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IDENTIFICATION OF PHYTASE PRODUCING YEAST AND OPTIMIZATION & CHARACTERIZATION OF EXTRACELLULAR PHYTASE FROM *CANDIDA PARAPSILOSIS*

*Ranjan, K. & Sahay, S.
*Department of Botany, Government College of Science & Commerce Benazir, Bhopal, MP, India
*email – drkamleshranjan@gmail.com; *Mobile - +91 9934501632

ABSTRACT
Among sixty one yeast isolates from various samples, twelve were capable of producing phytase. Out of twelve, 5 isolates showed maximum phytase activity. CB3 isolate was selected for further study. CB3 isolate was genetically characterized as *Candida parapsilosis*. The crude extract was optimized for maximum enzyme activity. The enzyme was stable between the pH 2 to 7 but the optimal pH was found to be 3. The enzyme was also stable between temperature ranges 25° C to 45°C but best temperature for enzyme activity was found to be 37° C. Enzymatic activity of crude extract was 20.6 U/ml after 24 h of incubation. Maximum phytase activity was increased to 56.2 U/ml at pH 3 after 24 h of incubation under optimal conditions. Total protein present was 0.6 mg/ml in crude extract by SMF but as high as 3.5 mg/ml by SSF.

KEY WORDS: Yeast; Phytase; Fermentation; *Candida parapsilosis*

INRODUCTION

The yeast most frequently found associated with plant leaves, flowers, soil, insects and the skin and intestinal tract of warm blooded animals [1]. A large number of industrial processes in the areas of industrial, environmental, clinical, food and pharmaceutical biotechnology utilize enzymes at some stage or the other. The major usage of microbial enzymes in food industry started in 1960s in starch industry. A large number of microorganisms, including bacteria, yeast and fungi produce different groups of enzymes [2]. Phytases are found naturally in plants and microorganisms, particularly fungi [3]. Yeasts have been found to be important sources of phytase [4,5]. Phytase (myo-inositol hexakisphosphate phosphorylase) catalyzes release of phosphate from its complexes phytate (myo-inositol phosphate), salt of phytic acid [myo-inositol (1, 2, 3, 4, 5, 6) hexakisphosphate; $C_6H_{18}O_{24}P_6$] and phytin (myo-inositol phosphate), calcium and magnesium salt of phytic acid. The Enzyme Nomenclature Committee of the International Union of Biochemistry recognizes three types of phytases based on the position of the first phosphate hydrolyzed, those are 3-phytase (E.C. 3.1.3.8), 4-phytase (E.C. 3.1.3.26) and 5-phytase (E.C. 3.1.3.72) and all these phytases are belong to the subfamily of histidine acid phosphatases [6].

Phytic acid is the major storage form of phosphorus in cereal grains, legumes, pollens, and oil seeds and thus in seed-based animal feed [7]. Phytates in legumes, in cereals, and in some animal tissues has been reported [8]. Phosphorus is stored in cereals, legumes, oil seeds, fodder and root crops as phytate and phytin which can only be digested by ruminants but nonruminants or monogastric animals such as pigs, poultry and humans are unable to utilize phytic acid phosphorus either due to lack of or insufficient amount of phytate degrading enzymes and since phytic acid cannot be resorbed and undigested phosphorus is released into the environment [9,10]. Accumulation of undigested phosphorus in soil and water is toxic and may cause eutrophication in water bodies. Therefore, pig and poultry feed commonly is supplemented with either inorganic phosphate or a phytase. Phytase can therefore play important role in controlling phosphate pollution. The supplementation of phytase in fodder improves the phosphorus bioavailability and also reduces its excretion in the areas of intensive livestock [11]. Thus, for both environmental and economic reasons phytases and phytase producing microbes are attracting significant industrial interest. Because of their great industrial significance there is an ongoing interest in isolation of new microbial isolates producing phytase and optimization of this enzyme. Hence the aim of the study is to isolate the yeasts with phytase producing ability and optimization of the enzyme production.

MATERIAL AND METHODS

All the chemicals, reagents and media were obtained from Sigma-Aldrich (USA), HiMedia (India), Fisher (India), SRL (India), Qualigens (India), Merck (India) and SD Fine Chemicals (India).

