A C T A A L I M E N T A R I A P O L O N I C A

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# PROPERTIES OF β-GALACTOSIDASE FROM CELLS OF *STREPTOCOCCUS LACTIS* 192 AND *LACTOBACILLUS LACTIS* F-16 STRAINS

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Key words: β-galactosidase activity, desintegration of cell thermal stability, lactose, influence of ions, *Streptococcus lactis*, *Lactobacillus lactis*.

The β-galactosidase synthetized by cells of *Str. lactis* 192 and *Lbc. lactis* F-16 in characterized on the basis of pH and temperature optimum for the enzyme's activity, thermal stability and the effect of selected ions. There were significant differences in β-galactosidase activity depending on the strain and on the kind of extract. The *Str. lactis* 192 enzyme demonstrated higher activity in lactose fermentation and lower thermal stability than the β-galactosidase of *Lbc. lactis* F-16. The effect of ions on enzyme activity was diversified and depended on the time of activity, the kind of extract and the bacteria strain.

## INTRODUCTION

The studies of lactic bacteria enzymes have been concentrated in recent years mainly on the evaluation of proteolytic activity and characteristics of proteolytic enzymes of selected strains. The activity of streptococci and lactobacilli in the hydrolysis and fermentation of lactose is considerably less known.

Works of numerous authors [2-6, 9, 12] show that β-galactosidase isolated from different bacteria strains has different properties. The β-galactosidase from *Leuconostoc citrovorum* displayed optimum pH of 6.5 and optimum temperature of 333 K (60°C) [12] while the analogous figures for the enzyme from *Streptococcus cremoris* H were 7.0 and 338 K (65°C), respectively [6]. In their study of the β-galactosidase of *Str. thermophilus*, Ramana Rao and Dutta [9] found that the enzyme was maximally active at pH 6.0 and 6.7 and at temperature of 330 K (57°C); the enzyme was most stable in the pH range 6.8-7.0.

Our studies of the fermentative activity of selected strains of lactic bacteria [1, 10, 11] have shown that the enzymatic extracts of the in-

vestigated strains caused coagulation of milk proteins and an increase of acidity to the constant level of pH 4.4.

The present attempts at characterizing β-galactosidase synthetized by cells of *Str. lactis* 192 and *Lbc. lactis* F-16 through a determination of optimum pH and temperature, thermal stability and the effect of selected ions, is a continuation of these studies.

## EXPERIMENTS AND METHODS

1. **Strains.** The two strains used in the experiments, *Str. lactis* 192 and *Lbc. lactis* F-16, came from the collection of Milk Biopreparations Production Plant in Olsztyn.

2. **Medium preparation.** The medium used was whey from milk that was partly hydrolysed with pepsin according to Rymaszewski et al. [11], enriched with additions of glucose (0.5%), salts ($MnSO_4$, $MgSO_4$, $ZnSO_4$, 0.12% of each), and yeast extract (0.5% volume).

3. **Biomass multiplication** was performed in a Biotec 1601 fermenter (5 l capacity) at 303 K (30°C) for *Str. lactis* 192 and 310 K (37°C) for *Lbc. lactis* F-16. The medium was inoculated with 5% of inoculum. Throughout the culture the pH of the medium was maintained on a constant level of 6.7 for *Str. lactis* 192 and 5.4 for *Lbc. lactis* F-16. In order to ensure constant pH the excess of milk acid was neutralized by additions of 25% $NH_4OH$ automatically dosed out with a pump coupled to a pH-stat. The milk bacteria cells were multiplied until the end of the logarithmic growth phase.

The biomass was separated from the medium by centrifugation on a Sharpless (Penwalt, England) centrifuge at 5500×g. Next the biomass was rinsed with sterile phosphate buffer 0.05 M with pH 7.0 three times and every time centrifuged for 15 min. The centrifuged biomass was frozen at 253 K (-20°C).

4. **Disintegration and obtaining of extracts.** The frozen biomass was disintegrated by the mechanical-pressure method with a Biotex X-25 (Sweden) disintegrator. After disintegration the sediment was dispersed in 40 ml of phosphate buffer (0.5 M with pH 7.0) in 1 : 4 volume ratio. Maceration lasted 4 hours, following which the sediment was centrifuged for 15 minutes on the Beckman J-21C centrifuge at 5500×g yielding intracellular extract and cell wall sediment. The sediment was macerated for 4 hours in 40 ml of 2M NaCl, and centrifuged as above, giving cell wall extract [11].

