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Kinetics of Xylanase Fermentation by Recombinant *Escherichia coli* DH5α in Shake Flask Culture

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Abstract: Problem statement: Interest in xylanase enzyme application has markedly increased in pulp and paper processing industries. The switch to xylanase-producing recombinant *Escherichia coli* DH5α pTP510 is seen here as an economic alternative towards higher productivity and easier downstream purification. Modeling of *E. coli* DH5α growth and enzyme secretion is thus desired for future optimization in fermentation process. **Approach:** Kinetics of intracellular xylanase fermentation by a recombinant *E. coli* DH5α was studied in shake flask culture. The effect of different medium formulations (complex, minimal and defined), initial pH (6.5, 7.0, 7.4 and 8.0) and agitation speeds (150, 200 and 250 rpm) on cell growth and xylanase production were evaluated. Mathematical models based on Logistic and Luedeking-Piret equations had been proposed to describe the microbial growth and xylanase production. **Results:** Highest xylanase production was obtained in defined medium. Based on medium formulation, the highest cell concentration (4.59 g L^{-1}) and xylanase production (2, 122.5 U mL^{-1}) was obtained when $(NH_4)_2HPO_4$ was used as the main nitrogen source, with an adjustment of the initial pH to 7.4 and agitation speed of 200 rpm. The maximum specific growth rate (μ_{max}), growth associated xylanase production coefficient (α) and non-growth associated xylanase production coefficient (β) was 0.41 h^{-1}, 474.26 U mg $cell^{-1}$and 0 U mg $cell^{-1}$ h^{-1}, respectively. **Conclusion:** Xylanase production was growth associated process and the enzyme secretion was greatly dependent on cell concentration and the specific growth rate of *E. coli* DH5α.

Key words: Recombinant *E. coli* DH5α, thermophilic xylanase, submerged fermentation, kinetics, mathematical model

INTRODUCTION

The polysaccharides of xylan typically associated with cellulose and lignins play an important structurally-supportive role in building up of plant cell walls[1]. Degradation of hemicellulose component which mainly consists of xylan requires an efficient xylanolytic enzymatic systems; consisting several enzymes including endo-β-1,4-xylanase, β-xylosidase, α-L-arabinofuranosidase, α-glucuronidase, acetyl xylan esterase, ferulic acid esterase and p-coumaric acid esterase. In recent years, there has been an increasing interest in xylanase enzyme fermentations regarding their wide applications in various industries, especially in paper and pulp industry.

The use of xylanases and side-chain splitting enzymes in pulp bleaching, especially at higher temperature, helps to improve specific indicators, like kappa number and brightness. In nature, filamentous fungi such as *Aspergillus* spp. and *Trichoderma* spp. are well known producers of extracellular xylanases[2]. Thermostable xylanase enzymes were also secreted by some bacterial species such as *Bacillus stearothermophilus*[3] and *Bacillus subtilis*[4]. The ability of *Penicillium purpurogenum* (yeast) to produce several xylanases has also been reported[5].

The secretions of xylanases from wild microorganisms (fungal, bacterial and yeast) were commonly associated with other enzymes such as cellulases and mannanases, where purification of the xylanases became problematic. The metabolic enzymes

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of xylanase producer such as proteases and transglycosidases also affect the actual yield of the xylanase enzyme[6,7]. In addition, the xylanases producing-fungi normally have slow growth rate thereby correspond to lower enzyme productivity which limits its application in commercial fermentation processes. Alternatively, enhanced enzyme production using recombinant strain such as *Escherichia coli* may be used to overcome these problems. Recombinant enzyme produced by *E. coli* generates higher productivity due to its faster growth rate compare to fungal producers.

Construction of the recombinant *E. coli* producing xylanases free of cellulolytic enzymes has been reported[8]. However, very little attention has been paid to its process development which normally related to optimization, kinetic and modeling as well as fermentation modes. Proteins and enzymes production by recombinant strains could also be enhanced through optimization of medium and culture conditions[9]. The mathematical model based on some equations can be used to describe the relationship of the experimental variables and explains the behavior of the system itself. The information gathered may be useful in optimization and designing of the fermentation process for improvement of the xylanase production.

