Modeling Growth of *Cellulomonas cellulans* NRRL B 4567 under Substrate Inhibition During Cellulase Production

R. Agarwal, B. Mahanty, and V. Venkata Dasu*
Department of Biotechnology, Indian Institute of Technology Guwahati, Guwahati-781039, Assam, India

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Cellulase production study was performed in shake flask and bioreactor system using *Cellulomonas cellulans* NRRL B 4567 for initial substrate concentration from γ_{S_0} = 2 to 12 g L^{-1}. The growth, substrate uptake profile and enzyme activity at different initial substrate concentrations were measured. The results inferred the presence of substrate inhibition kinetics. Various substrate inhibition models were tested and parameters were estimated, using non-linear regression analysis. Han-Levenspiel model was found to be the best fitted model for both shake flask and reactor study. The highest volumetric enzyme activity was observed at initial substrate concentration of γ_{S_0} = 12 g L^{-1} and 4 g L^{-1} in shake flask and bioreactor respectively.

Key words:
Cellulase production, substrate inhibition model, *Cellulomonas cellulans*

Introduction

Cellulose is the major component of plant biomass produced during photosynthesis. This makes it a cheap and abundant renewable carbon source. However, the need is to develop a low-cost technology by which we can use this abundant resource as a raw material in various processes. One of the promising strategies is the production of cellulolytic enzymes that could degrade this complex into glucose and thus be used for production of ethanol, organic acids and other chemicals.[1–3] Cellulase is one such cellulolytic enzyme which breaks down cellulose to beta-glucose. It is produced mainly by symbiotic bacteria in the ruminating chambers of herbivores.[4]

In recent years, the interest in cellulase has increased due to many potential applications, for example, in the formulation of washing powders in the textile industry, as a fabric softener in the laundry industry, for brightening and as a detergent in the pulp and paper industry.[5–7] Cellulase is also used for commercial food processing in coffee; it performs hydrolysis of cellulose during drying of beans. It is estimated that approximately 20 % of the > 1 billion US dollars of the world's sale of industrial enzymes consists of cellulase, hemicellulases and pectinases.[8]

Numerous fungi have been studied for their potential extracellular cellulase production on various simple to complex carbon as substrate.[9–13] In general, fungal cellulase is produced only in the presence of cellulose as a substrate, whereas bacterial cellulases are produced constitutively.[14] So, production of cellulase would be more advantageous from bacterial sources. Production of bacterial cellulase has been studied by various authors and *Cellulomonas cellulans* is considered one of the best cellulolytic aerobic gram-positive bacteria, possessing not only the capacity to degrade various carbohydrates, such as starch, xylan and cellulose, but crystalline cellulose as well, and have been most widely studied among bacterial species reported for cellulase production.[15–18] Glucanases of *Cellumonas* have been critically reviewed.[19] Particularly, *Cellumonas flavigena* and *Cellumonas fimi* are known to produce a diversity of extracellular cellulases when grown on variety of carbon sources.[20–22]

The kinetics of saccharification processes on various cellulosic substrates by different cellulase enzyme has been studied.[23–25] However, kinetics of microbial-production of those cellulase enzymes has not been largely dealt with, which is essential to evaluate, predict and optimize the enzyme production in any bioprocess system. Microbial production and specificity of cellulases have been shown to be dependent on the nature and concentration of substrate.[26–28]

In the present work, batch experiments in both shake flask and bioreactor at different initial concentration of cellulose inferred the presence of substrate inhibited growth, which was subsequently modeled into different known substrate inhibition models. A reliable kinetic model of the microbial process is essential for the large-scale production and for describing the process of microbial synthesis of cellulase.

