Research Article

RSM-Based Optimization of Fermentation Conditions and Kinetic Studies of Glutamic Acid and Lysine Production by *Corynebacterium glutamicum*

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Corynebacterium glutamicum is an authenticated microorganism that supports amino acid production consistent with dietary importance. Fermentation parameters like temperature, agitation speed, and carbon source concentration were optimized using response surface methodology. Surface response model suggested that optimal fermentation parameters including 30°C, 50 g/L glucose concentration, and shaking speed of 120 rpm furnished 14.2 g/L of glutamic acid and 5.1 g/L lysine, comparable with the predicted values. After optimizing fermentation parameters in shake flasks, the fermentation kinetics was studied in a stirred fermenter. The kinetic study revealed that the substrate consumption rate achieved a maximum level of 3.36 g/L/hour between 12 and 18 hours; afterwards, it decreased and fell to 1.9 g/L/hour. Average biomass yield over a period of 48 hours was 0.337 g/L; however, maximum biomass yield of 0.51 g/L was noted between 10 and 20 hours. Overall molar yield coefficient of CO_2 ($Y^C_{CO2/S}$) was found to be 0.234. The molar yield coefficient of biomass ($Y^C_{X/S}$) was 0.6 in exponential phase which decreased afterwards. This study indicates that the average biomass yield over a time of 48 hours was 0.337 g/L, with a maximum yield within 10 to 20 hours obtained under optimized conditions. The molar yield coefficient of CO_2 ($Y^C_{CO2/S}$) was found to be 0.234. The molar yield coefficient of biomass ($Y^C_{X/S}$) was 0.6 in exponential phase which decreased and fell to near zero at 48 hrs.

1. Introduction

Amino acids are the basic functional and structural units of proteins and play a major role in the regulation of vital metabolic pathways for the sustainable cultivation of microorganisms [1]. Amino acids are very helpful in promoting growth through several actions including efficient regulation of muscle protein, controlling growth and immunity of organisms, and optimizing the efficiency of food metabolism [2–4]. Since 1907, the production of monosodium glutamate

[5] has raised its demand in international market and gave a boost to amino acid production. A wide range of biochemical products have also multiplied as a result of their applications in industries particularly in the area of livestock as feed additive, as healthy ingredient in pharmaceuticals and cosmetics, and as seasoning in human nutrition [6]. Production of amino acids has been tremendously increased as these biomolecules have vast applications that include animal feed supplements, cosmetics, additives in food, chemicals in agriculture, and polymer materials.
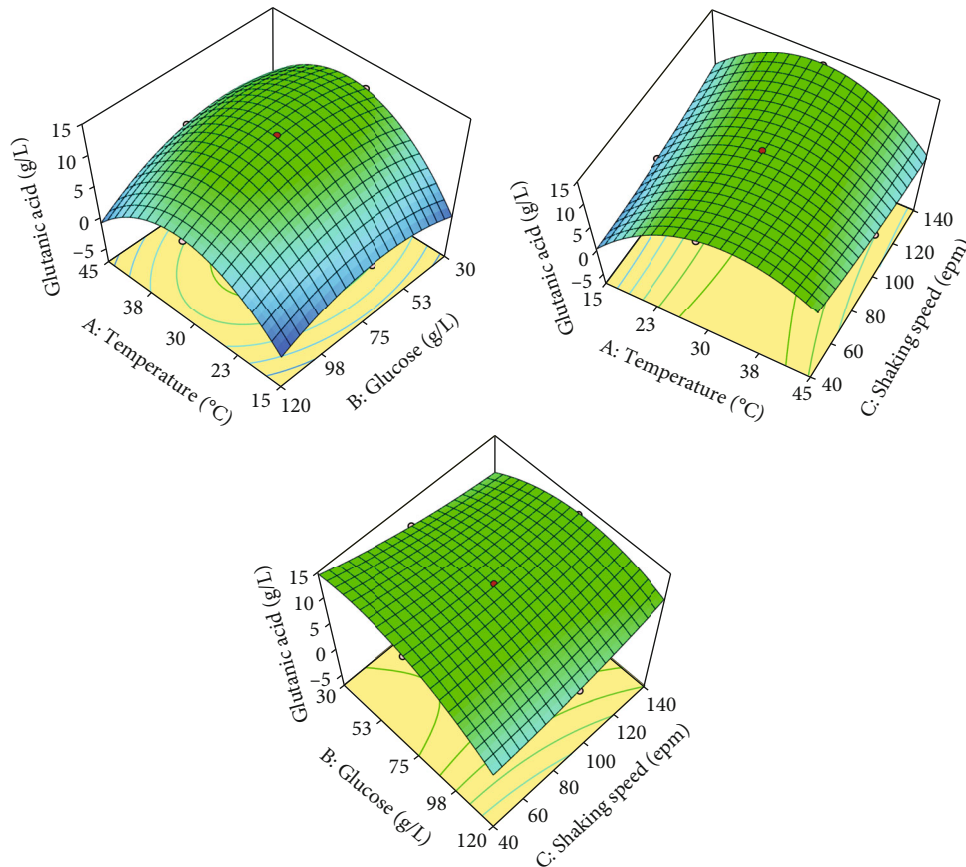
FIGURE 1: Synergism between parameters affecting glutamic acid production.

