Enhanced butanol production and reduced autolysin activity after chloramphenicol treatment of Clostridium acetobutylicum ATCC 824

Xiangdong Zhou* and Richard W. Traxler

Department of Food Science and Nutrition, University of Rhode Island, Kingston, RI 03881, USA

Received 2 October 1991/Accepted 17 February 1992

Summary. Release of autolysin during the late exponential growth phase of Clostridium acetobutylicum resulted in early lysis of the culture and reduction of solvent formation. A simple and effective way of reducing autolysin activity and increasing solvent production is partial inhibition of protein synthesis with chloramphenicol (CAP). The extracellular autolytic activity in the culture, determined by following loss of turbidity of washed clostridial cells in 0.04 M sodium phosphate buffer at 37 °C, was decreased by 40% after CAP treatment. This caused an extension of cell viability by 12 h and an increase in butanol production by 30%. The optimal time of CAP addition was 12 h of incubation, and the optimal antibiotic concentration was 120 μg/ml. The effects of CAP on the fermentation are due to the inhibition of protein synthesis leading to a decrease in autolysin level in the culture. The results obtained provide economic advantages for industrial production of solvents by minimizing autolysin activity and maximizing solvent yield during the critical solvent-producing phase.

Introduction

An intrinsic problem associated with acetone-butanol-ethanol fermentation by Clostridium species is that shortly before the end of the exponential growth phase an extracellular autolysin is produced that partially inhibits solvent production. Autolytic enzymes (or autolysins) destroy the bacterial cell wall by hydrolyzing peptidoglycan and cause cellular autolysis (Rogers et al. 1980; Yoshino et al. 1982). Autolysin activity in the culture of Clostridium acetobutylicum (van der Westhuizen et al. 1982; Soucaille et al. 1987) was found to correlate with butanol tolerance, since autolytic-deficient mutants that produce much less autolysins were observed to have greater butanol tolerance compared to the wild-type strains.

Reduction of autolysin production following inhibition of protein synthesis after the addition of chloramphenicol (CAP) or other protein synthesis inhibitors has been reported in Streptococcus faecalis (Pooley and Shockman 1970), Bacillus cereus (Chung 1967), and Escherichia coli (Leduc et al. 1982). Similar results were obtained in cultures of E. coli and S. faecalis with amino acid starvation (Goodell and Tomasz 1980; Pooley and Shockman 1970). Autolysin-defective mutants were found to autolyze much more slowly than the parent wild-type strains (Allcock et al. 1981; Pooley et al. 1972). This investigation was designed to determine whether addition of CAP to cultures of C. acetobutylicum could cause a reduction in autolysin activity and an increase in solvent yield.

Materials and methods

Microorganism and culture conditions

C. acetobutylicum ATCC 824 was grown in batch fermentations in Hi flasks containing 700 ml broth. Cultures were incubated anaerobically at 37 °C without agitation or pH control. The medium used for batch cultures contained the following ingredients (g/100 ml): Na₂SO₄, 0.018; K₂HPO₄, 0.0175; biotin, 0.001; p-aminobenzoic acid, 0.001; tryptone (BBL, Baltimore, Md., USA), 0.1; yeast extract (Difco, Detroit, Mich., USA), 0.5; glucose, 5.0; mineral salt solution, 0.1 ml; distilled water, 100 ml. The mineral salt solution contained the following in 11 distilled water: NaMoO₄·2H₂O, 0.24 g; CoCl₂·6H₂O, 0.24 g; CaCl₂·2H₂O, 1.5 g; FeCl₃·6H₂O, 2.7 g; CuSO₄·5H₂O, 0.25 g; ZnSO₄·0.29 g; MnSO₄·H₂O, 1.7 g; MgSO₄, 12 g; H₂SO₄ (6 M), 28 ml. Samples (10 ml each) were withdrawn periodically to determine pH, optical density (OD), cell dry weight, viable cell number, acids, solvents.

* Present address: Buckman Laboratories International, Inc., 1256 North McLean Boulevard, Memphis, TN 38108, USA
Correspondence to: R. W. Traxler
residual glucose and autolysin activity. CAP was purchased from Gibco Life Technologies (Chagrin Falls, Ohio, USA).

