Abstract

In the present study, economical fermentation medium process was developed to achieve the maximum production of L-methionine by Corynebacterium glutamicum through screening of different nutritional and physical parameters by Plackett-Burman design. A total of eleven process variables such as plantain as carbon source, groundnut as nitrogen source, CaCO₃, K₂HPO₄, KH₂PO₄, biotin, MgSO₄.7H₂O, inoculum size, agitation speed, volume ratio of medium/fermenter and pH were used for screening experiments. The PBD model results suggested that seven variables namely plantain as carbon source, groundnut as nitrogen source, CaCO₃, MgSO₄.7H₂O and KH₂PO₄ had shown a significant effect on L-methionine production, while remaining six variables didn’t show a much effect on L-methionine production. The R² value (0.99) of analysis of variance (ANOVA) recommended that the model used for response prediction is significant (p<0.05). In comparison with the unoptimized medium, 24% higher L-methionine production was obtained from the optimized medium and L-methionine production was found to be 5.6 g/l.

Keywords: L-methionine, Corynebacterium glutamicum, Optimization, Plackett-Burman Design.

Introduction

Methionine, alpha-L-amino-gamma-methylthio-nbutyric acid is nutritionally essential for mammals and fowls. It can’t be synthesized internally, but may be added to food and feed materials to improve the protein quality (1). Methionine is generally being produced by chemical and enzymatic methods, both are expensive, chemical method requires hazardous chemicals and enzymatic method requires expensive enzymes. Methionine can be produced economically by using fermentation, because many fermentation processes have been developed to produce many other amino acids inexpensively (1, 2 and 3).

Plant proteins are frequently deficient in methionine and consequently an exclusively vegetable diet may fail to meet nutritional requirements. Methionine deficiency has been linked to development of various diseases and physiological conditions including toxemia, childhood rheumatic fever, muscle paralysis, hair loss, depression, schizophrenia, Parkinson’s liver deterioration, and impaired growth (4). Deficiencies can be overcome by supplementing the diet with methionine and, therefore, methionine is of significant interest (5).

The history of species Corynebacterium as amino acid producer started in the 1950s when...
Dr. Kinoshita was the first to discover that Corynebacteria glutamicum is a superior amino acid producer (6, 7 and 8). Now a day’s L-glutamic acid, L-lysine, L-isoleucine, L-threonine, L-aspartic acid and L-alanine are produced by Corynebacteria in terms of high production rate and economical value.

In this work, we carried out the screening of critical medium fermentation components and conditions, which have been predicted to play a significant role on methionine production by Corynebacteria glutamicum using Plackett-Burman design. Design expert 8.0.7.1 (Stat-Ease) statistical software was used to carry out Plackett-Burman design, statistical analysis of results and coefficient of the effect estimate.

Materials and Methods

Chemicals

All the chemicals and reagents were purchased from Hi-Media, Mumbai. Plantain and groundnut were obtained from the local market in Guntur, Andhra Pradesh, India.

Microorganism and Culture conditions

L-Methionine producing strain of C. glutamicum MTCC 2745 obtained from the microbial type collection centre, Chandigarh, India was used throughout this study. It was maintained on nutrient agar slants (Beef extract 1 g/l; Yeast extract, 2 g/l; Peptone, 5 g/l; NaCl, 5 g/l; Agar, 15 g/l and pH was adjusted to 7.2 with 1N NaOH) and stored at refrigeration temperature 4°C for further analysis. 3 ml of 24 hour slant culture was used to inoculate a 100 ml Erlenmeyer flask containing 30 ml of seed medium.

Design of Experiments

The nutrient and physical parameters such as plantain as carbonsource, groundnut as nitrogen source, CaCO₃, KH₂PO₄, KH₄PO₄, biotin, MgSO₄.7H₂O, inoculum size, agitation speed, volume ratio of medium/fermenter and pH were used for experimental screening purpose. Fermentation (shake flask) experiments were designed as per the PBD matrix (Shown in Table.2) based on 30 mL of medium dispensed into 100 mL Erlenmeyer flask. Fermentation medium was sterilized at 121°C and 15 min. Upon cooling, the inoculum was added to the media and flasks were incubated in an orbital shaker at 170 rpm. All measurements were done in triplicates and average values were reported. The effect of individual components on L-Methionine production was calculated by following equation.

\[
E = \frac{2(\sum H^+ - \sum H^-)}{N}
\]

Where E is the effect of parameters and H^+ and H^- are responses of trails in which the parameter high and low levels respectively and N is the number of trails.

Analytical Techniques

Carbon sources (Preparation of starches)

Agriculture products utilized here for the preparation of starch is plantain. Starch was prepared according to the method portrayed by (9). Plantain samples were brought from Guntur (Andhra Pradesh, India) local market were first peeled, washed and cut into little pieces before being homogenized with water in Moulinex blender. Homogenate blended with excess water was tied in cheese cloth and placed on tripod stand overnight, to take into account extraction of starch into a clean plastic bowl. The supernatant was emptied and the sedimented starch dried at 50°C for 48 hours. The resultant chips were grounded into powder and utilized as starches.