5. **Characteristic of the extracts.** Extracts were characterized on the basis of the following determinations:

A. Protein content by the method of Lowry et al. [7].

B. Activity of β-galactosidase according to Hemme et al. [2]. A reactive mixture of 2 ml of 0.9% NaCl, 3 ml of distilled water and 6 ml of ONPG solution, was heated to 303 K (30°C) in the case of *Str. lactis* 192 and to 310 K (37°C) in the case of *Lbc. lactis* F-16, mixed with 4 ml of extract and incubated at suitable temperature. After 10, 20 and 30 minutes, 6-ml samples were taken and transferred to test-tubes with 0.6 ml of 1 M $Na_2CO_3$ which stopped the enzymatic reaction. Extinction was read at 420 nm (Beckman-25 spectrophotometer, 1 cm developing dish). The amount of o-nitrophenol was determined from the standard curve for the concentration range of $1\text{-}20\times10^{-5}$ M.

The specific activity of β-galactosidase was expressed as the amount enzyme of in 1 mg of protein in the extract needed to liberate 1 μM of o-nitrophenol from ONPG during 1 minute of reaction [2].

The β-galactosidase activity was also expressed in μM of o-nitrophenol liberated from ONPG in 1 minute of reaction by 1 mg of protein contained in the extract.

C. Optimum pH determination. 0.2 M phosphate buffer with pH of 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0 and 8.8 was used. The activity of β-galactosidase was determined as described above.

D. The optimum temperature was sought in the range from 293 K (20°C) to 328 K (55°C) using 5° intervals. 0.2 M phosphate buffer with pH 7.0 and 5.0 (optimum pH for *Str. lactis* 192 extracts) and 6.0 and 7.0 (optimum pH for *Lbc. lactis* F-16 extracts) was used.

E. Thermal stability was determined as follows: 4 ml of extract were heated at 328 K (55°C) for 0, 1, 2, 5 and 10 minutes, and then the enzyme activity was determined in optimum pH and temperature conditions as described above.

F. The effect of $Mn^{+2}$, $Mg^{+2}$, $Ca^{+2}$ and $SO_4^{-2}$ ions on enzyme activity was examined at 5 mM concentration. β-galactosidase activity was assayed immediately after the addition of ions, and after 24 and 48 hours of incubation at 279-280 K (6-7°C).

G. Determination of β-galactosidase activity after storage. One sample of *Str. lactis* 192 bacteria biomass was freeze-stored at 253 K (-20°C), and one was disintegrated, dissolved in 150 ml of 0.05 M phosphate buffer (pH 7.0), lyophilized (Heto, Denmark) and then stored at 279-280 K (6-7°C). β-galactosidase activity was determined in both the samples after one, two and three months of storage.

6. Determination of the kinetics of lactose solution fermentation. The substrates used were 0.5, 1.0-, 2.0-, 3.0- and 4.0-percent lactose solutions in 0.05 M phosphate buffer with pH optimum for the different extracts. 1 per cent of protein contained in the extracts was added to 50 ml of lactose solution which was then incubated at 303 K (30°C) in the case of *Str. lactis* 192 and at 310 K (37°C) in the case of *Lbc. lactis* F-16. The pH

changes and the level of absorption at 340 nm was determined in the separate solutions every 4 hours. After 24 hours the content of milk acid was also determined.

## DISCUSSION OF RESULTS

A clear dependence of β-galactosidase activity on the strain, the kind of extract and the pH of the substrate was observed. For intracellular extract from *Str. lactis* 192, the optimum pH was found to be 7.0. Two such optima were found for the cell wall extract: maximum activity of β-galactosidase was at pH 5.0, then a sharp drop to a minimum at pH 6.5, and another optimum at pH 7.5 (Fig. 1A).

Different results were obtained for *Lbc. lactis* F-16 extracts (Fig. 1B). The cell wall extract showed one clear optimum at pH 7.0, while the maximum activity of β-galactosidase was found at pH 6.0, and an activity about 1.5 times lower at pH 7.0.

The activity of β-galactosidase depended also on temperature. The cell wall extracts of both strains of milk fermentation bacteria had single optima: 308 K (35°C) (extract of *Str. lactis* 192, Fig. 2A) and 313 K (40°C) (*Lbc. lactis* F-16. Fig. 2B). The intracellular extract of *Str. lactis* 192 (Fig. 2A) displayed two temperature optima, namely 308 K (35°C) and 318-323 K (45-50°C). The intracellular extract of *Lbc. lactis* F-16 showed the lo-

