APPLIED AND ENVIRONMENTAL MICROBIOLOGY, June 1976, p. 807-812 Vol. 31, No. 6
 Printed in U.S.A.

Production of Extracellular α-Glucosidase by a Thermophilic *Bacillus* Species

YUZURU SUZUKI,* TAKASHI KISHIGAMI, AND SHIGEO ABE

Laboratory of Microbial Physiology and Applied Microbiology, Department of Agricultural Chemistry, Kyoto Prefectural University, Shimogamo, Sakyo-ku, Kyoto 606, Japan

Received for publication 8 December 1975

Production of extracellular α-glucosidase was studied with strain KP 1006 of a new species of thermophilic *Bacillus*, which was isolated from soil samples by enrichment at 65 C. α-Glucosidase production was maximum at 60 C and at an initial pH of 6.5. The final enzyme yield was increased by starch, maltose, glycerol, peptone, and yeast extract but reduced by acetate and gluconate. α-Glucosidase was formed in the cytoplasm and accumulated as a large pool during the logarithmic growth phase. At a midpoint of this period, the enzyme appeared in the culture broth, and its level increased until the end of the stationary phase.

Although a number of studies have been performed on α-glucosidase (α-D-glucoside glucohydrolase, EC 3.2.1.20) derived from various origins, only a few reports on bacterial α-glucosidase have been presented (1, 14, 18). These studies have been restricted to α-glucosidase from mesophilic organisms and have not yet been made on the enzyme of thermophilic microbes. Furthermore, it has never been demonstrated that α-glucosidase can be released outside of the cell. In the present report, we describe a method for isolating, from soil samples, thermophilic bacteria capable of producing extracellular α-glucosidase. We have found that these isolates are closely related to *Bacillus stearothermophilus*. However, they differ from the latter organism in several morphological and physiological characteristics, particularly higher productivity of α-glucosidase. In this paper, representative strain KP 1006 is investigated regarding factors affecting α-glucosidase production, such as initial pH of the medium, temperature and time of cultivation, carbohydrates, and complex organic nutrients.

MATERIALS AND METHODS

Isolation of amylolytic thermophiles. All media were sterilized by autoclaving at 15 lb/in² for 15 min. Cultivation in screening of thermophilic microorganisms was made at 65 C for 18 h. The 330 soil samples (each about 0.1 g) collected in the vicinity of Shimogamo, Kyoto, were added to test tubes (1.8 cm in diameter by 19 cm in length) containing 5-ml amounts of medium I, which was composed of 0.5% peptone (Mikuni Chemical Industries, Ltd., Tokyo; percent = grams per 100 ml of solution), 0.3% meat extract (Mikuni), 0.3% yeast extract (Oriental Yeast Industries, Ltd., Tokyo), 0.3% K_2HPO_4, and 0.1% KH_2PO_4, and the pH was adjusted to 7.0 with 2 N NaOH before autoclaving. The tubes were incubated leaned at an angle of about 10°. Aliquots (0.5 ml) of the heavily turbid cultures were transferred into the tubes containing 5-ml amounts of medium I and were cultivated. The 0.5-ml portions of the cultures were submitted for recultivation on the same media. Pure cultures were isolated by streaking samples of the last enrichments on plates of medium I solidified with 3% agar. The colonies were restreaked after grown on medium I. The 523 isolates obtained were inoculated on the agar plates supplemented with soluble starch (Nakarai Chemicals Ltd., Kyoto). An iodine solution (0.2% I_2 in 2% KI) was poured on the plates after cultivation, and the 131 amylolytic strains with starch digestion zones around their colonies were selected.

