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Optimization of Media Composition for α-amylase Production in Liquid State Fermentation of Bitter Cassava by *Aspergillus niger*

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A number of media components influencing α-amylase production by *Aspergillus niger O103A* were studied using local bitter variety of cassava (*Manihotes cuelenta*) as the main substrate. The media components (cassava, maltose, glucose, yeast extract, urea, NH_4NO_3, KH_2PO_4, $CaCl_2$, $MgSO_4.7H_2O$, NaCl, and $FeSO_4.7H_2O$) were analyzed using Plackett–Burman design. Optimal concentrations of the components contributing positively to enzyme production were further determined using one factor at a time (OFAT) and response surface methodology (RSM) method. Among different nitrogen and supplementary carbon sources tested, yeast extract 2 g/l and maltose 3 g/L were most effective for maximum production of enzyme (49 U/ml). The results show a favorable α-amylase production by applying statistical approach in optimization process.

Key words: α-amylase; response surface methodology; Bitter cassava; *Aspergillus niger*; Submerge fermentation.

The hydrolytic enzyme, α-amylases (EC 3.2.1.1) are extracellular enzymes that randomly cleave the 1,4-α-D-glucosidic linkages inside the polysaccharide chain[1,2]. These enzymes account for 65% of enzyme market in the world with microorganisms being the predominant sources for enzyme production. Fungal and bacterial amylases have been widely obtained from fermentation processes[3]. Due to their acceptable GRAS (Generally Recognized As Safe) status, enzyme production diversity and high yields of enzymes produced; filamentous fungi like *Aspergillus niger* is considerd one of the most used fungal strains in enzyme industry[4].

Enzyme production on industrial level is still faced by an obstacle represented by high production costs, hence, the search is always on for means to decrease the costs incurred by enzyme production processes. Utilization of agro-industrial residues like bitter cassava as a substrate for α-amylase production is economically convenient and will help in reducing production cost since it is abundant, cheap, not competing as food and environmentally friendly (represents a zero waste technology where cassava waste also can be utilized for ethanol production due to its high content of cellulose, hemicelluloses and starch[5,6]. In this study, an optimized α-amylase production from *Aspergillus niger* O103A using bitter cassava as main substrate was investigated.

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MATERIALS AND METHODS

Sample collection and preparation

Bitter cassava (tapioca-root) samples were collected from local farm in Kuantan, Pahang, Malaysia and immediately brought to the laboratorywashed, peeled, cut into smaller pieces, dried and ground into flour for use in fermentation.

Microorganism and inoculum preparation

Aspergillus niger O103A was obtained from Bioenvironmental Engineering Lab stock, International Islamic University Malaysia. The stock culture was maintained and subcultured on potato dextrose agar. Seven-day PDA plate of *Aspergillus niger* O103A was used to prepare spore suspension (10^6 spore/ml) as inoculum according to the method described by Alam *et al.*,[7].

Screening of important media components

Plackett–Burman (PB) design was used to screen medium constituents that influence α-amylase production. Experimental design matrix was generated using Design Expert 6.0.8 statistical software, each variable was examined at two levels; low level (–1) and high level (+1). The constituents studied include (Cassava, glucose and maltose) as carbon sources, (yeast extract, NH_4NO_3 and urea) as nitrogen sources and (KH_2PO_4, $CaCl_2$, $MgSO_4$, $FeSO_4$ and NaCl) as inorganic mineral sources. All experiments were carried out in triplicate and the averages of α-amylase activity were taken as response.

One factor at a time (OFAT) optimization

Optimization of various parameters was carried out using one factor at a time strategy (OFAT). The most influencing variables in PB design based on the statistical analysis were selected for the OFAT method and further design with the varied ranges of parameters for optimization. Fermentation trials were conducted for each variable with keeping the other positive parameters from the PB design fixed at constant values (the same positive values used in PB design).

Response surface methodology (RSM)

Based on the acquired results from Plackett-Burman design and OFAT experiments, face centered central composite design (FCCCD) under response surface methodology (RSM) was used to determine the optimal concentrations of two significant medium constituents (maltose and yeast extract).

