Production of Phytate-Degrading Enzyme from Malaysian Soil Bacteria Using Rice Bran Containing Media

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ABSTRACT

The aims of the study were to observe the effects of different concentration of rice bran in different media on phytase synthesis and to optimize the temperature and pH of the media for phytase production by those bacterial strains. Three bacterial strain isolates obtained from the soil of Malaysian maize plantation were used to produce phytase. In this study, the effects of different rice bran concentration, incubation temperature and initial pH-values of the media on phytase production were evaluated. Incorporation of 7.5% rice bran has the inducible effect on all the bacterial tested. In respect to phytase production, the best cultivation media and cultivation time for *Bacillus cereus* ASUIA260 was PFE with 7.5% rice bran after 3 days, whilst for *Pantoea stewartii* ASUIA271 and *Enterobacter sakazakii* ASUIA279, it was LB with 7.5% rice bran after 3 days and 5 days, respectively. The arrangement of those isolates according to their ability to produce phytases were *E. sakazakii* ASUIA279 > *P. stewartii* ASUIA271 > *B. cereus* ASUIA260. Production of phytase by those bacteria was triggered by the high content of organic phytate in the rice bran. Optimum temperature for phytase production of *B. cereus* ASUIA260 was 41 ºC compared to *P. stewartii* ASUIA271 and *E. sakazakii* ASUIA279 with 33 ºC and 37 ºC, respectively. Optimum initial pH for phytase production of *B. cereus* ASUIA 260 was pH 7.2, while *P. stewartii* ASUIA271 and *E. sakazakii* ASUIA 279 were both at pH 6.0.

Keywords: Bacterial phytase, *Bacillus cereus*, *Enterobacter sakazakii*, *Pantoea stewartii*, rice bran

ABSTRAK

Tujuan kajian ini adalah untuk mengesan kepekatan sekam padi yang berlainan, suhu inkubasi dan nilai pH media terhadap penghasilan phytase oleh strain bakteria tersebut. Tiga strain bakteria yang diperolehi dari tanah ladang jagung Malaysia telah digunakan untuk menghasilkan phytase. Penambahan 7.5% sekam padi telah memberi kesan aruhan kepada semua strain bakteria yang dikaji. Dalam menghasilkan phytase, media pertumbuhan yang terbaik untuk *Bacillus cereus* ASUIA260 ialah PFE dengan 7.5% sekam padi, selepas 3 hari, manakala untuk *Pantoea stewartii* ASUIA271 dan *Enterobacter sakazakii* ASUIA279, ialah LB dengan 7.5% sekam padi, selepas 3 hari dan 5 hari, secara berturutan. Susunan bakteria-bakteria tersebut mengikut kebolehan menghasilkan phytase adalah *E. sakazakii* ASUIA279 > *P. stewartii* ASUIA271 > *B. cereus* ASUIA260. Penghasilan phytase oleh bakteria tersebut adalah dicetus oleh kandungan phytate organik yang tinggi di dalam sekam padi. Suhu

stewartii ASUIA271 > *B. cereus* ASUIA260. Penghasilan phytase oleh bakteria tersebut adalah dicetus oleh kandungan phytate organik yang tinggi di dalam sekam padi. Suhu optimum untuk penghasilan phytase oleh *B. cereus* ASUIA260 ialah 41 ºC berbanding *P. stewartii* ASUIA271 dan *E. sakazakii* ASUIA279 iaitu 33 ºC dan 37 ºC, secara berturutan. Nilai pH awal optima untuk penghasilan phytase oleh *B. cereus* ASUIA260 ialah pH 7.2, dan untuk kedua-dua *P. stewartii* ASUIA271 dan *E. sakazakii* ASUIA279 ialah pada pH 6.0.

Kata kunci: Phytase bakteria, *Bacillus cereus*, *Enterobacter sakazakii*, *Pantoea stewartii*, sekam padi

INTRODUCTION

During the last 20 years, phytases have attracted considerable attention from both scientists and entrepreneurs in the areas of nutrition, environmental protection and biotechnology (Konietzny and Greiner, 2004). The interest in phytate-degrading enzymes and their application in the animal industry have advanced significantly over the past few years (Mullaney *et al.*, 2000). 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. 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 a 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; Abbelson, 1999).