Isolation and preservation of yeast isolates

The yeast strain was isolated from spoil vegetables, fruits, leaves, seeds, and other natural sources. The yeast isolates were identified up to the genus level based on the morphological and physiochemical properties of the isolates. The yeast cultures were maintained in eppendorf tubes containing glycerol stock and stored at -20ºC until phytase production and assay, which was done after thawing the strain and then prepared for extracellular fermentation.

Screening of phytate degrading yeast

All isolates were plated onto minimal medium contacting phytate as a sole source of phosphorus that was phytase screening medium (PSM) containing 1% glucose, 50 mg sodium phytate, 50 mg $MgSO_4.7H_2O$, 10 mg $CaCl_2.2H_2O$, 10 mg NaCl, 100 ml sterile water, pH adjusted at 6.5. (All chemicals were used of Hi Media Laboratories). A negative control without any phosphorus source and a positive control with 50 mg KH_2PO_4 were kept simultaneously. The isolates grown vigorously on PSM and not growing on phosphorus minus plate medium (negative control) were selected as phytase positive strains. The colonies exhibiting zones of clearance (translucent areas) were selected and streaked onto fresh PDA plates (Fig. 1 & 2).

Production of phytase

The yeasts isolates shown positive result for phytase in the screening test plus high growth rate, more thermal and pH tolerance were selected for the production of phytase. Fermenting medium was prepared in a 250 ml baffled flask containing 5 g sterilized wheat bran and 60 ml minimal broth (Glucose- 1%; Sodium Phytate- 50 mg; $MgSO_4.7H_2O$- 50 mg; $CaCl_2.2H_2O$- 10 mg; NaCl- 10 mg; Sterile water- 100 ml; KH_2PO_4 (as an inducer) - 0.1 mg, pH 6.5) and 2.5 ml micronutrients and then incubated at 25ºC for about 6 h on a rotary shaker. Inoculum in fermenting medium was inoculated with 1% (v/v) fermenting media. To minimize the variation of pH during yeast cultivation, HCL/NaOH was used as buffer in the fermenting media [12].

The main cultivation for the production of enzyme was performed for 72 h under the same conditions as with the fermentation culture. Cells were harvested by centrifugation at 5,000 x g for 10 min and then the supernatant are used as an extracellular phytase. All experiments were carried out at least twice until the optimal culture conditions were found; as such, the data shown in this paper are representative.

Phytase Assay

Effect of bile solution on selected phytase producing yeasts

Study the effect of bile solution on phytase producing yeast. The bile was extracted from sterile syringe and kept at 5^oC till use. Prepared different concentration of bile solution (1%, 5%, 10%, 50%, and 100%) and poured separately onto the Potato Dextrose Agar Medium (PDA) plates. All selected phytase producing yeast isolates were then inoculated onto this PDA plate. The plate was inoculated at 37^oC for 4 days and observed every day.

Effect of intestinal microflora on the growth of phytase producing yeasts

Study the effect of intestinal flora on phytase producing yeast. 1 ml aliquot was spread onto the poured PDA plate and the plate was left for 1 h to settle. All selected phytase producing yeast isolates were then inoculated onto this PDA plate. The plate was inoculated at 37^oC for 4 days and observed every day.

Effect of temperature

To determine the optimal incubation temperature for maximum phytase activity, crude extract was assayed at various temperatures Phytase was assayed at 4, 25, 37 and 45^oC for 7 days.

Thermostability test of phytase activity

Phytase from various isolates was assayed after incubating them at 37^oC for 12 h, 24 h, and 48 h.

Effect of pH

To determine the optimal incubation temperature for maximum phytase activity, crude extract was assayed at various pH 2, 3, 5, 6.8 and 11. For assaying enzyme at pH 3, 5, 6.8 and 11 the substrate was dissolved in 0.2 N sodium-acetate buffer (pH 3), 0.2 N sodium-acetate buffer (pH 5), 40mM sodium-phosphate buffer (pH 7) and glycine-NaOH buffer (pH 11) respectively and then assayed by the above method.

Effects of heavy metal ions

To determine the maximum phytase activity, crude extract was assayed in presence of metal ions such as Mn^{2+}, Fe^{2+}, Cu^{2+}, and Zn^{2+}.

