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Isolation and some properties of β-galactosidase from the thermophilic bacterium *Thermus thermophilus*

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The highest β-galactosidase activity in *Thermus thermophilus* cells was achieved after 40 h of cultivation at 70°C in a medium containing 0.8% peptone, 0.4% yeast extract and 0.2% NaCl. After the addition to the medium of 2% of lactose or galactose, enzyme synthesis in the cells increased by about 33% and 61% of its constitutive value. The thermostable β-galactosidase isolation and partial purification involved extraction in 0.01 M phosphate buffer at pH 7.2, ammonium sulphate precipitation, dialysis and gel filtration on Sephadex G-200 or lyophilization. The highest recovery yield of activity during the isolation process was about 72% of its total value in the extract. The gel filtration step resulted in an increase of β-galactosidase specific activity of two-fold more than the ammonium sulphate precipitate. The crude enzyme obtained exhibited highest activity at pH 6.6 and 87°C. Decimal reduction times of activity at 75, 80, 85, 90 and 95°C were 174, 50, 22, 5.5 and 0.9 h, respectively.

INTRODUCTION

Various β-galactosidases were investigated for the preparation of low lactose milk and dairy products (Kennedy, 1987). Interest is mainly due to the occurrence of lactose intolerance in some populations (Fabrizis *et al.*, 1997). Also, whey utilization for the production of many valuable products has been extensively studied (Horton, 1994). One of the major obstacles to whey utilization is lactose content, which causes crystallization at low temperatures, low sweetness and poor digestibility when used as food. Lactose is also less fermentable than other sugars (Holsinger, 1978). These problems can be solved if whey lactose is hydrolysed to glucose and galactose. The sweet syrup can be used as a sugar source or as a basis for further fermentation.

However, the released galactose is a competitive inhibitor of β-galactosidases. This inhibition increases during the course of lactose hydrolysis and substantially reduces the enzyme activity (Woychik and Wondolowski, 1973). More efficient lactose hydrolysis can be achieved using a continuous-flow reactor with immobilized enzyme as against batch treatments with free enzyme. These differences in amounts of lactose hydrolysed can be attributed to the fact that all of the free enzyme is exposed to increased levels of the galactose inhibitor, whereas in the immobilized enzyme system, the hydrolysis products are removed from the reactor operating at higher substrate concentration and lower levels of galactose inhibition. Immobilization of β-galactosidase reduces the cost of the enzyme and assures better control of the process conditions.

Thermostable β-galactosidases from moderate- and hyperthermophiles have maximum activity at temperatures ranging from 70 to 90°C. Such temperatures eliminate the hazard of undesired microbial contamination during the long operating time of the immobilized enzyme system. Such enzymes can also improve the economy of the lactose hydrolysis as a result of their greater stability (Cowan *et al.*, 1984). However, little information is available on the properties and β-galactosidase activity of enzymes from thermophilic bacteria. This article reports some properties of β-galactosidase from *Thermus thermophilus* (HB-8).

MATERIALS AND METHODS

Cell cultivation

Thermus thermophilus HB-8 (ATCC 27534) was cultivated on media composed of yeast extract (Difco

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Laboratories, USA), peptone bio-Trypcase (bio Merieux, France), NaCl and water in amounts listed in Table 1. The cultures were grown in 200 ml Erlenmeyer flasks containing 100 ml of liquid medium (pH 7.5) autoclaved for 30 min at 121°C. The flasks were inoculated with 1 ml of a *T. thermophilus* cell suspension (OD 1.2) and stirred in a water bath at 150 rpm at 70°C for 6–96 h without additional aeration.

To examine the induction of the thermostable β-galactosidase, up to 2% lactose or galactose was added to the medium after 5 h of *T. thermophilus* cultivation.

After the desired growth time, the cells were harvested by centrifugation at 12 000***g*** for 15 min. The pellet was washed with distilled water, centrifuged and stored at −20°C until use.

