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Optimization of Culture Conditions and Partial Purification of Phytase Enzyme Isolated from Three Lactic Acid Producing Bacteria

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ABSTRACT

Background: Phytic acid is an anti-nutritional agent in cereal foods that causes health problems in humans and animals by chelating minerals thereby reducing their intestinal uptake. This study investigated the effects of culture conditions on phytase enzyme production by three bacterial strains.

Methods: The three bacterial strains were isolated from traditional fermented foods and confirmed by 16S Ribosomal RNA as *Lactococcus lactis* LAC460, *Lactobacillus fermentum* SCB0035 and *Lysinibacillus macroides* DSM54. The phytase-producing bacteria strains were isolated using a phytase screening agar medium. Various factors that could enhance culture conditions for maximum production of phytase from bacteria strains were investigated.

Results: All the isolates attained maximum growth after 36 h of fermentation, and the absorbance was 0.85, 0.88, and 0.84 at 700 nm for *Lactococcus lactis* LAC460, *Lactobacillus fermentum* SCB0035, and *Lysinibacillus macroides* DSM54 respectively. The combination of 0.75% mannose, 0.75% sucrose and yeast extract were the most efficient carbon and nitrogen sources for phytase production by all the strains isolated while corn starch and soybean meal were the most efficient alternative carbon and nitrogen sources for phytase production. The optimum pH range and temperature for the activity of phytase were found to range from 6.5 to 7.5 and 40 to 60℃ for all the strains. The partially purified enzyme from *L. fermentum* SCB0035 was 50% stable for 1 h at 80°C. $CuSO_4$ and $FeSO_4$ inhibited phytase activity in a concentration-dependent manner while $CaCl_2$ and $MgSO_4$ increased phytase activity in a concentration-dependent manner.

Conclusion: Optimal conditions were pH 7.0, temperature at 50°C and absence of certain cations. This study suggests that the phytase enzyme obtained from the bacteria strains most especially *L. fermentum* SCB0035 can be exploited in upgrading the nutritional status of animal diets and for reducing phosphorus pollution in livestock production.

INTRODUCTION

Phosphorus is a limiting nutrient for animals because most of it in plant seeds occur in the form of phytic acid that cannot be digested by monogastric animals, and acts as an antinutritional factor hindering the uptake of a range of minerals (Rocky-Salimi *et al.,* 2016). Phytic acid occurs as negatively charged phytate often in the form of monovalent, divalent, or trivalent metal salts (Von Grebmer *et al.,* 2014). This is due to the varying affinity of phytic acid protons to dissociate in the pH range of the stomach and intestine (Sreedevi and Reddy, 2013). Phytic acid salts, also known as phytate, myo-inositol and hexakis dihydrogen phosphate, are the principal storage (1–5% by weight) form of both myo-inositol and phosphate in cereal grains, legumes, oilseeds, plant seeds, and nuts (Priyodip and Balaji, 2018). These phytic acid salts are not readily soluble under the pH conditions of the upper gastrointestinal tract, where most minerals are absorbed, and tend to precipitate with increasing pH along the intestine (Rutherfurd *et al.,* 2014).

Also, monogastric animals such as swine and poultry cannot metabolize phytate phosphorus owing to the lack of digestive enzymes that can hydrolyze the substrate, hence inorganic phosphate is added to their diet to meet the phosphorus requirement, while undigested phytate phosphorus is excreted in manure and causes a serious phosphorus pollution problem, leading to the eutrophication of surface water in areas of intensive livestock production. In addition, phytic acid also acts as an antinutritional agent by forming complexes with carbohydrates, proteins, lipids, and metal ions, leading to a decrease in the dietary bioavailability of these nutrients (Bindu *et al.,* 1998; Batal and Abdelkarim, 2001). It also interacts nonspecifically with enzymes such as trypsin, α-amylase, pepsin, and β-galactosidase, resulting in decrease of their activity.

Enzymes involved in hydrolysis of phytate are known as phytases. Phytases (myo-inositol hexakisphosphate phosphohydrolases; EC 3.1.3.8/EC 3.1.3.26) are the enzymes that catalyze the hydrolysis of phytate in a stepwise manner releasing solubilized forms of inorganic phosphate, myo-inositol and lower forms of inositol phosphate (Askelson *et al.,* 2014; Zeller *et al.,* 2015). This hydrolytic reaction plays an important role in energy metabolism, metabolic regulation, and signal transduction pathways in biological systems. Phytase is commonly used as an exogenous enzyme in the feed for monogastric animals. Phytase can reduce the antinutritional effect of phytate and improve the digestibility of phosphorous (P), calcium (Ca), amino acids and energy, as well as reduce the negative impact of inorganic P excretion on the environment. Phytases that hydrolyze phytate to less phosphorylated myo-inositols and inorganic phosphate can be used as feed supplements in the production of animal feed, processing and manufacturing of human food, and additives to improve mineral nutrition. This will go a long way in achieving food security, improving nutrition, and promoting sustainable agriculture as stipulated in one of the themes of Sustainable Development Goals (SDG).

