

ORIGINAL ARTICLES

Optimization of complex fermentation media for glucose oxidase production using statistical approach

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ABSTRACT

Production cost of enzyme is largely determined by the type of the strain and raw material used to propagate the strain. Hence, selection of the strain and raw materials is crucial in enzyme production. For Glucose oxidase (GOx), previous studies show *Aspergillus terreus* UniMAP AA-1 offers a better alternative as compared to the existing glucose oxidase sources. Thus, a lower production cost could be logically anticipated by growing the strain in a cheaper locally complex media such as molasses. In this work, sugar cane molasses, supplemented with urea and carbonate salt, and a new locally isolated strain *Aspergillus terreus* UniMAP AA-1 were used to produce a crude GOx enzyme in small scale. A statistical optimization approach namely Response Surface Methodology (RSM) through Central Composite Design (CCD) was used to optimize the media components for highest GOx activity. From the response surface plot and analysis, it was found that the highest GOx activity was achieved using a combination of molasses, carbonate salt and urea at concentration 32.51%(v/v), 4.58%(w/v) and 0.93 %(w/v) respectively. Fourier transform infrared spectroscopy (FTIR) analysis reflected that the GOx produced from the complex media under optimized media compositions is in-line with the typical commercial GOx properties.

Key words: Enzyme, Optimisation, Molasses, Urea, Glucose oxidase, *Aspergillus terreus*, FTIR, Fungus, RSM.

Introduction

Glucose oxidase is an enzyme belongs to oxidoreductase group which catalyse the oxidation of glucose and producing D-glucono-1,5-lactone and hydrogen peroxide. The enzyme has found several commercial application including removal of oxygen for food and beverage industries (Wong *et al.*, 2008), detection of glucose for development of biosensor (Yoo and Lee, 2010), and as an enzymatic electrode for biofuel (Kim *et al.*, 2009). For industrial scale production of an enzyme, isolation and characterization of new promising strains using cheap carbon and nitrogen sources is a continuous process (Parekh *et al.*, 2002). As the need of glucose oxidase increases for various fields, there is need to search for fungus glucose oxidase producer in the presence of economical carbon and nitrogen sources.

Extensive work has been made to improve the production of GOx from fungus (Bankar *et al.*, 2009). However, minimal effort has been focused on the improvement of GOx in terms of processing cost using locally available materials and statistical optimization approach.

In a previous paper, (Ahmad Anas & Arbain, 2012), we reported a new and promising glucose oxidase-producing strain, *Aspergillus terreus* UniMAP AA-1. This strain produces GOx extracellularly and exhibits a pelleted form in submerged fermentation, thus allowing easy product recovery and purification. These two typical behaviours of the strain would prove useful in lowering the manufacturing cost of the enzyme. However, having a good strain alone would not guarantee a cheaper production cost because a predominant cost factor of enzyme production and in many bio-based productions lies on the type of raw materials used. Typically, raw material cost in fermentation may reach to 60% of the total manufacturing cost. Thus, it is deemed important to select and to optimize the raw material used for a production scale of fermentation. As far as media is concerned, it is not economical to use a defined media for production scale. Instead, it is preferable to use complex media such as molasses, corn-steep liquor, meat extract and agricultural wastes. In this context, to further justify the usefulness of the newly isolated strain, *Aspergillus terreus* UniMAP AA-1 for GOx production, here we are reporting the optimization of complex fermentation media for production of GOx by *Aspergillus terreus* UniMAP AA-1. Thus, in this study, Molasses and urea, locally available materials are used as a relatively inexpensive and economic carbon and nitrogen source respectively alternative to synthetic media for the production of economic glucose oxidase enzymes. Molasses is reported in enhancing the enzyme activity up to 40 folds, in which it signifies economically attractive process for enzyme production (El-Sherbeny, 2005

and Hatzinikolaou & Macris, 1995). Apart from the molasses and urea, CaCO₃ is used in the production media to control the pH which is deemed necessary for optimal production of glucose oxidase (Petruccioli & Federici, 1993).

Response Surface Methodology (RSM) is used to statistically optimize the conditions of the fermentation media. RSM is used to obtain a predicted model and the model can be used in optimizing the fermentation media formulation and running simulation with model equation (Panda *et al.*, 2007). RSM is preferable than one-factor-at-a-time (OFAT) approach to optimize the fermentation media since the method is effective in terms of time, analysis and cost. It is thus expected that this approach will improve the production of GOx in terms of processing cost and productivity.

