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ORIGINAL ARTICLE

# Optimization of fermentation conditions for enhancing extracellular production of L-asparaginase, an anti-leukemic agent, by newly isolated *Streptomyces brollosae* NEAE-115 using solid state fermentation

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**Abstract** Optimization of the fermentation conditions for extracellular production of L-asparaginase by *Streptomyces brollosae* NEAE-115 under solid state fermentation was investigated. The Plackett–Burman experimental design was used to screen 16 independent variables (incubation time, moisture content, inoculum size, temperature, pH, soybean meal + wheat bran, dextrose, fructose, L-asparagine, yeast extract, $KNO_3$, $K_2HPO_4$, $MgSO_4.7H_2O$, NaCl, $FeSO_4$. $7H_2O$, $CaCl_2$) and three dummy variables. The most significant independent variables found to affect enzyme production, namely soybean + wheat bran ($X_6$), L-asparagine ($X_9$) and $K_2HPO_4$ ($X_{12}$), were further optimized by the central composite design. We found that L-asparaginase production by *S. brollosae* NEAE-115 was 47.66, 129.92 and 145.57 units per gram dry substrate (U/gds) after an initial survey using "soybean meal + wheat bran" as a substrate for L-asparaginase production (step 1), statistical optimization by Plackett–Burman design (step 2) and further optimization by the central composite design (step 3), respectively, with a fold of increase of 3.05.

**Keywords** *Streptomyces brollosae* NEAE-115 · L-asparaginase production · Solid state fermentation · Soybean meal · Plackett–Burman design and central composite design

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## Introduction

The enzyme L-asparaginase (L-asparagine aminohydrolase EC 3.5.1.1) is an effective chemotherapeutic agent used to treat a wide variety of tumors, especially acute lymphoblastic leukemia (ALL) and lymphosarcoma (Narta et al. 2007; Bhargavi and Jayamadhuri 2016). It is also gaining in importance as an industrial food-processing agent for reducing—or removing entirely—acrylamide in processed food products (Pedreschi et al. 2008). L-asparaginase catalyzes the hydrolysis of amide group of L-asparagine to form L-aspartate and ammonium, and this catalytic reaction is essentially irreversible under physiological conditions (Prakasham et al. 2007). L-asparagine is essential amino acid for the protein synthesis that can be produced within normal cells by enzyme asparagine synthetase. Unlike normal cells, however, certain tumor cells are unable to synthesize L-asparagine due the absence of L-asparagine synthetase and thus require an external source of L-asparagine for their growth and multiplication. Such cells can be selectively killed by L-asparagine deprivation in the presence of L-asparaginase (Pritsa et al. 2001).

The growth of a microorganism and its production of natural products are strongly influenced by medium composition; consequently, the optimization of media components and cultural conditions is an important component of bioprocess development (Suresh and Raju 2012). The production of L-asparaginase is greatly influenced by the composition of the fermentation media and culture condition factors such as temperature, pH, inoculum size, agitation rate and incubation time (Hymavathi et al. 2009). To date, no defined medium has been

established for the optimum production of L-asparaginase from different microbial sources—each organism has its own special conditions for maximum enzyme production.

Solid state fermentation (SSF) has several advantages over submerged fermentation, including superior productivity, lower energy requirements, low risk of bacterial contamination, lower need of water, ease of product extraction and fewer environmental concerns regarding the disposal of solid waste (Pandey et al. 2001). L-asparaginase production in SSF has been reported earlier on soybean meal (El-Bessoumy et al. 2004) and wastes from three leguminous crops, namely, bran of pigeon pea (*Cajanus cajan L.*), mungbean (*Phaseolus mungo*) and soybean (*Glycine max*) (Mishra 2006).

Nutritional requirement can be manipulated by conventional and statistical methods. The conventional approach involve changing one independent variable at a time while keeping the others at a certain fixed value (Liu and Tzeng 1998). The disadvantages of this method are that it is time consuming, tedious, laborious and expensive; in addition, it does not consider the interaction effects among the different variables employed (Gao et al. 2009). The statistical designs, such as the Plackett–Burman design and response surface method, are effective methods for optimization of the operational parameters (Wang et al. 2008) because they reduce the total number of experiments needed and provide a better understanding of the interactions among factors on the outcome of the fermentation (Revankar and Lele 2006). The Plackett–Burman design is usually used to screen important parameters (Levin et al. 2005) while ignoring interactions among variables. Response surface methodology (RSM) is a statistical technique based on the fundamental principles of statistics, randomization, replication and duplication which simplifies the optimization by studying the mutual interactions among the variables over a range of values in a statistically valid manner. It is an efficient technique for optimization of multiple variables in order to predict the best performance conditions with a minimum number of experiments and to explain the individual and interactive effects of test variables on the response (Panwal et al. 2011). Several researchers in biotechnology have applied these techniques for optimization of different parameters (El-Naggar et al. 2014a).

Most of the L-asparaginase produced by microorganisms is intracellular in nature, with the exception of a few which secrete L-asparaginase outside the cell wall (Narayana et al. 2008). For industrial and medical purposes, extracellular L-asparaginase is more advantageous than the intracellular type for a number of reasons, including higher accumulation in culture broth under normal conditions, easy extraction and downstream processing (Amena et al. 2010; El-Naggar et al. 2014b). In addition extracellular L-asparaginase produced by bacteria is protease deficient, the liberated protein exported to the medium is mostly soluble, biologically active and has an authentic N-terminus and it is relatively free from endotoxins, all properties which result in the minimization of adverse effects (Pradhan et al. 2013).

Actinomycetes are a good source for the production of L-asparaginase (Narayana et al. 2008). Among the actinomycetes, *Streptomyces* species are responsible for the production of about one-half of the bioactive secondary metabolites identified to date, notably antibiotics, anticancer compounds and enzymes (Manivasagan et al. 2013). Several *Streptomyces* species, such as *S. gulbargensis* (Amena et al. 2010), *S. olivaceus* NEAE-119 (El-Naggar et al. 2015a) and *S. parvus* NEAE-95 (El-Naggar 2015b), have been explored for L-asparaginase production.

The general objective of the study reported here was the optimization of fermentation conditions for the extracellular production of L-asparaginase under SSF conditions. A statistical approach was employed through which a Plackett–Burman design was used to identify significant variables influencing L-asparaginase production, and the levels of the significant variables were further optimized using RSM.

## Materials and methods

### Substrates

Wheat bran and soybean meal were used as the substrate for the production of the L-asparaginase. Soybean grains were ground in a Willey Mill to a particle size of about 1–3 mm.

### Microorganisms and cultural conditions

The *Streptomyces* sp. NEAE-115 used in this study was isolated from a soil sample collected from Brollos Lake, located along the Mediterranean coast of Egypt, using the standard dilution plate method procedure and petri plates containing starch nitrate agar medium (in g/L: starch, 20; $KNO_3$, 2; $K_2HPO_4$, 1; $MgSO_4.7H_2O$, 0.5; NaCl, 0.5; $CaCO_3$, 3; $FeSO_4.7H_2O$, 0.01; agar, 20; distilled water up to 1 L). T plates were incubated for a period of 7 days at 30 °C, and the isolate was maintained as spore suspensions in 20 % (v/v) glycerol at −20 °C for subsequent investigation. *Streptomyces* sp. NEAE-115 was previously identified as a novel species of the genus *Streptomyces*, for which we suggest the name *S. brollosae* NEAE-115 (El-Naggar and Moawad 2015).

### Screening of L-asparaginase production by plate assay

L-asparaginase production is accompanied by an increase in the pH of the culture filtrates (DeJong 1972). The plate assay performed in our study was based on the method described by Gulati et al. (1997) in which the pH indicator phenol red (prepared in ethanol) is incorporated in medium containing L-asparagine as the sole nitrogen source. Phenol red is yellow

at acidic pH, turning pink at alkaline pH; thus a pink zone is formed around microbial colonies producing L-asparaginase. Potential L-asparaginase-producing actinomycetes were screened using asparagine dextrose salts agar (ADS Agar; 1.0 % asparagine, 0.2 % dextrose, 0.1 % $K_2HPO_4$, 0.05 % $MgSO_4$, 1.5 % agar), adjusted to pH 6.8 and supplemented with phenol red as a pH indicator (final concentration 0.009 %) (Gulati et al. 1997), and sterilized at 1.5 atmospheric pressure for 20 min. Inoculated plates were incubated at 30 °C for 7 days and then examined for a change in the color of the medium from yellowish to pink due to changes in the pH, indicating positive asparaginase activity. Colonies with pink zones were considered to be L-asparaginase-producing strains, and those isolates exhibiting L-asparaginase activity were selected for further study.

