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Influence of culture conditions on production of phytase by *Zygosaccharomyces bailii* var. *bailii*

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Abstract

Microbial phytases are phosphohydrolytic enzymes which are gaining attention for their commercial exploitation in feed and food industry. In the present study, ten yeasts were isolated from different soil samples and screened for their phytase producing capability. Among these isolates, the most promising yeast strain was *Zygosaccharomyces bailii* var. *bailii* which produced highest phytase yield (6.36 U ml^{-1}) in malt yeast extract glucose peptone (MYGP) medium. In order to improve phytase production by *Zygosaccharomyces bailii*, different physio-chemical parameters were optimized. The optimal conditions for phytase production was found to be: incubation time-42 hr, temperature-30°C, medium pH-6.0 and substrate (calcium phytate) concentration-0.1%. Glucose at 0.5% concentration supported higher phytase production (13.75 U ml^{-1}) than other carbon sources tested. Metal ions (Ca^{++}, Na^{+}, K^{+}, Mg^{++}) and additives; ethylene diamine tetraacetate (EDTA), sodium dodecyl sulphate (SDS) and toluene did not affect enzyme production. However, Zn^{++}, Ni^{++}, Ba^{++}, Pb^{++} and detergents like Triton X-100 and Tweens strongly inhibited (>90%) phytase production. An overall 2.21-fold enhancement in phytase activity (6.36→14.03 U ml^{-1}) was attained after optimization studies.

Key words

Phytic acid, Phytase, Phytate hydrolyzing enzyme, Submerged fermentation, *Zygosaccharomyces bailii*

Introduction

Phytic acid (*myo*-inositol 1,2,3,4,5,6-hexakis dihydrogen phosphate) is a major storage form of organic phosphorus, which comprises 18% to 88% of total phosphorus content in edible legumes, cereal grains, oilseed meals, pollens and nuts (Vats and Banerjee, 2002). It occurs naturally in the form of mixtures of cationic salts of phytic acid, known as phytates in mature seeds and cereal grains. Phytate is considered as an anti-nutritional factor (ANF) since it causes mineral deficiency due to efficient chelation of metal ions such as Ca^{++}, Mg^{++}, Zn^{++} and Fe^{++} and unavailable nutritionally for monogastrics (poultry, pigs, fishes and humans). They lack high level of phytate degrading enzymes (phytases) in their gastrointestinal gut (Ranjan and Sahay, 2013). These unmetabolized phytates pass through the intestinal tract and are excreted by monogastric animals and cause environmental problems by eutrophication and algal blooms of surface aquatic bodies (Raboy *et al.*, 2001). In order to increase the bioavailability of essential dietary minerals and decrease environmental pollution, degradation of phytates in foods and feeds is of nutritional and environmental importance (In *et al.*, 2008).

Phytases (*myo*-inositol hexakisphosphate 3-phosphorylase and *myo*-inositol hexakisphosphate 6-phosphorylase) are a special class of phosphatases that catalyze hydrolysis of phytic acid to inorganic phosphate and *myo*-inositol phosphate derivatives in a stepwise manner, all utilizing a phosphohistidine intermediate in their phosphoryl transfer reaction (Vats and Banerjee, 2004). Phytases are produced by a wide range of microorganisms, plants and certain animal tissues. They have been studied most intensively in plant seeds (Greiner,

2002). However for commercial applications, industrial enzymes of microbial origin are preferred due to their multifold properties and easy extraction procedure. Most of the research on microbial phytase was reported on fungi belonging genus *Aspergillus* and bacteria: *Bacillus* and *Klebsiella* species (Pandey *et al.*, 2001; Vohra and Satyanarayana, 2003). Yeasts have been fairly well investigated for extracellular phytase production such as *Rohodotorula gracilis* (Bindu *et al.*, 1998), *Arxula adeninivorans* (Sano *et al.*, 1999), *Pichia spartinae* and *P. rhodanensis* (Nakamura *et al.*, 2000), *Candida krusei* (Quan *et al.*, 2001), marine yeast *Kodamaea ohmeri* BG3 (Hirimuthugoda *et al.*, 2006), *Debaryomyces castellii* CBS 2923 (Ragon *et al.*, 2008) and *Zygosaccharomyces bisporus* NCIM 3265 and 3296 (Pable *et al.*, 2013), while intracellular phytase is also known to be produced by some yeasts (Vohra and Satyanarayana, 2001, Olstorpe *et al*., 2009, Suyal and Tewari, 2013). The present paper deals with phytase production by yeast, *Zygosaccharomyces bailii* var. *bailii* and optimization of its culture conditions for phytase production.