Table 1. Composition of the media (g litre^{-1} of water solution) used for *Thermus thermophilus* cultivation

Medium no.	bio-Trypcase	Yeast extract	NaCl
1	8	4	2
2	6	6	2
3	4	8	2
4	8	4	0
5	8	4	1
6	8	4	5

Isolation of crude β-galactosidase

Approximately 10 g of frozen cells were disrupted for 15 min in a refrigerated mortar with 20 g of alumina (Sigma Chemical Co., St. Louis, MO, USA) and 70 ml of 0.01 M sodium phosphate buffer (pH 7.2) containing (per 1 ml) 1 μmol $MgSO_4$ and 50 μmol β-mercaptoethanol (buffer A). The resulting mixture was centrifuged at 12 000***g*** for 15 min to give a clear supernatant. The protein fraction with β-galactosidase activity was precipitated by ammonium sulphate added slowly over a period of 30 min with constant stirring up to a final concentration of 50% (w/v). The centrifuged precipitate (12 000***g***, 15 min) was redissolved in a small volume of buffer A, dialyzed twice at 4°C against 100 volumes of the same buffer overnight and lyophilized or applied for gel filtration.

Gel filtration

A concentrated solution of crude β-galactosidase (5 ml) was applied to a column (3 × 40 cm) of Sephadex G-200 equilibrated with 0.01 M Tris-acetate buffer (pH 7.2) containing (per 1 ml) 10 μmol $MgSO_4$, 10 μmol NaCl and 10 μmol β-mercaptoethanol (Craven *et al.*, 1965). The samples were eluted with the same buffer at a flow rate of 30 ml h^{-1} and 7 ml fractions were collected for the determination of enzyme activity and protein content.

Enzyme assay and definition of units

β-Galactosidase activity was determined by release of *o*-nitrophenol from a 0.03 M solution of *o*-nitrophenol-β-D-galactopyranoside (ONPG; Sigma) in 0.1 M sodium phosphate buffer (pH 6.6 adjusted at 70°C) using the modified Craven *et al.* (1965) procedure. The substrate (2.5 ml) was preincubated at 70°C or at a desired temperature and the reaction was initiated by the addition of 0.5 ml of crude enzyme solution. After 30 min of incubation, the reaction was stopped using 1 ml of 1 M sodium carbonate solution. A blank containing 1 ml of water instead of enzyme solution was used to correct the thermal hydrolysis of ONPG.

For the determination of β-galactosidase activity in whole cells, 5 ml of a suspension of a known amount of *T. thermophilus* cells in 0.1 M sodium phosphate buffer (pH 6.6) was pretreated with two drops of toluene and preincubated for 30 min at 70°C. The reaction was initiated by the addition 0.8 ml of 0.015 M ONPG solution in sodium phosphate buffer (pH 6.6) and terminated by 2 ml of 2 M sodium carbonate solution. The cell residue in the samples was removed by centrifugation at 12 000***g*** for 10 min.

Absorbances at 420 nm were converted to *o*-nitrophenol concentration using a molar absorption coefficient of $4.5 \times 10^3 M^{-1} \times cm^{-1}$.

One unit of β-galactosidase activity is defined as 1 nmol of *o*-nitrophenol liberated from ONPG per min under described conditions. Specific activity is defined as β-galactosidase units per 1 mg of protein determined as by Lowry *et al.* (1951).

pH profile and optimal temperature

The effect of pH on β-galactosidase activity was determined using 0.03 M ONPG solution in 0.1 M citrate-phosphate buffers in the pH range 2.5–8.5 adjusted at 70°C.

The effect of temperature on β-galactosidase activity was assayed using 0.03 M ONPG solution in 0.1 M sodium phosphate buffer (pH 6.6) at temperatures ranging from 30 to 98°C.

