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Optimal medium and conditions for phytase production by thermophilic bacterium, *Anoxybacillus* sp. MHW14

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Abstract

Phytase is one of several enzymes that widely used in feed industry. Feed enzymes are expected to have high thermostability to withstand the high temperature during feed processing. The main purpose of this research was to find out the cheap and optimal medium and condition for production of phytase from a new source of phytase producing bacterium, *Anoxybacillus* sp. MHW14. The suitable sources of phytic acid, carbon and nitrogen source were selected according to the highest enzyme activity. Rice bran, glucose and ammonium sulfate were found to be the proper components as mentioned, respectively. The least square linear regression according Plackett and Burman design revealed that rice bran and ammonium sulfate were significant positive factors ($p<0.05$) on phytase production. The quadratic model of central composite design generated reliable predicted equation for maximum phytase activity with optimum rice bran and ammonium sulfate levels of 10.35% and 2.64% (w/v), respectively. The validation of 105% was attained within 0.20 U/mL at 12 h of cultivation at 45^{o}C which confirmed the successful optimization. Moreover, *Anoxybacillus* sp. MHW14 was capable of producing the enzyme in broad pH (6.0-9.0) at the optimum temperature (45^{o}C) in optimized medium. The unique characters of the bacterium were interesting and it was the first report of phytase from *Anoxybacillus* sp.

Keywords: phytase, thermophilic bacteria, *Anoxybacillus*, response surface

1. Introduction

Phytic acid (myo-inositol-hexakisphosphoric acid) was the major storage form of phosphorus typically found in plant seeds including cereals, legumes and oilseeds from 90% of harvested area grown in the world (Keruvuo, 2000). One-third of organic phosphate appeared in the forage was digestible inorganic P while another was organic P in the form of mixed salt of inositol-hexakisphosphoric acid known as phytic acid or phytate, the salt form was commonly found in the nature (Vats and Banerjee, 2004).

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According to the structure of phytic acid, it was capable of chelating mono-, di- and trivalent cation as well as proteins and their mixtures to form insoluble complexes (Reddey et al., 1989). Therefore, phytate was biologically unavailable in some kinds of animal, particularly, monogastric animals such as poultry, pig and fish which had no phytase in their digestive tracts (Yoon, et al., 1996). In animal feed industries, high amount of inorganic P was added to feed to ensure sufficient phosphorus and promote animal growth. Fortunately, they could not be used all added. Excess inorganic P and indigested phytate were discharged to environment (Kerovuo, 1998) and led to phosphate pollution in environment like eutrophication in surface water (Casey and Walsh, 2003) and the death of marine animals (Singh and Satyanarayana, 2007). To avoid the problem, supplementation of phytate degrading enzyme in animal feed should be therefore considered.

Phytase (myo-inositol hexakisphosphate phosphohydrolase; EC 3.1.3.8 and EC 3.1.3.29) first discovered by Suzuki in 1907 (Wodzinski and Ullah, 1996). It catalyzed the hydrolysis of phytate to release lower myo-inositol phosphate, inorganic phosphate or free myo-inositol in some cases. The supplementation of phytase in animal feed did not only enhance the bioavailability of minerals and proteins (Singh and Satyanarayana, 2007) but also reduced the amount of feed additive phosphate that was usually the main cause of eutrophication. However, feed additive enzyme should have practically active property at high temperature according to feed processing particularly pelletization process that performs at 60-70°C (Wang et al., 2004). By the way, phytase was found in varieties of microbial systems including fungi (*Aspergillus niger*, *A. ficuum*, *A. terreus*), yeast (*Schwaniomyces castelli*, *Saccharomyces cerevisiae*, *Arxula adeninivorans*) and bacteria (*Bacillus subtilis*, *E. coli*, *Klebsiella* sp.) (Quan et al., 2002) but there were no publications reported *Anoxybacillus phytase*. Therefore, this strain was probably used as an alternative source of phytase for further utilization in industry.

This paper described a new source of thermostable phytase obtained from *Anoxybacillus* sp. isolated from hot springs and optimal medium composition and conditions for production of phytase using response surface methodology.