Materials and Methods

Calcium salt of phytic acid (Calcium phytate) of highest available purity was purchased from HiMedia Chemical Laboratories Private Limited Company, Mumbai. All other chemicals used were of analytical grade and obtained from leading manufacturers including BDH, HiMedia and Glaxo.

Phytase producing yeast culture *Zygosaccharomyces bailii* var. *bailii* was isolated from soil dumps of distillery waste, Meerut, India. Yeast culture was maintained at 4°C on MYGP agar medium which consisted of malt extract (0.3%), yeast extract (0.3%), glucose (1.0%), peptone (0.5%) and agar (1.5%).

Isolation, screening and identification of phytase producing yeast : Isolation of yeast was carried out from soil sample collected from soil dumps of five different distillery wastes of Meerut region, by serial dilution technique. Ten gram of soil sample was suspended in 90 ml of sterile distilled water. Suspension was shaken well at 200 rpm for 10 min and then soil particles were allowed to settle down. The suspension was diluted through a series of dilutions to obtain a final concentration of 1/10,000 times (10^{-4}). From each dilution, 0.1 ml was pipetted out and spread on the surface of malt yeast extract glucose peptone (MYGP) agar plates and incubated at 30°C. After 2-5 days of growth, when luxuriant growth was observed in different yeast cultures, the yeast colonies were subcultured on MYGP slants. These isolates were screened for their phytase producing capability both qualitatively and quantitatively.

The qualitative screening of phytase producing isolates was carried out by plate assay procedure on phytase screening medium (PSM) (Howson and Davis, 1983). Selective medium PSM contained (gl^{-1}): Glucose, 15.0; NH_4NO_3, 5.0; $MgSO_4.7H_2O$, 0.5; KCl, 0.5; $FeSO_4.7H_2O$, 0.01; $MnSO_4.7H_2O$, 0.01; calcium phytate, 5.0; agar, 15.0 (pH 5.5). Isolates producing zone of clearance around growth on PSM agar plates were selected as phytase producers. Acid-producing microbes also give false positive reactions with the plate assay method. To avoid this problem, two-step counterstaining technique was used to confirm phytate-hydrolysing ability of the isolated yeasts (Bae *et al.*, 1999). Cultures exhibiting zone of clearance after removal of chromogen was confirmed as phytase producers.These isolates were maintained on MYGP slants for further studies.

Yeast isolates exhibiting zone of clearance were selected and further subjected to quantitative screening for extracellular phytase production. Enzyme production was performed in 250 ml Erlenmeyer flasks containing 50 ml MYGP broth supplemented with 0.1% calcium phytate as the sole source of phosphorus with 1% (v/v) inoculum and incubated at 30°C in a rotary shaker (180 rpm) for 42 hr. Phytase activity of extracellular phytase from the culture supernatant was determined by performing phytase assay using phytic acid as substrate. Yeast isolate showing highest phytase activity was selected for further studies. Colonies and cells of selected yeast isolate was studied using a stereomicroscope and phase contrast microscope, respectively.

Phenotypic characterization of the strain was done at Microbial Type Culture Collection (MTCC), Institute of Microbial Technology (IMTECH), Chandigarh (India) and was identified as *Zygosaccharomyces bailii* var. *bailii*.

Biomass estimation : To determine yeast biomass, the culture was centrifuged at 10,000 rpm for 10 min at 4°C and the pellet was washed twice with distilled water. The washed pellet was inverted on tissue paper and kept at room temperature for 15 min in a preweighed centrifuged tube and expressed as gram wet weight/liter medium (gl^{-1}).

Phytase assay: Phytase activity was estimated by colorimetric method. Calcium phytate (0.2%) prepared in 0.1M acetate buffer (pH 5.0) was used as substrate. The reaction mixture was prepared by adding of 600 µl substrate with 150 µl of crude enzyme and incubated at 35ºC for 20 min. Enzymatic reaction was stopped by adding 750 µl of 5% (w/v) trichloroacetic acid solution and the released free orthophosphate (Pi) in the reaction mixture was measured by modification of the method of Fiske and Subbarow (Fiske and Subbarow, 1925). Colour reagent (750 µl), prepared freshly by mixing four volumes of 1.5% (w/v) ammonium molybdate in 5.5% (v/v) sulphuric acid solution and one volume of 2.7% (w/v) ferrous sulphate solution, was added to the sample solution (750 µl) and the production of phosphomolybdate was measured spectrophotometrically at 700 nm (Bae *et al.*, 1999). One unit is defined as the amount of enzyme that liberates one µg inorganic phosphate ml^{-1} min^{-1} under the assay conditions.

