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Characterization and Some Reaction-Engineering Aspects of Thermostable Extracellular β-Galactosidase from a New *Bacillus* Species

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ABSTRACT. A new strain of *Bacillus* sp. was isolated from a hot water spring in India. This strain generated a high activity of extracellular β-galactosidase at 37 °C in shake flasks. The β-galactosidase activity was found to increase continuously but the production rate was slower than with some other organisms reported in the literature. There were noteworthy differences in the time-domain profiles of bacterial concentration and β-galactosidase activity when the starting concentration of substrate (glucose) was tripled from 10 g/L. These differences may be explained in terms of the relative rates of enzyme synthesis and its diffusion across the cell wall. The enzyme produced by this organism is more stable than other β-galactosidases; its half-life is 408 h at 50 °C and 94 h at 55 °C, while the reported enzymes showed perceptible loss of activity within 2 h.

In view of their many applications, largely in the dairy sector, there are continuing efforts to isolate and engineer microorganisms that can efficiently synthesize β-galactosidases of high stability. This class of enzymes are best known for their ability to hydrolyze lactose to glucose and galactose, thereby improving the solubility and sweetness of the milk product (Becerra and Siso 1996; Gekas and Lopez-Leiva 1985; Greenberg and Mahoney 1981). Besides hydrolysis, β-galactosidase (EC 3.2.1.23) also catalyzes transglycosylation reactions. β-D-Galactopyranosides, such as lactose, are thereby converted to galactooligosaccharides by the mechanism described by Prenosil *et al.* (1987). These oligosaccharides are useful for human health as they promote the growth of bifidobacteria in the large intestine (Othsuka *et al.* 1989; Tanaka *et al.* 1983). In dairy industry, β-galactosidases are used to prevent the crystallization of lactose, to produce lactose-free milk for patients with low lactose tolerance and for the utilization of cheese whey, which would otherwise be an environmental pollutant (Siso and Doval 1994).

Even if both fungi and yeasts are known to produce β-galactosidases (Athes and Combes 1998; Becerra *et al.* 1998; Santos *et al.* 1998; Shaikh *et al.* 1997; Bailey and Linko 1990; Gekas and Lopez-Leiva 1985), bacterial sources are preferable because of ease of fermentation, high activities of the enzyme and good stability. *Escherichia coli* and *Bacillus* strains are the commonly used bacteria. Although it is possible to obtain good yields of β-galactosidase from recombinant *E. coli*, the fermentation requires complex control (Machida *et al.* 1998; Ye *et al.* 1994) and, for some strains, can be extremely sensitive to fluctuations in temperature (Nielsen *et al.* 1991). Moreover, the enzyme from *E. coli* tends to stay inside the cells whereas bacilli secrete it into the broth (Shaikh *et al.* 1997; Zukowski 1992). This feature and the fact that the efficacy and stability of β-galactosidases from *E. coli* strains have not yet been established make bacilli popular for β-galactosidase production. The most common bacilli used in the industry are *Bacillus licheniformis*, *B. amyloliquefaciens* and *B. subtilis*.

Regardless of the source organism, long-term stability of the enzyme continues to be a problem. Fig. 1 shows that the half-lives (the time required for deactivation to half of the initial activity) range from 1.3 to 116 min. However, not all authors who studied β-galactosidase synthesis have also evaluated its stability and even those who did have not gone beyond 2 h. The present work reports on a new species of *Bacillus* which generates high activities of extremely stable β-galactosidase.

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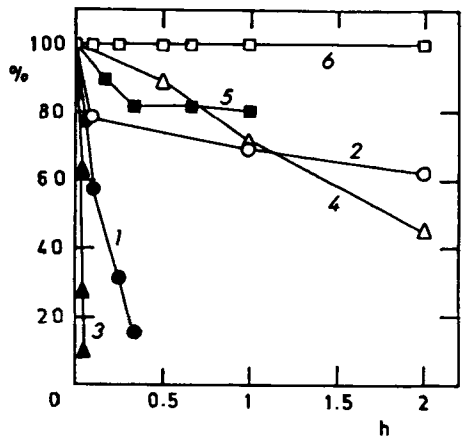

Fig. 1. Deactivation profiles of β-galactosidase activity (residual activity, %) of different strains reported in the literature; *1* — *Aspergillus oryzae* at 55 °C (Athes *et al.* 1998), *2* — *E. coli* at 55 °C (Athes *et al.* 1997), *3* — *K. lactis* at 55 °C (Athes *et al.* 1997), *4* — *K. marxianus* CCY eSY2 at 45 °C (Tomaska *et al.* 1995), *5* — *Bacillus* sp. TA-11 at 50 °C (Choi *et al.* 1995), *6* — *Rhizomucor* sp. at 50 °C (Shaikh *et al.* 1997).

