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Kinetics and modelling of batch fermentation for the production of organic solvent tolerant and thermostable lipase by recombinant E. coli

Organik çözücü toleranslı ve ısıya dayanıklı rekombinan E. coli lipaz üretiminin kinetiği ve grup fermentasyonu modellemesi

Abstract: Objective: Kinetics of organic solvent tolerant and thermostable lipase production by recombinant E. coli in shake flask level and 2 L stirred tank bioreactor level was studied to observe the variations of important kinetic parameters at two different levels of bioprocess.

Methods: Unstructured models based on Monod equation for growth and Luedeking-Piret equation for lipase production and glucose consumption were used to predict cell growth, lipase production and glucose utilization. The shake flask fermentation experiments were carried out at different initial glucose and yeast extract concentrations using recombinant bacterial strain E. coli BL21. Lipase production was also carried out using 2L stirred tank bioreactor for comparison.

Results: In all cases, the data fitted well to the proposed models. The highest growth and lipase activity were obtained at 25 g/L glucose and 25 g/L yeast extract. Cell growth (6.42 g/L) and lipase production (65.32 IU/mL) in 2 L stirred tank bioreactor was comparable to those obtained in shake flask fermentations. The calculated value of growth associated constant (9.874 IU/g/h) was much higher than that of non-growth associated constant (0.022 IU/g/h) in bioreactor as well as in shake flasks. The values of maximum specific growth rate (μₘₐₓ) and glucose saturation constant (Kₛ) for shake flask fermentations, calculated from Monod equation, were 0.476 h⁻¹ and 5.237 g/L respectively.

Conclusion: From the modelling exercise, it was concluded that the lipase production is dominantly growth associated process. The kinetic parameter values for fermentations in shake flask and 2L stirred tank bioreactor were comparable, indicating that the bioprocess could be transferred into larger scale.

Keywords: Lipase, recombinant E. coli, fermentation kinetics, Monod model, Luedeking-Piret equation

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Bulgular: Her durumda veriler önerilen modellere tam olarak uyum göstermiştir. Yüksek büyüme seviyesi ve lipaz aktivitesi 25 g/L glikoz ve 25 g/L maya ekstraktında elde edilmiştir. İlk litrelik karışımlı biyoreaktörün biyoreaktörüne dayalı doyuma hızı (0.022 IU/g/h) çok yüksek elde edilmiştir. Maksimum özgürlük büyüme oranı ($\mu_\text{m}$) ve karışımlı biyoreaktördeki biyoreaktörün biyoreaktöründe elde edilen hücre büyümesi (6.42 g/L) ve lipaz üretimi (65.32 IU/mL), karışımlı biyoreaktörden elde edilen veriler karşılaştırılabilir olarak bulunmuştur. Biyoreaktör ve karışımlı biyoreaktörün içindeki hesaplanan büyüme hızı 6.42 g/L olarak bulunmuştur.

