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Short Communication

Comparative evaluation of crude β-galactosidase enzyme extraction from *Lactobacillus acidophilus* using five different extraction methods

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Abstract

The aims of this study were twofold: firstly, to identify the *Lactobacillus* strain among seven commercial strains that produces the highest levels of β-galactosidase enzyme, and secondly, to compare the efficiency of five different extraction methods for obtaining crude enzyme extracts from selected highest enzyme producing strain. *L. acidophilus* ATCC 4356 was identified as having the highest β-galactosidase enzyme activity after screening by X-Gal method, and therefore, this strain was chosen for further investigation. After selecting *L. acidophilus* ATCC 4356, comparative evaluation of the crude enzyme extraction using five different extraction methods was performed. Highest enzyme activity in µmol/ mL/min was obtained by lysozyme method (48.27±0.38), followed by micro fluidization (37.41±0.52), sonication (33.29±0.61), toluene extraction (28.16±1.06) and lowest enzyme activity was observed with glass bead method (12.08±0.35). In conclusion, technology for extraction of crude β-galactosidase extract from *L. acidophilus* ATCC 4356 by using five different extraction methods has been developed successfully.

Keywords: Glass beads, Lysozyme, Micro fluidization, Sonication, β-galactosidase

Highlights

- After initial screening by X-Gal method *L. acidophilus* ATCC 4356 culture exhibited the highest β-galactosidase activity.
- Technology for extraction of crude β-galactosidase extract from *L. acidophilus* ATCC 4356 by using five different extraction methods had been developed successfully in this study.
- Highest enzyme activity was obtained by lysozyme method and lowest enzyme activity was observed with glass bead method.

β-galactosidase is a subcellular enzyme and thus needs mechanical disorganization and chemical permeabilization of the cell membrane for its release from microorganisms (Ustok, 2007). In the dairy industry, β-galactosidase helps in sorting out many problems like lactose crystallization in ice cream and sweetened condensed milk and an increase in sugar level naturally in the final product by hydrolyzing lactose to glucose and galactose (Murugan, 2013; Joon *et al.*, 2018). Furthermore, this helps in providing lactose-free food, i.e., providing food to lactose intolerant people. Also, hydrolysis of whey can be done through this which can help in environmental pollution control. Beneficial effects of β-galactosidase in industry are lactose elimination and lactose hydrolysis, which further forms galacto-oligosaccharides and improved dairy food characteristics like enhanced solubility, sweetening potency, monosaccharide production and helps in Maillard reaction. Also, in pharmaceutical industry, β-galactosidase is used in clinical preparation (Makwana *et al.*, 2019; Kaur *et al.*, 2023).

To extract the β-galactosidase, methods such as mechanical or chemical breakdown of the cell membrane of microbes are typically employed. The efficiency of these methods for disruptions differs in terms of microorganism's genera and strains. In general, rupturing cells employing various cell disruption procedures can drastically boost β-galactosidase activity in the media. In the literature, most research focuses on yeast disruption, with comparatively less information available for lactobacilli. The development of a cost-effective lactose hydrolysis technology, along with the identification of an appropriate microbial source, would enable increased production of lactose and lactose-containing dairy products. Crude enzymatic extract is one of the economical methods for lactose hydrolysis as enzyme purifying step is eliminated which is highly costly.

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The objectives of this study were to identify the *Lactobacillus* strain with the highest β-galactosidase production among seven commercial strains and to compare five different enzyme extraction methods, i.e. toluene, lysozyme, sonication, glass beads, and micro fluidization, in order to determine the most efficient method for extracting β-galactosidase from highest enzyme producer.

Commercial lactobacilli cultures for screening and extraction of β-galactosidase were procured from ATCC through Hi Media, Mumbai (Table 1). Stock cultures were preserved at -80ºC.

