

Article

Subcellular Localization of β -Galactosidase Protein from Probiotic *Limosilactobacillus fermentum* LF08 Strain: Probability of Cell Wall Association

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Abstract

Lactic acid bacteria exhibit high adaptability to their environment due to their wide variety of enzymes. Despite extensive knowledge of bacterial cell walls, the subcellular localization of β -galactosidase in many probiotic lactic acid bacteria, including *Limosilactobacillus fermentum*, remains unclear. Determining the cellular localization of such enzymes may improve insight into bacterial metabolic mechanisms and support the development of efficient downstream processes, as well as applications. In this study, three cell disruption strategies (mechanical homogenization and chemical disruption with different agents) were applied to assess the subcellular localization of β -galactosidase from the *Ll. fermentum* LF08 strain. Enzyme activity was measured in a ferment broth, a supernatant and cell-associated fractions. No and very low β -galactosidase activity was detected in the ferment broth and the supernatant, respectively, when either chemical or mechanical treatment was applied, whereas the main enzyme activity was assayed in the cell suspension fraction. Combined lysozyme and CTAB treatment resulted in a 21.4-fold increase in β -galactosidase activity in the supernatant fraction (2.14 U/mL), compared with CTAB treatment alone (0.10 U/mL). Bioinformatic analyses provided additional significant information to propose the potential cell wall association (maybe the outer side of the cell wall) of the subcellular localization of β -galactosidase. This feature may support understanding of the interactions between probiotic bacteria and host tissues, as well as the development of probiotic immobilized cell systems for applications such as the elimination of lactose, designing novel functional foods.

Keywords: β -galactosidase; cell disruption; lactic acid bacteria; bioinformatic tools; subcellular localization



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1. Introduction

Enzymes are biocatalysts crucial to enabling metabolic and biochemical reactions. They can be extracted and purified from various sources, with primary attention given to microorganisms [1]. β -galactosidase catalyzes the hydrolysis of lactose into glucose and galactose and plays an important role in both food science and nutrition, as well as waste management and utilizing dairy byproducts [2]. This enzyme can be extracted from different sources with different features, but in the industry, *Aspergillus* mould and *Kluyveromyces* yeast [3] are dominant.

Lactic acid bacteria are one of the most important microbial groups that have a GRAS status (Generally Recognized as Safe) [4], and are relevant in the food production system, including food preservation, the production of fermented foods, etc. The main advantage of lactic acid bacteria is their high adaptability towards their living environment because of their versatility in the production and secretion of different proteins, including extracellular, cytoplasmic, periplasmic or surface-associated proteins. These proteins can participate in a wide variety of processes, such as recognition binding, signal transduction, communication with the environment, and attachment of the bacterial cell to specific sites or surfaces [5]. Among the lactic acid bacteria, *Limosilactobacillus fermentum* has gained considerable attention due to its probiotic properties and potential applications in food technology. It has been isolated from various environments, including fermented foods, human milk, and the gastrointestinal tract [6]. In addition to its probiotic relevance, this strain has attracted interest as a potential producer of β -galactosidase.

In the last few years, studies have been conducted to understand the localization of this enzyme in lactic acid bacteria, and its intracellular nature [7–9] has been reported in most studies. The mode of operation between different cell disruption methods can give us valuable insights into the potential localization of the enzyme. Knowledge and understanding of bacterial cell envelope composition, as well as potential sites where the enzyme can be localized, is critical for the evaluation of experimental results. The bacterial cell envelope is a complex structure that serves a protective role and functions as a semipermeable membrane. It selectively permits the entry of nutrients from the external environment while facilitating the release of waste products from within the cell. The cell membrane complex in Gram-negative bacteria has a thin peptidoglycan layer surrounded by an outer membrane made from lipopolysaccharides. On the other hand, in Gram-positive bacteria, there is no outer membrane, but the layer of peptidoglycan is much thicker [10]. Proteins in Gram-positive bacteria can be targeted to the cytoplasm, cytoplasmic membrane, cell wall, or extracellular environment. Protein synthesis occurs in the cytoplasm, which suggests that there is a need for a mechanism that can transport the protein into the cytoplasmic membrane or through the cytoplasmic membrane. The proteins that are translocated are usually produced as precursor proteins with an amino-terminal signal peptide. Precursor proteins are recognized by targeting factors. Some proteins are attached to the membrane through lipid modification, while others are immobilized on the cell wall through electrostatic or covalent interactions [11]. In Gram-positive bacteria, after the synthesis of proteins in the cytoplasm, 5–10% are released outside the cytoplasmic membrane [12]. The majority of the translocated proteins in Gram-positive bacteria follow the general protein secretion (Sec) pathway, which includes SecA, SecY, SecE, and SecG, along with several supplementary proteins [13]. Certain proteins, for example, metabolic enzymes, can be attached to the surface, especially if the function of the enzyme is to dismantle nutrient polymers, which can afterwards be taken by the cell. However, not all proteins localized on the surface have a role in the utilization of the nutrients [14]. Despite extensive knowledge of Gram-positive cell envelope architecture and protein secretion pathways, the subcellular localization of β -galactosidase in the probiotic strains of

Limosilactobacillus fermentum remains poorly understood. Identifying whether this enzyme is predominantly cytoplasmic, membrane-associated, cell wall-associated, or extracellular is essential for understanding its physiological role and for developing efficient downstream recovery strategies. Therefore, this study aimed to investigate the subcellular localization of β -galactosidase in the probiotic strain *Limosilactobacillus fermentum* LF08 using different cell fractionation methods after disruption in combination with bioinformatic tools.

