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Glutamate excretion by *Corynebacterium glutamicum*: a study of glutamate accumulation during a fermentation course

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Summary. *Corynebacterium glutamicum* was used in fed-batch fermentation for glutamate production. Both intracellular and extracellular concentrations were determined which allowed us to study the repartition of the amino acid according to the culture conditions and in the presence or absence of surfactants. A decrease in cell volume was observed after addition of surfactants during the exponential phase of growth; glutamate accumulates in the cell, whereas in standard industrial conditions the glutamate concentration in the medium during the production phase can be 30-fold higher than that found inside the cell. The level of excretion is compatible with industrial production.

Introduction

Glutamate is by far the most important commercial amino acid. The world's production, obtained exclusively by fermentation, reaches 300,000 tonnes per year. The most widely used species for glutamate overproduction are *Corynebacterium* and *Brevibacterium*.

In both cases, an increase of permeability of the bacterial membrane is necessary to allow the excretion of the amino acid. This modification of permeability is obtained by biotin limitation (Shiio et al. 1962), by inhibiting cell wall synthesis with penicillin (Kinoshita and Tanaka 1973) or by addition of surfactants (fatty acids derivatives) (Takinami et al. 1965).

In the present study we examined the production of glutamate by *Corynebacterium glutamicum* in fed-batch fermentation. The excretion of the amino acid was induced by the addition of two surfactants at a very precise step during the exponential growth. Some effects of the surfactants are reported and their role on excretion triggering is discussed. We followed the glutamate repartition between intracellular and extracellular media during a fermentation course in various conditions leading to either high or low levels of excreted glutamate. The intracellular concentration of glutamate has been determined taking into account variations of the cell volume reflecting modifications of physiological state.

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Materials and methods

Inoculum preparation. An industrial strain of *C. glutamicum* was used. The inoculum medium contained (g/l): Beet molasses 80, H_3PO_4 3, urea 8, $MgSO_4$ 0.5, desthiobiotine 10^{-4}; pH was adjusted to 5.75 before autoclaving (20 mn at 121° C). The inoculum culture was grown overnight at 30° C on a rotary shaker.

Fermentations. Fermentations were carried out in a 20 l fermentor (Chemap) with a working volume of 15 l. The medium had the following composition (g/l): Beet molasses 150, H_3PO_4 1.65, $(NH_4)_2SO_4$ 1, $MgSO_4$ 0.5, $MnSO_4$ 0.005, desthiobiotine 10^{-4}. Culture conditions were pH 7.3, temperature +34.5° C, agitation 550 RPM, aeration 0.6 VVM. Surfactant S_1 was a polyethylene glycol acylated by stearic and palmitic acids. Surfactant S_2 was laurylamine.

The mode of fermentation was a fed batch. The initial concentration of sucrose in the medium was about 75 g/l; in the stage when the concentration fell to 30 g/l, a glucose solution (600 g/l) was supplied to keep the sugar concentration constant in the medium.

Analytical methods. The cell concentration was determined by absorbance at 650 nm (Spectrophotometer Perkin Elmer λ3) and by the dry matter method. The quantities of sucrose and glucose were determined by an enzymatic method with a Yellow Spring Instruments model 27 analyser. The method for lipid analysis has been described previously by Huchenq et al. (1984). The amount of glutamate was determined according to Beutler and Michal (1974) with the Boehringer kit.

The intracellular glutamate content was measured according to Uribelarrea et al. (1984) after breakage of the cells by

glass beads. Thermogravimetry experiments were done using a Sartorius thermobalance. Intracellular glutamate concentration could be determined by the following equation:

$$\frac{G_i}{G_e} = \frac{1}{\%i}\left(\frac{G_p}{G_e}(K+\alpha)-K\right)+1,$$

where

G_p = glutamate concentration in the pellet (g/l)
G_i = intracellular glutamate concentration (g/l)
G_e = intersticial glutamate concentration (g/l)
$\%i$ = intracellular water percentage in the pellet determined by thermogravimetry
$\%dm$ = dry matter percentage in the pellet determined by thermogravimetry
W_p = pellet weight (g)
V_w = water volume added for the breaking of the cells (ml)
K = $1-4\%dm$
α = $\frac{V_w}{W_p}$

Results

Fed Batch Fermentation and lipid composition

The courses of experimental curves are depicted in adimentional coordinates in Fig. 1. Surfactants were added during the exponential phase of growth.

