Biotechnological studies on phytate degrading lactic acid bacteria: screening, isolation, characterization and application

A THESIS SUBMITTED TO THE

UNIVERSITY OF MYSORE

FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

In

BIOTECHNOLOGY

By

PONNALA RAGHAVENDRA M. Sc.

Department of Food Microbiology

Central Food Technological Research Institute

Mysore - 570 020, India

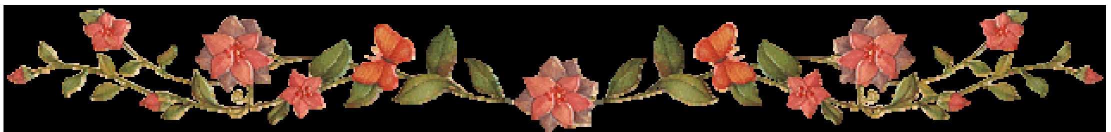
February 2011

ePrints@CFTRI

Dedicated to

My beloved

Shailu, Dheeru and Tillu



eprints@CFTRI

CERTIFICATE

I **Ponnala Raghavendra,** certify that this thesis is the result of research work done by me under the supervision of **Dr. Prakash M. Halami**, at **Food Microbiology Department, Central Food Technological Research Institute,** Mysore, India. I am submitting this thesis for possible award of Doctor of Philosophy (Ph.D.) degree in **Biotechnology** of the University of Mysore.

I further certify that this thesis has not been submitted by me for award of any other degree/diploma of this or any other University.

Signature of Doctoral candidate

Signed by me on …………………(date)

Signature of Guide

Date: *Date:*

Counter signed by

……………………………………………………

Signature of Chairperson/Head of Department/

Institution with name and official seal.

ePrints@CFTRI

Dr. Prakash M. Halami
Scientist-EII
Food Microbiology Department
Email: prakashalami@cftri.res.in

FT/FM/PMH/PhD/2011
Dated: February, 21, 2011

CERTIFICATE

I hereby certify that the thesis entitled **"Biotechnological studies on phytic acid degrading lactic acid bacteria: screening, isolation, characterization and application"** submitted to the University of Mysore for the award of the degree of DOCTOR OF PHILOSOPHY in BIOTECHNOLOGY by Mr. Ponnala Raghavendra, is the result of the research work carried by him in the Department of Food Microbiology, Central Food Technological Research Institute, Mysore under my guidance and supervision during the period of 2006–2009. This has not been submitted either partially or fully to any degree or fellowship earlier.

ePrints@CFTRI

PRAKASH M. HALAMI

(Guide)

ACKNOWLEDGEMENTS

I owe much of what I know about care as well as the ability to express it. My first and most earnest acknowledgment must go to my guide **Dr. Prakash M. Halami**, He has been instrumental in ensuring my academic, professional and moral well being ever since. His immense patience and freedom given to carryout experiments have helped me a lot to complete this work.

I am immensely grateful to **Dr. V. Prakash**, Director CFTRI, for allowing me to take up my Ph.D. at CFTRI and for providing facilities for carrying out my research work.
I am immensely thankful to **Dr. S. Umesh Kumar**, Head, FM, CFTRI, Mysore for permitting to use the infrastructure facilities throughout my research period.

With deep sense of gratitude, I thank **Dr. M. C. Misra**, former Head, FTBE, Mysore for his ideas, encouragement to dream this project. I can hardly imagine how my work would have evolved without his guidance.

I am indebted to the **Indian council for medical research** (ICMR) for providing med the fellowship which rendered me to carry out this work very successfully.

I thank **Dr. M.C. Varadaraj**, Head, HR department, CFTR, Mysore for his kind help during my tenure.

Far too many people to mention individually, hence assisted in so many ways during my work at CFTRI. They all have my sincere gratitude. I would like to thank **Dr. Vijayalakshmi, Dr. T.R. Shyamala, Dr. G. Venkateswaran, Dr. S.V.N. Vijayendra, Dr. A. Anu Appaiah, Dr. Kalpana platel, Dr. Krudachikar** and **Mr. Mukund** for their help and support in my work. I also salute the intellectual inputs of **Dr. Ushakumari** and **Mr. Anabalgan**.

I sincerely thank **Dr. Ratnasudha,** M.D., Uique Biotech Limited, Hyderabad, for her support to finish this thesis successfully.

ePrints@CFTRI

No words are enough to express my immense gratitude to **Praveen Reddy, Badri, Kiran (hero), Surya tammudu and Girish** who were with me during my research years.

Thanks to the concept of **Probiotics and phytate degradation**, because of which I enjoyed science and where I could see the wonder land of useful bacteria. I am indebted to the **IT** and **World Wide Web** for opening up a plethora of knowledge to carryout my work. **Google** had always been a shoulder to depend upon for answering my queries.

My sincere thanks to my former seniors: **Sarat anna**, **R.P. Rao**, **Mylarappa** and **Chetan** who were very helpful. The fun, enthusiasm and encouragement with them can never be forgettable.

My sincere thanks to my juniors **Manju**, **Nitya**, **Vrinda**, **Padmaja**, **Yogesh** as well as **Anusha**, **Chandrakanth**, **Ratish**, **Santhosh**, **Anila** and **Avinash** and other research fellows and project assistants in FM.

Last but not least I thank **staff** of CIFS and Library, accountants and other administrative departments who were ever ready to help me when required.

A penultimate thank you goes to my wonderful **parents** and **brothers (Pavan and chandu)** for always being there when I need them most and never once complaining about how infrequently I visit. They deserve far more credit than I can give them. The same also, to my **in-laws** who have been supportive and encouraging in all these years.

My final and most heartfelt acknowledgement must go to my wife **Shailaja**. Her patience, support, encouragement and companionship have turned my journey through life into pleasure. For all that, and for being everything I am not, she has my everlasting love. And a special thanks goes to my little one **Dheeraj**, who missed precious pleasured moments with me.

Ponnala Raghavendra

eprints@CFTRI

CONTENTS

PARTICULAR	**PAGE No.**
List of Abbreviations	viii-ix
List of Figures	x-xi
List of Tables	Xii
Synopsis	xiii-xx
Chapter 1 Introduction and Review of Literature	1-48
Scope of the Investigation	49
Chapter 2 Materials and Methods	50-88
Chapter 3	89-151
Section 1 Screening, isolation and characterizaton of phytate degrading lactic acid bacteria	89-109
Section 2 Characterization and evaluation of phytate degrading ability of lactic acid bacteria	110-129
Section 3 Application of phytate degrading lactic acid bacteria	130-151
Chapter 4 Summary and conclusion	152-156
Bibliography	157-186
Appendices	I-III

ePrints@CFTRI

List of Abbreviations

β-gal	β-galactosidase
μM	Micro Mole
AAS	Atomic Absorption Spectroscopy
bp	Base pair
Ca	Calcium
$CaCl_2$	Calcium chloride
CaP	Calcium phytate
CFS	Cell free supernatant
CFU	Colony forming unit
DEAE	Diethylaminoethyl cellulose
DNA	Deoxyribo Nucleic Acid
DPPH	2, 2-diphenyl-1-picrylhydrazyl
EDTA	Ethylene diamino tetra acetic acid
ESI	Electron Spray Inonization
Fe	Iron
HCl	Hydrochloric acid
HPLC	High performance liquid chromatography
IP6	*Myo*-inositol hexakisphosphate
kDa	Kilo Daltons
LAB	Lactic acid bacteria
Lb.	*Lactobacillus*
MALDI-TOF	Matrix Associated Laser Desorption Ionization- Time of Flight
MFSC	Malted Finger Millet Seed Coat
Mg	Magnesium
MTCC	Microbial type culture collection
NaCl	Sodium chloride
NaP	Sodium phytate
PA	Phytic acid
PAGE	Polyacrylamide gel electrophoresis

ePrints@CFTRI

PBS	Phosphate Buffered Saline
SDS	Sodium dodecyl sulfate
SEM	Scanning Electron Microscopy
TCA	Tri Chloro Acetic acid
UV	Ultraviolet
Zn	Zinc

ePrints@CFTRI

List of Figures

Title	Page No.
Figure 1.1 *Myo*-inositol hexa kis phosphate and its complex	5
Figure.1.2 Schematic representation of phytate hydrolysis by LAB	24
Figure 3.1.1 Phytate degradation by LAB cultures	91
Figure 3.1.2 SEM pictures of phytate degrading LAB	93
Figure 3.1.3 Carbohydrate utilization by isolated cultures CFR R35 and CFR R38	96
Figure 3.1.4 16S rRNA amplification and its analysis	96
Figure 3.1.5 Phylogenetic tree for the strains *Ped. pentosaceus* CFR R123, CFR R38 and CFR R35	97
Figure 3.1.6 Acid tolerance ability of LAB cultures at pH 2 and pH 2.5	100
Figure 3.1.7 Adhesion property of the phytate degrading LAB	105
Figure 3.1.8 β-gal activity of phytate degrading LAB	107
Figure 3.2.1 Quantitative analysis of phytate degradation by LAB at 50°C	111
Figure 3.2.2 Phytase activity of LAB cultures at 37°C and 50°C	113
Figure 3.2.3 Phytase activity of the LAB test strains grown in media 1 at 24 h	117
Figure 3.2.4 Phytase activity of the LAB test cultures grown in media 2 at 24 and 48 h	118
Figure 3.2.5 Phytase activity of the LAB test cultures grown in media 3 at 24 h	119
Figure 3.2.6 Phytase activity of the LAB test strains grown in media 4 at 24 h	119
Figure 3.2.7 Phytase activity of the LAB test strains grown in 4 different media at 24 h	120
Figure 3.2.8 Acid phosphatase activity of the LAB test strains grown in 4 different media at 24 h	121
Figure 3.2.9 Extracellular acid phosphatase activity of LAB cultures grown in media 2	123
Figure 3.2.10 Effect of temperature on phytase activity of *Ped. pentosaceus* CFR R38	125

ePrints@CFTRI

Figure 3.2.11 Effect of pH on phytase activity of *Ped. pentosaceus* CFR R38	125
Figure 3.2.12 Effect of substrate concentration on phyase activity of *Ped. pentosaceus* CFR R38	126
Figure 3.2.13 Phytic acid Analysis during *Ped. pentosaceus* CFR R38 fermentation process	127
Figure 3.3.1 Phytate degradation during MFSC fermentation by LAB at different temperatures	132
Figure 3.3.2 Phytate degradation analysis by HPLC	136
Figure 3.3.3 Magnesium content during MFSC fermentation	137
Figure 3.3.4 Bio-accessible calcium content during MFSC fermentation	139
Figure 3.3.5 Level of free calcium during MFSC fermentation	140
Figure 3.3.6 Bio-accessible zinc content during MFSC fermentation	142
Figure 3.3.7 Phytate degradation during soya milk fermentation by LAB cultures	144
Figure 3.3.8 Soya curd by *Ped. Pentosaceus* CFR R38	145
Figure 3.3.9 pH profile during soymilk fermentation by *Ped. pentosaceus* CFR R38 at 50°C	147
Figure 3.3.10 Phytate degradation during soya curd preparation	147

ePrints@CFTRI

List of Tables

Title	Page No.
Table 1.1 Phytic acid content in seeds and grains	3
Table 1.2 Phytate content in plant-derived foods	13
Table 1.3 Phytase producing LAB strains	20
Table 1.4 Properties of microbial phytases	21
Table 1.5 LAB isolated from various sources	26
Table 1.6 LAB and current taxonomic classification	28
Table 1.7 General approaches used in LAB identification	30
Table 1.8 LAB-supplemented foods currently available in different markets	44
Table 2.1 List of bacterial cultures engaged in this study	52
Table 2.2 16S rDNA PCR amplification conditions	61
Table 2.3 Composition of restriction digestion	62
Table 2.4 Composition of microbial culture media	70
Table 3.1.1 Lactic acid bacterial isolates obtained from different sources	89
Table 3.1.2 Growth of isolates at different physiological conditions	94
Table 3.1.3 Bile tolerance of phytate degrading LAB	102
Table 3.1.4 Antimicrobial activity of phytate degrading LAB	104
Table 3.1.5 Antibiogram of the selected phytate degrading LAB	108
Table 3.2.1 Phytase and acid phosphatase activities of potent phytate degrading LAB	112
Table 3.3.1 Analysis of phytate content in MFSC by HPLC	135
Table 3.3.2 Texture and structural properties of fermented soymilk at different time intervals by *Ped. Pentosaceus* CFR R38	146
Table 3.3.3 Sensory properties of soya curd by CFR R38	150

ePrints@CFTRI

Synopsis

ePrints@CFTRI

Tilte: **Biotechnological studies on phytic acid degrading lactic acid bacteria: screening, isolation, characterization and application**

Cereals, pulses and legume based commodities are rich and low-cost sources of nutrients for a large part of the World's population. But their nutritive value is limited by the presence of several antinutritional substances, like *myo*-inositol hexakisphosphate (IP6). Its negative charge make positively charged minerals unavailable for biological activities. Monogastric individuals do not contain the mechanism to hydrolyze IP6, hence needed processed food with lower levels of phytic acid for the improved nutritive substances ailable for biological activities.

Hydrolysis of IP6 into lower inositol phosphates can lead to mineral availability which can be employed through enzymes such as phytase (EC 3.1.3.8 and 3.1.3.26). The phytase enzyme is widely distributed in plants, microorganisms and animals, which helps in improved availability of nutritional factors by degrading IP6. Among the microorganisms members of lactic acid bacteria (LAB) such as Lactobacillus species was found to have phytase enzyme. Several studies on LAB conducted to examine their role in enhancing bio-accessible minerals during sourdough fermentation process. But the mechanism behind their role unrevealed. Hence, this study proposed with the following hypothesizes to find the role of phytate degrading LAB with improved mineral availability during different food fermentation processes.

ePrints@CFTRI

Objectives:

1. Screening and selection of phytic acid degrading Lactic Acid Bacteria (LAB) from different sources

2. Characterization and evaluation of selected phytate degrading lactic acid bacteria

3. Application of potent phytate degrading lactic acid bacteria in processes for the enhancement of trace element availability.

Thesis organization:

The out come of the work is presented in the form of a Ph.D. thesis. The thesis comprises of four chapters; the first chapter contains literature review on adverse effects of phytate content in our daily food, importance of phytase enzyme and the role of LAB in giving solution for malabsorption with phytase system through phytate degradation. Materials and methodologies used for the study were described in the chapter second. While, the chapter three was organized into three sections describing the results obtained with appropriate discussion. Section I embraces the results pertaining to the mode of isolation, screening and selection of phytate degrading LAB, their identification and characterization. Section II explains the evaluation of phytate degrading ability of LAB with evidences. The application of phytate degrading LAB in improving mineral availability during fermentative process was placed in section III. The Summary and conclusion of the study was portrayed as chapter four. The list of references cited in all the chapters compiled as a bibliography section.

ePrints@CFTRI

The salient features of the experimental work and the results enumerated in the thesis are as follows

CHAPTER I : **INTRODUCTION**

This chapter elucidates the scientific information on phytic acid and its role as an antinutritional factor, its degradation mechanism by phytase, importance of phytase and phytase producers in nature. The role of LAB in particular with the mode of phytic acid degradation, probiotic properties and beneficial attribute published in peer reviewed scientific journals, book chapters and popular articles with respect to mineral absorption, fermented food processes as well as functional foods. The scope of the work is briefly indicated in this chapter.

CHAPTER II: **MATERIAL AND METHODS**

Details of materials and methods used in the present study are discussed. It provides the brief methodologies; modified procedures as well as recent methodologies are described in detail and/or provided with suitable references. Bacterial strains procured from other laboratory and materials such as fine chemicals, reagents etc are also included in this section.

ePrints@CFTRI

CHAPTER III/Section-1: Screening, isolation and characterization of phytate degrading lactic acid bacteria

In search of phytate degrading LAB divergent sources like fermented food processes, vegetables, chicken and fish intestines and from culture collection centers were screened. There were 20, 28, 08, 07, 07 and 07 isolates obtained from chicken intestinal source, Idli batter, cabbage and fish intestine, red rice, wheat respectively. The screening of phytic acid degrading LAB was done by cobalt chloride plate assay method. All the isolated cultures showed ability to degrade 0.2% calcium phytate by producing phytase, whereas twelve cultures from chicken intestine and one culture each from raw milk and one from fermented rice showed the ability to degrade 0.2% sodium phytate. All the tested cultures showed the ability to degrade 0.2% sodium phytate in presence of 0.2% calcium chloride. In order to confirm the role of phytate degrading LAB ability is due to acid produced by the LAB or enzyme present in it. The staining method followed, clearly demonstrated that the use of cobalt chloride resulted in a hallow zone where the degradation was due to enzymatic way where as the nonspecific degradation occurred due to acid showed a precipitate zone around the hallow zone. Among screened isolates, 21 isolates selected as sodium and calcium phytate degrading LAB. Based on RFLP profile the selected 21 isolates were sorted into three groups and one representative culture from each group was selected. They were CFR R35, CFR R38 and CFR R123. The three isolates were identified by physiological, biochemical and molecular tools as *Pediococcus*

ePrints@CFTRI

pentosaceus. The respective 16S rRNA gene sequences were deposited in NCBI-GenBank under accession numbers FJ889048, FJ586350, FJ889049 for CFR R35, CFR R38 and CFR R123 respectively. For these three *P. pentosaceus* strains probiotic attributes were evaluated considering *Lactobacillus rhamnosus* GG as a positive control.

All the selected three isolates along with positive control strain were able to survive 55-45% when grown at pH 2 for 3 h. Among the tested strain, *P. pentosaceus* R38 and R123 were able to resistant to 0.3% bile and whereas strain *P. pentosaceus* R35 was 0.3% bile tolerant. *L. rhamnosus* GG was found to be 0.3% bile sensitive. Selected native and control strains were displayed antagonistic activity against *Listeria monocytogenes* Scott A, *E.coli*, *B. cereus* and *S. paratyphi.* All the selected three isolates were resistant to wide range of antibiotics such as ampicillin, penicillin, tetracycline, etc.

CHAPTER III/Section -2: Evaluation of phytate degrading ability of lactic acid bacteria

Here in this chapter, phytate degrading ability of the selected cultures were tested by biochemical analysis. For which 24 h old cultures grown in MRS broth were used for the assay. Cell pellet suspended in acetate buffer served as enzyme source. Sodium phytate at a concentration of 0.2 M prepared in acetate buffer was used as substrate. The selected cultures were able to degrade phytic acid up to

ePrints@CFTRI

70%, which resulted in 3-459 U of enzyme activity. The enzyme activity was expressed in Units/min/9 log CFU. Culture *Pediococcus pentosaceus* CFR R123 exhibited highest enzyme activity whereas *P. pentosaceus* CFR R38 and *P. pentosaceus* CFR R35 showed 215 and 89 U respectively. The selected cultures along with control culture *L. amylovorus* were grown in presence of different media conditions, and the obtained cell pellet was tested for their phytic acid degrading ability at different substrate concentrations, pH, and temperature conditions. The cell free supernatants were also analyzed, to find their ability was an extracellular effort. However, it was found that the degrading ability due to intracellular fraction. The optimal conditions for the enzyme studies to assess cultures phytate degrading ability, cultures grown in MRS media, acetate buffer (pH 5.6), 0.2 M sodium phytate as substrate and 50°C temperature. A good number of trials were attempted to isolate or extract enzyme from the cells, however protoplast sonication was found to be efficient for the enzyme extraction. Further the enzyme extracted was analyzed for its specificity by its zymogram in presence of sodium phytate and its molecular weight confirmed to be in the range of 40-50 kDa. The enzyme isolated was more fragile and needed proper storage and maintenance. The existence of phytase as an intracellular origin explains the phytate degrading ability of selected LAB. The degraded products of phytic acid were eluted through ion exchange chromatography and were subjected to HPLC and MS to confirm their molecularity.

ePrints@CFTRI

CHAPTER III/Section -3: Application of phytate degrading lactic acid bacteria

Selected potent phytate degrading LAB were observed for their phytic acid degrading ability during different fermented food processes. In this study malted finger millet seed coat (MFSC), millet industrial by-product was used. It is rich in calcium with high phytic acid content from which only 10% of calcium is bioavailable. The potent phytate degrading LAB *P. pentosaceus* CFR R123, *P. pentosaceus* CFR R38 and *P. pentosaceus* CFR R35 were assessed for their phytic acid degrading ability during MFSC fermentation. There was 5-12% phytate degradation observed which in turn resulted up to 125% increase in bio-available calcium levels when compared to the control. This elucidates the LAB role in MFSC fermentation. Apart from MFSC fermentation, the cultures were also tested for soya milk fermentation to study their role as phytate degrading LAB. Cultures *P. pentosaceus* CFR R123, *P. pentosaceus* CFR R38 and *P. pentosaceus* CFR R35 were able to ferment soya milk and the finished product was found to be in acceptable manner when it was done with CFR R38. There was 12% phytate degradation observed with CFR R123 resulted in 68% calcium availability, where as during *P. pentosaceus* CFR R38 fermented soya milk resulted in 50% decrease in phytate levels when compared to control resulted in increased bio-available calcium levels.

ePrints@CFTRI

CHAPTER –IV: Summary and conclusions

Among the isolated LAB, three isolates were able to exhibit phytate degrading ability through plate assay method. When these cultures *P. pentosaceus* CFR R123, *P. pentosaceus* CFR R38 and *P. pentosaceus* CFR R35 were subject to biochemical assay with their cell suspension as enzyme source, sodium phytate was degraded under standard conditions at 37°C and 50°C temperatures. The enzyme activity was found to be maximum at 50°C. The selected cultures were further tested for their enzyme existence with in them. Protoplast sonication was the method found to be significant in extracting enzyme and was assayed and found to degrade sodium phytate. When it was subjected to zymogram, at 40-50 kDa, region on native PAGE sodium phytate hydrolysis was observed. Further cultures, when tested for their phytate degrading ability during MFSC and soya milk fermented food processes; they were able to minimize phytate levels as well as improve bio-available minerals.

ePrints@CFTRI

The out come of this study explains that the phytate degrading ability of LAB is due its intracellular phytase enzyme. It also explains that the LAB, which could be an integral part of processed food, resulted in decreased levels of phytic acid for the improved nutritional factors. The resulted improved bio-available minerals during fermentative processes by LAB are independent of phytic acid degradation.

Chapter 1

Introduction and Review of literture

ePrints@CFTRI

1.0 INTRODUCTION

Improvement in both quantity as well as quality of food is needed to cope with the increasing human pressure. Green revolution in cereals, averted problems of starvation, has helped the humanity to a greater extent but it did not address the health problems related to the deficiencies of vitamins and minerals. The impaired absorption of trace minerals (Zinc, Iron and Calcium) besides proteins and vitamin B_{12} are consequences of the excess phytate content in cereals, nuts, legumes and oil seeds, which represent the mainstay of their food intake (Famularo *et al*., 2005; Maga, 1982). There exists a scope for improvement in quality of food (Guttieri *et al*., 2004). Phytic acid is widely distributed in seed as insoluble phytin (Ca-Mg salt of phytic acid) complex and also accounts for 60-85 % of seed total phosphorous (Raboy, 1997).

Several animal experimental studies reveal that the phytate content of some foods such as whole wheat products, wheat bran and soy products is a major determinant, which negatively influences the nutritional balance of trace minerals and proteins in subjects who are on regular vegetarian diet (FAO/WHO, 2001). Natural degradation (due to intrinsic enzymes) of phytic acid is almost impossible and chemical hydrolysis in the laboratory is very slow (Turner *et al*., 2002). However, the enzyme phytase found in plants, animals and microbes (extrinsic enzymes) can rapidly breakdown phyate (Mullaney and Ullah, 2003). Phytases are the hydrolases that initiate the step-wise removal of ortho-phosphate from phytate

ePrints@CFTRI

(Lei and Porres, 2003; Feng *et al*., 2009). Several microbial phytases have been reported in a number of bacteria. Among them, lactic acid bacteria (LAB) are the one which can participate in phytate degradation (Vohra and Satyanaraya, 2003; Haros *et al*., 2007). Exploring phytate degrading LAB in the preparation of cereal-pulse based fermented foods may help in improving the quality of food.

1.1 Phytic acid (PA)

Phytic acid (*myo*-inositol hexakisphosphate, IP6), is a major component of all plant seeds constituting 1-3% by weight of many cereals and oil seeds and accounting for 60-90% of the total phosphorus found in the plant commodities (Loewus, 2002). Table 1.1 illustrates the content of phytic acid in different plant based commodities. The daily intake of phytic acid has been estimated to be 200-800 mg in industrialized countries and 2 g in developing countries (Plaami, 1997). Complementary foods based on cereals are often one of the first semisolid foods introduced into the diet of infants. To improve protein quality, cereals are commonly combined with milk or legumes. However, both cereals and legumes contain relatively high amounts of PA that binds strongly to nutritionally essential minerals such as Ca^{2+}, Fe^{2+}, Mg^{2+}, Zn^{2+}, and other trace elements that can impair their bioavailability (Noureddini and Dang, 2008). The monogastrics or simple-stomached animals like swine, poultry and humans have little or no phytase activity in their gastrointestinal tract (Oloffs *et al*., 2000; Feil, 2001). There is a large body of evidence that minerals are less available from foods of plant origin

ePrints@CFTRI

as compared to animal based foods. Furthermore, phytate-phosphorus is less nutritionally available, since phytate is not hydrolysable quantitatively in the human gut (Sandberg, 1988).

Table 1.1 Phytic acid content in seeds and grains

Plant	Part	% Phytic Acid	Plant	Part	% Phytic Acid
Sesame	Dry seed	4.71	Beans	Dry seed	1.41
Pumpkin/squash	Embryo	4.08	Watermelon	Seed only	1.36
Flax (linseed)	Dry seed	3.69	Kiwi fruit	Fleshy fruit	1.34
Rapeseed (canola)	Dry seed	2.50	Broad beans	Dry seed	1.11
Sunflower	Embryo	2.10	Cucumber	Immature seed	1.07
Mustard	Dry seed	2.00	Sorghum	Dry grain	1.06
Cashew	Embryo	1.97	Cocoa beans	Dry seed	1.04
Peanut Seed	Shell	1.70	Barley	Dry grain	1.02
Tomato	Seed only	1.66	Oats	Dry grain	1.02
Soybean	Dry seed	1.55	Wheat	Dry grain	1.02
Almond	Dry embryo	1.42	Peas	Dry seed	1.00

Afinah *et al.*, 2010

ePrints@CFTRI

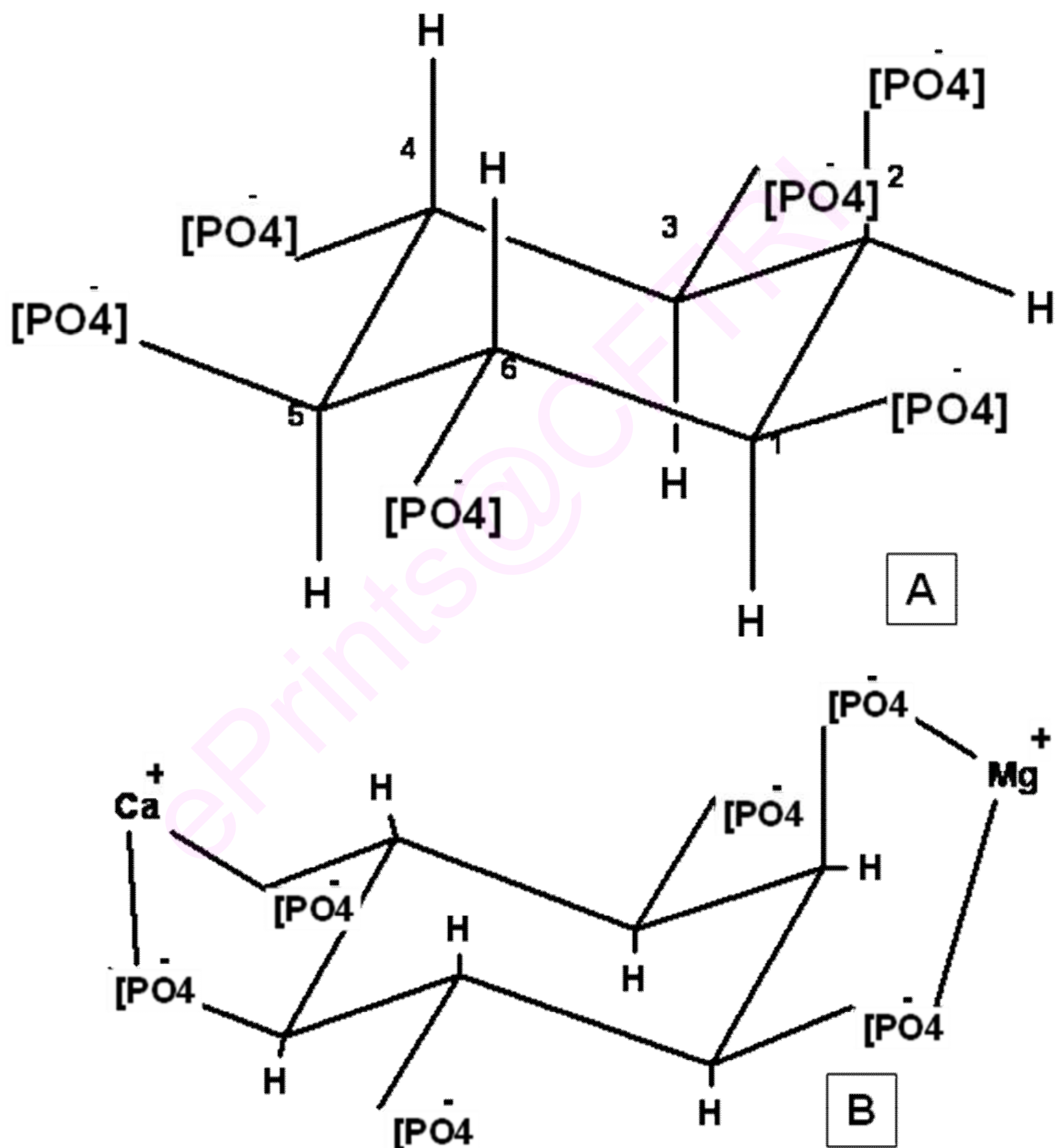
The physiologic roles of phytic acid in plants have been described by Cosgrove (1970). It serves as a phosphorus store, an energy store and a competitor for ATP during its rapid biosynthesis near maturity. It involves in the dormancy induction by inhibiting metabolism and also severs as a regulator of the level of inorganic phosphate. The lower phosphoric esters of *myo*-inositol appear freely in nature in small amounts as transient intermediates in biochemical reactions, i.e. cell signaling both in plants and mammalian cells (Angel *et al*., 2002).

ePrints@CFTRI

1.2 Phytic acid structure and chemistry

Phytic acid consists of a *myo*-inositol ring with six phosphate moieties attached (Graf and Eaton, 1993). The modern terminology given was hexakisphosphate of *myo*- inositol. Chemically, PA has six strongly dissociated protons (pKs 1.1 to 2.1) and six weakly dissociated protons (pKs 4.6 to 10.0). The formation of phytate-mineral (M) or peptide-mineral-phytate complexes exerts an effect on minerals and proteins. These complexes have stoichiometries of the M^+ (n)-phytate type (n=1-6). Phytate forms wide variety of insoluble salts with divalent and trivalent cations (Afinah *et al*., 2010). Hence it can be assumed that PA exists as free acid, phytate or phytin according to physiological pH and the metal ions present. Neuberg (1908) proposed a structure containing $C_6H_{24}O_{27}P_6$ with 18 acid hydrogens. However, Anderson (1914) proposed a structure containing 12 acid hydrogens $C_6H_{18}O_{24}P_6$. The naturally-occurring inositol hexakisphosphates have been synthesized in the laboratory. At higher pH

values, particularly in the presence of coordinating cations such as Na^+ and K^+, conformational inversion takes place to give the (5a/le) VII form found in the crystalline dodecasodium salt. The pictorial representation of phytic acid as well as its possible interaction with cations is given in 1.1.



ePrints@CFTRI

Figure 1.1 *Myo*-inositol hexa kis phosphate and its complex
A: Phytic acid; B: Phytin

The major concern about the presence of phytate in the diet is its negative effect on mineral uptake. Minerals of concern in this regard, include Zn^{2+}, $Fe^{2+/3+}$, Ca^{2+}, Mg^{2+}, Mn^{2+}, and Cu^{2+} (Oloffs *et al.*, 2000; Feil, 2001; Oberleas, 1983), also a negative effect on the nutritional value of protein by dietary phytate. In animals, it has been associated with reduced absorption of certain minerals especially iron. In human trials with radioactive or stable isotopes, PA has been reported to inhibit the absorption of iron (Hallberg *et al.,* 1989), zinc (Navert *et al.,* 1985), calcium (Weaver *et al.,* 1991), magnesium (Bohn *et al.,* 2004) and manganese (Davidsson *et al.,* 1995). Influence of PA on iron and zinc absorption is of great public health importance.

1.3 Interaction of phytic acid with different compounds

Phytate forms complexes with numerous divalent and trivalent metal cations. Stability and solubility of the metal cation phytate complexes depends on the individual cation, the pH-value, the phytate:cation molar ratio, and the presence of other compounds in the solution (Lonnerdal, 2002). The influence of negative charges on phytic acid provides space to bind one or more phosphate group of a single phytate molecule or bridge two or more phytate molecules. Phytate has six reactive phosphate groups and meets the criterion of a chelating agent (Oberleas, 1983). In fact, a cation can bind to one or more phosphate group of a single phytate molecule or bridge two or more phytate molecules (Reddy *et al.*, 1982). Most phytates tend to be more soluble at lower pH compared to higher

ePrints@CFTRI

(Torre *et al.*, 1991). Solubility of phytates increase at pH-values lower than 5.5–6.0 with Ca^{2+}, 7.2–8.0 with Mg^{2+} and 4.3–4.5 with Zn^{2+} as the counter ion. In contrast, ferric phytate is insoluble at pH values in the range of 1.0 to 3.5 at equimolar Fe^{3+}: phytate ratios and solubility increases above pH 4 (Greiner *et al.*, 2006).

The ability of IP6 to complex with multivalent cations is important from the nutritional point of view. Several studies of relative stabilities made using titration methods (Vohra *et al.*, 1965) and listed the order of stability at pH7.4 as $Cu^{2+} > Zn^{2+} > Ni^{2+} > Co^{2+} > Mn^{2+} > Fe^{3+} > Ca^{2+}$. Metallic ions such as Fe^{3+} and Cu^{2+} are known to be effective catalysts for reactions leading to oxidative spoilage in foodstuffs. For this reason the use of IP6 as a sequestering agent has been suggested as a means of reducing spoilage in soya bean oil, ascorbic acid component of soft drinks (Niwa *et al.*, 1967) and in wines (Posternak, 1965).

Minerals are necessary for the activation of intracellular and extracellular enzymes. They regulate metabolic reactions by keeping body fluids at critical pH levels and also maintain the osmotic balance between the cell and its environment. A deficiency of any one of the essential minerals can result in severe metabolic disorders and compromise the health of the organism (Ali *et al.*, 2010). There are numerous evidences that illustrate the anti-nutritional behavior of PA with reference to trace mineral availability.

ePrints@CFTRI

1.3.1 *Phosphorus availability*

The fact that the phosphorus of IP6 is almost unavailable to young chickens was first demonstrated by Common (1939). His data suggested that phosphorus is absorbed as the orthophosphate ion. The ability of various species of poultry to utilize phosphorus from IP6 will largely depend on their ability to hydrolyze the phosphoric ester. The enzyme prepared as an acetone-dried powder from culture fluid of the fungus *Aspergillus ficuum* NRRL 3135 was added to the diet at levels up to 3 g/Kg. At this level chickens utilized phosphorus from IP6 as efficiently as supplemental inorganic phosphate. The added phytase was active in the alimentary tract of the chicken and not in the feed prior to ingestion. It was described by Vohra and Satyanarayana, (2003) that, ruminants are able to utilize phosphorus from IP6. Rapid hydrolysis of IP6 takes place in the rumen (Reid *et al.*, 1947) and the pronounced phytase activity of rumen organisms suggests that this hydrolysis is not dependent on phytases present in the feed (Raun *et al.*, 1956).

ePrints@CFTRI

1.3.2 *Calcium availability*

The anti-calcifying properties of certain cereals were first noticed by Mellanby (1925), and the responsible agent was later identified as IP6 (Bruce and Callow, 1934). The interrelation of dietary calcium with IP6 has been reviewed by Widdowson (1970). Evidences show that the human intestine can absorb calcium from a low- calcium "high phytate" diet as in such a situation hydrolysis of IP6

takes place in the intestine. Presumably, it is postulated that in a low- calcium situation, the IP6 is more soluble and thus is hydrolyzed more readily by intestinal phytases. The tendency to regard the role of IP6 as an important factor in calcium nutrition in humans has been shown in the works of Walker, *et al* (1948).

1.3.3 *Zinc availability*

Zinc is one of the most essential trace mineral trapped by phytic acid and results in decrease in its availability. The first direct evidence that zinc deficiency may develop in animals fed a diet composed of natural materials was obtained by Tucker and Salmon (1955). Zinc deficiency in humans was first recognized by Prasad *et al.,* (1963). PA is also shown to inhibit zinc absorption, (Manary *et al.,* 2000). In 1957 it was reported that zinc in soybean protein was less available to chickens than that in casein and eventually it was accepted that the presence of IP6 in plant products was an important factor in the reduction of zinc absorption from food stuffs (Oberleas, 1973; O'Dell, 1969). Zinc complexes strongly with IP6 particularly at pH 6.0 and furthermore, in the presence of calcium a synergistic effect has been demonstrated (Greiner and Konietzny, 2006).

ePrints@CFTRI

1.3.4 *Iron availability*

Iron is also an essential mineral, whose deficiency can lead to anemia. The negative influence of PA on iron absorption was clearly demonstrated in both adults and in infants (Hurrell *et al.,* 2002). Although there is little doubt that the consumption of a diet containing added IP6 lowers iron levels in human subjects

(Turnbull *et al.*, 1962), the effect of the endogenous IP6 contained in brown bread or whole meal bread is less certain. Added Na- IP6 has been reported to have no effect or only a slightly depressing effect on utilization of iron by rats (Ranhotra *et al.*, 1974). The ability of the rat to utilize the iron naturally present in cereals has been attributed to secretion of intestinal phytase. Morris and Ellis (1976) have reported that the major portion of iron in wheat is present as a salt extractable monoferric salt of IP6 that has a high biological availability to rats.

1.3.5 *Protein complex*

Apart from cations, PA can bind to proteins and lipids (Posternak, 1965). When polyphosphates such as IP6 are added to protein solutions at a pH below the isoelectric point, precipitation takes place and the complex does not dissolve until the pH is lowered to less than 2.0. This observation has been made for IP6 on a large number of proteins, and it appears that the property is common to most of the globular proteins. The precipitation presumably results from an aggregation, by formation of salt-like linkages, of several amino groups in a protein molecule around a molecule of IP6. This leads to folding and a closer packing of the peptide chains and hence to the formation of an insoluble co-activate (Greiner *et al.*, 2006). The binding of IP6 to glycinin, a major globulin of soybean, has been investigated over the range of pH 2-10. The properties of protein-hexakisphosphate complexes are markedly affected by the amount of polyvalent cations present (Okubu *et al.,* 1976). Saio *et al.,* (1969) have studied extensively

ePrints@CFTRI

the effect of calcium levels on the properties and stability of tofu - gel (soybean curd) an important food stuff in Japan. Efforts are under to develop low phytic acid wheat bread for commercial production. In addition, phytate interacts nonspecifically with enzymes such as trypsin, α-amylase, pepsin, β-galactosidase, resulting in a decrease of their activity.

The PA degradation is of nutritional importance, because the degradation results in decrease in mineral binding strength and their solubility increases when phosphate groups are removed from the inositol ring, resulting in an increased bioavailability of essential dietary minerals (Afinah *et al*., 2010). IP6 can be degraded by enzymatic or non enzymatic hydrolysis (Brookes *et al*., 2001). Enzymatic hydrolysis generally occurs during biological processing and preparation of plant food/feed such as steeping, malting, hydrothermal processing, fermentation, and addition of phytase as well as degradation in the gastrointestinal tract (Sandberg, 2002). Table 1.2 demonstrates the phytate content of several plant derived food commodities at different processing conditions. Non-enzymatic hydrolysis usually takes place when food/feed is treated with strong acid or high temperature and pressure (Afinah *et al*., 2010). The enzymatic degradation is more selective and isomer specific (Sandberg, 2002).

ePrints@CFTRI

1.4 Phytase (EC: 3.1.3.8)

In biological system, hydrolysis of PA to *myo*-inositol and inorganic phosphate is an important reaction for energy metabolism, metabolic regulation and signal transduction pathways (Vats and Benergy, 2004). The reaction is catalysed by phytases (*myo*- inositol hexakisphosphate phosphohydrolase), that cleave phosphate group of phytate. During the last 15 years, phytases have attracted considerable attention from both scientists and entrepreneurs in the areas of nutrition, environmental protection, and biotechnology. Phytases have been identified in plants, microorganisms, and in some animal tissues (Konietzny and Greiner, 2002). Based on the catalytic mechanism, phytases can be referred to as histidine acid phytases, β-propeller phytases, cysteine phytases or purple acid phytases (Mullany and Ullah, 2003; Chu *et al*., 2004). Depending on their pH optima, phytases have been divided into acid and alkaline phytases. Based on the carbon in the *myo*-inositol ring of phytate at which dephosphorylation is initiated into it has been classified into 3-phytases (E.C. 3.1.3.8), 6-phytases (E.C. 3.1.3.26) and 5-phytases (E.C. 3.1.3.72). Phytases are histidine acid phosphatases (HAPs), a subclass of phosphatases, which catalyze the hydrolysis of phosphate moieties from PA, thereby, resulting in the loss of ability to chelate metal ions. The histidine residue has been proposed to serve as a nucleophile in the formation of covalent phosphoenzyme intermediates (van Etten *et al.,* 1982).

ePrints@CFTRI

Table 1.2 Phytate content in plant-derived foods

Food	PA (mg/g)	Food	PA (mg/g)
Cereal-based		*Legume-based*	
French bread	0.2–0.4	Chickpea (cooked)	2.9–11.7
Mixed flour bread (70 % wheat, 30 % rye)	0.4–1.1	Cowpea (cooked)	3.9–13.2
Mixed flour bread (30 % wheat, 70 % rye)	0–0.4	Black beans (cooked)	8.5–17.3
Sourdough rye bread	0.1–0.3	White beans (cooked)	9.6–13.9
Whole wheat bread	3.2–7.3	Lima beans (cooked)	4.1–12.7
Whole rye bread	1.9–4.3	Faba beans (cooked)	8.2–14.2
Unleavened wheat bread	10.6–3.2	Kidney beans (cooked)	8.3–13.4
Maize bread	4.3–8.2	Navy beans (cooked)	6.9–12.3
Unleavened maize bread	12.2–19.3	Soybeans	9.2–16.7
Oat bran	7.3–2.1	Tempeh	4.5–10.7
Oat flakes	8.4–12.1	Tofu	8.9–17.8
Oat porridge	6.9–10.2	Lentils (cooked)	2.1–10.1
Pasta	0.7–9.1	Green peas (cooked)	1.8–11.5
Maize	9.8–21.3	Peanuts	9.2–19.7
Cornflakes	0.4–1.5	Others	
Rice (polished, cooked)	1.2–3.7	Sesame seeds (toasted)	39.3–57.2
Wild rice (cooked)	12.7–21.6	Soy protein isolate	2.4–13.1
Sorghum	5.9–11.8	Soy protein concentrate	11.2–23.4
		Buckwheat	9.2–16.2
		Amaranth grain	10.6–15.1

(Greiner and Konietzny, 2006). PA: Phytic acid.

ePrints@CFTRI

1.4.1 *Phytase classification*

The International Union of Biochemistry and Molecular Biology (IUBMB) in consultation with the IUPAC- IUB, Joint Commission on Biochemical Nomenclature (JCBN) have listed two phytases:

1. *3-phytase, EC 3.1.3.8 (myoinositol hexakis phosphate-3-phosphohydrolase)* and
2. *6-phytase, EC 3.1.3.26, (myo-inositol hexakis phosphate-6-phosphohydrolase).*

The two enzymes differ only in the position from which they remove phosphate from the substrate i.e, 3-phytase, EC 3.1.3.8, that hydrolyzes the ester bond at the 3rd position of *myo*-inositol hexakis phosphate to D- *myo*- inositol 1, 2, 4, 5, 6 pentakisphosphate and orthophosphate and 6- phytase, EC 3.1.3.26 which first hydrolyzes the 6th position of *myo*- inositol hexakis phosphate to D- *myo*-inositol 1, 2, 3, 4, 5 pentakisphosphate and orthophosphate. Subsequent ester bonds in the substrate are hydrolyzed at different rates. Both the phytases are members of the hydrolase class of enzymes. In the presence of water they tend to hydrolyze the substrate PA resulting in the release of inorganic phosphate (Wodzinski and Ullah, 1996; Vats and Benergy, 2004). The enzyme, 3- phytase is a characteristic of microorganisms and 6- phytases of the seeds of higher plants (Cosgrove, 1970).

ePrints@CFTRI

1.4.2 *Phytase mechanism*

A 3-phytase (EC 3.1.3.8) first attacks phytate at the 3rd position (Johnson and Tate, 1969) of *myo*-inositol: *Myo*-inositol hexakisphosphate + H_2O = D-myoinositol 1,2,4,5,6-pentakisphosphate + orthophosphate.

