Statistical Evaluation of Medium Components Using Plackett-Burman Experimental Design and Kinetic Modeling of Lipase Production by *Bacillus sphaericus*

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Evaluation of twelve medium components on lipase production by *Bacillus sphaericus* MTCC511 in submerged batch fermentation was studied using the Plackett-Burman statistical experimental design. The most significant variables affecting lipase production were found to be glucose, olive oil, peptone, NaCl and $MnSO_4 \cdot H_2O$. Maximum lipase activity of 2.82 U ml^{-1} was obtained in the batch fermentation. The logistic model for cell growth, the Luedeking-Piret model for lipase production, and the modified Luedeking-Piret model for substrate utilization were capable of predicting the fermentation profile with high determination coefficient (R^2) values of 0.9977, 0.9497 and 0.9962 respectively. The results indicate that this statistical method is an efficient tool in detecting the most significant medium components with a minimum number of experimental trials, and the unstructured models were able to describe fermentation kinetics more effectively. The validity of the proposed model enables prediction of the optimal fermentation conditions.

Key words:
Bacillus sphaericus, submerged fermentation, lipase, Plackett-Burman experimental design, unstructured modeling

Introduction

Lipases, collectively known as triacylglycerol acylhydrolases (EC 3.1.1.3), catalyze the hydrolysis of water-insoluble esters and triglycerides at the interface between the insoluble substrate and water. Lipases are ubiquitous in nature, and lipases of microbial origin represent the most widely used class of enzymes in biotechnological applications. Lipases of various microbial origin, especially those from bacteria, exhibit the highest versatility and stability in catalyzing reactions in organic phase.[1] *Bacillus* species are among those microorganisms capable of promoting lipid conversion by means of their lipolytic system.[2] *Bacillus* species generally synthesize a variety of extracellular enzymes, the maximum synthesis of which normally occurs in the late exponential and early stationary phases of growth before sporulation, and are produced by submerged fermentation.[1] The stability, inexpensive manufacturing, as well as their broad synthetic potential make bacterial lipases ideal biocatalysts for oleo-chemical, detergent, oil, food, and paper industries. *Bacillus sphaericus,* a Gram-positive spore-forming bacteria, has been generally studied because of its ability to produce a protein parasporal body, which is highly toxic if eaten by susceptible mosquito larvae.[3,4] Lipase from *B. sphaericus* was stable, and activated by the organic solventsn-hexane and *p*-xylene by 3.5 and 2.9-fold respectively, at $\varphi = 25$ %.[3] There are only a few reports on the production of lipase by *B. sphaericus*.[3] So far there is no report on statistical medium optimization and kinetic modeling of lipase production by *B. sphaericus.*

The study of factors affecting the yield of the desired metabolic product is an important strategy for the bioprocess development. The cell growth and accumulation of metabolic products are influenced strongly by medium compositions, such as carbon sources, nitrogen sources and inorganic salts. Thus, it is difficult to search for the major factors and optimize them for biotechnological processes as several factors are involved.[5] The classical method of medium optimization involves changing one variable at a time by keeping the others at fixed levels. Being one-dimensional, this laborious and time-consuming method does not guarantee determination of optimal conditions. On the other hand, carrying out experiments with every possible factorial combination of the test variable is impractical because of the large number of experiments. Experimental design techniques present a more balanced alternative to the one-factor-at-a-time approach to fermentation improvement. The statistical approach

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for the optimization of media effectively tackles the problem, and involves a specific design of experiments, which minimizes the error in determining the effect of variables. The Plackett-Burman design allows reliable short-listing of a small number of ingredients for further optimization and allows for unbiased estimates of the linear effects of all factors with maximum accuracy for a given number of observations. This design is practical especially when the investigator faces a large number of factors and is uncertain of which settings are likely to produce optimal or near optimal responses.[6]

The rational design and optimization of the fermentation requires understanding production kinetics.[4] Although numerous papers have been published on the selection of lipase producers, not much information is available on the fermentation process. The kinetics of fermentation is important to identify optimal operating conditions for enzyme production. This study determines the effect of various medium components on lipase production by *B. sphaericus* using the Plackett-Burman design, while unstructured kinetic models were used to characterize the fermentation process.

