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Full Length Research Paper

Production of phytate-hydrolyzing enzymes by thermophilic moulds

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138 isolates of thermophilic/thermotolerant moulds were isolated from soil, straw and compost samples, collected from various regions of India. Among the thermophilic fungal isolates screened for the secretion of phytase (phytate-hydrolyzing enzyme), *Sporotrichum thermophile* BJTLR50 produced a very high enzyme titre at pH 5.0, 45°C and 250 rpm in 5 days with an inoculum level of 1 × 10^7 spores per 50 ml medium prepared from 6 days old culture. Glucose and ammonium sulphate supported higher phytase production than other carbon and nitrogen sources. The phytase of *S. thermophile* was optimally active at pH 5.0 and 60°C. An overall 2-fold improvement in the phytase production was achieved due to optimization.

Key words: Phytic acid, phytase, thermophilic moulds, *Sporotrichum thermophile*, *Humicola lanuginosa*, submerged fermentation, optimization.

INTRODUCTION

Phytic acid (*myo*-inositol-hexakis-dihydrogen phosphate) is an organic form of phosphorus, which accounts for 1 to 5% (by weight) of edible legumes, cereals, oilseeds, pollens and nuts (Vohra and Satyanarayana, 2003; Singh et al., 2011; Singh and Satyanarayana, 2011). Most foods of plant origin contain 50 to 80% of their total phosphorus as phytate (Harland and Morris, 1995), which acts as an anti-nutritional factor, since it causes mineral deficiency due to efficient chelation of metal ions such as Ca^{2+}, Mg^{2+}, $Zn^{2+}$and Fe^{2+}, forms complexes with proteins, affecting their digestion and also inhibits enzymes like α-amylase, trypsin, acid phosphatase and tyrosinase (Harland and Morris, 1995). This is considered to be partly responsible for the widespread human nutritional deficiencies in developing countries where the staple foods are plant derived (Manary et al., 2002). Due to the lack of adequate levels of phytases in monogastric animals (poultry, pigs, fishes and humans), phytic acid is excreted in faeces, which is degraded by soil micro-organisms releasing phosphorus in the soil that reaches aquatic bodies leading to eutrophication. Phytases (*myo*-inositol hexakisphosphate 3-phosphorylase, EC 3.1.3.8. and *myo*-inositol hexakisphosphate 6-phosphorylase, EC 3.1.3.26.) are classified as the family of histidine acid phosphatases that catalyze the hydrolysis of phytic acid to inorganic phosphate and myo-inositol phosphate derivatives in a stepwise manner (Mitchell et al., 1997; Singh et al., 2011; Singh and Satyanarayana, 2011). The reduction of phytic acid content in the food and feeds by enzymatic hydrolysis using phytase is desirable. This enzyme has, therefore, potential applications in feed and food industries. These enzymes are present in many plants and animal tissues, and they are also produced by many moulds, yeasts and bacteria (Shieh and Ware, 1968; Howson and Davis, 1983; Vohra and Satyanarayana, 2003; Singh et al. 2011; Singh and Satyanarayana, 2011). In the case of fungi, various species of *Aspergillus* (Shieh and Ware, 1968; Howson and Davis, 1983) and thermophilic moulds such as *Myceliopthora thermophila* (Mitchell et al., 1997), *Thermomyces lanuginosus* (Berka et al., 1998) and *Thermoascus aurantiacus* (Nampoothiri et al., 2004) are known to secrete phytase enzymes. Thermophilic mould phytases are relatively more thermostable than their mesophilic counterparts, and therefore, the present

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attempt was made to isolate and screen thermophilic moulds for extracellular phytase production. This paper describes isolation and screening of thermophilic moulds for extracellular phytase production and optimization of phytase production by *Sporotrichum thermophile* BJTLR50 in submerged fermentation. *S. thermophile* is an ubiquotous thermophilic mould found in soil and self-heating compost. It displays maximum growth between 45 and 50°C, and is an efficient decomposer of organic materials and would be a good source of genes encoding extracellular thermostable enzymes for biotechnological applications.

MATERIALS AND METHODS

Isolation and maintenance of thermophilic moulds

The thermophilic fungi were isolated from environmental samples, collected from different regions of India, on Emerson's YpSs (Emerson, 1941) agar (g L^{-1} Starch 15; Yeast extract 4; K_2HPO_4 1; $MgSO_4.7H_2O$ 0.5 pH 7.0 ± 0.2). The isolated fungi were maintained at 4°C on YpSs slops and -20°C in glycerol stocks.

Qualitative screening

The isolated thermophilic moulds were screened for extracellular phytase production using modified phytase screening medium (PSM) (Howson and Davis, 1983) containing g L^{-1}: D-glucose 15, Calcium phytate 3, NH_4NO_3 5, $MgSO_4.7H_2O$ 0.5, KCl 0.5, $FeSO_4.7H_2O$ 0.01, $MnSO_4.4H_2O$ 0.01, Agar 20; pH-5.5). All fungi were incubated at 45°C and observed for clear zone of calcium phytate hydrolysis. Acid producing fungi also solubilize calcium phytate. Therefore, the zone forming fungi were further cultivated on PSM containing sodium phytate instead of calcium phytate (Bae et al., 1999). After incubation, the zone forming fungi were subjected to double staining method (Bae et al., 1999).

Quantitative screening and phytase production

The fungi, which showed zone formation on PSM agar were later cultivated in 250 ml Erlenmeyer flasks containing 50 ml modified phytase screening broth (Howson and Davis, 1983) containing sodium phytate as sole source of phosphorus and incubated for 4 days at 45°C and 200 rpm in an incubator shaker. The medium with fungal biomass was filtered through filter paper and the cell-free culture filtrate was used in phytase assays.

Optimization of phytase production by *S. thermophile* BJTLR50

Phytase production by *S. thermophile* BJTLR50 was optimized in submerged fermentation by one variable at a time' approach, changing one variable while keeping all others at their constant level. Phytase production was optimized in the modified PSM containing (g/L) 5 g calcium phytate, 10 g sucrose, 2 g $(NH_4)_2SO_4$, 3 g tryptone, 2 g yeast extract, 0.5 g KCl, 0.5 g $MgSO_4$, 0.01 g $MnSO_4$, $5H_2O$, 0.01 g $FeSO_4$, and 1 g triton X-100. The effect of pH was assessed by adjusting pH of medium (pH 4.0 to 8.0). The effect of temperature and agitation was assessed by incubating flasks at different temperatures (30 to 60°C) and agitation speeds (0-300 rpm), respectively. Effect of incubation period (2 to 8 days), inoculum level (5×10^5 to 5×10^8), inoculum age (4 to 8 days) and different carbon and nitrogen sources was also checked for phytase production. Effect of different concentrations of glucose (1 to 5%), $(NH_4)_2SO_4$ (0.1 to 0.6%), phytic acid (0 to 0.5%) and $MgSO_4.7H_2O$ (0 to 0.25%) was also assessed on phytase production.

