*Int.J.Curr.Microbiol.App.Sci* (2017) *6*(12): 266-274

*International Journal of Current Microbiology and Applied Sciences*
*ISSN: 2319-7706* **Volume 6 Number 12 (2017) pp. 266-274**
Journal homepage: http://www.ijcmas.com

**Original Research Article** https://doi.org/10.20546/ijcmas.2017.612.033

# Autolysis of Rice Bran Phytate in Long-Term Study on Batch Fermentor

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**Keywords**

Autolysis, Fermentation, Phytate-degrading enzymes, Rice bran.

**Article Info**

***Accepted:***
04 October 2017
***Available Online:***
10 December 2017

**A B S T R A C T**

Microorganisms especially bacteria produce a diverse of phytate-degrading enzymes. Rice bran is excellent media for bacterial growth and enzymes secretion. The aim of the study was autolysis of rice bran phytate (6%) in long-term on batch fermentor (with constant agitator speed (300 rpm) and fixed air flow rate (0.5kg/cm$^2$). The phytase production in the fermentor was with gradual color change from initial light green to dense green during the fermentation processes. The pH and temperature changes during production of phytase in the rice bran media over 10 weeks were observed. Initial 3 weeks, a reduction in pH from pH 6 to pH 4.2. After the middle of 4$^{th}$ week and 5$^{th}$ week considerable increase in pH towards the neutral range was observed i.e. from pH 6.2 to pH 6.99. In the 5$^{th}$ and 6$^{th}$ weeks the pH range was found to be pH 7 to pH 7.9. Starting from the beginning of 8$^{th}$ week to 10$^{th}$ week pH was in the near alkaline range pH 8- pH 8.2. The temperature of the media during the initial stages of fermentation for first 3 weeks was 22-25°C. Increase in temperature was noticed after the end of the third week. The remaining weeks from 3 to 10 the temperature range was 25°C-29°C. The temperature of the media inside the fermentor was in between 22°C and 29°C throughout the study (environment temperature 20-40°C). Enzymatic partitional hydrolyzed of rice bran phytate into lower myoinsitol phosophates will have many health benefits applications.

## Introduction

Phytate (myo-inositol hexakisphosphates), a phosphorylated derivative of myo-inositol, is widely distributed in plants, particularly in cereals and legumes, such as corn, soybean, wheat bran, rice bran, cotton seeds, rape seeds and soybean with a concentration range between 0.4% to 6.4%. Phytases are capable of hydrolyzing phytates, the major storage form of phosphate in plant seeds and pollen (Konietzny and Greiner, 2002) to a series of lower phosphate esters of myo-inositol and phosphate. Phytase is an extracellular enzyme that can catalyse the hydrolysis of phytic acid to inorganic monophosphate and lower myoinositol phosphates. Phytases are

widespread in the nature because they can be found in the animals, plants, and microorganisms. Generally, phytase activity of animals is negligible compared to their plant and microbial counterparts (Weremko *et al.,* 1997).

Phytases decomposes Phytate, which is the primary storage form of phosphate in plants. All commercial phytase preparations contain microbial enzymes which are produced by fermentation. A wide variety of phytases were discovered and characterized in the last 10 years. Different formulation technologies are used to produce enzyme powders that retain enzyme activity, are stable in application, resistant against high temperatures, dust-free, and easy to handle (Mukesh Kumar, 2011).

Phytases are widely distributed in nature (Irving, 1980; Nayini and Markakis, 1986) for example in plants, microorganisms and certain animal tissues. Phytase supplementation has been found to increase not only the growth rate of monogastric animals but also the efficiency of phosphate utilization in feeds, which significantly reduces phosphorus excretion and the chances of environmental pollution (Kornegay, 1996). This is because the undigested Phytate phosphorus is excreted in manure and poses serious phosphorus pollution problem, contributing to the eutrophication of surface waters in areas of intensive livestock production (Reddy *et al.,* 1982; Wodzinski and Ullah, 1996). Phytase can be also applied as cosmetic additives and plant nutrition (Koshy *et al.,* 2012; Gujar *et al.,* 2013). Phytase acts as an anti-nutrient by binding to proteins and by chelating minerals (Cheryan, 1980; Reddy *et al.,* 1989)., The addition of phytase can improve the nutritional value of plant-based foods by enhancing protein digestibility and mineral availability through Phytate hydrolysis during digestion in the stomach or during food processing (Reddy *et al.,* 1989; Sandberg and Andlid, 2002).