Materials and methods

Microorganism and culture maintenance

B. sphaericus MTCC511 was obtained from Microbial Type Culture Collection of gene bank (MTCC), Institute of Microbial Technology, Chandigarh, India. Spirit blue agar (Himedia, India) was used for the detection of lipolytic activity and the stock cultures were maintained on nutrient agar slants containing (g l^{-1}): Beef extract, 1; Yeast extract, 2; Peptone, 5; NaCl, 5; Agar, 15.

Batch fermentation

The lipase production by *B. sphaericus* was conducted in a 250-ml Erlenmeyer flask with 100 ml of production medium. The medium was adjusted to the initial pH of 7.0 using 2 mol l^{-1} NH_4OH and sterilized (121 °C for 20 min). The production medium was inoculated with $\varphi = 5$ % of seed culture in its mid exponential phase at 10 h. The Erlenmeyer flask was incubated in an orbital shaker at $n = 120$ min^{-1} and 30 °C for the fermentation period of 48 h. Aliquot of sample (1.5 ml) from the fermentation broth was withdrawn at regular time intervals without much change in the culture volume to maintain constant oxygen transfer, and the cells were separated from the medium by centrifugation for 15 min at 4 °C and $a_c = 5030$ *g*. The clarified supernatant was used for the analysis of lipase activity, protease activity, total soluble protein and glucose mass concentration. The components of the production medium were tested at two levels, high (+) and low (–), to study their effect on lipase production using the Plackett-Burman statistical experimental design.

The medium components were evaluated using the 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.[14] Twelve factors were screened in sixteen combinations with three dummy variables, which will provide an adequate estimate of the error. 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. Thus, the effects of changing other variables cancel out when determining the effect of one 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. The Plackett-Burman experimental design is based on the first order model:

$$Y = M_0 + \Sigma M_i X_i,$$

where Y is the response (lipase activity), M_0 is the model intercept, and M_i 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 factor confidence levels above 90 % were considered the most significant factors affecting lipase production. The design of the 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 considered to affect lipase production. The factors tested were glucose, olive oil, peptone, yeast extract, Tween 80, CH_3COONa, NaCl, KCl, $CaCl_2 \cdot 2H_2O$, $MgSO_4 \cdot 7H_2O$, $MnSO_4 \cdot H_2O$, $FeSO_4 \cdot 7H_2O$ with three dummy variables, and the tested concentration range is given in Table 1. All the submerged batch fermentations were conducted in triplicate in shake flasks, and the results were the average of the three trials, while the response was measured in terms of lipase production.

Lipase activity assay

Lipase activity was estimated with an olive oil emulsion by the procedure of *Ota* and *Yamada*.[7] The olive oil emulsion was prepared by homogenizing 25 ml of olive oil and 75 ml of $w = 2$ % polyvinyl alcohol solution in a homogenizer for 6 min at $n = 20\,000$ min^{-1}. The reaction mixture composed of 2 ml olive oil emulsion, 2.5 ml 0.05 mol l^{-1} phosphate buffer of pH 7.0, and 0.5 ml enzyme solution

Table 1 – *Medium components at different levels used in Plackett-Burman experimental design*

Medium components with designation	Lower level (–1)	Higher level (+1)
A glucose, γ/g l^{-1}	10	20
B olive oil, σ/ml l^{-1}	2	10
C peptone, γ/g l^{-1}	5	10
D yeast extract, γ/g l^{-1}	5	10
E tween 80 σ/ml l^{-1}	5	10
F CH_3COONa, γ/g l^{-1}	5	10
G NaCl, γ/g l^{-1}	1	5
H KCl, γ/g l^{-1}	0.5	2
I $CaCl_2 \cdot 2H_2O$, γ/g l^{-1}	0.5	1
J $Mg\ SO_4 \cdot 7H_2O$, γ/g l^{-1}	0.01	0.5
K $MnSO_4 \cdot H_2O$, γ/g l^{-1}	0.01	0.5
L $FeSO_4 \cdot 7H_2O$, γ/g l^{-1}	0.01	0.1

was incubated at 37 °C for 15 min. The emulsion was destroyed by adding 10 ml acetone immediately after incubation and the liberated fatty acid was titrated against 0.05 mol l^{-1} 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.

Protease activity assay

Protease activity was assayed by the modified Anson method[8] using casein as the substrate. 2 ml of $w = 1$ % 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 mol l^{-1} trichloroacetic acid was added to arrest the reaction. The solution with the precipitate was filtered and 1 ml of filtrate, 5 ml of 0.4 mol l^{-1} Na_2CO_3 and 0.5 ml of folin reagent were added. After 10 min of incubation, the developed colour density 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 at 37 °C.

