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The Formation of Extracellular, Thermoactive Amylase and Pullulanase in Batch Culture by *Thermoanaerobacter finnii*

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ABSTRACT

Conditions were found in batch culture under which high levels of extremely thermoactive maltogenic amylase and pullulanase from *Thermoanaerobacter finnii* were formed and released into the culture fluid. A total of 3000 U/L of pullulanase and 2200 U/L of amylase was detected after 16 h of anaerobic cultivation at 65°C; the amounts secreted were 55% and 70%, respectively. Starch was completely hydrolyzed in the culture fluid within 12 h of cultivation. The major product, maltose, was hydrolyzed by an intracellular enzyme. The extracellular enzyme production was greatly influenced by variations in the concentration of the medium components such as yeast extract, tryptone, ammonium, phosphate, and carbon source. The extracellular protein pattern was also analyzed during the course of starch fermentation.

Index Entries: *Thermoanaerobacter finnii;* starch fermentation; thermostable amylase and pullulanase; enzyme secretion.

INTRODUCTION

Starch is a branched polysaccharide and it consists of glucose units that are bound by α-1,4- and α-1,6-glycosidic linkages. A variety of aerobic and anaerobic organisms produces amylolytic enzymes that are required for the degradation of these branched polysaccharides (*1*). Of great importance is the pullulanase (debranching enzyme), because it attacks spe-

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cifically the α-1,6-glycosidic linkages and causes the complete hydrolysis of various polysaccharides (*2–4*). Only recently a number of anaerobic thermophiles were investigated for their production of amylases and pullulanases (*5–8*). Although the extreme thermostability of these enzymes was documented, most were found unsuitable for industrial application. This is owing to the inability of most bacteria to efficiently secrete these enzymes into the culture fluid. Tedious methods like detergent treatment and cell breakage, therefore, had to be employed for their isolation. We have recently reported a process in continuous culture that allowed the overproduction and secretion of extracellular amylases and pullulanases from various anaerobic bacteria (*9–11*). These enzymes were found to be extremely thermoactive and displayed a temperature optima between 70 and 90°C. In this communication we report on factors affecting enzyme production during cultivation of *Thermoanaerobacter finnii* in batch culture. Conditions were found under which a high concentration of amylase and pullulanase were formed and released into the culture fluid.

MATERIALS AND METHODS

Organism and Growth Conditions

Thermoanaerobacter finnii DSM 3389 was obtained from the German Culture Collection, Braunschweig, FRG. Anaerobic techniques were employed for the cultivation of bacteria. Experiments were conducted either in 15-mL Hungate tubes containing 10 mL cultures or in 100-mL bottles containing 50 mL cultures. Growth experiments were performed in 1-L fermenters with 0.5 L culture volume. The fermenters were agitated at 300 rpm under strict anaerobic conditions at 65°C. The gas atmosphere used was sterile nitrogen. The pH of the medium was adjusted to 7.0. A compact BCC-Minibior fermenter was purchased from BCC-Göttingen, FRG. The glass electrode used was from Ingold (Zürich, Switzerland). The growth medium that was developed for optimal production of amylase and pullulanase is described in the Results section. In all experiments the concentration of the following components was kept constant (%, w/v): 0.016 $MgCl_2.H_2O$, 0.0012 $CoCl_2.H_2O$, 0.05 cysteine.HCl, and 1 mL each of vitamin solution (*12*) and trace element solution (*13*). The effect of various parameters was studied by the variation of the concentration of the following components: starch (0.2–2.0%), yeast extract (0.025–0.200%), tryptone (0.06–0.50%), phosphate (0.013–0.33%), and ammonium (0.001–0.5%).