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

Materials and methods

Bacterial strain and culture medium

Cellulomonas cellulans NRRL B 4567 obtained from National Center for Agricultural Utilization Research, USDA, Agricultural Research Service, USA was used in this work. It was maintained on agar slants/plates containing nutrient agar 15 g L^{-1}. All kinetic experiments were conducted with the medium containing (g L^{-1}): carboxymethyl cellulose (CMC) (degree of substitution: 0.6 ± 0.1), 2–12; K_2HPO_4, 1.0; KH_2PO_4, 1.0; KCl, 0.5; $MgSO_4 \cdot 7H_2O$, 0.5; $FeSO_4 \cdot 7H_2O$, 0.01; yeast extract, 2.0; and $NaNO_3$, 0.5. The seed-culture was prepared with the above medium (with glucose instead of CMC) by inoculating active culture from plate or slant aseptically.

Kinetic experiments

Shake flask studies were carried out in 250 mL Erlenmeyer flask containing 50 mL of the production medium was inoculated with 2 % of seed culture (OD = 0.6 at 600 nm) and incubated at θ = 35 °C and 160 rpm in an orbital shaker. The initial pH of the medium was adjusted to 7.1, however it was neither maintained nor monitored during the experimental period.

Aerobic batch fermentations (with 1 L medium) were carried out in a 3 L Applikon fermentor, equipped with pH, dissolved oxygen (DO) and temperature sensors. Temperature, pH, agitation and aeration rate were maintained at θ = 35 °C, 7.1, 350 rpm and 1.5 L min^{-1} respectively.

The sample from shake flask and fermentor were collected at every 2 h for cell growth, cellulose degradation and extracellular cellulase activity measurement. Specific growth rate was calculated by log-linear regression of biomass versus time in exponential growth phase. The biomass yield ($Y_{X/S}$) was determined as the ratio of a linear regression of biomass concentration (γ_X) versus substrate mass concentration (γ_S) during the exponential growth phase. The specific cellulose uptake rate (q_S), defined as the differential change in substrate concentration (γ_S) with respect to time (t), normalized by the biomass concentration during the corresponding period.

Mathematical models

Substrate inhibition occurs generally at high substrate concentrations. It is primarily caused by more than one substrate molecule binding to an active site, and/or often by different parts of the substrate molecules binding to different sub-sites within the substrate binding site. If the resultant complex is inactive, this type of inhibition causes a reduction in the rate of reaction. Different substrate inhibition models have been used to explain cell growth kinetics is presented in Table 1.

Table 1 – *Different models considered in this study*

Model	Equation	Reference
Andrew's	$\mu = \frac{\mu_{max}\gamma_S}{(K_S + \gamma_S)(1 + \gamma_S/K_I)}$	29
Luong's	$\mu = \frac{\mu_{max}\gamma_S}{K_S + \gamma_S}\left[1 - \frac{\gamma_S}{\gamma_S^*}\right]^n$	30
Han-Levenspiel	$r = k\left[\left(1 - \frac{\gamma_S}{\gamma_S^*}\right)^n\right]\frac{\gamma_S C}{\gamma_S + K_M\left[\left(1 - \frac{\gamma_S}{\gamma_S^*}\right)^m\right]}$	31
Haldane	$\mu = \frac{\mu_{max}\gamma_S}{K_S + \gamma_S + \frac{\gamma_S^2}{K_I}}$	32
Moser	$\mu = \frac{\mu_{max}\gamma_S^n}{K_S + \gamma_S^n}$	33
Aiba	$\mu = \frac{\mu_{max}\gamma_S}{K_S + \gamma_S}\exp\left(\frac{-\gamma_S}{K_I}\right)$	34

Monod's model is the most simple and fundamental model proposed to explain microbial growth which predicts a proportional relationship between specific growth rate and initial substrate concentration at low substrate concentration. At high substrate concentration, a constant and maximum value specific growth rate is reached.

Andrew's model is also used to explain inhibitory effects of substrate at higher concentrations.[29] It reduces to the conventional Monod's equation at very large inhibition constant.

Luong's model is a generalisation of Monod's kinetics to account for substrate inhibition on growth.[30] The Luong's model incorporates a term for substrate concentration above which growth is completely inhibited. The type of relation between specific growth rate and initial substrate concentration could be linear (n = 1), non-linear (concavity upward) (n > 1) or a concavity downward (n < 1) depending on the value of constant parameter (n).

