REDUCTION OF PHYTIC ACID IN FOOD USING SPECIFIC

STARTER CULTURES

A Thesis

Submitted in the partial fulfillment of the requirement for the award of the degree of

MASTER OF SCIENCE

IN

MICROBIOLOGY

Under the guidance of

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CANDIDATE'S DECLARATION

I hereby declare that the work which is being presented in the dissertation entitled, **"Reduction of Phytic acid in food using specific Starter Cultures"** in partial fulfillment of the requirement for the award of the degree of Masters of science in Microbiology, Department of Biotechnology, Thapar university, Patiala, Punjab; is an authentic record of my own work during a period of five months from January 2014 to May 2014, under the supervision of Dr.Abhijit Ganguli, Associate Professor, Department of Biotechnology, Patiala. The matter embodied in this thesis has not been submitted in part or full to any other university or institute for the award of any other degree.

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This is to certify that above statement made by the candidate is correct and true to the best of my knowledge.

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ABSTRACT

In the present study, twenty six strains of previously isolated Lactic acid bacteria (LAB) were examined for phytate degradation. A strain of *Lactococcus lactis* subsp. *lactis*, showed highest phytate reduction in de Man Rogosa Sharpe (MRS) media and was examined further. Incubation with optimized parameters such as temperature of 37˚C, inoculum size,(5%) sucrose, di hydrogen phosphate as nitrogen source and casein as protein source, agitation, pH of 5 resulted in an overall reduction of 40% phytate. Phytate reduction was mediated primarily by intracellular phytase. When composite dough (wheat flour +*Vigna mungo* flour) was fermented with *Lactococcus lactis*, same reduction of phytic acid was achieved at 37˚C and pH 5.0, no reduction of phytate could be observed in uninoculated composite sourdough. Phytate reduction remained unaltered in the composite sourdough matrix, indicating that phytase activity was unaffected by food matrix conditions. Phytate did not hamper biofilm producing capabilities of *Lactococcus lactis* and the reduction achieved was higher (50.26%) than that observed for plank tonic cells (40%). Thus the results suggest *Lactococcus lactis* as a potential phytate reducing strain.

Keywords: Lactic acid bacteria, phytate reduction, sourdough, *Lactococcus lactis*.

LIST OF ABBREVIATIONS

$CaCl_2$	Calcium Chloride
MPSN	Modified phytase screening media
LAB	Lactic acid bacteria
˚C	Degree Celsius
HCL	Hydrocloric acid
L.lactis	*Lactococcus lactis*
MRS	Man Ragosa sharpe
O.D.	Optical density
psi	Per square inch
Pi	Inorganic phosphate
NaOH	Sodium hydroxide

LIST OF SYMBOLS

%	Percentage
µl	Micro litre
µm	Micrometre (1×10^{-6}m)
^{0}C	degree(s) Celsius
Gm	Gram
Mg	Miligram
mg/ml	Milligram per milliliter
Min	Minutes
Ml	Mililitre
mM	Milimolar
U	Units

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CHAPTER 1
INTRODUCTION

1.1 PHYTIC ACID

Phytic acid also known as *myo*- inositol hexakisphosphate (IP6), or phytate when in salt form, a saturated cyclic acid, is the principal storage form of phosphorus in many plant tissues, especially bran and seeds.

For centuries phytic acid a component of whole wheat grain has been posed a nutritonal problem in world where these grains are the major food source. With increse the use of whole wheat flour in united state, there has also been an increased interst in reducing the levels of phytic acid in whole wheat food products.phytic acid bind mineral and other whole grains. The chelation of their products decrease their bioavaibality and comprise their nutritional status of individuals cosuming large amount of whole grains.the sourdough or the whole wheat dough used for the experiment is adjusted to pH 5.2 had agreater reduction in phytic acid than dough with a normal pH. Phytic acid was reduced by 40% in these dough after two hours incubation.The purpose of this study is to investigate the effect of pH, time, stirring, and yeast on reducing phytic acid in whole wheat dough after 4 hours incubation. Phytase hydrolyses phytic acid (phytate; *myo*-inositol hexakisphosphate) into *myo*-inositol (phosphate)n and inorganic phosphate (Pi) or (phytase)

$$\text{Phytic acid} + H_2O \longrightarrow \textit{myo}\text{-inositol (phosphate) n} + Pi$$

Alkaline phosphates (ALP) further hydrolyses *myo*-inositol (phosphate)n producing *myo*-inositol and Pi.(ALP)

$$\textit{myo}\text{-inositol (phosphate)n} + H_2O \longrightarrow \textit{myo}\text{-inositol} + Pi$$

Inositol phosphate and ammonium molybdate react to form 12-molybdophosphoricacid, which is subsequently reduced under acidic conditions to Molybdenum blue. Phytic acid is anti nutritional

in nature and able to induce hormonal as well as metabolic changes in animals because of its metal-binding or chelation ability Phytic acid when cleaved by phytase loses its ability to bind to metals, and also generation of inorganic phosphate, thus eliminating the need of extra addition of inorganic phosphate in feeds. Phytic acid is hydrolyzed, enzymatically by phytases, or chemically to lower inositol phosphates such as inositol pentaphosphate (IP5), inositol tetra phosphate (IP4), inositol triphosphate (IP3) and possibly the inositol di- and monophosphate during storage, fermentation, germination, food processing and digestion in the human gut (Ayet & Cuadrado, *et al*., 1995). Only IP6 and IP5 have a negative effect on a bioavailability of minerals, the other hydro-lytic products formed have a poor capacity to bind minerals, or the complexes formed are more soluble.

1.2DISADVANTAGES OF PHYTIC ACID

1.2.1 ANTINUTRITIONAL

The anti-nutritional aspect of phytate occurs due to the complexing action of phytate on other divalent and trivalent cations, namely Cu, Zn, Ni, Co, Mn, Fe, Se, Mg and Ca. Graf and Eaton explain that one phytate molecule can bind up to six divalent cations, and the metal ion may bridge at least two phytate molecules, depending on the redox state. They note that the effect of this chelation on the bioavailability of minerals depends on the ratio of metal-to-phytate, and the number of different cations which are in the complex. The problems of mineral chelation and subsequent deficiencies are real. Deficiencies of iron and zinc can cause anemia, poor psychomotor development, impaired growth and increased risk of diarrhea and respiratory infections. Other minerals are also involved with serious health consequences in deficiency states. Some studies have looked at the effects of phytate-reduced foods, while others concentrate on interactions between phytate and different methods of fortifying foods.

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 in preceding sourdough. The micro flora of such dough depends on the micro flora of the raw materials used and the prevailing hygienic

conditions, and is variable in terms of kind, origin and storage conditions of flour, as well as the technological parameters of the fermentation process applied.

The preparation of sourdough begins with a pre-ferment, (the "starter" or "levain", also known as the "chief", "chef", or "head"), made of flour and water. The purpose of the starter is to produce a vigorous leaven and to develop the flavor of the bread. In practice there are several kinds. The ratio of water to flour in the starter (the "hydration") varies and a starter may be a fluid batter or stiff dough.

When wheat flour comes into contact with water, naturally occurring amylase enzymes break down the starch into maltose; the enzyme maltase converts the maltose sugar into glucose, which yeast can metabolize.

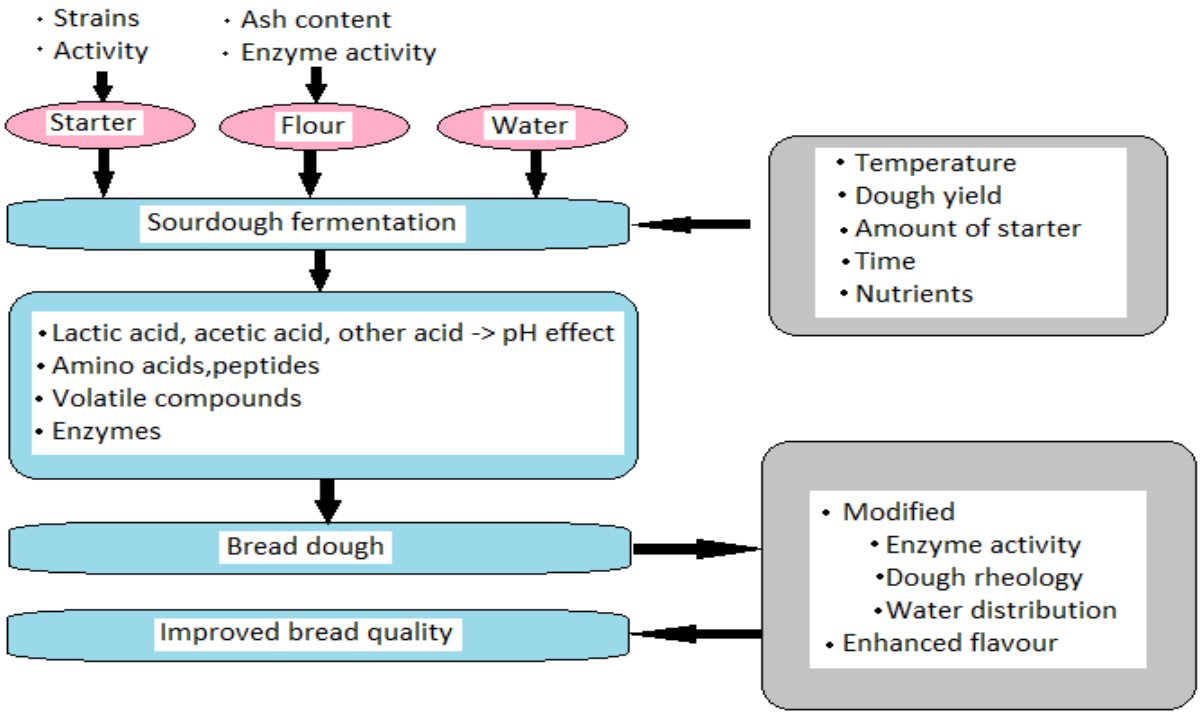


Figure 1.1: Sourdough fermentation and influencing factors.

