APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Sept. 1991, p. 2544–2548
0099-2240/91/092544-05$02.00/0

Vol. 57, No. 9

Isolation and Characterization of *Clostridium acetobutylicum* Mutants with Enhanced Amylolytic Activity

BASSAM A. ANNOUS AND HANS P. BLASCHEK*

Department of Food Science, University of Illinois, 905 South Goodwin Avenue, Urbana, Illinois 61801

Received 25 March 1991/Accepted 10 June 1991

***Clostridium acetobutylicum* mutants BA 101 (hyperamylolytic) and BA 105 (catabolite derepressed) were isolated by using *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine together with selective enrichment on the glucose analog 2-deoxyglucose. Amylolytic enzyme production by *C. acetobutylicum* BA 101 was 1.8- and 2.5-fold higher than that of the ATCC 824 strain grown in starch and glucose, respectively. *C. acetobutylicum* BA 105 produced 6.5-fold more amylolytic activity on glucose relative to that of the wild-type strain. The addition of glucose at time zero to starch-based P2 medium reduced the total amylolytic activities of *C. acetobutylicum* BA 101 and BA 105 by 82 and 25%, respectively, as compared with the activities of the same strains grown on starch alone. Localization studies demonstrated that the amylolytic activities of *C. acetobutylicum* BA 101 and BA 105 were primarily extracellular on all carbohydrates tested.**

The microbial fermentation of carbohydrates to acetone, butanol, and ethanol is well known (15, 19, 20). Currently, this value-added fermentation is viewed as potentially attractive for several economic reasons. Prominent among these economic factors is the current surplus of agricultural wastes or byproducts that can be utilized as inexpensive fermentation substrates. This is particularly true for mycotoxin-contaminated corn, which is unsuitable for use as animal feed and is difficult to dispose of.

Several schemes have been proposed for the fermentative production of butanol from various low-cost substrates (21, 23). One of these economically viable approaches depends on use of a stable, high-yielding strain of *Clostridium acetobutylicum*, low-cost corn substrate and an increased market for butanol (23).

Results from various laboratories suggested that amylolytic enzyme biosynthesis in *C. acetobutylicum* is subject to catabolite repression by glucose and induction by starch (1, 7, 10, 17). Lin and Blaschek (17) reported that higher starch utilization by *C. acetobutylicum* SA-1 (a butanol-tolerant strain) correlated with a higher α-amylase activity and butanol production. Also, they suggested that further amplification of the amylolytic enzyme production in *C. acetobutylicum* SA-1 may increase the final butanol concentration.

The development of *C. acetobutylicum* strains with improved starch-hydrolyzing ability and enhanced starch-to-butanol conversion efficiency should improve the economics of this value-added (corn-based) fermentation. This work was undertaken to develop glucose-derepressed and/or hyperamylolytic strains of *C. acetobutylicum* and to characterize these strains by examining the effect of the carbohydrate source on the regulation and localization of these enzymes.

MATERIALS AND METHODS

Culture maintenance, inoculum development, and experimental media. *C. acetobutylicum* ATCC 824 was obtained from the American Type Culture Collection. Unless otherwise indicated, all experiments were performed under anaerobic conditions as previously described (1). The medium, buffer, and distilled water were placed in an anaerobic chamber at least 24 h before use to ensure the removal of dissolved oxygen. Stock cultures were maintained as spores in sterile distilled and deionized water at room temperature. Chemically defined P2 medium (1) containing 5 g of glucose and 5 g of starch per liter was used for the routine transfer of *C. acetobutylicum* ATCC 824 stock cultures. The glucose analog 2-deoxyglucose (2-DOG) was substituted for glucose during the routine transfer of *C. acetobutylicum* BA 101 and BA 105 stock cultures. Culture development and the inoculation of the experimental media were as previously described (1).

Analytical procedures and chemical reagents. Growth, amylolytic activity, and protein concentration were determined as previously described (1). Amylolytic activity was reported in units per milligram of total protein. Biochemical characterization of all strains was accomplished by using the API-20A system (Analytab Products, Inc., Plainview, N.Y.).

All chemicals used were analytical reagent grade. Glucose and sodium acetate were obtained from Fisher Scientific, Fair Lawn, N.J. Soluble starch and calcium chloride were obtained from EK Industries, Inc., Addison, Il. 2-DOG, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG), dextrin type I, and HCl-cysteine were obtained from Sigma Chemical Co., St. Louis, Mo. Bacto-Agar, thioglycollate, yeast extract, and peptone were from Difco Laboratories, Detroit, Mich. Maltose was from ICN Biochemicals, Inc., Cleveland, Ohio, and Trypticase peptone was from BBL Microbiology Systems, Cockeysville, Md.