MATERIALS AND METHODS

Chemicals

2-Nitrophenyl β-D-galactopyranoside (ONPG), X-Gal (5-bromo-4-chloro-3-indolyl β-D-galactopyranoside), 4-nitrophenol and 3,5-dinitrosalicylic acid were obtained from *Sigma Chemical Company* (MO, USA). All other chemicals used were of analytical-grade purity.

Microorganism and cultivation conditions

The microorganism used for the production of β-galactosidase was identified to belong to the genus *Bacillus* by the *Microbial Type Culture Collection, Institute of Microbial Technology*, Chandigarh (India), and it has been deposited there under accession number MTCC 3088. It was screened from a hot spring (Manikaran, India) and maintained on nutrient agar plates containing 1 % (*W/V*) lactose. The medium used for the production of β-galactosidase had the following composition (g/L): glucose, 10; meat extract, 15; biopeptone, 5; yeast extract, 0.5 and sodium chloride, 1.5. The pH of the medium was adjusted to 7. The seed culture was prepared by inoculating a single colony from the maintenance plate into 100 mL of the culture medium in a 500 mL Erlenmeyer flask and incubating in a rotary shaker (37 °C, 3.3 Hz) for 18 h. A 5 % (*V/V*) of this culture was used to inoculate similar flasks containing 100 mL medium. To assess the effect of glucose concentration on cell growth and enzyme production, two sets of experiments were conducted, one with an initial concentration of 10 g/L and another with 30 g/L. The flasks were incubated in a temperature-controlled (37 °C) shaker at a frequency of 3.3 Hz. Each experiment was carried out in triplicate and samples were withdrawn at regular intervals and examined for growth, enzyme activity and residual substrate concentration.

Assay methods

β-Galactosidase activity was assayed according to the method of Nagano *et al.* (1992) by using ONPG as the substrate. The reaction mixture contained 2 mL ONPG (2 mmol/L in 0.1 mol/L potassium phosphate buffer, pH 7) and 1 mL appropriately diluted enzyme solution. The reaction was allowed to proceed for 20 min at 60 °C and stopped by adding 2 mL of 1 mol/L Na_2CO_3 solution. The absorbance was measured at 416 nm. Appropriate enzyme and substrate blanks were included. A calibration curve was prepared using 4-nitrophenol as standard. One unit of enzyme activity was defined as the amount of enzyme that liberated 1 μmol 4-nitrophenol per min. The culture broths were centrifuged (165 Hz, 5 min) and the supernatants with appropriate dilutions were assayed for β-galactosidase activity. Dry-cell mass of the *Bacillus* sp. was determined after washing the cell mass thoroughly with distilled water to remove any adhering material and drying overnight at 80 °C. The thermostability of β-galactosidase was established by incubating the enzyme solution at 50 and 55 °C and assaying the enzyme activity at regular intervals. Glucose concentration was assayed by the 3,5-dinitrosalicylic acid method.

RESULTS AND DISCUSSION

The time profiles of cell mass concentration and β-galactosidase activity for two concentrations of glucose are shown in Figs 2 and 3. Contrary to many other microbial sources, the present *Bacillus* sp. grew on both glucose and lactose (Fig. 2). However, glucose was preferred because, except during the start-up phase, it generated higher β-galactosidase activity throughout the fermentation period (Fig. 3). At 1 % (*W/V*) glucose in the starting medium the cell growth followed a normal pattern of increasing until the substrate was exhausted and then decreased. However, when 3 % (*W/V*) glucose was supplied, there was an initial sharp increase in cell mass, followed sequentially by an abrupt fall, a slower rate of increase and a mild decrease spanning many hours. Enzyme activity (Fig. 3), however, did not follow this pattern. It differed from the nature of the cell mass concentration in two important aspects. First, enzyme activity was detected in the medium after a lag period of about 40 h from the start, whereas no lag phase existed in the cell mass (Fig. 2). Secondly, β-galactosidase activity increased monotonically even while the cell mass passed through a peak and a trough.