Sonuç: Modelleme çalışmasına göre lipaz üretimine bakımdan bir büyüme ilişkisi süreç olduğu sonucuna varılmıştır. Karışımlı biyoreaktör ve 2 litrelik karışımlı biyoreaktörün biyoreaktörüne dayalı doyuma hızı (9.874 IU/g/h), büyüme olmayan sabite göre (0.022 IU/g/h) çok yüksek elde edilmiştir. Maksimum özgürlük büyüme oranı ($\mu_\text{m}$) ve karışımlı biyoreaktördeki biyoreaktörün biyoreaktöründe elde edilen hücre büyümesi (9.874 IU/g/h), büyüme olmayan sabite göre (0.022 IU/g/h) çok yüksek elde edilmiştir. Maksimum özgürlük büyüme oranı ($\mu_\text{m}$) ve karışımlı biyoreaktördeki biyoreaktörün biyoreaktöründe elde edilen hücre büyümesi (9.874 IU/g/h), büyüme olmayan sabite göre (0.022 IU/g/h) çok yüksek elde edilmiştir. Maksimum özgürlük büyüme oranı ($\mu_\text{m}$) ve karışımlı biyoreaktördeki biyoreaktörün biyoreaktöründe elde edilen hücre büyümesi (9.874 IU/g/h), büyüme olmayan sabite göre (0.022 IU/g/h) çok yüksek elde edilmiştir. Maksimum özgürlük büyüme oranı ($\mu_\text{m}$) ve karışımlı biyoreaktördeki biyoreaktörün biyoreaktöründe elde edilen hücre büyümesi (9.874 IU/g/h), büyüme olmayan sabite göre (0.022 IU/g/h) çok yüksek elde edilmiştir. Maksimum özgürlük büyüme oranı ($\mu_\text{m}$) ve karışımlı biyoreaktördeki biyoreaktörün biyoreaktöründe elde edilen hücre büyümesi (9.874 IU/g/h), büyüme olmayan sabite göre (0.022 IU/g/h) çok yüksek elde edilmiştir. Maksimum özgürlük büyüme oranı ($\mu_\text{m}$) ve karışımlı biyoreaktördeki biyoreaktörün biyoreaktöründe elde edilen hücre büyümesi (9.874 IU/g/h), büyüme olmayan sabite göre (0.022 IU/g/h) çok yüksek elde edilmiştir. Maksimum özgürlük büyüme oranı ($\mu_\text{m}$) ve karışımlı biyoreaktördeki biyoreaktörün biyoreaktöründe elde edilen hücre büyümesi (9.874 IU/g/h), büyüme olmayan sabite göre (0.022 IU/g/h) çok yüksek elde edilmiştir. Maksimum özgürlük büyüme oranı ($\mu_\text{m}$) ve karışımlı biyoreaktördeki biyoreaktörün biyoreaktöründe elde edilen hücre büyümesi (9.874 IU/g/h), büyüme olmayan sabite göre (0.022 IU/g/h) çok yüksek elde edilmiştir. Maksimum özgürlük büyüme oranı ($\mu_\text{m}$) ve karışımlı biyoreaktördeki biyoreaktörün biyoreaktöründe elde edilen hücre büyümesi (9.874 IU/g/h), büyüme olmayan sabite göre (0.022 IU/g/h) çok yüksek elde edilmiştir. Maksimum özgürlük büyüme oranı ($\mu_\text{m}$) ve karışımlı biyoreaktördeki biyoreaktörün biyoreaktöründe elde edilen hücre büyümesi (9.874 IU/g/h), büyüme olmayan sabite göre (0.022 IU/g/h) çok yüksek elde edilmiştir. Maksimum özgürlük büyüme oranı ($\mu_\text{m}$) ve karışımlı biyoreaktördeki biyoreaktörün biyoreaktöründe elde edilen hücre büyümesi (9.874 IU/g/h), büyüme olmayan sabite göre (0.022 IU/g/h) çok yüksek elde edilmiştir. Maksimum özgürlük büyüme oranı ($\mu_\text{m}$) ve karışımlı biyoreaktördeki biyoreaktörün biyoreaktöründe elde edilen hücre büyümesi (9.874 IU/g/h), büyüme olmayan sabite göre (0.022 IU/g/h) çok yüksek elde edilmiştir. Maksimum özgürlük büyüme oranı ($\mu_\text{m}$) ve karışımlı biyoreaktördeki biyoreaktörün biyoreaktöründe elde edilen hücre büyümesi (9.874 IU/g/h), büyüme olmayan sabite göre (0.022 IU/g/h) çok yüksek elde edilmiştir. Maksimum özgürlük büyüme oranı ($\mu_\text{m}$) ve karışımlı biyoreaktördeki biyoreaktörün biyoreaktöründe elde edilen hücre büyümesi (9.874 IU/g/h), büyüme olmayan sabite göre (0.022 IU/g/h) çok yüksek elde edilmiştir.

Anahtar Kelimeler: Lipaz, rekombinan E. coli, fermentasyon (mayalanma) kinetiği, Monod modeli, Luedeking-Piret denklemi

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Introduction

Lipases are important industrial enzymes that are commonly used for catalyzing many reactions such as hydrolysis, esterification, modification of fat, kinetic resolution and enantio selective group differentiation in industrial processes [1,2]. Industrial lipases are produced through microbial fermentations using low cost medium formulations [3]. Many natural isolates of bacteria and fungi are used for lipase production. Bacillus [4], Pseudomonas [5], Burkholderia [6], are most commonly used bacterial strains. Some thermophilic microbial strains that are capable to produce thermostable lipase have been reported [4,7,8]. However, these strains only produced small quantities of lipase. Therefore the lipase producing genes of these strains were cloned into host bacterial strains that can easily be grown at room temperature and able to produce lipase at higher quantity [9,10].

Batch fermentation is the simplest mode of bioprocess, which can be easily set up and operated with a limited knowledge of the related factors [11]. Kinetic modeling is an important step in the development of fermentation process that can be used to determine the optimal operating conditions for the production of the target product. The data obtained from batch fermentations may be used to generate the kinetic parameters, which are important for scaling-up and designing of other fermentation modes such as fed-batch and continuous. Two types of models including structured and unstructured are commonly used for kinetic and modeling of bioprocesses. Structured models are complex and used to describe a process at molecular level while unstructured models are relatively simple and easy to apply on many bioprocesses [12]. Monod and Luedeking-Piret equations are examples of unstructured models that are widely used to describe many fermentation processes [12–14].

Bacillus sp. 42, an organic acid tolerant and thermostable lipase (Lip 42) producing bacterial strain, has been isolated from palm oil mill effluent [4]. Lip 42 is stable in many organic solvents such as DMSO, DMF, acetone, methanol, heptanol and octanol. Therefore, Lip 42 has potential to be used as a biocatalyst for biodiesel production. Since, Bacillus sp. 42 produced very low quantities of lipase, the gene was overexpressed in E. coli BL21 [15] for enhancement of lipase production. Information on fermentation kinetics of thermostable lipase production by recombinant E. coli is rarely available in literature.

