Growth Kinetics and Production of Glucose Oxidase Using *Aspergillus niger* NRRL 326

N. Gera,[a] R. V. S. Uppaluri,[a] S. Sen,[b] and V. Venkata Dasu[c,*]

[a]Department of Chemical Engineering, Indian Institute of Technology Guwahati, Guwahati 781039, Assam, India

[b]Centre for the Environment, Indian Institute of Technology Guwahati, Guwahati 781039, Assam, India

[c]Department of Biotechnology, Indian Institute of Technology Guwahati, Guwahati 781039, Assam, India

Original scientific paper
Received: April 3, 2007
Accepted: February 27, 2008

In this paper, we demonstrate the substrate inhibition phenomena for growth kinetics of *Aspergillus niger* NRRL 326 grown on sucrose during glucose oxidase production. The initial set of experiments were carried out using three different substrates, *viz*., glucose, sucrose and raffinose of which it was observed that sucrose serves better for higher production of glucose oxidase. Experiments involving sensitivity studies conveyed that substrate inhibition became predominant when sucrose concentration was above $\gamma = 30$ g L^{-1} in the cultivation medium. The later part of the work was dovetailed towards validation of substrate inhibited growth kinetics with established models such as Haldane, Andrews, Luong, Han-Levenspiel and Aiba. Finally, it was observed that none of the classical models explains the kinetics well.

Key words:
Glucose oxidase, *Aspergillus niger*, sucrose, glucose, raffinose, substrate inhibition

Introduction

Glucose oxidase (GOx) is an important enzyme with a wide range of applications in both biochemical and food industries. Typical applications of GOx in the food industry include glucose removal from dried eggs and foods to improve colour, flavour, and shelf life[1] and oxygen removal from fruit juices, canned beverages and mayonnaise to prevent rancidity.[2] Furthermore, GOx is identified to catalyze the oxidation reaction of β-D-glucose to produce gluconic acid and hydrogen peroxide.[3] Utilizing oxygen as an electron acceptor, recently GOx is deployed as a component in biosensors to detect glucose concentration levels in blood sampling and analysis.[4]

To date, common substrates investigated for laboratory scale GOx production refer to glucose,[5] sucrose, corn steep liquor,[6] molasses[7] and rice polishing[8] using microbial sources such as *Aspergillus niger, Penicillium notatum, Penicillium glaucum, Penicillium amagasakiense* and *Penicillium purpurogenum*. Of these options, commercial interest points towards GOx production from glucose using *Aspergillus niger.*[9]

Hatzinikolaou and Macris[10] have focused towards regulation of GOx production using molasses and sucrose with *Aspergillus niger.* The authors reported that enzyme activity increased with carbon mass concentration in the fermentation medium and remained constant at mass fraction levels above 4 % using molasses and 10 % using sucrose and inhibitory factors are reported after these concentration levels. Kona *et al.,*[6] produced GOx from corn steep liquor and reported that above concentration levels of $\sigma = 20$ mL L^{-1}, enzyme synthesis is severely inhibited. Hamid *et al*.,[8] produced GOx from rice polishing and observed that maximum GOx production is attained at a substrate mass fraction of $w = 2$ % in the fermentation medium.

Existing research interests in GOx production is towards the identification of cheaper substrates and easily controllable process conditions using *Aspergillus niger.* It can be observed that existing industrial production of GOx could be further improved as the cost of sucrose is lower than the cost of glucose with the fact that an inversion process from the former commonly produces the latter. The objective of this work is to demonstrate the substrate inhibition phenomena for growth kinetics of *Aspergillus niger* grown on sucrose during glucose oxidase production. Furthermore, it can be observed in existing literature that substrate inhibition has not been studied in detail. However, from an industrial point of view, substrate inhibition phenomena translate into the efficiency of the bioreactor in terms of effective utilization of raw materials per unit GOx produced. Modelling efforts with respect to substrate inhibition too pay huge dividends in the form of sizing, cost estimation and economic assessment.

