DISTRIBUTION, ISOLATION AND CHARACTERIZATION OF BACTERIA WITH INDUSTRIAL POTENTIAL IN LAKE NAKURU, KENYA

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EGERTON UNIVERSITY

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DECLARATION AND RECOMMENDATION

DECLARATION

I declare that this thesis is my original work and has not been previously published or presented for the award of a degree in any university.

Signature: ..... Date: .....

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SM18/2267/08

RECOMMENDATION

This thesis has been submitted for examination with our recommendation and approval as University Supervisors.

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DEDICATION

This thesis is dedicated to my loving parents Mr and Mrs Musikoyo, my brothers and sisters. They all enthusiastically supported me throughout the entire period of my studies.

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ABSTRACT

Lake Nakuru is an economically important natural resource in Kenya. Despite the unique ecological characteristics exemplified by this lake, the industrial potential of microbes in the lake has barely been explored. The main objective of this study was to determine the distribution of bacteria, isolate and characterize bacteria with industrial potential. This was achieved through intensive ecological study of three sites on the lake for a period of six months (January to June 2010). Total bacterial counts were determined by direct epifluorescence microscopy of acridine orange stained samples on black polycarbonate filters. Heterotrophic plate counts were plated on 4% NaCl and 1 % Na_2CO_3 nutrient agar. Enzymatic activities of bacteria towards various macromolecules were investigated for 30 selected isolates by testing their ability to degrade such substrates as starch, cellulose, casein and lipids. The potential use of bacterial isolates for feather degradation was determined by allowing isolates to grow on a feather waste medium as the sole source of carbon and nitrogen. Genomic DNA of four isolates (LNS08, LNC09, LNC11 and LNC06) that showed highest potential to degrade macromolecules and feathers was amplified by PCR and subsequently sequenced. Temperature and pH variations in the lake were low throughout the sampling duration. Dissolved oxygen varied tremendously in the lake with a high value of about 18.4±5.1 mg/l in February and a low mean value of 2.08mg/l in April 2010 when a highest rainfall event occurred and consequently higher total counts (TC) and heterotrophic plate counts (HPC) were recorded. There were significant ($P<0.05$) temporal and spatial variations in terms of HPC and TC and this was factored by the rainfall event with the Northern side of the lake being significantly different from the South and Central points. Of the 30 bacterial isolates from Lake Nakuru, 24 showed ability to degrade macromolecules with 26.7% degrading starch, 20% proteins, 16.7% cellulose and 16.6% lipids while for 20% of isolates no enzymatic activity was recorded. DNA analysis revealed identity of four isolates with industrial potential to be closely related to known bacteria *viz*; *Nesterenkonia lacusekhoensis* (LNS08), *Bacillus agaradhaerens* (LNC06) and *Bacillus sp.* Acc No: AB043860.1(LNC09 and LNC11). *Bacillus agaradhaerens* showed best potential for industrial application in the degradation of chicken feathers. *Bacillus sp.* Acc No: AB043860.1has potential industrial production of sugars from starch and cellulose. *Nesterenkonia lacusekhoensis* is a lipid degrader. The results of this work indicated that Lake Nakuru is a rich source of many alkaliphilic bacteria which could be a good source of interesting enzymes from the industrial point of view.

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LIST OF ABBREVIATIONS

AO	Acridine orange
DO	Dissolved oxygen
DOC	Dissolved organic carbon
DOM	Dissolved organic matter
HCO_3^-	Bicarbonate ions
LNS	Lake Nakuru South
LNC	Lake Nakuru Central
LNN	Lake Nakuru North
LSD	Least significant difference
M	Molecular weight marker
OD	Optical density
PCR	Polymerase chain reaction
VLP	Virus like particles

CHAPTER ONE
INTRODUCTION

1.1 Background information

Lake Nakuru is an important natural resource located in the eastern arm of the Great Rift Valley in Kenya, at an altitude of 1759 meters above sea level and a geographical location of 00º 22'S, 36º 05'E. It is an alkaline saline lake characterized by extreme conditions of pH, alkalinity and salinity. Microorganisms which thrive in these environments offer us the opportunity to appreciate the range of adaptive possibilities that evolution can bring to bear on fundamental biological processes. Bacteria in particular constitute unique models for investigations on how biomolecules are stabilized when subjected to these extreme conditions. Alkaliphilic bacteria offer a multitude of actual or potential applications in various fields of biotechnology. Not only do many of them produce compounds of industrial interest, but they also possess useful physiological properties which can facilitate their exploitation for commercial purposes. Enzymes capable of degrading macromolecules have been isolated in some of the Rift Valley Lakes, especially Lake Bogoria and Lake Elementaita. The bacterial ecology of Lake Nakuru is not well studied even though a lot of studies on zooplankton ecology in Lake Nakuru have been undertaken by Yasindi *et al.,* 2002 and phytoplankton ecology by Oduor and Schagerl 2007. Lake Nakuru is a eutrophic water body with high phytoplankton biomass which contributes to the high organic substrate that is important for bacterial growth and metabolism. This causes high increase in the bacteria density since bacteria growth is substrate limited.

Bacteria are ubiquitous in their distribution, being able to survive under extreme conditions such as high or low temperatures, acidic conditions and saline habitats (Rheinheimer, 1991). Bacteria form important components of the microbial loop of the aquatic food webs (Azam *et al.,* 1983). Though most bacteria are heterotrophs, some are photoautotrophs and chemoautotrophs that contribute to the primary production, especially in oligotrophic lakes. Bacteria together with viral particles are sources of dissolved organic carbon to nanoplankton such as the flagellates and ciliates (Rheinheimer, 1991). This trophic relationship translates into the microbial loop where dissolved organic carbon is retained in the food web through conversion into bacterial biomass. In aquatic habitats and especially in the alkaline-saline systems, bacteria are considered to be substrate limited with their biomass being positively correlated to the biomass of the lake's

organic substrates which increases with increase in productivity of a lake (Steward *et al*., 1996). The highest concentration of bacteria has been observed in tropical alkaline-saline lakes such as Lake Elementaita, with values up to 3.6×10^8 bacteria per ml (Kilham, 1981). Low numbers have been found in acid dystrophic lakes with humic acid.

The role of bacteria in environment has received less attention especially in aquatic systems (Kilham, 1981). Bacteria and fungi play major roles in the degradation, remineralization and recycling of organic matter in aquatic environments. Through the microbial loop, bacteria are responsible for key processes regulating the function and productivity of ecosystems (Wommack and Colwell, 2000). Apart from regulation by virioplankton, bacterioplankton biomass is also consumed by nanoflagellates and ciliates (Lindström, 2001). However, in comparison to protists grazing on bacterioplankton, bacteriophage attack is more relevant in this regulation of bacterial biomass under more eutrophic conditions (Weinbauer and Peduzzi, 1995).

Limited studies have been done on microbial ecology of Lake Nakuru with most of these focused on quantification of the bacterioplankton and ciliates (Kilham 1981, Yasindi *et al.,* 2002). Jones and Grant *et al.* (1999) and Harper *et al*. (2003) described the microbial diversity of the Rift Valley soda lakes focusing mainly on L. Bogoria. Other researchers have focused on the aspect of cyanotoxins production in the Kenyan Rift Valley lakes due to the mass death of the flamingoes reported in the late 1990s (Ballot *et al.* 2003; Krienitz *et al*., 2003; Ballot *et al.* 2004). Recent studies have focused on Lake Elmentaita (Mwirichia *et al.*, 2009). However, no long term study has been done to analyze the dynamics of the microbial activities in these lakes. Generally, these tropical lakes still remain largely understudied when compared to temperate lakes in terms of the processes controlling distribution and abundance of microbial species and the general microbial ecology (Duckworth *et al*. 1996).

Some studies carried out on East African soda lakes have occasionally revealed novel species but information regarding the full extent or the roles played by individual organisms has not been wholly established (Grant *et al*., 1999). Such studies are important for understanding the ecological roles of microorganisms in aquatic environment. There is need to carry out microbial investigation with an aim of establishing the roles played by the bacterioplankton and benthic microbes in the ecological dynamics of these lakes. Bacteria with industrial applications have

been isolated in extreme environments in other lakes having similar characteristics to Lake Nakuru. Enzymes secreted by the halophiles and alkaliphiles have been found to possess abilities that can be utilized in industries for the degradation of macromolecules and in the making of medical equipment, oil recovery and surfactants for pharmaceuticals. Some of these enzymes have been used in the making of detergents which are stable to use and environmentally friendly (Van den Burg, 2003; Raja *et al.*, 2007).

Lake Nakuru has higher number of lesser Flamingoes with up to 500,000 flamingoes recorded at one particular time (Owino *et al.,* 2001). Inspite of these high numbers in the lake, their presence has never resulted in high accumulation of feather wastes that could cause menace in the lake. The bacteria in the lake seem to exhibit high degradative ability that reduces feather wastes in the lake. Many authors have isolated feather degrading bacteria from non-treated soils in natural compost and poultry feather wastes (Zerdani *et al.,* 2004 and Joshi *et al.,* 2007). No research has been done on feathers degrading bacteria from flamingo feathers and therefore this forms an important scientific opportunity to conduct research on these bacteria from Lake Nakuru. With this perspective, bacteria with protease enzyme from flamingo feathers were sought from the Lake Nakuru. Proteolytic bacteria from the lake were then tested on chicken feathers for hydrolytic activities.

Keratin in feathers is insoluble to most common protease enzymes such as papain, trypsin and pepsin (Matikevičienė *et al.,* 2009). *Bacillus* have been found to possess keratinolytic enzymes that could be utilized in hydrolyzing chicken feathers making the amino acids in feather leachate available as protein supplements to domestic animals. No study has been made to investigate and isolate microorganisms with potential industrial application in Lake Nakuru. This study has helped to enhance our knowledge on the availability and the potential use of such microbes from this lake.

1.2 Statement of the problem

Lake Nakuru exhibits unique conditions typical of soda lakes that support growth of various unique organisms. Despite the major roles played by bacteria in degradation of organic matter in such environments, minimal work focusing on the bacterial ecology has been undertaken in this ecosystem. It is however known that bacteria from such extreme environments as Lake Nakuru produce extracellular enzymes with industrial applications, a feature that has not been investigated in East African Soda lakes. This study investigated the distribution of bacteria in the lake, and isolated those bacteria with industrial potential by measuring their ability to degrade macromolecules such as starch, fat, cellulose, and protein. Furthermore, bacteria isolated were found to degrade poultry feathers a property that can be utilized for production of animal feeds from waste feathers. This discovery can go a long way in solving environmental problems associated with disposal of feathers from poultry farms and provision of proteins that may be used as feed supplements for domestic animals from such degraded feathers.

1.3 Objectives

1.3.1 General objective

To study the distribution of bacteria, isolate and characterize the physiological groups of bacteria with industrial potential in Lake Nakuru.

1.3.2 Specific objectives

1. To determine the spatial and temporal distribution of bacteria densities in Lake Nakuru.
2. To determine the population density and characterize cultivatable bacteria at different sites of Lake Nakuru
3. To measure the enzymatic degradative ability towards different macromolecules and to determine the influence of these macromolecules on growth of bacteria isolated from Lake Nakuru
4. To isolate, characterize and determine feather degrading ability of bacteria from the shores of Lake Nakuru

1.4 Hypotheses

1. There are no significant differences in the spatial and temporal distributions of bacteria densities in Lake Nakuru
2. There are no significant differences in the densities of cultivatable bacteria at different sites in Lake Nakuru
3. Cultivatable bacteria from Lake Nakuru do not degrade macromolecules and these do not significantly influence growth of bacteria isolated from Lake Nakuru
4. Bacteria isolated from degrading feathers of flamingoes at the shore of Lake Nakuru are incapable of degrading poultry feathers

1.5 Justification

Extreme environments offer unique biotopes inhabited by microorganisms with products of potential industrial use. The alkaline saline conditions in Lake Nakuru provide an extreme environment which may support such unique organisms. This study enhances our understanding of the nature of microbes found in this lake and how they affect the functioning of the lake ecosystem. The study helps in identifying those microorganisms with enzymatic degrading properties on the macromolecules and therefore the possibility of exploiting these microbes in industrial applications. This study has potential economic impacts. Also from past experience some enzymes from extremophiles isolated from Rift Valley lakes in Kenya in the 1990's have been used in detergent industry in USA but unfortunately benefits accruing from such studies are not evident in Kenya. Scientists in Kenya should therefore aggressively investigate the potential posed by such lucrative environments for their own industrial exploitation.

1.6 Definition of terms

Alkaliphiles: organisms which exhibit optimum growth in an alkaline environment that is generally in the range between pH 9 and 10

Alkalitolerants: organisms capable of growing at pH values more than 9 or 10, but with optimum growth rates at around neutral values

Amylolytic bacteria: starch degrading bacteria

Anaerobes: Organisms that grow in the absence of oxygen.

Cellulolytic bacteria: are cellulose degrading bacteria.

Haloalkaliphiles: Organisms that require both alkaline conditions and high concentration of salt (NaCl) for growth.

Halophiles: Organisms that grow in conditions with levels of salt greater than 1.0-1.5 M (salt usually presumed to be NaCl) and up to 5.2 M.

Halotolerants: Organisms that can grow well in salt concentrations of up to 1M

Lipolytic Bacteria: Lipids degrading bacteria

Proteolytic bacteria: Protein degrading bacteria.

CHAPTER TWO
LITERATURE REVIEW

2.1 Soda lakes and their formation

Soda lakes are naturally occurring alkaline-saline water bodies with unique abiotic and biotic conditions. Formation and properties of these lakes are described in Williams (1996). They are mostly located in arid and semi-arid regions and are mostly endorheic thereby resulting into the accumulation of salts washed in from their catchments through inflowing rivers and runoffs. The salts come from the ions dissolved from soluble rocks or from a slow chemical reaction with rocks in the catchment area. Such endorheic lake basins often exist in plateaus formed by the action of volcanicity, tectonic plate movements or by glacial erosion. Due to their location in semi-arid environments, they can be subjected to large volume of water changes according to the water inflow and water loss. Salt concentration increases when water loss occurs by evaporation with extreme dessication causing crystallization of salts. Salt lakes are estimated to account for 0.008% of the world's total water content while fresh water lakes account for a slightly higher volume with a figure of 0.009% of the world's total water (Grant *et al.,* 1990). The most extensively studied soda lakes are those of the East African Rift Valley, where detailed limnological investigations have been done over many years (Grant *et al*., 1990; Jones *et al*., 1998). The Great Rift Valley is an arid tropical zone where in many areas dissolved minerals have concentrated into alkaline brines with carbonate as the major anion, forming shallow soda lakes. The salinities of these lakes vary from around 5% (w/v) total salts to saturation (Grant *et al.,* 1990).

2.2 Microbial ecology of soda lakes

Soda lakes offer extreme environments in which only well adapted organisms inhabit. Such lakes have simple food web, and relatively simple microbial communities (Humayoun, 2003). The distribution and density of bacteria in these lakes depend on the lake's productivity. Lakes with high organic matter (substrate for microbial metabolism) have high numbers of bacteria of up to 10^8 bacteria per ml (Kilham, 1981). Saline water bodies which have high bacterial densities also tend to have high number of bacteriophages (Lindstrom and Bergstrom, 2004). Microbial studies in Mono Lake (USA) and Lake Bagaejinnor (Mongolia) have shown high number of cyanophages and overall elevation in microbial phylogenetic diversity which has been made

available by the constant utilization of bacteria by the cyanophages (Mesbah *et al.,* 2007). Succession patterns of the phytoplankton community in such lakes may also affect the concentration and biochemical composition of autochthonous organic matter available to bacteria (Pinhassi *et al.,* 2004; Kent *et al.,* 2007). This may then influence the diversity and abundance of other microbial communities that depend on this plankton as energy sources due to the nutrition quality changes that accompany community structure changes. The sinking detritus derived from blooms of phytoplankton provide an important substrate for the fermentative microorganisms at the lake bottom (Walker, 1975).

Limited studies have been done on the spatial and temporal distribution of bacterial and viral populations in Kenyan Rift valley soda lakes and on ascribing aspects of the biogeochemical cycles of soda lakes to these bacteria. Interest in soda lake microbiology has centered primarily on the ecology of bacteria and viruses (Jones *et al.,* 1998). The work by Kilham (1981) in which he observed that bacterioplankton biomass in Lake Elementaita and Lake Bogoria were very high, and Yasindi *et al* (2002) with their contribution on the study of the ecology of ciliated protozoa form the main literature on microbial ecology of these lakes. Research by Grant, (2006a) reveal that the abundant bacteria in Kenyan soda lakes are aerobic Gram-negative bacteria of the *Proteobacteria* lineage, including large numbers of types affiliated to the *Halomonadaceae* family (the *Halomonads*) of moderately halophilic bacteria found in a range of terrestrial and marine saline environments. These organisms constitute probably the single most important Gram-negative group in the soda lake environment, although other *Proteobacteria* related to *Pseudomonads* and enterics can also be isolated. *Bogoriella* is a novel genus of bacteria isolated from Lake Bogoria, *Streptomyces* genus is very common in soda lake environments such as Lake Nakuru that grows at pH 10 and hydrolyzes proteins and carbohydrates (Grant, 2006a).

Other unique microorganisms have been isolated from Lake Magadi which provides the most extreme alkaline environment at pH greater than 12 in the Kenyan Great Rift valley. The lake is dominated by bright red blooms of organotrophic haloalkaliphilic archae. *Halomonas magadiensis* (originally named *H. magadii*) (Duckworth *et al.,* 2000) has also been isolated from this lake, From other Kenyan Rift Valley alkaline saline lakes, the isolates include *Cellulomonas bogoriensis* (Jones *et al.,* 2005) and *Alkalimonas delamerensis* (Ma *et al.,* 2004) isolated from

Lake Bogoria, and another from Lake Elementaita where a type of strain, and *Dietzia natronolimnea* (Duckworth *et al.,* 1996) have been isolated. Other aerobic heterotrophic East African soda lake isolates include *Alcalimnicola halodurans* (Yakimov *et al.,* 2001), *Bacillus bogoriensis* (Vargas *et al.,* 2005), and *Bogoriella caseilytica* (Groth *et al.,* 1997). Information regarding the full extent of community structures or the roles played by individual organisms in these lakes has not been wholly revealed neither are reports on industrial application of strains isolated from these Kenyan soda lakes available from literature (Grant *et al*., 1999). This lack of information justifies urgent need to explore this line of research for maximum exploitation of the rich microbial biodiversity of Kenyan lakes.

2.3 Biology of halophiles

Microorganisms that can survive in extreme conditions of soda lakes are called halophiles. These microorganisms are categorized by the precise salinity requirements (Table 1) which takes into account the variety of salt ranges in which they survive in. Extreme halophiles can cope with NaCl levels as high as 5.2 M while halotolerant organisms grow in concentrations of up to 1 M.

