Proteolytic Profile of Lactic Acid Bacteria

A Thesis

submitted in the partial

fulfilment of the requirement

for the award of the degree of

MASTERS OF SCIENCE

IN

MICROBIOLOGY

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CANDIDATE'S DECLARATION

I hereby declare that the work which is being presented in the dissertation entitled "Proteolytic Profile of Lactic Acid Bacteria" in partial fulfillments of the requirements for the award of the degree of Masters of Science in Microbiology, Department of Biotechnology and Environmental Sciences, Thapar University, Patiala is an authentic record to my own work during a period of one year from July 2011 to July 2013, under the supervision of Dr. Abhijit Ganguli, Department of Biotechnology and Environment Sciences, Thapar University.

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CERTIFICATE

This is to certify that the thesis entitled "**Proteolytic profile of Lactic Acid Bacteria**" submitted by **Nippunpreet Kaur** in partial fulfillment of the requirement for the award of Degree of **Master of Science in Microbiology** to **Thapar University**, Patiala, is a record of student's own work carried out by him. The report has not been submitted for the award of any other degree or certificate in this or any other university or institute.

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…….DEDICATED TO MY PARENTS

To my parents, for all the support and motivation they have given me throughout my life...

Contents

List of Abbreviations

LAB	Lactic Acid Bacteria
MRS	de Man Rogosa Sharpe
DCM	Dry Cell Mass
OPH	Organophosphorus hydrolase
HCl	Hydrochloric Acid
TCA	Trichloroacetic Acid
NaOH	Sodium hydroxide
CO_2	Carbon dioxide
O_2	Oxygen

List of Symbols

°C	degree(s) Celsius
g	gram
h	hours
mL	millilitre
mg	milligram
U/mL	Units per millilitre
U/mg	Units per milligram
U/mL/g	Units per millilitre per gram
µL	microlitre
min	minute(s)
nm	nanometer
%	percentage
rpm	revolutions per minute
h^{-1}	per hour
M	Molar
cfu/g	colony forming units/gram

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ABSTRACT

In the present study, twenty six strains of previously isolated lactic acid bacteria (LAB) were screened for protease production and the isolate showing highest enzyme activity, *Lactococcus lactis* subsp. *lactis*, was evaluated furthur. The highest protease activity in de Man Rogosa Sharpe (MRS) media was 1.1 Units/mL at pH 7.0 and incubation temperature of 37ºC with inoculum size of 5%, sucrose as carbon source, yeast extract as nitrogen source and casein as protein source without any agitation. When sourdough, was supplemented with *L.* lactis, high protease activity was observed with *Vigna mungo* (20.26 Units/g) than plain dough (7 Units/g) at 37ºC and pH 7.0. Protease production remained unaltered by selected intrinsic and extrinsic factors (water activity, organophosphorus compounds and phytate content). Besides, it was found that water activity, phytate content and organophosphorus compounds (malathion) had negligible effect on protease activity of *L. lactis*. Visual and textural attributes indicated the overall general acceptability of dough containing *Vigna mungo* (8.15 ± 0.03) on a ten-point hedonic scale Thus, the results suggetsed the potential of *L. lactis* as a proteolytic LAB strain.

Keywords: Lactic acid bacteria, protease production, sourdough, *Vigna mungo*, *Lactococcus lactis*

CHAPTER 1- INTRODUCTION

1.1 Lactic acid bacteria

Lactic acid bacteria (LAB) consist of a number of bacterial genera within the phylum Firmicutes. The genera *Carnobacterium, Enterococcus, Lactobacillus, Lactococcus, Lactosphaera, Leuconostoc*, *Melissococcus, Oenococcus*, *Pediococcus, Streptococcus, Tetragenococcus, Vagococcus* and *Weissella* are recognized as LAB. Lactic acid-producing Gram-positive bacteria but belonging to the phylum Actinobacteria are genera such as *Aerococcus, Microbacterium*, and *Propionibacterium* as well as *Bifidobacterium*. LAB are gram-positive, nonsporulating, catalase-negative, and facultative anaerobic bacteria with a fermentative metabolism. They ferment glucose primarily to lactic acid, or to lactic acid, CO_2 and ethanol. All LAB grow anaerobically, but unlike most anaerobes, they grow in the presence of O_2 as "aerotolerant anaerobes".

Lactococcus lactis is the most extensively studied LAB organism and the second most studied gram-positive bacterium with respect to its genetics, physiology, and molecular biology. Its genome was the first LAB genome to be completely sequenced and annotated. It has a very limited biosynthetic capacity and is nutritionally fastidious. It has a relatively simple metabolism. In particular, *Streptococcus thermophilus*, *L. lactis*, *Lactobacillus helveticus*, and *Lactobacillus delbrueckii* subsp. *bulgaricus* (*L. bulgaricus*) are widely used dairy starters and are of major economic importance.

Lactic acid bacteria comprise an ecologically diverse group of microorganisms united by formation of lactic acid as the primary metabolite of sugar metabolism. Depending on the organism, metabolic pathways differ when glucose is the main carbon source: homofermentative bacteria such as *Lactococcus* and *Streptococcus* yield

two lactates from one glucose molecule, whereas the heterofermentative (i.e. *Leuconostoc* and *Weissella*) transform a glucose molecule into lactate, ethanol and carbon dioxide. Lactic acid bacteria are restricted to environments in which sugars are present. They have limited biosynthetic ability, having evolved in environments that are rich in amino acids, vitamins, purines and pyrimidines. So they must be cultivated in complex media that fulfill all their nutritional requirements. They are GRAS- Generally Regarded as Safe, due to their ubiquitous appearance in food and their contribution to the healthy microflora of human mucosal surfaces.

Celiac disease is an autoimmune disease which occurs in genetically predisposed people and caused after the consumption of wheat, rye and barley. CD has an incidence of 1 of 100-550 people in the European population. Gluten proteins, mostly gliadin, have high proline and glutamine content and they are partially cleaved by the enzymes of the human digestive tract. When gluten reaches the small intestine, it activates an immune response and mucosal damage occurs. Sourdough fermentation is considered as an alternative to gluten-free product variety by including proteolytic lactic acid bacteria. Besides, sourdough fermentation affects nutritional properties, texture and flavor, also changes in the protein structure occur with the help of LAB and wheat flour endogenous enzymes.

Organophosphates are organic esters of phosphoric acid, thiophosphoric acid and other phosphoric acids, which are widely used as insecticides and acaricides. Organophosphorus compounds exhibit a wide range of toxicity to mammals. They work on central nervous system inhibiting its normal function resulting in convulsions, paralysis and death.

1.2 Lactic acid bacteria benefiting health

A number of strains of Lactic Acid Bacteria (LAB) have been proposed and safely used as probiotics, which are defined as "live micro-organisms, which upon ingestion in certain numbers, exert health benefits beyond inherent basic nutrition". Probiotics are widely used to aid digestion and help restore gut bacterial balance after antibiotics. Lactic acid bacteria have been cited to be part of human and animal microbiota and are regarded as a major group of probiotic bacteria. Certain LAB strains, most notably the strains from the genera Lactobacillus, are increasingly marketed as health-promoting, i.e., probiotic bacteria, while certain strains are believed to produce bioactive health-beneficial peptides from milk proteins.

Lactic acid bacteria constitute an integral part of the healthy gastrointestinal (GI) microecology and are involved in the host metabolism. LAB along with other gut microbiota ferment various substrates like lactose, biogenic amines and allergenic compounds into short-chain fatty acids and other organic acids and gases. They have been found to control intestinal disorders, partially due to serum antibodies IgG, and secretory IgA and IgM enhancing immune response. Reports have been published on bacteriocin production by some probiotic bacteria targeting pathogenic bacteria in vitro. Reutericyclin, an antibiotic produced by *Lactobacillus reuteri* LTH2584, has recently been discovered to inhibit a broad range of bacteria. Its biological activity is comparable to that of nisin.

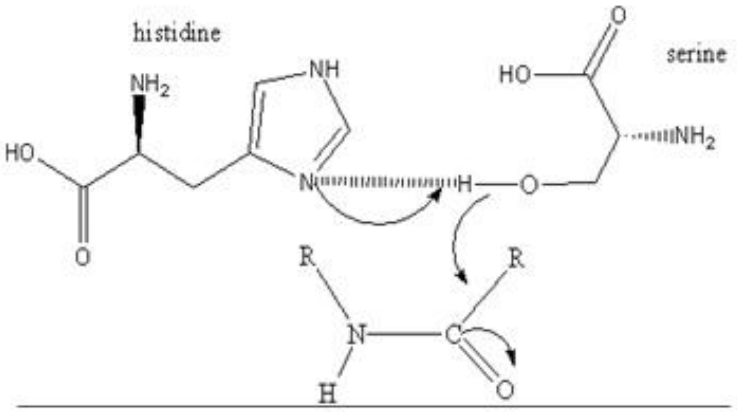
1.3 Proteases

Proteolysis is a process in which a protein is broken down partially, into peptides, or completely, into amino acids, by proteolytic enzymes or proteases, present in bacteria. Proteases, also termed proteinases or peptidases, are proteolytic enzymes

functioning as molecular knives that cut long amino acid sequences into fragments. This is essential for the synthesis of all proteins, controlling their size, composition, shape, turnover, and ultimate destruction. According to their catalytic mechanisms, proteases are classified into the following six types: aspartic, cysteine, glutamic, metallo, serine, and threonine proteases.

1.4 Structure

Serine proteases are proteases that have serine, an amino acid, bonded at the active site. Serine proteases are grouped depending on their structure. Major groups of serine proteases include alpha hydrolase, beta hydrolase, and signal peptidase. Serine proteases are grouped into clans that share structural homology and then further subgrouped into families that share close sequence homology. Aligned side chains of serine, histidine and aspartate build the catalytic triad common to most serine proteases. The active site of serine p oteases is shaped as a cleft where the polypeptide substrate binds. Many proteases are synthesized and secreted as inactive forms called zymogens and subsequently activated by proteolysis. This changes the architecture of the active site of the enzyme. Few examples are: Chymotrypsin, trypsin, and elastase



Figu re 1. Chemical Structure of Serine Protease

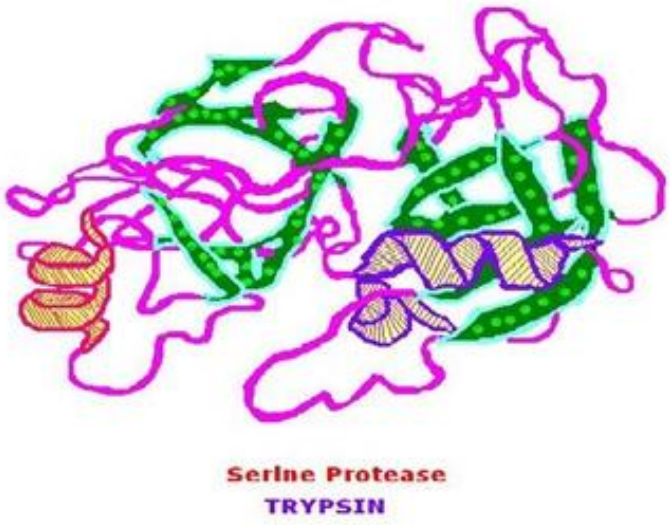


Figure2. Crystal structure of Trypsin, a serine protease

1.5 Functions/Importance of proteases

- Proteolysis by LAB is important for bacterial growth
- It forms metabolites that contribute significantly to flavour compunds as fermented /end products
- It brings about complete inactivation of undesirable enzymatic activity or removal of unwanted, damaged or abnormal proteins
- Microbial proteases are widely used in the detergent industry as detergent additives.
- They are an also used in food, pharmaceutical, leather, diagnostics, waste management and silver recovery.
- PrtP produce bitter peptides

1.6 Proteolytic systems in lactic acid bacteria

Lactic acid bacteria have a complex proteolytic system capable of converting milk casein to the free amino acids and peptides necessary for growth and acid production. The proteolytic system is essential for growth in milk and contributes

significantly to flavour development in fermented milk products where LAB are used as starter cultures. The proteolytic system is composed of a Proteinase, which is involved in the initial cleavage of casein, Peptidases, which hydrolyse the large peptides thus formed and Transport Systems, which are involved in the uptake of small peptides and amino acids. Their proteolytic systems are important as a means of making protein and peptide N available for growth and as part of the curing or maturation processes which give foods their characteristic rheological and organoleptic properties. The proteolytic system of lactococci is one of the best documented.

1.7 Proteolytic enzymes in lactic acid bacteria

1.7.1 Proteinases

1.7.1.1 Extracellular. Proteinases are secreted as "free" enzymes outside the cell. The same organism also produces an intracellular proteinase with some properties in common with the supposed extracellular enzyme. Actively growing or metabolizing cells release a constant amount of proteinase into the medium, irrespective of its Ca^{2+} content. Only the enzyme in the cell wall is affected by the Ca^{2+}.

1.7.1.2 Intracellular. Although extracellular proteinases are a vital part of the mechanism by which bacteria make external proteins available for growth, their intracellular equivalents are equally important in the turnover of denatured or defective proteins, the activation of zymogens, and the termination of newly synthesized proteins. Lactobacilli generally produce neutral proteinases active on α-, β- and κ-casein but the intensity of their activity is extremely variable from strain to strain. β-casein is the most preferred substrate.

