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Production of Thermostable α-Amylase, Pullulanase, and α-Glucosidase in Continuous Culture by a New *Clostridium* Isolate

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The production of α-amylase, pullulanase, and α-glucosidase and the formation of fermentation products by the newly isolated thermophilic *Clostridium* sp. strain EM1 were investigated in continuous culture with a defined medium and an incubation temperature of 60°C. Enzyme production and excretion were greatly influenced by the dilution rate and the pH of the medium. The optimal values for the formation of starch-hydrolyzing enzymes were a pH of 5.9 and a dilution rate of 0.075 to 0.10 per h. Increase of the dilution rate from 0.1 to 0.3 per h caused a drastic drop in enzyme production. The ethanol concentration and optical density of the culture, however, remained almost constant. Growth limitation in the chemostat with 1% (wt/vol) starch was found optimal for enzyme production. Under these conditions 2,800 U of pullulanase per liter and 1,450 U of α-amylase per liter were produced; the amounts excreted were 70 and 55%, respectively.

Recently, we isolated a new thermophilic *Clostridium* strain on starch and reported on its growth, amylolytic enzyme production, and excretion (8). This organism, strain EM1, produces high levels of a thermostable pullulanase. Carbohydrates containing maltose units induce the formation of this enzyme along with an α-amylase and α-glucosidase. Maximal amounts of these enzymes are formed in the presence of 0.5% substrate. Under these conditions more than 95% of α-amylase and 65% of pullulanase are cell bound (8). In the present study we report on continuous culture experiments with strain EM1 in a defined growth medium. We established optimal conditions for the production of high levels of α-amylase and pullulanase under which 55 and 70%, respectively, of these enzymes were released into the culture broth.

MATERIALS AND METHODS

Bacteria and growth medium. *Clostridium* sp. strain EM1, isolated from fruit juice waste products, was used (8). The strain was maintained on tryptone yeast extract medium supplemented with 0.5% (wt/vol) soluble starch at pH 6.5 as previously described (8). Batch and continuous culture experiments were conducted with a defined medium which contained the following components (by percentage [wt/vol]): KH_2PO_4, 0.68; $(NH_4)_2SO_4$, 0.1; $MgCl_2 \cdot 6H_2O$, 0.016; $CoCl_2 \cdot 6H_2O$, 0.0012; and cystein hydrochloride, 0.05. Samples of 1 ml each of vitamin solution and trace element solution were added per liter of medium (9, 10). The pH was adjusted to 6.5. The defined medium was supplemented with either starch, dextrin, maltose, or glucose.

Batch culture. Strict anaerobic culture techniques were used for growth experiments which were conducted in 100-ml cultures at 60°C supplemented with 1% (wt/vol) soluble starch. Samples (2 ml) were taken for the measurement of amylolytic enzyme activities, fermentation products, substrate concentration, optical density at 580 nm, and pH of the culture.

Continuous culture. Experiments were performed in 2- or 5-liter Biostat fermentors (Braun, Melsungen, Federal Republic of Germany) with 1- to 2-liter culture volumes at 60°C. The culture was kept anaerobic by a continuous flow of N_2 gas which was sterilized by passage through a sterilized cotton-wool filter. The culture was stirred at a rate of 150 rpm. The pH was measured with a glass electrode (Ingold, Zürich, Switzerland) and maintained at the desired value by automatic addition of 2 N KOH. After attaining steady state (at least six volume changes), three samples (10 ml each) were taken in 24-h intervals and the activities of the amylolytic enzymes and their location were determined (8). In addition the fermentation products, substrate concentration, optical density at 580 nm, and the pH of the culture were measured.

Enzyme assays. α-Amylase, pullulanase, and α-glucosidase were measured in the culture broth containing cells and culture supernatant and in the supernatant prepared by centrifugation of the culture broth. The cytoplasmic portion of the enzyme was determined after cell rupture by French press at 13,400 N/cm^2. Amylase and pullulanase activities were assayed by measuring the reducing sugars which were liberated during reaction on starch and pullulan, respectively (2). The exact conditions for enzyme determination have also been previously described (8). The activity of 1 U of amylase or pullulanase is defined as that amount of enzyme which liberates 1 μmol of reducing sugar as a maltose standard per min under the specified conditions. α-Glucosidase activity was measured as previously described (3, 8). α-Amylase, pullulanase, and α-glucosidase were measured at 60, 70, and 65°C, respectively. Protein was determined by the method of Lowry et al. (7).