Six different production medium were tested to obtain maximum production of phytase by *Zygosaccharomyces bailii*. Yeast culture was grown in these media for 2 days at 30°C in

orbital shaker. The composition of different media was as follows (gl^{-1}); Phytase screening medium (PSM): (Howson and Davis, 1983); Corn starch medium (CSM): Corn starch, 80.0; Glucose, 30.0; $NaNO_3$, 8.6; KH_2PO_4, 0.017; KCl, 0.5; $MgSO_4.7H_2O$, 0.5; $FeSO_4.7H_2O$, 0.1 (pH 5.0) (Volfova *et al*., 1994); Phytase screening (PS) broth: Glucose, 10.0; $(NH_4)_2SO_4$, 3.0; $MgSO_4.7H_2O$, 0.5; KCl, 0.5; $CaCl_2$, 0.1; Calcium phytate, 1.0 (pH 5.6); Glucose phosphate (GP) broth: Glucose, 10.0; $(NH_4)_2SO_4$, 3.0; $MgSO_4.7H_2O$, 0.5; KCl, 0.5; $CaCl_2$, 0.1; KH_2PO_4, 3.0; trace elements (pH 5.6); Peptone dextrose (PD) broth: Peptone, 10.0; Dextrose, 40.0; (pH 5.6); MYGP broth: Malt extract, 3.0; Yeast extract, 3.0; Glucose, 10.0; Peptone, 5.0; Calcium phytate, 1.0 (pH 5.6) (Vohra and Satyanarayana, 2001).

Effect of various physio-chemical parameters on phytase production : To determine the optimum conditions for phytase production, various physical and nutritional parameters were studied such as: incubation period; substrate concentration; medium of pH (pH 3.0-9.0) and temperature (25°-50°C). The effect of different carbon sources (1% w/v concentration), nitrogen sources (0.3% w/v concentration), metal ions (0.1% w/v) and additives (0.1% w/v) were also investigated.

Result analysis : All fermentations and assays were carried out in triplicate and mean values with standard deviation (SD) are presented.

Results and Discussion

Total ten yeast isolates were isolated from soil dumps of five different distillery wastes collected from Meerut (UP), India. Upon initial screening by plate assay method, out of ten isolates, five yeasts were selected as phytase producers. Further, extracellular phytase production from these cultures was studied in liquid medium (MYGP broth supplemented with 0.1% calcium phytate). On the basis of phytase activity, the yeast isolate 'M11' producing highest phytase activity (6.36 U ml^{-1}) was selected for phytase production. In preliminary morphological characterization, the colonies of yeast strain were observed smooth, round, convex and pale yellow coloured. Vegetative cells were ellipsoid and non-motile. It was identified as *Zygosaccharomyces bailii* var. *bailii* (MTCC Accession No.11852) and deposited at Microbial Type Culture Collection (MTCC), Chandigarh (India). Six production media were tested in an attempt to improve phytase production from *Zygosaccharomyces bailii*. Among different media tested, growth as well as enzyme production (6.36 U ml^{-1}) was found maximum in MYGP broth (supplemented with 0.1% calcium phytate) used initially. PSM and PS broth supported growth of yeast culture but not enzyme production (Table 1). Starch medium and GP broth did not favour growth as well as enzyme production, while no enzyme activity was detected in PD broth. The results suggested that phytase from *Zygosaccharomyces bailii* was inducible in nature, since all the media tested contained the source of phosphorus except PD broth. However, *Zygosaccharomyces bisporus* strains (NCIM 3265 and NCIM 3296) and *Zygosaccharomyces priorionus* showed highest phytase production in cane juice medium than MYGP medium (Pable *et al*., 2013).

Cell-bound phytase production by *Pichia anomala* was compared in synthetic glucose–beef extract and cane molasses media. An overall 86.6% enhancement in phytase yield was attained in optimized cane molasses medium using fed-batch fermentation (Vohra and Satyanarayana, 2004). Yeasts like *Pichia kudriavzevii* TY13, *Hanseniaspora guilliermondii* TY14 and TY20, isolated from Tanzanian togwa, produced highest activity in complex yeast medium YPD (Hellstrom *et al*., 2012).