Table 1: The classification of prokaryotes based on salt concentrations (Horikoshi and Grant, 1998)

Category	**Range**		**Optimum**	
	(g/l)	**w/v (%)**	**(g/l)**	**w/v (%)**
Non-halophile	0-58	0-5.8	<11.6	<1.16
Slight halophile	11.6-116	1.16-11.6	11.6-29	1.16-2.9
Moderate halophile	23.2-203	2.32-20.3	29-116	2.9-11.6
Borderline extreme halophile	81.2-232	8.12-23.2	116-174	11.6-17.4
Extreme halophile	116-301.6	11.6-30.2	>174	>17.4
Halotolerant	0->58	0->5.8	<116	<11.6
Haloversatile	0->174	0->17.4	11.6-29	1.16-2.9

Examples of halophilic organisms are found in archaea, bacteria, and eucarya domains in these hypersaline environments although the bacteria and archaea are much more prominent. With increasing salinity above 3 g/l, the composition of the bacteria of salt lakes, when considered in

detail, increasingly deviates from that of fresh waters species with their taxa becoming increasingly different (Horikoshi and Grant, 1998). In water bodies with salinities just above 3 g/l, i.e. between 3 and 20 g/l, most taxa present, at all taxonomic levels, may also be found in fresh water bodies and are essentially comprised of halotolerant freshwater forms. At salinities between 20 and 50 g/l, the proportion of halotolerant freshwater forms is much less, though not entirely absent with most of the biota comprising taxa restricted to inland saline waters of moderate salinity. At salinities greater than 50 g/l, the biota is almost entirely restricted to highly saline waters with only a few genera and even fewer species found in less saline waters. Since the range of salinity encountered in saline waters extends well beyond 50 g/l (up to 350 g/l and beyond in certain lakes), it is clear that throughout most of the range of salinity in salt lakes the biota of salt lakes is quite different from that of fresh water bodies.

The most characteristic elements of the biota of salt lakes are the archaeobacteria, of which six genera are presently recognized: *Halobacterium*, *Haloferax*, *Haloarcula*, *Halococcus*, *Natrobacterium* and *Natronococcus*. These taxa constitute the so-called halobacteria. The Archaeobacteria, other than in salt lakes, are typically found in thermal waters (Horikoshi and Grant, 1998). On the other hand the phototrophic purple and green Eubacteria [e.g. *Rhodospirillum* (a non-sulphur purple bacterium), *Chromatium* and *Ectothiorhodospira* (both purple sulphur bacteria) and *Prosthecochloris* (a green bacterium), and non-phototrophic halotolerant eubacteria are also present in salt lakes (Horikoshi and Grant, 1998).

Other than the soda lakes, halophiles may also be found in such environments as garden soil, presumably due to transient alkaline conditions caused by biological activity such as ammonification, sulphate reduction or photosynthesis. However, a richer source of a greater variety of halophilic organisms is the naturally occurring stable alkaline environments of the soda lakes (Jones *et al*. 1998). Halophiles, the majority of which are *Bacillus* species, have been isolated from non-saline environments as discussed by Horikoshi and Akiba, (1982). Despite the harsh environment, soda lakes are nevertheless the home to a large population of prokaryotes, a few types of which may dominate as permanent or seasonal blooms. The organisms range from alkaliphilic cyanobacteria to haloalkaliphilic archaeobacteria. It is not unusual to find common types of haloalkaliphilic organisms inhabiting soda lakes in various widely dispersed locations in various parts of the world such as in the East African Rift Valley, in the western U.S.A, Tibet,

China and Hungary. For example, Natronobacteria have been isolated and identified in soda lakes of China (Wang and Tang, 1989) and the western U.S.A (Morth and Tindall, 1985), in Tibet and India (Upasani and Desai, 1990).

2.4 Degradation of macromolecules in alkaline environments

Research on the biodegradation of materials is of great importance from both economic and ecological viewpoints. With their extra cellular enzymes, bacteria breakdown complex high molecular weight organic compounds into simpler forms that can be taken up by cells for metabolism. Sugars, proteins and starch are easily degradable while fats, waxes, cellulose, hemicellulose, pectin and lignin are more difficult to degrade but they are however usually broken down by microbes (Rheinheimer, 1991). Alkaline environments are colonized by a variety of bacterial populations which might play a role in the chemical breakdown of some of these macromolecules. Few studies in the tropical lakes have focused on alkaliphilic species ability to degrade macromolecules, and especially in the degradation of phenolic-lignin compounds (Horikoshi, 1999a). More extensive studies focused on the capabilities of such micro-organisms to degrade lignin polymer are clearly needed.

2.5 Industrial potential of halophiles

Reports of bacteria growing in high salt levels appeared in the early 1900's (LeFevre and Round, 1919) with the isolation of bacteria from the red, strong smelling salt associated with salt mines. Characterization of these halophilic organisms started in the 1930's when red pigmented bacteria was isolated from animal hides (Lochhead, 1934) and Gibbons who cultured a number of red pigmented halophiles from salted fish (Larsen, 1986).Since then, enzymes and organic compounds derived from environmental isolates have become commercially available. Little attention has been paid to isolation and characterization of individual microorganisms with potential industrial applications in Kenya. However, procedures to detect and identify halophilic organisms have become rapid and routine with DNA sequencing, particularly PCR technology (Duckworth *et al.,* 1996). Since soda lakes are environments of extreme physiochemical conditions, organisms living in these lakes may possess novel adaptations to these environments for example, transport systems, osmoregulatory compounds, or ectoenzymes. Some of these adaptations may involve enzymes or other physiological properties with potential commercial significance (Horikoshi and Akiba, 1982; Grant *et al.,* 1990). New species and novel genes may

offer solutions to some of the present environmental problems. Isolation of such novel organisms however is still problematic, because the majority of microbes have not or cannot be cultured presently using the culture media commonly used. This lack of complete range of viable cultures is a disadvantage in understanding prokaryotic physiology.

2.5.1 Enzymes produced by soda lake microorganisms

Soda lakes contain dense populations of aerobic organotrophic and alkaliphilic bacteria, some of which are potential sources of alkali-stable enzymes (Gessesse and Gashe 1997; Martins *et al*. 2001). Microbial lipases and proteases are among the most important group of enzymes with applications in detergents, manufacture of food ingredients, and pitch control in the pulp and paper industry in processes which use high level of salts where other enzymes would be denatured. These enzymes are also interesting as biocatalysts in organic media, for transesterification reactions and synthesis of chiral compounds (Van den Burg, 2003).

Members of diverse genera have been reported to produce different types of lipolytic enzymes. So far not many lipases with optimum activity under alkaline conditions have been studied. Lipase/esterase positive clones from genomic DNA libraries made from DNA or enrichment cultures isolated from Lake Elementaita in Kenya have been reported (Rees *et al.* 2004). Proteolytic enzymes are degradative enzymes which catalyze the cleavage of peptide bonds in other proteins. Proteases are classified on the basis of three major criteria; type of reaction catalyzed, chemical nature of the catalytic site and evolutionary relationship with reference structure. Alkaline proteases refer to proteolytic enzymes which work optimally in alkaline pH (Barett 1994; Gupta *et al.* 2002). The vast diversity of proteases, in contrast to the specificity of their action, has attracted worldwide attention in attempts to exploit their physiological and biotechnological applications, for example, in food and feed industry, peptide synthesis, leather industry, management of industrial household waste, photographic industry, medical usage, silk gumming and detergents industry (Poldermans 1990; Fox *et al*, 1991; Gupta *et al.* 2002).

Industrial applications of alkaliphiles have been investigated and some enzymes have been commercialized. Of the enzymes now available to industry, proteases, cellulases, lipases, pullulanases are by far the most widely employed and they still remain the target biomolecules for industrial activities. Detergent enzymes account for approximately 60% of total worldwide

enzyme production (Horikoshi, 1999a). These enzymes usually have a pH in solution of between 8 and 10.5. The main reason for selecting enzymes from halophiles for use in detergents is their long term stability in detergent products and the energy cost saving by lowering the washing temperatures. Due to the unusual properties of these enzymes they are expected to fill the gap between biological and chemical processes and have been greatly employed in laundry detergents. Many currently employed alkaliphile enzymes are useful as tools for biotechnological exploitation (Schaechter, 2003). There are a wide range of potential applications and many benefits to be gained from them which are yet to be exploited.

2.5.2 Other biotechnological uses of halophiles

Historically, salts have been obtained by evaporation of lagoons and man-made pan lakes (Wallace and Grant, 2008). Salt was so important to ancient cultures that it became a form of currency. Today, food, medical, biotechnological and industrial companies make use of halophile-related products in their businesses. Being able to survive and flourish in environments that limit the growth of most other organisms suggests their ability to produce substances that enable them to withstand the harsh environmental conditions. Some of these substances, e.g the Taq polymerase used in PCRs, may be harnessed and used for industrial activities. This therefore makes halophiles the potential microorganisms for investigation in bioprospecting. It is likely that halophiles will be the source of future biotechnological products though many of these applications are not yet of large economic value. Halophiles have already made an impact in the application of biotechnology for the manufacture of consumer products. Alkali-tolerant enzymes produced by haloalkaliphilic microorganisms have found use in industrial processes and have considerable economic potential (Wallace and Grant, 2008). Their enzymes are currently used in detergent compositions and in leather tanning. They are expected to find applications in the food, waste treatment and textile industries (Raja *et al.*, 2007).

The genes encoding these enzymes may be isolated, cloned and brought to expression in compatible expression hosts to provide a source of larger volumes of enzyme products which may be easily purified and used in various industrial applications, should the wild-type strain fail to produce sufficient amounts of the desired enzyme, or does not ferment well. The current significant commercial uses of halophiles include fermentation of soy and fish sauces and β-

carotene production (Van den Burg, 2003). Table 2 shows the large range of applications which exist for halophile products (Schaechter, 2003).

Table 2: Some biotechnological uses of halophiles and haloalkaliphiles (Schaechter, 2003)

Source of microbial product	Application/use
Halophiles	
Bacteriorhodopsin	Optical switches and photocurrent generators in bioelectronics
Polyhydroxyalkanoates	Medical plastics
Rheological polymers	Oil recovery
Eukaryotic homologues	Cancer detection, screening anti-tumour drugs
Lipids	Liposomes for drug delivery and cosmetic packaging, Heating oil
Compatible solutes	Protein and cell protectants in a variety of industrial uses, such us freezing, heating
Various enzymes, such as amylases, proteases	Various industrial uses, such as flavouring agents
β-Linoleic acid, β-carotene and cell extracts, such as Spirulina and Dunaliella	Health foods, dietary supplements, food colouring and feedstock
Micro organisms	Fermenting fish sauces and modifying food textures and flavours.
Membranes	Surfactants for pharmaceuticals
HaloAlkaliphiles	
Proteases, cellulases,, lipases and pullulanases	Detergents
Proteases	Gelatine removal on X-ray film
Elastases, keritinases	Hide dehairing
Cyclodextrins	Foodstuffs, chemicals and pharmaceuticals
Xylanases and proteases	Pulp bleaching
Pectinases	Fine papers, waste treatment and degumming
Alkaliphilic halophiles	Oil recovery

Novel halophilic biomolecules can be used for specialized applications. For example, bacteriorhodopsin is used for specialized novel applications for biocomputing, biosensors, desalination of seawater and optical processing (Van den Burg, 2003; Casuso *et al.*, 2007; Raja *et al.*, 2007). Gas vesicles from halophiles have been utilised as floating particles in bioengineering (Ventosa and Nieto, 1995).

Halophile pigments are employed in food coloring, and compatible solutes from halophiles are used as stress protectants. Industrially, biopolymers with surfactant and bioemulsifying properties are used in oil recovery for hypersaline brines found with geological formations, where petroleum reserves occur. Halophiles are useful in decontaminating industrial brine by biodegradation of waste containing toxic organic compounds. Biotechnology employs halophile genes in production of polysaccharide of biological interest and in genetic systems inserting osmoregulatory genes to protect crops in water shortage (Llamas *et al.*, 2000). Inspite of the growing interest in the use of halophilic enzymes for biotechnological application there are relatively few reports in literature about their production and characterization.

2.6 Chicken feather degradation by bacteria

World-wide, poultry processing plants produce millions of tons of feathers as a waste product annually, which consists of approximately 90% keratin. Feathers represent 5-7% of the total weight of mature chickens (Matikevičienė *et al.,* 2009). These feathers constitute a sizeable waste disposal problem with landfill disposal being the most common. Although combustion of feathers forms another means of its disposal, it involves high energy expenses and can cause contamination of air, soil and water. Feathers hydrolysed by mechanical or chemical treatment can be converted to feedstuffs, fertilizers and glues or used in the production of amino acids. Keratin is an insoluble protein that is resistant to normal protease enzymes disintegration such as pepsin. Various authors have reported that, among the keratinolytic microorganisms, some species of *Bacillus*, actinomycetes, and fungi are able to produce these keratinases and peptidases (Mazotto *et al.*, 2011).

Biodegradation of poultry waste by keratinases is an environment friendly biotechnological process, which converts this abundant waste into low-cost, nutrient-rich animal feeds (Matikevičienė *et al.,* 2009). Most researchers working on poultry feathers degrading bacteria

have isolated the keratinolytic bacteria from poultry feather waste (Joshi *et al.,* 2007; Matikevičienė *et al.,* 2009). Zerdani *et al.,* 2004 isolated *Bacillus licheniformis* from non-treated soils in natural composting wastes in Morocco from which he compared their keratinolytic activities with *Bacillus subtilis*. No research has been done on isolation of feather degrading bacteria from the flamingo feathers in the alkaline saline lakes of Rift Valley. This study sought to find out the possibility of using the bacteria in the flamingo feathers in Lake Nakuru as a potential for chicken feather degradation.

CHAPTER THREE
MATERIALS AND METHODS

3.1 Study Area

This study was carried out in Lake Nakuru, a shallow, endorheic, hyper-eutrophic alkaline saline lake. It occupies an area of approximately 40 km^2 with a mean depth of 1 m at an altitude of 1,759 m above sea level and a geographical position of 00° 22´ S, 36° 05´ E. The lake is fed by three rivers, the Njoro, Makalia and Enderit (Figure 1) which are highly seasonal. It also has two inflows from Baharini springs at the northern end and the sewage discharge from the town´s sewage treatment plant (Ballot *et al.*, 2004).

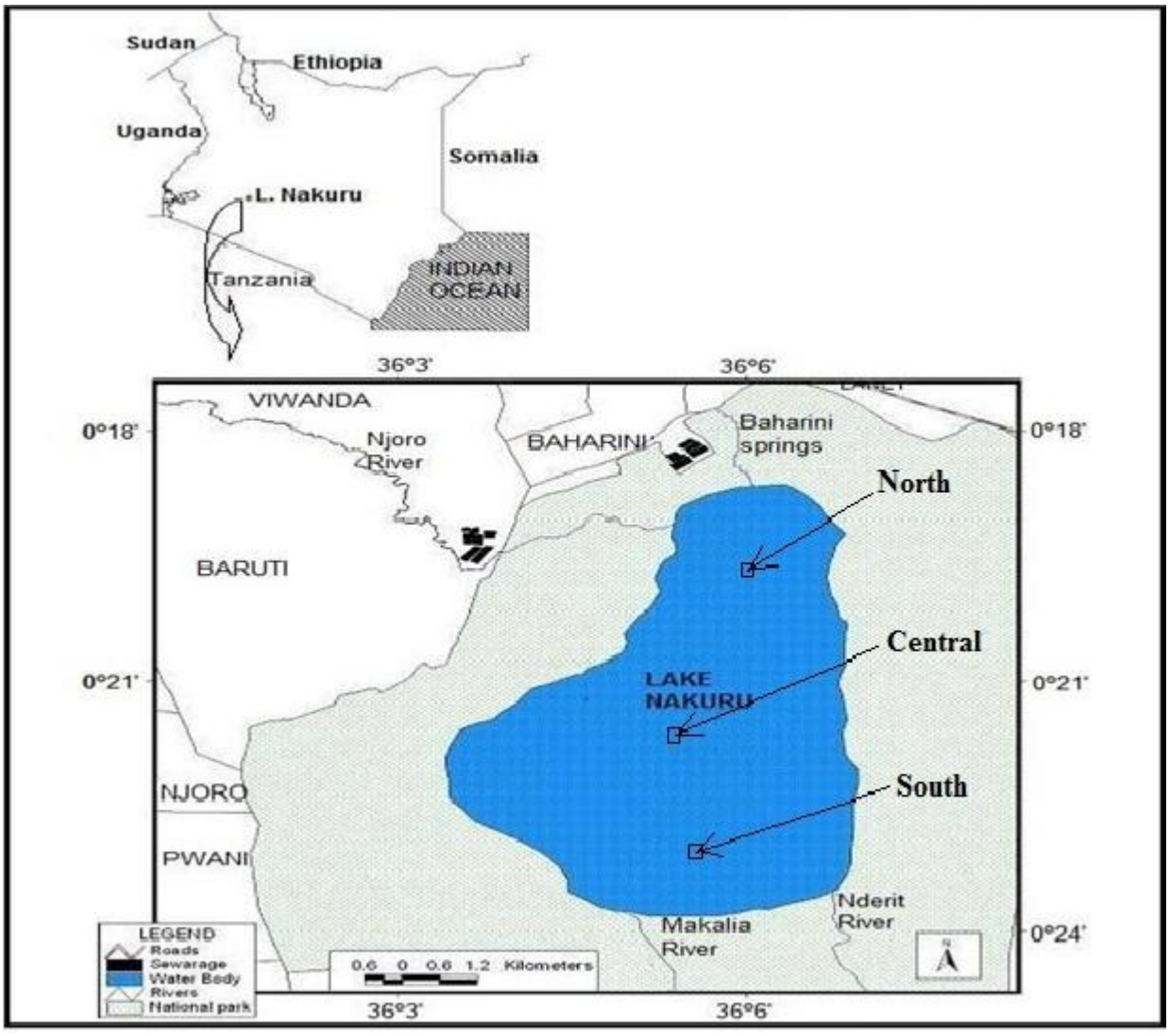

Figure 1: A map of Lake Nakuru showing the sampling points

Direct precipitation on the lake now appears to contribute more to its recharge than the river inflows since these rivers have become intermittent in their flow, flowing for less than three months in a year. The area of the lake varies greatly and is strongly influenced by the hydrological conditions in its catchment area. Though the lake offers unfavorable conditions for most aquatic life (pH 10.5, conductivity range of 76-80mS/cm) a few specially adapted species of aquatic flora and fauna form a very high producer and consumer biomass. As a simplified, discrete and productive system, Lake Nakuru offers a suitable object for research on microbial ecology.

3.2 Data collection

3.2.1 Nature of data collection

In this study, the data gathered was that relating to the four specific objectives aimed at meeting the broad objective of studying the distribution of bacteria, isolating and characterizing the physiological groups of bacteria with industrial potential in this lake. Data was collected for several variables that included the physico-chemical parameters of the lake namely temperature, pH, dissolved oxygen (DO) concentration, salinity, secchi depth and conductivity in order to assess the influence of these variables to the bacterial community within the lake. Similarly, data was collected on the densities of bacteria of both the bacteria and the cultivatable types to determine their distribution in the North, Central and Southern sampling points of the lake. The ability of the isolated bacteria in the degradation of macromolecules and feathers was measured and those bacteria with industrial potential were characterized by molecular means. The procedures used to achieve the objectives are as described below.