Determination of Substrates and Products

Starch was determined by acid hydrolysis (*14*) and the reducing sugars by the dinitrosalicylic acid method (*15*). Ethanol and acetic acid were determined with a gas chromatograph (Perkin-Elmer, Überlingen,

FRG). Gas chromatography was carried out at 150°C using a (2000×2 mm) column packed with Chromosorb 101 using nitrogen as carrier gas. Lactate concentration was measured enzymatically using lactate dehydrogenase assay (*16*). Glucose was determined enzymatically, as described previously (*17*). The products of starch hydrolysis in the culture fluid were analyzed by high pressure liquid chromatography using an oligosaccharide analysis column (Aminex HPX 87 C, Bio-Rad, Richmond, VA). After centrifugation of the cultures the supernatant was mixed for 15 min with an ion exchanger (Serdolit-MB, Serva, Heidelberg, FRG) and finally centrifuged at 4000 g for 10 min. The following sugars were used as references: glucose (DP_1), maltose (DP_2), maltotriose (DP_3), and maltotetraose (DP_4), and maltopentaose (DP_5).

Enzyme Assay

Amylase and pullulanase activities were measured in culture broth containing cells and supernatant (total enzyme activity), and in the supernatant (extracellular enzyme activity), which was prepared after centrifugation of the culture broth at 4000 g for 15 min at 4°C. Enzyme activities were assayed by measuring the amount of reducing sugars liberated during incubation with starch or pullulan (*15*). The activity of 1 U of amylase or pullulanase is defined as that amount of enzyme that is able to liberate 1 μmol of reducing sugar as a glucose standard per min under the specified conditions. Sodium acetate buffer (20 m*M*), pH 5.5 was used for enzyme assay at 80°C. The reaction was started by the addition of either starch (1%, w/v) or pullulan (0.5%, w/v) under aerobic conditions. The K_m-values of amylase and pullulanase for their substrates was calculated to be 0.11 and 0.09 m*M*, respectively.

Polyacrylamide Gel Electrophoresis

Analytical gel electrophoresis was carried out in 1.5 mm thick (5%, w/v) slab gels at pH 7.0 for 3 h at 4°C. The buffer used was tris/diethylbarbituric acid. Up to 20 μg of protein was mixed with some crystals of sucrose and applied to the gel. The silver staining of the protein bands was performed according to Morrissey (*18*). For the detection of protein bands displaying amylolytic activity the gel was first soaked for 30 min in acetate buffer (20 m*M*, pH 5.5) containing 1% starch and then was incubated at 80°C for another 5 min. Finally, the gel was stained by soaking in 0.15% I_2, 1.5% KI solution for 2 min at room temperature. The appearance of colorless spots in the vicinity of dark colored starch containing gel designated the presence of amylolytic enzyme activity.

Enzymes and Chemicals

Starch for the cultivation of bacteria was obtained from Fluka (Buchs, Switzerland) and the chemicals for gel electrophoresis were from Serva

(Heidelberg, FRG). Oligosaccharides and pullulan from *Aureobasidium pullulans* were from Sigma (St. Louis, MO). All other chemicals were obtained from Merck (Darmstadt, FRG).

RESULTS

The Effect of Starch and Complex Components

The influence of starch (0.2–2.0%), yeast extract (0.025–0.20%), and tryptone (0.06–0.50%) on growth, enzyme formation, and release was investigated during cultivation of *Thermoanaerobacter finnii* in batch culture under strick anaerobic conditions. As shown in Fig. 1, the optimal starch concentration required for the production of amylase and pullulanase ranged between 0.8–1.2%. The elevation of starch concentration up to 2.0% supported growth (from O.D. 2.0 to 2.8) but was accompanied by the reduction in the level of enzymes formed. At a starch concentration of 2.0% the level of reducing sugars and starch in the medium after 18 h of growth amounted to 0.7 and 0.5%, respectively. Unlike the results reported for most starch degrading thermophilic bacteria, around 55% of the total amylase and pullulanase were released into the culture fluid in batch culture. In addition, the concentration of thermostable enzymes produced was very high; it amounted to 3000 U/L of pullulanase and 2400 U/L of amylase. The maximal level of ethanol, lactate, and acetate formed was 80, 20, and 4 m*M*, respectively.

