UNIVERSITY OF NAIROBI

DEPARTMENT OF BIOCHEMISTRY

SCHOOL OF MEDICINE

ALKALINE ACTIVE α-AMYLASES FROM ALKALIPHILIC *BACILLUS* SP.: SCREENING AND ENZYME PROUCTION

HBC 305: RESEARCH PROJECT

BY

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H12/1581/2010

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A research dissertation submitted to the Department of Biochemistry in partial fulfillment for the requirement of Bachelor of Science Degree in Biochemistry, School of Medicine, University of Nairobi

OCTOBER 2014

DECLARATION

Declaration by the student

I hereby declare that this research dissertation is my original work and has not been presented to any other university for examination or any other award.

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Declaration by the supervisor

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ACKNOWLEDGEMENT

Praise be to GOD for seeing me through it all. All Glory, Honour and Highest praises be to His Son Jesus Christ, Lord and Saviour.

I wish to express my heartfelt thanks to my very hardworking and dedicated supervisor, Mr. Kevin R. Oluoch, for his guidance, motivation and optimistic outlook in the course of my research and dissertation writing.

I wish also to share this achievement with my fellow colleague Janemary Obiny. I would especially like to thank Mr. C. Wycliff, Nelson Khan, Irene Kiio, Mr. Osawa and Isemeki J. for assisting me with my research work. My sincere gratitude for the use of the facilities, cooperation and assistance rendered in one way or another.

My appreciation and thanks to Mama C for assistance with access to Dr. C. A. Omwandho's laboratory, to Dr. Muge for his concern and guidance, to the staff and my fellow colleagues undertaking MSc. for their kind generosity and hospitality in the course of my research in the Department of Biochemistry, University of Nairobi.

Last but not least, I thank God for my family who are my companion on this academic journey. In my many moments of disappointment and dismay, the thoughts of your prayers and moral support have helped me to keep my chin up and journey on in faith and hope. Thank you for being my sheltering trees.

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ABBREVIATIONS

α: alpha

β: Beta

CO_2: Carbon(IV) oxide

DNA: Deoxyribonucleic acid

U: Enzyme Unit

Fig: figure

$H^{+:}$ Hydrogen ion

H20: Water

$K_2HPO_{4:}$ Dipotasium hydrogen phosphate

LBW: Lake Bogoria Water

mg: miligram

Mg_2SO_4: Magnesium sulphate

MPa: Mega Pascal

Na_2CO_3: Sodium carbonate

NaCl: Sodium chloride

NaOH: Sodium hydroxide

pH: Hydrogen potential

sp: species

w/v: weight per volume

rpm: revolutions per min

ABSTRACT

The term alkaliphiles defines microorganisms that grow optimally at pH values above 9, often between 10 and 12, but are unable to grow or grow slowly at the near neutral pH value of 6.5. Consequently, their extremozymes such as amylases also have high catalytic efficiency and stability at an alkaline pH range of pH9- 11. These alkaline active amylases have potential applications for hydolysing starch under high pH conditions in the starch-based industries and also as ingredients in enzyme-based detergents. Potential application of alkaliphilic amylases has led to their bioprospecting from the microorganisms found thriving in naturally occurring alkaline environments such as soda lakes found along the East African Rift valley.

In this study, five alkaliphilic bacteria designated *Bacilus* sp. LBW 213, *Bacilus* sp LBW 2.719, *Bacilus* sp LBW 33, *Bacilus* sp LBW 35 and *Bacilus* sp LBW 313 previously isolated from Lake Bogoria, a soda lake found in the east African Rift Valley in Kenya were screened for the production of alkaline-tolerant amylases. The five *Bacilli* sp were grown on solid Horikoshi medium pH (10.5) for 72 h, after which the plates were flooded with Gram's iodine solution in order to identify amylase-producers. All 5 bacteria exhibited extracellular starch hydrolysing activities, as depicted by the presence of clearance zones or 'halos' around their colonies after staining with Gram's iodine solution. The ratios of the diameter of halos to that of colonies were then used as a semi-quantitative method for classifying the bacteria. *Bacillus* sp. LBW 33 and LBW 35 were considered as excellent producers while the rest were very good producers. Based on this analysis, all the bacteria were considered for further experiments.

The five *Bacilli* sp. from Lake Bogoria in the presence of soluble starch were cultivated in liquid broth at 37^{o}C, 100rpm in a shaker incubator for up to 48h, during which samples were withdrawn after every 12h to determine the cell growth by measuring their optical density at 600nm in order to generate characteristic bacterial growth curves. During each withdrawal, cells were harvested by centrifugation and the cell free culture supernatants used as crude enzyme source for extracellular amylase assay using a glucose standard. All the bacteria exhibited both growth and α-amylase enzyme production.

During the cultivation, all five bacteria exhibited a general growth profile, reaching optimal growth at 24 h with maximum OD at 600nm of 4.370(*Bacillus sp.*LBW 213), 4.670 (*Bacillus sp.* 27 19), 5.206(*Bacillus sp.*LBW 33), 6.10(*Bacillus sp.*LBW 35) and 6.310(*Bacillus sp.*LBW 313).

There was a general increase in α-amylase production by most of the bacteria, reaching optimum levels during late exponential phases of bacterial growth (24 h) for most of the bacteria except for *Bacillus sp.*LBW313, during cell death phase (36 h). Therefore, in this study the range of enzymatic activity for all the five bacteria was $5.5 \times 10^{-3} – 38 \times 10^{-3}$U/ml. Although production levels by the bacteria were low compared to those of other alkaliphilic bacteria studied, *Bacillus sp.* LBW 33 was the most promising candidate for enzyme production (highest producer), thus making it ideal for further studies.

CHAPTER 1

1.0. INTRODUCTION

Amylases are enzymes that digest glycosidic linkages in starch molecules. They are derived from several sources e.g. fungi, yeast, bacteria and actinomycetes. Fungal and bacterial sources, however dominated industrial applications. This is because they offer several advantages e.g. they meet industrial needs, have short growth periods, are biochemical diverse and the ease with which enzyme concentrations might be increased by environmental and genetic manipulation.

Starch-converting enzymes can be classified into four groups: endoamylases, exoamylases, debranching enzymes and Transferases: a) Endoamylases cleave the α-(1-4) glycosidic bonds present in the inner part of amylose and amylopectin polymers in a random fashion, by-passing the α-(1-6) branching points. The obtained products of hydrolysis are thus linear oligosaccharides of varying length, with an α-conformation, and branched oligosaccharides (α-limit dextrins). The most well know endoamylase is α-amylase (EC 3.2.1.1) b) Exoamylases act on the external glucose residues of amylose or amylopectin and may either exclusively cleave α (1-4) glycosidic bonds and, thus produce maltose and β-limit dextrin (β-amylase EC 3.2.1.2), or cleave both α,1-4 and α,1-6 glycosidic bonds and produce only glucose (glucoamylase EC 3.2.1.3) and α-glucosidase (EC 3.2.1.20) c). Debranching enzymes exclusively hydrolyze α (1-6) glycosidic bonds in amylopectin, glycogen and branched limit dextrins, thus leaving long linear polysaccharides as end products. Examples include isoamylase (EC 3.2.1.68) and pullulanase type I (EC 3.2.1.41) which also has the ability to hydrolyse Pullulan (a polysaccharide composed of repeating maltotriose units linked by α-(1-6) bonds to panose. In addition, pullulanase type II enzymes (also referred to as α-amylase-pullulanase or amylopullulanase) are also available. They hydrolyze both α-(1-4) and α-(1-6) glycosidic bonds. Their action on amylopectin results in the release of small sugars e.g. glucose, maltose and maltotriose. A remarkable example of this group of enzyme is neopullulanase, which can catalyse the formation of new α-(1-4) and α-(1-6) glycosidic bonds by transglycosylation d) Transferases cleave α-(1-4) glycosidic bonds of a sugar molecule (donor) and transfer part of it to another (acceptor), forming a new glycosidic bond -transglycosylation. Examples include amylomaltase (EC 2.4.1.25) and cyclodextrin

glycosyltransferase (CGTases) (EC 2.4.1.19) which form new α (1-4) glycosidic bonds and branching enzymes (EC 2.4.1.18) which form new α-(1-6) glycosidic bonds.
The first alkaline amylase was produced in Horikoshi II medium by Horikoshi after he cultivated an alkaliphilic B. pseudofirmus No. A-40-2 (Horikoshi, 1971).Several types of alkaline starch-degrading enzymes were subsequently discovered by cultivating alkaliphilic microorganisms. The alkaline amylases have also been classified into four types according to their pH activity curves: The type-I curve has only one peak at pH 10.5; type-I curve has two peaks at pH 4-4.5 and 9- 10; type-III curve has three peaks at pH 4.5, 7, and 9.5- 10; type-IV curve has one peak at pH 4 with a shoulder at pH 10 (Boyer and Ingle, 1972; Yamamoto, 1972).

In the starch conversion industry, alkaline α-amylases are used for starch hydrolysis in the starch liquefaction process that converts starch into fructose and glucose syrups. α-amylase of Bacillus stearothermophilus or Bacillus licheniformis are used.

Alkaline amylases are used in textile industry for desizing process. Sizing agents like starch are applied to yarn before fabric production to prevent breaking of the warp thread during the weaving process. Desizing involves the removal of starch from the fabric using α-amylases remove selectively the size and do not attack the fibres. Amylase from *Bacillus* stain has been employed for long in this industry.