3.2.2 Sample collection, fixation and storage for bacterial counting

Sampling was done from a boat. An integrated water sample between surface and 60cm depth was collected using a Schindler sampler for enumeration of bacteria from three sampling points in the lake along a transect running from the Northern to Southern end of the lake representing the South (0° 23' 5.19" S, 36° 5' 41.44" E), Central (0° 21' 41.01" S, 36° 5' 14.29" E) and North (0° 19' 43.39" S, 36° 6' 2.78" E) sampling points. The samples were collected between 10am to 12pm. Three replicates of the samples for culturing and isolation of bacteria were collected at the 3 sites using sterile sampling bottles. The samples were stored in a cool box under ice and transported to Egerton University where further analyses were done. Samples for bacterial

density determination were preserved with formaldehyde (4% final concentration) on site and placed on ice. Degrading flamingo feathers (shown by pink color of flamingo) were collected from the North, Central and Southern shores of the lake aseptically by use of sterilized pair of forceps and placed in sterile sampling bags for isolation of feather degrading bacteria. The fixed samples were stored at 4ºC in the refrigerator until slide preparations and staining were made for counting. The samples were analyzed within 24 hours of their collection.

3.2.3 Physico- chemical parameters

The physico-chemical parameters such as dissolved oxygen concentration, pH, conductivity, salinity and temperature were measured *in situ* in the three sampling points of the lake using a multimeter probe (Hydro lab-Quanta Water Quality Monitoring System model no. QD02233). The secchi depth was determined using a secchi disk of 20cm diameter which measured the transparency of water by considering the difference in depth at which the disk appeared and disappeared in the water.

3.2.4 Enumeration of bacteria

The formaldehyde preserved water samples were stained with 0.01% final concentration of acridine orange (AO) dye (Hobbie *et al*., 1977) and incubated for 2 minutes in the dark. The AO stained samples were then filtered through a 25 mm diameter black polycarbonate (GTBP Millipore, 0.25µm pore size) filter placed on a 25 mm diameter HA cellulose acetate (Millipore) supporting membrane over a filtration apparatus. The damp filter was then embedded in paraffin oil on a glass slide and the preparation examined with epifluorescence microscope (Mitotic: EX D480/30X, DM505DCLPand BA D535/40m model) under blue light excitation (Figure 2). The numbers of bacteria per milliliter of sample were estimated from a count of 30 randomly chosen microscope fields on each slide. The total number of bacteria per ml of sample was determined according to the formula:

$$N = \left(\frac{S \times 10^6 \times n}{s \times v}\right) \times Dilutionfactor$$

(Porter and Feigh, 1980).

Where, N = number of cells ml^{-1} of sample, S = surface area of the filter in mm^2, n = mean number of bacteria per working field, s = surface area of working field in μm^2, v = volume of sample filtered in ml. At least 600 cells were counted per slide.

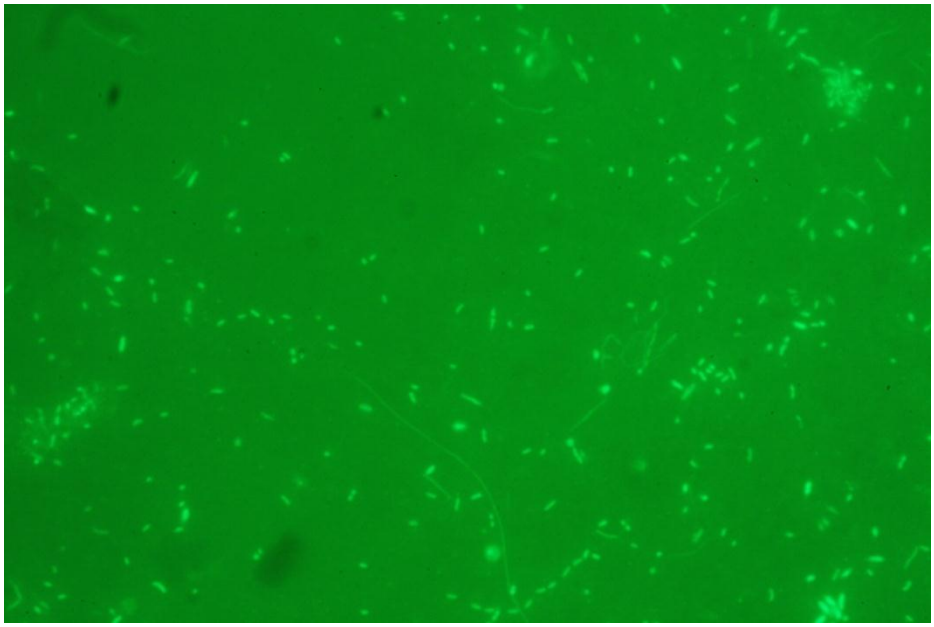

Figure 2: Bacteria stained by Acridine orange under epifluorescence microscopy

3.2.5 Isolation and pure culturing of bacteria

The media used in the isolation of bacteria was prepared using nutrient agar, 4% NaCl, and 1 % (w/v) Na_2CO_3 for pH adjustment as described by (Grant, 2006a). The media was autoclaved at 121°C for 15 min. Using a sterile pipette, 1 ml of the sample water was placed in 9 ml of sterile lake water to make dilution of up to 10^{-2}. A micropipette was then used to measure 0.1 ml of raw water and the diluted sample water which was inoculated on the media using spread plate method. The plates were incubated for 48 hours at 30°C. Colonies that appeared (Figure 3) were counted on dilutions with 30-300 colonies, mean number of colonies was multiplied with dilution factor to get number of CFU/ml of the lake water. The bacteria were then streaked on agar for pure culture isolation and characterization. Pure cultures were stored on agar slants in refrigerator at 4°C.

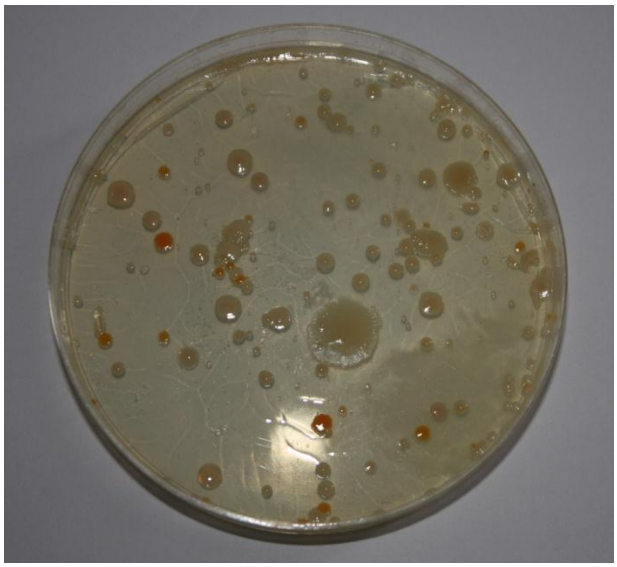

Figure 3: Culture growth of cultivatable bacteria from Lake Nakuru plated on nutrient agar

3.2.6 Characterization of isolates

Thirty (30) isolates of the bacteria from the spread plates that appeared different were characterized morphologically. Classification of the isolates as gram positive or gram negative was done by Gram stain reaction and KOH sensitivity test (Gregerssen, 1978). For Gram staining, the Color Gram 2 kit of bioMérieux (Marcy l'Etoile, France) was used while KOH sensitivity test a heavy mass of 24 hours bacteria and cells suspensions on a slide was mixed rapidly with circular motion with an inoculation loop for 15-30 seconds. The formation of a string (DNA) in 3 % (w/v) KOH indicated that the isolate is a gram negative organism. *Escherichia coli* and *Bacillus megaterium* were used as gram negative and gram positive controls, respectively. Cultural characteristics of bacteria were determined such as the colour of the colony, the texture, shape and measurement of colony diameter.

3.2.7 Test for presence of endospore

A wire loop was used to smear a colony of bacteria on a microscope slide and distilled water was used to spread the colony. A drop of malachite green was added and the slide was placed over a beaker containing boiling water. The wet stain condition was maintained for 10 minutes. The slide was then removed from the water bath and allowed to cool to room temperature. Once the slide was cooled to room temperature it was rinsed with tap water to remove the malachite green and was then counterstained with a drop of basic fuchsin (Beishir, 1991). After 1 minute the slide

was rinsed with dry bibulous paper. The slide was then examined on a microscope under oil immersion objective. Endospores appeared as green bodies within the cells.

3.3 Determination of extracellular enzyme production

All the strains isolated were screened for extracellular enzymatic activity as evidence of their ability to degrade macromolecules on solid media. The procedures for starch, cellulose, lipid and protein hydrolases are explained below.

3.3.1 Determination of protease producing bacteria

The isolates were screened for proteolytic activity using soft agar overlay containing casein 10g/l, agar 14g/l supplemented with 4% (w/v) of NaCl. 1% (w/v) of $Na2CO_3$ was added to adjust the pH to around 10.5. K_2HPO_4 1.0g/l, $MgSO_4.7H_2O$ 0.1g/l, yeast extract 1g/l and $CaCl_2.7H_2O$ 0.05g/l were added in the casein medium. Positive protease activity was detected by the presence of clear zone after incubation at 30˚C for 7 days (Rondon *et al.,* 2000). These colonies were isolated and streaked in fresh plates until single uniform colonies were obtained.

3.3.2 Determination of starch degraders

Cultures were screened for their ability to hydrolyze starch on agar medium with soluble starch 5g/l as substrate in which, agar 14g/l, 4% (w/v) NaCl and 1% (w/v) of Na_2CO_3 was added to adjust the pH to around 10.5 closer to that of Lake Nakuru water (Grant, 2006b). K_2HPO_4 1.0g/l, $MgSO_4.7H_2O$ 0.1g/l, yeast extract 1g/l and $CaCl_2.7H_2O$ 0.05g/l were added in the starch medium. The plates were incubated at 30-34ºC for 3 days (Rohban *et al.,* 2008). After incubation the plates were flooded with 1% iodine in 2% KI. The clear zones around the colony indicated the amylase activity (Collins and Lye, 1980).

3.3.3 Determination of lipolytic bacteria

The isolates were screened for lipase activity on olive medium containing olive oil 5ml/l, agar 14g/l, 4% (w/v) NaCl and 1% (w/v) Na_2CO_3. K_2HPO_4 1.0g/l, $MgSO_4.7H_2O$ 0.1g/l, yeast extract 1g/l and $CaCl_2.7H_2O$ 0.05g/l were added in the olive agar medium. The plates were incubated at 30ºC for 48hrs. A clear zone that developed around the colony in the olive agar medium indicated lipase activity (Collins, 1964).

3.3.4 Determination of cellulolytic bacteria

The isolates were screened for cellulase activity in agar medium with 5g/l carboxy methyl cellulose (CMC) as substrate, agar 14g/l, 4% (w/v) NaCl and 1% (w/v) Na_2CO_3, K_2HPO_4 1.0g/l, $MgSO_4.7H_2O$ 0.1g/l, yeast extract 1g/l and $CaCl_2.7H_2O$ 0.05g/l were added in the cellulose medium. The growth on incubated plates were stained with Congo red dye and destained with 1 M NaCl (Teather and wood, 1982). The positive cellulase activity was shown as presence of yellow hallo against red background.

3.4 Effects of macromolecule degradation on bacterial growth

The influence of macromolecules on growth of bacterial isolates showing positive results above was determined. Each flask containing known concentration of these macromolecules, starch, cellulose, proteins and lipids as the sole carbon source in the broth with inorganic salts that included; K_2HPO_4 1.0g/l, $MgSO_4.7H_2O$ 0.1g/l, yeast extract 1g/l and $CaCl_2.7H_2O$ 0.05g/l as inorganic nutrients was inoculated with the isolates. The inoculum was prepared by picking a loop from cultures on slants and suspending in saline water to have an optical density (OD_{600nm}) of about 0.4 giving a cell density of about 10^6 of the inoculum (Zerdani, *et al.,* 2004). The tubes were incubated at 34ºC. Increase in growth was measured by recording the turbidity of broths in the spectrophotometer every 24 hours interval for a period of 10 days.

3.5 Isolation of bacteria from flamingo feathers and determination of chicken feather degrading activity

The degrading flamingo feathers were placed in petri dish containing trypticase soy agar (TSA) with sterilized sample of the saline lake water with suitable pH close to that of the lake poised by the addition of 1% (w/v) of Na_2CO_3 in the TSA (Grant, 2006b). The feathers were then incubated at 30ºC for 24 hours. The bacteria that grew were streaked on agar for pure culture isolation and characterization. Pure cultures were then identified culturally and morphologically and then stored on agar slants in the refrigerator at 4°C.

To test for feather degradation, 4g of feathers (cut into 2 cm pieces) suspension used as the sole carbon source was incubated with the test bacteria in a flask at optimum pH of growth, salinity and temperature conditions for growth. An aliquot of the content in the flask was taken and the optical density of bacteria and feathers was measured at 600nm at given time intervals according

to Zerdani *et al*., 2004. An increase in OD was an indication of degradation and subsequent utilization of these feathers as carbon source by bacteria hence increase in numbers and turbidity resulting in the increased OD. The dry weight of feather was determined by collecting, a piece of feather from the feather suspension and placing it in the pre-weighed crucibles and then dried at 60^{o}C for 24 hours after which they were ashed at 500^{o}C for four hours to obtain the ash free dry mass. This was done after every 24 hours to the inoculated feathers. The dry weight and ash free dry weights were recorded. The decrease in weight of the feathers was an indication of growth of bacteria.

3.6 Characterization of bacteria with industrial potential

3.6.1 Biochemical tests

The biochemical tests described in Bergey's Manual of Systematic Bacteriology 2nd Edition Volume 2 (2005) were used to characterize the bacteria with the best ability to degrade the macromolecules and chicken feathers. The biochemical tests included catalase, gelatin hydrolysis, urease and citrate utilization.

3.6.1.1 Catalase test

Hydrogen peroxide in the quantity of 2-3ml was poured into a test tube and using a sterile glass rod, a good growth of the test organism was picked from a bacterial colony in Nutrient Agar and immersed in the hydrogen peroxide solution. Presence of immediate bubbling indicated a positive test.

3.6.1.2 Gelatin hydrolysis

A loop of bacterial colony was picked from bacterial cultures incubated on nutrient agar and inoculated by stab inoculation in Nutrient gelatin deep tubes. The tubes were incubated for 24-48 hours at 30°C after which they were placed into a refrigerator at 4°C for 30 minutes. Cultures that liquefied produced gelatinase and demonstrated gelatin hydrolysis.

3.6.1.3 Urease test

A loop of bacterial colony was picked from the bacterial cultures stored in glycerol and inoculated on urea broth and incubated for 24-48 hours at 30°C. Presence of deep pink color indicated a positive urease test.

3.6.1.4 Citrate utilization

A wire loop was used to inoculate 3-4 ml of sterile Koser citrate medium (oxoid) with a broth culture of bacteria. The inoculated broth was incubated at 34°C for up to 4 days. Presence of turbidity and blue color indicated citrate utilization.

3.6.2 Alkaliphily test

For the alkaliphily test, the isolates were inoculated into enriched liquid medium and suitable temperature and salinity adjusted at different pH values of 6, 7, 9, 10 and 11. The pH was adjusted by addition of HCl for acidic state and 1% (w/v) Na_2CO_3 for alkaline state and incubated at 30 °C for 72 hours. Their OD_{600nm} were measured before incubation and after, every 24 hours.

3.6.3 Temperature effect

The isolates were also grown on enriched liquid medium and suitable pH of 10 and salinities of 4% w/v NaCl at different temperature values of 25°C, 30°C, 35°C and 40°C. Their optical densities were measured at 600nm after every 24 hours for 3 days.

3.6.4 Molecular characterization of isolates

Extraction of DNA from isolates and PCR were carried out at Biotechnology Laboratory at KARI plant breeding station in Njoro. The procedure described by Doyle and Doyle (1990) was used for the extraction of bacteria DNA, while Qiagen kit was used for the DNA purification as per the manufacturer's instruction.

3.6.4.1 Extraction of DNA from bacteria colonies

The cells of isolates LNS08, LNC06, LNC09 and LNC11 were harvested from colonies in nutrient agar and re-suspended in 700µl of CTAB for 10 minutes. Centrifugation of these contents was done at 13000 rpm for 10 minutes and 650µl of supernatant was transferred to fresh tubes and equal volume of chloroform: isoamyl alcohol 24:1were added. The tubes were well shaken and then centrifuged at 13000rpm for 10 minutes. The content was vortexed gently and incubated at 65ºC for 30 minutes with the tubes inverted, 600µl aqueous phase was transferred to a fresh tube carefully avoiding the interphase. Ice-cold isopropanol was then added and the tubes inverted gently.

The content was then centrifuged at 14500 rpm for 10 minutes and the supernatant poured, 500µl of 70% ethanol was then added and centrifuged at 14500 rpm for five minutes. The supernatant was again poured and the pellets left to air dry for 30 minutes and re-suspended in 100µlof de-ionized water, 5µl of 1mg/ml of RNase was added and then incubated at 37°C for 15 minutes. After incubation, 10µl of 5mg/ml Proteinase K was added and incubated at 37°C for 1 hour. Centrifugation was done for 4 minutes at full speed in a micro-centrifuge. The aqueous (upper) phase was transferred into a new eppendorf tube and 100µl of 7.5 M NH_4OAc was added to precipitate the DNA. Ice cold absolute EtOH 800µl was added, mixed gently and incubated at -20°C for 30 minutes. The content was then centrifuged for 5 minutes in a microfuge and the supernatant poured off. The pellets were then washed with 1 ml 75% EtOH and centrifuged for 4 minutes in a microfuge and the supernatant poured off. The pellets were air dried for 30 minutes and then dissolved in 50µl TE-8.0.

3.6.4.2 PCR amplification of 16S rRNA genes

The genomic DNA of these isolates was extracted as above and 16S rRNA gene amplified using two primers set targeting intergenic regions of the bacterial DNA. The first set of primers is the universal primers PC3 Mod Forward (5’ - GGACTACAGGGTATCTAAT - 3') and P0 Mod Reverse (5’ - AGAGTTTGATCATGG - 3'). The other set of universal primers is Primer P1 Forward (5′ - AAGAGTTTGATCCTGGCTCAGGATT - 3′) and P6 Reverse (5′ - CGGTAGGGATACCTTGTTACGACTTA- 3′). Genomic DNA was subjected to amplification using a PCR Cycler (Biometra). Amplification reaction mixture contained 0.5 µl each of the forward and reverse primers, template DNA 2µl, GoTaq (DNA polymerase) 9µl, dNTP (10mM) 0.5µl, PCR buffer 1.75µl, $MgCl_2$ (50mM) 0.75 µl and sterile H_2O 5µl, in a final volume of 20µl.

