African Journal of Biotechnology Vol. 7 (17), pp. 3115-3120, 3 September, 2008
Available online at http://www.academicjournals.org/AJB
ISSN 1684–5315 © 2008 Academic Journals

Full Length Research Paper

Fermentative production and application of acid phytase by *Saccharomyces cerevisiae* CY strain

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Accepted 16 July, 2008

Improvement of phytase production from *Saccharomyces cerevisiae* CY strain was attempted by optimizing the culture medium. The optimum medium components for cell growth and enzyme production were found to be galactose, ammonium sulfate, sodium phytate, and magnesium sulfate. Galactose (5%) and ammonium sulfate (2%) were the most suitable carbon and nitrogen sources, respectively, for phytase production. The phytase activity was most active at pH 4.0 in the initial medium conditions. Phytate stimulated enzyme production at low concentration; however, phytase activities were decreased with increase of phytate concentration at above 0.002%. Maximum phytase activity was 135.09 mU/mg DCW after 72 h fermentation under optimal conditions. The harvested *S. cerevisiae* CY cells were used for the degradation of phytate in the soybean-curd whey. Over 60% of phytate in the soybean-curd whey were nearly linearly degraded with cells of 27.16 U/g-phytate dosage as phytase source after a hydrolysis time of 2 h.

Key words: Culture medium, fermentation, phytase, phytate degradation, *Saccharomyces cerevisiae.*

INTRODUCTION

Phytate (*myo*-inositol hexakisphosphate) is the common storage form of phosphorus in plant seeds and cereal grains (Reddy et al., 1982). Phytate is considered to be an anti-nutritional factor for humans and animals because of its high chelating ability with cations and complex formation with the basic amino acid group of proteins, thus decreasing the dietary bioavailability of these nutrients (Wodzinski and Ullah, 1996; Martinez et al., 1996). Phytate is not metabolized by monogastric animals, which have low levels of phytate-hydrolyzing enzymes in their digestive tracts. These unmetabolized phytates pass through the intestinal tract and are excreted outside and caused environmental problems by eutrophication of surface water resources (Raboy, 2001). In order to increase the bioavailability of essential dietary minerals and decrease environmental pollution, the degradation of phytate in foods and feeds is of nutritional and environmental importance.

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Phytases (*myo*-inositol hexakisphosphate phosphohydrolase, EC 3.1.3.8) catalyze the hydrolysis of phytate to the inorganic phosphate and less-phosphorylated *myo*-inositol derivatives (Konietzny and Greiner, 2002). This enzyme produces available phosphate and a non-metal chelator compound. Therefore, phytases are considered to be enzymes of great value in enhancing the nutritional quality of phytate-rich foods and feeds (Martinez et al., 1996; Oboh and Elusiyan, 2007). Phytases are present in plants, certain animal tissues, and microorganisms. They have been studied most intensively in the seeds of plants (Gibson and Ullah, 1988; Greiner 2002). Phytase activity in microorganisms has been found most frequently in fungi (Ullah and Gibson, 1987; Mullaney et al., 2000), bacteria (Kim et al., 1998; In et al., 2004; Oh and Lee, 2007) and yeast (Quan et al., 2002; Veide et al., 2006; Kaur et al., 2007). Among the bacterial phytases, the pH optimums for extracellular and intracellular phytases are 6.0-7.0 and 4.5-6.0, respectively. For industrial application, a phytase with a pH activity profile ideally suited for maximal activity in the digestive tract of monogastric animals is desirable. Because of its great practical importance, there is an ongoing interest in isolating new

and safe microbial strains producing novel and efficient phytases.

As *Saccharomyces cerevisiae* is generally recognized as safe (GRAS), it is frequently used in food and feed applications. Recently, we have reported the isolation of a *S.cerevisiae* CY strain producing acid phytase which was highly active at pH 3.5 (Seo et al., 2005). In this study, we attempted to optimize the cultural conditions, such as carbon, nitrogen, mineral sources, and culture pH, to increase the phytase production and to apply the degradation of phytate in soybean-curd whey.

