Statistical evaluation of medium components by Plackett-Burman experimental design and kinetic modeling of lipase production by *Pseudomonas fluorescens*

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The evaluation of medium components for lipase production by *Pseudomonas fluorescens* in submerged batch fermentation was studied using Plackett-Burman experimental design. Twelve medium components with three dummy variables were studied in this experimental design. The most significant variables affecting lipase production were found to be glucose, olive oil, yeast extract, dipotassium hydrogen phosphate and ferrous sulphate heptahydrate. Maximum lipase activity of 3.32 U mL\(^{-1}\) and maximum cell mass concentration of 2.15 g L\(^{-1}\) was obtained in the 32 h of fermentation using the optimized medium under optimized conditions of 30°C, with an initial pH of 7.0 at 120 rpm. Various unstructured kinetic models were analyzed to simulate the experimental values of cell growth, lipase activity and glucose concentration. Logistic model for cell growth, Luedeking-Piret model for lipase production and modified Luedeking-Piret model for substrate utilization were found to predict the fermentation profile more accurately with high determination coefficient \(R^2\) values of 0.9893, 0.9314 and 0.9765, respectively. The estimated values of the kinetic model parameters, \(\alpha\) and \(\beta\) for lipase production clearly indicate that the lipase production by *P. fluorescens* is growth-associated.

**Keywords:** *Pseudomonas fluorescens*, submerged fermentation, lipase, Plackett-Burman experimental design, unstructured kinetic modeling

**IPC Code:** Int. Cl. 5 C12N9/20

**Introduction**

Lipases are enzymes whose biological function is to catalyze the hydrolysis of triacylglycerols and are known as triacylglycerol acylhydrolases (EC 3.1.1.3). Lipases remain active in reaction mixtures containing high proportions of organic solvents and act on wide range of water insoluble carboxylic esters at the interface. Interfacial activation of lipases occurs at the lipid-water interface, a phenomenon that can be traced to the unique structural characteristics of this class of enzymes. Lipases contain a helical oligopeptide unit that shields the active site called ‘lid’, which upon interaction with a hydrophobic interface such as a lipid droplet, undergoes movement in such a way that exposes the active site providing free access for the substrate (interfacial activation). The active site is generally characterized by the triad composed of serine, histidine and aspartate, acyl–enzyme complexes being the crucial intermediates in all lipase-catalyzed reactions\(^1\). Traditionally, lipase has been used in the food industry for the ripening of cheese and in the detergent industries as laundry detergent additives. Recently, it has turn out to be worth to the chemical and pharmaceutical industries because of its ability to hydrolyze ester bonds, trans-esterify triglycerides, resolve racemic mixtures, and synthesize ester and peptide bonds\(^2,3\).

Lipases occur widely in nature, but only microbial lipases are commercially significant. Lipase production by microorganisms is mostly performed by batch cultivation processes and those from bacteria are widely used for a variety of biotechnological applications. It is known that a wide variety of Gram-positive and Gram-negative bacterial species produce lipases and most widely used lipases originate from the species of genus *Pseudomonas*\(^4\). *P. fluorescens*, a Gram-negative bacterium belonging to family I.3, is distinguished from others not only in amino acid sequence but also in its secreting ability by a three-component ATP-binding cassette (ABC) transporter mechanism\(^3\).

Lipase activity and production depends upon the composition of fermentation medium\(^5,6\). The study of factors affecting lipase productivity is an important

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strategy for the bioprocess development. The cell growth and the accumulation of metabolic products in bacteria are strongly influenced by medium compositions such as carbon sources, nitrogen sources and inorganic salts. Thus, it is difficult to search for the major factors and to optimize them for biotechnological processes as several factors are involved. The classical method of medium optimization involves changing one variable at a time by keeping the others at fixed levels. Being single dimensional, this laborious and time consuming method does not guarantee the determination of optimal conditions. On the other hand, carrying out experiments with every possible factorial combination of the test variable is impractical because of large number of experiments. Experimental design techniques offer a considerable advantage over the one-factor-at-a-time approach for fermentation improvement. Statistical approach for the optimization of media effectively tackles the problem, which involves specific design of experiments which minimizes the error in determining the effect of variables. Plackett-Burman design allows reliable short listing of medium components in fermentation for further optimization and allows one to obtain unbiased estimates of linear effects of all factors with maximum accuracy for a given number of observations.

