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Full Length Research Paper

Phytase production by the unconventional yeast *Pichia anomala* in fed batch and cyclic fed batch fermentations

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A marked enhancement in the production of cell-bound phytase in *Pichia anomala* was achieved in cyclic fed batch fermentation (47333 UL^{-1}) in comparison with that in batch (11569 UL^{-1}) and fixed volume fed batch (36911 UL^{-1}) fermentations in cane molasses medium. Phytase productivity was sustainable in cyclic fed batch fermentation over seven days, and therefore, this mode of fed batch fermentation appears to be a better approach for producing phytase in a cost-effective manner.

Key words: Fed-batch fermentation, cyclic fed batch fermentation, *Pichia anomala*, phytase.

INTRODUCTION

Phytic acid is the primary storage form of phosphate (30 to 50%) in plants. Phytates are considered as anti-nutritional factor (ANF) because they chelate metal ions like Mg^{2+}, Ca^{2+}, Fe^{2+} and Zn^{2+} making them unavailable, forms complexes with proteins and carbohydrates which are not easily digested and inhibit enzymes such as amylase, lipase, tyrosinase and others (Noureddini and Dang, 2009). Phytate phosphorus is not available to monogastric animals due to lack of adequate level of phytate-degrading enzymes. Pre-hydrolysis of phytates are, therefore, mandatory to liberate inorganic phosphates in order to make it available for monogastrics. Phytases (myo-inositol hexakisphosphate phosphohydrolase) include a class of acid phosphatases capable of liberating phosphate from phytate hydrolysis (Quan et al., 2001; Kerovou et al., 2000). International Union of Pure and Applied Chemistry and the International Union of Biochemistry (IUPAC–IUB) classified two groups of phytases, 3-phytase (EC 3.1.3.8) and 6-phytase (EC 3.1.3.28) on the basis of initiation of dephosphorylation at 3 or 6 positions, respectively. Thus, phytases not only promote the growth of monogastrics but also reduce the phosphorous excretion by up to 50% and consequently, mitigates the phosphorous associated pollution (Vohra and Satyanarayana, 2001; Wodzinski and Ullah, 1996).

Batch and fed batch fermentations have been extensively used for producing biomass (Ibrahim and Steinbuchel, 2010) and enzymes (Bailey et al., 2007; Sharma and Satyanarayana, 2011). Nonetheless, cyclic fed batch has not received adequate attention for enzyme production. Withdrawal of a fixed volume of culture medium and refilling it with the same volume of fresh sterilized medium can extend the life of fed batch cultivation (Ibrahim and Steinbuchel, 2010). Moreover, the residual culture acts as inoculum for the next cycle of fed batch that makes ensures the advantage of higher inoculum ratio.

In this investigation, different strategies of fed batch fermentation was attempted for producing biomass and phytase by the unconventional yeast *Pichia anomala* in cane molasses medium (CM medium). The use of soybean oil in overcoming the routine problem of foaming during fermentation has also been checked. This is the first report on the production of phytase by cyclic fed batch fermentation by *P. anomala.*

MATERIALS AND METHODS

Cultivation conditions

The yeast strain was isolated in our laboratory from dried flower buds of *Woodfordia fruticosa* and identified as *P. anomala* (Hansen)

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Kurtzman according to the study of Barnett et al. (2000) and maintained as described earlier (Vohra and Satyanarayana, 2001). The culture was deposited at the Microbial Type Culture Collection, Institute of Microbial Technology (Chandigarh, India) (MTCC-4133). The yeast *P. anomala* was grown in an Erlenmeyer flasks (250 ml) containing 50 ml of cane molasses medium (Kaur and Satyanarayana, 2005).

Analytical methods

The fermentation medium was drawn aseptically at desired intervals and analyzed for phytase and biomass production as well as, total sugar consumption as described earlier (Kaur and Satyanarayana, 2010). The cell culture was divided into two equal parts and harvested by centrifugation followed by washing of the cells with normal saline. One fraction of the cells were suspended in acetate buffer (100 mM, pH 4.0) and assayed for phytase. The other fraction was kept for drying at 80°C to constant weight in order to estimate yeast biomass.

Phytase assay

Phytase was assayed colorimetrically by measuring the release of inorganic phosphate from calcium phytate (HiMedia, India) according to the study of Heinonen and Lahti (1981). One unit of phytase is defined as the amount of enzyme that liberates one nanomole inorganic phosphate per second under the assay conditions.

Fermentation strategies

The yeast was grown in shake flasks (1 L) as well as in 7 L lab stirred tank laboratory fermenter (Applikon Biotechnology, The Netherlands) containing 200 ml and 4 L of CM medium, respectively and inoculated with 2% (v/v) 12 h old culture (10^{10} CFU/ml). The fermenter was operated at 25°C, 250 rpm with 1 vvm aeration. The pH was adjusted to 5.0 with 1 N NaOH/HCl. The samples were drawn aseptically at the desired intervals and analyzed for cell bound phytase and biomass. Two strategies were carried out for feeding the CM medium. The first strategy was the conventional fixed volume fed batch fermentation that was maintained at 75% dissolved oxygen (DO) (by supplementing the air with oxygen) with 1 vvm aeration. Feeding was done at every 24 h with concentrated CM medium to maintain the sugar level at 8.0%. The second strategy consisted of the concept of 'batchfill and draw system' for feeding, in which 2 L of culture medium was drawn out from the reactor every 24 h and filled with 2 L of 1.5X fresh cane molasses medium to maintain the sugar level. Samples were drawn at every 12 h for analytical studies. Foaming is a very frequent problem in all types of fed batch fermentations. Therefore, an attempt was made to run the cyclic fed batch fermentation by incorporating soybean oil (0.5% v/v) in CM medium.