Phytase assay

Phytase was assayed by measuring the amount of inorganic phosphate released from sodium phytate (Singh and Satyanarayana, 2006) at pH 5.0 and 60°C. The liberated inorganic phosphate was estimated according to Fiske and Subbarow method (1925). One unit of phytase is defined as the amount of enzyme required to liberate one nmole of inorganic phosphate s^{-1} mL^{-1} of enzyme under the assay conditions.

The dry biomass was estimated gravimetrically after drying fungal mats at 80°C to constant weight. Protein content of the culture filtrate was measured according to Lowry et al. (1951) using BSA as the standard.

Effect of temperature and pH on phytase activity

The effect of pH and temperature on phytase activity was assessed by using buffers [0.1 M glycine/HCl buffer (pH 2.0 to 3.0), 0.1 M acetic acid/sodium acetate buffer (pH 4.0 to 6.0), 0.1 M Tris/HCl (pH 7.0 to 8.0)] of pH between 2.0 and 8.0, and performing assays at different temperatures (25 to 80°C). All the experiments were carried out in triplicates and their mean values are presented.

RESULTS AND DISCUSSION

Thermophilic fungi had been isolated from various types of habitats like soils, composts, retting guayule, stored grains, birds and animal excreta and others (Cooney and Emerson, 1964; Satyanarayana et al., 1977). Nowadays, thermophilic moulds attract the attention of scientists because of their ability to produce thermostable enzymes with unique properties. 138 isolates of thermophilic moulds were isolated from different environmental samples and screened for phytase production.

All isolates of thermophilic moulds except a few were able to grow on PSM agar plates but only few fungi showed zone of calcium phytate hydrolysis. When these zone forming fungi were cultivated on PSM agar plates containing sodium phytate, only few thermophilic moulds showed zone of hydrolysis after double staining method (Bae et al., 1999). This could be explained due to the formation of various acids like acetic acid, malic acid and others, which solubilize calcium phytate resulting in zone formation. These acids lower the pH of the medium, and hence, increase the solubility of calcium phytate (Bae et al., 1999). The numbers of phytase producing fungi were reduced, when they were grown in PSM broth. Similar observation was made earlier (Shieh and Ware, 1968; Howson and Davis, 1983). The thermophilic mould showed varied titres of phytase secretion in PSM broth ranging between 0 and 4651 U L^{-1}. Based on enzyme production, two strains of *S. thermophile* (*S. thermophile* BJTLR50, and *S. thermophile* BJA64) and two strains of

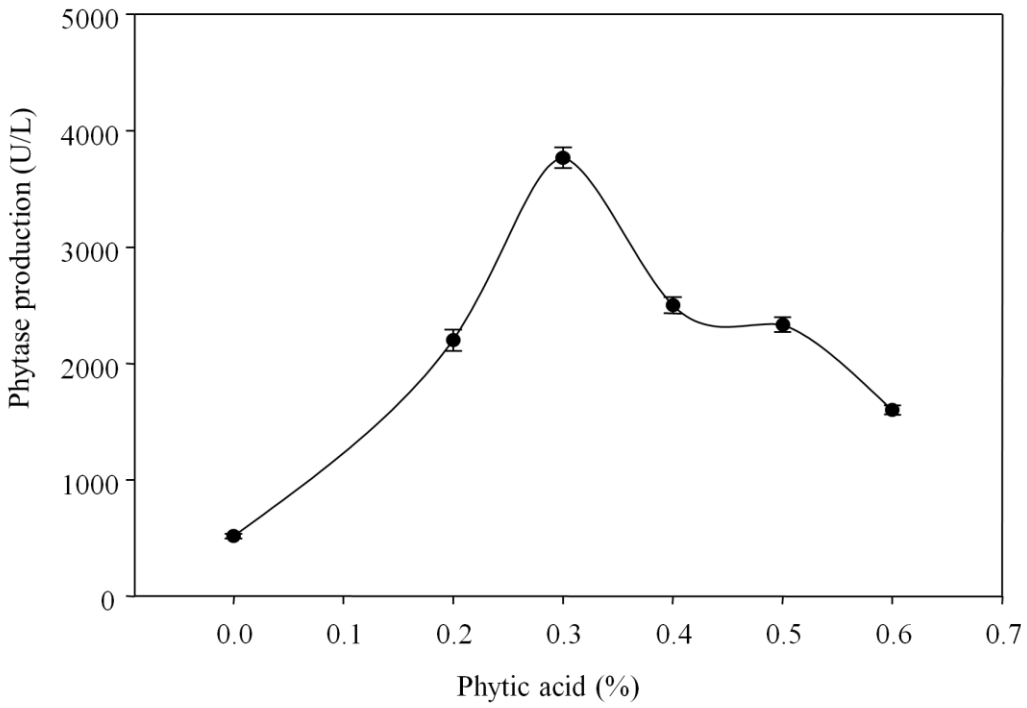

Figure 1. Effect of phytic acid concentration on phytase production by *S. thermophile* BJTLR50.

Humicola lanuginosa (*H. lanuginosa* DCC and *H. lanuginosa* BJPAT102), were selected for further screening in order to select a potent phytase producer. Based on repeated screening, *S. thermophile* BJTLR50 was selected for detailed investigation as it produced higher phytase titre (3.99 U mg^{-1} protein) than others.

The thermophilic mould, *S. thermophile* BJTLR50 secreted phytase in the basal medium containing glucose and ammonium sulphate within 5 days in shake flasks agitated at 250 rpm, 45°C and pH 5.0 (Figures 1 to 8). The enzyme produced by *S. thermophile* BJTLR50 is of inducible nature as the enzyme titres were enhanced many folds in the presence of sodium phytate in the medium (Figure 1). Substrates are known to induce, and thus, enhance enzyme production. High phytase production by *Klebsiella aerogenes* was achieved in the medium containing 2% sodium phytate (Tambe et al., 1994). Phytase production in *Lactobacillus amylovorus* (Sreeramalu et al., 1996) was induced by phytic acid. The phytase production was initiated during the early stage of cultivation, and reached a peak on the 5th day (Figure 2), and after that, it declined which might be due to the depletion of nutrients and release of protein degrading enzymes into the medium.