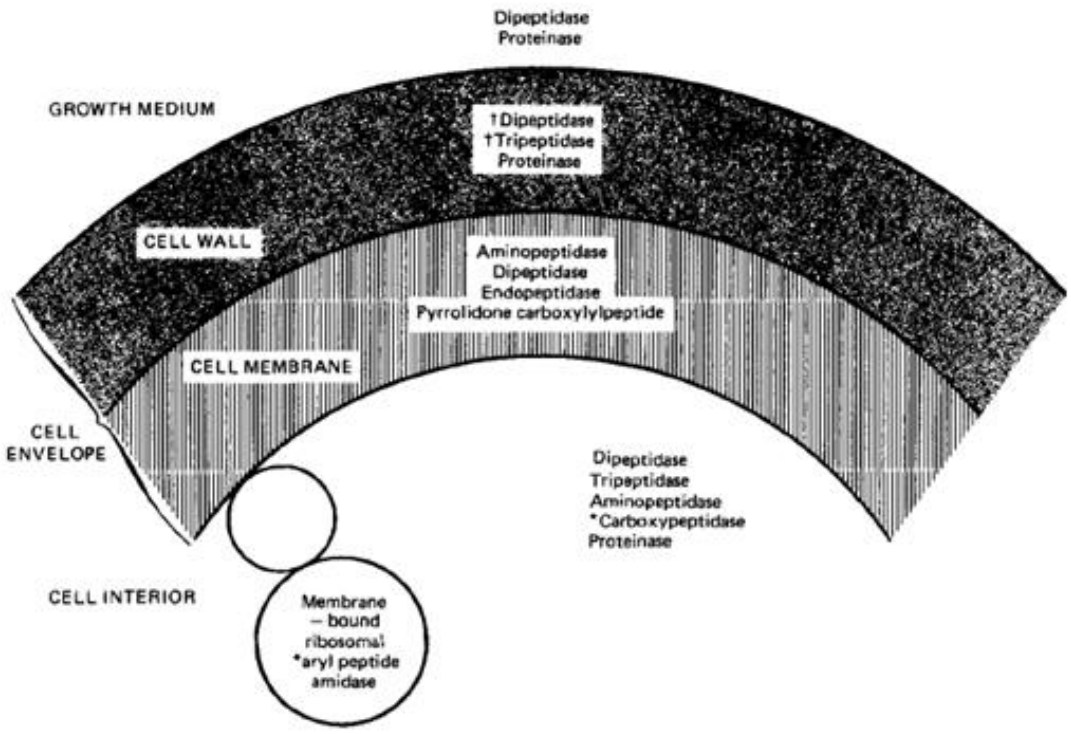


Figure3. Schematic representation of the possible cellular locations of proteinase and peptidase activities in lactic acid bacteria

*** Only lactobacilli,**

† Only *Streptococcus lactis*

1.7.2 Peptidases

1.7.2.1 Extracellular. Many classes of peptidases have been reported in every conceivable location in or on the cells of lactic acid bacteria. It refers to those enzymes whose activity manifests itself outside the cell membrane. Such enzymes have been recovered from culture supernatants.

1.7.2.2 Intracellular. Both the lactococci and the lactobacilli are well endowed with peptidases, which can be demonstrated in various intracellular locations. These are derived from cell-free extracts of mechanically disrupted cells. Some of the intracellular

peptidases of the group N streptococci are associated with subcellular structures. Peptidases in lactobacilli appear to have a wider range of activities than the lactococci.

Many peptidases have been named according to the substrates which they hydrolyse. These are Endopeptidases (degrade oligopeptides), Aminopeptidases (important for the development of flavour in fermented milk products), Dipeptidases (hydrolyse dipeptides), Tripeptidases (hydrolyse tripeptides) and Proline-Specific Peptidases.

1.8 Proteolytic system of *Lactococcus lactis*

The degradation of milk proteins is initiated by an extracellular proteinase, PrtP, that is bound to the cell wall.

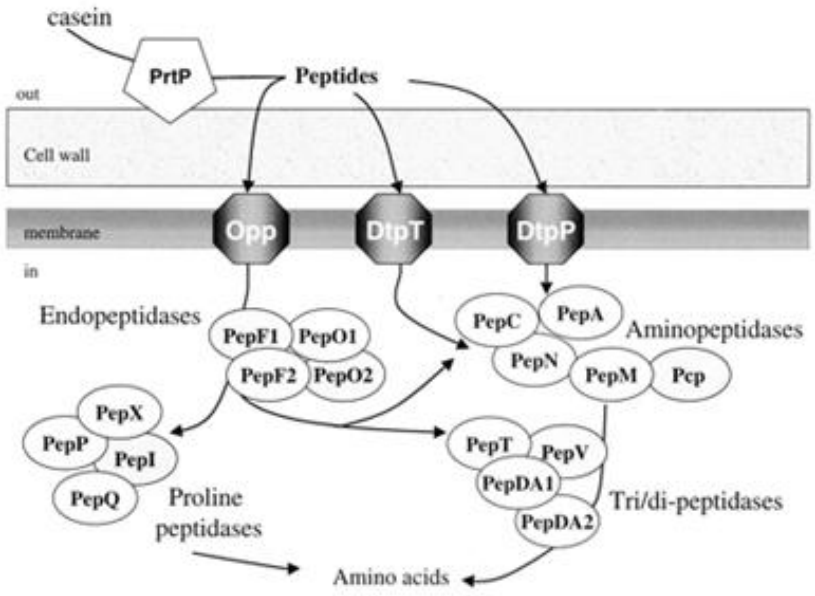


Figure4. Schematic representation of *L. lactis* proteolytic system. The cell wall proteinase (pentagon), three transport systems (hexagon), and 18 intracellular peptidases (oval) are represented in their relative locations in the cell. Peptidases are classified on the basis of their cleavage specificity. White and grey ovals represent peptidases that were included and not included, respectively, in this study.

Two types of proteinase, P_I and P_{III}, have been characterized in *Lactococcus lactis* subsp.*cremoris* on the basis of the casein degradation pattern. In *L. lactis*, peptides produced by the proteinase are internalized by three transporters. The Opp system takes up oligopeptides of 4 to 18 residues, while DtpT and DtpP transport hydrophilic and hydrophobic di- and tripeptides, respectively. Internalized peptides are further hydrolyzed by several intracellular peptidases that are classified depending on their cleavage specificity. Six aminopeptidases (PepN, PepC, PepP, PepX, PepA, and Pcp) generate dipeptides and free amino acids by cleaving the N-terminal end of oligopeptides. Endopeptidases such as PepO1, PepO2, PepF1, and PepF2 cleave internal peptide bonds of oligopeptides, and several other peptidases, such as PepV, PepQ, and PepT, cleave di- or tripeptides.

1.9 Sourdough

Sourdough is a process in which wheat flour and water (and other ingredients) are fermented with microbes. Spontaneous dough fermentation starts by mixing flour with water without adding a starter culture or portion of a preceding sourdough (mother dough). The microflora of such dough depends on the microflora of the raw materials used and the prevailing hygienic conditions, and is variable in terms of kind, origin and storage conditions of the flour, as well as the technological parameters of the fermentation process applied. Commercial sourdough processes do not rely on fortuitous flora but on the use of commercial starter cultures or a portion of the preceding sourdough (mother dough).

Sourdoughs are considered extremely complex ecosystems in which lactic acid bacteria (LAB) and yeasts represent the prevailing microflora. Sourdough has a natural, additive-free image and lactic acid bacteria have been used in food for thousands of years and are "generally regarded as safe". Sourdough is an ancient way to improve

flavour, texture and microbiological shelf life of bread. However, control of the acidity level of wheat sourdoughs is a premise for the improvement of quality.

Wheat is the most important cereal crop and constitutes the main component of millions of heads world over. It has therefore, become a normal agricultural practice to spray or fumigate stored grains, particularly prior to shipment with contact insecticides so that they reach the port of destination free from any pest infestation. A large number of pesticides are in common use as grain protectants. Some pesticides, such as malathion, have been used worldwide for over 30 years, whereas others such as bioresmethrin, bromophos, dichlorovos, fenitrothion and pirimiphos-methyl, have been in use for 10 - 22 years. Cereal grain might be contaminated with several pesticides, which can enter in food chain of human consumption with its consequential hazard. Therefore, it is deemed absolutely necessary to establish several reliable, rapid, inexpensive and effective analytical methods for simultaneous determination of the residues of many pesticides. The present method is based on the organophosphorus hydrolase activity of LAB, which is simultaneously induced and is responsible for the degradation of these toxic pesticides. This OPH activity of the bacteria allows them to degrade various proteins in dough without any restriction from these organophosphorus pesticides. These are the most toxic among the insecticides and are dangerous not only to insects but also to mammals. Many of these compounds are in the "super toxic" category of human poison, for example, Chlorpyriphos, Malathion, Parathion, Diarzinon, Guthion and Phenothoate.

1.10 Importance of proteolytic enzymes in sourdough

The sourdough process depends on numerous factors including the composition of microflora, fermentation and enzymatic activities and flour characteristics. Many factors simultaneously affect the processes involved in sourdough fermentation such as

the formation of acidity, the production of volatile compounds and the degradation of carbon and nitrogen compounds.

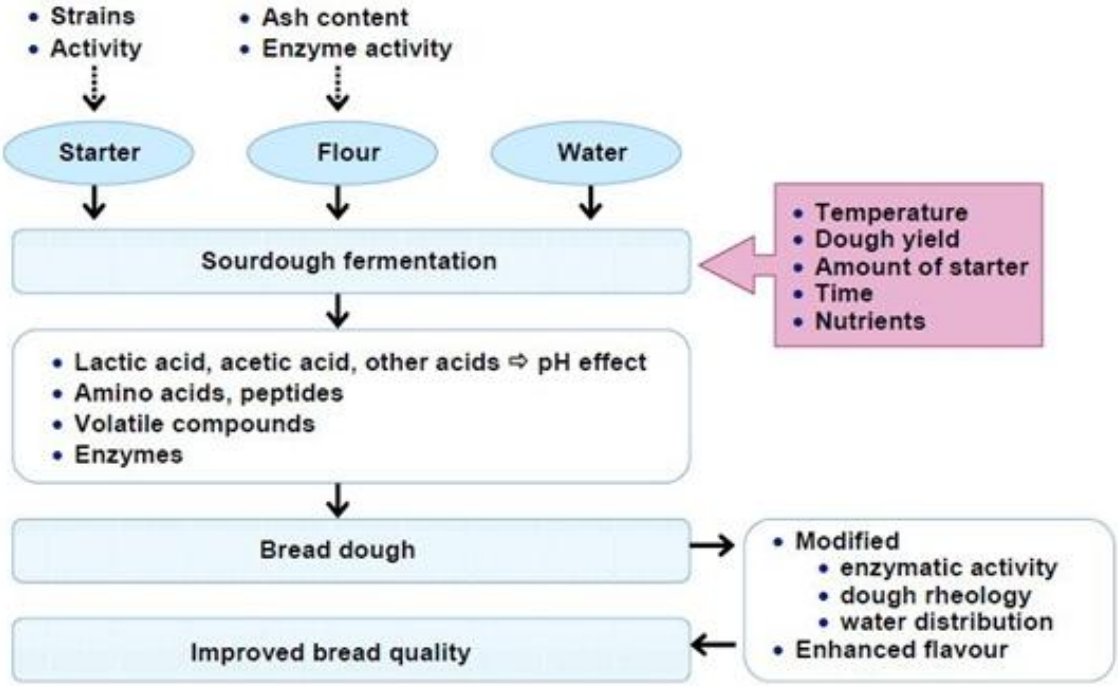


Figure5. Sourdough fermentation and influencing factors. (Katina *et al.*, 2005)

The proteolytic enzymes present in the sourdough system degrade various cereal proteins. This proteolysis produces free amino acids, which may act as flavour precursors. Gluten proteins determine, to a great extent, the rheological properties of wheat doughs and texture of wheat breads. Substantial hydrolysis of gliadinin and glutenin proteins occurs during sourdough fermentation due to pH-mediated activation of cereal enzymes. Furthermore, sourdough fermentation results in a solubilisation and depolymerisation of the gluten macropolymer. Proteolysis during sourdough fermentation is highly dependent on formation of acids. Lactic acid bacteria contribute to overall proteolysis during sourdough fermentation by creating optimum conditions for activity of cereal proteinases.

1.11 Advantages of sourdough

The use of sourdough offers a number of advantages in baked goods technology. A great part of these advantages is promoted by the decrease in pH during fermentation: gas retention and resistance of the gluten network, inhibition of flour amylases, water binding of gluten and starch granules, swelling of pentosans, solubilization of the phytate complex by endogenous phytases, and prevention of malfermentation and spoilage. However, few reports have been reported on protease activity of lactic acid bacteria in sourdough in Indian subcontinent.

CHAPTER 2- REVIEW OF LITERATURE

2.1 Occurrence of lactic acid bacteria

Lactic Acid Bacteria occur naturally in fermented food (Caplice and Fitzgerald, 1999) and have been detected in soil, water, manure and sewage (Holzapfel et al., 2001). Their natural habitats include milk and milk-related places, intact and rotting plants as well as intestinal tracts and mucous membranes of animals and humans (Hans, 1993). However, some LAB are part of the oral flora which can cause dental caries (Monchois et al., 1999; Sbordone and Bortolaia, 2003). LAB were first isolated from milk (Carr et al., 2002; Metchnikoff, 1908; Sandine et al., 1972) and have since been found in such foods and fermented products as meat, milk products, vegetables, beverages and bakery products (Aukrust and Blom, 1992; Caplice and Fitzgerald, 1999; Harris et al., 1992; Gobbetti and Corsetti, 1997; Jay, 2000; Liu, 2003; Lonvaud-Funel, 2001; O'Sullivan et al., 2002).

2.2 General and metabolic characteristics of LAB

Members of lactic acid bacteria share the property of being gram-positive bacteria (Fooks et al., 1999) that ferment carbohydrates into energy and lactic acid (Jay, 2000). Depending on the organism, metabolic pathways differ when glucose is the main carbon source: homofermentative bacteria such as *Lactococcus* and *Streptococcus* yield two lactates from one glucose molecule, whereas the heterofermentative (ie. Leuconostoc and Weissella) transform a glucose molecule into lactate, ethanol and carbon dioxide (Caplice and Fitzgerald, 1999; Jay, 2000; Kuipers et al., 2000). In addition, LAB produce small organic compounds that give the aroma and flavor to the fermented product. LAB have been used as a flavoring and texturizing agent as well as

a preservative in food for centuries and are now added as starters in food (Caplice and Fitzgerald, 1999). LAB, such as lactobacilli, *L. lactis*, and *Streptococcus thermophilus*, inhibit food spoilage and pathogenic bacteria and preserve the nutritive qualities of raw food material for an extended shelf life (Heller, 2001; O'Sullivan et al., 2002). Recently, the use of metabolites of LAB as biological preservatives in food packaging materials has been discussed (Pirttijärvi et al., 2001; Scannell et al., 2000).

