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# Dephytinization of Cereals and Pulses by Phytase Producing Lactic Acid Bacteria

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**A B S T R A C T**

The increasing health complications due to changes in the life style has paved path to search for a food supplement that can provide additional benefits other than the basic nutrients. Phytase is an enzyme that is extensively used in poultry and swine industries as feed additives. The enzyme also finds immense application in the human nutrition for dephosphorylation of phytic acid which otherwise forms insoluble complex with minerals such as Ca, Mg, Fe, Zn etc. Due to extensive utilization of Lactic acid bacteria in food fermentations, the present investigation aimed at screening of probiotic bacterial strains for phytase production. Of the 24 strains, ten strains such as *Lactobacillus plantarum* $L_{20}$, *Lactobacillus brevis* $L_{01}$, *Lactobacillus acidophilus* $L_{11}$, *Lactococcus* sp $B_1$, *Lactococcus* sp $B_4$, *Lactococcus* sp $C_5$, *Lactococcus* sp $C_6$, *Lactococcus* sp $BA_{242}$, *Pediococcus acidilactici* $K_7$ and *P. acidilactici* $C_{20}$ were found positive for phytase production. However, three hyper phytase producing strains like *Lactobacillus plantarum* $L_{20}$ (272 U/mg protein), *Lactobacillus brevis* $L_1$ (252 U/mg protein) and *Lactobacillus acidophilus* $L_{11}$ (198 U/ml protein), were used to reduce phytic acid extracted from selected cereals and pulses through submerged fermentation. Although results showed variation, highest rate of reduction in phytic acid content from chickpea (70.7%) was found with *Lactobacillus brevis* $L_1$.

## Introduction

Over two billion people that is about 30% of the world's population live with iron deficiency anaemia (Stoltzfus and Dreyfuss 1998) which may be attributed to the complexation of iron and phytate (Leenhardt et al. 2005). This may be one of the causes for malnutrition, a global issue particularly in developing countries (Barberena et al.

2008). Most of the plant products such as cereals and legumes consist of about 60-70% phytic acid as an anti-nutritional factor that chelates metal ions such as $Ca^{2+}$, $Mg^{2+}$, P, $Zn^{2+}$, $Fe^{2+}$, thus making them unavailable to monogastric animals (Ragon et al. 2009), as they lack phytase enzyme to hydrolyze phytate molecule (Oatway et al. 2001; Lassen et al. 2001; Wyss et al. 1999). This leads to the use of inorganic phosphates such as di-calcium or tri-calcium phosphate that might increase phosphorus pollution load in the environment (Suryanarayana, 2013). The bioavailability of essential dietary minerals can be improved by reducing the phytate content in foods and feeds (Turk et al. 2000).

Hence, numerous attempts have been made for the use of exogenous phytase, a class of phosphatases that catalyses hydrolysis of phytic acid into lower myo-inositol intermediates and improve the bioavailability of minerals to monogastric animals (Suryanarayana, 2013; Tamayo - Ramos et al. 2012). Phytases from fungi have been extensively used as animal feed to improve the bioavailability of minerals that are otherwise chelated by phytic acid (Haefner et al. 2005; Rao et al. 2009).

Due to these nutritional consequences, the degradation of phytate during food processing is desirable. Fermentation is widely used to improve the nutritional and functional qualities of food products (Fredrikson et al. 2002; Bergqvist et al. 2005) by way of reducing phytic acid from cereals (Gunashree et al. 2014).

Probiotics are reported to render potential health benefits in humans and animals due to their interaction with gastrointestinal tract (Chou and Weimer, 1999; Haros et al. 2009). Hence the present work aimed at screening of phytase producing probiotic strains for the reduction or elimination of phytic acid extracted from commonly used cereals and pulses to combat malnourishment and iron- deficiency disorders.

## Materials and Methods

### *Qualitative Screening of Probiotics for Phytase Production*

About 24 Lactic Acid Bacterial strains were obtained from the Microbial Culture Collection Centre at NDRI, Haryana, India and from Culture Collection Centre at Food Microbiology Department of CSIR- CFTRI, Mysore, Karnataka, India. The cultures so obtained were screened for phytase production by inoculating on to modified Mans Rogosa and Sharpe (MRS) medium supplemented with 0.5% calcium phytate and the plates were incubated at 37ºC for 20 h. The strains that produced zone of hydrolysis around the colonies were selected as positive cultures for phytase production.