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2. Materials and Methods

2.1 Microorganisms

Anoxybacillus sp. MHW14 isolated from hot spring in Chiang Mai area, Thailand (Kanpiengjai, 2008), was stored in 15% (v/v) glycerol at -80^{o}C. It was cultured in nutrient broth and streak to single colony prior to utilizing.

2.2 Optimal medium composition for production of phytase

2.2.1 Proper phytic acid source

The production medium was 100 mL in 250-mL Erlenmeyer flask basal medium modified from Atlas (1993) containing (%w/v) of 0.10 $(NH_4)_2SO_4$, 0.01 $MgSO_4$, 0.10 D-glucose and 0.01 Na-citrate and 1.0 phytic acid source. The liquid medium was adjusted to pH 7.0. Fermentation was carried out on 150 rpm rotary shaker at 45°C for 24 h. Culture broth was taken to determine phytase activity.

2.2.2 Screening of proper carbon source

The various types of carbon sources including glucose, sucrose, maltose, fructose, maltodextrin and starch were used as carbon source in the previous medium with the carbon source concentration of 0.1% (w/v). Fermentation was carried out on 150 rpm rotary shaker at 45°C for 24 h. Culture broth was taken for determination of phytase activity.

2.2.3 Screening of inorganic nitrogen source

The various types of nitrogen sources including $(NH_4)_2SO_4$, NH_4NO_3, peptone, yeast extract, beef extract and urea were used as nitrogen sources with the concentration of 0.10% (w/v) in the previous medium. The fermentation was carried out as previously described and the phytase activity was assayed.

2.2.4 Plackett and Burman design (PBD)

The main medium composition demonstrated main significant effects on phytase production were screened and evaluated using Plackett and Burman design (N=9). The high and low levels of each component were set and 12 treatments were generated based on fractional

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factorial design as shown in Table 1. The data were analyzed by using statistix program version 8.0 (Analytical Software, FL).

Table 1. Low and high levels of medium components for Plackett and Burman design.

Variables	**Levels**	
	Low	**High**
Rice bran (%w/v)	1	10
Glucose (%w/v)	0.1	2
$(NH_4)_2SO_4$ (%w/v)	0.1	2
Tween80 (%w/v)	0.1	0.5
$FeSO_4$ (%w/v)	0.0014	0.014
$CaCl_2$ (%w/v)	0.00074	0.0074
$CuSO_4$ (%w/v)	0.00125	0.0125

2.2.5 Central composite design (CCD)

To fine the best solution for maximum phytase production and proper quantity of the most important medium components affected to phytase production by *A. gonensis* MHW14. In this study, five levels of two significant components exhibited positive impacts on phytase production were created according to central composite design (CCD) as shown in Table 2. The 11 treatments were generated including 4 axial star points, 4 factorial points and 3 center points. Other components were fixed at their low and high level according to the least square regression analysis in the previous study. The data were analyzed by design expert version 7.0.0 (Stat-Ease, MN).

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Table 2 The 5 levels of the two positive significant medium components for central composite design

Variables	Symbols	Level				
		-1.414	-1	0	+1	+1.414
Rice bran (%w/v)	X_1	5.00	6.46	10.00	13.54	15.00
$(NH_4)_2SO_4$ (%w/v)	X_2	0.10	0.65	2.05	2.71	4.00

2.3 Validation of statistical optimized medium

To evaluate the reliability of the quadratic model and the best fit, time course of phytase production was performed using optimum quantities of the two variables suggested by quadratic equation. Sample was taken periodically for 24 h to determine phytase activity. Protein was assayed by Lowry method (Lowry, 1951).

2.4 Effect of pH and temperature on phytase production

The effect of temperature on phytase production was investigated by growing the bacterium in the optimized medium on 150 rpm rotary shaker at 37, 45, 50, 55, 60 and 65°C for 12 h. The sample was collected to determine phytase activity. The effect of initial pH on phytase production was investigated by growing the bacterium in the optimized medium adjusted pH to 5, 6, 7, 8, 9 and 10 on 150 rpm rotary shaker at 45°C for 12 h. The sample was collected to determine phytase activity.