While a 6-phytase (EC 3.1.3.26) first attacks phytate at the 6th position (Cosgrove, 1969; 1970) of *myo*-inositol: Myo-inositol hexakisphosphate + H_2O = D-myo-inositol 1,2,3,4,5-pentakisphosphate + orthophosphate.

The physicochemical characteristics and catalytic properties of phytases from various sources indicates it to be ester- hydrolyzing enzyme with an estimated molecular weight of 35- 700 kDa depending upon the source of origin and are usually active within a pH range of 4.5- 6.0 and temperature range at 45-60°C. Generally, phytases from bacteria have optimum pH in neutral to alkaline range while in fungi optimum pH range is 2.5- 6.0. It is demonstrated *in vitro* that, in the stomach where the pH is 2.5, phytase acts on phytin-Ca complex. In small intestine (pH 6.5); phytase does not act on phytin-Ca complex and thus forms a precipitate. Phytases are fairly specific for PA under the assay condition and it is possible to distinguish phytase from acid phosphatase that is incapable of degrading phytase.

The enzyme reaction is likely to proceed through a direct attack of the metal- binding water molecule on the phosphorus atom of a substrate and the

ePrints@CFTRI

subsequent stabilization of the pentavalent transition state by the bound calcium ions. The enzyme has two phosphate binding sites, the “cleavage site”, which is responsible for the hydrolysis of a substrate and the “affinity site, which increases the binding affinity for substrates containing adjacent phosphate groups. The existence of the two nonequivalent phosphate binding sites explains the formation of alternately dephosphorylated *myo*-inositol triphosphates from phytate and the hydrolysis of *myo*- inositol monophosphates (Vohra and Satyanarayana, 2003).

Since, the enzyme has the ability to cleave any of the phosphate groups of phytate, it is highly likely to hydrolyze Ins (1, 3, 5) P3 and Ins (2, 4, 6) P3 further at a rate comparable to that of hydrolyzing Ins P1s. There is no stearic limitation in the simulated binding of each of the Ins (1, 3, 5) P3 and Ins (2, 4, 6) P3 molecules to the active site. However, under *in vitro* condition, in which produced phosphate is not removed, further degradation of the inositol phosphates should be very slow, not only due to the reduced turn-over rate for the hydrolysis of non-adjacent phosphate groups, but also due to the increased susceptibility of the enzyme to the product inhibition. In a physiological situation, the less-phosphorylated *myo*-inositols could be further degraded by the enzyme, owing to the utilization of the produced phosphate ions (Vohra and Satyanarayana, 2003).

Similarly the other phosphatases like alkaline phosphatases and acid phosphatases, particularly purple acid phosphatases are metallo- enzymes. Purple acid phosphatases employ a nuclear Fe^{3+}/ Fe^{2+} or Fe^{3+}/ Zn^{2+} center to catalyze the

ePrints@CFTRI

hydrolysis of phosphate monoesters (Pinkse *et al.*, 1999). In alkaline phosphatases, two Zn^{2+} and one Mg^{2+} are closely bound in the active center (Coleman, 1992). Mg^{2+}ion in the enzyme probably acts only to orient the phosphate containing substrate (De Silva and Williams, 1991), whereas two Zn^{2+} ions together with an arginine and a reactive serine residue are involved in the actual catalysis. Phosphatases have been traditionally divided into alkaline, acid and protein phosphatases (Vincent *et al.,* 1992). Acid phosphatases exhibit an optimum pH of below 7 and can be further divided into three different subclasses: low molecular weight acid phosphatases (18,000), high molecular weight acid phosphatases (50,000) and purple acid phosphatases. Of various HAPs reported, *Phy*A and *Phy*B are the most extensively characterized representatives. They are shown to possess conserved active site sequence, RHGXRXP, which is unique to high molecular weight acid phosphatase (Ullah *et al,* 1991). *Phy*A is characterized by two pH optima (2.5 and 5.0), whereas, *Phy*B is referred to as pH 2.5 optimum acid phosphatase. This is attributed to differences in the charge distribution at the substrate specificity sites of *Phy*A and *Phy*B.

ePrints@CFTRI

1.4.3 *Substrate specificity of phytase*

Phytases from plant and microbiological sources have in general been described as non- specific acid phosphor mono esterases (Sloane- Stanley, 1961). Substrate selectivity studies showed that the phytate degradation was observed due to phytase at pH 2.5 and 6.0 optima but acid phosphatases were unable to

hydrolyze phytate at pH 5.0 (Ullah and Cummins, 1988). Ullah and Phillippy (1994) have reported that both phytase and acid phosphatase (2.5 pH optimum) can efficiently hydrolyze the tested forms of *myo*-inositol phosphates. The difference in pH profiles for these enzymes indicated that the catalytic domains are not identical.

1.4.4 *Plant and animal phytases*

Phytase has been reported in rice, wheat, maize, soybeans, corn seeds, lettuces, dwarf beans, mung beans, fababean, rye, and other legumes or oil seeds (Chang, 1967; Eskin and Wiebe, 1983; Gibson and Ullah, 1990). In germinating seeds or pollen, the phytase seems responsible for phytin degradation (Greene *et al.*, 1975). Plant phytases, however, may be partially or totally inactivated by over-heating or high steam-pelleting temperatures (Ravindran *et al.*, 1995). Phillippy (1999) also demonstrated that wheat phytase lost substantial activity when incubated with pepsin, a proteolytic digestive enzyme.

ePrints@CFTRI

The existence of animal phytase in calf liver and blood was described by Mc Collum and Hart (1908), further it was found to be not a successful finidnig. Phytase was detected in the blood of lower vertebrates such as birds, reptiles, fishes, sea turtle (Rapoport *et al.,* 1941). Because phytate acts as an antinutritional factor for animals, the presence of phytase in the gastrointestinal tract of various animals was investigated. Patwardhan (1937) first noted phytate hydrolysis in the rat intestine. Phytase activity was also observed in the intestine of pig, sheep, and

cow. Bitar and Reinhold (1972) reported partially purified phytase from rat, chicken, calf, and human intestines. About 30 times lower phytase activity was found in the human intestine when compared with that of a rat. The normal human small intestine has a limited ability to digest undegraded phytates (Igbal *et al.*, 1994). It does not seem to play a significant role in phytate digestion, but dietary phytase may be an important factor in phytate hydrolysis (Frolich, 1990). The ruminants probably digest phytate through the action of phytase produced by microbial flora in the rumen.

1.4.5 ***Microbial phytases***

Microbial sources of phytase are the most promising ones for the production of these enzymes on commercial level and for cereal based foods (De Angelis *et al*., 2003; Pandey *et al*., 2001). Phytases have been detected in some of the bacteria that include *Aerobacter aerogenes* (Greaves *et al.*, 1969), *Bacillus subtilis* (Powar and Jagannathan, 1982), *B. subtilis* N77 (natto) (Shimizu, 1992), *Escherichia coli* (Greiner *et al.,* 1993), *Klebsiella aerogenes* (Tambe *et al.*, 1994) and *Pseudomonas* sp. (Irving and Cosgrove, 1971). Phytase activity has also been shown in yeasts (Greenwood and Lewis, 1977) and in rumen microorganisms (Raun *et al.,* 1956). Soil microorganisms and mycorrhizal microorganisms (Greaves and Webley, 1969) have also been studied with respect to their phytase activity. Table 1.3 describes the phytase studied in several LAB. Table 1.4

ePrints@CFTRI

illustrates the properties of representative fungal, yeast, Gram positive and Gram negative bacterial phytases.

Table 1.3 Phytase producing LAB strains

Name of LAB strain	Source of isolation	Reference
Lb. acidophilus BS, *Lb. casei* 1K	Commercial fermented milk	Haros *et al*., 2008
Lb. casei DSM 20011	Cheese	-do-
Lb. fermentum DSM 20052	Fermented beets	-do-
Lb. gasseri DSM 20243, *Lb. johnsonii* DSM 10533	Human	-do-
Lb. plantarum JBPRS, *Lb. plantarum* W42	Plant	-do-
Lb. plantarum 110	Fermented plant food	-do-
Lb. reuteri DSM 20016	Intestine of adult	-do-
Lb. rhamnosus DSM 20021	Lymph node	-do-
Leu. mesenteroides KC51	Kimchi	Oh and In 2009
Ent. faecium A86, *Leu. gelidum* A16, *Lb. plantarum* T211	Pizza dough	Anastasio *et al*., 2009
Lb. plantarum H10, *Lb. plantarum* H5, *Lb. plantarum* L3	Sour dough	
B. adolescentis ATCC 15703, *B. angulatum* ATCC 27535, *B. animalis* DSM 10140, *B. animalis* DSM 20104, *B. breve* ATCC 15700, *B. catenulatum* ATCC 27539, *B. globosum* DSMZ 20092, *B. longum* ATCC 15707, *B. pseudocatenulatum* ATCC 27919	Chicken intestine	Haros *et al*., 2005
Lb.sanfranscisensis CB1	Sour dough	De Angelis *et al*., 2003
Lb. amylovorus B 4552	Plant source	Sreeramulu *et al*., 1996

Prints@CFTRI

Table 1.4 Properties of microbial phytases

Phytase source	pH	Optimum Temperature °C	Specific activity at 37°C U/mg	Reference
A. niger	2.2, 5.0–5.5	55-58	50-103	Wyss *et al.*, 1999
A. terreus	5.0-5.5	70	142-196	Wyss *et al.*, 1999
A. fumigatus	5.0-6.0	60	23-28	Wyss *et al.*, 1999
A. oryzae	5.5	50	11	Shimizu, 1993
E. coli	4.5	55-60	811-1800	Greiner, 1993; Golovan, 2000
K. terrigena	5.0	58	205	Greiner, 1997
K. pneumoniae	5.0-5.5	50,60	224, 297	Sajidan *et al.*, 2004
K. aerogenes	4.5-5.2	68	-	Tambe *et al.*, 1994
Lb. sanfranciscensis	4.0	45	-	De Angelis *et al.*, 2003
B. subtilis	6.5-7.5	55-60	9-15	Kerovuo *et al.*, 1998; Shimizu, 1992
B. amyloliquefaciens	7.0-8.0	70	20	Kim *et al.*, 1998
Lb. plantarum	5.0	50	-	Palacios *et al.*, 2005
Lb. plantarum	5.5	65	0.463	Zamudio *et al.*, 2001

A.: *Aspergillus*; ***E.***:*Escherichia*; ***K.:*** *Klebsiella*; ***Lb***.:*Lactobacillus*; ***B.***:*Bacillus*.

ePrints@CFTRI

Among food microorganisms, in particular yeasts and Bifidobacteria are noteworthy phytase sources due to their various applications and safety (De Angelis *et al*., 2003; Oh and Lee, 2007; In *et al*., 2008). Most LAB isolated from different food fermentations and ecosystems have shown to produce phosphatase activity with low levels of activity against phytate (Zamudio *et al*., 2001; Palacios *et al*., 2005). Several bacteria were able to degrade phytate during growth and produce either extracellular or intracellular phytases even if only few strains of LAB have shown consistent phytase activity (Sreeramulu *et al*., 1996; Zamudio *et al*., 2001; De Angelis *et al*., 2003). Microorganisms with phosphatase and phytase activities can be potentially used as starter cultures for cereals and legume fermentation, to improve dietary nutrients and phosphate.

ePrints@CFTRI

Previous studies also have demonstrated that the degradation of phytate in the stomach and intestine is mainly due to dietary phytases and, probably, to the metabolic activity of the colonic microflora (Sandberg, 2002). So far, the only phytic acid degrading bacteria identified in human faeces are members of the genera *Bacteroides* and *Clostridium* and the Gram-negative bacteria *E. coli* and *Klebsiella pneumoniea* (Steer *et al*., 2004). This biochemical property has not been attributed to intestinal isolates of the genera *Lactobacillus* and *Bifidobacterium*, which are important integrants of the gut microflora and the preferred source of probiotics (Haros *et al*., 2005). This activity has only been screened in *Lactobacillus* cultures isolated from food fermentations (De Angelis

et al., 2003). These isolates rarely produce phytase activity although they normally possess non-specific acid phosphatase activity (Zamudio *et al*., 2001). Recently, novel phytate degrading enzymes from bifidobacterial strains were incorporated in wheat dough as a fermentation starter replacing the common LAB. However, taking into account the phytate degrading activity besides the pH and the total titrable acidity of the resulting dough, the Bifidobacterium strains from infants could be good starter for being used in bread making (Palacios *et al*., 2008).

LAB are often used for food fermentation. These bacteria increase the shelf life and the nutritional value of many products, also contributing to their unique organoleptic characteristics (Palacios *et al*., 2005), and also provide health benefits to consumers (Tsangalis *et al*., 2002). LAB degrades phytic acid by means of acid hydrolysis as well as specific enzyme hydrolysis (Figure 1.2). Similarly, probiotics may help alleviate symptoms of lactose intolerance, intestinal atopic disorders, and celiac disease, and are used in the treatment and prevention of diarrhea, ulcerative colitis, and irritable bowel syndrome as well as for urogenital tract and *Helicobacter pylori* infections (Kolida *et al*., 2006). There have also been claims for cholesterol-lowering effects (Liong and Shah, 2005), anticarcinogenic actions (Commane *et al*., 2005), and augmentation of immune function (Reid, 2002).

ePrints@CFTRI

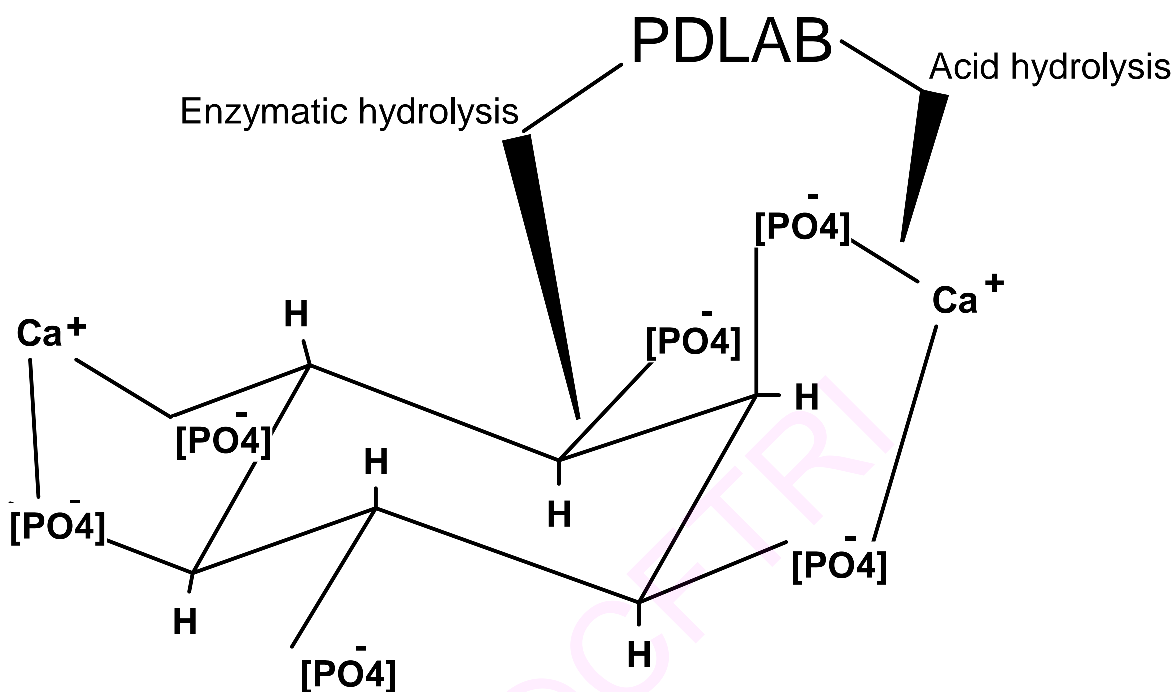


Figure.1.2 Schematic representation of phytate hydrolysis by LAB
PDLAB: phytate degrading lactic acid bacteria

ePrints@CFTRI

1.4.6 ***Advantages of microbial phytases over plant phytases***

There are several advantages have been listed for microbial phytases over plant phytase. Microbes producing phytases can be easily maintained in lab or commercial scale. They are easier to process and scale up and are also activate wide ranges of temperatures and pH. Down stream process for the microbial phytases are more comfortable than plant phytases (Vohra and Satyanarayana, 2003).

1.5 Lactic acid bacteria

In the late 19th century, microbiologists observed microflora in the GI tracts of healthy individuals that are different from those found in diseased individuals (Parvez *et al*., 2006). During beginning of 20th century, Elic Metchnikoff a Nobel laureate explained that the consumption of fermented milk has a beneficial effect to humans, these attributes includes, lactose digestion and production of bioactive metabolites and also noted that maintenance of proper equilibrium of microflora can be ensured by constant supplementation of beneficial microorganisms in the diet (Fuller, 1991). Fermentation has been used for many centuries throughout the world. Microorganisms, especially LAB, have been involved in many food fermentations including dairy and non-dairy products (De Angelis *et al*., 2003). LAB were used in various fermented foods since antiquity. The preservation and health benefits of such traditional foods have been recognized for thousands of years and accordingly lactic acid fermentation played an important role in the early years of Microbiology.

ePrints@CFTRI

1.5.1 *Isolation of LAB*

The isolation and screening of LAB from natural sources has an important means of obtaining useful and genetically-stable strains for industrial and probiotic applications (WHO/FAO 2002). As it occurs naturally in several sources such as human faeces, naturally fermented foods (Table 1.5) *etc*. have been considered for the isolation to study their probiotic properties (Rodriguez *et*

al., 2000). Table 5 demonstrates that the fermented products originated from several countries involve LAB as an integral part.

Table 1.5 LAB isolated from various sources

Source of isolation	**Lab strains**	References
Columbian dairy products	*Str. thermophilus* and *Lb. delbrueckii ssp. bulgaricus*	Velez *et al*., 2006
Morcilla (Blood Sausage)	*Leuconostoc, Pediococcus, Lb. sp, Weisella viridescens* and *Carnobacterium*	Santosa *et al*., 2005
Malaysian traditional fermented foods	*Lb. casei* and *Lb. plantarum*. *Lactococcus lactis* and *Lb. casei*	Adnan and Tan, 2007
Spontaneously fermented millet porridge and drink	*Lb. salivarius, Ped. pentosaceus, Ped. acidilactici* and *Lb. paraplantarum*	Lei and Jakobsen, 2004
Faecal sample from Rabbit	*Ent. faecalis* and *Ent. faecium*	Linaje *et al*., 2004
Chicken crop & intestine	*Lb. plantarum*	Lin *et al*., 2007
Wheat sourdoughs	*Lb. sanfranciscensis, Lb. fermentum, Lb. brevis, Lb. alimentarius, Lb. farciminis,* *Lb. plantarum, Lb. fructivorans,* and *Weissella confusus*	Corsetti *et al*., 2003
Tradition fermented food ('Boza')	*Lb. plantarum, Lb. rhamnous, Lb. pentosus, Lb. paracasei*	Todorov *et al*., 2007
Ghanaian fermented Maize	*Lb. plantarum* and *Lb.fermentum*	Jacobsen *et al*., 1999

ePrints@CFTRI

1.5.2 *Characterization of LAB*

Traditionally taxonomic characterizations of LAB have been carried out according to the Bergey's manual of Systematic Bacteriology. Species level identification can be achieved based on this classification method. Biochemical characterization include, Gram's staining, Catalase test, gas production from glucose, growth at different temperatures, pH and NaCl concentration, hydrolysis of arginine and utilization of various carbon sources, are widely used (Hamad *et al*., 1997).

The classification of LAB was initiated in 1919 by Orla-Jensen (Holzapfel et al., 2001) and was until recently primarily based on morphological, metabolic and physiological criteria. The taxonomic classification criteria are depicted in Table 1.6. LAB comprises a diverse group of Gram-positive, non spore forming, non motile rod and coccus shaped, catalase negative organisms. They are chemo organotrophic and only grow in complex media. Fermentable carbohydrates and higher alcohols are used as the energy source to form chiefly lactic acid (Savadogo *et al*., 2006). LAB degrades hexoses to lactate (homofermentatives) or lactate and additional products such as acetate, ethanol, CO_2, formate or succinate (heterofermentatives). They are widely distributed in different ecosystems and are commonly found in foods (dairy products, fermented meats and vegetables, sourdough, silage, beverages), sewage, on plants but also in the genital, intestinal and respiratory tracts of man and animals (Rodriguez *et al*., 2000).

ePrints@CFTRI

Table 1.6 LAB and current taxonomic classification

Genus	Shape	Catalase	Nitrite reduction	Fermentation	Current genera
Betabacterium	Rod	-	-	Hetero	*Lactobacillus* *Weissella*
Thermobacterium	Rod	-	-	Homo	*Lactobacillus*
Streptobacterium	Rod	-	-	Homo	*Lactobacillus* *Carnobacterim*
Streptococcus	Coccus	-	-	Homo	*Streptococcus* *Enterococcus*
Lactococcus *Vagococcus* *Betacoccus*	Coccus	-	-	Hetero	*Leuconostoc* *Oenococcus* *Weissella*
Microbacterium	Rod	+	+	Homo	*Brochothrix*
Tetracoccus	Coccus	+	+	Homo	*Pediococcus** *Tetragenococus*

Adopted from Holzapfel *et al*., 2001; *In genera *pediococci* are catalase negative but some strains produce a pseudocatalase that results in false positive reactions.

ePrints@CFTRI

Current methodologies used for classification of LAB mainly rely on 16S ribosomal ribonucleic acid (rRNA) gene analysis and sequencing (Olsen *et al*., 1994). Based on these techniques, Gram-positive bacteria are divided into two groups depending on their G + C content. The actinomycetes have a G + C content above 50 mol% and contain genera such as *Atopobium*, *Bifidobacterium*, *Corynobacterium* and *Propionibacterium*. In contrast, the *Clostridium* branch has a G + C content below 50 mol% and include the typical LAB genera

Carnobacterium, Lactobacillus, Lactococcus, Leuconostoc, Pediococcus and *Streptococcus* (Savadogo *et al*., 2006).

Although several biochemical or phenotypic tests are used in identification of LAB, there is a limit to identify or differentiate between strains. This led to the development of molecular tools for characterization of LAB. The following analysis are mainly used in identification of LAB and also reported in Table 1.7.

1.6 LAB as probiotic

Lactic acid bacteria were referred to as probiotics in scientific literature by Lilley and Stillwell (1965). Parker (1974) redefined it as organisms and substances that contribute to the intestinal microbial balance. The most recent and accurate description of probiotics was undertaken by Fuller (1989) who redefined it as 'a live microbial feed supplement beneficial to the host (man or animal) by improving the microbial balance within its body'. According to FAO/WHO (2000) it can also be defined as viable microbial food supplements which beneficially influence the health of the host.

ePrints@CFTRI

1.6.1 *Properties of probiotic LAB*

Probiotic is a microbial dietary adjuvant that beneficially affects the host physiology by modulating mucosal and systemic immunity, as well as improving nutritional and microbial balance in the intestinal tract. Whereas, probiotic - active substance is a cellular complex of LAB that has a capacity to interact with the host

mucosa and may beneficially modulate the immune system independent of LAB viability (Coudeyras *et al*., 2008).

Table 1.7 General approaches used in LAB identification

Study	Approach	Discriminatory power
Phenotypic methods Morphological analysis, Physiological analysis Biochemical characterization Protein profiling	Microscopic analysis Growth characteristics and simple tests Assimilation and fermentation pattern (API and Biolog) SDS-PAGE analysis of cellular proteins	Genus level Genus level Genus level or species level Species level
Genotypic methods Specific primers Sequencing RFLP AFLP RAPD-PCR Rep-PCR PFGE Ribotyping Hybridization probe	 PCR with group specific primers Determination of gene sequencing (16S rDNA) Restriction enzyme analysis (REA) of genomic DNA or PCR amplicons Combination of REA and PCR amplification Randomly primed PCR PCR targeting repetitive interspersed sequences REA and pulsed-field gel electrophoresis REA and oligonucleotide prove detection DNA-DNA hybridization using labeled probes	 Depending on primer used Genus to species level Species to strain level Species to strain level Species to strain level Species to strain level Strain level Species to strain level Genus to species level

RFLP: Restriction Length Polymorphism; **AFLP**: Amplified Fragment Length Polymorphism; **RAPD**: Random Amplified Polymorphic DNA; **PFGE**: Pulse Field Gel Electrophoresis; **SDS-PAGE**: Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (Source: Temmerman *et al*., 2004).

ePrints@CFTRI

LAB with probiotic activity is generally enteric flora, believed to play a beneficial role in the ecosystem of the human gastrointestinal tract. The probiotic spectrum of activity can be divided into nutritional, physiological and antimicrobial effects. These observations have led to the development of a variety of foods and feeds containing LAB cells for probiotic use in man and animals (Gerritse *et al.,* 1990). Some of the nutritional and therapeutic effects ascribed to LAB, viz., They improve the nutritional quality of food and feed. They also trigger the metabolic stimulus for the synthesis of vitamins and enzymes. LAB stabilizes the gut microflora and also excludes enteric pathogens, enhances innate host defenses by producing antimicrobial substances. By assimilating cholesterol helps in reducing serum cholesterol, reduces cancer by detoxification of carcinogens and with cell mediate immune system it is helpful in tumor suppression.

Recent global marketing trends of probiotics are based on expectations of a prophylactic effect and in many cases as an alternative to more conventional pharmaceutical preparations. Although used in humans and animals for generations, only recently, probiotics have been subjected to clinical research. The most common use of probiotics is as food in the form of fermented milk products. The list of probiotic effects and health claims with the use of LAB is expanding (Dicks and Botes, 2010; Coudeyras *et al*., 2008). There are several characteristics that are of importance for organisms used as probiotics (Hoves *et al*., 1999).

ePrints@CFTRI

These include: the organism should maintain viability and activity in the carrier food before consumption, should survive the upper gastrointestinal tract, be capable of surviving and growing in the intestine, be a normal inhabitants of the intestinal tract, and eventually produce beneficial effects when in the intestinal tract. Further, the organism must be non-pathogenic and non-toxic (Hoves *et al*., 1999).

1.6.2 *Survival of probiotics during journey in gastrointestinal tract*

Bacteria from food and the environment enter the mouth and are washed with saliva into the stomach. Most of the bacteria are destroyed in the stomach by gastric acid. The effect of LAB in the intestine requires that the bacteria or at least their enzymes survive the acid gastric content and are active after the passage of the stomach. Studies of orally administered LAB have demonstrated that the LAB counts in the small intestine increase significantly after ingestion (Robins-Browne *et al.*, 1981). Among many mechanisms operating in the gastrointestinal tract, gastric acid is a major host defense mechanism against infection from ingested pathogenic microorganisms. Gastric acid is also important in maintaining a sparse bacterial population in the upper small bowel because; only the most acid resistant organisms survive transit through the stomach. The small intestine constitutes a zone of transition between the sparsely populated stomach and the luxuriant bacterial flora of the colon. Intestinal motility and the inhibitory effects

ePrints@CFTRI

of bile salts are major host factors in preventing bacterial overgrowth in the small bowel (Zarate *et al*., 2000; Fernandez *et al*., 2003).

1.6.3 *Adherence property*

One of the main criteria for selecting probiotic strains is their ability to adhere to intestinal surfaces. Attachment to mucosa prolongs, during the period probiotics can influence the gastrointestinal immune system and microbiota of the host. Thus the ability to adhere to intestinal surfaces is thought to correspond to the efficacy of the probiotic strain. The antibody detected from the serum of people treated with probiotic bacteria has been shown to be directly correlated with the adherence ability of the used strain (Coudeyras *et al*., 2008). Bacterial adhesion is initially based on non-specific physical interactions between two surfaces, which enable specific interactions between adhesions (usually proteins) and complementary receptors (Beachey, 1981). Studying bacterial adhesion *in vivo* is difficult and *in vitro* models with intestinal cell lines are widely adapted methods for this assessment (Lehto and Salminen, 1997). The mucus covering the epithelial cells is the initial surface that ingested microorganisms confront in the human gut and is considered to be an important site for bacterial adhesion and colonization (Mikelsaar *et. al.,* 1998). Mucus is continually subjected to degradation; conversely new mucin glycoproteins (the major components of mucus) are constantly secreted. Adherence of probiotic strains has also been investigated using immobilised human intestinal mucus glycoproteins extracted

ePrints@CFTRI

from faeces (Kirjavainen *et al.,* 1998; Ouwehand *et al.,* 1999). The strains tested showed considerable variation in their degree of adhesion to intestinal mucus glycoproteins. However, the mechanisms involved in probiotic attachment to mucus glycoproteins are poorly known (Chauviere *et al,* 1992; Adlerberth *et al.,* 1996).

Thus, bacteria that are able to adhere to mucus but unable to reach the epithelial cells might be dislodged from the mucosal surface with the degraded mucin and washed away with the luminal contents. This may partly explain the transient pattern of colonization characteristic for most probiotic bacteria. On the basis of these remarks, an *in vitro* evaluation of the bacterial adhesion to human intestinal mucus provides a good additional model for studying the ability of probiotics to adhere to intestinal surfaces.

ePrints@CFTRI

1.6.4 *Antimicrobial property*

Several investigations have demonstrated that various species of LAB exert antagonistic action against intestinal and food borne pathogens (Gibson *et al.,* 1997). LAB are capable of preventing the adherence, establishment, replication and/or pathogenic action of specific enteropathogens (Savedra, 1995). These antagonist properties may be manifested by

1. Decreasing the luminal pH through the production of volatile short chain fatty acid (SCFA) such as acetic, lactic, or proprionic acid.

2. Rendering specific nutrients available to pathogens
3. Decreasing the redox potential of the luminal environment
4. Producing hydrogen peroxide under anaerobic condition
5. Producing specific inhibitory compounds such as bacteriocins (Havenaar *et al.,* 1992; Sanders, 1993).

A) Lactic acid and volatile acids

Fermentation involving LAB results in accumulation of organic acids, primarily lactic acid as a major end product of carbohydrate metabolism, generated from pyruvate by lactic acid dehydrogenase. The accumulation of lactic acid and the concomitant reduction in pH of the milieu results in a broad-spectrum inhibitory activity against Gram-positive and Gram-negative bacteria. The acidic pH, dissociation constant (pK value), and mole concentration are the factors that determine the inhibitory activity of lactic acid and acetic acid in the milieu (Ingram *et al,* 1958). Because of the high pK value, acetic acid (pK 4.75) has more antimicrobial activity than the lactic acid (pK 3.86) (Rasic and Kurmann, 1983). Lipophilic acids such as lactic acid and acetic acid in undissociated form penetrate the microbial cell membrane, and at higher intracellular pH dissociate to produce hydrogen ions that interfere with essential metabolic functions such as substrate translocation and oxidative phosphorylation (Baird-Parker, 1980).

ePrints@CFTRI

B) Hydrogen peroxide

In the presence of oxygen, LAB produces hydrogen peroxide (H_2O_2) through electron transport *via* flavin enzymes, and in the presence of H_2O_2, produces superoxide anions from destructive hydroxyl radicals. This process may lead to per-oxidation of membrane lipids (Morris, 1979), and increased membrane permeability (Kong and Davison, 1980). The resulting bactericidal effect of these oxygen metabolites has been attributed to their strong oxidizing effect on the bacterial cell as well as destruction of nucleic acids and cell proteins (Piard and Desmazeaud, 1992). Also, H_2O_2 could react with other cellular and milieu components to form additional inhibitory substances. H_2O_2 formation by LAB and its effect on various microorganisms has been documented for years (Dahiya and Speck, 1968). LAB strains have been reported to produce H_2O_2under aerobic conditions in a complex glucose based media.

ePrints@CFTRI

C) Bacteriocins

The gastrointestinal tract contains many antimicrobial proteins such as colicins, defensins, cercropins and magainins. These are low-molecular weight, cationic, amphiphilic molecules; tend to aggregate and are benign to the producing organism. LAB also produce wide range of similar antagonistic factors that include metabolic products, antibiotic like substances and bactericidal proteins, collectively termed bacteriocins. Bacteriocins vary in spectrum of activity, mode of action, molecular weight, genetic origin and biochemical

properties. Bacteriocins can be produced spontaneously or induced (Savadogo *et al*., 2006). The genetic determinants of most of the bacteriocins are located on the plasmids, with a few exceptions, which are chromosomally encoded. The release of bacteriocins requires the expression and activity of released proteins and the presence of detergent resistant phospholipase A in the bacterial outer membrane of the LAB. These antimicrobial agents are species specific and exert their lethal activity through adsorption to specific receptors located on the external surface of sensitive bacteria, followed by metabolic, biological and morphological changes, resulting in the killing of such bacteria (Savadogo *et al*., 2006).

1.6.4 Alleviation of lactose intolerance symptoms

Lactose maldigestion is present in approximately 70% of the population worldwide. In infants, primary lactose intolerance is virtually nonexistent. *Lb. bulgaricus* and other lactobacilli commonly used in the fermented milk industry present sufficient active β-galactosidase to significantly decrease the lactose in the product. Kilara and Shahani (1976) suggested that yoghurt containing *Lb. bulgaricus* and *Str. thermophilus* had a beneficial effect for lactose intolerant individuals because of the endogenous lactase. These findings were further supported by the research of Gilliland and Kim, (1984) Marteau *et al.,* (1990). This aspect has been reviewed extensively by Sanders (1994). Lin *et al.,* (1991) demonstrated the importance of selecting strains for their β-galactosidase activity.

ePrints@CFTRI

Martini *et al.,* (1991) indicated that lactose from yoghurt was digested better by lactose-deficient adults partly due to the microbial β-galactosidase activity. The yoghurt contained *Lb. delbrueckii* spp. *bulgarcius* and *Str. salivarius* spp. *thermophilus.* Shermak *et al.,* (1995) examined the effect of the consumption of yoghurt and milk on lactose absorption in 14 lactose-malabsorbing children aged 4 to 16 years. They found that considerably fewer children experienced symptoms of lactose maldigestion after consuming yoghurt containing active cultures of *Lb. bulgaricus* and *Str. thermophilus* as compared to only milk.

ePrints@CFTRI

1.6.5 *Supplementary effects*

There are numerous studies indicating that fermentation of food with LAB cultures increase the quantity, availability, and digestibility of nutrients. Yoghurt, like milk, is a good source of protein, riboflavin, folic acid, and calcium. The basis for this conclusion comes from direct measurements of vitamin synthesis and from increased feed efficiency when fermented products are fed to animals (Gorbach, 1990). Fermentation has been reported to increase folic acid in a variety of products, including yoghurt, bifidus milk, and kefir (Alm, 1982). There have also been studies showing an increase in niacin and riboflavin in yoghurt, B12 in cottage cheese, and pantothenic acid (B6) in Cheddar cheese (Deeth and Tomine, 1981; Alm, 1982). Thiamin and riboflavin have also been shown to increase during the preparation of LAB-fermented products.

1.6.6 *Production of vitamins*

Several LAB cultures synthesize certain vitamins (vitamin B) in fermented dairy products. In contrast, directly acidified dairy products do not exhibit such enhancement in vitamin B. Reddy *et al*., (1976) studied the effect of various factors on vitamin B content of cultured yoghurt and compared the vitamin B contents of cultured and direct acidified yoghurt. Incubation of yoghurt culture at 42°C for 3 h yielded maximum vitamin synthesis concurrent with optimal flavor and texture qualities. Acidified yoghurt showed a slightly higher content of certain B vitamins than the cultured yoghurt. Both cultured and acidified yoghurt showed good keeping quality and freedom from microbial contaminants during storage at 5°C for 16 days. However, folic acid and vitamin B12 content decreased 29 and 60% in cultured yoghurt and 48 and 54% in acidified yoghurt. Leim *et al*., (1977) found that the major source of vitamin BI2 in commercial tempeh (fermented soybean food product) was a LAB that co-exists with the mold during fermentation. Reinoculation of the pure LAB in dehulled, hydrated, and sterilized soybeans resulted in the production of vitamin B12. Similarly, nutritionally significant amounts of vitamin B12 were also found in the Indonesian fermented food (Leim *et al*., 1977).

1.7 Fermentation and LAB

Research findings have brought to light the invaluable attributes of fermented food products. It is now known that fermentation process leads to production of

ePrints@CFTRI

valuable products including flavour and aroma compounds; biomass proteins/amino acids; minerals; lipids; carbohydrates; vitamins and other products of the respiratory/biosynthetic process such as lactic acid, ethanol, acetylaldehydes, pyruvic acid, which help in altering the pH of food to levels that do not favor growth of pathogenic microorganisms (Au and Fields, 1981; Baghel *et al.*, 1985; Steinkraus, 1996; Deshpande and Salunke, 2000; Beaumont, 2002; Annan *et al.*, 2003; Kalui *et al.*, 2009). This in turn enhances food safety and increases food shelf life hence aiding in food preservation (Yasmine, 2002). The changes associated with the fermentation process are due to the action of enzymes produced by microorganisms (Pederson, 1979; Steinkraus, 2002). Fermentation could lead to reduction of toxic products (Steinkraus, 1983) and has been reported to improve the bioavailability of minerals such as iron and zinc by significantly reducing the phytate compounds present in fermented cereals (Sankara and Deosthale, 1983). Fermentation leads to production of acids and probable bacteriocins that prevent growth of microorganisms hence increasing shelf life of fermented products (Mbugua and Njenga, 1991; Chen and Hoover, 2003; Kalui *et al.*, 2009). This is a very valuable attribute especially in rural areas where advanced food preservation technologies such as refrigeration are not affordable and considering that people are beginning to appreciate more of naturally preserved than chemically preserved foods (Rolle and Satin, 2000).

ePrints@CFTRI

Fermented foods are associated with 'good bacteria' referred to as probiotics (Patricia *et al*., 2002; Helland *et al*., 2004). People with flourishing intestinal colonies of beneficial bacteria are better equipped to fight the growth of disease causing bacteria (Reid *et al*., 2003) Examples of probiotics that have found application in probiotic products include some strains of *Lactobacillus* genera (*Lb. plantarum, Lb. rhamnosus, Lb. acidophilus, Lb. reuteri, Lb. gasseri,* and *Lb. amylovorus*); *Bifidobacterium* genera *(B. adolescentis, B. animalis, B. bifidum, B. breve, B. infantis, B. lactis,* and *B. longum); Enterococcus (Ent. faecalis,* and *Ent. faecium)* (Holzapfel and Schillinger, 2002). Species of the genera *Lactobacillus* are the most widely studied for probiotic attributes (Mishra and Prasad, 2005).

LAB important in food technology include those of the genera *Lactobacillus, Lactococcus, Pediococcus,* and *Leuconostoc* (Harrigan and McCance, 1990). *Lb. fermentum, Ped. pentosaceus, W. confusus, Lb. plantarum, Lb. salivarius, Lb. casei, Lb. acidophilus,* and *Leuconostoc* spp are some species that have been reported isolated from cereal based fermented foods (Achi, 2005; Kalui *et al*., 2009). Examples of *Lactobacillus* spp involved in LAB fermentation of cereal based fermented foods include *Lb. plantarum, Lb. casei, Lb. sakei, Lb. acidophilus,* and *Lb. salivarius* among others (Jacobsen and Lei, 2004; Achi, 2005; Kalui *et al*., 2009). Kalui *et al*., (2009) reported isolation of *Lb. fermentum, Ped. pentosaceus, Lb. plantarum, W. confusus* and *Lb. rhamnosus* from ikii, a traditional fermented maize porridge. *Lb. fermentum* have been reported as the

ePrints@CFTRI

predominant species in *Kisra* a Sudanese sorghum fermented flat bread (Hamad *et al*., 1997). *Lb. fermentum* and *Lb. plantarum* have been reported to be the most commonly associated LAB species with spontaneous lactic acid fermentations of cereal products (Kunene *et al*., 2000).

1.8 Supplemented foods

The species of LAB used in the preparation of probiolic products include *Lb. bulgaricus, Lb. lactis, Lb. salivarius, Lb. plantarum, Str. thermophilus, Ent. faecium, Ent. fecalis,* and *Bifidobacterium* sp. Considerable attention has been given in recent years to the use of *Bifidobacteria* in probiotic foods, particularly in Japan and Europe (Ishibashi and Shimamura, 1993). Hughes and Hoover (1991) reviewed and summarized a number of probiotic applications for bifidobacteria. In the 1940s, bifidus milk was used as a treatment for infants with nutritional deficiencies, in Japan, the first bifidus product (low-fat fresh milk containing *B. longum* and *Lb. acidophilus)* was developed by Morinaga Milk Industry Company in 1971; full scale production began in 1977 when the company started a home delivery service (Ishibashi and Shimamura, 1993). By 1984, there were 53 Bifidus products in the market in Japan. Today, many products, including yoghurts, have been reformulated to include bifidus cultures; total yoghurt sales in Japan have nearly doubled from the 1980s to 1990s (Hughes and Hoover, 1991).

ePrints@CFTRI

Today, many products containing LAB are available worldwide. Probiotic preparations are manufactured in various forms (tablets or powders) and also incorporated in a number of foods (milk, chewing gums, fiber preparations, sweets, cakes, beer, and soymilk). Some of probiotic products available worldwide and the LAB used in their production are summarized in Table 1.8.