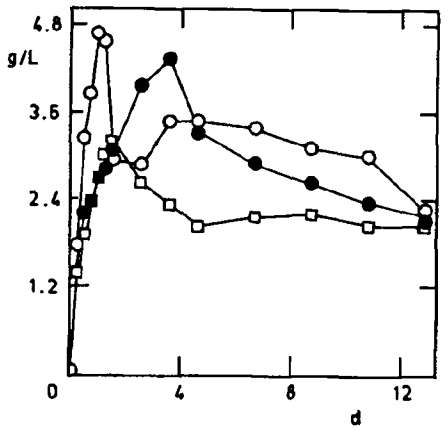

Fig. 2. Effect of glucose (*circles*) or lactose (*squares*) concentration (in g/L, *numbers at curves*) on the production of cell mass (g/L) by *Bacillus* sp. MTCC 3088 during the synthesis of β-galactosidase.

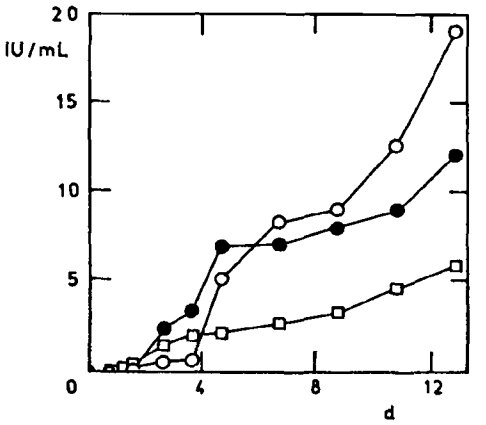

Fig. 3. Activity profiles of β-galactosidase (enzyme activity, IU/mL) during fermentation by *Bacillus* sp.; for symbols see Fig. 2.

These differences suggest two features of β-galactosidase production by this particular organism. First, β-galactosidase is an intermediate enzyme in the metabolic pathway and hence its synthesis can only begin after the precursor molecules have been generated. This may explain the initial lag between cell growth and enzyme activity. Secondly, it may be reiterated that the β-galactosidase activity reported here pertains to the extracellular liquid. This means that the activity was detected only after the enzyme had diffused out through the cell wall. Since diffusion rates are slower than the intracellular reaction rates (Vieth 1988), even during the period of cell decay the extracellular activity of β-galactosidase continued to increase because of the diffusive efflux of the enzyme that has accumulated inside the cells. There are interesting comparisons between the specific growth rate and the cell mass. For both concentrations of glucose, the specific growth rate was minimum (Fig. 4 *left*) shortly after the concentration had reached a peak. However, the bimodal concentration variation with 3 % glucose was not reflected in the growth rate. The bimodal shape of the cell mass profile at a high concentration of glucose (Fig. 3) is a manifestation of the Crabtree effect, which is well known for *E. coli* and *Saccharomyces* yeasts but is less prevalent in bacilli.

These differences become more vivid in a phase plane diagram of the specific growth rate against cell concentration (Fig. 4 *right*). The trajectories spiral inward but do not converge to a focus or a limit cycle. As expected, the cyclic behavior stabilizes at a constant specific growth rate as the stationary phase is reached. Between the two phase trajectories there are significant differences in the multiplicity pattern. At 1 % glucose, there can be just one or two specific growth rates for each value of cell mass concentration. However, when the glucose concentration is 3 %, the trajectory completes two separate cycles and the multiplicity pattern is 1→2→4→3→4→2→1→2 as one moves up from a low cell mass. Even though the cultivation time for this organism is more than those reported by other workers, this is compensated by the maximum activity attainable and its stability. Since the studies

