Production of phytase from *Lactobacillus paracasei* strain and its probiotic profile

Deepali Bhagat, Parvez Singh Slathia, Neelu Raina & Preeti Sharma*

School of Biotechnology, Shri Mata Vaishno Devi University, Jammu, Jammu & Kashmir-182 320, India

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Phytase, as an effective enzyme for phytic acid degradation, has significance in bioremediation, poultry and aquaculture. In view of such an environmental and industrial importance, phytase producing probiotic bacteria have gained attention. Here, we screened lactic acid bacteria (LAB) for their phytase producing potential. The strain showing maximum phytase activity was studied for its probiotic characteristics. Among 20 LAB isolated from Kalarei, an edible source, two isolates K.J (a) and K exhibiting maximum enzyme activity (5.18 U/mL and 5.0 U/mL) were selected. In optimization studies for production of phytase using ‘one-variable-at-a-time’ (OVAT) approach, isolate K showed maximum (5.92 U/mL) enzyme activity. The strain was identified by ribotyping as *Lactobacillus paracasei* and the sequence was submitted in NCBI GenBank under accession number MK280749. Further optimization studies for phytase production were carried out using Plackett–Burman design and central composite design (CCD) of response surface methodology (RSM). Statistically optimized four significant variables: incubation temperature, initial pH, maltose and peptone resulted in an increase (6.37 U/mL) in enzyme activity. The enzyme was purified 3.97 fold with a specific activity of 278 U/mg. The molecular weight of enzyme was 70 kDa and optimum activity was determined at 37°C, pH 5.5. The strain was designated as *Lactobacillus paracasei* SMVDUB1 and showed promising probiotic characteristics viz. survival rate above 80% (low pH, high bile salt concentration under simulated gastrointestinal conditions), hydrophobicity with chloroform (96.74%), autoaggregation (69.61%) and coaggregation ability (82.79%) with *Bacillus subtilis*.

**Keywords:** Central composite design (CCD), Kalarei, Lactic acid bacteria (LAB), OVAT approach, Phytic acid, Plackett–Burman design, Response surface methodology (RSM)

Plant based diets, common in the developing countries due to the economic, religious, traditional and cultural reasons¹, with cereals, legumes and oilseeds are the major energy sources². They contain high levels of phytic acid (myoinositol hexakisphosphate), a major storage form of phosphate, which accounts for more than 80% of the total phosphorus. Monogastric animals and human digestive systems are incapable to metabolize phytate³. Phytic acid in plant based diets exhibits a strong chelating effect on divalent minerals, such as Ca²⁺, Mg²⁺, Zn²⁺ and Fe²⁺, and thus, leads to major public health and environmental problems worldwide⁴. Further, eutrophication of nearby water bodies is another major problem in agricultural areas because of excessive phosphorous containing effluents. Phytic acid intake can result in micronutrient malnutrition including deficiency of iron, zinc and vitamins⁵,⁶. An effective phytic acid degradation can increase the bioavailability of essential minerals and form lower inositol phosphate derivatives having health potentials⁷. The effective degradation of phytic acid can be achieved enzymatically as well as non-enzymatically⁸. Nonenzymatic hydrolysis employs chemical and physical methods to hydrolyse phytate which is a costly process with further reduction in the nutritional value of diet. Therefore, enzymatic methods were adopted for phytate hydrolysis⁹. Enzymatic degradation is obtained by using phytase (myo-inositol hexakisphosphate phosphohydrolases) which disintegrates the phosphoric monoester bonds in phytic acid and phytate¹⁰.

Phytase enzyme is produced by various microorganisms including bacteria, fungi and yeasts⁴. Commercial utilization of phytase from *Aspergillus* has been reported as feed supplement in animals, poultry and aquaculture¹¹. Since the first commercial phytase product Natuphos® from *Aspergillus*, launched in 1991, the present market value has

*Correspondence:
Phone: +91 9419304654 (Mob.);
E-mail: preeti.res@gmail.com
reached €150 million and is likely to expand in coming years. Phytase from probiotic bacteria have a significant advantage, as the global demand for probiotics is expected to reach USD 96 billion by 2020. Giri et al. and Demir et al. have reported phytase production from *Lactobacillus plantarum* isolated from a traditional rice based fermented beverage “bhaatijaanr” and *Lactobacillus coryniformis* isolated from “Lorcheeze”, respectively. Presently, phytase production has been considered as one of the important characteristic of probiotic bacteria. In view of the industrial importance, optimization of production process is necessary for making phytase production economically feasible. The modern statistical method, response surface methodology (RSM) has emerged as the potential alternative to conventional ‘one-variable-at-a-time’ approach followed for optimization of enzyme production.

Although *Lactobacillus paracasei* has been widely appreciated for probiotic characteristics, there are very few reports on phytase production, optimization, and purification from this species till date. RSM studies in particular have been carried out to lesser extent with respect to optimization studies for production of phytase, further lacking the focus on phytase characterization. The objective of present study was to evaluate potential of lactic acid bacteria (LAB) from local edible fermented cheese product Kalarei for their phytate degrading ability, one of the essential probiotic properties. Maximum phytase production was reported after statistical optimization of fermentation conditions.