RESULTS AND DISCUSSION

Phytase production in the fermenter was 11569 UL^{-1} and it was ~1.12 fold higher than that in shake flasks (10329 UL^{-1}). The production time was reduced by 4 h in the fermenter, where the peak was attained in 20 h. The higher production of enzyme as well as, biomass achieved in the fermenter than in shake flasks could be due to proper regulation of pH and better mixing of contents in the fermenter. When yeast was cultivated with supplementation of oxygen, better phytase and biomass production were attained as compared to the control (cultivation of yeast at 1 vvm of aeration without supplementation of oxygen). Therefore, various runs of batch fermentation were carried out for optimizing dissolved oxygen by varying supplementation of dissolved oxygen (25, 50, 75 and 100%) in stirred tank fermenter to find out the favorable DO. Maximum phytase and biomass production were attained at 75% DO (data not shown). Therefore, subsequent cultivations were performed in the fermenter at 75% DO. An oxygen saturated medium contains only ~7.6 mg/dm^3 of O_2 at 30°C (Stanbury et al., 1997), which is not adequate for the complete oxidation of the carbon source. Moreover, cane molasses is a complex and highly viscous material, which contains more reduced form of sugars and at the same time demands high oxygen.

Phytase as well as, biomass production declined after 24 h in batch cultivation, and therefore, fed batch strategies were attempted. Sugar concentration was reduced by 50% during the cultivation of the yeast in 24 h. Thus, fresh concentrated medium was added every 24 h at 1.1 mL^{-1} min^{-1}. Higher production of inulinase (Hensing et al., 1994), protease (Beg et al., 2001), laccase (Bailey et al., 2007), α-amylase (Sharma and Satyanrayana, 2011), probiotic biomass (Aguirre-Ezkauriatza et al., 2010) and ethanol production (Zhang et al., 2010) have been attained in fed batch cultivation. Fed-batch process also acts as a microbial cell factory for the production of high cell density biomass by maintaining limiting substrate concentration at low level (Schmidt, 2005; Ibrahim and Steinbuchel, 2010). Phytase as well as, biomass production enhanced till 48 h in fed batch fermentation was followed by a decline (Figure 1), which could be due to the production of secondary metabolites, acidic products and their accumulation during the long run of fermentation (Rudolf et al., 2004; Radwan et al., 2011) or accumulation of phosphorus that represses phytase synthesis.

To dilute the accumulated metabolites and phosphorus, and to overcome the limitations of fixed volume fed batch, cyclic mode of fed batch was attempted. A high phytase (47333 UL^{-1}) and biomass (32 g L^{-1}) production were achieved after 72 h in cyclic fed batch fermentation and it was sustainable till 7 days of fermentation (Figure 2). The residual sugar in the spent medium was the lowest, signifying that sugar consumption is the highest in cyclic fed batch fermentation (Table 1). This fermentation strategy was commonly used for the production of secondary metabolites (Bushell, 1989), but not for enzyme production. The fermented medium was drawn out and filled with the same volume of concentrated fresh medium to attain quasy steady state by which sustainable biomass generation was attained throughout the run (Pirt, 1979). Cyclic mode of feeding extended sustainable phytase formation till the 7^{th} cycle followed by a decline thereafter:

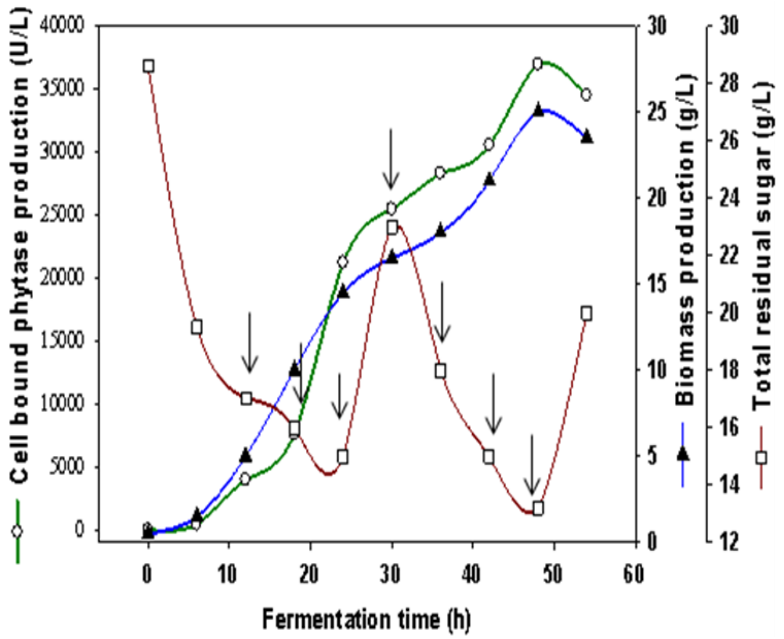

Figure 1. Fed batch fermentation profile for the production of phytase at 75% DO in a laboratory fermenter; →: indicate addition of fresh medium.