The rational design and optimization of the fermentation requires the understanding of production kinetics. A kinetic model can provide insight about the influence of operational parameters on cell growth, product formation rate and substrate utilization rate and thus ensures its economic viability. Although numerous papers have been published on selection of lipase producers, information on the fermentation process kinetics is meagre. This information is important in order to identify optimal operating conditions for the enzyme production.

In the present study, the effect of medium components on lipase production using *P. fluorescens* was studied using Plackett-Burman design and unstructured kinetic models were used to characterize the fermentation process.

**Materials and Methods**

**Microorganism**

*Pseudomonas fluorescens* MTCC 103 was obtained from the Microbial Type Culture Collection of gene bank (MTCC), Institute of Microbial Technology, Chandigarh, India. The medium components were procured from Himedia Ltd, Mumbai, India. Spirit blue agar was used for the detection of lipolytic activity of *P. fluorescens*.

**Maintenance of Culture**

The *P. fluorescens* stock culture was maintained on nutrient agar slants containing (g L⁻¹): Beef extract - 1; Yeast extract - 2; Peptone - 5; NaCl - 5 and Agar - 15. The 48 h old culture, maintained on nutrient agar was used to inoculate the seed culture medium (nutrient broth) in 250 mL conical flask with working volume of 100 mL and incubated at room temperature for 12 h. 5 mL of seed culture was used to inoculate 100 mL of the production medium in 250 mL conical flask. The organisms were subcultured at regular time intervals.

**Batch Fermentation**

The lipase production by *P. fluorescens* was conducted in 250 mL Erlenmeyer flask with 100 mL of the production medium. The production medium was adjusted to the initial pH of 7.0 using 2 M NH₄OH and sterilized at 121°C for 20 min. It was inoculated with 5% (v/v) of seed culture in the mid exponential phase at 12 h. The flasks were incubated in an orbital shaker at 120 rpm at 30°C for the fermentation period of 48 h. Aliquot of samples from the fermentation broth were withdrawn at regular intervals without much change in the culture volume to maintain constant oxygen transfer and the cells were separated from the medium by centrifugation at 5030g for 15 min. The clarified supernatant was used for the analysis of lipase activity, protease activity, total soluble protein and glucose.

The components of the production medium for lipase production by *P. fluorescens* were tested using Plackett-Burman statistical experimental design (Table 1). The medium components tested were, glucose, olive oil (emulsified), peptone, yeast extract, KH₂PO₄, K₂HPO₄, CaCl₂·2H₂O, NaNO₃, CuSO₄, MnCl₂·4H₂O, MgSO₄·7H₂O and FeSO₄·7H₂O. All the submerged batch fermentations were conducted in shake flasks (in duplicate) and the response was measured in terms of lipase production.

**Lipase Activity Assay**

Lipase activity was estimated with olive oil emulsion following the procedure of Ota and Yamada. One unit (U) of lipase activity is defined as 1 µmol of free fatty acid liberated per mL of enzyme
per min at 37°C. Olive oil emulsion was prepared by homogenizing 25 mL of olive oil and 75 mL of 2% polyvinyl alcohol solution in a homogenizer for 6 min at 20000 rpm. The reaction mixture composed of 2 mL olive oil emulsion, 2.5 mL 0.05 M phosphate buffer and 0.5 mL enzyme solution and the reaction mixture was incubated at 37°C for 15 min. The emulsion was destroyed by addition of 10 mL acetone immediately after incubation and the liberated fatty acid was titrated against 0.05 N NaOH.