Enzyme thermostability

The thermal stability of *T. thermophilus* β-galactosidase was investigated by incubation of enzyme solution in 0.1 M sodium phosphate buffer (pH 6.6) in sealed tubes at 75, 80, 85, 90 and 95°C for different periods. After heating, samples were cooled and the remaining activity was measured at 70°C. The decimal reduction time (*D*) was calculated by plotting the log of the residual activity vs time.

Statistical analysis

Analysis of variance and Tukey's studentized range test (Senecdor and Cochran, 1980) were used to determine

differences in mean values of the data from four replicates. Significance was determined at 95% probability.

RESULTS AND DISCUSSION

Figure 1 shows the cultivation profiles of *T. thermophilus*. The biomass yield increased rapidly up to 40 h of growth at 70°C and was only slightly influenced by the changes in the ratio of peptone and yeast extract (2:1, 1:1 and 1:2) in the media containing 0.2% NaCl. The final density of culture reached during this time was about 1.6 g of dry cells per litre of medium. However, the highest β-galactosidase activity in whole cells was achieved after 40 h of *T. thermophilus* growth on the medium containing 0.8% peptone, 0.4% yeast extract and 0.2% NaCl (Fig. 2). Prolonged cultivation time, up to 96 h, did not influence the amount of the enzyme in the cells. Moreover, the β-galactosidase activity measured in the cells was significantly lower after elimination of sodium chloride from the medium or after its replacement by $NaNO_3$ or KCl (Figs 2 and 3).

The β-galactosidase activity in *T. thermophilus* growing on the medium without inducers was $3.3 \pm 0.2\ U\ mg^{-1}$ of dry cells and was increased up to 4.4 ± 0.2 and $5.3 \pm 0.3\ U\ mg^{-1}$ after the addition to the medium of 2% lactose or galactose, respectively. It is difficult to know whether the β-galactosidase activity detected in the *T. thermophilus* cultivated without inducers was the result of constitutive synthesis or the result

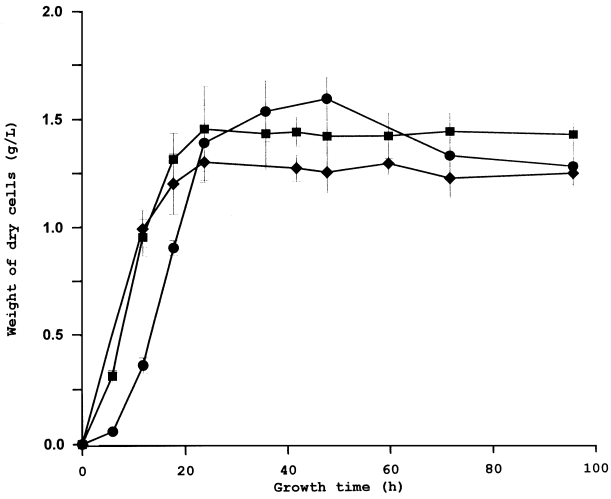

Fig. 1. Growth of *Thermus thermophilus* (HB-8) at 70°C on media listed in Table 1: no. 1 (●); no. 2 (■); no. 3 (◆). The results are the mean values of three determinations.

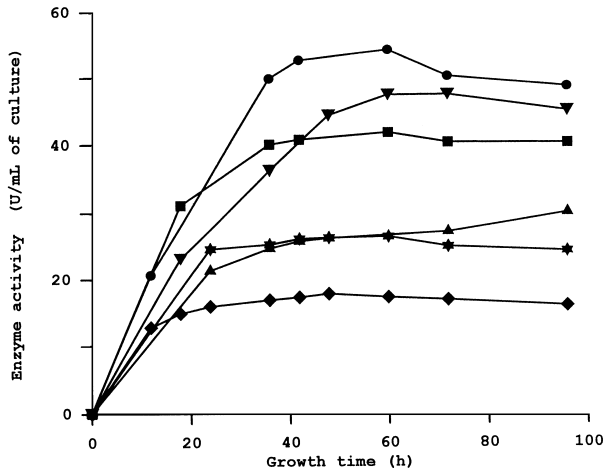

Fig. 2. β-Galactosidase synthesis in *Thermus thermophilus* (HB-8) cells cultivated at 70°C on media listed in Table 1: no. 1 (●); no. 2 (▼); no. 3 (▲); no. 4 (◆); no. 5 (■) and no. 6 (⋆). The results are the mean values of three determinations. Standard deviations did not exceed 8% of the recorded values.