RESULTS AND DISCUSSION

Screening of phytate degrading yeast

Among the sixty one isolates isolated from various samples, twelve yeast isolates showed positive result on Phytase Screening Medium (PSM) and from these isolates five yeast isolates showed maximum clear halo zone around the colonies on phytate containing minimal medium (Fig. 1). The morphology of CB3 was studied. The yeast CB3 showed orange colony, butyrus texture and arthrospores formation (Table 2). The isolate was urease and diazonium blue B positive and devoid of sexual reproduction (Table 3). Carbon and nitrogen test as an essential nutrition for growth has been done (Table 4). Out of the five isolates, isolate CB3 have hydrolytic zone of 40 mm and their crude extract showed maximum phytase activity of 20.6 U/ml (Table 1) was selected for biochemical characterization (Table 2-4).

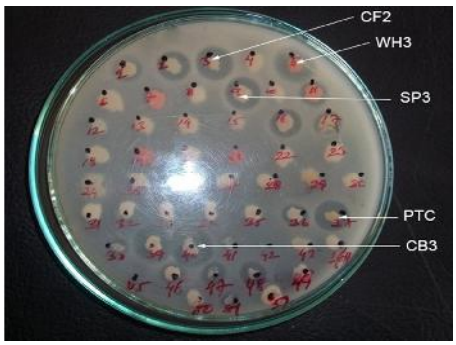

FIGURE 1: Zone of clearance by phytase producing yeasts on PSM

TABLE 1: Identification and screening of phytase producing yeast isolates

Yeast isolates	Identification	Habitat	Zone of hydrolysis of phytate	Total protein present in crude extract (mg/ml)		Specific enzyme activity
				SMF	SSF	
CB3	*Candida parapsilosis*	Spoiled *Brassica rapa* (Cabbage)	40	0.6	2.5	20
SP3	*Candida spp.*	Spoiled *Spinacea oleracea* (Spinach)	22	0.13	1.8	15
WH3	*Debaromyces spp.*	Wheat plant	30	0.16	2	5.7
CF2	*Dipodascus spp.*	Spoiled *Brassica oleracea* (Cauliflower)	24	0.44	3.5	20.6
PTC	*Dipodascus spp.*	Garden Soil	33	0.41	3	3.5

TABLE 2: Morphological characterizations of CB3

Characteristics	CB3
Colony colour	Orange
Colony texture	Butyrus
Growth pattern	Flat
Filamentation	No
Shape of cell	Oval
Budding	Unipolar
Special feature	Arthrospores
Ascospore	None
Basidiospore	None
Species	*Candida parapsilosis*

TABLE 3: Physiochemical characterizations of CB3

Aerobic use of carbon sources		Chemical Tests				Effect of Temperatures			Effect of pH		
Glucose Fermentation	Xylose fermentation	DBB	Urease	Iodine	Cyclohexamide	4°C	25°C	45°C	3	7	11
−	+	+	+	−	−	−	+	+	+	+	−

TABLE 4: Carbon and Nitrogen utilization by CB3 as a source of nutrition

Carbon source of nutrition	Result	Carbon source of nutrition	Result
D- Xylose	W	L+ Salicin	−
D+ Galactose	+	Succinate	+
D- Ribose	−	Citrate	−
L- Sorbose	+	Methanol	−
D- Arabinose	−	Ethanol	−
L+ Arabinose	+	Propane 1,2-diol	−
D+ Trehalose	+	Butane 2,3-diol	−
D- Mannose	−	Glycerol	−
D+ Cellobiose	−	α- D- Glucoside	−
L+ Rhamnose	−	D- Glucosamine	W
D+ Mellibiose	−	DL- Lactate	−
D+ Lactose	−	Starch	−
D+ Raffinose	−	Cyclohexamide	−
Maltose	−	Quinic Acid	−
Sucrose	−		
D- Melizitose	−	Nitrogen source of nutrition	Result
Ribitol	W	Nitrate	+
D- Xylitol	−	Nitrite	−
D- Sorbitol	+	Ethylamine	W
D- Mannitol	+	L-Lysine	−
Galactitol	+	Cadaverin	−
Myo-inositol	−	Creatine	−
Erythritol	−	Creatinine	−
L- Arabinitol	−	Glucosamine	−
L- Proline	−	Imidazole	−
D- Inulin	−	D+ Tryptophan	−

+ Positive; − negative; W weak growth

Phenotypic and genotypic identification of CB3

For molecular identification, the D1/D2 sequence of CB3 was cloned and compared with those available in the NCBI database. The phylogenetic tree based on D1/D2 sequence of CB3 showed its 100% similarity to type and many other strains of *Candida parapsilosis* (Fig. 2). Based on the morphological, physiological and molecular characteristics, isolates CB3 were named as *Candida parapsilosis*.

