Application of Statistical Experimental Designs for the Optimization of Medium Constituents for the Production of L-Asparaginase by *Serratia Marcescens*

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Abstract

Statistical experimental designs were applied for the optimization of medium constituents for L-Asparaginase production by *Serratia marcescens* NCIM 2919 in solid state fermentation (SSF) using sesame oil cake (SOC) as the sole substrate. Using Plackett–Burman design (PBD), moisture content of substrate, glucose and NaNO3 were identified as significant variables which highly influenced L-Asparaginase production and these variables were subsequently optimized using a Doehlert experimental design (DD). Besides reducing the number of experiments required for optimization, this technique allowed to quantify the amount of L-Asparaginase in any part of the experimental domain. The optimum conditions were found to be moisture content of the substrate 68.64 (%), glucose 3.093 (%w/w) and NaNO3 1.013 (%w/w). L-Asparaginase activity at these optimum conditions was 110.705 U/g ds (Units/g of dried sesame oil cake waste as substrate).

Keywords: L-Asparaginase; Sesame oil cake; Optimization; Plackett–Burman design; Doehlert experimental design; *Serratia marcescens*

Introduction

The field of cancer research has some good examples of the use of therapeutics. Recent studies show that PEGylated enzyme, Oncaspar (pegasparagase), already in use in the clinic has shown better results for the treatment of children with newly diagnosed standard-risk acute lymphoblastic leukemia (Vellard, 2003). L-Asparaginase is an effective antineoplastic agent, used in acute lymphoblastic leukemia chemotherapy (Azmi et al., 2007). The discovery of high L-Asparaginase activity with serum of guinea pig is turned out to be important for the development of L-Asparaginase as a potential antineoplastic agent. Intense interest in asparaginase has resulted since the demonstration that L-Asparaginase from *E.coli* has antitumor activity. Studies on the mechanism of antineoplastic action of this enzyme suggest that a number of tumor cells responding to it lack adequate L-asparagine synthetase activity and require an exogenous supply of L-asparagine. Upon the depletion of this amino acid by asparaginase, *in vitro* or *in vivo*, these tumor cells die.

Although L-Asparaginase has been found in various plant and animal species, due to the difficult extraction procedure of this enzyme, other potential sources like microorganisms were searched. Microorganisms like fungi and bacteria have proved to be very efficient variables, followed by estimation of optimal levels of these variables. Plackett–Burman design (Plackett and Burman, 1946) is one design which is well established and widely used statistical technique for

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screening of medium constituents (Sarat et al., 2010). Following the initial screening, the next step is optimizing the variables selected through Plackett–Burman design. The traditional ‘one-factor at a time’ technique used for optimizing a multivariable system is not only time consuming but also often easily misses the alternative effects between components. Also, this method requires to carry out a number of experiments to determine the optimum levels when the interactions are significant. These drawbacks of single factor optimization process can be eliminated by optimizing all the affecting parameters collectively by Doehlert experimental design (Doehlert, 1970) using response surface methodology (RSM). Recently, many statistical experimental design methods have been employed in bioprocess optimization. Among them, Doehlert designs (Ferreira et al., 2004) offer the following advantages over the other designs. This method has successfully been applied to optimize different process parameters (Ismail et al., 1999; Carvalheiro et al., 1999; Vanot et al., 2002; Dutra et al., 2006). Basically, this optimization process involves three major steps: performing the statistically designed experiments, estimating the coefficients in a mathematical model, predicting its response and checking the adequacy of the model. Several researchers in biotechnology have applied these techniques for optimization of different parameters (Francis et al., 2003; Bandaru et al., 2006; Singh and Satyanarayana, 2006; Sita and Narasimha, 2010). Hence, the present study reports the application of the Plackett–Burman design and Doehlert experimental design to optimize L-Asparaginase production from sesame oil cake by SSF using Serratia marcescens NCIM 2919.

Materials and Methods

Substrate

Sesame (Sesamum indicum) oil cake (SOC), procured from a local oil extracting unit (Jeevan Enterprises) of Vizianagaram, Andhra Pradesh, India was used as the substrate. It was dried at 60°C for 72h to reduce the moisture content to around to 5% and ground to a desired size.