In developing functional foods and neutraceuticals, food-grade LAB have been studied to select types with optimal qualities for fermentation (Fumalaro, *et al*., 2005). One study indicated that from 94 LAB strains isolated from fermented vegetable or bamboo products, 59% would degrade phytic acid. *Lactobacillus plantarum* exhibited particularly potent activity (Tamang *et al*., 2009). Soymilk is increasingly being consumed as a milk substitute by perimenopausal women, people with lactose intolerance, and vegans (Ryan-Borchers *et al*., 2006). To ensure that soymilk is nutritionally equivalent to cow's milk, it is often fortified with calcium. The bioavailability of added calcium, may however be compromised if high levels of phytate is present. This calcium availability study aimed to investigate the phytase activity of 7 strains of *Lactobacillus* spp. that are commonly used as probiotics in fermented foods. Their phytase activity was analyzed when they were incubated in culture media and also when they were fermented in a commercially available soymilk fortified with a proprietary phosphate of calcium fortificant (Tang *et al*., 2010).

ePrints@CFTRI

Table 1.8 LAB-supplemented foods currently available in different markets

Product or trade name	Origin	LAB culture
A B milk products	Denmark	*Lb. acidophilus, B. bifidum*
Acidophilus bifidus	Germany	*Lb. delbrueckii subsp. bulgaricus,*
Yoghurt	Europe	*Str. thermophilus Lb. acidophilus B. bifidum or B. longum*
B A®	France	*B. longum*
Bifidus milk	Germany	*B. bifidum or B. longum*
Bifidus milk with yoghurt flavor	UK	*B. bifidum, B. longum, or B. infantis*
Bifidus yoghurt	Many countries	*B. bifidum or B. longum*
Bifighurt®	Germany	*B. bifidum or B. longum*
Bioghurt®	Germany	*Lb. acidophilus, B. bifidum, S. thermophilus*
Biokys®	Czechoslovakia	*B. bifidum, Lb. acidophilus, Ped. acidilactici*
Biomild®	Germany-	*Lb. acidophilus, Bifidobacterium sp.*
Cultura®	Denmark	*Lb. acidophilus, B. bifidum*
Diphilus milk®	France	*Lb. acidophilus, B. bifidum*
Mil-Mil®	Japan	*B. bifidum, B. breve, Lb. Acidophilus*
Sweet acidophilus bifidus milk	Japan	*Lb. acidophilus, B. longum*
Sweet bifidus milk	Japan/Germany	*Bifidobacterium, sp.*
Prolife®	India	*Lb. acidophilus*
Yakult	India	*Lb. Shirota*

Source: Tamime *et al*., 1995

A more acceptable alternative in food production could be to use the enzymatic activity of phytase naturally occurring in the ingredients of cereal-based foods. Phytase in grains and seeds can be activated by traditional food

ePrints@CFTRI

processing methods such as soaking, germination and fermentation to decrease the PA content in complementary and other foods (Porres *et al*., 2001). However, these processing methods change the composition, viscosity and taste of the complementary foods considerably and might result in products with low consumer accessibility. In addition a complete PA degradation is necessary to improve mineral absorption (Hurrell *et al*., 1992) generally required prolonged fermentation and therefore might introduce problems of microbiological safety. There are several lactic bacterial strains which involves in bread making are evolved from *bifidobacterium* spp. to degrade PA (Lopez *et al*., 2002).

1.9 Functional foods

Scientific investigations have changed the view of the role of food as being beyond the provision of energy and body forming substances to having the extra role of possessing active substances that impart health benefits to the consumer (Grajek *et al*., 2005). Foods are now known to contain bioactive substances that prevent the initiation, promotion and development of allergies, diseases such as cancer, cardiovascular diseases, diarrhea, osteoporosis, among others (Sanders, 2003; Lei *et al*., 2008). This has led to the emergence of interest in functional foods which are defined as a part of an everyday diet and are demonstrated to offer health benefits and to reduce the risk of chronic diseases beyond the widely accepted nutritional effects.

eprints@CFTRI

Functional foods include: i) conventional foods that contain naturally occurring bioactive substances such as dietary fiber, ii) foods enriched with bioactive substances such as probiotics, antioxidants, iii) synthesized food ingredients introduced to traditional foods such as prebiotics. The useful components in functional foods include probiotics, prebiotics, soluble fiber, polyunsaturated fatty acids, antioxidants, vitamins, minerals (Grajek *et al*., 2005). Functional foods are not prescribed but are consumed as part of a normal everyday diet. Health benefits associated with functional foods include reduction of the risk of cancer, improvement of cardiovascular health, boosting of immune system, improvement of gastrointestinal health, maintenance of urinary tract health, anti-inflammatory effects, reduction of blood pressure, antibacterial and antiviral activities, anti-obese effects, reduction of osteoporosis, maintenance of vision, among other benefits (Grajek *et al*., 2005; Parvez *et al*., 2006; Shah, 2007; Nissen *et al*., 2009).

ePrints@CFTRI

1.9.1 *Soy food*

Phytate in soy appear to be unique, although it associated with protein bodies. They appear to be having no specific site of localization. PA content was reported in 15 soybean varieties as ranging from 1.0 to 1.47% dry weights, which represented between 51.4 and 57.1% of the total phosphorous. The PA content in several commercially available soy products was also reported. The potential for soy phytate to undergo enzymatic hydrolysis during bread making and phytase

activity of numerous commercially available soy products were evaluated, and all products were found to have little activity (Maga, 1982). Earlier, the addition of 10% product to the bread formulation resulted in phytate hydrolysis in excess of 80% based upon the initial PA levels of approximately 300mg/loaf, which was approximately twice as high as the no-soy control of 134mg/loaf. In contrast, hydrolysis of a whey-soy blend product was only 22%, probably due to high residual levels of calcium in the product (Maga, 1982).

Prebiotics are the food ingredients that can be utilized or can enhance the growth of probiotics. Soybeans and soy products have noted for the prebiotics like oligosaccharide. The combination of probiotics and prebiotic is called ‘synbiotic’ (Gibson and Roberfroid, 1995). Hence, fermented soymilk can be considered as a synbiotic product. It has been also reported that consumption of fermented soymilk is beneficial to the ecosystem of the intestinal tract by increasing the population of probiotics and reducing the colonization of unwanted bacteria. In addition, fermented soymilk may also provide other exclusive ingredients such as isoflavones and saponin that do not exist in dairy products (Cheng *et al*, 2004).

Soybean-based products contain rich proteins, lipids, carbohydrates, minerals and vitamins with only 0.1 to 0.4% of phytate content. In particular, fermented soymilk with LAB may be a distinctive functional food because it has growth stimulating factors, such as oligosaccharides, amino acids and peptides (Oh and In, 2009).

ePrints@CFTRI

1.10 Methods for the measurement of *myo*-inositol phosphates

The method used for quantification of the phytate present in the experimental diets was shown to be one factor responsible for the variability of the results obtained in mineral availability studies. In the past, phytate was mainly quantified by addition of a controlled amount of Fe^{3+} to an acidic sample extract to precipitate the phytate (Wheeler and Ferrel, 1971). Phytate is subsequently estimated either by determining the phosphate, inositol or iron content of the precipitate (direct method), or by measuring the excess iron in the supernatant (indirect method). These approaches are not specific for phytate due to the co-precipitation of partially phosphorylated myo-inositol phosphates (Xu *et al*., 1992) and should therefore be limited to the analysis of material which contains negligible amounts of phytate dephosphorylation products. If substantial amounts of partially phosphorylated *myo*-inositol phosphates are present such as in processed foods, the content of phytate will be overestimated by using phytate determination methods based on iron precipitation. The high performance liquid chromatography (HPLC) techniques have been introduced into phytate determination (Xu *et al*., 1992). Among these ion pair reverse-phase and anion-exchange chromatography are largely used today. These systems allow the simultaneous separation and quantification of *myo*-inositol tris- to hexakisphosphates (ion-pair reverse-phase chromatography) (Sandberg and Ahderinne 1986) or myo-inositol mono- to hexakisphosphates (anion- exchange chromatography) (Talamond, 2000).

ePrints@CFTRI

Scope of theInvestigatin

ePrints@CFTRI

In developed countries, there is clear interest in the health effects of food and increased use of whole grains. Recent epidemiological findings support the protective role of whole grain foods against several western diseases such as obesity, diabetes or cardiovascular diseases. However, whole products are suspected of impairing mineral absorption. Phytic acid present in these products is considered to be the major factor causing impaired absorption of nutritionally essential minerals and proteins. Effective reduction of phytic acid content can be obtained via the action of exogenous phytic acid degrading enzymes. Phytase supplementation has a promising role to play in the bioavailability of essential nutrients in monogastric feed/food. This enzyme catalyses the hydrolysis of phytic acid to release chelated phosphorus, other divalent cations and proteins. (use of microbial phytases). Phytase has a wide range of sources, of which microbes form the most extensive group for the production of phytases. Lactic acid bacteria are present in a number of fermented foods and constitute an integral part of healthy gastro-intestinal tract when ingested. Several LAB are known as probiotic and exert a positive influence on host health or physiology. The scope of the present investigation is the isolation and characterization of phytate degrading lactic acid bacteria and applying them in fermented food processes. Optimization of various physical, chemical and cultural conditions for the evaluation of phytate degrading ability are also aimed in improving mineral solubility during different food fermentation processes.

ePrints@CFTRI

Chapter 2

Materials and Methods

ePrints@CFTRI

2.0 MATERIALS

2.1 Chemicals and Reagents

2.1.1 ***Microbiological media***: de Mans, Rogosa and Sharpe medium, other media components like, yeast extract, beef extract, bacteriological peptone, M_{17} medium, brain heart infusion medium, etc. were obtained from HiMedia, Mumbai, India.

2.1.2 ***Molecular biology reagents*****:** *Taq* DNA polymerase, protein molecular mass kit, semi permeable membrane, sodium phytate, tetra butyl ammonium hydroxide (40% solution), agarose, 2- mercaptoethanol, lysozyme, proteinase K, 16S rDNA primers, Ox bile were from Sigma Chemicals, USA. Restriction enzymes such as, *Hae*II and *Alu I*, DNA loading markers, dNTPs mix were purchased from MBI fermentas, USA. Whatman filter papers (No.1, No.40 and No.42), pepsin, pancreatic, acrylamide, bisacrylamide, ammonium per sulphate, Tris (hydroxymethyl aminomethane), ethylene diamine tetra acetic acid (EDTA), sodium dodecyl sulphate (SDS), N, N, N', N'- tetramethyl ethylene diamine (TEMED), phenol, *O*-nitrophenyl-L-D-galactopyranoside (*O*NPG), trypsin, bovine serum albumin, coomassie brilliant blue G 250, bromophenol blue, ethidium bromide, *p*- nitrophenol, *p*- nitrophenyl phosphate, ammonium metavanadate were procured from Sisco Research Laboratory (SRL), India.

ePrints@CFTRI

2.1.3 ***Biochemical Reagents***: Glycine, E-strip antibiotic discs, carbohydrate kit (KB009 Hicarbohydrate ™ kit), calcium phytate, cholesterol, Gram staining kit used in the present study were also obtained from HiMedia. Ammonium nitrate, potassium dihydrogen phosphate, calcium chloride, disodium hydrogen phosphate, ammonium sulphate, sodium hydroxide, ammonium molybdate, citric acid, trichloroacetic acid (TCA), cesium chloride, cobalt chloride, sodium thio cyanate, sodium thiosulphate, sodium carbonate, silver nitrate, formaldehyde, ferrous sulphate, Tween- 80, solvents such as, isopropanol, acetone, phosphoric acid, glacial acetic acid, hydrochloric acid sulphuric acid were of analytical grade and HPLC grade solvent methanol was obtained from Qualigens India Pvt. Ltd., Mumbai, and Merck Chemicals, Mumbai. All other chemicals were of the highest purity and were procured from standard sources.

All the glass wares used in this study were procured from Borosil Glass, Mumbai, India Ltd.

ePrints@CFTRI

2.1.4 ***Bacterial strains and maintenance*****:** Bacterial cultures used in this study are listed in Table 2.1. LAB and pathogenic bacteria were maintained as a frozen stock at - 20°C in 10% (v/v) glycerol. LAB cultures were propagated in MRS broth under static and pathogenic bacteria in BHI broth under shaking. , two generation propagation of the cultures was carried out in respective broth at 37°C before use.

Table 2.1 List of bacterial cultures engaged in this study

Bacterial strains	Media	Source	Purpose
Standard cultures			
Lb. rhamnosus GG ATCC 51530	MRS	ATCC	Probiotic standard
Lc. lactis (*cremoris*) B 634	MRS	NRRL	Standard LAB
Lb. plantarum B 4496	MRS	NRRL	Standard LAB
Lb. helveticus B 4526	MRS	NRRL	Standard LAB
Lb. casei B 1922	MRS	NRRL	Standard LAB
Lb. amylovorus B 4552	MRS	NRRL	Standard LAB
Lc. lactis MTCC 3038	MRS	MTCC	Standard LAB
Leu. mesenteroides MTCC 107	MRS	MTCC	Standard LAB
Lb. acidophilus MTCC 447	MRS	MTCC	Standard LAB
Lb. casei MTCC 1423	MRS	MTCC	Standard LAB
Lb. fermentum MTCC 903	MRS	MTCC	Standard LAB
Lb. plantarum MTCC 1325	MRS	MTCC	Standard LAB
Ent. faecium MTCC 5153	MRS	MTCC	Starter culture

ePrints@CFTRI

Table 2.1 continued...

Pathogenic bacteria			
Y. enterocolitica MTCC 859	BHI	MTCC	Indicator
L. monocytogenes Scott-A	BHI	Scott A	Indicator
Sal. paratyphi FB254	BHI	FMCC	Indicator
B.cereus F 4810	NB	FMCC	Indicator
Sal. typhi FB231	BHI	FMCC	Indicator
Staph. aureus FRI 722	BHI	FMCC	Indicator
E. coli ATCC 31705	BHI	ATCC	Indicator
E. coli MTCC 108	BHI	MTCC	Indicator

***Lb.**: Lactobacillus*; ***Lc**: Lactococcus;* ***Leu.**: Leuconostoc*; ***Ent.**: Enterococcus;* ***L.**: Listeria;* ***Y.**: Yersinia;* **B.:** *Bacillus;* ***Sal.**: Salmonella*; ***Staph.**: Staphylococcus;* ***E.**: Escherichia;* **MRS**: de Mann, Rogosa and Shapre, **BHI**: Brain Heart Infusion; **ETEC**: Entero toxigenic *E. coli.* **FMCC:** Food Microbiology Culture Collection, CFTRI, Mysore, India; **MTCC**: Microbial Type Culture Collection, Chandigarh, India; **ATCC**: American Type Culture Collection, USA; **NRRL**:Northern Regional Research Laboratory, Peoria, USA.

eprints@CFTRI

2.2 Section I

2.2.1 *Sample collection*

For screening and selection of potent phytate degrading lactic acid bacteria (LAB), a wide range of sources were collected in and around Mysore, Karnataka, India. Sources includes samples of cereals and pulses *viz.,* red rice, white dosa rice, chenna dhal, wheat, ragi, bengal gram, green gram black gram, and cereal based traditional fermented food sample *idli* batter. The other LAB sources include the intestinal samples (chicken, fresh water fish and marine water fish) and other miscellaneous samples (vaginal swabs, cucumber, raw milk and cow dung) were used. The samples were collected in sterile containers or polythene covers and were stored under moisture free environment (cereals and pulses), whereas other samples were stored at refrigerated condition until use.

2.2.2 *Selection and isolation of LAB*

In order to enumerate the LAB from the above mentioned sources, the samples were prepared and fermented for a desired period. The samples such as cereals and pulses were grounded into fine flour and mixed with two different concentrations of NaCl solution (0.85 and 5%) and prepared into slurry (batter). Similarly, the same procedure was followed for the miscellaneous sources too. Whereas the intestinal samples were sliced, dissected and suspended in the same

ePrints@CFTRI

NaCl concentrations. All the above prepared samples were incubated at two different temperatures (room temperature and 37°C) for a period of 24-48 h.

At regular intervals (4 h), 1 ml of each sample was drawn and serially diluted in physiological saline, and the aliquots were pour plated on to MRS agar. The plates were incubated at 37°C for overnight. The representative individual colonies were selected based on their colony morphology.

2.2.3 *Growth and storage*

The individual colonies were picked and resuspened in sterile MRS broth, grown at 37°C overnight. Equal volumes of the grown cultures and glycerol (80%) were mixed and stored at -20°C, until use. The cultures from glycerol stock were propagated for two generations before any test could be performed.

2.2.4 *Preliminary identification of LAB*

In order to ensure that the isolated and purified cultures belong to LAB, following preliminary tests such as acid production, anaerobic growth and catalase tests were performed.

2.2.5 *Cell morphology*

Cell morphology of each isolate was determined using routine laboratory staining protocols. The stained cells were observed under oil immersion objective of a phase contrast microscope (Olympus, Germany). Cell shape and arrangements

ePrints@CFTRI

were recorded. Further, cultures cell morphology was also visualized by scanning electron microscopy.

2.2.6 Scanning Electron Microscopy

Overnight grown culture (1 ml) was harvested in micro centrifuge tube by spun at 8000 rpm for 10 min. The pellet was washed twice with phosphate buffer saline (PBS) at 7000 rpm for 10 min. To the washed cell pellet, 1ml of glutaraldehyde solution was added and incubated at 4°C overnight. The cell suspension was spun at 7000 rpm for 10 min. The pellet was dehydrated by washing in 10-100% alcohol in a stepwise fashion. To the pellet, 50 µl of absolute alcohol was added and mixed. A drop of the suspension was placed on cover slip, air dried and stored in a desiccator until use.

2.2.7 *Catalase test*

The assay was performed by picking a colony the surface of agar plate from and suspended in 0.2 ml of hydrogen peroxide (3%) solution contained test tube. The solution was observed for effervescence, the reaction observed within 10 sec was considered, positive.

2.2.8 Non-pathogencity assay

The cultures were grown overnight and streaked on blood agar plates containing 5% sheep blood. The plates were incubated overnight at 37°C and observed for any zone of clearance. The hemolytic reaction was recorded by observing clear

eprints@CFTRI

zone of hydrolysis around the colonies (β-hemolysis), partial hydrolysis and greening zone (α-hemolysis) or no reaction (γ-hemolysis).

2.3 Phytate degradation plate assay

All the LAB isolates obtained from the selected sources were screened for their phytate degrading ability by qualitative screening method as described by Bae *et al.,* (1999). In addition to all the isolates, standard cultures obtained from different collection centers (Table 2.1) were also investigated for phytate degradation.

Overnight grown cultures were harvested by centrifugation (8000 rpm for 15 min at 4ºC) and washed with 50 mM Tris-HCl (pH 6.5) buffer, and suspended in saline. From the cells suspension (10^8-10^9 CFU/ml), 3 µl was spotted on the surface of three different modified MRS agar. The MRS medium was modified by replacing inorganic phosphate (KH_2PO_4) and supplementing following substrate combinations

a) 2% Calcium phytate (Opaque media)

b) 2% Sodium phytate (Transparent media)

c) 2% Sodium phytate + 2% calcium chloride (Transparent media)

The plates were incubated at 37°C overnight. Post incubation, the cells were washed with sterile distilled water, subsequently were flooded with 2% (w/v) aqueous cobalt chloride solution and incubated for 5 min at room temperature. The solution (cobalt chloride) was replaced with counter stain (molybdate-vandate

ePrints@CFTRI

solution) for 5 min. Finally the molybdate-vanadate solution was removed and the plates were examined for clear halo zones.

2.4 Phenotypic and genotypic identification of LAB

This includes physiological, biochemical and molecular phylogenetic characterizations and followed by taxonomic conclusions.

2.4.1 *Physiological growth tests*

Among the 121 LAB cultures, the 21 isolates that had the ability to degrade sodium phytate in presence and absence of calcium were selected for further identification and strain designation. The physiological tests included the growth at various temperatures (15, 37 and 45°C), pH (3.5, 4, 4.8 and 8.6) and NaCl (6.5 and 10%), concentrations. The assays were performed as per the protocols outlined by Bergey's mannual. The observations for were made at the end of the respective incubations periods and results were recorded.

ePrints@CFTRI

2.4.2 *Carbohydrate utilization test*

A set of tests for carbohydrate utilization along with citrate, esculin and ONPG (*o*-nitrophenyl β-D-galactopyranoside) was carried out using KB009 Hicarbohydrate ™ kit. The test was performed as per the provider guidelines.

Kit contents: The kit has three parts, with media containing different carbohydrates and substrates viz., ONPG, citrate, esculin, etc.

PART A: lactose, xylose, maltose, fructose, dextrose, galactose, raffinose, trehalose, mellibiose, sucrose, L-arabinose and mannose

PART B: inulin, sodium gluconate, glycerol, salicin, glucosamine, dulcitol, inositol, sorbitol, mannitol, adonitol, α-methyl D-glucoside and ribose

PART C: rhamnose, cellobiose, melezitose, α-methyl D-mannoside, xylitol, ONPG, esculin, D-arabinose, citrate, \malonate, sorbose and control

Preparation of inoculum The culture grown in media 2 for overnight were harvested and washed with saline. Then cell suspension was prepared in saline. The cell density of suspension was adjusted to 0.5 O.D. at 600 nm. The kit was opened under aseptic conditions in laminar air flow. Each well was then inoculated with 50 µl of the suspension. Lid was replaced carefully and the kits loaded with test cultures were incubated at 37°C for 24-48 h. The results were interpreted as follows.

ePrints@CFTRI

i. **Carbohydrate utilization test:** Change in colour at wells from red to yellow indicates positive towards carbohydrate fermentation and no change indicates negative result.

ii. **ONPG test**: Media colour change from colourless to yellow colour indicates positive and no colour change is negative.

iii. **Esculin hydrolysis**: A colour change from cream to black show the positive and it remains cream indicates negative result

iv. **Citrate utilization**: Change in medium colour from yellowish green to blue shows positive

v. **Malonate utilization**: Colour of the medium changes from light green to blue indicates positive.

2.5 Molecular identification

2.5.1 *Isolation of genomic DNA*

The total DNA from the LAB was isolates according to Mora *et al*., (1998). The lactic acid bacterial cultures (1 ml) grown at 37°C for over night were harvested by centrifugation at 8000 rpm for 15 min at 4°C. The cells were lysed with lysozyme and the DNA was extracted with phenol: chloroform protocol. The total DNA obtained was dissolved in 50 µl TE buffer and was stored in -20°C till further analysis.

2.5.2 *Agarose gel electrophoresis of DNA*

The isolated DNA was electrophoresed on 0.8% agarose gel, stained with ethidium bromide and visualized under UV-transilluminator and the image was captured using gel documentation system (Biorad, USA). The concentration of the DNA was estimated spectrophotometric (Schimadzu, Japan) assay as described by Sambrook and Russell (2001).

2.5.3 *Amplification of 16S rRNA gene*

The 16S rRNA gene of the selected strains was amplified using the primer set namely, **BSF** (5´GAGTTTGATCCTGGCTCAGG3´) and BSR (5´ TCATCTGTCCCAC CTTCGGC 3´) (Halami, 2008), respectively. The PCR

ePrints@CFTRI

amplification reaction mix of 25 µl contained 100-200 ng of genomic DNA, 2.5 µl of 10 X *Taq* polymerase buffer, 0.25 mM of each dNTP (1 mM), 5 pico moles of each primer, 0.3 U of *Taq* DNA polymerase and autoclaved triple distilled water to make up the volume. Amplification program was followed as given in Table 2.2. Amplification was carried out with thermo cycler (MWG primus, Germany). A negative control (reaction mix without any DNA template) for PCR amplification was also maintained.

2.5.4 *Amplified Ribosomal DNA Restriction Analysis (ARDRA)*

For ARDRA analysis, the PCR amplified product of 16S rRNA gene was subjected to restriction digestion by incubating the amplicon with restriction endonucleases *AluI, Hae III* and *Alu I+Hae III* (Table 2.3). The reaction was carried out at 37°C for 1 h. Then the digested products were separated on 1.8% agarose gel, subsequently stained with ethidium bromide and visualized under UV trans-illuminator and photographed.

Table 2.2 16S rDNA PCR amplification conditions

Parameters	**Temperature (°C)**	**Time (min/sec)**
Initial denaturation	95	5 min
35 cycles (a-c)		
(a) Denaturation	94	40 sec
(b) Primer annealing	52	20 sec
(c) Extension	72	2 min
Final extension	72	10 min

Table 2.3 Composition of restriction digestion

Contents	**Volume (µl)**
Template (1.4 kb fragment of 16Sr RNA gene)	5
Restriction enzyme (10 U/µl)	1
10X restriction buffer	2
MilliQ water	12
Total	20

ePrints@CFTRI

2.5.5 *PCR product purification and Sequencing*

DNA from the preparative gel was extracted by QIAquick gel extraction kit (Qiagen, Germany) according to manufacturer's instructions. For determining the nucleotide sequence of 16S rDNA, the PCR amplified products were purified using PCR purification kit, ligated to the pGEM-T vector (Promega) and transformed into *E. coli* DH5α cells (NEB). Unidirectional DNA sequencing was carried out by dideoxy chain termination method using M13F () primer at the sequencing facility of Bangalore Genei (Bangalore, India). The gene sequences obtained were analyzed by using BLAST search programme (Altschul *et al.*, 1997) and sequences were compared with those available in the NCBI database. The sequences obtained were deposited in GenBank under the accession numbers FJ889048; FJ889049 and FJ586350.

2.6 Beneficial attributes

2.6.1 *Acid tolerance assay*

The acid tolerance of LAB was studied at different pH as described by Jacobsen *et al*., (1999). 10 ml of overnight (16 h) culture grown in MRS broth was harvested by centrifugation (8000 rpm at 4ºC for 15 min). The cell pellet was washed and resuspended in 10 ml of saline to make a concentration of cells 10^7-10^8 CFU/ml. MRS broth was adjusted to pH 2, 2.5, 3 and 3.5 with 0.1 N HCl. The tubes were inoculated with 10% of cell suspension and were incubated at 37ºC for 4h. During the incubation period, 1 ml of sample was drawn every 1 h and serially diluted (7-8 folds) in saline. The desired aliquots were spread plated on MRS agar and incubated at 37ºC for 24 h. The obtained colonies were counted and were recorded as colony forming units (CFU). The percentage of survival rate was calculated by using the equation

$$\% \text{ Survival} = \frac{\text{log number of viable cells survived (CFU/ml)}}{\text{log number of initial viable cell inoculated (CFU/ml)}} \times 100$$

ePrints@CFTRI

2.6.2 *Bile tolerance assay*

Bile tolerance of the isolates was carried out as reported by Gilliland *et al*., (1984). Overnight grown LAB cultures were harvested by centrifuging at 8000 rpm at room temperature for 15 min and suspended in saline. MRS broth containing 0.3% bile was inoculated with 5% cell suspension and a control was also kept where no bile was added. The samples were incubated at 37°C for 6 h.

At every 1 h interval, sample was drawn and optical density (O.D.) was observed at 600 nm using UV-visible Spectrophotometer (Shimadzu, Japan). Tolerance to bile was estimated by comparing the delay in time of the growth of the test cultures in presence and absence of bile.

2.6.3 *Bacterial adhesion to hydrocarbons (BATH) test*

Bacterial adhesion to hydrocarbons (BATH) test was performed using xylene as a hydrocarbon to assess the ability of adherence of the isolates as described by Canzi, *et al.*, (2005). Cells were washed once with phosphate-buffered saline (PBS: 140 mM NaCl, 3 mM KCl, 8 mM Na_2HPO_4, 2 mM KH_2PO_4 pH 7.2) and resuspended in the same buffer and adjusted to an absorbance (A) of 0.5 at 600 nm. To this, an equal volume of xylene was added. The two-phase system was thoroughly vortexed for 3 min. The aqueous phase was removed after 1 h incubation at room temperature and its absorbance (A_{600}) was measured. Adhesion percentage was calculated according to the formula

$$\text{Adhesion percentage} = \frac{A_0 - A}{A_0} \times 100$$

Where A_0 and A are absorbance before and after extraction with organic solvents, respectively.

ePrints@CFTRI

2.6.4 *Antibacterial activity*

For the detection of antibacterial activity, agar spot method was used according to Chen *et al*., (2002). Cells were harvested and suspension (10^6-10^7 CFU/ml) was prepared. A volume 3 µl of the suspension was point inoculated on to the surface of the MRS agar and incubated at 37°C for 24 h. After incubation, 1 ml of 4-6 h grown indicator (pathogenic strain) as mentioned in Table 2.1 were cultured in BHI and were mixed with 7 ml of soft BHI agar (0.8%) and poured over the spotted agar plates. The plates were further incubated at 37°C for 12-16 h and the zone of inhibition was measured in mm (diameter).

2.6.5 *β-Galactosidase assay*

β-Galactosidase activity was studied as described by Chen *et al.*, (2002) with slight modifications. Twelve hour old cultures were harvested by centrifugation, washed with 10 mM sodium phosphate buffer (pH 7.0) and suspended in the Z buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4 and 2.7 µl/ml β-mercaptoethanol). The reaction mixture, containing 100 µl of the cell suspension, 900 µl of Z buffer and 20 µl of toluene, was vortexed at high speed for 2 min followed by incubation at 37°C for 1 h to remove the toluene, prior to assay. To the reaction mixture, 200 µl of 200 mM *O*-nitrophenyl-L-D-galactopyranoside (ONGP) prepared in Z-buffer was added and incubated at 37°C for 30 min. The reaction was stopped by adding 500 µl of 1 M Na_2CO_3 and the concentration of *o*-

nitrophenol (ONP) released from ONPG was determined by measuring the absorbance at 420 nm using UV-visible Spectrophotometer. The activity was determined as Miller units (MU) and was calculated using following formula.

$$\text{Miller Units (MU)} = \frac{((\text{OD @ 420 nm} + \text{OD@550 nm})/2) - (1.75 \times \text{OD@ 550 nm})}{45 \times \text{volume taken } (0.1) \times \text{OD@600 nm}}$$

2.6.6 *Antibiotic susceptibility assay*

Antibiotic susceptibility of the selected LAB isolates was determined according to Danielsen et al. (2007). The selected LAB isolates were harvested as mentioned earlier and the cell suspension (100 µl of 10^6–10^7 CFU/ml) was pour plated using MRS agar. Antibiotic E-strips were placed on the surface of the media, prior to solidification and incubated overnight at 37°C. The zone at lowest concentration of antibiotic giving a complete inhibition of visible growth was considered as minimal inhibitory concentration (MIC) (Wright, 2005).

2.7 Quantitative analysis of phytic acid

The quantitative estimation of phytic acid was determined using the method described by Davies and Reid (1979). A volume of 0.2-1.0 ml of the filtrate (extract from the sample) or standard sodium phytate solution (90.2 mM) was diluted with distilled water to a final volume of 1.4 ml to which 1.0 ml of a solution of ferric ammonium sulphate was added (containing 50 µg Fe^{3+}). After mixing, the test-tubes were stoppered and placed in a boiling water bath for 20

ePrints@CFTRI

min. When cooled to room temperatures, 5 ml amylalcohol was added to each test-tube followed by 0.1 ml of a solution of ammonium thiocyanate (100g/l). The contents of the test-tubes were immediately mixed by inversion and shaking. After centrifuging for a short time at a low speed, the intensity of the colour in the amyl alcohol layer was determined at 465 nm using spectrophotometer, against an amyl alcohol blank exactly 15 min after addition of the ammonium thiocyanate. As the method is based on the observation that ferric ions complexed with phytate at pH 1-2 cannot react with thiocyanate ion to give the characteristic pink complex, the extinction at 465 nm in the amyl layer is inversely related to the phytate anion concentration. Under these conditions, an inverse linear relationship was found over a range of phytate concentrations from 40 to 200 nmol.

ePrints@CFTRI

2.8 Phytase and acid phosphatase assay

Reagents preparation

Ammonium molybdate: It was prepared by dissolving 1.5 g of ammonium molybdate in 100 ml of 1 M H_2SO_4.

Ferrous sulphate ($FeSO_4$): It was prepared by dissolving 2.7 g of ferrous sulfate in 100 ml of 1 M H_2SO_4.

Colour reagent: 50% ammonium molybdate solution + 50% ferrous sulphate solution.

Phytate degrading ability of the isolates grown in modified MRS broth (MRS-MOPS-NaP), in which inorganic phosphate (KH_2PO_4) was replaced by 0.65 g/l of sodium phytate and 0.1M 3-[N-Morpholino] propanesulfonic acid (MOPS)

was used for the study. The contents of glucose, yeast extract and beef extract were reduced to 10, 2 and 4 g/l, respectively to reduce the final phosphate content and to promote the enzyme synthesis. MRS-MOPS medium was inoculated with 5% (v/v) overnight culture propagated in same conditions for two generations and incubated until the stationary phase of growth was attained (16-24 h). Cells were harvested by centrifugation (8000 rpm for 15 min at 4ºC) and washed with 50 mM Tris-HCl (pH 6.5) buffer. The cell pellet (10^7-10^8 CFU/ml) thus obtained was suspended in 100 mM sodium acetate-acetic acid buffer (pH 5.5).

The assay was carried out with slight modifications as described by Haros *et al*., (2005) and Neilson *et al*., (2008). The reaction mixture consisted of 250 µl of 100 mM sodium acetate-acetic acid buffer (pH 5.5) containing 2 mM substrate and 250 µl of cell suspension (prepared in 100 mM acetate buffer (pH 5.5) containing 10^7-10^8 CFU/mL). The reaction was carried out at 50°C for 15 min and was stopped by adding 500 µl of 10% (w/v) trichloro acetic acid solution (TCA). A blank was also kept where the reaction mixture was added with 10% TCA to the enzyme prior to the addition of the substrate (sodium phytate). After incubation, the contents were brought to room temperature and centrifuged at 5000 rpm for 5 min. The inorganic phosphorous released was quantified in the supernatant using the ferrous sulphate- ammonium molybdate method according to Nielsen *et al*., (2008). The analysis was carried out in micro titre plates. For the analysis, 100 µl of enzyme reaction mixture with 100 µl of colour reagent was added and incubated

ePrints@CFTRI

at room temperature for 10 min, and the absorbance was read at 700 nm with in 10 min using microtitre plate reader (Molecular Device, USA). Phytase activity was determined by measuring the amount of liberated inorganic phosphate from sodium phytate. One unit of phytase activity (U) was defined as the amount of enzyme that produces one nanomol of inorganic phosphorous per min at 50ºC.

Acid phosphatase activity was determined using *p*-nitrophenyl-phosphate as substrate. The reaction mixture consisted of 250 µl of 100 mM sodium acetate-acetic acid (pH 5.5) containing 5 mM substrate and 250 µl of cell suspension. After 15 min of incubation at 50ºC, the reaction was stopped by adding 500 µl of 1 M NaOH. A blank was prepared by adding the enzyme followed by stop solution (NaOH) in the reaction prior to the addition of the substrate. The *p*-nitrophenol released was determined by measuring the absorbance at 405 nm. One unit of phosphatase activity (U) was defined as the amount of enzyme that produces 1 µmol of *p*-nitro phenol per min at 50ºC by Palacios *et al.*, (2008a).

ePrints@CFTRI

2.9. Media optimization to study phytate degrading ability of selected LAB

There were four media compositions made (Table 2.4) based on the nutritional factors. The media 1 contain MRS media compositions. The other three media *viz.*, 2, 3 and 4 were made by reducing their nutrient concentrations. Yeast extract, beef extract and glucose were reduced to 2, 4 and 10 g/l. In media 2 phosphate source was replace with 0.2% sodium phytate and also supplemented with buffering substance MOPS. Media 3 was designed without phosphate source

but supplemented with MOPS, whereas media 4 was completely devoid of phosphate sources as well as buffering agent. Buffering agent MOPS and Sodium phytate were filter sterilized prior to use.

Table 2.4 Composition of microbial culture media

Ingredients	Quantity (g/l)			
	Media 1	**Media 2**	**Media 3**	**Media 4**
Protease peptone	10.00	10.00	10.00	10.00
Yeast extract	5.00	2.00	2.00	2.00
Beef extract	10.00	4.00	4.00	4.00
Dextrose	20.00	10.00	10.00	10.00
Polysorbate 80	1.00	1.00	1.00	1.00
Ammonium citrate	2.00	2.00	2.00	2.00
Sodium acetate	5.00	5.00	5.00	5.00
Magnesium sulphate	0.10	0.10	0.10	0.10
Manganese sulphate	0.05	0.05	0.05	0.05
Dipotassium phosphate	2.00	-	-	-
MOPS (0.1 M)	-	20.926	20.926	-
Sodium phytate	-	0.65	-	-
Final pH (at 25°C)	6.5±0.2	6.5±0.2	6.5±0.2	6.5±0.2

ePrints@CFTRI

2.10 Phytase evaluation in phytate degrading *Pediococcus pentosaceus* CFR R38

Overnight MRS grown *Pediococcus pentosaceus* CFR R38 was harvested by centrifugation at 8000 rpm for 15 min at 4°C. The collected cell pellet was washed with 0.02 M Tris buffer (pH 7.0) followed by another wash with 0.02 M Tris buffer containing 10 mM calcium chloride. Further, the cell pellet was suspended in 0.5 ml of 0.5 M sodium acetate buffer with pH ranging from 3.6 to 5.6. An aliquot of 250 µL cell suspension was added to 250 µL substrate (5 mM sodium phytate) and the reaction mixture was incubated at 50°C for 30 min. At the end of incubation period, reaction was terminated by adding 500 µL of 10% TCA. Reaction mixture without substrate was taken as a control. After 30 min of 10% TCA addition, the contents were centrifuged at 8000 rpm for 5 min at room temperature, in order to avoid turbidity obtained. To estimate the released inorganic phosphates, 100 µl of supernatant was taken in the micro tire plate, to which 100 µl of color reagent was added. Optical density was observed at 700 nm using microtire plate reader (Shimadzu, Japan) within 10 min of colour reagent added.

2.11 Phytase isolation and characterization

2.11.1 *Isolation of phytase enzyme*

Isolation of phytase enzyme was carried out according to De Angelis *et al.*, (2003) with certain media. Twenty four hours old culture was harvested by

ePrints@CFTRI

centrifugation at 7000 rpm for 10min at 4°C. The resultant cell pellet was suspended in 5 ml of 0.05 M Tris-HCl (pH 7.5) containing 0.1 M $CaCl_2$ and centrifuged at 8000 rpm for 10 min at 4°C. The collected cell pellet was resuspened in 5 ml of 0.05 M Tris-HCl (pH 7.5) and incubated at 30°C for 30 min. Post incubation, it was centrifuged at 9000 rpm for 20 min at 20°C and resuspended in 10 ml of 0.05 M Tris HCl (pH 7.0) containing 24% sucrose and 10 mM $MgCl_2$ and incubated at 37°C for 30min. Further, 2 ml of lysozyme (20 mg/ml) was added and incubated at 37°C for 45min followed by centrifugation at 9000 rpm for 20 min at 20°C. The cell pellet was then resuspended in 0.02 M Tris-HCl (pH 7.5) at 4°C. The pellet was resuspened in 10 ml of 0.02 M Tris HCl containing 0.05 M KCl, 1 mM EDTA and 1% triton X-100. The suspended cells were disrupted by two cycle of sonication and then incubated for 30 min at 4°C. The cell debris was then removed by centrifugation at 14000 rpm for 20 min at 4°C and the clear supernatant collected was used for ammonium sulfate precipitation.

ePrints@CFTRI

2.11.2 ***Ammonium sulfate precipitation***

The protein from the sample was precipitated out at different concentrations of ammonium sulfate namely 20, 30, 40, 50 and 60 %. Appropriate amount of finely powdered ammonium sulfate was gently added to the sample with constant stirring at 4°C. The solution was kept stirring on a magnetic stirrer for overnight

at 4°C for protein precipitation. The precipitate was then collected by centrifugation to obtain the protein fraction obtained was dialyzed against 0.2 M sodium acetate (pH 5.6) with several changes of liquid to remove the sulphate salts. After dialysis, phytase assay was carried out for the dialysate. Further, the sample was concentrated by lyophylization for further analysis.

2.11.3 *Preparation of dialyzing bags*

Appropriate size of the semi permeable membrane tube was cut and washed with double distilled water. The membrane was boiled in double distilled water for 10min and a pinch of sodium citrate and sodium carbonate was added and boiled for 15 min. The membrane was then rinsed with double distilled water and was used as a bag for dialysis of the sample.

ePrints@CFTRI

Gel permeation Chromatography

Column Packing:

Column size:	0.7 diameter, 50 cm length
Stationary Phase:	Sephadex G-100
Mobile Phase:	Tris-HCl (pH 5.6)
Flow rate:	2 ml/20 min

Coloumn was set up by placing glass wool at the bottom. The sephadex G-100 beads, washed and soaked overnight in Tris-HCl buffer (pH 5.6) were packed into the column slowly and allowed to set as bed. The column was washed by

running Tris-HCl buffer (pH 5.6) and the flow rate was adjusted to 1 ml/min. Two ml of the sample was loaded onto the top of the bed in the column and then eluted with 0.05 M Tris-HCl (pH 5.6). A fraction (15 ml) was collected and the flow was stopped to facilitate efficient binding of the sample onto the column bed. After 15 min, the flow was resumed and a fraction of 35 ml was collected. The flow rate was adjusted to 0.1 ml/min and the different fractions were collected by the fraction collector. For each of the collected fraction phytase biochemical assay was done to confirm the presence of the phytase protein. The purified protein thus prepared was analyzed for its molecular weight by SDS-PAGE (Laemmli, 1970) and zymogram (enzyme activity staining was performed as per De Angelis *et al*., (2003).

2.11.4 ***Activity staining for the phytase***

The gel was first kept in 1% triton-100 at room temp for 30 min. Then the gel was washed with sodium acetate buffer (pH 5.6) at 4°C for 1 h, and it was incubated in the acetate buffer (pH 5.6) at 50°C for 16 h. Then the gel was stained with cobalt chloride (Bae *et al*., 1999) followed by ammonium molybdate coloring reagent and observed for clear zone of the phytase activity.

2.12 Phytase primer designing

The molecular evidences for the existance of gene responsible for the phytase activity was evaluated by designing the specific gene primers from the data available in NCBI data base on phytase in different *Bacillus* spp.

ePrints@CFTRI

2.13 Phytate degrading ability of LAB in different food fermentation food processes

2.13.1 *Malted Finger millet Seed Coat (MFSC)*

Malted finger millet seed coat was collected from Grain Science Technology Department, CFTRI, Mysore. The malted finger millet seed coat (MFSC) powder was packed in polyethylene bags and gamma irradiated at 1.5 kGy (20 min 28 sec at 23°C), 3 kGy (40 min 28 sec at 27°C) and 5 kGy (1h 21 min 4 sec at 22.8°C) when dosage rate was 4.4480 kGy per h. The gamma irradiated sample was stored for 6-8 months at 4°C. The storage stability depends on the moisture content of the material. Proximate analysis of the material was performed and used for the fermentation processes by LAB.