Protease Activity Assay
The protease activity was assayed by modified Anson method using casein as the substrate. One unit (U) of protease activity is defined as 1 µg of tyrosine liberated per min by 1 mL of enzyme at 37°C. 2 mL of 1% w/v casein solution was mixed with 0.5 mL of suitably diluted enzyme solution and incubated at 37°C for 30 min. 2.5 mL of 0.4 M trichloroacetic acid was added to arrest the reaction. The solution with precipitate was filtered and to the 1 mL of filtrate, 5 mL of 0.4 M Na₂CO₃ and 0.5 mL of Folin reagent were added. After 10 min of incubation, the colour density developed was determined at 660 nm.

Biomass, Glucose and Protein Determination
The bacterial cell growth was determined by measuring optical density at a wavelength of 600 nm. The biomass concentration was determined with a calibration curve made from the relationship between optical density at 600 nm and dry cell weight. The glucose concentration in the fermentation broth was determined by dinitrosalicylic acid method. The total soluble protein in the fermentation broth was determined by Lowry method.

Plackett-Burman Design
For screening purpose, various medium components have been evaluated using Plackett-Burman statistical design, which is a fraction of a two-level factorial design and allows the investigation of n-1 variables in at least n experiments. This design requires that the frequency of each level of a variable should be equal and that in each test the number of high and low variables should be equal. Then the effects of changing the other variables cancel out while determining the effect of particular variable.

The main effect was calculated as the difference between the average of measurements made at the high level setting (+1) and the average of measurements observed at low level setting (-1) of each factor. This design is practical especially when the investigator is faced with large number of factors and is unsure of which settings are likely to produce optimal or near optimal responses. Plackett-Burman experimental design was based on the first order model.

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<th>B</th>
<th>C</th>
<th>D</th>
<th>E</th>
<th>F</th>
<th>G</th>
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<th>K</th>
<th>L</th>
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A: Olive oil, B: Glucose, C: Yeast extract, D: Peptone, E: KH₂PO₄, F: K₂HPO₄, G: MgSO₄.7H₂O, H: NaNO₃, I: CaCl₂.2H₂O, J: FeSO₄.7H₂O, K: CuSO₄, L: MnCl₂.4H₂O
Y = β₀ + ΣβᵢXᵢ,

Where Y is the response (lipase productivity), β₀ is the model intercept and βᵢ is the variable estimates. The factor with confidence level above 95% is considered the most significant factor that affects the lipase production. Twelve factors were screened in sixteen combinations with three dummy variables and all the trials were performed in duplicate and the average of observation was used as the response of the design. Normally, three dummy variables will provide an adequate estimate of the error

Unstructured Model Development for Fermentation Kinetics

Various unstructured models were proved to be sufficient for characterizing the fermentation kinetics. In an unstructured model, the cellular representations are single component representations. The growth of cell is governed by a hyperbolic relationship and there is a limit to the maximum attainable cell mass concentration. Such growth kinetics is described by logistic equation as

\[
dX/dt = \mu_0 \left(1 - \frac{X}{X_{\text{max}}} \right) X
\]  \quad \ldots (1)

Where dX/dt is the growth rate (g L⁻¹ h⁻¹); X is the concentration of biomass (g L⁻¹); \mu is the specific cell growth rate (h⁻¹); \mu₀ is the initial specific growth rate (h⁻¹), and X_{\text{max}} is the maximum cell mass concentration (g L⁻¹). Equation (1) on integration using \( X \) gives a sigmoidal variation X (t) that may empirically represent both an exponential and a stationary phase.

\[
X(t) = \frac{X_0 e^{\mu_0 t}}{1 - \left( \frac{X_0}{X_{\text{max}}} \right) \left(1 - e^{\mu_0 t} \right)} \quad \ldots (2)
\]

The kinetic parameter, \( \mu_0 \) in this equation was determined by rearranging equation (2) as

\[
\mu_0 = \ln \left[ \frac{X_{\text{max}}}{X_0} \right] + \ln \left[ \frac{X}{1 - X} \right].
\]

Where \( X = \frac{X}{X_{\text{max}}} \) \ldots (3)

If the logistic equation describes the data suitably, then a plot of \( \ln \left[ \frac{X}{1 - X} \right] \) vs t should give a straight line of slope \( \mu_0 \) and intercept \( \ln \left[ \frac{X_{\text{max}}}{X_0} \right] \).