Determination of substrates and products. Starch, dextrin, and pullulan concentrations were determined by acid hydrolysis as previously described (6, 8). Reducing sugars were measured by the dinitrosalicyclic acid method (2). The concentrations of maltose was determined enzymatically with commercial maltase; the product glucose was determined enzymatically as described elsewhere (3). Ethanol and acetic acid in the culture supernatant were determined with a gas chromatograph (The Perkin-Elmer Corp., Überlingen, Federal Republic of Germany) equipped with a flame ionization detector (Shimadzu, Kyoto, Japan). Gas chromatography was carried out at 150°C with a glass column (2,000 by 2 mm) packed with Chromosorb 101. The

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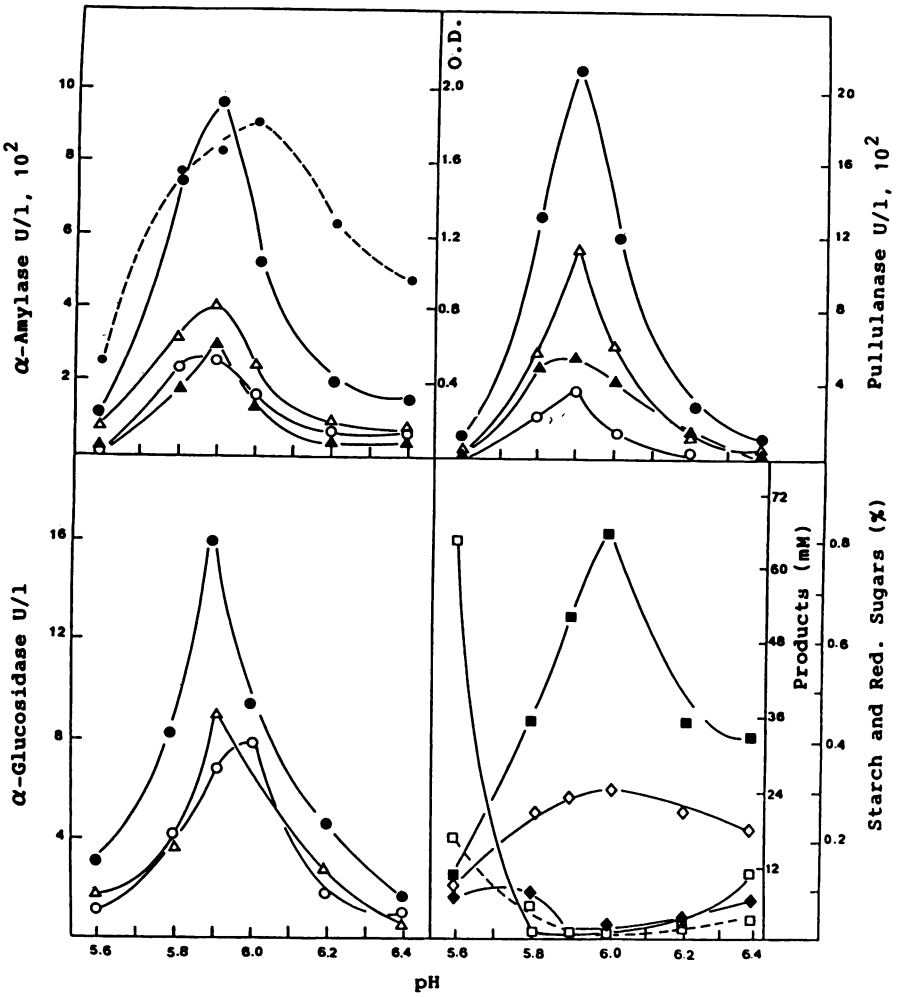

