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Release of β-galactosidase from Lactobacilli

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Summary

Lactobacillus brevis cells at the end of growth release intracellular β-galactosidase into the medium. Enzyme release begins immediately after the end of cell multiplication and is connected to cell autolysis and breakage of the cell wall. *Lactobacillus plantarum* cells did not release β-galactosidase because autolysis occurred in a different way. The cells initially collapsed, without showing signs of cell wall breakage, which became evident only after 30 or more days from the end of growth.

Key words: lactic acid bacteria, autolysis, β-galactosidase

Introduction

It is well known that autolysis in lactic acid bacteria (LAB) and other bacteria caused by so-called autolysins which are made up of four enzymes, each with a definite peptidoglycan hydrolase activity (*1–4*). Unlike the autolysis that occurs in cells of other organisms, *i.e.* yeasts, the breakage of the cell wall of LAB does not necessarily involve other intracellular structures. The compounds released, including enzymes, can be still biologically active. It is an accepted fact that by autolysis LAB and other bacteria release proteolytic enzymes that play an important role in cheese ripening (*5–14*).

Autolysis of LAB could also release other enzymes including β-galactosidase (EC 3.2.1.23) causing extracellular lactose hydrolysis and interesting consequences for the dairy industry. The presence of free β-galactosidase has been determined in yogurt (*15*), but there are many indications that this could also occur in other dairy products. The growth of non-lactose fermenting yeasts, such as *Saccharomyces cerevisiae* and *Sacch. unisporus*, has been reported in some types of cheese (*16*), deformed yogurt tubs (*17*) and fermented milk and whey (*18–20*). The growth of non-lactose fermenting yeasts could be correlated with the traits of some LAB such as *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus* which ferment only glucose and release galactose into the medium (*21,22*), probably with the release of β-galactosidase (from LAB autolysis) and hydrolysis of lactose in the medium.

In order to confirm the validity of this last hypothesis, the current research examined numerous strains of two of the most common lactobacilli, the homofermentative *Lactobacillus plantarum* and the heterofermentative *Lb. brevis*. The β-galactosidase activities of the cells and cell-free supernatant were determined at different times and the state of the cells was examined using a scanning electron microscope.

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Materials and Methods

Organisms

A total of 32 lactose fermenting strains of *Lb. brevis* and 15 strains of *Lb. plantarum* were used, mainly isolated from koumiss fermented milk (*20*), but also from other products such as fermented silage (*23*) and fermented sausages (*24*). These strains were compared with the strains of American Type Culture Collection: *Lb. brevis* ATCC 14869 and *Lb. plantarum* ATCC 14917.

Fermentation

The fermentation tests were carried out using 50 mL of MRS liquid medium (*25*) with 10 g/L glucose. The cultures were examined after 3 and 30 days of incubation at 30 °C.

Preparation of cells and cell-free supernatant

The cells of the entire cultures of 50 mL were collected by centrifugation, washed twice in distilled water and re-suspended in 10 mL of distilled water. Half the suspension (5 mL) was used for the determination of the dry weight of cells per mL using weighing bottles and oven drying at 100 °C until constant weight was reached. The dry weight of cells per mL of medium was found to be 0.7–0.9 mg. The other half of the suspension was diluted to a total of 25 mL using a 0.1 M sodium phosphate buffer (pH=7).

One mL of this suspension and 1 mL of the cell-free supernatant was used for measuring β-galactosidase activity.

Permeabilization of cells from 2 mL of the suspension was achieved by the addition of 50 μL of acetone-toluene (9:1 v/v), shaken vigorously for 7 min and 1 mL immediately assayed for β-galactosidase activity.

β-*galactosidase activity assay*

Release of *o*-nitrophenol (ONP) from *o*-nitrophenol-β-galactopyranoside (ONPG) was used to measure β-galactosidase activity (*26*). 1 mL of cell suspension or 1 mL of cell-free fermented broth was incubated with ONPG solution (5 mM ONPG in 0.05 M sodium phosphate

Table 1. β-galactosidase activity of 32 strains of *Lactobacillus brevis* cells and cell-free fermented broth after 3 and 30 days (values are means of three replicates)