Recently, phytases have been of interest for biotechnological applications, as environment-friendly feed additives in the feed manufacturing industry (Jaie *et al.*, 2003). Because 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).

Thus, in the past decade, there has been a great deal of interest on the study of microbial phytase production and the optimization of media and conditions for maximum production of the enzyme with the aim to increase yields to make it economical as a commercial product. The effects of nutritional and physical parameters have been studied after its production. An early study by Shieh and Ware (1968) showed that phytase production of *Aspergillus niger* NRRL 3135 was influenced by the source of starch used in the medium. Later, Ebune *et al.* (1995) found that supplementation of glucose up to 5.2% to a canola meal medium had a positive effect on phytase production. Sunitha *et al.* (1999) discovered that incorporation of glucose to Luria Bertani (LB) medium at a level of 2 g/L significantly increased the phytase production by *E. coli* BL21. According to Lan *et al.* (2002), the best carbon source for the production of phytase by *M. jalaludinii* was rice bran. Recently, three potential bacterial strains were isolated from Malaysian maize plantations and they have been shown to be able to produce phytase *in vitro* (Anis Shobirin *et al.*, 2007). However, there is no information on the influence the nutrient components and physical condition of the media on its phytase production. Thus, the objectives of the study were to observe the effects of different concentrations of rice bran in different media on phytase synthesis, and optimize the temperature and pH of the media for phytase production by those bacterial strains.

MATERIALS AND METHODS

Bacterial Strains

Pantoea stewartii ASUIA271, *Enterobacter sakazakii* ASUIA279, and *Bacillus cereus* ASUIA260 showing a high phytase activity were previously isolated from the endophyte of Malaysian maize plantation (Anis Shobirin *et al.,* 2007) and identified by using the genotypic method.

Behavioral Study in Different Concentration of Rice Bran Media

For the study on the effects of different concentrations of rice bran, a completely randomized design was used in a 4 x 5 factorial arrangement of treatments with four types of media: distilled water, Luria Bertani (LB), PFE and PMM and five concentrations of rice bran: 0%, 1%, 2.5%, 5% and 7.5%. LB broth was prepared from 10 g tryptone, 5 g yeast extract and 10 g NaCl, while PFE broth was prepared from 5 g peptone, 3 g meat extract, 20 mL glycerol, 150 mL soil extract, and 850 mL distilled water. PMM was prepared from 5 g $MgSO_4$, 5 g $MgCl_2$, 0.5 g KCl, 0.5 g $CaCl_2$, 24 g NaCl, 5 g peptone from casein (pancreatic digest), 5 g peptone from soya meal (papain digest), 5 g meat extracts, 10 g glucose, 150 mL soil extract, and 850 mL distilled water. To determine the effects of phytate and phosphate content on the phytase production, the bacterial strains were grown in LB + 0.1% sodium phytate and in Low Phosphate Media (LPM). LPM was prepared from 5 g $MgSO_4$, 5 g $MgCl_2$, 0.5 g KCl, 0.5 g $CaCl_2$, 24 g NaCl, 5 g peptone from casein (pancreatic digest), 5 g peptone from soya meal (papain digest), 5 g meat extracts, and 10 g glucose. All the components were dissolved and mixed, and the pH was adjusted to pH 7.0. Then, 10 mL of the medium was dispensed into universal bottles and autoclaved. The strains cultured in LB broth for 18 h at 37 ºC were used as inoculums (10% v/v). Cultivation was carried out at 37 ºC and sampling was done at 0, 1, 2, 3, 4, 5, and 7 days for phytase assay. Total plate count (TPC) was done from cultivated LB and LB + 7.5% rice bran. The experiment was repeated three times, each with triplicate.

Growth Effect of Different Temperature and Ph on the Production of Phytase

The LB with 7.5% rice bran was used as the basal medium to study the effects of temperature and pH on phytase production of the three strains. The media for determining the effects of temperature was kept at pH 6, while, the media for determining the effects of pH were adjusted to pH 6, 6.4, 6.8, 7.2 and 7.6, before autoclaving. The inoculums were prepared and inoculated as described above. Cultivation was conducted at 37 ºC for the pH effects study, while the temperature affects study was conducted at 29, 33, 37, 41 and 45 ºC. All the experiments were conducted twice, each with three replicates. Sampling was done at 0, 3, 4, 5, 6 and 7 days cultivation for phytase assay.