FIG. 1. Influence of pH on enzyme formation and export, biomass, and fermentation products of *Clostridium* sp. strain EM1 grown with 1% starch in continuous culture. $D = 0.075$ per h. The fermentor was continuously gassed with nitrogen, and the temperature was maintained at 60°C. The chemostat was starch limited in the range of pH 5.8 to 6.0 and was otherwise pH limited. Symbols: △, extracellular enzyme activity; ▲, cell-bound enzyme activity; ○, intracellular enzyme activity; ●, total enzyme activity; · ---- · , optical density (OD) at 600 nm; □——□, residual starch concentration; □----□, residual reducing sugars; ◇, acetate; ■, ethanol; ◆, lactate.

carrier gas was N_2. Lactate was determined enzymatically with lactate dehydrogenase (1a).

Enzymes and chemicals. Pullulan from *Aureobasidium pullulans*, dextrin, cyclodextrin, maltose, glucose, and chemicals were purchased from Sigma Chemical Co., St. Louis, Mo. Soluble starch was obtained from Fluka, Neu-Ulm, Federal Republic of Germany. Biochemicals and enzymes were from Boehringer GmbH, Mannheim, Federal Republic of Germany.

RESULTS

Batch culture. The defined medium described in Materials and Methods was developed for growth of the isolated *Clostridium* strain. Growth experiments were then conducted on this medium, which was supplemented with 1% (wt/vol) soluble starch at 60°C under anaerobic conditions. The results obtained from batch culture experiments (not shown) indicated that α-amylase and pullulanase were formed during the exponential growth phase. The total α-amylase and pullulanase levels reached a maximum of 320 and 250 U/liter after 10 h of growth, respectively. These values were comparable to the results obtained on tryptone-yeast extract medium (8). The pH of the culture decreased to 4. After reaching the beginning of the stationary phase of growth (10 h), both enzymes began to be extensively released into the culture medium. The total amount of the amylolytic enzymes remained constant. The observed excretion of extracellular enzymes was not accompanied by cell lysis, as confirmed by the microscopic examination of the culture. Approximately 90% of the amylolytic enzymes were present in the culture broth after 22 h of cultivation. Similar to the growth experiments on complex medium (8), growth in the presence of lower starch concentrations (0.5% soluble starch) was accompanied by an increase in the level of pullulanase but a decrease in the percentage of enzymes released.

Effect of pH on fermentation products and amylolytic enzymes formed in continuous culture. Continuous culture experiments were conducted in defined medium under starch or pH limitation, and the effect of pH (5.6 to 6.4) on biomass, enzyme production and excretion, and fermentation prod-