1.3 PHYTATE IN PLANT FOODS AND ITS RELATION TO LACTIC ACID BACTERIA:

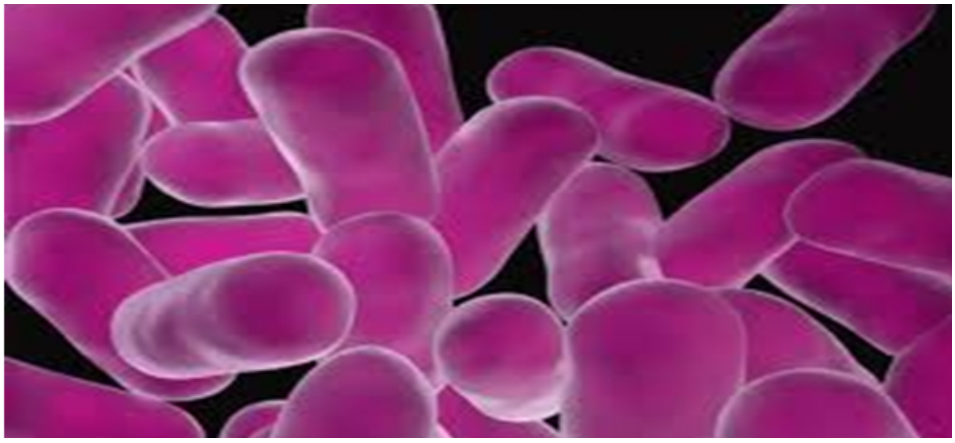


Figure 1.2 Lactic acid bacteria

The **lactic acid bacteria** (**LAB**) comprise a clade of Gram-positive, low-GC, acid-tolerant, generally non-sporulating, non-respiring rod or cocci that are associated by their common metabolic and physiological characteristics. These bacteria, usually found in decomposing plants and lactic products, produce lactic acid as the major metabolic end-product of carbohydrate fermentation. The industrial importance of the LAB is further evinced by their generally recognized as safe (GRAS) status, due to their ubiquitous appearance in food and their contribution to the healthy micro flora of human mucosal surfaces. The genera that comprise the LAB are at its core *Lactobacillus*, *Leuconostoc*, *Pediococcus*, *Lactococcus*, and *Streptococcus* as well as the more peripheral *Aerococcus*, *Carnobacterium*, *Enterococcus*, *Oenococcus*, *Sporolactobacillus*, *Tetragenococcus*, *Vagococcus*, and *Weisella*; these belong to the order Lactobacillales. The lactic acid bacteria (LAB) are either rod shaped (bacillus), or spherical (coccus), and are characterized by an increased tolerance to acidity (low pH range). This aspect helps LAB to outcompete other bacteria in a natural fermentation, as they can withstand the increased acidity from organic acid production (e.g., lactic acid). Laboratory media used for LAB typically include a carbohydrate source, as most species are incapable of respiration. LAB is catalase negative. They consist of the organelles of a simple bacterial structure. LAB is amongst the most important groups of microorganisms used in the food industry.

Lactococcus lactis is a Gram-positive bacterium used extensively in the production of buttermilk and cheese, but has recently also become famous as the first genetically modified organism to be

used alive for the treatment of human disease. *Lactococcus lactis* cells are cocci that group in pairs and short chains, and, depending on growth conditions, appear ovoid with typically 0.5 - 1.5 μm in length. *Lactococcus lactis* does not produce spores (non sporulating) and are not motile (nonmotile). They have a homo fermentative metabolism and have been reported to produce exclusive L-(+)-lactic acid. However, reported D-(−)-lactic acid can be produced when cultured at low pH. The capability to produce lactic acid is one of the reasons why *Lactococcus lactis* is one of the most important microorganisms in the dairy industry; based on its history in food fermentation *Lactococcus lactis* has the GRAS status (Generally Regarded As Safe).

CHAPTER2
REVIEW OF LITERATURE

2.1 Chemical structure of Phytic acid

The poly-valent structure of phytic acid contributes to the formation of insoluble salt with polyvalent cations (Cleamons, 1984). At present time the most accepted structure of phytic acid is that proposed by Anderson (1914) as shown in Figure 2. Phytic acid is considered the storage form of phosphorous in seeds. It occurs in legumes, grains and tubers. The reactive phosphate group of phytic acid can form compounds with mono-valent or poly-valent cations and proteins.

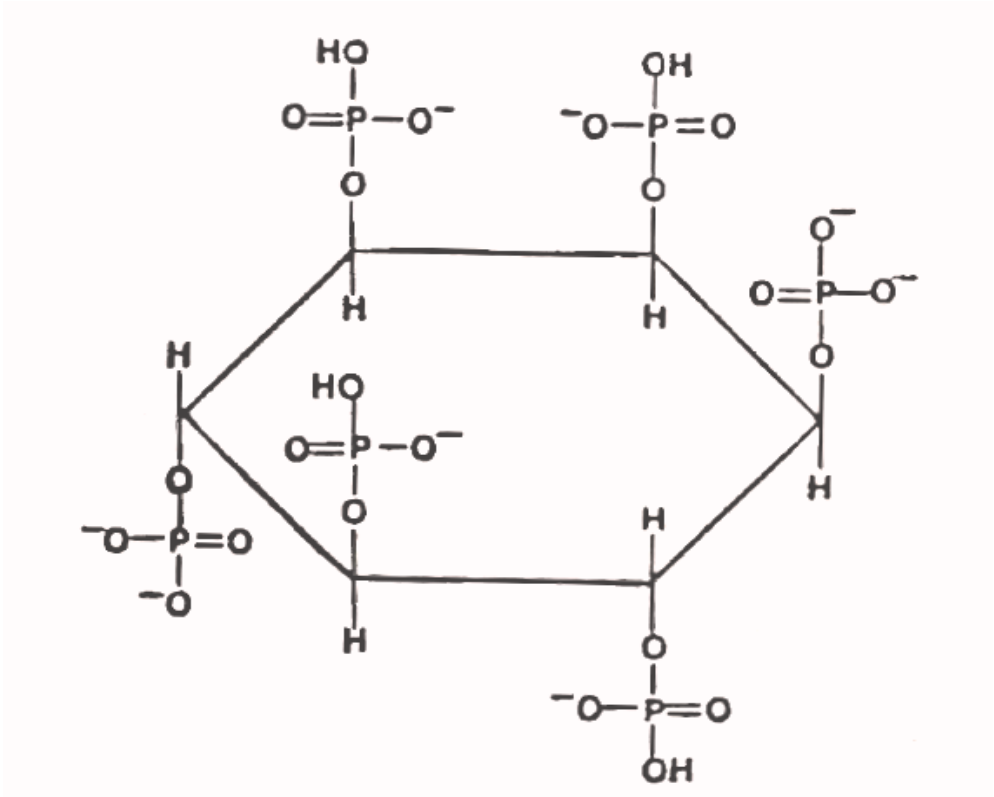


Figure 2.1 Chemical structure of Phytic acid

2.2 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 is the part of the oral flora which can cause dental caries (Monchois *et al.*, 1999). Lab were first isolated from milk (Carr *et al.*, 2002) and have since been found in such foods and fermented products as meat, milk products, vegetables, beverages and bakery products (Aukrust and Blom, 1992; Harris *et al.*, 1992).

2.3 General and metabolic characteristics of LAB

Members of lactic acid bacteria hare 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 into lactate, ethanol and carbon dioxide (Caplice and Fitzgerald, 1999; Kuipers *et al*., 2000).In addition, LAB produce small organic compounds that give the aroma and flavor to the fermented product.

2.4 Role and degradation of Phytate by Lactic acid bacteria

The phytate-degrading and phosphates activities of several *Lactobacillus* strains (Haros *et al*., 2007) belonging to different species and isolated from different ecosystems were tested. The specific activities against phytate (InsP6) varied from 0.024 to 0.531 U/mg proteins, being the highest for *Lactococcus lactis*. The studied strains hydrolyzed *p*-nitropheny phosphate at higher rates than phytate, except for *Lactococcus lactis*. The ability of the different strains to hydrolyze InsP6 and generate lower *myo*-inositol phosphates during growth was tested by spectrophotometric methods. The optimal pH and temperatures of phytate-degrading activity varied in the range from 5.0 to 7.5 and from 50 to 60°C, respectively. The incorporation of different types of carbon sources or inorganic phosphate to the growth medium modulated the synthesis of phytate-degrading enzymes in the studied strains. Further studies should be carried out to provide progress in the understanding of the potential nutritional and technological roles of the most active strains in the elaboration of whole sour breads.

2.5 Regulation of Phytate reduction in LAB

The phytase activity of 12 species of sourdough (Angelis *et al.*, 2007) lactic acid bacteria was screened. It was intracellular only, largely distributed among the species and strains of *Lactococcus lactis* possessed the highest levels of activity. A monomeric ca. 50-kDa phytase was purified to homogeneity from *Lactococcus lactis* by three chromatographic steps. *Lactococcus lactis* exhibited the highest hydrolyzing activity on Na-phytate after reaching the stationary phase of growth. Cells cultivated in the presence of maltose and fructose showed an increase of the phytase activity of 35% with respect to the other carbon sources used. The phytase was optimally active at pH 4.0 and 4.5. The enzyme was strongly inhibited by 2 mM of phenylmethylsulfonyl fluoride (PMSF), and 2 mM, Hg^{2+} and Fe^{2+}. It had a pI of ca. 5.0. The substrate specificity was dependent on the type of phosphate ester; a very low activity was detected on a-D-glucose-1-phosphate and D-fructose-6- and 1, 6-phosphate, while the highest hydrolysis was found towards adenosine tri-, di- and mono-phosphate. Compared to these substrates, the activity on Na-phytate was also relevant.

2.6 Phosphate and reduction of Phytase activity

Lactic acid fermentation of cereal flours (Reale *et al*., 2004) resulted in a 100 (rye), 95-100 (wheat), and 39-47% (oat) reduction in phytate content within 24 h. The extent of phytate degradation was shown to be independent from the lactic acid bacteria strain used for fermentation. However, phytate degradation during cereal dough fermentation was positively correlated with endogenous plant phytase activity (rye, 6750 mU g-1; wheat, 2930 mU g-1; and oat, 23 mU g-1), and heat inactivation of the endogenous cereal phytases prior to lactic acid fermentation resulted in a complete loss of phytate degradation. Phytate degradation was restored after addition of a purified phytase to the liquid dough. Incubation of the cereal flours in buffered solutions resulted in a pH-dependent phytate degradation. The optimum of phytate degradation was shown to be around pH 5.5.

