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Extracellular production of Phytases by a Native *Bacillus subtilis* Strain

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ABSTRACT

Phytases are the enzymes hydrolyzing phytic acid to less phosphorylated myo-inositol derivatives, releasing inorganic phosphate. Phytase has become an important industrial enzyme and is the object of extensive research. The objective of the present study was to isolate and characterize a potential phytase producing bacterial strain from boggy water, and production of phytase in a submerged fermentation system. The phytase producing bacteria were isolated from boggy water, and were screened using PSM plates, containing selectable media. The phytase producing strain was identified using 16S rDNA sequencing followed by BLAST analysis. Different fermentation parameters, including incubation time, temperature, pH, carbon source and nitrogen sources were optimized to enhance phytase production. The phytase enzyme was produced using shaken flask fermentation and purified using ethanol precipitation and chromatography. The enzyme was further characterized using SDS-PAGE and zymogram analysis. From the 21 bacterial isolates, one Bacillus subtilus strain (BPTK4) with high potential for phytase production was selected. The isolated Bacillus subtilis produces significant amount of phytase during 48th h of incubation at 32 °C with the pH of 6.5. Glucose is considered as suitable carbon source whereas yeast extract is for nitrogen. It has been concluded that pond water can also be used as the source for the isolation of phytase producing bacteria. The phytases produced can be used further for various applications.

Keywords: *Bacillus subtilis,* Phytases, Submerged Fermentation, 16S rDNA.

INTRODUCTION

Phytases (*myo*-inositol(1,2,3,4,5,6) hexakisphosphate phosphohydrolases are commonly used as animal feed additives for poultry and swine. The use of phytase as a feed additive has been approved as GRAS (Generally Regarded as Safe) in 22 countries [1].

Phosphorous is one of the major feed ingredients and is supplied to animals in required amounts through raw material and added phosphates. 50–80% of phosphorous is bound in phytates, which cannot be broken down by endogenous enzymes in poultry [2]. Phytate is the major storage form of phosphorus in seeds and found in diet of many animals and humans [3]. As a consequence, phosphorous from vegetable sources is poorly digested and cannot meet nutritional requirements of poultry regardless the fact that cereals, leguminous and oilseed plants contain 1–5% phytate. Phosphorous from vegetable sources must be hydrolyzed, with phytase as a catalyst, in order to become available to broiler chicks as inositols and inorganic phosphates which are readily absorbed in digestive tract [4]. Phytase added to corn-soybean pig diet converted approximately one-third of the unavailable phosphate to an available form [5].

Phytases are known to be found in plants, microorganisms, and in some animal tissues [6,7]. Phytase has been detected in various bacteria, e.g. *Aerobacter aerogenes* [8], *Pseudomonas* sp. [9], *Bacillus subtilis* [10], *Klebsiella* sp. [11] and *Enterobacter* sp. [12]. Phytase activity has also been detected in white mustard, potato, radish, lettuce, spinach, grass and lilly pollen.

A thermostable phytase could have potential as a novel biological agent to degrade phytic acid during pulp and paper processing. The exploitation of phytases in the pulp and paper process could be eco-friendly and would assist in the development of cleaner technologies [13].

Vegetarians, elderly people consuming unbalanced food with high amounts of cereals, people in undeveloped countries who eat unleavened bread and babies eating soy-based infant formulas take in large amounts of phytate [14]. Undigested phytate in the small intestine negatively affects the absorption of zinc, calcium, magnesium and iron. It also reduces the digestability of dietary protein and inhibits digestive enzymes. Additions of *A. niger* phytase to flour containing wheat bran increased iron absorption in humans [15]. However, more studies should be performed before accepting phytase as a food additive.

In the past decade, it has been paid attention towards the study on protease production and optimization for maximum yields to make phytase as an economical and commercial product [16]. Since the source of organism also important for the production of phytase, bacteria were isolated from various sources those days. In recent, three bacterial strains were isolated from the Malaysian maize plantations with the ability of phytase production [17].

In the present investigation also, the ability of locally isolated bacterial strain from Boggy water to produce extracellular phytase in a submerged fermentation was evaluated and their production conditions were characterized. It was essential to produce phytase on cost effective media. The organism was identified using the novel 16S rDNA sequencing.