The objective of the present study was to investigate the kinetics of cell growth and xylanase production by the recombinant *E. coli* DH5α using different medium formulations and culture conditions. The information obtained from this kinetic study may be useful to obtain better understanding of the fermentation process for the improvement of intracellular xylanase production by *E. coli* DH5α.

MATERIALS AND METHODS

Microorganism and cell maintenance: *E. coli* DH5α, which has been constructed to secrete intracellular xylanolytic enzymes was obtained from Department of Chemistry, Airlangga University, Surabaya, Indonesia and employed throughout this study. The recombinant strain encodes genes for exo-xylanase (exo-xyn), α-L-arabinofuranosidase (abfa) and β-xylosidase (xyl) from *Bacillus thermoleovorans* IT-08, isolated from Gunung Pancar Hot Spring, Bogor-West Java, Indonesia. The genes have been cloned into pTP510 and expressed in *E. coli* DH5α. The xylanase gene was targeted into intracellular space of *E. coli* DH5α which involved simple plasmid conduct using shotgun cloning technique. This recombinant *E. coli* DH5α was independent of molecular inducer such as IPTG for the xylanase production. The cell culture was streaked on the Luria Bertani (LB) agar plate containing 100 μg mL^{-1} ampicillin and incubated overnight at 37°C to obtain a single cell colony. For maintenance, the plate was subcultured at 1 week intervals and stored at 4°C.

Inoculums preparation: A single cell colony picked from the stock culture was 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 (150 rev min^{-1}) until the Optical Density (OD) of the 8 h culture reached a value of around 0.8. This culture was used as standard inoculum for all fermentations.

Medium and fermentation: Initially, the feasibility of using three different media (LB, defined and minimal medium) on growth of *E. coli* DH5α and xylanase production was investigated. LB medium consisted of (g L^{-1}): tryptone, 10.0; yeast extract, 5.0 and NaCl, 5.0. Composition of defined medium is as follows (g L^{-1}): K_2HPO_4, 13.2; KH_2PO_4, 2.6; $(NH_4)SO_4$, 4.1; NaCl, 2.0; $MgSO_4.7H_2O$, 0.5; $FeCl_3$, 0.025 and trace element solution (TES), 3.2 mL L^{-1}. TES constituted of (g L^{-1}): $CaCl_2.2H_2O$, 1.44; $AlCl_3.6H_2O$, 0.042; $ZnSO_4.7H_2O$, 0.87; $CoCl_2.6H_2O$, 0.16; $CuSO_4$, 1.6; H_3BO_3, 0.01; $MnCl_2.4H_2O$, 1.42; $NiCl_2.6H_2O$, 0.01 and $Na_2MoO_4.H_2O$, 0.02. Minimal medium only consisted of (g L^{-1}): NH_4Cl, 1.0; KH_2PO_4, 3.0; $Na_2HPO_4.2H_2O$, 6.0 and $MgSO_4$, 1.0. In all fermentations, the media was supplemented with glucose and 100 μg mL^{-1} ampicillin.

For the subsequent experiments to investigate the effect of various nitrogen sources on growth of *E. coli* DH5α and xylanase production, the defined medium was employed. The medium with the preferred nitrogen sources (NH_4Cl, NH_4NO_3, $(NH_4)_2HPO_4$ and $NaNO_3$) replacing $(NH_4)SO_4$ was used to investigate the effect of initial culture pH, ranging from pH 6.5-8, on growth of *E. coli* DH5α and xylanase production. For the effect of agitation speed (150, 200 and 250 rpm), the optimal medium with an initial culture pH of 7.4 was used. All experiments were conducted in triplicates and results given are the average value.

Analytical procedures: During the fermentation, samples were withdrawn at 2 h interval for the analysis. The samples were centrifuged at 10,000 rpm, 4°C, for 10 min (Centrifuge 5810R, Eppendorf, Germany). The cell pellets obtained were used for determination of 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 (Spectronic®20, GENESYS, USA). The cell suspensions were then filtered through dry membrane

filter and then dried in an oven for 24 h at 90°C for measurement of Dry Cell Weight (DCW). Correlation between DCW and OD was estimated from several batch experiments. One OD unit was approximately equivalent to 0.6042 g L^{-1} of cells.