Luedeking and Piret\(^{21}\) stated that the product formation rate depends upon both the instantaneous biomass concentration (X) and growth rate (dX/dt) in a linear fashion.

\[
\frac{dP}{dt} = \alpha \frac{dX}{dt} + \beta X
\]  \quad \ldots (4)

Where \( \alpha \) (gP gX⁻¹) and \( \beta \) (gP gX⁻¹ h⁻¹) are empirical constants that may vary with fermentation conditions (temperature, pH, etc.). The convenience of this model is that ‘\( \beta \)’ may be evaluated from stationary phase data (dX/dt = 0), leaving the single parameter \( \alpha \) to fit dP/dt throughout the earlier exponential phase. The lipase production kinetics was analyzed according to the Luedeking-Piret model using the method of Weiss and Ollis\(^{22}\), where equation (2) and (4) were combined and integrated with two initial conditions, (\( X_0 \), \( P_0 \)), a final condition, \( X_{\text{max}} \) and three parameters \( \mu_0 \), \( \alpha \) and \( \beta \).

\[
P_1 = P_0 + \alpha A(t) + \beta B(t) \quad \ldots (5)
\]

where \( A(t) = X_0 e^{\mu_0 t} \), \( B(t) = \frac{X_{\text{max}}}{\mu_0} \ln \left[ 1 - \frac{X_0}{X_{\text{max}}} \left(1 - e^{\mu_0 t} \right) \right] \)

The parameters \( \alpha \) and \( \beta \) in equation (5) are determined by plotting \( P_1 - P_0 / B(t) \) vs. \( A(t) / B(t) \) which is a straight line with slope ‘\( \alpha \)’ and intercept ‘\( \beta \).’

The substrate utilization kinetics is given by the following equation, which considers substrate conversion to cell mass, to product and substrate consumption for maintenance

\[
\frac{dS}{dt} = -\frac{1}{Y_{X/S}} \frac{dX}{dt} - \frac{1}{Y_{P/S}} \frac{dP}{dt} - k_e X \quad \ldots (6)
\]

Where \( Y_{X/S} \) and \( Y_{P/S} \) are yields of cell mass and product with respect to substrate and \( k_e \) is the maintenance coefficient for cells. Rearranging the substrate material balance equation (6),

\[
\frac{dS}{dt} = -\gamma \frac{dX}{dt} - \eta X \quad \ldots (7)
\]

where \( \gamma (\text{gS gX}^{-1}) = \frac{1}{Y_{X/S}} + \frac{\alpha}{Y_{P/S}} \) and

\[
\eta (\text{gS gX}^{-1} \text{hr}^{-1}) = \frac{\beta}{Y_{P/S}} + k_e \]
Equation (7) is the modified Luedeking-Piret equation for substrate utilization kinetics.

Substituting for \( \mu \) from (1) and integrating with initial conditions \( X = X_0 \) \((t=0)\) and \( S = S_0 \) \((t = 0)\) gives

\[
S_t = S_0 - \gamma m (t) - \eta n (t) \quad \quad \ldots \quad (8)
\]

Where \( S_0 \) and \( S_t \) are the substrate concentrations at initial time and at any time ‘t’ \((g L^{-1})\)

Where \( m (t) = \frac{X_o e^{\mu t}}{1 - \left(X_0/X_m\right)(1 - e^{\mu t})} - 1 \) and

\[
n (t) = \frac{X_o}{\mu_0} \ln \left[ 1 - \frac{X_0}{X_m} (1 - e^{\mu t}) \right]
\]

Kinetic parameters \( (\gamma, \eta) \) in equation (8) were determined by plotting \( \frac{S_0 - S_t}{n (t)} \) vs \( m(t)/n(t) \), which is a straight line with slope \( '\gamma' \) and intercept \( '\eta' \).