Mutagenesis, enrichment, and isolation of mutants. A late-exponential-phase TGY (1) culture of *C. acetobutylicum* ATCC 824 was centrifuged at 10,400 × *g* for 20 min at 2°C, washed once with PT buffer (0.1 g of peptone, 8.5 g of sodium chloride, and 1 g of sodium thioglycollate per liter), and suspended in fresh TGY medium containing 50 μg of NTG per ml to a final optical density at 600 nm of 1.0. After incubation for 15 min, the NTG-treated cells were washed three times with PT buffer to remove the residual NTG and suspended in an equal volume of fresh TGY medium for 7 h. The cells were then washed three times with PT buffer to remove the residual glucose, suspended in an equal volume of P2 medium containing 5 g of starch and 1 g of 2-DOG per

* Corresponding author.

liter for 20 h, and plated onto P2 agar medium containing 15 g of glucose and 5 g of starch per liter. After 3 days, colonies were picked at random and replica plated onto P2 agar medium containing 20 g of glucose per liter. After 2 days, the master plates were overlaid with an agar solution containing 10 g of starch per liter (pH 5), incubated for 2 h, and then stained with iodine vapor. The iodine vapor was generated by heating iodine crystals in a water bath at 80°C. The colonies exhibiting large clear zones on the iodine-stained plates (identified from the replica plates by comparing the colony positions) were subjected to four transfers in P2 medium containing 5 g of starch and 5 g of 2-DOG per liter. The cells from the fourth transfer were then plated onto P2 agar medium containing 5 g of starch and 5 g of 2-DOG per liter. Colonies were picked, replica plated, overlaid, stained, and identified from replica plates as above. Colonies exhibiting large, clear zones were tentatively identified as glucose-derepressed mutants.

Amylolytic enzyme production on various carbohydrate sources. P2 medium containing 20 g of either dextrin, glucose, maltose, or starch per liter as the carbohydrate source was inoculated with the appropriate strain of *C. acetobutylicum*. Samples were collected at various growth stages and assayed for total (intracellular as well as extracellular) and extracellular amylolytic activities as previously described (1).

Regulation of amylolytic enzyme production. P2 medium containing 20 g of starch per liter was inoculated with the appropriate strain of *C. acetobutylicum*. After growth for 0 or 12.5 h, glucose P2 medium was added to half the volume of the growth medium to give a final glucose concentration of 1%. An equal volume of P2 medium with no added carbohydrate was added to the other half to serve as a control. Samples were collected at different growth stages and used to determine total and extracellular amylolytic activities.

RESULTS

Mutagenesis, enrichment, and isolation of mutants. NTG treatment of *C. acetobutylicum* ATCC 824 resulted in 99.75% loss of cell viability. A total of 389 colonies of the 2.8×10^5 survivors were picked at random; 9 of these were further examined based on the production of large clear zones on starch-overlaid glucose plates. The amylolytic activities of the isolates when grown in P2 medium containing 20 g of glucose or starch per liter demonstrated considerable variability (Table 1). Two strains, designated as hyperamylolytic (BA 101) and catabolite derepressed (BA 105), were further examined. Amylolytic enzyme production by both isolates was stable after eight culture transfers. Although *C. acetobutylicum* BA 101 and BA 105 demonstrated Gram stain reactions and microscopic morphologies similar to those of the ATCC 824 strain, they acquired the ability to ferment melezitose, rhamnose, and trehalose. Also, BA 101 acquired the ability to ferment raffinose.

Effect of carbohydrate source on growth and total amylolytic activity. *C. acetobutylicum* ATCC 824 demonstrated a lag phase for outgrowth in starch-based P2 medium that was up to 18 h longer than those with the other three carbohydrate sources tested. The delayed growth on starch corresponded with a dramatic increase (8.3-fold) in the total amylolytic activity over that seen when this strain was grown on glucose (Table 1). Growth of *C. acetobutylicum* ATCC 824 on either dextrin or maltose had a slight stimulatory effect (up to twofold) on the total amylolytic activity relative to the activity when glucose was the carbohydrate source.