2.7 Phytate reduction by starter cultures in cereal products

Sourdough from lactic acid bacteria (*Lactococcus lactis, Lactobacillus reuteri*) with different dough yield (250 and 300) is made (Guerra *et al*., 2001) and incubated at 30℃ for 20 hour, then added to dough in the ratio of 10, 20 and 30% replacement. Breads that supplemented with *Lactococcus lactis* sourdough had lower phytic acid. Higher replacement of sourdough and higher DY cause higher decrease in phytic acid content. Sourdough from *Lactococcus lactis* DY = 300 and 30% replacement cause the highest decrease in phytic acid content (49.63 mg/100g). As indicated by panelists, *Lactococcus lactis* sourdough can present the greatest effect on overall quality score of the breads. DY reduction causes a decrease in bread quality score.

2.8 Extraction, purification and analysis of Phytate from food products

The effectiveness of acid concentration in extracting (Newsom *et al.,* 1999*)* phytate from rape seed protein concentrates. It is evident that a more complete extraction is obtained by using 2.4% HC1 compared to the 2-h extraction period with 1.2% HC1 recommended by (Harland and Oberleas *et al.,* 1977). At test at the 5% level showed a significant difference between the 1.2 and 2.4% acid extraction whereas no significant difference was evident between 2.4 and 4.8% HC1 extraction. The extraction time was tested by using 2.4% HCl over a 1-h period and was found to give the same results as the 4-h extraction, thus permitting a 40% reduction in extraction time. Recovery of sodium phytate following this procedure indicating that no losses occurred during the extraction and elution procedures described. The Wade reagent must be added to the 0.7 M NaCl eluant fraction within 0.5 h of passing through the column. Once the reagent is added, however, the mixture remains quite stable.

2.9 Phytic acid content in milled cereal products and breads

Phytic acid was determined (Guerra *et al*., 1999) in cereal (brans, ours and milled wheat-products) and breads. The method was based on com-plexometric titration of residual iron (III) after phytic acid precipitation. The cereal ours showed values ranged between 3±4 mg/g for soft wheats, 9 mg/g for hard wheat and 22 mg/g for whole wheat. Corn, millet and sorghum ¯ours reported a mean of 10 mg/g and oat, rice, rye and barley between 4 and 7 mg/g. Wheat brans had

wide ranges (25±58 mg/g). The phytic acid for oat brans was half that of wheat bran (20 mg/g) and higher value (58 mg/g) than that for rice bran. The milling products (semolinas) from hard wheat exhibited 10 mg/g and soft wheat a mean of 23 mg/g. The breads made with single or mixture cereal ours exhibited ranges between 1.5 and 7.5 mg/g. The loss of phytic acid relative to unprocessed ours was between 20% for oat bread and 50% for white bread.

2.10 Phytate degradation during bread making

Phytic acid has been considered to be an antinutrient (Kurent *et al.,* 2009) due to its ability to bind minerals and proteins, either directly or indirectly, thus changing their solubility, functionality, absorption, and digestibility. In this study, the influence of the flour type (type 500, type 850, and whole meal flour) and three different bread making procedures (direct, indirect, and with sourdough addition) on phytic acid was investigated. The results showed that the flour type influenced the phytic acid content. The phytic acid contents of flour type 500, type 850, and whole meal flour was 0.4380, 0.5756, and 0.9460 g/100 g dm, respectively. The dough and bread prepared from flour with a higher phytic acid content also contained higher amount of phytic acid. During fermentation and baking, degradation of phytic acid occurred. Phytic acid was also influenced by pH. Samples of lower pH had a lower phytic acid content. Dough prepared from flour type 500 and type 850 with 10% addition of sourdough had especially low phytic acid contents, and the bread prepared from the respective dough contained no phytic acid at all.

CHAPTER 3
OBJECTIVES

Few studies in India have attempted to examine phytate reduction through application of LAB especially in cereal based products. In a previous study, a composite sourdough using wheat flour and *Vigna mungo* flour was developed in our laboratory. The sourdough had important nutritional and therapeutic properties (Carlson *et al*., 2003).In the present study we attempted to examine the potential of starter cultures used for fermenting the composite dough in terms of removal of phytate an important antinutrient which decreases the nutritional value of this product. For this, twenty six lactic acid bacteria isolates were obtained from earlier studies (Singh *et al.,* 2011; Bhanwar *et al.,* 2013) and evaluated for phytate degradation activity. Efficient phytate degradation LAB was 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 Phytase degradation strains of lactic acid bacteria.
2. Optimization of Phytate degradation activity of selected strains,
3. Real time evaluation of Phytase activities of selected lactic acid bacteria in sourdough.

CHAPTER 4
MATERIALS AND METHODS

4.1 CHEMICALS

All chemicals and reagents were of highest purity and were purchased from Sigma (MO, USA). Bacteriological media was purchased from Hi media (Mumbai).Sodium phytate was purchased from Hi media (Mumbai).

4.2 Microorganisms and culture conditions:

Twenty six strains of lactic acid bacteria isolated in previous studies (Singh et al., 2001; Bhanwar et al., 2013) were examined for phytic acid reduction. Based on the highest reduction of phytic acid, *Lactococcus lactis* was selected. The *Lactococcus lactis* was activated from glycerol stock maintained at -20˚C. MRS (de Man Rogosa Sharpe) media was used for isolation and maintenance of LAB strains. The media was sterilized at 121˚C for 20 minutes and cooled at room temperature prior to use. The initial culture conditions were incubation temperature of 37˚C for 24 hours cultivation period, 1% inoculums and pH of medium unless otherwise stated.

4.3 Phytic acid estimation (AOAC, 2001)

One ml of phytic acid was taken from stock solution of 12mg/ml. It was mixed in 1.5 ml of working solution (20mM of ammonium heptamolybedate and 5mM of ammonium vanadate). Solution was incubated for 1 hour at 37˚C. The precipitation formed was removed by using centrifugation at 2000 rpm for 10 minutes. Then the supernatant were collected and absorbance was taken at 415 nm by spectrophotometer.

4.4 Tolerance of *Lactococcus lactis* to Phytic acid:

First the MPSN media was inoculated with 100µl of *Lactococcus lactis* culture and 4 ml of phytic acid was added to it. In another test tube 10 ml of MPSN media was taken and inoculated with 100µl of *Lactococcus lactis* culture. Both the test tubes were inoculated at 37˚C in incubator

shaker for 24 hours. Next day both the test tubes were centrifuged with phytic acid and without phytic acid at 8000 rpm for 5minutes at 4˚C. The pellet and the supernatant was separated. The pellet was washed with saline solution 2 times and dissolved in it. Both the pellet and the supernatant were stored in freezer at 4˚C. After every two hours the absorbance of both the samples was taken up to 32 hours. Before taking the absorbance of supernatant 150µl of sample was taken and 750µl of mixture of ammonium vanadate and ammonium molybedate was added to it. Then the sample was incubated at 37˚C for about 1 hour and the absorbance was taken at 600 nm up to 30 hours.

4.4.1 Optimization of cultures variables for Phytic acid degradation:

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

4.4.1.1 Effect of agitation: MRS media was used for cultivation of overnight grown culture of *Lactococcus lactis* and was incubated for 24 hours at 37˚C, with and without, to study its effect on the protease activity. Following incubation for 24 h at 37˚C, the supernatant were analyzed for phytic acid degradation.

4.4.1.2 Effect of pH: To study the effect of pH on phytic acid degradation MRS media previously set with different pH values (5, 6 and 6.5), using 1N HCL and 1N NaOH, was used for cultivation of overnight grown culture of *Lactococcus lactis* by following incubation for 24 hours at 37˚C.

4.4.1.3 Effect of temperature: To study the effect of temperature on the phytic acid degradation MRS media was used for cultivation of overnight grown cultures of *Lactococcus lactis* and was incubated at different temperatures (35˚C, 30˚C and 42˚C) for 24 hours.

4.4.1.4 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 followed by its incubation at different temperatures.

4.4.1.5 Effect of nitrogen source: For evaluation of effect of different nitrogen sources on enzyme activity in culture media the supernatants were analyzed for phytic acid reduction at 37˚C for 24 hours.

4.5 Intracellular and Extracellular enzyme.

One hundred µl overnight grown cultures of *Lactococcus lactis* was taken. Then 4ml of phytic acid and 6ml of MPSN media was added to it. This solution was incubated it at 37˚C for overnight. The overnight growing media containing culture was centrifuged at 8000 rpm for 10 minutes. The supernatant and pellet were separated and were placed in different autoclaved test tubes. The pellet was washed with 0.85%of saline solution. After that intracellular enzyme (solution containing pellet) were subjected to ultra sonication. After sonication both the test tubes containing intracellular and extracellular enzyme were placed at 4˚C.