The present work is a part of scaling up process for lipase production by recombinant E. coli. In the first step, process was shifted from shake flask to a small scale bioreactor. The scale up for each step is considered successful if the production is at least equal to the previous scale [16]. The hypothesis of the present study is that the lipase production in small scale stirred tank bioreactor could be achieved with similar or higher performance as compared to those obtained in the shake flask fermentations, if suitable process conditions and operating parameters are used. The main objective of this study is therefore to compare the kinetics of lipase (Lip 42) production by E. coli BL21 in batch fermentation at two different levels, shake flask and 2 L stirred tank bioreactor. The models based on Monod and Luedeking-Piret equations were used for the generation of kinetic parameters. The information
Materials and Methods

Microorganism and inoculum preparation

*E. coli* BL21 (DE3) pLysS [15] harbouring organic solvent tolerant and thermostable lipase gene of *Bacillus* sp. 42 [4] was used in this study. Inoculum was prepared in 50 mL LB broth by adding a single colony grown from LB agar plate and incubated at 37°C in a rotary shaker with a shaking speed of 200 rpm for 16–18 h.

Media

LB broth was used for inoculum preparation. Production medium consists of (g/L): NaCl, 3; glucose, 5 to 35; tryptone, 10, yeast extract, 5 to 30, potassium hydrogen phosphate, dibasic 9.4; and potassium phosphate monobasic 2.2. Ampicillin (50 µg/mL) and chloramphenicol (35 µg/mL) were also added to all media. Medium pH was adjusted to pH 7 using 0.1 M NaOH before sterilization. Isopropyl β-D-1-thiogalactopyranoside (IPTG) was used as an inducer at a concentration of 0.5 mM, which was added to the culture at mid log growth phase.

Lipase production

Shake flask experiments were conducted in 250 mL Erlenmeyer flasks containing 50 mL production medium. The flasks were inoculated with 4% v/v inoculum and incubated at 30°C in a rotary shaker with a shaking speed of 200 rpm for 16 h. All the experiments were carried out in triplicate. The values presented here are means of three observations for each parameter.

Lipase production was also carried out in 2 L stirred tank bioreactor (B-DCU Model, Sartorius, Germany) with 1 L working volume. The bioreactor was equipped with temperature, dissolved oxygen and pH control modules. A single six bladed Ruston turbine, agitated at 500 rpm, was used for agitation. Aeration was provided by sparging sterile air into the bioreactor at a flow rate of 1 vvm. To start the fermentation, the bioreactor was inoculated with 4% v/v inoculum. During the fermentation, the temperature within the bioreactor vessel was controlled at 30°C. The culture pH and dissolved oxygen tension (DOT) were not controlled but the changes during the fermentation were recorded.

Models

Unstructured models, based on Monod and Luedeking-Piret equations, were used for the modelling of growth, substrate consumption and product formation.

Growth rate

\[
\frac{\Delta X}{\Delta t} = \mu X
\]

Lipase production rate

\[
\frac{\Delta P}{\Delta t} = q_P X
\]

Substrate consumption rate

\[
\frac{\Delta S}{\Delta t} = q_S X
\]

Where \( X \) is cell concentration, \( P \) is lipase activity, \( S \) is glucose concentration, \( \mu \) is specific growth rate, \( q_p \) is volumetric lipase production rate and \( q_s \) is volumetric glucose uptake rate.

Monod model for growth is expressed in equation 4;

\[
\mu = \frac{\mu_{max} S}{K_S + S}
\]

Where \( \mu_{max} \) is the maximum specific growth rate and \( K_S \) is half saturation constant for glucose.

The Luedeking-Piret model for product formation and substrate consumption are given in equations 5 and 6;

\[
\frac{\Delta P}{\Delta t} = \alpha \frac{\Delta X}{\Delta t} + \beta X
\]

\[
\frac{\Delta S}{\Delta t} = m \frac{\Delta X}{\Delta t} + n X
\]

Where \( \alpha \) is the growth associated lipase production constant, \( \beta \) is the non-growth associated lipase production constant, \( m \) is growth associated glucose consumption constant and \( n \) non-growth associated glucose consumption constant.

By placing the values of \( \frac{\Delta P}{\Delta t} \) and \( \frac{\Delta S}{\Delta t} \) from equations 1 and 2 into equation 5 and the values of \( \frac{\Delta X}{\Delta t} \) and \( \frac{\Delta P}{\Delta t} \) from equations 1 and 3 into equation 6, equations 5 and 6 can be simplified into equations 7 and 8, respectively;

\[
q_p = \alpha \mu + \beta
\]

\[
q_s = m \mu + n
\]

Statistical analysis

Curve fitting toolbox of MATLAB (7.10.0.499 (R2010a) was used for simulation of data and solving the equations. Equation 4 was created and fitted by using non linear least
squares method. Linear polynomial regression was used for solving equations 7 and 8 and non-linear polynomial regression was used for the simulation of experimental data with linear least squares method for model fitting. Tukey’s Honestly Significant Difference (HSD) function of SPSS (version 17) was used for the analysis of variance of the calculated parameters. HSD is the most commonly used test to compare the effect of different treatments in fermentation processes [17,18]. In one way ANOVA test of lipase production parameters, two parameters including acetate concentration ($A_n$) and growth associated glucose consumption rate ($m$) were not homogeneous. Therefore, Kruskal-Wallis and pair wise Mann-Whitney tests were conducted as alternative to post hoc test for these two parameters. On the other hand, the fermentation data obtained from shake flask and bioreactor were compared using the independent sample T test of SPSS. Correlation analysis of $\mu$ with $q_p$ and $q_s$ was also conducted using SPSS.

