

Optimization of the Phosphate-Solubilizing Fungus, *Aspergillus japonicus* SA22P3406, in Solid-State Cultivation by Response Surface Methodology

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ABSTRACT

Phosphorus (P) is one of the essential macronutrients for plant growth and reproduction. Plants acquire P from the soil solution as phosphate anions. However, phosphate anions are extremely reactive and may be immobilized through precipitation with cations such as Ca^{2+} , Mg^{2+} , Fe^{3+} and Al^{3+} , depending on the particular properties of the soil and as a result, the phosphate is highly insoluble and unavailable to plants. Application of phosphate-solubilizing fungi (PSF) has been added as fertilizer to increase P uptake and plant growth. The aim of this study was to use response surface methodology to determine the optimal factors of the phosphate-solubilizing fungus *Aspergillus japonicus* SA22P3406 for use in solid state cultivation, specifically the pH, temperature and ratio of water to dry rice bran (RWB). *A. japonicus* SA22P3406 was screened from 24 soil samples. Primary screening was carried out by a 10-fold serial dilution of the soil samples at 10^{-4} to 10^{-6} and then spread on Pikovskaya agar supplemented with 1.0% w/v of tricalcium phosphate at 37°C for 3-5 d. One hundred and fifty five fungal isolates produced high ratios of clear zone to colony on Pikovskaya agar supplemented with 0.003% w/v of rose bengal at 37°C for 7 d. The ratios were calculated by dividing the area of the clear zone by the area of the colony. Secondary screening was performed by measuring the available phosphate in liquid medium from the 62 fungal strains by CRD and DMRT at 5% probability. Two fungal strains, *A. niger* SA07P3332 and *A. japonicus* SA22P3406, were selected as most suitable for solubilizing tricalcium phosphate and producing the highest available phosphate levels of 3.01 and 2.99 mg P_2O_5 ml^{-1} , respectively. *A. japonicus* SA22P3406 was cultivated in 5.0 g rice bran to study the optimal conditions using response surface methodology with a central composite design. The optimum conditions of pH, temperature and RWB were 7.37, 36.0°C and 82.10% v/w of dry rice bran, respectively. These conditions were used in the solid-state cultivation and a sporulation curve was determined. The fungal sporulation had a lower than average decadic logarithm of practical quantitative sporulation of 8.46 compared with the estimated quantitative sporulation of 8.53 at the 5th day of incubation.

Key words: phosphate-solubilizing fungi, response surface methodology, solid-state cultivation, rice bran, *A. japonicus*.

INTRODUCTION

Thailand is known to be one of the developing countries that is also the world's largest net food exporter. The use of biofertilizer can lessen dependence on chemical fertilizer, resulting in cost saving as well as reducing deleterious environmental and human effects (Damrongchai, 2003). Biofertilizer has been identified as an alternative to chemical fertilizer to increase soil fertility and crop production in sustainable farming (Wu *et al.*, 2005). Phosphorus (P) is one of essential macronutrients for plant growth and reproduction (Reddy *et al.*, 2002). Plants acquire P from soil solution as phosphate anions. However, they are extremely reactive and may be immobilized through precipitation with cations such as Ca^{2+} , Mg^{2+} , Fe^{3+} and Al^{3+} , depending on the particular properties of the soil. In these forms, phosphate is highly insoluble and unavailable to plants. Consequently, the amount of P available to plants is usually decreased, so that only a small proportion found naturally can be utilized by the plant (Forum for Nuclear Cooperation in Asia (FNCA), FNCA Biofertilizer Project Group, 2006). Phosphate-solubilizing microorganisms (PSMs) have been used as fertilizer to increase the P uptake and plant growth (Wang *et al.*, 2007). The principle mechanism for mineral phosphate solubilization is the production of organic acids. It is generally accepted that the major mechanism of mineral phosphate solubilization is the action of organic acids synthesized by soil microorganisms. Production of organic acids results in acidification of microbial cells and their surroundings (Forum for Nuclear Cooperation in Asia (FNCA), FNCA Biofertilizer Project Group, 2006). Many fungal strains can solubilize rock phosphate, aluminium phosphate and tricalcium phosphate, such as *Aspergillus* sp., *A. niger*, *A. tubingensis*, *A. fumigatus*, *A. terreus*, *A. awamori*, *Penicillium* sp., *P. italicum*, *P. radicum*, *P. rugulosum*, *Fusarium* sp., *F. oxysporum*, *Curvularia lunata*, *Humicola* sp., *Sclerotium*

