

PURIFICATION AND CHARACTERIZATION OF PHYTASE PRODUCED BY *BACILLUS AMYLOLIQUEFACIENS* AUPPB02 AND ITS PROBIOTICS POTENCY

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Abstract– The anti nutritional property of phytic acid is one of major challenge with food but phytase producing probiotic strain could be the best alternative for this issue. In the present study, phytase from *Bacillus amyloliquefaciens* AUPPB02 was isolated, purified by ammonium salt precipitation, ion exchange chromatography and gel filtration chromatography, which showed molecular mass of 47 kDa by SDS-PAGE. The purification fold of purified phytase was 5.13, with a specific activity of 10.15 IU mg⁻¹ protein. The purified enzyme showed its optimum activity at 7.0 pH and 40 °C temperature for sodium phytate, as specific natural substrate. To enhance the enzyme's activity, the additives, which were used (EDTA, BME, sodium azide, oxalate and citrate), didn't respond positively, hence they were not required. The K_m and V_{max} values of phytase produced by this strain were calculated from the Lineweaver - Burk plot, as 1.057 mM and 0.20 μmoles/sec respectively. Further, *B. amyloliquefaciens* AUPPB02 was evaluated for the probiotic activity of. It showed much resistance toward high acidic (pH 2 and 3) and bile concentration (0.3 – 2.0 %), with MIC of 2.0 % bile. It also exhibited good hydrophobicity, when treated with xylene (70.2 %), chloroform (65.69 %) and ethyl acetate (68.32 %). The performance of this strain was exceptionally good for autoaggregation (68.3 %) along with digestibility for casein and lipid. It has non haemolytic and non lecithinase producing ability. Subsequently, it revealed exceptional antibacterial activity against *S. aureus* (32 ± 0.72 mm) and *P. aeruginosa* (27 ± 0.21 mm). The strain *B. amyloliquefaciens* AUPPB02 also displayed susceptibility against commonly used antibiotics like, azithromycin, tetracycline, erythromycin, amoxicillin, norfloxacin and levofloxacin. Considering the outstanding biochemical qualities of isolated phytase, which *B. amyloliquefaciens* AUPPB02 possess, along with its significant probiotic traits, it could be a suitable candidate to be used in food and feed applications. Yet, in order to reap the promised benefits from the strain, secreting phytase, its *in vivo* research in animals is required.

INTRODUCTION

Probiotics are live microbes of non-pathogenic nature which provide certain health benefits to the host when given in sufficient amounts (Chen *et al.*, 2019). Probiotic applications and molecular processes are currently the subject of investigation of various studies. Improved intestinal mucosal barrier function, gut immunomodulation, neurotransmitter production and competitive exclusion of pathogens for adhesion sites are some of the mechanisms by which probiotic strains perform their action. The ability of probiotics to

regulate gut microbiota and modulate immune responses makes them a potential therapeutic option for the treatment of gastrointestinal disorders, hypertension and hypercholesterolemia (Latif *et al.*, 2023). According to Jezewska-Frackowiak *et al.* (2018), the *Bacillus* genus is the basis for a sizable collection of bacterial probiotics, comprising the frequently mentioned *B. subtilis*, *B. amyloliquefaciens*, *B. coagulans*, *B. pumilus*, *B. clausii* and *B. licheniformis*. Due to their widespread presence in fermented foods from Asia and West Africa, these species are a subject of growing scientific attention and have the potential for industrial applications (Gopikrishna *et*

al., 2021). The sporulation capacity shared among *Bacillus* species is a vital trait that allows them to withstand environmental stress and harsh conditions of growth, storage and preservation. These spore forming organisms exhibit remarkable resilience and tolerance to extreme temperatures, pH (even bile fluids), salt, dehydration, or poor nutrition (Jezewska-Frackowiak *et al.*, 2017, Yao *et al.*, 2020). These species also produce several extracellular enzymes that improve food and feed digestion, as well as bioavailability of nutrients (Kilik *et al.*, 2020). Among the extracellular enzymes, phytases are of major interest, for their action on phytic acid (PA) complexes.

PA (myo-inositol 1, 2, 3, 4, 5, 6-hexakis dihydrogen phosphate) is an organic storage form of phosphate, accounting for more than 80% of the total phosphorus in cereals and legumes. Due to its unusual molecular structure, it can also form complexes with other nutrients, such as metallic ions (Ca, Mg, Fe, Cu etc.), amino acids, proteins, lipids and vitamins, rendering them unavailable to organisms via lowering their solubility and bioavailability (Rizwanuddin *et al.*, 2023; Mishra *et al.*, 2024). The organically bound phosphate of PA is not metabolized by monogastric animals, such as pig, poultry and fish due to lack of phytase and consequently contributes to the phosphorus pollution problems in areas of intensive livestock production (Sreedevi and Reddy, 2012). This PA can only be degraded by phytases (myo-inositol hexakisphosphate phosphohydrolase enzyme), which catalyze the breakdown of PA to myo-inositol and phosphoric acid in a stepwise manner (Mishra *et al.*, 2024). The phytase supplementation as feed additive could be an exciting approach, as this enzyme has a positive effect on mineral and non-mineral nutrient availability (Attia *et al.*, 2021). It has been reported that several species of bacteria and fungi produce phytases. However, bacterial phytases are more preferred due to their higher substrate specificity, higher proteolysis tolerance, higher heat stability, better pH optimum and higher catalytic efficiency than their fungal counterparts (Ahmad *et al.*, 2023). Bacterial species like *Aerobacter aerogenes*, *Bacillus licheniformis*, *B. amyloliquefaciens*, *B. subtilis*, *Enterobacterium*, *Escherichia coli*, *Pseudomonas* sp. and *Lactobacillus sanfranciscensis* are among of the highest phytase producers (Konietzny and Greiner, 2004; Zhao *et al.*, 2021; Trivedi *et al.*, 2022; Mishra *et al.*, 2024).