Inoculum age and level are known to affect the microbial growth as well as enzyme production. An inoculum level of 1×10^7 spores per 50 ml of the medium prepared from a 6 day old sporulating culture supported a high enzyme production (Figure 3A and B). At higher inoculum levels, the production declined due to competition among the fungal population for the nutrients. In addition, very young cultures of the mould are non-sporulating, and therefore, it is difficult to prepare a suspension with high number of spores. In cultures older that 6 days, the spores entered into the dormant phase, and thus, took a longer time to germinate, leading to low enzyme titres.

S. thermophile BJTLR50 secreted phytase optimally at 45°C (Figure 4A), which is also the optimum temperature for its growth. Other fungi like *Aspergillus niger* (Howson and Davis, 1983) and *A. carbonarius* (Al-Asheh and Duvnjak, 1994) have also been reported to produce phytase maximally at their temperature optima for growth; 25 and 30°C, respectively. In contrast, *Arxula adeninivorans* secreted phytase optimally at 44°C, whereas yeast grows optimally at 28°C (Sano et al., 1999). The phytase production by *S. thermophile* increased when the pH of the medium was increased from 3 to 5 and thereafter, it declined (Figure 4B). Several investigators have reported that acidic to neutral pH range favours the phytase production (Howson and Davis, 1983; Shieh and Ware, 1968). *A. niger* produced phytase optimally at a pH 3.0 (Volfova et al., 1994) while *Bacillus* sp. DS11 (Kim et al., 1998) and *A. oryzae* K1 (Shimizu, 1993) produced phytase optimally at pH 6.5, and that in *A. adeninivorans*, (pH 5.5) supported high phytase production (Sano et al., 1999).

Agitation rates affect the distribution of nutrients and solubility of oxygen. Agitation rates affected phytase production as the yields were very low in the static

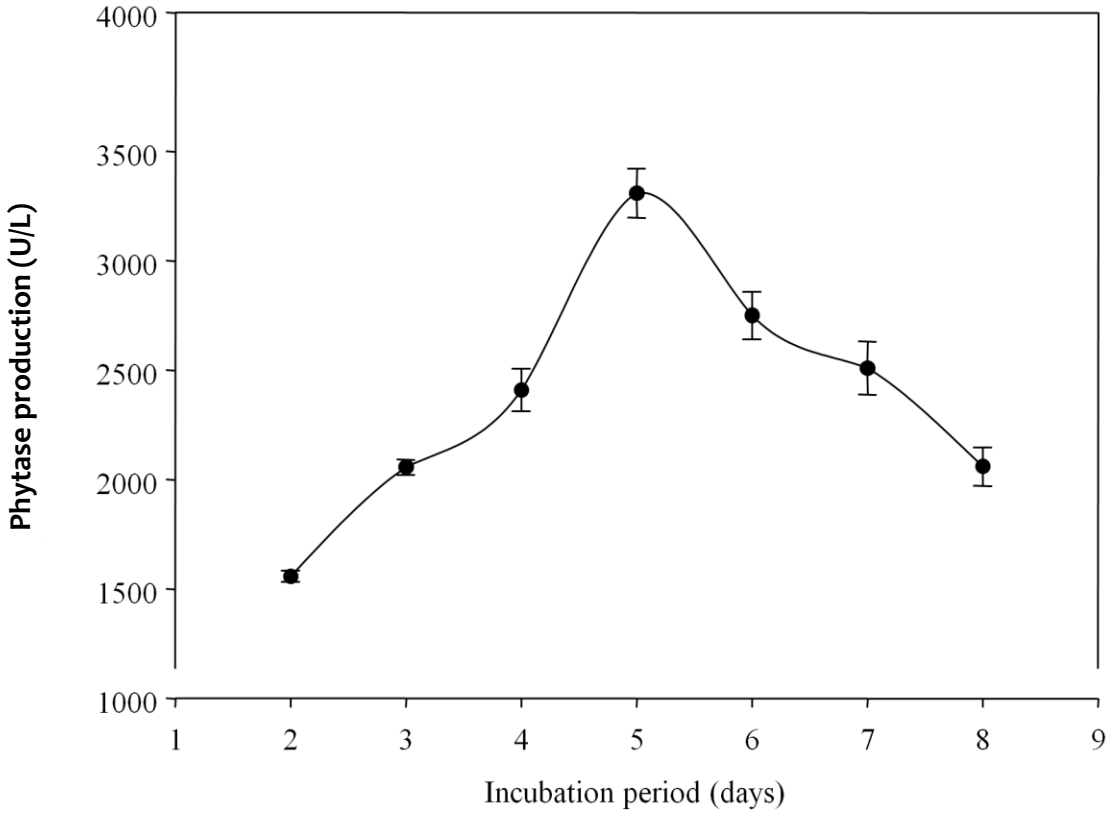

Figure 2. Effect of incubation period on phytase production by *S. thermophile* BJTLR50.

conditions, but there was a gradual rise in production with increasing agitation rates up to 250 rpm (Figure 5), due to improved availability of nutrients and oxygen. Further increase in agitation rates caused a fall in the enzyme production, which could be due to disruption of fungal mycelium at very high agitation speeds, as well as shorter contact between cells and the medium, thus hindering the uptake of the nutrients. *A. niger*, *Aspergillus ficuum* NRRL3135 and *A. terreus* produced high phytase at 270 rpm (Shieh and Ware, 1968), while *Bacillus* sp DS11 produced high phytase at 230 rpm (Kim et al., 1998).

Among various carbon sources tested, glucose supported high phytase production by *S. thermophile* as compared to other carbon sources (Figure 6A to B) as reported in *L. amylovorus* (Sreeramulu et al. 1996) and *Pichia anomala* (Vohra and Satyanarayana, 2001). In *A. adeninivorans*, galactose increased phytase production several folds in comparison with that in glucose (Sano et al., 1999). *A. niger* produced high phytase, when grown in a medium containing corn starch along with glucose (Volfova et al., 1994).