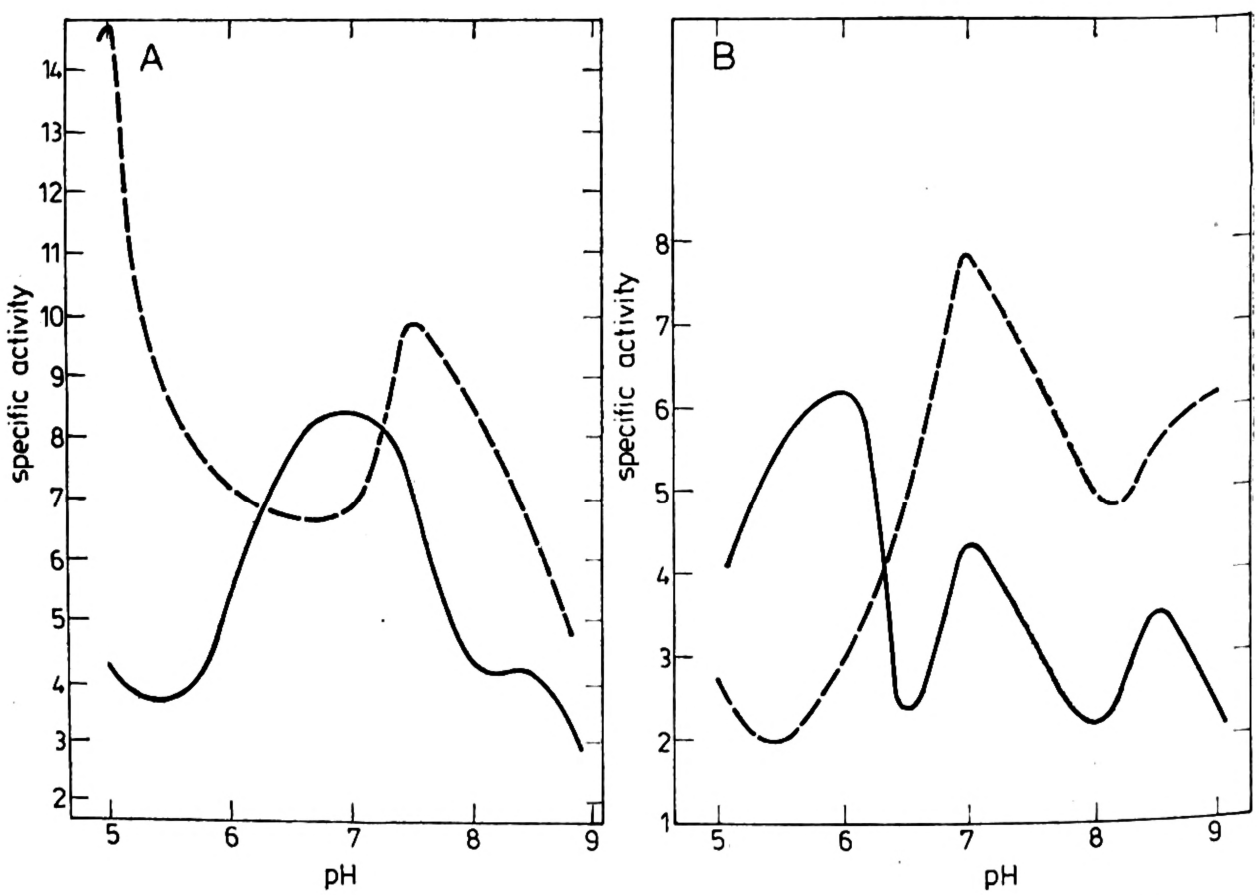


Fig. 1. Effect of pH on the activity of β-galactosidase of *Str. lactis* 192 (A) and *Lbc. lactis* F-16 (B). Solid line — intracellular extract, broken line — cell wall extract

west β-galactosidase activity at 298-303 K (25-30°C) and a steady growth of this activity from 303 K (30°C) to the maximum at 323 K (50°C) (Fig. 2B).

