Full Length Article

Enhanced Production of Thermophilic Xylanase by Recombinant *Escherichia coli* DH5α through Optimization of Medium and Dissolved Oxygen Level

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ABSTRACT

Enhancement of thermophilic xylanase production by recombinant *Escherichia coli* DH5α through suitable medium formulation was initially investigated using shake flask cultures. Thereafter the effect of dissolved oxygen tension (DOT) level on the performance of xylanase fermentation by *E. coli* DH5α was investigated in 2 L stirred tank bioreactor using the optimal medium. Among the two basal medium tested (complex medium of Luria Bertani & defined mineral medium), defined mineral medium gave the highest growth and xylanase production. The optimal glucose and $(NH_4)_2SO_4$ for xylanase production was obtained at 10 g L^{-1} and 2 g L^{-1}, respectively. Growth of *E. coli* DH5α and xylanase production was inhibited in oxygen limited fermentation, where dissolved oxygen tension level was controlled at 0% saturation. On the other hand, xylanase production was enhanced at DOT level controlled at 20% saturation, though growth was not significantly improved. Substantially high xylanase production (1784.57 U mL^{-1}) was obtained in fermentation using optimal medium composition and DOT level. These results indicate that efficient process control strategy is important for the mass production of xylanase enzyme by *E. coli* DH5α. © 2010 Friends Science Publishers

Key Words: Thermophilic xylanase; Xylan-degrading enzymes; Batch fermentation; *E. coli* DH5α; Kinetic; Modeling

INTRODUCTION

Interest in xylan-degrading enzymes has risen owing to the applications in kraft pulp processing and bioremediation of lignocellulosic materials. Xylanase in conjunction with other accessory enzymes, act synergistically to degrade xylan to component sugars. In nature, xylanases are readily produced by filamentous fungi such as *Aspergillus* spp. (Haq *et al*., 2004) and *Trichoderma* spp. (Seyis & Aksoz, 2005). Thermostable xylanase enzymes are also secreted by some bacterial species such as *Bacillus stearothermophilus*, *B. subtilis* (Saleem *et al.*, 2002) and *Paenibacillus* spp. (Khianngam *et al*., 2009). More recently, novel sources of acidophilic xylanase had been identified from the gut environment of subterranean termites, *Reticulitermes flavipes* (Smith *et al*., 2009) and brackish-water clam, *Corbicula japonica* (Sakamoto & Toyohara, 2009). The regulation of xylanase biosynthesis in fungi or bacteria requires certain inducers i.e., xylan or low molecular weight fragments as in xylose or sucrose supplemented by amino acids (Beg *et al*., 2001; Saleem *et al.*, 2002). Despite its complex regulation of xylanase synthesis, fungal xylanases are generally associated with cellulases, which are not favorable for industrial processes. Thus the most striking alternative is to employ recombinant strain, whereby xylanase may be produced constitutively without the presence of any inducers and free from other contaminating enzymes such as cellulases, mannanase and proteases. In addition, due to their stability at high temperatures and ease of bioseparation, the introduction of thermostable enzymes into the protein population of a mesophile by cloning offers a clear opportunity to use heat treatment method for purification (Schofield & Daniel, 1993). Heat treatment precipitated the labile host proteins and inactivated the mesophilic enzyme activities, which in turn facilitate the separation of the target thermophilic proteins or enzymes (Patchett *et al.*, 1989).

E. coli is the first-line system for producing recombinant proteins, because of its favorable traits i.e., readily available host-vector systems, ease in cultivation and high growth rate (Zhao *et al.*, 2005). Commercially, *E. coli* is classified as a generally recognized safe organism and proven to be an economical host cell line for producing proteinaceous products. However not all proteins are accumulated to maximal levels in *E. coli* and the production of the target proteins requires medium and bioprocess

optimization. Defined mineral media have been used for high cell density culture of *E. coli* (Yee & Blanch, 1992). A drawback of using minimal media for the production of recombinant proteins may be the misincorporation of amino acids. On the other hand, complex media usually support higher specific growth rate of the microorganism and decrease the metabolic burden.

The objective of the present work was to formulate suitable medium for growth of *E. coli* DH5α and xylanase production. Oxygen requirements during fermentation were also investigated using stirred tank bioreactor. The fermentation data were then evaluated with the proposed models to generate kinetic parameters values.

MATERIALS AND METHODS

Microorganism and inoculum preparation: The recombinant *E. coli* DH5α, has been constructed to secrete intracellular xylanolytic enzymes (Puspaningsih *et al.*, 2008). This strain encodes genes for exo-xylanase (exo-xyn), α-L-arabinofuranosidase (abfa) and β-xylosidase (xyl) from *B. thermoleovorans* IT-08, isolated from Gunung Pancar Hot Spring, Bogor-West Java, Indonesia.

The cell culture was streaked on the Luria Bertani (LB) agar plate containing 100 μg mL^{-1} ampicillin and incubated overnight at 37°C. A single cell colony picked from the plate was then inoculated into 100 mL LB medium with the addition of 100 μg mL^{-1} ampicillin in Erlenmeyer flasks (250 mL). The inoculated flasks were incubated at 37°C in rotary shaker (200 rpm) for 12 h. This culture was used as standard inoculum for all fermentations.