TABLE 1. Total amylolytic activity produced by *C. acetobutylicum* ATCC 824 and mutant strains grown in P2 medium containing 20 g of carbohydrate per liter

Strain	Total amylolytic activity[a] (U/mg of protein) of strains grown on:				Repression ratio[b]
	Starch	Glucose	Dextrin	Maltose	
ATCC 824	3.3	0.4	0.7	0.8	8.3
BA 101	6.0	1.0	1.1	1.6	6.0
BA 105	3.4	2.6	2.3	2.4	1.3
BA 109	0.9	0.5	—[c]	—	1.8
BA 217	1.2	0.3	—	—	4.0
BA 317	3.0	0.1	—	—	30.0
BA 2117	2.4	0.2	—	—	11.0
BA 2133	2.9	0.3	—	—	9.7
BA 2238	4.2	0.2	—	—	21.0
BA 7233	1.5	0.6	—	—	2.5

[a] Highest activity produced.
[b] Specific amylolytic activity produced by a culture grown on starch divided by the specific activity produced when the culture was grown on glucose.
[c] —, not determined.

C. acetobutylicum BA 101 demonstrated an 18-h shorter lag phase and a 1.8-fold increase in total amylolytic activity (Table 1) relative to those of the ATCC 824 strain grown in starch-based P2 medium. When grown in P2 medium containing either dextrin or maltose, *C. acetobutylicum* BA 101 demonstrated up to a twofold increase in total amylolytic activity (Table 1) over that seen with the ATCC 824 strain. However, despite the increase in amylolytic enzyme production by the BA 101 strain on the various carbohydrates, these enzymes still appeared to be subject to catabolite repression by glucose, as evidenced by the associated repression ratio (Table 1).

When grown in starch-based P2 medium, *C. acetobutylicum* BA 105, like BA 101, demonstrated a lag phase that was 18 h shorter than that of the ATCC 824 strain. *C. acetobutylicum* BA 105 also demonstrated a dramatic increase (6.5-fold) in total amylolytic activity (Table 1) over that seen with the ATCC 824 strain grown in glucose-based P2 medium. This finding, together with the corresponding repression ratio of 1.3 (the lowest value of the tested strains), suggests that amylolytic enzyme production in the BA 105 strain is not significantly catabolite repressed. The specific growth rates of *C. acetobutylicum* BA 105 on glucose, dextrin, maltose, and starch were 46, 27, 9, and 7% lower, respectively, than that of the ATCC 824 strain. This suggests that this isolate was negatively affected by the mutagenic treatment.

Regulation of amylolytic enzyme biosynthesis. The effect of the addition of 1% glucose to 2% starch-based P2 medium at time zero (0 h) and during the early-exponential phase (12.5 h) on the growth response and the total amylolytic activity of *C. acetobutylicum* BA 101 is shown in Fig. 1. The addition of glucose at time zero (Fig. 1A) reduced the lag phase of *C. acetobutylicum* BA 101 by ca. 12 h and the total amylolytic activity by 82%. A similar addition at 12.5 h (Fig. 1B) had no effect on the growth response of *C. acetobutylicum* BA 101 but reduced the total amylolytic activity by 13% relative to the values obtained without added glucose.

Figure 2 shows the effect of the addition of 1% glucose to 2% starch-based P2 medium at 0 and 12.5 h on the growth

