NUSANTARA BIOSCIENCE
Vol. 9, No. 1, pp. 68-76
February 2017

ISSN: 2087-3948
E-ISSN: 2087-3956
DOI: 10.13057/nusbiosci/n090112

Production and characterization of a neutral phytase of *Penicillium oxalicum* EUFR-3 isolated from Himalayan region

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Manuscript received: 6 January 2017. Revision accepted: 27 January 2017.

Abstract. *Kaur R, Saxena A, Sangwan P, Yadav AN, Kumar V, Dhaliwal HC. 2017. Production and characterization of a neutral phytase of Penicillium oxalicum EUFR-3 isolated from Himalayan region. Nusantara Bioscience 9: 68-76.* Micronutrient bioavailability from cereals is poor to monogastric animals due to presence of phytate as a chelating agent in seeds and an exogenous application of phytases is required to overcome antinutritional effect of phytate. In the present investigation, 40 fungal isolates including *Aspergillus, Penicillium, Trichoderma, Tolipospori*um, *Cladosporium, Talaromyces* etc. from different habitats were screened positive for phytase activity on specific media. On the basis of phytase production potential in solid state fermentation conditions, plant epiphytic fungi *Penicillium oxalicum* strain EUFR-3 has been observed with maximum phytase activity of 12.8 U/g. The biochemical properties i.e. activity and stability under optimum pH and temperature, and effect of modulators on phytase activity from this isolate were studied for its prospective application in human food. The *P. oxalicum* EUFR-3 phytase (PhyP-EUFR3) had maximum activity at pH 7.0 and temperature 40 °C. It was stable in pH range of 3-8 with more than 60 % activity throughout investigation. The thermostability of PhyP-EUFR3 was about 30 % residual activities after 10 min at 80 °C. Further purification, characterization and study of catalytic mechanism will be useful in better utilization of this phytase in human food for increased bioavailability of important metal ions.

Keywords: Micronutrient bioavailability, phytase, phytate, *Penicillium oxalicum*

INTRODUCTION

Phytate (*myo*-inositol 1,2,3,4,5,6-hexa*kis*phosphate; IP_6) is the major storage form of phosphorus (P), 65-80 % of total P in grains (Lott et al. 2000). It exists primarily as metal phytate complex with nutritionally important divalent cations (Bohn et al. 2008). Monogastric animals, e.g., human, pig, poultry and fish, poorly utilize this form of phytate due to lack of enzyme or the inadequate catalytic ability for hydrolysis of phytate complexes (Roy et al. 2009). Therefore, phytic acid cannot be absorbed in the digestive tract of monogastric animals and released into the environment and causes pollution. To meet their daily need, addition of external source of phosphorus and micronutrients in diet of these animals is required, which leads to increased cost of diets. The reduction of phytic acid content in food and feed by enzymatic method is therefore desirable as it improves the nutritional value of the food and feed besides removing the anti-nutritional properties of phytic acid with concomitant reduction in environmental phosphorus pollution (Singh 2013).

Phytases (myo-inositol 1,2,3,4,5,6-hexakisphosphate phosphohydrolase) are a special group of phosphatases which catalyzes the stepwise removal of phosphates from phytic acid or its salt phytate (Kumar and Agrawal 2014; Lei et al. 2013). The supplementation of diets with microbial phytases increases the bioavailability of phosphorus and micronutrients besides reducing the aquatic phosphorus pollution in the areas of intensive livestock production and combating environmental phosphorus pollution (Kumar et al. 2015). Phytases has tremendous economic impact in animal feed market and according to the report of first phytase summit in 2010, phytases share 60% of total feed enzyme market with a global phytase market of $350 million annually (Reddy et al. 2015). Phytases are widely distributed among plants (Hegeman and Grabau 2001; Wang et al. 2014), bacteria (Dan et al. 2015; Jain and Singh 2016; Kim et al. 1998b) and fungi (Bala et al. 2014; Howson and Davis 1983). Based on the sequence information and 3-D structures of phytases, these are classified into four major classes including histidine acid phytases (HAPhy), β-propeller phytases (BPPhy), PTP-like cysteine phytases (CPhy) and purple acid phytases (PAPhy) (Kumar et al. 2012; Kumar et al. 2016; Lei et al. 2007). Although various phytase preparations are commercially available, none of the commercial phytases assessed satisfied all of the criteria of an ideal phytase for use in animal feed (Boyce and Walsh 2006). For efficient industrial application, a phytase which has high production, good activity at 37 °C, high activity and stability at broad pH range and ability to tolerate the high temperature are desirable characteristics. Therefore, most of the research on phytase has been focused on isolation and characterization of novel phytase with desirable characteristics from different sources (Huang et al. 2009; Kalsi et al. 2016; Kumar et al. 2013; Mullaney et al. 2010). *Penicillium*

oxalicum has been reported as a promising phytase producing fungi with reports on characterization, heterogeneous expression and engineering of phytase from this fungi are available (Lee et al. 2007; Lee et al. 2015; Zhao et al. 2010). Filamentous fungi have been reported to good sources of phytases with higher production potential than bacteria and can lead to economic production because of their potential to grow on various agro-residues for the production of phytases in SSF (Salmon et al. 2012; Singh et al. 2015). Filamentous fungi may produce phytase directly on the selected feed/foods in SSF, or crude product may be mixed in feed rations as a value-added supplement (Bogar et al. 2003). Fungal product also contains accessory enzymes, fungal proteins and organic acids with phytase that increase feed digestibility and access to phytate in plant cells (Singh and Satyanarayana 2006). In this investigation, 40 fungal isolates with phytase positive response were isolated from different habitats and analyzed for phytase production. Further, phytase enzyme from fungal isolate *Penicillium oxalicum* strain EUFR-3 with maximum phytase production was characterized for important biochemical properties for determining its applicability in value addition of human food.

