Continuous Production of Thermostable β-Amylase with *Clostridium thermosulfurogenes*: Effect of Culture Conditions and Metabolite Levels on Enzyme Synthesis and Activity

A. NIPKOW,[1] G.-J. SHEN,[1] AND J. G. ZEIKUS[1,2]*

Michigan Biotechnology Institute, 3900 Collins Road, Lansing, Michigan 48909,[1] and Departments of Biochemistry and Microbiology, Michigan State University, East Lansing, Michigan 48824[2]

A β-amylase-overproducing mutant of *Clostridium thermosulfurogenes* was grown in continuous culture on soluble starch to produce thermostable β-amylase. Enzyme productivity was reasonably stable over periods of weeks to months. The pH and temperature optima for β-amylase production were pH 6.0 and 60°C, respectively. Enzyme concentration was maximized by increasing biomass concentration by using high substrate concentrations and by maintaining a low growth rate. β-Amylase concentration reached 90 U ml^{-1} at a dilution rate of 0.07 h^{-1} in a 3% starch medium. A further increase in enzyme activity levels was limited by acetic acid inhibition of growth and low β-amylase productivity at low growth rates.

The industrial production of sweeteners from corn starch requires thermostable enzymes. Four of the five main amylolytic enzymes used in the starch processing industry are sufficiently thermostable for the economic production of glucose, high-fructose corn syrup, and regular corn syrups: α-amylase, glucoamylase, glucose isomerase, and pullulanase (10). β-Amylase, which is used for the production of high-conversion syrups, high-maltose syrups, and extreme-high-maltose syrups, is available as a moderately thermostable enzyme (55 to 60°C) from various microorganisms and plants. However, it is expected that the production economics of high-maltose-containing syrups could be improved if an extremely thermostable β-amylase were available that could be custom-mixed with other extremely thermostable enzymes (pullulanase, α-amylase) for the production of high-maltose-containing syrups in a single conversion step (23).

Our laboratory has isolated a thermophilic bacterium, *Clostridium thermosulfurogenes* (24), that excretes an extremely thermostable β-amylase which has an optimum temperature of 75°C (13). *C. thermosulfurogenes* is an anaerobic species that forms ethanol, acetic and lactic acids, H$_2$, and CO$_2$ as end products of carbohydrate fermentation. Batch production of the enzyme was characterized (15), and an overproducing mutant was selected (14). We decided to investigate the feasibility of continuous production of β-amylase with the overproducing mutant to improve the productivity of the fermentation.

Economic incentives of continuous versus batch fermentation processes have stimulated a number of studies investigating the continuous production of extracellular enzymes over the past 20 years. The performance of the continuous production process is determined by how the process conditions affect repression, induction, limitation, and inhibition of enzyme synthesis. In some cases, process conditions can be adjusted in a favorable way and can cause an increase of enzyme concentration and productivity (1, 5, 7). In other cases, batch production is superior to continuous production in terms of product concentration (6, 8, 16, 22). Besides product concentration and productivity, the long-term sta-

bility of production is decisive for the application of continuous culture. Only a few reports comment on the stability of continuous enzyme production, and some enzyme production systems degenerated quickly (7, 11, 22), while others for protease (16) were stable for 26 days. The use of thermophilic organisms for the continuous production of extracellular enzymes has been studied in a few cases. Cellulase was produced with a *Thermomonospora* sp. (18), α-amylase was produced with *Bacillus stearothermophilus* (4) and *Bacillus caldolyticus* (5), and pullulanase was produced with a *Clostridium* sp. (1). We report here on the continuous culture production kinetics of thermostable β-amylase with *C. thermosulfurogenes* and demonstrate the limits of enzyme productivity and process stability.

MATERIALS AND METHODS

Organism and maintenance. A β-amylase-overproducing mutant, designated H12-1 (14), of *C. thermosulfurogenes* ATCC 33743 was used. It was periodically transferred in 26-ml anaerobic pressure tubes (Bellco Glass, Inc., Vineland, N.J.) that contained 10 ml of anaerobic TYE medium, 1% soluble starch, 0.25 ml of 2.5% cysteine sulfide reducing agent, and a headspace of N$_2$-CO$_2$ (95:5). Cultures were grown under early stationary conditions to stationary phase at 60°C and then were stored at 4°C.

