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Short Communication

Effect of carbohydrate source on alpha-amylase and glucoamylase formation by *Clostridium acetobutylicum* SA-1

Andrew Chojecki and Hans P. Blaschek*

Department of Food Science, University of Illinois, Urbana, IL 61801, U.S.A.

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SUMMARY

An examination into the effect of different carbohydrate sources indicated that the production of extracellular alpha-amylase and glucoamylase was under similar biosynthetic control in *Clostridium acetobutylicum* SA-1. Cell-associated starch-hydrolytic enzymes may be more important than extracellular enzymes in the processing of the starch molecule.

MATERIALS AND METHODS

Strain, media and experimental conditions

Clostridium acetobutylicum SA-1 (ATCC 35702) was obtained from the Department of Food Science at the University of Illinois. This butanol-tolerant strain was originally described by Lin and Blaschek [7]. Small-scale batch fermentation was carried out in 4.2% corn starch-soluble medium (CSSM) or 4.7% glucose-soluble medium (GSM). GSM and CSSM are modifications of the soluble medium described by Moreira et al. [8] with glucose (Sigma Chemical Co., St. Louis, MO) or corn starch (Sargent-Welch Co., Skokie, IL) as the primary carbohydrate sources, respectively. All experiments were carried out under anaerobic atmosphere (85% N_2, 10% CO_2 and 5% H_2) in a Coy Anaerobic Chamber (Coy Laboratory Products, Inc., Ann Arbor, MI) at 37°C. Sterile medium, 0.1% peptone (Difco, Detroit, MI) dilution tubes and distilled water were placed in the anaerobic chamber at least 24 h before use to ensure removal of dissolved oxygen. Plate counts were carried out using GSM medium solidified with 1.5% Bacto-agar (Difco).

Inoculum development

C. acetobutylicum SA-1 was maintained as described previously [7]. A 1.2 cc nunc cryotube (Vangard International, Neptune, NJ) containing 1.0 ml culture was taken out of liquid nitrogen storage and incubated for 30 min at 37°C in the anaerobic chamber. 9 ml of fresh medium (GSM or CSSM) was inoculated (10% v/v) with 1.0 ml culture. A late exponential phase culture was diluted 1:100 with either GSM or CSSM and used at the 2% (v/v)

* To whom correspondence should be addressed.

level to inoculate 400 ml GSM or CSSM, respectively.

Quantitative analysis of amylolytic enzymes

C. acetobutylicum SA-1 alpha-amylase was assayed using a modification of the methods described by Bernfeld [2], Fischer and Stein [6], Welker and Campbell [10], Ensley et al. [5], and Cork and Arnold [4]. One volume of cell-free supernatant was mixed with one volume of 1.0% soluble starch containing 0.1 M sodium acetate buffer and 0.02 M $CaCl_2$. The pH of the solution was adjusted to the 4.5 pH optimum for this enzyme. 1 ml of the solution was transferred to a borosilicate glass culture tube (18 × 150 mm; Kimble, Toledo, OH) and placed in a rotary bath shaker (G76, New Brunswick Scientific Co., Inc., New Brunswick, NJ). As a control, the amount of residual glucose in the spent medium was determined and used to correct the enzyme activity values. In the case of supernatants derived from GSM, the supernatant-to-buffer ratio was 1:20 in order to minimize the effect of residual glucose. After 20 min incubation at 37°C, the reaction was terminated by adding 1 ml of 3,5-dinitrosalicylic acid (3,5-DNSA; [2]). The resultant color change was a function of the reaction of liberated reducing groups with 3,5-DNSA during a 5 min boiling treatment. 10 ml of distilled water