Sample Preparation

Samples for enzyme activity assays were prepared by centrifugation of 1.5 mL bacterial culture at 13000 rpm for 1 min (Idriss *et al*., 2002). The cell-free supernatant was separated for phytase and phosphate assays.

Assay for Phytase

Phytase measurements were carried out at 50 ºC consisting of 250 µL of 0.1 M sodium acetate (pH 5.0), 100 µL of 3.6 mM sodium phytate and 50 µL enzyme preparation. The reaction was initiated

initiated with the addition of enzyme preparation. 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. Any cloudiness was removed by centrifugation prior to the measurement of absorbance at 355 nm. In order to quantify the phosphate released, a calibration curve was constructed over the range of 5 to 600 mM phosphate. The activity (U) was expressed as 1 µmol phosphate liberated per minute.

Statistical Analysis

The Minitab Release 14 was used for statistical analysis. Data were reported as the mean with *P* values determined by the analysis of variance (ANOVA).

RESULTS

Production of Phytase in Different Concentrations of Rice Bran Media

The addition of rice bran in different concentrations in various media showed a significant difference ($P < 0.05$) on the phytase production by all the bacterial strains tested. Figure 1 shows the phytase activity of the three bacteria in various concentrations of rice bran in specific media on the optimum cultivation days. *B. cereus* ASUIA260 and *P. stewartii* ASUIA271 showed the highest activity on the third day while *E. sakazakii* ASUIA279 on the fifth day. For all the media used, the phytase activities were gradually increased with the increment of rice bran concentrations. The induction effect of phytase production also depends on the basal media used as well as the bacterial strains involved. With *P. stewartii* ASUIA271 and *E. sakazakii* ASUIA279, the phytase production was highest in LB media with 7.5% rice bran, whilst *B. cereus* ASUIA260 preferred PFE media with 7.5% rice bran.

The highest phytase producer was *E. sakazakii* ASUIA279, with phytases activity up to 2.7 µm/L, 5.5 µm/L, 3.6 µm/L and 2.6 µm/L after 5 days cultivation in DW, LB, PFE and PMM with 7.5% rice bran, respectively. While *P. stewartii* ASUIA271, has phytase activity up to 1.6 µm/L, 2.9 µm/L, 1.8 µm/L and 1.3 µm/L and *B. cereus* ASUIA260 shows activity of 2.2 µm/L, 2.0 µm/L, 2.6 µm/L, and 1.4 µm/L after 3 days of cultivation in the previously mentioned media. Figure 2 shows the liaison between the biomass and the phytase production. It indicates that the production of phytase was continuous during the stationary stage of the bacterial growth and stopped when the bacterial count dropped. There was no difference ($P > 0.05$) in the bacterial count between the cultivated LB and LB + 7.5% rice bran media for all the strains. The bacterial count of *B. cereus* ASUIA260 and *P. stewartii* ASUIA271 decreased after 3 days of cultivation in contrast to *E. sakazakii* which decreased after 5 days of cultivation.

Figures 3 and 4 indicate the relationship of the phytase secretion by the bacterial strains and the content of phytate and phosphate in the media. It suggests that the secretion of phytase by those bacteria were triggered by the phytate content but not by the limitation of phosphate content in the media.