Media. A complex medium (TYE containing 1, 2, or 3% soluble starch) (no. 2630; Sigma Chemical Co., St. Louis, Mo.) was used. The 1% starch–TYE medium contained the following (liter^{-1}): Trypticase (BBL Microbiology Systems, Bethesda, Md.), 10 g; yeast extract, 3 g; NH$_4$Cl, 1 g; KH$_2$PO$_4$, 0.3 g; Na$_2$HPO$_4$ · 7H$_2$O, 2.1 g; MgCl$_2$ · 6H$_2$O, 0.2 g; vitamin solution (28), 10 ml; trace element solution (as described in reference 29 but with 26 mg of NiSO$_4$ · 6H$_2$O liter^{-1}), 10 ml; 2.5% FeSO$_4$ · 7H$_2$O solution (pH 1.2 with H$_2$SO$_4$), 25 μl; 0.2% resazurin, 1 ml; polypropylene glycol MW 2000, 50 μl. The 2% starch–TYE medium was modified to contain the following: yeast extract, 6 g; no NH$_4$Cl but (NH$_4$)$_2$SO$_4$, 2.5 g; MgCl$_2$ · 6H$_2$O, 0.4 g; polypropylene glycol MW 2000, 100 μl. The 3% starch–TYE medium was modified to contain the following: yeast extract, 9 g; no NH$_4$Cl but (NH$_4$)$_2$SO$_4$, 3.75 g; KH$_2$PO$_4$, 1 g; no NaH$_2$PO$_4$ · 7H$_2$O; MgCl$_2$ · 6H$_2$O, 0.6 g; FeSO$_4$ · 7H$_2$O

---

* Corresponding author.

(2.5% solution), 37.5 μl; polypropylene glycol MW 2000, 100 μl. The starch-TYE medium was used for pH and temperature studies. All three starch-TYE media were used for studies of growth and product formation at various dilution rates and substrate concentrations, as indicated in the text. Media were prepared in 18-liter glass bottles that were autoclaved for 90 min and immediately thereafter gassed with oxygen-free N_2 for a few minutes and then hooked to N_2 (20 kPa) during cooling and maintained at that pressure while they served as feed tanks to the continuous cultures. A 10-ml portion of 2.5% cysteine sulfide was added as a reducing agent to 16 liters of medium.

Culture conditions. Continuous cultures were grown at low stirrer speeds without gassing in Multigen fermentors (New Brunswick Scientific Co., Inc., Edison, N.J.) equipped with a customized sampling port and an overflow weir that maintained a working volume of 290 ml. Unless otherwise mentioned, pH was controlled at 6.0 with 1 to 3 N NaOH and temperature was maintained at 60°C with a heating tape wound around the vessel that supported the built-in heater. Before inoculation, an additional 3 ml of 2.5% cysteine sulfide was injected into the vessel, and it was flushed with N_2-CO_2 (95:5) to maintain anaerobiosis during start-up. Sulfide in the exhaust gas was trapped in a bottle containing 10% zinc acetate. After a modification in cultivation parameters was made, sampling for steady-state data began when six or more culture volume changes occurred.

Analytical procedures. To estimate biomass concentration, the optical density of an appropriately diluted culture was measured at 660 nm and converted to biomass dry weight by using a calibration curve. Dry weight was measured with either a centrifuged (10,000 × g) or filtered (0.2-μm-pore-size nylon membrane filter) culture sample that had been washed with deionized water and dried at 105°C for 20 h. Ethanol and acetate concentrations were determined on a Hewlett-Packard 5890A gas chromatograph by using Porapak or Super Q columns maintained isothermally at 170 and 190°C, respectively. Residual reducing sugars were determined by the dinitrosalicyclic acid (DNS) method (3) with maltose or glucose as a standard. To measure total reducing carbohydrates, 0.5 ml of a diluted culture broth supernatant was mixed with 50 μl of 5 M sulfuric acid solution in a screw-cap vial, steam heated for 3 h, and then neutralized with 35 μl of 10 N NaOH. Released reducing sugars were determined by the DNS method with glucose as a standard. Residual starch was calculated by subtracting the free residual sugars from the total reducing carbohydrate reading of a sample. β-Amylase activity was assayed by using a reaction mixture (5 ml) consisting of boiled soluble starch solution (2%), acetate buffer (50 mM, pH 6.0), and the appropriately diluted enzyme solution. After incubation for 30 min at 60°C, the reducing sugar liberated was measured by the DNS method with maltose as a standard.