For the extraction of the intracellular xylanase, the cell pellet was washed with 0.9% (w/v) NaCl and resuspended in phosphate citrate buffer (pH 7). The cells suspension was subjected to ultrasonication using sonicator (XL-2020, Heat System, New York) operated at 20 kHz, 15 sec 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 0.1 mL of substrate (1% w/v oat spelt xylan in citrate phosphate buffer, pH 7) and 0.1 mL of crude enzyme was incubated at 70°C in a shaking water bath for 1 h. The reaction was stopped by adding 0.6 mL of DNS (3, 5-dinitrosalicylic acid) reagent and the reducing sugar produced from the reaction was measured using modified DNS method as described elsewhere[10]. One unit of xylanase activity is the amount of enzyme in 1 mL sample solution producing 1 µg mL^{-1} of reducing sugar (xylose) in a minute incubation under the given experimental conditions. Residual glucose in the culture supernatant was analyzed using biochemistry analyzer (YSI 2700 Select Biochemistry Analyzer, YSI, Ohio).

Kinetics and mathematical models: The simplified batch fermentation kinetic models based on Logistic and Luedeking-Piret equations which have been described elsewhere[11] were used to describe growth of *E. coli* DH5α and xylanase production, respectively.

Cell growth:

$$\frac{dX}{dt} = \left[\mu_{max}\left(\frac{1-X}{X_{max}}\right)\right]X \quad (1)$$

Substrate consumption:

$$\frac{-dS}{dt} = \frac{1}{Y_{X/S}}\left(\frac{dX}{dt}\right) + m_s \cdot X \quad (2)$$

Xylanase production:

$$\frac{dP}{dt} = \alpha\left(\frac{dX}{dt}\right) + \beta X \quad (3)$$

Where:

X = The cell concentration (g L^{-1})
X_{max} = The maximum cell concentration (g L^{-1})
μ_{max} = Maximum specific growth rate (h^{-1})
S = The substrate concentration (g L^{-1})
$Y_{x/s}$ = Yield coefficient of cells on carbon substrate (g g^{-1})
m_s = Maintenance coefficient (g g^{-1} h^{-1})
α = Growth associated xylanase production coefficient (U mg $cell^{-1}$)
β = Non-growth associated xylanase production coefficient (U mg $cell^{-1}$ h^{-1})
t = The fermentation time (h)

Equations 1-3 were fitted to the experimental data by non-linear regression through Marquadt algorithm using MATLAB software (matlab release 14.0, mathworks, USA). The model parameter values were evaluated by solving Eq. 1-3 with the computer program applied as a search method to minimize the sum of squares of the differences between predicted and calculated values. The predicted values were then used to simulate the profiles of cell, substrate and product formation during the fermentation process.

Statistical analysis: The statistical significance of the cells and xylanase produced during various treatments was then analyzed using the SPSS program (SPSS Version 15, SPSS Inc., USA). The Tukey's Honestly Significant Difference (HSD) test multiple comparison method was used to compare the difference between treatment means.

RESULTS

Effect of basal medium on growth of *E. coli* DH5α and xylanase production: Comparison of growth of *E. coli* DH5α, xylanase production and glucose consumption during batch fermentation using three different basal media is shown in Fig. 1. Higher growth rate was determined in LB where lag phase was not observed as compared to defined and minimal media (Fig. 1a). In fermentation using defined medium, growth reached a stationary phase after 24 h while for LB medium it was reached only after 14 h. However, the final cell concentration attained at the end of fermentations was not significantly different with different media used. The final cell concentration for defined, minimal and LB media was 2.47, 2.22 and 2.12 g L^{-1}, respectively (Table 1). In all cases, xylanase production was increased concomitantly with growth, suggesting that the process is growth associated (Fig. 1c). It is interesting to note that the final xylanase activity obtained at the end of fermentations was significantly different with the different media used.