MATERIALS AND METHODS

Materials

The YPD broth and YM agar were products of Difco Laboratories (Detroit, MI). All other chemicals were of analytical grade. The soybean-curd whey was kindly obtained from a tofu manufacturing company, Doosol Co., Ltd. (Yesan, Korea).

Microorganism

The *S. cerevisiae* CY strain which was screened from mash of Korean traditional wine (Seo et al. 2005) was used for the microbial phytase source in this work. The stock culture was maintained on an YM agar (0.3% yeast extract, 0.3% malt extract, 0.5% peptone, 1% dextrose, 1.5% agar, pH 6.2) slant and subcultured monthly.

Growth and culture conditions

Seed culture was prepared in a 250 ml baffled flask containing 50 ml of YPD broth (1.0% yeast extract, 2.0% peptone, 2.0% dextrose, pH 6.5) incubated at 30ºC for about 6 h on a rotary shaker. Basal culture medium (1.0% yeast extract, 0.6% succinic acid, pH 6.0) was inoculated with 2% (v/v) seed culture. To minimize the variation of pH during yeast cultivation, succinic acid/NaOH was used as buffer in the basal media (Andlid et al., 2004). The main cultivation for the production of enzyme was performed for 48~72 h under the same conditions as with the seed culture. Cells were harvested by centrifugation at 5,000 x g for 10 min and then the pellet used for phytase activity. For the determination of dry cell weight (DCW), the pellet was washed twice with distilled water and dried overnight at 80ºC to a constant weight. All experiments were carried out at least twice until the optimal culture conditions were found; as such, the data shown in this paper are representative.

Phytase assay

Phytase assays were carried out using the Shimizu method (Shimizu, 1992) with some modification. The collected cells were resuspended in 0.2 M acetate buffer (pH 3.6) for the determination of phytase activity. The enzymatic reactions were initiated by incubating 0.1 ml of the cell suspension with 0.9 ml of 2 mM sodium phytate in 0.2 M sodium acetate buffer (pH 3.6). After incubation at 37ºC for 10 min, the reaction was stopped by adding 1.0 ml of 10% trichloroacetic acid. The liberated inorganic phosphate was measured according to the ammonium molybdate method (Heinonen and Lahti, 1981). One unit (U) of phytase activity was defined as the amount of enzyme that liberated one micromole of inorganic phosphate per minute under the assay conditions, and the phytase activity is expressed as a function of dry cell weight (mU/mg DCW).

Degradation of phytate

A glass vessel (5 x 5.5 cm) equipped with a water-jacket was used as the bioreactor system. Phytate in soybean-curd whey, the watery part of soymilk that is separated from the curd during the tofu manufacturing process, was used as substrate (pH 3.6). The yeast cell suspension was added to the vessel and kept at a temperature of 40ºC. The enzyme reaction was executed for 4 h with mixing at 150 rpm. The phytate concentration of the reaction product was measured by the Wade method (Latta and Eskin, 1980). The reaction solution (0.3 ml) was added to 2.7 ml DW and 1.0 ml of Wade reagent (0.03% $FeCl_3 \cdot 6H_2O$ and 0.3% sulfosalicylic acid in DW). The mixture was stirred on a vortex mixer and then filtered with 0.45 µm syringe filter; the absorbance of the filtrate was read at 500 nm.

RESULTS AND DISCUSSION

Effect of carbon sources

The effects of carbon sources on the yeast growth and phytase production by *S. cerevisiae* CY strain were investigated. Various carbohydrates (2.0%) were supplemented as the carbon source in the basal medium. The results were shown in Table 1. Among the tested media with various carbon sources, the highest cell growth (5.57 mg DCW/ml) and phytase production (11.30 mU/mg DCW) was obtained in the galactose supplemented medium. After 72 h cultivation, a high level of cell growth and phytase activities were obtained when fructose, galactose, glucose, mannose as monosaccharide and maltose and sucrose as disaccharide were used in the basal media. In spite of lactose being composed of galactose and glucose, the very poor performance of lactose appears to be related with the deficiency of lactose hydrolysis system in *S. cerevisiae* CY strain. The effect of concentration of galactose on phytase production was investigated. Concentration of galactose was changed from 1.0 to 10.0% in the basal media. As shown in Figure 1, phytase activity and cell growth increased with the increase of initial galactose concentration in the medium. The maximum phytase activity (60.72 mU/mg DCW) was obtained after 48 h of cultivation at 10.0% of initial galactose concentration. Galactose, the best carbon source for the production of phytase by *S. cerevisiae* CY, has been also reported to enhance the phytase production in the yeast *Arxula adeninivorans* CBS7377 (Sano et al. 1999). However, Kim et al. (1982) noted that galactose was less effective carbon source than glucose for phytase production by the fungus *Aspergillus niger*.