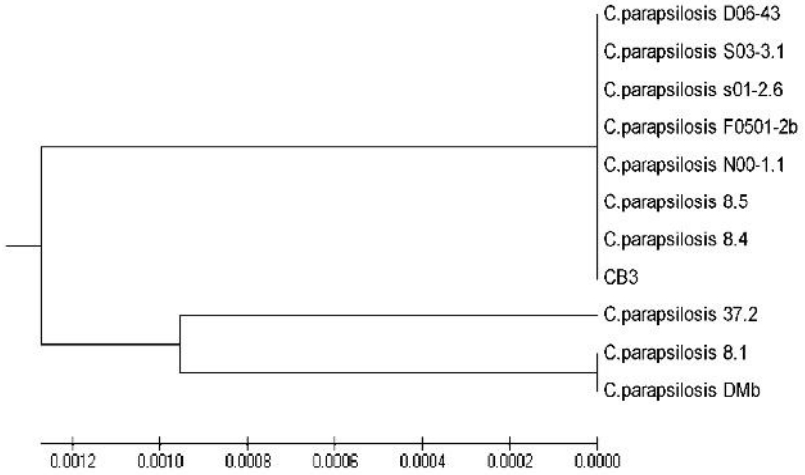

FIGURE 2: Phylogenetic tree constructed on the basis of 28s rDNA sequence of CB3 and those of other strains obtained from GenBank database

The D/D2 sequence has earlier been used to identify yeasts [13]. The nucleotide sequence of CB3 has been submitted to NCBI under accession number JN091166.

Effect of bile on the growth of selected phytase producing yeasts

All selected phytase producing yeasts were grown in presence of different concentrations of bile after 24 h of incubation at 37ºC (Table 5)**.**

TABLE 5: Growth of phytase producing yeasts in presence of different concentration (%v/v) of bile in the medium

Yeast isolates	Concentration of bile (%v/v) in the nutrient medium				
	1	5	10	50	100
CF2	+	+	+	+	+
SP3	+	+	+	+	+
WH3	+	+	+	+	+
CB3	+	+	+	+	+
PTC	+	+	+	+	+

Effect of intestinal microflora on the growth of selected phytase producing yeasts

All selected phytase producing yeasts were grown in presence intestinal bacteria after 24 h of incubation at 37ºC. The plates showed innumerable bacterial colonies after 48 h. But the growth of yeasts was not affected by the presence of this intestinal bacterial flora (aerobic). We did not perform this test in anaerobic condition (Table 6).

TABLE 6: Growth of yeast isolates in presence of aerobic intestinal microflora

Yeast isolates	Growth of yeast in presence of intestinal bacteria
CF2	+
SP3	+
WH3	+
CB3	+
PTC	+

Production of phytase

Protein was extracted from the culture of selected isolates by solid state fermentation (SSF) and submerged fermentation (SMF). The selected isolates were raised in submerged fermentation medium (SMF) and solid state fermentation medium (SMF). The extracts from both were assayed to quantitative determination of proteins which shows superiority of SSF, the later yields many times more proteins. The data shows a difference in yield about six times in case of CF2 and CB3 (Fig. 3).

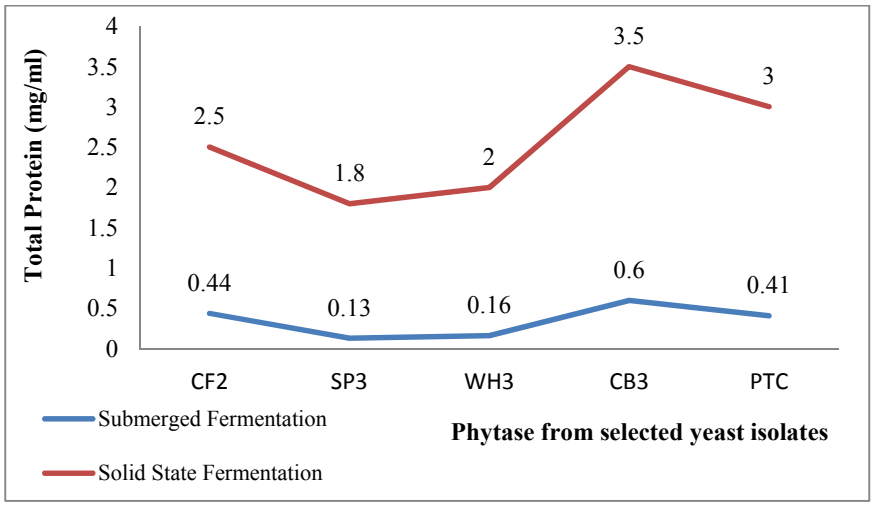

FIGURE 3: Total protein present in crude extract obtained by SMF and SSF

However, phytase activity was more in the extract from SMF indicating the presence of other proteins in higher proportion in case of SSF (Fig. 4).