Media: Initially the feasibility of two different media (complex medium of LB & defined mineral medium) on growth of *E. coli* DH5α and xylanase production was investigated. Complex LB medium consisted of (g L^{-1}): 10 tryptone, 5 yeast extract and 5 NaCl. Defined mineral medium consisted of (g L^{-1}): 15 glucose, 11.9 K$_2$HPO$_4$, 2.4 KH$_2$PO$_4$, 1.8 NaCl, 3 (NH$_4$)$_2$SO$_4$, 0.11 MgSO$_4$.7H$_2$O, 0.01 FeCl$_3$ and 0.72 mL L^{-1} of trace elements solution (Pinsach *et al.*, 2008). Each medium was supplemented with glucose and 100 μg mL^{-1} ampicillin.

For investigation on different initial concentrations of glucose and (NH$_4$)SO$_4$, the defined mineral medium was employed using shake flask fermentation. The 250 mL Erlenmeyer flasks containing 50 mL medium were inoculated with 10% (v/v) inoculum. The flasks were incubated on a rotary shaker at 37°C and agitated at 200 rpm.

Subsequent studies on the effect of DOT level were carried out in 2 L stirred tank bioreactor (Biostat® B, Sartorius, Germany) equipped with a single Rushton turbine impeller. Fermentation medium consisted of (g L^{-1}): 25 glucose, 11.9 K$_2$HPO$_4$, 2.4 KH$_2$PO$_4$, 1.8 NaCl, 3 (NH$_4$)$_2$SO$_4$, 0.45 MgSO$_4$.7H$_2$O, 0.02 FeCl$_3$ and 2.86 mL L^{-1} of trace elements solution. The trace elements solution (TES) composition contained (g L^{-1}): 1.44 CaCl$_2$.2H$_2$O, 0.042 AlCl$_3$.6H$_2$O, 0.87 ZnSO$_4$.7H$_2$O, 0.16 CoCl$_2$.6H$_2$O,

1.6 CuSO$_4$, 0.01 H$_3$BO$_3$, 1.42 MnCl$_2$.4H$_2$O, 0.01 NiCl$_2$.6H$_2$O and 0.02 Na$_2$MoO$_4$.H$_2$O.

The bioreactor containing 900 mL of sterilized medium was inoculated with 10% (v/v) inoculum. Fermentation was controlled at 37°C and pH was maintained at 7.00 ± 0.05. DOT level was controlled at the required values (0, 20 & 40% saturation) by the manipulation of agitation speed, while air flow rate was fixed at 1 L min^{-1} (1 vvm). All experiments were conducted in triplicates and results given were the average values.

Analytical methods: During fermentation, samples were withdrawn at time intervals and centrifuged at 10,000 rpm, 4°C, for 10 min (Centrifuge 5810 R, Eppendorf, Germany). The cell pellets obtained were used to determine cell growth and enzyme activity. The cells pellet was resuspended in 0.9% (w/v) NaCl and the OD was measured at 600 nm using spectrophotometer (Mini UV-1240, Shimadzu, Japan). The cell suspensions were then filtered through dry membrane filter and then dried in an oven for 24 h at 80°C, for measurement of dry cell weight (DCW). Correlation between DCW and OD was estimated from several experiments, which indicated that one OD unit was approximately equivalent to 0.5 g DCW L^{-1}.

To extract intracellular xylanase, the cell pellet was washed with 0.9% (w/v) NaCl and resuspended in phosphate citrate buffer (pH 7). The cell suspension was subjected to sonication (XL-2020, Heat System, New York) at 20 kHz, 15 s for cell disruption. The disrupted cell suspension was then incubated at 70°C for 1 h and then recentrifuged to obtain cell free extract or crude enzyme sample. For xylanase activity assay, a mixture of 0.2 mL of substrate (1% (w/v) oat spelt xylan in citrate phosphate buffer, pH 7) and 0.2 mL of crude enzyme was incubated at 70°C in a shaking water bath for 1 h. The reaction was stopped by adding 1 mL of DNS (3,5-dinitrosalicylic acid) reagent and the reducing sugar produced from the reaction was measured using modified DNS method (Miller, 1959). The absorbance of the reducing sugar was read at 540 nm. One unit of xylanase activity is the amount of enzyme in 1 mL sample solution producing 1 μg mL^{-1} of reducing sugar (xylose) after incubation for 1 min under the given experimental conditions. Residual glucose in the culture supernatant was analyzed using biochemistry analyzer (YSI 2700 Select Biochemistry Analyzer, YSI, Ohio).

Mathematical methods: The batch kinetic models based on logistic and Luedeking-Piret equations (Weiss & Ollis, 1980) were used to model the cells growth, substrate consumption rate and xylanase production.

E. coli DH5α cell growth:

$$dX/dt = \left[\mu_{max} \left(1 - X/X_{max} \right) \right] X \qquad [1].$$

Substrate consumption:

$$-dS/dt = \alpha \left(dX/dt \right) + \beta X \qquad [2].$$

Product formation:

$$dP/dt = m(dX/dt) + nX \qquad [3].$$

Where X is cell concentration (g L^{-1}), X_{max} is the maximum cell concentration (g L^{-1}), μ_{max} is maximum specific growth rate (h^{-1}), S is the substrate concentration (g L^{-1}), α is growth associated constant for substrate consumption (g substrate g $cell^{-1}$), β is the non-growth associated constant for substrate consumption (g substrate g $cell \cdot h^{-1}$), m is the growth associated constant for product formation (U xylanase g $cell^{-1}$), n is the non-growth associated constant for product formation (U xylanase g $cell \cdot h^{-1}$) and t is the fermentation time (h).