### *Submerged Fermentation for Phytase Production by Probiotics*

The phytase positive strains were cultivated in MRS broth in two sets, one with and the other without 0.5% calcium phytate to check for constitutive or inductive nature of the enzyme. About 100 ml aliquots of MRS broth were inoculated with overnight cultures of *Lactobacillus plantarum* $L_{20}$, *Lactobacillus brevis* $L_1$, *Lactobacillus acidophilus* $L_{11}$, *Lactococcus* sp $B_1$, *Lactococcus* sp $B_4$, *Lactococcus* sp $C_5$, *Lactococcus* sp $C_6$, *Lactococcus* sp $BA_{242}$, *Pediococcus acidilactici* $K_7$ and *P. acidilactici* $C_{20}$ at 1% level. The flasks were incubated for 72 h at 37ºC and 150 rpm in a shaker incubator. The culture broth was filtered through whatman No 1. filter paper and the filtrate was used as crude phytase preparation for further quantitative assay.

### *Enzyme Assay*

Phytase enzyme activity was determined by the method of Heinonen and Lahti (1981). In brief, the method proceeds as 1 ml suitably diluted crude enzyme sample mixed with 0.5 ml of 0.2 M acetate buffer and 0.5 ml of 15 mM Sodium phytate. The mixture was incubated for 40 minutes at 45ºC. Reaction was terminated by adding 2 ml of 15% Trifluroacetic acid. Aliquot of 0.5 ml was taken from the above reaction mixture and mixed with 4 ml of AAM solution (2: 1: 1, Acetone; Ammonium molybdate; Sulphuric acid). To the same mixture 0.4 ml of 1 M Citric acid was added, mixed well and the optical density was read at 355 nm. A standard graph was plotted using Potassium dihydrogen phosphate with working concentration ranging from 30- 360 µM. One Unit of phytase was defined as the amount of enzyme required to liberate 1 µM of inorganic phosphate (Pi) from the substrate under the assay conditions.

### *Soluble Protein Estimation*

Soluble protein in the phytase enzyme sample was estimated by the method of Bradford, (1976). 0.1ml of the protein sample was mixed with 5 ml of protein reagent and absorbance was read at 595 nm after 2 minutes against reagent blank prepared with 0.1 ml of distilled water. A standard curve was plotted using Bovine serum albumin with the working concentration ranging from 5-25 µg/mL.

### *Extraction and Estimation of Phytic Acid from Cereals and Pulses*

The cereals and pulses such as sorghum (*Sorghum bicolor*), wheat (*Triticum aestivum*), ragi (*Eleusine coracana*), foxtail millet (*Alopecurus*), bajra or pearl millet (*Pennisetum glaucum*), kodo millet (*Paspalum scrobiculatum*), bengal gram *(Cicer arietinum*), blackgram (*Vigna mungo*), black beans (*Phaseolus vulgaris*), cowpea (*Vigna unguiculata*), green gram (*Vigna radiata*) and soybean (*Glycine max*) were selected and their phytic acid was extracted using different organic compounds such as concentrated sulphuric acid, hydrochloric acid, Trichloroacetic acid and glacial acetic acid and distilled water.