Table 1 : Screening of different medium for maximum phytase production by *Zygosaccharomyces bailii* after 48 hr at 30°C

Medium*	Phytase activity (U ml^{-1})	Wet wt. of biomass (gl^{-1})
PSM	3.04 ± 0.31	1.12 ± 0.11
CSM	2.04 ± 0.28	0.96 ± 0.12
PS broth	3.64 ± 0.27	1.32 ± 0.12
GP broth	1.12 ± 0.20	0.78 ± 0.10
PD broth	0.0	0.69 ± 0.08
MYGP broth	6.36 ± 0.33	1.71 ± 0.11

*Phytase Screening Medium (PSM) (gl^{-1}): Glucose, 15.0; NH_4NO_3, 5.0; $MgSO_4.7H_2O$, 0.5; KCl, 0.5; $FeSO_4.7H_2O$, 0.01, $MnSO_4.7H_2O$, 0.01, calcium phytate, 5.0, Corn starch Medium (CSM) (gl^{-1}): Corn starch, 80.0; Glucose, 30.0; $NaNO_3$, 8.6; KH_2PO_4, 0.017; KCl, 0.5; $MgSO_4.7H_2O$, 0.5; $FeSO_4.7H_2O$, 0.1, Phytase Screening (PS) broth (gl^{-1}): Glucose, 10.0; $(NH_4)_2SO_4$, 3.0; $MgSO_4.7H_2O$, 0.5; KCl, 0.5; $CaCl_2$, 0.1; Calcium phytate, 1.0, Glucose Phosphate (GP) broth (gl^{-1}): Glucose, 10.0; $(NH_4)_2SO_4$, 3.0; $MgSO_4.7H_2O$, 0.5; KCl, 0.5; $CaCl_2$, 0.1; KH_2PO_4, 3.0, Peptone Dextrose (PD) broth (gl^{-1}): Peptone, 10.0; Dextrose, 40.0; Malt extract Yeast extract Glucose Peptone (MYGP) broth (gl^{-1}): Malt extract, 3.0; Yeast extract, 3.0; Glucose, 10.0; Peptone, 5.0; Calcium phytate, 1.0. Results are expressed as the mean of three replicated measurements ($n = 3$)

To determine the optimum incubation time for phytase production, the yeast culture was grown under submerged shaking conditions and the biomass as well as phytase activity were estimated at regular intervals of 6 hr. Phytase activity was initially detected at 18 hr of incubation, which progressively increased with time. As indicated in Fig.1, peak growth as well as phytase activity (7.52 U ml^{-1}) was observed at 42 hr of incubation. Thereafter, enzyme production slightly decreased. The growth and enzyme yield on prolonged incubation remained constant. Phytase yield from C*andida krusei* WZ-001 was found to be highest after 48 hr of incubation (Quan *et al*., 2001). Maximum extracellular phytase production was achieved by marine yeast *Kodamaea ohmeri* BG3 (Li *et al*., 2008) and *Saccharomyces cerevisiae* CY strain after 72 hr of incubation (In *et al*., 2008). The optimum extracellular phytase production from *Candida parapsilosis* was reported at pH 3 after 24 hr of incubation (Ranjan and Sahay, 2013).

Substrates are known to induce and thus enhance enzyme production. As evident from Table 1, phytase from *Zygosaccharomyces bailii* is inducible in nature; hence substrate concentration was optimized by supplementing the MYGP medium with calcium phytate in the range of 0.1-0.6%. The maximum enzyme production (8.65 U ml^{-1}) was found in the medium containing 0.1% calcium phytate (Fig. 2). Further increase in concentration led to sharp decrease in growth and therefore enzyme production. It may be the result of enzyme inactivation or inhibition of synthesis of phytases by high phytate content of the medium, which lead to low Pi availability (Lambrechts *et al.*, 1992). Similar results were obtained for phytase produced by *Candida krusei*. Phytase production was found to be controlled by phosphate concentration in the medium used and the optimum concentration was reported to be 0.5 mg phosphorus per 100ml (Quan *et al.*, 2001). Pavlova *et al.* (2008) reported maximum phytase production from *Cryptococcus laurentii* AL_{27} using inorganic phosphate source (KH_2PO_4) at 5 mgl^{-1}, while sodium phytate at 0.04% in the production medium.

The effect of incubation temperature on phytase production was studied in the temperature range of 25º-50ºC under submerged fermentation conditions. The optimum temperature for growth and phytase production from *Zygosaccharomyces bailii* was found to be 30ºC (Table 2). Further rise in temperature, decreased the production of phytase and negligible phytase activity was observed at 45ºC. Above 45ºC, the culture could not grow. Incubation at higher temperature affected the yeast harmfully, which was reflected on enzyme synthesis. Phytase production by yeast at 30ºC has been reported in several studies (Vohra and Satyanarayana, 2001; Quan *et al.*, 2001; In *et al.*, 2008). However, optimum phytase production from psychrophilic Antarctica yeast strain *Cryptococcus aurentii* AL_{27} was reported at 24°C (Pavlova *et al.*, 2008), while by *Candida parapsilosis* was reported at 37°C (Ranjan and Sahay, 2013), respectively.