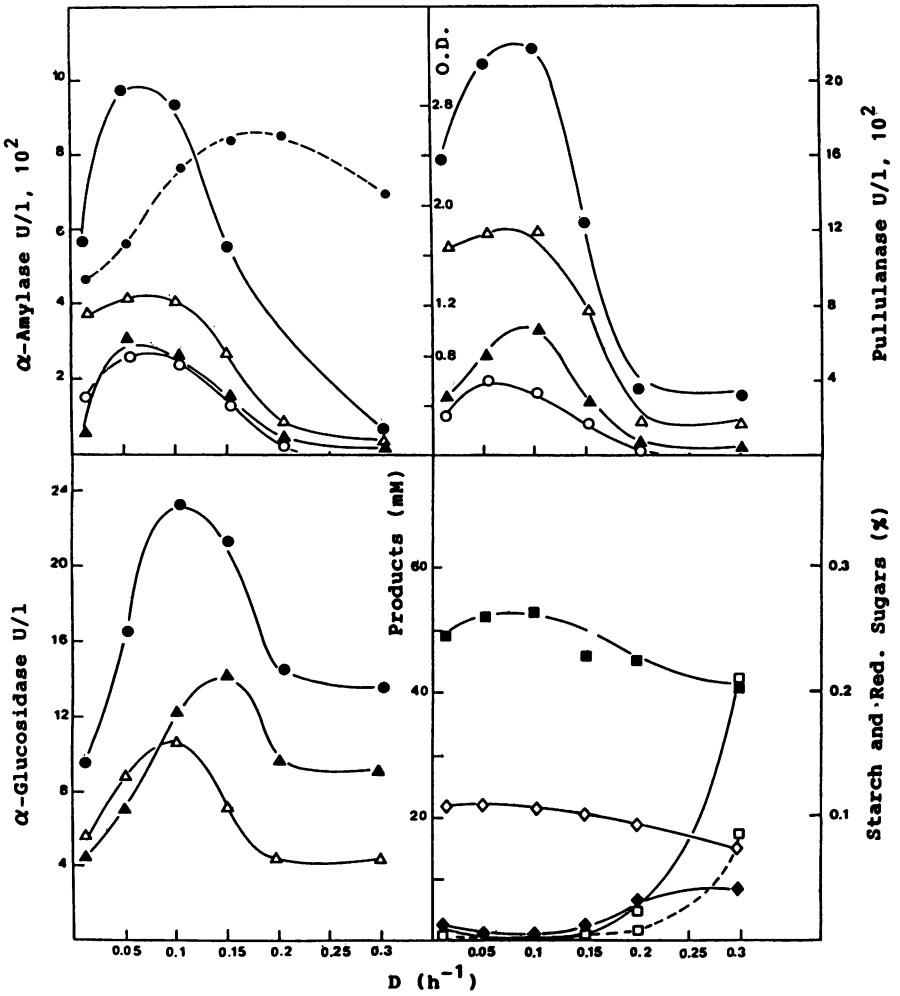

FIG. 2. Effect of dilution rate (D = 0.01 to 0.30 per h) on enzyme formation and release, biomass, and fermentation products. Experiments were conducted at pH 5.9 and 60°C. Symbols are defined in the legend to Fig. 1.

ucts was determined. It is apparent from Fig. 1 that the pH greatly influenced starch metabolism parameters. The optimal pH for enzyme production and excretion was 5.9. Enzyme production was drastically decreased at pH values of 6.2 and higher. The concentration of all three enzymes, α-amylase, pullulanase, and α-glucosidase, significantly decreased from 1,000, 2,200, and 16 U/liter at pH 5.9 to 200, 300, and 4 U/liter at pH 6.2, respectively. At pH values lower than 5.8 the biomass and the enzyme concentrations were also greatly reduced. Starch, as well as reducing sugars, were detectable below pH 5.8 and above pH 6.0. Therefore, under these conditions the culture was limited by pH but not by starch. Under optimal conditions 60% of pullulanase and α-glucosidase and 45 to 55% of α-amylase were continuously released into the culture broth. At pH values higher than 5.9 the ratio of ethanol to acetate concentration decreased from 3.5:1 to 1.5:1.

Effect of dilution rate on starch metabolism parameters. The effect of dilution rate (D = 0.01 to 0.3 per h) on enzyme formation and release, biomass, and fermentation products was determined at pH 5.9. Over the range for D of 0.01 to 0.1 per h, the total concentration of α-amylase, pullulanase, and α-glucosidase increased from 550, 1,500, and 9 U/liter to 1,000, 2,200, and 24 U/liter, respectively (Fig. 2). At dilution rates greater than 0.1 per h, the concentrations of the three enzymes started to decrease and were drastically lowered at a dilution rate of 0.3 per h. The levels of the enzymes measured were 50 U/liter for α-amylase, 350 U/liter for pullulanase, and 15 U/liter for α-glucosidase. The extra- and intracellular enzyme concentrations followed a similar trend. The optical density of the culture, however, decreased only slightly at dilution rates higher than 0.1 per h. Some starch and reducing sugar were detectable under these conditions. Both ethanol and acetate concentrations (52 and 21 mM, respectively) remained constant over the dilution range tested.

