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Characterization and Identification of Phytate Solubilizing Yeasts Isolated from Food Grains

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A B S T R A C T

Phytase activity of bacteria, yeasts and moulds isolated from different ecosystems have been identified and studied by several researchers. The present study focused on isolation and identification of phytate solubilizing yeasts associated with freshly soaked cereals, pulses, condiments and oilseeds. Isolation on MRS-Calcium phytate at pH 6.2 ±0.2 resulted in a high diversity of microorganisms developing clear zones of phytate hydrolysis. Microscopic study revealed identification of three groups of phytate solubilizing organisms (*viz.*, yeasts, bacteria and moulds); only yeasts were selected for the following study. Screening on phytase screening medium (phytate as the only source of P) confirmed the phytase activity of a few yeasts species within 2 to 4 h of inoculation. Morphological, physiological and 18SrRNA analysis confirmed that yeasts isolates belonged to the genera; *Meyerozyma*, *Saccharomyces*, *Geotrichum* and *Rhodotorula* species. This preliminary investigation has discovered a novel enzyme activity in *Meyerozyma*, *Geotrichum* and *Rhodotorula* which has not been reported earlier. Detail analysis of these identified strains with respect to phytate degradation *in vitro* and *in vivo* is being studied in our laboratory to check the potential application of its enzyme 'phytase' in the feed technology.

Introduction

Phytic acid is a complex organic compound originating in the plant system. It is the major storage form of phosphorus and other nutrients in plants. Phytate (*myo*-inositol hexakisphosphate) is formed naturally during maturation of plants and their seeds. Its content is quite high in cereals, oilseeds, condiments and nuts. Earlier several scientists isolated phytate from plants and seeds in the form of a 'globoid' and confirmed its main chemical contents to be carbon, phosphorus and metal ions. Winterstein (1897) named these globoids as inositol-phosphoric acid. Its chemical nature was later studied in detail and the name was revised as *'myo*-inositol (1, 2, 3, 4, 5 and 6) hexakis-dihydrogen phosphate' by IUPAC-IUB (1968). Phytic acid is considered as an anti-nutritional factor of plant based foods. It is not digestible by human beings and non-ruminant animals as their digestive system lacks the enzyme to solubilize them.

Hydrolysis of phytic acid is important in the human diet, especially to vegetarians. Phytase (*myo*-inositol hexakisphosphate phosphor hydrolases) is special class of enzyme

specifically hydrolyzing highly stable phytic acid (phytate), releasing free *myo*-inositol phosphates and minerals. Phytase is widespread in nature. It is occurring in every plant system, dry seeds and selected animal tissues.

Phytase activity is prevalent in microorganisms. Since the understanding of the significant role of enzyme phytase, industrial development in food and feed processing has created a potential of over $500 million market, mainly as feed additive in poultry farming (Mullaney *et al.,* 2000). During the last 50 years, research has led to an increased use of phytase supplemented soybean and corn meal along with other plant materials as protein and mineral sources in animal feeds.

Phytase activity has quite frequently been detected in fungi. Various species of yeasts and moulds have been identified to produce good extracellular enzyme activity. Strains of *Aspergillus ficuum* and *A. niger* have already been employed for industrial purposes. Other moulds like *Mucor piriformis* and *Rhizopus oligosporus* are also known to produce phytase (Bogar *et al.,* 2003a).

Yeasts are commonly found in the surrounding environment and are the common inhabitants of food as well as gastrointestinal tract of animals and human beings. Several yeast strains are known to produce this enzyme. Phytase activity has been detected in *Schwanniomyces occidentalis, Pichia anomala* and few *Saccharomyces* species (Nakamura *et al.,* 2000). According to Shamna *et al.,* (2012) since phytate in the small intestine negatively affects absorption of minerals like Zn, Ca, Mg and Fe and reduce digestibility of dietary proteins, using certain yeast species as probiotics would probably help to overcome negative effects of plant phytates. These probiotic yeasts can possibly be found occurring naturally in our daily food. Hence the main objective of this study was to isolate and characterize phytate solubilizing yeasts from different food grains like cereals, oilseeds, pulses and condiments. This would represent the significance of yeasts microbiota naturally present in food grains. Native yeast plays an important role in natural fermentation of sprouted grains and grain-flours.