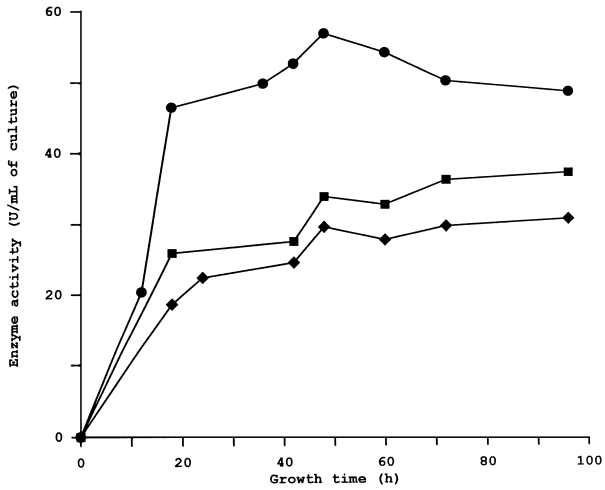

Fig. 3. The influence of 0.2% NaCl (●), KCl (■) or $NaNO_3$ (◆) in the medium on β-galactosidase synthesis during growth of *Thermus thermophilus* (HB-8). The results are the mean values of three replicates. Standard deviations did not exceed 7% of the recorded values.

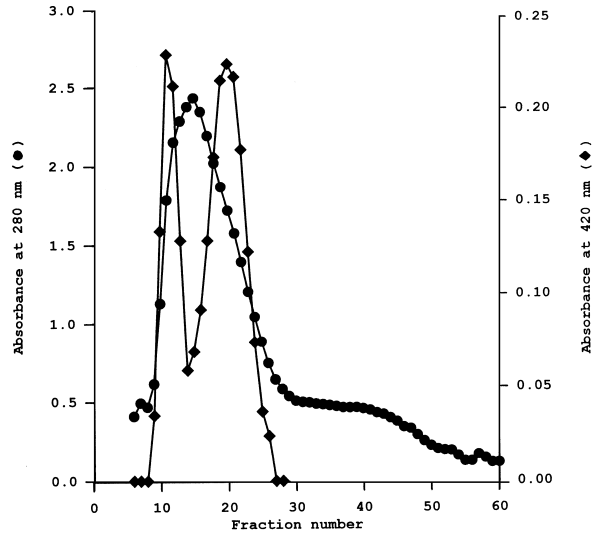

Fig. 4. Gel filtration of crude β-galactosidase on the column with Sephadex G-200. Absorbances at 280 nm and 420 nm represent changes in protein content and enzyme activity, respectively, between collected fractions (7 ml).

Table 2. Activity and protein recovery (calculated per 1 g of a dry basis of the cells) during isolation of crude β-galactosidase from *T hermus thermophilus*[a]

Component	Protein (mg)	Total activity ($10^3 \times$ U)	Specific activity (U mg^{-1} protein)	Recovery of activity (%)
Cell extract	426 ± 61	5.2 ± 0.5	12.3	100.0
Protein precipitated by $(NH_4)_2SO_4$	410 ± 11	3.8 ± 0.1	9.3	73.0
Supernatant after protein precipitation	16 ± 1	0.1 ± 0.00	–	2.5
Dialysed enzyme solution	400 ± 32	3.7 ± 0.3	9.3	72.0

[a] The results are the mean values of data from three separate samples (cultivated 48 h) ± standard deviation.

of induction due to the presence of trace amounts of sugar in the medium. Koyama *et al.* (1990) reported constitutive β-galactosidase activities for *T. thermophilus* (HB-27). Galactose is a better inducer of β-galactosidase activity than lactose. This agrees with the results from several strains of *Thermus* species investigated by Berger *et al.* (1995).