*Corresponding author: E-mail: veeranki@iitg.ernet.in
Tel: + 91-361-258 2212; Fax: + 91-361-258 2249

Material and methods

Organism and culture conditions

Aspergillus niger NRRL 326 was obtained from the United States Department of Agriculture (USDA). The culture was maintained on a slant of minimal media containing (in g L^{-1}) glucose – 25, KH_2PO_4 – 2, K_2HPO_4 – 2, $MgSO_4 \cdot 7H_2O$ – 1, peptone – 2, yeast extract – 2, KCl – 0.2, NH_4NO_3 – 0.6, agar – 15 at θ = 4 °C and sub-cultured every month. Production medium was prepared by using above said minimal medium without agar. Experiments have been performed using different substrates, sucrose and raffinose by replacing glucose in the medium. 50 mL of the production medium in V = 250 mL flask was inoculated with spore suspension containing approximately $5 \cdot 10^6$ spores of *A. niger* per mL. The spore suspension was prepared by washing a 3-day-old plate culture with 10 mL of sterile solution containing Tween 80 (σ = 0.25 mL L^{-1}) and NaCl (γ = 0.8 g L^{-1}). Flasks were incubated in an orbital shaker for t = 84 h at 150 rpm and at θ = 30 °C. Samples were taken for every t = 12 h and centrifuged at m = 11180 g for t = 10 min and analyzed for extracellular enzyme activity and substrate utilization. Kinetic experiments have been carried out with sucrose as sole carbon source.

Kinetic experiments

Experiments were performed according to the procedure described in the above section with various initial sucrose concentrations ranging from γ = 10 to 50 g L^{-1}. Samples were collected at different interval of time and measured cell mass (dry cell mass) and sucrose consumption. For each initial mass concentration of sucrose, specific growth rate was calculated in exponential phase. The specific growth rate (μ) in the exponential phase was calculated as the slope of plot drawn between ln (γ_x) vs. time. Where, γ_x is the dry cell mass obtained at a particular time. All experiments were performed in duplicate under identical conditions and all results had a standard deviation ± 4 % to ± 10 % about the mean.

Kinetic models

The substrate inhibition occurs generally at high substrate concentrations. Different substrate inhibition models, Haldane,[11] Andrews,[12] Han-Levenspiel,[13] Aiba[14] and Luong[15] are considered to explain the cell growth kinetics. The variables of different growth models considered and estimated using MATLAB® software are given in Table 1.

Table 1 – *Estimated parameters of various substrate inhibition models explaining the entire data*

Model	Equation	Parameters	R^2
Aiba[14]	$\mu = \frac{\mu_{max}\gamma_S}{K_S+\gamma_S}\exp\left(\frac{-\gamma_S}{K_I}\right)$	μ_{max} = 0.0204 K_S = 9.3673 K_I = 71.9628	0.9568
Andrews[12]	$\mu = \frac{\mu_{max}\gamma_S}{(K_S+\gamma_S)\left(1+\frac{\gamma_S}{K_I}\right)}$	μ_{max} = 0.0430 K_S = 21.2406 K_I = 20.2556	0.9282
Haldane[11]	$\mu = \frac{\mu_{max}\gamma_S}{\left(K_S+\gamma_S+\frac{\gamma_S^2}{K_I}\right)}$	μ_{max} = 0.0230 K_S = 12.2563 K_I = 35.4017	0.9324
Luong[15]	$\mu = \frac{\mu_{max}\gamma_S}{K_S+\gamma_S}\left[1-\frac{\gamma_S}{\gamma_S^*}\right]^n$	μ_{max} = 0.0138 K_S = 4.3818 γ_S^* = 59.5329 n = 0.2253	0.994
Han-Levenspiel[13]	$\mu = \mu_{max}\left(1-\frac{\gamma_S}{\gamma_S^*}\right)^n \frac{\gamma_S}{\gamma_S+C_M\left(1-\frac{\gamma_S}{\gamma_S^*}\right)^m}$	μ_{max} = 0.137 γ_S^* = 59.4901 C_M = 4.3523 n = 0.2291 m = 0.1064	0.9014

