

OPTIMIZATION OF FERMENTATION CONDITIONS FOR PHYTASE PRODUCTION BY THE NOVEL ISOLATE *KLEBSIELLA* SP.

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ABSTRACT

Phytate is the main storage form of phosphorous of plant parts but it acts as antinutrients for the herbivores and certain aquatic animals. Since, it is present in bound form, its bioavailability depends mainly on the hydrolysis of phytate.

In an attempt to screen phytase producing bacteria from aquatic soil environment, among 200 isolates on the basis of phytase production, SCTb2 strain was selected after primary and secondary screening. Following morphological, cultural and molecular characterization the isolate SCTb2 was identified as *Klebsiella pneumoniae* SCTb2. The environmental conditions, both physical and nutritional, were optimized for phytase production. The isolate could produce the phytase extracellularly in the shake flask condition. The maximum production of phytase by this isolate was observed after 72 hour incubation at 28°C and at pH 6.0. The best carbon and nitrogen sources for maximum phytase production were 1% sucrose and 0.1% ammonium nitrate, respectively.

INTRODUCTION

Phytic acid (myo-inositol-1, 2, 3, 4, 5, 6-hexakis di-hydrogen phosphate, IP6) is an organic form of phosphorous, of molecular weight 659.86 having molecular formula $C_6H_{18}O_{24}P_6$. It is the primary source of inositol and storage form of phosphorous in plants particularly in cereal grains and legumes that represent approximately 75% to 80% of the total phosphorous available in nature (Chunshan *et al.*, 2001; Tye *et al.*, 2002; Turner *et al.*, 2002; Chang *et al.*, 2004; Kaur and Satyanarayana, 2005; Huang *et al.*, 2009). Such plant materials are used as animal feed ingredients, of which the phytate (salts of phytic acid), as phosphorous, is not well digestible by monogastric animals, like pig, poultry and fish, due to lack of phytase. Consequently, it contributes to the phosphorous pollution as a fall out of indigestible food material from intensive livestock production (Cromwell *et al.*, 1995). Being an important mineral, phosphorous must be available to animal and human to meet their daily requirements. Presence of indigestible phosphorous or the excess use of dietary phosphorous becomes responsible for environmental problems (Raza *et al.*, 2010). Also, the reactive groups of the IP6, make it a strong chelating agent that binds to cations of certain metals and become the antinutritional factor by decreasing the dietary availability of nutrients for animals (Batal and Abdelkarim, 2001; Applegate *et al.*, 2003; Veum *et al.*, 2006; Bohn *et al.*, 2008).

To overcome the problem of phosphorous deficiency in animal feed and to control the environmental pollution, phytase seems destined to become increasingly important. Phytase or myo-inositol hexakis phosphate phosphohydrolase

(E.C 3.1.3.8), can catalyse the hydrolysis of phytic acid to inositol and orthophosphoric acid (Wodzinsky and Ullah, 1996; Liu *et al.*, 1998; Chunshan *et al.*, 2001; Cho *et al.*, 2003; Wang *et al.*, 2004; Casey and Walsh, 2004). Supplemental microbial phytase in diets for swine, poultry and fish effectively improves phytate phosphorous utilization, thereby decreasing excretory phosphorous pollution (Rutherford *et al.*, 2002; Augspurger *et al.*, 2003; Olukosi *et al.*, 2007). Thus, enzymatic hydrolysis using phytase improves nutritional value of the feed material.

Phytase can be derived from a number of sources, including plants animals and microorganisms however, microbial sources are most promising for the production of phytase on a commercial level. Several strains of bacteria, yeast and fungi are used for the phytase production (Vohra and Satyanarayana, 2003; Konietzny and Greiner, 2002; Pandey *et al.*, 2001). However, bacterial phytase are mostly cell associated with the exception of *Bacillus subtilis*, *Lactobacillus amylovorus* and *Enterobacter* sp (Vohra and Satyanarayana, 2003).

This paper deals with study of extracellular phytase production by *Klebsiella pneumoniae* SCTb2.

MATERIALS AND METHODS

Media used

For screening and maintenance of the isolates, phytase screening medium (PSM) as detailed in Table 1 was used. To select the phytase producing medium, several media were tested as presented in Table 1.

