doi: 10.5505/tjb.2013.81994

Research Article [Araştırma Makalesi]

Yayın tarihi 30 Eylül, 2013 © TurkJBiochem.com

[Published online 30 September, 2013]

Optimization of fed-batch fermentation for organic solvent tolerant and thermostable lipase production from recombinant *E. coli*

[Rekombinant *E. coli*'den organik çözücüye toleranslı ve ısıya dayanıklı lipaz üretimi için kesikli-beslemeli fermantasyon optimizasyonu]*

Rubina Nelofer[1],
Raja Noor Zaliha Raja Abd Rahman[2],
Mahiran Basri[3],
Arbakariya B. Ariff[1]

University Putra Malaysia, Faculty of Biotechnology and Biomolecular Sciences, Departments of [1]Bioprocess Technology, [2]Microbiology, Faculty of Science, [3]Department of Chemistry, Serdang, Selangor.

Yazışma Adresi
[Correspondence Address]

Dr. Rubina Nelofer

Pakistan Council of Scientific and Industrial Research
Biotechnology and Food Research Centre, Bfrc, Pcsir Labs Comlex
Lahore, 54600, Pakistan
Tel. 933336282257
E-mail. rubinanelofer@yahoo.com

*** Translated by [Çeviri]** Dr. Aylin Sepici Dinçel

Registered: 27 February 2013; Accepted: 12 June 2013
[Kayıt tarihi: 27 Şubat 2013; Kabul tarihi: 12 Haziran 2013]

ABSTRACT

Objective: Lipases are industrially important enzymes especially the thermostable and organic solvent tolerant lipases. This study was planned to enhance the production using fed batch fermentation technique.

Materials and Methods: The possibility of using fed-batch fermentation for improvement of organic solvent tolerant and thermostable lipase production by recombinant *E.coli* BL21 was studied in 2 L stirred tank bioreactor. Exponential fed-batch fermentations were operated at different specific growth rates (μ). The effect of antibiotics addition in feed medium, on the number of plasmid bearing cells was also investigated. Kinetics of the fed-batch fermentation was evaluated for the generation of kinetic parameter values. Effect of yeast extract in feed medium was also investigated.

Results: The number of plasmid bearing cells was increased from 13 % to 91 % with the addition of antibiotics (2 g/L ampicillin and 1.4 g/L chloramphenicol) in the feed medium. Among the different μ investigated (0.05, 0.10 and 0.15 h^{-1}), the highest cell concentration (30.32 g/L) and lipase production (130.5 IU/mL) were obtained in exponential fed-batch fermentation, where μ was controlled at 0.10 h^{-1} with 800 g/L glucose in the feed medium. No significant improvement was achieved on growth and lipase production in fed-batch fermentation with addition of yeast extract in feed medium, though μ was controlled at 0.10 h^{-1}.

Conclusion: Fed-batch was found a good technique for enhancement of lipase production from recombinant *E.coli* BL21 on the cost of increasing time of fermentation (fermentation time for batch fermentation was 16 h and for fed-batch was 26 h) and quantity of some medium ingredients as compared to the batch mode.

Key Words: fed-batch culture, thermostable lipase, recombinant, E. coli

Conflict of Interest: The authors declared that there was no conflict of interest in this work.

ÖZET

Amaç: Isıya dayanıklı ve çözücüye toleranslı lipazlar, endüstriyel olarak önemli lipazlardır. Bu çalışma kesikli-beslemeli fermentasyon tekniği kullanılarak lipaz üretimini arttırmak için planlanmıştır.

Gereç ve Yöntemler. Rekombinant *E.coli* BL21ile ısıya dayanıklı ve çözücüye toleranslı lipazların üretimini, kesikli-beslemeli fermentasyon kullanarak geliştirebilmek için 2 L'lik karıştırılmış biyoreaktör tankında çalışıldı. Üstel kesikli-beslemeli fermantasyon farklı özgül büyüme oranlarında işletildi (μ). Ayrıca besleme vasatına eklenen antibiyotiğin, plazmid içeren hücrelerin sayısına olan etkileri de incelendi. Kesikli-beslemeli fermantasyon kinetiği, kinetik değerleri oluşturmak için değerlendirildi. Maya ekstrelerinin de besleme vasatına etkileri incelendi.

Bulgular: Besleme vasatına antibiyotik (2 g/L ampisilin ve 1.4 g/L kloranfenikol) eklenmesi ile plasmid içeren hücre sayıları % 13'den % 91'e çıktı. Farklı özgül büyüme oranları (0.05, 0.10 ve 0.15 h^{-1}) arasında yapılan araştırmada, en yüksek hücre konsantrasyonu (30.32 g/L) ve lipaz üretimi (130.5 IU/mL) üstel kesikli-beslemeli fermantasyondan sağlandı. Bu durumda μ besleme vasatında 0.10h^{-1} de 800 g/L glukoz ile kontrol edildi. Kesikli-beslemeli fermantasyonda maya ekstresinin besleme vasatına ilavesi, μ 0.10 h^{-1} de kontrol edilmiş olmasına rağmen büyüme ve lipaz oluşumu üzerinde anlamlı bir ilerleme gerçekleştirmedi.

Sonuç: Kesikli-beslemeli fermentasyon, rekombinat *E.coli* BL21 den lipaz üretimini arttırması, fermentasyon zamanını uzatması (kesikli-beslemeli fermentasyon 26 saat ve kesikli fermentasyon 16 saat) ve vasat içeriğinin miktarı açısından kesikli model ile karşılaştırıldığında, daha iyi bir teknik olduğu görülmüştür.