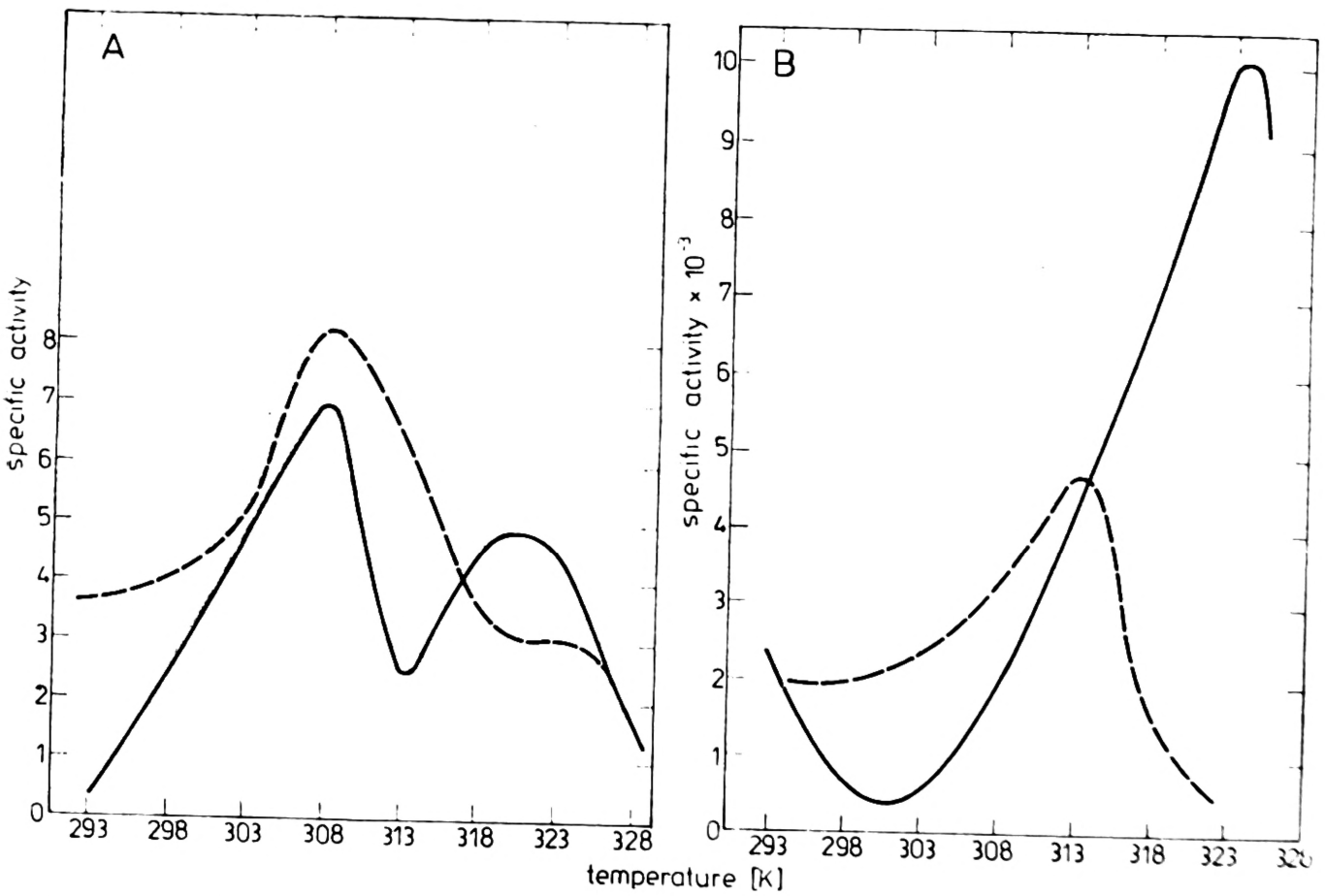


Fig. 2. Effect of temperature on the activity of β-galactosidase of *Str. lactis* 192 (A) and *Lbc. lactis* F-16. Solid line — intracellular extract, broken line — cell wall extract

As to thermal stability of β-galactosidase, it was found that the enzyme of *Str. lactis* 192 is more sensitive to temperature than that of *Lbc. lactis* F-16. A two-minute heating of intracellular extract of *Str. lactis* 192 at 328 K (55°C) inactivated the enzyme completely. On the other hand, the cell wall β-galactosidase demonstrated 11% of the initial activity after 1-2 minutes of heating at 328 K (55°C), and 22% of the initial activity after 10 minutes of heating (Fig. 3). Also the β-galactosidase from *Lbc. lactis* F-16 cell wall extract suffered a severe loss of activity (down to 30% of the initial activity) after 10 minutes at 328 K (55°C). The best thermal stability was demonstrated by intracellular β-galactosidase of *Lbc. lactis* F-16: after 1-2 minutes of heating at 328 K (55°C) the activity showed over 50%, and after 5-10 minutes of heating at this temperature it rose to 70% of the initial activity (Fig. 3B). The differences in thermal stability level between the investigated strains of milk fermentation bacteria strains must be due to the greater thermal immunity of lactobacilli.

Comparing the specific activities of the investigated *Str. lactis* 192 and *Lbc. lactis* F-16 extracts in optimum pH and temperature conditions, we

