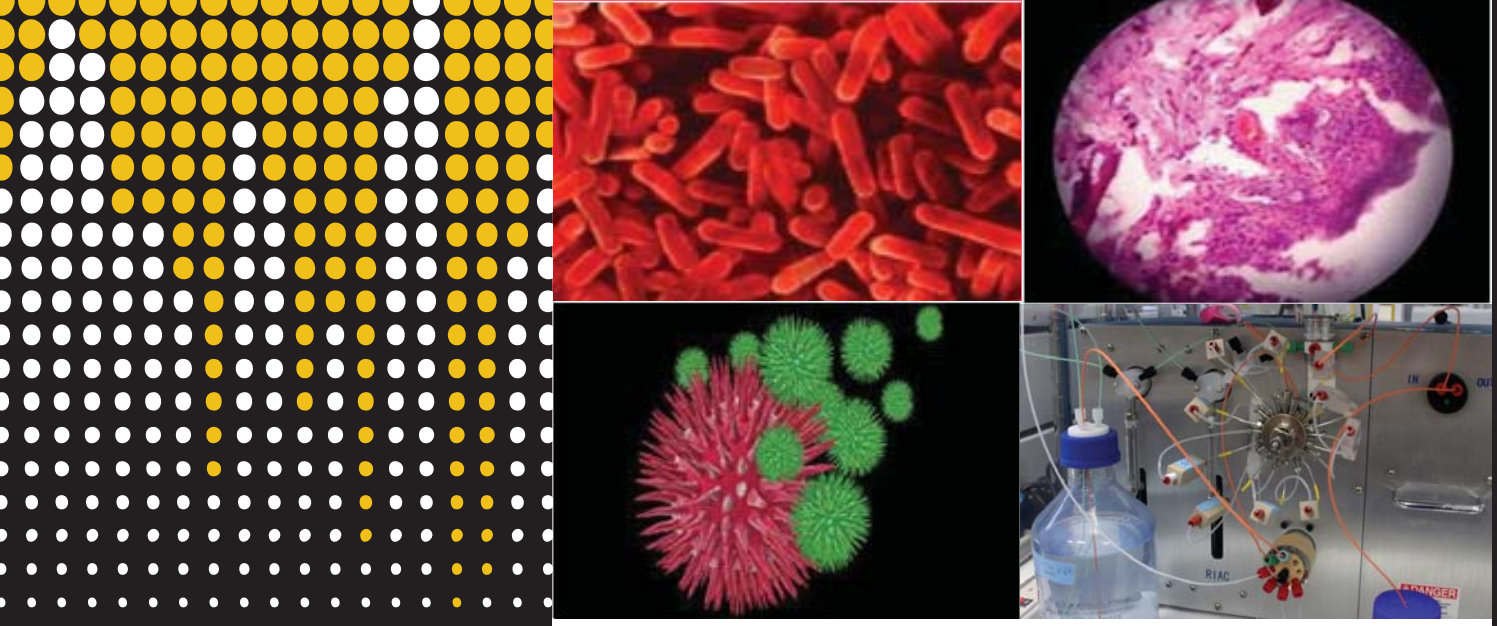

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Microbial & Biochemical Technology

Karanam et al. J Microbial Biochem Technol 2010, 2:1
http://dx.doi.org/10.4172/1948-5948.1000016

Research Article Open Access

Application of Doehlert experimental design for the optimization of medium constituents for the production of L-asparaginase from Palm Kernal cake (*Elaeis guineensis*)

