Statistical optimization of medium components for the production of lipase by Serratia marcescens SB08
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Citation

Abstract
The optimization of the fermentation medium and conditions for maximum lipase production was carried out using a new strain, Serratia marcescens SB08. The results of factorial design showed that CaCl₂, incubation time, pH and yeast extract were the key factors affecting lipase production. The optimal cultural conditions for lipase production obtained with central composite design was pH 7.0, incubation time 51 h, yeast extract 3.0 g/L and CaCl₂ 0.13 g/L. The model was also validated by repeating the experiments under the optimized conditions, which resulted in the lipase production of 243.91 U/mL (Predicted response 251.83 U/mL), thus proving the validity of the model. Lipase enzyme was purified and the molecular weight was found to be 52 KDa. In this work the use of a central composite design by determining the conditions leading to the high yield of enzyme production has been demonstrated. Thus, smaller and less time consuming experimental designs could generally suffice for the optimization of many fermentation processes.

INTRODUCTION
Lipases are extremely versatile enzymes, showing many interesting properties of industrial applications. They are a class of enzymes which catalyze the hydrolysis of long chain triglycerides and constitute the most important group of biocatalysts for biotechnological applications. Lipases can be divided generally into the following four groups according to their specificity in hydrolysis reaction: substrate specific lipases, regio-selective lipases, fatty acid specific lipases, and stereo-specific lipases. They are obtained from a variety of sources like plants, animals, yeast, bacteria; but among all, microbial lipases are the most popular for industrial use as they are easy to produce and are stable comparatively. Pancreatic lipase of porcine origin is one of the earliest recognized lipases and is still the best-known lipase. Plant lipases are not used commercially; the animal and microbial lipases are used extensively.

Although there have been many papers dealing with the lipase producing yeasts such as Candida cylindracea, Saccharomyces lipolytica, Geotrichum candidum and Trichosporon fermentans, only limited research has been directed towards the lipase producing organisms. The most productive fungi species belong to the genera Geotrichum, Penicillium, Aspergillus and Rhizomucor. Lipases from unicellular bacteria, mainly those produced by various species of the genus Pseudomonas, have also proved to be useful both in organic reactions and in the detergent industry. Several microorganisms, such as Candida rugosa, Candida antarctica, Burkholderia cepacia, and Pseudomonas alcaligens, can produce lipase efficiently and their lipases are commercially available.

A characteristic trait of many strains of Serratia marcescens is that they produce extracellular enzymes, including nuclease, protease, chitinase and lipase. Serratia species are gram-negative mesophils that have the capability to grow and produce extracellular lipase and protease during culture period. The early literature reported that S. marcescens isolated from raw milk produced lipase enzyme (Thomas, 1958; Witter, 1961). Recently, some workers have isolated psychrotrophs from refrigerated raw milk samples; about 3 to 6% of the isolated strains were S. marcescens (Milliere and Veillet-Poncet, 1985; Ahmed et al., 1989; Abdou, 1997). S. marcescens was found to have lipolytic and proteolytic activities (Abdou and Ohashi, 1996). The extracellular lipase from S. marcescens (triacylglycerol acylhydrolase [EC 3.1.1.31]) is an industrially important enzyme. It is stable in some organic solvents and is applicable to the asymmetric synthesis of chiral compounds by asymmetric hydrolysis.

The demand for the production of highly active preparations of lipolytic enzymes has led to research on lipase producing
microorganisms and on culture strategies (Lechner et al., 1988; Suzuki et al., 1988).

The interest in microbial lipase production has increased in the last decades, because of its large potential in industrial applications as additives in foods (flavor modification), fine chemicals (synthesis of esters), detergents (hydrolysis of fats), waste water treatment (decomposition and removal of oil substances), cosmetics (removal of lipids), pharmaceuticals (digestion of oil and fats in foods), leather (removal of lipids from animal skins) and medical (blood triglyceride assay) (Elibol and Ozer, 2000; Kamini et al., 2000).

Lipases represent an extremely versatile group of bacterial extracellular enzymes that are capable of performing a variety of important reactions, thereby presenting a fascinating field for future research. Wide and constant screening of new microorganisms for their lipolytic enzymes will open novel and simpler routes for the synthetic processes. Consequently, this may pave new ways to solve biotechnological and environmental problems. Hence the present study has been aimed with the objective of screening a novel lipase producing bacteria.

MATERIALS AND METHODS
MICROORGANISM AND CULTURE MAINTENANCE

The new strain Serratia marcescens SB08 was isolated from soil and the culture was maintained at 4°C and subcultured every two weeks. Nutrient broth medium was prepared and the pH of the medium was adjusted to 7.0 and was autoclaved at 121°C for 15 minutes. A loopful of bacterial culture (Serratia marcescens SB08) was inoculated and incubated at 30°C for 18 hrs.