Effect of nitrogen sources

To investigate the effect of nitrogen sources on the growth and production of phytase, several organic and inorganic nitrogen sources (1.0%) were added in the basal medium containing 3.0% galactose as a carbon source. As shown in Table 2, the cell growth was similar

Table 1. Effect of carbon sources on the cell growth and phytase production by *Saccharomyces cerevisiae* CY strain.

Carbon source	Cell growth (mg DCW/ml)	Phytase activity (mU/mg DCW)
Control[a]	0.30	0
Arabinose	0.40	2.51
Ribose	0.50	4.02
Xylose	0.60	5.02
Fructose	5.37	7.44
Galactose	5.57	11.30
Glucose	5.37	8.19
Inositol	0.40	5.02
Mannose	5.57	4.85
Lactose	0.40	2.51
Maltose	5.18	6.96
Sucrose	5.57	6.64
Dextrin	0.50	4.02

[a]Control medium: 1.0% yeast extract, 0.6% succinic acid, pH 6.0.

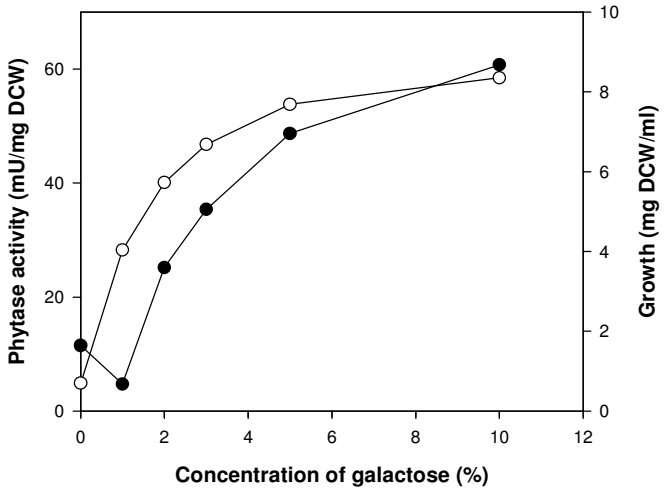

Figure 1. Effect of concentration of galactose on phytase activity (●) and cell growth (○) by *S. cerevisiae* CY strain. Fermentation was carried out in 500 ml baffled flask containing 50 ml medium (1~10% galactose, 1% yeast extract, 0.6% succinic acid, pH 6.0) for 48 h at 30ºC and 150 rpm.

in the media with organic and inorganic nitrogen sources. However, the highest phytase activity (52.66 mU/mg DCW) was obtained when ammonium sulfate was used in the media, followed by peptone (25.42 mU/mg DCW), yeast extract, potassium nitrate, and sodium nitrate. Ammonium nitrate was the worst nitrogen source for phytase production in this study. On the other hand, Ramachandran et al. (2005) stated that ammonium nitrate as inorganic nitrogen and peptone as organic nitrogen sources stimulated the phytase production in *Rhizopus* spp. and the former was more effective than

Table 2. Effect of nitrogen sources on the cell growth and phytase production by *Saccharomyces cerevisiae* CY strain.