Materials and Methods

Isolation of phytate solubilizing yeasts

Selected grains/seeds of cereals, pulses, condiments, oilseeds were soaked for 2 h in sterile water and isolations were carried out by standard plate count method. Food yeasts are fast growing common contaminants in the MRS medium (De Man *et al.,* 1960) which is used to isolate lactic acid bacteria from foods. Hence this medium was utilized to isolate yeasts from soaked grains/seeds. Potassium orthophosphate from MRS medium was eliminated, and 0.5% calcium phytate (Organic source- phytic acid calcium salt, HiMedia) was used as a substrate for the induction of phytase. Only zone forming colonies were picked up and purified by streak plate method using MRS medium (devoid of dipotassium orthophosphate). Pure cultures, both bacteria and yeasts were preserved in 15% glycerol (at -20°C) for further study. Only phytate solubilizing yeasts strains were considered for the following investigation and were coded as 1 to 13 (Table 1).

Characterization of phytate solubilizing yeasts

Morphological study

All the phytate degrading yeasts isolates were stained with lactophenol cotton blue (HiMedia) and observed under microscope at

40X and 100X magnifications. Morphological characters like colony type, cell shape, cell arrangement cell arrangement, size and budding nature was recorded by simple staining, without heat fixing. Yeast isolate selected as positive for solubilization of calcium phytate in MRS medium were characterized and tentatively identified as described by Deak and Beuchat (1996) and Van der Walt and Yarrow (1984). Each isolate was given group code as M, S, G and R (Table 2) and strains were coded as 1 to 13.

Physiological study

Phytate solubilizing yeast isolates were screened for the enzyme activity *viz*., amylase, caseinase, gelatinase and hemolytic activity by culturing them on starch agar (Harrigan and McCance, 1979), skim milk agar (Chalmers, 1962), gelatine medium (Smith *et al.*, 1952) and sheep blood agar (Mata *et al.*, 1969) respectively. Reference organism *Saccharomyces cerevisiae* positive for starch hydrolysis was inoculated in one section and phytate solubilizing yeast on the other half section of the starch agar plate. Amylase activity was tested by flooding iodine solution on 48 h incubated starch agar plates. Isolates representing clear zones were recorded as positive (+) and those appearing blue were recorded as negative (-).Casein hydrolysis was tested by observing clearance of white colour (milk) in the skim milk agar plates. Test tubes containing gelatin medium were allowed to solidify in the refrigerator. The medium was inoculated with phytate solubilizing yeasts by stab method and incubated at room temperature (28°C). Test tubes were transferred in refrigerator for 2 h to check gelatin hydrolysis after 72 h of incubation. The test tubes with molten state of gelatin after refrigeration were recorded as positive and those in solidified state were recorded as negative for gelatinase activity. Hemolysis test was carried out by aseptically adding freshly drawn sheep blood into molten SBA agar medium, mixed thoroughly, poured into the plates and allowed to solidify. The yeasts cultures were streaked on the plates and incubated at 37°C. Plates were observed for hemolysis of blood (indicated by the halo zone) on SBA medium after 48 h.

In sugar fermentation tests, phytate solubilizing yeasts isolates were inoculated in yeast extract sucrose (YES) broth that contained 20% sucrose and 2% yeast extract (Daouk *et al.*, 1995). The cultures were incubated in water bath shakers for 48 h at 32°C to visually inspect froth formation on the surface (CO_2 production). Later the same cultures were kept still (without shakers) for another 24 h to check alcohol production. *Saccharomyces cerevisiae* var., *ellipsoideus* that ferments sucrose to alcohol with rapid production of CO_2 (with accumulation of froth) was used as a reference organism.