Inoculum preparation

Inoculum was prepared in PSM (20 ml in 100 ml conical flask) (Table 1) and incubated at 37°C for 48 hr under shaking (210 rpm). Fermentation medium was inoculated with 1% inoculum (10⁹ CFU/ml).

Selection of producer organism

Phytase producing ability of the isolates was tested in petriplate containing Medium No. F. The plates were incubated at 37°C for 72 hr and observed the production by measuring the cleared zone formation, if any. Final selection was made through phytase assay (Engelen *et al.*, 2001) using cell free broth as crude enzyme source.

Medium optimization

Several media (Table 1) were tried to find out a suitable fermentation medium for growth and phytase production. In addition to Na-phytate, other common carbon sources were checked at 1% level and the selected carbon supplement also attempted at varying concentration. Different N₂ sources were tried, as nitrogen equivalent, of selected basal medium. Also, the concentration of selected nitrogen source was optimized.

Optimum fermentation period, temperature, pH, were determined in selected medium condition, considering the standardized factor in each case. Effect of surfactants and vitamins were optimized for growth and phytase production. In this experiment, the surfactants and vitamins were filter sterilized and added to the sterile medium aseptically to reach the desired concentration.

Each experiment was done in triplicates and presented as mean value.

Phytase assay

Phytase assay was performed as described by Engelen *et al* (2001). Reaction mixture contained 1.0 ml of enzyme and 2.0 ml of substrate (0.84 gm sodium phytate in 90 ml of acetate buffer, adjusted to pH 5.5 with acetic acid and made up to 100 ml with distilled water) and incubated for 65 minutes at 37°C. Reaction was stopped by adding 4 ml of color reagent [25 ml ammonium heptamolybdate (10% of ammonium molybdate in 0.25% ammonia solution) with 25 ml ammonium vanadate (2.35 g ammonium vanadate in one liter of 2% (v/v) nitric acid solution), adding 16.5 ml nitric acid (65%), mixing slowly and diluting to 100 ml with water]. Then cooled to room temperature and measured at 415 nm. The substrate mixture and coloring reagent were used as a blank.

One unit of the phytase activity was defined as the amount of enzyme able to hydrolyze phytate to give one mole of inorganic phosphate per min under the assay conditions. Specific activity was expressed in units of enzyme activity per milligram of protein.

Statistical analysis

The statistical analysis was conducted by using Microsoft office Excel, 2007 (Troesch *et al.*, 2009). Standard error of a set of values is formulated by the mean standard error (standard deviation/"n) for an n set of values.

16s rRNA gene sequencing

Bacterial 16S rRNA genes were amplified from boiled cell extract using specific primers f27 and r1492. The PCR amplification reactions were performed using high fidelity PCR master kit (Roche Applied Science) according to the manufacture's instruction. Primers at a concentration of 300 nM each were used with 25µl PCR master mix (provided by the manufacturer) in a final reaction volume of 50µl. The thermal cycles were: 94°C for 5 min and subjected to 30 cycles each consisting of 30 sec at 94°C; 30 sec at 60°C and 1 min at 72°C; and again for 7 min in same temperature to complete primer extension. The PCR products were electrophoresed on agarose gel in TAE buffer. After run, the gel excised and eluted using Qiagen gel elution kit. The sequence of PCR amplified 16S rDNA fragments were determined using universal primers 27f, 357f, 530f, 704f, 926f, 1242f, 321r, 685r, 907r, 1069r, and 1220r (Gerhardt *et al.*, 1994), terminator sequencing kit and an automated DNA sequencer ABI 377; Applied biosystem (Gerhardt *et al.*, 1994) for phylogenetic analysis (Pearson *et al.*, 1988) 16S rDNA sequence of the isolate was compared against those in the EMBL, Gen Bank and DDBJ database using FASTA (Version 3.4t23). Multiple alignment 16s rDNA sequences was done using the CLUSTALW program (Thompson *et al.*, 1994).

RESULTS

In an attempt to isolate phytase producing bacteria, 6 isolates were short listed out of about 200. From the short listed ones, the isolate SCTb2 was selected for its optimum production both in solid and liquid medium (Fig. 1a). Several media were tried to find out a suitable fermentation medium. It was found that the isolate (SCTb2) could grow and produce phytase in all the test media, but the Medium No. F was selected for optimum growth and production (Fig. 1b).