Analytical methods

The sugars were measured by the 3,5-dinitro-salycilic acid (DNS) method using glucose as the standard.[16] The same method was used for measuring extra cellular glucose oxidase activity i.e., measuring residual reducing sugars. Sucrose was measured after hydrolysis with φ = 40 % HCl to reducing sugars. Subsequently, GOx activity is evaluated as the amount of enzyme required for the conversion of 1 μmole of glucose per 30 min per mL at θ = 30 °C. The reducing sugars were treated with 3,5-dinitro-salicylic acid (DNS) which is reduced to 3-amino-5-nitro-salicylic acid. The latter was quantified by measuring absorbance at λ = 540 nm using a spectrophotometer (Make: Perkin Elmer, Model: Lambda 45). The DNSA reagent consisted of 1 g DNS dissolved in 20 mL 2 mol L^{-1} NaOH and 50 mL distilled water. Thirty grams of Rochelle salt (potassium sodium tartrate tetrahydrate: $KO_2CCH(OH)CH(OH)CO_2Na \cdot 4H_2O$) was added and the volume was brought up to 100 mL with distilled water. The reducing sugars were measured as follows: 0.2 mL reducing sugar solution (containing 1 ± 3.5 g L^{-1} reducing sugar), 1.8 mL distilled water and 2 mL DNS reagent were boiled for 5 min followed by cooling to room temperature and diluting to 24 mL. A standard curve was prepared using known concentrations of glucose. From the standard curve the concentration of reducing sugar was determined.

GOx activity was assayed using method of Kona *et al.*[6] The enzyme assay mixture consisted of 0.2 mL reducing sugar solution (2.5–5.0 g L^{-1}), 0.2 mL crude enzyme preparation, 1 mL citrate phosphate buffer (pH 5.6) and 0.6 mL distilled water. The citrate phosphate buffer contained 0.02 g L^{-1} sodium nitrate to inhibit catalase activity without affecting glucose oxidase activity. The reaction mixture was incubated at 30 °C for 30 min. The reaction was stopped by keeping the tube in boiling water. To measure residual sugar, 2 mL of DNS reagent was added to the above tube and the mixture boiled for 5 min followed by cooling to room temperature and diluting to 24 mL. The absorbance was read and glucose concentration was determined from the standard curve as described above. The blank (containing de-ionized water instead of crude enzyme) was immediately boiled after adding the enzyme preparation. One unit of glucose oxidase activity was expressed as that amount of enzyme which converts 1.0 μg of glucose per 30 min at 30 °C. Since the method of measuring glucose/reducing sugar by DNS method is accurate, it was possible to measure as low as 5 units of enzyme activity.

The cell mass was measured as dry mass. The filtered cell mass was washed with distilled water 2–3 times to remove medium ingredients and dried at 60 °C to a constant mass. Biomass concentration (γ_x) vs. fermentation time was plotted at different initial sucrose concentration ranging from γ = 10 g L^{-1} to 50 g L^{-1} to see the effect of initial substrate concentration on growth (Fig. 4). Natural logarithm of dry cell mass in g, ln (γ_x) is plotted against incubation time in h (Fig. 5). Specific growth rate was calculated from this plot.

Results and discussion

Production of glucose oxidase using different substrates

Fig. 1 presents GOx activity obtained at different fermentation times for glucose, sucrose and raffinose being used as the sole carbon source in the medium. Among these, glucose was well investigated substrate in GOx production[17–19] and the emphasis is to find alternate substrates that could produce GOx. It was observed that sucrose provided high activity (0.90 U) where as glucose (0.75 U) and raffinose (0.69 U) provided lower GOx activity comparatively. Therefore, sucrose serves as a better substrate for GOx production using *Aspergillus niger* rather than glucose and raffinose. The maximum production of glucose oxidase was observed in the earlier reports using sucrose as sole carbon source.[6,10,20,21]

Therefore, all the substrate inhibition studies and validation of mathematical models were performed using sucrose as the sole source of carbon.