MATERIALS AND METHODS

Isolation of phytate-hydrolyzing fungi

Phytase hydrolyzing fungi were isolated from different source samples (Poultry soil, Rhizospheric soils, Leaves, Fungal infected wheat spike, Compost and Degraded wood samples) taken from area in and around Baru Sahib, Sirmour, Himachal Pradesh (Latitude 30.8244, longitude 77.26855). The isolation of fungi isolates was achieved using direct tissue inoculation and serial dilution approach. Briefly, for each sample, 1 g of collected sample was added to 9 mL of sterile water in a test tube and mixed properly to get a dilution of 10^1 times. Further, 1 mL diluted sample from this tube again added to 9 mL sterile water in next tube and repeated thereafter upto a dilution of 10^5 times. From this dilution, 100 µL samples were plated directly onto Czapek Dox Agar (CDA) media [composition (g/l): Sucrose 30, $NaNO_3$ 2, K_2HPO_4 1, $MgSO_4$ 0.5, KCL 0.5, $FeSO_4$ 0.01 and pH 5.5] plates and incubated at 35 °C for 2-6 d.

Screening of phytate-hydrolyzing fungi

The isolates from all sources were culture purified through sub culturing and further inoculated on phytase screening media [composition of PSM (g/l): D-glucose 10, Na-phytate 4, NH_4NO_3 2, KCl 0.5, $MgSO_4.7H_2O$ 0.5, $FeSO_4$ 0.01, $MnSO_4.H_2O$ 0.01, Agar 15 and pH 5.5]. PSM plates inoculated with fungal spores were incubated at 37 °C for 5-6 d. The isolates forming hydrolysis zone from all sources were purified and further screened for their phytate hydrolysis potential. The plates were then examined for halo zone around fungal culture and solubilization Index (SI) was calculated as, SI = (colony diameter+halo zone diameter)/colony diameter (Singh et al. 2014). Pure cultures of phytase positive fungal isolates were maintained on CDA agar plates and slants. The isolates were given name as 'EUFR' followed by numerals.

Morphological, taxonomic and molecular identification of phytate-hydrolyzing fungi

Morphological identification of isolates was performed by preparation of slides of fungal spores and mycelium followed by their microscopic examination of morphology (shape, size, margins and pigmentation) in order to easy identification and maintenance of pure culture of the isolates. Fungal isolate EUFR-3 with highest phytase production was identified taxonomically using microscopic examination and molecular level using multigene sequencing of ITS region and beta-tubulin gene at National fungal collection facility, Agharkar Research Institute, Pune. For microbial and molecular identification of EUFR-3, fungal colonies were grown on MEA plates, and genomic DNA was isolated following the rapid salt-extraction method (Aljanabi and Martinez 1997). Fragments containing the region encoding the ITS 1-5.8S nrDNA-ITS 2 (ITS) were amplified using primer pairs ITS4 and ITS5 (White et al. 1990). A fragment of the *benA* gene was amplified using primer pairs Bt2a and Bt2b (Glass and Donaldson 1995). The PCR conditions, sequence alignment and subsequent phylogenetic analysis followed the methods of Houbraken and Samson (2011). The sequence searches were performed at NCBI using blastn and mycobank. The phylogenetic tree was constructed on the aligned datasets using the neighbor-joining method (Saitou and Nei 1987) implemented in the program MEGA 4.0.2 (Tamura et al. 2007). The partial ITS sequence was submitted to NCBI GenBank and the assigned accession number was KX865276.

Phytase production using solid state fermentation

Fungal culture and inoculum preparation

For the selection of fungal isolate with higher phytase activity using solid state fermentation, inoculum was prepared using mycelial blocks (5×5 mm) from an agar plate of fungal strains on CDA Media at 35 °C for 5 days to inoculate 100 mL of Czapek liquid media [Composition (g/l): sucrose 30, yeast extract 6, KH_2PO_4 1, $MgSO_4$ 0.5, $FeSO_4$ 0.01, pH 6.0] in a 250 mL shake flask and incubated at 35 °C and 250 rpm for 5 d in incubator shaker (Salmon et al. 2012).

Enzyme production using solid state fermentation

For production of phytase using solid state fermentation, air-dried wheat bran substrate (5g) was placed in Erlenmeyer 250 mL flasks, supplemented with 8 mL salt solution containing (g/l): $NaNO_3$ 2, K_2HPO_4 1, $MgSO_4$ 0.5, KCL 0.5, $FeSO_4$ 0.01 and 60 % moisture levels were adjusted with distilled water. The wet substrate was sterilized at 121 °C and 15 psi for 20 min. After it had cooled the substrate was inoculated with 1 mL spore suspension of the respective fungal spores. The contents were mixed completely and incubated at the 35 °C temperature under humidity conditions for 5 d. After fermentation, the fermented material was harvested and

assayed for phytase activity. All experiments were performed in triplicate.