Biomass, glucose and protein determination

Bacterial cell growth was determined by measuring OD at 600 nm. The biomass concentration was determined with a calibration curve made from the relationship between OD at 600 nm and dry cell mass. The glucose mass concentration in the cultivation broth was determined as described by *Miller*[9] using dinitrosalicylic acid. The total soluble protein in the medium was determined as described by *Lowry* et al.[10]

Unstructured model development for fermentation kinetics

Various unstructured models were proved efficient for characterizing the fermentation kinetics. In an unstructured model, the cellular representations are single component representations.[11] The exponential growth phase may be characterized by the following first order equation which states that the rate of increase of cell mass is proportional to the quantity of viable cell mass at any instant time,

$$\frac{d\gamma_X}{dt} = \mu\gamma_X \tag{1}$$

where $d\gamma_X/dt$ is the growth rate (g l^{-1} h^{-1}); γ_X is the instantaneous biomass concentration (g l^{-1}); μ is the specific cell growth rate (h^{-1}). The cell growth 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[11] as,

$$\frac{d\gamma_X}{dt} = \mu_0\left(1.0 - \frac{\gamma_X}{\gamma_{X,max}}\right)\gamma_X \tag{2}$$

where μ_0 is the initial specific growth rate (h^{-1}) and $\gamma_{X,max}$ is the maximum cell mass concentration (g l^{-1}). Eq. (2) on integration using $\gamma_{X,0} = \gamma_X$ ($t = 0$) gives a sigmoidal variation of γ_X that may empirically represent both the exponential and stationary phase.

$$\gamma_X = \frac{\gamma_{X,0}e^{\mu_0 t}}{1 - \left(\frac{\gamma_{X,0}}{\gamma_{X,max}}\right)(1 - e^{\mu_0 t})} \tag{3}$$

The kinetic parameter, μ_0 in this equation is determined by rearranging equation (3) as,

$$\mu_0 t = \ln\left[\frac{\gamma_{X,max}}{\gamma_{X,0}}\right] + \ln\left[\frac{\bar{\gamma}_X}{1 - \bar{\gamma}_X}\right], \tag{4}$$

where

$$\bar{\gamma}_X = \frac{\gamma_X}{\gamma_{X,max}}$$

If the logistic equation describes the data suitably, then a plot of $\ln\left[\frac{\bar{\gamma}_X}{1-\bar{\gamma}_X}\right]$ vs t should give a straight line of slope μ_0 and intercept $-\ln\left[\left(\frac{\bar{\gamma}_{X,max}}{\gamma_{X,0}}\right)\right]$.

Luedeking and *Piret*[12] states that the product formation rate depends upon both the instantaneous biomass concentration (γ_X), and the growth rate ($d\gamma_X/dt$) in a linear fashion.

$$\frac{d\gamma_P}{dt} = \alpha \frac{d\gamma_X}{dt} + \beta \gamma_X \quad (5)$$

where α and β are empirical constants that may vary with fermentation conditions (temperature, pH, etc.). Integrating equation (5) using equation (2),

$$\gamma_P = \gamma_{P,0} + \alpha\, A(t) + \beta\, B(t) \quad (6)$$

where $\gamma_{P,0}$ and γ_P are the product mass concentrations at initial time and at any time 't' respectively, $A(t) = \gamma_{X,0} \dfrac{e^{\mu_0 t}}{1 - \left(\dfrac{\gamma_{X,0}}{\gamma_{X,max}}\right)(1.0 - e^{\mu_0 t})} - 1.0$ and

$$B(t) = \frac{\gamma_{X,max}}{\mu_0} \ln\left[1.0 - \frac{\gamma_{X,0}}{\gamma_{X,max}}(1.0 - e^{\mu_0 t})\right].$$

The quantities α and β in eq. (6) are determined by plotting $\gamma_P - \gamma_{P,0}/B(t)$ *vs* $A(t)/B(t)$ which is a straight line with slope 'α' and intercept 'β'.

