Stimulatory effect of glycine betaine on L-lysine fermentation

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Summary. The growth rate, sugar consumption rate, and production rate of an L-lysine producing Brevibacterium lactofermentum mutant were stimulated by addition of exogenous glycine betaine. Glycine betaine stimulated the growth rate especially in media of inhibitory osmotic stress, and the stimulation was independent of any specific solute. Therefore growth stimulation by glycine betaine was considered to be an osmoprotective effect. A strong enhancement of the sugar consumption rate and the L-lysine production rate was observed even with resting cells under osmotic stress as well as in a fermentation with growing cells. These data indicated that the osmoprotective effects of glycine betaine on L-lysine production can be independent of protein synthesis.

Introduction

Numerous studies on the microbial production of amino acids have been carried out, and an increasingly large number of L-amino acids is now produced on an industrial scale using auxotrophs and regulatory mutants of bacteria (Hirose and Okada 1979). Brevibacterium lactofermentum is one of the L-glutamic-acid-producing bacteria, and various amino-acid-producing mutants have been derived from it. Recent improvement of L-amino-acid-producing mutants (Tosaka et al. 1978a, b) and studies on the optimum oxygen supply (Akashi et al. 1978, 1979a, b, c) have significantly increased the accumulation of L-amino acids.

Because a large amount of carbon and nitrogen source is necessary to obtain high accumulation of L-amino acids, the osmotic pressure of media for industrial amino acid fermentations is apt to be higher than that of media used generally for small-scale experiments, and thereby the growth of L-amino-acid-producing bacteria may be stressed. It is therefore considered that the osmotic pressure of media used for amino acid fermentations is one of the limiting factors of productivity.

We have investigated osmoregulation of the wild strain of B. lactofermentum (Kawahara et al. 1989, 1990) and have clarified that exogenous glycine betaine stimulates its growth rate in a medium of inhibitory osmotic strength. In this paper, we examine the effects of exogenous glycine betaine on L-lysine fermentation using a mutant of B. lactofermentum AJ12319.

Materials and methods

Organism and culture conditions. B. lactofermentum AJ12319, an L-lysine-producing mutant, was employed. Seed medium had the following composition (per litre): 50 g sucrose, 5 g urea, 1.0 g KH₂PO₄, 0.4 g MgSO₄·7H₂O, 0.01 g FeSO₄·7H₂O, 0.01 g MnSO₄·4H₂O, 50 ml soybean protein hydrolysate, 5 mg nicotinamide, 0.2 mg thiamine·HCl, and 0.05 mg biotin, pH 7.5 (with NH₄OH). Production medium had the following composition (per litre): 130 g or 160 g glucose, 23 g (NH₄)₂SO₄, 1 g KH₂PO₄, 1 g MgSO₄·7H₂O, 0.01 g FeSO₄·7H₂O, 0.01 g MnSO₄·4H₂O, 5 mg nicotinamide, 0.2 mg thiamine·HCl, 0.5 mg biotin, and 55 ml soybean protein hydrolysate, pH 6.5 (with NH₄OH). Complete medium had the following composition (per litre): 5 g glucose, 10 g yeast extract, 10 g peptone, 5 g NaCl, pH 7.2 (with KOH). Minimal medium had the following composition (per litre): 20 g glucose, 10 g (NH₄)₂SO₄, 1 g KH₂PO₄, 0.4 g MgSO₄·7H₂O, 0.01 g FeSO₄·7H₂O, 0.01 g MnSO₄·4H₂O, 0.2 mg thiamine·HCl, 0.5 mg biotin, 5 mg nicotinamide, 2.5 g urea, and 100 mg NaCl, pH 7.2 (KOH). The cultivation temperature was 31.5°C. Shaking flasks or test tubes were used for bacterial cultivation. The cultivation in production medium was performed with 1-l jar fermentors. The dissolved oxygen level was kept above 0.01 atm and the pH was kept at 6.5 with gaseous NH₃ throughout the cultivation in jar fermentors.

Measurement of oxygen uptake. Cells were grown in seed medium, harvested at late logarithmic growth phase, centrifuged at 4°C, washed twice with 50 mM potassium phosphate buffer, pH 7.0, and finally resuspended in the same buffer. Decrease in the dissolved oxygen level of cell suspensions was measured at 30°C in the sealed reaction vessel with a Clark (CABLE Co., Tokyo, Japan) oxygen electrode in the presence of 50 mM potassium phosphate buffer, pH 7.0.

