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Influence of acetic and butyric acid addition on polysaccharide formation by *Clostridium acetobutylicum*

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SUMMARY

The production of granulose (an intracellular reserve polygranule), capsule and exopolysaccharide was investigated in a synthetic medium in which the oxido-reduction level was modified by the addition of acetic or butyric acid. After addition of the acids, granulose synthesis increased from 150 to 300 mg glucose equivalents $\cdot l^{-1}$ and capsular synthesis decreased by 25%. Exopolysaccharide production was unchanged under these conditions. A hypothesis that attributes a role to the polymer in the oxido-reduction sequences is discussed.

INTRODUCTION

Many bacteria are capable of producing polysaccharides outside the cell wall. These exopolysaccharides may either take the form of a capsule or they may be present as extracellular slime apparently unattached to the bacterial surface [12]. Polysaccharides may be important to the bacteria (i.e., in adhesion, infection and protection) and can serve as physical barriers between the environment and the organism.

Häggström and Förberg [4] first demonstrated the production of a new extracellular polymer by *Clostridium acetobutylicum*. The presence of this polymer has been revealed by investigating material balances during the course of the fermentation. It seems to play a role in energy metabolism and when butyric acid is reassimilated simultaneously with butanol formation. It is suggested that this acetylated polymer could act as a sink for storage of non-reduced compounds when excess reducing power is required [4].

The addition of oxidized products such as acetic or butyric acids to batch cultures alters the oxido-reduction level. Their effects on solvent formation have been studied extensively [3,5–7] and their actions are well known.

The aim of the present study was to investigate

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the quantities of granulose, capsule and exopolysaccharide produced by *C. acetobutylicum* when oxido-reduction levels are modified by continuous inflow of acetic or butyric acids.

MATERIALS AND METHODS

Microorganism

The organism used was *C. acetobutylicum* strain ATCC 824. Spores of the culture were stored at 4°C in RCM medium (reinforced clostridial medium, Oxoid).

Medium

The synthetic medium had the following components per liter of distilled water: KH_2PO_4, 0.5 g; K_2HPO_4, 0.5 g; $MgSO_4 \cdot 7H_2O$, 0.2 g; $FeSO_4 \cdot 7H_2O$, 10 mg; $MnSO_4 \cdot 4H_2O$, 10 mg; NaCl, 10 mg; $(CH_3COO)NH_4$, 2.2 g; *p*-aminobenzoic acid, 1 mg; biotin, 0.01 mg; glucose, 60 g.

Continuous inflow of acid was maintained by means of a peristaltic pump. The flow rate (0.15 g $\cdot$ l^{-1} for acetic acid; 0.20 g $\cdot$ l^{-1} for butyric acid) was established after 24 h of growth and maintained until growth ceased. Penicillin G (K-Salts 1536 IU $\cdot$ mg^{-1}, Serva) (1 mg $\cdot$ l^{-1}) was also added after 24 h of growth.

Experiments were carried out in a 2 liter Biolafitte fermentor. The total volume of culture was 1.5 liters; the volume of the inoculum formed 10% of the total volume. The culture was stirred at a rate of 200 rpm. The pH of the medium was maintained at 4.8 by automatic addition of 2 M NaOH. The growth temperature was 35°C.

Methods of analysis

Cell concentration was estimated by cell dry weight measurement using a predetermined correlation between absorbance at 600 nm and cell dry weight.

Granulose was determined as glucose liberated by acid hydrolysis of cell-washed organisms. The suspension of the organism in 1 M H_2SO_4 was heated at 100°C for 3 h and neutralized with KOH [11]. Glucose was assayed using glucose oxidase (glucose diagnostic kits, Sigma No. 510).

Exopolysaccharide was assayed according to the method of Mian et al. [8]. Culture broth (10 ml) was mixed with 5 M NaCl (0.2 ml) and 0.5 M tetrasodium EDTA (0.2 ml), stirred for 30 min, and centrifuged at 11 000 $\times$ *g* for 30 min. The supernatant liquid was separated and dialysed against water for 18 h to remove free glucose. All dialysis tubing (Visking 30 μ) was pre-treated by repeating washing in 0.01 M EDTA/1% (w/v) Na_2CO_3, followed by washing in distilled water until contaminating carbohydrate material was undetectable. The amount of extracellular polymer produced was determined by the phenol-sulfuric acid method of Dubois et al. [2] with glucose as the standard [13].