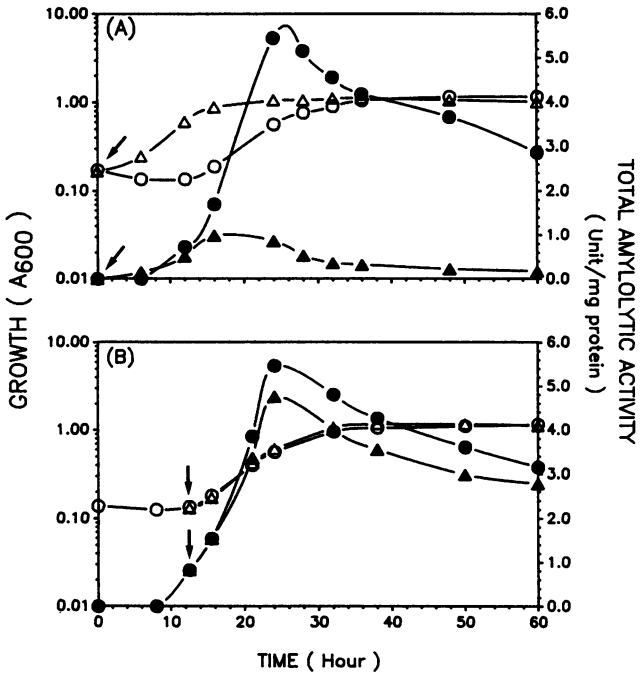

FIG. 1. Effect of the addition of 1% glucose to 2% starch-based P2 medium at time zero (A) and during the early-exponential phase (B) on the growth response (○, △) and the total amylolytic activity (●, ▲) of *c. acetobutylicum* BA 101. ○ and ●, 2% starch; △ and ▲, 2% starch plus 1% glucose.

response and the total amylolytic activity of *C. acetobutylicum* BA 105. The addition of glucose at time zero (Fig. 2A) reduced the lag phase by ca. 6 h, the specific growth rate by 50%, and the total amylolytic activity by 25%. The addition of glucose at 12.5 h (Fig. 2B) had no effect on the growth response and little effect on the total amylolytic activity.

Amylolytic enzyme biosynthesis by *C. acetobutylicum* BA 101 and BA 105 (Fig. 1 and 2, respectively) appears to be growth related, since these enzymes are primarily produced during the exponential phase, in agreement with earlier results with the parental strain, ATCC 824 (1).

Effect of carbohydrate source on the localization of amylolytic enzymes. Prior work (1) demonstrated that amylolytic activity was primarily intracellular when *C. acetobutylicum* ATCC 824 was grown in P2 medium containing 20 g of either glucose or maltose per liter. This activity was calculated as a percentage of the highest total amylolytic activity; 94, 90, 75, or 67% of this activity was extracellular for *C. acetobutylicum* BA 101, and 95, 92, 89, or 79% was extracellular for *C. acetobutylicum* BA 105, when these strains were grown in P2 medium containing starch, dextrin, maltose, or glucose, respectively.

Although the addition of 1% glucose to 2% starch-based P2 medium at time zero increased the intracellular amylolytic activity of *C. acetobutylicum* BA 101 and BA 105 by ca. sixfold over that obtained without the added glucose, this activity remained primarily extracellular. A similar addition of 1% glucose after 12.5 h of growth did not affect the secretion of these enzymes in either strain.

DISCUSSION

Recent reports (5, 6, 15, 28) indicated that indirect mutagens (e.g., UV radiation), which rely on the error-prone repair system, are poor mutagenic agents in *C. acetobutylicum*. These reports concluded that *C. acetobutylicum* lacks such a repair mechanism. On the other hand, NTG and ethylmethyl sulfonate, which act by a direct mutagenic mechanism to induce base substitutions or deletions, have been reported to be the most effective mutagenic agents in *C. acetobutylicum* (5, 9, 15, 16, 18). The variability in amylolytic activity values and the corresponding repression ratios of the mutant strains isolated in this study (Table 1) suggest alteration of the promoter region controlling amylolytic enzyme biosynthesis (30).

C. acetobutylicum BA 101 and BA 105 showed an alteration in carbohydrate metabolism. A similar alteration in the carbohydrate metabolism was reported by Lin and Blaschek (17) for butanol-tolerant *C. acetobutylicum* SA-1, which was isolated after 12 transfers of *C. acetobutylicum* ATCC 824 in media containing increasing concentrations of *n*-butanol. They reported that the SA-1 strain lost the ability to ferment esculin and acquired the ability to ferment raffinose, sorbitol, and melezitose. The similar results obtained herein for raffinose and melezitose suggest that these loci associated with carbohydrate fermentation may be mutational hot spots.

Catabolite repression of amylolytic enzymes by glucose has been previously reported for *Clostridium* species (1, 2, 7, 10, 12, 17, 22, 24). Recent work in our laboratory suggested that the amylolytic enzymes in *C. acetobutylicum* ATCC 824 are subject to catabolite repression by glucose at the level of transcription (1). This type of regulation of enzyme synthesis in clostridia has been shown to be mediated by the phosphoenolpyruvate-dependent sugar phosphotransferase system (3, 25). The phosphotransferase system is a widespread mechanism for carbohydrate uptake by bacteria (4, 14, 29).

