloth (leads to the production of high-quality fabrics - soft, smooth, flexible, and with good strength retention) (Agrawal, 2016). However, most of the desizing amylases are costly high-temperature enzymes that have to be imported from a few European and Asian countries (Yahya *et al*., 2021). The situation is worse in developing countries like Kenya where conventional aggressive chemicals e.g., alkaline hydrogen peroxide are used as desizing agents. Alkaline peroxide is risky to handle because it is corrosive to the eyes, skin and respiratory system. It can also attack the cloth during the treatment (air-induce cellulose degradation) and cause high fabric

weight losses (weak fabric) associated with low tensile strengths, rough 'feel', and high pollution loads [e.g., Biological Oxygen Demand (BOD), Chemical Oxygen Demand (COD) and Total Dissolved Solids (TDS)] in the effluent generated. This is a potential threat to the environment (Ul-Haq & Nasir, 2012; Saha *et al*., 2018).

Furthermore, desizing is expensive because large volumes of water are required to wash and remove the harsh chemicals from treated fabrics, but this generates large volumes of chemically contaminated wastewaters, which are not only harmful to the flora and fauna in potential receiving water bodies, but can also affect the underground drinking water supplies. Moreover, chemical desizing is a time-consuming process, implying that the turnover rate of the finished product is low, and this has a direct negative impact on the profits made [personal communication from Hosea Too, Rivatex (EA) Ltd (REAL), Kenya].

The objective of this study was to determine the physicochemical properties of crude amylase (designated Amy LBW 5117) from locally isolated alkaliphilic *Bacillus halodurans* LBW 5117 (Oluoch *et al*., 2018) and to carry out a preliminary investigation to demonstrate its potential to desize industrially woven cotton.

4.2 Materials and Methods

4.2.1 Materials

Starch from Irish potato (*Solanum tuberosum*) was purchased from Merck (Darmstadt, Germany) while those from sweet potato (*Ipomoea batatas*), tapioca (*Manihot esculenta*), rice (*Oryza sativa*), corn (*Zea mays*) and wheat (*Triticum aestivum*) were bought in the form of flour from a local store (Carrefour Supermarket, Ltd, Nairobi, Kenya). Precoated Kieselgel 60 F254 silica gel sheets were purchased from Macherey-Nagel GmbH & Co. KG (Düren, Germany) while industrially woven plain grey cotton (100 %), containing 10 % (w/v) corn-starch as the sizing material, was kindly donated by Rivatex (EA) Ltd, Eldoret, Kenya. Yeast extract, peptone, Tween 20, 3,5-Dinitrosalicylic acid, potassium sodium tartrate tetrahydrate, carboxymethyl cellulose

sodium salt, avicel, cellobiose, glucose (G1) and maltose (G2), were purchased from Sigma Aldrich (St. Louis, MO, USA.

4.2.2 Bacterial isolate and culture for amylase (Amy LBW 5117) production

Bacillus halodurans LBW 5117 was isolated in our laboratory from a soil sample collected from Lake Bogoria (00º 15'N and 36º 06' E), a soda lake found in the Kenyan Rift Valley area, and identified as an amylase producer (Oluoch *et al*., 2018). A stock of this isolate in 40 % (v/v) glycerol was retrieved from the freezer (-20 ºC) (Figure 4.1a) and used to inoculate solid Horikoshi II solid medium containing starch as the sole carbon source [pH adjusted to 10.0 using 20 % (w/v) Na_2CO_3] (Horikoshi, 1999).

The plate was incubated at 37 ºC for 12 h, after which a colony of the bacterium was obtained from the plate (Figure 4.1b) and used to inoculate 20 ml Horikoshi II pre-culture medium (without agar). The bacterium was cultured in a Gallenkamp thermoshaker incubator (London, UK) at 37 ºC and 100 rpm for 12 h (Figure 4.1c). This pre-culture was then used to inoculate 80 ml of the same (main) medium in a 500 ml conical flask and the bacterium cultured for 48 h under the same conditions (Figure 4.1 d). The cell culture was centrifuged at 5,000 x g and 4 ºC for 30 min to obtain the supernatant, which was then treated as a crude enzyme (Figure 4.1e). The appearance and odor of the enzymatic solution were noted, after which it was stored at -20 ºC until use.

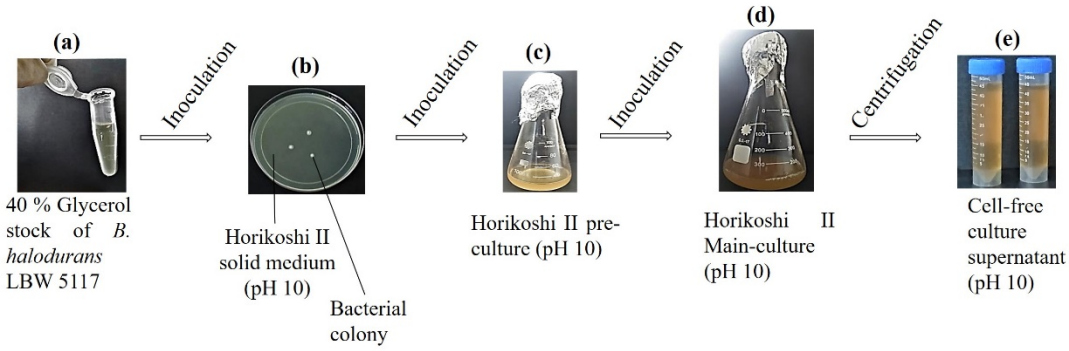


Figure 4.1: Schematic representation of the procedure that was used to culture *Bacillus halodurans* LBW 5117 for the production of Amy LBW 5117.

4.2.3 Enzyme assay

The catalytic activity of Amy LBW 5117) was determined by adding 0.1 ml enzyme solution to 0.4 ml 0.3 % (w/v) Irish potato starch in 50 mM glycine-NaOH buffer, pH 10. The reaction mixture was incubated in a LKB 2209 Multitemp heating/cooling circulating water bath (Colora Messtechnik GmbH, Lorch/Wűrtt, Germany) at 60 ^{o}C for 10 min, and the amount of reducing sugars formed was monitored by the method described by Wang *et al*. (1997). Heat-inactivated enzyme (95 ^{o}C for 30 min) was used as control. One unit of enzyme activity was defined as the amount of enzyme that liberated one µmol glucose/min under the established standard assay conditions.

4.2.4 Characterization of Amy LBW 5117 activity

The enzyme was characterized to determine its physicochemical properties e.g., a) appearance, and b) optimum storage and operational conditions. This was carried out as described below:

4.2.4.1 Effect of storage temperature on the stability of the enzyme

The stability of the enzyme was studied by storing it in a refrigerator at 4^{o} C and in separate LEEC research GA2000 incubator**s** (Akribis Scientific Limited, Cheshire, UK) at 20 and 30 ^{o}C, respectively**,** for six weeks. Samples were withdrawn after every two weeks for the determination of residual enzyme activity under the standard assay conditions.

4.2.4.2 Effect of pH on the activity of the enzyme

The effect of pH on the activity of the enzyme was investigated by carrying out the assays at t pH 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0, 10.0, 10.5, 11.0, 12.0 and 13.0, at 60 ^{o}C, under the standard assay conditions. Fifty (50) mM buffer systems consisting of: NaOAc/CH_3COOH (pH 3.0, 4.0 and 5.0); $NaHPO_4$/NaH_2PO_4 (pH 6.0, 7.0 and 8.0); Tris/HCl (pH 8.0 and 9.0); Glycine/NaOH (pH 9.0, 10.0 and 10.5); $NaHCO_3$ /NaOH (pH 10.5 and 11.0); Na_2HPO_4/NaOH (pH 11.0 and 12.0) and KCl/NaOH (pH 12.0 and 13.0) were prepared according to Gomori, (1955) and the web site

http://delloyd.50megs.com/moreinfo/buffers2.html (cited date 16 Feb 2023), and used in the assays.

4.1.4.3 Effect of temperature on the activity of the enzyme

The effect of temperature on the activity of the enzyme was determined by carrying out the assays at 30, 35, 40, 50, 55, 60, 70 and 90 ^{o}C) using 50 mM glycine-NaOH (pH 10) under the standard assay conditions.

4.2.4.4 Effect of metal ions on the activity of the enzyme

The effect of metal ions on the activity of the enzyme was studied by incubating the enzyme with KCl, $CaCl_2$, $MgSO_4$, $Fe_2(SO4)_3$, $CuSO_4$, NaCl, $MnCl_2$ and $ZnSO_4$ (final concentrations = 0 - 10 mM, respectively) at room temperature (29 ± 3^{o}C), for 30 min, and then determining the residual activities under the standard assay conditions.

4.2.4.5 Effect of surfactants on the activity of the enzyme

The effect of Tween 20, Triton X-100 and SDS on the activity of the enzyme was studied by incubating the enzyme with each surfactant (final concentration = 0.05, 0.1, 0.5, 1.0, 1,5, 1.75 and 2.0 mM, respectively) at room temperature (29 ± 3 ^{o}C) for 30 min, and then determining the residual activities under the standard assay conditions.

4.2.4.6 Effect of optimum temperature on the stability of the enzyme

The stability of the enzyme at its optimum temperature of activity is paramount if it is to be used to degrade starch optimally. Thus, Amy LBW 5117 was incubated at 60 ^{o}C in the absence and presence of Ca^{2+} and Tween 20 (final concentrations = 1.0 and 0.05 mM, respectively), individually, and together. The samples were withdrawn after 30 min, 1, 2, 3 and 4 h for the determination of residual activity of the enzyme under the standard assay conditions.

4.2.4.7 Effect of different substrates on the activity of the enzyme

This was carried out using both semi-quantitative and quantitative assays. For the semi-quantitative assays, 10 µl enzyme containing Ca^{2+} and Tween 20 (final concentrations = 1.0 and 0.05 mM, respectively) was aliquoted into small depressions (0.5 cm in diameter) made on the surfaces of agar media (pH 10.0) containing 0.3 % (w/v) Irish potato, sweet potato-, tapioca-, rice-, corn- and wheat- starch products, respectively. The plates were incubated in a LEEC Research GA2000 incubator (Akribis Scientific Limited, Cheshire, UK) at 60 ^{o}C for 6 h and then stained with iodine reagent (Yassin *et al*., 2021).

The activity of the enzyme [solubilization index (SI) value] was determined by calculating the size of the halos formed around each well (Luang-In *et al*., 2019). Similarly, 10 µl enzyme was aliquoted in depressions made on cellulosic plates containing 1.0 % (w/v): carboxymethyl cellulose (CMC) sodium salt, avicel and cellobiose, respectively. The plates were incubated at the same temperature and for the same duration after which, they were stained with Gram's iodine dye solution (Sinza *et al*., 2021). The presence of halos around the CMC sodium salt, avicel and cellobiose plates, if any, is indicative of endoglucanase, exoglucanase and cellobiase activities, respectively. For the controls, heat-inactivated enzyme (95 ^{o}C for 30 min) was used.

The effect of the different starch products on the activity of the enzyme was also studied quantitatively in the presence of Ca^{2+} and Tween 20 (final concentrations = 1.0 and 0.05 mM, respectively). This was carried out as described in the assay method (section 4.2.3) by using 0.3 % (w/v) of the substrates. In addition, the activities of endoglucanase, exoglucanase, and β-glucosidase in the crude enzyme were determined by using a modified version of the method described by Kiio *et al*. (2016). Five hundred (500) µl enzyme was added to 500 µl 1 % (w/v) carboxymethyl cellulose sodium salt, avicel, and cellobiose, respectively, prepared in 50 mM glycine-NaOH buffer pH 10. The mixtures were incubated at 60 ^{o}C for 1 h and the amount of reducing sugars formed followed using the method described by Wang *et al*. (1997). One unit

of enzyme activity was defined as the amount of enzyme that liberated one µmol glucose/min under the established standard assay conditions. The heat-inactivated enzyme (95 ^{o}C for 30 min) was used as a control.

4.2.4.8 Mode of action of the enzyme

The mode of action of the enzyme was determined according to Wang *et al.* (2019). An aliquot of 500 µl (0.5 U) Amy LBW 5117 was added to an equal volume of 1 % (w/v) starch (Irish potato) prepared in 50 mM glycine NaOH buffer (pH 10). The reaction mixture was incubated in a LKB 2209 Multitemp heating/cooling circulating water bath (Colora Messtechnik GmbH, Lorch/Wűrtt, Germany) at 60 ^{o}C, and samples (150 µl) were withdrawn after 0, 2, 4, 8 and 24 h. The samples were heated in a Buchi Type 311350 1200W water bath (Brinkmann Instruments, Flawil, Switzerland) at 95 ^{o}C for 5 min and then stored in a refrigerator at 4 ^{o}C until use. A defined amount of the samples (5 µl) were spotted on the bottom of a precoated Kieselgel 60 F254 silica gel aluminum sheet, left to dry and then developed with a mobile phase of 1-butanol: ethanol: water (5:3:2; v/v/v), with two ascents, in a saturated thin layer chromatography (TLC) chamber at room temperature. The sheet was air-dried, immersed in 15 % (v/v) H_2SO_4 (5 sec), air-dried again and finally developed by heating at 110 ^{o}C until spots appeared. Linear sugars (0.1 % (w/v) glucose (G1) and maltose (G2) were used as standards.

4.2.5 Desizing of woven cotton using Amy LBW 5117

A 5 cm x 5 cm piece of plain-woven grey cotton containing corn-starch as the sizing material was weighed in a Mettler Toledao AB54S high resolution analytical balance **(**City, Switzerland) and then subjected to desizing using Amy LBW 5117 in a batch process (Chinnammal & Arunkumar, 2013) (Figure 4.2). The fabric was first wetted to approximately 100 % in desizing liquor (1.0 mM Ca^{2+}, 0.05 mM Tween 20 and 3.9 U Amy LBW 5117 in 50 mM glycine NaOH buffer pH 10) in a Gallenkamp thermoshaker incubator (London, UK) set at 29 ±3 ^{o}C without agitation, for 30 min (material/liquor ratio was maintained at 1:20). Thereafter, the temperature of the

incubator was raised to 60 °C and 100 rpm for 4 h. The fabric was then removed from the desizing bath and washed in hot water (95 °C, 10 min) followed by several cold-water washes. The fabric was then dried in a Kottermann D 3165 oven (Hanigsen, Germany) at 105 °C for 1 h, cooled in a desiccator and re-weighed. As a control, a second piece of woven cotton (5 cm x 5 cm) was weighed, treated in a similar fashion, but with heat-inactivated enzyme (95 °C for 30 min), and finally re-weighed.

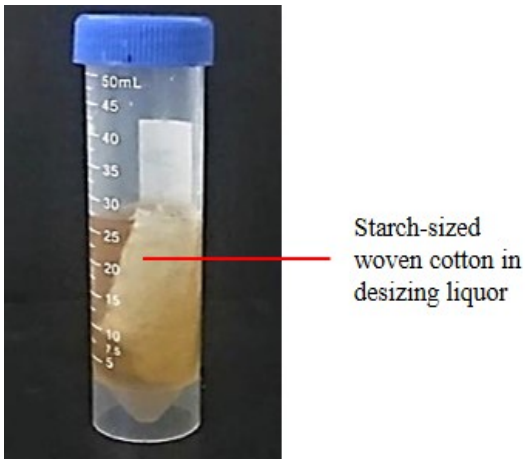


Figure 4.2: Enzymatic desizing of woven cotton (batch process) using Amy LBW 5117.

