CHARACTERIZATION OF β-GALACTOSIDASE BY LACTIC ACID BACTERIA FROM MILK AND TRADITIONALLY FERMENTED MILK PRODUCTS FROM IBADAN

BY

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ABSTRACT

Lactose intolerance (a condition in which man elucidates an immune reaction towards the presence of lactose due to inability to produce enzyme lactase) is a major nutritional deficiency among some adult consumers of milk and other dairy products worldwide. β–galactosidase hydrolysis of milk is one of the promising enzymatic applications in dairy industries for reducing lactose intolerance of milk products. However, plant and animal sources cannot meet the high demand of the enzyme in food industries. Hence, the aim of this study was to characterize β–galactosidase production by Lactic Acid Bacteria (LAB) isolated from locally fermented milk products.

Raw milk from Sokoto *Gudali* was collected from Fulani settlement in Ojoo, Ibadan along with some fermented milk products (‘’Nono’’ and ‘’Wara’’). LABs were isolated from them and identified using conventional methods. The ability of the isolates to hydrolyze 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) was used to screen for β–galactosidase production. Isolates with the best β-galactosidase production were selected. The enzyme was extracted and optimization of growth conditions (temperature, pH, nitrogen, carbon sources, inoculums size and inoculums age) for β-galactosidase production was carried out using o-nitrophenyl-β-D-galactopyranoside (ONPG). The enzyme produced was characterized using pH, temperature, metal and non-metal ions, and inhibitors. Purification of the enzyme was carried out using dialysis and chromatographic methods. Hydrolytic effects of the purified β–galactosidase were determined in different concentrations of lactose using standard method. Data were analyzed using descriptive statistics.

The isolated bacteria were identified as *Lactobacillus plantarum* (G11, E13 and E36), *L. brevis*, *L. casei*, *L. lactis*, *Leuconostoc lactis*, *Streptococcus* sp, *and Bacillus subtilis.* *Lactobacillus plantarum* (G11) had optimum growth value of 4.2 at 20°C and pH 7.0 with maximum enzyme value of 6.2U/mL at 30 hrs. The optimal β-galactosidase production occurred at neutral pH and 6% inoculum size. The best inoculums age varied between 18 hrs and 36 hrs. The best carbon source for enzyme production was raffinose with maximum value of 0.3U/mL while minimum activity was found in fructose with 0.2U/mL. The best nitrogen source was NH_4NO_3 with maximum value of 0.5U/mL and yeast extract had minimum value of 0.1U/mL. β–galactosidase activity increased with increase in molar concentration of the mono-valent chloride ions in which the highest was recorded in KCl at maximum value of 0.08U/mL while the minimum value of 0.001U/mL was obtained by NaCl at a concentration of 0.2 mmol respectively. The best sulfate ion was $CuSO_4$ with maximum activity value of 0.2 U/mL at 0.2 mmol concentration and minimum value of 0.007 U/mL at 0.1 mmol by $ZnSO_4$. The best enzyme inhibitor was KCN with maximum activity of 0.2U/mL at 0.2 mmol. The specific activity of β-galactosidase was 292.5 U/mg, 104.2 U/mg and 585.46 U/mg for G11, E13 and E36 respectively.

The hydrolytic effects of the purified β-galactosidase showed a maximum yield of 35.8% glucose, 19.3% galactose and 35.3% glucose and 18.5% galactose at 80% and 60% lactose concentration respectively.

β-galactosidase produced by *Lactobacillus plantarum* strain achieved lactose hydrolysis and could be of potential application for production of low lactose dairy products for consumption by lactose intolerant people.

Keywords: Lactose intolerance, Lactic acid bacteria, β-galactosidase production, Hydrolysis effect, Enzyme characterization

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Parkha, Oni AYODELE
June, 2013.

CERTIFICATION

I certify that this project work was carried out by Parkha Oni, AYODELE in the Department of Microbiology, University of Ibadan, Nigeria under my supervision.

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DEDICATION

This work is dedicated

To my children: Seun, Muyiwa, Owotomo , Omolara and Abiodun. You are my constant source of joy and praises to the almighty God.

To

My late mother

Madam Felicia Tonidunni Ayodele.

You insisted "I must be educated". You labored hard to give me necessary financial and material support up till your last day 2nd August, 2000, when you departed dramatically in your sleep. Mama, thank you even though you are no longer around to see your dream come true

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LIST OF ABBREVIATIONS

LAB	Lactic Acid Bacteria
BSA	Bovin Serum Albumin
β-gal	Beta-galactosidase
α-gal	alfa galactosidase
x-gal	5-bromo-4-chloro-3-indoxyl-β-D-galactopyranoside
LAC* P	Lactose Persistence
LAC* R	Normal adult lactase restriction
GOs	Galactooligosaccharides
TOs	Transgalactosylated Oligosaccharide
IPTG	Isopropyl-β-D-thiogalactosidase
MRS	de Man, Rogosa and Sharpe
MMRS	Modified de Man, Rogosa and Sharpe
OD	Optical density
ON PG	o-nitrophenyl-β-D-galactopyranoside
ONP	o-nitrophenol
PCR	Polymerase Chain Reaction
SCP	Single Cell Protein
SDS – PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electophoresis
YEA	Yeast Extract Agar
YEPA	Yeast Extract Peptone Agar

CHAPTER ONE

1.0 INTRODUCTION

1.1 General consideration

Microbial galactosidases have become a major biotechnogical tool in the development of viable bioprocessing for the conversion of many non-digestible carbohydrates in variety of natural products into utilizable forms (Vasilijevic *et al*., 2002). Glycosidases are enzymes that are capable of hydrolyzing glycoside bonds in oligo or polysaccharides and heteroglycosides (Jacobson, 1994). Among these group of enzymes, β-galactosidase (or β-D-galactosidase galactohydrolase EC.3.2.1.23), is a well known biocatalyst in the dairy industry as well as in synthetic glycochemistry (Karasova *et al.*, 2002). The importance of β-galactosidase is related to its use in milk and milk derivatives to decrease their lactose content, solving the problem of low lactose solubility and its low degree of sweetness (Somkuti *et al*., 1998). β-Galactosidase can catalyse two basic reactions: the hydrolysis of β-1,4-D-glycosidic linkage of lactose and other structurally similar substrates, and is also responsible for the formation of galacto-oligosaccharide (GOS) by transgalactosylation(Iqbal *et al*., 2011). This hydrolysation and transglycosylation activity enables them to synthesize oligosaccharides from the hydrolysis of natural products and catalyzes the hydrolysis of lactose into galactose and glucose (Furlan *et al.*, 2000). Oligosaccharides are water soluble, mildly sweet many of which are not digestible in the small intestine (Karasova *et al*., 2002). The most commonly found natural substrate for β-Galactosidase is lactose, the main sugar of milk and other dairy products (Ferguson, 1995). Some of the β-galactosidase are able to disrupt the B-1,4 bond and form other covalent bonds like β-1,6, β-1,3 and β-,2 (Mahoney, 1998). These compounds are indigestible, act as dietary fibres, and promote the growth of bifidobacteria in the intestine (Crittenden and playne, 1996; Sako *et al.*, 1999). Possible sources of the enzyme are plants, animal organs, bacteria, yeasts, fungi and moulds.

The problems with lactose fall within three major areas: Health, Food technology, and Environment which collectively constitute the main interest in β-galactosidase production, utilization and research (Mc Feters *et al*., 1969). Yeasts for example, usually

utilize only the fructose, (a trisaccharide moiety) in cane and beet molasses during production of alcohol after hydrolysis by invertase but not the residual disaccharide (Webb *et al*., 1976). There are many reports in the literature describing enzyme processes for hydrolysis of lactose by exploiting enzymes extracted from a number of species of microorganisms especially yeasts (of the genera *Kluyveromyces* and *Saccharomyces)* and fungi of the general *Aspergillus, Penicillum* and others (Roberto *et al.*, 1984). Lactic acid bacteria (LAB), which constitute a diverse group of Lactococci, Streptococci, and Lactobacilli have been studied intensively with respect to their enzymes for various different reasons especially food-related applications (Somkuti *et al*.,1998; Vasiljevic *et al.*, 2002).

During the last two decades, for example, the application and processing of β-galactosidase for the hydrolysis of lactose in dairy processing industries has gained increased attention because β-galactosidases are capable of synthesizing oligasoccharides during hydrolysis of natural substrates (Cortes *et al*., 2005). Moreover, glucose and galactose expands the use of lactose in food especially dairy industries, because of a marked increase in sweetness and solubility (Lifron *et al.*, 2000) which enhances frequent addition of oligosaccharides to beverages, infant milk powder, yogurts and other dairy products to improve their physico-chemical attributes. The lactose hydrolyzed milk may be consumed directly or may be used to produce many products including dehydrated products, concentrates, cheeses, yogurts, butter milk especially suitable for lactose intolerant individuals who are lactose maldigesters as observed amongst over 75% of the world population (Gekas and Lopez-leiva, 1985; Roy and Gupta, 2003; Panesar *et al.*, 2006). Hydrolysis of Lactose result in valuable changes on dairy products with increased solubility, broader fermentation possibilities, more ready fermentation of carbohydrate and reduced lactose concentration with associated diminished possibility of lactose crystallization (Roberto *et al* ., 1984).

Lactose hydrolysis in whey can help to solve the pollution problem caused by whey and whey permeates (Rachie, 1988). In the cheese industry, lactose is a waste which causes several economic and environmental problems (Grosova *et al.*, 2008). The problem arose because lactose is associated with high Biochemical and Chemical Oxygen Demand (BOD,

COD) and because of lactose uncertain solubility (Guimares *et al.*, 1992); the environment can thus be rendered inhabitable as a result of pollution. The production of microbial protein from whey for example reduces the BOD value of the effluent by converting lactose into protein. Moreso *et al.* (1980) reported that the alteration of *Kluyeveromyces fragilis* has the main advantage of making easier the final disposal of whey since it lowers the COD by more than 90%.

The lactose-fermenting yeast *Kluyveromyces maxianus* and *Kluyveromyces lactis* are both important industrial yeasts in classical applications with biomass conversion, ethanol, enzyme and single-cell protein production (Inchaurrondo *et al.*, 1994). *Kluyveromyces maxianus* offer great advantages such as (Belem and Lee, 1998);

(i) Good growth yield, which has an important economic impact in the food industry.

(ii) Acceptability as safe microorganism, an important technical aspect when considering that the fermented products have good or pharmaceutical applications and

(iii) Higher β-galactosidase activity than other yeasts.

Scopularriopsis sp (Pastor and Park 1980) and *Erwinia aroida* were equally screened for β-galactosidase production (Flores and Ighem-Alegre, 1996). The β-galactosidase from these different sources are well characterized with regards to their kinetic behaviour in the hydrolytic mode (Daniela *et al.*, 2006). They were all reported to cause hydrolysis of β-1, 4-glycosidic bond in lactose (4-0-β-D galactopyranosy l β-D-glucopyranose), in different β-D-galactosides, glycopeptides, and glucolipides.

However, there is a definite need for β-galactosidase that is stable at high and low temperatures and could be approved as GRAS (generally regarded as safe) for hydrolysis of lactose in milk and other dairy products (Kim and Raja Gospel, 2000). β-galactosidase is also known to have a stable enzymatic activity at temperatures up to 55^0C, preferable as low as 6^0C and specifically at 4^0C which corresponds to refrigerating/conservation temperature for dairy products. β-galactosidase hydrolyzes lactose in dairy products and

milk processing at low temperature and hinders the saprophytic activity and restricts their proliferation (Jelen *et al*., 2005). These oligosaccharides are not utilized by the mouth microflora (*Streptococcus immitants*) and as such limits the formation of caries (Karasova *et al.*, 2002). Many of them are not digested in the small intestine and this makes them suitable for use as low calorie sweeteners for consumption by individuals with diabetes. In recent years, the ability of oligosaccharides to promote the proliferation of bifido bacteria in the colon has been recognized. These disaccharides are selectively fermented by bifidobacteria such that the growth of undesirable bacteria is suppressed (Molder, 1994).

Flavor is an important attribute of grape-derived alcoholic beverages and many Variables contribute to it (Sarova and Nikolova, 2002). Among these, the enzymatic activities of β-galactosidase and esterase of yeast play an important role as they are involved in their breakdown. Fermandez *et al.*, (1997), have revealed the potential of indigenous wine yeasts to produce enzymes that improve the sensory properties of wine. This has encouraged studies on yeasts which possess these enzymes (). Although, many microbial β-galactosidase has been investigated for the aforementioned properties and potential beneficiary effects namely: assimilation of food containing lactose intoleranse, for formation of galacto-oligosaccharides during lactose hydrolysis. (Nizamudin *et al.*, 2008); β-galactosidase in bacteria and in yeast is generally intracellular (Wallenfels *et al*., 1972).

The major applications for Lactose hydrolysis can therefore be summaries as followed (Cortes *et al*., 2005):

(a) Lactose hydrolysis in liquid milk improves digestibility for lactose in tolerance consumers
(b) Lactose hydrolysis increases sweetness and enhances flavor
(c) Lactose hydrolyzes milk powders for dietetic uses, especially for infants with temporary β-galactosidase deficiency.
(d) In some cases, β-galactosidase used for the manufacture of cheese and yogurt can increase the rate of acid development and thus reduce processing time
(e) Lactose hydrolysis in concentrated milk products (e.g. sweetened condensed milk, ice cream), prevents crystallization of Lactose

(f) To serve as source of functional proteins and sweet carbohydrates used as food ingredients in bakery and confectionary products (USA patent No. 4, 657088: Kane Kramer).

In view of the very wide applications identified above, yeast and lactic acid bacteria (LAB) have gained great interest in the last decade because of their GRAS status and their long history of usage and association with milk (Kim and Raja Gospel, 2000). Amongst these groups, yogurt bacteria *Lactobacillus bulgaricus and streptococcus thermophilus* are already identified as the highest β-galactosidase producers (Kreft and Jelen, 2000). The β-galactosidase of this culture has been characterized, showing high stability and activity at high temperatures (Greenberg and Mahoney, 1982). As their importance in the food and dairy industries is already well known, and because the lactic acid and other organic acids produced by these bacteria act as natural preservatives as well as flavor enhancers (Salminen *et al*., 1998a). Lactic acid is an economically valuable product with different applications in pharmaceutics and other industrial products. Production has increased considerably due to the development of new applications and the current level of production in technology is based on microbial fermentation (Gaudreau *et al*., 2005). This has energized in the discovery of new microbial sources with novel characteristics (Datta and Henry, 2006), in particular its stability and ability to lasting enough to attain the desire effect. As in other microbial galactosidases that are thermolabile the most important characteristic of the enzyme is its low heat stability.

The classification of Lactic acid bacteria was initiated in 1919 by Orla-Jesen and was until recently primarily based on morphological, metabolic and physiological criteria (Orla-Jesen, 1919). Lactic acid bacteria are Gram- positive, usually none-motile, non-spore-forming rods or coci (Stiles *et al.,* 1997). Nutritionally, the lactic acid bacteria are extremely fastidious (John Lindquist, 1998). They are chemo-organotrophic and only grow in complex media.A medium that will support their growth must contain fermentable carbohydrate and many growth factors (De Man Rogosa *et al*., 1960). Lactic acid bacteria are classified by the fermentation pathway used to ferment glucose and by their cell morphology.They are widespread and can be isolated from many plant and animal sources (John Lindquist, 1998).

Current methologies used for classification of LAB mainly rely on 16S ribosomal ribonucleic acid (rRNA) analysis and sequencing (Olsen *et al.*, 1994). Based on these techniques, Gram-positive bacteria are divided into two groups depending on their G+C content (John Lindquist, 1998). The Actinomycetes have a G+C content above 50 mol% and contain genera such as *Atopobium, Bifidobacterium, Corynbacterium* and *Propionibacterium* (John Lindquist, 1998). In contrast, the Clostridium branches has a G+C content below 50 mol% and include the typical LAB genera *Carnobacterium, Lactobacillus, Lactococcus, Leuconostoc, Pediococcus* and *Streptococcus* (Berthier *et al*., 1999).

Lactic acid bacteria, including *Lactobacillus, Leuconostoc, Lactococcus, Pedicoccus* and *Bifidobacterium* spp., are prominent members of the comensal intestinal flora and are the commonly studied probiotics bacteria (He *et al*., 2008). They cause reduced lactose intolerance alleviation of some diarrhoeas, lowered blood cholesterol, increased immune response and prevention of cancer (Marteau and Ramband, 1993, 1996; Gilliland, 1996 and Salminen *et al.*, 1998a). The selection criteria for probiotic LAB include, human origin, safety, viability/activity in delivery vehicles, resistance to acid and bile, adherence to gut epithelial tissue, ability to colonize the gastro intestinal tract, production of antimicrobial substances, ability to stimulate a host immune response and the ability to influence metabolic activities such as vitamin production, cholesterol assimilation and lactose activity (Salminen *et al*., 1996).

1.2 Statement of problem

Lactose (or milk sugar) is a disaccharide sugar that is found exclusively in the milk of mammals (Nahvi *et al*., 2004). However, lactose is not actively absorbed in the intestine of all man in all regions unless it is first split into its constituent monosaccharides: glucose and galactose. Hydrolysis of Lactose is effected by the lactase enzyme .Many grown-up adults worldwide have lost the capability to synthesize β-galactosidase (or lactase) enzyme as this ability begins to drop between ages 2 to 5 years (McBeans *et al*, 1998). It is estimated that the phenomenon affects over 75 per cent of world adult population resulting in lactose intolerance or hypolactasia (Heyman, 2006). For this group of people, it is impossible to consume large quantities of mammalian milk or other milk products without

adequate treatment. Enzymatic hydrolysis of lactose is a preferred option because it permits wider operational conditions of both temperature and pH (Gekas and Lopez-leiva, 1985). The application of the process has thus created a potential market for the application of β-galactosidase. Microbial β-galactosidase from organisms such as yeasts, mold and bacteria still remain a better source for its commercial production (Vasiljevic and Jellen, 2001). The use of microorganisms in food production and /or fermentation has long been known especially when they have a long history of safe use and without extensive purification (Inchurrondo *et al*., 1994). The bacteria species currently used by the dairy industry for β-galactosidase enzyme production belong to the genera of *Lactobacillu and Bifidobacterium* and comprise a limited collection of strains (Heymann, 2006, He *et al*., 2008). Lactic acid bacteria have been used for centuries to produce fermented food products like pickles, Saussage, yogurt and cheese (Inchurrondo *et al*., 1994).

1.3 Justification for the work

Part of the federal government of Nigeria's policy objectives for Agricultural transformation Agenda is to modernize the agricultural system including the indigenization of the dairy industry that is presently import dependent (Adesina, 2012). As revealed lately, Nigeria spends over four billion dollars annually on the importation of milk and milk products (Adesina, 2012). To reverse this trend, steps must be taken to establish raw milk collection centres at some locations in dairy producing states in Nigeria such as Adamawa, Taraba and other states that may be identified in the Niger trough for this purpose. Milk as its well known is characterized by it high lactose content. The lactose content needs to be reduced to facilitate the utilization of milk by all consumers, due to the fact that some people are lactose intolerant. β-galactosidase is a well known enzyme that reduces lactose contents of milk and milk products. For this program to have any meaningful impact and to make Nigeria self sufficient in dairy products, the traditional way of milking the cows has to be upgraded.Among β-galactosidase-producing organisms, bacterial sources are preferable due to ease of fermentation, high enzyme activities and good stability (Vasijevic and Jelen, 2001) .Organisms so identified should have potential for high catalytic activity within a reasonable range of temperature and pH. Therefore, Lactic Acid Bacteria (LAB), which constitute a diverse group of *Lactococcus*, *Streptococcus*, and *Lactocobacilli* have

attracted a great deal of attentions in recent years as a source of β-galactosidase production for several reasons (Somkuti *et al*., 1998) including its long history of usage as stater cultures for fermentation in dairy foods and/or in food preservation for thousands of years. Moreso, because of its GRAS status as extensive purification of enzymes produced by them may not be necessary.

1.4 Aim of the Study

β-galactosidase hydrolysis of milk is one of the promising enzymatic applications in the dairy industries for reducing lactose intolerance of milk products.However, plant and animal sources cannot meet the high demand for the enzyme in food industries.Hence the aim of this study was to characterize β-galactosidase production by Lactic Acid Bacteria(LAB) isolated from locally fermented milk products.

1.5 Objectives of study

This study aims at achieving the following objectives:

1. Isolation screening and identification of beta galactosidase-producing microorganisms associated..
2. Growth and nutrient utilization of the isolates in various nutrient media.
3. Optimization of production conditions for beta galactosidase by the isolates
4. Purification and characterization of the beta galactoside produced by the isolates.
5. *In situ* application of the enzyme to different Nigerian foods.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Lactose

Lactose or milk sugar is a disaccharide sugar that is found exclusively in the milk of mammals and it is not found naturally in any other food aside from dairy products (Kemer, 1995). It was discovered in milk by Fabriccio Batoletti (1619) and identified as a sugar by Carl Wilhelm Scheele Bartoletto (1780). It was isolated and descried as essential salt without nitrogen in (1633). It is comprised of galactose and glucose linked together by β-D-galactoparanosyl-(1→4)-D-glucose. The glucose can be in either the α-pyranose form or the β-Pyranose form, whereas the galactose can only have the β-Pyranose form (Jacobson, 1994). Hence α-lactose and β-lactose refer to an anomeric form of the glucopyranose ring alone (Jellen, 2005).

Lactose makes up a little less than 80% (by weight) of the solids in milk, although the amount varies among species and individuals (Piverlik *et al*., 1992). It is also found to be the principal component of cow's milk. Indeed, it is a major constituent of the milk of all mammals except sea lion (Kretchmer, 1993) and as a primary source of energy for the neonate. The human milk is said to contain the highest lactose concentration of about 7%. Lactose levels in commonly milked animals such as camels, goats, sheep and cows run between 4 and 5 percent (Webb *et al*., 1976).

Because of their high nutritional value and bioactive properties, lactose products are increasingly used in products designed to improve health and wellness (Jellen *et al.,* 2002). Lactose offers a number of nutritional benefits not found in other sugar sources (Kim *et al*., 1983).

It also generates a prolonged energy supply due to its slow hydrolysis in the body (Flatz, 1987) and also increases the absorption of minerals such as calcium, magnesium, and zinc in laboratory animals and human infants (Jelen, 2005). According to the USA dairy council information details, lactose contributes to a healthy intestinal flora and has minimal effect on tooth decay compared to other sugars.

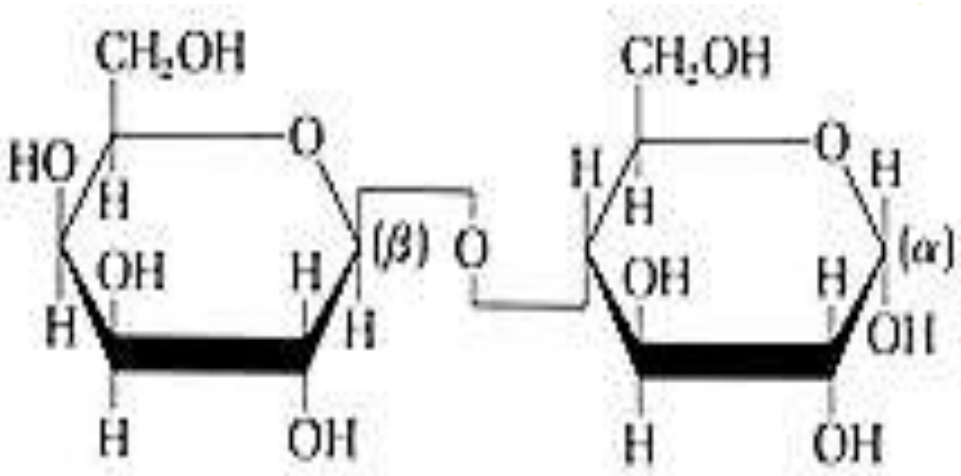


Fig 2.1: Molecular structure of lactose (Jellen, 2005).

Table 2.1: Species Concentration of lactose in milk of different mammals

Species	Lactose concentration	%water content
Human	7.1	87.1
Cow	4.6	87.3
Buffalo	4.8	82.8
Goat	4.3	86.7
Sheep	4.8	82.0

Source: Dairyforall. com, the milk and Dairy products website

2.1.1 Production of lactose

Several million tons of lactose are produced annually as by-product from the dairy industry animals.It is generally an excellent source of protein, calories in the form of fats and carbohydrates, vitamins and minerals (Kosikoski, 1982). Production of both pure lactose and lactose-containing dairy by-products, have markedly increased since the 1960s. For example, its bland flavour has lent to its use as a carrier and stabilizer of aromas and pharmaceutical products. Purified lactase can also be purchased as high calorie diet additives (Jellen, 2005). Lactose is little fermented by barker's yeast which is of advantage during brewing. An easily obtained source of lactase is lactaid pills which are sold over the counter to treat lactose intolerance (Mishkini, 1997).

According to Pavel Jellen, in 1996 a total of 425 Kilotons of lactose were produced in U.S for use as infant foods, confectionery and others (bakery, dry mixes and protein standardization). Another 175 kilotons were produced for pharmaceuticals, fermentation industries, feeds and other derivatives in Germany (Sahari and Friend, 1983). Whey is made up of 6.5% solids of which 4.8% is lactose (Hussain, 2007). Lactose has a relatively low glycemic index (Buller and Grand 1990) which makes the sugar beneficial for diabetics. The glycemic index of carbohydrate of food refers to the increase in blood glucose relative to a standard such as glucose or white bread (Sahi, 1994). Food with a low glycemic index causes a slow and modest rise in blood glucose levels. In contrast, foods with high glycemic index causes a faster and higher increase in blood glucose (Arola and Tem, 1994). Lactose has a lower glycemic index (65) than glucose (138), honey (104) or sucrose (87). Diets with glycemic index not only help decrease the risk of developing diabetics but such diets may also have other health benefits (Cambridge history of food science).

2.1.2 Probiotics effects of lactose

By stimulating the growth of beneficial intestinal bacteria such as Bifidobacteria and Lactobacilli and inhibiting pathogenic bacteria, lactose contributes to a healthy intestinal flora (Kemer, 1995). When the ingested lactose is not absorbed in the small intestine but rather become fermented by lactic acid bacteria in the large intestine, it

produces unknown volumes of carbon dioxide. Common symptoms of indigested lactose includes nausea, cramps, bloating, flatulence and diarrhea which begins about 30mins to 2hrs after drinking or eating foods containing lactose (Jellen, 2005).

Lactose is not actively absorbed by the intestine unless it is first split into its two monosaccharide components i.e glucose and galactose (Jellen *et al*., 2005). The hydrolysis of lactose is affected by the lactose enzyme produced by the epithelial cells in the brush border of the small intestine (Arola and Temm, 1994). Thus, the capacity of mammals to digest lactose depends on the level of lactase activity in the small intestine. Infacts and young children digest lactose with an enzyme lactase which splits this molecule in the small intestine into the two absorbable simple sugars of glucose and galactose. However, the majority of adults has lost this ability and is lactose malabsorbers (Buller and Grand, 1990).

2.1.3 History of lactose

Gastrointestinal distress in adults after milk consumption was described in ancient Greek and Roman texts (Cambridge History of food science) and there were isolated clinical reports in the late nineteenth and early twentieth centuries but the mechanisms controlling lactose production were disputed for many years as a result of which the problem was not widely studied until the development in the 1960s of new techniques to study enzymatic action in the intestine (Flatz, 1987). This prompted Dahlgvist (Dahlgvist *et al*., 1977) to describe the high prevalence of diminished lactase activity in healthy adults as a common worldwide phenomenon in adulthood. According to this finding, production of lactase begins to decline in most children between the ages of 2 to 5 years (around the time of weaning). Arola and Tam (1994) reported that most adults retain only about 10 percent of infant- level lactase activity.

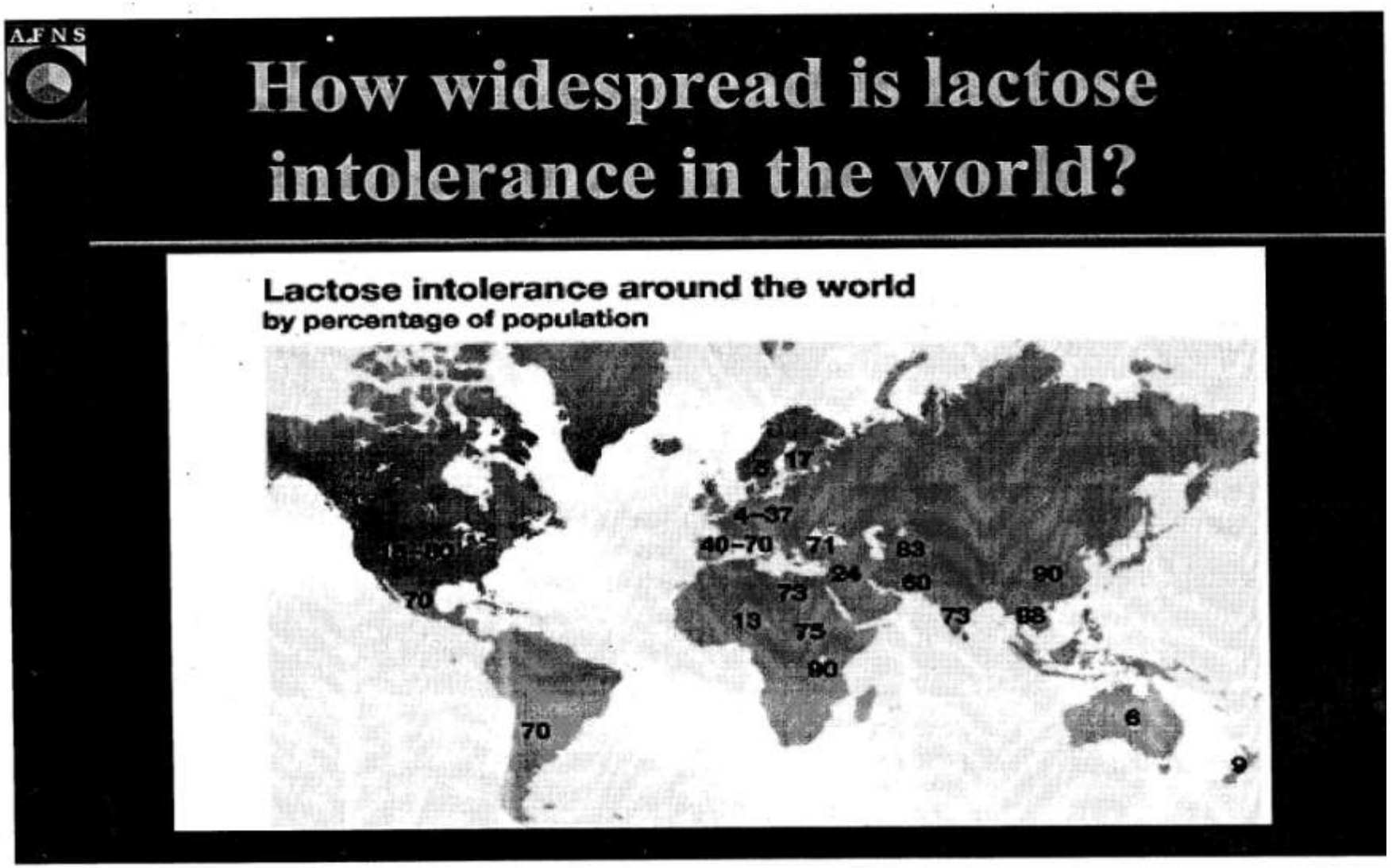


Fig 2.2: Lactose intolerance around the world by percentage of population (Pavel Paul Jellen, 2008)

2.1.4 Genetics of lactose intolerance

The mechanisms controlling lactase production were disputed for many years (Sahi, 1994). Some researchers, drawing on studies of gene regulation in bacteria, argued in the 1960's that lactase was a substrate-inducible enzyme; that is, that lactase production was believed to be stimulated by the presence of its substrate lactose (Fernendes *et al*., 1987). In this view, populations that did not use milk as adults lost the ability to produce lactase, whereas groups that did consume milk and milk products retain lactase capability (Bullar and Grand, 1990). Biochemical studies cast doubts on this theory, and family studies have demonstrated that lactase production is controlled by an autosomal gene, recently located on chromosome 2 (Bullar and Grand, 1990). Persistence of lactase production is a dominant trait.

Following the terminology suggested by Gebhard Flatz (1987), the two alleles are designated as LAC* P for lactase persistence and LAC* R for normal adult lactase restriction. The LAC locus appears to be a regulatory gene that reduces lactase synthesis by reducing the transcription of messenger RNA (Arola and Temm, 1994). Persons inheriting LAC* P from both parents would have lactase persistence into adulthood. Those getting LAC* R alleles from both parents would display lactase restriction as adults. Heterozygotes would get different alleles and be LAC* P/LAC*R, but since LAC* P is dominant, lactase activity and ability to digest milk would persist beyond childhood (called lactase persistence or lactase tolerance).Many people with ancestry in Europe, West Asia, India, and parts of East Africa, maintain lactase production in adulthood. Fredrick J. Simoons (1969, 1994), had advanced the thesis that lactase persistence is closely linked to dairying. His culture-evolution hypothesis that groups that kept cattle and other milk animals would gain a selective advantage if adults retain the ability to use milk and milk products as food (Sahi, 1994). In many of these areas, milk from mammals such as cattle, goats, and sheep is used as a large source of food. It was in these regions that genes for life long lactase production first evolved (Fernendes *et al*., 1987; Sanul, 1990). By descent more than 70 percent of Western Europeans can drink milk as adults compared with less than 30 percent of people from areas of Africa, East and South eastern Asia and the Oceania. Of the Thai, Chinese and African American populations due to a mutation which is believed to have occurred between 5 and 10 thousand years ago (Sahi, 1994) and involves a single base change in the promoter region to prevent binding of the

transcription factors, 97, 90 and 73 percent respectively are reported to be lactase intolerant, whereas 84 and 96 percent of the US white and Swedish populations respectively are tolerant (Fernendes *et al*., 1987).

The second center of adult lactase persistence lies in the land, of Arabia, the Sahara desert, and Eastern Sudan. In these regions lactase persistence characterizes only nomadic populations who heavily dependent on camels and cattle such as Bedovin Arabs; the Tuareg of the Sahara, the Fulani of the West African Sahel, and the Beja and Kabbabish of Sudan(Fernendez *et al*., 1987). Low rates among Nigerian Fulani may indicate a higher degree of genetic mixing with other peoples in their abode than among the Fulani of Senegal (Sahi, 1994).

2.1.5 Lactose and Human Health

Recent developments in the field of microbiology has stimulated increased awareness and instense search for human friendly microorganisms amongst bacterial species, fungi and yeasts that have ability to utilize lactose. Lactose is the main carbohydrate in milk it is unique to the milk of mammals (Kemer, 1995). Unfortunately, humans cannot absorb lactose for use as a nutrient. Gurr (1987) mentioned that to be efficiently absorbed from the gut lactose must be digested into its constituent sugars by the enzyme lactase. The term lactose intolerance (LI) describes the inability of individuals to digest significant amount of lactose resulting from the shortage of the enzyme lactase. Lactase is a protein produced in the cells of the epithelium of the small intestine. It is mot accentuated in the Muscosa cells of the brush bother of the Jejunum (Kim *et al.*, 1983; Buller and Grand, 1990; De Vresse, 2001). About 90-100 percent of Native Americans, Native Africans, Asians and Eskimos are lactose intolerant (Mishkin, 1997). As many as 80 percent of African-Americans, and Mexican-Americans experience lactose intolerance. It is estimated that over 75 percent of adults worldwide incure lactose intolerance or hypolactasia, or lactase deficiency.

The enzyme lactase is a disaccharide which acts selectively upon lactose contained in milk and dairy products hydrolyzing it into its two component monosaccharides, glucose and galactose (Ferguson, 1995). Lactase can be detected in the human fetus by the eighth week of gestation (Pray, 1999). Levels continue to rise during the remainder of

gestationperiod, peaking the second to third day post partum and remaining high during infancy. This lactase is critical in allowing the mammal to gain nutritional value from milk and other dairy products by allowing absorption and use of the component monosaccharide. Furthermore, if undigested, lactose on reaching the small or large intestine produces numerous troublesome symptoms that lead to abdominal pains.

However, what happens after infancy is determined by one's genetic make up. If man possesses the more common autosoma recessive gene, lactase activity begins to drop between the ages of 2 and 20 years (McBean *et al.,* 1998; Lee *et al.,* 1998). On the other hand (or chromosomal) if one possesses the less common autosoma dominant gene, lactase activity remain high into adulthood.

2.1.6 Manifestation of lactose intolerance

The problems relating to the inability to digest lactose occurs in several anatomic locations (Fernendes *et al*., 1987). In the small intestine, lactose exerts an Osmotic pull which prevents reabsorption of water into the body. The individual then suffers from accompanying abdominal pain, nausea, and rumblings which can lead to decreased quality of life and daily activities (Ferguson, 1995). Intestinal contents moved through more rapidly to the large intestine. Bacteria in the large intestine ferment the lactose, producing hydrogen, organic acids, and carbon dioxide. Abdominal pain and bloating become pronounced. Eventually the patient may suffer from watery diarrhea, flatulence, involuntary leakage and staining of underclothing and/or other inconveniences, even from a competent rectal sphincter (Ferguson, 1995). However, secondary β-galactosidase deficiency can occur at any age. It is a transient state of β-galactosidase deficiency due to the damage to the intestinal mucosa where β-galactosidase is produced.

2.1.7 Treatment of lactose intolerance

As lactose intolerant people do not have ability to synthesize β-galactosidase enzymes, the treatment of milk and its derivatives with β-galactosidase is required (Fernendes *et al*., 1987). Among the enzymes hydrolases are receiving increasing industrial applications, especially β-galactosidase or β-D-galactoside galactohydrolase (EC 3.2.1.23), which catalysis the hydrolysis of lactase into galactose and glucose has got

special attention (Furlan *et al*., 2000). The importance of β-galactosidase is related to its use in milk and milk derivatives to decrease their lactose content thereby solving the problem of low lactose solubility and degree of sweetness. Furthermore, the economic interest in this enzyme is related to its use in food and pharmaceutical industries because of the deficiency of β-galactosidase in many people (Gekas,1985). Therefore, products free of lactose or low lactose content can be consumed without any problems by lactose intolerant people (Furlan *et al.,* 2000). This has thus created a potential market for the application of β-galactosidase thereby increasing the demand for the discovery of new species of microorganism producing β-galactosidase with novel characteristics that will be of great value to the enzyme industry for different applications (Cortes *et al.*, 2005).

2.1.8 Lactose Hydrolysis Mechanisms

Lactose hydrolysis can be performed in two ways namely acid hydrolysis and enzymatic hydrolysis (Gekas and Lopez-leiva, 1985)).

2.1.8.1 Acid Hydrolysis of lactose

First way of lactose hydrolysis is acid hydrolysis (Gekas and Lopez-Leiva, 1985. It is carried out by a homogenous reaction in acid solution or in a heterogeneous phase with ion exchange resins (Gekas and Lopez-Leiva, 1985). Acid hydrolysis can be performed under harsh conditions. For example; 80% hydrolysis may be achieved in three minutes at pH 1.2 and 150^0C (Gekas and Lopez-Leiva, 1985). Although this procedure seems to be simple, it has several disadvantages the most important of which is protein denaturation due to low pH and high temperature. It causes reduction in the function of the proteins (Gekas and Lopez-Leiva, 1985). Thus, it prevents their uses in many products (Bury *et al.*, 2001). Moreover, the presence of salts in whey causes deactivation of acid and requires a demineralization step. Other disadvantages include off-colour and off-flavour formation. Due to many drawbacks of acid hydrolysis, enzymatic hydrolysis by β-galactosidase is the preferred method of lactose hydrolysis (Gekas and Lopez-Leiva, 1985).

2.1.8.2 Enzymatic hydrolysis of lactose

Lactose hydrolysis can be performed either by enzymatic catalysis carried out with β-galactosidase enzyme that permits wider operation conditions of both temperature and pH (Gekas and Lopez-leiva, 1985). The enzymatic hydrolysis of lactose offers some benefits mainly in three areas: Health, food technology and environment (Gekas and Lopez-Leiva, 1985).

Health: The health aspects are as described for lactose intolerance patients in the opening paragraph of this exposition (Furlan *et al*., 2000).

2.1.8.3 Food technology

The high lactose content in milk products like ice-cream, frozen milks, whey spreads and condensed milk, can lead to excessive crystallization resulting in products with a mealy, sandy or gritty texture (Zadow, 1993). Further more, the economic aspect of transport and storage require the concentration of milk whey by evaporation or ultrafilteraction (Zadow, 1993). The easy crystallization of lactose, however, represents a strong limitation in such processes (Zadow, 1993). Monosaccharides derived from hydrolysis are highly soluble and usually prevents the crystallization of the remaining lactose (Zadow, 1993). Using galactosidase to process such products could reduce lactose concentrations to acceptable value. This process can thus contribute significance improvement to technological and sensorial quality of those foods like increasing the digestibility, softness and creamness, etc (Zadow, 1993). Hydrolysis of lactose present in whey converts whey into very useful sweet syrup, which can be used in the dairy, confectionery, baking and soft drinks industries (Pivarinka *et al.,* 1995). In addition, several studies have demonstrated the opportunity to degrade whey lactose using β-galactosidases for manufacturing galacto-oligosaccharides (Foda and Lopez-leiva, 2000; Novalin *et al.,* 2005).

Table 2.2: Properties of β - galactosidase from microbial sources.

Sources	pH Optimum	Temperature optimum (^{0}C)	Activators	Inhibitors
A niger	3.0-4.0	55-60	None needed	None
A. oryzea	5.0	50-55	None needed	None
K.lactic	6.5-7.3	35	K^t, mg^{+2}	Ca^{+2}, na^+
K. Fragilis	6.6	37	K^t, Mn^{+2}, mg^{+2}	Ca^{+2}, Na^+
E. Coli	7.2	40	Na^t, K^+	
B. subtilis	6.3	50	None needed	Ca^{+2}
B. stearothermophilus	5.8-6.4	65	Mg^{+2}	
S. thermophilus	7.1	55	K^t, Mg^{+2}	
L. bulgaricus		42.45		
C. inaegualis	6.0	30.55		

Source: Gekas and Lopez-Leiva 1985, Mahoney, 1985

Enzymologists have attributed great importance to the galacto- syltransferase activity of β-galactosidase to obtain the synthesis of oligosaccharides with two or more galactose units starting from lactose (Rojaka *et al*., 2003). Solutions with high lactose concentrations submitted to the action of β -galactosidase and thereby suffer transgalactosylation reaction hence producing transgalactosylated oligosaccharide (TOS). These products act as functional foods with several beneficial effects for their consumers (Ferguson, 1995). Diets enriched with TOS significantly increase the population of *Bifidobacterium* like *Lactobacillus* and some species of *Streptococcus*, with a consequence decrease in the concentration of putrefactive bacteria in the gut of humans and animals. It is also known, however, that the number and amount of oligosaccharides formed from lactose depends on the origin of β-galactosidase (Ferguson, 1995**)**. Besides, TOS are non-carcinogenic sugars and can be widely used as additives in several infant formulations and in the manufacture of Candy, Pastry, etc, because of their heat stability (Ferguson, 1995).

This change in the intestinal flora (bifido effect) composition has been proposed to be responsible for the decrease of putrefactive products in the feces, lower blood cholesterol content, and higher Ca^{2+} absorption, a small loss of bone tissue in overiectomised rats and a lower incidence of Colon Cancer (Gaudreau *et al*., 2005).

(a) Reaction Mechanism

Enzymatic hydrolysis of lactose is one of the most important biotechnological processes in the food industry (Furlan *et al*., 2000). Lactose hydrolysis mechanism was explained by using the enzyme β-galactosidase obtained from *Escherichia coli* (Jacobson 1994).

A double-displacement reaction mechanism was proposed in which β -galactosidase formed and hydrolyzed a glycosyl-enzyme intermediate via carbonium ion galactosyl transition state (Wallfels and Malhrotra, 1961). It is proposed that the active site of β-galactosidase contains cysteine and histidine amino acids which function as proton donor and proton acceptor, respectively (Wallenfels, 1972). Cysteine amino acid contains the sulphydryl group acted as proton donor and histidine residues contains imidazole

group acted as neucleophile site to facilitate cleavage of the glycosidic bond, respectively, during the enzymatic hydrolysis procedure (Wallenfels, 1972)

The second reaction that occurred is called galactosyI (transgalactosyI) reaction. In this reaction, β galactosidase transferred the galactosyI moiety from the interdicted to an acceptor containing a hydroxyI group (Figure 2.4) (Mahoney, 1998). When this acceptor is water, free galactose is formed by hydrolysis. However, under certain conditions, other sugars are able to act as acceptors and give rise to oligosaccharide formation (Mahoney, 1998).

(b) Oligosaccharide formation

Enzymatic hydrolysis of lactose is accompanied by galactosyI transfer of other sugars, thereby reducing oligosaccharides (Furlan *et al*., 2000). The amount and nature of the oligosaccharide formed by transgalactosyI reaction depends mainly on the enzyme source and the nature and concentration of the substrate (Gekas *et al*., 1985).

(c) The substrate

The yield of oligosaccharides can be increased by using higher substrate and/or by decreasing the water content (Mahoney 1998). Quantitatively, the disaccharide allocatese is one of the major oligosaccharides production can be reached between 30-40% of the total sugars present (Huber *et al.*, 1976). However, at lower initial lactose levels, such as those found in milk and whey, transferase activity is reduced, where maximum oligosaccharide levels can reach 22 to 25% (Huber *et al.*, 1976).

Oligosaccharides are hydrolyzed slowly, both in vitro and in vivo (Nielsen *et al*., 1992). They can be considered to be physiologically functional foods which promote the growth of bifidobacteria in the colon and a wide variety of health benefits has been claimed in connection with this effect (Mahoney, 1998).

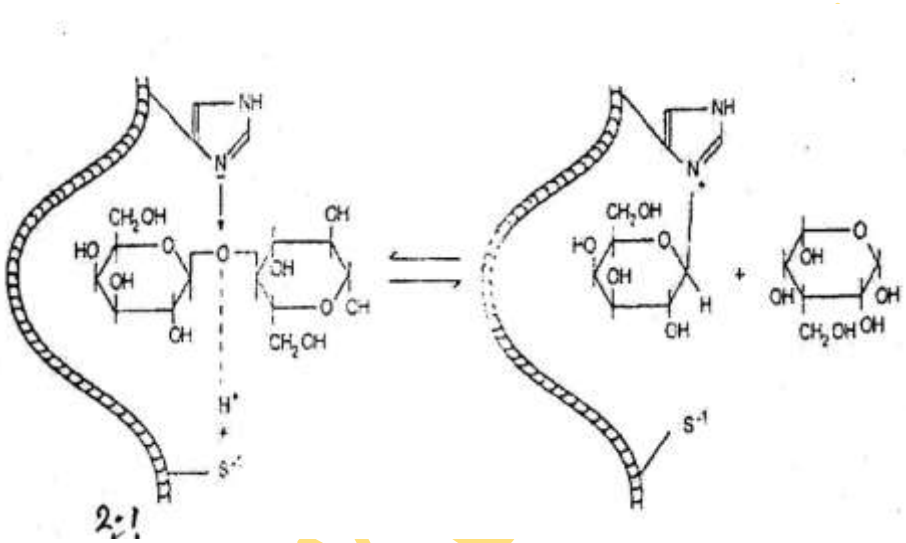


Figure 2.3: Proposed mechanism of lactose hydrolysis by β _ galactosidase (Source: Richmond *et al.*, 1981)

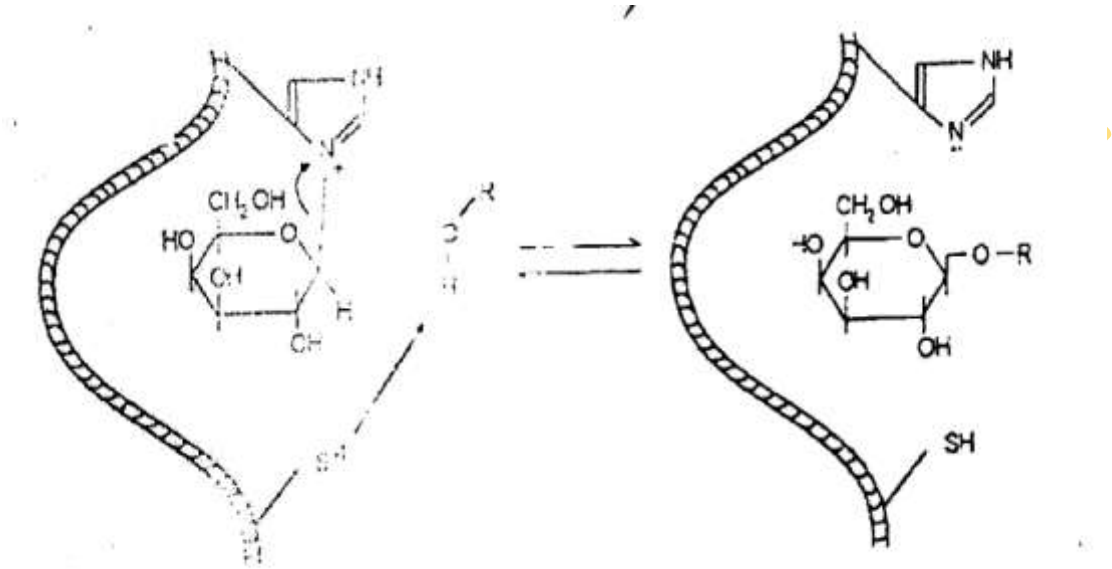


Figure 2.4: Proposed mechanism of galactosyl transfer reaction by β galactosidase.

(Source: Richmond *et al.*, 1981)

The mechanism for the transferase reaction is still not known but it is thought to involve a 1, 4-linked galactose being transferred to a 1, 6-linkage (Huber *et al*., 1976). The result of oligosaccharide formation is beneficial to human health (Welttagen *et al*., 1998). They have been added to infant formula as potential bifidus factors" to promote the growth and the establishment of bifid bacteria in the intestine (Hsu *et al.*, 2005). Moreover, other reported therapeutic benefits of oligosaccharide consumption include decreased serum cholesterol levels, enhanced absorption of dietary calcium and enhanced synthesis of B-complex vitamins (Onishi *et al.*, 1995).

2.2 Galactosidases

This is described as either of two types of Enzymes: α - (or alpha) galactosidase EC3.2.1.22, with a systematic name as: α -D-galactoside galactohydrolase; otherwise called *melibiase* (Guimares *et al*., 2001). It is an enzyme that hydrolytically removes terminal non-reducing α -D-galactose residues in α -galactosides including galactose, oligosaccharide, galactomannans, and galactolipids (Luonteri *et al.*, 1998, de vries and viser, 2011).

β -galactosidase EC3.2.1.23 with systematic name: β -D-galactoside galactohydrolase, also known as: lactose is an enzyme that removes terminal β -D-*galactose* residues in β -galactosides by hydrolysis.

2.2.1 Alpha Galactosidase (α -Gal)

Galactosidase or melibiose (α-D-galactosidase galactohyrolase, EC 3.2.1.22) is an exogalactosidase that cleaves the terminal non-reducing α-1-6-linked galactose residues from α-D-galactosides including galactose olligosaccharrides such as melibiase, raffinose and stachyose and branched polysaccharides such as galactomaannans and galactoglucomannans (Manzanares *et al.*, 1998, Shibuya *et al.*, 1995). The enzyme is widely distributed in nature: microorganisms, plants, and mammals (Naumoff, 2004 and Matrai *et al.*, 2000). They are of particular interest in view of much potential of their biotechnological and industrial applications. In plants, α-galactosidase has been greatly investigated from the seeds in relation of germination where it is involved in the mobilization of reserved carbohydrates like raffinose and stachyose (Mita and Steinkraus,

1975, Kang and Lee, 2001). They play a crucial role in upgrading nutritional values of legume-based foods: Industrially (food processing in particular), it is important in removing raffinase in beet sugar which inhibits normal crystallization of sucrose (Ohtakara and Mitsutomi, 1987). It is also used in the hydrolysis of raffinose and stachyose present in soybean milk (Guimaraes *et al.*, 2001). Transglycosidase activity was also demonstrated in some of the α-galactosidase (Anisha *et al.*, 2007). The galactooligosaccharides by the transferase action of this enzyme can be used as a Probiotic in functional foods. It also has interesting applications in the pulp and paper industries (Kang and Lee, 2001).

2.2.2 Beta Galactosidase (β -Galactosidase)

β -galactosidase (β -D-galactoside galactohydrolyse; E.C. 3.2.1.23), in other words lactase, (Yang *et al*., 1995) is a commercially important enzyme that catalyzes the hydrolysis of lactose into its constituent monosaccharides glucose and galactose (Figure 2.5)

The enzyme from several sources have been well characterized, especially the enzyme from *Escherichia coli* serves as a model for the understanding of the action of the enzyme (Jacobson 1994).

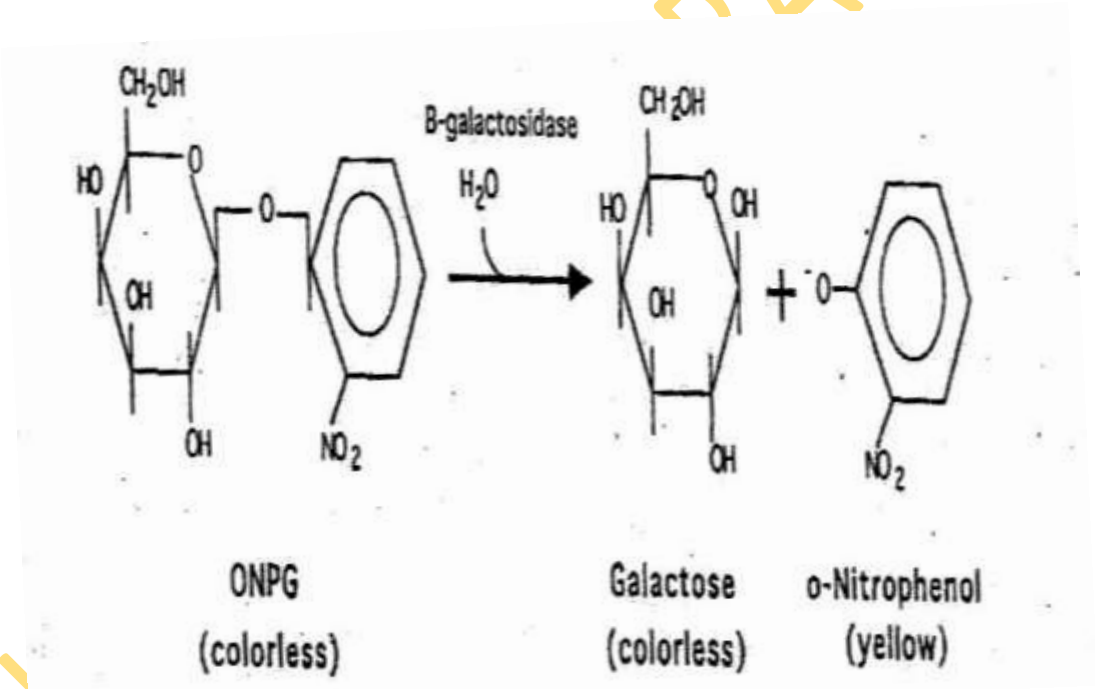


Fig 2.5 β -galactosidase structure (source: Yang *et al*., 1995)

2.2.3 Physicochemical Properties of β-Galactosidase

The enzymes derived from various microbial sources have different properties, such as protein chain length, and the position of the active site. However, it has been found recently that β-galactosidase from different sources have the same amino acid residue and glutamic acid, as their catalytic site, as shown in Table 2.3.