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Abstract

Doehlert experimental design (DD) was applied for the optimization of medium constituents for L-asparaginase production by *Yarrowia lipolytica* NCIM 3472 in solid state fermentation (SSF) using palm kernel cake as the substrate. From the results of preliminary experimental runs, the three variables (glucose, moisture content, L-asparagine) have been identified as the potential variables for the production of L-asparaginase. Fifteen experimental runs designed by DD are carried out and the process response is modeled using a polynomial equation as function of these parameters. The proposed quadratic model for DD fitted very well to the experimental data that it could be used to navigate the design space according to analysis of variance results. The experimental values were in good agreement with predicted values and the correlation coefficient was found to be 0.9988. The optimal set of conditions for maximum L-asparaginase activity was as follows: moisture content of the substrate: 54.8622 (%), glucose concentration: 11.9241 (%w/w) and L-asparagine concentration: 1.0758 (%w/w). L-asparaginase activity at these optimum conditions was 39.8623 U/gds. STATISTICA 6.0 was used for implementing Doehlert experimental design.

Keywords: L-asparaginase; Palm kernel cake; Optimization; Doehlert experimental design; *Yarrowia lipolytica*

Introduction

L-asparaginase (EC.3.5.1.1; asparagine amidohydrolase) is an enzyme that catalyzes the hydrolysis of L-asparagine to L-aspartic acid and ammonia. This enzyme is used for the treatment of selected types of haematopoietic diseases such as acute lymphoblastic leukaemia and non-Hodgkin lymphomas (Ravindranath et al., 1992). It is also using in food industry for the production of acrylamide free food (Pedreschi et al., 2008), model enzyme for the development of new drug delivery system (Teodor et al., 2009) and L-asparagine biosensor for leukemia (Verma et al., 2007). Studies on the molecular structure (Aung et al., 2000), catalysis (Kelo et al., 2002), clinical aspects (Narta et al., 2007), genetic determinants involved in regulation (Hüser et al., 1999) and stabilization to enhance biological half-life (Ó'Fágáin, 2003) of L-asparaginase have been reported. L-asparaginase production pattern was studied in *Escherichia coli*, *Erwinia cartovora*, *Corynebacterium glutamicum*, *Cylindrocarpon obtusisporum*, *Pseudomonas stutzeri*, *Rhodosporodium toruloids*, *Tetrahymena pyriformis*, *Pseudomonas aeuginosa* 50071, *Aspergillus tamari* and *Aspergillus terreus* (Hymavathi et al., 2009).

Recently, several investigations have been published indicating the application of this culture in upgrading the food and industrial wastes and in the production of fine chemicals and enzymes. In SSF, any type of substrate, including industrial wastes, could be used to enhance the production of enzymes because of their richness in fatty acids, triacylglycerols and /or sugars. The use of cheap raw materials would diminish the operating costs of the process. Moreover, total capital investment for L-asparaginase production has been reported to be significantly lower in solid state fermentation than in submerged fermentation (Castilho et al., 2000). The utilization of by-products and wastes from food and industrial sources has several advantages over submerged fermentation such as superior productivity, simple techniques, reduced energy requirements, low wastewater output, improved product recovery and the reduction in production costs (Ashok, 2003).

The global production of Palm Kernel Cake (PKC), a by-product of oil extraction from the nut of the palm tree, *Elaeis guineensis*, is ever increasing due to the tremendous growth of the oil palm industry in many parts of Asia and Africa (PORLA, 2000). World producers of palm kernel include Malaysia, Indonesia, Nigeria, Colombia, India, and Thailand. Palm trees are widely cultivated in the state of Andhra Pradesh, India. Currently, most of the PKC produced in Malaysia is exported at a low price to Europe for use as cattle feed concentrates in dairy cows. PKC has been successfully used as a feed ingredient for ruminants (Siew, 1989; Hassan and Yeong, 1999), and research has shown that it could also be incorporated at low levels (10–30%) in the diets of nonruminant livestock such as poultry (Yeong, 1985; Panigrahi and Powell, 1991) and pigs (Rhule, 1996; Agunbiade et al., 1999). The

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Received January 22, 2010; **Accepted** February 27, 2010; **Published** February 27, 2010