In order to examine the effect of medium pH the batch fermentation of *Zygosaccharomyces bailii* were conducted in shake flasks. The optimum pH for phytase production by yeast culture was found to be 6.0. Above and below this pH, the enzyme production reduced drastically (Table 3), however the culture could grow in the pH range of 5.0-8.0. Phytase production has mostly been reported in acidic to neutral pH range. Phytase production from *Saccharomyces cerevisiae* YS18 was also optimum at initial pH 6.0 (Andlid *et al.*, 2004). Different variables for phytase production from *Kodamaea ohmeri* BG3 were optimized and observed maximum enzyme yield at initial pH 6.3 (Li *et al.*, 2008). High phytase yield from *Saccharomyces cerevisiae* CY strain was obtained at pH 4.0 (In *et al.*, 2008), however from alkalophilic yeast strain, *Candida melibiosica* 2491 at pH 8.5 (Georgiev *et al.*, 2013).

The effect of different carbon sources on phytase production by *Zygosaccharomyces bailii* was investigated. Various sugars (1%) were supplemented as carbon source in the production medium. Among the different carbon sources tested, highest phytase activity (11.65 U ml^{-1}) was obtained with glucose,

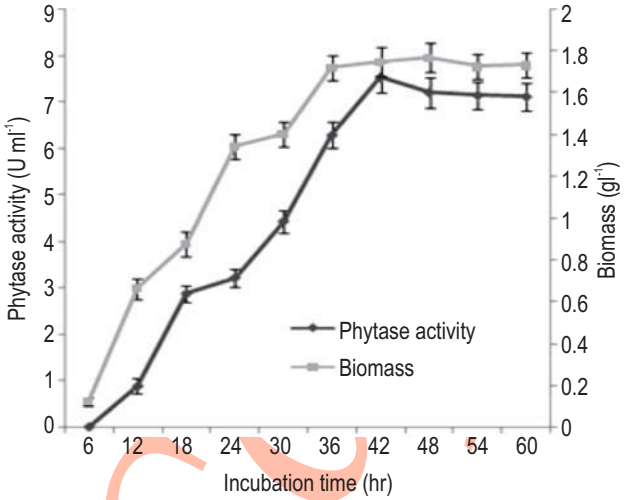

Fig. 1 : Effect of incubation period on phytase production from *Zygosaccharomyces bailii* at 30ºC and at medium of pH 6.0. Results are expressed as mean of three replicates ($n = 3$)

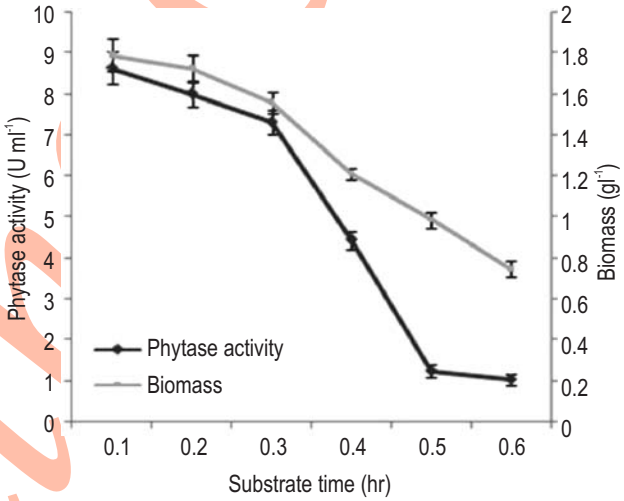

Fig. 2 : Effect of different substrate (calcium phytate) concentration of (0.1-0.6%) on phytase production by *Zygosaccharomyces bailii* at 30°C after 42 hr. Results are expressed as mean of three replicates ($n = 3$)

Table 2 : Effect of different incubation temperature on phytase production by *Zygosaccharomyces bailii* at constant pH after 42 hr incubation

Temperature (ºC)	Phytase activity (U ml^{-1})	Wet wt. of biomass (gl^{-1})
25º	7.78 ± 0.34	1.294 ± 0.16
30º	9.02 ± 0.36	1.799 ± 0.20
37º	8.71 ± 0.32	1.769 ± 0.18
40º	6.67 ± 0.31	1.732 ± 0.17
45º	1.22 ± 0.12	1.081 ± 0.12
50º	0.0	0.0

Results are expressed as mean of three replicates ($n = 3$)

followed by sucrose (10.65 U ml^{-1}) and maltose (10.54 U ml^{-1}). Lactose, galactose, cellulose and fructose were found to be poor carbon sources for phytase production in this study (Table 4). Further, the effect of different concentrations of glucose (0.5 to 5.0%) on phytase production was also studied. As compared to 1% (used initially), 0.5% concentration of glucose in production medium was most favorable for growth and enzyme production (Fig.3). Higher concentrations were inhibitory, both for biomass and production of enzyme.