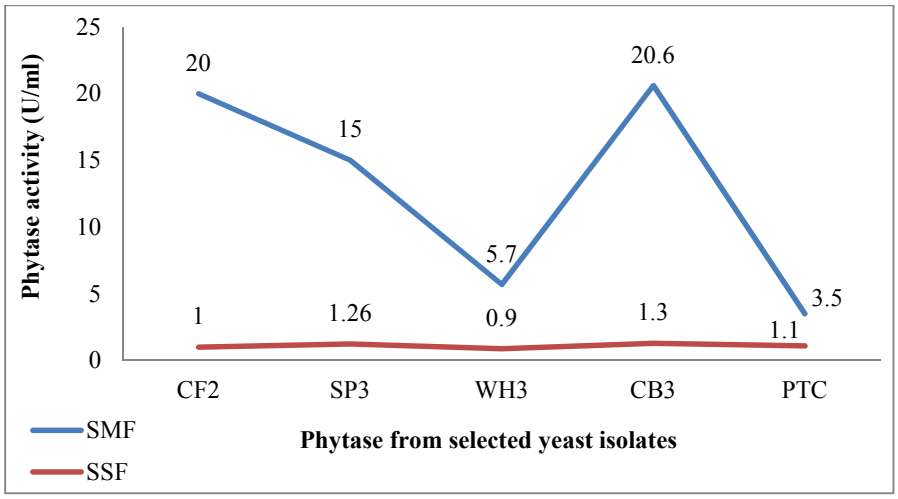

FIGURE 4: Phytase activity in crude extracts obtained by SSF and SMF

Effect of temperature

Phytase from SMF of selected isolates were assayed at various temperatures. Maximum activity was showed by CB3 (41.3 U/ml) while least activity was showed by WH3 (9 U/ml) at 37°C which was optimum temperature for phytases activity of selected yeast isolates. The range is very high which indicates the inherent difference in activity (Fig. 5).

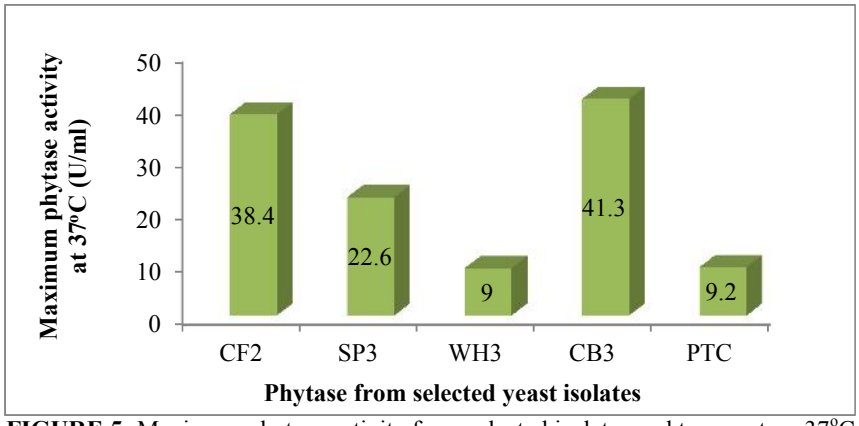

FIGURE 5: Maximum phytase activity from selected isolates and temperature 37°C

Thermostability of phytase

Phytase from various isolates was assayed after incubating them at 37°C for 12 h, 24 h, and 48 h. CB3 and SP3 showed a 66% and 65% of residual activity respectively after 48 h which were significant and render the enzyme reasonably stable. PTC was least stable, but level retained 61% of its activity which was also little significant. Overall, phytases from yeast isolates studied were stable (Fig. 6).