### Agar well diffusion technique

Aliquots (50 μl) of cell-free culture broth were poured into agar wells (diameter 8 mm) prepared in plates containing ADS Agar medium. The filtrate was allowed to diffuse into the medium for 12 h at 4 °C, and then the plates were incubated at 37 °C for 2 h. The diameter of the zone (mm) of L-asparaginase activity, as indicated by the formation of a pink-colored zone around the well against the yellow background, was measured. For further studies, cultures showing greater enzyme production were selected.

### Inoculum preparation

The spore suspension ($1.6 \times 10^9$ spores/ml) was prepared from a 7-day-old culture grown on starch nitrate agar slant by adding 10 ml of sterile production medium containing 0.01 % (v/v) Tween 80 and suspending the spores with a sterile loop (Lingappa and Babu 2005).

### Comparative evaluation of substrates for L-asparaginase production

A comparative study was conducted to determine the suitability of agro-industrial residues, such as wheat bran and soybean meal, as single and mixed substrates, respectively, as carbon sources for L-asparaginase production. In these experiments, 10 g of wheat bran, soybean meal and wheat bran + soybean meal mixture (1:1) was transferred into separate 250-mL Erlenmeyer flasks (3 flasks per substrate type) into which a supplemental 15 mL of freshly prepared ADS broth (1.0 % asparagine, 0.2 %, dextrose, 0.1 %, $K_2HPO_4$, 0.5 %, $MgSO_4$) was added to obtain the desired moisture level. Gulati et al. (1997) reported that L-asparagine was supplemented as an inducer for the synthesis of L-asparaginase. The contents of the flasks were mixed thoroughly and the flasks then autoclaved at 121 °C at 1.5 atmospheric pressure for 20 min. The cooled substrates were inoculated under aseptic conditions with 2 ml of spore suspension (final moisture content 70 %). The contents of the flasks were then mixed thoroughly using a sterile spatula and incubated at 30 °C for 5–7 days.

### Chemical analysis of soybean

Soybean meal was finely powered and subjected to chemical analysis.

**Estimation of total carbohydrates** The total carbohydrate content was estimated by the phenol sulfuric acid method (Dubois et al. 1956). Briefly, 100 mg of the sample was placed into a boiling tube and hydrolyzed with 5 ml of 2.5 N HCl in a boiling water bath for 3 h before being cooled to room temperature. The solution was neutralized with solid sodium carbonate until the effervescence ceased. The volume was made up to 100 ml. The tubes were centrifuged and the supernatant collected; 0.2 ml of the sample solution was pipetted out into two separate clean test tubes and the volume made up to 1 ml with water. The standards were prepared by taking 0 (blank), 0.2, 0.4, 0.6, 0.8 and 1 ml of the working standard into separate test tubes adding distilled water to a volume of 1 ml, following which 1 ml of 5 % phenol solution and 5 ml 96 % sulphuric acid were added to each tube. The tubes were shaken well for 10 min, heated for 20 min in a water bath at 25–30 °C and the optical density (OD) read at 490 nm. The standard to estimate the total carbohydrates was glucose (100 μg/ml). A standard graph was plotted with $OD_{490nm}$ versus concentration.

**Estimation of total protein, total fats and trace elements** Protein was analyzed using the macro-Kjeldahl method (AOAC Official Method 984.13; Latimer 2012) using a Foss Kjeltec 2300 automatic analyzer (Foss Tecator AB, Höganäs, Sweden). Crude fat was analyzed by AOAC method 945.16 (Latimer 2012) with ether as a solvent, and trace elements were analyzed using official AOAC methods (Latimer 2012).

### SSF and crude enzyme production

The fermentation media consisted of 5 g of soybean meal ground to particle sizes of about 1–3 mm mixed with 5 g of wheat bran as a substrate and 15 ml of 0.1 M sodium phosphate buffer (pH 7.0) in a 250-ml flask. The flasks were autoclaved for 15 min at 121 °C and 15 lb pressure, and then cooled. The sterilized fermentation media was inoculated with 2 ml of inoculum, mixed thoroughly and incubated at 30 °C for 5–7 days in stationary culture conditions. Crude L-asparaginase was recovered from the fermented substrate by mixing thoroughly the fermented substrate with 90 mL of 0.1 M sodium phosphate buffer (pH 7) and then agitating the contents for 1 h at room temperature in a rotary shaker at

150 rpm. At the end of this extraction period, 2 ml of the extract was transferred to the Eppendorf tube and centrifuged at 10,000 *g* for 10 min; the resulting clear filtrate was used for the L-asparaginase assay.

### Assay of L-asparaginase activity

L-asparaginase activity was determined by measuring the amount of ammonia released by Nesslerization according to the method described by Wriston and Yellin (1973). The reaction mixture (1.5 ml of 0.04 M L-asparagine prepared in 0.05 M Tris–HCl buffer, pH 8.6, and 0.5 ml of an enzyme, up to a total volume of 2 ml) was added to tubes and incubated at 37 °C for 30 min. The reaction was stopped by adding 0.5 ml of 1.5 M trichloroacetic acid (TCA) to the tubes. The blank was prepared by adding enzyme after the addition of TCA. The precipitated protein was removed by centrifugation at 10,000 *g* for 5 min, and the liberated ammonia in the supernatant was determined colorimetrically by direct Nesslerization by adding 1 ml Nessler's reagent into tubes containing 0.5 ml of clear supernatant and 7 ml distilled water followed by incubation at room temperature for 20 min. A yellow coloration indicates the presence of ammonia: at higher concentrations, a brown precipitate may form. The intensity of the yellow color was read using a UV-visible spectrophotometer (Optizen Pop–UV/Vis; Mecasys Co., Ltd., Daejon, Republic of Korea) at 480 nm. The amount of ammonia liberated was calculated using an ammonium (ammonium chloride) standard curve. One unit (U) of L-asparaginase was defined as the amount of enzyme which catalyzed the formation of 1 μmole of ammonia from L-asparagine per minute at 37 °C and pH 8.6. The enzyme activity was expressed in terms of units per gram dry substrate (U/gds).

### Selection of significant variables by the Plackett–Burman design

Different process parameters influencing enzyme yield during SSF were optimized. The Plackett–Burman statistical experimental design (Plackett and Burman 1946) is a two-factorial design which identifies the critical physicochemical parameters required for elevated production, and it is very useful for screening the most important factors with respect to their main effects (Krishnan et al. 1998). The total number of experiments to be carried out according to Plackett–Burman is $n + 1$, where $n$ is the number of variables. Each variable is represented at two levels, high and low, denoted by "+" and "−", respectively. The Plackett–Burman experimental design is based on the first order model:

$$Y = \beta_0 + \sum \beta_i X_i \quad (1)$$

$Y$ is the response or dependent variable (L-asparaginase activity) and always be the target variable of the prediction; $\beta_0$ is the model intercept; $\beta_i$ is the linear coefficient; $X_i$ is the level of the independent variables which are those variables that will help to explain L-asparaginase activity. All trials were performed in duplicate, and the average of L-asparaginase activity was treated as the response.

### Central composite design

This step involved optimization of the levels and the interaction effects between various significant variables which exerted a positive effect on the L-asparaginase activity by using central composite design (CCD). In this study, the experimental plan consisted of 20 trials, and the independent variables were studied at five different levels (−1.68, −1, 0, 1, 1.68). The center point was repeated six times in order to evaluate the curvature, and the experiment replication facilitated the pure error estimation so that the significant lack of fit of the models could be predicted. All of the experiments were conducted in duplicate, and the average of L-asparaginase activity obtained was taken as the dependent variable or response ($Y$). The experimental results of CCD were fitted via the response surface regression procedure using the following second order polynomial equation:

$$Y = \beta_0 + \sum_i \beta_i X_i + \sum_{ii} \beta_{ii} X_i^2 + \sum_{ij} \beta_{ij} X_i X_j \quad (2)$$

$Y$ is the predicted response, $\beta_0$ is the regression coefficients, $\beta_i$ is the linear coefficient, $\beta_{ii}$ is the quadratic coefficients, $\beta_{ij}$ is the interaction coefficients), and $X_i$ is the coded levels of independent variables.