The acylated surfactant S_1 did not significantly modify the growth curve, while the amine surfactant S_2 slowed it down and induced the excretion of glutamate. It was a second type fermentation according to the Gaden classification since growth and production are uncoupled.

Figure 2 shows the change of fatty acid composition of the membrane during fermentation. The addition of surfactant S_1 stopped fatty acid synthesis (Huchenq et al. 1984), the fatty acids of the surfactant (octadecanoic acid 18:0) being incorporated in the cell lipids. It is worth while noting that the incorporation took place between the two maximal values of the specific growth rate (μ) and the specific production rate (ν). This occured regardless of the level (high or low) of glutamate excretion. The highest excretion rate was obtained when the highest saturated/unsaturated ratio was reached. A short time after S_1 addition, cells return to a nearly normal fatty acid composition. At the same time the specific production rate decreased sharply, although some excretion was maintained during the last two thirds of the fermentation. Thus if the modification of the cell fatty acids seems sufficient to trigger excretion, this was not the only phenomenon that led to glutamate excretion. This was also suggested by the comparison of several cultures that did not reveal a correlation between the saturated fatty acid content of cells and the final glutamate production.

This has already been noted by Fukui et al. (1973). Moreover, good glutamate production (80 g/l) was obtained by adding the amine surfactant S_2 without any addition of the acylated surfactant. Since S_2 did not change the cell fatty acid composition, either in fed-batch cultures or in synthetic medium in Erlenmeyer flasks (Huchenq et al. 1984), it is clear that the fatty acid composition was not a requirement for glutamate excretion.

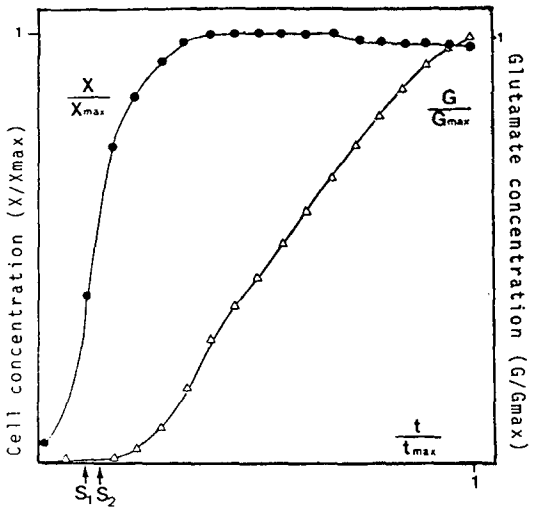

Fig. 1. Course of cells (X/X_{max}) and glutamate concentration (G/G_{max}) during a fed batch fermentation

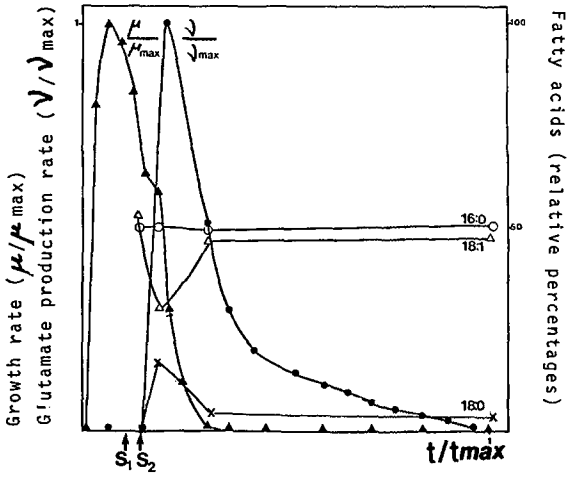

Fig. 2. Fatty acids composition of bacterial membrane. Growth and glutamate specific production rates. 16:0 = hexadecanoic acid; 18:0 = octadecanoic acid; 18:1 = octadecanoic acid