Fermentation is well thought-out as the most efficient, cost-effective, and beneficial technique in terms of amino acid synthesis. During fermentation, microorganisms use cheap raw materials as fermentation substrates. The process of fermentation is an advanced industrial method that uses several microorganisms to transform sugar substrates into a wide range of amino acids, both aerobically and anaerobically. A number of reasons contribute to the efficacy of amino acid synthesis by fermentation. First, as a result of fermentation, only the L-form stereoisomer of amino acids is produced which help escape any additional purification steps. Second, fermentation procedure is carried out at mild conditions which prevent any product degradation. Third, the maintenance costs are significantly less in comparison to the extraction processes [6, 7].

In recent years, varieties of technologies have been developed due to increased interest in amino acid production. Bacteria like *E. coli* and *C. glutamicum* are both useful for amino acid production through fermentation [7]. A variety of genetically engineered species have been applied under optimized conditions as amino acid producers. Lysine and glutamic acid are produced by genetically modified C. glutamicum with high yields up to 50% (*w/w*) [8, 9]. There are many strains of microorganisms that have been modified genetically and are used for amino acid production. *Corynebacterium glutamicum* is primarily aerobic, nonpathogenic, rod-shaped, Gram-positive bacterium, commonly recognized as a safe (GRAS) creature for manufacturing bioelements like amino acids and nucleotides [10–12]. *C. glutamicum* was recognized in the biotechnology industry for amino acid production on a business scale containing L-lysine and L-glutamate as flavor enhancers since 1960 [13–15]. Since half a century after its discovery, this microorganism has participated in the amino acid production by fermentation and has an extensive and effective record in the biotechnological formation of L-lysine and L-glutamic acid [16, 17]. *C. glutamicum* is genetically modified for the production of glutamic acid with maximal yield (50% ww^{-1}), while aromatic amino acids are produced by genetically modified *Escherichia coli* [18].