Material and Methods

Material:

Molasses are obtained from Fermpro Sdn. Bhd, bioethanol production factory located at Chuping, Perlis, Malaysia while urea and CaCO₃ are procured from grocery at Kangar, Perlis, Malaysia.

Microorganism:

Aspergillus terreus UniMAP AA-1 strain (Ahmad Anas & Arbain, 2012) is maintained on Malt Extract Agar (MEA) at 4°C at the culture collection of School of Bioprocess Engineering, University Malaysia Perlis.

Pre-treatment of molasses:

The pH of the molasses solutions was maintained at 3.5 by treating with 35ml of 1N H₂SO₄ per litre. Then, the treated molasses was heated at 60°C for 2 hours. After centrifugation at 8000g for 15 minutes, the supernatant was adjusted to pH 6.0 with 2M NaOH.

Production of crude GOx:

All the culture media were carried out in 100 ml Erlenmeyer flasks with 50 ml working volume. The culture media consist of molasses, CaCO₃ and urea. The flasks were inoculated with 1 ml (5.17 x 10⁷ spores/ml) of inoculums and incubated in a rotary shaker operating at 200 rpm and 30°C for 110 hours.

Assay of GOx activity:

Glucose oxidase activity in the supernatant was measured spectrophotometrically using the coupled *o*-anisidine-peroxidase reaction method explained by Bankar *et al.* (2008).

Optimization of Media Components by Response Surface Method (RSM):

The Central Composite Design (CCD) under RSM (Box & Wilson, 1951) was employed in order to find the optimum levels of significant media components. CCD was generated using a statistical analysis package Design-Expert Software (Stat-Ease Inc., Statistic made easy, Minneapolis, MN, USA, version 6.0.8) and the statistical analysis of experimental data was also performed using this software.

Molasses, CaCO₃ and urea were optimized and examined at three different levels (low, middle, high) concentration coded (-1, 0, +1) as shown in Table 1.

Table 1: CCD Experimental Variables with Respective Levels.

Factors	Unit	Symbols	Levels		
			-1	0	+1
Molasses	% v/v	X ₁	25.0	30.0	35.0
CaCO ₃	% w/v	X ₃	3.0	4.0	5.0
Urea	% w/v	X ₂	0.5	1.0	1.5

According to CCD for the three variables, 19 experimental runs were executed and observations were fitted to the following second order polynomial:

$$Y = \beta_0 + \beta_1A + \beta_2B + \beta_3C + \beta_{11}A^2 + \beta_{22}B^2 + \beta_{33}C^2 + \beta_{12}AB + \beta_{13}AC + \beta_{23}BC \quad (1)$$

where Y is the predicted response (enzyme activity U/mL), A, B and C are independent variable (molasses, CaCO₃ and urea); β_0 is the regression coefficient at center point; β_1 , β_2 and β_3 are linear coefficients; β_{11} , β_{22} and β_{33} are the quadratic coefficients; and β_{12} , β_{13} and β_{23} are the second order interaction coefficients.

Validation of the model:

In order to verify the adequacy of the developed CCD model, confirmation runs were performed.

Crude Glucose oxidase analysis by Fourier transforms infrared spectroscopy (FT-IR):

The formation of D-glucono-1,5-lactone as the result of GOx enzymatic reaction was analyzed using Spectrum 65 Perkin-Elmer FT-IR spectrophotometer. The formation of D-glucono-1,5-lactone was reflected in the peaks corresponding to the C=O and C-O bonds.

Result and Discussion

Optimization of media components by CCD:

Table 2 shows the result of GOx activity for the experimental data (using equation 1) for the respective parameters.

Table 2: Observe value of GO_x activity obtained from CCD.

Standard	Run	Parameters			Response
		Molasses, A (%)	CaCO ₃ , B (%)	Urea, C (%)	GO _x activity (U/ml)
1	14	25.00	3.00	0.50	0.612
2	18	35.00	3.00	0.50	0.642
3	17	25.00	5.00	0.50	0.633
4	11	35.00	5.00	0.50	1.032
5	10	25.00	3.00	1.50	0.762
6	3	35.00	3.00	1.50	0.779
7	8	25.00	5.00	1.50	0.688
8	5	35.00	5.00	1.50	1.005
9	2	25.00	4.00	1.00	0.632
10	7	35.00	4.00	1.00	1.018
11	9	30.00	3.00	1.00	0.897
12	1	30.00	5.00	1.00	0.985
13	19	30.00	4.00	0.50	0.875
14	15	30.00	4.00	1.50	0.899
15	12	30.00	4.00	1.00	1.032
16	16	30.00	4.00	1.00	1.028
17	13	30.00	4.00	1.00	1.047
18	6	30.00	4.00	1.00	1.025
19	4	30.00	4.00	1.00	1.039