4.5.1 Intracellular and extracellular enzyme for Phytic acid determination.

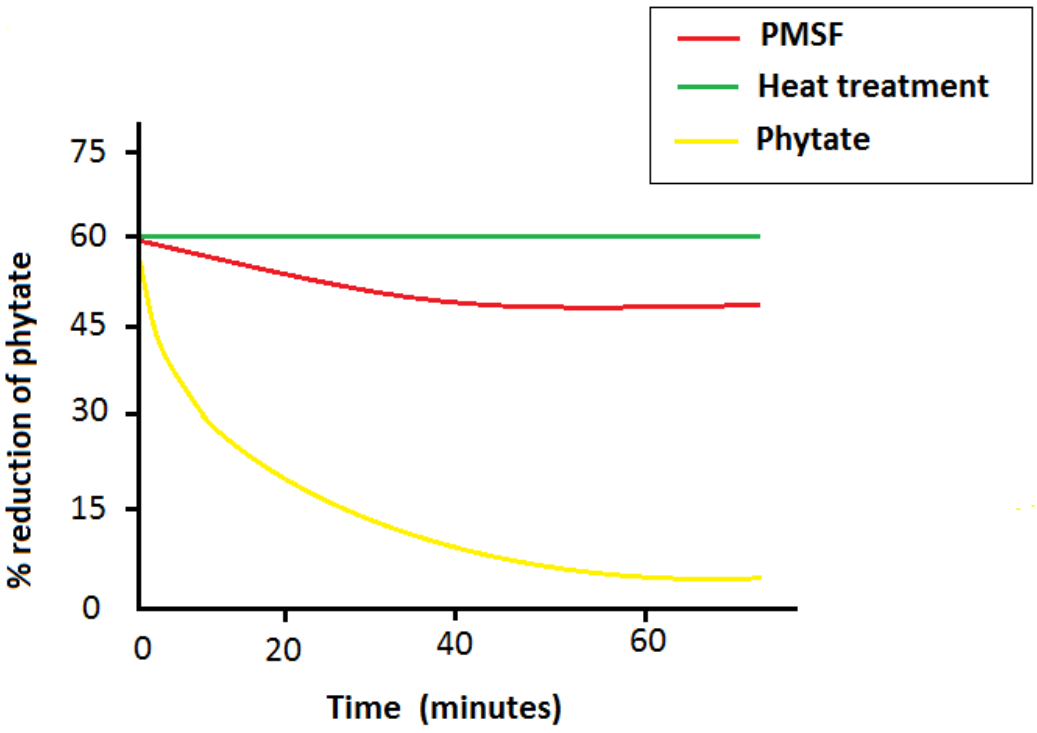
The 150 µl of both cell free extract and supernatant was taken and 600µl of 12 mg/ml concentration of phytic acid was added to it. Solution was incubated at 37˚C for 45 minutes. The reaction was terminated by adding 750µl of 5% trichloroacetic acid. Then 750µl of color reagent was added to it formed by mixing four volumes of 1.5 % (w/v) ammonium molybedate in 5.5% (v/v) of sulphuric acid solution and one volume of 2.7% (w/v) of ferrous sulphate solution. Finally the absorbance was recorded at 700nm by spectrophotometer.

4.6 Effect of PSMF on Phytic acid:

First the media was inoculated with culture of *Lactococcus lactis* and by incubating it overnight at 37˚C. The 6ml of MPSN media was taken and 4ml of phytic acid and 100 µl of overnight grown culture were added to it. The solution was incubated overnight in incubator shaker at 37˚C. The whole media containing culture was centrifuged at 8000 rpm for 5 minutes at 4˚C. The supernatant and pellet were separated. The supernatant was then stored in the freeze. The pellet was washed two times with saline solution and was stored in the freeze.

4.7 Effects of selected intrinsic and extrinsic factors on Phytic acid degradation:

The 2ml of intracellular sample was taken and heated at 100˚C for about 30 minutes in water bath. This solution was stored in the freeze at 4˚C. The 2ml of extracellular sample was taken and heated at 100˚C for about 30 minutes in water bath and stored in the freezer at 4˚C. After weighing 17.4mg of PMSF and dissolving it with 10 ml of iso propanol, the solution was added in intracellular sample. The sample was then stored at -20˚C. Out of 10 ml, 1ml of intracellular and extracellular enzyme was taken and 1ml of PMSF was added to it. The sample was incubated for about 30 minutes. The absorbance of extracellular sample was taken at 600nm. The150µl of Intracellular sample was added in 750µl of mixed solution of ammonium molybdate and ammonium vanadate. This sample was incubated for 1 hour at 37˚C. The absorbance was recorded at 600nm by using spectrometer.



4.8 Development of *Lactococcus lactis* biofilm with or without Phytate:

The MPSN media was inoculated overnight with *Lactococcus lactis* culture and incubated at 37°C with shaking. The first four wells of micro titer plate were inoculated with 117µl of MPSN

media + 3µl of L.L. culture and 180µl of 12mg/ml concentration of phytic acid. In next four wells 300µl of MPSN media was added which is kept under predefined control. In next four wells 297µl of MPSN media and 3µl of *Lactococcus lactis* culture were added to the solution. In another micro titer plate in first ten wells 117 µl of MPSN media and 3µl of *Lactococcus lactis* culture were added and after 3 days 180µl of phytic acid was added to it. Both the micro titer plates were incubated for about 3-4 days for bio-film development. After 3 days when the bio-film is developed, the micro titer plate was rinsed with PBS (pH 7.2) and 200µl of 1% crystal violet was added to it and was kept for 5 minutes. Then the crystal violet was decanted without disturbing the walls of the plate. Then the plate was washed with deionzed water. The water was decanted slowly and was allowed to dry in the laminar air flow chamber. Then 200µl of 30% acetic acid was added to it and was kept for 15 minutes. The 100µl of these samples were taken out and placed in the new micro titer plate. Absorbance was taken at 595 nm. An aliquot of 150 µl of sample were taken in test tube and 750µl of ammonium molybedate mixture and ammonium van date was added to it. The media was incubated at 37˚C after 1 hour. Another reading was taken after 2 hours incubation following the same procedure. Absorbance was recorded at 595 nm by using spectrophotometer (Murosaki *et al.*, 2000).

4.8.1 Phytate reduction in bio-film via culture:

In first six wells of micro titer plates 117 µl of MPSN media, 3µl of overnight grown *Lactococcus lactis* culture and 180µl of phytic acid was added. Then in these wells, 297µl of MPSN media and 3µl of *Lactococcus lactis* culture were added. These micro titer plates were incubated at 37˚C for 3 days. After 3 days the media was decanted and the plate was dried in L.A.F. The bio-film was scratched on an aluminum foil and weighed. The MPSN media containing *Lactococcus lactis* culture was centrifuged and decanted. The pellet was washed two times with saline solution and grows at constant temperature. Then 1ml of saline solution was added on pellet and the pellet was placed in L.A.F. chamber in order to dry it. First the empty eppendoff was weighed and then the eppendoff was weighed with pellet. Then the weight of the bio-film and the weight of the pellet was compared (Pestel *et al*., 2002).

4.9 Preparation of composite sourdough:

Lactococcus lactis was grown at 37 ºC in MPSN media, with shaking. The overnight grown culture of *Lactococcus lactis* was centrifuged at 8000 rpm for 5 minutes. The supernatant was discarded and the pellet was washed with 0.85% of saline solution. Sourdough was prepared by adding 5 gm of wheat flour and 5 gm of *Vigna Mungo* flour. Overnight grown culture containing saline was added to it and labeled it as with culture fermentation. Another sourdough was prepared by adding water in it and was labeled as without culture fermentation. The sourdough containing culture at 37˚C was placed for 4 hours and sourdough sample without culture was also placed in freeze at 4˚C for 4 hours. After fermentation both the sourdough samples were taken out and water was added for the extraction of phytic acid. Both the sourdough samples were placed in the water bath at 100˚C for fermentation. After extraction the extracted solution was filtered and placed in a tube and was stored at 4˚C in freezer.

CHAPTER 5
RESULTS AND DISCUSSION

5.1 Tolerance of *Lactococcus lactis* to Phytate

Figure 5.1 shows the tolerance of phytic acid is highest in concentration of 0 mg/ml i.e. control. After control the highest tolerance was shown in concentration of 2mg/ml. The rate of tolerance was decreases from concentration of 2mg/ml to 8mg/ml. The least tolerance of phytic acid was shown in concentration of 8mg/ml. Humans can tolerate a small to moderate amount of phytic acid in the range of 100 mg to 400 mg per day.

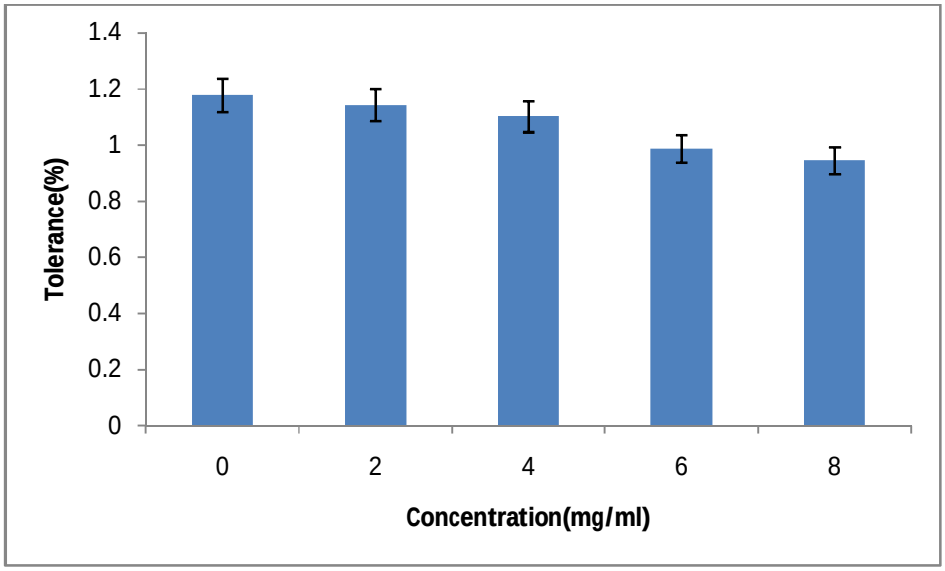


Figure 5.1 The tolerance of *Lactococcus lactis* to phytate. Error bar represents the standard deviation from the mean value of three experiments.

Fig 5.2 shows the reduction of phytic acid reduction in MPSN media. Concentration of 12 mg/ml of media showed greater reduction of phytic acid in MPSN media. In term of percentage 40%

reduction of phytic acid was reduced in media. Least reduction of phyic acid is occurring for the occurred in the concentration of 2mg/ml.