**Analyses**

**Biomass and substrate concentration.** OD was monitored with a Klett-Summersen photoelectric colorimeter equipped with a no. 42 filter. OD values were obtained by multiplying Klett units by the factor of 0.002 (Klett-Summersen Clinical Manual, Klett Manufacturing Co., New York, 1969). Cell dry weight was determined on a preweighed membrane (Nuclepore, Pleasanton, Calif., USA; 0.45 μm); culture broth was filtered through the membrane, the cells trapped on the membrane were washed twice with distilled water, the membrane and the cells were dried in a desiccator at 37°C until a constant weight was reached, and the membrane was then re-weighed. Viable cell numbers were obtained by the double-layer method in which 200 μl diluted cell culture was mixed with 4 ml of upper layer medium, containing 5% glucose, 1% trypotone, 3% yeast extract, mineral salts (as described above) and 0.75% agar at 42°C, and the entire contents were poured onto the basal medium (same as upper medium, but with 1.5% agar) in a petri dish. Plates were incubated in a Gas Pak jar (BBL, Baltimore, Md., USA) at 37°C for 2 days. Colonies were counted and the viable cell numbers (colony-forming units, cfu) were expressed as log_{10} cfu/ml. Residual glucose concentration was measured by the glucose oxidase method using a Model 23A Glucose Analyzer (Yellow Springs Instrument Co., Yellow Springs, Ohio, USA).

**Concentration of acids and solvents.** Acids and solvents were determined using a Hewlett-Packard model 5730A (Hewlett-Packard, Avondale, Pa., USA) gas chromatograph equipped with a flame ionization detector and a 2 mm by 3 m stainless steel column packed with Poropak Q 80/100 mesh Product Company (Supelco, Bellefonte, Pa., USA). Nitrogen with a flow rate of 42 ml/min was used as the carrier gas. The column temperature was held at 130°C for 3 min after injection then programmed at a rate of 16°C/min to 200°C with an 8 min final hold. The temperature for detector and inject port were 250°C and 200°C, respectively. The peak area was automatically integrated against an external standard with a Shimadzu C-R3A data processor (Shimadzu, Kyoto, Japan).

**Cellular autolysis test.** Cells from both control and CAP-treated cultures were harvested by centrifugation at 12100 g in a Sorvall RC-5B centrifuge (Sorvall, Wilmington, Del., USA) with an SS-34 rotor for 10 min at 4°C, washed twice with cold distilled water, and suspended in 0.04 M sodium phosphate buffer (pH 6.4). Cellular autolysis was monitored by following loss of turbidity of the cell suspension incubated at 37°C (Sayare et al. 1972). By this method, intracellular autolysin activity was indirectly assayed. Release of intracellular nuclear acids to the culture supernatant was also assayed as a direct test of cellular autolysis by measuring the concentration of autolysin activity by a modified procedure described previously (Allecoc et al. 1981). Exponential-phase C. acetobutylicum cells were harvested by centrifugation at 12100 g (SS-34 rotor) for 10 min (4°C), washed three times in distilled water, and suspended in 0.04 M sodium phosphate buffer (pH 6.3). Cell/buffer solution (8 ml and 2 ml) of autolysin sample (culture filtrate obtained by filtering the broth through a 0.45-μm membrane) were introduced into test tubes, mixed and incubated at 37°C. One unit of autolysin activity was defined as the amount of enzyme that causes a decrease in OD of the suspension by 0.1 unit/h. A control tube, to which 2 ml distilled water and 8 ml cell suspension were added, was used to subtract the amount of OD decrease caused by the test cell itself (including extracellular autolysins) from the assay system. By this method, only extracellular autolysin activity was measured.

**Results**

**Effect of time of adding CAP on the fermentation**

Addition of CAP (120 μg/ml) during the exponential growth phase (4 h or 8 h) suggested a certain degree of growth inhibition. However, such inhibition was not observed when CAP was added after the exponential phase (i.e. at 12 h and 16 h) since maximum growth was already achieved at 12 h in the control culture (Fig. 1A). The culture showed an extended stationary phase when treated with CAP at 12 h, whereas no such extension of the stationary phase was observed when treated with CAP at 16 h. Butanol production was greatly reduced when cultures were treated with CAP at 4, 8 and 16 h in comparison with the control (Fig. 1B). Therefore, it was concluded that the optimal time for adding CAP was at 12 h.

**Effect of concentration of CAP addition on the fermentation**

The optical density and butanol production of the culture treated with 10 μg/ml of CAP were almost identical to that of the control culture (Fig. 2A, B). When the concentration of CAP was increased to 60 μg/ml, reduction of autoysis and an increase in butanol production were achieved. Moreover, a greater cell number with an extension of cell viability and a greater increase in buta-

Fig. 1. Effect of time of chloramphenicol (CAP) addition on culture optical density (A), and butanol production (B). CAP (120 μg/ml) was added to growing cultures of Clostridium acetobutylicum at different fermentation times: O, control (no CAP added); □, CAP added at 4 h; +, 8 h; Δ, 12 h; ■, 16 h.