Simplex Lattice Mixture Design Approach for the Production of L-Glutaminase Using Oil Cake Mixtures

V. Sameera & K. Jaya Raju

1M.Tech Biotechnology, Department of Chemical Engineering, Centre for Biotechnology, Andhra University, Visakhapatnam.
2Professor, Department of Chemical Engineering, Centre for Biotechnology, Andhra University, Visakhapatnam

Abstract: L-glutaminase production from mixed substrate using Aspergillus wentii MTCC 1901 under solid state fermentation was reported. Two potential substrates, coconut oil cake and sesame oil cake were screened and mixed in different compositions according to simplex lattice mixture design using Design expert v.9.0.5.1. This study revealed the significance of mixed substrate compared to individual substrate in the production of L-glutaminase. Multiple linear regression analysis indicated that cubic model (low p value-0.0001, high F value- 3165.46 and R²-0.9999) was more significant compared to other models. Maximum of 123U/gds of L-glutaminase yield was obtained using Coconut oil cake and Sesame oil cake when mixed in the ratio of 1.25:3.75.

1. Introduction

Microbial enzymes are routinely used in many industrial sectors, as they are economic and environmental friendly. L-glutaminase is one among the several enzymes produced using microorganisms. The enzyme L-glutaminase (EC 3.5.1.2) is an amidohydrolase enzyme which generates L-glutamic acid and ammonia from L-glutamine [1]. This cellular enzyme deaminates L-glutamine and acts as a proteolytic endopeptidase, which hydrolyses the peptide bonds present in the interior of protein molecules.

In recent years L-glutaminase has attracted much attention with respect to proposed applications in pharmaceuticals as antileukemic agent [2] and in food industry as flavor enhancing agent [3]. Mixed substrate fermentation has been more advantageous for the production of enzymes than single substrate fermentation [4,5]. Mixed substrate fermentation was done experimentally using mixture designs which are a type of factorial designs that are used to find the best composition when there is a mixture of components.

The design of mixture experiments configures a special case in response surface methodologies using mathematical and statistical techniques [6,7]. The experimental designs largely follow from Scheffe’s simplex lattice and simplex centroid designs. The simplex is simply the projection of an n-dimensional space onto an n-1 dimensional coordinate system. Both the simplex-lattice and simplex-centroid may be generalized to any number of species [7].

In mixture experiments, the measured response is assumed to depend only on the relative proportions of the ingredients or components in the mixture and not on the amount of the mixture. The main distinction between mixture experiments and independent variable experiments is that with the former, the input variables or components are non-negative proportionate amounts of the mixture, and if expressed as fractions of the mixture, they must sum to one [8].

The designs require large numbers of design points. For example, a two component simplex-lattice design of degree two requires 5 design points. In mixture problems, the purpose of the experiment is to model the blending surface with some form of mathematical equation and to find the best mixture with respect to a well-defined response variable and an optimality criterion on it. The general equation is

\[
E(y) = \beta \cdot \beta^T + \sum_{i=1}^{p} \beta_i \cdot x_i, 0 \leq x_i \leq 1, \sum_{i=1}^{p} x_i = 1
\]

Eq 1

The usual assumptions made for regression models in general are also made for mixture experiments. While in usual factorial designs often ANOVA models are in use in mixture design, regression analysis is mainly used; especially linear, quadratic or cubic response surfaces are assumed in dependence on the mixture components. In a mixture experiment, the factors are different components of a blend.

Planning a mixture experiment typically involves selection of the mixture components, identifying any constraints on the mixture...
components to specify the experimental region, identifying the response variable to be measured, defining an optimality criterion for the construction of the design, proposing an appropriate model for modelling the response data as functions of the mixture components and select an experimental design.

Let \( p \) be the number of factors occurring in a mixture design. The unrestricted design region for mixture proportions is a simplex, a regularly sided figure of dimension \( p-1 \) with \( p \) vertices. For example, with two factors, the simplex is the line segment from \((0, 1)\) to \((1, 0)\).

Technically, there are no interaction terms in a Scheffe mixture model. However, there are terms that look like interaction terms. For example, in a 2-component mixture the term that looks like an interaction term, \( AB \) for the quadratic model and \( AB(A-B) \) for the cubic model.