The extraction and spectrophotometric estimation of phytic acid was carried out by the method of Gao et al. (2007). The procedure in brief: One g of ground powder was mixed thoroughly with 10 ml of different organic compounds such as Trichloroacetic acid (3, 6 and 9%), conc. sulphuric acid (3, 6 and 9%), conc. hydrochloric acid (3, 6 and 9%), glacial acetic acid (3, 6 and 9%) at various concentrations and distilled water. The tubes were shaken at 220 rpm for 16- 20 h. Centrifuged at 5000 rpm at 10$^{o}$C for 20 min and the crude extract collected for phytic acid determination was mixed with 0.5g of NaCl, shaken at 250 rpm for 20 min to dissolve the salt and allowed to settle at 4$^{o}$C for 1h or at -20$^{o}$C for 20 min. Mixture was centrifuged at 5000 rpm at 10$^{o}$C for 20 min. One ml of the supernatant was mixed with 4 ml of deionized dis. $H_20$ and 1.5 ml of diluted sample and 0.5 ml of Wade reagent (0.03g of ferric chloride and 0.3g of sulfosalicylic acid dissolved in distilled water and the volume made up to 100 ml) were mixed thoroughly on a vortex, centrifuged at 5000 rpm at 10$^{o}$C for 10 min and absorbance was read at 500 nm.

### Calculation

$$\text{Phytic acid (mg/100g)} = \frac{\text{Sample concentration} \times \text{Initial dilution (10ml)} \times \text{Final dilution (25ml)}}{\text{Weight of the sample (0.5g)} \times \text{Aliquot taken for further analysis (3 ml)}}$$

## Fermentation of Phytic Acid Extracts of Cereals and Pulses

Phytic acid extracted from all the selected cereals and pulses using 3% HCl were filter sterilized and varied volumes of each of the filtrates were added to MRS broth so as to make the concentration of phytic acid to 1% and the total volume to 50 ml. The flasks were then inoculated with 1% inoculum of 16-18 h old *Lactobacillus plantarum* $L_{20}$, *L. brevis* $L_1$ and *L. acidophilus* $L_{11}$ strains which were found to be hyper phytase producers. The flasks were incubated at 37°C for 72 h in an incubator shaker. Five ml each of the samples were drawn at every 0h, 12h, 24h, 36h, 48h, 60h and 72h of incubation time and estimated for phytase and phytic acid content.

### *Statistical Analysis*

Data are presented as standard error means (SEM). Comparisons between phytase production by the bacterial strains with and without calcium phytate were made with analysis of variance and considered significant at $p < 0.05$. All statistical tests were carried out using demo version of Graph Pad Prism software.

## Results and Discussion

In the present study, about twenty four lactic acid bacterial strains were screened by qualitative assay, out of which ten strains were found positive for phytase production. All the positive strains were further analysed for quantitative enzyme production.

*Lactobacillus plantarum* $L_{20}$ showed highest enzyme activity of 272 U/mg in the presence of phytic acid which is 78% higher when compared to its activity in the absence of phytic acid (152 U /mg) (Fig 1a). *Lactobacillus brevis* $L_1$ showed 252 U/mg in the presence of phytic acid and 155 U/mg in the absence of phytic acid which is 62% less (Fig 1b). *Lactobacillus acidophilus* $L_{11}$ had phytase content of 92 and 56 U/mg in the presence and absence of phytic acid respectively (Fig 1c). *Lactococcus* sp. $B_1$ showed 94 and 55 U/mg of phytase in the presence and absence of phytic acid (Fig 1d). *Lactococcus* sp. $B_4$ showed 72 and 62 U/mg of phytase in the presence and absence of phytic acid (Fig 1e). *Lactococcus* sp. C5 showed 76 and 72 U/mg (Fig 1f), phytase with and without phytic acid respectively. *Lactococcus* sp. C6 showed 72 and 49 U/mg of phytase activity with and without phytic acid (Fig 1g). *Pediococcus acidilactici* $K_7$ showed 54 and 20 U/mg (Fig 1h) with and without phytic acid respectively. *Pediococcus acidilactici* $C_{20}$ showed 46 and 36 U/mg at 48 and 24 h respectively (Fig 1i). *Lactococcus* sp. BA 242 (Fig 1j) showed 43 and 30 U/mg in the presence and absence of phytic acid respectively.

The results showed that all the ten positive probiotic strains produced increased amounts of phytase enzyme in the presence of phytic acid (calcium phytate). However, the quantity of enzyme or their activities variably increased among the strains by keeping the amount of calcium phytate constant at 0.5%. This indicates that the enzyme is produced inductively by all the strains studied.