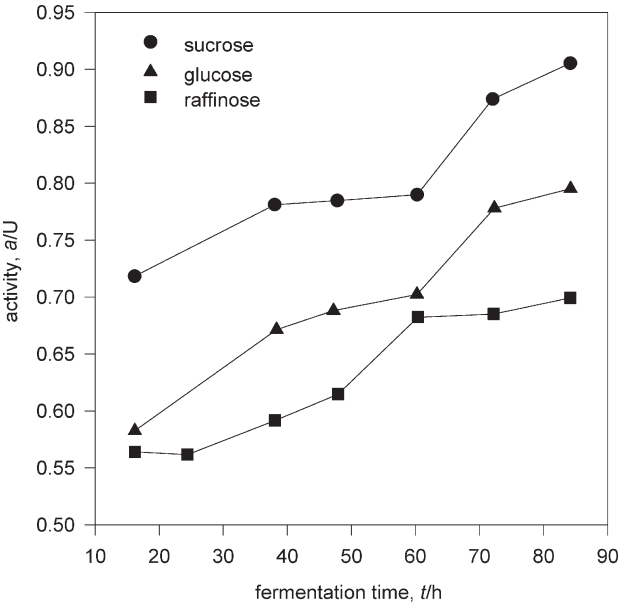
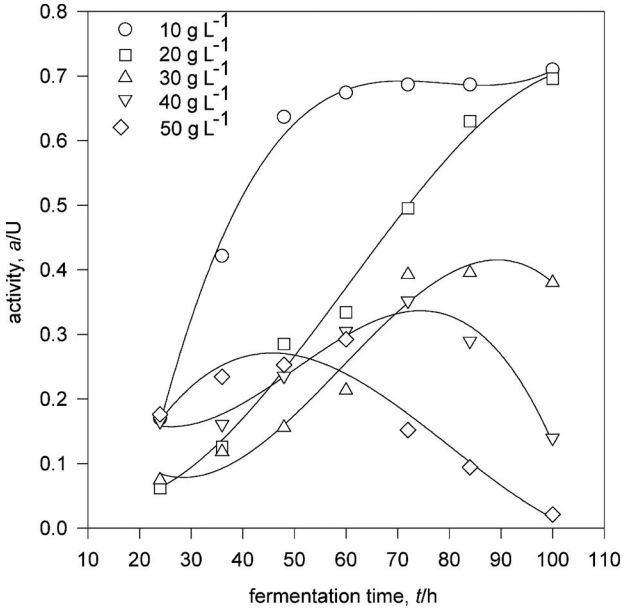
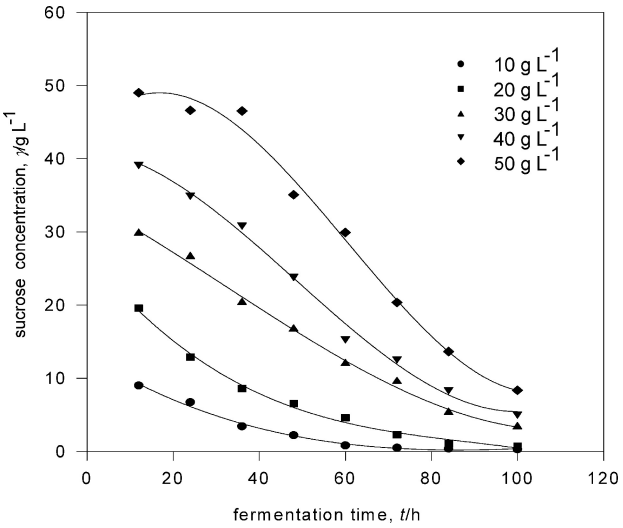


Fig. 1 – *Production of glucose oxidase from A. niger NRRL 326 using different carbon sources each at a concentration of 25 g L^{-1}*



F i g . 2 – *Effect of initial substrate (sucrose) concentration on the production of glucose oxidase using A. Niger NRRL 326*