2. Results and Discussion

2.1. RSM-Based Simultaneous Optimization of Temperature, Shaking Speed, and Carbon Source Concentration. Response surface methodology was followed for optimizing fermentation parameter including temperature, amount of glucose syrup (with 60% solids) in the fermentation medium, and rotational speed of an orbital shaking incubator. Many experiments were performed, and reaction conditions were carefully investigated to determine the optimum parameters for the production of glutamic acid (Figure 1) and lysine (Figure 2).
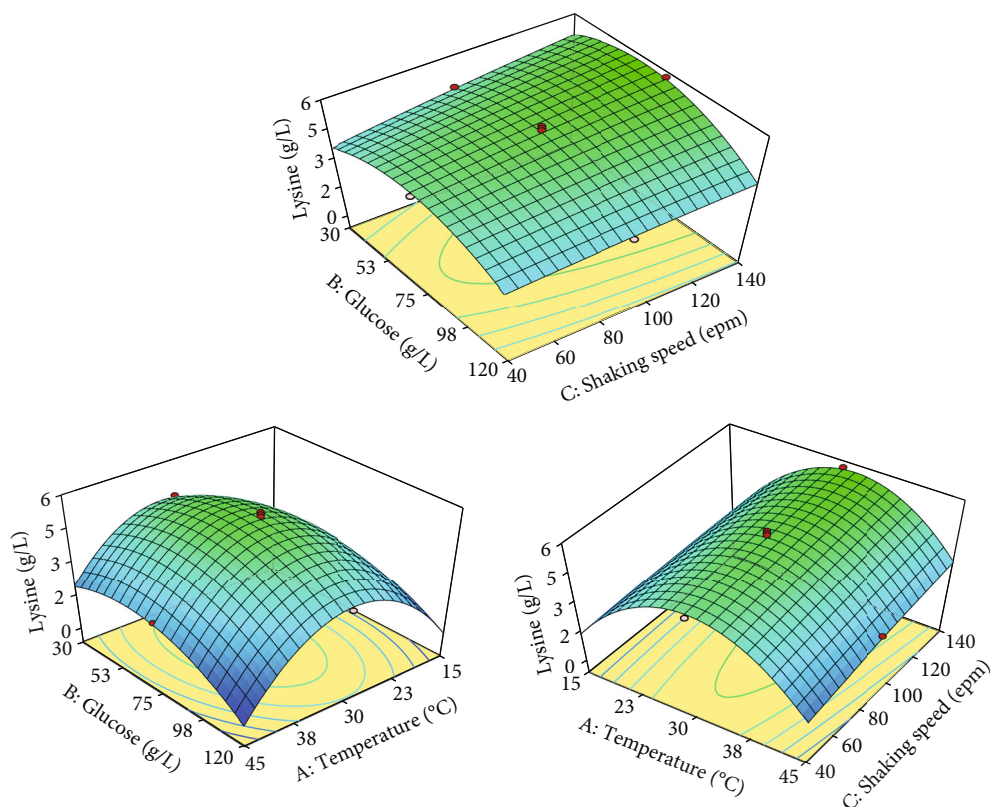
FIGURE 2: Synergism between parameters affecting lysine production.

The analysis of variance data indicated that the applied model has good fit over the selected range of fermentation conditions. Similarly, coefficient of variation range established that results produced are quite reliable and the experimental model can be effectively used to predict the responses. The high R^2 values of models for the optimization of glutamic acid and lysine production also affirm the validity of experimental models.

The analysis of variance data predicted that the temperature and glucose concentration significantly influence the production of glutamic acid as well as lysine while the effect of shaking speed was found to be nonsignificant for the production of amino acids with p values > 0.5.

Figures 1 shows that increasing the concentration of glucose up to 50 g/L, improved the production of glutamic acid up till it reached 65 g/L. Likewise, increasing the concentration of glucose up to 55 g/L improved the production of lysine to a level of 5.1 g/L and further increase in glucose concentration resulted in reduction in lysine yield.

Finally, validation experiments conducted under most desirable conditions revealed that the fermentation conditions embracing 30°C temperature, 50 g/L glucose concentration, and 120 rpm shaking speed furnished 14.2 g/L of glutamic acid and 5.1 g/L of lysine.

3. Fermentation Kinetics

3.1. Fermentation Pattern in a Stirred Fermenter. Figure 3 shows the conversion of the feather hydrolysate into micro-
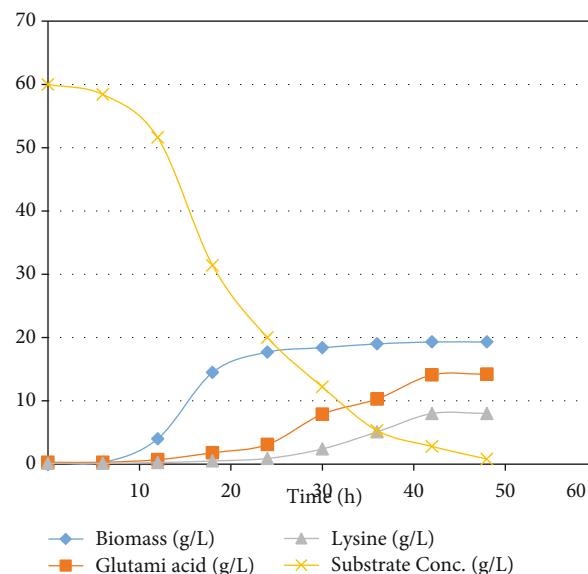