Although, numerous studies have demonstrated

the production of phytase from different bacterial species, few investigations have been carried out on the probiotic potential of a strain with a natural ability to produce phytase. In this regard, the present study emphasises on purification and characterization of phytase enzyme produced by *Bacillus amyloliquefaciens* AUPPB02, as well as its potency as probiotics using various assays. In the current study, *B. amyloliquefaciens* AUPPB02 (NCBI GenBank accession no. OR187307) was initially isolated from fermented lentil seeds and was then characterized and identified by referring to Bergey's manual of systematic bacteriology and 16S rRNA gene sequencing. Optimisation of strain for incubation time, pH, temperature, inoculum size, carbon and nitrogen source for phytase production was also carried out (Mishra *et al.*, 2024).

MATERIAL AND METHODS

Chemical and reagents

All of the chemicals used in the present work were of analytical grade and were purchased from Sigma, Merck, HiMedia and SRL India.

Bacterial strain and enzyme production

Phytase producing bacterial strain, *Bacillus amyloliquefaciens* AUPPB02 (NCBI Genbank accession no. OR187307) was obtained from Microbial Biodiversity Lab, Department of Botany, Patna University, Patna, Bihar, India. This strain was initially isolated from fermented (72 h) lentil seeds and was characterized, optimized and identified (Mishra *et al.*, 2024). The culture was maintained on Luria Bertani (LB) agar slant (10 g l⁻¹ tryptone, 5 g l⁻¹ yeast extract, 10 g l⁻¹ NaCl, pH 7.0) and stored at 4 °C for further studies. The inoculum was prepared by adding single colony of 24 h old bacterial culture by transferring aseptically in 100 ml of optimized MRS media (1.0 % lactose, 0.075 % sodium phytate, 1.0 % peptone, 1.75 % of calcium chloride (1M), 0.5 % sodium acetate, 0.2 % tri ammonium citrate, 2 % MgSO₄·7H₂O (100 mM), 0.5 % MnSO₄ (10 mM) 0.65 KCl (1M) 0.1 % Tween 80, pH 5). The culture flasks were incubated with 1.0% inoculum density at 37±2 °C in shaker cum incubator (Rivotek) for 48 h at 200 rpm.

Purification of phytase

The crude phytase was extracted after fermentation by centrifuging the bacterial samples at 12298×g for 10 min and then the supernatant was collected in the

McCartney bottle. The purification was carried out through salt precipitation, by using ammonium sulfate (0 - 80 %) and dialyzed. After that, the phytase was further purified by gel filtration chromatography. The dialyzed enzyme was loaded onto Sephadex G-100 matrix (30 x 1.5 cm²), equilibrated with sodium acetate buffer (100 mM, pH 4.0) and the protein (phytase) was eluted at a flow rate of 1.0 ml min⁻¹. 30 fractions of 3 ml each were collected. The active fractions were pooled and concentrated by polyethylene glycol (PEG). The purified phytase were characterized on the basis of molecular weight, effect of various physiochemical parameters.

Phytase and protein assay

Phytase activity was assayed according to the method, previously used by Mishra *et al.*, 2024. Released inorganic phosphate (Pi) was measured at 355nm, as per modified ammonium molybdate method of Heinonen and Lahti (1981). One unit of the enzyme activity was defined as the amount of the enzyme, able to hydrolyse PA resulting in liberation of 1 μmol of inorganic phosphorus per min per ml under the assay condition.

Protein concentration was determined by the Bradford's method (1996) at 595 nm and the specific activity of Phytase is expressed as Umg⁻¹ protein.

Determination of molecular weight

The molecular weight of purified phytase was estimated by SDS-PAGE, following Laemmli protocol, using 10% separating gel (pH 8.8) and 5% stacking gel (pH 6.8). The molecular weight of the purified enzyme was determined against standard protein markers after staining with Coomassie Brilliant Blue R-250 (Trivedi *et al.*, 2022).

Effect of pH on phytase activity

The effect of pH on the activity of purified phytase was determined at pH ranging from 5 to 10. Enzyme activity was determined using the standard phytase assay and results were expressed as percentage relative activity with respect to maximum activity, considered 100% for optimum.

Effect of temperature on phytase activity

The optimal temperature of phytase activity was determined by performing the enzyme assay at temperature, ranging from 20 – 80 °C at an interval of 10 °C at pH 5.0. Samples were withdrawn sequentially for phytase activity. The maximum

enzyme activity was considered as 100%.

Substrate specificity

Substrate specificity of purified phytase was determined in the presence of different substrates, such as sodium phytate, calcium phytate, phenyl phosphate and glycerol -2- phosphate at final concentration of 2 mM. Relative activity was expressed as the percent ratio of enzyme activity, determined against the substrate, to the enzyme activity obtained with sodium phytate.

Effect of various additives on phytase activity

1M stock solution in 0.1M sodium acetate buffer (pH 4.0) of EDTA, β-mercaptoethanol (BME), sodium azide, oxalate and citrate were used to determine the effect of various additive agents on the purified enzyme. Each compound (0.1 ml) was separately added to the enzyme mixture (0.1 ml), incubated for 30 min at 37± 2 °C and phytase activity was determined.

