

Full Length Research Paper

***In vitro* degradation of natural animal feed substrates by intracellular phytase producing Shiwalik Himalayan budding yeasts**

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Himalayas are the natural reservoir of complex and diversified gene pool. Three Shiwalik Himalayan intracellular phytase producing budding yeasts were assayed for *in vitro* degradation of natural animal feed substrates. Phosphorus availability was found to enhance upto 70% yeast cultures during *in vitro* biodegradation of natural animal feed substrates. A direct correlation between intracellular phosphate concentration and phytase activity suggested the use of whole cell preparations in place of purified enzymes. Zymogram analysis revealed the presence of single high molecular weight isoform of the enzyme phytase. Based on 5.8S-ITS-rDNA sequencing, using ITS1 and ITS4 primers, the cultures were identified as *Candida tropicalis* (B4), *Issatchenkia orientalis* (PA4) and *Pichia gluvermondii* (SS1). Indigenous *I. orientalis* strain PA4 was found superior among all the yeasts strains and therefore can be developed as successful inoculant for animal nutrition as well as environmental management under Himalayan ecosystems.

Key words: Phytase, Shiwalik Himalaya, phytase biodegradation, 5.8S-ITS rDNA, Yeast identification.

INTRODUCTION

Phosphorus (P), like nitrogen, is an essential element for all forms of life. But approximately 75 to 80% of the total P in nature is found in the fixed organic form- phytate (myo-inositol 1, 2, 3, 4, 5, 6-hexakisphosphate, IP6). The phytic acid is the primary storage form of P in plants; constitutes 3-5% of dry weight of seeds in cereals and legumes that are used as principal components of animal feeds. It acts as an anti-nutritional component in plant-derived feed; as a result they are undesirable for mono-gastric animals. The excess of P in the feed that remains unutilized is partly excreted in manure and results in pollution of ground water leading to eutrophication of freshwater bodies. Facing the problem of P deficiency in plants and animals feed, together with its pollution in areas of intensive livestock production, phytase seems

destined to become increasingly important. Phytase, myo-inositol 1,2,3,4,5,6-hexakisphosphate phospho-hydrolases (EC 3.1.3.8) belongs to a sub-class of the family of histidine acid phosphatase as it can catalyze hydrolysis of phytate to inositol and orthophosphoric acid (Guilan et al., 2009).

Himalayan regions are well known for their diversified flora and fauna. Yeasts from these icy heights are well studied and characterized (Sourabh et al., 2012). The distribution of phytase is widespread among bacteria, yeast, fungi, plants, and also in animals (Mittal et al., 2012). However, negligible information is available about the phytase producing Shiwalik Himalayan Yeasts. Present study describes the phytase producing potential of the indigenous Himalayan yeast strains.

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Supplementation of yeast to animal feed as bio-inoculants can be an alternative approach to tackle P unavailability effectively because many yeast strains are already being used as single cell protein (SCP). In this perspective, *Issatchenkia orientalis* strain PA4 as an intracellular phytase producing yeast is particularly well adapted to the fluctuating temperatures of the Himalaya and could be used effectively as a low cost bioinoculant in Himalayan livestock nutrition and environmental management.

MATERIALS AND METHODS

Yeast cultures and screening for phytase production

Standard culture of *Saccharomyces cerevisiae* ATCC-9763 was procured in freeze-dried form from MTCC Chandigarh, India. Three budding yeast isolates (SS1, B4 and PA4) used in this study were obtained from departmental culture collection and revived on yeast extract peptone dextrose (YPD). Initially, the cultures were isolated from *Musa acuminata* fruit surface (B4), *Malus domestica* fruit surface (PA4) and *Sorghum bicolor* stem juice (SS1) from Pantnagar (29.00°N/79.28°E), a subtropical region of Indian Shiwalik Himalayas. The active cultures were screened qualitatively for phytase production using phytase screening medium (PSM) as described by Lambrechts et al. (1992). Sodium phytate (2 gL⁻¹) was filter sterilized and added to the sterilized medium before pouring. Yeast cultures were pin-point inoculated on MPSM plates using tooth pick and incubated at 30±1°C for 24 to 48 h. The plates were visualized for the microbial growth and the clear (halo) zone formation around the colonies following the method described by Yanke et al. (1998).

Prediction of growth pattern in response to P availability

For determining growth pattern, active cultures were inoculated individually (at 5% v/v) in MPSM broth containing 0.3% KH₂PO₄ or 0.3% sodium phytate separately and incubated at 30±1°C. The samples were withdrawn periodically at an interval of 2 h, upto a period of 96 h, till the stationary phase was achieved. Yeasts growth rate were analyzed overtime according to the Gompertz equation modified by Zwietering et al (1990):

$$y = A \cdot \exp\left\{-\exp\left[\left(\frac{\mu_{\max} e}{A}\right) \cdot (\lambda - t) + 1\right]\right\} \quad (1)$$

Where, y is O.D. value at time t (h), A represents the maximum O.D. (when t = ∞), μ_{max} is the maximum specific growth rate (h⁻¹) and λ is the lag time (h).

Generation time (mean doubling time) was calculated using the following formula:

$$g = 0.693/\mu \quad (2)$$

Where, g = generation time and μ = growth rate constant.