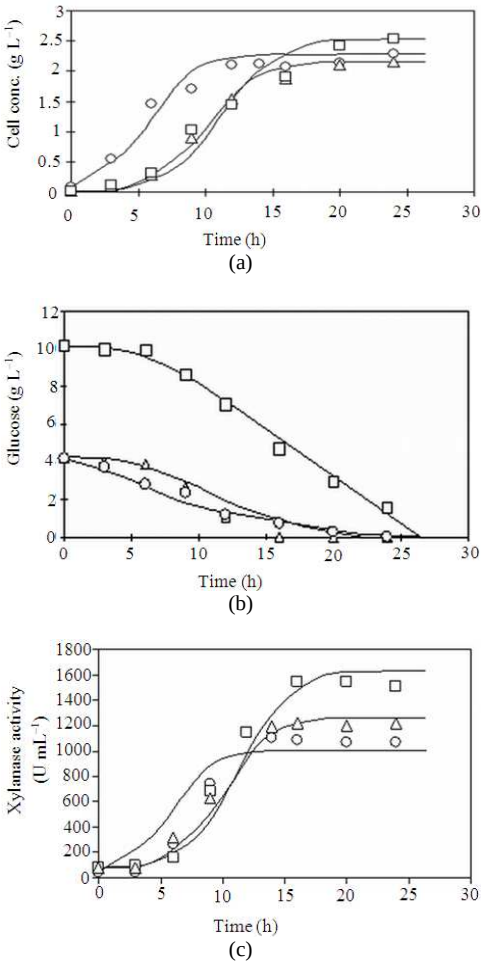

Fig. 1: Time course of xylanase fermentation by *E. coli* DH5α using different basal media. (a): Biomass; (b): Glucose consumption and (c): Xylanase activity: (○): LB; (□): Defined; (Δ): Minimal and (—): Simulated data calculated according to Eq. 1-3. Symbols represent the experimental data

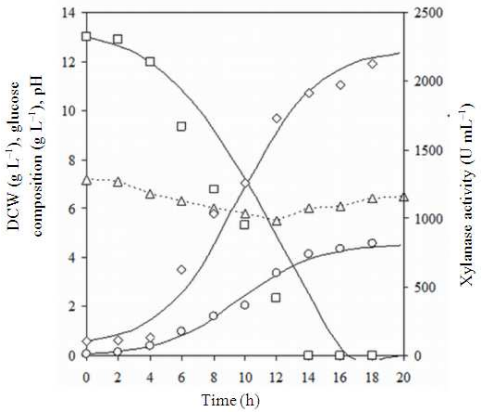

Fig. 2: The time course of xylanase fermentation by *E. coli* DH5α using the optimized medium. (○): Cell concentration; (◊): Xylanase production; (□): Glucose concentration; (Δ): Culture pH and (—): Simulated data calculated according to the proposed models Eq. 1-3. Symbols represent experimental data

Table 1: The performance of xylanase fermentation by *E. coli* DH5α using different types of basal media

	Medium		
Kinetic parameter values	LB	Defined	Minimal
Maximum cell concentration, X_{max} (g L^{-1})	2.220^{ab}	2.470^{b}	2.120^{a}
Initial cell concentration, X_o (g L^{-1})	0.090^{a}	0.020^{b}	0.020^{b}
Maximum specific growth rate, μ_{max} (h^{-1})	0.590	0.490	0.540
Initial xylanase production, P_o (U mL^{-1})	47.000^{a}	78.000^{b}	79.500^{b}
Maximum xylanase production, P_{max} (U mL^{-1})	1094.500^{a}	1526.500^{b}	1208.500^{c}
Maintenance coefficient, m_s (g glucose g cell^{-1} h^{-1})	0.051	0.202	0.092
Growth-associated xylanase production coefficient, α (U mg cell^{-1})	436.060	616.340	554.540
Non-growth associated xylanase production coefficient, β (U mg cell^{-1} h^{-1})	0.000	0.000	0.000
Cell yield, $Y_{x/s}$ (g cell g glucose^{-1})	0.520^{a}	0.290^{b}	0.510^{a}

$^{a, b \text{ and } c}$: Means values with the same letter are not significantly different. μ_{max}, m_s, α and β are calculated from the Eq. 1-3

The highest xylanase activity (1526.5 U mL^{-1}) was obtained in defined medium, followed by minimal medium (1208.5 U mL^{-1}) and LB medium (1094.5 U mL^{-1}). Glucose was consumed for growth and complete utilization of glucose was observed at the end of the cultivation (Fig. 1b).