Nitrogen source	Cell growth (mg DCW/ml)	Phytase activity (mU/mg DCW)
Control[a]	7.36	23.63
Ammonium chloride	7.86	5.09
Ammonium nitrate	7.76	1.03
Ammonium sulfate	5.37	52.66
Potassium nitrate	7.46	22.91
Sodium nitrate	7.76	21.51
Beef extract	8.06	1.74
Casamino acid	7.76	2.06
Malt extract	8.56	9.93
Peptone	8.46	25.42
Proteose peptone	8.46	21.40
8.46		16.79
Tryptone	8.46	4.26
Urea	7.27	18.45

[a]Control medium: 3.0% galactose, 1.0% yeast extract, 0.6% succinic acid, pH 6.0.

the latter. To determine the optimum concentration of ammonium sulfate and peptone, their concentrations in the basal medium with 3.0% galactose as carbon source were varied from 0 to 5.0%. As presented in Figure 2, the cell growth was higher in the peptone supplemented media than those of ammonium sulfate media, but the phytase activities were lower in the peptone supplemented media. The phytase activity peaked at 2.0% ammonium sulfate (106.23 mU/mg DCW) and at 1.0% peptone (58.65 mU/mg DCW) as the nitrogen source after 48 h of cultivation, respectively.

Effect of culture pH

In order to examine the effect of culture pH on phytase production, the batch cultures of *S. cerevisiae* CY were conducted in baffled flask at pH 4.0~6.1 using the basal media with 3.0% galactose and 2.0% peptone. The cell growth and phytase production for different pH during cultivation were shown in Figure 3. Due to succinic acid/NaOH buffer system (Andlid et al., 2004), the initial pH in the range 4.0 to 6.1 were slightly changed in 4.6 to 5.9 during cultivation (data not shown). Yeast growth slightly decreased but phytase production drastically decreased with the increase of initial pH of medium. The maximum amount (57.42 mU/mg DCW) of phytase production occurred at initial pH 4.0 after 48 h culture. In contrast, Andlid et al. (2004) stated that the degradation of phytate during *S. cerevisiae* YS18 cultivation in YPD broth was efficient at initial pH 6.0 and expression of phytase gene was controlled by medium pH.

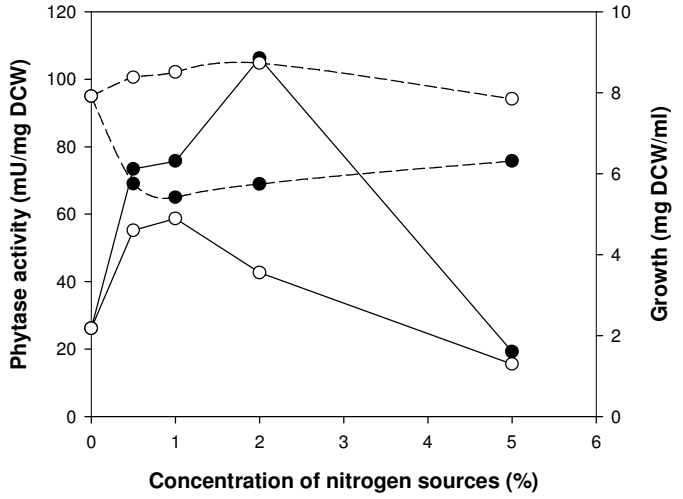

Figure 2. Effect of concentration of ammonium sulfate (●) and peptone (○) as nitrogen source on phytase activity (solid line) and cell growth (dashed line) by *S. cerevisiae* CY strain. Fermentation was carried out in 500 ml baffled flask containing 50 ml medium (3% galactose, 0~5% ammonium sulfate or peptone, 1% yeast extract, 0.6% succinic acid, pH 6.0) for 48 h at 30ºC and 150 rpm.