Although phytase producing microorganisms have been reported, most of them have limitations such as producing the enzyme intracellularly and having low enzyme yields (Palacious *et al.,* 2005; Sumengen *et al.,* 2013; El-Touky *et al.,* 2013). Therefore, scientists are searching to isolate novel phytase-producing microorganisms and equally optimize the culture conditions for enzyme. In this study, phytase enzyme was isolated from *Lactobacillus fermentum* SCB0035, *Lactococcus lactis* LAC460 and *Lysinibacillus macroides* DSM54. The culture conditions to produce the enzyme were optimized and the enzyme from the best-producing strain was partially purified and characterized.

MATERIALS AND METHODS

Microorganisms

Three bacterial strains were isolated from traditional fermented foods obtained from different markets in Umuahia metropolis. The bacterial strains were isolated using deMan Rogosa Sharpe Agar (MRS) and incubated anaerobically at 37^{o}C for 48 h in anaerobic jars with gas park inside (deMan *et al*., 1960).

Preliminary Assessment of Bacteria Isolates to Produce Extracellular Phytase

The screening for extracellular phytase production was carried out according to the method of Bae *et al.* (1999) using phytase screening agar (PSA) described by Taheri *et al.* (2009) with slight modifications. Approximately 10 g of traditional fermented food samples (White-Maize *Ogi*, Yellow-Maize *Ogi* and *Kunun-zaki*) were suspended in 90 mL of physiological saline and an aliquot (0.1 mL) of this suspension was streaked onto phytase specific medium [1.5% glucose, 0.5% $(NH4)_2SO4$, 0.05% KCl, 0.01% $MgSO_4.7H_2O$, 0.01% NaCl, 0.01% $CaCl_2.2H_2O$, 0.001% $FeSO_4$, 0.001% $MnSO_4$, pH 7.0 with 0.5% sodium phytate (Sigma-Aldrich USA). The medium was sterilized at 121°C for 15 min, poured into sterile plates and allowed to set. The isolates were streaked on the dried plates and the plates were incubated at 37°C for 48 h. Colonies that were surrounded by clear zones on the PSA medium were selected and flooded with 2% (w/v) aqueous cobalt chloride ($CoCl_2$) solution and incubated at room temperature for 30 min. To eliminate false positive results that may be caused by acid production, a freshly prepared mixture of 6.25% (w/v) ammonium molybdate $(NH4)_6Mo_7O_{24}$ and 0.42% (w/v) ammonium heptavanadate (NH_4VO_7) solutions were added to the plates. The plates were incubated at room temperature for 5 min. The colonies that maintained the zone of hydrolysis were selected (Bae *et al.,* 1999).

Determination of Phytase Enzyme Activity

The phytase enzyme assay was done using ammonium-molybdate blue method as described by Nielsen *et al.* (2008). The isolates were cultivated in sterile modified Phytase Screening Broth (PSB) with pH adjusted to 7 in test tubes (Taheri *et al.,* 2009). The culture supernatant was collected by centrifuging at 8000 *g* for 10 min. A 10 µL of the supernatant (sample) was transferred into a clean test tube. The test tube was allowed to stand in a water bath at 37℃ for 5 min and 350 µL of buffered sodium phytate (substrate) solution was added to the sample, mixed, and incubated in the water bath at 37℃ for 30 min to allow for reaction. A quantity (1 mL) of 5% trichloroacetic acid (TCA) was added to stop the reaction. Colour reagent (1 mL) freshly prepared from 10% (w/v) ammonium molybdate solution in 5 M sulfuric acid solution, was added and the mixture was shaken thoroughly. The mixture was then centrifuged at 8000 *g* for 10 min and allowed to stand at room temperature for 10 min before the absorbance was read at 700 nm using a spectrophotometer (Jenway 6310 model). The sample blank was prepared using the above description except that the stop solution was added before the substrate solution. In order to obtain the standard curve from which the amount of liberated inorganic phosphate was extrapolated, the above procedure was followed using potassium dihydrogen phosphate (KH_2PO_4) as the sample solution. A standard blank was also prepared for the sample blank and absorbance was read accordingly after which a graph of absorbance against concentration was drawn. One unit (U) of phytase activity was defined as the amount of enzyme that produced 1 µM of inorganic phosphorous per minute at 37°C. Phytase activities (U/mL) were calculated according to the equation.

$$Activity\ (U/mL) = \Delta Cp/\Delta t\ \times\ V_{Tot}/V_{Sample}$$

Where V_{Tot} is the total sample volume (mL); V_{Sample} is the volume of extracellular enzyme extract (mL); ΔCp is measured phosphate concentration during the time (mmol/mL); Δt is reaction time (min) (Cizeikiene *et al.,* 2015).