Microorganism

S. marcescens NCIM 2919 obtained from National Chemical Laboratory, Pune, India was used throughout the study.

Growth conditions

The culture was maintained on nutrient agar medium slants having the composition (%): beef extract 1.0, NaCl 0.5, peptone 1.0, agar agar 2.0. The pH of the medium was adjusted to 7.0–7.5 and culture was incubated at 30°C for 48h. Sub-culturing was carried out once in every 2 weeks and the culture was stored at 4°C.

Inoculum preparation

The Serratia strain was cultivated in a medium containing trypton 15g, soy bean meal 5g and sodium chloride 5 g/l of distilled water. The cells were cultivated in this medium at 30°C on a shaker at 180 rpm for 24h (Abdel-Fattah and Olama, 2002).

Media preparation

Ten grams of substrate was weighed into a 250 ml Erlenmeyer flask and to this a supplemental salt solution was added to the desired moisture level. The composition of the salt solution was as follows (% w/w): MgSO4·7 H2O 0.05; FeSO4·7 H2O 0.00 1; KCl 0.05; and K2HPO4 0.1. NaNO3 and glucose were taken as nitrogen and carbon sources having the composition (%): beef extract 1.0, NaCl 0.5, peptone 1.0, glucose 2.0. The pH of the medium was adjusted to 7.0–7.5 and culture was incubated at 30°C for 48h. Sub-culturing was carried out once in every 2 weeks and the culture was stored at 4°C.

Solid state fermentation

The sterilized substrate including media as shown in the above Section 2.5 was inoculated with 2 ml of inoculum. The contents were mixed thoroughly and incubated in a slanting position at 37°C. All the experiments were carried out in duplicate and samples were collected after 4 days of incubation.

Extraction of enzyme and assay method

The crude enzyme from the fermented material was recovered by simple extraction method. For this, the fermented substrate was mixed thoroughly with 100 ml of 0.01 M sodium phosphate buffer (pH 7.0) and the contents were agitated for 1h at room temperature in a rotary shaker at 150 rpm. At the end of extraction, the liquid was filtered off through Whatman No.1 filter paper and the resulting clear filtrate was used for enzyme assay. L-Asparaginase activity was assayed by the method Nesslerization (Geckil and Gencer, 2004). A standard curve was prepared with ammonium sulphate. One L-Asparaginase unit of activity (IU) was defined as the amount of enzyme that liberated 1μ mole of ammonia per gram of dry substrate per min under optimal assay conditions. (Mishra, 2006). The effect of each variable on L-Asparaginase production was calculated by using the following equation:

$$E_{i} = \frac{\Sigma Y_{i} - \Sigma Y_{i}}{L/2}$$

where, $E_{i}$ is the effect of levels of the tested variables, $\Sigma Y_{+1}$, and $\Sigma Y_{-1}$, are the L-Asparaginase production from the experimental runs in which the variables being tested are added to the medium at their maximum and minimum levels respectively and $L$ is the number of experiments carried out. When the value of concentration effect $(E_{i})$ of the tested variable is positive, the influence of the variable is greater at high concentration, and when it is negative, the influence of the variable is greater at low concentration.

Doehlert experimental design: Doehlert experimental design (Doehlert, 1970) was used in the optimization of L-Asparaginase production. The Plackett–Burman (Plackett and Burman, 1946) statistical experimental design is very useful for screening the most important variables and does not consider the interaction effects between the variables. The total number of experiments to be carried out is $K + 1$, where $K$ is the number of variables. Each variable is represented by two levels, high and low denoted by (+) and (–), respectively. The statistical software package (Stat-Ease Inc., Minneapolis, MN, USA) was used for analyzing the experimental data.

