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Evaluation and Optimization of Extracellular Digestive Enzymes from *Bacillus* spp. Isolated from Curds

Rashmi BS and Gayathri D*

Department of Microbiology, Davangere University, Shivagangothri, Davanagere-577002, India

Abstract

Objective: The aim of the present study was to evaluate the digestive enzyme activity of the four *Bacillus* spp. and to optimize the physical parameters.

Methods: The enzymes were produced by submerged fermentation supplementing enzyme specific substrates. The fermentation broths were centrifuged and the supernatants were used as source of crude enzyme. Amylase activity was determined by 3, 5-dinitro salicylic acid method using starch as substrate while copper soap method was used to evaluate lipase activity. Further, protease activity was measured by Lowry's method; whereas, phytase activity was assayed using sodium phytate as substrate. All the enzymes were optimized for pH, temperature and substrate concentration. The total protein content per one mL of the crude enzyme in the supernatant was quantified by Lowry's method.

Results: All the four tested isolates *B. subtilis GS 1, B. cereus GS 3, B. cereus GS 199 and B. subtilis GS 547* showed high extracellular digestive enzyme activity at pH range 5 to 8 and temperature 20 to 50°C.

Conclusion: The four tested *B. subtilis GS 1, B. cereus GS 3, B. cereus GS 199 and B. subtilis GS 547* could be promising extracellular digestive enzyme producing isolates. Further, evaluation of *in vivo* efficacy and safety in animal models and clinical trials would be helpful in assisting digestive enzyme deficiencies using these extracellular enzyme preparations or whole cell bacteria.

Keywords: Digestive enzymes; Amylase; Lipase; Protease; Phytase; *Bacillus*

Introduction

Microbial enzymes are being increasingly used as therapeutics that play an important role in alleviating the burden of several digestive and malabsorption disorders. Use of microbial enzymes to substitute pancreatic enzymes has been considered safe with very less side effects and also proven economic [1]. Further, microbial derived digestive enzymes were proven to exert efficient digestive property at very low doses and also can possess a broader pH range of activity than animal and plant based counterparts [2]. Furthermore, GRAS (generally regarded as safe) strains of bacteria and probiotics with extracellular digestive enzyme activity were also extensively studied for their oral administration in order to improve digestion in the host [3].

Several studies showed that lipase producing probiotics can be used to reduce cholesterol levels, to overcome malabsorption, aid in the proper digestion of fats in the diet in addition to adjunct therapy in the management and prevention of mental depression [4-9]. Further, probiotics have proven to be useful in the management of colorectal cancer as evaluated in mice system and also possess anticancer properties [10-12]. Interestingly, the treatment of steatorrhea by lipase supplementation therapy has become more successful in the last decade [13]. To consider, in an *in vivo* study, acid resistant lipase from fungi was used to treat steatorrhea in dogs. In the study 4800 U of fungal lipase was used and a significant reduction in stool bulking and fat excretion was observed [14]. Further, microbial proteases are known for efficient proteolytic activity and are extensively used in food processing for gluten intolerance, cow's milk allergy, soy allergy and also in other dietary protein intolerances [15,16]. Addition of microbial beta-galactosidases directly to milk represents a potential enzyme replacement therapy for primary lactase deficiency [17]. In addition, the use of probiotic fermented milk products also found beneficial in improving lactose digestion [18]. Further, biotechnological application of phytase in food is gaining importance, since phytate in the diet act as an anti-nutrient [19]. The addition of phytase or fermenting foods with phytase producing strains of GRAS and probiotics can improve nutritional value of plant based foods by improving protein digestion and also mineral bioavailability through phytate hydrolysis [20].

Compared to enzyme preparations, the use of whole microbial cells that secret extracellular digestive enzyme was found beneficial. Because, enzyme preparations must be supplemented at each meal time to ensure the digestion of ingested diet. In addition, there are several problems associated with the use of enzyme preparations that mainly includes *in vivo* efficacy, efficient delivery against gastric condition in the stomach, formulation and dosage [2,21,22].