Substrate concentration and productivity. The influence of the starch concentration on the formation of enzymes and fermentation products is depicted in Fig. 3. The optimal starch concentration in the inflowing medium for enzyme production was 1% (wt/vol) at a dilution rate of 0.075 per h and a pH of 5.9. Under these conditions 1,450 U of α-amylase per liter and 2,860 U of pullulanase per liter were formed. The concentration of enzyme released into the culture broth was 850 U of α-amylase per liter and 2,050 U of pullulanase per liter; the extracellular enzymes constituted 55% of the total α-amylase and 70% of the total pullulanase formed. It can also be seen from the results in Fig. 3 that, at concentrations below and above 1% starch, the productivity

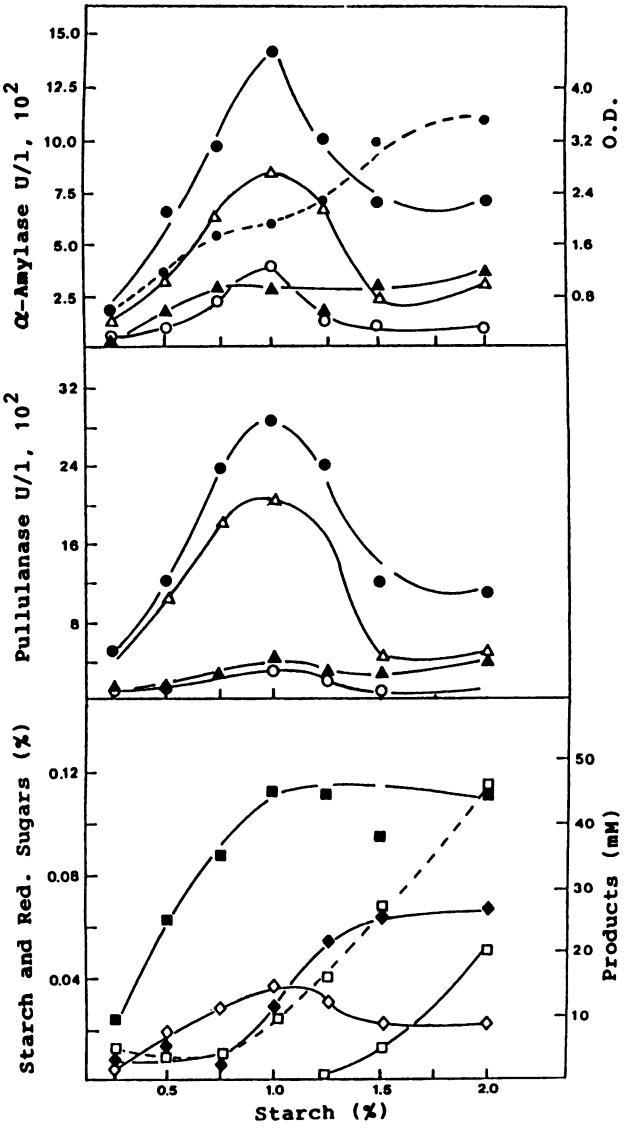

FIG. 3. Dependence of enzyme production, enzyme excretion, and fermentation products on the concentration of starch in the inflowing medium. The chemostat was run at a dilution rate of 0.075 per h and a pH of 5.9 at 60°C under anaerobic conditions. Symbols are defined in the legend to Fig. 1.

for amylolytic enzymes was drastically reduced. At higher substrate concentrations in the inflowing medium, the increase of the optical density of the culture from 2 to 3.5 was not paralleled by an increase in productivity with respect to the amylolytic enzymes. Starch could not be completely utilized at concentrations above 1.25%. Approximately 0.05% starch and 0.12% reducing sugars were detected at starch concentrations of 2%. The productivity of *Clostridium* sp. strain EM1 for α-amylase and pullulanase at pH 5.9 and different dilution rates was calculated. Maximal productivity (100 U/liter per h for α-amylase and 230 U/liter per h for pullulanase) was achieved at dilution rates of 0.075 to 0.2 per h. The ethanol concentration was raised up to 50 mM at a starch concentration of 1% and above. The ratio of ethanol to acetate increased from 3:1 (0.25% starch) to 6:1 (1.5 to 2% starch).