Enzymes Kinetics

Kinetic characteristics of *B. amyloliquefaciens* AUPPB02 were studied for sodium phytate as substrate. Kinetic properties of phytase were determined by evaluating the effect of substrate concentration [S] on phytase activity, i.e. reaction velocity (V). Enzyme assay was done with different substrates in the concentration range from 0.1 – 10 mM. Michaelis-Menten coefficient (Km) was determined by plotting Line-Weaver Burk plot for each substrate.

Validation of probiotic potential

Probiotic potential of *B. amyloliquefaciens* AUPPB02 was assessed under *in vitro* conditions, with reference to the known probiotic strain, *Lactobacillus acidophilus* (MTCC 10307).

The survival of selected bacterial strain in acidic condition was assessed, following the method of Pereira and Gibson (2002). Overnight grown cultures (10⁶ CFU ml⁻¹) were transferred to the modified MRS broth, amended with 1N HCl to maintain different pH values (2.0 and 3.0) and incubated at 37±2 °C for 3 h. At an interval of 1 h, the viable bacterial count was determined by plating on LB agar medium. Simultaneously, bacterial growth was measured at the absorbance of 600nm. The broth media with pH 5.5, inoculated with bacteria, served as control and survival percentage at different pH values were calculated.

The methodology of Hyronimus *et al.* (2000) was used to determine the bile tolerance of phytase producing bacterial strains, using bile concentrations of 0.3, 0.5, 0.7, 0.9, 1.1, 1.3, 1.5, 1.7 and 2.0%. Strains were grown in modified MRS broth at 37 ± 2 °C overnight. Saturated bile solutions were prepared separately by dissolving powdered bile extract (Oxoid) in DDW. Bile solutions were then filtered aseptically, through 4-micron filter, then added to the broth culture and re incubated at 37 ± 2 °C for 3 h. Viable counts of strains were determined by pouring plate counts of all the samples using 10-fold serial dilutions prepared in 0.1% peptone water, every hour. Simultaneously bacterial growth was monitored by measuring absorbance at 600 nm. All the experiments were repeated thrice. The minimal inhibitory concentration (MIC) of bile, against bacterial strains was determined as the lowest concentration of the bile totally inhibiting the growth, observed from a visual examination. Inoculated plates, without bile salt served as control.

Cell-surface hydrophobicity was determined, as per the method of Rosenberg *et al.* (1980). Briefly, overnight grown test culture and reference strains were pelleted through centrifugation (5000g, 15 min) washed twice with phosphate buffer solution (pH 7.3) and the pellet was resuspended in the same buffer having viable count of 10^8 CFU ml⁻¹. The OD of cell suspension was taken at 600 nm (A_0). Three part of cell suspension was mixed with one part of solvent, preincubated at room temperature for 10 min and vortexed for 2 min. The aqueous phase was pipetted out after incubation period of 20 min, at room temperature and absorbance was taken at 600 nm (A_1). The percentage of hydrophobicity of solvent was calculated, using the formula $(1 - A_1/A_0) \times 100$. Three different solvents were tested in this study: xylene, which is an apolar solvent; chloroform, a monopolar and acidic solvent and ethyl acetate, a monopolar and basic solvent (Kos *et al.*, 2003).

The autoaggregation assay was performed according to the method of Del Re *et al.* (2000). Overnight grown test culture and reference strain were pelleted through centrifugation (5000g, 15 min) washed twice with phosphate buffer solution (PBS, pH 7.3) and the pellet was resuspended in the same buffer having viable count of 10^8 CFU ml⁻¹. 4 ml cell suspension was vortexed for 10 sec and left behind for 15 min at room temperature. From that vortexed suspension, 0.1 ml was taken along with

3.9 ml of PBS and absorbance was taken at 600 nm (A_0). Remaining vortexed suspension was kept for 5 h at room temperature for the assay of autoaggregation percent. After completion of incubation period, OD was retaken and denoted as A. The autoaggregation percentage was expressed as: $(1 - A/A_0) \times 100$ (Kos *et al.*, 2003).

Nutrient agar, supplemented with 0.5% skimmed milk and 1% tributyrin were used for detecting the protein and lipid digesting capability, respectively (Kim *et al.*, 2007). The zone of hydrolysis around the colonies was considered as a positive test. While for the assessment of haemolytic activity, blood agar base supplemented with 5% defibrinated blood was inoculated with test culture and reference strain, separately (Patel *et al.*, 2010). In the similar way, for the lecithinase activity, lukewarm agar medium (pH 7.0) was supplemented with 8% egg yolk and inoculated with test culture and reference strain, separately (Bhat *et al.*, 2013). Plates were incubated at 37 ± 2 °C for 48 h and the clear halo zones, around the colonies were observed for their positive results.

The inoculums of *B. amyloliquefaciens* AUPPB02 and *L. acidophilus* MTCC 10307 (reference strain) were separately prepared to give the aliquot size of 10^8 CFU ml⁻¹ by using 0.5 McFarland turbidity standards, to evaluate their antimicrobial activity by well diffusion method (Hong, 2011), against target strains *Staphylococcus aureus* (MTCC 3160) and *Pseudomonas aeruginosa* (MTCC 741). Briefly, The MH agar plates were swabbed separately by *S. aureus* and *P. aeruginosa* (10^8 CFU ml⁻¹). In each plate, three wells of 10 mm were made by sterile corkborer and filled individually with 50 µl of test strain and reference strain. In the third well, amoxicillin (5 µg/ml) solution was filled as positive control. Plates were incubated at 37 ± 2 °C for 24 h. After the completion of incubation time, diameter of the inhibition zone was measured and tabulated (Fatima *et al.*, 2023).