2. Materials and Methods

2.1. Probiotic Culture

Probiotic bacterium *Limosilactobacillus fermentum* LF08 strain was obtained from Probiotical S.p.A (Novara, Italy). The strain was maintained in the de Man, Rogosa, and Sharpe (MRS) medium [15].

2.2. Production of β -Galactosidase

The inoculum was prepared by culturing bacterial cells in modified de Man, Rogosa, and Sharpe broth containing 2% (*w/v*) lactose (Reanal, Budapest, Hungary) as a carbon source at 37 °C for 24 h. The fermentation for the production of the enzyme was carried out using modified de Man, Rogosa, and Sharpe (mMRS) broth supplemented with 4% (*w/v*) lactose as a carbon source and inducer. The process was initiated by inoculating the medium with 5% (*v/v*) of the propagated cell suspension and carried out at 37 °C for 16 h. Then, the bacterial cells were harvested by centrifugation at 8400 g at 4 °C for 10 min. The cell biomass was washed twice with 66.7 mM Sorensen phosphate buffer (pH 6.5) and finally resuspended in the desired volume of the same buffer [16].

2.3. Cell Disruption

Three methods were applied for cell disruption: homogenization with a French press, chemical disruption using cetyltrimethylammonium bromide (CTAB) (VWR International Kft., Debrecen, Hungary), and a biological method with lysozyme. These methods provide complementary information for the evaluation of the subcellular localization of the β -galactosidase protein. After the cell disruption process, the activity of β -galactosidase was assessed in two fractions: a supernatant fraction (SF, obtained after the centrifugation of the disrupted cells), and a broken cell suspension (BCS, whole disrupted cells prior to centrifugation).

2.3.1. Mechanical Method

In the case of the mechanical method, homogenization with a French press, 12,700 PSI (pounds per square inch) pressure, was applied. After the completion of the cell disruption process, two fractions were separated by centrifugation at 8400 g for 10 min.

2.3.2. Chemical Method

The chemical method was performed by testing different ratios of cell suspension to CTAB (1:0.5, 1:1, and 1:2) in volume (*v/v*). The CTAB concentration was always 0.45 mg/mL, and the treatment time periods were 10, 20, 30, 60, and 120 min at room temperature.

2.3.3. Biological Method

Lysozyme from chicken egg white (Sigma-Aldrich, Budapest, Hungary) was used in the biological cell disruption method. The enzyme dose and the incubation time for the disruption of bacterial cells were optimized using a central composite experimental design (CCD). β -galactosidase activity (U/mL) was evaluated as a response dependent variable. The center point was set at an enzyme dose of 50 mg/mL and an incubation time of 150 min. In total, 13 experimental runs were performed in an incubator at a constant

temperature of 37 °C (Table 1). The results were analyzed using Minitab statistical software version 20.4 (Minitab, LLC., State College, PA, USA).

Table 1. The experimental results of β -galactosidase activity while treating the *Ll. fermentum* LF08 strain under various lysozyme concentrations and incubation times.

Run	Lysozyme Concentration (mg/mL)	Incubation Time (min)	Enzyme Activity (U/mL)
1	50	277	5.33
2	20	240	4.56
3	50	22	0.08
4	50	150	1.83
5	50	150	2.53
6	80	240	3.77
7	50	150	2.40
8	20	60	0.85
9	50	150	2.87
10	92.4	150	1.96
11	50	150	2.58
12	80	60	1.24
13	7.5	150	1.92

2.3.4. Combination of Biological and Chemical Methods

To ensure information on the subcellular localization of β -galactosidase protein, two cell disruption methods were combined separately and simultaneously. In the simultaneous combination, both CTAB and lysozyme were added initially to the cell suspension and incubated further for 120 min, while in the separated approach, the bacterial cells were first treated with CTAB for 120 min, followed by centrifugation and resuspension of the cell pellet in 66.7 mM Sorensen phosphate buffer (pH 6.5). Subsequently, lysozyme was added and incubated for an additional 120 min.

2.4. Enzyme Activity Assay

The artificial substrate p-nitrophenyl- β -D-galactopyranoside (Thermo Fisher Scientific Inc., Budapest, Hungary) was used in the assay of β -galactosidase enzyme activity [17]. Briefly, 0.3 mL buffer and 0.5 mL 1.5 mM substrate were pipetted into test tubes that were pre-incubated at 50 °C for 5 min. The enzyme reaction was initiated by adding 0.2 mL of the appropriately diluted enzyme sample. After 5 min, the reaction was stopped by adding 5.0 mL of 0.1 N Na₂CO₃, and the concentration of released p-nitrophenol was determined spectrophotometrically at 405 nm. A standard curve was prepared using p-nitrophenol prepared in the same Sorensen buffer. One unit of enzyme activity was defined as the amount of enzyme releasing 1 μ mol p-nitrophenol per minute under the defined reaction conditions.

