EFFECT OF CARBON SOURCES ON FORMATION OF α -AMYLASE BY *BACILLUS STEAROTHERMOPHILUS*¹

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ABSTRACT

WELKER, N. E. (Western Reserve University, Cleveland, Ohio) and L. LEON CAMPBELL. Effect of carbon sources on formation of α -amylase by *Bacillus stearothermophilus*. J. Bacteriol. **86**:681-686. 1963.—A chemically defined medium was devised for use in α -amylase induction studies. The addition of 0.1% casein hydrolysate to the chemically defined medium permitted growth on fructose, and with glucose, sucrose, maltose, starch, and glycerol it shortened the lag period and increased both the growth rate and the total enzyme produced. Growth did not occur when gluconate, acetate, or succinate were used as carbon sources. α -Amylase was produced during the logarithmic phase of growth; the amount produced was inversely proportional to the rate of growth. The poorer the carbon source for growth (glycerol, $k = 0.24$; glucose, $k = 0.26$; sucrose, $k = 0.42$), the higher was the amount of enzyme produced (glycerol, 109 units/ml; glucose, 103 units/ml; sucrose, 45 units/ml). Cells grown on technical-grade maltose ($k = 0.26$) or starch ($k = 0.42$) did not conform to this relationship in that unusually large amounts of α -amylase were produced (362 and 225 units/ml, respectively). Cells grown on fructose or sucrose had the same growth rate ($k = 0.42$), but smaller amounts of α -amylase were produced on fructose (fructose, 0 to 4 units/ml; sucrose, 45 units/ml). An intracellular α -amylase was not detected in *Bacillus stearothermophilus*.

Bacillus stearothermophilus produces a low molecular weight, thermostable, extracellular

α -amylase (Manning and Campbell, 1961). Although considerable information is available concerning the chemical and physical properties of this enzyme (Manning and Campbell, 1961; Manning, Campbell, and Foster, 1961; Campbell and Manning, 1961; Campbell and Cleveland, 1961), nothing is known about its biosynthesis. The present study is concerned with the effect of various carbon sources on α -amylase formation by *B. stearothermophilus*.

MATERIALS AND METHODS

Organism. The organism used in this study was an obligately thermophilic strain of *B. stearothermophilus* (1503-4) used previously (Manning and Campbell, 1961). Stock cultures were maintained on Difco nutrient agar slants.

Two types of colonies, "smooth" and "rough," were observed when this organism was grown at 55 C on 2% Trypticase Agar plates. When a smooth colony was picked and grown at 62 C on Trypticase Agar, all the colonies appeared rough. A rough colony, when picked and grown at 55 or 62 C, retained the rough appearance. No taxonomic or biochemical differences were observed between the rough and the smooth types, by the species characteristics of Smith, Gordon, and Clark (1952). Cells giving rise to rough colonies at 55 C were used in all subsequent experiments.

Minimal medium. Welker (1963) devised a chemically defined minimal medium which supports good growth of *B. stearothermophilus* 1503-4 at 55 C. The composition of the medium is given in Table 1. This strain has an absolute requirement for valine and nicotinic acid, and growth is stimulated by arginine, methionine, thiamine, and biotin.

Preparation of cell inoculum. The cell inoculum for growth studies was prepared as follows. Two nutrient starch-agar plates were spread with cells from a Trypticase Agar plate and incubated for 8 hr at 55 C. The cells from these plates were

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TABLE 1. *Chemically defined medium for Bacillus stearothermophilus 1503-4*

Compound	Amt
	mg/liter
L-Arginine (free base)	105.0
DL-Methionine	60.0
DL-Valine	144.0
Thiamine-HCl	0.15
Nicotinic acid	1.50
d-Biotin	0.009
Potassium acetate	500.0
KH ₂ PO ₄	1000.0
K ₂ HPO ₄	2500.0
NH ₄ Cl	1000.0
NaCl	1000.0
FeCl ₃ ·6H ₂ O	5.0
MgCl ₂ ·6H ₂ O	5.0
CaCl ₂ ·H ₂ O	5.0
Carbohydrate*	5000.0