The *Bacillus* species. The 131 amylolytic isolates were tested for ability to yield extracellular α-glucosidase after growth on medium III (see below). It was found that the six strains, KP 1006, KP 1012, KP 1013, KP 1014, KP 1019, and KP 1022, produced higher levels of α-glucosidase. These strains were aerobic, sporeforming, motile, amylolytic, thermophilic, long rod-shaped bacteria forming racket-shaped sporangia, which have been assigned to the microbes closely related to *B. stearothermophilus* (4, 5). However, these isolates were found to differ from the latter bacterium by the following set of characteristics: (i) light-brown, opaque colonies, including longer rod-shaped vegetative cells, 0.5 to 1.0 by 3.2 to 7.0 μm, after growth at 55 C for 18 h on bouillon agar slants; (ii) narrow pH and temperature ranges required for growth, from pH 6.0 to 8.5 and from 45 to 65 C; (iii) inability to grow in the presence of 2% NaCl and in the anaerobic medium containing 1% glucose; (iv) no production of acid from arabinose; and (v) higher potential to yield extracellular α-glucosidase (Table 1). On the basis of the above criteria, these strains were regarded as a new species (Y. Suzuki, T. Kishigami, and S. Abe, Proc.

TABLE 1. *Production of extracellular α-glucosidase by various strains of B. stearothermophilus (ATCC 7953-21365) and Bacillus strains KP 1006-1022*

Strain no.	α-Glucosidase (units/ml)
ATCC 7953	531
ATCC 8005	88
ATCC 10149	388
ATCC 12016	194
ATCC 12976	425
ATCC 12977	50
ATCC 12978	63
ATCC 12980	344
ATCC 15951	113
ATCC 15952	38
ATCC 21365	0
KP 1006	894
KP 1012	1,020
KP 1013	1,440
KP 1014	1,290
KP 1019	1,050
KP 1022	956

Annu. Meet. Jpn. Agric. Chem. Soc., p. 444, 1975). A taxonomic description will be published elsewhere.

Maintenance. Thermophilic bacteria with amylolytic activity were grown at 55 C for 15 h on 3% agar slopes of medium I with 1% soluble starch (referred to as medium II). Cultures were stored at 4 C as well as at −20 C and under lyophilization.

Fermentation experiments. Standard culture medium, medium III, contained 0.5% soluble starch, 2.5% peptone, 0.2% meat extract, 0.3% yeast extract, 0.3% K_2HPO_4, 0.1% KH_2PO_4, and distilled water, which was adjusted by 2 N NaOH to pH 7.0 before autoclaving. Cells grown on a fresh slant of medium II at 55 C for 15 h were suspended in 5 ml of 0.85% NaCl, followed by mixing. The suspension (0.5 ml) was inoculated to an absorbancy of 0.05 to 0.08 in L-shaped tubes (1.8 cm in diameter, 15.1 and 8.9 cm in length for the respective horizontal and vertical parts), each containing 5 ml of medium III. The tubes were shaken at 60 C for 15 h at 55 oscillations/min with an L-shaped tube shaker (Kyoto Rikagaku-kikai Co., Ltd., Kyoto) attached to a controlled thermobath. The tap water was sealed in the shaker bath by a liquid paraffin layer (0.4 cm) to avoid water evaporation. Growth was monitored by reading the absorbance increase at 660 nm in the L-tubes with a photoelectric colorimeter (model ANA-74, Tokyo Photoelectric Co., Ltd.). After cultures were centrifuged at 2,800 rpm with a Kokusan tube centrifuger (model H-103, Kokusan Enshinki Co., Ltd., Tokyo), the supernatant fluids were assayed for α-glucosidase activity, and final pH values of the fluids were measured. Dry cell weight was estimated from a standard curve, which correlated absorbance at 660 nm to weight of cells dried at 80 C for 25 h.

α-Glucosidase assay. *p*-Nitrophenol released from *p*-nitrophenyl-α-D-glucopyranoside by the action of α-glucosidase was determined photometrically with a Shimazu double-beam spectrophotometer (model UV-200). The reaction mixture (1 ml) in a 1.0-cm-lightpath cell contained: 33.3 μmol of potassium phosphate (pH 6.8), 2 μmol of *p*-nitrophenyl-α-D-glucopyranoside, and enzyme preparation (0.1 ml). After 0.1 ml of the substrate solution was mixed with 0.9 ml of the complete medium minus the substrate, the reaction proceeded at 60 C for 1 to 3 min. The increase in absorbance at 400 nm due to production of *p*-nitrophenol was recorded against the control from which the substrate was omitted. The velocity of the initial reaction was determined from the slope of the record progressing under linearity. A molar extinction of 9.6×10^3 at pH 6.8 was used in calculating the amount of product yielded (7). One unit of enzyme activity is defined as the amount of enzyme needed for hydrolysis of 1 nmol of the substrate per min under the conditions used.