The kinetic models (Equations 1-3) were fitted to the experimental data by the non-linear least square regression via Sigmaplot Ver. 11 (Systat Inc, USA) to solve for the unknown kinetic parameter values. The predicted values were then used to simulate the profiles of cells, substrate and enzymes concentrations during fermentation. To determine the significance of deviations between the experimental and calculated data, unpaired t-test analysis of the SAS Institute Inc (1988) was used with a significance probability of 5%.

The cell yield during fermentation, $Y_{x/s}$, is determined by Equation [4]:

$$Y_{x/s} = (X_{max} - X_i)/(S_i - S_o) \qquad [4].$$

Where X_i is the initial cell concentration (g L^{-1}), S_i is the initial substrate concentration (g L^{-1}) and S_o is the residual substrate concentration at the end of the fermentation (g L^{-1}).

Overall productivity, P (g $L^{-1}h^{-1}$) was determined by measuring the total amount of biomass formed over a period of cultivation time as shown in Equation [5]:

$$P = (X_{max} - X_i)/t \qquad [5].$$

RESULTS

Time course and modeling of batch xylanase fermentation by *E. coli* DH5α: Typical time course of batch fermentation by *E. coli* DH5α using glucose as a carbon source is shown in Fig. 1, which also includes the fitness of the calculated data according to Equations 1-3 to the experimental data. Growth of *E. coli* DH5α was very rapid from inoculation to 14 h of fermentation, where lag phase was not observed and growth reached a stationary phase after about 16 h. Glucose was consumed at very high rate during exponential growth phase and depletion of glucose was observed when growth reached a stationary phase. Xylanase production was increased concomitantly with growth, suggesting that the process is growth associated. DOT level decreased rapidly during the initial growth phase and became 0% saturation after 5 h, indicating that the fermentation was oxygen limited. The culture pH was maintained at around 7 throughout the fermentation process.

From t-test analysis, the deviations between the calculated and experimental data of cell concentration, glucose concentration and xylanase production in the culture are not significant at significance probability of 5%. This result suggests that the proposed models based on logistic and Luedeking-Piret equations are sufficient to describe growth of *E. coli* DH5α, glucose consumption and xylanase production.

Effect of basal medium on the xylanase production by *E. coli* DH5α: Growth of *E. coli* DH5α and xylanase production using two different basal media is shown in Table I. Although higher final cell concentration (3.42 g L^{-1}) was obtained in fermentation using complex (LB) medium than fermentation using a defined mineral medium (1.73 g L^{-1}), xylanase production (127.67 U mL^{-1}) was significantly reduced. High xylanase production (324.72 U mL^{-1}) obtained in fermentation using defined medium indicated that the presence of simple sugars and compounds which can be utilized easily by the bacterium would enhanced the cell built-up with high ability to express the gene for xylanase production. The measure of cell efficiency (P_{max}/X_{max}), calculated as xylanase activity per unit cell weight (187.92 U mg $cell^{-1}$) was about six times higher for fermentation using a defined medium as compared to that obtained in complex medium (37.32 U mg $cell^{-1}$). Although the cells yield ($Y_{x/s}$) for a defined medium was lower compared to complex medium, the enzyme product yield ($Y_{p/s}$) was higher. In addition, μ_{max} for growth in complex medium is about 50% higher compared to growth in defined medium.

Effect of initial glucose concentration on xylanase production by *E. coli* DH5α: The profile of cell concentration, glucose and xylanase activity for xylanase fermentation by *E. coli* DH5α using different concentrations of glucose are shown in Fig. 2, which also shows the fitness of the calculated data according to the proposed models to the experimental data. Summary of the fermentation performance and the kinetic parameter values is shown in Table II. The maximum cell concentration (X_{max}) increased with increasing glucose concentration from 0 to 25 g L^{-1}, though the maximum specific growth rate (μ_{max}) was not significantly different. However the highest xylanase production (1714.24 U mL^{-1}) was obtained at 10 g L^{-1} glucose. Reduced xylanase production with higher glucose concentration (> 15 g L^{-1}) was observed. The highest overall xylanase productivity (107.08 U $mL \cdot h^{-1}$), xylanase yield (0.17 U g $glucose^{-1}$) and P_{max}/X_{max} (370.90 U mg $cell^{-1}$) were obtained at 10 g L^{-1} glucose.

The proposed models (Equations 1-3) also fitted well to the experimental data. The highest value of growth associated constant (α) was obtained at 10 g L^{-1} glucose, which corresponded well to the highest efficiency of the cell in producing xylanase. In all cases, the value of non-growth associated constant was zero, confirming the initial assumption that xylanase production by *E. coli* DH5α is in fact growth-associated process.