MATERIALS AND METHODS

Isolation and screening of phytase producing bacteria

Phytase producing bacteria were isolated from boggy water sample from Chetpet soolai kutti (pond), Chennai, TN, India by the dilution spread plate technique [18] using PSM (Phytase Screening Medium) agar media composed of Galactose (2%), Threonine-L (0.5%), Calcium chloride (20mM), Magnesium chloride (20mM), Sodium phytate (20mM), Trace elements (0.1%), H_3BO_2 (5.7 mg), $Fe(NH_4)(SO_4)_3{\cdot}18H_2O$ (173.0 mg), $CUSO_4{\cdot}5H_2O$ (18.6 mg), $MnSO_4{\cdot}4H_2O$ (8.1 mg), $NH_4 6MO_7O_{24}{\cdot}4H_2O$ (3.6 mg), $ZnSO_4{\cdot}7H_2O$ (79.0 mg) prepared in 100 ml distilled water at pH 6.5. The plates were incubated at 37 °C for 24 h. To indicate the phytase

activity of the bacterial isolates, diameters of clear zone around colonies on PSM agar were measured. A bacterial isolate with the highest phytase activity was selected for the next studies.

Molecular identification of organism using 16S rDNA sequencing

For the sequence analysis, bacterial genomic DNA was extracted and purified using CTAB method [19]. Two primers annealing at the 5′ and 3′ end of the 16S rDNA were Forward:5′-AGAGTTTGATCCTGGCTCAG-3′, Reverse:5′-TACCTTGTTACGACTT-3′ [20]. PCR amplification was performed in a final reaction volume of 100 µl. The PCR reaction was run for 35 cycles in a DNA thermal cycler. The amplified PCR products were then analyzed in a 1.0 % (w/v) agarose gel, excised from the gel, and purified. The amplified DNA sequence was then sequenced on Chromous biotech, Hyderabad using automated sequencer. The 16S-rDNA gene sequence of the isolates was aligned with reference 16S-rDNA sequences of the GenBank using the BLAST algorithm [21, 22] available in NCBI.

Phytase production and activity assay

The production of enzyme was carried out in the production medium without addition of agar using Shaken flask fermentation method. The inoculum of the selected strain was produced using LB Broth. Five percent of inoculum was inoculated on 500 ml of production medium [23] taken in 1000 ml conical flask. The flask was then incubated at 30 °C for 48 h at shaken condition at 200 rpm for better aeration and growth of organism.

The amount of phytase produced was assayed using plate assay and chemical assay. The plate assay method was performed by pouring the culture filtrate on PSM medium, which confirms the production of phytase production by forming clear zones. The chemical assay was done by using ferrous sulphate molybdenum blue method [24]. Total protein content from the sample was determined using Bradford method [25].

Optimization of phytase production

In order to determine the effect of temperature on phytase production, the selected bacterial isolate was grown in production media and incubated at 28, 32, 37, 42, 47 and 52 °C for 48 h. Culture filtrates were later measured for phytase activity.

The effect of initial media pH on phytase production was conducted by adjusting the production media to pH 5.5, 6.0, 6.5, 7.0 and 7.5 before bacterial inoculation. After 48 h of incubation at 37 °C, culture filtrates were measured for phytase activity.

To evaluate the effect of incubation time on phytase production, the inoculated medium was incubated at 30 °C with shaking around 150 rpm. Around 20 ml of culture was aseptically drowned periodically at every 6 h time interval up to 72 h [26]. Culture filtrates were measured for phytase activity.

The effect of carbon and nitrogen source on phytase production was determined by adding the production media with different carbon and nitrogen sources and inoculated with test organism. The media was incubated for 24 h at 37 °C. Culture filtrates were later measured for phytase activity. In addition, the role of various natural substrates like Sugar cane baggage, rice bran and wheat bran on phytase production was also analysed.

Purification and characterization of phytase enzyme (SDS-PAGE and zymogram analysis)

The culture fluid from the production media was collected and centrifuged. The culture supernatant was collected as crude enzyme extract for purification. To the culture supernatant,

three volumes of 95% cold ethanol was added, and the mixture was maintained in ice for 1 h with agitation. The precipitated crude extract was harvested by centrifugation and dissolved in 0.1 M Tris–HCl buffer (pH 7.0). The precipitated enzyme was then subjected to column chromatography for further purification [27]. SDS PAGE was performed according to the Laemmli [28] with the 4% Acrylamide stacking gel and 10% Acrylamide separating gel to determine the molecular mass and purity of the protein. Staining was carried out with CBB staining method [29]. To conduct zymmogram analysis, PAGE was executed according to the method of Laemmli [30]. Electrophoresis was done under non-denaturing conditions. Gels were incubated at 22 °C in 50 mM NaCl (pH 9.6) and 2mM $CaCl_2$ with pNPP as substrate. Phytase release was detected by Malachite green staining procedure [31].