### Statistical analysis

The experimental data obtained was subjected to multiple linear regressions using Excel 2007 (Microsoft Corp., Redmond, WA) to evaluate the analysis of variance (ANOVA) and to estimate the main effect and obtain the $t$ values, $p$ values and confidence levels. The Student $t$ test was used to determine the significance of the regression coefficients of the parameters. The $p$ values were used as a tool to check the significance of the interaction effects, which in turn may indicate the patterns of the interactions among the variables (Montgomery 1991). The quality of fit of the regression model was expressed via the correlation coefficient ($R$), the coefficient of determination ($R^2$) and the adjusted $R^2$, and its statistical significance was determined by $F$ test. Optimal value of activity was estimated using the *solver* function of Microsoft Excel tools. The statistical software package, STATISTICA (ver. 8.0; StatSoft Inc., Tulsa, OK) was used to plot the three-dimensional surface plots in order to illustrate the relationship between the

responses and the experimental levels of each of the variables utilized in this study.

## Results and discussion

L-asparaginase-producing actinomycetes were isolated from soil samples collected in Egypt. All isolates were purified and screened for their L-asparaginase activity using the plate method in which the formation of pink zones around the colonies indicated the presence of L-asparaginase activity (Fig. 1a) and the agar well diffusion technique (Fig. 1b). Enzyme production is known to be largely dependent on the growth conditions and composition of the nutrient medium. Therefore, our aim was to optimize the composition of the medium in order to enhance the production of L-asparaginase. In SSF, the selection of a suitable solid substrate for fermentation is the most critical factor. The aim of industrial scale SSF systems is to increase the yield of L-asparaginase and minimize the cost of production by using cheaper substrate materials.

### Comparative evaluation of substrates as carbon source for L-asparaginase production under SSF conditions

A comparative study was conducted to determine the suitability of agro-industrial residues, such as wheat bran, soybean meal and a mixture of these, as carbon sources for L-asparaginase production. All ten of the morphologically different actinomycete strains that were identified as L-asparaginase producers on the initial screening by the plate assay were examined for L-asparaginase activity during SSF by a previously described method. The data clearly showed that the L-asparaginase enzyme was produced on both of the substrates when used separately but that maximum L-asparaginase yield was supported by the mixed wheat bran + soybean substrate (Table 1; Fig. 2). *Streptomyces brollosae* strains NEAE-115, NEAE-K and NEAE-99 showed the highest L-asparaginase activities (47.67, 44.82 and 41.86 U/gds, respectively) (Table 1). Based on these results, the soybean meal + wheat bran mixture (1:1; w/w) was selected for subsequent fermentation studies on L-asparaginase production under SSF. Among the tested strains, strain *S. brollosae* NEAE-115 was selected and used for L-asparaginase production.

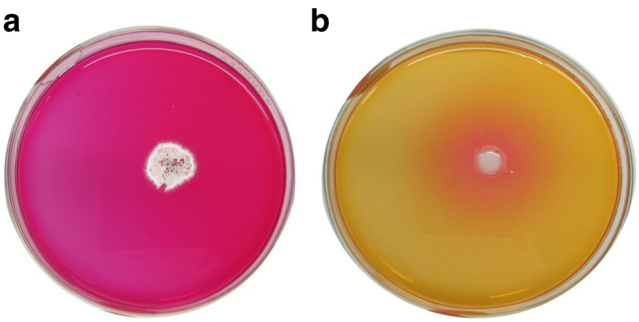


**Fig. 1** Production of L-asparaginase by *Streptomyces brollosae* NEAE-115 as detected by the plate assay, in which a color change in the medium from a standard yellow coloration to the presence of a pink zone around the colony after 5 days of incubation indicates the production of L-asparaginase (**a**), and by the agar well diffusion technique (**b**)

### Chemical analysis of soybean

Soybean meal was analyzed for total carbohydrate, protein, fat and trace element contents. The results revealed that the soybean meal used in the study contained a total carbohydrate, protein and fat content of 106.96, 362 and 183.10 g/kg, respectively. Nine trace elements were identified in the soybean meal by absorption spectrometry, revealing that soybean meal contained higher amounts of potassium (9820 mg/kg), magnesium (261.20 mg/kg), sodium (39.60 mg/kg) and iron (25.20 mg/kg) than other elements (in mg/kg: copper, 7.05; zinc, 6.40); (in g/kg: phosphorus, 1.50; calcium, 1.30).

Soybean meal was the best substrate for the production of L-asparaginase, possibly due to the presence of sufficient amounts of lipids, proteins, carbohydrates and minerals. In the present study, we supplied nutrient sources, such as carbon, nitrogen, phosphate and metal ions, to the soybean substrate to achieve higher yields of L-asparaginase under SSF conditions.

### Identifying the significant variables affecting L-asparaginase production by *S. brollosae* NEAE-115 using the Plackett–Burman design

This experiment was conducted in 20 runs, with each run screening one of 16 independent (assigned) variables (incubation time, moisture content, inoculum size, temperature, pH, soybean meal + wheat bran, dextrose, fructose, L-asparagine, yeast extract, $KNO_3$, $K_2HPO_4$, $MgSO_4.7H_2O$, NaCl, $FeSO_4.7H_2O$, $CaCl_2$) and three unassigned variables (commonly referred as dummy variables) in the Plackett–Burman experimental design (Table 2) in order to study the effect of each selected variable on the production of L-asparaginase. Dummy variables (Dummy 1, Dummy 2 and Dummy 3) are used to estimate experimental errors in data analysis. The design matrix selected for the screening of significant variables for L-asparaginase production and the corresponding response ($Y$) under SSF conditions are shown in Table 3. All trials were performed in duplicate, and the average L-asparaginase production was treated as the response. Maximum L-asparaginase activity (131.05 U/gds) was achieved in run number 17, while minimum L-asparaginase activity (58.02 U/gds) was observed in run number 10, possibly due to the low level of inoculum size, soybean + wheat bran and L-asparagine in the fermentation media.

**Table 1** Comparative evaluation of substrates as a carbon source for L-asparaginase production

| *Streptomyces* species and strain number | L-asparaginase activity (U/gds) | | |
|---|---|---|---|
| | Soybean meal | Wheat bran | Soybean meal + wheat bran (1:1) |
| *Streptomyces brollosae* strain NEAE-115 | 31.28 | 15.02 | 47.67 |
| *Streptomyces olivaceus* strain NEAE-119 | 2.10 | 1.17 | 27.76 |
| *Streptomyces parvus* strain NEAE-95 | 18.20 | 4.89 | 25.14 |
| *Nocardiopsis synnemasporogenes* strain NEAE-85 | 1.14 | 0.57 | 1.93 |
| NEAE 42 | 2.28 | 1.93 | 2.39 |
| NEAE- F | 11.60 | 1.25 | 24.46 |
| NEAE 99 | 27.99 | 18.09 | 41.86 |
| NEAE K | 21.84 | 16.49 | 44.82 |
| NEAE 111 | 8.76 | 6.48 | 8.99 |
| NEAE 82 | 2.28 | 1.71 | 5.35 |

The relationship between a set of independent variables and the response ($Y$) is determined by a mathematical model called the multiple regression model. Statistical analysis of the response was performed and the results are presented in Table 4. With respect to the main effect of each variable (Fig. 3), ten variables from the 16 tested, namely, incubation time, moisture content, inoculum size, soybean meal + wheat bran, L-asparagine, yeast extract, $KNO_3$, $K_2HPO_4$, $FeSO_4.7H_2O$ and $CaCl_2$, positively affected L-asparaginase production, whereas the remaining six variables, namely, temperature, pH, dextrose, fructose, $MgSO_4.7H_2O$ and NaCl, negatively affected L-asparaginase production. For further optimization by the CCD, we fixed the significant variables with positive effect on L-asparaginase production at a high level and those variables which exerted a negative effect were maintained at a low level.