2.13.2 *Phytates extraction and analysis*

Two grams of raw food material (malted finger millet seed coat) was suspended in 50 ml 0.5 M HCl and incubated at 37ºC for 7 h at 110 rpm on shaker incubator. The sample was centrifuged at 9000 rpm for 20 min at room temperature and the supernatant was evaporated to dryness at reduced pressure at 40ºC. The concentrate was dissolved in 5 ml distilled water. The inositol phosphates formed were separated by ion exchange chromatography using Glass column (70 cm x 1 cm) loaded with 10 ml resin (AG1-X8 200-400 mesh). Elution of 30 ml fraction of 0.05 M HCl was used to separate inositol mono and di phosphates from concentrated supernatant. Then linear gradient of HCl was used

ePrints@CFTRI

(0.05 M- 0.5 M) to separate inositol phosphates. Collected fractions were evaporated to dryness at reduced pressure and dissolved in 5 ml of mobile phase, for which HPLC was performed (Sandberg *et al*., 1999). 20 µl of the solution was injected into ODS-2 column (10 µm waters, 4.6 x 150 mm analytical) and inositol phosphates were detected using RID at 45ºC. The mobile phase used was 51% methanol and 49% 0.05 M formic acid containing 0.4 % tetra butyl ammonium hydroxide and the pH of the mobile phase was adjusted to 4.3 using 1 M H_2SO_4. The coloumn was run at a flow rate of 0.4 ml/min at 40°C. The HPLC fraction collected at respective RT (peak) and was injected to MS and the molecular weight was confirmed.

Malted finger millet seed coat was sterilized using 1.5 kGy and 3kGy gamma irradiation. 10% malted finger millet seed coat solution was prepared with sterile water and was inoculated with 1% over night old potent LAB and was fermented was performed for 24 h. The resulting fermented product was made up to 50 ml with 0.5 M HCl to extract phytates. Standard inositol phosphates were made using standard sodium phytate.

2.13.3 *Mineral availability tests*

Fermented and control samples were prepared as mentioned in phytate extraction procedure. Mineral availability was studied according to the method

ePrints@CFTRI

followed by Miller *et al*., (1981). The three steps involved in the procedure are as follows.

1. **Gastric Digest**: An aliquot of 20 ml of the sample was suspended along with 70 ml of water in a 250 ml conical flask, pH was adjusted to 2 with 6 N HCl. The solution was kept at room temperature for 5 min and the pH was monitored. To it 3mL of pepsin solution was added and the volume was made up to 100 ml using distilled water. The mixture was incubated at 37ºC for 2 h in an incubator shaker at 110 rpm. The gastric digest reaction was arrested by keeping at 0ºC for 90 min and then titratable acidity (TTA) was measured for an aliquot of 20 ml.

2. **Titratable acidity:** To measure the titratable acidity the gastric digest was brought to room temperature and an aliquot of 20 ml was taken and 5 ml pancreatin bile mixture was added. The mixture was titrated against 0.2 M sodium hydroxide till it attains pH of 7.5. TTA was defined as the amount of 0.2 M sodium hydroxide required to attain a pH of 7.5. The amount of sodium bicarbonate required to perform intestinal digest was calculated as per sodium hydroxide volume required for the titration.

Amount of sodium bicarbonate required = burette reading X Normality of sodium bicarbonate (0.1 N) X Molecular weight of sodium bicarbonate

3. **Intestinal digestion:** To carry out intestinal digestion, an aliquot of 20 ml gastric digest was taken in 100 ml conical flask and equilibrated at 37ºC for 10

ePrints@CFTRI

min. The segments of dialysis tube containing 25 ml of 0.1 M sodium bicarbonate (calculated from TTA) was placed in to the conical flask. It was incubated at 37ºC for 30 min or longer till the pH reached to 5.0. To the contents, 5 ml of pancreatic mixture was added, incubated at 37ºC on shaker for 3 h or till pH reach to 7.5. The dialysis bag was removed, surface was washed with distilled and the contents were measured. The dialysate was acidified with 5 ml warm concentrated HCl and the volume was made up to 50 ml with distilled water. The mineral content was determined using atomic absorption spectrophotometer (AAS).

Regeneration of Dialysis bags

a) Dialysis bags were boiled in water for 10 min.
b) A pinch of EDTA, $NaHCO_3$ were added to the double distilled water and boiled for 10-15 min
c) The water was drained and bags were further boiled in double distilled water for 2-3times.

ePrints@CFTRI

The bioavailability of minerals in the samples obtained after fermentation of malted finger millet seed coat with LAB cultures followed by gastric digestion and intestinal digestion was estimated by titrimetric method (for calcium) and by AAS (for Magnesium, Zinc).

2.13.4 ***Calcium estimation assay by titrimetric method***

It was performed according to AOCC (2000) protocol.

Reagents Required

1) Conc. HCl
2) **Bromocresol green**: The solution was prepared by dissolving 0.1 g of Bromocresol green in 14.3 ml of 0.01 M NaOH solution and the final volume was made up to 250 ml with double distilled water.
3) **20% Sodium acetate**: The solution was prepared by dissolving 20 g of sodium acetate in 70 ml of distilled water and the final volume was made up to 100 ml with double distilled water.
4) **3% Oxalic acid**: The solution was prepared by dissolving 7.5 g of oxalic acid in 200 ml of double distilled water. The final volume was made up to 250 ml with distilled water.
5) **Dil. H_2SO_4**: Prepared by adding 20 ml of Conc. H_2SO_4 slowly to 480 ml of distilled water with constant stirring.
6) **Standard $KMnO_4$ Solution:** Prepared by dissolving 15.8 g of $KMnO_4$ in 1000 ml of water.
7) **Ammonium hydroxide solution**: Prepared by adding 5 ml of NH_3 to 250 ml of double distilled water.

ePrints@CFTRI

Procedure

To the 25 ml of sample taken in a 500 ml glass beaker, 150 ml of double distilled water was added followed by 8-10 drops of bromocresol green indicator. Sodium acetate was added to the solution to bring down the pH to 5 (blue colour solution). The solution was heated to boiling point by covering it with watch glass. Oxalic acid (3%) solution was added carefully drop by drop till the colour of the solution changes to distinct green shade (pH 4.6). The mixture was then boiled for

2 min and the mixture was allowed to settle overnight. The following day, the supernatant was filtered through Whatman no. 42 filter paper. The beaker and the precipitate on filter paper were washed with small portions of ammonium hydroxide solution. The filter paper was pierced with a glass rod to wash the precipitate into a beaker using hot (80-90ºC) Dil.H_2SO_4. The above solution was titrated at 80°C with 0.05 N $KMnO_4$ until slight pink colour was obtained. Filter paper was added to the solution and titration was continued till pale pink colour was obtained. The amount of calcium in the sample was estimated by the following formula

Calcium content of the sample (mg/100g) =

$$\frac{(\text{Sample titre} - \text{Blank titre}) \times 1.002 \times 100 \times \text{Total volume of Solution}}{\text{Volume of ash Sol'n} \times \text{Weight of sample}}$$

ePrints@CFTRI

2.14 Application of phytate degrading *Pediococcus pentosaceus* CFR R38 in soya curd preparation

2.14.1 *Optimizing conditions*

In order to optimize the conditions for the phytic acid degradation to improve the nutritional quality, 5.5% inoculum of CFR R38 was inoculated into sterile soya milk and incubated at 37 and 50°C, respectively for a period required to form chock curd. The pH was observed before and after incubation, phytate content, and mineral availabilities were analyzed. Considering the optimal

conditions, the final product was prepared. The product was analyzed for its nutritional parameters and also evaluated for its sensory attributes.

2.14.2 *High Performance Liquid Chromatography (HPLC)*

Confirmation of phytate degradation was confirmed as described in section 2.13.2

2.14.3 *Soymilk Preparation*

About 100 g of soy beans was soaked in excess water in a glass container overnight. The following day, the seed coat was removed manually and seeds were ground in to a past with 300 mL of distilled water in a mixer grinder. The material was filtered through the pre-washed starch free muslin cloth. The material was completely squeezed until dry *okara* was obtained. The final volume was made up to 700 ml with distilled water. Soymilk so obtained was autoclaved at 121°C for 15 min.

2.14.4 *Mineral Analysis*

Mineral analysis of fermented soy milk as well as unfermented soymilk (control) was performed as described by Miller *et al*., (1981). After fermentation, the sample was drawn to extract phytates by acid extraction procedure. The extraction procedure was carried out for 3 h. The acid extracted samples were centrifuged at 8000 rpm for 20 min. Supernatant was passed through Whatman no. 2 filter paper then the filtrate obtained was further passed through Whatman no. 40

ePrints@CFTRI

filter paper. An aliquot of 10ml filtrate obtained was diluted with 40mL of triple distilled water. About 50 ml of the sample was run in an anion exchange chromatography (Dowex beads as stationary phase) column. The sample (50 ml) eluted with cations was collected and evaporated in crucibles by heating it on hot plate. The residue was kept in muffle furnace at 471°C for ashing. The heating was continued until white ash was obtained. The ash was suspended in 5mL of concentrated HCl to dissolve and the volume was made up to 50 ml with triple distilled water. The sample thus prepared was further analyzed for mineral content by using AAS.

2.14.5 *Product characterization*

Further product was prepared by studying the product in three different stages. These include Sensory evaluation, Functional properties and Chemical/Nutritive. Sensory evaluation was carried out for soy curd prepared by using *Ped. pentosaceus* CFR R38.

2.14.6 *Antioxidant property*

The method was followed as per standard laboratory protocols. The whey obtained from 250 µl of soy curd was taken in two separate test tubes. One was labelled as control and the other as test. The volume of test tubes was made up to 2ml with addition of 1.750ml of distilled water. In control, 2ml of methanol, whereas in test 2ml of DPPH (Diphenyl-picryl-1-hydrazine) was added. The contents were vortexed and incubated for 30 min at 37°C. The samples were read

ePrints@CFTRI

at 517 nm using water as blank. Antioxidant level can be known by calculating the percentage of free radical inhibition given by

Percentage of free radical inhibition =

$$1-\frac{Sample\ OD-Blank\ OD}{Control}\times 100$$

2.15 Chemical/Nutritive studies

In addition to the phytic acid content levels and mineral availability, moisture content, ash content, fat, protein and carbohydrates were estimated as follows.

Phytic acid and mineral availability were done as mentioned in earlier sections 2.13.2 and 2.13.3.

2.15.1 *Moisture content*

It was performed according to AACC (2000) protocol. Five gram of the food sample was weighed in an aluminum dish using a mettler balance and placed in a hot air oven maintained at 110±1°C for 16 h. It was cooled to room temperature in a desiccator and the loss in weight in percentage was reported as moisture content using the following formula.

Moisture content (%) =

$$\frac{(W2-W3)\times 100}{(W2-W1)}$$

Where W1 = initial weight of cup.
W2 = Weight of cup with sample (before drying)
W3 = Weight of cup with sample (after drying)

ePrints@CFTRI

2.15.2 *Ash content*

It was performed according to AACC (2000) protocol. About 5 to 10 g of the sample was weighed accurately in a tarred silica crucible (which has been previously heated to about 450°C and cooled). The crucible was placed on a heater and initially heated over a low flame till all the material was completely charred followed by heating in a muffle furnace for about 3 to 4 h at about 450°C, it was then cooled in a dessicator and weighed. To ensure completion of ashing, the crucible is again heated in a muffle furnace for a half an hour, cooled and weighed. This was repeated till two consecutive weights are the same and the ash is almost white ($MgNO_3$ was added to the solution to get white color and heat in muffle furnace) or grayish white in color was obtained.

Ash content (g/100g of sample) =

$$\frac{\text{Weight of ash} \times 10}{\text{Weight of sample taken}}$$

ePrints@CFTRI

2.15.3 *Fat extraction (Ether extraction)*

It was performed according to AACC (2000) protocol. Fat was estimated as crude ether extract of the dry material. The dry sample (5-10g) was weighed accurately into a thimble and plugged with cotton. The thimble was then placed in a soxhlet apparatus and extracted with anhydrous ether for about 16 h. The ether extract was filtered into a weighed conical flask. The flask containing the ether

extract was washed 4 to 5 times with small quantities of ether and the washing was also transferred. The ether was then removed by evaporation and the flask with the residue was dried in an oven at 80 to 100°C, cooled in a desiccator and weighed. Fat content was calculated by following formula.

$$\text{Fat content (g/100g sample)} = \frac{\text{Weight of the ether extract} \times 100}{\text{Weight of the sample}}$$

2.15.4 ***Nitrogen analysis for protein***

It was performed according to AACC (2000) protocol. To 0.5g of each of the sample taken in digestion tubes, to which, 0.5 g of $CuSO_4$, 5 g of K_2SO_4 and 10ml of conc. H_2SO_4 was added. The samples were digested for about 30 to 35 min till color changes to greenish blue. The digested samples were diluted with 5 times of distilled water and distilled in a distillation unit (Gerhardt, Vapodest-20) with 25ml of freshly prepared 2% boric acid containing 2 to 3 drops of mixed indicator. The distillate was collected and titrated against 0.1 N HCl. The experiment was repeated with a blank. The protein content was determined using the formula.

$$\textit{Percentage of } N_2 = \frac{(\textit{Sample reading} - \textit{Blank reading}) \times (N_2 \textit{ of titrant}) \times 1.4007}{\textit{Weight of the sample in gram}}$$

Protein value = % $N_2 \times C$

Where C = 6.25 (conversion factor)

ePrints@CFTRI

2.15.5 *Carbohydrate estimation*

It was performed as per AAAC (2000) protocol. To 100 mg of defatted food sample, 15ml of distilled water and 0.1ml of thermostable α-amylase (TARMAYC, sigma) was added and then cooked in boiling water bath for about 30 minutes with often stirring and made up the evaporation loss with distilled water. Contents were cooled to room temperature. Then 15ml of 0.2 M glycine HCl buffer (pH 2) containing 10 mg of porcine stomach pepsin (SRL, 1:3000 U) was added to the reaction mixture. The reaction was carried out by incubating at 37°C for 2 h in a shaking water bath. The pH of reaction mixture was adjusted to 6.8 with 0.2 M NaOH, to which 15ml of 0.05 M phosphate buffer (pH 6.8) containing 5 mg of porcine pancreatin enzyme (sigma, activity equivalent 4×USP) was added. Whole components were incubated for 2 h in shaking water bath at 37°C. Further, pH was adjusted to 4.8 with dilute acetic acid and was added with 15 ml acetate buffer (0.05 M) containing 20 mg of amyloglucosidase and incubated at 55°C for 2 h in shaking water bath. The contents were transferred to 100 ml volumetric flask and the volume was made up to 100 ml. An aliquot (about 10 ml) sample was withdrawn, centrifuged to collect turbid free supernatant. Glucose was estimated from the 2 ml of supernatant by glucose oxidase method. Calculated glucose released by comparing standard glucose curve, simultaneously prepared reagent blank. Calculated the percentage of carbohydrate hydrolysis during the reaction was done by following formula.

ePrints@CFTRI

Percentage of carbohydrate hydrolyzed = Equivalent glucose released × 0.9 × 100/ Weight of food sample

DNS preparation

One gram of 3,5-Dinitro salicylic acid dissolved in 80m of warm 30% sodium potassium tartar ate and 20 ml of 2 N sodium hydroxide (NaOH).

DNS estimation of sugars

To 2 ml of filtrate, 2 ml of DNS reagent was added then incubated at boiling water bath for exactly five minutes. The reaction components were made up to 20 ml with distilled water (16 ml). The colour developed due to reactants was observed at 540 nm. 1 mg of amyloglycosidase contains 42 U therefore one unit will liberate one mg of glucose from soluble starch in three minutes at pH 4.8 at 55°C.

2.16 Sensory evaluation

Quantitative Descriptive Analysis (QDA) was used to assess the sensory attributes of the samples by a trained panel test. The intensity of each attribute was quantified on a structured scale comprised of 15 cm line scale wherein 1.25 cm was anchored as 'Low' and 13.75 cm as 'High'. In the first phase of evaluation, a suitable score card was framed using 'Free Choice Profiling' method. This involved listing of appropriate terminology and describing individual quality attributes of the product. Using this scorecard, panelists were adequately trained to

ePrints@CFTRI

detect subtle differences in the perceived intensity of the attributes. Evaluation was carried out in ‘Sensory booths’ under standard conditions. Porcelain plates coded with three digit random numbers were used for serving the samples to avoid bias. Mean scores for all the attributes were calculated. These mean scores represented the panel’s judgment about the sensory quality of the samples.

ePrints@CFTRI

Chapter 3

Section-1

Screening, isolation and characterization of phytate degrading lactic acid bacteria

ePrints@CFTRI

3.1.1 Isolation of lactic acid bacteria

In search of LAB to investigate their ability to degrade phytic acid, a diverse set of sources were selected and were screened. The colonies obtained were isolated, purified and stored as mentioned in the section 2.1.4. The isolates were Gram positive, catalase negative, non-hemolytic and acid producing strains presumptive for LAB. The number of isolates obtained, from each source are listed in Table 3.1.1.

Table 3.1.1 Lactic acid bacterial isolates obtained from different sources

Source/Origin	Number of cultures isolated
Cereals & pulses	
Idli batter	28
Red Rice	07
White Dosa Rice	06
Chenna dhal	02
Wheat	07
Raagi	02
Bengal gram	02
Green gram	04
Black gram	03
Intestinal source	
Chicken intestine	20
Fish Intestine	07
Miscellaneous	
Vaginal swabs	01
Cucumber	05
Raw milk	06
Cow dung	01

ePrints@CFTRI

3.1.2 Screening of lactic acid bacteria for phytate degrading ability

A total of 101 LAB isolates recovered from several selected sources (Table 3.1.1) were tested for their phytate degrading ability by plate assay method. In addition, 13 LAB obtained from different culture collection centers were also investigated. Initially, when all the cultures (114) were tested on MRS agar medium containing calcium phytate, positive results were obtained with a translucent zone around the colony, indicating phytate hydrolysis. To avoid overestimate, the plates were stained with aqueous cobalt chloride solution that helps in elimination of false positive cultures and resulted in restriction of a clear zone to the spotted area (Figure 3.1.1A).

Further, all the native isolates along with standard cultures were also screened for their phytate degrading ability with sodium phytate as the substrate. The results observed were in contrast with those obtained when calcium phytate was supplemented as substrate. It was found that among all the isolates, only 20 cultures produced a clear halo zone when stained with aqueous cobalt chloride solution. These 20 isolates include 12 from chicken intestine, one each of marine fish intestine and raw milk and six from red rice (Figure 3.1.1B). Interestingly, all the test isolates in turn produced positive results (translucent halo zones, Figure 3.1.1C) on same sodium phytate containing when supplemented in the media along with calcium chloride. The representative cultures which, degrades both the phytate complexes (sodium and calcium) are given in Figure 3.1.1D and 3.1.1E.

ePrints@CFTRI

However, all the cultures from culture collections produced negative results for phytate degradation except B 4552.

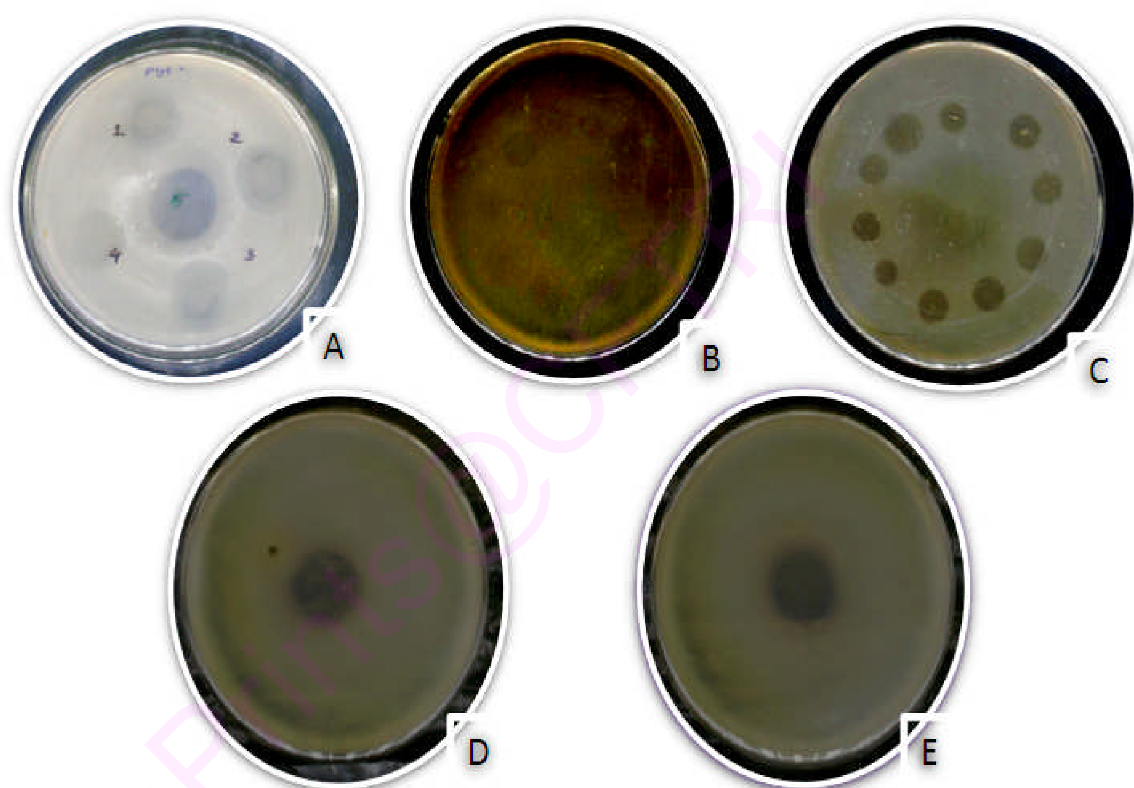


Figure 3.1.1 Phytate degradation by LAB cultures

A: Calcium phytate degradation;

B: Sodium phytate degradation;

C: Sodium phytate degradation in presence of calcium chloride;

D: Phytate degradation by CFR R123;

E: Sodium phytate degradation by CFR R38

Similar studies of phytase activity in LAB were carried out previously in *Lb. sanfrancisensis* (De Angelis *et al*., 2003). It was observed that calcium ions are required for the enzyme activity. It was concluded that calcium ions may not be involved in the reaction, but is required for enzyme activity. However, some of the lactic strains such as *Lb. plantarm* was found to degrade phytate in which, phytic acid was the sole source of phosphate (Marklinder *et al*., 1995). Thus, the positive results observed with all the 114 isolates, could be due to the presence of phytate specific enzyme. The results also revealed that the phytate degradation ability of the test isolates was due to enzyme activity and was not due to acid hydrolysis (Anastasio *et al*., 2009). This view can be supported by the fact that acid produced by LAB results in dissociation of metal ion (non-specific hydrolysis) blocked by phytin complex. Hence, the negative charge of phytic acid complex upon staining with cobalt chloride precipitates, producing reversible phytin complex with cobalt molecule (Bae *et al*., 1999). Whereas the phosphate molecule when cleaved by specific enzyme results in clear halo zone, that will neither binds to cobalt nor produced no precipitate. Hence, the results obtained in this assay clearly indicate that the phytic acid degradation by test isolates is through phytate specific enzyme supporting the view that LAB possessing phytate degrading ability.

ePrints@CFTRI

The selected 20 phytate degrading LAB, were subjected to preliminary identification (physiological, biochemical and molecular identification) and strain differentiation. The microscopic observation of selected isolates illustrated that the cultures obtained from chicken intestine (12), and fermented red rice (1) were cocci (Figure 3.1.2).

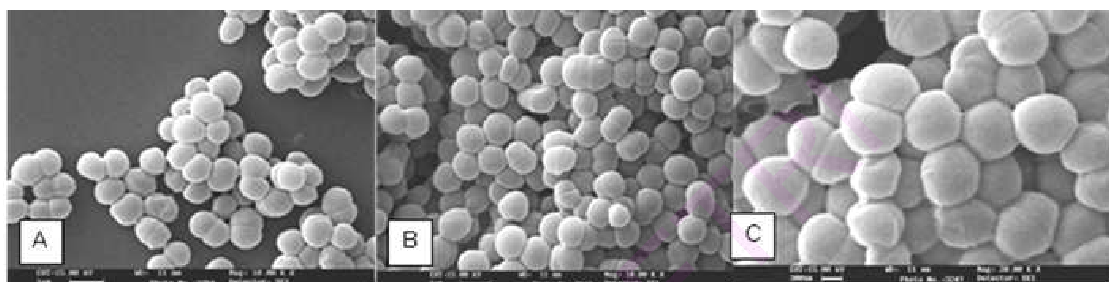


Figure 3.1.2 SEM pictures of phytate degrading LAB. A: CFR R38; B: CFR R35; C: CFR R123. (Magnification: 10000-12000 X)

ePrints@CFTRI

3.1.3 Physiological growth characteristics

Growth of the tested cultures at different physiological conditions is represented in Table 3.1.2. All the tested cultures were grown at different temperatures and luxurious growth was observed at 37°C. Though the cultures were able to grow at 45°C, a declined in growth was observed compared to that of 37°C. Poor growth was observed with all the strains at 10°C. All the three isolates were able to grow in presence of 6.5 % of sodium chloride but were unable to tolerate 10% sodium chloride. Growth at slightly elevated temperatures of 70°C for 15 min and at 65°C for 15 and 30 min, depicts one of the *Ped. pentosaceus* properties. All the isolates exhibited good growth at different pH (4,

4.8 and 8.6) except for pH 3.5, wherein poor or no growth was observed. The physiological properties of the three representative isolates from test strains are given in table 3.1.2.

Table 3.1.2 Growth of isolates at different physiological conditions

Conditions		CFR R35	CFR R38	CFR R123
Growth at different temperature °C	15	+	+	-
	37	+++	+++	+++
	45	++	++	+++
Heat tolerance	65°C (15 min)	-	-	-
	65°C (30 min)	-	-	-
	70°C (15 min)	-	-	-
NaCl concentration (%)	6.5% NaCl	+++	+++	+++
	10% NaCl	-	-	-
pH conditions	pH 3.5	+	+	++
	pH 4	++	++	+++
	pH 4.8	+++	+++	+++
	pH 8.6	+++	+++	+++

+=Delay in Growth, ++= Optimal Growth, +++= Very good growth/Very Good Tolerant, - = No growth/ No tolerance

3.1.4 Biochemical identification

The results of sugar fermentation are presented in Figure 3.1.3. Phytate degrading cultures CFR R35, CFR R38 and CFR R123 were used for the experiment. Among the carbohydrates tested, culture CFR R35 was unable to ferment, mellibiose, sodium gluconate, dulcitol, inositol, sorbitol, mannitol, adonitol, xylitol, alpha-methyl-mannoside and ONPG. Culture CFR R38 was unable to ferment raffinose, trehalose, mellibiose, sucrose, sorbitol, alpha-methyl-

ePrints@CFTRI

mannoside and ONPG, where as culture CFR R123 was unable to ferment xylose, L-arabinose, inulin, dulcitol, inositol, sorbitol, adonitol, alpha-methyl-D-glucoside, cellobiose, melzitose, D-arabinose. The phenotypic methods include morphological and physiological characterization, carbohydrate and fermentation pattern. Gonzalez, *et al*., (2000) identified LAB isolates from fresh water fish using 44 morphological and physiological tests. A high percentage (90%) of the isolates could only be identified at the genus level. Corsetti, *et al*., (2001) analyzed 317 presumptive LAB isolates from sourdoughs based on morphological and physiological characteristics, but only 38% of the isolates could be identified to the species level. In the present study, 90% similarity was found when compared with that of the Bergey's manual for the characterization of the LAB isolates.

ePrints@CFTRI

3.1.5 Molecular characterization

3.1.5.1 ARDA analysis: In addition to the physiological and biochemical characterization, the selected test isolates were further subjected to molecular characterization using ARDA and 16S rRNA gene sequence analysis. The 1.4 kb PCR product amplified from the internal regions of the 16S rRNA gene was subjected to restriction digestion using *Alu I* and *Hae III*. From the results illustrated in Figure 3.1.4, it can be deduced that three different pattern of bands were observed ranging from 300 -1000 bp. In order to further identify the isolates at strain level, a representative from each group was selected and subjected to 16S rRNA gene sequence analysis. The sequences obtained were aligned with the gene sequences from NCBI, matched with the group of *Ped. pentosaceus*. Further, phylogenetic tree was constructed and analyzed.

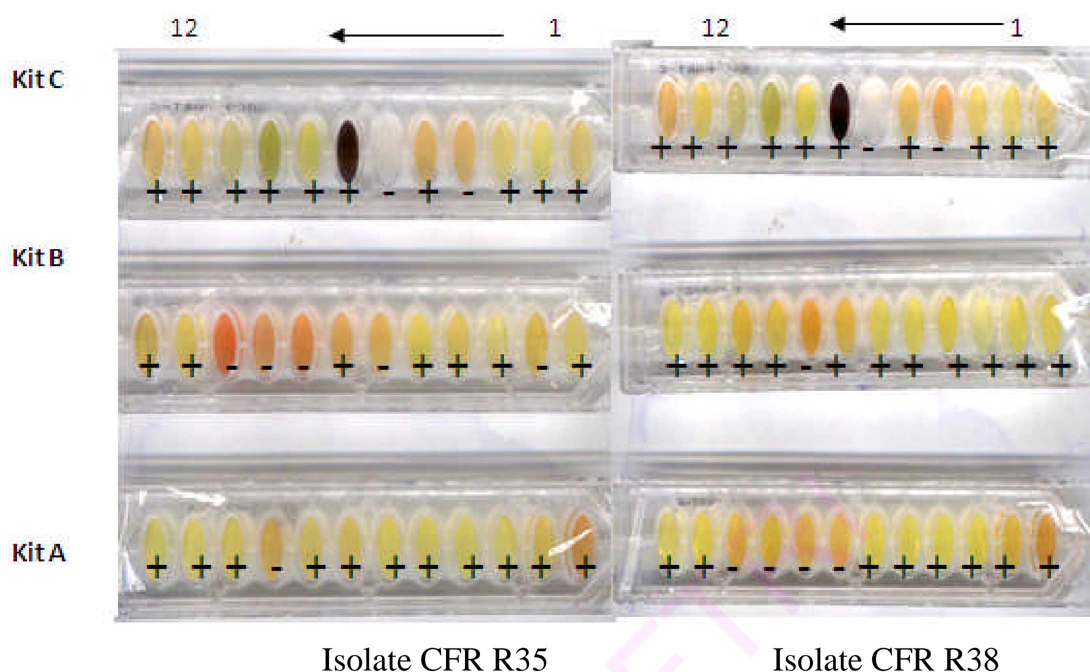


Figure 3.1.3 Carbohydrate utilization by isolated cultures CFR R35 and CFR R38

PART A: 1.Lactose, 2. Xylose, 3. Maltose, 4. Fructose, 5. Dextrose, 6. Galactose, 7. Raffinose, 8. Trehalose, 9.Mellibiose, 10. Sucrose, 11. L-Arabinose, 12. Mannose

PART B: 1.Inulin, 2. Sodium gluconate, 3. Glycerol, 4. Salicin, 5. Glucosamine, 6. Dulcitol, 7. Inositol, 8. Sorbitol, 9. Mannitol, 10. Adonitol, 11. α-methyl D-glucoside, 12. Ribose

PART C: 1. Rhamnose, 2. Cellobiose, 3. Melezitose, 4. α-methyl D-mannoside, 5. Xylitol, 6. ONPG, 7. Esculin, 8. D-Arabinose, 9. Citrate, 10. Malonate, 11. Sorbose, 12. Control

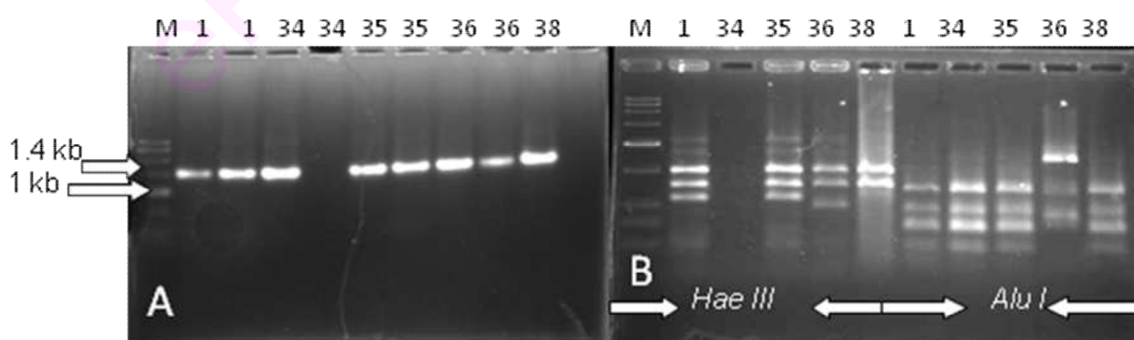


Figure 3.1.4 16S rRNA amplification and its analysis (A) 1.4 kb 16S rRNA PCR product and (B) ARDA analysis of 16S rRNA amplicon by *Hae III* and *Alu I. M=3 kb Marker; 1,34, 35, 36 and 38 were LAB isolates*

ePrints@CFTRI

3.1.5.2 Taxonomical identification

The phylogenetic tree was constructed using MEGA 5.0 version, where Neib-joining method was followed. Standard reference sequences from NCBI data base were taken from three different species of genera *Pediococcus,* and compared with that of test strains. Three major clusters were obtained on the dendrogram (Figure 3.1.5) each relating to the respective species. The test strains CFR R123, CFR R38 and CFR R35 were clustered with *Ped. pentosaceus* group, clearly differentiating these strains at their taxonomical level.

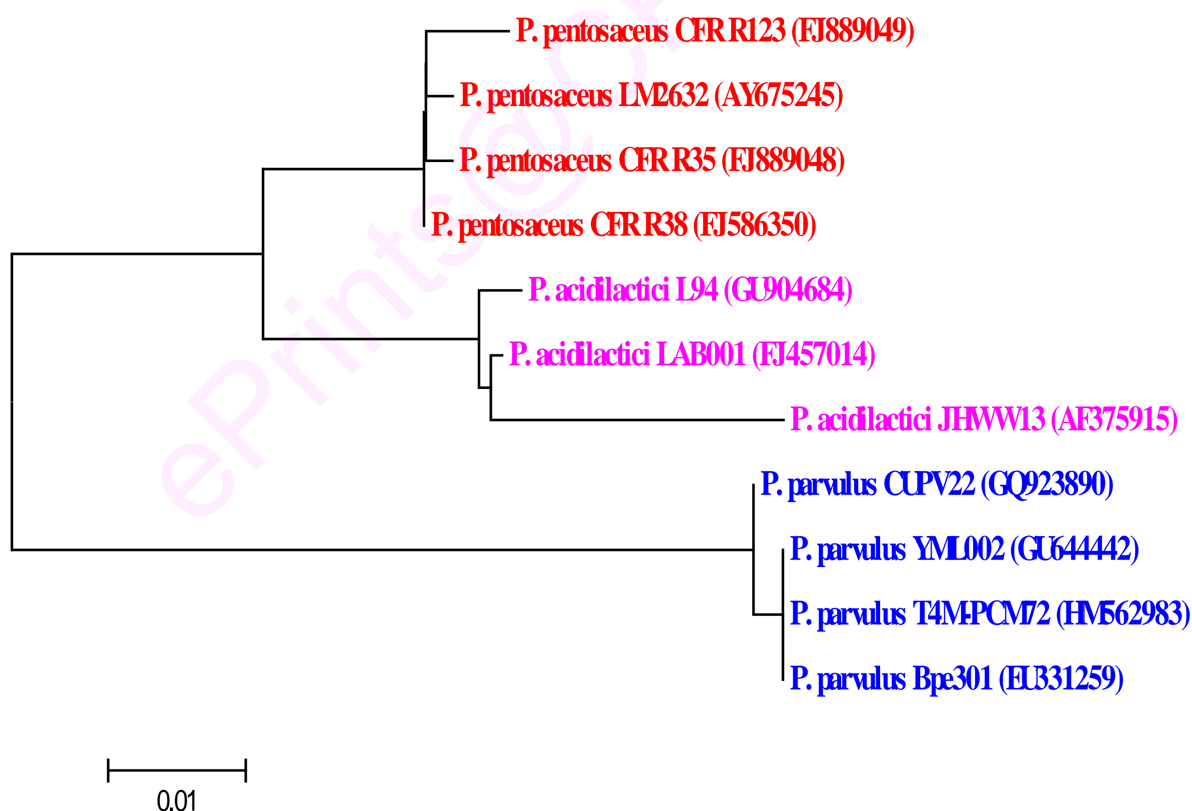


Figure 3.1.5 Phylogenetic tree for the strains *Ped. pentosaceus* CFR R123, CFR R38 and CFR R35

ePrints@CFTRI

Pediococci are lactic acid bacteria commonly found in fermented vegetables, dairy products, and in meat (Pederson, 1949; Raccacham, 1987). Although eight species of *Pediococcus* were listed in the last edition of the Bergey's manual (Garvie, 1986), more recent information indicates that only five species belong to the genus: *Ped. acidilactici, Ped. damnosus, Ped. dextrinicus, Ped. parvulus*, and *Ped. pentosaceus* (Back and Stackebrandt, 1978; Bosley *et al*., 1990). The association of pediococcal isolates with human infections has recently been described, but their identification in the clinical laboratory can be incorrect due, in part, to difficulties in differentiating them from physiologically similar bacteria (Colman and Efstratiou, 1987; Facklam *et al*., 1995).

Among the five recognized species, *Ped. acidilactici* and *Ped. pentosaceus* have been isolated from sterile and nonsterile sites in immunocompromised patients, but their role in the pathogenesis of infections remains unclear (Maugein *et al*., 1992). Recovery of *Ped. acidilactici* is more frequent than *Ped. pentosaceus*, and *Ped. acidilactici* has also been more frequently associated with cases of invasive infections, such as bacteremia, than *Ped. pentosaceus* (Mastro, 1990). Furthermore, the members of the genus *Pediococcus*, as well as some other LAB, such as *Leuconostoc* and *Lactobacillus* spp., are intrinsically resistant to vancomycin, a characteristic that increases the need for a correct identification of these microorganisms (Facklam *et al*., 1995).

ePrints@CFTRI

3.1.6 Beneficial attributes of selected LAB

LAB is considered model probiotics as they enhance lymphocyte proliferation, augment innate and adaptive immune responses, and stimulate anti-inflammatory cytokines (Famularo *et al*., 2005). Hence the selected phytate degrading *Ped. pentosaceus* CFR R35, CFR R38 and CFR R123 were also evaluated for their beneficial characteristic features by *in vitro* methods.

3.1.6.1 Acid tolerance

The primary barrier of microorganisms in the stomach is the gastric acid with the intensity of the inhibitory action being related to pH and hydrochloric acid concentration. It also seems that, the key factor determining microbial survival in gastric juice is the pH (about 2-2.5), but components in the gastric juice may confer some protective effect on the cells (Fernandez *et al*., 2003). Hence, tolerance to the acidic environment in the stomach is required for the bacteria to survive passage through stomach (Henriksson *et al*., 1999; Lee and Salminen, 1995). Thus, one of the main criteria for selection is survival at low pH (Cebeci and Guakan, 2003). In this study survivability of selected LAB cultures CFR R35, CFR R38 and CFR R123 were investigated along with reference probiotic strain *Lb. rhamnosus* GG ATCC 531530. The results obtained are presented in Figure 3.1.6.

ePrints@CFTRI

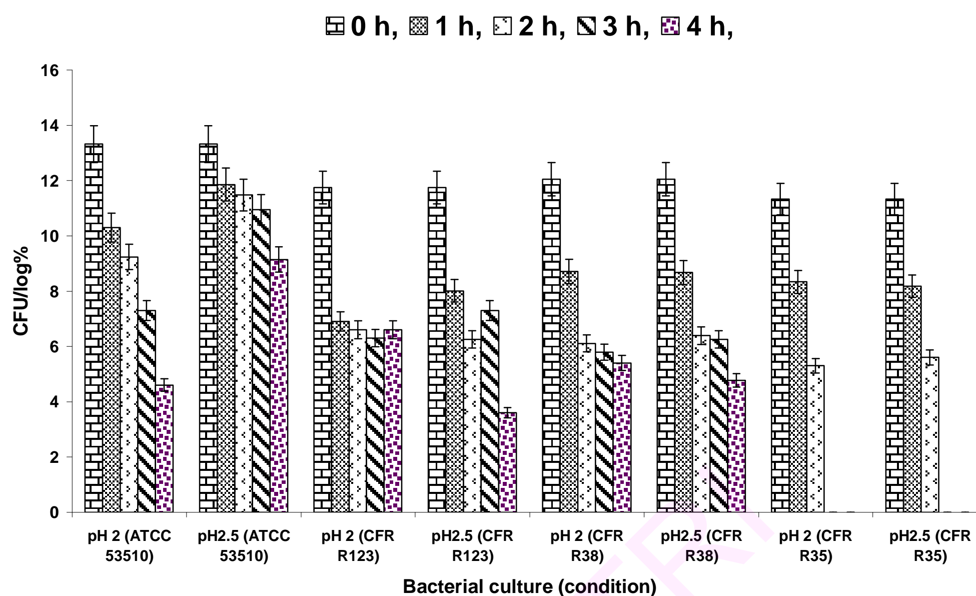


Figure 3.1.6 Acid tolerance ability of LAB cultures at pH 2 and pH 2.5 (CFR R35, CFR R38 & CFR R123:*Ped.pentosaceus*; *Lb. rhamnosus* GG ATCC 53510)

As shown in the Figure 3.1.6, the survival rate of 53 and 62% was observed for CFR R123, respectively at the end of 3 h of incubation at pH 2 and pH 2.5, respectively. The strains CFR R38 and CFR R35 exhibited a survival of 48 and 46% at pH 2 and 52 and 49% at pH 2.5 after two hours of incubation. However the commercial probiotic strain GG proved its endurance capacity of 55% at pH 2 and 82% at pH 2.5 after 3 h. Such survival studies were also carried out in *Lb. acidophilus* isolated from chicken intestine (Jin *et al*., 1998) and *Lb. rhamnosus* GG (Goldin *et al*., 1992) reporting survival of 50% at pH3. In contrary to these observations, a complete loss of viability in *Lb. casei* 212.3 and F19 strains and *Lb. rhamnosus* GG (Charteris *et al*., 1998) at pH 2.5 for 3 h. Such similar observations were also made in spore forming LAB and in a group of 44

ePrints@CFTRI

Lactobacillus spp. where with no replication at pH 2.5 (Hyronimus and Rafter, 2000 and Jacobsen *et al*., 1999). However, the studies carried out by Berrada *et al*., (1991) showed a different profile of survivability among the different strains of *Lb. casei*. The above observations indicate that the survival rate of LAB at different pH is strain specific.