Specific activity of the cell-free extract was about 12.3 U mg^{-1} protein and slightly decreased to 9.3 U mg^{-1} after ammonium sulphate precipitation (Table 2). This phenomenon can be explained by partial inactivation of the enzyme and by co-precipitation with β-galactosidase of about 97% of proteins found in the cell extract. The total activity yield (75%) after ammonium sulphate precipitation was quite similar to the value (74%) measured by Cowan *et al.* (1984) during the isolation of β-galactosidase from *Thermus* (4-1A) strain. The obtained specific activity of β-galactosidase from *T. thermophilus* corresponds to the data reported by Berger *et al.* (1995).

During gel filtration on Sephadex G-200, two maxima of β-galactosidase activity were observed (Fig. 4). This is perhaps due to the dissociation of enzyme molecules into subunits or by their partial aggregation by disulphide bridges in the presence of oxygen. This effect was also reported by Hu *et al.* (1962) for β-galactosidase from *Escherichia coli*. An alternative explanation is that part of the enzyme remains particle-bound, probably to cell debris, membrane fragments, etc. Similar to purification of enzyme from *E. coli* (Craven *et al.*, 1965), the gel filtration step of *T. thermophilus* β-galactosidase resulted in an increase in specific activity of two-fold over the ammonium sulphate precipitate.

The optimum pH for the hydrolysis of ONPG by the investigated enzyme was 6.6 (Fig. 5). Similar results have been reported for several β-galactosidases from yeast and bacteria which have optimum pHs in the range 6.5–7.5 (Wierzbicki *et al.*, 1974; Khare and Gupta, 1988; Pisani *et al.*, 1990; Wang *et al.*, 1996). The retention of about 50% of maximal activity at pH values of 5.0 and 7.4 indicates that the enzyme is suitable for hydrolysis of lactose in milk and sweet whey.

The optimum activity of *T. thermophilus* β-galactosidase was observed at 87°C. However, at higher temperatures, the enzyme activity sharply declined due to enzyme denaturation (Fig. 6). The β-galactosidase from *T. thermophilus* is very stable at temperatures up to

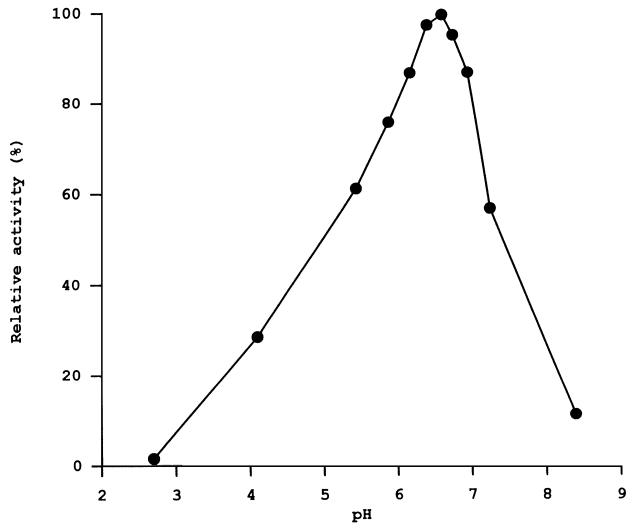

Fig. 5. Effect of pH at 70°C on the activity of β-galactosidase from *Thermus thermophilus* (HB-8). The results are the mean values of three replicates. Standard deviations did not exceed 5% of the recorded values.