rolfsii, *Pythium* sp., *Aerothecium* sp., *Phoma* sp., *Cladosporium* sp., *Rhizoctonia* sp., *Rhizoctonia solani*, *Cunninghamella* sp., *Rhodotorula* sp., *Candida* sp., *Schwanniomyces occidentalis*, *Oideodendron* sp., *Pseudonymnoascus* sp. (Isbelia *et al.*, 1999; Sparks, 1999; Whitelaw *et al.*, 1999; Didiek *et al.*, 2000; Helen *et al.*, 2002; Reddy *et al.*, 2002; Achala *et al.*, 2007). Response surface methodology (RSM) is a combination of statistical and mathematical techniques useful for developing, improving and optimizing processes. Optimization of the response determines the conditions that optimize the process. A second-order model can be used to estimate the response in a narrow region and from examination of the response surface, the optimum levels or condition for process variables can be chosen (Boonorm, 2003). When the region of the optimum response has been located, curvature can be produced. Three factors (k) and three levels of factorial experiment are often conducted in order to fit such response surfaces. The total number of test runs (n) in a central composite design (CCD) is based on a complete 2^k factorial, which is $n = 2^k + 2k + m$, where m is the number of replications at a center point of design. This number of treatments is usually less than 3^k , so that fewer observations are required than in a 3^k factorial. The CCD can be rotated by choosing $\alpha = F^{1/4}$, where F is the number of factorial points (e.g., $F = 2^k$ when a complete factorial is used) (Mason *et al.*, 2003).

MATERIALS AND METHODS

Materials

Rice bran was purchased from a rice mill in Nong Nae, Phanom Sarakham district, Chachoengsao province, Thailand and tricalcium phosphate media was purchased from Hi-Media (Mumbai, India). All other reagents were of analytical grade and were purchased from Merck (Germany), Sigma (St. Louis, MO, USA) and BDH (England).

Microorganism

From 24 soil samples, 62 fungal isolates were selected from the first screening. Two isolates obtained from soil collected from Huai Khayeng, Thong Pha Phum district in Kanchanaburi province, Thailand, could solubilize tricalcium phosphate well in the second screening tests. These isolates were identified as *A. niger* and *A. japonicus* designated as SA07P3332 and SA22P3406, respectively. The *A. japonicus* SA22P3406 was selected to study the optimal conditions for quantitative sporulation in solid-state cultivation. This fungus was maintained in potato dextrose agar slant and potato dextrose broth supplemented with 10% v/v of glycerol at 4°C and 80°C, respectively.

Soil sample collection and the first fungal screening

Fungal strains were screened from 19 Kanchanaburi soils, 2 Chaiyaphum soils, 2 Trang soils and 1 Krabi soil in Thailand. Soil samples (approximately 2 kg) were collected from the areas where any plants has flourished (Hu *et al.*, 2006) by taking the soil sample from a depth of 0-15 cm and storing at 4°C in a refrigerator (Coyne, 1999). One gram of soil sample was added to 9 ml of 0.85% w/v of physiological saline. Ten-fold serial dilution was made to drop 0.1 ml of 10^{-4} - 10^{-6} on plates containing the Pikovskaya agar. The composition of the Pikovskaya agar medium was 0.5, 0.5, 0.3, 0.3, 0.03, 0.02, 10.0, 10.0 and 15.0 g/l for $(\text{NH}_4)_2\text{SO}_4$, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, NaCl, KCl, $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, $\text{MnSO}_4 \cdot \text{H}_2\text{O}$, $\text{Ca}_3(\text{PO}_4)_2$, glucose and agar, respectively. The plates were spread and incubated at 37°C for 3-5 days. The different colonies were picked and grown on Pikovskaya agar again. The interesting strains were purified on the same medium (Hu *et al.*, 2006). The 155 isolates of phosphate-solubilizing fungi (PSF) were purified on Pikovskaya agar supplemented with 0.003% w/v of rose bengal. The area of the clear zone and the colony were measured after incubation at 37°C for 3, 5 and 7 d (Taweenut,