Extraction of phytic acid from the selected cereals and pulses using different organic acids showed varied amounts. The phytic acid extracted from different cereals and pulses, irrespective of the concentration found in the extract were used at a constant level of 1% to study their reduction through probiotic fermentation using three hyper phytase producing strains.

**Table.1** Extraction of Phytic Acid from Different Cereals and Pulses using Various Organic Compounds

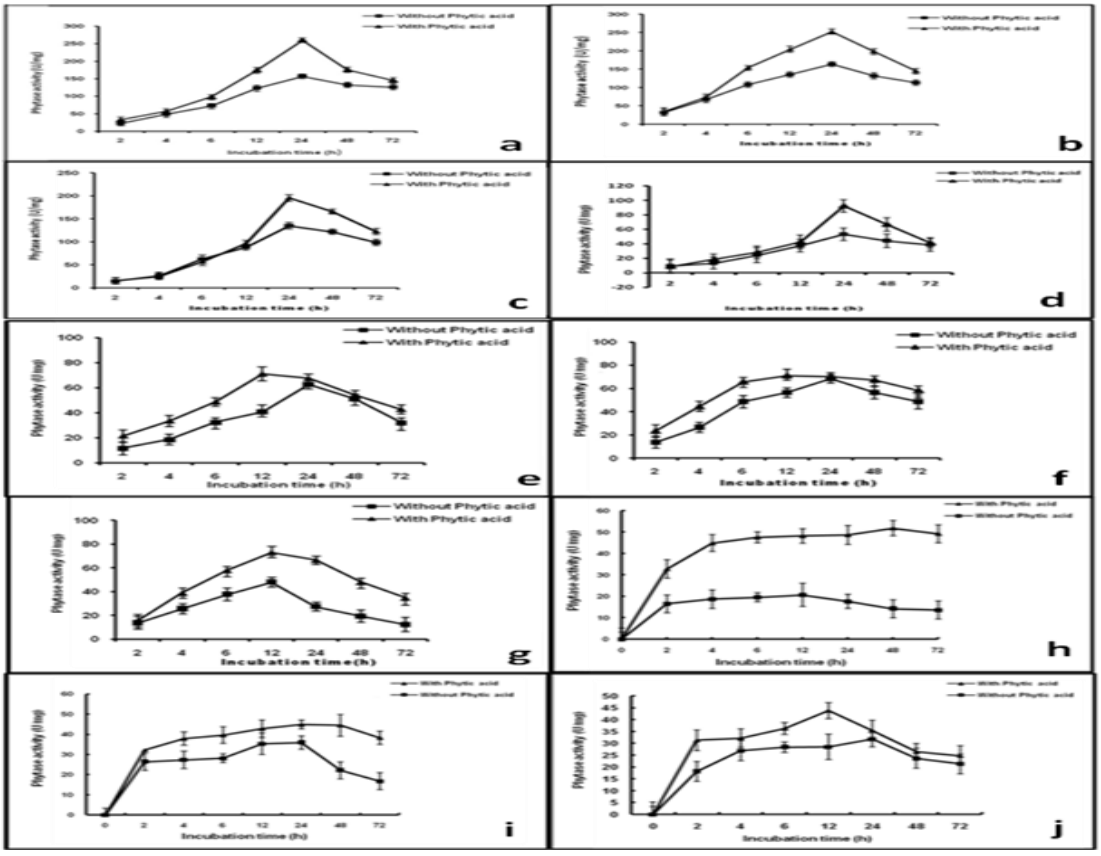
| Cereal/pulse | $H_2O$ | $H_2SO_4$ (µg/ml) | | | HCL (µg/ml) | | | TCA (µg/ml) | | | $CH_3COOH$ (µg/ml) | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | (µg/ml) | 3 | 6 | 9 | 3 | 6 | 9 | 3 | 6 | 9 | 3 | 6 | 9 |
| Finger millet | 75.0 | 170.3 | 993.3 | 1015.0 | 846.6 | 840.0 | 1001.7 | 221.7 | 625.0 | 930.0 | 326.7 | 301.7 | 301.7 |
| Pearl millet | 83.3 | 159.2 | 913.3 | 891.7 | 760.0 | 870.0 | 981.7 | 468.3 | 786.7 | 1010.0 | 163.3 | 216.7 | 216.7 |
| Wheat | -3.3 | 166.5 | 903.3 | 773.3 | 910.0 | 975.0 | 976.7 | 315.0 | 621.7 | 1041.7 | 343.3 | 405.0 | 405.0 |
| Kodo millet | 548.3 | 172.5 | 940.0 | 968.3 | 791.6 | 968.3 | 1006.7 | 271.7 | 368.3 | 1063.3 | 468.3 | 491.7 | 491.7 |
| Foxtail millet | 195.0 | 153.1 | 858.3 | 771.6 | 868.3 | 898.3 | 716.7 | 555.0 | 743.3 | 1026.7 | 671.7 | 766.7 | 766.7 |
| Chickpea | -176.7 | 178.1 | 970.0 | 996.7 | 935.0 | 931.7 | 1020.0 | 308.3 | 655.0 | 921.7 | 370.0 | 236.7 | 236.7 |
| Green gram | -96.7 | 179.0 | 983.3 | 990.0 | 780.0 | 946.7 | 1000.0 | 311.7 | 680.0 | 1016.7 | 435.0 | 510.0 | 510.0 |
| Black bean | 315.0 | 171.5 | 880.0 | 265.0 | 728.3 | 950.0 | 990.0 | 495.0 | 800.0 | 961.7 | 315.0 | 230.0 | 230.0 |
| Black gram | -28.3 | 148.5 | 815.0 | 836.7 | 736.6 | 770.0 | 901.7 | 581.7 | 741.7 | 830.0 | 285.0 | 316.7 | 316.7 |
| Cowpea | 146.7 | 168.7 | 910.0 | 881.7 | 776.6 | 971.7 | 978.3 | 735.0 | 946.7 | 966.7 | 355.0 | 395.0 | 395.0 |
| Sorghum | 368.3 | 168.4 | 905.0 | 778.3 | 826.6 | 823.3 | 880.0 | 388.3 | 631.7 | 1050.0 | 518.3 | 526.7 | 526.7 |
| Soybean | 205.0 | 115.1 | 453.3 | 146.6 | 878.3 | 523.3 | 765.0 | 216.7 | 481.7 | 586.7 | 725.0 | 381.7 | 381.7 |