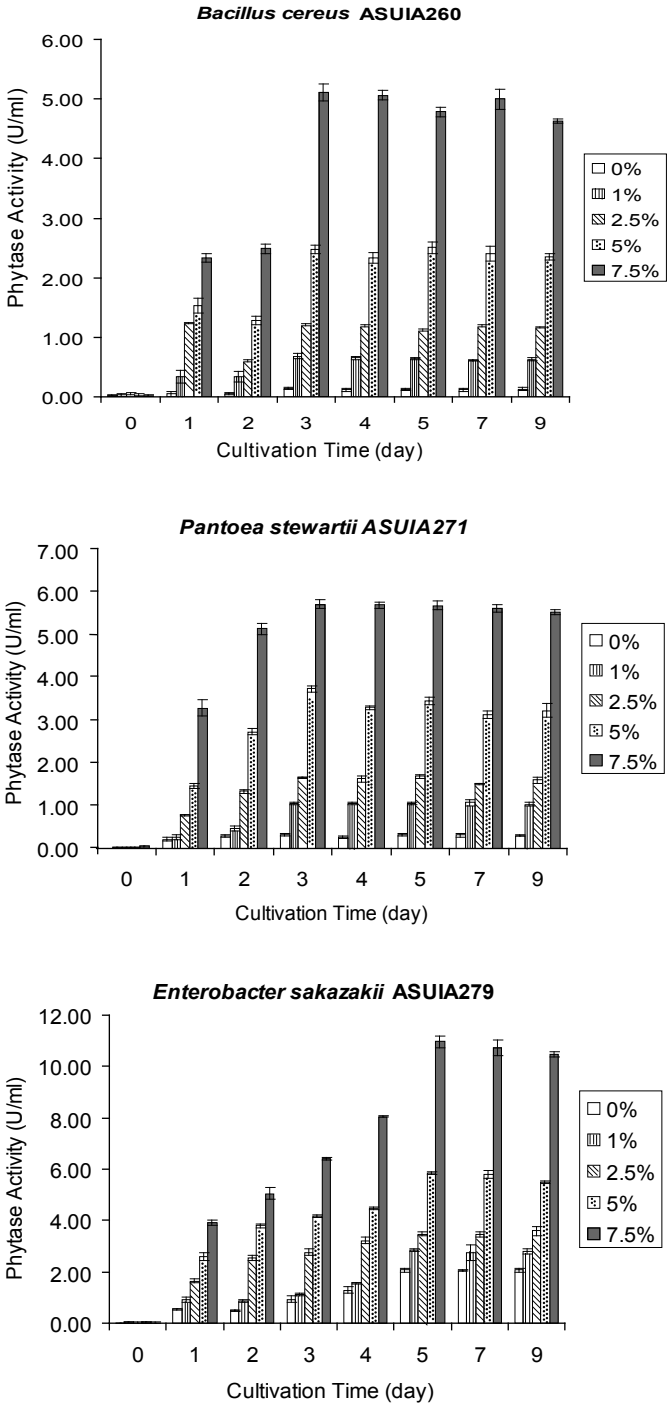

Fig. 1. Phytate-degrading enzyme production by the bacterial strains in the media with different percentage of rice bran. Error bar shows mean ± standard deviation.