Estimation of Growth and Phytase Production

A loopful of bacteria culture was transferred to 150 mL of phytase production medium in a 250-mL Erlenmeyer flask, and the culture was incubated at 37°C with shaking at 150 rpm. For the determination of the growth curve, 5 mL of the culture medium was withdrawn at regular intervals of 12 h and the cell density determined at 700 nm up to 72 h (Hosseinkhani *et al.,* 2009).

Effects of Culture Conditions on Phytase Production

Effect of carbon sources on phytase production

The effect of carbon sources was studied to optimize the production of the phytase enzyme from the isolates. Different carbon sources including maltose (1.5%), sucrose (1.5%), mannose (1.5%), maltose and sucrose (0.75% each), maltose and mannose (0.75% each), and sucrose and mannose (0.75% each) were added instead of the original carbon source (glucose) in the phytase production medium and the effect on the phytase production was observed.

Effect of agricultural substrates on phytase production

Isolates were also inoculated into different phytase screening broths containing 5% agricultural substrates including corn starch, cassava starch, sweet potato starch, rice starch, and yam starch prepared according to the method of Ahmed *et al.* (2022) and incubated at 37ºC anaerobically for 24 h. During incubation, the flask was shaken twice a day to achieve homogeneity before the enzyme production level in the culture supernatant was measured. All the experiments were carried out in triplicate.

Effect of nitrogen sources on the phytase enzyme production

Different nitrogen sources including beef extract, yeast extract, ammonium sulphate and ammonium chloride each at 0.5% concentration were used. Also, soybean meal and groundnut cake prepared according to the method of Ahmed *et al.* (2022) were used in the phytase production medium to optimize the production of the phytase enzyme from the isolates.

Effect of temperature on the phytase enzyme production

To study the optimal incubation temperature for maximum phytase production, the flask containing the production medium was incubated at 20-70°C at intervals of 10°C for 24 h keeping all other conditions at their optimum level.

Effect of pH on the phytase enzyme production

Phytase screening media with different pH values of 4.5, 5.5, 6.5, 7.5, 8.5 and 9.5 were prepared to produce phytase enzyme produced from the isolates. The different pH values were prepared using different kinds of buffer, depending on the effective pH ranges; a citrate buffer for pH 4.5-5.5, a tris-maleate buffer for pH 6.5, a tris-HCl buffer for pH 7.5-8.5, and a carbonate-bicarbonate buffer for pH 9.5 (Temizkan and Arda, 2008).

Partial Purification of Phytase Enzyme

The crude enzyme preparation obtained from *Lactobacillus fermentum* SBC0035 was precipitated with ammonium sulphate (80% saturation) as suggested by Fu *et al.* (2008). The protein precipitate was dissolved in a minimal volume of double-distilled water and the resulting enzyme was dialyzed against buffer A (tris-HCl buffer, 0.025 M, pH 8.0) overnight at 4℃. The dialyzed sample was subjected to diethyl-aminoethyl cellulose (DEAE cellulose) chromatography (Merck, Bangalore, India) using buffer A, and the bounded protein was eluted with a linear gradient of NaCl (0-1 M). The fractions having phytase activity were combined and concentrated using ammonium sulphate

(80% saturation) and dialyzed against buffer B (tris-HCl buffer, 0.05 M, pH 8.0). The concentrated sample was loaded on a pre-equilibrated Sephadex G-75 column (Amersham Biosciences, SE-751 84, Uppsala, Sweden) and eluted with buffer B.

Thermal Stability of the Phytase Enzyme

The thermal stability of the purified phytase was determined by incubating the enzyme with 10 mM sodium phytate in a concentration of 100 mM sodium acetate buffer pH 5 at 50 to 90°C at intervals of 10°C for different incubation periods of 0, 1, 4, 8, 16 and 24 h (El Toukhy, 2001).

Effect of Metal Ions on the Phytase Enzyme Activity

The effect of metal cations, in the form of metal salts ($CuSO_4$, $FeSO_4$, $CaCl_2$, and $MgSO_4$) on the phytase enzyme activity was determined. Ten microlitres of each metal salt was added at a concentration of 0.5, 1, 1.5, 2, 5, to 10 mM of the partially purified phytase enzyme. The mixture was incubated at 37°C for 1 h. After incubation, a reaction was started by adding 100 mM sodium citrate buffer (pH 5) containing 2 mM sodium phytate, and the enzyme activity was analyzed under standard conditions (Sümengen *et al.*, 2012).

Statistical Analysis

To discern meaningful disparities among strains, the phytase activity data were subjected to ANOVA with isolates as a fixed factor, followed by comparative analysis involving the Duncan test (Ohaegbu *et al*., 2022).

RESULTS

Phytase Enzyme Activity by the Bacteria Isolate Assay

Lactobacillus fermentum SBC0035 had the highest phytase activity (706 U/mL), while *Lysinibacillus macroides* DSM54 had the least phytase activity (650 U/mL) as shown in Table 1.