Han and Levenspiel model is extension of the Monod's model to account for cell, product and substrate inhibition, which is able to explain the type of inhibition as competitive, non-competitive or uncompetitive depending upon the values of two constant parameters (n, m).[31] This model assumes a value for critical inhibitory concentration of substrate above which growth completely ceases.

The Haldane growth model is widely accepted due to its mathematical simplicity and wide accep-

tance for representing the growth kinetics of inhibitory substrates, incorporating both substrate affinity constant and the substrate-inhibition constant.[32] When the inhibition constant is infinitely large, the kinetics follows simple Monod's model.

Moser model is a modified Monod equation with power function of substrate concentration.[33] The value of the power determines the degree of inhibition. However, it does not indicate any critical substrate concentration or inhibition constant.

The Aiba growth inhibition model was originally proposed for product inhibition in alcohol fermentation, where specific growth rate decreases as the product concentration increases.[34] Exponential term to take care of the product inhibition could be well replaced with substrate concentration. Aiba's exponential model, though has been widely used to analyze product inhibition, fails to give the critical value of inhibitory substrate/product concentration.

The parameters of different growth models were estimated from experimental results, using GraphPad PRISM® software. Since the models had non-linear coefficients, the parameters were estimated iteratively with non-linear least square algorithm.

Analytical procedures

Because of the presence of cellulose, cell dry mass concentration could not be measured directly. Intracellular protein concentration was measured instead.[35] Samples were centrifuged, washed twice with distilled water. The cells were then lysed in 3 mL of c = 0.2 mol L^{-1} NaOH, at 100 °C for 20 min. The protein concentration of the lysate was then measured by the standard Lowry method.[36] Relationship between the intracellular protein concentration and cell dry mass concentration was established in batch fermentation with glucose as the sole carbon source. Samples taken at different stages of the fermentation were analyzed for both cell dry mass concentration and intracellular protein concentration. The relationship was established as:

$$\text{Cell dry mass concentration (g L}^{-1}) = \\ = \text{Intracellular protein content (g L}^{-1}) \cdot 8.0 \quad (1)$$

Cellulase assay was performed by earlier reported method.[37] In a reaction mixture containing 500 μL of w = 2 % sodium carboxy methyl cellulose in c = 150 mmol L^{-1} citrate buffer (pH 4.8), 500 μL of enzyme preparation was added and incubated at 50 °C for 30 min on stirred conditions. The reaction was terminated by adding 1 mL of potassium-sodium tartarate to the reaction mixture. A control was taken where the enzyme was deactivated by adding 1 mL of potassium-sodium tartarate before beginning the incubation. 1 mL of 3,5-dinitro-salicylic acid reagent was added to each tube and boiled for 5 min in boiling water bath, cooled to 30 °C and then 5 mL of distilled water was added. Intensity of color formed due to the release of glucose was measured at 540 nm by a spectrophotometer. One unit of cellulase activity was defined as the amount of enzyme needed to liberate 1 μmol of glucose equivalent reducing sugars per minute under assay conditions.

While cellulose was estimated by the method of Viles and Silverman, where acid hydrolysis of cellulose and subsequent anthrone reagent treatment and colorimetric measurement at 630 nm are the key steps.[38] As in this present study we are not concerned about product inhibition kinetics, no attempt was made to estimate concentration of glucose formed in the culture medium.

Results and discussions

Effect of initial substrate concentration on specific growth rate

The cell growth increased with increase in initial concentration of cellulose for both shake flask and bioreactor. For all initial cellulose concentrations in shake flask there was a lag of about 2 h, but time to reach stationary phase increased with increasing initial cellulose concentration. The specific growth rate as a function of initial substrate concentration for both shake flask and bioreactor is presented in Fig. 1. It was inferred that above initial substrate concentration γ_S = 4 g L^{-1}, the specific growth rate (μ) has a continuously decreasing trend, indicating possibility of substrate inhibition kinetics.