Anahtar Kelimeler: kesikli-beslemeli kültür, ısıya dayanıklı lipaz, rekombinant, E. coli

Çıkar Çatışması: Yazarlar çıkar çatışması olmadığını beyan ederler.

Introduction

Thermostable and organic solvent tolerant lipases are in high demand in many industrial reactions. Production of these types of enzymes could be enhanced using recombinant microorganisms by expressing the genes isolated from the wild strain to the host cell. *E. coli* has been successfully used as the host cell to express lipase genes [1-3].

Recombinant proteins produced by *E. coli* are mostly intracellular and therefore the production is normally proportional to cell concentration [4]. The production of recombinant protein by *E. coli* depends on many factors including medium composition, time of induction, physical conditions and mode of fermentation. In general, three modes of bioreactor operation (batch, fed-batch and continuous) could be used for the production of biotechnology product through fermentation. The selection of mode for bioreactor operation depends on various factors such as the microorganisms employed, the target products and the kinetics of growth and product formation. Batch fermentation is mostly used for the preliminary studies of the process development such as selection and optimization of medium composition and other physical culture conditions. In industry, it is also used for simple fermentation process where complicated control strategy may not be required for the production of the target product. The main limitations of batch fermentation are the use of high initial substrate concentration and accumulation of undesired by-products, which may inhibit growth and the production of the target product excessively. In addition, longer operation time such as time for cleaning, reloading and sterilization between batches reduces the overall productivity of the process. In theory, continuous fermentation has many advantages such as high productivity and ability to control fermentation parameters accurately. However, this mode of fermentation is complex, difficult to setup and to maintain the sterility and stability for long operation period.

Fed-batch fermentation is widely used for recombinant protein production by *E. coli*, especially when glucose is used as a carbon source. The use of high initial concentrations of glucose in batch fermentation of *E. coli* leads to the accumulation of acetate even in aerobic conditions [5], which inhibit growth and the production of recombinant protein even at very low levels. In fed-batch fermentation, glucose in the culture could be controlled at low levels by appropriate glucose feeding strategy that meet the requirement of the growing culture. Thus, accumulation of acetate shall be inhibited to enhance growth and product yields. The use of fed-batch fermentation for high cell density culture of *E. coli* and the overproduction of recombinant proteins have been reported [6-8].

An organic solvent tolerant and thermostable lipase (Lip 42) gene from a local *Bacillus* sp. 42 [9] has been cloned in *E. coli* BL21 (DE3) by a research group [10]. The composition of medium and the culture conditions for Lip 42 fermentation by *E. coli* have been optimized in shake flasks [11, 12] and 1 L stirred tank bioreactor. From kinetic analysis, it was found that the specific Lip 42 production rate was proportionally increased with increasing specific growth rate (Nelofer et al., 2013, work in publishing process), suggesting that the exponential fed-batch fermentation where the specific growth rate could be controlled at certain values may be used to improve the production of this enzyme.

Only a limited number of research articles are available on fed-batch fermentation for the production of lipase by recombinant *E. coli* in bioreactor. A research group in University Putra Malaysia isolated an organic solvent tolerant and thermostable lipase (Lip 42) producing bacterial strain, *Bacillus* sp.42 from oil mill effluent [9]. The lipase gene from *Bacillus* sp. was over expressed in *E. coli* BL21 [10]. The recombinant lipase production process was statistically optimized in shake flasks using batch mode of fermentation [11, 12]. The next step of the study was the development of a fed-batch process for Lip 42 production. The objective of the present study was therefore to investigate the possibility of using exponential fed-batch fermentation for the improvement of lipase (Lip 42) production by recombinant *E. coli* BL21 in stirred tank bioreactor. The specific growth rate (μ) of *E. coli* in fed-batch fermentation was controlled at several required values by glucose feeding strategy according to the algorithm developed from the mass balance equations.

Material and methods

Microorganism and medium

Recombinant *E. coli* BL21 [10] hosting a thermostable and organic solvent tolerant lipase gene from *Bacillus* sp. 42 [9] was used in the study. Luria Bertani (LB) medium was used for inoculum preparation. The production medium used in this study consisted of (g/L); glucose, 10; tryptone, 10; yeast extract, 25; NaCl, 3; potassium hydrogen phosphate, dibasic 9.4; potassium phosphate, monobasic 2.2. Medium pH was adjusted to 7 before sterilization. Ampicillin 50 µg/mL and chloramphenicol 35 µg/mL were added to all media to avoid contamination. The feed medium for fed-batch fermentation consisted of 800 g/L glucose with and without antibiotics (2 g/L ampicillin and 1.4 g/L chloramphenicol).