Phytase is very sensitive to pH and temperature, the utilization of phytase in fish feed is still on its first stage compared with that of in poultry and swine feed (Ling and Wang, 2007). The Phytate-degrading enzymes (Hussain *et al.,* 2009, 2010, 2015) also can be divided into two types based upon their optimal pH. These are the acid Phytate-degrading enzymes with a pH optimum around 5.0, and the alkaline Phytate-degrading enzymes with a pH optimum around 8.0 (Vucenik and Shamsuddin, 2003; Farouk *et al.,* 2015).

Most of the Phytate-degrading enzymes belong to the acid type. However, it has to be taken into account that microbial phytases of different sources can differ in their bio-efficacy per unit (Konietzny and Greiner, 2002; Hussain *et al.,* 2009, 2010, 2015). According to Lan, *et al.,* 2002 proteins and minerals in standard and purified rice bran phytic acid Rice bran, consisting of pericarp, aleurone and germ, has a high concentration of Phytic acid ranging from 5.94 to 6.09 g 100 $g^{-1}$ Rice bran is considered as a rice industry by-product and corresponds to 10g 100$g^{-1}$ of integral rice grain. It is used for oil extraction, feed production and also as an ingredient in the formulation of food products because of its protein, lipid, mineral and antioxidant (Parrado *et al.,* 2006). Rice bran was stated as the best carbon source for the production of phytase (Lan *et al.,* 2002).

Salts of phytic acid, designated as phytates, are regarded as the primary storage form of both phosphate and inositol in plant seeds and grains. Phytate is formed during maturation of the plant seed and in dormant seeds it represents 60–90 % of the total phosphate (Loewus, 2002). Phytate is therefore a common constituent of plant-derived foods. Depending on the amount of the plant--derived foods in the diet and the grade of food processing; the daily intake of phytate can be as high as 4500 mg. On an average, daily

intake of phytate was estimated to be 2000–2600 mg for vegetarian diets as well as diets of inhabitants of rural areas in developing countries and 150–1400 mg for mixed diets (Reddy, 2002).

Rice bran, a by-product of rice milling, is usually either discarded as a waste or used to feed poultry and livestock (Yusoff *et al.*, 2010). The Crude protein content in defatted rice bran is higher (20.02%) than corn (9.53%) and lesser than soya bran (20.02%), where Crude fiber content in rice bran is higher (10.73%) than both corn (4.78%) and soya bran (3.32%) (Moreira, 2003). The Phytate-P contents in rice bran and Wheat bran are 14.17 g/kg and 8.36 g/kg and their proportion % was 79.5 and 76.3 respectively (Cao *et al.*, 2007).

*Aspergillus oryza* has pH optimum 5.5 and temperature optimum 50°C with specific activity at 37°C was 11U/mg was stated (Greiner and Konietzny, 2006; Weremko *et al.,* 1997). The Content of organic, inorganic and total phosphorus, total nitrogen, phosphorus, total nitrogen, soluble and total proteins and minerals in standard and purified rice bran phytic acid were analysed by Canan (2011).

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In 2010 the technical enzymes generated was $1.10 billion and the world market for industrial enzymes surpassed $6 billion in 2016 (Anon, 2012). The continuously growing bio-processing industry demands inexpensive and renewable material. A study of microbial phytase production in the rice bran of media in a batch fermentor was carried out. The effect of pH, temperature and dissolved oxygen content during production were observed. Phytase production was influenced by the pH and temperature since it has sensitive protein nature of denaturation. In whole grain cereals such as corn, wheat and rice, the ranges of phytic acid is from 1.5 to 6.4% while defatted and dehulled oilseed meals such as soy, peanut and sesame contain 1.5% or more of the compound (Grases, 2004). Phytic acid is primarily found in the outer layers of rice bran. Rice bran is a by-product of rice milling to produce white rice to fulfill its desirability.

## Materials and Methods

### Rice bran media

For the autolysis of rice bran study, six percent of rice bran (source?) was used in fermentation for 10 weeks in a batch fermentor (model, country).

The changes of pH, temperature and dissolved oxygen content in the 6% rice bran without any additives such as tryptone, yeast, NaCl, peptone, meat extract, $MgSO_4$, $MgCl_2$, KCl, $CaCl_2$, casein (pancreatic digest) was selected to know the natural outcome. The media's initial pH was adjusted to pH 7. The reduction in pH i.e. acidic range was noticed in the initial stages of fermentation and the shoot up at the later stage denoted the production of active phytase.

The temperature (ºC), pH and dissolved oxygen (DO) observation was carried out for 10 weeks in the batch fermentor and results are shown in Figure 2 and 3 respectively.