Citation: Karanam SK, Medicherla NR (2010) Application of Doehlert experimental design for the optimization of medium constituents for the production of L-asparaginase from Palm Kernal cake (*Elaeis guineensis*). J Microbial Biochem Technol 2: 007-012. doi:10.4172/1948-5948.1000016

low cost and availability of PKC have recently generated much interest in its potential use in fish diets. In recent years, the cost of imported feed ingredients used in commercial aqua feeds in many developing countries in Asia has continued to rise due to increased global demand and fluctuation in foreign currency exchange. There is currently a great interest within the animal feed industry to reduce costs by using locally available feed ingredients like Palm kernel cake. Hence an attempt is made in this investigation to utilize the palm kernel cake as a substrate for L-asparaginase production as well as to carry out an initial process optimization.

After screening of the important variables the conventional method used for optimization is the "one factor at a time" method in which a single variable or one independent variable is varied while fixing all others at a specific level. This may lead to unreliable results and less accurate conclusions. This method requires us to carry out a large number of experiments which might result in predicting the untrue optimum values. These drawbacks of single parameter optimization process can be eliminated by optimizing all the affecting parameters collectively by Doehlert experimental design (DD) (Doehlert, 1970) of Response Surface Methodology (RSM). Basically, this optimization process involves three major steps: performing the statistically designed experiments, estimating the coefficients in a mathematical model, predicting the response and checking the adequacy of the model. Several researchers have applied these techniques for optimization of different process parameters (Vanot et al., 2002; Dutra et al., 2006; Imandi et al., 2007). Hence, the present study reports the application of Doehlert experimental design to optimize L-asparaginase production from palm kernel cake (*Elaeis guineensis*) by SSF using *Yarrowia lipolytica* NCIM 3472.

Materials and Methods

Substrate

Palm Kernel Cake (*Elaeis guineensis*), obtained from a local palm oil distillery (Jeevan Enterprises) unit of Vizianagaram, India, was used as the substrate. It was dried at 60°C for 72 h to reduce the moisture content to around 5 %, and ground to the desired size (2 mm).

Chemical analysis of PKC

Palm kernel cake is the major byproduct of oil palm kernel oil extraction. PKC, when powdered, gives meal or flour. As such there is no significant alteration in the content of nutrients when the cake is converted to meal or flour. Palm kernel meal contains 20 % protein which is the lowest among the oil seed meals. Carbohydrates are the major constituents of palm kernel meal than most of the oil seed meals. It is characterized by higher fiber content. The contents of nitrogen free extract (NFE) and crude fiber depend on the method of oil extraction from palm kernel. PKC contains a good amount of minerals: 0.69 % phosphorus, 0.42 % potassium, 0.29 % calcium, and 0.017 % iron (Imandi, 2008).

Microorganism and growth conditions

Yarrowia lipolytica NCIM 3472, obtained from National Chemical Laboratory, Pune, India, was used throughout the study. The culture was maintained on MGYP slants having the composition (%): malt extract 0.3, glucose 1.0, yeast extract 0.3, peptone 0.5 and agar agar 2.0. The pH of the medium was adjusted to 6.4–6.8 and culture was incubated at 30°C for 48 h. Subculturing was carried out once in 2 weeks and the culture was stored at 4°C.

Inoculum and media preparation

The *Yarrowia* strain was cultivated in a medium containing peptone 5 g, yeast extract 3 g and sodium chloride 3 g per liter of distilled water. The cells were cultivated in this medium at 30°C on a shaker at 200 rpm for 24 h (Imandi et al., 2008). Ten grams of substrate was weighed into a 250 ml Erlenmeyer flask into which a supplemental salt solution was added to get the desired moisture level. The composition of the salt solution was as follows (% w/w): $Na_2HPO_4.2H_2O$: 0.6; KH_2PO_4: 0.3; $MgSO_4.7H_2O$: 0.05; $CaCl_2.2H_2O$: 0.00015; NaCl: 0.05 (Kumar et al., 2010). From the results of preliminary experimental runs, glucose and L-asparagine were identified as the suitable carbon and nitrogen sources respectively for the production of L-asparaginase. The contents were thoroughly mixed and autoclaved at 121°C (15 psi) for 20 min.