Antibiotic sensitivity of test and reference strains was determined by adjusting the density of bacterial suspension equal to 0.5 McFarland standards. The inoculum was spread evenly with a sterile swab over the entire surface of MH agar plates (Fatima *et al.*, 2023). Subsequently, each antibiotic (azithromycin, tetracycline, erythromycin, ampicillin, norfloxacin, levofloxacin) of 5 µg ml⁻¹ was put in well made by sterile corkborer, incubated at 37 ± 2 °C for 24 h, zone of inhibition was observed and measured.

RESULTS

Purification of Phytase

Phytase from *Bacillus amyloliquefaciens* AUPPB02 was purified through three steps, i.e., ammonium sulphate precipitation (60 - 80 %), ion exchange chromatography and gel filtration, summarized in Table 1. The crude extract containing 0.834 mg ml⁻¹ protein with specific activity of 1.98 IU mg⁻¹ protein was precipitated by ammonium sulfate with a saturation range of 80 %. It enhanced the specific activity (IU mg⁻¹ protein) to 4.85, which was 2.45 fold higher than the crude extract, with yield of 15.12 %. Further, by ion exchange chromatography, the enzyme was purified with specific activity (IU mg⁻¹ protein) to 5.10, which was 2.58 fold higher than the crude extract with yield of 10.17 %. Lastly, the enzyme was purified by gel filtration method with approximately 5.13 purification fold, 4.25 % yield with a specific activity of 10.15 IU mg⁻¹ protein.

This purified enzyme is further studied for its molecular mass and physiochemical factors, affecting its activity.

Molecular weight determination

The SDS – PAGE analysis of purified phytase is shown in Fig. 1A. Molecular weight of purified phytase was found to be 47 kDa, which is in range of other studied *Bacillus* phytase of 47.5 - 38 kDa (Tran *et al.*, 2011; Fasimoye *et al.*, 2014)

Effect of pH on phytase activity

The effect of pH on enzyme activity was determined at various pH values, ranging from 5.0 – 10.0. (Fig. 1B). Results indicated that the enzyme was more active at pH range of 6.0 to 9.0, with an optimum activity at pH 7.0 (1.64±0.04). Relative activities of the enzyme at pH 6.0, 8.0 and 9.0 were found to be 82.92 %, 97.56 % and 85.63 %, respectively. Purified enzyme maintained more than 80% of maximum activity within the pH range of 6.0 – 9.0, but below

Table 1. Summary of various steps involved in the purification of phytase from *B. amyloliquefaciens* AUPPB02.

Purification step	Total activity (IU mg ⁻¹)	Total protein (mg ml ⁻¹)	Specific activity (IU mg ⁻¹ protein)	Yield (%)	Purification fold
Crude extract	1.65	0.834	1.98	100	1
Ammoniumsulfate precipitation(60- 80%) and dialysed	1.57	0.324	4.85	15.12	2.45
Ion exchange chromatography	1.50	0.294	5.10	10.17	2.58
Gel filtration chromatography	1.38	0.136	10.15	4.25	5.13

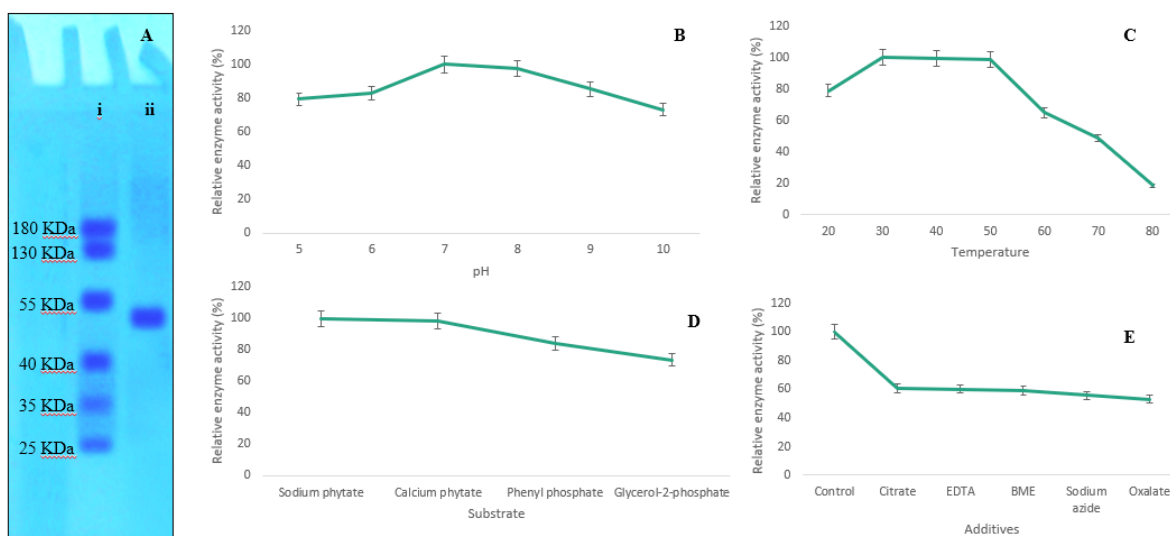


Fig. 1. (A) Determination of molecular weight by SDS-PAGE. (i) protein marker and (ii) Sephadex G-100 purified phytase. (B) Effect of pH on phytase activity. (C) Effect of temperature on phytase activity. (D) Substrate specificities of purified phytase. (E) Effect of various additives on phytase activity.

and above this pH, enzyme activity was reduced abruptly. These results indicated that purified phytase was more active and stable at neutral pH.