Inoculum preparation and fermentation

Inoculum was prepared by inoculating a single colony from LB agar plate into a 250 mL screw capped bottle containing 40 mL LB medium. The bottle was incubated at 30°C on a rotary shaker agitated at 200 rpm for 12-16 h. All fed-batch fermentations were carried out using a 2 L stirred tank bioreactor (BIOSTAT DCU-200, B. Braun Biotech International, Germany). The

initial batch fermentation was started with a volume of 1L and inoculated with 4 % (v/v) inoculum. The fed-batch fermentation was started when significant changes in the culture pH and dissolved oxygen tension (DOT) were observed, about 6 h from the start of the experiment. The feeding strategy was performed according to the algorithm (Eq.1) at different required μ values. At mid log phase, the culture was induced with 0.5 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) to enhance the expression of the gene. Initially, the fed-batch fermentation was conducted with the required μ of 0.15 h^{-1}, without induction with 0.5 mM IPTG to identify the growth profile. In the subsequent experiments, the fed-batch fermentations were conducted with and without antibiotics in the feed medium to investigate the effect of antibiotics on plasmid stability and lipase production. For kinetic studies, fed-batch fermentations were carried out with feeding strategy at different required μ values. The effect of yeast extract in the feeding medium on Lip 42 production by *E. coli* was also studied. In this experiment, the exponential fed-batch fermentation was performed with a required μ of 0.10 h^{-1} and the feed medium consisted of 800 g/L glucose, 200 g/L yeast extract and antibiotics.

Feeding strategy

The exponential feeding strategy was calculated according to Eq. 1. Derivation of this equation has been described elsewhere [13].

$$F = \frac{X_0 V_0 \mu e^{\mu t}}{Y_{x/s} S_0} \qquad 1$$

Where F is the feed flow rate (mL/h), X_o (g cell/mL) is cell concentration at 0 h of fed-batch fermentation, V_o is the initial culture volume (mL) of fed-batch fermentation, $Y_{x/s}$ is the cell yield (g cell/g substrate) of substrate from the batch fermentation experiment and S_o is glucose (g substrate/mL) concentration in the feed solution.

The performance of exponential fed-batch fermentation for Lip 42 production was investigated using three different required μ values (0.05, 0.10 and 0.15 h^{-1}). The required μ values selected are lower than the μ_{max} value (0.431 h^{-1}) for recombinant *E. coli* BL21, calculated from batch fermentation data using batch fermentation model based on Monod equation (data not shown). The feed flow rate was controlled by a microtube pump MP-3N (EYELA, Tokyo Rikakikai Co, LTD, Japan). A standard curve was drawn for the pump flow rate with the set points.

Kinetic models

Mass balance equations used for batch fermentation;

Growth rate

$$\frac{\Delta X}{\Delta t} = \mu . X \qquad 2$$

Lipase production rate

$$\frac{\Delta P}{\Delta t} = q_p . X \qquad 3$$

Substrate consumption rate

$$\frac{\Delta S}{\Delta t} = -q_S . X \qquad 4$$

Where, X, P and S is cell concentration, lipase activity and glucose concentration, respectively. Specific growth rate is denoted by μ, while volumetric lipase production rate and volumetric glucose uptake rate is expressed as q_p and q_S, respectively.

Mass balance equations used for fed-batch fermentation;

Rate of increase in volume

$$\frac{dV}{dt} = F \qquad 5$$

Growth rate

$$\frac{dX}{dt} = \mu X - \frac{F}{V} \cdot X \qquad 6$$

Glucose consumption rate

$$\frac{dS}{dt} = -\sigma X + \frac{F}{V}(S_0 - S) \qquad 7$$

Lipase production rate

$$\frac{dP}{dt} = \pi X - \frac{F}{V} P \qquad 8$$

Where, μ, σ and π is the specific growth rate, substrate consumption rate and lipase production rate in fed-batch fermentation, respectively. V is the culture volume and F is the feeding rate.

Statistical analysis

Tukey Honestly Significant Difference (HSD) test was used to compare the means of kinetic parameters using SPSS (version 17).

Analytical procedures

Samples withdrawn at time intervals from the bioreactor were centrifuged at 10700 × g for 10 minutes at 4 °C (TA-14-50, Allegra®250R, Beckman Coulter, USA). The supernatant was used to analyse acetate and glucose concentration while the cell pellet was resuspended in 20 mM phosphate buffer at pH 7 for enzyme extraction. The cells were disrupted using a sonicator for 2 minutes on ice and re-centrifuged, where the supernatant obtained was used for the determination of lipase activity. Lipase activity was determined according to the method used earlier [11]. One unit of lipase is defined as 1 μM free fatty acids released per minute. Glucose was analyzed using YSI biochemistry analyzer (Model 2700). Acetate was analyzed using HPLC (Shimadzu, model JUBR 121) with 300 mm X 7.8 mm Aminex® organic acid column (HPX-87H) and UV detector. The mobile phase (4 mM H_2SO_4) at a flow rate of 0.6 mL/min was used and the detection was read at 210 nm after 15 min retention time.

In order to check the plasmid stability, the culture sample was diluted in a series and was grown on LB agar plate with and without the presence of antibiotics. The plates were incubated at 37 °C for 24 hours and the colonies appeared on the plates were counted. The percentage of plasmid stability was calculated as the number of colonies on plate with antibiotics divided by the number of colonies in plate without antibiotics, and multiplied with 100.

Results

Effect of antibiotics in feed medium

The fed-batch fermentation was started after a drastic changes in pH and DOT level (after about 6 h of initial batch fermentation), which indicated that the substrates were utilized for active growth of *E. coli*. The culture was induced in the mid exponential phase, which was after 12 h of fermentation. The performance of exponential fed-batch fermentation of lipase by *E. coli* BL21, with and without induction with IPTG, as well as with and without the presence of antibiotics in feed medium is shown in Table 1. The specific growth rate was controlled at 0.15 h^{-1} and fermentation time is 26 h. Lipase production in exponential fed-batch fermentation without induction and antibiotics in feed medium was significantly lower than that obtained in batch fermentation, though the cell concentration was about three times higher.