Table 1: Maximum phytase activity of bacteria isolates

Isolate	Phytase activity (U/mL)
Lactobacillus fermentum SCB0035	706
Lactococcus lactis LAC460	665
Lysinibacillus macroides DSM54	650

Growth of Isolates and Phytase Production

All the isolates attained maximum growth after 36 h of fermentation (Figures 1, 2, 3). Growth and phytase production declined with increasing time of incubation. The isolates reached stationary phase after 48 h.

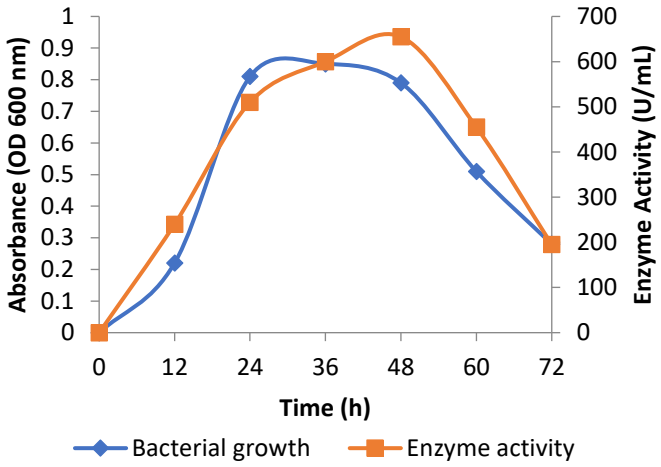

Figure 1: Growth and phytase production of *Lactococcus lactis* LAC460 in phytase screening medium.

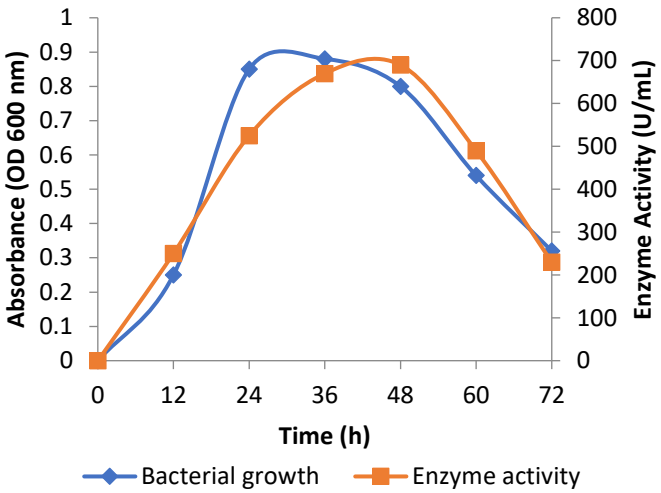

Figure 2: Growth and phytase production of *Lactobacillus fermentum* SCB0035 in phytase screening medium.

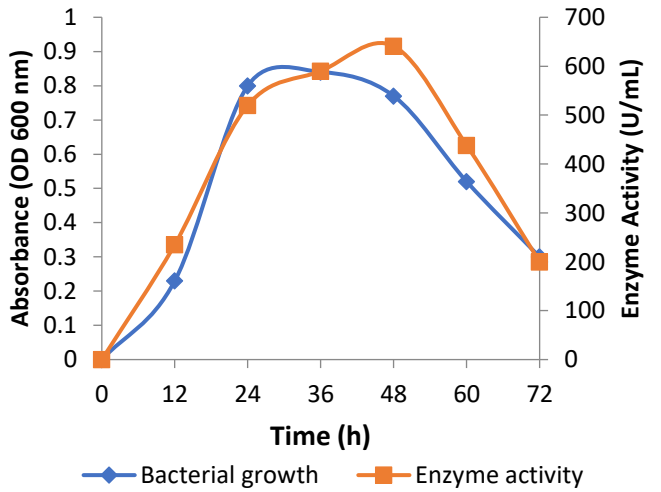

Figure 3: Growth and phytase production of *Lysinibacillus macroides* DSM54 in phytase screening medium.

Effects of Culture Conditions on Phytase Production

Effect of carbon sources on phytase production from the isolates

Carbon source represents the energy source that will be available for the growth of microorganisms. Table 2 shows the effect of different carbon sources on phytase production. Although all carbon sources exerted a positive effect on phytase synthesis by the bacterial culture, the phytase production was maximum (716±0.68 U/mL) in the presence of 0.75% mannose plus 0.75% sucrose.

Table 2: Effect of carbon sources on phytase production from the isolates.