The efficiency of the desizing process was assessed by determining the extent to which the size material was enzymatically removed from the fabric and then comparing the result with that from the control fabric. This assessment was carried out as described below:

4.2.5.1 Surface property determination

The sample was handled or felt freely for: a), softness/firmness b) roughness/smoothness and c) flexibility/stiffness (Grinevičiūtė & Gutauskas, 2004).

4.2.5.2 Weight loss (%) determination

The weight loss (%) that the fabric incurred after the treatment was calculated using equation below:

$$\text{Weight loss (\%)} = \frac{W1 - W2}{W1} \text{ x } 100\ \% \quad \text{..... (Au \& Holme, 1999)}$$

Where $W_1$and W_2 are the weights of the fabric before and after treatment, respectively.

4.2.5.3 Iodine-stain test

This was carried out according to Au and Holme, (1999). The fabric was immersed in 0.005 M iodine solution in a 250 ml beaker. After 1 min, it was removed, washed with cold water, mangled, air dried and immediately observed for color changes. A deep bluish/purple/black color indicates the presence of a significant amount of starch while a brownish/beige color signifies its absence or near absence (Halim & Zhou, 2018).

4.2.5.4 TEGEWA rating and residual starch (%) content determination

The color of the stained fabric observed in section 4.2.5.3 was visually compared with those on the TEGEWA violet scale [Figure 2.11], and its closest match identified, along with its numerical value (TEGEWA rating) and corresponding approximate residual starch (%) content.

4.2.6 Data presentation and analysis

The data for characterization of Amy LBW 5117 with respect to its catalytic activity and stability, and kinetic parameters are presented in graphical or tabular forms, and were conducted in duplicate with calculated means. The data for substrate specificity studies of the enzyme is presented in both pictorial and graphical forms, with the latter conducted in duplicate with calculated means. Finally, the data for the enzyme's mode of action and that for the desized cotton are presented in either tabular or pictorial forms or both, and were conducted once.

4.3 Results and Discussion

4.3.1 Physicochemical properties of Amy LBW 5117

The enzyme was characterized in order to determine its physicochemical properties, including appearance, and operational and storage conditions. This was carried out as described below;

4.3.1.1 Appearance and catalytic activity

Amy LBW 5117 was a brown liquid that exhibited a slight fermentation odor. These are some of the typical physical characteristics of crude enzymatic preparations that are used for commercial desizing [http://www.sunsonenzyme.com/Products/Textile/Desizing/ (cited date 16 Feb 2023)], [https://biosolutions.novozymes.com/en/textiles/products/desizing/aquazym (cited date 16 Feb 2023)]. The catalytic activity of the enzyme was (0.41 U/ml) is similar to that produced by *Bacillus subtilis* after utilizing different substrates as carbon sources e.g., treated wheat straw (0.46 U/ml), maize straw (0.47 U/ml), and rice straw (0.44 U/ml) (Abd El Mageed *et al*., 2022), but much lower than those of known commercial desizing enzymes (Table 4.1). Thus, if Amy LBW 5117 is to be used as a desizing agent, its catalytic activity must be enhanced e.g., through optimization of the culture conditions that were used to grow the bacterium (Pandey *et al*., 2000).

Table 4.1 Comparison of some physicochemical properties of Amy LBW 5117 with those of commercially available desizing amylases.

Enzyme	Catalytic activity (U/ml)	Storage temp (°C)	pH of activity		Temperature of	Activity
			Operational	Optimum	Operational	Optimum
LTAA31°	≥ 3000	5 - 25, for 12 months	5 – 7	6	20 – 90	80
HTAA25L°	≥ 25000	5 - 25, for 12 months	5.5 - 9.0	5.5 - 8.0	80 – 110	95
Coenzyme® LTAA3P°	≥ 3000	5 - 35, for 6 - 9 months	6.0 - 7.0	6.0	35 – 70	60
Coenzyme® DD990L°	≥ 6000	5 - 25, for 12 months	5.5 - 7.5	6.5	40 – 120	80
HTAA190L°	≥ 190000	5 - 25, for 12 months	5.5 - 10.0	5.8 - 8.0	50 – 110	95
Aquazyme® Prime 12000L•	-	-	5.0 - 10.0	5.0 - 10.0	35 – 90	50 - 80
Aquazyme® Ultra 1200N•	-	-	5.0 - 7.5	5.0 - 7.5	40 – 95	55 - 70
Amy LBW 5117	0.41	4 - 30, for 6 weeks	7.0 - 10.5	10.0	50 – 63	60

°Commercial desizing amylases from Sunsonzymes, Sunson Industry Group Co., Ltd, Suite 2302, Zhong'an Shengye Building, Chaoyang District, Beijing, China (100101) [http://www.sunsonenzyme.com/Products/Textile/Desizing/ (cited date 16 Feb 2023)],

• Commercial desizing amylases from Novozymes **A/S,** Krogshoejvej 36, 2880 Bagsvaerd, Denmark [https://biosolutions.novozymes.com/en/textiles/products/desizing/aquazym (cited date 16 Feb 2023)].

4.3.1.2 Storage stability

Temperature is an important limiting factor for the transportation and storage of enzymes following their production. Industrial enzymes are often produced in bulk liquid (or powder) form, with each container carrying 25 or 30 Kg of the enzyme. This is because emphasis is laid on catalysis rather than purity [https://www.sunsonzymes.com/product/9/97/160/detail (cited date 07 July 2023]. These bulk liquid enzymes are therefore not suitable for storage in frozen form and are instead stabilized to enable them withstand wide ranges of temperature e.g., 5 – 25 ^{o}C during their transportation and storage [https://www.sunsonzymes.com/product/9/97/160/detail (cited date 07 July 2023)]. This is cost-effective in terms of time- and energy-consumed because freezing is avoided. For these reasons, Amy LBW 5117 was stored at various temperatures in liquid state and its catalytic activity was monitored every two weeks over a period of 6 weeks. As seen in Figure 4.3, the activity of the enzyme declined gradually, reaching 93, 76 and 70 % of the activity after 6 weeks of storage at 4, 20 and 30 ^{o}C, respectively. This shows that the site and tertiary structure of the enzyme was not adversely affected by the storage temperatures investigated, implying that the enzyme can be produced and used within 6 weeks of storage at the said temperatures. In the future, it would be interesting to determine the catalytic activities of the enzyme beyond this storage period, e.g., up to 12 months, and then compare the results obtained with those from commercially available desizing amylases (Table 4.1).

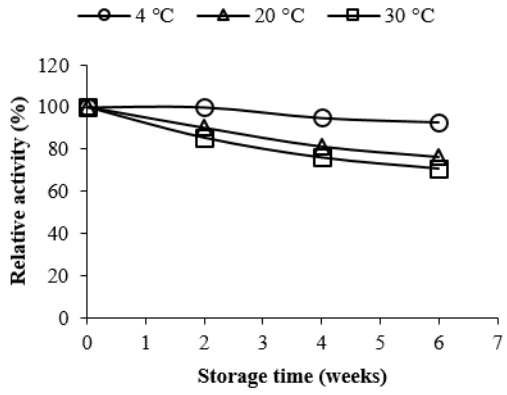


Figure 4.3: Effect of storage temperature on the activity of Amy LBW 5117 at pH 10.0. Enzyme activity corresponding to 100 % was 0.41 U/ml.

4.3.1.3 Optimum pH of enzyme activity

Another important limiting factor that affects the activity of enzymes is pH. For this reason, the activity of Amy LBW 5117 was measured using different 50 mM buffer systems at 60 ºC. As seen in Figure 4.4, the activity of the enzyme increased rapidly with increase in pH from 3.0 - 4.0. This was followed by a gradual increase in the activity as the pH increased to 10. The activity declined sharply thereafter at higher pH values, reaching 0 at pH 13. The enzyme exhibited an optimum pH of 10 in 50 mM glycine NaOH buffer, with over 80 % of its original activity displayed in the pH range of 7.0 - 10.5 (operational range). Most commercially available desizing amylases are however optimally active at the near-neutral pH range (Table 4.1). This implies that Amy LBW 5117 has an added advantage over them, since it can be used to hydrolyze starch optimally under alkaline conditions and thus, help minimize contamination from neutrophilic microorganisms that might end up producing extracellular cellulases that can potentially attack the cellulosic structures of the cloth and thus, lead to the production of poor quality fabrics with increased pollution loads in the effluents generated.

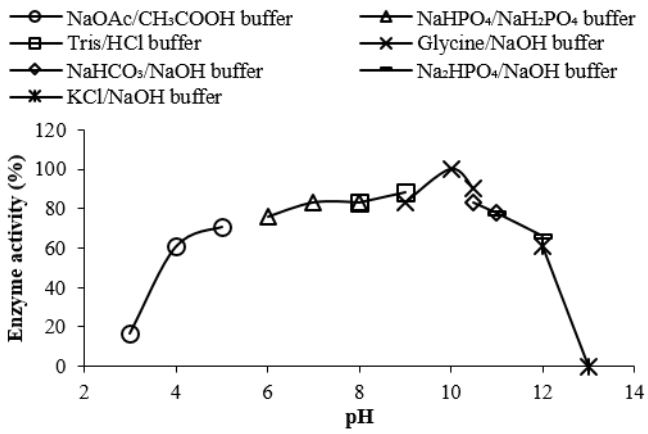


Figure 4.4: Effect of pH on the activity of Amy LBW 5117 at 60 ºC. The enzyme activity corresponding to 100 % was 0.41 U/ml at pH 10.0.

4.3.1.4 Optimum temperature of enzyme activity

Temperature is yet another important limiting factor that affects the activity of enzymes. The activity of Amy LBW 5117 was thus, investigated at different temperatures in 50 mM glycine-NaOH buffer (pH 10). As shown in Figure 4.5, the activity of the enzyme increased rapidly with increase in temperature, reaching optimum at 60 ºC, and then declining rapidly thereafter to reach 0 at 90 ºC. The enzyme exhibited optimum temperature at 60 ºC, with over 80 % of the activity falling between 50 and 63 ºC (operational range). The optimum temperature of activity of the enzyme falls within those listed as low-temperature commercial desizing amylases e.g., COENZYME® LTAA3P and NOVOZYME AQUAZYME® ULTRA 1200 N (Table 4.1). Amy LBW 5117 can therefore, also be considered a low-temperature amylase. This comes with numerous advantages e.g., low energy costs and savings on the purchase of specialized equipment that is resistant to heat (Yahya *et al*., 2021).

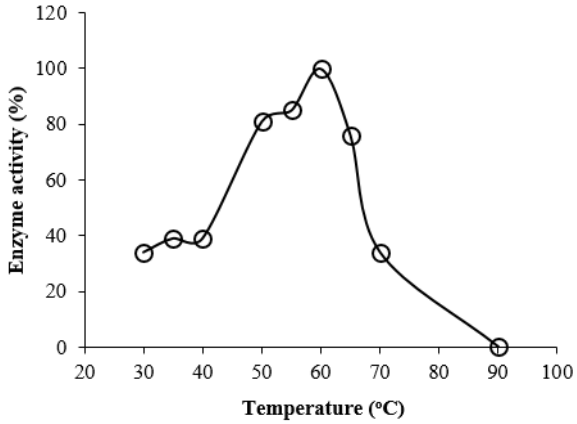


Figure 4.5: Effect of temperature on the activity of Amy LBW 5117 at pH 10.0. The enzyme activity corresponding to 100 % at optimum pH was 0.41 U/ml.

4.3.1.5 Effect of metal ions on the activity of the enzyme

Metal ions play an important role in the functioning of enzymes, with the most notable ones being the alteration of their structures to improve their stability and/or by taking part in their catalytic functions (Sudha, 2012). The type and concentration of the metal ion must however be selected carefully or else the enzyme can become inactivated. In this regard, the effect of different concentrations of metal ions on the activity of Amy

LBW 5117 was studied. The activity of the enzyme was stimulated; a) either stimulated or was insignificantly affected in the presence of up to 10 mM concentrations of most metal ions tested, and b) insignificantly affected by Cu^{2+} and partially inhibited by Mn^{2+} at low concentrations (Table 4.2). This shows that Amy LBW 5117 requires some metal ions, particularly Ca^{2+} at 1 mM. Ca^{2+} ions play the important roles of: a) linking starch to the active site of amylases to help maintain the enzyme's catalytic activity b) interacting with the negatively charged amino acid residues (glutamic- and aspartic- acid) and resulting in the stabilization and maintenance of the enzyme conformation and c) salting out the hydrophobic amino acid residues to force the enzyme to acquire a compact structure that can resist extreme pH and temperature (Sudha, 2012).

On the other hand, the complete inhibitory effect on the activity of the enzyme by ≥ 5 mM Cu^{2+} is ascribed to the fact that this metal ion binds to His- amino acid residues at the active site of enzymes, and this perturbs the proton shuttling effect in which the amino acid participates during catalysis (Chang *et al*., 2003). The inhibitory effect exerted by ≥ 1 Mn^{2+} could be due to its ability to outcompete or displace the more essential metal ions that bind to the enzyme's metal binding sites thus, leading to a modification of the enzymatic activity (Lévêque *et al*., 2000). Overall, these results show that the activity of Amy LBW 5117 can either be stimulated or may not be significantly affected by the metal ions present in cotton fibers and tap water, both of which the textile industry heavily depends on for desizing woven cotton (Brushwood & Perkins, 1994), [Chrome-extension://efaidnbmnnnibpcajpcglclefindmkaj/https://wasreb.go.ke/downloads/Water_Quality_&_Effluent_Monitoring_Guidelines.pdf, 2008 (cited date 16 Feb 2023]. This implies that if the enzyme is to be used for such an application, a high turnover rate of desized fabrics can be realized, which means more profits while savings are also made on what would have been used to purchase metal ion chelators. No information is available on the effect of metal ions on commercially available desizing amylases

[https://biosolutions.novozymes.com/en/textiles/products/desizing/aquazymmylases (cited date 16 Feb 2023], [https://biosolutions.novozymes.com/en/textiles/products/desizing/aquazym (cited date 16 Feb 2023)].

Table 4.2 Effect of metal ions on the activity of Amy LBW 5117. The activity of the enzyme is expressed as a percentage of the untreated enzyme (enzyme activity corresponding to 100 % activity was 0.41 U/ml).