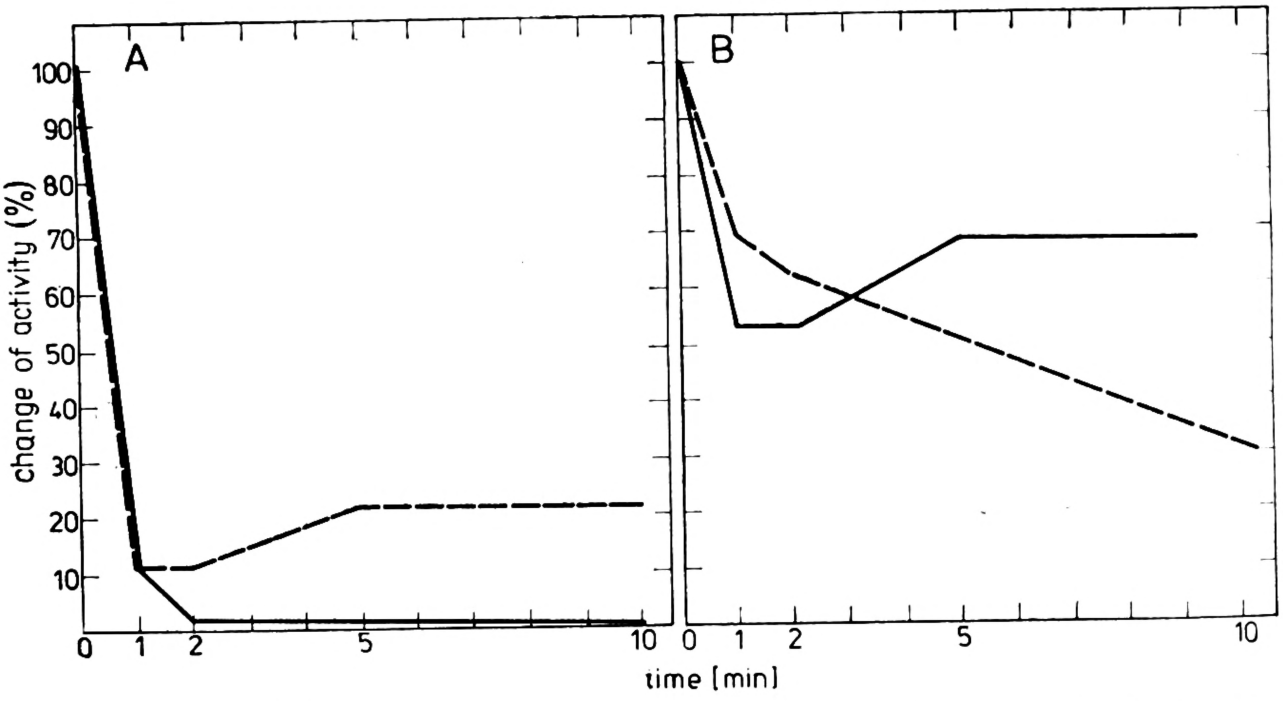


Fig. 3. Thermal stability of β-galactosidase of *Str. lactis* 192 (A) and *Lbc. lactis* F-16 (B). Solid line — intracellular extract, broken line — cell wall extract

see that the highest β-galactosidase activity is demonstrated by the cell wall extract of *Str. lactis* 192 (37 mg of protein per 1 μM o-nitrophenol), and the lowest activity by the cell wall extract of *Lbc. lactis* F-16 (210 mg of protein per 1 μM o-nitrophenol). The β-galactosidase activity in the intracellular extract of *Lbc. lactis* F-16 was twice higher than in the cell wall extract. For the lactobacilli this dependence was reversed: the cell wall enzyme was three times more active that the intracellular enzyme.

The fermentative ability of *Str. lactis* 192 and *Lbc. lactis* F-16 was next examined by checking pH changes in 0.5-, 1.0-, 2.0-, 3.0 and 4.0-per-cent lactose solutions with extract additions during 24 hours of incubation. Significant pH changes during incubation were found in the case of *Str. lactis* 192. Enzymes of the intracellular extract decreased pH by 0.98 on the average. The effect of lactose concentration on the level of hydrolysis and fermentation was observed (Fig. 4A, a). Over the same period, i.e. during 24 hours, the pH of the 0.5-per cent solution dropped by 0.473, and in the 4.0-per cent solution by 1.24. It may thus be said that the activity of β-galactosidase and of lactose-fermenting enzymes contained in the intracellular extract of *Str. lactis* 192 was clearly stimulated by the concentration of substrate in the solution. On the other hand, no dependence between lactose concentration in the solution and ultimate changes of pH was observed in the *Str. lactis* 192 cell wall extract.

The fermentative ability of the *Lbc. lactis* F-16 extract is different. The intracellular extract reduced pH by an average of 0.567 and the cell wall extract by a more 0.088 (Fig. 4B). The concentration of lactose had no effect on pH reduction in either of the *Lbc. lactis* F-16 extracts.

The effect of selected ions on the activity of β-galactosidase was studied. The activity of the enzyme was assayed directly after the ions were

added and the obtained value was taken as 100%; subsequent measurements were performed after 24 and 48 hours of incubation. As is shown in Table 1, the effect of ions on the hydrolytic activity of β-galactosidase was very diverse. Each ion acted differently in dependence on the time of reaction and on the kind of extract. In the case of intracellular extract of *Str. lactis* 192 a 5 mM addition of $Mn^{+2}$ ions lowered β-galactosidase activity by 12% after 24 hours of incubation and then raised it by 16% after 48 hours (Table 1). $Mn^{+2}$ and $Ca^{+2}$ ions stimulated β-galactosidase activity in cell wall extracts of both *Lbc. lactis* F-16 and *Str. lactis* 192. $Mg^{+2}$ ions stimulated the activity of enzymes in both cell wall extracts,