3.1.6.2 Bile tolerance

Bile resistance is an essential characteristic in considering a culture as a dietary adjunct (Walker and Gilliland, 1993; Gilliland and Walker, 1990). The physiological concentration of bile acids in the small intestine is between 5000 to 20,000 μmol (Hofmann, 1991). However, a concentration of 0.3% or 0.15% of bile salts is considered to study the probiotic properties (Zarate *et al*., 2000; Fernandez *et al*., 2003). In this study, ability of selected LAB cultures CFR R35, CFR R38 and CFR R123 along with ATCC 53510 to withstand physiological bile condition was evaluated *in vitro*. The time delay in the growth of the test strain in presence of bile was compared to that of the control (absence of bile) and the results are given in Table 3.1.3. As observed, the strain CFR R123 and CFR R38 showed a time delay in growth of 6.25 and 10 min, respectively illustrating their resistance to 0.3% bile. The strain CFR R35 was tolerant to such bile concentration with a time delay of 40 min. However, the reference strain, ATCC 53510 exhibited no growth at tested bile concentration (0.3%) and was thus sensitive. Bile resistance is an important factor for an organism to grow in the

ePrints@CFTRI

intestinal tract (Gilliland *et al*., 1984; Suscovic *et al*., 1997). The results of the present study indicated that the two strains resistance to 0.3% bile (CFR 123 and CFR R38) suggests their ability to proliferate in the intestine and decipher their beneficial attributes to the host.

Table 3.1.3 Bile tolerance of phytate degrading LAB

Bacterial culture	Delay in growth	Result
Ped. pentosaceus CFR R35	40 min	Tolerant
Ped. pentosaceus CFR R38	10 min	Resistant
Ped. pentosaceus CFR R123	6.25 min	Resistant
Lb. rhamnosus GG ATCC 53510	no growth	non tolerant

3.1.6.3 Antimicrobial activity

One of the major criteria for probiotic LAB is its inhibitory effect on the growth of pathogenic bacteria (Lin *et al*., 2007) as it prevents the infection and/or invasion of pathogenic bacteria. All the four test strains were evaluated for their antimicrobial activity against indicator organisms listed in Table 3.1.4. All the test cultures were able to inhibit the growth of indicator strains with difference in zone of inhibition ranging from 10-30 mm dia (Table 3.1.4). A maximum antibacterial activity was observed with CFR R 38 against enterotoxigenic *E. coli* with a inhibition zone of 30 mm in dia. Similarly, CFR 38 was also exhibited in a range of 21 to 30 mm against other indicator strains. Comparatively, CFR R35 showed good antibacterial activity against *E.coli* MTCC 108 with reduced activity against other indicator strains. The inhibition of growth of *L. monocytogenes* Scott A was

ePrints@CFTRI

also observed with CFR R123. However, its antimicrobial activity was comparatively less against other indicator strains. The reference strain ATCC 53510 showed inhibitory action, but was least compared to other strains.

The inhibitory action of LAB on most microorganisms could be due to the production of H_2O_2, organic acids, specific bacteriocin or non-bacteriocin by LAB (Jacobsen *et al*., 1999; Lin *et al*., 2007). In view of this, the nature of the antimicrobial compound responsible for their antagonistic activity was evaluated by agar well diffusion assay. Initially the cell free extracts (culture supernatant) of the test isolates examined against indicator strains expressed no antimicrobial activity except for *Listeria*. However, when the culture filtrate was treated with trypsin, the antimicrobial activity against *L. monocytogenes* was also lost, suggesting the proteinaceous nature of the antimicrobial compound in the culture filtrate. The antagonistic activity of the selected isolates against different groups such as Gram positive and Gram negative can have added benefit in utilizing such LAB in food applications or as probiotic in elimination of intestinal pathogens.

ePrints@CFTRI

Table 3.1.4 Antimicrobial activity of phytate degrading LAB

Indicator strains	Bacterial cultures			
	CFR R38	**CFR R35**	**CFR R123**	**ATCC 53510**
E. coli MTCC 108	+++	+++	+	++
B. cereus F 4810	+++	++	++	+
L. monocytogenes Scott A	+++	++	+++	++
Y. enterocolitica MTCC 859	+++	++	+	+
Sal.pParatyphi	+++	++	+	+
Staph. aureus FRI 722	++	+	+	++

+: poor activity (≤ 10 mm); ++: moderate activity (10-30 mm); +++: potent activity (≥30 mm)
CFR R35, CFR R38, CFR R123: *Ped. pentosaceus;* ATCC 53510: *Lb. rhamnosus* GG

3.1.6.4 Adhesion activity

The ability to adhere mucosal surfaces has been suggested to be an important property of bacterial strains used as a probiotics. In addition, bacterial aggregation is of considerable importance in several ecological niches, especially in the human gut, where probiotics are to be active (Aswathy *et al*., 2008). Hence, it is considered as a pre-requisite of probiotic applications in order to confer certain health promoting effects (Canzi *et al*., 2005). Bacterial adhesion can also determine the colonization capability of a microorganism (Aswathy *et al*., 2008). Adhesion and colonization of tissues by probiotic microorganism can prevent pathogen access by steric interaction or specific blockage on cell receptors (Aswathy *et al*., 2008). The BATH test has been extensively used for measuring cell surface hydrophobicity in LAB (Vinderola *et al*., 2004; Canzi *et al*., 2005).

ePrints@CFTRI

Thus, the objective was to study the cell surface properties such as hydrophobicity by BATH test. In this test, the hydrocarbon, xylene was used to study the cell wall hydrophobicity and evaluate adhesion property of LAB *in vitro*. The data obtained are demonstrated in Figure 3.1.7. From the results obtained, it can be observed that test isolate CFR R123 exhibited high adhesion property of 62.8% compared to the other test strains. This was followed by the reference strain ATCC 53510 and CFR R38 with 58 and 54.6%, respectively. Among the tested isolates, the least adherence was observed in CFR R35 (44.8%). Hence it can be concluded that the adhesion property of the test isolates were moderate to good level.

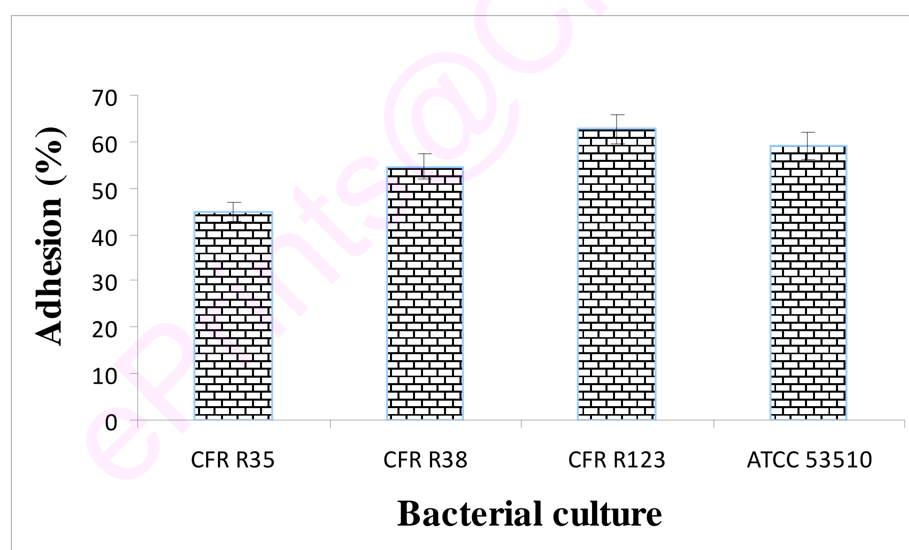


Figure 3.1.7 Adhesion property of the phytate degrading LAB (CFR R35, CFR R38 & CFR R123: *Ped. pentosaceous; Lb. rhamnosus* GG ATCC 53510)

ePrints@CFTRI

3.1.6.5 β-Galactosidase activity

Lactose intolerance is a term used to describe the discomfort that occurs after digestion of milk. This condition results from insufficient amounts of β-galactosidase to digest lactose in the intestines. Because of discomfort, intolerant people prefer to delete milk from the diet (Cebeci and Guakan, 2003). Generally, LAB contains intracellular β-gal that catalyzes lactose hydrolysis which has wide applications in dairy industries (Gilliland, 1989; Cebeci and Guakan, 2003). Therefore, testing for the production of this enzyme by LAB is essential to evaluate them as probiotics. The existence of β-gal in phytate degrading LAB isolates was evaluated *in vitro* by biochemical assay in presence of glucose or lactose. The obtained results are illustrated in Figure 3.1.8. It was observed that in the presence of glucose, the enzyme activity was negligible or nil in all the tested strains including the reference strain. Intrestingly, when cultures were grown in presence of lactose, β-gal activity varied among the cultures with the highest being observed in CFR R35 with 613 MU. The other strains such as CFR R38 and CFR R123 displayed an activity of 580 MU and 413 MU respectively. Comparatively, the reference strain exhibited a least β-gal activity to that of with test strains. The β-gal is less common in *Ped. pentosaceus* strains. There are very few reports available on this. It also been demonstrated that the β-gal vary among the strains studied so far in pediococci, enterococci, etc. (Badarinath and Halami, 2010).

ePrints@CFTRI

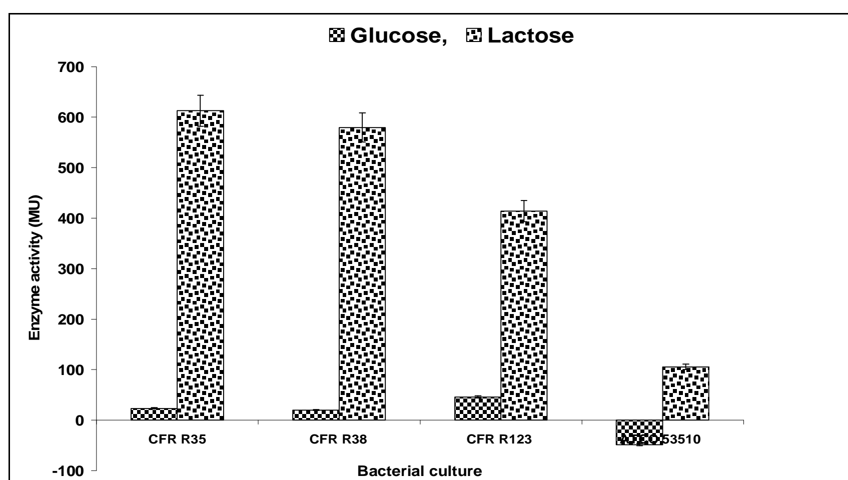


Figure 3.1.8 β-gal activity of phytate degrading LAB

3.1.6.6 Antibiotic sensitivity pattern

LAB widely used as probiotics or in starter cultures have the potential to serve as a host of antibiotic resistance genes with the risk of transferring the genes to other LAB and pathogenic bacteria. Vancomycin resistant enterococci (VRE) have emerged in the last decade as a frequent cause of nosocomial infections. Of considerable concern is the possibility that VRE, selected and enriched by the use of avoparcin (with cross resistance to vancomycin) as a growth promoter in animal husbandry, are spread via the food chain (Wegener *et al*., 1997; Klein *et al*., 1998). In view of this, the responses of tested isolates to the varied number of antibiotics were evaluated for antibiotic susceptibility by E-test. Results pertaining to this observation are given in Table 3.1.5. Based on European commission (2005), the cultures were demonstrated sensitive (S) and resistant (R) by observing the inhibitory zone against tested antibiotics taking into consideration the clinical break points presented by the FEEDAP panel (European

ePrints@CFTRI

commission, 2005). The table 3.1.5 demonstrates MIC values obtained for the 8 antibiotics tested against *Ped. pentosaceus* test strains CFR R38, CFR R123 and CFR R35. It was observed that all the isolates were sensitive to six antibiotics with MIC values within the clinical breakpoints range. However all the three test strains showed a similar range of MIC value to polymyxin B. The test strains sensitive to the tested medically important antibiotics reveals no acquired resistance. Hence these isolates are safe that can be used as starter cultures as well as in the functional food preparations, as they pose no threat in transfer of resistance genes.

Table 3.1.5 Antibiogram of the selected phytate degrading LAB

Antibiotic	**Minimum inhibitory concentration in µg**		
	CFR R38	CFR RR35	CFR R123
	Inhibitors of cell wall synthesis		
Ampicillin	2 (S)	2 (S)	2 (S)
Cephalotin	4.0 (R)	0.5 (S)	4.0 (R)
	Inhibitors of protein synthesis		
Chloramphenicol	0.5 (S)	0.5 (S)	0.5 (S)
Gentamycin	2.0 (S)	5.0 (S)	2.0 (S)
Erythromycin	0.25 (S)	0.25 (S)	0.25 (S)
Tetracyclin	0.01 (S)	8 (R)	2.0 (S)
Streptomycin	5.7 (R)	30 (S)	30 (S)
	Inhibitors of cytoplasmic functions		
Polymyxin B	32 (R)	32 (R)	32 (R)

S: sensitive; **R**: resistance; **CFR R35, CFR R38, CFR R123**: *Ped. pentosaceus*

ePrints@CFTRI

3.1.7 Conclusion

The qualitative screening resulted in the selection of three phytate degrading LAB strains with their ability to degrade different phytic acid substrates. The isolates were identified and characterized as *Pediococcus* strains. The isolated strains exhibited a spectrum of acid and bile tolerance and were capable of producing antimicrobial compounds along with moderate to good levels of adherence efficiency when tested *in vitro*. The strains exhibited antibiotic sensitivity pattern within the clinical break points. They also illustrated their β-galactosidase activity. The phytate degrading LAB with beneficial attributes can serve as a good starter culture in different food fermentation processes, in which they involved. The characterized phytate degrading LAB cultures CFR R35, CFR R38 and CFRR123 were deposited in the repository of Food Microbiology department of the institute. Further, the cultures were assessed for their phytate degrading ability by quantitative analysis and their specific enzyme existence.

ePrints@CFTRI

Chapter 3

Section -2

Characterization and evaluation of phytate degrading ability of lactic acid bacteria

ePrints@CFTRI

The three potent phytate degrading strains of *Ped. pentosaceus* CFR R123, CFR R38 and CFR R35 obtained during preliminary screening were investigated for phytate degrading ability. The evaluation was carried out employig three methods *viz.*, qunatitative analysis of degraded phytic acid by biochemical assay, estimating the enzyme activiteis (phytase and acid phosphatase) and determining the mass of degraded PA by HPLC-MS. In addition, media optimization was carried to demonstrate the role of substrate, phosphate source, buffering agent at different time intervals for enzyme activity of potent phytate degrading cultures. Further, using selective media for CFR R38, its ability towards phytate degradation at different temperature, pH and substrate concentrations was elucidated. Intacellular nature of phytate specific enzyme from the CFR R38 cell lysate was evaluated at optimal conditions. Finally, phytate degrading ability was confirmed by estimating the resulted products of post phytic acid degradation by HPLC-MS and phytase activity by zymography.

ePrints@CFTRI

3.2.1 Phytate degrading ability of the LAB

The phytate degrading ability of the isolates was evaluated by quantifying the retained phytic acid using spectrophotometric analysis. Sodium phytate at a concentration of 2 mM was used as a substrate. Available literature (Sreeramulu *et al.*, 1996) demonstrates that *Lb. amylovorus* B 4552 is a phytase producing LAB, hence it was used as a reference strain in this study. The enzyme activity was evaluated at two different temperatures i.e., 37 and 50°C. The absorptiometric analysis illustrated that all the test cultures including reference strain had phytic acid degrading ability at 50°C. On the other hand, this was found to be negligible at 37°C in all the test strains. As shown in Figure 3.2.1, the phytate content reduced at 50°C was 46, 44.4, 34.4 and 17% with CFR

R123, CFR R38, CFR R35 and B 4552 in 15 min, respectively. However, at the end of the 60th min, the phytate content reduction was 70% as observed in CFR R123. The other strains, namely, CFR R38, CFR R35 and B 4552, displayed a decrease in phytate content of 65.3, 53.28 and 22.8%, respectively.

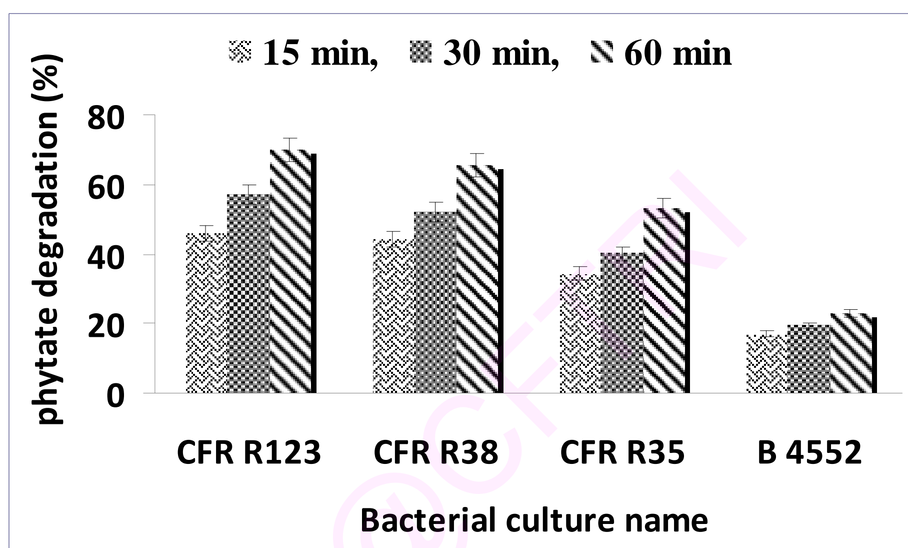


Figure 3.2.1 Quantitative analysis of phytate degradation by LAB at 50°C (*Ped. pentosaceus* CFR R35, CFR R38 and CFR R123 and *Lb.* amylovorus B 4552)

ePrints@CFTRI

3.2.2 Evaluation of phytase and phosphatase activities

Although microbial phytases are considered as of a great value in upgrading the nutritional quality of plant foods, the studies dealing in this particularl area are meagre. The three test cultures employed in the present study exhibited phytate degrading ability both qualitative and quatitative analysis. The whole cell suspension of the test cultures were used in the phytase and phosphatase activity analysis. The phytase activity of the test isolates was investigated at 37 and 50°C by estimating the liberated inorganic phosphates spectrophotometrically (700 nm). The observed results indicated that the phytase activity ranged from 3-459 U/ 9Log CFU/ml (Table 3.2.1). At 37°C, the phytase

activity was highest in CFR R123 (40 U) and the least being observed in CFR R38 (4.4 U). The other isolates scuh as ATCC 53510, B 4552 and CFR R35 demonstrated 27, 15 and 12 U, respectively. Similarly, at 50°C, high phytase activity of 459 U in CFR R123 was observed and the least with 3 U for the reference strain (B 4552) (Table 3.2.1). However, CFR R38, CFR R35 and ATCC 53510 exhibited 213, 89 and 6 U of phytase activity, at the same temperature (50°C). The experimental analysis was carried out in microtitre plates with 200 µl reaction and the enzyme activity was described in nkatal as described earlier by Neilson *et al*., (2008), The results obtained are given in nkatal (Figure 3.2.2).

Table 3.2.1 Phytase and acid phosphatase activities of potent phytate degrading LAB

Name of the strain	Phytase activity U[ϒ]		Acid phosphatase activity at 50°C U [*]
	37°C	50°C	
Ped. pentosaceus CFR R38	4.4	**213**	1.9
Ped. pentosaceus CFR R35	12	89	1.05
Ped. pentosaceus CFR R123	**40**	**459**	4
Lb. rhamnosus GG ATCC 53510	27	6	**15.1**
Lb. amylovorus B 4552	15	3	8.1

[ϒ] One unit of phytase activity (U) was defined as the amount of enzyme that produces one nano mole of inorganic phosphorous per min at 50ºC. * One unit of phosphatase activity (U) was defined as the amount of enzyme that produces 1 µmol of *p*-nitro phenol per min at 50ºC. Highest enzyme activities of the culture are highlighted.

ePrints@CFTRI

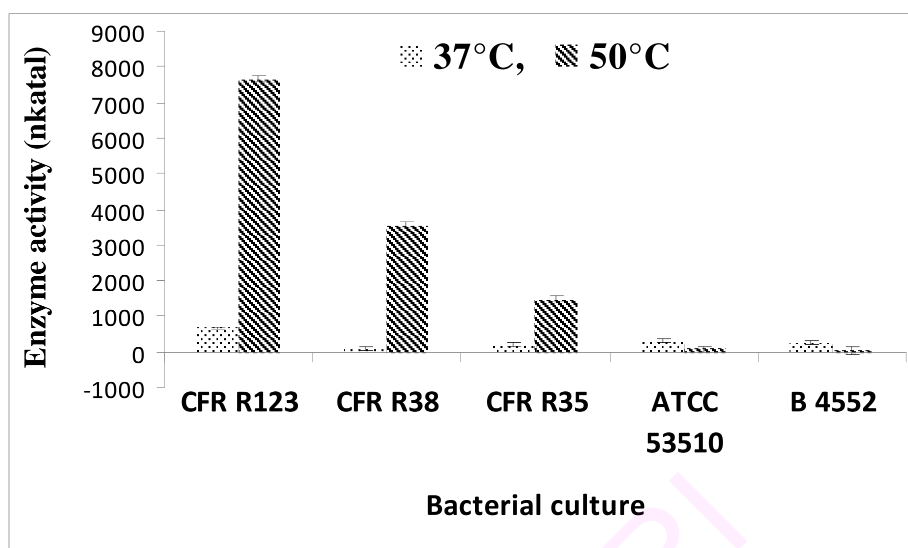


Figure 3.2.2 Phytase activity of LAB cultures at 37°C and 50°C. (CFR R35, CFR R38 and CFR R123: *Ped.pentosaceus*;ATCC 53510: *Lb. rhamnosus* GG; B 4552: *Lb. amylovorus*)

The phytase activity of LAB has been considered to be intracellular (De Angelis *et al*., 2003) and in certain cases it is extracellular (Sreeramulu *et al*., 1996, Vohra and Satyanarayana 2003). In the present study, no extracellular activity was observed for either of the phosphatases. Among the 3 test isolates, it was observed that the strain CFR R123 from red rice exhibited high phytase activity at 50°C compared to the other isolates (CFR R38 and CFR R35) which are of chicken intestinal origin. The high phytase activity at 50°C observed in CFR R123 (459 U) and CFR R35 (213 U) showed a negligible acid phosphatase activity at the same temperature. These observations are in accordance with the previous work carried out in *Lb. sanfranciscensis* CB1 by De Angelis *et al*., (2003). In contrast, phosphatase activity of 15.1 U at 50°C was observed in *Lb. rhamnosus* GG., which showed least phytase activity at the same temperature. Studies on phytase activity was carried out using whole cells of LAB such as *Lb. plantarum*, *Lb. acidophilus, Leu. mesenteroides subsp. mesenteroids* in white flour

medium (Lopez *et al*., 2000) and in *Bifidobacterium* sps. (Haros *et al*., 2008, 2007 and Palacios *et al*., 2008). These LAB were reported to exhibited low intracellular phyase activity. Thus intracelluar phytase activity might be found in almost every cell since phytate is a common cellular constituent with a significant turnover (Shears *et al*., 1998). However, it is very unlikely that intracellular phytases are involved in extracellular phytate dephosphorylation and it cannot be ruled out that phytate is taken up by bacterial cells. This view can be supported by the work carried out on *Pseudomonas* strains, that lack extracellular phytase activity. It was observed that the isolates could grow on phytic acid containing medium with no readily utilisable phosphate source, suggesting the transport of phytate into the bacterial cells (Richardson *et al*., 1997). However, contradiction always exist on phytases of LAB and the existing report suggesting the phytic acid degradation of LAB due to non-specific acid phosphatases (Zamudio *et al*., 2001, Palacios *et al*., 2005).

Acid phosphatase (E.C.3.1.3.2) is a member of histidine group of phosphatases that has broad substrate specificity. Acid phosphatase act on a large number of phosphate compounds and release lower intermediates. Simultaneously, acid phosphatase activity was also observed among the tested isolates of the present study. The specific activities against *p*-nitrophenol phosphate varied from 1.05 U to 15.1 U (Table 3.2.1). At the tested conditions, maximum activity was observed to be 15.1 U for ATCC 53510 and lowest for CFR R35 with 1.05 U. Other strains CFR R38, CFR R123 and B 4552 expressed 1.9, 4 and 8.1 U respectively. The studies on two phosphatases showed remarkable differences among the tested strains in their activities. At 50ºC, the phytase

ePrints@CFTRI

activity was increased to several folds (~12). In contrast, the enzyme activity in the reference cultures was lower at 50 ºC when compared to 37 ºC.

Phosphatases are ubiquitous enzymes of broad specificity that have been recently found in LAB, while phytases are a particular subgroup of phosphatases, with preference for phytate as they do not seem to be common in this bacterial group (Zamudio *et al*., 2001). The studies on ability of LAB to degrade phytate and its derived products are however limited. This property (phytase activity) has often been detected in LAB strains from plant origin, but not in dairy environments (Sreeramulu *et al*., 1996; De Angelis *et al*., 2003). In general, phosphatases and phytase activities are measured at optimal acid pH as reported in previous studies (Palacio *et al*., 2005; Abdallah *et al*., 1998). However, these activites are only detected in cell suspensions but not in the extracellular medium. Acid phosphatases showed high hydrolysis rates with monophosphorylated compounds but low levels of activity against phytic acid (Vohra and Satyanaryana, 2003). The phytases usually show broad substrate specificity,showing the highest preference for IP6 and only a few have shown to have little or no activity on phosphate esters such as *p*-nitrophenol phosphates (Zamudio *et al*., 2001; De Angelis *et al*., 2003; Vohra and Satyanarayana, 2003).

3.2.3 Evaluation of optimal growth conditions for phytase activity

The aim of the experiment was to optimize the growth media conditions for the potent phytate degrading native isolates. LAB have complex growth requirements and the MRS components like yeast extract, meat extract, and peptone are required for their growth. Phosphates are an integral part of several MRS media components, which were

ePrints@CFTRI

retained in the culture medium until 7 days. The inorganic phosphates can have profound effect on the production of enzymes such as phosphatases and phytases (Vohra and Satyanarayana, 2003). Hence, in search of a suitable media components, MRS medium was modified by altering the media composition from which four media were formulated (Table 2.1). Cultures grown in respective media for 48 h and harvested at different intervals were subjected for determining their phytase activity. A cells concentration of 9 log CFU/ml were used in the assay and subsequently activity was calculated per 9 log CFU/ml/nkatal.

The test cultures *Ped. pentosaceus* CFR R123, CFR R38 and CFR R35, as well as reference *Lb. rhamnosus* GG ATCC 53510 and *Lb.amylovorus* B 4552 were propogated in media 1 and were analyzed for phytase actitiy at 24- 48 h. At 24 h, all the test cultures exhibited good growth and intracellular phytase activity. However, difference in their phytase activity was observed with the highest recorded in CFR R123 (4900 U) (Figure 3.2.3). The other isolates CFR R38, CFR R35 and ATCC 53510 exhibited a phytase activity of 4321, 3482 and 2025 respectively. Although there was a reduction in the phytase activity in all the test cultures from 24-48 h, the cells could retain the activity at the end of 48 h. However, one of the reference strains (*Lb.amylovorus)*, showed a least activity (1234 U) at 24 h and showed no activity at 48 h.

ePrints@CFTRI

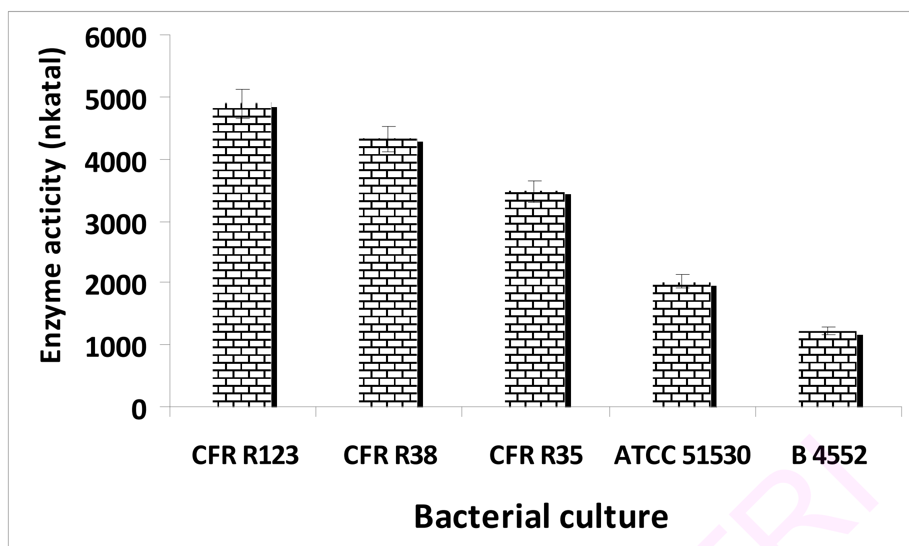


Figure 3.2.3 Phytase activity of the LAB test strains grown in media 1 at 24 h (CFR R123, CFR R38 and CFR R35:*Ped. pentosaceus*; ATCC 53510: *Lb. rhamnosus* GG, B 4552: *Lb. amylovorus*).

ePrints@CFTRI

The growth and phytase activity of the tested strains (CFR R123, CFR R38, CFR R35, *Lb. rhamnosus* GG ATCC 53510 and *Lb. amylovorus* B 4552) were also evaluated in media 2., in which the inorganic phosphate was replaced with sodium phytate and a buffering agent, MOPS. Phytase activity was observed in the cells that were grown for 24, 48, 52, 60 and 72 h. It was observed that there was an increase in activity from 24 to 48 h and thereby a gradual decline in the activity was observed till 72 h.

The phytase activity of the cultures grown for 24 and 48 h are presented in Figure 3.2.4, and the activity is expressed in nkatal. From the figure, it can inferred that the among all the tested isolates, CFR R38 showed a highest activity with 4900 and 5700 nkatal in 24 and 48 h, respectively. The other cultures CFR R123, CFR R35, ATCC 53510 and B 4552 expressed an activity, that was lower than CFR R38 with the values 3718, 3718, 3579 and 3718, respectively in 24 h and 4622, 3962, 3162 and 3197 in 48 h.

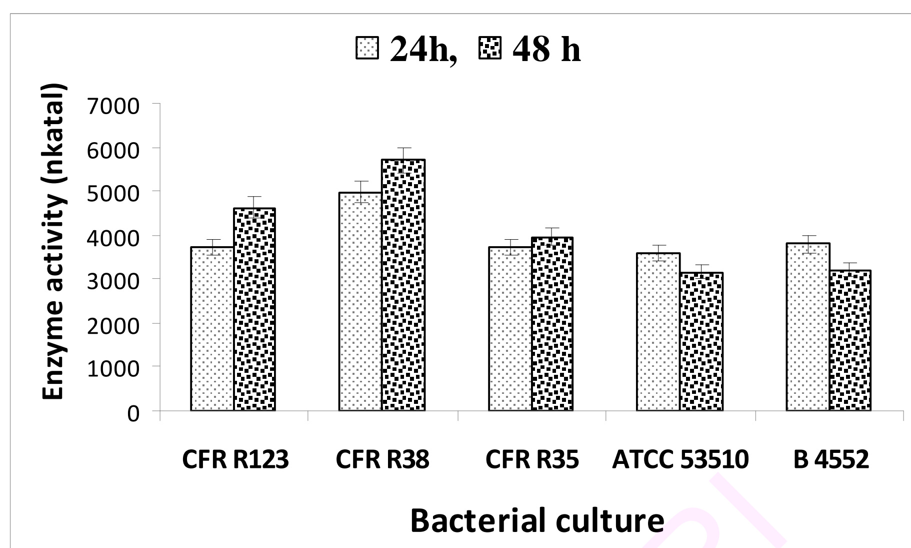


Figure 3.2.4 Phytase activity of the LAB test cultures grown in media 2 at 24 and 48 h (CFR R123, CFR R38 and CFR R35:*Ped. pentosaceus*; ATCC 53510: *Lb. rhamnosus* GG, B 4552: *Lb. amylovorus*).

The phytase activity of the tested isolates was also evaluated in the modified MRS media with 0.1 M MOPS, where either of the phophate source (sodium pytate or KH_2PO_4) was supplemented (media 3). It was found that the enzyme activity observed in all these isolates was quite negligible compared to the activity observed in meda 1 and 2. However, difference in the enzyme activity among the isolates presumed with the highest being observed in CFR R123 with 270 nkatal The observed enzyme activites for CFR R123, CFR R38, CFR R35, ATCC 53510 and B 4552 are given in Figure 3.2.5. In absence of either of the pohosphate source, cultures expressed very negligible activity. The enzyme activites were observed to be 167 nkatal with CFR R123 which is the highest among the tested cultures. All the other isolates exhibited an activity of ≤116 nkatal (Figure 3.2.5.)

ePrints@CFTRI

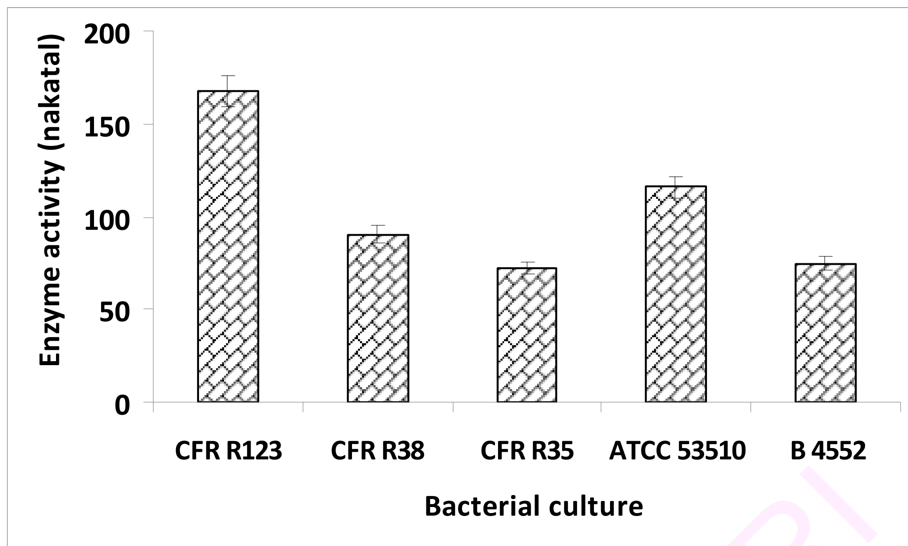


Figure 3.2.5 Phytase activity of the LAB test cultures grown in media 3 at 24 h (CFR R123, CFR R38 and CFR R35:*Ped. pentosaceus*; ATCC 53510: *Lb. rhamnosus* GG, B 4552: *Lb. amylovorus*).

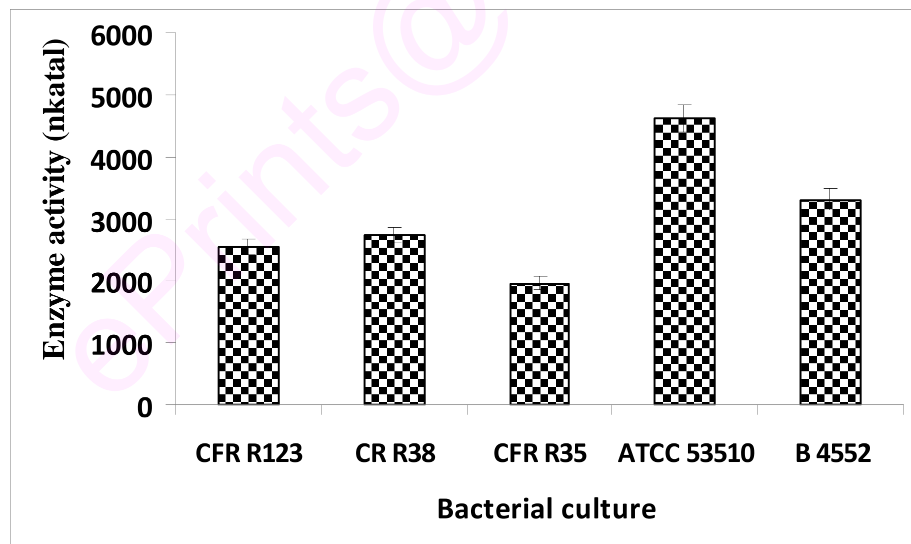


Figure 3.2.6 Phytase activity of the LAB test strains grown in media 4 at 24 h (CFR R123, CFR R38 and CFR R35:*Ped. pentosaceus*; ATCC 53510: *Lb. rhamnosus* GG, B 4552: *Lb. amylovorus*).

ePrints@CFTRI

In order to examine the combined effect of MOPS and substrate on the enzyme activity of the cultures, media 4 was formulated which was devoid of both MOPS and either of the phosphate source (sodium phytate or KH_2PO_4). Interestingly, the results obtained are in contrast to those observed with other media formulations. It was found that all the isolates exhibited an activity of ≥ 2200 nkatal (Figure 3.2.6). Figure 3.2.7 illustrates the differences among the enzyme activites of test cultures grwon in four different media.

ePrints@CFTRI

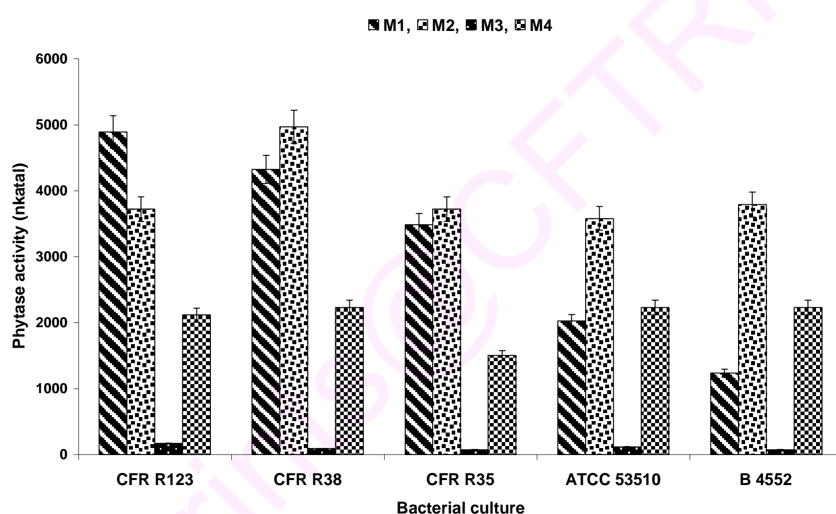


Figure 3.2.7 Phytase activity of the LAB test strains grown in 4 different media at 24 h *(Ped. pentosaceus* CFR R123, CFR R38 and CFR R35 and *Lb. rhamnosus* ATCC 53510, *Lb. amylovorus* B 4552)

All the tested isolates along with *Lb. amylovorus* and *Lb. rhamnosus* GG grown in four different medias were also observed for their specificity towards *p*-nitrophenol phosphate andd the results are shown in Figure 3.2.8. None of the isolates exhibited intracellular enzyme activity except for reference strains ATCC 53510 and B 4552. These results also correlate with those obtained during the prliminary screening for acid phosphatase activity.

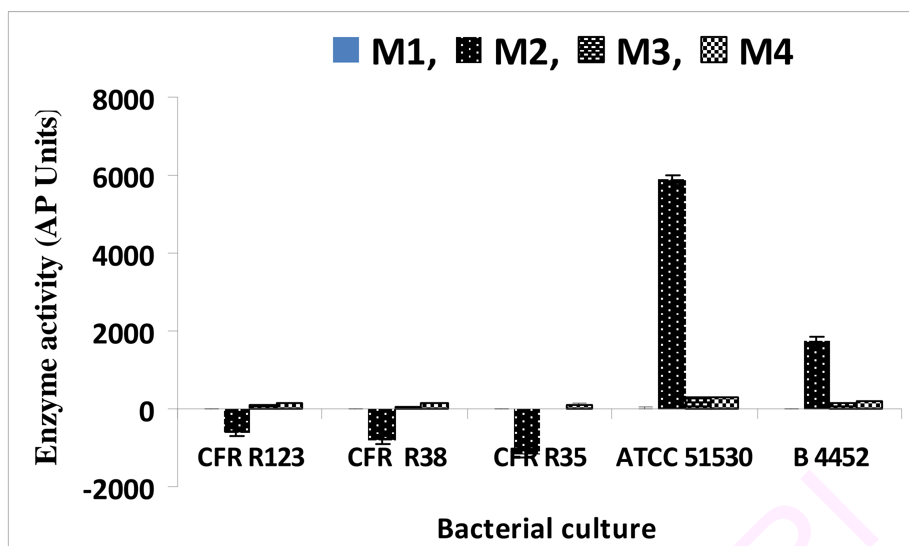


Figure 3.2.8 Acid phosphatase activity of the LAB test strains grown in 4 different media at 24 h

As shown in Figure 3.2.8, cultures grown in media 2 exhibited no significant acid phosphatase activity, except for ATCC 51530 (5900 U) and B 4552 (1738 U). However with the other media formulations there was negligible or no enzyme activity observed with CFR R123, CFR R38 and CFR R35 cultures. Whereas the two reference strains showed an activity which was least when compared to the values obtained in media 2.

The phosphorous and carbohydrate sources used in the growth medium are some of the known environmental factors that regulate the synthesis of microbial phytases (Haros *et al*., 2005). Optimizinig the conditions for the phytate degradrading microorganisms is cumbersome because phytate degrading enzymes exhibit different catalytic properties depending on the source of origin. Moreover, a failure to detect phytase activity could be difficult to find the advantageous culture conditions for microorganisms under investigation (Konietzny and Greiner 2003). The synthesis of phytases is generally known to be induced when limiting concentrations of phosphorous

ePrints@CFTRI

present in the growth medium of yeast, moulds and bacteria (Sreeramulu *et al*., 1996; Pandey *et al*., 2001; Vohra and satyanarayana, 2003). Carbon source and its concentrations are critical factors for phytase production in other bacteria, with 1-2% (w/v) being normally the preferred substrate concentration (Sreeramulu *et al*., 1996; Vohra and Satyanaraya 2003).

Bacterial phosphatases and phytases are either periplasmic or cell associated enzymes, with the exception of the phytases described in *Bacillus subtilis*, *Lb. amylovorus* and *Enterobacter* sp4., which are extracellular (Vohra and Satyanarayana, 2003). Over all, the activities were maximal at the onset of the stationary phase as described for the phytase of *Lb. amylovorus* and *Lb. sanfranciscensis* CB1 (Sreeramulu *et al*., 1996; De Angelis *et al*., 2003). According to Dasa *et al*., (1982), acid phosphatase from Gram-negative bacteria were also induced when cultures enter the stationary phase. This report is similar representative for the acid phosphatase data produced by ATCC 53510 culture in media 2 and 3. The specific activities were maximal at the lowest glucose concentration (0.5%), suggesting that the synthesis of the enzyme(s) can respond to limiting concetrations of carbon source. Moreover, the biomass was reduced in the presence of 0.5% glucose and therefore the total activity recovered was higher on adding 1.0% of glucose in the culture medium. The inhibitory effect caused by the presence of inorganic phosphate in the growth medium could be partially restored by the simultaneous NaP, indicating that substrate could act as an inducer. On the other hand NaP did not exert a stimulatory effect on the enzyme production in yeast (Segueiha *et al*., 1993). The repression of the phytase synthesis by inorganic phosphorous seems to be less significant with higher medium composition complexities. It is however unknown,

ePrints@CFTRI

what components in the complex media accounts for the reduced repression (Fredrikson *et al*., 2002).