FIGURE 3: Fermentation profile of amino acid production in a stirred fermenter.

bial cell mass and amino acids as the function of fermentation period in the fermenter. The figure indicates that the rate of substrate consumption and biomass production was high for the first 30 hours of fermentation process. But amino acids were progressively released into the fermentation medium at
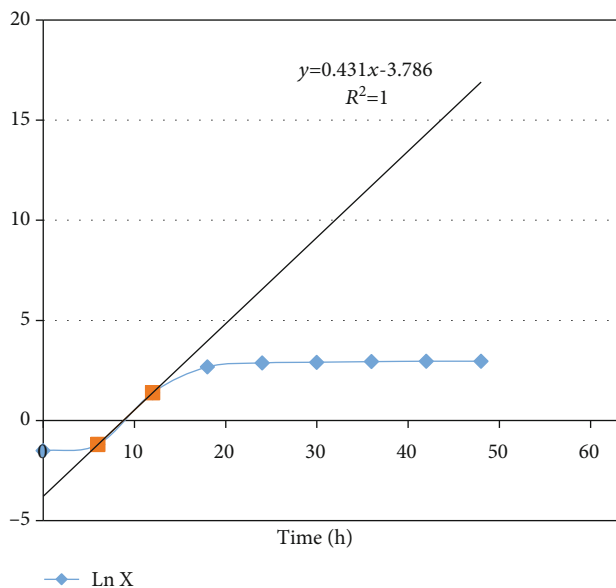
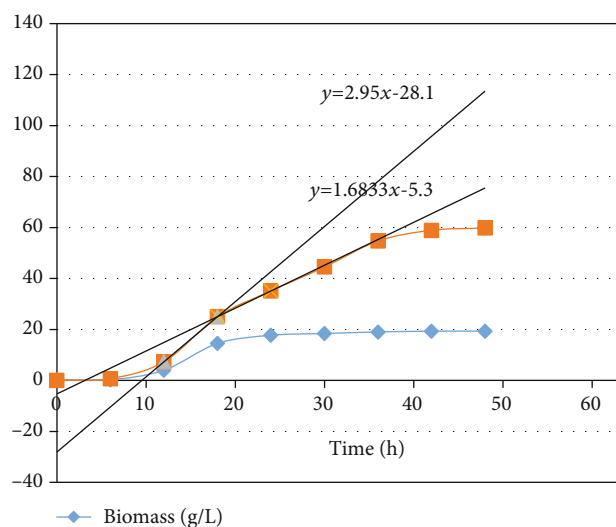FIGURE 4: Growth curve showing specific growth rate.

FIGURE 5: Concentration time graphs for substrate consumption rates.

an admirable level till 48 hours of submerged fermentation and beyond this, decline phase started.