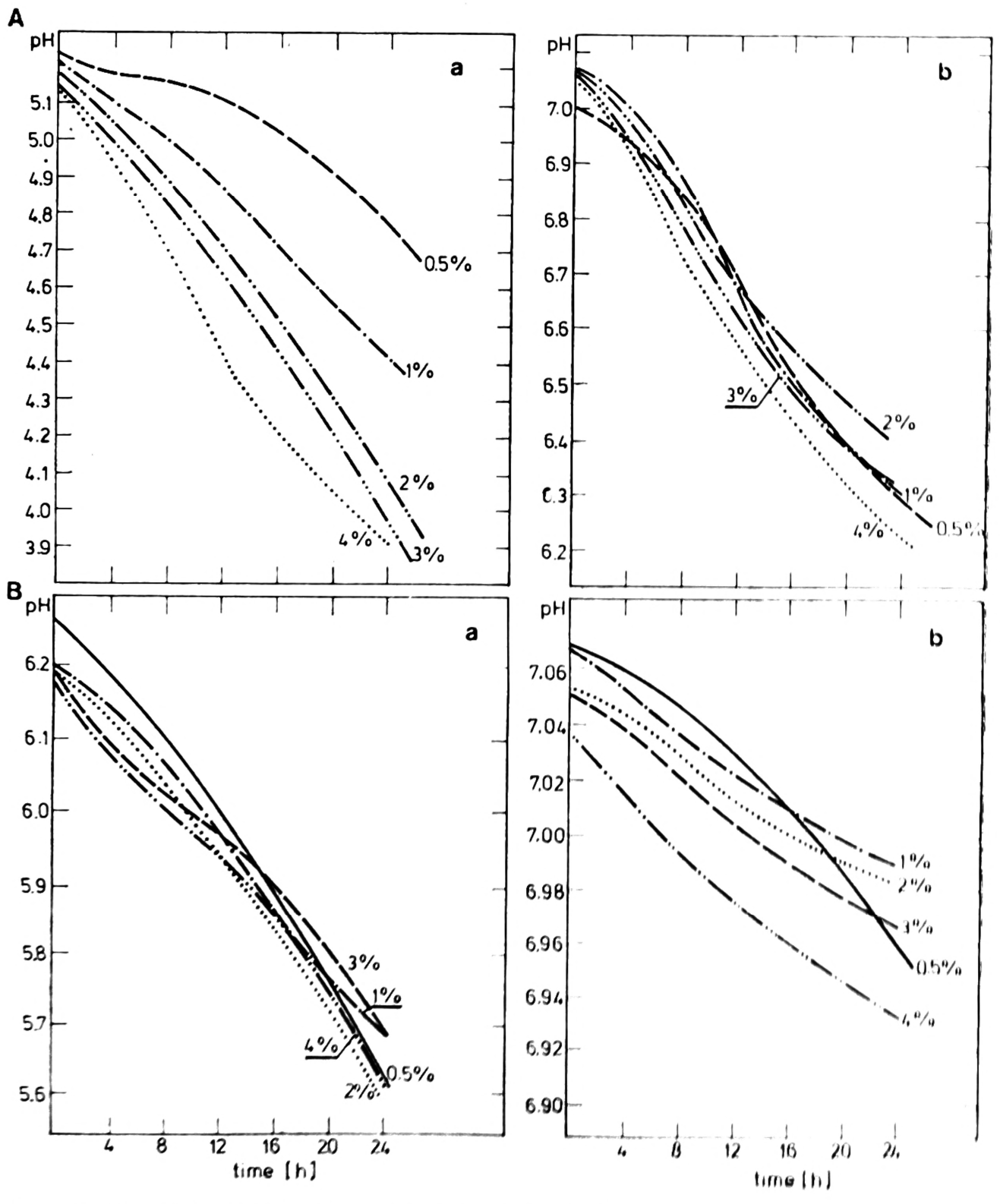


Fig. 4. Kinetics of lactose fermentation by intracellular extract (a) and cell wall extract (b) of *Str. lactis* 192 (A) and *Lbc. lactis* F-16 (B)

Table 1. Effect of selected ions on the activity of β-galactosidase of *Str. lactis* 192 and *Lbc. lactis* F-16

