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研究报告

Evaluation of Medium Components by Plackett-Burman Statistical Design for Lipase Production by *Candida rugosa* and Kinetic Modeling

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Abstract: Lipase production by *Candida rugosa* was carried out in submerged fermentation. Plackett-Burman statistical experimental design was applied to evaluate the fermentation medium components. The effect of twelve medium components was studied in sixteen experimental trials. Glucose, olive oil, peptone and $FeCl_3 \cdot 6H_2O$ were found to have more significance on lipase production by *Candida rugosa*. Maximum lipase activity of 3.8 u mL^{-1} was obtained at 50 h of fermentation period. The fermentation was carried out at optimized temperature of 30ºC, initial pH of 6.8 and shaking speed of 120 r/min. Unstructured kinetic models were used to simulate the experimental data. Logistic model, Luedeking-Piret model and modified Luedeking-Piret model were found suitable to efficiently predict the cell mass, lipase production and glucose consumption respectively with high determination coefficient(R^2). From the estimated values of the Luedeking-Piret kinetic model parameters, α and β, it was found that the lipase production by *Candida rugosa* is growth associated.

Keywords: lipase, *Candida rugosa*, Plackett-Burman experimental design, submerged fermentation, unstructured kinetic modeling

Lipases(EC 3.1.1.3) comprise a group of hydrolytic enzymes which catalyze reversibly the hydrolysis and synthesis of triacylglycerides in the oil water interface[1]. Enormous interest on lipase production has been evolved due to its widespread applications in oleochemial, detergent, food processing and in fine chemical manufacturing industries[1,2]. Particularly the non specific lipase produced by *Candida rugosa* was employed in synthesizing enantiopure drug formulations in pharmaceutical industries because of its high sterioselective and organic solvent tolerant nature[3]. Lipases are ubiquitous in nature and *C. rugosa* is a well known industrial lipase producer[4–6].

The study of factors influencing the production of biomolecules is very much essential in any bioprocess development. Generally a higher productivity has been achieved by culture medium optimization[7]. The classical practice of changing one variable at a time while keeping others at a constant level was found inefficient. This single dimensional task does not explain interaction effects among the variables and their effect on the fermentation process. Moreover it is a time consuming laborious practice because of the large number of experiments[8–10]. Conversely, rapid statistical approach enables us to obtain the physicochemical parameters and factors influencing the fermentation process with limited number of planned experiments[11]. One such approach is Plackett-Burman design that allows efficient screening of key variables for further optimization. For the given number of observation the linear effect of all factors can be screened with maximum accuracy. This design is practical when investigating large number of factors to produce optimal or near optimal response.

Although numerous reports are available on fermentative production of lipase by *Candida rugosa*[4–6], kinetic modeling on the production process is very sparse. Statistically optimized media design along with kinetic models characterizes the fermentation behavior more rapidly to achieve maximum lipase productivity. The use of good reliable model is essential to develop better strategies for the optimization of the fermentation process[12]. In the present study, Plackett-Burman statistical design was used to evaluate the fermentation medium components for lipase production by *Candida rugosa* and various unstructured models are used to describe the kinetics of lipase fermentation profile.

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1 Materials and methods

1.1 Microorganism

Candida rugosa NCIM 3462 was obtained from National Chemical Laboratories, Pune, India. The medium components were procured from Himedia Ltd, Mumbai, India. All chemicals used in the experiment were of analytical grade. Spirit blue agar was used for the detection of lipolytic activity of *Candida rugosa* NCIM 3462.

1.2 Culture maintenance

The *Candida rugosa* stock culture was maintained on MGYP agar slants containing (g L^{-1}): Malt extract, 3.0; Glucose, 10.0; Yeast extract, 3.0; Peptone, 5.0 and Agar, 10.0. The 48 h old culture, maintained in MGYP agar was used to inoculate the seed culture medium (MGYP broth) in 250 mL Erlenmeyer flask with working volume of 100 mL and incubated at 30°C for 24 h. 5 mL of seed culture was used to inoculate 100 mL of the production medium in 250 mL Erlenmeyer flask.

