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Extraction, purification and application of phytase

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ABSTRACT

Microbial phytases can improve the nutritional quality of food by hydrolyzing phytic acid and releasing phosphate groups. *Aspergillus niger* producing the enzyme phytase was isolated from soil sample. The media, pH and temperature requirement for maximum production and activity of phytase was standardized. The enzyme was purified by acetone precipitation and NATIVE PAGE and immobilized using sodium alginate entrapment. The enzyme was used to treat sprouted legumes to increase the nutritive value.

KEYWORDS

Phytase;
Aspergillus niger;
Enzyme purification.

INTRODUCTION

The food industry plays an important role in our present day society. As we know without the food industry, urbanization would not have been possible. Presently, the food industry employs biobased process which account 95% of the industrial applications of living systems. There can be little doubt that the new biotechnologies are having and will continue to have significant impact on the practice of food processing, agriculture and related research and development.

The nutritional quality of food depends on the total amount of nutrients present in the food consumed, as well as the levels of antinutritive factors. Some nutritional stress factors present in foods are Phytates, Oxalates, Avidin, etc. Traditional processing methods can improve nutritional quality by increasing food palatability and digestibility or increasing nutrient availability by destroying toxic factors or minimizing their effects. Enzymatic treatment can reduce the level of nutritive stress factors in food. For example, raffinose and stachyose present in soyabeans cause flatulence in humans and animals because there is no alpha- galactosidase activity in the mammalian intestine, thus decresing the intake of soy products. Treatment of soyabean meal with invertase and alpha- galactosidase resulted in the conversion of these oligosaccharides to monosaccharides[9].

Phytase is an enzyme that hydrolyzes phytic acid to myoinositol and phosphoric acid in a stepwise manner forming myoinositol phosphate intermediates. Phytic acid, major storage form of phosphorus in the mature seeds of both monocot and dicot plants, acts as an antinutritional factor. The International union of Biochemistry (1979) listed two phytases: (1) 3-phytase,EC 3.1.3.8 which hydrolyzes the ester bond at the 3 position of myoinositol hexaphosphate to D-myoinositol 1,2,4,5,6-pentakisphosphate and orthophosphate and (2) 6-phytase, EC 3.1.3.26 which hydrolyzes the 6- position of myoinositol hexakisphosphate to D-myoinositol 1,2,3,4,5-pentakisphosphate and orthophosphate.

Phytase activity has been detected in *Aerobacter aerogens, Klebsiella aerogens*, culture filterates of *Bacillus subtilis* and also Lactobacillus and Streptococcus.There are 84 fungi from 25 species that

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are used for phytase production. Of all the organisms surveyed, *Aspergillus niger* NRRL 3135 produced the most active extracellular phytase. Phytases thus extracted has various applications in (1) Animal feed industry (2) feed trials (3) pollution abatement (4) Food industry (5) Antioxidant etc[8].

MATERIALS AND METHODS

Isolation of the organism

The soil sample was collected and about 1gm of soil was weighed and mixed with 100ml of distilled water (stock solution) was prepared. 10 test tubes were taken and marked as (10^{-1} to 10^{-10}) 9ml of distilled water was added, to the first tube 1ml of the stock solution was added, similar method was performed up to 10^{-10}. The last four dilutions ($10^{-6}$to 10^{-10}) was plated on to Nutrient agar (For isolation of Bacteria) and Potato dextrose agar (For isolation of Fungi). The Nutrient agar plates were incubated at 37°C for 24hrs. The Potato dextrose agar was incubated at room temperature for 3 to 5 days. The different colonies observed were stained using Gram Staining technique and Lacto phenol cotton blue staining, based on the colony morphology and microscopic examination *Aspergillus niger* was isolated.

Preparation of corn starch media

The media required for the production of Phytase enzyme was Corn starch media.about 3gms of Corn was weighed and added to 10ml of Distilled water and autoclaved The *Aspergillus niger* culture was inoculated in the media and was incubated at room temperature for about 3 to 5 days. The medium was filtered and the filtrate was assayed for the production of enzyme.

Estimation of phosphate-fiske and subbarow method

Principle

The Phosphomolybdic acid formed by the addition of acid molybdate solution to the Phosphate it is reduced by the addition of addition of 2,4- amino napthol sulphonic acid (ANSA) reagent to produce a blue colour the intensity of colour is proportional to the amount of Phosphate present[1].

Materials

10N Sulphuric acid, Molybdate solution, aminonapthol sulphonic acid (ANSA) reagent.

