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Production of Phytase by Three Thermophilic Fungi

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Abstract: Production of phytase by three thermophilic fungi, *Thermomyces lanuginosus*, *Talaromyces luteus* and *Rhizomucor pusillus* under different cultural conditions was assessed. Temperature of 45°C, pH-6.0 were optimum for phytase production by the all three fungi under investigation. Carbon and nitrogen sources for production of phytases by the three thermophilic fungi varied with the fungus. When *T. lanuginosus* opted for D-glucose followed by D-fructose, *T. luteus* preferred D-glucose, D-mannose and mannitol for production a phytase. On the other hand, *R. pusillus* produced maximum phytase during its growth on mannitol and maltose as carbon source. L-asparagine, L-arginine and L-asparatic acid were preferred nitrogen sources for production of phytase by *T. lanuginosus*. On the other hand *T. luteus*, opted for L-asparagine, L-glutamic acid and L- glycine for the activity of phytase. *R. pusillus* produced maximum phytase in medium containing L-argine, L-asparagine and L-asparatic acid.

Keywords: thermophilic fungi, phytase, temperature, pH, carbon and nitrogen source

Introduction:

Phytase (myo-inositol hexakisphosphate hydrolases), causes release of phosphate from phytic acid, is one of the considerable importance in animal nutrition, environmental protection and biotechnology [1]. Phytic acid which is a major form of phosphorus present in the bound form in the seed based feed cannot be utilized by monogastric animals like pigs and poultry. Further, most of the phytic acid is an anti-nutrient, which complexes with protein by variety of divalent metallic cations and decreases the availability of these nutrients. Therefore, phytase has received increased

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attention in animal feeds which upgrades feed quality by liberating phosphorus, proteins and minerals. Phytases required for commercial feed production must be thermostable with high specific activity and active over a wide range of pH and temperature [2]. Though mesophilic fungi have been reported to produce phytases useful in feed production [3,4], only limited information is available on production of phytases by thermophilic fungi [5]. Therefore, in the present investigations production of phytases by three thermophilic fungi under different environmental conditions was investigated and discussed in this communication

Materials and Methods:

Isolation and identification of thermophilic fungi:

An extensive and intensive survey of thermophilic fungi substrates such as coalmine soil, composting pits, birds nests, herbivores animals and soils were surveyed for the presence of thermophilic fungi. different samples were collected aseptically in a sterile polythene bags and analyses within 24 hrs.

The thermophilic fungi were isolated by the paired petriplate method as suggested by Cooney and Emerson [6]. The samples thus obtained were subjected to serial dilution and 0.1 ml of the diluted samples was placed in a sterilized petriplates and 15-20 ml poured aseptically and covered immediately covered with petriplates and allowed to cool to room temperature. the paired petriplates was dixed with cellophane tape and incubated at $40 \pm 1^\circ\text{C}$ in a incubator. the fungal colonies arising after 4-6 days were pickedup for isolation and purification. they were identified with the help of manual. they were categorized into thermophilic and thermotolerant based on the temperature requirment.

Phytase production:

Influence a different media on growth and phytase production was studied by growing three organisms in the following media

1. Yeast Extract Starch medium (A): Yeast extract 5.0g, Starch 15g, MgSO_4 0.5g, K_2HPO_4 0.5g, agar agar 20 g and distilled water 1 liter).
2. Yeast Extract Starch medium (B): Yeast extract 5.0g, Starch 15g, MgSO_4 0.5g, K_2HPO_4 0.5g, agar agar 20 g and distilled water 1 liter). + 0.1 % Ca Phytate.
3. Yeast Extract Glucose medium (C): Yeast extract 5.0 g, Glucose 15g, MgSO_4 0.5g, K_2HPO_4 0.5g, agar agar 20 g and distilled water 1 liter).
4. Yeast Extract Glucose medium (D): Yeast extract 5.0 g, Glucose 15g, MgSO_4 0.5g, K_2HPO_4 0.5g, agar agar 20 g and distilled water 1 liter)+0.1% Ca phytate.
5. Modified Czepek dox (E): Glucose 2.0g, L-asparagine 10.0g, K_2HPO_4 1.52, KCl 0.52g, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.52g, CuNO_3 , 3 H_2O trace, ZnSO_4 trace, Agar Agar 20g and 1 liter distilled water + 0.1% Ca phytate.

6. PSM: Phytase Screening Medium (F): 5g calcium phytate; 10g sucrose, 2g (NH₄)₂SO₄, 3g tryptone, 2g yeast extract, 0.5g KCl, 0.5g MgSO₄, 0.01 g MnSO₄ .5H₂O, 0.01g FeSO₄, 1 g triton X-100.