LAB play an important role in processing animal feeds like silage (Aukrust and Blom, 1992; Driehuis and Elferink, 2000; Holzer et al., 2003). The antimicrobial effect of LAB is mainly due to their lactic and organic acid production, causing the pH of the growth environment to decrease (Caplice and Fitzgerald, 1999; Kuipers et al., 2000). Low pH induces organic acids to become lipid soluble and diffuse through the cell membrane into the cytoplasm (Gottschalk, 1988). LAB also produce acetaldehyde, hydrogen peroxide, diacetyl, carbon dioxide, polysaccharides and bacteriocins (Caplice and Fitzgerald, 1999; de Vuyst and Degeest, 1999; Rodrígues et al., 2003), some of which may act as antimicrobials.

Lactic Acid Bacteria can be divided into two groups based upon the products produced from glucose fermentation:

Homofermentative organisms ferment glucose to 2 moles of lactic acid, generating a net of 2ATP (Adenosine Triphosphate) per mole of glucose metabolized, lactic acid is the major product of this fermentation (Axellsson, 1998).

Heterofermentative organisms ferment 1 mole of glucose to 1 mole of lactic acid, 1 mole of ethanol and 1 mole of CO2. A mole of ATP is generated per mole of glucose, resulting in less growth per mole of glucose metabolized. Due to the low energy yields, lactic acid bacteria often grow more slowly than microbes capable of respiration and thus produce smaller colonies of between 2 - 3 mm (Axellsson, 1998).

Since they do not use oxygen in their energy production, lactic acid bacteria readily grow under anaerobic conditions, but they can also grow in the presence of oxygen, and are thus microaerophiles. They are protected from oxygen by products like hydrogen peroxide because they have peroxidases, they are also differentiated from other organisms by their ability to ferment hexoses to lactic acid, hence their name (Eugene *et al.,* 1998).

2.3 Proteases, occurrence and roles

Proteases defined as enzymes, that break peptide bonds between amino acids of proteins in a process called proteolytic cleavage (Hooper, 2002; Barrett *et al.,* 2003), are the most important industrial enzymes, representing a worldwide sale of about sixty-percent of total enzyme market (Woods *et al.,* 2001). They find commercial applications in a number of industries like the leather industry where serine proteases are used to dehair hides and softer leather due to their collagenolytic activity (George *et al.,* 1995), pharmaceutical industry in combination with broad spectrum antibiotics for treating festering and wet wounds by offering a gentle and selective debridement while supporting natural healing process, and also as an antiplaque and antitartar components of toothpastes (Hernandez and Maria 1996), cosmetics (Ohta *et al.,* 1996), also for the recovery of silver from used X-ray films (Ishikawa *et al.,* 1993).

Due to their ability to remain stable in the presence of surfactants, they aid the removal of proteinaceous dirt from laundries (Godfredson, 1990). Metalloproteases have found immense use in the brewery industries as well as in alcohol production while the acid proteases find uses in the manufacture of cheese and the baking industries (Bjorkling *et al.*, 1991). Solid substrate fermentation has the potential for higher protease yield because economically this type of fermentation processes many advantages including superior volumetric productivity, use of simpler machinery, use of

an inexpensive substrate, simpler down-stream processing, low energy requirements and low waste water output (Malathi and Chakraborty, 1991).

2.4 Protease purification

Several research works have been carried out on lactic acid bacteria proteases, likewise several types have been purified and characterized. Several studied using *Streptococci* and *Lactococci* extracellular or cell wall-associated proteases have also been conducted (Thomas and Mills, 1981; Kunji *et al.,* 1996). Julliard *et al.,* (1995) found that cell wall associated extracellular protease produced by *Lactococcus lactis* subsp. *cremoris* is a P-1 type that hydrolyses casein. An extracellular cysteine proteinase produced by *Micrococcus* sp. INIA 528 was also purified by chromatography using sephadex G-100 and G-50 pre-equilibrated with 50mmol-sodium phosphate buffer to achieve a 29-fold increase and 28% recovery of the proteinase activity (Fernandez *et al.,* 1996). Microbial proteases play an important role in biotechnological processes accounting for approximately 59 percent of total enzymes used (Maugh, 1984). The use of microorganisms to produce enzymes has enormous economic and technical advantages, thus methods of production, extraction and purification are of much concern to scientists (Alvarez-sanchez *et al.,* 2000; Gianna *et al.,* 2001; Thangam and Suseela, 2002).

2.5 Regulation of protease activity in LAB

Lactococci, gram-positive, facultatively anaerobic bacteria, depend on the availability of a proteolytic system for their growth on milk. The enzymes of the proteolytic system composed of the extracellular cell wall-bound serine proteinase (PrtP) and various well characterized peptidases, supply essential amino acids by concerted action in degradation of the casein (Jensen *et al.*, 1993; Juillard *et al.*, 1995). The proteolytic system has an important role in the generation of casein peptides, which

contribute to organoleptic changes in fermented milk products (Kok *et al.*, 1993; Laan *et al.*, 1989; Thomas *et al.*, 1987; Visser *et al.*, 1993). Meijer *et al.* (1995) demonstrated the regulation of proteolytic enzyme activity in two different *Lactococcus lactis* host strains, *L. lactis* subsp. *lactis* MG1363 and *L. lactis* subsp. *cremoris* SK1128, both containing plasmid pNZ521, which encodes the extracellular serine proteinase (PrtP) from strain SK110, were used to study the medium and growth-rate-dependent activity of three different enzymes involved in the proteolytic system of lactococci. Enzyme activity was used as a measure of total enzyme production. A direct relationship between PrtP activity and PrtP production has been demonstrated (Bruinenberg *et al.*, 1992; Hugenholtz *et al.*, 1987).

In cheese manufacture, the proteolysis of casein is thought to play a pivotal role because amino acids resulting from proteolysis are the major precursors of specific flavour compounds, such as various alcohols, aldehydes, acids, esters and sulphur compounds (Smit *et al.*, 2005). It was recently proposed that food grade strains of *L. lactis* expressing *L. helveticus* CNRZ32 PepO2 and Pep O3, in combination with PepN, can be used to reduce bitterness in cheese (Sridhar *et al.*, 2005). Kholif *et al.* (2011) evaluated the proteolytic activity of 8 lactobacilli strains. Among the tested strains, *Lactobacillus rhamnosus*, *Lactobacillus helveticus*, *Lactobacillus plantarum* and *Lactobacillus delbrueckii* ssp. *bulgaricus* were the highest producers. Maximum protease activity appeared at the beginning of stationary phase (Kawai *et al.*, 1999; Wang *et al.*, 2007).

2.6 Implications of protease activity

Lowe *et al.* (2005) applied three strains of lactic acid bacteria during the malting process to evaluate the impact on malt and wort quality. Increased β-glucanase activities were found in malts where LAB and FG LA had been applied to the barley

kernels. Malted barley contains several endogenous β-glucanases including at least two isoenzymes of (1-3,1-4)-β-D-glucan 4-glucanohydrolase (Fincher *et al.*, 1986; Litts *et al.*, 1990; Manners *et al.*, 1969; Slaseki *et al.*, 1990). Haikara *et al.* (1993) employed starter cultures of *Lactobacillus plantarum* and *Pediococcus pentosaceus* during malting to restrict the growth of *Fusarium* moulds and their mycotoxins in order to prevent gushing.

Pescuma *et al.* (2011) evaluate the growth and proteolytic activity of sixty-four strains of lactic acid bacteria in whey to further formulate a starter culture for the development of fermented whey-based beverages. Whey is a source of vitamins, minerals and high quality proteins compared with caseins, egg and soy proteins (Dralic´ *et al*., 2005; Smithers, 2008). The major constituents of bovine whey proteins are β-lactoglobulin (β-lg) and α-lactalbumin (α-la), which constitute 55-60 and 15-20% of total proteins, respectively. β-Lg is not degraded by pepsin and may cause allergenicity problems especially in children less than three years of age. Lactic acid bacteria (LAB) are commonly used as starter cultures in the fermented food industry mainly due to their acidifying capacity but also to their metabolic activity on proteins, sugars and lipids, thus contributing to food digestibility and preservation as well as to the texture and sensory characteristics of the end product (Khalid and Marth, 1990; Wood, 1997). On the whole, the majority of the assayed strains digested the protein fractions of 69 and 50 kDa the most; *Str. thermophilus* CRL 808 showed the maximum degradation percentage for the 50 kDa fraction. The strains which showed the highest hydrolysis percentages of β-lg were *Lb. acidophilus CRL 636* (16-18%) and *Lb. delbrueckii* subsp. *bulgaricus* CRL 656 (12-14%). Ohmiya and Sato (1969) studied the ability of *Lb. helveticus, Lactococcus lactis* and *Lb. delbrueckii* subsp. *bulgaricus* strains to degrade milk proteins and found different casein and whey protein degradation degrees for

strains of each species. In addition, Khalid and Marth (1990) reported degradation of different protein fractions between strains of *Lb. plantarum* and *Lb. casei*.

Moslehishad *et al.* (2013) evaluated the proteolytic activities of 14 strains of lactic acid bacteria and their impact on sensory characteristics of the resultant fermented cow and camel milk. Controlled fermentation using some strains of lactic acid bacteria (LAB) improves fermented milk, especially traditional camel's milk products (Hassaïne *et al.*, 2007; Panesar 2011). Proteolytic system of LAB lets them use milk protein as a source of nitrogen, which has an essential role in the production of different types of metabolites in fermented food products (Shihata and Shah 2000; Liu *et al.*, 2010). This ability can also cause the release of a wide range of biologically active peptides such as hypotensive peptides, opioid agonists, antagonist peptides, antibacterial and immunomodulatory peptides (Donkor *et al.*, 2007). Recently, the application of proteolytic food-grade LAB has been considered as a new strategy for producing novel functional food rich in bioactive peptides (Hayes *et al.*, 2007). Sensory evaluation of dairy products is crucial for consumer acceptance and quality control (Karagul-Yuceer and Drake, 2006). In all cases, except *P. acidilactici* PTCC 1424 and PTCC 1602, the protease activity of the cell-free supernatant was lower than that obtained in bacterial cells. The flavour acceptance of fermented cow milk was in the range of 1.05 ± 0.23 to 3.28 ± 0.12. The mean values for flavour analysis of fermented camel milk were lower than 3.47 ± 0.15.

Rizzello *et al.* (2006) showed the capacity of selected sourdough lactic acid bacteria to hydrolyze wheat and rye allergens. The clinical classification of food allergy diseases is based mainly on the presence or absence of food specific immunoglobulin E (IgE) antibodies (Johansson *et al.*, 2001). It has been shown that gastrointestinal enzymes (pepsin, pancreatin and trypsin) are not able to degrade cereal allergens which

reach unaltered the intestine where they elicit the immune response (Astwood *et al.*, 1996). Previously, it had been shown that a pool of selected lactic acid bacteria had the capacity to extensively degrade wheat gliadins during sourdough fermentation and to decrease the human intolerance to gluten (celiac disease) (Cagno *et al.*, 2004). Proteolysis is one of the mechanisms by which probiotic bacteria are involved in the management of food allergy (Weid *et al.*, 2002). The results of this study showed that long-time fermentation (24 h at 37 ◦C) with selected sourdough lactic acid bacteria favoured the degradation by digestive enzymes of the IgE-reactive epitopes which persisted after baking wheat and rye breads.

M'hir *et al.* (2008) selected strains with proteolytic activity of *Enterococcus faecalis* on wheat gliadin, previously isolated from Tunisian fermented wheat dough. LAB which are used in the sourdough process, exhibit proteolytic activity (Di Cagno et al. 2002; Zotta et al. 2007). This proteolytic activity may be used as a tool to reduce gliadin, which is involved in coeliac disease and is present in wheat-baked foods (Rolla´n et al. 2005; Rizzello et al. 2006). The optical density values (absorbance at 340 nm: OPA value), determined after the OPA spectrophotometric assay, was used to quantify proteolysis by selected LAB cultures. Higher OPA values indicate higher proteolysis (Madreau et al. 2006). Only the LAB *E. faecalis* ND3, HM3C and G32 showed proteolytic activity in GGB, giving OPA values higher than 5. The hydrolysis percentage for individual *Enterococcus* strain was 59%, 23% and 31%, respectively, for G32, ND3 and HM3C.

Wehrle *et al.* (1999) evaluated the proteolytic breakdown of gluten by lactic acid bacteria and enzyme preparations. Enzymes play a major role in the production of bakery products. The modification of the gluten network improves the dough's machinability. Additionally it affects aroma, crust colour and crumb texture of the final

products (Damm *et al.*, 1992). Proteolytic activity in sourdoughs has been detected by measuring the increase in free amino acids during fermentation of sourdough in comparison with dough systems without starter cultures (Collar *et al.*, 1993; Collar *et al.*, 1991; Gobbetti *et al.*, 1994). All *L. pentosus* strains and two *Pediococci* strains were proteolytically active on gluten; the highest activities were found for *L. pentosus 03A*.

Thiele *et al.* (2004) studied gluten hydrolysis and depolymerization during sourdough fermentation. Evidence for the impact of specific metabolic activities on bread quality was provided, for instance, concerning the generation of flavour precursors and flavor volatiles (Thiele *et al.*, 2002). The formation of expolysaccharides in dough by *Lactobacillus sanfranciscensis* improves wheat bread texture (Korakli *et al.*, 2001). Substantial hydrolysis of gliadin and glutenin proteins occurs during sourdough fermentation. Proteolysis in sourdoughs and the rheological consequences of gluten degradation are mainly based on the pH-mediated activation of cereal enzymes (Thiele *et al.*, 2003; Clarke *et al.*, 2004; Loponen *et al.*, 2004). During sourdough fermentation, glutenin subunits were hydrolyzed and the resulting peptides, with lower molecular weights, remained associated with the gluten macropolymer.

The above discussion implies ample scope of exploiting beneficial aspects of lactic acid bacteria with a view of improving food and food products. In this regard, few LAB of indigenous origin have been studied especially, for their proteolytic properties in sourdough or other fermented food products consumed in the Indian subcontinent.

