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Statistical optimization and mutagenesis for high level of phytase production by *Rhizopus oligosporus* MTCC 556 under solid state fermentation

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Abstract

The present study deals with production of phytase from *Rhizopus oligosporus* MTCC 556 by solid state fermentation (SSF) using different (ADT27, IR20, PAIYUR1, KG, and RASI) rice bran varieties, in which ADT27 rice bran yield maximum of 6.2 U gds^{-1} phytase. Statistical optimization was employed by Central Composite Design (CCD); the results showed that 3.0 g dextrose, 2.5 g ammonium nitrate, substrate size of 80 mesh, 10 mg calcium chloride was 116 hr at optimal for phytase production by SSF, with maximum of 23.14 U gds^{-1}. Phytase production improved by 4 fold (31.3 U/gds) due to chemical mutagenesis (mutant *Rhizopus oligosporus* MTCC 1116) in optimized media composition. Partially purified phytase showed approximately 90 kDa of molecular mass and was optimally active at 5.5 pH and 50°C temperature. Substrate specificity exhibited in sodium phytic acid and phytase activity was stimulated by Zn^{2+} and Ca^{2+}.

Key words

Phytase, *Rhizopus oligosporus*, Rice bran variety, Solid state fermentation

Introduction

Phytic acid (myo-inositol 1,6 hexakis phosphate) is the major storage form of phosphorus in cereals, grains and oilseeds (Kumar *et al.,* 2010). It acts as an anti nutritional agent by chelating with several metal ions and insoluble form of protein complex (Andriotis and Ross, 2003). Therefore, it cannot be metabolized by monogastric animals due to lack of phytic acid degrading enzyme present in their intestine. These consequences contribute to mineral deficiencies and phosphorus pollution in livestock industries (Vohra and Satyanarayana, 2003). Addition of phytase is the best way to degrade phytic acid phosphorus that can reduce negative nutritional effects and phosphorus pollution. Fungal phytase is one of the commercially prime enzymes for food and feed industries, and is also known to deliver desirable characteristics comparable to bacteria and yeast (Pandey *et al.,* 2000). Among fungi, *Rhizopus* species is the best phytase producing fungi that has been under intensive research for higher yield (Bogar *et al.,* 2003 a; Ramachandran *et al.,* 2005; Sabu *et al.,* 2002). Hence, efforts are needed to produce phytase at an affordable cost so that it can be used for hydrolyzing phytic acid with high economic potential.

Rhizopus oligosporus has been reported to reduce phytic acid content during fermentation and its phytase characteristics are industrially important (Casey and Walsh, 2004). Due to its rapid growth rate and generally regarded as safe (GRAS) strain for biological studies, it is mainly used for tempeh production (Feng *et al.,* 2007). In recent decades, commercial phytases are produced by fungi, and are often enhanced through mutagenesis and screening (Shah *et al.,* 2009; Bhavsar *et al.,* 2013). Hence, an effective physical and chemical mutagenesis was induced to enhance phytase production ability of *R. oligosporus* followed by comparative study of wild and mutant strains. The process of solid state fermentation is simple, involving low cost and less energy consumption, and is a potential tool for fungal studies than submerge fermentation (Pandey, 2003). In the present study, rice bran varieties were exploited as raw material for the

production of phytase under SSF. Rice bran consists of pericarp, aleurone and germ layer and high concentration of phytic acid (Canan *et al.,* 2011). However, the phytic acid content range depends on local rice cultivation conditions (Liu *et al.,* 2005). Besides its utilization for phytase production, rice bran serves to be a good source of carbon and nitrogen for fungal growth.

Optimization of culture medium variables by statistical designs like response surface methodology (RSM) has been employed. Multiple number of variables can be studied with RSM simultaneously that will greatly reduce time (Bhavsar *et al.,* 2011). The time consuming statistical optimization and mutagenesis trials were therefore carried out in counterpart because it may be practically expected that a mutant strain may also be a gain from the optimized media formulation attained with the parent strain. However, there is no literature reported to optimize the culture parameters for phytase production using *R. oligosporus* under SSF.

The present study aimed at phytase production from *R. oligosporus* MTCC 556 using different rice bran varieties under solid substrate fermentation. Statistical optimization for the cultural variables and strain improvement studies enhanced phytase production followed by purification and characterization of phytase to investigate its suitability in the food and feed industries.