**Materials and Methods**

All the chemicals and biochemicals employed in present study were purchased from Hi-Media, Sigma and S.D Fine Chemicals, India. Standard bacterial strains: *Bacillus subtilis*, *Mycobacterium smegmatis*, *Staphylococcus aureus*, *Proteus vulgaris* and *Escherichia coli* were procured from Microbial Type Culture Collection (MTCC), CSIR-Institute of Microbial Technology, Chandigarh, India.

**Isolation of lactic acid bacteria (LAB)**

LAB strains were isolated from an edible source, Kalarei, a fermented cheese product. Twelve samples of Kalarei were collected from local market of different regions of Jammu, India and each sample has been given a code based on the region of collection (KA Akhnoor, K1 Battal, K2 & K4 Rajouri, K5 Ramban, K6 Poonch, KB Billawar, KJ Jammu, KP Panthal, KU & K Udhampur). For isolation of LAB, 1.0 g of sample was dissolved in 9.0 mL of (0.85%) normal saline solution. De Man, Rogosa and Sharpe (MRS) medium was used for isolation, purification and maintenance of LAB isolates. Samples were enriched in MRS broth and incubated at 37±0.1°C for 24 h. For single colony isolation, plate dilution method was adopted, using MRS agar medium. The MRS petriplates containing dilutions were incubated at 37±0.1°C for 48 h under anaerobic conditions.

**Qualitative and quantitative estimation of phytase**

Phytase assay was carried out by growing the bacterial cultures in modified MRS broth (MRS-MOPS) as reported in the literature. For qualitative screening, plate assay method was followed for identifying phytate degrading isolates, both supernatant and saline cell suspensions were used. Phytase screening media (PSM) was used in qualitative screening as illustrated in previous reports. The methodology adopted ensured the phytate hydrolysis rather than acid production by the LAB isolates, as given by Chanderman et al. For quantitative phytase assay, the amount of liberated inorganic phosphate from sodium phytate was measured. The presence of crude extracellular phytase was determined using a reaction mixture consisting of 800 µL sodium phytate (1% w/v) prepared in sodium acetate buffer (0.2M, pH 5.5) and 200 µL of crude enzyme extract. After incubation at 37±0.1°C for 1 h, the reaction was stopped by heating the reaction mixture at 100°C for 10 min. The liberated phosphate ions were quantified by spectrophotometric method. One unit of phytase activity was defined as the amount of enzyme that released 1 µmol of phosphate per min under the assay conditions.

**Optimization of parameters for phytase production using ‘one-variable-at-a-time’ (OVAT) approach**

Media optimization studies were carried out by varying the composition of MRS broth. Initially, MRS broth medium was used for growth of bacterial cultures. Sodium phytate (0.1%) was then used as phosphorus source in place of KH₂PO₄ in MRS broth (MRS-SP) to reduce the final phosphate content and to induce the enzyme synthesis. Subsequently, the MRS broth was modified in which contents of glucose, yeast extract and beef extract were reduced to 10, 2.0 and 4.0 g/L, respectively and KH₂PO₄ was replaced by 0.65 g/L of sodium phytate (MOD MRS).
The next medium used was modified MRS broth with 3-[N-morpholino] propanesulfonic acid (MRS-MOPS)\(^7\). PSM medium\(^9\) containing 1.5% glucose, 0.1% sodium phytate, 0.2% ammonium citrate, 0.05% KCl, 0.05% MgSO\(_4\).7H\(_2\)O, 0.03% MnSO\(_4\), 0.03% FeSO\(_4\).7H\(_2\)O, 1% peptone, 0.5% sodium acetate, 1% beef extract, 0.5% yeast extract and 1% tween 80 was also used for media optimization studies.

For studying the nutritional effect on phytase production, carbon sources used were: glucose, sucrose, mannose, maltose, fructose and galactose, added at 1% concentration. For nitrogen sources: peptone (1%), beef extract (0.4%), yeast extract (0.2%), ammonium citrate (0.2%) were used eliminating one nitrogen source at a time from the production media. Cultivation parameters studied included incubation temperature (30-45°C) and incubation period (24-96 h) that might affect phytase production from LAB. For optimizing fermentation conditions, OVAT approach was used followed by statistical optimization.

**Molecular characterization of selected LAB isolate**

The genomic DNA extraction of potential phytase producing LAB isolate (grown in MRS medium) was carried out by using DNA purification kit. Identification was based on 16S rRNA gene sequencing. The universal primers used were lac1–27F 5'-AGAGTTTGATC CTGGCTCAG-3' and lac1-1492R 5'-TACGGYTAC CTGGTTACGACT-3'.

PCR studies were carried out under the following conditions: initial denaturation (94°C for 2 min), denaturation (94°C for 30 s), annealing (55°C for 1 min), extension (72°C for 1 min) and final extension (72°C for 10 min). The PCR product obtained was sequenced by AgriGenome Labs Pvt. Ltd., Kerala. Amplicon was studied for sequence homology with those already deposited in the NCBI GenBank DNA database using the Basic Local Alignment Search Tool (BLAST) program (https://blast.ncbi.nlm.nih.gov/BLAST.cgi) and the sequence was submitted to GenBank.

