ELSEVIER

Bioresource Technology 85 (2002) 207–211

BIORESOURCE TECHNOLOGY

Short communication

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

Nereida Coello [a,*], Edie Montiel [b], Milagros Concepcion [a], Pierre Christen [c]

[a] *Laboratorio de Procesos Biotecnológicos, Instituto de Biología Experimental (IBE), Universidad Central de Venezuela, AP 47279, Caracas 1041-A, Venezuela*

[b] *Instituto de Zoología Tropical, Universidad Central de Venezuela, Caracas 1041, Venezuela*

[c] *Laboratoire de Microbiologie IRD-ESIL, Case 925, 163 Luminy, 13288 Marseille, Cedex 9, France*

Received 5 September 2000; received in revised form 8 March 2002; accepted 13 March 2002

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.

Keywords: L-lysine; Fish silage; Medium optimisation; *Corynebacterium glutamicum*

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

* Corresponding author. Fax: +58-212-753-58-97.
E-mail address: mcoello@reacciun.ve (N. Coello).

0960-8524/02/$ - see front matter
PII: S0960-8524(02)00084-6

work. Biochemical tests conducted on the GIGO strain in our laboratory were confirmed by the Pasteur Institute of France, which characterised the isolate as *C. glutamicum*. It was maintained on 25% (v/v) glycerol at −70 °C by CVCM-IBE strain culture collection (Venezuela).

2.2. Culture media and fermentation conditions

The inoculum (10% (v/v)) and fish silage medium was based on a previous study by Coello et al. (2000) and had the following composition per liter: fish silage, 40 g; glucose, 140 g; $(NH_4)_2SO_4$, 75 g; K_2HPO_4, 1 g; KH_2PO_4, 1 g; $MgSO_4 \cdot 7H_2O$, 0.4 g; $MnSO_4 \cdot 4H_2O$, 10 mg; $FeSO_4 \cdot 7H_2O$, 10 mg; thiamine–HCl, 300 μg; biotin, 400 μg. Fish silage was prepared as described by Bello et al. (1993). Fermentation runs were performed in 500 ml-baffled Erlenmeyer flasks (20 ml culture volume) at 30 °C for 72 h on a rotary shaker at 250 rpm. Samples were withdrawn at intervals to measure dry cell mass, L-lysine production, organic acids excretion and residual glucose. The original and optimised media were compared two-fold in triplicate using the same conditions. The purity and auxotrophy characteristics of the strain were determined each time by culture on appropriate agar plates.

Table 1
Assigned concentrations of each compound at different levels of the fractional factorial design (experimental design 1)

Variable	Compound	Levels		
		−1	0	+1
X_1	$MnSO_4$	5	10	15
X_2	$FeSO_4$	5	10	15
X_3	$MgSO_4$	0.20	0.40	0.60
X_4	Thiamine	0.20	0.30	0.40
X_5	Glucose	140	150	160
X_6	Fish silage	30	40	50
X_7	$(NH_4)_2SO_4$	55	65	75
X_8	K_2HPO_4	0.75	1.0	1.25
X_9	KH_2PO_4	0.75	1.0	1.25
X_{10}	Biotin	0.30	0.40	0.50

Concentrations are given in g/l, except for $MnSO_4$, $FeSO_4$, thiamine and biotin expressed in mg/l.

2.3. Experimental designs

The optimisation of the culture medium was performed in two steps. First, 10 compounds were considered within a 2^{10-6} FFD, in order to determine which compounds had a major influence on L-lysine production (Table 1). Each variable was evaluated at a high (+1), a low (−1), and a central level (0), that was repeated four times to estimate the experimental variability. Data were fitted to a first-order model (Table 2). In a second step, five of the 10 initial compounds were selected and their influence was studied with a central composite design with five coded levels (Tables 3 and 4). A two-blocks design was built as follows: a 2^{5-1} fractional factorial design (16 cube points) augmented with one replication of the centre point (all factors at level 0) and the 11 star points having for one factor an axial distance to the centre of $\pm\alpha$, whereas the other factors were at level 0. The axial point α coded was ±2.27519. A second-order polynomial equation obtained by multiple regression was used to fully describe the response surface. Then, a canonical analysis was employed to in-