Fermentation medium preparation and α-amylase production

α-amylase production medium was prepared according to the statistical design of experiments. The initial pH was adjusted to pH 6 and autoclaved at 121°C and 15 psi for 15 min. (1 % v/v) of the prepared inoculum was added to 100 ml medium in 250 ml Erlenmeyer flasks. The flasks were incubated for 3 days at 28°C and 150 rpm agitation. After the incubation, the culture broth was filtered using whatman filter paper no.1 and the cell-free filtrate was used as a source of extracellular α-amylase.

Colorimetric α-amylase enzyme Assay

Enzyme activity for α-amylase was determined by DNS method[8] described by Gupta *et al.*,[9] using soluble starch as the substrate. Assay were conducted in triplicate and one unit of enzyme activity was defined as the amount of enzyme required to release one µmol of reducing sugar from 1% soluble starch under assay conditions per minute.

RESULTS AND DISCUSSION

Screening of medium constituents using Plackett-Burman design

Results obtained from statistical screening with Plackett-Burman design (Table 1) showed that glucose and sodium chloride (NaCl) affected the response at a negative level (Fig. 1). As shown in Table 1, production of α-amylase varied from 0.84 to 35 U/ml indicating a strong influence of medium components on the production of the enzyme. The highest production of α-amylase (35 U/ml) was achieved in Run 5, where medium components (cassava, maltose, yeast extract, urea, $MgSO_4$ and KH_2PO_4) were at their highest level, while runs 1 and 3 showed the lowest α-amylase activity of 0.84 and 1 U/ml respectively, This could be related to the absence of nitrogen sources in media used for both runs.

Analysis of main effects of medium components on α-amylase production is shown in Fig. 1. Results obtained through analysis of main effects showed that out of 11 media components screened with PB design only glucose and NaCl affected the response at a negative level. The negative effect of glucose on amylases production comes in agreement with previous studies where

Table 1. Screening of eleven medium components using Plackett-Burman design with actual and coded values for α-amylase production by *Aspergillus niger* O103A

Run	Cassava (g/L)	Maltose (g/L)	Glucose (g/L)	Y. extract (g/L)	Urea(g/L)	NH4NO3 (g/L)	$MgSO_4$ (g/L)	NaCl (g/L)	$CaCl_2$ (g/L)	$FeSO_4$ (g/L)	KH_2PO_4 (g/L)	α-amylase (U/ml)
1	10 (-1)	0 (-1)	0 (-1)	0 (-1)	0 (-1)	0 (-1)	0.00 (-1)	0.0 (-1)	0.0 (-1)	0.0 (-1)	0 (-1)	0.84
2	10 (-1)	2 (+1)	2 (+1)	1 (+1)	0 (-1)	1 (+1)	0.05 (+1)	0.0 (-1)	0.5 (+1)	0.0 (-1)	0 (-1)	32
3	30 (+1)	0 (-1)	2 (+1)	0 (-1)	0 (-1)	0 (-1)	0.05 (+1)	0.5 (+1)	0.5 (+1)	0.0 (-1)	1 (+1)	1.0
4	30 (+1)	2 (+1)	0 (-1)	1 (+1)	0 (-1)	0 (-1)	0.00 (-1)	0.5 (+1)	0.5 (+1)	0.5 (+1)	0 (-1)	26.5
5	30 (+1)	2 (+1)	0 (-1)	1 (+1)	1 (+1)	0 (-1)	0.05 (+1)	0.0 (-1)	0.0 (-1)	0.0 (-1)	1 (+1)	35
6	10 (-1)	0 (-1)	0 (-1)	1 (+1)	1 (+1)	1 (+1)	0.00 (-1)	0.5 (+1)	0.5 (+1)	0.0 (-1)	1 (+1)	25
7	30 (+1)	0 (-1)	2 (+1)	1 (+1)	0 (-1)	1 (+1)	0.00 (-1)	0.0 (-1)	0.0 (-1)	0.5 (+1)	1 (+1)	33.5
8	30 (+1)	0 (-1)	0 (-1)	0 (-1)	1 (+1)	1 (+1)	0.05 (+1)	0.0 (-1)	0.5 (+1)	0.5 (+1)	0 (-1)	33.5
9	30 (+1)	2 (+1)	2 (+1)	0 (-1)	1 (+1)	1 (+1)	0.00 (-1)	0.5 (+1)	0.0 (-1)	0.0 (-1)	0 (-1)	4.5
10	10 (-1)	2 (+1)	0 (-1)	0 (-1)	0 (-1)	1 (+1)	0.05 (+1)	0.5 (+1)	0.0 (-1)	0.5 (+1)	1 (+1)	25
11	10 (-1)	2 (+1)	2 (+1)	0 (-1)	1 (+1)	0 (-1)	0.00 (-1)	0.0 (-1)	0.5 (+1)	0.5 (+1)	1 (+1)	21.5
12	10 (-1)	0 (+1)	2 (+1)	1 (+1)	1 (+1)	0 (-1)	0.05 (+1)	0.5 (+1)	0.0 (-1)	0.5 (+1)	0 (-1)	25.5