For modeling with Gompertz equation, the means of three replicates and two repetitions were used (Tofalo et al., 2009). In all the cases, the variability coefficient of raw data (cell load as O.D.) was <5%. The data relative to the growth kinetics were subjected to student's test to identify significant differences between yeast species using "Non-linear Regression Analysis" (NLREG, USA).

Estimation of phytase activity

For crude enzyme preparation, the cultures were grown in MPSM medium at 30±1°C (120 rpm) in an incubator shaker for 96 h.

Samples withdrawn periodically at an interval of 24 h, were centrifuged at 10,000 rpm (4°C) for 10 min. The culture filtrate was used as crude enzyme for determining extracellular phytase activity while cell pellet obtained as above was used for determining intracellular whole cell enzyme activity (Shimizu, 1992). The cell extracts for determining cell free intracellular phytase activity were prepared by the sonication protocol (Ruiz et al., 1999). Cell pellet were washed twice with normal saline (0.1% NaCl) and suspended in phosphate buffer (0.1 M, pH 7.0) in 1:1 ratio. Each cell suspension was sonicated on ice, at wave amplitude of 2 μm, for 6 cycles (30 s each) with 10 s interval after each cycle. PMSF was added to the final concentration of 1 mM. The sonicated suspension was centrifuged at 10,000 rpm for 10 min at 4°C. Supernatant was used as crude enzyme for quantification. The phytase activity was assayed following the method described by Bae et al. (1999). One unit of phytase activity was defined as the amount of enzyme that released 1 μmol phosphate per min at 37°C. All determinations were performed in triplicate.

Partial purification of enzyme

The sonicated suspension, prepared as above was centrifuged at 10,000 rpm (4°C) for 30 min and passed through bacterial filters. The filtrate was concentrated to 1/5 of the original volume by ultra filtration through OmegaTM polyether sulfone ultra filtration membrane capsule of 10 KDa molecular weight cutoffs (MWCO) using MinimateTM Tangential Flow Ultra filtration system (Pall life science, USA). Protein concentration was measured by Bradford's method (Bradford, 1976) with bovine serum albumin as standard.

Electrophoresis and phytase zymogram analysis

The ultra filtered purified phytase samples were fractionated by polyacrylamide gel electrophoresis. Zymogram technique was used for phytase isoform analysis and activity bands were visualized by Native PAGE through activity staining as described earlier (Laemmli and Favre, 1970). The gel photograph was documented using gel documentation system (GelDocMEGA, UK).

Phytate biodegradation assay

The protocol for phytate biodegradation was adopted from the method described by Quan et al. (2001). Natural animal feed samples (wheat bran, rice bran, and sweat sorghum baggase) were autoclaved for 20 min at 120°C to inactivate any phytase present in them. 10 g of the autoclaved sample was suspended in 100 ml of acetate buffer (0.2 M, pH 5.5). The suspension was incubated with 1 ml of cell suspension (prepared as described in section 2.3) at 30°C on an incubator shaker at 110 rpm. The reaction was stopped at various incubation periods by adding an equal volume of 10% trichloroacetic acid. The amount of liberated inorganic phosphate was measured by taking absorbance at 700 nm.

Molecular characterization and identification of yeasts

All the three isolates viz. SS1, B4, and PA4 were characterized on the basis of 5.8S-ITS rRNA gene sequences. The polymerase chain reaction (PCR) amplification and sequencing of partial 5.8S-ITS rRNA gene was carried out with the standard primer set ITS1 (forward, 5' TCCGTAGGTGAACCTGCGG 3') and ITS4 (reverse, 5' TCCTCCGCTTATTGATATGC 3') as described earlier (Bruns et al., 1991). The sequences (5.8S-ITS rRNA gene) were then analyzed by Basic Local Alignment Tool (BLAST) at NCBI database. Phylogenetic tree was constructed based on 5.8S-ITS rRNA gene sequences by using MEGA4x1.BETA2 software (N-J plot method).

RESULTS AND DISCUSSION

Qualitative screening for phytase production

All the isolates (SS1, B4 and PA4) along with standard culture of *S. cerevisiae* showed good growth on MPSPM medium during plate assay. This indicates their capabilities to hydrolyze sodium phytate through phytase activity and utilize it as their sole P source. However, none of them showed the formation of any clear zone (halo zone) around the colonies, indicating thereby the intracellular location or cell surface attachment of the enzyme. Yeast have been well reported as rich genetic resources for production of extracellular (*Schwanniomyces castellii* and *S. cerevisiae*) and intracellular (*S. cerevisiae*, *Cryptococcus laurentii* and *Candida krusei*) phytases (Man-Jin et al., 2008).

The isolate PA4 showed maximum biomass yield (1.79 g L^{-1}) that was significantly higher than the other cultures tested under P-deficient condition. Comparatively lower biomass yield for all sodium phytate grown cultures was recorded as compared to that in the presence of available P source (KH_2PO_4). Sodium phytate is a complex organic phosphorus source that is not readily available and therefore growth rate of yeast on sodium phytate depends upon its phytase producing potential. A direct correlation between growth phase and biomass yield of the cultures was observed during growth on sodium phytate (Figure 1). The isolate PA4 showing maximum growth and biomass yield on sodium phytate was found superior among all the cultures tested including standard culture (*S. cerevisiae*). A strong correlation between biomass yield and the growth phase of the cultures indicates strong growth-associated phytase production.