Analysis

Culture samples withdrawn from the fermentation at time intervals were centrifuged at 10,700xg (TA-14-50, Allegra®250R, Beckman Coulter, USA) for 10 min. The cell pellets obtained were washed and resuspended in 20 mM phosphate buffer at pH 7 and then lysed by sonication for 2 min on ice. Supernatant obtained after the removal of cell debris by centrifugation at 10,700xg was used for the determination of lipase activity.

The method proposed by Hamid [15], which is a modified method of Kwon and Rhee [19] was used for the determination of lipase activity. Cell concentration was determined by dry cell weight method. The cell pellets obtained from the known volume of culture samples were dried at 80°C for 24 h and the weight of dry cell was determined using electronic balance. Glucose was analyzed using YSI biochemistry analyzer (Model 2700). Acetate was analyzed using HPLC (Shimadzu, model JUBR 121) with 300mm×7.8mm Aminex® organic acid column (HPX-87H) and UV detector. The mobile phase (4mM H$_2$SO$_4$) at a flow rate of 0.6 mL/min was used and the detection was read at 210 nm after 15 min retention time.

Results

Time course of lipase fermentation by *E. coli* and modelling

The typical time course of batch lipase fermentation by recombinant *E. coli* BL21 in shake flask culture and 2 L stirred tank bioreactor are shown in Figures 1 and 2, representing the fitness of the proposed models (equations 7 and 8) to the experimental data. Lipase was not produced before the induction, and the production was increased enormously after the induction. The lag growth phase was very short followed by a long exponential growth phase. The amount of acetate accumulated in the culture was
gradually increased with concomitant decrease in culture pH during the fermentation process. The fermentation data fitted well to the models, suggesting that the proposed models are sufficient to describe lipase fermentation by recombinant *E. coli*. The proposed models also fitted well with variation in fermentation parameters such as glucose and yeast extract concentrations and the results are discussed in details in the following sections.

**Effect of glucose concentration**

The performance and the kinetics parameter values for batch fermentation of lipase by recombinant *E. coli* in shake flask using different initial glucose concentrations (5, 10, 15, 20, 25, 30 and 35 g/L) are shown in Table 1 and Figure 3. The highest maximum cell concentration ($X_m$) (4.68 g/L), maximum lipase activity ($P_m$) (56.84 IU/L) and glucose consumption ($S_m$) (16.7 g/L) was observed in fermentation with 25 g/L glucose. From the comparison of means, it was observed that these three parameters are significantly different at different initial sugar concentrations. Acetate was not accumulated in fermentation with initial glucose concentrations of 5 and 10 g/L, indicating that the results of these two values are statistically similar. The concentration of acetate accumulated in fermentations with 20 and 25 g/L glucose was not significantly different. The concentration of acetate accumulated in fermentations with other glucose concentrations was significantly different. The highest concentration of acetate (9.72 g/L) accumulated in the culture was observed at initial glucose concentration of 35 g/L.

The highest cell yield ($Y_{X/S}$) (0.291 g/g), lipase yield ($Y_{P/S}$) (3.405 IU/g) and cell productivity ($Y_{P/X}$) (12.152 IU/g) was also obtained at 25 g/L glucose. The lowest $Y_{X/S}$ (0.203 g/g) was observed at 35 g/L glucose and the value was also significantly different from $Y_{X/S}$ for the fermentation with other glucose concentrations. The values of $Y_{P/S}$ were significantly different at different glucose concentrations. The lowest $Y_{P/S}$ (2.476 IU/g) was obtained at 35 g/L glucose. Cell productivity ($Y_{P/S}$) was not significantly different for fermentation with initial glucose concentration of 5, 10 and 15 g/L and statistically similar in fermentation with 20, 25 and 30 g/L glucose.

The highest $\mu_{\text{max}}$ (0.489 h$^{-1}$) was observed at 25 g/L glucose and this value was not significantly different from $\mu_{\text{max}}$ (0.487 h$^{-1}$) obtained in fermentation with 20 g/L glucose. The smallest $\mu_{\text{max}}$ (0.246 h$^{-1}$) was obtained at 5 g/L glucose. The $q_P$ and $q_S$ values were significantly different at different glucose concentrations. The highest $q_P$ (8.278 IU/g/L/h) and $q_S$ (1.241 g/g/L/h) was obtained at 25 g/L glucose while the lowest values for $q_P$ (3.755 IU/g/L/h) and $q_S$ (0.7 g/g/L/h) were obtained at 5 g/L glucose. The value of $\alpha$ was varied from 6.305 to 17.332 IU/g with the variation in initial glucose concentration. The highest $\alpha$ value (17.332 IU/g) was obtained at 25 g/L glucose while the lowest $\alpha$ value (6.305 IU/g) was obtained at low initial glucose concentration (5 g/L). For all initial glucose concentrations tested in this study, the values of $\beta$ were very low, ranging from 0.012 to 0.044 IU/g/h. Similarly, the values of $n$ for all glucose concentrations tested were also very low (0.015 to 0.08 IU/g/h). On the other hand, the values
Table 1: Batch fermentation kinetics of lipase production by recombinant E. coli carried out in shake flasks using different initial glucose concentrations.