Molecular weight of β-galactosidase varies between organisms as well. *E. coli* β-galactosidase has a molecular weight of 116, 353 kDa per monometer (approximately 540 kDa per molecule). Molecular weights of other bacteria's β-galactosidase are presented in Table 2.4

The effects of mono-and divalent cations have been well documented (Greenberg and Mahoney, 1982; Garman *et al.,* 1996; Kreft and Jelen, 2000; Vasiljevic and Jelen, 2002). Divalent cations such as magnesium and manganese cations may have a positive or negative effect (Garman *et al.,* 1996). The rate of lactose hydrolysis by β-galactosidase from each species was enhanced by Mg^{+2} (Vasiljevic and Jelen, 2002). However, the effect of K^{+} and Na^{+} differed from strain to strain. In another study, manganese was found to be the most effective cation followed by magnesium for the maximum β-galactosidase activity of *Streptococcus thermophilus* (Greenberg and Mahoney, 1982).

Calcium ($Ca^{+2)}$ is known as an inhibitor of β-galactosidase (Greenberg and Mahoney, 1982). However, all of the calcium in milk is bound to casein. As it is not free in solution, it does not inhibit β-galactosidase activity (Garman *et al*., 1996).

2.2.4 Applications of β-galactosidase

β-galactosidase enzyme used in the hydrolysis of lactose is one of the most important biotechlogical processes in the food industry. It has potentially beneficial effects on the assimilation of foods containing lactose, as well as the possible technological advantages as below: (Gekas and Lapez-leiva 1985, Jurado *et al.,* 2002)

1. Elimination of lactose intolerance by encouraging the utilization of lactose as an Energy Source

2. Formation of galacto-oligosaccharides during lactose hydrolysis to favour the growth of intestinal bacterial micro flora.
3. Improvement in the technological and sensorial characteristics of foods containing hydrolyzed lactose from milk or whey: increased solubility, greater sweetening power, formation of monosaccharide which are easier to ferment certain products such as yoghurt and reduction of the maillard reaction.
4. Greater biodegradability of whey.

Many organisms have been known to have high β-galactosidase production for commercial use. Although, yeasts (intracellular enzyme), fungi or molds (extra cellular enzyme) are known to produce β-galactosidase (Gekas and Lopez-leiva, 1985). Bacterial sources have gained more importance and they are preferable due to ease of fermentation. High activities of exploited sources of β-galactosidase have been of microbial origin (mainly yeast and moulds) (Shibuya *et al*., 1995).

Although properties of the microbial β-galactosidase vary with the organism, suitable enzymes must be selected according to their application of an enzymes can be determine by its operational pH enzymes from yeast and bacteria (Shibuya *et al*., 1995). Acid pH enzymes from fungi and neutral-pH enzymes from yeast and permeate whereas; neutral-pH enzymes from yeast and bacteria are generally suitable for milk and sweet whey process (Mahoney, 1985)

Fungal enzymes have pH optima in the range of 3 to 5 and are therefore, suitable for processing acid whey and permeate. They also have relatively high optimum temperature which is between 55 ^{O}C -60 ^{O}C. (Flores *et al*., 1996) However, fungal β-galactosidase is not as pure as from a yeast source and it may contain other enzymes such as protease, lipase or amylase (Mahoney, 1985). As a consequence of these limitations, fungal applications of β-galactosidase have been limited as high acid products and pharmaceutical preparations (Mahoney, 1985).

Table 2.3 Active sites and other properties of β-galactosidase from various microbial origins. (Source: Zhou and Chen 2001).

Enzyme Origin	***K.lactis***	***E.coli***	***A.niger***
Length (AA)	1025	1023	1006
Proton donor	Glu^{482}	Glu^{461}	Glu^{200}
Nucleophile/base	Glu^{551}	Glu^{537}	Glu^{298}

Table 2.4 Molecular weights of β-galactosidase from bacterial sources

Organism	**Molecular Weight (kDa)**	**Source**
S. solfataricus	240	Grogan (1991)
S. thermophilus	530	Greenberg and Machoney (982)
L.acidophilus	570	Nielsen and Gilliland (1992)
L.lactis	500	McFEters *et al*., (1969)

2.2.5 Commercial procedures

Enzymatic hydrolysis of lactose by β-galactosidase is one of the most popular technologies to produce lactose-reduced milk for consumption by lactose-intolerant people (Gekas and Lopez-leiva, 1985). Evolution of lactose-products began when the enzyme β-galactosidase became accessible to the consumer (Kligerman, 1981). The consumer could add this enzyme to a glass of fresh milk to hydrolyse the lactose. After the overnight incubation at refrigerator temperature, lactose content is reduced by 70% or more depending on the enzyme amount added (Kligerman, 1981). Most lactose intolerant people could then consume the "lactose-reduced" milk without adverse effects (Kligerman, 1981).

With the developments in processing techniques, hydrolyzing lactose before packaging, certain dairy products have become more prevalent (Gilliland *et al*., 1996). Through the advances in research, the procedure has become more cost effective due to increased understanding of the process and its effects on characteristics of the product (Holsinger and Kligerman, 1991). With these new advancements, various types of lactose-reduced foods such as milk. American cheese, cottage cheese and ice cream have made their way into this growing market (Reiter, 1991). Many research efforts have been focused on ways to reduce or remove the lactose in dairy products (Kocak and Zadow, 1989). One of the more common ways to accomplish this is through the use of enzymes. Three techniques may be utilized to accomplish enzymatic lactose hydrolysis (Holsinger and Kligerman, 1991):

1. "Single-use" or "throw-away" batch systems
2. Recovery systems (β-galactosidase reuse systems)
3. Immobilized enzymes which are systems where the enzyme is chemically bound to an inert matrix (Holsinger and Kligerman, 1991).

The advantages of immobilized enzymes are clear: ease in enzyme recovery and reuse, ease in product recovery and continuous operation (Bury *et al*., 2001). The behavious of immobilized enzyme reactors have been extensively studied as a critical point in the industries process production (Li *et al*., 2007).

It has been proposed that the "single-use" enzyme batch system could be used conveniently during the development of new lactose reduced products, whereas the latter systems are better for large-scale operations (Holsinger and Kligerman, 1991). The method ultimately used is mainly dependent on a number of factors including pH of the product, maximum temperature the product will reach and how long it will remain there, contact time, activity of the enzyme, substrate, and cost (Zadow, 1986).

2.3 Food grade microorganisms

Although β-galactosidases (lactase) have been found in numerous biological systems, microorganisms such as yeasts, mold and bacteria groups still remain the only sources for its commercial production (Vasiljevic and Jelen, 2001). The use of microorganisms in food production is accepted when they have a long history of safe use. The bacterial species currently being used by the dairy industry which produce β-galactosidase enzyme, belong to the genera of *Lactobacillus* and *Bifidobacterium* (Heyman *et al.*, 2002). It comprises a limited collection of strains (Fernandez *et al.*, 1999; Heyman, 2006; He *et al.*, 2008). These bacteria have become a focus of scientific studies for three (3) particular reasons (Somkuli *et al.*, 1998): (a) lactose maldigesters may consume some fermented dairy products with little or no adverse effects, (b) these bacteria are Generally Regarded As Safe (GRAS) so the β-galactosidase enzymes derived from them might be used without extensive purification (Vasiljevic and Jelen, 2001), (c) some strains have probiotic activity such as improved digestion of lactose and a suitable strain selection must be carried out to manufacture probiotic dairy products (Vinderola and Reinheimer, 2003).

β-galactosidase enzyme therefore, is one of the glycosidases that is widely used in dairy industry and is produced by most lactobacilii and yeasts (Karasova *et al.*, 2002; Corral *et al.*, 2006; Nguyen *et al.*, 2007). It has two enzyme activities: one is responsible for the hydrolysis of lactose and also cleaves cellobiose, cellotriose, cellotetrose and to certain extent cellulose and the other, β-glycosides (Troelsen, 2005; Heyman, 2006). However, the β-galactosidase from thermophilic Lactic acid bacteria is an intracellular enzyme (Kreft *et al*., 2000). Its release from microorganisms may be obtained either by

mechanical disruption or by chemical permeabilization of the cell membrane (Kreft *et al*., 2000). The effectiveness of the various disruption methods differs for different microbial genera and strains. The literature tends to focus more on the distruption of yeasts with much less information being available on the disruption of lactobacilli (Bury *et al.*, 2005).

2.3.1 Lactic acid bacteria

These are Gram-positive, usually non-motile, non-spore forming rods and cocci, naturally present in raw food materials and in the human gastrointestinal tract (Salminen *et al*., 1998a). They lack the ability to synthesize cytochrome and porphyrins and therefore cannot generate ATP by creation of a proton gradient. The lactic acid bacteria can only obtain ATP by fermentation, usually of sugars (Chammes *et al*., 2006). Since they do not use oxygen in their energy production, lactic acid bacteria happily grow under anaerobic conditions, but they can also grow in oxygen's presence as they are protected from oxygen by-products (e.g H_2O_2) because they have peroxidases (Kim *et al*., 1983). Hence they are referred to as aerotolerant anerobes. They differ from other organisms by their ability to ferment hexoses to lactic acid (Frazir *et al*., 1978).

Lactic acid bacteria can be divided into two groups based on the products produced from the fermentation of glucose (John Lindquist, 1998). **Homofermentative** Organisms ferment (i) glucose to two moles of lactic acid, hence lactic acid is the major product of this fermentation. **Heterofermentative** lactic acid bacteria ferment 1 mole of glucose to 1 mole of lactic acid, 1 mole of ethanol, and 1 mole of $C0_2$ (John Lindquist, 1998). The homofermenters possess the enzyme aldolase and they are able to ferment glucose more directly to lactic acid than the heterofermenters (John Lindquist, 1998). The heterofermenters use the alternative Pentose Monophosphate Pathway, converting six carbon sugars hexoses to five carbon sugars (pentoses) by the enzyme phosphoketolase, producing in the process both aldehyde and diacetyl-highly desirable aromatic and flavor-enhancing substances (Car *et al.*, 2002). The heterofermenters are often use in dairy industry because of these flavor-enhancing substances (Wood and Holzapfel, 1995 and Car *et al.*, 2002). Because of the low energy yields, lactic acid bacteria often grow more slowly than microbes capable of respiration, and produce smaller colonies of 2-3mm. http://www:bact.wisc.edu/Microtextbook/rodex.

2.3.1.1 The genera of lactic acid bacteria

Lactic acid bacteria are classified by the fermentation pathway used in fermenting glucose and by their cell morphology. *Lactobacilli are* rod-shaped organisms that can be either hetero-or homofementative (John Lindquist, 1998). *Lactobacilli* are more tolerant to acid than the other genera of lactic acid bacteria and this property makes them important in the final phases of many food fermentations when other organisms are inhibited by the low pH (Axelsson, 1998).

Leuconostoc are ovoid cocci, often in chains (Wood and Holzaphel, 1995). All bacteria of this genus have a heterofermentative mode of metabolism. *Pediococcus* are cocci often found in pairs and tetrads that are strictly homofermentative (Axelsson, 1998). Their habitat is restricted mainly to plants. *S.cerevisiae* has been used as a starter culture for the fermentative of some sausages with great success (Wood and Holzaphel, 1995, Axelsson, 1998, Carr *et al*., 2002). *Streptococcus* are cocci in chains that are distinguished from the *Leuconostoc* by their strictly homofermentative metabolism (Wood and Holzaphel, 1995). These organisms can be Isolated from oral cavities of animals, the intestinal tract, skin and any food that comes in contact with these environments. *Enterococcus* and *Lactococcus* are two recent taxonomic divisions of lactic acid bacteria (John Lindquist, 1998). They were created to re-organize the large and divergent *Streptococcus* genus into smaller, more related groups of bacteria. *Enterolcoccus* are gram-positive cocci that form pairs or chains. They are distributed widely in the environment particularly in faeces of vertebrates (Jacobson, 1994). *Lactococcus* includes strains that are gram-positive, spherical cells, occurring in pairs or chains. They have a strict homofermentative metabolism and are found in dairy and plant products (Wood and Holzaphel, 1995; Axelsson, 1998).

Lactic acid bacteria have been used for centuries to produce fermented food products including Pickles, Sauerkraut, Sausage, Yogurt, Cheese, Butter milk and soy sauce (Rachi, 1988). They are widely used by man a/or in food preservation. They are widely used as starter cultures for fermentation in dairy, meat and other food industries. They remain the main factors of fermentation and protection of fermentative foods (Chammas *et al.*, 2006).

These food grade bacteria can also improve the safety, shelf-life nutritional values as well as other qualities as enumerated above (Kim and Raja Gospel, 2000). In addition lactic acid bacteria can be used as cell factory for the production of food additives and as well function as probiotics thereby contribute to the general health of the consumer upon consumption (Kim and Raja Gospel, 2000).

2.3.2 Food grade yeasts

Yeasts have been considered the predominant microbial enzyme source for food applications (Ah *et al.*, 2002). For centuries man has applied yeasts as starter cultures in Cheese, Bread, Wine, Beer, and other alcoholic fermentation products (Lowes *et al.*, 2000). Different varieties of yeasts are the world's premier industrial microorganisms which has wide exploitation in the production of food beverages and pharmaceuticals (Savora, 2002). Yeasts can grow at room temperature and can utilize (assimilate and/or ferment) a variety of carbohydrates (Nahvi and Moeini, 2004), such as pentose, hexose, disaccharides, and rarely, polysaccharides (Barnette *et al.*, 1999). They are regarded as important component of the mioroflora of many cheese varieties because of the low pH, low moisture content, high salt concentration and refrigerated storage condition of these products (Fleet, 1990; Deveyol, 1990).

Yeasts are widely distributed in nature in almost all niches and are therefore often found as contaminants, in both commercial and traditionally fermented milk (Nahvi and Moeini, 2004). In some circumstances, however, like in the production of Kefin and Kumis, where they are deliberately introduced into milk to bring about the desired aroma and flavour of the final product (Nahvi and Moeini, 2004).

As is commonly observed, the dairy products are especially favourable medium for growth of yeast due to the acidic reaction of the medium. Another important condition for their development is their ability to grow at low temperature (low pH value and low moisture content) which are unsuitable for many other microorganisms especially bacteria (Sarova, 2002). Cultured milk products create favourable conditions for microbial growth (Viltoen *et al.*, 2003). The changes of cultured milk products caused by metabolic activity of yeasts depend on degradation of lactose or compounds of its hypolysis, secretion of lipolytic and

proteolytic enzymes, assimilation of organic salts and the ability to multiply at low temperature (5-10^{0}C) during product storage (Fleet, 1992; Norvhus, 1996).

2.3.2.1 Yeasts and their coexistence with lactic acid bacteria

Yeasts usually adapt to coexistence with lactic acid bacteria as one of the groups gain the upper hand over the other or both groups grow intensively together (Ah *et al*., 2002). By this process, specific interactions take place. The yeasts play favourable effects upon the bacteria due to the changes in pH value in the medium and the secretion of biologically active substances such as vitamins, enzymes, amino acids, etc. In some cases, the yeasts significantly increase the viability of the lactic acid bacteria (Roberto *et al*., 1984). The investigations of the microflora found in the dairy drinks Kefir and Kumis proved the presence of lactic acid bacteria and yeasts coexisting as the later play stimulating effects on the bacteria (Gekas and Lopez-Leiv, 1985).

2.3.2.2 Influence of yeast growth on the maturation process and final quality of cheese

There are several mechanisms by which yeast growth is thought to influence the maturation process and quality of cheese (Sarova, 2002). Fermentation of residual lactose within the curd by species of *Kluyveromyces maxianus* produce secondary (flavour) metabolites as well as carbondioxide gas that opens up the curd texture (Rachie, 1988). Furthermore, the activities of extracellular proteases and lipases produced by some yeast species such as *Candida lipolytica* could alter curd flavour and texture. According to Fleet (1992), the release of autolytic products by yeasts can influence these cases. The high proteolytic and lipolytic activity of yeasts does not allow the cheese surface to form: the yeast may produce substances that stimulate surface growth of both lactic acid bacteria and aerobic bacteria (Welthagen and Viljoen, 1998). They may ferment residual lactose, metabolize the lactose and influence flavour formation by producting volatile acids and carbonyl compounds (Fleet, 1992).

2.3.2.3 Role of yeasts in Yogurt production

Yogurt is considered as a potential source of different metabolites of biotechnological interest (Bralasiewicz *et al.*, 2001). Hence numerous studies have been

undertaken to isolate and screen potentially important microbes in particular yeasts from yogurt (Bralasiewicz *et al.,* 2001). The presence and predominance of certain yeast species in yogurt is associated with their chemico-physical properties including their ability to ferment variety of sugars, and to produce several enzymes that hydrolyze milk sugars, fats, and proteins (Gekas and Lopez-Leiv, 1985). Such enzymes could have multidimentional applications in pharmaceutical industries. β-galastosidase produced by lactose fermenting yeast species of *Kluyveromyces* group is indeed been utilized for the treatment of lactose intolerance (Gekas and Lopez-Leiv, 1985). Furthermore, yeast isolates from yoghurt shown bactericidal activity against *Helicobacter pylori* suggesting its potential application as probiotics (Ah *et al.*, 2002).

2.3.2.4 The importance of yeast in the treatment of whey wastes

Whey is the aqueous fraction of milk generated as a by-product of cheese manufacturing (Rachie, 1988). The main solute in cheese whey is lactose, present at a concentration of 4.5-5%. Because of its high organic content, dumping it directly to the environment causes serious contamination problems (Rohm *et al*., 1992). As a solution, bioconversion of whey into Single Cell Protein (SCP) or ethanol has been adopted in several countries ((Nahvi and Moeini, 2004). This is achieved by employing yeasts of different species including *Kluyveromyces, candida* and *Trichosporous* as they are naturally able to metabolize lactose. Single cell protein can be used as a dietary supplement in feeding domestic fowl and farm animals.As they can grow at between 25-30^{o}C (Sarova and Nikolova, 2002), yeast can be exploited to manage lactose pollution. This attributes can be exploited to manage the biodegradable wastes of the food, and beverage industries that is non friendly in the environment (Nahvi and Moeini, 2004).

2.3.2.5 Yeast as a spoilage organism

Yeasts play a dual role: in some cheese types, they make a positive contribution to the development of flavour and texture during maturation; while in other varieties they can be regarded as spoilage organisms. The main defects caused by spoilage yeasts are fruity, bitter or yeasty off-flavours, gas production, discolouration changes and loss of texture quality due to gas-production and packaging (Seiler and Busse, 1990; Rohm *et al*., 1992)

contamination by yeast in yogurts is generally related to the fruits added and/or poor hygienic practices during packaging operations (Bralasiewicz *et al.,* 2001).

2.4 Milk

2.4.1 General Overview

Milk, according to F.A.O. (1990) is a unique biological fluid secreted by mammary glands of mammals for the primary purpose of nourishing their young.It is believed to be nature's most nearly perfect simple food (Ihokoronye and Ngody, 1998). Milk has been used as food since antiquity. Although a liquid, it contains greater amounts of solids than many other food materials that occur in nature in solid forms (Vedamuthu, 1983). This unique feature is made possible by well known physico-chemical forces in nature (Vedamuthu, 1979). Milk is designed to satisfy the nutrient requirement of the young and rapidly growing mammals.

2.4.2 Nutritional significance

Milk is often regarded as being nature's most complete food. It earns this reputation by providing many of the nutrients which are essential for the growth of the human body (Sarova, 2002). Being an excellence source of protein and having an abundance of vitamins and minerals, particularly calcium, milk can make a positive contribution to the health of a nation (Bralasiewicz *et al.,* 2001). The realization of its nutritional attributes is clearly illustrated by the implementation of numerous "school milk programs" worldwide.Nearly every civilization has consumed cultured milk of one type or another and these products have been and are still of extreme importance in the nutrition of people throughout the world(Anonymuos,1987). Dairy products provide about 76% of Calcium, 45% of Riboflavin, 25% of protein, 12% of vitamin and 10% of thianin proportional to the recommended dietary allowances in the human diet (Brink *et al.* ,1969). In traditional milk-drinking populations milk has always been converted to concentrated forms that retain their feed value for a longer period (Chamberlain,1990). The shortage of milk and dairy products in most developing tropical countries is due primarily to low level of milk production and lack of effective processing and preservation techniques (Sarova, 2002).

2.4.3 Types, Sources and Production

Milk is inherently a highly perishable food.Fermentation of milk occurred naturally in surplus milk set aside for later use, which were later observed that in some cases to form smooth curd with pleasing acid, odour and flavor, became putrid and cause sickness when consumed as such (Bulut *et al*., 2005). From this observation, the preparation of fermented or cultured milk products became an art which was handed down from one generation to another (Shahari and Friend, 1983). Cultured dairy products such as cheese, yogurt, Kefir and clabbered milk have been part of human diet since before recorded time (Datta *et al*., 2006). These are all forms of cultured, soured or fermented milk. At no point in 5,000 years of recorded history was there ever a time when milk and its products were not a significant part of the human diet (Fox and Mulrihill, 1982). This is what Sally Fallon (1982) in her book "Nourishing Traditions" calls scene of these highly nourishing foods that the human race through history to this point in time. Since fresh raw milk is loaded with numerous beneficial bacterial that when left to their own devices for more than a few hours at room temperature will begin the process of fermentation, its probably safe to extrapolate that early shepherds discovered that souring not only acts as a means of preservation but also improves its digestibility and nutrient availability (Molder, 1994).

Milk however, is a highly perishable food and there is a high risk of transmitting food poising bacteria to consumers (Anon., 1978). It is stressed therefore, that milk processing of any kind must be done under carefully controlled hygienic conditions.

2.4.4. Nutritional Composition and Values of milk

Milk contains three major proteins, the principal among them being Casein (Gill *et al*., 1994). When milk is converted into curd either by acid coagulation (e.g Lactic acid fermentation) or by enzyme clotting (renette curd), the solid mass is formed by Casein (Gill *et al.*, 1994). The watery exudates, whey, contain the other two: lacto-talbumin and lactoglobulim. It is for this reason that the two are called "Whey proteins" (Fox and Mulvihill, 1982).

In addition to this, the micromolecular aggregates or casein "micelles" also contain small ions such as Calcium, phosphate, magnesium and aerate referred to as Colloidal Calcium phosphate (Cap) (Christensen, 1975). According to O'mahomy (1988), lactose is

the principal Carbohydrate of milk, and it is responsible for souring of milk and cream. Its value in milk may be as high as 5.5% and as low as 3.5% (Webb *et al.*, 1976). Lactose occurs in true solutions in milk. It is a disaccharide made up of glucose and galactose linked together by a β -1, 4-linkage (Fox *et al.*, 1982). Microorganisms capable of fermenting lactose produce lactic acid as a by-product (Fox *et al.*, 1982). And to the cheese maker, lactose is the single most important milk component. According to Fox *et al.*, (1982) it is the source of a steady rate of acid accumulation in the cheese vat which is absolutely necessary for curd formation, moisture expulsion, texture and flavour development. At present, the milk of dairy cows, sheep, goats, camels, water buffalo, reinder Yaks and mares is used in various parts of the world (Chirstensen, 1975).

It is mandatory to ferment and preserve milk to avoid outbreaks of food-borne illness and intoxications that could be introduced through the use of raw milk in making (e.g. Yogurt or cheese).

2.4.4.1 The quality of milk

The type of animals, its quality, and its diet can lead to differences in the colour, flavour, and composition of milk (Gill *et al.*, 1994). Infections in the animal which cause illness may be passed directly to the consumer through milk. It is therefore extremely important that quality-control tests are carried out to ensure that the bacterial activity in raw milk is of an acceptable level, and that no harmful bacteria remain in the processed products.

2.4.4.2 Processed milk products

Although Cow's milk is the most popular in many countries, milk can be obtained from many different sources. For example, milk from goats and sheep makes a substantial contribution to the total milk production in countries of Eastern and Southern Europe, Malawi and Barbadoes, whereas the water buffalo is a common source of milk in much of Asia. Table 2.5 illustrates some of the differences in composition between these milks.

2.4.4.3 Local/indigenous milk products

The degree to which milk consumption and processing occurs will differ from region to region. It is depended upon a whole host of factors, including geographic and climatic conditions, availability and cost of milk, food taboos and religious restrictions where processing does exist many traditional techniques can be found for producing indigenous milk products (Cortes *et al*., 2005). These are more stable than raw milk and provide a means of preservation as well as adding variety to the diet (Kolars *et al*., 1984). In addition, the introduction of Western-style dairy products and the subsequent setting up of small scale dairies has provided more choice of dairy products to the consumer (Verdamuthu, 1978).

2.4.4.4 Fermented-milk products

Such as yogurt and soured milk contain bacteria from the Lactobacilli group (Bralasiewicz *et al.*, 2001). These bacteria occur naturally in the digestive tract and have a cleansing and healing effect (Ah *et al*., 2002). Therefore, the introduction of fermented products into the diet can help prevent certain yeasts and bacteria which may cause illness (Cortes *et al*., 2005).

Many people suffer from a condition known as 'lactose intolerance'. This means they are unable to digest the milk lactose. Such people however, tolerate milk if it is fermented to produce foods such as yogurt. During fermentation lactic acid producing bacteria break down lactose, and in doing so eliminate the cause of irritation.

Table 2.5 Average (%) Composition of milk in Different Species of Mammals

	Percentage of Composition				
Species	**Water**	**Fat**	**Protein**	**Lactose**	**Ash**
Ass	90.0	1.3	1.7	6.5	0.5
Buffalo	84.2	6.6	3.9	5.2	0.8
Camel	86.5	3.1	4.0	5.6	0.8
Cat	84.6	3.8	9.1	4.9	0.6
Cow	86.6	4.6	3.4	4.9	0.7
Dog	75.4	9.6	11.2	3.1	0.7
Elephant	67.8	19.6	3.1	8.8	0.7
Ewe	79.4	8.6	6.7	4.3	1.0
Goat	86.5	4.5	3.5	4.7	0.8
Guinea pig	82.2	5.5	8.5	2.9	0.9
Human	87.7	3.6	1.8	6.8	0.1
Liama	86.5	3.2	3.9	5.6	0.8
Mare	89.1	1.6	2.7	6.1	0.5
Porpoise	41.1	45.8	11.2	1.3	0.6
Reindeer	68.2	17.1	10.4	2.3	1.5
Sow	89.6	4.8	1.3	3.4	0.9
Whale	70.1	19.6	9.5	--	1.0
Sheep	80.71	7.9	5.23	4.81	0.9
Horse	89.04	1.59	2.69	6.14	0.51

Source: 2011 Dairy for all. Com, the milk and Diary products website

2.4.4.5 Other Fermented Milk Beverages

According to official legislation, fermented products derived from milk result from fermentation of lactose by means of different bacterial strains (Roginsky, 1988). The most recognized fermented product, yogurt, is defined by Codex Alimentarius of 1992 as a coagulated milk product that results from fermentation of glucose to lactose by *Lactobacillus bulgaricus* and *Streptococcus thermophilus* (Rosodo *et al*., 1992). Other lactic acid bacteria (LAB) particularly from the general *Lactobacillus*, *Streptococcus* and *Bifidobacterium* can be combined with yogurt starters to produce fermented milks with specific desirable characteristics (Kolars *et al*., 1984). LABS have been use for thousands of years to produce fermented food and milk products.

2.4.4.6 Cultured/fermented milk products

Yoghurt is one of the oldest fermented products that is believed to have originated from Bulgaria several centuries ago. It was thought to have evolved by allowing naturally-contaminated milk to sour at a warm temperature. Yoghurt is a highly nutritious acidic product of milk of various forms that is of plant or animal origin (Frazier and Westhoff, 1978). Yogurt can be unsweetened or sweetened, set, or stirred. Curd is the name given to a yoghurt-type product made from buffalo milk. Yoghurt according to Vanthamathu (1978), may be defined microbiologically as the end product of a controlled fermentation of high solids whole milk with a symbiotic mixture of *Streptococcusthermophilus* and *Lactobacillusbulgaricus.*To obtain good flavoured yoghurt, starter strains are selected with a view to obtaining good symbiosis (Moon andReinbold, 1976). The balance of these organisms in the culture is important for quality production.The milk which is the raw material used in the manufacturing of yoghurt may be obtained from various sources (Drissen *et al*., 1977). The distinctive flavor is contributed by blactic acid, which is odourless and with trace amounts of acetaldehyde, diacetyl and acetic acid, which are volatile and have strong odours (Verdamuthu, 1978). Apart from the plain yoghurt, fresh yoghurt presently marketed is sold in different forms for different applications. The varieties include flavoured yoghurt without fruit (like lemon and vanilla), yoghurt with

fruit purees whole or sliced fruits mixed uniformly throughout (Swiss style) or filled in the bottomof cup or on top of the yoghurt to mixed by the consumer (Sundae style), soft or hard –frozen yoghurt sticks, fluid yoghurt drinks and fruit topped pies field with yoghurt products are also sold for making up baking mixes, soup, salad dressing and confectionery (Vedamuthu and Neville, 1978).

The principles of preservation for yoghurt are (Vedamuthu and Neville, 1978);

- Pasteurization of the raw milk to destroy contaminating microorganisms and enzymes
- An increase in acidity due to the production of lactic acid from lactose. This inhibits the growth of food-poisoning bacteria.
- Storage at a low temperature to inhibit the growth of microorganisms.

Yoghurt types and products include (Kolars *et al*., 1984):

- Sweetened stirred style yogurt with fruit preparation
- Fruit-on-the-bottom set style: fruit mixture is layered at the bottom followed by inoculated yogurt, incubation occurs in the sealed cups
- Soft-serve and Hard Pack frozen yogurt
- Probiotic yogurts: it has become quite common to add probiotic bacterial strains to yogur (those with proven health-promoting benefits, in addition to ST and LB). These could include *lactobacillus acidophilus, Lactobacilus casei,* or *Bifidobacterium* spp. When probiotics are added, it has also become common to add ingredients known as prebiotics, such as inulin, which will, after digestion, aid in the growth of the probiotics in the colon (Ah *et al*., 2002).

2.4.4.7 Pasteurization

Pasteurization is carried out by heating to 65^0C for a period of 30 minutes (Driessen *et al*., 1977). This product was originally fermented by product of butter, but today it is more common to produce cultured buttermilks from skim or whole milk (Driessen *et al*., 1977). The culture most frequently used *Lactococcus lactis*, perhaps also subsp. *Cremoris* or *diacetylactis.* Milk is usually heated to 95^0C and cooled to 20-25^0C before the addition of the starter culture. Starter is added at 1-2% and the fermentation is allowed to proceed for

16-20 hours, to an acidity of 0.9% lactic acid. This product is frequently used as an ingredient in the baking industry, in addition to being packaged for sale in the retail trade (Viljoen *et al*., 2003).

2.4.4.8 Other Processed milk products.

Ice cream

Ice cream is a frozen mixture which contains milk, sugar, fat, and optical thickeners (e.g. pectin or gelatin), colouring, and flavouring. It may be sweetened and flavoured in numerous ways with nuts, fruit pieces, and natural or artificial flavours and colours (Hastings *et al*., 1987).

The principles of preservation are (Kreft *et al*., 2000);

(a) pasteurization to destroy most micro-organism and enzymes

(b)freezing to inhibit microbial growth

Acidophilus milk

Acidophilus milk is traditional milk fermented with *Lactobacillus acidophilus* (LA), which has been thought to have therapeutic benefits in the gastrointestinal tract (Gilliland *et al*., 1996). Skim or whole milk may be used. The milk is heated to high temperature, e.g., 95^0C for 1 hour, to reduce the microbial load and favour the slow growing LA culture (Sraidhar *et al*., 1989).

2.4.5 Cheese

Cheese is a wholesome article of food, made from the curd of milk coagulated by rennet, separated from the whey and pressed into a solid mass (Singh and Rachie, 1988).Cheese is a solid curd with some or all of the whey drained off, and either used in its present form or dried with further maturation taken place in storage (Rachie, 1989). The process is achieved by the combined action of lactic acid bacteria and the enzyme rennin. Just as cream is a concentrated form of milk fat, cheese is a concentrated form of milk-protein (Rachie, 1989). The curd can be formed by activities of lactic acid bacteria, organic acids such as lemon juice, vinegar or rennet, sometimes in the presence of heat (Chamberlain, 1996). The differences in cheeses that are produced in different regions

results from variations in the composition and type of milk, variations in the process, and the bacteria used (Chamberlain, 1996). The different cheese varieties can be classified as either hard or soft. They can also be classified on the basis of moisture content, into very hard, semi hard and soft cheese (Muir, 1997).

In many parts of West Africa, including Nigeria, the normadic Fulani produce the bulk of milk from cattle and goat. Traditional cheese making as practiced by the Fulani house wives is probably the most important technique of preserving surplus in rural communities in Nigeria and in Benni republic (Aworh, 1987).”Warankasi” in Nigeria is a soft un-ripened cheese made from milk. It is regarded as an important source of protein as it ay replace meat of fish or be used in various food recipes(Aworh and Egunolety, 1985).

2.4.6 Phenotypic identification of fermentative microflora

This procedure is time consuming and often problematic due to ambiguous biochemical or physiological traits(Hasting and Holzapfel,1987; Hugas *et al.*, 1993).In the last few years, the development of molecular typing methods has offered the possibility of accelerating a great deal of bacterial identification. Moreover, direct sampling in complex matrices, such as food, may avoid biases related traditional methods. Several molecular techniques have been applied for the identification of the main bacterial population isolated from meat products such as Sodium Dodecyl Sulfate polyacrylamide gel electrophoresis (SDS-PAGE) of whole cell proteins(Samelis *et al.*, 1995); hybridization with rRNA probes (Nissen and Dainty, 1995); restriction fragment length polymorphism analysis of 16SrRNA gene(Sanz *et al.*, 1998), randomly applied polymorphism DNA-PCR analysis(Berthier *et al.*,1998; Andrighetto *et al.*,2001); PCR-temperature gradient gel electrophoresis(Cocolin *et al.*, 2000) and species-specific PCR (Yost *et al.*, 2000).

Species-specific PCR is a rapid and reliable molecular technique for the characterization of bacterial communities without colony isolation. However, the sensitivity of PCR in foods can be reduced due to the complexity of the food matrix and the presence of many PCR inhibitors. Substances which have been proven to be PCR inhibitory, such as Proteinase (Powell *et al.*, 1994), heme compounds (Akane *et al.*,1994),chelating agents and proteins (Buffone *et al.*,1991) may be present in fermented sausage homogenates. Thus many sample preparation methods such as dilution, centrifugation, filteration,

aqueous two-phase systems adsorption methods, and DNA extraction have been developed to overcome the effects of PCR inhibitory substances (Lantz *et al.*, 1994).

2.4.7 Microbial Physiology

Growth in the microbial world is usually referred to as an orderly increase in the numbers of individuals and the quantity of the cellular constituents which depends on the ability of the cells to form new protoplasm from the available nutrients in the environment.In most bacteria species this process involves increase in cell mass and duplication of organelles, synthesis of new cell wall, partitioning of the two chromosomes, septum formation all of which result in binary fission. Growth can therefore be measured in terms of changes in cell mass and cell number.

Physiological characterization of microbial isolates is usually carried out by the determination of morphological and biochemical characteristics. The use of physiological methods in microbial identification and characterization in combination with molecular identification and typing techniques yields the desired results especially in microbial ecosystems where the variety of the different strains is of interest. Of equal importance are the various responses to Temperature, acid tolerance test (pH), Oxygen potential, growth on different Carbon sources which are all beneficial in arriving at a complete physiological profile of an organism.

2.4.7.1 Enzyme purification

Enzymes are the catalysts of biological processes. The activities of enzymes have been recognized for thousands of years. Most workers have employed the principle of Centrifugation of microbial cultures to obtain the enzyme fractions from the culture broth or media. The products obtained there from are labeled as “crude enzyme” whose activities could be accurately characterized through progressive purification procedures. Some of those steps would involve ammonium sulphate precipitation, Acetone precipitation, Dialysis through a permeable membrane and passage through columns of different mesh/ pore sizes

2.4.7.2 Electrophoresis

Electrophoresis refers to the migration of ions in an electric field, a property widely employed for the analytical separation of biological molecules. Proteins constitute a major fraction of any biological system even though its presence may either be dominant or sparse. To isolate these proteins they have to be in solution. Different proteins move at different rates as a result of their diverse charges and frictional coefficients so that the reading and trailing edges of the migrating protein columns of each species form separate moving boundries in a buffer solution. Moving boundary electrophoresis was one of the few powerful analytical techniques commonly employed in the early years of protein chemistry. However, because of its many drawbacks such as the convective mixing of the sample which often limits the resolution to be achieved, other techniques like zone electrophoresis in which the sample is constrained to move in a solid support such as filter papers, cellulose or a gel. Moreover the small quantity of material that is used in zone Electrophoresis permits the various sample components to migrate as descrete bands.

2.4.7.3 Gel-electrophoresis

Gel- electrophoresis is among the most powerful and most conveniently used methods for micro-molecular separation. The gel in common use, Polyacrilamide and Agarose, have pore of different molecular dimensions whose sizes can be specified. The molecular separations are therefore based on gel filteration as well as the electro-phoretic mobility of the molecules being separated. Sodium dodecyl sulfate (SDS), a detergent that is often used in biochemical preparations, binds quite firmly to proteins causing them to assume a rod-like shape. Consequently, the electrophoresis of proteins in an SDS- containing polyacrilamide gel, separates them in order of their molecular masses because of gel filtration effects.

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Sample Collection

Twenty five packs of different brands of yogurt manufactured by three different companies, namely; Ice creams from Mr Biggs and Tantalizer, yogurt was collected from Fanyogo, Cedaa yogurt and De-real yogurt while local cheese 'wara' were sourced and purchased from Ojoo and Sasa market with different retail outlets in Ibadan. All samples were transported in sterilized ice-packed containers to the laboratory and stored in deep freezers (4℃) till further processing.

3.2 Media preparation

For isolation of Bacteria 2g of cheese and 2 mL of milk was added to 5 mL of MRS broth and incubated anaerobically (24 hrs at 37^{o}C).Then 5ml of each were spread onto MRS agar. Plates were incubated in anaerobic jars at 37^{o}C for 48 hrs.

3.2 Isolation and culture methods

3.2.1 Isolation and enumeration of yeasts and moulds

Content of each sachet of yogurt was uniformly mixed and a 10 mL sample was aseptically withdrawn and mixed in a flask containing 90 mL of 0.1% sterile peptone solution. Ten fold serial dilutions (Harrigan and MacCance, 1960) were made to a power of 10 for counting purposes. One mL of each dilution level was introduced into sterile Petri dishes to which 20 mL sterile molten agar (SDA, YEA and MRS) was added and the mixture gently swirled to ensure even distribution of the inoculum.

For enumeration of total fungal count 1 mL of each serial dilution was mixed with Saboraud Dextrose Agar (SDA). The medium was allowed to solidify and then incubated at 37^{o}C for 24-48hr (Moreira *et al.*, 2001)

Lactic acid bacteria were isolated on De Mann Rogosa Sharpe (MRS) agar incubated under appropriate anaerobic conditions at 37^{o}C for 24h. Bacterial isolates were examined by Gram-stain and identified by standard bacteriological and biochemical methods (Sneath *et al*., 1984). Acid production from carbohydrates in MRS broth base was evaluated. Their ability to produce lactic acid was also studied by titrating 10 mL of homogenized samples against 0.25 mol/L NaOH using 1 mL of phenolphthalein indicator (0.5% in 50%alcohol).

3.2.2 Characterisation of obtained isolates

3.2.3 Microbial morphology

The morphological and colonial appearance of isolates were observed and recorded. Colony and cultural appearances were examined with the aid of magnifying lens. The forms and sizes of the isolated organisms were observed. The parameters used depended on the shape, colour, elevation, margin, texture, size. Microbial growth in broth culture tubes was also observed and examined for uniformity, granular sructure or a uniform growth with deposit or surface pellicle.

The microscopic study was carried out to enhance distinct identification. This included the size, shape and type of cell wall staining. Photo-micrographs of important isolates were carried out at high magnifications (×400).

3.2.4 Screening of Isolates for β-galactosidase Production

In situations where N, N-dimenthyl formamide could not be readily obtained, 0.1mM IPTG (isopropyl-thio-β-D-galatophyranoside) was used as an inducer.

X-gal (5-bromo-4-chloro-3-indoxyl-β-D-galactopyranoside) is a chromogenic substrate which has been used to screen β-galactosidase positive organisms.One milliliter(1ml) of culture samples was serially diluted and plated on MRS agar containing 0.01% X-gal (5-bromo-4-chloro-3-indolyl-B-D- galactopyaranoside) and 0.1mM IPTG (Isopropyl-thio-β-

D-galactopyranoside) as an inducer. Plates were anaerobically incubated at 37^{o}C for 24-48h for colour formation. Colonies producing β-galactosidase were green (Vinderola and Reinheimer, 2003).

3.3 Beta -Galactosidase production

3.3.1 Inoculum Preparation

The inoculum cultures were grown on a medium composed of modified MRS broth. Inoculum was prepared using 24h-old cultures on agar slants. This was rinsed with 5ml of sterile distilled water and thoroughly mixed so that the cells become evenly distributed. Thereafter, 1ml of the bacterial cell was transferred into another sterile bottle and stained with a drop of crystal violet.

3.3.2 Determination of Inoculum Size

Single colonies of the different selected bacterial isolates were inoculated into freshly prepared and sterilized modified MRS broth. Each broth culture was serially diluted out in sterile distilled water (Harrigan and McCance, 1966) and a mL quantity of each dilution fold was introduced into sterile petri dishes. Sterile molten agar was added by the pour plate method and the dish thoroughly swirled to ensure adequate and even mixing of the cells. The number of cells per petri dish was counted and extrapolated to determine what contained in the initial culture broth. This broth is now diluted out to use at a 10^{-6} colony forming unit per ml of culture broth.

3.3.3 B-galactosidase production from lactic acid bacteria

Modified MRS broth (MMRS) which has the same composition with MRS broth except that 2% lactose was replaced with 2% glucose was used for β-galactosidase production. Fermentation were started with the introduction of 10% of inoculums into 150 mL culture medium in 250 mL Erlenmeyer flasks. This was incubated under anaerobic conditions for 24 hours. Isolates were sub-cultured successivefully in MRS broth and again in modified MRS broth for the period of cultivation. Optical Density (OD_{600nm}) of culture inoculum (10 mL) was measured before the inoculation into 600ml modified MRS medium at pH 7.0. Inoculum culture (1 mL) was diluted with peptone water (9 mL) before reading the OD at 600nm.

3. 3. 4 Intracellular enzyme extraction

For intracellular extraction of enzyme, 10 mL of fermented broth (MMRS) is harvested by centrifugation at 7000 rpm and 4^0C for 15 min. The pellet obtained was washed with 10 mL sodium phosphate buffer (pH 7.0). Two milliliter (2 mL) of Isoamyl alcohol was added into the tube containing 10 mL of fermented broth. After gentle mixing, the content was put on rotary shaker and shaken at 180 rpm for 15 min. The mixture was again incubated at 37^{o}C for 15 mins. This solution was then centrifuged at 7000 rpm and 4^{o}C for 15mins. Finally the pellet was re-suspended in 9 mL 0.05M sodium phosphate buffer pH 7.0. The supernatant was immediately transferred into eppendorf tubes for enzyme assay. Supernatant was stored at 4^{o}C until the assay time (Barberis and Gentina, 1998). This solution was labeled "Cell Free Crude Extract".

3.3.5 Enzyme assay

The modified method method method method method method method method method method method method described by Food Chemical Codex (1993) was used, whereby 2.5×10^{-3} mg of the chromogenic substrate ONPG (o-nitrophenyl-β-D-galactopyranoside), (Sigma-Aldrich) was dissolved in 0.2M sodium phosphate buffer (pH 7.0). The crude permeabilized cell suspension (1.5 mL) was incubated with 2 mL of the above substrate (ONPG) mixture at 37^{o}C for 15min {Miller, 1998}. The reaction was terminated by the addition of 1ml of 10% cold Na_2Co_3. The liberated o-nitrophenol (ONP) was measured spectrophotometrically at 420 nm absorbance. ONP concentration is calculated using an extinction coefficient.One unit enzyme activity (U) is defined as µmoles of o-nitrophenol formed per ml per min. Folin Lowry method was used for protein estimation using BSA as standard. Protein content within all culture filtrates was tested for by the method of Lowry et al.(1951) using bovine serum albumin as standard. The agents required were solution A: 1% $CuSO_4.5H_2O$, solution B: 2%Na-K-Tartarate and solution C: 2% Na_2CO_3 in 0.1M.Ratio 1:1:98 of solutions A, B and C were mixed.

3.3.6 Determination of Enzyme activity

One unit of enzyme activity (U) was defined as the amount of enzyme that liberated 1mMol of o-nitrophenol per minute under the assay condition (Kuby and Lard, 1953). The results are expressed as U/ml. Specific activity was defined (Ug^{-1}) as the number of enzyme units per gram of cell biomass. The biomass concentration was measured gravimetrically as dry cell mass (gm^{-1}) by centrifuging, 20 mL of original culture broth at

4000rpm for 20 min; followed by drying the cell in a pre-heated oven-dryer at 100^0C overnight at appropriate temperature overnight.

A volume of resuspended cells in 0.2M phosphate buffer (pH 7.0) amounting to 10-20mg of dry cell mass was mixed with 5 mL isoamyl alcohol and diluted up to 25 mL with 0.2M phosphate buffer pH 7.0. The mixture was shaken for 15 min. at room temperature to make the cell envelope permeable and used for the enzyme assay as stated under section 3.3.5.

3.3.7 Determination of pH, lactic acid, acetic acid

3.3.7.1 Quantitative Estimation of Lactic acid: The production of Lactic acid was determined by titrating 10 mL of the homogenized sample against $0.25mol^{-1}$ NaOH using 1ml of phenolphthalein indicator (0.5% in 5% alcohol).Each milliliter of 1N NaOH is equivalent to 90.08mg of lactic acid. The titratable acid was calculated according to A.O.A.C method (1990).

$$\text{Titratable acidity} = \frac{\text{MI NaOH} \times \text{N NaOH} \times \text{M.E} \times 100}{\text{Volume of sample used}}$$

The lactic acid was determined in MRS broth cultures by titrating 25ml against 0.1M NaOH in a 100 mL conical flask, to the first trace of pink colouration. This was repeated two times and the average titre value was taken. Where: MI NaOH = Volume of NaOH used; N NaOH = normality of NaOH solution; M.E = Equivalence factor.1ml of 0.1M NaOH=9.008mg lactic acid (Mante *et al*., 2004)

3.4. Determination of Growth

Modified MRS (MMRS) medium was used during growth studies for enzyme production. The growth studies were performed in 500 mL volume of MMRS, which includes calculation of change in pH, change in turbidity of medium and total β-galactosidase activity of the isolates and measurement of change in turbidity of medium for construction of growth curve of the isolates.

The number of mole of ONP (O-nitro phenol) Liberated was determined from the standard curve of ONP. (One unit (U) of β-galactosidase activity was defined as the amount of enzyme required to release 1.0 µmol of o-nitrophenol (ONPG) per minute under standard

assay conditions. The extinction coefficient of dissolved o-nitro phenol at 420 nm was found to be (1.3546) mL µmol-1 cm-1.

Each experiment was done at least twice. Then the results were taken.

Activities were Calculated as described by Baran formula (Baran, 1996)

$$U/mL = \frac{\Delta A_{420}/min}{\varepsilon}, \text{ where:}$$

Ɛ= (extinction coefficient): 1.3546 mL µmol-1 cml.

The rate of absorbance change (ΔA_{420}/ mm) was calculated from linear position of the curve by using linear regression.

The rate of absorbance change (ΔA_{420}nm/mm) was calculated as follows

(ΔA420/min) =Δ A420/min (Enzyme reaction) _ΔA420/min (blank).

A specific activity was calculated as follows (Baran, 1996),

$$U/mg\ protein = \frac{\Delta A_{420}/min}{\varepsilon\ (Extinction) \times mg\ protein\ /mL\ reaction\ medi}$$

3.5 Biochemical characterization of isolates

Various biochemical tests were carried out for possible identification of the isolates. Young cultures (24-48hours old) were used for each biochemical test inoculation except where otherwise stated.

3.5.1 Catalase test

This test detects the presence or absence of catalase in the isolates. Catalase is an enzyme found in most bacteria and is known to catalyze the breakdown of hydrogen peroxide (H_2O_2) to lobe oxygen as (O_2) in this process.

$$2H_2O_2 \longrightarrow 2H_2 + 2O_2$$

The test was carried out using the method of Kiss (1984). To a loopful of the culture was added few drops of 3% hydrogen peroxide on a clean sterile slide and observed for

bubbles. Evolution of gas or white froth indicates catalase positive reactions while absence of froth indicates negative reactions.

3.5.2 Oxidase test

This detects the presence or absence in bacteria of certain oxides that will catalyze the transport of electrons between electron donors in the bacteria and redox dye. A few drops of 1% aqueous solution of tetramethyl-p- phenylene-diamine hydrogen peroxide were added to a piece of whatman No. 1 filter paper with the aid of glass rod to moisten the filter paper. The filter paper was then smeared with the different isolates at different positions. A purple colour (within 5-10 seconds) was regarded as positive and a delayed reaction or no colouration at all was negative (Seeley and Vandemark, 1972) or delayed/weak positive reactions (Kics, 1984).

3.5.3 Methyl Red Test

Glucose phosphate peptone broth was prepared as described by Harrigan and Mc Cance (1976). Ten milliliters of the broth were dispensed into screw cap tubes and sterilized by autoclaving. The test organisms were then inoculated into the broth and incubated. A few drops of methyl red indicator were added to the cultures after the incubation period. Development of red colouration was considered positive; otherwise it was negative (Harrigan and Mc Cance 1966).

3.5.4 Indole Production

Two percent (2%) (w/v) peptone broth was prepared and 10 mL was dispensed into each screw-capped tube and then sterilized by autoclaving. Inoculation of the tubes with the isolates was carried out and incubated for 5-7 days at 35^{o}C. To 1ml of the broth culture, 1 mL of 6% α-naphtol solution and 1ml of 40% potassium hydroxide (kovac's reagent) was added in a sterile tube. Development of red colour within 5mins was considered positive (an indication of indole production) while absence of such colouration indicated a negative result.

3.5.4 Oxidative/fermentative test

The medium of Hughes and Leifson (1953), containing bromothymol blue, was used. Ten (10.0) mL of the solution medium was dispensed into screw-capped bottles and then

sterilized by autoclaving. Two replicates of each tube were inoculated with each of the test organisms. One of the tubes was covered with sterile paraffin and the tubes were then incubated for 5-8 days at 35^{o}C. Acid production is shown by colour change from blue to yellow. Fermentation organisms have acid produced in both tubes while oxidative organism produces acid only in tube without paraffin seal.

3.5.6 Homo fermentative/hetero fermentative test

As obligate fermenters of carbohydrates, the lactic acid bacteria are universally able to ferment glucose. These can be divided into two groups one can culture the test organism in a medium containing glucose and, after good growth is obtained, plunge a red-hot inoculating loop into the broth culture: this will cause release of CO_2 which will be seen as an eruption of very fine bubbles rising to the surface of the broth and forming a noticeable ring if a test organism is heterofermentative.based on the end products formed during the fermentation of glucose. Homofermentative lactic acid bacteria produce lactic acid as the sole or major end product, while the heterofermentative lactic acid bacteria produce equivalent amounts of lactic acid, CO_2 and ethanol. The hot-loop test can be used to differentiate these two groups. As CO_2 is very soluble in water at high pH, a Durham tube was unable to detect its production. If, however, the temperature of the solution is increased, CO_2 will become insoluble and will be released in the gaseous form.

3.5.7 Nitrate Reduction Test

This test was carried out to investigate the ability of the bacterial isolates to reduce nitrate compounds to nitrite and ammonia. Nitrate peptone water containing peptone water with addition of 0.1% KNO_3 (potassium nitrate) was the medium used for the test. Ten milliters of the medium was dispensed into test tubes with inverted Durham tubes. These were autoclaved, allowed to cool and inoculated with test organisms. An uninoculated tube served as control. The tubes were then incubated at 30^{0}C for 5-7 days and the presence of nitrite was determined by adding to each tube, 0.5 ml of 1% sulphanilic acid in 5 ml acetic acid followed by 0.5 ml of 0.6% N, N-dimethyl 1-P-diamise dichloride in 5.0 ml acetic acid. Development of red colouration gives a positive result while the presence of gas in Durham tubes indicates production of Nitrogen (Payne, 1973).

3.5.8 Growth in sodium chloride (NaCl)

Growth of isolates in 0.01% (w/v) phenol red glucose peptone water (GPW) containing 6.5% (w/v) and 4.0% (w/v) NaCl, each, was carried out and examined after 3-4days of incubation at 30^{o}C. Colour change from red to yellow indicates positive result, otherwise result is negative (Harrigan and McCance, 1966). Uninoculated tubes served as control.

3.5.9 Production of ammonia from arginine

The production of ammonia from arginine was tested using the method of Doring (1988). A modified nutrient broth (Nutrient-arginine broth) was used. The nutrient broth without arginine was used as control. 24hrs old cultures were inoculated into 4% arginine broth (5mls) in test tubes plugged with cotton wools and incubated at 30^{o}C for 5-7 days. Few drops of Nesslers reagent were then added after the incubation period to drop of the broth in small size test tubes. Development of deep yellow or brown colour indicates positive reactions (Harrigan and McCance, 1966).

3.5.10 Oxygen relationship of isolates

This involves oxygen requirements of the isolates for the utilizations of compounds. It is used extensively to show the decomposition of simple carbohydrates (Holding and Collee, 1971). 10.0 ml of Yeast Extract peptone agar (YEPA) was dispensed into each test tube and sterilized by autoclaving. The tubes were later inoculated with the test organisms at 4^{o}C for 7 days. Areas of growth along the tubes were noted after the incubation period. Growth at the surface indicates obligate aerobes while obligate anaerobes grow at the bottom of the tubes. Those along the length of the tubes are facultative anaerobes while microaerophilic organisms grow near the surface.

3.5.11 Gelatin Hydrolysis

Gelatin is a protein which can be metabolized only by those microorganisms which are capable of producing proteolytic enzymes that breaks it down. When broken down, gelatin loses its gelling qualities. Gelatin broth was prepared by suspending 10g of gelatin in 100 ml of nutrient broth to give a broth of 10% (w/v) gelatin. Nine ml of this distributed into each test tube plugged with cotton wool and sterilized by autoclaving at 121^{o}C for 10 min. A loopful of the isolates inoculated into the tubes and incubated at 4^{o}C for 10days. Gelatin hydrolysis was tested for by cooling the tubes in freezer for about 15 minutes after

which the tubes were observed for gelatin hydrolysis. The tubes in which gelatin hydrolysis did not take place had their gelatin content in solidified state, while a positive reaction was indicated by the gelatin remaining in the liquid state without solidification (Harrigan and McCance, 1966).