Growth behavior in response to P availability

Various growth parameters of the cultures *viz.* maximum logarithmic growth phase (A_{max}), growth rate constant (μ_{max}), lag phase (λ) and generation time (g) were calculated in response to P availability (Table 1). Among the four cultures, the isolate PA4 showed maximum log phase value (A_{max} , 2.35) with lowest generation time (6.30 h^{-1}), while growing at the expense of sodium phytate (Figure 2). A general decline in O.D. observed in the presence of complex organic P source, might be attributed to a slower proliferation of yeast cells due to the P stress. Similar type of growth decline due to abiotic stress in yeast has also been reported earlier (Sourabh et al., 2012). An extended lag phase (λ_{max} 14.25) under P starvation condition indicated clearly that the yeast takes longer adaptation time in the stress conditions, but once adapted, it starts growing exponentially at the expense of phytate that was hydrolyzed through the phytase activity.

Intracellular phytase activity

The intracellular enzyme activity (U mg^{-1} wet biomass)

varied from 4.85 to 31.26 in whole cell and 4.43 to 30.97 for cell free preparations at 96 and 48 h of incubation period, respectively (Table 2). Slightly higher whole cell intracellular enzyme activity as compared to the cell free activity might be attributed to the mechanical loss of the enzyme during the enzyme preparation procedure especially sonication (Ruiz et al., 1999). The isolate PA4 showed maximum phytase activity (31.26 U mg^{-1} wet biomass) at 48 h of incubation period. A slight difference in the whole cell and cell free intracellular enzyme activities observed during present investigation reveals the better use of whole cell preparations in place of purified enzymes. The cytoplasmic phosphate content of phytate grown cells increased gradually up to 72 h and then it decreased invariably in all the cases and varied from 69.75 (SS1) to 189.00 (PA4) $\mu\text{g ml}^{-1}$ at 24 and 72 h, respectively. It is because of the better availability of phosphate in culture medium due to solubilization of sodium phytate through phytase activity of yeast cells. A direct correlation was thus observed between the phytase activity and increasing P-content in cells with respect to time (Figure 3). However, a decrease in P content of the cells after 72 h might be correlated with higher rate of phosphate requirement and its uptake by the cells as compared to its availability in the medium due to poor phytase activity. Inhibition of phytase activity upon prolonged incubation might be due to the inhibitory effects of some intermediates produced during growth on phytate and/or possibly cells might have entered into the stationary phase, switching off expression of the enzyme (Hatzack et al., 2000).

In vitro biodegradation assay

In vitro biodegradation system (wheat bran, rice bran and sweet sorghum bagasse) consisted of autoclaved samples in acetate buffer (0.2M, pH 5.0) inoculated with cell suspensions and phytate hydrolysis was allowed to occur at 37°C for four days to simulate the conditions of the digestive tract; phosphate released in various samples was measured (Figure 4). Once biodegradation began, the phytate in the samples was gradually hydrolyzed and phosphate liberated. The increasing phosphate concentration with respect to incubation period in all the feed samples indicates clearly the potentiality of all the cultures to hydrolyze phytate through phytase activity. Among the four samples evaluated, maximum and significantly higher phosphate release (0.71 mg g^{-1} of sample) was shown by the isolate PA4 in rice bran. Since yeast cultures are used as starter cultures for leavening of bread dough, therefore, the isolates having intracellular phytase activity at longer time can have a better application in bread making with long leavening time (Bohn et al., 2008). Moreover, as reported earlier also, the exogenous phytase is not primarily required rather whole yeast cells can also be used for phytate degradation during bread making, unless whole grains are added to the mixture (Bohn et al., 2008).