	Phytase Activity (U/mL) in various Carbon Sources					
Isolates	Mannose	Sucrose	Maltose	Mannose + Sucrose	Mannose + Maltose	Mannose + Maltose
L. lactis LAC460	668±0.58^{b}	651±1.15^{d}	637±0.58^{f}	672±0.78^{a}	646±1.28^{e}	660±0.26^{c}
L. fermentum SCB0035	704±1.15^{b}	674±1.15^{c}	651±1.73^{f}	716±0.68^{a}	660±0.78^{e}	669±0.98^{d}
L. macrolides DSM54	673±1.15^{b}	623±0.58^{f}	665±0.58^{c}	674±1.15^{a}	633±1.73^{e}	646±1.12^{d}

Values are means ± standard error of means of three replicates. Values in each row followed by different superscripts within each row are significantly different at $P< 0.05$.

Effect of agricultural substrates on phytase production

Table 3 indicates that phytase enzyme can be produced using alternative carbon sources for example with corn starch; the phytase production was 415±1.15 for *Lactobacillus fermentum* SCB0035 and 401±0.58 for *Lysinibacillus macroides* DSM54.

Table 3: Effect of agricultural substrates on phytase production

	Phytase Activity (U/mL) in various Agricultural Substrates				
Isolates	Potato starch	Cassava starch	Corn starch	Yam starch	Rice starch
L. lactis LAC460	302±1.15^{c}	334±0.58^{b}	386±1.73^{a}	292±0.58^{d}	284±1.15^{e}
L. fermentum SCB0035	314±2.31^{c}	386±1.15^{b}	415±1.15^{a}	305±0.58^{d}	298±1.73^{e}
L. macrolides DSM54	308±0.58^{c}	323±0.58^{b}	401±0.58^{a}	299±0.58^{d}	288±0.58^{e}

Values are mean ± standard error of mean of three replicates. Different superscripts within each row are significantly different at

Effect of nitrogen source

The quality of nitrogen may regulate phytase production. Six different kinds of nitrogen sources (beef extract, yeast extract, soybean meal, groundnut cake, NH_4SO_4 and NH_4Cl) were analyzed for optimal enzyme production (Table 4). These nitrogen sources were added at the level of 0.5% w/w in the phytase medium. Although all the nitrogen sources supported the growth of the isolates and enzyme productivity, the highest enzyme activity was produced in the presence of yeast extract (710±1.16 U/mL).

Table 4: Effect of nitrogen sources on phytase production from the isolates

	Phytase Activity (U/mL) in various Nitrogen Sources					
Isolates	Yeast extract	Beef extract	Soybean meal	Groundnut cake	NH_4Cl	NH_4SO_4
L. lactis LAC460	669±1.16^{a}	656±1.73^{b}	651±2.31^{c}	642±0.58^{d}	568±1.73^{f}	597±2.31^{e}
L. fermentum SCB0035	710±1.16^{a}	669±1.73^{b}	656±0.58^{c}	628±2.89^{d}	554±1.15^{f}	592±1.73^{e}
L. macroides DSM54	660±1.16^{a}	637±2.31^{b}	628±0.58^{c}	545±1.16^{d}	521±0.58^{e}	518±1.16^{f}

Values are mean ± standard error of mean of three replicates. Different superscripts within each row are significantly different at $P< 0.05$.

Effect of temperature on the phytase activity of the isolates

Temperature is a critical factor in the production of phytase enzymes. Table 5 shows the effects of temperature (20^{o}C to 70^{o}C) on the activity of the phytase positive isolates. The results revealed that phytase activity was maximal at 50^{o}C. An increase in temperature above 50^{o}C resulted in a gradual decrease in the phytase activity of the isolates.

Table 5: Effect of temperature on the phytase activity of the isolates

	Phytase Activity (U/mL) at Different Temperatures					
Isolates	20°C	30°C	40°C	50°C	60°C	70°C
L. lactis LAC460	325±0.58^{f}	506±1.16^{d}	642±1.73^{c}	675±0.58^{a}	652±1.16^{b}	405±0.58^{e}
L. fermentum SCB0035	365±1.16^{f}	557±1.16^{d}	712±0.58^{b}	721±1.16^{a}	697±1.16^{c}	423±1.73^{e}
L. macroides DSM54	321±1.16^{f}	510±0.58^{d}	648±1.73^{c}	682±0.58^{a}	674±1.16^{b}	413±1.16^{e}

Values are mean ± standard error of mean of three replicates. Different superscripts within each row are significantly different at $P< 0.05$.

Effect of pH on the phytase activity of the isolates

The phytase activity of the isolates at different pH (4.5, 5.5, 6.5, 7.5, 8.5 and 9.5) was examined. Table 6 shows that the phytase activity of the isolates was clearly affected by the different pH values. Phytase activity was maximal at pH 7.5 for all the isolates. A decrease in pH below 7.5 and an increase in pH above 7.5 resulted in gradual decreases in phytase activity for all the isolates.