Most of the commercially available digestive enzymes are from *Aspergillus* and *Bacillus* spp. *Bacillus* spp. continue to be dominant bacterial workhorses in microbial fermentations due to their stability in wide range of pH and temperature [23]. The GRAS strains of *Bacillus* mainly include *Bacillus subtilis, Bacillus cereus, Bacillus licheniformis, Bacillus pumilus, Bacillus clausii* and *Bacillus coagulans* [24]. Given this, in the present study, the four isolates namely: *Bacillus subtilis GS 1, B. cereus GS 3, B. cereus GS 199 and B. subtilis GS 547* isolated

***Corresponding author:** Gayathri D, Department of Microbiology, Davanagere University, Shivagangothri campus, Davangere-577002, India, Tel: +919448823876; Fax: +918192208008; E-mail: gayathridevaraja@gmail.com

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from curd samples were evaluated and optimized for their extracellular digestive enzyme activity.

Materials and Methods

Inoculum preparation

In the present study, the four isolates namely *Bacillus subtilis GS 1, Bacillus cereus GS 3, Bacillus cereus GS 199,* and *Bacillus subtilis GS 547* isolated from curd samples were tested for their extracellular hydrolytic enzyme activity. The isolates were grown overnight in nutrient broth at 37°C. After incubation, the cells were harvested by spinning at 7000 rpm/10 min, washed thrice and resuspended in sterile distilled water to obtain OD 1.2 at A_{600} and used as inoculum.

Quantitative assay for amylase, protease, lipase and phytase from *Bacillus* spp.

Spectrophotometric assay of Amylase by DNS method: The amylase was produced by submerged fermentation in the medium containing lactose 40 gl^{-1}, yeast extract 20 gl^{-1}, KH2PO4 0.05 gl^{-1}, MnCl2 .4H2O 0.015 gl^{-1}, MgSO4.7H2O 0.25 gl^{-1}, CaCl2.2H2O 0.05 gl^{-1}, FeSO4.7H2O 0.01 gl^{-1} [25]. 100 mL of autoclaved production medium was inoculated with 2% inoculum and incubated at 37°C/24 h with continuous shaking (200 rpm). After incubation, the broth was centrifuged at 7000 rpm/15 min in a cooling centrifuge. Then the supernatant obtained was used as source for estimation of amylase activity by DNS (3, 5-dinitro salicylic acid) method by monitoring the amount of reducing sugar liberated from starch. To the one milliliter of crude enzyme, 0.5 mL of 1% soluble starch (prepared in 0.1 M phosphate buffer of pH 6) was added and incubated at 37°C for 20 min. Further, 1 mL of DNS reagent was added and boiled for 10 min to stop the reaction. Then, the final volume was made up to 5 mL by adding distilled water and absorbance was measured at 540 nm. One unit of amylase activity was defined as the amount of enzyme per mL of culture supernatant that released 1 µg of maltose per minute. In the assay, heat killed culture supernatant was served as control. The experiment was repeated in triplicates and results were expressed as mean ± standard deviation.

Estimation of *Bacillus* spp. lipase activity using copper soap method: The lipase enzyme was produced by submerged fermentation and quantified using olive oil as a substrate according to Veerapagu. In brief, after 72 h of submerged fermentation, the broth was centrifuged at 1000 rpm/20 min/4°C and supernatant was used as source of enzymes. To the 1 mL of culture supernatant, 2.5 mL of olive oil was added and incubated for 5 min at 37°C. Enzyme activity was arrested by adding 1 mL of 6N HCl and 5 mL benzene. Further, 4 mL of upper layer was carefully collected and to which 1 mL of cupric acetate pyridine was added. In the assay, reaction mixture with heat inactivated supernatant served as control. The lipase activity was determined by measuring the absorbance of free fatty acids dissolved in benzene at 715 nm and compared with oleic acid standard curve. One unit of lipase activity is defined as the amount of enzyme that liberated 1 µmol FFA in 1 min at 37°C.

Spectrophotometric assay of extracellular protease activity of *Bacillus* spp: The submerged fermentation for protease production was carried out according to Ikram-Ul-Haq and Mukthar (2006) and protease activity was assayed by Lowry's method. In brief, after 24 h of incubation at 37°C with continuous shaking at 200 rpm, the fermentation broth was centrifuged at 5000 rpm for 10 min and supernatant was assayed for protease activity. The reaction mixture containing 1 mL of casein (1% solution in 0.1 M phosphate buffer of pH 6) and 1 mL of culture supernatant was incubated at 37°C/30 min. Control tubes contained supernatants boiled for 10 min. The enzyme activity was arrested by adding 5 mL of 5% trichloro acetic acid and further incubated at 37°C/10 min. Then the reaction mixture was centrifuged at 8000 rpm/10 min to remove insoluble particles and to which 1 mL of 1:1 Folin and Ciocalteau reagent and water was added. After 30 min of incubation, absorbance was measured at 700 nm. The amount of amino acids released was compared with tyrosine standard curve for the determination of protease activity.