Extraction and estimation of phytase

Crude enzyme was extracted by mixing the fermented substrate with 50 mL sodium acetate buffer (0.2 M, pH 5.5) and fermented slurry was filtered through and centrifuged at 10,000 rpm for 10 min. The supernatant was used for the assay of enzyme activities (Ramachandran et al. 2005). Phytase activity was determined by following the method of Engelen et al. (1994). In detail procedure, the reaction mixture contained 100 µL of enzyme sample and 900 µL of 0.1M acetate buffer, pH 5.5. It was followed by addition of 500 µL of 5 mM sodium phytate (Prepared in 0.1M acetate buffer, pH 5.5) as substrate. The reaction was carried out at 37ºC for 30 min, and then stopped by adding 500 µL freshly prepared colour reagent [The colour reagent was prepared by mixing 25 mL ammonium molybdate solution (10 g Ammonium molybdate tetrahydrate, 90 mL distilled water, 1 mL NH_3 (25 %) adjusted to 100 mL) with 25 mL ammonium vanadate solution (0.235 g ammonium vanadate, 40 mL distilled water at 60ºC, slowly added 2 mL of 65 % nitric acid before adjustment to 100 mL) and stirred while slowly adding 16.5 mL nitric acid (65 %). The colour reagent mixture was then cooled to room temperature and the volume adjusted to 100 mL]. In blank reaction, colour reagent was added prior to the incubation and substrate solution (added after incubation). The colour developed from the phytase activity was determined at 415 nm. One unit was defined as the amount of enzyme that released 1 µM of inorganic phosphate in 1 min. The amount of phosphate released was calculated based on standard curve of KH_2PO_4. Amount of protein in samples was estimated by Bradford method to calculate the specific phytase activity (Bradford 1976).

Biochemical characterization of PhyP-EUFR3 phytase

Biochemical properties of enzymes are important for their industrial and other applications. Therefore, *P. oxalicum* strain EUFR-3 phytase, named as 'PhyP-EUFR3', was characterized for its biochemical properties i.e., pH optima and stability, temperature optima and stability. As reported, enzymes are affected by metal ions and some other modulators; effect of metal ions and modulators was also estimated on concentrated phytase extracted from these isolates.

Effect of pH on enzyme activity and stability

The optimum pH for the activity of the fungal isolates were determined by carried out standard phytase assay with following buffers (0.1M); glycine-HCl (pH 2.0-3.0), sodium acetate buffer (pH 4.0-6.0) and Tris-HCl buffer (pH 7.0-9.0). Maximum enzyme activity was taken as 100 % and relative activity in other was calculated. Graphs were plotted between pH vs enzyme activity (% Relative activity). To determine pH stability of PSB phytates, 350 µL of the enzyme was preincubated with 350 µL of different buffers such as 0.1 M glycine-HCl buffer (pH 2.0-3.0), 0.1M acetate buffer (pH 4.0, 5.0, 6.0 and 6.5), 0.1M Tris-HCl (pH 7.0 and 9.0) at 4 °C for 2 h, in absence of phytic acid. After incubation, phytase activity was estimated in incubated samples using standard phytase assay with sodium acetate buffer. The residual enzyme activity was calculated. Graph was plotted between pH vs % residual activity.

Effect of temperature on enzyme activity and stability

The optimum temperature for phytase activity of fungal isolates was determined by performing the routine phytase assay at different temperatures i.e., 30 °C, 40 °C, 50 °C, 60 °C, 70 °C and 80 °C. The enzyme activity was calculated as % relative activity in comparison to maximum activity obtained at a particular temperature. For estimation of thermostability, enzyme in acetate buffer (0.1M, pH 6.0) was incubated at 80ºC for different time intervals (10 min and 20 min). After incubation, 500 µL of sodium phytate substrate solution (5mM, prepared in 0.1M Acetate Buffer pH 6.0) was added in each test tube. Reaction was carried out at 37ºC for 30 min and the residual activity of the enzyme was calculated as per standard protocol. Graph was plotted between incubation time vs % residual activity.

Effect of Metal ions and Modulators on enzyme activity

The effects of Metal ions NaCl, $CuCl_2$, $MgCl_2$, $CaCl_2$ and $FeCl_2$, enzyme modulators SDS, ascorbic acid, EDTA, β-mercaptoethanol, DTT and urea on phytases activity were measured in the reaction mixture as described. The 100 µL enzyme was pre-incubated with the 900 µL acetate buffer with respective modulator for 30 min at 37 °C, followed by the standard enzyme assay as described earlier. The activity assayed in the absence of the modulators was defined as the control.

RESULTS AND DISCUSSION

Isolation, screening and identification of phytate-hydrolyzing fungi

In present study, fungal strains from different sources (Poultry soil, Rhizospheric soils, Leaves, Fungal infected wheat spike, Compost and Degraded wood samples) directly isolated and screened on PSM plates. Initially, multiple colonies were observed on PSM plates with zone of hydrolysis. From these colonies, depending on number of colonies with zone on each plate, culture was taken randomly from 40 colonies forming zone of hydrolysis. These were purified and again grown on PSM plates (4 isolates on each plate) to reconfirm their phytase production ability. Initially screened isolates again shown repeated results on PSM plates, which confirm their phytate solubilization potential. Further, in order to determine extracellular phytase production potential in production media, all 40 isolates were cultured in production media under SSF conditions. It has revealed extracellular phytase production potential with varying amount of phytase activity units per gram of carbon source (Table 1). Isolate EUFR-3 was reported with highest phytase activity (12.8 U/g) followed by EUFR-16 (10 U/g), EUFR-2 (9.13 U/g), EUFR-1 (7.9 U/g) and EUFR-24 (7.1 U/g). Further selection for potential isolates was made on

the basis of higher solubilization index on PSM plates as well as phytase activity after SSF and a total number of 8 isolates (EUFR-1, EUFR-2, EUFR-3, EUFR-10, EUFR-15, EUFR-16, EUFR-19 and EUFR-24) were selected (Phytase activity of selected isolates is shown in bold (Table 1). The list of fungal isolates along with their isolation sources are given in Table 1.