The use of alkaline α-amylases in the pulp and paper industry is for the modification of starch of coated paper, i.e. for the production of low-viscosity, high molecular weight starch. The coating treatment serves to make the surface of paper sufficiently smooth and strong, to improve the writing quality of the paper.

The addition of α-amylase with an activity around alkline pH to the dough results in enhancing the rate of fermentation and the reduction of the viscosity of dough, resulting in improvements in the volume and texture of the product. Moreover, it generates additional sugar in the dough, which improves the taste, crust colour and toasting qualities of the bread. Besides generating fermentable compounds, α-amylases also have an anti-staling effect in bread baking, and they improve the softness retention of baked goods, increasing the shelf life of these products

thermostable maltogenic amylase of *Bacillus stearothermophilus* is used commercially in the bakery industry.

Another important application is the industrial production of cyclodextrin by alkaline cyclomaltodextrin glucanotransferase. This enzyme has reduced the production cost and paved the way for cyclodextrin use in large quantities in foodstuffs, chemicals, and pharmaceuticals.

The use of enzymes in detergents formulations enhances the detergents ability to remove tough stains and making the detergent environmentally safe. These enzymes are used in detergents for laundry and automatic dishwashing to degrade the residues of starchy foods .Amylases have activity at lower temperatures and alkaline pH, maintaining the necessary stability under detergent conditions and the oxidative stability of amylases is one of the most important criteria for their use in detergents where the washing environment is very oxidizing (Kirk, 2002).

1.1. PROBLEM STATEMENT

Conventional detergents are synthetic. They often contain ingredients such as builders, surfactants, optical brighteners, preservatives, active oxygen bleaches and fabric softeners. However, they also contain enzymes such as proteases, amylases, cellulases and also lipases. Currently, the amylases incorporated in detergents are inefficient and costly. Owing to the fact that they are obtained from mesophilic microorganisms and so the secreted enzymes are not stable and efficient over a wide range of alkaline pH in the detergent formulation and industrial processes requiring high pH. There has also been serious public concern about the ecological problems arising from the use of such synthetic detergents on a large scale. Owing to the fact that these synthetic detergents are corrosive, toxic and exhibit a slow rate of biodegradation, their extensive usage leads to the formation of slumps, creating unhygienic conditions in the surroundings. On individuals these have negative health effects ranging from skin and throat irritation to carcinogenicity.

Enzyme-based technology has enabled ingredient replacement or a reduction of surfactants, builders or other chemicals, without compromising performance. Enzyme-based detergents have found a wide range of applications in laundry, dishwashing, textile and other such industries. The

alkaline enzyme preparations like proteases, amylases, lipases and cellulases are considered as indispensable ingredients in these detergents mainly due to the synergistic action exhibited by the different detergent ingredients and the enzyme-preparations. Enzyme detergents are proving extremely useful in keeping a check on environmental pollution. They offer a suitable option to the synthetic detergents with regard to their biodegradability, low toxicity, non-corrosiveness, environment-friendliness, enhanced cleaning properties as well as increased efficiency and stability in different formulations. The optimized formulations make it possible to:

• Replace costly and toxic surfactants such as linear alkyl sulfonates used in traditional cleaners.

• Eliminate builders, such as phosphates, e.g. sodium tripolyphosphate (STPP).

• Replace other toxic or harmful substances with all-natural ingredients.

. Low volumes of enzymes are required to replace high-volume ingredients such as surfactants and builders.

• Improve performance. The synergistic effect of combining several enzymes results in improved stain removal and overall cleaning beyond what single enzymes and traditional surfactants can achieve.

• Energy-efficient product. Enzymes work well even at low temperatures, allowing wash temperature reductions and thereby energy savings.

• Enzyme cleaners are naturally occurring elements so they won't negatively affect the environment.

1.2. JUSTIFICATION OF THE RESEARCH

The alkaliphiles are unique microorganisms, with great potential for microbiology and biotechnological exploitation. Alkaliphilic extremozymes are stable and active at high pH hence their application in processes requiring high pH would increase efficiency and reduce production cost. Therefore, bioprospecting for new microorganisms that can be used for alkaline stable amylase production is a necessary continuous process for industrial development and environmental conservation.

1.3. HYPOTHESIS

Alkaliphilic bacillus species isolated from Lake Bogoria, a soda lake found in the east African Rift valley, Kenya, produce alkaline active- and stable- amylases that can be incorporated in detergents for more efficient cleaning of starch-stained garments and dishes.

1.4. RESEARCH OBJECTIVES

1. Screening alkaliphilic bacillus species for alkaline-active amylase producers
2. cultivating the positive amylase producers in broth medium while monitoring both cell growth and enzyme production levels

CHAPTER 2

2.0 LITERATURE REVIEW

2.1 EXTREMOPHILES

Extremophiles are microorganisms that grow and thrive in extreme environments which were formerly considered too hostile to support life (Gomes, 2004). The term "extreme environment" is however, a relative term since environments that are extreme for one organism may be essential for the survival of another organism. These environments include those with high (55 ^{o}C to 121 ^{o}C) or low (-2 ^{o}C to 20 ^{o}C) temperatures, high salinity (at least 1M salt concentration), high or low pH (pH > 8 and pH < 4, respectively), high pressure (38-110 MPa), high radiation levels, toxic waste, organic waste and harmful heavy metals. Others include conditions that man considers unusual such as living in rocks in the interior of the earth, cold deserts, or extremely dry areas with very low water activity and nutrient concentration, or from the absence of oxygen (Madigan, 1997).

Extremophiles are classified according to the conditions in which they thrive: thermophiles (organisms that grow optimally at high temperatures (55-85 ^{o}C), hyperthermophiles (organisms that grow optimally at very high temperatures (85-110 ^{o}C), psychrophiles (organisms that grow best at low temperatures (-2 ^{o}C-20 ^{o}C), acidophiles (organisms that grow optimally at acidic pH (0-3), alkalophiles (organisms optimally adapted to alkaline pH (10-12), piezophiles (organisms that grow optimally under high pressure (38-110 MPa), and halophiles (organisms that require 2-5M NaCl for optimal growth) (Pabulo, 2013). In addition, there is another special group of organisms called polyextremophiles. These are organisms that have adapted to live in environments that have a combination of extreme physico-chemical parameters, such as acidic- or alkaline- hot springs, deep ocean where it is generally cold, oligotrophic and pressure is high, and hyper saline lakes which are not only saline, but also very alkaline.

2.2 ALKALIPHILES

Several microorganisms exhibit more than one optimum pH for growth depending on the growth conditions, in particular, metal ions, nutrients and temperature. This eliminates precise definitions of what characterizes an alkaliphilic or alkali-tolerant organism. Therefore, the term

alkaliphiles defines microorganisms that grow optimally at pH values above 9, often between 10 and 12, but are unable to grow or grow slowly at the near neutral pH value of 6.5.

2.2.1 Distribution

Alkaliphiles require an alkaline pH of 9 or more for their growth, but generally have an optimal growth pH of around 10. Human industry activity by processes such as cement manufacture, mining operations, paper and pulp production, and food processing effluents all generate examples of highly alkaline environments often in excess of pH 11. Natural geothermal processes such as weathering of silicate minerals can lead to alkaline water about pH 11 because of calcium hydroxide (Grant, 1992; Grant et.al, 1996; Bath et.al., 1987). In volcanic areas, alkaline hot springs have been reported with pH 9 (Hansel, 1997) where alkalinity is also generated by decomposition of silicates.

Therefore, alkaline environments are placed into several categories depending on the nature of the process generating alkalinity. They depend on a continuous process, either microbial or chemical, to maintain an alkaline pH and also counter the buffering capacity of CO_2 which tends to maintain a more neutral or acidic pH.

2.2.2: The East African Rift valley and the soda lakes found therein

Soda lakes and soda deserts are the most stable highly alkaline environments (Table 1). They represent the most stable high pH environments on earth, where large amounts of carbonate minerals can generate pH values greater than 11.5.

Table 1: Examples of some alkaline environments (Grant, 2004)

Continent	Country	Location
Africa	Libya	Lake Fezzan
	Egypt	Wadi Natrun
	Ethiopia	Lakes Aranguadi, Kilotes, Abiata, Shala, Chilu, Hertale, Metahara
	Sudan	Dariba Lake
	Kenya	Lakes Bogoria, Nakuru, Elmenteita, Magadi, Simbi, Crater Lake
	Tanzania	Lakes Natron, Eyasi, Magad, Manyara, Balangida
North America	Canada	Manito
	USA	Ragtown Soda Lakes, Alkali Valley, Albert Lake Lenore, SoapLake

Weathering processes known to occur on earth suggests that alkalinity is likely to arise as result of excess sodium and calcium ions in basaltic minerals, resulting in carbonate-rich and therefore alkaline aqueous environments (Mills and Sims, 1995). The East African Rift Valley is part of a

huge volcanic rift that stretches from north of Africa with an eastern branch through Kenya and Tanzania. The climate of the rift is semi-arid or arid with a geology dominated by Na^+ trachyte lavas resulting from vulcanism. The floor of the rift has a considerable number of highly alkaline soda lakes, ranging in salinity from around 5% (w/v) total salts, e.g. L. Bogoria and Elementaita to saturation with respect to NaCl and Na_2CO_3, e.g. Lakes Magadi and Natron (Fig 1). They also range in pH values from 10.5-11 for the more dilute lakes e.g. L. Bogoria to values in excess of 11.5 for the hypersaline types e.g. L. Magadi. This alkalinity is as a consequence of the high Na^+ and, low Mg^{2+} and Ca^{2+} ions. Following evaporative concentration in the absence of significant amounts of Mg^{2+} and Ca^{2+} ions, alkaline sodium carbonate brine develops, typically presented by the East African soda lakes. The Mg^{2+} and Na^+ ions usually buffer the aquatic environment by the precipitation of insoluble carbonates (Grant, 1990).

