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Isolation, characterization and optimization of extracellular phytase producing *Bacillus amyloliquefaciens* AUPPB02

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A B S T R A C T

Lentils (*Lens culinaris* Medik.) are one of the important pulse crops in the world, because of their nutritional quality. They are rich sources of carbohydrates, proteins, iron, dietary fibres, vitamins, minerals and high energetic value. Unfortunately, the acceptability of lentils as staple food is limited, due to presence of various antinutritional factors. One of them is phytic acid (myo-inositol hexakisdihydrogen phosphate), which forms complexes with other nutrients, such as metallic ions (Ca, Mg, Fe, Cu etc.), amino acids, proteins, lipids and vitamins, rendering them unavailable to plants via lowering their solubility and bioavailability. The phytic acid (PA) can only be degraded by the enzyme, phytases (myo-inositol hexakisphosphate phosphohydrolase enzyme), which break it into to myo-inositol and phosphoric acid. The present study gives information about PA, Iron (Fe) and protein present in lentils. It also elaborates isolation, characterization and optimization of phytase producing PA degrading bacteria. Wet food processing methods (soaking, germination and fermentation) were employed to minimise the inhibitory effect of PA, as well asto increase Fe and protein in samples. The contents of PA (Wade Method), iron (Bathophenathroline method) and protein (Bradford's method)were estimated by their respective standard curves, using double beam UV/VIS spectrophotometer (Systronic, 119) at 500 nm, 535 nm, 595 nm, respectively. Among all the wet processes, fermentation supported best for degrading PA (62.35 %) at 72 h than 48 h and 24 h. At this fermentative stage of 72 h, as a result of PA degradation, maximum increment of Fe (245%) and protein (42%) were observed. A total of 11 PPB (AUPPB01-AUPPB011) were isolated from fermented lentil sample and screened out. Among them AUPPB02 exhibited a halozone of 19 mm, having 89.47% hydrolysis efficiency and 1.68 U/ml of enzyme producing capacity. On the basis of morphological, physiological and genotypical characteristics, the isolate was identified as *Bacillus amyloliquefaciens* AUPPB02 (Accession no.- OR187307). The optimized conditions for phytase production were 48h of incubation time, 5.0 pH, 37 °C temperature, 1% inoculum size, 1% of lactose as carbon and 1% of peptone as nitrogen source. The strain showed increment of 21.33% phytase production in optimised media (1.76± 0.10 U/ml) than in pre-optimised media (1.45±0.12 U/ml).

Introduction

Being an essential macronutrient, phosphorous (P) is less abundant (0.1% of total) in the Earth's crust. It is utilized by plants through various metabolic processes, such as photosynthesis, respiration and cell division (Karpagam and Nagalakshmi, 2014; Motamedi, 2016). The concentration of P, present in soil is totally dependent upon the content of its original source. While, considering the total P in soil, organic P accounts for approximately 30-65% and on the other hand inorganic P constitutes for approximately 30-75% (Rizwanuddin *et al.,* 2023). One of the most prevalent organic forms of P present in soil is Phytic acid (PA) or phytate and is chemically named as myo-inositol hexakisdihydrogen phosphate (Dahiya, 2016). This primary source of inositol is present in a lot of cereals (wheat, barley, rice, maize, etc.), legumes (soyabean, lentils, pulses, etc.) and nuts in the form of minerals as well as storage (Mittal *et al.,* 2011; Kim *et al.,* 2015; Baruah *et al.,* 2017). Despite, its presence in soil and seeds of various crops, it remains in unavailable form for plants (Lazali *et al.,* 2013; Mittal *et al.,* 2012; Shivange *et al.,* 2012), because of its complexity. This compound has strong binding capacity to mono or divalent cations in soil (Cerino Badone *et al.,* 2012). 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 (Selle *et al.,* 2012; Shim and Oh, 2012), rendering them unavailable to plants via lowering their solubility and bioavailability. This limits the absorption of these nutrients in them (Savita *et al.,* 2017; Rizwanuddin *et al.,* 2023).

Normally, chemical and physical methods are used to degrade PA, which in turn reduce the nutritive value of food and feed products (Olika *et al.,* 2019). Thus, to overcome the said problem, alternative biological food processing and preparation techniques, such as fermentation, sprouting and soaking, along with the exogenous enzymes are used (Sharma and Shukla, 2020; Suri and Tanumihardjo, 2016; Palacios *et al.,* 2008).

Moreover, various studies have shown that the fermented products facilitate the bioavailability of minerals by reducing the PA content (Nionelli *et al.,* 2018; Yildirim and Arici, 2019). These 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.

Phytase can be categorised into two main classes, (i) location of dephosphorylation on the inositol ring and (ii) the catalytic mechanism (Ghorbani Nasrabadi *et al.,* 2018; Greiner *et al.,* 2007; Mullaney and Ullah, 2003). The first class of phytase, which is present on the location of dephosphorylation on the inositol ring, are types of (a) 3-phytase (EC 3.1.3.8), produced primarily by microbes; (b) 4/6-phytase (EC 3.1.3.26) by plants; and (c) 5-phytase (EC 3.1.3.72) by many legumes, such as *Pisum sativum, Phaseolus vulgaris* and *Medicago sativa* (Bhavsar and Khire, 2014; Greiner and Carlsson, 2006). The second class of phytase enzymes, which are based on the catalytic mechanism are also classified into (a) acidic (EC 3.1.3.2) and (b) alkaline (EC 3.1.3.8) types. Acidic phytases include histidine acid phosphatase, cysteine acid phosphatase and purple acid phosphatase while, alkaline phytase comprises β-propeller phytase. Phy A-3-phytase, Phy B-3-phytase, Phy C-6-phytase belong to a subclass of histidine acid phosphatase, while Phy D-3-phytase is subclass of β-propeller phytase, produced by Bacillus sp. (Mullaney and Ullah, 2003; Nagar, 2021).

Due to wide range of actions, high activity, economic benefits at various scales and quick production turnaround times, microbial phytases have developed keen interest of researchers over the time. According to Dahiya (2016), phytase is known to enhance bone health in animals, decrease mineral deficiencies, decrease nonutilized PA and boost mineral absorption and bioavailability. The use of microbial phytase can be an innovative approach that orchestrate the path of food safety, through balancing antinutritional factors.

Phytases have been found in various microorganisms, such as *Aspergillus fumigatus* (Vasudevan *et al.,* 2017), Enterobacter sp.4 (Vasudevan *et al.,* 2019), *Bacillus amyloliquefaciens* (Selle and Ravindran, 2007), *Lactobacillus sanfranciscensis* (Raghavendra and Halami, 2009), *Bacillus* sp. (Choi *et al.,* 2001) and others.