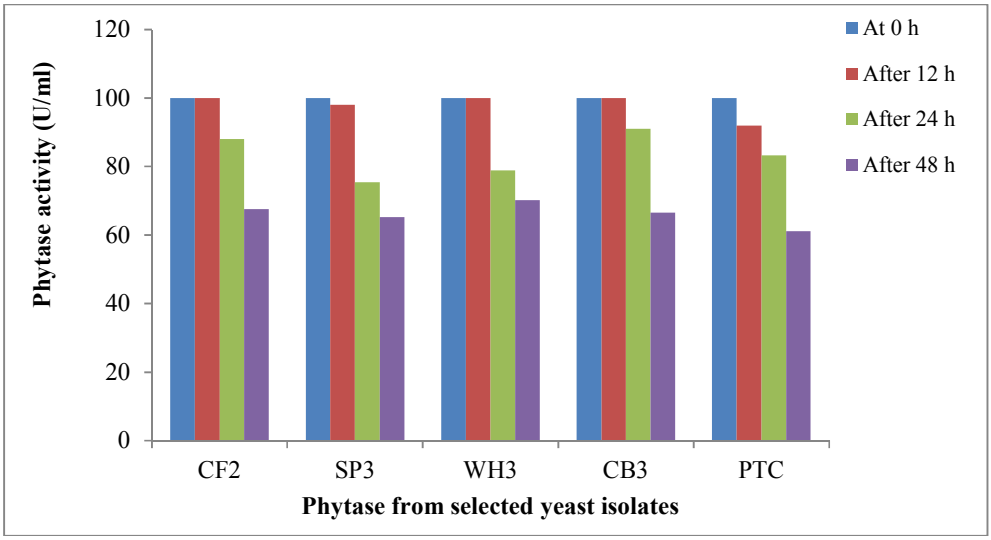

FIGURE 6: Phytase activity shown from selected isolates in different incubation period at 37°C

Effect of pH

The data showed two optimal pH; pH 3 for phytases from the isolates CF2, CB3 and PTC and pH 5 for phytases from SP1 and WH3. Phytase from CB3 showed maximum activity (56.2 U/ml) at pH 3 while phytase from WH3 and PTC showed least activity (9.5 U/ml) at pH 5 and 3 respectively under optimal conditions (Fig. 7).

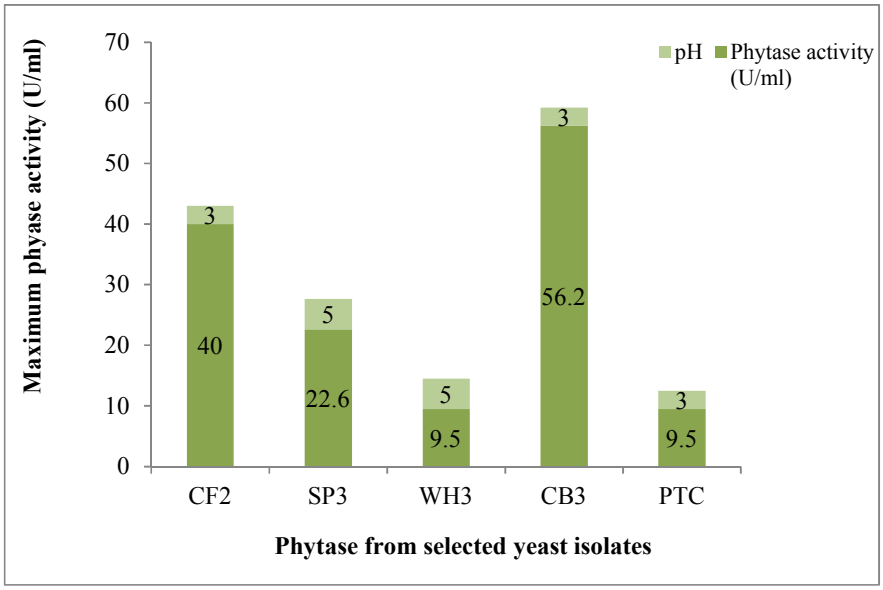

FIGURE 7: Maximum phytase activity from selected isolates at their optimum pH

Effect of metal ions

Phytase was assayed in presence of metal ion such as Mn^{2+}, Fe^{2+}, Cu^{2+} and Zn^{2+} to optimize its activity. Except phytase from PTC which showed maximum activity in presence of Fe^{2+} (120 U/ml), the phytases from all other isolates showed maximum activity in presence of Mn^{2+}. Phytase from CF2 was most inhibited by the presence of Zn^{2+} while phytase from all other yeast isolates were inhibited to maximum level by the presence of Cu^{2+} (Fig. 8).