The percentage of plasmid bearing cells in this fermentation was 27 %. Increase in lipase production was observed in fed-batch fermentation with induction and without antibiotic, though cell concentration and the percentage of plasmid bearing cells were reduced. In exponential fed-batch fermentation with the addition of antibiotics (ampicillin and chloramphenicol) in the feed medium and induced with IPTG, the percentage of plasmid bearing cells was more than 90 % while lipase activity (82.83 IU/mL) was increased by more than two times higher as compared to fermentation without antibiotics (31.18 IU/mL). The lipase production in the fed-batch with antibiotics was also higher than lipase activity obtained in batch mode, but the difference was not as prominent as with other experiments. However cell mass concentration was more than two times as compared to the batch fermentation (Table 1).

Effect of specific growth rate on lipase production

A typical time course of exponential fed-batch fermentation, using high glucose concentration (800 g/L) in the feed medium, is shown in Fig. 1. For fed-batch fermentation operated at required μ of 0.05 h^{-1}, the culture pH was increased up to pH 8.5, while for fed-batch fermentation operated at μ of 0.15 h^{-1}, the culture pH was decreased to 5.4. The culture pH for fed-batch fermentation operated at μ of 0.10 h^{-1} μ was remained almost constant around pH 7 throughout the fermentation.

During fed-batch fermentations, DOT was varied from 25 to 40 % saturation, though the DOT was planned to control at 30 % saturation. Big variations in agitation speed to maintain the DOT level at 30 % saturation were observed for fed-batch fermentation operated at different required μ value. In all cases, during the initial batch fermentations the change in agitation was almost similar (Fig. 2). The agitation was started to increase within 2 h of fermentation, increased gradually up to 4 h and then the agitation was stated to decrease for the next two hours. This was the time when the feeding was started to initiate fed-batch fermentation. During the time course of fermentation (including both batch and fed-batch parts), the agitation speed was varied from 350 to 900 rpm, 350 to 1050 rpm and 350 to 850 rpm for the required μ of 0.05 h^{-1}, 0.10 h^{-1} and 0.15 h^{-1}, respectively.

Eq. 2, 3 and 4 were used to calculate cell growth, lipase production and glucose consumption rates for initial batch fermentation, respectively. On the other hand, Eq. 6, 7 and 8 were used for the calculation of these rates for fed-batch fermentation. Table 2 shows the performance and kinetic parameter values of exponential fed-batch fermentation of lipase by *E. coli* BL21 carried out at different μ values. Among the three different μ tested, the highest lipase production (130.5 IU/mL) and cell concentration (30.32 g/L) were observed in fed-batch fermentation, where μ was controlled at 0.10 h^{-1}. In this fermentation run, residual glucose in the culture was

Table 1. The performance of exponential fed-batch fermentation of lipase by *E. coli* BL21, with and without induction with IPTG, as well as with and without the presence of antibiotics in the feed medium. The specific growth rate was controlled at 0.15 h^{-1}. (Fermentation time is 26 h).

Variable (units)	Fed-batch fermentation without antibiotic and induction	Fed-batch fermentation with induction and without antibiotic	Fed-batch fermentation with induction and with antibiotic	Batch fermentation
Lipase (IU/mL)	0.13 ± 0.07	31.18 ± 1.24	82.83 ± 1.70	73.85 ± 2.04
Cell concentration (g/L)	23.85 ± 0.84	20.63 ± 1.53	17.94 ± 1.27	7.55 ± 0.75
Plasmid bearing cells (%)	27.00 ± 1.73	13.00 ± 0.76	91.00 ± 1.41	93.00 ± 1.74

Values after ± are the standard deviation of triplicate data.

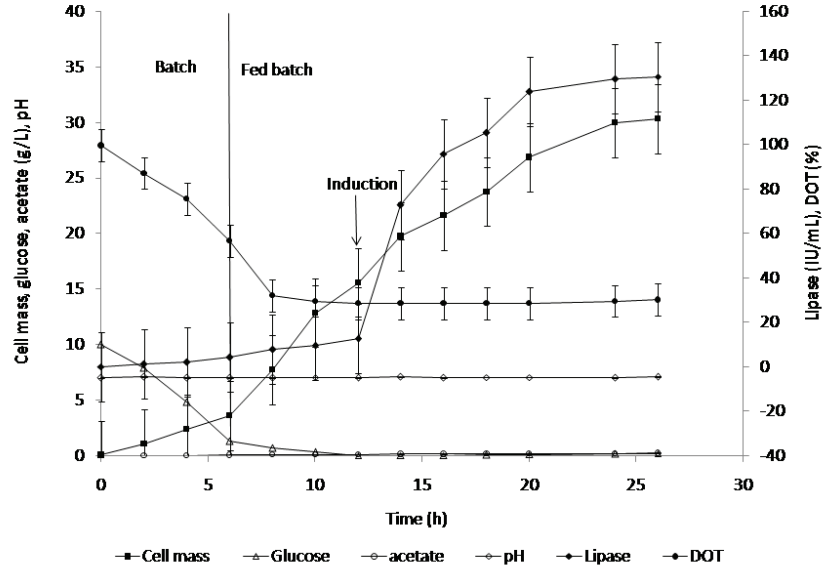

Figure 1. Time course of fed-batch fermentation for lipase production by *E. coli* BL21, operated at the required μ of 010 h^{-1}.