The following conditions were used in the amplification of 16S rRNA gene: 94°C for 3 min, followed by 35 cycles of heat denaturation at 94°C for 30 sec, primers annealing at 50°C for 30 sec, and DNA amplification at 72°C for 30 sec. Final extension of the amplified DNA was done at 72°C for 2 min. The 600-bp PCR product was visualized by electrophoresis on 1.8% (w/v) agarose gel containing 0.02% final concentration of ethidium bromide.

3.6.4.3 DNA sequencing

The PCR products were purified from agarose gel using a QIA quick gel extraction kit according to the instructions of manufacturer (Qiagen, Docking, Uk). Sequencing of the PCR products was carried out in Macrogen Lab in Korea (908 World Meridian Venture Center, #60-24, Gasan-dong, Geumchun-gu, Seoul 153-781, Korea). Analysis of the reaction mix was performed in a DNA sequencer (Applied Biosystems). The sequence was assembled using the 'sequencer' program, edited with the BioEdit program. The sequence data was subjected to BLAST (http://www.ncbi.nlm.nih.gov/BLAST/) and analyzed against the genebank 16S r RNA database.

3.6.4.4 Phylogenetic analysis

Phylogenetic analysis was performed using the software package MEGA Version 5 (Tamura *et al.,* 2011). Subsequently, CLUSTALW alignment tool in MEGA Version 5 was used to align the sequences from the database. The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura *et al.,* 2004). Clustering was performed using the neighbor joining method (Saitou and Nei 1987). With the help of phylogenetic tool in MEGA Version 5 the phylogenetic tree was visualized.

3.7 Data analyses

The computer-based Statistical Package for the Social Sciences (SPSS version 17 for windows) was used for data analysis to yield various statistics that helped to explain the objectives for this study.

The collected data on the density of bacteria from the 3 sites were computed to find the mean densities at each sampling site for the whole lake. ANOVA was then used in comparing the means of these densities. To find out if there was any significant difference between the 3 sites, the significant means of bacterial densities in ANOVA test were separated using the LSD as the Post Hoc test. ANOVA was also used to compare means between the months of sampling for number of cultivatable bacteria, physical chemical parameters such as pH, temperature, salinity, DO and conductivity. Correlations between the total counts and heterotrophic bacteria were done. Regression analysis was used to determine relationships between optical density, ash free dry weights and dry weight of chicken feathers.

CHAPTER FOUR

RESULTS

4.1 Physico-chemical parameters

4.1.2 Spatial variation in physico-chemical parameters

The means and standard deviations of physico-chemical variables in Lake Nakuru are summarized in Table 3. The temperature values recorded during the study did not show any significant variation between the Southern and Central sampling points. However, the southern point of the lake had the highest value of 27.3°C±1.7, and this was significantly different from the Northern point of the lake. The dissolved oxygen concentration in the lake ranged from 11.3-15.1 mg/l. The Northern part of the lake had the lowest dissolved oxygen (11.3±8.2 mg/l) which was significantly different from that of the Southern and Central parts of the lake.

The pH value was 10.3±0.1 in all the 3 sampling points. The conductivity and salinity values recorded in the Southern point of the lake were 35.5±15.8 mS/cm and 21.6±11.2‰ respectively (Table 3). The variations in conductivity and salinity were not significant between the 3 sampling points. The Northern sampling point had the lowest conductivity and salinity values of 31.0±16.7 mS/cm and 19.0± 11.5‰ respectively compared to the other sampling points. The secchi depth values ranged from 24.5-25.3 cm with no significant variation between the 3 sites.

Table 3: Spatial variations of physico-chemical variables in Lake Nakuru (mean ± SD) from January to June, 2010.

Sites	Temp (°C)	Oxygen (mg/l)	pH	Cond (mS/cm)	Salinity (‰)	Secchi depth(cm)
South	27.3± 1.7^{b}	15.1± 7.1^{b}	10.3± 0.1^{a}	35.5± 15.8^{a}	21.6±11.2^{a}	25.2± 11.8^{a}
Central	26.3± 2.0^{ab}	15.1± 8.4^{b}	10.3± 0.1^{a}	32.7± 19.7^{a}	21.2±14.9^{a}	24.5± 12.9^{a}
North	26.0± 3.3^{a}	11.3± 8.2^{a}	10.3± 0.1^{a}	31.0± 16.7^{a}	19.0± 11.5^{a}	25.3± 8.6^{a}

Means sharing the same superscript letter notations are not significantly different from each other, α=0.05.

4.1.3 Temporal variations in physico-chemical parameters

The temperature of the lake during this study ranged between 25.3 and 28.8 °C with the highest value being recorded in April which was significantly different from the other months as shown in Table 4.

The highest dissolved oxygen concentration of 18.4±5.1 mg/l was recorded in the month of February during the onset of the rainfall in the lake. This concentration decreased sharply in April with a mean value of 2.0±1.3 mg/l.

The pH values in the lake ranged between 10.1±0.1 and 10.3±0.1 with no significant temporal variations during this study period. The conductivity and salinity values decreased from January to June with a range of 55.5±14.2-16.6±5.7ms/cm for conductivity and a range of 38.0±11.9-9.6±3.4 ‰ salinity.

The secchi depth of the lake varied between 13.0±1.9 cm and 38.7±10.1cm. It was highest in the month of June which was significantly different from all the other months (Table 4).

Table 4: Temporal variations of physico-chemical variables in Lake Nakuru from January to June, 2010

Months	Temp (ºC)	Oxygen (mg/l)	pH	Cond (ms/cm)	Salinity (‰)	Secchi depth (cm)
January	26.2± 0.9^{a}	15.9± 6.7^{c}	10.1± 0.1^{a}	52.3± 15.1^{d}	35.5±14.6^{d}	14.6± 0.7^{a}
February	25.3± 2.8^{a}	18.4± 5.1^{d}	10.3± 0.1^{a}	55.5± 14.2^{d}	38.0±11.9^{d}	13.0± 1.9^{a}
March	26.5± 2.1^{a}	13.9± 2.7^{b}	10.2± 0.0^{a}	44.9± 12.5^{c}	27.9± 8.1^{c}	16.0± 1.1^{a}
April	28.8± 2.2^{b}	2.0± 1.3^{a}	10.3± 0.0^{a}	34.7± 2.6^{b}	19.8± 0.7^{b}	28.8± 2.2^{c}
May	26.7± 2.4^{a}	17.3± 8.2^{dc}	10.3± 0.1^{a}	20.0± 4.4^{a}	11.5± 2.9^{a}	23.2± 2.4^{b}
June	25.9± 1.9^{a}	16.9± 7.1^{dc}	10.3± 0.0^{a}	16.6± 5.7^{a}	9.6± 3.4^{a}	38.7± 10.1^{d}

Means sharing the same superscript letter notations are not significantly different from each other, α=0.05.

4.2 Bacterial densities in the lake

The total counts of bacteria in the lake were high as compared to the heterotrophic plate counts (Table 5). Spatial and temporal variations in bacterial densities were observed in the lake as described in the following sections.

4.2.1 Spatial variation in total counts (TC) and heterotrophic plate counts (HPC)

Generally, the Northern part of the lake had the highest number of TC as indicated by its mean value of $1.8\times10^{8}\pm 7.3\times10^{7}$/ml. The Southern and Central points were not significantly different (Table 5). There was a significant difference in the total counts of bacteria between the 3 sites (One way ANOVA, $F_{(2,99)}=3.926$, $P<0.05$).

Post Hoc analysis indicated significant difference in terms of TC between the Central and North and South and North sampling sites ($P<0.05$) . The South and Central sampling points were not significantly different ($P>0.05$) and they recorded lower numbers compared to the North sampling point.

There were no significant differences in heterotrophic plate counts between the 3 sampling sites (One way ANOVA, $F_{(2,99)}= 1.815$ $p>0.05$). The Northern point of the lake had the highest number of cultivatable bacteria with a mean value of $1.4\times10^{5}\pm1.4\times10^{5}$CFU/ml while the Central point had the lowest mean number of bacteria $9.2\times10^{4}\pm 9.6\times10^{4}$CFU/ml.

Table 5: Spatial variations in total counts and heterotrophic plate counts in Lake Nakuru from January to June, 2010.

Sites	Mean HPC CFU/ml	Mean TC/ml
South	$9.8\times10^{4}\pm 1.1\times10^{5a}$	$1.4\times10^{8}\pm 6.9\times10^{7a}$
Central	$9.2\times10^{4}\pm 9.6\times10^{4a}$	$1.4\times10^{8}\pm 4.5\times10^{7a}$
North	$1.4\times10^{5}\pm 1.4\times10^{5a}$	$1.8\times10^{8}\pm 7.3\times10^{7b}$

Means sharing the same superscript letter notations are not significantly different from each other, $\alpha=0.05$.

4.2.2 Temporal variations in total counts of bacteria

Results for temporal variation in total bacterial counts are shown in Figure 4. From January to March, the Southern point had the lowest numbers of TC with values as low as 8.1×10^{7}/ml recorded on 4/02/2010. The TC then picked steadily (within the three months) ranging from 8.8-9.0×10^{7}/ml in the month of April when it reached the peak. Total counts of bacteria were highest in the month of April with a mean value of $2.4\times10^{8}\pm5.1\times10^{7}$/ml as shown in Table 6.

At the beginning of the study, the Central point had a low TC value of 9.9×10^7/ml. This number increased steadily up to 1.4×10^8/ml on 10/03/2010. A slight decrease was recorded on 25/03/2010 which then increased sharply in the month of April and then dropped drastically in the month of May. A sharp increase in TC was recorded towards the beginning of the month of June which again dropped in the middle and at the end of the month with the lowest value of 7.5×10^7/ml being recorded.

In the Northern sampling point of the lake, a higher value of 1.4×10^8/ml was recorded in February compared to the Central and Southern sampling points. There was an increase in TC at the beginning of March in the North of the lake which again dropped during the end of March but picked sharply in April when the highest number of TC in the lake (3.3×10^8/ml) was recorded.

Generally the northern sampling point had the highest bacterial counts for both HPC and TC with a mean TC of 1.8×10^8/ml as compared to 1.4×10^8/ml for Southern and Central sampling points. The number of TC at this site then dropped steadily from the end of April and reached the lowest value of 5.3×10^7/ml towards the end of June (Table 6).

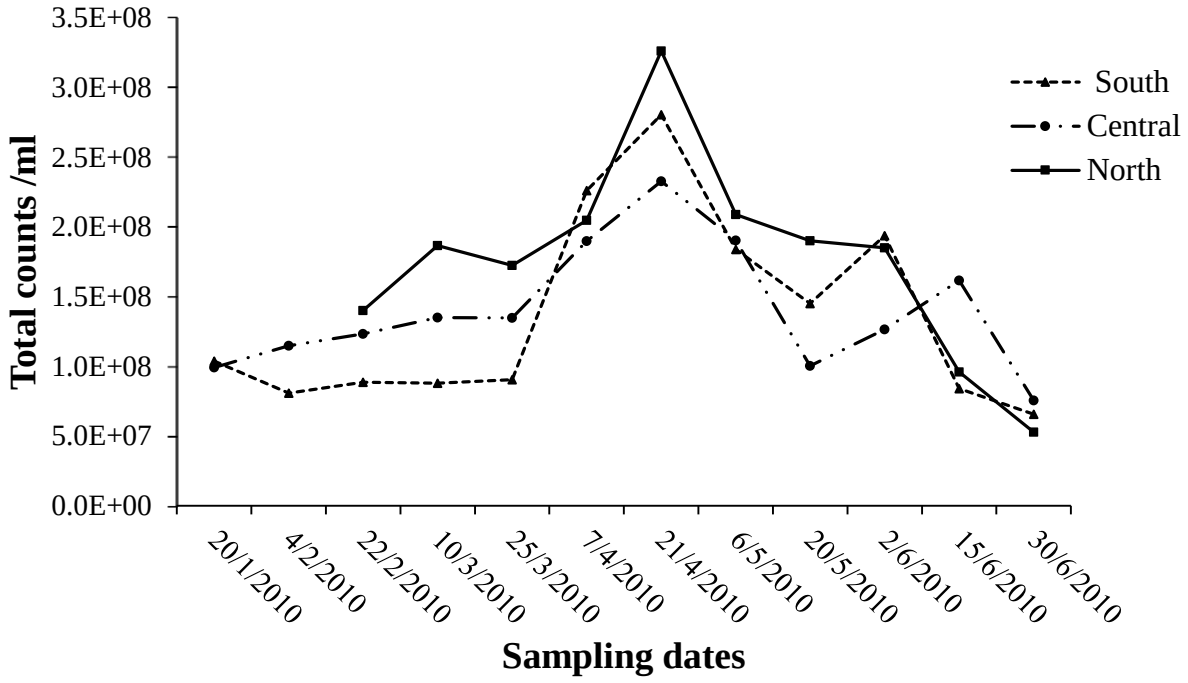

Figure 4: Temporal trends of total counts of bacteria in Lake Nakuru from January to June 2010

4.2.3 Temporal variations of heterotrophic plate counts

The data on temporal variations of HPC is shown in Figure 5. There was no notable variation in HPC from January to February between the Southern and Central sampling points. Although sampling in the North began towards the end of February, there was no major variation in HPC numbers for the 3 sampling sites from February to April. The numbers increased sharply at the beginning of April with the Northern point having the highest HPC of 5.0×10^5CFU/ml while the South and Central had 3.8×10^5CFU/ml and 3.6×10^5CFU/ml respectively. The HPC number in the Northern point decreased sharply towards the beginning of the month of May and continued with this decrease up to the end of the study.

Although the number of HPC in the lake decreased towards the month of June, South and Central sampling points exhibited similar trends with slight increase in HPC values in mid-June, followed by a drop in numbers at the end of the month.

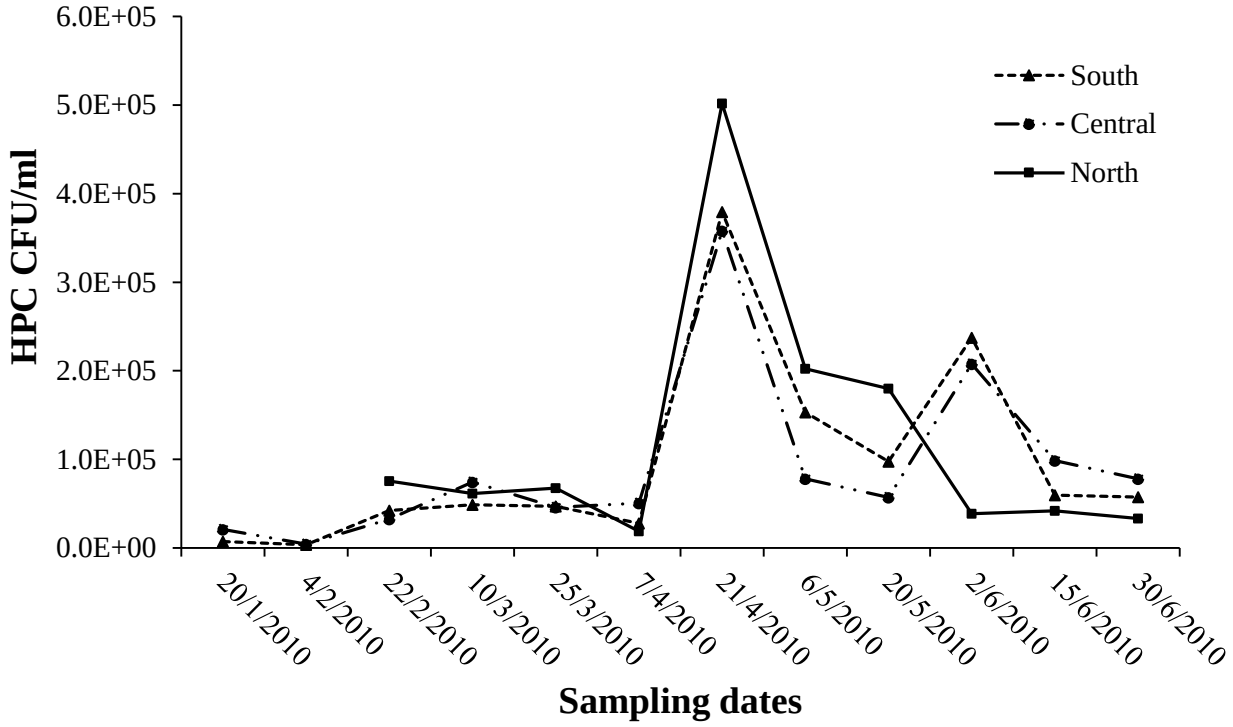

Figure 5: Temporal trends of heterotrophic plate counts in Lake Nakuru from January to June 2010.

There was significant temporal variations in HPC (One way ANOVA, $F_{(5, 96)}=8.832$, $p<0.005$) as shown in Table 6. Post hoc test indicated significant difference between all the months in terms of HPC except for the months of May and June which were statistically the same in post hoc analysis (LSD $P> 0.05$).

In terms of the total counts, there were significant differences between the months of sampling and the total counts in the lake (One way ANOVA, $F_{(5,96)}=24.588$, $p<0.005$). Post hoc analysis indicated significant difference between the months of April and May with the other months while January, February, March and June had no significant differences ($P>0.05$).

Table 6: Temporal variations of heterotrophic plate counts (HPC) and total counts (TC) in Lake Nakuru from January to June 2010.

Months	**Mean HPC CFU/ml**	**Mean TC/ml**
January	$1.4\times10^{4}\pm 7.8\times10^{3a}$	$1.0\times10^{8}\pm6.9\times10^{6a}$
February	$3.2\times10^{4}\pm 2.9\times10^{4b}$	$1.1\times10^{8}\pm 2.5\times10^{7a}$
March	$5.4\times10^{4}\pm 1.3\times10^{4c}$	$1.3\times10^{8}\pm 4.0\times10^{7a}$
April	$2.2\times10^{5}\pm 2.0\times10^{5d}$	$2.4\times10^{8}\pm 5.1\times10^{7d}$
May	$1.3\times10^{5}\pm 5.6\times10^{4e}$	$1.7\times10^{8}\pm 4.4\times10^{7b}$
June	$1.2\times10^{5}\pm 8.2\times10^{4e}$	$1.2\times10^{8}\pm 5.3\times10^{7a}$

Means sharing the same superscript letter notations are not significantly different from each other, $\alpha=0.05$.

The number of heterotrophic plate counts increased with increase in total counts of bacteria in the lake giving a positive correlation between the two parameters (Figure 6). Densities of TC were 3 powers of magnitude higher than HPC.