The equation for a Cubic model is:

\[
E(y) = \sum_{i=1}^{p} \beta_i x_i + \sum_{i<j} \beta_{ij} x_i x_j + \sum_{i<j<k} \beta_{ijk} x_i x_j x_k
\]

From a geometric perspective each term in the model contributes to the shape of the response surface. A 2-component cubic mixture model has 4 model terms – linear (A and B), quadratic (AB), and cubic (AB (A-B)).

The objective of this work was to determine the best composition of the substrates to get high yield. Oil cakes rich in fibre, protein and energy content were used as substrate. They offer potential benefits in developing bioprocesses for the production of industrial enzymes. The bioprocess utilizing oil cakes has been attractive due to relatively cheaper availability of the oil cakes throughout the year making it favourable when economics is considered [4]. In the present study two types of oil cakes were mixed in different proportions according to the simplex lattice mixture design to produce L-glutaminase using Aspergillus wentii MTCC 1901.

2. Experimental procedure

The substrates used were two different cheap agro residues namely Coconut oil cake (COC) and Sesame oil cake (SOC). The cakes were sun dried and ground to fine powder. The powder was sieved and used in the study. Aspergillus wentii MTCC 1901 obtained from Microbial Type Culture Collection, Chandigarh, India was used. The culture was maintained on PDA agar slants. 39gm of PDA medium and 0.1gm agar-agar were weighed in 1000ml distilled water and used as growth medium. The culture was incubated at 28°C for 120h. Sub culturing was carried out once in every 15 days and the culture was stored at 4°C. Inoculum preparation was done by scrapping the spores of the fungi, Aspergillus wentii using Tween 80 solution.

A mixture augmented-simplex lattice design with two components (COC and SOC) was used. A total of nine experiments with four replicates were generated using Design Expert statistical software (v.9.0.5.1). The points chosen were the pure components, the center point, and check blends (to augment the design). All data collected according to the simplex lattice design were analyzed using the software [9]. In this design, 2 factors were evaluated by changing their weights simultaneously and keeping their total weight constant. The range was fixed from 0 to 5 (actual values) and the composition sums up to 5. Substrates were weighed separately, moistened with 2ml of moistening medium (distilled water used here) and were autoclaved at 121°C (15lb) for 20 min, cooled to room temperature and then inoculated with 2ml of inoculum. The inoculated flasks were incubated at 28°C in an incubator for 120h. After fermentation, extraction for crude enzyme was done using phosphate buffer of pH 8. The enzyme assay was carried out for the crude enzyme after filtration for the enzyme activity and enzyme yield by following the method used by Imada et al., 1973 [10]. The activity of L-glutaminase was determined by estimating the amount of ammonia liberated during the assay process.

3. Results and discussion

Table 1 presents the varying compositions of the solid substrates used during fermentation process and the amount of L-glutaminase yield for each experiment. The glutaminase production yield varied from 47.83 to 123U/gds. This variation in glutaminase yield with different substrates under similar fermentation conditions suggested the importance of substrate composition on fermentative glutaminase production. The low fraction limit (0.25) for Coconut oil cake (thus the high limit for Sesame oil cake in the mixture was 0.75) was determined to be the best composition for high yield of the enzyme.
Table 1: Simplex lattice mixture design experimental layout and L-glutaminase yield

<table>
<thead>
<tr>
<th>Run</th>
<th>Space Type</th>
<th>Component 1 A: coc gm</th>
<th>Component 2 B: soc gm</th>
<th>Response L-glutaminase Yield U/gds</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Coded</td>
<td>Real</td>
<td>Coded</td>
</tr>
<tr>
<td>1</td>
<td>Vertex</td>
<td>1</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>Vertex</td>
<td>1</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>AxialCB</td>
<td>0.75</td>
<td>3.75</td>
<td>0.25</td>
</tr>
<tr>
<td>4</td>
<td>Vertex</td>
<td>0</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>5</td>
<td>Vertex</td>
<td>0</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>6</td>
<td>Center</td>
<td>0.5</td>
<td>2.5</td>
<td>0.5</td>
</tr>
<tr>
<td>7</td>
<td>Vertex</td>
<td>0</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>8</td>
<td>AxialCB</td>
<td>0.25</td>
<td>1.25</td>
<td>0.75</td>
</tr>
<tr>
<td>9</td>
<td>Vertex</td>
<td>1</td>
<td>5</td>
<td>0</td>
</tr>
</tbody>
</table>

Data from the experimental design was further analyzed by employing a multiple-linear regression using L-glutaminase yield as the response. Sequential F-tests, on the linear to cubic solutions were performed for appropriate model selection (based on highest F-statistics significance) suitable for glutaminase production. ANOVA results of all three models are presented in Table 2. The cubic model showed a high F value (3165.46) and a low p value (0.0001). The simplex lattice design for a 2-component system is represented by a line segment when observed and predicted values were plotted as shown in Figure 1.