1.3 Batch fermentation

The lipase production by *Candida rugosa* was conducted in 250 mL Erlenmeyer flask with 100 mL of the production medium. The production medium was adjusted to the initial pH of 6.8 using 2 mol/L NH_4OH and sterilized (121°C for 20 min). The production medium was inoculated with 5%(*V*/*V*) of seed culture in its mid exponential phase at 24 h. The flasks were incubated in an orbital shaker at 120 r/min and 30°C for the fermentation period of 72 h. Aliquot of sample from the fermentation broth was withdrawn at regular time interval without much change in the culture volume to maintain constant oxygen transfer and the cells were separated from the medium by centrifugation at 10 000 r/min 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 were tested at two levels, high (+) and low (−), to study the effect of medium components on lipase production using Plackett-Burman statistical experimental design (Table 1). The design of experiments was formulated for 12 factors using, The Unscrambler, version V.8.0.5, CAMO process AS, Norway. Sixteen experiments were generated for the 12 factors selected in this study, which were considered to affect the lipase production and is illustrated in Table 2. The medium components tested were, glucose, olive oil (emulsified), peptone, KH_2PO_4, yeast extract, $(NH_4)_2SO_4$, $MgSO_4 \cdot 7H_2O$, NaCl, $CaCl_2 \cdot 2H_2O$, $FeCl_3 \cdot 6H_2O$, thiamine HCl, biotin and three dummy variables. Inositol (0.000 004 g L^{-1}) was added to all the fermentation trials. The submerged batch fermentation was conducted in shake flasks (in duplicate) and the response was measured in terms of lipase production.

1.4 Lipase activity assay

Lipase activity was estimated with olive oil emulsion by the procedure of Ota and Yamada[13]. 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 20 000 r/min. The reaction mixture composed of 2 mL olive oil emulsion, 2.5 mL 0.05 mol/L phosphate buffer and 0.5 mL enzyme solution 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 mol/L NaOH. One unit (u) of lipase activity was defined as 1 μmol of free fatty acid liberated per mL of enzyme per minute at 37°C.

1.5 Protease activity assay

The protease activity was assayed by modified Anson method[14] using casein as the substrate. 2 mL of 1%(*W*/*V*) casein solution was mixed with 0.5 mL of enzyme solution and incubated at 37°C for 30 min. 2.5 mL of 0.4 mol/L 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 mol/L Na_2CO_3 and 0.5 mL of folin reagent were added. After 10 min of incubation, the colour density developed was determined at 660 nm. One unit (u) of protease activity was defined as 1 μg of tyrosine liberated per minute by 1 mL of enzyme.

1.6 Biomass, glucose and protein determination

The bacterial cell growth was determined by measuring the optical density at a wavelength of 600 nm (Double beam UV-Vis Spectrophotometer, Elico India Limited, India). 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[15]. The total soluble protein in the medium was determined by Lowry *et al*[16] method.

1.7 Plackett-Burman experimental design

The medium components were evaluated using Plackett-Burman statistical design[17]. This is a fraction of a two-level factorial design and allows the investigation of '*n*-1' variables with at least '*n*' experiments. Twelve factors were screened in sixteen combinations with three dummy variables which will provide an adequate estimate of the error[18] and all the trials were performed in duplicate and the average of observation was used as the response of the design. 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 effect of changing the other variables was cancelled out when determining the effect of particular variable. The main effect was calculated as the difference between the average of measurements made at the high setting (+1) and the average of measurements observed at low setting (−1) of each factor [19]. Plackett-Burman experimental design is based on the first order model

$$Y = \beta_0 + \sum \beta_i X_i$$

where Y is the response (lipase productivity), β_0 is the model intercept and β_i is the variable estimates. This model describes no interaction among factors and it is used to screen and evaluate the important factors that influence lipase production. The factors that have confidence level above 95% are considered the most significant factors that affect the lipase production.

1.8 Unstructured models for fermentation kinetics

1.8.1 *Cell growth 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[9]. There are several mathematical expressions that could be used to describe this sigmoidal relationship between μ and S. The kinetic model tested was logistic equation for cell growth[9].

$$\frac{dX}{dt} = \mu_0 \left(1.0 - \frac{X}{X_{\max}}\right) X \quad (1)$$

1.8.2 *Lipase production kinetics*

Luedeking and Piret[20] states that the product formation rate depends upon both the instantaneous biomass concentration (X) and growth rate (dX/dt) in a linear fashion.

$$\frac{dP}{dt} = \alpha \frac{dX}{dt} + \beta X \quad (2)$$

The lipase production kinetics was analyzed according to the Luedeking-Piret model using a method similar to Weiss and Ollis[21].Integration of equation (2) using equation (1) for $X(t)$ gives an equation with two initial conditions, (X_0, P_0), a final condition, $X_{\max}$ and three parameters μ_0, α and β[9]

$$P_t = P_0 + \alpha A(t) + \beta B(t) \quad (3)$$

where $A(t) = X_0 \dfrac{e^{\mu_0 t}}{1 - \left(\dfrac{X_0}{X_{\max}}\right)\left(1.0 - e^{\mu_0 t}\right)} - 1.0$ and $B(t) = \dfrac{X_{\max}}{\mu_0} \ln\left[1.0 - \dfrac{X_0}{X_{\max}}\left(1.0 - e^{\mu_0 t}\right)\right]$

