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EXTRACELLULAR PHYTASE PRODUCTION BY *BACILLUS* SP. T4 USING SOLID STATE FERMENTATION

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ABSTRACT

Wheat bran, soybean meal, corn flour and the combinations of these individual substrates with nutritive supplements containing 1% casein hydrolysate, 0.2% $CaCl_2 2H_2O$, 0.4% $(NH_4)_2SO_4$, 0.02% $MgSO_4 7H_2O$, 0.05% KH_2PO_4 and0.04% K_2HPO_4 were evaluated to select an optimal medium in solid state fermentation (SSF) to produce extracellular phytase from a bacterial strain, *Bacillus* sp. T4. The combination of corn flour with nutritive supplements resulted in best phytase synthesis and was used for further SSF explorations. Maximum phytase production (20787±39 U/g) was observed at a growth period of 84 h, 55.5% moisture content, and 4% inoculum density. Optimum pH for phytase production was 7.0. Enzyme activity was enhanced ($P<0.05$, 16496±187 to 18304±187 U/g) in the presence of glucose and galactose as a carbon source. The additional nitrogen sources impaired ($P<0.05$) the phytase activity, and corn steep liquor and sodium nitrateseverely inhibited the enzyme synthesis. T4 phytase produced in SSF may be a promising strategy for upgrading the nutritional quality and combating environment pollution in the feed industry.

Key words: Solid state fermentation, phytase, *Bacillus* sp., feed industry.

INTRODUCTION

Phytate (*myo*-inositol 1,2,3,4,5,6-hexakis-dihydrogen phosphate) is the major storage form of phosphorus in plant-based feed ingredients such as grains, legumes and oilseeds (Lestienne *et al*., 2005; Dost and Tokul, 2006). However, it is poorly metabolizable to monogastric animals including swine and poultry, due to a lack of an intrinsic phytate hydrolyzing enzyme in their digestive tracts (Comon, 1989; Wodzinski and Ullah, 1996) and is even considered to be an anti-nutritional factor by chelating vital divalent cations such as Ca^{2+}, Fe^{2+}, Zn^{2+} and Mg^{2+}, thereby decreasing their bioavailability (Erdman and Poneros, 1989). Moreover, this non-digestible phytate causes phosphorus pollution as it accumulates in areas of intensive livestock production, resulting in eutrophication and algal blooms (Mallin, 2000). Phytase (EC 3.1.3.8 or 3.1.3.26), a special type of phosphatase that catalyzes the hydrolysis of phosphate moieties from phytate, can ultimately improve phosphorus digestibility of the substrate, block the anti-nutritional effects and reduce environmental pollution (Yano *et al*., 1999; Bhavsar *et al*., 2011). Currently, a large number of commercial phytases are available and almost depend on fungal sources (Vohra and Satyanarayana, 2003; Sulabo *et al*., 2011; Almeida *et al*., 2013; Awad*et al*., 2014).

In light of the production of industrial enzymes, solid-state fermentation (SSF), in which cheap and abundant agro-industrial residues can be used as substrates (Shankar and Mulimani, 2007), has several advantages over conventional submerged fermentation (SmF), which include lower wastewater output, high product concentration, improved product recovery, simple cultivation equipment and lower plant operational cost (Becerra and Siso, 1996; Pandey *et al*., 2000, 2001). In fact, the existing commercial microbial phytases produced by SmF are expensive because of diluted product, production using recombinant strains, and high product recovery costs (Krishna and Nokes, 2001). Moreover, phytase may be produced in SSF by filamentous fungi on selected feed ingredients and the crude product may be mixed in feed rations as a value-added supplement (Pandey*et al*., 2001; Bogar *et al*., 2003). To date, phytase research on SSF has been conducted solely with fungal strains (Roopesh *et al*., 2006; Javed *et al*., 2010; Bhavsar *et al*., 2011). There is no report on phytase production under SSF by bacterial *Bacillus* strains, despite the fact that they are recently considered promising candidates for application in animal feed (Oh *et al*., 2004; Fu *et al*., 2008). Therefore, the objective of the present study is to describe the production of phytase under SSF by mesophilic *Bacillus* sp. T4.