Effect of temperature on phytase activity

The effect of temperature on the purified phytase was estimated at the range of 20 °C to 80 °C, using modified ammonium molybdate method (Heinonen and Lahti, 1981). This was summarized in Fig. 1C. The purified enzyme was more active between 30 °C–50 °C, exhibiting optimal activity at 40 °C (1.68 ± 0.03). The relative activity of purified enzyme was more than 80 % for temperature range from 20 - 50°C, but after that enzyme activity fall abruptly.

Substrate specificity

Effect of various substrates was investigated for the phytase activity (Fig. 1D). The optimum hydrolytic rate was observed by sodium phytate (1.67 ± 0.12), as most suitable substrate, followed by slight decrease in calcium phytate (1.64 ± 0.12), phenyl phosphate (1.40 ± 0.034) and minimum in glycerol -2- phosphate (1.23 ± 0.12).

Effect of various additives on phytase activity

The effect of various additives on the activity of the purified enzyme was examined at optimum pH (pH7). All investigated additives show negative impact on phytase activity (Fig. 1E). Citrate showed least reduction (1.30 ± 0.04), followed by EDTA (1.20 ± 0.08), sodium azide (1.11 ± 0.07), BME (1.10 ± 0.01) and oxalate (1.09 ± 0.08).

Enzyme kinetics

The K_m and V_{max} values of phytase produced by *B. amyloliquefaciens* AUPPB02 were calculated from the Lineweaver - Burk plot, as 1.057 mM and 0.20 μ moles/sec, respectively (Fig. 2).

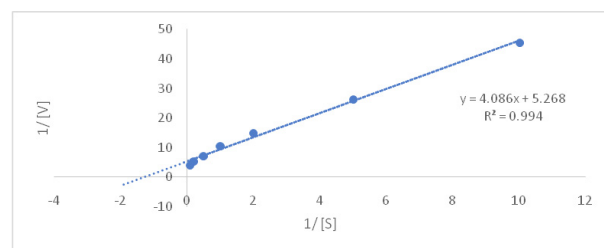


Fig. 2. Lineweaver-Burk graph, reflecting apparent K_m and V_{max} values for *B. amyloliquefaciens* AUPPB02

HPLC analysis

The HPLC analysis was done to identify the end products, formed upon hydrolysis of sodium

phytate by crude phytase of isolates *B. amyloliquefaciens* AUPPB02. The results given below are system generated and compared with two standards that are IP6 and IP5, having retention time (min) of 10.96 and 6.105, respectively. *B. amyloliquefaciens* AUPPB02 showed two major peaks at the retention time of 10.977 min and 6.018 min, which were identified as IP6 and IP5, respectively. Presence of IP5 and IP6 in PA hydrolysate of all selected strains confirmed them to be phytase producers (Fig. 3).

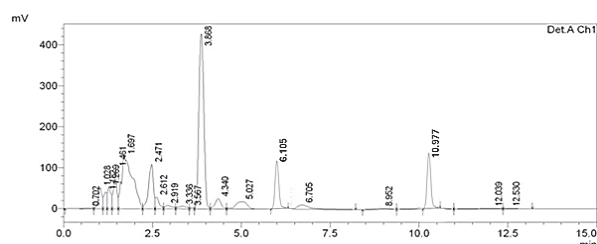


Fig. 3. HPLC chromatogram of phytase produced by *B. amyloliquefaciens* AUPPB02.

Validation of probiotic potential

The acid tolerance test of *B. amyloliquefaciens* AUPPB02 exhibited 89.67, 82.42 and 77.96 % survival at pH 2 and subsequently, 94.06, 86.89 and 82.31 % survival at pH 3 after 1, 2 and 3 h of incubation periods, respectively. On other hand the reference probiotic strain *L. acidophilus* (MTCC 10307) exhibited survival rate of 62, 51 and 49 % (pH 2), followed by 69, 61 and 52 % (pH 3) at same incubation periods of 1, 2 and 3 h, respectively (Fig. 4A).

In the bile tolerance test, the isolated strain *B. amyloliquefaciens* AUPPB02 and reference probiotic strain *L. acidophilus* (MTCC 10307) were grown in modified MRS media, supplemented with the concentration range of 0.3 to 2.0 % bile salt. The isolated strain showed tolerance against bile concentration, upto 2.0 %, while the reference strain showed bile tolerance, only upto 0.7 % (Table 2).

The hydrophobicity (%) of *B. amyloliquefaciens* AUPPB02 and reference strain was investigated in xylene, chloroform and ethyl acetate, represented in Fig. 4B. The isolated strain exhibited significant hydrophobicity (%) of 70.2, 65.69 and 68.32 in xylene, chloroform and ethyl acetate, respectively. In contrast, reference strain showed limited hydrophobicity (%) of 40.54, 54.23 and 55.18 in xylene, chloroform and ethyl acetate, respectively. Hence, isolated strain showed enhanced hydrophobicity (%) over reference strain.

