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OPTIMIZATION OF ALPHA AMYLASE PRODUCTION FROM RICE STRAW USING SOLID-STATE FERMENTATION OF *Bacillus subtilis*

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Abstract: The current study optimized the production of α-amylase by *Bacillus subtilis* using solid state fermentation (SSF) process. The agricultural by-product of rice straw was utilized as a substrate support for the fermentation process. The characterization of untreated and NaOH-treated rice straw was conducted using Scanning Electron Microscope (SEM) and Energy Dispersive X-ray Analysis (EDX). The optimization of α-amylase production was investigated under several influences including incubation time, incubation temperature and the additional carbon and nitrogen sources using a statistical analysis Central Composite Design (CCD) of Response Surface Methodology (RSM). The highest enzyme activity obtained was 345 U/g at 50 ºC with very limited additional glucose (0.02 g/g) and yeast extract (0.01 g/g).

Keywords Alpha-amylase, Optimization, Response Surface Methodology, Rice straw.

1.0 Introduction

Malaysia is one of the largest countries which produce paddy rice annually. From the production of this agricultural products, a plenty of derivatives in terms of rice husks, rice straw and ashes have been yielded as by-products. Rice straw is considered to account for the largest portion of available biomass feedstock in the world, i.e. 7.31 x 10^{14} kg of dry rice straw per year, and Asia contributes about 90 % of the annual global production [1]. Rice straw has been proven to provide a valuable source of carbon of about 70% [2] and used as significant solid substrate support for enzyme production [3].

Enzymes are among the largest production in biotechnology industries especially for food, pharmaceuticals, detergents and textile. Enzymes are very important compounds because they function mainly as catalysts in biological and chemical processes. One of the widely studied

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and produced enzymes is the alpha (α)-amylase. The α-amylase enzyme is very important in the field of biotechnology especially in the application of food industry, alcoholic compounds production, textile and paper industry [1]. Previous literatures reported that α-amylase was excellently produced through microbial fermentation for example by *Bacillus sp*. [4, 5, 6, 7 & 8], and *Aspergillus sp*. [9 & 10]. It is found that α-amylase can almost completely replace chemical hydrolysis of starch in the starch-processing industry [11].

Process optimization of variables for enzymes production seems to be very imperative in lab-scale research study due to the fact that the α-amylase is vigorously produced in industrial scale owing to its vast use in biotechnology industries. The most common use of statistical approach for optimization study is Taguchi and Response Surface Methodologies. These methods provide exceptional analysis approach for example factorial designs, analysis of variance and etc., easy-to-be-used and have high flexibility for the researcher in terms of data manipulation. The current study aims to optimize the effect of incubation time, incubation temperature, additional carbon and nitrogen sources on the production of α-amylase by *B. subtilis* using Response Surface Methodology.

2.0 Materials and Methods

2.1 Solid Substrate Preparation and Pre-treatment

This study used solid-state fermentation (SSF) process with rice straw as solid substrate support. Rice straw was collected from Kampung Tok Pulau, Perlis. The rice straw was soaked in distilled water and washed to remove soils attached to the rice straw. It was then dried at 45 - 50°C. The dried rice straw will be cut into small pieces of about 1 - 2 cm long using a pair of scissor.

The rice straw was soaked inside 2.0% (w/v) NaOH and heated at 86°C for 3 h. The treated rice straw was then filtered and washed with distilled water until no traces of acid or alkali could be detected and dried in an oven at 60°C for 2 days.

2.2 Microorganism

Bacillus subtilis used in this study was obtained from the Bioprocess Laboratory of the School of Bioprocess Engineering, UniMAP. The culture was maintained and sub-cultured in the nutrient agar and stored at 4ºC.

2.3 Characterization of Rice Straw

The surface structures of rice straw were characterized using Scanning Electron Microscope (SEM) before and after NaOH treatment. The untreated and treated rice straws were also

characterized using Energy Dispersive X-ray Analysis (EDX) to study the significant contents of silica and lignin.

2.4 Inoculum Preparation and Batch Experiment of SSF

The nutrient broth was prepared inside a 250-ml Erlenmeyer flask. The culture *B. subtilis* was inoculated in the nutrient broth for 8 hours of optimum growth. 10 mL of inoculated broth was centrifuged at 4000 rpm for 10 minutes. The cell pellet was re-suspended with 5 mL sterile distilled water and added to the 250-ml Erlenmeyer flasks containing 4 g pre-treated rice straw. 10 mL of fermentation media comprised of $MgSO_4$. $7H_2O$ (0.2 g/L), $CaCl_2$ (0.02 g/L), KH_2PO_4 (1.0 g/L), $NH_3H_2PO_4$ (1.0 g/L), NH_4NO_3 (1.0 g/L), $FeCl_3$ (0.05 g/L) and glucose, was evenly mixed with the rice straw and cells. The initial pH of fermentation media was maintained throughout the experiments at pH 7. Each experiment of SSF was carried out in duplicate sets.