**Statistical optimization of phytase production**

**Plackett–Burman design**

Relevant factors were recognized by Plackett–Burman design. Various parameters involved in phytase production medium were taken into consideration which included initial pH, maltose, and peptone. The other fermentation conditions such as inoculum age, inoculum size, incubation temperature and fermentation period were also considered. Each parameter was examined at two levels (high level and a low level) as illustrated in (Table 1) and these levels were selected on the basis of OVAT approach and literature studies. The experiment was generated and analysed by using Design Expert 6 software (StateEase Inc., Minneapolis, USA).

**Response surface methodology (RSM)**

Four significant variables: incubation temperature, initial pH, maltose and peptone were identified for phytase production, based on Plackett-Burman design and were further chosen for statistical studies using CCD of RSM. Table 2 represents low and high levels of selected variables. A total of 30 experiments were performed as designed by Design Expert 6 software. Validation of the statistical model for phytase production was carried out on the basis of point prediction method.

**Phytase purification**

For protein purification, extracellular enzyme (48 h grown culture) was precipitated at 4°C using different concentrations of ammonium sulphate ranging from 10-100%. The fractions were collected, after centrifugation at 15,000×g for 30 min at 4°C. The precipitates were dissolved in sodium acetate buffer (0.2M, pH 5.5) followed by overnight dialysis in the same buffer at 0.05M concentration. Fractions obtained were analyzed for phytase as well as for protein content. The fractions showing enzyme activity were subjected to hydrophobic column chromatography. For purification of enzyme, linear decreasing gradient was followed at a flow rate of 1.0

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mL/min. Phenyl-Sepharose CL-4B column matrix was calibrated with 0.2M concentration of sodium acetate buffer at pH 5.5. Purification of the phytase protein was confirmed by SDS-PAGE analysis as described by Laemmli. The protein concentration was determined according to the Bradford method using bovine serum albumin.

**Effect of pH and temperature on phytase activity**

Phytase activity was determined using buffer with pH range of 2.5-12.5. The buffers used were: glycine-HCL buffer (pH 2.5), acetate buffer (pH 3.5-5.5), citrate buffer (pH 6.5), glycine-NaOH buffer (pH 8.5-10.5) and KCl-NaOH buffer (pH 12.5). To study the effect of temperature, enzyme substrate mixture was incubated at different temperatures viz. 20, 30, 37, 50 and 60°C. Thermostability of phytase was determined by preincubating the enzyme at 50 and 60°C for 3 h, and then the residual activity was calculated after a regular interval of 30 min.

**Enzyme Kinetics and substrate specificity**

To determine $K_m$ and $V_{max}$, the phytase activity was measured at various substrate (sodium phytate) concentrations ranging from 0.1-2.4 mM in sodium acetate buffer (0.2 M) at pH 5.5. Lineweaver-Burk plot was used for determination of kinetic parameters viz. $K_m$ and $V_{max}$. In order to determine the substrate specificity of the purified enzyme, various phosphorylated substrates i.e. glucose-6-phosphate, sodium $\beta$-glycerophosphate, p-nitrophenyl phosphate were used at 3 mM concentration.

**Probiotic properties**

*Tolerance to simulated gastrointestinal tract conditions*  
To study low pH tolerance, cultures were incubated in 10 mL of phosphate buffered saline (PBS) solution containing 9.0 g/L NaCl, 9.0 g/L Na$_2$HPO$_4$, 2H$_2$O, 1.5 g/L KH$_2$PO$_4$ at 37±0.1°C, pH 6.2 for a period of 3 h and kept as experimental control. The pH 2.0 and 3.0 were taken for studying the tolerance of phytate degrading LAB. To study bile tolerance, bacterial cultures were centrifuged at 4°C (10,000 rpm) for 10 min. The cell pellets were washed and resuspended in PBS solution. 100 µL of cell suspension was added to 900 µL of 0.3% and 0.5% Ox-bile concentrations followed by incubation at 37±0.1°C for a period of 4 h. Zero percent bile concentration was taken as control. Enumeration of the colonies was carried out as stated by Tulumoglu et al. The total survival rate was calculated as described by Guo et al.

For simulated gastrointestinal conditions pepsin (3.0 mg/mL, pH 3.0) and trypsin (1.0 mg/mL, pH 8.0) were used and transits were determined as stated by Guo et al. Pepsin (1:10,000) and trypsin (1:250) were used in the study. Survival rate in the simulated gastric conditions was determined after 3 h of incubation period. For simulated intestinal conditions, the studies were carried out after 4 h of incubation period. MRS agar medium was used to determine the total viable counts of LAB. The incubation conditions were anaerobic and the cultures were kept at 37±0.1°C for 48 h.