* Starch and glycerol were used at concentrations of 1%.

used to inoculate a 2800-ml Fernbach flask containing 500 ml of the following medium: nutrient broth (Difco), 8 g; fructose, 5 g; K₂HPO₄, 2.5 g; KH₂PO₄, 1.0 g; NH₄Cl, 1.0 g; NaCl, 1.0 g; FeCl₃·6H₂O, 5 mg; MgCl₂·6H₂O, 5 mg; CaCl₂·H₂O, 5 mg per liter of distilled water. The pH of the medium was adjusted to 7.3. The inoculated flask was incubated at 55 C in a New Brunswick Gyrotory Water Bath Shaker at a speed of 133 rev/min, describing a 0.5-in. diameter circle.

After 3 to 4 hr of growth, the cells were removed by centrifugation, washed two times with, and resuspended in 10 ml of a sterile minimal salt buffer solution. The minimal salt buffer contained K₂HPO₄, 2.5 g; KH₂PO₄, 1.0 g; NaCl, 1.0 g; NH₄Cl, 1.0 g; FeCl₃·6H₂O, 5 mg; MgCl₂·6H₂O, 5 mg; CaCl₂·H₂O, 5 mg per liter of distilled water. This buffer is referred to as M buffer.

Growth studies and α-amylase formation. Growth was measured in a Bausch and Lomb Spectronic-20 colorimeter at 525 mμ. Cell density was determined from a standard curve relating dry weight to absorbancy.

Growth studies were carried out in 250-ml nephelometer flasks containing 30 ml of the minimal medium or minimal medium supplemented with 0.1% casein hydrolysate (MCH medium) at pH 7.3. The carbon source employed depended on the experiment. Flasks were in-

oculated to an absorbancy of 0.1 to 0.15 and incubated at 55 C in a rotary water-bath shaker.

The growth rate is expressed by the constant (k), defined by the equation $(dA/dt) = kA$. A is the absorbancy of the culture, and t is the time expressed in hours. The values for k were obtained from the linear portion of semilogarithmic plots of growth.

The effect of various carbohydrates on α-amylase formation was quantitated by measuring the differential rate of synthesis (K) of enzyme (E), where the increase in enzyme is directly proportional to the increase in cell mass. The rate is expressed as follows: $(\Delta E/\Delta A) = K$.

Enzyme assay. α-Amylase activity was measured by the rapid colorimetric determination of reducing groups by the dinitrosalicylic acid procedure of Fischer and Stein (1961). Culture supernatant fluids were diluted twofold with 0.2 M sodium acetate buffer (pH 4.5). A 1% solution of soluble starch (Pfanstiehl Chemical Corp., Waukegan, Ill.) was prepared in 0.1 M sodium acetate buffer (pH 4.6). To 50 ml of the buffered starch solution were added 2.5 ml of 0.5 M CaCl₂ and 10 ml of distilled water. The final concentration of Ca⁺⁺ was 0.02 M. The buffered starch (1.0 ml) was placed in each of two tubes (18 by 150 mm) and equilibrated at 65 C. The diluted enzyme (1.0 ml) was added to one tube, and the reaction was carried out for the desired time. The reaction was stopped by adding 3,5-dinitrosalicylic acid reagent (2.0 ml) to both the experimental and the undigested starch control tube. Diluted enzyme (1.0 ml) was then added to the control tube.

The two tubes, along with a reagent blank, were heated in a boiling-water bath for 5 min, immersed in an ice bath, and brought to a volume of 24 ml with water. The tubes were thoroughly mixed, and the resulting orange-colored solutions were decanted into colorimeter tubes. The absorbancy was read at 540 mμ in a Bausch and Lomb Spectronic-20 colorimeter.

The amount of reducing sugar (in mg) was calculated from a standard curve previously calibrated against maltose. Enzymatic activity was measured as mg of maltose released in 3 min at pH 4.6 and 65 C. One unit of α-amylase activity was defined as that amount of protein which will release 0.01 mg of reducing groups, as maltose, in 3 min, under the specified conditions.