Figure 2 shows the time course of xylanase fermentation by the recombinant *E. coli*, which also includes the comparison between the calculated data according to Eq. 1-3 with the experimental data. The calculated data fitted well to the experimental data. The result suggests that the proposed models based on Logistic and Luedeking-Piret equations were sufficient to describe growth of *E. coli* DH5α and xylanase production respectively. The kinetic parameter values for the fermentations employing three different basal media are shown in Table 1. The highest maximum specific growth rate (μ_{max}) (0.59 h^{-1}) was obtained in LB medium, followed by minimal medium (0.54 h^{-1}) and defined medium (0.49 h^{-1}). Although defined medium gave the lowest μ_{max}, but this medium gave the highest growth-associated xylanase production coefficient (α). The α value for fermentation using defined medium (616.34 U mg cell^{-1}) was significantly higher than those obtained in minimal medium (436.06 U mg cell^{-1}) and LB medium (554.54 U mg cell^{-1}). In all fermentations, the value of non-growth associated xylanase production coefficient (β) was 0 U mg cell^{-1} h^{-1}.

Table 2: Kinetic parameter values of xylanase fermentation by *E. coli* DH5α using different types of inorganic nitrogen sources

Kinetic parameters	NH_4Cl	$(NH_4)_2SO_4$	NH_4NO_3	$(NH_4)_2HPO_4$	$NaNO_3$
Maximum cell concentration, X_{max} (g L^{-1})	2.99[c]	3.02[c]	2.64[b]	4.47[d]	1.00[a]
Maximum xylanase production, P_{max} (U mL^{-1})	1475.00[c]	1632.50[d]	1244.50[b]	1772.00[e]	696.00[a]
Specific xylanase activity (U mg $cell^{-1}$)	479.64[b]	540.66[c]	471.40[b]	396.47[a]	696.00[d]
Maximum specific growth rate, μ_{max} (h^{-1})	0.69	0.46	0.35	0.36	0.52
Growth-associated xylanase production coefficient, α (U mg $cell^{-1}$)	430.42	417.83	397.16	490.70	655.94
Non-growth associated xylanase production coefficient, β(U mg $cell^{-1}$ h^{-1})	0.00	0.00	0.00	0.00	0.00
Cell yield, $Y_{x/s}$ (g g^{-1})	0.28[b]	0.26[b]	0.22[a]	0.37[c]	0.23[a]
Yield of xylanase, $Y_{p/sec}$ (U mg $glucose^{-1}$)	133.32[b]	157.80[c]	105.92[a]	154.09[c]	135.94[b]
Cell productivity (g L^{-1} h^{-1})	0.23[c]	0.21[bc]	0.16[ab]	0.32[d]	0.09[a]
Xylanase productivity (U mL^{-1} h^{-1})	123.50[b]	116.71[b]	69.70[a]	128.29[b]	58.58[a]
Fermentation time	12.00	14.00	18.00	14.00	12.00

[a, b, c, d and e]: Means values with the same letter are not significantly different. α, β and μ_{max} are calculated from the Eq. 1-3

Table 3: Kinetic parameter values of xylanase fermentation by *E. coli* DH5α at different initial pH using $(NH_4)_2HPO_4$ as nitrogen source

	Initial culture pH			
Kinetic parameters	6.50	7.00	7.40	8.00
Maximum cell concentration, X_{max} (g L^{-1})	1.46[a]	3.66[b]	4.92[d]	4.63[c]
Maximum xylanase production, P_{max} (U mL^{-1})	738.00[a]	1248.50[b]	1854.50[c]	1724.00[d]
Specific xylanase activity (U mg $cell^{-1}$)	529.25[b]	341.62[a]	376.92[a]	372.79[a]
Maximum specific growth rate, μ_{max} (h^{-1})	0.44	0.42	0.43	0.52
Growth-associated xylanase production coefficient, α (Umg $cell^{-1}$)	521.60	338.69	402.63	385.42
Non-growth associated xylanase production coefficient, β (U mg $cell^{-1}$ h^{-1})cell yield, $Y_{x/s}$ (g g^{-1})	0.00	0.00	0.00	0.00
	0.22[a]	0.47[b]	0.44[b]	0.44[b]
Yield of xylanase, $Y_{p/s}$ (U mg $glucose^{-1}$)	95.83[a]	158.35[b]	169.21[b]	165.12[b]
Cell productivity (g L^{-1} h^{-1})	0.11[a]	0.25[b]	0.28[b]	0.31[b]
Xylanase productivity (U mL^{-1} h^{-1})	43.56[a]	83.58[b]	103.05[bc]	115.36[c]
Fermentation time	18.00	16.00	18.00	16.00