| Performance/Kinetic parameter value | Glucose concentration (g/L) (Mean±SD) |
|------------------------------------|----------------------------------------|
|                                    | 5  | 10  | 15  | 20  | 25  | 30  | 35  |
| Maximum cell concentration, \( X_m \) (g L\(^{-1} \)) | 1.27±0.919 | 2.58±0.134 | 3.9±0.378 | 4.17±0.472 | 4.68±0.632 | 4.28±0.75 | 3.35±0.624 |
| Maximum lipase activity, \( P_m \) (IU mL\(^{-1} \)) | 15.14±1.57 | 30.77±1.25 | 46.53±0.65 | 50.61±1.20 | 56.84±1.22 | 51.88±0.87 | 40.12±0.83 |
| Glucose consumed, \( S_m \) (g L\(^{-1} \)) | 5±0.852 | 10±0.793 | 14.8±0.768 | 15.3±0.579 | 16.7±0.824 | 16.6±0.748 | 16.2±0.651 |
| Acetate formed, \( A_m \) (g L\(^{-1} \)) | 0±0.00 | 0±0.00 | 0.35±0.152 | 1.82±0.465 | 2.67±0.587 | 6.01±1.308 | 9.72±1.183 |
| Cell mass yield, \( Y_{x/s} \) (g g glucose\(^{-1} \)) | 0.254±0.042 | 0.258±0.027 | 0.263±0.032 | 0.273±0.031 | 0.291±0.035 | 0.239±0.041 | 0.203±0.024 |
| Lipase yield, \( Y_{p/s} \) (IU g glucose\(^{-1} \)) | 3.03±0.205 | 3.077±0.085 | 3.144±0.16 | 3.307±0.307 | 3.405±0.241 | 3.12±0.092 | 2.476±0.253 |
| Cell productivity, \( Y_{x/s} \) (IU g cells\(^{-1} \)) | 11.92±0.863 | 11.926±0.681 | 11.93±0.752 | 12.137±0.684 | 12.152±0.872 | 12.121±0.573 | 11.976±0.916 |
| Maximum specific growth rate, \( \mu_m \) (h\(^{-1} \)) | 0.246±0.064 | 0.414±0.047 | 0.462±0.038 | 0.487±0.039 | 0.489±0.052 | 0.484±0.071 | 0.48±0.086 |
| Glucose consumption rate, \( q_s \) (g g\(^{-1} \) h\(^{-1} \)) | 0.7±0.075 | 0.747±0.036 | 0.878±0.081 | 1.123±0.074 | 1.241±0.067 | 1.134±0.082 | 0.912±0.049 |
| Lipase production rate, \( q_p \) (IU g\(^{-1} \) h\(^{-1} \)) | 3.755±0.536 | 6.337±0.831 | 7.79±0.826 | 8.185±0.754 | 8.278±0.586 | 7.696±0.467 | 7.566±0.658 |
| Growth associated lipase production constant, \( \alpha \) (IU g\(^{-1} \)) | 6.305±0.475 | 14.362±0.538 | 16.527±0.962 | 16.855±0.853 | 17.332±0.686 | 16.233±0.925 | 14.162±0.894 |
| Non growth associated lipase production constant, \( \beta \) (IU g\(^{-1} \) h\(^{-1} \)) | 0.012±0.005 | 0.025±0.002 | 0.031±0.003 | 0.035±0.005 | 0.044±0.003 | 0.025±0.004 | 0.024±0.005 |
| Growth associated glucose consumption constant, \( m \) (g g\(^{-1} \)) | 2.847±0.095 | 3.167±0.364 | 3.949±0.510 | 4.605±0.089 | 4.643±0.483 | 3.83±0.0623 | 2.977±0.371 |
| Non growth associated glucose consumption constant, \( n \) (g g\(^{-1} \) h\(^{-1} \)) | 0.015±0.004 | 0.045±0.005 | 0.054±0.003 | 0.069±0.006 | 0.08±0.004 | 0.046±0.005 | 0.025±0.003 |

a, b and c: Means values with the different letters are significantly different.
Table 2: Batch fermentation kinetics of lipase production by recombinant E. coli BL21 carried out in shake flasks using different yeast extract concentrations.