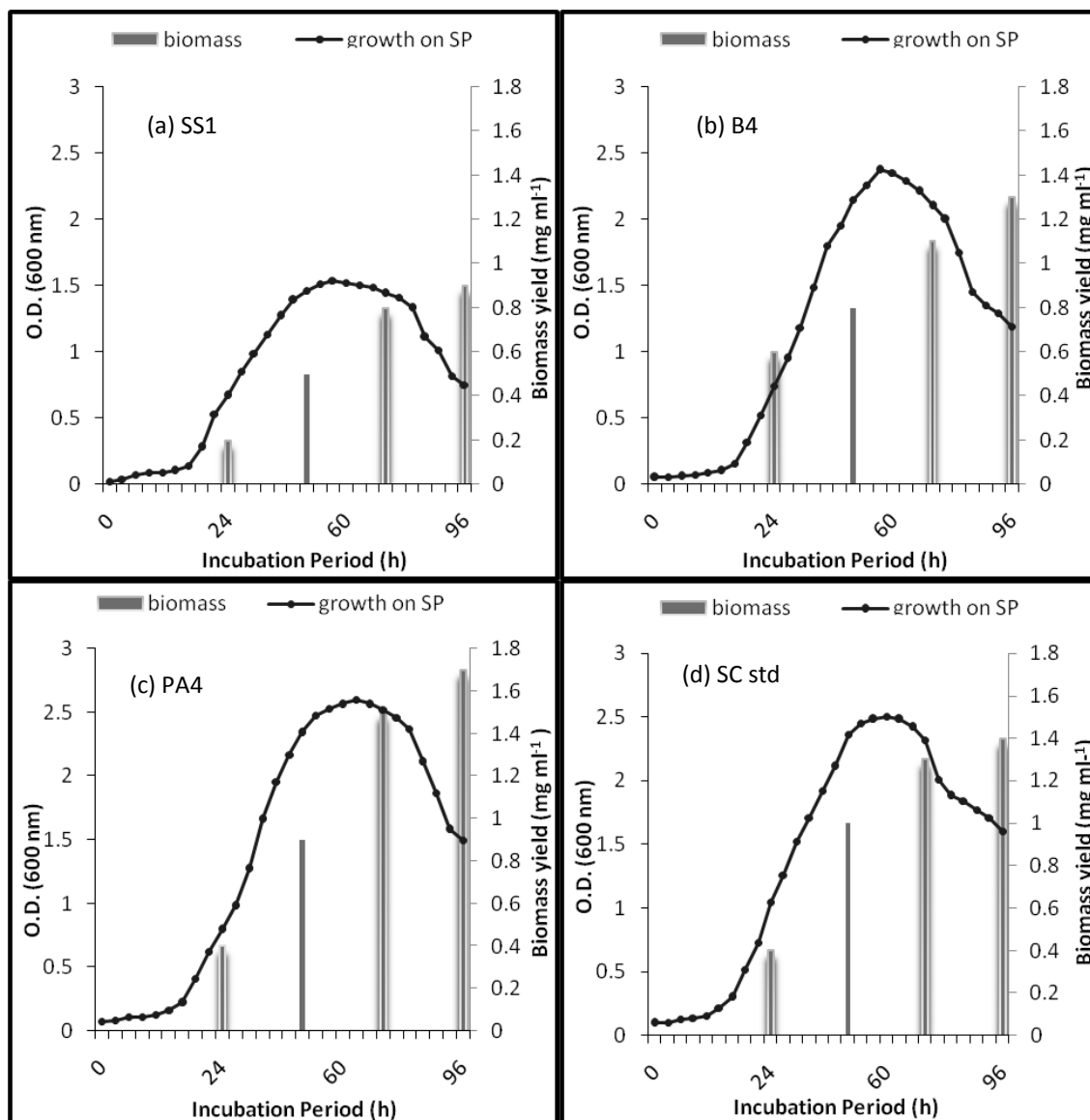


Figure 1. Correlation between biomass yield and growth of SS1 (a); B4 (b); PA4 (c) and SC-std (*S. cerevisiae*) (d).

Table 1. Growth parameters of yeast isolates with respect to phosphorus availability.

P source	Culture/ Isolate	Growth parameter			
		A_{max} (O.D.)	μ_{max} (h^{-1})	λ (h)	Generation time (g)
Unavailable P (sodium phytate)	SS1	1.35±0.06	0.07±0.01	11.52±0.11	9.90
	B4	1.73±0.08	0.09±0.03	14.42±0.13	7.70
	PA4	2.35±0.06	0.11±0.02	14.27±0.13	6.30
	SC std	2.31±0.03	0.10±0.03	13.44±0.15	6.93
Available P (KH_2PO_4)	SS1	3.08±0.02	0.10±0.01	5.92±0.09	6.93
	B4	3.22±0.07	0.11±0.01	4.13±0.12	6.30
	PA4	3.31±0.06	0.12±0.03	8.05±0.11	5.77
	SC std	3.31±0.03	0.13±0.02	7.95±0.13	5.30