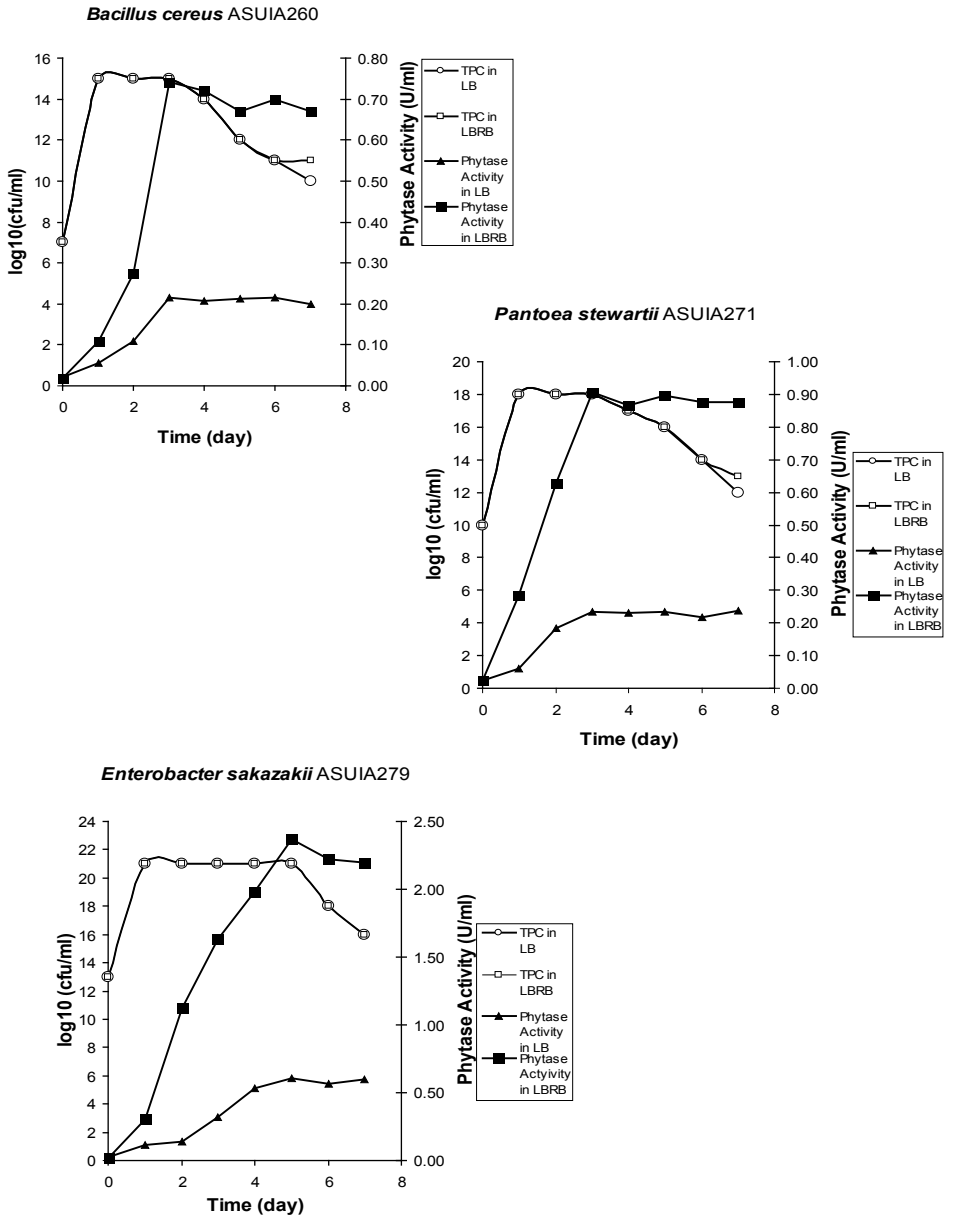

Fig. 2. The phytate-degrading enzymes production by the bacterial strains versus the biomass when cultivated in LB and LBRB (LB + 7.5%).

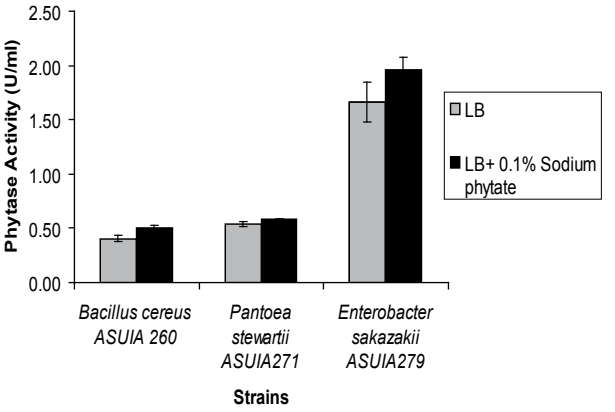

Fig. 3. Comparison of phytate-degrading enzyme production of the bacterial strains in LB and LB + 0.1%. sodium phytate. Error bar shows mean ± standard deviation.

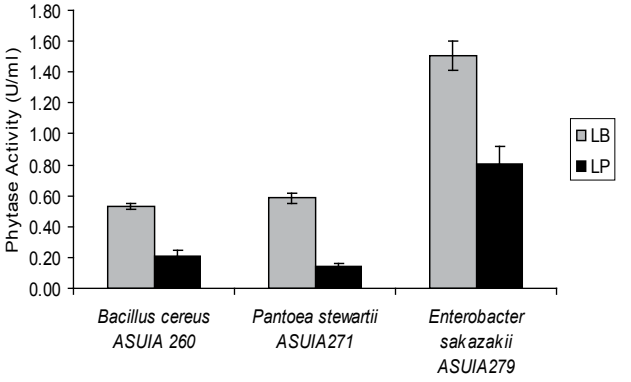

Fig. 4. Comparison of phytate-degrading enzyme production by the bacterial strains in LB and low phosphate media. Error bar shows mean ± standard deviation.