RESULTS

Stability of continuous culture. Long-term stability of β-amylase productivity is a prerequisite for an implementation of a continuous culture process for enzyme production. We inspected this need from two viewpoints. First, stability was checked in chemostat runs over periods of 292 to 432 h at constant dilution rates by comparing initial versus final enzyme levels and biomass concentrations in either 1 or 2% starch-TYE medium (Table 1). Continuous β-amylase production appeared quite stable because enzyme yield decreased only slightly (<4%) after long periods on either 1 or

TABLE 1. Stability of *C. thermosulfurogenes* β-amylase production in continuous culture at constant dilution rates[a]

Starch content (%)	Time (h)	β-Amylase activity (U ml^{-1})	Biomass (g liter^{-1})	β-Amylase yield (U mg of cells^{-1})	Dilution rate (h^{-1})
2					
Initial	25	55	1.43	38	0.28
Final	292	62	1.83	34	0.28
1					
Initial	46	32	0.82	39	0.285
Final	432	27	0.78	35	0.285

[a] Experimental conditions: pH, 6; temperature, 60°C; TYE medium with 1 or 2% soluble starch. Steady-state conditions were achieved prior to the initial time indicated.

2% starch–TYE medium. Second, stability was judged over even longer periods (up to 26 weeks) by comparing steady-state values that were obtained under identical conditions at different times during a run or under the same conditions in other runs. Values in Table 2 are based on measurements of β-amylase activity during a wide variety of different culture conditions (i.e., dilution rate and starch concentrations were varied during a given continuous culture run). The highest of the values obtained at identical conditions (dilution rate, medium composition, pH, temperature) was assigned 100%, and all of the other values measured under these conditions in the same run or in other runs are given in percentages thereof. Runs 2 and 3 maintained full capacity of β-amylase production at long time periods. Run 4 was atypical of repeat experiments; initially, it only produced 25% of the maximal activity under the specific conditions, and then the activity improved to 98% after 3 weeks and then declined again.

Optimal pH and temperature. By using 1% starch–TYE medium and a dilution rate of $D = 0.28$ h^{-1}, pH and temperature were varied to maximize biomass concentration and β-amylase activity. During pH studies, temperature was maintained at 60°C. Figure 1 shows that pH 5.8 to 6.0 is optimal for the formation of both biomass and β-amylase. Moreover, specific β-amylase productivity is also highest at this pH. For temperature studies, pH was kept at 6.0. Figure 2 indicates that 60°C is the optimum temperature for growth of the organism as well as production of β-amylase. Again, specific β-amylase activity peaked at the same value. For

TABLE 2. Stability of *C. thermosulfurogenes* β-amylase production in continuous culture during four representative runs[a]

Age of culture (wk)	Stability of production (%) during run no.:			
	1	2	3	4
1	80	72	100	25
1.5	80	100	100	53
2	100	100	100	80
2.5	100	100	100	75
3	100	100	100	98
4	100	100	100	92
6	100	100		79
7		100		85
18		100		
26		63		

[a] Experimental conditions: pH, 6; temperature, 60°C; TYE medium with 1, 2, or 3% soluble starch; various dilution rates. Values indicate what percentage of the potential β-amylase productivity was achieved under given combinations of substrate concentrations and dilution rates (see text for details).
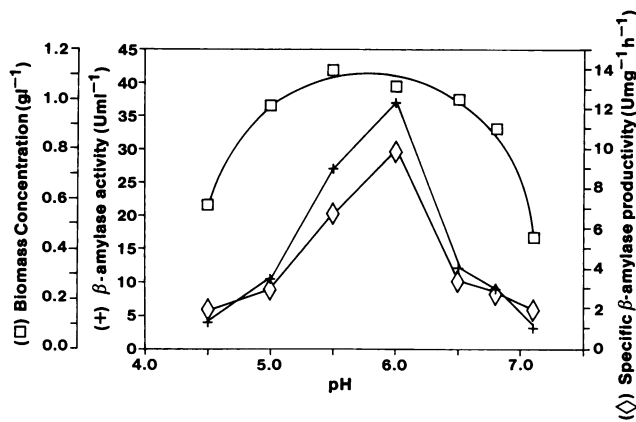
FIG. 1. Effect of pH on biomass formation, β-amylase production, and specific β-amylase productivity of *C. thermosulfurogenes*. Data are from continuous cultivation on 1% starch–TYE medium at 60°C; dilution rate, 0.28 h⁻¹.

subsequent studies, these optimized process conditions were applied.