Like the carbon sources, nitrogen sources also affect the phytase production by the microbes. Ammonium sulphate supported high phytase production by *S. thermophile* BJTLR50 as compared to other organic and inorganic nitrogen sources (Figure 7A and B). Similar observations were reported in *S. castellii* (Lambrechts et al., 1992) and *A. ficuum* NRRL 3135 (Shieh and Ware, 1968; Howsion and Davis, 1983). Ammonium nitrate in *Bacillus* sp. DS11 (Kim et al., 1998) supported high phytase titers. In contrast, organic nitrogen sources (beaf extract and peptone) supported high phytase production by *P. anomala* (Vohra and Satyanarayana, 2001) and *A. adeninivorans* (Sano et al., 1999), respectively.

The optimum pH for the activity of phytase of *S. thermophile* was 5.0 (Figure 8A), which is very close to those of other fungi and yeasts (Vohra and Satyanarayana, 2003). Two distinct pH optima were identified for the phytases of *A.ficuum* NRRL 3135 with the highest activity being at pH 5.0 to 6.0 and a small second peak at pH 2.5 to 2.8 (Shieh and Ware, 1968; Howson and Davis, 1983). Phytases of thermophilic fungi *Thermomyces lanuginosus* (Berka et al., 1998) and *Thermoascus aurantiacus* (Nampoothiri et al., 2004) exhibited optimal activity at pH 7.0 and 5.0, respectively. Phytase of *S. thermophile* was optimally active at 60°C (Figure 8B), as reported for most fungal and yeast phytases (50 to 70°C) (Vohra and Satyanarayana, 2003), while those of *T. aurantiacus* (Nampoothiri et al., 2004) and *T. lanuginosus* (Berka et al., 1998) were optimally active at 55 and 65°C, respectively. Thermostability is a desirable feature of phytase because there is a stage in feed pellet development where it is held at 85°C for a few

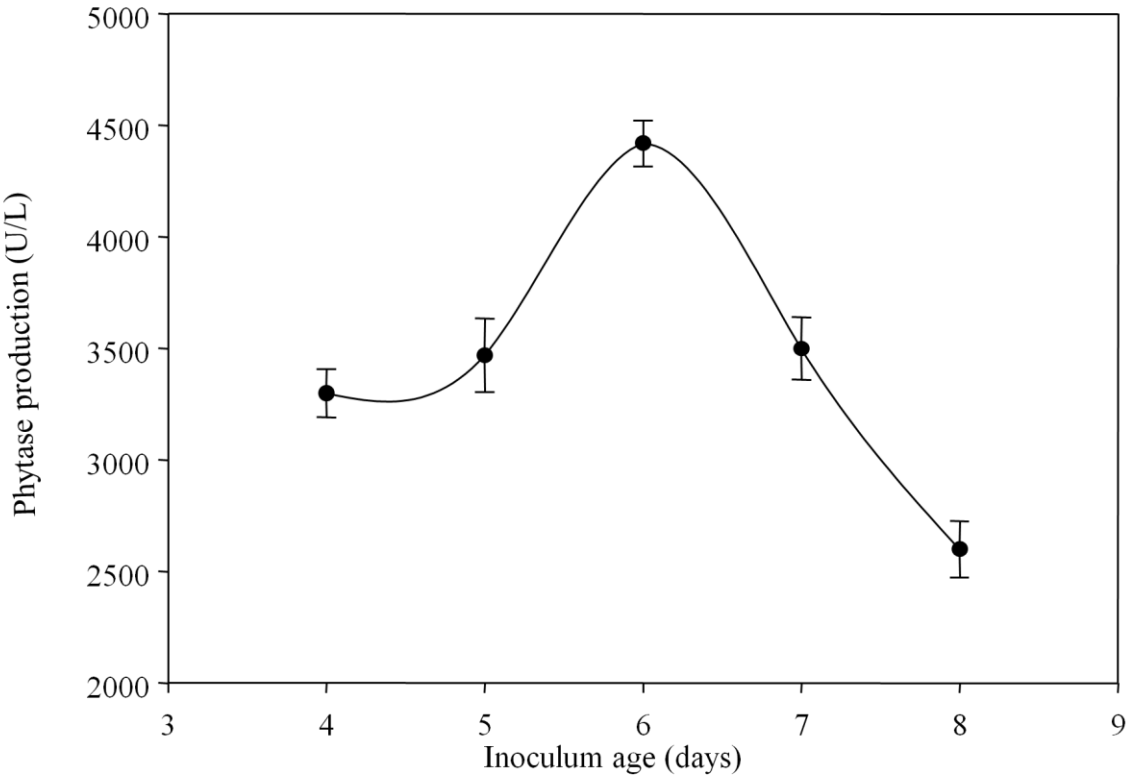

Figure 3A. Effect of inoculum age on phytase production by *S. thermophile* BJTLR50.