The phytase-producing bacteria have been isolated from many terrestrial and aquatic habitats, but less information is available about their occurrence from fermented food, which is the natural habitat of diverse microbial community. This study includes about impact of various food processing methods on PA, iron (Fe) and protein contents, as well as the isolation and characterization of potent phytase producing bacterial (PPB), under optimised conditions.

Materials and Methods

Chemicals

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

Seed sampling and processing

Seed sample of Lentil (*Lens culinaris* Medik.) was procured from farmyard of Patna, Bihar. It was pre-processed for the isolation and assessment of its nutritional properties through various wet methods. The wet processing methods: i) soaking, ii) germination and iii) fermentation were used to study their impacts on PA, Fe and protein contents in the selected samples. All the experiments were done in triplicates. During soaking, 100 g of seeds were cleaned, washed and soaked in 500ml of sterile double distilled water (DDW) at (~25°C) for 24h under ambient laboratory conditions. At the end of period, the water was drained, soak dried on blotting paper and crushed in mortar and pestle. In germination process, the soaked seeds (24 h) were allowed to germinate in wet muslin cloth for 72 h and were sprinkled with sterile DDW every day. The seeds with radicle were picked up, soak dried on blotting paper and crushed. For fermentation method, dry seeds were grounded to powder and mixed with sterile DDW (1:5 w/v) left to ferment in 250 ml conical flasks separately, for 24 h, 48 h and 72 h at 30±2°C in shaker cum incubator (Rivotek).

Quantitative estimation of PA, Fe and protein

Effect of different wet processes on the sample along with the control (dry raw seeds) were analysed by estimating their PA, Fe and protein contents quantitatively, using double beam UV/VIS spectrophotometer (Systronics, 119). PA estimation was carried out by Wade method (Latta and Eskin, 1980) at 500nm and the concentration was calculated through extrapolating the value on standard curve of sodium phytate. Fe estimation was carried out by modified bathophenathroline method (Cowart *et al.,* 1993), measuring only the non-haem-iron at 535 nm. The Fe concentration was calculated with reference to the standard curve of Mohr's salt. Protein estimation was carried out by Bradfords method (Bradford, 1996) at 595 nm. The protein concentration was calculated with reference to the standard curve of BSA. Among all the wet processing methods, the fermentation was more supportive for microbial growth and thus selected for isolation.

Isolation of phytase producing bacteria (PPB)

For isolation of bacteria, 5 g of powdered samples were weighed and mixed with 20 ml sterile DDW in conical flasks (n=3) and were allowed for fermentation at 30±2°C for 24 h, 48 h and 72 h in shaker cum incubator. To continue with isolation, 1ml of fermented sample was added in 9ml of physiological saline (0.85%) solution, vortexed vigorously and serially diluted (10^{-1} to10^{-7}). The aliquots (0.1 ml) of each dilution were spread on nutrient agar (NA) plate and incubated at 30±2°C for 72 h under regular observation. After incubation, colonies showing different morphology (variations in shape, colour and regularity) were picked up and further sub-cultured on same media to get pure isolates and were maintained at 4°C for further studies (Aneja, 2018).

Screening of phytase producing bacteria

Qualitative screening for phytase production by bacterial isolates were carried out through streaking on phytase screening medium (PSM), containing 15 gl^{-1} Glucose, 5.0 gl^{-1} NH4 NO3, 0.5 g^{l-}1KCl, 0.5 gl-1 MgSO4. 7H2O, 0.01 $gl^{-1}FeSO_4.7H_2O$, 0.01 $gl^{-1}MnSO_4.7H_2O$, 0.3 % Ca-PA,20.0 gl^{-1}agar; pH 5.5 and incubated at 30±2°C for 24-72 h. Bacterial colonies showing zone of hydrolysis on solid plates, indicated for positive phytase activity (Mussa *et al.,* 2023). Those PPB were again qualitatively confirmed on PSM plates by counterstaining with 2% cobalt chloride solution for 5 min, followed by addition of equal volumes of aqueous ammonium molybdate (6.25% w/v) and ammonium vanadate (0.42% w/v) solution. PPB was identified by the presence of a clear halo zone around the colony (Bae *et al.,* 1999). The positive isolates were stored at 4°C on Luria- Bertani (LB) slants (10 g^{l-}1tryptone, 5 g^{l-}1yeast extract, 10 gl^{-1} NaCl, pH 7.0) for further studies.

Preparation of bacterial inoculum

The LB broth medium (10 ml) was inoculated with bacterial isolate, incubated overnight in a shaker cum incubator (200 rpm) at 30±2°C. 2ml of the bacterial culture having OD_{600} of 0.1 (1 x 10^8 $CFUml^{-1}$) was centrifuged at 10,000 rpm. The cell pellet was transferred

into 250 ml flask containing 100 ml of MRS (2.0% glucose, 0.075 % sodium phytate, 0.2 % $(NH_4)_2SO_4$, 1.75 % of calcium chloride (1M), 0.5 % beef extract, 0.5 % sodium acetate, 0.2 % tri ammonium citrate, 2 % $MgSO_4.7H_2O$ (100 mM), 0.5% $MnSO_4$ (10 mM) 0.65 KCl (1 M) 0.1% Tween 80, pH 5.5) and incubated at 30±2°C and 200 rpm for 48 h. Now the incubated sample (10 ml) was centrifuged for 15 min at 1400 rpm, pellet was washed twice with 0.1 M acetate buffer (pH 5.5) and suspended in 0.2 M acetate buffer (pH 4.5-6.3) for estimation of phytase activity.

Phytase assay

For the assay of phytase activity, the bacterial suspension (0.1 ml) from above section was added with 0.9 ml substrate (2 mM sodium phytate in 0.2 M acetate buffer) and incubated at 35°C for 60 min. After the completion of incubation period, the reaction was stopped by adding 1ml of (10%) aqueous trichloroacetic acid (TCA). The liberated orthophosphate was measured by a modified ammonium molybdate method (Heinonen and Lahti, 1981). This method was chosen, since it uses the direct detection of the yellow phosphomolybdic acid, without reduction to molybdenum blue. 0.5 ml assay mixture (cell suspension + substrate + trichloroacetic acid) was mixed with 4ml of freshly prepared stop solution of (acetone:5 N H_2SO_4:10 mM ammonium molybdate in the ratio of 2:1:1 (v/v)) and vortexed. Further, 0.4 ml citric acid (1 M) was added to the assay mixture, which complexes with excess molybdate. Any cloudiness was removed by centrifugation and absorbance was taken at 355 nm.

The assay mixture of blank contained 0.1 ml buffer (0.2 M acetate buffer), substrate and TCA. In order to calculate the enzyme activity, a calibration curve was drawn over the range of 100-1500 µg/ml orthophosphate. Enzyme activity was expressed in international units (U). 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.