[a, b, c and d]: Means values with the same letter are not significantly different. α, β and μ_{max} are calculated from the Eq. (1-3)

This result confirmed that the xylanase production by *E. coli* DH5α was growth associated process. It is also interesting to note the fermentation that produced the highest xylanase showed the highest maintenance coefficient (m_s). The m_s value for defined medium (0.202 g substrate g $cell^{-1}$ h^{-1}) was about 2 and 4 times higher than those calculated for LB and minimal medium, respectively.

Effect of nitrogen source on growth of *E. coli* DH5α and xylanase production: Table 2 shows the kinetic parameter values for xylanase fermentation by *E. coli* DH5α using different types of nitrogen source. The kinetic parameter values were calculated from the modeling exercise using the data obtain from the time courses of the fermentation runs (data not shown). In all cases, the formulation was based on defined medium. Both, cell growth and xylanase production were greatly influenced by the type of nitrogen source used. The highest final cell concentration (X_{max}) (4.47) was obtained in fermentation using $(NH_4)_2HPO_4$ as nitrogen source, followed by $(NH_4)_2SO_4$ (3.02), NH_4Cl (2.99), NH_4NO_3 (2.64) and $NaNO_3$ (1.00 g L^{-1}). This is corresponded well to the final xylanase activity obtained at the end of fermentation, where the highest activity (1772 U mL^{-1}) was obtained in fermentation using $(NH_4)_2HPO_4$. The highest xylanase productivity (128.29 U mL^{-1} h^{-1}) was also obtained in fermentation using $(NH_4)_2HPO_4$. The xylanase yield based on glucose consumed was not significantly different among the nitrogen sources tested. The lowest xylanase activity (696 U mL^{-1}) was obtained in fermentation using $NaNO_3$. However, the specific xylanase activity was the highest for this fermentation. The maximum specific growth rate (0.69 h^{-1}) was observed with NH_4Cl while the lowest observed with NH_4NO_3 (0.35 h^{-1}).

Effect of initial pH on growth of *E. coli* DH5α and xylanase production: Effect of initial culture pH on growth of *E. coli* DH5α and xylanase production is summarized in Table 3. The highest final cell concentration (4.92 g L^{-1}) was obtained at initial pH of 7.4 and corresponded to the highest xylanase production (1854.0 U mL^{-1}). Inhibition of growth and xylanase production was observed with a slight reduction in pH to 7: and greatly inhibited at pH 6.5. Final cell concentration and xylanase activity obtained at pH 6.5 were about 3 times lower than those obtained at pH 7.4. Only slight reduction in growth and xylanase production was observed at alkaline conditions (pH 8).

In terms of cell yield ($Y_{x/s}$) and xylanase yield ($Y_{p/s}$), the values were not significantly different at pH ranged from 7-8, but greatly reduced at pH 6.5. On the other hand, xylanase productivity (103.05 U mL^{-1} h^{-1}) obtained at pH 7.4 was comparable to that obtained at pH 8, but significantly reduced at pH 6.5 and 7.

Table 4: Kinetic parameter values of xylanase fermentation by *E. coli* DH5α at different agitation speeds on rotary shaker

	Agitation speed (rpm)		
Kinetic parameters	150.00	200.00	250.00
Maximum cell concentration, X_{max} (g L^{-1})	4.92[b]	4.59[a]	4.48[a]
Maximum xylanase production, P_{max} (U mL^{-1})	1854.50[a]	2122.50[b]	2120.00[b]
Specific xylanase activity (U mg $cell^{-1}$)	376.92[a]	462.42[b]	473.21[c]
Maximum specific growth rate, μ_{max} (h^{-1})	0.43	0.42	0.41
Growth-associated xylanase production coefficient, α (U mg $cell^{-1}$)	402.63	474.26	501.73
Non-growth associated xylanase production coefficient, β (U mg $cell^{-1}$ h^{-1})	0.00	0.00	0.00
Cell yield, $Y_{x/s}$ (g cell g $glucose^{-1}$)	0.44[b]	0.35[a]	0.33[a]
Yield of xylanase, $Y_{p/s}$ (U mg $glucose^{-1}$)	169.21[a]	160.66[a]	161.02[a]
Cell productivity (g L^{-1} h^{-1})	0.28[ab]	0.26[a]	0.28[b]
Xylanase productivity (U mL^{-1} h^{-1})	103.50[a]	117.92[b]	117.78[b]
Fermentation time	18.00	18.00	18.00

[a, b and c]: Means values with the same letter are not significantly different. α, β and μ_{max} are calculated from Eq. 1-3

From the results of this study it can be suggested that initial culture pH 7.4 was the preferred pH for growth of *E. coli* DH5α and xylanase production.