Solid state fermentation and extraction of L-asparaginase

The sterilized substrate including media as shown in the above Section was inoculated with 2 ml of inoculum. The contents were mixed thoroughly and incubated in a slanting position at 30°C. All the experiments were carried out in duplicate and samples were withdrawn after 4 days of incubation. The crude enzyme from the fermented material was recovered by simple extraction method. The fermented substrate was mixed thoroughly with 50 ml of 50 mM phosphate buffer (pH 7.0) and the contents were agitated in a rotary shaker (180 rpm) for 60 min at 30°C, a temperature high enough to increase the extraction efficiency without causing enzyme denaturation. The raw extract was obtained by pressing the mixture and subsequent centrifugation. The resulting clear filtrate was used for L-Asparaginase assay.

L-asparaginase assay

L-asparaginase enzyme assay was performed by a colorimetric method, according to Wriston and Yellin (Wriston and Yellin, 1973) at 37°C, using UV-visible spectrophotometer, by estimating the ammonia produced during L-asparagine catalysis using Nessler's reagent. Reaction mixture consisting of 0.5 ml of 0.08 mM L-asparagine, 1.0 ml of 0.05 mM borate buffer (pH 7.5) and 0.5 ml of enzyme solution. There action was terminated by the addition of 0.5 ml of 15 % trichloroacetic acid solution after 30 min of incubation. The liberated ammonia was coupled with Nessler's reagent and was quantitatively determined using a calibration curve from standard solutions of ammonia. One unit of the L-asparaginase (IU) is defined as that amount of enzyme capable of producing 1 µ mol of ammonia per minute at assay conditions. Enzyme activity was expressed as units/gram of the initial dry substrate (U/gds).

Doehlert experimental design

Once the variables having the statistically significant influence on the responses were identified, a Doehlert experimental design [Doehlert, 1970] was used to optimize the levels of these variables. The number of experiments required (N) is given by $N = n^2 + n + n_0$, where n is the number of variables and n_0 is the number of center points. Replicates at the central level of the variables are performed in order to validate the model by means of an estimate of experimental variance. For statistical calculations the variables X_i were coded as x_i according to Equation (1)

$$x_i = \left(\frac{X_i - X_{oi}}{\Delta X_i} \right) \alpha_i \quad (1)$$

where x_i is the coded value of the i^{th} variable, X_i the natural value, X_{oi} the value at the center point, ΔX_i the step change value, and α_i is the maximum value of the coded variable (i.e. 1.0, 0.866 and 0.816 for five levels, seven levels and three levels, respectively).

The second degree polynomial (Equation. (2)) was fitted to the experimental data by using the statistical package STATISTICA 6.0 (Stat-Ease Inc., Tulsa, OK, USA) to estimate the response of the dependent variable and the regression coefficients.

$$Y = b_0 + b_1X_1 + b_2X_2 + b_3X_3 + b_{11}X_1^2 + b_{22}X_2^2 + b_{33}X_3^2 + b_{12}X_1X_2 + b_{23}X_2X_3 + b_{13}X_1X_3 \quad (2)$$

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 the coefficients for linear effects, b_{11}, b_{22}, b_{33} are the coefficients for squared effects and b_{12}, b_{23}, b_{13} are the coefficients for interaction terms.

Results and Discussion

Optimization of the medium constituents using DD

The selection of the range for process variables is extremely important when planning the experimental design; otherwise, after completion of the experimental runs, the optimal conditions, obtained either by response surface methodology, might not be found inside the experimental region. The following experiments were carried out to study the variables in such a range so that reasonable production of L-asparaginase would be achieved within that range.