Phenotypic characterization of isolate

The selected isolate was morphologically, physiologically and biochemically characterized. For morphological characterization, colony morphology, Gram-staining, endospore-forming ability, motility, etc. were studied. Temperature (20, 30, 40, 50, 60, 70°C), pH (3, 4, 5, 6, 7, 8 and 9) and NaCl (0.5, 1.5, 2.5, 3.5, 4.5, 5.5, 6.5, 7.5 % w/v) tolerance assay contributed the physiological tests. Biochemical tests were conducted by referring the Bergey's manual of Determinative Bacteriology (9th ed., 1993). It included tests of methyl red, Voges Proskauer, citrate utilization, catalase, oxidase, urease, nitrate reduction, starch hydrolysis, gelatin hydrolysis, H_2S production and carbohydrate fermentation.

Molecular characterization of isolates

16S rDNA sequencing

The bacterial isolate was further characterized by 16S rDNA gene sequencing. Genomic DNA from the selected PPB was isolated. The amplification of 16 S rRNA gene was carried out by using 50µl reaction mixture, containing 2.5 mM each of four dNTP (4µl), 10X Taq DNA polymerase assay buffer (10µl), 3U/ml Taqpolymerase enzyme (1µl), template DNA (1µl) and 2µl each primer of forward (16s F: GGATGAGCCCGCGGCCTA) and reverse (16s R: CGGTGTGTACAAGGCCCGG) oligonucleotide, along with millipore water (30µl). The amplification was set as initial denaturation at 94 °C for 3min followed by 30cycles of denaturation (94 °C for 1min), annealing (50 °C for 1min) and extension (72 °C for 2min). The final extension was at 72 °C for 7min. The sequencing was performed according to manufacturer's protocol using Big Dye Terminator Cycle Sequencing Kit (v3.1, Applied Biosystem) and analysed in an applied biosystems analyzer. Sequenced data were aligned and analysed to find the closest homolog of the isolates using the BLAST search tool (http://www.ncbi.nlm.nih.gov). A phylogenetic tree was made in MEGA 11 software using the Neighbor-joining method with Bootstrap analysis to obtain information on their molecular phylogeny. The partial 16S rDNA sequence of isolate has been deposited in the NCBI Genbank database to obtain the accession number.

Optimization of culture condition for phytase production

Various parameters viz., incubation period, temperature, pH, inoculum density, carbon (C) and nitrogen (N) sources were investigated for optimum phytase production. Optimization was done with one variable at a time. First of all, the effect of different incubation times (24, 48, 72 and 96 h) was studied in MRS media at 30±2

°C. Thereafter, effect of various temperatures (20, 25, 30, 37 and 42°C) and pH of MRS media (2, 2.5, 3, 3.5, 4, 4.5, 5 and 5.5) were studied. Effect of inoculum density varying from 0.1 to 5% in the growth media was also optimised. The study about impact of different C-sources by replacing mannose to glucose, fructose, maltose, sucrose, lactose, starch, carboxy methyl cellulose (CMC), xylan, wheat bran and sugarcane bagasse were also done. Similarly, the effect of N-sources was studied by replacing $(NH_4)_2SO_4$ to peptone, yeast extract, beef extract, sodium nitrate, urea and malt extract. Along with above studies, the optimization of C and N concentrations were also carried on for optimum phytase production.

Statistical analysis

The data obtained were statistically analysed by MS-Excel 2019 and GraphPad Prism 8 software, graphically represented as the mean±standard deviation (n=3).

Results and Discussion

Estimation of PA, Fe and protein in collected lentil seed sample

Concentration of PA was calculated by standard curve of sodium phytate (R^2=0.9953). Fe was estimated by the standard curve of Mohr's salt (R^2=0.9937) and protein by BSA (R^2=0.9944).

During different processing methods, levels of Fe content and protein were gradually increased but at same time, there was decrease in PA level. The maximum decline of PA was observed under fermentation of 72 h (62.35 %) followed by 48 h (53.93 %), 24 h (43.61 %), germination (20.93 %) and soaking (13.56 %). On the contrary, there was increment of Fe and protein contents in fermentation at 72 h (242.85%, 45.32%), which lagged behind by fermentation of 48h (165.04 %, 35.98 %), 24 h (93.31 %, 26.25 %), germination (69.60 %, 14.18 %) and soaking (26.44 %, 7.12%), respectively when compared with control i.e., raw seeds (Table 1).

It could be due to microbial activities, which played a great role in improving nutritional quality by degrading anti-nutritional component like, PA. This also concluded that PA was negatively correlated with Fe and protein. Such result was also supported by early research of Kumari *et al.,* (2020), who concluded that soaking and germination increased the amount of iron and protein, while fermentation maximised them and negatively correlated with PA in lentil. The pH of the fermented lentil was found to be 6.4 in control, which decreased to reach the pH of 5.0 after the fermentation of 24 h, followed by 4.5 at 48 h and 4.3 at 72 h. This is due to production of microbial acid, which resulted the decrement of pH during fermentation.

Isolation of phytase producing bacteria

In the present work, total of twenty-five bacterial colonies, corresponding to 24 h (08), 48 h (08) and 72 h (09), which appeared on NA plates were picked up for phytase activity.

Screening of bacterial isolates for phytase activity

Qualitatively, screening of PA degrading bacteria was carried out on PSM by single line streak technique. Among 25 isolates, eleven showed, a diameter of 8 to 22 mm clear halo zone, around their colony. Those phytase positive bacterial isolates were designated as AUPPB01-AUPPB11. One of the isolates, designated as AUPPB02 exhibited considerable halo zone of 19mm on PSM plate (Fig. 3b) was selected for further study.

Phenotypic characterization of isolate

The isolate AUPPB02 appeared as small, having irregular margin, lobate, creamy mucoid whitish colony (Fig. 3a). The microscopic observations revealed it as Gram positive, motile, rods, arranged singly, having ellipsoidal sub terminal endospore. The physiological observations of the isolate, revealed its tolerance towards temperature upto20 to 60 °C, pH upto 3 to 7 and salinity upto 0.5 to 6.5 % (w/v). Various biochemical tests, pertaining to the isolate's carbohydrate utilization have been listed in the Table 3 as positive or negative results. On the basis of morphological, various physiological and biochemical tests, the isolate AUPPB02 was identified as Bacillus sp., following the Bergey's manual of Determinative Bacteriology (9th ed., 1993).

Genotypic characterization of isolate

Genotypic characterization of the isolate (AUPPB02) was done by 16S rDNA gene sequencing. Such analysis showed its 99.63% homology with the type strain *Bacillus amyloliquefaciens* in the database. On the basis

of homology, a phylogenetic tree was drawn, which identified the isolate as *Bacillus amyloliquefaciens* AUPPB02. The aligned partial sequence data (1347 bp) shown in Fig. 3 has been deposited in Genbank of NCBI with the accession number OR187307. The sequenced analysis of *Bacillus amyloliquefaciens* AUPPB02 revealed its close relationship with *B. amyloliquefaciens* strain NBRC 15535; Class: Bacilli, Order: Bacillales, Family: Bacillaceae.

