http://www.pjbs.org

PJBS

ISSN 1028-8880

Pakistan Journal of Biological Sciences

ANSInet
Asian Network for Scientific Information
308 Lasani Town, Sargodha Road, Faisalabad - Pakistan

Pakistan Journal of Biological Sciences 16 (18): 960-964, 2013
ISSN 1028-8880 / DOI: 10.3923/pjbs.2013.960.964

Optimization of Complex Fermentation Media for Glucose Oxidase Production Usimg Statistical Approach

Ahmad Anas Nagoor Gunny, Dachyar Arbain and Logachanthirika Sithamparam
School of Bioprocess Engineering, Universiti Malaysia Perlis, Kompleks
Pusat Pengajian Jejawi 3, 02600 Arau, Perlis, Malaysia

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 showed *Aspergillus terreus* UniMAP AA-1 offers a better alternative to the existing sources. Thus, a lower production cost could be logically anticipated by growing the strain in a cheaper complex media such as molasses. In this work, sugar cane molasses, supplemented with urea and carbonate salt and a locally isolated strain *Aspergillus terreus* UniMAP AA-1 were used to produce a crude GOx enzyme in a small scale. A statistical optimization approach namely Response Surface Methodology (RSM) was used to optimize the media components for highest GOx activity. It was found that the highest GOx activity was achieved using a combination of molasses, carbonate salt and urea at concentration 32.51, 4.58 and 0.93% (w/v), respectively. This study provides an alternative optimized media conditions for GOx production using locally available raw materials.

Key words: Enzyme, optimisation, molasses, urea, glucose oxidase, *Aspergillus terreus,* ftir, fungus, RS

INTRODUCTION

Glucose oxidase (Gox) oxidizes glucose to 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). 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*., 2009a). However, minimal effort has been focused on the improvement of GOx in terms of processing cost using locally avaliable materials and statistical optimization approach.

Ahmad and Arbain (2012), reported a locally isolated 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 locally 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 found to be an attractive economic component as it has been reported to enhance enzyme activity up to 40 folds (El-Sherbeny *et al*., 2005;

Corresponding Author: Dachyar Arbain, School of Bioprocess Engineering, Universiti Malaysia Perlis, Kompleks Pusat Pengajian Jejawi 3, 02600 Arau, Perlis, Malaysia Tel: +604-979-8848 Fax: +604-979-8755

Hatzinikolaou and Macris, 1995). Apart from the molasses and urea, $CaCO_3$ is used in the production media to control the pH which is deemed necessary for optimal production of glucose oxidase (Petruccioli and Federici, 1993).

Response Surface Methodology (RSM) is a statistical approach used to optimize the conditions of the fermentation media by using a generated model (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 $CaCO_3$ are procured from grocery at Kangar, Perlis, Malaysia.

Microorganism: *Aspergillus terreus* UniMAP AA-1 strain (Ahmad and 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 35 mL of 1N H_2SO_4 per litre. Then, the treated molasses was heated at 60°C for 2 h. After centrifugation at 8000 g for 15 min, the supernatant was adjusted to pH 6.0 with 2 M 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, $CaCO_3$ and urea. The flasks were inoculated with 1 mL (5.17×10^7 spores mL^{-1}) of inoculums and incubated in a rotary shaker operating at 200 rpm and 30°C for 110 h.

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*. (2009b).

Optimization of media components by response surface method (RSM): The Central Composite Design (CCD) under RSM (Box and 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.

Table 1: Values of independent variables at different levels of CCD

			Levels		
Factors	Unit	Symbols	-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

Molasses, $CaCO_3$ and urea were optimized and examined at three different levels (low, middle, high) concentration coded (-1, 0, +1) as shown in Table 1. The center point value was set at molasses; 30.0% (v/v), $CaCO_3$; 4.0 % (w/v) and urea; 1.0% (w/v).

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^{-1}), A, B and C are independent variable (molasses, $CaCO_3$ 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 Eq. 1) for the respective parameters. The highest GOx activity was found from the center point value. This indirectly reflects the closest of the values to optimal media conditions.

Regression analysis: The experimental data through polynomial regression analysis was used to develop the following second-order empirical model:

Table 2: Observe value of GO_x activity obtained from CCD

		Parameters			Response
Standard	Run	Molasses, A (%)	$CaCO_3$, B (%)	Urea, C (%)	GO_x activity (U mL^{-1})
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

*Gox activity (U mL^{-1}) was expressed as the mean of 3 replicates

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
A^2	0.054	14.98	0.0038
B^2	1.669E-003	0.46	0.5138
C^2	0.017	4.69	0.0586

$p<0.05$ indicate the model terms are significant

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

where, GOx production as yield (Y) is a function of molasses (A), $CaCO_3$ (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 R^2, 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.