3.1.1. Specific Growth Rates. A graph (Figure 4) between natural log of biomass and fermentation time during the amino acid production revealed that there was a six-hour lag in growth in the stirred fermenter. Lag phase was followed by a brief logarithmic phase In the log phase, the specific growth rate was $0.43\,h^{-1}$ which was gradually decreased. After 12-hour fermentation, the specific growth started to slow down, indicating the termination of true exponential phase. The generation time and the number of generations during the exponential growth phase were $1.6\,h$ and 3.73, respectively.
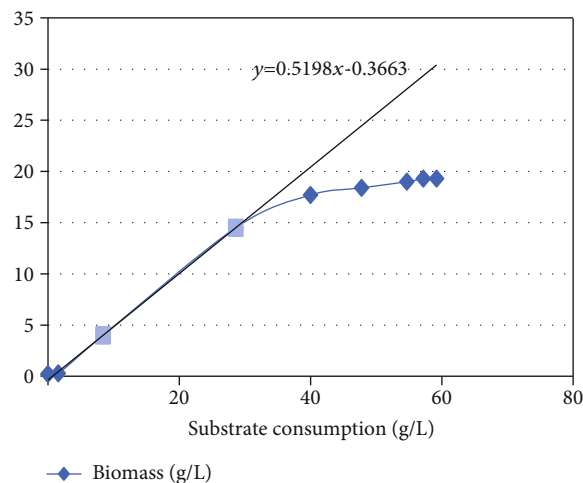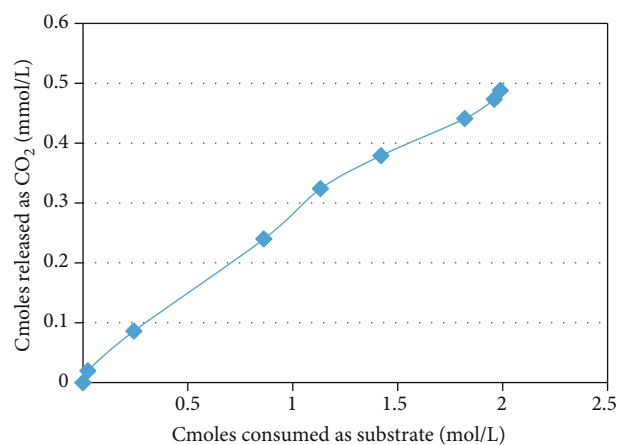

FIGURE 6: Biomass growth yield.

FIGURE 7: Molar yield coefficient of CO_2.

3.1.2. Substrate Consumption Rate and Specific Substrate Uptake Rate. Concentration-time graph (Figure 5) for the substrate consumption shows substrate consumption rate at different levels. It is evident that initially, the rate of substrate consumption was very low and then, it increased with biomass as a function of time and achieved a maximum level of $3.36\,g/L/hour$ between 12 and 18 hours; afterwards, it decreased and fell to $1.9\,g/L/hour$. This may be related to reduction in growth activity which in turn dropped the consumption rate. This may also be related to high value of Monod constant which does not allow complete utilization of the substrate. Average biomass yield over a period of 48 hours was $0.337\,g/g$; however, maximum biomass yield of 0.51 was noted between 10 and 20 hours as shown in Figure 6.

3.1.3. Growth Yield ($Y_{X/S}$) and Product Yield ($Y_{P/S}$). It is pronounced from Figure 7 that biomass yield ($Y_{X/S}$) remained 0.52 during the log phase along with economic coefficient: $1.96\,g$ substrate/g biomass. During the logarithmic phase, nearly 59.8% carbon of the consumed substrate was converted
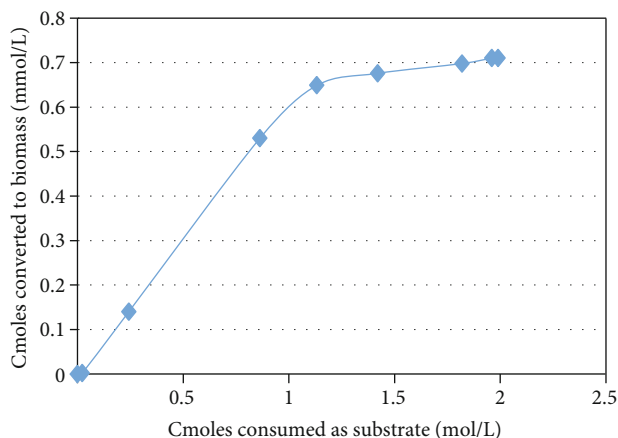
FIGURE 8: Molar biomass yield.

into biomass. However, after 24 hours, the metabolic activities slowed down and the substrate used participated mainly in providing the maintenance energy through oxidation resulting in a molar yield coefficient of CO_2 ($Y^C_{CO2/S}$) equal to 0.234.

Figure 8 shows that molar yield coefficient of biomass ($Y^C_{X/S}$) was 0.6 in the exponential phase which decreased afterwards. At the end of 48 hours, instantaneous conversion of substrate to microbial biomass fell to near zero.

4. Materials and Methods

4.1. Microorganism. Corynebacterium glutamicum was obtained from Biotechnology and Food Research Centre, PCSIR Lahore, and was maintained on preprepared nutrient agar slants. The cultured slants were refrigerated at 4°C.