On the basis of microscopic examination of spore and mycelium morphology, the isolates were identified as *Penicillium* sp. (10 isolates), *Aspergillus* sp. (9 isolates), *Trichoderma* sp. (4 isolates), *Alternaria* sp. (3 isolates), *Stachybotrys* sp. (2 isolates), *Actinomycetes* sp. (3 isolates), *Cladosporium* sp. (2 isolates), *Cephalosporium* sp. (2 isolates), Yeast sp. (2 isolates), *Toliposporium* sp. (1 isolate), *Seclerotia* sp. (1 isolate) and *Talaromyces* sp. (1 isolate) (Table 1). The morphological feature of fungal spores and mycelium of the selected isolates is shown in figure 1. Based on molecular characterization, the isolate EUFR-3 was identified as *Penicillium oxalicum* strain EUFR-3 (Accession No. KX865276), with 99 % similar to *P. oxalicum* (Accession No. HM05477) ITS sequence. The phylogenetic tree of ITS sequence of *P. oxalicum* with the ITS sequences of other phytase producing fungal isolates is given in figure 2. The phytate solubilization index of selected 8 isolates was in the range of 0.35 to 2.1 at different time interval of 24 to 72 h (Table 2). The halo zone diameter and solubilization index by different isolates are shown in Table 2. The maximum solubilization index was reported by EUFR-15 after 24 h, EUFR-16 after 48 h and EUFR-1 after 72 h.

Biochemical and kinetic properties of PhyP-EUFR3 phytase

Analysis of PhyP-EUFR3 phytase for its optimum activity at varying pH (using different pH buffers of pH 2.0-9.0) during phytase assay and its stability under treatment by different pH revealed that PhyP-EUFR3 phytase was active under broad range of pH with maximum activity at neutral pH 7 and more than 60 % activity at other pH in the range of 3-9. It was highly stable at pH range of 3-9 with more than 60 % residual activity throughout experimental analysis, taking maximum residual activity as 100 % (Figure 3).

Further analysis of PhyP-EUFR3 phytase for its optimum activity at varying temperature (using different temperature from 30-80 °C) during incubation at the time of phytase assay revealed that its activity varied from 30 °C to 80 °C under given conditions. PhyP-EUFR3 phytase had highest activity at 40 °C which decreased gradually towards higher temperature and no activity was reported at 80 °C (Figure 4).

The thermal stability of EUFR-3 phytase and the ability of the heat-denatured enzyme to refold were investigated at 80 °C. The temperature stability at 80 °C, when treated for 10 min/20 min before enzyme assay, the stability of EUFR-3 phytase was very less (23.45 % residual activity) at 80 °C after treatment for 10 min and after treatment at same temperature for 20 min, its % residual activity was 5.35 % of untreated enzyme.

Table 1. Isolation sources, morphological identification and phytase activity of 40 microbial isolates

Fungi	Isolation source	Identified as	Phytase activity (U/g)
EUFR-1	Wheat	*Penicillium* sp.	7.988
EUFR-2	Chilli seeds	*Talaromyces* sp.	9.140
EUFR-3	Soil	*Penicillium oxalicum*	12.80
EUFR-4	Poultry soil	*Trichoderma* sp.	0.707
EUFR-5	Guggal	*Aspergillus ochraceus*	1.930
EUFR-6	Maize Rhizosphere	*Aspergillus* sp.	0.085
EUFR-7	Bajra Rhizosphere	*Aspergillus* sp.	4.903
EUFR-8	Shilazit	*Trichoderma* sp.	0.058
EUFR-9	Shilazit	*Actinomycetes* sp.	0.150
EUFR-10	Bajra seed	*Tolyposporium* sp.	0.781
EUFR-11	Soybean Rhizosphere	*Actinomycetes* sp.	1.098
EUFR-12	Infected seed of flower	*Alternaria* sp.	0.903
EUFR-13	Parthenium plant	*Alternaria* sp.	0.058
EUFR-14	Plant bark	*Yeast* sp.	0.142
EUFR-15	Parthenium plant	*Penicillium crustosum*	1.220
EUFR-16	Infected wheat plant	*Ustilago* sp.	10.09
EUFR-17	Soil	*Penicillium* sp.	4.718
EUFR-18	Soil	*Aspergillus* sp.	2.951
EUFR-19	Bajra Rhizosphere	*Aspergillus* sp.	5.951
EUFR-20	Soybean Rhizosphere	*Aspergillus* sp.	1.171
EUFR-21	Wax	*Penicillium* sp.	2.075
EUFR-22	Wax	*Actinomycetes* sp.	0.488
EUFR-23	Plant bark	*Penicillium* sp.	0.258
EUFR-24	Wood	*Trichoderma viridae*	7.157
EUFR-25	Wood	*Cephalosporium* sp.	0.403
EUFR-26	Asrol	*Penicillium* sp.	0.703
EUFR-27	Lab contaminated media	*Aspergillus* sp.	0.988
EUFR-28	Garlic covering	*Alternaria* sp.	1.313
EUFR-29	Badam	*Cladosporium* sp.	0.039
EUFR-30	Lab contaminated media	*Cladosporium* sp.	0.077
EUFR-31	Wood	*Trichoderma* sp.	1.659
EUFR-32	Plant leaf	*Penicillium* sp.	1.198
EUFR-33	Kari patta leaves	*Secletoria* sp.	1.269
EUFR-34	Wood	*Stachybotrys* sp.	1.622
EUFR-35	Wood	*Penicillium* sp.	0.058
EUFR-36	Wood	*Cephalosporium* sp.	2.727
EUFR-37	Shilazit sample	*Stachybotrys* sp.	0.806
EUFR-38	Maize Rhizosphere	*Aspergillus* sp.	0.064
EUFR-39	Baker yeast	Yeast sp.	0.180
EUFR-40	*Zymograma bicalrata* Beetle	*Penicillium* sp.	0.012