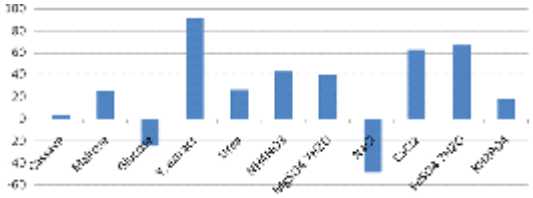

Fig. 1.Main effects of the medium constituents on *A. Niger O103A* α-Amylase production by the Plackett–Burman experimental results

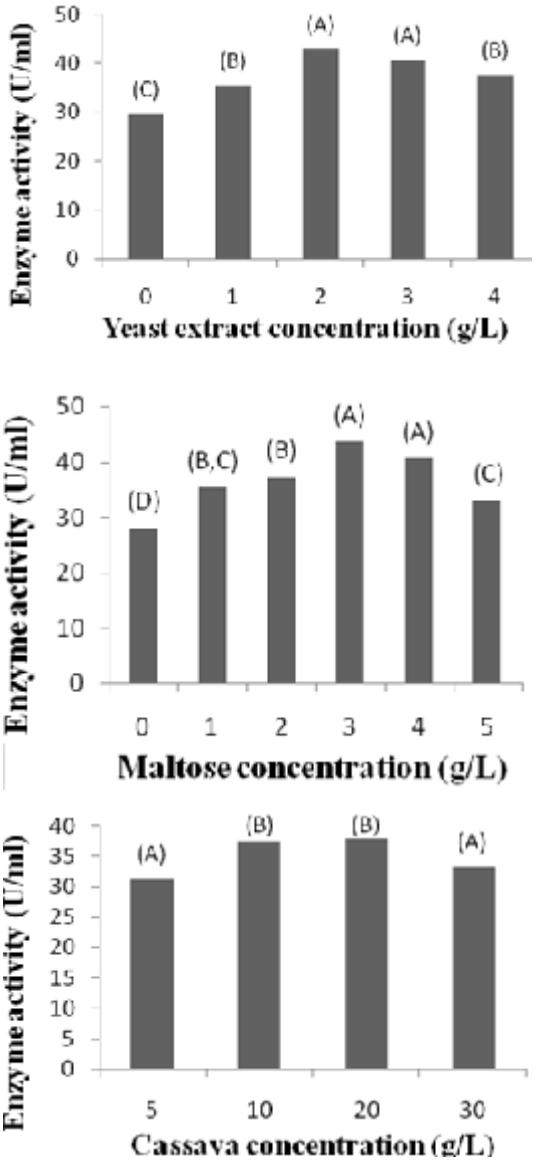

Fig. 2. Effect of different concentrations of yeast extract (A), maltose (B) and cassava (C) on α-amylase activity. Mean values sharing a letter are significantly indifferent based on ANOVA tukey test ($p \leq 0.05$)

glucose was found to be of inhibitory effect on amylolytic enzymes production by *Aspergillus* species due to catabolite repression[10]. The negative effect of glucose on á-amylase production was also reported by Suganthi et al[11] and Monga et al[12]. Also, a similar observation that Sodium has an inhibitory effect on amylase production was reported by Reyed[13]and Varalakshmi *et al.*,[14].