| Performance/Kinetic parameter value | Yeast extract concentration (g/L) (Mean±SD) |
|------------------------------------|---------------------------------------------|
|                                    | 5   | 10  | 15  | 20  | 25  | 30  |
| Maximum cell concentration, $X_m$ (g L$^{-1}$) | 4.13±0.875 | 4.52±0.684 | 4.82±0.759 | 5.03±0.596 | 5.46±0.753 | 5.17±0.643 |
| Maximum lipase activity, $P_m$ (IU mL$^{-1}$) | 50.21±2.848 | 54.98±1.562 | 58.64±3.836 | 61.92±2.72 | 67.82±3.042 | 58.36±1.961 |
| Glucose consumed, $S_m$ (g L$^{-1}$) | 14.18±1.631 | 15.42±0.964 | 15.83±1.47 | 16.36±1.320 | 16.97±1.518 | 16.15±1.293 |
| Acetate formed, $A_m$ (g L$^{-1}$) | 4.82±0.836 | 4.15±0.691 | 3.95±0.758 | 3.64±0.642 | 3.15±0.593 | 3.12±0.731 |
| Cell mass yield, $Y_{x/s}$ (g cells g glucose$^{-1}$) | 0.291±0.017 | 0.293±0.035 | 0.304±0.031 | 0.307±0.025 | 0.322±0.016 | 0.320±0.028 |
| Lipase yield, $Y_{p/s}$ (IU g glucose$^{-1}$) | 3.54±0.815 | 3.565±0.572 | 3.704±0.743 | 3.75±0.716 | 3.996±0.637 | 3.614±0.594 |
| Cell productivity, $Y_{p/x}$ (IU g cells$^{-1}$) | 12.157±0.962 | 12.164±0.857 | 12.168±0.895 | 12.31±1.063 | 12.42±0.958 | 11.28±0.89 |
| Maximum specific growth rate, $\mu_m$ (h$^{-1}$) | 0.386±0.063 | 0.408±0.039 | 0.412±0.062 | 0.427±0.071 | 0.431±0.059 | 0.415±0.063 |
| Glucose consumption rate, $q_S$ (g g$^{-1}$ h$^{-1}$) | 2.965±0.418 | 3.184±0.536 | 3.195±0.372 | 3.266±0.385 | 3.373±0.517 | 2.333±0.492 |
| Lipase production rate, $q_p$ (IU g$^{-1}$ h$^{-1}$) | 2.290±0.254 | 2.445±0.352 | 2.465±0.237 | 2.532±0.265 | 2.669±0.318 | 2.5±0.273 |
| Growth associated lipase production constant, $\alpha$ (IU g$^{-1}$) | 2.108±0.536 | 13.974±0.974 | 14.756±0.895 | 16.184±1.052 | 18.726±1.136 | 18.726±1.207 |
| Non growth associated lipase production constant, $\beta$ (IU g$^{-1}$ h$^{-1}$) | 0.011±0.002 | 0.014±0.001 | 0.016±0.001 | 0.025±0.003 | 0.029±0.002 | 0.025±0.003 |
| Growth associated glucose consumption constant, $m$ (g g$^{-1}$) | 1.779±0.537 | 1.809±0.392 | 1.906±0.467 | 2.469±0.471 | 2.494±0.316 | 2.304±0.452 |
| Non growth associated glucose consumption constant, $n$ (g g$^{-1}$ h$^{-1}$) | 0.009±0.001 | 0.01±0.002 | 0.01±0.002 | 0.015±0.002 | 0.06±0.003 | 0.054±0.002 |

a, b and c: Means values with the different letters are significantly different.
of m varied from 2.847 to 4.643 g/g with different initial glucose concentrations. The lowest value of m (2.847 g/g) was obtained in fermentation with 5 g/L glucose while the highest (4.643 g/g) was obtained in fermentation with 25 g/L glucose. However the value of m was not significantly different from that obtained in fermentations with 15 and 20 g/L glucose.

**Effects of yeast extract concentration**

The values of $X_a$, $P_m$ and $S_m$ were significantly different at different initial yeast extract concentrations used in lipase fermentation by recombinant *E. coli* (Table 2 and Figure 3). The highest values of $X_a$, $P_m$ and $S_m (5.46 g/L, 67.82 IU/mL and 16.7 g/L respectively) were obtained at 25 g/L yeast extract while the lowest values (4.13 g/L, 50.21 IU/mL and 14.18 g/L respectively) were obtained at 5 g/L. The amount of acetate accumulated in the culture was greatly influenced by the variation of yeast extract concentration added into the culture medium. The highest accumulation of acetate was detected at 5 g/L yeast extract and the lowest accumulation was observed at 30 g/L yeast extract.

Lipase yield ($Y_{p/s}$) was also significantly different at different initial yeast extract concentrations. The highest value of $Y_{p/s}$ (3.996 IU/g) was observed at 25 g/L yeast extract while the lowest (3.54 IU/g) was obtained at 5 g/L yeast extract. The smallest value of $Y_{p/x}$ observed at 30 g/L yeast extract, which was not significantly different with that obtained at 20 g/L yeast extract. The values of $Y_{p/x}$ for fermentation with 5, 10 and 15 g/L yeast extract were statistically similar.

The highest $\mu_{max}$ (0.431 h$^{-1}$) was observed at 25 g/L.
yeast extract but it was not significantly different with the μₘₐₓ calculated for fermentation with 10, 15, 20 and 30 g/L yeast extract. The values of μₘₐₓ for fermentation with 5, 10, 15 and 30 g/L yeast extract were almost similar. The values of qₚ were statistically different at different initial yeast extract concentrations. The highest value of qₚ (3.373 g/g/h) was obtained at 25 g/L yeast extract while the lowest value of qₔ (2.333 g/g/h) was obtained at 30 g/L yeast extract. The highest value of α (18.726 IU/g) was observed at 25 and 30 g/L yeast extract. The values of β for all concentrations of yeast extract tested in this study were very low (0.011 to 0.029 IU/g/h) and were not significantly different. The value of m was the highest at 20 g/L (2.467 g/g/h) and 25 g/L (2.494 g/g/h) yeast extract. The values of n for all yeast extract concentrations were ranged from 0.009 to 0.054 g/g/h.