To optimize the carbon supplementation, several carbon sources were tried in the selected medium for growth and phytase production by the isolate. No other than sucrose supplementation showed enhancement of growth and phytase production (Fig. 2a) and 1% sucrose found optimum (Fig. 2b). Several N₂ sources (as nitrogen equivalent) were attempted in optimum medium condition and all of them were utilized for growth and production. However, ammonium nitrate was found as most suitable (Fig. 3a). Hence different concentrations of ammonium nitrate were tested and 0.1% was selected as optimum (Fig. 3b) for phytase production by the selected isolate.

While selecting the optimum fermentation period the experiment was conducted up to 96 hr, it was found that the isolate SCTb2 could produce maximally at 72 hr of fermentation (Fig. 4a). In addition, the fermentation was carried out in different temperature condition. The isolate could grow and produce suitably in the test range. However, it showed optimum production at 35°C (Fig. 4b). Fermentation was also conducted in a range of pH condition and found its compatibility to the test range, but to produce maximally at pH 6.0 (Fig. 4c).

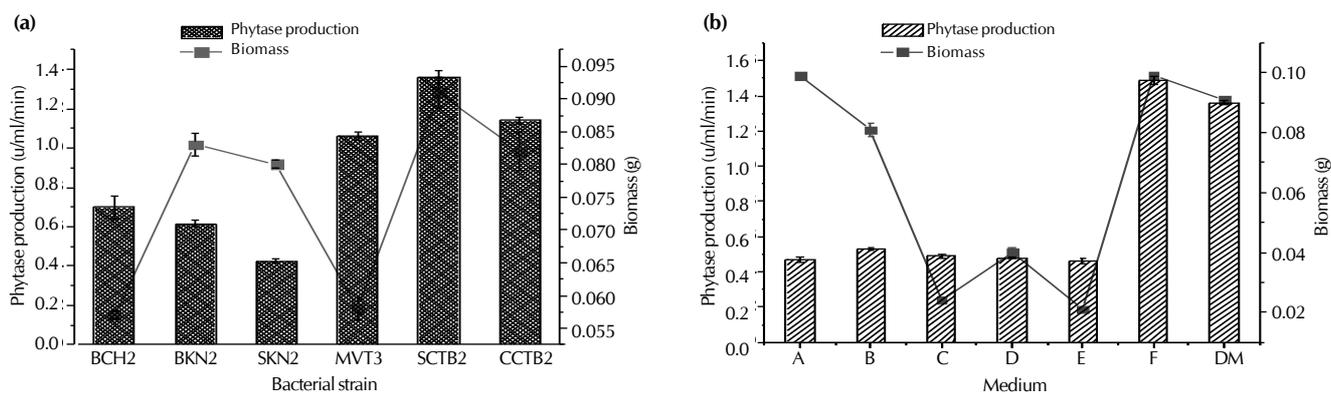
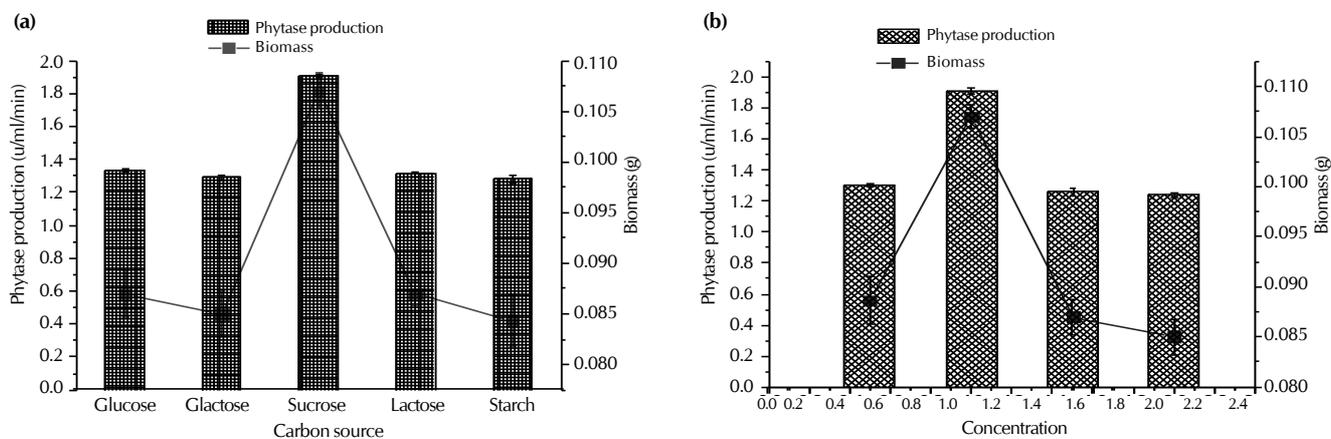
To study the effect of surfactants for enhancement of production, several surfactants were used. Inhibitory effect