Effect of agitation speed on growth of *E. coli* DH5α and xylanase production: Table 4 shows the effect of orbital shaker agitation speed on growth of *E. coli* DH5α and xylanase production in shake flask culture. The X_{max} was not significantly different with variation in agitation speed. However, xylanase production was slightly improved at higher agitation speeds (200 and 250 rpm). The highest xylanase activity (2122.5 U mL^{-1}) was obtained at 200 rpm. This is corresponded well with a slight increase in xylanase productivity. Other kinetic values such as $Y_{x/s}$, $Y_{p/s}$ and μ_{max} were not significantly different with variation in agitation speed.

DISCUSSION

Production of intracellular xylanase by *E. coli* DH5α was influenced by the medium composition and culture conditions. LB medium is commonly used for recombinant gene expression on laboratory scale. The effect of each nutrient component in LB medium on growth of the recombinant strain and metabolite production cannot be clearly identified. The use of defined medium permitted better understanding of nutrient requirement for growth and metabolism of the required product. Results from this study indicated that the type of nitrogen source was the important factor that determined growth rate of *E. coli* DH5α and xylanase production. Besides, the initial culture pH and oxygen supply were also another important factors that affect growth of *E. coli* DH5α and xylanase production. From the modeling study, it was found that the xylanase production was associated to growth of *E. coli* DH5α and xylanase production was proportionally increased with cell biomass. In other words, improvement of xylanase production was very much related to cell built-up, though some factors such as nutrients concentration, pH and dissolved oxygen level may enhance the ability of cell to express the xylanase genes. It is interesting to note that the specific growth rate also gave a profound effect on the final cell concentration and xylanase activity. This means that the specific production rate of xylanase was influenced by the specific growth rate. A moderate specific growth rate of *E. coli* DH5α (0.36 h^{-1}) gave higher cell yield (0.37 g g^{-1}) and xylanase yield (154.09 U mg^{-1}) based on glucose consumed. It can be deduced that cultivation of cells at lower growth rate allowed them to allocate more cellular resources to express the xylanase gene.

The concentration of each nutrient in the defined medium such as glucose, ammonia, magnesium, phosphorus and zinc that inhibited growth and metabolite production shall be determined. The knowledge obtained from this study is very useful for further optimization of medium composition, as well as development of fed-batch culture, to enhance growth of *E. coli* DH5α and xylanase production. Specific nutrient could be controlled below inhibition levels in fed-batch culture to maintain high cell growth of *E. coli*[12]. For example, efficient production of truncated thermostable xylanase from *Rhodothermus marinus* in *E. coli* had been achieved in fed-batch culture employing complex medium[13]. Moreover, optimization of medium component using response surface methodology for phytase production by *E. coli* has also been reported[14].

The maximum specific growth rate obtained in fermentation using NH_4Cl and $NaNO_3$ as an individual nitrogen source was 0.69 h^{-1} and 0.52 h^{-1}, respectively. High maximum specific growth rate of recombinant *E. coli* with a value of 0.67 $h^{-1}$and 0.44 h^{-1} was also obtained in fermentation using NH_4Cl and $NaNO_3$, respectively[15]. They also claimed that the ammonia-grown cultures of *E. coli* yielded a greater specific growth rate, cell yield and lipopolysaccharide production efficiency and a more hydrophilic surface than nitrate grown cultures. The use of $(NH_4)_2HPO_4$ exhibited high cell concentration of *E. coli* (4.47 g L^{-1}) and corresponded to the highest xylanase activity (1772 U mL^{-1}). Most probably, the energy uptake by the cells was influenced by ionic strength of the substances and a well buffering system by $(NH_4)_2HPO_4$. NH_4NO_3 and $NaNO_3$ were found not suitable as the main nitrogen source for growth of *E. coli* DH5α and xylanase production due to significant reduction in productivity.