Spectrophotometric assay of extracellular phytase activity of *Bacillus* spp: The extra cellular phytase was produced as described by Sreeramulu et al., with slight modification [26]. After fermentation for 72 h, the broth was centrifuged at 6000 rpm/30 min/4°C and supernatant was used for phytase assay. The assay mixture consisted of 1 mL of acetate buffer (pH 5.5) containing 6.82 mM sodium phytate, 0.2 mL of culture supernatant and 0.2 mL 100 mM $MgSO_4$ and incubated at 37°C for 30 min. Reaction mixture containing heat inactivated culture supernatant served as control. Immediately after stopping the reaction by adding 1 mL of 10% trichloroacetic acid, 1 mL of Taussky shorr color reagent solution prepared as described by Tungala was added and absorbance was measured at 660 nm. Then the absorption values were compared with the standard graph of potassium dihydrogen phosphate (0.1 to 0.5 mg/mL) to determine phytase activity. One unit of phytase activity was defined as the amount of enzyme required to liberate 1 mole of phosphate per min under assay conditions.

Total Protein Estimation

The total protein content was estimated by Lowry's method.

Optimisation of pH, temperature and substrate concentration for amylase, lipase, protease and phytase from *Bacillus* spp.

The optimum pH for all the four enzyme assay was determined by incubating the enzyme–substrate at various pH from 3 to 10 in the following buffers: 50 mM sodium acetate buffer (pH 3–5), 50 mM potassium phosphate buffer (pH 5–7), 50 mM Tris–HCl buffer (pH 7–9), and 50 mM glycine–NaOH buffer (pH 9–10). Enzyme activity in each buffer was measured using the standard assays as described previously. Further, to determine the optimum temperature for the amylase, lipase, protease and phytase, the reaction mixtures were incubated at various temperature ranged from 10 to 60°C and then absorbance were measured according to the standard assays. Furthermore, to determine the effect of substrate concentration on enzyme activities, culture supernatants were incubated with various volumes of specific substrate solutions viz., starch, olive oil, casein and sodium phytate for amylase, lipase, protease and phytase respectively.

Statistical Analyses

All the experiments were conducted in triplicates and results were expressed as mean ± standard deviation. The data were analyzed using two-tailed paired t-test and for all tests, the level of significance was set at $p<0.05$ (GraphPad Prism version 5.02 for Windows, GraphPad Software, San Diego, California, USA).

Results and Discussion

In the present study the extracellular amylase, lipase, protease and phytase activity of four *Bacillus* spp. were evaluated and optimized. The total protein content and specific activity of all the four extracellular digestive enzymes evaluated (Table 1).

Amylases break down α-linked sugar units in the ingested diet to

	Amylase		Lipase		Protease		Phytase	
Isolates	**Protein mg/mL**	**Specific activity (IU)**	**Protein mg/mL**	**Specific activity (IU)**	**Protein mg/mL**	**Specific activity (IU)**	**Protein mg/mL**	**Specific activity (IU)**
B. subtilis GS 1	0.101	1262.04	0.05	653.2	0.098	1602.229	0.046	1337.064
B. cereus GS 3	0.106	1082.77	0.194	142.57	0.093	1512.646	0.038	2138.007
B. cereus GS 199	0.105	994.13	0.045	785.11	0.091	1456.121	0.052	1056.65
B. subtilis GS 547	0.119	759.034	0.193	207.25	0.095	1394.164	0.0403	1966.63

Table 1: Total protein and specific activity.