(Fig 1): Map showing the some of the highly alkaline lakes found in the east African Rift valley

Alkaliphiles thrive in such soda lakes, although they have been isolated mainly from alkaline soils laden with carbonates and, sometimes even from acidic soils pH 4 and neutral soils (Horikoshi, 1991).

2.2.3 Microbial diversity of soda lakes

Soda lakes have been clearly defined as lakes that contain higher amounts of Na_2CO_3 and NaCl salts compared to other salts in the water. Microbiological analyses done on these lakes have recorded a vast number of microorganisms that are of biotechnological importance to the society. There is a diversity of prokaryotes with alkaliphiles represented in most of the major taxonomic groups. Many of the microorganisms so far characterized from soda lakes have relatives in salt lakes except that they are all alkaliphilic or at least highly alkali-tolerant (Grant, 1990). Table 2 shows some of the microorganisms that have been isolated from soda lakes.

Table 2: Microorganisms from Soda Lake (Grant et al., 2003)

Bacteria	**Strain**
Cyanobacteria	*Spirulina* spp., *Cyanospirarippkae*, *Synechocystis* sp.
Corynebacteria	*Bogoriellacasielyticus*, *Dietzianatronolimnaea*
Micrococcocci	Lake Bogoria isolate 69B4
Streptomyces	Lake Nakuru isolate 11AG8
Bacillus/Clostridium	*Clostridiumparadoxum, Anaerobrancagottshalkii, Tindalliamagadiensis,and Natronincola histidinovarans*
Holoanaerobes	*Natroniellaacetigena,Halonatronumsaccarohilum,Thermosyntrophlypolyticum,* and *Anaerobrancahorikoshiia*
Heliobacteria	*Heliorestisdurensis*, *Heliorestisbaculata, and Heliospira dauricaa*
Proteobacteria	*Pseudomonassp,,Halomonas sp.*
Sulfur oxidizers	*Thioalkalimicrobiumsibericum, Thioalkalimicrobiumaerophilum, Thioalcalivibrioversutus, Thioalcalivibrioniitratus, and Thioalkalivibriojannaschiia*
Nitrifiers	*Nitrobacteralakalicus*
Sulfate-reducing bacteria	*Desulfonatronovibro*

2.2.4 Isolation

Isolation of alkaliphiles must be carried out in alkaline medium containing Na_2CO_3, K_2CO_3, or $Na_2B_4O_7$ at recommended concentrations of 0.5- 2.0% (Horikoshi, 1991). The pH of the medium is maintained at about 8.5- 11.0. Na_2CO_3, normally autoclaved separately, is often the salt of choice, as it occurs in most naturally occurring alkaline environments, and provides the much required Na^+ ions for growth, germination and sporulation of most alkaliphilic *Bacillus* strains (Horikoshi, 1996). Nitrogen sources other than ammonium should be used because at high pH

the equilibrium between NH_4^+ and NH_3 results in the volatalisation of ammonia from the media containing ammonium salts. A typical isolation media for alkaliphiles is shown in table 3:

Table 3: A typical basal isolation media for alakliphilic microorganisms

Ingredient(g/L)	Horikoshi-I	Horikoshi-II
Glucose	10	0
Soluble starch	0	10
Yeast extract	5	5
Polypeptone	5	5
K_2HPO_4	1	1
$MgSO_4.7H_2O$	0.2	0.2
Na_2CO_3	10	10
Agar	20	20

2.2.5 Special physiological features of alkaliphiles

2.2.5.1 Internal pH

The cell surface is a key feature in maintaining the intracellular neutral environment separate from the extracellular alkaline environment. Alkaliphiles use proton pumps to maintain a neutral pH internally, and so the intracellular enzymes from these microorganisms need not to be adapted to extreme growth conditions. The Na^+/H^+ antiporter protein present on the plasma membrane enables cells to adapt to a sudden upward shift in pH and to maintain a cytoplasmic pH that is 2 to 2.3 units below the external pH in the most alkaline range of pH for growth.

2.2.5.2 Cell Walls

Alkalophiles have a peptidoglycan cell wall, although in addition to this they have negatively charged cell wall polymers. Alkaliphilic *Bacillus* species contain certain acidic polymers, such as galacturonic acid, gluconic acid, glutamic acid, aspartic acid and phosphoric acid (Aono, 1983). These function as a negatively charged matrix and reduce the pH values at the cell surface because the cell membrane is very unstable at alkaline pH values below the optimum pH for growth and therefore must be kept below 9. Therefore, it helps to stabilize the cell membrane (Gomes, 2003 *and* Horikoshi, 1999**)**. They may give the cell its ability to adsorb sodium and hydronium ions and repulse hydroxide ions and, therefore, may assist cells to grow in alkaline environments. Their cellular fatty acids contain predominantly, saturated and monosaturated

straight chain fatty acids (Gomes, 2003; Horikoshi, 1999). These also enable the cell to adsorb Na^+ and H_3O^+ ions, and repulse OH^- ions, thus maintaining the cells internal pH.

The composition of peptidoglycan in alkaliphilic *Bacillus* species have an excess of hexosamines and amino acids in the cell walls compared to that of neutrophilic *Bacillus sp***.** Glucosamine, muramic acid, D- and L- Alanine, D-Glutamic acid, meso-diaminopimelic acid, and acetic acid were found in hydrolysate contents of amide is also observed in their peptidoglycan. These components also provide the negative charge density on the cell surface, thus giving the cell its ability to adsorb Na^+ and H_3O^+ ions, and repulse OH^- ions and, therefore, may assist cells to grow in alkaline environments.

2.2.5.3 Na^+ ions and membrane transport

Alkaliphiles require Na^+ ions for growth and they have exhibited vigorous growth at pH range of pH 9 to 11. According to the Chemiosmotic theory, the proton motive force in the cells is generated by the Electron Transport Chain or by excreted H^+ derived from ATP metabolism by ATPase. H^+ is then reincorporated into the cells with co-transport of various substrates.

In Na^+ ion–dependent transport systems, the H^+ ion is exchanged with Na^+ by Na^+/H^+ antiporter systems, thus generating a sodium motive force, which drives substrates accompanied by Na^+ into the cells. Incorporation of substrates at pH 9 is observed to greatly increase and the presence of Na^+ significantly enhances the incorporation. However, other cations including Li^+, K^+, NH_4^+, Cs^+ and Rb^-, showed no effect, nor did their concentrations.

Plasma membranes play a role in maintaining pH homeostasis by using Na^+/H^+ antiporter systems, K^+/H^+ antiporter and ATPase drives H^+ expulsion.

2.2.5.4 Mechanisms of cytoplasmic pH regulation

The Na^+/H^+ antiporter protein present on the plasma membrane enables cells to adapt to a sudden upward shift in pH and to maintain a cytoplasmic pH that is 2 to 2.3 units below the external pH in the most alkaline range of pH for growth. These mechanisms of these membrane proteins play a key role in keeping the intracellular pH value in the range between 7 and 8.5 (Horikoshi, 1999).

2.2.5.5 Biotechnological application of alkaliphiles

Alkaliphilic microorganisms, in particular *Bacillus* sp., have attracted a lot of interest in the last few decades, due to their ability to produce extracellular enzymes that are active and stable at high pH values (alkaline active enzymes). The unusual properties of the enzyme offer a potential opportunity for their utilization in processes demanding such extreme conditions. One such application of great impact has been the inclusion of alkaline active enzymes in laundry and dish washing detergents, comprising about 30 % of the total world enzyme production. (Horikoshi, 1996). Other applications of alkaline active enzymes are leather tanning, paper-pulp bleaching, production of CDs, and treatment of agricultural and food processing wastes (Aguilar et al., 1998; Horikoshi, 1996; Horikoshi, 1991; Horikoshi, 1999; Horikoshi and Akiba, 1982; Sharp and Munster, 1986; Grant, 1993; Grant and Horikoshi, 1992; Krulwich and Guffanti 1989 and Grant et al., 1990). Several enzymes such as proteases, amyalses, CGTases, cellulases, lipases, mannases, pectinases and xylanases have been purified, purified characterized and evaluated for industrial use. Alkaline active β-lactamase, catalase, chitinase, D-xylose isomerase, and DNase and RNase have also been purified and investigated (Horikoshi, 1996; Horikoshi, 1999; Sharp and Munster, 1986 and Krulwich and Gruuanti, 1989). The use of microbial enzymes from alkaliphiles and haloalkaliphiles (both from Bacteria and Archaea) in biotransformation at high pH, synthesis of pure enantiomers, and region specific conversion has been receiving increased attention (Grant et al., 1990).

Although alkaline active enzymes monopolize the biotechnological interest of alkaliphiles, the production of metabolites by alkaliphiles is also worth exploring. Carotenoids, 2-phenylamine, siderophores, cholic acid derivatives, organics acids antibiotics, and enzyme inhibitors are known to be produced by alkaliphilic *Bacillus* sp., and other alkaliphilic microorganisms and have great potential use (Aguilar et al., 1998; Horikoshi, 1999; Sharp and Munster, 1986 and Grant, 1993). For example, the alkaliphilic cyanobacteria of *spirulina* sp. is an extraordinary source of carotenoids, vitamins, essential fatty acids, exopolymers, an dprobably antibiotics (Grant, 1993)

Another potential application of alkaliphiles is in the construction of secretion vectors. The gene encoding penicillinase from an alkaliphilic *Bacillus* sp. has been shown to drive the excretion of cell products by *E. coli* (Horikoshi, 1990).