Complete utilization of cellulose was observed within 6 h for all initial cellulose concentration except for γ_{S_0} = 12 g L^{-1}, where it took 8 h for complete utilization. Using the substrate uptake profile and biomass profile of the bacteria at different ini-

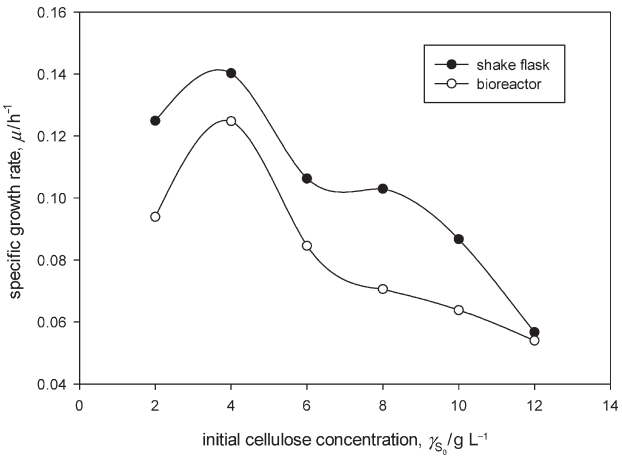


Fig. 1 – *Specific growth rate as a function of initial substrate (cellulose) concentration*

Table 2 – *Yield and specific cellulose uptake rate at different initial substrate concentrations in reactor study*

γ_{S_0}/g L^{-1}	2	4	6	8	10	12
$Y_{X/S}$	0.61	0.36	0.17	0.10	0.07	0.07
q_S/h^{-1}	0.12	0.23	0.38	0.52	0.66	0.52

tial substrate concentration the yield and specific cellulose uptake rate was determined. The results obtained are presented in Table 2. The highest yield of $Y_{X/S}$ = 0.61 was obtained at initial substrate concentration of γ_{S_0} = 2 g L^{-1} comparable with results of Rajoka and Malik who calculated yield of 0.5 g g^{-1} carbohydrate utilized.[18]

Enzyme activity at different initial substrate concentration

Extracellular enzyme activity was measured at different initial substrate concentrations both in shake flask and bioreactor operated under batch mode. The results are presented in Fig. 2 and Fig. 3 for shake flask and bioreactor respectively. The highest volumetric enzyme activity was observed at initial substrate concentration of γ_{S_0} = 12 g L^{-1} and 4 g L^{-1} in shake flask and bioreactor respectively. Ortega *et al.* in their CMC degradation kinetic study have found the typical saturation kinetics for the enzyme where at cellulose concentration above 0.5 % substrate inhibition was observed.[25] Such kinds of inhibition are common in enzymatic hydrolysis of cellulose.[39]

Mathematical modeling of growth kinetics of *Cellulomonas cellulans*

The growth kinetics of *Cellulomonas cellulans* was modeled using the substrate inhibition models and the parameters estimated for both shake flask and bioreactor are presented in Table 3. The variation of experimental specific growth rate as a function of initial substrate concentration and the fitted curves is presented in Fig. 4 and Fig. 5 for shake flask and bioreactor respectively. Parity plot showing estimated specific growth rate by different mod-

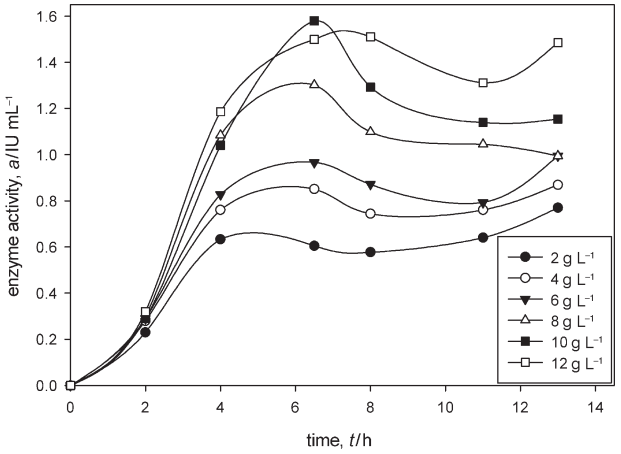


Fig. 2 – *Volumetric enzyme activity obtained at different initial substrate concentrations in shake flask*