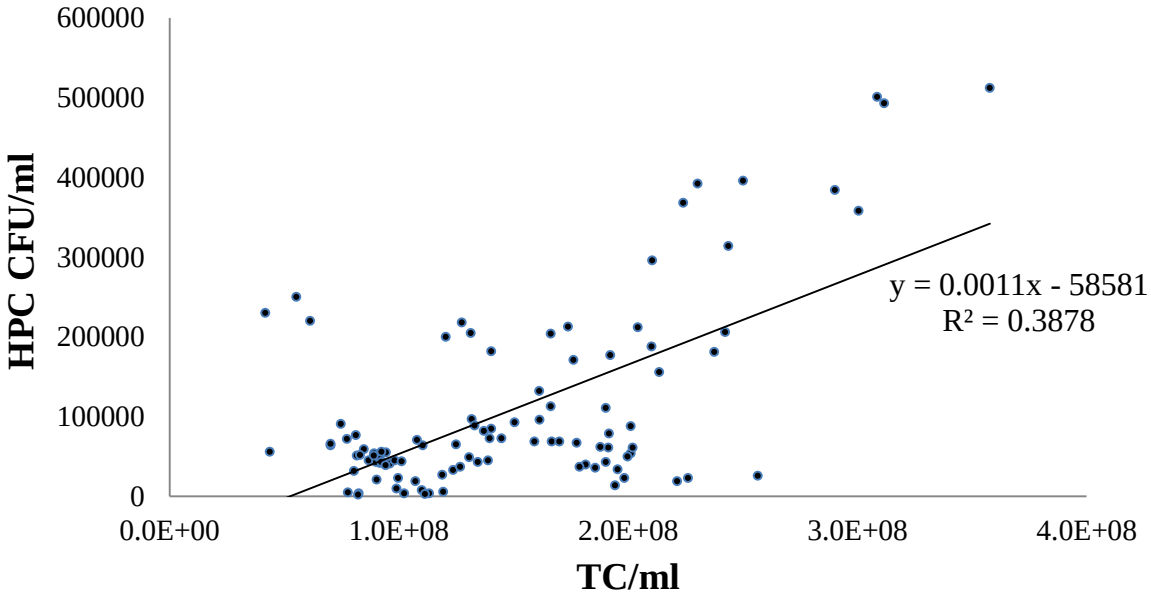

Figure 6: Relationship between total counts and heterotrophic plate counts in Lake Nakuru from January to June 2010.

4.3 Bacterial isolates from Lake Nakuru

Thirty isolates were selected, with most of them being circular in form, entire and cream in colour. Among these bacteria, gram negative isolates occurred more frequently than gram positive types. There were 19 gram-negative forms and 11 gram-positive rods (Table 7). The Central part of the lake had the highest number of isolates with 14 isolates including the ones isolated from the flamingo feathers. Bacteria isolated from the flamingo feathers were gram-positive endospore forming rods.

Table 7: Morphological characteristics of the isolates from Lake Nakuru

Code	Form	Size (mm)	Edge	Optical Characteristics	Texture	Colour	Gram reaction	3% KOH	Shapes	Endospores
LNS05	circular	0.5	entire	translucent	Rough	orange	-	+	Cocci	absent
LNS02	circular	2	entire	translucent	Smooth	egg/york yellow	-	+	Cocci	absent
LNS06	circular	1	entire	opaque	smooth	orange	-	+	Rods	absent
LNS04	circular	1	entire	opaque	rough	cream	-	+	Cocci	absent
LNC03	irregular	4	undulate	opaque	rough	yellow	-	+	Spiral	absent
LNC02	circular	1	entire	opaque	smooth	orange	-	+	Cocci	absent
LNC01	irregular	2	undulate	opaque	smooth	orange	-	+	Spiral	absent
LNS07	filamentous	3	filamentous	opaque	smooth	yellow	-	+	Rods	absent
LNS01	circular	2	entire	translucent	smooth	white	-	+	Rods	absent
LNS03	circular	4	entire	clear/translucent	rough	colourless	-	+	Cocci	absent
LNS08	circular	1.5	entire	opaque	smooth	Bright yellow	-	+	Short rods	absent
LNN02	circular	3	entire	translucent	smooth	white	-	+	Cocci	absent
LNN07	circular	7	entire	opaque	smooth	yellow	-	+	Cocci	absent
LNN08	irregular	1	undulate	opaque	smooth	yellowish	-	+	Rods	absent
LNN01	circular	1	entire	opaque(granules)	smooth	cream	-	+	Cocci	absent
LNN03	circular	3	entire	opaque	rough	cream white	-	+	Cocci	absent
LNC10	circular	4	undulate	opaque	smooth	cream	+	-	Rods	present
LNC11	circular	5	undulate	translucent	smooth	cream	+	-	Rods	present
LNC12	circular	3	undulate	opaque	smooth	white	+	-	Rods	present
LNC13	circular	1	entire	opaque	smooth	yellow	+	-	Rods	present
LNC14	circular	3.5	entire	opaque	rough	cream	+	-	Rods	present
LNC15	circular	4	entire	opaque	smooth	cream	+	-	Rods	present
LNN05	circular	2.5	entire	opaque	smooth	orange	-	+	Cocci	absent
LNN06	circular	2	entire	translucent	smooth	white	-	+	Cocci	absent
LNN04	circular	9	entire	translucent	Smooth/shiny	cream	-	+	Rods	absent
LNC06	irregular	27	undulate	opaque	rough	white	+	-	Rods	present
LNC07	irregular	18	undulate	opaque	rough	white	+	-	Rods	present
LNC08	irregular	15	entire	opaque	rough	white	+	-	Rods	present
LNC09	circular	5	undulate	translucent	smooth	cream	+	-	Long rods	present
LNC05	circular	7	undulate	opaque	rough	white	+	-	Cocci	present

4.4 Hydrolytic activity of isolates

The ability to produce 4 different hydrolases was tested among the isolates. A total of 8, 6, 5 and 5 isolates were able to produce amylase, protease, lipase and cellulase enzymes, respectively (Table 8). From the hydrolytic tests of the isolated bacteria on the four different macromolecules, out of the tested 30 isolates, 26.7% showed positive results in degradation of starch. Gram staining revealed that out of the 8 isolates that tested positive for amylolytic activity, 4 of them were gram positive and the other 4 were gram-negative. 20 % of the isolates were able to utilize protein as the sole carbon with 3 of them being gram-positive and the other 3 being gram-negative. Lipase and cellulase isolates represented 16.7 % of the isolates. One of each tested isolate for the lipase and cellulase enzyme was gram-positive while the other 3 were gram negative. The other 20 % did not show any enzyme activity (Table 8).

Table 8: A summary of the hydrolytic activities of bacterial isolates from Lake Nakuru from January to June 2010.

Enzymes	**Gram Positive**	**Gram Negative**	**Total**	**Percentage (%)**
Protease	3/11	3/19	6/30	20
Amylase	4/11	4/19	8/30	26.7
Cellulase	1/11	4/19	5/30	16.7
Lipase	1/11	4/19	5/30	16.7
No enzyme	2/11	4/19	6/30	20

The study also showed combined hydrolytic activities in certain isolates. Isolate LNN05 showed 3 hydrolytic activities, being able to degrade proteins, cellulose and lipids. Isolate LNS04 exhibited ability to hydrolyze all the tested four macromolecules. Four isolates tested showed ability to hydrolyze two macromolecules while several isolates were able to hydrolyze one form of macromolecule, especially starch.

Among the 3 best proteolytic isolates, LNC06 showed highest activity on protein media than LNN03 and LNC07 as indicated by the mean diameter of the colony size and the zone of activity (Figure 7). Isolate LNC09, LNC11 and LNC05 had the highest starch activity exhibited by the size of the colony and the zones of activity and thus selected for the degradation of starch as sole carbon source.

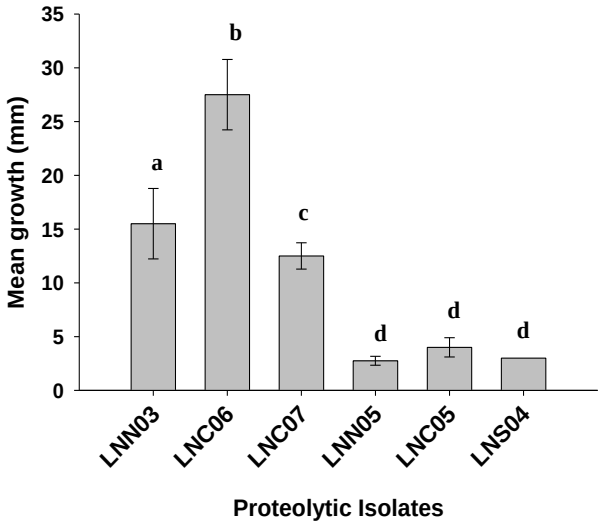

Figure 7: The mean diameter (mm) of the zones of activity of various proteolytic isolates from L. Nakuru within10 days of incubation.

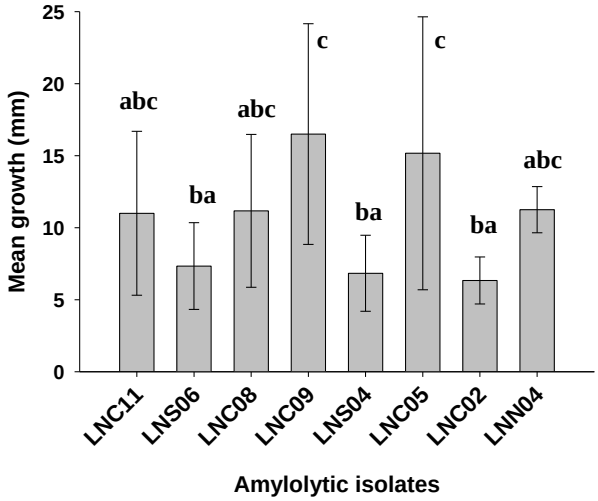

Figure 8: The mean diameter (mm) of the zones of activity of various amylolytic isolates from L. Nakuru within10 days of incubation.

Vertical Bars=Means± Standard deviation, bars sharing the same letter indicate no significant difference in the diameter of zones of activity at $P<0.05$

In the degradation of cellulose, isolate LNC09 showed the highest activity (Figure 9). Isolate LNS04, LNS05 and LNC09 were selected for the degradation of cellulose because of their higher growth rate as compared to the other cellulase positive isolates.

Isolate LNN04, LNS04 and LNS08 were selected for testing their effect on lipid broth. The 3 isolates were selected based on the diameter of the zone of activity on the lipid solid media an indication of high growth and adaptability (Figure 10).

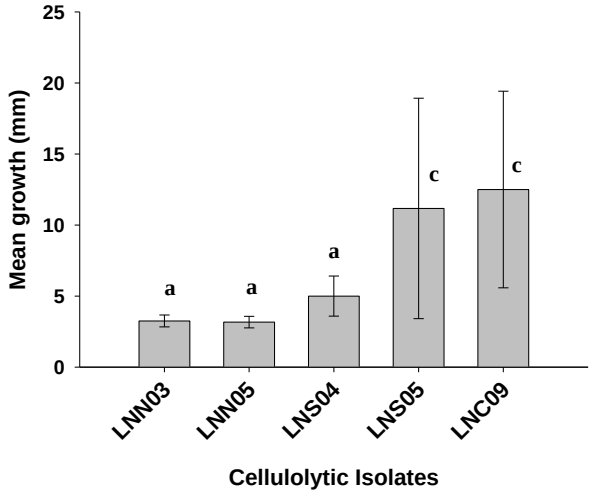

Figure 9: The mean diameter (mm) of the zones of activity of various cellulolytic isolates from L. Nakuru within 10 days of incubation.

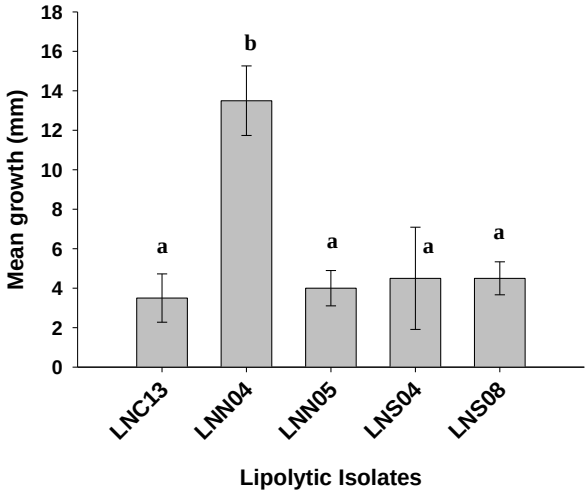

Figure 10: The mean diameter (mm) of the zones of activity of various lipolytic isolates from L. Nakuru within 10 days of incubation.

Vertical Bars=Means± Standard deviation, bars sharing the same letter indicate no significant difference in the diameter of zones of activity at $P<0.05$

4.5 Utilization of macromolecules by selected isolates

The optical densities observed in the starch broth were high indicating high activities of the isolates in the degradation of starch. The mean optical densities ranged from 0.928-1.951. Isolates with cellulolytic ability showed the least activity in these experiments giving low optical density values that ranged between 0.406 and 0.564. All the tested isolates showed positive activity in the degradation of different macromolecules but the overall degree of activity varied especially in starch which was the highest, followed by proteins then lipids and lastly cellulose.

4.5.1 Protein utilization

From the start of the protein hydrolysis experiment up to the 7th day the activities on protein substrate by the 3 isolates showed no noticeable variations. After the 7th day onwards, the degradation of protein by isolate LNC06 increased faster, from an optical density of 0.555 to 0.939 at day 10. The optical densities of isolate LNN03 and LNC07 changed minimally (Figure 11).

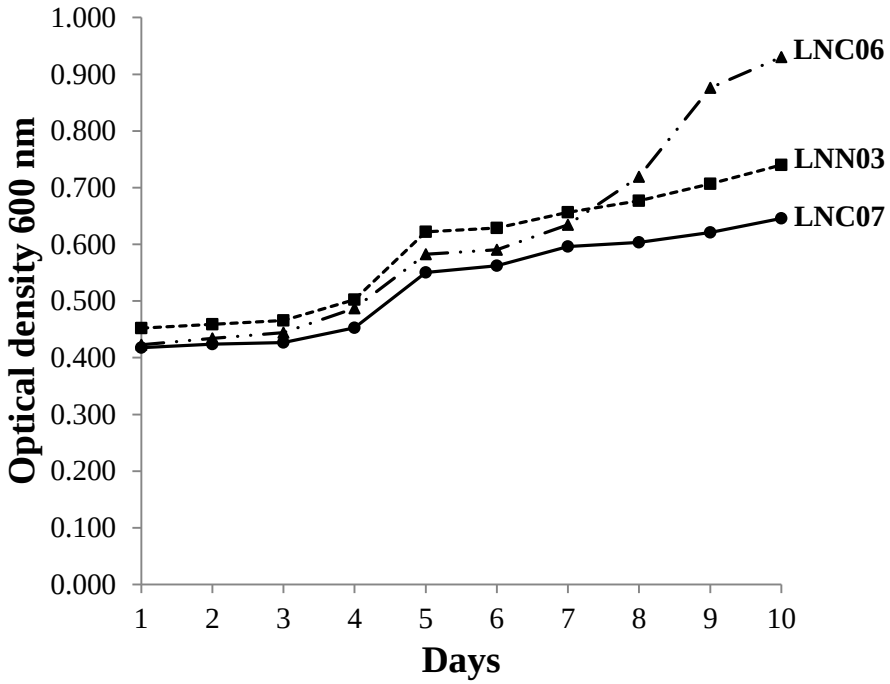

Figure 11: Pattern of degradation of protein containing broth by isolate LNN03, LNC06 and LNC07 in Lake Nakuru

4.5.2 Starch utilization

There was no significant difference in the degradation of starch by isolates LNC11, LNC05 and LNC09. However, isolate LNC05 showed the lowest activity (Figure 12). Isolates LNC09 and LNC11 had almost equal activity in the degradation of starch. The 3 isolates were the most important starch degraders. All these isolates (LNC05, LNC09 and LNC11) were isolated from the Central sampling point of the lake.

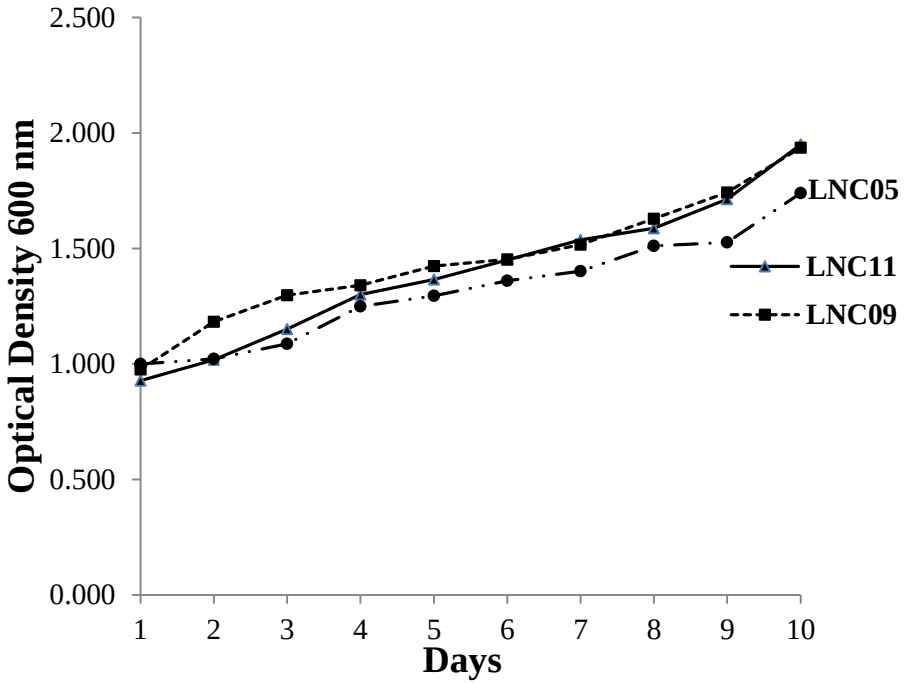

Figure 12: Pattern of degradation of starch containing broth by isolate LNC11, LNC09 and LNC05 in Lake Nakuru

4.5.3 Cellulose utilization

In the degradation of cellulose, isolate LNC09 showed the greatest activity during the 10 days of experiment with a mean OD_{600nm} of 0.564. There was no significant variation in the rate of degradation of cellulose between LNS05 and LNS04, both of which are also active in cellulose degradation.

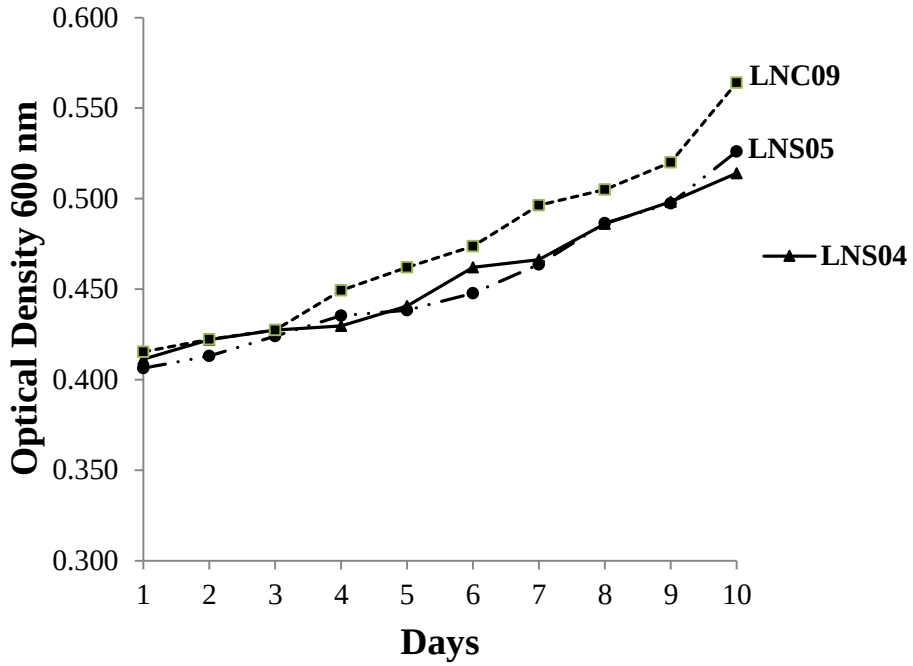

Figure 13: Growth of isolates LNS04, LNS05 and LNC09 from Lake Nakuru on cellulose containing broth.