2.2.6 Alkaliphilic *Bacillus* sp. and their alkaline active enzymes

2.2.6.1 Alkaliphilic Bacillus sp.

In Microbiology, the term *bacillus* means any rod-shaped microbe. However *Bacillus*, written with a capital letter and italicized, refers to a specific genus of bacteria. In the laboratory, *Bacillus* sp. can be isolated and readily grown, since they are flexible chemo-heterotrophs and therefore, capable of utilizing a variety of simple organic compounds e.g., simple sugars (glucose, amino acids and organic acids), and also complex sugars (starch and cellulose). Primary isolation can be performed on nutrient agar- or J agar (Tryptone 5g/L, Yeast extract 15g/L, K_2HPO_4 3g/L, glucose 2g/L, agar 20g/L, pH 7.4) – medium, although media components may be manipulated to best suit the growth of the *Bacillus* sp. in question (Kenneth, 2008). Under optimal conditions of growth they exhibit a generation time of 25 min following 24 h duration to several days of cultivation. Typical bacillus colonies have been observed to have various pigmentations, mucoid textures, opaque with smooth surfaces and grow in an elevated manner. The cells are rod shaped, motile and endospore-forming. Endospores may be central, terminal or sub terminal, and they can be round or oval. Most *Bacillus* sp. can be grown in defined or relatively complex media. Primary isolation can be performed on either nutrient agar or J- agar (Tryptone 5g/L, Yeast extract 15g/L, K_2HPO_4 3g/L, glucose 2g/L, agar 20g/L, pH 7.4), although the media components can be manipulated to best suit the growth of the *Bacillus* sp. microorganism grown (Kenneth, 2008). Furthermore, they are also able to degradation almost all substrates obtained from plant and animal sources.

In Bergy's manual of systematic Bacteriology (2^{nd} ed.2004) phylogenetic classification schemes outlined the two most prominent types of endospore forming bacteria as belonging to the classes clostridia and bacilli (Kenneth, 2008). The family Bacilaceae has more than 37 genera, including genus *Bacillus*. Great diversity has been observed within this genus, and analysis of their 16S rRNA genes by oligonucleotide sequencing, has led to the establishment of its phylogenetic tree. The taxonomy hierarchy therefore is:

1. Kingdom: Bacteria
2. Phylum: Firmicutes
3. Class: Bacilli
4. Order: Bacillales

5. Family; Bacilaceae
6. Genus *Bacillus*

Bacillus sp. may also be classified based on the extreme conditions they thrive in. Majority are mesophiles with growth temperature optima between 30-50 ^{o}C, and some are thermophiles with optima as high as 65 ^{o}C. Others are halophiles with ability to thrive in high salt concentrations of 2-5 M NaCl, and acidophiles and alkaliphiles being able to thrive in low and high pH, respectively.

In the past decade a full revision of the alkaliphilic *Bacillus sp.* classification was done with respect to the phylogenetic analysis and phenotypic diversity with proposal of 13 validated alkaliphilc *Bacillus* species (Spanka and Fritze, 1993; Agnew et al., 1995; Nielsen et al., 1995; Yumoto et al., 1998; Fritze et al., 1990 and Nielsen et al., 1994).

2.2.6.2 Alkaline active enzymes from alkaliphilic Bacillus sp.

Bacillus strains are well known to produce extracellular enzymes of industrial importance such as amylases, xylanases, cellulases, and pullulanases. The ability to secrete these enzymes into the medium, together with high growth rate, and the possibility of manipulation of their environment have given *Bacillus* sp. an extraordinary advantage in the production of enzymes of industrial interest (Ingle and Boyer (1976). Approximately half of the present commercial production of bulk enzymes is derived from *Bacillus* sp. strains.

Alkaliphilic and alkalitolerant Bacillus sp. have been shown to produce several active extracellular enzymes, the majority being relatively thermostable, with potential biotechnological applications (Table 4), and many of them have already been commercialized. The very first alkaline enzyme was a protease from an alkaliphilic *Bacillus* sp. strain reported by Horikoshi in 1971 (Horikoshi, 1971). Since then, several alkaline active enzymes from alkaliphilic Bacillus sp. strains have been purified and studied (Horikoshi, 1991; Horikoshi, 1999; Horikoshi and Akiba, 1982).

Table 4: Potential applications of alkaline active enzymes from alkaliphilic *Bacillus* sp. (Horikoshi, 1996; Takami and Horikoshi, 2000; Ito et al., 1998; Horikoshi, 1999; Horikoshi and Akiba, 1982; Grant, 1993; Grant et al., 1990; Jensen, 1972; Kelly and Fogartty, 1976; Ito, 1997 and Hoondall et al., 2002)

Alkaline active enzymes	Applications
Protease	- Detergent industry (removal stains of blood, milk, egg, body secretions etc - Leather tanning/dehairing - Decomposition of gelatinous coating of x-ray films, with silver recovery
Amylase	- Detergent industry (removal of starch based stains) - Desizing of denims and paper
Cellulase	- Detergent industry (removal of grass stains from cotton garments - Fabric softening and color brightening - Waste treatment containing cellulose and hemicellulose
Lipase	- Low energy detergents (removal of fats)
CGTase	- Cyclodextrins production
Mannase	- Hydrolysis of mannans used as food additives or products - Manufacture of Japanese mannan-based foods (cognac, guar gum)
Pectinase	- Japanese paper manufacture
Xylanase	- Hydrolysis of xylan into useful products - Rayon modification - Waste treatment of industrial containing xylan - Pulp and paper processing - Production of beverage water soluble dietery fiber

2.2.6.3 Amylases from alkaliphiles

Alkaliphiles can be isolated from normal environments such as garden soil, although viable counts of alkaliphiles are higher in samples from alkaline environments.

The first alkaline amylase was produced in Horikoshi II medium by Horikoshi after he cultivated an alkaliphilic *B. pseudofirmus* No. A-40-2 (Horikoshi, 1971). From its characterizationit was determined that it was most active at pH 10.0 to 10.5 and retains 50% of its activity between pH 9.0 and 11.5. The enzyme is not inhibited by 10 mM EDTA at 30°C but is completely inactivated by 8 M urea. The enzyme can hydrolyze 70% of starch to yield glucose, maltose, and maltotriose, and it is a type of saccharifying α-amylase.

Boyer and Ingle reported an alkaline amylase in strain NRRL B-3881, which was the second report of an alkaline amylase(Boyer and Ingle, 1972). The B-3881 amylase has its optimum pH for enzyme action at 9.2. It yields maltose, maltotriose, and a small amount of glucose and maltotetraose, all of which have a β-configuration.

McTigue et al. studied the alkaline amylases of three alkaliphilic *Bacillus* strains. *Bacillus halodurans* A-59 (ATCC 21591), *Bacillus* sp. strain NCIB 11203, and *Bacillus* sp. strain IMD370 produced alkaline α-amylases with maxima for activity at pH 10.0 (1994).

Nakamura and Horikoshi discovered many alkaliphilic *Bacillus* strains producing CGTases. The crude enzyme of *Bacillus* sp. strain 38-2 was a mixture of three enzymes: acidic CGTase, having an optimum pH for enzyme action at 4.6, neutral CGTase, having an optimal pH at 7.0, and alkaline CGTase, having an optimal pH at 8.5 (1976)).

Kelly et al. found that alkaliphilic *B. halodurans* A-59 (ATCC 21591) produced three enzymes, α-amylase, pullulanase, and α-glucosidase, in culture broth (Kelly, 1985). These three enzymes were separately produced, and the levels of α-glucosidase and pullulanase reached their maxima after 24 h of cultivation at the initial pH 9.7. Although this pullulanase was not purified, the indicated pH optimum was at 7.0.

Many alkaline enzymes are very unstable under alkaline condition, compared to their mesophilic counterparts which are destroyed when exposed to such harsh conditions.

When industrial production of β-CD (cyclodxtrins) began in USA and Japan, they used *B. macerans* CGTase. However, there were two serious problems in both production processes: (i) the yield of CD from starch was not high, and (ii) Toxic organic solvents such as trichloroethylene, bromobenzene, and toluene were used to precipitate CD owing to the low conversion rate. The use of CGTase of alkaliphilic *Bacillus* sp. strain 38-2 overcame all these weak points and led to the mass production of crystalline α-, β-, γ-CD at low cost without using any organic solvents (1975).

2.3 STARCH

Starch is a polysaccharide produced by green plants as carbon and energy storage material. Leaves trap sunlight energy and through a cascade of physico-chemical processes involving CO_2 and water, translate this into glucose. In itself, glucose is too mobile to act as a long-term energy storage molecule. Therefore the plant's solution is to immobilize the glucose by forming a condensation polymer in which glucose chains are linked together by a condensation polymerization reaction, resulting in the formation of starch, an anhydroglucose polymer that is laid down as insoluble, compact and microscopic semi-crystalline granules in the repository sites of the plant- seeds, tubers and roots.

2.4.1 Structure

A starch molecule contains two types of polymers; Amylose, a linear polymer made up of about 500 – 1000 α-D-glucopyranosyl units linked up by α-(1-4) glycosidic linkages and amylopectin, a branched polysaccharide composed of hundreds of short α-(1-4) -glucan chains that are interlinked by up to 30 glucose units with α-(1-6) linkages (French, 1984, Galliard; Bowler, 1987; Murphy, 2000) **(**Fig 2**)** (Murphy, 2000).The fraction of amylose and amylopectin varies depending on the plant sp. but generally consists of 15-30% amylose.