CHAPTER 3- OBJECTIVES

In the current work, twenty six lactic acid bacteria isolates obtained from earlier studies (Singh *et al.*, 2011; Bhanwar *et al.*, 2013) were evaluated for proteolytic activity. Efficient proteolytic producer was then further studied under various intrinsic and extrinsic cultural variables as well as in sourdough. The following objectives were framed to achieve the above:

1) Screening of potential proteolytic strains of lactic acid bacteria
2) Optimization of proteolytic activity of selected strain(s)
3) Real time evaluation of proteolytic activities of selected lactic acid bacteria in sourdough

CHAPTER 4- MATERIALS AND METHODS

4.1 Microorganisms and culture conditions

Twenty-six strains of lactic acid bacteria isolated in previous studies (Singh *et al.*, 2011; Bhanwar *et al.*, 2013) were examined for protease production. Higher protease producer was selected. Lactic acid bacteria cultures were activated from glycerol stocks maintained at -20ºC. MRS (de Man Rogosa Sharpe) media was used for isolation and maintenance of LAB strains. (Composition of MRS is given in annexure 1). The media was sterilized at 121ºC for 20min. and cooled to room temperature prior to use. The initial culture condition was incubation temperature of 37ºC for 24h cultivation period, 1% inoculum volume, 7.2 initial pH of medium. Unless otherwise stated, the following tests were conducted under the above conditions.

4.2 Screening of potential proteolytic LAB

LAB strains were screened using a rapid, high throughput assay for protease production. 50 µL of the supernatant of centrifuged (8000 rpm) bacterial cultures in MRS broth medium was loaded into the 6.5-mm-diameter wells of skim milk agar plates as described by Moslehishad *et al.* (2013). (Composition of skim milk agar is given in annexure 1) .Protease activity was determined by estimating the diameter of clear zone area after 24 h. PBS was used as control. (Composition of PBS is given in annexure 2). Out of 26 strains, a strain of *Lactococcus* showed highest activity. This culture was characterized in a previous survey as *Lactococcus lactis* ssp. *lactis* and used for further studies.

4.3 Growth profile

Lactococcus lactis (1%) was inoculated aseptically in autoclaved MRS broth and incubated at 37ºC for 24 h with shaking. 1mL sample was withdrawn after every 2 h interval of time. The samples were centrifuged at 12,000 rpm for 5 min. at 4ºC. Pellet was suspended in 1mL saline. Absorbance of each sample was measured at 600nm. Growth curve was plotted between absorbance and time.

4.4 Protease assay (Shimogaki *et al*., 1991)

Protease activity was carried out using casein as the substrate. For this, overnight grown culture of *L. lactis* was centrifuged at 11,000 *g* for 10 min at 4°C. Enzyme activity, both extracellular and cell bound, was estimated. The activity obtained with washed pellet (cell bound) was negligible. Therefore, only the culture supernatants were assayed for protease activity. 0.5ml of enzyme solution (culture supernatant) was added to 3.0ml of substrate solution (0.6% casein in 0.1 M Tris-HCl buffer, pH 8.0) (composition given in annexure) and the mixture was incubated at 30°C for 20min. The reaction was stopped by addition of 3.2ml of TCA mixture (containing 0.11M trichloroacetic acid, 0.22M sodium acetate and 0.33M acetic acid) (composition given in annexure) and kept at room temperature for 30 min followed by filtration through Whatman filter paper No. 1. The absorbance of the filtrate was then measured at 280nm. One unit of protease activity is defined as the amount of enzyme required to produce 1g of tyrosine per minute under the conditions described above. The standard curve is given in annexure.

4.5 Kinetics of protease production

Optimization of the growth conditions (agitation, pH, temperature, inoculums size, carbon source, nitrogen source and protein source) was carried out for obtaining enhanced protease production.

4.5.1 Effect of agitation: MRS media was used for cultivation of overnight grown culture of *L. lactis* and was incubated for 24 h at 37ºC, with and without agitation, to study its effect on the protease activity. Following incubation for 24 h at 37ºC, the supernatants were analyzed for protease activity.

4.5.2 Effect of pH: MRS media previously set with different pH values of 3.5, 7.2 and 8, using 1N HCl and 1N NaOH, was used for cultivation of overnight grown culture of *L. lactis* to study the effect of pH on the protease activity. Following incubation for 24 h at 37ºC, the supernatants were analyzed for protease activity.

4.5.3 Effect of temperature: MRS media was used for cultivation of overnight grown culture of *L. lactis* and was incubated at different temperatures *viz.* 4ºC, 28ºC, 37ºC and 45ºC for 24 h to study the effect of temperature on the protease activity. Following incubation for 24 h, the supernatants were analyzed for protease activity.

4.5.4 Effect of inoculum size: For evaluation of effect of different inoculum sizes on enzyme activity, MRS media was inoculated with different inocula of overnight grown culture of *L. lactis viz.* 1%, 5% and 10%. Following incubation at 37ºC for 24 h, the supernatants were analyzed for protease activity.

4.5.5 Effect of carbon source: For evaluation of effect of different carbon sources on enzyme activity in culture media, three different carbon sources *viz*. glucose, sucrose and fructose (2% w/v) were added to MRS broth. Following incubation at 37ºC for 24 h, the supernatants were analyzed for protease activity.

4.5.6 Effect of nitrogen source: For evaluation of effect of different nitrogen sources on enzyme activity in culture media, four different nitrogen sources including Yeast extract, glutamic acid, peptone and potassium nitrate (0.5%) were added to MRS broth. Following incubation at 37ºC for 24 h, the supernatants were analyzed for protease activity.

4.5.7 Effect of protein source: For evaluation of effect of different protein sources on enzyme activity in culture media, three different protein sources including casein, gliadin and gelatine (1%) were added to MRS broth and used for cultivation of *L. lactis*. Following incubation at 37ºC for 24 h, the supernatants were analyzed for protease activity.

4.5.8 Protease activity under optimised culture conditions: The present investigation was aimed at optimization of medium components which have been predicted to play a significant role in enhancing the protease activity. So proper combination of various cultural conditions were established in MRS media, i.e. the optimum pH, temperature, carbon source, nitrogen source, protein source and with/without agitation and then cultivated with the best inoculum size of *L. lactis* for enhanced protease activity. Following incubation for 24 h, the supernatants were analyzed for protease activity.

4.6 Measurement of dry cell mass (Park *et al.*, 2010)

Overnight grown culture of *L. lactis* was centrifuged in pre-weighed eppendorf at 12,000 rpm for 10 min. at 4ºC. The supernatant was removed and the pellet was dried at 37ºC overnight in an incubator to a constant weight. After drying, it was again weighed to determine the dry cell mass (DCM) of the bacteria.

4.7 Protease assay with malathion:

Overnight grown culture of *L. lactis* along with 80ppm malathion (10mg/ml) in MRS media was taken. *L. lactis* culture without malathion was used as control. Samples were taken at different time intervals and stored at 4ºC. The samples were centrifuged at 11,000*g* for 10 min at 4°C. Protease activity was determined in the supernatants using casein as the substrate (Shimogaki *et al*., 1991).

4.8 Behaviour and proteolysis in sourdough

4.8.1 Preparation of dough extracts:

Wheat flour (high grade Aashirvaad, ITC Ltd.) and *Vigna mungo* (urad dal), purchased from the market. *Vigna mungo* and flour were crushed and blended in a ratio of 1:1. Dough was prepared with resuspended pellet of overnight grown culture of *L. lactis* with sterile water as described by Bhanwar *et al.* (2013). Dough as control was also prepared with sterile water (without culture) and incubated for fermentation at 37ºC for 4h. Wheat flour without *Vigna mungo* was used to prepare dough with culture (test) and without culture (control) and fermented as above. Samples (1g) were withdrawn every hour for measuring viable count, quantitative estimation of protein content and protease activity. For extraction, samples were boiled in a water bath at 90ºC for 45min. and were centrifuged at 10,000 rpm for 5min at 4ºC. The supernatants obtained were then stored at 0ºC until analysis.

4.9.2 Survival of *Lactococcus lactis* in dough:

Samples (1g) were withdrawn after every hour from prepared dough (wheat flour + *Vigna mungo*) and diluted 10 fold with sterile PBS. After that, 0.1 mL of aliquot was spread on MRS agar plate and incubated for 24 h at 37ºC. Viable colonies were counted and expressed as log cfu/g (colony forming unit/g).

4.9.3 Determination of protein content in dough: (Lowry *et al*., 1951)

Protein content in sourdough samples with urad dal and without adding urad dal from different hours of fermentation was estimated by using Folin Lowry method. 3 mL of previously stored supernatants were taken and centrifuged them at 8000 rpm for 5 min. at 4ºC. The pellet obtained was washed and resuspended in saline. The samples were again centrifuged at 8000 rpm for 5 min. at 4ºC. The pellet obtained was

resuspended in 1N NaOH and boiled for 5 min. at 100ºC. The boiled samples were again centrifuged at 8000 rpm for 5 min. at 4ºC. Then, 2 mL alkaline solution was added to the supernatants obtained and incubated for 10 min. at room temperature. Then 0.2 mL of folin's reagent was added to each sample and incubated for 30 min. at room temperature. The absorbance of each sample was measured at 660 nm. Composition of alkaline solution is given in annexure.

4.9.4 Protease Assay:

Dough was inoculated with overnight grown culture of *L. lactis*. Dough without culture was also prepared as control. The prepared doughs were incubated at 37ºC for 4 h. Samples were taken at different time intervals and stored at 0ºC. The samples were centrifuged at 11,000*g* for 10 min at 4°C. Protease activity of the dough extracts (with and without urad dal) was determined in the supernatants by using casein as the substrate as described before.

4.10 Optimization of Protease Activity in Sourdough

Optimization of the growth conditions (time, inoculum size, pH and temperature) was carried out for obtaining enhanced protease production in sourdough made from wheat flour and *Vigna mungo*.

4.10.1 Effect of incubation period: Sourdough inoculated with *L. lactis* was incubated at 37ºC for 4 h. Samples were withdrawn after each interval of 1 h to determine the effect of incubation period of fermentation on protease activity and stored at 4ºC. Dough extracts were prepared as described by Bhanwar *et al.* (2013). The supernatants were analyzed for protease activity.

4.10.2 Effect of inoculum size: Sourdough was inoculated with different volumes of *L. lactis viz.* 1%, 5% and 10% and incubated at 37ºC for 4 h to determine the effect of inoculum size on protease activity and stored at 4ºC. Dough extracts were prepared as

described by Bhanwar *et al.* (2013). The supernatants were analyzed for protease activity.

4.10.3 Effect of temperature: Sourdough inoculated with *L. lactis* was incubated at different temperature conditions *viz.* 4ºC, 28ºC, 37ºC and 45ºC for 4 h to determine the effect of temperature on protease activity. Dough extracts were prepared as described by Bhanwar *et al.* (2013). The supernatants were analyzed for protease activity.

4.10.4 Effect of pH: For evaluation of the effect of pH on protease activity, sourdough was previously set with different pH values of 4, 7 and 8, using 1N HCl and 1N NaOH. It was then used for cultivation of *L. lactis*. Following incubation for 4 h at 37ºC, the supernatants were analyzed for protease activity.

4.10.5 Effect of selected intrinsic and extrinsic factors on protease activity:

4.10.5.1 Water Activity: For evaluation of the effect of water activity on protease activity, dough was prepared with different water activity *viz.* 0.94, 0.96 and 0.99. The water activity was measured with the help of Pa_wkit Water Activity Meter. Then, the dough was inoculated with overnight grown culture of *L. lactis* and dough extracts were prepared as described by Bhanwar *et al.* (2013). Following incubation at 37ºC for 4 h, the supernatants were analyzed for protease activity.

4.10.5.2 Organophosphorus compounds: For evaluation of the effect of organophosphorus compounds on protease activity, different concentrations of malathion (10ppm, 20ppm, 40ppm and 80ppm) were added to the prepared dough. Then, the dough was inoculated with *L. lactis* and dough extracts were prepared as described by Bhanwar *et al.* (2013). Following incubation at 37ºC for 4 h, the supernatants were analyzed for protease activity.

4.10.5.3 Phytate Content: Since, *Vigna mungo* contains high content of phytate, a principal storage form of phosphorus in many plant tissues but it is not digestible to

humans or non-ruminant animals. Phytate (myo-inositol hexakisphosphate) is an indigestible, organic form of phosphorus that is found in grains and oil seeds. Thus, three different concentrations of phytate *viz.* 5 mg/g, 6 mg/g and 7 mg/g were spiked in sourdough.

CHAPTER 5- RESULTS AND DISCUSSION

5.1 Screening of potential proteolytic LAB

Protease activity of twenty six LAB isolates was assessed based on their ability to produce clear zone on skim milk agar. Skim Milk is a source of lactose and casein and other nutrients required for the growth of LAB. A high throughput assay was developed for this purpose. Briefly, overnight cultures were grown in microtitre plate. 25 µl culture supernatant was taken by a multichannel pipette and loaded in the wells of skim milk agar. Out of 26 isolates, one strain, namely *Lactococcus lactis*, was selected for further studies as it exhibited highest proteolytic activity amongst all the isolates. The results indicated that protease was probably extracellular and was proved upon by assaying supernatants. Boiled or PMSF (phenylmethylsulfonyl fluoride, a serine protease inhibitor) treated supernatants lacked the activity. This is in confirmation with the results given by Lowe *et al.* (2005) who demonstrated that *L. amylovorus* FST 1.1 exhibited protease activity when tested on skim milk agar plate.