4.5.4 Lipids utilization

Isolate LNS08 showed the most rapid growth compared to LNS04 and LNN04 in the degradation of lipids (olive oil). It had the highest OD of 1.337 while LNS04 had 1.063 and LNN04 had 1.079 at the end of the 10 days incubation. The rate of lipolytic degradation was generally low until the fifth day when the rate of degradation increased rapidly. There was no difference in the degradation rate of lipids by isolate LNS04 and LNN04, although LNS04 had slightly higher degradation rate than LNN04.

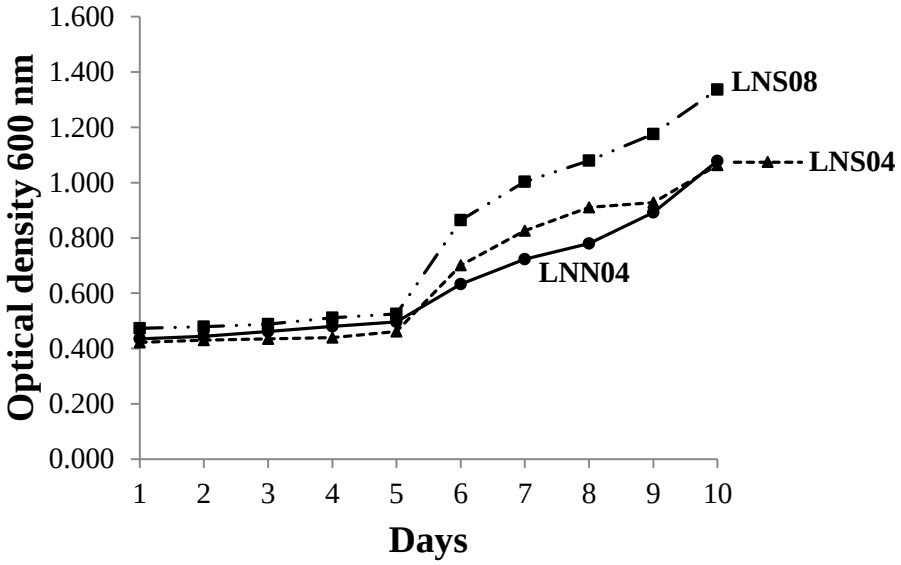

Figure 14: Pattern of degradation of lipids containing broth by isolates LNN04, LNS04 and LNS08 in Lake Nakuru

4.6 Feather degradation

Isolates LNC06 and LNN03 with the highest ability to degrade proteins were tested on clean chicken feathers as the sole carbon source. The two isolates were capable of degrading chicken feathers but isolate LNC06 was best in the degradation of feathers than LNN03. It degraded all the feathers in the inoculated broth (Figure 16).

Figure 15: Negative control with chicken feathers and no bacteria inoculum after 10 days of incubation.

Figure 16: Chicken feather degradation by isolate LNC06 after 10 days in feather broth.

Isolate LNC06 was the fastest feather degrading isolate from Lake Nakuru. There was no significant change in optical density for isolate LNN03 and LNC06 after 24 hours of inoculation in the feather broth medium. The mean OD value for isolate LNN03 was 0.401 with an increase in degradation being observed from day 3 to day 10. Isolate LNC06 showed a growth pattern higher than that of the isolate LNN03. The optical densities of bacteria started showing noticeable increase from day 2 with isolate LNC06 exhibiting remarkable growth as from day 4 to day 10 (Figure 17).

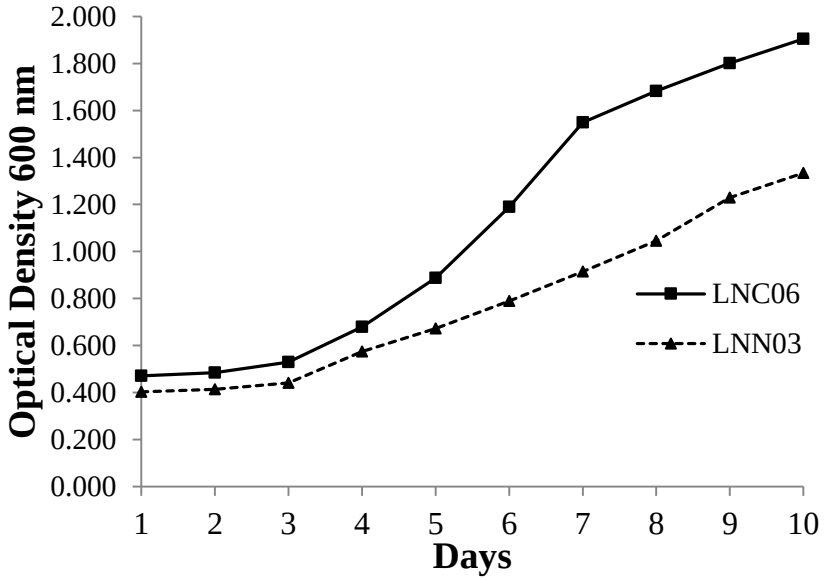

Figure 17: Growth patterns of LNC06 and LNN03 on chicken feather

The mean dry weight of feathers decreased with increase in growth of bacteria in the feather medium. The feathers in the medium inoculated with isolate LNC06 had the highest decrease in weight during the period of incubation. The results in Figure 18 show that at the beginning of the experiment, isolate LNN03 seemed to degrade the feathers faster than isolate LNC06 as shown by the decrease in dry weight from 0.107g to 0.059g in day 4. Isolate LNC06 started degrading the feathers actively from Day 4 to Day 10 where the loss in weight of feather was higher than that of isolate LNN03.

From day 5 to day 10, there was a higher loss in weight of feathers in medium inoculated by isolate LNC06 by 0.041g compared to that of isolate LNN03 which only lost 0.032g during the same period of time.

The two isolates exhibited similar pattern of decrease in the dry weight of feathers with incubation time. Although at the start of the experiment, degrading activity was higher for isolate LNN03 than LNC06, the overall performance was better with isolate LNC06 due to the higher loss in the dry weight of chicken feathers at the end of the experiment.

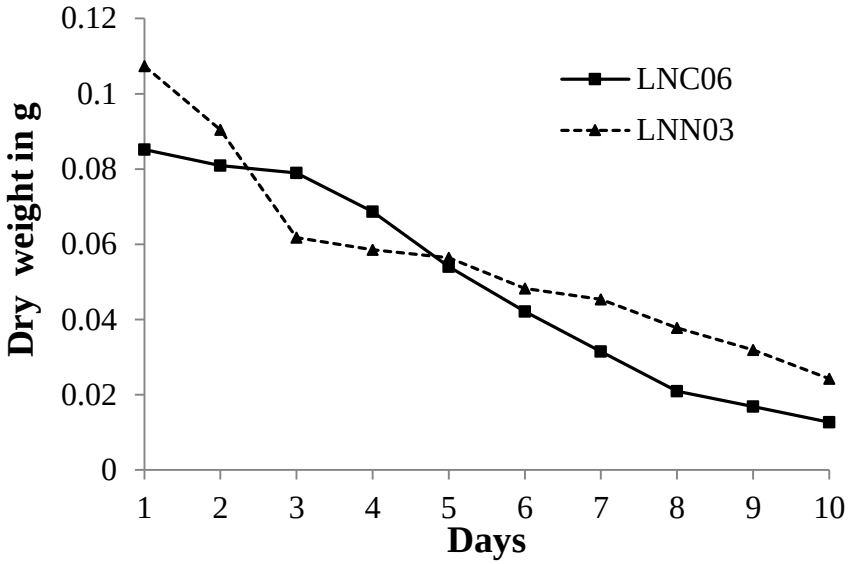

Figure 18: Profiles showing changes in dry weight of chicken feathers during the assay inoculated with isolates LNC06 and LNN03 from Lake Nakuru for 10 days.

The mean ash free dry weight of the inoculated feathers decreased with time of incubation. The ash free dry weight of the feathers in the beginning of the experiment was approximately 0.022-0.019g. This weight reduced to 0.0006 g in the medium inoculated by isolate LNC06. Both isolates were good feather degraders though isolate LNC06 appeared to degrade the feathers more effectively than LNN03 as indicated by the greater decrease in ash free dry weight of feathers. Degradation may have attained optimum levels as from day 5 onwards.

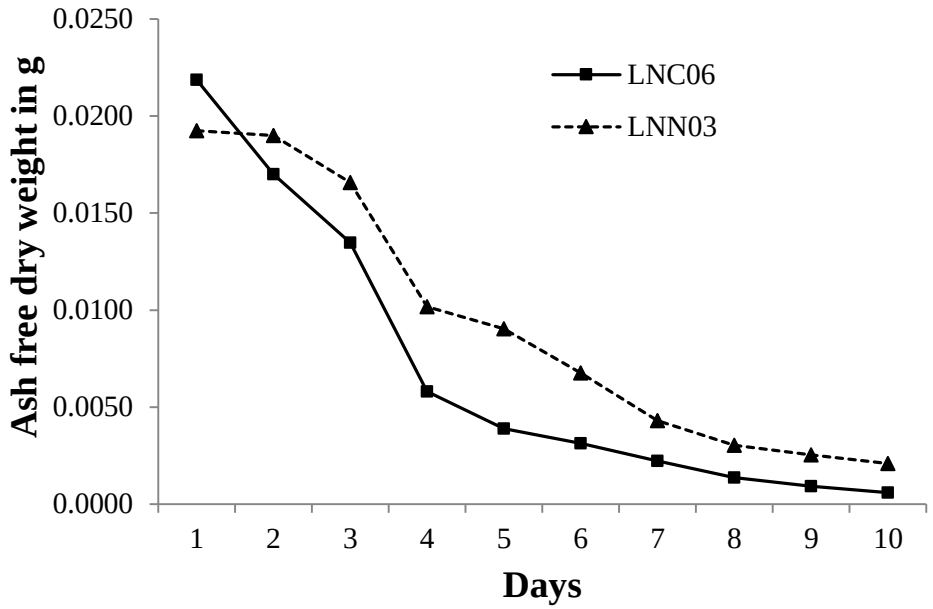

Figure 19: Profiles showing changes in ash free dry weight of chicken feathers during the assay inoculated with isolates LNC06 and LNN03 from Lake Nakuru for 10 days.

There was statistically significant difference in the feather degrading ability of the two isolates LNC06 and LNN03 (Independent t-test t=2.536, df= 58, $p<0.05$). The dry weight and ash free dry weights of the degraded chicken feathers were not statistically different between the isolates LNC06 and LNN03 (Independent t-test t=-0.737,df=58, $p>0.05$ and Independent t-test t=-1.217, df=58, $p>0.05$ respectively).

Increase in the optical density of the feather broth medium had an inverse relationship with the dry weight of the feathers as shown in Figure 20 (R^2= 0.536). Pearson correlation analysis indicated significant negative correlation (R^2 = -0.732, $p <0.01$, n = 60) between the optical density and the dry weight of chicken feathers.

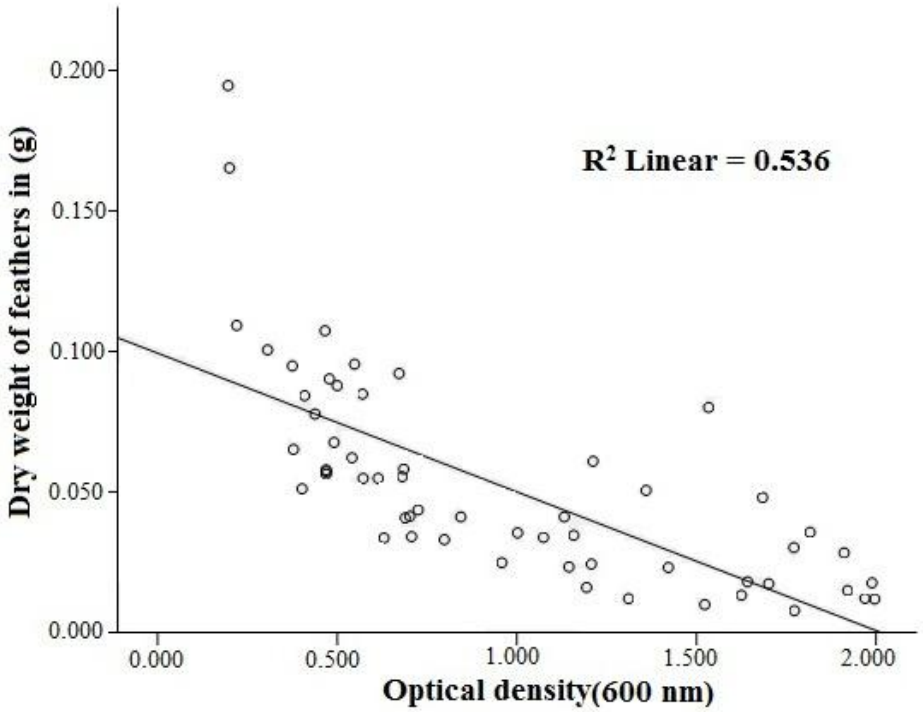

Figure 20: Relationship between optical density (600 nm) and dry weight of chicken feathers

There was a positive correlation between the dry weight and the ash free dry weight of the feathers (R^2= 0.496) Figure 21. Pearson correlation analysis also indicated significant positive correlation between the dry weight and ash free dry weight of chicken feathers (R^2=0.704, $p<0.01$, n=60). As the dry weight of feathers reduces as a result of feather degradation by bacteria, the ash free dry weight of the feather consequently reduced.

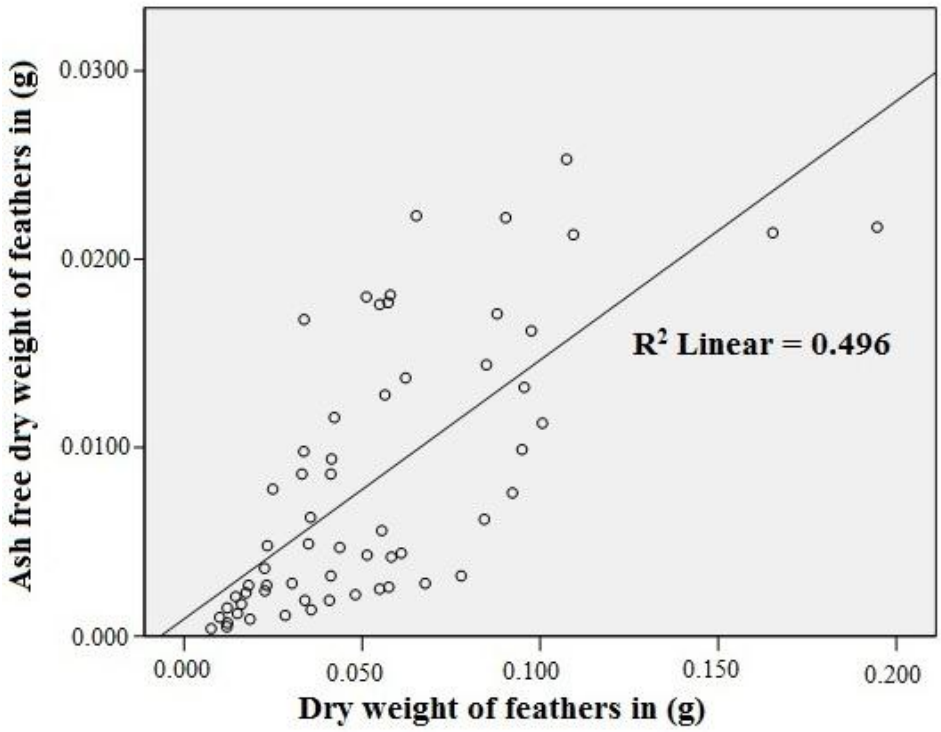

Figure 21: The relationship between dry weight and ash free dry weight of chicken feathers

4.7 Characterization of the four industrial potential isolates

The four selected isolates with industrial potential were characterized using biochemical tests, growth rates at different pH and temperature regimes and their DNA was sequenced to find out which organisms they were.

4.7.1 Biochemical tests for the isolates with industrial potential

The biochemical tests for the isolates that tested positive for casein hydrolysis, gelatin hydrolysis and keratin degradation showed no positive result for citrate utilization and urea degradation. A positive result was recorded for cellulose degradation by isolate LNC09 while isolate LNS08 was able to degrade lipids. Table 9 below shows the results of the various biochemical tests on the four isolates.

Table 9: Biochemical tests for isolates

Tests	LNS08	LNC09	LNC11	LNC06
Catalase Test	-	+	+	-
Gelatin Hydrolysis	-	-	-	+
Urease Test	-	-	-	-
Citrate Utilization	-	-	-	-
Cellulase	-	+	-	-
Casein	-	-	-	+
Starch	-	+	+	-
Lipase	+	-	-	-
Keratin	-	-	-	+

4.7.2 Growth of isolates in different pH

At a pH of 6 the growth of isolates was lowest as indicated by the low optical density of 0.4. Isolate LNS08 and LNC09 exhibited optimal growth at pH 9-10. At pH less than 7 the growth of these two species was poor as indicated by the low optical density values (Figure 22).

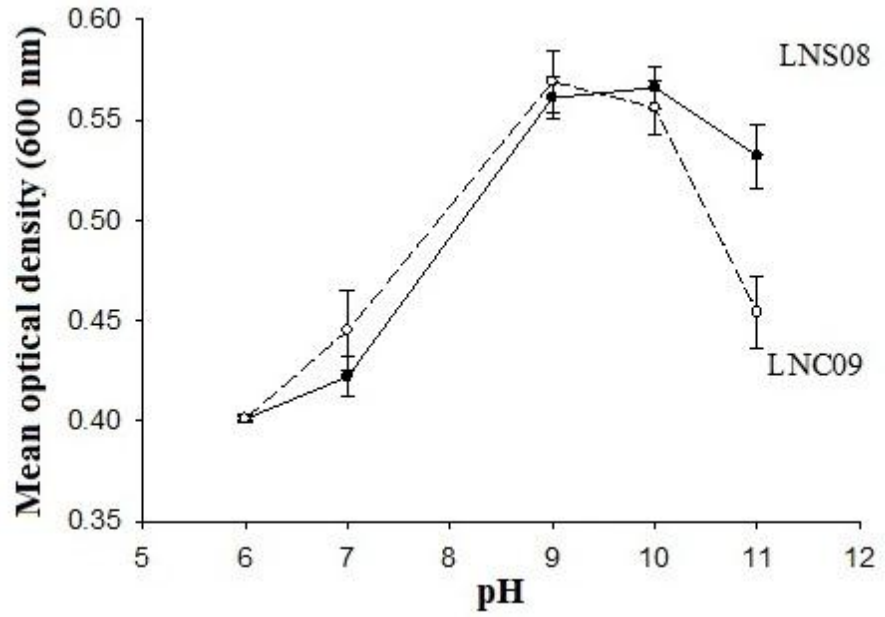

Figure 22: Effect of pH on growth of isolates LNS08 and LNC09. Vertical lines are ± STDEV

Isolate LNC06 and LNC11 showed optimal growth at pH 10. pH less than 9 did not favor the growth of isolate LNC06. Growth was recorded for pH 7 and above for the isolate LNC11

(Figure 23). At alkaline conditions with pH above 11, no good growth occurred as shown by the decreasing optical densities with increasing pH.