2.5 Solid Substrate Moisture Content

The moisture content of the solid substrate was estimated by drying 4 g of solid substrate to a constant weight at 70 °C for 24 h and the dry weight was recorded. To fix the initial moisture content of the solid medium, 4 g rice straw was soaked with 5-mL inoculums and 10-mL fermentation media. After soaking, the solid substrate was again dried as described above and the percent moisture content was calculated using Eq. 1 as follows:

$$\text{Initial moisture content } (\%) = \frac{W_{final} - W_{initial}}{W_{initial}} \text{ x } 100\% \qquad \text{Eq. 1}$$

where W_{final} is the weight of the dried solid substrate after soaking and W_{initial} is the weight of dried solid substrate before soaking. From the above procedure, it was found that the initial moisture content of rice straw was about 20 %.

2.6 Effect of Incubation Time, Incubation Temperature, Additional Carbon and Nitrogen Sources.

The SSF experiment was carried out to determine the incubation time (day) required for the optimum α-amylase production. The flask containing the mixture of fermentation media, cells and solid substrate was incubated at 37°C. The α-amylase enzyme was extracted every day for 5 days. The optimum day of incubation was then applied to study the effect of incubation temperature, additional carbon and nitrogen sources.

The different incubation temperatures, additional carbon and nitrogen sources were the parameters to be optimized and their concentrations and range are listed in Table 1.

2.7 Enzyme Extraction

α-Amylase enzyme was extracted by mixing 50-mL of 0.1 M phosphate buffer (pH 7) with the whole solid substrate and shake on a rotary shaker at 250 rpm for 30 minutes. The buffer containing enzyme was separated from solid substrate through filter paper. The filtrate was centrifuged at 4000 rpm for 20 minutes. The clear brown supernatant was used as the enzyme source for the enzyme assay analysis.

Table 1 The parameters used for optimization studies

Parameters	Materials	Conditions
Effect of additional carbon sources	Glucose Xylose Fructose Sucrose Maltose	0.02 g/g dry substrate
Effect of additional nitrogen sources	Sodium nitrate Ammonium sulfate Yeast extract Urea	0.01 g/g dry substrate
Incubation temperature		35°C 45°C 55°C 65°C

2.8 α-Amylase Enzyme Assay

α-Amylase activity was determined by the procedure of Bernfeld using soluble starch as a substrate [12]. The reaction mixture containing 200 µL of 1% substrate (soluble starch) (w/v) in 300 µL 0.1 M phosphate buffer (pH 7) and 150 µL of enzyme solution was incubated at 37°C for 30 minutes. The reaction was stopped by adding 400 µL of 3,5-dinitrosalicylic (DNS) acid solution followed by heating in a boiling water bath for 5 min and cooling at room temperature. Then, distilled water was added until the solution volume was 12 mL. Absorbance of each solution was measured at 489 nm using a UV-Visible spectrophotometer. The initial reading was prepared by boiling the enzyme solution first in the hot water bath for 20 minutes to denature the enzyme protein structure.

The α-amylase enzyme activity (EA) calculation was based on the amount of glucose released from the degradation reaction of α-amylase enzyme on the substrate soluble starch as in the enzyme assay procedure. One Unit (U) of α-amylase activity was defined as the

amount of enzyme that releases 1 μmol of reducing sugars as glucose per minute, under assay conditions of pH 7 and incubation temperature of 37°C with phosphate buffer solution. The enzyme activity was expressed in U/g of solid substrate.

2.9 Optimization of Incubation Temperature, Additional Carbon and Nitrogen Sources

Response Surface Methodology (RSM) was used for determining the optimized independent variables including incubation temperature, additional carbon and nitrogen sources. The values and the level of the independent variables were determined by considering the optimized values taken from the previous SSF process. The optimization of the variables for the production of α-amylase by *B. subtilis* was conducted using the Central Composite Design (CCD). Variables with 3 centre points (low "-1", moderate "0", and high "+1") were used in CCD which gives the total of 20 experiments. The maximum values of activity of α-amylase were taken as the response of the design experiment. Statistical analysis of the model was performed using the analysis of variance (ANOVA).

3.0 Results and Discussion

3.1 Characterization of Rice Straw using SEM and EDX

Fig. 1 shows the untreated rice straw (a) and NaOH-treated rice straw (b). It can be observed that the surface of the untreated rice straw has a layer of substances mostly composed of lignin, silica, and other non-cellulosic substances on the outer surface [13]. Fibres in the rice straw are composed of a bundle of single cells held together by lignin and other binding materials [13]. From Fig. 1(b), the alkali NaOH-treatment eliminated most of the surface substances, which resulted a smoother morphological surface. In addition, the silica content of treated rice straw was reduced from 17 - 31 % to 0.7 - 1.3 % and the carbon content was increased by 35%.