Intracellular and extracellular activities of α-glucosidase. After a defined period of cultivation, the culture (5 ml of medium III) was centrifuged at 10,000 rpm for 15 min at 0 C with a refrigerated centrifuger (model RS-18GL; Tomy Seiko Co., Ltd., Tokyo), followed by three washes of the cells with 5-ml portions of 0.85% NaCl. The supernatant solution was mixed with the washing to determine α-glucosidase activity present outside the cell. The cell paste suspended in 5 ml of 66.6 mM potassium phosphate (pH 6.8) was subjected to disintegration under 19,000 cycles/s at 0 C for 5 min with an ultrasonic oscillator (model T-A-42; Kaijo Denki Co., Ltd., Tokyo), after which the suspension was centrifuged. The cell debris collected was washed three times with the same buffer and dispersed in 5 ml of the buffer. The cell-free extract and the washings were combined and analyzed for enzyme activity, which revealed the activity to be located in the cytoplasm. α-Glucosidase activity associated with the cell debris was determined according to the method described above, except that after incubation for 5 min the reaction medium was immediately centrifuged at 8,000 rpm for 10 min at 0 C and then the increase in absorbance at 400 nm was measured. The enzyme activity indicated in Fig. 4 is expressed as the total amount in each fraction.

RESULTS

Effect of temperature on extracellular α-glucosidase formation. Strain KP 1006 was able to grow at 45 to 65 C in medium III but not at 40 and 70 C (Fig. 1). The final yields of cell and α-glucosidase were maximum at 60 C. However, α-glucosidase production was not parallel with growth. There was a sharp peak in the enzyme yield at 60 C, with an abrupt fall at higher and lower temperatures. Although a 74% of the maximal growth was obtained at 50 C, the enzyme yield exhibited only 21% of the highest value. A detectable amount of α-glucosidase was not formed at 45 C.

Effect of initial pH on extracellular α-glucosidase formation. Strain KP 1006 grew well and produced α-glucosidase effectively only

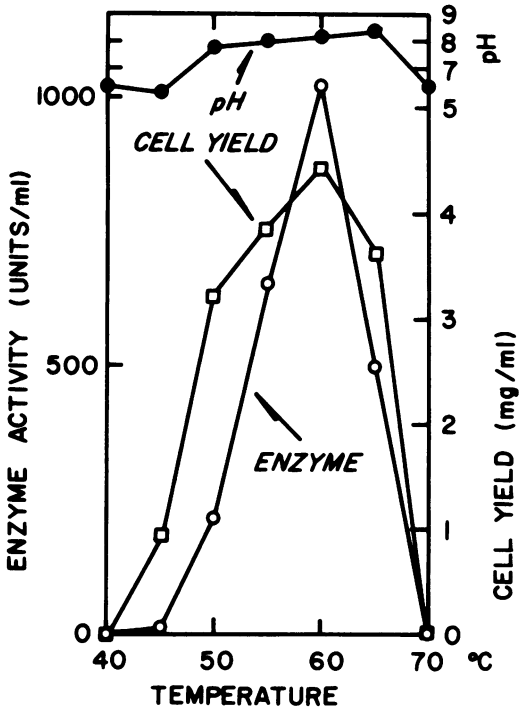

FIG. 1. *Effect of temperature on growth and production of α-glucosidase by Bacillus strain KP 1006 and change of final pH of culture medium. Symbols: (○) α-glucosidase activity; (□) cell yield; (●) final pH.*