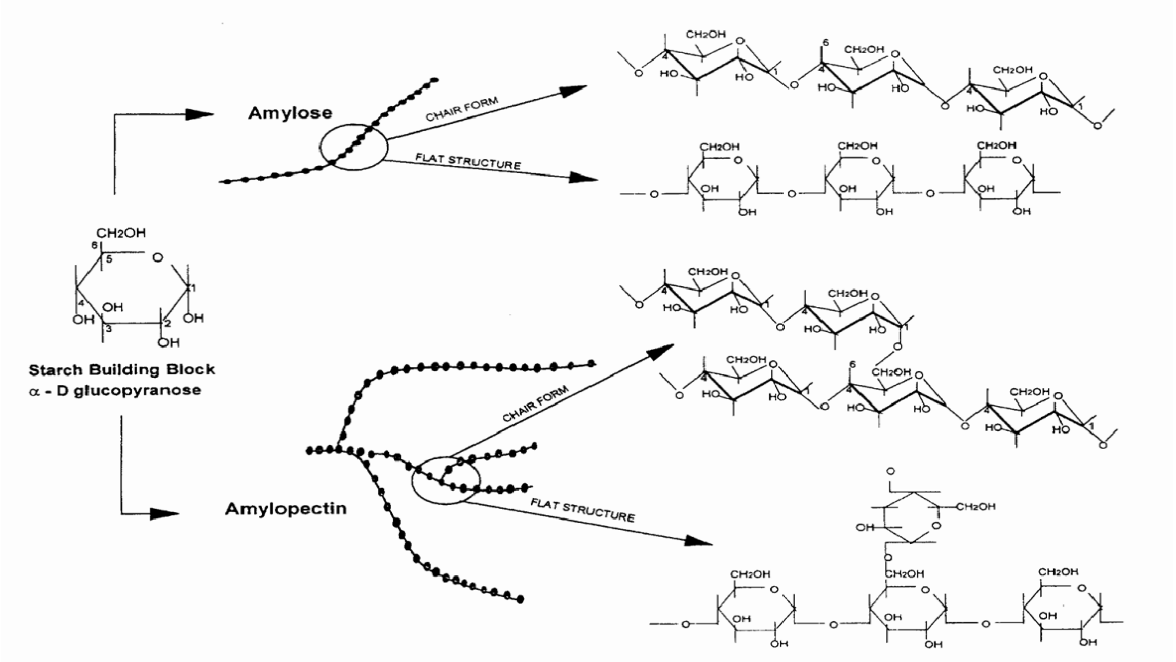


Fig 2: Linear and branched starch polymers

2.3.1 Physico-chemical properties

2.3.2.1 Iodine-staining properties

Iodine has the ability to detect small amounts of starch (as little as 1µg/ml) and reveal any changes in the starch's degree of polymerization caused by enzymes and chemical treatment (Peat, 1953). The blue color of the stain is due to the amylose component of starch. Amylopectin gives a red-purple colour which is much less intense than the amylose stain. When hydrolysed by acid or amylase, both polymers gradually lose the capacity to stain with iodine. The blue colour becomes purple then red, brown, and finally disappears. Screening microorganisms for amylase

producers is a key step for production of the enzyme. Starch hydrolysis is usually detected directly on agar medium containing starch as clear zones around bacterial colonies. The area of the clear zone is in turn related to the potency of the amylase (Dhawale et al., 1982).

2.3.2.2 Solubility

Starch granules are insoluble in water at room temperature due to hydrogen bonds between the hydroxyl groups in the glucose monomers within starch molecules. These bonds are weak and many, which makes it difficult for water molecules to enter and dissolve the starch in water. However, these inter- and intra-hydrogen bonds can become weaker as the temperature of the suspension is raised to that of boiling water or by autoclaving the suspension under pressure at 121 ^{o}C. Therefore, water is absorbed and the granules swell. This process is commonly called gelatinization because the solution formed has a gelatinous, highly viscous consistency. Although they still have some structural integrity, a further increase in temperature causes them to swell and eventually burst, releasing the amylase and amylopectin into the aqueous solution.

2.3.2.3 Hydrolysis

Starch may be hydrolysed chemically or enzymatically:

a) Chemical hydrolysis

Hydrolysis of starch to glucose by the usual acids such as H_2SO_4 must be followed by neutralization and the subsequent production of large amounts of salts. Acidic hydrolysis of glycosidic bonds in starch takes place in two phases (Fig 3):

1. Phase one - Formation of glucoside components that contains protons (activation complex)
2. Phase two - Splitting of the (1-4) glycosidic linkages in the glycoside residues to form glucose and water.

NOTE: The splitting of the glycoside bonds (phase 1) undergoes decomposition simultaneously with the splitting of the glycoside bonds (phase 1) (Bunton and Lewis, 1955).

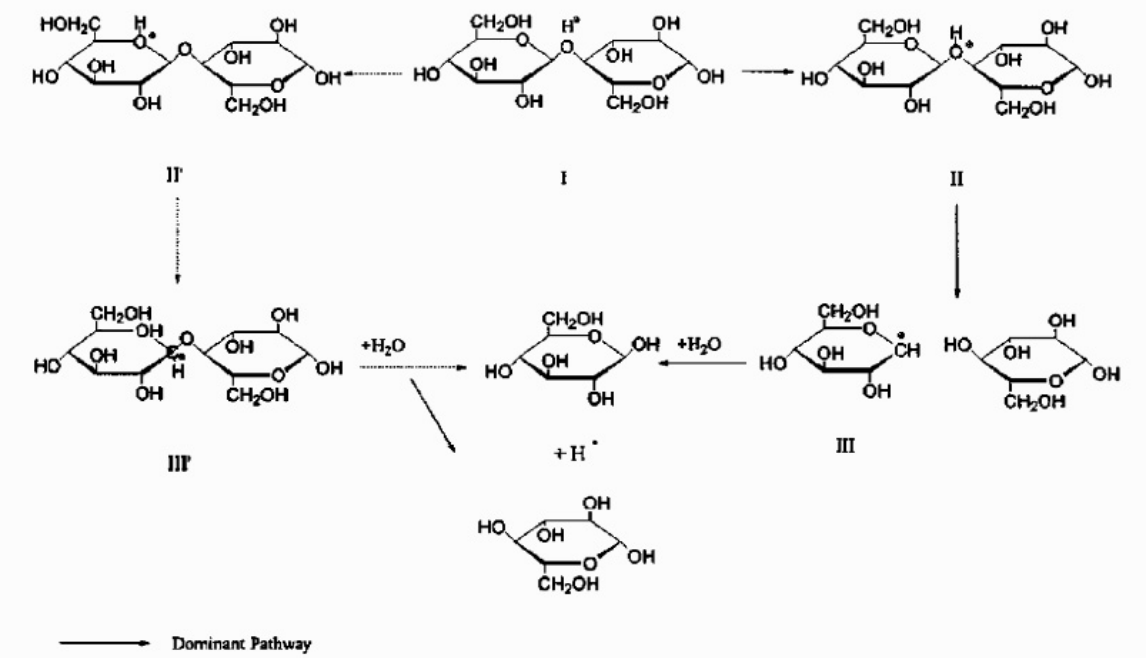


Fig 3: Mechanism of action for chemical hydrolysis starch

This approach of hydrolysis of starch in industries has however, greatly decreased in paper and pulp bleaching, textile and detergent industries, and in the developed countries its application ceased. This is due to the highly concentrated waste resulting from its application which exposes the workers to risk as well as pollutants in the environment. The method is also accompanied by high energy consumption and expensive waste treatment which increases the production costs.

b) Enzymatic hydrolysis

Enzymatic hydrolysis of starch can be compared to the acidic hydrolysis in respect to the probability of splitting of the glycosidic bonds. Bacterial α-amylase, being an endo-enzyme, catalyzes the hydrolysis of α-(1, 4) glycosidic linkages located in the inner regions of amylose and amylopectin in starch molecules. These enzymes don't hydrolyze the (1, 6) - α branch points in amylopectin, but can bypass them (Outtrup, 1984). Acid hydrolysis appears to be a totally random process which is not influenced by the presence of α-(1, 6) glycosidic linkages.

Amylases are a class of enzymes that are capable of digesting these glycosidic linkages found in starches. They can be derived from a variety of sources. They are present in all living organisms, but they vary in activity, specificity and requirements from species to species and even from tissue to tissue in the same organism.

The effective hydrolysis of starch demands the action of many enzymes due to its complexity, although a prolonged incubation with one particular enzyme can lead to (almost) complete hydrolysis.