Table I: Comparison of the performance and the kinetic parameter values of xylanase production in batch culture by *E. coli* DH5α using two different types of basal medium

Kinetic parameter values	Complex medium (Luria Bertani, LB)	Defined medium (Pinsach et al., 2008)
Maximum specific growth rate, μ_{max} (h^{-1})	1.027	0.600
Maximum cell concentration, X_{max} ($g\,L^{-1}$)	3.42	1.73
Maximum xylanase activity, P_{max} ($U\,mL^{-1}$)	127.67	324.72
Cell efficiency, P_{max}/X_{max} ($U\,mg\,cell^{-1}$)	37.32	187.92
Growth-associated constant, α ($U\,g\,cell^{-1}$)	27.92	108.31
Non-growth associated constant, β ($U\,g\,cell\cdot h^{-1}$)	0	0
Cell yield, $Y_{x/s}$ (g cell g glucose^{-1})	0.13	0.07
Product yield, $Y_{p/s}$ (U g glucose^{-1})	5.03	7.55
Overall biomass productivity ($g\,L\cdot h^{-1}$)	0.23	0.14
Overall xylanase productivity ($U\,mL\cdot h^{-1}$)	6.29	11.79
t (h)	20	16

Table II: Comparison of performance and kinetic parameter values of xylanase production in batch fermentation of *E. coli* DH5α using defined medium with different concentrations of glucose in shake flask fermentation

Kinetic parameter values	Glucose concentration ($g\,L^{-1}$)					
	0	5	10	15	20	25
Maximum specific growth rate, μ_{max} (h^{-1})	1.42	0.61	0.48	0.55	0.39	0.41
Maximum biomass concentration, X_{max} ($g\,L^{-1}$)	0.26	2.88	4.62	5.79	6.01	7.01
Maximum xylanase activity, P_{max} ($U\,mL^{-1}$)	0.26	885.34	1714.24	1341.89	1354.38	1176.76
P_{max}/X_{max} ($U\,mgcell^{-1}$)	0.99	307.66	370.98	231.95	225.33	167.81
Growth-associated constant, α ($U\,g\,cell^{-1}$)	0.19	261.55	342.81	232.67	251.39	198.50
Non-growth associated constant, β ($U\,g\,cell\cdot h^{-1}$)	0	0	0	0	0	0
Cell yield, $Y_{x/s}$ (g cell g glucose^{-1})	ND	0.57	0.46	0.36	0.48	0.54
Product yield, $Y_{p/s}$ (U g glucose^{-1})	ND	0.177	0.171	0.089	0.068	0.047
Overall biomass productivity, P_x ($g\,L\cdot h^{-1}$)	0.022	0.202	0.285	0.359	0.329	0.433
Overall xylanase productivity, P_{xyl} ($U\,mL\cdot h^{-1}$)	0.01	63.21	107.08	64.99	66.92	59.52
Maintenance coefficient, m_s (g glucose. g cell^{-1})	ND	0.070	0.008	0.071	0.142	0.221
Time to reach maximum xylanase activity, t (h)	10	12	14	18	18	18

Table III: Comparison of performance and kinetic parameter values of xylanase production in batch fermentation of *E. coli* DH5α using defined medium with different concentrations of $(NH_4)_2SO_4$ in shake flask fermentation

Kinetic parameter values	$(NH_4)_2SO_4$ concentration ($g\,L^{-1}$)					
	0	1	2	3	4	5
Maximum specific growth rate, μ_{max} (h^{-1})	0.46	0.49	0.44	0.45	0.45	0.45
Maximum biomass concentration, X_{max} ($g\,L^{-1}$)	1.45	2.56	3.91	4.74	4.96	4.47
Maximum xylanase activity, P_{max} ($U\,mL^{-1}$)	568.95	929.75	1196.19	810.41	777.10	724.37
P_{max}/X_{max} ($U\,mgcell^{-1}$)	391.68	363.24	306.17	171.02	156.71	162.13
Growth-associated constant, α ($U\,gcell^{-1}$)	366.66	391.47	280.48	143.54	125.30	144.05
Non-growth associated constant, β ($U\,g\,cell\cdot h^{-1}$)	0	0	0	0	0	0
Overall biomass productivity, P_x ($g\,L\cdot h^{-1}$)	0.059	0.109	0.171	0.291	0.306	0.273
Overall xylanase productivity, $P_{xylanase}$ ($U\,mL\cdot h^{-1}$)	21.45	37.85	49.96	74.94	33.92	45.19
Time to reach maximum xylanase activity, t (h)	22	22	22	10	18	14

Effect of initial $(NH_4)_2SO_4$ concentration on xylanase production by *E. coli* DH5α: The profile of cells concentration and xylanase activity using different concentrations of $(NH_4)_2SO_4$ are shown in Fig. 3 together with the fitness of the calculated data according to the proposed models data. While Table III shows the summary of fermentation performance and the kinetic parameter values. The maximum cell concentration (X_{max}) increased significantly with increasing $(NH_4)_2SO_4$ concentration up to 4 g L^{-1}. A slight inhibition of growth was observed at 5 g L^{-1} $(NH_4)_2SO_4$. However, the highest maximum xylanase activity (1196.19 U mL^{-1}) was obtained at 2 g L^{-1} of $(NH_4)_2SO_4$. Xylanase production was inhibited at $(NH_4)_2SO_4$ concentration higher than 3 g L^{-1}.

The maximum specific growth rate (μ_{max}) did not vary significantly with increasing $(NH_4)_2SO_4$ concentration from 0 to 5 g L^{-1}. On the other hand, the cell efficiency (P_{max}/X_{max}) in producing xylanase was reduced with increasing $(NH_4)_2SO_4$ concentration, suggesting that ammonium gave inhibition effect to xylanase production by *E. coli* DH5α. This is in agreement with the variation in the value of growth associated constant for product formation (α) with variation in $(NH_4)_2SO_4$ concentration. High value of α was obtained at low $(NH_4)_2SO_4$ concentration (0 to 2 g L^{-1}) and reduced significantly from 3 g L^{-1} to 5 g L^{-1}.