F i g . 3 – *Substrate (sucrose) assimilation during fermentation*

Effect of initial substrate concentration on glucose oxidase production

Fig. 2 illustrates the effect of initial substrate concentration on GOx activity obtained at different fermentation time for initial sucrose concentrations varying from γ = 10 g L^{-1}–50 g L^{-1}. It can be observed from the figure that when the sucrose concentration was low (10 g L^{-1}), GOx activity increased steadily with time. A similar trend was observed when the initial sucrose concentration was maintained at 20 g L^{-1}. However, initial sucrose concentration in between γ = 20–30 g L^{-1} indicated the onset of substrate inhibition phenomena with the activity profile ending with a slight dip after a maximum value. GOx activity profiles observed for initial sucrose mass concentrations of γ = 40 g L^{-1} and 50 g L^{-1} clearly indicate a dip in GOx activity after indicating a peak value. These observations conclude that initial sucrose concentration in between γ = 20–30 g L^{-1} indicates transition state for substrate inhibition phenomena and from substrate concentration of γ = 30 g L^{-1} result in strong substrate inhibition contributing to lower GOx activity. Hatzinikolaou *et al*.,[10] reported increase in enzyme activity with increasing sucrose concentration and remained practically constant at levels above w = 10 % using sucrose as a carbon source. Favela-Torres *et al*.,[22] and Rogalski *et al*.,[7] reported similar study with glucose as carbon source.

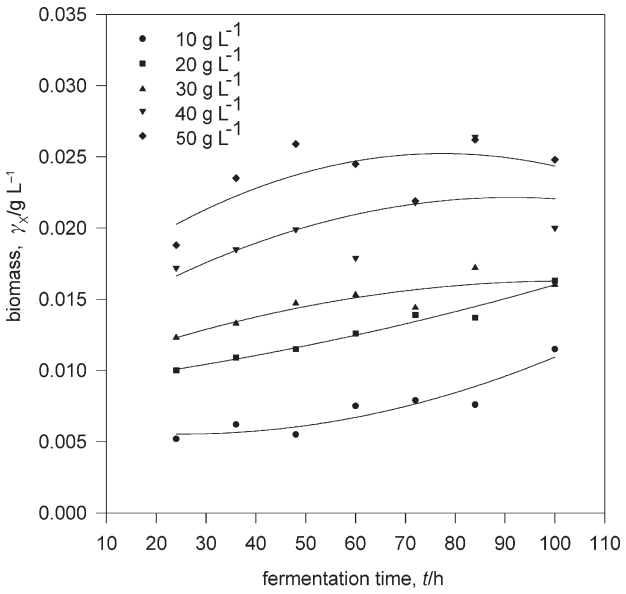
Substrate utilization during fermentation

Fig. 3 illustrates assimilation of sucrose during the fermentation for varying initial sucrose concentration indicating the rapidity of sucrose assimilation in the process. The rate of sucrose utilization increased with sucrose concentrations up to γ = 20 g L^{-1} indicating substrate limitations at low sugar concentrations. However, the rate decreased with increasing sugar concentrations above γ = 30 g L^{-1} due to substrate inhibition at high sucrose concentrations (Fig. 3). Similar type of substrate utilization profile was previously reported in case of ethanol fermentation by yeast.[22,23]

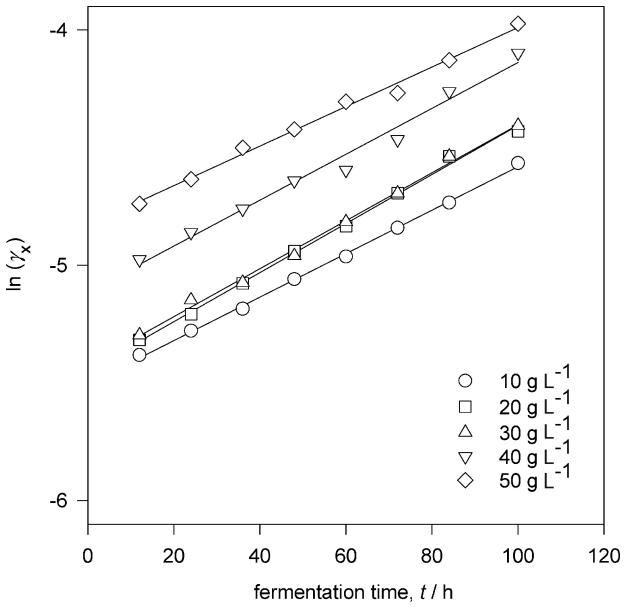
These profiles indicate non-linear assimilation of sucrose during the fermentation. Favela-Torres *et al*.,[22] reported complete uptake of glucose from the media at the lowest concentrations employed (γ = 30 and 50 g L^{-1}). This decreased to only 50 % at 100 g L^{-1} and to about 20 % at the highest concentration of 200 g L^{-1}.