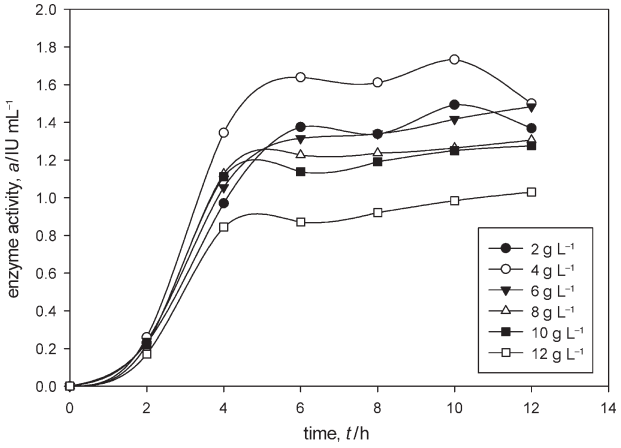


Fig. 3 – *Volumetric enzyme activity obtained at different initial substrate concentrations in bioreactor*

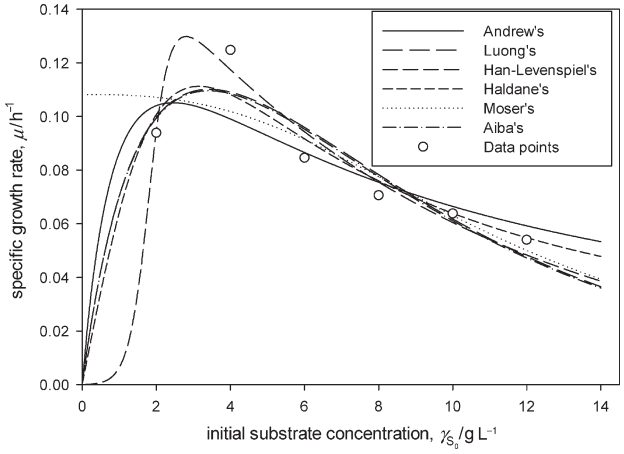


Fig. 4 – *Specific growth rate as a function of initial substrate (cellulose) concentration in bioreactor*

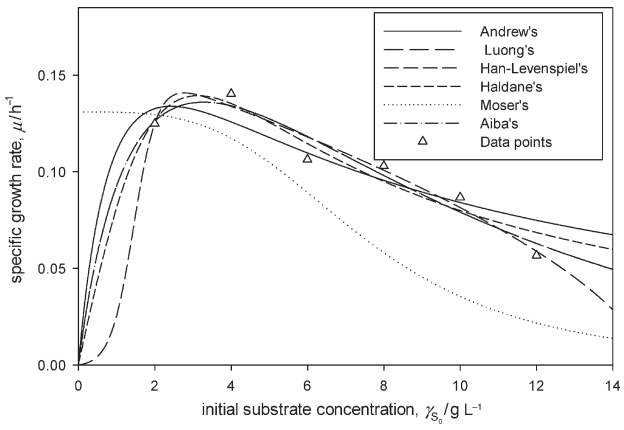
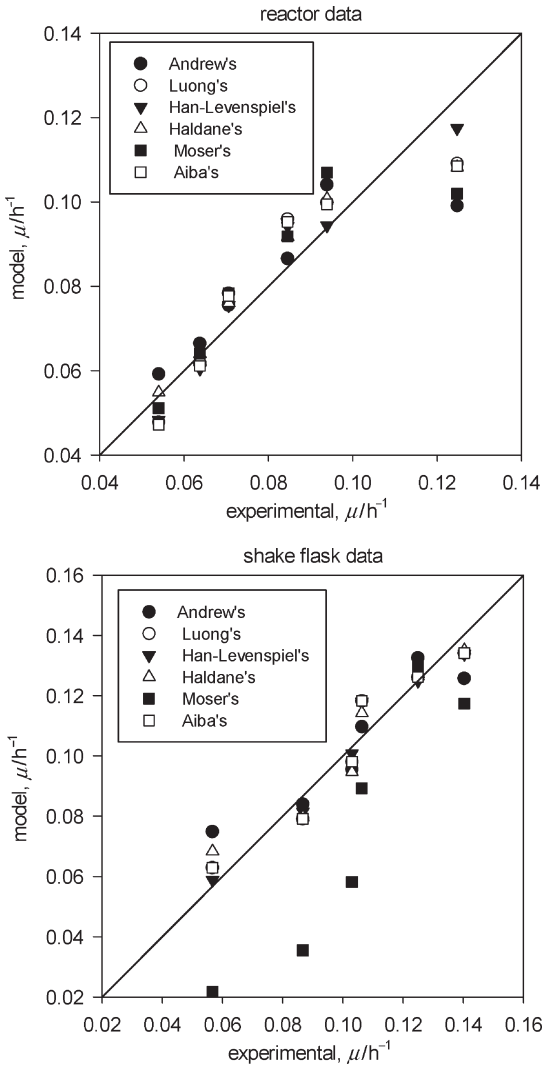