From the results of preliminary experimental runs, the three variables (glucose, moisture content, L-asparagine) have been identified as the potential variables for the production of L-asparaginase. Based on the results of preliminary experimental runs, the three variables (glucose, moisture content, L-asparagine) have been identified as the potential variables for the production of L-asparaginase. Out of them, glucose had shown stronger effect on L-asparaginase activity and hence it was assigned seven level, followed by moisture content of substrate was assigned five level and L-asparagine was assigned three levels. A summary of the independent variables and their range and levels was presented in Table 1.

Fifteen experimental runs (Table 2) including three replicates at the center point were carried out for four days of incubation time. By using multiple regression analysis (STATISTICA 6.0) the coefficients of equation (3) was estimated, and gave the following equation.

$$Y = -90.8822 + 2.9287X_1 + 6.7432X_2 + 15.9142X_3 - 0.0234X_1^2 - 0.2189X_2^2 - 6.1156X_3^2 - 0.0287X_1X_2 - 0.0988X_2X_3 - 0.0159X_1X_3 \quad (3)$$

The predicted L-asparaginase activity resulted from equation (3) are in close agreement with the experimental values as evident from last column of Table 4, and hence the above equation was deemed to be adequate in representing the solid state fermentation of L-asparaginase production under the specified range of experiments. For quadratic models, the optimum point can be characterized as maximum, minimum, or saddle. It is possible to calculate the coordinates of the optimum point through the first derivate of the mathematical function, which describes the response surface and equates it to zero. The above

Variables	Range and levels						
Coded variable, x_1 `	–1	–0.5	0	0.5	1		
Moisture content, X_1 (%)	40	50	60	70	80		
Coded variable, x_2	–0.866	–0.577	–0.288	0	0.288	0.577	0.866
Glucose, X_2 (%w/w)	4	6	8	10	12	14	16
Coded variable, x_3	–0.816	0	0.816				
L-asparagine, X_3 (%w/w)	0.5	1.5	2.5				

Table 1: Experimental range and levels of the variables.

Experiment number	Coded values			Natural values			L-asparaginase activity (U/gds)	
	x_1	x_2	x_3	X_1	X_2	X_3	Experimental	Predicted
1	1	0	0	80	10	1.5	23.12	22.8487
2	–1	0	0	40	10	1.5	30.27	30.5412
3	0.5	0.866	0	70	16	1.5	24.99	25.1762
4	–0.5	–0.866	0	50	4	1.5	23.25	23.0637
5	0.5	–0.866	0	70	4	1.5	22.16	22.6575
6	–0.5	0.866	0	50	16	1.5	32.96	32.4625
7	0.5	0.288	0.816	70	12	2.5	20.41	20.4950
8	–0.5	–0.288	–0.816	50	8	0.5	31.19	31.1050
9	0.5	–0.288	–0.816	70	8	0.5	28.95	28.7237
10	0	0.577	–0.816	60	14	0.5	32.89	33.2012
11	–0.5	0.288	0.816	50	12	2.5	25.58	25.8062
12	0	–0.577	0.816	60	6	2.5	20.79	20.4787
13	0	0	0	60	10	1.5	36.09	36.0633
14	0	0	0	60	10	1.5	35.98	36.0633
15	0	0	0	60	10	1.5	36.12	36.0633

X_1 = moisture content (%), X_2 = glucose (%w/w), X_3 = L-asparagine (%w/w)

Table 2: Doehlert three variable experimental design along with experimental and predicted values.