1.8.3 *Glucose utilization kinetics*

The substrate utilization kinetics is given by the following equation, which considers substrate conversion to cell mass, to product and substrate consumption for maintenance[21].

$$\frac{dS}{dt} = -\frac{1}{Y_{X/S}}\frac{dX}{dt} - \frac{1}{Y_{P/S}}\frac{dP}{dt} - k_e X \quad (4)$$

Substituting $r_{fP} = -Y_{P/S}\ r_{fs}$ in equation (4) and rearranging the substrate material balance equation,

$$\frac{dS}{dt} = -\gamma \frac{dX}{dt} - \eta X \quad (5)$$

where r_{fP} is the rate of product formation r_{fs} is the rate of substrate utilization,

. and $\eta\left(\mathrm{gS/gX \cdot h}\right) = \dfrac{\beta}{Y_{P/S}} + k_e$

Equation (5) is the modified Luedeking- Piret equation for substrate utilization kinetics.

Rearranging equation (5)

$$-\frac{dS}{dt} = \gamma + \frac{\eta}{\mu} \quad (6)$$

Substituting for μ from (1) and integrating with initial conditions

$X = X_0$ (t = 0) and $S = S_0$ (t = 0) gives

$$S_t = S_0 - \gamma m(t) - \eta n(t) \quad (7)$$

where $m(t) = \left[\dfrac{X_0 e^{\mu_0 t}}{1.0 - \left(X_0 / X_m\right)\left(1 - e^{\mu_0 t}\right)} - 1.0\right]$ and $n(t) = \dfrac{X_m}{\mu_0} \ln\left[1.0 - \dfrac{X_0}{X_m}\left(1.0 - e^{\mu_0 t}\right)\right]$.

2 Results and discussion

2.1 Statistical evaluation of fermentation medium components by Plackett-Burman design

The effect of twelve medium components of the fermentation for lipase production by *C. rugosa* was examined using Plackett-Burman statistical design. The main effect of the medium components, regression coefficient, F values and P values of the factors investigated in the present study is illustrated in Table 2. The activity of lipase enzyme synthesized by *Candida rugosa* was found to vary from 1.1 u mL^{-1} to 3.62 u mL^{-1} in the sixteen experiments due to the strong influence of medium components on lipase enzyme production. On analysis of regression coefficient of twelve medium components, glucose, olive oil, peptone, yeast extract, $(NH_4)_2SO_4$, $CaCl_2 \cdot 2H_2O$, $FeCl_3 \cdot 6H_2O$, thiamine HCl and biotin showed positive effect for lipase activity, whereas KH_2PO_4, $MgSO_4 \cdot 7H_2O$ and NaCl showed negative effect in the tested range of concentration.

Table 1 Plackett–Burman experimental design for evaluation of 12 variables with coded values for lipase production by *C. rugosa* and response of the design

Run Number	A	B	C	D	E	F	G	H	I	J	K	L	Lipase activity (u mL^{-1})	Cell mass (g L^{-1})	Protease activity (u mL^{-1})
1	+	+	+	+	–	+	–	+	+	–	–	+	3.10	2.83	1.83
2	+	+	+	–	+	–	+	+	–	–	+	–	2.80	3.32	2.13
3	+	+	–	+	–	+	+	–	–	+	–	–	2.60	3.21	0.82
4	+	–	+	–	+	+	–	–	+	–	–	–	3.20	3.00	0.65
5	–	+	–	+	+	–	–	+	–	–	–	+	1.84	3.15	1.54
6	+	–	+	+	–	–	+	–	–	–	+	+	2.00	2.77	2.54
7	–	+	+	–	–	+	–	–	–	+	+	+	3.40	3.21	1.21
8	+	+	–	–	+	–	–	–	+	+	+	+	3.62	2.72	1.30
9	+	–	–	+	–	–	–	+	+	+	+	–	2.54	3.56	3.15
10	–	–	+	–	–	–	+	+	+	+	–	+	2.40	2.45	0.98
11	–	+	–	–	–	+	+	+	+	–	+	–	1.86	2.40	1.10
12	+	–	–	–	+	+	+	+	–	+	–	+	2.70	2.17	2.15
13	–	–	–	+	+	+	+	–	+	–	+	+	2.06	3.26	0.84
14	–	–	+	+	+	+	–	+	–	+	+	–	2.40	3.00	0.85
15	–	+	+	+	+	–	+	–	+	+	–	–	2.30	3.08	2.40
16	–	–	–	–	–	–	–	–	–	–	–	–	1.10	2.61	0.65