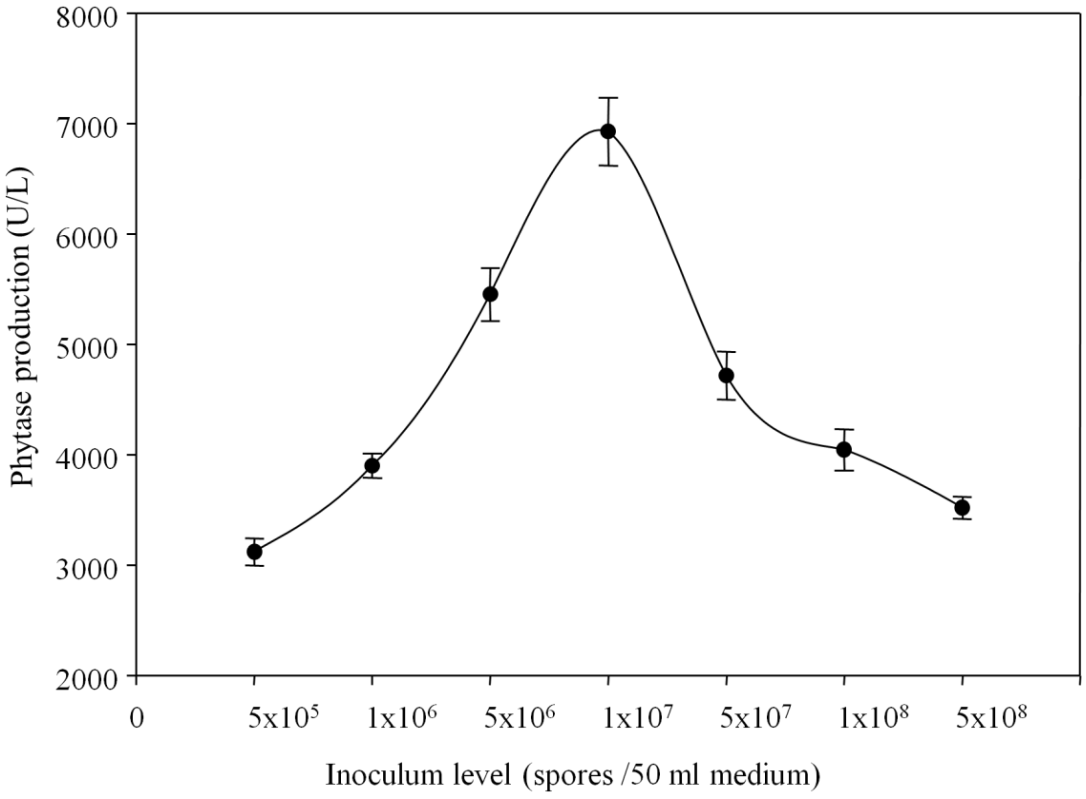

Figure 3B. Effect of inoculum level on phytase production by *S. thermophile* BJTLR50.

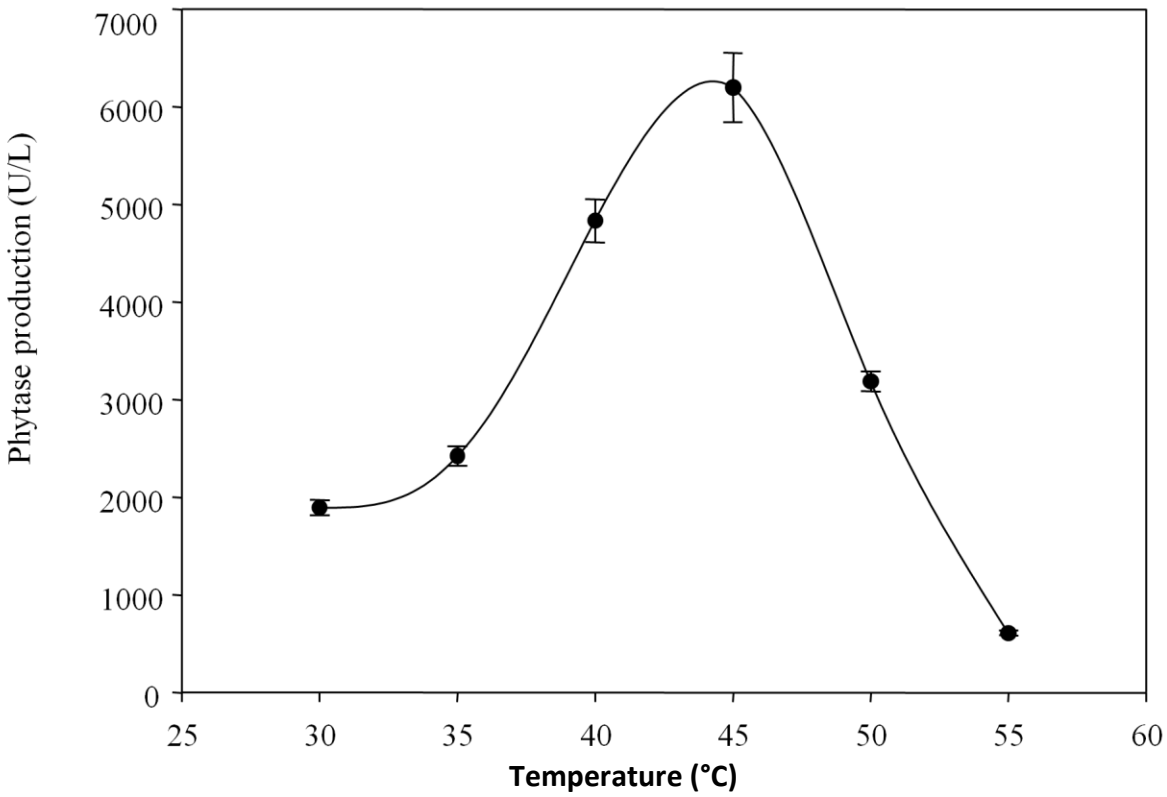

Figure 4A. Effect of temperature on phytase production by *S. thermophile* BJTLR5.