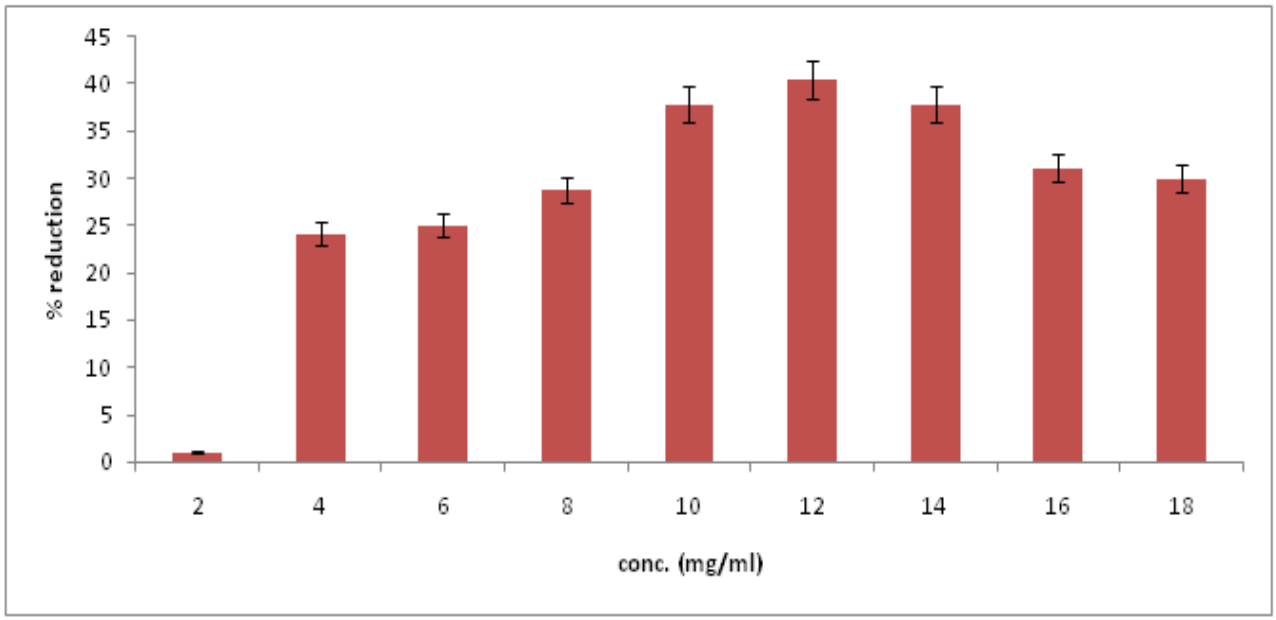


Figure 5.2: Percentage reduction of Phytic acid in MPSN media.

Growth kinetics in presence and absence of Phytic acid:

The comparison of kinetics of growth curves in presence and absence of phytic acid is shown in Figure 5.3. The growth curve with blue line shows the growth kinetics in presence of phytic acid and the red curve shows the growth kinetics in absence of phytic acid. The media containing phytic acid form more stable curve then the media which do not contain any phytic acid. The media containing phytic acid shows the reduction by *Lactococcus lactis* during the course of its growth. The lag phase of *Lactococcus lactis* appears after 2 hours 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 synthesizing the necessary proteins, co-enzymes and vitamins needed for their growth. *Lactococcus lactis* exhibited pronounced lag phase until approximately 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 specific growth rate of

Lactococcus lactis with phytase was 0.069 /h. Although, as *Lactococcus lactis* continue to grow and achieve high cell density at 18 h, the growth remains constant for a while as it reaches stationary phase. *Lactococcus lactis* has a stationary phase until approximately of 22 hours of growth. Phytase reduction increases during this phase. The maximum phytase activity found during this phase was 38 U/ml, which is agreement with results of (Kholif *et al.*, 2011) who observed the maximum phytase reduction (2.0620 U/ml) of *Lactobacillus rhamnous* NRRI-445 appeared at the beginning of stationary phase.

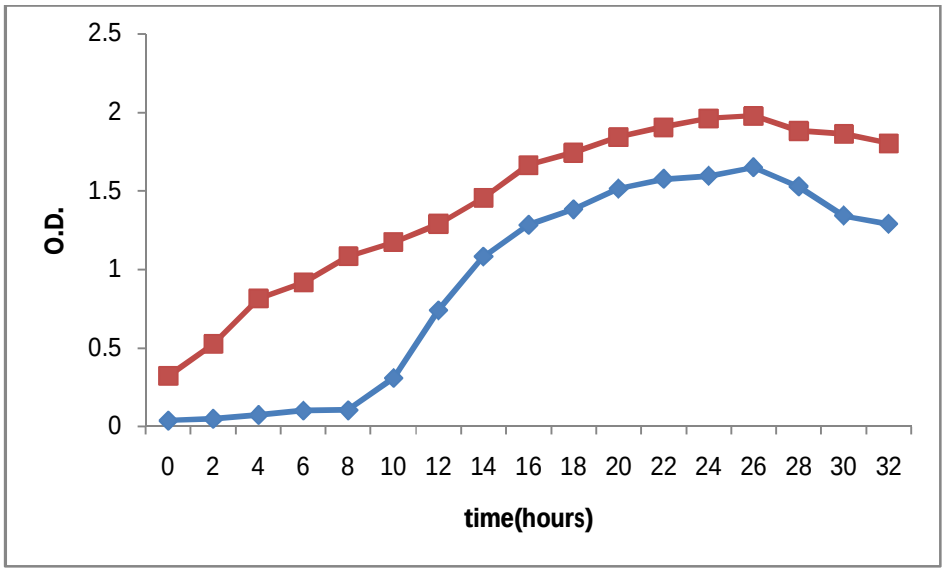


Figure 5.3 The growth kinetics in presence and absence of Phytic acid. Error bars represents the standard deviation from the mean values of three experiments.

5.2 Kinetics of Phytic acid degradation

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

5.2.1 Effect of agitation:

Agitation rate is one of the indispensable parameter for proper oxygen transfer and homogenous mixing of the nutrients in fermentation system. Oxygen acts as a terminal electron acceptor for oxidative reactions to provide energy foe cellular activities. The variation in agitation speed influences the extent of mixing in the shake flasks and also affects the nutrient availability. The effect of agitation on phytase reduction activity was investigated by incubating the inoculated MRS media on shaking (20 rpm, 80 rpm, 100 rpm, 120 rpm, and 250rpm) and static conditions.

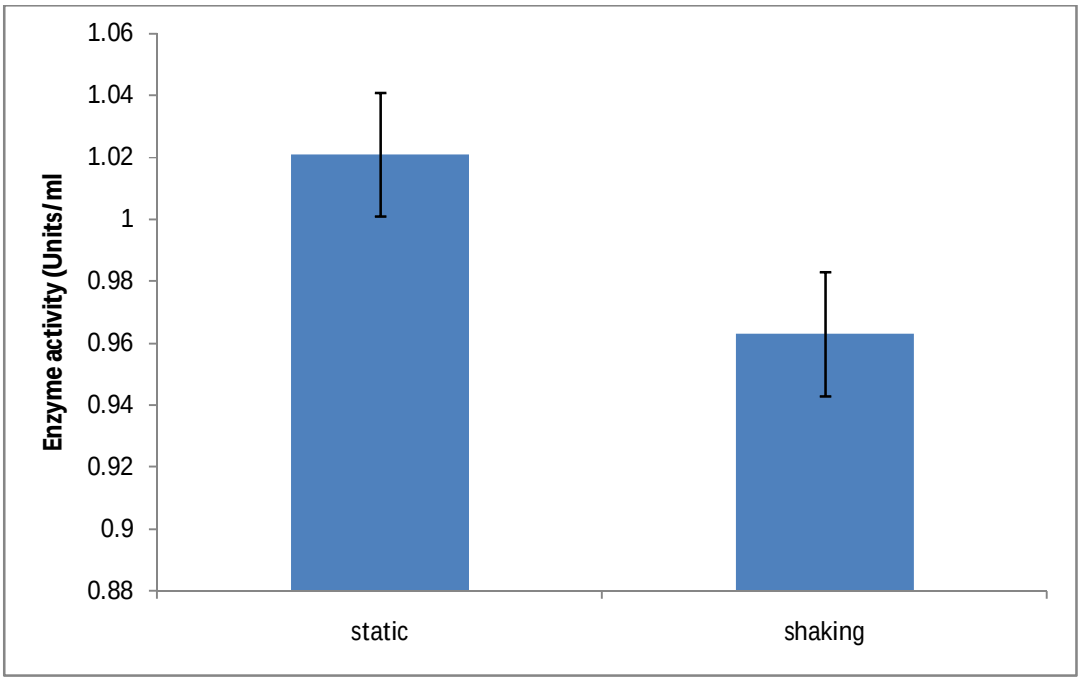


Figure 5.4: Effect of agitation on Phytase reduction activity by *Lactococcus lactis*. Error bars represent the standard deviation from the mean value of three experiments.

As shown in figure 5.4, phytase activity is maximum under static conditions and is less under shaking conditions. The data that under agitation conditions there was notable decrease in the phytate reduction activity. Hence, the optimal production of cell biomass and the highest phytase reduction yield (0.92 U/ml) were obtained at static conditions. Agitation reduced the cell

growth and phytate production due to sheer stress and heterogenous 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)

5.2.2 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 phytase activity of *Lactococcus lactis*, the initial pH of the media were adjusted to 5, 6 and 6.5, respectively. Culture supernatant solutions were examined for the presence of phytase reduction activity after adjustment of all samples.