Materials and Methods

Rhizopus oligosporus MTCC 556 was procured from Microbial Type Culture Collection (MTCC), Chandigarh (India), and maintained on potato dextrose agar slants at 4°C. Inoculum was prepared from 7 day-old-culture with 0.1% Tween80. Final spore suspension was ~$5x10^6$ CFU ml^{-1} (colony forming units per milliliter), and was used as inoculum for fermentation process.

Spore suspension (~$3x10^6$ CFU ml^{-1}) of *R. oligosporus* MTCC 556 strain was treated with Ultra Violet-irradiation (UV) (HEBER MULTILAMP TYPE PHOTO REACTOR) of 254 nm for 10 min at a distance of 25 cm. Chemical mutagen of ethyl methyl sulphonate (EMS) was used at a concentration of 0.3% (v/v). Spore suspensions subjected to mutations were incubated at room temperature for 24 hr. Mutated spore suspensions were subsequently plated on modified phytase screening medium (PSM). The plates were incubated at 25°C for 96 hr and the mutant strains were selected as a result of an enhanced zone of hydrolysis.

Evaluation of agro-residues by solid state fermentation: Ten grams of each rice bran variety were taken in a 250 ml Erlenmeyer flask along with the mineral salt solution containing (w/v): 0.5% NH_4NO_3, 0.05% $MgSO_4.7H_2O$ and 0.05% NaCl: pH 5.5. The medium was sterilized at 121°C for 15 min and 1 ml of spore suspensions was inoculated, and incubated at 30°C for 96 hr. Fermented samples were drawn and extracted with 0.1% Tween80 at 30°C in an orbital shaker (200 rpm) for 2 hr. Filtration was carried out with double layered muslin cloth followed by centrifugation at 10,000 rpm for 20 min. Supernatant was collected and taken as crude enzyme for extracellular phytase activity assay.

Phytase assay: Phytase activity was determined by estimating the amount of inorganic phosphate released from sodium phytic acid by the method adopted by Harland and Harland (1980). One unit of phytase activity is defined as the amount of enzyme required to liberate 1 µ mole of inorganic phosphate per minute under standard assay conditions. Phytase yield was expressed as units per gram of dry substrate (U gds^{-1}).

Protein estimation: Protein content of fermented sample was determined by Lowry's method (1951) using Bovine Serum Albumin as standard. The estimated protein content was expressed as milligram of protein per gram of dry substrate (mg gds^{-1}).

Statistical optimization for phytase production: Optimization of phytase production was carried out by *R. oligosporus* MTCC 556. All the culture flasks were maintained with 60% of moisture at 30°C and pH 5.5.

Central composite design (CCD) : To determine the selected factors (A) Dextrose (g) (B) Ammonium nitrate (g) (C) Substrate size (rice bran ADT27 was milled in five different sizes using USA standard testing sieve, particle size was expressed as mesh) (D) $CaCl_2.7H_2O$ (mg) and (E) Incubation time (hr) were chosen for optimization by RSM using CCD, with this design, each factor was set at five levels coded as -2, -1, 0, +1, and +2. The five factors included 26 experimental runs and these responses (phytase activity U/gds) are listed in Table.1. The behavior of the system was clarified by the following quadratic polynomial equation:

$$Y = b_0 + b_1x_1 + b_2x_2 + b_3x_3 + b_4x_4 + b_5x_5 + b_{11}x_1^2 + b_{22}x_2^2 + b_{33}x_3^2 + b_{44}x_4^2 + b_{55}x_5^2 + b_{12}x_1x_2 + b_{13}x_1x_3 + b_{14}x_1x_4 + b_{15}x_1x_5 + b_{23}x_2x_3 + b_{24}x_2x_4 + b_{25}x_2x_5 \ldots \quad (1)$$

where, Y is predicted response, b_0 is intercept, b_1, b_2, b_3, b_4, b_5 are linear coefficients, b_{11}, b_{12}, b_{13}, b_{14}, b_{15} are squared coefficients, b_{23}, b_{24}, b_{25} interaction coefficients and x_1, x_2, x_3, x_4, x_5, x_1^2, x_2^2, x_3^2, x_4^2, x_5^2, x_2x_3, x_2x_4, x_2x_5 are the independent variables. Statistical design software package "Design Expert" (8.0.6.1, Stat Ease Inc., Minneapolis, MN, USA) was used for experimental design.