The Pareto chart illustrates the order of significance of the variables affecting L-asparaginase production in the Plackett–Burman experimental design (Fig. 4). Among the variables screened, the most effective factors positively affect L-asparaginase production, as indicated by the Pareto chart, were soybean meal + wheat bran (17.46 %), L-asparagine (7.97 %) and $K_2HPO_4$ (7.37 %). These were identified as most significant variables affecting L-asparaginase production and

**Fig. 2** Growth of *Streptomyces brollosae* NEAE-115 during the experiment on L-asparaginase production under solid state fermentation (SSF) conditions after inoculation and incubation for 5–7 days at 30 °C. View is from above

**Table 2** Experimental independent variables at two levels used for the production of L-asparaginase by *Streptomyces brollosae* NEAE-115 using the Plackett–Burman design

| Variable code | Variables | Levels | |
|---|---|---|---|
| | | −1 | +1 |
| $X_1$ | Incubation time (days) | 5 | 7 |
| $X_2$ | Moisture content (%) | 50 | 70 |
| $X_3$ | Inoculum size (mL/10 gds) | 2 | 4 |
| $X_4$ | Temperature (°C) | 30 | 35 |
| $X_5$ | pH | 7 | 8.5 |
| $X_6$ | Soybean meal + wheat bran (g; 1:1) | 10 | 15 |
| $X_7$ | Dextrose (g/L) | 2 | 4 |
| $X_8$ | Fructose (g/L) | 2 | 4 |
| $X_9$ | L-asparagine (g/L) | 7 | 10 |
| $X_{10}$ | Yeast extract (g/L) | 0 | 2 |
| $X_{11}$ | $KNO_3$ (g/L) | 0 | 1 |
| $X_{12}$ | $K_2HPO_4$ (g/L) | 1 | 2 |
| $X_{13}$ | $MgSO_4.7H_2O$ (g/L) | 0.1 | 0.5 |
| $X_{14}$ | NaCl (g/L) | 0.1 | 0.5 |
| $X_{15}$ | $FeSO_4.7H_2O$ (g/L) | 0.01 | 0.02 |
| $X_{16}$ | $CaCl_2$ (g/L) | 0 | 0.01 |

g/ds, Grams per dry substrate

**Table 3** Twenty-trial Plackett–Burman experimental design for evaluation of independent variables with coded values along with the observed L-asparaginase activity

| Run/trail | Incubation time | Moisture content | Inoculum size | Temperature | pH | Soybean meal + wheat bran | Dextrose | Fructose | L-asparagine | Yeast extract | $KNO_3$ | $K_2HPO_4$ | $MgSO_4.7H_2O$ | NaCl | $FeSO_4.7H_2O$ | $CaCl_2$ | Dummy 1 | Dummy 2 | Dummy 3 | L-asparaginase activity (U/gds) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 1 | −1 | 1 | 1 | −1 | −1 | 1 | 1 | 1 | 1 | −1 | 1 | −1 | 1 | −1 | −1 | −1 | −1 | 1 | 85.09 |
| 2 | −1 | 1 | 1 | −1 | −1 | 1 | 1 | 1 | 1 | −1 | 1 | −1 | 1 | −1 | −1 | −1 | −1 | 1 | 1 | 116.95 |
| 3 | 1 | 1 | 1 | 1 | −1 | 1 | −1 | 1 | −1 | −1 | −1 | −1 | 1 | 1 | −1 | 1 | 1 | −1 | −1 | 95.56 |
| 4 | −1 | −1 | 1 | 1 | −1 | 1 | 1 | −1 | −1 | 1 | 1 | 1 | 1 | −1 | 1 | −1 | 1 | −1 | −1 | 104.66 |
| 5 | −1 | −1 | −1 | −1 | 1 | 1 | −1 | 1 | 1 | −1 | −1 | 1 | 1 | 1 | 1 | −1 | 1 | −1 | 1 | 104.20 |
| 6 | −1 | −1 | 1 | 1 | 1 | 1 | −1 | 1 | −1 | 1 | −1 | −1 | −1 | −1 | 1 | 1 | −1 | 1 | 1 | 103.75 |
| 7 | 1 | 1 | −1 | 1 | −1 | 1 | −1 | −1 | −1 | −1 | 1 | 1 | −1 | 1 | 1 | −1 | −1 | 1 | 1 | 121.72 |
| 8 | −1 | 1 | −1 | 1 | −1 | −1 | −1 | −1 | 1 | 1 | −1 | 1 | 1 | −1 | −1 | 1 | 1 | 1 | 1 | 101.93 |
| 9 | 1 | 1 | −1 | −1 | 1 | 1 | 1 | 1 | −1 | 1 | −1 | 1 | −1 | −1 | −1 | −1 | 1 | 1 | −1 | 117.63 |
| 10 | −1 | −1 | −1 | 1 | 1 | −1 | 1 | 1 | −1 | −1 | 1 | 1 | 1 | 1 | −1 | 1 | −1 | 1 | −1 | 58.02 |
| 11 | −1 | 1 | −1 | −1 | −1 | −1 | 1 | 1 | −1 | 1 | 1 | −1 | −1 | 1 | 1 | 1 | 1 | −1 | 1 | 78.72 |
| 12 | 1 | −1 | −1 | −1 | −1 | 1 | 1 | −1 | 1 | 1 | −1 | −1 | 1 | 1 | 1 | 1 | −1 | 1 | −1 | 111.49 |
| 13 | 1 | −1 | 1 | −1 | 1 | −1 | −1 | −1 | −1 | 1 | 1 | −1 | 1 | 1 | −1 | −1 | 1 | 1 | 1 | 89.19 |
| 14 | 1 | 1 | 1 | −1 | 1 | −1 | 1 | −1 | −1 | −1 | −1 | 1 | 1 | −1 | 1 | 1 | −1 | −1 | 1 | 103.75 |
| 15 | 1 | −1 | −1 | 1 | 1 | 1 | 1 | −1 | 1 | −1 | 1 | −1 | −1 | −1 | −1 | 1 | 1 | −1 | 1 | 105.11 |
| 16 | −1 | −1 | −1 | −1 | −1 | −1 | −1 | −1 | −1 | −1 | −1 | −1 | −1 | −1 | −1 | −1 | −1 | −1 | −1 | 93.97 |
| 17 | −1 | 1 | 1 | −1 | 1 | 1 | −1 | −1 | 1 | 1 | 1 | 1 | −1 | 1 | −1 | 1 | −1 | −1 | −1 | 131.05 |
| 18 | −1 | 1 | 1 | 1 | 1 | −1 | 1 | −1 | 1 | −1 | −1 | −1 | −1 | 1 | 1 | −1 | 1 | 1 | −1 | 84.18 |
| 19 | 1 | 1 | −1 | 1 | 1 | −1 | −1 | 1 | 1 | 1 | 1 | −1 | 1 | −1 | 1 | −1 | −1 | −1 | −1 | 91.46 |
| 20 | 1 | −1 | 1 | −1 | −1 | −1 | −1 | 1 | 1 | −1 | 1 | 1 | −1 | −1 | 1 | 1 | 1 | 1 | −1 | 126.50 |

The "−1" sign corresponds to the minimum value and the "+1" sign corresponds to the maximum value of the input parameter range