Metal ion	Enzyme activity (%)				
	0.5 mM	1.0 mM	5 mM	10 mM	180 mM
None	100	100	100	100	100
K^+	ND	146	153	147	78
Ca^{2+}	ND	200	176	106	ND
Mg^{2+}	ND	131	145	96	ND
Fe^{3+}	125	159	162	102	ND
Cu^{2+}	99	96	0	0	ND
Na^+	ND	101	153	116	ND
Mn^{2+}	74	0	0	0	ND
Zn^{2+}	103	96	95	96	ND

ND - Not Determined

4.3.1.6 Effect of surfactants on the activity of the enzyme

The penetration of enzymes into untreated fabric is often difficult and slow during desizing (Agrawal, 2016). Therefore, surfactants are added into desizing baths in order to accelerate the wetting of the fabric and penetration of the enzyme. The type and concentration of the surfactant must however be selected carefully otherwise the enzyme can become inactivated (Agrawal, 2016; Sethi *et al.*, 2016; Yahya *et al.*, 2021). In this context, the effect of different Tween 20 and Triton X-100 and SDS surfactants on the activity of Amy LBW 5117 was investigated under optimized pH and temperature.

The activity of the enzyme increased to 115 and 104 % in the presence of 0.05 mM Tween 20 and 0.1 mM Triton X - 100, respectively, and declined gradually thereafter to just below the 95 % activity mark at 2 mM concentration of both surfactants (Figure 4.6). Both surfactants are classified as nonionic or mild detergents and do not, therefore, interact strongly with enzyme 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). Instead, they lower the surface tension of the aqueous solution thus, facilitating wetting of the fabric surface. This results in improved penetration of the water solution and enzyme within the starch structure thus, speeding up the reactions (Wasinger, 1995; Agrawal, 2016). However, when used at concentrations that are higher than that of their respective CMC values, they form micelles, which interact with enzyme surfaces to form colloidal detergent-protein suspensions, which lower the catalytic function of enzymes by promoting: a) conformational changes in their active sites, b) partial unfolding of enzyme or c) disruption of binding of substrate (Agrawal, 2016; Sethi *et al.*, 2016; Yahya *et al*., 2021).

On the other hand, increasing the concentration of SDS resulted in a decrease in the activity of the enzyme from the onset, until 0 % at 1 mM SDS (Figure 4). SDS is an anionic surfactant, and may have therefore interacted strongly with the enzyme to induce a conformational change in its active site in a concentration-dependent manner that resulted in its inactivation. Similar results were obtained by other researchers (Ikemiya, 1957; Hou *et al*., 2020). The overall result shows that Tween 20, at a concentration of 0.05 mM, was the most compatible wetting agent for use with Amy LBW 5117.

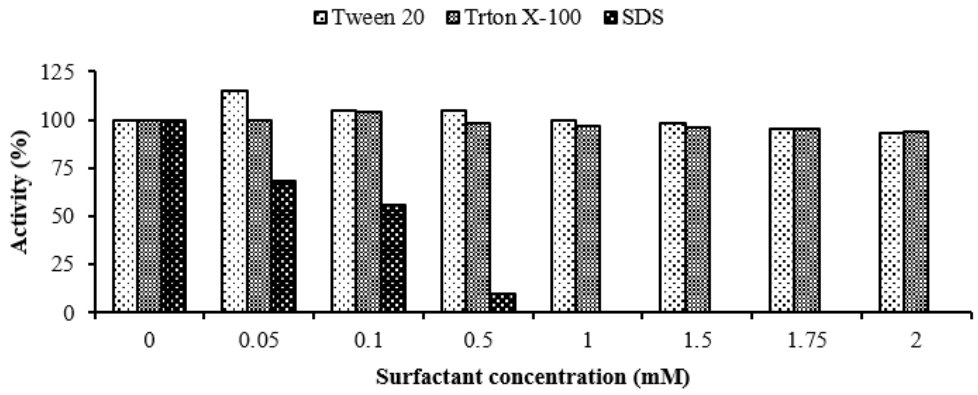


Figure 4.6: Effect of different concentrations of various surfactants on the activity of Amy LBW 5117 at pH 10.0 and 60 ºC. Enzyme activity corresponding to 100 % was 0.41 U/ml in the absence of the additives.

4.3.1.7 Thermostability of the enzyme

In addition to exerting a stimulatory effect on the activity of amylases, Ca^{2+} ions and Tween 20 have been reported to be good thermostabilizers of enzymes (Deb *et al*., 2013; Komori *et al*., 1993). For this reason, the stability of Amy LBW 5117 was studied with and without these additives at optimized temperature and pH. The enzyme exhibited initial activities of 110, 115 and 190 % in the presence of 1.0 mM Ca^{2+}, 0.05 mM Tween 20 and both additives together, respectively, and retained 71, 76 and 96 % of these activities 3 h later (Figure 4.7). Furthermore, it retained 70 % of its original activity in the presence of both additives upon extending the incubation period to 20 h (data not shown). On the contrary, the control enzyme (no additive added) exhibited an initial activity of 100 %, but lost all the activity after 3 h (Figure 5). This result shows that the enzyme is most stable in the presence of both Ca^{2+} and Tween 20, and can therefore be used to hydrolyze starch repeatedly for a as long as it is active. This can help reduce the frequency of its purchase (more savings).

Calcium (Ca^{2+}) ions contribute to the stability of amylases by: a) interacting with the negatively charged amino acid residues (e.g., glutamic- and aspartic- acid) and result in the maintenance of the enzyme conformation and b) salting out the hydrophobic amino acid residues to force the enzyme to acquire a compact structure that can resist extreme temperature (Sudha, 2012). On the other hand, Tween 20 may have contributed to the stability of the enzyme by preventing it from dissociating into its respective monomers or inhibiting the removal of essential cofactors (or prosthetic groups) from its active sites (Komori *et al*., 1993).

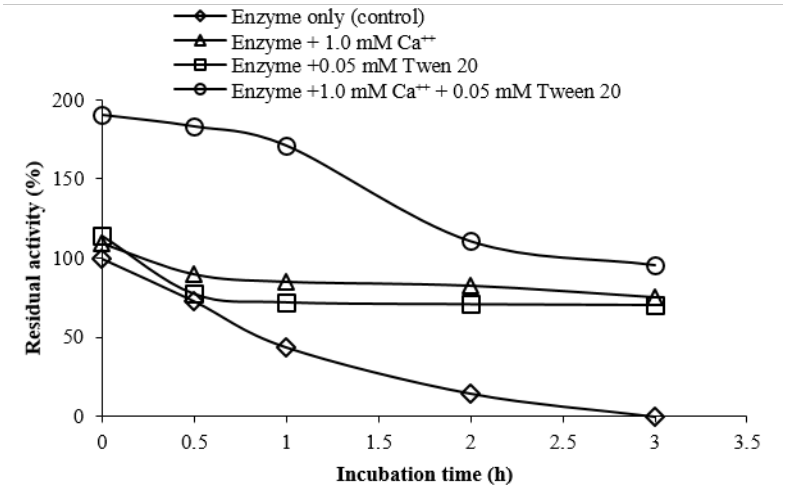


Figure 4.7: Effect of 1.0 mM Ca2+ and 0.05 mM Tween 20 on the stability of Amy LBW 5117 at 60 ºC and pH 10.0. Enzyme activity corresponding to 100 % was 0.41 U/ml [Enzyme only (control)].

4.3.1.8 Substrate specificity

For the semi-quantitative assays, Amy LBW 5117 hydrolyzed all the starch products as depicted by the formation of halos around the depressions on the starch plates [Fig 4.8A (i)]. The largest halo (SI = 6.4) was observed on the plate containing tapioca starch, and this was therefore taken to represent 100 % activity. Using this as a reference point, the activities of the enzyme were observed to decrease in the order: Tapioca > Irish potato > wheat > rice > sweet potato > corn [Figure 4.8A (ii)]. This variation is attributed to the difference in the chemical compositions of the various substrates (e.g., moisture, ash, lipid, protein, amylose and amylopectin contents), which in turn affects the accessibility of the enzyme and its subsequent conversion to the product once bound (Adeleye *et al*., 2014; Soto, 2012). On the other hand, no halos were observed on the cellulosic plates [Figs 4.8A (i)] to indicate that none of the cellulosic enzymes - endoglucanase (CMC plate), exoglucanase (avicel plate) and β-glucosidase (cellobiose plate) were present in the crude enzymatic preparation [Fig 4.8A (ii)].

To confirm these results, the more sensitive liquid assay was performed using the crude enzymatic preparation on the same substrates (quantitative assay). Like the results that

were obtained with the semi-quantitative assay method, the enzyme hydrolyzed all the starch products tested (Fig 4.8B). However, the enzymatic titers, which were in the order: Irish potato > sweet potato > tapioca > rice > wheat/corn, did not correlate with those obtained using the semi-quantitative assay method. Differences in amylolytic activities expressed by the same microorganism in solid and liquid media have also been reported by Castro *et al*. (1993). In addition, the enzyme did not hydrolyze the cellulosic products in the liquid assay thus, confirming that the crude enzymatic preparation was free from the cellulosic enzymes (Figure 4.8B).

This result confirms that Amy LBW 5117 has the potential to be used as a textile desizing agent, where the degradation and elimination of different types of starch-based sizing agents, from woven cotton, is required without damaging the cellulosic structures of the cloth. This can yield high quality desized fabrics that exhibit a softer- and smoother- feel, improved flexibility, and high TEGEWA rating with a corresponding low residual starch content (Saha *et al*., 2018). Commercial desizing amylases exhibit a similar property [http://www.sunsonenzyme.com/Products/Textile/Desizing/ (cited date 16 Feb 2023)], [https://biosolutions.novozymes.com/en/textiles/products/desizing/aquazym (cited date 16 Feb 2023)].

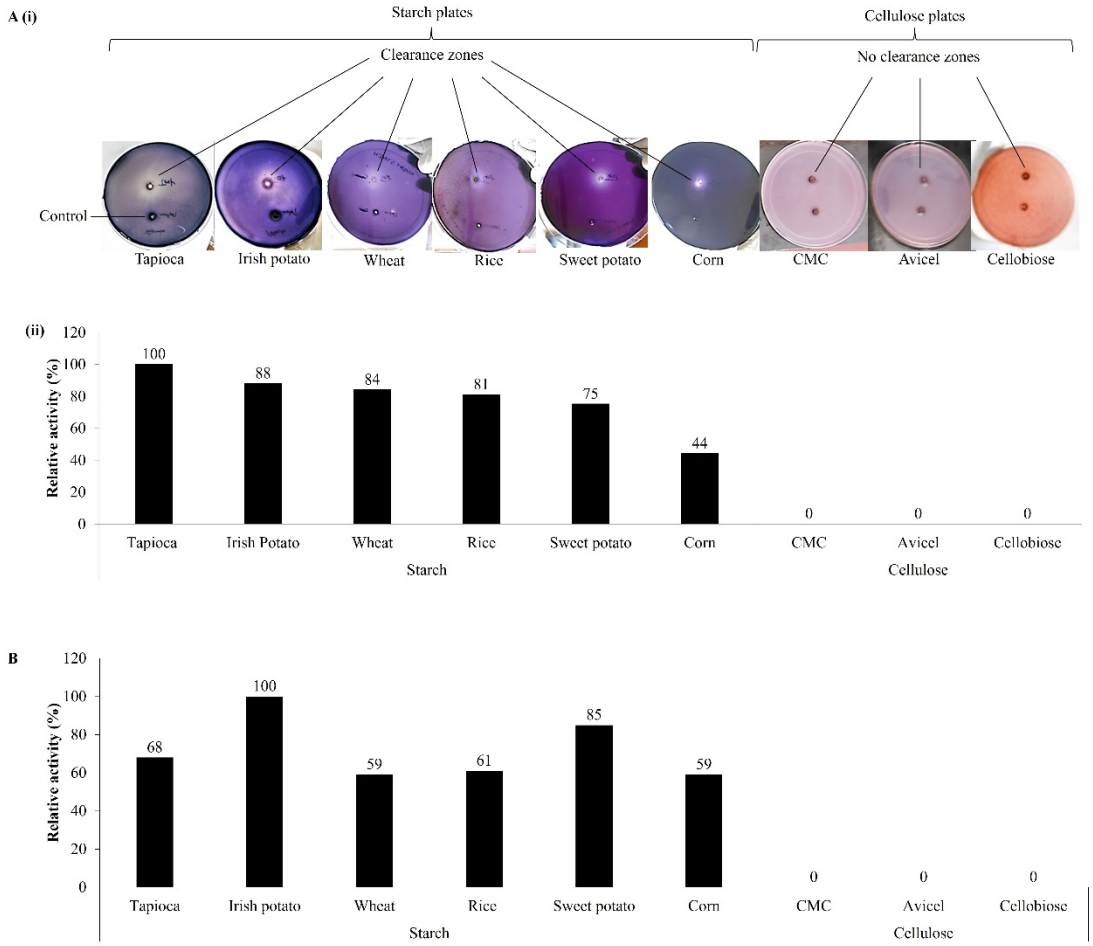


Figure 4.8: Effect of different starch- and cellulosic- products on the activity of Amy LBW 5117 in the presence of 1.0 mM Ca2+ and 0.05 mM Tween 20 at pH 10,0 and 60 ºC. The activities were determined: a) semi-quantitatively, and are seen as halos around the depressions on solid starch plates (i), and are expressed as a percentage of that of tapioca starch (activity corresponding to 100 % was 6.4 SI) (ii), and b) quantitatively, and are expresses as a percentage of that of Irish potato (activity corresponding to 100 % was 0.41 U/ml). No halo formation around the depressions on the cellulosic plates suggests the absence of cellulases in the crude enzymatic preparation.

4.3.1.9 Enzyme's mode of hydrolysis

An investigation into the products formed by the action of Amy LBW 5117 on the hydrolysis of starch is shown in Figure 4.9. A mixture of low and high α-maltooligosaccharides and α-dextrins was formed after 2 h of reaction time, the yields of which increased and decreased, respectively with time. After 24 h, another set of degradation products i.e., moderate α-maltooligosaccharides and moderate α-dextrins

were formed. The main final product in all the cases was maltose. This profile is typical of endo-α-1-4 amylase, which is a group of starch-degrading enzymes that randomly cleave multiple internal α- (1,4) glycosidic linkages in starch, thereby disintegrating it to water-soluble linear α-maltooligosaccharides of varying lengths, α- dextrins and a small amount of glucose (Savaner & Sohani, 2020). Based on this fact, Amy LBW 5117 is an endo-α-1-4 amylase (endoamylase or α-amylase).

Commercially available desizing enzymes are also endo-α-1-4 amylases. They are used to degrade starch sizes present on the warp yarns of woven cotton into varying lengths of water-soluble α-maltooligosaccharides and α-dextrins, which can then easily be removed from the fabric through washes (desizing) [http://www.sunsonenzyme.com/Products/Textile/Desizing/ (cited date 16 Feb 2023)], [https://biosolutions.novozymes.com/en/textiles/products/desizing/aquazym (cited date 16 Feb 2023].