Table 2
Experimental design and results of L-lysine production

Run no.	X_1	X_2	X_3	X_4	X_5	X_6	X_7	X_8	X_9	X_{10}	Lysine measured (g/l)
1	−1	1	1	−1	−1	−1	1	1	1	−1	9.44
2	−1	1	−1	−1	1	1	−1	1	−1	−1	23.00
3	1	1	1	−1	1	−1	−1	−1	−1	1	11.49
4	0	0	0	0	0	0	0	0	0	0	16.89
5	−1	1	1	1	−1	1	−1	−1	−1	−1	27.06
6	1	−1	1	−1	−1	1	−1	1	1	−1	18.58
7	1	1	−1	1	−1	−1	−1	1	−1	1	8.73
8	0	0	0	0	0	0	0	0	0	0	16.04
9	−1	−1	−1	1	−1	1	1	1	−1	1	20.45
10	1	−1	−1	−1	1	−1	1	1	−1	−1	10.40
11	−1	−1	1	1	1	−1	−1	1	1	1	9.32
12	−1	−1	−1	−1	−1	−1	−1	−1	1	1	9.39
13	−1	−1	1	−1	1	1	1	−1	−1	1	16.50
14	−1	1	−1	1	1	−1	1	−1	1	−1	10.29
15	0	0	0	0	0	0	0	0	0	0	16.47
16	1	−1	−1	1	1	1	−1	−1	1	−1	18.62
17	1	1	−1	−1	−1	1	1	−1	1	1	14.39
18	1	1	1	1	1	1	1	1	1	1	13.76
19	1	−1	1	1	−1	−1	1	−1	−1	−1	9.31
20	0	0	0	0	0	0	0	0	0	0	16.40

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	$FeSO_4$	4	10	15	20	26
X_2	$(NH_4)_2SO_4$	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 $FeSO_4$ 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

vestigate 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 ($(NH_4)_2SO_4$ and glucose) on L-lysine production.

3.1.2. Experimental design 2

Fish silage, $(NH_4)_2SO_4$ 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 ± 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; $(NH_4)_2SO_4$, 60 g; K_2HPO_4, 1 g; KH_2PO_4, 1 g; $MgSO_4 \cdot 7H_2O$, 0.4 g; $MnSO_4 \cdot 4H_2O$, 10 mg; $FeSO_4 \cdot 7H_2O$, 10 mg; biotin, 500 μg; thiamine–HCl, 300 μg. 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,