MATERIALS AND METHODS

Reagents: The substrate, phytatedodecasodium salt (sodium phytate; Na-InsP_6) for phytase assay was purchased from Sigma-Aldrich (St. Louis, MO, USA). Peptone, tryptone, yeast extract, tryptic soy broth and bacto agar were purchased from BD Biosciences (San Jose, CA, USA). All other chemicals used in this study were of analytical grade and were also procured from Sigma-Aldrich.

Microorganism and inoculum: A phytase-producing bacterium, *Bacillus* sp. T4, described previously (Park *et al*., 2012) was used for enzyme production. The strain was routinely grown on tryptic soy agar consisting of tryptic soy broth and 1.5% bacto agar at 37ºCfor 24 h for inoculum preparation. A single fresh colony of the growth was activated in a 50 mL Falcon conical tube (BD Biosciences) containing 5 mL of tryptic soy broth at 37ºC for 12 h on a rotary shaker (220 rpm) and 1% of the 12 h culture was then transferred into 250 mL Erlenmeyer flask containing 50 mL of the medium, followed by incubation at 37ºC for 24 h. This preculture was used as inoculum for further solid state fermentation.

SSF: Commercial quality wheat bran, soybean meal and corn flour were obtained from the local supplier and initially evaluated for selection of an appropriate solid substrate in the fermentation. Briefly, 10 g of individual substrate were placed in 250 mL Erlenmeyer flasks or together with nutritive supplements [casein hydrolysate: 10 g, $(NH_4)_2SO_4$: 4 g, $CaCl_22H_2O$:2 g, $MgSO_47H_2O$: 0.2 g, KH_2PO_4: 0.5 g, K_2HPO_4: 0.4 gper liter]. The initial pH and moisture content was 5.5-6.0 and 50%, respectively. After autoclaving at 121ºC for 15 min, the flasks were inoculated with a 4% inoculum and incubated at 37ºC with vigorous shaking (220 rpm).

Unless otherwise stated, the SSF was performed using 10 g corn flour (55.5% moisture content) and the above mentioned nutritive supplements in 250 mL Erlenmeyer flasks with 4% inoculum, followed by incubation at 37ºC for 84 h. The effects of various physicochemical parameters including moisture content (33.3-80%) of the substrate, incubation time (0-168 h), inoculum size (1-25%) and pH (5-8.5) were investigated for the optimum production of phytase by *Bacillus* sp. T4. Additionally, studies were conducted to evaluate the influence of different carbon (starch, glucose, sucrose, fructose, galactose, lactose, maltose, molasses and sodium phytate; final concentration 1%) and nitrogen (peptone, tryptone, yeast extract, urea, corn steep liquor, sodium nitrate and ammonium sulfate; final concentration 1%) sources on enzyme production.

Enzyme extraction: Crude enzymes were extracted by mixing a weighed quantity of the fermented matter with 40 mL of cold distilled water and then shaking the mixture on a rotary shaker (220 rpm) at 37ºC for 1 h. The suspension was spun down by centrifugation (4ºC, 10 min and 5,000 rpm) and the supernatant used for phytase assay.

Enzyme assay: Unless otherwise stated, phytase activity was assayed at 40ºC for 1 h in a reaction mixture consisted of 0.8 mL 50 mMTris-HCl (pH 7.4), 0.1 mL 10 mM sodium phytate and 0.1 mL the crude enzyme. The released inorganic phosphates were measured by a modified method of Heinonen and Lahti (1981), with a freshly prepared acetone ammonium molybdate (AAM) reagent consisting of acetone, 5 N sulfuric acid and 10 mM ammonium molybdate (2:1:1, v/v). Two milliliters of the AAM solution and, thereafter 0.2 mL of 1 M citric acid were added to the phytase assay mixture. Absorbance was read at 355 nm after blanking the spectrophotometer with an appropriate control. One unit (U) of enzyme activity was defined as the amount of enzyme required to produce 1 nmol of inorganic phosphate per second under the given assay conditions and expressed as U/g of dry substrate in SSF.

