J. Mycopathol, Res, **50(1)** : 93-96 (2012)

Production of phytase by submerged fermentation

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Received : 28. 04. 2011 RMs Accepted : 20. 12. 2011 Published : 30.04.2012

Extracellular phytase, a phytate hydrolyzing enzyme, was produced from *Bacillus subtilis* by submerged fermentation (SmF) using modified nutrient broth.Various environmental factors affecting phytase production, such as, fermentation time, fermentation temperature, age of inoculum, volume of inoculum and shaking speed were optimized for highest production of phytase. It was found that 10% of 24 hrs old inoculum gave highest phytase activity at 48 hrs of fermentation at 30°C and 140 r.p.m shaking speed.

Key words : Submerged fermentation, phytase, *Bacillus*

INTRODUCTION

Phytate (salt of phytic acid) is the predominant form of phosphorous in cereals, legumes, oilseeds and is also the most frequent form of phosphorous in soil (Ehrlich *et al.*,1993). It chelates vital ions, forms complexes with proteins. Thus, high intakes of phytate cause poor utilization of nutrients and hence it is an antinutrient (Graf,1983).. Moreover, monogastric animals are unable to use phytate phosphorous lacking endogenous phytase activity. As a result, they excrete large amounts of phosphorous into the environment causing pollution (Nasi.,1990). Therefore, the hydrolysis of phytic acid by phytase enzyme (E.C. 3.1.3.26) into less-phosphorylated myo-inositol derivatives in the intestine of monogastric animals is desirable (Kerovuo *et al.*,1998). Many attempts to enzymatically hydrolyze phytic acid have been made to improve the nutritional value of feed and to decrease the amount of phosphorous excreted by animals (Lambrechts *et al.*,1992). There have been reports of partially purified microbial phytase preparations from a variety of microbial species (Ghareib,1990).There are previous reports on production and partial purification of phytase from *Bacillus subtilis* (Powar and Jagannathan,1982 ; Choi *et al.*,1999 ; Shimizu 1992). Phytase gene from *Bacillus amyloliquefaciens* has been cloned in *Bacillus* expression vector for large scale production of phytase and the yield has been 100-fold higher than wild strain of *Bacillus amyloliquefaciens* DS11 (Kim *et al.*,1999). It has been reported that the analysis of phytate hydrolysis by *Bacillus* phytase revealed some novel reaction mechanism of phytase. The enzyme seemed to follow two alternative pathways for the hydrolysis of phytic acid, resulting into two different myo-inositol triphosphate end products (Kerovou *et al.*, 2000). There is report of phytase production by recombinant *Bacillus subtilis* BD 170 harboring a plasmid pGT44 [phy C] carrying the phytase gene (phy C) and a phosphate depletion inducible pst-promoter (Vuolanto *et al.*, 2001).

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MATERIALS AND METHODS

Microorganism

Bacillus subtilis, maintained in Nutrient Agar slant by monthly subculturing and stored at 4°C.

Inoculum preparation

Inoculum was prepared by inoculating 30 ml sterile fermentation medium with two loopful of *B. subtilis* fresh slant culture and incubated at 30°C and 140 r.p.m for 24 hrs. Concentration of cells in the inoculum was 10^8 cells/ml.

Fermentation Medium

Composition (g/L) : Peptone, 5 ; NaCl, 5 ; Beef Extract, 3 ; Glucose,10;.

Chemicals & reagents

All the chemicals and reagents used were commercially available and of analytical grade.

Fermentative production of phytase

Phytase was produced from *Bacillus subtilis* by submerged fermentation. Submerged fermentation was done by shake flask culture method taking 50 ml fermentation medium in 250 ml Erlenmeyer flask. Medium was sterilized by autoclaving at 121°C temperature and 15 lb pressure for 15 minutes, then, after cooling it to room temperature, cell suspension was added to it aseptically on 10% (v/v) basis (final concentration 10^7cells/ml) and mixed well. Then the flasks were kept at 30°C in shaker incubator at 140 r.p.m. for up to 72 hrs. Samples were removed at every 24 hrs interval and cell free fermentation broth was isolated by centrifugation at 10,000 r.p.m. for 10 minutes at 4°C. It was used as crude enzyme. This crude enzyme was used to determine enzyme activity and residual sugar. Cellular growth was also studied by measuring dry cell weight.

Different environmental parameters, such as, time of fermentation, temperature of fermentation, culture volume, age of culture, shaking speed, were optimized so as to get maximal yield of phytase.