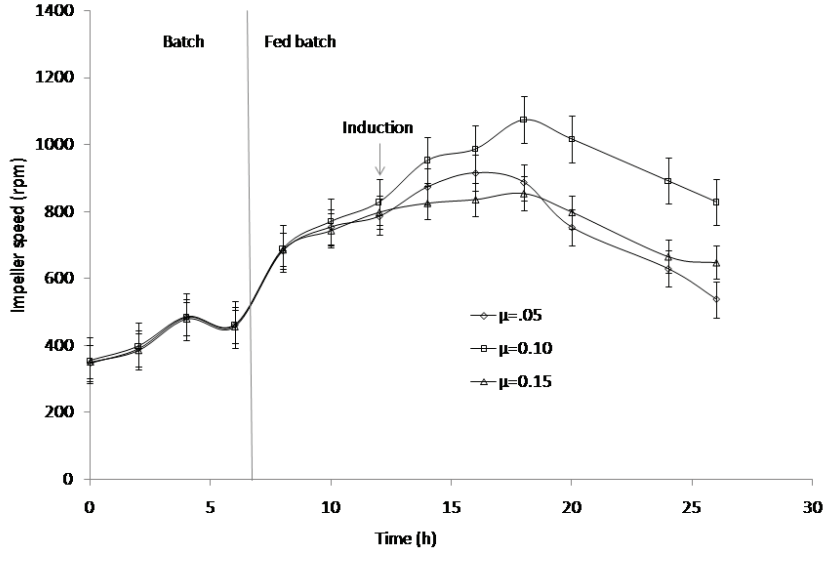

Figure 2. Variation in impeller speed to maintain the DOT at 30 % during exponential fed-batch fermentation conducted with three different required μ values.

maintained at very low level (0.19 g/L) and very low amount of acetate was accumulated in the culture (0.32 g/L).

In fed-batch fermentation operated at required μ of 0.05 h^{-1}, residual glucose and acetate were not detected in the culture. However, lipase activity (66.74 IU/mL) and cell concentration (15.63 g/L) obtained in this fermentation were the lowest among the three different μ tested in this study. Substantially high glucose (104.85 g/L) and acetate (6.52 g/L) were detected in fed-batch fermentation operated with the highest required μ value (0.15 h^{-1}), which resulted to significantly lower lipase activity (82.83 U/mL) and cell concentration (17.31 g/L) as compared to that obtained in fed-batch fermentation operated at the required μ of 0.10 h^{-1}. High cell concentration obtained in fed-batch fermentation operated at the required μ of 0.10 h^{-1} was related to the highest agitation speed applied (350 to 1050 rpm) to maintain the DOT level at 30 % saturation.

Most of the kinetic parameter values were statistically similar for the initial batch fermentation of three experiments with different μ values (Table 2). In contrast, most of the kinetic parameter values are significantly different for fed-batch fermentation operated at three different μ values. The actual μ values for fed-batch fermentation carried out at the required μ values of 0.05

Table 2. Kinetic parameters for the batch and fed-batch parts of exponential fed-batch production of lipase by recombinant *E. coli* BL21 conducted with different required μ values. (Fermentation time is 26 h).

Kinetic parameters	Specific growth rate, μ (h^{-1})					
	Initial batch fermentation			Fed-batch fermentation		
	0.05	0.10	0.15	0.05	0.10	0.15
X_m (g/L)	3.53±0.35[a]	3.59±0.26[b]	3.61±0.41[c]	15.63±1.06[a]	30.32±1.16[c]	17.31±0.92[b]
P_m (IU/mL)	3.79±0.72[a]	4.10±0.75[b]	4.00±0.63[b]	66.74±0.96[a]	130.5±1.76[c]	82.83±0.84[b]
Total glucose consumed (g/L)	8.50±0.59[a]	8.70±0.82[b]	8.98±0.71[c]	10.09±0.85[a]	48.16±0.95[b]	78.97±1.20[c]
Residual glucose (g/L)	--	--	--	--	0.190±0.074	104.85±1.53
Acetate accumulated (g/L)	0.030±0.005[a]	0.0400±0.0038[a]	0.0300±0.0027[a]	0.00±0.00[a]	0.320±0.051[a]	6.52±0.83[b]
μ (h^{-1})	0.47±0.06[a]	0.48±0.062[a]	0.46±0.028[a]	0.049±0.005[a]	0.120±0.007[b]	0.160±0.031[c]
q_p or π (IU/g/h)	0.040±0.007[a]	0.0400±0.0053[a]	0.0500±0.0092[a]	0.58±0.037[a]	0.790±0.014[c]	0.620±0.008[b]
q_S or σ (g/g/h)	0.800±0.052[a]	0.830±0.036[b]	0.840±0.025[b]	0.0700±0.0015[a]	0.120±0.006[b]	0.150±0.010[c]

Values with the different letters (a, b and c) are significantly different.
Values after ± are the standard deviation of triplicate data.

h^{-1}, 0.10 h^{-1} and 0.15 h^{-1} were 0.049 h^{-1}, 0.12 h^{-1} and 0.16 h^{-1}, respectively. This result suggests that the feed strategy employed in this study was capable to control the μ of *E. coli* during fed-batch fermentation at the required μ values. The highest lipase production rate, π, (0.79 IU/g/h) was observed in fed-batch fermentation operated at the required μ of 0.10 h^{-1}. In general, the values of π for all fed-batch fermentations were significantly higher than those calculated for initial batch fermentation. Glucose consumption rate (σ) was increased with increasing μ and the values for fed-batch fermentation were significantly lower than the values for initial batch fermentation.