Effect of Temperature and pH on the Production of Phytase

Phytase production by *B. cereus* ASUIA260, *P. stewartii* ASUIA271 and *E. sakazakii* ASUIA279 was affected by the incubating temperature and the initial pH of the cultivation media. Figures 5 and 6 show the phytase activity produced by all strains in different incubating temperatures and different initial pH of media. The phytase production was significantly higher at 41 ºC for *B. cereus* ASUIA260, 33 ºC for *P. stewartii* ASUIA271 and 37 ºC for *E. sakazakii* ASUIA279. Meanwhile, the optimum initial pH for *B. cereus* ASUIA260 was pH 7.2, while for *P. stewartii* ASUIA271 and *E. sakazakii* ASUIA279 it was pH 6.0.

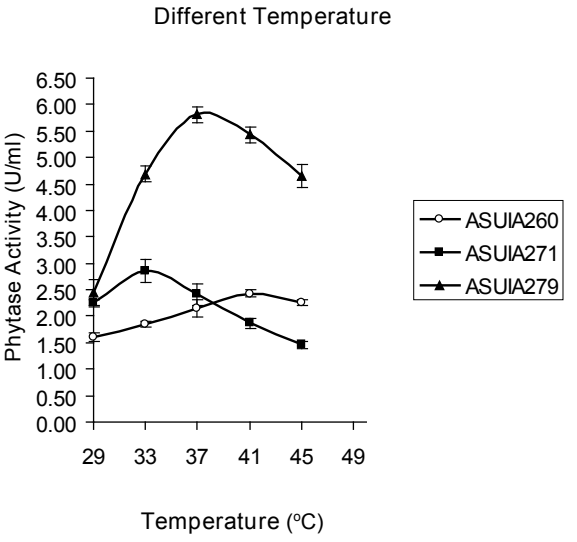

Fig. 5. Phytate-degrading enzyme production by the bacterial strains cultivated at different incubation temperatures. Error bar shows mean ± standard deviation.

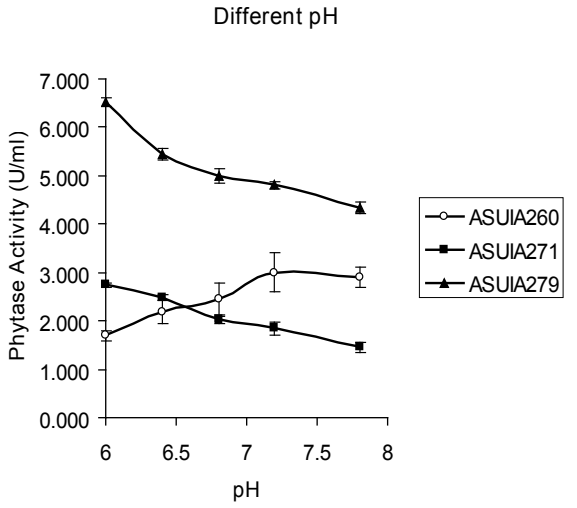

Fig. 6. Phytate-degrading enzyme production by the bacterial strains cultivated in media with different pH. Error bar shows mean ± standard deviation.