*GO_x activity (U/ml) was expressed as the mean of 3 replicates

Regression analysis:

The optimal values for production of GOx were predicted by fitting a polynomial model to the experimental results using the Design Expert software as follows:

$$Y = -4.185 + 0.3A - 0.174B + 1.099C + 0.017AB - 4.75E^{-3}AC - 0.065BC - 5.629E^{-3}A^2 - 0.025B^2 - 0.315C^2 \quad (2)$$

where, GOx production as yield (Y) is a function of molasses (A), CaCO₃ (B) and urea (C). Summary of the analysis of the variance (ANOVA) is presented in Table 3. The fit of the model was checked by the coefficient of determination R², which was calculated to be 0.9035. This indicates that the model is able to explain 93.50% of total variations and only 6.5% is not explained. Besides, the goodness of the model was also supported by the high adjusted coefficient of determination that is 0.87. This high value indicated the significance of the model (Goshal *et al.*, 2011).

ANOVA result reveals that the linear effect of molasses (A) and CaCO₃ (B) and the interactive terms and quadratic terms of AB and molasses (A²) respectively were significant at the level p<0.05. These significant variables indicate that they can act as limiting media components and the small changes of the components in the fermentation media could affect the glucose oxidase activity. The result indicates the linearly-formed

molasses has the highest F-value and lowest p-value which indicates the significance of molasses in maximizing the production of glucose oxidase.

Table 3: ANOVA of Response Surface Model, RSM .

Source	Sum of Squares	F value	p – value
Model	0.47	14.39	0.0002
Molasses, A	0.13	36.55	0.0002
CaCO ₃ , B	0.042	11.73	0.0076
Urea, C	0.011	3.18	0.1082
AB	0.056	15.49	0.0034
AC	1.128E-003	0.31	0.5899
BC	8.385E-003	2.32	0.1620
A2	0.054	14.98	0.0038
B2	1.669E-003	0.46	0.5138
C2	0.017	4.69	0.0586

*p<0.05 indicate the model terms are significant

Effect of interaction between media components on GOx activity:

3D response surface allows the user to study the effects of the selected media components and their mutual interaction on the GOx production. From figure 1, the response surface is elliptical in the entire region where this result reflected the perfect interaction between the molasses and urea. The maximum predicted value of glucose oxidase activity is referred by the surface confined in the smallest ellipse in the diagram where the concentration of molasses is near 32.50 %(v/v) and urea near 1.00 % (v/v) (Figure 1).

Similar observation is found in the effect of molasses and CaCO₃ on maximum production of glucose oxidase at their center and onward values (Figure 2). An increment in CaCO₃ concentration when molasses concentrations near the value of 32.50 % (w/v), the GOx productions increased. Likewise in Figure 3, where an increment in CaCO₃ concentration at urea concentrations near 1.0 % (w/v), increased the production of GOx. CaCO₃ is in turn, significant in GOx production because the addition of CaCO₃ prevents Ph reduction during cultivation for optimal GOx production (Petruccioli *et al.*, 1995). This result also is in agreement with Liu *et al.*, (2001) and Hatzinikolaou and Macris (1995) who found that addition of CaCO₃ being a strong inducer in GO_x production by *A.niger*. Thus, CaCO₃ strongly induced the enzyme production by *Aspergillus* sp.