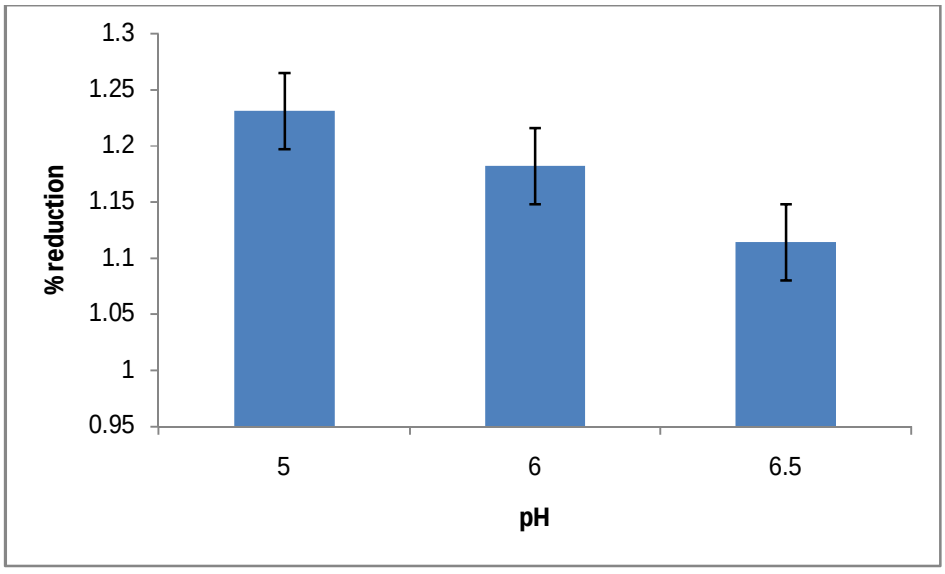


Figure 5.5: Effect of pH on Phytase activity by *Lactococcus Lactis*. Error bar shows the standard deviation of three experiments.

As shown in Figure 5.5, pH has the noticeable effect on the phytase activity. A pH- dependant decrease in the phytase activity of the both samples was noted. An increase in the culture pH

from 5 to 6.5 resulted in a decrease in maximum phytase activity from 45% to 15%. At pH 5 maximum phytase activity was more severely affected than the pH 6.5. The specific phytase activity at pH 6.5 was approximately 50% lowered compared to cultures controlled at pH 5.With increase in pH from 5 to 6, the phytase activity decreased from 2.14 U/mL to 1.47 U/mL. So, maximum enzymatic activity was obtained at pH 5. Higher or lower pH may lead to partial loss of phytase activity of *Lactococcus lactis*. However, Akinkugbe *et al.*, (2013) reported that the phytase activity of *Lactococcus lactis* exhibited a series of descending and ascending before stabilizing at its peak both pH 4.5 and 5.5.

5.2.3 Effect of temperature:

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

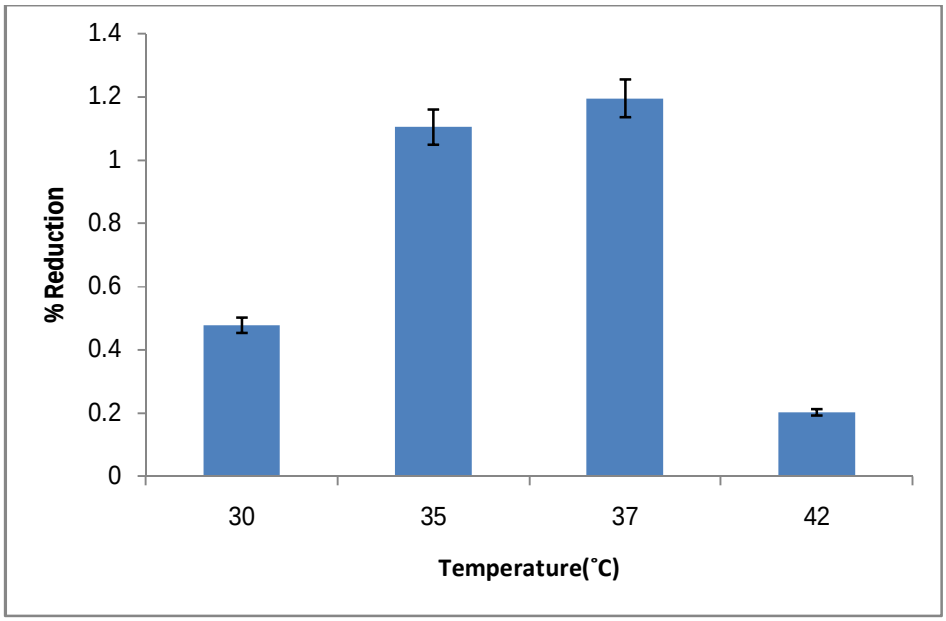


Figure 5.6: Effect of Temperature on Phytase reduction activity by *Lactococcus lactis*. Error bar represents the standard deviation from the mean value of the three experiments.

Figure 5.6 shows considerable variation in the phytase activity under different temperature conditions viz. at 30˚C, 35˚C, 37˚C and 42˚C. It was observed that the enzymatic activity was

maximum at 37˚C (40%). The activity rapidly decreased 30˚C and became negligible at 42˚C. When the temperature was increased to 42˚C the enzymatic activity was reduced significantly. So, an increase in growth temperature to 42˚C causes further decrease in phytase 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 decrease in phytase activity. When the temperature was decreased to 30˚C, the enzymatic activity was observed to be decreased. The data indicated that the optimal temperature was 37˚C and was beneficial for phytase activity as the activity was highest at this temperature. By comprehensive consideration of the above data 37^0C was selected to be optimum temperature for phytase activity for further studies.

5.2.4 Effect of Carbon source:

Carbon source considerably influence the enzyme production and a wide range of them serve as energy source. With different carbon sources impact on growth and production of extracellular phyatse was studied. To investigate the effect of concentration of different carbon sources on the phytase activity of *Lactococcus lactis*, the MPSN media were slightly modified and supplemented with different concentration of carbon sources viz. 5, 15 and 20gm/lt.

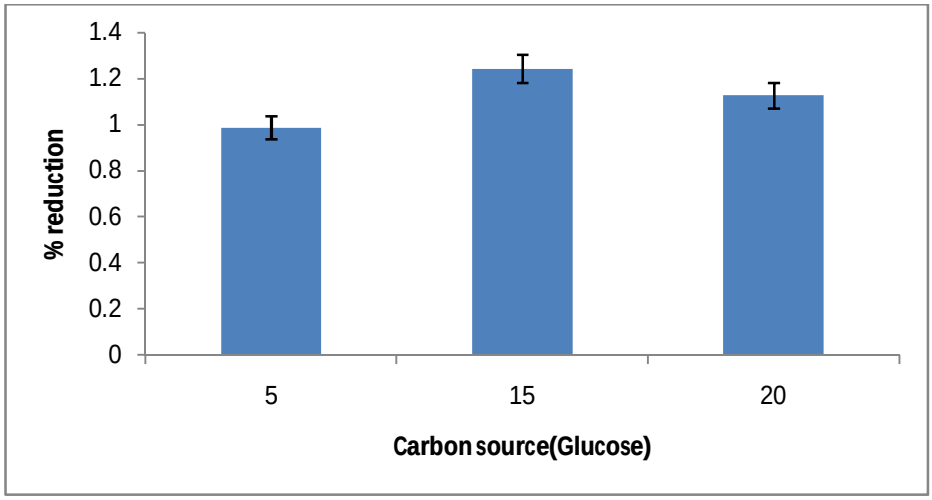


Figure 5.7 Effect of carbon source on Phytase activity by *Lactococcus lactis*. Error bars represents the standard deviation from the mean value of three experiments.

Figure 5.7 shows the highest reduction was obtained when the media was supplemented with glucose (concentration 15 gm/l). The activity decreased with glucose (concentration 5 gm/lt) as carbon source and was minimum with concentration of glucose (20 gm/lt).These findings indicate that the glucose (with concentration 15 gm/lt) is the best carbon source for phytase degradation by *Lactococcus lactis*. However Hebert et al. (2008) reported that the phytase specific activity values for *L. delbrueckii* subsp. *Lactis* CRL 581 were independent of the carbon source.

5.2.5 Effect of the Nitrogen source:

The type of nitrogen source also effects the enzyme production (Jacobsen et al., 2011). Medium supplemented with organic nitrogen source supported higher phytase production when compared to inorganic nitrogen source (Narayana et al., 2008). The authors studied the effect of nitrogen sources like peptone, beef extract, casein, yeast extract, tryptone, $NaNO_3$ and KNO_3 on production of phytase by *Streptomycin albidoflavus*. Organic nitrogen sources were superior to inorganic nitrogen sources. To study the effect of different nitrogen sources on the phytase activity of *Lactococcus lactis*, the MPSN media were slightly modified and supplemented with nitrogen source viz. ammonium sulphate of different concentrations 5, 10 and 15 gm/lt.

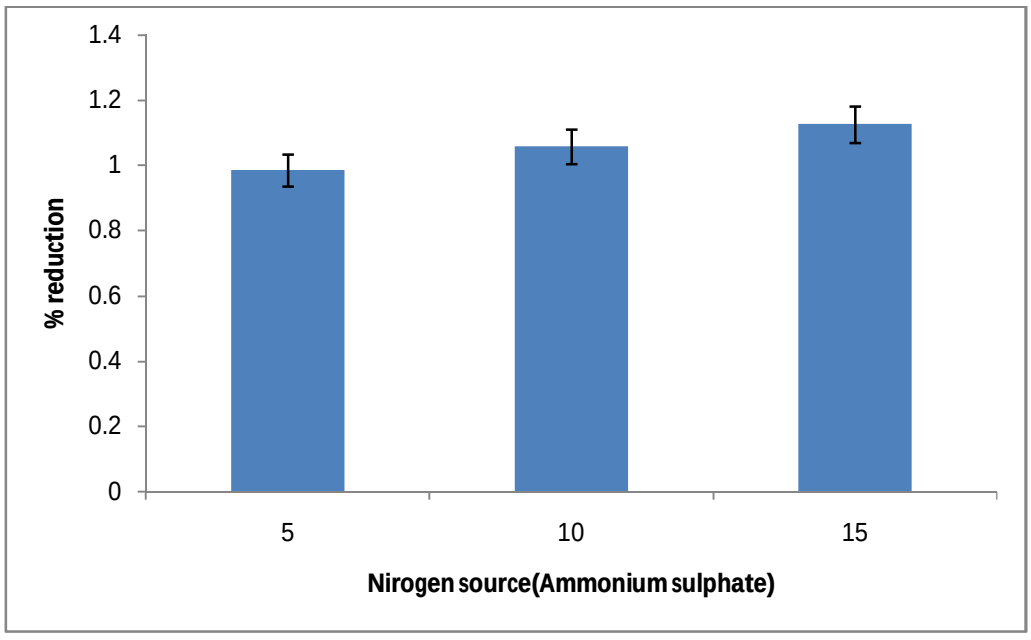


Figure 5.8: Effect of nitrogen source on Phytase reduction by *Lactococcus lactis*. Error bars represents the standard deviation from the mean value of three experiments.