glucose, maltose, and maltotriose which further undergo fermentation to produce various short chain fatty acids. In case of pancreatic insufficiency, microbial amylases are of best choice in improving the carbohydrate digestion. In the present study, the extracellular amylase activity of the four *Bacillus* spp. were studied and optimized for various physical parameters. As shown in the Figure 1, the amylolytic activity of the tested isolates were ranged between 87 and 128 U. Amongst, *Bacillus subtilis GS 1* showed highest activity (127.66 ± 8.02U, $p<0.05$). Further, in optimization studies, the four *Bacillus* spp. tested showed optimum activity at 40°C ranging between 87 and 130 U/mL and subsequently decreased at 50°C with activity ranging from 50 to 73 U/mL. Whereas in another study, *Bacillus* amylase activity as 34 U/mL at 37°C/pH 7 [25], however, Abd-Elhalem et al. reported that the amylase activity of *B. amyloliquefaciens* was 72.5 U/mL with an increased activity at 50°C [27]. During optimization for pH, *B. subtilis GS 1* and *B. cereus GS 3* showed maximum activity at pH 6 while *B. subtilis GS 547* and *B. cereus GS 199* showed highest activity at pH 7. Further, the V_{max} value for *B. subtilis GS 1* was 149 ± 4.05 U/mL at a substrate concentration 2 mg/mL/1 mL enzyme solution while for *B. cereus GS 3* the V_{max} was 122 ± 3.87 U/mL at 1 mg/mL/ 1 mL enzyme solution. However, for *B. subtilis GS 547* and *B. cereus GS 199* V_{max} values were 108 ± 5.32 and 95 ± 3.69 U/mL, respectively at a substrate concentration 1.5 mg/mL/ 1 mL enzyme solution. In another study, V_{max} for *B. subtilis* was 100 U/mL which was in agreement with the present study [28] (Figures 1-4).

Lipase hydrolyzes fats into fatty acids and glycerols. In the present study, the crude lipase activity of the four tested *Bacillus* spp. ranged between 27 and 40 U/mL using olive oil as substrate. Amongst, *Bacillus subtilis GS 547* showed highest activity 40 ± 2.13 U/ml ($p<0.05$). However, *Bacillus subtilis* showed 6.921 U/mL in soyabean oil, while *Bacillus amyloliquefaciens* exhibited 6.506 U/mL using ground nut oil as substrate at optimum pH 7.5 and temperature 45°C [29]. On storage at temperature above 40°C, pancreatic lipases lose their activity, however, microbial lipases are more resistant to heat inactivation and therefore, thermal stability is one of their desirable characteristics and which was corroborated in the present study [30]. The maximum lipolytic activity was found to be 40°C for all the four isolates tested and indicating the thermal stability at a temperature range between 20 to 50°C and decreased beyond 60°C. Further, optimum pH was 6 for all the isolates except *B. subtilis GS 2* (pH 7), however, lipase activity was stable at a pH range between 5 and 8 for all the four isolates tested. Further, the maximum rate of reaction V_{max} for *B. cereus 3* and *B. subtilis 547* were found to be 31 ± 1.42 and 41 ± 2.36 U/mL respectively. Whereas for *B. subtilis GS 1* and *B. cereus GS 199* V_{max} values were 32 ±3.4 and 34 ± 2.1 U/mL respectively at a substrate concentration 0.5 mL/mL (v/v)/1 mL enzyme solution. However, Hasan et al. (2007) reported the V_{max} value of *B. subtilis* as 0.416 U/mL/min which was quite lower the maximum rate of reaction of the four tested *Bacillus* spp. (Figures 5-7).

The enzyme protease breakdown protein into smaller fragments as peptides and further to amino acids. In the present study, the protease activity of four tested *Bacillus* spp. were ranged between 120 and 137 U/mL. Amongst, *B. subtilis GS 1* showed the highest enzyme activity

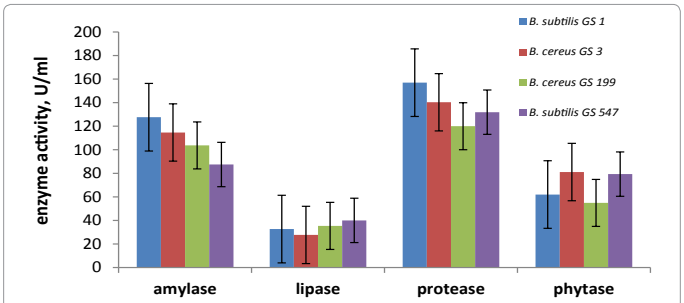

Figure 1: Extracellular digestive enzyme activity of *B. subtilis GS 1, B. cereus GS 3, B. cereus GS 199* and *B. subtilis GS 547*.