Strain	β-galactosidase activity (units*) after					
	3 d			30 d		
	dry cells	cell-free supernatant	total activity	dry cells	cell-free supernatant	total activity
1026	0.796	0.356	1.152	0.499	0.549	1.048
1028	0.869	0.044	0.913	0.140	0.639	0.779
1066	0.803	0.109	0.912	0.038	0.742	0.780
1167	0.782	0.348	1.130	0.276	0.747	1.023
1190	0.798	0.091	0.889	0.239	0.533	0.772
1198	0.794	0.151	0.945	0.281	0.527	0.808
1217	0.810	0.374	1.184	0.264	0.659	0.923
1222	0.796	0.292	1.088	0.253	0.534	0.787
1230	0.770	0.356	1.026	0.253	0.663	0.916
1250	0.788	0.374	1.162	0.341	0.654	0.995
1269	0.780	0.021	0.801	0.014	0.684	0.698
1277	0.790	0.28	1.070	0.270	0.657	0.927
1278	0.691	0.463	1.154	0.467	0.620	1.087
1288	0.795	0.330	1.125	0.250	0.610	0.860
1295	0.821	0.493	1.314	0.243	0.767	1.010
1310	0.679	0.621	1.300	0.317	0.783	1.100
1317	0.775	0.335	1.110	0.410	0.583	0.993
1320	0.826	0.010	0.836	0.330	0.458	0.788
1322	0.735	0.527	1.162	0.251	0.713	0.964
1334	0.728	0.439	1.167	0.138	0.791	0.929
1336	0.836	0.335	1.171	0.217	0.582	0.799
1338	0.712	0.401	1.113	0.205	0.761	0.966
1348	0.831	0.305	1.136	0.247	0.721	0.968
1357	0.808	0.374	1.182	0.249	0.756	1.005
1386	0.775	0.122	0.897	0.353	0.538	0.891
1391	0.626	0.489	1.015	0.187	0.733	0.920
1392	0.635	0.476	1.011	0.316	0.582	0.998
1397	0.703	0.488	1.191	0.265	0.630	0.895
1404	0.836	0.354	1.190	0.114	0.690	0.804
1409	0.712	0.114	0.826	0.236	0.417	0.753
1410	0.831	0.381	1.212	0.187	0.694	0.881
1411	0.834	0.229	1.063	0.054	0.830	0.884
Average	0.774	0.336	1.080	0.235	0.655	0.902
± SD	0.059	0.145	0.124	0.088	0.155	0.078

* unit definition see in the chapter β-*galactosidase activity assay*

buffer, pH=7) at 37 °C for 15 min. Colour development was stopped by adding 5.0 mL of cold 0.5 M sodium carbonate to the reaction mixture. Absorbance was measured at 420 nm. The μmoles of ONP liberated were determined from a standard curve measuring the change in absorbance produced by various ONP concentrations. The amount of ONP released/min by the cells or the cell-free supernatant was directly proportional to the quantity of enzyme. One unit of enzyme was equivalent to 1 μmole of ONP liberated from ONPG/(min/mL) of cell suspension or cell-free supernatant.

Statistical evaluation of data

Data for β-galactosidase activity were evaluated by analysis of variance. The least-significant-difference test was used to compare means.

Results and Discussion

β-galactosidase activity

A preliminary trial carried out without replica showed that 32 strains of *Lb. brevis* and 15 strains of *Lb. plantarum*, with the exception of *Lb. brevis* ATCC 14869 strain, were fermenting lactose and possessed constitutional β-galactosidase. A cell suspensions, 3 days old, grown in glucose produced ONP from ONPG. The two lactobacilli were, however, widely different concerning enzyme release into the medium.

With *Lb. brevis*, the release of β-galactosidase began very quickly, immediately after 3 days of growth. Indeed, β-galactosidase activity of the 3-day-old cell-free supernatant was already positive, though with widely differing intensity in different strains. This activity was at a barely measurable level but definitely positive in three of the 32 strains tested and highly positive in the remaining 29 strains with a maximum value found in strain 1310 (Table 1). The sum of the activity of cells contained in 1 mL of medium and in 1 mL of cell-free supernatant expressed the total β-galactosidase capacity of each strain.

After 30 days, all the strains had released β-galactosidase and the medium showed high positive activity. At the same time, with increase in broth activity, there was a considerable reduction in cellular activity. After 30 days, the overall activity (cells+broth) showed an average reduction of 17–18 % compared with that at 3 days.

The 3-day-old *Lb. plantarum* cells demonstrated lower activity than those of *Lb. brevis* and did not release the enzyme because the fermented broth was completely activity-free (Table 2). The 30-day-old cells showed a distinct drop in β-galactosidase activity and even the fermented broth was completely inert (data not shown).