2.5 Phytase activity assay

Phytase activity was determined by mean of ascorbic acid method modified from Kim et al. (1998) using sodium phytate as a substrate. Reaction mixture consisted of 50 µL enzyme solution and 200 µL of sodium phytate in 0.1 M Tris-HCl buffer pH 7.5. The reaction mixture was incubated at 55°C for 30 min and then stopped by adding 250 µL of 10%(w/v) trichloroacetic acid (TCA). The released inorganic phosphate was measured by incubating with 2 mL of color reagent (1:1:1:2 of 6 N H_2SO_4: 2.5% (w/v) ammonium molybdate: 10%(w/v) ascorbic acid: H_2O) at 55°C for 30 min. The absorbance at 820 nm was measured. One unit of enzyme was defined as the amount of enzyme required to liberate 1 µmole of inorganic phosphate per minute under the assay condition.

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3. Results and discussion

3.1 Selection of phytic acid source

In microbes, expression of inducible phytate-degrading enzyme was subjected to a complex regulation, but their formation was not controlled uniformly among different microorganisms (Greiner, 2007). However, substrate induction was necessary and also affected to production of bacterial phytase such as *Mitsuokella julaludinii* phytase (Lan et al., 2002) and several *Bacillus* spp. phytases (Powar and Jagannathan, 1982; Kerovuo et al., 1998; Kim et al., 1998 and Greiner, 2007). In this study, rice bran and wheat bran were chosen to determine the proper one since they were agricultural waste and also contained high amount of phytic acid (Gualberto et al., 1997). In addition, Greiner (2007) also suggested that bran was excellent substrate for production of extracellular phytate degrading enzyme in microorganisms. The phytate in bran was less soluble than the synthesized phytate like sodium-phytate, phosphates were therefore released more slowly than that from bran phytate. As the reason, phytase production from bran was less inhibited than that from solubilized phytate. Although rice bran induced higher phytase production by *A. gonensis* than wheat bran particularly at 12 and 24 h of enzyme production, there was a paper reported no difference of phytate content in both rice bran and wheat bran (Selle et al., 2000). Considering rapid enzyme production, rice bran was interestingly selected for further study as the highest phytase activity of 0.08 U/mL (Fig. 1).

3.2 Screening of carbon source

Expression of phytate-degrading enzymes depended on the nature of carbon source, the initial pH and temperature used for cultivation of microorganisms. Optimal concentration of glucose was required form some microorganisms. Low glucose levels had phytate-degrading activity decreased because of reduction of biomass production (Greiner, 2007). The presence of glucose caused high level of phytate-degrading activity in *E. coli* (Touati et al., 1987) and Lactobacillus amylovoras (Sreemulu et al., 1996). In this study, various carbon sources including glucose, sucrose, starch, maltose, fructose and maltodextrin, were added into minimal medium. The maximum phytase activity obtained from minimal medium containing glucose and fructose as carbon source (Fig. 2).

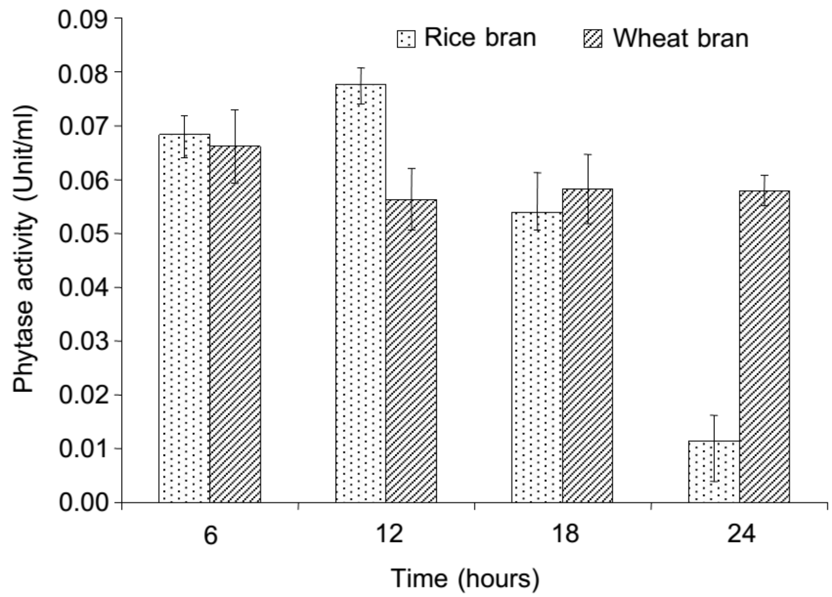

Figure 1. Effect of rice bran and wheat bran on phytase production.