### Production

The enzyme production was carried out by batch fermentation using production broth (rice bran extract - 6%; pH - 6.5, distilled water – 7000 ml). The medium was autoclaved at 121°C at 15 lbs. Fermentation under room temperature (25-30ºC) for 48 days at agitator speed at 300 rpm and at fixed air flow rate (0.5 kg/cm2). The temperature study was carried out for 10 weeks in the batch fermentor and the results are shown in Figure 1.

## Sample preparation

Samples for phytase enzyme activity assays were prepared by centrifugation of 1.5 mL bacterial culture from the fermentor at 13000 rpm for 1 min (Idriss *et al.,* 2002). The cell-free supernatant was separated for phytase assay. Phytase measurements carried out at 28ºC. The reaction was initiated with the addition of phytase enzyme from each day. After 30 minutes incubation, the liberated inorganic phosphate was measured using a modification of the ammonium molybdate method (Heinonen and Lahti, 1981). A freshly prepared solution of acetone: 5 N sulfuric acid: 10 mM ammonium molybdate (2:1:1 v/v) and thereafter 100 µL of 1.0 M of citric acid were added to 400 µL of the phytase assay mixture. The cloudiness was removed by centrifugation in a centrifuge at 13000 rpm for 10 min prior to the measurement of absorbance at 355 nm in a UV double beam spectrophotometer In order to quantify the phosphate released; a calibration curve was constructed over the range of 5 to 1200 mM phosphate**.**

## Production of phytase in rice bran media

The agitator speed was selected at 300 rpm since the rupture of bacterial cells may happen above this speed. Autolysis of rice bran showed the initial pH of the cultivation media which is different according to the each day of fermentation. Figure 2 shows the pH at different days and Figure 4 shows the fermentor samples variation in the absorption at 355 nm. The absorption was significantly higher in the 6$^{th}$ week at 26ºC with pH 7.62. The pattern was nearly similar on 7$^{th}$ week but the absorption values decreased in after 8$^{th}$ week to 10$^{th}$ weeks. It is assumed from study that the phytase production was significantly higher at 26ºC.

## Results and Discussion

The results of the present study showed that in this selected composition of rice bran (6%), production of phytase was significant. In beginning the phytate degrading enzymes from rice bran were active in the first six hours of the process. Then the bacteria of rice bran start to grow and produce more bacterial phytate degrading enzymes. Production of phytase was significant during the 6$^{th}$ week. The production of phytases started as soon as the cultures entered the stationary phase and it increased in the middle of the 5th week. As phytate in rice bran occurs as a less soluble potassium-magnesium salt, usually combined with protein, or enclosed by starch and other carbohydrates, the rate of rice bran phytate being hydrolyzed could be lower. The lower rate of hydrolysis ensures that phytase production is continuously induced during the whole fermentation process and end-product inhibition is prevented, thus leading to increased phytase production was also reported by Papagianni *et al.,* (1999).

The optimum temperature for the production of phytases from most of the microorganisms lies in the range of 25 to 37ºC (Vohra and Satyanarayana, 2003) also comes to true in this study of autolysis of rice bran. Awad *et al.,* (2014) also reported that 27 °C was the optimal temperature for phytase production. The optimum temperature range for incubation of microorganisms for high

phytase (Gautam *et al.*, 2002). However, Hussin *et al.*, (2011) found that 33°C was the optimum temperature for *P. stewartii* to produce phytase.

**Fig.1** Change in Temperature during the Production of Phytase in a batch fermentor. Liberation of P from action rice bran phytase on phytate showed effect on raising the residual temperature from 45 to 53 days

