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Production of an Extracellular Maltase by Thermophilic *Bacillus* sp. KP 1035

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Production of extracellular maltase was studied with thermophilic *Bacillus* sp. KP 1035, which was selected as the organism producing the highest levels of maltase. The final enzyme yield was increased by maltose, peptone, and yeast extract but reduced by succinate and fumarate. Maximum enzyme production was achieved at 55°C and at an initial pH of 6.2 to 7.0 on a medium containing 0.3% maltose, 1% peptone, 0.1% meat extract, 0.3% yeast extract, 0.3% K_2HPO_4, and 0.1% KH_2PO_4. Maltase was synthesized in cytoplasm and accumulated as a large pool during the logarithmic growth phase, which preceded sporulation. At the end of this phase, the enzyme appeared in the culture broth, and its accumulation increased in parallel with a rise in the extracellular protein level. Maltase was stable for 24 h at 60°C over a pH range of 5.6 to 9.0 and retained 95% of the original activity after treatment for 20 min at 70°C at pH 6.8.

Production of extracellular α-glucosidase responsible for hydrolysis of *p*-nitrophenyl-α-D-glucopyranoside has been previously studied with strain KP 1006 of a new species of thermophilic *Bacillus* (10; Y. Suzuki, T. Kishigami, and S. Abe, Proc. Annu. Meet. Jpn. Agric. Chem. Soc., p. 444, 1975). The purified enzyme has been found to be extraordinarily thermostable and to resemble yeast isomaltase in its substrate specificity (Suzuki et al., Proc. Annu. Meet. Jpn. Agric. Chem. Soc., p. 445, 1975; Y. Suzuki, T. Kishigami, and S. Abe, Proc. Annu. Meet. Jpn. Agric. Chem. Soc., p. 30, 1976). Maltose is not entirely hydrolyzed by the enzyme. Very recently, formation of an extracellular maltase by *Bacillus subtilis* P-11 was demonstrated (12). However, the maltase accumulation is very low. Any available information has never been presented on maltase of thermophilic bacteria. In the present report, 48 thermophilic isolates from soil were screened for productivity of extracellular maltase. Of these isolates, two strains, KP 1035 and KP 1036, produced the most maltase; these have been identified as the strains of thermophilic *Bacillus* (1, 2; Y. Suzuki, T. Tsuji, T. Ikemoto, and S. Abe, Proc. Annu. Meet. Jpn. Agric. Chem. Soc., p. 239, 1976). In this paper, studies on the effects of organic complex nutrients, carbon sources, initial pH of the medium, and temperature of cultivation on maltase production are presented. Also, the relationship between maltase accumulation and sporulation is studied. It was found that the enzyme is extremely thermostable.

MATERIALS AND METHODS

Organisms. Screening tests were conducted on 48 thermophilic isolates from soil (10) and 12 strains of *Bacillus stearothermophilus*, all from the collection in this laboratory. Maintenance of these organisms has been described (10).

Media. Agar slant (8 ml/tube, 1.8 cm in diameter by 19 cm in length) contained 1% soluble starch (Nakarai Chemicals, Ltd., Kyoto; percent = grams per 100 ml of solution), 0.5% peptone (Mikuni Chemical Industries, Ltd., Tokyo), 0.3% meat extract (Mikuni), 0.3% yeast extract (Oriental Yeast Industries, Ltd., Tokyo), 0.3% K_2HPO_4, 0.1% KH_2PO_4, 3% agar, and distilled water. Medium I, used for screening tests, consisted of 0.5% maltose (Nakarai), 2.5% peptone, 0.2% meat extract, 0.3% yeast extract, 0.3% K_2HPO_4, 0.1% KH_2PO_4, and distilled water. Medium II, used in fermentation experiments, had the same nutrients as medium I, except that the concentrations of maltose, peptone, and meat extract were 0.3, 1, and 0.1%, respectively. Unless otherwise stated, the pH of all media used was adjusted to 7.0 with 2 N NaOH before autoclaving at 15 lb/in² for 15 min.