The substrate utilization kinetics is given by the following equation, which considers substrate conversion to cell mass, to product and substrate consumption for maintenance[13]

$$\frac{d\gamma_S}{dt} = -\frac{1}{Y_{X/S}} \frac{d\gamma_X}{dt} - \frac{1}{Y_{P/S}} \frac{d\gamma_P}{dt} - k_e \gamma_X \quad (7)$$

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 eq. (7),

$$\frac{d\gamma_S}{dt} = -\zeta \frac{d\gamma_X}{dt} - \eta \gamma_X \quad (8)$$

where

$$\zeta = \frac{1}{Y_{X/S}} + \frac{\alpha}{Y_{P/S}} \text{ and } \eta = \frac{\beta}{Y_{P/S}} + k_e$$

Eq. (8) is the modified Luedeking-Piret equation for substrate utilization kinetics.

Substituting for μ from (2) and integrating gives

$$\gamma_S = \gamma_{S,0} - \zeta\, m(t) - \eta\, n(t) \quad (9)$$

where $\gamma_{S,0}$ and γ_S are the substrate concentrations at initial time and at any time 't' respectively,

$$m(t) = \left[\frac{\gamma_{X,0} e^{\mu_0 t}}{1 - (\gamma_{X,0}/\gamma_{X,max})(1 - e^{\mu_0 t})} - 1\right]$$

and

$$n(t) = \frac{\gamma_{X,max}}{\mu_0} \ln\left[1 - \frac{\gamma_{X,0}}{\gamma_{X,max}}(1 - e^{\mu_0 t})\right]$$

Kinetic quantities (γ,η) in eq. (9) are determined by plotting $\dfrac{\gamma_{S,0} - \gamma_S}{n(t)}$ *vs* $\dfrac{m(t)}{n(t)}$ which is a straight line with slope 'ζ' and intercept 'η'.

Results

Evaluation of culture medium components using Plackett-Burman design

The lipase enzyme synthesized by *B. sphaericus* was found to vary from 0.64 U ml^{-1} to 2.82 U ml^{-1} in the sixteen experiments conducted (Table 2), which shows the strong influence of medium components on lipase enzyme production. The main effect, regression coefficient, F values and P values of the factors were calculated for lipase production using the Unscrambler, version V.8.0.5, CAMO process AS, Norway (Table 3). Based on the statistical analysis of the experimental data it was found that the significant medium components were glucose, olive oil, peptone, NaCl and $MnSO_4 \cdot H_2O$.

Kinetics of lipase production

The sixteen trials for lipase production by *B. sphaericus* were carried out by keeping the fermentation conditions constant. The fermentation profile for the maximum lipase production trial (Experiment 6) is given in Fig. 1. The lipase production

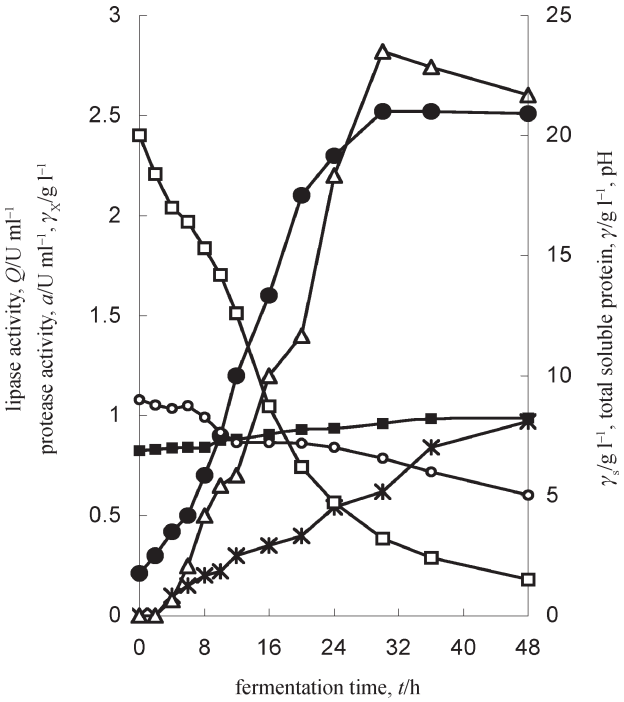

Fig. 1 – *Experimental time evolution of lipase activity (ρ), protease activity (∗), pH (ν), cell mass concentration (●), glucose concentration (□) and total soluble protein (○) by Bacillus sphaericus (Experiment run 6) in batch fermentation*

Table 2 – *Plackett-Burman experimental design for evaluation of 12 variables with coded values for lipase production by Bacillus sphaericus and response of the design*