RESULTS AND DISCUSSION

Microbial enzymes meet industrial demands; a large number of them are available commercially [32]. Enzymes from fungal and bacterial sources have dominated applications in industrial sectors. Fungal sources are confined to terrestrial isolates, mostly to *Aspergillus* and *Penicillium* species [32,33]. Most of the bacterial strains present in the muddy soil are having the ability to produce phosphatase and phytase.

A total of 21 bacterial strains were isolated from boggy water sample, from them, 8 strains were found to be positive for phytase production by their zone forming ability in PSM. From those 8 phytase positive strains the better zone forming strain was chosen and was assigned strain name as BPTK4. In 2000, a report showed that among 77 bacterial isolates, an isolate CMU4-4 exhibited the highest enzyme activity whereas its clear zone was smaller than other isolates [34]. The phytase production has been reported in variety of organisms including *Bacillus* [35], *Escherichia coli* [36], *Lactobacillus* [37], *Pseudomonas* [38] and *Aspergillus* [39].

The nucleotide sequence of the isolate was deposited in the GenBank database (Genbank accession no. EF077669-72). The BLAST analysis of the sequence of 16S rDNA gene showed that the strain BPTK4 had 100% homology with *Bacillus subtilis* strains. 16S rDNA analysis is more advanced and accurate since the difference in properties between the bacterial strains such as *B. anthracis*, *B. cereus*, and *B. thuringiensis*, are <1%. Such small differences cannot be analysed using conventional methods. The study made by Claudio [20] clearly demonstrates that such small differences also might be important for species identification. DNA hybridization studies have shown that these three *Bacillus* species are closely related and probably represent a single species [40–42]. If the three were classified as a single species, 16S rDNA sequencing appears to have the potential ability to differentiate strains at the subspecies level.

Since growth study was essential for the production of extracellular enzymes, it was studied by shaken flask fermentation method [21]. The stationary phase of growth was reached after about 48 h. The production of phytase was detected after 36 h of cultivation and increased during growth and reached maximum level (109 U/mg) at 48th h. The production of phytase was considerably low before 36th h and after 48th h of production (Fig. 1). It was considered as the log phase and its variation also depends on the nutrient present in the medium and the cultural condition of the organism. The environmental parameter also influences the maintenance time of the bacteria. The plate assay and chemical assay confirmed the production of phytase.