**Table.2** Phytic Acid Content in Cereals and Pulses after Bacterial Fermentation

| Cereals/ Pulses | % reduction in Phytic acid | | | |
|---|---|---|---|---|
| | ***Lactobacillus plantarum* L20** | ***Lactobacillus brevis* L1** | ***Lactobacillus acidophilus* L11** | **Without probiotic** |
| Finger millet | 54.4 ± 0.008 $^{b}$ | 65.6 ± 0.004 $^{a}$ | 55.8 ± 0.011$^{b}$ | 16.8 ± 0.010 $^{C}$ |
| Pearl millet | 30.7 ± 0.010 $^{b}$ | 46.5 ± 0.012 $^{a}$ | 45.5 ± 0.013 $^{a}$ | **25.4** ± 0.008 $^{C}$ |
| Wheat | 42.3 ± 0.006 $^{b}$ | 67.1 ± 0.008 $^{a}$ | 63.1 ± 0.007 $^{a}$ | 16.4 ± 0.007 $^{C}$ |
| Kodo millet | **67.1** ± 0.003 $^{a}$ | 54.5 ± 0.009 $^{a}$ | 29.4± 0.008 $^{b}$ | 22.3 ± 0.006 $^{C}$ |
| Foxtail millet | 22.8 ± 0.008 $^{C}$ | 47.3 ± 0.010 $^{b}$ | **63.2** ± 0.009 $^{a}$ | 25.8 ± 0.004 $^{C}$ |
| Chickpea | 58.7 ± 0.007 $^{b}$ | **70.7** ± 0.007 $^{a}$ | 46.2 ± 0.004 $^{C}$ | 43.3 ± 0.007 $^{C}$ |
| Green gram | 53.3 ± 0.012 $^{a}$ | 58.6 ± 0.005 $^{a}$ | 33.1 ± 0.010 $^{b}$ | 18.2 ± 0.008 $^{C}$ |
| Black bean | 57.1 ± 0.009 $^{a}$ | 69.1 ± 0.009 $^{a}$ | 43.4 ± 0.007 $^{b}$ | 23.3± 0.005 $^{C}$ |
| Black gram | 37.1 ± 0.013 $^{b}$ | 56.7 ± 0.007 $^{a}$ | 28.0 ± 0.006 $^{b}$ | 15.2 ± 0.007 $^{C}$ |
| Cowpea | 44.6 ± 0.006 $^{b}$ | 59.6 ± 0.008 $^{a}$ | 23.1 ± 0.008 $^{C}$ | 19.7 ± 0.010 $^{C}$ |
| Sorghum | 11.1 ± 0.012 $^{C}$ | 64.1 ± 0.003 $^{a}$ | 17.8 ± 0.012 $^{b}$ | 15.7± 0.006 $^{b}$ |
| Soybean | 51.6 ± 0.009 $^{a}$ | 60.5 ± 0.011 $^{a}$ | 20.6 ± 0.010 $^{b}$ | 19.5 ± 0.012 $^{C}$ |