Factors influencing phytase production:

Effect of pH on production of phytase was studied by adjusting the pH of the medium by adding HCL, NaOH so as to get pH of the basal medium pH 4.0, 5.0, 6.0, 7.0, 8.0 and 9.0. Similarly effect of incubation, temperature was studied by incubating the fungal inoculated cultures at 35, 40, 45, 50 and 55°C. Influence of carbon and nitrogen source was studied by substituting gulcose/ potassium nitrate source separately so as to get equal amount of carbon/nitrogen concentration respectively.

Twenty eight fungal strains covering 21 thermophilic and 7 thermotolerant isolated from different thermogenic substrates according to Cooney and Emerson [6] were screened for phytase production on solidified PSM medium (Phytase Screening Medium) with calcium phytate as carbon source. However, detailed investigations were carried out on monosporic cultures of *T. lanuginosus*, *T. luteus* and *R. pusillus* isolated from birds nest, zoo wastes and coalmine soils respectively. The fungi were grown in 25 ml medium contained in Erlenmeyer conical flask for 8 days at 40±2°C with initial pH 6.0. The experiment was carried out in triplicate. At the end of 8 days incubation period, the cultures were harvested on previously dried and weighed Whatman filter paper No. 42. The filter papers along with mycelial mat were dried at 65-70°C in an oven for 48 hrs. The filter papers along with mycelial mat were cooled to room temperature in desiccator for 24 hrs and weighed to a constant weight in an analytical balance. The mycelial mat weight was determined and an average of three values was taken as standard. The culture filtrate thus obtained was dialysed overnight against distilled water and employed as an enzyme. The other details are as described earlier [7,8,10]. The phytase activity was assayed as suggested by Wyas et al. [9].

Assay of phytase:

Phytase activity was measured in an assay mixture containing 0.5% sodium phytate and 0.2 M sodium acetate (pH 5.0) and 1.0 ml dialyzed enzyme sample. The reaction was stopped by adding equal volume of 15% trichloroacetic acid after 30 min of incubation at 37 °C. The liberated phosphate ions were quantified by mixing of assay mixture with 9 ml of 0.76M HSO₄, 2.5% ascorbic acid and 0.06% ammonium molybdate. The reaction of after 20 min incubation at 30°C, absorbance was measured at 820nm in spectrophotometer. One phytase unit was defined as the activity that released 1 ml of inorganic phosphorus from sodium phytate per 1 minute under assay conditions.

Results and discussion:

Out of 28 species belonging to 21 thermophilic and 7 thermotolerant fungi screened for production of phytase revealed that except *H. fuscoatra*, which did not produce phytase, all other fungi found to be have potential for production of phytase. However, the degree of phytase

production varied with the fungus. *T. lanuginosus* and *R. pusillus* proved to be good producers, while *T. luteus* was poor producer. Production of phytase by these three thermophilic fungi under different environmental conditions was investigated.

i. Influence of medium

Out of six media tried, the yeast extract starch (medium B) containing calcium phytase was most suitable for production of phytase for all three fungal species under study (fig.1). The medium F was the next preferred medium. In general medium containing phytic acid was conducive for phytase production, suggesting its adaptive nature. Similarly, Chadha et al. [10] have also recorded increased production of phytase in medium containing calcium phytate. Eight days incubation was optimum for enzyme production by all present three thermophilic fungi. Medium H was poor substratum for the all the three fungi understudy which may be attributed to the presence of starch.

Fig.1. Effect of different synthetic media on phytase production by three thermophilic fungi

ii. Influence of pH and temperature

pH 6.0 was optimum for production of phytase for all the three species under study. Production of phytase decreased both with increase of acidity or alkalinity. *T. luteus* failed to grow at pH 9.0 (table 1). Incubation temperature of 45°C was optimum for production of phytase by the fungi under investigation. Production of phytase decreased progressively with the increase of incubation temperature (table 2). Singh and Satyanarayana [11] also recorded maximum phytase production at 45 °C by the fungi studied by them.

Table 1: Effect of pH on growth and phytase*production (in 8 days) by three thermophilic fungi

Name of the Organism	pH					
	4.0	5.0	6.0	7.0	8.0	9.0
<i>T. lanuginosus</i>						
Dry weight (µg/ml)	177.2	181.3	188.4	194.2	166.2	145.2
pH	4.7	5.2	6.3	5.8	7.6	7.7
Phytase (in units)	18.0	38.0	65.0	60.0	38.0	22.0
<i>T. luteus</i>						
Dry weight (µg/ml)	--	160.2	177.2	172.3	155.3	--
pH	--	5.7	5.3	5.6	5.3	--
Phytase (in units)	--	36.0	58.0	48.0	28.0	--
<i>R. pusillus</i>						
Dry weight (µg/ml)	213.7	247.5	265.8	233.6	210.2	155.6
pH	4.4	5.3	6.4	6.3	7.5	7.6
Phytase (in units)	32.0	48.0	72.0	40.0	14.0	16.0

*1 unit of phytase activity (IU) is expressed as the amount of enzyme that liberates 1 mole phosphorus per minute under assay conditions.