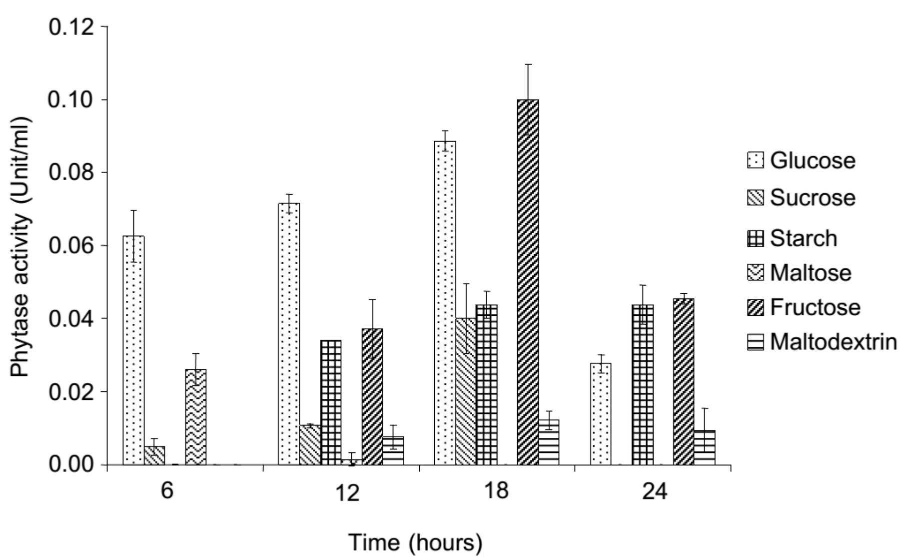

Figure 2. Effect of different carbon sources on phytase production.

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However, glucose was selected to be carbon source for further study because of an economical reason. Other papers also reported the effect of glucose on enhancement of phytase production. Ebune et al., (1995) reported the glucose was very often used as a nutrient for growing of microorganisms and production of enzyme and found that glucose of 5.2% (w/v) or lower had a positive effect on the rate of biomass growth, enzyme production and the reduction of phytic acid.

3.3 Screening of nitrogen source

Nitrogen source was an important factor influenced bacterial growth and enzyme production. Effect of different nitrogen sources on phytase production was performed in order to attain proper nitrogen source. Among organic and inorganic nitrogen sources applied in basal medium, $(NH_4)_2SO_4$ was found to be the best nitrogen source because of the highest enzyme activity from 6 to 18 h of cultivation (Fig. 3). $(NH_4)_2SO_4$ was great inorganic nitrogen source according to its cheapness and availability. It was moreover observed that organic nitrogen source did not have higher effect on phytase production by *A. gonensis* MHW14 than organic nitrogen source as presented in many cases of bacteria.