2.5. Bioinformatic Evaluation of the Localization of the Enzyme in the Bacterial Cells

The subcellular localization of β -galactosidases produced by different lactic acid bacteria was predicted by three software programs: CELLO v2.5 (Molecular Bioinformatics Center, National Yang Ming Chiao Tung University, Taiwan, Accessed: February 2026), DeepLoc v2.1 (Department of Health Technology, Technical University of Denmark, Denmark, accessed: February 2026), and Gpos-Ploc (<http://www.csbio.sjtu.edu.cn/bioinf/Cell-PLoc/>, accessed on 8 June 2026). A total of 12 protein sequences and encoded genes from *Lactobacillus helveticus*, *Lactobacillus acidophilus*, *Lacticaseibacillus casei*, *Limosilactobacillus fermentum*, *Lactiplantibacillus plantarum*, and *Limosilactobacillus reuteri*, respectively, were collected from the European Bioinformatics Institute (ebi.ac.uk) and analyzed.

2.6. Statistical Analysis

The fermentation experiments, cell disruption, and β -galactosidase activity assay were generally carried out in at least triplicate (three independent runs). The data were processed in a Microsoft Excel spreadsheet and expressed as the mean $\pm$ SE of different independent replicates. One-way analysis of variance (one-way ANOVA) and an experimental design were conducted using Minitab software (version 20.4, Minitab LLC., State College, PA, USA) to test the differences between variances and to analyze the experimental results of CCD, respectively. Data were considered significant at $p < 0.05$.

3. Results and Discussion

Cell mass of the *Limosilactobacillus fermentum* LF08 strain was produced by growing this bacterium in mMRS. The ferment broth was then separated into two fractions by centrifugation: cell biomass and broth fraction. β -galactosidase activity was checked in the broth fraction, and no activity was detected. It led to the conclusion that the probiotic *Limosilactobacillus fermentum* LF08 strain does not secrete β -galactosidase extracellularly; instead, it may synthesize this enzyme intracellularly or may be cell-wall-associated.

The cell suspension was subjected to disrupt using a French press in three cycles and finally it was subsequently separated into two fractions by centrifugation. The broken cell fraction was resuspended in buffer to its original volume, and it was designated as BCS, while the supernatant fraction was designated as SF. The enzyme activity in the BCS fraction was 16 U/mL, whereas the enzyme activity of SF was only 4 U/mL (Figure 1).

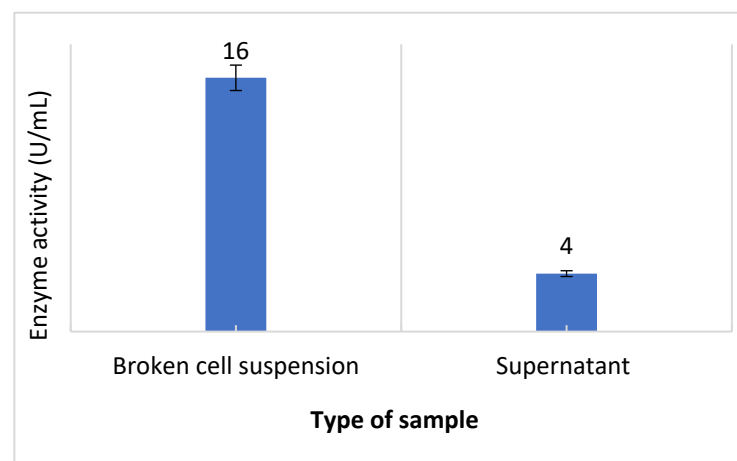


Figure 1. β -Galactosidase enzyme activity in different fractions obtained by the French press cell disruption method.

The mechanism of French press cell disruption used a hydraulic press to force a liquid cell suspension through a tiny, manually controlled needle valve at extreme pressure, thus breaking the cell wall and disrupting cells [18], releasing a cytoplasmic liquid fraction. After three cycles, less than 20% of enzyme activity was released from the cytoplasmic liquid (SF), while the main portion of the produced β -galactosidase enzyme was found in the BCS (associated with the cell wall). This observation initially suggested a possible association of the enzyme with cell wall structure, because if the enzyme is of a cytoplasmic nature (synthesized in the cytoplasm), the activity in the SF must be significantly higher than that in the BCS. Additionally, the incomplete disruption by the French press method cannot result in higher activity in the BCS than in the SF, because the cytoplasmic protein in the untouched cells is also not accessible in the activity assay (not assayable). Our results led to the proposal that β -galactosidase may associate with the cell wall, and there was

initial evidence of the subcellular localization of β -galactosidase from the *Ll. fermentum* LF08 strain.