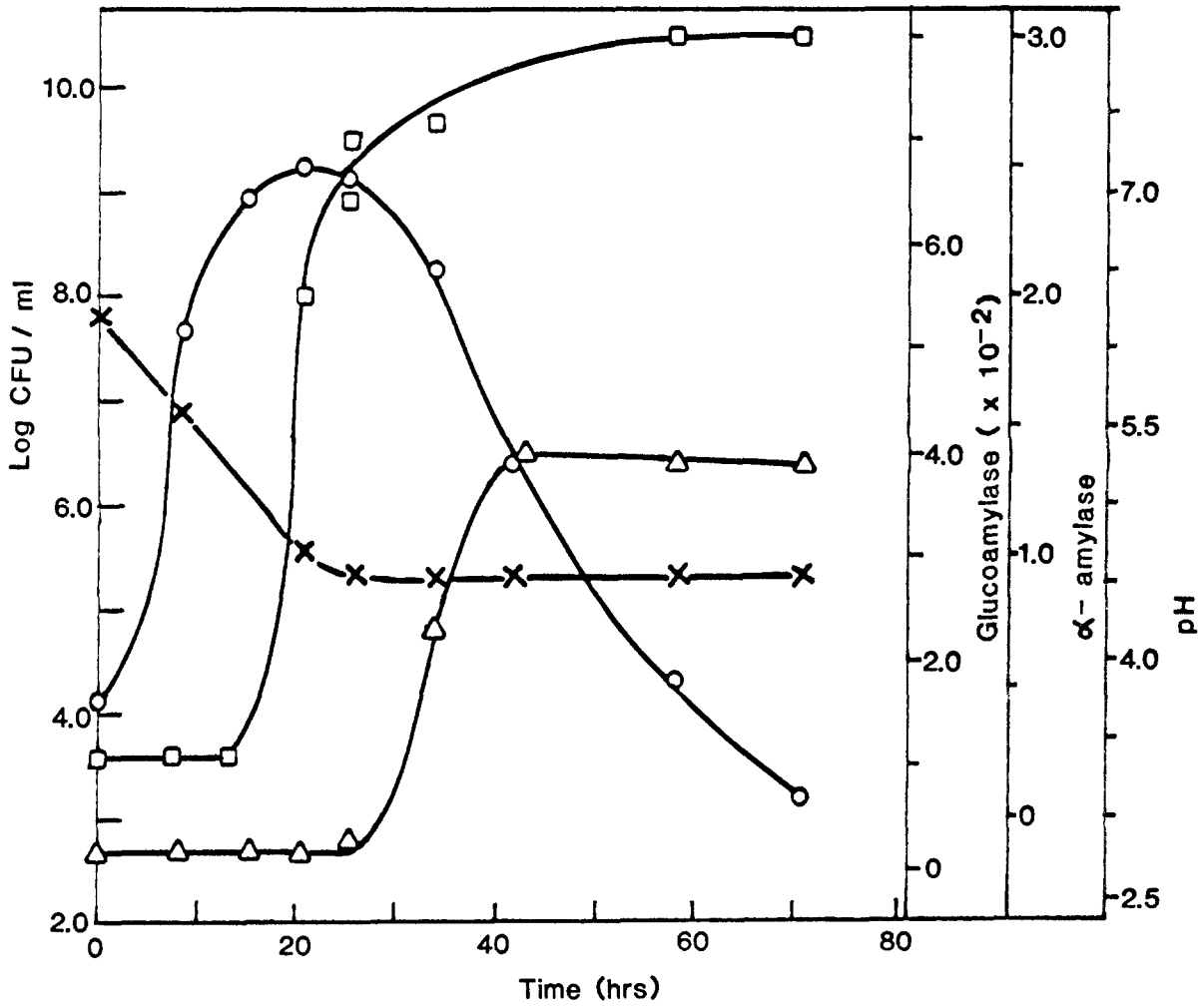

Fig. 1. Growth response (○), pH (×), α-amylase activity (□) and glucoamylase activity (△) of *C. acetobutylicum* SA-1 in 4.2% CSSM.

were added to the cooled solution and mixed thoroughly. The solution was further diluted 1:8 and the absorbance was measured at 540 nm (A_{540}) using a Spectronic 20 spectrophotometer (Bausch and Lomb, Inc., Rochester, NY). The absorbance value was corrected for dilution and the amount of maltose was calculated from the standard curve. Amylase activity was measured as μmol of maltose released in 20 min at pH 4.5 and 37°C. One unit of alpha-amylase activity was defined as the amount of enzyme which released 1 μmol of maltose per min per ml of supernatant under the above-described reaction conditions.