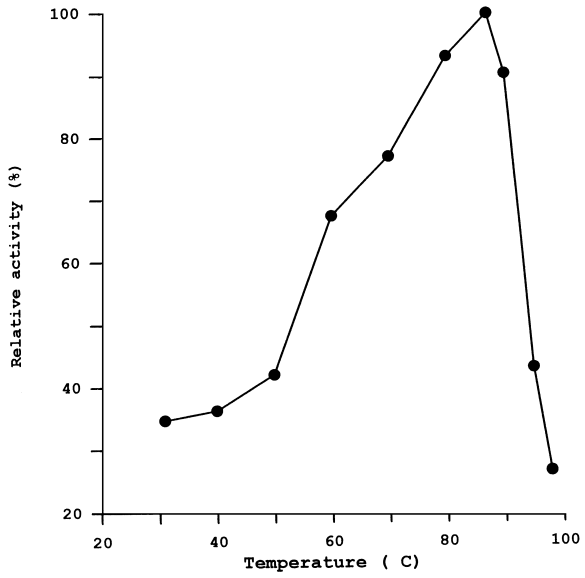

Fig. 6. Effect of temperature at pH 6.6 on the activity of β-galactosidase from *Thermus thermophilus* (HB-8). The results are the mean values of three replicates. Standard deviations did not exceed 5% of the recorded values.

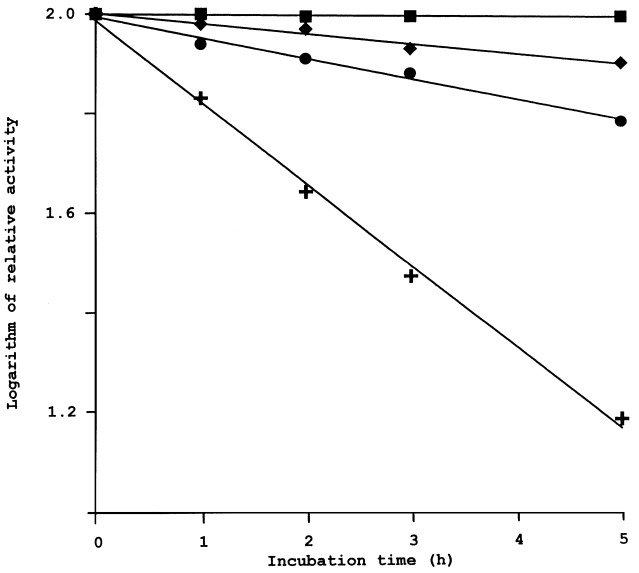

Fig. 7. Thermal stability of *β*-galactosidase from *Thermus thermophilus* (HB-8) incubated at pH 6.6 at temperature: 75°C (■), 80°C (◆), 85°C (●) and 90°C (×). The results are the mean values of three replicates. Standard deviations did not exceed 5% of the recorded values.

85°C and the activity loss after 5 h of enzyme incubation at 75 and 85°C does not exceed 2 and 20%, respectively (Fig. 7). The decimal reduction times of activity (*D*) at 75, 80, 85, 90 and 95°C were 174, 50, 22, 5.5 and 0.9 h, respectively.

A K_m of 2.6 mM was calculated from a Lineweaver-Burk plot of activity as a function of ONPG concentration. The measured value was similar to the K_m of *β*-galactosidases from *T. aquaticus* (4 mM), *Sulfolobus solfataricus* (2.6 mM) and *Pyroccocus furosus* (0.15 mM) and shows that the enzyme from *T. thermophilus* has a strong affinity for the substrate (Cowan *et al.*, 1984; Pisani *et al.*, 1990; Kengen *et al.*, 1993).

In conclusion, the thermal stability and pH optimum of *β*-galactosidase from *T. thermophilus* suggest that this enzyme can be used for milk and sweet whey processing in an immobilized system operating for a long time without undesired microbial contamination. However, *T. thermophilus* is less effective for *β*-galactosidase synthesis, as compared with other *Thermus* strains investigated by Berger *et al.* (1995).

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