A: Glucose; B: Olive oil; C: Peptone; D: KH_2PO_4; E: Yeast Extract; F: $(NH_4)_2SO_4$; G: $MgSO_4{\cdot}7H_2O$; H: NaCl; I: $CaCl_2{\cdot}2H_2O$; J: $FeCl_3{\cdot}6H_2O$; K: Thiamine HCl; L: Biotin

Table 2 Statistical analysis of medium optimization using Plackett-Burman design for lipase production by *C. rugosa* in submerged fermentation

Variables with designate		Lower level (–1)	Higher level (+1)	Main effect	β-coefficients	F value	P value	Confidence level /%
A	Glucose/(g L^{-1})	5	20	0.6625	0.331	29.023	0.0125	98.75
B	Olive oil/(mL L^{-1})	2	10	0.4025	0.201	10.713	0.0467	95.33
C	Peptone/(g L^{-1})	3	6	0.4225	0.211	11.804	0.0414	95.86
D	KH_2PO_4/(g L^{-1})	2	6	–0.2675	–0.134	4.732	0.1179	88.21
E	Yeast extract/(g L^{-1})	2	4	0.2275	0.114	3.422	0.1614	83.86
F	$(NH_4)_2SO_4$/ (g L^{-1})	0.5	1	0.3525	0.176	8.216	0.0642	93.58
G	$MgSO_4{\cdot}7H_2O$/(g L^{-1})	0.1	0.5	–0.3225	–0.161	6.877	0.0788	92.12
H	NaCl/(g L^{-1})	0.1	0.5	–0.0675	–0.0338	0.301	0.6213	37.87
I	$CaCl_2{\cdot}H_2O$/(g L^{-1})	0.05	0.2	0.2925	0.146	5.657	0.0977	90.23
J	$FeCl_3{\cdot}6H_2O$/(g L^{-1})	0.001	0.002	0.4875	0.244	15.715	0.0287	97.13
K	Thiamine HCl/(g L^{-1})	0.0002	0.0004	0.1675	0.0838	1.855	0.2664	73.36
L	Biotin/(g L^{-1})	0.000 002	0.000008	0.3025	0.151	6.051	0.0909	90.91

The lipase activity was found to be maximum at 3.62 u mL^{-1} for the medium having the following composition in experimental run number 8 in Table 1: glucose 20 g L^{-1}; olive oil 10 mL L^{-1}; peptone 3 g L^{-1}; KH_2PO_4 2 g L^{-1}; yeast extract 4 g L^{-1}; $(NH_4)_2SO_4$ 0.5 g L^{-1}; $MgSO_4{\cdot}7H_2O$ 0.1 g L^{-1}; NaCl 0.1 g L^{-1}; $CaCl_2{\cdot}2H_2O$ 0.2 g L^{-1}; $FeCl_3{\cdot}6H_2O$ 0.002 g L^{-1}; thiamine HCl 0.0004 g L^{-1}; biotin 0.008 mg L^{-1} and Inositol 0.004 mg L^{-1}. The maximum lipase production of 3.62 u mL^{-1} was obtained mainly due to the higher concentration of olive oil. The

cell mass production and protease enzyme activity was found to be 2.72 g L^{-1} and 1.30 u mL^{-1} respectively. The low protease activity in this medium which in turn caused a less inhibitory action on lipase activity and resulted in a higher lipase production.

The maximum cell mass concentration of 3.56 g L^{-1} was achieved in the medium composition in run number 9 of the Table 1 containing the following composition: glucose 20 g L^{-1}; olive oil 2 mL L^{-1}; peptone 3 g L^{-1}; KH_2PO_4 6 g L^{-1}; yeast extract 2 g L^{-1}; $(NH_4)_2SO_4$ 0.5 g L^{-1}; $MgSO_4 \cdot 7H_2O$ 0.1 g L^{-1}; NaCl 0.5 g L^{-1}; $CaCl_2 \cdot 2H_2O$ 0.2 g L^{-1}; $FeCl_3 \cdot 6H_2O$ 0.002 g L^{-1}; thiamine HCl 0.0004 g L^{-1}; biotin 0.002 mg L^{-1} and Inositol 0.004 mg L^{-1}. But the lipase enzyme activity was found to be 2.54 u mL^{-1} which is nearly 40% less than the maximum lipase activity obtained in experiment run number 8 as given in Table 1. Although the cell mass concentration was high, a comparatively less lipase production was obtained. This might be due to the low concentration of inducer olive oil in the medium which was responsible for the extracellular lipase production.