Substrate concentration. To produce high concentrations of β-amylase, the concentration of biomass had to be increased. Initially, biomass concentration was measured in a chemostat as a function of dilution rate by using our traditional 1% starch–TYE medium (Fig. 3). The highest biomass concentration was 1.0 g liter⁻¹ at a dilution rate of 0.26 h⁻¹. The maximal growth rate was >1.5 h⁻¹. A starch pulse injected into the culture at $D = 0.15$ h⁻¹ revealed that growth of the organism was starch limited. Therefore, we designed 2 and 3% starch–TYE media to increase biomass concentration. At low dilution rates, the 2% starch–TYE medium yielded almost double the biomass concentration of the 1% medium. However, the 3% medium did not produce an appreciable increase over the 2% medium. From a series of medium component pulses into cultures, it was concluded that growth was neither substrate limited nor substrate inhibited. The culture was checked for metabolite inhibition because a decline in biomass concentration was observed at a high dilution rate in 1 and 2% starch–TYE media (20).

End product inhibition. Concentrations of the main energy catabolites, ethanol and acetate, as a function of dilution rate
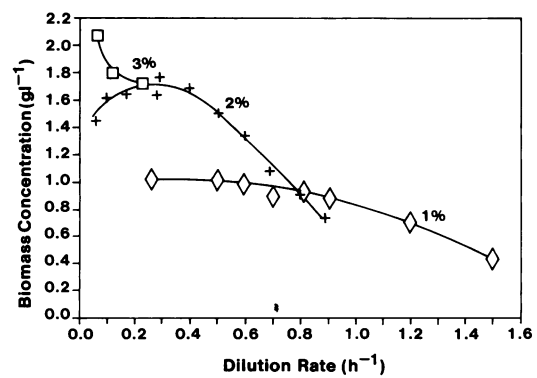
FIG. 3. Effect of starch concentration on biomass levels of *C. thermosulfurogenes* in continuous cultures. TYE medium containing 1, 2, or 3% starch at 60°C, pH 6.0, was used.

and medium concentration are given in Fig. 4. These metabolites were detected at relatively low levels in continuous culture (i.e., <5 g liter⁻¹ for ethanol and <3 g liter⁻¹ for acetate). A series of ethanol injections into cultures that were grown in either a 1 or a 2% starch–TYE medium at very high dilution rates or in a 3% starch–TYE medium at low dilution rate had no effect on growth rate (Table 3). A series of pulse experiments with acetate, however, demonstrated the inhibitory effect of acetic acid on growth rate under these culture conditions. The growth rate decreased after acetate was pulsed into 1, 2, and 3% media at dilution rates of 0.88, 0.70, and 0.29 h⁻¹, respectively, but it was unaffected at $D = 0.28$ h⁻¹ in the 1 and 2% starch–TYE media. As an example, Fig. 5 shows the effect of an acetate pulse into a culture on a 2% starch–TYE medium at $D = 0.69$ h⁻¹. The immediate decrease of the growth rate is reflected in the declining biomass concentration. After acetate had washed out to below 2 g liter⁻¹, the biomass started to rise to its initial value again. Acetic acid (i.e., acetate) was titrated to pH 6.0 with NaOH either before or during the injection to avoid a pH change in the culture. The notion that the sodium ion instead of the acetic acid was the effector was refuted by the ineffectiveness of a pulse of 2.9 g of NaCl liter⁻¹ under the conditions in question. In general, acetic acid-inhibited cultures seemed to be saccharide sufficient (>2 g of free reducing sugars liter⁻¹ as measured by the DNS method).

Substrate utilization. It was expected from batch experiments (15) that utilization of the carbon source, soluble
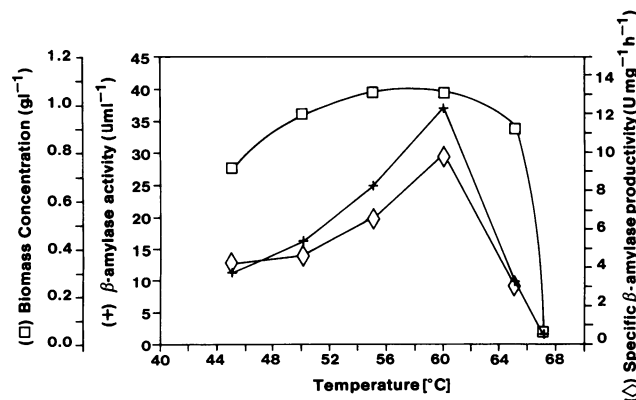