The chemical cell disruption method used cetyltrimethylammonium bromide (CTAB) to search for new evidence for ensuring the localization of β -galactosidase. CTAB belongs to the group of cationic surfactants, which comprises a long alkyl chain and a quaternary-ammonium cation. As a cationic detergent, CTAB's amphipathic structure binds to and solubilizes lipid bilayers, causing cell lysis and releasing intracellular contents (cytoplasmic fraction). This surfactant, depending on the provided quantity, can be used for biomass harvesting or the extraction of bioproducts [19]. The method for cell lysis based on utilization of the CTAB lysis buffer for subsequent purification of DNA from rumen fluid, plant cells, as well as from pure cultures of Gram-positive and Gram-negative bacteria, has also been reported in the literature [20]. It has been outlined that the DNA yield with the implementation of this method exceeds the yield obtained with commercial kits.

In our case, different volume ratios of CTAB to cell suspension were tested (1:0.5, 1:1, and 1:2), with a maximum CTAB concentration of 0.45 mg/mL for various treatment times. The chosen dose of CTAB is also important for the effectiveness of the experiments. The dose of 0.45 mg/mL used during our experiments was in line with previously performed studies [21]. After incubation at room temperature at time intervals, two fractions—the broken cell suspension (BCS) and the supernatant fractions (SF)—were separated by centrifugation. The β -galactosidase activity of both fractions was assayed and is presented in Figure 2.

Figure 2a demonstrates that in the broken cell fraction, the highest enzyme activities were recorded at treatment times of 30 and 60 min, with values of 29 U/mL and 27 U/mL, respectively, when the ratio of cell suspension to CTAB was 1:1. Further increase in the treatment time to 120 min resulted in a decrease in activity to 14 U/mL. In the case when the ratio of cell suspension to CTAB was 1:0.5, the maximum enzyme activity (16 U/mL) was observed at a treatment time of 20 min and then decreased slightly to 10 U/mL. Interestingly, with an increase in the ratio of CTAB to cell suspension (2:1), more time was needed to damage the cell wall. In this case, the enzyme activity of BCS peaked (17 U/mL) when the treatment time was 120 min.

The activities corresponding to supernatant fractions are presented in Figure 2b. During experiments, a similar pattern was observed as with the mechanical methods. All tested ratios of CTAB to cell suspension resulted in very low β -galactosidase activity (≈ 0.1 U/mL) in the supernatant fraction (SF). The activity peak of the SF was only 0.100 U/mL at 120 min of treatment time. This value is significantly lower than that of BCS (from 10 U/mL to 28 U/mL). It can be explained by two cases: the low efficacy of the CTAB method, and the cell-wall-associated localization of the enzyme protein. In the first case, CTAB binds and breaks down only the lipid bilayers of the cell wall of the bacterium *Ll. fermentum* LF08 strain, but it was not enough to release intracellular (cytoplasmic) content. In the second case, although the lysis of the cell wall by CTAB was well done, the cytoplasmic content was accessible, but the low enzyme activity indicates that there is no enzyme protein in the cytoplasm. Low activity in the SF and high activity in the BCS led to the proposal that the enzyme protein may associate with the cell wall, thus strengthening the previous evidence.

In order to confirm the subcellular localization of β -galactosidase from the *Ll. fermentum* LF08 strain, the biological cell disruption method with lysozyme was used. This enzyme was originally discovered by Alexander Fleming, and it is widely distributed in the tears, saliva, blood serum, egg white of birds, and human and cow milk [22]. The lysozyme aids the hydrolysis of β -(1,4) bonds between N-acetylglucosamine (NAG) and N-acetylmuramic acid (NAM) units [22,23] in the glucan chains that are the main component in the peptidoglycan layer. This layer is the strongest layer in the bacterial cell

wall, especially in Gram-positive bacteria. A central composite design (CCD) was used to evaluate the effect of lysozyme concentration and incubation time on the efficiency of the disruption method. A total of 13 experimental runs were conducted, with lysozyme concentrations ranging from 7.5 to 92.4 mg/mL and incubation times varying between 22 and 277 min (Table 1) as independent factors. The temperature was maintained at a constant 37 °C. During each measurement, β -galactosidase activity in the supernatant was determined, and the results as a dependent factor were statistically analyzed using Minitab software version 20.4.