Preparation of cell lysates and spheroplasts.

Cells were washed twice with 0.067 M sodium phosphate buffer (pH 7.4), suspended in 10 ml of buffer containing 0.15% egg-white lysozyme, and incubated at 37 C for 90 min (Downey, 1962). The cell debris, depending on the experiment, was either removed by centrifugation or left in the suspension.

Spheroplast formation was accomplished by adding 0.25 M sucrose to the incubation mixture. The formation of spheroplasts was followed with a phase-contrast microscope until less than 1% of the cells remained as rods.

Preparation of cell-free extracts. Cells grown for 12 to 16 hr in the minimal medium with maltose as a carbon source were removed by centrifugation and washed two times with M buffer. Cell-free extracts were obtained by passing a 50% cell suspension in M buffer through a French pressure cell (20,000 lb/in.²). Cell debris was removed by centrifugation at $27,000 \times g$ for 20 min.

Ascending chromatography of sugars. Technical-grade maltose solutions were diluted with water to a final concentration of 0.05%. Supernatant fluids, from cultures grown on MCH medium with starch as a carbon source, were treated as follows. To each 3 ml of supernatant fluid were added 0.5 to 1.5 g of Amberlite MB-3 (mixture of Amberlite IR-120 and IRA-410). The tubes were shaken for 5 min, and the resin was removed by centrifugation. The deionized solutions were lyophilized in a freeze dryer (Virtis Co., Inc., Gardiner, N.Y.). The samples were taken up in 0.5 ml of distilled water; 0.1-ml portions were applied to sheets (30 by 30 cm) of filter paper (no. 598, Schleicher and Schuell Co., Keene, N.H.) 4 cm from one side and 4 cm apart. The filter paper was stapled into a cylinder and placed in a large glass cylinder, sealed, and placed in the dark at ambient room temperature. The multiple ascending chromatographic procedure of Pazur and French (1952) was used. The developing solvent was *n*-butanol-pyridine-water (6:4:3). After each ascent (four to six ascents), the chromatogram was removed and air-dried.

Reducing sugars were detected by the silver-dip method of Mayer and Lerner (1959), modified as follows. Solution A was prepared by diluting 1.0 ml of saturated AgNO₃ to 6.0 ml with water and then to 200 ml with acetone. Solution B contained 1 volume of 10% aqueous NaOH and 5 volumes of methanol. Solution C was an aqueous 0.5 M solution of NaS₂O₃. The solutions were

poured into Pyrex baking dishes, and the chromatogram was dipped into solution A and allowed to air dry. The chromatogram was next dipped into solution B until the characteristic "black spots" appeared. After washing with water, it was placed in solution C until the background coloration disappeared. A final washing with water gave a stable chromatogram.

Carbon sources. Glucose (analytical reagent; Mallinkrodt Chemical Works, St. Louis, Mo.), fructose (chemically pure; Mann Research Laboratories, New York, N. Y.), and sucrose (from a local grocer) were sterilized by Millipore filtration. These sugars were found to be chromatographically free of other reducing and nonreducing sugars. Glycerol (chemically pure; Colgate-Palmolive Co., New York, N.Y.), maltose (Pfanstiehl, technical grade; and Eastman Kodak Co., Rochester, N.Y., organic reagent grade), and starch (Pfanstiehl) were autoclaved at 121 C for 20 min.

RESULTS

Production of α -amylase during growth of *B. stearothermophilus*. Figure 1 shows that α -amylase was produced during the logarithmic phase of growth.

The separate additions of casein hydrolysate

FIG. 1. Production of α -amylase by *Bacillus stearothermophilus* growing on MCH medium plus glucose (0.5%). Growth, $\circ$; α -amylase, Δ .