Table 1. List of commercial lactobacilli purchased

Sl. No.	Name of lactic acid bacteria
1	*Lactobacillus acidophilus* ATCC 314
2	*Lactiplantibacillus plantarum* ATCC 8014
3	*Lactobacillus sakei* ATCC 15521
4	*Lactobacillus gasseri* ATCC 19992
5	*Lacticaseibacillus rhamnosus* ATCC 7469
6	*Lacticaseibacillus casei* ATCC 393
7	*Lactobacillus acidophilus* ATCC 4356

Prior to each assay, strains were revived by transferring stock cultures into MRS medium and incubating at 37°C for 24 hours. Gram staining and catalase test confirmed that all cultures were pure. Cultures were stored at temperatures below 5°C between transfers.

MRS agar plates containing 60 µL of X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside, 20 mg/mL DMF (N-N dimethyl formamide)) and 10 µL of IPTG (iso-propyl-thio-β-D-galactopyranoside) were prepared. A single colony of each *Lactobacillus* strain was streaked on the above plates and then further grown at 37°C in the incubator for 24 hours for 24-72 h. Microorganisms having blue-green colonies were found to be β-galactosidase producers.

The method of Tari *et al.* (2010) was used for enzyme extraction by using lysozyme with slight modifications. The microbe was incubated at 37°C in 100 mL MRS-Lac broth for overnight. At 12000xg and 4°C, the cell biomass was centrifuged for 10 minutes. The pellet was rinsed with 0.05 M Na-phosphate buffer (pH 6.8) after the supernatant was removed. After the washing procedure, the pellet was again centrifuged at 12,000xg for 10 minutes. Pellet obtained was dissolved in 5.0 mL of 0.05 M Na-phosphate buffer (pH 6.8) by forceful mixing. 10 mg/mL concentration of lysozyme was added in the centrifugal tube, and this was then maintained at 37°C in the incubator for 15 minutes. 0.5 mL of 4M NaCl was supplemented to this and further placed in the incubator maintained at 37°C for a duration of 50 minutes. After that, centrifugation was done at 12,000×g for 10 minutes, and the supernatant thus obtained was used for the β-galactosidase estimation assay.

The method of Bury *et al.* (2000) was used for enzyme extraction by using glass beads. Pellet was obtained in a similar way as explained in the lysozyme extraction method. Five grams of glass beads were added to the suspension, followed by vortexing for 10 operating cycles (1 operating cycle = 1 minute of operation + 30 seconds of cooling on ice). After the 8th cycle, centrifugation was done at 10,000xg for a duration of 15 minutes, and the resulting supernatant was obtained and further analyzed for the β-galactosidase estimation assay.

The method of Feliu and Villaverde (1994) was used for enzyme extraction by using sonication. Pellet was obtained in a similar way as explained in the lysozyme extraction method. Cell suspension obtained was now sonicated (60% amplitudes, 750 watts and 20 KHz) using the ultra-sonicator (Ultrasonic Processor, USA) for 10 operating cycles, i.e., 1-minute operation and 30 seconds cooling on ice. Cooling was done by use of an ice water bath. Centrifugation conditions were kept same as that of lysozyme method and supernatant thus obtained was used for the β-galactosidase estimation assay.

The method of Choi *et al.* (1997) was used for enzyme extraction by using a micro fluidizer (Microfluidics M-110P, Newton, USA). The strain was inoculated in 500 mL of MRS-Lac broth for 24 h. Pellet was obtained in a similar way as explained in the above lysozyme extraction method. Pellet was re-suspended in 300 mL of 0.05 M Na- phosphate buffer. Cell disruption was done with the usage of this solution with the help of the micro fluidizer three times at 15,000 Pa. Centrifugation conditions were kept the same as that of the lysozyme method, and the supernatant thus obtained was used for the β-galactosidase estimation assay.

For analysis of β-galactosidase enzyme activity, 300 µL of cell suspension was taken in a test tube. To this, 2.7 mL of phosphate buffer and 600 µL ONPG (Ortho-nitrophenyl beta-D-galactopyranoside, Himedia, Mumbai) substrate was added. The samples were immersed in a steam bath at 37°C for 15 minutes. After that period the reaction was terminated with the help of 2.25 mL of 1M Na_2CO_3 and absorbance of mixture was checked at 420 nm. Single unit of β-galactosidase activity was defined as the amount of β-galactosidase needed to release 1 µmol of ONP from its substrate per minute under the same assay environmental conditions (Kaur *et al.*, 2023).