2003). The ratios of clear zone to colony were calculated by dividing the area of the clear zone by the area of the colony. The 62 ratios were arranged according to frequency to construct a normal distribution curve, which was used to calculate the z score at the 95% confidence level (Equation 1):

$$Z_\alpha = \frac{\bar{X} - \mu}{\sigma/\sqrt{n}} \quad (1)$$

The second screening of tricalcium phosphate solubilization and final pH in liquid cultivation

Sixty two strains had a high ratio value of 5% of clear zone were used to determine tricalcium phosphate solubilization. The inoculation ratio of 10^6 spore ml^{-1} was prepared to suspend *A. japonicus* SA22P3406 slant with 0.85% w/v of physiological saline. One ml of the inoculation ratio was added to 50 ml Pikrovskaya medium in a 250 ml Erlenmeyer flask. The triplicate repetition was incubated at 37°C with 200 rpm for 3, 5 and 7 d. Autoclaved and uninoculated medium were used as controls. The cultures were harvested by filtration with Whatman paper filter no. 42. The supernatants were analyzed for available phosphate (P_2O_5) with the molybdovanado-phosphate method (AOAC, 1997) and final pH was determined. The highest available phosphates were calculated by complete randomized design (CRD) and Duncan's multiple range test (DMRT) at 95% confidence levels.

Methodology

The fungus, *A. japonicus* SA22P3406, gave the highest available phosphate when cultivated in submerged medium supplemented with 1% w/v of tricalcium phosphate. The *A. japonicus* SA22P3406 slant was suspended with 0.85% w/v of physiological saline to obtain the concentration of 10^6 spore ml^{-1} . One ml of spore dilution was added to 5 g rice bran in a 250 ml Erlenmeyer flask for optimization. The quantitative sporulation was counted under a 400x compound microscope after the cultivation had

been inoculated for 5 d. Three factors and three levels of central composite design (CCD) were used to determine the optimal factors of fungal cultivation with three repetitions. A central composite design with a quadratic model was employed (Boonorm, 2003; Mason *et al.*, 2003). Three independent variables, namely pH (x_1), temperature (x_2) and ratio of water to dry rice bran, RWB (x_3) were chosen. Each independent variable had three levels, which were -1, 0 and +1. A total of 15 different combinations was chosen in random order according to the central composite design configuration for the three factors. The coded values of the independent variables were found from Equation 2 and are presented in Table 1.

$$OL_{1.682} = OL_0 \pm \Delta OL_{1.682} \quad (2)$$

where,

$$\Delta OL_{1.682} = (OL_1 - OL_0) \times 1.682$$

OL_0 is the original level at zero coded level.

OL_1 is the original level at one coded level.

$OL_{1.682}$ is the original level at 1.682 coded level.