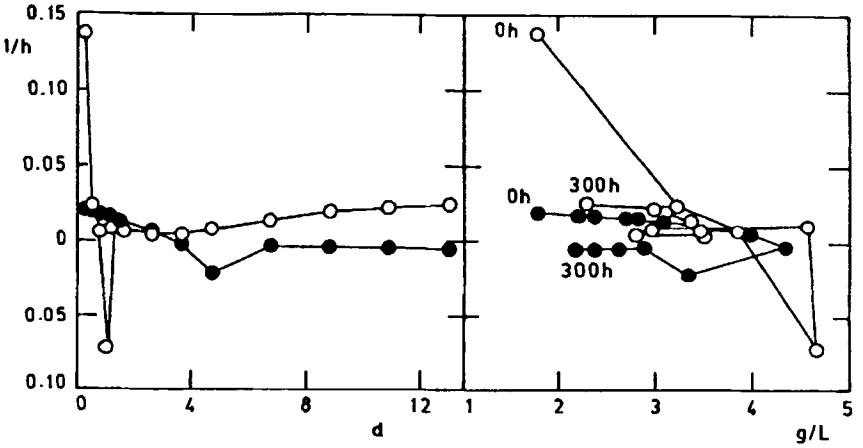

Fig. 4. *Left*: Specific growth rate (1/h) profiles of the *Bacillus* sp. *Right*: Phase plane trajectories of the specific growth rates (1/h) of *Bacillus* sp. during β-galactosidase production (cell mass, g/L); for symbols see Fig. 2.

reported in the literature differ in the substrates used, the cultivation conditions and the units of β-galactosidase activity, the results of Shaikh *et al.* (1997) provide the most reasonable comparison. For fermentation with a *Rhizomucor* sp. (NCIM 1253; *National Chemical Laboratory*, Pune, India) and 1 % lactose in the starting medium, the β-galactosidase activity reached a peak value of about 6 IU/mL after 5 d and then decreased. In the present study, the activity had reached 12 IU/mL for 1 % glucose and 19 IU/mL for 3 % glucose by 300 h and had not yet attained a peak (Fig. 3). Even at the end of 5 d (120 h), the present enzyme activity was by 1 IU/mL (17 %) more than obtained by Shaikh *et al.* (1997) for the same concentration of substrate.

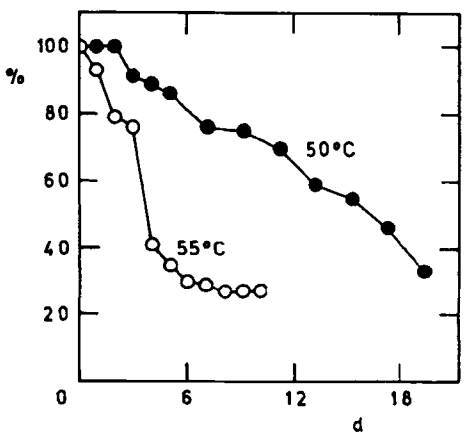

Fig. 5. Deactivation profiles of β-galactosidase activity by *Bacillus* sp. (*closed symbols*) 50 °C and (*open symbols*) 55 °C.

This enzyme is also extremely stable, its half-life being 408 h at 50 °C and 94 h at 55 °C (Fig. 5). Comparison with other sources of the enzyme should be done cautiously because not all authors who have generated β-galactosidase have also studied its stability and, as Fig. 1 shows, changes in activity have been measured over much shorter time spans (1–2 h) than in our experiments. Based on these limited data, it might appear that the enzymes obtained by Choi *et al.* (1995) and Shaikh *et al.* (1997) are more stable because the slow inactivation rate suggests a long predicted half-life. This inference, however, should be qualified. First, the time span in Fig. 1 is just 2 h, during which period the enzyme obtained in the present work also loses little activity at 55 °C (Fig. 5). Activity measurements over a longer duration are important because deactivation becomes significant only after about 70 h. Secondly, the β-galactosidase obtained by Shaikh *et al.* (1997) deactivated rapidly at 60 °C, losing more than 30 % of its initial activity in 2 h; extrapolating their data to 50 % loss of activity indicates a half-life of 2.5 h, compared to 94 h for the present enzyme at 55 °C. These observations indicate that the *Bacillus* sp. strain MTCC 3088 is a potent source of stable extracellular β-galactosidase. Its main weakness is the slow rate of enzyme synthesis; experiments with improved cultivation conditions in a laboratory bioreactor are in progress to increase this rate and consequently the approach to the peak activity.

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REFERENCES

ATHES V., COMBES D.: Influence of additives on high pressure stability of β-galactosidase from *Kluyveromyces lactis* and invertase from *Saccharomyces cerevisiae*. *Enzyme Microb.Technol.* **22**, 532–537 (1998).

ATHES V., DEGRAEVE P., CAVAILLE-LEFEBVRE D., ESPEILLAC S., LEMAY P., COMBES D.: Increased thermostability of three mesophilic β-galactosidases under high pressure. *Biotechnol.Lett.* **19**, 273–276 (1997).