Term	Coefficient	Value	Standard error of coefficient	*t*-value	*p*-Value
Constant	b_0	–90.8822	4.861603	–18.6939	0.000008*
Moisture content	b_1	2.9287	0.132246	22.1460	0.000003*
Glucose	b_2	6.7432	0.317095	21.2654	0.000004*
L-asparagine	b_3	15.9142	1.788421	8.8984	0.000298*
Moisture content × moisture content	b_{11}	–0.0234	0.001037	–22.5782	0.000003*
Glucose × glucose	b_{22}	–0.2189	0.008644	–25.3257	0.000002*
L-asparagine × L-asparagine	b_{33}	–6.1156	0.262424	–23.3041	0.000003*
Moisture content × glucose	b_{12}	–0.0287	0.003788	–7.5682	0.000639*
Glucose × L-asparagine	b_{23}	–0.0988	0.069155	–1.4280	0.212666
L-asparagine × moisture content	b_{31}	–0.0159	0.023956	–0.6644	0.535834

* Significant at $p \leq 0.05$

Table 3: Model coefficients estimated by multiple linear regression (significance of regression coefficients).

quadratic equation obtained for three variables as described below:

$$\frac{\partial Y}{\partial X_1} = 2.9287 - 0.0468X_1 - 0.0287X_2 - 0.0159X_3 = 0 \quad (4)$$

$$\frac{\partial Y}{\partial X_2} = 6.7432 - 0.4378X_2 - 0.0287X_1 - 0.0988X_3 = 0 \quad (5)$$

$$\frac{\partial Y}{\partial X_3} = 15.9142 - 12.2312X_3 - 0.0988X_2 - 0.0159X_1 = 0 \quad (6)$$

Thus, to calculate the coordinate of the optimum point, it is necessary to solve the first grade system formed by Equations (4), (5) and (6) and to find the $X_{1,\text{opt}}$, $X_{2,\text{opt}}$ and $X_{3,\text{opt}}$ values. The optimum values of the medium constituents for maximum L-asparaginase activity can be attained at 54.8622 % of moisture content, 11.9241 (% w/w) of glucose, and 1.0758 (% w/w) of L-asparagine. At these optimum medium constituents, maximum L-asparaginase activity of 39.8623 U/gds (Units/ grams of dried palm kernel cake waste as substrate) was obtained.

The significance of each coefficient in equation (4) was determined by student's *t*-test and *p*-values which were also listed in Table 3. The larger the magnitude of the *t*-value and smaller the *p*-value, the more significant is the corresponding coefficient (Lazic, 2004). This implies that the linear and quadratic effects of moisture content of substrate, glucose and L-asparagine were highly significant as is evident from their respective *p*-values. 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. The interaction effect of moisture content of substrate and glucose was found to be significant ($p \leq 0.05$). The remaining two interaction terms i.e. glucose × L-asparagine and L-asparagine × moisture content were found to be insignificant ($p > 0.05$) which were also presented in Table 3. The parity plot (Figure 1) showed a satisfactory correlation between the experimental and predicted values of L-asparaginase activity, wherein, the points cluster around the diagonal line which indicated the good fit of the model because the deviation between the experimental and predicted values is less.

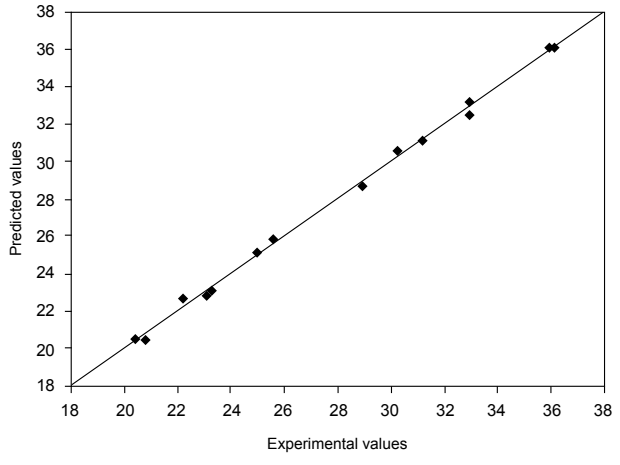

Figure 1: Parity plot showing the distribution of experimental vs. predicted values of L-asparaginase activity.