The study was carried out according to a central composite design and the experimental treatments used according to the design are shown in Table 2. A second-order polynomial equation was used to express the average decadic logarithm of quantitative sporulation as a function of independent variables, where y in Equation 3 represents the average decadic logarithm of quantitative sporulation. The coefficients of the response surface equation were determined using linear regression functions in the SPSS for WINDOWS software, version 11.5.

$$y = b_0 + b_1x_1 + b_2x_2 + b_3x_3 + b_{12}x_1x_2 +$$

$$b_{13}x_1x_3 + b_{23}x_2x_3 + b_{11}x_1^2 + b_{22}x_2^2 + b_{33}x_3^2$$

RESULTS AND DISCUSSION

After the 20 experimental treatments had been inoculated with *A. japonicus* SA22P3406 in rice bran for 5 d, the average decadic logarithm of quantitative sporulation was counted (Table 2). The average decadic logarithm of quantitative sporulation and original levels were plotted as a response surface and contour graphs by the STATISTICA software, version 7 of Statsoft Inc. Both the pH and temperature had maximum points for the pH (7.37) and temperature (36.0°C) and an average decadic logarithm of quantitative sporulation of 8.10 (Figure 1). Figure 2 shows the broad peak of 7.37 for the pH and 82.10% v/w for the percentage of water to dry rice bran, with a value of 7.51 for the average decadic logarithm of quantitative sporulation. Temperature and the ratio of water to dry rice bran also had broad peaks of 36.0°C and 82.10% v/w of dry rice bran, respectively, with a value of 8.31 for the average decadic logarithm of quantitative sporulation.

A second-order polynomial equation was used to express the average decadic logarithm of quantitative sporulation as a function of the independent variables given in Equation 4. The equation was analyzed by regression using 5% probability with the SPSS for WINDOWS software, version 11.5. The ANOVA and coefficients of the equation determined by regression analysis are shown in Tables 3 and 5, respectively. The quality of fit of the second-order regression equation was checked using the

Table 1 Original and coded levels of the independent variables.

Independent variables	Coded levels				
	-1.682	-1	0	+1	+1.682
pH	1.95	4.0	7.0	10.0	12.05
temperature (°C)	15.1	24.0	37.0	50.0	58.9
ratio of water to dry rice bran (%v/w of dry rice bran)	66.36	80.00	100.00	120.00	133.64

coefficient of determination (R^2) and calculated to be 0.785, indicating 78.50% of the variability in the response could be explained by the models. The effect of three variables is presented in Table 4. The model F value of 426.075 showed that the model was significant and the adequacy of second-order regression model. Statistical analysis demonstrated all three additives had a significant effect on sporulation within the tested ranges at a probability level of 95% and the lack of fit was insignificant. According to the linear coefficients of the second-order equation, pH and the ratio of water to dry rice bran showed a positive effect, while temperature had a negative influence.

The optimum conditions of pH (7.37), temperature (36.0°C) and the ratio of water to dry rice bran (82.10% v/w of dry rice bran) are shown in Figures 1, 2 and 3. Therefore, these values were used in the second-order polynomial equation (Table 6) to calculate a value of the average decadic logarithm of quantitative sporulation of 8.53. These conditions were used in solid-state cultivation for quantitative sporulation in rice bran. In Figure 4, for *A. japonicus* SA22P3406 the value is 8.46 for the average decadic logarithm of quantitative sporulation at the fifth day. Therefore, the practical average decadic logarithm of quantitative sporulation was 99.18% of the estimation.

Table 2 The experimental treatments according to the central composite design with three independent variables (original and coded levels).