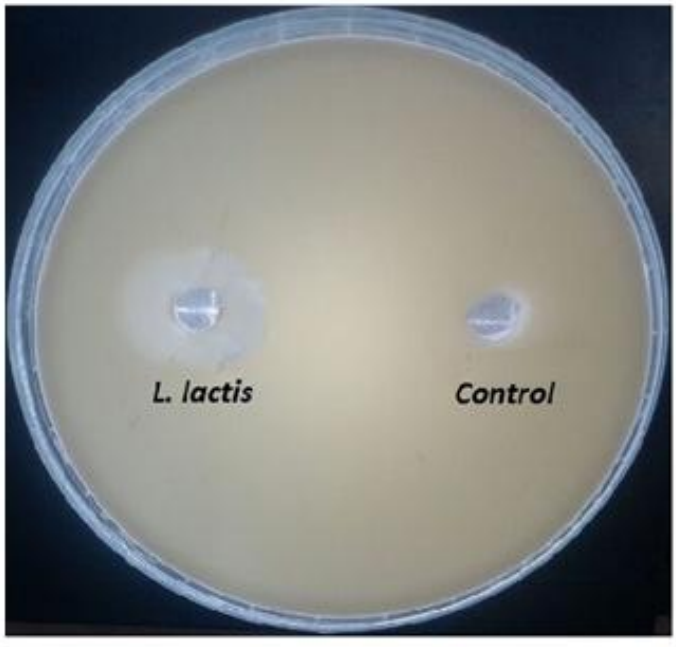
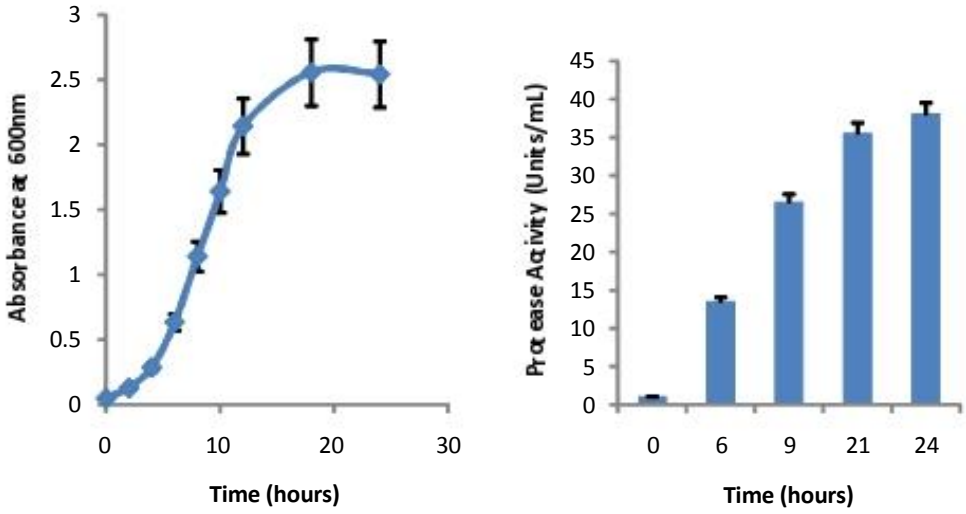


Figure6. Proteolytic Activity of *Lactococcus lactis* on Skim Milk Agar.

5.2 Growth Profile



(a) (b)

Figure 7. (a) Kinetics of growth and (b) extracellular protease production of *Lactococcus lactis*. Error bars represent the standard deviation from the mean values of three experiments.

To study the kinetics of protease production, the viable cells of the bacterium were inoculated on to the sterile MRS broth and incubated at 37ºC for 24 h. The dynamics of the bacterial growth can be studied by plotting the cell growth (absorbance) versus the incubation time. Fig.1 shows the protease production by *Lactococcus lactis* during the course of its growth. The lag phase of *L. lactis* appears after 2 h of growth but is negligible. The cellular metabolism is accelerated, cells increase in size, but the bacteria are not able to replicate and therefore no increase in cell mass. The bacteria start synthesising the necessary proteins, co-enzymes and vitamins needed for their growth. However, protease production was minimum at this stage (1 U/mL). *L. lactis* has a log phase until approximately 12 h of growth. During this phase, the microorganisms are in a rapidly growing and dividing state. The growth

medium is exploited at the maximal rate, the culture reaches the maximum growth rate and the number of bacteria increases logarithmically (exponentially). The protease activity also increased notably during this phase (26.5 U/mL). The specific growth rate of *L. lactis* during this phase was 0.069 h^{-1}. Although, as *L. lactis* continues to grow and achieve high cell density at18 h, the growth remains constant for a while as it reaches stationary phase. *L. lactis* has a stationary phase until approximately 22 h of growth. Protease activity was also observed to be increasing in this phase. The maximum protease activity found during this phase was 38U/mL, which is in agreement with results of Kholif *et al.* (2011) who observed that the maximum protease activity (2.620 U/mL) of *Lactobacillus rhamnosus* NRRl B-445 appeared at the beginning of stationary phase. Later, the growth declined at the end of stationary phase due to the accumulation of waste materials, toxic metabolites and inhibitory compounds such as antibiotics in the medium. At this phase, the protease activity remains constant.

5.3 Kinetics of protease production

Optimization of culture conditions was done to obtain maximum growth and protease production of *Lactococcus lactis*. Effect of different physical parameters was tested such as agitation, temperature, pH, inoculum size, carbon source, nitrogen source and protein source.

5.3.1 Effect of agitation:

Agitation rate is one of the indispensable parameter for proper oxygen transfer and homogeneous mixing of the nutrients in fermentation system. Oxygen acts as a terminal electron acceptor for oxidative reactions to provide energy for cellular activities. The variation in the agitation speed influences the extent of mixing in the shake flasks and also affects the nutrient availability. The effect of agitation on proteolytic activity was investigated by incubating the inoculated MRS media on

shaking (20 rpm, 80 rpm, 100 rpm, 120 rpm, 200 rpm and 250 rpm) and static conditions.

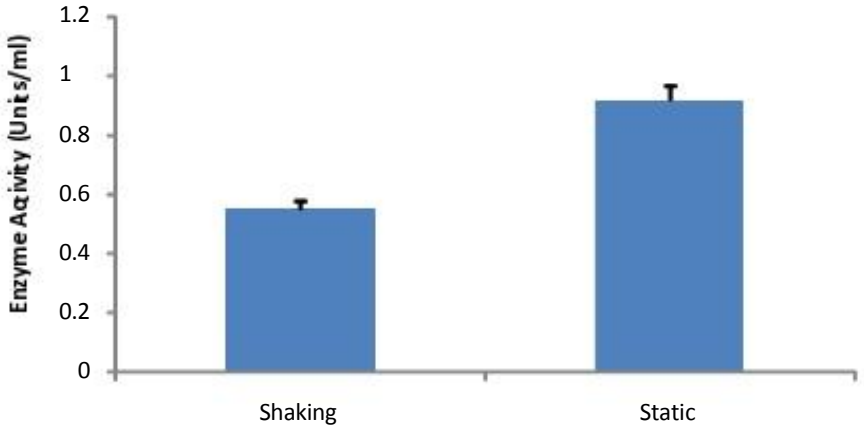


Figure 8. Effect of Agitation on Protease Activity by *L. lactis*. Error bars represent the standard deviation from the mean values of three experiments.

As shown in figure 8, protease activity is maximum under static conditions and is less under shaking conditions. The data shows that there was notable decrease in the proteolytic activity under agitating conditions. Hence, the optimal production of cell biomass and the highest protease yield (0.92 U/mL) were obtained at static conditions. Agitation reduced the cell growth and protease production due to sheer stress and heterogeneous mixing effects. Agitation affects both air bubble dispersion and mixing of nutrients during fermentation process. It increases the oxygen pressure (pO_2) of the system but does not increase production, probably because the structure of enzyme is altered. (Abusham *et al.*, 2009).

5.3.2 Effect of pH:

Microbial strains depend on the extracellular pH because culture pH strongly influences many enzymatic processes and transport of various components across the cell membranes, which in turn support the cell growth and product formation. To

investigate the effect of pH on the proteolytic activity of *Lactococcus lactis*, the initial pH of the media were adjusted to 4, 7 and 8, respectively. Culture supernatant solutions were examined for the presence of proteolytic activity after adjustment of all samples.

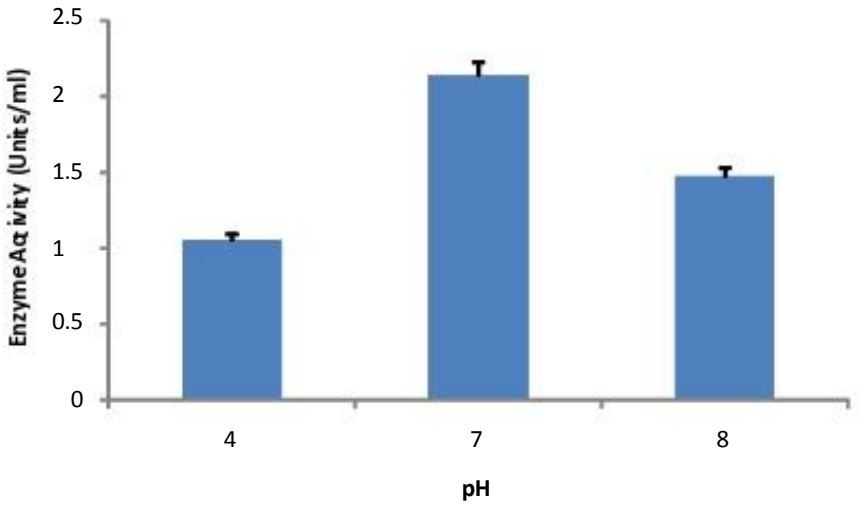


Figure 9. Effect of pH on Protease Activity by *L. lactis*. Error bars represent the standard deviation from the mean values of three experiments.

As shown in Figure 9, pH has a notable effect on the protease activity. A pH-dependent decrease in the protease activity of the broth samples was noted. An increase in the culture pH from 4 to 7 resulted in an increase in maximum protease activity from 1.05 U/mL to 2.14 U/mL. At pH 4, maximum protease activity was more severely affected than at pH 8. The specific protease activity at pH 4 was approximately 50% lower compared to cultures controlled at pH 7. With increase in pH from 7 to 8, the protease activity decreased from 2.14 U/mL to 1.47 U/mL. So, maximum enzymatic activity was obtained at pH 7. Higher or lower pH may lead to partial loss of protease activity by *L. lactis*. However, Akinkugbe *et al.* (2013) reported that the proteolytic activity of *L. lactis* exhibited a series of descending and ascending before stabilizing at its peak (2.90) both pH 5.5 and 6.0.

5.3.3 Effect of temperature:

Temperature is a critical parameter which needs to be controlled as it was found to influence the secretion of extracellular enzymes, possibly by changing the physical properties of the cell membrane.

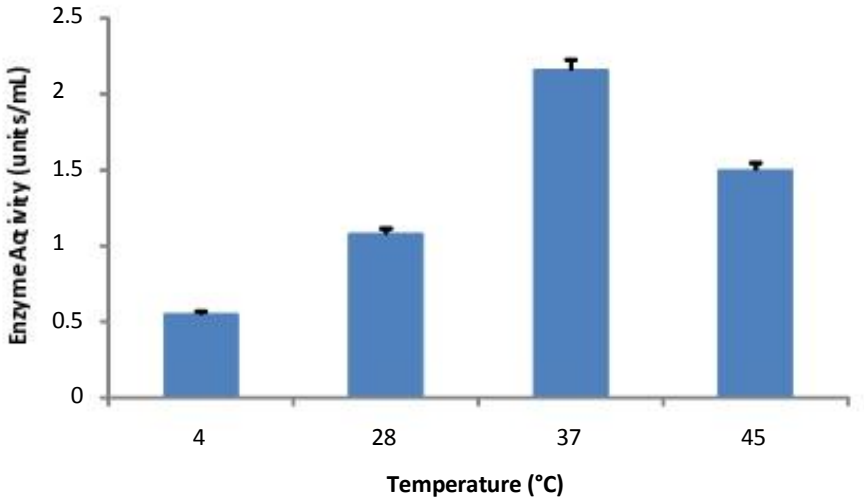


Figure 10. Effect of Temperature on Protease Activity by *L. lactis*. Error bars represent the standard deviation from the mean values of three experiments.

Figure 10 shows considerable variation in the protease activity under different temperature conditions *viz*. 4ºC, 28ºC, 37ºC and 45ºC. It was observed that the enzymatic activity was maximum at 37ºC (2.16 U/mL). The activity rapidly decreased at 45ºC, 28ºC and became negligible at 4ºC. When the temperature was increased to 45°C, the enzyme activity was reduced significantly to 1.5 U/mL ($p<0.05$). So, an increase in growth temperature to 45°C caused a further decrease in protease activity. Higher temperature is found to have some adverse effects on metabolic activities of microorganism and cause inhibition of the growth. The enzymes become denatured by losing its catalytic properties at high temperature. A decrease in growth temperature also led to a decrease in protease activity. When the temperature was decreased to 28ºC and 4ºC, the enzyme activity decreased to 1.08 U/mL and 0.55 U/mL, respectively. The

data indicated that the optimal temperature was 37ºC and was beneficial for protease activity as the activity was the highest at this temperature. By comprehensive consideration of the above data, 37ºC was selected to be optimum temperature for proteolytic activity for further studies. Akinkugbe *et al.* (2013) reported that the proteolytic activity of *L. lactis* reached its peak (2.32, peak height) at 28ºC, descended at 30ºC and re-ascended at 35ºC.

5.3.4 Effect of inoculum size:

To ensure a high production of enzyme in the limited volume of medium, the bacterial inoculum size should be controlled. Size of inoculum is an important biological factor in the production of the enzyme which determines biomass production in fermentation. Hence, a balance between the proliferating biomass and available materials will yield maximum enzyme production.

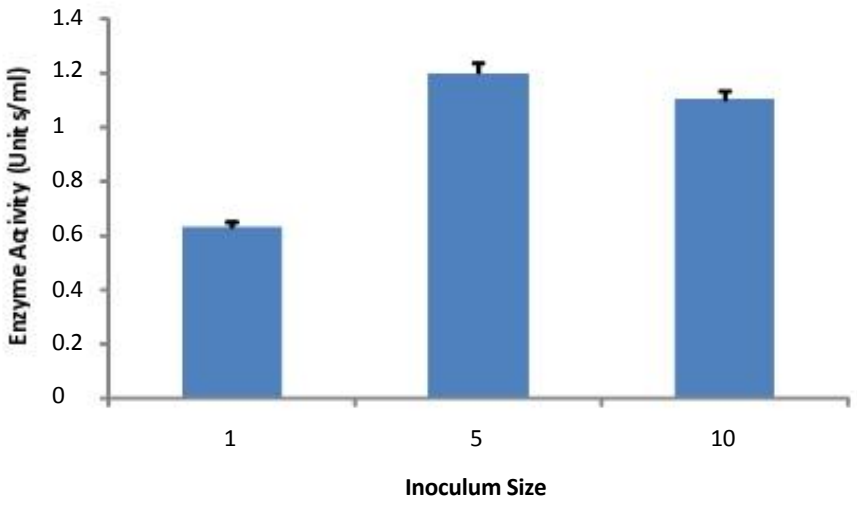


Figure 11. Effect of Inoculum Size on Protease Activity by *L. lactis*. Error bars represent the standard deviation from the mean values of three experiments.

