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Production of Phytase by *Mitsuokella jalaludinii* in Semi-Solid State Fermentation of Agricultural by-products

(Pengeluaran Fitase oleh *Mitsuokella jalaludinii* dalam Fermentasi Separa Pepejal Produk Sampingan Pertanian)

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ABSTRACT

Phytase activity and growth of anaerobic rumen bacterium, Mitsuokella jalaludinii *were investigated by semi-solid state fermentation. Carbon source (rice bran, yam and cassava), nitrogen sources (soya bean, offal meal, fish meal and feather meal) and growth factors (hemin, L-cysteine hydrochloride and minerals) were evaluated in a one-factor-at-a-time approach. Rice bran and fish meal produced better growth and phytase enzyme activity. The removal of L-cysteine hydrochloride and minerals significantly decreased ($p<0.05$) phytase activity from 1178.72 U to 446.99 U and 902.54 U, respectively. The response surface methods (RSM) was conducted to optimize the phytase production and the results showed the combination of 7.7% of rice bran and 3.7% of fish meal in semi-solid state fermentation gave the highest phytase activity. Maximum phytase production and optimum growth of bacteria were detected at 12 h incubation in both MF medium (control) and agro-medium. In this agro-medium,* M. jalaludinii *produced 2.5 fold higher phytase activity compared to MF medium.*

Keywords: Agro-medium; Mitsuokella jalaludinii*; phytase activity; response surface methods; semi-solid state fermentation*

ABSTRAK

Aktiviti fitase dan pertumbuhan bakteria rumen anaerob, Mitsuokella jalaludinii *dikaji dengan teknologi fermentasi separa pepejal. Sumber karbon (dedak beras, keladi dan ubi kayu), sumber nitrogen (kacang soya, mil organ, mil ikan dan mil bulu pelepah) serta faktor pertumbuhan (hemin, L-cystein hidroklorida dan mineral) dinilai dengan kaedah satu faktor pada satu masa. Dedak beras dan mil ikan didapati mampu menghasilkan pertumbuhan sel dan aktiviti fitase yang baik. Penyingkiran L-cysteine hidroklorida dan mineral menyebabkan penurunan aktiviti fitase yang ketara ($p<0.05$) masing- masing dari 1178.72 U ke 446.99 U dan 902.54 U. Kaedah gerak balas permukaan (RSM) dijalankan untuk mengoptimumkan penghasilan fitase dan keputusan menunjukkan gabungan penggunaan 7.7% dedak beras dan 3.7% mil ikan dalam proses fermentasi separa pepejal memberikan aktiviti fitase yang tertinggi. Penghasilan fitase dan pertumbuhan maksimum bakteria untuk kedua-dua media MF (kawalan) and agro-medium berlaku dalam masa eraman 12 jam.* M. jalaludinii *berupaya menghasilkan 2.5 kali ganda aktiviti fitase dalam agro-medium berbanding dengan media MF.*

Kata kunci: Agro-media; aktiviti fitase; fermentasi separa pepejal; kaedah gerak balas permukaan; Mitsuokella jalaludinii

Introduction

Phosphorus (P) is an essential nutrient for the formation and maintenance of animal skeletal structure and for ensuring rapid and efficient growth (Proszkowiec-Weglarz & Angel 2013). Most of the P in seeds and cereals are in the form of phytate. However, due to the lack of digestive enzymes, monogastric animals such as poultry, swine and even humans are unable to fully utilize this nutrient (Ravindran et al. 1995). The hydrolysis of phytate in the gastrointestinal tract can only occur either through the enzymatic hydrolysis by bacteria or non-enzymatic hydrolysis by gastric acid in the gastrointestinal tract (Bohn et al. 2008). The former is generally more efficient (Bhavsar & Khire 2014) and therefore enzyme supplementation is a common practice in animal husbandry. For instance, phytase is commonly used to release P from the grain component of the animal feed. This approach replaces the need for inorganic P supplementation in the animal feed, which is non-renewable, expensive and not environmentally friendly (Lei & Porres 2003). Apart from having the ability to release the phytate-bound P from the grains, phytase also improves the absorption of some important dietary nutrients such as calcium, amino acids, protein and starch for monogastric animals by intercepting the chelating effect of phytate (Bhavsar & Khire 2014). Phytases not only exist in plants and animal tissues, but also in microorganisms (Konietzny & Greiner 2002). Although various sources of phytase have been studied, microbial phytases offer certain advantages for commercial production when compared

to other sources (Pandey et al. 2001). It was reported by Bhavsar and Khire (2014), that some plants like wheat and barley are rich in intrinsic phytase, but they are less effective in comparison to the microbial phytases due to the low heat stability and narrow pH spectrum of activity. In addition, the bio-efficacy of plant phytases was only 40% of that microbial phytases. Numerous species of bacteria, yeast, and fungi are found to produce phytase. Fungal and yeast phytases are commonly used in animal husbandry (Gontia-Mishra et al. 2013), although bacterial phytases display superior catalytic efficiency, substrate specificity and ability to resist proteolysis than fungal phytases (Konietzny & Greiner 2004). *Mitsuokella jalaludinii*, an anaerobic bacteria isolated from the rumen of a local cattle in Malaysia was found to be a good producer of phytase enzyme (Lan et al. 2002a). Previous studies showed that *M. jalaludinii* produced cell-bound phytase and whole cell *M. jalaludinii* preparation significantly ($p<0.05$) improved the growth performance of broiler chickens. The effects were found to be comparable to the commercial phytase enzyme Natuphos® (Lan et al. 2012).