Reaction with resting cells. Basal reaction mixture of the following composition was used (per litre): 20 g glucose, 10 g (NH₄)₂SO₄,
1 g KH₂PO₄, 0.4 g MgSO₄·7H₂O, 100 mg chloramphenicol, 50 g CaCO₃. The concentrations of glucose and (NH₄)₂SO₄ were changed as described. Cells were grown in production medium, harvested at late logarithmic growth phase, centrifuged at 4 °C, washed twice with 50 mM potassium phosphate buffer, pH 7.0, and finally resuspended in the reaction mixture. The reaction was carried out at 31.5°C on a shaker. The sugar consumption rate and the L-lysine production rate were determined by measuring residual sugar concentration and L-lysine concentration every 2 h during the reaction.

Other analyses. Osmotic pressure of media was measured by an Advanced wide range osmometer 3W2 (Advanced Instruments Needham Heights, Mass., USA), and expressed as osmoles of solute per kilogram of solution. Bacterial cell growth was determined by the optical density at 562 nm or 620 nm after diluting appropriately. Quantitative determination of L-lysine was conducted by the acidic ninhydrin method (Chinard 1952). The concentration of glucose was determined by the glucose C test (Wako Chemical Co., Tokyo, Japan).

Chemicals. Peptone and yeast extract were obtained from Daigo Eiyou Kagaku Co., Tokyo, Japan, Nicotinamide, thiamine-HCl, biotin, and glycine betaine were obtained from Tokyo Kasei Co., Tokyo, Japan. Other chemicals used here were obtained from Junsei Kagaku Co., Tokyo, Japan.

Results

Influence of osmotic pressure on the growth of an L-lysine-producing mutant

The influence of osmotic pressure on the growth rate of an L-lysine-producing mutant of *B. lactofermentum* AJ12319 was examined. Table 1 shows the relationship between the osmotic pressure of complete medium and the maximum specific growth rate of AJ12319. As the osmotic pressure of the media was increased by adding sorbitol, the maximum specific growth rate of the mutant decreased.

Effect of glycine betaine on L-lysine production

The effects of glycine betaine on L-lysine production were investigated by cultivation in a jar fermentor (Fig. 1). When the initial sugar concentration of the production medium was 130 g/l, the addition of glycine betaine stimulated the growth rate of AJ12319, but L-lysine accumulation did not vary. Then, with 160 g/l initial sugar concentration (Fig. 2), greater accumulation of L-lysine was observed. Furthermore, the addition of glycine betaine into the medium significantly stimulated the growth rate, the sugar consumption rate, and the L-lysine production rate of the mutant.

**Table 1.** Influence of osmotic pressure on the growth of *Brevibacterium lactofermentum* AJ12319

<table>
<thead>
<tr>
<th>Sorbitol (M)</th>
<th>Osmotic pressure (osmol/kg)</th>
<th>Maximum specific growth rate (h⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0</td>
<td>0.50</td>
<td>0.285</td>
</tr>
<tr>
<td>0.5</td>
<td>1.10</td>
<td>0.280</td>
</tr>
<tr>
<td>1.0</td>
<td>1.67</td>
<td>0.214</td>
</tr>
</tbody>
</table>

Cultivation was carried out in complete medium. Cell growth was determined every 1 h by the optical density (OD) at 620 nm after 26-fold dilution. The specific growth rate was calculated based on the following equation: \( \frac{dX}{dt} = (\text{specific growth rate}) X \), where \( X = \text{OD} \). Determination and definition of osmotic pressure are described in Materials and methods.

**Fig. 1.** L-Lysine produced in production medium with 130 g/l initial glucose in the presence of 10 mM glycine betaine (solid symbols) or in the absence of glycine betaine (open symbols) as described in Materials and methods. Optical density (OD) at 620 nm was measured after 51-fold dilution: O, OD; Δ, L-lysine HCl (LysHC1); □, glucose

**Fig. 2.** L-Lysine produced in production medium with 160 g/l initial glucose in the presence of 10 mM glycine betaine (solid symbols) or in the absence of glycine betaine (open symbols) as described in Materials and methods: O, OD; Δ, L-lysine HCl; □, glucose

Effect of glycine betaine on the growth of the L-lysine-producing mutant

We investigated effects of exogenous glycine betaine on the growth of AJ12319 with minimal medium in order to confirm that it is an osmoprotective compound of the mutant (Fig. 3). The addition of glycine betaine into