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Excretion of lysine by *Micrococcus glutamicus*

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Abstract. Analysis of intracellular and extracellular lysine concentration during lysine fermentation by *Micrococcus glutamicus* AEC RN–13–6/1 indicated that lysine excretion occurs against a concentration gradient towards the end of the fermentation period. The capacity to excrete lysine against a concentration gradient may be a factor contributing to the high yield of lysine.

Keywords. *Micrococcus glutamicus;* lysine production.

Introduction

Analysis of intracellular and extracellular amino acids in biotin-sufficient and biotin-deficient cells of *Micrococcus glutamicus* indicated that a deficiency of biotin stimulated the synthesis of glutamate and the excretion of amino acids (Kimura, 1963). Similar analyses indicated that cells of *Brevibacterium flavum* release intracellular amino acids into wash solutions more readily when grown under biotin-deficient conditions (Shiio *et al.,* 1962).

This paper describes investigations on lysine excretion by a lysine over producing strain of *M. glutamicus* by measurement of the concentration of free lysine and of the total free amino acids inside and outside the cells during lysine fermentation.

Materials and methods

All amino, acids and their derivatives (except threonine which was used in growth media), dried cells of *Bacillus cadaveris* (used as a source of lysine decarboxylase), Dowex ion-exchanger and Blue Dextran B-2000 were purchased from Sigma Chemical Company, St. Louis, Missouri, USA. L-threonine used in the growth media was a product of Kyowa Hakko Kogyo Limited, Tokyo, Japan. Polyvinyl alcohol (Cellacell grade 198) was from Cellulose Products of India Limited, Ahmedabad. All other chemicals were of the highest purity available.

M. glutamicus AEC RN-13-6/1 was the gift of Dr V. C Vora, Central Drug Research Institute, Lucknow; this is ahomoserine-requiring, S-(2-aminoethyl)-L cysteine (AEC)-resistant mutant of . *glutamicus* ATCC 13059 and was maintained on slants of medium B (Sano and Shiio, 1967) containing S-(2-aminoethyl)-L cysteine. HCl (2 mg/ml), L-threonine (2 mg/ml) and DL-methionine (50 pg/ml). Cells were grown in a medium containing clarified corn steep liquor (Lakshman, 1980). Cells in the log phase obtained by two transfers into clarified corn steep liquor medium were used as inoculum (10%, v/v).

Viable counts were made on nutrient agar plates after appropriate dilution of the sample with 0.9% saline. Total bacterial counts were made in a haemocytometer with 0.4% polyvinyl alcohol in 0.9% saline containing 0.5% formalin as the suspending medium. Samples were diluted as required with 0.9% saline containing 0.5% formalin. Individual cells in a cluster were mounted.

The volume of a pellet of packed cells is the sum of the volume occupied by the cells (or cell volume) and the intercellular volume. The intracellular volume and the volume of a pellet of packed cells were measured essentially as described by Roberts *et al.* (1955). Cells were packed by centrifugation at 27,000 *g* for 20 min. The concentration of Blue Dextran-2000 in solution was measured by its absorbance at 630 nm. The cell volume, calculated by difference, was correlated to cell mass and number as described by Roberts *et al.* (1955) except that total cell counts were made.

For the extraction of intracellular amino acids, wet-packed cells from 48 ml of broth were washed (twice) rapidly with 20% sucrose (20 ml). The washed cells were extracted repeatedly, by shaking with aliquots (30 ml) of 70% ethanol for 15 min at 270 rpm, until a 100 µl aliquot was ninhydrin-negative. The pooled extracts were evaporated to dryness *in vacuo* at 50° C. They were then taken up in 10 ml water and desalted on a column (1×5 cm) of Dowex 50-H^+ (X8, 200-400 mesh). The resin was washed with water and the amino acids eluted with 2N NH_4OH. The ammoniacal effluent was dried *in vacuo* at 60°C. It was taken up in a known volume of water. Lysine was separated from other amino acids by high voltage electrophoresis at 2500 V on Whatman No. 3 MM filter paper (28×60 cm) in acetic acid-formic acid buffer pH 1.8 in a Savant apparatus (Blackburn, 1965). After electrophoresis, the paper was dried at 80° C for 60 min. Lysine was quantitated using ninhydrin-cadmium acetate reagent (Blackburn, 1965). Total amino acids were estimated against standard leucine by the method of Spies (1952). Lysine was also identified by decarboxylation by using the lysine decarboxylase of *Bacillus cadaveris* (Dickerman and Carter, 1962).

The culture filtrate was deproteinized by the addition of 0.5 volume of 50% perchloric acid. Amino acids in 1.5 ml of deproteinized culture filtrate were desalted on a column (1.4×5.4 cm) of Dowex 50-H^+. Lysine and total amino acids were estimated as above.

Results and discussion

The growth pattern of *M. glutamicus* is shown in figure 1.