| Strain | Ions in 5 mM concentration | Intracellular extract | | | | Cell wall extract | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | μmol of o-nitrophenol liberated from ONPG in 1 min by 1 mg of protein | change in activity (%) | μmol of o-nitrophenol liberated from ONPG in 1 min by 1 mg of protein | change in activity (%) | μmol of o-nitrophenol liberated from ONPG in 1 min by 1 mg of protein | change in activity (%) | μmol of o-nitrophenol liberated from ONPG in 1 min by 1 mg of protein | change in activity (%) |
| *Str. lactis* 192 | $Mn^{+2}$ | 0.375 | −12 | 0.435 | + 16 | 0.115 | − 2 | 0.275 | +140 |
| | $Mg^{+2}$ | 0.310 | + 5 | 0.285 | − 9 | 0.025 | + 40 | 0.060 | +140 |
| | $Ca^{+2}$ | 0.380 | − 4 | 0.375 | − 1 | 0.050 | + 40 | 0.050 | + 1 |
| | $SO_4^{-2}$ | 0.300 | +12 | 0.312 | + 4 | 0.048 | − 42 | 0.025 | − 47 |
| *Lbc. lactis* F-16 | $Mn^{+2}$ | 0.075 | −90 | 0.025 | − 67 | 0.060 | 0 | 0.102 | + 70 |
| | $Mg^{+2}$ | 0.048 | −89 | 0.072 | + 52 | 0.020 | +275 | 0.040 | +100 |
| | $Ca^{+2}$ | 0.182 | −68 | 0.075 | − 59 | 0.042 | +241 | 0.102 | +141 |
| | $SO_4^{-2}$ | 0.025 | +70 | 0.050 | +100 | 0.058 | + 35 | 0.080 | + 39 |

while $SO_4^{-2}$ ions stimulated the activity of β-galactosidase contained in the intracellular extract of *Str. lactis* 192 and the cell wall extract of *Lbc. actis* F-16.

Also examined was the effect of storage of *Str. lactis* 192 cells, in frozen and lyophilized state, on β-galactosidase activity. Three-month storage of frozen lactobacillus cells at 253 K (-20°C) did not decrease enzyme activity to any serious extent. It remained on the level of 71 mg of protein per 1 μm of o-nitrophenol in the intracellular extract, and 27 mg of protein per 1 μm of o-nitrophenol in the cell wall extract (Table 2). On the other hand, in lyophilized cells the activity of intracelluar enzyme dropped after three months of storage from 77 to 122 mg of protein per 1 μm of o-nitrophenol. In the cell wall the activity of β-galactosidase increased during the same period from 36 to 33.5 mg of protein per 1 μM of o-nitrophenol (Table 2).

Table 2. Effect of storage on activity of *Str. lactis* 192 β-galactosidase

| Storage | Time of storage (months) | Intercellular extract | | Cell wall extract | |
|---|---|---|---|---|---|
| | | μmol of o-nitrophenol liberated from ONPG in 1 min by 1 mg protein | specific activity (mg of protein per 1 μmol of o-nitrophenol) | μmol of o-nitrophenol liberated from ONPG in 1 min by 1 mg protein | specific activity (mg of protein per 1 μmol of o-nitrophenol) |
| In frozen state | 1 | 0.310 | 75 | 0.165 | 29 |
| | 2 | 0.155 | 70 | 0.095 | 26 |
| | 3 | 0.147 | 74 | 0.103 | 27 |
| In lyophilized state | 1 | 0.150 | 77 | 0.080 | 36 |
| | 2 | 0.115 | 105 | 0.115 | 24 |
| | 3 | 0.105 | 122 | 0.090 | 33.5 |

The drop of β-galactosidase activity in lyophilized preparations was probably due to the fact that the cells subjected to lyophilization have previously been disintegrated; moreover, no protective substances have been used during freezing and freeze drying.

## DISCUSSION

The results of this work are largely compatible with the available data from the literature. Hemme et al. [2-5] as well as other authors studying the activity of β-galactosidase of various bacteria strains (*Str. thermophilus*, *Str. cremoris* H, *Leuconostoc citrovorum*) have demonstrated it to be maximal in various pH and temperature conditions. The results obtained here are in agreement with these findings.

The studies of thermal stability of β-galactosidase of ten strains of *Str. thermophilus* performed by Hemme et al. [2] demonstrated that after 5 minutes heating at 328 K (55°C) four strains retained 60-80% of the initial activity, five others from 25% to 38%, and one strain less than 5% of the initial activity. The study of the properties of β-galactosidase of *Str. lactis* 192 and *Lbc. lactis* F-16 brought similar results. The enzyme of lactic streptococcus, a mesophylic strain, was more susceptible to the action of temperature.

According to Jagota et al. [6] and Ramana Rao [9], the activity of β-galactosidase of *Str. cremoris* H and *Str. thermophilus* was stimulated by $Mn^{+2}$, $Mg^{+2}$ and $K^{+1}$, but severely inhibited by $Hg^{+2}$, $Cu^{+2}$ and, to a lesser degree, by $Ca^{+2}$.

On the other hand, Singh [12] reports that the activity of β-galactosidase of *Leuconostoc citrovorum* was inhibited by $Mg^{+2}$, $Mn^{+2}$, and especially severely by $Hg^{+2}$ and $Ni^{+2}$.

Hemme et al. [3-5] studied ten strains of *Str. thermophilus* and found that Mg ions inhibit enzyme activity in two cases but stimulate it quite considerably in ally the others. The $SO_4^{-2}$ ions inhibit β-galactosidase of all the strains.