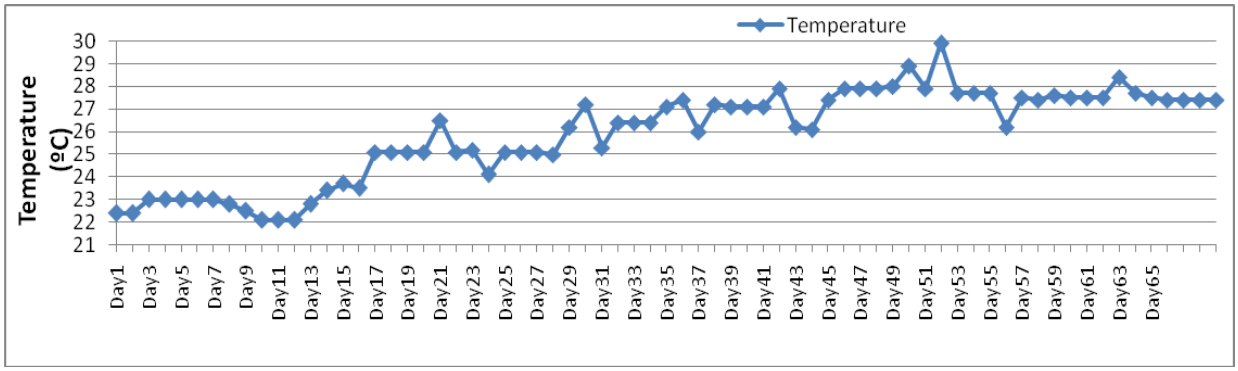



**Fig.2** Change in pH during the Production of Phytase in a batch fermentor indicate the microbial growth (bacterial) during the phytate hydrolysis. The pH was turned from pH 5.0 to 4.2 (Acidic) during the first 3 weeks. After 3 weeks till 37 days pH have fast increased to 7.0. During the last 3 weeks till 69 days pH turned to 7.8 (Alkaline)

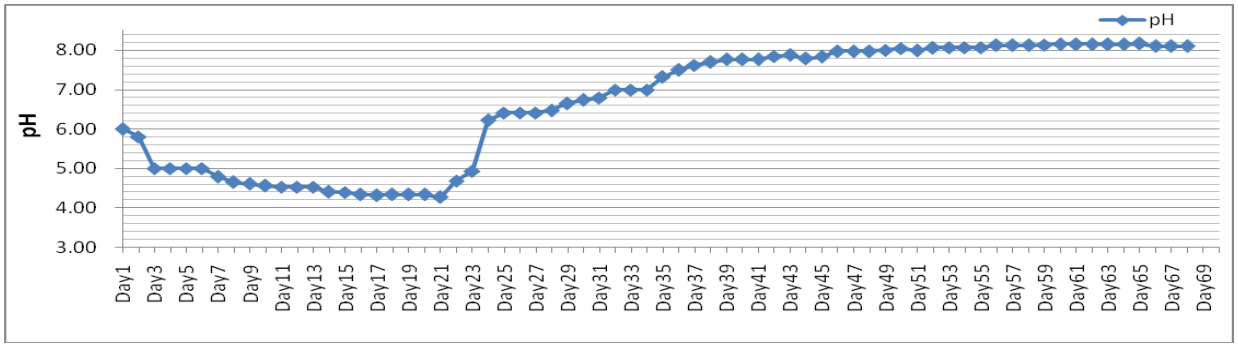



**Fig.3** Change in dissolved oxygen (DO) of Rice bran phytase production for 67 days in a batch Fermentation. The DO have decreased after 3 weeks and reach steady low after 57 days

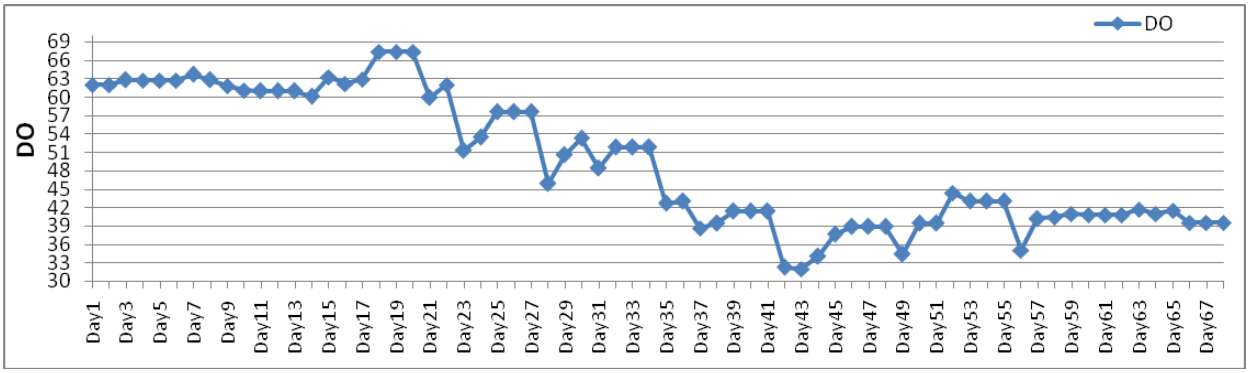
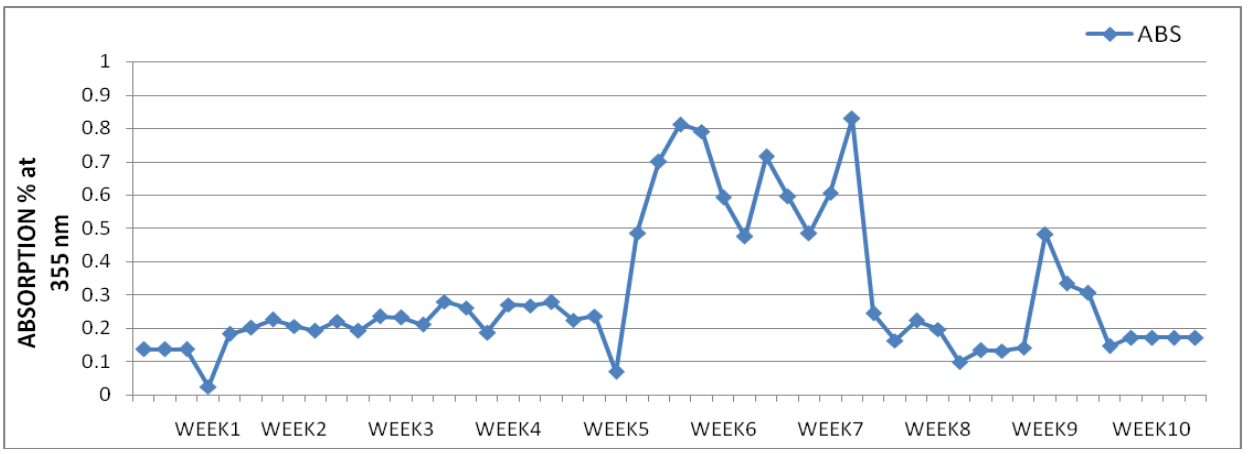