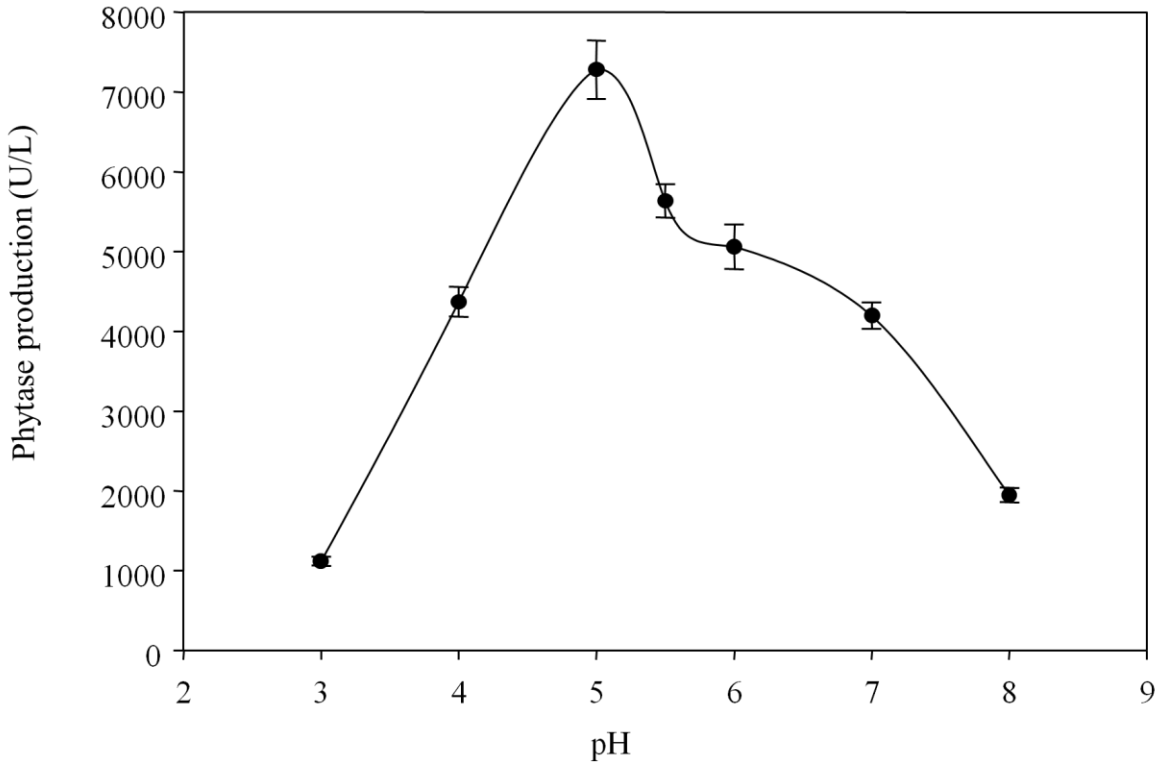

Figure 4B. Effect of pH of medium on phytase production by *S. thermophile* BJTLR50.

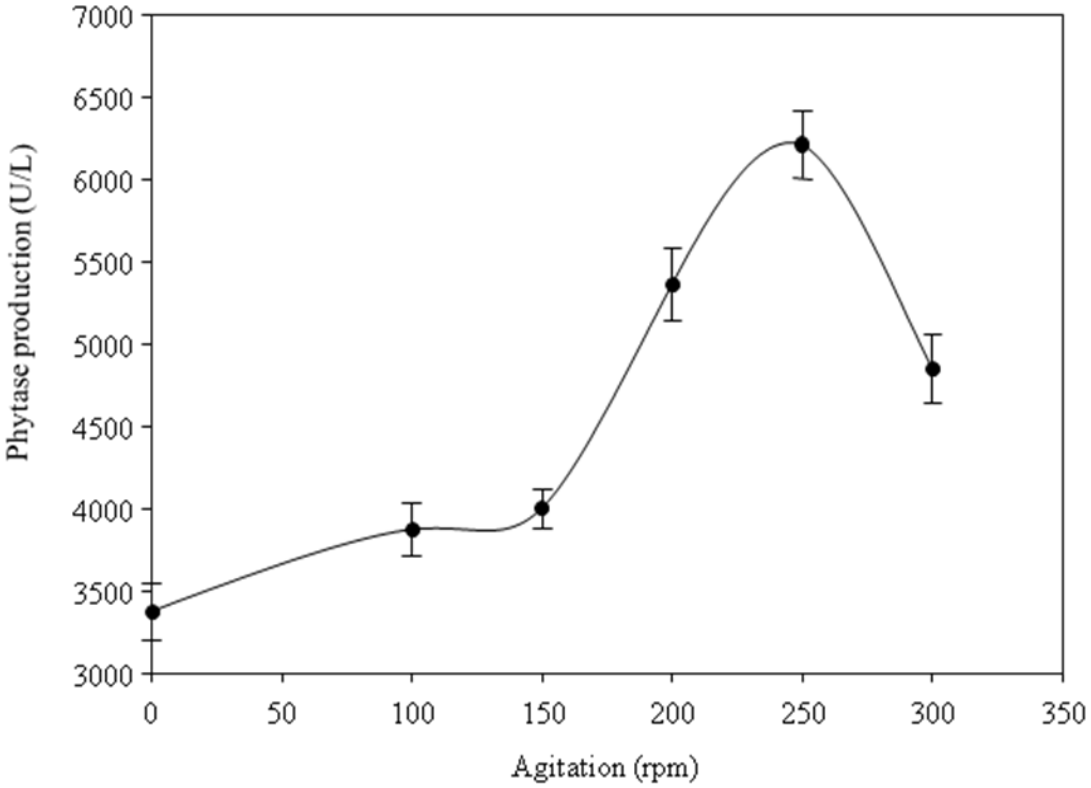

Figure 5. Effect of agitation on phytase production by *S. thermophile* BJTLR50.

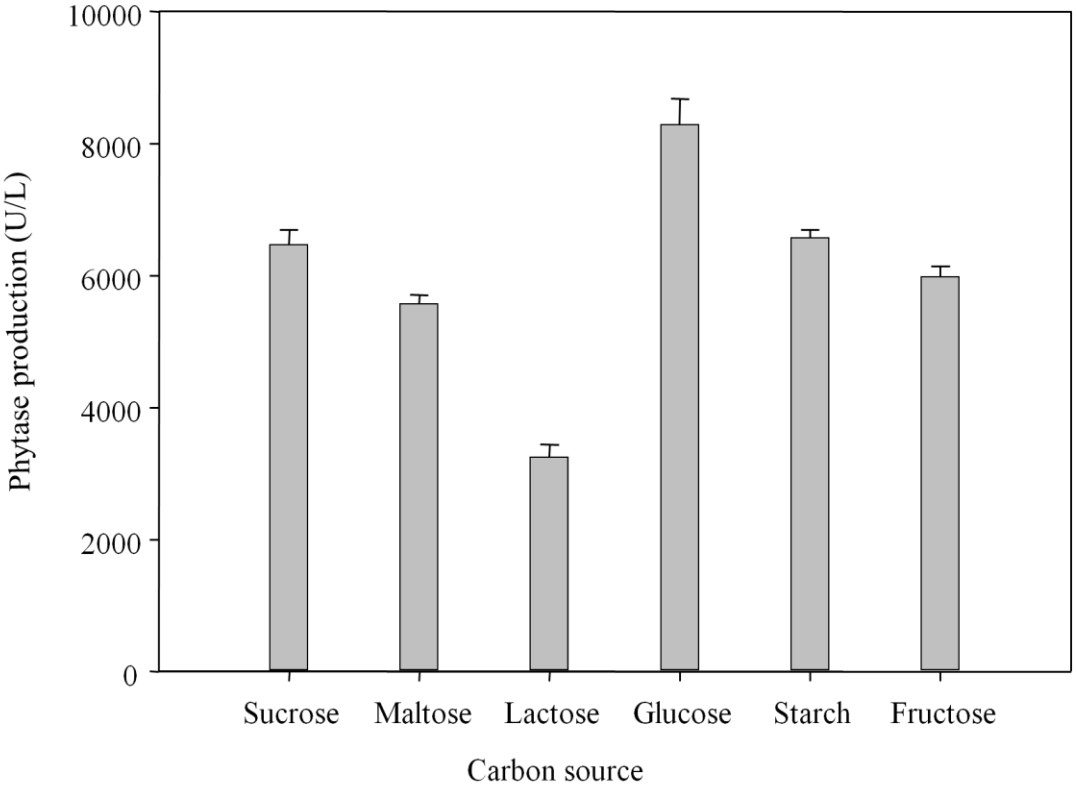

Figure 6A. Effect of different carbon sources on phytase production by *S. thermophile* BJTLR50.