LIPASE ASSAY
PRODUCTION OF LIPASE

One hundred ml of nutrient broth was inoculated with 1 ml of the above 18 hrs bacterial inoculum and was incubated at 30°C for 24 hours. After incubation the crude enzyme was obtained by centrifugation of the culture broth at 10,000 rpm for 10 minutes. The cell free supernatant was assayed for lipase activity.

ENZYME ASSAY

The lipase assay was carried out by the modified method of Satarik (1991). The fermented broth was centrifuged at 10,000 rpm for 15 minutes. The supernatant was taken for determination of lipase activity. For this, an aliquot of olive oil (250 mg) was transferred into a test tube containing 2 ml of phosphate buffer (pH 6.5) and 1 ml of crude enzyme was added to it. The mixture was vortexed for 15 seconds and incubated at 37°C in a water bath under static conditions for 30 minutes. After stopping the reaction by adding 1 ml concentrated HCl and vortexing for 10 seconds, 3 ml of benzene was added and after vortexing for 90 seconds, the aqueous phase and organic phases were allowed to separate. From this 2 ml of benzene layer was withdrawn and transferred to a tube containing 1 ml of aqueous solution of cupric acetate (5 %) and the mixture after vortexing for 90 seconds was centrifuged at 5,000 rpm for 10 minutes to obtain a clear organic phase. The organic layer (Benzene layer) was withdrawn and used to estimate the liberated free fatty acid by measuring the optical density (OD) against distilled water at 715 nm using spectrophotometer (Model – 3210, Hitachi, Japan). One unit (U) of lipase activity is equal to one micromole of free fatty acid liberated per minute per ml using the assay condition.

OPTIMIZATION OF PROCESS PARAMETERS
SCREENING OF IMPORTANT NUTRIENT COMPONENTS USING PLACKETT – BURMAN DESIGN

This study was done by Plackett - Burman design for screening medium components with respect to their main effects and not their interaction effects (Plackett and Burman, 1946) on enzyme production by Serratia marcescens SB08. The medium components were screened for eleven variables at two levels, maximum (+) and minimum (-). According to the Plackett – Burman design, the number of positive signs (+) is equal to (N+1) / 2 and the number of negative signs (-) is equal to (N-1) / 2 in a row. A column should contain equal number of positive and negative signs. The first row contains (N+1) /2 positive signs and (N-1) /2 negative signs and the choice of placing the signs is arbitrary. The next (N-1) rows are generated by shifting cyclically one place (N-1) times and the last row contains all negative signs. The medium was formulated as per the design and the flask culture experiments for lipase enzyme was assayed as described earlier. Response was calculated as the rate of enzyme production and expressed as U/mL. All experiments were performed in triplicates and the average of the rate of enzyme production was considered as the response.

The effect of each variable was calculated using the following equation
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Figure 1

\[
E = \frac{\sum M_+ - \sum M_-}{N},
\]

Where E is the effect of tested variable, M<sub>+</sub> and M<sub>-</sub> are responses (enzyme activities) of trials at which the parameter was at its higher and lower levels respectively and N is the number of experiments carried out.

The standard error (SE) of the variables was the square root of variance and the significance level (p – value) of each variables calculated by using Student’s t – test.

Figure 2

\[
t = \frac{E_{xi}}{SE}
\]

where E<sub>xi</sub> is the effect of tested variable. The variables with higher confidence levels were considered to influence the response or output variable.

OPTIMIZATION OF CONCENTRATIONS OF THE SELECTED MEDIUM COMPONENTS USING RESPONSE SURFACE METHODOLOGY

Response surface methodology is an empirical statistical modeling technique employed for multiple regression analysis using quantitative data obtained from factorial design to solve multivariable equations simultaneously (Rao et al., 2000). The screened medium components affecting enzyme production were optimized using central composite design (CCD) (Box and Wilson, 1951; Box and Hunter, 1957).

According to this design, the total number of treatment combinations is 2<sup>k</sup> + 2<sup>k</sup> + n<sub>0</sub> where ‘k’ is the number of independent variables and n<sub>0</sub> the number of repetitions of the experiments at the center point. For statistical calculation, the variables X<sub>i</sub> have been coded as x<sub>i</sub> according to the following transformation:

Figure 3

\[
x_i = \frac{X_i - X_0}{\delta X}
\]

where x<sub>i</sub> is dimensionless coded value of the variable X<sub>i</sub>, X the value of the X<sub>i</sub> at the center point, and \(\delta X\) is the step change. A 2<sup>k</sup>-factorial design with eight axial points and six replicates at the center point with a total number of 30 experiments was employed for optimizing the medium components.