The purpose of the first optimization step was to identify which ingredients of the medium have a significant effect on L-Asparaginase production. The Plackett–Burman designed experiment (Doehlert, 1970) was used in the optimization of L-Asparaginase production. The moisture level of substrate ($X_{1}$), %, glucose ($X_{2}$, %/w/v), and NaNO3 ($X_{3}$, %/w/v) of fermentation were chosen as the independent input variables, amount of L-Asparaginase (Y, U/gds) was used as the dependent output variable and were shown in Table 3 and Table 4. For statistical calculations the variables Xi were coded as xi according to Eq. (2)

$$x_{i} = \frac{X_{i} - X_{o}}{\Delta X_{i}}$$

where, $X_{i}$ is the coded value of the $i$th factor, $X_{o}$ the natural value, $X_{o}$ the value at the center point, $\Delta X_{i}$ the step change value, and $z_{i}$ is the maximum value of the coded factor (i.e. 1.0, 0.866 and 0.816 for five levels, seven levels and three levels, respectively).
A three variable Doehlert experimental design involving a total number of 15 experiments including three replicates at the center point (Table 4) was employed for the optimization of the parameters. The model terms with highest are chosen as the independent input variables and L-Asparaginase activity was used as the dependent output variable. A DD was employed to analyze the interactive effect of these parameters and to arrive at an optimum. The three variables which influence the fermentative production of L-Asparaginase highly were moisture content of substrate, glucose and NaNO₃. Out of them glucose had shown stronger effect on L-Asparaginase production and hence it was assigned seven levels (Ferreira et al., 2004).

Experiments were carried out as per the design, and the L-Asparaginase obtained after 4 days fermentation with 15 experiments of different combinations of moisture content, glucose and NaNO₃ was estimated (Table 3 and Table 4). By applying multiple regression analysis on the experimental data, the following second degree polynomials (Eq. (3)) were calculated with the statistical package (Stat-Ease Inc., Minneapolis, MN, USA) to estimate the response of the dependent variable.

\[
Y = b_0 + b_1X_1 + b_2X_2 + b_3X_3 + b_{12}X_1X_2 + b_{13}X_1X_3 + b_{23}X_2X_3 + b_{123}X_1X_2X_3
\]  

where \(Y\) is predicted response, \(X_1, X_2, X_3\) are independent variables, \(b_0\) is offset term, \(b_1, b_2, b_3\) are linear effects, \(b_{12}, b_{23}, b_{13}\) are squared effects and \(b_{123}\) are interaction terms.

### Results and Discussion

Identification of important medium constituents using Plackett–Burman design

A total of seven medium components were screened in eight experimental runs and the corresponding Plackett–Burman experimental design matrix for screening of important variables for L-Asparaginase production were shown in Table 1. The resulting effects (Eᵢ) of the variables on the responses, the associated \(t\)-values and significant levels were shown in Table 2. A \(p\)-value less than 0.05 for the three variables viz., moisture content of substrate \(X_1\), glucose \(X_2\) and NaNO₃ \(X_3\) indicates that they are significant. The same was confirmed from the pareto graph (Figure 1) which was also used to show the effect of all variables on L-Asparaginase production. These variables had confidence level above 95% in comparison to other variables and thus were considered to be significant for L-Asparaginase production. These significant variables were further optimized by DD involving RSM.

### Optimization of the selected medium constituents using doehlert experimental design

Based on the results of the Plackett–Burman design, moisture content of substrate, glucose and NaNO₃ were chosen as the independent input variables and L-Asparaginase activity was used as the dependent output variable. A DD was employed to analyze the interactive effect of these parameters and to arrive at an optimum. These variables had confidence level above 95% in comparison to other variables and thus were considered to be significant for L-Asparaginase production. These significant variables were further optimized by DD involving RSM.