Influence of various carbohydrates on enzyme and product formation. The effect of starch, dextrin, maltose, and glucose on starch metabolism parameters in chemostat at 60°C (at a dilution rate of 0.075 per h) was investigated. From the results summarized in Table 1, it is apparent that the highest concentrations of α-amylase and pullulanase were detected in the presence of 1% starch or 1% dextrin in a defined medium. Thus, approximately 2,800 to 3,100 U of pullulanase per liter and 1,300 to 1,450 U of α-amylase per liter were formed. In the presence of starch as substrate, 60% of the total α-amylase and about 70% of the total pullulanase were released into the culture broth. The specific activities of released α-amylase and pullulanase were 1,700 and 4,110 U/g of protein, respectively. The addition of 0.2% yeast extract into the inflowing medium containing 1% starch did not enhance the production of amylolytic enzymes. The optical density was raised up to 2.7. Interestingly, only 28% of the α-amylase and 50% of the pullulanase were present as extracellular enzymes. Similarly maltose was shown to be a less effective inducer than starch or dextrin. Under glucose limitation, however, a drastic drop of α-amylase and pullulanase levels to 140 and 215 U/liter, respectively, was observed. The pronounced effect of glucose was not abolished by the addition of starch (Table 1). The concentration of the fermentation products was not significantly influenced by the carbohydrates tested. Compared with the data from batch culture experiments, the levels of α-amylase and pullulanase in a chemostat operated under substrate limitation was increased 5 and 13-fold, respectively.

Enzyme properties. In contrast to the commercially available pullulanases, the extracellular enzyme from the newly isolated strain was very stable in the absence of metal ions under aerobic conditions. At 60°C and pH 5, two commercial pullulanases were completely inactive after 5 h of incubation. The third enzyme was inactive after 4 days. In contrast to this for the enzyme from *Clostridium* sp. strain EM1, no loss of enzyme activity was observed after 5 days at 60°C. The enzyme was less stable when incubated at pH values of 4.5 and 6.0, respectively; 50% of pullulanase activity was detected after 2 days at 60°C. Under the same conditions the commercial enzymes were completely inactive. At 90°C and pH 5.0, 20% of the pullulanase activity was still measurable after 1 h of incubation. It is worth mentioning that this extracellular enzyme was more stable than the cell-bound pullulanase from the same organism (8). The influence of α- and β-cyclodextrin (1 to 10 mM) and metal ions (5 mM) on α-amylase and pullulanase activity was also investigated (Table 2). α-Cyclodextrin up to a concentration of 5 mM did not influence the activities of the amylolytic extracellular enzymes from the newly isolated strain. Inhibition (3 and 22%, respectively) of pullulanase and amylase activity was observed in the presence of 10 mM α-cyclodextrin. In contrast to this, the effect of β-cyclodextrin on α-amylase and pullulanase was more pronounced. In the presence of 10 mM of this compound, 45% of α-amylase and 7% of pullulanase activity were measured.

The following metal ions did not have any stimulating effect on the activity of both enzymes: Ca^{2+}, Mg^{2+}, Mn^{2+}, Ni^{2+}, and Mo^{2+}. On the other hand, in the presence of Fe^{2+} and Fe^{3+} (5 mM), only 13 and 68% of α-amylase and 48 and 50% of pullulanase activity could be detected. The independence of the amylolytic enzyme activities from metal ions was further documented by the addition of 10 mM EDTA; under these conditions, 70 to 80% of the enzyme activity was measurable.

TABLE 1. Influence of various carbohydrates on enzyme formation and excretion by *Clostridium* sp. strain EM1 grown under substrate limitation

Substrate (%)[a]	Total activity (U/liter)		Extracellular enzyme (U/liter)[b]		Productivity (U/liter per h)		Product concn (mM)		Optical density
	Amylase	Pullulanase	Amylase	Pullulanase	Amylase	Pullulanase	Ethanol	Acetate	
Starch (1)	1,425	2,867	852 (55)	2,058 (70)	109	215	46	15	1.9
Starch (2)	740	1,150	350 (48)	610 (53)	55	8	45	10	3.5
Starch (1) + yeast extract (0.2)	1,007	2,237	284 (28)	1,131 (50)	75	168	62	23	2.7
Dextrin (1)	1,295	3,100	685 (53)	1,692 (55)	97	232	51	16	1.5
Maltose (1)	730	1,924	310 (42)	634 (33)	55	144	57	21	1.5
Glucose (1)	140	215	106 (75)	160 (75)	10	16	43	13	1.6
Starch (1) + glucose (1)[c]	416	592	242 (58)	400 (68)	31	44	48	7	2.9
Starch (1) + maltose (1)[c]	820	1,411	455 (55)	715 (50)	61	106	51	11	2.5

[a] The inflowing defined medium contained 1 or 2% (wt/vol) supplements of various carbohydrates.