Effect of initial substrate concentration on specific growth rate of *A. niger*

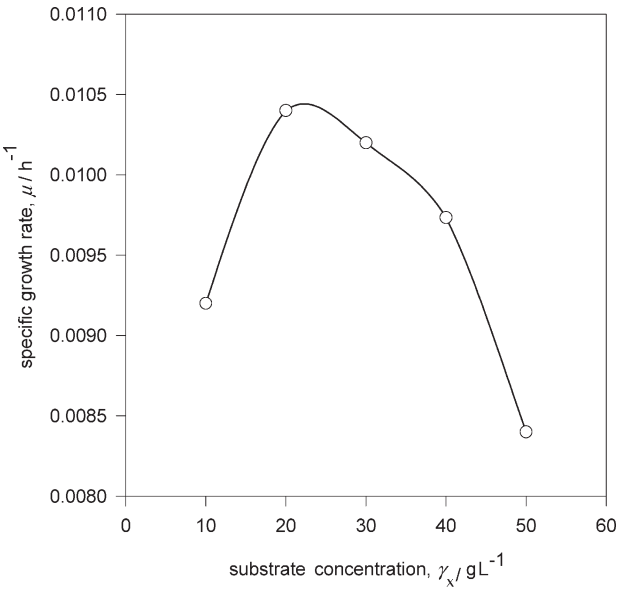
Specific growth rate of (μ) for each initial sucrose concentration were calculated from the plot of ln γ_x vs. time (Fig. 5) in the logarithmic phase as described in the materials and methods. Fig. 6 presents variation of specific growth rate with initial sucrose concentration (γ = 10–50 g L^{-1}). The onset of substrate inhibition phenomena was observed in between γ = 20–30 g L^{-1} of sucrose being used in the fermentation. This is because the growth profiles obtained for sucrose concentrations of 30 g L^{-1}, 40 g L^{-1} and 50 g L^{-1} correspond to declining trend, whereas other concentrations range (10–20 g L^{-1}) indicates a steadily increasing specific growth rate. The similar types of substrate inhibited growth profile are also reported by several researchers for different microorganisms grown on different substrates used for various purposes.[24–27]



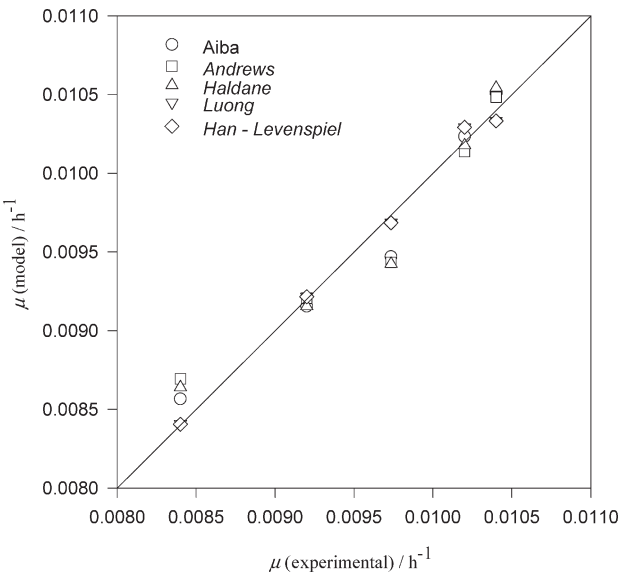
F i g . 4 – *Plot of biomass concentration (γ_x) vs. fermentation time for different initial substrate concentration viz., (in g L^{-1}) 10, 20, 30, 40 and 50*