Note: *Fungal isolates in bold were identified at ARI Pune

Table 2. Phytate solubilization index obtained by selected PSB Isolates

Fungi	Solubilization index		
	24 h	48 h	72 h
EUFR-1	1.33	1.37	2.1
EUFR-2	1.37	1.65	2
EUFR-3	1.05	1.1	1.4
EUFR-10	1.04	1.3	1.5
EUFR-15	1.41	1.5	1.7
EUFR-16	1	1.85	1.9
EUFR-19	0.45	1	1.2
EUFR-24	0.35	0.6	0.75

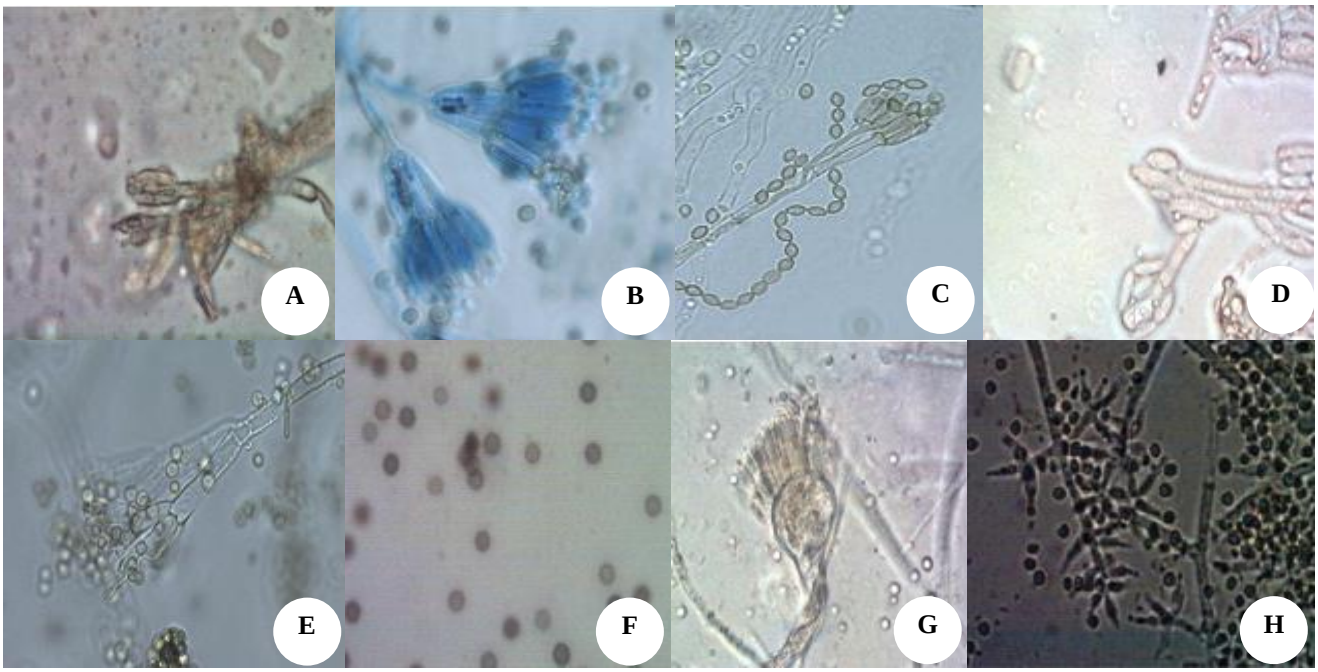

Figure 1. Light microscope (100X) based mycelium and spore structures of selected isolates for their morphological identification. A. *Penicillium* sp.EUFR-1, B. *Talaromyces* sp. EUFR-2, C. *Penicillium oxalicum* EUFR-3, D. Long smut fungi EUFR-10 (*Tolyposporium* sp.), E. *Penicillium crustosum* EUFR-15, F. Loose smut fungi EUFR-16 (*Ustilago* sp.), G. *Aspergillus* sp. EUFR-19, H. *Trichoderma viridae* EUFR-24