No.*	Original levels			Coded levels			Observed values****	Predicted values****
	pH	Temp.**	Ratio***	pH	Temp.	Ratio		
1	4.00	24.0	80.00	-1	-1	-1	6.013 ± 0.0833	6.579 ± 1.1388
2	4.00	24.0	120.00	-1	-1	+1	6.212 ± 0.0884	6.684 ± 1.1388
3	4.00	50.0	80.00	-1	+1	-1	5.719 ± 0.0895	6.262 ± 1.1388
4	4.00	50.0	120.00	-1	+1	+1	5.811 ± 0.1846	6.319 ± 1.1388
5	10.00	24.0	80.00	+1	-1	-1	5.933 ± 0.3191	6.465 ± 1.1388
6	10.00	24.0	120.00	+1	-1	+1	5.993 ± 0.0594	6.490 ± 1.1388
7	10.00	50.0	80.00	+1	+1	-1	5.965 ± 0.0517	6.532 ± 1.1388
8	10.00	50.0	120.00	+1	+1	+1	6.035 ± 0.2032	6.508 ± 1.1388
9	1.95	37.0	100.00	-1.682	0	0	8.485 ± 0.0774	7.744 ± 1.1388
10	12.05	37.0	100.00	+1.682	0	0	8.536 ± 0.1252	7.807 ± 1.1388
11	7.00	15.1	100.00	0	-1.682	0	5.841 ± 0.1665	5.113 ± 1.1388
12	7.00	58.9	100.00	0	+1.682	0	5.604 ± 0.1458	4.862 ± 1.1388
13	7.00	37.0	66.63	0	0	-1.682	7.807 ± 0.0728	6.995 ± 1.1388
14	7.00	37.0	133.64	0	0	+1.682	7.722 ± 0.0409	7.064 ± 1.1388
15	7.00	37.0	100.00	0	0	0	8.526 ± 0.1066	8.540 ± 1.1388
16	7.00	37.0	100.00	0	0	0	8.527 ± 0.0500	8.540 ± 1.1388
17	7.00	37.0	100.00	0	0	0	8.569 ± 0.0494	8.540 ± 1.1388
18	7.00	37.0	100.00	0	0	0	8.548 ± 0.1709	8.540 ± 1.1388
19	7.00	37.0	100.00	0	0	0	8.372 ± 0.0099	8.540 ± 1.1388
20	7.00	37.0	100.00	0	0	0	8.448 ± 0.1103	8.540 ± 1.1388

Notes: *No. = experimental treatments were performed in random order.

**Temp. = temperature (°C)

***Ratio = ratio of water to dry rice bran (%v/w of dry rice bran)

****Observed and predicted values = average decadic logarithm of quantitative sporulation when cultivation had been inoculated for 5 d.

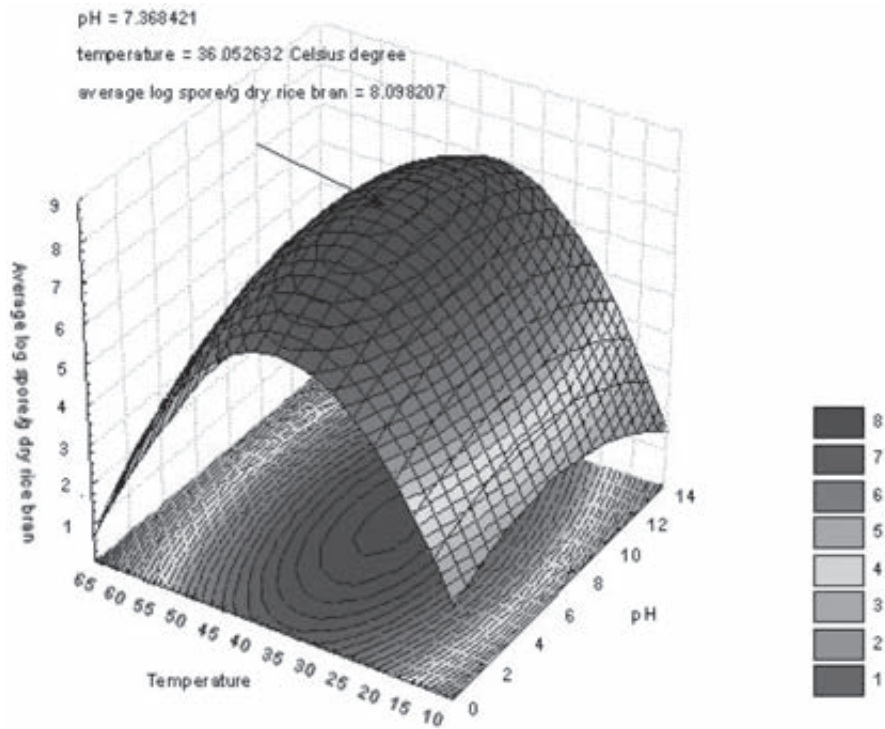


Figure 1 Surface and contour graphs plotted for pH and temperature.