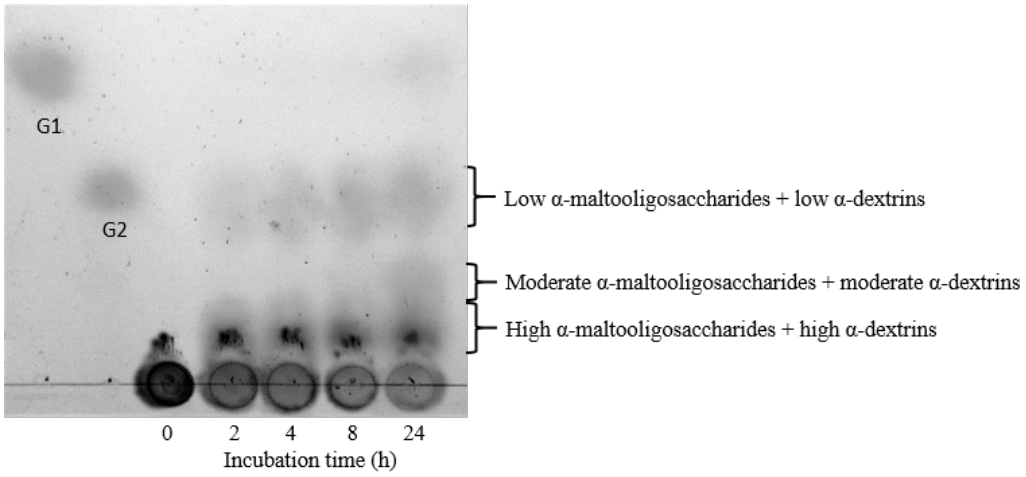


Figure 4.9: TLC analysis of starch hydrolytic products generated by Amy LBW 5117 over a 24 h incubation period. G1 and G2 are the standards of glucose and maltose, respectively.

4.3.2 Assessment of the Amy LBW 5117 desized fabric

The extent to which the starch sizing material was removed from the fabric by Amy LBW 5117 was assessed by determining its properties e.g., a) surface-handle or 'feel' b) weight loss (%) c) stainability with iodine dye solution d) TEGEWA rating and e)

residual starch content (%), and then comparing the results with those from the control fabric (treated with denatured enzyme).

4.3.2.1 Surface properties and weight loss (%)

As seen in Table 4.3, the fabric acquired new surface-handle or 'feel' characteristics to indicate absence or near absence of starch compared to the control, which exhibited the characteristics of a starched fabric (Grinevičiūtė & Gutauskas, 2004). Furthermore, it incurred a weight loss of 9.5 % compared to that of the control, which lost only 1.3 %. Assuming that the weight loss incurred by the latter was due to the loss of small particles as well as the removal of fringe yarns from its surface and edges, respectively, then the same can be argued for the former. This means that the fabric that was enzymatically treated incurred an additional weight loss of 8.2 %. This relatively high weight loss was attributed to the removal of a significant amount of the starch from the fabric, which went on to acquire the new surface handle properties observed earlier on. Although no reports are available on weight loss measurements incurred by enzymatically desized fabrics at the commercial level, a similar weight loss was reported elsewhere at the lab-scale level (Halim & Zhou, 2018).

Table 4.3 Comparison of some physical properties of the Amy LBW 5117 desized fabric with that of the control (undesized) fabric.

Property	**Fabric treated with:**	
	Active enzyme	**Denatured enzyme (control)**
Surface handling 'feel'	Softer, smoother, and more flexible	Firm, rough, and stiff
Weight loss (%)	9.5 (8.2)	1.3

4.3.2.2 Iodine-stain, TEGEWA rating and residual starch content (%)

That the enzyme was responsible for the removal of starch from the fabric was confirmed by performing the iodine-stain test on it. As seen in Figure 4.10a, the desized fabric presented with a light brown color to signify the absence/near absence of starch on it (desized) while the control fabric presented with a black color to indicate

the presence of starch (undesized) [Figure 4.10b)]. This result confirms that Amy LBW 5117 was responsible for the removal of the starch from the fabric by degrading it into soluble low molecular weight α-anomeric maltooligosaccharides and dextrins, which were subsequently eliminated from the fabric through several washes. The colors of the two fabrics were then compared with those on the TEGEWA violet scale (Figure 2.11). The color of the desized fabric matched with one that exhibited a high TEGEWA rating of 7 - 8 on the scale thus, confirming that most of the starch was removed from it and it now had a residual starch estimate of only 0.0725 %. On the other hand, the color of the control fabric matched with one that exhibited a low rating of 1 on the TEGEWA scale, implying that most of the starch was still intact on the fabric (residual starch > 1.0 %).

The recommended commercially acceptable amount of residual starch on desized fabrics is 0.125 – 0.085 % (rating of 6 - 7) (Harane & Adivarekar, 2017). This is important because improperly desized fabrics can develop different shades of color when subjected to subsequent processing steps e.g., dyeing (Agrawal, 2016). This result shows that Amy LBW 5117 can degrade and eliminate a significant amount of starch-sizing material from woven cotton. Therefore, it has the potential to be a good fabric-desizing agent.

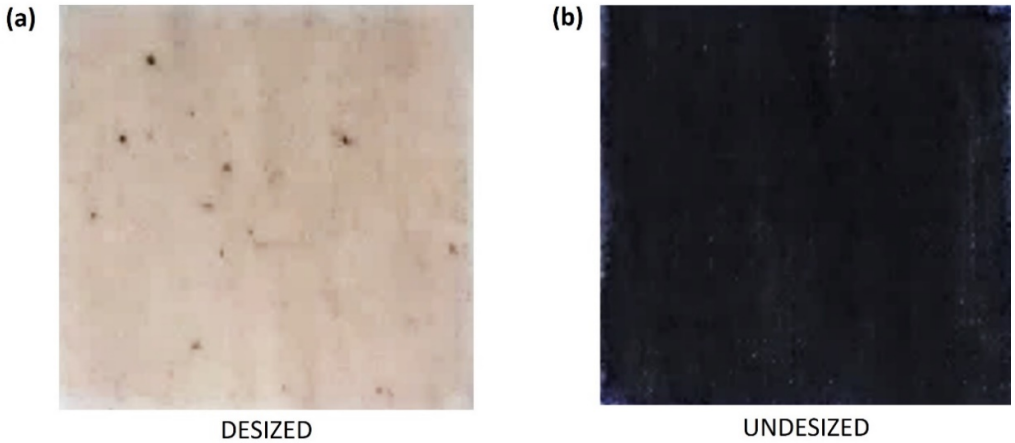


Figure 4.10: Photograph of the iodine - stained fabrics; (a) enzymatically treated and (b) control.

4.4 Conclusions and Recommendations

The present findings show that Amy LBW 5117 is a cellulase-free surfactant compatible metallo endo-α-1-4-amylase that can hydrolyze different types of starch products under alkaline [pH 8.0 - 10.5 (optimum pH 10)] and relatively low temperature [50 - 63 ^{o}C (optimum 60 ^{o}C)] conditions. These properties make it suitable for an application that involves the desizing of woven cotton at low operational costs (low consumption of energy, no need to purchase heat resistant equipment or metal ion chelators) without damaging the fabric by both enzyme and cellulase-producing neutrophilic contaminants. Moreover, the the enzyme exhibited good thermal stability property during use (albeit in the presence of thermostabilizers) and storage (4, 20 and 30 ^{o}C), implying that it can be produced in bulk, stored appropriately and used within 6 weeks in such an application. This can help reduce the frequency of its purchase,which is a boost to savings. A preliminary investigation into the biotechnological application of the enzyme, under its optimum operating conditions, revealed that it could effectively desize woven cotton, and in the process, yield a fabric with a commercially acceptable amount of residual starch (0.06 - 0.085 %) (TEGEWA rating 7 - 8). This shows that Amy LBW 5117 is potentially a good desizing agent.

It is recommended that an Amy LBW 5117 desized fabric be subjected to the next pretreatment step using the pectinase (bio-scouring) from alkaliphilic *Bacillus halodurans* LBW 5117 (**chapter one)** under the enzyme's optimum operating conditions. This work is described in the next chapter **(chapter five).**

CHAPTER FIVE

5.0 CHARACTERIZATION OF AN ALKALINE ENDO-POLYGALACTURONASE FROM ALKALIPHILIC *BACILLUS HALODURANS* LBW 5117 AND DEMONSTRATION OF ITS BIOSCOURING POTENTIAL

The work described in this chapter was accepted in the *Journal of Microbiology, Molecular Biotechnology and Food Sciences* (https://doi.org/10.55251/jmbfs.9945) and appears as **paper III** in the 'list of papers' in this thesis (page xxi).

5.1 Introduction

Pectic substances are complex, heterogeneous and acidic glycosidic polysaccharides that are predominantly found in the middle lamella and primary cell wall in plants (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 high c) 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 & Abate, (2017) classified pectinases into three major groups, depending on their mode of action: a) protopectinases (no EC number assigned), which degrade insoluble protopectin aggregates, resulting in the formation of highly polymerized soluble pectins, b) esterases (also called pectinesterase (PE) or pectin methylesterase (PME) (EC 3.1.1.11), which catalyzes the de-esterification of pectin by removing the methoxyl esters in the presence of water thereby,

converting it into pectic acid (polygalacturonic acid) and methanol and c) depolymerases, which act on pectic substances by two different mechanisms i.e., hydrolysis, where they catalyze the cleavage of α-(1,4) glycosidic bonds linking the galacturonic acid residues with the introduction of water across the oxygen bridge and trans-elimination lysis, where they break the glycosidic bonds via trans-elimination reaction without any participation of water molecule.

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. These are:-

(i) Polygalacturonases (PGase) and polymethylgalacturonase (PMGase), which depolymerize polygalacturonic acid and pectin chains, respectively, by catalyzing the hydrolytic cleavage of the α-(1,4) glycoside bonds in their respective chains and include endo-PG (EC 3.2.1.15) and endo-PMGase (EC number not defined). They randomly cleave the internal α-(1,4) glycoside bonds in their respective galacturonan chains to liberate oligo- and methyl oligo- galacturonates, respectively. Exo- PGase I (EC 3.2.1.67), exo-PGase II (EC 3.2.1.82) and exo-PMGase (EC number not defined), cleave the glycosidic bonds at the non-reducing ends of their respective galacturonan chains to liberate mono-, di- and methyl mono- galacturonates respectively.

(ii) Pectate lyase (PecL) and pectin lyases PNL, which depolymerize polygalacturonic acid and pectin chains, respectively, but via β-elimination of the α-(1,4) glycoside bonds in their respective chains, and include endo-pectate lyase (endo-PecL) and endo PNL, which randomly cleave the internal α-(1,4) linkages of their respective chains to liberate 4,5 unsaturated oligo- and 4,5 unsaturated methyl oligo- galacturonates, respectively, and exo-PecL and exo-PNL, which cleave the linkages at the non-reducing end of their respective chains to liberate

4,5 unsaturated mono- and 4,5 unsaturated methyl mono- galacturonates respectively.

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, particularly 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 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).

Furthermore, these enzymes are biodegradable and do not require the use of harsh chemicals hence, are both fabric- and eco-friendly. However, most commercial bioscouring alkaline endo-PecLs are expensive because they have to be imported from a) Novozym**es** A/S, Krogshoejvej 36, 2880 Bagsvaerd, Denmark, and (https://www.novozymes.com/en/solutions/leather-textile/textile-enzymes/biopreparation), and b) Sunson Industry Group., Ltd, Suite 2302, Zhong'an Shengye Building Chaoyang District, Beijing, China

(https://www.sunsonzymes.com/products/Textile/Bio_scouring), etc, using foreign reserves. The situation is worse in a developing country like Kenya, where chemical methods of scouring are practiced at high temperatures (95 ^{o}C) thus, leading to the production of damaged fabrics at a high cost while generating high pollution loads (personal communication from Hose Too of Rivatex (EA) Limited). Therefore, there is a need to replace the chemical scouring method used in the Kenyan textile industry with a more cost-effective fabric- and eco-friendly bioscouring process that utilizes locally produced alkaline pectinases. However, for such enzymes to be effective, they must perform optimally with respect to pH, temperature, metal ions, and 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 overall objective of the present study was to determine the physicochemical properties crude PGase LBW 5117 from a locally isolated alkaliphilic bacterial strain (*Bacillus halodurans* LBW 5117), and to demonstrate its potential to scour industrially woven cotton under the established operating conditions.

5.2 Materials and Methods

5.2.1 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 (e.g., DE ≤ 25, 20 - 34, 50 - 75 and ≥ 85 %), peptone, yeast extract, potassium sodium tartrate tetrahydrate, Tween 20, 3,5-Dinitrosalicylic acid, carboxy methyl cellulose, avicel and cellobiose were purchased from Sigma-Aldrich (St.

Louis, MO, USA). Rivatex (EA) Ltd (Eldoret, Kenya), donated the 100 % industrially woven plain grey cotton (contained corn-starch sizing material) used in the study.

5.2.2 Bacterial isolate and cell culture for polygalacturonase (PGase LBW 5117) production

A stock of alkaliphilic *Bacillus halodurans* LBW 5117 in 40 % (v/v) glycerol was retrieved from -20 ºC freezer (Figure 5.1a) and used to inoculate pectin 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 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 (Figure 5.1b) 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 (Figure 5.1c) . The seed-culture was used to inoculate 80 ml main broth culture medium (prepared as described above) and the bacterium grown for 48 h using the same culture conditions (Figure 5.1d). The cell culture was centrifuged at 5,000 g for 30 min at 4 ºC to obtain the cell-free culture supernatant, which was treated as the crude enzyme (Figure 5.1e). The appearance and odor of the enzymatic solution was noted, after which it was stored at -20 ºC until use.

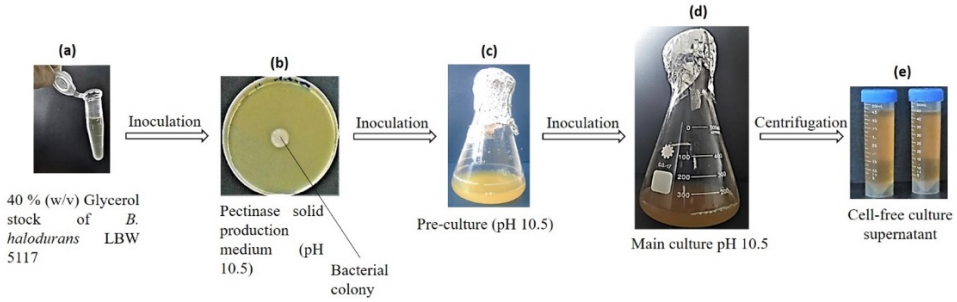


Figure 5.1: Schematic representation of the procedure used to culture *Bacillus halodurans* LBW 5117 for the production of PGase LBW 5117.

5.2.3 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) in 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 ^{o}C for 30 min). The assay plate was incubated at 50 ^{o}C 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 20 ^{o}C. 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 $\leq$ 25 %) prepared in 50 mM glycine-NaOH buffer, pH 10.5. The reaction mixture was incubated at 50 ^{o}C 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 ^{o}C 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 ^{o}C, 30 min) was used instead of the active one.

5.2.4 Characterization of PGase LBW 5117 activity

The crude enzyme was subjected to both physicochemical characterization in order to determine its optimum storage and operating conditions. These was done as described below:

5.2.4.1 Effect of storage temperature on the stability of the enzyme

One (1 ml) of the enzyme was stored in a freezer (-20 ^{o}C), refrigerator (4^{o} C) and in two separate incubators at 20 ^{o}C and 30 ^{o}C, respectively. The samples were retrieved after one year and the residual activities of the enzyme determination under the standard assay conditions.

