

Short communication

# Optimisation of a culture medium containing fish silage for L-lysine production by *Corynebacterium glutamicum*

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## Abstract

In a first step the effects of 10 components of a culture medium designed for L-lysine production were evaluated with a  $2^{10-6}$  factorial design. Among them, glucose, fish silage, and ammonium sulphate showed a significant effect. In a second step, an orthogonal–central composite experimental design and response surface methodology was performed with five from the 10 initial compounds. The determination coefficient ( $R^2$ ) of the fitted second-order model was 0.990. L-lysine production with the optimised medium increased 2.6 times as compared with the original medium.

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## 1. Introduction

L-lysine is the third most abundantly produced amino acid at the industrial scale. Eighty per cent is produced by fermentation and 20% by chemical synthesis. In the fermentative processes, the culture medium is the major financial input and N-source is usually the most expensive component. Potential N-sources include various inorganic (ammonium or nitrate salt) or organic compounds (yeast extract, corn steep liquor, soybean protein hydrolysate), and various other extracts of vegetal and animal tissues (Bisaria et al., 1997).

By-products of seafood have a high potential as cheap N-sources for microbial growth (Vecht-Lifshitz et al., 1990). Silage produced from fish wastes (whole heads, fins, skeletal debris, shrimp by-catch captures, and under-utilised whole species of fish) results in a stable product containing peptides and amino acids with high nutritional value (Raa and Gildberg, 1982). The use of fish silage instead of yeast extract has been reported for L-lysine microbial production (Coello et al., 2000).

In the last decades, statistical experimental methods have been applied to media optimisation for industrial

purpose. These designs include the blocking and factorial experiments for determining the path of steepest ascent, in order to identify the effect of individual factors and to approach the neighbourhood of the optimum response (Box et al., 1978). Furthermore, response surface methodology (RSM) is suitable for describing a near-optimum region and thus for exactly investigating optimum conditions for a multifactorial system. RSM and central composite designs has been successfully used to produce enzymes, biomass, and metabolites (Udeh and Achremowicz, 1993; Saval et al., 1993; Ergum and Mutlu, 2000).

We used two successive fractional factorial designs (FFDs) followed by a RSM to optimise a medium containing fish silage, glucose, and eight inorganic salts for L-lysine production by *Corynebacterium glutamicum*. A comparison between the original and the optimised media for biomass and L-lysine productions, and glucose consumption, is presented.

## 2. Methods

### 2.1. Microorganism

The L-lysine producing strain, designated as GIGO, isolated from soil in aerobic conditions, was used in this

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Table 3  
Assigned concentrations of each compound at different levels of the central composite design (experimental design 2)

| Variable | Compound  | Levels     |     |     |     |          |
|----------|---|------------|-----|-----|-----|----------|
|          |   | Star point |     |     |     |          |
|          |   | –2.27519   | –1  | 0   | +1  | +2.27519 |
| $X_1$    | FeSO <sub>4</sub>                               | 4          | 10  | 15  | 20  | 26       |
| $X_2$    | (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> | 47         | 60  | 70  | 80  | 93       |
| $X_3$    | Biotin  | 0.17       | 0.3 | 0.4 | 0.5 | 0.63     |
| $X_4$    | Fish silage                                     | 32         | 45  | 55  | 65  | 78       |
| $X_5$    | Glucose   | 127        | 140 | 150 | 160 | 173      |

Concentrations are given in g/l, except for biotin and FeSO<sub>4</sub> expressed in mg/l.

Table 4  
Experimental design and results of the orthogonal–central composite design together with predicted values for L-lysine production estimated from the model equation