Table 6: Effect of pH on the phytase activity of the isolates

	Phytase Activity (U/mL) in Various pH					
Isolates	4.5	5.5	6.5	7.5	8.5	9.5
L. lactis LAC460	325 ± 2.89^{f}	406 ± 1.16^{d}	642 ± 1.73^{b}	659 ± 1.16^{a}	597 ± 1.16^{c}	405 ± 1.73^{e}
L. fermentum SCB0035	365 ± 1.16^{f}	457 ± 1.16^{d}	656 ± 0.58^{b}	701 ± 1.16^{a}	608 ± 2.31^{c}	423 ± 1.16^{e}
L. macroides DSM54	321 ± 1.73^{f}	410 ± 0.58^{e}	648 ± 1.73^{b}	651 ± 1.16^{a}	621 ± 1.16^{c}	413 ± 2.31^{d}

Values are mean ± standard error of mean of three replicates. Values in each row followed by different superscripts within each row are significantly different at $P< 0.05$.

Effects of purification steps on some parameters of phytase from *Lactobacillus fermentum* SCB0035

Table 7 shows the purification steps and yield of phytase enzyme extract of the bacteria isolates at 660 nm prior to its purification based on its solubility, size, and polarity.

Table 7: Effects of purification steps on some parameter of phytase from *Lactobacillus fermentum* SCB0035

Purification Steps	**Total enzyme activity (U/mg)**	**Total enzyme protein (mg)**	**Specific Enzyme activity (U/mg)**	**Purification (fold)**	**Yield (%)**
Crude extract	706	524	1.347	1	100
80% $(NH4)_2SO_4$	618	306	2.020	1.50	87.53
DEAE cellulose	502	145	3.462	2.57	71.10
Sephadex G-75	412	97	4.247	3.15	58.36

Thermal stability of the purified phytase enzyme

The thermal stability of the purified phytase showed that the enzyme retained 76% of the maximal activity when incubated at 50°C for 8 h, 69% of the maximal activity at 60°C for 8 h, and 55% of the maximal activity at 70°C for 4 h. The maximal activity of the purified phytase decreased with increasing temperature, for example, at 50°C, the purified phytase retained 51% of its original activity after 24 h. The enzyme retained less than 50% of the maximal activity at 90°C (Table 8).

Table 8: Thermal stability of the purified phytase enzyme

	Phytase Stability (%) at Various Temperatures				
Time (h)	50°C	60°C	70°C	80°C	90°C
0	100 ± 0.00^{a}	97 ± 0.58^{a}	84 ± 0.58^{a}	52 ± 0.58^{a}	46 ± 1.73^{a}
1	97 ± 0.58^{ab}	95 ± 1.16^{ab}	72 ± 1.16^{b}	50 ± 0.58^{a}	40 ± 1.16^{b}
4	95 ± 1.16^{b}	92 ± 0.58^{b}	55 ± 0.58^{c}	45 ± 1.16^{b}	35 ± 0.58^{c}
8	76 ± 1.73^{c}	69 ± 1.73^{c}	43 ± 0.58^{d}	35 ± 1.73^{c}	24 ± 0.58^{d}
16	61 ± 1.16^{d}	51 ± 0.58^{d}	30 ± 1.16^{e}	23 ± 1.16^{d}	11 ± 0.58^{e}
24	51 ± 1.16^{e}	46 ± 1.16^{e}	23 ± 0.58^{f}	16 ± 0.58^{e}	6 ± 1.16^{f}

Values are mean ± standard error of mean of three replicates. Different superscripts within each column are significantly different at $P< 0.05$.

Effect of metal ions on phytase enzyme activity

Divalent metal ions affect enzyme activity. $CuSO_4$ and $FeSO_4$ showed an inhibition effect on the phytase activity and the effect was concentration-dependent. However, $CaCl_2$ and $MgSO_4$ showed an increase in phytase activity till the addition of 1.5Mm where the phytase activity began to decrease (Table 9).

Table 9: Effect of metal ions on phytase enzyme activity

	Phytase Activity (U/mL) in Various Salt Solutions			
Concentrations	$CuSO_4$	$FeSO_4$	$CaCl_2$	$MgSO_4$
0.01	338 ± 0.58^{a}	371 ± 1.73^{a}	382 ± 0.58^{d}	387 ± 0.58^{d}
0.1	315 ± 2.3^{b}	357 ± 1.16^{b}	397 ± 1.16^{c}	391 ± 1.16^{c}
0.5	214 ± 1.16^{c}	348 ± 0.58^{c}	408 ± 1.73^{b}	410 ± 1.73^{b}
1.0	205 ± 0.58^{d}	311 ± 1.16^{d}	418 ± 0.58^{a}	421 ± 1.16^{a}
1.5	122 ± 1.73^{e}	297 ± 1.16^{e}	322 ± 0.58^{e}	325 ± 0.58^{e}
2.0	86 ± 0.58^{f}	187 ± 0.58^{f}	214 ± 1.16^{f}	219 ± 1.16^{f}
5.0	35 ± 1.16^{g}	35 ± 0.58^{g}	196 ± 1.73^{g}	187 ± 0.58^{g}

Values are mean ± standard error of mean of three replicates. Different superscripts within each column are significantly different at $P< 0.05$.