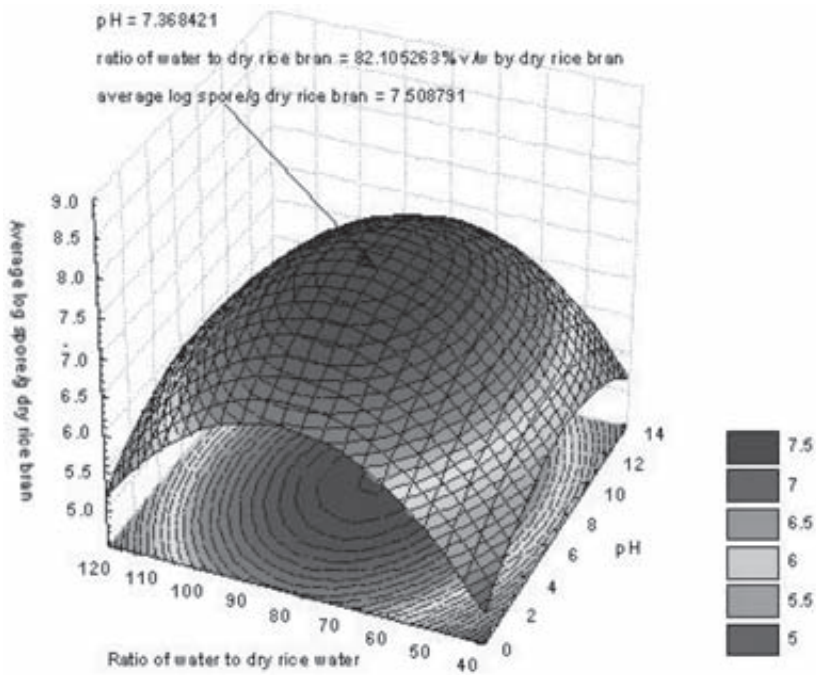


Figure 2 Surface and contour graphs plotted for pH and ratio of water to dry rice bran.

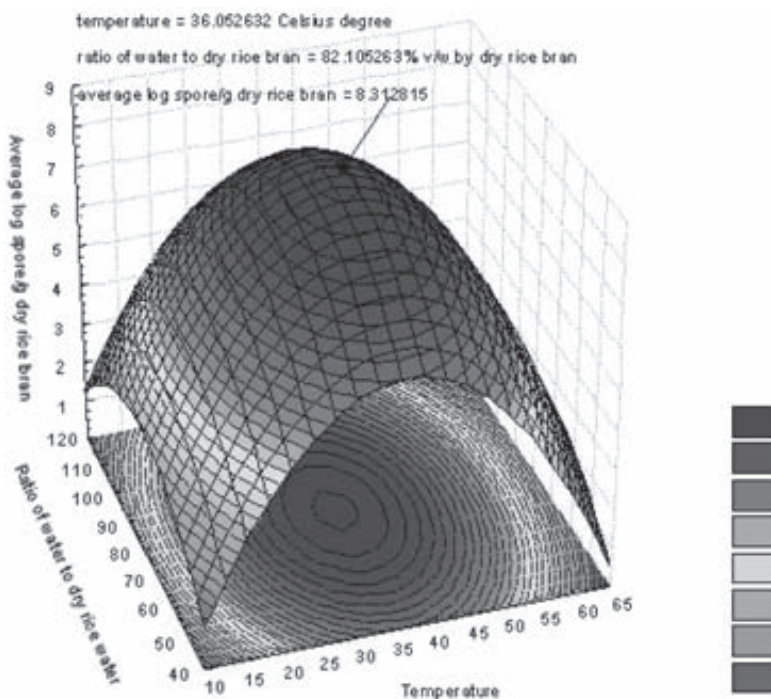


Figure 3 Surface and contour graphs plotted for temperature and the ratio of water to dry rice bran.