No satisfactory scientific evidences showing degradation of phytate by a wild type LAB has been presented with regard to phyase production. In this study the tested LAB strains seem to require less phosphorous for growth than the other strains. LAB are adapted to environments, rich in nutrients and energy and, therefore, have dispensed with their biosynthetic capacity (Axelsson, 1998). Due to rich environments where LAB exist, there may never have been an evolutionary selection of LAB with respect to phytate degrading capacity. Thus to date it is uncertain whether there are any wild type LAB with the ability to produce a phytate degrading enzyme. In the present study, an extracellular acid phosphatase activity was observed in cultures grown in media 2 at 24 hr (Figure 3.2.9) with nearly ~600 U in *Lb. amylovorus* and ~368 U for *Lb. rhamnosus* ATCC 53510. However, there was a negligible or no enzyme activity observed with CFR R35 as well as for CFR R123 and CFR R38.

ePrints@CFTRI

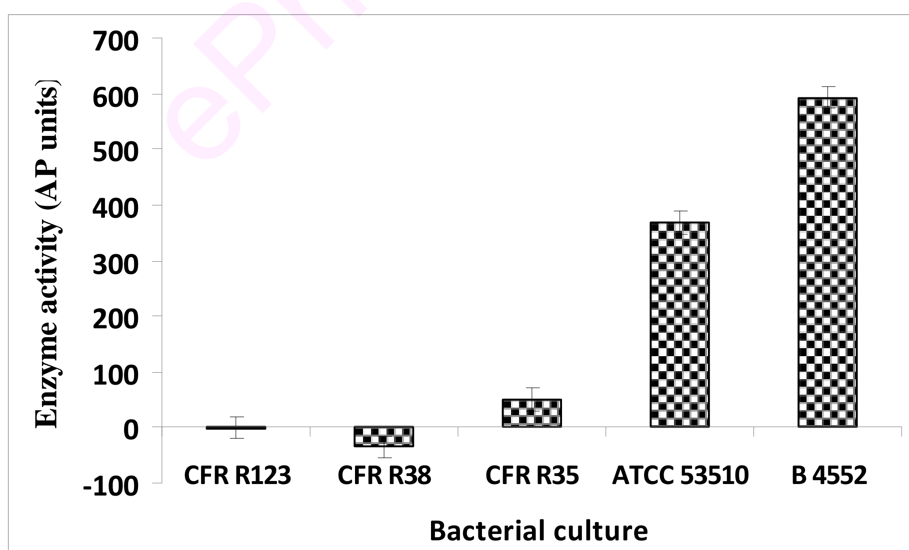


Figure 3.2.9 Extracellular acid phosphatase activity of LAB cultures grown in media 2

3.2.4 Effect of physiological parameters on phytate degrading ability of LAB

Among the tested media combinations *Ped. pentosaceus* CFR R38 and CFR R123 were able to grow in media 1, 2 and 4. However, the phytase activities of these two strains in the same media were different with maximum being observed in CFR R38 (media2). Thus it was found that media 1 is suitable for CFR R123 where as media 2 for CFR R38. Although, media 2 was suitable for the growth of CFR R35, the activities expressed are less than the other two *Pediococcus* strains. Further, CFR R38 was selected and propagated in media 2 and the obtained cell suspensions were tested for phytase activity under different concentrations of substrate, pH and temperature.

Temperature is one of the vital physical factors that play a role in growth and metabolism of all the organisms. An optimum temperature exists for every activity, which may enhance the metabolic activities. In the present study, enzyme activity for CFR R38 was optimum at 50°C (Figure 3.2.10). The strain displayed very less or negligible activity at 37°C. As the temperature increases from 50°C, there was a gradual decrease in the phytase activity. The obtained results are in agreement with the available reports where *Bifidibacterium* sps. expressed negligible activity at 37°C but retained activity of ~7 % at 50°Cs (Haros *et al*., 2007). According to De Agelis *et al*., (2003), *Lb. sanfranciscensis* CB1 expressed its activity at an optimum temperature of 40-45°C. In general, the optimal temperature of phytate degrading enzymes vary from 35-77°C, whereas, the optimal temperature from bacterial phytases are comprised between 50-70°C (Konietzy and Griener, 2002; Vohra and Satyanarayana, 2003, Oh *et al*., 2004). The optimal temperature required for the enzyme activity of the strain CFR R38 is with in the range found for phytases of bacteria.

ePrints@CFTRI

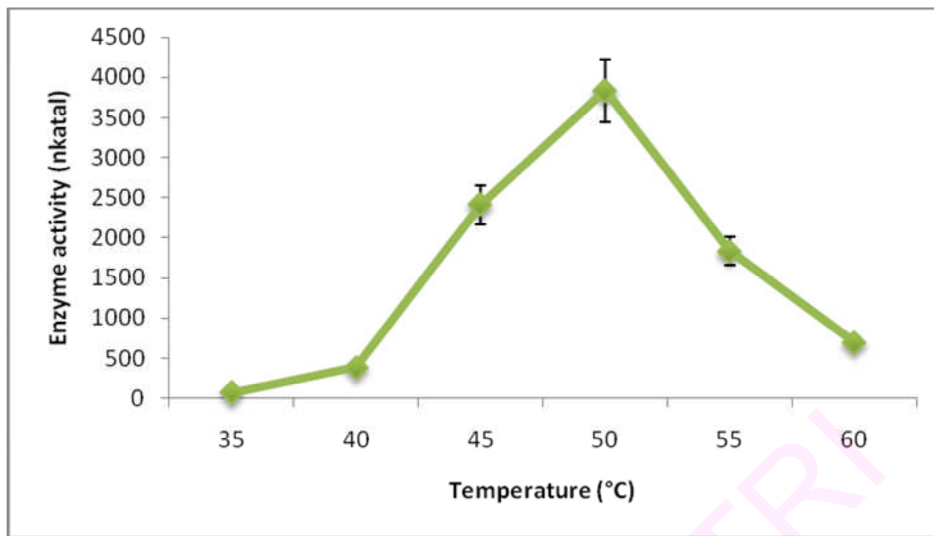


Figure 3.2.10 Effect of temperature on phytase activity of *Ped. pentosaceus* CFR R38

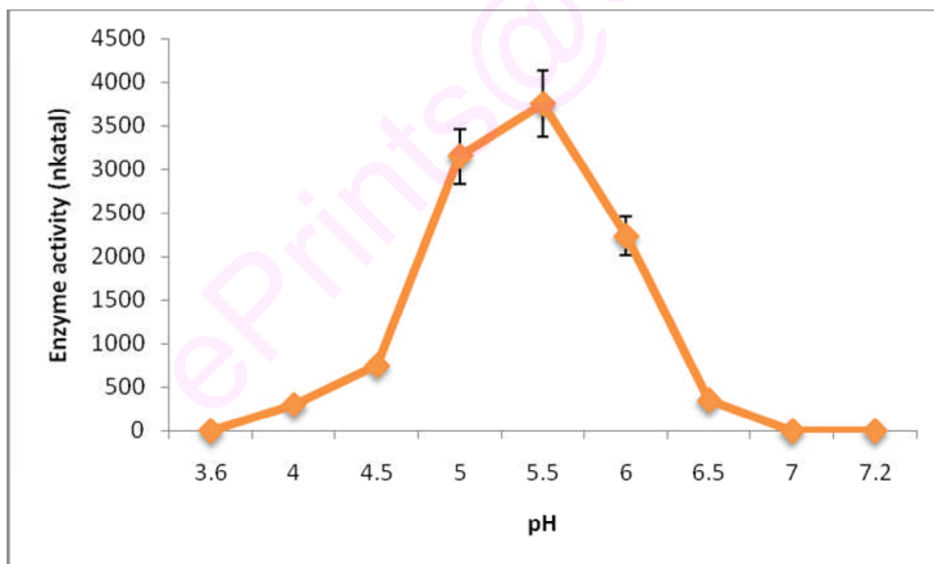


Figure 3.2.11 Effect of pH on phytase activity of *Ped. pentosaceus* CFR R38

ePrints@CFTRI

The optimal pH for phytate degrading activity in CFR R38 was found to be 5-6.0 under the standard assay conditions (Figure 3.2.11). The strain CFR R38 expressed its activity at acidic pH but not below pH 4.0. Two main types of phytate degrading enzymes had been identified: acid phytate degrading enzymes with an optimum pH of 4.5-6.0 and alkaline phytate degrading enzymes with 7.0-8.0 pH (Konietzny and Greiner, 2002; Oh *et al*., 2004). According to this classification, the test strain seems to produce an acid phytate degrading enzymes. Earlier, a pH of 4.0 was found to be optimal for the phytase activity of *Lb. sanfranciscensis* CB1 (De Angelis *et al*., 2003). Whereas several phytate degrading *Bifidobacterium* sp. studied by Haros *et al* (2007) and Palacios *et al*., (2008b) exhibited the activity in the pH range of 6.0-6.5. Under optimal temperature and pH conditions, an optimum concentration of 2 mM substrate showed maximum enzyme activity (Figure 3.2.12).

ePrints@CFTRI

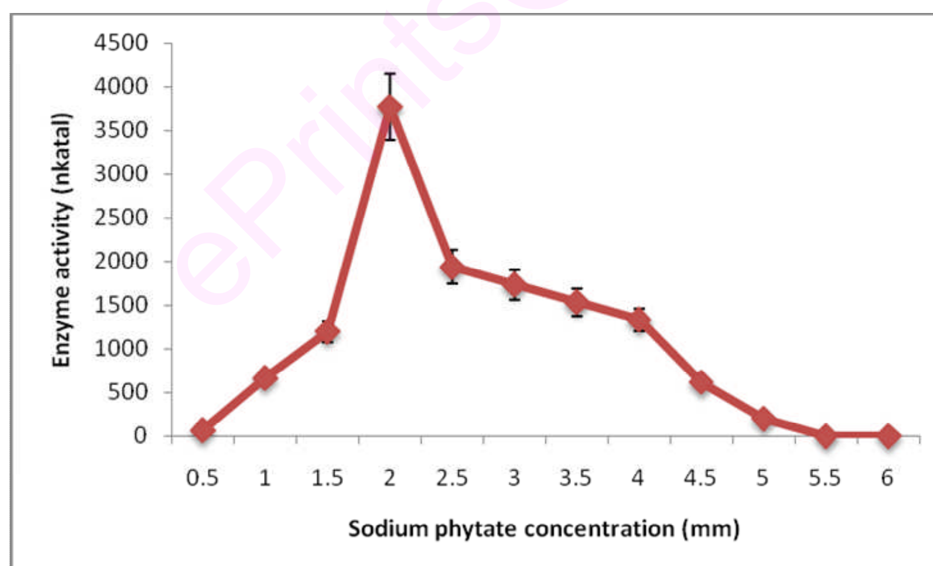


Figure 3.2.12 Effect of substrate concentration on phyase activity of *Ped. pentosaceus* CFR R38

3.2.5 Evaluation of phytate degradation by HPLC

In order to confirm the phytate degradation by LAB, HPLC method was adopted. Though, this method is very y sensitive, Refractive Index Detector (RID) is not so efficient or sufficient to confirm the degraded products. Hence, the pure fractions obtained through HPLC were further injected to MALDI-TOF MS for the molecular mass determination. Based on the corresponding molecular mass, the peaks were selected and phytate degradation was confirmed. There was 50% IP6 degradation, that was observed (Figure 3.2.13), which can be inferred from the IP5 peak which is the first product formed as a result of phytase reaction. The concentration of IP6 was gradually decreased and resulted in IP5 content and other lower inositol phosphates (IP4 and IP3).

ePrints@CFTRI

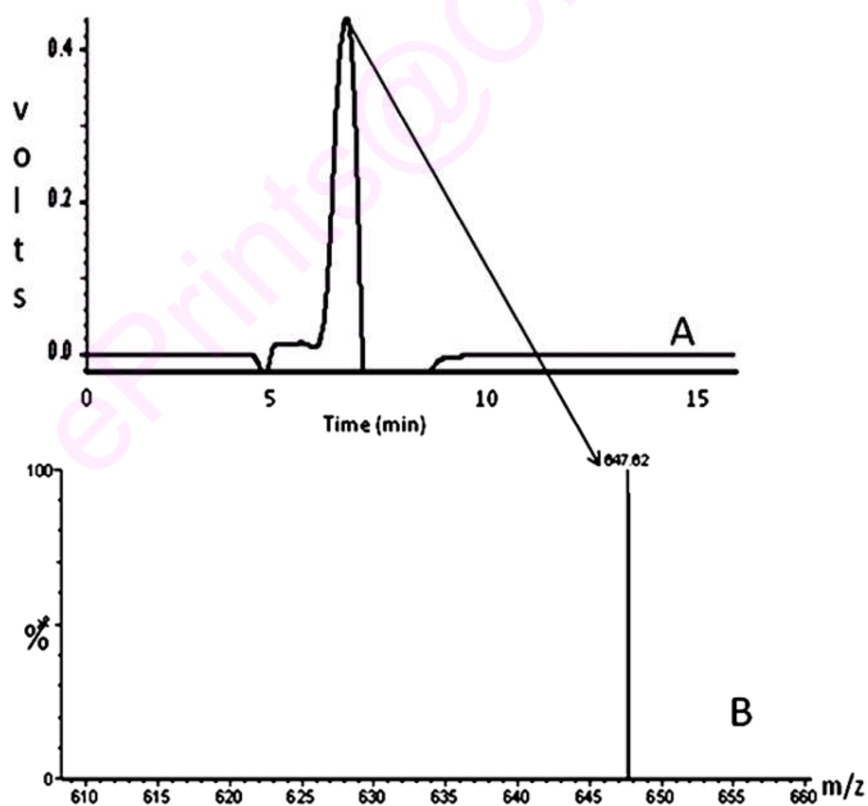


Figure 3.2.13 Phytic acid Analysis during *Ped. pentosaceus* CFR R38 fermentation process. A: HPLC chromatogram; B: Mass Spectra analysis

3.2.6 Phytase in CFR R38

There are several reports on phytase gene identified from the *Bacillus* strains and the gene sequences are available in GenBank. Based on available sequences, primers were designed with an expected amplicon of 600 bp, but no positive amplification was observed. It may be the reason the bacillus and LAB cultures possess different characteristic features. Cell suspension was tested for enzyme activity at different pH conditions at 50°C temperature, pH 5.6 was found to be optimum from a range of 3.6 to 6.0. The strain CFR R38 was able degrade sodium phytate up to 70% during 30 minutes of reaction. Several trials were attempted to isolate or extract enzyme responsible for phytate degradation. Liquid nitrogen, protoplast lysis, protoplast sonication were followed. Among them, protoplast sonication was found to be suitable to extract the enzyme. The crude extract was assayed for the enzyme activity and was found to possess phytate degrading ability. The crude enzyme was ammonium precipitated and the extract was analyzed on SDS PAGE. As determined by SDS-PAGE, the apparent molecular mass of the enzyme was ~45 kDa.

Overall, microbial phytase are considered as monomeric proteins ranging from 40 to 100 kDa (Pandey *et al*., 2001). The bacterial phytases characterized from *Bacillus subtilis, E. coli* and *Klebsiella terrigena* had apparent molecular masses of 36 to 45 kDa (Pandey *et al*., 2001). The only other nonspecific acid phosphatase enzyme purified from a LAB had an apparent molecular mass of 52 kDa (Zamudio *et al*., 2001). The molecular mass determined for the enzyme of *Lb. pentosus* CECT 4023 differs from those reported for other bacteria. Most of the characterized bacterial phosphatases such as those from *Lb. plantarum* DPC 2739, *Lb. curvatus* and enteric bacteria having a

ePrints@CFTRI

molecular mass of 100-110 kDa have been reported. Similarly, the corresponding enzymes of other strains of *Lb. plantarum* and for *Lb. sanfranciscensis* and *Lc. lactis*, the enzymes appeared to be monomers (Zamudio *et al*., 2001; De Angelis *et al*., 2003).

3.2.7 Conclusion

In this study the selected phytate degrading *Ped. pentosaceus* strains were able to degrade sodium phytate up to 70% by expressing their phytase activities in a range of 3-459 U of enzyme activity at 50°C. The tested strains expressed only intracellular enzyme activity. Among the tested media combinations, Media 2 containing sodium phytate as phosphate source along with MOPS was found to be suitable for CFR R38 whereas Media 1 was studied for CFR R123. The strains expressed poor acid phosphatase activity except reference strains B 4552 and ATCC 53510. For *Ped. pentosaceus* CFR R38, the optimum temperature of 50°C, pH 5.5 of acetate buffer containing 0.2 M sodium phytates were found to be optimal for the enzyme activity. Further the enzyme extracted was analyzed for its specificity by its zymogram in presence of sodium phytate and its molecular weight confirmed to be in the range of ~40-50 kDa. The enzyme isolated was more fragile and require proper storage and maintenance. The existence of phytase as an intracellular origin explains the phytate degrading ability of selected LAB. The degraded products of phytates were further confirmed by HPLC and MS. Phytase by *Ped. pentosaceus* is a new finding, not reported so far. The native isolates obtained during this investigation can be exploited for their possible application in phytate degradation in different food systems.

ePrints@CFTRI

Chapter 3

Section -3

Application of phytate degrading lactic acid bacteria

ePrints@CFTRI

3.3.1. Role of phytate degrading LAB in functional food formulations

In order to study the phytic acid degradation of the isolates in the food system, Malted Finger millet Seed Coat (MFSC) and soymilk (SM) were selected and conditions were optimized for the maximum degradation. The main objective of this study was to identify the culture that has maximum growth and ability for maximum degradation of phytin complexes in food system which in turn results in increase in the concentration of bio-accessibility of minerals bound to it.

Two types of malted finger millet seed coat materials (fine and coarse) were used in the study. MFSC is a by-product of malted ragi industry which is recently exploited for an ingredient of ragi biscuits at CFTRI, Mysore. MFSC is a rich source of phytic acid (0.09g/100g) as well as calcium (700 mg/100g). Due to the high content of calcium, MFSC can be used as an ingredient in the biscuit preparation. However, only 10% of calcium is bio-accessible due to the presence of high content of phytic acid and dietary fiber (Ratish et al., 2010) Hence, selected potent phytate degrading strains were applied for their ability to degrade phytic acid during MFSC fermentation.

Initially, fine and coarse MFSC powders were autoclaved, and slurry was prepared by adding 10 ml of water to 1 g of sample followed by inoculation with overnight grown cultures. When analyzed for phytic acid content, after 24 h of incubation at 37°C, there was 22% decrease. The results indicated that autoclaving can degrade phytic acid, which is in accordance with the available literature

ePrints@CFTRI

(Ologhobo and Fetuga 1984). At the same time, cultures were unable to degrade much phytate in coarse material than in fine material. Therefore, only fine MFSC powder was considered for the study.

To overcome the degradation of phytic acid by steam sterilization and contamination, gamma irradiation was employed. MFSC was packed in polyethylene bags and then sterilized by gamma irradiation at 1.5 kGy (20 min 28 sec at 23°C) and 3 kGy (40 min 28 sec at 27°C) when dosage rate was 4.4480 kGy per h was applied to evaluate the effect on the microbial load as well as phytate content was also analyzed. No microbial count was observed in 3kGy conditions but was observed for 1.5 kGy at a CFU of 10^2. Gamma irradiation was resulted in 2.1 and 3.0 % phytic acid degradation at 1.5 and 3.0 kGy respectively. Among the tested conditions, 3kGy was the effective dose to remove contaminants as well as not much effect was found on phytate degradation during the dosing period. The gamma irradiated sample was stored for 6-8 months at 4°C. It was also found that the storage stability of the packed MFSC material depends on its moisture content and hence was stored at <5% moisture content.

Gamma irradiated MFSC powder was fermented with 1% inoculation of overnight grown cultures of *Ped. pentosaceus* CFR R123, CFR R38 and CFR R35 and *Lb. amylovorus* B 4552. The fermentation resulted in 3% phytate degradation compared to the control sample, with a decline of pH indicating that, the cultures were able to grow in this food system. In view of this, to speed up the

ePrints@CFTRI

fermentative process and as the material used is not a rich source of glucose, inoculum size was increased to 5%. For all the experiments, a parallel control was kept without the inoculation of any culture. The results obtained are portrayed in Fig 3.3.1.

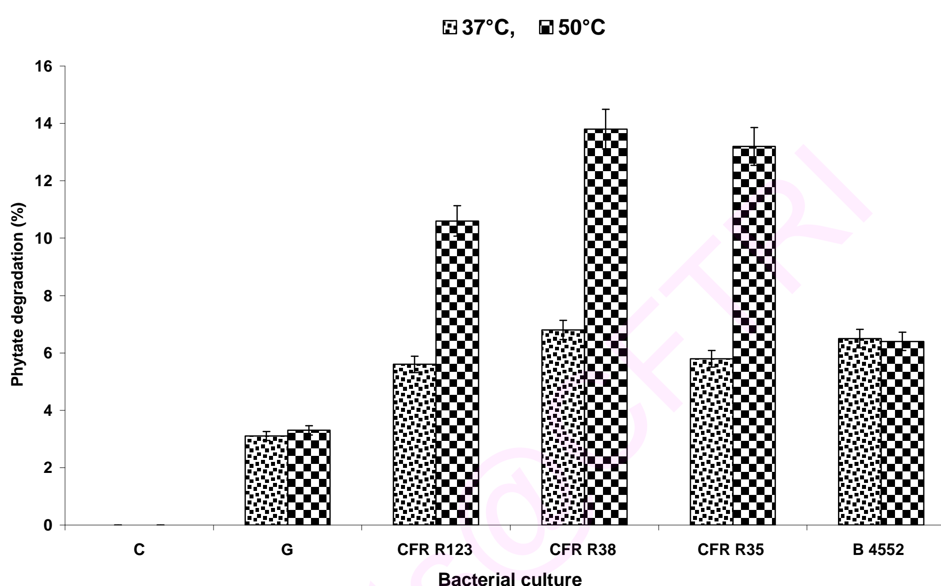


Figure 3.3.1 Phytate degradation during MFSC fermentation by LAB at different temperatures. (C: control unfermented; G: Gamma irradiated unfermented: CFR R123, CFR R38 & CFR R35: *Ped. Pentosaceus*; B 4552: *Lb. amylovorus*)

In this study, all the three potent phytate degrading isolates along with the extracellular phytase producing *Lb. amylovorus* were taken. When MFSC fermentation was carried out with tested cultures at 37°C, phytate degradation was observed in gamma irradiated sample without inoculation (G), when compared to the unfermented control (C). Among the tested strains, 5.6 (2.42 mM/100g), 6.8 (2.39 mM/100g), 5.8 (2.42 mM/100g) and 6.5% (2.39 mM/100g) phytic acid degradation was observed at 37°C for CFR R123, CFR R38, CFR R35 and *Lb.*

ePrints@CFTRI

amylovorus B 4552 respectively, compared to the control value. There was 2-3% decrease, compared to the gamma irradiated sample.

It was known that, various food processing and preparation methods reduce the phytate content. The decrease in phytic acid content was observed during boiling and steaming at temperature around 100°C, but degradation was greatest in processes in which phytase is activated by any means (Ologhobo and Fetuga, 1984; Bullock *et al*., 1993). Lease (1966) and de Boland *et al*., (1975) suggested that, the rate of phytate destruction is low when it is associated with the proteins and/or cations in natural products. Toma and Tabekhia (1979) noted that cooking rice in domestic tap water resulted in no significant loss of phytic acid, whereas, cooking in distilled deionized water reduced the phytic acid content by two-thirds. This difference can be attributed to the ability of phytic acid to form salt complexes.

Fermentation of food changes or creates unique flavours and digestibility. Kingsley (1995) found that, the concentration of phytates was 76% lower in fermented African oil beans than in raw beans. In some traditional Indian fermented foods (fermented and steamed dhokla), almost all phytic acid may be hydrolyzed, although, in most of the foods 50% or less of the phytate remains (Reddy *et al*., 1994). The optimal conditions for the better phytase activity for the isolates was found to be 50°C, hence further fermentation of MFSC was also carried out. The results are given in Figure 3.3.2. There was no change in the

ePrints@CFTRI

phytate content in gamma irradiate MFSC un-inoculated control (2.47 mM/100g) compared to unfermented sample (2.57 mM/100g). However, there was a remarkable decrease in phytate content in the fermented batter over unfermented one. About 13% decline in phytate concentration was observed when MFSC fermentation was carried out with CFR R38 (2.31mM/100g) and with CFR R35 (2.26 mM/100g). The phytate degradation observed with CFR R38 and CFR R35 was ~10% high at 50°C compared to the fermentation carried out at 37°C. However, a 5% decrease in the phytate content in fermented batter was observed at 50°C compared to that at 37°C for CFR R123 (2.48 mM/100g). No change in the phytate content was observed during fermention process of *Lb. amylovorus* B 4552 at both the tested temperatures.

Regarding the importance of LAB phytase for phytate degradation during sourdough fermentation, the scientific data are interpreted supporting the hypothesis that either LAB phytases are significantly involved in phytate degradation during sourdourgh fermentation (Lopez *et al*., 2000; Reale *et al*., 2007; De Angelis *et al*., 2003) or the intrinsic creal phytases are responsible for phytate degradation after activated by a fall in pH due to lactic acid production by the LAB (Marklinder *et al*., 1995; Fredrikson *et al*., 2002; Leenhardt *et al*., 2005). To act on phytate, phytases must have access to the phytates present in the dough. The MFSC was a by product of malting, which may be exposed to heat during

ePrints@CFTRI

drying and milling, hence there will be no possibility of retaining the endogenous phytase.

3.3.2 Analysis of phytate degradation during MFSC fermentation

Further, phytate degradation was also evaluated by analyzing the MFSC fermented extracts through HPLC. Fractions collected from HPLC were injected into MS and their mass was correlated with that of the fractions obtained from standard phytic acid. The HPLC column was loaded with standard PA samples (40%, 1:1 and 1:2 dilutions) and graph was plotted against peak area and concentration of the sample. From the graph, it can be inferred that as the concentration of phytic acid increases, the peak area also increases. Retention time for IP6 was found to be 5.5-5.7 min. The sample was collected from HPLC and analyzed by mass spectra, showed 647 Da for phytic acid, which was similar to that of the standard phytic acid.

ePrints@CFTRI

Table 3.3.1 Analysis of phytate content in MFSC by HPLC

Bacterial culture/sample	**Retention time for IP6 (min)**
C	5.71
G	5.667
Ped. pentosaceus CFR R123	5.6
Ped. pentosaceus CFR R38	5.738
Ped. pentosaceus R35	5.627
Lb. amylovorus B 4552	5.571

C: control unfermented; G: Gamma irradiated unfermented

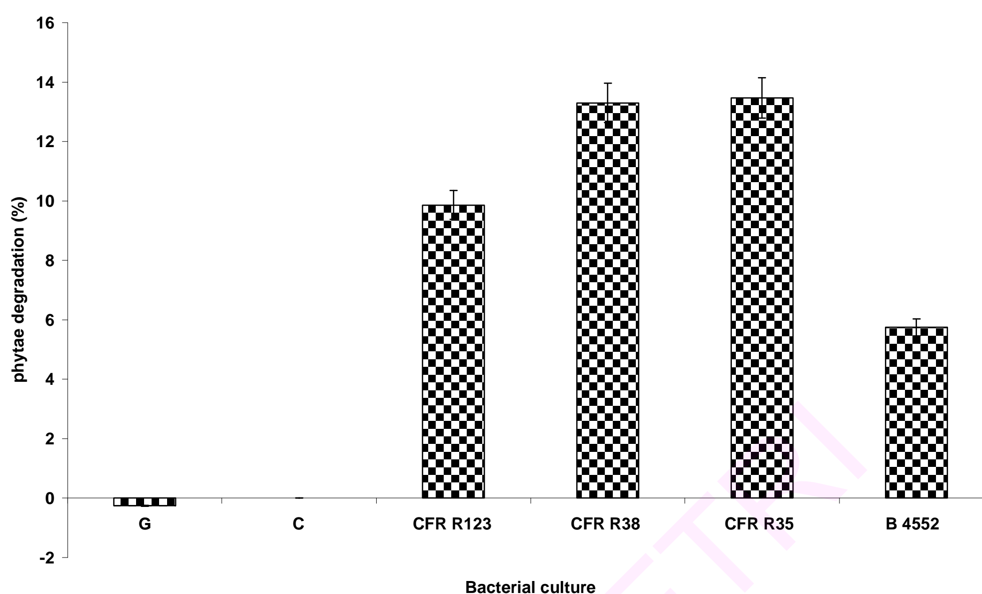


Figure 3.3.2 Phytate degradation analysis by HPLC (C: control unfermented; G: Gamma irradiated unfermented: CFR R123, CFR R38 & CFR R35: *Ped. Pentosaceus*; B 4552: *Lb. amylovorus*)

ePrints@CFTRI

During the growth phase (stationary culture) at different time intervals, LAB count was recorded and was found to be 1 million CFU/gm of tested fermented MFSC sample, where there samples were inoculated with the test strains. pH decreased to 3.6 from 6.5 for CFR R123 and CFR R35, whereas it was 4.0 and 4.2 with CFR R38 and *Lb. amylovorus* respectively after in 24 h of fermentation. It can be concluded that, the cultures tested have the ability to degrade food grade phytin complexes. As MFSC is a fiber rich source, LAB may directly utilize it for their energy source, but when fermentation was carried out in the presence of glucose, no change in the phytate content was observed.

Effect of phytate degrading LAB on mineral solubility was evaluated during MFSC fermentation. The minerals such as magnesium; zinc and calcium were studied during the fermentation processes.

3.3.3 Bio-accessibility of magnesium during MFSC fermentation by LAB cultures

Due to its negative changes phytic acid shares more space with divalent magnesium ions. Hence, during MFSC fermentation process, solubility of magnesium was addressed. The samples inoculated with bacterial cultures when compared to the control (without inoculum) after fermentation, showed considerable increase (~35-40) in the amount of free magnesium (Fig. 3.3.3). There was 7-8 mg/100g of free magnesium increased compared to the unfermented MFSC. This could be due to the action of phytic acid degrading LAB on phytin complex which resulted in the degradation of phytin complex and/or action of produced acid resulted in release of free magnesium into fermented batter.

ePrints@CFTRI

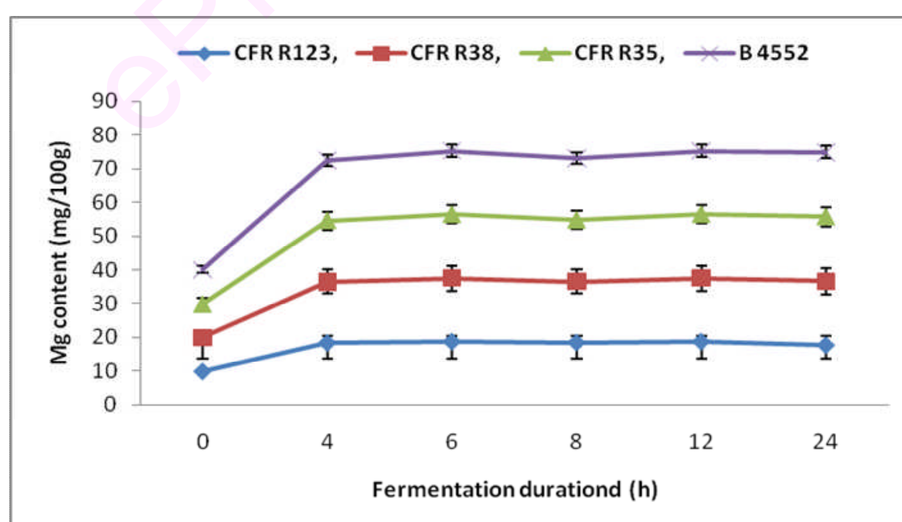


Figure 3.3.3 Magnesium content during MFSC fermentation (CFR R123, CFR R38 & CFR R35: *Ped. Pentosaceus*; B 4552: *Lb. amylovorus*)

In the samples inoculated with the LAB cultures CFR R38, CFR R35, and *Lb. amylovorus* B 4552, the availability of free magnesium was high in 24 hr incubation. With R123 bacterial culture, it was observed that, as the incubation time increases (4 h to 6 h), there was increase in the availability of free magnesium. However, during the 8th h of fermentation there was decrease in magnesium bioavailability. The reason could be that, the organism itself would have utilized the magnesium for its metabolic activities. It was also observed that, the availability of free magnesium was less when carbon source such as glucose was added to the medium. This is may be due to the fact that, the organism first depends on the carbon source that was supplied to it for its growth. Only after the complete utilization of glucose by the organism, it has to depend on MFSC for energy and hence it would have degraded phytic acid, which indirectly leads to the release of magnesium into the medium. The condition in which glucose was added, where, organism depends on the source of material for its energy, which leads to the increase in the bio-accessible magnesium.

3.3.4 Bio-accessible calcium during MFSC fermentation by LAB cultures

Phytic acid forms complexes with calcium and made them unavailable. In this regard during MFSC fermentation calcium solubility was assessed. The samples inoculated with bacterial cultures compared to the control (without inoculum) after fermentation, showed considerable increase in the amount of free calcium in the fermented MFSC batter. This may be due to the acid produced by

ePrints@CFTRI

the bacteria or during the fermentation process by the action of the enzyme phytase i.e., due to the degradation of phytin complex.

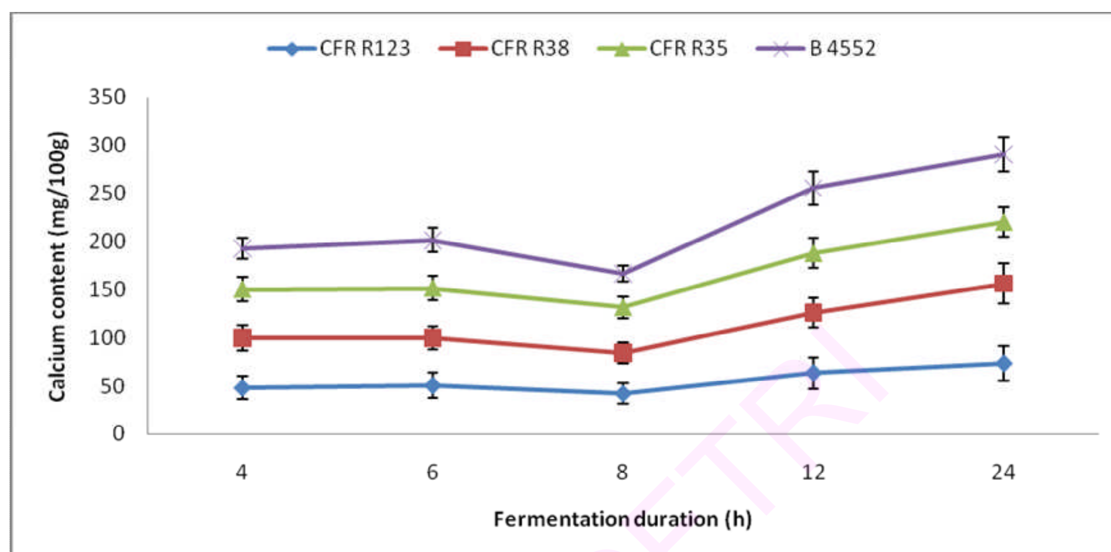


Figure 3.3.4 Bio-accessible calcium content during MFSC fermentation (CFR R123, CFR R38 & CFR R35: *Ped. Pentosaceus*; B 4552: *Lb. amylovorus*)

ePrints@CFTRI

For all the tested cultures, it was observed that, as the incubation time increased from 4 to 6 h, there was an increase in free calcium levels. There was a slight decrease in calcium at 8 h of incubation which increased further on incubation up to 24 h. At early hours of incubation, the increase in bio-accessible calcium may be due to the acid produced by the LAB cultures. It can be inferred from Fig. 3.3.4, that, there was a considerable increase in the bio-accessible calcium when MFSC was fermented with CFR R123, CFR R38, CFR R35 and B 4552 cultures. Increase in calcium content was found to be high with the culture CFR R38 (83 mg/100g), as it has potency towards phytase production which was confirmed by the plate assay, followed by CFR R123, B 4552 and CFR R35.

Further, bio-accessible calcium content both in the presence and absence of glucose during MFSC fermentation was observed. The same trend as magnesium expressed was repeated for CFR R123. When compared to control (without inoculation), the bioavailability of calcium is considerably increased when inoculated with LAB cultures. It was observed that the bio-accessible calcium is less when carbon source such as glucose was added to the MFSC for fermentation. This could be the reason that the organism first depends on the carbon source that is supplied to it for its growth. Only after the glucose completely utilized by the organism, it may switch to MFSC for their energy source and hence involves in phytate degradation, which may indirectly leads to the release of free calcium into the fermented batter as explained to that of Mg^{2+}. The level of bio-accessible calcium in MFSC compared to the unfermented control sample is presented in Figure 3.3.5.

ePrints@CFTRI

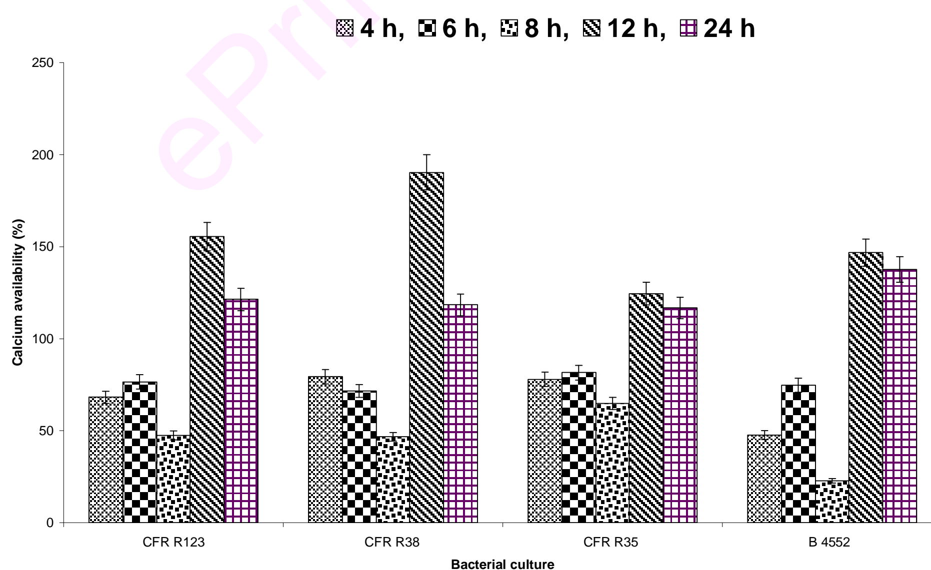


Figure 3.3.5 Level of free calcium during MFSC fermentation. (CFR R123, CFR R38 & CFR R35: *Ped. Pentosaceus*; B 4552: *Lb. amylovorus*)

3.3.5 Bio-accessible zinc during MFSC fermentation

Zinc is an essential trace element required by the humans. Due its efficiency towards binding with negatively charged PA make them unavailable. In this view of this it solubility during MFSC fermentation by LAB was also investigated. The gamma irradiated MFSC slurry was inoculated with CFR R123, CFR R38, CFR R35 and *Lb. amylovorus.* Compared to the control (without inoculum) after fermentation, these samples showed substantial increase in the amount of free zinc in the fermented batter. The results pertaining to zinc content during MFSC fermentation are described in Figure 3.3.6. For R123, R38 bacterial cultures, it was observed that, as the incubation period increased from 4 to 24 h, there was an increase in the bio-accessible zinc ions, but the increase was substantial till 6 h of incubation period and then it gradually decreased till 12 h. There after this there was a slight increase in free zinc ions. Similar trend was observed for CFR R38, but there was a decrease in zinc levels till 24 h beyond 6 h of incubation period. For CFR R35 and B 4552 it was observed that, as the incubation time increased from 4 to 8 h, there was a slight increase in the bio-accessible zinc ions followed by gradient decrease. Malted finger millet seed coat was inoculated with different cultures and incubated for 24 h both in the presence and absence of glucose and the bio-accessible zinc ions was assessed. It was observed that, the availability of free zinc was less when carbon source such as glucose was added to the fermenting material.

ePrints@CFTRI

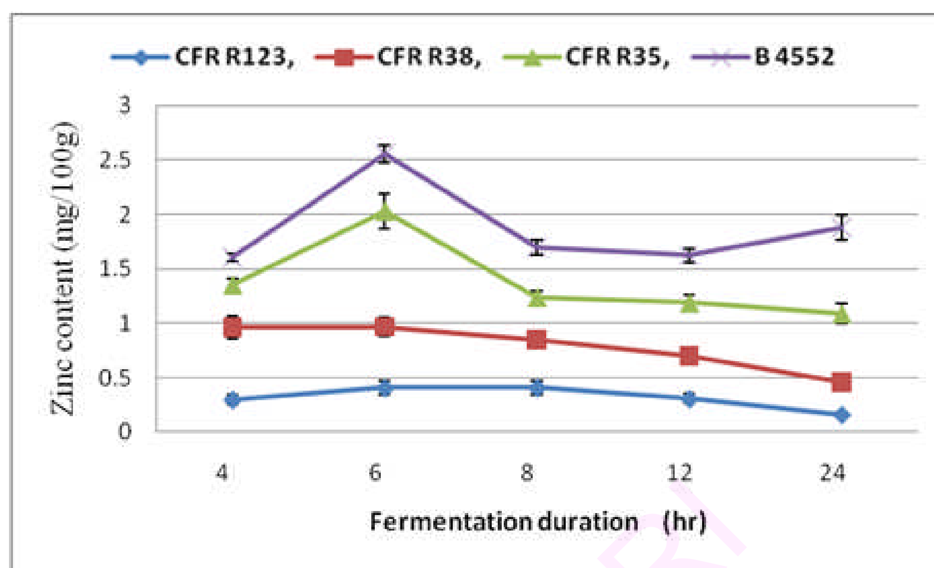


Figure 3.3.6 Bio-accessible zinc content during MFSC fermentation. (CFR R123, CFR R38 & CFR R35: *Ped. Pentosaceus*; B 4552: *Lb. amylovorus*)

Though, there were mixed type of results obtained for bio-accessible minerals, it can be still concluded that, a good percentage of minerals are bio-accessible due to sourdough fermentation by the selected potent phytate degrading LAB.

3.3.6 Soya milk fermentation by phytate degrading LAB

Apart from MFSC, Soya milk was also been considered as one of the food based phytate source. The amount of phytic acid present in soya milk is about 0.04 -0.09 mg/100g. Soya milk is a protein rich food and is a versatile product from soya beans.