Source of variation	Sum of squares (SS)	Degree of freedom (d.f.)	Mean squares (MS)	*F*-value	Probe>*F*
Model	462.2621	9	51.36246	248.6102	0.000004
Error	1.0330	5	0.20660		
Total	463.2951	14			

R= 0.99888455; R^2= 0.99777034; Adjusted R^2= 0.99375695

$P_{\text{model}} > F = 0.000004$

Table 4: ANOVA for the entire quadratic model.

The results of the second order response surface model fitting in the form of Analysis of Variance (ANOVA) were given in Table 4. It is required to test the significance and adequacy of the model. The Fisher variance ratio, the *F*-value ($= S^2_r / S^2_e$), 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 ANOVA of the regression model demonstrates that the model is highly significant, as is evident from the Fisher's *F*-test ($F_{\text{model}} = 248.6102$) and a very low probability value ($P_{\text{model}} > F = 0.000004$).

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 variables 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. In this case, the value of the determination coefficient ($R^2 = 0.9977$) indicates that 99.77 % of the variability in the response could be explained by the model. In addition, the value of the adjusted determination coefficient (Adj $R^2 = 0.9937$) is also very high to advocate for a high significance of the model. Also

a higher value of the correlation coefficient (R=0.99888) justifies an excellent correlation between the independent process variables.

Figures 2–4 represent the response surface plots for the optimization of medium constituents of L-asparaginase production. Figure 2 shows the interaction effect of moisture content of substrate and glucose on the L-asparaginase activity at the fixed L-asparagine concentration (1.5 %w/w). It was evident that the L-asparaginase activity significantly increased with the glucose concentration up to about 12 (%w/w), and reached the maximum L-asparaginase activity at some 11.5–12.5 (% w/w) glucose, but decreased slowly beyond this concentration. Similarly, the L-asparaginase activity drastically increased with increase in the moisture content of substrate in the range 50–60 (%), and then steadily decreased beyond that range. Figure 3 depicts the response surface plot of the effects of L-asparagine and moisture content on L-asparaginase activity at a fixed glucose concentration (10 %w/w). As can be seen from Figure 3, with the increase of moisture content from 50 to 60 (%), the L-asparaginase activity increased gradually from 35 to 38 (U/gds) but the trend is negligible beyond 60 (%). Figure 4 shows the effects of interaction of glucose and L-asparagine on the L-asparaginase activity at a fixed moisture content of substrate (60 %). With the concentration of L-asparagine increasing, the L-asparaginase activity increased in the glucose concentration of 10.5–12.5 (% w/w), but decreased slowly beyond that range.

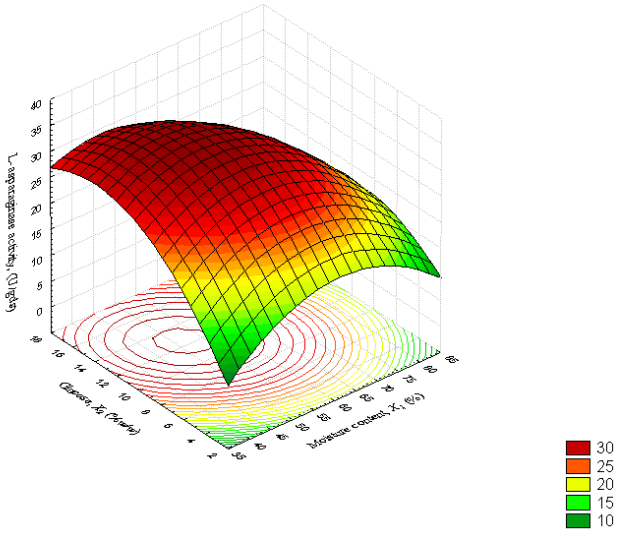

Figure 2: Response surface and contour plot of moisture content vs. glucose on L-asparaginase activity (L-asparagine concentration was kept constant at 1.5 (%w/w)).