It is known that aliphatic amines are potent respiratory inhibitors and bacteriostatic compounds. S_2 could thus induce glutamate excretion either by uncoupling the glutamate uptake system, as was postulated for excretion induced by S_1 (Clement et al. 1984), or by lowering the protonmotive force, or both.

Another beneficial effect of S_2 for the glutamate yield is the bacteriostatic effect: biomass production slows down after amine addition, giving a higher yield of sucrose conversion to glutamate.

Modification of Corynebacterium glutamicum cells during a fermentation

The intracellular water percentage of the cell and the dry weight percentage evaluated by thermogravimetry varied according to the culture stages between 25% and 30% and between 12% and 25%, respectively. The ratio $\%i/\%dm$ could be associated with the physiological state of bacteria. We have found that this ratio decreases four times during the course of fermentation which means that the addition of the surfactants S_1 and S_2 causes a diminution of the intracellular volume of *C. glutamicum,* this phenomenon being accentuated by the ageing of the culture (data not shown).

Glutamate repartition between the cell and the external medium

Glutamate intracellular concentrations (G_i) were evaluated taking into account physiological modifications of bacteria during a fermentation and compared with extracellular concentrations (G_e). We compared the results obtained in a culture where the final glutamate concentration was 7.5 g/l (Assay A: Table 1) with those obtained in a culture where the final glutamate concentration was 100 g/l (Assay B: Table 2). The low value of glutamate concentration in assay A was related to an early addition of surfactants S_1 and S_2. In both assays, intracellular glutamate concentrations were higher than the extracellular concentrations during the exponential phase of growth but they became lower during the production phase to be stabilized at a value of 3 g/l. Between the addition of surfactants and the end of the fermentation, the ratio G_e/G_i was multiplied by a factor 170 in assay A and by a factor 2200 in assay B. In the latter assay, the glutamate concentration in the medium was then 30 times higher than in the interior of the cell, giving rise to levels compatible with industrial production.

Table 1. Repartition of glutamate between the intracellular (G_i) and the extracellular (G_e) media during a fermentation when the maximal glutamate concentration is 7.5 g/l

t (h)	$\frac{t}{t_{max}}$	G_e (g/l)	G_i (g/l)	$\frac{G_e}{G_i}$	Exit of glutamate against a concentration gradient
5	0.14	0.2	15.1	0.014	–
9	0.25	2.2	9.6	0.230	–
12	0.33	4.7	3.5	1.350	+
26	0.72	6.9	3.1	2.220	+
36	1	7.5	3.1	2.400	+

Table 2. Repartition of glutamate between the intracellular (G_i) and the extracellular (G_e) media during a fermentation when the maximal glutamate concentration is 100 g/l

t (h)	$\frac{t}{t_{max}}$	G_e (g/l)	G_i (g/l)	$\frac{G_e}{G_i}$	Exit of glutamate against a concentration gradient
4	0.11	0.2	14.6	0.014	–
5	0.14	1.8	17.1	0.105	–
10	0.28	10.9	6.2	1.760	+
18	0.50	45.1	3.3	13.670	+
24	0.67	66.2	2.9	22.830	+
36	1	98.7	3.2	30.840	+

Discussion

Some authors studied the phenomenon of intracellular accumulation of glutamate in various conditions such as limitation by biotin (Shiio et al. 1962; Fukui and Ishida 1973) or addition of penicillin (Kinoshita and Tanaka 1973).