BAILEY M.J., LINKO M.: Production of β-galactosidase by *Aspergillus oryzae* in submerged bioreactor cultivation. *J.Biotechnol.* **16**, 57–66 (1990).

BECERRA M., CERDAN E., SISO M.I.G.: Micro-scale purification of β-galactosidase from *Kluyveromyces lactis* reveals that dimeric and tetrameric forms are active. *Biotechnol.Biotech.* **12**, 253–256 (1998).

BECERRA M., SISO M.I.G.: Yeast β-galactosidase in solid-state fermentation. *Enzyme Microb.Technol.* **19**, 39–44 (1996).

CHOI Y.J., KIM H., LEE B.H., LEE J.S.: Purification and characterization of β-galactosidase from alkalophilic and thermophilic *Bacillus* sp. TA-11. *Biotechnol.Appl.Biochem.* **22**, 191–201 (1995).

GEKAS V., LOPEZ-LEIVA M.: Hydrolysis of lactose: a literature review. *Proc.Biochem.* **20**, 2–12 (1985).

GREENBERG N.A., MAHONEY R.R.: Immobilization of lactase (β-galactosidase) for use in dairy processing: a review. *Proc. Biochem.* **16**, 2–8 (1981).

MACHIDA S., YU Y., SINGH S.P., KIM J.D., HAYASHI K., KAWATA Y.: Overproduction of β-galactosidase in active form by an *Escherichia coli* system coexpressing the chaperonin GroEL/ES. *FEMS Microbiol.Lett.* **159**, 41–46 (1998).

NAGANO H., OMORI M., SHOJI Z., KAWAGUCHI T., ARAI M.: Purification and characterization of β-galactosidase from *Enterobacter cloacae* GAO. *Biosci.Biotech.Biochem.* **56**, 674–675 (1992).

NIELSEN J., PEDERSEN A.G., STRUDSHOLIN K., VILLADSEN J.: Modeling fermentation with recombinant microorganisms: formulation of a structured model. *Biotechnol.Bioeng.* **37**, 802–808 (1991).

OTHSUKA K., BENNO V., ENDO K., OZAWA O., VEDA H., UCHIDA T., MITSUOKA T.: Effects of 4'-galactosyllactose intake on human fecal microflora. *Bifidus* **2**, 143–149 (1989).

PRENOSIL J.E., STUKER E., BOURNE J.R.: Formation of oligosaccharides during enzymatic lactose hydrolysis. 1. State of art. *Biotechnol.Bioeng.* **30**, 1019–1025 (1987).

SANTOS A., LADERO M., GARCIA-OCHOA F.: Kinetic modeling of lactose hydrolysis by a β-galactosidase from *Kluyveromyces fragilis*. *Enzyme Microb.Technol.* **22**, 558–567 (1998).

SHAIKH S.A., KHIRE J.M., KHAN M.I.: Production of β-galactosidase from thermophilic fungus *Rhizomucor* sp. *J.Ind.Microbiol. Biotechnol.* **19**, 239–245 (1997).

SISO M.I.G., DOVAL S.S.: *Kluyveromyces lactis* immobilization on corn grits for milk whey lactose hydrolysis. *Enzyme Microb. Technol.* **16**, 303–310 (1994).

TANAKA R.H., TAKAYAMA M.M., KUROSHIMA T., UEYAMA S., MATSUMOTO K., KURODA A., MUTAI M.: Effects of administration of TOS and *Bifidobacterium breve* 4006 on the human fecal flora. *Bifidobacteria Microflora* **2**, 17–24 (1983).

TOMASKA M., STREDANSKY S., GEMEINER P., STURDIK E.: Improvement of the thermostability of β-galactosidase from *Kluyveromyces marxianus*. *Process Biochem.* **30**, 649–652 (1995).

VIETH W.R.: *Membrane Systems: Analysis and Design*. Hanser, Munich (Germany) 1988.

YE K., JIN S., SHIMIZU K.: Fuzzy neural network for the control of high cell density cultivation of recombinant *E. coli*. *J.Ferment. Bioeng.* **77**, 663–673 (1994).

ZUKOWSKI M.M.: Production of commercially valuable products, pp. 311–337 in R.H. Doi, M. McGlouglin (Eds): *Biology of Bacilli: Applications to Industry*. Butterworth–Heinemann, Oxford (UK) 1992.