Most of the commercial phytases are produced by using submerged fermentation method, a process which is expensive (Satyanarayana et al. 2012). Solid state fermentation is a more economical alternative but it is not suitable for bacteria due to the low moisture content in which the growth of bacteria will be restricted and cellular metabolism will be affected (Bhargav et al. 2008). Hence, higher moisture semi-solid state fermentation is more appropriate for the production of bacterial phytase (Economou et al. 2010). One advantage of the method is that substrates used in semi-solid state fermentation can be cheap by-products or agricultural residues which contain carbohydrates, nitrogen, and minerals that could be utilized for bacterial fermentation (Naveena et al. 2004). To date, there is limited information on phytase enzyme production by bacteria through semi-solid state fermentation. Thus, the objective of this study was to determine suitable agricultural products for the production of phytase enzyme by *M. jalaludinii* in semi-solid state fermentation. Conventional 'one-factor-at-a-time' approach was used to screen for suitable substrates and growth factors for *M. jalaludinii*, whereas central composite design (CCD), a standard response surface methodology (RSM) approach was applied for the optimization process.

Materials and Methods

Bacterial Strain and Cultivation

Mitsuokella jalaludinii (DSM 13811^T; ATCC BAA-307^T), is a strict anaerobic phytase producing bacterium which was previously isolated from the rumen of cattle in Malaysia (Lan et al. 2002a). The bacterium was routinely maintained in modified M10 medium (MF medium) supplemented with 0.4% sodium phytate as described by Caldwell and Bryant (1966) but without the addition of volatile fatty acid. The culture was maintained at 39°C and sub-cultured every 12 h. The bacterial stock was kept in MF medium at –80°C. All the media preparation and sub-culturing procedures were performed according to the strict anaerobic technique of Hungate (1969).

Preparation of Media for Semi-Solid State Fermentation

The carbon and nitrogen sources of MF medium were modified using agriculture based-products. The carbon sources normally used in the formulation of growth medium (D- glucose, cellobiose and soluble starch) were replaced with rice bran, yam, or cassava whereas the nitrogen sources (trypticase peptone and yeast extract) were replaced with soya bean meal, offal meal, fish meal or feather meal (Table 2). Yam and cassava were chopped and ground to pass through 1 mm sieve after drying. All carbon sources were dried to constant weight at 110°C. Rice bran was provided by Serba Wangi, Sekinchan, Malaysia, while yam and cassava were purchased from the local markets. The feather, soya bean and offal meals were supplied by the Malaysian Agricultural Research and Development Institute (MARDI). The modified M media (M) were prepared by using strict anaerobic technique (Hungate 1969). The media contained substrate (agro-product) and water at 1:12 ratio. All components listed in media M1, M2, M3, M4, M5, M6 & M7 (Table 1) except sodium carbonate and L-cysteine hydrochloride were mixed well. After the pH was adjusted to pH8.2, sodium carbonate was added and the media were heated to boiling point. Subsequently, L-cysteine hydrochloride was added and the media were boiled again. The media were then flushed with oxygen-free carbon dioxide to make the solution anaerobic for 10 min before autoclaving at 121°C, 15 psi for 15 min. MF medium served as the control for all experiments carried out in this study.

Semi-Solid State Fermentation

Three percent (v/v) of 12 h culture of *M. jalaludinii* in MF medium were inoculated into each medium (Table 2) in Schott bottle (Duran, Germany) and the fermentation process was carried out for 12 h at 39°C under an agitation speed of 130 rpm. Each medium contained 83 g (about 1:12 ratio) of agro-products as the solid substrate. The fermented media were then centrifuged at 10,000 × *g* for 20 min. The pellet obtained was washed twice with 0.1 M sodium acetate buffer (pH5.0) and re-suspended in equal volume of the same buffer. The re-suspended residue (1 mL) was used for the determination of the total number of viable cells by using the roll tube method as described by Hungate (1969) and 0.2 mL was used for the determination of phytase activity. The best carbon and nitrogen sources identified were then used for subsequent experiments.