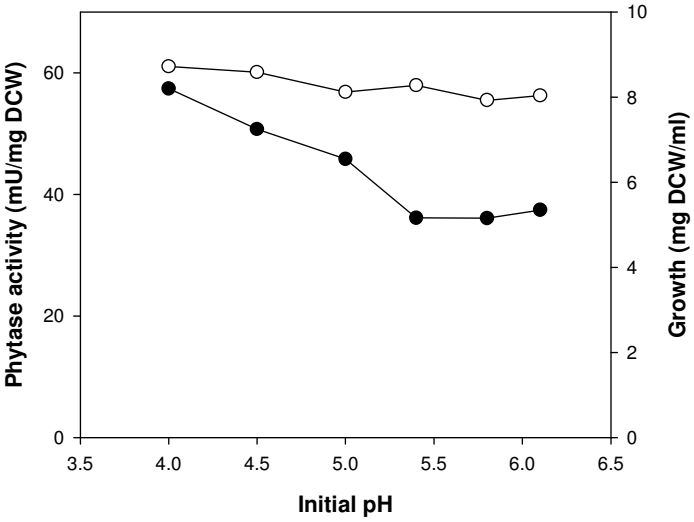

Figure 3. Effect of initial medium pH on phytase activity (●) and cell growth (○) by *S. cerevisiae* CY strain. Fermentation was carried out in 500 ml baffled flask containing 50 ml medium (3% galactose, 2% peptone, 1% yeast extract, 0.6% succinic acid, pH 4.0~6.1) for 48 h at 30ºC and 150 rpm.

Effect of phosphate and mineral sources

The effects of phosphorous sources on the yeast growth and phytase production by *S. cerevisiae* CY were investigated. When potassium phosphate (KH_2PO_4) was added to the medium at the concentration of 0.01~0.07%, phytase activity showed very low activity of about 1~3 mU/mg DCW (data not shown). *S. cerevisiae* CY strain was cultured in a baffled flask containing basal media

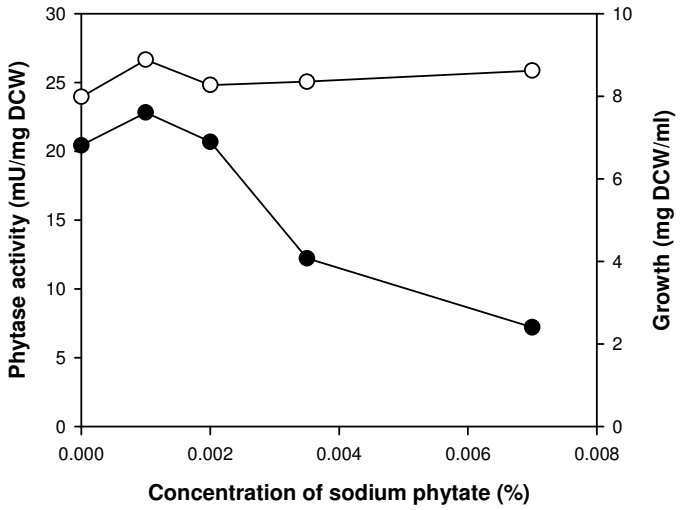

Figure 4. Effect of concentration of sodium phytate on phytase activity (●) and cell growth (○) by *S. cerevisiae* CY strain. Fermentation was carried out in 500 ml baffled flask containing 50 ml medium (3% galactose, 1% yeast extract, 0.6% succinic acid, 0~0.007% sodium phytate, pH 4.0) for 48 h at 30ºC and 150 rpm.

with 3% galactose, 0.001~0.007% sodium phytate (pH 4.0). Phytase activity in the non-KH_2PO_4 added control medium showed 20.42 mU/mg DCW as a control group. On the other hand, the maximum phytase activity was 22.81 mU/mg DCW in the supplemented medium with 0.001% sodium phytate (Figure 4). However, phytase activity decreased with the increase of sodium phytate concentration at above 0.002%. After 48 h cultivation in 0.007% sodium phytate conditions, inorganic phosphate concentration in broth was determined to 0.0062%, which was enough to repress the phytase production (Ogawa et al., 2000). Although the presence of inorganic phosphorus is an essential ingredient of phytase production medium (Soni and Khire, 2007), increasing the concentration of inorganic phosphorus above 0.005% shows adverse effect on phytase production (Kim et al., 1998; Bhavsar et al., 2008). Similarly, phytase production was strongly inhibited in *Schwanniomyces castellii* when phosphorus levels exceeded 0.132% (Lambrechts et al., 1992). High levels of inorganic phosphorus repress the biosynthesis of phytase (Ogawa et al., 2000; Andlid et al., 2004). In the case of *Bacillus subtilis*, minimal medium containing inorganic phosphate and phytate did not induce phytase production but defined medium containing phytate as sole source could (Kerovuo et al., 1998). The observed result in the *S. cerevisiae* CY strain may be shown that phytate at lower concentration stimulates the phytase synthesis, but some phosphate liberated from phytate by phytase during cultivation significantly inhibits phytase production at high phytate concentration. Therefore, to minimize the repression by phosphate, 0.001% of sodium phytate was suitable for phytase production.