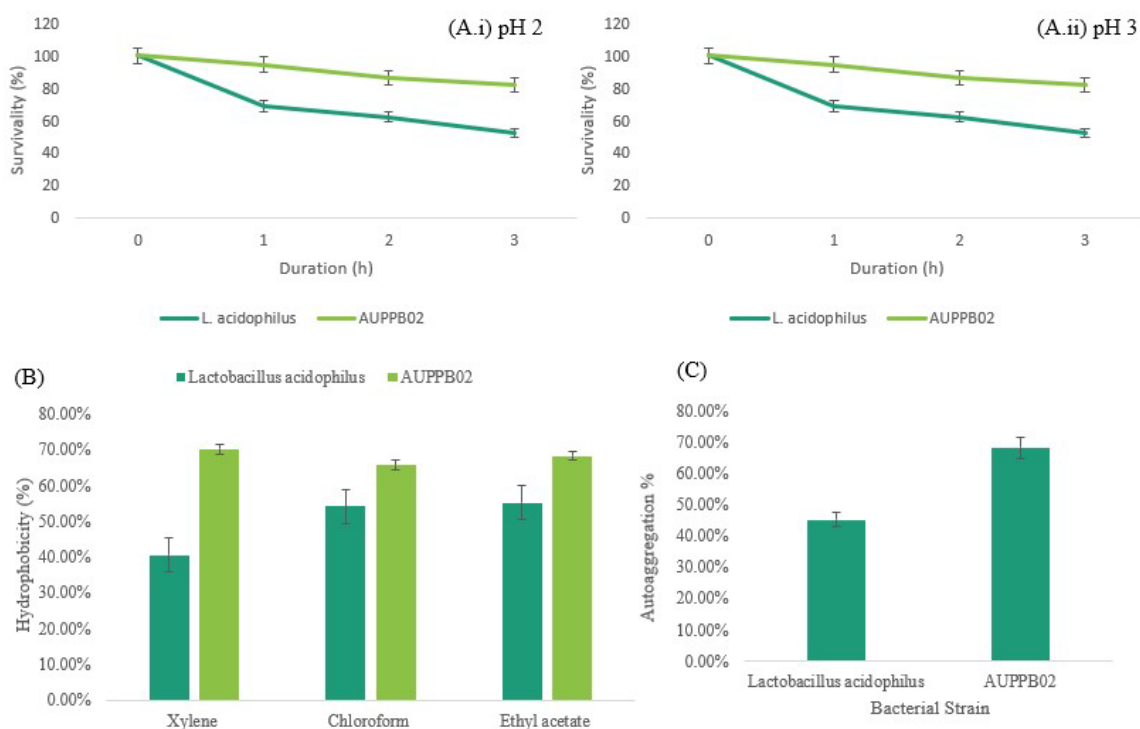


Fig. 4. (A) Acid tolerance of reference strain and AUPPB02 at pH 2 (i) and pH 3 (ii) (B) hydrophobicity (%) of AUPPB02 and reference strain (C) autoaggregation (%) of AUPPB02 and reference strain.

Autoaggregation property was examined, and was found that isolated strain (*B. amyloliquefaciens* AUPPB02) possesses significant autoaggregation (%) of 68.3 while reference strain has value of 45.3%. Hence isolated strain showed enhanced autoaggregation (%) of 50.77 over reference strain (Fig. 4C).

The casein and lipid digestibility of *B. amyloliquefaciens* AUPPB02 was studied on NA modified plate, containing substrates of skimmed milk and tributyrin, respectively. The result, represented in Fig 5A and 5B that isolated strain can digest skimmed milk and tributyrin, exhibiting its positivity for casein and lipid digestibility.

In the test for haemolytic and lecithinase activity, any type of zone formation was not observed,

around point inoculation by isolated strain and reference strain, which emphasized that both strains don't hydrolyse RBCs (Fig. 5C) and lecithin. This observation also concluded that *B. amyloliquefaciens* AUPPB02 is non-pathogenic strain and can be a potential probiotic candidate.

The antimicrobial activity of both, isolated and reference strains were tested against *S. aureus* and *P.*

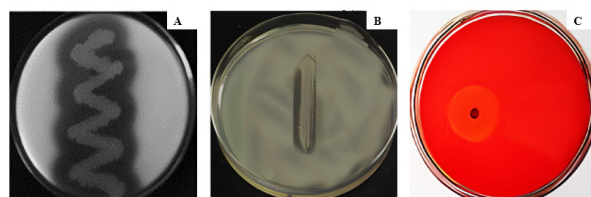


Fig. 5. (A) Casein hydrolysis (B) Lipid hydrolysis and (C) Haemolytic plate of AUPPB02.

Table 2. Bile tolerance of phytase producing *B. amyloliquefaciens* AUPPB02 and reference strain (*L. acidophilus*) with their MIC values.

Bacterial sample	Bile Concentration (%)									MIC (%)
	0.3	0.5	0.7	0.9	1.1	1.3	1.5	1.7	2.0	
<i>B. amyloliquefaciens</i> AUPPB02	+	+	+	+	+	+	+	+	+	2.0
<i>L. acidophilus</i>	+	+	+	-	-	-	-	-	-	0.8

(+), growth in the presence of bile; (-), no growth in the presence of bile.

aeruginosa, keeping Amoxicillin (5 μ g) as control. Due to action of amoxicillin, there was zone formation of 14.45 \pm 0.10 mm and 12 \pm 0.09 mm, against *S. aureus* and *P. aeruginosa*, respectively. The isolated strain, *B. amyloliquefaciens* AUPPB02 had more effectivity, as 133.33 % and 108 %, against tested pathogens than reference strain. The action by isolated strain enhanced the zone upto 221.45 % (32.00 \pm 0.09) and 225 % (27.00 \pm 0.12) for both the pathogens in respective manner, while through the reference strain, zone of inhibition was enhanced by 166.09 % (24 \pm 0.15) and 208.33 % (25.00 \pm 0.10), as compared to control (Table 3, Fig. 6A).