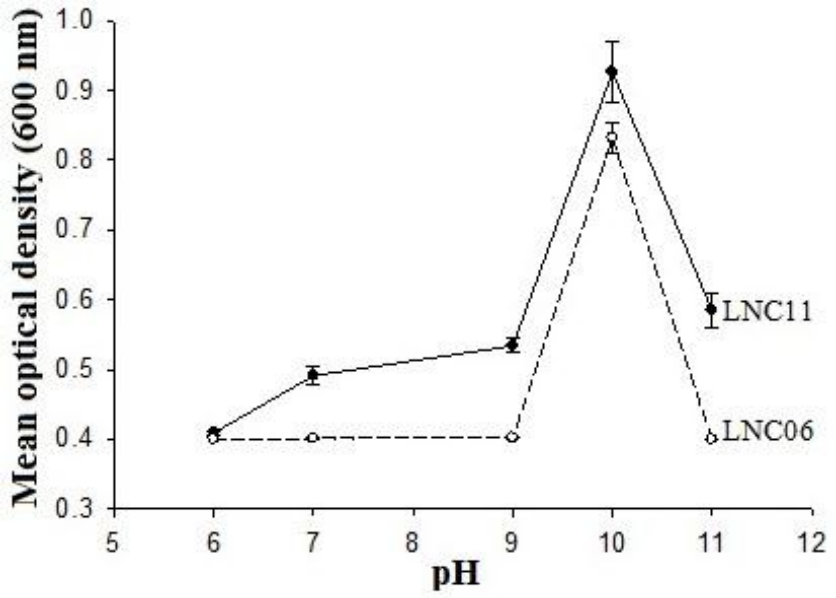

Figure 23: Effect of pH on growth of isolates LNC11 and LNC06. Vertical lines are ± STDEV

4.7.3 Growth of isolates at different temperatures

The isolates were subjected to different temperature regimes to find out the best temperature for optimal performance in enzyme production and growth. Isolate LNS08 grew well as the temperature increased towards 40°C. Optimum temperature for isolate LNC09 was 35°C.As the temperature increased to 40°C the growth of isolate LNC09 reduced as shown by the fall in the optical density values (Figure 24).

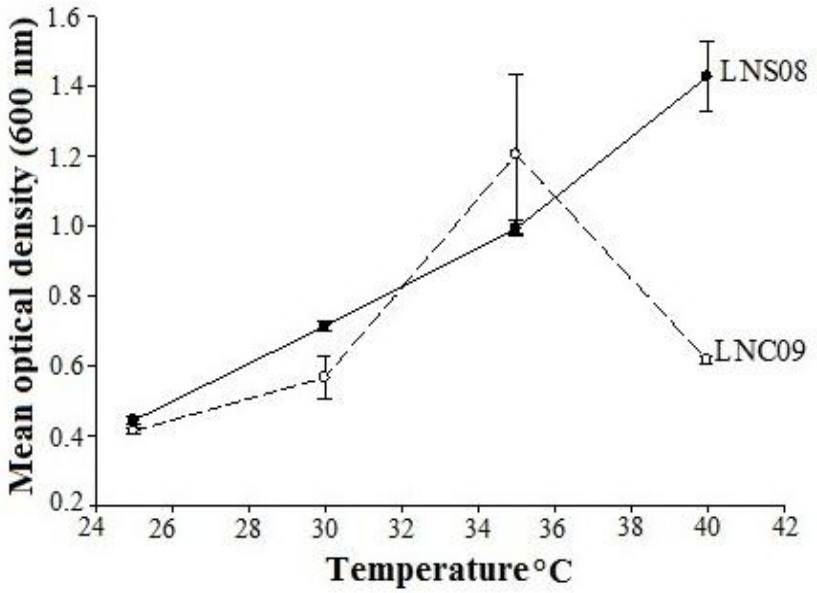

Figure 24: Effect of temperature on growth of isolates LNS08 and LNC09. Vertical lines are ± STDEV

Isolates LNC11 and LNC06 exhibited similar patterns in terms of temperature effect on their growth (Figure 25). These two isolates were able to grow at temperatures above 20°C, with optimum temperature for growth being about 35°C (Figure 25). However, as temperatures increased their growth reduced as indicated by the lowering of the optical density values.

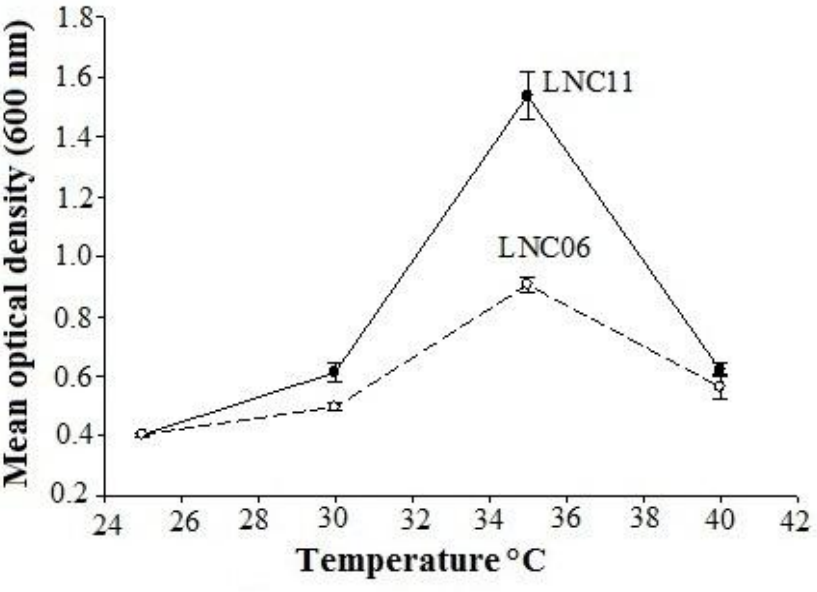

Figure 25: Effect of temperature on growth of isolates LNC11 and LNC06. Vertical lines are ± STDEV

4.7.4 Molecular identification of bacteria isolates

The DNA quality check on 1.8% w/v agarose gel stained by ethidium bromide (0.02% final concentration) revealed 3 samples with visible DNA. Samples 1: LNS08, 2:LNC09 and Sample 4:LNC06 (Figure 26).

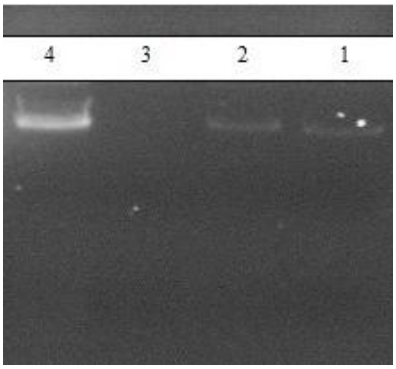

Figure 26: DNA quality check on samples one to four; 1-LNS08, 2-LNC09, 3-LNC11 and 4-LNC06

Two sets of primers were used in the sequencing of the four samples. *Bacillus subtilis* was used as a positive control while negative control was distilled water. Isolates LNC09 (2) and LNC11 (3) appeared to be similar as shown by the gel electrophoresis bands (Figure 27). The two sets of primers successfully targeted the intergenic DNA of the fours samples.

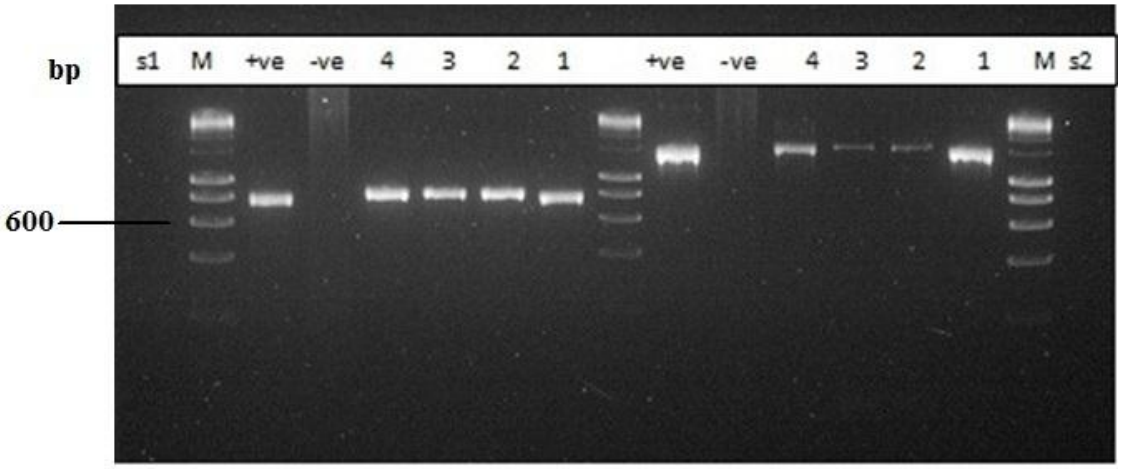

Figure 27: Agarose gel (1.8% w/v) electrophoresis and ethidium bromide staining 16S rDNA PCR-amplification products of DNA of bacteria isolated from Lake Nakuru.

The results of the BLAST analysis also indicated that the two isolates LNC09 and LNC11 were similar with close relatedness of 99% to *Bacillus sp*. (Accession No AB043860.1). Isolate LNS08 was probably *Nesterenkonia lacusekhoensis* with a maximum identity of 98% and E. value of 0.0. Isolate LNC06 was *Bacillus agaradhaerens* strain DSM 8721 with a maximum identity value of 99% (Table 10).

Table 10: Identification of bacteria through BLAST analysis

Code	Bacteria	Accession No	E.Value	Max Identity
LNS08	*Nesterenkonia lacusekhoensis*	NR 028928.1	0.0	98 %
LNC09	*Bacillus sp*. S2 gene for 16S rRNA	AB043860.1	0.0	99%
LNC11	*Bacillus sp*. S2 gene for 16S rRNA	AB043860.1	0.0	99%
LNC06	*Bacillus agaradhaerens* strain DSM 8721	NR_026142.1	0.0	99%

4.8 Phylogenetic analysis

LNS08 had a sequence identity with *Nesterenkonia lacusekhoensis*. Isolate LNC06 was closely related to *Bacillus agaradhaerens* with 99% similarity. Sequences of isolates LNC09 and LNC11 were similar to *Bacillus sp* AB043860.1 and *Bacillus sp*. S2 gene for 16S r RNA AB043860.1 respectively which were more or less the same. *Bacillus* species isolated from Lake Nakuru showed similarity in origin (Figure 28).

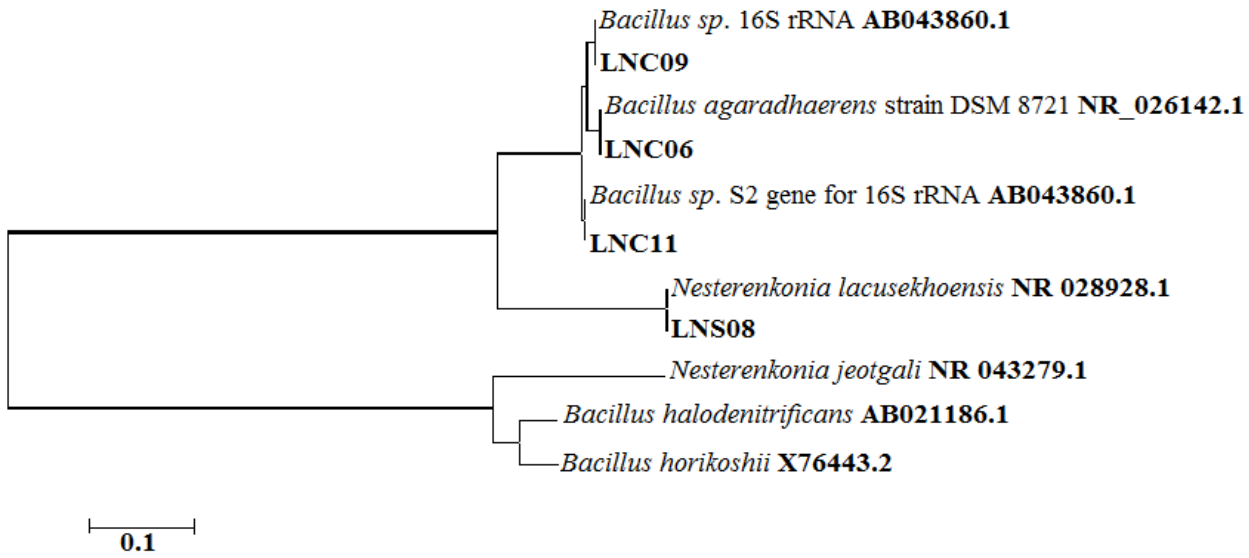

Figure 28: Phylogenetic tree of Soda Lake isolates with industrial potential based on 16S rRNA gene sequence using the neighbor-joining method (Saitou and Nei 1987).

CHAPTER FIVE
DISCUSSION

5.1 Physico-chemical parameters

From the results obtained, the pH, dissolved oxygen and high conductivity values are typical of shallow alkaline saline lakes in the Rift Valley region of East Africa (Oduor and Schargel 2007). Shallow lakes found in semi-arid areas experience high fluctuation in their physico-chemical parameters which are strongly influenced by hydrology of the inflowing rivers and the climatic changes especially rainfall patterns in the catchment area (Talling, 2001). Lake Nakuru shows such fluctuations causing temporal and spatial variations in their physico-chemical characteristics as shown by this study. Rainfall pattern both in the lake and the catchment area was believed to be instrumental in driving these temporal and spatial fluctuations, causing major changes especially in the chemical environment of the lake, a feature also observed in this lake and other neighbouring lakes by Oduor and Schargel (2007).

The salinity and conductivity fluctuations are influenced by changes in the concentration of ions in the water. Lake Nakuru, being a shallow flat pan morphologically loses water more rapidly through the rapid daily heating of its surface and sediment which enhances the evaporation and consequently increasing the concentration of ions. High conductivity values in the beginning of the year coincided with dry spell in catchment area with low water availability in the lake coupled with high temperatures and rapid water loss through evaporation which leaves the solutes in the lake that enhances solute concentration.

At the beginning of the rainy season in March, entry of freshwater in the lake as precipitation and through the River Njoro diluted the lake water reducing the conductivity and salinity as shown in Table 4. These results agree with those of Kirschner *et al.,* (2002) on the effect of rainfall on dilution of shallow pools which affected the water chemistry of the pools by lowering their conductivity.

The pH of Lake Nakuru was stable with very minimal variations despite water level changes in the lake as a result of the climatic changes particularly the rainfall patterns. Stability in pH in the lake was due to the efficient carbonate buffering system in the lake (Oduor & Schargel, 2007). The pH and dissolved oxygen of Lake Nakuru are typical of alkaline saline lakes. The rainfall in

the month of January to July impacted a lot on the chemistry of the Lake as indicated by the physico-chemical parameters especially salinity, conductivity and temperature

5.2 Bacterial density

Bacterial abundance in Lake Nakuru, as in most aquatic systems is influenced by the organic matter concentration in the lake. In Lake Nakuru, the total counts of bacteria were three log units more than the heterotrophic plate counts and the fact that only 0.1-1% of bacteria from natural environments are culturable appears to be a common characteristic of tropical lakes as also observed by William *et al.,* (1986). Abundance of bacteria in Lake Nakuru is among the highest reported values for eutrophic lakes. Being very shallow, this lake is subjected to re-suspension of bacteria from the sediments and has a richer supply of organic materials from the sediments on which the bacteria thrive, and also from the rich phytoplankton bloom of *Arthrospira fusiformis.*

The increase in the bacterial total counts in the lake during the period of increased rainfall may be attributed to the influx of organic matter and nutrients by the inflowing rivers which resulted into enhanced growth of the lake flora and increase in organic matter. The temporal variations in bacterial total counts coincided with the hydrologic pattern of dry and wet season in the catchment area. In the dry period, the amount of water and nutrients in the lake was low. High conductivity and salinity values at this time of the year reduced the survival of bacteria in the lake, a pattern that was also observed by Barcina *et al.,* (1997) in one of their studies on survival of allochthonous bacteria in aquatic systems.

The high bacterial density observed during the months of April and May, though with significant temporal variations as shown in Figure 4, 5 and Table 6, were not sustained through June. This may be as a result of decreased substrate availability, lower temperatures, or a combination of both brought about by the increase in water level in the lake (Wiebe *et al*., 1993, Kirchman & Rich 1997). The comparatively high abundance of bacteria in the northern part of the lake in relation to the other two sampling points may be attributed to the fact that most of the inlets to the lake such as River Njoro, Baharini springs and Nakuru municipal sewage are situated in the Northern point of the lake (Figure 1). They bring in high organic matter content on which bacteria feed and therefore contribute to the high numbers of heterotrophic bacteria in Northern point. These findings agree with the results of the study by Kilham, (1981) who observed that

allochthonous material entering the lake from the catchment area are an important substrate for bacterial utilization and colonization. On the other hand the reason for the lower number of heterotrophic bacteria and total counts in the Central part of the lake is because of a stable water quality with limited influence from the inlets and only the bacteria that are able to adapt to this conditions do well out competing the others. Barcina *et al.,* (1997) observed that extreme aquatic conditions such as high salinity and pH with no much external influence reduces the survival of bacteria in the aquatic environment and only those that adapt well flourish.

A number of biotic and abiotic factors are known to reduce the survival of bacteria in aquatic environments (Barcina *et al*., 1997). For instance, the dissolved oxygen concentration showed temporal variations in Lake Nakuru especially in the month of April with a mean value of 2.0±1.3mg/l when we had the highest population of total counts and the heterotrophic counts. This low dissolved oxygen concentration could have been resulted from high biological oxygen demand impacted by bacteria as a result of breakdown of organic matter brought in by runoff from the terrestrial lands. The change in dissolved oxygen could also have been influenced by the phytoplankton biomass in the lake (Oduor and Schargel, 2007)

There was no spatial variation in heterotrophic plate counts in the lake and this could be explained by the possibility of lake water mixing due to the openness of the lake and the shallow depth. However, the higher numbers of HPC and TC in the Northern part of Lake Nakuru could possibly have occurred due to the influence of organic matter entry into the lake from the Nakuru municipal sewage. Northern point also normally has high number of mammals coming to drink freshwater from Njoro river and droppings from the mammals enhance organic matter content in this area.