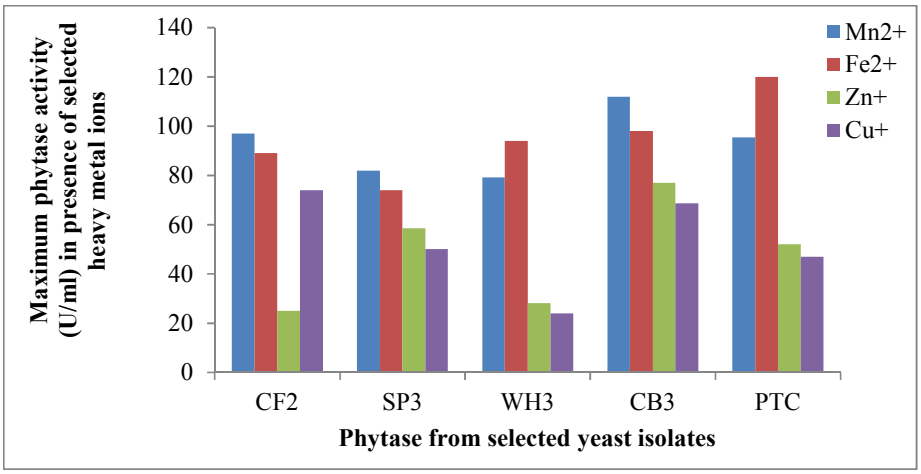

FIGURE 8: Maximum phytase activity in presence of selected heavy metal ions

Optimized phytase activity of *Candida parapsilosis* (CB3)

Phytase from CB3 had maximum activity 112 U/ml in presence of $Mn^{2+,}$ which was double than its optimal activity but showed least activity 68.7 U/ml in presence of Cu^{2+} (Fig. 9).

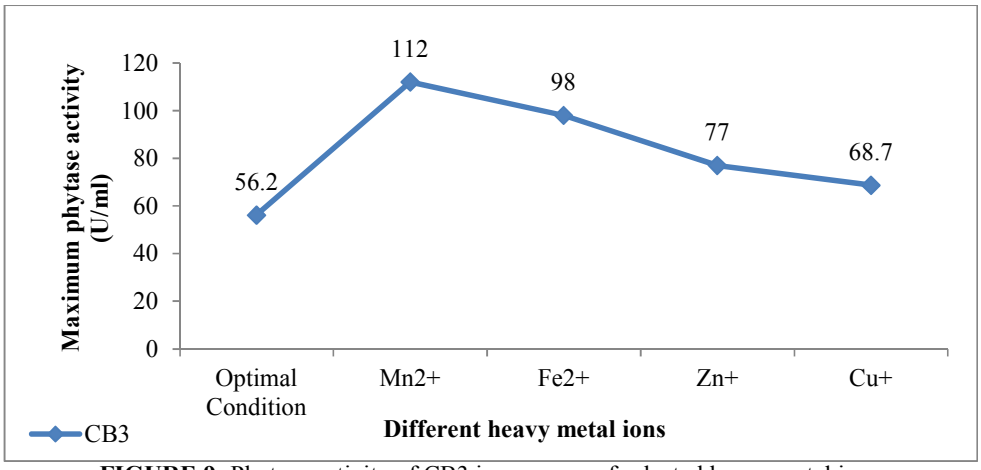

FIGURE 9: Phytase activity of CB3 in presence of selected heavy metal ions

CONCLUSION

This work reports the production of extracellular phytase from Candida parapsilosis and other yeast isolates. Maximum protein present in crude extract isolated by solid state fermentation (SSF), but more phytase activity was shown by the crude extract from submerged fermentation (SMF) indicating the presence of other proteins in higher proportion in case of SSF. The phytase activity of the crude enzyme from CB3 was 20.6 U/ml and this was increased to 56.2 U/ml in optimum condition. Temperature 37°C and pH 3.0 was optimum for maximum phytase activity of *Candida parapsilosis* but enzyme was also tolerable at broad range of temperature (25° to 45°C) and pH (2 to 6.8). Phytase activity of CB3 phytase was doubled due to optimization (112 U/ml). The enzyme being thermostable and acid stable, can find application in animal feed industry for improving nutritional status of the feed and combating environmental phosphorus pollution.

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