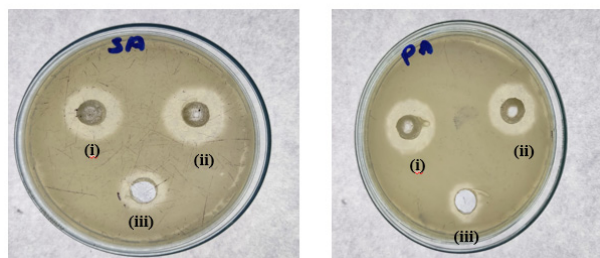


Fig. 6A. Antimicrobial activity of reference strain (i) and AUPPB02 (ii) against *Staphylococcus aureus* and *Pseudomonas aeruginosa*, with positive control (iii) Amoxicillin (5 μ g)

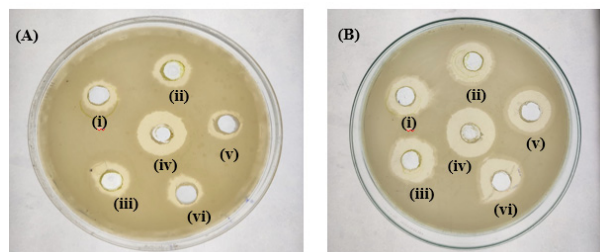


Fig. 6B. Antibiotic susceptibility of (A) reference strain and (B) AUPPB02 against (i) azithromycin, (ii) tetracycline, (iii) erythromycin, (iv) amoxicillin, (v) norfloxacin, (vi) levofloxacin (5 μ g)

The antibiotic susceptibility of *B. amyloliquefaciens* AUPPB02 and *L. acidophilus* are depicted in Fig 6B. This show that isolated strain was more sensitive against all tested antibiotics (azithromycin, tetracycline, erythromycin, amoxicillin, norfloxacin, levofloxacin) than reference strain.

DISCUSSION

Phytases are the enzymes, which catalyze the breakdown of PA into myo-inositol and phosphoric acid. They are one of the major interests in food and feed industry. In the present study, extracellular phytase was isolated and further purified from *Bacillus amyloliquefaciens* AUPPB02, after gel filtration as monomeric protein of 47 kDa molecular weight. However, the relative molecular mass of our purified enzyme was in range as compared to the other *Bacillus* phytases, viz. *Bacillus* sp. MD2, 47.5 kDa (Tran *et al.*, 2011), *B. subtilis* CF92, 46 kDa (Hong, 2011); *B. nealsonii* ZJ0702, 43 kDa (Yu and Chen, 2013); *B. subtilis* natto N-77, 38 kDa (Shimizu, 1992); *B. subtilis* MJA, 38 kDa (El-Toukhy *et al.*, 2013) and *B. licheniformis* PFBL-03, 38 kDa (Fasimoye *et al.*, 2014). Purified phytase showed activity of more than 80 % at the pH range (6.0 – 9.0) and temperature range (30–50 °C), with optimum activity at 7 pH and 40 °C temperature. In accordance with our finding, research report of Boukhris *et al.* (2015) also reflected the pH 9 and temperature 37 °C, as optimum conditions for phytase activity in *B. amyloliquefaciens* US573. In our study, the highest enzymatic activity was recorded against sodium phytate (100 %), followed by calcium phytate (98.2 %). However, there was a sharp decline in enzymatic activity, against phenyl phosphate and glycerol -2-phosphate. Such findings were also reported by various researchers during their study on bacterial phytases (Choi *et al.*, 2001; Farhat *et al.*, 2008; Yu and Chen, 2013). These results conclude that *Bacillus* phytase had narrow substrate specificity, which predominantly hydrolyzed their natural substrate, phytate. All tested additives like EDTA (27.27 %), BME (33.33 %), sodium azide (32.72 %), oxalate (33.93 %) and citrate (21.21 %) showed decline in phytase activity. Hence, no additives are required for optimum phytase activity in *B. amyloliquefaciens* AUPPB02. Purified phytase showed K_m and V_{max} value of 1.057 mM and 0.20 μ moles/sec, respectively. K_m represents the enzyme affinity for a specific substrate and this value can alter according to the environmental conditions, e.g., pH, temperature and

Table 3. Antimicrobial activity of *B. amyloliquefaciens* AUPPB02 and *L. acidophilus*

Pathogen	<i>Staphylococcus aureus</i>	<i>Pseudomonas aeruginosa</i>
Amoxicillin (5 μ g)	14.45 \pm 0.10 mm	12.00 \pm 0.09 mm
<i>B. amyloliquefaciens</i> AUPPB02	32.00 \pm 0.72 mm	27.00 \pm 0.21 mm
<i>L. acidophilus</i>	24.00 \pm 0.15 mm	25.00 \pm 0.82 mm

ionic strength. If the K_m value is less, it demonstrates about the enzyme to reach its saturation, with a small amount of substrate, while a large K_m denotes, the requirement of higher amount of substrate concentration, to reach maximum reaction velocity. The K_m value of phytase was observed from 0.08 to 10 mM. The maximum velocity of an enzymatic reactions can be represented as V_{max} , when the binding site is saturated with substrate (Dokuzparmak *et al.*, 2016).

Such physio-biochemical properties of phytase, produced by *B.amyloliquefaciens* AUPPB02, make it suitable for feed and food application and could be helpful in catalysis of phytic acid, present in feed and food components of monogastric animals, under gastrointestinal (GIT) environment. The *Bacillus* species are normally not found in the GIT environment, but these effectively keep a favorable balance of microbiota there and thus, there may be availability of improved performance of nutrients and health in animals (Kritas and Morrison, 2005).