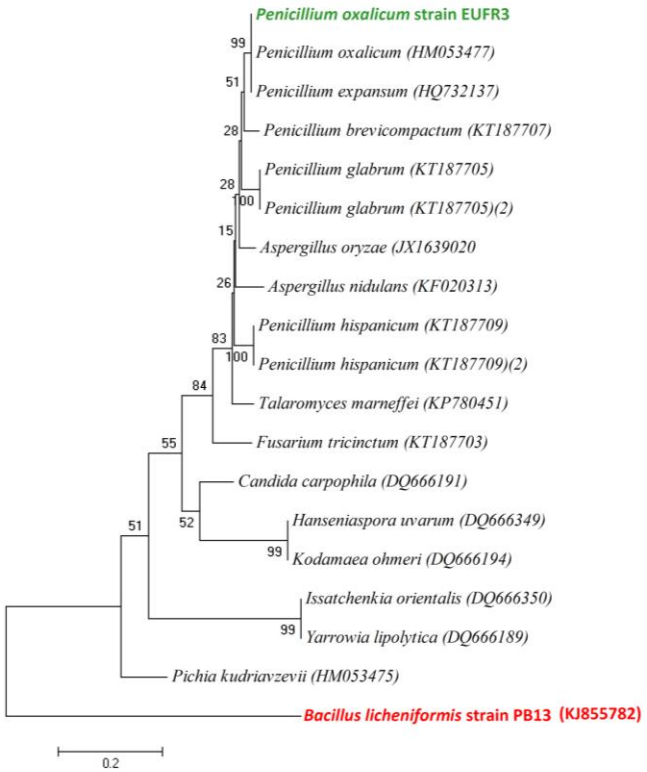

Figure 2. Phylogenetic tree showing the relationship phytase producing fungus, ITS sequences with reference sequences obtained through BLAST analysis. The sequence alignment was performed using the CLUSTAL W program and trees were constructed using Neighbor joining with algorithm using MEGA4 software (Tamura et al. 2007). The tree was rooted using *Bacillus licheniformis* strain PB13 (KJ855782) a phytase producing bacteria as the out group

Effect of metal ions and modulators on phytase activity

In this study, chloride ions of different metals i.e., sodium chloride, copper chloride, magnesium chloride, calcium chloride, ferrous chloride and several modulators (Ascorbic acid, EDTA, β-mercaptoethanol, DTT, Urea and SDS) were used to check activity of phytases in presence of these metal ions and modulators. On incubation with these metal ions and modulators, variation in activity of EUFR-3 phytase was reported (Figure 5). Maximum inhibition of about 46 % was reported by β-mercaptoethanol followed by ascorbic acid (~43 %), DTT (~29 %) and NaCl (~17 %). Other metal ions and modulators decreased phytase activity in the range of 5-10 % as compared to control, where no metal ion or modulator was added to assay buffer (considered 100 % activity). No modulators and metal ions under investigation reported to increase the EUFR-3 phytase activity.

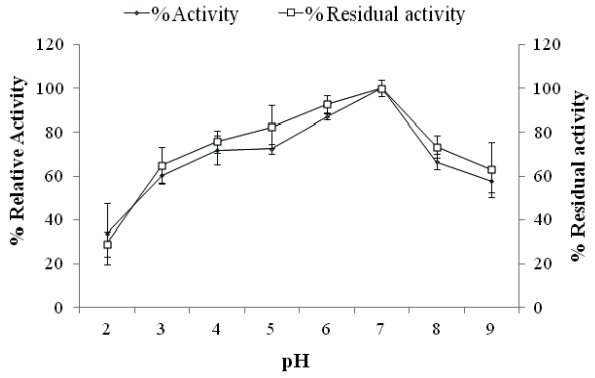

Figure 3. Effect of change in pH during phytase assay on phytase activity

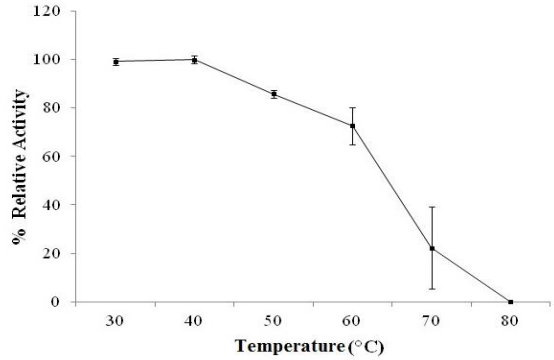

Figure 4. Effect of different incubation temperatures on phytase activity

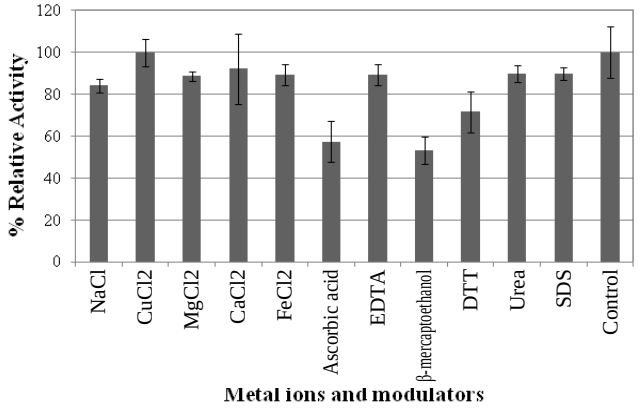

Figure 6. Effect of different metal ions and modulators on phytase activity during phytase assay

Discussion

Phytic acid is the major storage form of P in cereals and considered as an antinutritional factor due to its interaction with divalent cations including Fe^{2+} and Zn^{2+}. Absence of suitable enzyme system in digestive system of monogastric animals makes them incapable to hydrolyze and utilize this complex form of P and metals ions. Therefore, phytate decrease the bioavailability of these metal ions to monogastric animals including humans, especially in the case of population in developing countries largely depend on cereal based foods. Decreased bioavailability of Fe and Zn leads to their deficiency in large segment of world population and causing severe health and growth problems. As an intervention to alleviate this problem, supplementation of an enzyme capable of hydrolyzing this phytate-mineral complex in human food is a promising approach. Phytase is an enzyme chemically known as myo-inositol hexakisphosphate phosphohydrolase. It hydrolyzes phytate-mineral complex to myo-inositol, inorganic P and free divalent cations in a stepwise manner. Phytases were originally proposed as an animal feed additive to enhance nutritional quality of plant material in the feed for monogastric animals by liberating phosphate (Mitchell et al. 1997). In earlier studies, known phytate degrading organisms that include aerobic bacteria, anaerobic bacteria and fungi were isolated from different environmental conditions but fungi as a source of the higher phytase activity and cheaper than the other sources. Survey of microorganisms for selection of potential phytase producers was the subject of study by several workers (Hill et al. 2007; Kalsi et al. 2016; Kumar et al. 2014b; Kumar et al. 2013; Kumar et al. 2016; Mukesh et al. 2004; Yoon et al. 1996). Different workers used multiple sources for isolation of phytase positive isolates including different kinds of soils, animals, plants and geographical locations. Although several strains of bacteria and yeast can produce phytase, but fungal phytase has received more attention due to high production yields and acid tolerance for feed production (Kim et al. 1998a).