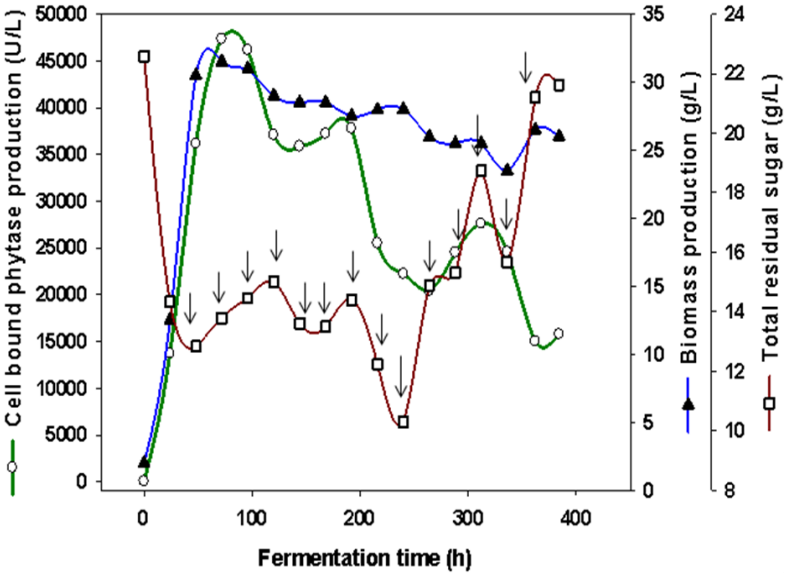

Figure 2. Phytase production profile in cyclic fed batch fermentation at 75% DO in a laboratory fermenter; →: indicate removal of fermented medium and addition of fresh medium.

this could be due to the accumulation of inhibitory metabolites (Queener and Swatz, 1979) and non-producing variants (Staunbury et al., 1997). A Similar enhancement was also reported in sorbose by *Gluconobacter oxydans* (Giridhar and Srivastava, 2001), human serum albumin from the recombinant *Pichia pastoris* (Bushell et al., 2003) and citric acid by *Yarrowia lipolytica* (Moeller et al., 2011) due to repeated fed batch fermentations. Furthermore, cyclic fed batch fermentation was not limited by working capacity of fermenter, and therefore, several cycles can be run. Repetitive sterilization, inoculation and washing of fermenter can be avoided in cyclic fed batch, which

Table 1. Phytase production by *P. anomala* in various fermentation strategies.

Fermentation strategy	Phytase production (UL^{-1})	Biomass production (g^{-1} DYB)	Total residual sugar ($g\ L^{-1}$)
Batch fermentation (Shake flask)	10329 ± 100	12.1 ± 1.2	18.4 ± 1.2
Batch fermentation (Lab fermenter)	11569 ± 105	13.5 ± 0.9	15.3 ± 1.1
Fed batch fermentation (sparging with air)	16040 ± 130	17.5 ± 0.9	17.5 ± 1.9
Fed batch fermentation (Sparged with O_2 supplemented air)	36911 ± 97	25.0 ± 1.1	13.2 ± 1.6
Cyclic Fed batch fermentation (sparged with O_2 supplemented air)	47333 ± 103	32.0 ± 1.2	7.4 ± 1.9

± Standard deviation; DYB: dry yeast biomass.

consequently, enhances the overall productivity and makes it a cost-effective and efficient fermentation strategy.

Vegetable oils have successfully been used as defoamers. Soybean oil comes under the category of fatty acid derivatives, which rupture foam film by reducing the surface tension (Reit and Van Sonsbeck, 1992). Soybean oil (0.5%, v/v) was effective in coping with the foaming problem for sustainable phytase and biomass production during fed batch fermentation. Soybean oil can thus be a cheaper alternative to silicon to overcome the problem of foaming.

Since spent molasses medium contained 7 to 14 $g\ L^{-1}$ total sugar, vitamins and other growth factors, it was recycled for reducing nutrient levels by cultivating the yeast *P. anomala*, thermophilic bacterium *Geobacillus thermoleovorans* and thermophilic mould, *Thermomucor indicae–seudaticae* for the production of phytase, α-amylase and glucoamylase, respectively (Kaur et al., 2011). These enzymes are useful in starch saccharification (Kaur and Satyanarayana, 2004) and in extending the shelf life of bread (Rao and Satyanarayana, 2007).

Conclusion

The cyclic fed-batch fermentation represents an easy process with simple feeding strategy involving withdrawal and refilling of the medium. A marked improvement in phytase production in cyclic fed batch fermentation signifies its importance and can markedly lower the cost of phytase production.

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