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Effect of glycine betaine on osmoadaptation of Propionibacterium acidipropionici cultivated in elevated osmolarities

Abstract The sensitivity of industrial strains Acetobacter aceti, Gluconobacter frateurii, and Propionibacterium acidipropionici to osmotic stress was studied. Growth of A. aceti and G. frateurii was totally inhibited at 0.4 M NaCl concentration, but P. acidipropionici was able to grow on a medium containing 1.2 M NaCl. Addition of glycine betaine to the medium had no detectable osmoprotective effect on A. aceti and G. frateurii cultivations in elevated NaCl concentrations, but it enabled cells of P. acidipropionici to achieve faster the maximum specific growth rate after the prolonged lag phase and therefore to gain faster the final biomass and product concentrations. The final concentrations of biomass and product of P. acidipropionici were the same as for the cultivations of the bacterium without NaCl and glycine betaine present in the medium. Intracellular accumulation of glycine betaine was detected in P. acidipropionici cells cultivated in the medium containing glycine betaine. The amount accumulated increased with NaCl concentration, suggesting that glycine betaine plays an important role in the osmoadaptation.

Introduction

The economic feasibility of fermentation processes is often limited by low productivity due to low cell densities. In fermentation processes, cells are exposed to osmotic stress caused by medium components and metabolic products. Cells respond to increased osmolarity by spontaneous water loss, which may inactivate the enzymatic reactions inside the cells and thus be a limiting factor for both growth and production. Several process alternatives have been proposed to avoid substrate and product inhibition, including stepwise substrate addition by a fed batch process and continuous withdrawal of the product by a cell recycle process. Although improvements in productivity have been achieved, the cell loss due to osmotic stress has not been avoided.

Bacteria minimize water loss by adjusting their turgor pressure to the external osmotic pressure through the accumulation of inorganic ions and compatible organic solutes. Compatible organic solutes, called osmolytes, when accumulated at high intracellular concentrations are not deleterious to essential biochemical and metabolic functions of the cells. Osmolytes are either synthesized de novo (endogenous osmolytes) or taken up from the environment (exogenous osmolytes). Glycine betaine, choline, proline, ectoine, trehalose, and 3-dimethylsulfiniopropionate (DMSP) are the most studied among the known exogenous osmolytes (Beumer et al. 1994; Cosquer et al. 1999; Jebbar et al. 1995; Skjerdal et al. 1996). Endogenous osmolytes synthesized by bacteria include a few amino and imino acids such as glycine betaine, glutamate, proline, and ectoine; polyol glycerol; and the disaccharides trehalose and sucrose (Gouli et al. 1999).

Glycine betaine is a compatible solute found in large amounts in some bacterial cells living in environments with high osmolarity. A transport system specific for glycine betaine has been found in both halophilic (Canovas et al. 1996) and nonhalophilic bacteria (Baë et al. 1993; Park et al. 1995; Peter et al. 1998; Pichereau et al. 1999; Skjerdal et al. 1995) as well as in methanogenic archaea (Proctor et al. 1997). Accumulation of glycine betaine increases cell tolerance to high salinity and drought (Beumer et al. 1994; Jebbar et al. 1995; Kets et al. 1996; Peter et al. 1998; Skjerdal et al. 1996). Besides acting as a compatible solute, glycine betaine has an osmoprotective function in some bacteria, stimulating their growth in hyperosmotic environments (Csonka 1989). In addition to glycine betaine transport, Bacillus subtilis (Boch et al. 1994) and Halomonas elongata

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(Canovas et al. 1996, 1998) reportedly are able to synthesize glycine betaine from exogenously added precursor choline, and they possess an uptake system specific for choline.

Improvement of the productivity of fermentation processes requires an enhancement of the osmotic tolerance of the production strains. It is of utmost importance therefore to understand the mechanism of osmoadaptation of the bacteria and the osmoprotective effect that the osmolytes have on these bacteria.

The objective of the present study was to establish the effects of glycine betaine addition on bacterial cell cultivations in elevated osmolarities created through the addition of NaCl. We examined the osmosensitivity of commercially promising bacterial strains Acetobacter aceti, Gluconobacter frateurii, and Propionibacterium acidipropionici and the effect of glycine betaine addition on their osmotolerance. In addition, we studied the uptake of glycine betaine by resting cells of P. acidipropionici with 14C-labeled glycine betaine.

Materials and methods
Organisms, maintenance, and inocula preparation

Propionibacterium acidipropionici ATCC 4875, Acetobacter aceti IFO 3281, and Gluconobacter frateurii IFO 3254 were stored as frozen stock cultures containing 10% (v:v) glycerol in 2-ml ampoules at −80 °C. Inocula for osmosensitivity studies was prepared by cultivating P. acidipropionici, A. aceti, and G. frateurii in 250-ml shake flasks containing 50 ml complex medium at 30 °C for 20–24 h. A. aceti and G. frateurii were shaken at 200 rpm during cultivation in a rotary shaker (Cerzetron R, B. Braun Biotech International, Germany).

Inocula for bioreactor cultivation were prepared by cultivating P. acidipropionici first in 10-ml test tubes on complex medium for 24 h at 30 °C and then in 250-ml shake flasks containing 50 ml complex medium for 24 h without shaking. Cells were separated by centrifugation (8,000 rpm, 10 min, Sorval RC 5 C Plus, Du Pont, USA), washed and suspended in 30 ml deionized water to give an initial biomass concentration of 0.14–0.34 g cell dry weight (CDW) per liter in a bioreactor.