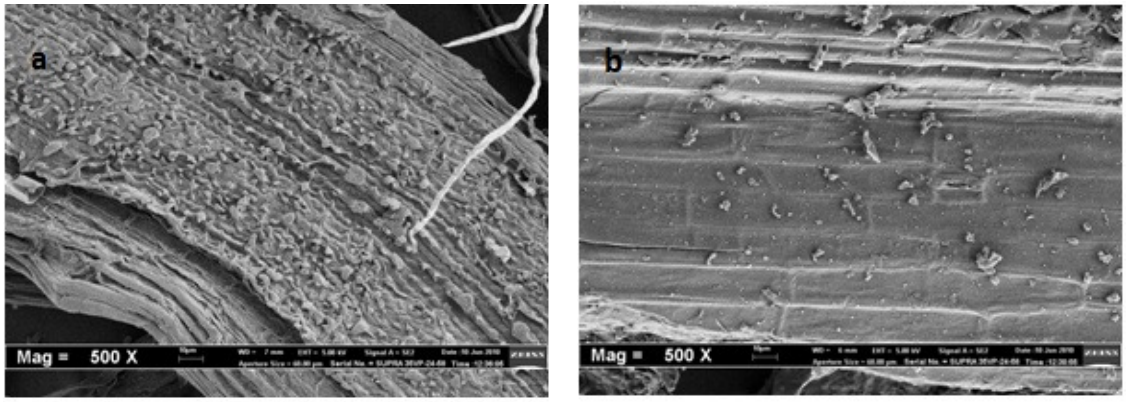

Fig. 1 SEM pictures of surface of untreated (a) and treated rice straw (b).

It could be suggested that the detachment of silica bodies, lignin and other substances on the surfaces of rice straw using NaOH pre-treatment might have better penetration of bacteria and increase the utilization of the fibers' nutrients by *B. subtilis*, especially the carbon contents as major source of the growth, thus yield higher production of α-amylase. Therefore, the treated rice straw was used to further study the effect of incubation time, temperature, additional carbon and nitrogen sources in optimizing the production of α-amylase.

3.2 Effect of Incubation Time

The time course for the production of α-amylase by *B. subtilis* in the SSF process using rice straw as the substrate is depicted in Fig. 2. α-Amylase activity increased during the growth phase of the culture and the optimum incubation time was reached after 48 h. α-Amylase production declined after 81 h and reached the minimum level after 120 h. A study using *B. cereus* produced amylase enzyme on wheat bran support reported that the maximum enzyme production occurred after the third day of incubation [5]. Other studies also suggested the highest production of α-amylase by several *Bacillus sp*. occurred after 2–4 days of incubation time [6, 7 & 14]. The lesser fermentation time (24 – 48 h) using *B. subtilis* to produce α-amylase will lead to significant reduction of processing cost and energy.

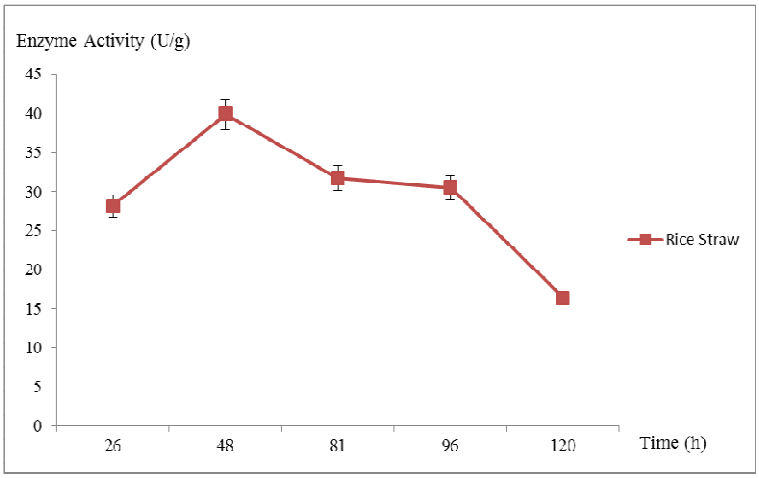

Fig. 2 Effect of incubation time for the maximum production of α-amylase

3.3 Effect of Incubation Temperature

The effect of incubation temperature for enzyme production could provide the information on whether the microorganism used is mesophilic or thermophilic. Several studies reported that most *Bacillus sp*. for example *B. amyloliquefaciens, B. subtilis, B. licheniformis* and *B. stearothermophilus* produced α-amylase at temperature range from 37 till 60°C [8, 15].

Fig. 3 shows the highest enzyme activity of 120 U/g occurred at 55°C. The enzyme activity dropped considerably at 65°C due to enzyme denaturation which led to degradation of its activity. This finding agrees with Anto [5] that the optimum temperature for the highest α-amylase activity on wheat bran was observed at 55°C. Further increase of temperature decreased the enzyme activity by 10 % [5].