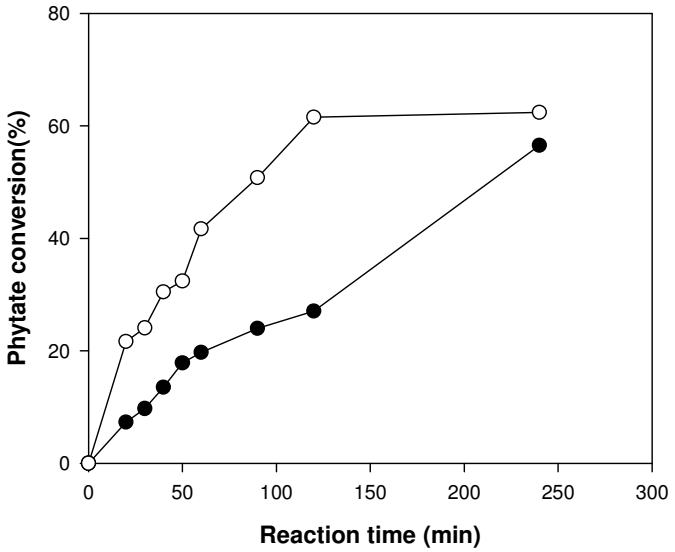

Figure 5. Time course for the conversion of phytate dissolved in soybean-curd whey mixture using different enzyme dosages. The reactor was kept at 40ºC and the initial concentration of phytate was 0.73 mg/ml. Enzyme dosages (U/g-phytate): ●, 13.58; ○, 27.16.

To investigate the effect of Mg^{2+} and Ca^{2+} on phytase production by a batch culture of *S. cerevisiae* CY strain, the fermentation supplemented with 0~0.2% of $MgSO_4$ or $CaCl_2$ were performed in basal media containing 3.0% galactose. Magnesium ion stimulated the phytase production at 0.025% but calcium ion faintly lowered the enzyme production and cell growth (data not shown). The addition of 0.025% of $MgSO_4$ enhanced enzyme production by about 1.4-fold compared with the phytase activity of non-added $MgSO_4$ media.

From above results, the optimum medium composition for the phytase production by *S. cerevisias* CY strain was 5% galactose, 1% yeast extract, 2% ammonium sulfate, 0.6% succinic acid, 0.025% $MgSO_4$, and 0.001% sodium phytate (pH 4.0) in this work. Maximum phytase activity in optimized medium reached to 135.09 mU/mg DCW after 72 h cultivation. The phytase production in the optimized medium resulted in as much as around 6-fold increase in phytase production when compared with the phytase activity in the basal medium with 3% galactose.

Degradation of phytate

The extent of phytate breakdown by the *S. cerevisiae* CY phytase was determined in a stirred tank bioreactor. The harvested CY cells and phytate in soybean-curd whey were used as phytase source and substrate, respectively. Soybean-curd whey generally contains 0.05~0.1% phytate because raw soymilk contains 0.1~0.4% phytate (Ishiguro et al., 2003). The concentration of phytate in soybean-curd whey was determined as 0.073% in this work. The decrease in phytate content was expressed as the conversion of phytate. The changes in conversion with reaction time, for the different dosages of *S. cerevisiae* CY phytase, are presented in Figure 5. Phytate conversion increased with increasing reaction time, and with enzyme dosage, in the soybean-curd whey mixture. Between the two dosages in the soybean-curd whey, the 27.16 U/g-phytate dose exhibited the faster conversion, which reached a plateau (over 60% conversion) for 2 h of hydrolysis. Similarly, phytate was degraded up to 50% when the immobilized *S. cerevisiae* CY cells were incubated in soybean-curd whey (In et al., 2007).

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