[b] The values in parentheses represent the amount of enzyme released into the culture broth as a percentage of total enzyme formed (total enzyme = extracellular + cell-bound + intracellular enzyme activities). Under most of these conditions, the chemostats were run under substrate limitation; at 2% starch and 1% dextrin, 0.05% starch and 0.15% dextrin could be detected in the chemostats.

[c] In addition to 1% starch, either maltose or glucose was added. The chemostat was run at a dilution rate of 0.075 per h at pH 5.9 and 60°C; the optical density was measured at 580 nm.

DISCUSSION

The results reported here document the superiority of continuous culture as compared with batch culture with respect to the production of α-amylase and pullulanase by the newly isolated thermophilic *Clostridium* sp. strain EM1. A decrease of the total activity of the enzyme with starch concentrations higher than 0.5% had already been observed in batch culture experiments. Therefore, it could be expected that continuous culture under substrate limitation would lead to much higher activities as compared with those of batch culture. This was indeed the case: the activity per volume increased up to 12-fold. A similar effect was also observed for the hydrogenase from *Alcaligenes eutrophus* (4). Extracellular enzymes produced by anaerobes often remain associated with the cells. This is true for the thermostable pullulanase from clostridia described recently (5, 6). The same has been observed with *Clostridium* sp. strain EM1 (8). However, conditions have been found now under which the enzyme is released into the culture broth. This is achieved by an increase of the substrate concentration in batch culture which, however, is accompanied by a considerable diminution of total enzyme activity. The more favorable alternative is to grow the organism in continuous culture in a defined medium containing growth-limiting amounts of starch. This leads to overproduction of the amylolytic enzymes, to a partial disintegration of the surface layer of the cells, and to a proliferation of the cytoplasmic membrane which is associated with the formation of blebs and extracellular vesicles (1). Consequently, conditions have been found under which an anaerobic thermophile becomes an enzyme producer of industrial interest.

Several questions arise in this connection. Are the ultrastructural changes leading to enzyme excretion by strain EM1 steered by a specific gene product? Can other anaerobic thermophiles also be forced to excrete certain enzymes? Further research in our laboratory will be aimed at answering these questions. It will also focus on the isolation of pullulanases more stable than those currently used in industry.

An invention report covering the thermostable amylolytic enzymes from the newly isolated strain and the process for the excretion of thermostable amylolytic enzymes from anaerobic thermophiles has been filed at the German Patent Office (No. 3639267.7, Nov. 1986).

TABLE 2. Influence of metal ions and cyclodextrins on activities of amylase and pullulanase from *Clostridium* sp. strain EM1[a]

Condition	Amylase (%)	Pullulanase (%)
No addition	100	100
Ca^{2+}	100	110
Mg^{2+}	94	106
Mn^{2+}	102	98
Ni^{2+}	100	97
Mo^{2+}	94	112
Fe^{2+}	13	48
Fe^{3+}	68	50
EDTA (10 mM)	80	70
α-Cyclodextrin (mM)		
1	95	99
2	110	100
5	113	101
7.5	82	102
10	78	97
β-Cyclodextrin (mM)		
1	80	31
2	88	18
5	64	15
7.5	63	12
10	45	7

[a] Values represent the average of five measurements. Samples containing extracellular enzymes were incubated in 20 mM sodium acetate buffer, pH 5.5. Metal ions (5mM and cyclodextrins in different concentrations were added, and the reaction was conducted at appropriate termperatures.

ACKNOWLEDGMENTS

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