Statistical analysis: The data reported in the present study were presented as the mean±standard error from three experiments (n=3). All results were subjected to a one-way analysis of variance using PROC GLM (SAS 9.3, SAS Institute Inc, Cary, NC) to test for significant differences between treatments with the Duncan's multiple range test (Duncan, 1965). The probability levels used for statistical significance were $p<0.05$ for all tests.

RESULTS AND DISCUSSION

Selection of an appropriate medium for phytase production in SSF: As shown in Table 1, of only three solid substrates, wheat bran was the most effective for phytase production by *Bacillus* sp. T4. Previously, wheat bran also served as the best carbon source for phytase production by a thermophilic fungus, *Sporotrichum thermophile*, as compared to rice husk, rice bran, fish meal, corn seed and corn gluten, because it might offer adequate amounts of nutrients such as carbohydrates, proteins, fats, calcium, phosphorus, potassium and amino acids necessary for high yield enzyme production in the presence of oxygen supply (Javed *et al*., 2010). Meanwhile, the combinations of individual substrate with nutritive supplements containing 1% casein hydrolysate, 0.2% $CaCl_22H_2O$, 0.4% $(NH_4)_2SO_4$, 0.02% $MgSO_47H_2O$, 0.05% KH_2PO_4 and0.04% K_2HPO_4 remarkably enhanced ($P<0.05$) the phytase synthesis and, in particular, maximal activity was observed in the combination of corn flour with nutritive supplements (Table 1). These findings may be somewhat related with earlier report that considerable phytase production was induced within 24 h

with casein hydrolysate as the main nitrogen source under SmF by *Bacillus subtilis* (Powar and Jagannathan, 1982). Subsequently, the combination of corn flour with nutritive supplements was used for further SSF study by *Bacillus* sp. T4.

Phytase production at different periods of fermentation: The time course of phytase synthesis by *Bacillus* sp. T4 was shown in Fig. 1. Phytase activity was highest ($P<0.05$, 12030±84 U/g–12088±229 U/g) after 84-96 h of incubation and was decreased ($P<0.05$) thereafter, which could have been due to the depletion of the nutrients or denaturation of the enzyme caused by the interaction with other components in the medium or change in the pH of the medium (Mahanta *et al*., 2008). Similarly, maximum phytase yields were observed after 96 h of incubation at 30ºC and 45ºC during SSF of wheat bran and oil cakes, and wheat bran alone by *Mucorracemosus* and *Sportrichum thermophile*, respectively (Roopesh*et al*., 2006; Javed *et al*., 2010).

Effect of initial moisture content on phytase production: Generally, bacterial cultures were considered unfavorable for SSF due to high water activity requirement (Mahanta *et al*., 2008). Initial moisture content critically affects microbial growth and enzyme production in SSF, and an optimum moisture level is essential for appropriate growth and enzyme production (Shankar and Mulimani, 2007). As shown in Fig. 2, maximum enzyme production was obtained with 55.5% moisture content ($P<0.05$, 14331±39 U/g). A low moisture content leads to sub-optimal growth and a lower degree of substrate swelling which also decreases enzyme production (Mahadik *et al*., 2002), while excessively high moisture content causes a reduction in substrate porosity and a decrease of the air content of the substrate, which in turn inhibits enzyme production and the microbial activity (Gautam *et al*., 2002; Baysal *et al*., 2003). Roopesh *et al*. (2006) observed maximum phytase production from *Mucorracemosus* at 60% moisture content with wheat bran and sesame oil cake as mixed substrates.