Fermentation experiments. Cells grown on a fresh slant 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 0.04 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 I or medium II. The tubes were shaken at 60°C for 24 h at 56 oscillations/min with an L-shaped tube shaker, as described previously (10). Growth was followed by measuring the absorbance increase at 660 nm in the L-tubes with a photoelectric colorimeter (model ANA-74, Tokyo Photoelectric Co., Ltd.). Cultures were centrifuged at 15,000 rpm with a refrigerated

centrifuge (model RS-18GL, Tomy Seiko Co., Ltd., Tokyo). The supernatant solutions were assayed for enzyme activity, and final pH values of the solutions were determined. Dry cell weight was estimated from a standard curve, which correlated absorbance at 660 nm with weight of cells dried at 83°C for 32 h.

Maltase assay. Glucose formed from maltose by the action of maltase was determined by using a Beckman oxygen electrode and glucose oxidase (7). The electrode detected O_2 consumed in glucose oxidation by glucose oxidase. The potential applied between a rhodium cathode and a silver anode via a polarographic circuit (Beckman 100800 oxygen analyzer) was 0.53 V. The maltase reaction proceeded for 10 min at 60°C in a medium (2 ml) containing 66.7 μmol of potassium phosphate (pH 6.8), 100 μmol of maltose (Nakarai), and enzyme preparation (0.2 ml of culture filtrate was diluted with 33.3 mM potassium phosphate [pH 6.8, 0.8 ml], and a 0.2-ml portion of the diluted solution was used). The reaction was stopped by heating at 95°C for 4 min. A 0.9-ml aliquot of the medium was mixed with 2 ml of 0.9 M tris(hydroxymethyl)aminomethane-hydrochloride (pH 7.6) and subsequently equilibrated with air at 30°C to allow O_2 dissolved in 230 μM in a vessel to which the electrode assembly was fitted. Glucose oxidase (0.1 ml, 1 mg of protein with activity of 14.7 U, dissolved in 1.5 M tris(hydroxymethyl)aminomethane-hydrochloride at pH 7.0; Sigma Chemical Co.) was added to the medium. A decrease in the O_2 level was recorded with a Shimazu recorder (model U-125MU). Glucose was assessed by calibrating the reciprocal of the initial velocity of O_2 consumption on a standard Lineweaver-Burk plot constructed with the known levels of glucose (0.06 to 1.5 mM). One unit of enzyme activity was defined as the amount of enzyme needed for the formation of 1 μmol of glucose per min at 60°C under the conditions used.

Intracellular and extracellular protein fractions and maltase activities. Cells were cultivated for a defined period (Fig. 5) on medium II (5 ml), and then the culture was centrifuged at 15,000 rpm for 15 min at 0°C, 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 protein and maltase activity present outside the cell. The cell paste suspended in 5 ml of 66.7 mM potassium phosphate (pH 6.8) was subjected to sonic oscillation under 19,000 cycles/s at 0°C for 5 min, using an ultrasonic oscillator (model T-A-42; Kaijo Denki Co., Ltd., Tokyo). The homogenate was centrifuged, and the cell debris collected was washed three times with the same buffer. The cell-free extract and the washings were combined and analyzed for enzyme activity and protein, which revealed the activity and protein to be located in the cytoplasm (10). The enzyme activity and protein indicated in Fig. 5 are expressed as the total amounts in each fraction. Protein was determined spectrophotometrically. One milliliter of the protein solution was vigorously mixed with 3 ml of 5% trichloroacetic acid. Protein was assessed from absorbance at 660 nm of the suspension with egg albumin as a standard (0.1 to 5 mg of protein per ml), using a Hitachi spectrophotometer (model 101).

Vegetative cells, sporangia, and free spores. Vegetative cells, sporangia, and free spores were counted with a hematometer after dilution of the culture with 0.85% NaCl. Their total numbers are presented in Fig. 5.

Heat treatment. The culture filtrate (1.41 mg of protein and 8.09 U of maltase activity per ml) was diluted fivefold with 33.3 mM potassium phosphate (pH 6.8). One-milliliter portions were incubated for 20 min or 24 h at 4, 55, 60, 65, 70, and 75°C, respectively, after which they were immediately cooled. The solutions were analyzed for enzyme activity recovered. The pH stability of the enzyme was examined as described above, except that the incubation was performed at 60°C for 24 h and the culture filtrate was diluted with the following buffers: McIlvain buffer (pH 3.0 to 8.5), 0.2 M borate buffer (pH 9.0), 0.2 M Na_2CO_3-$NaHCO_3$ (pH 9.7 and 10.1), and Ringer buffer (pH 10.8).