FIG. 2. Effect of temperature on biomass formation, β-amylase production, and specific β-amylase productivity of *C. thermosulfurogenes*. Data are from continuous cultivation on 1% starch–TYE medium at pH 6.0; dilution rate, 0.28 h⁻¹.
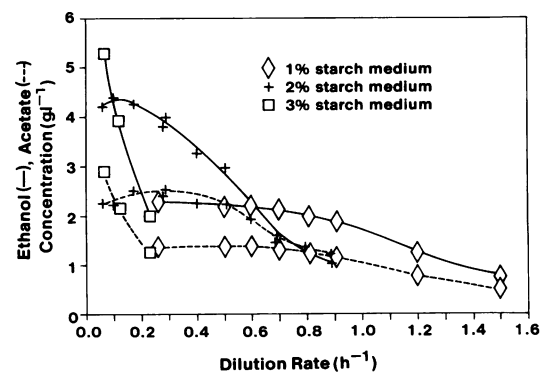

FIG. 4. Effect of starch concentration on ethanol and acetate produced by *C. thermosulfurogenes* in continuous cultures. TYE medium containing 1, 2, or 3% starch at 60°C, pH 6.0, was used.

TABLE 3. Effects of ethanol and acetate pulses into continuous cultures of *C. thermosulfurogenes* grown on TYE-starch media[a]

Starch content (%)	Dilution rate (liters h⁻¹)	Biomass concn (g liter⁻¹)	Ethanol concn (g liter⁻¹)	Ethanol pulse (g liter⁻¹)	Acetate concn (g liter⁻¹)	Acetate pulse (g liter⁻¹)	Effect[b]
1	0.99	0.89	1.69	1.2			−
	0.99	0.89	1.69	1.9			−
	0.28	0.99			1.35	2.5	−
	0.88	0.91			1.17	2.5	+
2	0.96	1.11	1.60	2.0			−
	0.96	0.94	1.60	4.8			−
	0.28	1.60			2.48	3.0	−
	0.70	1.04			1.47	3.0	+
3[c]	0.29	1.79	4.07	4.0			−
	0.15	1.79			2.15	3.0	−
	0.28	1.51			2.15	4.6	+
	0.29	1.79			2.03	3.0	+

[a] Experimental conditions: pH controlled at 6.0; temperature, 60°C; no gassing.
[b] +, Decreased growth rate after pulse; −, no effect on growth rate after pulse.
[c] Medium without Trypticase and vitamin solution.

starch, would be incomplete. Even at low dilution rate and optimum conversion, 40% of the incoming carbon left the reactor unutilized, mainly as nondegraded starch, which is assumed to be β-limit dextrin (Fig. 6). Except for dilution rates which caused acetate inhibition, glucose concentration in the effluent was below 0.2 g liter⁻¹. This was expected because *C. thermosulfurogenes* is not known to excrete glucoamylase or other amylase activities. Concentrations of free reducing carbohydrates (as measured by the DNS method) were below 2 g liter⁻¹ at low dilution rates and increased at high dilution rates.

β-Amylase production. The effect of substrate concentration on the activity levels of β-amylase is shown in Fig. 7. In the 1, 2, and 3% starch–TYE media the peak β-amylase activities reached approximately 45, 85, and 90 U ml⁻¹, respectively. Since high product concentration is most desirable in biotechnological production, a combination of low dilution rate and high substrate concentration is the most viable process condition.

Figure 8 illustrates the effect of dilution rate on the volumetric productivity of β-amylase in 1, 2, or 3% starch–TYE medium; maximal volumetric productivities occurred at dilution rates of 0.5 and 1.0 h⁻¹ for 1 and 2% starch–TYE media, respectively. Since high volumetric productivity is a process advantage, the data in Fig. 7 and 8 suggest that 2% starch–TYE medium and dilution rate below 0.5 h⁻¹ are

optimal for β-amylase productivity and concentration. Specific β-amylase productivity was positively correlated with specific growth rate, as demonstrated by plotting steady-state specific productivities of cultures in noninhibitory or limited media against dilution rate (data not shown).

DISCUSSION

These fermentation findings on stability of β-amylase productivity, yield, and activity levels demonstrate that continuous production of an extracellular, thermostable enzyme under anaerobic and thermophilic conditions is feasible. Stability of β-amylase production at 80 to 100% of the maximal productivity can be expected in continuous culture. Fermentation operating parameters such as pH, temperature, substrate concentration, and dilution rate were optimized with regard to high β-amylase activity levels and high productivity. The fact that optimal pH (6.0) as well as temperature (60°C) are identical for both growth of the organism and production of β-amylase means that the cultivation in a single-stage chemostat does not compromise growth or production.
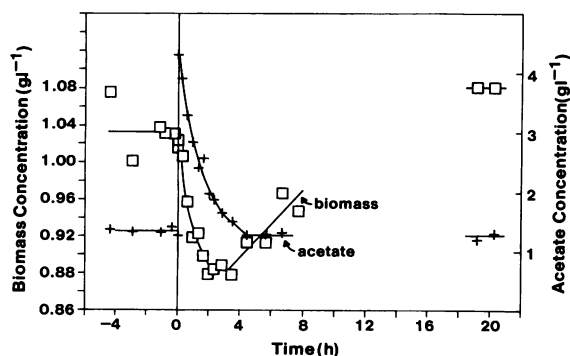