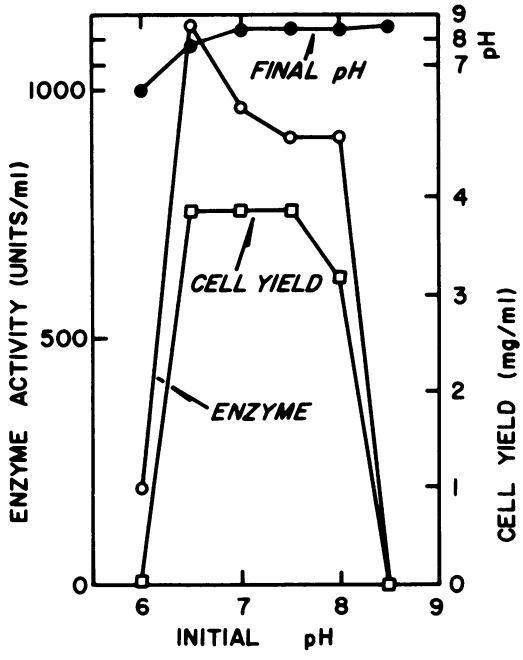

FIG. 2. *Effect of initial pH of culture medium on growth and production of α-glucosidase by Bacillus strain KP 1006 and change of final pH. Initial pH of the medium was varied with 2 N NaOH or 2 N HCl. Symbols: (○) α-glucosidase activity; (□) cell yield; (●) final pH.*

when the organism was inoculated in medium III having initial pH values between 6.5 and 8.0 (Fig. 2). Cell yields were similar, within the pH range from 6.5 to 7.5. Enzyme production was optimum at an initial pH of 6.5 and diminished slowly as the pH rose to 8.0.

Effect of carbohydrates on extracellular α-glucosidase formation. Strain KP 1006 was allowed to grow on medium III, in which starch was replaced with one of the various carbohydrates. Acetate, gluconate, and tartarate, each at 0.6%, depressed the final amounts of α-glucosidase production, whereas glycerol and starch increased the enzyme yields as their concentrations increased, respectively, to 1.2 and 1.5% (Table 2). These compounds failed to prevent growth within the levels tested. The effects of these substances are made clearer when the quantity of enzyme formed is expressed per unit of cell weight. Acetate, gluconate, and tartarate gave lower specific enzyme yields, whereas starch and glycerol gave higher values, compared with that shown in the absence of these substances (Table 2). An increase in α-glucosidase production took place by fructose,

TABLE 2. *Effect of carbon sources on production of extracellular α-glucosidase by Bacillus strain KP 1006*

Carbon source	%	α-Glucosidase (units/ml)	Cell yield (mg/ml)	α-Glucosidase/cell yield (units/mg)	Final pH
None		258	1.81	142	8.4
Acetate	0.2	196	1.92	102	8.4
	0.6	90.3	2.02	44.7	8.4
Gluconate	0.2	232	1.81	128	8.4
	0.6	142	2.18	50.5	8.4
Tartarate	0.2	263	1.85	142	8.3
	0.6	212	1.81	117	8.3
Fructose	0.2	317	1.70	186	8.3
	0.6	157	0.42	374	5.6
Sucrose	0.2	325	1.81	180	8.4
	0.6	121	0.26	465	5.5
Glucose	0.2	255	2.18	117	8.3
	0.6	59.3	0.26	228	5.6
Maltose	0.2	454	1.81	251	8.4
	0.4	330	0.38	868	5.6
	0.6	253	0.38	666	5.6
Glycerol	0.2	292	2.06	142	8.5
	0.6	356	2.13	167	8.0
	1.2	459	2.06	223	6.4
Starch	0.5	815	2.30	354	8.4
	1.0	1,020	2.88	354	8.4
	1.5	759	2.15	353	5.4

sugars did not block growth. The same content of glucose did not inhibit growth and α-glucosidase formation. On the contrary, the cell and enzyme yields were strongly reduced by elevation of the sugar levels to 0.6%. Both reductions were not parallel, since the high values of specific enzyme yield were obtained, respectively, with fructose, sucrose, maltose, and glucose (Table 2). Among the carbohydrates tested, the highest production of α-glucosidase was achieved by starch at 1%.