Cells autolysis

As β-galactosidase is an intracellular enzyme, the activity determined on whole cells, which were not permeabilized, could be the cause of the difference observed between the two bacterial species and among their strains. The test was repeated in triplicate with the strains that, with reference to the preliminary trial, highlighted the larger differences in activity and the β-galactosidase activity in whole cells permeabilized with organic solvents was assayed.

Table 2. β-galactosidase activity of 16 strains of *Lactobacillus plantarum* cells and cell-free fermented broth after 3 and 30 days (values are means of three replicates)

Strain	β-galactosidase activity (units*) after			
	3 d		30 d	
	dry cells	cell-free supernatant	dry cells	cell-free supernatant
ATCC 14917	0.352	0	0.032	0
1021	0.663	0	0.120	0
1038	0.879	0	0.135	0
1051	0.913	0	0.240	0
1056	0.247	0	0.044	0
1063	0.256	0	0.024	0
1094	0.542	0	0.056	0
1101	0.489	0	0.111	0
1110	0.529	0	0.089	0
1157	0.965	0	0.210	0
1164	0.303	0	0.064	0
1168	0.278	0	0.097	0
1173	0.981	0	0.185	0
1142	0.261	0	0.035	0
1290	0.280	0	0.065	0
1347	0.768	0	0.132	0
Average ± SD	0.544 ± 0.271	0	0.102 ± 0.063	0

* unit definition see in the chapter β-*galactosidase activity assay*

The results shown in Table 3 confirmed the differences in behaviour of the two species and the difference in β-galactosidase activity.

Examined with a scanning electron microscope, some 3-day-old *Lb. brevis* cells showed the presence of pores, a clear sign of autolysis (Fig. 1a). In the 30-day-old cells, the existing autolysis was shown very clearly with holes, fractures and cell wall laceration (Fig. 1b). The damage of the cell walls was in almost all the cells.

Table 3. β-galactosidase activity of permeabilized cells of some strains of *Lactobacillus brevis* and *Lactobacillus plantarum* (values are means of three replicates)

	β-galactosidase activity (units*) after	
	3 d	30 d
Lb. brevis strain		
1190	1.490	0.580
1288	1.336	0.610
1397	1.443	0.630
Lb. plantarum strain		
14917 ATCC type strain	0.715[a]	0.110[a]
1056	0.700[a]	0.120[a]
1110	0.918[b]	0.300[b]
1173	1.610[c]	0.410[c]

[a, b, c] Values in the same column followed by different superscript letters were significantly different: $P<0.05$