**Table 4** Statistical analysis of the Plackett–Burman design showing coefficient values, *t* test, *P* values and confidence levels for each variable affecting L-asparaginase production

| Variables | Coefficients | Main effect | *t* Statistic | *P* value | Confidence level (%) |
|---|---|---|---|---|---|
| Intercept | 101.25 | 202.49 | 89.53 | 0.000 | 99.99 |
| Incubation time | 3.50 | 7.01 | 3.10 | 0.053 | 94.66 |
| Moisture content | 3.05 | 6.10 | 2.70 | 0.074 | 92.60 |
| Inoculum size | 2.82 | 5.64 | 2.49 | 0.088 | 91.19 |
| Temperature | −6.10 | −12.20 | −5.39 | 0.013 | 98.75 |
| pH | −2.41 | −4.82 | −2.13 | 0.122 | 87.73 |
| Soybean meal + wheat bran | 9.97 | 19.93 | 8.81 | 0.003 | 99.69 |
| Dextrose | −4.69 | −9.37 | −4.14 | 0.026 | 97.45 |
| Fructose | −3.46 | −6.92 | −3.06 | 0.055 | 94.49 |
| L-asparagine | 4.55 | 9.10 | 4.02 | 0.028 | 97.24 |
| Yeast extract | 0.25 | 0.50 | 0.22 | 0.839 | 16.09 |
| $KNO_3$ | 1.09 | 2.18 | 0.97 | 0.405 | 59.46 |
| $K_2HPO_4$ | 4.21 | 8.42 | 3.72 | 0.034 | 96.62 |
| $MgSO_4.7H_2O$ | −3.53 | −7.05 | −3.12 | 0.053 | 94.75 |
| NaCl | −5.32 | −10.65 | −4.71 | 0.018 | 98.19 |
| $FeSO_4.7H_2O$ | 1.80 | 3.59 | 1.59 | 0.210 | 78.98 |
| $CaCl_2$ | 0.34 | 0.68 | 0.30 | 0.783 | 21.75 |
| Analysis of variance (ANOVA) | | | | | |
| | *df* | *SS* | *MS* | *F* test | Significance *F* (*P* value) |
| Regression | 16 | 5791.39 | 361.96 | 14.15 | 0.025 |
| Residual | 3 | 76.73 | 25.58 | | |
| Total | 19 | 5868.12 | | | |

*t* Student's test, *P* corresponding level of significance, *df* Degree of freedom, *SS* Sum of squares, *MS* Mean sum of squares, *F* Fishers's function, *Significance F* corresponding level of significance

Multiple *R* 0.9934, $R^2$ 0.9869, Adjusted $R^2$ 0.9171

selected for further optimization using the CCD to determine the optimal range of these variables.

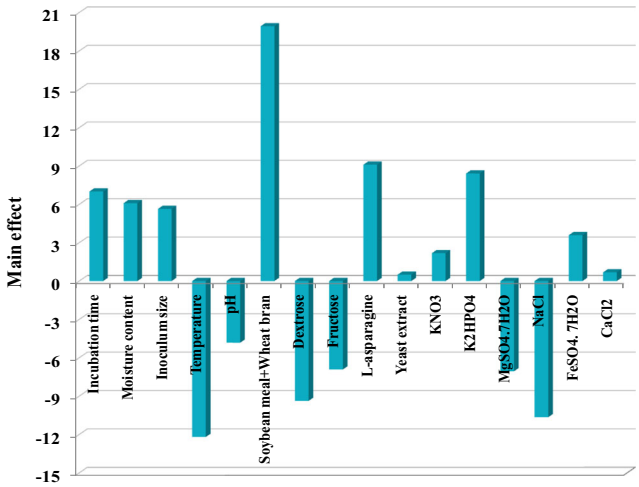


**Fig. 3** Main effects of the fermentation conditions on L-asparaginase production according to the Packett–Burman experimental results

The model's goodness of fit was checked using the determination coefficient ($R^2$). In this case, the value of the determination coefficient ($R^2 = 0.9869$) indicates that 98.69 % of the variability in the response was attributed to the given independent variable and that only 1.31 % of the total variation is not explained by the independent variables. In addition, the value of the adjusted determination coefficient (Adj. $R^2 = 0.9171$) is also very high, which indicates a high significance of the model. A higher value of the correlation coefficient ($R = 0.9934$) denotes a better correlation between the experimental and predicted responses.

The significance of each coefficient was determined by Student's *t* test and *P* values, which are listed in Table 4. In the current experiment, variables with *P* values of <0.05 (confidence levels of >95 %) were considered to have significant effects on L-asparaginase activity. Soybean meal + wheat bran, with a probability value (*P*) of 0.003, a *t* value of 8.81 and confidence level of 99.69, was determined to be the most significant factor, followed by temperature (*P* value 0.013, *t*

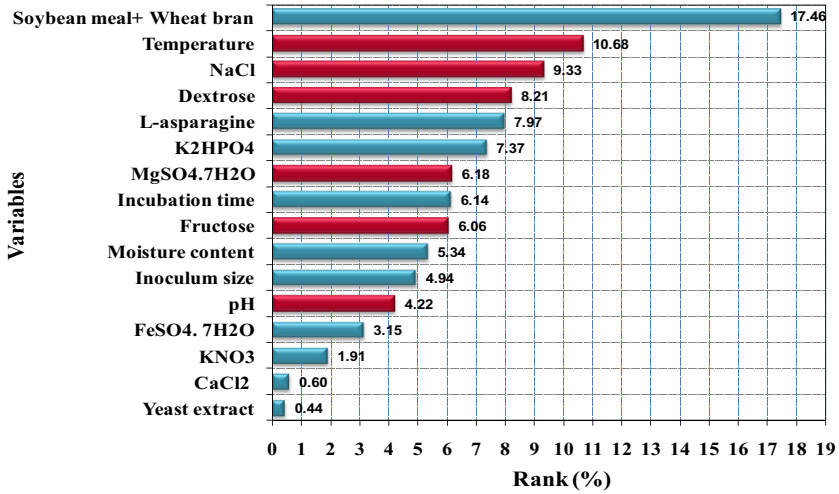


**Fig. 4** Pareto chart illustrates the order of significance of the variables affecting L-asparaginase production by *Streptomyces brollosae* strain NEAE-115. *Red color* represents those variables with negative effects, *blue color* represents those variables with positive effects. Ranking (%) values ranged from 0.44 to 17.46)

value −5.39, confidence level 98.75), NaCl (*P* value 0.018, *t*value −4.71, confidence level 98.19), dextrose (*P* value 0.026, *t* value −4.14, confidence level 97.45), L-asparagine (*P* value 0.028, *t* value 4.02, confidence level 97.24) and $K_2HPO_4$ (*P* value 0.034, *t* value 3.72, confidence level 96.62).

The model *F* value of 14.15 (Table 4) implies that the model is significant and *F* (*P*) values of <0.05 (0.025) indicate that the model terms are significant. By neglecting the terms that were insignificant ($P > 0.05$), we derived the first order polynomial equation representing L-asparaginase production as a function of the independent variables:

$$\mathbf{Y}_{(\text{L-asparaginase production})} = 101.25 – 6.10(X_4) + 9.97(X_6) – 4.69(X_7) + 4.55(X_9) + 4.21(X_{12}) – 5.32(X_{14}) \quad (3)$$

*Y* is the response, and $X_4$, $X_6$, $X_7$, $X_9$, $X_{12}$ and $X_{14}$ are temperature, soybean meal + wheat bran, dextrose, L-asparagine, $K_2HPO_4$ and NaCl, respectively. It can be seen from Eq. 3 that soybean meal + wheat bran, L-asparagine and $K_2HPO_4$ exerted a positive effect on L-asparaginase production by *S. brollosae* NEAE-115, while other factors exerted a negative effect.

A plot of predicted versus observed values of the response (Fig. 5a) shows a satisfactory correlation between the experimented values and predicted values wherein the points gathered around the diagonal line indicate the good fit of the model. The residual plot in Fig. 5b shows equal scatter of the residual data above and below the *x*-axis, indicating that the variance was independent of L-asparaginase production, thus supporting the adequacy of the model fit. Figure 5c shows a plot of normal probability of the experimental results. The residual was plotted against a theoretical normal distribution of the model in such a way that the points should form an approximate straight line for L-asparaginase production. Departures from this straight line indicate departures from normality. A linear pattern demonstrated that the errors are normally distributed and are independent of each other. The normal probability plot of the residuals shows the points close to a diagonal line; therefore, the residuals appear to be approximately normally distributed, indicating that the model was well fitted with the experimental results.

In an experiment to evaluate the accuracy of the Plackett–Burman design, as confirmation, we tested a medium with a different composition (in g/L: soybean meal + wheat bran, 15; dextrose, 2; fructose, 2; L-asparagine, 10; yeast extract, 2; $KNO_3$, 1; $K_2HPO_4$, 2; $MgSO_4.7H_2O$, 0.1; NaCl, 0.1; $FeSO_4.7H_2O$, 0.02; $CaCl_2$, 0.01) at an incubation time of 7 days, moisture content of 70 %, inoculum size of 4 mL, temperature of 30 °C and pH of 7. We obtained an L-asparaginase activity of 129.92 U/gds, which is more than threefold higher than the result obtained before applying the Plackett–Burmanby design (47.66 U/gds). The experiment was performed in duplicate, and the average L-asparaginase activity was treated as response.