The supplementation of the growth medium with complex components like yeast extract and tryptone was necessary for growth and enzyme production. These results are depicted in Fig. 2. The increase in the concentration of yeast extract (up to 0.15%) and tryptone (0.37%) was paralleled by growth and by an increase in the total amount of amylase and pullulanase formed. The complete utilization of 1% starch was achieved after 18 h of growth in the presence of 0.2% yeast extract and 0.5% tryptone. From the results shown in Fig. 2 it is apparent that under various conditions the concentration of reducing sugars in the medium after cultivation of bacteria was almost constant; it amounted to 0.4%. Analysis of these samples with an Aminex HPX 87 C column showed that the smallest sugar formed in the culture fluid during starch hydrolysis was maltose (DP_2). The absence of glucose in the medium indicated the formation of maltogenic enzyme by *T. finnii* and lack of extracellular maltase. For the detection of glucose, HPLC analysis and enzymatic methods were employed (*17*). Interestingly, maltose concentration was highest if the medium was supplemented with high levels of complex components (Table 1). At low concentrations of yeast extract and tryptone, the sugars DP_2, DP_3, DP_4, and DP_5 were found in equal ratios.

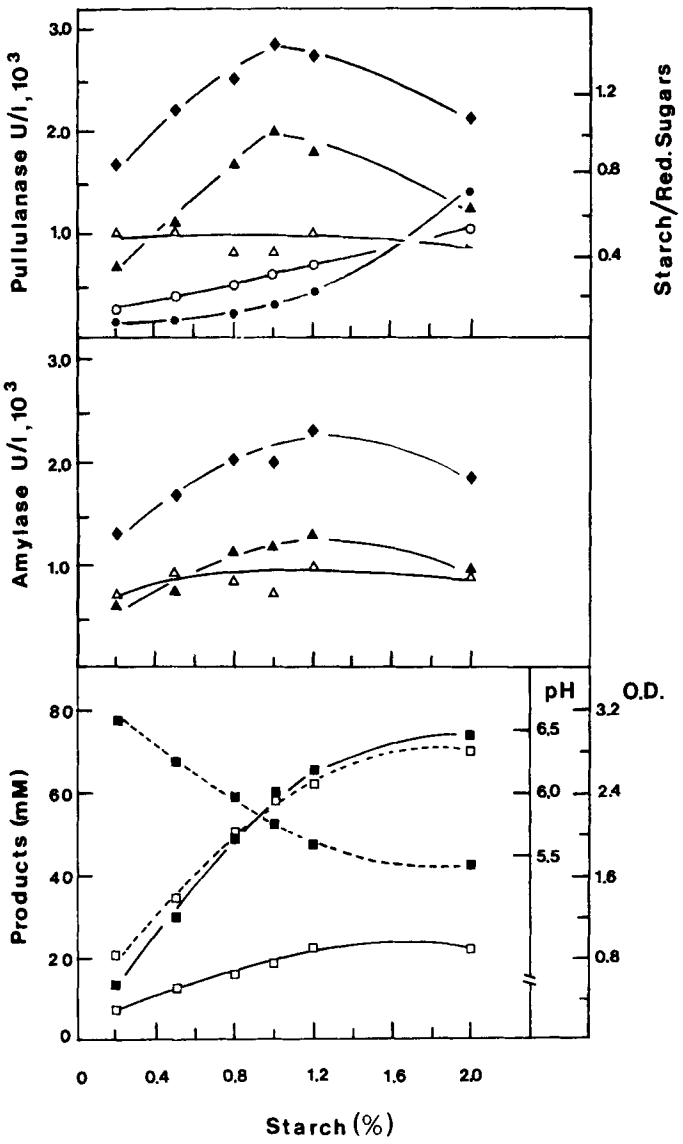

Fig. 1. The influence of starch concentration (0.2–2.0%, w/v) on growth, enzyme production, and product formation after cultivation of *T. finnii* for 18 h. Experiments were conducted in 100-mL bottles containing 50 mL of medium under strict anaerobic conditions at 65°C. The concentration of yeast extract and tryptone was 0.2 and 0.5%, respectively. Total enzyme activity (◆), cell-bound, enzyme activity (△), extracellular enzyme activity (▲), residual starch concentration and reducing sugars (●, ○) optical density at 580 nm (□———□), broth pH (■———■), ethanol (■——■), lactate (□——□).