* unit definition see in the chapter β-*galactosidase activity assay*

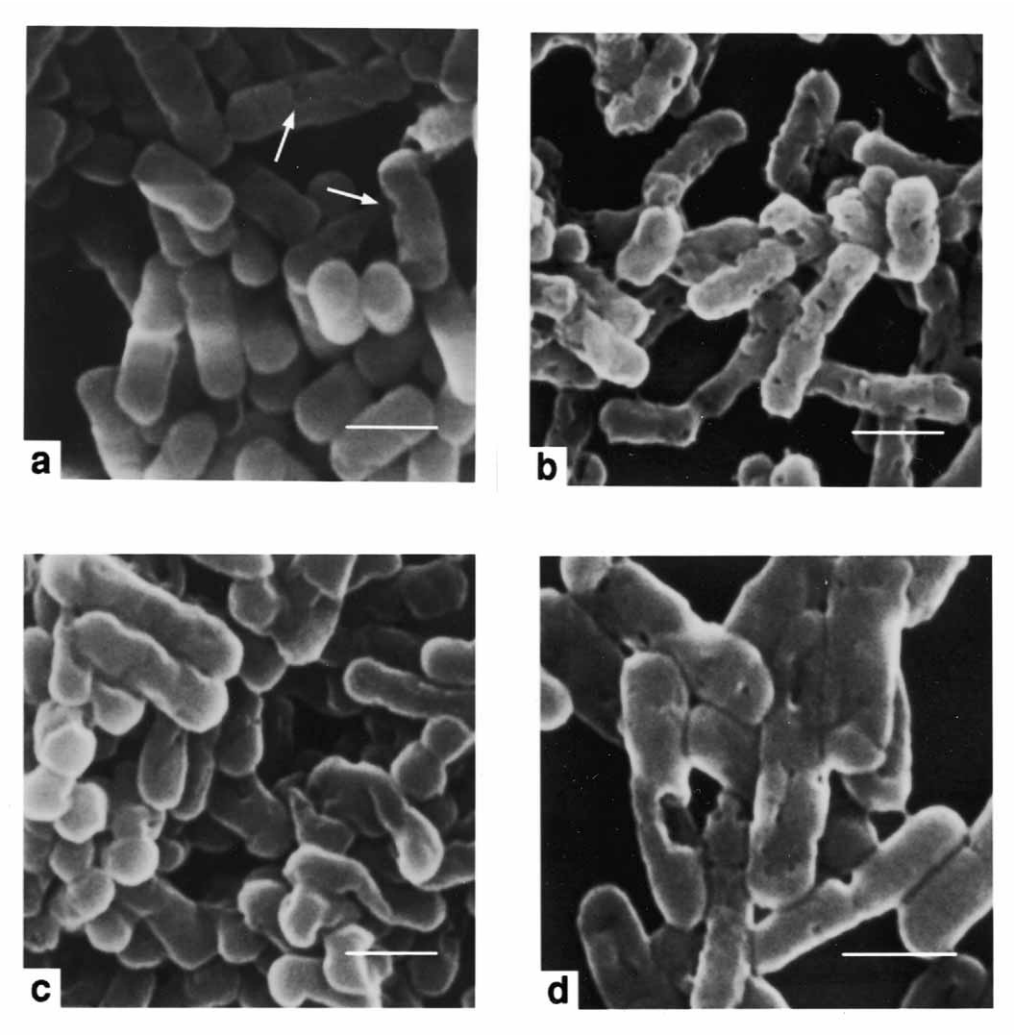

Fig. 1. Three and thirty-day-old *Lb. brevis* and *Lb. plantarum* cells examined with a scanning electron microscope: a) three-day-old *Lb. brevis* cells: only some cells have lesions in the cellular wall; b) thirty-day-old *Lb. brevis* cells: most of the cells are autolysed with lesions and holes in the cellular wall; c) three-day-old *Lb. plantarum* cells: only some cells are collapsed; d) thirty-day-old *Lb. plantarum* cells: the cells are collapsed or show clear signs of lesions on the wall. All scale bars, 1 μm

In *Lb. plantarum*, the signs of cell wall fractures appeared much later and were preceded by cell collapse. In the young cultures, *i.e.* after 3 days of growth, some cells showed cell walls which had collapsed but were still whole and without porosity or evident surface damage (Fig. 1c). In the 30-day-old cultures, the cells with collapsed walls were numerous and some showed clear signs of fractures (Fig. 1d).

The appearance of the cells viewed under scanning electron microscope could explain the differences in the behaviour of the cells to release β-galactosidase. In *Lb. brevis* the release of β-galactosidase into the medium was due to the rapid autolysis with cell-wall breakage. The release of β-galactosidase did not occur in *Lb. plantarum* though, because cell autolysis occurred in a different way. It seems very likely in this last species that compounds with high molecular weight, such as enzymes, may have been hydrolysed before release in a manner similar to that occurring during autolysis of other microorganisms such as yeasts.

Conclusions

Autolysis and the way it comes about is certainly important from a technological point of view and as the selection criteria for bacteria, because the bacterial activity may continue even after the end of fermentation. In particular, the release of β-galactosidase can have a strong effect on the characteristics of fermented foods, especially dairy products. The above mentioned cases of seemingly anomalous development and often the prevalence of non-lactose fermenting yeasts in fermented milk such as koumiss, kefir and others can be explained by lactobacilli autolysis. In these products the pH is low and not optimal for the stability and activity of the β-galactosidase; nevertheless, not optimal pH conditions are compensated by the graduality of autolysis and the consequent gradual release of the enzyme (*27*).

As a result of the autolysis and the way this occurs, the LAB can release compounds other than enzymes. For example *Lb. plantarum* can release compounds which have an inhibiting action on mould (*28*). The same ther-

apeutic action attributed to koumiss and other fermented milks could be a consequence of the autolysis of LAB as reported by Metschnikoff (*29*), Robinson (*30*), Antoine (*31*), Patel and Renz-Schauen (*32*) and others.

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Otpuštanje β-galaktozidaze iz laktobacila

Sažetak

Stanice *Lactobacillus brevis* na kraju rasta otpuštaju intracelularnu β-galaktozidazu u podlogu. Otpuštanje enzima započinje neposredno nakon završetka staničnog razmnožavanja, a povezano je s autolizom stanica i oštećenjem staničnog zida. Stanice *Lactobacillus plantarum* nisu otpuštale β-galaktozidazu jer se autoliza drukčije provodila. Na početku su se stanice urušile na pokazujući znakove oštećenja staničnog zida, što se vidjelo tek nakon 30 ili više dana od završetka rasta.