**Results**

**Effect of Culture Medium Components on Lipase Production**

Twelve medium components were examined using Plackett–Burman statistical experimental design. The main effect of the medium components examined in the present study is given in Table 2. The regression coefficient, F values and P values of the factors were calculated for lipase production using the statistical software, The Unscrambler, version V.8.0.5, CAMO process AS, Norway. The lipase enzyme synthesized by *P. fluorescens* was found to vary from 1.15 U mL\(^{-1}\) to 3.2 U mL\(^{-1}\) in the sixteen experiments conducted which shows the strong influence of medium components on lipase enzyme production. On analysis of regression coefficient of twelve medium components (Table 2), olive oil, glucose, peptone, yeast extract, \( K_2HPO_4 \), \( K_2HPO_4 \), \( CaCl_2\cdot2H_2O \) and \( FeSO_4\cdot7H_2O \) showed a positive effect for lipase activity, whereas \( MgSO_4\cdot7H_2O \), \( NaNO_3 \), \( CuSO_4 \) and \( MnCl_2\cdot4H_2O \) showed negative effect in the tested range of concentration. The Pareto chart as shown in Fig. 1 offers a convenient way to view the results obtained by Plackett-Burman design. The Pareto chart illustrates the order of significance of the variables affecting the microbial lipase production. The order of significance as indicated by Pareto chart is olive oil, \( K_2HPO_4 \), glucose, yeast extract, \( FeSO_4\cdot7H_2O \), \( NaNO_3 \), \( MgSO_4\cdot7H_2O \), \( K_2HPO_4 \), \( CaCl_2\cdot2H_2O \), \( CuSO_4 \), \( MnCl_2\cdot4H_2O \) and peptone. Fig. 2 illustrates the response surface plots showing the effects of the various combinations of independent variables (a) Olive oil and glucose (b) Olive oil and yeast extract (c) glucose and yeast extract (d) \( K_2HPO_4 \) and \( FeSO_4\cdot7H_2O \) on lipase production by *P. fluorescens* with all the remaining factors kept constant at the middle level of the Plackett-Burman experimental design. This plot is very useful in determining the lipase activity at intermediate levels of different combinations of independent variables and also for the optimization of microbial production of lipase economically and efficiently.

**Kinetics of Lipase Production**

The lipase activity, protease activity, cell mass concentration, \( pH \) and soluble protein concentration profile is given in Fig. 3 for the optimized medium using Plackett-Burman experimental design. The optimized medium contains, olive oil, 10 mL L\(^{-1}\); glucose, 10 g L\(^{-1}\); yeast extract, 10 g L\(^{-1}\); peptone, 10 g L\(^{-1}\); \( KH_2PO_4 \), 6 g L\(^{-1}\); \( K_2HPO_4 \), 2 g L\(^{-1}\); \( MgSO_4\cdot7H_2O \), 0.2 g L\(^{-1}\); \( NaNO_3 \), 0.1 g L\(^{-1}\);

<table>
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<th>Variables with designate</th>
<th>Lower level (-1)</th>
<th>Higher level (+1)</th>
<th>Main effect</th>
<th>( \beta )-Coefficients</th>
<th>F value</th>
<th>P-value</th>
<th>Confidence level (%)</th>
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<td>3.116</td>
<td>0.1757</td>
<td>82.43</td>
</tr>
<tr>
<td>H  NaNO(_3) (g L(^{-1}))</td>
<td>0.1</td>
<td>1</td>
<td>-0.3125</td>
<td>-0.169</td>
<td>7.861</td>
<td>0.0676</td>
<td>93.24</td>
</tr>
<tr>
<td>I  CaCl(_2)\cdot2H(_2)O (g L(^{-1}))</td>
<td>0.05</td>
<td>0.1</td>
<td>0.0975</td>
<td>0.0488</td>
<td>0.656</td>
<td>0.4772</td>
<td>52.28</td>
</tr>
<tr>
<td>J  FeSO(_4)\cdot7H(_2)O (g L(^{-1}))</td>
<td>0.01</td>
<td>0.05</td>
<td>0.4275</td>
<td>0.201</td>
<td>11.181</td>
<td>0.0443</td>
<td>95.57</td>
</tr>
<tr>
<td>K  CuSO(_4) (g L(^{-1}))</td>
<td>0.001</td>
<td>0.01</td>
<td>-0.0625</td>
<td>-0.0188</td>
<td>0.097</td>
<td>0.7758</td>
<td>22.42</td>
</tr>
<tr>
<td>L  MnCl(_2)\cdot4H(_2)O (g L(^{-1}))</td>
<td>0.001</td>
<td>0.01</td>
<td>-0.0525</td>
<td>-0.0263</td>
<td>0.19</td>
<td>0.6922</td>
<td>30.78</td>
</tr>
</tbody>
</table>
CaCl$_2$·2H$_2$O, 0.1 g L$^{-1}$; FeSO$_4$·7H$_2$O, 0.05 g L$^{-1}$; CuSO$_4$·0.001 g L$^{-1}$ and MnCl$_2$·4H$_2$O, 0.001 g L$^{-3}$. The lipase production was found to increase gradually after 6 h of the fermentation period when the growth of the microorganisms reaches the exponential phase. The maximum lipase activity was found in the late exponential phase and early stationary growth phase of P. fluorescens as reported earlier$^{23}$. The maximum lipase activity of 3.32 U mL$^{-1}$ was obtained at the 32 h of fermentation and the activity reduces sharply after 32 h, probably this might be due to the increased activity of the protease after 30 h in the stationary phase of the microorganism.