Procedure

The phosphate content of corn (control) was estimated by adding 1ml of Ammonium molybdate and 0.4ml of ANSA.The filtrate obtained after inoculation of the culture was also estimated using 1ml of Ammonium molybdate and 0.4ml of ANSA. The optical density was measured at 660 nm by colorimetry.

pH variation

To standardize the optimum pH at which the organism grows and produces the enzyme, Corn starch media was prepared and the pH was adjusted to 4.5, 5.5, 6.5 and 7.5 using acid (Hydrochloric acid) or base (Sodium hydroxide). The media was autoclaved and *Aspergillus niger* was inoculated, and incubated at room temp for about 3 to 5 days. The medium was filtered and estimated for the amount of Phosphate by Fiske and Subbarow method.

Temperature variation

To standardize the optimum temperature for the growth of the organism and the production of the enzyme, the corn starch media was prepared and the organism was inoculated, then the flasks were incubated at various temperatures (17, 27, 37 and 47) for 3 to 5 days. The medium was filtered and assayed for the amount of Phosphate- Fiske and Subbarow method.

Substrate variation

To the standardize activity of the enzyme, various substrates such as Corn, wheat, oats, rice were used. About 3gm of each were weighed and added to 10ml of distilled water and autoclaved. Then they were inoculated with *Aspergillus niger,* and incubated for 3 to 5 days. The medium was filtered and assayed for the amount of Phosphate- Fiske and Subbarow method.

Purification of the enzyme

The enzyme was purified by Acetone precipitation and later by Gel filtration chromatography. And then the purified enzyme was run on the gel by performing native polyacrylamide gel electrophoresis. The optimum pH and temperature of the purified enzyme was also

determined[4].

Enzyme immobilation

The purified enzyme was immobilized for future use by Sodium Alginate Gel Entrapment method[13].

Application of enzyme[14]

The sprouted grains were mixed with enzyme and incubated for 24 hours and the amount of phosphate before and after the treatment was estimated.

RESULT AND DISCUSSION

Isolation of the organism

Lacto phenol cotton blue staining was performed and *Aspergillus niger* was isolated based on Macroscopic and microscopic examination. The *Aspergillus niger* spores were also isolated from bread sample.

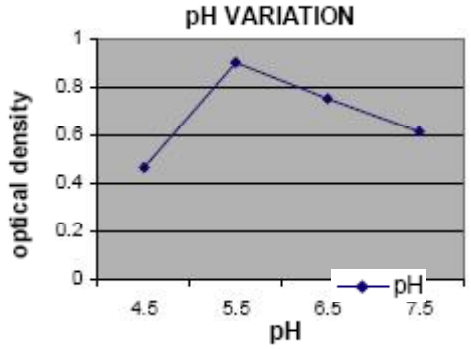

Figure 1 :Effect of pH on enzyme production

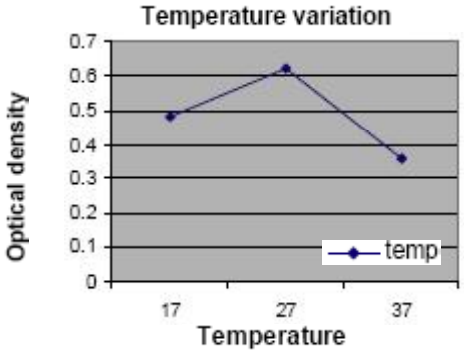

Figure 2 : Effect of temperature on enzyme production

Assay for enzyme production

For the production of enzyme the organism-*Aspergillus niger* was inoculated in Corn starch media. After 3-5 days of incubation, the medium filtrate was assayed for the amount of Phosphate by Fiske and Subbarow method.Increase in the phosphate content was observed due to the hydrolysis of phytic acid present in Corn. To confirm this standard graph was plotted with varying phosphate concentration (4-20μg). Within the standard value unknown was extrapolated to determine the amount of phosphate. In comparison with the amount of phosphate in corn, the filtrate assayed after inoculation of the organism the amount of phosphate was found to increase. The concentration of phosphate in the given solution was found to be 18.40 mg of phosphate, when compared with the control it was estimated to be 10.50mg of phosphate(TABLE 1).

pH variation (Figure 1)

The organism increased the production of phytase with increase in pH and maximum enzyme was produced at pH 5.5 as the enzyme may be an acidic protein. Similar results have been reported where the enzyme was produced maximum at acidic range of pH.

Temperaturature variation (Figure 2)

The organism increased the production of phytase with increase in temperature and maximum enzyme was produced at 27^{0}C.Similar results have been reported where the enzyme was produced maximum at lower temperature[6].