Starch-converting enzymes can be classified into four groups: endoamylases, exoamylases, debranching enzymes and Transferases:

a) Endoamylases cleave the α-(1-4) glycosidic bonds present in the inner part of amylose and amylopectin polymers in a random fashion, by-passing the α-(1-6) branching points. The obtained products of hydrolysis are thus linear oligosaccharides of varying length, with an α-conformation, and branched oligosaccharides (α- limit dextrins). The most well know endoamylase is α-amylase (EC 3.2.1.1)

b) Exoamylases act on the external glucose residues of amylose or amylopectin and may either exclusively cleave α (1-4) glycosidic bonds and, thus produce maltose and β-limit dextrin (β-amylase EC 3.2.1.2), or cleave both α,1-4 and α,1-6 glycosidic bonds and produce only glucose (glucoamylase EC 3.2.1.3) and α-glucosidase (EC 3.2.1.20)

c) Debranching enzymes exclusively hydrolyze α (1-6) glycosidic bonds in amylopectin, glycogen and branched limit dextrins, thus leaving long linear polysaccharides as end products. Examples include isoamylase (EC 3.2.1.68) and pullulanase type I (EC 3.2.1.41) which also has the ability to hydrolyse Pullulan (a polysaccharide composed of repeating maltotriose units linked by α-(1-6) bonds to panose. In addition, pullulanase type II enzymes (also referred to as α-amylase-pullulanase or amylopullulanase) are also available. They hydrolyze both α-(1-4) and α-(1-6) glycosidic bonds. Their action on amylopectin results in the release of small sugars e.g. glucose, maltose and maltotriose. A remarkable example of this group of enzyme is neopullulanase, which can catalyse the formation of new α-(1-4) and α-(1-6) glycosidic bonds by transglycosylation

d) Transferases cleave α-(1-4) glycosidic bonds of a sugar molecule (donor) and transfer part of it to another (acceptor), forming a new glycosidic bond -transglycosylation. Examples include amylomaltase (EC 2.4.1.25) and cyclodextrin glycosyltransferase (CGTases) (EC 2.4.1.19) which form new α (1-4) glycosidic bonds and branching enzymes (EC 2.4.1.18) which form new α-(1-6) glycosidic bonds. Cyclodextrin glycosyltransferases (CGTase) have a very low hydrolytic activity and make cyclic oligosaccharides with 6, 7, or 8 glucose residues and highly branched high molecular weight dextrins, the cyclodextrin glycosyltransferase limit dextrins.

Amylomaltases are very similar to cyclodextrin glycosyltransferases with respect to the type of enzymatic reaction. The major difference is that amylomaltase performs a transglycosylation reaction resulting in a linear product while cyclodextrin glycosyltransferase gives a cyclic product.

Depending on the relative location of the bond under attack as counted from the end of the chain, the products of this digestive process are dextrin, maltotriose, maltose, and glucose, etc. Dextrins are shorter, broken starch segments that form as the result of the random hydrolysis of internal glucosidic bonds. A molecule of maltotriose is formed if the third bond from the end of a starch molecule is cleaved; a molecule of maltose is formed if the point of attack is the second bond; a molecule of glucose results if the bond being cleaved is the terminal one; and so on(Suhaila, 2004).

CHAPTER 3

3.0 MATERIALS AND METHODS

3.1 MATERIALS

Five alkaliphilic bacteria designated *Bacillus sp.*LBW 213, *Bacillussp.*LBW 2.719, *Bacillussp.*LBW 33, *Bacillussp.*LBW 35 and *Bacillussp.*LBW 313 were provided by Mr. Kevin R. Oluoch. The microorganisms were previously isolated from Lake Bogoria, a soda lake found in the east African Rift Valley in Kenya, and stored as glycerol stocks at -80 ^{o}C in our laboratory at the Department of Biochemistry, University of Nairobi.

The reagents and chemicals used in the research included: tryptone, yeast extract, $MgSO_4$, iodine, potassium sodium tartarate (Rochelle salt), DNS, Na_2CO_3, Glucose, glycine, ethanol and NaOH, which were purchased from Sigma-Aldrich(Missouri, USA). Others included K_2HPO_4, and agar from Kobian (Kenya), and glucose and soluble starch from BDH chemicals (Poole, England) and MercK (Dermsdat, Germany), respectively.

3.2 RESEARCH SITE

The research work described here-in was carried out in Prof. Francis J. Mulaa's laboratory at the Department of Biochemistry, University of Nairobi.

3.3 METHODOLOGY

3.3.1 Screening for amylase producers

Screening for amylase producers among the 5 alkaliphilic bacillus species was carried out as described by Castro, 1983. Briefly, the bacteria were inoculated in Horikoshi II agar medium (Horikoshi, 1971), pH 10.5. The medium consisted of (g/l): starch 10g, yeast extract 5g, peptone 5g, K_2HPO_4 1g, $MgSO_4$ 0.2g and agar, 20g dissolved in 950 ml distilled water and 50 ml 20% (w/v) Na_2CO_3. Both were autoclaved separately at 121^{o}C, 20Psi for 15minafter which the former was added asceptically to the latter to raise the pH to 10.5. 20 ml of the sterile alkaline medium was then poured asceptically in a sterile petri dish and allowed to solidify at room temperature. The five bacillus species were each inoculated in separate spots on the plate, allowed to dry and

the plate incubated at 37^{o}C in a incubator for 72h. Amylase production by the bacteria was determined by flooding the plate with Gram's iodine solution (1.27g iodine in 10ml distilled water containing 2g potassium iodide, and dilute to 300ml with distilled water). The presence of clear zones around the colonies is an indication of starch hydrolysis, which signifies extracellular amylase production (Dhawale et al., 1982).

3.3.2 Cultivation of amylase producing bacteria

Positive amylase-producing bacterial colonies were selected and inoculated in separate 100 ml conical flasks containing 20 ml Horikoshi II broth mediumpH10.5, prepared as described above, but without agar. Cultures were grown for 48h at 37^{o}C and 100 rpm in a shaker incubator, during which samples were withdrawn after 12, 24, 36 and 48 h of cultivation. I ml of the withdrawn sample was used to determine bacterial cell growth while the remaining 19 ml was viewed as crude enzyme source and was kept frozen at – 80 ^{o}C when not in use.

3.3.3 Analytical methods

3.3.3.1 Screening for amylase producers

The size of the clearance zone around each colony was determined semi-quantitatively as the ratio of the halo to that of its colony, which was in turn determined by measuring the diameter of the clearance zone and that of the colony. The measurements were taken and recorded in duplicates and their averages obtained.

3.3.3.2 Cell growth determination

Samples of the bacterial cultures were withdrawn after 12, 24, 36 and 48h of cultivation and the absorbance of the cells measured at 600 nm (in triplicates) using a spectrophotometer in order to generate characteristic bacterial growth curves.

3.3.3.3 Enzyme assay

Cell cultures were sampled after 12, 24 , 36 and 48 h of growth, centrifuged (5000rpm, 4^{o}C for 30min) (Hanil science industries, Korea) and cell-free supernatants assayed (in triplicates) for amylase activity using a glucose standard, prepared as described below:

Glucose standard curve

A stock glucose solution (2.5 mg/ml) was prepared by dissolving 250mg D-glucose (w/v) in a minimum volume of 50 mM NaOH-Glycine buffer pH 10.4 and then topping up to 100ml using the same buffer. A working glucose solution was prepared from the stock solution by aliquoting 10ml of the stock solution into a clean conical flask and then topping up to 100ml using the same buffer. Standard glucose solutions were prepared by pipetting the working glucose solution into test tubes in the range of 0-1ml at intervals of 0.2ml in triplicates and the final volumes in the test tubes made up to 1ml with the buffer. The standard curve was first generated by determining the absorbances, at 540 nm, of glucose concentrations in the range 0-0.25mg/ml using the DNS method.

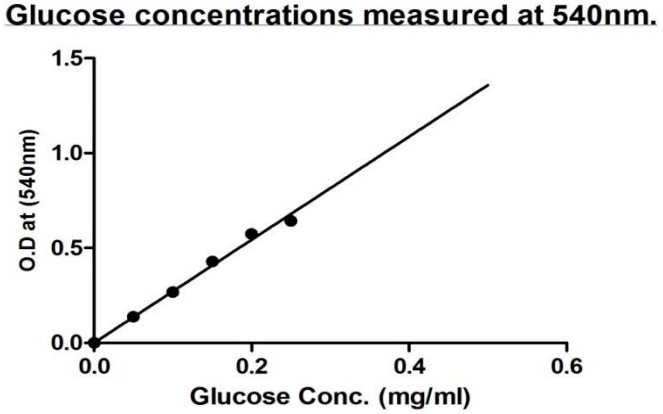


Fig 4: Glucose standard curve

α- Amylase activity

α-Amylase activityin the crude culture supernatants was determined from the amount of reducing sugars formed using a modification of the dinitrosalicylic acid (DNS) method (Miller, 1959) with D-glucose as the calibration standard.50µl cell-free culture supernatant was incubated

with 450µl 0.3% (w/v) soluble starch in 50mM glycine/NaOH pH 10.5 at 40^{o}Cin a water bath (Memmert Company, Shwadach, Germany) for 10 min. The reaction was stopped by the addition of 1ml DNS reagent (2g phenol, 10g dinitrosalicylic acid, 0.5g sodium sulphite, 10g NaOH and, top up to 1000ml with ditilled water), after which the samples were placed in a boiling water bath at 90^{o}C for 10 min. 0.3ml 40% (w/v) Rochelle salt(Potassium sodium tartarate) was then added to the samples, after which they were left to cool at room temperature for 30 min to allow for colour development, and then absorbance read at 540nm using a spectrophotometer. These readings were used to calculate enzyme activity after quantifying the amounts of sugars formed using the D-glucose calibration curve. One unit of enzyme activity was defined as the amount of enzyme releasing 1µmol reducing sugars per min under the standard assay conditions. The samples were assayed in triplicates. The control samples used in the assay comprised of 450µl 0.3% (w/v) soluble starch in 50mM glycine/NaOH pH 10.5 and 50µl heat deactivated (90^{o}C for 10 min) crude enzyme.