Figure 5.8, shows considerable variation in the phytase reduction with different nitrogen sources. The phytase reduction was maximum with nitrogen concentration of 15gm/lt. Moderate to good levels of phytase activities were obtained with nitrogen concentration of 10g/l. The phytase activity was decreased with nitrogen concentration of 5gm/lt.

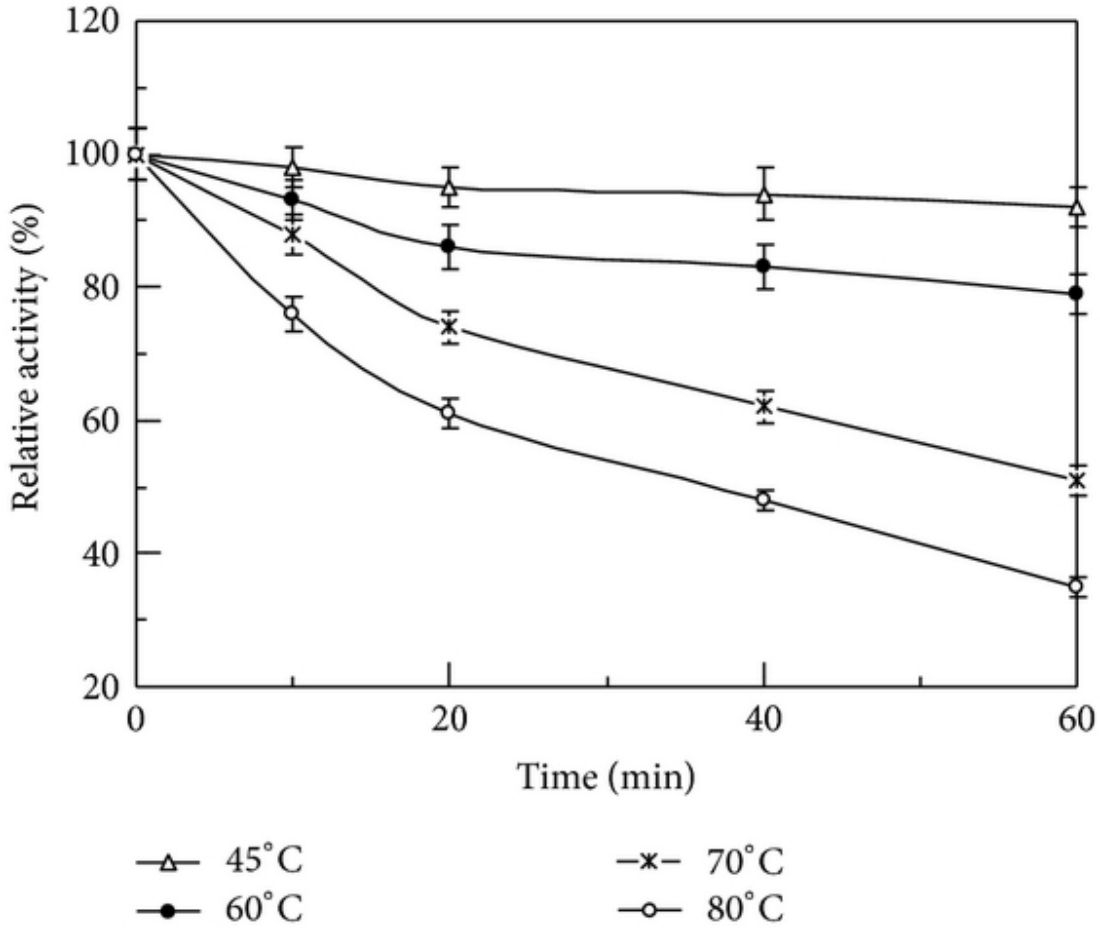


Figure 5.9 Thermostability of intracellular Phytase from *Lactococcus lactis*; activity at 0 minute is defined as 100%.Results are presented as mean ± SD (n=3).

From Figure 5.9 The crude phytase had a temperature optimum of 37°C, but lower than those reported for other phytases reported. Most of them had a temperature optimum in a temperature

range of 45–70°C including *V.volvacea* (45°C*), F. velutipes* (45°C*), Aspergillus niger* PhyA (58°C), *Aspergillus niger* PhyB (60°C), *Aspergillus ficcum* PhyB (65°C), and *Schwanniomyces castellii* (77°C).*Lactococcus lactis* phytase also demonstrated considerable thermostability with about 60%. manifested the desirable features of relative pH tolerance and thermostability, which makes it a promising candidate with more potential applications. (Ullah *et al*., 2000)

5.3 Biofilm production:

Biofilm is a communities of microorganisms attached to a surface. It is clear that microorganisms undergo profound changes during their transition from planktonic organisms to cells that are part of a complex, surface-attached community. These changes are reflected in the new phenotypic characteristics developed by biofilm bacteria and occur in response to a variety of environmental signals. Recent genetic and molecular approaches used to study bacterial and fungal biofilms have identified genes and regulatory circuits important for initial cell-surface interactions, biofilm maturation, and the return of biofilm microorganisms to a planktonic mode of growth. The current findings indicate that presence of phytate do not inhibit biofim formation of *L*actococcus *lactis* implying that colonization by this microorganism in gut probably is not inhibited by phytate in plant foods.

Table 5.1: Biofilm production in presence and absence of phytate

Biofilm	% reduction
Biofilm production in presence of Phytic acid	50.16%
Biofilm production in absence of phytic acid	40.06%

From Table 5.1 we conclude the percentage of biofilm production. In presence of phytic acid there is 50.16% biofilm development as compare to in absence of phytic acid. In the absence of phytic acid the biofilm development is 40.06% .Biofilm is present in the gut of human and when we ingest the food containing phytic acid and help in mineral absorption.

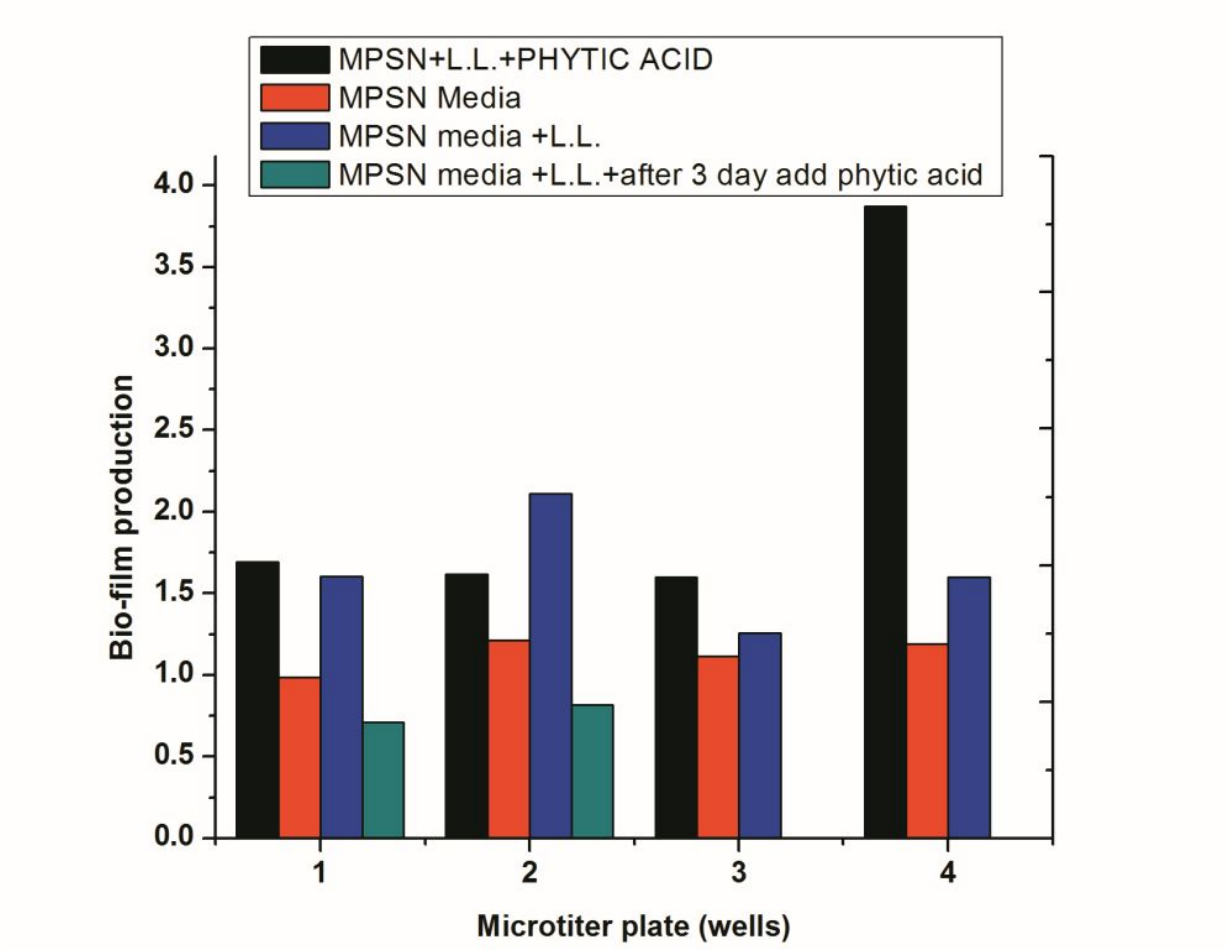


Figure 5.10: Biofilm production in presence and absence of Phytate and *Lactococcus lactis*

5.4 Phytate reduction in dough

By using the culture of *Lactococcus lactis* about 40% reduction of phytic acid was observed in sourdough within a period of 4 hours at ambient temperature (Table: 5.2). In sourdough lacking *Lactococcus lactis* starter culture phytate content remained unaltered, minor reduction was observed and could be attributed to the indigenous phytase activity of cereals (Kopzar *et al*., 2009).

Table 5.2: Percentage reduction of Phytate in sourdough using *Lactococcus lactis*.