Screening of selected isolates

Screening was carried out to check the growth and confirm phytase activity of the isolates on chemically defined phytase screening agar medium [PSM] (Howson and Davis, 1983). Calcium phytate (0.5%) was added to medium as the only source of phosphorus. PSM agar plates were marked into quadrants and loop full of yeast culture was placed directly on four different spots marked in PSM plates. The plates were observed for clear zones every 2 h and up to 24 h of incubation (at 32°C). Strains that showed good zone of solubilization within 2- 4 h were designated as the best strains. From each group one strain was selected for further identification.

Identification of effective phytate solubilizing yeasts by 18SrRNA

One species from each group of tentatively identified yeasts genera (Table 1) was selected for 18SrRNA analysis. Total genomic DNA from yeast was isolated by N-

Cetyl- N, N, N-trimethyl- ammonium bromide (CTAB) method. Quantity of the isolated DNA was checked in UV-VIS spectrophotometer (Vivaspec Biophotometer, Germany). From the stock solution, 1µl DNA was mixed with 49 µl sterile distilled water to get 50 times dilution. The A260/A280 ratio was recorded to check purity of DNA preparation. Polymerase chain reaction **(**PCR) amplification of internal transcribed spacer (ITS) region was done in 20 µl of reaction mixture. BIOTRON (German make) PCR instrument was used for amplification.

Oligonucleotide primers used:

Oligonucleotide	Sequence (5'-3')	GC %	T_m value	Length	Product size
18S FP	GCCTGTCTCAAAGATTAAGCC	48	52.4	C21	700bp
18S RP	CACCTACGGAGACTTTGTTAC				

GC: Guanine and cytosine, Tm: melting temperature, FP: forward primers, RP: reverse primers

PCR temperature profile maintained is as follows

Process	Temperature	Time
Initial denaturation	94°C	2 min
Denaturation	48°C	50 s
Annealing	48°C	30 s
Extension	72°C	1 min 30 s
Final extension	72°C	6 min

30 cycles

BLAST analysis

BLAST program was used for the data submitted to NCBI Gene-Bank database to assess the DNA similarities. Multiple sequence alignment of 18SrRNA sequence was conducted followed by Phylogram using CLUSTALAW multiple sequence alignment tool.

Results and Discussion

Isolation of phytate solubilizing yeasts

Yeasts were found to be growing luxuriously in the MRS medium of pH 6.2 ± 0.2. The visible zones of clearance (Ca- phytate hydrolysis) around the colonies in the MRS isolation plates aided in recognizing phytate degrading yeasts from the other species.

Characterization of phytate solubilizing yeasts

Morphological study

The morphology study (Table 1) resulted in presumptive identification of four different species of yeasts *viz*., *Meyerozyma* sp., *Saccharomyces* sp., *Rhodotorula* sp. and *Geotrichum* sp.

Physiological study

Yeasts revealed different enzyme activity other than phytase. Growth was observed in all the media (starch agar, milk agar, gelatin and sheep blood agar), but the strains showed negative test for casein, gelatin and hemoglobin hydrolysis (hemolysis). Most of the yeasts isolated from food grains were positive for starch hydrolysis except one

strain of *Meyerozyma* (M_3) and two strains of *Geotrichum* (G_{11} and G_{12}). The addition of iodine to starch agar plates inoculated with yeast isolates exhibited wide zone of starch degradation indicating good amylase activity. Phytate solubilizing yeast isolates showed good turbidity in YES medium and population significantly increased within 24 h of inoculation Group-S (*Saccharomyces* sp.) and reference organism *Saccharomyces cerevisiae* var., *ellipsoideus* produced profuse froth on the surface of the broth which was an indication of rapid CO_2 production. It also showed presence of alcohol. However, group-M (*Meyerozyma*), G (*Geotrichum*) and R_{13} (*Rhodotorul*a) did not produce any froth on the surface of the broth and there was no production of alcohol.