Table 2: Effect of temperature on growth and phytase*production (in 8 days) by three thermophilic fungi

Name of the Organism	Temperature (°C)					
	35	40	45	50	55	60
<i>T. lanuginosus</i>						
Dry weight (µg/ml)	176.2	177.3	189.3	165.3	153.8	145.2
pH	6.4	6.5	6.3	6.4	7.6	7.7
Phytase (in units)	28.0	40.0	60.0	48.0	28.0	22.0
<i>T. luteus</i>						
Dry weight (µg/ml)	158.3	164.4	182.3	155.2	138.7	--
pH	6.6	5.7	5.3	5.8	5.3	--
Phytase (in units)	38.0	55.0	55.0	38.0	28.0	--
<i>R. pusillus</i>						
Dry weight (µg/ml)	213.7	245.2	268.3	245.3	231.2	155.6
pH	6.1	6.1	6.4	6.3	6.5	7.6
Phytase (in units)	34.0	48.0	68.0	52.0	34.0	16.0

*1 unit of phytase activity (IU) is expressed as the amount of enzyme that liberates 1 mole phosphorus per minute under assay conditions

iii. Influence of carbon source:

D-glucose followed by D-mannose was preferred carbon sources for the production of phytase by *T. lanuginosus*. Borges et al. [12] also recorded maximum phytase production in the presence of glucose as carbon source by *M. racemosus*. Enzyme secretion was minimum in medium containing succinic acid followed by D-ribose. Rest of the carbon sources induced intermediate amount of phytase production. D-glucose followed by maltose and mannitol were favorable carbon sources for the production of phytase by *T. luteus*, while D-xylose and D-ribose were poor substrates (table 3). Rest of the carbon sources supported intermediate degree of phytase production by *T. luteus*. Mannitol followed by maltose and sucrose were the good carbon sources for production of phytase by *R. pusillus*. Sucrose and starch were almost equal in induction of phytase in *R. pusillus*. L-sorbose, D-ribose and D-galactose were least preferred carbon sources for production of phytase by *R. pusillus*. Vats and Benerjee [13] reported starch as a better carbon source for phytase production by *Aspergillus niger*. The vegetative growth of *T. lanuginosus* and *T. luteus* was maximum in medium containing glucose, while *R. pusillus* preferred starch for mycelial growth.

Table 3: Influence of carbon sources on growth and Phytase*production (in 8 days) by three thermophilic fungi

Carbon source	<i>T. lanuginosus</i>			<i>T. luteus</i>			<i>R. pusillus</i>		
	Dry wt (µg/ml)	pH	phytase (in units)	Dry wt (µg/ml)	pH	phytase (in units)	Dry wt (µg/ml)	pH	phytase (in units)
D-glucose	198.5	6.4	68.0	180.7	6.2	58.0	245.3	6.2	42.0
D-fructose	188.4	6.3	54.0	171.5	6.4	44.0	242.8	6.6	48.0
D-galactose	175.3	6.4	48.0	168.4	6.2	48.0	234.8	6.4	36.0
D-mannose	178.3	6.4	60.0	178.5	6.3	56.0	233.8	5.8	44.0
L-sorbose	158.5	6.4	48.0	172.5	6.3	48.0	224.7	6.2	32.0
D-ribose	158.4	6.3	38.0	162.4	6.2	42.0	228.6	6.2	34.0
D-xylose	158.2	6.2	42.0	158.5	5.4	36.0	223.4	6.2	38.0
Sucrose	177.3	6.3	54.0	166.8	6.4	48.0	244.8	6.2	60.0
Maltose	165.2	6.2	58.0	166.4	6.2	56.0	242.6	6.1	66.0
Lactose	172.6	6.2	52.0	166.2	6.2	44.0	241.5	6.2	56.0
Citric acid	--	--	--	--	--	--	186.3	3.4	36.0
Succinic acid	134.2	5.2	22.0	146.8	4.7	42.0	191.6	3.8	32.0
Mannitol	178.4	6.2	52.0	178.2	6.0	52.0	158.3	6.1	70.0
Glycerol	158.4	6.2	48.0	162.4	6.2	46.0	243.2	5.8	48.0
Starch	168.3	6.6	52.0	162.8	6.2	44.0	247.9	6.3	60.0