Saccharification of starch

Saccharification of starch took after the method illustrated by (2). A 500 ml flask containing a mixture of 30g of starch and 100 ml of water was heated for 15 min at 95°C in a water bath to gelatinize starch. The beaker was covered with aluminium foil after adding 1 ml of α-amylase and again heated in water bath for 10 min at 95°C to impact liquefaction. After cooling liquefied starch to 60°C, 1 ml amyloglucosidase enzyme was added before replacing the beaker in the water bath at 60°C for 48 hr for saccharification to takes place.
Nitrogen sources: Preparation of defatted proteins

The protein utilized here from agricultural products as nitrogen sources is groundnut. For preparation of defatted protein took after the strategy explained by study (2). Groundnut was crushed in a blender and then some division of homogenized proteins was defatted by soxhlet extraction method using diethyl ether. The meals obtained after extraction were oven dried at 34-35°C for 20 hr and afterward ground into fine powder.

L-Methionine Assay

Quantitative determination of L-methionine in the culture broth without purification was carried out by the modified calorimetric method (10). A 5 ml volume of the culture broth was centrifuged at 5,000xg for 20 minutes and the cell free supernatant was assayed for L-methionine.

1 ml of 5N NaOH was added to a test tube followed by the addition of 0.1ml of 10% sodium nitroprusside solution with thorough mixing. The mixture was allowed to stand for 10 min. Then two milliliters of 3% aqueous solution of glycine was added to the reaction mixture with frequent shaking over a period of 10 min. After an additional 10 min interval, 2ml of concentrated orthophosphoric acid was added drop wise to the mixture with shaking. Colour development was allowed to proceed for 5 min and the colour intensity measured at 540nm in a spectrometer. A blank containing distilled water and all other reagent served as the 100% transference standard. Results obtained with the test samples were interpolated on a standard methionine curve.

Estimation of Reducing Sugar

The reducing sugar (glucose) in the time-course fermentation broth was estimated by the modified method described by (11). A 1ml volume of dinitrosalicylic acid was added to 1ml of the supernatant in a test tube and the mixture heated in boiling water for 10 minutes. The test tube was cooled rapidly under tap water. 1ml of 4% potassium sodium tartarate was added and the volume was adjusted to 12 ml with distilled water. A blank containing 1 litre of distilled water and 1 ml of dinitrosalicylic acid was similarly prepared. The optical density of the sample was read against the blank in a spectrophotometer at 540nm. The concentration of the reducing sugar in the supernatant was estimated from a standard glucose curve.

Results and Discussion

Effect of process parameters on L-methionine production

The effect of 11 process parameters such as plantain as carbon source, groundnut as nitrogen source, CaCO₃, KH₂PO₄, KH₂PO₄, biotin, MgSO₄·7H₂O, inoculum size, agitation speed, volume ratio of medium/fermenter and pH on L-methionine production by _C. glutamicum_ were examined. In this work, 12 experimental runs were carried out to screen 11 parameters using Plackett-Burman design. The low and high levels of these parameters are used in PBD were shown in Table.1. The PBD matrix for influences of 11 parameters on L-methionine production and their responses are shown in Table.2.

Results from Table.2 indicated that highest concentration of L-methionine (4.5 g/L) achieved in run no.8 with the composition: plantain as carbon source- 25 g/L, groundnut as nitrogen source-15 g/L, CaCO₃- 25 g/L, KH₂PO₄- 0.5 g/L, KH₂PO₄- 0.5 g/L, biotin- 75 μg/L, MgSO₄·7H₂O- 2 g/L, inoculum size- 3 ml, agitation speed- 200 rpm, volume ratio of medium/fermenter- 35 %mL/mL and pH- 6.00 and the lowest concentration of L-methionine (1.6 g/L) achieved in run no.10 with the composition: plantain as carbon source- 15 g/L, groundnut as nitrogen source-5 g/L, CaCO₃- 15 g/L, KH₂PO₄- 1 g/L, KH₂PO₄- 0.5 g/L, biotin- 125 μg/L, MgSO₄·7H₂O- 2 g/L, inoculum size- 3 ml, agitation speed- 200 rpm, volume ratio of medium/fermenter- 35 %mL/mL and pH- 8.00.

ANOVA of PBD results (Table.3) suggested that only 5 variables namely plantain as carbon source, groundnut as nitrogen source, CaCO₃, MgSO₄·7H₂O and KH₂PO₄ had shown significant effect (p<0.05) on L-methionine production. The

Plackett-Burman design for L-methionine production
remaining 6 variables not had shown significant contribution (p>0.05) to L-methionine production. There is a close agreement between experimental and theoretical values of L-methionine. The R² value of 0.99 proved that PBD model was significant in estimating the effects of variables on L-methionine production by *C. glutamicum*.