Effects of Hemin, L-Cysteine and Mineral Solution on Phytase Activity

Mitsuokella jalaludinii were cultured in the modified M media without hemin, L-cysteine hydrochloride, or mineral solution (Table 2) to evaluate the effect of this particular

TABLE 1. Media used to study the growth and phytase activity of *M. jalaludinii*

Component	Medium							
	MF	M1	M2	M3	M4	M5	M6	M7
D- Glucose (g)	10	-	-	-	10	10	10	10
Cellobiose (g)	4	-	-	-	4	4	4	4
Soluble starch (g)	4	-	-	-	4	4	4	4
Rice bran (g)	-	83	-	-	-	-	-	-
Yam (g)	-	-	83	-	-	-	-	-
Cassava (g)	-	-	-	83	-	-	-	-
Tryticase peptone (g)	10	10	10	10	-	-	-	-
Yeast extract (g)	4	4	4	4	-	-	-	-
Fish meal (g)	-	-	-	-	83	-	-	-
Feather meal (g)	-	-	-	-	-	83	-	-
Offal meal (g)	-	-	-	-	-	-	83	-
Soya bean meal (g)	-	-	-	-	-	-	-	83
Minerals solution* (mL)	100	100	100	100	100	100	100	100
8 % (w/v) Na_2CO_3 (mL)	50	50	50	50	50	50	50	50
0.05 % (v/v) Hemin (mL)	1	1	1	1	1	1	1	1
Cysteine•HCl•H_2O	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5
Distilled water (mL)	815.5	833.5	833.5	833.5	829.5	829.5	829.5	829.5

Mineral solution (per liter)*: 0.4466 g NaCl; 4.4941 g $(NH_4)_2SO_4$; 0.25 g $CaCl_2$; 0.9366 g $MgSO_4.7H_2O$; 3.4643 g KCl

TABLE 2. Media used to study the effects of hemin, L-cysteine hydrochloride and mineral solution on phytase production

Component	medium	without hemin	without L-cysteine	without mineral solution
Rice bran (g)	99.36	99.36	99.36	99.36
Fish meal (g)	52.64	52.64	52.64	52.64
Minerals solution* (mL)	100	100	100	-
8 % (w/v) Na_2CO_3 (mL)	50	50	50	50
0.05 % (v/v) Hemin (mL)	1	-	1	1
Cysteine•HCl•H_2O (g)	1.50	1.50	-	1.50
Distilled water (mL)	847.5	848.5	849.0	947.5

component in bacterial growth and enzyme activity. All the fermentation and post-fermentation procedures were as described above.

OPTIMIZATION OF CULTURE CONDITION USING RESEARCH SURFACE METHODS

Parameters such as agitation speed, moisture content, amount of carbon and nitrogen were subjected to two-level full factorial design according to the response surface methodology (RSM) for optimization of phytase production. A total of 32 sets of experiments were carried out (Table 3) based on the combination of 4 factors and 2 levels designed using the statistical Design Expert software version 9 (Stat-Ease, Minneapolis, MN, USA). Each independent variable factor was tested at two levels, represented with the standard notation of high (1) and low (–1) level where the phytase activity was the dependent variable or response in this study. Factors that significantly affected and showed high impact on the phytase production were further optimized by using central composite design (CCD) under RSM. In the response surface design, each factor was varied over five levels, at ±1, 2(2) axial points and a centre point. Thirteen experiments were conducted with $k= 2\ (2k + 2k + 5)$, where k was the number of factors. The data were analyzed based on phytase activity of samples obtained after 12 h of fermentation process by fitting it to the second- order polynomial regression equation as below:

$$y = \beta_0 + \sum_{i=1}^{k}\beta_i x_i + \beta_i x_i^2 + \sum_{i=1}^{k}\sum_{j=1}^{k-1}\beta_{ij} x_i x_j,$$

where y was the response variable; β_0 referred to the intercept; β_i, β_{ii}, β_{ij} were the regression coefficients variables for linear, quadratic and interaction, respectively, whereas x_i and x_j were independent variables.

TABLE 3. Experimental design for two- level full factorial design

Run	Agitation speed (rpm)	Moisture (mL)	Nitrogen (g)	Carbon (g)
1	-1	-1	-1	-1
2	-1	-1	-1	-1
3	1	-1	-1	-1
4	1	-1	-1	-1
5	-1	1	-1	-1
6	-1	1	-1	-1
7	1	1	-1	-1
8	1	1	-1	-1
9	-1	-1	1	-1
10	-1	-1	1	-1
11	1	-1	1	-1
12	1	-1	1	-1
13	-1	1	1	-1
14	-1	1	1	-1
15	1	1	1	-1
16	1	1	1	-1
17	-1	-1	-1	1
18	-1	-1	-1	1
19	1	-1	-1	1
20	1	-1	-1	1
21	-1	1	-1	1
22	-1	1	-1	1
23	1	1	-1	1
24	1	1	-1	1
25	-1	-1	1	1
26	-1	-1	1	1
27	1	-1	1	1
28	1	-1	1	1
29	-1	1	1	1
30	-1	1	1	1
31	1	1	1	1
32	1	1	1	1