5.2.4.2 Effect of pH on the activity of the enzyme

This was studied by carrying out the assays using 0.5 % (w/v) polygalacturonic acid sodium salt (substrate) prepared in different 50 mM buffers (pH 3.0 - 13.0). The buffers used in the study were prepared according to Gomori, (1955) and the website http://delloyd.50megs.com/moreinfo/buffers2.html (cited date 12 Dec 2022). 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).

5.2.4.3 Effect of temperature on the activity of the enzyme

The effect of temperature on the activity of PGase LBW 5117 was investigated by performing the assays at 30, 35, 40, 45, 50, 55, 60, 65, 70, 80 and 90 ^{o}C using glycine/sodium hydroxide buffer, pH 10.5.

5.2.4.4 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^{o}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 Ca^{2+} ions in a similar fashion.

5.2.4.5 Effect of surfactants on the activity of the enzyme

The enzyme PGase LBW 5117 was incubated in the presence of Tween 20 (final concentrations of; 0, 0.025, 0.05, 0.1 and 0.15 mM, respectively) at 29 ± 3 ^{o}C. 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 concentrations of; 0, 0.1, 0.2, 0.25 and 0.3 mM, respectively, and b) sodium dodecyl sulphate (SDS) final concentration of; 0, 1, 1.5, 2 and 2.5 mM, respectively.

5.2.4.6 Effect of optimum temperature on the stability of the enzyme

The enzyme PGase LBW 5117 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 ^{o}C, for 8 h. Samples were withdrawn after 0.5, 1, 2, 3, 4, 6 and 8 h for determination of residual activity of the enzyme under the standard assay conditions.

5.2.4.7 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 Heidarizadeh *et al*., (2018) was used. Ten (10) µl enzyme containing $CaCl_2$ and Tween 20 (final concentrations = 1.5 and 0.5 mM, respectively) was aliquoted in small depressions (diameter = 0.5 cm) on the surfaces of agar media prepared using 0.5 % (w/v): low degree of esterified pectin [DE < 25, DE 20 - 34 %)] (polygalacturonic acid), medium degree of esterified pectin (DE 50 - 75 %) and high degree of esterified pectin (DE ≥ 85 %), respectively. Similarly, the enzyme was inoculated in another set of depressions on solid media containing 1 % (w/v) CMC sodium salt, 1 % (w/v) avicel and 1 % (w/v) cellobiose, respectively. All the depressions were made using a sterile cork borer. The plates were incubated at 50 ^{o}C 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 PGase 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 ^{o}C 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). Five hundred (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 ^{o}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 enzyme (95 ^{o} C, 30 min) were used as controls.

5.2.4.8 Mode of action of the enzyme

A modified version of the method described by Anand *et al*., (2016) was used to determine whether the enzyme hydrolyzes polygalacturonic acid in an exo- or 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 ^{o}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 1.0, 2.0, 4.0, 8.0 and 18 h, heated at 95 ^{o}C for 5 min (to denature the enzyme), centrifuged (2,600 g, 5 min) and the supernatants stored at 4 ^{o}C until use. Five (5 µl) samples were spotted on a precoated Kieselgel 60 F254 silica gel aluminium sheet. A similar volume of 0.1 % (w/v) monogalacturonic acid and polygalacturonic acid sodium salt were also spotted, as standards. 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 ^{o}C until spots appeared. The sheet was then photographed.

5.2.5 Bioscouring of woven cotton using PGase LBW 5117

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 ^{o}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*. (accepted in the *Journal of Applied Biology and Biotechnology*) followed by washing, drying and trimming it along its edges to obtain a fringe-free desized woven cotton, which was then weighed.

The desized fabric was subjected to bioscouring in a batch process (Rajendran *et al*., 2011). The fabric was initially wetted to approximately 100 % using bioscouring liquor [0.05 mM Tween 20, 1.5 mM Ca^{2+} and 40.5 U of enzyme (pH 10.5)] at room temperature (29 ± 30 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 in a similar fashion, but with heat-inactivated PGase LBW 5117 (95 ºC for 30 min) instead of the active one. Finally, the fabric was re-weighed.

The efficiency of the process or preparedness of the bioscoured fabric towards absorption of water and dye in subsequent stages of processing was determined and the result compared with that from the control fabric. This assessment was carried out as described below:

5.2.5.1 Weight loss (%) determination

The weight loss (%) that the fabric incurred after the treatment was calculated using equation stated below:

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

5.2.5.2 Absorbency test

The fabric was cut into two equal parts measuring 2.5 cm x 5 cm. One of the fabric samples was subjected to the drop penetration test and the other to the capillary rise test as described below:

5.2.5.3 Drop penetration test

Water absorbency was evaluated using AATCC technical manual 39 - 1980 edition (wettability, evaluation of) (Tzanov *et al*., 2001). The fabric was placed over the top of a beaker so that the centre is 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 is the process. This procedure was repeated at a different spot 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 bioscoured 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 on different spot on the surface of the fabric by dropping it from a height of 1 cm, and the size and shape of the absorbed area was determined and compared with that from 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 did not take place.

5.2.5.4 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 alternative fabric was hung (using a 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 from the control fabric.

5.2.6 Data presentation and analysis

The data for characterization of the enzyme with respect to 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 bioscoured cotton fabric is presented in both tabular and pictorial forms, and were conducted once.

5.3 Results and Discussion

5.3.1 Physicochemical properties of PGase LBW 5117

The enzyme was characterized in order to determine its physicochemical properties, including appearance, and optimum operational and storage conditions. This was carried out as described below;

5.3.1.1 Appearance, detection, identification and catalytic activity

The crude PGase LBW 5117 was was a brown liquid that exhibited a slight fermentation odor. These are some of the typical physical characteristics of crude enzymatic preparations that are used for bioscouring woven cotton commercially [https://biosolutions.novozymes.com/en/leather-textiles/textiles/biopreparation (cited date 04 July 2023)], [http://www.sunsonenzyme.com/Products/Textile/Bio_scouring

(cited date 04 July, 2023)], [https://biosolutions.novozymes.com/en/leather-textiles/textiles/biopreparation (cited date 04 July 2017)], [https://microbiosci.creative-biogene.com/enzyme-application/textile-industry-1283.html (cited date 04 July 2023)].

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 5.2, a pale hydrolyzation zone was formed around the depression on the gel where the enzyme was aliquoted (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 & Wade, 1980).

To identify the type of pectinase that was present in the crude enzymatic solution, quantitative assays were performed for both enzymes using polygalacturonic acid (pH 10.5) as the substrate. The catalytic activity of the enzyme 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 polygalacturonase (PGase LBW 5117). The catalytic activity of this enzyme is similar to that produced by *Bacillus* sp. P4.3 (0.3 U/mml) (Soares *et al*., 1999), but much lower than that of the commercial bioscouring pectinase COENZYME SPA-5 (10,000 U/ml) (http://www.sunsonenzyme.com/Products/Textile/Bio_scouring). Thus, if the enzyme is to be used for an industrial application such as that of bioscouring, its catalytic activity will have to be enhanced several-fold e.g., through optimizing the culture conditions that were used to grow the microorganism (Haile & Ayele, 2022) and concentrating it thereafter.

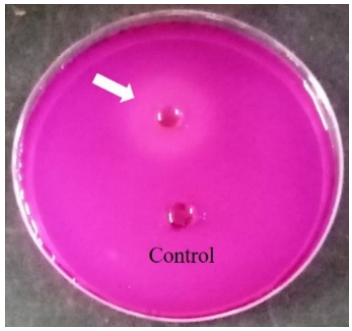


Figure 5.2: Detection of pectinase in the crude culture supernatant using the cup-plate assay method. The pale zone around the depression on the gel (indicated by arrow) depicts polygalacturonase- or pectate lyase- activity, or both.

5.3.1.2 Storage stability

The stability of the enzyme was studied by storing it frozen (-20 ºC) and in liquid forms (4, 20 and 30 ºC) for a period of one (1) year. The frozen enzyme retained 67 % of the original activity while those stored in liquid state (had a foul smell) retained over 90 % of the activity (Figure 5.3). The relatively high loss of activity exhibited by the frozen enzyme could be due to the high freezing rate that it might have encountered on 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 thus, leading to denaturation, unfolding and aggregation (Park *et al*., 2021).

On the other hand, the relatively high activity the enzyme retained after storage at higher temperatures can be attributed to the good thermostability property that alkaline enzymes are known to exhibit at high pH. This is contributed to by 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 PGase LBW 5117 can be produced, transported and stored in liquid state

at 4 - 30 °C and used within a guaranteed period of one (1) year in an application that involves the degradation of pectin. This is important because enzymes that are destined for industrial use are often produced in bulk e.g., 25 or 30 kg because emphasis is laid on catalysis rather than purity.

For this reason, they are not suitable for transportation and storage in frozen form (https://www.sunsonzymes.com/product/9/97/160/detail). This is cost-effective in terms of time- and energy consumed because freezing is avoided. It would however be desirable to concentrate and add preservatives to the enzyme following production to help reduce its volume and (storage space) and foul smell. Most of the commercially available alkaline bioscouring pectinases are also produced and stored in liquid state under similar conditions (Table 5.1).

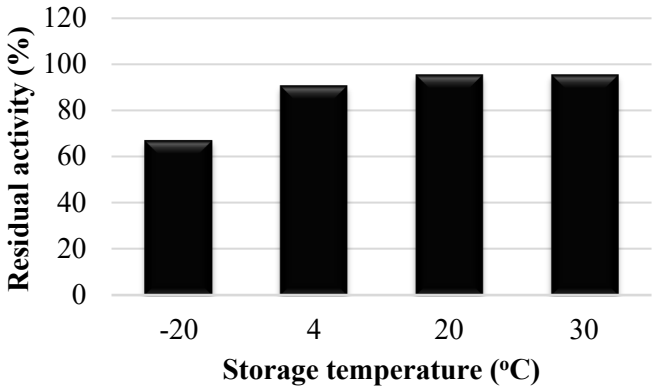


Figure 5.3: Effect of storage temperature on the activity of PGase LBW 5117 at pH 10.5. Enzyme activity corresponding to 100 % was 0.21 U/ml.

Table 5.1 Comparison of some physical properties of PGase LBW 5117 with those of commercially available bioscouring pectinases.

Enzyme	Appearance	Storage (^{o}C)	pH of activity		Temperature of activity (^{o}C)		Reference
			Operational	Optimum	Operational	Optimum	
Coenzyme® SDT-1	White powder	< 30, 3-6 months	-	-	-	80, 1h	a
Coenzyme SPA-5 (PG, PE and PL)	Brown liquid	Dry& cool place, for 9 months	7.0 - 10.0	9.8	45 – 65	58	a
BioPrep 3000L (PL)	Brown liquid	-	-	8.5	-	55	b
Scourzyme L (PL)		-	7.5 - 8.5	7.5 - 8.5	25 – 60	55	c
Alkaline pectinase (MBS-SE22)	-	5 - 35, for 9 - 12 months	-	-	-	-	d
Alkaline pectinase (MBS-1851)	-	-20	-	-	-	-	d
Alkaline pectinase (MBS-SE23)	-	5 - 35, for 6 - 9 months	-	-	-	-	d
PGase LBW 5117	Brown liquid	4 - 30	10.2 - 11.0	10.5	45 – 57	50	This study

[a]: http://www.sunsonenzyme.com/Products/Textile/Bio_scouring (cited date 15 January 2023)
[b]: https://biosolutions.novozymes.com/en/leather-textiles/textiles/biopreparation (cited date 15 January 2023)
[c]:https://www.textiletoday.com.bd/introducing-bio-scouring-a-cost-effective-and-eco-friendly-process-for-the-generation-next/ (cited date 15 January 2023)
[d]: https://microbiosci.creative-biogene.com/enzyme-application/textile-industry-1283.html (cited date 15 January 2023).

5.3.1.3 Optimum pH of enzyme activity

The pH of an enzyme is another important factor that affects its activity. The activity of PGase LBW 5117 was thus, measured using different 50 mM buffer systems at 50 ^{o}C. The activity of the enzyme remained constant at 19 % between pH 3 - 4, and increased gradually thereafter to 33 % at pH 8 (Figure 5.4). At higher pH values, the activity of the enzyme increased rapidly, reaching 100 % at pH 10.5 and then declined 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 5.1). This would mean a reduction of incidents of contamination from cellulose-producing neutrophilic microorganisms during its use. This implies production of higher quality fabrics with intact cellulosic structures and lower pollution loads in the generated effluents.

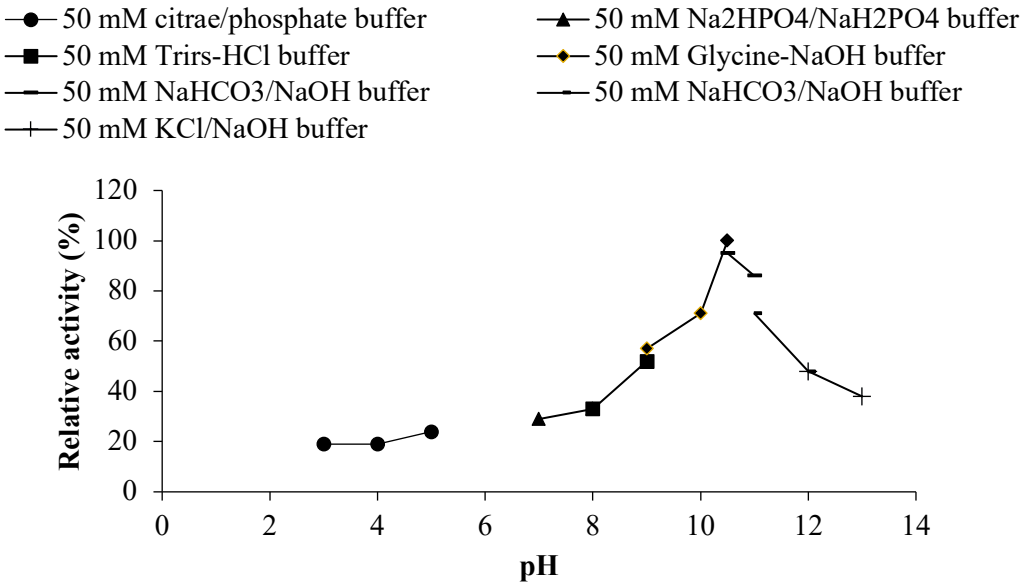


Figure 5.4: Effect of pH on the activity of PGase LBW 5117 at 50 ^{o}C. The enzyme activity corresponding to 100 % was 0.21 U/ml at pH 10.5.

5.3.1.4 Optimum temperature of enzyme activity

Temperature is yet another important factor that affects the activity of enzymes. The activity of PGase LBW5117 was, therefore, investigated at different temperatures using 50 mM glycine-NaOH buffers (pH 10.5). There was a rapid increase in the activity of the enzyme from 57 % at 30 ^{o}C to 100 % at 50 ^{o}C (Figure 5.5). This was followed by a rapid decline in the activity, reaching 11 % at 90 ^{o}C. The enzyme exhibited optimum pH at 50 ^{o}C, with over 80 % of its original activity falling within the temperature range 45 - 57 ^{o}C (operational range). The optimum temperature of the enzyme falls below those of known commercial pectins that are used for bioscouring (Table 1). PGase LBW 5117 can therefore be considered a low temperature enzyme, implying lower energy costs during its use and savings on the purchase of specialized equipment that is resistant to heat.