The behavior of the system was explained by the following quadratic equation:

Figure 4

\[
Y = \beta_0 + \sum \beta_i x_i + \sum \beta_{ii} x_i^2 + \sum \beta_{ij} x_i x_j
\]

where Y is the predicted response, \(\beta_0\) the intercept term, \(\beta_i\) the linear effect, \(\beta_{ii}\) the squared effect, and \(\beta_{ij}\) is the interaction effect. The regression equation was optimized for maximum value to obtain the optimum conditions using Design Expert Version 7.1.5 (State Ease, Minneapolis, MN).

VALIDATION OF THE EXPERIMENTAL MODEL

The statistical model was validated with respect to lipase under the conditions predicted by the model in shake flask conditions. Samples were withdrawn at the desired intervals and lipase assay was determined as described above.

PURIFICATION OF THE LIPASE

Purification of the lipase from Serratia marcescens SB08 was done by ammonium sulphate precipitation followed by dialysis. The enzyme from the cell free supernatant was precipitated by ammonium sulphate at 80% saturation and kept overnight at 4°C. The precipitate was collected by centrifugation at 15,000 rpm for 15 min and was dissolved in a minimum quantity of 0.2M phosphate buffer at pH 7.5. The solution was dialyzed against the same buffer overnight at 4°C. The dialyzed enzyme was used for further studies.

DETERMINATION OF MOLECULAR WEIGHT BY SDS PAGE

The homogeneity of the dialysate was checked by Sodium Dodecyl Sulphate Poly Acrylamide Gel Electrophoresis (SDS PAGE). SDS PAGE was performed as described by Laemmli (1970). Ten percent acrylamide gel was prepared according to standard procedure and the gel was run at 60 volts till the dye crossed the stacking gel margin and then increased to 140 volts till the end. The unit was switched off when the dye front reached the bottom of the running gel. The gel was then transferred to distilled water and then to Coomassie Brilliant Blue staining solution. The staining was carried out for 2 hrs followed by the transfer of gel to the destaining solution. Destaining was carried out until the bands became clear and the background completely destained.
Molecular weights of the enzymes were determined by interpolation from a linear semi-logarithmic plot of relative molecular weight versus the Rf value (relative mobility) using standard molecular weight markers.

RESULTS

PLACKETT – BURMAN DESIGN

The influence of eleven medium factors namely pH, temperature, agitation, inoculum concentration, incubation time, sucrose, peptone, KH$_2$PO$_4$, yeast extract, NaCl and CaCl$_2$ in the production of lipase was investigated in 12 runs using Plackett – Burman design. Table 1 represents the Plackett–Burman design for 11 selected variables and the corresponding response for lipase production. Variations ranging from 41.46 to 236.09 U / mL in the production of lipase in the 12 trials were observed by Plackett – Burman design.

The Pareto chart illustrates the order of significance of the variables affecting lipase production (figure 1). Among the variables screened, the most effective factors with high significance level indicated by Pareto chart were in the order of CaCl$_2$, incubation time, pH and yeast extract. They were identified as most significant variables in lipase production and selected for further optimization while temperature, agitation, inoculum concentration, sucrose, peptone, KH$_2$PO$_4$, and NaCl which exhibited less significance level were omitted in further experiments.

Statistical analysis of the Plackett – Burman design demonstrates that the model F value of 0.87 is significant. The values of p < 0.05 indicate model terms are significant (Table 2).

Regression analysis was performed on the results and first order polynomial equation was derived representing lipase production as a function of the independent variables.

Lipase = 99.00 + 10.83 A + 17.83 E + 7.67 J + 18.33 L

The magnitude of the effects indicates the level of the significance of the variable on lipase production. Consequently, based on the results from this experiment, statistically significant variables i.e. CaCl$_2$, incubation time, pH and yeast extract with positive effect were further investigated with central composite design to find the optimal range of these variables.

CENTRAL COMPOSITE DESIGN

Based on Plackett – Burman design CaCl$_2$, incubation time, pH and yeast extract were selected for further optimization using response surface methodology. To examine the combined effect of these factors, a central composite design (CCD) was employed within a range of -2 to +2 in relation to production of lipase. The results obtained from central composite design are given in Table 3.