TABLE 2. *Effect of carbon sources on growth rate (k) and total yield of α -amylase produced by *Bacillus stearothermophilus***

Carbon source†	k		α -Amylase (units/ml)	
	Minimal medium	MCH medium	Minimal medium	MCH medium
Glycerol.....	0.02	0.24	28	109
Glucose.....	0.11	0.26	26	103
Sucrose.....	0.21	0.42	23	45
Maltose (Pfanstiehl).....	0.15	0.26	280	362
Maltose (Eastman Kodak).....	0.15	0.26	139	174
Fructose.....	0	0.42	—	4
Starch (Pfanstiehl).....	0.17	0.42	106	225
K-gluconate.....	0	0	—	—
Na-succinate.....	0	0	—	—
Na-acetate.....	0	0	—	—

* Experimental details are described in Materials and Methods.

† All carbon sources were used at a concentration of 0.5%, except starch and glycerol which were used at a concentration of 1.0%.

TABLE 3. *Effect of carbon source on growth rate (k) and differential rate (K) of α -amylase synthesis**

Carbon source	k	K
Glycerol.....	0.24	240-350†
Glucose.....	0.26	210-290
Fructose.....	0.42	0-25
Sucrose.....	0.42	40-87

* Cells grown in MCH medium.

† Range of values obtained from experiment to experiment.

or vitamins to cultures, which had just entered the logarithmic phase of growth, failed to stimulate further increases in α -amylase formation. In separate experiments, varying the glucose concentration from 0.125 to 0.5% had no effect on the rate of growth, kinetics of enzyme formation, or the total amount of enzyme formed. Growth was proportional to glucose concentration over a range of 0.005 to 0.1%.

Effect of carbon sources on growth and α -amylase formation. The effect of different carbon sources on growth and α -amylase production was tested in the minimal medium and in the minimal medium supplemented with 0.1% casein hydrolysate, MCH medium (Table 2). The addition of

0.1% casein hydrolysate to the minimal medium permitted growth on fructose, and with the other sugars it shortened the lag period and increased both the growth rate and the total enzyme produced. The organism did not grow on MCH medium without a carbon source. Gluconate, acetate, or succinate did not serve as carbon sources.

Table 2 shows that with some carbon sources an inverse relationship exists between the rate of growth and the total amount of α -amylase produced. Maltose (technical or reagent grade), starch, and fructose did not conform to this relationship in that maltose and starch stimulated (induced), while fructose inhibited α -amylase formation. Ascending paper chromatography (four to six ascents) of technical-grade maltose and reagent-grade maltose revealed the presence of four contaminating oligosaccharides and glucose in the former, and one contaminating oligosaccharide and glucose in the latter. This suggests that either maltose or the contaminating oligosaccharides present in technical- and reagent-grade maltose were responsible for the stimulation of α -amylase formation.

When starch was used as a carbon source, a lag period of 45 to 60 min was observed, even when 0.1% casein hydrolysate was added. Growth, once initiated, proceeded at the same rate as that observed on sucrose ($k = 0.42$). Ascending chromatography of supernatant fluids from the late lag phase and the early logarithmic phase of growth revealed the presence of the same oligosaccharides found in technical-grade maltose. Maltose was not found in large amounts in these samples.

Differential rate of α -amylase formation. Table 3 shows that the differential rate (K) of α -amylase formation was also inversely proportional to the rate of growth, except in the case of fructose. The K value for cells growing on sucrose was always less than 60% of that obtained with cells growing on glucose or glycerol. Variation in the K values, within a given range, occurred from experiment to experiment. Similar variations in K values have been reported for a β -galactosidase-constitutive mutant of *Escherichia coli* (Mandelstam, 1962).

*Attempts to demonstrate intracellular α -amylase in *B. stearothermophilus*.* Pollock and Richmond (1962) suggested that, although the α -amylase of *B. stearothermophilus* might be an extracellular enzyme, it might also appear in culture filtrates

as a result of cell autolysis. In an attempt to find intracellular α -amylase in *B. stearothermophilus*, we examined cell-free extracts (prepared in a French pressure cell) for the presence of α -amylase. No activity was detected.