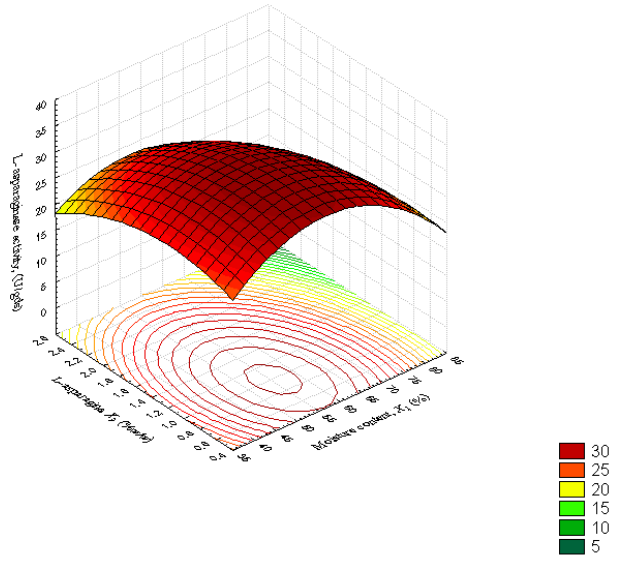

Figure 3: Response surface and contour plot of moisture content vs. L-asparagine on L-asparaginase activity (glucose concentration was kept constant at 10 (%w/w)).

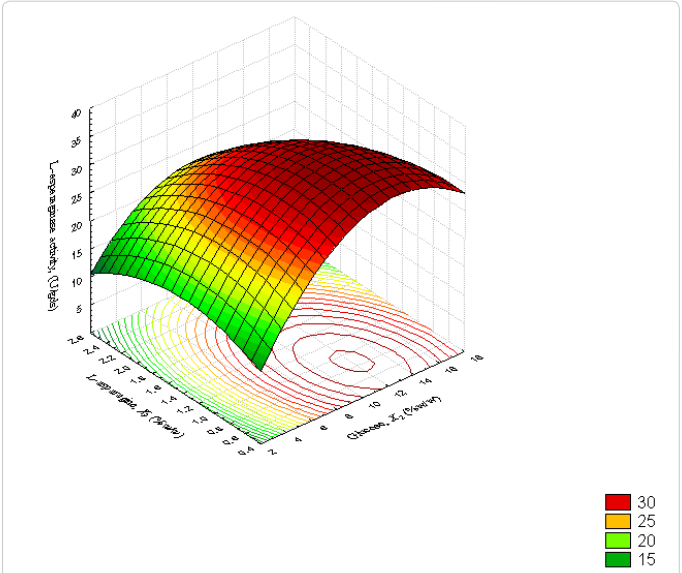

Figure 4: Response surface and contour plot of glucose vs. L-asparagine on L-asparaginase activity (moisture content of substrate was kept constant at 60 %).

Conclusion

The present study involves the use of Doehlert experimental designs to optimize medium constituents of the fermentation medium for the production of L-asparaginase from Palm kernel cake by SSF using *Yarrowia lipolytica* NCIM 3472. Three variables viz., moisture content of substrate, glucose and L-asparagine were identified by preliminary experimental runs as significant for L-asparaginase activity. These variables are optimized with Doehlert experimental design using STATISTICA 6.0 for the necessary computations. All the three variables showed significant influence on the L-asparaginase activity. The significant interactions between the three variables were also observed from the response surface and contour plots. The maximum activity of L-asparaginase produced from palm kernel cake was predicted to be 39.8623 U/gds when the optimized medium constituents of the fermentation medium were set as follows: moisture content of substrate 54.8622 %, glucose concentration 11.9241 (%w/w), and L-asparagine concentration 1.0758 (%w/w). The high L-asparaginase activity achieved in conjunction with the abundantly available Palm kernel cake in the state of Andhra Pradesh, India, paved a way for the industrial exploitation of this substrate under solid state fermentation using the indigenous *Yarrowia lipolytica* NCIM 3472 as a suitable microorganism.

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