| Run no. | Block | $X_1$    | $X_2$    | $X_3$    | $X_4$    | $X_5$    | L-lysine       |                 |
|---------|-------|----------|----------|----------|----------|----------|----------------|-----------------|
|         |       |          |          |          |          |          | Measured (g/l) | Predicted (g/l) |
| 1       | 1     | 1        | 1        | 1        | 1        | –1       | 25.01          | 24.4362         |
| 2       | 1     | –1       | –1       | –1       | 1        | 1        | 32.74          | 32.0336         |
| 3       | 1     | 1        | –1       | –1       | 1        | –1       | 30.24          | 30.5045         |
| 4       | 1     | –1       | 1        | 1        | 1        | 1        | 24.83          | 23.8713         |
| 5       | 1     | 1        | –1       | 1        | –1       | –1       | 22.13          | 22.4257         |
| 6       | 1     | –1       | 1        | –1       | 1        | –1       | 25.48          | 25.2652         |
| 7       | 1     | –1       | –1       | 1        | 1        | –1       | 34.38          | 33.3940         |
| 8       | 1     | 1        | 1        | –1       | 1        | 1        | 22.86          | 22.9019         |
| 9       | 1     | 0        | 0        | 0        | 0        | 0        | 24.00          | 23.8658         |
| 10      | 1     | 1        | 1        | 1        | –1       | 1        | 15.31          | 15.6330         |
| 11      | 1     | 1        | 1        | –1       | –1       | –1       | 17.32          | 18.3870         |
| 12      | 1     | –1       | –1       | 1        | –1       | 1        | 22.97          | 22.8808         |
| 13      | 1     | 1        | –1       | –1       | –1       | 1        | 20.65          | 21.5613         |
| 14      | 1     | –1       | –1       | –1       | –1       | –1       | 20.94          | 21.5947         |
| 15      | 1     | 1        | –1       | 1        | 1        | 1        | 32.84          | 32.1106         |
| 16      | 1     | –1       | 1        | –1       | –1       | 1        | 15.53          | 16.2120         |
| 17      | 1     | –1       | 1        | 1        | –1       | –1       | 16.04          | 16.1064         |
| 18      | 2     | 0        | 0        | 2.27519  | 0        | 0        | 14.60          | 15.7821         |
| 19      | 2     | 0        | 0        | 0        | 0        | 2.27519  | 13.98          | 14.0798         |
| 20      | 2     | 2.27519  | 0        | 0        | 0        | 0        | 14.72          | 14.1430         |
| 21      | 2     | 0        | 0        | –2.27519 | 0        | 0        | 16.31          | 15.1019         |
| 22      | 2     | –2.27519 | 0        | 0        | 0        | 0        | 14.56          | 15.1110         |
| 23      | 2     | 0        | 0        | 0        | 0        | 0        | 15.22          | 15.3496         |
| 24      | 2     | 0        | –2.27519 | 0        | 0        | 0        | 24.29          | 24.4379         |
| 25      | 2     | 0        | 0        | 0        | –2.27519 | 0        | 6.07           | 4.36758         |
| 26      | 2     | 0        | 0        | 0        | 0        | –2.27519 | 15.60          | 15.4743         |
| 27      | 2     | 0        | 0        | 0        | 2.27519  | 0        | 22.52          | 24.1965         |
| 28      | 2     | 0        | 2.27519  | 0        | 0        | 0        | 9.34           | 9.16621         |

investigate the nature of the surface and to obtain the canonical equation (Box et al., 1978). Statgraphics, version 5.0 (Statistical Graphics Corp., USA), was used for the regression analysis of the data.

#### 2.4. Analytical determinations

Biomass dry weight, extracellular amino acids, glucose concentration and organic acids were estimated by the same procedures described previously (Coello et al., 2000).

### 3. Results and discussion

#### 3.1. Optimisation of the culture medium

##### 3.1.1. Experimental design 1

The mean L-lysine production in the 20 experiments was 14.83 g/l (Table 2). The contrast coefficients demonstrated (pareto chart not shown) that three variables had a strong positive (fish silage) or negative impact ((NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> and glucose) on L-lysine production.

### 3.1.2. Experimental design 2

Fish silage,  $(\text{NH}_4)_2\text{SO}_4$  and glucose were used to perform the second optimisation step, together with iron, and biotin, because of their reported role in L-lysine production (Messenger and Ratledge, 1985; Tosaka et al., 1979). Their effect was explored with a  $2^{5-1}$  orthogonal–central composite design. The other five compounds were used at zero level as indicated in Table 1. This design included two star points ( $+\alpha$  and  $-\alpha$ ) for each variable and a distance between levels of  $\pm 2.27519$ . These additional eleven star points allowed the evaluation of five concentration levels for each variable. Table 3 shows the five independent variables and their concentration at different levels of the central composite design. Table 4 shows the design for the 28 runs and the experimental and predicted values for L-lysine production.

The best L-lysine production (34.38 g/l) was obtained with the concentrations corresponding to run 7. This value was 2.6 fold higher than that obtained with the original medium (13.22 g/l). The contrast coefficients revealed, as for the first experimental design, that fish silage had a strong positive effect on L-lysine production (figure not shown). The contrast coefficient for ammonium sulphate was strongly negative, indicating that L-lysine production resulted from both the supplement of a complex nitrogen source (fish silage) and an adequate balance between the N-sources that guarantees an optimum C/N ratio (Hadj Sassi et al., 1996).

The model fitted satisfactorily to the experiment ( $P < 0.004$ ); the determination coefficient ( $R^2$ ) was 0.990. The canonical equation was:

$$\hat{Y} = 32.3819 - 0.0684375X_1^2 - 0.195312X_2^2 + 0.0365625X_3^2 - 0.0140625X_4^2 - 0.110616X_5^2$$

The coefficients of the canonical equation were negative except for biotin, indicating that the surface explored had a saddle shape, without maximum or minimum point.