Optimization of culture condition for phytase production

The optimum incubation period for phytase production by *B. amyloliquefaciens* AUPPB02 was 48 h. It was initiated at 24 h (0.25 ± 0.02 U/ml), maximised at 48 h (1.56 ± 0.43 U/ml), followed by decline upto after 72 h (1.15 ± 0.79 U/ml) of incubation period (Fig.6).

Such trend of result was also observed by Olajuyigbe (2016) in *B. amyloliquefaciens* PFB-02. In his strain, the phytase production was initiated slowly from 12 h of cultivation, reached maximum at 48 h and drastically declined thereafter.

There was a minor level of increase in phytase production upto 30 °C, which reached to its optimum production level at 37 ˚C, followed by steep decline at 42 °C (Fig. 7), depicting the optimum temperature of 37 ˚C for phytase production (1.65 ± 0.36 U/ml). In the same manner, Olajuyigbe (2016) also reported in *B. amyloliquefaciens* PFB-02, which grew maximally upto 40 °C with drastic decline after and above that temperature (40 °C), where maximum phytase was produced at the temperature range of 30-40 °C. Similar optimum temperature was also reported by an earlier worker, Kerovuo *et al.,* (1998), who studied on *B. subtilis* and achieved the highest yield of phytase at 37 °C.

Phytase production was initiated from pH 2 with gradual increment over a broad range of 5.5, being optimum at pH 5 (1.65± 0.31 U/ml). There was decrease in phytase content, as well as in bacterial growth, when pH goes above the optimum level (Fig. 8).

Work of Olajuyigbe (2016) supported our result that *B. amyloliquefaciens* PFB-02, also can grow maximally over a narrow pH range of 4.0 to 6.0 with highest growth at pH 5.0. Gulati *et al.,* (2006) also reported similar optimum pH of 5.5 for phytase yield of 1.80 U/ml from *Bacillus laevolacticus*. On the contrary, optimum phytase production from *Bacillus* sp. at alkaline pH values of 7.5 and 8.0 were reported by Demirkan *et al.,* (2014) and Fu *et al.,* (2011), respectively. Such results reflect that if there is any decrease or increase in optimum pH level of any microorganism, it results poor microbial growth, leading to the reduction in level of metabolites.

Phytase production was initiated at the inoculum density of 0.1%, which ranged broadly with a density of 2.0%. Within the range of 0.1%-2.0% (Fig. 9) the optimum inoculum density was obtained as 1% with enzyme activity of 1.64 ± 0.47U/ml at 48 h.

There was decrease in phytase content, as well as in bacterial growth, when inoculum density goes beyond the optimum level. This may be due to increased competition among the bacterial population for nutrient uptake and creation of nutrient imbalance. In the like manner, Trivedi *et al.,* (2017) reported maximum phytase production in *B. subtilis* P6, at the inoculum density of 2.5% (v/v) and incubation period of 20 h.

Different C sources, used in growth media, worked differently, as far as their support in phytase production is concerned. Maximum phytase was produced by using lactose (1.59 ± 0.65 U/ml), followed by maltose (1.55 ± 0.43 U/ml), fructose (0.9 ± 0.19 U/ml), starch (0.83 ±0.08 U/ml), CMC (0.74 ± 0.07 U/ml) and xylan (0.63 ± 0.34 U/ml).

The two agricultural wastes i.e., wheat bran (0.66 ± 0.28 U/ml) and sugarcane bagasse (0.3 ± 0.06 U/ml) also assisted positively in phytase production (Fig.10). Report of Olajuyigbe (2016), stated about positive role of wheat bran as C source, for phytase production in *B. amyloliquefaciens* PFB-02. While working on *Bacillus* sp., Demirkan *et al.,* (2014) found lactose as best carbon source among other used.

Among all the studied C sources, lactose was best supporter (1.59 ± 0.65 U/ml) and thus its optimised concentration in media was found as 1%, to produce 1.63 ± 0.96 U/ml phytase enzyme.

With gradual increase of lactose source in media from 0.25% to 1%, there was an enhanced phytase production, but by increasing the concentration to 1.5% there was a decline in phytase production, as well as in bacterial population (Fig. 11).

Table.1 Amount of PA, Fe and Protein in collected seed sample

Sl. No.	Processing Methods	PA (mg/100gm) (p≤ 0.005)	Fe (mg/100gm) (p≤ 0.005)	Protein (mg/gm) (p≤ 0.005)
1.	Raw	63.19±1.3	3.29±0.29	43.77±0.426
2.	Soaking	54.62±0.03	4.16±0.02	46.89±0.874
3.	Germination	49.96±0.13	5.58±0.43	49.98±0.746
4.	Fermented (24h)	35.63±0.98	6.36±0.76	55.26±0.808
5.	Fermented (48h)	29.11±0.88	8.72±0.014	59.52±0.433
6.	Fermented (72h)	23.79±1.01	11.28±0.26	63.61±2.31

Table.2 Qualitative and quantitative phytase assay

Isolate	Halozone diameter[a]	Colony diameter[b]	Hydrolysis efficiency*	Quantitative of phytase
AUPPB02	19mm	2mm	89.47%	1.45 ± 0.12

* Hydrolysis efficiency = $\frac{a-b}{a} \times 100$

Table.3 Biochemical characterization of isolate

Sl. No.	Biochemical test	Result	Sl. No.	Biochemical test (Carbohydrate utilization)	Result	Sl. No.	Biochemical test (Carbohydrate utilization)	Result
1.	Catalase test	+	11.	D-galactose	+	21.	D-fructose	+
2.	Oxidase test	+	12.	D-glucose	+	22.	D-maltose	+
3.	Urease	-	13.	D-lactose	+	23.	D-sucrose	+
4.	Methyl Red test	-	14.	D-mannose	+	24.	Inulin	+
5.	Voges Proskauer test	+	15.	D-xylose	+	25.	D-raffinose	+
6.	Citrate	+	16.	D-ribose	+	26.	Starch	+
7.	H_2S	-	17.	D-mannitol	+	27.	Glycogen	+
8.	Gelatin hydrolysis	+	18.	D- cellobiose	+			
9.	Nitrate reduction	+	19.	D-arabinose	-			
10.	Casein hydrolysis	+	20.	Inositol	-			

+: positive, -: negative

Figure.1 Schematic diagram of isolation, characterization and optimization of phytase producing bacteria.