PHYTATE ACTIVITY AND GROWTH OF *MITSUOKELLA JALALUDINII* IN MF AND SELECTED M MEDIUM

Three percent (v/v) of 12 h culture of *M. jalaludinii* in MF medium were inoculated into a series of 50 mL MF and agro-medium inside serum bottles. Sodium phytate at 0.4% was added into MF medium to induce the production of phytase. The culture was allowed to be fermented for 24 h at 39°C under static condition. For every 4 h interval, a MF culture sample and an agro-medium culture sample were removed and centrifuged at 10,000 × g for 20 min. The pellet obtained was washed twice with 0.1 M sodium acetate buffer (pH5.0) and re-suspended in equal volume of the same buffer. One mL of each sample was added into 9 mL of dilution blank for serial dilution to determine bacterial growth based on the roll tube method of Hungate (1969). Each sample (0.2 mL) was also used for phytase assay.

PHYTASE ACTIVITY

Phytase assay was performed according to the method described by Yanke et al. (1998). Each sample (0.2 mL) was mixed with 0.8 mL of 0.4% (w/v) sodium phytate solution prepared in 0.1 M sodium acetate buffer (pH5.0). The mixture was incubated at 39°C for 15 min with an agitation of 180 rpm. Then, 1 mL of 5% (v/v) trichloroacetic acid (TCA) was added to terminate the reaction and the released P (in the form of phosphate) was determined by using the ammonium molybdate method (Heinonen & Lahti 1981). One unit of phytase activity (U) was expressed as the amount of phytase enzyme needed to release one nanomol of phosphate per min per mL.

STATISTICAL ANALYSIS

All the experiments, except RSM, were conducted three times with three replicates and the data collected were analyzed by using statistical software, IBM® SPSS for Microsoft Windows® version 22.0. Data were subjected for analysis of variance (ANOVA), and the means were tested by Duncan multiple range test for significant difference at $p<0.05$. The results were reported as means ± standard error (SE). For RSM study, the data were analyzed by using the statistical Design Expert software version 9 (Stat-Ease, Minneapolis, MN, USA).

Results and Discussion

EFFECTS OF CARBON AND NITROGEN SOURCES ON BACTERIAL GROWTH AND PHYTASE PRODUCTION

Figure 1 shows the total viable cell count (TVCC) and phytase activity of *M. jalaludinii* grown in different media. The growth of *M. jalaludinii* in M1 (RB) (11.5 log CFU/mL), where rice bran served as the sole carbon source was significantly higher ($p<0.05$) in comparison to the other two media. The population of *M. jalaludinii* in M2 (yam) and M3 (cassava) reached 11.1 and 11.2 log CFU/mL, respectively, were not significantly different ($p>0.05$). The highest phytase production (1012.61 U) was observed in M1 (RB) medium. Although the growth of *M. jalaludinii* was not significantly different in M2 (yam) and M3 (cassava), the phytase production in M3 (cassava) (195.39 U) was significantly lower ($p<0.05$) than M2 (yam) (378.72 U).

The bacterial growth and phytase activity of *M. jalaludinii* in media supplemented with different nitrogen sources are shown in Figure 2. The growth and phytase activity of *M. jalaludinii* were significantly different ($p<0.05$) in all media. Significantly higher ($p<0.05$) bacterial growth (12.5 log CFU/mL) and phytase activity (222.80 U) were observed in medium containing fish meal as the nitrogen source (M4). The growth of *M. jalaludinii* for other media in descending order was offal meal, soya

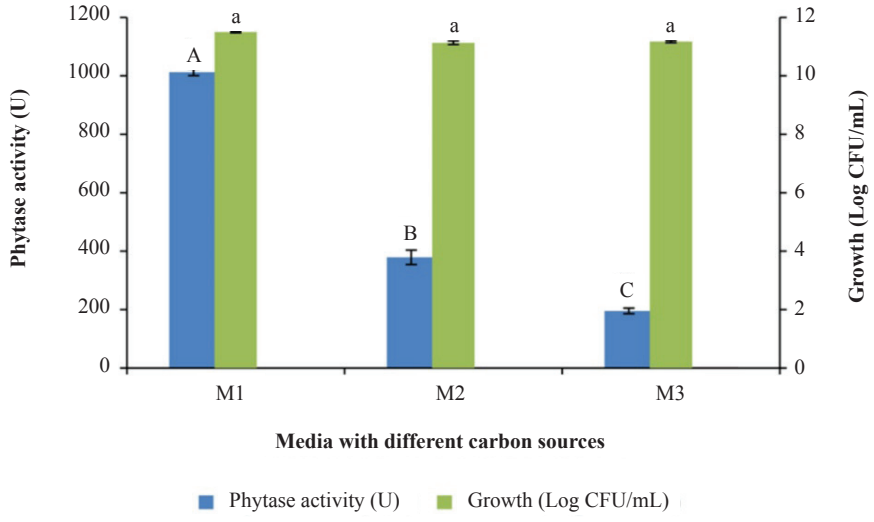


FIGURE 1. Phytase activity versus viable cell count of *M. jalaludinni* grown in media with different carbon sources. (M1: medium with rice bran; M2: medium with yam; M3: medium with cassava).
[A-C] Bars with different superscripts differ significantly (p<0.05)