All values are mean of triplicates with SEM significant at $p < 0.05$. The column not sharing same alphabets in the table are significant

**Fig.1** Phytase activity (U/ml) of (a) Lactobacillus plantarum L020, (b) Lactobacillus brevis L01, (c) Lactobacillus acidophilus L011, (d) Lactococcus sp B1, (e) Lactococcus sp. B4, (f) Lactococcus sp. C5, (g) Lactococcus sp. C6, (h) Pediococcus acidilactici K7, (i) Pediococcus acidilactici C20, (j) Lactococcus sp BA242



The results showed significant levels of reduction in the phytic acid content after fermentation with probiotic strains when compared to natural fermentation which was insignificant (Table 2). However, an overall highest reduction in the phytic acid content was with *Lactobacillus brevis* $L_1$ and the rate of reduction varied among different cereals and pulses.

Fermentation of cereals and legumes by lactic acid bacterial fermentation is one of the well established processing methods in Asia and Africa for the production of various foods (Charalampopoulos et al. 2002).

The incorporation of different types of carbon sources or inorganic phosphates to the growth medium is shown to modulate the synthesis of phytate- degrading enzyme in the studied strains (Monika et al. 2008). The phytate-degrading activity of *Lactobacilli* seems to be due to a non-specific acid phosphatase (Palacios et al. 2005; Zamudio et al. 2001). Phytase activity has been detected in *L. amylovorus*, *L. sanfranciscensis* (De Angelis et al. 2003) and *Lactobacillus salivarius* FC113 (Lee et al. 2013). The activity of *L. sanfranciscensis* led to a 64-74% decrease of phytates in sour dough after 8 h of fermentation at 37°C. On the other hand, Lopez et al. (2000) reported that all the tested strains of lactic acid bacteria isolated from sourdough expressed phytase activity and were able to degrade ≈30% of phytate in only 2 h. *L. casei* DSM 20011, *L. casei* 40W, *L. fermentum* DSM 20052, *L. plantarum* JBPRS, *L. plantarum* W42 and *L. plantarum* 110 showed the highest phytase activities and the highest degradation of InsP6 was carried out by *L. plantarum* W42 (8.53%) and *L. plantarum* 110 (8.83%), followed by *L. casei* 40W (6.56%) (Monika et al. 2008).

In conclusion, it is important to confirm the specificity of phytase for different salts of phytic acid extracted from various cereals and pulses in this case. The minimal concentration of calcium phytate inducing phytase production may be optimized. Further confirmation is also necessary to find out whether the decrease in phytic acid content is due to only phytase or the combined effect of phytase and acid phosphatases. There are many earlier reports showing bacteria producing intracellular phytase, on the contrary, in the present study, all the positive cultures produced extracellular phytase. However, the rate of enzyme production may be improved further by increasing the permeability of the cells. The whole cereals and pulses may be fermented using a potent strain and employed in the formulation of a nutritionally superior composite food to combat malnutrition.

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