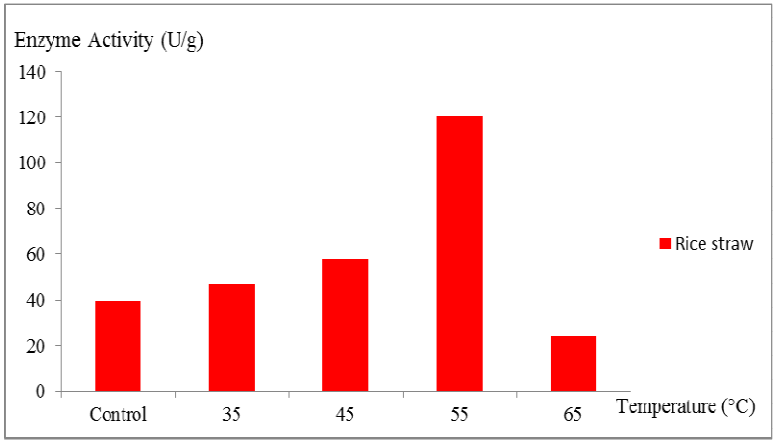

Fig. 3 Effect of incubation temperature for the maximum production of α-amylase

3.4 Effect of Additional Carbon Sources

Fig. 4 denotes the production of α-amylase on various additional carbon sources during the fermentation process. Among the five carbon sources added, glucose exhibited the highest enzyme activity which is 276 U/g. It was found that the supplementation of starch, glucose and peptone would lead to increasing enzyme synthesis in the fermentation process [14 & 16]. Glucose is a simple sugar (monosaccharide) which already degraded from complex sugars, has higher accessibility and suitability for the bacteria to utilize and subsequently produce larger amount of enzyme. It could be suggested that the enzyme production was growth associated and the presence of simple sugars such as glucose in the medium stimulated the increased production of α-amylase [16].

The additions of maltose, xylose, sucrose and fructose to the fermentation system have little effect in increasing the production of α-amylase. Maltose and sucrose are complex sugars which relatively more difficult to be utilized by *B. subtilis* and required longer time to be decomposed into simpler sugar. In addition, it was found that sucrose and lactose did not exert any effect on the *A. oryzae*'s activity of enzyme synthesis [16].

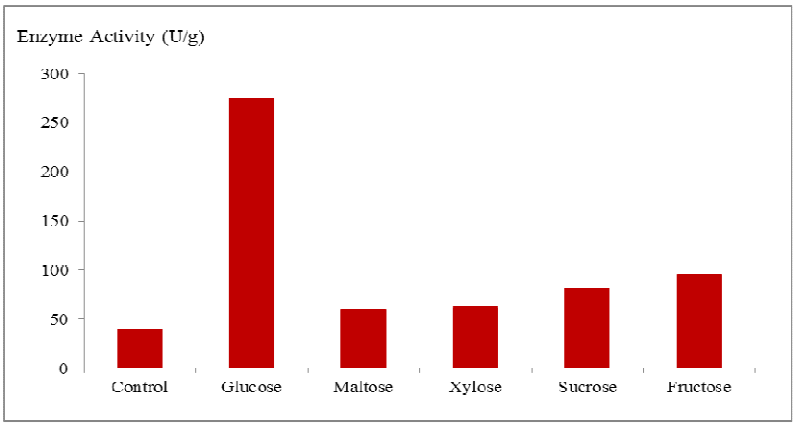

Fig. 4 Effect of additional carbon sources for the maximum production of α-amylase

From the results of the effect of additional carbon sources, the types of saccharide molecules which have 6 or 5 carbon atoms shows mix trends to the production of α-amylase. In other words, the utilization of carbon sources by *B. subtilis* did not give a significant meaning to the types of molecules whether they have 5 or 6 carbon atoms.

3.5 Effect of Additional Nitrogen Sources

As shown in Fig. 5, it can be observed that yeast extract affected the highest in the production of α-amylase, followed by urea. It was reported that yeast extract resulted in significant α-amylase yield by *Thermomyces lanuginosus* [17]. This result shows that the organic nitrogen sources i.e. yeast extract and urea are more preferable than inorganic nitrogen sources including ammonium sulfate and sodium nitrate.

Yeast extract as undefined media, contain high nutritional amino acids for instance, glutamic acid. Glutamic acid is found to be significant for the cellular metabolism and could provide sufficient energy for the better growth of *B. subtilis* [18]. Furthermore, yeast extract might contain enough and compatible nitrogen sources to support the growth of *B. subtilis*, in addition to other valuable nutrients which stimulate the enzyme activity. Urea also yielded high and comparable enzyme activity with yeast extract. This can be explained by the unique structure of urea itself. According to its chemical formula, urea is the combination of ammonia and carbon dioxide. The readily nitrogen source inside the ammonia which composes the urea was easily to be utilized and supplemented to *B. subtilis* thus induced greater amount of enzyme production.

The addition of ammonium sulfate showed a quite significant effect to the enzyme activity. The nitrogen source inside the ammonia which contains in the ammonium sulfate was readily and easily supplied to the growth of *B. subtilis* similar to that of urea. Pederson [9] reported

that the supplementation of yeast extract along with ammonium sulfate also gave significant enzyme productivity (110 %) by *A. oryzae*.

Among those four additional nitrogen sources, the addition of sodium nitrate to the rice straw substrate showed negative influence to the production of α-amylase as only half of the amount produced in the control flask. This could be proven by the finding of Ramachandran [16]. The depressing effect to the α-amylase production is because of the nitrate (NO_3^-) compound that is more difficult to be degraded compared to the ammonium (NH_4^+) salt. Nitrate needs to be degraded into a simpler compound of nitrite (NO_2^-) and subsequently into ammonium. As in ammonium sulfate, the nitrogen source is readily to be utilized by *B. subtilis*, thus the consumption of nitrogen sources in ammonium sulfate is faster and more effective than that in sodium nitrate.