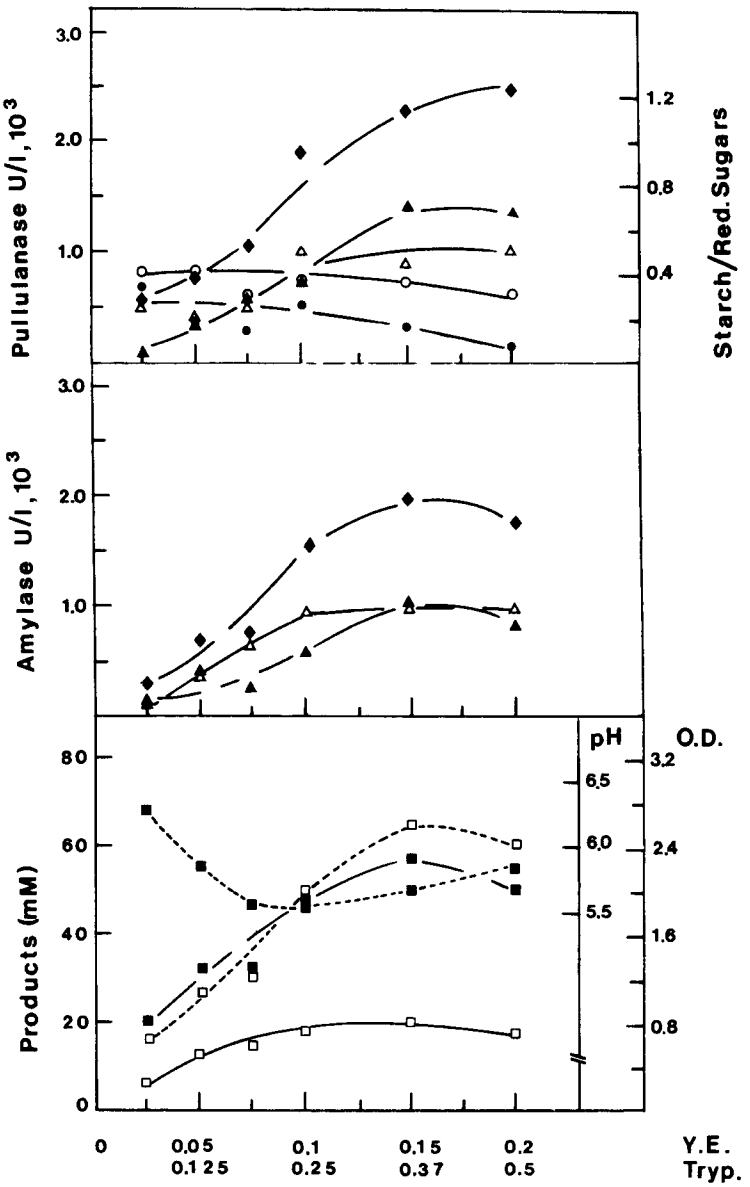

Fig. 2. The effect of various concentrations of yeast extract and tryptone on growth, enzyme formation, and fermentation products in the presence of 1% starch. All parameters were measured after growth of *T. finnii* for 18 h at 65°C. Symbols are described in the legend to Fig. 1.