Effect of inoculum size on phytase production: As shown in Fig. 3, an appropriate inoculum concentration was 4%, giving the maximum enzyme yield of 20787±39 U/g ($P<0.05$). High inoculums densities inhibited the phytase production and minimum enzyme activity (8932±289 U/g) was obtained with the highest inoculum concentration (25%). Higher inoculums concentrations can lead to the exhaustion of nutrients, followed by severe competition for carbon source and nutrients, which results in reduced enzyme production (Ramachandran *et al*., 2005). Moreover, these conditions could also significantly increase the moisture content (Baysal *et al*., 2003). Hence, the free excess liquid present in an unabsorbed form will give rise to an additional diffusional barrier together with that imposed by the solid nature of the substrate, and lead to a decrease in growth and enzyme production (Krishna and Chandrasekaran, 1996).

Effect of initial pH of the medium on phytase production: The pH is an important factor affecting the growth and enzyme production during solid-state fermentation (Kunamneni *et al*., 2005; Mahanta *et al*., 2008). As shown in Fig. 4, the phytase synthesis occurred in a relatively wide pH range (5.0-8.5). It is assumed that the solid substrate contributes to a better buffering capacity (Wang *et al*., 2004). Although enzyme activity is not quite different ($P>0.05$) from 5.5 to 7.0, phytase yield was optimalat pH 7.0, which is in accordance with lipase produced by *Pseudomonas aeruginosa*PseA in SSF using *Jatrophacurcas* seed cake as substrate (Mahanta*et al*., 2008).

Effect of supplementation of additional carbon sources on phytase production: Enzyme activity was enhanced ($P<0.05$) in the presence of glucose and galactoseas compared with control (Fig. 5), even if other carbon sources had no positive impact on the phytase synthesis. Previously, glucose was also an efficient carbon source for phytase production in SSF by *Aspergillusniger* CFR 335 (Gunashree and Venkateswaran, 2008) and *Mucorracemosus* (Roopesh*et al*., 2006). In contrast, galactose did not support the phytase production in SSF by *Aspergillusniger* CFR 335 (Gunashree and Venkateswaran, 2008).

Effect of supplementation of additional nitrogen sources on phytase production: As shown in Fig. 6, none of the additional nitrogen sources enhanced the phytase activity, and corn steep liquor and sodium nitrate severely inhibited the enzyme synthesis. Strong repression of enzyme was observed in the presence of nitrogen source, which could have been due to an imbalance in the carbon/nitrogen ratio required for enzyme production (Roopesh*et al*., 2006). Similarly, corn steep solid was also highly inhibitory for phytase synthesis in SSF by *Mucorracemosus* (Roopesh *et al*., 2006).

Table 1.Phytase synthesis by *Bacillus* sp. T4 on different media in SSF

Media	[+]Enzyme activity (U/g)
Wheat bran	285±17[c]
Soybean meal	124±21[c]
Corn flour	177±20[c]
Wheat bran + [*]nutritive supplements	1462±183[ab]
Soybean meal + [*]nutritive supplements	1325±62[b]
Corn flour + [*]nutritive supplements	1651±17[a]

The[*]nutritive supplements consisted of casein hydrolysate 10 g/L, $CaCl_2 2H_2O$ 2 g/L, $(NH_4)_2SO_4$ 4 g/L, $MgSO_4 7H_2O$ 0.2 g/L, KH_2PO_4 0.5 g/L, K_2HPO_4 0.4 g/L. [+]Enzyme activity was assayed at 30ºC by the method described in the text. Data were expressed as mean±standard error from three experiments.
[a~c]Means lacking common superscripts differ (P<0.05).