Table 1: List of media used

Media components(%w/v)	A	B	C	D	E	F	PSM
(NH ₄) ₂ SO ₄	0.03	-	0.03	0.03	-	0.03	0.3
MgSO ₄	0.05	0.05	0.05	0.015	0.01	0.02	0.05
KH ₂ PO ₄	-	-	0.1	0.1	0.1	0.1	-
CaCl ₂	0.01	-	-	0.01	-	0.01	0.01
FeSO ₄	0.001	0.001	-	-	-	-	0.01
Asparagine	-	0.1	-	-	-	-	-
KCl	-	0.05	-	-	-	-	-
K ₂ HPO ₄	-	-	-	-	0.7	-	-
KNO ₃	-	-	-	-	0.1	-	-
Na- phytate	0.2	0.2	0.2	0.2	0.2	0.2	0.2

Table 2: Effect of vitamins on growth kinetics and phytase production by *Klebsiella pneumoniae* SCTb2

vitamin	Concentration(μg/ml)	Biomass (g)	Phytase production(U)
Pyridoxine	1	0.104	1.84
	10	0.108	1.85
	100	0.112	1.86
Pentathenic acid	1	0.103	1.85
	10	0.105	1.86
	100	0.103	1.87
Riboflavin	1	0.104	1.85
	10	0.103	1.88
	100	0.104	1.9
Thiamine	1	0.108	2.21
	10	0.115	2.4
	100	0.123	3.151

**Figure 1: Growth kinetics and phytase production: (a) Selection of isolate and (b) Selection of medium****Figure 2: Growth kinetics and phytase production by *Klebsiella pneumoniae* SCTb2: (a) Effect of carbon supplements and (b) Concentration of selected carbon source**

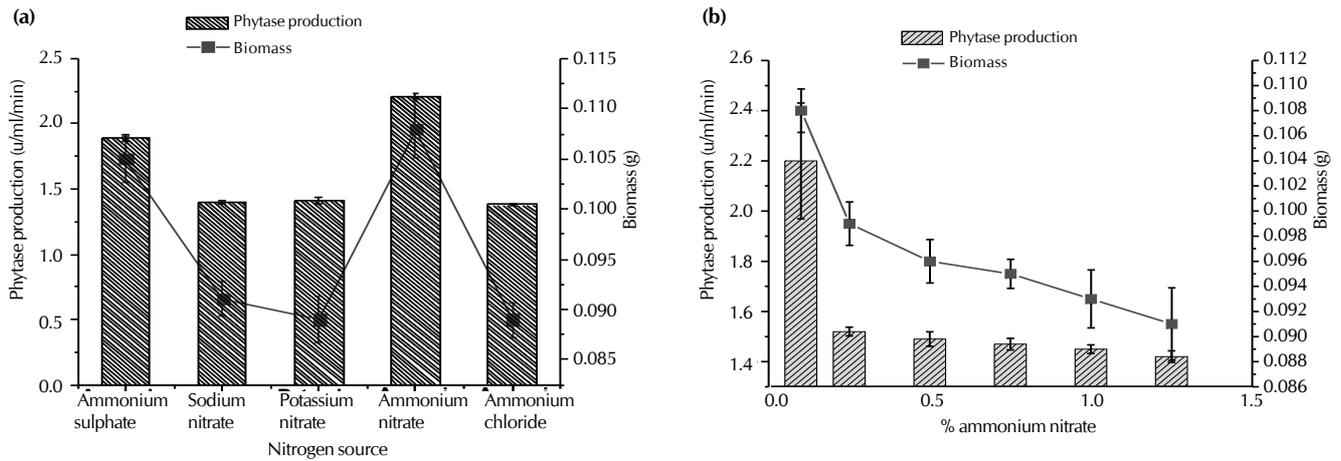


Figure 3: Growth kinetics and phytase production by *Klebsiella pneumoniae* SCTb2: (a) Effect of nitrogen source and (b) Concentration of selected nitrogen source