### Table 1: Plackett–Burman experimental design matrix for screening of important variables for L-Asparaginase production.

| Run no. | Levels | Glucose (%w/w) | Maltose (%w/w) | (NH₄)₂SO₄ (%w/w) | Urea (%w/w) | NaNO₃ (%w/w) | Casein (%w/w) | Moisture content (%) | L-Asparaginase activity (U/gds) |
|---------|--------|----------------|----------------|-----------------|-------------|--------------|---------------|---------------------|-----------------|
| 1       | +      | 5              | 5              | 2               | 2           | 2            | +             | 80                  | 71.29           |
| 2       | +      | 5              | 5              | 2               | 2           | +            | −             | 30                  | 84.56           |
| 3       | +      | 5              | −              | 2               | +           | −            | −             | +                   | 76.32           |
| 4       | −      | +              | −              | 2               | −           | +            | −             | −                   | 80.19           |
| 5       | −      | +              | +              | 2               | +           | −            | +             | −                   | 78.92           |
| 6       | +      | −              | +              | −               | +           | −            | +             | −                   | 82.19           |
| 7       | +      | +              | +              | +               | +           | +            | +             | −                   | 76.56           |
| 8       | +      | +              | +              | +               | +           | +            | +             | −                   | 82.16           |

| Variables | Effect (b) | %| P-value |
|-----------|------------|---|---------|
| Glucose   | 6.50250    | 41.727 | 0.015254* |
| Maltose   | −0.43250   | −2.775 | 0.220161 |
| (NH₄)₂SO₄ | 1.86750    | 11.984 | 0.053000 |
| Urea      | −1.76750   | −11.342 | 0.055983* |
| NaNO₃     | −2.06750   | −13.267 | 0.047893* |
| Casein    | −0.76250   | −4.893  | 0.128340 |
| Moisture content | 2.93250 | 18.818 | 0.033798* |

*Significant at p ≤ 0.05, Standard error=0.155833

### Table 2: Effects for L-Asparaginase production from the results of Plackett–Burman design.

| Factors                        | Range and levels |
|--------------------------------|------------------|
| Coded variable, \(X_1\)        | −1               |
| Moisture content, \(X_1\) (%)  | 0.5              |
| Coded variable, \(X_2\) (%)    | 0.5              |
| Glucose, \(X_2\) (%w/w)        | 1.67             |
| Coded variable, \(X_3\) (%)    | 0.5              |
| NaNO₃, \(X_3\) (%w/w)          | 1.0              |

### Table 3: Experimental range and levels of the factors.

### Figure 1: Pareto graph showing effect of various variables on L-Asparaginase production based on the observations of Plackett Burman design.
order polynomial equation was found to represent the L-Asparaginase production adequately.

\[ Y = -136.651 + 4.720X_1 + 23.730X_2 + 92.030X_3 - 0.034X_1^2 - 3.089X_2^2 - 48.273X_3^2 - 0.339X_1X_2 - 0.534X_1X_3 + 0.109X_2X_3 \] (4)

The coefficients of the regression model (Eq. (4)) calculated are listed in Table 5, which contain three linear, three quadratic and three interaction terms and one block term. The significance of each coefficient was determined by Student’s t-test and p-values, which are listed in Table 5. The larger the magnitude of the t-value and smaller the p-value, the more significant is the corresponding coefficient (Akhazanrova and Kafarov, 1982; Khuri and Cornell, 1987). This implies that the first order and second order main effects of moisture content of substrate, glucose and NaNO_3 are highly significant as is evident from their respective p-values. They are more significant at the second order. This indicates that they can act as limiting nutrients and small variations in their concentration will alter either growth rate or product formation rate or both to a considerable extent. All the interaction effects were found to be insignificant (p > 0.05). The parity plot (Figure 2) showed a satisfactory correlation between the experimental and predicted values (obtained from Eq. (4)) of L-Asparaginase, wherein, the points cluster around the diagonal line which indicates the good fit of the model, since the deviation between the experimental and predicted values was minimal.

The results of the second order response surface model fitting in the form of ANOVA were given in Table 6. It is required to test the significance and adequacy of the model. The Fisher variance ratio, the F-value (= S_p^2 / S_e^2), is a statistically valid measure of how well the factors describe the variation in the data about its mean. The greater the F-value is from unity, the more certain it is that the factors explain adequately the variation in the data about its mean, and the estimated factor effects are real. The analysis of variance of the regression model demonstrates that the model is highly significant, as is evident from the Fisher’s F-test (F_{model}=32.55999) and a very low probability value (p_{model}>F=0.000651). Moreover, the computed F-value (F_{0.01}=32.55999) is greater than the Table F-value at the 1% level, indicating that the treatment differences are significant.