The maximum protease activity of 3.15 u mL^{-1} was observed in run number 9 as indicated in Table 1. But low lipase activity of 2.54 u mL^{-1} and a high cell mass concentration of 3.56 g L^{-1} were observed in this medium. This might be due to the presence of high concentration of glucose but with a low concentration of olive oil in this medium. This caused an increase in cell mass concentration and protease activity but resulted in low lipase enzyme activity. *C. rugosa* was capable of secreting significant amount of protease enzyme in addition to the lipase production under substrate limited condition. The protease enzyme production was found to dominate in the stationary phase of the microorganism and resulted in the less production of lipase to certain extent. By altering the fermentation conditions and medium compositions the lipase activity in the medium could be increased with less protease activity.

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 glucose, $FeCl_3 \cdot 6H_2O$, peptone, olive oil, $(NH_4)_2SO_4$, $MgSO_4 \cdot 7H_2O$, biotin, $CaCl_2 \cdot 2H_2O$, KH_2PO_4, yeast extract, thiamineHCl and NaCl. These significant factors identified by Plackett-Burman design were considered for the next stage in the medium optimization using response surface optimization technique for the future study. Fig. 2 and Fig. 3 illustrate the response surface plots showing the effects of various combinations of the independent variables on lipase production by *C. rugosa* 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 without performing the experiments and also it is useful for the microbial production of lipase economically and efficiently. Thus these plots find applications in the design of enzyme reactors by knowing the kinetics of microbial lipase production.

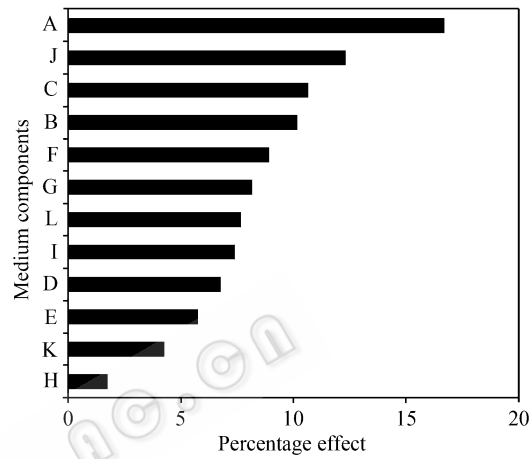

Fig. 1 Pareto-Plot for Plackett-Burman parameter estimates for twelve medium components

The carbon sources, glucose and olive oil were found to have high confidence levels of 98.75% and 95.33% respectively. They were found to exhibit positive effect in the tested range of concentration. Thus it is evident that the glucose and olive oil are essential for the growth and production of lipase by *Candida rugosa*. Olive oil along with a carbon source is commonly used as inducer for lipase secretion, but the role of inducer is not clearly understood[5]. Lipase encoding genes in *Candida rugosa* have grouped into two classes, one of which is constitutively expressed and the other is induced by fatty acids[22]. Basal level production of lipase was observed when glucose was used as the sole carbon source[23]. This result indicates the complex regulatory pattern of gene expression according to the composition of the medium[22]. Although simple carbohydrates like glucose do not contribute to lipase secretion but they can support the growth of yeast[23,24]. Presence of insoluble substances with ester bonds was found to increase the lipase production, indicates the necessity of lipid source for enzyme secretion[5,23]. Several reports on lipase production by olive oil as sole carbon source was available[6,9,18]. Benjamin *et al*[4] has reported a proportional increase in enzyme activity by the addition of 10% olive oil. In a comparative analysis for maximizing lipase production by *Candida rugosa*, increased biomass and lipase activity