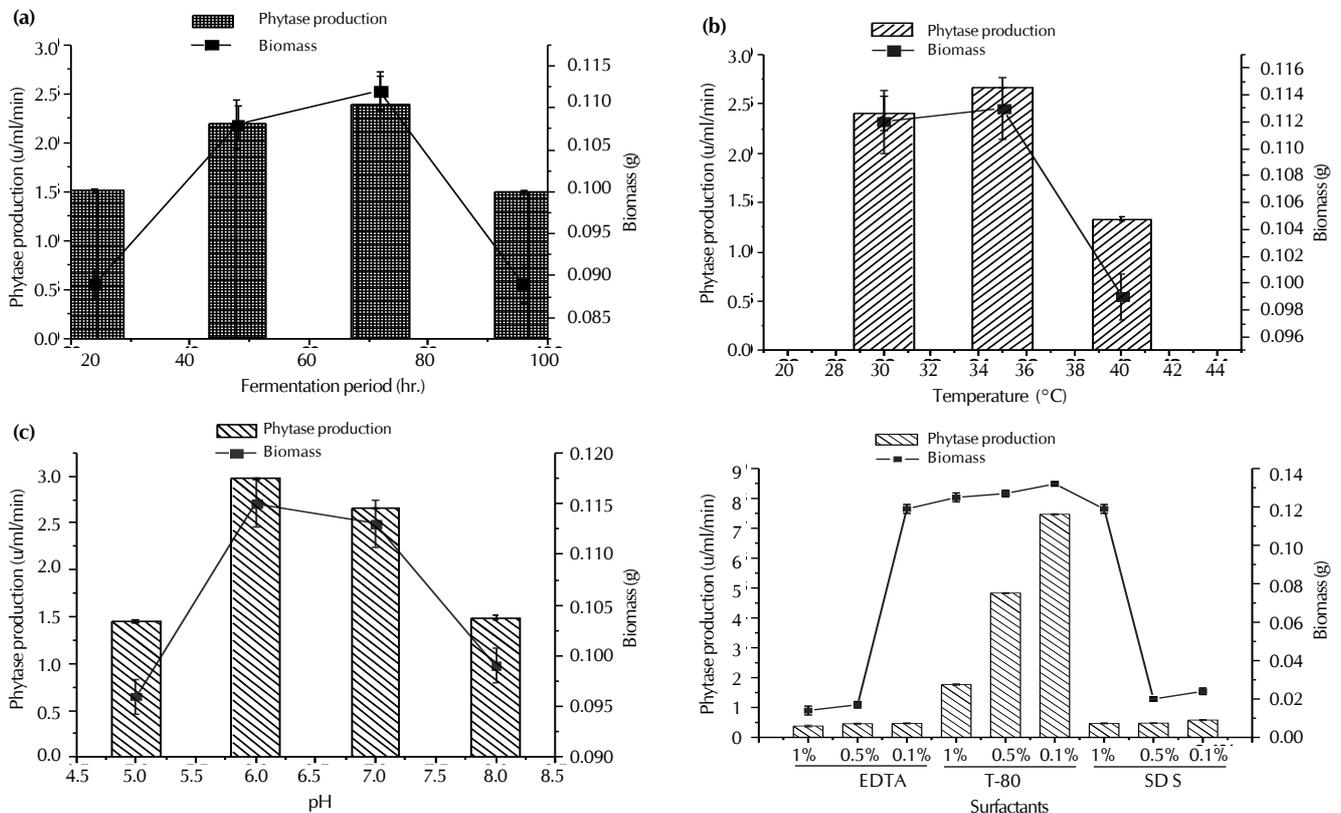


Figure 4: Growth kinetics and phytase production by *Klebsiella pneumoniae* SCTb2: (a) Effect of fermentation period, (b) Effect of temperature and (c) Effect of pH

was observed with EDTA, where as no significant effect with SDS, but Tween 80 (0.1%) showed certain enhancement for phytase production (Fig. 5a). Of the different vitamins tested, most of them did not impart any significantly positive effect except thiamine at 100 µg/ml (Table.2).