Screening of selected isolates

PSM screening confirmed the phytase enzyme activity of all the strains selected from the isolation plates. The zone of phytate solubilization was observed in some strains within 4 h of inoculation in PSM medium (Table 2). These strains were selected for 18SrRNA analysis.

Identification of effective yeast strains by 18SrRNA

BLAST analysis of the gene sequence (601bp) of phytate solubilizing budding yeast isolate selected from the group- M (Table 1) identified M_4 as *Meyerozyma guilliermondii* with 99% homology. The gene sequence (610 bp) of oval shaped budding yeasts S_8 characterized as *Saccharomyces* was identifed as *S. cerevisae* with 100% homology. The gene sequence (519 bp) of phytate degrading pseudo-mycelial, white powdery yeast G_{12} was identified as *Geotrichum candidum* with 99% homology. The gene sequence (581bp) of the pink pigmented yeast isolate R_{13} (Table 1) was identifed as *Rhodotorula mucilaginosa* with 98% homology.

Yeasts are always associated with cereals and pulses and are predominantly involved in the fermentation of sprouted-grains (beer production) and grain-flours (*idli /dosa* batter fermentation). In this study, isolation in MRS-Phytate medium resulted in various groups of phytate utilizing yeasts. Phytate solubilizing microorganisms have been isolated from different food sources by researchers on MRS medium enriched with phytate as it is known to induce phytase activity in the growth medium (Haros *et al.,* 2008 and Raghavendra and Halami, 2009).

Varied colony morphology as well as phytase activity was observed within 24 h of isolation indicating rich diversity of phytate solubilizing yeasts inhabiting food grains. Phytase activity of yeasts like *Saccharomyces* sp. isolated from cereal based foods and beers have been reported by Nuobariene *et al.* (2011). Presence of different groups of native yeasts in food grains and their significant role in fermentation process has been reviewed in detail by Poutanen *et al.,* (2009). *Geotrichum candidum* is an important yeast in agro food industries in Western countries. It is always found associated with grains, fruits and diary products (Gente *et al.,* 2006). Association of *Meyerozyma* (earlier referred as *Candida*), *Geotrichum* and *Rhodotorula* with foods has been reported by several researchers (Tamang *et al*., 2016).

Table.1 Morphological study of phytate solubilizing yeasts isolated from food grains/seeds

Source	Strain code	Strains exhibiting similar colony morphology	Strains exhibiting similar cell morphology (Shape and Size [µm])	Presumptive identification
Black gram	1	Creamy, off- white, circular and convex/flat colonies. Smooth surface with entire colony margin. No mycelia or pseudohyphae observed. Fast growing in MRS- phytate medium.	Single, oval to ellipsoidal and scattered cells. Cells elongate extensively while budding. Single cell av. size 3.65x2.25 µm. No spores seen.	*Meyerozyma* sp. (group code M)
Corn	2			
Beans	3			
Lentil	4			
Wheat	5			
Barley	6	Moist, opaque or creamy, circular and flat colonies Smooth or rough surface colonies. No mycelia, however some older strains showed pseudohyphae. Fast growing in MRS -phytate medium.	Single, oval to ellipsoidal and scattered cells. Cells elongate slightly during budding. Single cell av. size on 2.58x1.50 µm. No spores seen.	*Saccharomyces* sp. (group code S) .
Fenugreek	7			
Mustard	8			
Sorghum	9			
Wheat	10			
Beans	11	Fast growing, large, off- white circular colonies and sticky mass in MRS -phytate medium. Powdery and white cottony appearance in PSM medium. Filaments visible at 10X. Indication of mycelia or branched hyphae.	Large cylindrical/rectangular cells in chains or singles, cells appear rounded at ends. Hollow filaments/hyaline seen. No budding observed. Cell av. size 4.65x2.50 µm.	*Geotrichum* sp. (group code G)
Corn	12			
Chick pea	13	Pink/orange pigmented circular and convex colonies Smooth glossy surface. Slow growing in MRS -phytate medium. No mycelia or pseudohyphae observed.	Round to oval and single. Cells adhering to each other (presence of mucilaginous substance), budding observed. Single cell av. size 3.20x3.10 µm.	*Rhodotorula* sp. (group code R)