Soya milk fermentation by LAB was carried out at 37°C and the percentage of phytate degradation is potrayed in Figure 3.3.7. A decline in the

eprints@CFTRI

phytate concentration was observed in soya milk due to the effect of the tested cultures. Among the tested strains, highest reduction in the phytate concentration at about 16.4% (0.327 mM/100g) was observed with CFR R38 compared to control (unfermented soya milk) which was 0.391 mM/100g. CFR R35, *Lb. amylovorus* and CFR R123 degraded 15.3 (0.331 mM/100g), 12.14 (0.334 mM/100g) and 12.11% (0.344 mM/100g) of phytates during 24 h soya milk fermentation at 37°C, respectively. The phytate content retained after fermentation are given in Table 3.3.3. The optimal condition for the better phytase activity of the isolates was found to be 50°C; hence soya milk fermentation was carried out at this particular temperature and the extent of phytate degradation was also evaluated. *Ped. pentosaceus* CFR R38 cultivation in soymilk resulted in a significant reduction of phytate content (Figure 3.3.7). No change in the phytate content of unfermented soy milk was observed at 50°C. However, compared to 37°C, the decrease was 5%. The phytate content in the soymilk fermented by CFR R38 was reduced to 46% (0.211 mM/100g) as observed from the initial phytic acid content in the unfermented sample. The strains, CFR R123, CFR R35 and *Lb. amylovorus* B 4552 reduced phytate content by 38 (0.243), 37(0.246) and 28%(0.282) during fermentation process.

The endogenous soybean phytase was inactivated by autoclaving at 121°C for 15 min such as inactivation of intrinsic cereal phytase (Reale *et al*., 2007), and hence the phytate content decreased as a consequence of the activity of the CFR

ePrints@CFTRI

R38 phytase during fermentation. In this study, a 50% breakdown in initial phytate content that of the recent reports of the phytate degradation ~40-60% observed in soybean-curd whey as result of *Saccharomyces cerevisiae* CY phytase (In *et al*., 2008) and there was 46% of phytate degradation by *Leu. mesenteroides* KC51 in a similar fashion (Oh and In, 2009).The phytate reduction was not complete in the cases and the possible reasons may be that, the optimal pH for the activation of phytase (or microorganism) lasted only for a short period. It was also reported (Oh and In, 2008) that, the pH of fermented soymilk decreased below 4.5 after 12 h of fermentation with *Leu. mesenteroides* KC51 strain (Oh and In, 2009), which plays the major in elevating optimal condition for the LAB phytase.

ePrints@CFTRI

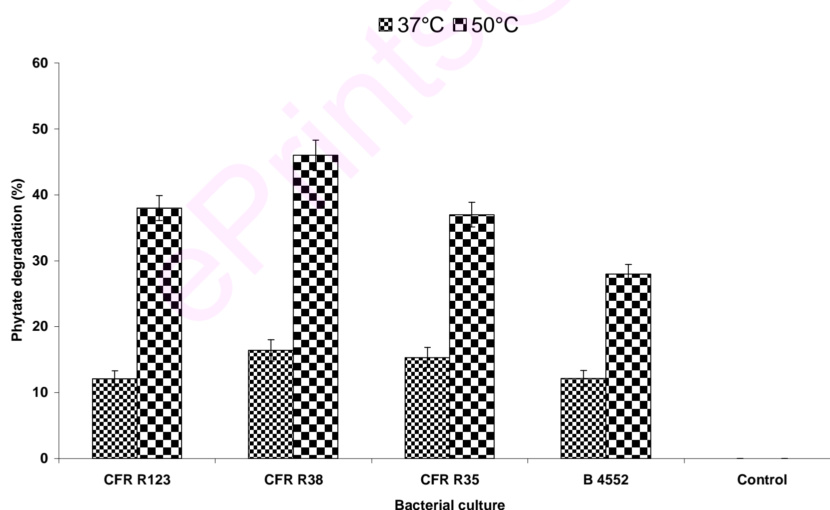


Figure 3.3.7 Phytate degradation during soya milk fermentation by LAB cultures (Control: unfermented soy milk; CFR R123, CFR R38 & CFR R35: *Ped. Pentosaceus*; B 4552: *Lb. amylovorus*)

In order to assess the role of phytate degrading LAB, soya milk fermentation by CFR R123, CFR R38, CFR R35 and *L. amylovorus* were studied.

All the tested cultures were able to degrade phytic acid. Among the tested strains, CFR R38 was found to be appropriate to carry out further studies because of its potency to degrade phytate during soymilk fermentation.

3.3.7 Soya Curd preparation

The strain CFR R38 was able to ferment soya milk when 5.5% inoculum was used. The pH profile was observed at two different temperatures 37 and 50ºC. The culture had the ability to get soya curd set (Figure 3.3.8) in 12 h, and decrease in pH was observed with the same period. However, there was no much change in the pH pattern at 24 h fermentation. Soymilk fermented at 50ºC was found to be suitable for decrease in phytate levels (figure 3.3.9) in short span of time.

cftri-theses@CFTRI

Figure 3.3.8 Soya curd by *Ped. Pentosaceus* CFR R38

Table 3.3.2 Texture and structural properties of fermented soymilk at different time intervals by *Ped. Pentosaceus* CFR R38

Property	Control									*Ped. pentosaceus CFR R38*							
Fermentation period (h)	0	2	4	6	8	10	12	16	24	2	4	6	8	10	12	16	24
Flavour	-	-	-	-	-	-	-	-	-	+	+	++	++	++	+++	++	++
Colour	W	W	W	W	W	W	W	W	W	CW	CW	CW	CW	CW	CW	CW	CW
Viscosity	-	-	-	-	-	-	-	-	-	+	+	++	++	++	+	+	+
Whey production	-	-	-	-	-	-	-	-	-	-	-	+	+	++	+++	+++	+++
Hardness	-	-	-	-	-	-	-	-	-	-	+	+	++	++	+++	+++	+++

-: Negative; +: fair; ++: Good; +++: Excellent, W-White; CW – Creamy White

ePrints@CFTRI

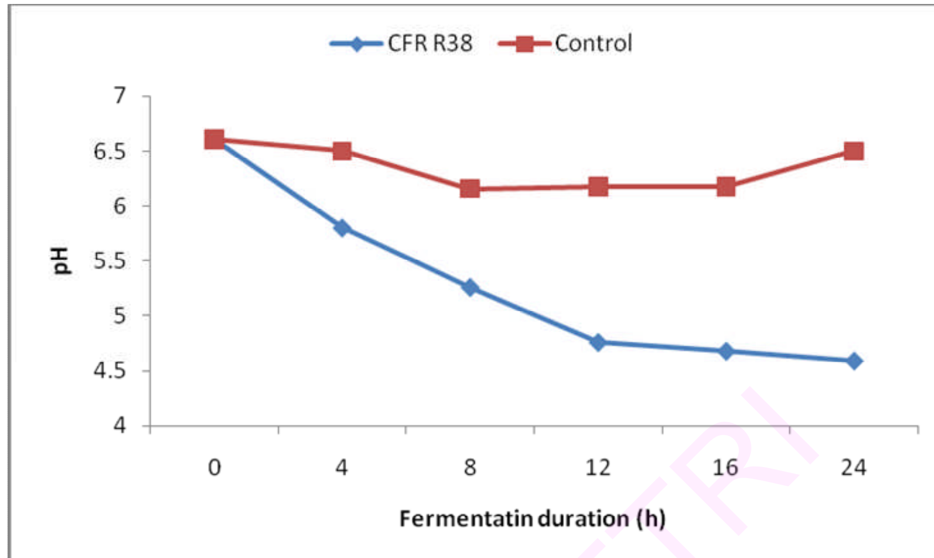


Figure 3.3.9 pH profile during soymilk fermentation by *Ped. pentosaceus* CFR R38 at 50°C.

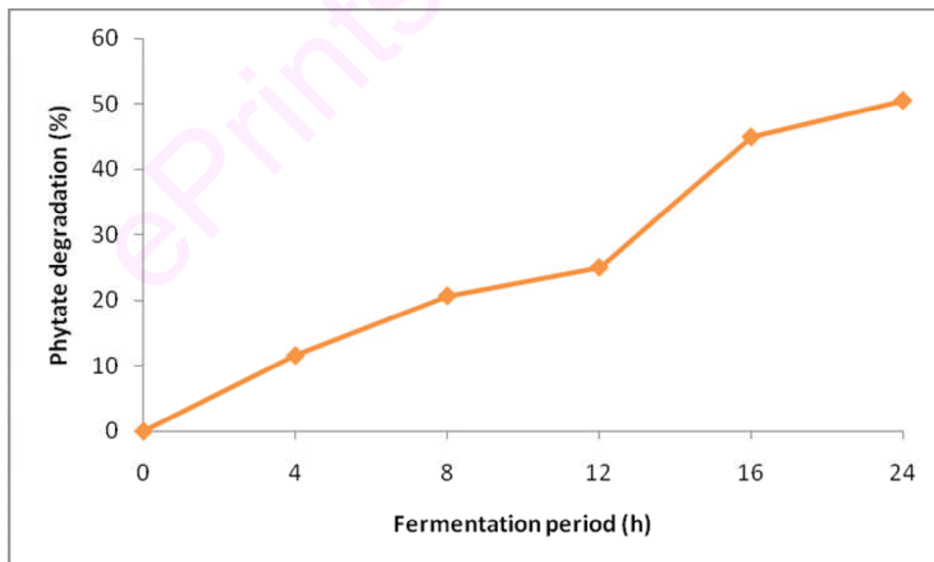


Figure 3.3.10 Phytate degradation during soya curd preparation

ePrints@CFTRI

Table 3.3.2 gives the mean sensory scores of soy curd samples. The attributes used for soy curd were colour – buff, Consistency – set, mouth coating, aroma – beany, pulsey, fermented, sourish, taste – sour, aftertaste – beany and overall quality. Difference in sensory quality was seen in some of the attributes used for the three samples. The change in sensory attributes did not influence the overall quality. Overall, the quality score of 6.7 indicates that R38 fermentation needed some improvement in the taste and colour, hence it was fairly acceptable. There was an increase in the soluble calcium observed. The product was observed to be a high energy provider and possessed 55% antioxidant and other improved soluble minerals and nutritive values.

The demand for alternatives to dairy products is growing due to problems with intolerance and allergy, desire for vegetarian alternatives, and so on, and hence the interest in soy-based foods is in demand. Probiotic yogurts are now being marketed, and consequently it would be desirable to know if probiotic bacteria can also be incorporated into soy-based yogurt-type fermentations (Farnworth *et al*., 2007).

Probiotic products developed with soy extrat mixed with fruit juices are the new generation of foods on the market, which is a convenient way to include soy protein in the basic diet (Champagne and Gardner, 2008). From 1992 to 2008, soy foods sales world wide have increased from US$ 300 million to most US$ 4 billion. This increase can be attributed to new soy food categories being

ePrints@CFTRI

introduced, repositioned in the marketplace, new customers selecting soy for health, and philosophical reasons (Granato *et al*., 2010)

Soy and its derivatives have received attention from researchers world wide, mainly due to the amount and quality of its protein. Soy protein presents a good amino acid profile; however, cysteine, cystin and methionine are limiting. Moreover, soy is a source of soluble fiber, magnesium, phosphorous, vitamins K, riboflavin, thiamine and folic acid. Soy contains several oligosaccharides-raffinose and stachyoe that are not digested by humans and therefore can cause flatulence. However, these α-galactosides are sources of carbon for the growth of various *Lactobacillus* species, such as *Lb. acidophilus* and *Lb. delbruecki* subsp. *Bulgaricus* as well as *Bifidobacterium* species. (Granato *et al*., 2010). Therefore, soy products can be a good culture medium for inoculation and growth of probiotic strains. There is every reason to believe that soy beverages and yogurts will be the next food category for which the healthy bacteria will make their mark (Granato *et al*., 2010). Hence in this view supplementation of phytate degrading LAB with probiotic properties in such plan-based food products would results in improved nutritional factors thus exerting the health benefits to the consumers.

ePrints@CFTRI

Table 3.3.3 Sensory properties of soya curd by CFR R38

Attributes	R38
Buff	5.4
Consistency	8.5
Mouthcoating	5.4
Beany	9.4
Pulsey	8.1
Fermented	7.6
Sourish	7.1
Sour	7.3
After taste	6.0
Overall Quality	6.7

ePrints@CFTRI

3.3.8 Conclusion

In this investigation the potent phytate degrading *Ped. pentosaceus* CFR R123, CFR R38 and CFR R35 were able to degrade phytates in MFSC by 5-12 % in 24 h. The fermentation of MFSC with tested strains resulted in increase of bio-accessible calcium up to 125% when compared to the control. The three strains also exhibited their ability to ferment soy milk. There was 12% decrease in the phytate levels observed with CFR R123 which in turn resulted in 68% bio-accessible calcium availability. *Ped. pentosaceus* CFR R38 fermented soya milk

showed 50% decrease in phytate levels with improved bio-accessible calcium compared to the control.

The culture CFR R38 was potential in soya milk fermentation that resulted in good set of curd. The resulted product was revealed low level of phytates and appreciable increase in bio-accessible mineral content. Over all, the product attained 6.7 score and found to be high energy provider. The soycurd by CFR R38 possessed 55% antioxidant property along with improved nutritive values.

ePrints@CFTRI

Chapter 4

Summary and Conclusions

ePrints@CFTRI

Phytic acid present in the whole grain products are suspected of impairing mineral absorption of Zn, Fe or Ca. Recent epidemiological findings support the protective role of whole grain foods against several diseases. Hence, effective reduction of phytic acid content can increase the bioavailability of the minerals and can be cheived through exogenous phytic acid degrading enzymes (phytase) of microbes.

In search of phytate degrading LAB divergent sources like fermented food processes, vegetables, chicken and fish intestines and LAB cultures from culture collection centers were screened. All the test strains displayed calcium phytate degrading ability and sodium phytate in presence of calcium chloride. Among the tested cultures could degrade sodium phytate with out any calcium source. All the selected cultures showed ability to degrade 0.2% calcium phytate by producing phytase, whereas twelve cultures from chicken intestine and one culture each from raw milk and one from fermented rice showed the ability to degrade 0.2% sodium phytate. All the tested cultures showed the ability to degrade 0.2% sodium phytate in presence of 0.2% calcium chloride. Among the screened isolates, 21 isolates were selected as sodium and calcium phytate degrading LAB.

Based on RFLP profile the 21 selected isolates clustered sorted into three groups and one representative culture from each group was selected. There were CFR R35, CFR R38 and CFR R123. The three isolates were identified by physiological, biochemical and molecular tools as *Ped pentosaceus.* The

respective 16S rRNA gene sequences were deposited in NCBI-GenBank under accession numbers FJ889048, FJ586350, FJ889049 for CFR R35, CFR R38 and CFR R123 respectively. For these three *Ped. pentosaceus* cultures probiotic attributes were also evaluated considering *Lb. rhamnosus* GG as a positive control.

The selected 3 LAB isolates along with positive control strain has survival of 55-45% when grown at pH 2 for 3 h. Among the tested strain, *Ped. pentosaceus* R38 and R123 were able to resistant to 0.3% bile, whereas strain *Ped. pentosaceus* R35 was 0.3% bile tolerant. *Lb. rhamnosus* GG was sensitive to be 0.3% bile sensitive. Selected native and control strains were displayed antagonistic activity against *L. monocytogenes* Scott A, *E.coli*, *B. cereus* and *S. paratyphi.* Antibiotic sensitivity pattern of the strains against tested antibiotics were within the break point concentrations.

The selected cultures were able to degrade phytic acid up to 70%, which resulted in 3-459 U of enzyme activity. The enzyme activity was expressed in Units/min/9 log CFU. Culture *Ped. pentosaceus* CFR R123 exhibited highest enzyme activity whereas *Ped. pentosaceus* CFR R38 and *Ped. pentosaceus* CFR R35 showed 215 and 89 U respectively. The selected cultures along with control culture *Lb. amylovorus* were grown in presence of different media conditions. Media 1 containing MRS composition was found to suitable for CFR R123, where as media 2 containing decreased nutrient content and sodium phytate as

ePrints@CFTRI

phosphorous source along with buffering agent found to suitable for CFR R38. It was found that the phytate degrading ability was due to intracellular fraction. The temperature 50°C, pH of 5.5 with acetate buffer containing 0.2 M sodium phytates were found to be optimal for the enzyme activity of the culture CFR R38. Further the enzyme extracted was analyzed for its specificity by its zymogram in presence of sodium phytate and its molecular weight confirmed to be in the range of 40-50 kDa. The enzyme isolated was more fragile and needed proper storage and maintenance. The existence of phytase as an intracellular origin explains the phytate degrading ability of selected LAB. The degraded products of phytic acid were eluted through ion exchange chromatography and subjected to HPLC and MS to confirm their molecular masses.

Selected potent phytate degrading LAB were observed for their phytic acid degrading ability during different fermented food processes. In this study malted finger millet seed coat (MFSC), millet industrial by-product was used. It is rich in calcium with high phytic acid content from which only 10% of calcium is bioavailable. The potent phytate degrading LAB *viz*., *Ped. pentosaceus* CFR R123, *Ped. pentosaceus* CFR R38 and *Ped. pentosaceus* CFR R35 were assessed for their phytic acid degrading ability during MFSC fermentation. There was 5-12% phytate degradation observed which in turn resulted up to 125% increase in bio-available calcium levels when compared to the control. This elucidates the LAB role in MFSC fermentation. Apart from MFSC fermentation, the cultures

ePrints@CFTRI

were also tested for soya milk fermentation to study their role as phytate degrading LAB. Cultures *Ped. pentosaceus* CFR R123, *Ped. pentosaceus* CFR R38 and *Ped. pentosaceus* CFR R35 were able to ferment soya milk and the finished product was found to be in acceptable manner when it was done with CFR R38. There was 12% phytate degradation observed with CFR R123 resulted in 68% calcium availability, where as during *Ped. pentosaceus* CFR R38 fermented soya milk resulted in 50% decrease in phytate levels when compared to control resulted in increased bio-available calcium levels.

The phytate degrading isolates were further evaluated in soya milk fermentation. All the cultures were able to reduce phytic acid content in soya milk during fermentation. This resulted in increased mineral solubility of calcium and zinc. The culture CFR R38 was potential in soya milk fermentation that resulted in good set curd. The product was revealed low level of phytates and appreciable increase in bio-accessible mineral content. Over all, the product attained 6.7 score and the product was a high energy provider and possessed 55% antioxidant property along with improved nutritive values.

The outcome of this study explains that the LAB exhibits phytate degrading ability also explained that the activity was due to its intracellular phytase enzyme. It also explains that the LAB, which could be an integral part of processed food, resulted in decreased levels of phytic acid for the improved nutritional factors. The results

ePrints@CFTRI

obtained on bio-accessible minerals during fermentative processes by LAB are independent of phytic acid degradation.

Future perspective

The inclusion of exogenous in food medium and reduction of phytate levels in plant based foods has been observed as a promising agent. In the present investigation, several phytate degrading LAB isolates with their probiotic attributes have been optimized for their phytate degradation in food system. These pilot plant studies can be further enhanced to food processing industries involving these phytate degrading LAB. The MFSC and soymilk fermentation with probiotic LAB can have a promising influence in promoting health effects through food systems where plant based products are used as raw material. Although phytate degrading LAB in food applications seems to be gifted approach, characterization of phytases at their biochemical level in potent probiotic strains is an immense important. Hence a vigorous screening in isolating novel and best phytate degrading probiotic LAB and their catalytic features would generate an idyllic phytase for functional food applications.

ePrints@CFTRI

Bibliography

ePrints@CFTRI

1. AACC. (2000) Approved methods of the American Association of Cereal Chemists. Moisture (Method 35-75), Protein (Method 08-76), Fat and Ash (Method 13-61), Dietary fiber (Method 16-91), (10th ed.), AACC, St Paul.
2. Abdallah, A. A., Tinay, A. H. and Mohammed, B. E. (1998) Effect of traditional processes on phytate and mineral content of pearl millet. *Food Chemistry*, **63**:79-84.
3. Achi, O. K. (2005) The potential for upgrading traditional fermented foods through biotechnology. *African Journal of Biotechnology*, **4**:375-380.
4. Adlerberth, I., Jalil, F., Carlsson, B., Mellander, L., Hanson, L. A., Larsson, P., Khalil, K. and Wold, A. E. (1998) High turnover rate of *Escherichia coli* strains in the intestinal flora of infants in Pakistan. *Epidemiology and Infection,* **121**:587-598.
5. Adnan, A. F. M. and Tan, I. K. P. (2007) Isolation of lactic acid bacteria from Malaysian foods and assessment of the isolates for industrial potential. *Bioresource Technology*, **98**:1380-1385.
6. Afinah, S., Yazid, A. M., Anis Shobirin, M. H. and Shuhaimi, M. (2010) Phytase: application in food industry. *International Food Research Journal,* **17**:13-21.
7. Ali, M., Shuja, M. N., Zahoor, M. and Qadri, I. (2010) Phytic acid: How far have we come? *African Journal of Biotechnology*, **9**:1551-1554.
8. Alm, L. (1982) Effect of fermentation on B-vitamin content of milk in Sweden. *Journal of Dairy Science,* **65**: 353-359.
9. Altschul, S. F., Madden, T. L., Schäffer, A. A., Zhang, J., Zhang, Z., Miller, W. and Lipman, D. J. (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Research,* **25**:3389-3402.
10. Anastasio, M., Pepe, O., Cirillo, T., Palomba, S., Blaiotta, G. and Villani, F. (2009) Selection and use of phytate-degrading LAB to improve cereal-based products by mineral solubilization during dough fermentation. *Journal of Food Science*, **75**:M28-M35.

eprints@CFTRI

11. Anderson, R. J. (1914) A contribution to the chemistry of phytin. I. composition of barium phytateand phytic acid. II. A study of the properties of phytic acid and its decomposition products.Eighth paper on phytin. *Journal of Biological Chemistry,* **17**:171-190.
12. Angel, R., Tamim, N. M., Applegate, T. J., Dhandu, A. S. and Ellestad. L. E. (2002) Phytic acid chemistry: influence on phytin-phosphorus availability and phytase efficacy. *The Journal of Applied and Poultry Research*. **11**:471-480.
13. Annan, N. T., Poll, L., Sefa-Dedeh, S., Plahar, W.A. and Akobsen, M. (2003) Volatile compounds produced by *Lactobacillus fermentum, Saccharomyces cerevisiae* and *Candida krusei* in single starter culture fermentations of Ghanaian maize dough. *Journal of Applied Microbiology*, **94**:462-474.
14. AOAC (2000), Official methods of analysis. association of official analytical chemists. calcium (method 965.09), (17th ed.), AOAC, Gaitherburg.
15. Aswathy, R. G., Ismail, B., John, R. P. and Nampoothiri, K. M. (2008) Evaluation of the probiotic characteristics of newly isolated lactic acid bacteria. *Applied Biochemistry and Biotechnology*, **151**:244-255.
16. Au, P. M. and Fields, M. L. (1981) Nutritive quality of fermented sorghum. *Journal of Food science*, **46**:652-654.
17. Axelsson, L. (1998) Lactic acid bacteria: classification and physiology, pp. 1-72. In, S. Salminen and A. Von Wright (eds). Lactic acid bacteria: microbiology and functional aspects, 2nd edition. Marcel Dekker, Inc, New York.
18. Back, W., and Stackebrand, E. (1978) DNS/DNS homologies studied in her lb der Gattung *Pediococcus*. *Archives of Microbiology*, **118**:79-85.
19. Badarinath, V. and Halami, P.M. (2011) Purification of new β-galactosidase from *Enterococcus faecium* MTCC 5153 with transgalactosylation activity. Food Biotechnology (in press)

20. Bae, H. D., Yanke, L. J., Cheng, K. J., Selinger, L. B. (1999) A novel staining method for detecting phytase activity. *Journal of Microbiological Methods*, **39**:17-22.

21. Baghel, R. P. S., Netke, S. P. and Bajpai, L. D. (1985) Nutritive value of kangni. *Poultry Guide*, **22**:28-29.

22. Baird-Parker, A. C. (1980) Organic acid, in microbial ecology of foods. Siliker, J. H., Elliott, R. P., Baird-Parker, A. C., Bryan, F. L., Christian, J. H. B., Clark, D. S., Olson, J. C. and Roberts, T. R. (Eds.), Academic Press, New York. pp126-135.

23. Beachy, E. H. (1981) Bacterial adherence. adhesin-receptor interactions mediating the attachment of bacteria to mucosal surfaces. *Journal of Infectious Diseases,* **143**:325-345.

24. Beaumount, M. (2002) Flavouring composition prepared by fermentation with bacillus species. *International Journal of Food Microbiology*, **75**:189-196.

25. Bergey's mannual of systemiic bacteriology. Vol. 2. Peter, H. A., Sneath, Mair, N. S., Sharpe, M. E. and Holt, J. G. (Eds.). *Williams & Wilkins publishers*, Waverly Press, pp1208-1234.

26. Berrada, N., Lemelan, L. M., Laroche, G., Thouvenot, P. and Piaia, M. (1991) *Bifidobacterium* from fermented milks: survival during gastric transit. *Journal of Dairy Science*, **74**:409-413.

27. Bitar, K. and Reinhold, J. G. (1972) Phytase and alkaline phosphatase activities in intestinal mucosae of rat, chicken, calf, and man. *Biochimica et Biophysica Acta,* **268**:442-452.

28. Bohn, T., Davidsson, L., Walczyk, T. and Hurrell, R.F. (2004) Phytic acid added to white-wheat bread inhibits fractional apparent magnesium absorption in humans. *American Journal of Clinical Nutrition,* **79**:418-423.

29. Bosley, G. S., Wallace, P. L., Moss, C. W., Steigerwalt, A. G., Brenner,D. J., Swenson,J. M., Hebert, G. A. and Facklam, R. R. (1990) Phenotypic characterization, cellular fatty acid composition, and DNA relatedness of

ePrints@CFTRI

aerococci and comparison to related genera. *Journal of Clinical Microbiology*, **28**:416-421.

30. Brooks, S. P. J., Oberleas, D., Dawson, B. A., Belonje, B. and Lampi, B. J. (2001) Proposed phytic acid standard including a method for its analysis. *Journal of AOAC International*, **84**:1125-1129.

31. Bruce, H.M. and Callow, R. K. (1934) Interaction of vitamin D and dietary factors in the healing of rickets in rats. *Biochemical Journal,* **28**:512-516.

32. Bullock, J. I., Duffin, P. A. and Nolan, K. B. (1993) *in vitro* hydrolysis of phytate at 95°C and the influence of metal ion on the rate. **63**:261-263.

33. Canzi, E., Guglielmetti, S., Mora, D., Tamagnini, I., Parini, C. (2005) Conditions affecting cell surface properties of human intestinal Bifidobacteria. *Antonie van Leeuwenhoek*, **88**:207-219.

34. Cebeci, A. and Guakan, C., (2003). Properties of potential probiotic *Lactobacillus plantarum* strains. *Food Microbiology,* **20**:511-518.

35. Champagne, C. P. and Gardner, N. J. (2008) Effect of storage in a fruit drink on subsequent survival of probiotic lactobacilli to gastro-intestinal stresses. *Food research International,* **41**: 539-543.

36. Chang, C. W. (1967) Study of phytase and fluoride effects in germinating corn seeds. *Cereal Chemistry,* **44**:129–142.

37. Charteris, W. P., Kelly, P. M., Morelli, L. ad Collins, J. K. (1998) Development and application of an *in vitro* methodology to determine the transit tolerance of potentially probiotic Lactobacilli and *Bifidobacterium* species in the upper human gastrointestinal tract. *Journal of Applied Microbiology*, **84**:759-768.

38. Chauviere, G., Coconnier, M. H. and Kerneis, S. (1992) Adhesion of human *Lactobacillus acidophilus* strain LB to human enterocyte-like Caco-2 cells. *Journal of General Microbiology*, **138**:1689-1696.

39. Chen, H. and Hoover, D. G. (2003) Bacteriocins and their food applications. *Comprehensive Reviews in Food Science and Food Safety*, **2**:82-99.

ePrints@CFTRI

40. Chen, Y. M., Betzenhauser, M. J., Snyder, J. A. and Burne, R. A. (2002) Pathways for lactose/galactose catabolism by *Streptococcus salivarius*. *FEMS Microbiology Letters*, **209**:75-79.

41. Cheng, S. Y, Shaw, N. S, Tsai, K. S., Chen, C. Y. (2004) The hypoglycemic effects of soy isoflavones on postmenopausal women. *Journal of Women's Health*, **13**:1080-1086.

43. Cheryan, M. (1980) Phytic acid interactions in food systems. *Critical Reviews in Food Science and Nutrition*, **13**:297-335.

44. Christensen, S., Harbak, H. (1990) Serial separation of inositol phosphates including pentakis and hexakisphosphates on small anion-exchange column. *Journal of Chromatography*, **533**:201–206.

45. Chu, H. M., Guo, R.T., Lin, T.W., Chou, C.C., Shr, H.L., Lai, T.Y., Tang, K. J., Cheng, B. L., Selinger, A. H. J. and Wang. (2004) Structures of *Selenomonas ruminantium* phytase in complex with persulfated phytate: DSP phytase fold and mechanism for sequential substrate hydrolysis. *Structure,* **12**:2015–2024.

46. Coleman, J. E. (1992) Structure and mechanism of alkaline phosphatase. *Annual* Review of *Biophysics and Biomolecular Structure,* **21**:441-483.

47. Colman, G, Efstratiou, A. (1987) Vancomycin-resistant leuconostocs, lactobacilli and now pediococci. *Journal of Hospital Infection,* **10**:1-3.

48. Commane, D, Hughes, R. Shortt, C. and Rowland, I. *(*2005*)* The potential mechanisms involved in the anti-carcinogenic action of probiotics. *Mutation Research* **11**:276-289.

49. Common, R. H. (1939) Phytic acid and mineral metabolism in poultry. *Nature,* **143**:379-380.

50. Corsetti, A., De Angelis, M., Dellaglio, F., Paparella, A., Fox, P. F., Settanni, L. and Gobbetti, M. (2003). Characterization of sourdough lactic acid bacteria based on genotypic and cell-wall protein analyses. *Journal of Applied Microbiology,* **94**:641-654.

ePrints@CFTRI

51. Corsetti, A., Lavermicocca, P., Morea, M., Baruzzi, F., Tosti, N. and Gobbetti, M. (2001) Phenotypic and molecular identification and clustering of lactic acid bacteria and yeast from wheat (species *Triticum durum and Triticum aestivum*) sourdoughs of southern Italy. *International Journal of Food Microbiology*, **64**:95-104.

52. Cosgrove, D. J. (1969) Ion exchange chromatography of inositol polyphosphates. *Annals of the New York Academy of Sciences,* **165**:677–686.

53. Cosgrove, D. J. (1970). Inositol phosphates of microbial origin. Inositol phosphate intermediates in the dephosphorylation of the hexaphosphates of myoinositol, scyllo-inositol, and D-chiro-inositol by a bacterial (*Pseudomonas* sp.) phytase. *Australian Journal of Biological Sciences,* **23**:1207-1220.

54. Cosgrove, D. J. (1980) Inositol phosphates: Their chemistry, biochemistry and physiology, *Elsevier Science Publishing* Co., New York, NY.

55. Coudeyras, S., Marchandin, H., Fajon, C. and Forestier, C. (2008) Taxonomic and strain-specific identification of the probiotic strain *Lactobacillus rhamnosus* 35 within the *Lactobacillus casei* group. *Applied and Environmental Microbiology*, **74**:2679-2689.

56. Dahiya, R. S. and Speck, M. L. (1968) Hydrogen peroxide formation by lactobacilli and its effect on *Staphylococcus aureus. Journal of Dairy science,* **51**:1068-1072.

57. Danielsen, M., Simpson, P. J., O'Connor, E. B., Ross, R. P. and Stanton, C. (2007) Susceptibility of *Pediococcus* spp. to antimicrobial agents. *Journal of Applied Microbiology,* **102**:384-389.

58. Davidsson, L., Almgren, A., Juillerat, M.A and Hurrell, R.F. (1995) Manganese absorption in humans: the effect of phytic acid and ascorbic acid in soy formula. *American Journal of Clinical Nutrition,* **62**:984-987.

59. Davies, N. T. and Reid, H. (1979) An evaluation of the phytate, zinc, copper, iron and manganese contents of calcium and Zn availability from, soy-based

ePrints@CFTRI

textured-vegetable-protein meat-substitutes or meat extenders. *British Journal of Nutrition*, 41: 579-589.

60. De Angelis, M., Gallo, G., Corbo, M.R., Mc Sweeney, P.L., Faccia, M., Giovine, M., Gobbetti, M. (2003) Phytase activity in sourdough lactic acid bacteria: purification and characterization of a phytase from *Lactobacillus sanfranciscensis* CB1. *International Journal of Food Microbiology*, **87**:259-270.
61. de Boland, A. R., Garner, G. B. and O'dell, B. L. (1975) Identification and properties of phytate in cereal grains and oilseed products. *Journal of Agricultural Food Chemistry*. **23**:1186.
62. De Silva, J.J.R.F and Williams, R.J.P. (1991) The biological chemistry of the elements, 310-311, Oxford University Press, Oxford.
63. Deeth, H. C. and Tomine, A. Y. (1981) Yogurt: nutritive and therapeutic aspects. *Journal of Food Protection,* **44**:78-86.
64. Deshpande, S. S. and Salunkhe, D. K. (2000) Grain legumes, seeds and nuts: rationale for fermentation. Fermented grains legumes, seeds and nuts: a global perspective. *FAO Agricultural Services Bulletin*, **142**:1-32.
65. Dicks, L. M. T. and Botes, M. (2010) Probiotic lactic acid bacteria in the gastro-intestinal tract: health benefits, safety and mode of action. *Beneficial Microbes*, **1**:11-29.
66. Eskin, N. A. M. and Wiebe, S. (1983) Changes in phytase activity and phytate during germination of two faba bean cultivars. *Journal of Food Science*, **48**:270-271.
67. European Commission. (2005) Opinion of the FEEDAP panel on the updating of the criteria used in the assessment of bacteria for resistance to antibiotics of human or veterinary importance. *European Food Safety Authority Journal*, **223**:1-12.
68. European Food Safety Authority.(2004) EFSA scientific colloquium summary report. QPS: qualified presumption of safety of microorganisms in food and feed. European Food Safety Authority, Brussels, Belgium.

eprints@CFTRI

69. Facklam, R., Pigott, N., Franklin, R. and Elliott, J. (1995) Evaluation of three disk tests for identification of *Enterococci, Leuconostocs*, and *Pediococci*. *Journal of Clinical Microbiology*, **33**:885-887.
70. Famularo, G., De Simone, C., Pandey, V., Sahu, A. R., Minisola, G. (2005) Probiotic lactobacilli: an innovative tool to correct the malabsorption syndrome of vegetarians? *Medical Hypotheses,* **65**:1132–1135.
71. FAO/WHO Working Group Report. (2002) Guidelines for the evaluation of probiotics in food. London, Ontario, Canada.
72. Franworth, E. R., Mainville, I., Desjardins, M. P., Gardner, N., Fliss, I. and Champagne, A.C. (2007) Growth of probiotic bacteria and befidobacteria in a soy yoghurt formulation. *International Journal of Food Microbiology*, **116**:174-181.
73. Feil, B. (2001) Phytic acid. *Journal of New Seeds*, **3**:1-35.
74. Feng, H. Zuo, R.Y., Chang, J., Zheng, Q.H. and Yin, Q.Q. (2009) Phytase expressed by pIA_8 and pGAPZ_A vectors and analysis of its biochemical characters. *Open access Biotechnology Journal*, **3**:19-23.
75. Fernandez, M. F., Boris, S. and Barbes, C. (2003) Probiotic properties of human lactobacilli strains to be used in the gastrointestinal tract. *Letters in Applied Microbiology*, **94**:449-455.
76. Fredrikson, M., Andlid, T., Haikara, A. and Sandberg, A. S. (2002) Phytate degradation by microorganisms in synthetic media and pea flour. *Journal of Applied Microbiology*, **93**:197-204.
77. Frolich, W. (1990). Chelating properties of dietary fibre and phytate. The role for mineral availability. *Advances in Experimental Medicine and Biology,* **270**:83–93.
78. Fuller, R. (1991) Probiotics in human medicine. *Gut,* **32**:439-442.
79. Fuller, R. (1989) Probiotics in man and animals. *Journal of Applied Bacteriology*, **66**:365–378.
80. Garvie, E. I. (1986) Genus *Pediococcus*, p. 1075–1079. In P. H. A. Sneath, N. S. Mair, M. E. Sharp, and J. G. Holt (ed.), Bergey's manual of Systematic Bacteriology. vol. 2. The Williams & Wilkins Co., Baltimore, Md.

ePrints@CFTRI

gastrointestinal enzymes. *Journal of Agricultural and Food Chemistry* **47**:1385-1388.

81. Gerritse, K, Posno, M., Schellekens, M, Boersna, W, Claassen, E. (1990) Oral administration of TNP-*Lactobacillus* conjugates in mice: a model for evaluation of mucosal and systemic immune response and memory formation elicited by transformed lactobacilli. *Research in Microbiology* **141**:955-962.
82. Gibson, D. M. and Ullah, A. B. J. (1990) Inositol Metabolism in Plants, pp.77-92,Wiley-Liss, Chichester,
83. Gibson, G. R., Saavedra, J. M., Macfarlane, S. and Macfarlane, G. T. (1997) Probiotics and intestinal infections. In: probiotics: therapeutic and other beneficial effects (Fuller, R., ed.), pp. 10–39. Chapman & Hall, London,UK.
84. Gibson, G. R and Roberfroid, M. B. (1995) Dietary modulation of the human colonic microbiota: introducing the concept of prebiotics. *Journal of Nutrition*, **125**:1401-1412.
85. Gilliland, S. E. and Walker, D. K. (1990) Factors to consider when selecting a culture of *Lactobacillus acidophilus* as a dietary adjunct to produce a hypocholesterolemic effect in humans. *Journal of Dairy Science*, **73**:905-911.
86. Gilliland, S. E. (1989) Acidophilus milk products: a review of potential benefits to consumers. *Journal of Dairy Science*, **72**:2483-2494.
87. Gilliland, S. E. and Kim, H. S. (1984) Effect of viable starter culture bacteria in yogurt on lactose utilization in humans. *Journal of Dairy Science,* **67**:1-6.
88. Goldin, B. R. and Gorbach, S. L. (1992) Review of Probiotics available to modify gastro intestinal flora. *International Journal of antimicrobial Agents*, **67**:3045-3051.
89. Golovan, S., Wang, G., Zhang, J. and Forsberg, C.W. (2000) Characterization and over production of the *Escherichia coli appA* encoded bifunctional enzyme that exhibits both phytase and acid phosphatase activities. *Canadian Journal of Microbiology,* **46**:59-71.

eprints@CFTRI

90. González, C. J., Encinas, J. P., García-López, M. L. and Otero, A. (2000) Characterization and identification of lactic acid bacteria from freshwater fishes. *Food Microbiology,* **17**:383-391.
91. Gorbach, S. L. (1990) Lactic acid bacteria and human health. *Annals of Medicine* **22**:37-41.
92. Graf, E. and Eaton, J. W. (1993) Suppression of colonic cancer by dietary phytic acid. *Nutrition and Cancer*, **19**:11-19.
93. Graf, E. and Eaton, J. W. (1990) Antioxidant functions of phytic acid. *Free Radical Biology and Medicine,* **8**:61–69.
94. Graf, E. (1983) Calcium binding to phytic acid. *Journal of Agricultural and Food Chemistry*, **31**:851-855.
95. Grajek W, Olejnik A, Sip A (2005) Probiotics, prebiotics and antioxidants as functional foods. *Acta Biochimica Polonica,* **52**:665-671.
96. Granato, D., Branco. G. f., Nazzaro, F., Cruz, A. G. and Faria, J. A. F. (2010). Functional foods and nondairy probiotic food development: trends, concepts, and products. *Comprehensive Reviews in Food Science and Food Safety,* **9**:292-302.
97. Greaves, M.P and Webley, D.M. (1969) The hydrolysis of *myo*-inositol hexaphosphate by soil microorganisms. *Soil Biology and Biochemistry.* **1**:37-43.
98. Greaves, M. P., Anderson, G. and Webley, D. M. (1967) The hydrolysis of inositol phosphates by *Aerobacter aerogenes*. *Biochimica et Biophysica Acta*, **132**:412- 418.
99. Greene, D. A., De Jesus, P. V. and Winegrad, A. I. (1975) Effects of insulin and dietary *myo*-inositol on impaired peripheral motor nerve conduction velocity in acute streptozotocin diabetes. *The Journal of Clinical Investigation,* **55**:1326-1336.
100. Greenwood, A.J and Lewis, D.H. (1977) Phosphatases and the utilisation of inositol hexaphosphate by soil yeasts of the genus *Cryptococcus. Soil Biology and Biochemistry,* **9**:161-166.
101. Greiner, R., Konietzny, U. and Jany K. D. (2006) Phytate - an undesirable constituent of plant-based foods? *Journal für Ernährungsmedizin,* **8**:18-28.

ePrints@CFTRI

102. Greiner, R., Haller, E., Konietzny, U. and Jany, K. D. (1997) Purification and characterization of a phytase from *Klebsiella terrigena*, *Archives of Biochemistry and Biophysics.* **341**:201–206.

103. Greiner, R., Konietzny, U and Jany, K. D. (1993) Purification and characterization of two phytases from *Escherichia coli*. *Archives of Biochemistry and Biophysics.* **303**:107-113.

104. Guttieri, M., D. Bowen, J. A. Dorsch, E. Souza, and V. Raboy. (2004) Identification of low phytic acid wheat. *Crop Science*, **44**:418-424.

105. Halami, P. M. (2008) Production of polyhydroxyalkanoate from starch by the native isolate *Bacillus cereus* CFR06. *World Journal of Microbiology and Biotechnology,* **24**:805-812.

106. Hallberg, L., Brune, M. and Rossander, L. (1989) Iron absorption in man: ascorbic acid and dose-dependent inhibition by phytate. *American Journal of Clinical Nutrition,* **49**:140-144.

107. Hamad, S. H., Dieng, M. C., Ehrmann, M. A. and Vogel, R. F. (1997) Characterisation of the bacterial flora of Sudanese sorghum flour and sorghum sourdough. *Journal of Applied Microbiology,* **83**:764–770.

108. Haros, M., Bielecka, M., Honke, J. and Sanz Y. (2008) Phytate-degrading activity in lactic acid bacteria. *Polish Journal of Food and Nutrition Sciences*, **58**:33-40.

109. Haros, M., Bielecka, M., Honke, J., Sanz, Y., (2007) Myo-inositol hexakisphosphate degradation by *Bifidobacterium infantis* ATCC 15697. *International Journal of Food Microbiology*, **117**:76-84.

110. Haros, M., Bielecka, M. and Sanz, Y. (2005) Phytase activity as a novel metabolic feature in *Bifidobacterium. FEMS Microbiology Letters,* **247**:231–239.

111. Harrigan, W. F. and McCance, M. E. (1990) Laboratory Methods in Foods and Dairy Microbiology. Academic Press, London.