3.5.12 Motility Test

Test organisms were grown in sterile peptone water broth in McCartney bottles at 4^{o}C for about 7-8 days. About a drop or two from the micropipette of the broth was then placed in a hollow in a clean cavity slide and with cover slides using Vaseline seals to place the cover slide in position to exclude air bubbles. The slides were then examined under the lower power of the microscope to observe for any possible motility of the different organisms on slides. Motility was recorded as positive for any movement noticed of the isolates, otherwise motility was negative (Seeley and Van Demark, 1972).

3.5.13 Starch Hydrolysis

Starch agar plates as described by Seeley and Van Demark, 1972, were used. One isolate was streaked on each of the plates and then incubated for 5 days at 30^{o}C. Starch hydrolysis (due to amylase activity of the isolates) was tested for by flooding the plates with dilute Gram's iodine solution. Occurrence of clear zones along the line of streak was a positive result.

3.15.14 Voges Proskauer Test

This is a test for the production of acetyl methyl carbinol from glucose. Cultures of methyl red broth were tested to know whether the isolates after producing acid from glucose are capable of producing acetyl methyl carbinol from the acid by adding 1.0 ml of 6% NaOH and 0.5 ml of 5% solution of ∝-naphthol in absolute ethanol to 1ml of 72 hrs culture of the isolates, after 3 – 5 days of incubation at 4^{o}C. Appearance of a pale pink colouration within 5mins was recorded as positive but negative results could be left for up to 3-5hrs to check for slow reaction.

3.5.15 Production of Hydrogen Sulphide

Some bacteria are capable of decomposing sulphur compounds such as cystine or reduced inorganic sulphur compounds to produce hydrogen sulphide. Lead acetate paper strips in

tubes containing the thiosulphide broth (nutrient broth plus 0.01% thiosulphate) was used. Production and liberation of hydrogen sulphide causes blackening of the lead acetate paper strips.

3.15.16 Sugar Fermentation Test

This is a test to confirm the ability of the isolated microorganisms to utilize various sugars as carbon sources. The products of sugar or carbohydrates metabolism depend largely upon the type of enzyme possessed or produced by the organism. Sugar utilization is coupled with acid production which can be confirmed by the addition of an indicator to the medium. Gas may also be formed during the reaction and this may be detected by inserting Durham tube in an inverted position into the reaction tube

Preparation of Sugar Solutions

One percent (w/v) of each of the peptone water and sugar was prepared to result in peptone water-sugar broth. (Peptone 1.0% NaCl (0.1%), fermentable sugar (1.0%)). Phenol red (0.01% w/v) was added to the broth as indicator. The sugars used include lactose, mannitol, sorbitol, dulcitol, sucrose, dextrin, maltose, glucose, saccharose, xylose, arabinose, inulin, raffinose, cellulose, mannose, salicin, rhamnose, fructose, sorbose, galactose, ribose, and inositol.

3.6 Identification of isolates

Results of the various biochemical tests were utilized in identifying the isolates with particular reference to Bergey's Manual of Determinative Bacteriology (Sneath, 1986). However some of the isolates could not be identified to the species level. The API 50 CH (for rods and cocci) was used for further identification of some of the isolates using the manufacturer's online software (www.apiweb.biomerieux.com).

3.7 Molecular analysis of Isolates

3.7.1 Extraction of genomic DNA of LAB isolates

DNA extraction from the LAB isolates was carried out using a modified GES (5 M guanidine thiocyanate (Fisher Scientific BPE221-250), 0.1 M EDTA Sigma E-5134), and 0.5% N-lauroyl-sarcosine sodium salt (Sigma L-5777) (w/v) DNA extraction method (Pitcher *et al*.,1989). Aliquots of 1.5 mL of overnight cultures grown in appropriate broth

were centrifuged (Biofuge, Heraeus, Germany) in eppendorf tubes at 13,000g for 1min. Pellets obtained were washed in 1 mL of ice cold lysis buffer (25mM Tris-HCl (Sigma T-6066), 10 mM EDTA , 50 Mm sucrose (BDH GPR 302997J) pH 8.0). The pellets were re-suspended in 100 µl of lysis buffer in addition to 50 $mgml^{-1}$ lysozyme (Sigma L-6876) and incubated at 37°C for 30min. volumes of 0.5 mL GES solution were added and mixed well. This was incubated at room temperature for 15 min. The lysate was then cooled on ice for 2 min and 0.5 mL of 7.5 M ammonium acetate (Fisher Scientific A3440/60) (also cooled on ice) was added, voltexed and incubated on ice for 10 min.

Aliquots (0.5ml) of 24:1 chloroform: isoamylalcohol (Sigma CO549-1QT) were added, voltexed and centrifuged for 10 min at 13,000g. Aliquots of 800 µl of the upper phase were removed quantitatively and placed into a clean eppendorff tube. Cold isopropanol (Fisher Scientific P/7500/21) (0.54 vol) was added and mixed for 1 min. This was then centrifuged at 13,000g for 5min and the supernatant removed from the pellet. The pellet was washed three times in 500 µl of 70% ethanol and dried at 37^0C for 15 min.Aliquots (50µl) of TE buffer (10 mM Tris-HCl, 1mM EDTA, pH 7.5) were added and 5 µl of the DNA were checked on 1% agarose (Biogene,Kimboltton,UK) gels in 200 ml 1X TAE (4.84 g Tris base, 1.14 g glacial acetic acid, 2 ml 0.5 M EDTA, pH 8) buffer and the DNA samples were then stored at -20^0C for future use.

3.7.2 PCR amplification of 16S rDNA gene

The modified method of Bulut *et al*., 2005, was used. Amplification of 16S rDNA gene-ITS region, was performed by using the following primers: order to determine the phylogenetic grouping of the six bacterial isolates,their 16S rDNA genes were amplified by standard PCR using universal Operon primer series **:**OPA1,OPA2 OPA3,OPG16 And OPF17 Respectively Designed To Target The Conserved Region Of Bacteria 16S rDNA (Macrogen Corp,9700 Great Seneca Highway,Rockville MD 20850 USA) Forward (16S ITS For), 5'-AGAGTTTGATCCTGGCTCAG-3' and reverse (16S its Rev), 5'-CAAGGCATCCACCGT-3'; 16S rDNA v3, Forward, 5'-CCTACGGGAGGCAGCAG-3' and 16s rDNA v3 Reverse 5'-ATTACCGCGGCTGCTGG-3'. The v3 primer was used for ease of sequencing of the gene, using the variable region 3 (v3), for the genetic identification of the isolates.

Each of the PCR was performed in a 50 µl reaction volume containing 50 ng genomic DNA as the template (equivalent to 1 µl), 0.2 mM- deoxynucleoside triphosphates, dNTPs, (Promega, U120A-U13A, Madison, WI, USA), 2.5 mM-$MgCl_2$ 10 pmo each (0.1 µl volume) of the DNA primers in 1 X PCR buffer (Promega, UK), and 1.25 units Taq DNA polymerase (Promega, UK). Amplification conditions were as follows: an initial denaturation step of 5 min at 94^0C, 40 amplification cycles, each consisting of 1 min denaturation at 90^0C, 1 min annealing at 42^0C, and 1 min elongation at 72^0C. PCR amplifications were performed in a thermocycler (Techneprogen, Cambridge, UK).

3.7.3 Gel electrophoresis of 16S rDNA PCR products

Electrophoresis of the amplified 16S rDNA PCR products was performed on the Bio-rad contour- Clamped Homogenous Electric Field (CHEF) DRII electrophoresis cell.This was done through 1.5% (w/v) Agarose gel (general purpose,Biogene) in 0.5 X TAE buffer at 84 volts for 1.5-2 hrs. A 100bp (promega G210A and 1Kb DNA ladder (promega G571A) were used as a molecular size markers.

3.8 Sequencing and Analysis of 16S rDNA gene

3.8.1 Purification of PCR 16S rDNA gene

Seventy five microlitre of the PCR 16S rDNA amplified products were resolved in 1% agarose gels with the conditions earlier described .The resulting bands in agarose gel were carefully excised with sterile scarpels and then purified using purification kit (Wizard PCR preps DNA purification kit, USA). The purification process involved the introduction of the excised bands into 1.5 mL Eppendorf tubes and suspended in water bath that was maintained at 65^0C. One mL of PCR preps purification resin was added and tubes were incubated for about 5 min or until the agarose gel melted completely. The DNA/ purification resin mix was pipette into the syringe barrel and the syringe plunger was used to slowly push the slurry into the attached mini column. The mini column was then washed with 2 mL of 80% isopropanol to remove contaminants from the DNA. The Eppendorff tubes, on which the columns were mounted, were then centrifuged at 10,000 x g for 2 minutes at 4^0C, to remove the remaining resin and isopropanol from the purified DNA in the columns. The minicolumns were transferred into new Eppendorff tubes and 40 µl TE buffer (pH 7.5) was added into each of the columns, and left to stand for 1 min.

Centrifuge the minicolumns at 10,000 x g at 4 ^{0}C for 20 secs,to elute the DNA into the centrifuge tubes. The purified DNA was kept at 4 ^{0}C until use.

3.8.2 Drying of the purified 16S rDNA genes

To 1mlo f the purified DNA, 0.1 volume of Sodium acetate (3 M, pH 5.0) and 2.0 mL of 100% ethanol were added. This was then incubated at -20 ^{0}C for 1 hr. It was brought out and left to stand at room temperature for 5 minutes, and centrifuged at 13,000 x g at 4 ^{0}C for 45 miniutes.The liquid was removed, leaving only the DNA in the Eppendorff tubes. The DNA was then dried at 37 ^{0}C for 30 minutes.

3.8.3 Sequencing of 16S rDNA gene

The dry DNA samples (obtained using the v3 primer) were sequenced using a computer analytical sequencer (MGW-Biotech, Germany) with the v3 primer Rev, acting as the basis. The generated nucleotide sequences were subjected to analysis.

3.8.4 Analysis of the 16S rDNA gene sequences

The generated sequences of the 16S rDNA genes were subjected to alignment in the databases at the BLAST. Basic Local Alignment and Search Tool, website http://www.ncbi.nlm.nih.gov/blast. The isolates were then identified based on the result of the analysis. The isolates were then identified based on the result of the analysis .A similarity of > 98% to 16S rDNA sequences of type strains was used as the criterion for identification (Altschul *et al*., 1997).

3.9 Effect of incubation time on growth and β-galactosidase production

3.9.1 Measurement of growth with time

Growth of the test organisms was determined by taking the optical density readings (OD) at 600nm.Twenty milliliter of sample was incubated at 4^{o}C for 15 min to stop further growth. Growth was measured turbidimetrically at 600nm and cells diluted with water when the OD was higher than 0.8. Cell turbidity at OD_{600} was kept between 0.4 and 0.8 by appropriate dilutions. pH of the medium is measured by pH meter.

3.10 Optimization of growth conditions for β-galactosidase production

Optimization studies were carried out on the growth of the isolates to determine base requirements for optimal galactosidase production. Optimization parameters will include the pH, optimum temperature, and effect of agitation on enzyme production

3.10.1 Effect of Temperature on β-galactosidase production

Twenty milliliters (20ml) of the modified MMRS basal medium was dispensed into each screw-capped tube and then sterilized by autoclaving. The colour change and turbidity of each bottle is noted as a simple indicator of growth or no growth. Each treatment is tested with triplicate bottles.

3.10.2 Effect of pH on β-galactosidase production

The basal MMRS medium was adjusted with appropriate quantities of Sodium dibasic hydrogen phosphate and monosodium dihydrogen phosphate to prepare the requied pH. The screw-capped bottles were placed in water bath with reciprocal shaking. The pH range determined were 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5 and 8.0 respectively while the temperature was kept constant at 37 ^{0}C.

3.10.3 Effect of different carbon sources on β-galactosidase production.

In` this study, galactose in the basal medium was replaced by 1% of: Raffinose, Melibiose, Arabinose, Xylose and Glucose separately keeping constant the rest of the media composition.A control experiment consisted of inoculated MRS without sugar additions.

The effect of combination of raffinose (0.5%) with galactose (0.5%) and melibiose (0.5%) with galactose (0.5%) on β-galactose production were also studied.

3.10.4 Effect of different Nitrogen sources on β-galactosidase production.

In this study, yeast extract and ammonium sulphate in the basal medium were replaced with different organic compounds (Yeast extract, peptone, malt extract, tryptone and ammonium nitrate) as nitrogen source. The rest components of the media composition remained the same. The concentration of organic and inorganic compounds added in the medium are: 01% and 0.3% respectively

3.10.5 Effect of agitation on enzyme production

This was determine by agitating the culture flasks inoculated with 25 hours old test isolates in a rotary shaker at; 60, 80, 100, 120, 140, 160 and 180 rpm for a period of 6 hours each after which the samples are then analysed for enzyme production total protein, and cell growth.

3.11 Characterization of Crude Enzyme.

3.11.1 Effect of temperature

To find the optimum temperature for β-gal production, the fermentation medium after inoculation was incubated at temperatures in the range of 16 - 42^{o}C (16, 20, 28, 37 and 42^{o}C) in an automatic incubator for 36 hrs and then check for the level of enzyme production at the respective temperatures.A controlled experiment consisted of an uninoculated broth maintained at that temperature.

3.11.2 Effect of pH

To investigate the effect of pH on β-gaactosidase production, the initial pH of the basal medium was adjusted with Na_2HPO_4 and NaH_2PO_4 in a prepared pH range of 3 – 8.0 (3.0, 4.0, 5.0, 6.0, 7.0, and 8.0). It was then inoculated with a 16 hrs old culture and incubated at 37^{o}C for 36h.A controlled experiment consisted of an un-inoculated broth maintained at the test pH.

3.11.3 Effect of Inoculums size

To determine the effect of inoculums size on enzyme production, different inoculum levels are prepared as follows: 4, 6, 8, 10 and 12% (4 – 12% v/v) each of which was separately added to the fermentation medium and incubated at 37^{o}C for 36 hrs.

3.11.4 Effect of Inoculum age

To determine the effect of the inoculums age on enzyme production, fermentation medium was inoculated with 12-24 hrs old culture at 12, 16, 20, 24, 28, and 32, 36 hrs each and incubated at 37ºC after which they were withdrawn and analysed for enzyme production and cell growth.

3.11.5 Effect of incubation period

To determine the optimal incubation time for maximum enzyme production, the fermentation medium was inoculated with 16 hrs old culture and incubated for 36 hrs: 20,

24, 28, 32, 36 hrs. Samples were out every 4 hrs and analysed for β-galactosidase activity and cell growth.

3.11.6 Effect of different inhibitors

The inhibitors used were Ethylene diamine tetra acetic acid (EDTA), potassium cyanide (KCN), benzoic acid and Urea. The stock solution of each was prepared using a range of concentrations (0.1mol, 0.2mol, 0.3mol, 0.4mol and 0.5mol). From the stock, 0.1ml from each was added to 1ml of substrate ONPG in sodium phosphate buffer (pH 7.0) and 0.5ml of enzyme extract. The reaction was stopped by the addition of 1ml of cold 10% $NaCO_3$ solution.

3.11.7 Effect of different cations

Stock solutions of different cations were prepared at different concentrations (0.1mol, 0.2mol, 0.3mol, 0.4mol and 0.5mol). The cations used were $CaCl_2$, $MgSO_4$, $MnSO_4$, KNO_3 and NaCl. One millilitre of each of the stock solution of the different cations was added to 1ml of 1% ONPG in phosphate buffer (pH 7.0). To this mixture was added 0.5ml of enzyme extract after which β-galactosidase activity was determined.

3.11.8 Effect of different anions

The anions used were $NaNO_3$, $FeSO_4$, $FeCl_2$, NH_4NO_3 and NH_4Cl. The stock solution was prepared by varying the concentration from 0.1m to 0.5m. 1ml each from the stock solution of the different anions concentrations was added to 1ml of 1% ONPG in phosphate buffer (pH 7.0) and 0.5ml of enzyme extract after which the β-galactosidase was assayed for.

3.11.9 Effect of enzyme concentration

Effect of enzyme concentration on β-galactosidase activity was determined by varying the concentration of enzyme extract (0.5%, 1%, 1.5%, 2% and 2.5%). This was added to different test tubes containing Sodium phosphate buffer (pH 7.0).

One unit of β-galactosidase activity was defined as the amount of enzyme that librated 1µ-mole of o-nitrophenol per minute under the assay conditions (Haider and Hussain, 2008 b).

3.12 Construction of ONP Standard Curve

In this assay, free ONP at different levels was used to construct the standard curve. 0.033383g of ONP was mixed with 1ml of 0.05M phosphate buffer (pH 7.0). Final volume of the mixture was set at 4ml. This mixture was agitated until all the solid particles were dissolved.This mixture was transferred into a preheated water bath at 45^0C. It was labeled as ONP stock solution. Several dilutions (0.5%, 1%, 2%, 2.5% and 3%) were carried out in 0.05M sodium phosphate buffer (pH 7.0) respectively under assay conditions. 0.5 mL of the enzyme extract is added to 1ml of the solution and the enzyme assay is carried out. Absorbance was read at 420nm.

Blank solution was prepared by mixing 3ml alcohol and 1ml sodium phosphate buffer 0.05M (pH 7.0). For each set of dilutions of stock solution, blank solution was made. Absorbance values were read against the blank solutions in UV-spectrophotometer .All readings were carried out as duplicate experiments in three independence trials. The average of each set of data was plotted against respective concentrations.

3.13 Intracellular Enzyme extraction

Extraction was carried out by the addition of 0.2g of 1.5 mm glass beads and stirred vigorously in a vortex for 20 secs and placed on ice 20 secs.The harvested cells were suspended in 10ml of Sodium phosphate buffer and then centrifuged at 10,000 rpm for 15 min. at 4^0C, Sieved using watman paper and washed again with the same buffer.

3.14 Purification of β-galactosidase

Enzyme purification steps were sequentially carried out using Ammonium Sulphate, Enzyme dialysis in Viskin tubes and Column Chromatography with Sephadex resin first with G-100 and later with G-50 of varing sizes between 50 -100 mesh size.

3.14.1 Ammonium Sulphate precipitation

The crude enzyme extract was concentrated with the addition of requisite weight of ammonium sulphate in a "salting-out process" up to 100% saturation (Dixon and Webb,1971). Enzyme saturation was gradually achieved by using 24.3g; 28.5g and 15.7g to achieve 0 - 40% and 40 - 80% saturation respectively. Each quantity of ammonium sulphate was weighed and dissolved in 100 mL of enzyme sample with constant stirring. The mixture was maintained at 4^0C overnight in a cold room. Thereafter, the solution was centrifuged at 7000 rpm for 15 min and the precipitate stored at 4^0C. All precipitates were

pooled and dissolved in 0.2M sodium phosphate buffer (pH7.0) and made up to 100ml. The precipitated enzyme samples were introduced into dialysis tubes (Spectra/por® molecular porous membrane tube, Spectrum medical Industries Inc., Texas), and dialysed extensively against three changes of the phosphate buffer for 24 hrs at 4^0C. The dialyzing buffer was thereafter, discarded and the enzyme stored in sterile screw capped tubes at 4^0C for further purification by column Chromatography.

3.14.2 Dialysis

The precipitates for each batch were re-suspended to initial volume of culture filtrate with 0.05M Sodium phosphate buffer pH 7.0 and dialysed in a tubular cellulose membrane against one litre of the same buffer for 24 hrs at 4^0C .The fractions were pooled together and used as partially purified enzyme for enzyme assay.

3.15 Column chromatography

3.15.1 Preparation of resin

Ten grammes of Sephadex G 100 resin was weighed into a glass beaker containing 100ml sterile distilled water with 0.97% sodium azide (to prevent microbial contamination) and 200ml 0.05M phosphate buffer (pH 7.0). The gel was allowed to swell for 3days with constant gentle stirring at 4^0C before loading. The gel was allowed to swell for 3 days with constant shaking.

The column of Sephadex G-100 was prepared according to the method described by Olutiola and Cole (1980) and was surrounded by water jacket at 4^0C. The column was equilibrated with 0.05M sodium phosphate buffer pH 7.0.

3.15.2 Loading of the Column

After the third day of soaking, the resin was loaded into a clean glass chromatography column (25x190mm) and allowed to compact while preventing air from being trapped along the column. The Sephadex was allowed to compact in liquid on top was sucked out. More gel slurry was introduced until the column was fully loaded to the zero point of the column. This was done for 48 hr before 2ml of the dialysed enzyme sample was loaded. The column was first eluted with the same buffer followed by a linear gradient of Sodium Chloride from 0.1M l^{-1} to 0.5M l^{-1} at a flow rate of 25ml/sec, and the eluent was collected in 5ml fractions. The enzyme activities and protein content in each 5ml fraction were determined by procedures previously stated and protein content was calculated by the

method of Lowry *et al*., (1951) using BSA as **standard.** The active fractions were pooled and used for electrophoretic determination

3.15.3 Fractionation on Sephadex G-100

The column of Sephadex G-100 was prepared and loaded according to the method described by Olutiola and Cole (1980) and was surrounded by water jacket at 4^0C. The column was equilibrated with .05M sodium phosphate buffer pH 7.0. Two milliliter (2ml) of the enzyme concentrate was loaded into the column and eluted with sodium phosphate buffer. The eluted fractions were collected in calibrated 5ml tubes for thirty fractions. Each eluted fraction was analysed for β-galactosidase activity. Protein estimation was also carried out.

3.15.4 Fraction of Sephadex C-50

Fractions which showed appreciable activity after passing through Sephadex G-100 were pooled together and applied to the column of Sephadex C-50 ion-exchange column(1.7 x 1.8cm) previously equilibrated with the same buffer.The flow rate was adjusted to 17.5 ml/hr. Two milliliters (2ml) of each fraction was applied to the column and eluted with sodium phosphate buffer (pH 7.0). The column was first eluted with the same buffer followed by a linear gradient of Sodium Chloride from 0.1M l^{-1} to 0.5M l^{-1}. The eluted fractions were collected as above and analysed for β-galactosidase activity .Protein estimation was also carried out.

3.16 Protein Estimation

Protein concentration was estimated according to the procedure described by Lowry *et al.*, (1951) Bovin Serum Albumin was used as standard. The reagents required were: solution A. 1% $CuSO_4$. $5H_20$; solution B, 2% Na- K-Tartarate and solution C: 2% Na_2Co_3 in 0. 1m NaOH. Ratio 1:1:98 of solutions: A, B and C were mixed. Folins reagent was diluted in equal volumes with water.Absorbance was read at 540nm.

3.17. Electrophoresic separation of protein samples/SDS-PAGE

3.17.1 Electrophoretic Separation of Extracellular β-galactosidase

Native polyacrylamide gel **electrophoresis** (PAGE) and denaturing sodium dodecyl sulfate polyacrylamide electrophoresis (SDS-PAGE) were performed on a Phast System Unit using precast polyacrylamide gels (Phast HGel8-25, Amersham). For SDS-PAGE,the enzyme was pre-incubated with Laemuli buffer for 5minutes at 90^0C for Coomasaie blue staining and 60^0C for active staining.Coomassie brilliant blue R-250 was used for

visualization of protein bands,and active staining was performed by using 4-Methylumbelliferyl-β-D-galactosidase (MUG) as substrate which was added directly onto the gel.Samples that were applied to the individual lanes contained approximately 1-2µg of the total proteien.Isoelectric focusing(pI: or isoelectric point) was performed in the range of pH 3-10 using the Multiphor System and precast dry polyacrylamide gels rehydrated with carrier ampholytes as recommended. The broad pI marker protein kit, pH 3-10 was2 used to compare and determine the pI value.

b. **Stacking gel buffer**

All the components were dissolved in 500ml distilled water in a 1 litre standard volumetric flask. The pH was adjusted to 6.8 with Potassium sulphate solution. The solution was made up to mark with distilled water and stored at 4^{o}C.

c. **Running gel buffer**

1.5M Tris, 8mM EDTA, 0.4% SDS pH 8.8 water in a 1 litre standard volumetric flask. The pH was adjusted to 8.8 with hydrochloric acid. The solution was then made up to mark with distilled water and stored at 4^{o}C.

e. **Electrode buffer:** Glycine, SDS, sodium salt EDTA

All the components were dissolved in 4 litres distilled water with constant stirring. The solution was kept at 4^{o}C.

f. **Sample buffer**

6ml glycerol was mixed with 1.2ml of potassium phosphate buffer pH 6.8, 1.5g of sodium dodecyl sulphate (SDS), 30mg dithiothreitol (DDT) and 10mg bromophenol blue. Distilled water was added to make 20ml. The mixture was dispensed into test tubes in aliquots of 1ml each and stored at 20^{o}C until needed.

3.17.2 Gel-electrophoresis

The stacking gel and running gel were prepared on the day of the experiment. The running gel solutions were poured into the gel assembles to a level of about 4cm below the maximum filling level. Distilled water was layered on the gel surface using a Pasteur pipette to ensure an even surface and also to avoid evaporation of the gel while the polymerizing gel was allowed to polymerize for 1 – 2 hours.

The water on the gel was poured off and the stacking gel was poured onto the polymerized running gel and a comb (1.5 mm thick) was gently inserted to obtain wells.

The protein samples were prepared by mixing sample buffer (in a dilution of 1:1 (v/v) with enzyme samples. The mixtures were place in a dry bath and heated for 4 minutes at 95^{o}C. Equal volume of samples was applied to the bottom of the sample wells with a Hamilton syringe. Molecular weight standards (Bio Rad high and low ranges) were applied into wells alongside the samples.

Gel electrophoresis was conducted at 120volts in a vertical electrophoresis apparatus for about 1.5 hours or until the bromophenyl blue dye has migrated to the bottom of the gel.

At the end of the electrophoresis, the gel was carefully removed from the glass plates and stained by soaking in the staining solution for 20 minutes with gentle agitation. Excess stain was removed by immersing the gel for 1 hour in several changes of the destining solution each lasting for 15 – 20 minutes. The gel was removed and allowed to dry.

3.17.3 Effect of different inhibitors on purified enzyme

The inhibitors used were Ethylene diamine tetra acetic acid (EDTA), potassium cyanide (KCN), benzoic acid and Urea. The stock solution of each was prepared using different concentrations (0.1M, 0.2M, 0.3M, 0.4M and 0.5M). From the stock, 0.1 mL from each was added to 1 mL of substrate in sodium phosphate buffer (pH 7.0) and 0.5 mL of enzyme extract. The reaction was stopped by the addition of 1 mL of cold 10% $NaCO_3$ solution.

3.10.2 Effect of different cations on purified enzyme

Stock solutions of different cations were prepared at different concentrations (0.1M, 0.2M, 0.3M, 0.4M and 0.5M). The cations used were $CaCl_2$, $MgSO_4$, $MnSO_4$, KNO_3 and NaCl. 1ml of each of the stock solution of the different cations was added to 1 mL of 1% ONPG in phosphate buffer (pH 7.0). To this was added 0.5 mL of enzyme extract respectively after which β-galactosidase activity was determined.

3.10.3 Effect of different anions on purified enzyme

The anions used were $NaNO_3$, $FeSO_4$, $Fecl_2$, NH_4NO_3 and NH_4Cl. The stock solution was prepared by varying the concentration from 0.1M to 0.5M. 1 mL each from the stock solution of the different anions concentrations was added to 1 mL of 1% ONPG in phosphate buffer (pH 7.0) and 0.5 mL of enzyme extract after which the β-galactosidase was assayed for.

3.10.4 Determination of percentage Lactose hydrolysis using β-galactosidase obtained from LAB-isolates

Determining the hydrolytic effect of purified β-galactosidase. Rotary evaporator was used to obtain different concentrations of the raw milk. By this method, percentage milk concentrations of 100%, 80%, 60%, 40% and 20% respectively prepared. To known volumes of the enzyme concentrate as follows: 1.5, 2.0, 2.5 and 3.0 mL respectively. The best volume with the highest hydrolysis was determined using Lactose hydrolysis by Spectrophotometry method.

$$\% \text{ Lactose Hydrolysis} = \frac{\text{Absorbance} \times \text{Average gradient} \times \text{DF}}{10{,}000}$$

CHAPTER FOUR

4.0 RESULTS

4.1 Sample collection and isolation of bacteria

A total number of thirty strains of lactic acid bacteria and one strain of *Bacillus* were isolated from raw milk and dairy products. The isolates were predominantly from local cheese(wara). Results of biochemical characterization tests revealed that the isolates belong to the genera of lactic acid bacteria. The isolates were characterized and identified by their cultural and morphological features as well as physiological and biochemical properties. The lactic acid bacteria were identified as *Lactobacillus plantarum, Lactobacillus brevis, Lactobacillus casei, Leuconostoc lactis, Lactobacillus lactis, Streptococcus spp* and *Bacillus subtilis. Lactobacillus plantarum* had the highest percentage of occurrence (35%), followed by *L. brevis* (20%) while the least is recorded in *Streptococcus* spp. and *Bacillus* spp (5%) (Table 4.1).

All the lactic acid bacteria isolates were small colonies and the cell shape revealed medium short rods to long rods. Some were cocci, non-motile, non-sporing, gram-positive, methyl red,catalase positive, oxidase negative, nitrate reduction negative, Voges Proskauer tests negative and some strains hydrolysed starch while the *Bacillus* spp was catalase positive, short rods, Gram-positive, spore former. The isolates were facultative anaerobes and were fermentative rather than oxidative in nature (Table 4.2).

Table 4.3 shows the result of the corfirmation of the isolates using molecular techniques. *Lactobacillus plantarum* (G11) with nucleotide length 869 displayed 99% homology and was identified as such. In the same sequence are isolates G12 and E13, 890 nucleotide length, 99% homology, E36: 890 nucleotide length, 99% homology. However, isolte F6 had nucleotide length of 920 and 100% homology with *Lactoccocus lactis* while isolate F26 with nucleotide length 817 had 100% homology *with Bacillus subtilis.*

Table 4.1: Percentage Occurrence of bacterial isolates in the different samples

Probable Organisms	Number of Occurrence	Percentage of Occurrence
Lactobacillus plantarum	7.0	35.0
Lactobacillus casei	3.0	15.0
Lactobacillus brevis	4.0	15.0
Lactococcus lactis	2.0	10.0
Leuconostoc lactis	3.0	15.0
Streptococcus spp	1.0	5.0
Bacillus subtilis	1.0	5.0
Total	21.0	100.0

Table4.2: Biochemical Characterization of the Isolates

Isolates	Saccharose	Glucose	Xylose	Sorbitol	Lactose	Arabinose	Mellibiose	Raffinose	Maltose	Mannose	Sucrose	Fructose	Mannitol	Trehalose	Ribose	Inositol	Gram reaction	Shape	Nitrate reduction	Arginine hydrolysis	Aesculin hydrolysis	Endospore formation	Oxidase	Catalase	Voges-Proskauer test	H2S production	Indole Test	Probable Organisms
F28	+	+	+	-	+	+	+	-	+	+	+	+	-	+	+	-	+	rod	-	+	-	-	-	-	-	-	-	*L. brevis*
E11	+	+	+	-	+	+	+	-	+	+	+	+	-	+	+	-	+	rod	-	+	-	-	-	-	-	-	-	*L. brevis*
G31	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	-	+	cocci	-	+	-	-	-	-	-	-	-	*Streptococcus* spp
E13	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	rod	-	-	-	-	-	-	-	-	-	*L. plantarum*
E2	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	-	+	rod	-	-	-	-	-	-	-	-	-	*L. casei*
E1	+	+	+w	+	+w	-	+w	+	+	+	+	+	-	+	+	-	+	rod	-	-	-	-	-	-	-	-	-	*L. casei*
E36	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	rod	-	-	-	-	-	-	-	-	-	*L. plantarum*
F5	+	+	+	+	+	-	+	+w	+	+	-	+	-	+	+	-	+	cocci	-	-	-	-	-	-	-	-	-	*Lactococcus lactis*
G12	+	+	+	+	+	+	+w	-	+	+	+	+	-	+	+	-	+	cocci	-	-	-	-	-	-	-	-	-	*Leuconostoc lactis*
G32	+	+	+	+	+	+w	+	+	+	+	+	+	+	+	+	-	+	cocci	-	-	-	-	-	-	-	-	-	*Streptococcus* spp
14E	+	+	+	+	+	+	+	-	+	+	+	+	+w	+	+	-	+	rod	-	-	+	-	-	-	-	-	-	*L. plantarum*
E30	+	+	+	+	+	-	+w	+	+	+	+	+	-	+	+	-	+	cocci	-	-	+	-	-	-	-	-	-	*Lactococcus lactis*
F6	+	+	+	+	+	+w	+w	-	+	+	+	+	-	+	+	-	+	cocci	-	-	+	-	-	-	-	-	-	*Leuconostoc lactis*
E17	+	+	+	+	+	+w	+w	-	+	+	+	+	-	+	+	-	+	cocci	-	-		-	-	-	-	-	-	*Leuconostoc lactis*
F33	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	rod	-	-	+	-	-	-	-	-	-	*L. plantarum*
E22	+	+	+	+	+	+	+w	-	+	+	+	+	+w	+	+	-	+	rod	-	-	+	-	-	-	-	-	-	*L. plantarum*
G11	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	rod	-	-	+	-	-	-	-	-	-	*L. plantarum*
F26	+	+	+	-	+	+	+	-	+	+	+	+	-	+	+	-	+	rod	-	+	-	-	-	-	-	-	-	L. brevis
F26*	+	+	+	-	+	+	+	-	+	+	+	+	-	+	+	-	+	rod	-	+	-	-	-	-	-	-	-	L. brevis
ME	+	+	+	+	+	+	+	+w	+	+	+	+	+	+	+	-	+	rod	-	-	+	-	-	-	-	-	-	*L. plantarum*

Table 4.3: Phylogenetic identity of the isolates (blast sequence match)

Bacteria division	Isolate code	Length of the Nucleotide	Best phylogenetic match (accession no)	% similarity	Name of the organisms
Lactobacillus	G_{11}	869	JF 965385.1	99	*L. plantarum*
Lactobacillus	G_{12}	890	JF 965387.1	99	*L. plantarum*
Lactobacillus	E_{13}	890	JF 757224.1	99	*L. plantarum*
Lactobacillus	E_{36}	896	AB 601179.1	99	*L.plantarum subsp. plantarum*
Lactobacillus	F6	920	Jq 723699.1	100	*Lactococcus lactis*
Bacillus	F_{26}	817	Jq 835018.1	100	*Bacillus subtilis*

Screening of the isolates for β-galactosidase production on solid agar

The LAB isolates were screened for β-galactosidase production on solid agar using X-gal (5-bromo-4-chloro-3-indoxyl-β-D-galactopyranoside) as an indicator. Table 4.4 shows the ability of the LAB strains to produce β-galactosidase on solid agar. The isolates which were able to produce β-galactosidase were observed to have green coloration after anaerobic incubation at 37^0C for 36 hrs. Out of the 30 isolates screened, 12 had the ability to utilize X-gal within 24 hours of incubation as shown in Table 4.3.

The isolates are classified as very good, weak or poor producers of β-galactosidase. Based on the result, 7 LAB strains were termed very good producers, 4 were weak producers while only 1 was a poor producer. However, 19 isolates were negative to β-galactosidase production.

Table 4.5 shows the result of the screening of lactic acid production (Table 4.3) in MRS broth medium. Lactic acid production ranged from 7.134 mg/L in isolate E13 after 24 hrs of incubation to 15.240 mg/L after 48hrs in which highest production was recorded in isolate F26 at 36 hrs of incubation while isolate E13 had the least production at 24hrs of incubation

Table 4.4: Screening of lactic acid bacterial isolates for β-galactosidase Production on solid agar

Serial No	Isolate Code	x-gal Reaction	Serial No	Isolate Code	x-gal Reaction
1	3E	-	17	E39	-
2	4E	-	18	14E	+
3	E1	+	19	F5	+
4	E2	-	20	F6	+
5	E11	-	21	F26	+
6	E13	+	22	F27	-
7	E14	+	23	F28	-
8	E17	-	24	F33	-
9	E18	-	25	G8	+
10	E19	-	26	G11	+
11	E20	-	27	G12	+
12	E22	-	28	G31	-
13	E28	-	29	G32	-
14	E29	-	30	G37	-
15	E30	+	31	G38	-
16	E36	+			

+ = has potential for β-galactosidase production

- = absence of β-galactosidase production

Table 4.3: Screening of lactic acid bacterial isolates for lactic acid production in broth

Isolates code	Incubation Time (hrs)/lactic acid concentration (mg/L)		
	24	48	72
G11	9.2964 [c]	9.4584 [b]	11.1012 [a]
F26	8.9718 [c]	15.1332 [a]	11.1684 [b]
E36	9.18816 [c]	14.3226 [a]	10.9476 [b]
G12	11.4042 [c]	14.0826 [a]	10.554 [b]
F6	11.3502 [a]	10.6476 [b]	7.7184 [c]
E13	7.134 [c]	14.8092 [a]	11.6964 [b]
14E	10.9176 [c]	15.2406 [a]	11.7042 [b]

Figures with different superscripts are significantly different using Duncan's Multiple Range Test.

Screening of the selected LAB strains for Extracellular and Intracellular β-galactosidase production using submerged fermentation under static and agitation

The ability of the LAB strains to produce extracellular and intracellular β- galactosidase using submerged fermentation under static and agitation conditions was investigated.

Extracellular β- galactosidase production under static and agitated condition is shown in Figure 4.1a. β -galactosidase production ranged from 0.0172 U/ml in isolate G12 to 0.2145U/ml in isolate 14E under static condition, while production ranged from 0.0053 U/ml in E13 to 0.1296 U/ml in isolate 14E. Isolate 14E had the highest β-galactosidase production under static condition, while Isolate G12 had the least production under static condition.

Intracellular β-galactosidase production by the isolates ranged from 0.2057 in isolate F6 to 7.8892U/ml in isolate G11 under static condition. Under agitated condition β-galactosidase production ranged from 0.3270 U/ml in isolate F6 to 8.5612U/ml in G11. *L.plantarum* had the highest production under agitation condition as shown in Figure 4.2b. It was observed that *L.plantarum* had highest intracellular enzyme production was recorded under agitated condition except for isolate *L.plantarum* (E13) which produced the highest value under static condition as shown in Figure 4.1b

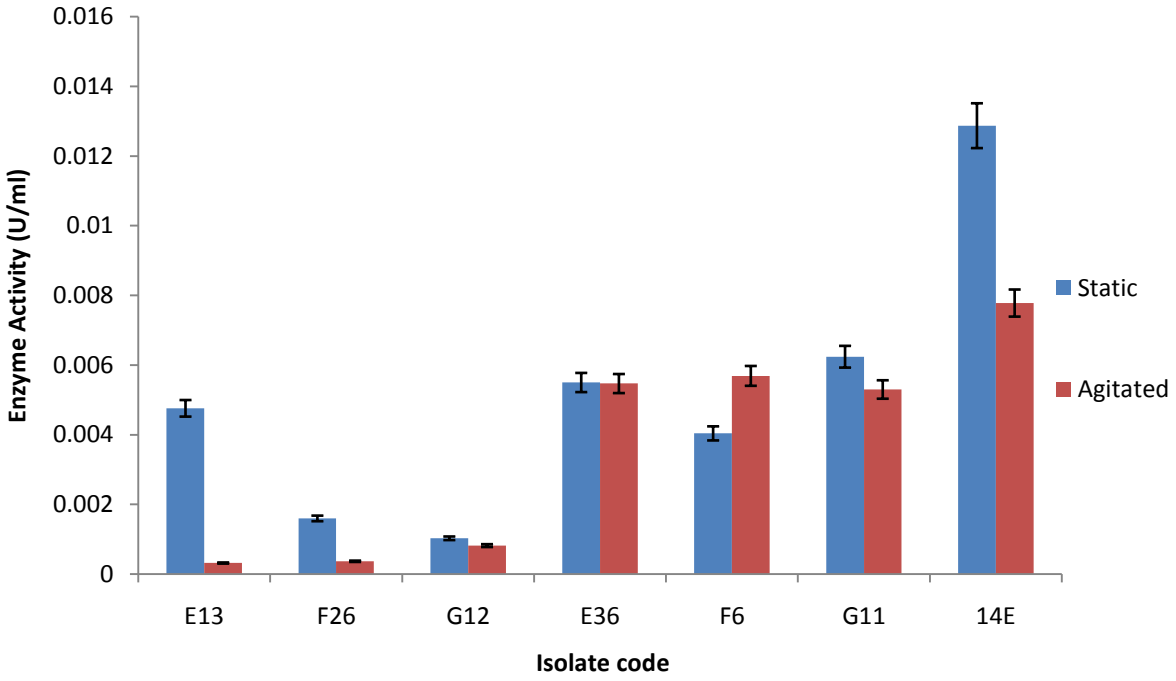


Figure 4.1a: Extracellular production of β-galactosidase by the selected LAB isolates

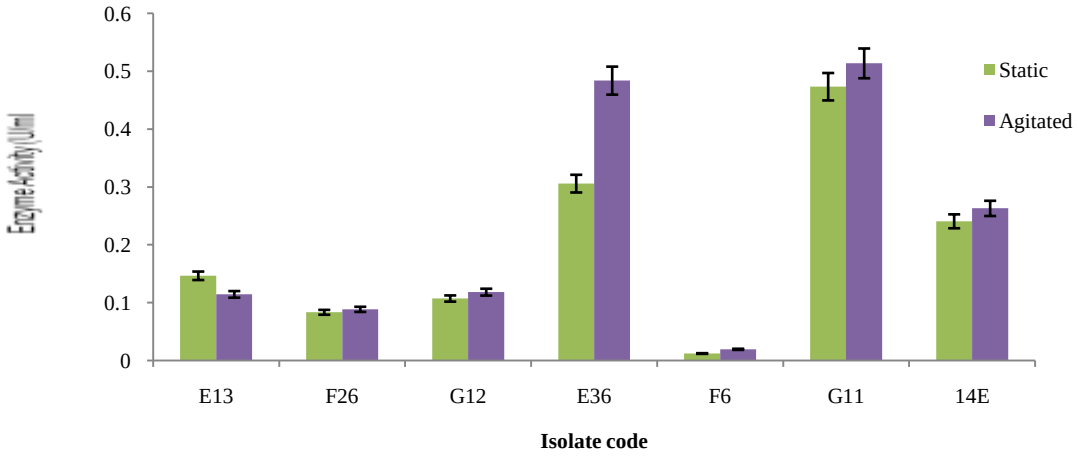


Figure 4.1b: Intracellular β-galactosidase production potential of selected Isolates

Effect of incubation time on growth and β- galactosidase production by the selected LAB strains

Figure 4.2a shows the effect of varying incubation time on growth by *L.plantarum (G11)*. It ranged from 1.1066 to 3.035OD_{600nm} in which the highest growth was attained after 18 hrs of incubation. There was a gradual increase in growth with increase in incubation time up to and including 30 hrs. This was followed by a sharp drop at 36hrs. Stability was reached at 42 hrs.

Comparatively, Figure 4.2b, shows the effect of incubation time on growth and β-galactosidase production by *L. plantarum* (G11) which ranged from 1.1066/ml to 3.035OD_{600nm} and 4.0566-7.6267U/ml respectively in which the highest β- galactosidase production was recorded between 18 and 30hrs after incubation. Growth was observed to increase gradually from inception up to 12 hours of incubation and attained peak after 30 hours of incubation and thereafter entered a sharp decline as incubation progresses. Similar trend was observed in β-galactosidase production, showing a correlation between peak microbial growth and maximum enzyme production. The exponential phase lasted for 6 hours and then entered progressive increase in enzyme activity for another 18 hours of incubation as shown in the figure.

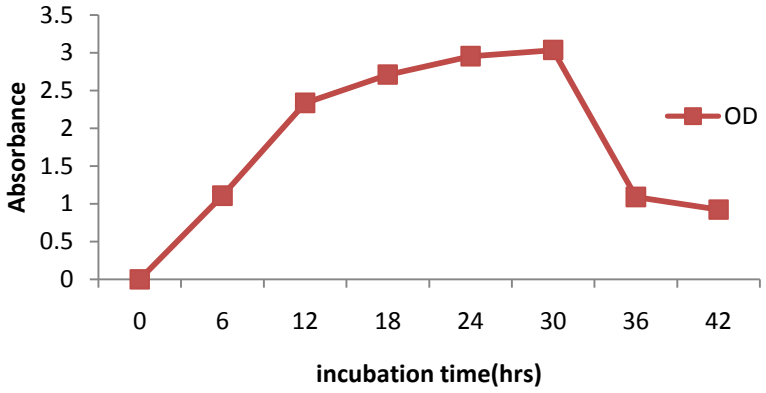


Figure 4.2a:Effect of incubation time on growth of isolate G11

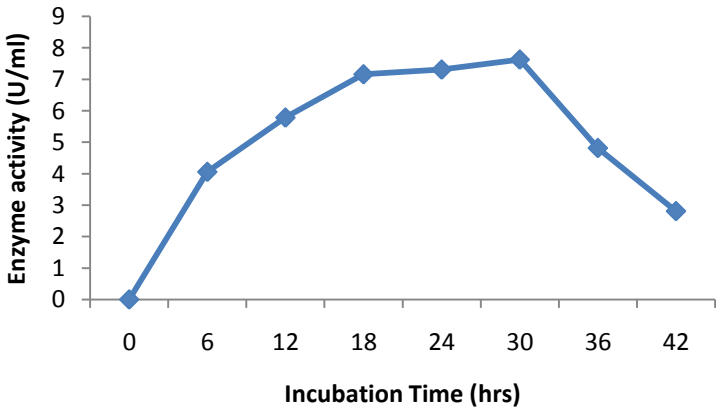


Figure 4.2b: Effect of incubation time on β-D-Galactosidase production by the isolate G11

Figure 4.3a shows the effect of incubation time on growth by *L.plantarum* isolate E13. Rapid growth was observed in the first 6hrs after which was followed by a gradual slow down.Growth ranged from 0.5706-0.8090OD_{600nm} in which the highest growth (1.889 OD_{600nm}) was attained at 24hrs of incubation.This was followed by a gradual decline in growth which eventually resulted into a sharp decline at 36hrs when no further increase in growth take place again even with increase in incubation time.

Figure 4.3b shows the effect of incubation time on β-galactosidase production by *L.plantarum* G11.It shows a correlation between growth and β-galactosidase production.Production was rapid during the early parts until the 6^{th} hr which was followed by a gradual decline between the 6^{th} and the 12^{th} hrs.Therafter, production gradually increases up till the 30^{th} hr when maximum β-galactosidase production (4.953 U/ml) was attained. This was followed by a sharp decline in production with increase in incubation time

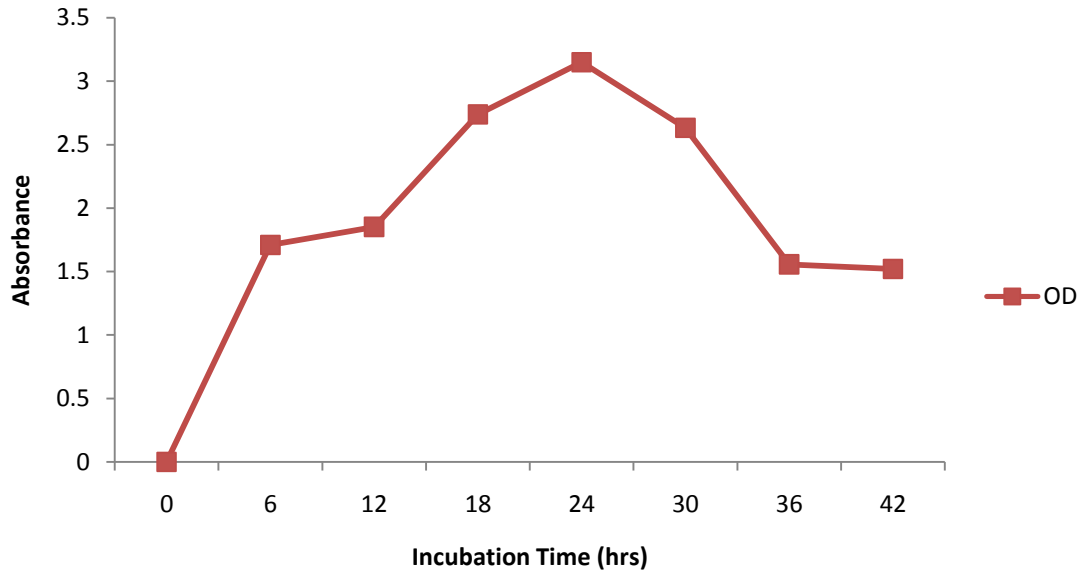


Figure 4. 3a:Effect of incubation time on growth of *L.plantarum* isolate E13

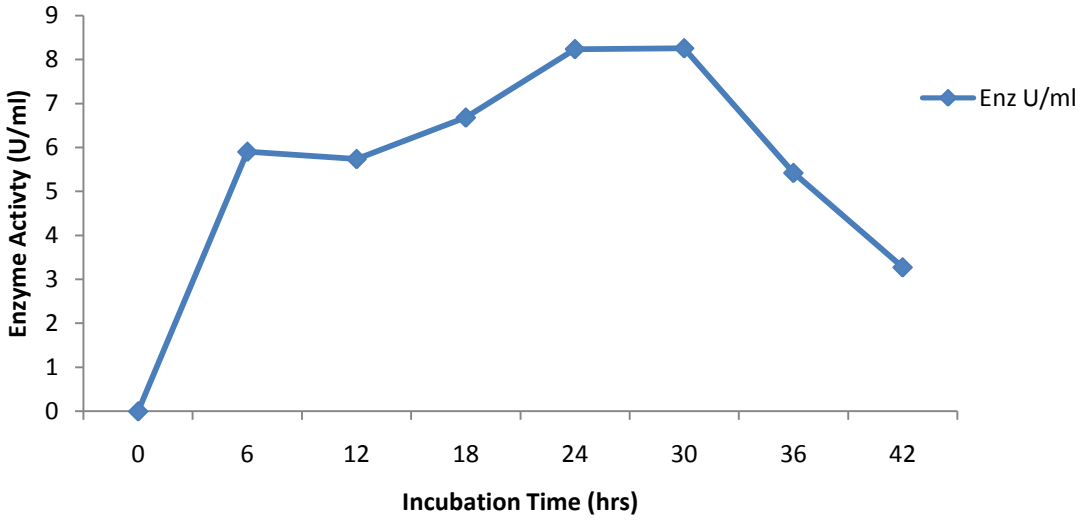


Figure 4.3b: Effect of incubation time on β-D-Galactosidase production by *L.plantarum* isolate E13

Effect of incubation time on growth and enzyme production by *L.plantarum subsp.plantarum*

Figure 4.4a shows the growth of *L.plantarum* subsp.*plantarum*incubated at different time intervals (hrs).Growth ranged from 1.5542 to 3.3242_{600nm} in which the highest was recorded at 30 hrs of incubation beyond which drop in growth was observed.

The figure also shows that β- galactosidase production by*L.plantarum* subsp.*plantarum* has two log phases in which the first lasted for 6hrs during which there was gradual increase in production. After 6hrs, a momentary lag phase was observed before the second exponential phase was attained between 12-18hrs of incubation which gradually entered into the stationary phase between 18-30hrs.This was then followed by a sharp drop at 36hrs and stability was reached at 42hrs.

Figure 4.4b shows a correlation between growth and and β- galactosidase production by *L. plantarum subsp.plantarum* where increase in growth also results in corresponding increase in production. β- galactosidase production by *L.plantarum* subsp.*plantarum* ranged from 2.9496 to 9.3708 U/ml within the time of incubation in which the highest was recorded at 30hrs. It shows that β- galactosidase production by *L. plantarum* subsp.*plantarum* started shortly after incubation and reached peak production of 9.371 U/ml at 30hrs beyond which drop in production was observed as incubation time continued.

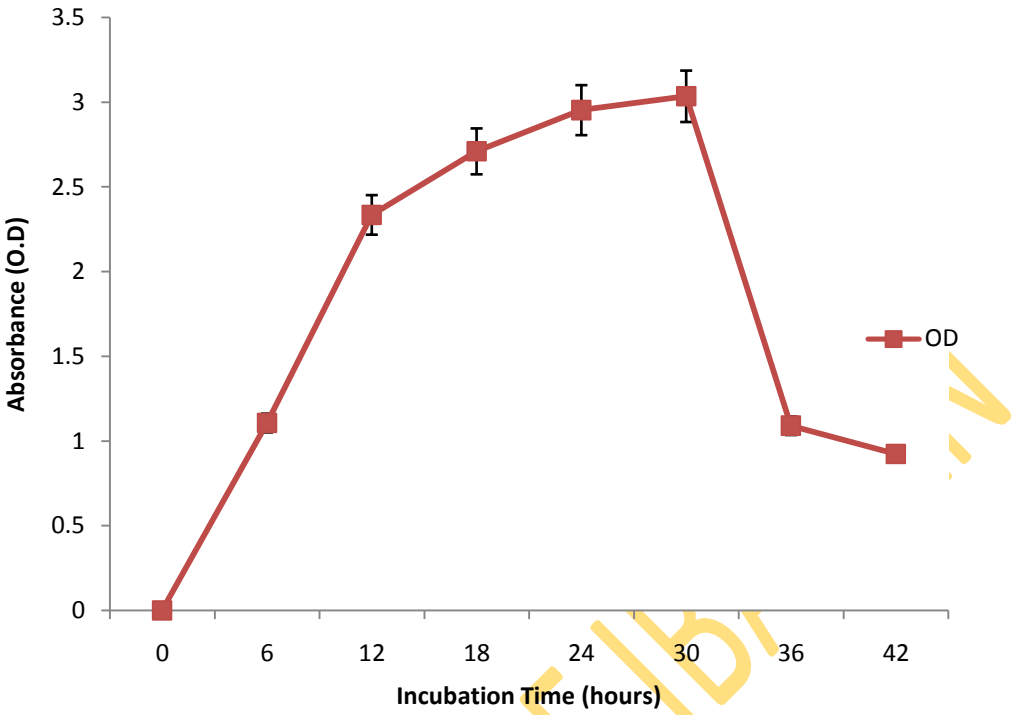


Figure 4.4a: Effect of incubation time on growth of *L. plantarum subsp. plantarum (E36)*

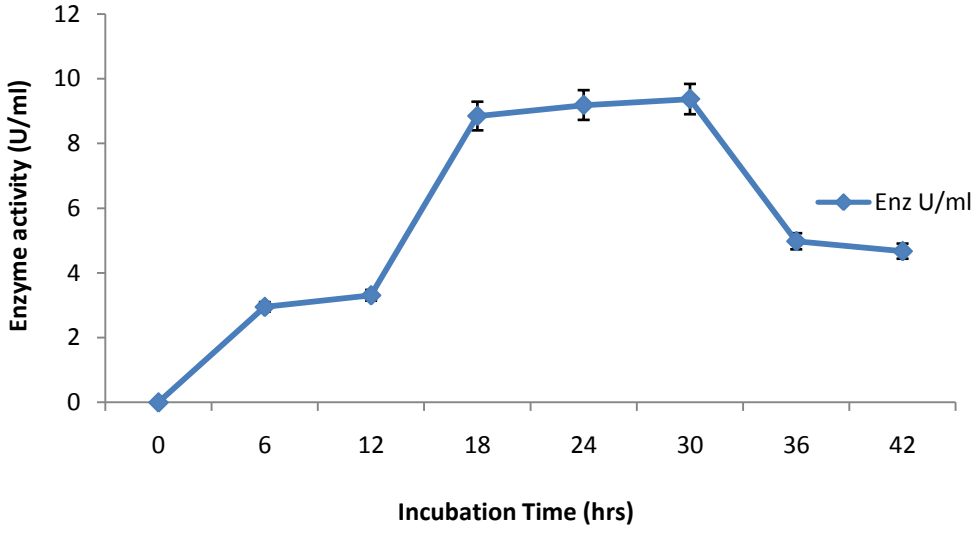


Figure 4.4b: Effect of incubation time on β-galactosidase Production by *L.plantarum* subsp.*plantarum(E36)*

Effect of temperature on growth and β-galactosidase production by the selected LAB isolates

Figure 4.5a shows the effect of temperature on growth by*L.plantarum*(G11). Gradual increase in growth was observed between 20-30^{0}C.Growth ranged from 0.7733 to 1.0568 OD_{600nm}. The highest growth was recorded at 40^{0}C. A gradual decrease in growth was observed in *L.plantarum*G11with increase in temperature above 35^{0}C. Maximum growth was best between the temperature of 30^{0}C and 40^{0}C with a peak absorbance of 1.0569. This was followed by a sharp drop at 40hrs.