Phytase activity assay

Enzyme assay was performed as per Sigma protocol; Enzyme-substrate reaction mixture contains Deionized water, 0.4 ml ; 0.2 M Sodium acetate (pH-5), 2 ml ; 0.1 M Magnesium sulphate, 0.1 ml ; 6.82 mM Sodium phytate, 1 ml ; Crude enzyme, 1 ml. The reaction was carried out at 55°C for 30 minutes and reaction was stopped by 10% of 6.1 N Trichloroacetic acid. The inorganic phosphate released hydrolytically during the enzyme-substrate reaction was measured by King's method (King,1932). The optical density values obtained were converted to concentrations of inorganic phosphate using standard curve of potassium phosphate.

One unit of enzyme is defined as the amount of phytase that liberates 1 micromole of phosphate per minute, under the assay condition.

Measurement of residual Sugar

Residual sugar of the cell free fermentation broth was measured using Dinitrosalicylic acid. A standard curve was prepared with different concentrations of glucose and using this curve, residual sugar concentrations were determined from the optical density values obtained.

Measurement of cell weight

After centrifugation, cell pellet was collected on previously weighed aluminium cup and washed well with distilled water and then kept in hot air oven at 80°C till constant weight.

RESULTS AND DISCUSSION

The time course of fermentation, cell growth and utilization of sugar were studied and presented in Fig 1. It was observed that highest enzyme production occurred at 48 hrs of fermentation. Dry cell weight values and residual sugar level showed that maximum cell growth as well as utilization of sugar occurred at 48 hrs of fermentation.

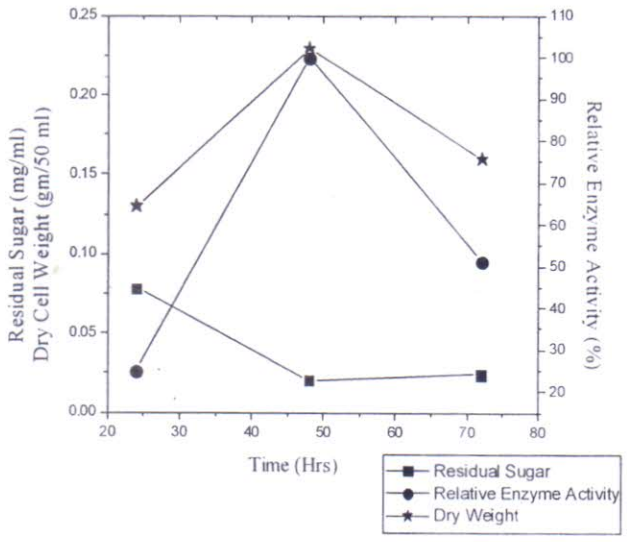

Fig. 1: Enzyme activity, cell growth & residual sugar content at different hrs of fermentation (0.77 U/ml phytase activity was considered as 100%).

In Fig. 2, enzyme activity, cell growth and residual sugar content with respect to different ages of inoculum were presented. It was found that the highest enzyme production as well as cell growth and utilization of sugar occured at 48 hrs of fermentation using 24 hrs old inoculum.

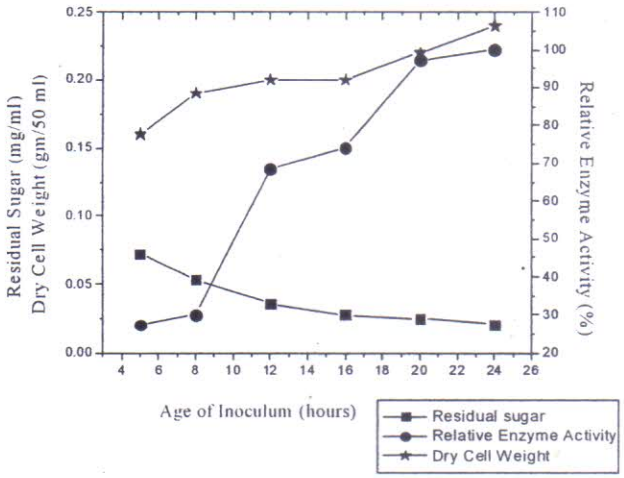

Fig. 2: Enzyme activity, cell growth & residual sugar content with respect to different age of inoculum (0.73 U/ml phytase activity was considered as 100%).

In Fig 3, enzyme activity, cell growth and residual sugar content with respect to different volumes (%) of inoculum were presented. It was found that the highest enzyme production as well as cell growth and utilization of sugar occurred at 48 hrs of fermentation when 10% of 24 hrs old culture was used as inoculum.