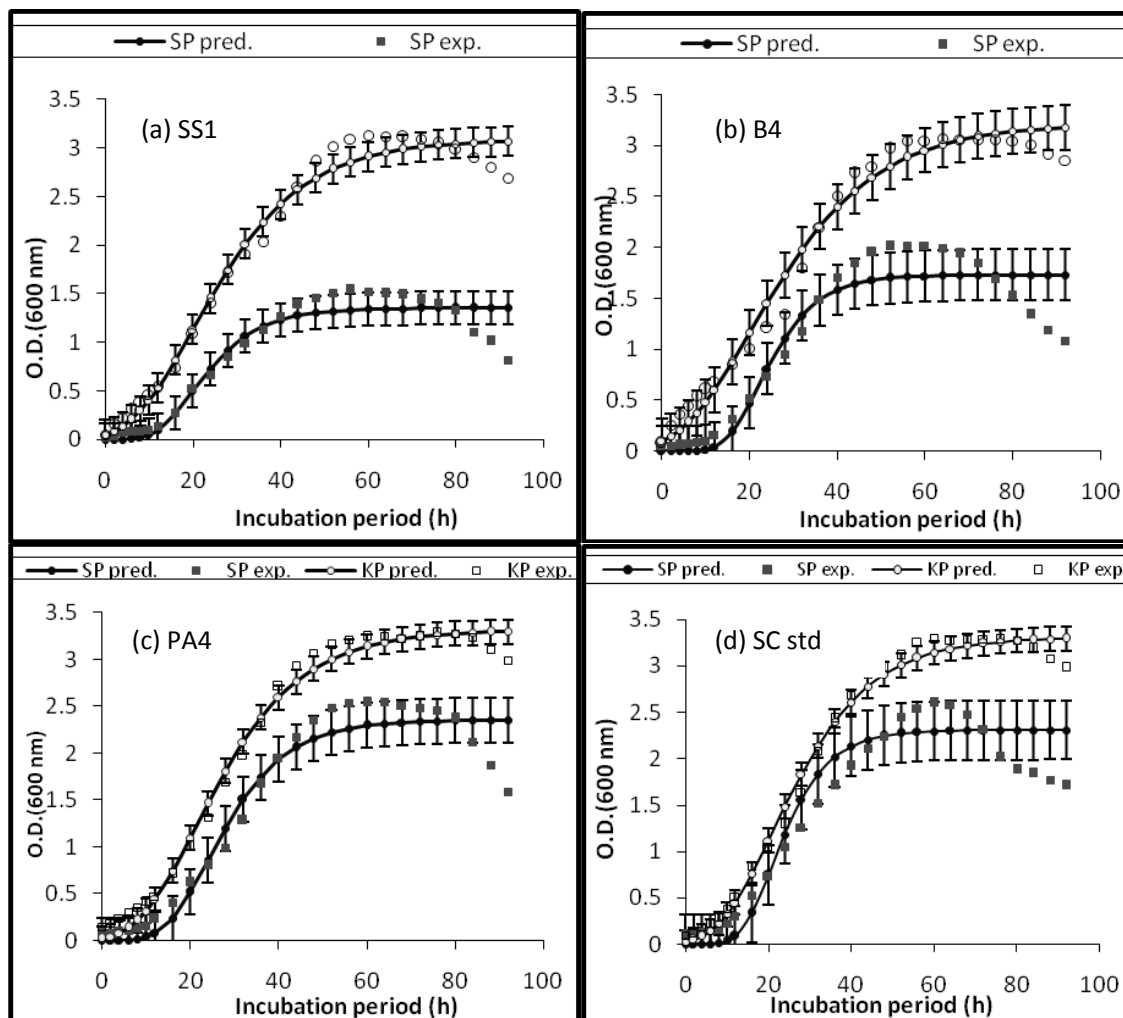


Figure 2. Growth behavior of various yeast cultures under P-stress (sodium phytate) isolates and SC-std (*S. cerevisiae*, d) in MPSM medium and P-sufficient (KH_2PO_4) conditions in MPSM broth. SP: Sodium Phytate; KP: KH_2PO_4 ; Exp: Experimental; Pred: Predicted.

Table 2. Intracellular (whole cell and cell free) phytase enzyme activities of various cultures of yeast grown on sodium phytate mineral medium (MPSM).

Culture/ Isolate	Intracellular Phytase activity (U mg^{-1} wet weight of biomass) at different time intervals (h)							
	Whole cell activity				Cell free activity			
	24	48	72	96	24	48	72	96
SS1	10.00	20.24	10.92	6.52	9.42	18.10	10.68	6.14
B4	16.68	23.11	15.45	6.55	13.61	22.63	15.40	5.94
PA4	15.32	31.26	13.00	4.85	13.27	30.97	12.52	4.44
<i>S. cerevisiae</i> (SC std)	12.60	23.14	13.13	5.03	12.35	22.55	12.97	4.43
CD at 5% a (Isolates)	0.12401		b (Interval) 0.13125		a*b (Interaction) 0.25526			

Zymogram analysis

The partially purified intracellular protein samples when analyzed for phytase isoforms by activity staining through

native PAGE, only one sharp band could be visualized in each test sample (Figure 5a). Results suggest that the molecular mass of native protein from different cultures fall between 100-135 KDa with different band intensities.