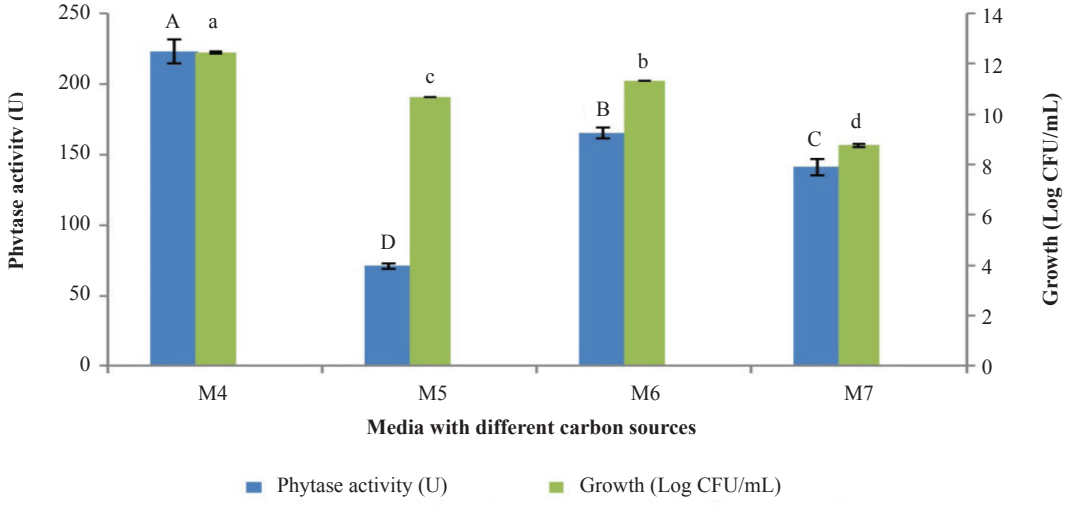


FIGURE 2. Phytase activity versus viable cell count of *M. jalalludinni* in media with different nitrogen sources. (M4: medium with fish meal; M5: medium with feather meal; M6: medium with offal meal; M7: soya bean meal)
[A-D] Bars with different superscripts differ significantly (p<0.05)
[a-d] Bars with different superscripts differ significantly (p<0.05)

bean meal and feather meal. The phytase activity was significantly lower, particularly in M5 (feather meal) medium (70.85 U).

Carbon and nitrogen are the basic nutrients required for growth of all living organisms. They are the building blocks of the cell structure, leading to efficient growth and production of metabolic products (Sanchez & Demain 2008). Important aspects to consider for the production of commercially feasible enzymes from microorganisms are cost and ease of production. Media using easily available resources and efficient production protocol are preferred. In most low-cost media formulation, agro- products or their byproducts are commonly good sources of carbon and nitrogen. Agro-substrates that commonly served as carbon and nitrogen sources are wheat bran, rice bran, cotton cake, coconut cake and ground nut cake (Bhavsar et al. 2011). Microorganisms which grew in these media were reported to have better growth and activity in comparison to the synthetic media. For instance, Lan et al. (2002b) showed that the yield of phytase by *M. jalaludinii* was significantly higher (p<0.05) when using rice bran and soybean milk in the medium compared to the use of synthetic medium. Similarly, in the present study, rice bran was the best substrate to induce the production of phytase by *M. jalaludinii*. The phytase production was 5 fold and 2.7 fold higher in M1 (rice bran) in comparison to M3 (cassava) and M2 (yam), respectively. The phytase production was 3.1, 1.6 and 1.3 fold higher in medium containing fish meal when compared to medium contained feather, soya bean and offal meal, respectively.

Animal proteins contain higher concentration of essential amino acid (Young & Pellett 1994) when compared to plant proteins, which might be the reason contributing to higher phytase enzyme of *M. jalaludinii* grown in fish meal medium. The lowest phytase activity was detected in *M. jalaludinii* grown in feather meal even though feather meal is categorized as animal protein. This is probably due to the presence of keratin protein that is packed tightly in the feather meal, making it difficult to be hydrolysed by the microorganisms (Kim et al. 2002). The type of carbon and nitrogen sources is important to stimulate the enzyme production. As observed in the present study (Figure 1), the bacteria grew well, but the phytase activity was low.