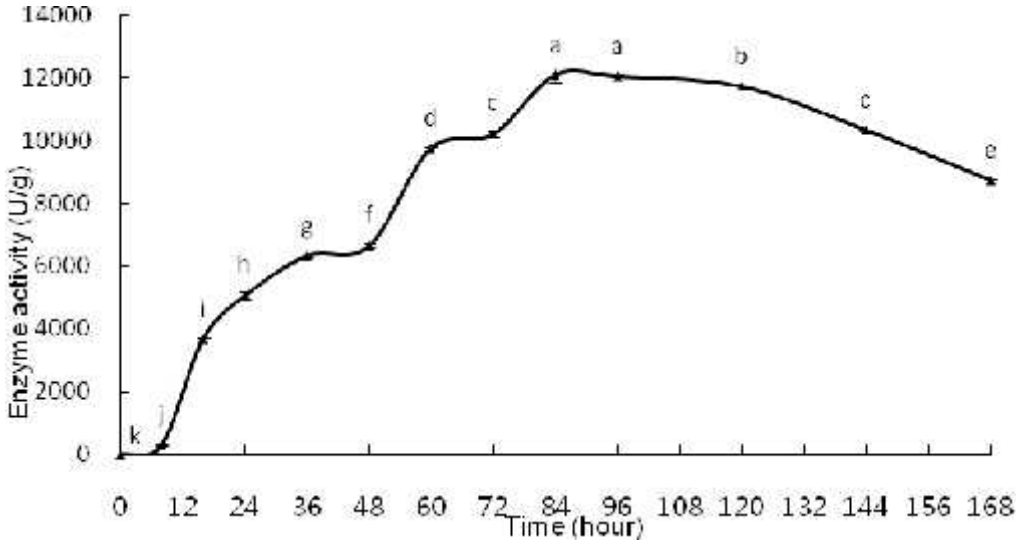

Fig. 1: Time course of T4 phytase production in SSF (37ºC, 55.5% moisture). [a~k]Means lacking common superscripts differ (P<0.05). Data were expressed as meanïstandard error from three experiments.

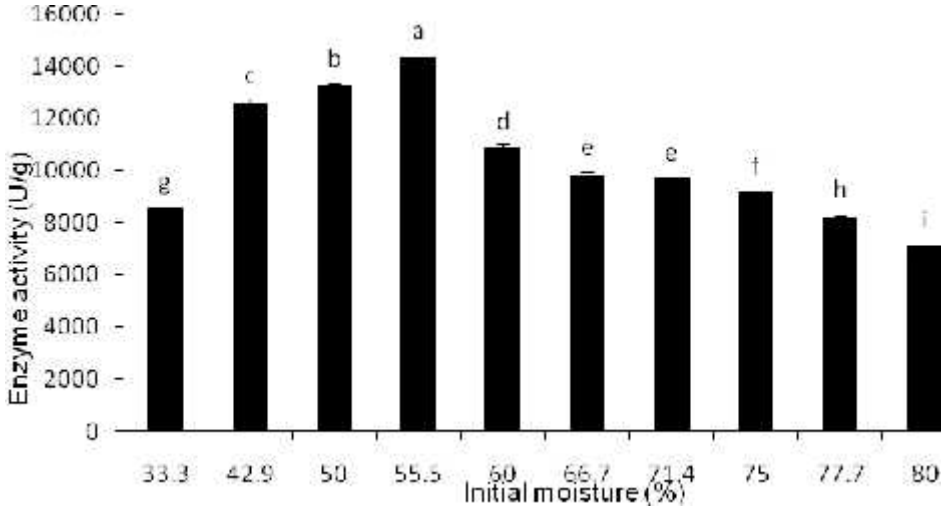

Fig. 2: Effect of initial moisture content on T4 phytase production in SSF (37ºC, 96 h cultivation). [a~i]Means lacking common superscripts differ (P<0.05). Data were expressed as meanïstandard error from three experiments.