The identification of the selected strain was carried through preliminary characterization (data not shown) 3 up to 16s rDNA sequencing. The complete 16s rDNA gene sequences of the organism were compared with those available in the EMBL, Gen Bank and DDBJ databases and FASTA analyses

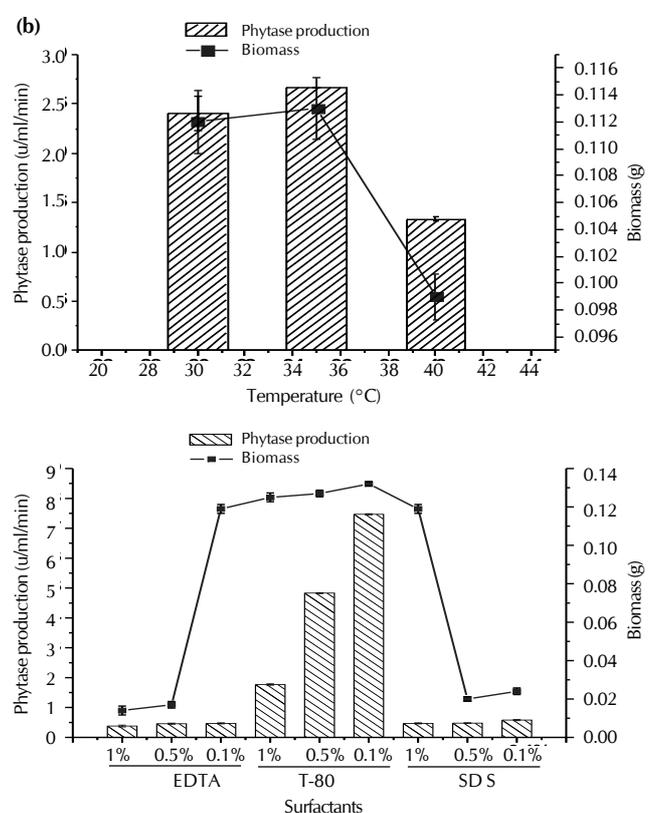


Figure 5: Effects of surfactants on growth kinetics and phytase production by *Klebsiella pneumoniae* SCTb2

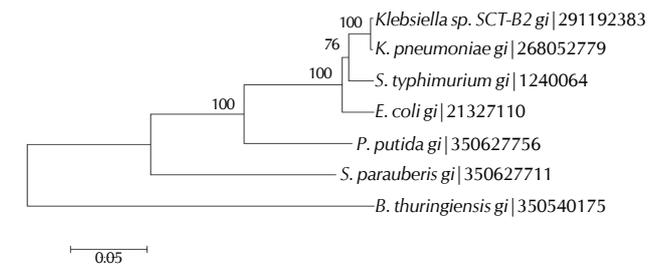


Figure 6: The phylogenetic tree construct of isolate (SCTb2) based on 16SrDNA sequences

with those of existing species. Results showed that the isolate SCTb2 had maximum identity (99-100%) with *Klebsiella pneumoniae*. Multiple alignment of those sequences revealed that selected strain SCTb2 and *Klebsiella pneumoniae* were identical to each other (Fig. 6). Further, the higher value of 16s rRNA gene sequence similarity observed between isolate SCTb2 and related species of all the close genera, it appeared that the isolate SCTb2 represent the same phylogenetic lineage along with *Klebsiella pneumoniae*.

DISCUSSION

Since last decade, phytase shows a potential demand, particularly as feed supplements (Troesch *et al.*, 2009). Microbial phytases are considered valuable in upgrading the nutritional quality of plant based feed ingredients. Phytase from several species of bacteria, yeast and fungi have been studied (Pandey *et al.*, 2001 Konietzny and Greiner, 2002).