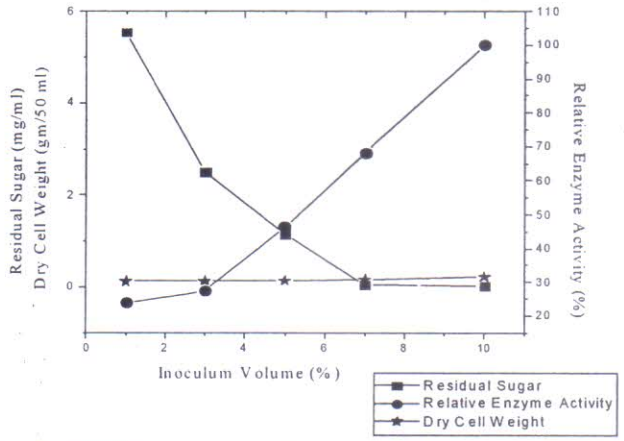

Fig. 3: Enzyme activity, cell growth and residual sugar content at different volume of inoculum (0.75 U/ml phytase activity was considered as 100%).

It is seen from Fig. 4 that different temperatures affected enzyme production, cell growth and utilization of sugar. It was found that highest enzyme production as well as cell growth and utilization of sugar occurred at 30°C.

It was also observed that the shaking speed (r.p.m) influenced fermentation and the highest enzyme production as well as cell growth and utilization of sugar occurred at 140 r.p.m as observed from Fig.5.

In our present study, the yield of phytase from *Bacillus subtilis* was encourageous (0.77 U/ml/min) compared to the reports of other *Bacillus* sp. (0.2 U/ml/min) (Choi *et al.*, 1999); (0.24 U/ml/min) (Power *et al.*, 1982). There are differences at fermentation hours where maximum yield of phytase was obtained; our strain gave highest production at 48 hrs of fermentation, whereas, other strains required more fermentation time - *Bacillus* sp KHU-10, 96 hours (Choi *et al*, 1999 and 2001); *Bacillus subtilis* 2712, 72 hours (Power *et al.*, 1982) and *Bacillus subtilis* N-77, 120 hrs (Shimizu, 1992). These differences might be due to different experimental conditions. The phytase obtained from our *Bacillus subtilis* strain showed maximum activity at 55°C and pH 5.0 and these features are common to phytases from other *Bacillus* sp. (Choi *et al.*, 2001). Further works on production and characterization of phytase are in progress.

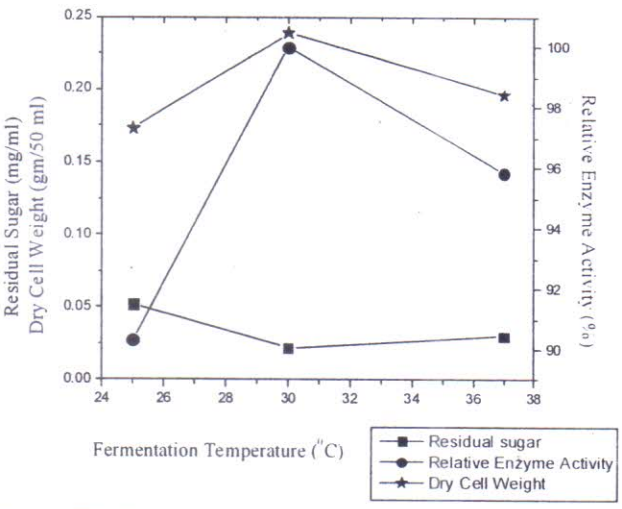

Fig. 4: Enzyme activity, cell growth & residual sugar content with respect to different fermentation temperature (0.72 U/ml phytase activity was considered as 100%).

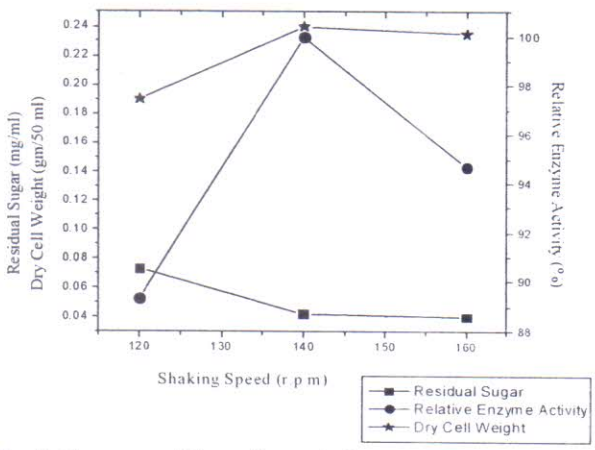

Fig. 5: Enzyme activity, cell growth & residual sugar content at different shaking speed (0.75 U/ml phytase activity was considered as 100%).

ACKNOWLEDGEMENT

Authors gratefully acknowledge the financial support obtained from "UGC-CAS I", New Delhi, for carrying out the study.

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