DISCUSSION

Our study has shown that the bacteria isolates (*Lactococcus lactis* LAC460, *Lactobacillus fermentum* SCB0035 and *Lysinibacillus macroides* DSM54) can produce phytase enzyme and the process can be optimized. In the quantification of phytase enzyme production, the isolates showed phytase activity ranging from 605 U/mL to 706 U/mL. *Lactobacillus fermentum* SCB0035 had the highest phytase activity of 706 U/mL, while *Lysinibacillus macroides* DSM54 had the least phytase activity of 605 U/mL. Sumengen *et al.* (2012) have determined the extracellular phytase activity of *Lactobacillus brevis* to be 886.18 U/mL suggesting that the amount of phytase production differed between species and varied among strains of the same species.

In the estimation of growth and phytase production by the isolates, phytase was expressed on the onset of late log phase. The reduction in enzyme yield after the optimum period was probably due to the depletion of nutrients that are available to the isolates. This result is in accordance with the observation made by Vijayaraghavan *et al.* (2013) and Bellaouchi *et al.* (2021).

Phytase production is therefore an important property of lactic acid bacteria (Sharma *et al*., 2020). However, the low activity of phytase produced by most lactic acid bacteria may prevent it from becoming commercially available, for which at least a hundred times higher production would be required. To achieve the production of phytase with high activity acceptable for its commercial use, optimization of the conditions for phytase production such as carbon source, nitrogen source, growth temperature and pH, is a prerequisite (Qasim *et al*., 2017). The effects of carbon sources alternative to glucose in the phytase production of the isolates were studied. The incorporation of sugars instead of glucose used in the original medium caused a significant reduction in the phytate-degrading activity in all the isolates compared to the original medium. This was probably due to the more efficient use of glucose than other sugars as an energy source or precursor for enzyme synthesis in the cell. These results suggest that regulation of the biosynthetic pathway of phytase may be dependent on carbohydrate source (Petry *et al.,* 2000). Easily metabolizable sugar e.g., glucose has been reported to increase phytase production in both bacteria and fungi (Sasirekha *et al.,* 2012). This confirms the report by Haros *et al.* (2008) that glucose is the most preferred substrate for phytase production. However, the carbon source effect changes with the production strain and other conditions. The cost of phytase enzyme primarily depends on the raw material especially of carbon and nitrogen sources which play a major role in the economics of phytase production (Lee *et al.,* 1997; Dailin *et al.,* 2019). Generally, sugars like glucose, sucrose, mannose, and maltose are being used as carbon sources for the production of phytase (Zhang *et al*., 2021). The use of these sugars in their pure form increases the production cost because they are the key raw materials for enzyme production. As a result, we also carried out the production of phytase by lactic acid bacteria using alternative carbon sources such as corn starch, cassava starch, sweet potato starch, rice starch and yam starch. All substrates supported microbial growth and phytase production, with corn starch producing a higher phytase yield than the other substrates, perhaps due to the nutritional composition of the substrate for the growth of the organism (Moongngarm *et al.,* 2012; Ramadoss and Muthukumar, 2014). Rice starch has a high content of cellulose and

because of this; it might have been difficult for microorganisms to utilize it as a nutrient (Abdul Razack *et al*., 2013). The lower phytase production by the isolates when cultivated in the other agro-industrial byproducts (rice starch, yam starch, potato starch) suggests a limited use of nutrients by these organisms, or the repression of the enzyme synthesis by compounds from these substrates. The yield of enzymes from agro-industrial byproducts varies and depends on the species of the cultured microorganism (Bala *et al.,* 2014; Awad *et al.,* 2014).

Various nitrogen sources were observed for their effects on phytase production by the isolates. Organic nitrogen sources were found to yield a higher amount of phytase than inorganic nitrogen sources. Yeast extract was found to produce the maximum activity followed by beef extract. Yield was generally retarded using inorganic nitrogen sources such as ammonium sulphate (NH_4SO_4) and ammonium chloride (NH_4Cl) when compared to using organic nitrogen sources. Our result agrees with that of Singh *et al.* (2013). Wu *et al.* (2008) suggested that certain essential amino acids cannot be synthesized from inorganic nitrogen components. As a result, bacteria cells might neither fully grow nor undergo metabolism hence the deterioration of enzyme yield. It was also reported that the primary role of most bacteria is classically considered to be the decomposition and mineralization of dissolved particulate organic nitrogen (Al Nahas *et al.,* 2011). This might be an obvious cause of higher production of phytase by lactic acid bacteria using organic nitrogen sources.