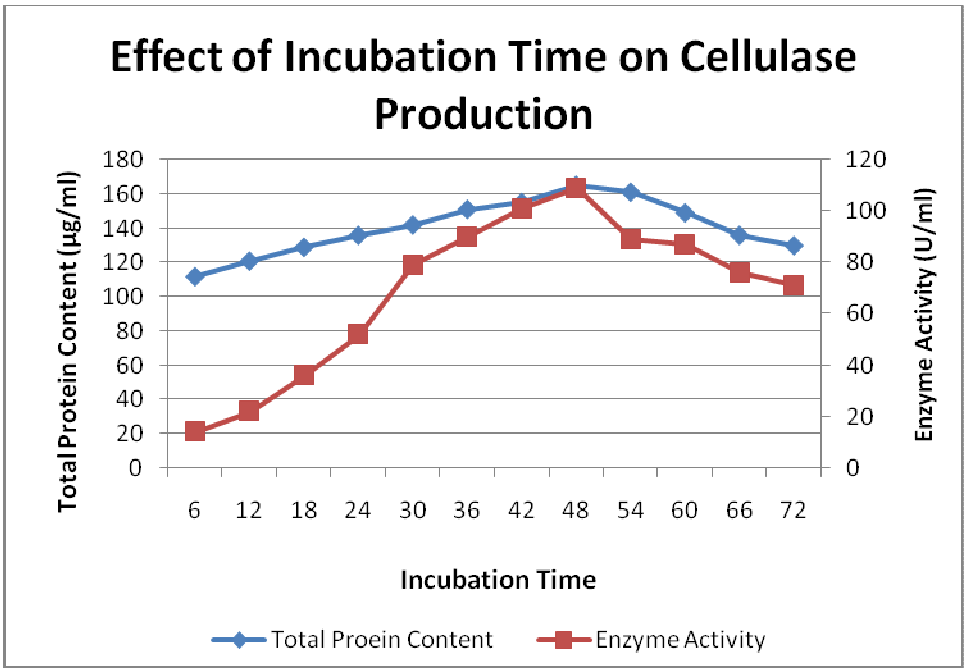

Fig. 1: Effect of incubation time on Phytase production

To evaluate the optimum temperature for the phytase production, the fermentation was done on different temperatures. Like other mesophilic bacteria, the isolated strain, BPTK4 also shown higher phytase activity at 32 °C (116 U/mg) (Fig. 2). Depending upon the source of the bacteria isolated, their optimum temperature also varies. Vohra and Satyanarayana [43] observed high phytase production at pH 6.0 and lowered to 4.0 after incubation for 2 days at 30 °C. It has been found that most of the phytases produced by microorganisms possesses the optimal temperature from 25 to 37 °C [44].

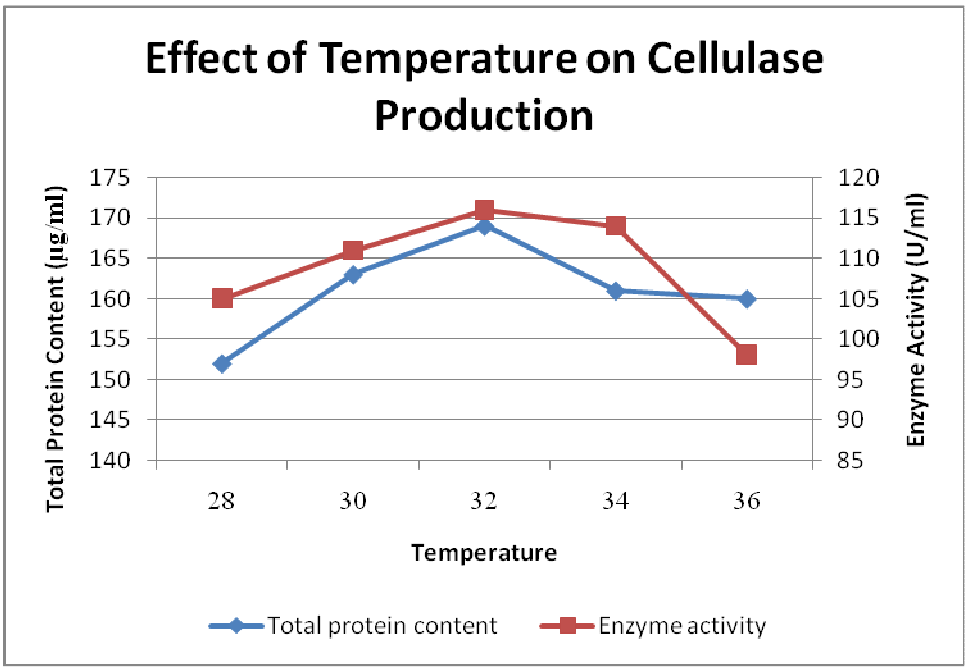

Fig. 2: Effect of temperature on Phytase production

Next to temperature, pH is found to be the important parameter for phytase production. The considerable production of phytase was observed at the pH of 6.5 (114 U/mg) (Fig. 3). It was found that the strain BPTK4 requires alkaline pH for phytase production. Most isolated phytases have their pH optima in the range of 4.5–6. But, phytase from *Bacillus sp.* have neutral or alkaline pH optima [45]. The better incubation time for the phytase production was found to be 36–48°C. The better production was achieved at 37°C with the pH of 7.0. *B. subtilis* (pH 6.0–6.5 and 60°C) [46], *B. amyloliquefaciens* (pH 7.0–8.0 and 70°C) [47], and *E. coli* (pH 5.0 and 70°C) [44]