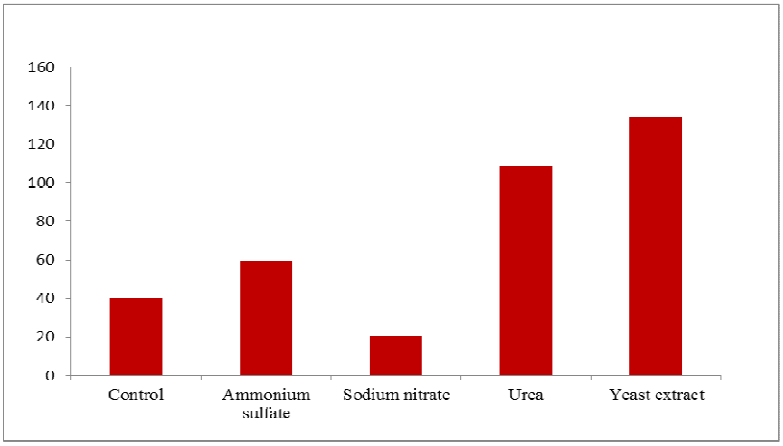

Fig. 5 Effect of additional nitrogen sources for the maximum production of α-amylase

3.6 Optimization using Response Surface Methodology (RSM)

The incubation temperature, i.e. 45, 50 and 55°C was selected since the enzyme activities were the highest in this temperature range. Glucose and yeast extract as the additional carbon and nitrogen sources, respectively, were chosen because both gave the highest enzyme activities among the sources studied.

3.6.1 Analysis by ANOVA

Coded factors A, B, and C represent temperature, glucose and yeast extract, respectively. Since the ratio of the maximum (385.98 U/g) to the minimum (42.82 U/g) enzyme activity in this design was 9.01 which is less than 10, therefore the transformation by using square root, inverse, natural log or others, is not required [20].

Table 2 shows the analysis of variance (partial sum of squares). The Model F-value of 9.62 implies the model is significant. There is only a 0.05 % chance that a Model F-Value could

occur due to noise. Values of Prob > F less than 0.0500 indicate model terms are significant. In this case, B and A2 are significant model terms since their Prob > F values are 4.39 and 0.95%, respectively.

Values greater than 0.1000 indicate the model terms are not significant. This shows that the glucose and incubation temperature have direct relationship with the production of α-amylase in SSF. On the other hand, the concentration of yeast extract may be the limiting nutrient in the design. If there are many insignificant model terms (not counting those required to support hierarchy), model reduction may improve the model. The Lack of Fit F-value of 4.75 implies the Lack of Fit is not significant. There is a 5.42% chance that a "Lack of Fit F-value" this large could occur due to noise. The non-significant "Lack of Fit F-value" of 5.42% showed that the quadratic model is valid and adequate for optimizing the parameters for obtaining the optimum α-amylase production [19].

Table 2 Analysis of Variance (ANOVA) depicted from CCD

Response: Enzyme activity

ANOVA for Response Surface Reduced Quadratic Model

Analysis of variance table [Partial sum of squares]

Source	Sum of Squares	DF	Mean Square	F Value	Prob > F	
Model	2.129E+005	8	26617.08	9.62	0.0005	significant
A	*69.64*	*1*	*69.64*	*0.025*	*0.8768*	
B	*14314.87*	*1*	*14314.87*	*5.17*	*0.0439*	
C	*6631.66*	*1*	*6631.66*	*2.40*	*0.1498*	
A^2	*27186.40*	*1*	*27186.40*	*9.83*	*0.0095*	
B^2	*11765.13*	*1*	*11765.13*	*4.25*	*0.0636*	
C^2	*9557.56*	*1*	*9557.56*	*3.45*	*0.0900*	
AB	*2753.56*	*1*	*2753.56*	*1.00*	*0.3399*	
AC	*3642.31*	*1*	*3642.31*	*1.32*	*0.2756*	
Residual	30433.27	11	2766.66			
Lack of Fit	*25889.36*	*6*	*4314.89*	*4.75*	*0.0542*	*not significant*
Pure Error	*4543.91*	*5*	*908.78*			
Cor Total	2.434E+005	19				

Std. Dev.	52.60	R-Squared	0.8750
Mean	209.09	Adj R-Squared	0.7840
C.V.	25.16	Pred R-Squared	0.5858
PRESS	1.008E+005	Adeq Precision	8.298

The Squared regression cerrelation coefficient, R^2 is 0.8750 showing that the model presented relatively a high determination coefficient which explains 87.5% of the variability in response [19]. The predicted R^2 of 0.5858 is in reasonable agreement with the adjusted R^2 of 0.7840 since the difference between both R^2 is less than 0.200. Adequate Precision measures the signal to noise ratio. A ratio greater than 4 is desirable. The ratio of 8.298 indicates an adequate signal. Therefore, this model is reliable in optimizing the chosen process variables for α-amylase production.

In analysing the effect of variables, normally the 3-D contour plots (Fig. 6 and Fig. 7) were used. 3-D contour plots represent the relationship of response surface function of two variables; meanwhile another variable is maintained at zero level [20]. The coordinates of the central point within the highest contour levels in these figures represent the optimum condition and concentrations of respective parameters [21].