CHAPTER 4

4.0 RESULTS

4.1 Plate test assay for screening for amylase producing bacteria

The five alkaliphilic bacillus species were grown on Horikoshi agar medium pH 10.5) for 72 h, after which the plate was flooded with Gram's iodine solution in order to identify amylase-producers. All five colonies were circular, flat, and smooth with entire margins. Pigmentation of the colonies of isolate LBW 2.13 and LBW 2.719 were white, opaque and dull. LBW 33 and LBW 313 had cream, translucent and shiny pigmentation, and LBW 3.5 had white, translucent and shiny. Similar results were obtained for these bacteria(personal communication from Mr. Oluoch, K. R).All 5 bacteria exhibited extracellular starch hydrolysing activities, as depicted by the presence of clearance zones or 'halos' around their colonies after staining with Gram's iodine solution(Fig 5). The ratios of the diameter of halos to that of their respective colonies was then used as a semi-quantitative method for classifying the bacteria as; excellent producers of starch hydrolysing enzymes (halos > 1.5), very good producers (halo size > 1 but < 1.5), good producers (halo size > 0.5 but < 1) and poor producers when no clear zones were observed (Table 1). Based on this criterion, *Bacillus* sp. LBW 33 and LBW 35 were considered as excellent producers while the rest were very good producers. All the bacteria were considered for further experiments.

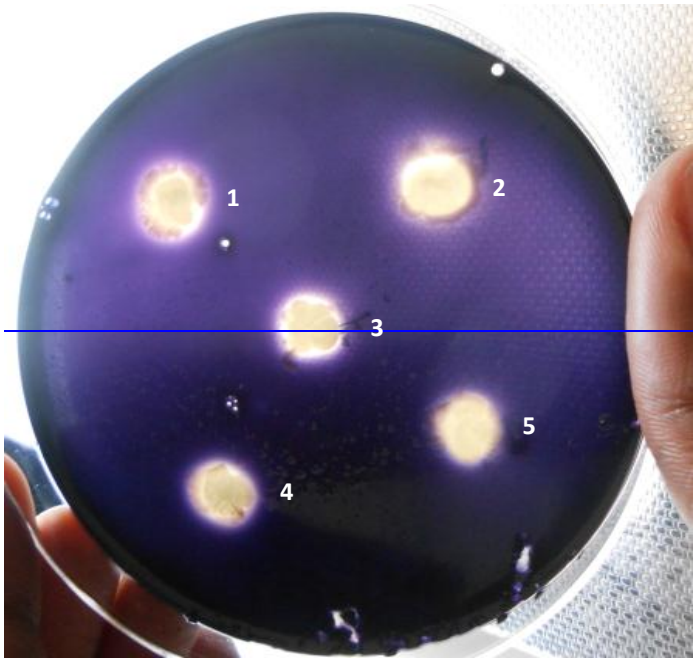


Fig 5: Picture of the bacterial colonies on a Horikoshi agar plate containing starch as carbon source, followed by assay of depolymerized starch (depictedby halos formed around the colonies after flooding with Gram's iodine): 1 = *Bacillus* LBW 213, 2 = *Bacillus* LBW 2.719, 3 = *Bacillus* LBW 33, 4 = *Bacillus* LBW 35 and 5 = *Bacillus* LBW 313

Table 5: Size of clearance zone, ratio of halo diameter to that of colony diameter (cm)

***Bacillus* sp.**	**Horizontal diameter (cm)**		**Vertical Diameter (cm)**		**Average diameter (cm)**		**Clearance size** **Average halo diameter: Average colony diameter**	**Classification**
	Colony	**Halo**	**Colony**	**Halo**	**Colony**	**Halo**		
LBW 213	1.1	1.1	1.0	1.2	1.05	1.15	1.1	Very good
LBW 2.719	0.8	1.0	1.1	1.2	0.95	1.1	1.2	Very good
LBW 33	0.7	1.1	0.8	1.1	0.75	1.1	1.5	Excellent
LBW 35	0.7	1.5	0.8	1.3	0.75	1.4	1.9	Excellent
LBW 313	0.9	1.2	0.9	1.1	0.9	1.15	1.3	Very good

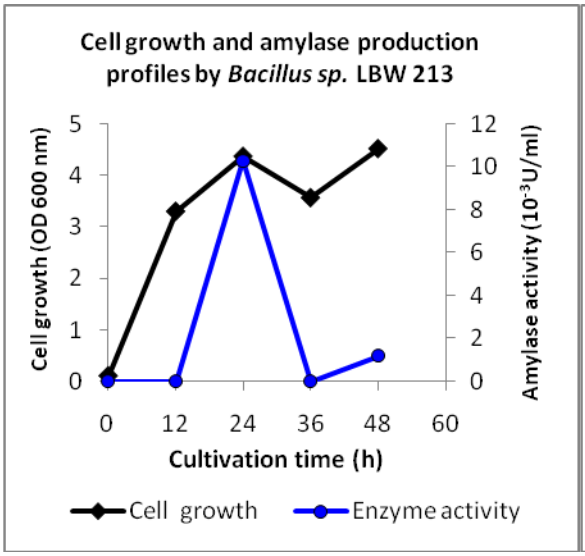
4.2 Extracellular starch hydrolysing activities of Lake Bogoria isolates

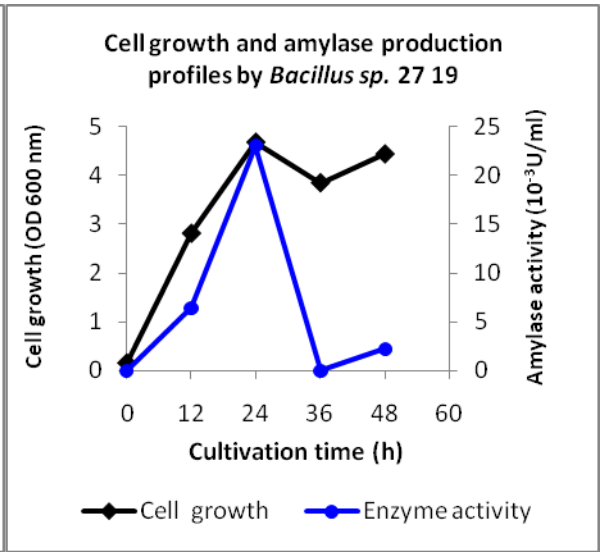
The five alkaliphilic bacillus species were cultivated separately in Horikoshi broth medium containing starch as the sole carbon source for a period of 48h. During this period, samples were withdrawn every 12 h and used to determine both cell growth and amylase activity. Table 6 shows the average OD 600 nm readings for the bacteria and enzyme activities they exhibited over the entire cultivation period. These readings were used to generate both characteristic bacterial growth curves (OD 600 nm) and enzyme production profiles for all the bacteria (Fig 6). The standard curve was first generated by determining the absorbances, at 540 nm, of glucose concentrations in the range 0-0.25mg/ml using the DNS method and then using the standard curve to calculate the enzyme activities by determining the quantities of reducing sugars produced following enzymatic hydrolysis of soluble starch during the assays. The glucose standard curve was then used to calculate the specific enzyme activities of the five *Bacillus* sp. as shown in the Table.

Table 6: O.D(600 nm) readings taken after 0, 12, 24, 36 and 48 h of bacterial cultivation and the corresponding amounts of reducing sugars and enzyme obtained after the assay

***Bacillus* sp**.	**Cultivation time(h)**	**Average O.D 600 nm**	**Glucose conc.(mg/ml)**	**Enzyme activity(U/ml)**
LBW 213	0	0.1	0	0
	12	3.300	0.0000	0.0000
	24	4.370	0.0370	0.0103
	36	3.565	0.0000	0.0000
	48	4.520	0.0044	0.0012
LBW 2.719	0	0.15	0	0
	12	2.805	0.0232	0.0064
	24	4.670	0.0830	0.0230
	36	3.840	0.0000	0.0000
	48	4.430	0.0081	0.0022
LBW 33	0	0.1	0	0
	12	3.660	0.0375	0.0104
	24	5.206	0.0140	0.0389
	36	4.650	0.0500	0.0139
	48	4.190	0.0000	0.0000

LBW 35	0	0.2	0	0
	12	3.142	0.0221	0.0061
	24	6.100	0.0420	0.0117
	36	4.073	0.0350	0.0097
	48	3.665	0.0000	0.0000
LBW 313	0	0.25	0	0
	12	1.940	0.0000	0.0000
	24	6.310	0.0078	0.0022
	36	3.218	0.0199	0.0055
	48	4.650	0.0000	0.0000