F i g . 5 – *Plot of natural logarithm of biomass concentration ln(γ_x) vs. fermentation time for different initial substrate concentration viz., (in g L^{-1}) 10, 20, 30, 40 and 50*



F i g . 6 – *Plot of specific growth rate (μ) vs. initial substrate concentration (various initial sucrose conversation viz. (in g L^{-1}) 10, 20, 30, 40 and 50)*



F i g . 7 – *Parity plot for the prediction of specific growth rates (μ) by various substrate inhibited that fitted for the entire data. Standard deviation was calculated for experiments carried out in duplicates under indicated conditions.*

Growth kinetics of *A. niger* grown on sucrose

The growth kinetics of *Aspergillus niger* was employed using different substrate inhibition models available in the literature and the values of variables were estimated using MATLAB (Table 1). Fig. 7 shows the models fitted to the entire data for specific growth rate vs. initial substrate concentration. Although we could find no earlier attempt reported on the modelling of growth kinetics of *Aspergillus niger* during production of glucose oxidase, a similar approach was taken earlier by different scientists for other purposes.[26–30] Parity plot showing the estimated specific growth rate by different models that fit to the entire data vs. experimental specific growth rate is shown in Fig. 7. The kinetic variables $\mu_{max,}$ and K_S estimated using the kinetic models, Haldane,[11] Andrews,[12] and Aiba[14] did not match with the experimental values. Even though, the kinetic variables (μ_{max} , K_S) estimated better both by Luong and Han-Levenspiel model (Table 1), the growth rates given by Luong model lies very close to the experimental values (Fig. 7).

In addition, the R^2 value of Luong model is higher (0.994) than that of Han-Levenspiel model (0.9014). The latter also gives very close approximation of the kinetic variables for growth. Though the two models Luong and Han-Levenspiel model give very high R^2 values, the discrepancies in K_I value tells that none of the model explains well the kinetics in this study. Further studies are required to establish a perfect model for this system. Hence, experiments are being carried out for that purpose.

Conclusions

For the first time, this work targeted concentration and process regimes that involve substrate inhibition phenomena for growth of *Aspergillus niger* during glucose oxidase production using sucrose as substrate. In this work, inhibitory substrate concentrations ranging from 20 g L^{-1} and above have been observed for GOx production. The kinetic parameters were evaluated and the data did not fit well with good confidence with any of the classical models discussed here. Hence in conclusion, it was inferred from this study that classical inhibition models are not suitable here. Further work is needed to establish a model.

Abbreviations

GOx – glucose oxidase
X – biomass
DNS – 3,5-dinitro-salycilic acid

List of symbols

a – activity, U
K_s – substrate affinity constant, g L^{-1}
K_I – inhibition constant, g L^{-1}
C_M – Monod's constant, g L^{-1}
θ – temperature, °C
t – time, min, h
V – volume, mL, μL
μ – specific growth rate, h^{-1}
μ_{max} – maximum specific growth rate, h^{-1}
γ_{S_0} – initial substrate concentration, g L^{-1}
n – constant which accounts the relationship between μ and γ_s
m – constant which accounts the relationship between C_M and γ_s
γ – mass concentration, g L^{-1}
σ – volume concentration, mL L^{-1}
φ – volume fraction, %
w – mass fraction, %