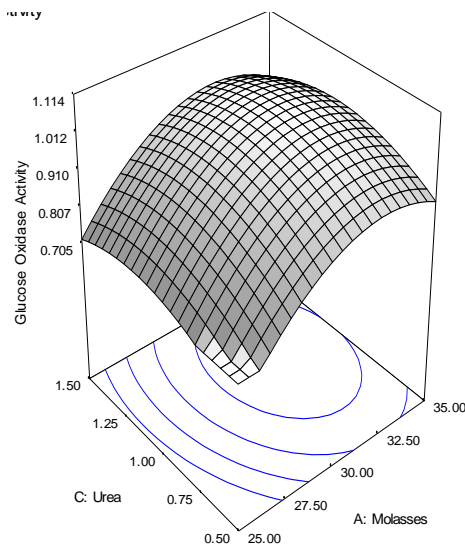


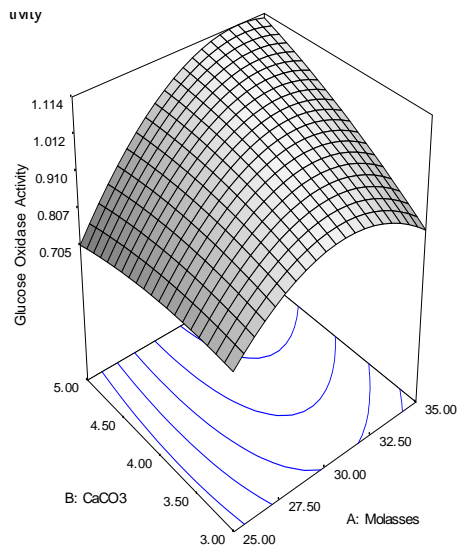
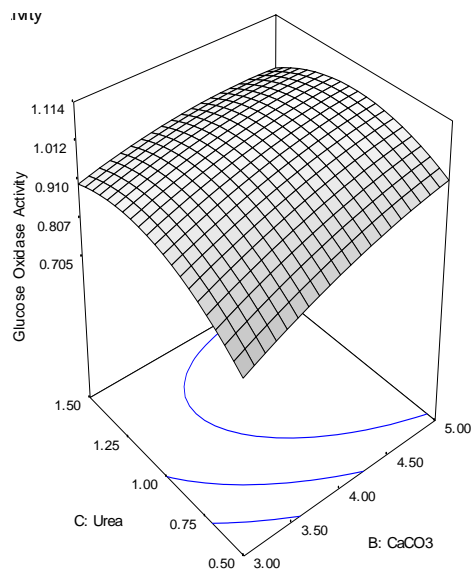
Fig. 1: 3D plot of interaction between molasses, A (%) and urea, C (%) at constant CaCO₃, B (4.00%) on GO_x production, (U/ml)

Validation of the model:

The results of optimum media compositions are presented in Table 4. The experiment yields 1.077 U/ml as the highest GOx activity obtained from the optimized media conditions. The experimental value is lower than the predicted value by 4.09 % only which is a very good agreement. These results confirm the validity of the model. This small error is probably due to the nature of the microorganism and its biochemical reaction with complex media comprising of molasses, CaCO₃, and urea.

Table 4: Validation and optimum media compositions for developed quadratic model with predicted and observed results.

Run	Molasses, A (%)	CaCO ₃ , B (%)	Urea, C (%)	GO _x activity (U/ml)		
				Predicted	Observed	Error (%)
1	33.96	4.56	0.83	1.061	1.056	0.47
2	32.51	4.58	0.93	1.077	1.033	4.09
3	33.34	4.52	0.82	1.060	1.041	1.76
4	33.92	4.54	0.84	1.059	1.032	2.55
5	32.27	4.31	0.97	1.055	1.036	1.80

**Fig. 2:** 3D plot of interaction between molasses, A (%) and CaCO₃, B (%) at constant urea, C (1.00%) on GO_x production, (U/ml).**Fig. 3:** 3D plot of interaction between CaCO₃, B (%) and urea, C (%) at constant molasses, A (30.00%) on GO_x production, (U/ml).

Analysis of crude glucose oxidase, GO_x by Fourier Transform Infrared Spectroscopy (FT-IR):

The ability of the crude GO_x to catalyze the oxidation of β-D-glucose into D-glucono-1,5-lactone was analyzed by FT-IR spectra. The spectra in Figure 4 indicate that the product formation resulted from the enzymatic reaction of crude glucose oxidase with β-D glucose. The spectra at 1728 cm⁻¹ and at 1204 cm⁻¹ show

the presence of C=O and C-O band respectively as a reflection of the formation of D-glucono-1,5-lactone in the enzymatic reaction.

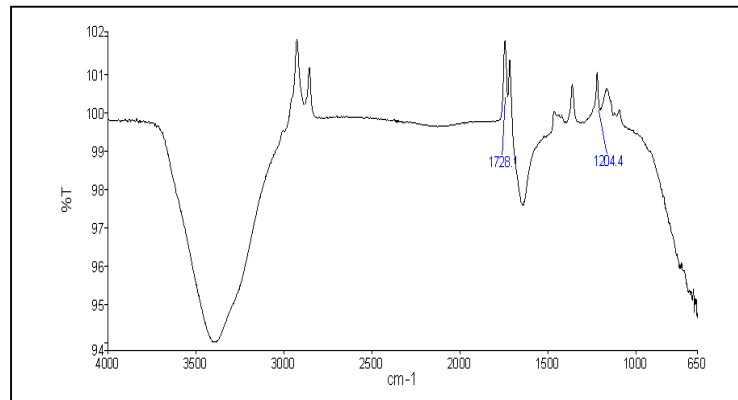


Fig. 4: FT-IR spectra of the enzymatic reaction where spectra shows the wavelength within the range of 4000 – 650 cm^{-1} .