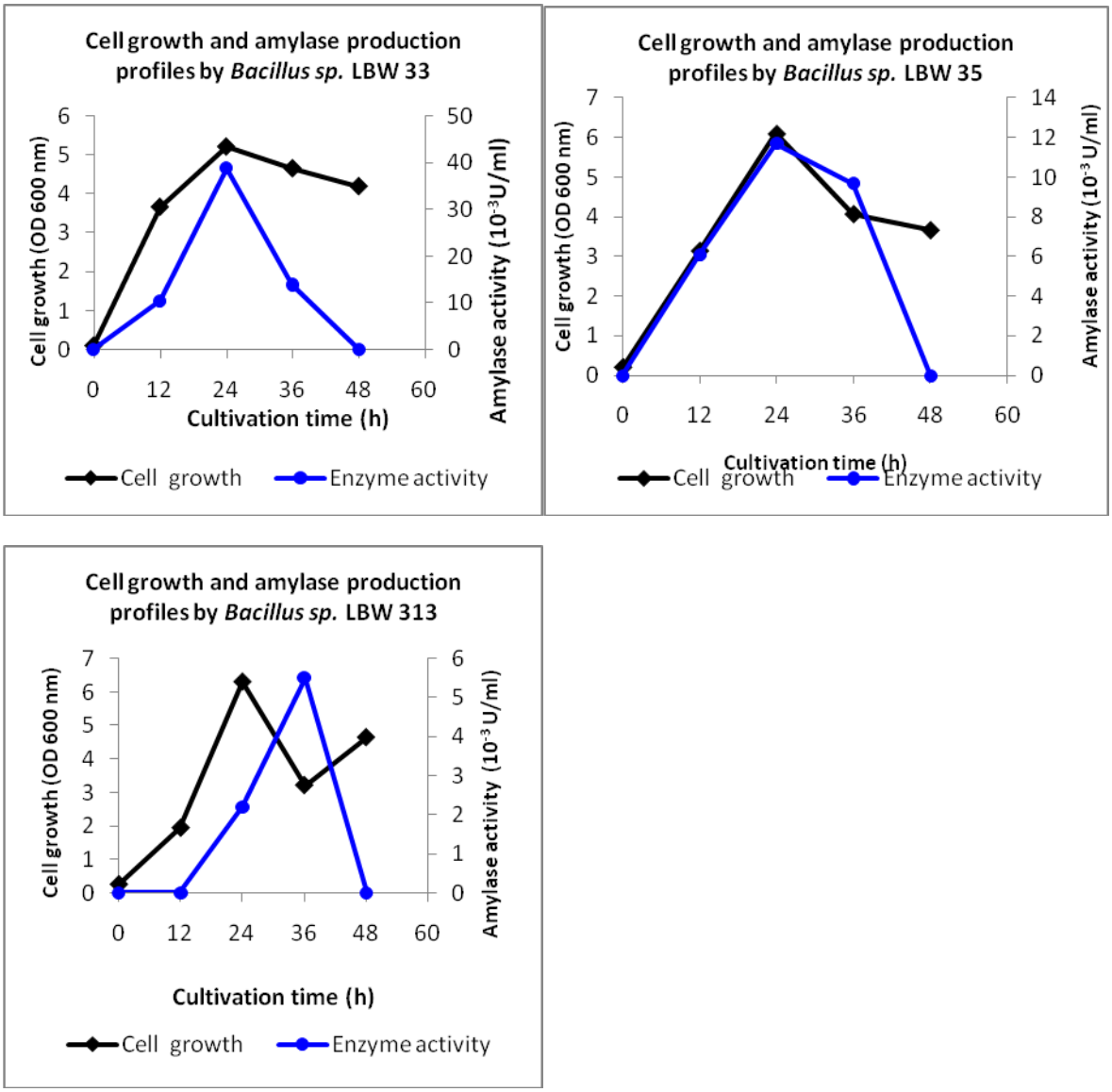


Fig 6: characteristic bacterial growth curves (OD 600 nm) and enzyme production profiles for all the alkaliphilic Baillus sp bacteria.

During the cultivation, all five bacteria exhibited a general growth profile, reaching optimal growth at 24 h with maximum OD at 600nm of 4.370(*Bacillus sp.*LBW 213), 4.670 (*Bacillus sp.* 27 19), 5.206(*Bacillus sp.*LBW 33), 6.10(*Bacillus sp.*LBW 35) and 6.310(*Bacillus sp.*LBW 313) (Fig 6curve profiles).Thereafter, growth declined gradually for *Bacillus sp.* 33 and *Bacillus sp.* 34, reaching OD 600 nm of 4.2 and 3.7, respectively, at the end of the cultivation period (48 h) (Fig 6). The remaining bacteria showed a similar pattern only until the 36th h, after which growth

started to increase unexpectedly. This unexpected result may be attributed to pipetting errors encountered during the research.

Production of α-amylase by the 5 bacterial species was also followed during the cultivation period. There was a general increase in α-amylase production by most of the bacteria, reaching optimum levels of 10.3 x 10^{-3} (*Bacillus sp.* LBW 213), 23 x 10^{-3} (*Bacillus sp.* LBW 27 19), 38 x 10^{-3} (LBW*Bacillus sp.* 33), and 11 x 10^{-3} (*Bacillus sp.* LBW35) during late exponential phases of bacterial growth (24 h), and 5.5 x 10^{-3}(*Bacillus sp.*LBW313) during cell death phase (36 h) (Fig 6). Therefore, in this study the range of enzymatic activity for all the five bacteria was 5.5 x 10^{-3} – 38 x 10^{-3}IU/ml. Other alkaliphiles have been reported to produce various amounts of α-amylases: The alkaliphilic *Bacillus halodurans* LBK34 was shown to exhibit a much higher α-amylase activity in the range 1.2- 1.8 U/ml when cultured under optimum temperature conditions (55- 65^{o} C)(Hashim, 2004).Indira, in his study on α-amylase production from *Bacillus sp.* SI- 136 isolated from sodic alkaline soil reported that log phase was reached by the 20th h when cultivation in Horikoshi-II brothmedium at 60^{o}C(Indira, 2012),but maximum amylase production (2000U/ml) wastowards late log-stationery phase as reported in an earlier study (Annamalai et al., 2011 (Indira, 2012). Pardeep (2011) reported that during determination of optical production of alkaline α-amylase from newly isolated Bacillus *sp.* DLB 9, maximum enzyme synthesis was recorded at 24 h in pH 10 at 37 ^{o}C (10 +/-0.283Uml/min). *Bacillus sp.* DLB 9 had maximal enzyme activity at 24 h, pH 9, at 37 ^{o}C (12.2+/-0.283U/ml/min) although the production and enzyme activity was considerably high at 24 h, pH 10, 37 ^{o}C (10+/-0.0.848U/ml/min). *Bacillus sp.* DLB 9 also had enzyme activity at 24 h, pH 9 at 37 ^{o}C of (12.2+/-0.424 U/ml/min) although its optimum temp was 60 ^{o}C (16.2+/-0.141) and at 50 ^{o}C it had (16.0+/-0.283U/ml/min) indicating an increase in enzyme activity with increase in temperature up to 60^{o}C.

CHAPTER 5

5.0 DISCUSSION

Alkaline active amylases have potential applications in the detergent (removal of starch based stains) and textile industries (de-sizing of denims and paper). Currently, these industries utilize environment unfriendly chemicals or less active- and/or unstable- amylases to manufacture their respective products, thus leading to environmental pollution and high cost of production. Alkaliphilic microorganisms are known to possess alkaline active and stable enzymes. One such enzyme is α-amylase which can find applications in both the detergent and textile industries.The study therefore focused on bioprospecting for alkaline-active amylases from alkaliphilic bacillus species, previously isolated from a Kenyan soda lake.

The research started with the screening of five alkaliphilic bacteria for amylase production. All the bacteria exhibited extracellular starch hydrolysing activities, as depicted by the presence of clearance zones around their colonies after staining with Gram's iodine solution.Bacterial α-amylase, being an endo-enzyme, catalyzes the hydrolysis of alpha-(1, 4) glycosidic linkages located in the inner regions of amylose and amylopectin in starch molecules resulting in the production of α-limit dextrins. Iodine has the ability to detect small amounts of starch and reveal any changes in the starch's degree of polymerization caused by enzymes. Hence, in the absence of starch around the 5 bacterial colonies, iodine does not stain.

The 5 bacterial species were subjected to growth in separate Horikoshi broth media and both cell growth and α-amylase production levels followed. The general increase in growth of the bacterial cells followed a typical sigmoid curve, with very short lag phases (not determined) followed by exponential-, stationary- and death- phases in that order. Enzyme production by the bacteria correlated to cell growth - the higher the cell growth the higher the enzyme production. Thus, with no limiting factor, enzyme production was enhanced when most of the bacteria entered their exponential phases of growth, reaching optimum levels at the log-stationary phases. The only exception was *Bacillus sp.* 313 whose unexpected increase in enzyme activity was enhanced in the death phase of that bacterium when limiting factors such as lack of nutrients and oxygen rain in. As expected, enzyme activity generally declined in the death phases of all the bacteria, a fact that is attributed to the depletion of nutrients, oxygen and change in pH during growth (decreased growth translates to decreased enzyme production).

The general low level of α-amylase production by all the bacteria under this study was probably due to un-optimized culture conditions (pH, temperature, concentration of carbon and nitrogen, among others). Assay conditions (e.g. incubation temperature) are other factors to take into consideration.

CHAPTER 6

6.0 CONCLUSION

The five alkaliphilic bacteria were able to grow under alkaline conditions and produce extracellular alkaline active α-amylases (Due to limited resources, I was not able to identify other types of amylases e.g. β-amylase, amyloglucosidase or glucoamylase, CGTases, pullulanase, isoamylase, and amylolactose). Although production levels by the bacteria were low compared to those of other alkaliphilic bacteria studied, *Bacillus sp.* LBW 33 was the most promising candidate for enzyme production (highest producer), thus making it ideal for further studies.

6.1 RECOMMENDATIONS

1. Assay for other amylases in the crude culture supernatants frozen at – 80 ^{o}C.
2. Optimize culture conditions to maximize enzyme yields for best amylase producer.
3. Characterize crude secreted by best amylase producer with respect to temperature, pH, surfactants, chelators and other additives in order to determine factors required to maintain enzyme activity and stability during its application in the target industry.
4. Carry out a pilot study to evaluate the efficiency of the enzyme with respect to an application in the detergent or textile industry.
5. Establish strong collaborations with potential partners in the relevant industry.
6. Identify the alkaliphilic amylase-producing *Bacilli* sp. used in this study.

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