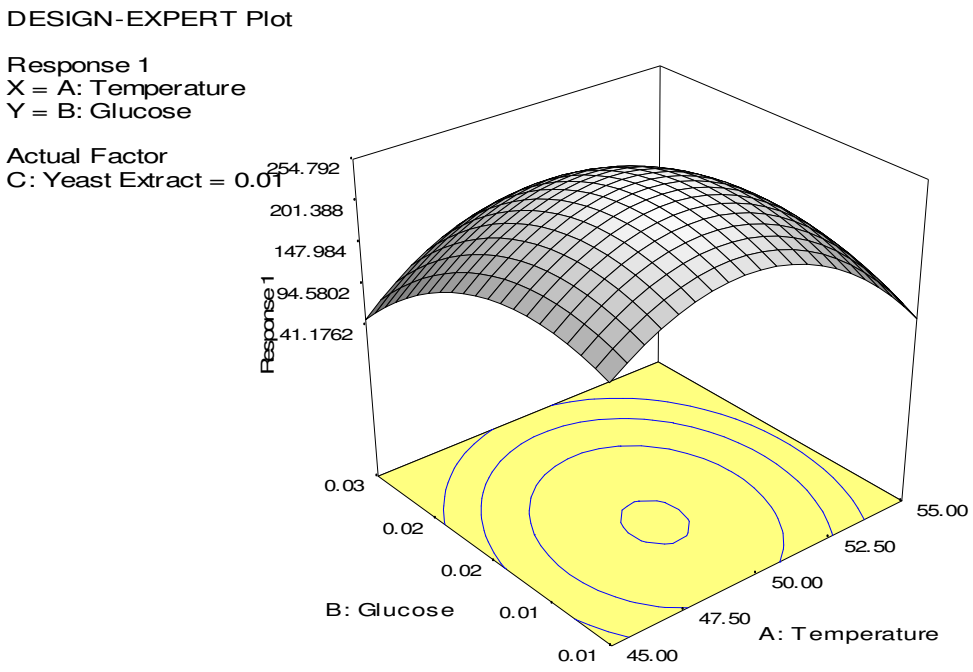

Fig. 6 Response surface plot showing the effect of glucose concentration and incubation temperature

From Fig. 6, it can be observed that the enzyme activity was increased upon the temperature at the range of 47.5 to 50°C. The optimum glucose concentration was about 0.015 g/g substrate. Any further increases in temperature and glucose concentration would lead to the decrease in enzyme activity. This result proved the findings of Swain [22] where the optimum temperature of producing α-amylase by *B. subtilis* on cassava fibrous residue lied at

44 – 50°C. In the degree of significance between the incubation temperature and glucose, the incubation temperature seems to be more significant than glucose in enhancing the α-amylase activity as the central contour points are heading toward temperature level.

The optimization of the α-amylase activity was also improved by the interconnection between the temperature and yeast extract. As in the result shown by Fig. 7, the optimum temperature is still at 50°C. The optimum concentration of yeast extract is at 0.01 – 0.0125 g/g. Further increase in concentration of yeast extract would decrease the enzyme secretion. Tanyildizi [20] reported that increasing the yeast extract concentration from 0 to 2 g/L had resulted in the increasing enzyme activity. However, at higher concentration, yeast extract may inhibit the enzyme synthesis. It was found that the supplementation of yeast extract in optimizing the α-amylase enzyme by *B. circulans* yielded very high value of the probability of the linear effect coefficient [23]. However, the additional of yeast extract in increasing the α-amylase activity was not entirely overruled as its interaction with other parameters did affect the enzyme activity.