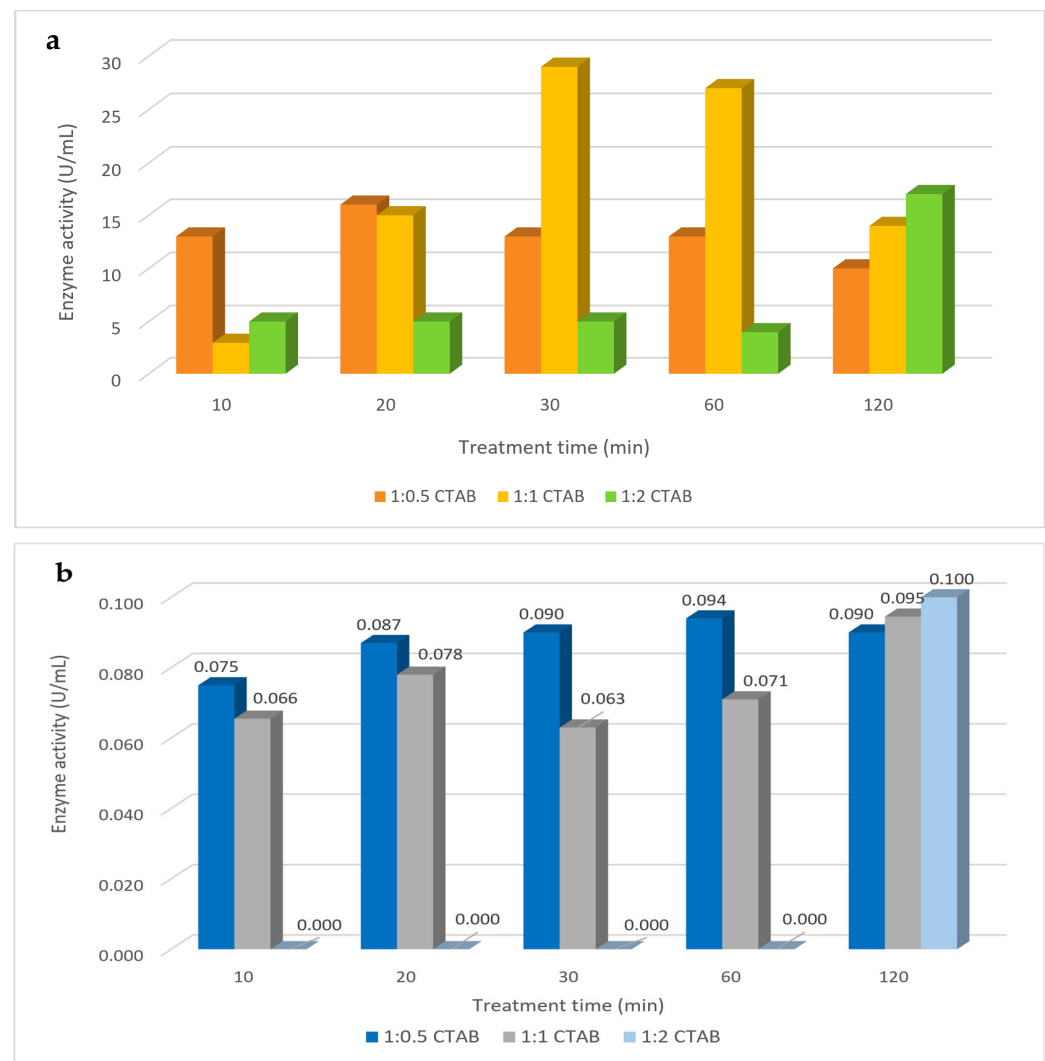


Figure 2. β -Galactosidase activity (a) in the broken cell suspension and (b) in the supernatant fractions after cell disruption with CTAB in various ratios and treatment times.

The CCD data were analyzed by regression, and the results are presented in Table 2. The developed model, as well as linear members, were statistically significant at 95% level ($p < 0.05$). The quadratic and interaction members of the model were significant at 78% and 85%, respectively.

The model obtained from the regression analysis is presented in Equation (1):

$$Y = -1.132 + 0.0359 X_1 + 0.01717 X_2 - 0.000204 X_1^2 + 0.000025 X_2^2 - 0.000112 X_1 X_2 \quad (1)$$

where:

Y = β -galactosidase activity;

X_1 = Lysozyme concentration (mg/mL);

X_2 = Incubation time (minutes).

Table 2. Analysis of variance (ANOVA) for the central composite design (CCD) model.

Source	DF	Adj SS	Adj MS	F-Value	p-Value
Model	5	24.4285	4.8857	36.51	<0.001
Linear	2	23.4665	11.7332	87.67	<0.001
Lysozyme concentration (mg/mL)— X_1	1	0.0134	0.0134	0.10	0.761
Incubation time (min)— X_2	1	23.4530	23.4530	175.25	<0.001
Quadratic	2	0.5944	0.2972	2.22	0.179
Lysozyme concentration— X_1^2	1	0.2340	0.2340	1.75	0.228
Incubation time— X_2^2	1	0.2831	0.2831	2.12	0.189
Interaction	1	0.3677	0.3677	2.75	0.141
Lysozyme concentration $\times$ Incubation time— $X_1 X_2$	1	0.3677	0.3677	2.75	0.141
Error	7	0.9638	0.1338	-	-
Lack-of-fit	3	0.3450	0.1150	0.78	0.565
Pure error	4	0.5918	0.1480	-	-
Total	12	25.3653	-	-	-

The obtained value of R^2 of the model was 96.31%, which suggested that the predicted model strongly fit the experimental data.