Substrate variation (Figure 3)

Different substrates were tried to help the growth of the organism and production of phytase and when

TABLE 1 : Estimation of phosphate-fiske and subbarow method

S.no.	Reagents	B	S_1	S_2	S_3	S_4	S_5	U_1	U_2
1.	Volume of phosphate (ml)	-	0.5	1.0	1.5	2.0	2.5	-	-
2.	Concentration of phosphate(μg)	-	4	8	12	16	20	-	-
3.	Volume of unknown(ml)	-	-	-	-	-	-	0.5	1.0
4.	Volume of water(ml)	4.6	4.1	3.6	3.1	2.6	2.1	4.1	3.6
5.	Volume of ammonium molybdate (ml)	1	1	1	1	1	1	1	1
6.	Volume of ANSA	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4
	Incubate for 10 minutes in room temperature and read at 660nm.								
7.	Optical density at 660nm	0.00	.49	0.50	0.56	0.66	0.77	0.54	0.63

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tried it was found that the Corn gave maximum amount of the enzyme[5].

Purification of phytase

Acetone precipitation is included in the procedure for obtaining crude enzyme. The obtained phytase enzyme preparation was concentrated by Gel filtration and kept in the refrigerator at 5^0C[4,11].

Figure 3 : Growth on different substrate

Figure 4 : Enzyme treated sprouts

TABLE 2: Substrate variation

S.no	Substrate	Optical density
1.	oats	0.36
2.	corn	0.72
3.	wheat	0.58
4.	rice	0.50

Native page

Native page was performed using 10% acryl amide gel and the proteins were stained with Coomassie brilliant blue. The band for the sample was found to correspond with marker at 62KDa[12].

Effect of pH on phytase

The stability of enzyme at different pH was examined. It was found that the enzyme activity was best at pH 5.5 clearly indicating that the enzyme works better in acidic pH[7].

Effect of temperature on enzyme

The stability of the enzyme was examined at different temperature (17^0C, 27^0C, 37^0C and 47^0C) in phosphate buffer at constant pH. When the graph was plotted the peak value (maximum activity) was observed at 27^0C[3].

Immobilisation of enzyme by gel entrapment

Enzyme was successfully immobilized by gel entrapment via Sodium Alginate. This is an efficient method and it can be used for further large scale application. The immobilized enzyme activity was assayed by estimation of phosphate-Fiske and Subbarow method[10].

Application of enzyme

The sprouted gram was treated with the enzyme and was left over night. It was found that the amount of phosphate was increased when compared with control (without treatment with the enzyme). The results indi-

TABLE 3: Effect of pH on enzyme activity

Sr.no.	Reagents	B	T_1	C_1	T_2	C_2	T_3	C_3	T_4	C_4
			4.5		5.5		6.5		7.5	
1	Buffer (ml)	-	1	1	1	1	1	1	1	1
2	Substrate (ml)	1	1	1	1	1	1	1	1	1
3	Sodium chloride(ml)	1	1	1	1	1	1	1	1	1
	Incubate at room temperature for 10 minutes									
4	Enzyme (ml)	-	0.2	-	0.2	-	0.2	-	0.2	-
	Incubate at room temperature for 10 minutes									
5	DNSA (ml)	2	2	2	2	2	2	2	2	2
6	Enzyme (ml)	-	-	0.2	-	0.2	-	0.2	-	0.2
	Incubate in boiling water bath for 10 minutes									
7	Sodium potassium tartarate (ml)	1	1	1	1	1	1	1	1	1
	Incubate for 5 minutes at room temperature									
8	Optical density	0 .0	0.14	0 .0	0.32	0 .0	0.22	0.02	0.11	0.0
9.	Difference in OD		0.14		0.32		0.20		0.11	

TABLE 4: Effect of temperature on enzyme activity

	Reagents	B	T_1	C_1	T_2	C_2	T_3	C_3	T_4	C_4
			17		27		37		47	
1	Buffer (ml)	-	1	1	1	1	1	1	1	1
2	Substrate (ml)	1	1	1	1	1	1	1	1	1
3	Sodium chloride(ml)	1	1	1	1	1	1	1	1	1
	Incubate at room temperature for 10 minutes.									
4	Enzyme (ml)	-	0.5	-	0.5	-	0.5	-	0.5	-
	Incubate at room temperature for 10 minutes									
5	DNSA (ml)	2	2	2	2	2	2	2	2	2
6	Enzyme (ml)	-	-	0.5	-	0.5	-	0.5	-	0.5
	Incubate in boiling water bath for 10 minutes									
7	Sodium potassium tartarate (ml)	1	1	1	1	1	1	1	1	1
	Incubate for 5 minutes at room temperature									
8	Optical density	0	0.12	0.02	0.46	0.01	0.23	0.03	0.18	0.03
9	Difference in OD		0.10		0.45		0.20		0.15	

cated that the mineral composition had greatly been increased by the enzyme treatment[2].

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