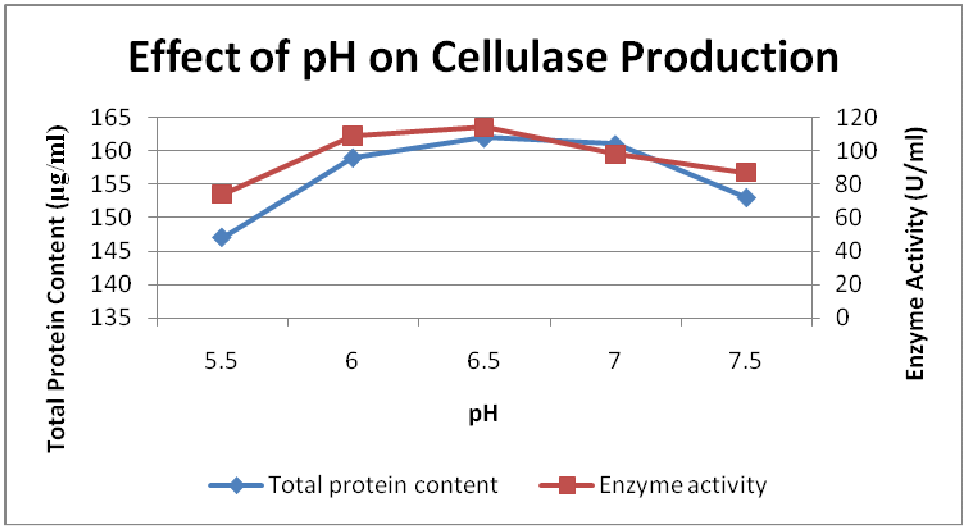

Fig. 3: Effect of pH on Phytase production

Next to the environmental parameters, nutrient sources such as carbon and nitrogen sources plays major role in phytase production carbon is found to be the primary energy source required by most of the organisms. The production of phytase using different carbon sources' including Glucose, lactose, sucrose and galactose was analyzed. Glucose was found to be the best carbon source for the phytase production (Fig. 4).

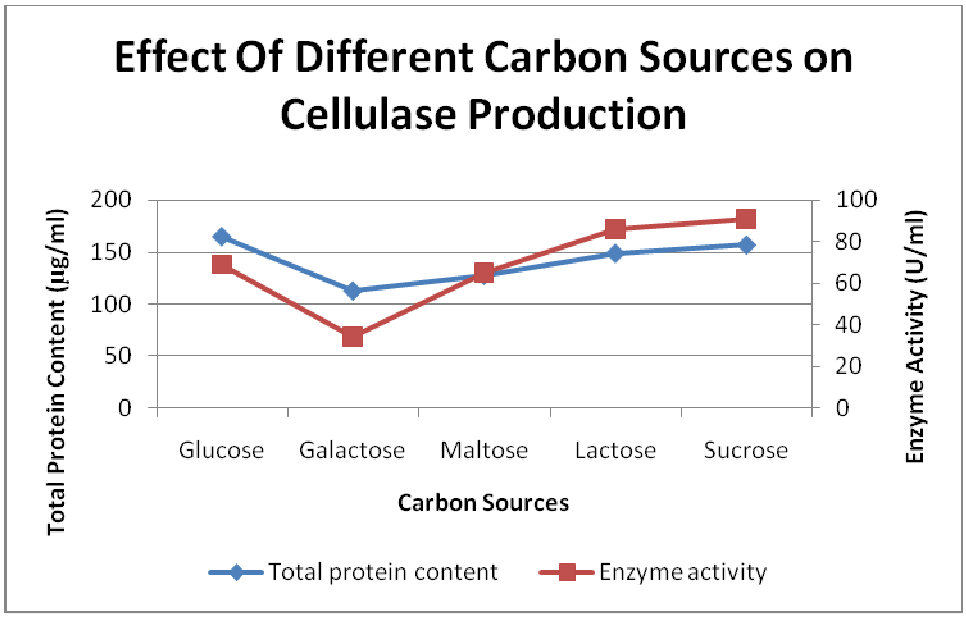

Fig. 4: Effect of Carbon sources on Phytase production

Nitrogen is considered as the secondary energy source for organisms for growth and production of enzymes. Yeast extract was found to be the better nitrogen source for the production of phytase using the strain BPTK4 (Fig. 5). Besides, fungal and bacterial phytases, the optimisation of yeast, *Pichia anomala* was performed by Satyanarayana [45]. His work supported that galactose as best carbon source with peptone as the best nitrogen source and Fe^{2+} served as a key intermediate in enzyme activity. From those it is clear that the simple monosaccharide Glucose with yeast extract were found to be the better carbon and nitrogen sources for the phytase production.