DISCUSSION

In some bacteria, phytase is an inducible enzyme and its expression is subjected to a complex regulation, but phytase formation is not controlled uniformly among different bacteria (Liu *et al.*, 1998). The phytase production by *B. cereus* ASUIA260, *P. stewartii* ASUIA271 and *E. sakazakii* ASUIA279 were significantly affected by the supplementation of rice bran. The results of the present study showed that the composition of rice bran in the media were very important to stimulate the expression of phytase by those bacterial strains. The incorporation of 7.5% rice bran has an inducible effect on all the bacterial strains tested. Different isolates showed a different fold of phytase formation. It definitely depends on the type of media that was incorporated with the rice bran and how long the strains could survive in the media. The production of phytases started as soon as the cultures entered the stationary phase and it increased gradually until reaching the maximum state, as the biomass dropped, because of the limitation of nutrients in the media. From the comparison of phytase production in the media supplemented with phytate and in low phosphate media, it is possible that some of the constituents in rice bran, particularly phytate and their intermediates from myo-inositol phosphates, may be responsible for inducing phytase production in these bacteria. This was compatible with *Klebsiella* sp. where the phytase was produced only in the presence of phytate (Shah and Parekh, 1990; Tambe *et al.,* 1994; Greiner *et al.*, 1997). According to Greiner *et al.* (1997), substrate induction for phytase varies among microorganisms. Among the nutrient limitations studied by Greiner *et al.* (1997), only carbon starvation was able to provoke an immediate synthesis of the *Roultella terrigena* phytase. This situation is different from the production of phytate-degrading enzyme in *Escherichia coli.* Their synthesis has been shown to be stimulated by a limitation of inositol phosphate or anaerobiosis (Greiner *et al.*, 1993).

There was a production of phytase in the media where rice bran was the only nutrient source (rice bran in distilled water), but at a low rate. This shows the favorability of these bacterial phytase to hydrolyze not only chemically pure soluble sodium phytate, but also natural phytate in feeds. This is practically important, because phytate in plants exists as an insoluble phytic acid salt form and this indicates that these phytase may improve the nutritional quality of some grains such as rice bran, wheat bran and soy bean meal as animal feeds. According to Lan *et al.* (2002), the best carbon source for production of phytase by *M. jalaludinii* was rice bran compared to supplementing 0.5% sodium phytate to MF_1 medium (MF_{15}). 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. Papagianni *et al.* (1999) found that when wheat bran (20 g/L) was included in a semisynthetic medium comprising of cornstarch, glucose and peptone, the biomass and phytase production of *Aspergillus niger* increased, and they suggested that the increased phytase production might be due to the low release of phosphorus from wheat bran or phytase induction by the presence of phytate.

The presence of other carbon and nitrogen sources also have a positive effect on phytase synthesis such as rice bran in LB and PFE based media compared to rice bran in distilled water (control). However, all the strains cultivated in rice bran with PMM as the based media had the lowest phytase activity. This can be explained by the high content of glucose (10 g/L) in this media. There was a corresponding decrease in phytase production by *M. jalaludinii* as the glucose concentration increased (Lan *et al.,* 2002). According to Sunitha *et al.* (1999), a 2 g/L of glucose source was the best for phytase production by *E. coli*. But, these results are in contrast with some reported data. In a solid-state fermentation study with *A. ficum*, Ebune *et al.* (1995) found that better phytase production was observed in canola meal medium containing added glucose of up to 5.2%. The differences in results may be due to the different microorganisms, media and fermentation conditions used.

Surprisingly, the optimum temperature for phytase production of *B. cereus* ASUIA260 was 41 ºC when compared to *P. stewartii* ASUIA271 and *E. sakazakii* ASUIA279 which were 33 ºC and 37 ºC, respectively. The optimum temperature for the production of phytases from most of the microorganisms lies in the range of 25 to 37 ºC (Vohra and Satyanarayana, 2003). However, the optimal temperature for phytase production of *M. jalaludinii* was about 39 ºC, as it was the rumen temperature of cattle. So, the optimal temperature of 41 ºC could be close to the temperature of the Malaysian soil during day time.

The pH has a profound effect on the production of the enzyme. The present study was conducted under batch fermentation condition. Under this condition, phytase synthesis was obviously influenced by the pH of the medium. The optimum initial pH for phytase production of *B. cereus* ASUIA260 was pH 7.2, whilst *P. stewartii* ASUIA271 and *E. sakazakii* ASUIA279 were pH 6.0. Vohra and Satyanarayana (2003) reported that for phytase production, the optimum pH of most bacteria and fungi is in the range between 5.0 and 7.0.

At least one concentration of the rice bran seems to have an inducing effect on the expression of phytate-degrading enzymes in the three soil bacteria studied. Expression of phytate-degrading enzymes is regulated differently by the physical parameters in different microorganisms. Ongoing study is being carried out in order to purify and characterize the enzymes produced by these bacteria.

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