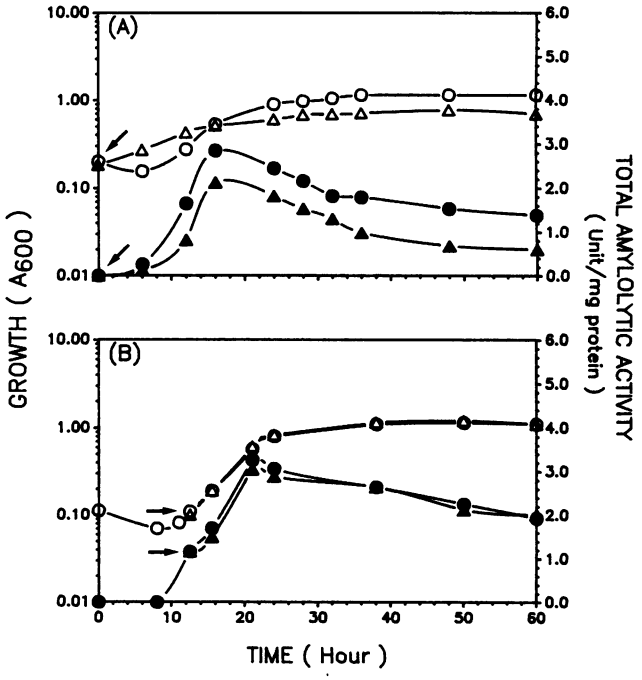

FIG. 2. Effect of the addition of 1% glucose to 2% starch-based P2 medium at time zero (A) and during the early-exponential phase (B) on the growth response (○, △) and the total amylolytic activity (●, ▲) of *c. acetobutylicum* BA 105. ○ and ●, 2% starch; △ and ▲, 2% starch plus 1% glucose.

The amylolytic enzymes produced by *C. acetobutylicum* ATCC 824 appear to be subject to induction by starch and catabolite repression by dextrin, glucose, or maltose (Table 1). The amylolytic activity produced on either dextrin, glucose, or maltose by *C. acetobutylicum* ATCC 824 may be considered as a low basal level. A similar observation was previously reported for different strains of clostridia (1, 10, 13, 24) and bacilli (14, 26). The growth of *C. acetobutylicum* ATCC 824 on starch demonstrated a lengthened lag phase that corresponded to a dramatic increase in total amylolytic activity when compared with growth on either dextrin, glucose, or maltose. Since starch cannot penetrate the bacterial cell wall, the lengthened lag phase is a reflection of the time needed to hydrolyze the starch to metabolizable subunits. It has been proposed that this may be achieved by the continual secretion of a low basal level of hydrolyzing enzymes (11, 22, 26). When starch is present in the environment, it is degraded by these enzymes with the release of low-molecular-weight molecules, which in turn will enter the cell and further effect induction of the amylolytic enzymes (11, 26). This phenomenon has been observed in bacterial and fungal systems (8, 11). This response was also expected when *C. acetobutylicum* ATCC 824 was grown on dextrin, but the presence of as little as 0.4% (wt/vol) glucose (data not shown) may be the reason for the rapid cell outgrowth and the production of relatively low levels of amylolytic enzymes (Table 1) on this carbohydrate. The repression of amylolytic enzyme production when *C. acetobutylicum* ATCC 824 and BA 101 were grown on maltose is consistent with the observation that maltose is a sugar of the phosphotransferase system in the clostridia (3).

When 1% glucose was added at time zero to 2% starch-based P2 medium, the amount of total amylolytic enzymes produced by *C. acetobutylicum* BA 101 (Fig. 1A) and BA 105 (Fig. 2A) was the same as that observed when these strains were grown on glucose alone (Table 1). Enzyme synthesis by *C. acetobutylicum* BA 101 (Fig. 1B) and BA 105 (Fig. 2B) continued for 12 h at a rate similar to that of the control after the addition of glucose at the early-exponential phase. This response may be due to the presence of an mRNA pool that continues to support the de novo synthesis of these enzymes, as observed earlier for *C. acetobutylicum* ATCC 824 (1). This type of regulatory control was also previously reported for bacilli (14, 27).

When compared with that of *C. acetobutylicum* ATCC 824, the specific growth rate of *C. acetobutylicum* BA 105 appears to be affected by the NTG treatment. This may indicate that the sugar phosphotransferase system in *C. acetobutylicum* BA 105 is defective. Priest (26) reported that the catabolite derepression of amylase synthesis in *B. licheniformis* RM10 in the presence of glucose was due to a defect in glucose uptake.