**Fig.4** The Optical density at 355 nm values measured the liberated P from rice bran during 10 weeks of fermentation process. The highest P was liberated during weeks 6, 7 and 9




The optimum initial pH for phytase production of *B. cereus* ASUIA260 was pH 7.2 (Vohra and Satyanarayana, 2003), the optimum pH of most bacteria and fungi is in the range between pH 5.0 and pH 7.0. In this study the optimum value was at pH 7.5. The stability of most plant phytases decreased dramatically at pH values below 4 and above pH 7.5, while the majority of the corresponding microbial enzymes are stable even at pH values above 8.0 and below 3.0 (Greiner and Konietzny, 2006). In our present study the pH variations were found, above pH 8 the stability decreased. Most phytases have an optimal pH in the range of 4.5–6.0 and a temperature range of 45–60ºC. Outside the optimal range of pH and temperatures the action of phytase is reduced (Turk *et al.*, 1996).

The majority of the plant phytases are irreversibly inactivated at temperatures above 70ºC within minutes, whereas most of the corresponding microbial enzymes retain significant activity even after prolonged incubation times (Singh and Krikorian, 1982). The majority of the so far characterized phytases showed maximal phytate-degrading activity in the acid pH range. Alkaline phytases have been purified from different *Bacillus* species. In this study at pH 7.62 a sharp pH optimum was noticed. Anyhow repeated and further studies required. Enzyme activity increases with temperature up to a maximal temperature. A further increase in temperature results in a heat-induced denaturation of the enzymes. Depending on the enzyme source, optimal temperature for phytate hydrolysis varies from 35 to 80°C (Greiner and Konietzny, 2006). In this study the temperature range was 22-29°C throughout the experiment. Further study needed to know the behaviors at elevated temperatures.

The Figure 2 shows the pH at different days and Figure 4 shows the phytase activity. The phytase production was significantly higher at pH range 7.62. The phytase production was significantly higher at temperature 26ºC. The phytase production was significantly higher at $6^{th}$ week. Further study can be conducted by increasing/decreasing the rpm of the agitator, changing the dissolve oxygen (D.O) content, % of rice bran, the volume of the media, changing the impeller design and at elevated temperatures. The nature for phytases with more constructive properties should be studied for food applications and engineering phytases in order to optimize their catalytic

and stability features are suitable approaches to make a proper phytase available for a specific application in food processing. An improved method for production of phytase and yields can be studied for defatted rice bran/enriched rice bran/at different temperature/varying the periods of fermentation with known concentration of rice bran though many times the concentration has little effect compare to other factors.

## Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

## Acknowledgement

The authors express their gratitude to Taif University, Al-Hawiya 888, Kingdom of Saudi Arabia and the support of the Dean of Science. Also, the authors extend their sincere gratitude for the support of the Biotechnology Department of Taif University, Saudi Arabia for its support for these research efforts.

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**How to cite this article:**

Abd-ElAziem Farouk, N. Thoufeek Ahamed, Anis Shobirin Meor Hussin and Othman AlZahrani. 2017. Autolysis of Rice Bran Phytate in Long-Term Study on Batch Fermentor. *Int.J.Curr.Microbiol.App.Sci.* 6(12): 266-274. doi: https://doi.org/10.20546/ijcmas.2017.612.033