Fig. 5 – *Specific growth rate as a function of initial substrate (cellulose) concentration in shake flask*

T a b l e 3 – *Estimated parameter of different models in shake flask and reactor*

Model	System	Parameter	R^2
Andrew's	Shake flask	μ_{max} = 0.536, K_s = 2.43, K_i = 2.42	0.843
	Bioreactor	μ_{max} = 0.412, K_s = 2.40, K_i = 2.5	0.745
Luong's	Shake flask	μ_{max} = 0.367, K_s = 2.48, γ_s^* = 1681, n = 220.1	0.9297
	Bioreactor	μ_{max} = 0.385, K_s = 3.7, γ_s^* = 103736, n = 15718	0.8215
Han-Levenspiel	Shake flask	μ_{max} = 0.162, γ_s^* = 14.91, n = 0.622, m = 34.98, K_M = 58.73	0.9496
	Bioreactor	μ_{max} = 0.182, γ_s^* = 388.5, n = 42.27, m = 1202, K_M = 544.6	0.9333
Haldane	Shake flask	μ_{max} = 11.29, K_s = 125.3, K_i = 0.078	0.9193
	Bioreactor	μ_{max} = 27.52, K_s = 388.3, K_i = 0.0256	0.8753
Moser	Shake flask	μ_{max} = 0.131, K_s = 0.001, n = –3.43	0.9092
	Bioreactor	μ_{max} = 0.108, K_s = 0.0015, n = –2.66	0.7478
Aiba	Shake flask	μ_{max} = 0.369, K_s = 2.49, K_i = 7.59	0.9296
	Bioreactor	μ_{max} = 0.383, K_s = 3.69, K_i = 6.569	0.8412



F i g . 6 – *Parity plot for the predictions of specific growth rate by various substrate inhibition models*

els that fit to the entire data versus experimental specific growth rate is shown in Fig. 6. The best fit is obtained for Han-Levenspiel model in both shake flask and bioreactor with the R^2 value is 0.9496 and 0.9333 respectively. According to the Han-Levenspiel model, the maximum substrate concentration at which the culture ceased to grow was 388.5 g L^{-1} and 14.91 g L^{-1} of cellulose (CMC) in bioreactor and shake flask respectively. This large difference indicates the better performance of reactor system, when substrate inhibition kinetics is associated with growth.