EFFECTS OF HEMIN, L-CYSTEINE HYDROCHLORIDE, MINERAL SOLUTION ON PHYTASE ACTIVITY

The removal of hemin from the M medium with rice bran and fish meal as carbon and nitrogen sources did not significantly reduced the phytase enzyme activity (p>0.05) (1138.44 U). However, the phytase activity was significantly decreased (p<0.05) from 1178.72 U to 446.99 U and 902.54 U with the removal of L-cysteine hydrochloride and mineral solution, respectively (Figure 3).

It is well known that hemin and L-cysteine hydrochloride are commonly included in anaerobic media in which hemin functions as a factor to stimulate cell growth whereas L-cysteine hydrochloride serves as

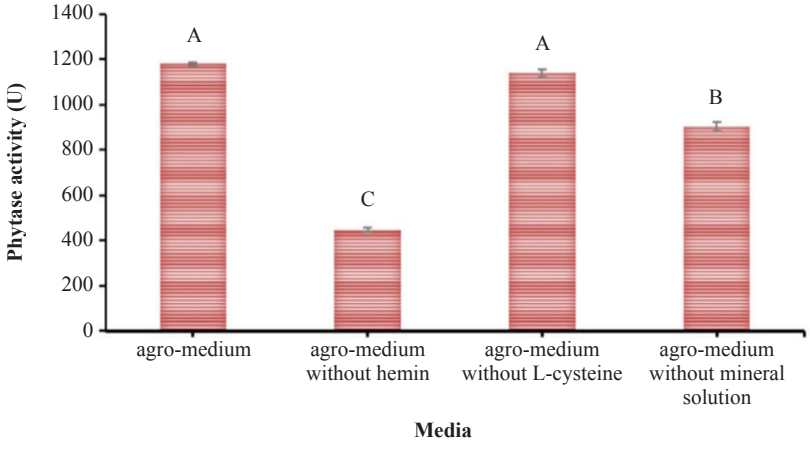


FIGURE 3. Phytase activity of *M. jalaludinii* in agro-medium containing rice bran and fish meal, without hemin, without L-cysteine or without mineral solution
[A-C] Bars with different superscripts differ significantly (p<0.05)

TABLE 4. ANOVA for phytase production by *M. jalaludinii* using 2 level full factorial design

Source	Sum of Squares	DF	Mean Square	F value	Prob> F	
Model	3.615 E + 006	8	4.518 E + 005	247.01	< 0.0001	Significant
A	337.74	1	337.74	0.18	0.6714	
B	48349.62	1	48349.62	26.43	< 0.0001	
C	1.174 E + 006	1	1.174 E + 006	641.79	< 0.0001	
D	1.392 E + 006	1	1.392 E + 006	760.99	< 0.0001	
BC	3.564 E + 005	1	3.564 E + 005	194.84	< 0.0001	
BD	11881.88	1	11881.88	6.50	0.0180	
CD	3.954 E + 005	1	3.954 E + 005	216.13	< 0.0001	
BCD	2.363 E + 005	1	2.363 E + 005	129.19	< 0.0001	
Residue	42073.14	23	1829.27			
Lack of Fit	33596.05	7	4799.44	9.06	0.0001	Significant
Pure Error	8477.09	16	529.82			
Cor Total	3.657 E + 006	31				
Std. Dev.	42.77					
Mean	288.96					
R^2	0.9885					
Adj R^2	0.9845					
Pred. R^2	0.9777					
Adeq Precision	46.571					

A= agitation speed; B= moisture; C= nitrogen; D= carbon
Abbreviation: DF, degree of freedom

a reducing agent. This indicated that the components of the agro-medium could replace the function of hemin. Since *M. jalaludinii* is a strict anaerobic bacterial species, supplementation with L-cysteine hydrochloride is necessary to ensure the medium is reduced and the anaerobic condition is maintained to promote anaerobic bacterial growth (Rymovicz et al. 2011). It was reported that the used of L-cysteine hydrochloride had the significant effect on the growth of *Bifidobacterium bifidum* (Shu et al. 2013). In addition, the mineral solution is also important for cell growth as minerals affect the bacterial metabolic processes (Kim et al. 2000).

RESPONSE SURFACE METHODOLOGY (RSM)

ANOVA result obtained from two-level full factorial design showed that the model was significant based on the F-value of 247.01 (Table 5).