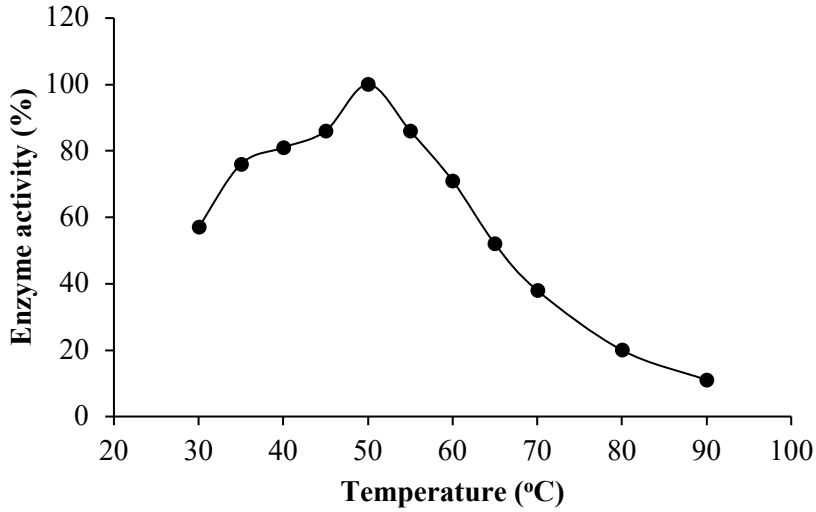


Figure 5.5: Effect of temperature on the activity of PGase LBW 5117 at pH 10.5. Enzyme activity corresponding to 100 % was 0.21 U/ml.

5.3.1.5 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. All the metal ions tested either stimulated the activity of PGase LBW 5117 at 1 mM or did not have a significant effect on it, except for Mn^{2+}, which partially inhibited the activity (Table 5.2). On increasing the concentration of the metal ions to 5 and 10 mM, only K^+ and Ca^{2+}stimulated the enzyme 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 has a requirement for 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 linking the substrate to the active site of enzyme to help stimulate its catalytic activity (Li *et al*., 2008) and also act directly on the pectin molecule by 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 raw cotton fibers (Brushwood & Perkins, 1994) and in tap water [Chrome-extension://efaidnbmnnnibpcajpcglclefindmkaj/https://wasreb.go.ke/downloads/Water_Quality_&_Effluent_Monitoring_Guidelines.pdf, 2008 (cited date 22 January 2023), both of which the industry heavily depends on for scouring woven cotton.

Table 5.2 Effect of metal ions on the activity of PGase LBW 5117. 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 5.2), 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 5.6, 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 enhances 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)

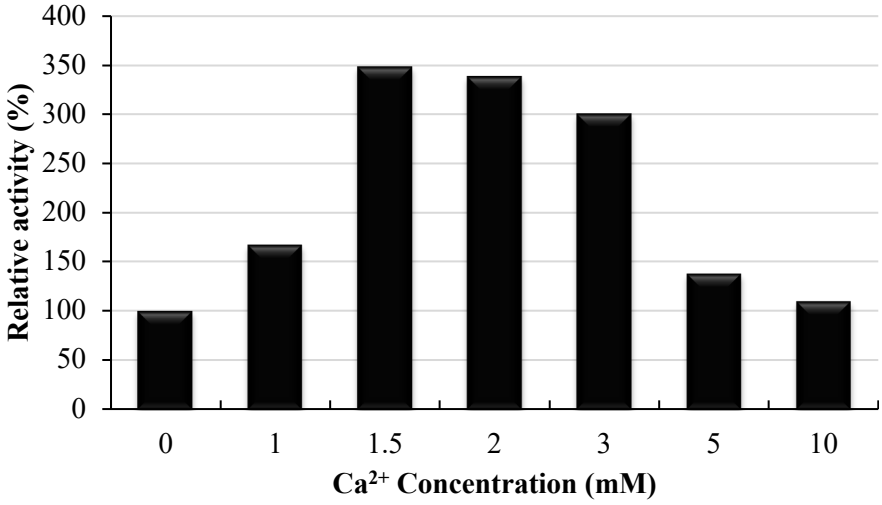


Figure 5.6: Effect of Ca^{2+} concentration on the activity of PGase LBW 5117 at pH 10.5 and 50 ^{o}C. Enzyme activity corresponding to 100 % was 0.21 U/ml (Control).

5.3.1.6 Effect of surfactants on the activity of the enzyme

The use of surfactants in industrial processes such as in bioscouring is important because they help facilitate the wetting of fabrics and thus, make them more accessible to the scouring enzymes (Rocky, 2012; Hamaki *et al*., 2019). The type and concentration of the surfactant required must however be selected carefully otherwise the enzyme can become inactivated. The activity of PGase LBW 5117 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 (Figures 5.7a and 5.7b). 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 5.7c).

Tween 20 and Triton X - 100 are non-ionic 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 (Figures 5.7a and 5.7b) (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 enzyme, partial unfolding of enzyme or disruption of substrate binding. This lowers the activity of the enzyme (Figures 5.7a and 5.7b) (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 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.

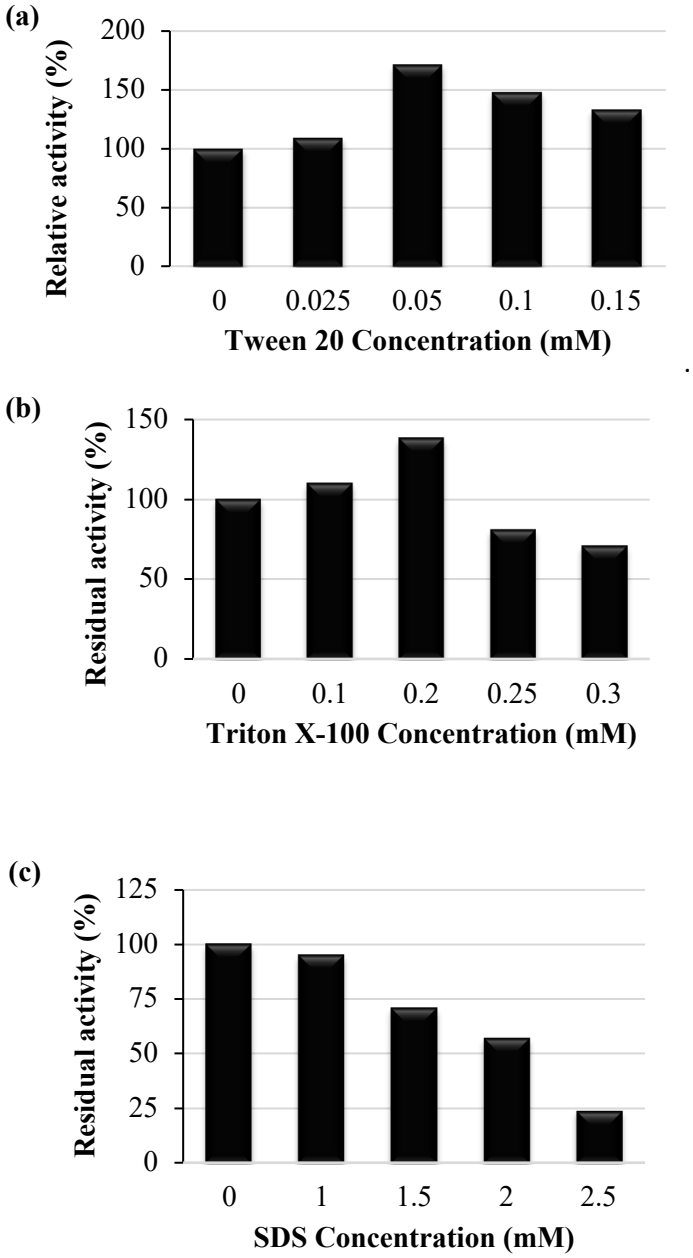


Figure 5.7: Effect of different surfactant concentrations on the activity of PGase LBW 5117 at pH 10.5 and 50 ^{o}C. Enzyme activity corresponding to 100 % was 0.21 U/ml (control).

5.3.1.7 Thermostability of the enzyme

The effect of optimum temperature on the stability of PGase LBW 5117 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 5.8, 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 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 (Komori *et al*., 1993; Bennamoun *et al.*, 2016). A stable PGase LBW 5117 means that the enzyme can be reused and thus, help reduce the frequency of its purchase (more savings).

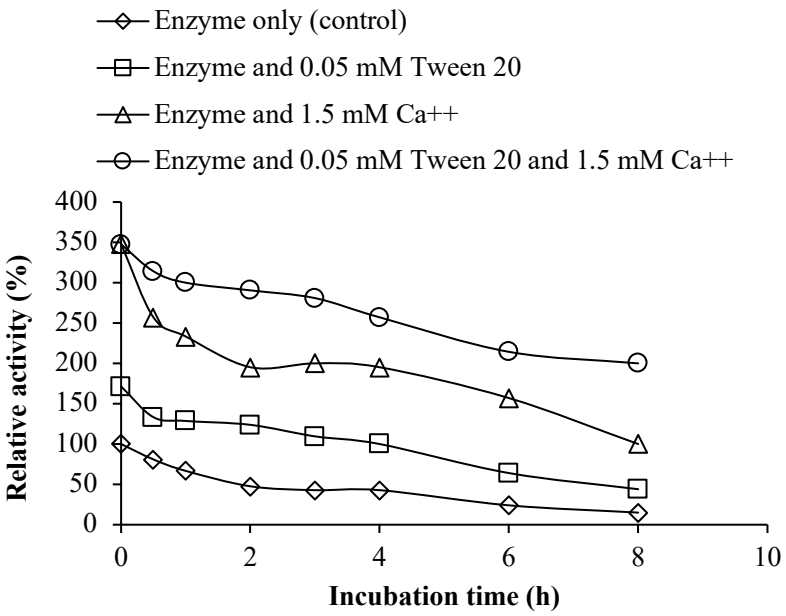


Figure 5.8: Effect of 1.5 mM Ca^{2+} and 0.05 mM Tween 20 additives on the thermostability of PGase LBW 5117 at pH 10.5 and 50 ºC. Enzyme activity corresponding to 100 % was 0.21 U/ml (control), 0.36 U/ml in the presence of 0.05 mM Tween 20 and 0.73 U/ml in the presence of both Ca^{2+}, and Ca^{2+}/Tween 20.

5.3.1.8 Substrate specificity

Substrate specificity studies of PGse LBW 5117 were carried out by performing semi-quantitatively 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 5.9 A(i)] 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 5.9 A(ii)].

A similar pattern of enzymatic activities was observed when the assays were carried out using the more sensitive quantitative liquid assay method (Figure 5.9B). Overall, these results show that the amount of methyl-ester groups have 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 5.9A and 5.9B), 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. The results confirmed that none of the three cellulases were detected in the enzymatic solution (Figure 5.9B). 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 (cited date 02 January, 2023)], [https://biosolutions.novozymes.com/en/leather-textiles/textiles/biopreparation (cited date 02 January, 2023)], [https://www.textiletoday.com.bd/introducing-bio-scouring-a-cost-effective-and-eco-friendly-process-for-the-generation-next/ (02 January, 2023)], [https://microbiosci.creative-biogene.com/enzyme-application/textile-industry-1283.html (cited date 02 January, 2023)].

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 degrade and eliminate all types of pectins including polygalacturonic acid, which is commonly found in cotton fibers and in the process also remove the undesired hydrophobic non-cellulosic substances that adhere to it, without damaging the desired hydrophilic cellulosic structures that make up cloth. This can lead to the production of high-quality cellulosic fabrics with better softness and good absorbency properties (Kohli & Gupta, 2015).

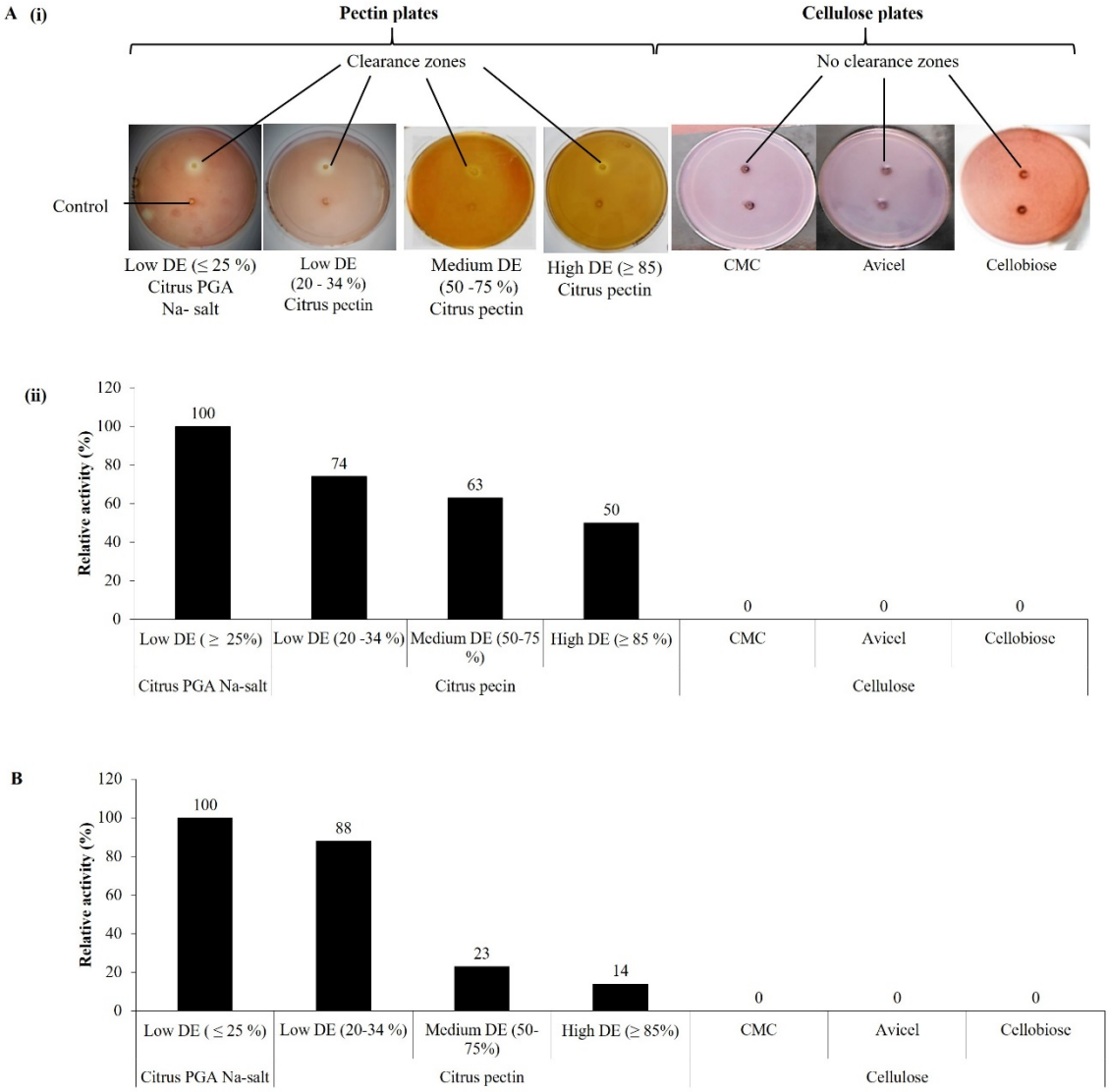


Figure 5.9: Effect of different pectin- and cellulosic- products on the activity of PGase LBW 5117 in the presence of 1.5 mM $Ca^{2+}$and 0.05 mM Tween 20 at pH 10.5 and 50 ^{o}C. The activities were determined semi-quantitatively, and are seen as halos around the depressions on solid pectin plates [A (i)], and are expressed as a percentage of that of citrus PGA sodium salt (activity corresponding to 100 % was 5.4 SI) [A (ii)]. The activities were also determined quantitatively, and are expressed as a percentage of that of citrus PGA sodium salt (activity corresponding to 100 % was 0.73 U/ml) (B). No halo formation around the depressions on cellulosic plates suggests the absence of cellulases in the crude enzymatic preparation.