**Hydrophobicity**

Adhesion of bacterial cultures to organic solvents (ethyl acetate, xylene or chloroform) was determined by the method given by Andrab et al. 24 h grown culture in MRS broth was centrifuged, the pellets were washed twice and resuspended in Ringer solution (6% NaCl, 0.0075% KCl, 0.01% CaCl$_2$ and 0.01% NaHCO$_3$). The absorbance was determined at 580 nm ($A_{580}$ initial). The ratio of organic solvent to cell suspension was 1:3. For determining the adhesive properties, the bacterial cultures were incubated at room temperature for 10 min followed by vortexing and allowed to stand for 30 min. The absorbance of the aqueous phase was measured at 580 nm and hydrophobicity percentage was calculated using the formula given below:

$$\text{Hydrophobicity} \% = \frac{A_{580}(\text{solvent}) - A_{580}(\text{initial})}{A_{580}(\text{initial})} \times 100$$

**Autoaggregation and coaggregation assays**

Autoaggregation (AAg) ability of bacterial cells was analysed by performing the experiments as reported in literature. The cells were suspended in 4.0 mL of PBS solution, thoroughly mixed by vortexing and incubated at 37±0.1°C for 24 h. Aliquots (0.1 mL of the upper layer of cell suspension) were taken after regular intervals viz. 0, 1, 2, 3 to 4 h and after 24 h. The absorbance was measured at 600 nm and autoaggregation percentage was measured using the formula given below:

$$\text{AAg} \% = \left[ 1 - \frac{A_t}{A_0} \right] \times 100$$

where, $A_0$ represents the absorbance at 0 h and $A_t$ represents absorbance after the time interval of 0, 1, 2, 3, 4 and 24 h.

For coaggregation studies bacterial strains used were: *Bacillus subtilis* (MTCC 121), *Mycobacterium smegmatis* (MTCC 994), *Staphylococcus aureus* (MTCC 3160), *Proteus vulgaris* (MTCC 426) and *Escherichia coli* (MTCC 1652). The selected strain
was grown in MRS broth but the test organisms were grown in nutrient broth at 37±0.1°C for 24 h in order to attain viable counts of approximately 10^7-10^8 cfu/mL (A_{600} 0.85-0.9). Two milliliter of experimental cell suspension was mixed with a bacterial strain and incubated at 37±0.1°C for 24 h. Aliquots (0.1 mL of upper layer) were taken at regular time intervals from 0 h - 4 h and after 24 h. The absorbance was measured at 600 nm. Coaggregation percentage was calculated using the following formula:

\[
\text{Coaggregation (\%)} = \left( \frac{A_{\text{pat}} - A_{\text{pat} + \text{probio}}}{A_{\text{pat} + \text{probio}}/2} \right) \times 100
\]

where, A_{\text{pat}} represents the absorbance of pathogenic and A_{\text{probio}} represents the absorbance of probiotic strains, and A_{\text{pat} + \text{probio}} is the absorbance (A_{600 nm}) of mixed bacterial suspensions taken after the time intervals of 0, 1, 2, 3, 4 and 24 h.

**Susceptibility to antibiotics**

The antibiotic susceptibility for LAB was determined by agar overlay disc diffusion method. Hi-Media Octadisc Combi 505 [cefoperazone (75 µg), cefpodoxime (30 µg), cefazidime (30 µg), cefepime (30 µg), imipenem (10 µg), gentamycin (10 µg), amikacin (30 µg), moxifloxacin (5 µg)]; Hi-Media Octadisc combi III [ampicillin (10 µg), cefotaxime (30 µg), cephalexin (30 µg), co-trimoxazole (25 µg), gentamycin (10 µg), nalidixic acid (30 µg), nitrofurantoin (300 µg), norfloxacin (10 µg)] were used. Antibiotics, streptomycin (25 µg), tetracycline (30 µg), erythromycin (15 µg), rifampicin (5 µg), amoxicillin (10 µg), penicillin G (10 µg), and vancomycin (30 µg) were also tested to check the antibiotic susceptibility of experimental strain. MRS media was used for growing experimental strain and the Petriplates were incubated at 37±0.1°C for a period of 24 h. The Petriplates were examined for the presence of inhibition zones around the antibiotic disks.

**Results**

**Isolation and selection of phytase producing lactic acid bacteria**

Twenty LAB isolates capable of growing on MRS medium were isolated from Kalarei samples. These Gram positive and catalase negative strains were screened for phytate hydrolysing potential. For qualitative analysis plate assay method was adopted. Phytase producing LAB formed clear zones on PSM agar media (containing 0.1% sodium phytate as substrate). Out of twenty LAB isolates, screened for phytate degrading activity four strains failed to show clear zones on PSM agar while no intracellular activity was observed in all the LAB isolates. For quantitative analysis, modified MRS (MRS-MOPS) medium was used with 0.065% of sodium phytate. Isolate K.J (a) and isolate K revealed highest extracellular phytase activity (5.18±0.09 and 5.0±0 U/mL, respectively) compared to other strains (Fig. 1) and these two isolates were selected for further studies.