Effect of complex organic nutrients on extracellular α-glucosidase formation. Both peptone and yeast extract were more effective than meat extract for α-glucosidase production by strain KP 1006, although a full complement of these nutrients was needed for optimal enzyme formation (Table 3). When either peptone or yeast extract was omitted from medium III, the final enzyme yield was decreased by 66 to 74%, but inhibition of growth was only 29 to 38%. In this case, the quantity of enzyme formed per unit of cell mass was extremely depressed. Removal of meat extract induced 18 and 19% reductions, respectively, in the yields of cell and enzyme, without affecting the specific enzyme yield. Figure 3 reveals the effect of peptone level on growth and α-glucosidase production. Peptone selectively promoted the enzyme production, which was maximum at 2.5% peptone when the medium contained 0.5% starch. The cell yield was almost constant at 0.5 to 3.0% peptone, and a small increase in the yield was observed at a level up to 0.5%. When starch was increased to 1.0%, demand of peptone for the optimal enzyme formation was diminished to 1.5%.

Change of extracellular and intracellular levels of α-glucosidase during cultivation. Strain KP 1006 continued in logarithmic

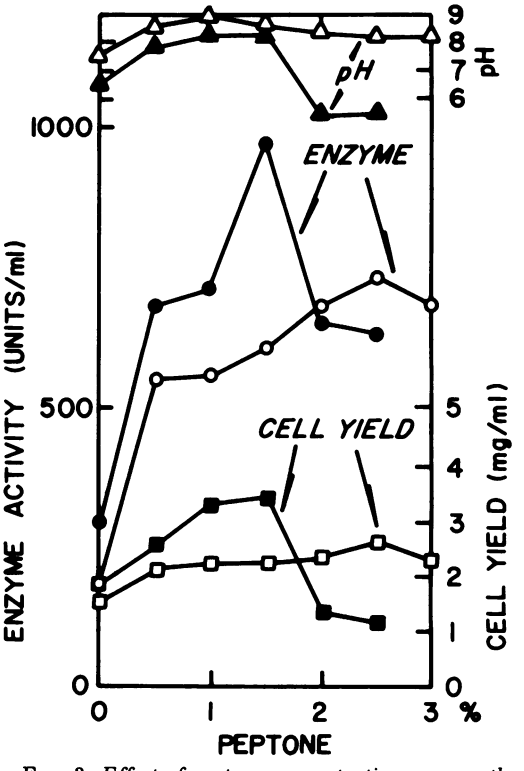

FIG. 3. *Effect of peptone concentration on growth and formation of α-glucosidase by Bacillus strain KP 1006 in the presence of 0.5 or 1.0% starch and change of final pH of culture. The medium (pH 7.0) contained 0.3% K_2HPO_4, 0.1% KH_2PO_4, 0.3% yeast extract, 0.2% meat extract, 0.5 or 1.0% starch, and varied amounts of peptone. Final pH, growth, and α-glucosidase activity are indicated, respectively, with the symbols (△), (□), and (○) when the culture contained 0.5% starch, or with the symbols (▲), (■), and (●) when 1.0% starch was present.*

TABLE 3. *Effect of complex nutrients on formation of extracellular α-glucosidase by Bacillus strain KP 1006*

Medium no.	Additions[a]			α-Glucosidase (units/ml)	Cell yield (mg/ml)	α-Glucosidase/cell yield (units/mg)
	Peptone (2.5%)	Yeast extract (0.3%)	Meat extract (0.2%)			
1	+	+	+	730	2.44	299
2	+	+	−	599	1.98	303
3	+	−	+	248	1.74	143
4	+	−	−	219	1.24	177
5	−	+	+	190	1.50	127

[a] The medium (pH 7.0) contained 0.5% starch, 0.3% K_2HPO_4, 0.1% KH_2PO_4, and the above nutrients.

sucrose, and maltose, each at 0.2%, but these growth for the first 12 h of cultivation in medium II (Fig. 4). After this time, a stationary phase was initiated and continued for 12 h, after which lysis of the cell began. α-Glucosidase formation progressed quickly in cytoplasm during the logarithmic phase and reached a maximum at 8-h cultivation, 4 h before onset of the stationary phase. However, a sudden decrease in the enzyme level occurred within 4 h after the maximal level was reached and then the level decreased in a linear fashion. α-Glucosidase appeared in the culture broth at 4-h cultivation. This time was the midpoint of the logarithmic phase. The enzyme accumulated continuously throughout the remainder of cultivation. Maximal accumulation was achieved at the end of this period. α-Glucosidase associated with cell debris obtained by disintegration