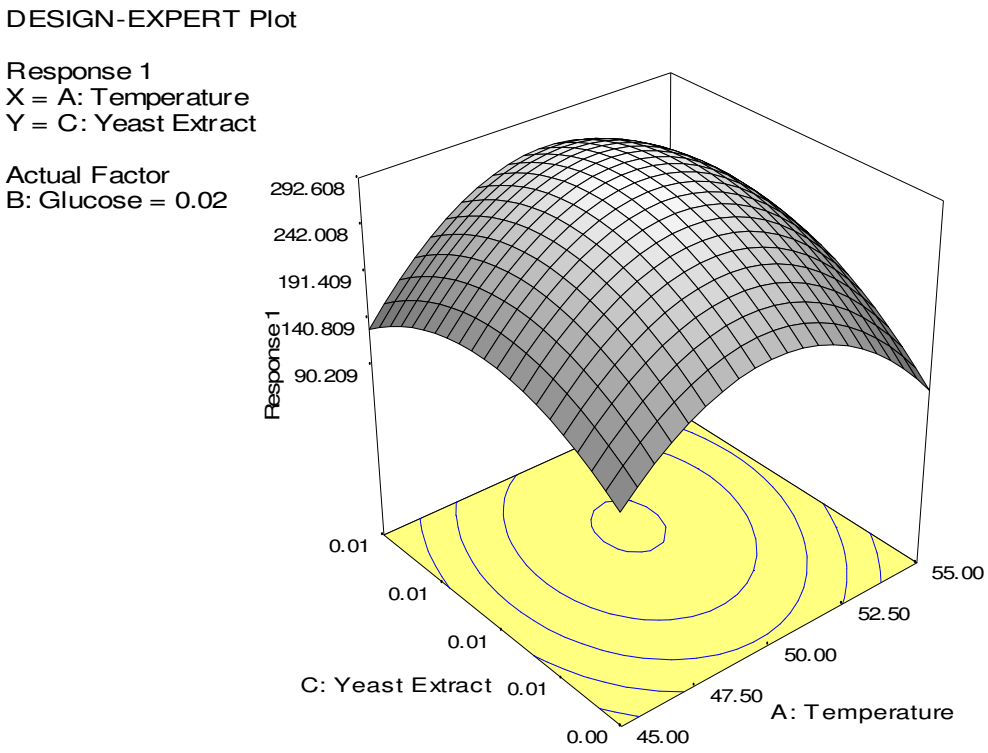

Fig. 7 Response surface plot showing the effect of yeast extract concentration and incubation temperature

It can be said that the ranges of temperature, glucose and yeast extract in optimizing the α-amylase production are 47.5 – 50°C, 0.015 – 0.02 g/g and 0.01 – 0.0125 g/g, respectively. For those three effects, further increase in temperature or concentrations would decrease the enzyme production. This happened due to the system of solid state fermentation which is related to water content in the solid medium. When the water quantity was not enough for the growth of *B. subtilis*, the diffusion of solutes and gas throughout the medium was hindered. This condition will slow down the cell metabolism due to the lack of substrates or through too high concentration of inhibitive metabolites in or near the cells [24, 25]. Therefore, decrease or further increase in concentration of both nutrients may lead to side metabolites production which inhibits the enzyme activity.

Table 3 shows the solution for the optimization of α-amylase production depicted from RSM. By setting the temperature, additional glucose and yeast extract concentrations in range, and the response which is the enzyme activity in maximum yield, the values of variables are 49.92 °C, 0.02 g/g and 0.01 g/g respectively. The optimized enzyme activity predicted from varying these parameters is 329.3 U/g. The desirability of 83.5 % which is close to 100 % shows that the value of response is highly favourable and tendency in obtaining the respective value of enzyme activity by using those variables is high.

Table 3 Solution for optimizing α-amylase production

Name	Goal	Lower Limit	Upper Limit	Lower Weight	Upper Weight	Importance
Temperature	is in range	45	55	1	1	3
Glucose	is in range	0.01	0.03	1	1	3
Yeast Extract	is in range	0.005	0.015	1	1	3
Response 1	maximize	42.82	385.98	1	1	3

Solutions

Number	Temperature	Glucose	Yeast Extract	Response 1	Desirability	
1	49.92	0.02	0.01	329.299	0.835	Selected

To validate the predicted optimized conditions obtained from RSM, a triplicate SSF experiments were carried out by using those values of variables including 49.92°C (≈ 49.9°C) for incubation temperature, 0.02 g/g for additional glucose and 0.01 g/g for additional yeast extract. From the result tabulated in Table 4, it was proven that the predicted values from

RSM can be used in optimizing the α-amylase production by *B. subtilis* on rice straw solid substrates as the experimental values obtained are close to those predicted.

Table 4 The validation of α-amylase production using predicted optimized conditions i.e. incubation temperature (49.92°C); additional glucose (0.02 g/g) and additional yeast extract (0.01 g/g)

Run	Enzyme activity (U/g) (**Experimental**)	Enzyme activity (U/g) (**Predicted**)	Percentage difference (%)
1	339.1	329.30	2.98
2	303.9	329.30	7.71
3	344.9	329.30	4.74

4.0 Conclusions

The production of α-amylase by *B. subtilis* has been examined by using rice straw in solid state fermentation process. The highest α-amylase enzyme activity of 39.9 U/g was produced after 48 h of incubation time. For the results of incubation temperature, rice straw yielded 120 U/g at 55°C. The additional glucose gave the highest enzyme activity of 275.7 U/g among other carbon sources including maltose, xylose, sucrose and fructose. In studying the effect of additional nitrogen sources, yeast extract was observed to yield the highest enzyme activity of 134.3 among other nitrogen sources including urea, ammonium sulfate, and sodium nitrate. RSM was applied in optimizing the parameters of incubation temperature (45, 50 and 55°C), additional glucose concentrations (0.01, 0.02, 0.03 g/g substrate) and additional yeast extract concentrations (0.005, 0.01, 0.015 g/g substrate). The reduced quadratic model gave a significant response to the variables by having Prob > F value of 0.05 % and R^2 of 0.8750. The interactions of temperature-glucose and temperature-yeast extract were observed to be significant model terms which ended up of final optimized parameters of incubation temperature of 49.92°C; glucose 0.02 g/g and yeast extract 0.01 g/g with optimum enzyme activity of 385.98 U/g.

References

[1] Kim, S. and Dale, B.E. (2004). Global potential bioethanol production from wasted crops and crop residues. *Biomass and Bioenergy*, 26, 361–375.

[2] Reddy, N. and Yang, Y. (2005). Biofibers from agricultural byproducts for industrial applications. *Trends in Biotechnology*, 23(1), 22-27.