RESULTS

Selection of productive strains. It was found that among 48 thermophilic amylolytic isolates, 38 strains formed extracellular maltase after growth on medium I. However, only six strains (KP 1016, KP 1018, KP 1033, KP 1035, and KP 1036) yielded more than 10 U of maltase per ml of culture (or 1 U/mg of cell weight) (strains KP 1035 and KP 1036 gave the highest values, each 15.6 U/ml [2.06 U/mg of cell]). These organisms were compared by enzyme productivity with 12 strains (ATCC 7953, ATCC 8005, ATCC 10149, ATCC 12016, ATCC 12976, ATCC 12977, ATCC 12978, ATCC 12979, ATCC 12980, ATCC 15951, ATCC 15952, and ATCC 21365) of *B. stearothermophilus* and six strains (KP 1006, KP 1012, KP 1013, KP 1014, KP 1019, and KP 1022) of the thermophilic *Bacillus* capable of producing an extracellular α-glucosidase (10). Most of the strains of the latter two bacilli failed to yield the enzyme, although five strains (ATCC 8005, ATCC 12978, KP 1006, KP 1013, and KP 1022) produced 2.7 to 5.3 U of enzyme per ml (0.18 to 0.48 U/mg of cell).

Effect of peptone, meat extract, and yeast extract on extracellular maltase formation. Figure 1 shows the effects of peptone, meat extract, and yeast extract levels on growth and maltase production of strain KP 1035. Enzyme production was strongly dependent on peptone and yeast extract. Although the cell concentration rose as the peptone level increased, optimum enzyme production occurred at 0.5 to 1.0% peptone (Fig. 1A). The enzyme was not formed with 0 to 0.1% yeast extract, but its formation was enhanced by yeast extract above 0.1%. The maximum enzyme production was observed at 0.3% yeast extract (Fig. 1C). Final cell concentrations were almost similar at 0.05 to 0.3% yeast extract. Meat extract was less effective than peptone and yeast extract for maltase pro-

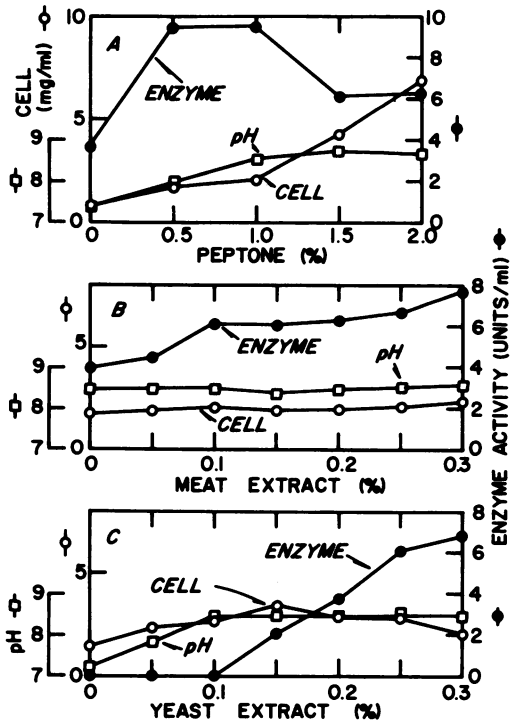

FIG. 1. *Effects of peptone (A), meat extract (B), and yeast extract (C) on growth and production of extracellular maltase by Bacillus sp. KP 1035, and change in final pH of the culture medium. Cells were grown on medium (pH 7.0) containing 0.3% maltose, 0.3% K_2HPO_4, 0.1% KH_2PO_4, peptone, meat extract, and yeast extract. In (A), the peptone level was varied from 0 to 2.0%, and meat extract and yeast extract levels were fixed at 0.1 and 0.3%, respectively; in (B), the meat extract concentration was changed from 0 to 0.3%, and peptone and yeast extract concentrations were maintained at 1 and 0.3%, respectively; in (C), the yeast extract level was varied from 0 to 0.3%, and the respective amounts of peptone and meat extract were fixed at 1 and 0.1%. Symbols: (●) Maltase activity; (○) cell concentration; (□) final pH.*

duction. When grown on the medium minus meat extract, the organism produced a considerably quantity of enzyme (Fig. 1B). The enzyme yield rose as meat extract increased to 0.1%, and the yield was constant at 0.1 to 0.2% meat extract. This nutrient did not block growth within the levels tested.