The profiles of kinetic parameter values for fed-batch fermentation operated at the required μ of 0.10 h^{-1} are shown in Fig. 3. The σ and μ were decreased drastically during the initial batch fermentation. The rates remained almost constant during the fed-batch fermentation. The π was increased gradually during the initial batch fermentation and slightly reduced during the initial

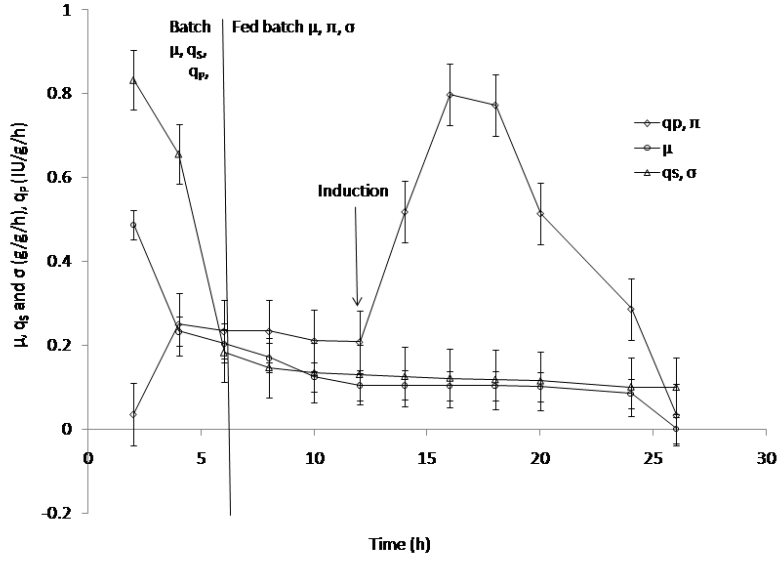

Figure 3. Profile of specific growth, specific lipase production rate and glucose consumption rate during fed-batch fermentation using *E. coli* BL21 for lipase production, where the specific growth rate (μ) was controlled at 0.10 h^{-1}.
μ: Specific growth rate, q_S: Specific glucose consumption rate in batch part, q_p: Specific lipase production rate in batch part, π: Specific lipase production rate in fed-batch part, σ: Specific glucose consumption rate in fed-batch part.

stages of fed-batch fermentation. The π was increased sharply after the induction and reached its maximum value after 2 h and then decreased drastically with time.

Effect of yeast extract in the feed medium

The time course of fed-batch fermentation for Lip 42 production by recombinant *E. coli* with the supplement of yeast extract in the feed medium is presented in Fig. 4. This fed-batch fermentation was conducted with the required μ of 0.10 h^{-1} and the feed medium consisted of 800 g/L glucose, antibiotics and 200 g/L yeast extract. Initial yeast extract concentration in the medium for initial batch fermentation was 5 g/L. In this fermentation run, glucose and acetate were maintained at very low levels. However, reduced cell concentration (28.3 g/L) and lipase production (90.03 U/mL) were observed in this fermentation run as compared to fed-batch fermentation without the addition of yeast extract in the feed medium and operated at the same required μ value (0.10 h^{-1}).

Discussion

The natural producer of Lip 42 was *Bacillus* sp. 42. It produced very low quantities of lipase, even highest amount obtained was less than 1 IU/mL [9]. The gene responsible for lipase production was therefore over expressed in *E. coli* BL21 [10]. The batch lipase production process was then optimized statistically in shake flasks [11, 12]. In batch process, large concentrations of acetate were observed in the medium, which inhibit the cell growth and lipase production. Therefore it was assumed that the slow feeding of substrate in a fed-batch mode may increase the growth and lipase production.

In the present work the fed-batch process for lipase production was studied in 1L bioreactor. In fed-batch fermentation without the addition of antibiotics, cells without plasmid grew faster than the cells with plasmid. This was indicated by the low percentage of plasmid bearing cell in fed-batch fermentation without antibiotics in the feed, which resulted to substantial reduction Lip 42 production. That is why, the reduced lipase (Lip 42) production by recombinant *E. coli* BL21 was observed in exponential fed-batch fermentation when antibiotics were not added into the feed medium. Similar phenomena has also been observed for other recombinant proteins production [14]. The recombinant *E. coli* BL21 is ampicillin resistant and degradation of ampicillin during high cell density cultivation has been reported [14, 15]. The addition of antibiotics throughout the fed-batch fermentation has also been successfully used to maintain plasmid stability for recombinant protein production [16]. However, Goyal [14] claimed that the addition of antibiotics in fed-batch fermentation failed to increase plasmid stability in *E. coli* system when the DOT level was fluctuated from 5 to 55 % saturation. The problem was recovered when the DOT level was successfully controlled accurately at 50 % saturation throughout the fermentation. In this study, the DOT level was fluctuated between 25 to 40 % saturation and the problem of plasmid stability was solved by the addition of antibiotics in the feed. The result suggests that the DOT level may not contribute to the plasmid instability of *E. coli* BL21 for Lip 42 production. Induction was performed at mid log phase, which has been claimed as the appropriate induction point for improvement of recombinant protein yields [17]. Lipase activity observed in fed-batch with antibiotics was not much higher than the batch fermentation mode in the present study. On the other hand the cell mass concentration for fed-batch was more than double as compared to the batch mode. The small difference in the lipase activities and big difference in cell mass produced in batch and fed-batch