Figure 3 demonstrates that the variation in enzyme activity as a function of incubation time and lysozyme concentration becomes evident. Meanwhile, the data indicated that extended incubation time leads to a greater release of β -galactosidase ($p < 0.05$), whereas the lysozyme concentration appears to have no significant effect ($p > 0.05$), suggesting that the applied lysozyme concentration was sufficient to lyse the bacterial cell wall. In the literature, typical lysozyme concentrations for cell lysis are 0.1–1 mg/mL, depending on the source of lysozyme and the type of bacteria. The increase in treatment time can be explained by the dynamics of absorption of the enzyme into the cell wall, which is a slow process [23]. It is worth noting that the use of lysozyme, which directly hydrolyzes the peptidoglycan layer of the *Ll. fermentum* LF08 strain cell wall, resulted in significantly higher β -galactosidase activity (even >5 U/mL) in the supernatant fraction after 255 min of treatment. These values are significantly higher than those obtained by the French press and the CTAB treatment. Based on these findings, it can be proposed that β -galactosidase from the *Ll. fermentum* LF08 strain may associate with structures located within or closely connected to the peptidoglycan layer, and not to the membrane layers; thus, the degradation achieved by lysozyme facilitates the enzyme release. Thus, lysis of lipid membrane layers did not result in the release of the β -galactosidase protein into the supernatant fraction. It is worth noting that when the lysozyme was applied in combination with CTAB, a 21.4-fold increase in β -galactosidase activity in the supernatant fraction was observed (2.14 U/mL), compared with CTAB alone (0.1 U/mL). However, the activity detected in this supernatant fraction in this case is still lower than that in some cases of lysozyme alone (3.77–5.33 U/mL). It can be explained by the incubation time of lysozyme. In the cases of the combination of the biological and chemical methods, only 120 min were applied to the incubation time for lysozyme, while in the cases of lysozyme alone, higher β -galactosidase activities were assayed in the cases of longer incubation times (150–277 min).

Bioinformatic tools were used in order to understand the subcellular localization of the enzyme protein, focusing on lactic acid bacteria (Table 3). According to CELLO v2.5, the subcellular localization of β -galactosidases produced by *Lactobacillus helveticus* (UOE24092.1, UOE24093.1) and *L. acidophilus* (AGK94524.1 and AGK94623.1), except AGK94619.1 proteins produced by *Lactobacillus acidophilus*, is cytoplasmic (intracellular),

whereas the β -galactosidase proteins from *Lactiplantibacillus plantarum* (QHM27543.1 and QHM27544.1) and *Limosilactobacillus reuteri* (CUU13181.1 and CUU13182.1) are extracellular and transmembrane-associated. Three proteins (AGK94619.1 and AGK94624.1 from *Lactobacillus acidophilus* and Q03C30_LACP3 from *Lactocaseibacillus casei*) are predicted to be cell-membrane- or periplasmic-associated. The distributions of the β -galactosidase protein (WGW20706.1) from *Ll. fermentum* for cytoplasmic, extracellular, and cell membrane were 1.005, 1.286, and 0.889, respectively. These values are very close together; thus, it cannot clearly state the localization of this enzyme.

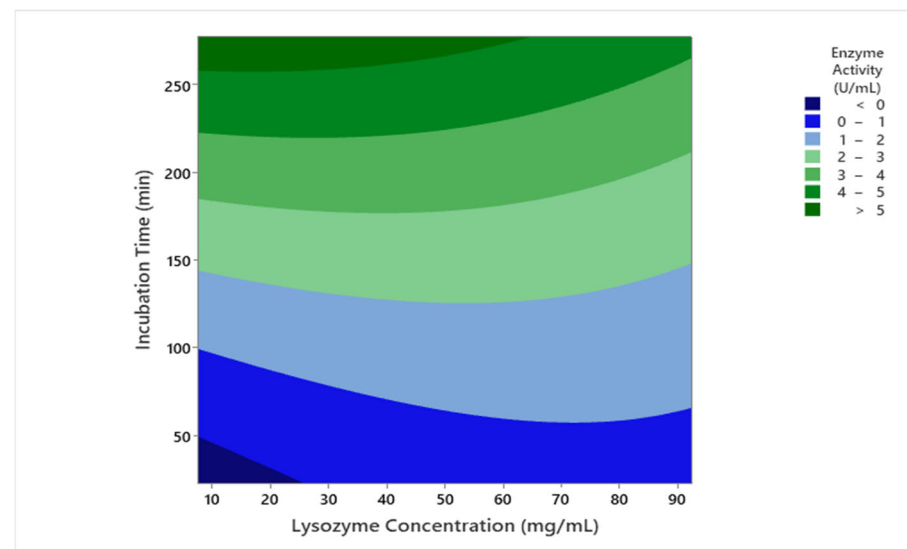


Figure 3. Contour plot of the change in the released β -galactosidase activity in response to the treatment of *Ll. fermentum* LF08 cells under different incubation times and lysozyme concentrations.

Table 3. Predicted localization of β -galactosidases from different lactic acid bacterial strains based on the protein sequence.