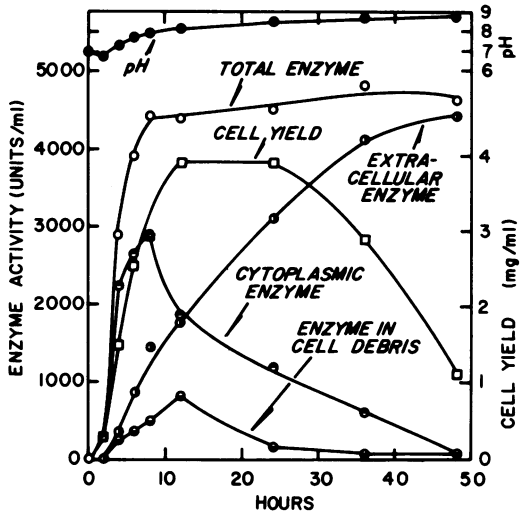

FIG. 4. *Change of respective α-glucosidase levels present in extracellular, cytoplasmic, and cell debris fractions during cultivation of Bacillus strain KP 1006 and change of cell yield and pH of culture medium. Total activity of α-glucosidase (○) is revealed as the sum of the enzyme activities present in extracellular (◑), cytoplasmic (◒), and cell debris (◒) fractions. Symbols: (□), cell yield; (●), pH of culture.*

rose until 12-h cultivation. After this point, the activity decreased rapidly to a negligible amount during the 12 h of stationary phase. The enzyme activities present in cytoplasm, culture broth, and cell debris fractions increased parallel with growth, but their accumulation stopped approximately when growth ceased. The sum of the enzyme levels was constant during the stationary phase.

DISCUSSION

Recently, we have confirmed that strain KP 1006 contains a single α-glucosidase, which is responsible for hydrolysis of *p*-nitrophenyl-α-D-glucopyranoside. The highly purified enzyme (molecular weight, 55,000) is quite stable at 30 to 60 C. Inactivation emerges slowly at temperatures above 60 C, and the activity is completely destroyed at 72 C (Y. Suzuki, T. Yuki, T. Kishigami, and S. Abe, Proc. Annu. Meet. Jpn. Agric. Chem. Soc., p. 445, 1975). Such an instability could have an immediate effect on the final quantity of enzyme produced during fermentation. Indeed, as shown in the present work, the enzyme yield is suddenly reduced at higher temperatures than 60 C. It has been observed that the purified enzyme fails to undergo inactivation at 60 C over pH 6.0 to 8.5. This pH range is wider than the range to which α-glucosidase production is restricted. Strain KP 1006 produces α-glucosidase well at an initial pH between 6.5 and 8.0, and the final pH of the culture is 7.7 to 8.2 after cultivation (Fig. 2).

The present investigation shows that α-glucosidase production by the strain is strongly stimulated by maltose and starch, similar to α-amylase synthesis (8–11, 16). Welker and Campbell have suggested that maltodextrins (maltotriose to maltohexose) are preferable as direct inducers of α-amylase of *B. stearothermophilus* (17). They have provided evidence that the inductive effect by maltose and starch is due to these maltodextrins, which are contaminated as impurities in technical-grade maltose or formed from starch during culture. The purified α-glucosidase of strain KP 1006 has been found to resemble yeast isomaltase (6, 15). Isomaltase is inducible specifically by methyl-α-D-glucopyranoside in *Saccharomyces cerevisiae* (15), but this substance is not a natural product.

α-Glucosidase of strain KP 1006 accumulates in medium throughout the logarithmic and stationary phases of growth, which is like the secretion pattern of α-amylase in *B. stearothermophilus* and in a mutant of *Bacillus licheniformis* (11, 16). However, a striking distinction is present. A preformed pool of α-glucosidase exists during the logarithmic phase in cytoplasm of the former microbe, whereas such a pool of α-amylase fails to be detected in the latter bacillus. α-Amylase secretion is simultaneously coupled with de novo protein synthesis, which seems to be a common aspect of exoenzyme production (2, 3, 12, 13). It is possible that α-glucosidase might appear in the culture broth as a result of cell lysis.