Effect of inoculum size on proteolytic activity was investigated by incubating the inoculated MRS media with different amounts of inoculums *viz*.1%, 5% and 10%. The optimal inoculum size was found to be 5%. The maximum enzyme production was

observed with this inoculum size was 1.20 U/mL. Further increase in inoculum volume to 1% resulted in the decrease of protease activity to 1.1 U/mL. High inoculum sizes do not necessarily give higher protease yield probably due to as increase in inoculum volume caused overcrowding of spore that decreased the enzyme activity. As shown in Figure 11, inoculum size has a notable effect on the protease activity. It was observed that the enzymatic activity was maximum at 5%. Possibly, the increase in the production of protease using small inoculum sizes was due to the higher surface area to volume ratio, which resulted in the increased production of protease. The activity rapidly decreased at 1% to 0.63 U/mL. The above data indicated that efficient protease activity needed high cell density. If the inoculum sizes are too small, insufficient number of bacteria would then lead to a reduced amount of secreted protease. Akinkugbe *et al.* (2013) reported that at 0.5 mL inoculum concentration, protease production of *L. lactis* decreased after starting off with a high value at pH 4.0. At 1.0 mL inoculum concentration, it reached its peak at pH 5.0 and maintained it. At an inoculum concentration of 1.5 mL, it gradually rose to peak respectively at pH 5.0 and 5.5 to both become stable afterwards. With 2.0 mL concentration, protease production increased gradually, till a peak at 5.5 (peak height) was attained.

5.3.5 Effect of carbon source:

Carbon sources greatly influenced the enzyme production and a wide range of them serve as energy source. With different carbon sources impact on growth and production of extracellular protease was studied. To investigate the effect of different carbon sources on the protease activity of *Lactococcus lactis*, the MRS media were slightly modified and supplemented with different carbon sources *viz*. Glucose, Sucrose and Fructose.

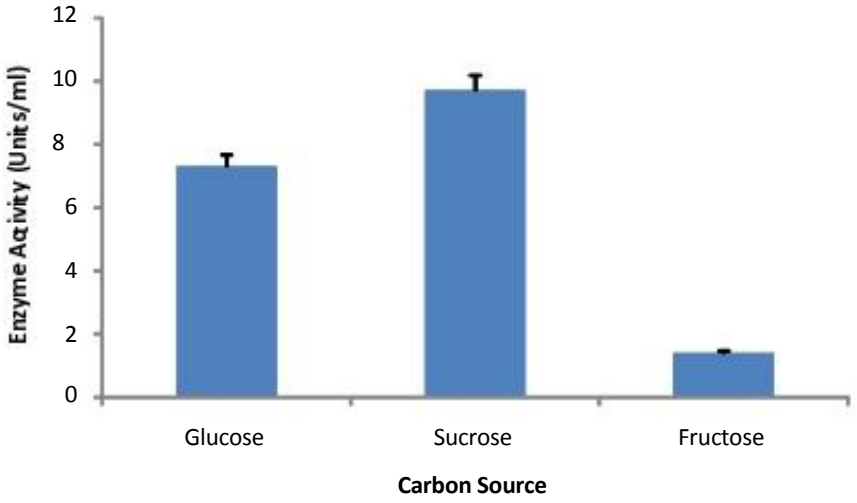


Figure 12. Effect of carbon source on Protease activity by *L. lactis*. Error bars represent the standard deviation from the mean values of three experiments.

Figure 12 shows that highest enzyme activity (9.7 U/mL) was obtained when the media was supplemented with sucrose among other carbon sources. Higher amount of protease is synthesized when carbon sources used is poorly utilized for growth purposes. The activity decreased with glucose (7.3 U/mL) as carbon source and was minimum with fructose (1.4 U/mL). These findings indicate that sucrose is the best carbon source for protease production by *L. lactis*. However, Hebert *et al.* (2008) reported that the proteinase specific activity values for *L. delbrueckii* subsp. *lactis* CRL 581 were independent of the carbon source.

5.3.6 Effect of Nitrogen Source:

The type of nitrogen sources also affects enzyme production (Sharma *et al.*, 2011). Medium supplemented with organic nitrogen sources supported higher protease production when compared to inorganic nitrogen sources (Narayana *et al*., 2008). The authors studied the effect of nitrogen sources like peptone, beef extract, casein, yeast extract, tryptone, $NaNO_3$, KNO_3 and NH_4Cl on production of protease by *Streptomycin albidoflavus*. Organic nitrogen sources were superior to inorganic nitrogen sources. To

study the effect of different nitrogen sources on the protease activity of *Lactococcus lactis*, the MRS media were slightly modified and supplemented with different nitrogen sources *viz*. potassium nitrate (KNO_3), peptone, glutamic acid and yeast extract.

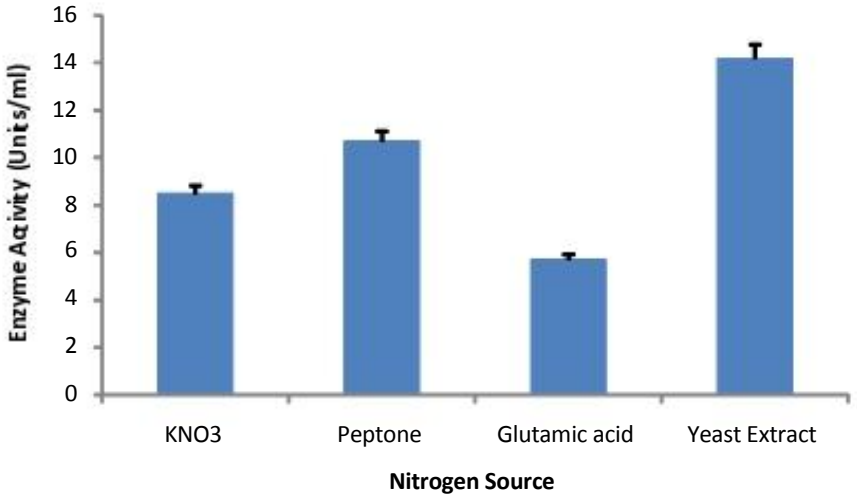


Figure 13. Effect of nitrogen source on Protease Activity by *L. lactis*. Error bars represent the standard deviation from the mean values of three experiments.

Figure 13 shows considerable variation in the protease activity with different nitrogen sources. The enzyme activity was maximum with yeast extract (14.2 U/mL). Moderate to good levels of enzyme activities were obtained when peptone (10.7 U/mL) was used as nitrogen source. The activity decreased with KNO_3 (8.49 U/mL) and was least with glutamic acid (5.7 U/mL). Hebert *et al.* (2000) reported that the proteinase (PrtH) activity levels of *L. helveticus* CRL 1062 cells grown in MRS broth and in SCDM supplemented with Casitone were approximately 32- and 11-fold lower, respectively, than those from cells grown in basal SCDM. The decrease of PrtH production was less marked in cells grown in SCDM supplemented with β-casein (about a 1.7-fold reduction) than in cells grown in SCDM supplemented with Casitone.

5.3.7 Effect of protein source:

To study the effect of different protein sources on the protease activity of *Lactococcus lactis*, the MRS media were supplemented with different protein sources *viz*. Gliadin, Casein and Gelatin.

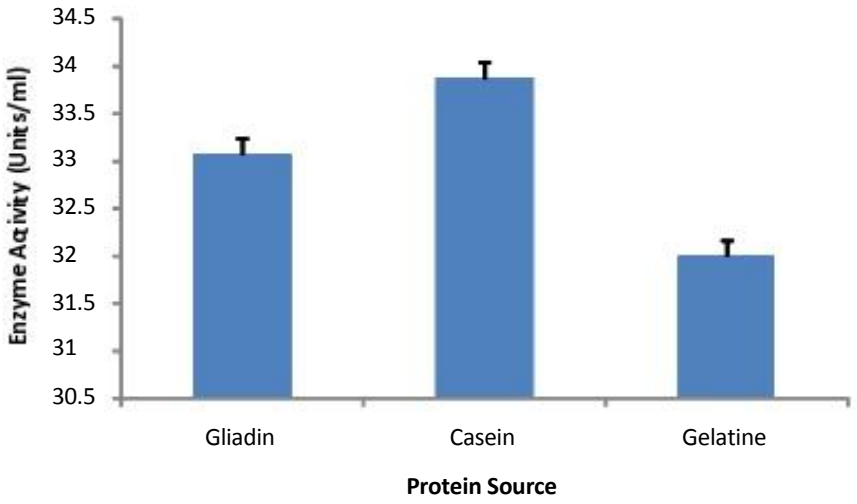


Figure 14. Effect of Protein Source on Protease Activity by *L. lactis*. Error bars represent the standard deviation from the mean values of three experiments.

Figure 14 depicts that highest enzyme activity was obtained when the media was supplemented with Casein (33.87 U/mL). It is possible that casein as a high molecular weight protein induces an increase in the protein production to degrade the substrate to an available form for utilization by the organism. The activity decreased with Gliadin (33.07 U/mL) as protein source and was minimum with Gelatin (32 U/mL).

5.3.8 Protease activity under optimized culture conditions:

To study the overall effect of the optimized parameters i.e. the optimum pH, temperature, inoculum size, carbon source, nitrogen source and protein source on the protease activity of *Lactococcus lactis*, the MRS media was modified and incubated using complete conditions described above.

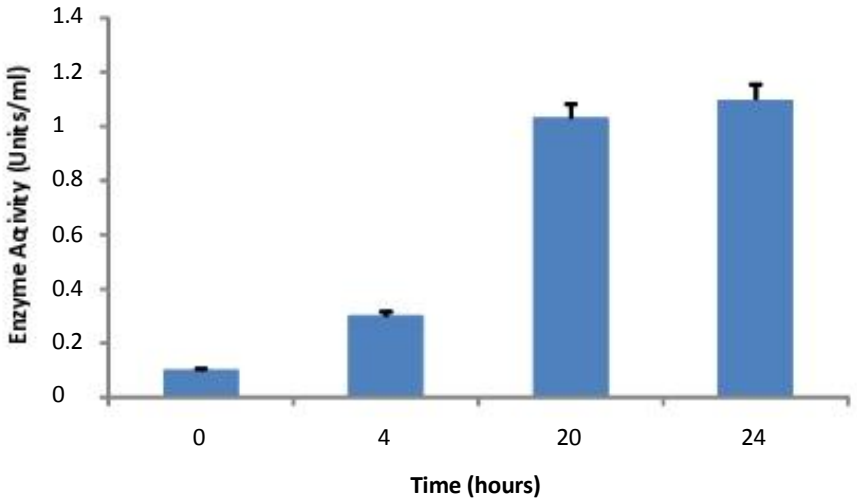


Figure 15. Effect of Optimized Parameters on Protease Activity by *L. lactis*. Error bars represent the standard deviation from the mean values of three experiments.

Figure 15 depicts that maximum protease activity with all the optimized parameters was achieved at 24 h (1.1 U/mL). The results indicated that the highest specific protease activity was observed at the end of the exponential growth phase. An extracellular nature of protease has been documented by other studies as well. For instance, the protease enzyme produced by *L. lactis* ssp. *lactis* is an extracellular serine proteinase (PrtP). Meijer *et al.* (1996) used two different *Lactococcus lactis* host strains, *L. lactis* subsp. *lactis* MG1363 and *L. lactis* subsp. *cremoris* SK1128, both containing plasmid pNZ521, which encodes the extracellular serine proteinase (PrtP) from strain SK110, to study the medium and growth-rate-dependent activity of proteinase enzyme involved in the proteolytic system of lactococci. The dry cell weight of *L. lactis* culture was measured to estimate the amount of protease activity present. The dry cell weight was found to be 2 mg. Hence, it can be said that 2 mg of *L. lactis* culture contains 1.1 U/mL protease activity. So, the amount of enzyme activity present would be 550 U/mL/g.

5.4 Protease assay with malathion:

The exploitation of micro-organisms especially lactic acid bacteria due to its fast growth and metabolism for successful degradation of organophosphorus pesticides has gained much importance. Keeping in view the importance of microbes in detoxification and the possible contamination of food matrix in relation to protease, experiment was carried out to degrade malathion by *L. lactis* in MRS media.

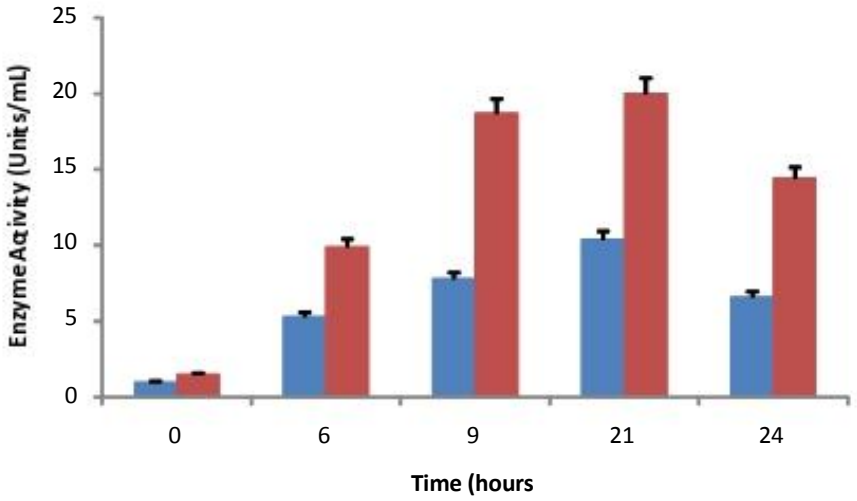


Figure 16. Protease Activity in MRS without Malathion (■) and with Malathion (■). Error bars represent the standard deviation from the mean values of three experiments.

Maximum protease activity appeared at the end of exponential phase, both in MRS without malathion and MRS with malathion. Protease activity increased with the increase in incubation time till 21 h incubation, and then the specific activity decreased. However, enzyme activity was even higher with malathion indicating the proteolytic activity of culture on malathion. The highest activity was found at 21 h (20 Units/mL), then the specific activity decreased. This can be due to the beginning of cell autolysis. The factor responsible for the proliferation of protease activity in the presence of malathion is organophosphorus hydrolase (OPH) activity in *L. lactis* which is simultaneously induced (Result for OPH activity given in annexure 2). This activity of

the bacteria allows it to degrade various proteins in dough without any restriction from organophosphorus pesticides, which are usually sprayed on wheat, black gram and other crops to control a variety of outdoor pests in both agricultural and residential settings.