Table 3 ANOVA of the average decadic logarithm of quantitative sporulation analyzed with a regression of 5% probability.

Model	Sum of squares	Degree of freedom	Mean square	F	Significant
Regression	76.515	9	8.502	24.888	0.000
Residual	17.080	50	0.342		
Total	93.595	59			

Note: $R^2 = 0.818$ and adjusted $R^2 = 0.785$

Table 4 Effect of three variables on the production of spores of *A. japonicus* SA22P3406 in dry rice bran.

Source	Sum of square	Mean square	F value	Significant
Model	92.824	7.140	426.075	0.000
x_1	0.015	0.007	0.440	0.647
x_2	0.228	0.114	6.812	0.003
x_3	0.077	0.039	2.304	0.111
x_1x_2	0.221	0.221	13.187	0.001
x_1x_3	0.010	0.010	0.585	0.448
x_2x_3	0.003	0.003	0.208	0.651
x_1^2	0.000	-	-	-
x_2^2	0.000	-	-	-
x_3^2	0.000	-	-	-
Lack of fit test	0.005	0.005	0.309	0.581

Table 5 Coefficient of the second-order polynomial equation determined by regression analysis.

Factors	Coefficients	Un standardized coefficients		t	Significant
		B	Standard error		
Constant	b ₀	8.5400	0.1376	62.0523	0.000
x ₁	b ₁	0.0187	0.0913	0.2049	0.838
x ₂	b ₂	-0.0746	0.0913	-0.8166	0.418
x ₃	b ₃	0.0204	0.0913	0.2234	0.824
x ₁ x ₂	b ₁₂	0.0960	0.1193	0.8043	0.425
x ₁ x ₃	b ₁₃	-0.0202	0.1193	-0.1694	0.866
x ₂ x ₃	b ₂₃	-0.0120	0.1193	-0.1009	0.920
x ₁ ²	b ₁₁	-0.2703	0.0889	-3.0418	0.004
x ₂ ²	b ₂₂	-1.2557	0.0889	-14.1290	0.000
x ₃ ²	b ₃₃	-0.5339	0.0889	-6.0075	0.000

Table 6 The average decadic logarithm of estimated quantitative sporulation calculated by the second-order polynomial equation.

No.*	Original levels			Code levels			Predicted values****
	pH	Temp.**	Ratio***	pH	Temp.	Ratio	
1	7.37	36.0	82.10	0.123	-0.077	0.105	8.540
2	7.37	38.9	82.10	0.123	0.146	0.105	8.498

Note: *No. = experimental treatments were performed in random order.

**Temp. = temperature (°C)

***Ratio = ratio of water to dry rice bran (%v/w of dry rice bran)

****Predicted values = average decadic logarithm of estimated quantitative sporulation when cultivation had been inoculated for 5 d.

Singal *et al.* (1994) found *A. japonicus* and *A. foetidus* solubilized five types of Indian rock phosphate at pH 8 and 9. Solubilization of rock phosphate was the result of organic acid release and pyrite oxidation. Solid-state fermentation (SSF) has become a very attractive alternative to submerged fermentation (SMF) for specific applications, due to the recent improvements in reactor designs. The application of SSF has been used to produce several metabolites relevant to the food processing industry, namely flavours, enzymes (α -amylase, fructosyl transferase, lipase, pectinase), organic acids (lactic acid, citric acid) and xanthan gum (Couto and Sanroman, 2006). Response surface methodology was used as an optimization procedure by central composite design with four factors (concentrations of maltrin and corn steep liquor (CSL), agitation speed and