The use of calcium phytate as the sole phosphorus and carbon source and formation of halo zone on PSM plates and phytase activity in media was effectively used for selection of phytase secreting / phytate solubilizing fungi and has been adopted in several previous studies (Singh and Satyanarayana 2012), where each produced a positive response to the plate clearing as well as an enzyme activity assay. It is proved to be a useful and effective strategy for such studies on screening and isolation of phytase positive isolates as growth on plates is usually easier than in a liquid medium (Choi et al. 2001; Lim et al. 2007), hence no further assessment need to be conducted on isolates that could not grow or form halo zone on PSM plates. Further, there was no direct correlation was found between halo zone on PSM plates to the phytase activity in liquid media which might be due to different specificity of various phytases for sodium phytate and calcium phytate as in liquid media sodium phytate was used compared to calcium phytate in PSM plates. Some strains produced clearance zone but the diameter of the clearance zone and growth of the organism were comparatively very less, which could be a reflection of the isolates inability to degrade phytate or perhaps, merely an indication of unfavorable conditions for phytase production. Isolation of diverse group of fungi capable of producing phytase enzyme in present study reveals that there is tremendous potential in this microbial community for possibility of an ideal phytase and its industrial application after characterization of produced enzyme. Ability of fungal isolates from varying habitats to produce extracellular phytase reveals their high diversity

and further strengthens chances of getting potential isolates for a suitable phytase with desired properties. Variation in amount of phytase produced by different isolates is suggested to be because of variation in optimum growth and media conditions for each isolate, substrate specificity of phytases and characteristics of individual strains. The PhyP-EUFR3 phytase with good phytate solubilization index, highest extracellular phytase production was found to be interesting and could be utilized for further application purposes.

The use of filamentous fungi for the production of commercially important products through solid-state fermentation (SSF) has gained much research interest during recent years (Pandey et al. 2000; Singh and Satyanarayana 2012). SSF has several advantages over submerged fermentation (SmF), which include lower wastewater output, reduced energy requirements, simpler fermentation media, easier aeration, reduced bacterial contamination etc. (Pandey et al. 2000). The phytase enzymes produced by conventional submerged fermentation are expensive and commercial preparations are highly priced, potentially increasing feed costs by US $2–3 per metric ton. Therefore, solid state fermentation has gained significance as an economical production alternative (Bogar et al. 2003). Ideally the solid substrate should be a cheap agricultural by-product rich in fibre with available starch and protein in addition to providing a large surface area for mycelial growth, so in this study, wheat bran was used as substrate. In the present work, we demonstrated a good phytase activity production by *P. oxalicum* strain EUFR-3 in SSF culture conditions and using wheat bran as the sole carbon source. The production of phytase enzyme by strain EUFR-3 was comparable to *P. oxalicum* recombinant phytase production by Lee et al. (2007). Further optimization of production media could lead to enhanced production of enzyme.

The ability of a phytase to hydrolyze phytate in the digestive tract is determined by its enzymatic properties. Since the temperature and pH of the initial phytase screening were fixed, the screening did not always accurately determine the phytase activity of all the isolates. As the stomach is the main functional site of supplemental phytase, an enzyme with an acidic pH optimum is certainly desirable. The main area of concern for the phytase is the low activity and stability under different conditions of pH. Most microbial phytases studied so far show their optimum activity in the acidic pH range (Pandey et al., 2001; Vats and Banerjee, 2004; Rao et al., 2009). Phytases from fungal origin exhibit optimal activity at pH 4.5 to 5.5, while some bacterial enzymes at pH 6.5 to 7.5. From a physiologically relevant standpoint, these phytases displays significant activity and stability between pH 3.0 and 6.0, a range necessary to facilitate phytate degradation in the salivary gland (pH 5.0–7.0), stomach (fed state pH 6.5, reducing to 3.5–4.5 upon stimulation of acid secretion) and upper part of the duodenum (pH 4.0–6.0). Activity and stability of selected PhyP-EUFR3 phytase was good in different range of pH. Phytase from this study with more than 60 % stability at lower pH and even higher stability at higher pH values will be significant for its usability under condition of low pH in stomach. The loss of phytase activity under high alkaline and acidic conditions might be due to the protein structure of phytase changed under the strong alkali or acid conditions (Jorquera et al. 2008). The PhyP-EUFR3 phytase with very good activity at neutral pH could also be used as an additive in aqua and poultry feed. The pH optima of PhyP-EUFR3 phytase was comparable to phytases of *T. lanuginosus* (Berka et al. 1998) and *A. fumigatus* (Pasamontes et al. 1997) which were optimally active at pH 6.0 to 6.5. The optimal pH for EUFR-3 phytase activity was more than other reported phytase from *P. oxalicum* which were active in the pH range 4.5-5.5 (Lee et al. 2007; Lee et al. 2015; Zhao et al. 2010).