Effect of dissolved oxygen tension (DOT) level on xylanase production by *E. coli* DH5α: Fig. 4 shows the profile of cell concentration and xylanase activity at

Table IV: Comparison of performance and kinetic parameter values of xylanase production in batch fermentation of *E. coli* DH5α cultivated with defined mineral medium at different dissolved oxygen tension (DOT) levels in 2 L fermenter

| Kinetic parameter values	Dissolved oxygen tension (%)			
	0	10	20	30
Maximum specific growth rate, μ_{max} (h^{-1})	0.60	0.49	0.38	0.47
Maximum biomass concentration, X_{max} (g L^{-1})	1.73	5.36	6.26	6.06
Maximum xylanase activity, P_{max} (U mL^{-1})	324.72	1654.12	1784.57	1185.08
P_{max} /X_{max} (U mgcell^{-1})	187.91	308.92	285.02	195.53
Growth-associated constant, α (U gcell^{-1})	133.19	250.47	268.16	180.52
Non-growth associated constant, β (U gcell·h^{-1})	0	0	0	0
Biomass yield, $Y_{x/s}$ (g cell g glucose^{-1})	0.111	0.355	0.417	0.599
Xylanase yield, $Y_{p/s}$ (U xylanase g glucose^{-1})	0.0217	0.1103	0.1190	0.1185
Overall biomass productivity, P_x (g L·h^{-1})	0.139	0.333	0.341	0.375
Overall xylanase productivity, $P_{xylanase}$ (U mL·h^{-1})	11.79	79.87	91.43	72.95
Time taken to reach maximum xylanase activity, t (h)	16	18	18	14

different DOT levels together with the fitness of the calculated data according to the proposed models to the experimental data. Comparison of the performance and kinetic parameter values of xylanase production by *E. coli* DH5α at different DOT levels is shown in Table IV. The X_{max} was increased almost 3 times with increasing DOT level from 0 to 10% saturation. On the other hand, X_{max} was only slightly increased with increasing DOT level from 10 to 30% saturation. However the μ_{max} value was not significantly different at various DOT levels.

Substantial increase in xylanase production, about 5 times higher, was obtained with increasing DOT level from 0% to 10% saturation. However xylanase production was not significantly different in fermentation with controlled DOT at 10% and 20% saturation. Reduced xylanase production was observed at high DOT level (30%). The highest xylanase production (1784.60 U mL^{-1}) was obtained at DOT level of 20%, which also gave the highest yield (0.12 U xylanase g glucose^{-1}) and growth associated constant (268.16 U g cell^{-1}).

DISCUSSION

One of the major factors that affect the enzyme production and level is the carbon source used in the production medium (Seyis & Aksoz, 2005). The carbon source in the medium exerted a significant effect on expression of genes for xylanase synthesis by *E. coli* DH5α. Some carbon sources supported good growth with reduced enzyme synthesis, while others supported good growth as well as enzyme secretion (Satyanarayana *et al.*, 2007). The bacterial "Crabtree effect", which occurs in the presence of glucose under aerobic condition, not only represses the formation of citric acid cycle enzymes, but also represses the formation of the plasmid-encoded product (Rinas *et al.*, 1989). High glucose level in the culture leads to "Crabtree effect", resulting in the formation of acetate and other metabolic by-products (Anderson & Von Meyenburg, 1980). Accumulation of acetate in the culture medium is concomitant with low levels of recombinant enzyme production (Meyer *et al.*, 1984). Thus, glucose in the culture
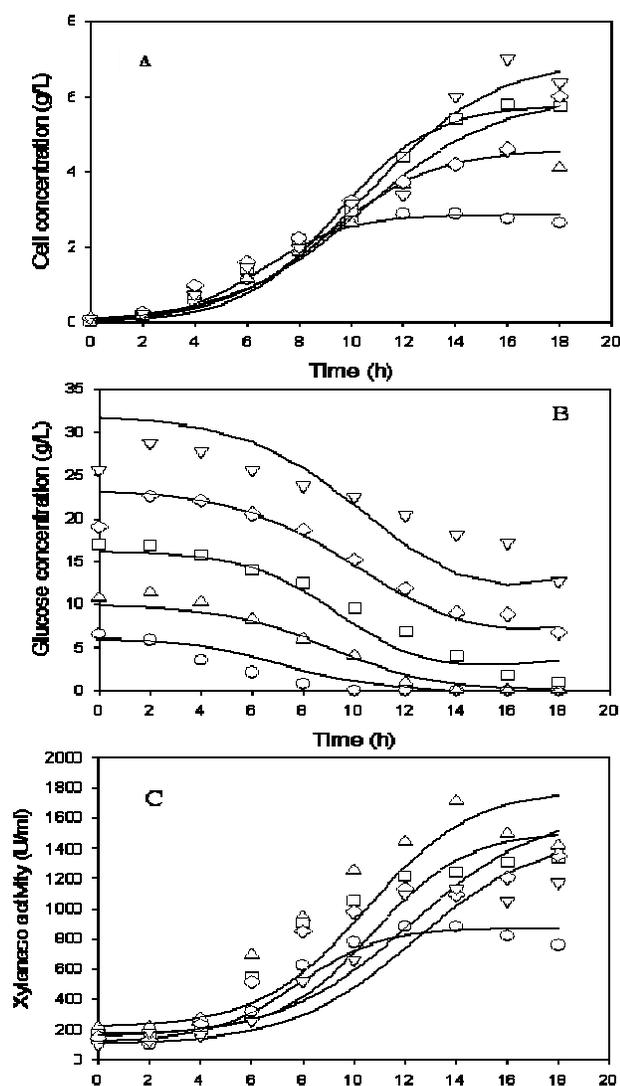
Fig. 1: Time course of batch cultivation of *E. coli* DH5α in 2 L stirred tank bioreactor, showing the comparison of calculated data to the experimental data. (○) cell concentration; (□) glucose concentration; (△) xylanase activity; (---) dissolved oxygen tension; (——) calculated data of cell, glucose concentrations and xylanase activity according to kinetic models (Equations 1–3)
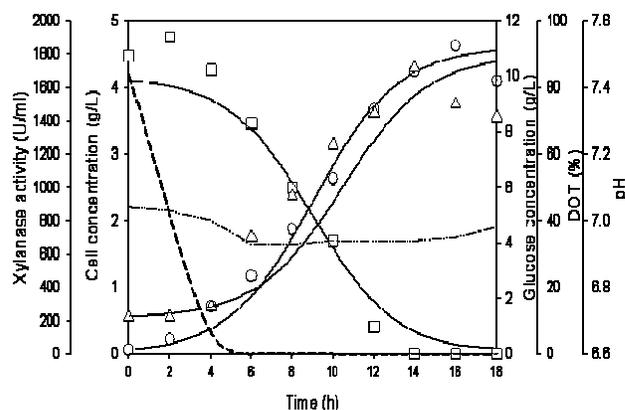
should be maintained at low level to prevent overflow metabolism of pathways, which may lead to the formation of by-products such as acetic acid and reduce the final cell concentration as well as recombinant protein production.