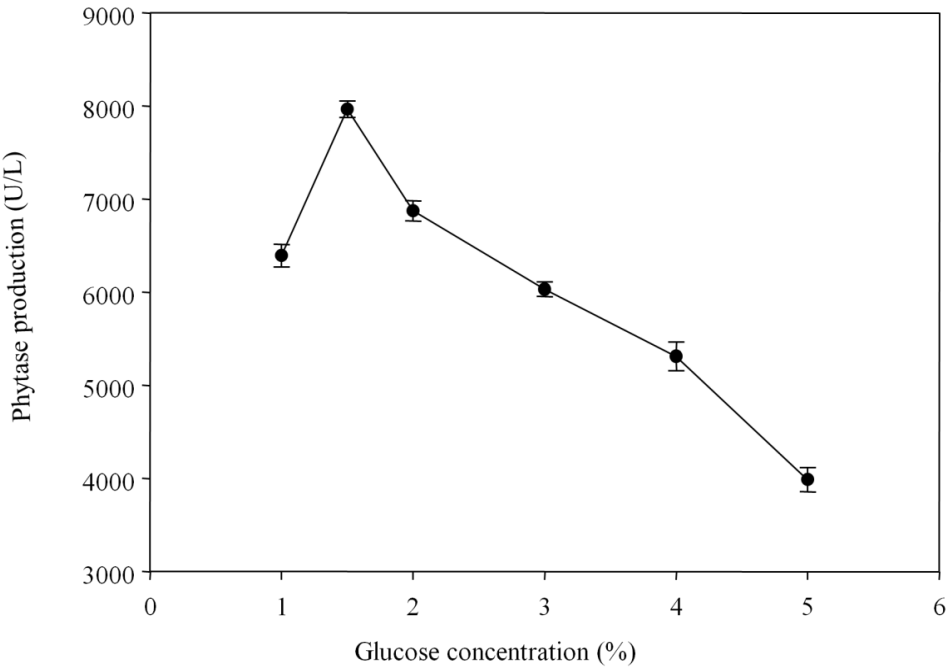

Figure 6B. Effect of glucose concentration on phytase production by *S. thermophile* BJTLR50.

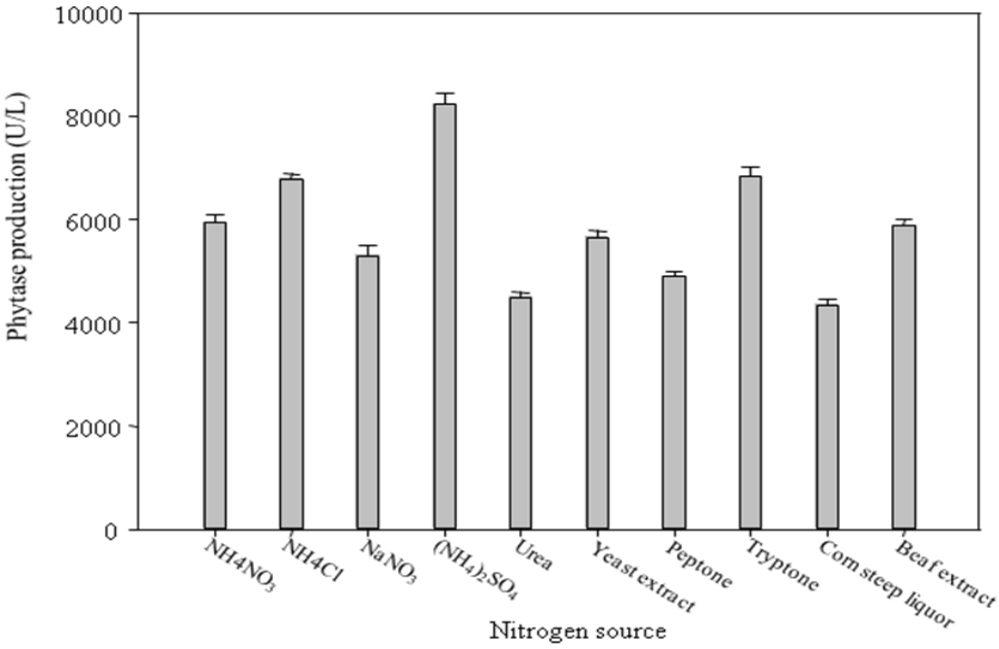

Figure 7A Effect of different inorganic and organic nitrogen sources on phytase production.

seconds; this step is meant for eliminating Salmonellae.

Conclusion

Most of the isolates of thermophilic moulds were capable of growing on PSM agar plates, but only a few secreted phytase into the PSM broth. The formation of zones of hydrolysis by most of the fungi could be due to the production of organic acids which solubilize calcium phytate. *S. thermophile* secreted a high phytase titre at pH 5.0, 45°C and 250 rpm in five days in an inducible