Comparison of fermentation in shake flask and 2L stirred tank bioreactor

The typical time course of lipase production in stirred tank bioreactor is represented in Figure 2. Simulated data are represented by the solid lines and the experimental data are represented by the broken lines. The performance and the kinetic parameter values for lipase fermentation by recombinant E. coli at two different scales, shake flask and 2L stirred tank bioreactor, are represented in Table 3. All the calculated parameters are not significantly different except Pₘₐₓ, α and n, as observed from the results of T test. The values of Pₘₐₓ, α and n are found higher in stirred tank bioreactor as compared to those in shake flask. It is interesting to note that the amount of acetate accumulated in the culture was higher in shake flask as compared to that accumulated in bioreactor.

The values of μₘₐₓ and Kₛ and the predicted fermentation data were calculated by solving equation 4 in MATLAB software. The goodness of the fit between the predicted and experimental data is represented in Table 4. The proposed models fitted very well to all experimental data, as shown by the values of correlation coefficient (R²) and adjusted coefficient correlation determination (adjusted R²) (Table 4). Almost all R² and adjusted R² values are close to 1.

Figure 3 represents the positive relationship between specific growth rate with lipase production rate and glucose consumption rate, calculated using equations 7 and 8 for fermentation in shake flasks and 2L stirred tank bioreactor. The predicted values according to the models and the calculated values from the observed data are in good agreement, as shown by correlation coefficient analysis (Figure 4). The values of correlation coefficient (r²) of μ with qₚ and qₔ are positive and very close to 1 for both fermentation levels, shake flask and stirred tank bioreactor. This positive relationship supported the assumption that the lipase production by E. coli BL21 is growth associated process. The results represented in Figure 4 showed that the behaviour of the E. coli strain was almost similar during fermentation in shake flask and stirred tank bioreactor. The curves are steeper for shake flask fermentation than that observed for fermentation in stirred tank bioreactor. In addition, the r² values for the shake flask fermentations are more close to 1 than the values for fermentation in the bioreactor.

Discussion

The preliminary development of fermentation process is usually conducted in Shake flasks, where the effect of large number of process variables such as medium components and culture conditions on the process performance to be evaluated [20]. A large number of experiments could be conducted for fermentation using shake flask in a short period of time, without involving high cost and complicated equipment [21,22]. Moreover small volumes can be easily handled with almost similar good results or sometimes even better than those obtained in bioreactors [16,23]. However, shake flask fermentation could not be carried out with large volumes due to high flask volume ratio and limitation in oxygen supply rate [24]. For large scale production, the fermentation process has to be shifted to bioreactor such as stirred tank bioreactor. Therefore in the present work lipase production process was first studied in shake flasks and then after calculating all the necessary parameters the process was tested in 2L stirred tank bioreactor with 1 L working volume. The observed parameters in the two different fermentation levels were comparable.

Variations in the process output parameters including cell mass and glucose consumed were seen within 2 hours of inoculation. However, lipase production was detected after 4 hours. E. coli BL21 (DE3) pLysS with lipase producing gene from a local isolate was used in this study [15]. Commercially available pET51b vector (Novagen, USA) was used to insert the lipase gene in the strain. The vector contains lac I gene from the lac operon that codes for the lac repressor (LacI). Therefore it needs lactose to induce the lipase expression. IPTG is a molecular mimic of allolactose, a lactose metabolite that triggers transcription of the lac operon. Therefore IPTG is commonly used
to induce protein expression where the gene is under the control of the lac operator. IPTG, unlike allolactose, is not metabolized by the bacterial cells. Therefore IPTG is required in very small quantities as compared to lactose. IPTG was used in all experiments as expression inducer after two hours of inoculation (almost at the mid log phase). Due to this reason, lipase activity was observed after 4 hours of inoculation for both levels of fermentations in shake flasks and in stirred tank bioreactor. Similar effects have also been observed in fermentation employing E. coli induced with IPTG for the production of various recombinant proteins [25,26].

Production of thermostable and solvent tolerant lipase by recombinant E. coli BL21 in batch fermentation was greatly influenced by the initial glucose and yeast extract concentrations. LB broth is a minimal medium commonly used as medium for fermentation employing E. coli. For industrial fermentation process, this medium needs to be supplemented with suitable carbon and nitrogen sources. Glucose is the most commonly used carbon source in fermentations employing recombinant E. coli, where the concentration needs to be optimized to enhance growth and the production of the target proteins [27–30]. Although glucose is the preferred carbon source for enhancement of growth but at high concentrations it also initiate acetate accumulation in the culture that may inhibit growth of E. coli [27]. Therefore, suitable initial glucose concentration must be identified in batch fermentation to avoid problems related to acetate accumulation and to make the process feasible. There is always a need to study the behaviour of a system with variation in substrate concentrations and other fermentation conditions in addition to optimization. Kinetics and modelling studies shall be employed to get better understanding of the bioprocess.