**Optimization of process parameters for phytase production using classical approach**

Initially, phytase production was optimized using OVAT approach. The effect on production of phytase was studied using different media, carbon and nitrogen sources, incubation temperatures and fermentation period. Modified MRS-MOPS served as the best phytase production media with enzyme activity 5.18±0.09 and 5.0±0 U/mL, in case of isolate K.J (a) and K, respectively (Fig. 2A). Maximum phytase production was observed at 37±0.1°C, after an incubation period of 48 h. While replacing glucose for maltose in MRS-MOPS, phytase production increased from 5±0 U/mL to 5.923±0.09 U/mL in case of isolate K which was higher than that of K.J (a) (Fig. 2B). Production media lacking peptone showed maximum decline in phytase production from 5.923±0.09 to 1.2±0.09 U/mL for isolate K which was higher than that of K.J (a) (Data not shown), thus peptone among different nitrogen sources found to be the most influential on phytase production. Maltose as a carbon source and peptone as nitrogen source were used in the subsequent experiments. Phytase production from isolate K was further optimized using statistical approach.

**Identification of isolate K**

Based on morphological and biochemical characteristics, isolate K showing highest (5.923±0.09)
phytase production after OVAT approach, was identified as Gram-positive, non-spore forming, homofermentative bacteria. 16S rRNA sequence analysis of the strain K showed high level of similarity with that of available strains of *Lactobacillus paracasei* in NCBI GenBank database and was identified as *L. paracasei*, and marked as *L. paracasei* SMVDUDB1 (Fig. 3). The sequence has been deposited in GenBank nucleotide sequence database under the accession number MK280749.

Optimization of process parameters for phytase production using statistical approach

**Plackett–Burman design (PBD)**

PBD was employed in order to determine the most effective variables that can influence the phytase production from *L. paracasei* SMVDUDB1. The effect of fermentation period, inoculum age, inoculum size, incubation temperature, initial pH, maltose and peptone was examined by the PBD experiment using a set of 12 runs (Suppl. Table 1. All supplementary data are available only online along with the respective paper in CSIR-NISCAIR repository NOPR at http://nopr.res.in). The p-values of fermentation period, inoculum size, incubation temperature, initial pH, maltose and peptone were less than 0.05. Therefore, these were recognised as significant model terms. The result of analysis of the variance (ANOVA) for the experimental data illustrated the significance of the model (Suppl. Table 2). The analysis of regression coefficient values depicted that incubation temperature, initial pH, maltose and peptone had affected the phytase production positively whereas fermentation period and inoculum size had a negative impact on phytase production. Therefore, incubation temperature, initial pH, maltose and peptone parameters were taken for further studies.

**Response surface methodology (RSM)**

RSM using CCD was applied in order to determine the optimal levels of the variables. The interactive effects of the four selected variables, peptone (A), temperature (B), pH (C) and maltose (D) were studied for enhanced phytase production in case of *L. paracasei* SMVDUDB1. The corresponding response of 30 experimental runs is given in Table 3. The result of analysis of variance (ANOVA) is depicted in Table 4.

The response surface (3D) plot was generated among two positive interacting variables (peptone and pH) and Fig. 4 confirms that phytase activity increases with increase in peptone concentration and pH value. The regression equation generated by the design-expert software is given below (obtained in terms of coded factors):

\[
Y = +5.89 - 0.43 (A) - 0.066 (B) + 0.077 (C) - 0.60 (D) - 0.38 (A^2) - 0.11 (B^2) - 0.38 (C^2) - 0.53(D^2) - 0.059 (AB) - 0.32 (AC) + 0.13 (AD) + 0.014 (BC) + 0.044 (BD) + 0.20 (CD)
\]

where, *Y* represents the predicted response of phytase production and *A*, *B*, *C* and *D* represent peptone, temperature, pH and maltose, respectively. The *F* value, 9.16 of model describes that the model is significant. There is only a 0.01% chance that a large “model *F* value” could occur due to noise. The values of *P* < 0.05 indicate that the model terms were
significant. The “Lack of Fit F-value” of 4.29 signified that lack of fit was not significant relative to the pure error. The coefficient of variation (CV) value of 9.86 denotes that the experiments are reliable. The goodness of the fit model was explained by the determination of coefficient, i.e. R square. The R square obtained was 0.895, which is closer to the value of 1.0, thus showing high correlation between the observed and predicted response. Adequate precision measures the signal to noise ratio and a ratio greater than 4 is desirable. The present model designated a ratio of 10.047, thus indicating an adequate signal. The results attained evidently suggest that the present quadratic model is significant to explain the phytase production process. Point prediction tool of RSM was used for validation of the statistical model. Optimum value of four variables, peptone (1.12%); temperature (37°C); pH (6.27) and maltose (1.39%) were employed and experiment was conducted which revealed that the predicted response for phytase production (6.252 U/mL) was in good agreement with the actual response (6.375 U/mL), indicating the suitability of the model.