Conclusion

Cellulomonas cellulans NRRL B 4567 can utilize cellulose, as the sole carbon source by secreting cellulase, for the cellulose concentration between 2 to 12 g L^{-1}. The growth kinetics of *Cellulomonas cellulans* NRRL B 4567 does not follow a simple Monod's kinetics. Substrate inhibition is exhibited in batch experiments carried out in both shake flask and bioreactor and it was found that Han-Levenspiel model was able to describe the growth kinetics. The maximum yield of this bioprocess in batch fermentation was found to be 0.6109 g g^{-1} at γ_{S_0} = 2 g L^{-1} for the initial cellulose concentration between γ_{S_0} = 2 to 12 g L^{-1}, while the maximum possible specific growth rate, which can be achieved, was found to be μ = 0.15 h^{-1} at initial cellulose concentration of γ_{S_0} = 3.2 g L^{-1} in shake flask and μ = 0.13 h^{-1} at γ_{S_0} = 3.5 g L^{-1} in bioreactor.

List of symbols

a – enzyme activity, IU mL^{-1}
c – concentration, mol L^{-1}
K_i – inhibition constant for cellulose, g L^{-1}
K_s – half saturation constant for cellulose, g L^{-1}
k – rate coefficient, min^{-1}
K_M – Monod's constant, g L^{-1}
r – reaction rate, mol L^{-1} min^{-1}
q_s – specific cellulose uptake rate, h^{-1}
w – mass fraction, %
$Y_{X/S}$ – yield, (biomass (g mL^{-1}) formed/substrate (g mL^{-1}) utilized)
t – time, h, min

Greek letters

γ_S – cellulose concentration, g L^{-1}
γ_{S_0} – initial cellulose concentration, g L^{-1}
γ_S^* – critical cellulose concentration, g L^{-1}
γ_X – biomass concentration, g L^{-1}
μ – specific growth rate of biomass, h^{-1}
μ_{max} – maximum specific growth rate of biomass, h^{-1}
θ – temperature, °C