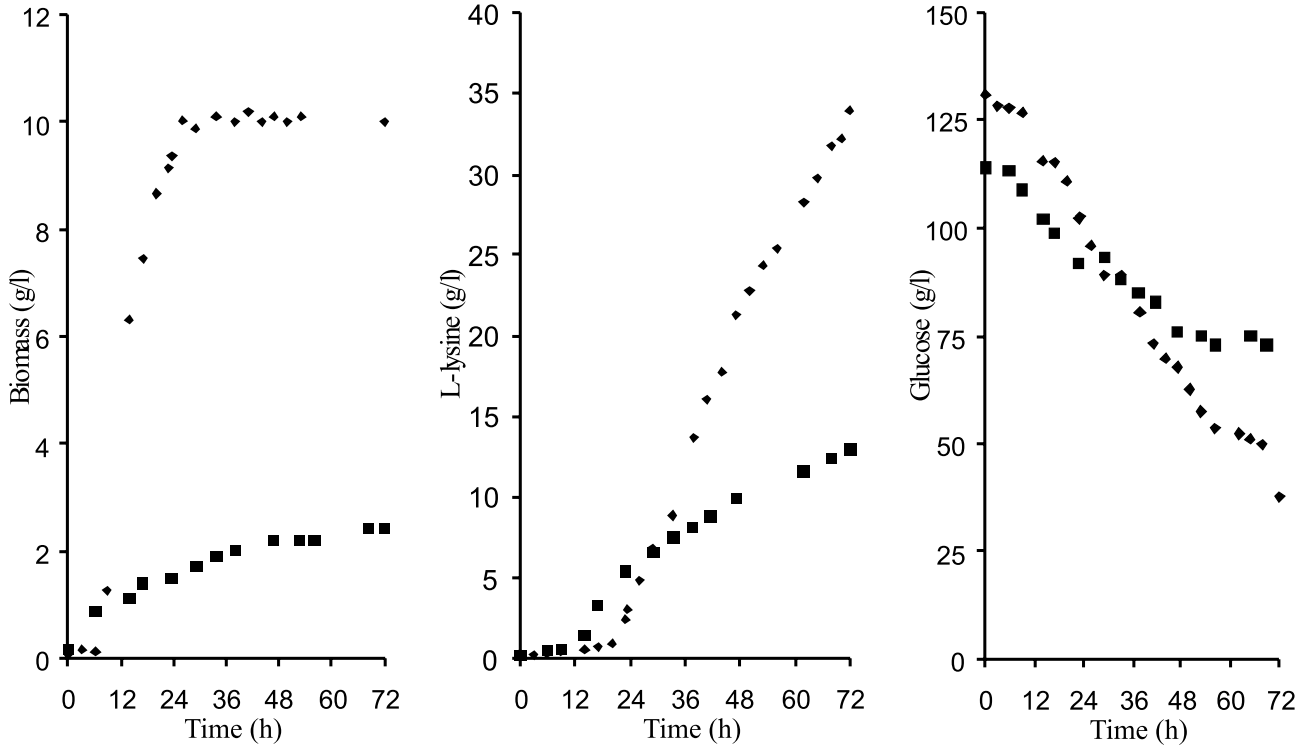

Fig. 1. Changes in biomass, L-lysine produced and glucose consumed during fermentation of *C. glutamicum* in Erlenmeyer baffled flasks at 30 °C. Optimised medium (◆) and original medium (■). Each value is a mean of three experiments.

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.

Acknowledgements

Financial support with grants BTI-88 and 03.33.4163.01 from Consejo Nacional de Ciencia and Tecnología and Consejo de Desarrollo Científico y Humanístico of Central University of Venezuela, respectively, are gratefully acknowledged. The authors wish to thank Dr. P. Roger (IRD, France) for his helpful comments on the manuscript.

References

Bello, R., Cardillo, E., Martínez, R., 1993. Microbial fish silage production from several fish species. Arch. Latinoamer. Nut. 43 (3), 221–227.

Bisaria, R., Madan, M., Vasudevan, P., 1997. Utilisation of agro-residues as animal feed through bioconversion. Bioresource Technol. 59, 5–8.

Box, G.E.P., Hunter, W., Hunter, J.S., 1978. Statistics for experimenters. J. Wiley & Sons, New York (Chapters 12 and 15).

Coello, N., Brito, L., Nonus, M., 2000. Biosynthesis of L-lysine by *Corynebacterium glutamicum* grown on fish silage. Bioresource Technol. 73, 221–225.

Ergum, M., Mutlu, F., 2000. Application of a statistical technique to the production of ethanol from sugar beet molasses by *Saccharomyces cerevisae*. Bioresource Technol. 73, 251–255.

Hadj Sassi, A., Deschamps, A., Lebeault, J.M., 1996. Process analysis of L-lysine fermentation with *Corynebacterium glutamicum* under different oxygen and carbon dioxide supplies and redox potentials. Process Biochem. 31, 493–497.

Messenger, A.J.M., Ratledge, C., 1985. Siderophores. In: Moo-Young, M. (Ed.), Comprehensive Biotechnology, vol. 3. Pergamon Press, Oxford, UK, pp. 275–295.

Raa, J., Gildberg, A., 1982. Fish silage: a review. Crit. Rev. Food Sci. Nutr. 16, 383–419.

Saval, S., Pablos, L., Sanchez, S., 1993. Optimization of a culture medium for streptomycin production using response-surface methodology. Bioresource Technol. 43, 19–25.

Tosaka, O., Marioka, H., Takinami, K., 1979. The role of biotin-dependent pyruvate carboxylase in L-lysine production. Agric. Biol. Chem. 43, 1513–1519.

Udeh, K., Achremowicz, B., 1993. Optimization of cultivation medium composition of an L-lysine producing mutant: the use of response surface methodology. Acta Microbiol. Pol. 42, 171–180.

Vecht-Lifshitz, S.E., Almas, K.A., Zomer, E., 1990. Microbial growth on peptones from fish industrial wastes. Lett. Appl. Microbiol. 10, 183–186.