Conclusion:

The result clearly shows that a relatively cheaper complex material such as molasses can be used as a substrate for a new locally isolated strain for production of GOx. The use of statistical approach such as RSM is also proved helpful for obtaining the optimum concentration of the complex media for highest GOx production. Additionally, apart from the assay used to monitor the GOx production, FTIR spectra provides support for the oxidation of glucose by the crude enzyme excreted by *A. terreus* UniMAP grown in molasses as a complex media supplemented with urea and carbonate salt. The highest GOx activity was achieved using a combination of molasses, carbonate salt and urea at 32.51% (v/v), 4.58% (w/v) and 0.93% (w/v) respectively. To this end, although no attempt has been made to actually compare the production cost between the combination strain and media used in this study and the combination used in commercial production of GOx, but nonetheless, this study provides an alternative production of GOx which explore some benefits carried by locally isolated strain and a locally obtained complex substrate.

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References

- Ahmad Anas, N.G. and D. Arbain, 2012. *Aspergillus terreus* UniMAP AA-1: A Newly Isolated Extracellular Glucose Oxidase-Producing Strain. *World Applied Science Journal*, 17: 86-90.
- Bankar, S.B., M.V. Bule, R.S. Singhal and L. Ananthanarayan, 2009. Glucose oxidase An overview. *Biotechnology Advance*, 27: 489-501.
- Bankar, S.B., M.V. Bule, R.S. Singhal and L. Ananthanarayan, 2008. Optimization of *Aspergillus niger* fermentation for the production of glucose oxidase. *Food and Bioprocess Technology*, 2: 344–352.
- Box, G. and K. Wilson, 1951. On the Experimental Attainment of Optimum Conditions. *Journal of Royal Statistic Society (Series B)*, 13:1-45.
- El-Sherbeny, G.A., 2005. Optimization of various factor affecting glucose oxidase activity produced by *Aspergillus niger*. *International Journal of Agriculture Biology*, 7: 953- 958.
- Hatziukolaou, D. and B.J. Macris, 1995. Factors regulating production of Glucose oxidase by *Aspergillus niger*. *Enzyme and Microbial Technology*, 17: 530-534.
- Kim, J., J. Parkey, C. Rhodes and A. Gonzalez-Martin, 2009. Development of a biofuel cell using glucose-oxidase- and bilirubin-oxidase-based electrodes. *Journal of Solid State Electronics*, 13: 1043-1050.
- Liu, J.Z., Y.Y. Huang, J. Liu, L.P. Weng and L.N. Ji, 2001. Effects of metal ions on simultaneous production of Glucose oxidase and catalase by *Aspergillus niger*. *Letters in Applied Microbiology*, 32: 16-19.
- Panda, B.P., M. Ali and S. Javed, 2007. Fermentation process optimization, *Research Journal of Microbiology*, 2(3): 201-208.

- Parekh, S., V.A. Vinei and R.J. Stroobel, 2002. Alkaline protease production by batch culture of *Bacillus* sp. *Applied Microbiology and Biotechnology*, 54: 287-301.
- Petruccioli, M., M. Fenice, P. Piccioni and F. Federici, 1995. Effect of stirrer speed and buffering agents on the production of Glucose oxidase and catalase by *Penicillium* variabile (P16) in benchtop bioreactor. *Enzyme and Microbial Technology*, 17: 336-339.
- Petruccioli, M. and F. Federici, 1993. Glucose oxidase production by *Penicillium* variabile PI6: effect of medium composition. *Journal of Applied Bacteriology*, 75: 364-372.
- Wong, C.M., K.H. Wong and X.D. Chen, 2008. Glucose oxidase: natural occurrence, function, properties and industrial applications. *Applied Microbiology and Biotechnology*, 78: 927-938.
- Yoo, E.H. and S.Y. Lee, 2010. Glucose Biosensors: An Overview of Use in Clinical Practice. *Sensors*, 10: 4558-4576.