Bacterial phytases are mostly cell associated, with the exception of *Bacillus subtilis*, *Lactobacillus amylovorus*, and *Enterobacter* sp.4 (Vohra and Satyanarayana, 2003). However, certain bacteria (*Bacillus*, *Staphylococcus*, *Brevibacterium*, *Pseudomonas* and *Kocuria*) are capable of producing phytate degrading enzyme extracellularly under specified conditions and *Staphylococcus lentus* ASUIA 279 showed a good amount of extracellular phytase production (Hussin *et al.*, 2007). There are four groups of potential phytase producing bacterial isolate, including *Pseudomonas* spp. (Richardson *et al.*, 1997; Kim *et al.*, 2002; In *et al.*, 2004). In this study, *Klebsiella pneumoniae* SCTb2 found as the best phytase producer among the six short listed ones, through quantitative screening. Hill *et al.*, (2007) screened 10 typical soil bacteria that released orthophosphate from phytate within 21hr of fermentation and 6 of them belonged to the genus *Pseudomonas*.

Identification of the isolate (SCTb2), based on the result of morphological, cultural, biochemical characterization and also 16s rDNA sequencing to identify it as a strain of *Klebsiella pneumoniae*.

The energy required and the physical support for an organism to grow and to produce the desire metabolite is primarily provided by the substrate (Pandey *et al.*, 2001; Spier *et al.*, 2008). The selected medium (Medium F) was taken as a basal medium and the process parameters under study were varied. While using supplemental carbon and phosphorous sources other than Na-phytate significant phytase production was observed and sucrose appeared as the best carbon supplement at 1% level. Result indicated that additional carbon source was utilized favorably for the production of phytase by this organism. To initiate growth and metabolism, bacteria require carbon sources at easy available form. Further, the effect of supplemental carbon sources (Glucose, Galactose, Sucrose, Lactose, and Starch) to the fermentation medium on enzyme production was evaluated (Hosseinkhani *et al.*, 2009). Impact of inorganic nitrogen sources to the fermentation medium on enzyme production was evaluated by incorporating various nitrogen sources in the medium (Spier *et al.*, 2008; Hosseinkhani *et al.*, 2009). It was found that

0.1% ammonium nitrate supported for optimum production and growth but further increment in amount of nitrogen inhibited the yield of phytase.

While optimizing the physical parameters, the experimental results suggested that phytase production increased progressively along with the increase in fermentation period until 72 hr and was decreased thereafter, probably due to the reduced nutrient level of medium, affecting enzyme synthesis. Declined enzyme yield could also be due to poisoning or denaturation of the enzyme under changed environmental condition (Sabu *et al.*, 2002). Temperature found to influence the rate of phytase production by the isolate. The maximal phytase production was observed at 35°C and the enzyme production decreased with further increase in temperature. Optimal temperature for production of most phytases varies from 30 to 80°C (Hara *et al.*, 1985; Wang *et al.*, 2004). The pH of the medium is the other most important factor for a fermentation process. Optimum pH required for maximal phytase production during shake flask method was evaluated under varied pH levels (Hara *et al.*, 1985; Ullah *et al.*, 2008). The enzyme production was maximum at pH 6.0 for *Klebsiella pneumoniae*, further increase of pH of the fermentation medium reduced the growth and enzyme production (Wang *et al.*, 2004).

Vitamins are the important growth factor for every organism. The potential exists for vitamins and feed enzymes to counteract some metabolic disorders and challenges in poultry (Broz and Ward, 2007). In this study, thiamine (100ig/ ml) supplementation showed positive effect. Like vitamins, surfactants are known to affect the growth and enzyme production (Nampoothiri *et al.*, 2004). A high phytase titre was attained in the medium containing sodium oleate and non-ionic detergents (Tween-20, 40 and 80). However, Triton X-100 (a non-ionic detergent) and SDS (an anionic detergent) inhibited enzyme production in *Sporotrichum thermophile*. Probably due to increase of viscosity of the medium that resulted in decrease in the rate of oxygen transfer or due to the toxic effect of surfactants (Rao and Satyanarayana, 2003). The surfactants may also act as detergents to solubilize membrane proteins thereby increasing membrane permeability that lead to the concomitant enhancement in the secretion of biomolecules (Ne'eman *et al.*, 1971; Rao and Satyanarayana, 2003). Similarly, Tween as well as oleic acid increased phytase production by *Aspergillus niger* (El-Batal and Abdel Kareem, 2001) and in *A. carbonarius* (Ebune *et al.*, 1995). In thermophilic mould *Thermoascus aurantiacus*, Tween-20 and Tween-80 are reported to enhance phytase production (Nampoothiri *et al.*, 2004). In this study, fermentation with Tween-80 (0.1%, vD v) showed satisfactory result.

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