Inocula for glycine betaine uptake studies with resting cells were prepared by cultivating P. acidipropionici on 50 ml complex medium for 48 h at 30 °C. Cells were separated by centrifugation (8,000 rpm, 10 min, Sorval RC 5 C Plus, Du Pont, USA). Glycine betaine taken up by the cells from the complex medium was washed off with Sörensen buffer solution and washed cells were suspended in deionized water.

Media
Complex medium contained yeast extract (Difco, USA) 10 g/l, trypticase peptone (BBL = Becton Dickinson and Company, USA) 10 g/l, KH2PO4 0.125 g/l, MgSO4·7H2O 0.25 g/l and glucose (Fluka, Switzerland) or glycerol (BBL = Becton Dickinson and Company, USA) 10 g/l. The pH of the medium was adjusted to 5.8 for A. aceti and G. frateurii and to 6.8 for P. acidipropionici cultivations.

Mineral medium was prepared according to Verduyn et al. (1992) and contained NH4SO4 5.0 g/l, KH2PO4 3.0 g/l, MgSO4·7H2O 0.5 g/l, EDTA 15.0 mg/l, ZnSO4·7H2O 4.5 mg/l, CoCl2·6H2O 0.5 g/l, MnCl2·4H2O 1.03 g/l, CuSO4·5H2O 0.3 g/l, CaCl2·2H2O 4.5 g/l, FeSO4·7H2O 3.0 g/l, NaMoO4·2H2O 0.4 g/l, H3BO3 1.0 mg/l, KI 0.1 mg/l, and silicone antifoaming agent.

331512 K 0.05 mg/l (BDH = BDH Laboratory Supplies, England). Glycerol (10 g/l) was added as a carbon source for A. aceti and G. frateurii cultivations while glucose (10 g/l) was used in P. acidipropionici cultivations. After sterilization (20 min at 120 °C), a filter-sterilized vitamin solution was added, giving a final concentration of biotin 0.05 mg/l, calcium pantothenate 1.0 mg/l, nicotinic acid 1.0 mg/l, myoinositol 25 mg/l, pyridoxal hydrochloride 1.0 mg/l, and para-aminobenzoic acid 0.2 mg/l. An additional vitamin supplement was used for acetate acid bacteria cultivation to give final concentrations of biotin 1.05 mg/l, nicotinic acid 1.5 mg/l, pyridoxal hydrochloride 1.5 mg/l, para-aminobenzoic acid 1.0 mg/l, riboflavin 1.5 mg/l, folic acid 1.0 mg/l, and vitamin B12 1.0 mg/l. The pH of the medium was adjusted to 5.8 for A. aceti and G. frateurii and to 6.5 for P. acidipropionici cultivations.

Osmosensitivity studies

Growth of P. acidipropionici, A. aceti, and G. frateurii was studied in Bioscreen C (Bioscreen C, Labsystems, Finland) microcultivation equipment. In Bioscreen C, the changes in optical density in the culture medium due to growth of micro-organisms are measured kinetically with a vertical photometer in which the light beam passes up through the bottom of the cuvette and through the sample suspension to a detector. The incubator of the Bioscreen C consists of an incubator tray, a cover for the incubator tray, a temperature control system, and a shaker. Bioscreen C permits 200 samples to be run at a time, with samples placed in the wells of two honeycomb plates, each with 100 wells of 500 μl volume.

In our studies 50 μl inoculum was added to 350 μl mineral medium and incubated at 30 °C for 4 days. The optical density of the cultivation medium was measured automatically at 800 nm once an hour. P. acidipropionici cultivation plates were shaken 20 s before each optical density measurement and A. aceti and G. frateurii cultivations were continuously shaken. The NaCl concentration of the medium was adjusted to 0, 0.2, 0.4, 0.8, or 1.2 M and glycine betaine concentration to 0 or 20 mM. Glycine betaine (Betafin, anhydrous, pharmaceutical grade) was a kind gift from Finnfeeds, Finland. Results of these experiments are averages of measurements in five to ten different wells. The standard deviation of the optical density measurements of the samples was less than 10%.

Bioreactor cultivations of P. acidipropionici

Bioreactor cultivations were carried out in a 1-dm³ bioreactor (Biotest Q, Braun Biotech International, Germany) with a working volume of 500 ml and automatic pH and temperature control. The temperature of the cultivation was 30 °C and stirrer speed 400 rpm. The culture pH was set to 6.5. The NaCl concentration of the medium was adjusted to 0, 0.4 or 0.8 M. The growth was measured as cell dry weight from the culture medium samples. All experiments were done in duplicate. The deviation between the dry weight samples of the parallel cultivation was less than 10%.

Dry weight measurement

Culture samples (10 ml) were vacuum-filtered through a pre-weighed nitrocellulose filter (0.45 μm, Schleicher & Schuell, Germany), washed with Milli-Q water, and dried in a microwave oven for 20 min (Igins, Japan). Two parallel measurements were done from one sample and the standard deviation of the technique was determined to be less than 3%.

Substrate and metabolite analysis

Samples of 1 ml from the bioreactor cultivations were centrifuged at 10,000 rpm for 10 min (Heraeus Sepatech, Biofuge A, Germany) and the supernatant was stored at −20 °C for further analysis. Glucose, acetate, and propionate concentrations were determined by high-performance liquid chromatography (HPLC). Sample components were separated on an HPX-87H Aminex ion-exclusion