Organophosphorus pesticide residues are frequently encountered in urad dal and wheat. Minimum residue limits (MRLs) recommended by WHO/FAO (Greene & Pohanish, 2005) for malathion, chlorpyriphos, bifenthrin and permethrin, respectively in cereal grains (wheat) are 8 mg/kg, 1 mg/kg, 2 mg/kg and 1 mg/kg, respectively as reported by Khan *et al*. (2007). MRL is the legal limit of pesticide allowed in food or animal feeds and it is expressed in mg/kg produce.

5.5 Behaviour and proteolytic activity in sourdough

5.5.1 Preparation of dough extracts:

Sourdough is a process in which wheat flour and water (and other ingredients) are fermented with microbes. The preparation of sourdough begins with a pre-ferment, (the "starter"), made of flour and water. The purpose of the starter is to produce a vigorous leaven and to develop the flavour of the bread. Inoculation of the sourdough with a starter increases the number of lactic acid bacteria to 10^7-10^8 CFU/g, which gives little possibility for growth of contaminating organisms, including those which are imported from flour. Dough extracts from different hours of fermentation *viz.* 1 h, 2 h, 3 h and 4 h were prepared as described by Bhanwar *et al.* (2013) under sterile conditions and were stored in freezer at 0ºC for future use.

5.5.2 Survival of *L. lactis* in dough:

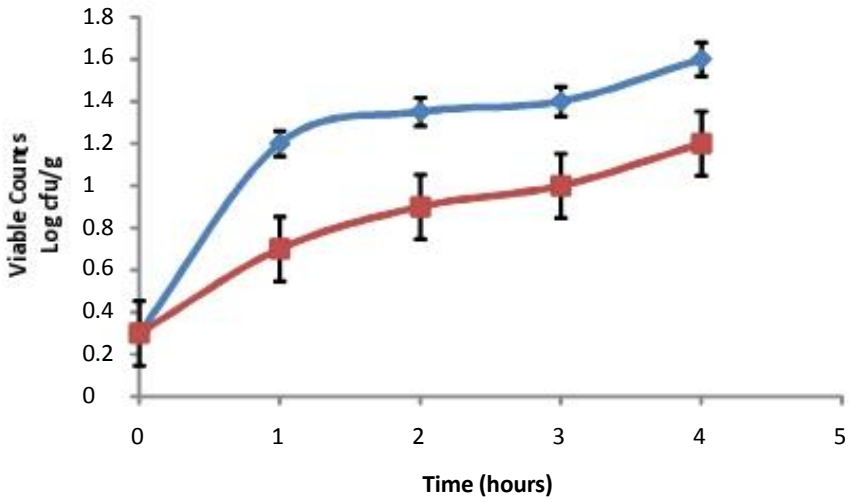


Figure 17. Viable Count (CFU/g) of sourdough prepared with (■) control (without culture) and (♦) *Lactococcus lactis*. Error bars represent the standard deviation from the mean values of three experiments.

Figure 17 depicts viable counts (cfu/g) in dough which is maximum with culture of *Lactococcus lactis* than without it. At 0 h, bacterial colonies were not observed as the fermentation process has just started. Following incubation at 37ºC, cells start growing and viable colonies are observed after 1 h (1.2×10^9 cfu/g). Viable colonies are observed after 4 h (1.5×10^9 cfu/g).

5.5.3 Determination of protein content in dough:

Protein content in sourdough samples (wheat flour + urad dal) from different hours of fermentation was estimated by using Folin Lowry method. Figure 18 below shows the different amount of protein content present in inoculated and uninoculated dough samples at different intervals of time of fermentation. It was observed that the protein content in uninoculated samples was almost negligible and was constant throughout the fermentation process. On the other hand, in inoculated dough samples, the protein content was maximum (1.53 mg/g) at the beginning of fermentation and

gradually decreased with the commencement of the process. After 2 h of fermentation, the protein was completely degraded as the protein content present was negligible, i.e. 0.24 mg/g.

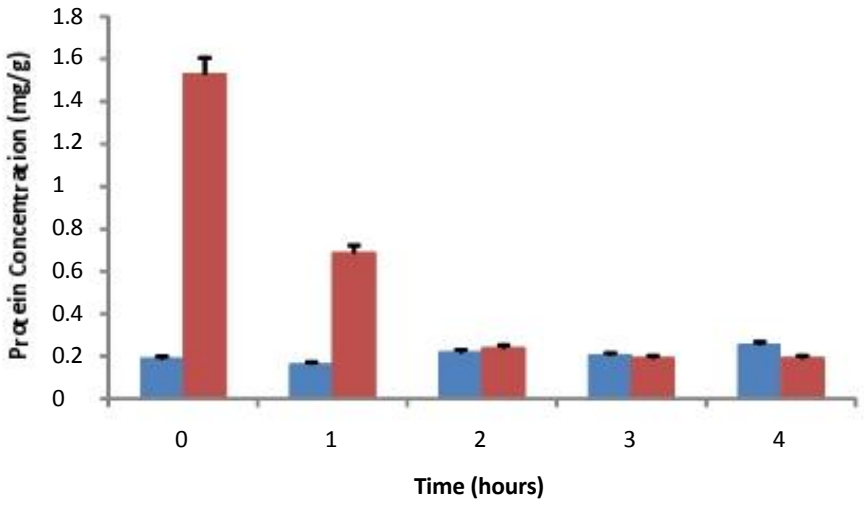


Figure 18. Determination of Protein Concentration in inoculated (■) and uninoculated (■) dough samples (wheat flour + *Vigna mungo*). Error bars represent the standard deviation from the mean values of three experiments.

The pH of the dough samples was also estimated. The pH values obtained for different dough samples were: after 0 h incubation, uninoculated-7.0, inoculated-7.0; after 2 h incubation, uninoculated-7.0, inoculated-4.5. There was a decrease in pH of inoculated dough after 2 h incubation. Maximum protein degradation is also observed in this sample. Komen *et al.* (2001) determined the pH and acidity profiles of sourdough samples. They reported that at the end of fermentation, pH decreased from 6.1-6.2 range to 3.8-3.5, total titratable acidity was reached the 13.49-17.34 range, and the LAB population was 10^8-10^9 cfu/g dough.

Protein content in sourdough samples (wheat flour) lacking urad dal from different hours of fermentation is shown in figure 19 below. It was observed that the protein content in uninoculated samples remained almost constant and negligible. On

the other hand, in inoculated dough samples, the protein content was maximum (1 mg/g) at the beginning of fermentation and gradually decreased with the commencement of the process. After 2 h of fermentation, the protein was completely degraded as the protein content present was negligible, i.e. 0.15 mg/g. But the protein content is less in dough samples lacking *Vigna mungo* (1 mg/g) than the dough samples containing it (1.53 mg/g). The protein content of the flour of two wheat (*Triticum aestivum* L.) cultivars was also determined by Arumugam (2012).

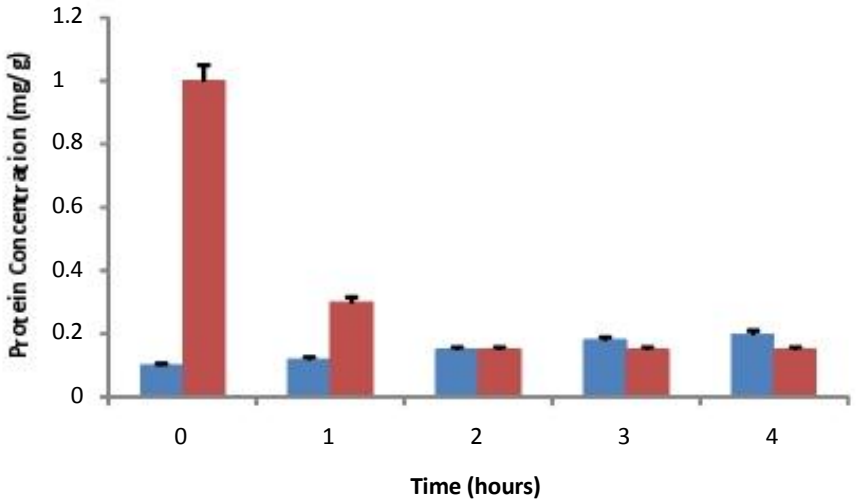


Figure 19. Determination of Protein Concentration in inoculated (■) and uninoculated (■) dough samples (wheat flour). Error bars represent the standard deviation from the mean values of three experiments.

Thus, *Vigna mungo* (black gram) provides a better composition to the sourdough and makes it nutririonally better than wheat dough. Better degradation of protein was also observed with dough containing *Vigna mungo* than with dough without it due to higher protease activity. Moreover, black gram serves as the best plant source of protein. It contains iron, folic acid, calcium, magnesium, potassium, and B vitamins but contains lesser amount of fat and high calories. It is also rich in fibre content and thereby enhances digestion. Consumption of black grams also helps to reduce

cholesterol and improves cardiovascular health. It contains high levels of magnesium and folate which aids in blood circulation.

5.5.4 Protease assay:

Sourdough fermentations were carried out using pure culture of *L. lactis*. Proteolysis due to LAB, in fact, increases the total concentration of free amino acids, especially aliphatic, dicarboxylic, and hydroxy amino acids which are stimulatory for bacterial growth. This was confirmed by evaluating the free protein content of dough samples. Generally, the concentration of protein decreased during fermentation and roughly became negligible within 2 h. In dough, many proteolytic enzymes are attached to the gluten but also that they are not very stable and can therefore easily be destroyed. To study the protease activity in sourdough, with and without black gram (*Vigna mungo*), it was inoculated with *L. lactis* and incubated at 37ºC for 4 h to carry out fermentation.

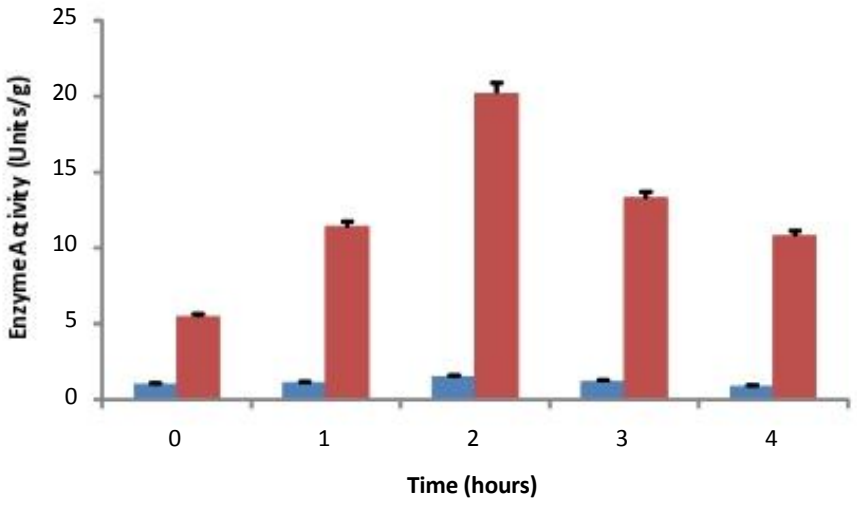


Figure 20. Protease Activity in Sourdough (wheat flour + urad dal) (inoculated ■ and uninoculated ■). Error bars represent the standard deviation from the mean values of three experiments.

Figure 20 depicts the enzyme activity in inoculated and uninoculated dough samples of sourdough containing *Vigna mungo* from different hours of fermentation. The activity was negligible in uninoculated dough. There was notable enzyme activity observed in inoculated dough samples. The activity was minimum at 0 h (5.48 U/g), reached maximum value at 2 h (20.26 U/g) and started decreasing afterwards.

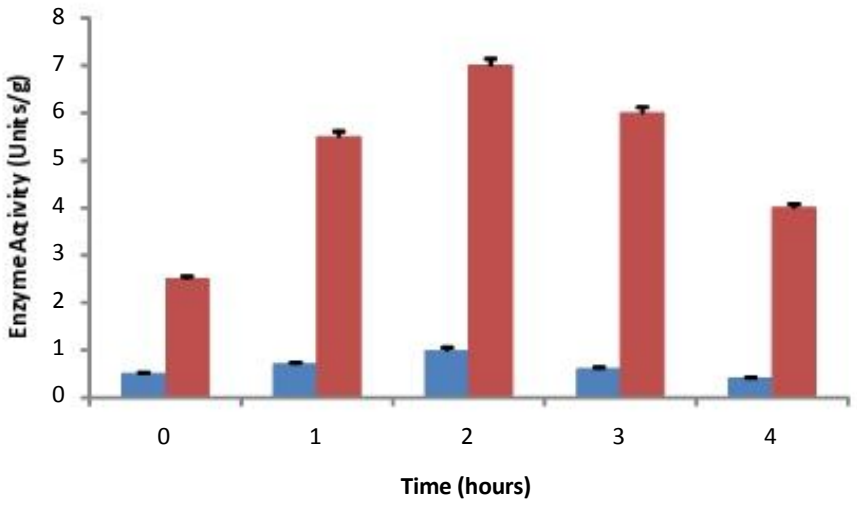


Figure 21. Protease Activity in Sourdough (Wheat flour) (inoculated ■ and uninoculated ■). Error bars represent the standard deviation from the mean values of three experiments.

Figure 21 shows the enzyme activity in inoculated and uninoculated dough samples of sourdough without *Vigna mungo* from different hours of fermentation. The activity was negligible in uninoculated dough. The activity in inoculated dough was minimum at 0 h (2.5 U/g), reached maximum value at 2 h (7 U/g) and started decreasing afterwards. This is in congruence with the observations of Wehrle *et al.* (1999) who reported that incubation of the enzymes with twice the amount of casein resulted in a doubling of the values of absorption within a 2 h incubation time. They also observed that after 2 h of fermentation, the concentration of product in relation to substrate suppressed the enzymatic reaction and absorption remained constant.

Rollán *et al.* (2005) evaluated the capacity of *Lactobacillus plantarum* CRL 759 and CRL 778 strains to hydrolyse synthetic peptides, homologous to the α-gliadin 31-43 amino sequence. After 4 h of incubation, hydrolysis of the 31-43 peptide of *α*-gliadin by enzyme preparations of *L. plantarum* CRL 759 and CRL 778 was 73% and 36% (expressed as percentage reduction of the peak area of the untreated substrate) respectively.