Phosphate and Ammonium Ion Concentration

In order to study the effect of other parameters like phosphate and ammonium concentration, further growth experiments were conducted in the presence of 1% starch, 0.075% yeast extract, and 0.185% tryptone. Phosphate concentration in the growth medium was varied from 1 to 20

Table 1
The Detection of Maltose (DP_2) and other Glucose Polymers in the Growth Medium of *Thermoanaerobacter finnii* after 18 h of Anaerobic Cultivation in the Presence of Different Concentrations of Yeast Extract (YE) and Tryptone (Tryp)[a]

	DP_2	DP_3	DP_4	DP_5	DP_n
0.2% YE 0.5% Tryp.	50	4	9	4	32
0.15% YE 0.37% Tryp.	30	10	16	10	34
0.1% YE 0.25% Tryp.	22	14	16	10	38
0.075% YE 0.187% Tryp.	16	14	18	10	42
0.05% YE 0.125% Tryp.	12	12	8	8	60
0.025% YE 0.060% Tryp.	8	10	8	8	66

[a]The starch concentration for cultivation of bacteria was 1% (w/v). For the detection of sugars in the medium samples from the supernatant were treated with an ion exchanger and then applied to an Aminex HPX 87 C column. All values are given in percent. $DP_n = > DP_5$.

m*M*. Under all these conditions, around 80% of starch was consumed after 18 h of growth. The decrease of phosphate concentration from 20 to 1 m*M* did not influence the rate of growth of bacteria. The final O.D. after 18 h of growth was measured to be between 1.4–1.6. The decrease of phosphate concentration to 1 m*M* was accompanied by the secretion of amylase and pullulanase into the culture fluid; but also by a decrease (to 50%) of the total amount of enzyme formed. The concentration of extracellular amylase and pullulanase could be raised from 50 to 75%. A similar effect that favors enzyme release was observed during cultivation of *Clostridium thermosulfurogenes* under starch and phosphate limitation in continuous culture (*9*).

The addition of 0.03% of ammonium sulfate into the growth medium containing 1% starch, 0.075% yeast extract, and 0.185% tryptone was necessary for growth and optimal production of enzymes. The increase of ammonium concentration above 0.03% was accompanied by a significant decrease in the amount of enzyme formed. Only 60% each of amylase and pullulanase activity were detected if ammonium concentration was raised to 0.25%. The detection of lower concentration of starch hydrolyzing enzymes in the culture fluid was not owing to the inhibition of their activity by ammonium sulfate. In vitro experiments have shown that an increase of ammonium concentration up to 0.5% did not influence the activity of these enzymes. Comparable results were also obtained if ammonium sulfate was replaced by ammonium chloride.

Table 2
The Influence of Carbon Source on Growth, Enzyme Formation, and Secretion During Growth of *T. finnii* at 65°C[a]

Substrate, 0.5%	Pullulanase, U/L			Amylase, U/L			O.D.
	Total	Extracellular, %		Total	Extracellular, %		
Starch	2180	1365	(62%)	1815	865	(47%)	1.6
Maltodextrin	2690	1460	(54%)	2105	1140	(54%)	2.1
Amylopectin	2490	1190	(47%)	2020	720	(35%)	1.8
Glycogen	2395	1380	(57%)	1980	1015	(51%)	2.0
Dextran	0	0	0	0	0	0	0
Pullulan	2185	1305	(59%)	1670	920	(55%)	1.8
Amylose	168	0	0	555	150	(27%)	0.7
Maltose	600	240	(40%)	545	215	(39%)	0.7
Glucose	0	0	0	0	0	0	1.9
Lactose	0	0	0	0	0	0	1.0
Galactose	0	0	0	0	0	0	1.0
Fructose	0	0	0	0	0	0	1.8

[a] *T. finnii* was grown in the newly developed medium for 16 h under strict anaerobic conditions. The values in parenthesis represent the amount of extracellular enzyme in percent measured in the culture fluid.