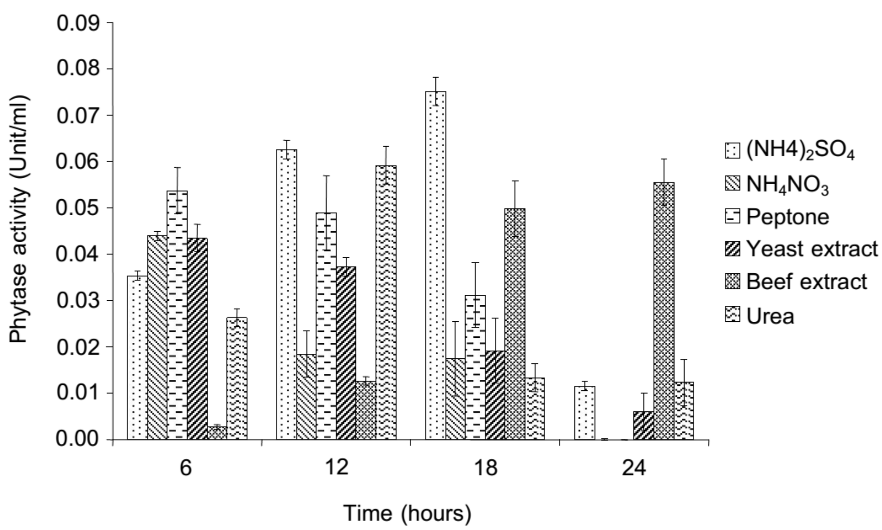

Figure 3. Effect of different nitrogen sources on phytase production.

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3.4 Most significant medium components

Six medium components including phytic acid source, carbon source, nitrogen source and mineral source were screened for the most effective factors influenced phytase production through fractional factorial design. The main effects were analyzed by the least squares linear regression. The results demonstrated that rice bran and $(NH_4)_2SO_4$ were two positive factors significantly affected to phytase production at *p*-value less than 0.05 while other components did not exhibit significant impacts as shown in Table 3. Therefore, both rice bran and $(NH_4)_2SO_4$ were selected to fine the optimum quantity that was capable of maximizing phytase activity by response surface methodology and other components were fixed at their high and low levels corresponding to their impacts. It was generally known that phytase was an inducible enzyme typically produced by using phytic acid containing substrates as inducers such as agricultural residues (wheat bran, rice bran and other legumes) and salts of phytic acid (sodium phytate and calcium phytate). Therefore, it was reasonable that high positive impact came from rice bran. Lan et al. (2002) found that sodium-phytate induced phytase production by *Mitsuokella jalaludinii* but a combination of glucose, rice bran and soybean milk inhibited the production. Another significant component was $(NH_4)_2SO_4$, a single organic nitrogen source in the medium which revealed its importance. It was suggested that nitrogen source was necessary for bacterial growth and *A. gonensis* MHW14 may require proper ratio of phytic acid and nitrogen source. Not many recent publications studied on medium optimization for phytase production by phytase producing bacteria. Most of them widely optimized medium for production the enzyme from fungal particularly *Aspergillus* sp. phytases. Ponpanich et al. (2003) used rice bran and soybean meal extract to maximize phytase production of soil bacterium strain PH01. The result was best explanation of the relation between phytic acid and nitrogen source. Kammoun et al. (2012) found that methanol and yeast extract were the most significant factors affected to phytase production by *B. subtilis* US417. Li et al. (2008) studied for optimization of medium composition and condition for phytase production by yeast, *Kodamaea ohmeri* BG3 using experimental design and oats and $(NH_4)_2SO_4$ was found to have significant positive impacts.

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Table 3 The least squares linear regression analysis of data obtained from Plackett and Burman design.

Variables	Coefficient	T	P
Constant	0.11752	20.67	0.0000
Rice bran	0.01815	3.19	0.0332
Glucose	2.833E-04	0.05	0.9626
$(NH_4)_2SO_4$	0.01590	2.80	0.0490
Tween80	-0.00428	-0.75	0.4932
$FeSO_4$	0.00548	0.96	0.3895
$CaCl_2$	-0.00823	-1.45	0.2212
$CuSO_4$	-0.00538	-0.95	0.3974
R-squared (R^2)	0.8491		
Adjusted R-squared	0.5849		

3.5 Optimal medium composition and validation of experimental model

Five levels of rice bran and $(NH_4)_2SO_4$ were varied according to quadratic model of central composite design (CCD). The data were analyzed by analysis of variance (ANOVA) as presented in Table 4. It was found that the data fitted to quadratic model significantly at *p*=0.0094, the lack of fit of 0.4678 and R-square of 0.9184 revealed that the model was considerably reliable and it could be therefore used to predict the optimum level of rice bran and $(NH_4)_2SO_4$ as the equation below. The response surface plots were demonstrated in both contour and 3D surface plot (Fig. 4A and 4B) as shown in Table 4.

$$Phytase\ activity = 0.18 + 0.021A - 0.015B + 0.025A * B - 0.009227 * A^2 - 0.025B^2$$