The amylolytic activities of *C. acetobutylicum* BA 101 and BA 105 were primarily extracellular on all carbohydrate sources tested. Recent reports (2, 22) indicated that an increase in the secretion of the amylolytic enzymes into the growth medium by *Clostridium* sp. strain EM1 grown on starch was accompanied by ultrastructural changes in the cell envelope that caused the cells to be leaky. Also, it was reported that such changes were not apparent when this microorganism was grown on glucose (2). We recently indicated that the amylolytic enzymes of *C. acetobutylicum* ATCC 824 were primarily intracellular (membrane bound) when this microorganism was grown on glucose or maltose and extracellular when it was grown on starch or dextrin (1). Although the addition at time zero of 1% glucose to 2% starch-based P2 medium increased the intracellular amylolytic activity in *C. acetobutylicum* BA 101 and BA 105 by ca. sixfold, these enzymes remained primarily extracellular. This finding may be due to an alteration in the cell envelope ultrastructure of *C. acetobutylicum* BA 101 and BA 105, which may allow for secretion of these enzymes into the growth medium.

The earlier onset times of amylolytic enzyme production by *C. acetobutylicum* BA 101 and BA 105 (Fig. 1 and 2, respectively) and the higher extracellular activity and the higher basal levels of these enzymes (Table 1) may explain the 18-h-shorter lag phase of these strains relative to that of *C. acetobutylicum* ATCC 824 (1) grown in starch-based P2 medium.

Both *C. acetobutylicum* BA 101 and BA 105 may be useful for the optimization of this starch-based butanol fermentation. Furthermore, these isolates may be useful in exploring alternative carbohydrate sources as substrates for this value-added fermentation. Work is currently underway to evaluate the solvent production capability of *C. acetobutylicum* BA 101 and BA 105 in starch-based complex media and to examine the altered sugar phosphotransferase system.

ACKNOWLEDGMENTS

This work was supported in part by grant ICMB 89-0044-03 from the Illinois Corn Marketing Board, by University of Illinois Agricultural Experiment Station Hatch grant 50-313, and by a UIUC Foundation University Scholar grant to H.P.B.

REFERENCES

1. **Annous, B. A., and H. P. Blaschek.** 1990. Regulation and localization of amylolytic enzymes in *Clostridium acetobutylicum* ATCC 824. Appl. Environ. Microbiol. **56**:2559–2561.
2. **Antranikian, G., C. Herzberg, F. Mayer, and G. Gottschalk.** 1987. Changes in the cell envelope structure of *Clostridium* sp. strain EM1 during massive production of α-amylase and pullulanase. FEMS Microbiol. Lett. **41**:191–197.
3. **Booth, I. R., and W. J. Mitchell.** 1987. Sugar transport and metabolism in the clostridia, p. 165–185. *In* J. Reizer and A. Peterkofsky (ed.), Sugar transport and metabolism in gram-positive bacteria. Ellis Horwood Ltd., England.
4. **Botsford, J. L.** 1981. Cyclic nucleotides in procaryotes. Microbiol. Rev. **45**:620–642.
5. **Bowring, S. N., and J. G. Morris.** 1985. Mutagenesis of *Clostridium acetobutylicum*. J. Appl. Bacteriol. **58**:577–584.
6. **Carrasco, A.** 1989. Photoreactivating capacity in *Clostridium butyricum* and *Clostridium acetobutylicum*. Lett. Appl. Microbiol. **8**:131–134.
7. **Chojecki, A., and H. P. Blaschek.** 1986. Effect of carbohydrate source on alpha-amylase and glucoamylase formation by *Clostridium acetobutylicum* SA-1. Ind. Microbiol. **1**:63–67.
8. **Clementi, F., J. Rossi, L. Costamagna, and J. Rosi.** 1980. Production of amylase(s) by *Schwanniomyces castelli* and *Endomycopsis fibuligera*. Antonie van Leeuwenhoek J. Microbiol. Serol. **46**:399–405.
9. **El Kanouni, A., M. Junelles, R. Janati-Idrissi, H. Petitedemange, and R. Gay.** 1989. *Clostridium acetobutylicum* mutants isolated for resistance to the pyruvate halogen analogs. Curr. Microbiol. **18**:139–144.
10. **Ensley, B., J. J. McHugh, and L. L. Barton.** 1975. Effect of carbon sources on formation of alpha-amylase and glucoamylase by *Clostridium acetobutylicum*. J. Gen. Appl. Microbiol. **21**:51–59.
11. **Fogarty, W. M., and C. T. Kelly.** 1980. Amylases. amyloglucosidases and related gluconases, p. 115–170. *In* A. H. Rose (ed.), Economic microbiology—microbial enzymes and bioconversions, vol. 5. Academic Press, Inc., New York.
12. **Hockenhull, D. J. D., and D. Herbert.** 1945. The amylase and maltase of *Clostridium acetobutylicum*. Biochem. J. **39**:102–106.