inoculation ratio) to investigate the effect of these parameters on the polygalacturonase (PG) enzyme activity and mycelial growth (biomass) of *A. sojae* ATCC 20235. The optimization step improved the PG activity by 74% and the biomass by 40% (Tari *et al.*, 2007). *A. niger* NRRL 567 was grown in an inert support material for citric acid production. Optimization of the medium components, including ethanol, methanol, phytate, olive oil and surfactant was carried out using “one-factor-at-a-time” and central composite design methods. Optimization using “one-factor-at-a-time” was performed using 30 g/kg dry peat moss (DPM) for citric acid production. However, the optimization of phytate, olive oil and methanol were selected to produce citric acid using CCD. The three variables were identified to have significant effects on the maximum citric acid

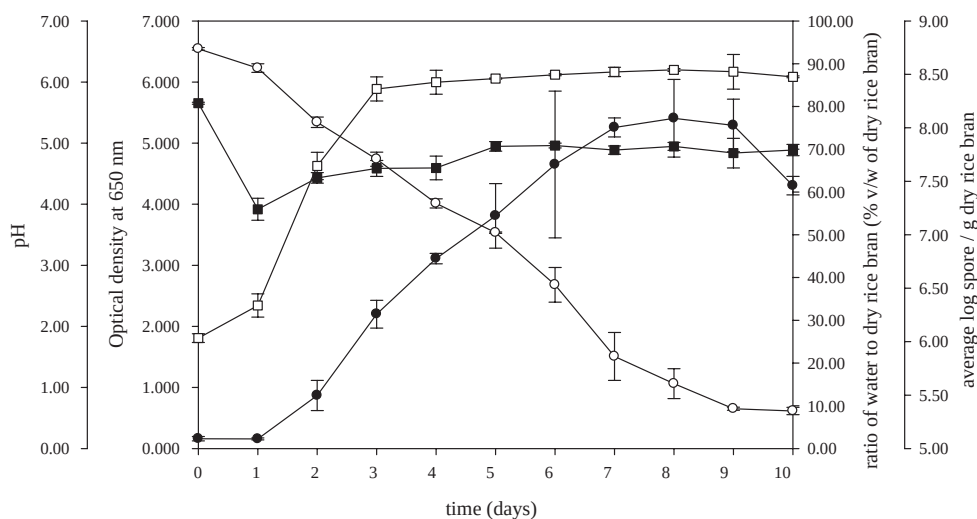


Figure 4 The sporulation curve, final pH, final ratio of water to dry rice bran and optical density at 650 nm of fungal *A. japonicus* SA22P3406 cultivated in 5 g rice bran with the initial pH of 7.37, temperature of 36.0°C and ratio of water to dry rice bran of 82.10% v/w of dry rice bran. ((□) log spore / g dry rice bran; (■) final pH was measured by mixture of 45.0 ml of distilled water pH 7.0±0.1; (○) final ratio of water to dry rice bran calculated as the difference in mass before being warmed in a hot air oven at 80°C for 24 h; and (●) optical density of spore suspended at 650 nm.)

production of 354.8 g/kg DPM. The maximum citric acid production in optimized conditions by CCD represented about a 2.7-fold increase compared to that obtained from control before optimization (Barrington and Kim, 2008).

CONCLUSION

From the results of the present study, the optimum conditions of pH, temperature and the ratio of water to dry rice bran were 7.37, 36.0°C and 82.10% v/w of dry rice bran, respectively. *A. japonicus* SA22P3406 was grown in rice bran and effectively produced the highest average decadic logarithm of quantitative sporulation of 8.54 on the eighth day. A statistically based method using CCD was applied to develop a second-order regression model. The high adequacy of the model was proven by the good fit between the experimental and predicted values. Response surface methodology was a better choice to

optimize the biological process than that of a “one-condition-at-a-time” because response surface methodology has factor interaction analysis and fewer treatments than a full factorial design (Barrington and Kim, 2008; Mason et al., 2003).

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