Acetate accumulation is one of the major considerations during growth of *E. coli* on glucose, particularly when growth is performed for recombinant protein biosynthesis, since acetate concentrations of above 2.4 g L^{-1} affect growth and possibly recombinant protein production (Kleman & Strohl, 1994). When *E. coli* is grown on glucose, acetate is produced from acetyl-CoA by phosphotransacetylase (pta) and acetate kinase (ackA) from pyruvate by pyruvate oxidase (poxB). The acetate formed can be converted back to acetyl-CoA by acetyl-CoA synthetase (acs) and by reversing the pta-ackA pathway. The acetyl-CoA is metabolized through the TCA cycle and the glyoxylate shunt pathway by isocitrate lyase (aceA) and malate synthetase (aceB). Acetate accumulation is triggered by high carbon flux through glycolysis exceeding the TCA
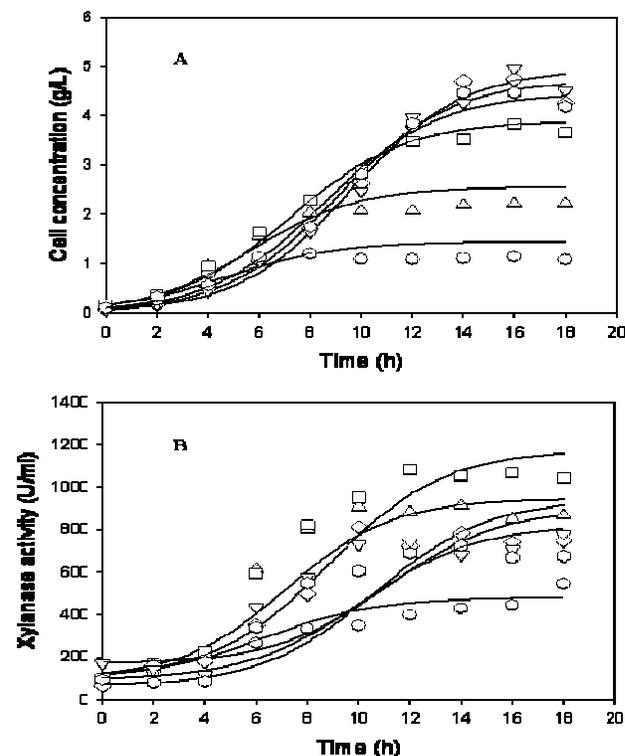
Fig. 2: Effect of different initial glucose concentrations on xylanase production by *E. coli* DH5α in shake flask culture, (A) Cell concentration; (B) Glucose concentration; (C) xylanase activity, ○ – 5 g L⁻¹; △ - 10 g L⁻¹; □ – 15 g L⁻¹; ◇ - 20 g L⁻¹; ▽ - 25 g L⁻¹; (—) Simulated data

Fig. 3: Effect of different ammonium sulphate concentration on xylanase production by *E. coli* DH5α in shake flask culture, (A) Cell concentration; (B) xylanase activity, ○ – 0 g L¹; △ - 1 g L¹; □ – 2 g L¹; ◇ - 3 g L¹; ▽ - 4 g L¹; - 5 g/L; (—) Simulated data

(1985) reported that the toxicity of ammonium to cells was observed at concentration higher than 3 g L⁻¹. Lee (1996) also agreed that ammonium concentration should not exceed 3 g L⁻¹ in the production medium to avoid reduction in the recombinant enzyme production level.