Glucose was mostly found as the best carbon source for yeast growth and phytase production (Zyla *et al.*, 1994; Vohra and Satyanarayana, 2001; Quan *et al.*, 2001). Galactose, in place of glucose, enhanced phytase production to several folds from *Arxula adeninivorans* (Sano *et al.,*1999) and *Saccharomyces cerevisiae* CY strain (In *et al.*, 2008), while sucrose was reported as the most suitable carbon source for phytase production by *Cryptococcus laurentii* AL_{27} (Pavlova *et al.*, 2008). Sarlin and Philip (2013) investigated four different media (M1, M2, M3 and M4) prepared by incorporating substrates such as glucose, sucrose, rice water and molasses as carbon sources for production of phytase from four marine yeasts: *Fenneropenaeus indicus viz. Debaryomyces hansenii* (S8), *D. hansenii* (S100), *Candida sake* (S165) *and C. tropicalis* (S186). Molasses was the most preferred carbon source and supplemented with peptone (0.75%), yeast extract (0.5%) and $MgSO_4$ (0.25%) supported maximum growth of four yeast strains tested (Sarlin and Philip, 2013).

To investigate the effect of nitrogen sources on phytase production, several organic and inorganic nitrogen (0.3%) sources were supplemented in the production medium. None of the nitrogen source tested individually enhanced phytase yield as compared to control (13.91 U ml^{-1}). Hence, combination of malt extract, yeast extract and peptone being the constituent of MYGP medium, was the most suitable nitrogen supplements for optimum phytase production (Table 5). These results are in accordance with Table 1, in which highest phytase activity was obtained in MYGP medium. All the other media which consisted of inorganic nitrogen sources produced lower titres of phytase. Yeast extract and peptone proved to be the best nitrogen source for *Arxula adeninivorans* phytase (Sano *et al.*, 1999), whereas enhanced enzyme production by *Pichia anomala* was observed using beef extract (1%) in the production medium (Vohra and Satyanarayana 2001). Highest phytase production with ammonium sulfate as the nitrogen source was observed by In *et al.* (2008) and Li *et al.* (2008).

Ries and Macedo (2011) investigated different concentrations of urea (0; 0.15 and 0.30% w/v) to determine the best concentration that promotes the production of phytase from *Saccharomyces cerevisiae*, and observed highest phytase activity (0.41 U ml^{-1}) at concentration of 0.15% urea.

Table 3 : Effect of initial medium pH on phytase production *Zygosaccharomyces bailii* at 30°C after 42 hr incubation

pH	Phytase activity (U ml^{-1})	Wet wt. of biomass (gl^{-1})
3.0	0.0	0.321 ± 0.07
4.0	1.24 ± 0.10	0.892 ± 0.10
5.0	8.66 ± 0.33	1.453 ± 0.14
6.0	10.93 ± 0.38	1.787 ± 0.16
7.0	8.21 ± 0.31	1.729 ± 0.15
8.0	2.88 ± 0.17	1.699 ± 0.11
9.0	0.90 ± 0.05	0.993 ± 0.06

Results are expressed as the mean of three replicated measurements (*n* = 3)

Table 4 : Effect of different carbon sources supplemented in MYGP medium for phytase production from *Zygosaccharomyces bailii*

Carbon source (1%)	Phytase activity (U ml^{-1})	Wet wt. of biomass (gl^{-1})
Glucose	11.65 ± 0.45	1.993 ± 0.14
Sucrose	10.65 ± 0.44	1.765 ± 0.11
Maltose	10.54 ± 0.41	1.821 ± 0.12
Lactose	3.55 ± 0.20	0.872 ± 0.10
Galactose	4.76 ± 0.20	0.998 ± 0.11
Cellulose	5.54 ± 0.31	0.985 ± 0.11
Cellobiose	2.33 ± 0.16	0.754 ± 0.10
Starch	9.66 ± 0.40	1.534 ± 0.13
myo-Inositol	8.80 ± 0.38	1.500 ± 0.13
Fructose	3.98 ± 0.18	0.993 ± 0.10
Glycerol	3.98 ± 0.14	1.078 ± 0.12

Results are expressed as mean of three replicates (*n* = 3). Phytase activity was determined after 42 hr incubation from culture filterate

Table 5 : Effect of different nitrogen sources supplemented in MYGP medium for phytase production from *Zygosaccharomyces bailii*