Run number	A	B	C	D	E	F	G	H	I	J	K	L	Lipase activity a/U ml^{-1}
1	+	+	+	+	-	+	-	+	+	-	-	+	2.03
2	+	+	+	-	+	-	+	+	-	-	+	-	2.80
3	+	+	-	+	-	+	+	-	-	+	-	-	1.64
4	+	-	+	-	+	+	-	-	+	-	-	-	1.48
5	-	+	-	+	+	-	-	+	-	-	-	+	1.51
6	+	-	+	+	-	-	+	-	-	-	+	+	2.82
7	-	+	+	-	-	+	-	-	-	+	+	+	1.50
8	+	+	-	-	+	-	-	-	+	+	+	+	2.13
9	+	-	-	+	-	-	-	+	+	+	+	-	1.25
10	-	-	+	-	-	-	+	+	+	+	-	+	1.60
11	-	+	-	-	-	+	+	+	+	-	+	-	1.98
12	+	-	-	-	+	+	+	+	-	+	-	+	1.58
13	-	-	-	+	+	+	+	-	+	-	+	+	1.62
14	-	-	+	+	+	+	-	+	-	+	+	-	1.45
15	-	+	+	+	+	-	+	-	+	+	-	-	2.08
16	-	-	-	-	-	-	-	-	-	-	-	-	0.64

Table 3 – *Statistical analysis of Plackett-Burman experimental design showing F values, P values and confidence level of each variable for lipase production by Bacillus sphaericus*

Medium components	Main effect	Coefficients	F-value	*P*-value	Confidence level (%)
glucose, γ/g l^{-1}	0.418	0.209	11.10	0.0446	95.54
olive oil, σ/ml l^{-1}	0.403	0.202	10.32	0.0489	95.11
peptone, γ/g l^{-1}	0.426	0.213	11.50	0.0427	95.73
yeast extract, γ/g l^{-1}	0.086	0.043	0.47	0.5418	45.82
tween 80, σ/ml l^{-1}	0.148	0.074	1.40	0.3218	67.82
CH_3COONa, γ/g l^{-1}	–-0.193	–0.096	2.37	0.2208	77.92
NaCl, γ/g l^{-1}	0.516	0.258	16.87	0.0261	97.39
KCl, γ/g l^{-1}	0.036	0.018	0.08	0.7918	20.82
$CaCl_2 \cdot 2H_2O$, γ/g l^{-1}	0.028	0.014	0.05	0.8338	16.62
$MgSO_4 \cdot 7H_2O$, γ/g l^{-1}	–0.206	–0.103	2.69	0.1993	80.07
$MnSO_4 \cdot H_2O$, γ/g l^{-1}	0.373	0.187	8.84	0.0589	94.11
$FeSO_4 \cdot 7H_2O$, γ/g l^{-1}	0.183	0.091	2.13	0.2399	76.01

was found to increase gradually after the 6 h of the fermentation period, when the growth of the microorganism reached the exponential phase. The maximum lipase activity was found in the late exponential phase and early stationary growth phase of *B. sphaericus* as reported earlier.[15] The maximum lipase activity of 2.82 U ml^{-1} was obtained at 30 h of fermentation and the activity reduced sharply after 30 h, this might be due to the increase in the protease activity during the stationary phase of the microorganism. The protease activity reached a maximum value of 0.97 U ml^{-1} at 48 h. The cell mass concentration reached a maximum of 2.52 g l^{-1} at 30 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 6 h to 24 h. This observation clearly indicated that the maximum lipase production was at the early stationary phase and post exponential phase, and the lipase production was found to be growth associated product. The pH of the medium increased gradually from the initial pH of 6.8 to 8.2 due to the cellular excretion of ammonium ions by utilizing the various nitrogen sources in the medium. This rise in the pH may also be due to the free amino acid synthesis during the enzyme production by *B. sphaericus*.[4] The rate of glucose utilization by the microorganism increased rapidly after 8 h of fermentation when the microorganism reached the mid exponential phase. The rate of glucose utilization was proportional to biomass concentration. Almost 90 % of the glucose was depleted in 48 h of fermentation. Mathematical analysis of the data showed that the unstructured models could satisfactorily explain the kinetics of the fermentation process for production of lipase by *B. sphaericus.*