The expression of more than 200 genes by *E. coli* is dependent on the availability of oxygen (Unden *et al.*, 1995) with DOT controlled at 10 to 50% saturation is found to be optimal for the expression of heterologous proteinaceous products (Goyal *et al.*, 2009). Thus it is important to control the supply of oxygen as a key parameter for growth of facultative anaerobes. Primary metabolism of *E. coli* responds quickly to oxygen limitation, changes in pH and acetate concentration, which hinder the optimal growth conditions over a long culture period. This explains the discrepancy between cell densities obtained in stirred tank bioreactors as compared to shake flask cultivation. Agitation and aeration are generally used to meet the oxygen demand, uniform mixing and distribution of nutrients during fermentation process. Increased agitation helps to overcome resistance to the transfer of oxygen into the medium and then into the microbial cells. Providing a large gas-liquid surface area could lead to an increase in the rate of oxygen transfer in the fermentation broth but at the expense of creating higher shear effect on the circulating cells.

cycle capacity, especially when glucose is in excess. Gradual addition of glucose to the culture using fed-batch culture technique and the development of mutant *E. coli* strains with altered metabolic patterns has been implemented to limit acetate accumulations during the fermentation.

Bacteria assimilate a variety of inorganic nitrogen sources. However *E. coli* assimilates only ammonia aerobically (Reitzer, 2003). Ammonium ions enhanced the growth rate as well as improved the recombinant protein expression by mediating ammonia-assimilating enzymes (Wang *et al.*, 2009) and therefore considered as the preferred nitrogen source for *E. coli*. Thompson *et al.*
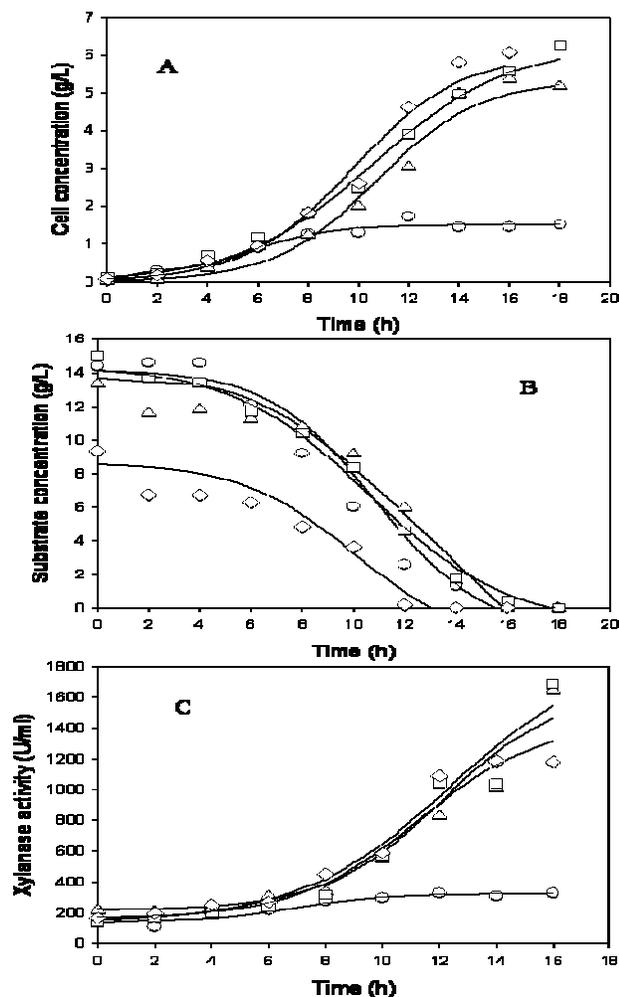
Fig. 4: Effect of different oxygen concentration on xylanase production by *E. coli* DH5α in 2 L stirred tank fermenter, (A) Cell concentration; (B) Glucose concentration; (C) xylanase activity, ○ – non-control oxygen; △ - 10 %; □ – 20%; ◇ - 30%; (—) Simulated data

LB medium consists mainly of nitrogen-containing complex compounds, the cells are forced to use these as energy sources. When LB medium is supplemented with glucose, the DOT level is crucial for the attainable intracellular product formation. Oxygen limiting conditions result in an accumulation of metabolic by-products, which cannot be degraded and consequently, in a low intracellular concentration of recombinant protein. Accumulation of intracellular enzymes was enhanced in fermentation under non-limiting oxygen.

CONCLUSION

Xylanase production by recombinant *E. coli* DH5α was significantly improved using optimal medium composition and DOT level. The defined mineral medium consisted of 10 g L^{-1} glucose and 2 g L^{-1} $(NH_4)_2SO_4$ was found optimal for xylanase production. Using this optimized medium together with DOT level controlled at 20% saturation throughout the fermentation period, the final xylanase activity of 1784.57 U mL^{-1} was obtained in the 2 L stirred tank fermenter. Most importantly, the xylanase production by *E. coli* DH5α was constitutive without the presence of any inducers. The proposed models based on logistic and Luedeking-Piret models were found sufficient to describe growth of *E. coli* DH5α and xylanase production in different medium formulations and DOT levels. Thus an efficient process control strategy is important for the mass production of xylanase enzyme by *E. coli* DH5α and the kinetic parameter values obtained in this study might be used to design the suitable fermentation mode for enhancement of the process performance. Enhanced xylanase production by *E. coli* DH5α using exponential fed-batch culture is being investigated in our laboratory.