Where A and B represented code values of rice bran and $(NH_4)_2SO_4$, respectively

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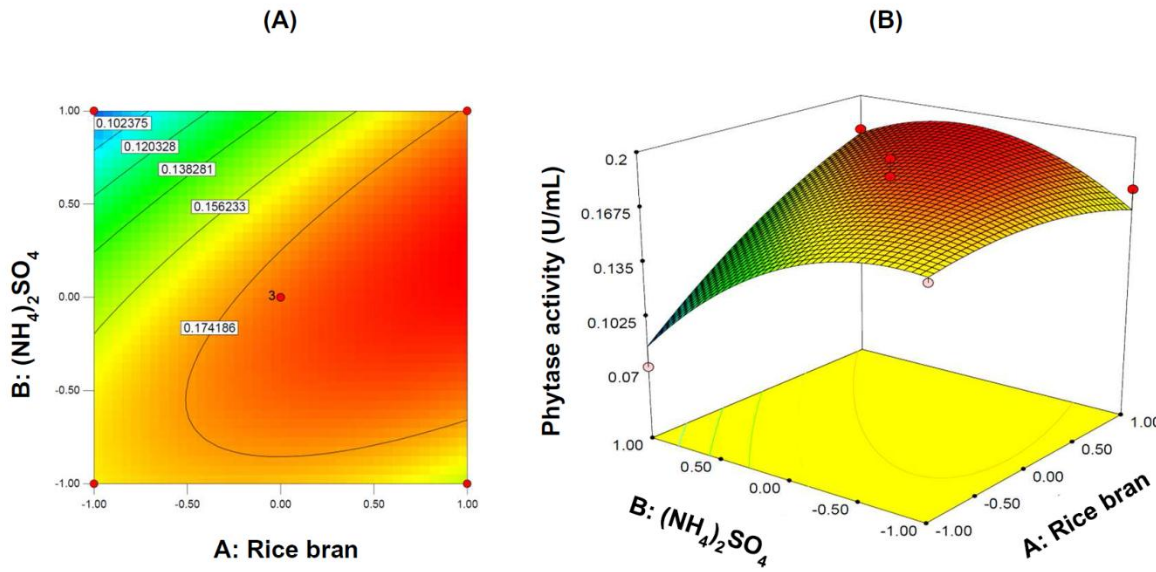

Figure 4. Contour and 3D response surface plot represented relation between rice bran and $(NH_4)_2SO_4$ and phytase activity.

Considering the ANOVA table and predicted equation, rice bran and $(NH_4)_2SO_4$ still had significant influence for phytase production at *p*-value less than 0.05. These two variables had positive interaction to phytase production significantly. The higher rice bran had negative effect on phytase production particularly $(NH_4)_2SO_4$ which gave negative effect significantly. From the equation, 30 solutions were suggested with maximum phytase activity approximately 0.19 U/mL when rice bran ranging from 6-12% (w/v) and $(NH_4)_2SO_4$ ranging from 0.9-3.5% (w/v) was applied in the medium. Only one solution was selected to validate the model fitting. Rice bran of 10.35% (w/v) and $(NH_4)_2SO_4$ of 2.64% (w/v) was applied to the medium composition and *A. gonensis* MHW14 seed culture was transferred to the production medium and carried out at 45^{o}C for 24 h.

Table 4 Analysis of variance (ANOVA) of phytase activity obtained from central composite design.

Source	Sum of squares	df	Mean square	F-value	p-value Prob >F	
Model	0.011	5	2.28E-03	11.26	0.0094	significant
A-Rice bran	3.50E-03	1	3.50E-03	17.32	0.0088	
B-$(NH_4)_2SO_4$	1.85E-03	1	1.85E-03	9.17	0.0292	
AB	2.43E-03	1	2.43E-03	12	0.018	
A^2	4.81E-04	1	4.81E-04	2.38	0.1836	
B^2	3.58E-03	1	3.58E-03	17.72	0.0084	
Residual	1.01E-03	5	2.02E-04			
Lack of Fit	6.63E-04	3	2.21E-04	1.28	0.4678	not significant
Pure Error	3.47E-04	2	1.73E-04			
Cor Total	0.012	10				