4.2. Fermentation. The fermentation parameters were optimized in shake flasks using a shaking incubator (New Brunswick, USA) set at 120 rpm, whereas the fermentation kinetics was studied in a bioreactor of 7.5 L capacity (New Brunswick). Half liter of 18 h old vegetative inoculum was shifted to the bioreactor containing 4.5 L sterile fermentation medium. The medium already optimized in shake flasks consisted of (g/L), glucose syrup (60DS): 100; feather hydrolysate: 20; $CaCl_2$:2.0; NaCl:2.0; $MgSO_4.7H_2O$:0.5; yeast extract: 5.0; KH_2PO_4:v0.5; ammonium sulphate: 20; and K_2HPO_4: 1.0. The pH was set at 7.0 ± 0.2 which was maintained using 1 N phosphoric acid and 12.5% aqueous ammonia along with this temperature which was set at 30°C. During the period of fermentation, the rate of aeration was kept constant at 0.55/L/min and by controlling the speed of stirrer from 100 to 130 rpm, the dissolved oxygen (DO) was maintained at 20%.

4.3. Optimization of Fermentation Process. Response surface methodology was carried out for the optimization of fermentation process. The parameter selected for this purpose were as follows: temperature in the range of 15-45°C, amount of glucose syrup (with 60% solids) in the fermentation medium within the range of 30-120 g/L, and the rotational speed of the orbital shaking incubator between 40 and 140 rpm. Various experiments were carried out, and reaction conditions were cautiously monitored to find out the optimum conditions for the production of glutamic acid and lysine. The experimental data was checked against different statistical models, i.e., linear quadratic and factorial, and the model which best fitted on data was chosen for further optimization studies; the selection was made on the bases of different statistical parameters (i.e., sequential p value, lack of fit p value, R^2 value, and the normality as well as predicted vs. actual plots). The parameters and their interaction terms which could significantly affect the amino acid yield were determined by the help of ANOVA and response surface plots.

4.4. Biomass Estimation. Two methods were used to determine the microbial biomass that includes total dry cell mass and optical density measurements. Using a spectrophotometer, the optical density was set at 600 nm which helped in the determination of total cell concentration [19]. Culture tubes of 1 mL capacity were taken to measure dry cell weight in triplets. Tubes were centrifuged at room temperature for about 15 minutes with a speed of 13,000 rpm (ScanSpeed Mini, Denmark), and sterilized containers were used to collect the supernatant. Supernatant was built up for further analysis. 50 mM PB of pH 7.2 was taken in where stored pallets were resuspended and centrifuged in preweighed culture tubes and finally dried at 80°C until constant weight.

4.5. Amino Acid Analysis. The concentration of amino acids in the fermentation media was determined by the method of [20] using HPLC system with C-18 column and a photodiode array detector at 338 nm. Samples were eluted with a linear gradient of solvent A (40 mmol/L Na_2HPO_4, pH 6.8) and solvent b (10% H_2O, 45% methanol, 45% acetonitrile) at the flow rate of 2 mL/min.

4.6. Estimation of Protein Content. Total protein content was estimated according to the Lowry protocol. Bovine serum albumin was used as a standard in this method [21].

4.7. Estimation of Glucose Content. The glucose content was estimated by Miller reagent, which was based on the reduction of 3,5-dinitro-2-hydroxybenzoic acid to an orange-red complex, under alkaline conditions. The absorbance was taken at 540 nm.

4.8. Determination of CO_2. Evolution of carbon dioxide gas from the fermenter was determined by absorbing the gas in a known volume of 1.0 molar KOH solution [22]. The unused KOH was titrated against 0.1 M HCl. In this way, moles of carbon dioxide produced during a definite period of time were calculated. The rate of CO_2 evolution was determined as

$$\text{Rate of } CO_2 \text{ evolution} = \frac{\text{moles of } CO_2 \times 44}{\text{period of evolution(hours)}}. \quad (1)$$

Data Availability

The data used to support the findings of this study are included within the article.

Conflicts of Interest

The authors declare no conflict of interest.

Acknowledgments

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