[3] Gangadharan, D., Sivaramakrishnan, S., Nampoothiri, K.M. and Pandey, A. (2006). Solid Culturing of *Bacillus amyloliquefaciens* for Alpha Amylase Production. *Food Technology and Biotechnology*, 44, 269–274.

[4] Rajagopalan, G. and Krishnan, C. (2009). Optimization of agro-residual medium for α-amylase production from a hyper-producing *Bacillus subtilis* KCC103 in submerged fermentation. *Journal of Chemical Technology and Biotechnology*, 84, 618-625.

[5] Anto, H., Trivedi, U. and Patel, K. (2006). Alpha amylase production by *Bacillus cereus* MTCC 1305 using solid-state fermentation. *Food Technology Biotechnol*ogy, 44 (2), 241-245.

[6] Ikram-ul-Haq, Ashraf, H., Iqbal J. and Qadeer, M.A. (2003). Production of alpha amylase by *Bacillus licheniformis* using an economical medium. *Bioresource Technology*, 87, 57–61.

[7] Baysal, Z., Uyar, F. and Aytekin, C. (2003). Solid state fermentation for production of α-amylase by a thermotolerant *Bacillus subtilis* from hot-spring water. *Process Biochemistry*, 38, 1665- 1668.

[8] Syu, M.J. and Chen, Y.H. (1997). A study on the α-amylase fermentation performed by *Bacillus amyloliquefaciens*. *Chemical Engineering Journal*, 65, 237-247.

[9] Pederson, H. and Nielson, J. (2000). The influence of nitrogen sources on the α-amylase productivity of *Aspergillus oryzae* in continuous cultures. *Applied Microbiology and Biotechnology*, 53, 278-281.

[10] Sivaramakrishnan, S., Gangadharan, D., Nampoothiri K.M., Carlos Ricardo Soccol, C. R. and Pandey, A. (2006). α-Amylases from Microbial Sources – An Overview on Recent Developments. *Food Technology and Biotechnology*, 44 (2), 173–184.

[11] Pandey, A., Nigam, P., Soccol, V.T., Singh, D. and Mohan, R. (2000). Advances in microbial amylases. *Biotechnology and Applied Biochemistry*, **31**, 135-152.

[12] Bernfeld, P. (1955). *Amylases, α and β. In: Methods in enzymology*. New York: Academic Press.

[13] Reddy, N. and Yang, Y. (2006). Properties of High-Quality Long Natural Cellulose Fibers from Rice Straw. *Journal of Agricultural and Food Chemistry*, 54, 8077-8081.

[14] Ramesh, M.V. and Lonsane, B.K. (1991). *Biotechnology Letter*, 13, 355.

[15] Mishra, S., Noronha, S.B., and Suraishkumar, G.K. (2005). Increase in enzyme productivity by induced oxidative stress in *Bacillus subtilis* cultures and analysis of its mechanism using microarray data. *Process Biochemistry*, 40, 1863-1870.

[16] Ramachandran, S., Patel, A.K., Nampoothiri, K.M., Chandran, S., Szakacs, G., Soccol, C. R. and Pandey, A. (2004). Alpha amylase from a fungal culture grown on oil cakes and its properties. *Brazilian Archives of Biology and Technology*, 47, 309-317.

[17] Nguyen, Q.D., Rezessy-Szabo, J.M. and Hoschke, A. (2000). Optimisation of Composition of media for the production of amylolytic enzymes by *Thermomyces lanuginosus* ATCC 34626. *Food Technology and Biotechnology*, 38, 229-234.

[18] Tarek, El Banna, A. Abd Aziz, M.I Abou-Dobara, and Reham I. Ibrahim. (2007). Production and Immobilization of α-amylase from *Bacillus subtilis*. *Pakistan Journal of Biological Sciences*, 10(12), 2039-2047.

[19] Montgomery, D.C. (1997). *Design and Analysis of Experiments*. New York: John wiley & Sons Inc.

[20] Tanyildizi, M.S., Selen, V. and Ozer, D. (2009). Optimization of α-amylase production in solid substrate fermentation. *The Canadian Journal of Chemical Engineering*, 87, 493-498.

[21] Tanyildizi, M. S., Ozer, D. and Elibol, M. (2005). Optimization of α-Amylase production by *Bacillus* sp. Using Response Surface Methodology. *Process Biochemistry*, 40, 2291-2296.

[22] Swain, M.R. and Ray, R.C. (2007). Alpha-amylase production by *Bacillus subtilis* CM3 in solid state fermentation using cassava fibrous residue. *Journal of Basic Microbiology*, 47, 417–425.

[23] Dey, G., Mitra, A., Banerjee, R. and Maiti, B.R. (2001). Enhanced production of amylase by optimization of nutritional constituents using response surface methodology. *Biochemical Engineering Journal*, 7, 227-231.

[24] Gervais, P. and Molin, P. (2003). The role of water in solid state fermentation. *Biochemical Engineering Journal*, 13, 85-101.

[25] Tanyildizi, M.S., Ozer, D. and Elibol, M. (2007). Production of bacterial α-Amylase production by *B. amyloliquefaciens* under solid substrate fermentation. *Biochemical Engineering Journal*, 37 (3), 294-297.