The protease activity reached a maximum value of 0.52 U mL$^{-1}$ at 40 h. The cell mass concentration reached a maximum of 2.15 g L$^{-1}$ at 40 h during the stationary phase and there was no further increase in the cell mass concentration. The exponential phase of the microorganism was observed from 8 h to 30 h. This observation clearly indicates that the maximum lipase production was at the post exponential phase and growth associated. The pH of the medium decreased gradually from the initial pH of 6.9 to 4.97. This decrease in the pH may be due to some organic acid production during the enzyme production by P. fluorescens. The rate of glucose utilization by the microorganism was found to increase rapidly after 12 h of fermentation when the microorganism reached the mid exponential phase. Almost 90% of the glucose was depleted in 48 h of the fermentation. Mathematical analysis of the data showed that the unstructured models could satisfactorily explain the kinetics of fermentation process for production of lipase by P. fluorescens.

**Discussion**

**Evaluation of Medium Constituents for Lipase Production by P. fluorescens**

The most important factor for the expression of lipase activity has always been carbon source since lipases are inducible enzymes and are generally produced in the presence of a lipid source such as oil$^{24}$. Microorganisms grow on glucose and are important for fast lipase production$^{25}$ although Fatima Silva Lopes et al$^{26}$ reported that glucose did not influence the maximum lipase activity reached in *Lactobacillus plantarum* batch culture. Shaeh and Zahrann$^{27}$ have reported that, as the glucose concentration is increased there is a considerable reduction in synthesis of extracellular lipase by P. fluorescens. High glucose concentration supported growth and repressed lipase production by P. fluorescens 2D$^{24}$. Contrary to these results, in this study the glucose had a positive effect on lipase activity in the concentration range studied and has a confidence level of 96.61%, even though the maximum lipase activity recorded in the Plackett-Burman experimental runs was found in the trial number 4 which contains low level of glucose concentration. Lipase and biomass production by *Yarrowia lipolytica* 681 was enhanced significantly in the presence of glucose and olive oil$^{29}$. While Dalmau et al$^{30}$ demonstrated the inhibition of lipase production by glucose, Chang et al$^{31}$ does not report any repressive effect of glucose on *Candida rugosa*.