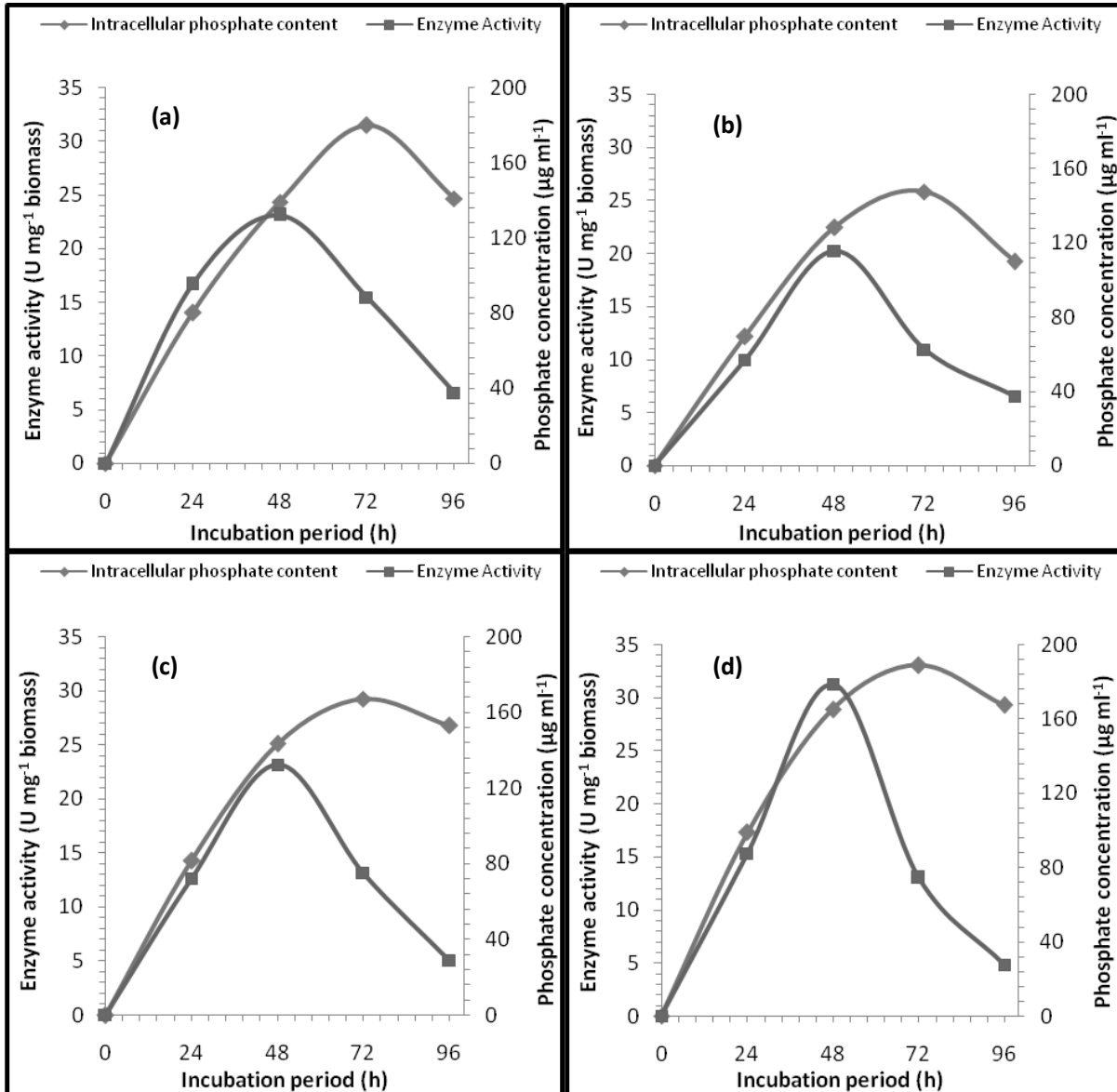


Figure 3. Intracellular phosphate concentration with respect to phytase production in MPSM grown yeast isolates: SS1 (a); B4 (b); PA4 (c) and SC-std (*S. cerevisiae*) (d).

The SS1 phytase showed a very faint band in comparison to others, while the intensities of B4 and PA4 phytase bands were higher than *S. cerevisiae* phytase band on zymogram. Single band pattern on zymogram shows that all the test strains of yeast produce only one isozyme for the phytase enzyme. The molecular weight of several yeast phytases such as *Debaryomyces castellii* and *Schwanniomyces castellii* have also been reported to be in the range of 72 to 125 KDa (Wyss et al., 1999).

Molecular characterization

PCR products showed a high length variation in this

region for different yeast cultures, approximately 850 bp for *S. cerevisiae* (SC-std, lane-1), 600 bp for the isolate PA4 (lane-2), 650 bp for the isolate B4 (lane-3) and 700 bp for the isolate SS1 (lane-4) (Figure 5b). Variable length of the 5.8S-ITS rDNA PCR product obtained for different cultures indicates clearly that they belong to different yeast genera; because, PCR products from different strains of the same species and from different species of the same genus should have identical or very similar molecular size (Sourabh et al., 2012). Variable size of the PCR product of 5.8S-ITS rDNA gene for different yeast cultures belonging to different genera and species has also been reported for different yeast genera viz *Candida*, *Pichia*, etc (Guillaman et al., 1998).