References

1. *Olsson, L., Hahn-Hägerdal, B.,* Enzyme Microb. Technol. **18** (1996) 312.
2. *Luo*, *J.*, *Xia*, *L. M.*, *Lin*, *J. P.*, *Cen*, *P. L.*, Biotechnol. Progress. **13** (1997) 762.
3. *Cao*, *N. J.*, *Xia*, *Y. K.*, *Gong*, *C. S.*, *Tsao*, *G. T.*, Appl. Biochem. Biotechnol. **63–65** (1997) 129.
4. *Lee*, *R. L.*, *Paul*, *J. W.*, *Willem*, *H. V. Z.*, *Isak*, *S. P.*, Microbiol. Mol. Biol. Rev. **66** (2002) 506.
5. *Lawford*, *H. G.*, *Rousseau*, *J. D.*, Appl. Biochem. Biotechnol. **105** (2003) 457.
6. *El-Hawary*, *F. I.*, *Mostafa*, *Y. S.*, *Laszlo*, *E.*, Acta. Aliment. Hung. **30** (2003) 281.
7. *Taguchi*, *F.*, *Yamada*, *K.*, *Hasegawa*, *K.*, *Taki-Saito*, *T.*, *Hara*, *K.*, J. Ferm. Bioeng. **82** (1996) 80.
8. *Bhat*, *M. K.*, Biotechnol. Adv. **18** (2000) 355.
9. *Wen*, *Z.*, *Liao*, *W.*, *Chen, S.*, Process Biochem. **40** (2005) 3087.
10. *Wen*, *Z.*, *Liao*, *W.*, *Chen, S.*, Bioresour. Technol. **96** (2005) 491.
11. *Domingues*, *F. C.*, *Queiroz*, *J. A.*, *Cabral*, *J. M. S.*, *Fonseca*, *L. P.*, Enzyme Microb. Technol. **26** (2000) 394.
12. *Weber*, *J.*, *Agblevor*, *F. A.*, Process Biochem. **40** (2005) 669.
13. *Ikeda*, *Y.*, *Hayashi*, *H.*, *Okuda*, *N.*, *Park*, *E. Y.*, Biotechnol. Prog. **23** (2007) 333.
14. *Suto*, *M.*, *Tomita*, *F.*, J. Biosci. Bioeng. **92** (2001) 305.
15. *Hanif*, *A.*, *Yasmeen*, *A.*, *Rajoka*, *M. I.*, Bioresour. Technol. **94** (2004) 311.
16. *Akila*, *G.*, *Chandra*, *T. S.*, FEMS Microbiol. Lett. **219** (2003) 63.
17. *Kang*, *S. W.*, *Park*, *Y. S.*, *Lee*, *J. S.*, *Hong*, *S. I.*, *Kim*, *S. W.*, Bioresour. Technol. **91** (2004) 153.
18. *Rajoka*, *M. I.*, *Malik*, *K. A.*, Bioresour. Technol. **59** (1997) 21.
19. *Chaudhary*, *P.*, *Kumar*, *N. N.*, *Deobagkar*, *D. N.*, Biotechnology Advances **15** (1997) 315.
20. *Sánchez-Herrera*, *L. M.*, *Ramos-Valdivia*, *A. C.*, *de la Torre*, *M.*, *Salgado*, *L. M.*, *Ponce-Noyola*, *T.*, Appl. Microbiol. Biotechnol. **77** (2007) 589.
21. *Ponce-Noyola*, *T.*, *de la Torre*, *M.*, Bioresour. Technol. **78** (2001) 285.
22. *Sami*, *A. J.*, *Akhtar*, *M. W.*, Enzyme Microb. Technol. **15** (1993) 586.
23. *O'Dwyer*, *J. P.*, *Zhu*, *L.*, *Granda*, *C. B.*, *Holtzapple*, *M. T.*, Bioresour. Technol. **98** (2007) 2969.
24. *Al-Zuhair*, *S.*, Bioresour. Technol. (*in press*) Available online 1 November 2007.
25. *Ortega*, *N.*, *Busto*, *M. D.*, *Perez-Mateos*, *M.*, Int. Biodeterior. Biodegradation **47** (2001) 7.
26. *Hari Krishna*, *S.*, *Sekhar Rao*, *K. C.*, *Suresh Babu*, *J.*, *Srirami Reddy*, *D.*, Bioprocess Eng. **22** (2000) 467.
27. *Jørgensen*, *H.*, *Mørkeberg*, *A.*, *Krogh*, *K. B. R.*, *Olsson*, *L.*, Enzyme Microb. Technol. **36** (2005) 42.
28. *Jørgensen*, *H.*, *Olsson*, *L.*, Enzyme Microb. Technol. **38** (2006) 381.
29. *Andrews*, *J. F.*, Biotechnol. Bioeng. **10** (1968) 707.
30. *Luong*, *J. H. T.*, Biotechnol. Bioeng. **29** (1987) 242.
31. *Han*, *K.*, *Levenspiel*, *O.*, Biotechnol. Bioeng. **32** (1988) 430.
32. *Haldane*, *J. B. S.*, Enzymes, MIT Press, Cambridge, 1965, pp. 84.
33. *Moser*, *A.*, Kinetics of batch fermentations, in *Rehm*, *H. J.*, *Reed*, *G.* (Eds.), Biotechnology Fundamentals of Biochemical Engineering, Verlaag Chemie, Weinheim, 1985, pp. 243–283.
34. *Aiba*, *S.*, *Shoda*, *M.*, *Nagatani*, *M.*, Biotechnol. Bioeng. **10** (1968) 845.
35. *Zhang*, *Q.*, *Lo*, *C. M.*, *Ju, L. K.*, Bioresour. Technol. **98** (2007) 753.
36. *Lowry*, *O. H.*, *Rosebrough*, *N. J.*, *Farr*, *A. L.*, *Randall*, *R. G.*, J. Biol. Chem. **193** (1951) 265.
37. *Miller*, *G. L.*, Anal. Chem. **31** (1959) 426.
38. *Viles Jr*, *F. J.*, *Silverman*, *L.*, Anal. Chem. **21** (1949) 950.
39. *Zhang*, *S.*, *Wolfgang*, *D. E.*, *Wilson*, *D. B.*, Biotechnol. Bioeng. **66** (1999) 35.