The Influence of Various Carbohydrate Substrates

It is apparent from Table 2 that the highest concentration of amylase and pullulanase was detected if the branched polysaccharides (starch, maltodextrin, amylopectin, glycogen, and pullulan) were used as substrates. In the presence of sugars that are exclusively bound by α-1,4-linkages, like amylose and maltose, the level of total and the extracellular enzymes was greatly reduced. The enzyme production was apparently induced by glucans containing α-1,4- or α-1,6-linkages. No growth, however was observed in the presence of dextran; a polysaccharide that is exclusively bound by α-1,6-glycosidic linkages. The level of starch hydrolyzing enzymes in the presence of other sugars like glucose, lactose, galactose, and fructose was negligible.

Growth Experiments

Based on the above-mentioned experiments, a growth medium could now be developed that supported growth and was optimal for the production of high concentrations of cell-free enzymes. This newly developed medium contained in addition to 1% (w/v) starch the following components (by percentage (w/v)): 0.15 yeast extract, 0.370 tryptone, 0.03 $(NH_4)_2$ SO_4, 0.33 KH_2PO_4, 0.016 $MgCl_2.6H_2O$, 0.0012 $CoCl_2.H_2O$, 0.05 cysteine HCl as well as 1 mL each of vitamin solution (*12*) and trace element solution (*13*) at pH 7.0. The synthesis and secretion of amylase and pullulanase during growth of bacteria on starch in the newly developed

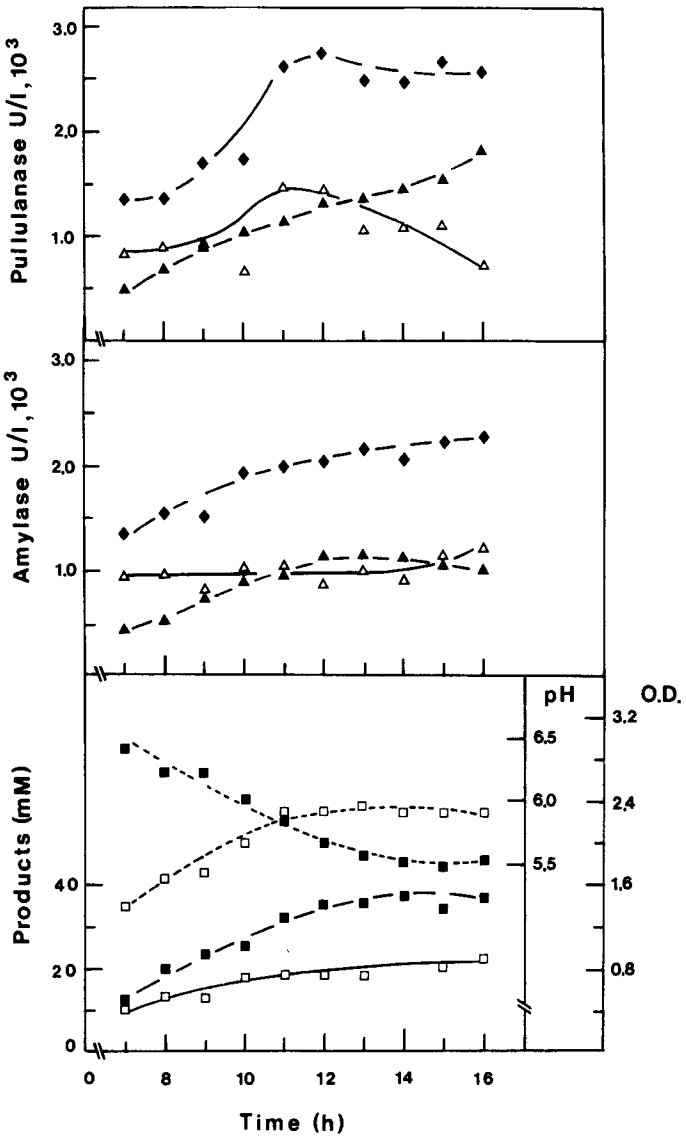

Fig. 3. Starch metabolism time course of *T. finnii*. Experiments were conducted at 65°C in 1-L fermenters containing 500 mL of the newly developed medium described in the Results section. Anaerobic conditions were maintained by continuous gassing of the fermentor with nitrogen. Twenty-five mL samples were taken at the time intervals indicated in the figure. Symbols are defined in the legend to Fig. 1.