Results from this study indicated that the performance of lipase fermentation by recombinant E. coli, measured in term of $X_m$, $P_m$ and $S_m$, was increased with increasing initial glucose concentration. The strain used in this study was E. coli BL21, which was able to grow at high glucose concentrations with little problem related to acetate accumulation and inhibition [31]. It was observed that the values of all kinetic parameter were the highest at glucose concentration of 25 g/L, which was optimal for enhancement of growth and lipase production. Different kinetic parameter values observed at different glucose concentrations indicate that glucose has a significant influence on lipase production by E. coli BL21. At high glucose concentrations (>30 g/L), the fermentation performance was gradually decreased, possibly due to the accumulation of acetate at concentration above the bacterial tolerance level. The higher values of $\alpha$ and $m$ from $\beta$ and $n$ (which are close to 0 IU/g/h and 0 g/g/h respectively, for all concentrations) shows that the lipase production and glucose consumption are growth associated, as observed for other intracellular proteins production by recombinant E. coli [17].

Although the higher values of $\alpha$ than $\beta$ suggest that the lipase production by recombinant E. coli BL21 can be considered as growth associated process. However, the $\beta$ values are not equal to 0 for the confirmation of this assumption. The small value of $\beta$ may be due to the fact that lipase production was only started after the induction of the culture with IPTG. On the other hand, rapid cell growth was observed shortly after the inoculation but lipase activity was not detected before the induction. This observation indicates that non growth associated behaviour was observed in the early stages of fermentation prior to induction.

Yeast extract is widely used as nitrogen source for the production of many recombinant proteins by E. coli [32,33]. Results from this study demonstrated that lipase production by recombinant E. coli BL21 was greatly influenced by yeast extract concentration. The amount of acetate accumulated in the culture was significantly decreased with increasing yeast extract. Acetate assimilation and growth enhancement of recombinant E. coli were also observed in the presence of yeast extract in the medium [34]. Increase in cell concentration and lipase production was observed with increasing initial yeast extract concentration and reached at maximum value of 25 g/L yeast extract. The effects of nitrogen source on the performance of recombinant protein production by E. coli have been reported [32]. Yeast extract, at a concentration of 24 g/L, was found to be the preferred and optimal for the production of recombinant protein by E. coli. Yeast extract is also the source of some vitamins and minerals to enrich the cultivation medium required for growth of E. coli [35].

Most of the fermentation parameters are similar for shake flasks and bioreactor studies except $P_{max}$, $\alpha$ and $n$. These parameters may be different due to different equipment used in the fermentation experiments at two different scales. The higher values of the three parameters in stirred tank bioreactor indicated that the production of lipase in stirred tank bioreactor was enhanced due to the use of control parameters such as dissolved oxygen tension and the culture pH which cannot be controlled in shake flask fermentations. In the present study, the fermentations carried out in bioreactor were not optimised and the data obtained were only used to compare the performance with fermentations in shake flask. Optimization of bioreactor process parameters will be carried out in the future study for further improvement of recombinant
lipase production by *E. coli* BL21.

The performance of the modelling for a process is usually expressed by coefficient of correlation determination $R^2$. Since the value of $R^2$ for all the studied parameters (growth, lipase production and glucose consumption) are close to 1, thus it can be suggested that the proposed models are sufficient to describe lipase fermentation by recombinant *E. coli* BL21. The values of $R^2$ for simulated data also indicate the goodness of fit. In this study, Monod model was used for cell growth while Luedeking-Piret model was used for lipase production and glucose consumption. Monod model has been successfully used by many researchers for the modeling of growth of recombinant *E. coli* during the fermentation [36,37]. Similarly, many researchers have also used Luedeking-Piret equation to model the substrate consumption and product formation with good $R^2$ values using *E. coli* and other microbial strains with sufficient fitting [17,38–40].

The observed relationship of $\mu$ with $q_p$ and $q_s$ was positive as indicated by the correlation analysis and was better to some extent for the shake flasks as compared to that obtained from fermentation in stirred tank bioreactor. The optimized fermentation conditions used in shake flask may be one of the possible explanations. The fermentation performance of stirred tank bioreactor may be improved with optimization of all the possible parameters using statistical methods such as response surface methodology and artificial neural network, which are currently carried out in our laboratory.

**Conclusion**

The mathematical models proposed in this study for lipase production by *E. coli* BL21 were found suitable for describing the fermentation process using various glucose and yeast extract concentrations as obvious from the $R^2$ values. The lipase production and glucose uptake was found to be growth associated process according to the calculated parameters. Most of the kinetic parameters values observed in 2 L stirred tank bioreactor were found comparable to that obtained in shake flask. In addition, some parameter values for fermentation in stirred tank bioreactor are better than those obtained in shake flask. Thus, it can be suggested that the scaling-up of the lipase production by *E. coli* BL21 is feasible for fermentation in stirred tank bioreactor. The information obtained from this study is also very useful for optimization, simulation and scaling-up of lipase production by *E. coli* BL2 in large scale bioreactors.

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