Partial purification of phytase : Crude enzyme extracted from fermented sample (*R. oligosporus* MTCC 1116) was used for purification by ammonium sulfate precipitation (up

to 80% of saturation) under constant stirring. Samples were collected after dialysis by passing them through 30 kDa ultrafiltration membranes (Millipore, USA). The collected fractions were suspended in 200 mM sodium acetate buffer at pH 5.0 and salt was removed by passing through Sephadex G-25 column. Fractions from the column were assayed for phytase activity by measuring spectrophotometrically at 280 nm. Molecular weight was determined by SDS-PAGE analysis as described by Laemmli (1970), followed by zymography to confirm the molecular weight of the enzyme.

Characterization of purified phytase

Effect of temperature and pH of purified phytase : To determine optimal temperature phytase activity was measured by incubating the reaction mixture at different temperatures (25−80°C) for 30 min using 2 mM of sodium phytic acid as substrate while optimum pH was determined by measuring phytase activity at various pH (2.0 – 9.0) values at 50°C. Maximal phytase activities at optimal pH and temperature were defined as 100% relative phytase activity. Thermal stability was determined by measuring relative phytase activity for 5 min of incubation in 100 mM sodium acetate buffer (pH 5.5) 70°C and 80°C. pH stability was determined by measuring the relative phytase activity after 5 h of incubation at 4°C.

Substrate specificity : To determine the substrate specificity, purified phytase in 100 mM sodium acetate buffer (pH 5.0) was incubated with different substrates viz., (2 mM) sodium phytic acid, disodium pyrophosphate, Adenosine Triphosphate (ATP), Adenosine Monophosphate (AMP), Guanosine Triphosphate (GTP), Glucose-1-phosphate (G-1-P) and Glucose-6-phosphate (G-6-P) at 50°C for 30 min.

Effect of metal ions : To investigate the effect of different metal ions on phytase activity different salts (Mg^{2+}, Hg^{2+}, Mn^{2+}, Zn^{2+}, Cu^{2+}, Ca^{2+}, Fe^{2+}, Na^{2+}) were added to the reaction mixture at a concentration of 5 mM. Reactions were carried out at pH 5.5 and 50ºC.

Results and Discussion

Agricultural residues of five different rice bran varieties (KG, PAIYUR1, IR20, RASI, and ADT27) were used as solid substrates for phytase production by *R. oligosporus* MTCC 556 with an incubation period of 96 hr. Maximal phytase production was obtained with 6.2 U gds^{-1} at 30°C in rice bran ADT27, followed by IR20 (5.82 U gds^{-1}) and PAYUR1 (5.11 U gds^{-1}) which showed moderate phytase yield. RASI (4.53 U gds^{-1}) and KG (3.11 U gds^{-1}) obtained lower phytase yield as compared to ADT27 rice bran variety. This kind of wavering was attained due to presence of phytic acid and protein content in rice varieties that were either species dependent or variety dependent (Wei *et al.,* 2007). Hence, ADT27 rice bran was selected as a suitable substrate for phytase production in SSF.

Statistical optimization for phytase production using *R. oligosporus* MTCC 556 was carried out in SSF. Factors including dextrose (A), ammonium nitrate (B), substrate size (ADT27 rice bran) (C), $CaCl_2.7H_2O$ (D) and Incubation time (E) were studied. Culture parameters selected were optimized by CCD (Table 1). Application of multiple regression analysis on experimental data resulted in the following quadratic polynomial equation, clearly explaining the production of phytase:

$$Y = 21.20 + 1.50A - 0.54B + 0.26C + 4.38D + 3{,}32E + 0.92AB + 1.13AC - 2.49AD - 0.94AE + 3.11BC - 0.25BD + 1.83BE - 1.02CD + 2.55CE - 1.81DE - 0.68A^2 - 0.59B^2 - 2.85C^2 - 3.00D^2 - 1.21E^2 \quad (2)$$

where, Y represents the predicted response and A, B, C, D, and E are coded factors mentioned above as followed respectively. In this case, A, B, C, D, E, AB, AC, AD, AE, BC, BE, CD, CE, DE, A^2, B^2, C^2, D^2, E^2 were significant model terms.