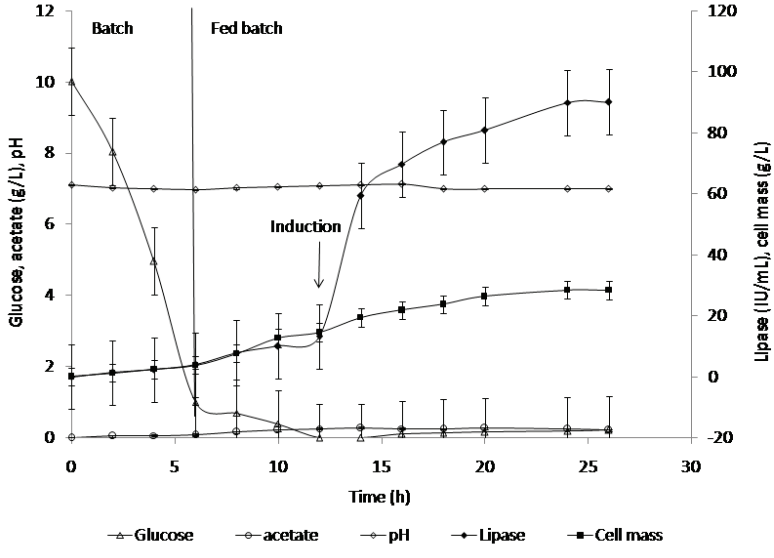

Figure 4. A typical time course of exponential fed-batch fermentation using *E. coli* BL21 for lipase production, where the feed medium consisted of a mixture of yeast extract and glucose was used and the required μ was set at 0.10 h^{-1}.

modes probably indicate the need of optimization of fed-batch process for lipase production enhancement.

In fed-batch fermentation by *E. coli* for the production of recombinant proteins including enzymes, carbon source such as glucose in the feed medium was normally kept at very high concentration to avoid substantial increase in culture volume due to the addition of large volume of feed [18]. Glucose concentration in the feed of up to 700 g/L [19, 20] and 800 g/L [21, 22] have been used in fed-batch fermentation of *E. coli*. Very high final cell concentration (134 g/L) has also been achieved in fed-batch fermentation of *E. coli* by feeding with solid glucose powder [23]. The use of very high glucose concentration (800 g/L) in the feed could also be used to overcome the problem related to substantial increase in culture volume in exponential fed-batch fermentation by recombinant *E. coli* BL21 for lipase production.

Exponential fed-batch fermentation carried out at three different required μ values showed that the required μ of 0.10 h^{-1} was most suitable for Lip 42 production by *E. coli* BL21. Fed-batch fermentation with low required μ value (0.05 h^{-1}) resulted in elevated pH, indicating the limitation of carbon source. Reduced cell concentration and lipase production were observed due to carbon source limitation. Decrease in culture pH with higher residual glucose and acetate accumulation in the culture was observed in fed-batch fermentation where the required μ was set at higher value (0.15 h^{-1}). This means that this fed-batch fermentation can be considered as over fed. Due to higher glucose concentration than that needed by the growing culture of *E. coli* BL21, acetate accumulated as a result of overflow metabolism, a well known phenomena at higher glucose concentrations [5]. The accumulation of acetate reduced the culture pH, which in turn, reduced the cell growth and lipase production. Fed-batch fermentation with controlled μ at 0.10 h^{-1} has been used for the improvement of the production of other recombinant proteins by *E. coli* [20, 24]. Yeast extract has a positive role in controlling the acetate accumulation in *E. coli* fermentation [25]. However, the addition of yeast extract in the feed did not improve Lip 42 production by recombinant *E. coli* BL 21.

Exponential fed-batch fermentation developed in this study was able to improve the final cell concentration and Lip 42 production by about 4 times and 1.8 times as compared to conventional batch fermentation (Table 1), respectively.

Conclusion

Exponential fed-batch fermentation has been developed for improvement of Lip 42 production, an organic solvent tolerant and thermostable lipase, by recombinant *E. coli* BL21. To overcome the problem of antibiotics degradation, their sufficient quantities were added in the feed medium. For optimal production of Lip 42 by recombinant *E. coli* BL21, fed-batch fermentation was operated with controlled μ at 0.10 h^{-1}. The total lipase activity (130.5 IU/mL) and cell concentration (30.32 g/L) obtained in optimal fed-batch fermentation were significantly higher than the batch fermentation (Lipase activity 73.85 IU/mL and cell concentration 7.55 g/L).

Acknowledgements: The research work is funded by and conducted in University Putra Malaysia.

Conflict of interest: The authors declared that there was no conflict of interest in this work.

References

[1] Kim SS, Kim EK, Rhee JS. Effects of growth rate on the production of *Pseudomonas fluorescens* lipase during the fed-batch cultivation of *Escherichia coli*. Biotechnol Prog 1996; 12(5): 718-722.

[2] Narayanan N, Chou CP. Alleviation of proteolytic sensitivity to enhance recombinant lipase production in *Escherichia coli*. Appl Environ Microbiol 2009; 75(16): 5424-5427.