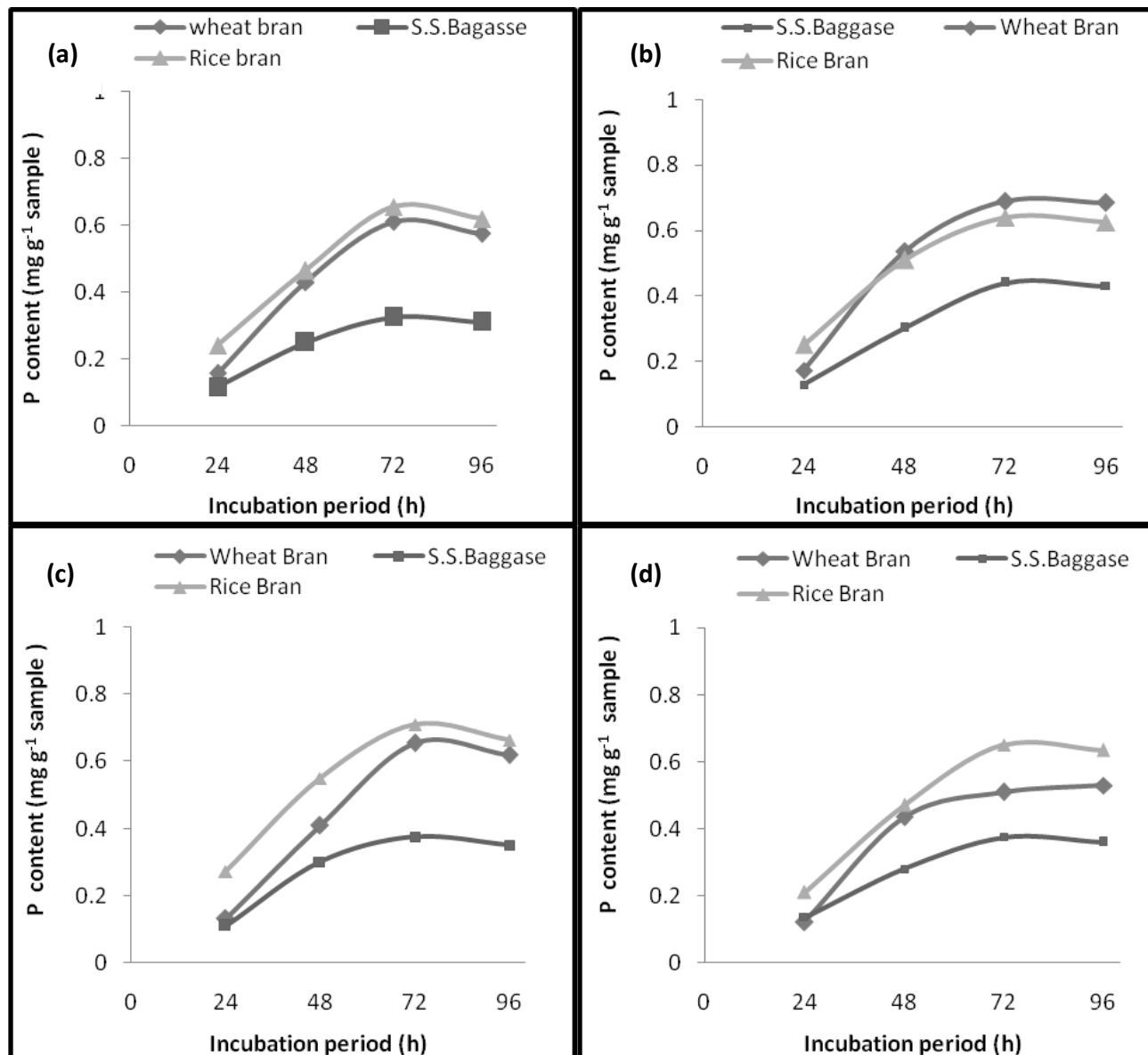


Figure 4. Phosphate released upon biodegradation of phytate in natural feed substrates by various yeast cultures: SS1 (a); B4 (b); PA4 (c); and SC-std (*S. cerevisiae*) (d).

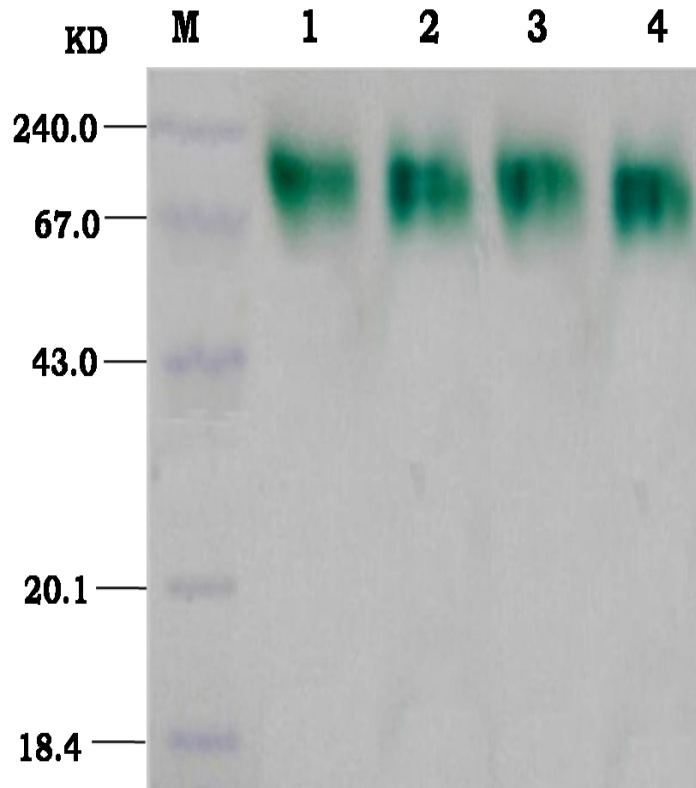
Identification of yeast isolates

NCBI-BLAST sequence similarity search based on 5.8S-ITS rRNA gene sequence identified the isolates B4, SS1 and PA4 as *C. tropicalis* (EF190223.1), *P. guilliermondii* (DQ088676.1) and *I. orientalis* (FJ697171.1), respectively (Table 3). Phylogenetic tree was constructed based on 5.8S-ITS rRNA gene sequence alignment using MEGA4 software (N-J plot method) (Figure 6).