ACKNOWLEDGMENT

We are deeply indebted to H. Samejima, Director of Tokyo Research Laboratories, Kyowa Hakko Kogyo Co., Ltd., for his kindness in supplying strains of *B. stearothermophilus*. We are grateful to F. Masui for her skillful technical assistance in isolating thermophilic bacteria.

LITERATURE CITED

1. **Amemura, A., T. Sugimoto, and T. Harada.** 1974. Characterization of intracellular α-glucosidase of *Pseudomonas* SB 15. J. Ferment. Technol. 52:778–780.
2. **Boethling, R. S.** 1975. Regulation of extracellular protease secretion in *Pseudomonas maltophila*. J. Bacteriol. 123:954–961.
3. **Eisenstadt, J. M., and H. P. Klein.** 1961. Evidence for the de novo synthesis of the α-amylase of *Pseudomonas saccharophila*. J. Bacteriol. 82:798–807.
4. **Gibson, T., and R. E. Gordon.** 1974. Description of the species of genus *Bacillus*, p. 531–550. *In* R. E. Buchanam and N. E. Gibbons (ed.), Bergey's manual of determinative bacteriology, 8th ed. The Williams & Wilkins Co., Baltimore.

5. **Gordon, R. E.** 1973. The genus *Bacillus*, p. 71-88. *In* A. I. Laskin and H. A. Lechevalier (ed.), Handbook of microbiology, vol. 1. The Chemical Rubber Co., Cleveland.
6. **Gorman, J., and H. Halvorson.** 1966. Isomaltase from yeast, p. 562-565. *In* E. F. Neufeld and V. Ginsburg (ed.), Methods in enzymology, vol. 8. Academic Press Inc., New York.
7. **Halvorson, H., and L. Ellias.** 1958. The purification and properties of an α-glucosidase of *Saccharomyces italicus* Y1225. Biochim. Biophys. Acta 30:28-40.
8. **Kuo, M. J., and P. A. Hartman.** 1966. Isolation of amylolytic strains of *Thermoactinomyces vulgaris* and production of thermophilic actinomycete amylase. J. Bacteriol. 92:723-726.
9. **Markovitz, A., and H. P. Klein.** 1955. Some aspects of the induced biosynthesis of alpha-amylase of *Pseudomonas saccharophila*. J. Bacteriol. 70:641-648.
10. **Markovitz, A., and H. P. Klein.** 1955. On the sources of carbon for the induced biosynthesis of alpha-amylase of *Pseudomonas saccharophila*. J. Bacteriol. 70:649-655.
11. **Saito, N., and K. Yamamoto.** 1975. Regulatory factors affecting α-amylase production in *Bacillus licheniformis*. J. Bacteriol. 121:848-856.
12. **Schaeffer, P.** 1969. Sporulation and the production of antibiotics, exoenzymes, and exotoxins. Bacteriol. Rev. 33:48-71.
13. **Stinson, M. W., and J. M. Merrick.** 1974. Extracellular enzyme secretion of *Pseudomonas lemoignei*. J. Bacteriol. 119:152-161.
14. **Sugimoto, T., A. Amemura, and T. Harada.** 1974. Formation of extracellular isoamylase and intracellular α-glucosidase and amylase(s) by *Pseudomonas* SB15 and a mutant strain. Appl. Microbiol. 28:336-339.
15. **Terui, G., H. Okada, and Y. Oshima.** 1959. Studies on the correlation of alpha-glucosidase formation with genotype composition in *Saccharomyces* (I). Technol. Rep. Osaka Univ. 9:237-259.
16. **Welker, N. E., and L. L. Campbell.** 1963. Effect of carbon source on formation of α-amylase by *Bacillus stearothermophilus*. J. Bacteriol. 86:681-686.
17. **Welker, N. E., and L. L. Campbell.** 1963. Induction of α-amylase of *Bacillus stearothermophilus* by maltodextrine. J. Bacteriol. 86:687-691.
18. **Yamasaki, Y., and Y. Suzuki.** 1974. Purification and properties of α-glucosidase from *Bacillus cereus*. Agric. Biol. Chem. 38:443-454.