Since it could be argued that exposure of cells to pressure might inactivate any intracellular α -amylase present, we also used a more gentle method of breaking cells. Cells from mid-log phase cultures growing on glycerol (1%) or pure maltose (0.1%), which were producing extracellular α -amylase at differential rates of 250 and 370, respectively, were removed by centrifugation and washed two times with 0.067 M phosphate buffer. Lysozyme lysates were prepared as described in Materials and Methods. Samples of the uncentrifuged lysates were examined for enzyme activity. The cell debris was then removed by centrifugation, and the supernatant fluids were assayed for α -amylase activity. No activity was detected in either case, even after 2 hr of incubation at 65 C. In a control experiment, lysozyme (0.15%) did not inhibit α -amylase activity.

In a parallel experiment, instead of allowing the lysozyme-treated cells to lyse, spheroplasts were prepared as described in Materials and Methods. The spheroplasts were removed by centrifugation at $3000 \times g$ for 30 min. No α -amylase activity was found in the supernatant fluids. The spheroplast preparations were suspended in 25 ml of a 0.03 M potassium phosphate buffer (pH 6.7)-0.15 M NaCl-0.25 M sucrose solution. Pure maltose (10^{-3} M) was added as an inducer, and the mixture was incubated at 55 C on a rotary shaker. Samples (2.0 ml) were removed at hourly intervals for 5 hr and assayed for α -amylase activity. No enzyme activity was detected. In a control experiment, 0.25 M sucrose did not inhibit α -amylase activity.

DISCUSSION

Mandelstam (1962) has shown in *E. coli* that, under appropriate physiological conditions, any compound which bacteria can use as a source of carbon and energy may repress constitutive β -galactosidase. He showed that the amount of constitutive β -galactosidase produced was correlated with the doubling time of the culture; the poorer the carbon source the higher the enzyme activity. It was concluded that the synthesis of inducible β -galactosidase in wild-type *E. coli*

was controlled by two distinct types of repressor: the repressor suggested by Pardee, Jacob, and Monod (1959), and the carbon-metabolite repressor. The latter was still able to function in the constitutive mutant. Our data also show that an inverse relationship exists between the rate of growth and the amount of α -amylase formed by *B. stearothermophilus*. A more complete study of this relationship was not possible, since this organism grows only on a limited number of carbon sources (glycerol, glucose, and sucrose) which do not stimulate α -amylase formation. Since fructose inhibits α -amylase formation, it is possible that it is a carbon-metabolite repressor of this enzyme in *B. stearothermophilus*.

Nomura, Maruo, and Akabori (1956) reported that *B. subtilis* strain N does not produce α -amylase in the logarithmic phase of growth, but produces large amounts of enzyme after the culture enters the stationary phase of growth. In contrast, Fukumoto, Yamamoto, and Tsuru (1957) and Coleman and Elliott (1962) have shown, with different strains of *B. subtilis*, that the formation of extracellular α -amylase increases approximately in parallel with increase in mass and levels off when the stationary growth phase is reached. Our data show that the formation of α -amylase by *B. stearothermophilus* occurs during the logarithmic phase of growth.

Pollock (1962) listed three criteria for establishing the true extracellularity of enzymes. The formation of α -amylase by *B. stearothermophilus* fulfills all three criteria in that (i) the enzyme appears in the culture medium during the logarithmic phase of growth, (ii) cell autolysis does not occur during enzyme formation, and (iii) there is no detectable intracellular α -amylase, as determined by three different techniques. We therefore conclude that the α -amylase of *B. stearothermophilus* is a true extracellular enzyme.

Maltose and starch were shown to stimulate (induce?) α -amylase formation by *B. stearothermophilus*. Markovitz and Klein (1955a, b) reported that maltose and starch are inducers of the α -amylase of *Pseudomonas saccharophila*. Since both compounds contain contaminating oligosaccharides, it is not known whether the stimulation (induction) of α -amylase is due to the maltose or starch, or whether it is due to one or more of the contaminating oligosaccharides. In this connection, it is interesting to note that Mandels and Reese (1959) and Mandels, Parrish, and Reese (1962) found that glucose produced by

acid hydrolysis of corn starch contains sophorose as an impurity which is a powerful inducer of cellulase in *Trichoderma viride*.

The accompanying paper reports the isolation and characterization of the oligosaccharides present in maltose and their function as inducers of the α -amylase of *B. stearothermophilus*.

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