Figure 4.5b shows the effect of temperature on β-galactosidase production by *L.plantarum*.G11 It was observed that enzyme production ranged from 3.6752 to 4.919 U/ml. Temperature of 20^{0}C to 35^{0}C favored β-glactosidase production with optimal yield of 4.919 U/ml at 35^{0}C. β-galactosidase production was maintained within the temperature range of 30 - 35^{0}C and at 35^{0}C as the temperature increases there was reduction in β-galactosidase production which is an indication that β-galactosidase produced by this species of *L.plantarum* is temperature sensitive, stable at low temperature and therefore thermo-labile. Fig 4.5c shows the effect of temperature on protein production by *L. plantarum* (G11). It shows highest production at 25^{0}C followed by a sharp drop at 30^{0}C. Thereafter, a gradual a decrease in production a temperature increases. Stability was reached at 45^{0}C.

Effect of temperature on growth and β-galactosidase production by*L.plantarum* (E13) was investigated as shown in Figure 4.6a. Growth ranged from 0.825 to 1.3483 OD_{600nm} in which the highest growth was recorded at 35^{0}C, the results showed that *L.plantarum* (E13) maintained gradual growth with increase in temperature. A sharp increase in growth activities was observed from 25^{0}C to 30^{0}C (a short exponential phase) which gradually enter into a stationary/growth phase between temperature of 30 and 35^{0}C. Peak absorbance values of 1.348was supported by 35^{0}C.Further increase in temperature (above 35^{0}C) did not support further increase in growth of the isolate.

Figure 4.6b shows the effect of varying temperatures on β- galactosidase production by *L.plantarum*(E13). From the result, it was observed that β- galactosidase production ranged from 1.9066 to 2.220U/ml. The results indicated that temperature of 20^{0}C best

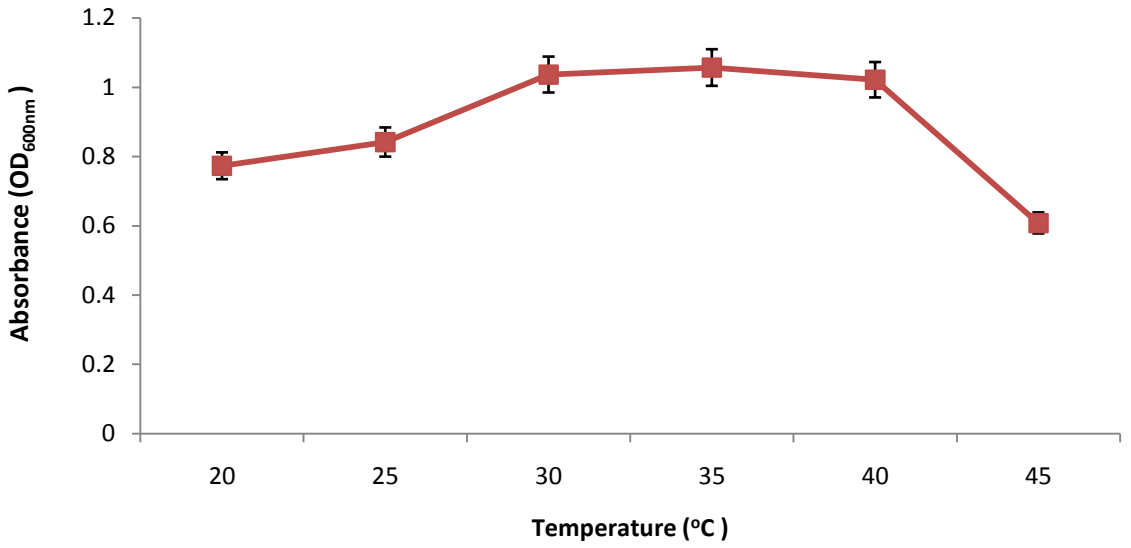


Figure 4.5a: Effect of temperature on growth of *L.plantarum*(G11).

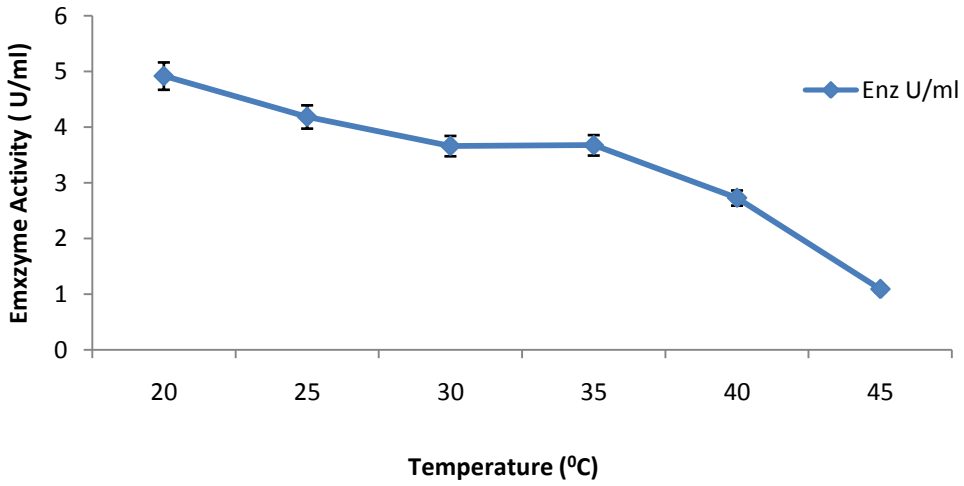


Figure 4.5b: Effect of temperature on β-galactosidase production by *L.plantarum*(G11).

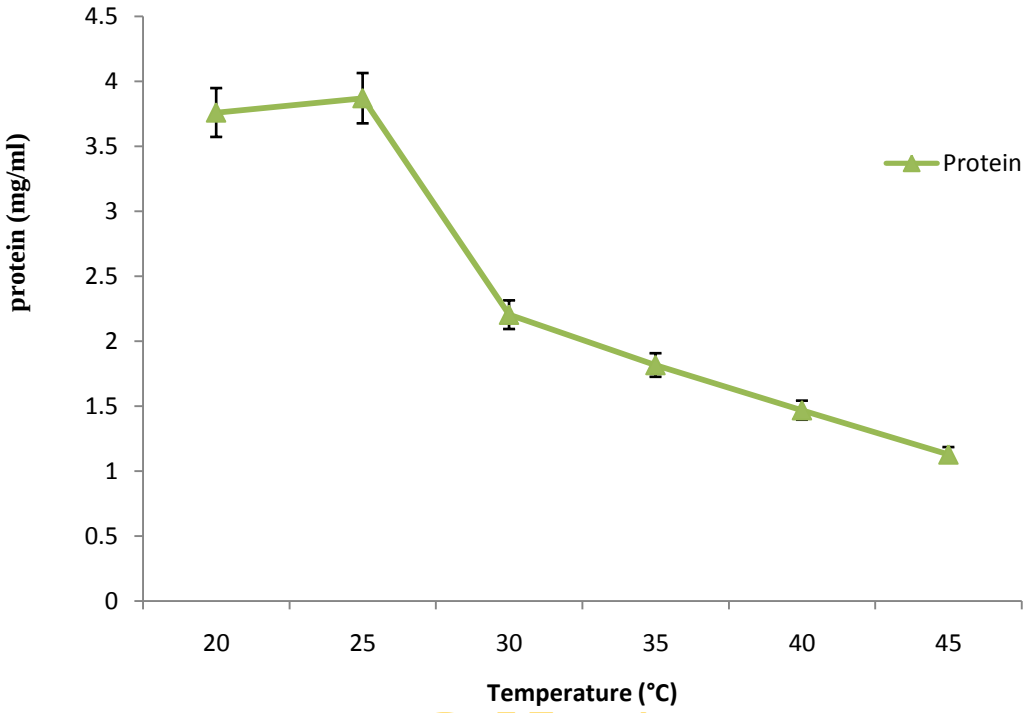


Figure 4.5c: Effect of Temperature on protein production by *L. plantarum* (G11).

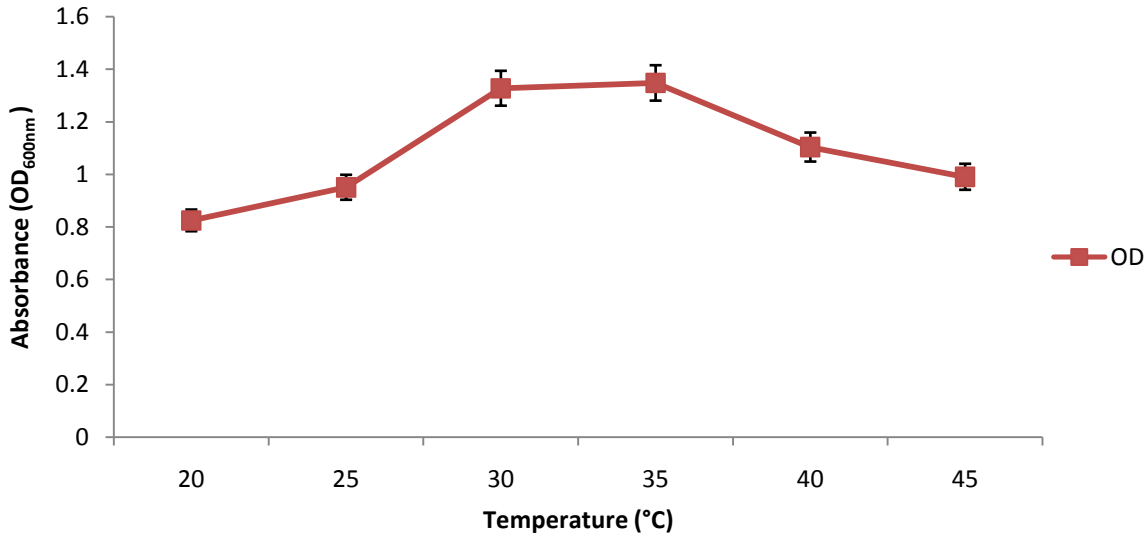


Figure 4.6a: Effect of temperature on growth of *L. plantarum* (E13).

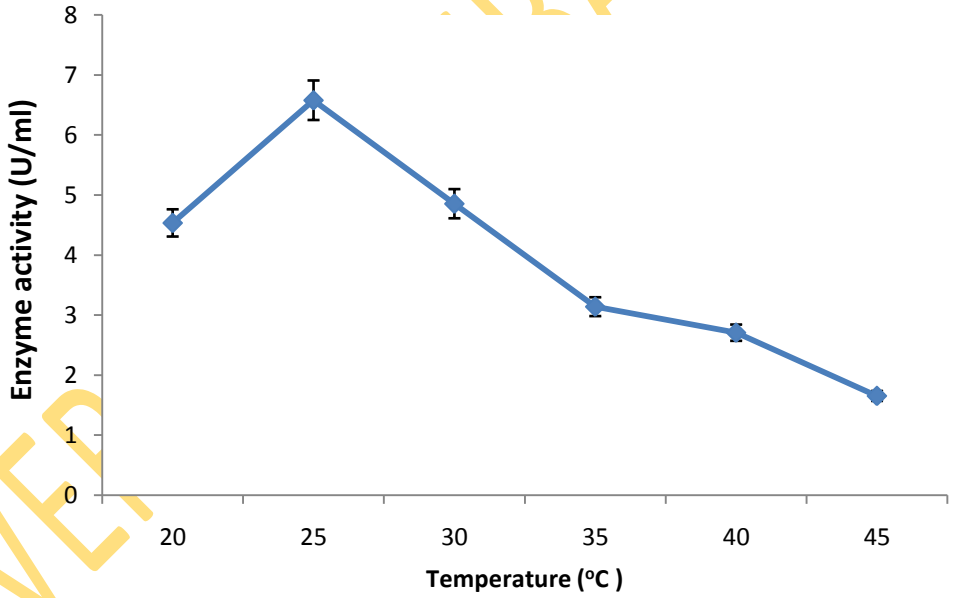


Figure 4.6b: Effect of temperature on β- galactosidase production by *L. plantarum* (E13).

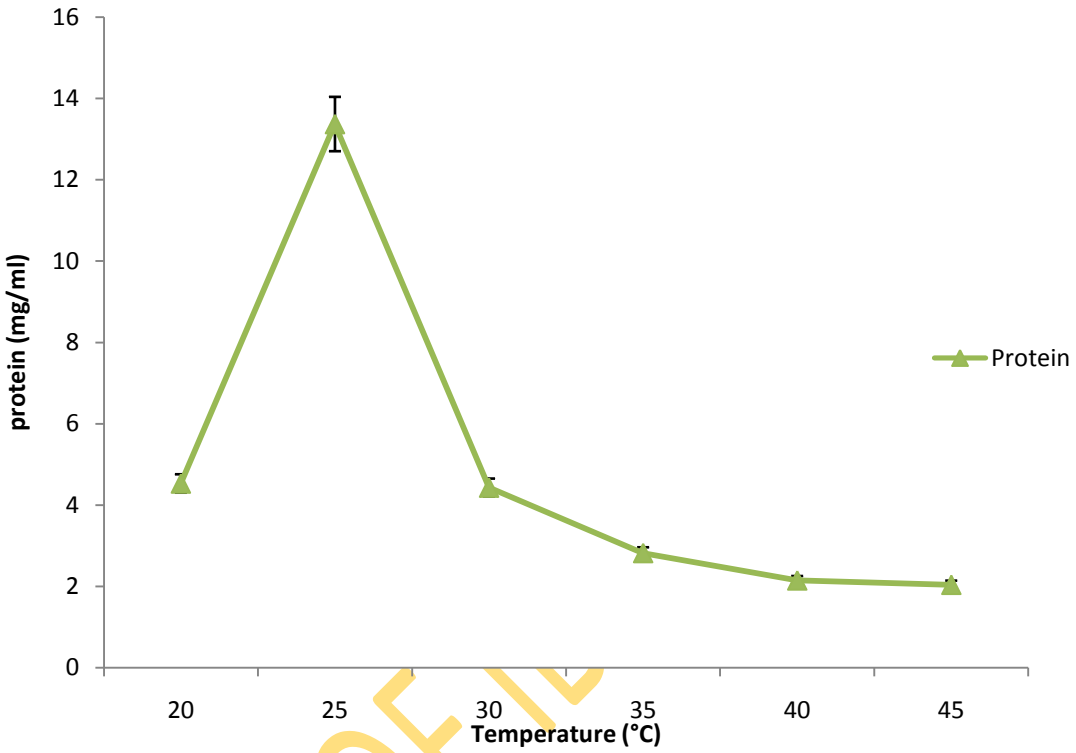


Figure 4.6c: Effect of temperature on protein synthesis by *L. plantarum* (E13).

supported β- galactosidase production by*L.plantarum* (E13). β-galactosidase production was highestbetween 25^0C and 35^0C, thereafter, a gradual decline was observed between 35^0C and 40^0C. Further increase in temperature resulted in decrease in β- galactosidase production.

Figure 4.7a shows the effect of varying temperatures on growth by *L.plantarum subsp.plantarum.* It shows that there was a sharp decline in growth at low temperature from 20^0C-25^0C. There was a gradual increase in growth with increase in temperature.Steady growth was maintained between 25-40^0C

Figure 4.7bshows that low temperature of 20^0C supported highest β-galactosidase production which ranged from 4.4385 to 7.3176U/ml in which the highest was recorded at 35^0C.A gradual drop in β-galactosidase production then follows. Thereafter production declined gradually with increase in temperature. This showed that β-galactosidase from *L. plantarum* subsp. *plantarum* (E36) is thermo labile.

Figure 4.7c shows the effect of varying temperature on protein profile of *L. plantarum subsp. plantarum*. The results indicated that the protein production was maximally supported by low temperature up to 25^0C with peak production value of 3.371 mg/ml. Further temperature increase resulted into sharp decrease (4.430 mg/ml at 30^0C) in enzyme production. Gradual reduction in protein level was observed as the temperature increases. This showed that high temperature was not favorable to protein stability by *L. plantarum subsp. plantarum*.

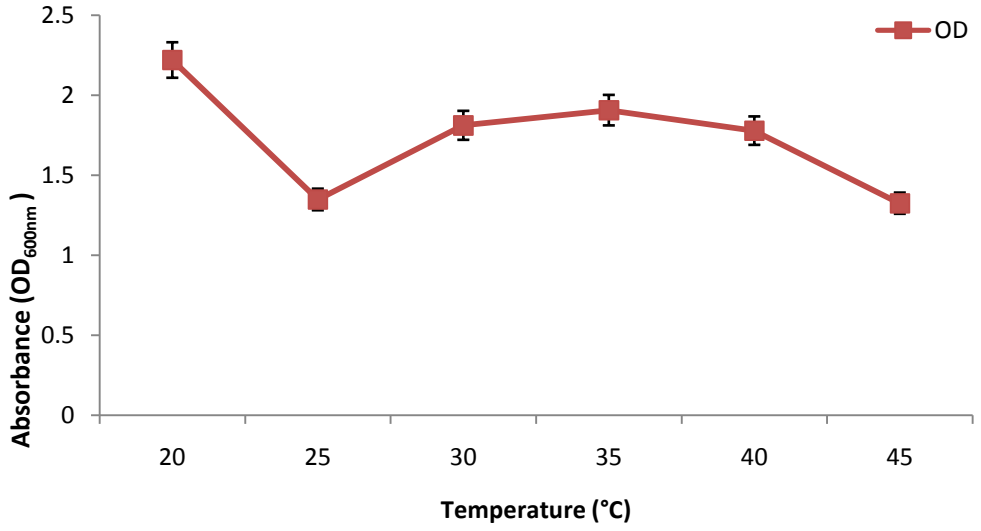


Figure 4.7a: Effect of Temperature on Growth for *L. plantarum* subsp.*plantarum* (E36)

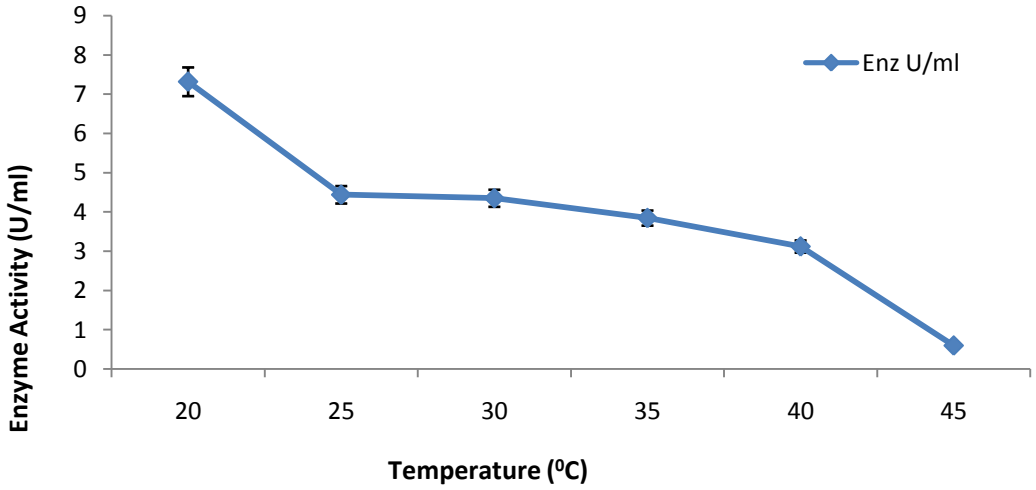


Figure 4.7b: Effect of Temperature on β-galactosidase production for *L. plantarum* subsp.*plantarum* (E36).

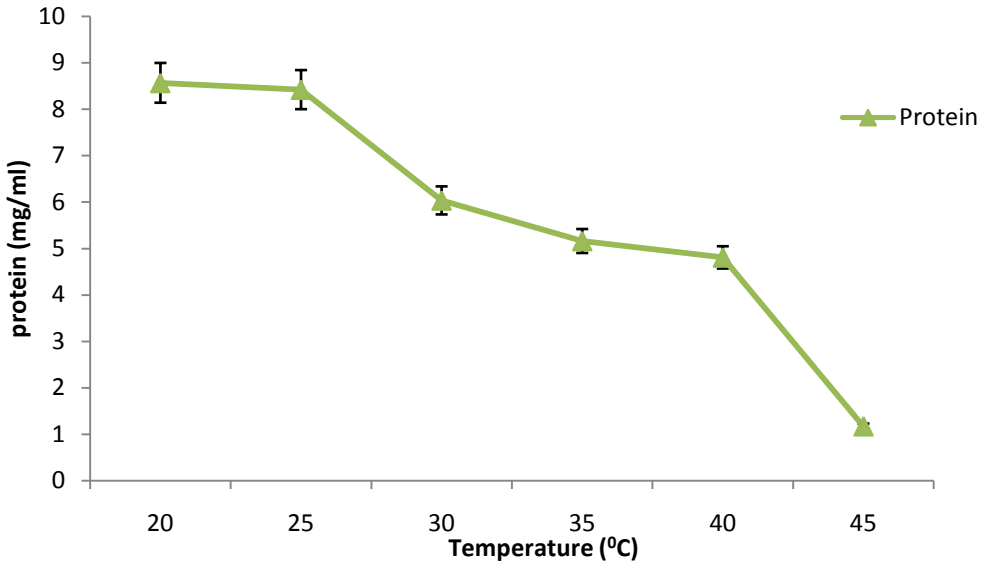


Figure 4.7c: Effect of temperature on protein profile of *L. plantarum subsp.plantarum*

Effect of pH on β- galactosidase productionby the selected isolates in an optimised medium

The effect of pH on β-galactosidase production by the three *L .plantarum*.Strains were investigated Figure 4.8a.Shows the effect of pH on β-galactosidase production by *L. plantarum* (G11). Production ranged from 0.1545 U/ml to 0.5729 U/ml.The highest β-galactosidase production (0.5627U/ml) was observed to occur at pH 7.5 Thereafter,a gradual decrease in β-galactosidase production was observed as pH increases.

Figure 4.8b profiles β-galactosidase production by *L. plantarum* (E13). Peak β-galactosidase production (0.4679U/ml)was attained at pH 7.0. Thereafter, production gradually declined as pH increases.

Figure 4.8c shows β-galactosidase production activity by *L. plantarum* subsp. *plantarum* (**E36)** in which pH 7.0 supported highest β-galactosidaseproduction (0.5729U/ml) thereafter production decrease sharply with increase in pH.Production activity by this strain follow similar trend as observed in strain (E13).

Out of all pH regimes tested for, pH 5 least supported β-galactosidase production. A neutral to alkaline broth environment favored β-galactosidase production by all the strains.

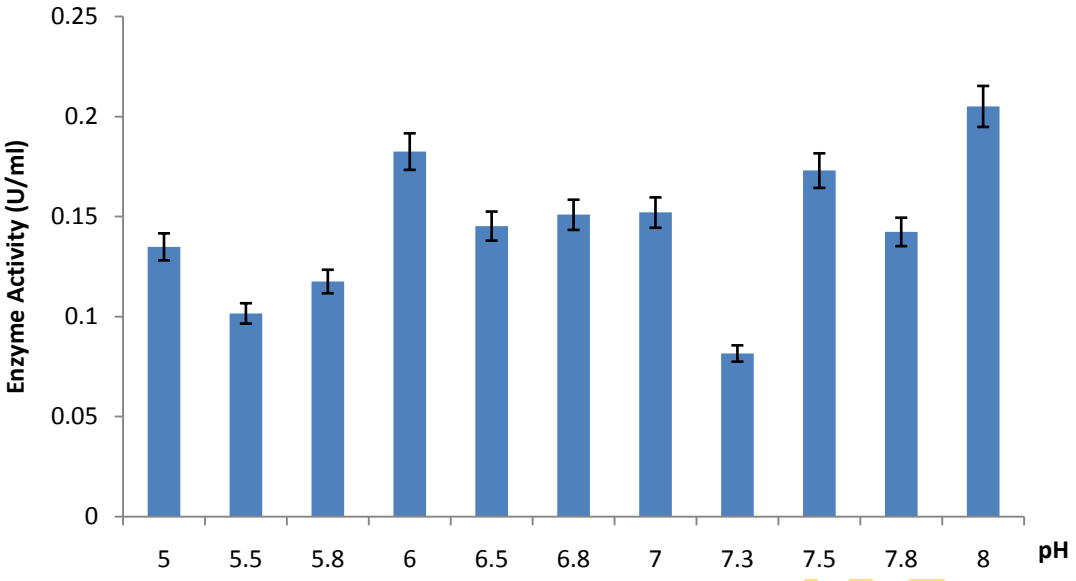


Figure 4.8a: Effect of pH on β-galactosidase production by*L.plantarum* (G11)

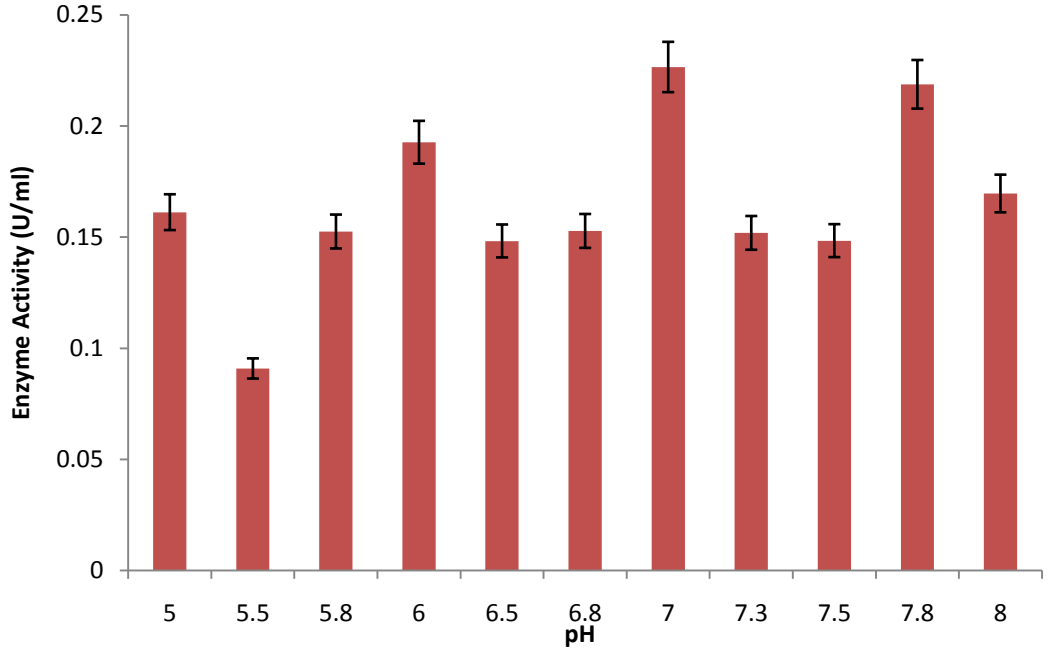


Figure 4.8b: Effect of pH on β-galactosidase production by *L.plantarum*(E13).

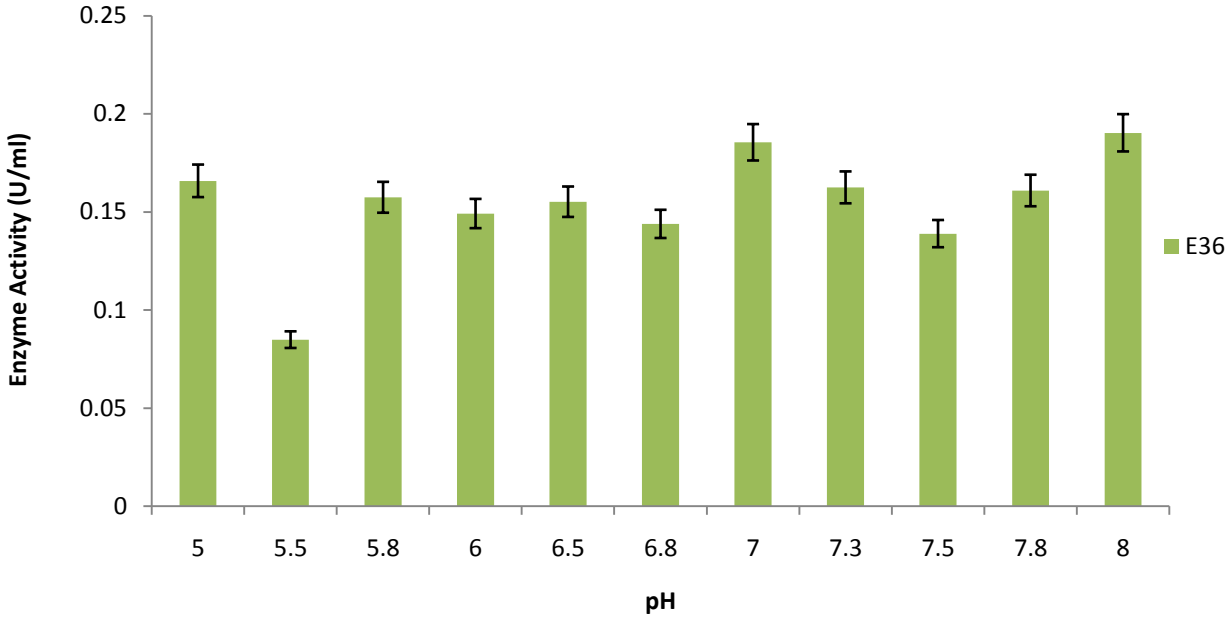


Figure 4.8c: Effect of pH on β-galactosidase production by *L. plantarum subsp. plantarum* (E36).

Effect of agitation speed on β-galactosidase production.

The effect of agitation on β-galactosidase production by the selected LAB isolates was investigated. Figure 4.9 shows the effect of agitation on β -galactosidase production by the isolates. β-galactosidase production by the different isolates ranged from 0.004 U/ml (0 rpm) to 0.186 U/ml (100rpm).The result showed that highest production was favoured at speeds less than 100rpm(between 60-120 rpm) rather than at higher speeds as shown by *L. plantarum*. (G11), *L. plantarum*. (E13) and *L. plantarum subsp. plantarum* (E36) respectively (Table 4).The speed of agitation increased enzyme production to a maximum limit of 60rpm by *L. plantarum* (G11). There was no further increase in production even with increase in the agitation speed. The effect of agitation on β- galactosidase production by *L .plantarum* (G11) showed that 60rpm supported maximum production of 0.184 U/ml. For *L. plantarum* (E13) agitation speed of 120 rpm give maximum production of 0.182 U/ml. Further increase in agitation speed has no effect on production of β-galactosidase**.** **I**ncrease in the speed of agitation for β-galactosidase production does not result into changes in the amount of production.Observations of the three strains of *plantarum* shows that each of the strains has different agitation speeds for β-galactosidase production.

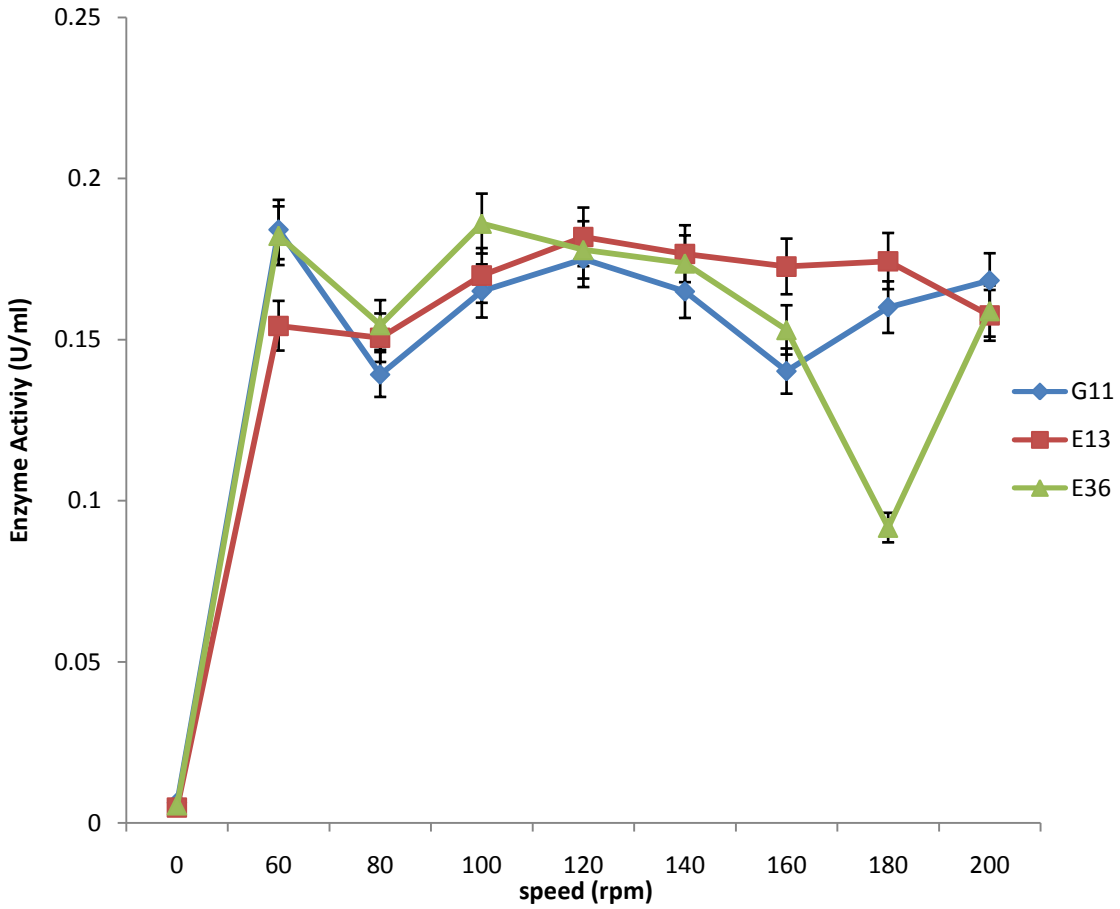


Figure 4.9: Effect of agitation on β-galactosidase production by LAB isolates.

Effect of carbon sources on β-galactosidaseproduction.

Figures 4.10a - 4.10d show the effect of different carbon sources on β-galactosidase production by the selected LAB isolates. All the fermentable sugars supported β-galactosidase production in all the selected isolates. Glucose, Galactose, Maltose and Mellibiose maximally enhanced β-galactosidase production. For *L.plantarum* (G11), Raffinose was found to be the best carbon source for β-galactosidase production having the highest enzyme activity of 0.305U/ml. This was followed in decreasing orders by mellibiose, galactose and saccharose. The least supporting carbon source was found to be fructose(0.1692U/ml) for the single sugars, while in the case of combined sugars, maltose + galactose(0.2959U/ml) was 14.47% better than galactose and 0.74% than maltose as single sugars respectively (Figure 4.10a).

For *L. plantarum* (E13), all carbon sources tested poorly on average. Only mellibiose, (0.2786U/ml) showed significant effectiveness. Galactose (0.2698U/ml) and maltose (0.2786U/ml) appreciably supported β-galactosidase production while glucose and trehalose either had close to zero or very low activity among the single sugars. The same trend of activity was observed in the case of combined sugars where maltose + Galactose combination was 11.65% and 5.47% better than Raffinose + Galactose and Trehalose + galactose combinations respectively (Figure 4.10b).

All sugar combinations specifically raffinose + galactose and Trehalose + Galactose performed appreciably well at 4.63% and 1.11% higher than Mellibiose as a single sugar respectively. Combining effect of the sugars showed that Galactose and Maltose are the best carbon source for *L. plantarum* (G11) and *L. plantarum* (E13). As for *L. plantarum subsp. plantarum* melibiose, galactose and saccharose at 0.2982U/ml,0.2827U/ml and 0.2689U/ml respectively best supported β- galactosidase production as single sugars while raffinose and galactose best supported β- galactosidase production as combined sugars (Fig.4.10a).

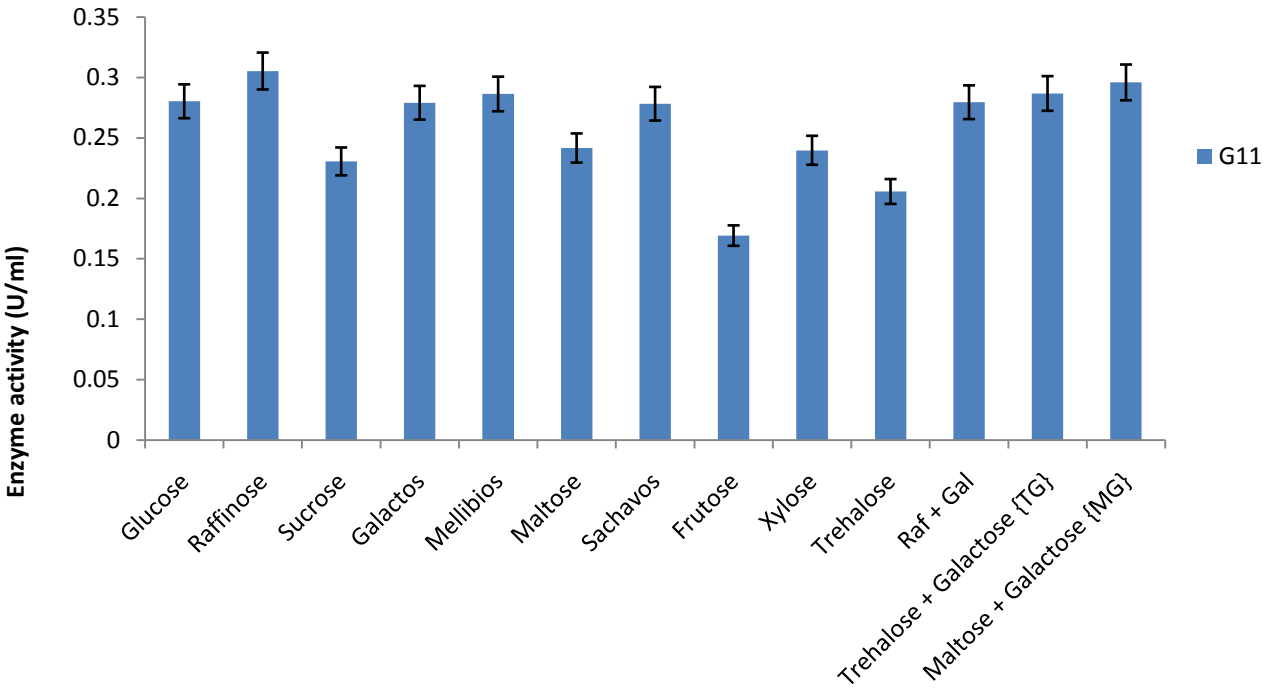


Figure 4.10a: Effect of different carbon sources onβ-galactosidase production by *L.plantarum*. (G11).

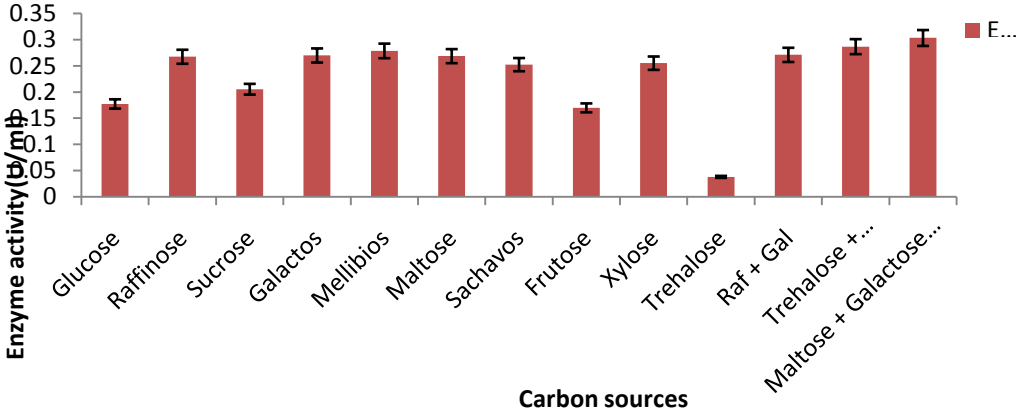


Figure 4.10b: Effect of different carbon sources onβ- galactosidase production by *L. plantarum* (E13)

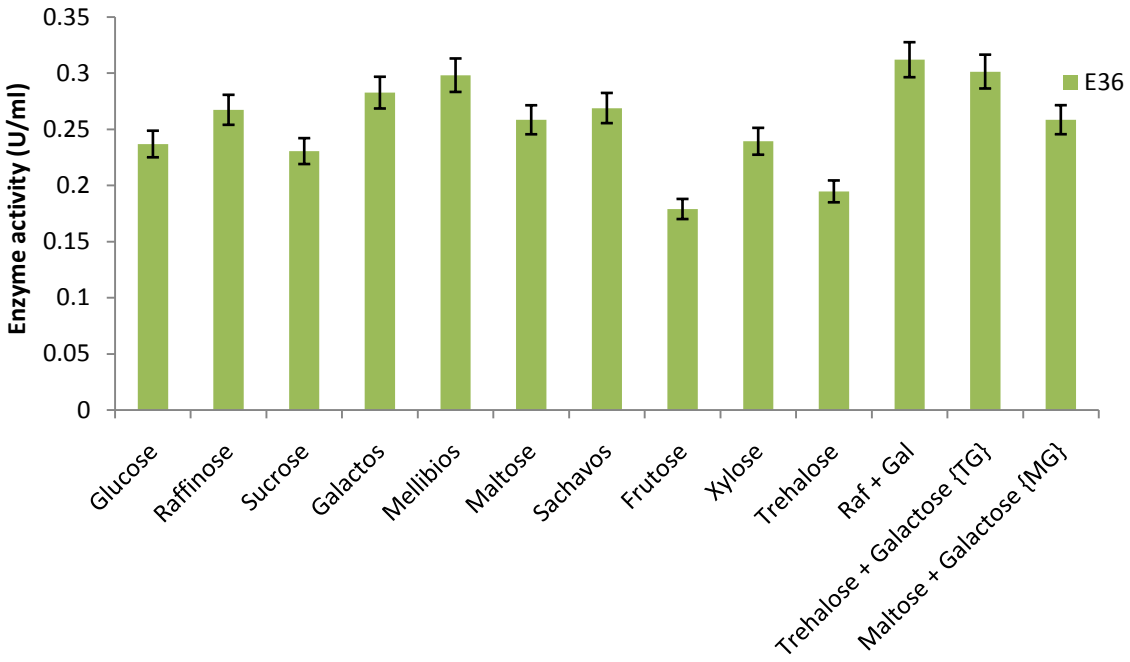


Figure 4.10c: Effect of different carbon sources on Growth β- galactosidase production by *L. plantarum subsp. plantarum* (E36)

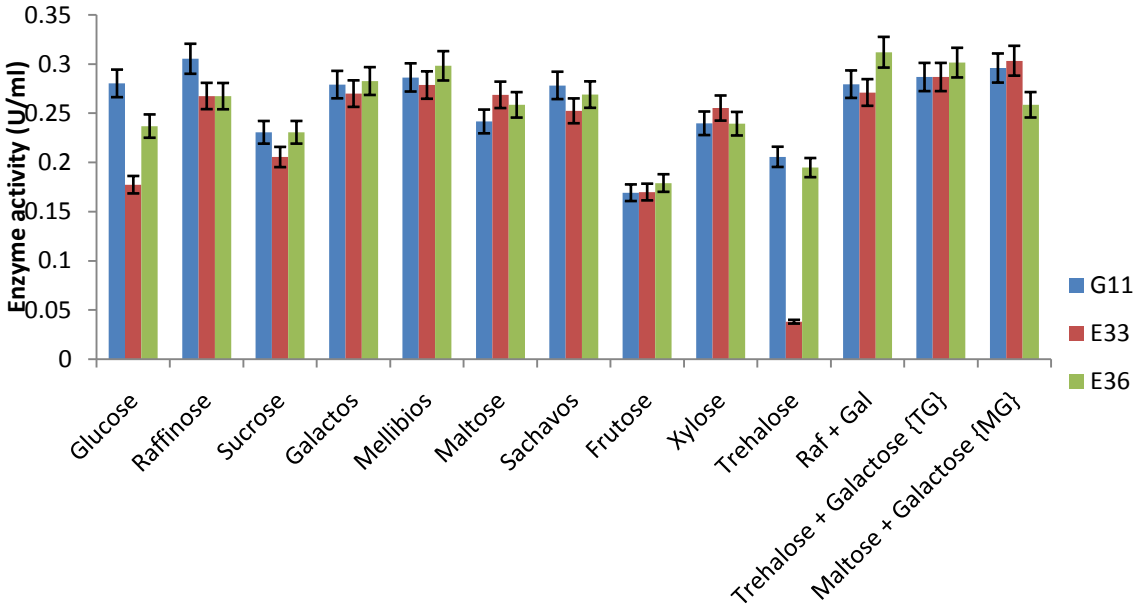


Figure 4.10d: Effect of different carbon sources on β- galactosidase production by LAB isolates.

Effect of organic and inorganic nitrogen sources on β-galactosidaseproduction

The effect of organic and inorganic nitrogen sources on β-galactosidase production by *Lactobacillus plantarum* strains was investigated (Figure 4.11). Nine (organic and inorganic) nitrogen sources were used. The results are as shown in the following figures, it was observed that casein and yeast extracts as organic nitrogen sources were the best enhancer for β-galactosidase production in both *L.plantarum*. (G11) and *L. plantarum* (E13) whereas all the inorganic nitrogen sources best supported β- galactosidase production with Potassium nitrate and Ammoniumnitrate salts recording the highest production of 0.2806U/ml and 0.2619U/ml respectively by *L. plantarum* (G11).

Also β- galactosidase production by *L. plantarum* (G11) was enhanced in broth augmented with malt extract, yeast extract, casein, urea and ammonium salts. A similar production trend was also observed with *L.plantarum* (E13) reaching peak production of 0.45583U/ml with ammonium nitrate. However, almost all the salts performed poorly in the case of *L.plantarumsubsp. plantarum* (E36) as none of the organic salts significantly boosted β-galactosidase production except ammonium nitrate. It was observed from fig. 4.10c that all the salts performed poorly in supporting β-galactosidase production by *L plantarum* subsp. *plantarum* (E36) just as observed with *L. plantarum* (E13).

The profile of the effect of the salts on β- galactosidase productionby the three strains tested isbest represented in Figure 4.11d.

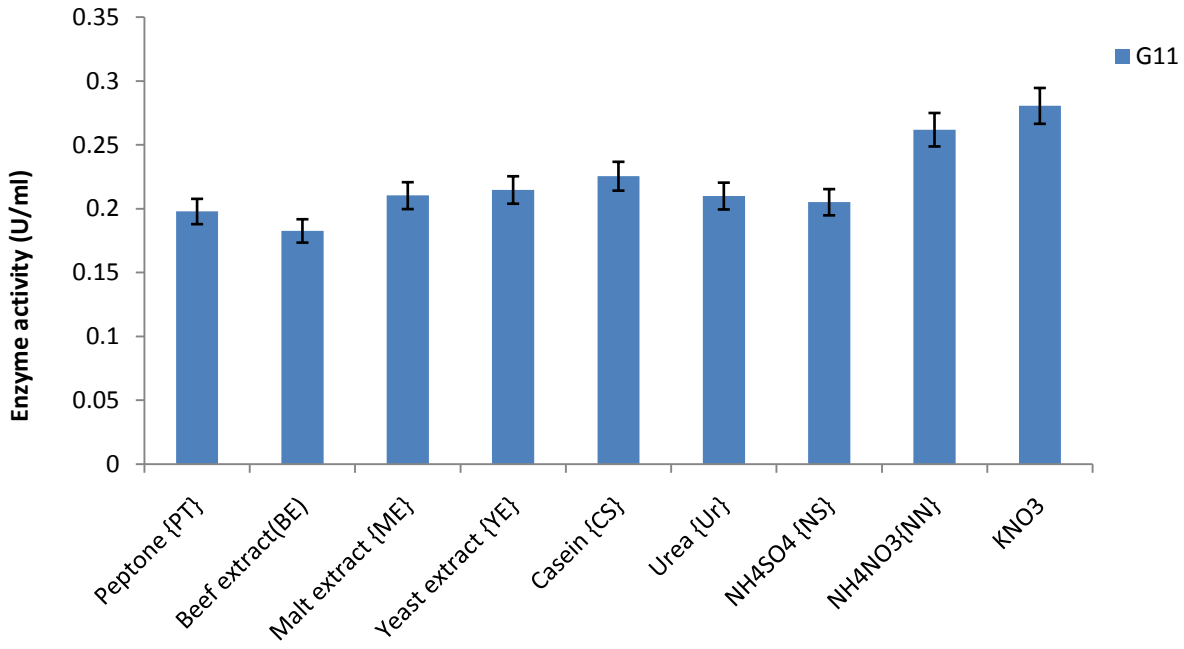


Figure 4.11a: Effect of different nitrogen sources on β- galactosidase production by *L. plantarum* (G11).

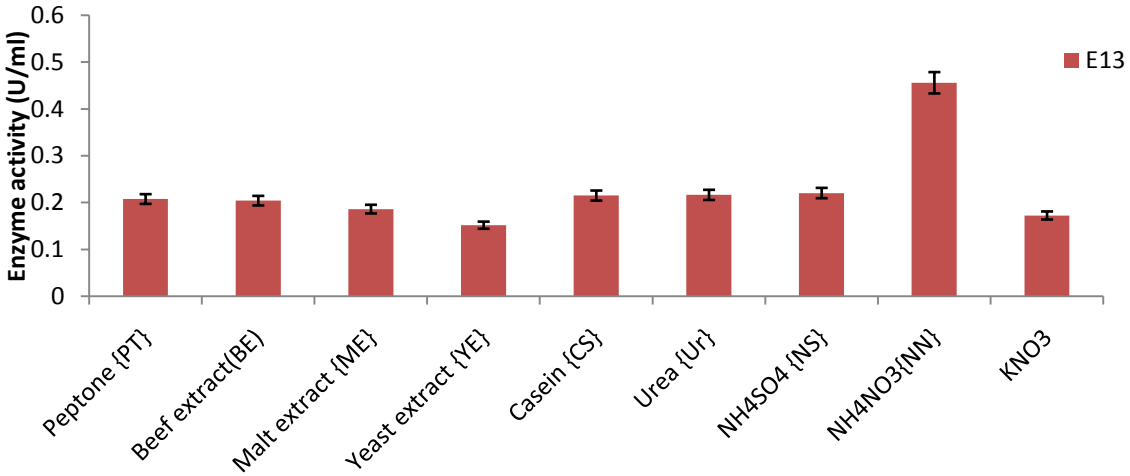


Figure 4.11b: Effect of different nitrogen sources on β- galactosidase production by *L. plantarum* (E13).

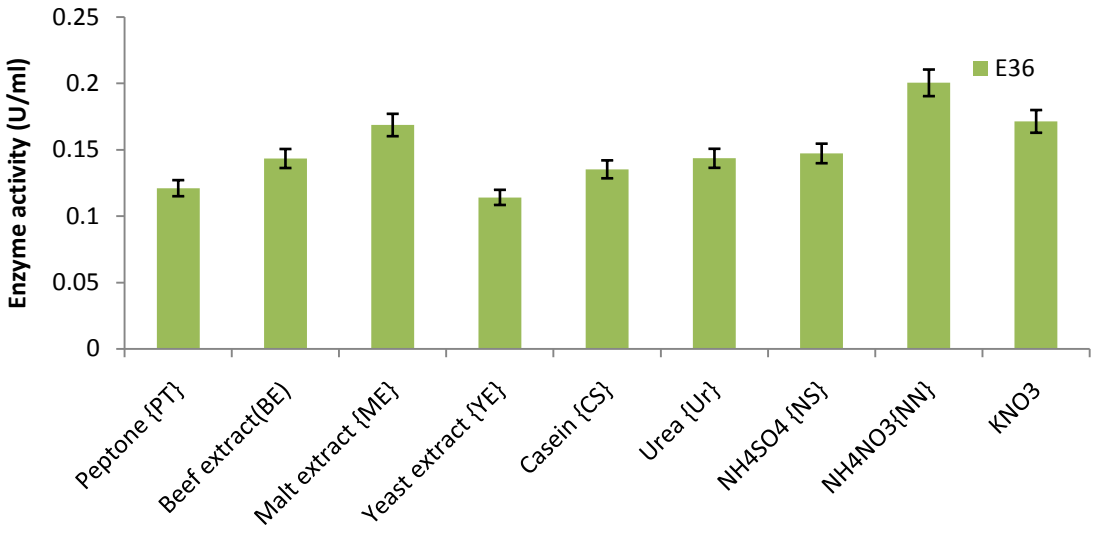


Figure 4.11c: Effect of different nitrogen sources on β- galactosidase production *L. plantarum subsp. plantarum* (E36)

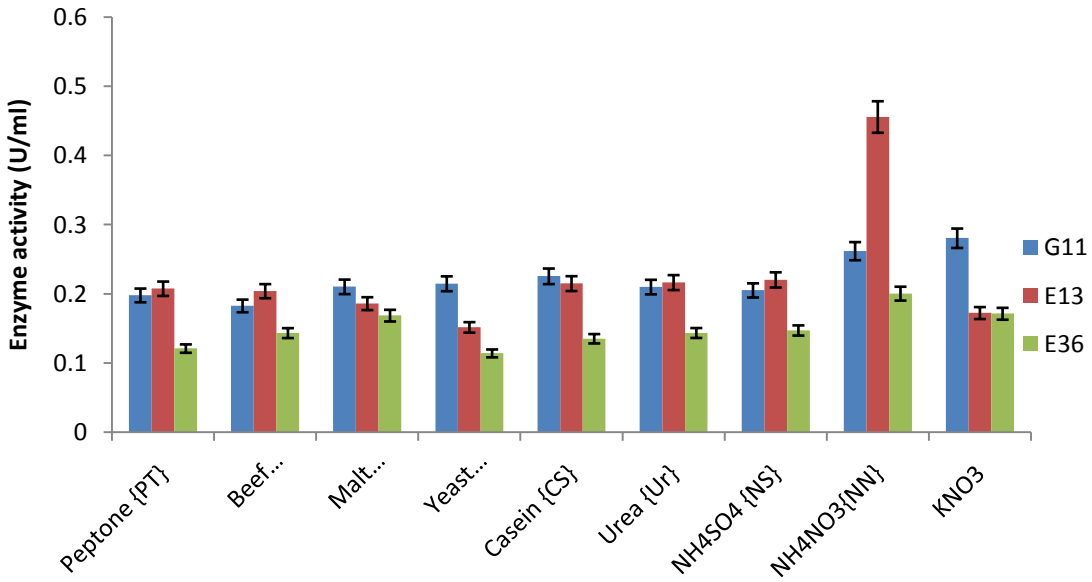


Figure 4.10d: Effect of different nitrogen sources on β- galactosidase production by selected LAB isolates.