Table.2 Physiological study of phytate solubilizing yeasts isolated from food grains/seeds

Tentatively Identified groups (with strains 1 to13)		Starch Hydrolysis	Casein Hydrolysis	Gelatine Hydrolysis	Hemolytic Activity	YES Broth		Screening (zones within hours)
						Froth	Alcohol	
Meyerozyma (M)	M_1	+	-	-	-	-	-	6 h
	M_2	+	-	-	-	-	-	4 h
	M_3	-	-	-	-	-	-	4h
	M_4	+	-	-	-	-	-	**2 h**
	M_5	+	-	-	-	-	-	4 h
Saccharomyces (S)	S_6	+	-	-	-	+	+	8h
	S_7	+	-	-	-	+	+	8 h
	S_8	+	-	-	-	+	+	**4 h**
	S_9	+	-	-	-	+	+	6 h
	S_{10}	+	-	-	-	+	+	8 h
Geotrichum (G)	G_{11}	-	-	-	-	-	-	2 h
	G_{12}	-	-	-	-	-	-	**2 h**
Rhodotorula (R)	R_{13}	+	-	-	-	-	-	8 h

Note: Numericals indicate phytate solubilizing strains isolated from soaked grains. PSM screening observations recorded every 2 h and upto 24 h. YES broth-Yeast extract sucrose; PSM-Phytase screenng medium. Highlighted strains indicate efficient phytate solubilizing yeast in each group.

However their phytase activity has not been so far reported by any investigators. Thus, the study reveals the novel enzyme activity of yeasts associated with food grains. *Saccharomyces* sp. isolated from sprouts or soaked grains are well studied for their amylase activity. Hence, they find an effective application in fermentation of cereals. Most of the yeasts studied have led to negative results for casienase and gelatinase activities. Negative results for hemolysis indicated that organisms do not lyse red blood cells, hence could be considered as non-pathogenic. Strains displaying accumulation of lather (rapid CO_2 production) on the surface of YES broth with alcohol production were considered as *Saccharomyces* sp. as these characteristics are commonly observed in *S. cerevisiae* (Deak and Beuchat, 1996). Screening on phytase screening medium (PSM) revealed the effectiveness of the yeast strains in hydrolyzing calcium phytate within 2-4 h. Screening by PSM plate assay has been characteristically used to screen phytate solubilizing microorganisms (Sreedevi and Reddy, 2012). PSM is chemically defined minimal nutrient medium with phytate (organic compound) as the only source of P. It was observed that PSM did not support vigorous growth of yeasts cells but induced phytase activity, displaying maximum diffusion ability of the enzyme phytase within 24 h. This was probably due to minimum nutrients accessibility. It is a known fact that cells under P- stress induce phytase activity in the presence of phytate and absence of inorganic P source (Kim *et al.*, 1999).

Strain M_4 identified as *Meyerozyma guilliermondii*, G_{12} identified as *Geotrichum candidum* and S_8 identified as *Saccharomyces cerevisiae* by 18SrRNA analysis were selected as competent phytate solubilizing yeast for further study. The pink pigmented

strain R_{13} identified *as Rhodotorula mucilaginosa* was not considered as the strain was slow growing and showed delayed zone of phytate solubilization. Detail analysis of these identified strains with respect to phytate solubilization/degradation *in vitro* and *in vivo* is being studied in our laboratory to check the potential application of its enzyme 'phytase' in the feed technology.

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