ANOVA result presented in Table 3 reveals that the linear effect of molasses (A) and $CaCO_3$ (B) and the interactive terms and quadratic terms of AB and molasses (A^2), respectively were significant at the level $p<0.05$. These significant variables indicate that they can act as limiting factors for GOx production and the small changes of the components in the fermentation media could affect the glucose oxidase activity. The result shows the

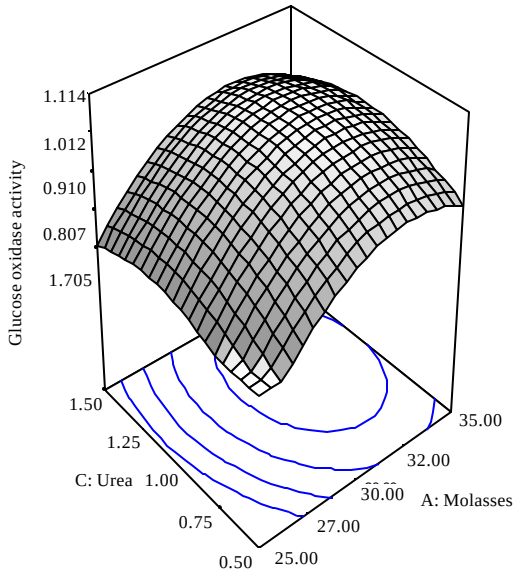

Fig.1: 3D plot of interaction between molasses, A (%) and urea, C (%) at constant $CaCO_3$, B (4.00%) on GO_x production (U mL^{-1})

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.

Effect of interaction between media components on GOx activity: A 3D response surface allows the user to study the effects of the selected media components and their mutual interaction on the GOx production. From Fig. 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) (Fig. 1).

Similar observation is found in the effect of molasses and $CaCO_3$ on maximum production of glucose oxidase at their center and onward values (Fig. 2). An increment in $CaCO_3$ concentration when molasses concentrations near the value of 32.50% (w/v), the GOx productions increased. Likewise in Fig. 3, the production of GOx is increased when an increment in $CaCO_3$ concentration at urea concentrations near 1.0% (w/v). $CaCO_3$ is in turn, significant in GOx production because the addition of $CaCO_3$ prevents pH reduction during cultivation for optimal GOx production (Petrucoili *et al.*, 1995). This result

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^{-1}) 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

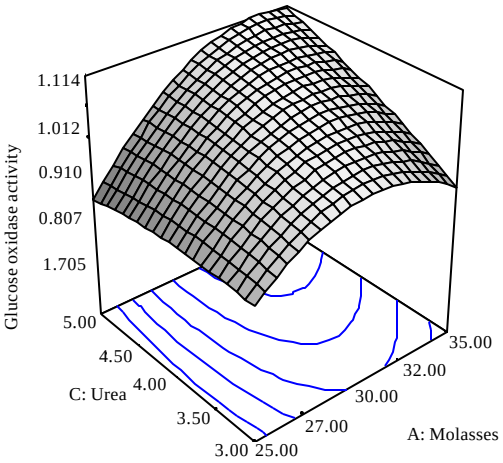

Fig. 2: 3D plot of interaction between molasses, A (%) and $CaCO_3$, B (%) at constant urea, C (1.00%) on GO_x production, (U mL^{-1})

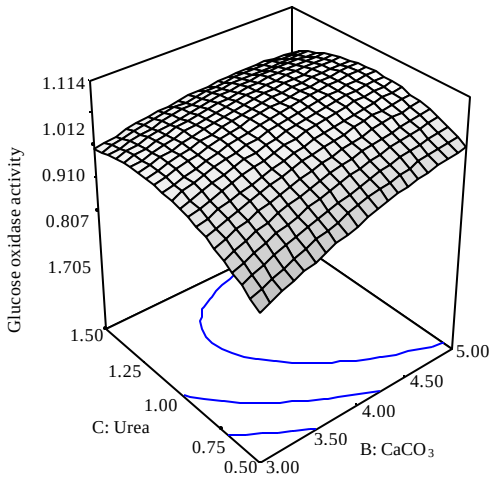

Fig. 3: 3D plot of interaction between $CaCO_3$, B (%) and urea, C (%) at constant molasses, A (30.00%) on GO_x production, (U mL^{-1})

also is in agreement with Liu *et al.* (2001) and Hatzinikolaou and Macris (1995) who found that addition of $CaCO_3$ being a strong inducer in GO_x production by *A.niger*. Thus, $CaCO_3$ strongly induced the enzyme production by *Aspergillus* sp.