**Phytase purification**

Extracellular phytase from *L. paracasei* SMVDUDB1 was purified using ammonium sulphate precipitation for partial purification and hydrophobic column chromatography. The ammonium sulphate fractions 30-40, 40-50 and 50-60% showing phytase activity were pooled and 94.86% of the total protein was removed. Specific activity observed was 157.451 U/mg proteins with 11.582% yield (Table 5). The partially
puriﬁed phytase from *L. paracasei* SMVDUDB1 was then subjected to hydrophobic interaction chromatography using Phenyl-Sepharose CL-4B column. About 97.33% reduction in total protein was achieved after this technique. Extracellular phytase was puriﬁed approximately 4 fold from the crude extract with speciﬁc activity of 278 U/mg protein and 10.6% enzyme yield (Table 5). The purity of phytase was conﬁrmed by SDS-PAGE which revealed the appearance of a single protein band (Fig. 5). The molecular weight of extracellular phytase was found to be 70 kDa.

**Effect pH and temperature on phytase activity**

*Lactobacillus paracasei* SMVDUDB1 exhibited maximum phytase activity at 37°C and pH 5.5, the enzyme activity was quite stable between temperatures 30 and 60°C and pH between 3.5 and 6.5 (Data not shown).

Moreover, puriﬁed phytase revealed excellent thermostability up to 60°C as no reduction in the enzyme activity was observed at 50 and 60°C after an incubation period of 1 h. However, only 23.80 and 39.68% reduction in activity was observed at 50 and 60°C after an incubation of 3 h, respectively (Suppl. Fig. 1).

**Enzyme kinetics and substrate speciﬁcity**

Kinetic parameters *Km* and *Vmax* were determined at various substrate concentrations of sodium phytate (0.1-2.4 mM) using Lineweaver-Burk plot. *L. paracasei* SMVDUDB1 showed *Km* and *Vmax* values of 0.51 mM and 6.046 μmol/min, respectively (Suppl. Fig. 2).

The relative enzyme activity of *L. paracasei* SMVDUDB1 was studied to dephosphorylate substrate in comparison to sodium phytate. The puriﬁed enzyme exhibited a relative activity of 70.01% with p-nitrophenyl phosphate as substrate and 44.29% with glucose-6-phosphate while no activity was observed with sodium β-glycerophosphate (Suppl. Table 3).

**Probiotic properties**

**Tolerance to simulated gastrointestinal tract conditions**

In case of *L. paracasei* SMVDUDB1, survival rate observed was maximum at 0.3% bile salt concentration (93.82±0.12) and pH 3.0 (99.8% ±0.04) under *in vitro* simulated gastrointestinal tract conditions while a slight decrease in the survivability was observed at 0.5% bile salt concentration (83.56±0.12) and pH 2.0 (88.36±1.24). *L. paracasei* SMVDUDB1 showed tolerance to gastrointestinal conditions when enzymes like pepsin and trypsin were used, without a significant decrease in survival rate, as shown in Table 6.

**Hydrophobicity, autoaggregation (AAg) and coaggregation**

*Lactobacillus paracasei* SMVDUDB1 has shown highest surface hydrophobicity (96.74±0.09%) with chloroform followed by ethyl acetate and xylene (Table 6). AAg and coaggregation percentage was calculated after regular time intervals viz. 1, 2, 3, 4 and then 24 h. AAg ability was above 50% at 37±0.1°C after an incubation period of 4 and 24 h. AAg increased gradually with the time of incubation. *L. paracasei*SMVDUDB1 exhibited high variability in coaggregation with standard strains from MTCC, belonging to Gram positive and Gram negative groups (Table 6). Considerable coaggregation (60-70%) was observed with *M. smegmatis*, *P. vulgaris* and high coaggregation ability (80-90%) was observed with *B. subtilis*, *S. aureus* and *E. coli* at 37±0.1°C after an incubation period of 1, 2, 3, 4 and then 24 h. Coaggregation increased with time of incubation and maximum coaggregation was observed at 24 h incubation period.

<table>
<thead>
<tr>
<th>Steps of purification</th>
<th>Volume (mL)</th>
<th>Activity (U/mL)</th>
<th>Protein (mg/mL)</th>
<th>Total activity (U)</th>
<th>Total protein (mg)</th>
<th>Specific activity (U/mg)</th>
<th>Purification fold</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude enzyme</td>
<td>100</td>
<td>6.294</td>
<td>0.090</td>
<td>629.4</td>
<td>9</td>
<td>69.933</td>
<td>1</td>
<td>100</td>
</tr>
<tr>
<td>Ammonium sulphate precipitation (30-60%)</td>
<td>2.5</td>
<td>29.16</td>
<td>0.185</td>
<td>72.9</td>
<td>0.463</td>
<td>157.451</td>
<td>2.251</td>
<td>11.582</td>
</tr>
<tr>
<td>Hydrophobic interaction Chromatography</td>
<td>12</td>
<td>5.56</td>
<td>0.020</td>
<td>66.72</td>
<td>0.24</td>
<td>278</td>
<td>3.975</td>
<td>10.60</td>
</tr>
</tbody>
</table>