Total carbohydrate content was determined by the method of Dubois et al. [2] in the broth culture previously heated in a boiling water bath for 5 min to prevent granulose degradation [1] and dialysed against water for 18 h. Capsular polysaccharide was determined by the difference between the total carbohydrate content and the granulose and exopolysaccharide.

RESULTS AND DISCUSSION

Granulose, capsular and exopolysaccharide production by C. acetobutylicum on a synthetic medium

Growth of *C. acetobutylicum* on a synthetic medium (66 g $\cdot$ l^{-1} glucose) in batch culture resulted in polysaccharide being produced throughout the fermentation (Fig. 1). Granulose – an intracellular reserve polygranule – accumulated in the cells and reached a concentration of 150 mg glucose equivalents $\cdot$ l^{-1} at the end of the exponential growth phase. *C. acetobutylicum* synthesized a capsular material continuously during the exponential phase and until growth ceased. Exopolysaccharide formation was elevated during the first 30 h but continued when growth ceased (Fig. 1).

Influence of acetic acid, butyric acid and penicillin on polysaccharide formation

The effects of acetic acid (0.15 g $\cdot$ l^{-1} $\cdot$ h^{-1} after 24 h of growth), butyric acid (0.20 g $\cdot$ l^{-1} $\cdot$ h^{-1} after 24 h of growth) and penicillin G (1 mg $\cdot$ l^{-1} after 24 h of growth) on polysaccharide production are il-

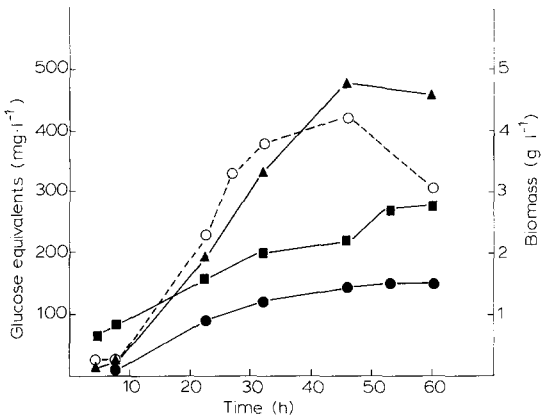

Fig. 1. Evolution of granulose (●), capsule (▲), exopolysaccharide (■) production and biomass formation (○) during glucose fermentation on a synthetic medium (66 g · l^{-1} glucose) by *C. acetobutylicum* ATCC 824.

lustrated in Fig. 2. The fermentation shown in Fig. 1 was used as reference for this study.

Addition of acetic or butyric acid (Fig. 2A and 2B) had similar effects on polysaccharide production by doubling granulose formation. Exopolysaccharide production was not affected but capsular production was decreased (350 mg eq. glucose compared to 475). Growth was slightly enhanced by addition of the acids.

The production of polysaccharide was also investigated in cultures treated with penicillin G (Fig. 2C). On the basis of the reference fermentation, after penicillin addition capsular, granulose and exopolysaccharide synthesis continued despite inhibition of growth. At the end of the fermentation – after 45 h when glucose consumption had stopped – a decrease in granulose and capsular synthesis occurred.

Under our experimental conditions, *C. acetobutylicum* ATCC 824 grown on a glucose medium produced granulose, capsule and exopolysaccharide.

In *C. acetobutylicum* ATCC 824, granulose synthesis was maximal after 45 h and remained constant (Fig. 1) which is contrary to what happens with *C. acetobutylicum* P 262 [9] where granulose is mobilized during spore development. Reysenbach