Fermentation temperature plays a major role in enzyme production by lactic acid bacteria (Wang *et al*., 2023). The result on the effects of temperature on phytase activity by the isolates revealed that phytase activity was increased with increase in temperature reaching its maximum value at 50ºC for all the isolates. Zuo *et al.* (2010) reported that the optimal temperature for phytase from *Lactobacillus casei* was 70°C. The optimal temperature for phytase enzyme from *L. plantarum* was 30°C (Saribuga *et al.,* 2014). Alcaligenes phytase was highly active (100% activity) at 60°C (Vijayaraghavan *et al.,* 2013). Sasirekha *et al.* (2012) reported that maximum phytase yield from *Pseudomonas* species was at 37°C. Optimal temperatures in which most phytases are active vary from 37°C to 77°C (Hara *et al.,* 1985). An increase in temperature above 50ºC resulted in a gradual decrease in the phytase activity of the isolates.

The results on the effect of pH on phytase production by the lactic acid bacteria indicated that the optimal pH to produce phytase was 7.0. A decrease in phytase activity was observed at pH above 7.0. The extracellular phytase of *L. plantarum* possessed optimal activity at pH 5.5 and 65°C (Zamudio *et al*., 2001). On the other hand, the extracellular phytase of *Lactobacillus plantarum* KV1 was most active at pH 5.5 and 37°C (Luechai and Dharmsthiti, 2010). The maximal phytase activity of *Lactobacillus casei* was obtained at pH 5.0 and the optimum temperature of phytase was observed at 70°C (Zuo *et al.,* 2010). Haros *et al.* (2008) reported that the optimum activity of phytase isolated from *Lactobacillus plantarum* W42 and *L. plantarum* JBRS was at pH 6.0-6.5, and at pH 7.5, is the optimum activity of phytase isolated from *L. plantarum* 110. These findings are similar to our observations in the present study regarding the three lactic acid bacteria. Increase in pH affects the charges on the amino acids within the active site such that the enzyme is not able to form an enzyme-substrate complex. Thus, there is a decrease in enzyme activity (Sasirekha *et al.,* 2012). Most phytases have an optimal pH in the range of 4.5–6.0 and a temperature range of 45–60°C. Outside the optimal range of pH and temperatures the action of phytase is reduced (Lei and Stahl, 2000).

The thermal stability of the phytase enzyme purified from *Lactobacillus fermentum* SCB0035 was measured in the range of 50-90°C at certain intervals. We discovered that the enzyme was stable up to 70°C of denaturing temperature (Table 8). This result shows that *Lactobacillus fermentum* SCB0035 phytase is relatively thermostable and is preferable for use in the feed industry. It is known that stability at high temperature is important for the phytases as they are widely used to increase nutritional value for the pretreatment of animal feeds that are processed at high temperatures. Therefore, we propose that *Lactobacillus fermentum* SCB0035 phytase is suitable and advantageous for animal husbandry and related fields.

The effect of certain metal ions such as Cu^{2+}, Fe^{2+}, Ca^{2+}, and Mg^{2+} on the phytase enzyme activity purified from *Lactobacillus fermentum* SCB0035 was also studied. It was found that Cu^{2+} and Fe^{2+} inhibited pure phytase enzyme activity while Ca^{2+} and Mg^{2+} activated the enzyme. Shimizu (1992) found that the activity of the extracellular phytase enzyme purified from *Bacillus subtilis* (natto) N-77 strains is inhibited by the addition of ethylenediaminetetraacetic acid (EDTA), Zn^{2+}, Ba^{2+} Cu^{2+} and Fe^{2+}. Yoon *et al.* (2011) showed that extracellular phytase enzyme from *Enterobacter* species was inhibited by 4.1 mM Zn^{2+}, Cu^{2+}, Ba^{2+} Al^{3+} and EDTA. Yanke *et al.* (1999) have reported that 5 mM each of Fe^{2+}, Fe^{3+}, Cu^{2+}, Zn^{2+} and Hg^{2+} inhibits the phytase enzyme of *Selenomonas ruminantum* whereas Pb^{2+} activates the enzyme. Also, Choi *et al.* (2001) found that the activity of extracellular phytase enzyme from *Bacillus* species KHU-10 is inhibited by EDTA and metal ions such as Ba^{2+}, Cd^{2+}, Cr^{3+}, Cu^{2+}, Hg^{2+} and Mn^{2+}. These previous studies support our present finding on the inhibitory role of cations on the activity of phytase enzyme.

CONCLUSION

All three bacteria isolates: *Lactococcus lactis* LAC460, *Lactobacillus fermentum* SCB0035, and *Lysinibacillus*

macroides DSM54 had varying capacities of phytase production. Optimal conditions were pH 7.0, temperature at 50ºC and absence of certain cations. Optimization of production conditions, including carbon and nitrogen sources, temperature, and pH, with glucose and organic nitrogen sources can enhance enzyme production for improved nutritional value and applications in various industries.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHOR'S CONTRIBUTION

All authors conceptualized the study. CGO, OKA and EN were responsible for the methodology, analysis, data curation and manuscript preparation. CGO and ACN were responsible for writing, critical review and editing. All authors agreed on the content before submission.

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