112. Havenaar, R., Brink, N. G. and Huis In't Ved, J. H. J. (1992) Selection of strains for probiotics use. In: Fuller, R. (Ed.), Probiotics, the Scientific Basis. Chapman & Hall, London, pp. 210–224.

ePrints@CFTRI

113. Helland. M. H., Wicklund, T. and Narvhus, J. A. (2004) Growth and metabolism of selected strains of probiotic bacteria in maize porridge with added malted barley. *International Journal of Food Microbiology*, **91**:305-313.

114. Henriksson, A. Khaled, A. K. D. and Conway, P. L. (1999) *Lactobacillus* colonization of the gastrointestinal tract of mice after removal of the non-secreting stomach region. *Microbial Ecology Health and Disease*, **11**:96-99.

115. Hofmann, A. (1991) Enterohepatic circulation of bile acids. In: Schultz, S.G., isolates from strains of *Lactobacillus acidophilus*. *Journal of Dairy Science*, **82**:23-31.

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

117. Hoves, H., Norgaard, H. and Mortensen, B. P. (1999) Lactic acid bacteria and human gastrointestinal tract. *European Journal of Clinical Nutrition*, **53**:33-350.

118. Hughes, D. B. and Hoover, D. G (1991) Bificobacteria: their potential for use in American dairy products. *Food Technology,* **45**:74-83.

119. Hurrell, R. F., Reddy, M. B., Burri, J. and Cook, J. D. (2002) Phytate degradation determines the effect of industrial processing and home cooking on iron absorption from cereal-based foods. *British Journal of Nutrition.* **88**:117-123.

120. Hurrell, R. F., Juillerat, M. A., Reddy, M. B., Lynch, S. R., Dassenko, S. A. and Cook, J. D. (1992) Soy protein, phytate and iron absorption in man. *American Journal of Clinical Nutrition,* **56**:573–578.

121. Hyronimus, B., Le Marrec, C., Hadj Sassi, A. and Deschamps, A. (2000) Acid and bile tolerance of spore-forming lactic acid bacteria. *International Journal of Food Microbiology*, **61**:193-197.

122. Igbal, T. H., Lewis, K. O. and Cooper, B. T. (1994) Phytase activity in the human and rat small intestine. *Gut* **35**:1233-236.

ePrints@CFTRI

123. In, M., Seo, S. and Oh, N. (2008) Fermentative production and application of acid phytase by *Saccharomyces cerevisiae* CY strain. *African Journal of Biotechnology*, **7**:3115-3120.

124. INACG (International Nutritional Anemia Consultative Group) (1993) The nutrition foundation, Washington DC. Barre, R and van Huot. N. (1965) *Bull. Soc. Chem. Biol.* **47**:1399-1417.

125. Ingram, P. L., Shillam, K. W., Hawkins, G. G. M. and Roy J. H. B. (1958) The nutritive value of colostrum for the calf. *British Journal of Nutrition*, **12**:203-215.

126. Irving, G.C.J and Cosgrove, D.J. (1971) Inositol phosphate phosphatases of microbiological origin. Some properties of a partially purified bacterial (*Pseudomonas* sp.) phytase. *Australian Journal of Biological Sciences,* **24**:547-557.

127. Ishibashi, N. and Shimamura, S. (1993) Bifidobacteria: research and development in Japan. *Food Technology* June: 126-135.

128. Jacobsen, CN., Rosenfeldt, V., Nielsen, Hayford, AE., Moller, PL., Michaelsen, KF., Paerregaard, A., Sandstro, M., Tvede, M. and Jakobsen, M. (1999) Screening of probiotic activities of forty-seven strains of *Lactobacillus* spp. by *in vitro* techniques and evaluation of the colonization ability of five selected strains in humans. *Applied and Environmental Microbiology*, **65:**4949-4956.

129. Jin, L. Z., Marquardt, R. R. and Zhao, X. (1999) Antagonism of *Lactobacillus spp.*, *Streptococcus* spp and Bifidobacteria spp. Against Enterotoxigenic *Escherichia coli*. 1st Edn., University of Manitoba, Winnipeg, MB., Canada, pp: 24-25.

130. Jin, L. Z., Ho, Y. W., Abdullah, N. and Jalaludin, S. (1998) Acid and bile tolerance of *Lactobacillus* isolated from chicken intestine. *Letters in Applied Microbiology*, **27**:183-185.

131. Johnson, L. F. and Tate, M. E. (1969) The structure of myo-inositol pentaphosphates. *Annals of the New York Academy of Sciences,* **165**:526–532.

ePrints@CFTRI

132. Jordan, W.H., Hart, E. B and Patten, A. J. (1906) A study of the metabolism and physiological effects of certain phosphorus compounds with milch cows. *American Journal of Physiology,* **16**:268-313.

133. Kalui, C. M., Mathara, J. M., Kutima, P. M., Kiiyukia, C. and Wongo, L. E. (2009) Functional characteristics of *Lactobacillus plantarum* and *Lactobacillus rhamnosus* from ikii, a Kenyan traditional fermented maize porridge. *African Journal of Biotechnology*, **8**:4363-4373.

134. Kerovuo, J., Lauraeus, M., Nurminen, P., Kalkkinen, N. and Apajalahti, J. (1998) Isolation, characterization, molecular gene cloning, and sequencing of a novel phytase from *Bacillus subtilis. Applied and Environmental Microbiology,* **64**:2079-2085.

135. Kilara, A. and Shahani, K. M. (1975) Lactase activity of cultured and acidified dairy products. *Journal of Dairy Science*, **59**:2031-2035.

136. Kim, H. S. and Gilliland, S. E. (1983) Effect of viable starter culture bacteria in yoghurt on lactose utilization in humans. *Journal of Dairy Science*, **67**:1-6.

137. Kim, Y. O., Kim, H. K., Bae, K. S., Yu, J. H. and Oh, T. K. (1998) Purification and properties of a thermostable phytase from *Bacillus* sp. DS11, *Enzyme and Microbial Technology,* **22**:2-7.

138. Kingsley, M. O. (1995) Effect of processing on some anti-nutritive and toxic components and on the nutritional composition of the African oil bean seed (Pentaclethra-*Macrophylla benth*), *Journal of the Science of Food and Agriculture*, **68**:153-158.

139. Kirjavainen, P. V., Ouwehand, A. C., Isolauri, E. and Salminen, S. J. (1998) The ability of probiotic bacteria to bind to human intestinal mucus. *FEMS Microbiology Letters* **167**:185–189.

140. Klein, G., Pack, A., Bonaparte, C. and Reuter, G. (1998) Taxonomy and physiology of probiotic lactic acid bacteria. *International Journal of Food Microbiology*, **41**:103-125.

141. Kolida, S., Saulnier, D. M. and Gibson, G. R. (2006) Gastrointestinal microflora: Probiotics. *Advances in Applied Microbiology*, **59**:187-219.

ePrints@CFTRI

142. Kong, S. and Davison, A. J. (1980) The role of interactions between O_2, H_2O_2, OH, e^- and O_2- in free radical damage to biological systems. *Archives of Biochemistry and Biophysics*, **204**:13-29.

143. Konietzny, U., and Greiner, R. (2003) Phytic acid: Nutritional impact. In B. Caballero, L. Trugo, & P. Finglas (Eds.),Encyclopaedia of food science and nutrition (pp. 4555–4563). London, UK: Elsevier.

144. Konietzny, U. and Greiner, R. (2002) Molecular and catalytic properties of phytare-degrading enzymes (phytases). *International Journal Food Science and Technology*, **37**:791-812.

145. Kunene, N. F., Ifigenia, G., Alexander, Von H and Hastings, J. W. (2000) Characterization and determination of origin of lactic acid bacteria from a sorghum-based fermented weaning food by analysis of soluble proteins and amplified fragment length polymorphism fingerprinting. *Applied and Environmental Microbiology*, **66**:1084-1092.

146. Laemmli, U. K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T_4. *Nature,* **227**:680-685.

147. Lease, J. G. (1966) The effect of autoclaving sesame meal on its phytic acid content and on the availability of its zinc to the chick. *Poultry Science*, **45**:237-241.

148. Lee, Y. K., and S. Salminen. (1995). The coming of age of probiotics. *Trends in Food Science and Technology,* **6**:241-244.

149. Leenhardt, F., Levrat-verny, M. A., Chanliaud, E. and Reämeäsy, C. (2005) Moderate Decrease of pH by Sourdough Fermentation Is Sufficient To Reduce Phytate Content of Whole Wheat Flour through Endogenous Phytase Activity, *Journal of Agricultural and Food Chemistry*, **53**:98-102.

150. Lehto, E. M. and Salminen, S. (1997) Adhesion of two *Lactobacillus* strains, one *Lactococcus* one *Propionibacterium* strain to cultured human intestinal Caco-2 cell line. *Bioscience and Microflora* **16**:13-17.

151. Lei, V. and Jakobsen, M. (2004) Microbiological characterization and probiotic potential of koko and koko sour water, African spontaneously fermented millet porridge and drink. *Journal of Applied Microbiology*, **96**:384-397.

eprints@CFTRI

152. Lei, X, G. and Porres, J. M. (2003) Phytase enzymology, application and biotechnology. *Biotechnology Letters,* **25**:1787-1794.

153. Lei, X. G., Ku, P. K., Miller, E. R., Ulrey, D. E and Yokoyama, M. T. (1993) Supplemental microbial phytase improves bioavailability of dietary zinc to weanling pigs. *Journal of Nutrition,* **123**:1117- 1123.

154. Lei. V, Friis, H. and Michaelsen, K. F. (2008) Spontaneously fermented millet product as a natural probiotic treatment for diarrhoea in young children: An intervention study in Northern Ghana. *International Journal of Food Microbiology*, **110**:246-253.

155. Leim, I. T., Steinkraus, K. H. and Cronk, T. C. (1977) Production of vitamin B-12 in tempeh, a fermented soybean food. *Applied and Environmental Microbiology,* **34**:773-776.

156. Lilley, D. M. and Stillwell, R. H. (1965) Probiotics growth promoting factors produced by microorganisms. *Science,* **147**:747-748.

157. Lin, M. Y., Savaiano, D. and Harlander, S. (1991) Influence of nonfermented dairy products containing bacterial starter cultures on lactose maldigestion in humans. *Journal of Dairy Science*, **74**:87-95.

158. Lin, W. H., Yub, B., Jangc, S. H. and Tsend, H. Y. (2007) Different probiotic properties for *Lactobacillus fermentum* strains isolated from swine and poultry. *Anaerobe*, **13**:107-113.

159. Linaje, R., Coloma, M. D., Perez-Martinez, G. and Zuniga, M. (2004) Characterization of faecal enterococci from rabbits for the selection of probiotic strains. *Journal of Applied Microbiology*, **96**:761-771.

160. Liong, M. T. and Shah, N. P. (2005) Optimization of cholesterol removal by probiotics in the presence of prebiotics by using a response surface method. *Applied and Environmental Microbiology*, **71**:1745-1753.

161. Loewus, F. A. (2002) Biosynthesis of phytate in food grains and seeds. In: Reddy NR, Sathe SK (Eds.). Food Phytates. *CRC Press*, Boca Raton Florida, 53-61.

162. Lönnerdal, B. (2002) Phytic acid-trace element (Zn, Cu, Mn) interactions. *International Journal of Food Science and Technology,* **37**:749-758.

ePrints@CFTRI

163. Lopez, H. W., Ouvry, A., Bervas, E., Guy, C., Messager, A., Deminne, C. and Remesy, C. (2000) Strains of lactic acid bacteria isolated from sour dourghs degrade phytic acid and improve calcium and magnesium solutbility from whole wheat flour. *Journal of agricultural food chemistry*, **48**:2281-2285.

164. Maga, J. A. (1982) Phytate: its chemistry, occurrence, food interactions, nutritional significance, and methods of analysis. *Journal of Agricultural and Food Chemistry*, **30**:1-9.

165. Manary, M. J., Hotz, C., Krebs, F., Gibson, R. S., Westcot, J. E., Arnold, T., Broadhead, R. L. and Hambidge, K. M. (2000) Dietary Phytate Reduction Improves Zinc Absorption in Malawian Children Recovering from Tuberculosis but Not in Well Children. *Journal of Nutrition* **130**:2959-2964.

166. Marklinder, I. M., Larsson, M., Fredlund, K. and Sandberg, A. S. (1995) Degradation of phytate by using varied sources of phytases in an oat-based nutrient solution fermented by *Lactobacillus plantarum* strain 299 V. *Food Microbiology*, **12**:487-490.

167. Marteau, P., Flourie, B., Pochart, P., Chastang, C., Desjeux, J. F. and Rambaud, J. C. (1990) Effect of the microbial lactase (EC 3.2.2.23) activity in yoghurt on the intestinal absorption of lactose: an *in vitro* study in lactase deficient humans. *British Journal of Nutrition.* **64**:71-79.

168. Martini, M. C., Kukielka, D. and Savaiano, D. A. (1991) Lactose digestion from yogurt: Influenceof a meal and additional lactose. *American Journal of Clinical Nutrition*. **53**:1253-1258.

169. Mastro, T. D., Spika, J. S., Lozano, P., Appel, J. and Facklam, R. R. (1990) Vancomycin-resistant *Pediococcus acidilactici*: nine cases of bacteremia. *Journal of Infectious Diseases*. **161**:956–960.

170. Maugein, J., Crouzit, P. Mahkoul, P. C. and Fourche. J. (1992) Characterization and antibiotic susceptibility of *Pediococcus acidilactici* strains isolated from neutropenic patients. *European Journal Clinicial Microbiology and Infectious Diseases,* **11**:383-385.

ePrints@CFTRI

171. Mbugua, S. K. and Njenga, J. (1991) The antimicrobial activity of fermented uji. *Ecology of Food and Nutrition,* **28**:191-198.

172. Mc Collum and Hart, E.B. (1908) On the occurrence of a phytin-splitting enzyme in animal tissues. *Journal of Biological Chemistry,* **4**:497-500.

173. Mellanby, E. (1925) *Spec. Rept. Med. Res. Council,* London, No. 93.

174. Mikelsaar, M. and Ma¨ndar, R. Sepp, E., (1998). Lactic acid microflora in the human microbial ecosystem and its development. Lactic acid bacteria: microbiology and functional aspects, 2nd edn. Marcel Dekker, New York, pp. 279– 342.

175. Miller, D. D., Schricker, B. R., Rasmussen, R. R., and Van Campen, D. (1981) An *in vitro* method for estimation of iron availability from meals. *American Journal of Clinical Nutrition*, **34**:2248-2256.

176. Mishra, V. and Prassad, D. N. (2005) Application of in vitro methods for selection of *Lactobacillus casei* strains as potential probiotics. *International Journal of Food Microbiology*, **103**:109-115.

177. Mora, D., Parini, C., Fortina, M.G. and Manachini, P.L. (1998) Discrimination among pediocin AcH/PA-1 producer starins by comparision of *ped*B and *ped*D amplified genes and by multiplex PCR assay. *Systemic and Applied Microbiology,* **21**:454-460.

178. Morris, E.R and Ellis, R. (1976) Isolation of monoferric phytate from wheat bran and its biological value as an iron source to the rat. *Journal of Nutrition* **106**:753-760.

179. Morris, J. G. (1979) Nature of oxygen toxicity in anaerobic microorganisms, In: Strategies of microbial life in extreme environments, Shilo, M. (Ed.), *Verlag Chemi*, Berlin, pp 149-162

180. Mullaney, E. J. and Ullah, A. H. J. (2003) The term phytases comprises several different classes of enzymes. *Biochemical and Biophysics Research Communication,* **312**:179-184.

181. Navert, B., Sandstrom, B. and Cederblad, A. (1985) Reduction of the phytate content of bran by leavening in bread and its effect on zinc absorption in man. *British Journal of Nutrition* **53**:47-53.

eprints@CFTRI

182. Nes, I. F., Diep, D.B., Havarstein, L. S., Bruberg, M.B., Eijsink, V. and Holo, H. (1996) Biosynthesis of bacteriocins in lactic acid bacteria. *Antonie van leewenhoek*, **70**:113-128.

183. Neuberg, C. (1908) The connection of the circular inositol to the aliphatic sugar. *Biochemical Journal,* **9**:557-560.

184. Nielsen, M. M., Damstrup, M. L., Hansen, A., (2008) An optimised micro-titer plate method for characterisation of endogenous rye phytase under industrial rye bread making conditions. *European Food Research and Technology*, **227**:1009-1015.

185. Nissen, Chingwaru, W., Sgorbatie, B., Biavati, B. and Cencic, A. (2009) Gut health promoting activity of new putative probiotic/protective *Lactobacillus* spp. Strains: A functional study in the small intestinal cell model. *International Journal of Food Microbiology*, **139**:288-294.

186. Niwa, S., Jimbo, Y., Katayama, R., Katayama, N., Hattori, I. and Ishida, A. (1967) *Bitamin,* **36**:28.

187. Noureddinin, H. and Dang, J. (2008) Degradation of phytase in distillers' grains and gluten feed by *Aspergillus niger* phytase. *Applied biochemistry and Biotechnology*, **159**:11-23.

188. O'Dell, B. L.(1969) Effect of dietary components upon Zinc availability. *American Journal of Clinical Nutrition.* **22**:1315-1322.

189. Oberleas, D. (1983). Phytate content in cereals and legumes and methods of determination. *Cereal Foods World*, **28**:352-357.

190. Oberleas, D. (1973) In: Toxicants occurring naturally in foods. National Academy of Sciences, Washington DC, *2nd ed*, Ch 17.pp. 363.

191. Oh, N. and In, M. (2009) Phytate degradation by *Leuconostoc mesenteroides* KC51 cultivation in soymilk. *African Journal of Biotechnology*, **8**:3023-3026.

192. Oh, N. S. and Lee, B. H. (2007) Phytase properties from *Bifidobacterium animalis*. *Food Science and Biotechnology*, **16**:580-583.

ePrints@CFTRI

193. Okubo, K., Myers, D. and Iacobucci, G. A. (1976) Binding of phytic acid to glycinin. *Cereal Chemistry,* **53**:513-524.

194. Oloffs, K., Strobel, E. and Jeroch, H. (2000) Changes of phytate-phosphorus content and phytase activity in germinated feedstuffs. *Bodenkultur,* **51**:41-48.

195. Ologhobo, A. D. and Fetuga, B. L. (1984) Distribution of phosphorus and phytate in some nigerian varieties of legumes and some effects of processing. *Journal of Food Science*, **49**:199-201.

196. Olsen, G. J., Matsuda, H., Hagstrom, R. and Overbeek, R. (1994). FastDNAml: a tool for construction of phylogenetic trees of DNA sequences using maximum likelihood. *Computer and Applied Biosciences,* **10**:41-48.

197. Ouwehand, A. C., Niemi, P. and Salminen, S. J. (1999). The normal faecal microflora does not affect the adhesion of probiotic bacteria *in vitro. FEMS Microbiology Letters*. **177**:35-38.

198. Palacios, M. C., Haros, M., Sanz, Y. and Rosell, C. M. (2008a). Phytate degradation by *Bifidobacterium* on whole wheat fermentation. *Food Microbiology,* **25**:169-176.

199. Palacios, M. C., Haros, M., Sanz, Y. and Rosell, C. M. (2008b). Selection of lactic acid bacteria with high phytate degrading activity for application in whole wheat breadmaking. *Journal of Food Science and Technology.* **41**:82-92.

200. Palacios, M. C., Haros, M., Rosell, C. M. and Sanz, Y. (2005) Characterization of an acid phosphatase from *Lactobacillus pentosus*: regulation and biochemical properties. *Journal of Applied Microbiology*, **98**:229-237.

201. Pandey, A., Szakacs, G., Soccol, C. R., Rodriguez-Leon, J. A., Soccol, V. T., (2001) Production, purification and properties of microbial phytases. *Bioresource Technology*, **77**:203-214.

202. Parker, R. B. (1974) Probiotics, the other half of the antibiotic story. *Animal Nutrition and Health*, 29:4-8.

ePrints@CFTRI

203. Parvez, S., Malik, K. A., Ah Kang, S. H-Y. (2006) Probiotics and their fermented food products are beneficial for health. *Journal of Applied Microbiology*, 100:1171-1185.

204. Patricia, R. M., Jeroen, H. and Pieternela, Z. (2002) An overview of the functionality of exopolysaccharides produced by lactic acid bacteria. *International Diary Journal* **12**:163-171.

205. Patwardhan, V. N. (1937) The occurrence of a phytin-splitting enzyme in the intestines of albino rats. *Biochemical Journal,* **31**:560-564.

206. Pederson, C. S. (1949) The Genus Pediococcus. *Microbiology and Molecular Biology Reviews*, **13**:225-232.

207. Pederson, C. S. (1979) Microbiology of food fermentation. 2nd edition. Connecticut: AVI

208. Phillippy, B. Q. (1999) Susceptibility of wheat and *Aspergillus niger* phytases to inactivation by gastrointestinal enzymes. *Journal of Agriculture and Food Chemistry* **47**:1385-1388.

209. Piard, J. C. and Desmazeaud, M. (1992) Inhibiting factors produced by lactic acid bacteria. II. Bacteriocins and other antibacterial substances. *Lait*, **71**:525-541.

210. Pinkse, M.W., Merkx, H. M. and Averill, B. A. (1999) Fluoride inhibition of bovine spleen purple acid phosphatase: characterization of a ternary enzyme−phosphate− fluoride complex as a model for the active enzyme−substrate−hydroxide complex. *Biochemistry*, **38**:9926-9936.

211. Plaami, S. (1997) Myoinositol phosphates: analysis, content in foods and effects in nutrition. *Food Science and Technology,* **30**:633-647.

212. Porres, J. M., Etcheverry, P., Miller, D. D. and Lei, X. G. (2001) Phytase and citric acid supplementation in whole-wheat bread improves phytate-phosphorus release and iron dialyzability. *Journal of Food Science,* **66**:614-619.

213. Posternak, T. (1965) The Cyclitols, Hermann, Paris, Ch. 12.

ePrints@CFTRI

214. Powar, V. K. and Jagannathan, V. (1982) Purification and properties of phytate-specific phosphatase from *Bacillus subtilis*. *Journal of Bacteriology* **151**:1102-1108.

215. Prasad, A. S., Miale, A., Farid, Z., Sandstead, H. H., Schubert, A. R and Darby, W.J. (1963) *Archives of Internal Medicine,* **111**:407-428.

216. Raboy, V. (1997) Accumulation and storage of phosphate minerals. In: Larkins BA, Vasil IK (eds) Cellular and Molecular Biology of Plant Seed Development. Kluwer Academic Publishers, Netherlands pp.441-477.

217. Raccachm, M. (1987) Pediococci and Biotechnology. *Critical Reviews in Microbiology*, **14**:291-309.

218. Ranhotra, G.S., Loewe, R. J and Puyat, L.V. (1974) Effect of dietary phytic acid on the availability of iron and phosphorous. *Cereal Chemistry.* **51**:323-329.

219. Rapoport, S., Leva, E. and Guest, G. M. (1941) Phytase in plasma and erythrocytes of various species of vertebrates. *Journal of Biological Chemistry,* **139**:621-632.

220. Rasic, J. L. and Kurmann, J. A. (1983) Bifidobacteria and their role. In: experiential supplementum, 39; Birkhauser Verlag, Basel.

221. Rateesh, K., Ushakumari, S. R., Sai, M. and Malleshi, N. G. (2008) Quality characteristics of biscuits from finger millet seed coat based composite flour. In:6th International Food Convention. AFSTi, Mysore, India, pp.44.

222. Raun, A., Cheng, E. and Burroughs, W. (1956) Ruminant nutrition, phytate phosphorus hydrolysis and availability to rumen microorganisms. *Journal of Agriculture and Food Chemistry,* **4**:869-871.

223. Ravindran, V., W. L. Bryden, and E. T. Kornegay, (1995) Phytates: occurrence, bioavailability, and implications in poultry nutrition. *Poultry and Avian Biology Reviews*, **6**:125-143.

224. Reale, A., Konietzny, U., Coppola, R., Sorrentino, E. and Greiner, R. (2007) The importance of lactic acid bacteria for phytate degradation during cereal dough fermentation. *Journal of Agricultural and Food Chemistry*, **55**:2993-2997.

ePrints@CFTRI

225. Reddy, K. P., Shaani, K. M. and Kulkarni, S. M. (1976) B-complex vitamins in cultured and acidified yogurt. *Journal of Dairy Science,* **59**:191-195.

226. Reddy, N. R., Sathe, S. K., Salunkhe, D. K. (1982) Phytates in legumes and cereals. *Advances in Food Research,* **28**:1–92.

227. Reid, G., and Bruce, A. W. (2006) Probiotics to prevent urinary tract infections: The rationale and the evidence. *World Journal of Urology*, **24**:28-32.

228. Reid, G. and Burton, J. (2002) Use of *Lactobacillus* to prevent infections by pathogenic bacteria. *Microbial Infections*,**56**:117-137.

229. Reid, R. L., Franklin, M. C and Hallsworth, E. G. (1947) The utilization of phytate phosphorus by sheep. *Australian Veterinary Journal,* **23**:136-140.

230. Richardson, A.E. and Hadobas, P.A. (1997) Soil isolates of *Pseudomonas* spp. That utilize inositol phosphates. *Canadian Journal of Microbiology*, **43**:509-516.

231. Robins-Browne, R. M., Path, F. F. and Levine, M. M. (1981). The fate of ingested lactobacilli in the proximal small intestine. *American Journal of Clinical Nutrition,* **34**:514-519.

232. Rodriguez, E., Gonzalis, B., Gaya, P., Nunez, M. and Medina, M. (2000) Diversity of bacteriocins produced by lactic acid bacteria isolated from raw milk. *International Dairy Journal,* **10**:7-15.

233. Rolle R. and Satin, M. (2000) Basic requirements for the transfer of fermentation technologies to developing countries. *International Journal of Food Microbiology*, **75**:181-187.

234. Ryan-Borchers, T. A, Soon Park, J., Chew, B. P., McGuire, M., Fournier, L. R. and Beerman, K. A. (2006) Soy isoflavones modulate immune function in healthy postmenopausal women. *American Journal of Clinical Nutrition*, **83**:1118-1125.

235. Saio, K., Koyama, E., Yamazaki, S and Watanabe, T. (1969) Food processing characteristics of soybean 11S and 7S proteins. Part I. Effect of difference of protein components among soybean varieties on formation of tofu-gel. *Agricultural and Biological Chemistry,* **33**:1301-1308.

ePrints@CFTRI

236. Sajidan, A., Farouk, A., Greiner, R., Jungblut, P., Mu¨ ller, E.-C.and Borriss, R. (2004) *Applied Microbiology and Biotechnology*, **65**:110-118.

237. Sambrook, J. and Russell, D. W. (2001) Molecular cloning: A laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

238. Sandberg, A. S. (2002) *In vitro* and *in vivo* degradation phytate. In: R. Reddy and S.K. sathe (Eds.). Food phytates (pp-134). London: *CRC Press* LLC.

239. Sandberg , A. S., Brune, M., Carlsson, N. G., Hallberg, L., Skoglund, E. and Rossander-Hulthen, L. (1999) Inositol phosphate with different numbers of phosphate groups influence iron absorption in humans. *American Journal of Clinical Nutrition*, **70**:240-246.

240. Sandberg, A. S. and Andersson, H. (1988) Effect of dietary phytase on the digestion of phytate in the stomach and small intestine of humans. *Journal of Nutrition*, **118**:469-473.

241. Sandberg, A. S. and Ahderinne, R. (1986) HPLC method for determination of inositol tri-, tetra-, penta-, and hexaphosphates in foods and intestinal contents. *Journal of Food Science*, **51**:547-550.

242. Sanders, M. E. (1994) Lactic acid bacteria as promoters of human health. In: Functional foods: designer foods, pharma foods, nutraceuticals, Goldber, I. (Eds.), *Chapman & Hall*, New York, pp294-322.

243. Sanders, M. E. (2003) Probiotics: considerations for human health. *Nutritional Reviews,* **61**:91-99.

244. Sanders, M. E. (1993) Summary of conclusions from a consensus panel of experts on health attributes of lactic cultures: signify chance to fluid milk products containing cultures. *Journal of Dairy Science,* **76**:1819-1828.

245. Sankara, R. D. S. and Deosthale, Y. G. (1983) Mineral composition, ionizable iron and soluble zinc in malted grains of pearl millet and ragi. *Food Chemistry*, **11**:217-223.

246. Santosa, E. M., Jaimeb, I., Rovirab, J., Lyhsc, U., Korkealac, H. and Bjorkrothc, J. (2005) Characterization and identification of lactic acid bacteria in morcilla de Burgos. *International Journal of Food Microbiology*, **97**:285-296.

ePrints@CFTRI

247. Savadogo, A., Ouattara, C. A. T., Bossole, I. H. N. and Traore, S. A. (2006) Bacteriocins and lactic acid bacteria- a mini review, *African Journal of Biotechnology*, **5**:678-683.

248. Savendra, J. (1995) Microbes to fight microbes: A not so novel approach to controlling diarrhoeal disease. *Journal of Pediatric Gastroenterology and Nutrition,* **21**:125-129.

249. Schrezenmeir, J. and De Vrese, M. (2001) Probiotics, prebiotics and synbiotics : approaching a definition. *American Journal of Clinical Nutrition*, **73**:361S-364S.

250. Shah, N. P. (2007) Functional cultures and health benefits. *International Dairy Journal*
17:1262-1277.

251. Shears, S. B. (1998) The versatility of inositiol phosphates as cellular signals. *Biochimical et Biophysics Acta,* **1436**:49-67.

252. Shermak, M. A., Saavedra, J. M., Jackson, T. L., Huang, S. S., Bayless, T. M. and Perman, J. A. (1995) Effect of yogurt on symptoms and kinetics of hydrogen production in lactose malabsorbing children. *American Journal of Clinical Nutrition,* **62**:1003-1006.

253. Shimizu, M. (1993) Purification and characterization of phytase and acid phosphatase produced by *Aspergillus oryzae* K1, *Bioscience Biotechnology and Biochemistry,* **57**:1364-1365.

254. Shimizu, M. (1992) Purification and characterization of phytase from *Bacillus subtilis* (natto) N-77 *Bioscience Biotechnology and Biochemistry,* **56**:1266-1269.

255. Sloane-stanley, G. H. (1967) A Simple Procedure for the Estimation of Very Small Amounts of Nitrogen in Lipids. *Biochemical Journal,* **194:**293-295.

256. Sreeramulu, G., Srinivasa, D. S., Nand, K. and Joseph, R. (1996) *Lactobacillus amylovorus* as a phytase producer in submerged culture. *Letters in Applied Microbiology* **23**:385-388.

eprints@CFTRI

257. Steer, T. E., Gee, J. N., Johnson, I. T. and Gibson, G. R. (2004) Biodiversity of human faecal bacteria isolated from phytic acid enriched chemostat fermenters. *Current Issues in Intestinal Microbiology,* **5**:23-39.

258. Steinkraus, K. H. (1983) Lactic acid fermentation in the production of foods from vegetables, cereals and legumes. *Antonie van Leeuwenhoek*, **49**:337-348.

259. Steinkraus, K. H. (2002) Fermentations in World Food Processing. *Comprehensive Reviews in Food Science and Food Technology,* **2**:23-32.

260. Steinkraus, K. H. (1996) Handbook of indigenous fermented foods. Marcel Decker, New York.

261. Suscovic, J., Brkic, B., Matosic, S. and Maric, V. (1997) *Lactobacillus acidophilus* M92 as potential probiotic strain. *Milchwissenschaft,* **52**:430-435.

262. Tabekhia, M. M. and Luh, B. S. (1979) Effect of milling on macro and micro-minerals and phytate of rice. *Dusch. Lebensm. Rundsch*. **75**:57.

263. Talamond, P., Doullbeau, S., Rochette, I., Guyot, J-P. and Treche, S. (2000) Anion-exchange high-performance liquid chromatography with conductivity detection for the analysis of phytic acid in food. *Journal of Chromatography A*, **871**:7-12.

264. Tamang, J. P., Tamang, B., Schillinger, U., Guigas, C. and Holzapfel, W. H. (2009) Functional properties of lactic acid bacteria isolated from ethnic fermented vegetables of the Himalayas. *International Journal of Food Microbiology*, **135**:28-33.

265. Tambe, S. M., Kaklij, G. S., Keklar, S. M. and Parekh, L. J. (1994) Two distinct molecular forms of phytase from *Klebsiella aerogenes*: evidence for unusually small active enzyme peptide, *Journal of Fermentation and Bioengineering,* **77**:23-27.

266. Tamime, A. Y., Marshall, V. M. E. and Robinson, R. K. (1995) Microbiological and technological aspects of milks fermented by bifidobacteria. *Journal of Dairy Research* **62**:151-187.

ePrints@CFTRI

267. Tang, A. L., Wilcox, G., Walker, K. Z., Shah, N. P., Ashton, J. F. and Stojanovska, L. (2010) Phytase activity from *Lactobacillus* spp.in calcium-fortified soymilk. *Journal of Food Science*, **75**:M373-M376.

268. Temmerman, R., Scheirlinck, I., Huys, G. and Swings, J. (2003) Culture-independent analysis of probiotic products by denaturing gradient gel electrophoresis. *Applied and Environmental Microbiology,* **69**:220-226.

269. Todorov, S. D., Nyati, H., Meincken, M. and Dicks, L.M.T. (2007) Partial characterization of bacteriocin AMA-K, produced by *Lactobacillus plantarum* AMA-K isolated from naturally fermented milk from Zimbabwe. *Food Control*, **18**:656-664.

270. Toma, R. B. and Tabekhia, M. M. (1979) Trials to reduce soluble oxalates in home prepared spinach. *Dtsch Lebensmitt Rundsch*, **75**:212–215.

271. Torre, M., Rodriquez, A. R., and Saura-Calixto, F. (1991) Effects of dietary fiber and phytic acid on mineral availability. *Critical Reviews in Food Science and Nutrition*, **1**:1-22.

272. Tsangalis, D., Ashton, J. F., Mcgill, A. E. J., Shah, N. P. (2002) Enzymic transformation of isoflavone phytoestrogens in soymilk by β-glucosidaseproducing Bifidobacteria. *Food Microbiology Safety,* **67**:3104-3113.

273. Tucker, H. F. and Salmon, W. D. (1955) Parakeratosis or zinc deficiency disease in the pig. *Proceedings of the* Society *for Experimental Biology and Medicine*, **88**:613-616.

274. Turnbull, A., Cleton, F., Finch, C. A., Thompson, L. and Martin, J. (1962) *Journal of Clinical Investigation,* **41**:1897-1907.

275. Turner, B. L., Paphazy, M. J., Hagarth, P. M., Mc Kelvie, I. D. (2002) Inositol phosphates in the environment. *Philosophical Transactions of the Royal Society* London. B. **357**:449-469.

276. Ullah, A. H. J. and Cummins, B. J. (1988) *A. ficuum* extracellular phytase: Immobilization on glutaraldehyde-activated silicate. *Annals of the New York Academy of Sciences,* **542**:102-106.

ePrints@CFTRI

277. Ullah, A. H. J. and Phillippy, B. Q. (1994) Substrate selectivity in *Aspergillus ficuum* phytase and acid phosphatases using *myo*-inositol phosphates. *Journal of Agriculture and Food Chemistry,* **42**:423-425.

278. Ullah, A. H. J., Cummins, B. J. and Dischinger, H. C. Jr. (1991) Cyclohexanedione modification of arginine at the active site of *Aspergillus ficuum* phytase. *Biochemical and Biophysics Research Communication*, **178**:45-53.

279. van Etten, R. L. (1982) Human prostatic acid phosphatase: a histidine phosphatase. *Annals of the New York Academy of Sciences,* **390**:27-51.

280. Vats, P. and Banerjee, U. C. (2004) Production studies and catalytic properties of phytases (*myo*inositolhexakisphosphate phosphohydrolases): an overview. *Enzyme and Microbial Technology* **35**:3-14.

281. Velez, M. P., Hermans, K., Verhoeven, T. L. A., Lebeer, S. E., Vanderleyden, J. and De Keersmaecker, S. C. J. (2006) Identification and characterization of starter lactic acid bacteria and probiotics from columbian dairy products. *Journal of Applied Microbiology*, **103**:666-674.

282. Vincent, J. B., Crowder, M. W. and Averill, B. A. (1992) Hydrolysis of phosphate monoesters: a biological problem with multiple chemical solutions. *Trends in Biochemical Sciences,* **17**:105-110.

283. Vinderola, C. G. and Reinheimer, J. A. (2003) Lactic acid starter and probiotic bacteria: a comparative -*in vitro* study of probiotic characteristics and biological barrier resistance. *Food Research International*, **36**:895-904.

284. Vohra, A. and Satyanarayana, T. (2003) Phytases: microbial sources, production, purification, and potential biotechnological applications. *Critical Reviews in Biotechnology*, **23**:29-60.

285. Vohra, P., Gray, G. A and Kratzer, F. H (1965) Phytic acid and metal complexes. *Proceedings of Society for Experimental Biology and Medicine.* 120:447-449.

286. Walker, A. R. P., Fox, F.W and Irving, J. T. (1948) Studies in human metabolism. *Biochemical Journal,* **42**:452-462.

287. Walker, D. K. and Gilliland, S. E. (1993) Relationships among bile tolerance, bile salt deconjugation and assimilation of cholesterol by *Lactobacillus acidophilus. Journal of Dairy Science*, **76**:956-961.

288. Walker, K. A. (1974) Changes in phytic acid and phytase during early development of *Phaseolus vulgaris* beans. *Planta,* **116**:91-98.

289. Weaver, C. M., Heaney, R. P., Martin, B. R. and Fitzsimmons, M. L. (1991) Human calcium absorption from whole wheat products. *Journal of Nutrition,* **121**:1769-1775.

290. Wegener, H. C., Madsen, M., Nielsen. N. and Aarestrup, F. M. (1997) Isolation of vancomycin resistant *Enterococcus faecium* from food. *International Journal of Food Microbiology*, **35**:57-66.

291. Wheeler, E. L. and Ferrel, R. E. (1971) A method for phytic acid determination in wheat and wheat fractions. *Cereal Chemistry*, **48**:312–320.

292. Widdowson, E. M. (1970) Inter-relation of dietary calcium with phytates, phosphates and fats. *Bibl. Nutrition and Dieta.* No **15**:38-47.

293. Wodzinski, R. J and Ullah, A. H. J. (1996) Phytase. *Advances Applied Microbiology,* **42**:263-303.

294. Wright, A. V. (2005) Regulating the safety of probiotics-the european approach. *Current Pharmaceutical Design*, **11**:17-23.

295. Wyss, M. R., Brugger, A., Kronenberger, R., Remy, R., Fimbel, G., Oesterhelt, M., Lehmann, A. P. G. M. and van Loon. (1999) Biochemical characterization of fungal phytases (*myo*-inositol hexakisphosphate phosphohydrolase): catalytic properties, *Applied and Environmental Microbiology,* **65**:367-373.

296. Xu, P., Price, J. and Aggett, P. J. (1992) Recent advances in methodology for analysis of phytate and inositol phosphates in foods. *Progr Food Nutr Sci,* **16**:245-262.

297. Yasmine, M. (2002) Impact of small scale fermentation technology on food safety in developing countries. *International Journal Food Microbiology*, **75**:213-229.

ePrints@CFTRI

298. Zamudio, M., Gonza´lez, A., Medina, J.A. (2001) *Lactobacillus plantarum* phytase activity is due to non-specific acid phosphatase. *Letters in Applied Microbiology,* **32**:181-184.

299. Zarate, G., Perez C., A., Gonzalez, S. and Oliver, G. (2000). Viability and -galactosidase activity of diary *propionibacteria* subjected to digestion by artificial gastric and intestinal fluids. *Journal of Food Protection*, **63**:1214-1221.

300. Zoetendal, E. G., von Wright, A., Vilpponen-Salmela, T., Ben-Amor, K., Akkermans, A. D. and de Vos, W. M. (2002) Mucosa-associated bacteria in the human gastrointestinal tract are uniformly distributed along the colon and differ from the community recovered from feces. *Applied and Environmental Microbiology,* **68**:3401-3407.

ePrints@CFTRI

Appendices

ePrints@CFTRI

List of publications

1) **Raghavendra, P.,** and P. M. Halami. (2009) Screening, selection and characterization of phytic acid degrading lactic acid bacteria from chicken intestine, *International journal of Food Microbiology,* **133:** 129-134.

2) **Raghavendra, P.,** Rao, S., C., T. and Halami, P.M. (2010) Evaluation of beneficial attributes for phytate-degrading *Pediococcus pentosaceus* CFR R123. *Beneficial Microbes*, **1**: 259-264.

3) Vure Badarinath, **Ponnala Raghavendra** and Prakash M. Halami. (2010). Characterization of lactic acid bacteria isolated from *Okara* for probiotic properties. *International Journal of Probiotics and Prebiotics*. 5(3), 149-156.

4) **Raghavendra, P.,** S. R. Ushakumari and P. M.Halami (2010) Phytate-degrading Pediococcus pentosaceus CFR R123 for application in functional foods, *Beneficial Microbes* (In press).

5) S. M. Devi, **Raghavendra, P.,** and P. M. Halami (2011) Random Amplified Polymorphic DNA (RAPD) of plasmid DNA to identify the pediocin PA-1 isolated from different sources (Communicated).

ePrints@CFTRI

List of papers presented

1. Poster entitled "Role of phytate degrading lactic acid bacteria on availability of Calcium from malted finger millet seed coat" **Ponnala Raghavendra,** Usha Kumari S. R and Prakash M. Halami, presented at 8th International food convention–2008 (IFCON-2008) conducted by AFSTi, at Mysore, Karnataka India during December 15-19, 2008. Abstract CP-21
2. Poster entitled " Development of defined starter culture for food fermentation" Sangeetha K, **Ponnala Raghavendra**, V. Badarinath, S.V.N.Vijayendra and Prakash M. Halami, presented at National Science Congress at CFTRI, during December, 2007
3. Poster entitled "Screening of lactic acid bacteria from different sources for phytase like activity" **Ponnala Raghavendra** and Prakash M. Halami, Presented at 48th AMI Annual conference, held at IITM Chennai during December 18-21, 2007. Abstract FG-2
4. Poster entitled "Probiotic properties of phytate degrading *Pediococcus pentosaceus CFR* R38 isolated from chicken intestine" **Ponnala Raghavendra,** Vure Badarinath and Prakash M. Halami, presented at 3rd International Conference on fermented foods conducted by SASNET at Anand, Gujarat during December 13-16, 2007. Abstract B20.
5. Poster entitled "Functional food formulation using bacteriocinogenic phytate degrading Lactic Acid Bacteria" Chandrakanth N, **Ponnala Raghavendra**, Amudha Senthil and Prakash M. Halami. Presented at ICFOST 2009, Mysore. Abstract FF11.

ePrints@CFTRI

6. **<u>Raghavendra, P</u>**., and Halami, P. M., (2010) Studies on phytic acid degrading lactic acid bacteria for functional food formulation and their application in agriculture and environment. Abstract P24 of the paper presented on plenary lecture at International symposium of lactic acid bacteria (ISLAB-2010). University of Putra, Malaysia, July 25-27, 2010.

ePrints@CFTRI