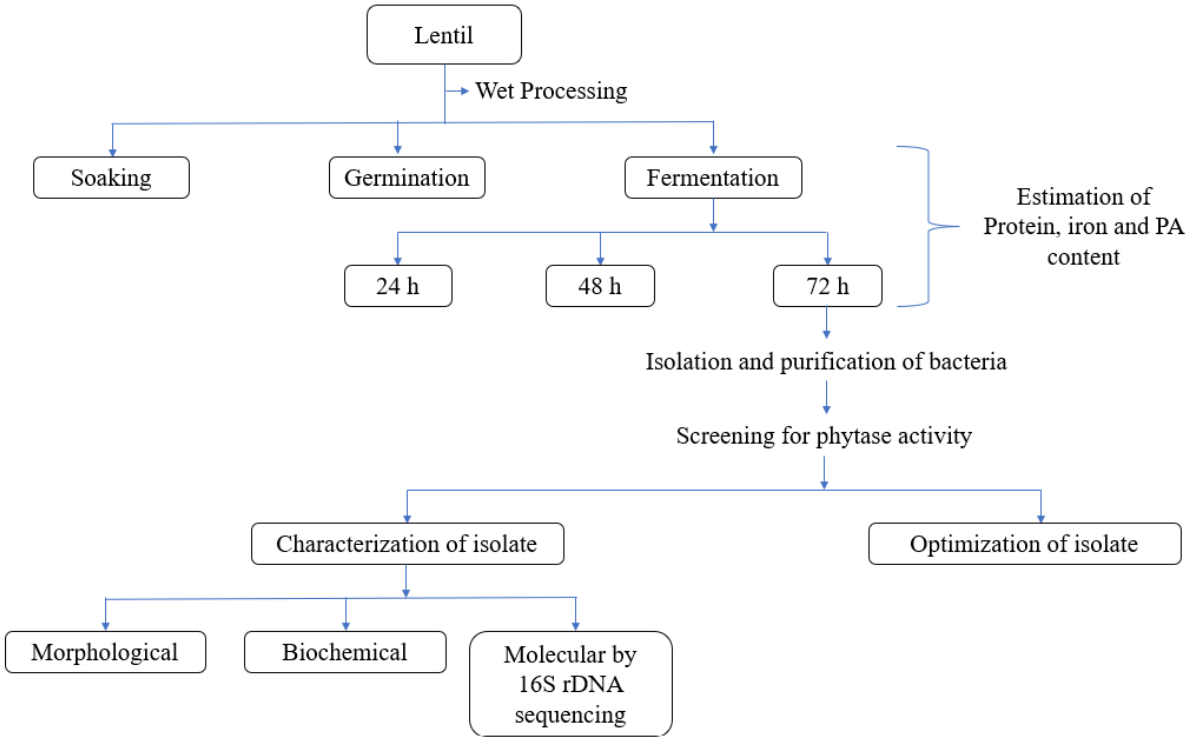


Figure.2 Relative % content of PA, Fe and proteins during different processing methods (1- raw, 2- soaking, 3- germination, 4- fermentation 24 h, 5- fermentation 48 h, 6- fermentation 72 h).

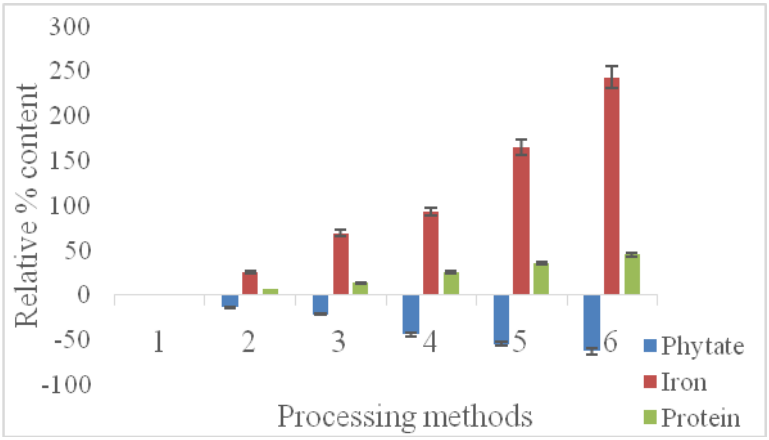
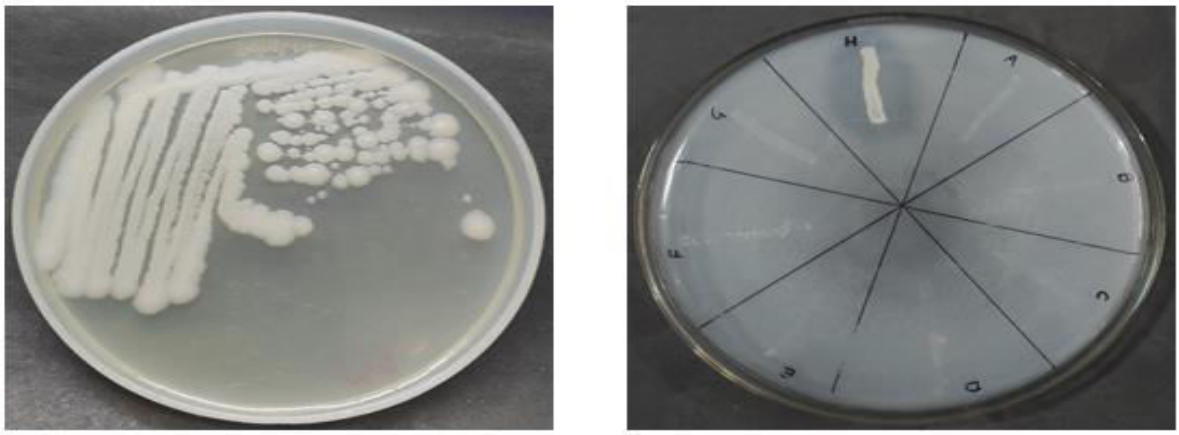


Figure.3 (a) Bacterial isolates (b) appearance of halozone on PSM.



(a) (b)

Figure.4 *Bacillus amyloliquefaciens* AUPPB02 16S ribosomal RNA gene, partial sequence.