References

1. *Dayal*, *R., Godjevargova*, *T.*, Enzyme Microb. Technol. **39** (2006) 1313.
2. *Atkinson*, *B.*, *Mavituna*, *F.*, Biochemical Engineering and Biotechnology Handbook, Stockton Press, New York, 1991, pp. 548.
3. *Wilson*, *R.*, *Turner*, *A. P. F.*, Biosens. Bioelectron. **7** (1992) 165.
4. *Petruccioli*, *M.*, *Piccioni*, *P.*, *Federici*, *F.*, Enzyme Microb. Technol. **21** (1997) 458.
5. *Eun-Ha*, *P.*, *Young-Mi*, *S.*, *Young-Yi*, *L.*, *Tae-Ho*, *K.*, *Dae-Hyuk*, *K.*, *Moon-Sik*, *Y.*, J. Biotechnol. **81** (2000) 35.
6. *Kona*, *R. P.*, *Qureshi*, *N.*, *Pai*, *J. S.*, Bioresour. Technol. **78** (2001) 123.
7. *Rogalski*, *J.*, *Fiedurek*, *J.*, *Szczordrak*, *J.*, *Kapusta*, *K.*, *Leonowicz*, *A.*, Enzyme Microb. Technol. **10** (1988) 508.
8. *Hamid*, *H. M.*, *Rehman*, *K.*, *Zia*, *M. A.*, *Asgher*, *M.*, Biotechnol. J. **2** (2003) 1.
9. *Betancor*, *L.*, *Lopez-Gallego*, *F.*, *Hidalgo*, *A.*, *Alonso-Morales*, *N.*, *Dellamora-Ortiz*, *G.*, *Guisan*, *J. M.*, *Fernandez-Lafuente*, *R.*, J. Biotechnol. **121** (2006) 284.
10. *Hatzinikolaou*, *D.*, *Macris*, *B. J.*, Enzyme Microb. Technol. **17** (1995) 530.
11. *Haldane*, *J. B. S.*, Enzymes, MIT press, Cambridge, MA, 1965, pp. 84,
12. *Andrews*, *J. F.*, Biotechnol. Bioeng. **10** (1968) 707.
13. *Han*, *K.*, *Levenspiel*, *O.*, Biotechnol. Bioeng. **32** (4) (1988) 430.
14. *Aiba*, *S.*, *Shoda*, *M.*, *Nagatani*, *M.*, Biotechnol. Bioeng. **10** (1968) 845.
15. *Luong*, *J. H. T.*, Biotechnol. Bioeng. **29** (2) (1987) 242.
16. *Miller*, *G. L.*, Anal. Chem. **31** (3) (1959) 426.
17. *Pitt*, *D.*, *Mosley*, *M. J.*, *Barnes*, *J. C.*, Trans. Br. Mycol. Sot. **81** (1983) 21.
18. *Caridis*, *C. A.*, *Christakopoulos*, *P.*, *Macris*, *B. J.*, Appl. Microbial. Biotechnol. **34** (1991) 794.
19. *Fiedurek*, *J.*, *Rogalski*, *J.*, *Ilczuk*, *Z.*, *Leonowicz*, *A.*, Enzyme Microb. Technol. **8** (1968) 734.
20. *Zetelaki*, *K. Z.*, *Vas*, *K.*, Biotechnol. Bioeng. **10** (1968) 45.
21. *Zetelaki*, *K. Z.*, Biotechnol. Bioeng. **12** (1970) 379.
22. *Favela-Torres*, *E.*, *Cordova-Lopez*, *J.*, *Garda-Rivero*, *M.*, *Gutirrrez-Rojas*, *M.*, Process Biochem. **33** (2) (1998) 103.
23. *Ozmihci*, *S.*, *Kargi*, *F.*, Bioresour. Technol. (2006) (article in press).
24. *Ruey-Shin*, *J.*, *Shang-Yuan*, *T.*, Biochem. Eng. J. **31** (2006) 133.
25. *Abuhamed*, *T.*, *Bayraktar*, *E.*, *Mehmetoglu*, *T.*, *Mehmetoglu*, *U.*, Process Biochem. **39** (2004) 983.
26. *Gokulakrishnan*, *S.*, *Gummadi*, *S. N.*, Process Biochem. **41** (2006) 1417.
27. *Govindaswamy*, *S.*, *Vane*, *L. M.*, Bioresour. Technol. **98** (2007) 677.
28. *Johng-Hwa*, *A.*, *Sangyoung*, *L.*, *Seokhwan*, *H.*, Process Biochem. **40** (2005) 1363.
29. *Bainotti*, *A. E.*, *Setogaichi*, *M.*, *Nishio*, *N.*, J. Fermentation Bioeng. **81** (4) (1996) 324.
30. *Starzak*, *M.*, Chem. Eng. J. **54** (1994) 221.