13. **Hyun, H. H., and J. G. Zeikus.** 1985. Regulation and genetic enhancement of β-amylase production in *Clostridium thermosulfurogenes*. J. Bacteriol. **164**:1162–1170.
14. **Ingle, M. B., and R. J. Erickson.** 1978. Bacterial α-amylase. Adv. Appl. Microbiol. **24**:257–278.
15. **Jones, D. T., and D. R. Woods.** 1988. Genetic manipulation of *Clostridium acetobutylicum* and other species of *Clostridium*, p. 255–276. *In* J. A. Thomson (ed.), Recombinant DNA and bacterial fermentation. CRC Press, Inc., Boca Raton, Fla.
16. **Lemmel, S. A.** 1985. Mutagenesis in *Clostridium acetobutylicum*. Biotechnol. Lett. **7**:711–716.
17. **Lin, Y.-L., and H. P. Blaschek.** 1983. Butanol production by a butanol-tolerant strain of *Clostridium acetobutylicum* in extruded corn broth. Appl. Environ. Microbiol. **45**:966–973.
18. **Linden, J. C., and R. H. Kuhn.** 1989. Biochemistry of alcohol effects on clostridia, p. 271–291. *In* N. Van Uden (ed.), Alcohol toxicity in yeasts and bacteria. CRC Press, Inc., Boca Raton, Fla.
19. **Linden, J. C., A. R. Moreira, and T. G. Lenz.** 1986. Acetone and butanol, p. 915–931. *In* M. Y. Murray (ed.), Comprehensive biotechnology, vol. 3. Pergamon Press, Inc., Elmsford, N.Y.
20. **Lovitt, R. W., B. H. Kim, G.-J. Shen, and J. G. Zeikus.** 1988. Solvent production by microorganisms. Crit. Rev. Biotechnol. **7**:107–185.
21. **Maddox, I. S.** 1980. Production of *n*-butanol from whey filtrate using *Clostridium acetobutylicum* NCIB 2951. Biotechnol. Lett. **2**:493–498.
22. **Madi, E., G. Antranikian, K. Ohmiya, and G. Gottschalk.** 1987. Thermostable amylolytic enzymes from a new *Clostridium* isolate. Appl. Environ. Microbiol. **53**:1661–1667.
23. **Marlatt, J. A., and R. Datta.** 1986. Acetone-butanol fermentation process development and economic evaluation. Biotechnol. Prog. **2**:23–28.
24. **Melasniemi, H.** 1987. Effect of carbon source on production of thermostable α-amylase, pullulanase and α-glucosidase by *Clostridium thermohydrosulfuricum*. J. Gen. Microbiol. **133**:883–890.
25. **Mitchell, W. J., and I. R. Booth.** 1984. Characterization of the *Clostridium pasteurianum* phosphotransferase. J. Gen. Microbiol. **130**:2193–2200.
26. **Priest, F. G.** 1987. Regulation of extracellular enzyme synthesis in bacilli, p. 3–12. *In* J. Chaloupka, and V. Krumphanzl (ed.), Extracellular enzymes of microorganisms. Plenum Publishing Corp., New York.
27. **Priest, F. G.** 1977. Extracellular enzyme synthesis in the genus *Bacillus*. Bacteriol. Rev. **41**:711–753.
28. **Rogers, P.** 1986. Genetics and biochemistry of *Clostridium* relevant to development of fermentation process. Adv. Appl. Microbiol. **31**:1–60.
29. **Saier, M. H.** 1989. Protein phosphorylation and allosteric control of inducer exclusion and catabolite repression by the bacterial phosphoenolpyruvate:sugar phosphotransferase system. Microbiol. Rev. **53**:109–120.
30. **Weickert, M. J., and G. H. Chambliss.** 1990. Site-directed mutagenesis of a catabolite repression operator sequence in *Bacillus subtilis*. Proc. Natl. Acad. Sci. USA **87**:6238–6242.