According to Shiio et al. (1962), the ratio G_e/G_i (where G_e is the glutamate concentration in the medium and G_i is the glutamate intracellular concentration) was 340-fold higher in a biotin-poor medium than in a biotin rich medium. In the case of addition of penicillin an inversion of the ratio G_e/G_i occured within 4 h. The ratio G_e/G_i was increased 800-fold during fermentation. Table 3 shows that in spite of various fermentation protocols leading to different values of intracellular concentrations of glutamate, a ratio G_e/G_i of more than 1 was always observed when the conditions of excretion were set up. However, the variation of this ratio was much higher in our experiments, corresponding to the maximal level of excreted glutamate.

Table 3. Maximal variation of the ration G_e/G_i and maximal concentrations of glutamate excreted in the medium when excretion is induced by biotin limitation (Shiio et al. 1962) penicillin addition (Kinoshita and Tanaka 1973) and surfactants additions (this work)

	Biotin limitation	Penicillin addition	Surfactants addition
$\frac{(G_e/G_i)_{max}}{(G_e/G_i)_{min}}$	340	800	2200
Maximal concentration of glutamate excreted (g/l)	8.5	7	100

As long as no surfactant was added, glutamate could be accumulated inside the cell by an active mechanism (Clement et al. 1984). The addition of surfactants induces a reversal of the polarity of transport leading to the excretion of glutamate into the medium.

The hypothesis of impairment of the function of the cell wall by surfactant addition by Fukui and Ishida (1973) could be explained by an uncoupling of the uptake system. In this case the partition ratio of glutamate (intracellular/extracellular concentration) may be evaluated on the basis of a Nernst equilibrium between both sides of the membrane.

It is likely that inside the cell, glutamate bears a net negative charge, a 30-fold concentration outside could be reached for a transmembrane potential difference of 87 mV (positive excretion); this value is compatible with the known potential differences measured in various bacteria (around 100 mV) (Krulwich et al. 1981).

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References

Beutler HO, Michal G (1974) L-glutamate determination with glutamate dehydrogenase, diaphorase and tetrazolium salts. In: Bergmeyer HU (ed) Methods of enzymatic analysis 2nd edition Vol. 4. Verlag Chemie, Weinheim/Bergstr., pp 1708—1713

Clement Y, Escoffier B, Trombe MC, Laneelle G (1984) Is glutamate excreted by its uptake system in *Corynebacterium glutamicum*? A working hypothesis. J Gen Microbiol 130:2589—2594

Demain AL, Birnbaum J (1968) Alteration of permeability for the release of metabolites from the microbial cell. Current Topics Microbiol 46:1—25

Fukui S, Ishida M (1973) Biomembrane permeability and the accumulation of amino acids. In: Yamada K (ed) The microbial production of amino acids. John Wiley, pp 123—137

Huchenq A, Marquet M, Welby M, Montrozier H, Goma G, Laneelle G (1984) Glutamate excretion triggering mechanism: a reinvestigation of the surfactant induced modification of cell lipids. Ann Microbiol (Inst. Pasteur) 135B:53—67

Kinoshita S, Tanaka K (1973) Glutamic acid. In: Yamada K (ed) The microbial production of amino acids. John Wiley, pp 263—324

Krulwich TA, Gruffanti AA, Maudel KG (1981) The role of the membrane in the bioenergetics of bacterial cells. In: Organization of prokaryotic cell membranes. Vol 2. Ghosh RH (ed) C.R.C. Press, Inc., pp 53—71

Shiio I, Narui K, Yabaha N, Takahashi M (1962) Free intracellular amino acid of a glutamate-forming Bacterium, Brevibacterium flavum N° 2247. J Biochem 51:109—111

Takinami K, Yoshii H, Tsuri H, Okada H (1965) Biochemical effects of fatty acid and its derivatives on L-glutamic acid fermentation. III Biotin — tween 60 relationship in the accumulation of L-glutamic acid and the growth of *Brevibacterium lactofermentum*. Agric Biol Chem 29:351—359

Uribelarrea JL, Pacaud S, Goma G (1985) New method for measuring the cell water content by thermogravimetry. Biotechnol Lett 7:75—80

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