Since lipases are inducible enzymes, they are generally produced in the presence of lipid sources such as oil. Out of the different oils added to fermentation medium, olive oil was found to be the best inducer for lipase secretion$^{31}$. Valero et al$^{32}$ reported that olive oil as a single carbon source produced maximum lipase activity and is associated with the growth of the microorganism. Approximately two fold increase in activity by B. coagulans BTS3 is by olive oil as an inducer$^{33}$. In this study, olive oil showed highest confidence level of 97.8% indicating that it is the key factor influencing lipase production. Generally, organic nitrogen is preferred type (such as peptone and yeast extract) of nitrogen source for lipase production by various *Pseudomonas* species$^{34}$. Thermostable lipase of *Pseudomonas* sp KW 1-56 was produced in a medium that contained peptone (2% w/v) and yeast extract (0.1% w/v) as nitrogen
The results obtained in this study are concurrent with the results reported earlier and yeast extract showed confidence level of 95.84%. Metal ions influence the lipase synthesis by microorganisms. FeSO₄·7H₂O, in this study showed a confidence level of 95.57%. Iron concentration was found to be critical for the production of lipase by Pseudomonas species G6. K₂HPO₄ has a confidence level of 97.8% and its influence was well supported by the report that K₂HPO₄ is one of the important variables influencing the lipase production by newly isolated Pseudomonas.
species H18 and showed a confidence level above 85% on primary screening. Based on the calculated P values it was found that the medium components glucose, olive oil, yeast extract, K$_2$HPO$_4$, and FeSO$_4$.7H$_2$O are the most significant variables as these factors have confidence level more than 95%.

Table 3—Unstructured kinetic model parameters evaluated using batch data for lipase production by *P. fluorescens*

<table>
<thead>
<tr>
<th>Kinetic model parameters</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\mu_o$ (h$^{-1}$)</td>
<td>0.135</td>
</tr>
<tr>
<td>$X_m$ (g L$^{-1}$)</td>
<td>2.15</td>
</tr>
<tr>
<td>$X_0$ (g L$^{-1}$)</td>
<td>0.34</td>
</tr>
<tr>
<td>$\alpha$ (U g X$^{-1}$)</td>
<td>1.53</td>
</tr>
<tr>
<td>$\beta$ (U g X$^{-1}$ h$^{-1}$)</td>
<td>0.008</td>
</tr>
<tr>
<td>$\gamma$ (gS gX$^{-1}$)</td>
<td>4.45</td>
</tr>
<tr>
<td>$\eta$ (gS gX$^{-1}$ h$^{-1}$)</td>
<td>0.018</td>
</tr>
</tbody>
</table>

Unstructured Model Equation of Best Fit

The unstructured models provide a good approximation of the fermentation profile even though the complete cell mechanism is not considered in the models. In Fig. 4, the unbroken lines show the estimated responses of the models. Table 3 shows the estimated parameters of the logistic model for the cell growth, lipase production by Luedeking-Piret model and substrate concentration by modified Luedeking-Piret model. The values of the kinetic parameters, $\alpha$, $\beta$ and $\mu$ were found to be 1.53, 0.008 and 0.135 h$^{-1}$, respectively. Since the magnitude of the growth associated parameter ‘$\alpha$’ is much greater than the magnitude of non-growth associated parameter ‘$\beta$’ in the product formation model the lipase production is growth associated.

The coefficient of determination, $R^2$ is a measure of the strength of the linear relationship between the experimental and predicted values. The greater the proportion of explained variation, the stronger the degree of linear relationship. Fig. 5 shows the comparison of experimental and model predicted cell mass, lipase activity and glucose utilization respectively and their respective $R^2$ values. The logistic model for cell growth gave $R^2$ value of 0.9893, Luedeking-Piret model for lipase production gave $R^2$ value of 0.9314 and modified Luedeking-Piret model for substrate utilization gave $R^2$ value of 0.9765, hence it was concluded that the unstructured models are well suited for describing the fermentation profile of *P. fluorescens*.

Conclusion

The statistical design of experiment offers efficient methodology to identify the significant variables and to optimize the factors with minimum number of experiments for lipase production by *P. fluorescens*. These significant factors identified by Plackett-Burman design were considered for the next stage in
the medium optimization by using response surface optimization technique and the studies in bioreactor in the future study. The unstructured kinetic models logistic model for cell growth, Luedeking-Piret model for lipase production and modified Luedeking-Piret model for substrate utilization were effective in predicting the fermentation profile with higher accuracy. The estimated values of the kinetic model parameters for lipase production using Luedeking-Piret model clearly indicate that the lipase production by \textit{P. fluorescens} is growth-associated.

**Acknowledgement**

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**References**

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