The obtained results were analyzed using analysis of variance (ANOVA). The analysis showed that regression was statistically significant ($P<0.0001$); the results of ANOVA are presented in Table 2. Statistical significance of the model equation (2) was supported by model high Fisher (*F)* value. Model *F*-value of 524.55 implied that the model was significant. Values of P>*F* less than 0.05 indicated that the model terms were significant. "Predicted R-Squared" of 0.7897 was not as close to "Adjacent R-Squared" of 0.9976 as one might normally expect. Adequate precision measures the signal-to-noise ratio and a ratio greater than 4 is desirable. The ratio of 87.76 indicated adequate signal. This model can be used to navigate the design space. A high value of coefficient of determination (R^2) was 0.999 for phytase production, suggesting that 99.9% of feasibility was explained in the model. The quality of fit of regression model proves an excellent correlation between independent factors. For a strong relation between the experimental and predicted values of phytase production, an adjusted R^2 value of 0.9976 was also determined. A low value of CV (1.60%) concluded that the experiments were highly reliable and were performed with better precision.

In the present study, the enhancing effect of the substrate size for maximum phytase production showed counter intuitive findings. A higher yield of phytase was obtained in the substrate size of 80 mesh (23.14 U gds^{-1}). Phytase yield was comparatively decreased, with 11.18 U gds^{-1} for 40 mesh, and 12.13 U gds^{-1} for 120 mesh of ADT27 rice bran size. This might be due to the fact that with larger

Table 1 : Experimental design of CCD with responsible and predicted values

Run	Dextrose (w/w %)	Ammonium nitrate (w/w %)	Substrate size (mesh)	$CaCl_2.7H_2O$ (mg)	Incubation time (hr)	Phytase activity (U gds^{-1})		
						Experimental	Predicted	Error
1	3.00	2.50	120.0	10.0	80	12.13	12.24	0.11
2	3.00	2.50	80.0	1.0	80	03.18	03.29	0.11
3	1.75	3.80	100.0	5.0	100	17.10	17.04	0.06
4	3.00	2.50	80.0	10.0	80	21.43	21.20	0.23
5	3.00	2.50	80.0	10.0	80	21.03	21.20	0.17
6	4.25	1.20	100.0	5.0	100	16.14	16.08	0.6
7	3.00	2.50	80.0	10.0	44	11.03	11.14	0.11
8	4.25	3.80	100.0	5.0	60	10.12	10.06	0.6
9	3.00	2.50	80.0	10.0	116	23.14	23.25	0.11
10	1.75	3.80	100.0	15.0	60	11.00	10.94	0.6
11	0.72	2.50	80.0	10.0	80	16.13	16.24	0.11
12	3.00	4.87	80.0	10.0	80	18.16	18.27	0.11
13	3.00	0.13	80.0	10.0	80	20.11	20.22	0.11
14	4.25	1.20	100.0	15.0	60	12.16	12.10	0.6
15	3.00	2.50	80.0	10.0	80	21.33	21.20	0.13
16	3.00	2.50	80.0	10.0	80	21.16	21.20	0.6
17	5.28	2.50	80.0	10.0	80	21.58	21.69	0.11
18	4.25	3.80	60.0	15.0	60	13.14	13.08	0.6
19	1.75	3.80	60.0	15.0	100	17.13	17.07	0.6
20	1.75	1.20	100.0	15.0	100	18.19	18.13	0.6
21	3.00	2.50	80.0	20.0	80	19.13	19.24	0.11
22	4.25	1.20	60.0	15.0	100	15.14	15.08	0.6
23	3.00	2.50	40.0	10.0	80	11.18	11.29	0.11
24	3.00	2.50	80.0	10.0	80	21.43	21.20	0.23
25	4.25	3.80	60.0	5.0	100	11.13	11.07	0.6
26	1.75	1.20	60.0	5.0	60	07.11	06.99	0.2

particles, filamentous fungi needed to penetrate more efficiently into the substrate and as a consequence, the enzyme produced encountered a larger surface of diffusion into the opened micro pores of particles for nutrient uptake (Silva *et al.,* 2009). The optimum values of tested parameters were dextrose (3.0 g) and ammonium nitrate (2.5 g) which enhanced phytase production. Bhavsar *et al.* (2011) also identified dextrin and ammonium nitrate as important variables for phytase production by *Aspergillus niger* NCIM 563 by using response surface analysis. Another interesting result was observed in the present study was that higher phytase yield with calcium chloride (10 mg) showed strongest positive influence on phytase production with respect to incubation time of 116 hr. This might be due to removal of inorganic phosphate by precipitating with $CaCl_2$ to enhance phytase production (Singh and Satyanarayana, 2006).