From Figure 1, it can be observed that medium constituents had different effects on α-amylase production by *Aspergillus niger* O103A. Among carbon sources, maltose as supplementary carbon source showed to have a higher positive effect on α-amylase production . Also, it was observed that complex organic nitrogen sources like yeast extract showed the highest positive effect on α-amylase production compared to the use of simple nitrogen sources like urea and ammonium nitrate (NH_4NO_3).

Determination of optimum levels of medium components using One-Factor-at-a-Time (OFAT) method

One factor at a time optimization (OFAT) was conducted to determine the possible optimum levels for other parameters that affected the response at a positive level; namely (maltose, yeast extract, urea, ammonium nitrate and the other inorganic salts mentioned previously). Using (OFAT) method; optimum concentration of cassava was found to be (10 g/L) and among all variables only maltose and yeast extract had a significant effect on α-amylase production. The optimum concentrations of maltose and yeast extract were 3 g/L and 2 g/L respectively (Fig. 2)., it was observed that organic nitrogen sources like yeast extract (2 g/L) supported maximum production of enzyme compared to the use of inorganic sources like urea and ammonium nitrate (NH_4NO_3) alone. Similar to the findings of this study, Mohamed, *et al*.,[15] reported yeast extract concentration of 2 g/L was also reported to be optimum in supporting maximum amylase activity by *Candida famata.* Furthermore, Baysal, *et al.*,[16] observed that variation in yeast extract concentration in the medium led to variation in α-amylase productivity by *Bacillus subtilis*, and that increasing the concentration of yeast extaract above 1.5 % w/v inhibited enzyme production significantly. Since cassava is poor in protein content[17] any supplementation with nitrogen sources would be expected to enhance both growth of *Aspergillusniger* and production of amylases. The effect of variation in mineral salts concentration led to a minimal increase in á-amylase production (data not shown). Similar to our findings, Varalakshmi, et al[14] reported that 1% maltose as a carbon source significantly increased á-amylase production. In another study to produce amylase from *Aspergillus awamori* using wheat bran as the main carbon source, it was observed that addition of maltose (1.5 g) as supplementary carbon source into the fermentation medium allowed 48% higher production. however, further addition of maltose reduced the activity of glucoamylase[18].

Based on OFAT experiments, yeast extract and maltose was found to be the most contributing, as such they were chosen for further optimization using response surface methodology (RSM).

Optimization of medium components by response surface methodology

Based on the acquired results from Plackett-Burman design and OFAT experiments, face centered central composite design (FCCCD) under response surface methodology (RSM) was used to determine the optimal concentrations of two significant medium constituents (maltose and yeast extract). For each run, the experimental results along with the predicted α-amylase activity obtained from the regression equation for the 13 combinations are shown in Table 2. The results showed that highest production of α-amylase (47.2 to 49.5 U/ml) by *Aspergillus niger* O103A was noticed in the center points (runs 3, 5, 6, 7 and 9) while the lowest production of α-amylase produced was noticed in run 2 (34.3 U/ml) where maltose were at high concentration and yeast extract were at low concentration This indicates that α-amylase and production was further improved by the statistical design matrix.