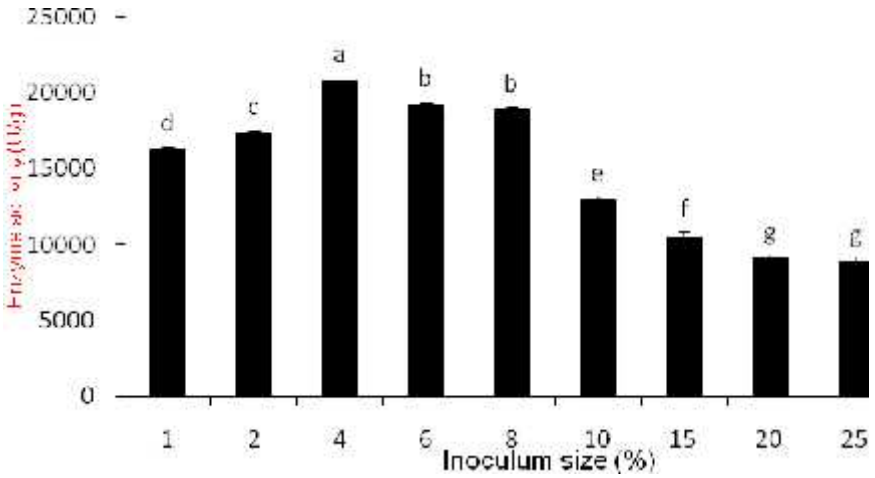

Fig. 3: Effect of inoculum size on T4 phytase production in SSF (55.5% moisture, 37ºC, 84 h cultivation). [a-g]Means lacking common superscripts differ (*P*<0.05). Data were expressed as meanïstandard error from three experiments.

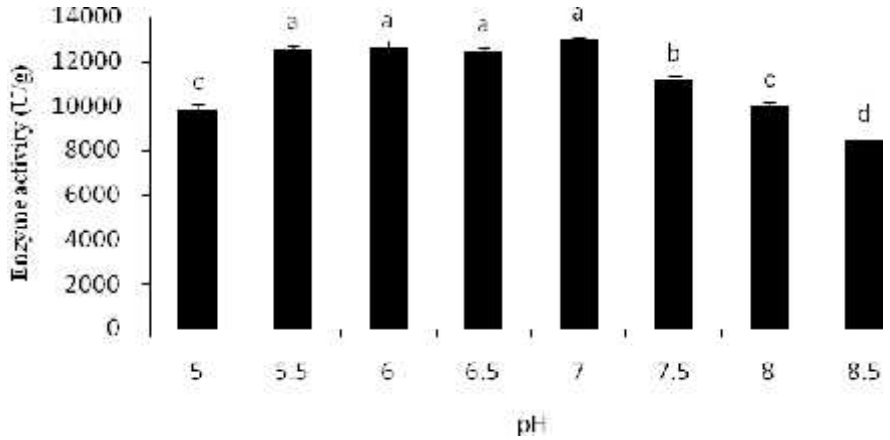

Fig. 4: Effect of initial pH of the medium on T4 phytase production in SSF (71.4% moisture, 37ºC, 84 h cultivation). [a~d]Means lacking common superscripts differ (*P*<0.05). Data were expressed as meanïstandard error from three experiments.