Carbohydrates are generally used as carbon sources in microbial fermentation processes. The energy for the growth of desired microorganisms during industrial fermentation is derived either from the oxidation of medium components or from light (Stanbury et al. 2013). Carbon sources in the

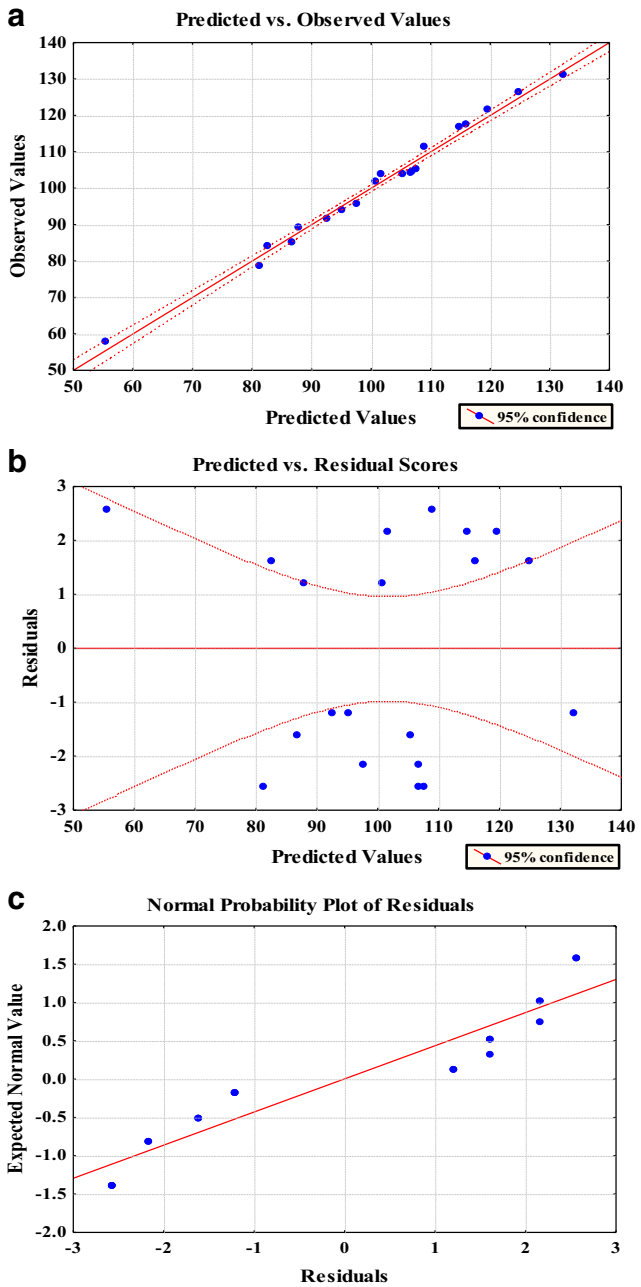


**Fig. 5** **a** Correlation between the observed and predicted values for L-asparaginase activity of *Streptomyces brollosae* NEAE-115 determined by the first-order polynomial equation. **b** Plot of residuals against predicted values for L-asparaginase production. **c** The normal probability plot of the residuals

medium are used to enhance growth, and in our study subsequently resulted in higher enzyme production, which is normally observed in the synthesis of primary metabolites, such as enzymes. Poor growth in an SSF system is associated with poor nutritional level in terms of solid substrates. We observed that the carbon concentration had a positive effect on L-asparaginase production and that high titers could be obtained in a medium rich in a carbon source. Various researchers have reported the positive effect that supplementation of different carbon sources has on L-asparaginase production (Kumar et al. 2009, 2010).

The effect of carbon source on growth and metabolite production is dependent upon several factors, such as carbon source concentration (Chen et al. 2008). Khamna et al. (2009) reported maximum L-asparaginase activity by *Amycolatopsis* strain CMU-H002 when the strain was cultivated in ADS broth amended with soluble starch (0.2 %). Kumar et al. (2009) reported maximum L-asparaginase activity when 0.2 % glucose was used as a carbon source, and Gurunathan and Sahadevan (2011) reported that 0.6 % glucose was found to be best carbon source for maximum L-asparaginase production by *Aspergillus terreus* MTCC 1782 when grown on a modified Czapek-dox media containing soyabean flour as a substrate. Among the tested carbon sources, L-asparagine or the combination of L-asparagine + glucose was found to be the most suitable carbon sources to maximize the production of L-asparaginase (Kumar et al. 2010). Maximum L-asparaginase activity was obtained by isolated *Bipolaris* sp. BR438 using the modified Czapek-Dox media containing 1 % L-asparagine and 0.4 % glucose (Lapmak et al. 2010).

Soyabean meal has been shown to be the best nitrogen source for L-asparaginase production (Sivasankar et al. 2013). El-Bessoumy et al. (2004) reported that *Pseudomonas aeruginosa* produced L-asparaginase under SSF conditions when soyabean meal was used as substrate, as also demonstrated by Dharmsthiti and Luechai (2011) in *Aspergillus niger* AK10 under SSF conditions. That soyabean has been shown to be the best substrate for the production of L-asparaginase may be due to the presence of sufficient amounts of lipids, proteins, carbohydrates and minerals. Enhanced production of L-asparaginase was observed in *Streptomyces albidoflavus* grown on medium with maltose as a sole carbon source and yeast extract as the sole nitrogen source (Narayana et al. 2008).

Maltose (0.5 %) and L-asparagine (0.5 %) proved to be the best carbon and nitrogen sources, respectively, for the production of L-asparaginase in *Streptomyces gulbargensis* (Amena et al. 2010). Additional nitrogen supplements, exclusively organic nitrogen in L-asparagine broth medium, were found to induce maximum L-asparaginase activity, whereas supplementation of inorganic nitrogen sources lowered L-asparaginase production drastically (Venil and Lakshma naperumalsamy 2009). L-asparaginase expression increases in the presence of L-asparagine, suggesting that it is nitrogen regulated and inducible, as observed in other microorganisms (Sarquis et al. 2004). L-asparagine acts as sole nitrogen source and also as an inducer of L-asparaginase production; consequently, variations in its concentration will have some impact on enzyme production. Sreenivasulu et al. (2009) reported that L-asparagine provided in the medium at 2 % (w/v) was the optimum concentration for L-asparaginase production (16.5 U/ml). The effect of varying the concentration of L-asparagine (0.5–2.0 %) as a sole nitrogen source on L-

asparaginase production was studied in *Streptomyces* ABR2, and the optimum concentration was determined to be 1 % (Sudhir et al. 2012). In another report, Gurunathan and Sahadevan (2011) showed that 1 % (w/v) of L-asparagine in the media induced maximum production of L-asparaginase by *Aspergillus terreus* MTCC 1782. The activity of L-asparaginase decreased with increase in the concentration of L-asparagine above 1 %, possibly due to substrate inhibition. L-asparagine was reported to be suitable nitrogen source for L-asparaginase production by *Streptomyces karnatakensis* and *S. venezuelae* (Mostafa 1979). Maximum L-asparaginase production using 0.1 % (w/v) L-asparagine as the sole source of nitrogen has been observed in *Aeromonas* sp. (Pattnaik et al. 2000).

$KH_2PO_4$ has been found to have a significant influence on L-asparaginase production (Kumar et al. 2009). Baskar and Renganathan (2009) have mentioned di-potassium hydrogen phosphate as one of the important factors for L-asparaginase production. Phosphate is required in a certain amount for growth and metabolism, and it plays a key role in the regulation of enzymes. The absence of mineral sources of phosphorous in the medium is known to cause a substantial drop in the activity and a decrease in the intensity of growth of the culture, which is not only due to the significance of phosphorous as an element of nutrition, but also to the buffering capacity of its salts. In addition to its role as an important constituent of cellular biomolecules, such as cyclic-AMP, nucleic acids, phospholipids and co-enzymes, phosphate is known to play a regulatory role in the synthesis of primary and secondary metabolites in microorganisms (Weinberg 1974).