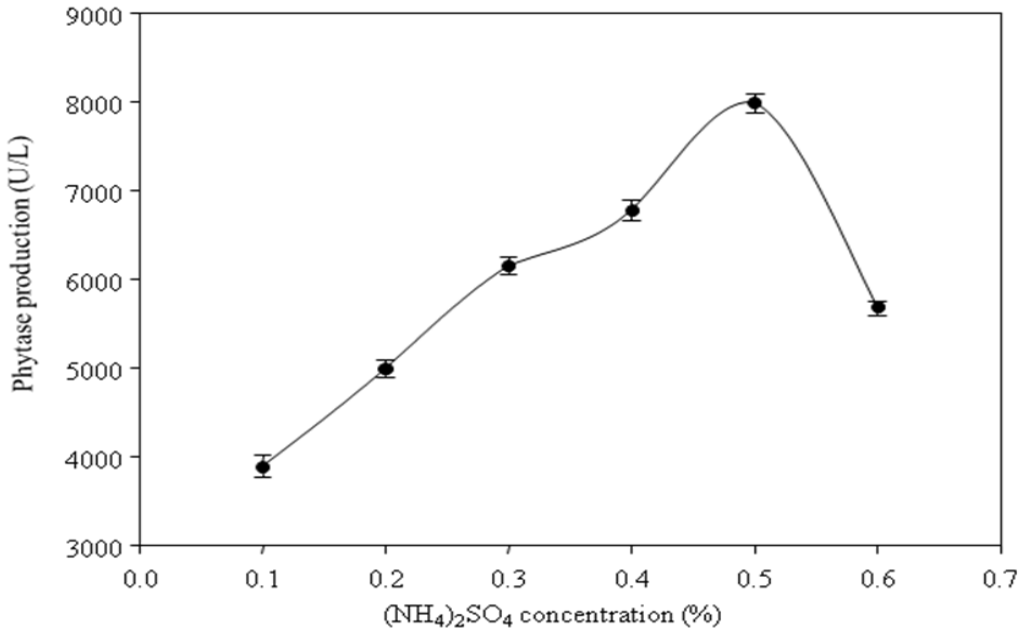

Figure 7B. Effect of $(NH_4)_2SO_4$ concentration on phytase production by the thermophilic mould.

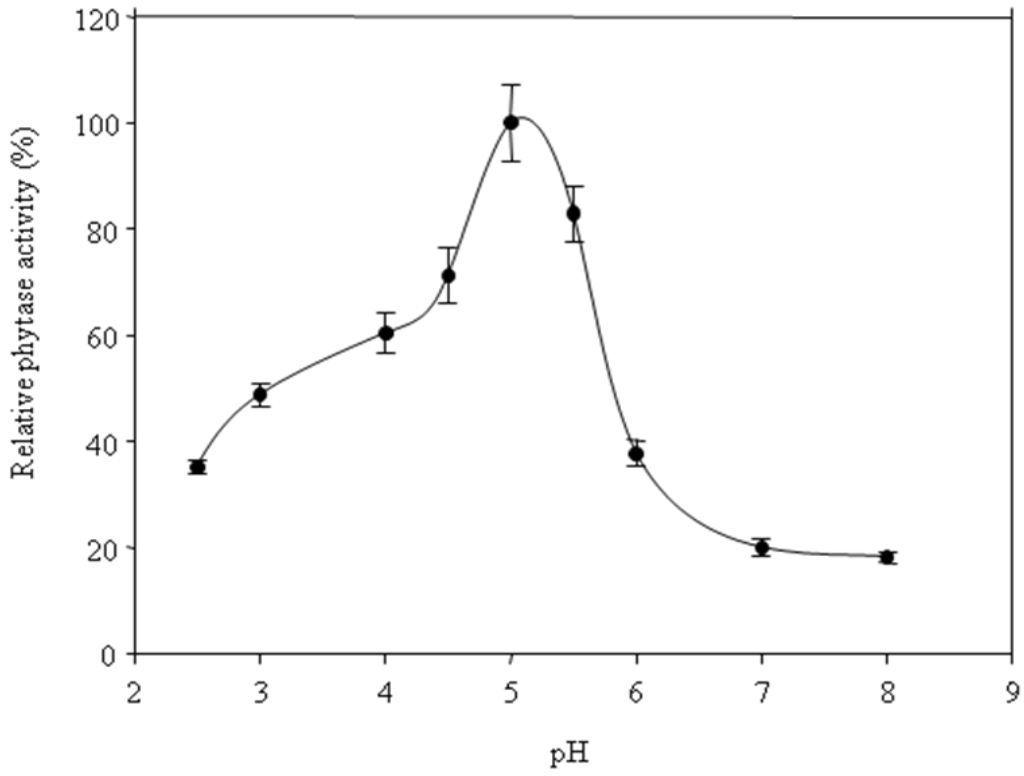

Figure 8A. Effect of pH on phytase activity.

manner, and the phytase of this mould has the requisite properties like activity at acidic pH and high temperature for its use as a feed or food supplement.

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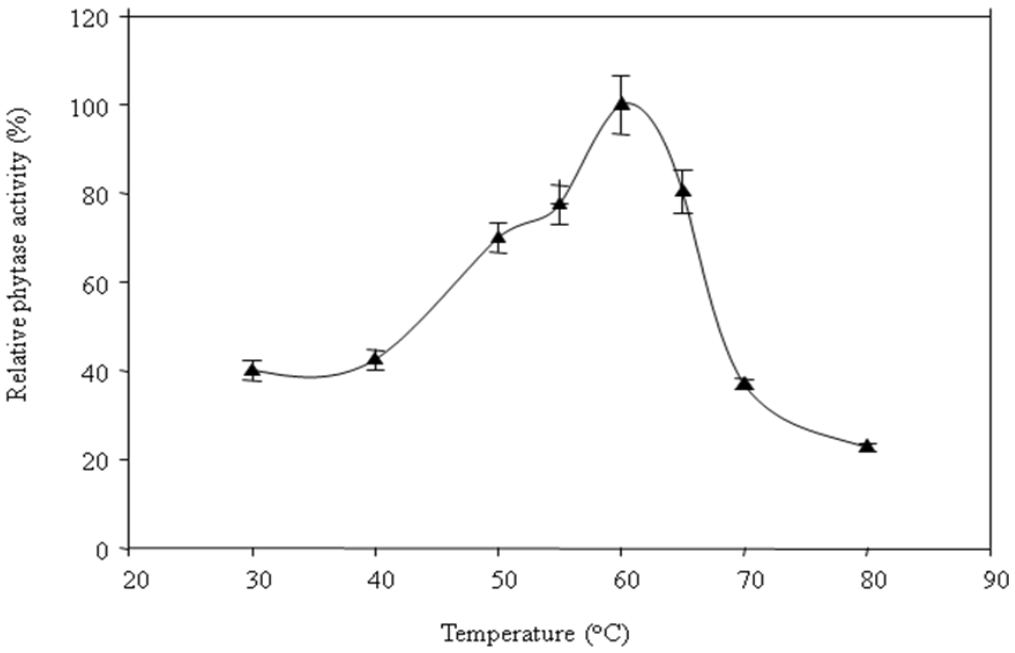

Figure 8B. Effect of temperature on phytase activity.

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