Table 2: ANOVA for significance of regression models

<table>
<thead>
<tr>
<th>Source</th>
<th>Sequential p-value</th>
<th>F Value</th>
<th>Sum of Squares</th>
<th>df</th>
<th>Mean Square</th>
<th>R-Squared</th>
</tr>
</thead>
<tbody>
<tr>
<td>Linear</td>
<td>0.2831</td>
<td>1.35</td>
<td>5357.59</td>
<td>3</td>
<td>1785.86</td>
<td>0.1618</td>
</tr>
<tr>
<td>Quadratic</td>
<td>0.0002</td>
<td>67.54</td>
<td>437.10</td>
<td>2</td>
<td>218.55</td>
<td>0.9316</td>
</tr>
<tr>
<td>Cubic</td>
<td>&lt; 0.0001</td>
<td>3165.46</td>
<td>0.69</td>
<td>1</td>
<td>0.69</td>
<td>0.9999</td>
</tr>
</tbody>
</table>

Figure 1: Comparison between observed and predicted values

The analysis of $R^2$ value revealed that the cubic model have higher fit ($R^2_{cubic}$ 0.9999). This data suggest that the cubic model is the most significant. The noticed $R^2$ value of the cubic model is 0.9999 indicating that 2 substrate components altogether would explain about 99.99% of the variability in
the response leaving only 0.01% of the variability remaining unexplained. Therefore further data analysis was performed using only cubic model.

In the present study, a good co-relation was identified between predicted and experimental glutaminase production. The empirical relationship between glutaminase production and substrate variables in coded units is obtained by the application of second order model as per the following equation

\[
\text{Yield} = +47.86A + 68.36B + 234.59AB - 164.00AB(A-B)
\]

Eq 3

Where Y is glutaminase production in U/gds response and A, B, AB and AB(A-B) were the model terms.

A reasonable agreement between the predicted R² and adjusted R² values were sought for each analysis as shown in Table 3. The Predicted R-Squared of 0.9989 is in reasonable agreement with the Adjusted R-Squared of 0.9998 i.e. the difference is less than 0.2. A probability plot of the Studentized residuals was checked for normality of residuals. Studentized residuals versus predicted values were verified for constant error. The model was suitable as R² values were close to 1 at the selected level of significance (P < 0.05) and an insignificant lack of fit (P > 0.05). An insignificant LOF value means that extra design points other than the design points used to predict the response fit the model.

<table>
<thead>
<tr>
<th>Source</th>
<th>R-Squared</th>
<th>Adjusted R-Squared</th>
<th>Predicted R-Squared</th>
</tr>
</thead>
<tbody>
<tr>
<td>Linear</td>
<td>0.1618</td>
<td>0.0421</td>
<td>-0.2466</td>
</tr>
<tr>
<td>Quadratic</td>
<td>0.9316</td>
<td>0.9088</td>
<td>0.8577</td>
</tr>
<tr>
<td>Cubic</td>
<td>0.9999</td>
<td>0.9998</td>
<td>0.9989</td>
</tr>
</tbody>
</table>

4. Conclusion

Overall, the present study emphasizes the role of mixed substrate for optimized production of L-glutaminase in mixture design fermentation using oil cakes such as coconut oil cake (COC) and sesame oil cake (SOC) with spores of Aspergillus wentii MTCC 1901 as inoculum under solid state fermentation. An optimum L-glutaminase production of 123U/gds could be obtained with a 1.25:3.75 ratio of COC : SOC respectively. The yield when compared with predicted value showed a linear curve indicating the observed values are in acceptance with experimental values. Further work can be done for optimization of the yield of L-glutaminase with optimum process parameters which affect the rate of enzyme yield.

5. References