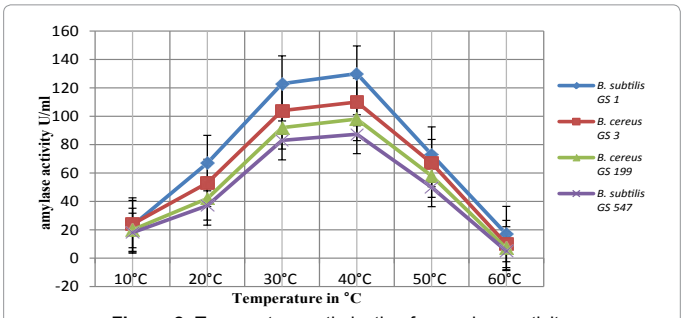

Figure 2: Temperature optimization for amylase activity.

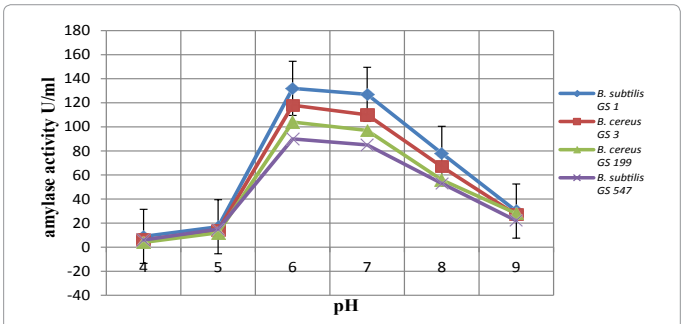

Figure 3: pH optimization for amylase activity by *Bacillus* spp.

157 ± 3.60 U/ml ($p<0.05$) at 37°C. Further, the optimum temperature was found to be 40°C and moreover, enzyme activity was stable in the temperature range 20 to 50°C for all the isolates and was in agreement with the report of Kotlar et al. [31]. Further, pH 8 was optimum and the activity was stabilized between pH 6 and 9 for all the tested isolates. Furthermore, the maximum rate of reaction Vmax were 160 ± 2.34, 145 ± 1.45 and 130 ± 3.72 U/mL for *B. subtilis GS 1, B. cereus GS 3* and *GS 9* respectively at a substrate concentration 2 mg/mL/1 mL enzyme solution. However, Nadeem et al. reported Vmax value of 61.58 U/ml for *B. licheniformis* [32]. The stability at wide range of temperature and pH suggested that the protease from tested *Bacillus* spp. may function

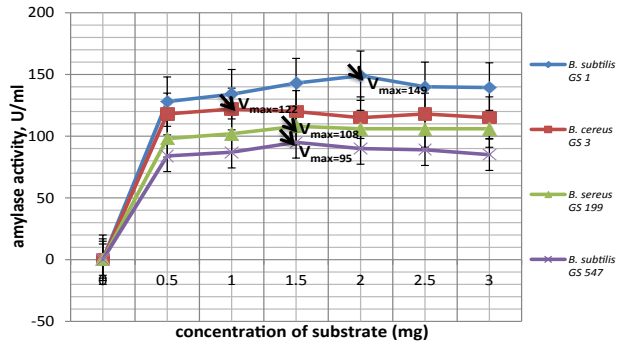

Figure 4: Effect of substrate concentration on amylase activity.

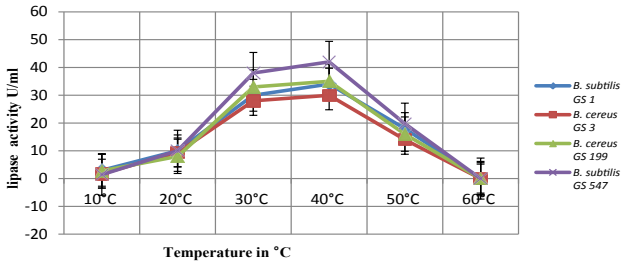

Figure 5: Temperature optimization for lipase activity.