Effect of inoculum size on β-galactosidase production activity by selected LAB isolates.

Figure 4.12a: is showing the effect of different inoculum sizes on β-galactosidase production.As shown in the figures, the inoculum sizes tested ranged from 4 – 12% as to determine the best inoculum size that best augment isolate growth and β-galactosidase production by LAB strains (Table 13) under optimised conditions. Inoculum size 6% best supported enzyme production for all isolates. Growth and enzyme activity was best supported by low (%) inoculum size attaining peak activities at 6%.This was followed in decreasing orders by sizes 8%,10% and 12% respectively.This trend were best demonstrated by *L. plantarum* (E13) and *L. plantarum subsp. plantarum* (E36) 12% increase in inoculum level have no effect on β-galactosidase production by the LAB isolates.

Figure 4.12b profiles the effect of inoculum size on β-galactosidase production by by *L. plantarum* (E13). It shows a gradual decrease in β-galactosidase production as percentage inoculums size increases.

Figure 4.12c shows the effect of inoculums size on β-galactosidase production by *L. plantarum* subsp. *plantarum* (E36). Six percent 6% inoculums size supported highest β-galactosidase production, Thereafter, gradual decrease in production was observed as percentage inoculums size increases.

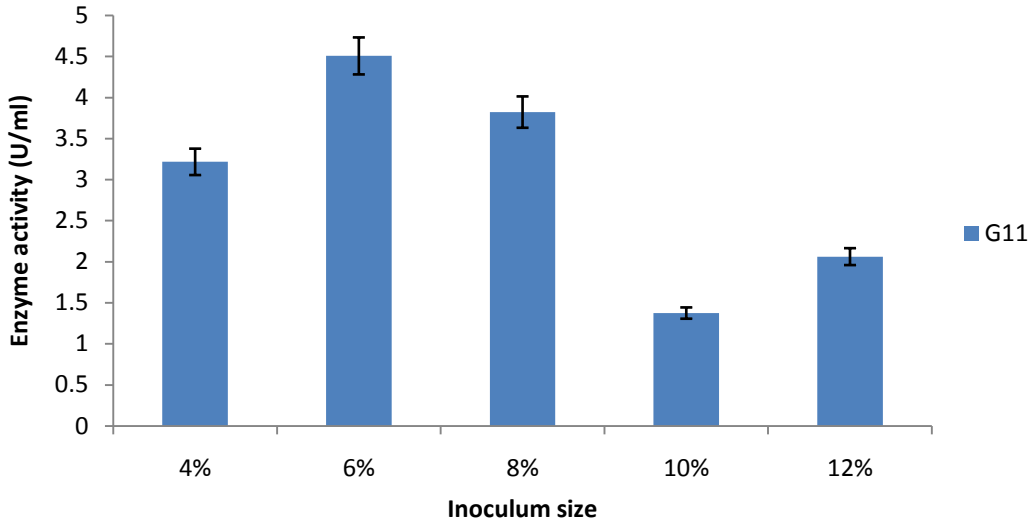


Figure 4.12a: Effect of inoculums size on β-galactosidase production by *L. plantarum* (G11).

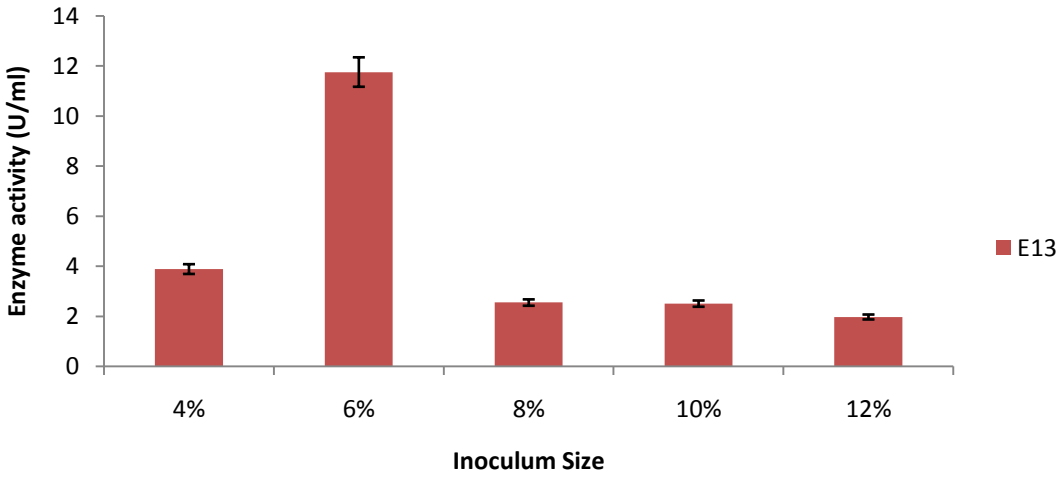


Figure 4.12b: Effect of inoculum size on β-galactosidase production by *L. plantarum* (E13).

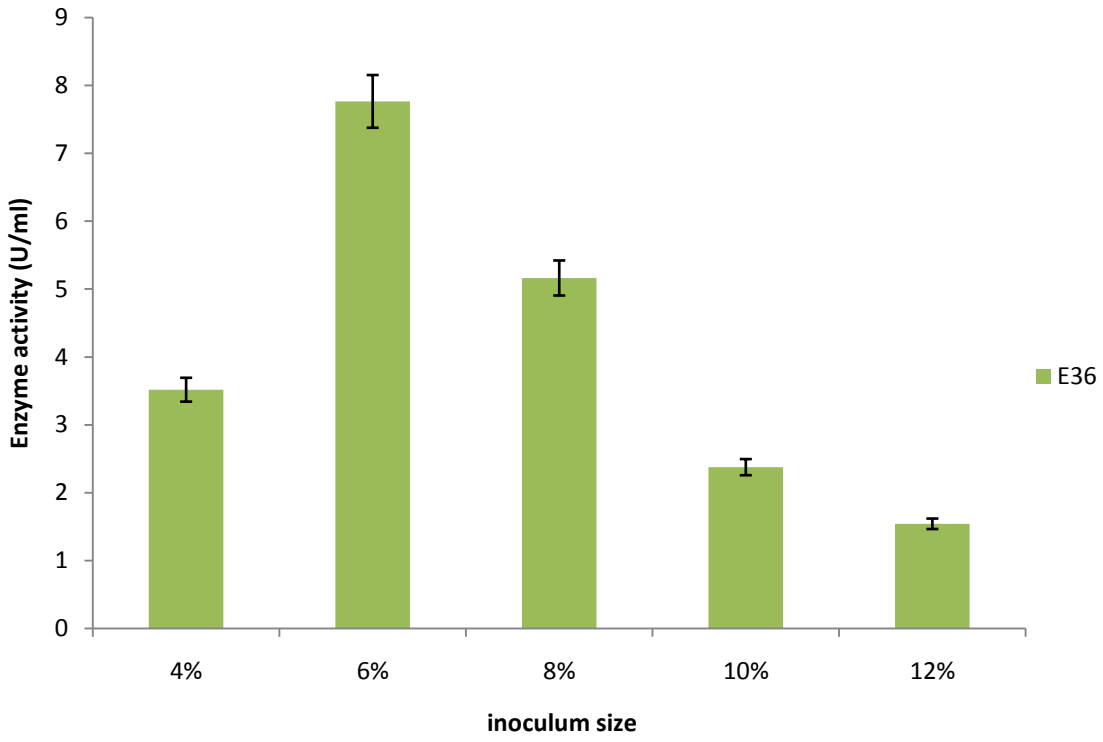


Figure 4.12c: Effect of inoculum size on β-galactosidase production by *L .plantarum subsp. plantarum* (E36)

Effect of inoculum age on β-galactosidase production

The effect of inoculum age on enzyme production was determined as shown in Figure 4.13a. The age of the cultures ranged from 6 – 42 hours to determine the best inoculums age that was suitable for peak β-galactosidase production. It was observed that β-galactosidase prodction ranged from 0.0851U/ml to 0.2965U/ml for all the LAB isolates.

Figure 4.13a shows the effect of varying inoculums age on β-galactosidase production. There was gradual increase in inoculum age from 6-18 hrs. The highest β-galactosidase amount (0.2697U/ml) was achieved at 18hrs. There was reduced production at 24hrs which immediately attained another peak(0.2614U/ml) again at 30hrs. This was followed by a gradual decrease in β-galactosidase production with increase in time. Therefore *L. plantarum* (G11) attained maximum production with 18hrs old inoculums.

Figure 4.13b shows the effect of varying inoculums age on β-galactosidase production by *L. plantarum* (E13). It shows high activity at 6hrs (0.2916U/ml) which gradually decreases with increase in time until reaching peak production (0.2965U/ml) at 24hrs. This was followed by decrease in production with increase in time.stability was reached at 42 hrs. The best inoculums age for β-galactosidase production was 24hrs old.

Figure 4.13c shows the effect of varying inoculum age on β-galactosidase production by *L. plantarum subsp. plantarum* (E36). It shows minimal β-galactosidase activity at 6hrs and 12hrs old inoculum even though what was produced at 6hrs was higher than at 12hrs. However, a sharp increase was observed at 18hrs (0.2619U/ml) with minimal decrease in activity at 24hrs but attained peak production with 30hrs old inoculums. Therafter, gradual decrease in production with increase in time was observed.Stability was achieved with 42hrs inoculum.

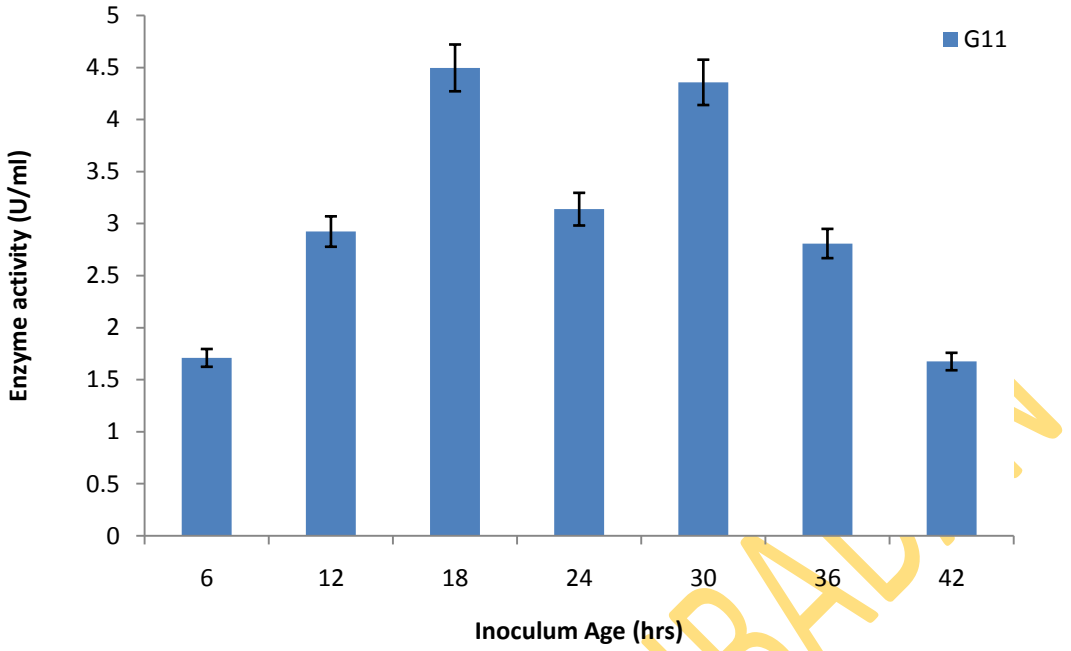


Figure 4.13a: Effect of inoculum age on β-galactosidase production at different time intervals for *L. plantarum* (G11)

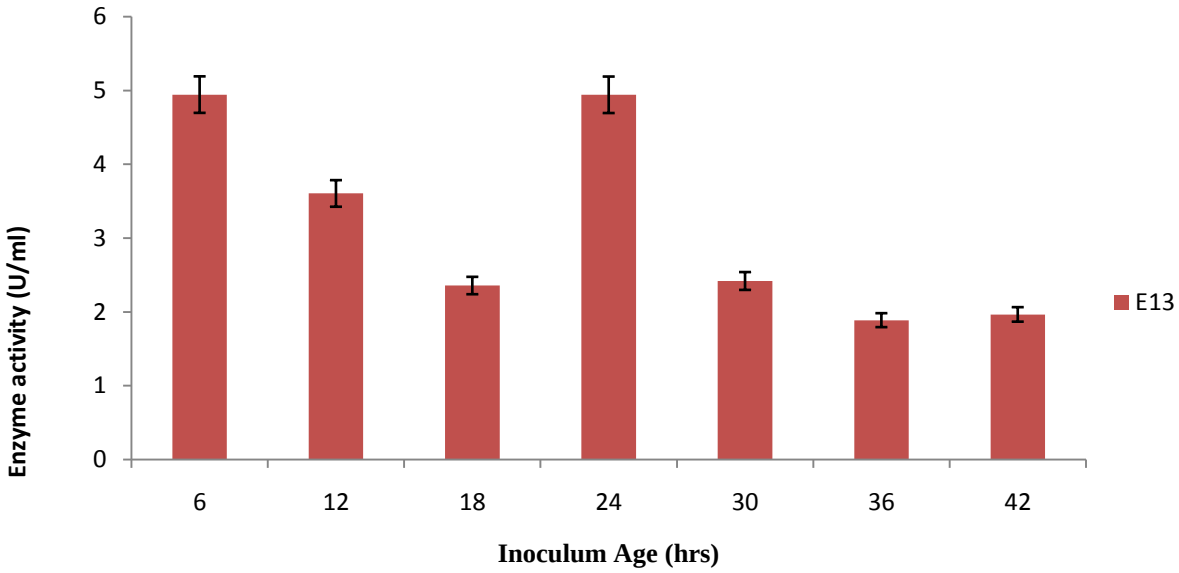


Figure 4.13b: Effect of inoculum age on β-galactosidase production at different time intervals for *L. plantarum* (E13).

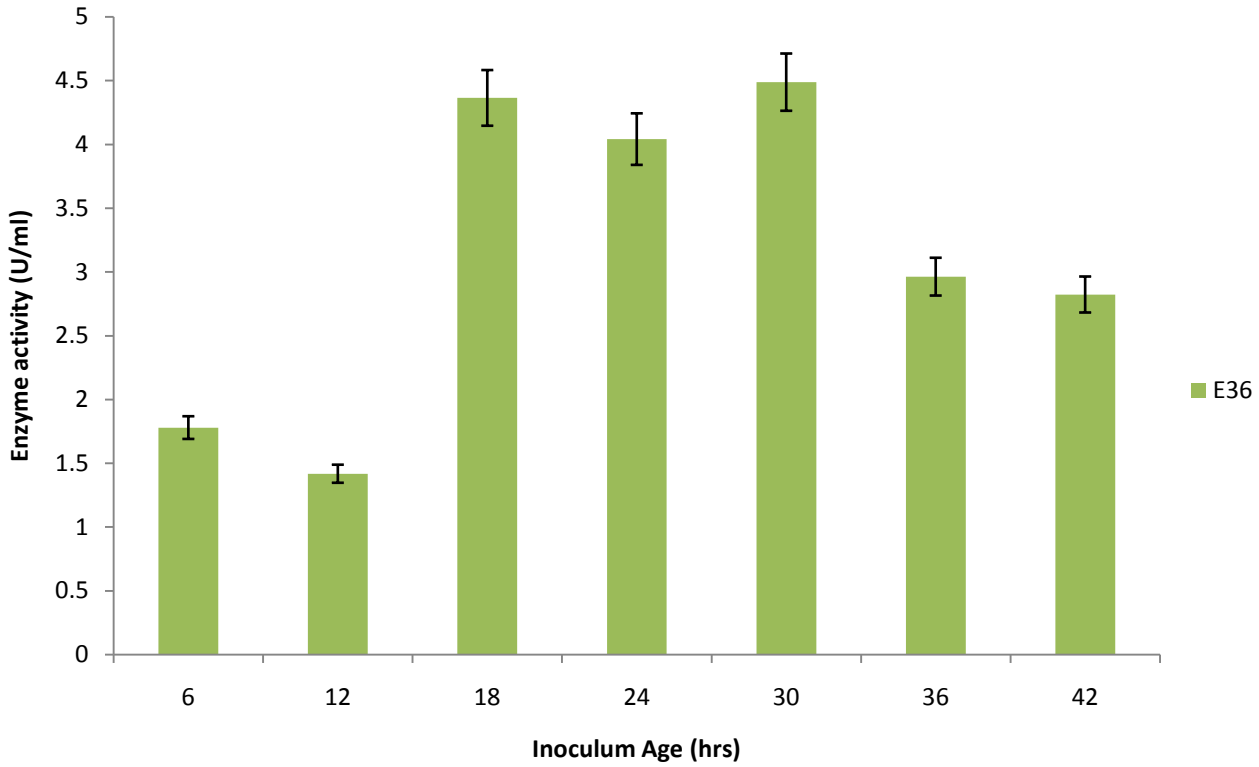


Figure 4.13c: Effect of inoculum age on β-galactosidase production at different time intervals by *L. plantarum subsp. plantarum* (E36)

Enzyme characterization

Effect of temperature on enzyme activity

The effect of varying temperature on the activity of β-galactosidase by the selected LAB isolates was determined. Figure 4.14a shows the effect of temperature on *L.plantarum* within the range of 15^0C -50^0C on β-galactosidase activity. β-galactosidase activity ranged from 0.00135U/ml-0.1225Uml. It shows that the lowest activity (0.00135 U/ml) was recorded by *L.plantarum*(G11) at 15^0C in which the highest activity (0.1225U/ml) was recorded at 45^0C. A gradual increase in β-galactosidase activity was observed between 15^0C and 20^0C. This was displayed better temperature stability than others as relative stability followed by a sharp drop at 250C followed by a stationary phase up to 30^0C. Another gradual increase in β-galactosidase activity now follows attaning a peak (0.1253U/ml) as stability reaches 50^0C.

In figure 4.14b β-galactosidase production by *L.plantarum* (E13) shows better temperature stability between 20^0C- 45^0C. A sharp increase in β-galactosidase production (0.04711 - 0.1285 U/ml)was observed from 15^0C-20^0C. This was followed by a gradual drop in β-galactosidase activity with increase in incubating temperature up to and including 30^0C. This was followed by a sharp increase at 35^0C. Increase in β-galactosidase activity increases gradually with increase in temperature until peak activity (0.1536U/ml) attained at 40^0C. A drop in activity was observed at 50^0C.

Figure 4.14c Profiles of β-galactosidase activity by *L. plantarum subsp. plantarum* (E36) within the temperature ranged tested. Gradual increase in β-galactosidase activity was observed between 15^0C-30^0C as β-galactosidase activity increases with with temperature. Maximum β-galactosidase activity (0.1681U/ml) was obtained at 30^0C. This followed by a sharp drop at 35^0C. Another sharp increase immediately occurred at 40^0C, attaining peak activity (0.1328U/ml) at 45^0C before finally recording another sharp drop at 50^0C

The characteristic behavior of these isolates and β-galactosidase production by each showed that *L. plantarum* (G11) has thermophilic properties by supporting optimal β-galactosidase activity at 45^0C while *L. plantarum subsp. plantarum* (E36) can be said to be mesophilic as temperature of 30^0C supported maximum β-galactosidase activity of 0.168U/ml

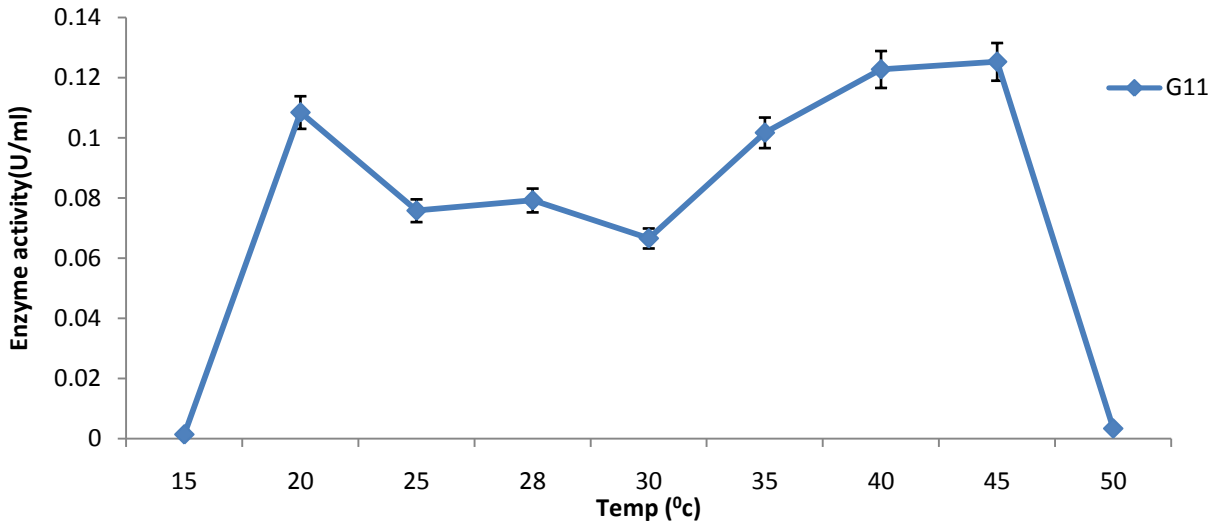


Figure 4.14a: Effect of temperature on β-galactosidase activity by (G11) *L. plantarum*

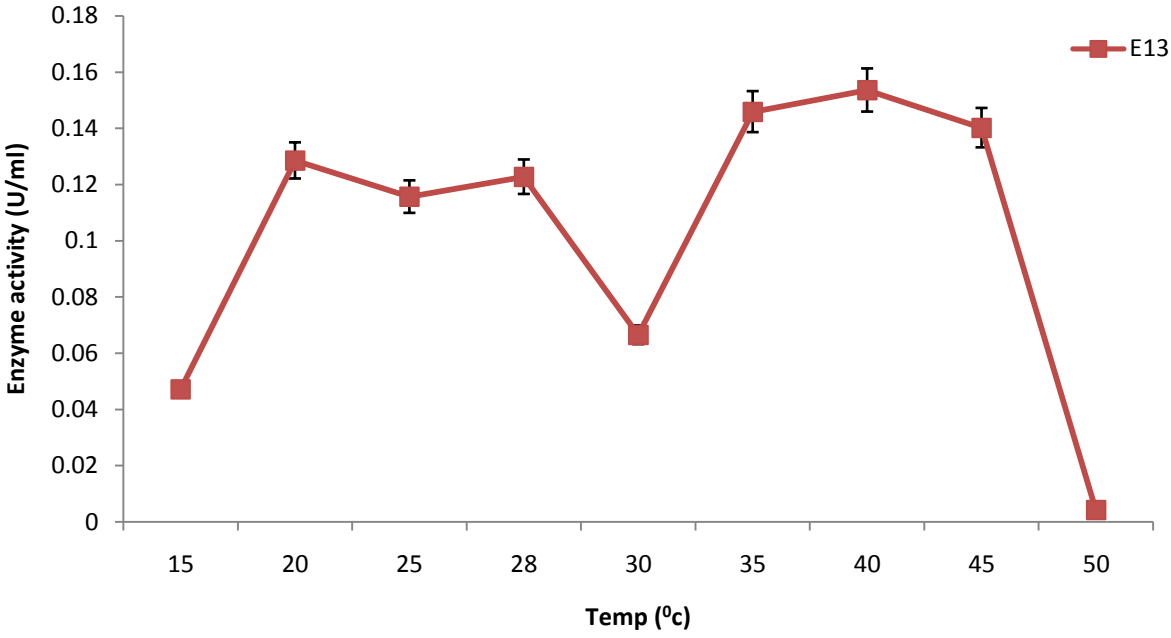


Figure 4.14b: Effect of temperature on β-galactosidase activity by *L. plantarum* (E13)

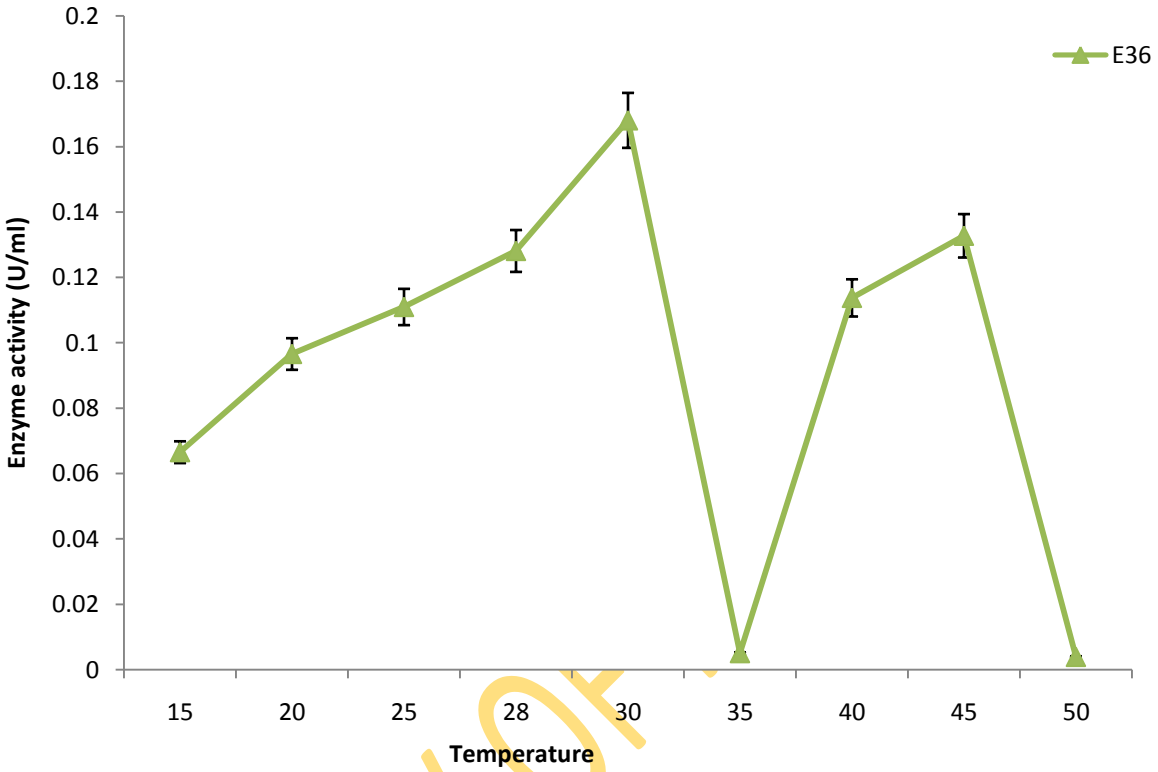


Figure 4.14c: Effect of temperature on β-galactosidase activity by *L. plantarum subsp. plantarum* (E36)

Effect of pH on β-galactosidase activity

Figure 4.15a shows the effect of varying pH on β-galactosidase activity as expressed by the selected LAB Isolates within pH range of 5.0 and 8.0. The highest β-D-galactosidase activity of 0.182 U/ml was produced by *L. plantarum* (G11) at pH 6.0.Highest β-galactosidase activity was observed at pH 5.0. It shows a gradual drop at5.5 which gradually increased to an initial peak activity (0.1825U/ml) at pH 6.0. A gradual drop in activity was observed at pH 6.5 which remain sustained up to and including pH 7.0. This was followed by a sharp drop at pH 7.3 followed by a sharp increase at pH 7.5. Highest β-galactosidase activity (0.1903U/ml) was recorded at pH. Hence *L. plantarum* is pH tolerant.

Figure 4.15b shows the profile effect of pH on the activity of β-galactosidase. At pH 5.0, very high β-galactosidase activity (0.1612U/ml) was observed followed by a sharp drop at pH 5.5. A gradual increase in activity was again seen at pH 6.0 attaining maximum β-galactosidase activity (0.2265U/ml) at pH 7, that is, it expressed peak activity at neutral pH.

Figure 4.15c shows the effect of pH on the activity of β-galactosidase produced by *L.plantarum subsp.plantarum* (E36). High β-galactosidase activity(0.1659U/ml)was observed at pH 5.0 which shows a sharp decline at pH 5.5. A gradual increase in activity was observed up to and including pH 7.0. This was followed by a gradual decrease in β-galactosidase activity until maximum activity was attained (0.1903U/ml) at pH 8.0.

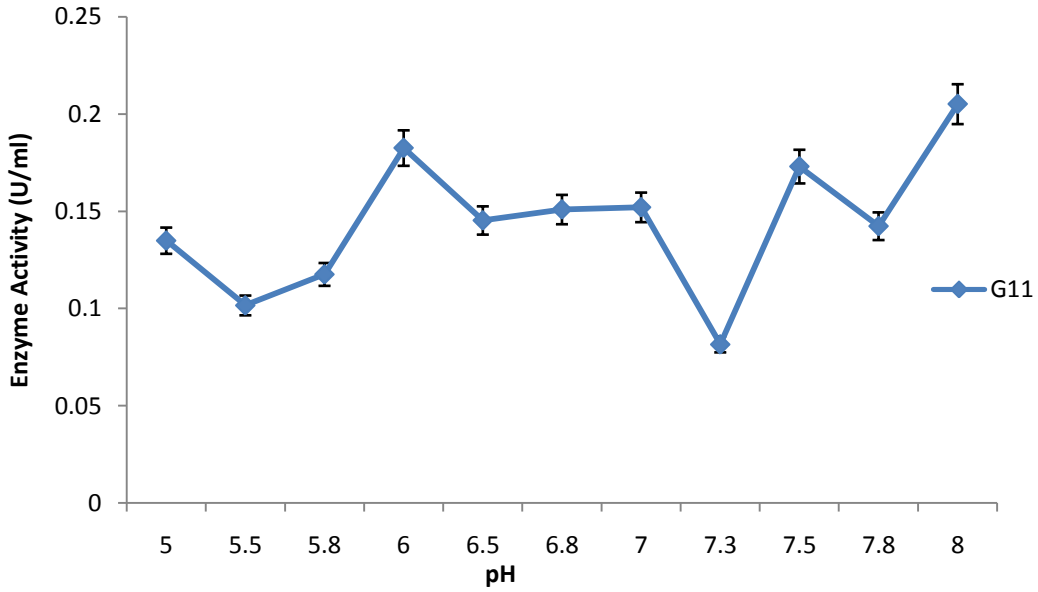


Figure 4.15a: Effect of pH on β-galactosidase production by *L. plantarum* (G11).

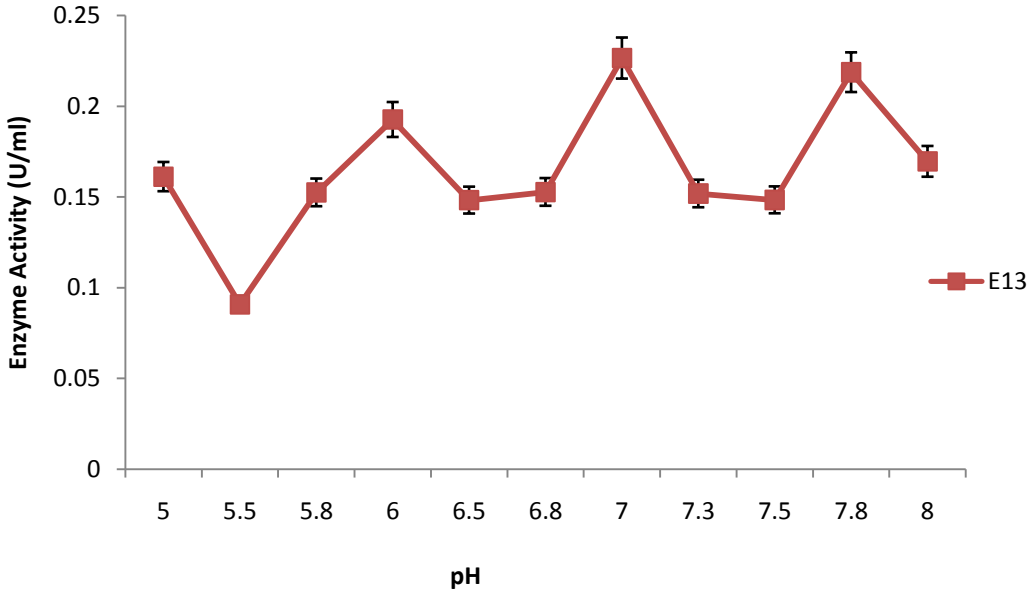


Figure 4.15b: Effect of pH on β-galactosidase production by *L .plantarum* (E13).

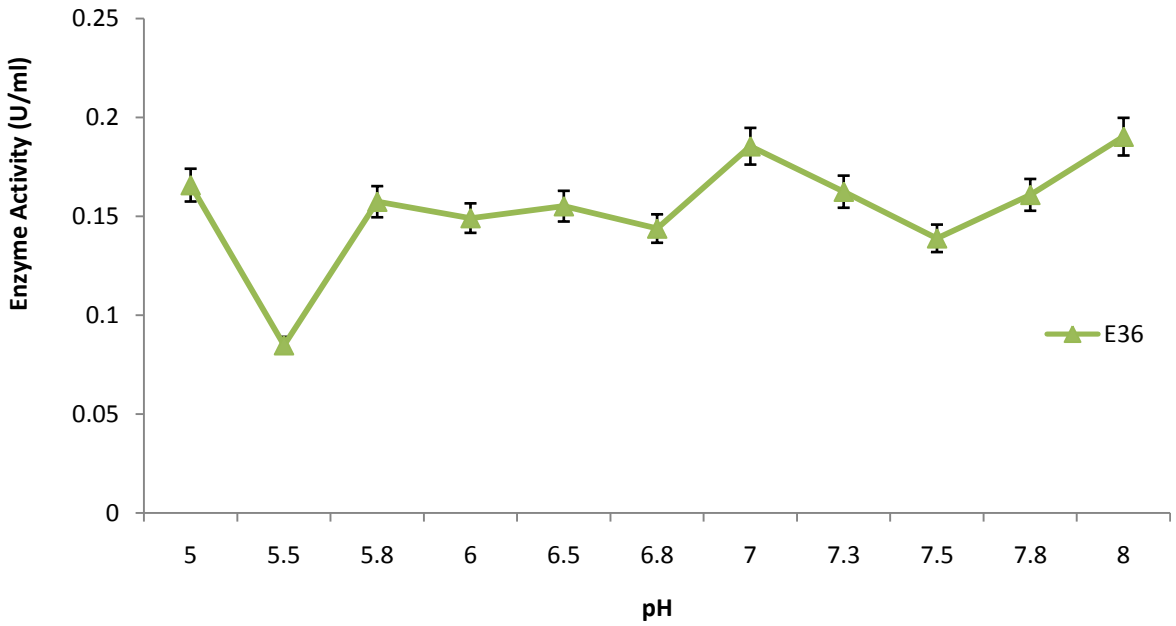


Figure 4.15c: Effect of pH on β-galactosidase production by *L. plantarum subsp. plantarum* (E36)

Stability of β-galactosidase activity at different temperatures

Stability of β-galactosidase activity at different temperature was investigated using temperature range from 20^0C - 75^0C as shown in Figure 4.16a. It is observed that the temperature profiles of the hydrolytic activity of LAB β-galactosidase produced by the different isolates. It shows that *L. plantarum* (G11) has its highest activity with ONPG as substrate at 25^0C (0.066U/mg). This was followed a gradual decrease in activity with increase in temperature.

A figure 4.16b show that L.plantarum (E13) has low hydrolytic activity at ordinary temperature.Sharp increase in activity was observed at 40^0C reaching maximum (0.032U/mg)at 45^0C. This was followed by a sharp drop in activity at 50^0C. A gradual decrease in activity was observed as temperature increases.Stability was achieved at 75^0C.

Figure 4.16c shows the effect of temperature on the hydrolytic capability of *L. plantarum subsp. plantarum* (E36) at different temperature regimes. A sharp drop in activity was observed at 20^0C with minimal increase in activity at 30^0C. This was followed by a sharp increase in activity (0.035U/mg) at 40^0C. A further drop in activity was again observed at 45^0C followed by another sharp increase in activity at 50^0C. Gradual decrease in activity then follows as the temperature increases. Stability was achieved at 75^0C.

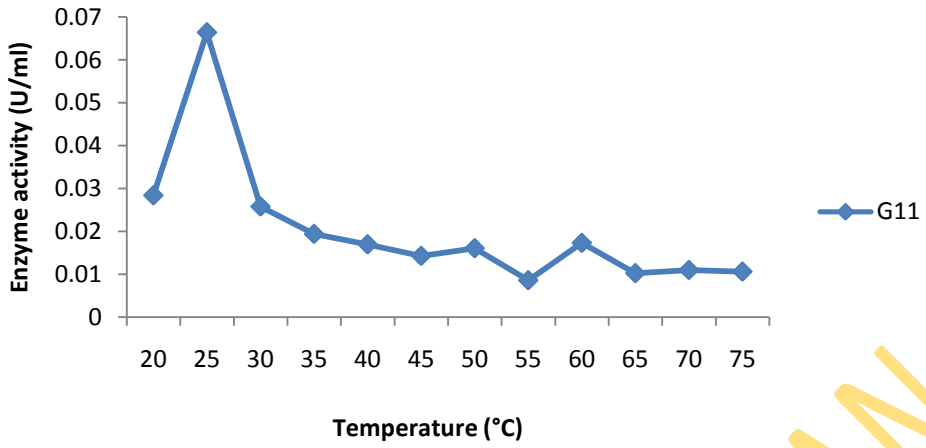


Figure 4.16a: Stability of β- galactosidase produced by *L. plantarum* (G11) at different temperatures

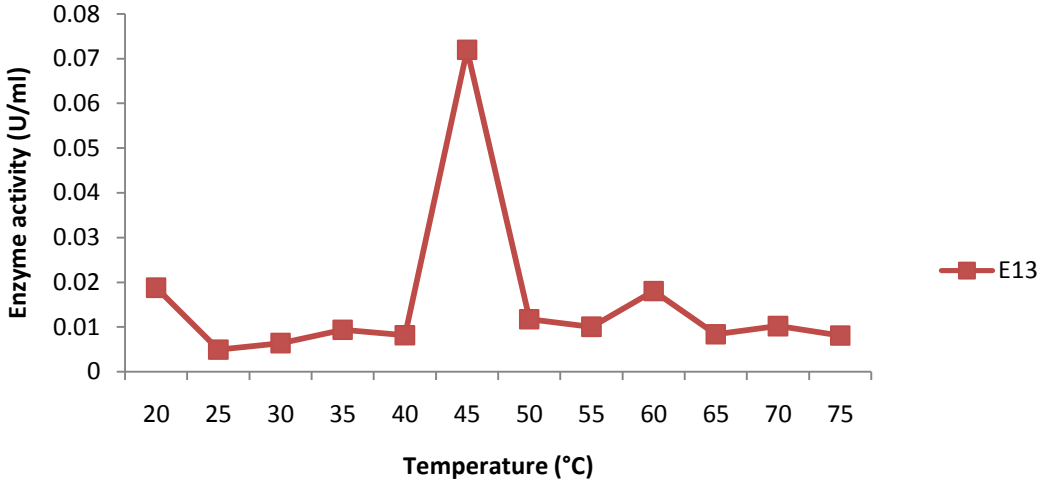


Figure 4.16b: Stability of β- galactosidase produced by *L. plantarum* (E13) at different temperatures

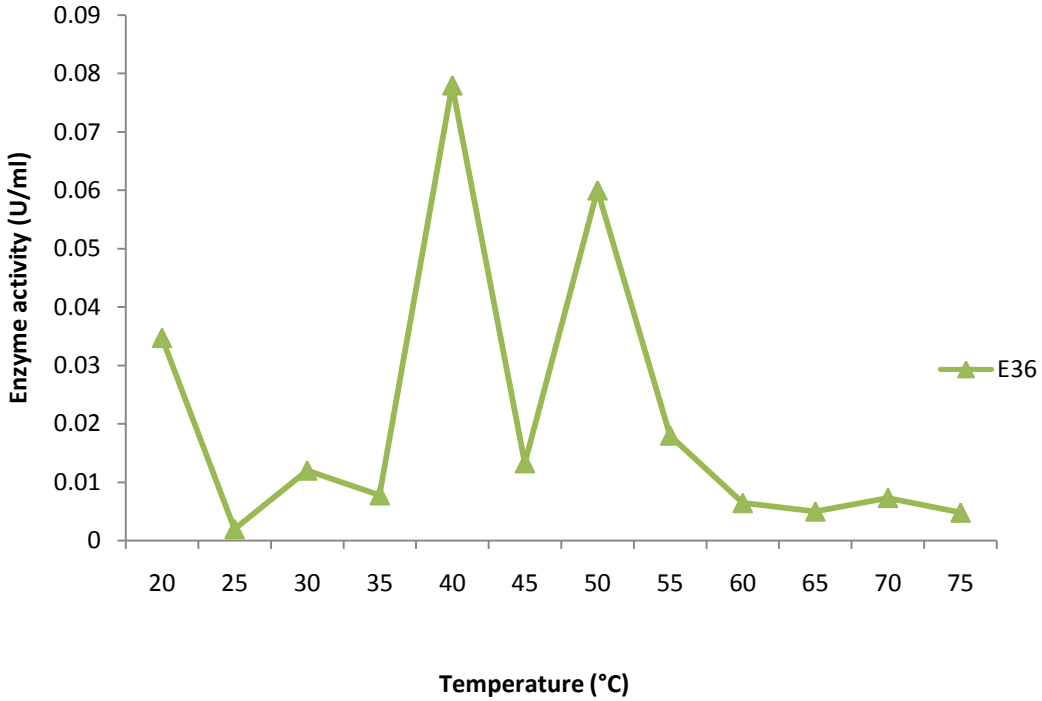


Figure 4.16c: Stability of β- galactosidase produced by*L.plantarum subsp.plantarum* (E36) at different temperatures.

Effect of molar concentrations of cations on β-galactosidase activity

Figure 4.17a shows the effect of different concentrations of cations on β-galactosidase activity produced by *L. plantarum* (G11). For KCl high activity was observed at 0.1molar concentration (0.0643U/ml). This was folloed by gradual decrease at 0.2mM. A maginal increase in activity was again observed as molar concentration again increases until peak activity was attained (0.0718U/ml) at 0.4mM. Thesame trend in activity was also observed with $CaCl_2$where β-galactosidase activity increases with increase in molar concentration. A sharp increase in activity was observed from 0.1-0.2molar concentration. There was another drop in activity 0.2-0.3mol. This was followed by a gradual increase in activity up to 0.5mol with peak activity of 0.0056U/ml.Molar concentration of 0.3-0.4mol was best for NaCl with the best activity of 0.0188U/ml at 0.4 molar concentration.NH_4Cl displays gradual increase in β-galactosidase activity as molar concentration increases. Highest activity (0.0319U/ml) was recorded at 0.2mol. Thereafter, gradual decrease in activity was observed as molar concentration increases.$MgSO_4$showed supportive activity in all concentration though with decreasing activity as concentration increases. $MnSO_4$ shows gradual increase in activity from 0.1-0.3molar concentration with peak activity of 0.0282U/ml. Thereafter, gradual decrease in activity with increase in concentration was observed.

Figure 4.17b profiles the effect of different molar concentrations of cations on β-galactosidase activity produced by *L. plantarum* (E13). The different concentration of KCl followed the same trend as observed by *L. plantarum* (G11). Highest activity (0.0625U/ml) was observed at 0.3molar concentration.This was followed by a sharp drop at 0.4 molar concentration with peak increase (0.0548U/ml) at 0.5molar concentration. The highest activity by β-galactosidase activity against different molar concentration of $CaCl_2$was recorded at 0.1 molar concentrations (0.0529U/ml). This was followed by a gradual decline in activity as concentration increases. With different concentration of NaCl salt, 0.4 molar concentrations was observed to produced the highest activity (0.0445U/ml). Although, 0.1molar concentration produced high activity but a gradual drop in activity was observed with increase in molar concentration.NH_4Cl salt also produce high activity at 0.1molar concentration(0.0367U/ml). However, gradual decrease in activity was observed as concentration increases.A sharp drop was produced at 0.4 molar

concentrations. This was followed by a sharp increase in activity at 0.5molar concentration. Different concentration of $MgSO_4$shows that 0.2mol. has the best activity forβ-galactosidase. Gradual decrease in activity was observed as concentration increases. $MnSO_4$ produced maximum activity at a concentration 0.2mM.Thereafter,gradual decrease in activity was observed as concentration inceases.

Figure 4.17c shows the effect of different molar concentrations of cations on β-galactosidase activity produced by *L. plantarum subsp. plantarum* (E36) 0.2mM of KCl produced the highest activity (0.0831U/ml). This was followed by a gradual decrease in activity at 0.3mM.A sharp drop in activity was observed at 0.4Mmol which was followed by a gradual increase at 0.5mM.CaCl salt has best activity at 0.1mM (0.0507U/ml)followed by a gradual drop at 0.2mM. There was another gradual increase at 0.3molar concentration which there after entered into gradual decrease as molar concentration increases.As for NaCl,0.2mM produced highest activity (0.0186U/ml) followed by gradual decrease as molar concentration increases.The best activity for different concentrations of NH_4Cl (0.0288U/ml) was produced at 0.4molar concentration.There was however an appreciable activity at all concentrations.The best activity recorded for magnesium sulphate was at 0.3Mm (0.0286U/ml).All other concentrations performed poorly. $MnSO_4$was best at 0.1molar concentration (0.0163U/ml).These was followed by gradual decrease in activity as concentration increases.

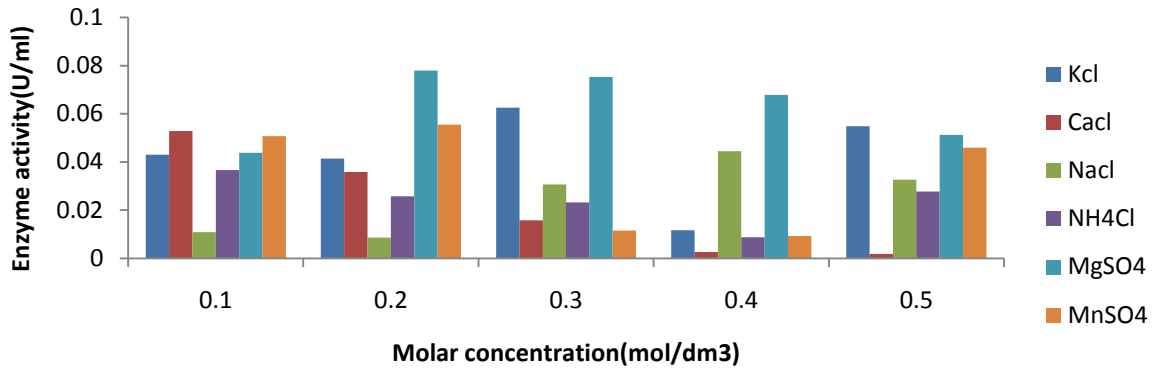


Figure 4.17a: Effect of different molar concentrations of cations on β-galactosidase activity by *L. plantarum* (G11).

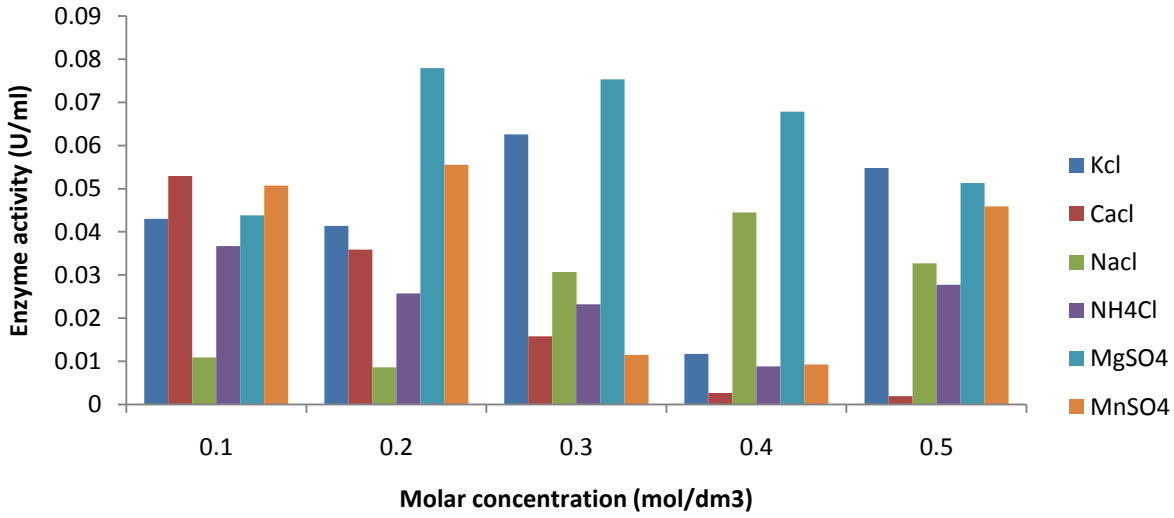


Figure 4.17b: Effect of different molar concentrations of cations on β-galactosidase activity by *L. plantarum* (E13).

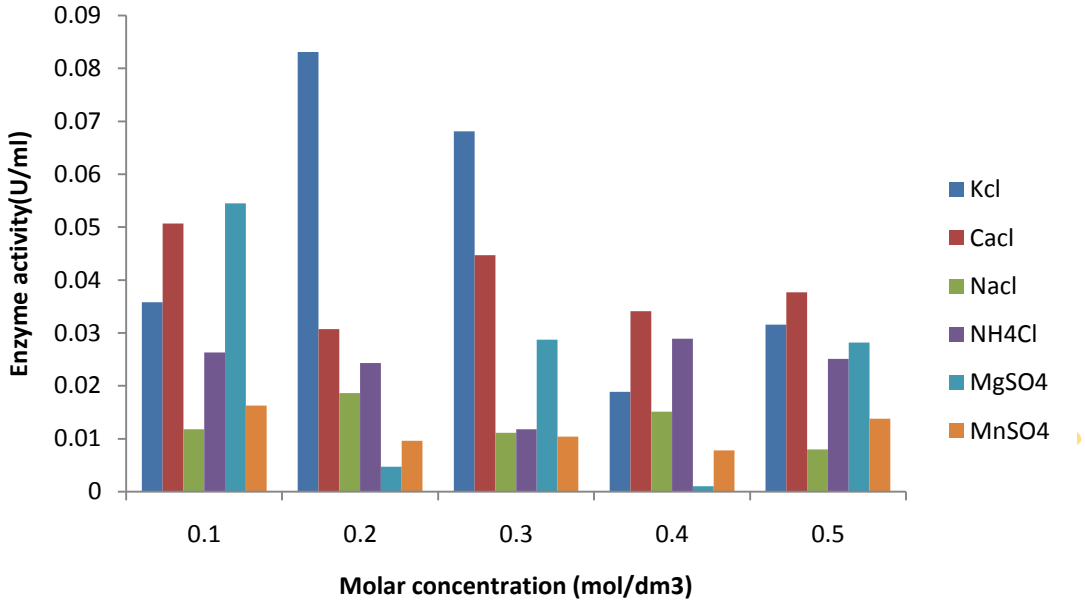


Figure 4.17c: Effect of different molar concentrations of cations on β-galactosidase activity by *L. plantarum subsp. plantarum* (E36)

Effect of varying molar concentrations of anions on β-galactosidase activity.

The effect of Anions on β-galactosidase activity using varying molar concentrations on selected bacterial isolates was determined as shown in figure 4.18a- 4.18c. The anions tested include copper sulphate, sodium nitrate, potassium nitrate and zinc sulphate with molar concentration ranging from (0.1-0.5M). β-galactosidase activity ranged from lowest activity of 0.00698 U/ml (in 0.1M) to optimum activity of 0.19031 U/ml (in 0.3M). β-galactosidase activity increases as molar concentration of the anions increases and thereafter declined.

Figure 4.18a shows the effect of molar concentrations of anion salts on β-galactosidase activity produced by *L.plantarum* (G11). From the figure β-galactosidase activity increases with increase in molar concentrarion of sulphate ions of copper. Gradual increase is observed from 0.1-0.3Mmol with peak activity (0.19303U/ml) at 0.3Mmol. This was followed by a gradual decrease in activity as concentration increases. As for the sodium nitrate salts, asharp increase at 0.2Mmol was observed. This was followed by a gradual decrease in activity withanother increase at 0.5Mmol concentration. KNO_3 salt had supportive activity in all concentrations. Highest activity was recorded at 0.1Mmol. This was followed by a gradual decrease as concentration increases except at 0.4molar concentration when marginal increase was observed. Different concentration of $ZnSO_4$ solutions show increase activity as concentration increases with peak activity at 0.2Mmol (0.0294U/ml). A shrp drop in activity was observed at 0.3Mmol, followed by a gradual increase in activity with increase in concentration.

Figure 4.18b profiles the effect of molar concentrations of anions on β-galactosidase activity produced by *L .plantarum* (E13). The figure reviewed a gradual increase in β-galactosidase activity as $CUSO_4$molar concentrations increases. The highest activity was observed at 0.5molar concentration. With different concentration $NaNO_3$, highest activity was attained at 0.1 molar concentrations. However, there was enhanced performance at all concentrations. KNO_3 has it highest activity at 0.1Mmol. This was followed by a gradual drop in activity as molar concentration increases. The same trend in activity was observed with similar concentrations of $ZnSO_4$ salt. The highest activity was recorded at 0.1Mmol.

Figure 4.18c shows the effect of molar concentrations of the different anion salts on β-galactosidase activity produced by *L. plantarum* subsp. *plantarum* (E36). $CUSO_4$ shows supportive activity on β-galactosidase activity at all concentration tested. The highest avtivity (0.1559U/ml) was recorded at 0.3Mmol. Marginal drop in activity was observed 0.4molar concentration followed by gradual increase at 0.5Mmol. The $NaNO_3$performedpoorly in all concentration tested except for 0.1 and 0.2 molar concentrations. Solutions of KNO_3 performed relatively better than that of $NaNO_3$. Sharp increase in activity was observed at 0.2molar concentration (0.0833U/ml). This was followed by a gradual decrease in activity. Stability was achieved with higher concentrations. $ZnSO_4$ cocentrations performed poorly except at 0.2Mmol where the highest activity (0.1002U/ml) was achieved.