5.3.1.9 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 5.9) 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 LBW 5117 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.

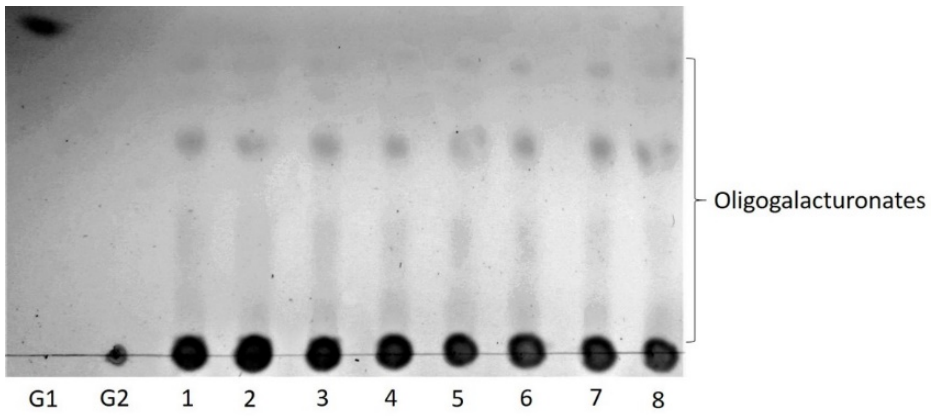


Figure 5.10: TLC profile of hydrolytic action of PGase LBW 5117 on polygalacturonic acid sodium salt: G1 and G2 are monogalacturonic acid and polygalacturonic acid standards, respectively; Lanes 1 - 3 are the reaction products after 15, 30 and 45 min, and Lanes 4 - 8 are the reaction products after 1, 2, 4, 8 and 18 h, respectively.

5.3.2 Assessment of the PGase LBW 5117 scoured fabric

The extent to which the pectin (and other natural hydrophobic impurities that adhered to it) was removed from the fibers of the woven cotton by PGase LBW 5117 was

assessed by determining its properties, including a) surface-handle or 'feel' b) weight loss (%) and c) absorbency, and then comparing the results with those from the control fabric (treated with denatured enzyme).

5.3.2.1 Surface properties and weight loss (%)

As seen in Table 5.3, the fabric acquired new surface-handle or 'feel' characteristics to indicate absence or near absence of pectin and other impurities compared to the control, which exhibited the characteristics of an unscoured fabric. Furthermore, it incurred a weight loss of 0.7 % compared to that of the control, which lost only 0.06 %. Assuming that the weight. 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 can be attributed to the enzymatic degradation and elimination of pectin (and other natural hydrophobic impurities that adhered to it) from it. This indicates that the fabric was bioscoured. Although there are no reports available on the measurements of weight losses incurred by fabrics that have been commercially bioscoured with pectinases, Rajendran *et al*. (2011) reported a slightly higher weight loss (0.89 %) from a piece of fabric that they bioscoured in their laboratory.

Table 5.3 Comparison of some physical properties of the bioscoured fabric with that of the control (unscoured) fabric.

Property	**Fabric treated with:**	
	Bioscoured	**Control (unscoured)**
Surface handling 'feel'	Extremely soft, smoother, and greater flexibility	Soft, smooth, flexible
Weight loss (%)	0.74 (0.68)	0.06

5.3.2.2 Absorbency of the bioscoured fabric

5.3.2.2.1 Drop penetration test

When two drops of water were added on separate spots on the surface of the enzymatically treated fabric, it took an average of 10 sec for them to be absorbed into the fabric compared to the control fabric, which did not absorb the water-drops even after extending the waiting period to 300 sec. This confirms that the enzyme degraded and eliminated the pectin and other hydrophobic non-cellulosic structures from the fabric, leading to the exposure of the desired hydrophilic cellulosic structures that easily absorbed the water-drops. Although a standard absorbency time of < 5 sec is recommended (Teli & Adere, 2016), this result is acceptable, considering that the study was a demonstration of the bioscouring potential of PGase LBW 5117, and was thus not carried out under optimized process conditions. Moreover, when a drop of malachite green dye was added on a separate point on the same fabric, it was absorbed and the colored area appeared as a big green circle to indicate that the fabric was evenly bioscoured and the process was complete (Figure 5.11a) (Halim & Zhou, 2018). On the other hand, the control fabric did not absorb the dye thus, indicating that it was not bioscoured (Figure 5.11b).

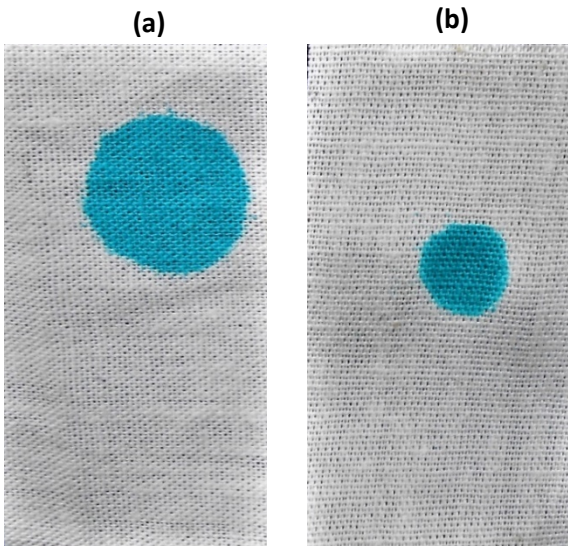


Figure 5.11: Photograph of woven cotton showing the size and shape of the colored dye (malachite green) after performing the absorbency test on them using the drop penetration method; (a) enzymatically treated - and (b) control - fabrics.

5.2.2.2.2 Capillary rise (wicking) test

On the onset of the experiment (time t = 0), the baseline of both the enzymatically treated- and control- fabrics were on the surfaces of malachite green dye in separate beakers (Figure 5.12A). However, after 30 min, the wicking height of the enzymatically treated fabric had risen to 28 mm while that of the control fabric hardly left its baseline (Figure 5.12B). This result further confirms that the enzymatically treated fabric became more absorbent due to the degradation and elimination of the pectin and other non-cellulosic substances from it by the enzyme. Although Halim & Zhou (2018) obtained a better wicking height of 40 cm under similar bioscouring conditions, this result is acceptable because PGase LBW 5117 was used under unoptimized process conditions.

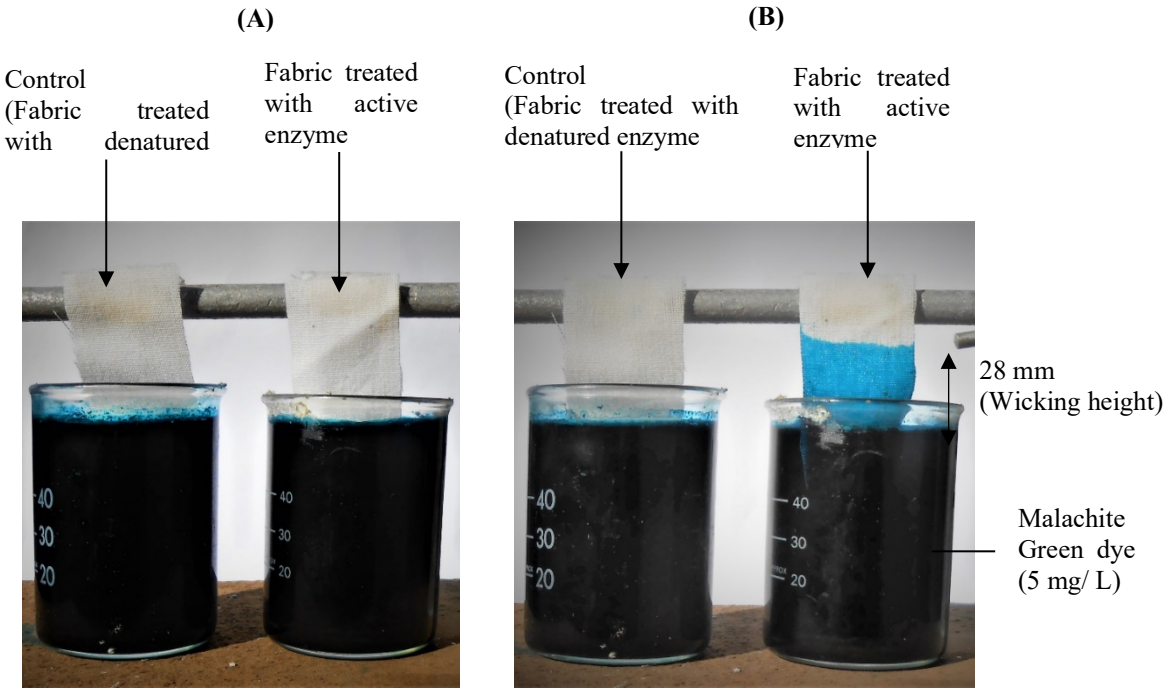


Figure 5.12: Photograph of the capillary rise (wicking) test performed on woven cotton using malachite green dye; (A) onset of the experiment (time t = 0) and (B) end of the experiment (time t = 30 min).

5.4 Conclusion and Recommendations

The present findings show that PGase LBW 5117 is a cellulase-free surfactant compatible metallo endo polygalacturonase that can hydrolyze pectin containing various degrees of methyl esters under alkaline [pH 10.2 - 11.0 (optimum, 10.5)] and relatively low temperature [45 - 57 ^{o}C (optimum 50 ^{o}C)] conditions. These properties make it suitable for use in an application such as that of scouring woven cotton at low operational costs (low energy consumption and no need to purchase expensive heat-resistant equipment or metal ion chelators) without worrying about damage to the fabrics by both enzyme and cellulase-producing neutrophilic contaminants.

Moreover, the the enzyme exhibited good stability properties during operation (albeit in the presence of thermostabilizers) and storage (4, 20 and 30 ^{o}C), implying that it can be produced in bulk, stored appropriately and used within 12 months in such an application. This can help reduce the frequency of its purchase,which is a boost to savings. A preliminary investigation into the bioscouring potential of the enzyme, under its optimum operating conditions, revealed that it could scour an Amy LBW 5117 desized fabric, and in the process, yield a more hydrophilic fabric with improved wettability (drop test = 10 sec) and dye-ability (capillary rise test = 28 mm after 30 min) properties. This shows that the enzyme is potentially a good bio-scouring agent.

It is recommended that the enzymatic de-sizing and bio-scouring processes described in **chapters 4** and **5**, respectively, be optimized to enhance their efficiencies afterwhich, their effectiveness will be compared with those of conventional chemical methods of desizing and scouring that are practiced on Kenya.

CHAPTER SIX

6.0 GENERAL DISCUSSION, CONCLUSIONS AND RECOMMENDATIONS

6.1 General Discussion

In recent years, there has been much interest in finding new microorganisms as biotechnological sources of enzymes with improved operational and storage properties as an alternative to the a) costly high-temperature de-sizing and scouring enzymes that not only operate at or near neutral pH, but also have to be imported from a few countries that manufacture them e.g., Denmark, China, and the US, and b) fabric-damaging and environmentally unfriendly de-sizing and scouring chemicals. To address these challenges, low-temperature alkaline enzymes from indigenous (Kenyan) alkaliphilic microorganisms should be sought, and utilized for de-sizing (amylases) and scouring (pectinases and/or proteases) under optimized conditions. This study, therefore, aimed to initially determine the optimum operating conditions of an alkaline protease, pectinase, and amylase obtained from an alkaliphilic microorganism that was isolated from Lake Bogoria (soda lake), Kenya, and to demonstrate their potential to desize (amylase) and scour (pectinase or protease) woven cotton.

Using microbiological techniques, as shown in chapter three of this study, one hundred and ninety (190) alkaliphilic microorganisms were isolated from the soda lake samples. Eighteen of these isolates were a) screened for protease-, pectinase- and amylase- activities, b) identified using phenotypic and molecular characterization, and c) cultured for the production of the enzymes. Sixteen (16) isolates were identified as *B. halodurans* (Group 1), with four (4) members of this group (sub-group I) exhibiting similar growth characteristics to those of *Bacillus* sp. C-125 (re-identified as *B. halodurans* C-125), *Bacillus* sp. AH-101 (re-identified as *Bacillus halodurans*) and *Bacillus halodurans* M29 (Takami and Horikoshi, 1999), (Takami *et al*., 1999), (Mei *et al*., 2013) and the remaining twelve (12) (sub-group II) exhibiting similar growth

characteristics to that of *Bacillus halodurans* DSM 497 type strain (Ghojavand *et al.*, (2008) and Nielsen, *et al*, (1995). This result is supported by the differences that were observed earlier on in the enzyme production profiles (Table 3.3) and colony/cell morphological characteristics (Figure 3.4, Table 3.4, and Figure 3.5a) of the isolates, and therefore, suggests that the *B. halodurans* isolates classified under sub-group I are a variant of those in sub-group II or the type strain. This could have occurred to enable them to adapt to their 'new' ecological niche (hot spring wells) where the temperatures were relatively higher thus, giving rise to a variant with different phenotypic characteristics (colony switching) and gene expression profiles (Rodríguez, 1995; Sousa *et al*., 2011). The remaining two (2) isolates were identified as *B. pseudofirmus*. Both *B. halodurans* and *B. pseudofirmus* species are among the Gram-positive alkaliphiles community commonly found in the Kenyan Rift Valley soda lakes (Duckworth *et al*., 1996). The limited number of alkaliphilic bacterial species isolated in this study can be attributed to the sampling method (purposive) and limited isolation conditions employed in the field and laboratory, respectively.

All *B. halodurans* and *B. pseudofirmus* isolates were individually grown in 50 ml broth media for the production of alkaline proteases, pectinases, and amylases. All the *B. halodurans* and *B. pseudofirmus* isolates produced amylases while pectinases were only produced by *B. halodurans* isolates. Proteases were produced by all the *B. pseudofirmus* isolates as well as *B. halodurans* isolates classified under sub-group II.