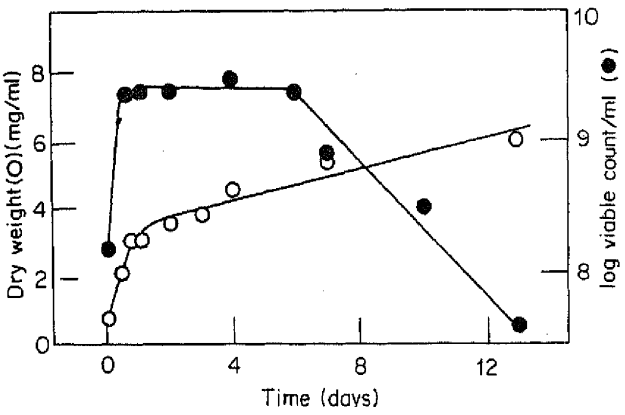

Figure 1. Growth pattern of *M. glutamicus* AEC RN–13–6/1

On an average it was found that 1 mg dry weight of 24 h cells corresponded to 4.45 mg wet weight, 0.52 µl cell volume and 1.07×10^9 cells (or 0.86×10^9 viable cells).

The intracellular and extracellular concentrations of lysine and total amino acids during the fermentation period are shown in table 1. Comparison of the data in figure 1 and table 1 shows that the viable counts increased rapidly during the first 24 h and remain constant for around 7 days. During this time, the cells synthesize lysine, most of which accumulates in the medium. The inference that most of the lysine arises by biosynthesis is based on the known amino acid composition of corn steep liquor (Cardinal and Heedrick, 1948; Aurich, 1960), on the basis that the corn steep liquor medium initially contained only 2 µmol lysine per ml (unpublished data) and because the specific activities of aspartokinase, dihydrodipicolinate synthetase and diaminopimelate decarboxylase differed only slightly (10% increase, 22% decrease and 22% increase respectively) between the 16th and 144th hour of fermentation (unpublished data).

Although the intracellular concentration of lysine is greater than the extracellular concentration in the initial stages of fermentation, it is less towards the end of the fermentation period (table 1). Thus, lysine is excreted against a concentration gradient towards the end of the fermentation period. Further support to this conclusion is lent by the observation that lysine is accumulated within the cells after viable counts start falling off (figure 1, table 1).

It may be assumed that in cells which can no longer divide, the biosynthetic and energy yielding machinery have become less organised. The viability of several bacteria is reported to decrease when the adenylate charge in the cells decreases to values below 0.8 (Chapman and Atkinson, 1977). The physiological changes occurring in cells that cannot divide would contribute significantly to those observed in the 'death phase'. It has been established that bacteria accumulate amino acids in cells to levels several times higher than those in the growth medium (Harold, 1972). Eftlux of intracellular amino acids occurs upon inhibition of energy-generating mechanisms either by the use of uncouplers, by the inhibition of

Table 1. Intracellular and extracelluar concentrations of amino acids during lysine fermentation.

Time (h)	Intracellular			Extracellular		
	Lysine [a] (nmol/mg dry weight)	Total amino acids (nmol/mg dry weight)	Mole fraction of lysine	Lysine [b] (μmol/ml or mM)	Total amino acids (μmol/ml)	Mole fraction of lysine
0	1 (2)	150	0.007	2	18	0.11
8	5 (10)	180	0.028	3	18	0.17
16	19 (37)	590	0.032	5	22	0.23
24	26 (52)	650	0.40	13	23	0.57
48	16 (31)	460	0.035	31	57	0.54
72	19 (37)	430	0.044	37	69	0.54
96	21 (40)	370	0.056	54	90	0.60
168	15 (29)	250	0.060	83	93	0.89
240	28 (54)	180	0.155	94	144	0.65
312	40 (77)	160	0.250	81	148	0.55

Values in parenthesis represent intracellular concentration (mM i.e., μmol/ml cell volume). 1 mg dry weight of cells was taken to be 0.52 μl cell volume.

[a] The estimated lysine was completely decarboxylated by lysine decarboxylase (*B. cadaveris,* Sigma Chemical Co., USA).

[b] Intracellular and extracellular lysine concentration as estimated by ion-exchange chromatography (Durrum Microbore Fluoropa Automatic Analyzer, Kit MBF, Durrum Chemical Co., USA) at the 24, 72 and 168th h of fermentation agreed with the electrophroetec value to within 5%.

electron transport or by the starvation of organisms such as *Streptococcus lactis,* which have no energy reserves (Brown, 1971; Kessel and Lubin, 1962; Thomas and Batt, 1969). Therefore, increase in the intracellular lysine concentration in the phase of logarithmic decrease in viable counts suggests that the concentration of lysine outside the cell was maintained at a higher level than the intracellular lysine concentration, at the expense of cellular energy, before the viable counts started falling off.

Copious excretion of primary metabolites such as citric acid, amino acids or nucleoside bases by microorganisms is well documented (Rose, 1978). The extracellular concentration attained in these fermentation are very high. It is not known whether excretion occurs against a concentration gradient in these fermen tations also. There is a report of 'active' excretion of lysine occuring in an AEC-resistant mutant of *Brevibacterium lactofermentum* (Nakayama, 1976). The details of the experiments leading to this inference are not available.

The possible advantage in having a transport system capable of excreting an amino acid to levels higher than those found within the cell is that excretion can continue uninterrupted to any extent, while the intracellular concentration is

maintained at low, presumably 'non-toxic' levels, levels low enough to prevent cessation of its synthesis or not high enough to stimulate its breakdown.

Further experimentation is required to show whether the cells of *M. glutamicus* have active lysine uptake systems functioning in the early stages of fermentation (table 1) which start functioning in the direction of active exit or whether the cells have a transport system for lysine in which coupling to cellular energy occurs in the reverse direction, so that it is able to establish a concentration gradient with higher concentration outside.

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