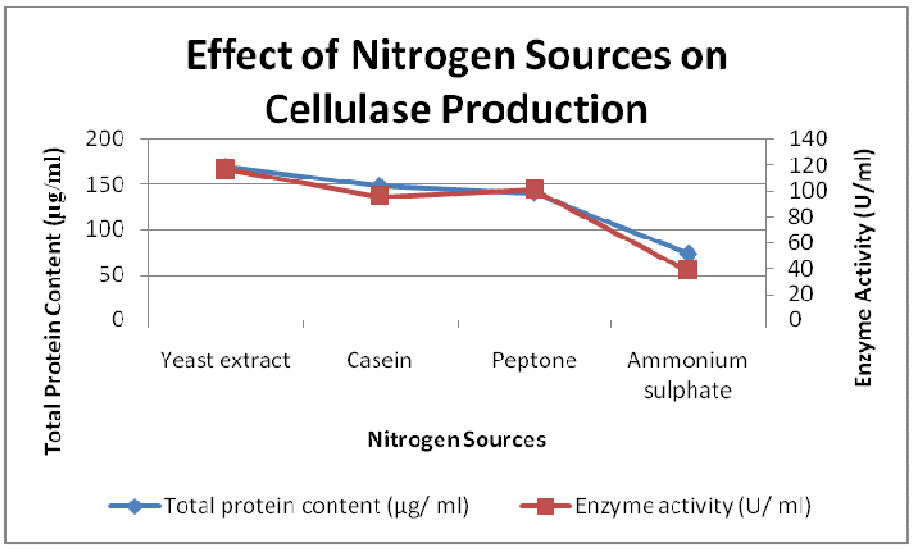

Fig. 5: Effect of Nitrogen sources on Phytase production

The real and beneficial production of phytase was done by using cheap natural substrates and industrial wastes. For the same several natural substrates were tested for their role in phytase production. Out of which, wheat bran is founded to be the best substrate for phytase production using the isolate BPTK4 (Fig. 6).

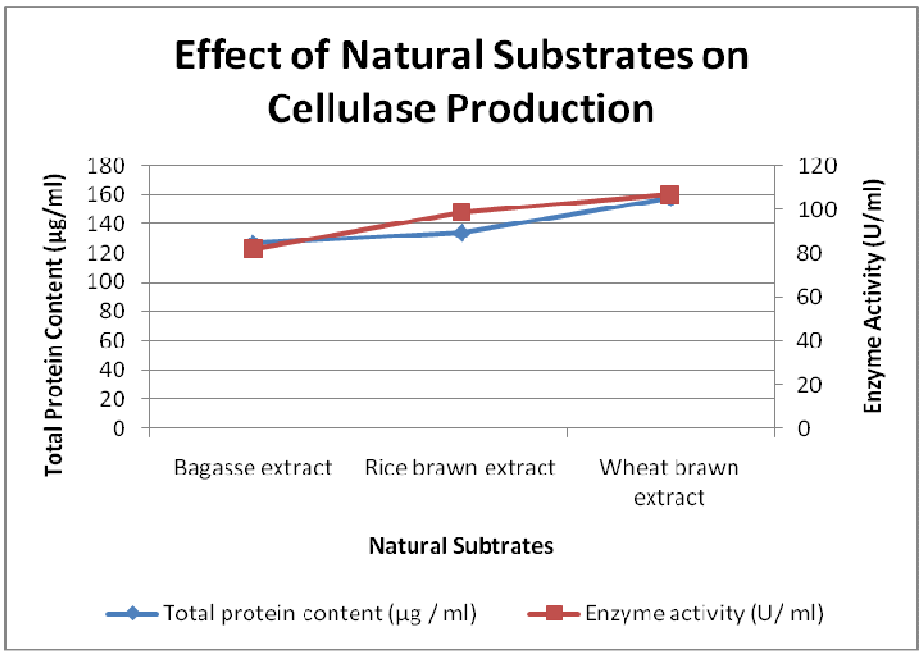

Fig. 6: Effect of Natural substrates on Phytase production

The phytase enzyme produced was extracted and purified using ethanol precipitation and chromatography [27]. Electrophoretic analysis of purified phytase has been carried out. We found a single band with approximate molecular mass of 40 kDa. The homodiameric forms of the crude enzyme, with a molecular mass of 54 kDa have been previously reported *E. pyrococcus abyssi* [47–49]. The SDS-PAGE of *B. stearothermophilus* enzyme showed a single protein band of 32 kDa [50]. The protease isolated was similar to the size of *Escherichia coli* (45 kDa) [36]. Bacterial phytases are found to be smaller than fungal phytases, including those from *Aspergillus niger* (84 kDa) [39].

The zymogram of the present study shows the presence of phytase as green band due to staining with malachite green solution. In 2004, Chang-Chih et al [29] purified phytase using acetone and ammonium sulfate precipitation and partially purified enzyme was visualized by SDS-PAGE and zymogram analysis using Triton-X-100.

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