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   1 ggagcttgct ccctgatgtt agcggcggac gggtgagtaa cacgtgggta acctgcctgt
  61 aagactggga taactccggg aaaccggggc taataccgga tgcttgtttg aaccgcatgg
 121 ttcagacata aaaggtggct tcggctacca cttacagatg gacccgcggc gcattagcta
 181 gttggtgagg taacggctca ccaaggcaac gatgcgtagc cgacctgaga gggtgatcgg
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 301 gcaatggacg aaagtctgac ggagcaacgc cgcgtgagtg atgaaggttt tcggatcgta
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1321 ggccttgtac acaccgcccg tcacacc
```

Figure.5 Phylogenetic tree showing genetic relationship of *B. amyloliquefaciens* with taxonomically similar strain, species and genus based on 16S rRNA gene sequences. Gene bank accession number of each isolate is given in parentheses. Bootstrap values based on 1000 replicates are shown next to the branches phylogenetic tree.

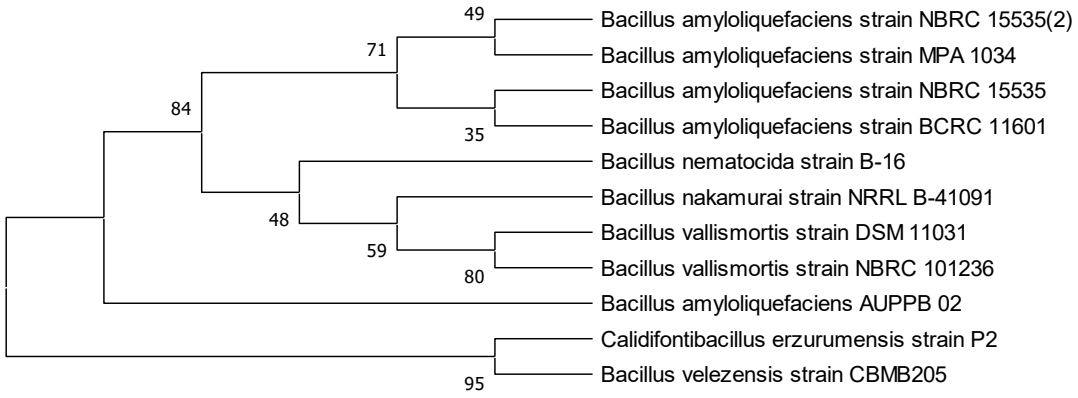


Figure.6 Effect of incubation period on phytase production from *B. amyloliquefaciens* AUPPB02 (OR187307). Bars represent mean ± SE (n=3).

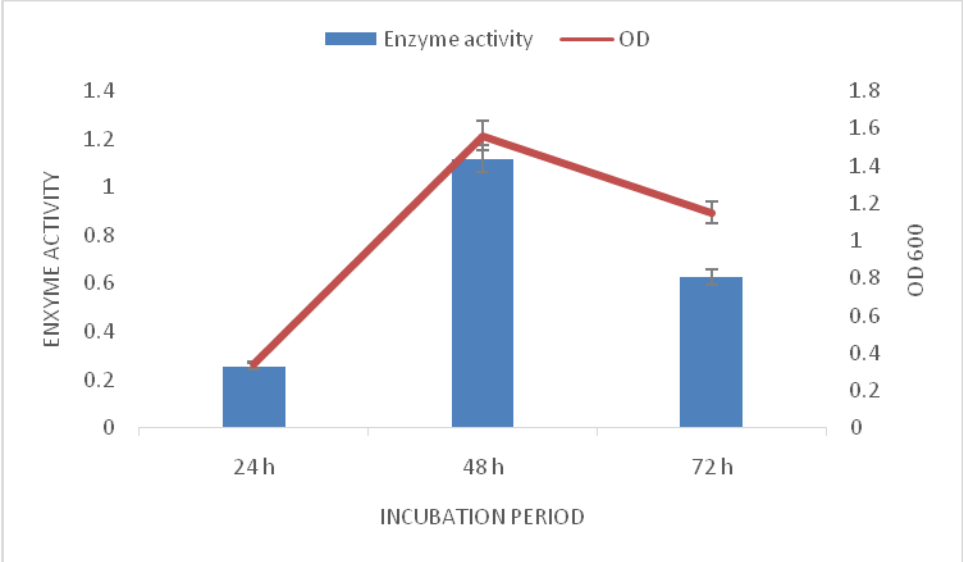


Figure.7 Effect of temperature on phytase production from *B. amyloliquefaciens* AUPPB02 (OR187307). Bars represent mean ± SE (n=3).

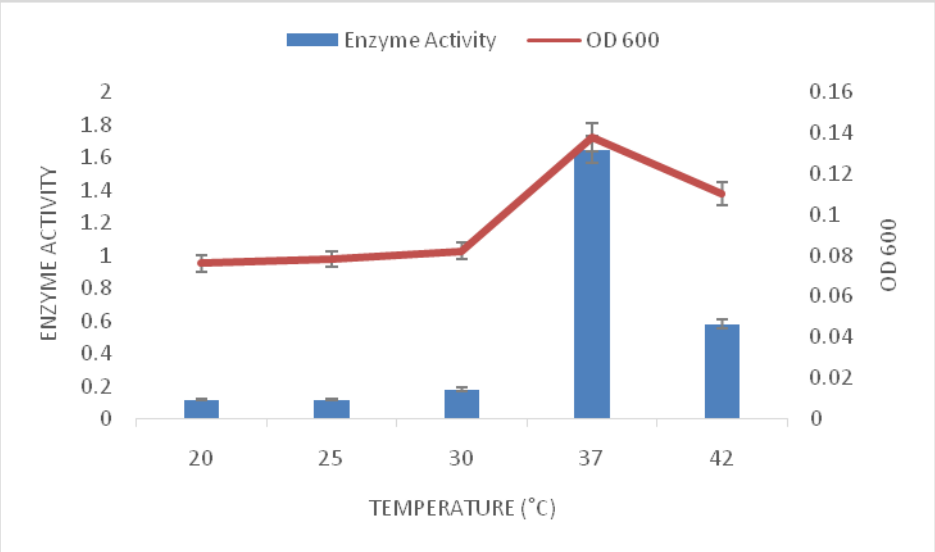


Figure.8 Effect of pH on phytase production from *B. amyloliquefaciens* AUPPB02 (OR187307). Bars represent mean ± SE (n=3).

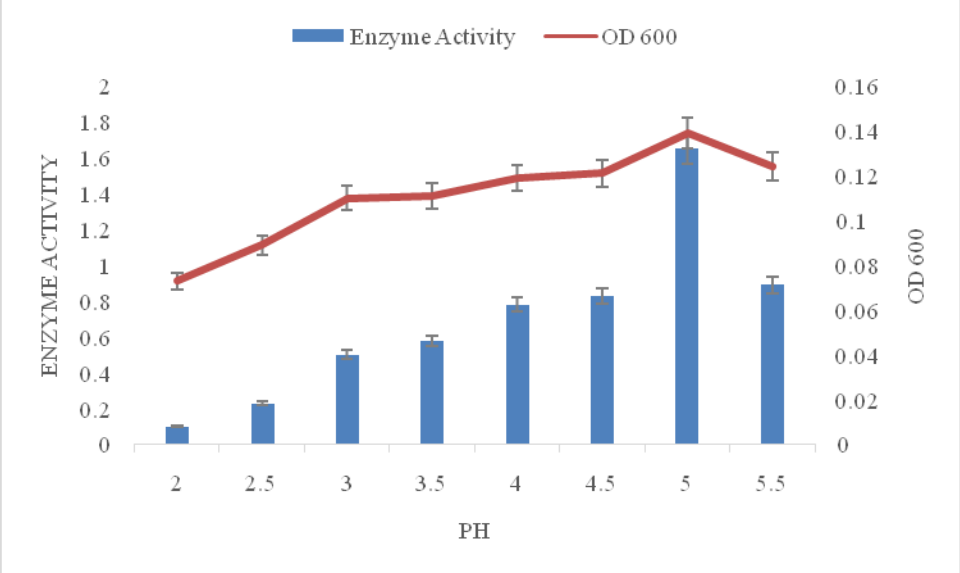


Figure.9 Effect of inoculum density on phytase production from *B. amyloliquefaciens* AUPPB02 (OR187307). Bars represent mean ± SE (n=3).

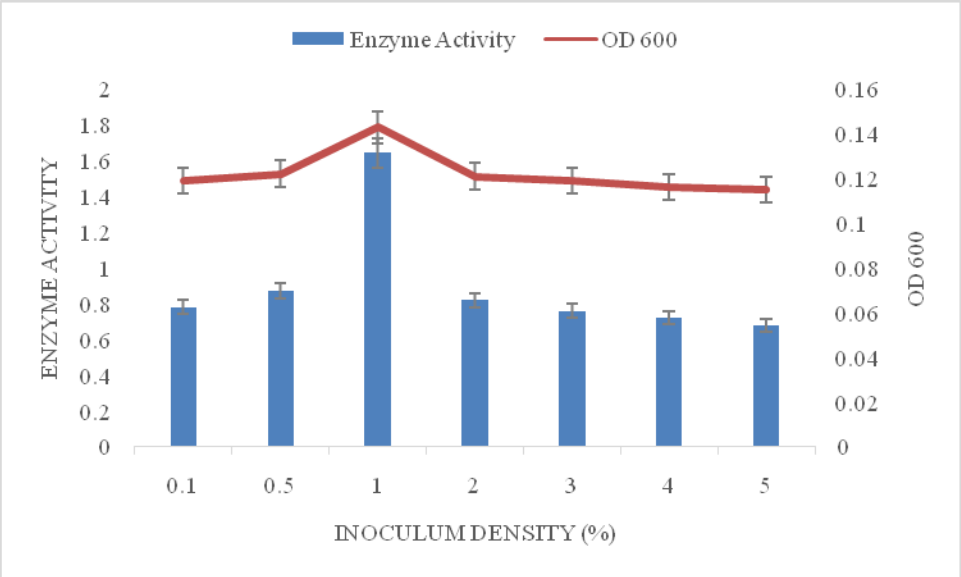


Figure.10 Effect of various carbon source on phytase production from *B. amyloliquefaciens* AUPPB02 (OR187307). Bars represent mean ± SE (n=3).

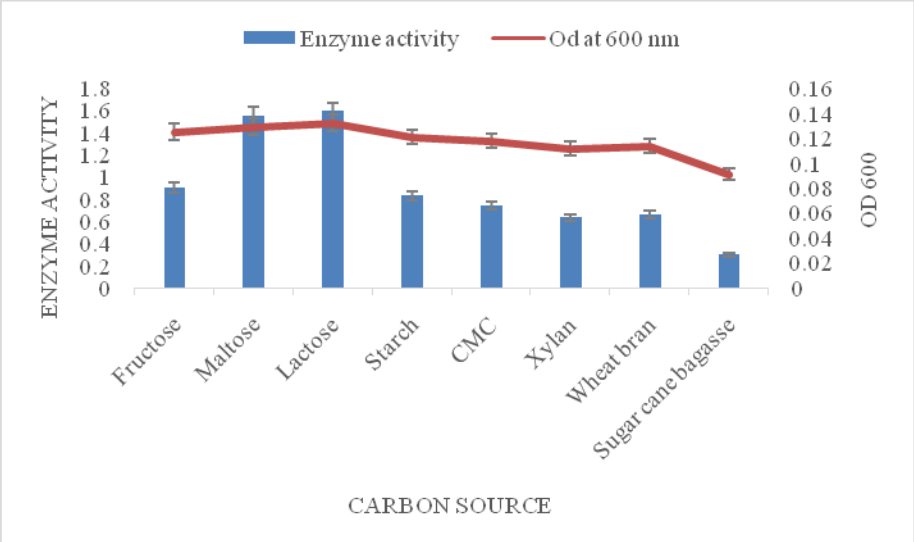


Figure.11 Effect of lactose concentration on phytase production from *B. amyloliquefaciens* AUPPB02 (OR187307). Bars represent mean ± SE (n=3).

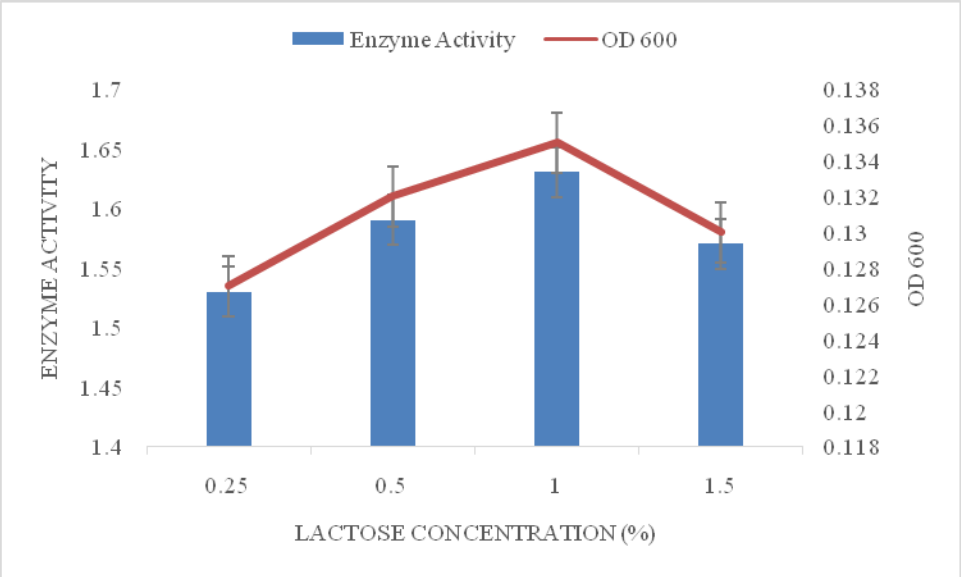