*1 unit of phytase activity (IU) is expressed as amount of enzyme that liberates 1 mole phosphorus per minute under assay conditions

iv. Influence of nitrogen source:

L-asparagine induced maximum phytase in *T. lanuginosus*, while L-arginine and L-aspartic acid were next preferred nitrogen source for the production of phytase. L-methionine and L-histidine were responsible for poor growth of *T. lanuginosus*. Amine form of nitrogen was superior source of nitrogen over the acid form for all the three fungi under investigation. Ammonium compounds were inferior source of nitrogen for induction of phytase in all the three fungi under study. L-asparagine followed by L-glutamic acid and L-glycine were responsible for maximum production of phytase by *T. luteus*, while ammonium chloride, L-methionine and ammonium nitrate were poor nitrogen sources. Rest of the nitrogen sources supported intermediate amount of phytase production by *T. luteus*. *R. pusillus* produced maximum phytase in medium containing L-arginine, L-asparagine and L-aspartic acid, while ammonium salts were poor nitrogen sources for phytase production (table 4). Ashes and Duvnjak [14] recorded significant influence of nitrogen source on phytase production by the fungi studied by them. Response of all the three fungi was uniform towards ammonium nitrate. Tyrosine was a good source of nitrogen for *T. luteus* and *R. pusillus*. On the other hand *Aspergillus niger* produced maximum phytase on peptone followed by ammonium nitrate and ammonium sulphate [15]. The vegetative growth of *T. lanuginosus* and *T. luteus* was maximum in medium containing L-asparagine, while *R. pusillus* recorded maximum growth in medium containing L-arginine as a nitrogen source. Eight days of incubation period was optimum for production of phytase on all the nitrogen sources tried. The pH changes were minimum in all the nitrogen sources tried and the final pH was near neutral.

The present studies on thermophilic fungi confirmed the observations of [3,15,16] on *Myceliophthora thermophila*, *Aspergillus fumigatus* and *Mucor racemosus* respectively.

Table 4: Influence of nitrogen sources on growth and Phytase *production (in 8 days) by three thermophilic fungi

Nitrogen source	<i>T. lanuginosus</i>			<i>T. luteus</i>			<i>R. pusillus</i>		
	Dry wt (µg/ml)	pH	phytase (in units)	Dry wt (µg/ml)	pH	phytase (in units)	Dry wt (µg/ml)	pH	phytase (in units)
Ammonium chloride	156.2	5.6	24.0	148.5	5.8	24.0	212.6	6.2	26.0
Ammonium nitrate	154.2	5.5	30.0	155.8	5.8	30.0	226.8	5.6	30.0
Ammonium sulphate	155.2	5.5	28.0	152.6	5.8	38.0	234.6	5.8	30.0
L-arginine	187.3	5.7	54.0	174.8	5.7	48.0	265.8	6.6	72.0
L-asparagine	189.4	6.4	60.0	178.3	6.4	58.0	255.7	6.4	64.0
L-aspartic acid	168.5	5.8	42.0	162.4	6.4	36.0	245.6	6.3	52.0
L-glutamine	167.3	6.2	40.0	154.3	5.8	44.0	236.4	5.8	36.0
L-glutamic acid	175.3	6.0	30.0	172.4	6.2	52.0	221.8	5.5	34.0
L-Glycine	183.7	6.2	38.0	165.4	5.9	48.0	211.8	5.8	48.0

L-methionine	155.2	5.5	24.0	154.2	5.4	24.0	202.4	5.6	38.0
L-histidine	147.3	5.8	28.0	164.2	6.4	38.0	212.4	6.3	32.0
L-lysine	158.3	6.2	38.0	162.4	6.2	32.0	232.4	6.2	38.0
L-tryptophan	159.3	6.0	38.0	144.8	5.8	38.0	241.2	6.3	48.0
L-tyrosine	166.4	5.8	28.0	155.8	6.2	44.0	227.3	5.8	41.0
Yeast extract	182.5	6.2	54.0	177.3	6.4	52.0	254.3	6.4	48.0

*1 unit of phytase activity (IU) is expressed as amount of enzyme that liberates 1 mole phosphorus per minute under assay conditions.

Conclusion:

Present studies *R. pusillus* is a versatile fungus and produces phytase in good amount. its ability to grow and produce phytase a pure synthetic medium is a desirable quality and can be exploited economically in a large scale fermentation. Further, the pH changes were also minimum with good amount mycelial growth is an added advantage. other two fungi through produced phytase their specificity to substrates and pH changes during their growth submerged conditions is an disadvantage. more in depth studies under varied condition and catalytic properties of phytase are needed.

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Conflict of interest statement: Authors have no conflicts of interest to declare.

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