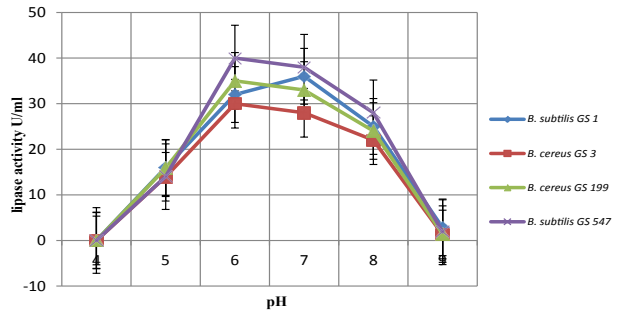

Figure 6: pH optimization for lipase activity.

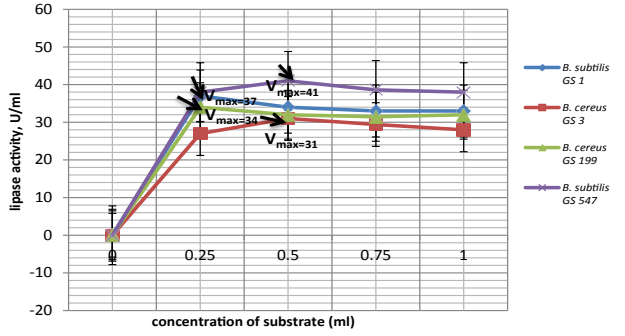

Figure 7: Effect of substrate concentration on lipase activity.

at different pH conditions *in vivo* and also can withstand varying temperature during processing (Figures 8-10).

Phytase catalyzes the hydrolysis of phytic acid into *myo*-inositol and inorganic phosphates. In the present study, all the four tested *Bacillus* spp. showed phytase activity using sodium phytate as

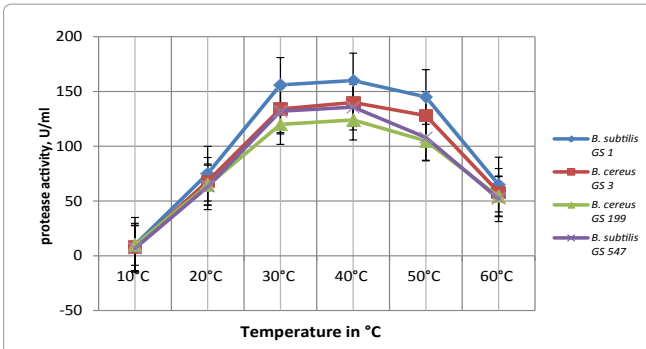

Figure 8: Temperature optimization for protease activity.

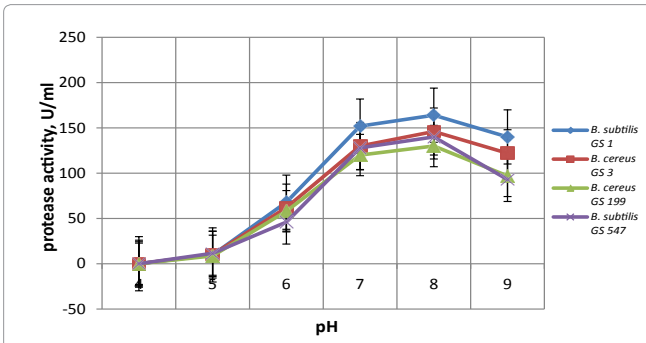

Figure 9: pH optimization for protease activity.

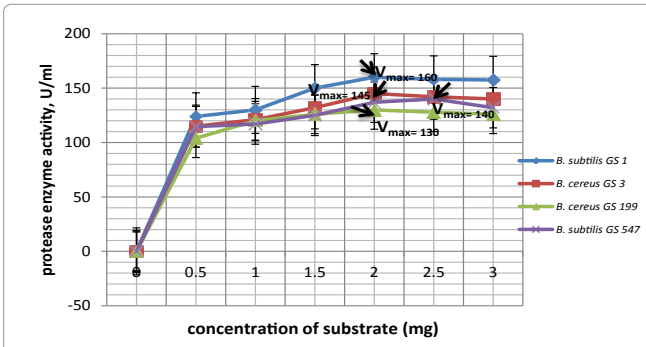

Figure 10: Effect of substrate concentration on protease activity.