[3] Sifour M, Saeed H, Zaghloul T, Berekaa M, Abdel-Fattah Y. Isolation of lipase gene of the thermophilic *Geobacillus stearothermophilus* strain-5. Biotechnol 2010; 9: 55-60.

[4] Lee S. High cell-density culture of *Escherichia coli*. Trend Biotechnol 1996; 14(3): 98-105.

[5] Lee SY, Shiloach J, Rinas U. Glucose and acetate metabolism in *E. coli*–system level analysis and biotechnological applications in protein production processes of *Escherichia coli*. Systems Biology and Biotechnology of *Escherichia coli*: Springer Netherlands; 2009. p. 377-400.

[6] Krause M, Ukkonen K, Haataja T, Ruottinen M, Glumoff T, et al. A novel fed-batch based cultivation method provides high cell-density and improves yield of soluble recombinant proteins in shaken cultures. Microbial Cell Factories 2010; 9(1):11.

[7] Henes B, Sonnleitner B. Controlled fed-batch by tracking the maximal culture capacity. J Biotechnol 2007; 132(2): 118-126.

[8] Zhang H, Zheng Y, Liu Q, Tao X, Zheng W, et al. Development of a fed-batch process for the production of anticancer drug TATm-survivin (T34A) in Escherichia coli. Biochem Engin J 2009; 43(2): 163-168.

[9] Eltaweel M, Rahman R, Salleh A, Basri M. An organic solvent-stable lipase from *Bacillus* sp. strain 42. Anal Microbiol 2005; 55(3): 187-192.

[10] Hamid T, Eltaweel M, Rahman R, Basri M, Salleh A. Characterization and solvent stable features of Strep-tagged purified recombinant lipase from thermostable and solvent tolerant *Bacillus* sp. strain 42. Anal Microbiol 2009; 59(1): 111-118.

[11] Nelofer R, Ramanan R, Rahman R, Basri M, Ariff A. Sequential optimization of production of a thermostable and organic solvent tolerant lipase by recombinant *Escherichia coli*. Anal Microbiol 2011; 61(3): 535-544.

[12] Nelofer R, Ramanan RN, Rahman RNZRA, Basri M, Ariff AB. Comparison of the estimation capabilities of response surface methodology and artificial neural network for the optimization of recombinant lipase production by *E. coli* BL21. J Ind Microbiol Biotechnol 2012; 39(2): 243-254.

[13] Bäcklund E, Markland K, Larsson G. Cell engineering of Escherichia coli allows high cell density accumulation without fed-batch process control. Bioprocess Biosyst Eng 2008; 31(1): 11-20.

[14] Goyal D, Sahni G, Sahoo DK. Enhanced production of recombinant streptokinase in *Escherichia coli* using fed-batch culture. Bioresour Technol 2009; 100(19): 4468-4474.

[15] Ensley BD. Stability of recombinant plasmids in industrial microorganisms. Crit Review Biotechnol 1986; 4(3): 263-277.

[16] Voulgaris I, Arnold SA, Speight R, Harvey LM, McNeil B. Effects of dissolved oxygen availability and culture biomass at induction upon the intracellular expression of Monoamine Oxidase by recombinant *E. coli* in fed-batch bioprocesses. Process Biochem 2010; 46(3): 721-729.

[17] Jeong KJ, Lee SY. High-level production of human leptin by fed-batch cultivation of recombinant *Escherichia coli* and its purification. Appl Environ Microbiol 1999; 65(7): 3027-3032.

[18] Shiloach J, Fass R. Growing *E. coli* to high cell density--a historical perspective on method development. Biotechnol Advan 2005; 23(5): 345-357.

[19] Caspeta L, Flores N, Perez NO, Bolivar F, Ramirez OT. The effect of heating rate on Escherichia coli metabolism, physiological stress, transcriptional response, and production of temperatureâ€ induced recombinant protein: A scale down study. Biotechnol Bioeng 2009; 102(2): 468-482.

[20] Luo Q, Shen YL, Wei DZ, Cao W. Optimization of culture on the overproduction of TRAIL in high-cell-density culture by recombinant *Escherichia coli*. Appl Microbiol Biotechnol 2006; 71(2): 184-191.

[21] Fan DD, Luo Y, Mi Y, Ma XX, Shang L. Characteristics of fed-batch cultures of recombinant Escherichia coli containing human-like collagen cDNA at different specific growth rates. Biotechnol Letter 2005; 27(12): 865-870.

[22] Xiufu H, Daidi F, Yan'e L, Xi Z, Huijuan S, Yu M, et al. Kinetics of high cell density fed-batch culture of recombinant *Escherichia coli* producing human-like collagen1. Chin J Chem Eng 2006; 14(2): 242-247.

[23] Matsui T, Yokota H, Sato S, Mukataka S, Takahashi J. Pressurized culture of Escherichia coli for a high concentration. Agricul Biol Chem 1989; 53(8): 2115-2120.

[24] Kim BS, Lee SC, Lee SY, Chang YK, Chang HN. High cell density fed-batch cultivation of *Escherichia coli* using exponential feeding combined with pH-stat. Bioprocess Biosyst Eng 2004; 26(3): 147-50.

[25] Nancib N, Branlant C, Boudrant J. Metabolic roles of peptone and yeast extract for the culture of a recombinant strain of *Escherichia coli*. J Ind Microbiol Biotechnol 1991; 8(3): 165-169.