Referring the above data to the results of this work, we may say that the selected ions did indeed have a various effect on the activity of β-galactosidase of different bacteria strains. The ions also acted differently on one and the same strain in dependence on the kind of substrate. For example, an addition of $Mn^{+2}$ ions is decidedly inhibitory towards the enzyme in the intracellular extract of *Lbc. lactis* F-16, but it stimulates the enzyme in cell walls. In turn, an addition of $Ca^{+2}$ ions markedly reduces the activity of the intracellular enzyme of *Lbc. lactis* F-16 but clearly boosts this activity in the cell walls. A comparison of our results with those of other authors is difficult because of differences in preparation of the study material.

Hemme et al. [2, 3] experimented with crude enzymatic extracts. Jagota et al. [6], Ramana Rao et al. [9] and Singh et al. [12], on the other hand, characterized partly refined enzymes from a purely biochemical viewpoint which doubtless increases the cognitive value of their work.

However, in industrial technology we use bacteria of refined cultures in the form of starters and thereby have to do with simultaneous action of the separate enzymatic groups. The direction of changes occurring in the product is thus a resultant of enzymatic processes, a situation that is not reflected in the activity of a pure enzyme. Hence, the study of the properties of unrefined enzymes is not without sense if only because a wide-scale application of pure enzymes in milk technology is not likely to occur soon; indeed, it is not certain that it would be at all justified.

## CONCLUSIONS

1. Significant differences in β-galactosidase activity were determined between the studied milk fermentation bacteria and the substrates that were used. The optima of β-galactosidase activity were the following:

— in the intracellular extract of *Str. lactis* 192: temperatures of 308 K (35°C) and 318-323 K (45-50°C), pH 7.0;

— cell wall extract of *Str. lactis* 192: temperature of 308 K (35°C), pH 5.0;

— in intracellular extract of *Lbc. lactis* F-16: temperature 323 K (50°C), pH 6.0;

— in the cell wall extract of *Lbc. lactis* F-16: temperature of 313 K (40°C), pH 7.0.

2. The thermal stability of β-galactosidase of *Str. lactis* 192 was lower than of *Lbc. lactis* F-16.

3. The extracts of *Str. lactis* 192 demonstrated a higher activity in fermenting lactose to milk acid than the respective extracts of *Lbc. lactis* F-16.

4. The effect of ions on β-galactosidase activity was diversified and depended on the time of activity, the kind of extract and the bacteria strain. The $Mn^{+2}$ and $Ca^{+2}$ ions stimulated the activity of β-galactosidase in cell walls of both strains of lactic bacteria, $Mg^{+2}$ ions were stimulatory in all four extracts while $SO_4^{-2}$ ions stimulated the activity of the intracellular extract of *Str. lactis* 192 and of cell wall extract of *Lbc. lactis* F-16.

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# WŁAŚCIWOŚCI β-GALAKTOZYDAZY Z KOMÓREK SZCZEPÓW *STREPTOCOCCUS LACTIS* 192 I *LACTOBACILLUS LACTIS* G-16

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Streszczenie

Podjęto próbę charakterystyki β-galaktozydazy syntetyzowanej przez komórki *Str. lactis* 192 i *Lbc. lactis* F-16 na podstawie wyznaczenia optimum pH i temperatury działania enzymu, stabilności termicznej i wpływu wybranych jonów. Stwierdzono istotne różnice w aktywności β-galaktozydazy pomiędzy badanymi szczepami, a także rodzajami ekstraktów. Optimum działania β-galaktozydazy zawartej w odpowiednich ekstraktach przypadało w warunkach:

— ekstrakt wewnątrzkomórkowy *Str. lactis* 192: temp. 308 K (35°C) i 318-323 K (45-50°C), pH 7.0,

— ekstrakt ściany komórkowej *Str. lactis* 192: temp. 308 K (35°C) i pH 5,0,

— ekstrakt wewnątrzkomórkowy *Lbc. lactis* F-16: temp. 323 K (50°C) i pH 6,0,

— ekstrakt ściany komórkowej *Lbc. lactis* F-16: temp. 313 K (40°C) i pH 7,0.

Enzym *Str. lactis* 192 wykazał wyższą aktywność fermentacji laktozy oraz niższą stabilność termiczną niż β-galaktozydaza *Lbc. lactis* F-16. Wpływ jonów na aktywność enzymu był zróżnicowany i zależny od czasu działania, rodzaju ekstraktu i szczepu bakterii. Oceniono również wpływ przechowywania komórek bakterii *Str. lactis* 192 w stanie zamrożonym i liofilizowanym na aktywność β-galaktozydazy. Zaobserwowano większy spadek aktywności β-galaktozydazy w przypadku preparatów liofilizowanych. Fakt ten należy tłumaczyć tym, że liofilizacji poddano komórki uprzednio zdezintegrowane, a ponadto nie stosowano żadnych substancji ochronnych podczas zamrażania i suszenia sublimacyjnego.