Table 5 — Purification steps for *L. paracasei*SMVDUDB1 phytase

Fig. 5 — SDS-PAGE analysis of phytase from *L. paracasei* SMVDUDB1. Lane 1: protein molecular weight marker, mass (in kDa) and Lane 2: purified enzyme after hydrophobic column chromatography.
Phytases are widely distributed among different sources including plants, yeast, bacteria, fungi, and certain animal tissues \(^ {32,28,29}\). However, phytase from microbial sources are the most promising for commercial production due to their higher production potential. Microbial phytases particularly from filamentous fungi being the widely studied ones \(^ {33,31}\), have found their place in market. Bacterial phytases, owing properties as high temperature stability, substrate specificity and stability at wide pH range make it a better alternative source for phytase production \(^ {10}\). Probiotic strain with phytase activity is a desirable characteristic for reducing the antinutritional effects of phytate rich diets and to improve the quality of food/feed \(^ {16,26}\), as phytase production by probiotics showing high survival rate in adverse gut conditions, can meet the criteria that have been interesting to the researchers \(^ {32,33}\). But several phytase producing bacteria while passing through harsh gastrointestinal tract (GIT) conditions lose their viability and ability to produce enzymes at low pH and high bile salt concentrations \(^ {34}\). \textit{L. paracasei} is regarded as GRAS (Generally Recognised as Safe) organism, and a potential strain having high survival rate in GIT conditions, and thus can be used in food processing \(^ {35}\).

In the present study, \textit{L. paracasei} SMVDUDB1 was isolated from the local fermented milk product Kalarei. As per earlier reports, probiotic bacteria like \textit{Weissella kimchii} R-3 (1.77 U/mL) \(^ {26}\) and \textit{Lactobacillus plantarum} (6.3 mU/mL) \(^ {36}\) have been reported for extracellular phytase production but the present study has shown maximum production of the enzyme (6.375 U/mL). Optimization of fermentation parameters: incubation temperature, initial pH, maltose and peptone have played an important role in evaluating total efficiency of phytase production from \textit{L. paracasei} SMVDUDB1. In our case study, modified MRS-MOPS supported maximum phytase yield which was in accordance with the earlier reports where phytase production was reported from \textit{L. pentoses} \(^ {37,38}\) and \textit{Pediococcus pentosaceus} \(^ {17}\). As per available literature, maltose has been reported as...
the best carbon source for *L. sanfranciscensis*<sup>30</sup> and *L. pentoses*<sup>38</sup> while peptone as the best nitrogen source for *L. casei*<sup>39</sup>. Our results are in accordance with the earlier reports. OVA T studies have shown that incubation at 37±0.1°C for a period of 48 h is optimal for extracellular phytase production from *L. paracasei* SMVDUDB1, similar reports were observed for *L. casei*<sup>39</sup>. Optimization of phytase production from *L. paracasei* using Plackett–Burman design and RSM has been reported in present study. After OVA T and RSM studies, the optimal values of significant variables: incubation temperature, 37°C; initial pH, 6.27; maltose, 1.39 and peptone, 1.12% increased the phytase production from 5.0 to 6.375 U/mL which is almost double than that of the phytase produced (3.248 U/mL) from *Thermomyces lanuginosus*<sup>40</sup> using RSM.

Nitrogen in the production media played a crucial role in metabolite production including enzymes<sup>41</sup>. Reports have shown that peptone plays a significant role in phytase production from *Enterobacter* sp.<sup>18</sup> but maltose as a carbon source increased the biomass yield in probiotic strains like *L. fermentum*<sup>42</sup> and *L. lactis*<sup>43</sup>. Temperature is another important parameter that has critical role on microbial growth, maintenance of activity and structural stability of enzyme and thus ultimately the cost of enzyme<sup>44</sup>. In microbial fermentations, pH has played a strong influencing factor. The optimal pH 6.27 was observed for phytase production from *L. paracasei* SMVDUDB1 which fairly matches with the pH of MRS media used for the cultivation of *Lactobacilli*<sup>38,45</sup>.

Enzyme purification is an important step to isolate a protein of interest and to eliminate other proteins. Moreover, it assists in acquiring information regarding biochemical properties, structural-functional relationship of purified enzyme and to predict its application. The enzyme was purified 3.975 fold from the crude extract with 10.60% yield and exhibits specific activity of 278 U/mg (Table 5). As per the earlier reports, phytase enzymes were purified 9.53 and 4.93 fold from *L. coryniformis*<sup>15</sup> and *Geobacillus* sp. TF16<sup>20</sup> with yield 2.60 and 3.13%, respectively, while specific activities were 202.25 and 1080.07 U/mg.

As per the available literature, biochemical characterization of phytases depends upon the microbial source from where it is reported and so the molecular weight differs from 35-700 kDa<sup>46</sup>. The purified enzyme from *L. paracasei* SMVDUDB1 has shown an apparent molecular mass of 70 kDa on SDS-PAGE gel (Fig. 5) which is in accordance with the molecular weight of earlier reports i.e. phytase reported from *L. pentosus* CFR<sup>38</sup>. Phytases with different molecular mass have been reported in case of *L. sanfranciscensis* (50 kDa)<sup>30</sup> and *Enterobacter* sp. ACSS (62 kDa)<sup>19</sup>. *L. paracasei* SMVDUDB1 phytase exhibited maximum activity at 37°C and pH 5.5 which is similar to the characteristic pH of stomach after initial ingestion of feed/food<sup>47</sup> at normal body temperature. Optimum temperature and pH for most of the phytase degrading enzymes ranged between 35 and 77°C; and pH 4.5-6.0, respectively<sup>28</sup>. Thermostability of phytase is an important criterion required for industrial application of enzyme. *L. paracasei* SMVDUDB1 phytase was quite stable at 50 and 60°C after an incubation period of 3 h.