A second order regression equation illustrated the reliance of enzyme production on concentration of maltose and yeast extract in the medium. The parameters of the equation have been obtained by multiple regression analysis of the experimental data. An empirical relationship between the response and the screened variables was expressed in terms of second order polynomial equations:

Y (α-amylase activity, U/ml) = -46.54 + 51.86A +

Table 2. Experimental design using FCCCD of two independent variables with their actual and coded values and six center points showing the experimental and predicted response

Run	Maltose (g/L)	Yeast extract (g/L)	α-amylase activity (U/ml)	
			Experimental	Predicted
1	3 (0)	1 (-1)	42	43.09
2	4 (+1)	1 (-1)	34.3	33.55
3	3 (0)	2 (0)	49	48.14
4	3 (0)	3 (+1)	45	45.49
5	3 (0)	2 (0)	47.2	48.14
6	3 (0)	2 (0)	48.4	48.14
7	3 (0)	2 (0)	49.5	48.14
8	4 (+1)	3 (+1)	36	35.55
9	3 (0)	2 (0)	48.2	48.14
10	2 (-1)	1 (-1)	35.5	35.15
11	2 (-1)	2 (0)	40	40.39
12	2 (-1)	3 (+1)	38	37.95
13	4 (+1)	2 (0)	37.2	38.39

Table 3. Analysis of variance for quadratic model for α-amylase production

Source	Sum of squares	F-value	*p*-value
Model	392.3856	73.61764	< 0.0001
Maltose, A	6	5.628466	0.0494
Yeast extract, B	8.64	8.104991	0.0248
A^2	211.2084	198.1299	< 0.0001
B^2	40.82841	38.30022	0.0005
AB	0.16	0.150092	0.7100
Lack of Fit	4.430069	1.948139	0.2638

$$17.18B - 8.74A^2 - 3.84B^2 - 0.20AB \quad ...(1)$$

Where the response (Y) represents enzyme activity while A and B represents the concentrations of Maltose and Yeast extract respectively.

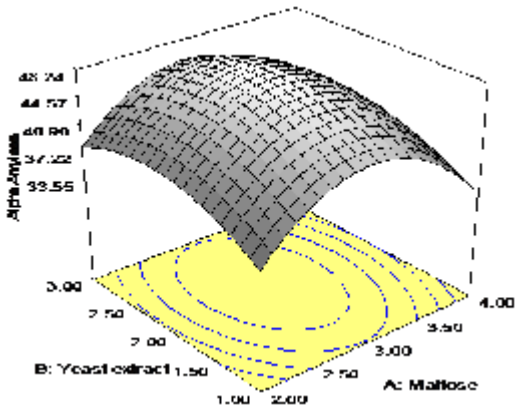

Fig. 3. 3D response surface curves of the combined effects of maltose and yeast extract on production of α-amylase by *Aspergillus niger* O103A

Analysis of variance (ANOVA) was used to inspect the adequacy of the model, the results are shown in Table 3, the F value of 73.61 and p-value of <0.0001 revealed that the model is significant, suggesting that there is only 0.01% that the model F value could occur due to noise. The interactive pattern between maltose and yeast extract and their effect on α-amylase production is represented in Figure 3. Optimized composition of medium components exhibited a collaborative effect on enzyme production. It can be concluded that a well-defined optimum production for α-amylase was achieved at the center points for maltose (3 g/L) and yeast extract (2 g/L) while higher and lower levels of maltose and yeast extract led to lower enzyme production. The 3D plot shown in Figure 2 is based on the function of concentrations of the two variables; maltose (A) and yeast extract (B). Significance of the interactions between the corresponding variables is indicated by an elliptical or saddle nature of the contour plots[19].

CONCLUSION

Based on the above findings it can be concluded that cassava can be a good substrate for the production of α-amylase and can be helpful in reducing the production cost of the enzyme. Maximum production of α-amylase was 49 U/ml achieved by optimizing medium constituents alone. A statistically design experiment is an efficient technique that can save time and material during batch culture studies. The use Plackett-Burman design and RSM allowed a rapid screening of large experimental domains in search of optimal fermentation conditions and helped revealing the influence of concentrations of medium components on enzyme productivity. This research provides a detailed study that used statistical analysis to determine the optimal levels and interaction of different medium components on α-amylase production by *Aspergillus niger* O103A.

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