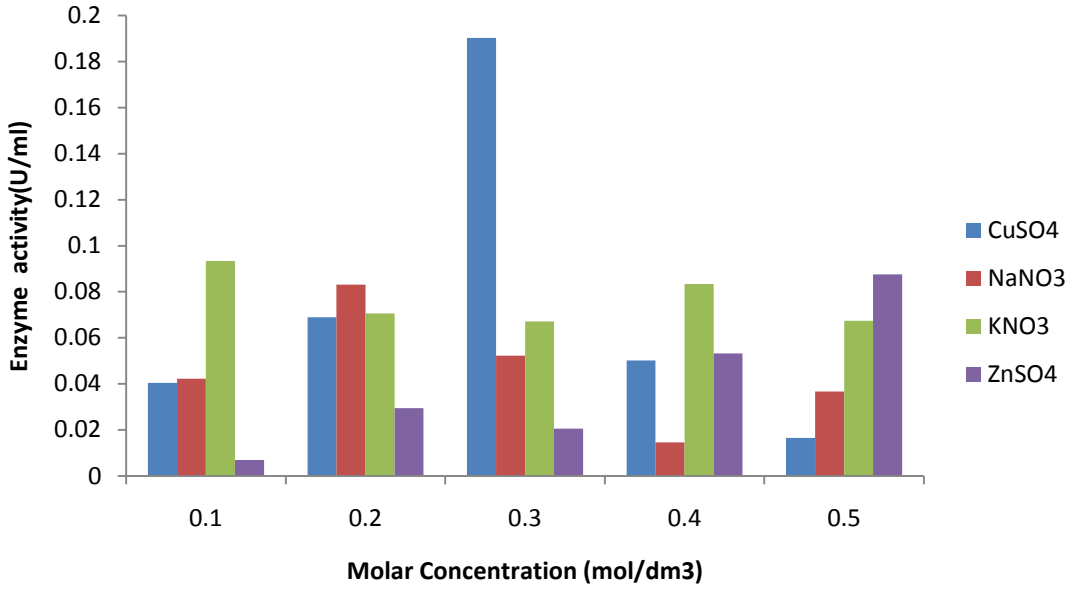


Figure 4.18a: Effect of different molar concentrations of anions on β-galactosidase activity produced by *L. plantarum* (G11).

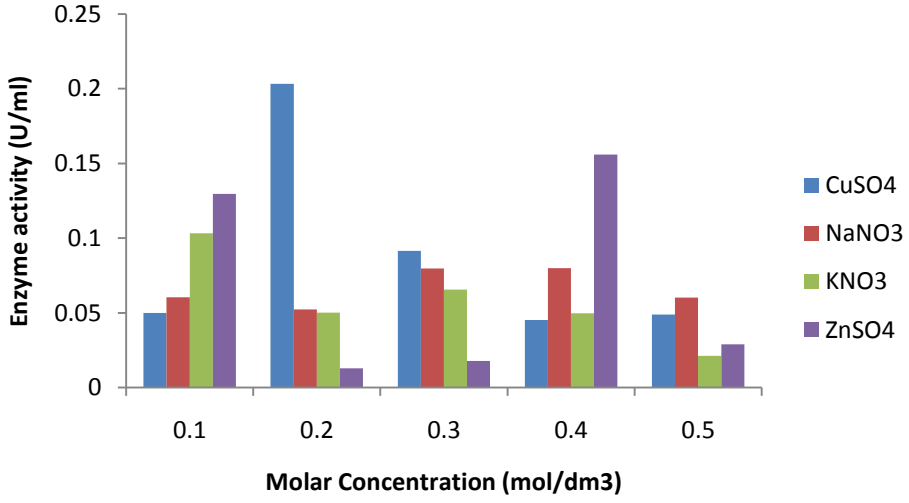


Figure 4.18b: Effect of different molar concentrations of anions β-galactosidase activity produced by *L. plantarum* (E13).

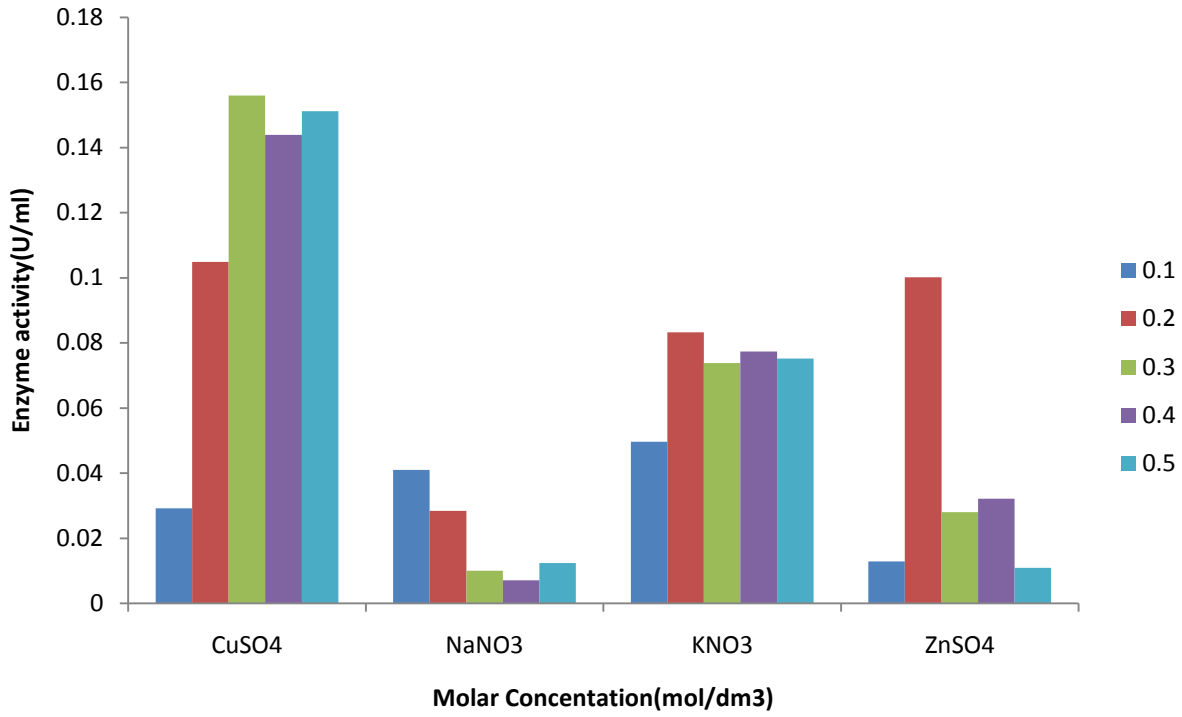


Figure 4.18c: Effect of different molar concentrations of anions on β-galactosidase activity produced by *L . plantarum subsp. plantarum* (E36).

Effect of different molar concentrations of inhibitors of β-galactosidase activity.

Figures 4.19-4.19c shows the effect ofvarying molar concentration (0.1-0.5M) of selected Inhibitors on β-galactosidase activity produced by the selected LAB isolates. Inhibitors tested on the β-galactosidase activity were Urea, EDTA and Potassium cyanide. β-galactosidase activity was found to range from 0.00966 U/ml (0.3M) to 0.17444 U/ml (0.2M).

Figure 4.19a shows the effect of different molar concentration of different inhibitors on β-galactosidase activity produced by *L. plantarum* (G11). Activity of inhibitors shows sharp increase from the lowest concentration of 0.1-0.2Mmol with marginal activity 0.0454U/ml for urea. The highest activity (0.0725U/ml) was obtained at 0.4Mmol, this was followed by a gradual decrease in activity. EDTA shows supportive activity at all concentrations except at 0.2 molar concentrations where a sharp drop was observed. This was followed by a gradual increase in activity with maximum (0.1587U/ml) at 0.5Mmol. KCN also gave enhanced activity at all concentrations attaining highest activity (0.1744U/ml) at 0.2Mmol. This was followed by a gradual decrease in activity as molar concentration increases.

Figure4.19b profiles the effect of inhibitors on β-galactosidase activity produced by *L .plantarum* (E13). Urea shows high activity (0.0762U/ml) at 0.1Mmoland then followed by gradual drop at 0.2molar concentration and another sharp drop 0.3Mmol was observed followed by a sharp increase (0.0953U/ml) was observed as the highest activity by urea. The highest activity by EDTA(0.0887U/ml) was attained 0.3 molar concentration, this was followed by a sharp drop in activity at 0.4Mmol which was followed by increase with increase in concentration. KCN shows supportive activity at all concentration tested. Highest activity (0.1692U/ml) was attained at 0.2Mmol.

Figure 4.19c shows the effect of different molar concentrations of the inhibitors onβ-galactosidase activity produced by *L.plantarum* subsp. *Plantarum*. It shows that different concentrations of urea have low activity at low concentrations. The highest activity (0.0599U/ml) was recorded at 0.3 molar concentrations. This was followed by agradual decrese in activity with increase in concentration.EDTA produced the highest activity (0.0857U/ml) at 0.2 molar concentration. This was followed by a gradual decrease up to but including 0.4Mmol. Another peak activity was observed at 0.5 molar concentrations.

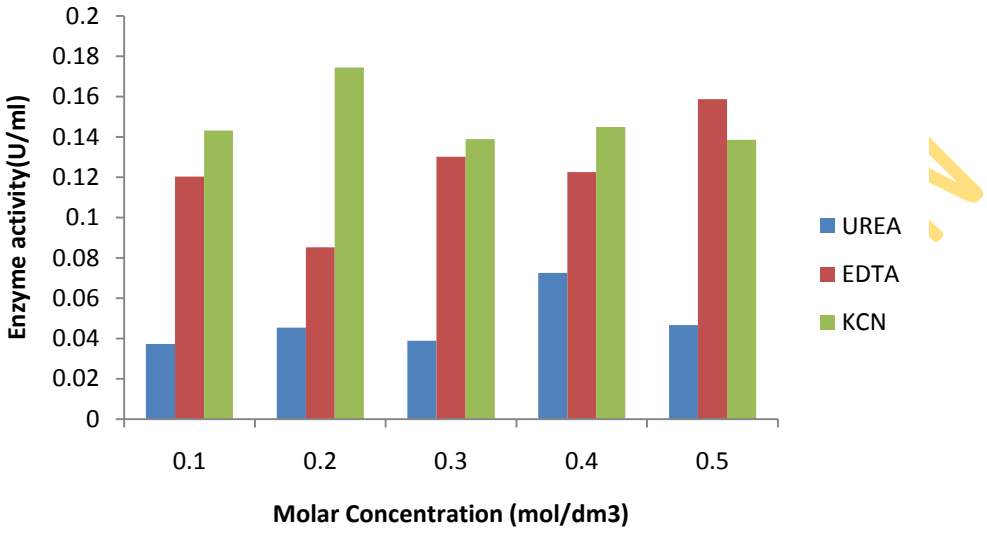


Figure 4.19a: Effect of different molar concentrations of inhibitors on β-galactosidase activity produced by *L. plantarum* (G11)

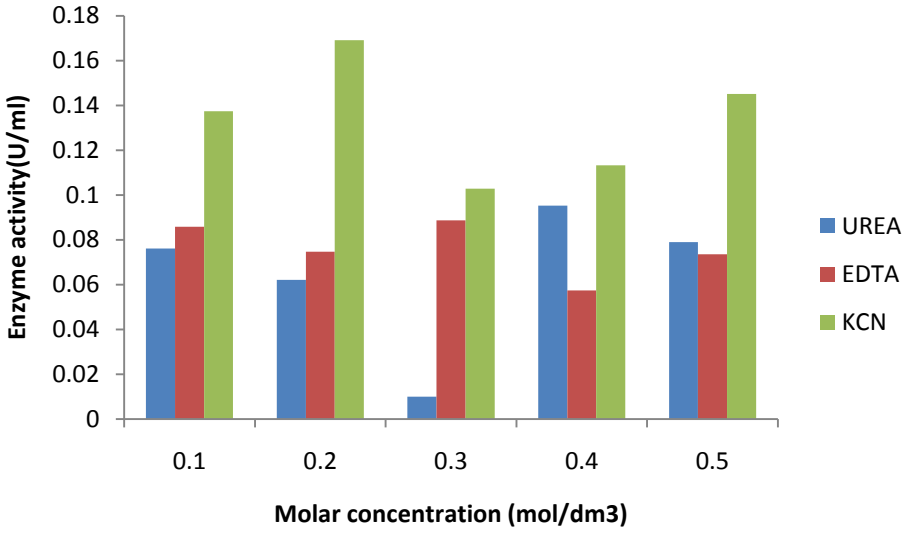


Figure 4.19b: Effect of different molar concentrations of Inhibitors on β-galactosidase activity produced by *L. plantarum* (E13).

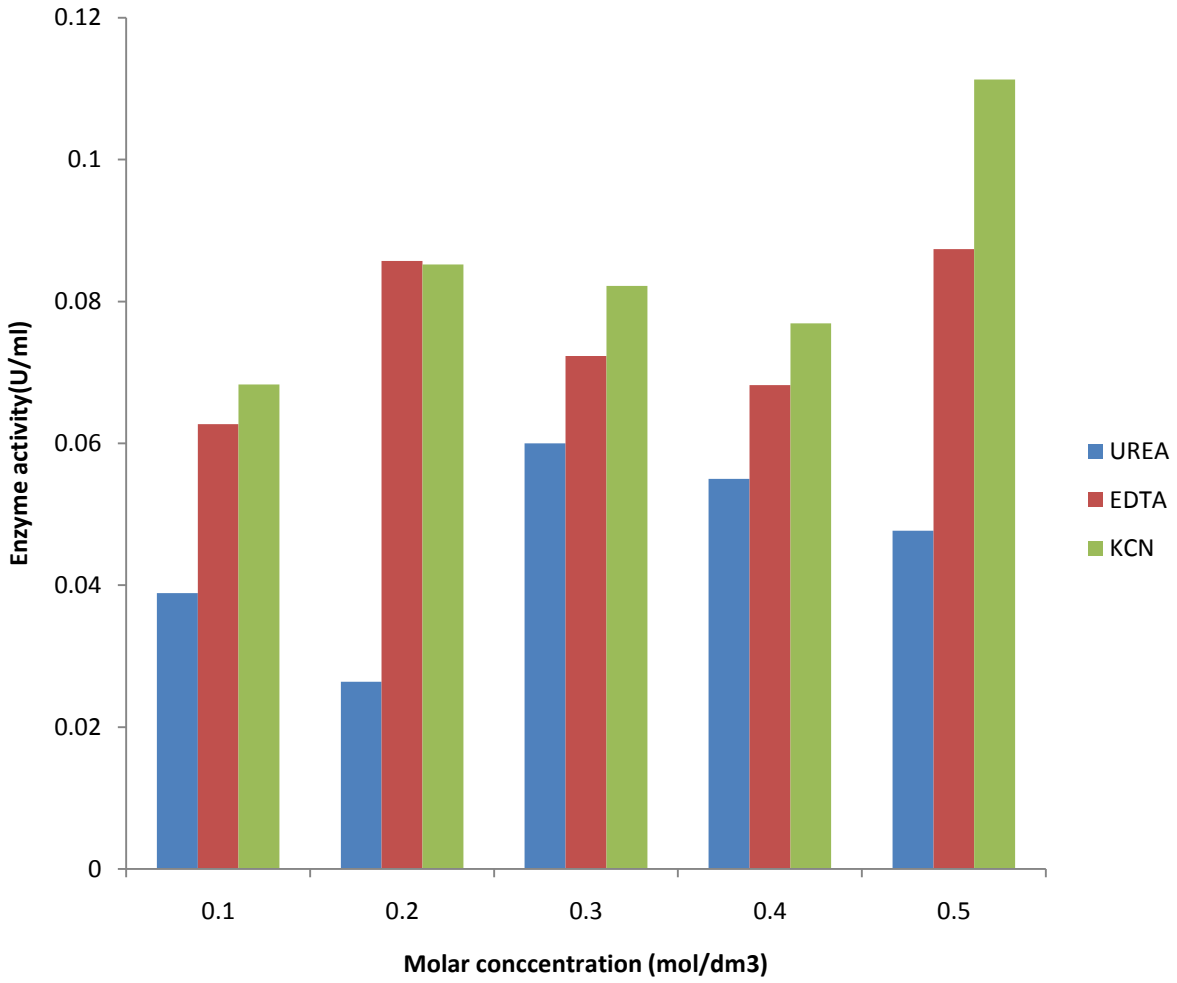


Figure 4.19c: Effect of different molar concentrations of inhibitors on β-galactosidase activity produced *L. plantarum* subsp. *plantarum* (E36).

KCN gave a supportive activity at concentrations tested. However, the best concentration for β-galactosidase activity is 0.5Mmol with equivalent activity of 0.1113U/ml.

Effect of different Substrates and Enzyme concentration

The Michaelis constant (K_m) of the purified enzyme was determined using different concentrations of the enzyme and the substrate. Values were obtained from double reciprocal plotting as described by Lineweaver and Burk (1934). The enzymatic kinetics of the LAB isolates was outlined in Figures 4.20 through. The Michaelis constant for *L. plantarum* (G11) β-galactosidase was determined. V_{max} was found to be 349.8572nkat or 0.35nkat and K_{max} to be 2.887mg/100ml.Michaelis constant for *L. plantarum* (E13) β-galactosidase was found to be 63.784nkat and V_{max} of 1.73882mg/100ml. *L. plantarum* subsp *plantarum* E36 has V_{max} of 40.03894µg/sec and K_{max} of 2.57753mg/100ml (Figure 4.20).

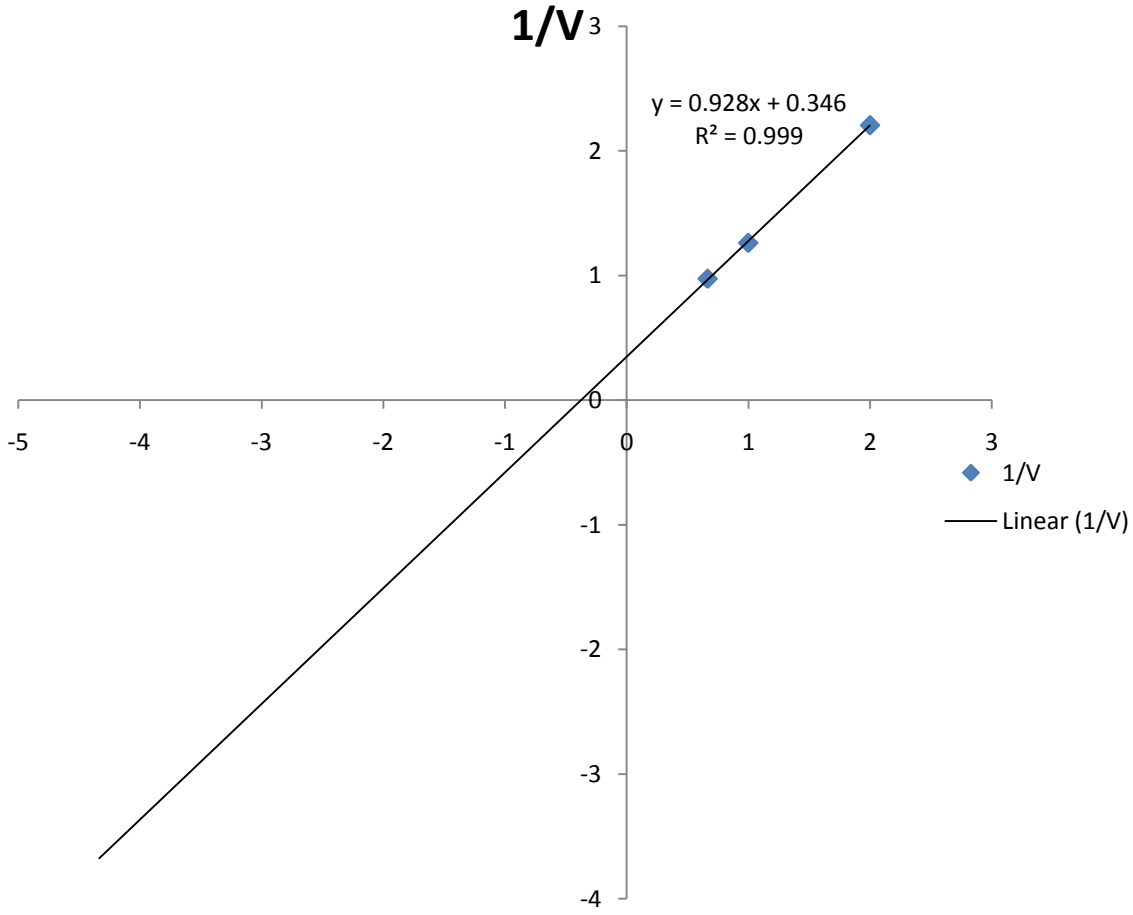


Figure 4.20a: Lineweaver-Burk plot of the enzymatic activity of isolate G11 at different Substrate Concentrations

In mM concentrations, the constant (0.003684uM, same as 3.684nKat) will be used to multiply absorbances equivalent to mg concentrations

V_{max} = **349.8572nKat**

K_{max} = **2.887mg/ml**

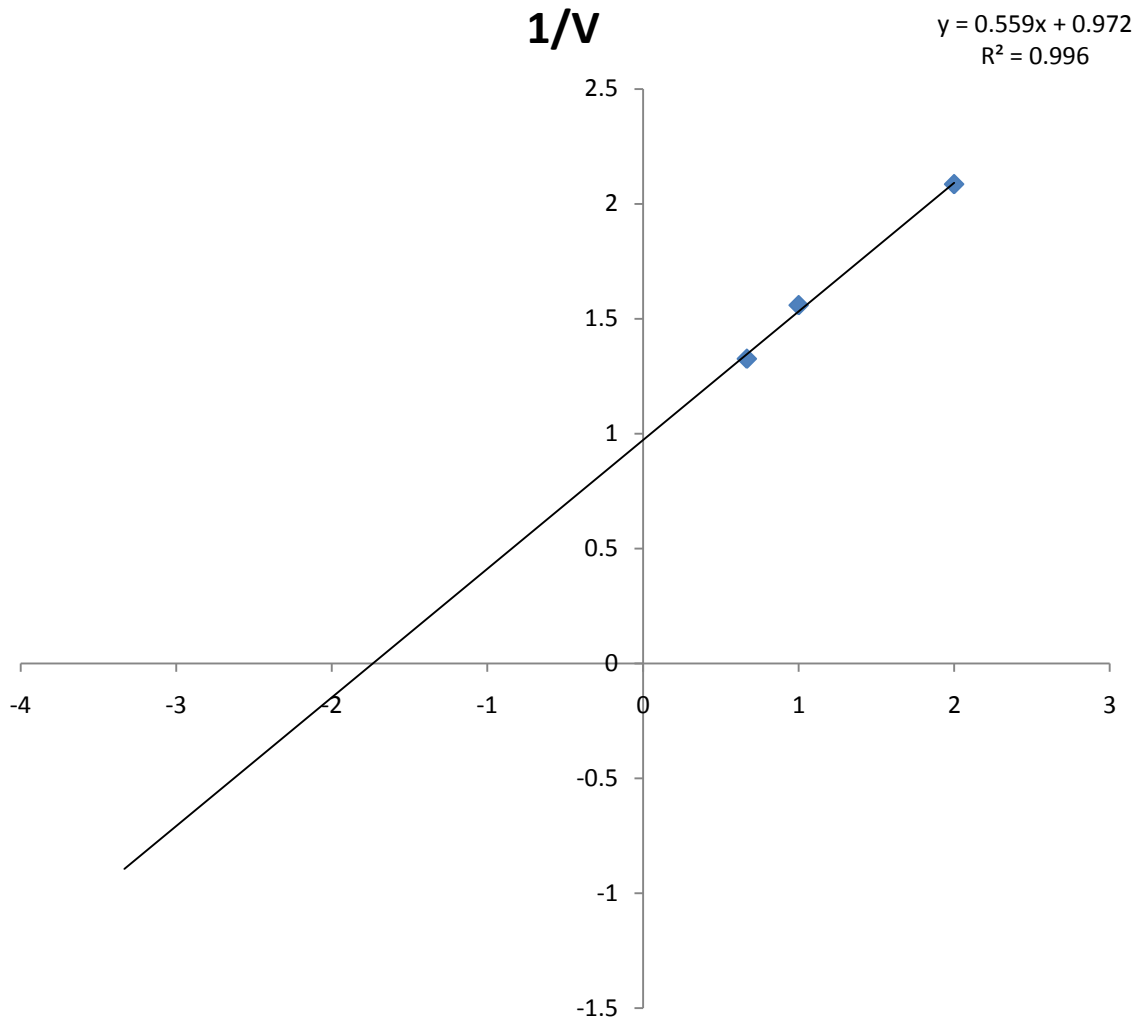


Figure 4.20b: Lineweaver-Burk plot of the enzymatic activity of isolate E13 at different Substrate Concentrations

In mM concentrations, the constant (0.003684uM, same as 3.684nKat) will be used to multiply absorbances equivalent to mg concentrations

V_{max} is equal to 63.784nKat

K_{max} is 1.73882 mg/100ml

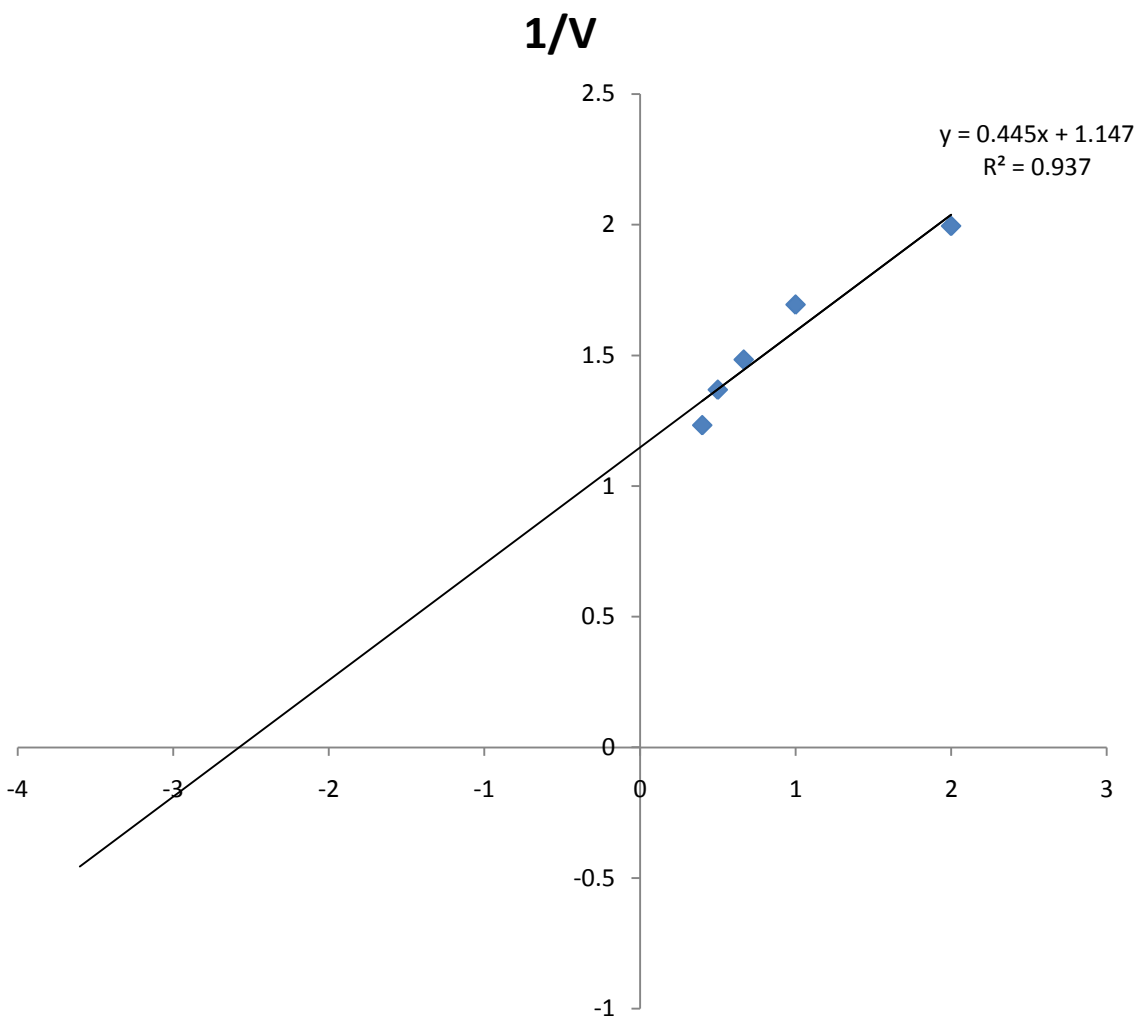


Figure 4.20c: Lineweaver-Burk plot of the enzymatic activity of isolate E36 at different Substrate Concentrations

In mM concentrations, the constant (0.003684uM, same as 3.684nKat) will be used to multiply absorbance equivalent to mg concentrations

V_{max} is equal to **40.03894nKat**

K_{max} is = **2.57753 mg/100ml**

Fractionation patterns of β-galactosidase produced by selected LAB isolates on Sephadex G-100 column Chromatography.

Figures 4.21a shows varied activity peaks from the fractions collected. Figure 4.21b showed the two β-galactosidase activity peaks of *Lactobacillus plantarum* isolate G11. The first major activity peak recorded was from fractions 9-12 which revealed an activity equivalent to 0.0304nMgalactose unit/ml/sec and a corresponding protein peak of 1.599mg **.**The second high peak recorded from fractions 24-27 yielded 0.0313nM galactose unit/ml/sec and 1.679mg protein respectively. Figure 4.21c shows the purification pattern of *L.plantarum* (E13) β-galactosidase. The two distinct peaks recorded were from fraction numbers 8-13 and 19-23 respectively. The first major peak revealed an activity of 0.0296nM galactose unit/ml/sec and a corresponding protein peak of 1.642 mg while the second activity peak revealed an activity equivalent of 0.0057nM galactose unit/ml/sec corresponding to 1.366mg of protein. Figure 4.21d shows the characteristic patterns of β-galactosidase peaks from isolate E36.Peak values from fractions: 12-14 and 20-24 respectively were determined. First peak enzyme activity was 0.2802 nMgalactose unit/ml/sec corresponding to peak protein value of 1.576mg, while fraction points 23 to 27 produced peak enzyme value of 0.2628nM galactose unit/ml/sec and corresponding protein value of 1.9503 mg protein.

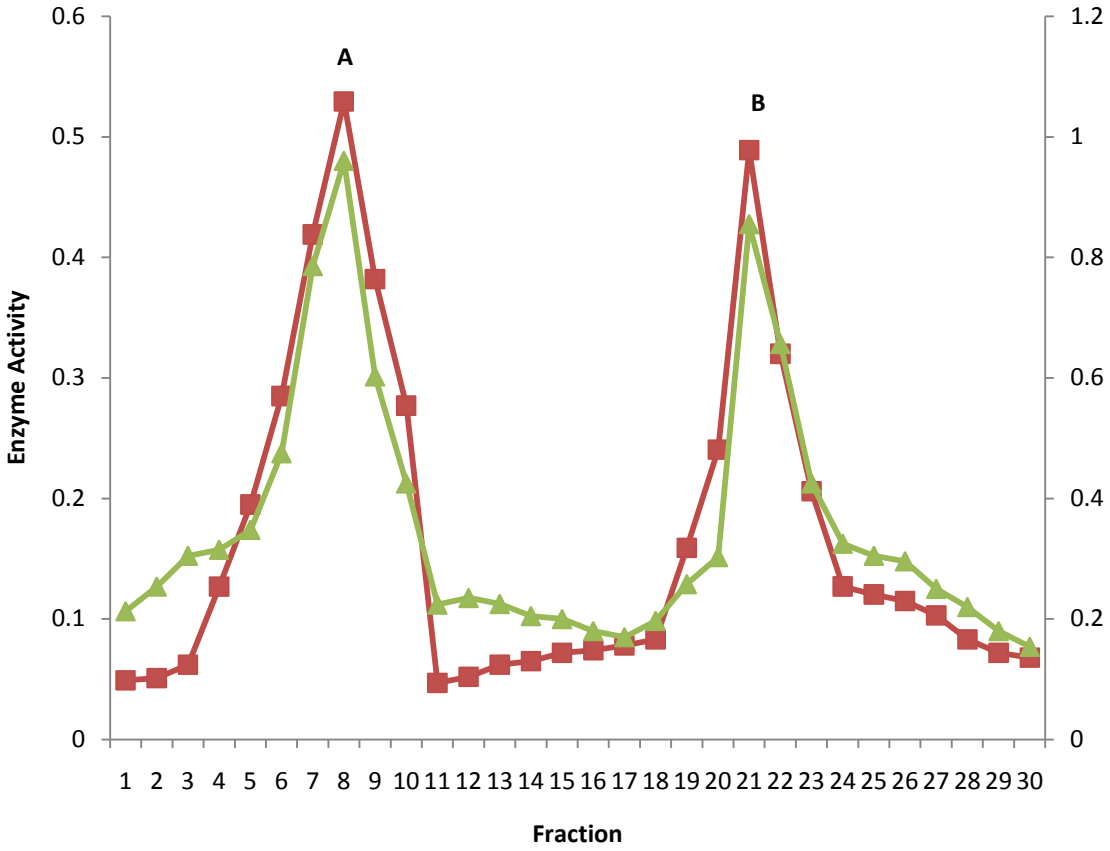


Figure 4.21a: Separation of High molecular weight proteins of *L. plantarum* isolate G11 using Sephadex G-100 and the enzymic activity of the eluants/fractions towards ONPG

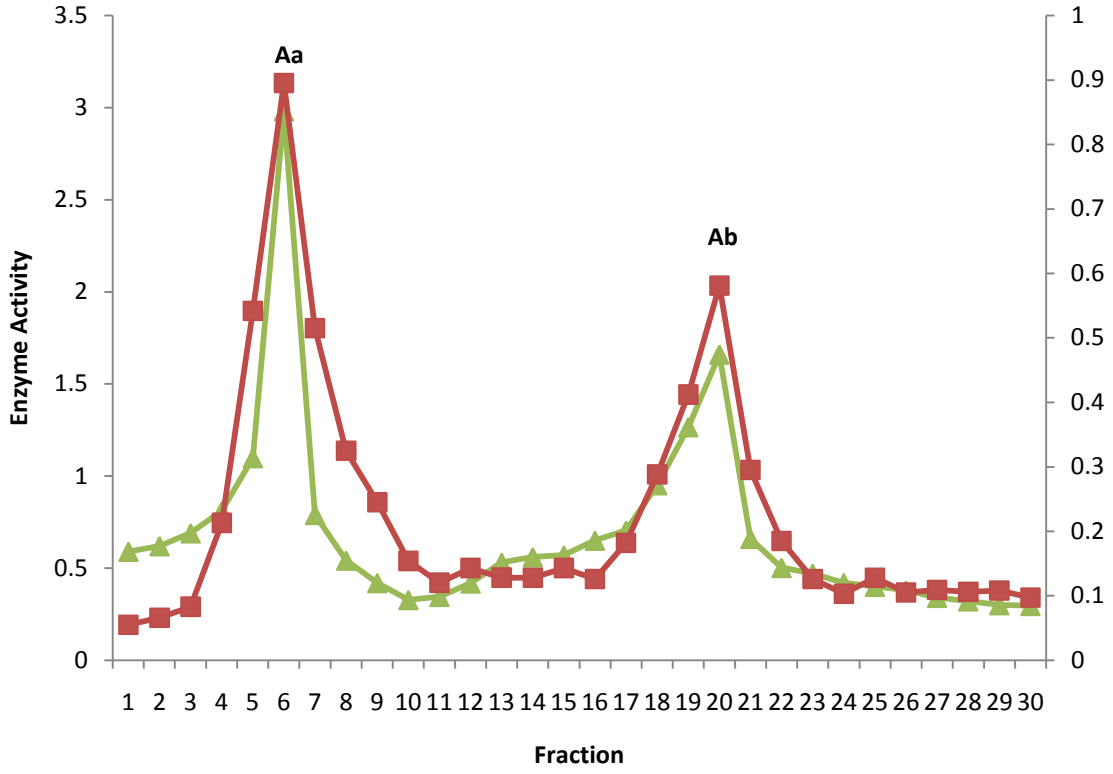


Figure 4.21b: Separation of High molecular weight proteins (Fractions 3-11) of *L. plantarum* isolate G11 using sephadex G-50 by ion-exhcnage and the enzymic activity of the eluants/fractions towards ONPG

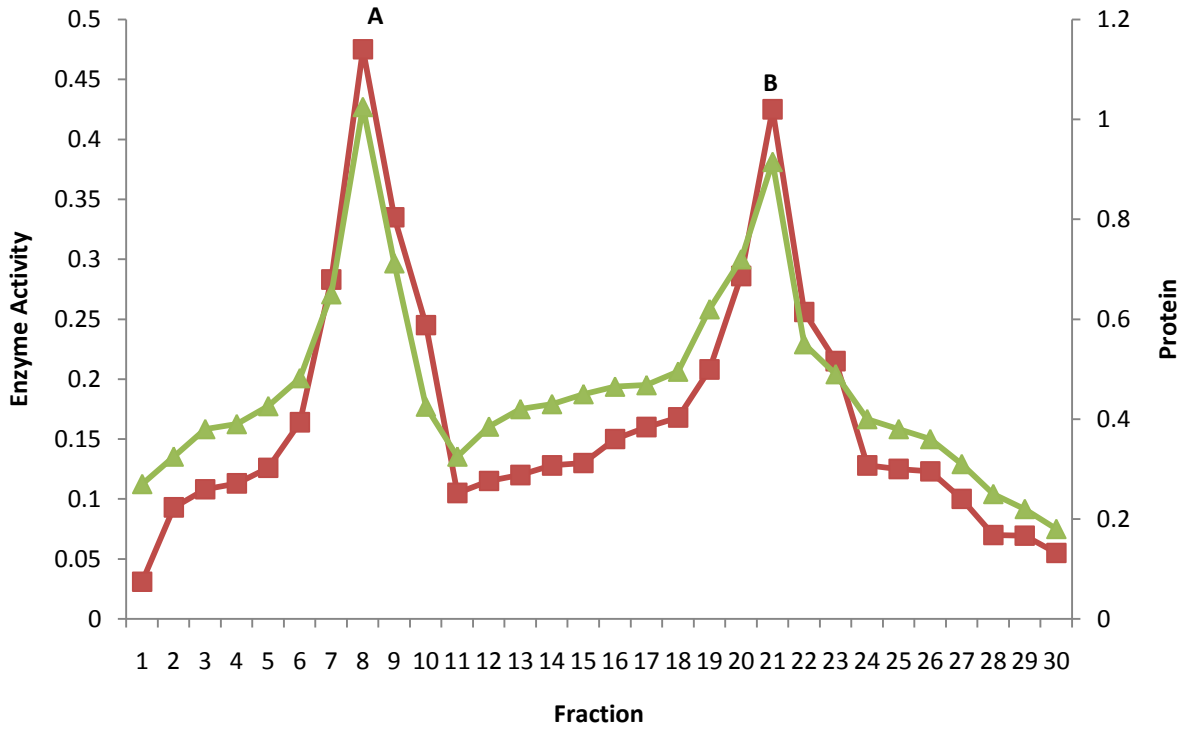


Figure 4.21c: Separation of proteins of *L. plantarum* isolate E13 using sephadex G-100 by ion-exhcnage and the enzymic activity of the eluants/fractions towards ONPG

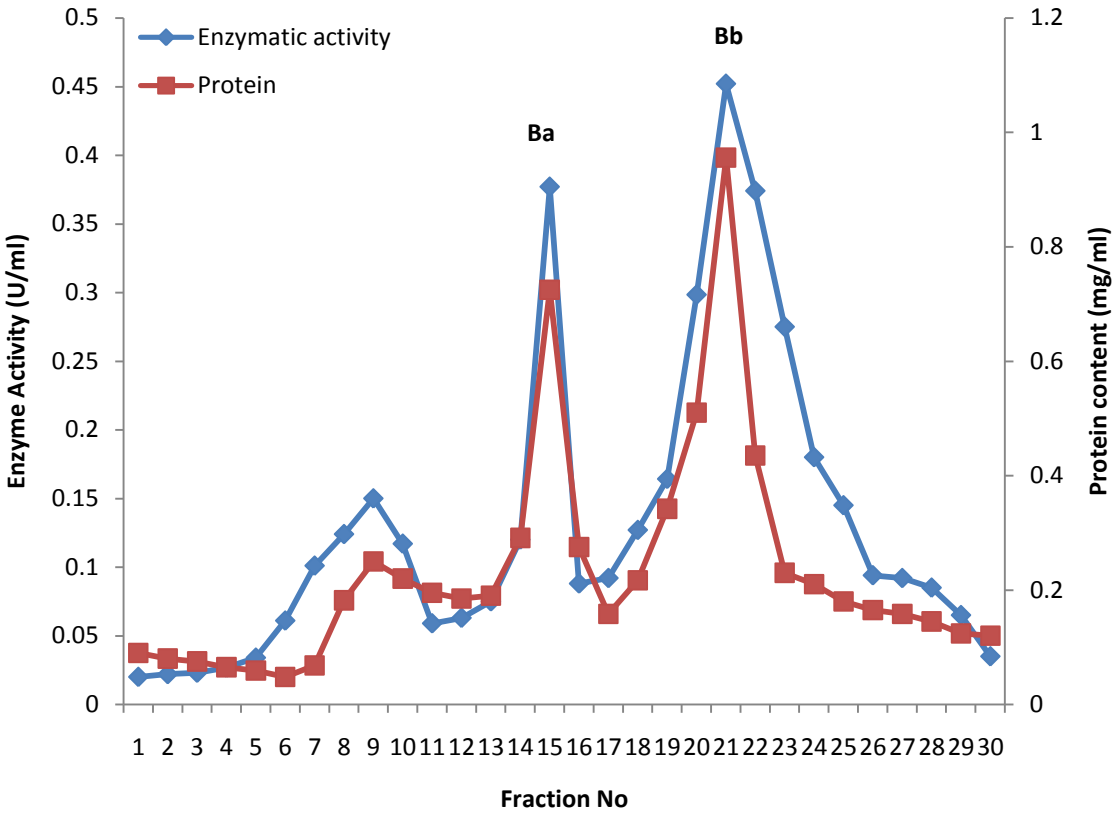


Figure 4.22a: Separation of Low molecular weight proteins (Fractions 5-11) of *L. plantarum*isolate E13 using sephadex G-50 by ion exchange and the enzymic activity of the eluants/fractions towards ONPG.

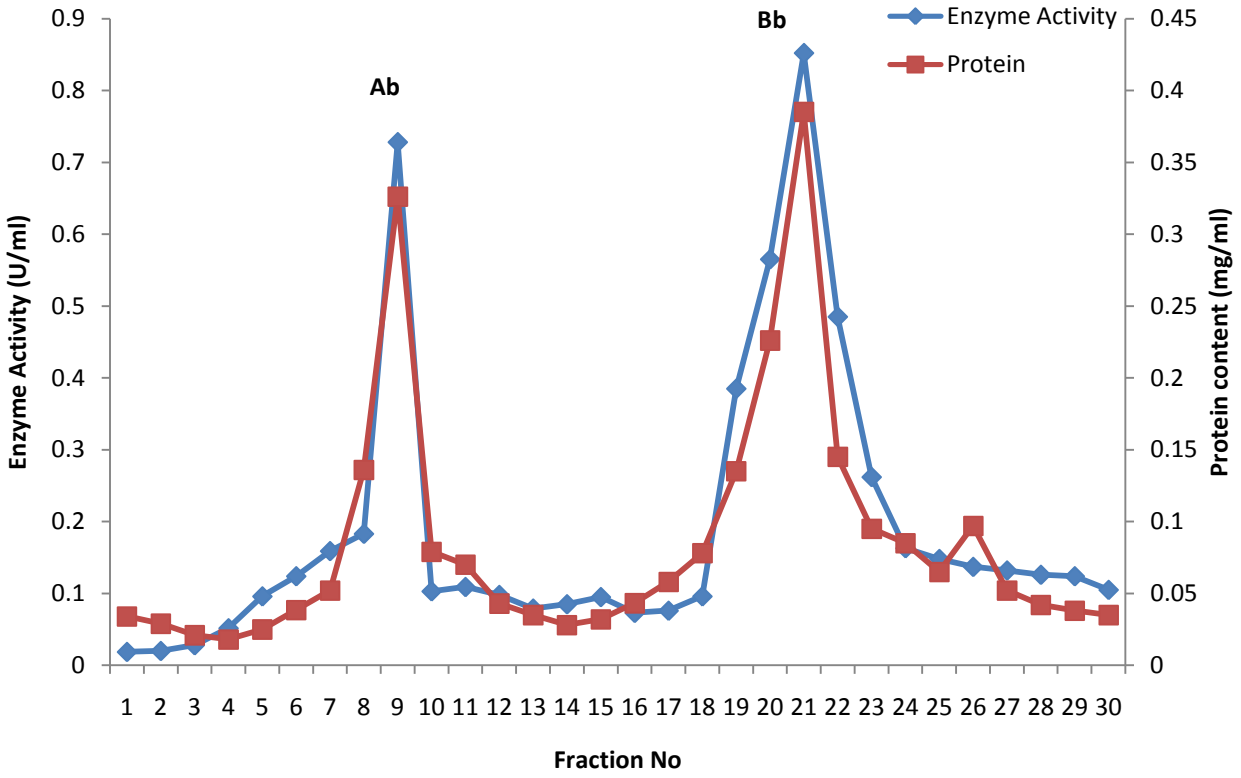


Figure 4.22b:Separation of High molecular weight proteins (Fractions 5-11) of *L.* plantarum isolate E13 by ion-exchange using sephadex G-50 and the enzymic activity of the eluants/fractions towards ONPG

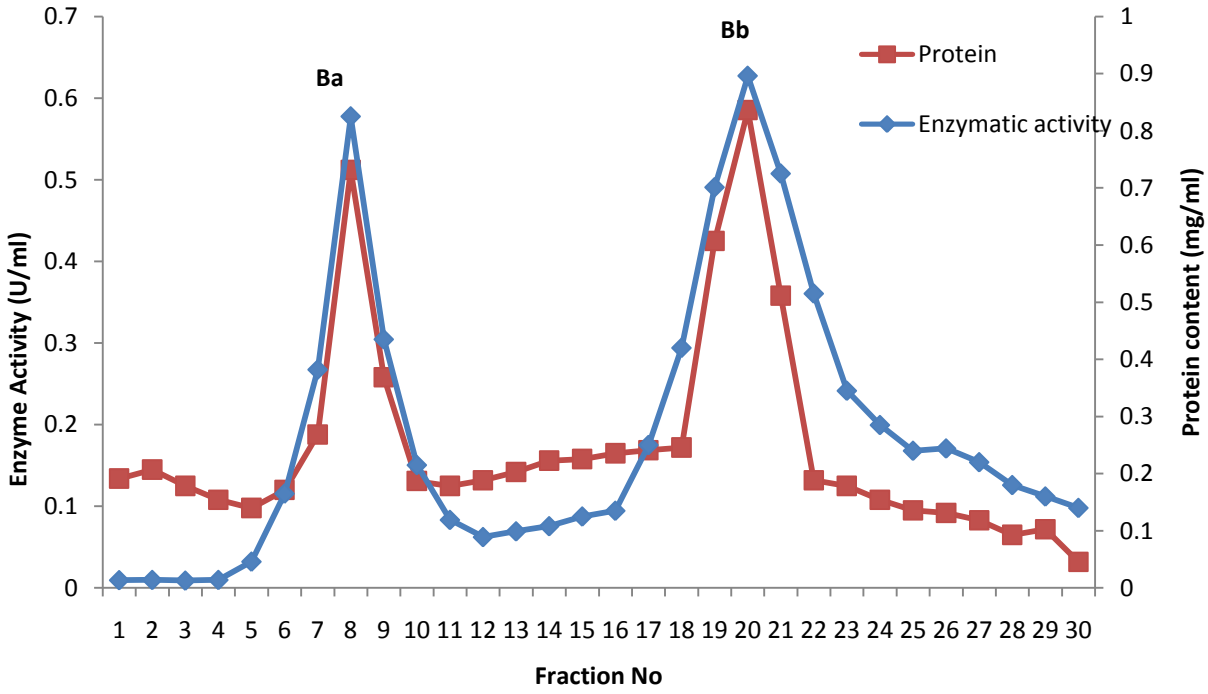


Figure 4.22c:Separation of Low molecular weight proteins (Fractions 17-24) of *L. plantarum* isolate E13 by ion-exchange using sephadex G-50 and the enzymic activity of the eluants/fractions towards ONPG.

Result of hydrolysis experiment

Figure 4.23a shows the percentage lactose hydrolysis by β-galactosidase produced by L.plantarum (G11) at different lactose concentrations. It shows a gradual increase in both glucose and galactose hydrolysis with increase in lactose concentration up to 40%. The highest % hydrolysis (35.8) for glucose was attained at 80% galactose and 19.3 glucose respectively. A sharp decline in glucose hydrolysis at 80% lactose concentration while steady galactose hydrolysis was maintained eith increase in concentration.

Figure 4.23b shows the hydrolytic effect of β-galactosidase produced by L.plantarum (E13) at different lactose concentrations. It shows a sharp increase in glucose hydrolysis at 40%. The highest hydrolytic activity (34.6% hydrolysis) was attained at 60% lactose concentration. This was followed with gradual decrease as lactose concentrations increases. A similar trend in activity was observed with respect to galactose hydrolysis. The highest concentration (20.2% hydrolysis) was achieved at 60% lactose concentration which equally decreases with incease lactose concentration.

Figure 4.23c profiles the hydrolytic effect of β-galactosidase produced by*L.plantarum* subsp. *plantarum*(E36) in which % hydrolysis ranged 13.5- 31.3%. The figure shows a gradual increase in glucose hydrolysis from 0-40% followed by a marginal decline before attaining maximum hydrolysis of 31.3% at 60% lactose concentration. Gradual decrease in glucose hydrolysis as lactose concentration increase. Similar pattern in hydrolytic was observed for galactose with maximum % hydrolysis (16.4%) observed at 60% lactose concentration. This was followed by gradual decrease in hydrolysis as lactose concentration increases.

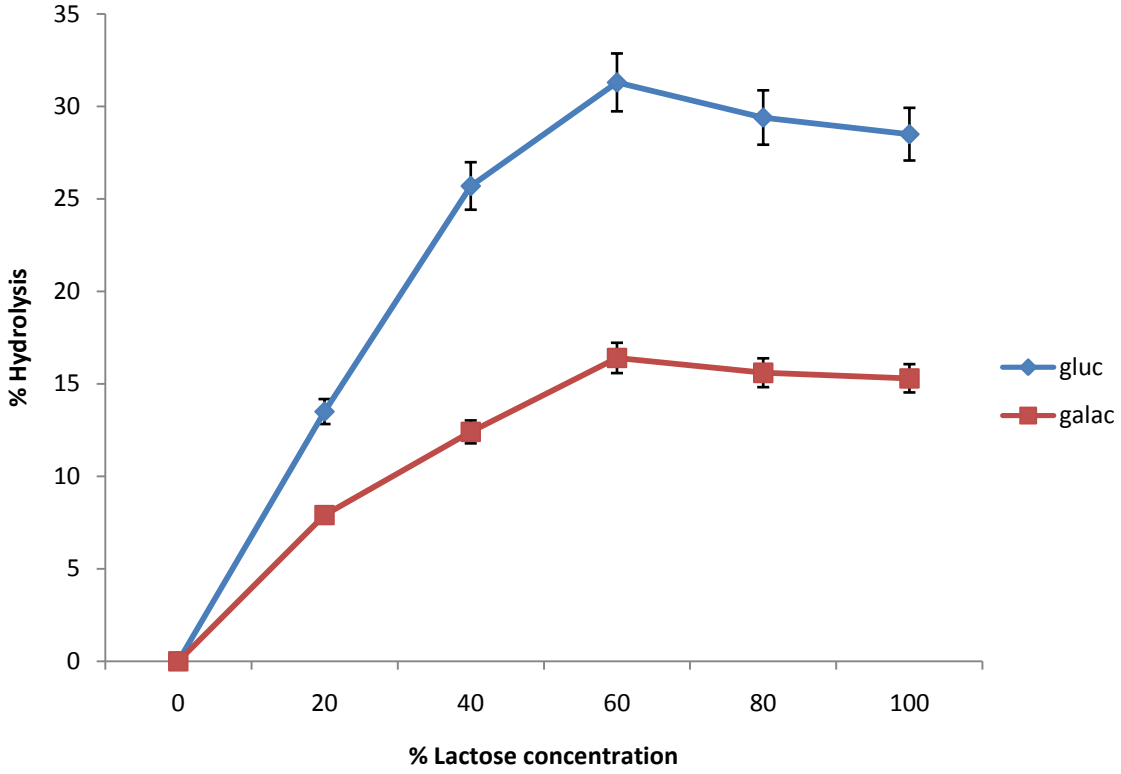


Figure 4. 23a: Percentage Lactose Hydrolysis by *Lactobacillus plantarum* G11 at different Lactose concentrations.