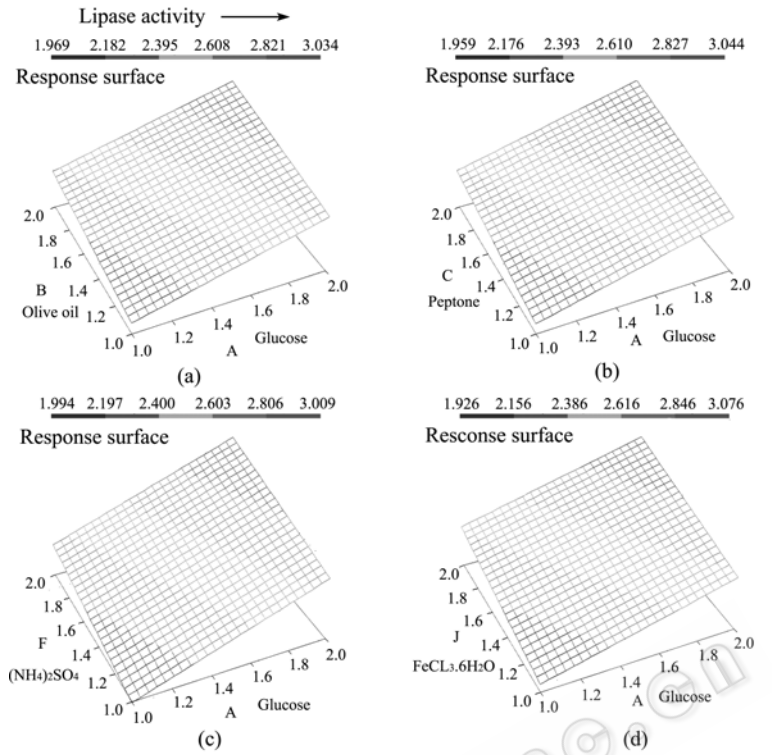

Fig. 2 Response surface plots showing the effects of the most significant independent variables on lipase production by *C. rugosa* with all the remaining factors kept constant at the middle level of the Plackett-Burman experimental design.

(a) Glucose and Olive oil (b) Glucose and Peptone (c) Glucose and $(NH_4)_2SO_4$ (d) Glucose and $FeCl_3 \cdot 6H_2O$. 1 signifies the lower level and 2 signifies the higher level of the independent variables in the experimented range

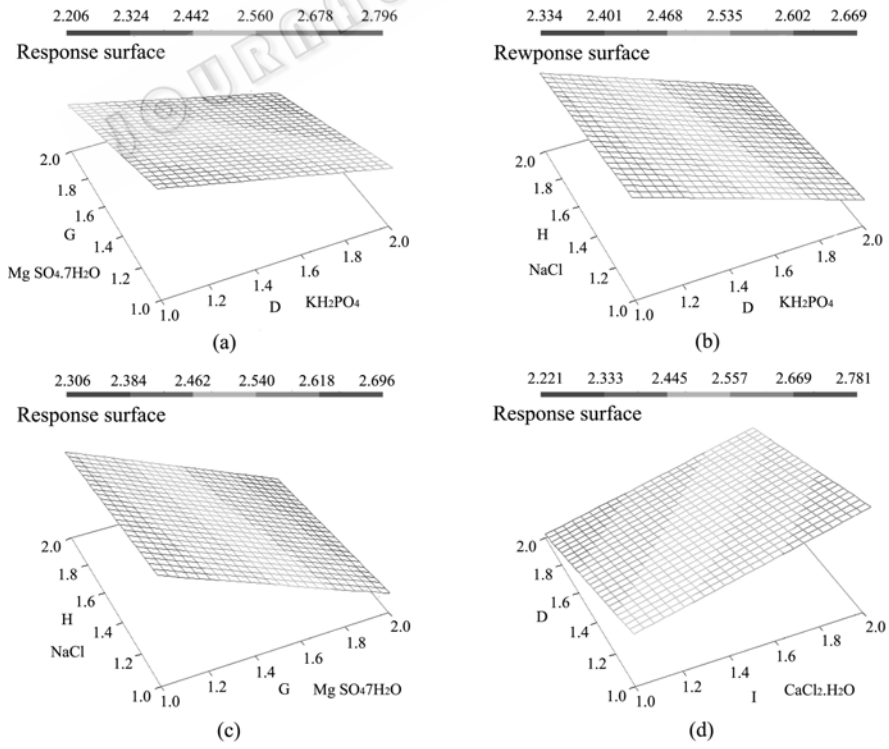

Fig. 3 Response surface plots showing the effects of the less significant independent variables on lipase production by *C. rugosa* with all the remaining factors kept constant at the middle level of the Plackett-Burman experimental design

(a) KH_2PO_4 and $MgSO_4 \cdot 7H_2O$; (b) KH_2PO_4 and NaCl; (c) $MgSO_4 \cdot 7H_2O$ and NaCl; (d) KH_2PO_4 and $CaCl_2 \cdot H_2O$. 1 signifies the lower level and 2 signifies the higher level of the independent variables in the experimented range

was observed in media supplemented with olive oil[22]. This is similar to the results obtained by Dalmau *et al*[25].