### 3.2. Comparison between original and optimised media

Fig. 1 shows the time courses of biomass and L-lysine production, and glucose consumption for the original and the optimised media. The composition of the optimised medium was that of run 7 of the second experimental design, and contained per liter: glucose, 140 g; fish silage, 65 g;  $(\text{NH}_4)_2\text{SO}_4$ , 60 g;  $\text{K}_2\text{HPO}_4$ , 1 g;  $\text{KH}_2\text{PO}_4$ , 1 g;  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.4 g;  $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$ , 10 mg;  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ , 10 mg; biotin, 500  $\mu\text{g}$ ; thiamine–HCl, 300  $\mu\text{g}$ . The original medium is given in the methods section.

The final biomass and L-lysine concentration increased by 2.5 and 2.6 times, respectively in the run 7 as compared with the original medium. L-lysine production for the optimised medium started around the 16th h,

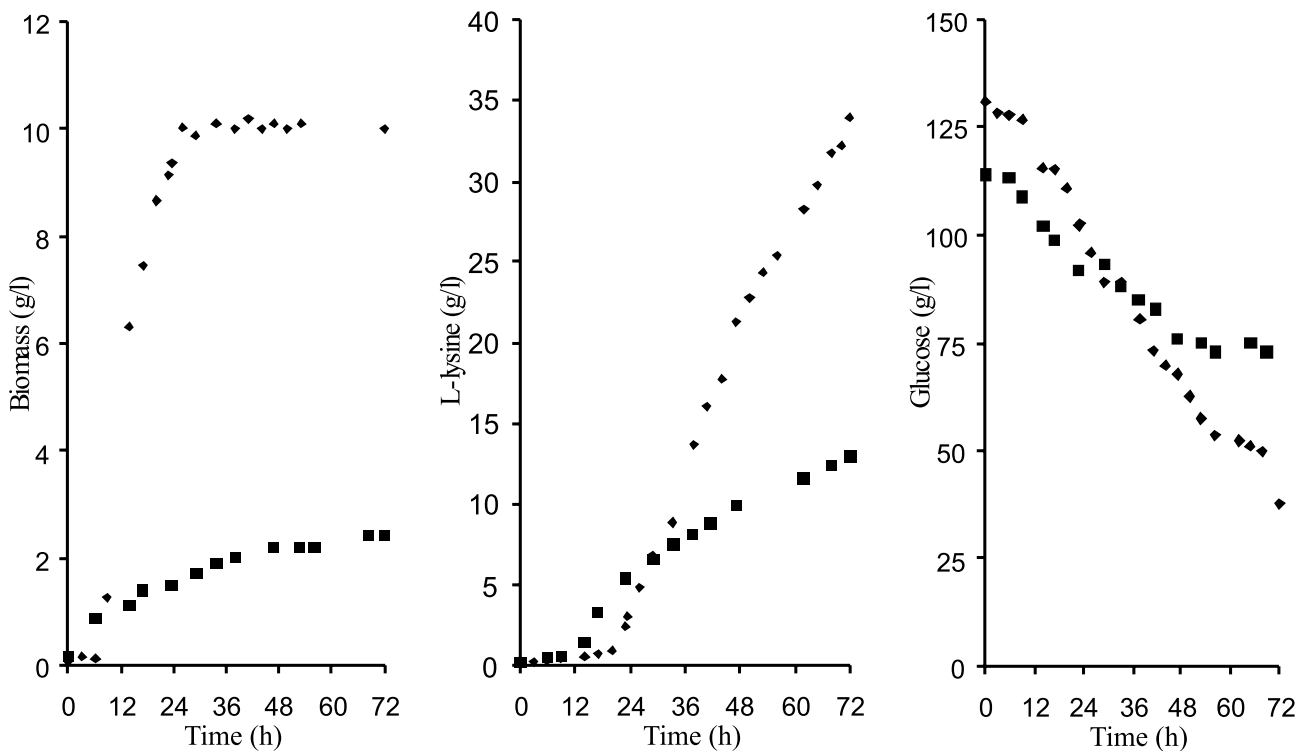


Fig. 1. Changes in biomass, L-lysine produced and glucose consumed during fermentation of *C. glutamicum* in Erlenmeyer baffled flasks at 30 °C. Optimised medium (◆) and original medium (■). Each value is a mean of three experiments.

later than with the original medium. The amount of glucose consumed was consistently higher in the optimised medium. These results showed that the optimised medium promoted the microbial growth and consequently L-lysine production better than the original medium.

The L-lysine volumetric productivity significantly increased in the optimised medium (0.47 g/l/h) as compared with the original media (0.18 g/l/h). However, the specific production at 72 h was similar in both cases (above 3 g lysine/g cells). The optimised medium allowed production of more biomass with a slightly enhanced substrate conversion yield ( $Y_{P/S} = 0.42$  g/g against 0.32 g/g for the original medium). In agreement with this, the production of by-products such as lactic and acetic acids was 70 and 10 mM respectively in the original medium, in contrast with the optimised medium where lactic acid reached 60 mM and acetic acid was not detected.

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