Glucoamylase was measured using a modification of the method described by Ensley et al. [5]. One volume of cell-free supernatant was mixed with one volume of 2.0% soluble starch in 0.1 M sodium acetate buffer. The pH of the solution was adjusted to the 6.5 pH optimum for this enzyme. A 0.5 ml volume was transferred to a culture tube and incubated in a rotary bath shaker for 60 min at 37°C. The amount of residual glucose in the spent medium was determined and used to correct enzyme activity values. In the case of GSM medium, the supernatant-to-buffer ratio was 1:10 to 1:40 in order to minimize the effect of residual glucose. The glucoamylase enzyme was inactivated by placing the reaction tubes in boiling water for 2 min. The amount of glucose liberated was quantitated using the colorimetric glucose oxidase-peroxidase (GOP) system described previously [1]. GOP reagent consisted of an ethanolic solution of 3,3-dimethoxybenzidine citrate-phosphate buffer, purified glucose oxidase and horseradish peroxidase (Sigma). A 0.5 ml volume of distilled water and 1.0 ml GOP reagent were added to the hydrolyzed starch solution. After incubation for 5 min at 30°C, 5 ml of 10 N sulfuric acid were added to stop the reaction. The absorbance of the solution was measured at 525 nm (A_{525}) using a Spectronic 20 spectrophotometer. The absorbance value was corrected for dilution and the amount of glucose was calculated from the standard curve. Glucoamylase activity was measured as μmol of glucose released in 60 min at pH 6.5 and 37°C. One unit of glucoamylase activity was defined as the amount of enzyme which released 1 μmol of glucose per min per ml of the supernatant under the above-described reaction conditions.

Determination of pH optima

The pH optimum for alpha-amylase and glucoamylase from *C. acetobutylicum* SA-1 was determined by adjusting the pH of a 4.2% CSSM culture supernatant to values between 2.0 and 9.0, using either HCl or NaOH. Enzyme activity was assayed as described above.

RESULTS AND DISCUSSION

The effect of carbon source on the formation of extracellular alpha-amylase and glucoamylase by *C. acetobutylicum* SA-1 can be seen in Figs. 1 and 2. When starch was the carbohydrate source (Fig. 1), alpha-amylase was excreted into the medium once the culture entered the stationary phase. The highest alpha-amylase activity was 3.0 U/ml, while the highest glucoamylase activity was $4.0 \cdot 10^{-2}$ U/ml. These values are 3.7- and 28.5-fold greater than the values reported by Ensley et al. [5] for alpha-amylase and glucoamylase activities of *C. acetobutylicum* NRRL-B592, respectively. The stimulation of alpha-amylase formation by cultivation of *C. acetobutylicum* on starch is in agreement with previous reports [3,10]. Scott and Hedrick [9] indicated that the 'beta-limit dextrin' in starch induced alpha-amylase synthesis in *C. acetobutylicum*. but glucose or sorbital did not stimulate the production of alpha-amylase. Induction of glucoamylase has been attributed to maltose or isomaltose [1]. This is consistent with the roughly 15 h lag in the production of extracellular glucoamylase behind alpha-amylase (Fig. 1). It is likely that breakdown products resulting from alpha-amylase action on starch are responsible for stimulating glucoamylase formation. In spite of a pH optimum of 6.5 for *C. acetobutylicum* SA-1 glucoamylase, this enzyme retains 50% of maximal activity at pH 4.5 (data not shown). The retention of activity by glucoamylase at low pH suggests that the conversion of starch to utilizable glucose subunits is possible throughout the normal course of this fermentation.