FIG. 5. Effect of acetate addition on biomass concentration of *C. thermosulfurogenes* in continuous culture. Conditions: pH controlled at 6.0; dilution rate, 0.69 h⁻¹; TYE medium with 2% starch; 60°C. Acetate (39 g liter⁻¹) was injected at time zero.
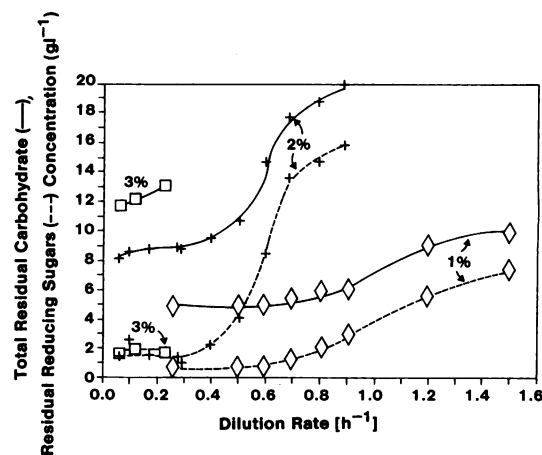

FIG. 6. Effect of dilution rate on total residual carbohydrates (mainly β-limit dextrin) and residual reducing sugars (mainly maltose) in continuous cultures of *C. thermosulfurogenes*. TYE medium with 1, 2, or 3% starch at 60°C, pH 6.0, was used.
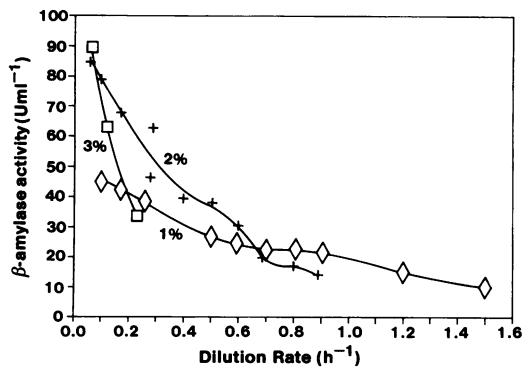
FIG. 7. Effect of starch concentration on β-amylase activity levels in continuous cultures of *C. thermosulfurogenes*. TYE medium containing 1, 2, or 3% starch at 60°C, pH 6.0, was used.

Increasing the starch concentration from 1 to 2 and then to 3% increased the maximal β-amylase concentrations from 45 to 85 to 90 U liter^{-1}, respectively. Volumetric productivities at these peak enzyme activity levels were relatively low, because they were attained at low dilution rates (0.07 h^{-1}). If productivity was to be enhanced in the 1% starch medium, this would be achieved by increasing the dilution rate at the expense of lower enzyme activity levels. In the 3% starch medium, this concept would not work because of severe acetate inhibition and would result in an undesirable sharp decrease in enzyme activity levels.

The low acetic acid tolerance of this organism obviously limits the prospects of using this organism alone for β-amylase fermentation. Biomass formation is limited to <2.3 g of acetate liter^{-1}, and therefore the β-amylase concentration obtained is low. Moreover, enzyme productivity is restrained because a low dilution rate has to be used.

End product inhibition has been studied in other ethanol- and acetate-producing thermophilic clostridia. The predominately ethanol-producing wild-type strains of *Clostridium thermohydrosulfuricum* (17) and *Clostridium thermocellum* (12) are inhibited by final ethanol concentrations of <20 g liter^{-1}. Wang and Wang (27) claimed that growth of *Clostridium thermoaceticum* was completely inhibited by 50 g of total acetic acid liter^{-1} (undissociated acetic acid plus acetate ion) at pH 6.0. In another study (25), the same species produced a maximum of 15 g of total acetic acid liter^{-1} at pH

6. Similar results were obtained by Reed et al. (21), who also reported that *Clostridium thermoautotrophicum* tolerated 14.5 g of total acetic acid liter^{-1} at pH 5.75. The end product tolerance of *C. thermosulfurogenes* of less than 5 g of acidic acid liter^{-1} is very low and quite different from those of the homoacetogenic species listed above. Acetic acid is generally thought to inhibit anaerobes as an uncoupler of the proton motive force (2). The very low tolerance of *C. thermosulfurogenes* to total acetate and acetic acid suggests that this inhibition probably involves aspects of the organism physiology other than just dissipation of the proton motive force.