Nitrogen sources (0.3%)	Phytase activity (U ml^{-1})	Wet wt. of biomass (gl^{-1})
MYGP medium (control)	13.91 ± 0.55	2.16 ± 0.22
Yeast extract	10.31 ± 0.42	2.00 ± 0.21
Peptone	9.12 ± 0.40	1.54 ± 0.18
Malt extract	9.83 ± 0.50	1.61 ± 0.16
Beef extract	7.01 ± 0.42	1.32 ± 0.15
Tryptone	6.31 ± 0.29	1.02 ± 0.09
Soybean meal	5.52 ± 0.25	1.27 ± 0.17
Casein	4.81 ± 0.22	0.97 ± 0.10
Ammonium sulphate	5.19 ± 0.35	1.02 ± 0.19
Ammonium nitrate	4.33 ± 0.21	0.88 ± 0.14
Ammonium chloride	3.18 ± 0.17	0.76 ± 0.18
Ammonium acetate	2.69 ± 0.20	0.91 ± 0.16
Sodium nitrate	4.47 ± 0.23	0.65 ± 0.09

Results are expressed as mean of three replicates (*n* = 3). Phytase activity was determined from culture filterate after 42 hr incubation

The effect of metal ions (0.1% w/v) on phytase production was studied by adding various salts in the production medium and found that the metal ions Ca^{++}, K^{+}, Na^{+}, Mg^{++} and Mn^{++} did not affect enzyme production. Zn^{++}, Ba^{++}, Ni^{++} and Pb^{++} inhibited enzyme production to about 50% while Co^{++}, Hg^{++} and Ag^{++} strongly inhibited growth and enzyme activity (Table 6). Phytase production from *Candida krusei* was improved the presence of Cu^{++} and Pb^{++} while was inhibited by Cd^{++}, Ba^{++}, Co^{++} and Hg^{++} (Quan *et al.*, 2001). However, phytase production from *Pichia anomala* enhanced in the presence of Fe^{++} (0.15mM) followed by Ca^{++} and inhibited by Co^{++}, K^{+}, Mn^{++}, Zn^{++} (Vohra and Satyanarayana, 2001).

Among various additives (0.1% w/v) tested, SDS (13.29 U ml^{-1}), EDTA (12.03 U ml^{-1}) and toluene (10.32 U ml^{-1}) did not affect growth and production of phytase by *Zygosaccharomyces bailii*, whereas Tweens and Triton X-100 strongly inhibited enzyme production. Glycerol, β-mercaptoethanol and Dithiothretiol (DTT) also had inhibitory effect (Table 7). In contrast, cell-associated phytase activity was decreased by 40%-50% in presence of 0.2% SDS in *Zygosaccharomyces bisporus* NCIM 3265, *Williopsis saturnus* NCIM 3298 and *Zygosaccharomyces prioriorus* NCIM 3299 while by about 70%-80% decreased in *Zygosaccharomyces bisporus* NCIM 3296 and *Schizosaccharomyces octoporus* NCIM 3297. However, presence of EDTA did not show inhibitory effect on cell-associated phytase activity of these yeast strains (Pable *et al.*, 2013).

Table 6 : Effect of different metal ions on phytase enzyme production from *Zygosaccharomyces bailii*

Metal ions (0.1%)	Phytase activity (U ml^{-1})	Wet wt. of biomass (gl^{-1})
Control	13.83± 0.58	2.089± 0.20
Na^{+}	10.08± 0.45	2.123± 0.23
K^{+}	12.03± 0.51	1.993± 0.17
Ca^{++}	13.32± 0.55	1.921± 0.19
Mg^{++}	10.09 ± 0.43	1.567± 0.16
Mn^{++}	9.78 ± 0.39	1.043± 0.19
Zn^{++}	8.78 ± 0.29	1.098± 0.18
Ni^{++}	6.78± 0.28	0.898± 0.11
Co^{++}	0.0	0.456± 0.08
Ba++	7.21± 0.31	0.985± 0.11
Pb^{++}	4.32 ± 0.21	0.678± 0.07
Hg^{++}	0.0	0.431± 0.08
Ag^{++}	0.0	0.502± 0.08

Results are expressed as mean of three replicates (*n* = 3). Phytase activity was determined after 42 hr incubation from culture filterate

Table 7 : Effect of additives on phytase enzyme production from Zygosac*charomyces bailii*