Figure.12 Effect of various nitrogen source on phytase production from *B. amyloliquefaciens* AUPPB02 (OR187307). Bars represent mean ± SE (n=3).

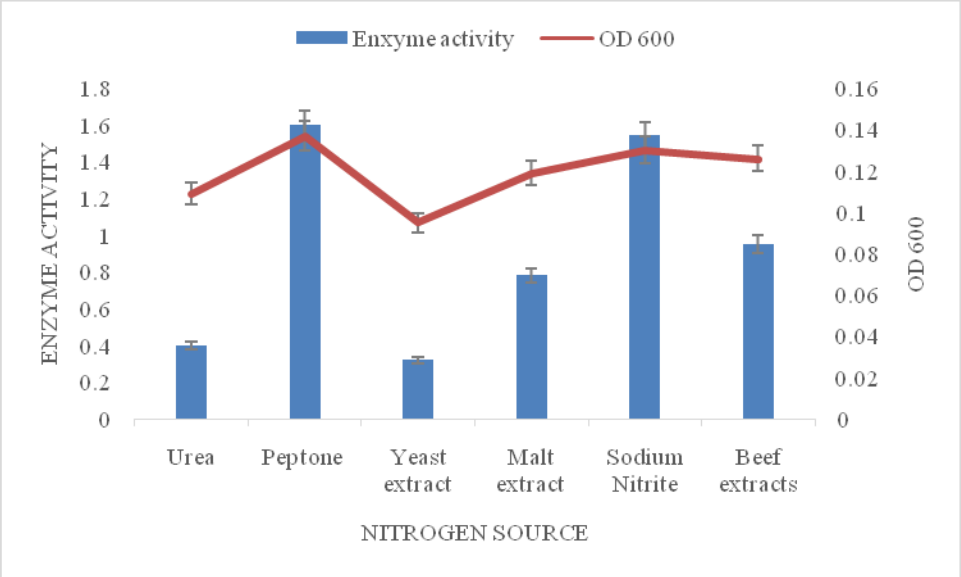
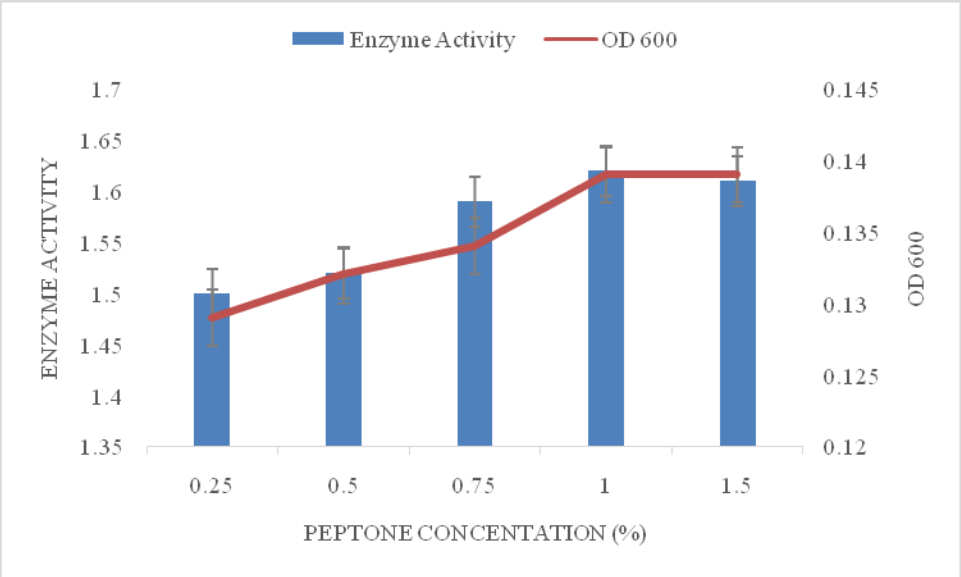


Figure.13 Effect of peptone concentration on phytase production from *B. amyloliquefaciens* AUPPB02 (OR187307). Bars represent mean ± SE (n=3).



Various N sources, such as urea, peptone, yeast extract, malt extract, sodium nitrite and beef extract were studied for phytase production, by replacing ammonium nitrite in media (Fig.12). Maximum phytase was produced by using peptone (1.6 ± 0.47 U/ml), followed by sodium nitrite (1.54 ± 0.01 U/ml), beef extract (0.95 ± 0.07 U/ml), malt extract (0.78 ± 0.31 U/ml), urea (0.4 ± 0.04 U/ml) and yeast extract (0.32 ± 0.02 U/ml).

Such result was also supported by many early researchers, who considered the organic form of N, as best source for maximum phytase production in many bacterial forms, and among them, Sharma *et al.,* (2019) also found peptone as the best N supporter for phytase production in *Lactobacillus paracasei* SMVDUDB1.

In other work by Singh *et al.,* (2013) yeast extract was found as best source of N for *B. subtilis* DR6. In our study also *B. amyloliquifaciens* utilised the yeast extract as N source for phytase production at the rate of 0.32 ± 0.02 U/ml, but of course, at a lesser rate.

The concentration of N source in media was also optimised by taking 0.25%, 0.5%, 1% and 1.5% of peptone, in which the isolate shows maximum phytase production (1.60 U/ml). With gradual increase of N source in media from 0.25% to 1% of peptone, there was increment in phytase production, but when its level goes to 1.5% there was decrement in phytase as well as in bacterial population (Fig. 13). Hence, 1 % of peptone was observed as best concentration of N source for optimum level of phytase production (1.62 U/ml).