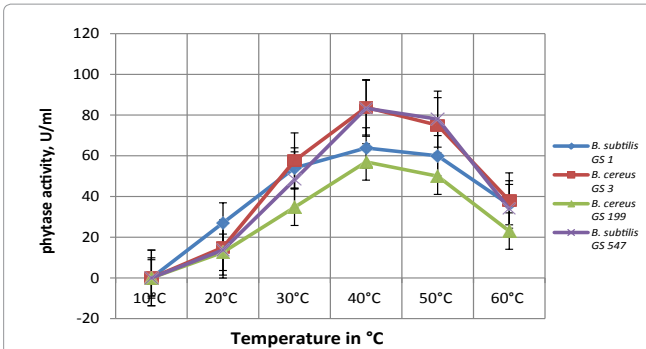

Figure 11: Temperature optimization for phytase activity.

substrate. As shown in the Figure 1, the phytase activity of the four isolates ranged between 54 to 81 U/mL. Out of four isolates evaluated,

B. cereus GS 3 showed highest activity (81.07 ± 2.47 U/mL, $p<0.05$). In addition, optimum temperature was 40°C, although activity remained fairly stable over temperature range of 30 to 50°C. Interestingly, the activity was found to stabilize at different pH range from 4 to 7 with an optimum pH 5 except for *B. subtilis GS 547* (pH 6). Further, V_{max} values for *B. subtilis GS 1* and *B. cereus GS 3* were 67.64 and 85.87 U/mL for 4.8 mg/mL/1 mL enzyme solution. Whereas for *B. cereus GS 199* and *B. subtilis GS 547,* the Vmax values were 60.75 and 93.47 U/mL for 6 mg/mL/1 mL enzyme solution. However, in an another study, the optimum pH and temperature for phytase from *B. subtilis* were 7 and 60°C respectively and K_m and V_{max} values for sodium phytate were 0.42 mM and 4.35 µmol/min, respectively. The activity of phytase at optimum pH 4 indicated that the enzyme from the four tested isolates could be used *in vivo* to improve the bioavailability of phosphate from ingested food (Figures 11-13).

Conclusion

In the present study, *B. subtilis GS 1, B. cereus GS 3, B. cereus GS 199* and *B. subtilis GS 547* were evaluated and optimized for their extracellular digestive enzymes viz., amylase, lipase, protease and phytase *in vitro.* The digestive enzymes of all the four isolates were active at wide temperature and pH range. Further, purification and characterization of these digestive enzymes would pave for the development of therapeutic enzyme formulations to alleviate the burden of pancreatic insufficiency and other digestive disorders. In addition, further *in vitro* and *in vivo* evaluation of the safety of tested *Bacillus* spp. and their validation as GRAS (generally regarded as safe) would also helpful in oral administration of these isolates as source of digestive enzymes in improving digestion.

Acknowledgements

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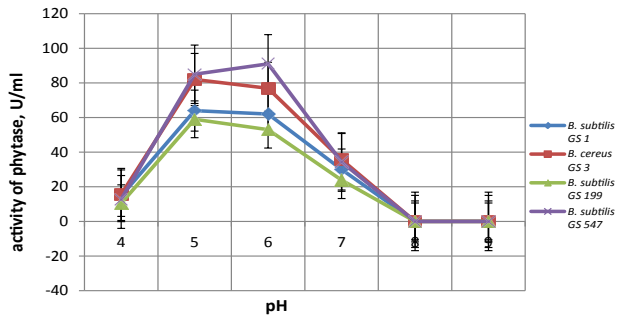

Figure 12: pH optimization for phytase activity.

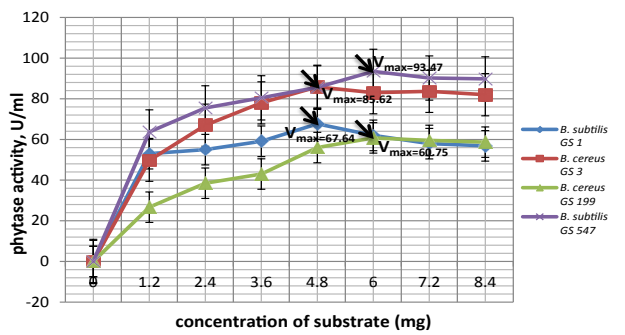

Figure 13: Effect of substrate concentration on phytase activity.

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