Gene	Protein Accession Number	Cytoplasmic		Extracellular		Cell Mem-brane/Periplasmic		Membrane Association	
		DeepLoc	CELLO	DeepLoc	CELLO	DeepLoc	CELLO	Type	Probability
LheBG1	UOE24092.1	0.7147	2.853	0.3255	0.840	0.1269	0.959	Soluble	0.7930
LheBG2	UOE24093.1	0.7636	2.792	0.1324	0.695	0.2456	1.033	Soluble	0.8340
LacBG1	AGK94524.1	0.6379	2.739	0.0658	0.878	0.1587	0.560	Soluble	0.7730
LacBG2	AGK94619.1	0.5575	0.938	0.0490	1.049	0.1761	2.361	Soluble	0.7840
LacBG3	AGK94623.1	0.7698	2.688	0.1132	1.231	0.2290	0.516	Soluble	0.8350
LacBG4	AGK94624.1	0.6816	1.438	0.2880	0.950	0.1517	1.997	Soluble	0.7730
LcaBG1	Q03C30_LACP3	0.6851	0.619	0.1954	1.311	0.2994	1.492	Soluble	0.8230
LfeBG1	WGW20706.1	0.6300	1.005	0.0219	1.286	0.4214	0.889	Transmemb-rane	0.6320
LplBG1	QHM27543.1	0.6127	0.997	0.0219	1.293	0.5950	0.892	Transmemb-rane	0.7850
LplBG2	QHM27544.1	0.5989	1.003	0.0236	1.286	0.4331	0.892	Transmemb-rane	0.6950
LreBG1	CUU13181.1	0.6207	0.993	0.0652	1.299	0.4967	0.890	Transmemb-rane	0.6990
LreBG2	CUU13182.1	0.6282	0.991	0.0392	1.304	0.6032	0.887	Transmemb-rane	0.7650

According to the evaluation from DeepLoc, it is clearly shown that the proteins from *L. helveticus*, *L. acidophilus*, and *Lc. casei* are cytoplasmic (intracellular), and the location of proteins from *Ll. fermentum*, *Lp. plantarum* and *Ll. reuteri* is not so clear. The probabilities of cytoplasmic and periplasmic proteins are almost equal. In the case of the *Ll. fermentum*, transmembrane association with a probability of 0.632 was predicted. While prior research suggests that the β -galactosidase enzyme is predominantly located in the cytosol, the one from the *Limosilactobacillus fermentum* LF08 strain would be associated with the cell wall,

presenting intriguing proof. This can indicate that the localization of the enzyme can also be species- and even strain-dependent.

4. Conclusions

In conclusion, our investigation provided some significant evidence that helps to clarify the subcellular localization of β -galactosidase from the *Limosilactobacillus fermentum* LF08 strain. A high probability of cell-wall-associated localization was proposed. The localization of β -galactosidase in the cell wall of the *Limosilactobacillus fermentum* LF08 strain is very important and provides some functional benefits, as well opens new opportunities for innovation. Cell-wall-associated localization of the enzyme can provide significant advantages over the cytoplasmic protein in the process of lactose hydrolysis. It can be exploited in the development of immobilized cells for removing lactose from milk or the treatment of cheese whey. The understanding gained from this study can be especially important in developing innovations in probiotic formulations, as well as functional foods. This positioning not only allows for improved substrate accessibility but also suggests potential interactions with host tissues that may positively influence gut health. Additionally, understanding cell-wall-associated β -galactosidase can lead to new research opportunities regarding protein behavior in Gram-positive bacteria.

5. Limitations

Although this study proposed the cell-wall-associated localization of β -galactosidase from the *Limosilactobacillus fermentum* LF08 strain, the evidence obtained was only from subcellular fractionation methods (mechanical, chemical, and biological) and the application of bioinformatic tools (deep learning tools), such as DeepLoc, CELLO, and Cell-PLoc. Some other approaches, such as fluorescent protein tagging, proximity labelling, or immunofluorescence, signal peptide recognition, etc., should be used to confirm and understand the mechanism of synthesis of this enzyme. Additionally, the interaction between the enzyme protein and peptidoglycan layer in the cell wall is still unknown and needs to be clarified.