Std. Dev.	0.014	R-Squared	0.9184
Mean	0.15	Adj R-Squared	0.8369
C.V. %	9.2	Pred R-Squared	0.5561
PRESS	5.50E-03	Adeq Precision	10.124

The result showed that *A. gonensis* MHW14 produced phytase activity of 0.20 U/mL at 12 h of cultivation (Fig. 5) which was calculated to 105% validation. Therefore, the optimization was successful. Although the yield was still low, it was suggested that this enzyme was associated with the growth of the bacterium and it moreover had great merit in term of short time consuming in order to maximize the enzyme production as the highest activity was obtained within 12 h of cultivation. The optimal medium contained (%w/v); 10.35 rice bran, 1.05 glucose, 2.64 $(NH_4)_2SO_4$, 0.10 Tween80, 0.014 $FeSO_4 \cdot 7H_2O$, 0.00074 $CaCl_2 \cdot 2H_2O$ and 0.00125 $CuSO_4 \cdot 5H_2O$ adjusted pH to 7.0.

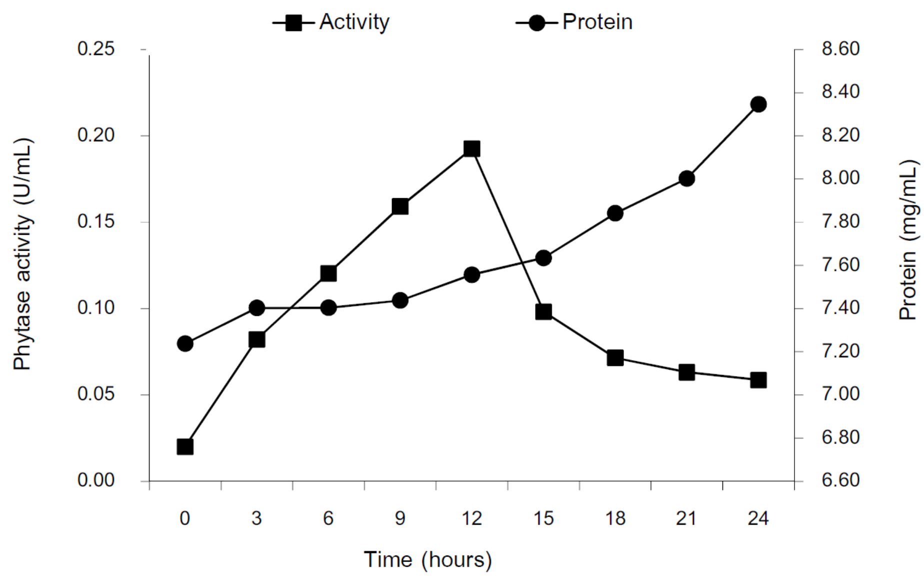

Figure 5. Profile of phytase activity and protein content produced by *A. gonensis* MHW14 during cultivation at 45°C using optimized medium.

3.6 Effect of pH and temperature

The temperature ranging from 37 to 70°C was used to cultivate *Anoxybacillus* sp. MHW14 for production of phytase using optimized medium. The result revealed that the highest enzyme activity obtained from culture in which incubated at 45 and 50°C significantly at $p<0.05$. The temperature as high as 55 and 60°C decreased the production of the enzyme and obviously decreased the enzyme activity at 65°C. It was moreover found that the bacterium had less capability in production of phytase at 37°C as shown in Fig. 6a. Although the temperature for growth of this strain was from 55 to 60°C, this might be too high temperature for the enzyme to tolerate for long time (Kanpiengjai, 2008). The least phytase activity obtained from medium that incubated at 37°C due to the bacteria was unable to grow well as it was reported to be moderately themophilic bacterium. Buldez et al. (2003) reported that *A. gonensis* and *A. flavithermus* had optimum temperature for growth at 55 to 60°C and 60-65°C, respectively. Colak et al. (2004) characterized a thermo-alkalophilic esterase from *Anoxybacillus gonensis* G2. They had grown the bacteria in the medium and incubated at 60°C. Kahar et al. (2013) produced amylopullanase from *Anoxybacillus* sp. SK3-4 at 55°C and purified the enzyme. They found optimum temperature of the