TABLE 5. Design matrix for CCD and phytase activity of *M. jalaludinii*

Run	Carbon (g)	Nitrogen (g)	Phytase activity (U)
1	7.00	4.00	1643.50
2	7.00	4.00	1406.72
3	7.00	4.00	1503.67
4	7.00	4.00	1554.50
5	7.00	4.00	1354.50
6	4.00	2.00	995.75
7	10.00	2.00	876.50
8	4.00	6.00	215.50
9	10.00	6.00	1309.50
10	2.76	4.00	241.50
11	11.24	4.00	967.50
12	7.00	1.17	1342.83
13	7.00	6.83	889.50

Carbon and nitrogen sources and moisture content were the key parameters that had been identified to influence the phytase production as p values are less than 0.0500 (p<0.05). Central composite design was used to further optimize the percentage of carbon (rice bran) and nitrogen source (fish meal) which showed a significant effect (p<0.05) on phytase enzyme production in semi-solid state fermentation. Semi-solid state fermentation is the modification of solid state fermentation by increasing the water content from the absence or nearly absence of free water in the latter. Table 4 shows that the presence of moisture was essential (p<0.05) in order for *M. jalaludinii* to produce the enzyme. Moisture content had been proven as a significant parameter for enzyme production (Kumari Chitturi & Lakshmi 2016; McKinney et al. 2015). It was true for bacteria as low moisture limit the metabolism and growth of microorganisms (Bhargav et al. 2008). However, moisture factor was not included in the CCD optimization as the amount of moisture in the medium was dependent on the percentage/ ratio of carbon and nitrogen sources after optimization. No significant effect was observed for the phytase production when the medium was fermented under shaking condition (p=0.6714). Table 5 shows the response, which was the phytase activity of *M. jalaludinii* based on 13 different combinations of carbon and 276 nitrogen amount from the design matrix.

The response of the CCD design was fitted with a second order polynomial equation as follows:

$$\text{Phytase activity} = 1492.58 + 250.18A - 123.54B - 446.79A^2 - 190.96B^2 + 303.31AB$$

where, A and B were the coded factors of carbon and nitrogen source, respectively.

The equation above shows that carbon source had a positive effect on phytase activity while nitrogen source had a negative effect on phytase activity. All the linear, quadratic and interaction terms were statistically significant (p<0.05). Contour plot in Figure 4 explores the relationship between the three variables: carbon concentration, nitrogen concentration and the phytase activity in two dimensions where the response variable (phytase activity) was represented by contours. Both contour and 3D response plot are plotted based on the regression model where both actually showing the response surface in a clearer graphical way. According to the second order polynomial equation as well as the contour and 3D response plot of phytase production, it was observed that increasing the percentage of carbon source from 4.00% to 7.70% could promote the yield of phytase. The increment of nitrogen source from 2.00% to 3.72% also increased the yield of phytase; however, the yield of phytase was reduced once the percentage of nitrogen source exceeded 3.72%.

Based on the results presented in Table 6, our model exhibited F-value of 54.31 and significant (p<0.0001). In addition, main effect of Factor A (carbon), Factor B (nitrogen), interaction between A and B, as well as the quadratic terms of A^2, B^2 were also statistically significant. The interaction between carbon and nitrogen was statistically significant (p<0.05) in improving the production of phytase enzyme. The lack of fit test is a measurement of the failure of the model to represent data in the regression (Rastogi & Rashmi 1999). The 'Lack of Fit F-value' of 0.29 suggested that the Lack of Fit was not significant relative to the pure error which means that the model fit to the data.

PHYTASE ACTIVITY AND GROWTH OF *M. JALALUDINII* IN MF MEDIUM AND AGRO-MEDIUM AT DIFFERENT INCUBATION PERIODS

Growth and phytase activity profile of *M. jalaludinii* in both MF and agro-medium at every 4 h interval is shown in Figure 5. The bacterial growth showed a similar trend in which it increased gradually until it reached the maximum at 12 h incubation before it started to decline. Nevertheless, the growth of *M. jalaludinii* was found to be significantly higher (p<0.05) in agro-medium (12.1 CFU/mL). The population of *M. jalaludinii* in agro-medium was 0.16-2.54 log CFU/mL higher than the growth in MF medium. The phytase production was in accordance with the growth of the bacteria in which significant higher

TABLE 6. ANOVA for response surface quadratic model

Source	Sum of Squares	DF	Mean Square	F Value	Prob > F	
Model	2.504E+006	5	5.008E+005	54.31	< 0.0001	significant
A	5.007E+005	1	5.007E+005	54.30	0.0002	
B	1.221E+005	1	1.221E+005	13.24	0.0083	
A^2	1.389E+006	1	1.389E+006	150.60	< 0.0001	
B^2	2.537E+005	1	2.537E+005	27.51	0.0012	
AB	3.680E+005	1	3.680E+005	39.91	0.0004	
Residual	64546.30	7	9220.90			
Lack of Fit	11374.36	3	3791.45	0.29	0.8346	not significant
Pure Error	53171.95	4	13292.99			
Cor Total	2.569E+006	12				
Std. Dev.	96.03					
Mean	1100.11					
C.V.	8.74					
PRESS	1.640E+005					