Temperature optima for PhyP-EUFR3 activity was in accordance to optimum temperatures reported by other workers i.e. 40 °C for that from *Bacillus sp*. KHU-10 (Choi et al. 2001), 45 °C for that from *Obesumbacterium proteus* (Zinin et al., 2004). It was less than that reported as 55C of *P. oxalicum* (Lee et al. 2007; Lee et al. 2015), 58 °C of *A. niger* 11T53A9 (Greiner et al. 2009), 60 °C of *Bacillus licheniformis* (Kumar et al. 2014a). Phytase of *A. fumigatus* (Pasamontes et al. 1997) and *A. niger* NRRL 3135 (Howson and Davis 1983) exhibited optimum activity at 37 °C and 55 °C, respectively. Thermal stability of phytase and the ability to refold to an active conformation are important properties from a commercial perspective. Enzyme thermal stability is pertinent in animal feed applications (Pandey et al. 2001), where the enzyme preparations are normally incorporated into the grains before pelleting. The pelleting of feed helps animals to have a balanced diet and also preserved from feed borne pathogens. However, during the pelleting process the enzyme is exposed to temperatures around 65–95 °C with holding times from 1-10 min (Bedford et al. 2001). Therefore, all feed enzymes need to be heat stable to avoid substantial activity loss during this process. Although phytase inclusion using an after-spray apparatus for pelleted diets and/or chemical coating of phytase may help bypass or overcome the heat destruction of the enzyme, phytases resisting high temperatures will no doubt be better candidates for feed supplements. As revealed from results, EUFR-3 did not possessed desired thermostability at higher temperature. It retained 25 % of its original activity at 80 °C for 10 min. In this regard, determination of thermostability of EUFR-3 phytase under varying temperature conditions and estimation of effect of different effectors on thermostability might lead to development of a bioprocess for its efficient application with improved stability under food processing conditions.

There are various reports in which metal ions have been shown to modulate phytate- degrading activity. Phytases from different microbes differed in their requirement for metal ions for their activity. The inhibitory effect of metal ions on phytase activity might be ascribed to the strong chelating ability of phytate, where binding of metal ions on phytate might impair access of the enzyme to the functional group on the phytate molecule leading to decreased activity (Maenz et al. 1999). The observations from effect of modulators inferred that β-mercaptoethanol was maximum effective in reducing the activity of PhyP-EUFR3 phytase.

As reducing reagents, β-mercaptoethanol and dithiotreitol (DTT), ascorbic acid have major effects on PhyP-EUFR3 phytase suggesting that this enzyme either have free and accessible sulfhydryl groups which might play an important role in the enzyme activity and structure. In case of urea, it inhibit PhyP-EUFR3 phytase activity >10 % suggesting that as concentration of urea increases, the kinetics of inactivation and the denaturation by urea increases linearly (Xiang et al. 2004). PhyP-EUFR3 phytase was found to be 57 % active with ascorbic acid, while phytase with other remaining metal ions and modulators was more than 60 % active. Ascorbic acid forms protonated chelates by coordinating with metal ions at low intermediate pH, while unprotonated chelates at higher pH (Martell 1982). Yanke et al. (1999) also reported that the mixtures containing 5 mM Fe^{2+}, Cu^{2+}, Zn^{2+} and Hg^{2+}, phytase activity of *Selenomonas ruminantium* was strongly inhibited. Phytase from *Enterobacter* sp. 4 was inhibited by each addition of 1 mM Zn^{2+}, Ba^{2+}, Cu^{2+}, Al^{3+} and EDTA (Yoon et al. 1996). The phytase producing bacterial strain, *Bacillus licheniformis* ONF2 was isolated from the proximal intestine of the freshwater fish, Nile tilapia, *Oreochromis niloticus.* The activity of the enzyme was moderately inhibited by 5 mM Mn^{+2}, Mg^{2+} and K^{+} and largely affected by the metal ions Cu^{2+}, Hg^{2+}, Zn^{2+}, Co^{2+} and EDTA but, in the presence of 1 mM $CaCl_2$, the inhibitory effect was less intense (Dan et al. 2015).

In conclusion, PhyP-EUFR3 phytase exhibiting stability over a broad pH range (2–8) and pH optima of 7.0 is of crucial importance in food applications as the pH of animal gastrointestinal tract ranges from 2.6 to 7.0, while phytases from *Aspergillus* sp. are active only under acidic pH range (2–5). Therefore, it is concluded that high phytase activity production and broad pH stability with desirable biochemical properties, *P. oxalicum* strain EUFR-3 phytase can be suitably applied in human food applications after further characterization and purification, and high phytase activity make this fungal strain potential isolate for industrial applications. Increasing activities of phytases from selected isolates using different approaches and evaluating their applications in increasing bioavailability of important minerals in human food would be of prime importance and focus on future studies.

ACKNOWLEDGEMENTS

The financial support provided by the Department of Biotechnology (DBT), Govt. of India (Grant No. BT/AGR/BIOFORTI/PHII/NIN/2011) for carrying out this work and Ministry of Food Processing Industries (MoFPI), Govt. of India for 'infrastructural facility development' in Akal College of Agriculture is duly acknowledged. Authors are grateful to Dr. P.K. Gill, Assistant Professor, Eternal University for technical advices during isolation of fungal isolates and Dr. KC Rajesh Kumar, Scientist C, Biodiversity & Palaeobiology Group, Agharkar Research Institute, Pune for his sincere help in molecular and microscopic identification of isolates. It is declared that there is no conflict of interest in publication of this work.

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