Discussion

Effect of medium constituents on lipase production by *B. sphaericus*

The analysis of regression coefficient for twelve medium components is illustrated in Table 3. The major carbon sources, glucose, and the inducer olive oil, were found to have confidence levels of 95.54 % and 95.11 % respectively, and they had a positive effect in the tested concentration range. The results apparently showed that glucose and olive oil were essential for the growth and lipase secretion by *B. sphaericus*. The most important factor for the expression of lipase activity was found to be the carbon source since lipases are inducible enzymes[16] and are generally produced in the presence of a lipid source such as oil.[3,17,18,19] Glucose was found to be the important carbon source for higher lipase production rate as the growth of microorganisms was phenomenal,[17] although *Fatima Silva Lopes* et al.[5] reported that glucose did not influence the maximum lipase activity in *Lactobacillus plantarum* batch culture. Lipase and biomass production by *Yarrowia lipolytica* 681 was enhanced significantly in the presence of glucose and olive oil.[20] *Valero* et al.[21] 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 lipase activity by *B. coagulans* BTS3 was by olive oil as an inducer.[19] While *Dalmau* et al.[22] demonstrated the inhibition of lipase production by glucose, *Chang* et al.[23] does not report any repressive effect of glucose on *Candida rugosa.*

Experimental results reveal that peptone and yeast extract have a positive effect on lipase production. However, peptone was found to have more influence on lipase production as it had a higher confidence level of 95.73 %. Generally, organic nitrogen sources (peptone and yeast extract) were preferred for lipase production by various *Bacillus* species[1]. Different types of nitrogen sources were tested to determine their effect on the production of lipase by alkaliphilic *B. coagulans BTS-3*. The maximum lipase production was obtained with peptone ($w = 0.5$ %) and yeast extract ($w = 0.5$ %) in the production broth.[19] Tween 80 was found to have a positive influence on lipase production by *B. sphaericus*. The presence of long-chain fatty acids and the emulsifying action of Tween 80 induced the lipase production, and thus had maximum positive effect among the various factors tested using Plackett-Burman statistical design for lipase production by a novel *Bacillus* species.[6] *Nthangeni* et al.[24] cultured *B. licheniformis* in liquid medium and found that only Tween 80 induced a significant level of lipase activity. These reports are in concurrence with the results obtained, but Tween 80 had a confidence level of 67.82 % and therefore was not statistically significant for lipase production by *B. sphaericus*. The inorganic salts NaCl, KCl, $CaCl_2 \cdot 2H_2O$, $MnSO_4 \cdot H_2O$ and $FeSO_4 \cdot 7H_2O$ showed a positive effect for lipase activity, whereas CH_3COONa and $MgSO_4 \cdot 7H_2O$ showed negative effect in the tested concentration range. Among the inorganic compounds tested, NaCl and $MnSO_4 \cdot H_2O$ were found to have confidence levels of 97.39 % and 94.11 % respectively, and they are highly significant. *Abdel-Fattah* et al.[6] evidenced NaCl as the most important factor which significantly increased lipase production by a novel thermophilic *Bacillus* sp. *Sharma* et al.[25] reported the stimulation effect of calcium chloride in lipase production from *Bacillus* sp RSJ1. Lipase production by a thermophilic *Bacillus* sp. was increased several-fold when magne-

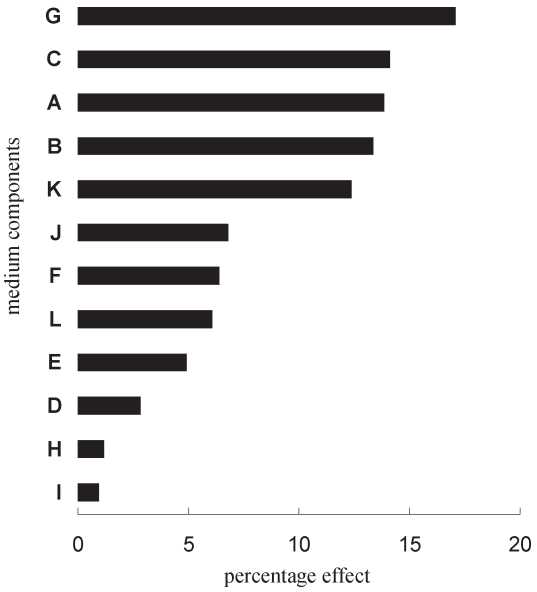

Fig. 2 – *Pareto-Plot for Plackett-Burman parameter estimates for twelve medium components*