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References

1. Anbu, P.; Gopinath, S.C.B.; Chaulagain, B.P.; Lakshmipriya, T. Microbial Enzymes and Their Applications in Industries and Medicine 2016. *BioMed Res. Int.* **2017**, *2017*, 2195808. [\[CrossRef\]](#)
2. Whitaker, J.R.; Voragen, A.G.J.; Wong, D.W.S. *Handbook of Food Enzymology*; Marcel Dekker: New York, NY, USA, 2003.
3. Zhou, Q.Z.K.; Chen, X.D. Effects of Temperature and PH on the Catalytic Activity of the Immobilized β -Galactosidase from *Kluyveromyces lactis*. *Biochem. Eng. J.* **2001**, *9*, 33–40. [\[CrossRef\]](#)
4. Kalathinathan, P.; Sain, A.; Pulicherla, K.; Kodiveri Muthukaliannan, G. A Review on the Various Sources of β -Galactosidase and Its Lactose Hydrolysis Property. *Curr. Microbiol.* **2023**, *80*, 122. [\[CrossRef\]](#)
5. Zhou, M.; Theunissen, D.; Wels, M.; Siezen, R.J. LAB-Secretome: A Genome-Scale Comparative Analysis of the Predicted Extracellular and Surface-Associated Proteins of Lactic Acid Bacteria. *BMC Genom.* **2010**, *11*, 651. [\[CrossRef\]](#) [\[PubMed\]](#)
6. Hossain, T.J. Functional Genomics of the Lactic Acid Bacterium *Limosilactobacillus Fermentum* LAB-1: Metabolic, Probiotic and Biotechnological Perspectives. *Heliyon* **2022**, *8*, e11412. [\[CrossRef\]](#) [\[PubMed\]](#)
7. Carević, M.; Vukašinović-Sekulić, M.; Grbavčić, S.; Stojanović, M.; Mihailović, M.; Dimitrijević, A.; Bezbradica, D. Optimization of β -Galactosidase Production from Lactic Acid Bacteria. *Hem. Ind.* **2015**, *69*, 305–312. [\[CrossRef\]](#)
8. Selvarajan, E.; Mohanasrinivasan, V. Kinetic Studies on Exploring Lactose Hydrolysis Potential of β Galactosidase Extracted from *Lactobacillus plantarum* HF571129. *J. Food Sci. Technol.* **2015**, *52*, 6206–6217. [\[CrossRef\]](#)
9. Vasiljevic, T.; Jelen, P. Production of β -Galactosidase for Lactose Hydrolysis in Milk and Dairy Products Using Thermophilic Lactic Acid Bacteria. *Innov. Food Sci. Emerg. Technol.* **2001**, *2*, 75–85. [\[CrossRef\]](#)
10. Silhavy, T.J.; Kahne, D.; Walker, S. The Bacterial Cell Envelope. *Cold Spring Harb. Perspect. Biol.* **2010**, *2*, a000414. [\[CrossRef\]](#) [\[PubMed\]](#)
11. Buist, G.; Ridder, A.N.J.A.; Kok, J.; Kuipers, O.P. Different Subcellular Locations of Secretome Components of Gram-Positive Bacteria. *Microbiology* **2006**, *152*, 2867–2874. [\[CrossRef\]](#)
12. Chapot-Chartier, M.P.; Kulakauskas, S. Cell Wall Structure and Function in Lactic Acid Bacteria. *Microb. Cell Fact.* **2014**, *13*, S9. [\[CrossRef\]](#) [\[PubMed\]](#)
13. Tjalsma, H.; Bolhuis, A.; Jongbloed, J.D.H.; Bron, S.; Maarten, J.; Dijl, V. Signal Peptide-Dependent Protein Transport in *Bacillus Subtilis*: A Genome-Based Survey of the Secretome. *Microbiol. Mol. Biol. Rev.* **2000**, *64*, 515–547. [\[CrossRef\]](#)
14. Wiley, W.; Schneewind, O. Surface Proteins of Gram-Positive Bacteria and Mechanisms of Their Targeting to the Cell Wall Envelope. *Microbiol. Mol. Biol. Rev.* **1999**, *63*, 174–229. [\[CrossRef\]](#) [\[PubMed\]](#)
15. Mourad, R.; Csehi, B.; Friedrich, L.; Nguyen, Q.D.; Bujna, E. Investigating the shelf-life of probiotics fermented egg white-based beverage using prebiotics. *Prog. Agric. Eng. Sci.* **2023**, *19*, 105–111. [\[CrossRef\]](#)
16. Rauen, H.M. *Biochemisches Taschenbuch*; Springer: Berlin/Heidelberg, Germany, 1964.
17. Nguyen, V.D.; Styevkó, G.; Ta, L.P.; Tran, A.T.; Bujna, E.; Orbán, P.; Dam, M.S.; Nguyen, Q.D. Immobilization and some properties of commercial enzyme preparation for production of lactulose-based oligosaccharides. *Food Bioprod. Process.* **2018**, *107*, 97–103. [\[CrossRef\]](#)
18. Singh, R.S. A Comparative Study on Cell Disruption Methods for Release of Aspartase from *E. coli* K-12. *Indian J. Exp. Biol.* **2013**, *51*, 997–1003. [\[PubMed\]](#)
19. Lai, Y.J.S.; Zhou, Y.; Eustance, E.; Straka, L.; Wang, Z.; Rittmann, B.E. Cell Disruption by Cationic Surfactants Affects Bioproduct Recovery from *Synechocystis* sp. PCC 6803. *Algal Res.* **2018**, *34*, 250–255. [\[CrossRef\]](#)
20. Minas, K.; Mcewan, N.R.; Newbold, C.J.; Scott, K.P. Optimization of a High-Throughput CTAB-Based Protocol for the Extraction of QPCR-Grade DNA from Rumen Fluid, Plant and Bacterial Pure Cultures. *FEMS Microbiol. Lett.* **2011**, *325*, 162–169. [\[CrossRef\]](#) [\[PubMed\]](#)
21. Orban, J.I.; Patterson, J.A. Modification of the Phosphoketolase Assay for Rapid Identification of Bifidobacteria. *J. Methods Microbiol. J. Microbiol. Methods* **2000**, *40*, 221–224. [\[CrossRef\]](#)
22. Khorshidian, N.; Khanniri, E.; Koushki, M.R.; Sohrabvandi, S.; Yousefi, M. An Overview of Antimicrobial Activity of Lysozyme and Its Functionality in Cheese. *Front. Nutr.* **2022**, *9*, 833618. [\[CrossRef\]](#)
23. Sedov, S.A.; Belogurova, N.G.; Shipovskov, S.; Levashov, A.V.; Levashov, P.A. Lysis of *Escherichia coli* cells by lysozyme: Discrimination between adsorption and enzyme action. *Colloids Surf. B Biointerfaces* **2011**, *88*, 131–133. [\[CrossRef\]](#)

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