Validation experiments were carried out for phytase production under conditions predicted by RSM. The results showed that strong agreement existed between maximum predicted response and experimental response of phytase activity was 23.25 and 23.14 U gds^{-1}, respectively thus supporting high fitness of the model. Interestingly, in the present study with initial screening, these media compositions were highly significant for phytase production by *R. oligosporus* MTCC 556, resulting in 2.73 fold increase in phytase yield. Previous studies using statistical optimization by SSF showed 1.85 fold of increased phytase production with *Mucor racemosus* (Bogar *et al.,* 2003b) and 2.6 fold higher phytase production with *Sporotrichum thermophile* (Singh and Satyanarayana, 2008). The study suggested that choosing an appropriate substrate coupled with process level optimization improved enzyme production markedly. Developing a phytase production process, based on rice bran as a substrate in SSF, is economically attractive as it is a cheap and readily available raw material in agriculture-based countries. This result is of significant interest due to low cost and abundant availability of residues.

In parallel with media optimization studies, mutagenesis studies were conducted using UV and EMS for obtaining a hypersecretory strain. Mutants were selected on the basis of large zone of hydrolysis on sodium phytic acid plate as compared to the parent strain (Shah *et al.,* 2009). Hence, all the positive mutants were quantified and confirmed for phytase production, using statistically

Table 2 : ANOVA analysis of regression model

Source	SS[a]	Df[b]	MS[c]	Coef[d]	F-value	*P*-value
Model	671.9	20	33.59	21.2	524.55	<0.0001
A	14.85	1	14.85	1.5	231.9	<0.0001
B	1.9	1	1.9	-0.5	29.69	0.0028
C	0.45	1	0.45	0.26	7.05	0.0452
D	127.2	1	127.2	4.38	1986.2	<0.0001
E	73.33	1	73.33	3.32	1145	<0.0001
AB	2.56	1	2.56	0.92	40.05	0.0015
AC	3.68	1	3.88	1.13	60.05	0.0006
AD	18.89	1	18.89	-2.5	294.91	<0.0001
AE	2.7	1	2.7	-0.9	42.09	0.0013
BC	29.54	1	29.54	3.11	461.28	<0.0001
BD	0.19	1	0.19	-0.3	2.91	0.149
BE	10.24	1	10.24	1.83	159.82	<0.0001
CD	3.2	1	3.2	-1	49.94	0.0009
CE	19.89	1	19.89	2.55	310.64	<0.0001
DE	10.01	1	10.01	-1.8	156.24	<0.0001
A2	9.44	1	9.44	-0.7	147.48	<0.0001
B2	7.23	1	7.23	-0.6	112.9	0.0001
C2	167.9	1	167.9	-2.9	2621.2	<0.0001
D2	186.1	1	186.1	-3	2906.2	<0.0001
E2	30.28	1	30.28	-1.2	472.82	<0.0001
Residual	0.32	5	0.064			
Pure error	0.12	4	0.031			
Cor. Tot[e]	672.2	25				

R^2 = 99.95%, R^2 (adjusted) = 99.76%, Coefficient of variation (CV) =1.60%; [a] Sum of squares, [b] Degree of freedom, [c] Mean square, [d] Coefficient estimate; [e]Correlation (Total)

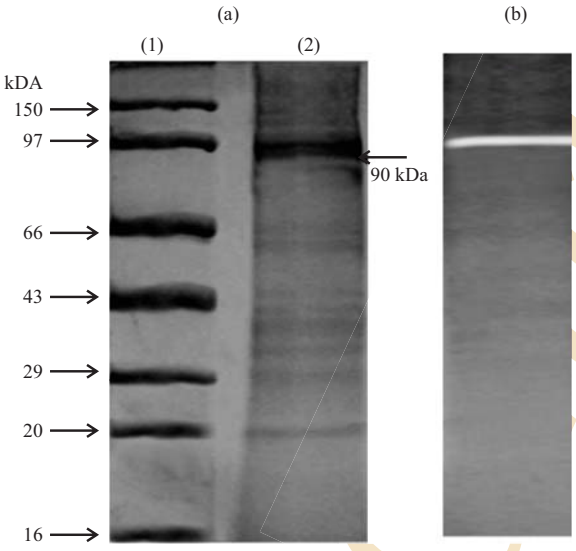

Fig. 1: Electrophoretic analysis of phytase (a) SDS-PAGE of the purified phytase [Lane 1: Molecular marker; Lane 2: Mutant strain enzyme]. (b) Zymogram developed for phytase activity