## Optimization by CCD

The CCD was employed to study the interactions among the significant factors and also to determine their optimal levels. The most significant positive independent variables affecting L-asparaginase production (in the Plackett–Burman experiment), namely, soybean meal + wheat bran ($X_6$), L-asparagine ($X_9$) and $K_2HPO_4$ ($X_{12}$), were selected and further investigated using CCD; the other variables in the study were maintained at a constant level; this design resulted in maximal yield in the Plackett–Burman experiments. We ran a total of 20 experiments with different combinations of the three independent variables; the predicted and observed values for L-asparaginase production together with the design matrix are presented in Table 5. The central point was repeated six times (trial number: 3, 11, 13, 14, 18 and 20). The results were analyzed by ANOVA and show considerable variation in L-asparaginase activity. The minimum L-asparaginase activity (74.20 U/gds) was observed in run number 5, while maximum L-asparaginase activity (149.65 U/gds) was achieved in run number 14.

**Table 5** Central composite design, representing the response of L-asparaginase production as influenced by soybean meal + wheat bran L-asparagine and $K_2HPO_4$ along with the predicted L-asparaginase production and residuals and the levels of variables

| Trials | Variables | | | L-asparaginase activity (U/gds) | | Residuals |
|---|---|---|---|---|---|---|
| | $X_6$ | $X_9$ | $X_{12}$ | Experimental | Predicted | |
| 1 | 0 | −1.68 | 0 | 85.09 | 88.02 | −2.92 |
| 2 | −1 | 1 | 1 | 125.06 | 115.91 | 9.16 |
| 3 | 0 | 0 | 0 | 145.56 | 146.74 | −1.18 |
| 4 | 0 | 1.68 | 0 | 104.66 | 113.65 | −8.99 |
| 5 | 1 | −1 | 1 | 74.20 | 71.87 | 2.34 |
| 6 | −1 | −1 | 1 | 114.51 | 109.99 | 4.52 |
| 7 | 0 | 0 | −1.68 | 134.69 | 139.45 | −4.76 |
| 8 | 1 | −1 | −1 | 101.93 | 102.68 | −0.75 |
| 9 | −1 | 1 | −1 | 121.95 | 115.88 | 6.07 |
| 10 | 0 | 0 | 1.68 | 106.44 | 113.59 | −7.15 |
| 11 | 0 | 0 | 0 | 147.53 | 146.74 | 0.80 |
| 12 | 1 | 1 | −1 | 131.16 | 127.28 | 3.88 |
| 13 | 0 | 0 | 0 | 149.10 | 146.74 | 2.36 |
| 14 | 0 | 0 | 0 | 149.65 | 146.74 | 2.92 |
| 15 | 1.68 | 0 | 0 | 81.00 | 81.87 | −0.87 |
| 16 | −1.68 | 0 | 0 | 93.28 | 104.32 | −11.04 |
| 17 | −1 | −1 | −1 | 107.78 | 102.17 | 5.61 |
| 18 | 0 | 0 | 0 | 144.18 | 146.74 | −2.55 |
| 19 | 1 | 1 | 1 | 91.46 | 88.66 | 2.80 |
| 20 | 0 | 0 | 0 | 146.50 | 146.74 | −0.23 |
| Level | g/L | g/L | g/L | | | |
| −1.68 | 8 | 8 | 1.5 | | | |
| −1 | 12 | 10 | 2 | | | |
| 0 | 16 | 12 | 2.5 | | | |
| 1 | 20 | 14 | 3 | | | |
| 1.68 | 24 | 16 | 3.5 | | | |

$X_6$ Code for soybean meal + wheat bran, $X_9$ code for L-asparagine, $X_{12}$ code for $K_2HPO_4$

## Multiple regression analysis and ANOVA

Multiple regression analysis was used to analyze the data. The results of the ANOVA, which is required to test the significance and adequacy of the model and model coefficients, are presented in Table 6. The determination coefficient ($R^2$) values, which are always between 0 and 1, provide a measure of how much variability in the observed response values can be explained by the experimental factors and their interactions. The closer the $R^2$ value is to 1, the stronger the model is and the better it predicts the response (Kaushik et al. 2006). The determination coefficient ($R^2$) of the model was 0.9574, indicating that 95.74 % of variability in the response could be

**Table 6** Statistical analysis of face-centered central composite design showing coefficient values, main effect, *t* test and *P* values

| Variables | Coefficients | Main effect | *t* Statistic | *P* value | |
|---|---|---|---|---|---|
| Intercept | 146.73 | 293.47 | 50.38 | 0.000 | |
| Soybean meal + wheat bran ($X_6$) | −6.68 | −13.37 | −3.46 | 0.006 | |
| L-asparagine ($X_9$) | 7.63 | 15.26 | 3.95 | 0.003 | |
| $K_2HPO_4$ ($X_{12}$) | −7.70 | −15.40 | −3.98 | 0.003 | |
| $X_6X_9$ | 2.72 | 5.44 | 1.08 | 0.307 | |
| $X_6X_{12}$ | −9.66 | −19.32 | −3.83 | 0.003 | |
| $X_9X_{12}$ | −1.95 | −3.90 | −0.77 | 0.458 | |
| $X_6X_6$ | −19.00 | −38.01 | −10.09 | 0.000 | |
| $X_9X_9$ | −16.26 | −32.53 | −8.63 | 0.000 | |
| $X_{12}X_{12}$ | −7.16 | −14.32 | −3.80 | 0.004 | |
| ANOVA | | | | | |
| | *df* | *SS* | *MS* | *F* test | Significance *F* (*P*) |
| Regression | 9 | 11476.79 | 1275.20 | 25.01 | 1.05001E-05 |
| Residual | 10 | 509.97 | 51.00 | | |
| Total | 19 | 11986.75 | | | |

$X_6$, Code for value of soybean meal + wheat bran, $X_9$ code for value of L-asparagine, $X_{12}$ code for value of $K_2HPO_4$

Multiple *R* 0.9784, $R^2$ 0.9574, Adjusted $R^2$ 0.9191; see footnote to Table 4 for definitions

explained by the model and that only 4.26 % of the total variance could not be explained by the model. A regression model with an $R^2$ value of >0.9 was considered is considered to have a very high correlation (Chen et al. 2009). Therefore, the $R^2$ value found in our study reflects a very good fit between the observed and predicted responses and implies that the model is reliable for the production of L-asparaginase. A higher value of the correlation coefficient ($R = 0.9784$) signifies an excellent correlation between the independent variables, indicating a good correlation between the experimental and predicted values (Box et al. 1978). In addition, the value of the adjusted determination coefficient (Adj. $R^2 = 0.9191$) is also very high in our study, which indicates a high significance of the model (Akhnazarova and Afarov 1982). Thus, we considered the analysis of the response trend using the model to be reasonable.

Interpretation of the data presented in Table 6 was based on the signs (positive or negative effect on the response) and statistical significance of coefficients ($P < 0.05$). Interactions between two factors could appear as an antagonistic effect (negative coefficient) or a synergistic effect (positive coefficient). The probability values of the coefficient suggest that the linear and quadratic effects of soybean meal + wheat bran ($X_6$), L-asparagine ($X_9$) and $K_2HPO_4$ ($X_{12}$) and the interaction between soybean meal + wheat bran ($X_6$) and $K_2HPO_4$ ($X_{12}$) are significant in terms of improving L-asparaginase production. It can be seen from the degree of significance that the interaction between soybean meal + wheat bran ($X_6$) and L-asparagine ($X_9$) and the interaction between L-asparagine ($X_9$) and $K_2HPO_4$ ($X_{12}$) are not significant ($P$ value > 0.05),

A second-order polynomial model (Eq. 4) was proposed to evaluate the relationship between L-asparaginase production and independent variables and to determine the maximum L-asparaginase production corresponding to the optimum levels of soybean meal + wheat bran ($X_6$), L-asparagine ($X_9$) and $K_2HPO_4$ ($X_{12}$). The maximum L-asparaginase production can be described as a function of the optimum levels of these variables:

$$Y_{(\text{L-asparaginase production})} = 146.73 - 6.68X_6 + 7.63X_9 - 7.70X_{12} + 2.72X_6X_9 - 9.66X_6X_{12} - 1.95X_9X_{12} - 19.00X_6^2 - 16.26X_9^2 - 7.16X_{12}^2 \quad (4)$$

The $Y$ is the predicted response, $X_6$ is the coded value of soybean meal + wheat bran, $X_9$ is the coded value of L-asparagine and $X_{12}$ is the coded value of $K_2HPO_4$.