Further improvement of the production could be achieved by optimizing the $(NH_4)_2HPO_4$ concentration, to over-express the xylanase gene efficiently.

In most fermentations employing *E. coli*, the initial culture pH was adjusted to pH ranging from 7.0[13,16] to 7.4[15,17] for enhancement of growth. In this study, initial pH 7.4 was found optimal for the growth of *E. coli* DH5α and xylanase production. On the other hand, initial pH 6.5 strongly inhibited the growth of *E. coli* DH5α as well as xylanase production. Acidic condition was found not sufficient for initial *E. coli* fermentation due to its adaptation phase. Duffy *et al*.[18] also claimed that the initial pH 5.8 was not suitable for the growth of *E. coli*. During the fermentation with optimal initial pH, the pH decreased with fermentation time up to 12 h due to increased in proton electrochemical gradient (data not shown). Glucose consumption was the highest when the proton electrochemical gradient was the highest. After 14 h of fermentation, the culture pH started to increase due to H^+ transport into the cells and depletion of glucose in the culture. The changes in proton electrochemical gradient resulted in differences in the metabolism[19]. The highest specific growth rate of *E. coli* DH5α and xylanase production rate were occurred at pH 6.4, while glucose consumption rate was the highest at pH 5.8. This result indicates that manipulation of culture pH during the fermentation at different phases may enhance growth of *E. coli* DH5α and xylanase production, as reported for many fermentation processes[19].

E. coli is a facultative bacterium which can grow either in the presence of oxygen or absence of oxygen. Higher growth rate of *E. coli* DH5α and xylanase production was obtained in shake flask fermentation agitated at high speed (200-250 rpm), indicating that the oxygen transfer rate may influence the process. Previous researchers found that the mass transfer rate of nutrients and rate of oxygen supply to the cells were enhanced by small cell cluster with bigger interfacial areas, which in turn, improve the cultivation performance of *E. coli*[20]. Oxygen supply to the shake flask culture was obtained by surface aeration, where the quantity of oxygen supplied was increased with increasing agitation speed. Improvement in xylanase production at higher agitation speeds (200 and 250 rpm) may be related to the level of dissolved oxygen in the culture. Vigorous agitation (250 rpm) may provide benefits for cell growth and protein production. This is in contrast to the findings from this study which indicated that moderate agitation (200 rpm) exhibit high xylanase activity and productivity. In shake flask culture incubated on rotary shaker, the agitation speed of 200 rpm was found optimal for growth of recombinant *E. coli*.[21,22]

The xylanase production obtained in this study (2122.5 U mL^{-1}) was significantly higher than those reported in the literature. For example, the highest xylanase production obtained in submerged cultivation of *Aspergillus niger* B03 was 996.3 U mL^{-1}[23] and 592.7 U g/substrate by *Trichoderma longibrachiatum*[24]. The highest activity of themostable cellulase-free xylanase in shake-flask fermentations by *Bacillus subtilis* was 1056 U mL^{-1}[4]. The advantages of intracellular as compared to extracellular xylanases include higher product yield and reduced degradation by proteolytic enzymes present in the culture. Further improvement of xylanase production by *E. coli* DH5α by refining the optimization of medium composition using Response Surface Methodology (RSM) is being carried out in our laboratory. In addition, the fed-batch culture technique, with various controlled parameters such as specific growth rate, nutrients level and the culture pH, is also being developed in our laboratory for efficient large scale fermentation process for xylanase production by the recombinant *E. coli* DH5α.

CONCLUSION

Results from this preliminary study showed that nutrient composition and optimum cultural conditions played an important role in growth of *E. coli* DH5α and intracellular xylanase production. Optimization of medium and culture conditions, as well as development of a suitable fermentation technique is required for enhancement of xylanase production by *E. coli* DH5α. From the kinetic and modeling study, it was found that the xylanase production by *E. coli* DH5α was growth associated process. Although the xylanase activity was increased concomitantly with increasing cell concentration in the culture, the growth associated xylanase production coefficient (α) was also depended on the maximum specific growth rate (μ_{max}) of the *E. coli* DH5α.

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