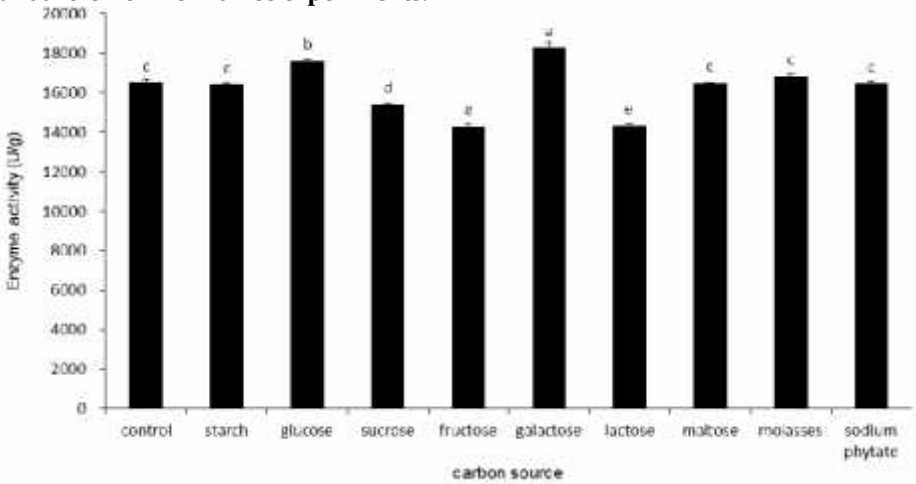

Fig. 5: Effect of different carbon sources on T4 phytase production in SSF (55.5% moisture, 37ºC, 84 h cultivation). Fermentation medium without any carbon supplementation was taken as control. [a~e]Means lacking common superscripts differ (*P*<0.05). Data were expressed as meanïstandard error from three experiments.

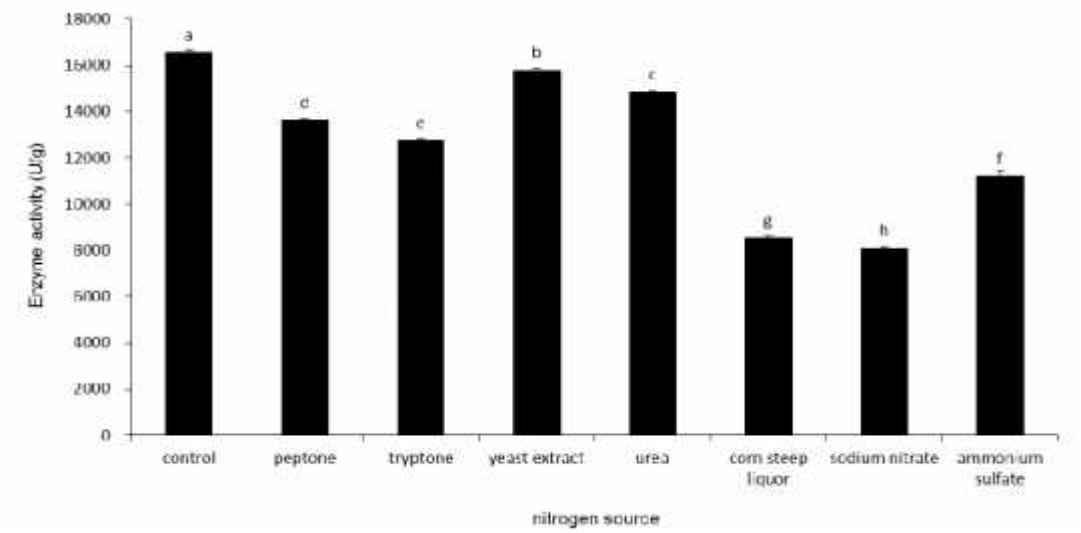

Fig. 6: Effect of different nitrogen sources on T4 phytase production in SSF (55.5% moisture, 37ºC, 84 h cultivation). Fermentation medium without any nitrogen supplementation was taken as control. $^{a\sim h}$Means lacking common superscripts differ (P<0.05). Data were expressed as meanïstandard error from three experiments.

Conclusion: Nowadays, the rapidly growing global market for animal feed enzymes is largely ascribed to increased use of exogenous phytases by the feed industry (Sulabo*et al*., 2011). The potential demand for phytase in cattle and poultry feed is around 4,000 tons per annum (Gunashree and Venkateswaran, 2008). In view of increasing demand for phytase, SSF can undoubtedly contribute to producing phytase in a cost-effective manner. Hence, the T4 phytase produced in SSF may be a promising strategy for upgrading the nutritional quality and combating environment pollution in the feed industry.

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