medium was then investigated. The synthesis of both enzymes was closely related to the growth of bacteria (Fig. 3). After 12 h of cultivation, starch was completely degraded and around 2800 U/L of pullulanase and 2200 U/L of amylase were formed. Under these conditions, 1000 U/L of pullulanase were detected in the culture fluid. Interestingly, and in contrast to the amylase, further cultivation of bacteria was accompanied by extensive

release of pullulanase from the cell surface. More than 2000 U/L of pullulanase (above 70%) were extracellular after 16 h of growth.

During the course of starch degradation samples were also taken for the detection of various sugars in the culture fluid. In the exponential growth phase the products of starch hydrolysis DP_2, DP_3, DP_4, DP_5, and DP_6 were present in equal ratio. In the stationary phase of growth the sugar spectrum in the medium was significantly changed; DP_2 and DP_4 being the major products. From these results it is apparent that the amylase of *T. finnii* is a maltogenic enzyme and it causes, in combination with the debranching enzyme, the complete degradation of starch. The product maltose has to be transported into the cell in order to be further hydrolyzed by an intracellular enzyme that was detected in this microorganism.

Analysis of the Secreted Proteins

Samples from different stages of growth were taken and analyzed using one-dimensional polyacrylamide gel electrophoresis. As shown in Fig. 4 no alteration in the pattern of total protein and the pattern of proteins exhibiting amylolytic enzyme activity was observed. It can be assumed that the presence of various bands exhibiting amylolytic activity was not owing to proteolytic degradation of these enzymes in the late stationary phase. Each band exhibiting amylolytic activity was cut and the enzyme was eluted and analyzed (paper in preparation). The incubation of each of these proteins with pullulan resulted in the complete hydrolysis of this polysaccharide, with maltotriose as the sole product of hydrolysis. These enzymes, therefore, are classified as pullulanases. In addition to their debranching activity, α-1,4-linkages in polysaccharides like starch and amylose were also hydrolyzed, but at a slower rate. The major products being maltose and maltotetraose. The product pattern of polysaccharide degradation shows clearly that this maltogenic amylase is an endo acting enzyme.

From these data it is possible to assume that an intracellular maltose hydrolyzing enzyme has to be present in *T. finnii*. Cells, therefore, were harvested after 16 h of growth on starch, and cell-free extracts were prepared and maltase activity was measured as previously described (*6*). The level of maltase activity in the supernatant was negligible. High concentration of maltase activity, on the other hand, was detected in the cells. The specific activity of this intracellular enzyme amounted to 0.10 U/mg of protein.

DISCUSSION

Conditions have been found now under which extremely thermoactive enzymes exhibiting amylolytic and pullulytic activities were released by cultivation of *Thermoanaerobacter finnii* in batch culture. It is, however, unknown whether this strain produces two distinct proteins with amylase