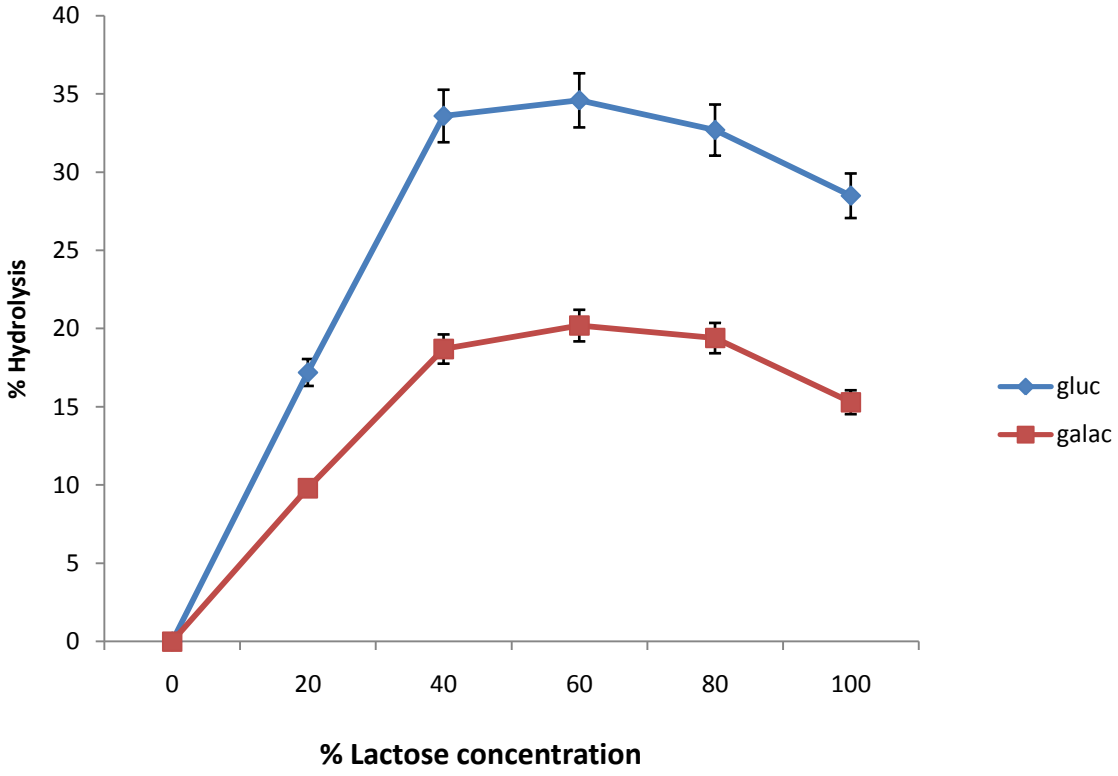


Figure 4.23b: Percentage Lactose Hydrolysis by *Lactobacillus plantarum* E13 at different Lactose concentrations

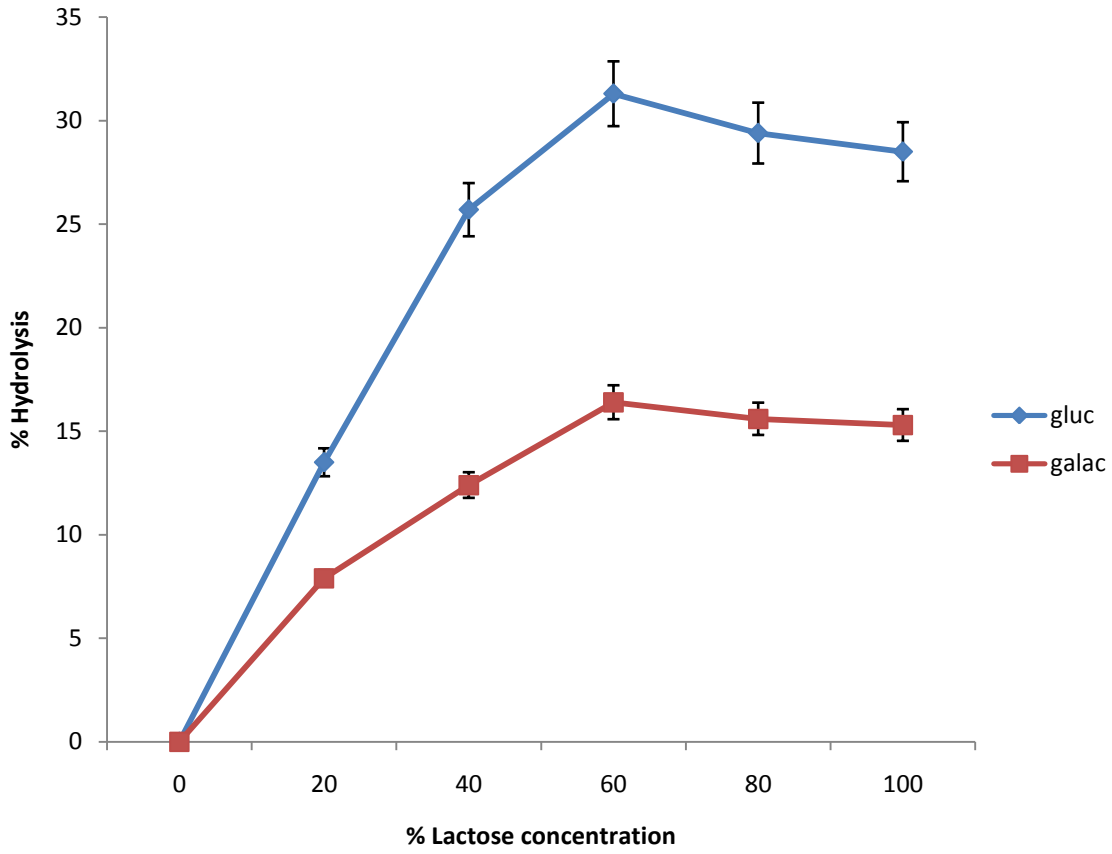


Figure 4.23c: Percentage Lactose Hydrolysis by *Lactobacillus plantarum subspp plantarum* E36 at different Lactose concentrations.

CHAPTER FIVE

5.0 DISCUSSION

Thirty one bacterial isolates were obtained from milk and traditionally fermented milk products. Nineteen out of these tested Gram-positive, Catalase-negative, oxidase-negative and were either rod-shaped or cocci. They were, therefore, classified as lactic acid bacteria based on their morphological, metabolic and physiological criteria (Holzapfel, 1995; Axelsson, 1998; Car *et al*., 2002). Their cultural and biochemical properties agreed with the descriptions of Schlegel (1997) and conformed with Bergey's Manual of Systematic Bacteriology (Sneath, 1986).

The most predominant LAB species isolated from milk and locally fermented dairy products were *Lactobacillus lactis, L.bulgaricus, L.casei, L.fermentum, L. plantarum, L. delbrueckii, L. brevis and Leuconostoc mesenteroides*. This agreed with the findings of Car *et al.* (2002). Lactic acid bacteria are indigenous to milk and milk products. They are hardly found in air or soil (Schlegel, 1997). Their main function in the fermentation of dairy products is to perform acidification of the product by fermentation of lactose into lactic acid (Solem *et al*., 2008). Lactic acid bacteria as a group play significant role in food fermentation processes because of their ability to produce high level of lactic acid as well as been able to survive under high acidic conditions (Wood, 1977). Their usefulness in dairy industry have been widely reported (Shah and Jelen, 1991; 1995; Somkuti *et al.*, 1998; Chammas *et al.* 1999; 2006,) and have been grouped as either homofermentative or heterofermentative (Shuler *et al*., 2003) based on the end products of their fermentation. These parameters are often employed in dairy industry because of the flavour-enhancing aldehyde and diacetyl they produce which are regarded as highly desirable aromatic substances. Various metabolic and enzymatic activities of LAB lead to the production of volatile substances which contribute to the flavor, aroma and in some cases, texture development in fermented dairy products (Sneath, 2002).

Lactic acid bacteria constitute an important group of organisms with beneficial attributes that have been extensively exploited in the preservation and processing of foods such as milk, meat and vegetables. For these purposes, they have a widespread use for their enhancement of nutritional values of foods, retardation of spoilage and reduction of

contamination and lots of other beneficial effects in human health (Mckay and Baldwin, 1990). Lactic acid bacteria which constitute a diverse group of lactococci, streptococci and lactobacilli have been studied intensively with respect to their enzymes for various reasons including their generally recognized as safe (GRAS) status (Stiles and Holzapfel,1997). Previous works on the characterization of enzyme status produced by lactic acid bacteria in the treatment of health- related problems used emerging. Greenberg and Mohoney (1982) had characterized β-galactosidase of yogurt mixed culture consisting *L.delbrueckii subsp. Bulgaricus*and found that they display high activity and stability at temperature above 50^0C and had inferred that such conditions can enhance the rate of lactose hydrolysis.From literature, there is scarce information on previous works in respect of the characterization of β-galactosidase produced by any group of lactic acid bacteria that is indigenous to any of our locally fermented foods.

Lactobacillus plantarum have been the most predominant lactic acid bacteria species implicated in the fermentation of food preparations of different plant origins (Daeschel *et al*., 1987). *Lactobacillus plantarum* requires less complex nutritional requirements compared to other *Lactobacillus* species (Hammes *et al*., 1992). *Lactobacillus plantarum* is a versatile lactic acid bacterium which is encountered in a range of environmental niches including dairy, meat and especially vegetable fermentation (DeVries *et al*., 2006).

The different isolates of lactic acid bacteria obtained for this work is an indication of the diversity of the group that are indigenous to milk and locally fermented milk products. The PCR specific primers were able to identify the selected species of lactic acid bacteria. Blast search analysis using whole 16S rDNA sequence resulted in the identification of the species above 99% level which is close to the considered cut-off value indicating species identity (Stackebrandt and Goebel, 1994). Hence, the genetic method of characterization of the isolates correlated with their biochemical classification.

More recently, thermophilic bacteria have become an object of interest for the commercial production of β-galactosidase (Petzel-bauer *et al*., 1999). Among these, special attention has been paid to lactic acid bacteria (LAB) because of their GRAS status (Stiles and Holzapfel, 1997). Their potential uses as a source of enzyme especially β-galactosidase have been shown to be promising (Murad, 1998)

A number of bacteria have been considered as potential β-galactosidase sources such as *L. lactis, L. acidophilus, L. bulgaricus* (Bury and Jelen, 2000; Vasiljevic and Jelen, 2001; Guimondeetal 2002; Akolkar *et al*., 2005).

Of the nineteen lactic acid bacteria screened for β-galactosidase production, 35% were identified as *Lactobacillus plantarum*, 15% *L. brevis*, 15% *L. casei*, 15% *L. lactis*, 10% are *Leuconostoc lactis*, 5% as *Streptococcus* spp. And another 5% as *Bacillus subtilis*. Six of the *Lactobacillus plantarum* strains showed high production of β-galactosidase under standard assay conditions and were selected for further studies.

Most procedures for assaying β-galactosidase are chromogenic galactosidase. As Wallenfels and Weil (1972) summarized, o-nitrophenyl-B-D-galactopyranoside(ONPG) was described as a sensitive and conversant assay that have been widely accepted and used most extensively.

However, 5-bromo-4-chloro-30indolyl-B-D-galactopra-noside (or X-Gal) was used as substrate for detecting β-galactosidase activity. It is generally used to screen for β-galactosidase activity on solid mediums. Favier *et al.*,1996, first reported a method of detecting bacteria strains with β-galactosidase activity by use of X-gal.He observed that lactic acid bacteria colonies growing on X-gal medium shows green color and are regarded as bacteria with β-galactosidase enzyme (Favier et al. ,1996).Greenish color was accepted as positive β-galactosidase activity. In order to extract intracellular products it is required to select suitable methods to breakdown cellular structures.

It is well known that β-galactosidase from lactic acid bacteria is an intracellular enzyme, and it is not released to the outside of cells under conventional fermentation conditions (Bury *et al*., 2001) Therefore, one should consider several factors during selection of suitable processes. In this study, Isoamyl alcohol and bead meals were used under appropriate laboratory conditionsfor cell disruption and β-galactosidase extraction. Almost all the cell-free extracts of *Lactobacillus* species show high β-galactosidase activity in which *L. plantarum*(G_{11}) produced the highest initial activity of 7.889 μ/ml while the lowest activity (0.2057 μ/ml) was recorded by isolate F_6.

During the study of growth characteristics and β-galactosidase production, the different *Lactobacillus plantarum* strains G11, E13 and E36 were subjected to

fermentations. Obtained data revealed by all the strains under study shows positive correlation between growth and β-galactosidase activity. It was further observed that maximum total β-galactosidase activity corresponds to the late stationary phase of growth for the three strains. This is in line with the findings of Rojaka *et al*.(2003),that there is a proportional relationship presenting growth-associated product formation kinetics. A decline in total enzyme activity was observed after the late stationary phase during the growth experiments. The decline in total enzyme activity could be considered to result from inhibition of cellular functions due to high pH, depletion of a nutritional factor from the growth medium, deactivation of the enzyme due to low pH catabolite repression or/and inducer exclusion. (Firat, 2004). Lactic acid bacteria maintain a cytoplasm that is more alkaline than the growth medium which becomes acidified during growth by secretion of lactic acid (Beshkova *et al*., 2002). Hence, any decrease in cytoplasmic pH below a certain level inhibits cellular functions and the intracellular enzymes can be deactivated (Kashket, 1987). In this study, specific growth rates for the respective isolates as determined from the growth curves were $0.170h^{-1}$, $0.083h^{-1}$ and $0.250h^{-1}$ respectively for *L. plantarum* (G11), *L. Plantarum* (E13) and *L. plantarum* subsp. *plantarum*(E36) respectively. These figures were lower when compared to a study performed by El Demardash *et al*.(2006) using *Streptococcus thermophilus*. (EL Demarclash *et al* . 2006).

Highest enzyme production was achieved at 24 hrs for strains G11, E13 and 30 hrs for E36 as seen in the figures. As stated earlier, positive correlation between growth and enzyme production was observed. The incubation time of 24hrs correspond to the stationary growth phase. This has been stated by many researchers which confirm our findings (Rojakat *et al.*, 2003).

In the comparison of the agitation speed on β- galactosidase enzyme production, definite differences were observed between the strains. For example *Lactobacillus plantarum* (strain G_{11}) agitation at a speed of 60rpm promoted β- galactosidase production (0.184U/ml) more than with no agitation (0.004U/ml) (control). However, for *L. plantarum* strains E13, agitation speed of 120rpm supported maximum activity of 0.184U/ml. While agitation speed of 100 rpm supported maximum activity of 0.186u/ml for*L. plantarum* strain E36. This observation is said to be closely related to the type of

strain and its tolerance to oxygen which is transferred by means of agitation (Beshkova *et al*., 2002). As it is very well known, most lactic acid bacteria are facultative anaerobes with preference for anaerobic conditions. They can, therefore, be differentiated on this basis indeed the effect of agitation cannot be only related to the effect of oxygen. It is also closely related to the nutrient mass transfer which is enhanced with agitation (Beshkova *et al*., 2002). This phenomenon has to do with the nutrient requirements of each strain and the more fastidious characters among them.

During the optimization of the culture medium for β - galactosidase production, the organisms were grown in a temperature range of between 20^{o}C to 45^{o}C. *Lactobacillus plantarum* (strain G_{11}) produced maximum initial β - galactosidase (2.952U/ml) at 20^{o}C followed by a significant decline in enzyme production with further increase in the incubation temperature (figures 4.7a, b,and c). The same trend in the production of β - galactosidase by *L. plantarum* strain E_{13} and *L. plantarum* subsp. *plantarum* (strain E_{36}) were observed with their maximum production (3.947 U/ml) at 25^{o}C and (4.391 U/ml) at 20^{o}C respectively. These results are in contrast to the findings of Hsu *et al*. (2005) who observed that the activity of β - galactosidase by Bifidobacteria increased as the cultivation temperature increased from 22^{o}C to 37^{o}C. Further increases in the cultivation temperature led to a reduction in the final viable population. It also differs from those of Friedurek and Szczodrak (1994) as well as Smith *et al*. (1985)-which demonstrated that the highest β - galactosidase production by *B. longum* was obtained at 37^{0}C. The differences could result from the organisms under study being totally mesosphilic but partially psychrotolerants.

The effect of initial pH on β - galactosidase production by the isolates under study were determined during the optimization studies. A pH range of 5.0 to 8.0 was used. It was found that the highest β - galactosidase production (9.379 u/ml) by *L. plantarum* (strain G_{11}) was at pH 7.5, while pH 6.0 was best (7.799 U/ml) for *L. plantarum* (strain E_{13}) and pH 7.0 for *Lactobacillus plantarum* subsp plantarum (strain E_{36}) with an optimum of 9.549 U/ml .The effect of the initial pH of the medium on enzyme production by *S. thermophilus* was studied over a pH range of 4.0 to 9.0 by Ramana Rao and Dutta (1977) and they reported that maximum enzyme production was observed between pH 6.5 and

7.5. Sridhar and Dutta (1991) worked on the production of β - galactosidase from *Streptococcus cremoris* on whey. They reported that the optimum pH was between 6.5 and 7.0. The result presented by Murad (1998) showed that the highest enzyme production by *L. bulgarious* was obtained at pH 5 while Hsu *et al*, (2005) reported the optimum initial pH for β - galactosidase production by *Bifidobacterium* sp to be 6.5. It is obvious that there is a great similarity in the effect of optimum initial pH (6.0-7.5) on the production of β - galactosidase by the strains under study and those reported previously by Ramana Rao and Dutta (1977), Sridhar and Dutta (1991) and Hsu *et al*. (2005).

The effect of carbon source was determined on the production of β - galactosidase by the isolates in the cultivation medium. Ten sugars were employed in this experiment by using them singly or in combinations. For *Lactobacillus plantarum* strain $G_{11,}$ Raffinose supported the optimum production (5.088 U/ml) of β - galactosidase as a single sugar while mellibiose supported the highest β - galactosidase production for *Lactobacillus plantarum* strain E_{13} and *Lactobacillus plantarum subsp plantarum* strain E_{36}. In a study carried out by Vasiljevic and Jelen (2001), the highest β - galactosidase activity was obtained when unsupplemented skin milk was used as cultivation media (Vasiljevic and Jelen, 2001).

In a study carried out by Hickey *et al.* (1986), they observed a significant decline in β - galactosidase activity of several *Lactobacillus bulgaricus* strains upon addition of small amounts of glucose to the growing culture (Hikey *et al*., 1986). In another study carried out by Smart *et al*. (1993), they found that 19 out of 21 *Lactobacillus* strains contain very low and non-detectable β - galactosidase activity when cultivated on media containing glucose or glucose + lactose (Smart *et al*., 1993). Vasiljevic and Jelen explored this by partial repression of the lac operon caused by glucose presence in MRS. which resulted in lower β - galactosidase activity of the culture. The moderate amount of β - galactosidase activity obtained in this study was comparable to many studies cited in literature (Montanari *et al.,* 2000; Gaudreauer *et al*., 2005) and are very promising for industrial applications.

The effect of nitrogen sources on β - galactosidase production was also determined. It was found that $(NH4)_2$ NO_3 and KNO_3 were best for all the *Lactobacillus*

plantarum under study. Potassium Nitrate supported the optimum β - galactosidase production (4.676u/ml) *Lactobacillus plantarum* strain G_{11} while Ammonium nitrated was best for (7.597U/ml) *Lactobacillus plantarum* strain E_{13} and *Lactobacillus plantarum* subsp plantarum (Srain E_{36}). This agreed with what was reported by Murad (1998) the highest production of β-galactosidase by *L. bulgarcius* was obtained using $(NH_4)_2HPO_4$. He further detected that no activity in the presence of $(NH_4)_2SO_4$. This fact was confirmed by Selvin and EL- Divary (1985) and Fiedurek and Szczodrak (1994) who reported that some salts such as $(NH_4)_2SO_4$ showed an inhibiting effect on β - galactosidase produced by *K. fragilis.* On the contrary, Rammana Rao and and Dutta (1997) found that the best nitrogen source for the production of β - galactosidase by *S. thermophilus* was peptone, followed by ammonium sulphate. Furthermore, Joker and Karbassi (2009) maximized β - galactosidase production by *L. delbruekii* when grown in permeate-based medium enriched with a combination of yeast extract, whey powder and wheat steep liquor as organic nitrogen sources.

Hydrogen ion concentration (pH) is known to influence the velocity of enzymes-catalyzed reactions. The effect of pH on β-galactrosidase activity produced by the three *Lactobacillus plantarum* strains under investigation showed that optimum pH was attained between pH 6.0 and 7.0. β–galactosidase from the three *Lactobacillus plantarum* strains showed above 80% activity (an average) between the pH ranges. The enzyme was essentially stable (83-95% over the pH range tested from the data, it was revealed that isolate G_{11} had maximum residual activity of 94.8% at pH 7.5 while *Lactobacillus plantarum* strains E_{13} and E_{36} showed highest residual activity of 117.5% and 83.6% respectively at pH 7.0. These findings are in accordance with several earlier studies in literature. In a study performed by Greenberg and Mahoney (1982), optimum pH for β-galactrosidase from S. *thermophilus* was found to be 7.0. The same findings were reported in another study by Kreft and Jelem (2000)

The effect of temperature on activity and stability of β-galactosidase produced from the *Lactobacillus plantarum* strains under study was determined between the temperature range of 20^0C to 75^0C β-galactosidase from *Lactobacillus plantarum* strain G_{11} and E_{13} were found to have optimum temperature of 20^0C,*Lactobacillus plantarum* strain G_{11} and *Lactobacillus plantarum* strain E_{13} retained over 50% of their activity between the

temperature range of 40-60$_0$C while *Lactobacillus plantarum* subs.*plantarum* (strain E_{36}) showed optimum activity only at temperature extremes and at 35^0C. This finding is in contrast to the findings of Shah and Jelen (1991) in which they observed optimum temperature of β-galactosidase from *L.delbruekii* subsp *bulgaricus* 11842 to be between 45-50^{0}C. Thermal stability is the ability of an enzyme to resist thermal unfolding in the absence of substrates (Bhatti *et al.,* 2006). The inactivation of enzyme during heating occurs mostly during the initial stages of mild heating (Yemenicioghi *et al*., 1999; Rodriguez-Lopez *et al.,* 1999; Yemencoglu *et al.,* 2002) and is generally attributed to conformational changes in the enzymes active site (Rodriguez-Lopez *et al*., 1999).

The effect of both monovalent and divalent metal ions on the activity of β-galactosidase was determined. The cations tested are Monovalent chloride salts of Potassium, Calcium, Sodium Ammonium and Sulphate salts of Mg^{2+}, Mn^{2+}, Cu^{2+} and Zn^{2+}. The anions tested are $CuS0_4$, $NaN0_3$, $KN0_3$ and $MnS0_4$. The monovalent chloride salts increased β-galactrosidase activity with increase in concentrations up to 0.4mmol for all the three *Lactobacillus plantarum* strains. Reduced activity was observed when the molar concentration was increased above 0.4mmol. Ammonium and Potassium salts have the best activity on β-galactosidase. Among the anions, $ZnS0_4$ produced the best optima for *Lactobacillus plantarum* (strain G_{11}) for all concentrations, $CuS0_4$ had optimum activity (0.1903U/ml) at 0.03mmol while $KN0_3$ as best at 0.1mmol. In the case of *lactobacillus plantarum* (strain E_{13}), $CuS0_4$ at 0.2mmol was best (0.2032h/ml), $KN0_3$ was best at 0.1mm (0.1033μ/ml) and $CuS0_4$ had optimum activity at 0.4mmol (0.1559μ/ml). This result that the addition of monovalent cations in appropriate concentration, show positive effects on β-galactosidase activity. The divalent ions, Mg^{2+} and Mn^{2+} showed strong activation of β-galactosidase activity.

Our observation of the effects of the various cations are in agreement with the reports on the requirements for movalent and divalent metal ions for optimal activity and stability for a number of different β-galactosidases (Nakayima and Amachi, 1999; Nguyen *et al*., 2006; Nguyen *et al*., 2007a). Ca^{2+} is a known inlibitor of some β-galactosidase (German *et al.,* 1996). Igbal *et al*.,(2011) observed that 10mmol of Cu^{2+}, Zn^{2+} and Fe^{2+} inactivated β-galactosidase activity by 100%, 94% and 66% respectively. Cu^{2+} was reported to have strong inhibitory effect on β-galactosidase activity even at low concentrations. In fact the

effects of these ions were observed to be concentration- dependent. In various studies reported in literature, different results were obtained depending on the ion used and the source of β-galactosidase. The effect of monovalent cations (K+ and Na+) varies with the enzymes from different sources (Jurado *et al*., 2002). For the enzymes from *Streptococcus thermophilus* and with ONPG as substrate, no effect was found in one case (Somkuti and Steinberg, 1979). However, slight activation was observed in another (Rao and Dutta, 1981). Similarly, presence or absence of Mg^{2+} had been shown to display different effects. In a study by Bhowmik *et al.* (1987), β-galactosidase from *L .acidophilus* was stimulated with Mg^{2+} whereas in another study by Itoh *et al*. (1992) it had no effect in the case of *L.kefironofaciens* (Itoh *et al*., 1992).

The results of the purification of β-galactosidase from *Lactobacillus plantarum* strain G11, *Lactobacillus plantarum* E_{13}, and *Lactobacillus plantarum* subsp *plantarum* showed that the elution profile display two β-galactosidase activity peaks with the first peak representing high molecular weight filtrates and the second peak representing low molecular weight proteins. Enzymes purity and efficiency were estimated by polyacrylamide gel electrophorensis under non-denaturing conditions. The specific activity of β-galactosidase obtained from the *Lactobacillus plantarum* strains are 292.5mol/mg, for strain G_{11}, 104.21mol/mg for strain E_{13} and 585.46 mol/mg for strain E_{36} respectively. This result indicated that β-galactosidase from *Lactobacillu splantarum* strains has high affinity for lactose. In this study, K_{max} and V_{max} values were calculated. V_{max} represents the rate at which substrates are converted to products while K_{max} (or Michealis constant) is the concentration that will make the enzyme act at its maximum velocity. Hence the V_{max} for *L.plantarum* strain G_{11} was 349.857 nkat (08 0.35Ukat) while the K_{max} was 2.89mg/100ml. and K_{max} of 1.739mg/100ml. for *L. plantarum* (E13). L. *plantarum* subsp *plantarum* strain E_{36} has it V_{max} as 40.039 nkat and K_{max} of 2.578mg/100ml-respectively.

Shegel (1975) summarized the importance of K_{max} and V_{max} as numerically equivalent to the substrate concentration that yields half-maximal velocity. Therefore, the numerical value of K_{max} is of interest for many reasons but most importantly, since K_{max} is a constant for a given enzyme, its numerical values provides a means of comparative enzymes from different organisms or from different tissues or the same organisms. In this way it can be

determined whether enzymes A is identical to enzyme B, or whether there are different proteins that catalyze the same reaction.

The application of the purified β-galactosidase enzyme on the different concentrations of Lactose shows a maximum percentage hydrolysis of 35.8% glucose and 19.35% galactose was achieved when 2.5ml of the enzyme was applied to 10ml of 80% lactose concentration. Furthermore a 35.35 glucose and 18% galactose was achieved when the same amount of β-galactosidase enzyme was introduced to the same volume of 60% lactose concentration.

Much research is focused upon microorganisms that produce beta-galactosidases with improved quality for production of galacto-oligosachridases. Hsu *et al.* (2007) have studied the production of GOs by transgalactosylation using β-galactosidase of β-longum BCRC 15708. Two types of GOs, tri-and tetrasaccharides, were formed after β-galastosidase action on 40% lactose. Trisaccharides were the major type of GOs formed in general, an increase of the initial lactose concentration in the reaction mixture resulted in a higher GOs production A higher yield of 32.5% (w/w) GOs could be achieved from 40% lactose solution at 45^0C.

In 2008, synthesis of GOs from 36% lactose using a recombinant beta-galactrosidase of β infants in *Pichia pestoris* was investigated. The trangalactosylation ratio reached up to 25.2% with 83.1% conversion of initial lactose and 8.76% monosaccharides.

Igbal *et al.* (2010) have observed that a recombinant beta-galactrosidase from *Lactobacillumsplantarum* has a high transgalactosylation activity and was used for the synthesis of prebiotic Gos. The maximum GOs yield was 41% (w/w) of total sugars at 85% lactose conversion.

Conclusion β-gal's capability has been realized by the plethora of products like biosensors, digestive supplements. Etc. thus research and development of beta-galastosidase founds applications in several industries.

CONCLUSION AND RECOMMENDATION

In this study, *L. plantarum* strains were successfully isolated and identified from raw milk and fermented milk products. The LAB isolates successfully produced β-galactosidase enzyme which was able to hydrolyse lactose sugar to simple sugars (glucose and galactose) in known concentration samples of milk.Percentage lactose hydrolysis which ranged between 31.3%- 35.8% was achieved .This compared favourably with results by other species of *Lactobacillus plantarum* from other regions of Asia. Therefore β-galactosidase from these strains can be subjected to further screening and future development and possibly deployed in the hydrolysis of milk and milk products for possible.utilization by lactose- intolerant people

β-galactosidase enzyme is a very important product to dairy industry and very costly to acquired. It is therefore recommended that more efforts be made in embarking on further search and screening for better β-galactosidase- producing species. Other β-galactosidase producing microorganisms should also be identified for deployment and application in our home grown dairy industries. This will, however, require financial support either governmental or by private organizations interms of sponsorship, laboratory equipments and chemical reagents.

REFERENCES

Adeshina, A.A. 2012. Minister of Agriculture, Federal Republic of Nigeria in FRCN 4:00pm News Discuss.

Ah. J and panesar, P.S. 2002. Whey- an alternative substrate for production of bakers yeast. *Indian Food Industry*, **12**:41-43.

Akane, A. K., Matsubara, H., Nakamura, S., Takahashi, S. and Kimura K. 1994. Identification of the heme compound co-purified with deoxyribonucleic acid (DNA) from blood stain. A major inhibitor of polymerase chain reaction (PCR) amplification. *J. Forensic Sc*. **39**: 362-372.

Akolkar, S.K., Sajgure, A., Lele, S.S., 2005. "Lactose production from Lactobacillus," *World Journal of Microbiology and Bacteriology* , **21:** III9-1122.

Albayrak, N. and Yang, S.T. 2002. Production of galactooligosacharide from lactose by *Aspergillus oryzae* β-galactosidase immobilized on cotton. *Cloth.Biotechnology and Bioengineering*, **77**(1)**:** 8-19.

Altschul S.F., Madden T.L., Schaffer A., Zhang J., Zhang Z., Miller W. and Lipman D.J. 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Research*, **25**: 3389-3402.

Andrightto, R. and pal, D. 2001. Influence of buttermilk solids on the physcio- chemical and sensory properties of market milks. *Asian . J. Dairy Res*. **2**: 129-135.

Anisha, G.S., P. Prema. 2007. Selection of optimal growth medium for synthesis of α-galactosidase from Mangrove Actinomyces. *Indian J. Biotechnology* **5**: 373-379.

Anon 1978 Milk Facts. Washington D.C. Milk Industry Foundation.

AOAC (1990). Official Methods of Analysis (15th ed). *Association of Official Analytical Chemists*, Washington, D. C. Pp. 1113.

Arol, H., and Tamm, A. 1994. Metabolism of lactose in the human body. *Scandinavian Journal of Gastroenterology*; **202**:21-25.

Arola, A. and Temm, B. C. 1994. Lactose/whey utilization and ethanol production by transformed *Saccharomyces cerevisiae* cells. *Biotechnology and Bioengineering*; **39**:799-805.

Artolozaga, M. J., Jones, R., Schneider, A. L., Furlan, S. A. and Carvalho-Jones, M. F. 1998. One step partial purification of β, D-galactosidase from *Kluyveromyces marxianus* CDB002 using steamline-DEAE. *Bioseparation*; **7**:137-143.

Aworh, O. C. 1987. Upgrading traditional techniques of food processing and preservation cheese making in University of Ibadan, Ibadan, Nigeria. *West African Journal of Archaeology*; **20**.

Aworh, O. C. and Egounlety, N. G. 1985. Preservation of West African soft cheese by chemical treatment. *Journbal of Food Science and Technology*; **52**:189-90.

Axelsson, L. 1998. Lactic acid bacteria: Classification and Physiology. *Lactic acid bacteria: Microbiology and Functional Aspects*. 2nd edition. Marcel Dekker, Inc, New York. Pp.**1**:1-72.

Baran, T. E 1996. Comparison of different methods of Beta-galactosidase immobilization on/in poly (2-Hydroxyethyl methacrylate) supports. Masters Thesis Biochemistry Department of Middle East Techanical University.

Barberries, S. and Gentina, J. C. 1998. Effect of dissolved oxygen level on lactase production by *Kluyveromyces fragilis*. *Journal of Chemical Technology and Biotechnology*; **74**:(1) 71-73.

Barnerjee, R., Mukherjee, G. and Patrar, K.C. 2005. Microbial tansformation of tannin-rich substrate to gallic acid through co-culture method. *Bioresource Technology*; **96**(8): 949-953.

Barry, T. Colleran, G., Glennon, M., Dunican, L. and Gannon, F. 1991. The 16s/23s ribosomal spacer region as a target for DNA probes to identify *Eubacteria*. *PCR Methods Applied*; **1**:51-56.

Bayles, T. M., Paige, D. M. and Ferry, G. D. 1971. Lactose intolerance and milk drinking habit. *Gastroenterology*; **60**:605-608.

Becerra, M., Cerdan, E. and Siso, M. G. 1998. *Biological procedures*; **12**:253-256.

Becerra, M., Cerdan, E. and Siso, M. G. 1998. *Biological procedures*; **1**:48-58.

Becerva, M. and Gonzalenz, Siso, M. I. 1996. Yeast β-galactosidase in solid state fermention. *Enzyme and Microbial Technology*; **19**(1): 39-44.

Belem, M. A. E. and Lee, B. H. 1998. Production of bioingredients from *Kluyrevromyces Marxianus* grown on whey: an alternative.*Critical Reviews in Food Science and Nutrition*; **38**:565-598.

Bernstein, S. C., Tzeng, H., and Sisson, D. 1977. The commercial fermentation of cheese whey for the production of protein and/or alcohol . *Biotechnology and Bioengineering*; **7**:1-9.

Berthier, F. and Ehrlich, S. D. 1999. Genetic diversity within *Lactobacillus sake* and *Lactobacillus curvatus* pud design of PCR primers for its edition using randomly amplified polymorphic DNA. *International Journal of systematic Bacteriology*;**49**:997-1007.

Beshkova, B. M, Simova, E. D., Frengova, G. I., Simov, Zh. I., Spasov, Z. N. 2002. Effect of oxygen on batch yoghurt cultures. *World Journal of Microbiology and Biotechnology*; **18**:361-365.

Bhowmik, T., Johnson, M. C. and Ray, B. 1987. Factors influencing synthesis and activity of β-galactosidase in *Lactobacillus acidophilus*. *Journal of Industrial Microbiology*; **2**:1-7.

Boon, M. A., Riet, Vant, K. and Janssen, A. E. M. (2000). Enzymatic synthesis of oligosaccharides product removed during a kinetically controlled reaction. *Biotechnology and bioengineering*; **70**(4): 411-420.

Bradford, M. (1976). A rapid and sensitive method for the quantitation of microorganism quantities of protein utilizing the principle of protein dye binding. *Analytical Biochemistry*; **72**:248-254.

Brandy, D., Marchant, L., Mc Hale, and Mc. Hale, A. P. 1995. *Enzyme Microbiology and Technology*; **17**:696-699.

Braslasiewicz, A. 2001. Fermentation of lactose by yeast cells screening recombinant fungal lactase. *Applied Environmental Microbiology*; **59**:4230-4235.

Bufone, G. J., Demmler, G. J., Schimbor, C. M. and Greer, J. 1991. Improved amplification of cytomegalovirus DNA from urine after purification of DNA with glass beads. *Clinical Chemistry*; **37**:1945-1949.

Buller, H. A. and Richard, J. G. 1990. Lactose intolerance. *Annual Review of Medicine*; **41**:141-148.

Bulut, C., Gunes, H., Okuklu, B., Harsa, S., Kilic, S., Caban, H., Yenidunya, S. and Peyniri, A. F. 2005. Homofermentative lactic acid bacteria of traditional cheese 'comlek peyniri' from coppodocia region III. *J. Dairy Res*. **72**:19-24.

Bury, D. and Jelen, P. 2000. Lactose hydrolysis using a distrupted dairy culture: Evaluation of technical and economical feasibility. *Canadian Agricultural Engineering*; **43**:75-80.

Bury, D., and Jelen, P. 2005. Lactose hydrolysis using a distrupted dairy culture: Evaluation of technical and economical feasibility. *Canadian Agriculture Engineering*; **42**:75-80.

Bury, D., Jelen, P. and Kalab, M. 2001. Distruption of *Lactobacillus delbruekil* ssp. *Bulgaricus* 11842 cells for lactose hydrolysis in dairy products: a comparison of sanication, high pressure homogenization and bead mill innovative. *Food Science and Emerging Technologies*; **2**:23-29.

Calab, E. and Duran, A. Simple and sensitive procedure for screening yeast Mutants that lyse at non-permissive temperatures. *Journal of Bacteriology*; **124**:1604-1606.

Carr, F. J., Frank, J., Chill, D. and Miada, N. 2002. The Lactic acid bacteria: A literature survey. *Critical Reviews in Microbiology*; **28**:281-370.

Casca, B., Mauca De Nadra, M. C., Strasser De Saad, A. M., Pesce De Ruiz Holgado, A. and Oliver, G. 1984. β-Galactosidase of *Lactobacillus* Species. *Folia Microbiology*; **29**:288-294.

Chamberlain, A. 1990. Milk production in the tropics. Longman Science and Technical U.K.

Chammas, G. I., Saliba, R., Corrieu, G. and Beal, C. 2006. Characterisation of lactic acid bacteria isolated from fermented milk "laban". *International Journal of Food microbiology*; **110**:52-61.

Chang, B. S. and Mahoney, R. R. 1989. Purification and thermostability of Beta-galactosidase (Lactose) from an autolytic strain of *Streptococcus salivarus subsp. Thermophitus*. *Journal of Dairy Res.*; **56**(1): 117-127.

Christensen, A. C. 1975. Production and utilization of milk protein products: In development in dairy chemistry. Ed. P.F. Fox. *Elsevier Applied Science*; 369-404.

Church, F. C. and Meyer, S. P. 1980. α-galactosidase from *Pichia guillermondii*. *Mycological*; **72**:279-257.

Clarke, L. H., Davidson, K., Rixon, J. E., Halstead, J. R., Fransen, M. P., Gilbert, H. J., Hazlewood, G. P. 2000. A composition of enzyme aided bleaching of softwood power pulp using combinations of Xylanase, Mannanse.

Cocolin, L., Manzano, M. and Comi, G. 2000. Development of a rapid method for the identification of *Lactobacillus* spp isolated from naturally fermented Italian sausages using a polymerase chain reaction-temperature gradient gel electrophoresis. *Letters in Applied Microbiology*; **30**:126-129.

Corral, E. J., Zayas, J. F. and Yadav, P. L. 2006. Methods in dittering Mechanisms in bitter Peptides of enzymic hydrolysates with exceptionally high content of essentially amino acids. *Agricultural and Food Chemistry*; **26**:742-756.

Cortes, C. E., Messia, M. C. and Candigliota, T. 2005. Assessment of quality and technological characterization of lactose-hydrolyzed milk. *Food Chemistry*; **102**(2):679-684.

Crittenden, R. G. and Playne, M. J. 1996. Production, properties and application of food-grade oligosaccharides. *Trends Food Science and Technology*; **7**:353-361.

Daeschel, M. A., McKenny, M. C. and McDonald, L. C. 1987. Characterization of a bacteriocin from *Lactobacillus plantarum*. *Abstract of the annual meeting of the American society for Microbiology*; **86**:277.

Dahlqvist, A. 1997. The basic aspects of the chemical background of lactose deficiency. *Postgraduate Medical Journal*; **53**:57- 64.

Daniela, T. B. 2000. An efficient two-step purification and molecular characterization of β-galactosidases from *Aspergillus oryae*.

Daniela, T. B., Ivanka, S., Stoyanka, G., Mookambeswaran A. and Vijayalakshmi. 2006. E-mail mookambeswaram Avijayalakshmi.

De Vrese, M., Stegelmann, A., Ritcher, B., Fenselau, S., Laue, C., and Schrezenmeir, J., 2001. Probiotics-compensation for lactose insufficiency."*American Journal of Clinical Nutrition*; **40**:1151-1154.

De Vrese, M., Stegelmann, A., Richter, B., Fenselan, S., Lave, C. and Schrezenmier, J. 2001. Probiotics-compensation for lactase insufficiency. *American Journal of Clinical Nutrition*; **42**:15-95.

De Vries Ronald, P. and Vissier, J. 2001. Aspergillus enzymes involved in degradation of plant cell wall polysaccharides. *Microbiology and Molecular Biology Review*; **65**(4):497-533.

De Vries, W., Kapteijn, W. M. C., Van Der Beek, E. G., and Stouthamer, A. H. 1970. Molar growth yield and fermentation balances of *Lactobacillus casei* L3 in batch culture and in continuous cultures. *Journal of General Microbiology*; **63**:33-45

Devoyod, J. J. 1990. Yeasts in cheese making. In: F. J. Spercer, D. M. Spencer (Eds). *Yeast Technology Springer-Verlag Berlin*. Pp. 228-245.

Dickson, R. C., Dickson, and Markin, L. R. 1979. *Journal of Bacteriology*; **137**:51-61.

Dixon, M. and Webb, E.C. 1971. Enzymes. William Clowes and Son, London, UK. Pp. 950.

Dotta, R., and Henry, M. 2000. Lactic acid recent advances in products processes and technologies: a review. *Journal of Chemical Technology and Biotechnology*; **18**:119-1129.

Driessen, F. M., Ubbels, I. and Stadhouders, J. 1977. Continuous manufacturer of yoghurt I. Optimal conditions and kinetics of the prefermentation process. *Biotechnology and Bioengineering*; **22**:263-266.

Dubois, M., Gilles, K. A. and Hamilton, J. K. 1966. Colorimetric method for determination of sugars and related substances. *Analytical Chemistry*; **28**:350-356.

Ducvet, A., Trani, M. and Lortie, R. 2002. Screening of Various glycosidases for the Synthesis of β- glucoside. *Biotechnology and Bioengineering*; **77**:752-757.

Ekwelor, I. A. and Obera, J. A. N. 2007. Effect of vitamins and bivalent metals on lysine yield in *Bacillus megaterium*. *African Journal of Biotechnology*; **6**(11):1348-1351.

El-Demardash, H. A., Oxman, J., Heller, K. J. and Geis, A. 2006. Yoghrt fermentation at elevated temperatures by strains of *Streptococcus thermophilus* expressing a small heat shock protein application of a two-plasmid system for constructing food grade strains of *Streptococcus thermophilus. Biotechnology Journal*; **1**:398-404.

FAO. 1990. Utillization of Tropical foods: *FAO food and nutrition papers* 47/5, Rome.

Favier, C., Mizon, C., Cortot, A., Clombel, J. F. and Mizon, J. 1996. Differentiation and identification of human faecal anaerobic bacteria producing β-galactosidase (a new methodology). *Journal of Microbiological Methods*; **27**:25-31.

Ferguson, E. J. 1995. Recent progress on research and application of non-digestible galacto-oligosaccharides. *International Dairy Journal*; **7**:80-85.

Fernandes C. F., Shahani, K. M. and Amer, M. A. 1987. Therapeutic role of dietary Lactobacilli and Lactobacillic fermented dairy products. *FEMS Microbiology Reviews*; **46**:343- 356.

Fernandez, J., Andrews, L. and Mische, S. M.1994. An improved procedure for enzymatic digestion of polyvinylidene diflouride-bound proteins for internal sequence analysis. *Analytical Chemistry*; **218**:112-117.

Fiedurek, J. and Szczodrak, J. 1994. Selection of strain culture conditions and extraction procedures for optimum production of β-galactosidase from *Kluyveromyces fragilis. Acta Microbiology Pol.*; **43**:57-65.

Firat, K. 2004. Release and characterization of β-galactosidase *from Lactobacillus plantarum*. An umpublished work, Graduate School of Natural and Applied Sciences Middle East Technical University.

Flata, G.1987. Genetics of lactose digestion in humans. *Advances in Human Genetics*; **16**:1-77.

Fleet, G. H. and Mian, M.A. 1987. The occurrence and growth of yeasts in dairy products. *International Journal of Food Microbiology*; **4**:145-155.

Fleet, G. H. 1990. Yeasts in dairy products a review. *Journal of Applied Bacteriology*; **68**:199-211.

Flores, M. V., Voget, C. E and Artola, R. J. J. 1996. Permeabiliration of yeast cell (*Kluyveremyces lactis*) with organic solvents. *Enzyme and Microbial Technology*; **16** (4):340-346.

Fox, P. F. 1982. Proteolysis in Milk and dairy products. *Biochem.Soc .Trans*.; **10**:280-281.

Frazier, W. C. and Westhoff, D. C. 1978. Lactic acid bacteria. Inc: *Food Microbiology* - TATA MC McGraw-Hill Publishers, New Delhi.

Furlan, S. A., Schneider, A. L. S., Merkle, R. and Carvallo-Jonas, M. F. 2002. Formulation of lactose-free, low-cost culture medium for production of Beta-galactosidase by *Kluyveromyces maxianus*. *Biotechnology Letters*; **22**:589-593.

Garman, J., Coolbear, T. and Smart, J. 1996. The effect of cations and the hydrolysis of lactose and the transferase reactions catalysed by beta-galactosidase from six strains of lactic acid bacteria. *Applied Microbiology and Biotechnology*; **46**:22-37.

Gaudreau, H., Champagne, C. P. and Jelen, P. 2005. The use of crude cellular extracts of *Lactobacillus delbrueckii* ssp. *bulgaricus* 11842 to stimulate growth of a probiotic *Lactobacillus rhamnosus* culture in milk. *Enzyme and Microbial Technology*; **36**:83-90.

Geciova J., Bury D. and Jelen, P. 2002. Methods for distruption of microbial cells for potential use in the dairy industry-review. *International Dairy Journal*; **12**:541-553.

Geciova, J., Giesova, M., Jelen, P. and Plockova, M. 2002. Distruption of *Streptococcus thermophilus* 143 culture by three mechanical methods for increase Beta-galactosidase activity. *Milchwiss*; **57** (a/10):509.

Gekas, V. and Lopez-Leiva, M. 1985. Hydrolysis of lactose. *Process biochemistry*; **2**:2-12.

German, J., Coolbear, T. and Smart, J. 1996. The effect of cations on the hydrolysis of lactose and the transferase reactions catalysed by β-galactosidases from six strains of lactic acid bacteria. *Applied Microbiology and Biotechnology*; **46**:22-37.

Gill, J. S. P. 1994. Quantitative bacteriological survey of milk and milk products. *Indian Journal of Diary Science*; **47**:8.

Gilliland, S. E., and Rich, N. 1996. Technological and commercial applications of lactic acid bacteria: Health and nutritional benefits in dairy products. *Applied Environmental Microbiology*; **49**:377-381.

Giraud, E., Gassoline, L., Marin, B., Parada, J. L and Raimbault, M., 2004. Purification and characterization of an extracellular amylase from *Lactobacillus Plantarum* strains A6.

Greenberg, N. A. and Mahoney, R. R. 1982. Production and characterization of β-galactosidases from *Streptococccus thermophilus. Journal of Food science*; **47**:1824-1828.

Grogan, D. X. 1991. Evidence that β-galactosidase of *Sulfolobus solfatancus* is only one of several activities of thermo-stable β-D-glucosidose II. *Applied Environmental microbiology*; **57**:1644-1749.

Grosova, Z, Rosenberg, M., Godwin, M., Slavikova, L. and Rebros, M. 2009. Production of D-galactose using β-galactosidase and *Saccharomyces cerevisiae* entrapped in poly(vinylalcohol) hydrogel. *Food Chemistry*; **116**: 96-100.

Guamaraes, P., Klein, J., Domingues, L. and Teixeria, J. A. 2005. Fermantation performance of recombinant lactose-consuming flocculating strain. *Brazillian Journal of Food Technology*; 34-39.

Gueimonde, M., Corzo, N., Vinderola, G., Reinheimer, J. and De Los Reyes-Gavilan, C.G., 2002. *Journal of Dairy Research*; **69:**125-137.

Guimaraes, W. V., Dudey, G. L. and Ingram, L. O. 2005. Fermentation of sweet whey by ethanologenic *Escherichia coli*. *Biotechnology and Bioengineering*; **40**:41-45.

Guimares, V. M., Rezende S., de Tavares Morera, M. A., Barros, E., de Gon, E. and Felix, C. R. 2001. Characterisation of α-galactosidase from germinating soybean

seed and their use for hydrolysis of oligosaccharides. *Phytochemistry*; **58**(1):67-73.

Gurr, M. I. 1987. Nutritional aspects of Fermented milk products. *FEMS Microbiology Reviews*; **46**:337-342.

Halm, M. and Olsen, A. 1996. The inhibitory potential of dominating yeasts and moulds in maize fermentation. In: *Traditional Food Processing in Africa.* 3rd Biennial Seminar on African Fermented Food. Eds. Halm, M. and Jakobsen, M. KVL, Copenhagen. Pp. 33-39.

Harrigan, W. F and McCance, M. E. 1966. Laboratory Methods in Food and Dairy Microbiology. Academic press, London, U.K. Pp. 452.

Hastings, J. W., and Holzapfel, W. H. 1987. Conventional taxonomy of lactobacilli surviving radiation of meat. *Journal Applied Bacteriology*; **62**:209-216.

He, T., Priebe, M. G., Zhong, Y., Huang, C., Harmsen, H. J. Rangs, G. C., Antoine, J. M., Welling, G. W. and Vonk, R. J. 2008. Effects of yoghurt and bifidobacteria supplementation on the colonic microbiota in lactose intolerant subjects *Journal applied Microbiology*; **104**(2):595-604.

Heyman, M., and Menard, S. 2002. Review Probiotic Microorganisms: How they affect intestinal pathophysiology.

Heyman, M. B. 2006. The committee on nutrition: lactose intolerance in infants, children and adolescents. *Journal of Pediatrics*; **118**:1297-1286.

Heyman, M. 2000. Effect of lactic acid bacteria on diarrhea diseases. *Journal of American Call. Nutrition*; **19**:137s-146s.

Hickey, M. W., Hillier, A. J., Jago, G. R. 1986. Transport and Metabolism of lactose, glucose and galactose in homofermentaive lactobacilli. *Applied Environmental Microbiology*; **51**(4):825-831.

Holsinger, V. H. and Kligerman, A. E, 1991. Applications of lactose in dairy foods and other foods containing lactose. *Food Technology*; 92-95.

Hsu, C. A., Yu, R. C., and Chou, C. C. 2005. Production of β-galactosidase by bifidobacteria as influenced by various culture conditions. *International Journal of Food Microbiology*; **104**:197-206.

Huber, R. E., Kurz, G. and Wallenfels, K. 1976. A quantitation of the factors which affect the hydrolase and trans-galactosylase activities of Beta-galactosidase (E.coli.) in lactose. *Biochemistry*; **15**(9):1994-2001.

Hughes, D. A. F., Rochat, S. P., Aeschilmann, J. M. and Schifrin, E. J. 1999. Modulation of non-specific mechanisms of defense lactic acid bacteria. *Journal of Dairy Science*; **88**:863-869.

Hughes, R. and Leifson, E. 1953. The taxonomic significance of fermentative versus oxidative metabolism of carbohydrates by various gram negative bacteria. *Journal of Bacteriology*; **66**:24-26.

Hussain, M., Muhammed, T. K., Abdul, W. and Sheikh Ajaz, R. 2008. Technological characterization of indigenous enteroccocal population for probiotic potential. *Pakistani Journal of Botany*; **40**(2):867-875.

Hussain, Q. 2010. β-galactosidase and their potential application; a review critique. *Reviews in Biotechnology*; **30**:41-62.

Ihekoronye, A. T. and Ngoddy, P. O. 1985. Integrated food science and technology for the tropics. London. Macmillan IX. Pg. 386.

Inchaurrondo, V. A., Yantorno, O. M. and Voget, C. E., 1994. Yeast growth and β-galactosidase production during aerobic batch cultures in lactose-limited synthetic medians. *Proceedings in Biochemistry*; **29**:47-54.

Iqbal, S., Nguyen, T. H., Nguyen, T. T., Maischberger, T. and Haltrich, D. 2010. B-galactosidase from *Lactobacillus plantarum* WCFSI biochemical characterization and formation of probiotic galactooligosaccharides. *Carbohydr. Res. Doi*; **10**:028.

Ismail, A. Linder, M. and Ghoul, M. 1999.Optimization of butyl-galactosidase synthesis by β-galactosidases from *Aspergillus oryzae. Enzyme Microbiology and Technology*; **25**:208-213.

Itoh, I., Suzuki, M. and Adachi, S.1982. *Agric. Biol. Chem.*; **46**:899-904.

Itoh, K., Toba, T., and Adachi, S. 1992. Properties of Beta-galactosidase of *Lactobacillus kefirranofaciens* K-1 isolated from kefir grains. *Letters in Applied Microbiology*; **15**:232-234.

Jacobson, R. H., hang, X. J., Dabose, R. F. and Matthews, B. W. 1994. Three dimension structure of β- galactosidase from *E. coli. Nature*; **369**: 761-766.

John, L. 1998. Laboratory Manual for food Microbiology Laboratory. Published by University of Wisconsin-Madison.

Jokar, A. and Karbassi, A. 2009. Determination of proper conditions for the production of crude β-galactosidase using *Lactobacillus delbruekii* subsp. *bulgaricus. Journal of Agricultural Science and Technology*; **11**:301-308.

Jurado, E., Camacho, F., Luzen, G., and Vicaria, J. M. 2002. A new kinetic model proposed for enzymatic hydrolysis of lactose by a Beta-galactosidase from *Kluyveromyces fragilis*. *Enzyme and Microbial Technology*; **31**:309-311.

Kaneko, S. and Hideyuki, K. 2002. Purification and characterization of extracellular β-galactosidases from *Oryzae sativa* (Rice) Cells. *Bioscience, Biotechnology and Biochemistry*; **67**(3):627-630.

Kang, E, C. and Lee, Q. T. 2001. Enzyme technology in food and health Industries. *India Food Industries*; **34**:22-37.

Karasova, P. Spiwok, V. Mala, S., Kralova, B. and Russel, N. J. 2002. Beta-galactosidase activity in psychrotrophic microorganisms and their potential use in food.*Czech. Journal of Food Science*; **20**(20):43-47.

Kengen, S. W. M., Luesink, E. J., Stains, A. J. M. and Zehnderi, A. J. B. 1993. *European. Journal of Biochemistry*; **213**:305-312.

Kewer, B. E., Kapil, V., Dodeja, A. K. and Sarma, S. C. 1995. Lactose manufacturer: A review. *Indian Food Packer*; **3**:51-53.

Kim, H.S., and Gilliland, H. 1983. *Lactobacillus acidophilus* as a dietary adjunct for milk to aid lactose digestion in humans. *Journal Dairy Science*; **66**:959-966.

Kim, J. W. and Rajagospal, S. J. 2000. Isolation and characterization of β-galactosidase from *Lactobacillus crispatus*. *Folia Microbiology*; **45**:29-34.

Kiss, T. and Kiss, L. 1984. Purification and characterization of an extracellular β-D-xylosidase from *Aspergillu scarbonarius. World Journal Microbiology and Biotechnology*; **16**:465-470.

Kligerman, A. E. 1981. Development of lactose-reduced milk products. In *Lactose Digestion*. Eds. D. M. Paige and T. M. Bayless. John Hopkins University Press. Baltimore. Pp. 252-280.

Kocak, H. R. and Zadow, J. G. 1989. The effect of lactose hydrolysis and subsequent low temperature-inactivation treatment on age gelation of UTH whole milk. *Australian Journal of Dairy Technology*; **44**:802-803.

Koizumi, K. T., Tanimoto, Y., Okada, K., Hara, H. O., Fujita, H., Hashimot, S. and Kitahara. 1995. Isolation and characterization of novel heterogenous branched cyclomatto-oligosaccharides (cydodextrin) produced by transgalactosylation with α-galactosidase from coffee bean. *Carbohydrate Research*; **278**:129-142.

Kolars, J. C., Levitt, M. D., Aouji, M. and Saviano, D. A. 1984. Yoghurt: an anti-digesting source of lactose. *New England Journal of Medicine*; **310**:1-3.

Kosikowski, M.1982. Influence of technologically harmful microflora of milk in the development of Bifidobacteria. *International Dairy Congress*; **1**:324.

Kreft, M. E. and Jelen, P. 2000. Stability and activity of Beta-galactosidase in sonicated cultures of *Lactobacillus delbrueckii* ssp bulgaricus 11842 affected by temperature and ionic environments. *Journal of Food Science*; **65**:1364-1368.

Ku, M. and Hang, Y. D. 1992. Production of yeast lactase from sauerkraut brine. *Biotechnology Letters*; **14**:925-928.

Kurby, S. A and Lardy, H. A. 1953, Purification and kinetics of Beta-galactosidase from *E. coli* strains K-12. *Journal of American Chemists*; **75**:890-896.

Le Blank, J. G., Piard, J. C., Sesma, F., Giori, G. S. 2005. *Lactobacillus* fermentation CRL 722 is able to deliver active α-galactosidase activity in the small intestine of rats. *FEMS Microbiology letters*; **248**(2):177-182.

Le, J. S., Yi, S. H., Kwon, S. J. Ahn, C. and Yoo, J.Y. 1997. Isolation, identification, and cultural conditions of yeasts from traditional "Maju" *Korean Journal of Applied Microbiology Biotechnology*; **25**:435-441.

Li, M. C, Zhang, Z., Li and Meng, J. 2005a. The analysis of the biological characters in hexaploid hybrids derived from *Brassia carinata* and *B. rapa*. *Acta Agronomica sinica*; **15:**1579-1585.