In conclusion, the present study revealed that lentil has significant amount of Fe, protein and antinutrient PA, where PA reduces the bioavailability of nutrients to the human body. Through our study, it was inferred that fermentation is the best method of wet processing, followed by germination and soaking, through which PA is reduced and simultaneously there is increase in Fe and protein contents. The isolated strain *Bacillus amyloliquefaciens* AUPPB02 (OR187307) was optimized for phytase production. The optimized conditions for phytase production in production media were 48h of incubation time, 5.0 pH, 37 °C temperature, 1% inoculum size, 1% of lactose as carbon and 1% of peptone as nitrogen sources. There was 21.33% enhanced phytase production in optimised media (1.76 ± 0.10 U/ml) than pre-optimised media (1.45 ± 0.12 U/ml). The enzyme phytase contributes towards break down of PA into myo-inositol and phosphorous, along with other divalent chelated ions, thus its bioavailability increases. Microbial phytases have been applied mainly to animal and human food stuff in order to improve mineral bioavailability and food processing. The use of microbial phytase can be an innovative approach that orchestrate

the path of food safety, through balancing antinutritional factors.

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Abbreviations

BLAST: Basic Local Alignment Search Tool; BSA: Bovine Serum Albumin; CMC: Carboxymethyl Cellulose; DDW: Double Distilled Water; Fe: Iron; M: Molarity; NCBI: National Centre for Biotechnology Information; NA: Nutrient Agar; P: Phosphorous; PSM: Phytase Screening Media; PA: Phytic Acid; PPB: Phytase Producing Bacteria.

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Author Contributions

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

Data Availability

The datasets generated during and/or analysed during the current study are available and attached as supplementary material.

Declarations

Ethical Approval Not applicable.

Consent to Participate Not applicable.

Consent to Publish Not applicable.

Conflict of Interest The authors declare no competing interests.

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