During the month of April the Northern sampling point had the highest bacterial density both heterotrophic and total counts when we had persistent rainfall in the lake as compared to the Central and the Southern sampling points. Rainfall was important in dilution of the lake water thereby reducing the salinity and conductivity and thus increasing the diversity of the bacteria and consequently their numbers. A similar phenomenon was recorded by Kirschner *et al.,* 2002 while working with extremely productive microbial communities in shallow saline pools. Another explanation for the higher abundance of bacteria in the Northern part of the lake could

be as a result of re-dissolution of immobilized nutrients from the dry areas of the sediment by the rise of the water level as postulated by Kirschner *et al.*, 2002 working in shallow productive saline pools similar to Lake Nakuru. Secondly, the higher depth of the lake at the Central point as compared to the North and Southern points of the lake could have also played a role in determining the lower numbers of bacteria at this point of the lake. This depth hindered efficient mixing of the lake water causing stratification that partitioned the top from bottom zone of the lake therefore hindering the entry or the renewal of nutrients into the epilimnion zone that could be utilized by the bacteria in metabolism and in number increase.

The colour of water at the Northern point of the lake was green most of the time during the sampling period and rich in phytoplankton. Phytoplankton production in the lake is very important in sustaining the aquatic life. The organic matter produced by the phytoplankton is utilized by the zooplankton such as the ciliates and rotifers which enter the food chain and thus sustaining the lake ecosystem (Rheinheimer, 1991). High phytoplankton production in the lake correlate positively with bacteria numbers possibly due to the availability of substrate from the senescence of algae for bacteria colonization. The Northern part of the lake harbored a high number of the lesser flamingoes because of the availability of food for these birds. The high number of lesser flamingoes in the Northern zone of the lake has implication in the nutrients status and organic matter flow in the lake. Flamingo wastes also contribute to the high organic matter in this part of the lake which provides substrate for the existence of the high heterotrophic counts and total counts in this part of the lake.

Input of allochthonous organic matter to lakes is of great importance for ecosystem structure and function (Lindström and Bergström, 2004). Intuitively, it can be stated that a large input of allochthonous organic matter changed the composition of bacteria community in the Northern part of Lake Nakuru which had many inflowing water sources. The influence of these rivers and inlets in terms of nutrient entry into the northern and southern parts of this lake therefore played an important role in the observed high numbers of bacteria in these sections.

Not all aquatic bacteria react in an equally sensitive manner to changes in the environment (Arbačiauskienė, 2002). Experiments have shown that even in the presence of high organic pollution, certain taxonomic groups of bacteria are not able to survive under conditions of

increased concentration of stressors, but are also able to actively participate in the processes of metabolism and thus increasing their numbers. The results of this study showed that anthropogenic pollution exerted a positive effect on the total number of bacteria in the lake. However, certain bacteria species are able to adapt to the stressful environment and remain active.

5.3 Macromolecules degradation

This study revealed the presence of bacterial isolates in the lake that have industrial potential through their extracellular enzyme production. Several isolates were found to possess the ability to degrade macromolecules. Macromolecules degrading enzymes from alkaliphilic organisms have attracted scientific attention due to their potential for use in industrial application (Kebede *et al.,* 1994). With this perspective, several isolates were cultured from Lake Nakuru and this study revealed the industrial potential of bacterial isolates from the lake and the possibility of harnessing their extra-cellular enzyme.

Presence of bacteria with amylase activity observed in this lake tends to be common in alkaline-saline water bodies for both gram positive and gram negative bacteria. Rohban *et al.,* 2008, observed similar bacteria in a study carried out in Howz Soltan Lake in Iran. In Lake Nakuru isolates LNC11 and LNC09 showing the highest amylolytic activity were sequenced and found to show 99% relatedness to the *Bacillus sp*. (Accession No: AB043860.1). Generally, amylolytic bacteria occurred in larger numbers in the lake with most of the heterotrophic bacteria found being capable of degrading starch. Starch is a simple form of nutrient as compared to proteins and cellulose (Grant, 2006a). It is simple in structure and easily assimilable by most aquatic bacteria.

Although isolate LNC09 degraded cellulose while LNC11 did not, all other tests subjected to these isolates confirmed their similarity. *Bacillus sp*. (Accession No: AB04360.1) which also degraded cellulose grows optimally at a pH of 9.5-10, 4% w/v NaCl and a temperature of 35°C. Grant, (2006b), identified suitable conditions of pH, temperature, salinities, nutrient and media requirements for optimal growth and isolation of *Bacillus* species from alkaline saline lakes of East Africa.

DNA sequencing of isolate LNC06 revealed that this isolate had 99% relatedness to *Bacillus agaradhaerens* which exhibited faster chicken feather degradation. *Bacillus agaradhaerens* also degraded casein and therefore has great potential for industrial application. Martin *et al.,* (2001) corroborated these findings on her study of starch hydrolyzing bacteria from Ethiopian Soda lakes where she reported that *Bacillus pseudofirmus* and *Bacillus agaradhaerens* are organisms with industrial potential. The alkaline amylases from the former have been isolated and characterized (Horikoshi, 1971).

Results from this study showed that *Bacillus agaradhaerens* exhibited slow growth on the solid lipid media but stabilized and exhibited higher growth rates in lipid broth media. This could be explained by the fact that aquatic bacteria do require a water environment to grow optimally. Their growth in the solid medium is limited to absence of the aquatic environment that tends to provide a stable pH and temperature condition for good growth. However, these findings tend to disagree with those of Martins *et al.,* 2001 who found out that overall amylase activity by *Bacillus sp* were lower in liquid medium but higher in solid medium.

While the activity of *Bacillus agaradhaerens* on casein was observed in this study as well as on the study by Nielsen *et al.,* (1995), other workers reported no activity by this bacteria on casein (Nogi *et al.,* 2005). In this study *B. agaradhaerens* was able to degrade casein and hydrolyze gelatin thereby strongly agreeing with Nielsen *et al.,* (1995) who proposed that this bacteria was a novel species. The ability of this bacteria to degrade casein and keratin in chicken feathers, indicate the possible industrial use of this bacteria's enzyme in degrading feathers and producing amino-acids from these feathers.

The isolate LNS08 from this lake was identified as *Nesterenkonia lacusekhoensis,* a bacterium which consists of short rods with a bright yellow colony with hydrolysis ability on lipids. It requires an optimal pH and temperature for growth of 9.5-10 and 30-40°C respectively (Collins *et al.,* 2002). Older cultures of this species appear coccoid as observed in this study. 16S rRNA of this isolate showed 98% relatedness to *N. lacusekhoensis* with an E-Value of 0.0 implying that the chances of this organism not being *Nesterenkonia lacusekhoensis* is zero. It is generally accepted that organisms displaying 16S rDNA sequence similarity values of 97% or less do not belong to the same species (Stackebrandt & Goebel, 1994). It is therefore evident from the

description data in Table 10 that the isolates in this study are represented by the species obtained from the BLAST analysis.

B. agaradhaerens and *N. lacusekhoensis* isolated in this lake grow well in alkaline environment and are known to be alkaliphilic (Martins *et al.,* 2001). This study confirmed findings of Nielsen *et al.,* 1995 on the alkaliphilic nature of *Bacillus agaradhaerens*.

In this study, the bacteria isolated are biased by the means of sampling and culture conditions used. Selection of enzyme producing isolate grown on solid media was done by the extent of indicator activity as a result of the enzymatic effect on specific macromolecules.

In the degradation of protein and the chicken feathers, pH of 10, salinity of 4% w/v (NaCl) and temperature of 34°C were optimal for enzyme production as observed by the results in this study and also supported by Grant, (2006b). *Bacillus agaradhaerens* has the property of being stable and makes it the best candidate in the biotechnological industry. *Bacillus* enzymes have the advantage of optimal activities at high salt concentrations (Ventosa *et al*., 1995).

5.4 Feather degradation

Most isolated bacteria in the lake that had industrial potential abilities were isolated from the Southern and Central points of the lake where there was minimal influence from the terrestrial environment. *Bacillus agaradhaerens* which was faster in the degradation of feathers was isolated from the Central point of the lake and at the shores from the flamingo feathers. Isolate LNN03 isolated from the open waters in the North of Lake Nakuru had positive effect in the degradation of protein and keratin thus indicating its potential for production of protease enzyme. The bacteria with protease enzymes utilized the keratin in the chicken feathers as metabolites for its growth and reproduction (Zerdani *et al.,* 2004).

The isolated *Bacillus agaradhaerens* with capability of hydrolyzing keratin in feathers was found to grow in natural media without any special requirements a part from alkaline pH a feature also confirmed by Zerdani *et al.,* 2004. This ability can be exploited in the degradation of chicken feathers which are produced in huge amounts in cities and farms.

Chicken feathers are poorly disposed in landfills. Combustion forms the major way of disposal of these feathers and this has had an adverse effect on the environment. Enzymatic hydrolysis of feather wastes could be a safe method of recycling this organic matter into a form that can be utilized by animals as protein feeds supplements. *Bacillus agaradhaerens* grows optimally at a temperature of 34°C and therefore would require less energy in a controlled process for efficient and faster degeneration of chicken feathers. Zerdani *et al.,* 2004 studied *Bacillus licheniformis* feathers degrading ability and concluded that its ability to grow well in thermophilic temperature is very important in efficient degradation of feathers which in this study was different with *Bacillus agaradhaerens.*

Feather degradation in this study was determined by the increase in optical density in the feather broth when the bacteria were inoculated. Increase in the turbidity of the feather broth was an indication of bacteria growth and subsequent degradation. Previous studies on bacterial degradation of feathers include those demonstrated by William *et al.,* 1990; Zerdani *et al.,* 2004 and Joshi *et al.,* 2007 and who isolated *Bacillus licherniformis* from a feather waste digestor. Feather degradation by *Bacillus agaradhaerens is* confirmation that these bacteria possess a protease capable of reducing disulphide bonds of keratin (William *et al.,* 1990).

Bacillus is more adaptable to the environment they inhabit than the other bacteria isolated in this study as shown by the dominance of *Bacillus sp* isolated in the whole study. Out of the other bacteria, the genera, *Bacilli* adaptability is much greater to varying environmental conditions (Park *et al.*, 2003).

CHAPTER SIX
CONCLUSION AND RECOMMENDATION

6.1 Conclusion

The following conclusions were drawn from this study;

1) There were spatial variations in total bacteria counts in Lake Nakuru with the Northern side of the lake which had influence from Nakuru municipal sewage, high flamingo numbers and inlet rivers showing significantly higher densities than the South and Central parts of the lake.
2) Temporal variations were observed for bacteria total counts in the lake with highest number of bacteria being recorded in the month of April and May when there occurred high rainfall and organic matter loading in the lake.
3) There was no significant spatial variation in heterotrophic plate counts in Lake Nakuru probably due to mixing of the lake water.
4) Temporal variations recorded for heterotrophic plate counts were factored by the rainfall period in the lake with highest values in April and May.
5) This study identified four native microorganisms from Lake Nakuru as potential sources for lipolytic, amylolytic, proteolytic and cellulolytic enzymes with diverse properties. *Bacillus agaradhaerens* (LNC06) was able to degrade proteins and chicken feathers. *Nesterenkonia lacusekhoensis* (LNS08) was able to degrade lipids while *Bacillus sp.* Accession No: AB04360.1 (LNC09 and LNC11) was able to degrade starch and cellulose.
6) The results of this work indicated that Lake Nakuru could be a rich source of alkaliphilic bacteria producing enzymes that could be put into industrial use as exhibited by the four isolates characterized in this study.

6.2 Recommendation

This study isolated and identified four bacterial isolates with industrial potential. There is need to carry out further studies on these isolates for the following industrial applications:

- Studies are needed particularly, in investigating the potential of *Bacillus sp.* Accession No: AB043860.1 (LNC09 and LNC11) cellulolytic strains obtained from this study in the management of paper wastes from paper industries and also in the production of sugars from the paper waste that can be utilized in food industries.
- Further studies and experiment are recommended to establish degradation of the chicken feathers and other keratin containing wastes for production of animal feed nitrogen supplements using *Bacillus agaradhaerens* (LNC06) isolated from this study.
- The purification and molecular properties of each enzyme isolated from the lake is a very important area that needs to be studied.
- Further work should be done on *Nesterenkonia lacusekhoensis* (LNS08), *Bacillus agaradhaerens* and *Bacillus sp.* Accession No: AB043860.1 (LNC09 and LNC11) especially in the evaluation of their enzymes as catalysts in biotechnological applications involving bioremediation and hydrolytic reactions.

All in all further microbial ecological studies are recommended on Lake Nakuru and other soda lakes for microbial diversity and biotechnological potential of these isolates since the potential of these lakes are unexploited.

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APPENDICES

Appendix 1: Summary of the density of HPC in Lake Nakuru from January to June, 2010.

Heterotrophic plate count CFU/ml			
Date	**South**	**Central**	**North**
20/1/2010	7.3×10^{3}	2.1×10^{4}	-
4/2/2010	3.7×10^{3}	4.3×10^{3}	-
22/2/2010	4.2×10^{4}	3.2×10^{4}	7.5×10^{4}
10/3/2010	4.9×10^{4}	7.4×10^{4}	6.3×10^{4}
25/3/2010	4.7×10^{4}	4.6×10^{4}	6.8×10^{4}
7/4/2010	2.8×10^{4}	5.1×10^{4}	1.9×10^{4}
21/4/2010	3.8×10^{5}	3.6×10^{5}	5.0×10^{5}
6/5/2010	1.5×10^{5}	7.8×10^{4}	2.0×10^{5}
20/5/2010	9.7×10^{4}	5.7×10^{4}	1.8×10^{5}
2/6/2010	2.4×10^{5}	2.1×10^{5}	3.9×10^{4}
15/6/2010	6.0×10^{4}	9.9×10^{4}	4.2×10^{4}
30/6/2010	5.7×10^{4}	7.8×10^{4}	3.3×10^{4}
Mean	$\mathbf{9.8\times10^{4}}$	$\mathbf{9.2\times10^{4}}$	$\mathbf{1.2\times10^{5}}$
STDEV	$\mathbf{1.1\times10^{5}}$	$\mathbf{9.8\times10^{4}}$	$\mathbf{1.5\times10^{5}}$

Appendix 2: Summary of the Total counts of bacteria in Lake Nakuru from January to June, 2010.

	Total counts per ml		
Date	**South**	**Central**	**North**
20/1/2010	1.0×10^{8}	9.9×10^{7}	-
4/2/2010	8.1×10^{7}	1.1×10^{8}	-
22/2/2010	8.9×10^{7}	1.2×10^{8}	1.4×10^{8}
10/3/2010	8.8×10^{7}	1.4×10^{8}	1.9×10^{8}
25/3/2010	9.0×10^{7}	1.3×10^{8}	1.7×10^{8}
7/4/2010	2.3×10^{8}	1.9×10^{8}	2.0×10^{8}
21/4/2010	2.9×10^{8}	2.3×10^{8}	3.3×10^{8}
6/5/2010	1.8×10^{8}	1.9×10^{8}	2.1×10^{8}
20/5/2010	1.5×10^{8}	1.0×10^{8}	1.9×10^{8}
2/6/2010	1.9×10^{8}	13×10^{8}	1.8×10^{8}
15/6/2010	8.4×10^{7}	1.6×10^{8}	9.6×10^{7}
30/6/2010	6.6×10^{7}	7.5×10^{7}	5.3×10^{7}
STDEV	$\mathbf{6.9\times10^{7}}$	$\mathbf{4.5\times10^{7}}$	$\mathbf{7.3\times10^{7}}$
Mean	$\mathbf{1.4\times10^{8}}$	$\mathbf{1.4\times10^{8}}$	$\mathbf{1.8\times10^{8}}$

Appendix 3: Table showing the mean optical densities (±SD) of different isolates subjected to 4 separate macromolecules as the sole carbon source for 10 days.

	Mean optical densities ($OD_{(600nm)}$) ± SD									
Isolates	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8	Day 9	Day 10
Amylolytic										
LNC11	0.928	1.018	1.151	1.300	1.366	1.450	1.537	1.588	1.714	1.951
SD	±0.141	±0.117	±0.033	±0.098	±0.120	±0.148	±0.148	±0.139	±0.201	±0.024
LNC09	0.976	1.182	1.298	1.340	1.424	1.453	1.517	1.629	1.743	1.937
SD	±0.016	±0.055	±0.118	±0.109	±0.150	±0.174	±0.160	±0.123	±0.149	±0.258
LNC05	0.999	1.022	1.088	1.249	1.296	1.360	1.401	1.512	1.526	1.740
SD	±0.160	±0.125	±0.025	±0.053	±0.010	±0.042	±0.043	±0.083	±0.072	±0.240
Proteolytic										
LNN03	0.452	0.459	0.466	0.503	0.622	0.629	0.657	0.677	0.707	0.740
SD	±0.011	±0.012	±0.016	±0.009	±0.048	±0.048	±0.019	±0.036	±0.063	±0.089
LNC07	0.418	0.424	0.427	0.453	0.550	0.562	0.596	0.603	0.621	0.646
SD	±0.004	±0.004	±0.006	±0.008	±0.033	±0.021	±0.049	±0.051	±0.057	±0.059
LNC06	0.423	0.434	0.444	0.487	0.582	0.590	0.634	0.719	0.876	0.930
SD	±0.027	±0.025	±0.029	±0.015	±0.077	±0.082	±0.114	±0.240	±0.497	±0.568
Cellulolytic										
LNS04	0.411	0.422	0.427	0.430	0.441	0.462	0.466	0.486	0.498	0.514
SD	±0.003	±0.006	±0.010	±0.009	±0.010	±0.032	±0.034	±0.049	±0.060	±0.059
LNS05	0.406	0.413	0.424	0.435	0.438	0.448	0.464	0.486	0.497	0.526
SD	±0.002	±0.003	±0.009	±0.019	±0.019	±0.016	±0.013	±0.010	±0.005	±0.031

LNC09	0.415	0.422	0.427	0.449	0.462	0.474	0.496	0.505	0.520	0.564
SD	±0.010	±0.006	±0.005	±0.010	±0.016	±0.012	±0.032	±0.035	±0.027	±0.040
Lipolytic										
LNN04	0.435	0.444	0.461	0.480	0.496	0.633	0.724	0.780	0.892	1.079
SD	±0.017	±0.011	±0.021	±0.032	±0.030	±0.084	±0.047	±0.071	±0.156	±0.201
LNS04	0.422	0.431	0.435	0.440	0.462	0.702	0.827	0.910	0.928	1.063
SD	±0.022	±0.017	±0.017	±0.011	±0.031	±0.132	±0.149	±0.128	±0.128	±0.319
LNS08	0.473	0.479	0.488	0.511	0.525	0.864	1.004	1.079	1.176	1.337
SD	±0.011	±0.011	±0.015	±0.018	±0.021	±0.154	±0.059	±0.075	±0.087	±0.195