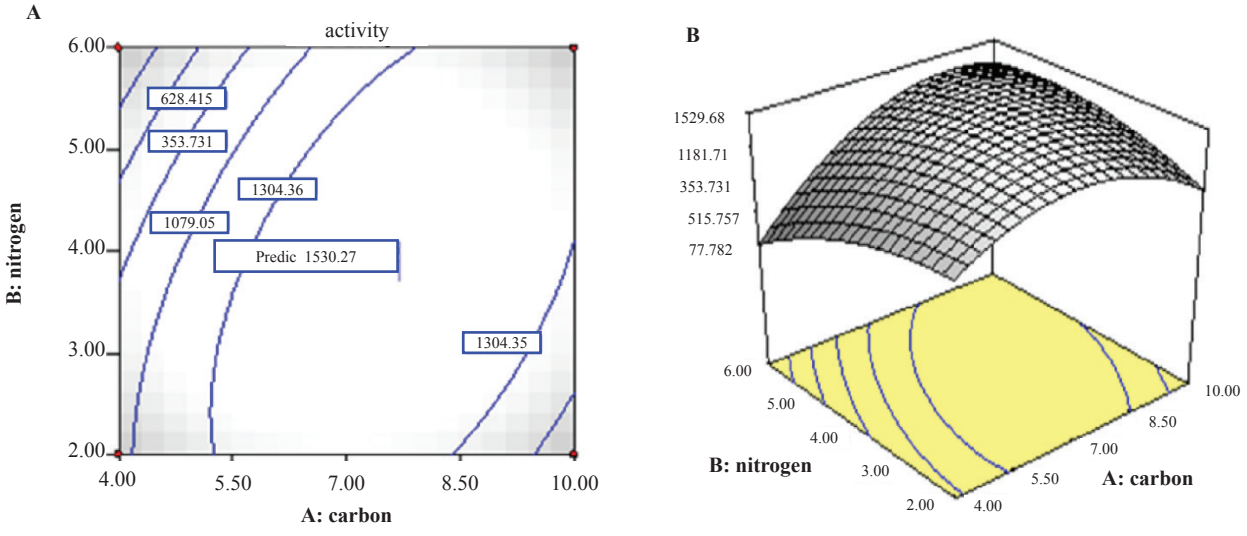
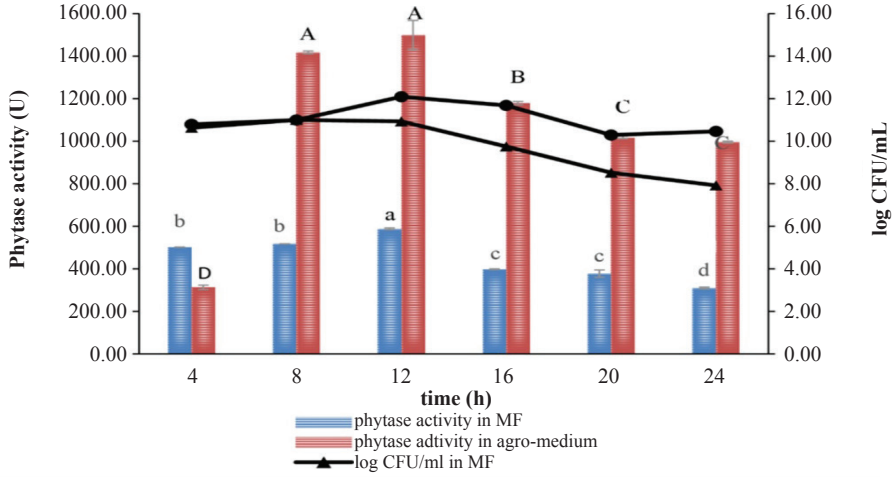


FIGURE 4. (A) Contour plot and (B) 3D response surface plot showing the phytase production by *M. jalaludinii* in semi-solid state fermentation



A-D Bars with different superscripts differ significantly (p<0.05)
a-d Bars with different superscripts differ significantly (p<0.05)

FIGURE 5. Phytase activity versus viable cell count of *M. jalalludinni* in MF medium and agro-medium at different times interval

(p<0.05) activity was observed at 12 h. The phytase enzyme activity was 2.5 fold higher in the agro-medium. The phytase enzyme activity (1498.58 U) was 2.5 fold higher than the highest activity (588.45 U) obtained in MF medium. Abd-Elhalem et al. (2015) also found that the used of agro-industrial by-products was able to give better enhancement of amylase enzyme production in comparison to the use of the basal medium. The same situation was observed by Sibi (2015) where the biomass and lipid production were improved when cultivating microalgae by using sweet sorghum stem and rice straw as carbon and nitrogen sources instead of the basal media with glucose.

CONCLUSION

The newly formulated agro-medium which contained rice bran and fish meal as carbon and nitrogen sources and without hemin was able to provide better growth and higher phytase enzyme production by *M. jalaludinii*.

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