REFERENCES

Anderson, K.B. and K. Von Meyenburg, 1980. Are growth rates of *Escherichia coli* in batch cultures limited by respiration? *J. Bacteriol.*, 144: 114–123

Beg, Q.K., M. Kapoor, L. Mahajan and G.S. Hoondal, 2001. Microbial xylanases and their industrial applications: a review. *Appl. Microb. Biotechnol.*, 56: 326–338

Goyal, D., G. Sahni and D.K. Sahoo, 2009. Enhanced production of recombinant streptokinase in *E. coli* using fed-batch culture. *Bioresour. Technol.*, 100: 4468–4474

Haq, I., M. Tasneem, K. Raana, A. Khan, H. Mukhtar and M. Javed, 2004. Optimization of cultural conditions for the production of xylanase by chemically mutated strain of *A. niger* GCBCX-20. *Int. J. Agric. Biol.*, 6: 1115–1118

Kleman, G.L. and W.R. Strohl, 1994. Acetate metabolism by *E. coli* in High-Cell-Density Fermentation. *Appl. Environ. Microbiol.*, 60: 3952–3958

Khianngam, S., S. Tanasupawat, J.S. Lee, K.C. Lee and A. Akaracharanya, 2009. *Paenibacillus siamensis* sp. nov., *Paenibacillus septentrionalis* sp. nov. and *Paenibacillus montaniterrae* sp. nov., xylanase-producing bacteria from Thai soils. *Int. J. Syst. Evol. Microbiol.*, 59: 130–134

Lee, S.Y., 1996. High cell-density culture of *E. coli*. *Trends Biotechnol.*, 14: 98–105

Meyer, H.P., C. Leist and A. Fiechter, 1984. Acetate formation in continuous culture of *E. coli* K12 D1 on defined and complex media. *J. Biotechnol.*, 1: 355–358

Miller, G.L., 1959. Use of dinitrosalicylic acid reagent for determination of reducing sugar. *Anal. Chem.*, 31: 426–428

Patchett, M.L., T.L. Neal, L.R. Schofield, R.C. Strange, R.M. Daniel and H.W. Morgan, 1989. Heat treatment purification of thermostable cellulolytic and hemicellulolytic enzymes expressed in *E. coli*. *Enz. Microb. Technol.*, 11: 113–115

Pinsach, J., C. De Mas and J. Lopez-Santin, 2008. Induction strategies in fed-batch cultures for recombinant protein production in *E. coli*: Application to rhamnulose 1-phosphate aldolase. *Biochem. Eng. J.*, 41: 181–187

Puspaningsih, N.N.T., A. Suwanto, M.T. Suhartono, S.A. Suminar, Yogiara and T. Kimura. 2008. Cloning, sequencing and characterization of the xylan degrading enzymes from *Geobacillus thermoleovorans* IT-08. *J. Basic Sci.*, 9: 177–187

Reitzer, L., 2003. Nitrogen assimilation and global regulation in *E. coli*. *Annu. Rev. Microbiol.*, 57: 155–176

Rinas, U., H.A. Kracke-Helm and K. Schügerl, 1989. Glucose as a substrate in recombinant strain fermentation technology. *Appl. Microbiol. Biotechnol.*, 31: 163–167

Sakamoto, K. and H. Toyohara, 2009. Putative endogenous xylanase from brackish-water clam *Corbicula japonica. Comp. Biochem. Physiol. B: Biochem. Mol. Biol.,* 154: 85–92

Saleem, M., M.S. Akhtar and S. Jamil, 2002. Production of xylanase on natural substrates by *Bacillus subtilis. Int. J. Agric. Biol.,* 4: 211–213

Satyanarayana, T., 2007. Thermophiles. *Curr. Sci.,* 93: 1340–1342

Schofield, L.R. and R.M. Daniel, 1993. Purification and properties of a β-1.4-xylanase from a cellulolytic extreme thermophile expressed in *E. coli. Int. J. Biochem.*, 25: 609–617

Seyis, I. and N. Aksoz, 2005. Effect of carbon and nitrogen sources on xylanase production by *Trichoderma harzianum* 1073 D3. *Int. Biodeterior. Biodegrad.*, 55: 115–119

Smith, J.A., M.E. Scharf, R.M. Pereira and P.G Koehler, 2009. Comparisons of gut carbohydrolase activity patterns in *Reticulitermes flavipes* and *Coptotermes formosanus* (Isoptera: Rhinotermitidae) workers and soldiers. *Sociobiology*, 53: 113–124

Thompson, B.G., M. Kole and D.F. Gerson, 1985. Control of ammonium concentration in *E. coli* fermentations. *Biotechnol. Bioeng.,* 27: 818–824

Unden, G., S. Becker, J. Bongaerts, G. Holighaus, J. Schirawski and S. Six, 1985. O_2-sensing and O_2-dependent gene regulation in facultatively anaerobic bacteria. *Arch. Microbiol.*, 164: 81–90

Weiss, R.M. and D.F. Ollis, 1980. Extracellular microbial polysaccharides I. Substrate, biomass and product kinetic equations for batch xanthan gum fermentation. *Biotechnol. Bioeng.,* 22: 859–864

Wang, H., F. Wang and D. Wei, 2009. Impact of oxygen supply on rtPA expression in *E. coli* BL21 (DE3): Ammonia effects. *Appl. Microbiol. Biotechnol.,* 82: 249–259

Yee, L. and H.W. Blanch, 1992. Recombinant protein expression in high cell density fed-batch cultures of *E. coli. Nat. Biotech.*, 10: 1550–1556

Zhao, K., M. Liu and R.R. Burgess, 2005. The global transcriptional response of *E. coli* to induced σ^{32} protein involves σ^{32} regulon activation followed by inactivation and degradation of σ^{32} *in vivo. J. Biol. Chem.,* 280: 17758–17768