optimized media for the parent strain in solid state fermentation condition. Among hypersecretory mutants, mutant strain of *R. oligosporus* MTCC 1116 (0.3% EMS) exhibited 31.3 U gds^{-1} phytase activity at 116 hr, and improved the yield 4 fold as compared to production by the parent strain with ADT27 rice bran variety, whereas the mutant strain was employed for phytase production in unoptimized conditions, and a result of (1.9 fold) 18.2 U gds^{-1} was obtained. This was comparatively lower yield under optimized media conditions. Therefore, optimized media conditions were suitable for mutant strain. In the present study can have remarkable value addition from phytic acid feed-conversion and environmental point-of-view. A significant variance on strain improvement was delay in sporulation time of the mutant strain (9^{th} day) as compared to the parent (4^{th} day). The morphological molds of the mutant strain mycelial morphology, sporangium shape, and sporangium size were assessed using optical microscopy under 100X magnification studied variability morphological Bhavsar *et al.* (2013) and suggested that the mutated strain show modified mycelia and sporangia with different shapes and sizes which also effectively influenced phytase production.

Shivanna and Govindarajulu (2009) treated *A. niger* CFR 335 in physical and chemical mutagenesis, and found improvement in phytase productivity of mutant strain from 0.85 U mg^{-1} to 1.26 U mg^{-1}.

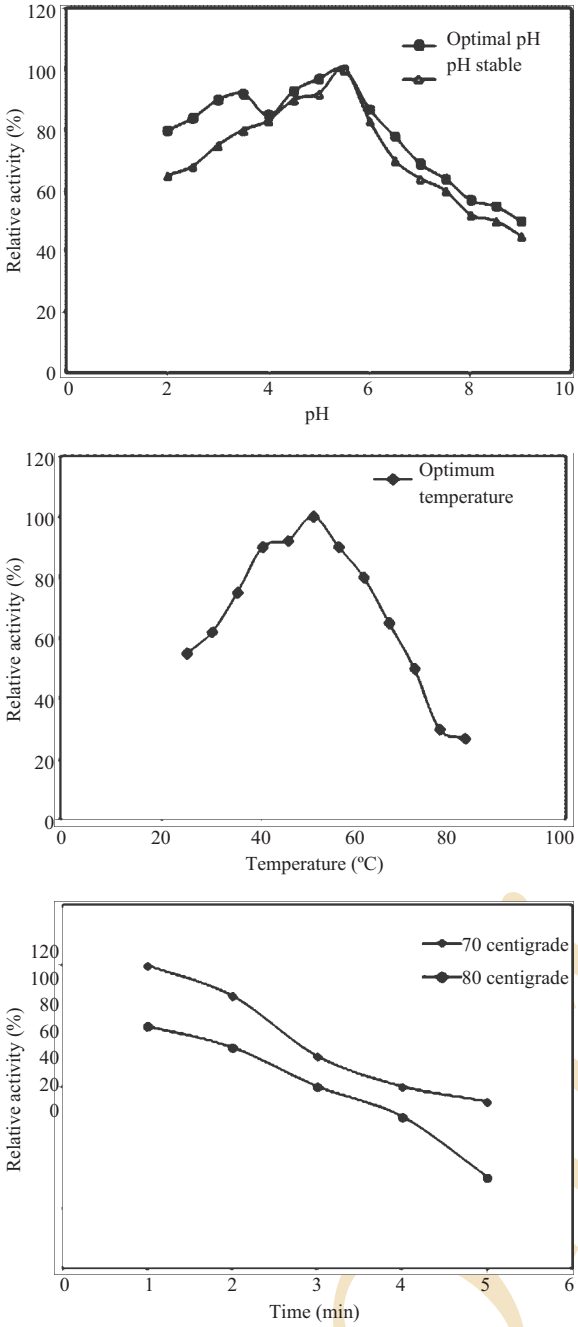

Fig. 2 : Effects of pH and temperature on activity and stability of phytase. (a) Effect of pH on enzyme activity and stability. (b) Effect of temperatures on enzyme activity. (c) Effects of Thermo stable on enzyme activity