Effect of carbon sources on extracellular maltase formation. Strain KP 1035 was cultivated on medium II, in which maltose was replaced with one of various carbon sources such as glucose, fructose, sucrose, starch, dextrin, gluconate, acetate, citrate, succinate, fumarate, and glycerin. These carbon sources except citrate failed to inhibit growth at the levels tested (0.2, 0.5, and 1.0%), but maltase production was greatly affected by these substances. Maltose and dextrin increased the final amounts of enzyme production, whereas succinate, fumarate, and acetate depressed the production. These effects are clearer if the amount of enzyme formed is expressed per unit of cell weight. Maltose and dextrin (1%) gave higher specific enzyme yields, 2.04 and 0.634 U/mg of cell, respectively, whereas succinate, fumarate, and acetate (1%) gave respective lower values of 0.02, 0.023, and 0.025 U/mg of cell, compared with the value, 0.303 U/mg, shown in the absence of carbon source. Figure 2 shows the effect of maltose concentration on maltase synthesis. Maltose strongly stimulated synthesis, which was maximum at 0.3% maltose. Final cell concentrations were almost unchanged within 0 to 0.4% maltose.

Effect of initial pH on extracellular maltase formation. Strain KP 1035 grew well and produced maltase effectively only when the microbe was inoculated in medium II having initial pH values between 6.2 and 8.2 (Fig. 3). Enzyme production was optimum at pH 6.2 to 7.0. Cell concentrations were similar within the pH range of 6.2 to 8.4, and a small increase in concentration was observed at pH 6.6

Effect of temperature on extracellular maltase formation. Strain KP 1035 was able to grow at 40 to 70°C in medium II but not at 35 and 75°C (Fig. 4). Growth exhibited the highest value at 50°C, with a slow fall at higher and lower temperatures. Although 79% of the maximal growth was achieved at 40°C, a detectable amount of maltase was not formed. Enzyme production was maximum at 55°C and diminished slowly as the temperature rose to 65°C. The enzyme was not produced at 70°C.

Change of extracellular and cytoplasmic

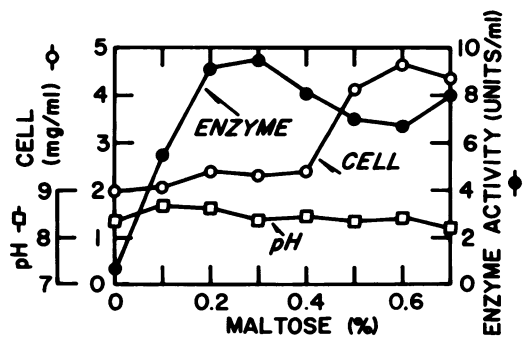

FIG. 2. *Effect of maltose on growth and production of extracellular maltase by Bacillus sp. KP 1035, and change in final pH of the culture medium. The medium had the same components as medium II, except that the maltose level was varied from 0 to 0.7%. Symbols are as in Fig. 1.*

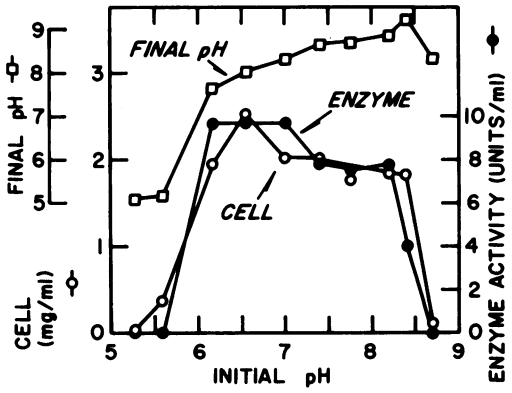

FIG. 3. *Effect of initial pH of the culture medium on growth and production of extracellular maltase by Bacillus sp. KP 1035, and change in final pH. The initial pH of the medium was varied with 2 N NaOH or 2 N HCl. Symbols are as in Fig. 1.*