The problem of incomplete starch utilization in the β-amylase fermentation can be solved in two ways. If a more hydrolyzed substrate such as maltodextrin DE 10 or high-maltose, low-glucose syrup is used instead of soluble starch, utilization will be improved (unpublished data). The use of glucose would be questionable because of the risk of selecting, for glucose repression, sensitive mutants that would produce little β-amylase.

The specific β-amylase productivity could be much higher if the cell concentration could be raised. This appears to be limited by acetate inhibition, which perhaps could be overcome by the use of a mutant strain. It is unfortunate that the enzyme productivity is extremely low at the very low dilution rates required to yield the highest product concentration. If the potential of the cells for high enzyme productivity at high growth rates could be exploited at low dilution rates, much higher β-amylase concentrations could be achieved. Improvements in complex N- and C-nutrient sources which have successfully stimulated specific productivities of extracellular enzymes in many processes (9, 19, 26) may also improve this fermentation. Nonetheless, an anaerobic fermentation process for enzyme production is not warranted unless this thermostable β-amylase demands a high price relative to other industrial amylases. Since this does not appear to be the case, genetic cloning and production of this β-amylase in a food-safe aerobic host (e.g., *Bacillus* species) would result in much higher yields and conversions of substrate into product than could be achieved even in an optimized anaerobic fermentation.
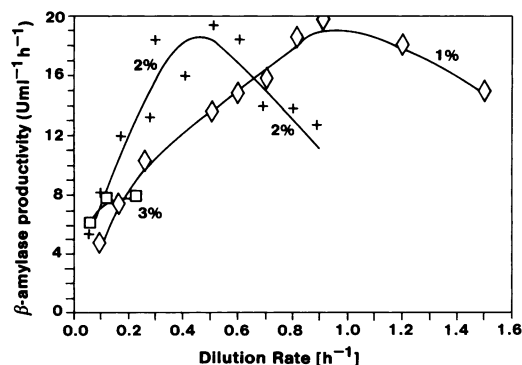

FIG. 8. Effect of starch concentration on volumetric β-amylase productivity of *C. thermosulfurogenes* cultures as a function of dilution rate. TYE medium containing 1, 2, or 3% starch at 60°C, pH 6.0, was used.

LITERATURE CITED

1. **Antranikian, G., and G. Gottschalk.** 1987. Production of thermostable enzymes for starch hydrolysis, p. 261–263. *In* O. M. Neijssel, R. R. Van der Meer, and K. C. A. M. Luyben (ed.), Proceedings of the 4th European Congress on Biotechnology, vol. 3. Elsevier, Amsterdam.
2. **Baronofsky, J. J., W. J. A. Schreurs, and E. R. Kashket.** 1984. Uncoupling by acetic acid limits growth of and acetogenesis by *Clostridium thermoaceticum*. Appl. Environ. Microbiol. **48:** 1134–1139.
3. **Bernfeld, P.** 1955. Amylases α and β. Methods Enzymol. **1:**149–158.
4. **Davis, P. E., D. L. Cohen, and A. Whitaker.** 1980. The production of α-amylase in batch and chemostat culture by *Bacillus stearothermophilus*. Antonie van Leeuwenhoek J. Microbiol. **46:**391–398.
5. **Emanuilova, E., and K. Toda.** 1984. α-Amylase production in batch and continuous cultures by *Bacillus caldolyticus*. Appl. Microbiol. Biotechnol. **19:**301–305.
6. **Fabian, J.** 1970. Synthesis of the extracellular protease by *Bacillus pumilus*. Folia Microbiol. **15:**160–168.
7. **Fencl, Z., and J. Pazlarova.** 1982. Production of extracellular enzymes in continuous culture. Folia Microbiol. **27:**340–349.
8. **Fencl, Z., J. Ricica, and J. Kodesova.** 1972. The use of the multi-state chemostat for microbial product formation. J. Appl. Chem. Biotechnol. **22:**405–416.
9. **Grueninger, H., B. Sonnleitner, and A. Fiechter.** 1984. Bacterial

diversity in thermophilic aerobic sewage sludge. Appl. Microbiol. Biotechnol. **19:**414–421.