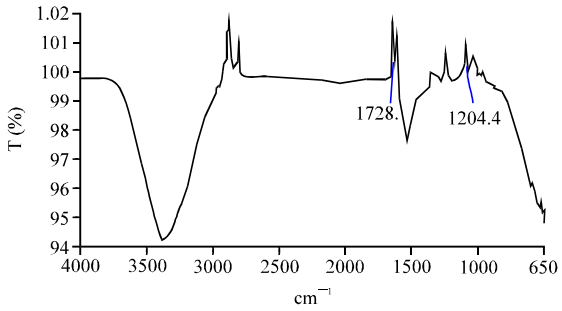

Fig. 4: FT-IR spectra of the enzymatic reaction where spectra shows the wavelength within the range of 4000-650 cm^{-1}

Validation of the model: The results of optimum media compositions are presented in Table 4. The experiment yields 1.056 U mL^{-1} as the highest GOx activity obtained from the optimized media conditions. The experimental value is lower than the predicted value by 0.47% 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, $CaCO_3$ and urea.

Analysis of crude glucose oxidase, GO_x by Fourier Transform Infrared Spectroscopy (FT-IR): The ability of the crude Gox to catalyze the oxidation of β-D-glucose into D-glucono-1,5-lactone was analyzed by FT-IR spectra. The spectra in Fig. 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.

CONCLUSION

The result is 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 33.96, 4.56 and 0.83% (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.

ACKNOWLEDGMENT

The authors thank to the Ministry of Higher Education (MOHE) for financing this research under Fundamental Research Grant Scheme (FRGS 9003-00268) and to School of Bioprocess Engineering, Universiti Malaysia Perlis for their support.

REFERENCES

Ahmad, N.G. and D. Arbain, 2012. *Aspergillus terreus* Unimap AA-1: A newly isolated extracellular glucose oxidase-producing strain. World Applied Sci. J., 17: 86-90.

Bankar, S.B., M.V. Bule, R.S. Singhal and L. Ananthanarayan, 2009a. Glucose oxidase: An overview. Biotechnol. Adv., 27: 489-501.

Bankar, S.B., M.V. Bule, R.S. Singhal and L. Ananthanarayan, 2009b. Optimization of *Aspergillus niger* fermentation for the production of glucose oxidase. Food Bioprocess Technol., 2: 344-352.

Box, G.E.P. and K.B. Wilson, 1951. On the experimental attainment of optimum conditions. J. R. Statist. Soc., 13: 1-45.

El-Sherbeny, G.A., A.A. Shindia and Y.M.M.M. Sheriff, 2005. Optimization of various factor affecting glucose oxidase activity produced by *Aspergillus niger*. Int. J. Agric. Biol., 7: 953-958.

Hatzinikolaou, D.G. and B.J. Macris, 1995. Factors regulating production of glucose oxidase by *Aspergillus niger*. Enzyme Microbiol. Technol., 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. J. Solid State Electrochem., 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*. Lett. Applied Microbiol., 32: 16-19.

Panda, B.P., M. Ali and S. Javed, 2007. Fermentation process optimization. Res. J. Microbiol., 2: 201-208.

Petruccioli, M. and F. Federici, 1993. Glucose oxidase production by *Penicillium variable* P16. Effect of medium composition. J. Applied Bacteriol., 75: 369-372.

Petrucoili, M., F. Fedeerici, P. Piccioni and M. Fenice, 1995. Effect of Stirrer speed and buffering agents on the production of glucose oxidase and catalase by *Penicillium variable* (p16) in bench-top bioreactor. Enzyme Microbial. Technol., 24: 336-339.

Wong, C.M., K.H. Wong and X.D. Chen, 2008. Glucose oxidase: Natural occurrence, function, properties and industrial applications. Applied Microbiol. Biotechnol., 78: 927-938.

Yoo, E.H. and S.Y. Lee, 2010. Glucose biosensors: An overview of use in clinical practice. Sensors, 10: 4558-4576.