The Pareto chart (Fig.1) reveals the order of significance of parameters affecting L-methionine production and shows an easy way to view the results achieved in PBD experiment. From Fig.1 the order of most significant variables was shown as plantain as carbon source (A), groundnut as nitrogen source (B), CaCO₃ (C), MgSO₄.7H₂O (G)
and KH2PO4 (E) and remaining six variables had not shown a significant effect on L-methionine production.

Linear equations representing L-methionine production to process parameters (input variables) shown by PBD model as follows:

\[
L\text{-}\text{methionine yield} = 3.10 + 0.49 \times \text{plantain as carbon source} + 0.34 \times \text{groundnut as nitrogen source} + 0.32 \times \text{CaCO3} + 0.24 \times \text{MgSO4} \cdot 7\text{H2O} - 0.025 \times \text{K2HPO4} + 0.2 \times \text{KH2PO4} + 0.24 \times \text{MgSO4} \cdot 7\text{H2O} \times 0.05 \times \text{inoculum size} - 0.14 \times \text{volume ratio of medium to fermenter} - 0.24 \times \text{pH} + 0.837 \times \text{MgSO4} \cdot 7\text{H2O} + 0 \times \text{agitation speed}
\]

Fig. 2 reveals normal plot for L-methionine production to estimate the significant factors. In the normal plot of effects, the points which do not exist near the line are significant. Important effects are far away from the fitted line than unimportant effects. Unimportant effects tend to be smaller and centered on zero. In this case, important effects are plantain as carbon source, groundnut as nitrogen source, CaCO3, MgSO4·7H2O and KH2PO4 for L-methionine production by C. glutamicum.

**Optimization of process parameters for maximizing L-methionine production**

Fig. 3 shows the ramps of PBD model estimates the optimum values of input process parameters for maximum production of L-methionine. As per the PBD model, the optimum values of input parameters were predicted as plantain as carbon source- 18.25 g/L, groundnut as nitrogen source- 14.1 g/L, CaCO3- 9.25 g/L, K2HPO4- 0.57 g/L, MgSO4·7H2O- 1.9 g/L, inoculum size- 1 mL, agitation speed- has no effect, volume ratio of medium/fermenter- 31.5 and pH- 7.0. Experiments were carried out with optimum values and obtained the L-methionine production as 5.6 g/L. In comparison with unoptimized medium 24% increase in L-methionine yield was obtained.

In Plackett-Burman screening plantain and groundnut were two of most significant variables on L-methionine production and in the earlier experiments.
works plantain and groundnut were reported by (12) using Bacillus cereus S8 from agricultural products and produced 2.05 mg/ml of methionine in submerged fermentation. In 2008, Adoki used plantain waste as carbon source for yeast growth and protein production by Candida species (13). Another significant variable MgSO<sub>4</sub>·7H<sub>2</sub>O has a positive impact on L-Methionine production, this is mostly due to methionine is sulphur containing amino acid addition of MgSO<sub>4</sub>·7H<sub>2</sub>O to the medium improved the growth and L-methionine production and many workers (Kase et al. 1975, Banik et al.

<table>
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<th>Maximum value (+1)</th>
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Table 2. Plackett-Burman Design matrix for L-Methionine production by C. glutamicum with experimental and predicted values.

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<th>B (g/L)</th>
<th>C (g/L)</th>
<th>D (g/L)</th>
<th>E (g/L)</th>
<th>F (µg/L)</th>
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<th>H (ml)</th>
<th>I (rpm)</th>
<th>J (%)</th>
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Table 1. Low and high levels of nutrient (chemical) and physical process variables used in Plackett-Burman design for the production of L-Methionine by C. glutamicum.
Calcium carbonate is also one of significant variables for maximum production of L-methionine. It balances the pH of fermentation broth by eliminating lag phase of cell growth thus reduction in fermentation time. As nutrients are consumed and converted into products during fermentation process, the pH changes significantly in the absence of suitable control mechanism. In order to keep optimal pH, reagents such as calcium carbonate should be added to the fermentation medium at the starting of the fermentation [17]. The other variable KH₂PO₄ also affects the production of L-methionine. Due to the potential applications of methionine in feed, food, pharmaceutical and health industry, the results presented here are promising and can be used as insights to enhance production of L-methionine by fermentation at industrial level.

**Conclusions**

The Plackett-Burman Design was efficiently used for screening of process parameters for L-methionine production by *C. glutamicum*. Results of PBD confirmed that five variables such as plantain as carbon source, groundnut as nitrogen source, CaCO₃, MgSO₄·7H₂O and KH₂PO₄ were found to be most significant on L-methionine production by *C. glutamicum*. And the remaining six variables had not shown a significant effect on L-methionine production. In comparison with the unoptimized medium, 24% increases in L-methionine production was obtained from the optimized medium and L-methionine production was found to be 5.6 g/L. Owing to the potential applications of methionine in feed, food, pharmaceutical and health industry, the results presented here are promising and can be used as insights to enhance production of L-methionine by fermentation at industrial level.

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applications of L-methionine in feed and food industries, results presented here are very promising and provides new insights for enhancing L-methionine production at industrial level.

References