The interaction effects and optimal levels of the variables were determined by plotting the response surface curves (shown in Fig. 6a–c) when one of the variables was fixed at

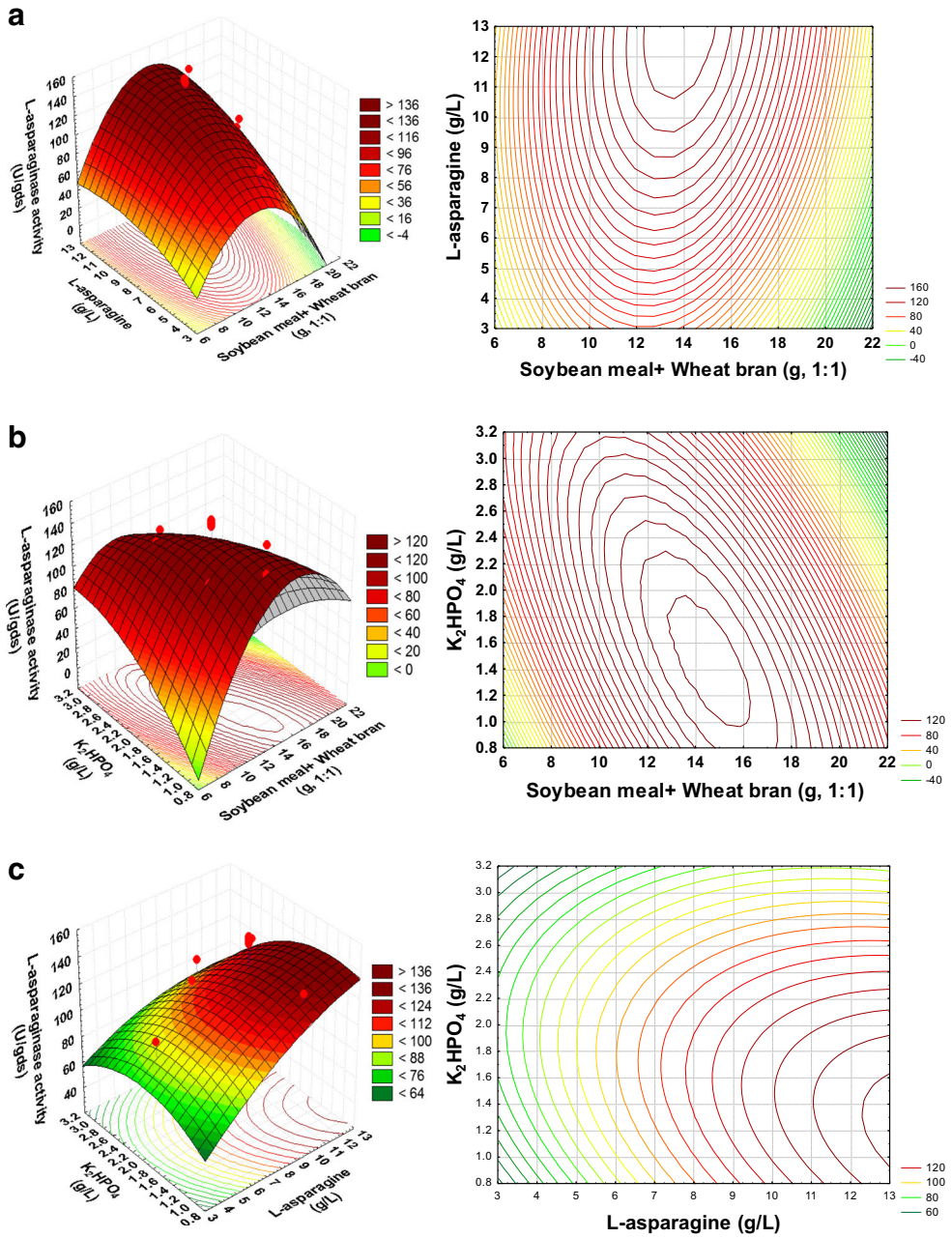


**Fig. 6 a-c**. Three-dimensional response surface plots and Contour plots showing the effect of the variables and their mutual effects on L-asparaginase production. **a** Effects of changes in soybean meal + wheat bran, L-asparagine and $K_2HPO_4$ level is fixed at 2 g/l. **b** Effects of soybean meal + wheat bran, $K_2HPO_4$ and L-asparagine level is fixed at 12 g/l. **c** Effects of L-asparagine, $K_2HPO_4$ and soybean meal + wheat bran (1:1) are fixed at 16 g/l

its optimum value and the other two were allowed to vary. Figure 6a represents L-asparaginase activity as a function of soybean meal + wheat bran ($X_6$), L-asparagine ($X_9$) by keeping $K_2HPO_4$ ($X_{12}$) fixed at optimum value. The plot shows that lower and higher levels of soybean meal + wheat bran support relatively low levels of L-asparaginase activity, with the highest value of L-asparaginase activity obtained at the middle range of soybean meal + wheat bran levels. Maximum L-asparaginase activity was attained at high levels of L-asparagine, with L-asparaginase activity increasing with increasing L-asparagine concentration. Interaction between soybean meal + wheat bran ($X_6$) and L-asparagine ($X_9$) is synergistic.

Figure 6b represents L-asparaginase activity as a function of soybean meal + wheat bran ($X_6$) and $K_2HPO_4$ ($X_{12}$) by keeping L-asparagine ($X_9$) at its optimum value. Maximum L-asparaginase activity was attained at moderate levels of both soybean meal + wheat bran and $K_2HPO_4$, with further

increases in the levels of both resulting in a gradual decrease in L-asparaginase activity. Figure 6c shows that the maximum L-asparaginase production was attained at higher levels of L-asparagine ($X_9$) and middle levels of $K_2HPO_4$ ($X_{12}$), where higher levels of $K_2HPO_4$ resulted in a decrease in L-asparaginase production. This means that the interaction between $K_2HPO_4$ and L-asparagine is antagonistic.

### Verification of the experimental model

In order to determine the accuracy of the model and to verify the result, an experiment was performed under the optimal conditions determined from the CCD–response surface methodology and the results compared with the predicted data. The measured L-asparaginase activity obtained from the experiment (145.57 U/gds) was very close to the response (149.97 U/gds) predicted by the regression model, revealing a high degree of accuracy. This verification revealed a high degree of accuracy of the model (97.06 %), indicating model validation under the tested conditions. The predicted optimal levels of the process variables for L-asparaginase production by *Streptomyces brollosae* NEAE-115 were soybean meal + wheat bran (g; 1:1) (16 g/L), L-asparagine (12 g/L) and $K_2HPO_4$ (2 g/L).

## Conclusion

The Plackett–Burman experimental design was used to screen parameters affecting the production of L-asparaginase by *Streptomyces brollosae* NEAE-115 during SSF, and the most significant independent variables affecting enzyme production were further optimized by the CCD. Based on these findings, we identified the optimum culture conditions for *Streptomyces brollosae* NEAE-115 in terms of extracellular L-asparaginase production. These are: an incubation time of 7 days, a moisture content of 70 %, an inoculum size of 4 ml, an incubation temperature of 30 °C, medium pH 7 and a revised medium (in g/L: soybean meal + wheat bran, 16; dextrose, 2; fructose, 2; L-asparagine, 12; yeast extract, 2; $KNO_3$, 1; $K_2HPO_4$, 2; $MgSO_4.7H_2O$, 0.1; NaCl, 0.1; $FeSO_4.7H_2O$, 0.02; $CaCl_2$, 0.01). L-asparaginase production by *S. brollosae* NEAE-115 was 47.665, 129.928 and 149.972 U/gds after the initial survey using soybean meal + wheat bran as a substrate for L-asparaginase production (step 1), after statistical optimization by Plackett–BurmanDesign (step 2) and after CCD (step 3), respectively, with a fold of increase 3.15.

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