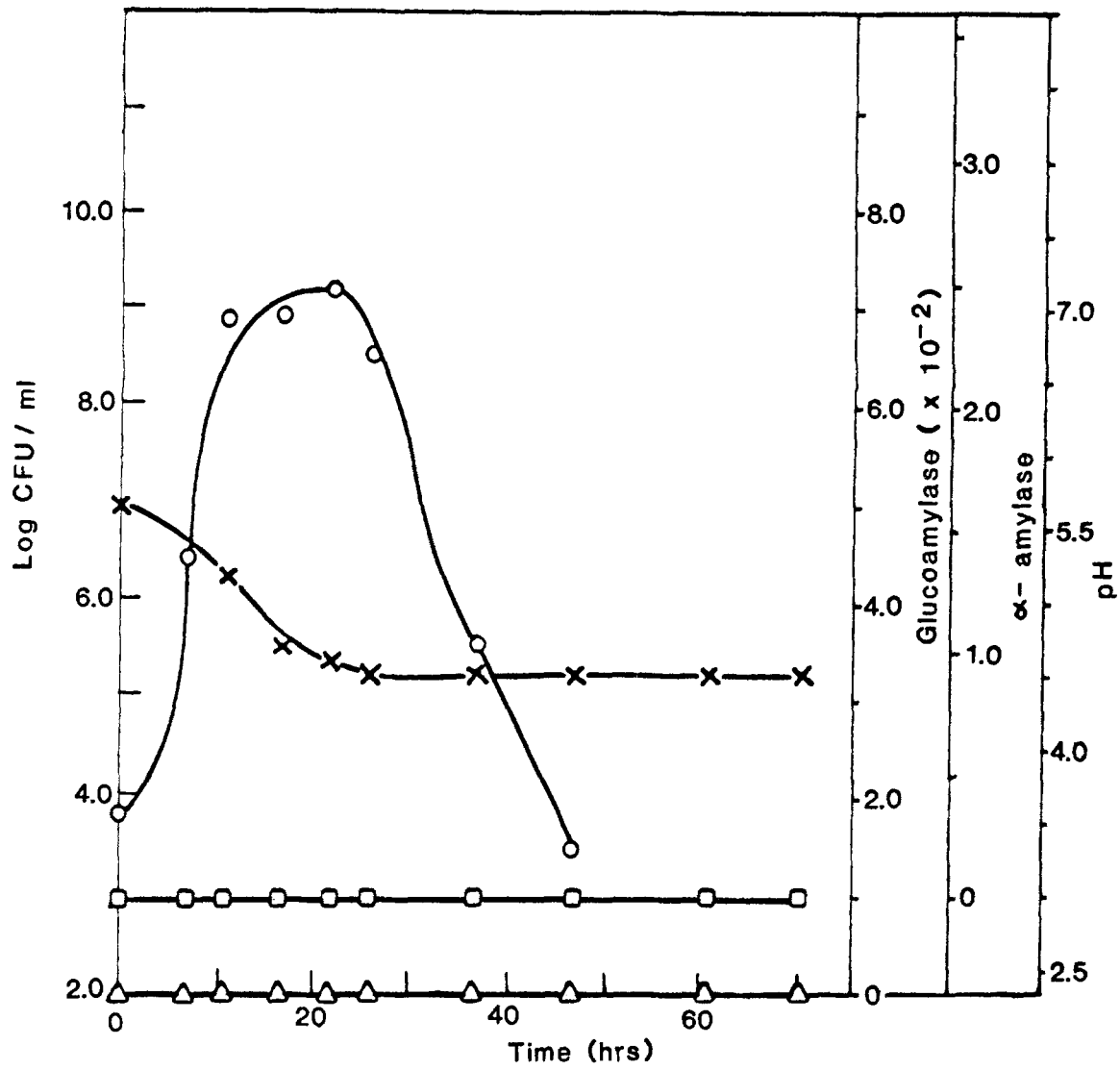

Fig. 2. Growth response (○), pH (×), α-amylase activity (□) and glucoamylase activity (△) of *C. acetobutylicum* SA-1 in 4.7% GSM.

When glucose was the sole carbohydrate source, *C. acetobutylicum* SA-1 did not produce any alpha-amylase or glucoamylase (Fig. 2). This suggests that biosynthesis of both of these hydrolytic enzymes is affected by catabolite repression and that the formation of alpha-amylase and glucoamylase is under a common biosynthetic control mechanism. These results differ from that reported by Ensley et al. [5] who found that induction of glucoamylase occurred when growth of *C. acetobutylicum* NRRL-B592 was on glucose.

The results suggest that starch as the carbohydrate source does not adversely affect the growth response of *C. acetobutylicum* SA-1 when compared to growth on glucose. The similar growth response of *C. acetobutylicum* SA-1 in GSM and CSSM suggests that this microorganism can utilize these substrates equally well. However, since the extracellular starch hydrolytic enzymes are produced only as the culture enters stationary phase, plus the fact that starch cannot penetrate the cell wall, suggests the early involvement of cell-associated hydrolytic

enzymes in the processing of the starch polymer to utilizable subunits. During cellular localization studies with a 3.5-h-old culture of *C. acetobutylicum* NRRL-B592, Ensley et al. [5] reported that 41% of total alpha-amylase and 33% of total glucoamylase were associated with the carbohydrate moieties at the surface of the cell.

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