The results obtained from the central composite design were
fitted to a second order polynomial equation to explain the dependence of lipase production on the medium components.

\[ Y = 176.17 + 3.75 A + 8.08 B + 3.08 D + 3.63 AB + 8.25 AC + 0.38 AD + 13.12 BC - 1.00 BD + 13.38 CD -1.71 A^2 - 25.08 B^2 - 35.46 C^2 - 11.83 D^2 \]

where \( Y \) is the predicted response of lipase production, \( A, B \), \( C \) and \( D \) are the coded values of pH, incubation time, yeast extract and \( \text{CaCl}_2 \) respectively.

**Figure 8**

Table – 3 Experimental plan for optimization of lipase production using central composite design

**Figure 9**

Table – 4 ANOVA for the experimental results of the central composite design (quadratic model)

The analysis of variance of the quadratic regression model suggests that the model is very significant as was evident from the Fisher’s F – test (Table 4). The model’s goodness of fit was checked by determination coefficient (R2). In this case, the value of R2 value (0.915) (multiple correlation coefficient) closer to 1 denotes better correlation between the observed and predicted responses. The coefficient of variation (CV) indicates the degree of precision with which the experiments are compared. The lower reliability of the experiment is usually indicated by high value of CV. In the present case a low CV (3.48) denotes that the experiments performed are highly reliable. The p values denotes the significance of the coefficients and also important in understanding the pattern of the mutual interactions between the variables.

The fitted response for the above regression model was plotted in figure 2. 3D graphs were generated for the pair wise combination of four factors for lipase production. Graphs highlight the roles played by various factors affecting the production of lipase. The 3D response surface plots described by the regression model were drawn to illustrate the effects of the independent variables and combined effects of each independent variable upon the response variable.

**VALIDATION OF THE MODEL**

The maximum experimental response for lipase production was 241.06 U/mL whereas the predicted value was 251.83 U/mL indicating a strong agreement between them. The optimum values of the tested variables are pH 7.0, incubation time 51 h, yeast extract 3.0 g/L and \( \text{CaCl}_2 \) 0.13 g/L as shown in perturbation graph (Figure 3). The model was also validated by repeating the experiments under the
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optimized conditions, which resulted in the lipase production of 243.91 U/mL (Predicted response 251.83 U/mL), thus proving the validity of the model.

To optimize industrial conditions for lipase production, scale-up study was carried out in a jar fermentor by using medium under optimum conditions. The maximum production of 262.91 U/mL lipase was achieved. The results are encouraging for optimization under pilot scale or industrial scale conditions

**Figure 10**

Figure – 2 Three dimensional response surface plot for the effect of (A) pH, yeast extract; (B) incubation time, yeast extract; (C) yeast extract, CaCl on lipase production by SB08

**Figure 11**

**Figure 12**

MOLECULAR WEIGHT OF LIPASE

The partial purification of the lipase crude extract that was affected by the ammonium sulfate (80%) precipitation showed that most of the enzyme activity was preserved in the precipitate. SDS-PAGE showed that the enzyme is one band with electrophoretic mobility of 0.48. By using different standard proteins with known molecular weights, it was discovered that the apparent molecular weight of Serratia marcescens SB08 lipase was 52 KDa.

**DISCUSSION**

Enormous interest on lipase production has been evolved due to its widespread applications in oleochemical, detergent, food processing and in fine chemical manufacturing industries (Macrae and Hammond, 1985; Pandey et al., 1999). 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.
(Montesinos et al., 1995). Lipase synthesis was found to increase in the presence of organic nitrogen sources (Fadiloglu and Erkmen, 2002). Yeast extract was found to be very effective for the production of lipase in Cryptococcus sp S2 (Kamini et al., 2000).

Lipase production by Serratia marcescens SB08 were subjected to response surface methodology and found pH, incubation time, yeast extract and CaCl₂ as positive factors. Incubation time has a significant role in enzyme production. Extended period of incubation might lead to the decomposition of enzyme due to interaction with other components in the media (Ramesh and Lonsane, 1987). Yeast extract is the key nutrient material which controls the biosynthesis of this enzyme. This fact has also been suggested previously during other enzyme production experiments on nitrogen repression effects (Cruegar and Cruegar, 1984; Frankena et al., 1986; Kole et al., 1988; Giesecke et al., 1991). The Ca²⁺ ions are important for enzyme activity, for its thermal stability (Bosh and Das, 1996; Egas et al., 1998).

In this study, with optimum factors designed by statistical methods, the maximum production of 241.06 U/mL was observed in Serratia marcescens SB08. This study demonstrates the presence of lipase in Serratia marcescens SB08 and reveals the organism can produce optimum quantity of this enzyme.

ACKNOWLEDGEMENTS

The authors are thankful to Bharathiar University, Coimbatore, Tamil Nadu, India for providing the infrastructure facilities for this study.

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Statistical optimization of medium components for the production of lipase by Serratia marcescens SB08 as positive factors.


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