Li, X., Quinn, Z. K. and Zhona, X. D. C. 2007. Pilot scale lactose hydrolysis using β-galactosidase immobilized on cotton fabric. *Chemical Engineering and processing*; **46:** 497-500.

Li, Y., Hugenholtz, J., Sybesma, W., Tjakko, A. and Molenaar, D. 2005. Using *Lactococcus lactis* for glutathione overproduction. *Applied Microbiology and Biotechnology*; **67**(1)83-90.

Lifron, G. and Horton, B. S. 2000. Commercial utilization of major milk components in the health and food industries. *Journal of Dairy Science*; **96**:3568-3573.

Liu, D. L., Yao, D. S., Liang, Y. Q., Zhou, T. H., Song, Y. P., Zhao, L. and Ma, L. 2001. Production, purificiation and characterization of an intracellular afllatoxin-detoxifizyme from *Armllariella tabescenes* (E.20). *Food and Chemical Toxicology*; **39**(5):461-466.

Loveland, J., Gutshall, K., Kasmir, J., Prema, P. and Brenchley, J. E. 1994. Characterization of psychrophilic microorganisms producing β-galactosidase activities. *Applied and Environmental Microbiology*; **60**:12-18.

Lowes, K. F., Shearman, C. A., Payne, J., Mackenzie, K., Arhcer, D. B., Merry, R. J. and Gasson, R. J. 2000. Prevention of yeast spoilage in feed and food by the yeast mycocin HMK. *Applied Environmental Mcriobiology*; 1066-1076.

Lowry, O. H. Rosebrough, N. J., Far, A L. and Randall, R. J. 1951. Protein measurement with the folin phenol reagent. *Journal of Biological Chemistry*; **193**:265-275.

Luonteri, E., TEntanen, M.; and Viikari, L. 1998. Substrate specificities of *Penicillium simplicissimum* α-galactosidase. *Enzyme Microbiol. Technol*. **22**: 192-198.

Mahoney, R.R. 1998. Galactosyl-oligosaccharide formation during lactose hydrolysis, a review.*Food Chem*. **63**:147-154.

Mantari,E.B., Kanda, H., Wang, H.L., and warner, K. 2000. Fermentation of blanched been Soymilk with lactic cultures. *J. Food Protect*. **60**:260-265.

Manzanares, P., De Graaff, L. H. and Visser, J. 1998. Characterization of galactosidase from *Aspergillus niger:* Purification of a novel α-galactosidase activity enzyme. *Microbial Technology*; **22**(5):383-390.

Margesin, R. and Schinner, F. 1994. Properties of cold-adapted microorganisms and their potential role in Biotechnology. *Journal of Biotechnology*; **33**:1-14**.**

Marteau, P. R., de Vrese, M., Cellier, C. J. and Schrezenmeir, J. 2001. Protection from gastrointestinal diseases with the use of probiotic. *American Journal of Clinical Nutrition*; **73**:430-436.

McFeters, C. A., Sandine, W. E., Becker, R. R. and Elliker, P. R., 1969. Some factors affecting associations of β-galactosidase from *Streptococcus lactis* 7962 C. M. *Journal of Microbiology*; **15**:105-110.

Mckay, L. L. and Baldwin, K. A. 1990. Application for biotechnology present and future improvements in lactic acid bacteria. *FEMS Microbology Reviews*; **87**:3-14.

Miller, G., Jarvis, J. and Mc Bean, L. D. 1995. Lactose intolerance. *Handbook of Dairy Foods and Nutrition*. C.R.C Press, Boca Raton. Pp.187-220.

Miller, J. H. 1972. Experiments in Molecular genetics.Cold spring Harbour Laboratory, New York. Pp. 352-360.

Mishkini, C. E., Gibson, G. R. and Roberfroid, M. B. 1997. Dietary modulation of the human colonic microbiology: introducing the concept of prebiotics. *Journal of Nutrition*; **125**:1401-1413.

Mital, B. K. and Steinkraus, K. H. 1974. Growth of actic acid bacteria in Soymilk. *Journal of Food Science*; **39**:1018.

Moeini, H., Vallian, S. and Nahvi, I. 2004. Isolation and identification of yeast strains capable of producing single cell protein from whey in co-cultures with *S. cerevisa*e. *Iranian Journal of Biotechnology*; **2**(1):13-18.

Molder, H. W. 1994. Bifidogenic factors-sources, metabolism and applications. *International Dairy Journal*; **4**:383-407.

Montanari, G., Zambonell, C., grazia, L., Benevelli, M. and Chiavari, C. 2000. Release of β-galactosidase from lactobacilli. *Food technology and Biotechnology*; **38** (2):125-133.

Moon, W. J. and Reinbold, G. W. 1976. Commensalism and competition in mixed cultures of *L.bulgaricus* and *S. thermophilus. Journal of Milk and Food Technology*; **39**:337-341.

Moresi, M., Patete, M. and Trunbio, A. 1980. UHT-processed beverage paved way for economic utilization of whey. *Applied Microbiology and Biotechnology*; **31**:495-501.

Moriera, S. R., Schwann, R. F., de Carvalho, E. P. and wheals, A. E. 2001. Isolation and identification of yeast and filamentous fungi from yoghurts in Brazil. *Brazil Journal of Microbiology*; **32**:117-122.

Morita, R. Y. 1975. Psychrophitic bacteria. *Bacteriology Reviews*; **39**:144-167.

Murad, H. A. 1998. Utilization of utrafilteration permeates for production of β-galactosidase from *Lactobacillus bulgaricus. Milchwissenchaft*; **53**:273-276.

Murray, M. G. and Thompson, W. F. 1980. Rapid isolation of high molecular weight DNA. *Nucleic Acids Research*; **8**:4321-4325.

Nahvi, I. and Moeini, H. 2004. Isolation and identification of yeast strains with high Beta-galactosidase activity from dairy products. *Bacteriology*; **3**(1):35-40.

Nakanya, T. and Amachi, T. 1999. β-galactosidase, enzymology. In: *Encylopedia of Bioprocess Technology Fermentation, Biocatalysis and Bioseparation.* Mc. Hickinger, S. W. Drew. Wiley, New York. Pp.1290-1305

Nakayo, H., Takansishi, S. and Watanaber, Y. 1987. Substrate specificity of several B-galactosidases toward a series of P-1-4-1miked galactooligosaccharides. *Agriculture, Biology and Chemistry*; **51**:2267.

National Academy of Science. 1996. Food Chemical Codex (4th ed.) National Academy Press, Wahsington, D.C. Pp. 802-803.

Naumoff, D. G. 2004. Phylogenetic analysis of ∝-galactosidases of the GH27 family, *Molecular Biology*; **38**:388-399.

Nguyen, T. H., Spechton, B., Yamabha, M., Haltrich, D. and Poterbauer, C. 2007. Cloning and expression of the β-galactosidases genes from *Lactobacillus reuteri* in *Eschericha coli*. *Journal of Biotechnology*; **129**:581-591.

Nguyen, T. H., Splechtna, B., Steinbock, M., Kneifel, W., Lettner, H. P. S., Kulbe, K. D., Haltrich, D. 2006. Purification and characterization of two novel β-galactosidases from *Lactobacillus reuteri*. *Journal of Agriculture and Food Chemistry*; **54:**4989-4998.

Nguyen, T. H., Splechtna, B., Krasteva, S., Kneifel, W., Kulbe, K. D., Divine, C. and Haltrich, D. 2010. Characterisation and molecular cloning of a heterodimeric β-galactosidases from the probiotic strain *Lactobacillus acidophilus* R22. *FEMS Microbiology Letters*; **269**:136-144.

Nielsen, J. W. and Gilliland, S. E., 1992. The lactose hydrolyzing enzyme from *Lactobacillus acidophilus*. *Cultural Dairy Production Journal*; 20-29.

Nielsen, V. H. 1994. Butter utilization of whey. *American Dairy Review*; **36**:8-10.

Nizamudin, A. N. and Suresh, K. B. 2008. Process utilization for preparation of beverages from lactose hydrolyzed whey permeate. *Journal of Food Science and Technology*; **41**:27-32.

Novalini, S., Neuhaus, W., Kulbe, K.D. 2005.A new Innovative process to produce lactose-reduced skin milk.*Journal of Biotech* **119**: 212-218.

O' Mahony, F. 1988. Rural dairy technology experiences in Ethiopia. *ILCA Manual No* **4** Addis-ababa.

Ogushi, S., Yoshimoto, T. and Tsuru, D. 1980. Purification and comparison of two types of β-galactosidases from *Aspergillus Oryzae*. *Journal of Fermentation Technology*; **58**(2):115-122.

Ohtakara, A. and Mitsutomi, M. 1987. Immobilization of thermostable ∝-galactosidae from *Psycnoporus cinnabarinus* on chitosan beads and its application to the hydrolyisis of raffinose in beet sugar molasses. *Journal of Fermentation Technology*; **65**:493-498.

Okuyama, H. 1988. Cold-adapted microorganisms for use in food biotechnology. Eds. R. Margesin and F. Schinner. In: *Biotechnological Applications of Cold-Adapted Organisms*. Springer, Berlin: Pp.101-117.

Olsen, G. J., Ubese, C. R. and Overbeck, R. 1994. The wind of (evolutionary) change: breaking new life into microbial. *Journal of Bacteriology*; **176**:1-6.

Olukoya, D. K., Tichaezek, P. S., Butsch, A., Vogel, R. F. and Hammes, W. P. 1993. Characterization of the bacteriocins produced by *Lactobacillus pentosus* Dk7 from fufu. *Chemisch Microtechnology L: ebeensmith*; **16**:65-69.

Olutiola, P. O. and Cole, O. O. 1980. Extracellular invertase from *Aspergillus flavus*. *Physiologia plantarum*; **50**:26-31

Onishi, N., Yamashiro, A. and Yokozeki, K. 1995. Production of galactoligosaccharate from lactose by *Stengmatomyces elviae* CBS 8119. *Applied Environmental Microbiology*; **61**:4022-4025.

Oyewole, O. B. 1990. Optimization of cassava fermentation for fufu production: Effects of single starter cultures. *Journal Applied Bacteriology*; **68**:49.

Panesar, P. S. R., Panesar, R. S., Singh, J., Kennedy, F. and Kumar, H. 2006. Microbial production, immobilization and applications of β-D-galactosidase. *Journal of Chemistry, Technology and Biotechnology*; **81**:530-543.

Panesar, R. P. S. Panesar, R. S., Singh, J., Kennedy, F. and Bera, M. B. 2007. Production of lactose hydrolyzed milk using ethanol permeabilized yeast cells. *Food Chemistry*; **101**:786-790.

Pariza, M. W. and Johnson, E. A. 2001. Evaluating the safety of microbial enzyme preparation used in food processing: update for a new century. *Regal Toxicol. Pharmol.*; **33**(2):173-186.

Pastor, G. M. and Park, Y. K. 1980. Purification and characterization of ∝-galactosidase from *Scopulariopsie* sp. *Journal of Fermentation Technology*; **58**(1):79-81

Payne, W. J. 1973. Reduction of nitrogenous oxide by microorganism. Academy Press. New York. *Bacteriology Reviews*; **37**:409.

Pinheiro, R., Belo, I. and Mota, M. 2003.Growth and ∝-galactosidase activity in cultures of *Kluyveromyces marxianus* under increased air pressure. *Letter of Applied Microbiology*; **37**:438-442.

Pinthong, R., Macrae, R. and Rothwell, J. 1980. The development of soybean yoghurt. *Journal of Food Technology*; **15**:647-667.

Pivarnik, L. and Rand, A. G. 1992. Assay conditions effect on β-galactosidase activity from *Kluyveromyces lactis*. *Journal of Food Science*; **57**(4):1020-1021.

Powell, H. A., Gooding, C. M., Garrett, S. D., Lund, B. M. and McKee, R. A. 1994. Proteinase inhibition of the detection of *Listeria monocytogenes* in milk using the polymerase chain reaction. *Letters of Applied Microbiology*; **18**:59-61.

Prashanth, S. J. and Mulimani, V. H. 2005. Soymilk oligosaccharide hydrolysis by *Aspergillus oryzae* ∝-galactosidase immobilised in calcium alginate. *Process Biochemistry*; **40**(3-4):1199-1205.

Pray, W. S. 2000. Lactose intolerance: The Norm among the world's peoples. *American Journal of Pharmaceutical Education*; **64**:205-207.

Purohit, J. S., Dutta, J. R., Nanda, R. K. and Banerjee, R. 2006. Strain improvement for tannase production from co-culture of *Aspergillus foetidus* and *Rhizopus oryzae*. *Bioresource Technology*; **97**(6):795-801

Rahim, K. A. A. and Lee, B. H. 1991. Specificity, inhibitory studies and oligosaccharide formation by β-galactosidase from psychrotropic *Bacillus subtilis* KL 88. *Journal of Dairy Science*; **74**:1773.

Ramirez, A. O. and Matheus, N. R. 2003. Production and partial characterization of β-galactosidases from *Kluveromyces lactis* grown in deprotineale whey, *Arch. Latinoam. Nutrition*; **53**:194-201.

Rammania, Rao, M. V. and Dutta, S. M. 1977. Production of β-galactosidase from *Streptococcus thermophilus* grown in whey. *Applied and Environmental Microbiology*; **34**:185-188.

Rao, M. V. and Dutta, S. M. 1981. Purification and properties of beta-galactosidase from *Streptococcus thermophilus*. *Journal of Food Science*; **46**:1419 -1423

Reichelt, J. R. 1983. *Industrial Enzyme*. In Godfrey, R.J. (Ed.), Nature Press, New York, Pg. 138.

Reiter, J. 1991. Small niche, big potential. *Dairy Foods*; 31-32.

Richmond, M. L., Gray, J. I. and Stine, C. M. 1981. β-galactosidase: Review of recent research related to technological application, nutritional concerns and immobilization. *Journal of Dairy Science*; **64:**1759-1771.

Rivero-Urgell, M. A. and Santamaria, O. 2001. Oligosaccharides: Applications in infant food. *Early Human Development (Suppl 2)***65**:43-52.

Roberto, P. E. and Vilott, J. l. 1984. Lowering the Milk lactose content: potential interests and physiological consequences. *Reproduction Nutrition Development*; **12**:182-187.

Rodriguez, A. P., Leiro, R. F., Triko, M. C., Gerden, M. E., Siso, M. I. G. and Bacerra, M., 2006. Secretory and properties of a hybrid *Kluyveromyces lactis-Aspergillus niger* β-galactosidase. *Microbial Cell Factories*; **5**(41):1-13.

Rodriguez-Nogales, J. M. and Delgadillo-Lopez, A. 2006. A novel approach to develop β-galacotosidase entrapped in liposomes in order to prevent an immediate hydrolysis of lactose in milk. *International Dairy Journal*; **16**:354-360.

Roginsky, H. 1988. Fermented milks. *Australian Journal of Dairy Technology*; **7**:39-46

Rohm, H., Lechner, F. and Lehner, M. 1992. Microflora of Austrian natural-set Yoghurt. *Journal of Food Protection*; **53**:478-480.

Rojaka, M. I., Khan, S. and Shalid, R. 2003. Kineteics and regulation studies of the production of β-galactosidase from *Kluyveromyces maxianus* growth on different substrates. *Food Technol. Biotechnol.* **4**(4):315-320.

Romirez-Matheus, A. O. and Rivas, N. 1989. Production and partial characterization of β-galactosidase from *Kluyveromyces lactis* grown in deproteinised whey. *Arch. Latinoam Nutrition*; **53**:194:201.

Rosado, J. L., Solomons, N. W. and Allen, L. H. 1992. Lactose digestion from unmodified low-fat and lactose-hydrolysed yoghurt in adult lactose mal-digesters. *European Journal of Clinical Nutrition*; **46**:61-67.

Rosado, J. L. C., Gonzalez, M. E. and Valencia. 1994. Lactose mal-digestion and milk intolerance: A study in rural and urban Mexico using physiological doses of milk. *Journal of Nutrition*; **124**:1009-1052.

Rosenweig, N. 1969. Adult human milk intolerance and intestinal lactose deficiency: A review. *Journal of Dairy Science*; **52**:585-587.

Roy, I. and Gupta, M. N. 2003. Lactose hydrolysis by lactozyme T M immobilized on cellulose beads in batch and fluidized bed modes. *Process Biochemistry*; **39**:325-332.

Rubio-Texeira, M. 2006. Endless versality in the biotechnological applications of *Kluyveromyces Lac*. Genes. *Biotechnol. Adr*.; **24**:212-225.

Saharie, C. and Friend, H. 1983. Formation of oligosaccharides during enzymatic lactose hydrolysis and their importance in a whey hydrolysis process: part II experimental. *Biotechnology and Bioengineering*; **30**:1026-1031.

Sahi, T. 1994. Genetics and epidemiology of adult type hypolactasia. *Scandinavian Journal of Gastroenterology*; **202** (supplement):7-20.

Sakai, Y., Nakagawa, T., Shimase, M. and Kato, N. 1998. Regulation and physiological role of the DASI gene encoding dihydroxyacetone synthase in the methylotrophic yeast *Candida boidimic*. *Journal of Bacteriology*; **180**:5885-5890.

Salminen, S., Deighton, M. A., Benno, Y. and Gaback, S. L. 1998. Lactic acid bacteria in health and disease. In: S. Saminen, A. Vanwright (eds). *Lactic acid bacteria; microbial and functional aspects* 2nd edition. New York: Marcel Decker Inc. 211-254.

Samelis, J., Tsakelidan, E., Metaxoprulos, J. and Kalantzopoulos, G. 1995. Differentiation of *Lactobacillus sake* and *Lactobacillus curvatus* isolated from naturally fermented Greek dry salami by SDS-PAGE of whole-cell proteins. *Journal of Applied Bacteriology*; **78**:157-163.

Sanaullah, I., Thu-Ha N., Hoang A. N., Tien, T. N., Thomas, M., Roman, K. and Dietmar, H. 2011. Characterization of a Heterodiemetric GH2 β-galactosidase from *Lactobacillus sakei* Lb 790 and formation of prebiotic Galacto-oligosaccharides. *Journal of Agricultural and Food Chemistry*; **59**:3803-3811.

Sanni, A. I. Ahrne, S. and Onilude, A. A. 1995. Production of alpha-galactosidase by *Lactobacillus plantarum* isolated from diverse sources. *Journal of Basic. Microbiology*; **29**(6):427-432.

Santos, A., Ladero, M. and Garcia-Ochoa, E. 1998. Kinetic modeling of lactose hydrolysis by a β-galactosidase from *Kluyveromyces fragilis Enzyme and Microbial Technology*; **22**:(7)558-569.

Sanul, A. R. 1990. Laktaz, Yetmezligi tanisinda degisik laktoz iceren test yemekleri ile alinan. Gastroenterologi Tezi.

Sarova, P. J. 2002. Influence of processins on functionality of milk and dairy proteins. *Advances in Food Nutrition Research*; **53**:1-20.

Saviano, D. A. and Levitt, M. D. (1987). Milk intolerance and microbes containing dairy foods. *Journal Dairy Science*; **70**:397.

Schlegel, H. 1997. *General Microbiology*. Cambridge University Press, Cambridge London. **7**:300-303.

Schneider, A. L. S., Merkle, R., Carvalho-Jonas, R. and Furlan, S. 2001. Oxygen transfer can β-D-galactosidase production by *Kluyveromyces marxianus* using sugar cane molasses as carbon source. *Biotechnology Letters*; **23**(7):547-550.

Seeley, H. W. and Van Demark, P. J. 1972. Microbes in action. *A Laboratory Manual of Microbiology*.2nd Edt. W.A. Freeman and co. San Francisco, USA. Pg. 361.

Seiler, H. and Busse, M. 1990. The yeasts of cheese brines. *International Journal of Food Microbiology*; **11**:289-304.

Selim, M. H. and El-Diwang, A. J. 1985. *Chem. Mikrobiol. Technol. Lesbensm*. **9**:81-86. Cited in Murad, H.A. 1998. Utilization of ultrafilteration permeate for production of β-galactosidase from *Lactobacillus bulgaricus.Milchwissenchaft* **53**: 273-276.

Shah, A. R., Shah, R. K. and Madamwar, D. 2006. Improvement of the quality of whole wheat bread by supplementation of Xylanase from *A. foetidus. Bioresource Technology*; **97**(16):2007-2053.

Shaik, S. A., Khire, J. M. and Khan, M. I. 1977. Production of β-galactosidase from thermophilus fungus *Rhizomucor* sp. *Journal Industrial Microbiology Biotechnology*; **19**:239-245.

Sheikh, S. A., Khire, J. M. and Khan, M. I. 1997. Production of β-galasidase from their mesophilic fungus *Rhizomucor sp. Journal of Industrial Microbiology & Biotechnology*; **19**(4):239-245.

Shibuya, H., Koboyashi, H., Park, G. G., Komatsu, Y., Sato, T., Kaneko, R., Nagasaki, H., Yoshida, S., Kasamo, K. and Kusakabe, I. 1995. Purification and some properties of α-galactosidase from *Penicillin purpurogenum. Bioscience, Biotechnology and Biochemistry*; **59**(12):2333-2335

Shuler, L. M. and Kargi, F. 2003. Bioprocess engineering basic concepts. *Methods in Enzymology*; **182**(3):50-69

Sieber, R., Stransky, M. and De Vrese, M. 1997. Lactose intolerance and consumption of milk and milk products. *Zeitschrift fur Ernahrungswisenschaft*; **36**:375-393.

Silveira, S. T. 2006. Optimization of glucoamylase production by *Aspergillus niger* in solid state fermentation. *Applied Biology and Biotechnology*; **128**:131-140.

Simoon, F. J. 1969. Primary adult lactose intolerance and the milking habit: A problem in biological and cultural interrelations review of the medical research. *American Journal of Digestive Diseases*; **15**:695-710.

Singh, K., and Rachie, P. J. 1988. The utilization of cheese whey and its components. *Advances in Biochemical Engineering Springer Berlin*; **2**:25-60.

Skegel, I. H. 1975. Enzyme Kinetics. 2nd Edition John Wiley & Sons. Inc. Car. Pg. 199.

Smith, P. K., Krohn, R. I., Hermanon, G. T., Maltia, A. K., Gartner, P. H., Provenzano, M. D., Pufimoto, E. K., Goeke, N. H., Olson, B. J. and Klenk, D. C. 1985. Measurement of protein using bicinchoninic acid. *Analytical Biochemistry*; ***150***:76

Sneath, P. H. A. 1986. Endospore-forming gram-positive rods and cocci. In: P. H. A. Sneath, F. G. Priest, M. Goodfellow, G. Todd. (eds.). *Bergey's Manual of Determinative Bacteriology*; **2**:1104-1207.

Sneath, P. H. A. 1986. *Bergey's Manual of Systematic Bacteriology*. Williams and Wilkins, Bathmore. Pp.1075-1079.

Sneath, P. H. A. 2002. Characterization and development of metabolic activities of lactic acid bacteria for application in the dairy sector. *Research Network for Lactic Acid Bacteria.*Montreal(Quebec) H2P2M6 Novalait Inc. 925. Chemin St Louis, Bur 450 Quebec G1KICI.

Solem, C., Koebmann, B. and Jensen, P. R. 2008. The extent of co-metabolism of glucose and galactose by *Lactococcus lactis* changes with the expression of the lacSZ operon from *Streptococcus thermoplilus*. *Biotechnology Applied Biochemistry*; **50**:35-40.

Somiari, R. I. and Balogh, E. 1995. Properteis of an extracellular glucosidase of *Aspergillus niger* suitable for removal of oligosaccharides from cowpea meal. *Enzyme Microbiology* and *Technology*; **17**(4):311-316.

Somkuti, G. A., Dominiecki, M. E. and Stemberg, D. H. 1998. Permeabilization of *Streptococcus thermophillus* and *Lactobacillus delbrueckii subsp. bulgaricus* with ethanol current. *Microbiology*; **36**:202-206.

Somkuti, G. A. and Steinberg, D. H. 1979. β-D-galactosidase galactohydrolase of *Streptococcus thermophilus*: Induction, purification and properties. *Journal of Applied Biotechnology*; **1**:357-368

Sridhar, N. and Dutta, S. M. 1989. Factors affecting production of β-galactosidase of *Streptococcus thermophilus* (H) grown in Whey. *Indian Journal of Dairy Science* **42**(3):538-542.

Stackebrandt, E. and Goebel, B. M. 1994. Taxonomic note; a place for DNA-DNA reassociation and 16SRA sequence analysis in the present species definition in bacteriology. *International Journal of Systematic Bacteriology*; **44**:846-849.

Stred'nsky, M., Tomaska, M., Sturdik, E. and Kremnicky, L. 1993. Optimization of β-galactosidase extraction from *Kluyveromyes marxianus*. *Enzyme Microbiology and Technology*; **15**:1063-1065.

Suarez, F. L., Savaiano, D. A. and Levitt, M. D. 1993. The treatment of lactose intolerance. *Alimentary Pharmacology Ther.*; **9**:389-597.

Surez, F. L., Saviano, D. A. and Levitt, M. D. 1995. A comparison of symptoms after the consumptions of milk or lactose-hydrolysed milk by people with self-reported severe lactose intolerance. *New England Journal of Medicine*; **333**:1-4.

Takenishi, S., Watanabe, Y., Miwa, T. and Kobayashi, R. 1983. Purification and some properties of β-galactosidase from *Penicillium multicolor*. *Agriculture Biology and Chemistry*; **47**(11):2538-2540.

Tamm, A. 1994. Management of lactose intolerance. *Scandinavian Journal of Gastroenterology*; **202** (supplement):55- 63.

Tari, C., Gogus, N. and Tokatli, F. 2007. Optimization of biomas pellet size and polygalacturonase production by *Aspergillus sojae* ATCC 20235 using response surface methodology. *Enzyme and Microbial Technology*; **40**(5)1108-1116.

The Cambrigde World History of Food 2012. Lactose from historical persectives. Eds. F. K. Keneth and C. C. Krimlaild. File///C/Users/Desktop/FUNADE/. html. Retrieved July 2012.

Thigiel, A. A. and Deak, T. 1989. Selection of strains and extraction procedures for optimum production of galactosidase from *Kluyveromyces strains Zbl. Microbiology*; **144**:465-471.

Thompson, J. 1987. Regulation of sugar transport and metabolism in lactic acid bacteria *FEMS Microbiology Reviews*; **46**:221-231.

Thompson, J. 1988. Lactic acid bacteria: Model systems for in vivo studies of sugar transport and metabolism in Gram-positive organisms. *Biochemistry*; **70**:325-356.

Toba, T., Hayasaka, I., and Taguchi. 1990. A new method for manufacture of lactose-hydrolyzed fermented milk. *Journalof Science Food Agriculture*; **52**:403-407.

Troelsen, J. T. 2005. Adult-type hypolactosia and regulation of lactose expression. *Journalof Biochemical and Biophysical Acta*; **1723**:19-32.

Tuohy, K. M., Probert, H. M., Smejkai, C. W. and Gibson, G. R. 2003.Using probiotics and prebiotics to improve gut health. *Drug Discovery today*; **8**:692-700.

Uma, M., Rao, J. L. and Satyanarayana, T. 2007. Improving production of hyperthermostable and high maltose forming∝-amalyse by an extreme thermophile *Geobacillus thermoleovorans* using response surface methodology and its applications. *Bioresource Technology*; **98**(2):345-352.

Vallier, M. J., Gunata, Z. and Barre, P. 1998. Purification, characterization and substrate specificity of a novel highly glucose-tolerant β-galactosidase from *Aspergillus oryzae*. *Applied Environmental Microbiology*; **64**:3607-3614.

Vasiljeric, T. and Jelen, P. 2001. Production of β-galactosidase for lactose hydrolysis in milk and dairy products using thermophilic lactic acid bacteria. *Innovations in Food Science and Emerging Technology*; **2**(1):75-85.

Vasiljevic, T. and Jelen, P. 2002. Lactose hydrolysis in milk as affected by neutralizers used for the preparation of crude β-galactosidases from *L. Bulgaricus* 11842. *Environmental and Food Science Emerging Technology*; **3**(2):75-85.

Vedamuth, R. and Ritcher, R. L. 2001. Microbiological examination of Food. 4th Edition. *American Public Health Association*; 483-485

Vedamuthu, E. R. and Neville, J. M. 1986. Involvement of a plasmid in production of ropiness (Mucoid in milk cultures by *Streptococcus cremoris* NIS). *Applied Microbiology and Biotechnology*; **29**:61-66.

Vedamuthu, E. R. 1982. Fermented milks in economic microbiology. In: *Fermented Foods*; **8**:241-217. Academic Press Inc.

Vesa, T. H., Marteau, P. and Korpela, R. Lactose intolerance. *Journal of American Coll. Nutrition*, M. **19**, 1655-1755.

Viljoen, B. C., Analie, L. H., Bridget, I. and Gabor, P. 2003. Temperature abuse initiating yeast growth in yoghurt. *Food Research International*; **36**:193-197.

Vinderola, C. G and Reinheimer, J. A. 2003. Lactic acid starter and prebiotic bacteria: A comparative *in vivo* study of probiotics characteristics and biological barrier resistance. *Food Research International*; **36**:895-904.

Wallenfels, K. and Malhotra, O. P. 1972. Beta-galactosidase. In: The Enzymes. Ed. P. D. Boyer. Academics Press New York.

Wallenfels, K. and Weil, R. 1972. β-galactosidases. In: The Enzyme. Academic Press, New York, NY. Pg. 617.

Wang, X. D. and Rakshit, S. K. 1999. Improved extracellular transferase enzyme production by *Aspergillus foetidius* for synthesis of Isooligosaccharides. *Bioprocess Engineering*; **20**(5):429-434.

Wang, C. L., Li, D. F., Lu, W. Q., Wang, Y. H. and Lai, C. H. 2004. Influence of cultivating conditions on the galactosidase biosynthesis from a novel strain of *Penicillium sp* in solid state fermentation. *Letters in Applied Microbiology*; **39**:369-374.

Wei, J. C. 1979. *Manual of Fungal Identification*. Shanghai, china: Shanghai Scientific and Technical Publishers.

Welttagen, J. J. and Viljoen, B. C. 1998. Yeast profile in Gouda cheese during processing and ripening. *International Journal of food Microbiology*; **41**:185-194.

Wendoff, W. L., Amundson, C. H., Olson, N. F. and Arver, J. C. 1971. Use of yeast beta-galactosidase in milk and milk products. *Journal of Milk and Food Technology*; **34**:294.

Wood, B. J. B. and Holzapfel, W. H. 1995. The genera of Lactic acid bacteria. Blackie Academic and Professional, Chapman and Hall, NZ. Pp. 1-105.

Yang, S. T. and Silva, E. M. 1995. Novel products and new tehcnologeis for use of a mamiliar carbohydrate, milk lactose. *Journal of Dairy science*; **78**:2541-2562.

Yemenicioglu, A. 2002. Control of polyphenol oxidase in white potatoes by low temperature blanching. *Enro. Food Technology*; **214**:313-319.

Yemenicioglu, A., Ozkan, M. and Cemeroglu, B. 1999. Heat inactivation Kinetics of apple polyphenoloxidase and activation of its latent form. *Journal of Food Science*; **62**:508-510

Zadow, J. C. 1993. Economic considerations related to the production of lactose and lactose-by products. *IDF Bulletin*; **289**:10.

Zekai, T. and Edogan, K. 2003. Physical, chemical, microbiological and sensory characteristics of some fruit flavored yoghurt YYU. *Vet. Fak. Derg*.; **14:**10-14.

Zhou, Q. Z. K. and Chen, X. D. 2001. Effects of temperature and pH on the catalytic of the immobilized Beta-galactosidase from *Klyverumyces lactis*. *Biochemical Engineering Journal*; **9**:33-40.

APPENDIX I

Table 4.1: Percentage of Occurrence

Probable Organisms	Number of Occurrence	Percentage of Occurrence
Lactobacillus plantarum	7.0	35.0
Lactobacillus casei	3.0	15.0
Lactobacillus brevis	4.0	20.0
Lactococcus lactis	2.0	10.0
Leuconostoc lactis	3.0	15.0
Streptococcus spp	1.0	5.0
Total	20.0	100.0

Table 4.10: Growth of G11 in MMRS broth at different pH

pH	Absorbance	OD_{600}	Protein
5.0	2.6422 ^{f}	1.061^{g}	1.096 ^{f}
5.5	2.9053 ^{d}	1.183^{f}	1.128 ^{e}
6.0	2.7792 ^{e}	1.675^{e}	1.153 ^{d}
6.5	2.5955 ^{g}	1.780^{c}	1.553 ^{c}
7.0	4.2653 ^{b}	2.909^{b}	1.935 ^{a}
7.5	9.3790 ^{a}	3.662^{a}	1.902 ^{b}
8.0	4.2099 ^{c}	1.694^{d}	0.329 ^{g}

Table 4.11: Growth of E13 in MMRS broth at different pH

pH	Absorbance	OD_{600}	Protein
5.0	2.7572±0.5 ^{g}	1.541±0.2 ^{g}	0.902±0.2 ^{g}
5.5	3.4523±0.5 ^{f}	1.546±0.2 ^{f}	0.988±0.2 ^{e}
6.0	7.7986±1.0 ^{a}	2.943±0.5 ^{a}	1.410±0.5 ^{c}
6.5	7.1638±1.0 ^{b}	2.923±0.5 ^{b}	1.818±0.5 ^{a}
7.0	4.8051±0.5 ^{c}	2.774±0.5 ^{c}	1.599±0.7 ^{b}
7.5	4.8013±0.5 ^{d}	1.843±0.3 ^{d}	1.311±0.7 ^{d}
8.0	4.1525±0.5 ^{e}	1.633±0.3 ^{e}	0.932±0.2 ^{f}

Table 4.12: Growth of E36 in MMRS broth at different pH

pH	Absorbance	OD_{600}	Protein
5.0	2.6212±1.0 [e]	1.310±0.2 [f]	1.420±0.5 [c]
5.5	3.8533±1.0 [c]	1.990±0.5 [b]	1.804v0.5 [b]
6.0	3.0793±1.0 [d]	1.663±0.5 [d]	1.081±0.5 [e]
6.5	2.5262±1.0 [f]	1.497±0.5 [e]	0.909±0.2 [f]
7.0	9.5492±2.0 [a]	4.214±1.0 [a]	3.578±1.0 [a]
7.5	4.7949±2.0 [b]	1.887±0.2 [c]	1.223±0.5 [d]
8.0	2.3529±1.0 [g]	1.181±0.2 [g]	0.686±0.2 [g]

Table4.7: Enzyme Activity Isolate G11

Time (hrs)	Absorbance	OD_{600}
0	0 [h]	0 [h]
6	2.434±1.0 [f]	0.664±0.2 [e]
12	3.469±1.0 [c]	1.401±0.5 [d]
18	4.495±2.0 [a]	1.626±0.5 [c]
24	3.138±1.0 [d]	1.772±0.5 [b]
30	4.357±2.0 [b]	1.821±0.5 [a]
36	2.888±1.0 [e]	0.654±0.2 [f]
42	1.685±0.5 [g]	0.554±0.2 [g]

Growth Analysis of Isolate G11 in MMRS incubated at 37°C. The following parameters were evaluated; OD600, total enzyme activity.

Table 4.8: Enzyme Activity Isolate E13

Time (hrs)	Absorbance	OD_{600}
0	0 [h]	0 [h]
6	3.954±0.5 [d]	1.026±0.2 [e]
12	3.442±0.5 [e]	1.111±0.2 [d]
18	4.008±1.0 [c]	1.643±0.5 [b]
24	4.942±1.0 [b]	1.889±0.5 [a]
30	4.953±1.0 [a]	1.579±0.5 [c]
36	3.253±0.5 [f]	0.934±0.2 [f]
42	1.964±0.2 [g]	0.912±0.2 [g]

Growth Analysis of Isolate E13 in MMRS incubated at 37°C. The following parameters were evaluated; OD600, total enzyme activity.

Table 4.9: Enzyme Activity Isolate E36

Time (hrs)	Absorbance	OD_{600}
0	0 [h]	0 [h]
6	1.769±0.5 [f]	1.325±0.3 [d]
12	1.418±0.5 [g]	0.872±0.3 [f]
18	5.308±1.0 [c]	1.889±0.1 [c]
24	5.512±1.0 [b]	1.931±0.5 [b]
30	6.223±0.5 [a]	1.995±0.5 [a]
36	2.986±0.5 [d]	0.989±0.3 [e]
42	2.803±0.5 [e]	0.380±0.1 [g]

Growth Analysis of Isolate E36 in MMRS was read at 37°C. The following parameters were evaluated; OD600, total enzyme activity.

SUGAR UTILISATION

Table 4.13 Raffinose utilization by G11

Concentration	Absorbance	OD_{600}	Protein
0.5	3.2628±0.5 [e]	0.813±0.2 [a]	0.612±0.1 [d]
1.0	0.5344±0.1 [f]	0.570±0.2 [b]	0.863±0.2 [a]
1.5	7.2118±2.0 [b]	0.487±0.1 [c]	0.650±0.2 [c]
2.0	5.2671±1.0 [c]	0.426±0.1 [d]	0.764±0.2 [b]
2.5	4.2513±1.0 [d]	0.360±0.1 [e]	0.612±0.1 [d]
3.0	8.1530±2.0 [a]	0.327±0.2 [f]	0.541±0.2 [f]

Table 4.14 Maltose + Galactose Utilization by G11

Concentration	Absorbance	OD_{600}	Protein
0.5	5.0863±1.0 [c]	0.565±0.1 [c]	0.567±0.1 [e]
1.0	4.0862±1.0 [e]	0.774±0.2 [a]	0.770±0.1 [a]
1.5	4.9882±1.0 [d]	0.623±0.2 [b]	0.716±0.2 [b]
2.0	5.8198±2.0 [b]	0.487±0.1 [d]	0.695±0.2 [c]
2.5	3.5846±0.5 [f]	0.264±0.1 [e]	0.637±0.1 [d]
3.0	7.7528±2.0 [a]	0.209±0.05 [f]	0.496±0.1 [f]

Table 4.15 Mellibiose Utilization by E13

Concentration	Absorbance	OD_{600}	Protein
0.5	6.4094±1.0 [a]	0.248±0.05 [f]	0.664±0.1 [d]
1.0	5.7228±1.0 [b]	0.289±0.05 [e]	0.652±0.1 [e]
1.5	3.3327±0.5 [f]	0.428±0.1 [d]	0.747±0.2 [c]
2.0	4.8007±0.5 [d]	0.485±0.2 [b]	0.929±0.1 [a]
2.5	4.1898±0.5 [e]	0.520±0.2 [a]	0.824±0.2 [b]
3.0	4.9300 ±1.0 [c]	0.443±0.1 [c]	0.590±0.1 [f]

Table 4.16 Maltose + Galactose Utilization by E13

Concentration	Absorbance	OD_{600}	Protein
0.5	4.8692±0.5 [c]	1.009±0.5 [c]	0.810±0.2 [d]
1.0	6.2422±0.5 [b]	1.334±0.5 [b]	2.806±0.5 [a]
1.5	6.6428±1.0 [a]	1.890±.0.5 [a]	1.162±0.5 [b]
2.0	4.4923±1.0 [d]	0.312±0.05 [d]	0.109±0.05 [f]
2.5	2.4667±0.2 [e]	0.278±0.05 [e]	0.824±0.2 [c]
3.0	0.7923±0.2 [f]	0.277±0.05 [e]	0.757±0.2 [e]

Table 4.17 Mellibiose Utilization by E36

Concentration	Absorbance	OD_{600}	Protein
0.5	7.3304±0.5 [a]	0.499±0.1 [f]	0.938±0.2 [a]
1.0	6.1876±0.5 [d]	0.854±0.2 [e]	0.866±0.2 [c]
1.5	7.0874±0.5 [b]	0.883±0.2 [d]	0.788±0.1 [d]
2.0	4.4650±0.1 [e]	1.457±0.5 [c]	0.542±0.1 [f]
2.5	3.3185±0.1 [f]	1.496 ±0.5 [b]	0.677 ±0.1 [e]
3.0	6.4490±0.5 [c]	1.749 ±0.5 [a]	0.877±0.2 [b]

Table 4.18 Trehalose + Galactose Utilization by E36

Concentration	Absorbance	OD_{600}	Protein
0.5	7.5984±2.0 [a]	0.595±0.1 [f]	0.798±0.2 [a]
1.0	5.8598±2.0 [c]	0.865±0.2 [e]	0.445±0.1 [c]
1.5	5.1163±1.0 [d]	0.921±0.1 [d]	0.489±0.2 [b]
2.0	4.7607±1.0 [f]	0.942±0.2 [c]	0.374±0.1 [d]
2.5	5.0930 ±1.0 [e]	0.961±0.2 [b]	0.365±0.1 [e]
3.0	6.1552 ±2.0 [b]	1.061±0.5 [a]	0.314±0.1 [f]

Table 1: Effects of varying Substrate Concentrations on β -D-Galactosidase Activity.

Substrate Concentration (%)	**Enzyme Activity (U ml^{-1})**		
	G_{11}	**E_{13}**	**E_{36}**
0.5	0.4534±0.1^{d}	0.4796±0.2^{b}	0.5014±0.1^{f}
1.0	0.7924±0.1^{b}	0.4163±0.2^{c}	0.5904±0.1^{e}
1.5	1.0262±0.2^{a}	0.5452±0.1^{a}	0.6741±0.2^{d}
2.0	0.0522±0.01^{f}	0.2713±0.1^{e}	0.7308±0.1^{c}
2.5	0.4126±0.1^{e}	0.1995±0.05^{f}	0.8112±0.2^{b}
3.0	0.7724±0.2^{c}	0.3475±0.1^{d}	0.9835±0.2^{a}

Table 2: Effect of Varying Enzyme Concentration on β -D-Galactosidase Activity.

Enzyme Concentration (%)	**Enzyme Activity (U ml^{-1})**		
	G_{11}	E_{13}	E_{36}
0.5	0.0327±0.01^{c}	0.02464±0.01^{e}	0.04321±0.01^{e}
1.0	0.01995±0.01^{e}	0.03123±0.01^{c}	0.03382±0.01^{f}
1.5	0.04604±0.02^{b}	0.02794±0.01^{d}	0.05573±0.01^{d}
2.0	0.02453±0.01^{d}	0.0545±0.02^{a}	0.09385±0.03^{a}
2.5	0.06764±0.02^{a}	0.03751±0.02^{b}	0.07009±0.02^{b}
3.0	0.02458±0.01^{d}	0.02492±0.01^{e}	0.06128±0.02^{c}

Table 3: Effect of Thermal Stability on β-D-Galactosidase Activity

Temperature (^{O}C)	**Enzyme Activity (U ml^{-1})**		
	G_{11}	E_{13}	E_{36}
20	0.2839±0.1^{c}	0.3188±0.1^{b}	0.3473±0.1^{b}
25	0.6641±0.2^{a}	0.0496±0.02^{i}	0.0197±0.01^{g}
30	0.1579±0.05^{f}	0.0641±0.02^{h}	0.0012±0.001^{h}
35	0.0194±0.05^{j}	0.0938±0.05^{f}	0.0777±0.01^{d}
40	0.1697±0.05^{d}	0.0818±0.05^{g}	0.0078±0.001^{g}
45	0.1428±0.05^{g}	0.3200±0.1^{b}	0.1326±0.01^{c}
50	0.1606±0.05^{e}	0.1177±0.02^{d}	0.0066±0.001^{g}
55	0.0859±0.05^{i}	0.1006±0.02^{e}	0.0018±0.001^{h}
60	0.1730±0.05^{d}	0.1805±0.02^{c}	0.0644±0.01^{e}
65	0.1025±0.02^{h}	0.0839±0.02^{g}	0.0499±0.01^{f}
70	0.1498±0.02^{g}	0.1022±0.02^{e}	0.0731±0.01^{d}
75	0.4611±0.1^{b}	0.4811±0.1^{a}	0.4304±0.1^{a}

Table 5: Effect of Agitation on β-D-Galactosidase Activity.

Agitation Speed (rpm)	Enzyme Activity (U ml^{-1})		
	G_{11}	E_{13}	E_{36}
0	0.00624±0.001^{f}	0.00476±0.001^{f}	0.0055±0.001^{e}
60	0.18419±0.05^{a}	0.15433±0.02^{de}	0.18227±0.005^{a}
80	0.13919±0.05^{e}	0.15061±0.02^{e}	0.15455±0.02^{c}
100	0.16512±0.05^{c}	0.16994±0.02^{c}	0.18604±0.05^{a}
120	0.17509±0.02^{b}	0.18191±0.05^{a}	0.17786±0.02^{b}
140	0.16499±0.02^{c}	0.17668±0.05^{ab}	0.17369±0.02^{b}
160	0.14025±0.01^{d}	0.17273±0.05^{b}	0.15303±0.02^{c}
180	0.16009±0.05^{c}	0.1744±0.05^{b}	0.09167±0.01^{d}
200	0.16841±0.05^{c}	0.15755±0.02^{d}	0.15889±0.05^{bc}

Table 6: Effect of Temperature on β-D-Galactosidase Activity.

Temperature (^{O}C)	Enzyme Activity (U ml^{-1})		
	G_{11}	E_{13}	E_{36}
15	0.00135±0.001^{f}	0.04711±0.01^{f}	0.06658±0.01^{f}
20	0.10843±0.01^{b}	0.12851±0.05^{c}	0.09661±0.01^{e}
25	0.07578±0.01^{c}	0.11564±0.05^{d}	0.11099±0.05^{d}
28	0.06656±0.02^{d}	0.12272±0.05^{c}	0.12812±0.05^{c}
30	0.10168±0.02^{b}	0.06649±0.02^{e}	0.16806±0.05^{a}
35	0.12274±0.02^{a}	0.14587±0.05^{ab}	0.00511±0.001^{g}
40	0.12528±0.02^{a}	0.15356±0.05^{a}	0.11377±0.05^{d}
45	0.00332±0.001^{e}	0.14017±0.05^{b}	0.13276±0.05^{b}
50	0.00135±0.001^{f}	0.00421±0.001^{g}	0.00396±0.001^{h}

Table 7: Effect of pH on β-D-Galactosidase Activity

pH	Enzyme Activity (U ml^{-1})		
	G_{11}	E_{13}	E_{36}
5.0	0.1349±0.05^{f}	0.1617±0.02^{c}	0.1658±0.05^{c}
5.5	0.1016±0.05^{g}	0.0908±0.02^{f}	0.0848±0.05^{g}
5.8	0.1178±0.05^{g}	0.1524±0.02^{d}	0.1574±0.07^{d}
6.0	0.1825±0.02^{b}	0.1926±0.05^{b}	0.1491±0.07^{de}
6.5	0.1453±0.02^{e}	0.1482±0.05^{e}	0.1551±0.07^{d}
6.8	0.1509±0.05^{d}	0.1527±0.05^{d}	0.1438±0.05^{e}
7.0	0.1520±0.5^{d}	0.2264±0.05^{a}	0.1854±0.05^{b}
7.3	0.0815±0.02^{h}	0.1518±0.05^{d}	0.1624±0.05^{c}
7.5	0.1730±0.05^{c}	0.1486±0.02^{e}	0.1389±0.05^{f}
7.8	0.1423±0.05^{e}	0.2187±0.02^{a}	0.1608±0.02^{c}
8.0	0.2051±0.05^{a}	0.1695±0.05^{c}	0.1903±0.02^{a}

APPENDIX II

Composition of MRS both used for the Cultivation of LAB

Peptone from Casein	10g
Meat extract	8g
Yeast extract	4g
K_2HP0_4	2g
D(+) glucose	1g
Tween ® 80	5.0g
Sodium acetate	5.0g
di-Ammonium citrate	2.0g
$MgSO_4$ 7 H_20	0.2g
Mn $S0_4$ $4H_20$	0.04g
Water	1000ml

pH was measured before autoclaving

Modified MRs broth

All components are same with MRS broth except that 2% lactose was replaced with 2% glucose.

MRS Agar: All components are same with MRS broth except that 15g agar per Liter of de-mineralized water was added to MRS broth solution. It was boiled and autoclaved

Preparation of o-nitrophenol-β-D-galactopyranoside (ONPG) Solution

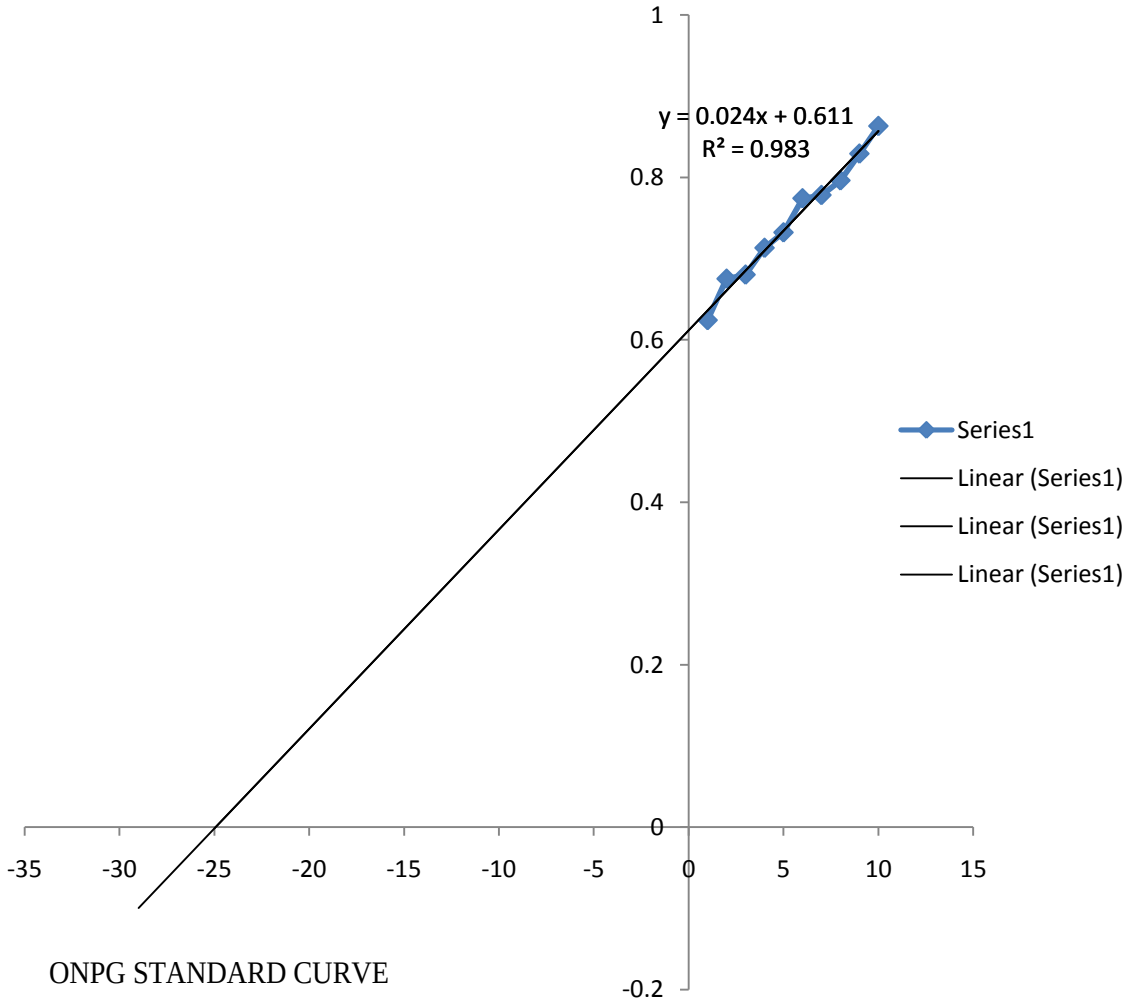
ONPG solutions were prepared by the addtion of solid ONPG to appropriate buffer solution (0.05M sodium phosphate solution) under the assay conditions. ONPG has low solubility, therefore, it should be properly mixed. The solution should be freshly prepared and used.

Molecular weight of ONPG= 301.26g/L.

Sodium Carbonate

0.05m is obtained by dissolving 5.3g in 1000ml deionised water.

MNa_2C0_3 = 106g/L.



ONPG STANDARD CURVE

LIST OF CHEMICALS

Chemicals	Supplier
Tween 80	BDH
5-bromo-4 chloro-3-Indolyl-β-D-galactopyranoside (x-gal).	Sigma
o-nitrophenyl-β-D-galactopyranoside (ONPG)	Sigma
Isoamyl alcohol	Sigma
Sodium chloride	Sigma
Magnesium sulfate	Sigma
Ammonium sulfate	
Potassium Nitrate	
di-sodium Hydrogen sulphate	
Sodium di-hydrogen sulphate	
Manganese sulfate	
Potassium di-hydrogen sulfate	
Sodium carbonate	
Ammonium chloride	
Ammonium nitrate	
MRS agar	
MRS broth	Lab M Limited
Yeast extract	Lab M Limited
Meat extract	Lab M Limited
Peptone from casein	Lab M Limited
Lactose	Lab M Limited
	Lab M Limited

3.5.4

Production of ammonia from peptone

Some bacteria are able to produce deaminase enzymes which enable them to break down peptone or other proteins to release ammonia. This is termed deamination.

Deaminase medium

Peptone water **APPENDIX III**

Peptone 1.0g

Sodium chloride 0.1%

Fermentable sugar 1.0%

Distilled water 100ml

Nitrate medium

Composition and preparation

1. Peptone water plus 0.1% potassium nitrate
2. Distribute the medium into tubes, each with an inverted Durham tube, and sterilize at 121^{0}C for 15mins and allowed to cool before inocubation with the isolates. Uninoculated tudes served as control.

Tubes were incubated at 30^{0}C for 4days. The ability of the isolates to reduce nitrate to nitrite,ammonia or free nitrogen was determined by adding to each tube 0.5ml of 0.6% dimethyl-naphthy lamine in 5.0ml acetic acid. The development of a red coloration indicated a positive result and the presence of gas in the Durham tube indicated the production of nitrogen gas.

Com position growth in 4% NaCl

MRS broth containing 4% Sodium Chloride is used

Arginine broth

For the Identification of some Streptococci (enterococci) and gram negative rods.

Composition

Tryptone 0.5g

Yeast extract 0.2g

K_2PO_4 0.2g

L arginine monohydrate 0.3g

Dextrose 50mg

Distilled water 200ml

Preparation: 1. Suspend the component in the water and heat to dissolve totally.

2. Sterilize at 121^{0}C for 15mins

Yeast Extract Peptone Agar

Composition

1. Yeast extract 20g
2. Peptone 5g
3. Agar 20g
4. Distilled water 500ml

Nutrient gelatin (broth)

Composition

1. Nutrient broth containing 10-15% gelatin
2. This is sterilized at 121^{0}C for 15mins

Nutrient broth

Peptone from meat 5.0g

Meat extracts 3.0g

Suspend 8g in 1litre of deminiralized water, dispense into small containers, autoclave at 121^{0}C for 15mins.

pH 7 0±0.2 at 25^{0}C

0.1% peptone water :

Dissolve 1g of peptone in 100ml of distilled water

Autoclave at 121^{0}C for 15mins

Casein hydrolysis medium

For detecting casein-hydrolysis organisms

Composition

Skim milk 1part

Nutrient broth (pH 7.4) 1part

Agar 1%

Dialyse the skim milk solution to rid it of lactose

Add the component together and steam to dissolve

Autoclave at 121^{0}C for 15mins