Sample	Amount of Phytate (µg/ml)	Percentage reduction of Phytate
In presence of *Lactococcus lactis*+ fermentation	5.84	38.91%
In absence of *Lactococcus lactis*+ fermentation	9.56	39%
In presence of *Lactococcus lactis* +no fermentation	0.05	ND
In absence of *Lactococcus lactis*+ no fermentation	0.08	ND

*ND Not detected

5.5 Biochemical analysis of Phytate degradation:

We observed both the intracellular (cell free extracts) and supernatant responsible for phytate degradation but it shows maximum extracellular activity. The heating of the intracellular and extracellular extracts at 100^0C for 5 min inhibited the activity of intracellular and extracellular phytase. The phytate reduction was strongly inhibited by 2 mM of phenylmethylsulfonyl fluoride (PMSF) (Hirayama *et al.*, 2001) treatment of both cell free and extracellular extracts .A clear kinetics of phytate reduction was observable upon incubation of both intracellular and extracellular extracts (Fig 5.3).

Table 5.3: Intracellular and extracellular phytase activity.

Time (hours)	***Cell free extract+Phytate+PSMF**	***Cell free extract (heated @100˚c for 1h 30 min.+Phytate**	***Cell free extract+ Phytate**	***Cell extract +Phytate+PMSF**	***Cell extract (heated @100˚c for 1h 30 min.) +Phytate**	***Cell extract+ Phytate**
Phytate conc. (μg/ml) after **1hour**	10.57±0.01	7.56±0.01	7.18±0.01	11.03±0.01	8.04±0.01	7.39±0.01
Percentage reduction	11%	37%	40.16%	8%	38.41%	38%

*(cell free extract and dialyzed, concentrated supernatant were used for assay: protein quantities in each were measured by Folin Lowry's method and appropriate quantities used for assay)

Overall the result demonstrated that the ability of *Lactococcus lactis* to degrade phytate effectively under in situ, conditions (by considering phytase). More studies are required to explain the role and attributes of the phytase elaborated by *Lactococcus lactis*.

CONCLUSION

1. In this study, phytase degradation by a strain of lactic acid bacteria, *Lactococcus lactis* subsp. *lactis* was evaluated.
2. Phytase activity of this strain was primarily extracellular and was highest under the following optimized conditions: pH-5, temperature-35˚C, inocula-5%, carbon source-dextrose, nitrogen source- dihydrogen phosphate and with or without agitation, in MPSN.
3. Adequate viability of *Lactococcus lactis* subsp. *lactis* in sourdough amended with *Vigna mungo* flour was observed and yielded high phytase reduction.
4. Phytate degradation of *Lactococcus lactis* remained unaffected under sourdough matrix factors over a period of 4 hours of incubation.

ANNEXURE

1. **MRS Media Composition (for 250ml):**

Peptone with casein –	2.5gm
Beef extract –	2.5gm
Yeast –	1.25gm
Dextrose –	5gm
Dipottassium hydrogen phosphate –	0.5gm
Tween 80 –	0.25gm
Triammonium citrate –	0.5gm
Sodium acetate –	1.25gm
Magnesium sulphate –	0.05gm
Magnese sulphate –	0.02 gm
Distilled water –	250 ml
pH _	7.0
Autoclave _	121°C at 15psi

2. **MPSN (Modified phytase screening media) Media composition (for1000 ml):**

Glucose –	10 gm
Ammonium sulphate –	1gm
Urea –	10gm
Citric acid –	3gm
Sodium citrate –	2gm
Magnesium sulphate –	1gm
Sodium phytase –	3gm
Ferrous sulphate -	0.01gm

3. **Reagents**

Anisaldehyde reagent composition (for 100 ml):

Anisaldehyde - 0.5ml

Glacial acetic acid – 10 ml

Methanol - 50 ml

Concentrated sulphuric acid - 4.5 ml

PROTEIN ESTIMATION (Lowry's method):

Reagent A: 2% of sodium carbonate in 0.1N of NaOH

Reagent B: 1% of NaK tartarate in water

Reagent C: 0.5% of copper sulphate in water.

Reagent 1:

48 ml of reagent A +1ml of regent B+ 1ml of reagent C

Reagent2:

1 part of folin-phenol [2N]: I part of water

BSA standard-1mg/ml

REFRENCES

Abusham H, Doesburg K, Iwasaki T and Mierau I (2001). Screening of lactic acid bacteria for bile salt hydrolase activity. Journal of Dairy Science, 82, 2530–2535.

Anderson RJ (1914). A contribution to the chemistry of Phytin. Journal Biological. Chemistry. 17, 171-174.

Angelis A, Maragkoudakisa (2007). Georgia Zoumpopoulou. Identifcation and properties of phytate in cereal grains and oilseed products. Journal of Agricultural and Food Chemistry, 23, 1186±1189

Anna R, Ursula K (2004). Centre for Molecular Biology, Effect of milling extraction on the inositol phosphates of wheat our and bread. Journal of Food Science, 48, 1384±1387.

Aukrust T and Blom H (1992). Transformation of Lactobacillus strains used in meat and vegetables fermentations, Food Research International 25, 253-261.

Bhanwar S, Bamnia M, Ghosh M and Ganguli A (2012). Use of *Lactococcus Lactis* to enrich sourdough bread with γ- aminobutyric acid, International Journal of Food Sciences and Nutrition, 17, 1-5.

Caplice E and Fitzgerald GF (1999). Food fermentation: role of microorganisms in food production and preservation, International journal of Food Microbiology, 50, 131-149.

Carr FJ, Hill D and Maida N (2002). the lactic acid bacteria: A literature survey, Critical Reviews in Microbiology 28, 281-370.

Cleamons M (1980). Phytic acid interactions in food systems. CRC critical Reviews in Food Science and Nutrition 66, 297-302.

Fooks LJ, Fuller R and Gibson GR (1999). Prebiotics, probiotics and human gut microbiology, Intenational dairy Journal, 9, 53-61.

Guerra R.M, Simpson BK, Idowu JS and Oke OL (2001). Effect of local food processing on phytate levels in cassava, yam, cocoyam, maize, sorghum, rice, cowpea, and soybean. Journal of Agricultural and Food Chemistry, 38, 1580-1585.

Hans KJ (1993). Probiotic bacteria in fermented food: product characteristics and starter organism, American Journal of Clinical Nutrition, 73, 374-379.

Haros JH, Lopez F, Leenhardt C, Coudray C and Remesy G (2002). International Jouranal of Food Science Technology 37, 727-73.

Harris LJ, Fleming HP and Klae Food fermentation:nhammer TR (1992). Characterization of two nisin producing *Lactococcus lactis* subsp. Lactis strains isolated from a commercial sauerkraut fermentation, Applied environmental Microbiology, 58, 1477-1483.

Hebert JM, Facklam RR and Thornsberry C (2008). Antimicrobial susceptibility of vancomycin resistant *Leuconostoc, Pediococcus* and *Lactobacillus* species. Antimicrobial Agents and Chemotherapy, 34, 543–547.

Hirayama DI, McCartney AL and Gibson GR (2001). A study of the probiotic potential of a bile-salt-hydrolyzing *Lactobacillus fermentum* strain, and determination of its cholesterol-lowering properties. Applied Environmental Microbiology, 69, 4743–5472.

Hoessinkhani YW, Gallagher DJ, Wilferd DJ (2001). Phytase production by *Aspergillus ficuum* on semisolid substrate. Journal Indian Microbiology 2, 195-200.

Holzapfel WH, Haberer P, Geisen R, Bjorkroth J and Schillinger U (2001) Taxonomy and important features of probiotic microorganisms in food nutrition, American Journal of Clinical Nutrition, 73, 365-373.

Jacobsen CN, Nielsen AE, Moller PL, Michaelsen KF, Paerregaard(2004). A, Sandstrom B, Tvede M and Jacobsen M (1999) Screening of probiotic activities of forty-seven strains of Lactobacillus sp. by in vitro techniques and evaluation of the colonization ability of five selected strains in humans. Applied and Environmental Microbiology, 65, 4949–4956.

Jay JM (2000). Fermentation and fermented dairy products, International Journal Modern Food Microbiology, 6, 113-130.

Kholif WP, Kelly PM, Morelly L and Collins JK (1998). Development of an in vitro methodology to determine the transit tolerance of potentially probiotic *Lactobacillu*s and *Bifidobacterium* species in the upper human gastrointestinal tract. Journal of Applied Microbiology, 84, 759–768.

Murosaki Aiba Y, Suzuki N, Kabir AM, Tagaki A and Koga Y (1998). Lactic acid mediated suppression of Helicobacter pylori by the oral administration of Lactobacillus salivarious as a probiotic in a gnotobiotic murine model. American Journal of Gastroenterology, 93, 2097–2101.

Narayana S, Lievin V, Bernet-Camard MF and Servin AL (1997). Antagonistic activity exerted in vitro and in vivo by *Lactobacillus casei* (Strain GG) against *Salmonella typhimurium* C5 infection. Applied and Environmental Microbiology, 63, 513–518.

Oberleas R, Clarkin RA, Minear S, Kim F and Elwood JW (2007). Environment. Science Technology. 26, 232-239.

Oberleas WP, Kelly PM, Morelli L and Collins JK (1998). Development of an in vitro methodology to determine the transit tolerance of potentially probiotic *Lactobacillus* and *Bifidobacterium* species in the upper human gastrointestinal tract. Journal of Applied Microbiology, 84, 759–768.

Pestel MF, Lievin V, Brassart D, Neeser JR, Servin AL and Hudault S (1997). The human *Lactobacillus acidophilus* strain LA1 secretes a non bacteriocin antibacterial substance(s) active in vitro and in vivo. Applied Environmental Microbiology, 63, 2747–2753.b

Sraco, E., & Dintzis, F. R. (1982). Determination of phytic acid in foods by high performance liquid chromatography. Journal of Agricultural and Food Chemistry, 30, 724±727

Singh MS (2004). Bhatura recipe from “A taste of Palace Life Royal Indian Cookery. Available at http.//www.food.com/recipe/bhatura-107130.Downloaded on 15/3/2014.