10. **Hebeda, R. E.** 1987. Corn sweeteners, p. 501–534. *In* S. A. Watson and P. E. Ramstad (ed.), Corn: chemistry and technology. American Association of Cereal Chemists, St. Paul, Minn.

11. **Heineken, F. G., and R. J. O'Connor.** 1972. Continuous culture studies on the biosynthesis of alkaline protease, neutral protease and α-amylase by *Bacillus subtilis* NRRL-B3411. J. Gen. Microbiol. **73:**35–44.

12. **Herrero, A. A., and R. F. Gomez.** 1980. Development of ethanol tolerance in *Clostridium thermocellum*: effect of growth temperature. Appl. Environ. Microbiol. **40:**571–577.

13. **Hyun, H. H., and J. G. Zeikus.** 1985. General biochemical characterization of thermostable extracellular β-amylase from *Clostridium thermosulfurogenes*. Appl. Environ. Microbiol. **49:** 1162–1167.

14. **Hyun, H. H., and J. G. Zeikus.** 1985. Regulation and genetic enhancement of β-amylase production in *Clostridium thermosulfurogenes*. J. Bacteriol. **164:**1162–1170.

15. **Hyun, H. H., and J. G. Zeikus.** 1985. Simultaneous and enhanced production of thermostable amylases and ethanol from starch by cocultures of *Clostridium thermosulfurogenes* and *Clostridium thermohydrosulfuricum*. Appl. Environ. Microbiol. **49:**1174–1181.

16. **Jensen, D. E.** 1972. Continuous production of extracellular protease by *Bacillus subtilis* in a two-stage fermentor. Biotechnol. Bioeng. **24:**647–662.

17. **Lovitt, R. W., R. Longin, and J. G. Zeikus.** 1984. Ethanol production by thermophilic bacteria: physiological comparison of solvent effects on parent and alcohol-tolerant strains of *Clostridium thermohydrosulfuricum*. Appl. Environ. Microbiol. **48:**171–177.

18. **Meyer, H. P., and A. E. Humphrey.** 1982. Cellulase production in continuous culture with the mutant *Thermomonospora* sp. N-35. Chem. Eng. Commun. **19:**149–156.

19. **Ohba, R., and S. Ueda.** 1980. Optimum medium components and culture conditions for the production of intra- and extracellular pullulanase by *Aerobacter aerogenes*. J. Fac. Agric. Kyushu Univ. **25:**135–151.

20. **Pirt, S. J.** 1975. Principles of microbe and cell cultivation. John Wiley & Sons, Inc., New York.

21. **Reed, W. M., F. A. Keller, F. E. Kite, M. E. Bogdan, and J. S. Ganoung.** 1987. Development of increased acetic acid tolerance in anaerobic homoacetogens through induced mutagenesis and continuous selection. Enzyme Microbiol. Technol. **9:**117–120.

22. **Rutten, R., and A. J. Daugulis.** 1987. Continuous production of α-amylase by *Bacillus amyloliquefaciens* in a two stage fermentor. Biotechnol. Lett. **9:**505–510.

23. **Saha, B. C., and J. G. Zeikus.** 1987. Biotechnology of maltose syrup production. Proc. Biochem. **22:**78–82.

24. **Schink, B., and J. G. Zeikus.** 1983. *Clostridium thermosulfurogenes* sp. nov., a new thermophile that produces elemental sulphur from thiosulphate. J. Gen. Microbiol. **129:**1149–1158.

25. **Schwartz, R. D., and F. A. Keller.** 1982. Acetic acid production by *Clostridium thermoaceticum* in pH-controlled batch fermentations at acidic pH. Appl. Environ. Microbiol. **43:**1385–1392.

26. **Takasaki, Y.** 1976. Productions and utilizations of β-amylase and pullulanase from *Bacillus cereus* va. mycoides. Agric. Biol. Chem. **40:**1515–1522.

27. **Wang, G., and D. I. C. Wang.** 1984. Elucidation of growth inhibition and acetic acid production by *Clostridium thermoaceticum*. Appl. Environ. Microbiol. **47:**294–298.

28. **Wolin, E. A., M. J. Wolin, and R. S. Wolfe.** 1963. Formation of methane by bacterial extracts. J. Biol. Chem. **238:**2882–2886.

29. **Zeikus, J. G., P. W. Hegge, and M. A. Anderson.** 1979. *Thermoanaerobium brockii* gen. nov. and sp. nov., a new chemoorganotrophic, caldoactive, anaerobic bacterium. Arch. Microbiol. **122:**41–48.