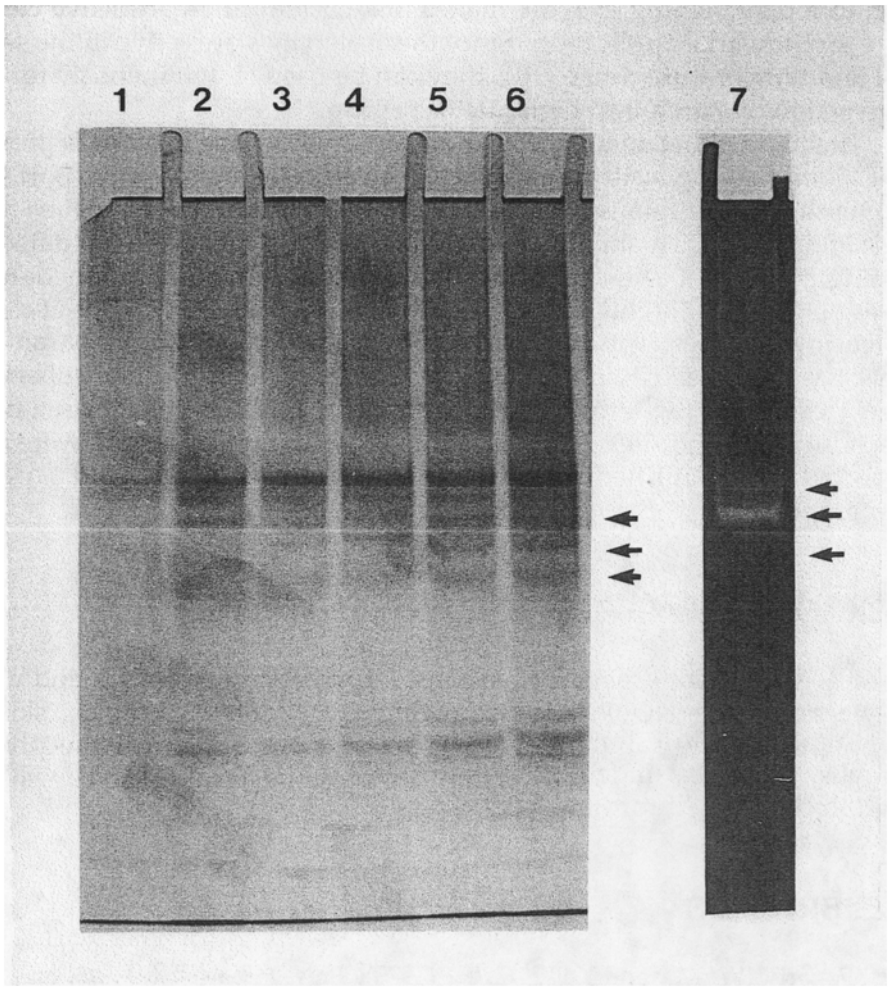

Fig. 4. Electrophoretic analysis of extracellular proteins formed by *T. finnii* in various stages of growth (experiment described in Fig. 3). The supernatants containing 0.1 U of amylase were concentrated using a membrane of 10,000 Da cutoff and applied to polyacrylamide gels (5%). Lanes 1 to 6: proteins detected by silver stain. Lane 7: Bands exhibiting amylolytic activity. Lanes 1,2,3,4,5 and 6 represent the samples that were taken after growth of bacteria for 7, 9, 11, 12, 14, and 16 h.

and pullulanase activities. It is possible that a single protein exhibits both amylolytic as well as pullulytic activities. The productivity with respect to the starch hydrolyzing enzymes (300 $U/1^{-1}.h^{-1}$ for pullulanase and 220 $U/1^{-1}.h^{-1}$ for amylase) is comparable to the results obtained by cultivation of the same strain in continuous culture (*9,10*). These investigations can also be extended now to other polysaccharide fermenting anaerobic bacteria for which the formation of small amounts of cell free enzymes was reported. The formation of an extracellular maltogenic amylase in addi-

tion to a debranching enzyme makes this bacterium an attractive candidate for industrial application. Since these enzymes are active in the same pH and temperature range (*10*), they can be used simultaneously for the conversion of starch into high maltose syrup.

The finding that an intracellular maltose hydrolyzing enzyme is present in *T. finnii* also suggests the existence of an efficient maltose transport system in this microorganism. In contrast to the situation in *Clostridium thermosulfurogenes* EM 1, amylase and pullulanase activities were not detected during cultivation of bacteria with glucose (*6,9*). Enzyme production by T. finnii could apparently be induced by glucans containing α-1,4-or α-1,6-glycosidic linkages. The inability of this organism to grow on dextran emphasizes the specificity of starch hydrolyzing enzymes for their substrate. The presence of α-1,4-linkages in the vicinity of α-1,6-linkages seems to be of utmost importance for enzymatic activity. Similar results were also reported for the purified enzymes from *C. thermosulfurogenes* EM 1 (paper in preparation).

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