The organic nitrogenous sources, peptone and yeast were found to have positive effect over the lipase production with 95.86% and 93.58% confidence level respectively. Lipase synthesis was found to increase in the presence of organic nitrogen sources[26] which is in contrast to the report by Montesinos *et al* [7]. In *Aspergillus niger, Mucor racemosus* and *Rhizopus nigiricans* lipase yield was found to be maximum when supplemented with (2%) peptone[3]. Yeast extract and peptone increases the lipase production when compared to inorganic salts in Y*arrowia lipolytica* 681[24]. Yeast extract was found to be very effective for the production of lipase in *Cryptococcus sp.* S-2[27]. The inorganic nitrogenous compound, $(NH_4)_2SO_4$ was found to have positive effect with 93.58% confidence level. $(NH_4)_2SO_4$ significantly increased the lipase activity in *Candida rugosa* medium optimization studies[4]. When the media with $(NH_4)_2SO_4$ was used, increased lipolytic activity and yield was observed in fed batch fermentative production of lipase[28]. Positive effect was observed for the minerals $CaCl_2{\cdot}2H_2O$ and $FeCl_3{\cdot}6H_2O$ whereas KH_2PO_4, $MgSO_4{\cdot}7H_2O$ and NaCl were found to have negative effect on lipase productivity. $FeCl_3.6H_2O$ with high confidence level of 97.13% was found to enhance the lipase production by *Candida rugosa* and similar results was obtained in most of the fermentation reported for lipase production[4,6,28,29]. The micronutrients thiamine HCl and biotin were found to have positive effect among which Biotin influence the fermentation process with the confidence level of 90.91%. In all the lipase fermentation process by *Candida rugosa*, the addition of micronutrients resulted in much higher lipase productivity[6, 28, 29].

2.2 Kinetics of lipase production

The kinetics of lipase production using *C. rugosa* was studied using the optimized medium obtained from the PB experimental design at 30°C, initial pH 6.8 and 120 r/min. The optimized medium obtained using PB experimental design is given below: glucose 20 g L^{-1}; olive oil 10 mL L^{-1}; peptone 6 g L^{-1}; KH_2PO_4 2 g L^{-1}; yeast extract 4 g L^{-1}; $(NH_4)_2SO_4$ 1 g L^{-1}; $MgSO_4{\cdot}7H_2O$ 0.1 g L^{-1}; NaCl 0.1 g L^{-1}; $CaCl_2{\cdot}2H_2O$ 0.2 g L^{-1}; $FeCl_3{\cdot}6H_2O$ 0.002 g L^{-1}; thiamine HCl 0.0004 g L^{-1}; biotin 0.008 mg L^{-1}; Inositol 0.004 mg L^{-1}.

The results of the kinetic study namely lipase activity, protease activity, cell mass concentration, substrate utilization, pH and total soluble protein concentration with respect to time using the optimized medium is given in Fig. 4. The lipase production was found to increase gradually from the 8 h of the fermentation period when the growth of the microorganism reaches the early exponential phase being growth associated product. The maximum lipase activity was found in the late exponential phase and early stationary growth phase of *C. rugosa* as reported earlier[6]. The maximum lipase activity of 3.8 u mL^{-1} was obtained at the 50 h of fermentation and the activity reduces sharply after the 50 h. This might be due to the increased protease activity which inhibits the lipase production during the stationary phase of the microorganism and also due to the unavailability of the substrate. The protease activity reached a maximum value of 2.50 u mL^{-1} at 72 h. The cell mass concentration reached a maximum of 3.91 g L^{-1} at 60 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 32 h of fermentation. The pH of the medium was found to decrease gradually to pH 4.2 from the initial pH of 6.8. This decrease in pH might be due to the free fatty acid and organic acid synthesis during growth of *C. rugosa*. The rate of glucose utilization by the microorganism was found to increase rapidly after 8 h of the fermentation when the microorganism reaches the exponential phase and almost 92% of the glucose was consumed at 72 h of fermentation and maximum lipase production was obtained at this stage correspondingly. Later the lipase production was found to decrease due to the inhibitory action of the protease enzyme whose production was pronounced in the stationary phase of the microorganism.

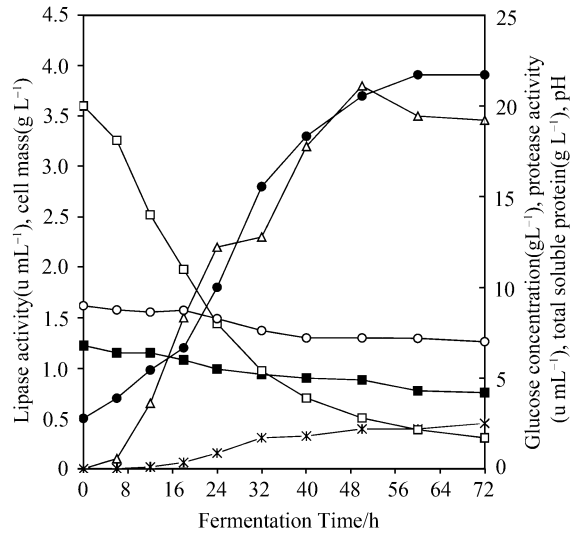

Fig. 4 Fermentative production of lipase by *C. rugosa* in submerged fermentation

Profile of lipase activity (△), protease activity (∗), pH (■), cell mass concentration (●), glucose concentration (□) and total soluble protein (O) in the optimized medium composition determined by Plackett-Burman statistical experimental design