The goodness of the fit of the model was checked by the determination coefficient (R^2). The R^2 value provides a measure of how
much variability in the observed response values can be explained by the experimental factors and their interactions. The \( R^2 \) value is always between 0 and 1. The closer the \( R^2 \) value is to 1, the stronger the model is and the better it predicts the response (Cochran and Cox, 1957). In this case, the value of the determination coefficient (\( R^2 = 0.98322 \)) indicates that 98.32% of the variability in the response could be explained by the model. The \( R^2 \) value also indicates that only 1.678% of the variation is not explained by the model. In addition, the value of the adjusted determination coefficient (Adj \( R^2 = 0.95302 \)) is also very high to advocate for a high significance of the model. Also a higher value of the correlation coefficient (\( R = 0.9915 \)) justifies an excellent correlation between the independent variables (Box and Hunter, 1978). The predicted optimum levels of moisture content of substrate, glucose and NaNO\(_3\) were obtained by applying the regression analysis to the Eq. (4).

Figures 3–5 represent the response surface and contour plots for the optimization of medium constituents of L-Asparaginase production. The effect of the moisture content and glucose on the L-Asparaginase production was shown in Figure 3. An increase in moisture content with glucose up to the optimum point, increased the L-Asparaginase production to a maximum level, but with a further increase in the moisture content with glucose, the trend is reversed. The interaction effect of moisture content and NaNO\(_3\) on L-Asparaginase production in Figure 4 clearly indicates a proper combination for production of L-Asparaginase. An increase in the NaNO\(_3\) with moisture content increased the L-Asparaginase production gradually, but at a higher NaNO\(_3\) and moisture content, the trend is reversed. The optimum for maximum L-Asparaginase production lies near the center point of the NaNO\(_3\) and moisture content. A similar effect on the response was observed for glucose at any level of NaNO\(_3\). An increase in glucose with NaNO\(_3\) up to the optimum point increased the L-Asparaginase production to maximum level, but a further increase in the glucose with NaNO\(_3\) decreased the L-Asparaginase production as shown in Figure 5. Therefore, an optimum was observed near the central value of glucose, moisture content and NaNO\(_3\). The optimum medium constituents for higher L-Asparaginase production can be attained at the concentration of 68.64% of moisture content, 3.093 (%w/w) of glucose and 1.013 (%w/w) of NaNO\(_3\). At these optimum medium concentrations, maximum L-Asparaginase activity 110.795 U/gds was obtained.

**Conclusion**

The present study involved the use of statistical experimental designs to optimize medium constituents of the fermentation medium for the production of L-Asparaginase from sesame oil cake by SSF using *S. marcescens* NCIM 2919. Three variables: moisture content of substrate, glucose and NaNO\(_3\) were identified as significant by Plackett–Burman design for L-Asparaginase production. These variables were further optimized using Doehlert experimental design involving RSM. Among the three variables tested for the correlation between their concentrations and the production of L-Asparaginase,
all the three variables showed significant influence on the enzyme production. The significant interactions between the three variables were also observed from the contour plots. The maximum amount of L-Asparaginase produced from sesame oil cake was predicted to be 110.795 U/gds (Units/g of dried sesame oil cake) when the optimized medium constituents of the fermentation medium was set as follows: moisture content of substrate 68.64 (%), glucose 3.093 (% w/w) and NaNO₃ 1.013 (% w/w). Applying statistical experimental designs to optimize the selected factors for maximal production is an efficient method that tests the effect of factors interaction with minimum number of experiments. The methodology as a whole proved to be adequate for the design and optimization of the bioprocess for obtaining a therapeutically valuable product like L-Asparaginase from an abundantly available, low grade agro-industrial waste like sesame oil cake. Thus, it is useful to advise the microbial industry sponsors to apply such experimental designs to maintain high efficiency and economic bioprocess.

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