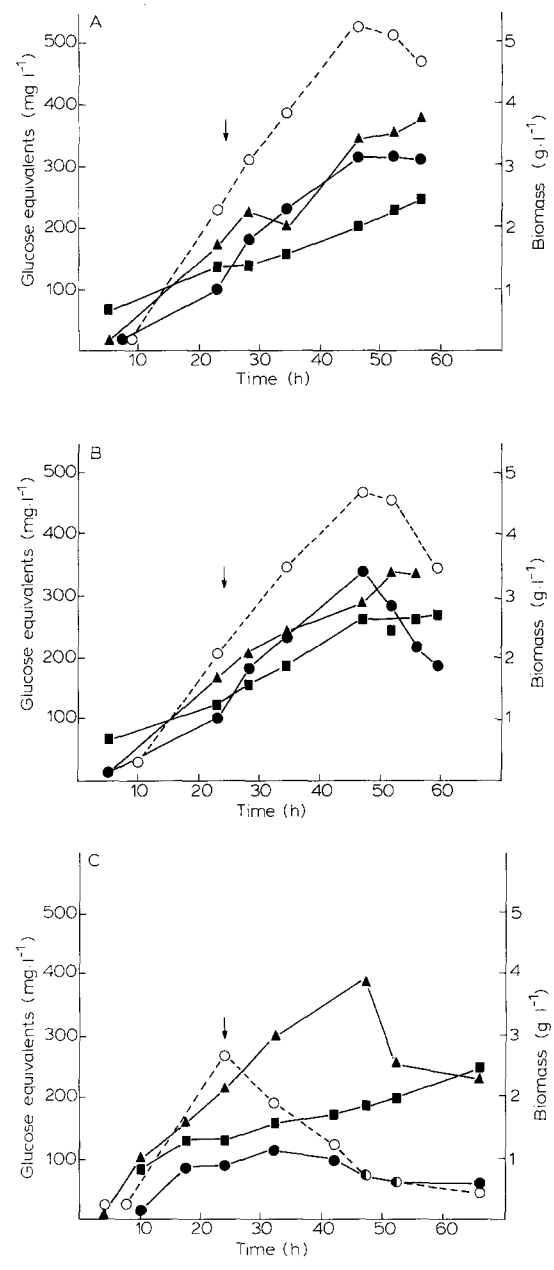

Fig. 2. Influence of the addition of acetic acid (A), butyric acid (B) and penicillin (C) on granulose (●), capsule (▲), exopolysaccharide production (■) and biomass formation (○) during glucose fermentation by *C. acetobutylicum*. For experimental conditions see Materials and Methods.

et al. [9] noted that granulose is not essential for sporulation but it may result in more effective sporulation under appropriate conditions. The absence

of granulose mobilization is probably due to the presence of exogenous glucose [10].

Penicillin affects peptidoglycan synthesis by inhibiting the crosslinking reaction. We have shown that after penicillin addition, capsular and exopolysaccharide synthesis continues despite inhibition of growth. These results suggest that in batch culture it is possible to obtain a higher specific polysaccharide formation rate and that some regulatory mechanisms exist.

By investigating material balances Häggström and Förberg [4] suggested that the polysaccharide is produced during the growth and acid production phase, and also when butanol is produced simultaneously from glucose and reassimilated butyric acid. The excess glucose consumed during butyric acid uptake is used to produce extracellular polysaccharide. When butanol and butyric acid are produced at the same time, reutilization of the produced polymer occurs. This acetylated polymer can act as a sink for storage of non-reduced compounds when excess reducing power is required [4] and it could be related to the oxidation and reduction levels.

It has been previously demonstrated that acetic acid added to the culture medium is metabolized by *C. acetobutylicum* and is the precursor of oxidized products such as acetone. When butyric acid is added to the culture medium, the organism requires extensive amounts of reducing power and the utilization of NADH is maximal [6,7]. Acetic or butyric acid addition modifies the oxido-reduction levels by enhancing formation of either oxidized or reduced compounds, but exopolysaccharide production is not greatly affected. Moreover the predicted polysaccharide uptake did not occur during the first phase of growth. The addition of acetic or butyric acid results in decreased capsular polysaccharide biosynthesis and in increased granulose formation. Reysenbach et al. [9] reported that granulose production is associated with the accumulation of threshold concentrations of acid end product.

The hypothesis of Häggström and Förberg [4] is based on material balance. We have shown that exopolysaccharide is produced by *C. acetobutylicum*. The maximal production is about 250 mg glucose equivalents $\cdot l^{-1}$ (Fig. 1). Such quantities cannot explain the imbalance between sugar consumption and product formation. At pH 6 acetoïn and lactate are produced in large quantities (respectively 4.4 and 3.2 g $\cdot l^{-1}$) (unpublished data) and could explain the deficit observed.

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