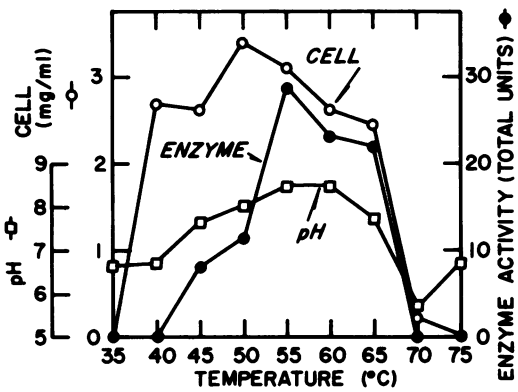

FIG. 4. *Effect of temperature on growth and production of extracellular maltase by Bacillus sp. KP 1035, and change in final pH of the culture medium. Symbols are as in Fig. 1.*

levels of maltase, and sporulation during fermentation. Vegetative cells of strain KP 1035 increased for the first 8 h of cultivation in medium II, after which the cell number fell rapidly for 16 h (Fig. 5C). Sporulation started at 6 h of cultivation. Sporangia (slightly swelled at terminals but not racket-shaped) increased until 24 h of culture, and a slow decrease in the number was observed after this point. Free spores appeared only after 12 h of incubation, and an abrupt increase took place during the following 12 h. Maltase synthesis quickly progressed in the cytoplasm during the logarithmic phase (Fig. 5B) and reached a maximum at 6 h of fermentation, 2 h before vegetative cells were maximal, and then the enzyme level fell in a linear fashion. The enzyme appeared in the culture broth at 4 h of cultivation. This point was the time when extracellular proteins were detected in the broth (Fig. 5A). The enzyme continuously accumulated until 36 h of cultivation, and maximal accumulation was achieved 12 h later. The extracellular protein level rose until 24 h of culture and was almost constant throughout the remainder of fermentation (Fig. 5A).

Stability of extracellular maltase. Stability of maltase was examined with culture filtrate. The enzyme was quite stable at 60°C for 24 h (Fig. 6). A rapid inactivation took place at temperatures higher than 70°C. After incubation for 20 min at 75°C, only 17% of the original activity was recovered. The enzyme retained 95% of the activity at 70°C for 20 min, whereas the activity recovered fell to 34% after 24 h. Maltase was fairly stable at 60°C for 24 h within

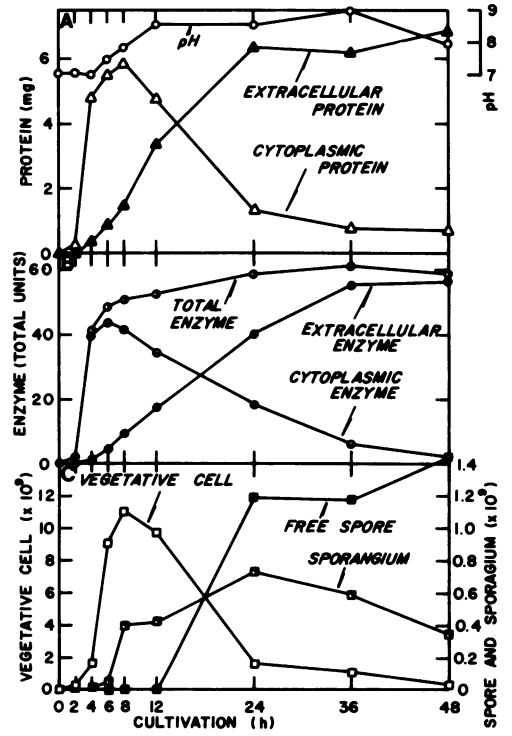

FIG. 5. *Change in protein (A) and maltase (B) levels present in extracellular and cytoplasmic fractions, and change in pH of the culture medium and numbers of vegetative cells, sporangia, and free spores (C) during cultivation of Bacillus sp. KP 1035. Total activity of maltase (◐) is revealed as the sum of the enzyme activities present in extracellular (●) and cytoplasmic (○) fractions. Symbols: (▲) Extracellular protein; (△) cytoplasmic protein; (□) vegetative cell; (■) free spore; (◪) sporangium; (○) pH of culture.*

a pH range of 5.6 to 9.0, but underwent a rapid inactivation below pH 5.6 and above pH 9.0 (Fig. 7).