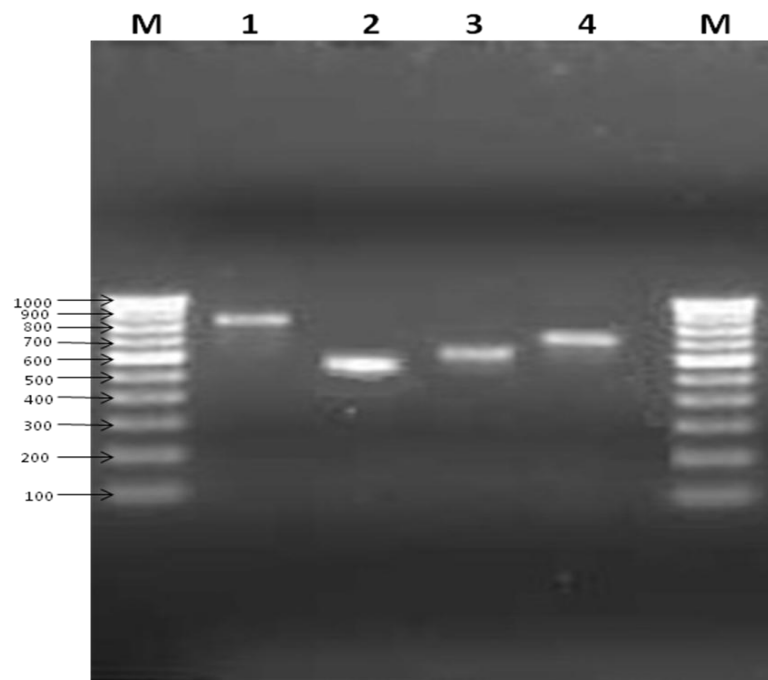
Conclusion

The study reveals a vast potential of Western Indian

Himalayan budding yeast belonging to diverse genera, *Candida*, *Pichia* and *Issatchenkia* for organic phosphorus solubilization. The high intracellular phytase activity along with high protein content of various isolates further emphasizes the use of intact cells as feed additive in place of purified enzymes for enhancing mineral bioavailability and thereby minimizing the cost of ensilage production. *I. orientalis* strain PA4 showing maximum intracellular phytase activity and phosphorus bioavailability in animal feed substrates indicates future prospects for this culture to be developed as a bioinoculant for animal feeds and various other industrial applications.



(a)



(b)

Figure 5. Electrophoretic analysis. **(a)** isoforms by Native PAGE through activity staining: Lane M, protein marker; lane 1, SC, std (*S. cerevisiae*); lane 2, SS1; Lane 3, B4; lane 4, PA4. **(b)** PCR amplified 5.8S, ITS rRNA gene products of various yeast cultures: lane M, 100 bp DNA ladder; lane 1, SC std; lane 2, PA4; lane 3, B4; lane 4, SS1.

Table 3. List of 5.8S rRNA gene sequences from gen bank data base along with the isolate SS1, B4,PA4 and SC-std. Sequence comparison of the gene by EMBOSS Alignment (<http://www.ebi.ac.uk/EMBOSS/align/>).

Gen bank accession number	Origin	Gene name	Identity (%)			E value
			SS1	B4	PA4	
EF190223.1	<i>Candida tropicalis</i>	5.8S rRNA	63.3	99	50.7	0
DQ088676.1	<i>Pichia guilliermondii</i>	5.8S rRNA	99.3	68.9	41	0
AB369918.1	<i>Issatchenkia orientalis</i>	5.8S rRNA	55.3	55.6	99	0
AB533542.1	<i>Saccharomyces cerevisiae</i>	5.8S rRNA	46.1	48.2	34.4	0
JF920159	SS1	5.8S rRNA	100	69.4	41	-
JF300164	B4	5.8S rRNA	69.4	100	57.6	-
JF920160	PA4	5.8S rRNA	41	57.6	100	-

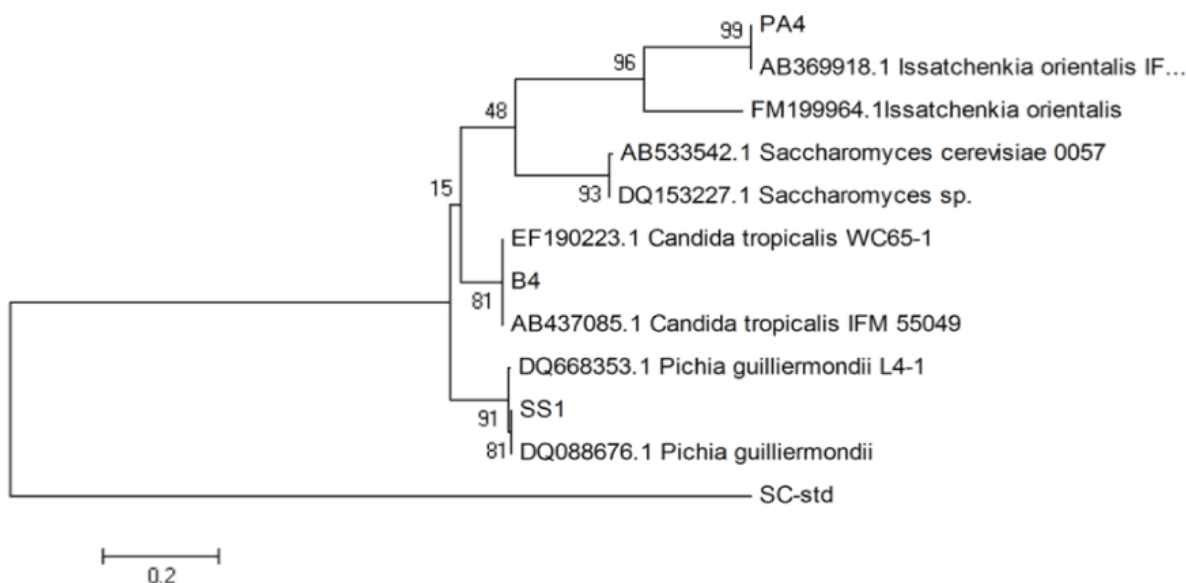


Figure 6. Phylogenetic tree showing evolutionary relatedness of the yeast isolates with closely related yeast species.

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