Phytase produced from mutant *R. oligosporus* MTCC 1116 in SSF was purified using ammonium sulfate precipitation (at the fraction of 60%), followed by ultrafilltration and Sephadex G-25 gel filtration column. The molecular weight of purified phytase was confirmed by SDS-PAGE. The estimated molecular weight was found to be approximately 90 kDa, as shown in figure 1(a), and it was confirmed by zymogram analysis (figure 1(b)). It suggested that the phytase from *R. oligosporus* MTCC 1116 was a monomeric protein. This is in accordance with the characteristics of phytases from filamentous fungi, which is likely to be between 70 and 100 kDa (Casey and Walsh, 2003). The molecular mass of phytase was found to be lesser than the previously reported phytase value (120 kDa) from *R. oligosporus* (Casey and Walsh, 2004). This occurrence might be due to varied media composition (phytase inducing-medium) used, and also signaling peptide responsible for expression of phytase gene (Martin *et al.,* 2006).

The effect of various temperatures on purified phytase ranged from 25°C to 80°C. A optimum of 100% relative phytase activity was determined at 50°C (figure 2(b)) and 85% relative phytase activity at 40°C, whereas above 60°C phytase activity decreased drastically. Phytase derived from mesophilic fungi significantly utilized the feed, for commercial products, in the range of 50 - 60°C (Casey and Walsh, 2004). Thermal stability of phytase was also checked at 70°C and 80°C. Fig. 2(c) represents 100% relative phytase activity at 70°C for 1 min, and by the end of 5 min, the activity was decreased to 55%. While at 80°C, 80% of the relative phytase activity was obtained for 1 min, at the end of 5 min, 30% relative phytase activity was observed. Phytase produced by *R. oligosporus* MTCC 1116 was found to be thermostable at 80°C, with 30% relative phytase activity. This was comparatively higher than the phytase activity obtained from commercial enzymes, in which their activity was lost in 4 min (Casey and Walsh, 2004).

The effects of pH from purified phytase were assayed using different buffer ranging from 2.0 to 9.0 pH. Optimum phytase activity was obtained at pH 5.5 with 100% relative phytase activity (Figure 2(a)). Phytase obtained from *R. oligosporus* MTCC 1116 also showed 80% and 85% of relative phytase activity at pH 3.5 and 5.5. pH stability was expressed as relative phytase activity (figure 2(a)), in which, after 5 hrs, phytase lost its activity from pH 6.0 to 9.0, and its stability was found to be better in acidic environment as pH variation in the stomach, intestine and duodenum ranged from pH 2.0 to 6.5, which was suitable for feed applications (Casey and Walsh 2004; Mullaney *et al.,* 2000).

From the results of substrate specificity, a relative phytase activity of 100% was obtained when sodium phytic acid was used as substrate. Various other substrates showed lower relative phytase activity of 90% for Glucose-1-phosphate, 87% for disodium pyrophosphate, 63% for Glucose-6-phosphate, 26% for ATP and 14.7% for GTP. The

influence of various metal ions on relative activity of phytase was studied, and it showed inhibitory effect of relative phytase activity (65%) Fe^{2+}, (60%) Cu^{2+}, (70%) Mn^{2+}, (30%) Hg^{2+}. On the contrary Zn^{2+} (117%), Ca^{2+} (110%), Na^{2+} (100%) and Mg^{2+} (100%) ions showed higher relative phytase activity than other metal ions studied, resulting in an enhanced phytase activity. In practical applications, these metal ions play a significant role as they are present in the animal feed (Gulati *et al.,* 2007).

In the present study, agricultural residues of rice bran varieties were transformed into value-added products by fermentation, using *R. oligosporus* MTCC 556. Highest phytase yield was obtained with ADT27 rice bran variety, and the applicability of RSM for optimizing phytase production by SSF indicated 2.7 fold increase. Statistically optimized condition was implemented for mutant strain, and it showed a remarkable rise in phytase production yield upto 4 fold. Along with high yield, pH tolerance and temperature stability characteristics phytase appears to be a viable option for use as food and feed in industrial applications.

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