*L. paracasei* SMVDUDB1 phytase exhibited low *K*<sub>m</sub> value of 0.51 mM for sodium phytate which is in accordance with the literature studies<sup>15</sup>. Wide range of substrate specificity is a desirable characteristic feature of enzyme for potential commercial applications. Phytase used in present study exhibited wide range of substrate specificity towards sodium phytate, glucose-6-phosphate and *p*-nitrophenyl phosphate. Literature studies have shown that phytase from *L. sanfranciscensis* CB1 have shown highest activity with sodium phytate and *p*-nitrophenyl phosphate.<sup>30</sup>

To consider a bacteria to be a potential probiotic, it should tolerate and survive harsh GIT conditions like tolerance to acidic pH (2.0-3.0), bile salt concentrations (0.3-0.5%) and to simulated gastric (pepsin at 3.0 mg/mL, pH 3.0) and intestinal conditions (trypsin at 1.0 mg/mL, pH 8.0). Similar to the current study, *L. paracasei* K5 isolated from Feta-type cheese exhibited high survival at low pH from 2.0-4.0 (6.1-9.3 log cfu/mL), bile concentration of 0.5% w/v (8.5 log cfu/mL) and in the presence of pepsin (6.1 log cfu/mL) and pancreatin enzymes (7.8 log cfu/mL).<sup>48</sup> According to Zhang et al.<sup>49</sup> different strains of *L. casei* exhibited variability in survival rate 86-97% at pH 3.0 while at pH 2.0, 81-93% survival rate was reported. Surface characteristics viz. hydrophobicity, autoaggregation and coaggregation ability of the cell are essential prerequisites for probiotic strain that determine its adhesion ability to epithelium and provide health benefits to the host.<sup>50</sup> *L. paracasei* SMVDUDB1 exhibited excellent hydro-phobicity.
potential with chloroform (96.74±0.09%) which is much higher than that reported previously in *L. acidophilus* M92 (36.06%)\(^5\). In the present study, minimum hydrophobicity was observed with xylene while *L. rhamnusos* strains isolated from human milk showed high hydrophobicity of 68-69% with xylene\(^5\). Coaggregation ability of probiotics prevents adhesion of pathogens on enteric epithelium, thus helps in eliminating enteric pathogens from the gastrointestinal tract\(^1\). *L. paracasei* SMVDUDB1 in the present study exhibited highest coaggregation (82.79±0.18%) with *B. subtilis*. Resistance of the probiotic strains to antibiotics could be used for both preventive and therapeutic purposes in controlling intestinal infections\(^5\). However, sensitivity of LAB towards antibiotics is one of the important criteria for the safety of probiotics\(^4\). *L. paracasei* SMVDUDB1 exhibited susceptibility towards commonly used antibiotics like erythromycin and streptomycin similar results for *L. paracasei* F08 were reported by Wang et al.\(^5\). *L. paracasei* SMVDUDB1 has shown resistance against vancomycin. Intrinsic resistance to vancomycin has been reported in *L. plantarum*, *L. paracasei* and *L. salivarius*\(^3\). Recently, many probiotic bacteria because of low phytase activity and having GRAS status have been effectively used for recombinant phytase expression\(^1\). Besides having benefits, there are serious challenges in employing these recombinant probiotic phytases which include high species diversity, obscurity in the route of administration, and stringent monitoring due to lack of clinical trial data\(^5\). Hence, *L. paracasei* SMVDUDB1 having innate phytase producing ability which can be preferred over recombinant probiotic phytases.

**Conclusion**
Phytase production from *L. paracasei* SMVDUDB1 was enhanced by optimizing various physico-chemical factors using OVAT and response surface methodology. The extracellular phytase from *L. paracasei* SMVDUDB1 has molecular weight of 70 kDa. The hydrophobic interaction chromatography has shown 3.975 fold purification of enzyme with specific activity of 278 U/mg. It was found that optimal pH (5.5) and temperature (37°C) for phytase from *L. paracasei* SMVDUDB1 matches well with the normal physiological conditions of the body. *L. paracasei* SMVDUDB1 also exhibited desired probiotic properties viz. hydrophobicity, autoaggregation and coaggregation with survival rate above 80% at low pH, high bile salt concentrations under simulated GI conditions. All these characteristics of the probiotic strain *L. paracasei* SMVDUDB1 contribute towards efficient phytase activity in the gastrointestinal tract revealing its potential to possibly address mineral deficiency in feed/food industry.

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**Conflict of Interest**
The authors declare no conflict of interest.

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