2.3 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. Fig. 5 shows the experimental and model predicted values of lipase activity by the Luedeking-Piret model. Table 3 shows the estimated kinetic parameter values of the unstructured models. The predicted and the experimental values are demonstrated by the determination coefficient (R^2) for cell growth, lipase production and substrate concentration kinetics profile of the batch fermentation. The coefficient of determination is the measure of the strength of the linear relationship between the experimental and predicted values and was analyzed to find the fitness of these models for enzyme production process. The R^2 values were found to be 0.9981, 0.9585 and 0.9984 for Logistic model for cell growth, Luedeking- Piret model for lipase production and modified Luedeking- Piret model for glucose utilization respectively. Thus the proposed empirical kinetic model provides an accurate approximation of the fermentation kinetics. Since the magnitude of the growth associated parameter 'α' is much greater than the magnitude of non-growth associated parameter 'β ' in the product formation model, the lipase production is found to be growth associated. The unstructured models are relatively simple, easy to apply and highly flexible for all other fermentation processes also. The parameters described in the models can be exploited for designing the bioreactor operations effectively. The validity of the proposed model enables us to predict the optimal conditions for the microorganism cultivation and for the maximum yield of the product.

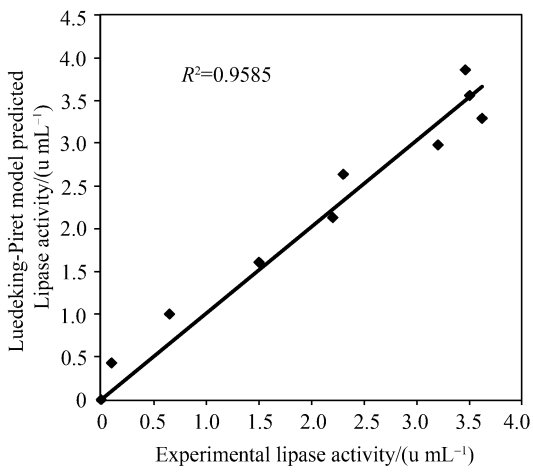

Fig. 5 Comparison of experimental and predicted values of Lipase activity by Luedeking-Piret model

Table 3 Estimated kinetic model parameters for lipase production by *Candida rugosa* in batch fermentation

Kinetic model Parameters	
μ_o (h^{-1})	0.12
X_m (g L^{-1})	2.72
X_0 (g L^{-1})	0.48
α (u gX^{-1})	0.7
β (u gX^{-1} h^{-1})	0.015
γ (gS gX^{-1})	6.9
η (gS gX^{-1} h^{-1})	0.03

3 Conclusion

The evaluation of the medium components for lipase production was done using the Plackett-Burman statistical method. The effect of twelve medium components were studied and among them glucose, olive oil, peptone and $FeCl_3 \cdot 6H_2O$ were found to be the significant variables for lipase production by *Candida rugosa* as the percentage confidence level was more than 95%.The Plackett-Burman statistical method offers the efficient and economical procedure by which the significant variables were identified. The significant factors identified by Plackett-Burman design are considered for the next stage of the medium optimization using response surface optimization technique for the future study. Maximum lipase activity of 3.8 u mL^{-1} was obtained in the optimized medium at the optimized condition of 30ºC, initial pH of 6.8 and at 120 r/min. The unstructured models Logistic model for cell growth, Luedeking-Piret model for lipase production and modified Luedeking-Piret model for glucose utilization were found suitable to describe the lipase production by *Candida rugosa*. All the models tested were able to predict the cell growth profile with higher R^2 values. From the estimated parameters of Luedeking-Piret model it was found that lipase production by *Candida rugosa* is growth associated.

The statistical design of experiment offer efficient methodology to identify the significant variables and to optimize the factors with minimum number of experiments for lipase production by microorganism. 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 fermentor.

Nomenclature

μ Specific growth rate (h^{-1})
μ_m Maximum specific growth rate (h^{-1})

μ_o Initial specific growth rate (h^{-1})

X_m Maximum cell mass concentration (g L^{-1})

X_0 Initial cell mass concentration (g L^{-1})

α Growth associated rate constant for product formation (u gX^{-1})

β Non growth associated rate constant for product formation (u $gX^{-1} \cdot h^{-1}$)

γ Growth associated rate constant for glucose consumption (gS gX^{-1})

η Non growth associated rate constant for glucose consumption (gS $gX^{-1} \cdot h^{-1}$)

$Y_{X/S}$ Yields of cell mass with respect to substrate

$Y_{P/S}$ Yields of product with respect to substrate

K_e Maintenance coefficient for cells

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