*Additive*s (0.1%)	Phytase activity (U ml^{-1})	Wet wt. of biomass (gl^{-1})
Control	14.03 ± 0.57	2.201 ± 0.27
Tween-20	4.54 ± 0.25	0.993 ± 0.20
Tween-40	3.21 ± 0.21	0.902 ± 0.16
Tween-60	0.0	0.332 ± 0.08
Tween-80	0.0.	0.221 ± 0.06
Triton X-100	2.43 ± 0.19	0.702 ± 0.17
SDS	13.29 ± 0.59	2.002 ± 0.28
β-mercaptoethanol	3.42 ± 0.26	0.673 ± 0.13
Dithiothretiol	3.34 ± 0.23	0.549 ± 0.09
EDTA	12.03 ± 0.51	2.231 ± 0.26
Toluene	10.32 ± 0.51	1.882 ± 0.27
Glycerol	8.93 ± 0.47	1.654 ± 0.29

Results are expressed as mean of three replicates (n = 3). Phytase activity was determined after 42 hr incubation from culture filterate

Optimization of various physical and nutritional conditions have led to improved phytase production starting from 6.36 U ml^{-1} reaching upto 14.03 U ml^{-1}. Phytase yield, therefore, enhanced to 2.21-fold as compared to initial culture conditions (Fig. 4). In an attempt to optimize cultural conditions for maximizing the production of phytase from *Pichia anomala* in synthetic medium (Vohra and Satyanarayana, 2002) and marine yeast *Kodamaea ohmeri* BG3 (Li *et al.*, 2008) in a cost-effective oats medium, an overall 1.75-fold and 9-fold (62.0→575.5 U ml^{-1}) enhancement in phytase activity respectively, was attained.

The results of the present study showed that optimum conditions for maximum phytase production were as follows: incubation time 42 hr; incubation temperature 30°C; substrate concentration (calcium phytate) 0.1%; pH 6.0. The best carbon source was found to be glucose at 0.5% concentration. The optimization study resulted into 2.21-fold increase in phytase yield. Strain *Zygosaccharomyces bailii* var. *bailii* therefore, had potential to be used as a source of phytase.

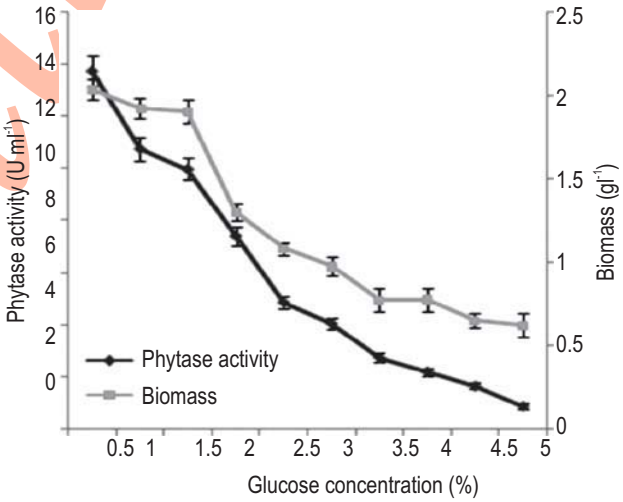

Fig. 3 : Effect of different glucose concentration (0.5-5.0%) on phytase production by *Zygosaccharomyces bailii* at 30°C after 42 hr. Results are expressed as mean of three replicates (*n* = 3)

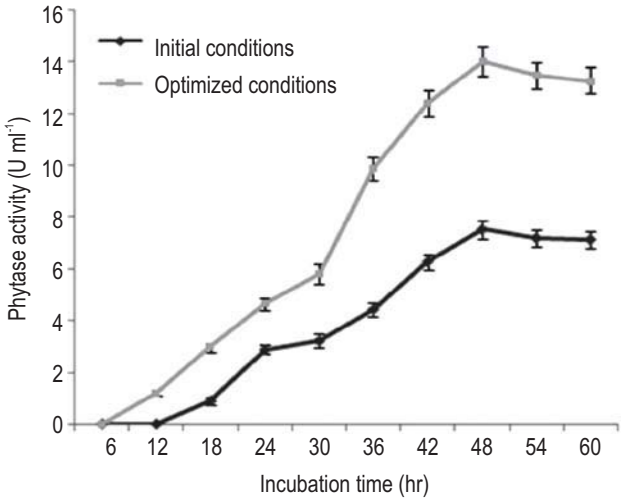

Fig. 4 : Time course of phytase production by *Zygosaccharomyces bailii* under initial and optimized culture conditions; Initial conditions: MYGP broth with 0.1% calcium phytate, 36 hr incubation, pH 5.5, 1% glucose as carbon source; Optimized conditions**:** MYGP broth with 0.1% calcium phytate, 42 hr incubation time, pH 6.0, 0.5% glucose as carbon source. Results are expressed as mean of three replicates (*n* = 3)

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