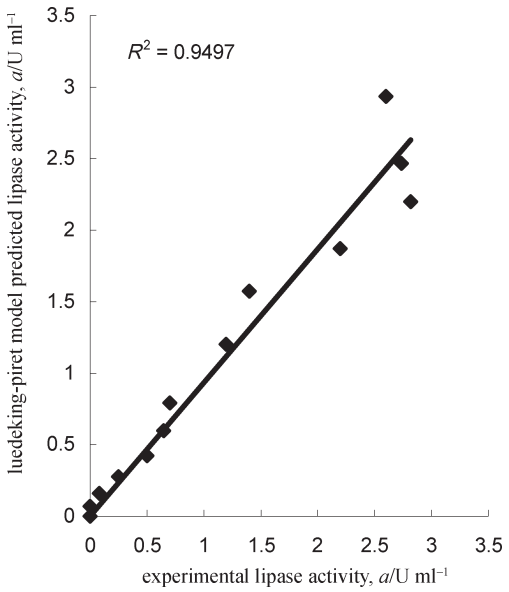

Fig. 3 – *Comparison of observed and predicted values of Lipase activity by Luedeking-Piret model*

sium, iron and calcium ions were added to the production medium.[26] *Rathi* et al.[18] observed stimulation of lipase production by *Burkholderia cepacia* in the presence of Ca^{2+} and Mg^{2+}. Exclusion of the magnesium ions from the production medium using *Pseudomonas aeruginosa* caused approximately 50 % reduction in lipase production.[27] Contradictory to the results reported for the influence of magnesium ions on lipase production, it was found that the magnesium ions have a negative effect on lipase production by *B. sphaericus*. Sodium ion in the form of NaCl was found to have a positive effect with high confidence level but CH_3COONa had a negative effect.

Based on the calculated *P* values it was found that the medium components glucose, olive oil, peptone, NaCl and $MnSO_4 \cdot H_2O$ as the most significant variables and these factors have confidence levels higher than 90 %. The Pareto chart (Fig. 2) offers a convenient view of the results obtained by Plackett-Burman design. These significant factors identified by the Plackett-Burman design are considered for the next stage in the medium optimization using response surface optimization technique for the future study.

Unstructured model equation of best fit

The unstructured models provide a good approximation of the fermentation profile even though the complete cell mechanism was not considered in the models. Table 4 shows the estimated parameters of the unstructured models by using batch fermentation data. Fig. 3 shows the experimental and estimated responses of lipase production using Luedeking-Piret model. The predicted and experimental values are demonstrated by the determination coefficient (R^2), which is a measure of the strength of the linear relationship between the experimental and predicted values. The R^2 values of 0.9977, 0.9497 and 0.9962 were obtained for Logistic model, Luedeking-Piret model and modified Luedeking-Piret respectively. Thus, the proposed empirical kinetic model provides an accurate estimation of the fermentation kinetics. Since the magnitude of the growth-associated parameter 'α' was much greater than the magnitude of non-growth-associated parameter 'β' in the product formation model, the lipase production was found to be growth-associated. The unstructured models are relatively simple, easy to apply and highly flexible to use for other fermentation processes also. The parameters described in the models can be used for designing the bioreactor operations effectively. The findings of this work will be important for further studies in optimizing lipase production by *B. sphaericus* and other bacteria.

Table 4 – *Unstructured kinetic model parameters evaluated using batch data for lipase production by Bacillus sphaericus*

Kinetic model parameters	
μ_0/h^{-1}	0.19
$\gamma_{X,max}$/g l^{-1}	2.52
$\gamma_{X,0}$/g l^{-1}	0.21
α/U g^{-1}	0.7
β/U g^{-1} h^{-1}	0.015
ζ/g g^{-1}	6.9
η/g g^{-1} h^{-1}	0.03

List of symbols

μ – specific growth rate, h^{-1}
μ_0 – initial specific growth rate, h^{-1}
$\gamma_{X,max}$ – maximum cell mass concentration, g l^{-1}
$\gamma_{X,0}$ – initial cell mass concentration, g l^{-1}
α – growth-associated empirical constant for product formation, U g^{-1}
a_c – real acceleration, m s^{-2}
g – standard acceleration, m s^{-2}
β – non-growth-associated empirical constant for product formation, U g^{-1} h^{-1}
ζ – growth-associated empirical constant for glucose consumption, m_S/m_X, g g^{-1}
η – non-growth-associated empirical constant for glucose consumption, g g^{-1} 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
w – mass fraction, %
φ – volume fraction
n – stirring speed, min^{-1}
σ – volume concentration, ml l^{-1}

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