DISCUSSION

Thermostable maltase is potentially useful for production of glucose from starch when used in combination with thermostable α-amylase. Such a maltase could be synthesized by thermophilic microbes. Our attempt has been to isolate thermophiles high in extracellular maltase production, since the advantage of extracellular enzyme is the ease of isolation and purification. In the present study we have selected bacteria suitable for this purpose, which have been characterized as strains of thermophilic *Bacillus* (1, 2). These strains are definitely distinguished in maltase productivity from *B. stearothermophilus* and the thermophilic *Bacillus* sp. characterized as a producer of an extracellular α-glucosidase (10). Most of the strains of these two bacilli groups tested failed to produce extracellular maltase, although several strains produced very small amounts of enzyme.

The present investigation shows that maltase production by *Bacillus* strain KP 1035 is stimulated by maltose, similar to isomaltase synthesis by *Bacillus* sp. KP 1006 (10). Simultaneous induction of both enzymes by maltose or methyl-α-D-glucoside has been demonstrated in yeast (5, 6, 8, 11). However, maltases from *Myxobacter* sp. (4) and *Pseudomonas fluorescens* (3) can be selectively induced by maltose

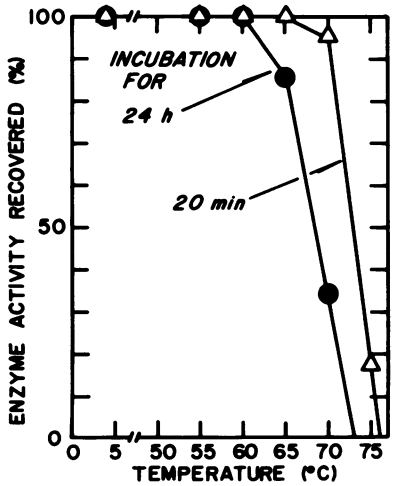

FIG. 6. *Effect of temperature on stability of maltase activity of Bacillus sp. KP 1035. The culture filtrate was incubated for 20 min (△) or 24 h (●) at varied temperatures. The activity observed before the incubation is expressed as 100%.*

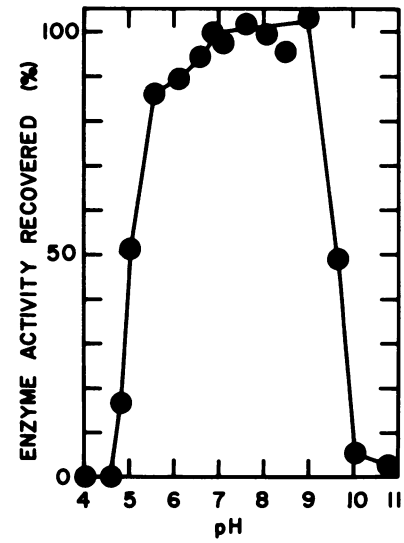

FIG. 7. *Effect of pH on stability of maltase activity of Bacillus sp. KP 1035. The culture filtrate was treated at various pH values for 24 h at 60°C. The activity found at pH 6.8 before the treatment is indicated as 100%.*

but not methylglucoside, whereas *B. subtilis* produces the enzyme when grown on a medium containing methylglucoside as sole carbon source (12). Maltase was not synthesized when strain KP 1035 was grown on fumarate and succinate, similar to production of *P. fluorescens* enzyme (3).

The enzymes responsible for hydrolysis of disaccharides are usually localized in the cytoplasm or tightly bound to the cell membrane. Recently, Wang and Hartman have shown production of an extracellular maltase by *B. subtilis* (12). However, it is not definite whether the enzyme appears as a result of cell lysis or is secreted like bacterial α-amylase (9). Maltase of strain KP 1035 exists as a large pool in cytoplasm during the logarithmic phase, before its appearance in the culture. Enzyme accumulation is nearly parallel with the increase in protein concentration in the broth, during which vegetative cells are reduced. It is possible that cell autolysis causes the enzyme accumulation. Maltase synthesis, however, might not be directly connected with sporulation, since the synthesis is completed before sporulation starts.

Maltase of strain KP 1035 is a thermostable protein. The enzyme is fairly stable at 60°C for 24 h over pH 5.6 to 9.0. When held at 70°C for 20 min, 95% of the initial activity remains. In contrast, most maltases of mesophiles undergo

a sudden inactivation at such elevated temperatures (12).

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