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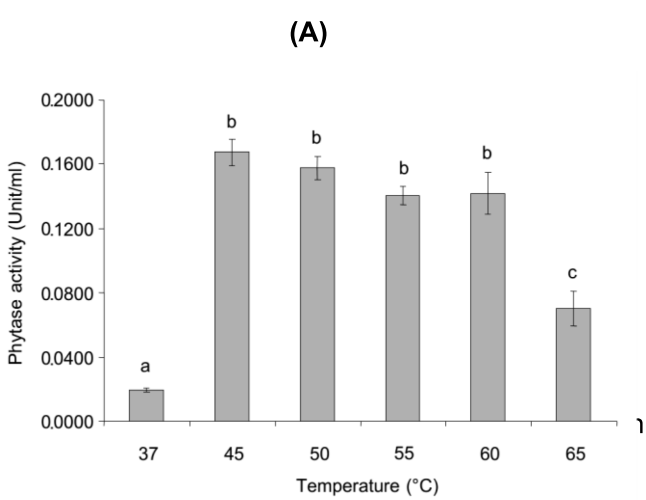
purified enzyme at 60°C and the enzyme was stable at this temperature longer than 4 h but lost more than 90% residual activity within 1 h at 70°C. However, there were no researches that studied the effect of temperature on enzyme production from this species but most of research usually grew the bacteria using the temperature between 50 and 60°C (Belduz et al., 2003; Colak et al., 2004; Dulger et al., 2004; Kevbrin et al., 2005; Kahar et al., 2013).



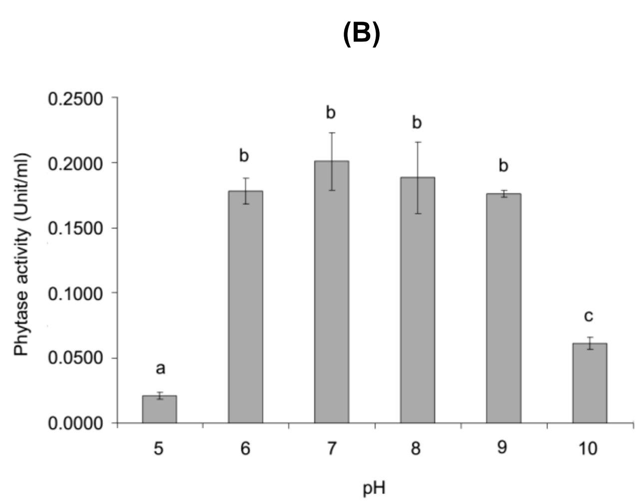


Figure 6. Effect of termperature (A) and pH (B) on phytase production.

The optimum pH for production of phytase was found in the medium adjusted to pH ranging from 6.0-9.0 since the enzyme production yield did not have significant difference as shown in Fig. 6b. It was explained that such a range of pH was optimum pH for growth of *Anoxybacillus* sp. Buldez et al. (2003) first reported a novel species known as A. gonenesis which had broad optimum pH ranging from 5.5-9.5 while *A. flavithermus* grew well at pH from 5.5-9.0. *A. kamchatkensis* also had broad pH optimum of 6.8-9.5 (Kevbrin et al., 2005). As reasons, effect of pH on production of phytase was probably corresponded to the optimum pH for the bacterial growth.

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4. Conclusion

Anoxybacillus sp. MHW14 isolated from hot spring had potentiality in production of phytase. The optimization of medium composition was also successfully performed by using Plackett and Burman design and Central composite design. This medium was cheap and practically used for phytase production by this strain as it contained rice bran, a cheap and available material and $(NH_4)_2SO_4$, as a cheap organic nitrogen source. The maximum phytase activity of 0.20 U/mL was attained from the optimized medium. It was interesting that the bacterium was capable of producing phytase in broad pH medium. Moreover, our study demonstrated not only new source of thermostable phytase by newly isolated strain but also the first report of phytase producing *Anoxybacillus* sp.

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