The enzymatic titers of both proteases and pectinases appeared to correlate well with those of their respective reference strains as well as those produced by the same isolates during the screening process thus, supporting the fact that *Bacillus halodurans* isolates classified under sub-group I was a variant of those in sub-group II or *B. halodurans* DSM 497 type strain. On the contrary, the amylase titers produced by both *B. halodurans* and *B. pseudofirmus* isolates were within range of those produced by their respective type strains but did not correlate with those produced by the same isolates on solid starch medium (Table 3.3). This can be attributed to the differences

in the a) uptake of nutrients by the isolates in the two types of media and b) activity detection rule e.g., in the solid medium the disappearance of the substrate is measured while in the liquid medium, the appearance of the product is measured, as was also reported by Castro *et al.* (1993). *Bacillus halodurans* LBW 5117 was among the most potent producers of both amylases (0.32 U/ml) and pectinases (0.09 U/ml).

Bacillus halodurans LBW 5117 was cultured in 100 ml broth media for the individual production of Amy LBW 5117 and PGase LBW 5117. The crude enzymes were then subjected to physicochemical characterization to determine their optimum operating and storage conditions and after that, evaluated for their ability to desize (Amy LBW 5117) and scour (PGase LBW 5117) woven cotton under their respective optimized operating conditions (chapters four and five, respectively). Like many industrial enzymes, both enzymes were brown liquids that exhibited a slight fermentation odor. These are typical characteristics of extracellular enzymatic preparations that are destined for industrial use. Such enzymes are often prepared in bulk and then subjected to only a few purification steps e.g., a) clarification; to remove cell biomass, and b) concentration; to enhance the activity of the enzyme. This is because emphasis is laid on catalysis rather than purity (Headon & Walsh, 1994), [https://biosolutions.novozymes.com/en/textiles/products/desizing/aquazym (cited date 14 March 2023)], [https://biosolutions.novozymes.com/en/textiles/products/biopreparation/bioprep (cited date 14 March 2023)].

Amy LBW 5117 was identified as α-amylase, a GH 13 that randomly cleaves multiple internal α-(1,4)- glycosidic linkages in starch, resulting in the rapid production of varying lengths of soluble α-maltooligosaccharides and α-limit dextrins (Van Der Maarel *et al*., 2002). On the other hand, PGase LBW 5117 was an endo polygalacturonase, a GH 28 that randomly cleaves the internal α-(1,4) glycosidic linkages in polygalacturonic acid to liberate soluble α-oligogalacturonates (Jayani *et al*., 2005). Commercially available de-sizing and scouring enzymes are also α-

amylases and endo pectate lyases (or endo polygalacturonases), respectively (Tables 4.3 and 5.3). Like endo polygalacturonase, pectate lyases also cleave the α-(1,4) glycosidic linkages in polygalacturonic acid, but via β-elimination to liberate soluble methyl-α- oligogalacturonates. In addition, both Amy LBW 5117 and PGase LBW 5117 exhibited catalytic activities of 0.41 U/ml and 0.21 U/ml, respectively. These activities are within the range of those produced by other *Bacillus* sp. in the laboratory but are much lower than those exhibited by commercial de-sizing and scouring enzymes (Abd El Mageed *et al*., 2022) (Soares *et al*., 1999) (Tables 4.1 and 5.1). It is, therefore, obvious that if Amy LBW 5117 and PGase LBW 5117 are to find applications in the textile industry, their catalytic activities will have to be improved.

This can be achieved by utilizing cheap agro-industrial waste products that are rich in pectin (e.g., pineapple-, orange- and lemon- peels, and coffee-, rice- and wheat- husks, etc) and starch (cassava and potato- peels, etc) as C-sources during cell culture (solid state fermentation) followed by scaling up the fermentation processes to pilot- and eventually to industrial- scale level (Haile & Ayele, 2022; Pandey *et al*., 2000). Alternatively, the activities may be improved by cloning and expressing.

On the issue of storage, it was established that the optimum temperature for storing Amy LBW 5117 was 4 ^{o}C for a guaranteed period of 6 weeks (retained 93 % of its original activity) (Figure 4.3) whereas that for PGase LBW 5117 was 4 - 30 ^{o}C for a guaranteed period of 12 months (retained over 90 % of its original activity) (Figure 5.3). This implies that both enzymes have the potential to be produced and stored in a liquid state for the specified durations before use. This finding is significant because a) consumers in the textile sector prefer using liquid enzyme solutions that are simple to dose using automated dosing systems in order to eliminate product wastage and lead to cost savings, and b) the enzymes exhibited remarkable stability in the absence of stabilizers during storage. Most enzymes are often formulated with additives that stabilize their 3-D structures and thus help preserve their catalytic activities during storage. However, the stabilizers must be custom tailored keeping in mind the best

stability and performance of the enzyme. Another important component of the formulation is preservatives, which exhibit anti-microbial action (http://www.enzyme-india.com/formulation.html)

As far as the operational conditions for Amy LBW 5117 and PGase LBW 5117 are concerned, both enzymes were found to operate optimally at relatively higher pH and lower temperatures than those of their counterparts commercially used for de-sizing and scouring woven cotton (operate optimally at or near neutral pH and at high temperatures). This implies that if they were to be used as de-sizing (Amy LBW 5117) and scouring (PGase LBW 5117) agents, there would be a) production of higher quality fabrics with reduced pollution loads due to limited contamination from cellulase-producing neutrophilic microorganisms, b) lower cost of energy, and c) no need to purchase heat-resistant equipment (savings).

Furthermore, metal ions commonly found in raw cotton fibers and tap water (K^+, Ca^{2+}, Mg^{2+}, Fe^{3+}, Cu^{2+}, Na^+, $Mn^{2+,}$ and Zn^{2+}), and nonionic surfactants/wetting agents (Triton X-100 and Tween 20), either stimulated the catalytic activities of the enzymes or did not affect them significantly [chrome-extension://efaidnbmnnnibpcajpcglclefindmkaj/https://wasreb.go.ke/downloads/Water_Quality_&_Effluent_Monitoring_Guidelines.pd (cited date 17 July 2023); Brushwood & Perkins, 1994; Agrawal, 2016). This finding is therefore attractive for the textile industry, which heavily relies on raw cotton fibers, tap water, and surfactants/wetting agents for de-sizing and scouring because it implies fast pretreatment times, which translates to great production (high profits) and savings (no need to purchase metal ion chelators).

Among the additives that stimulated the activities of both enzymes significantly were Ca^{2+} and Tween 20. These additives also improved the stabilities of both enzymes, implying that the latter can be reused in batch and continuous de-sizing- and scouring-processes for as long as they are catalytic active. This means savings in terms of

purchasing the enzyme. At the time of writing this thesis, it is not clear whether commercial de-sizing and scouring enzymes are reused. Furthermore, both Amy LBW 5117 and PGase LBW 5117 enzymes were free from cellulases, with the former also being able to hydrolyze different starch products (Irish potato, sweet potato, tapioca, rice, corn, and wheat)- starch) and the latter doing the same for pectins with various degrees of methyl esters (DE < 25, DE 20 - 34, DE 50 – 75, and DE ≥ 85 %). This shows that Amy LBW 5117 and PGase LBW 5117 have the potential to remove the different types of starch size- and pectin- impurities, respectively, from woven cotton, without damaging the cellulosic structures of the cloth thus, leading to the production of high-quality fabrics with low pollution loads in the generated effluents. Commercial de-sizing amylases and pectinases also exhibit a similar property [http://www.sunsonenzyme.com/Products/Textile/Desizing/ (cited date 16 Feb 2023)], [https://biosolutions.novozymes.com/en/textiles/products/desizing/aquazym (cited date 16 Feb 2023)].

A preliminary investigation into the biotechnological application of Amy LBW 5117 revealed that it could hydrolyze and eliminate up to 8.2 % corn starch-sizing material (8.2 %) from an industrially sized piece of woven cotton, at high pH (10.0), moderate temperature (60 ^{o}C) and in the presence of a) catalytic stimulant and b) thermostabilizer (Ca^{2+} and Tween 20), and in the process, yield a fabric with a commercially acceptable amount of residual starch (0.06 - 0.085 %) (TEGEWA rating 7 - 8) (Harane & Adivarekar, 2017). Similarly, PGase LBW 5117 could hydrolyze and eliminate up to 0.68 % pectin and other non-cellulosic substances that adhere to it, from an Amy LBW 5117 desized piece of woven cotton, at high pH (10.5), moderate temperature 50 ^{o}C) and in the presence of a) catalytic stimulant and b) thermostabilizer (Ca^{2+} and Tween 20), and in the process, yield an evenly bioscoured fabric with improved water and dye absorbency properties.

This shows that both Amy LBW 5117 and PGase LBW 5117 can be used to effectively desize and scour woven cotton, respectively, without worrying about a) cellulase-

producing neutrophilic contaminants, b) high cost of energy and heat-resistant equipment, and c) purchasing of metal ion chelators (savings). On the contrary, commercial de-sizing and scouring enzymes operate optimally at a) or near neutral pH, implying that the fabrics are susceptible to damage by cellulases from neutrophilic contaminants, and b) higher temperatures, implying higher cost of energy (Tables 4.1 and 5). This result shows that both amylase (Amy LBW 5117) and pectinase (PGase LBW 5117) have good operational properties with potentially better economic prospects compared to their commercial de-sizing and scouring enzymatic counterparts.

6.2 Conclusions

- A total of sixteen (16) alkaliphilic *Bacillus halo*durans isolates with alkaline protease, pectinase, and amylase activities, and two (2) *Bacillus pseudofirmus* isolates with protease and amylase activities, were isolated from samples collected in Lake Bogoria and its surrounding hot springs.
- An alkaline amylase (Amy LBW 5117) from *Bacillus halodurans* LBW 5117 in the collection of alkaliphilic microorganisms above exhibited good operational properties with potentially better economic prospects compared to the enzymes that are today used for commercial de-sizing. Furthermore, it could hydrolyze and eliminate starch sizing material from woven cotton, and in the process, yield a fabric with a commercially acceptable amount of residual starch (0.06 - 0.085 %) (TEGEWA rating 7 - 8). This demonstrates that it is an effective de-sizing agent.
- An alkaline pectinase (PGase LBW 5117) from the same microorganism mentioned above also exhibited good operational properties with potentially better economic prospects compared to the enzymes that are today used for commercial scouring. Furthermore, it could hydrolyze and eliminate pectin and other non-cellulosic impurities from an Amy LBW 5117 desized piece of woven cotton and, in the process, yield a fabric with improved water- and dye-

absorbency properties. This demonstrates that it is an effective bio-scouring agent.

6.3 Recommendation

Due to the promising potential of Amy LBW 5117 and PGase LBW 5117 as de-sizing and bio-scouring agents, respectively, it is recommended that:

- The de-sizing conditions of Amy LBW 5117 be optimized to improve the efficiency of the process and then compare the effectiveness of the process with that of standard oxidative de-sizing method in terms of quality of fabric produced produced and pollution loads generated in the effluents.
- The scouring conditions of PGase LBW 5117 be optimized to improve the efficiency of the process and then compare the effectiveness of the process with that of standard caustic scouring method in terms of quality of fabric produced and pollution loads generated in the effluents.
- The slight loss of catalytic activities exhibited by both enzymes and the foul smell that they emitted during and after long storage periods in liquid state be addressed e.g., by formulating them with stabilizers and preservatives, respectively.
- Their catalytic activities be improved to match those of their commercial desizing and scourng counterparts. This can be achieved through solid state fermentation i.e., growing the source of the enzymes (*B. haloduans* LBW 5117) on separate solid media, one containing a cheap agro-waste product that are rich in a) starch (amylase production) and the other pectin (pectinase production) at lab-scale level. If the results are promising, then both the processes can be up-scaled to pilot and later on to industrial levels.

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APPENDICES

Appendix 1: Composition and preparation methods of some reagents used in the study.

a) Trace elements solution

Ingredient	g/l
$CaCl_2 \cdot 2H_2O$	1.7
$FeSO_4 \cdot 7H_2O$	1.3
$MnCl_2 \cdot 4H_2O$	15.1
$ZnSO_4 \cdot 7H_2O$	0.25
H_3BO_3	2.5
$CuSO_4 \cdot 5H_2O$	0.125
$Na_2MoO_4 \cdot 2H_2O$	0.125
$CoNO_3 \cdot 6H_20$	0.23
95 - 97 % H_2SO_4	(2.5 ml)

b) DNS

Dissolve 0.502 g 3, 5 Dinitrosalicylic acid, 0.252 g sodium sulfite and 0.988 g phenol in 300 ml NaOH (prepared by dissolving 5 g NaOH in 300 ml distilled water). Top up with distilled water to 500 ml.

Appendix 2: Absorbance readings obtained for the purified 16S rDNA samples, their concentrations and degrees of purity.

Module:	**Nucleic Acid**						
Path:	**10 mm**						
Software:	**3.7.1**						
Firmware:							
Sample ID	**User ID**	**Date**	**Time**	**Concentration (ng/ul)**	**Absorbance (260nm)**	**Absorbance (280nm)**	**Purity* (260/280)**
1	Default	27/01/2015	12:19	52.67	1.053	0.489	2.15
2	Default	27/01/2015	12:20	61.21	1.224	0.582	2.1
3	Default	27/01/2015	12:20	57.91	1.158	0.502	2.31
4	Default	27/01/2015	12:21	77.42	1.548	0.822	1.88
5	Default	27/01/2015	12:21	51.93	1.039	0.482	2.15
6	Default	27/01/2015	12:22	75.55	1.511	0.653	2.32
7	Default	27/01/2015	12:22	71.29	1.426	0.646	2.21
8	Default	27/01/2015	12:23	69.39	1.388	0.687	2.02
9	Default	27/01/2015	12:23	37.94	0.759	0.32	2.37
10	Default	27/01/2015	12:24	38.01	0.76	0.354	2.15
11	Default	27/01/2015	12:24	60.09	1.202	0.545	2.2
12	Default	27/01/2015	12:25	28.85	0.577	0.248	2.33
13	Default	27/01/2015	12:26	66.08	1.322	0.616	2.15
14	Default	27/01/2015	12:26	47.1	0.942	0.434	2.17
15	Default	27/01/2015	12:27	60.88	1.218	0.63	1.93
16	Default	27/01/2015	12:27	47.44	0.949	0.486	1.95
17	Default	27/01/2015	12:27	60.47	1.209	0.59	2.05
18	Default	27/01/2015	12:29	42.23	0.845	0.369	2.29

* All DNA samples exhibited A260/A280 ratios that were approximately 1.8 and were therefore, considered as pure.

Key:

1. LBW 2719	LBW 39
2. LBW 226	LBW 7526b
3. LBW 318	LBW 328
4. LBS 77	LBS 16
5. LBW 261	LBW 625
6. LBW 4512	LBW 446
7. LBW 327	LBW 5117
8. LBW 434	LBW 313
9. LBW 7526a	LBW 317