5.5 Optimization of protease activity in sourdough

5.5.1 Effect of incubation period:

The incubation period of a fermentation process has a direct relationship with the growth of microorganism and production of enzymes.

Table1. Protease Activity in Sourdough at different intervals of incubation time.

Time of Incubation (hours)	Protease Activity (Units/mL) (Mean Value ± S.D.*)
0	5.48±0.01
1	11.4±0.03
2	20.26±0.02
3	13.3±0.05
4	10.8±0.01

*Standard Deviation

Table 1 shows the different amount of protease activity encountered in dough samples at different intervals of incubation time. It was found that the maximum production of protease (20.26 U/mL) was achieved after 2 h of incubation. It is clear from the results that the incubation period of more or less than 2 h did not show the

promising results as far as production of the enzyme is concerned. Moreover, there was a decrease in the amount of protease activity by *L. lactis* after 2 h of incubation.

5.5.2 Effect of inoculum size:

To ensure a high production of enzyme in the limited volume of medium, the bacterial inoculum size should be controlled. Effect of inoculum size on proteolytic activity was investigated by incubating the inoculated sourdough with different amounts of inoculum *viz*.1%, 5% and 10%. The optimal inoculum size was found to be 1%. The maximum enzyme production was observed with this inoculum size was 18.2 U/mL. Further increase in inoculum volume to 5% and 10% resulted in the decrease of protease activity to 14.1 U/mL and 10.5 U/mL. High inoculum sizes do not necessarily give higher protease yield probably because increase in inoculum volume caused overcrowding of spore that decreased the enzyme activity. Possibly, the increase in the production of protease using small inoculum sizes was due to the higher surface area to volume ratio, which resulted in the increased production of protease.

Table2. Protease Activity in Sourdough with different inoculum sizes.

Inoculum Size (%)	Protease Activity (Units/mL) (Mean Values ± S.D.*)
1	18.2±0.04
5	14.1±0.05
10	10.5±0.03

*Standard Deviation

5.5.3 Effect of temperature:

Variation in the protease activity under different temperature conditions *viz*. 4ºC, 28ºC, 37ºC and 45ºC was determined. It was observed that the enzymatic activity

was maximum at 37ºC (20.16 U/mL). The activity rapidly decreased at 45ºC, 28ºC and became negligible at 4ºC. When the temperature was increased to 45°C, the enzyme activity was reduced significantly to 10.5 U/mL. Higher temperature is found to have some adverse effects on metabolic activities of microorganism and cause inhibition of the growth. The enzymes become denatured by losing its catalytic properties at high temperature. A decrease in growth temperature also led to a decrease in protease activity. When the temperature was decreased to 28ºC and 4ºC, the enzyme activity decreased to 10.08 U/mL and 5.55 U/mL, respectively. The data indicated that the optimal temperature was 37ºC and was beneficial for protease activity as the activity was the highest at this temperature. Rollán *et al.* (2005) examined the effect of pH on the activities of enzymes (AP, DP, TP and EP) of *Lactobacillus plantarum* CRL 759 and CRL 778 at pH 7.0 in the temperature range of 30º-60ºC. They reported that optimum temperature values for all the peptidase activities were 37º-45ºC and high peptidase activities at the temperatures (30º-35ºC) of sourdough fermentation.

Table3. Protease Activity in Sourdough at different temperatures

Temperature (ºC)	**Protease Activity (Units/mL) (Mean Values ± S.D.*)**
4	5.55±0.02
28	10.08±0.05
37	20.16±0.03
45	10.5±0.01

*Standard Deviation

5.5.4 Effect of pH:

To investigate the effect of pH on the proteolytic activity of *Lactococcus lactis* in sourdough, the initial pH of was adjusted to 4.0, 7.0 and 8.0, respectively. Dough

extracts were examined for the presence of proteolytic activity after adjustment of all samples. An increase in the culture pH from 4.0 to 7.0 resulted in an increase in maximum protease activity from 10.05 U/mL to 20.14 U/mL. At pH 4.0, protease activity was more severely affected than at pH 8.0. With increase in pH from 7.0 to 8.0, the protease activity decreased from 20.14 U/mL to 10.47 U/mL. So, maximum enzymatic activity was obtained at pH 7.0.

Table4. Protease Activity in Sourdough at different pH values

pH	Protease Activity (Units/mL) (Mean Values ± S.D.*)
4.0	10.05±0.01
7.0	20.14±0.03
8.0	10.47±0.04

*Standard Deviation

Rollán *et al.* (2005) examined the effect of pH on the activities of enzymes (AP, DP, TP and EP) of *Lactobacillus plantarum* CRL 759 and CRL 778 at 37ºC in the pH range of 4.0-9.0. The optimum pH values for all the peptidase activities were 6.0-8.0 and high peptidase activities were observed at the pH 4.5-5.5.

5.5.5 Effect of selected intrinsic and extrinsic factors on protease activity:-

5.5.5.1 Water Activity:

Water is required to combine ingredients, which then binds the ingredients and forms a dough mass. It acts as a lubricant to disperse ingredients uniformly, and combines with flour to form gluten. Additionally, the amount of water affects the fermentation rate, which maintains the quality and strength of finished products. The results indicated that the water activity of 0.94, 0.96 and 0.99 had a little impact on

protease activity which is in agreement with those of Troller *et al.*, 1981 who observed that the minimal water activity ($a_{w)}$, at which growth of either *Streptococcus cremoris* or *Streptococcus diacetilactis* occurred was 0.95.

5.5.5.1 Organophosphorus compounds:

Organophosphates are organic esters of phosphoric acid, thiophosphoric acid and other phosphoric acids, which are widely used as insecticides and acaricides. Organophosphorus compounds exhibit a wide range of toxicity to mammals. They work on central nervous system inhibiting its normal function resulting in convulsions, paralysis and death. The pesticide tolerance set by the Food and Drug Administration ranges from 1 to 8 gmL^{-1} for all these pesticides. When different concentrations of malathion (10ppm, 20ppm, 40ppm and 80ppm) were added to the prepared dough, protease activity was observed probably due to organophosphorus hydrolase (OPH) activity, which is simultaneously induced in *L. lactis*.

5.5.5.3 Phytate Content:

L. lactis, which has been shown to produce phytase, a phosphatase enzyme that catalyzes the hydrolysis of phytic acid and releases a usable form of inorganic phosphorus (Bhanwar *et al.*, 2013, unpublished observation), spiking three concentrations of phytate *viz.* 5 mg/g, 6mg/g and 7 mg/g in dough changes the protease activity.

5.6 Sensorial Analysis of Sourdough:

After the completion of fermentation, acceptability of the dough was tested by a panel of 5 persons. A high scale acceptibilty of 8.15±0.08 of sourdough suggested suitability for further use either for frying or baking.

Table.5 Sensorial analysis of Sourdough with *Lactococcus lactis*

Visual Attributes	Sourdough
Texture(9)	7.5±0.05
Colour and Appearance(9)	8.5±0.05
Aroma(9)	8.6±0.05
Acidity(9)	8±0.05
Overall acceptability(9)	8.15±0.08

*compared with standard (commercially available sourdough)

Overall, the *Lactococcus lactis* strain was shown to be a good protease producer as determined by its profile under culture conditions as well as in a composite cereal matrix. The strain, thus, may be exploited further as a starter.

CONCLUSION

1. In this study, a high protease producing strain of lactic acid bacteria, *Lactococcus lactis* subsp. *lactis* was evaluated.
2. Protease activity was primarily extracellular and was highest under the following optimized conditions; pH-7.0, temperature-37ºC, inocula-5%, carbon source-sucrose, nitrogen source-yeast extract, protein source-casein and without agitation, in MRS.
3. Adequate viability of *L. lactis* subsp. *lactis* in sourdough amended with *Vigna mungo* flour was observed and yielded high protease activity.
4. Protease activity of *L. lactis* remained unaffected under selected intrinsic and extrinsic factors namely, phytate content, water activity, temperature and organophosphorus pesticides (malathion).

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ANNEXURE 1

1. Media

Composition of MRS medium

Peptone	10.0 g
Beef extract	10.0 g
Yeast extract	5.0 g
Glucose	20.0 g
Na_2HPO_4	2.0 g
Sodium acetate	5.0 g
Triammonium citrate	2.0 g
$MgSO_4.7H_2O$	0.2 g
$MnSO_4.4H_2O$	0.2 g
Tween 80	1.0 mL
Distilled water	1000 mL

Composition of skim milk agar

Skim milk powder	10.0 g
Agar	5.0 g
Distilled Water	300 mL

2. Chemicals and reagents

0.1 M Phosphate buffer saline (PBS):

NaCl	8.01 g
KCl	0.2 g
$Na_2HPO_4.2H_2O$	0.17 g
KH_2PO_4	0.27 g
Distilled water	1000 mL

Protein estimation:

Solution A- Sodium potassium tartrate	0.1g/10mL + 0.05g $CuSO_4$
Solution B- Alkaline sodium carbonate solution	2%Na_2CO_3 in 0.1 N NaOH
Alkaline Solution	4mL A + 200mL B

Protease assay:

Substrate solution: 0.788 g tris buffer was dissolved in 50 mL distilled water. Adjusted the pH to 8.0 with 1N NaOH and made up the volume to 100 mL with distilled water. 0.6 g casein was dissolved in the above solution.

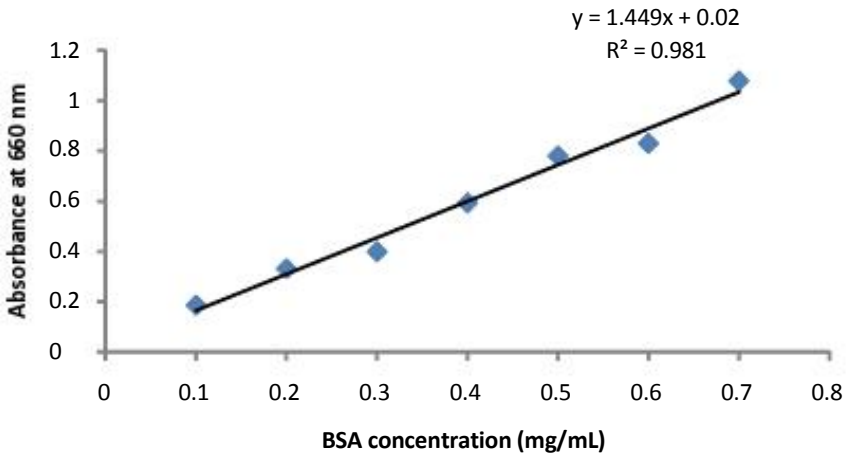
TCA mixture: 0.89 g trichloroacetic acid (0.11 M), 0.9 g sodium acetate (0.22 M) and 0.99 mL acetic acid (0.33M) were dissolved in 50 mL distilled water and made up the volume to 150 mL with distilled water.

Malathion: Weighed 10 mg malathion and dissolved in 100 µL glacial acetic acid and made up the volume to 10 mL with acetic acid.

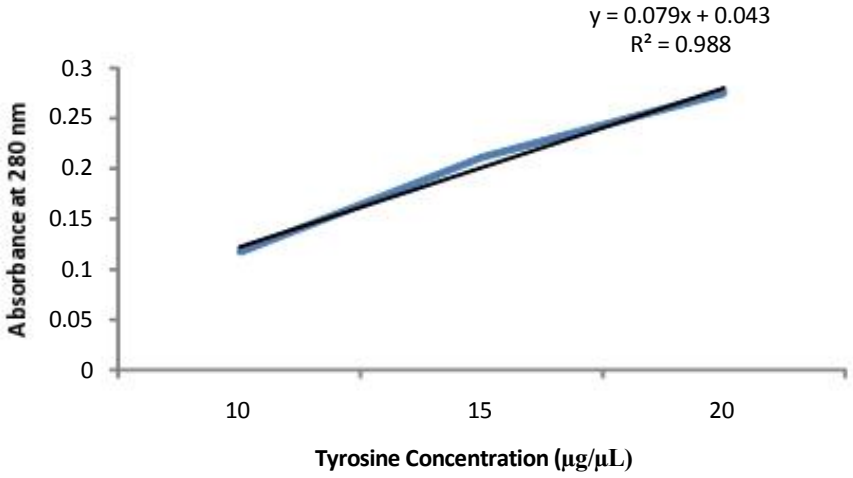
ANNEXURE 2

Standard Curves

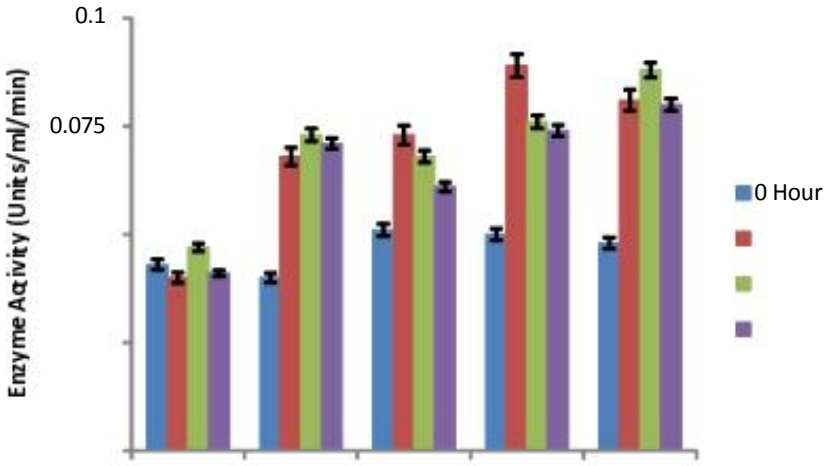
Protein estimation:

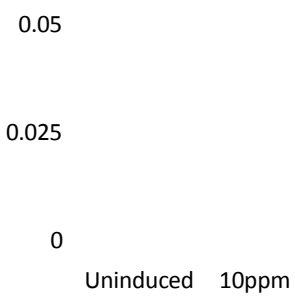


Protease assay:



Organophosphorus Activity:




0.05
0.025
0
Uninduced
10ppm

6 hours

18 Hours

24 Hours

40ppm 80ppm 100ppm

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