Therefore, the isolated *B.amyloliquefaciens* AUPPB02 compelled us to evaluate its probiotic potency, against *L. acidophilus* (MTCC 10307), which was earlier established as probiotic strain (Gao *et al.*, 2022). The gut acidity is the most important, determining factor that severely affects the growth and viability of any probiotic strain (Pan *et al.*, 2009). The survival rate of isolated strain was higher than reference strain, at acidic pH of 2 and 3. This is due to the fact that *L. acidophilus* is a non-spore forming bacterium, while *B.amyloliquefaciens* AUPPB02 forms spores that support better survivability, even under acidic environment of GIT. Such result was also reported by Ghelardi *et al.* (2022), while working on *Bacillus clausii*. After GIT acid tolerance, bile salt also affects the growth of microbes, due to production of bile salt hydrolase (Mandal *et al.*, 2006). The bile tolerance of isolated strain was remarkably resistant, as 0.3 % to 2.0 %, which is much higher to the reference strain (0.3 % - 0.7 %). Such results from *Bacillus* group have been reported earlier by (Gilliland *et al.*, 1984; Kristoffersen *et al.*, 2007; Wang *et al.*, 2010). Hydrophobicity is a positive character of probiotics and can be defined as a measure of bacterial colonization to various solvents (Krausova *et al.*, 2019). The hydrophobic ability of the isolated and the reference strains were examined against xylene, chloroform and ethyl acetate. The result showed an upsurged percentage of 73.16, 21.13 and 23.81 in the values of hydrophobicity by the isolated strain, as compared to the reference strain, in

accordance with the earlier reports given by many researchers in various *Bacillus* sp. (Sidira *et al.*, 2015; De Souza *et al.*, 2019; Falah *et al.*, 2019). Similar to hydrophobicity, being a positive property, autoaggregation of probiotic strain is necessary for adhesion with intestinal epithelial cells (Tuo *et al.*, 2013). In this regard, the result showed that *B. amyloliquefaciens* AUPPB02 (68.30 %) had an increase of 50.77 % in auto aggregation than *L. acidophilus* (45.30 %). Manvelyan *et al.* (2023), while working on *B.amyloliquifaciens* and the reference strains *Lactobacillus delbrueckii* also obtained, enhanced autoaggregation in their isolated strain. Many other studies have also observed enhanced autoaggregation in the isolated *Bacillus* strains, as compared to the reference strains (Zulkhairi Amin *et al.*, 2019; Algburi *et al.*, 2020; Mazlumi *et al.*, 2022). The isolated bacteria also secrete enzyme that help in digestion of protein and lipid digestibility while probiotic reference strain only help in digestion of protein but not lipid. Protein and lipid are chief component of food and isolated bacteria help in digestion of its, made them more suitable as probiotics.

Apart from considering the above-mentioned properties for probiotic strains, their haemolytic activity assessment should be taken seriously, as a safety consideration for human and animal consumption (FAO and WHO, 2002). None of the strains that we had tested, showed positive haemolytic activity, placing the *B. amyloliquefaciens* AUPPB02 at a safe state. These results are also supported by early researchers, who found that *B. subtilis*, *B. licheniformis*, *B. cereus*, and *B. amyloliquefaciens*, do not exhibit haemolytic activity and are employed in commercial enzyme production (Pedersen *et al.*, 2002; Trivedi *et al.*, 2022). According to Rowan *et al.* (2001), lecithinase is known to be a potential virulence factor, needed by invasive bacterial pathogens to cause systemic infections. No precipitation zone surrounding the inoculation, suggested negative lecithinase production. These data support the harmless nature of isolated strain. Although, Matarante *et al.* (2004) observed positive lecithinase activity in *B. pumilus*, contradicting our finding. However, the research work of Wang *et al.* (2022) stating *B. amyloliquefaciens*, as negative lecithinase producer, comes in our favour.

The antibacterial study of isolated and reference strains against *S. aureus* and *P. aeruginosa*, showed greater activity by isolated strain. This antibacterial

property is beneficial for competing with GIT pathogens. One of the important factors in choosing a probiotic strain of bacteria is antibiotic resistance. The relationship between the use of antibiotics in farm animals and the rise of antibiotic-resistant bacteria in humans has received a lot of attention lately (Salyers *et al.*, 2004). The isolated strain did not show resistance to tested antibiotics, according to results of antibiotic susceptibility, indicating that it is safe to use in feed and food applications.

CONCLUSION

This piece of work deals with isolation, purification and characterization of phytase from *Bacillus amyloliquefaciens* AUPPB02 (NCBI accession no. OR187307), along with its probiotic status. The physiobiochemical results concluded that enzyme might work optimally in monogastric animals, including human beings. It was also observed that isolated strain was much ahead of reference strain in probiotic tests. Hence, this strain can improve food and feed digestibility by releasing phytase that combat the problems caused by PA and can live in GIT.

To achieve its anticipated effects, strain secreting phytase must be investigated in animals under *in vivo* condition also.

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Conflict of Interest

The authors declare that there is no conflict of interest.

Authors' Contribution

Ushakar Mishra: Conceptualization, Methodology, Validation, Investigation, Data curation, Formal analysis, Writing – original draft. Sushma Kumari: Conceptualization, Methodology. Benazir Fatima: Formal analysis, Visualization, Investigation, Data curation, Writing – review and editing. Abha Singh: Writing – review and editing, Validation, Resources, Supervision.

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