Under the supervision of a statistician, data gathered from numerous experiments during the screening and

comparative analysis process were analyzed for two-way analysis of variance (ANOVA) and t-test using SAS 9.3 version. Microsoft Excel was used to calculate the mean and standard error of data when needed.

During this study, all the lactobacilli, namely, *L. acidophilus* ATCC 314, *L. plantarum* ATCC 8014, *L. sakei* ATCC 15521, *L. gasseri* ATCC 19992, *L. rhamnosus* ATCC 7469, *L. casei* ATCC 393 and *L. acidophilus* ATCC 4356 were found to be Gram-positive, rod-shaped bacteria under a microscope. All the lactobacilli were also found to be catalase-negative.

The strains possessing β-galactosidase activities have shown blue-green color colonies on X-Gal MRS plates. These bluish-green color colonies confirm that all the

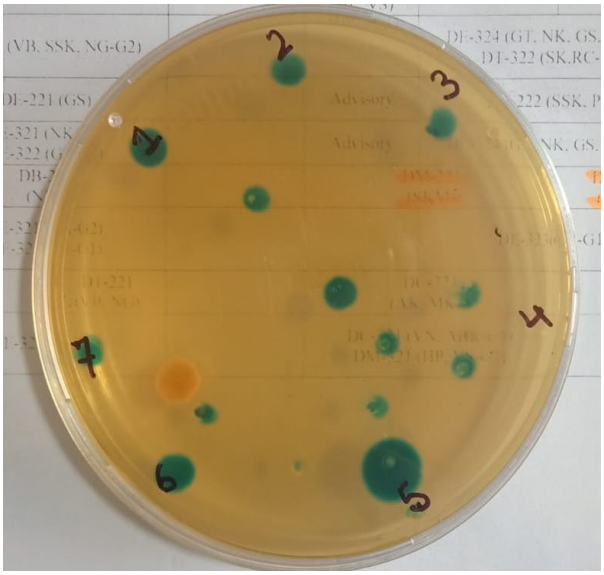

Fig. 1. Blue-green colonies of *Lactobacillus* strains on X-gal plates

lactobacilli possess β-galactosidase activity (Fig. 1).

Few strains produced strong and rapid color change, while few strains showed low and slow enzyme activity by taking more time. Few lactobacilli (41%) produced intense blue-green colonies after 24 h incubation, showing that they produced high enzymatic activity, while few (59%) had slow enzymatic activity and changed color after 2-4 days of incubation. The *L. acidophilus* ATCC 4356 culture exhibited the highest β-galactosidase activity and was therefore selected for further studies.

Here, five diverse β-galactosidase extraction techniques such as toluene, lysozyme, sonication, glass beads and micro fluidization were used to find out the efficient extraction method for β-galactosidase from previously selected *L. acidophilus* ATCC 4356 culture. Enzyme activity units obtained by using five distinct methods from the previously selected strain *Lactobacillus acidophilus* 4356 are given in Table 2. The highest enzyme activity was obtained by the lysozyme method (48.27±0.38), followed by micro fluidization (37.41±0.52), sonication (33.29±0.61), toluene extraction (28.16±1.06) and the lowest enzyme activity was observed with glass bead method (12.08±0.35).

Cell disruption is necessary for the extraction of subcellular ingredients, and it has a considerable impact on subsequent extraction and purification processes. It is necessary to choose appropriate ways to break down cellular structures for the isolation of subcellular products. Favier *et al.* (1997) first provided a method for the detection of microbes with β-galactosidase activity using X-gal. Bacteria containing β-galactosidase enzyme changes the color of MRS-X-gal medium to bluish green color.

The low activity observed with the glass bead and sonication methods may be due to the partial disruption of the cell membrane. Since β-galactosidase is a large enzyme, complete cell wall disruption is essential to release it effectively. One more fact for achieving low enzyme activity units by glass bead and sonication method was the exposure to the high amount of heat generation that may be the possible reason for enzyme inactivation (Ismai *et al.*, 2010). Mostly the literatures are engrossed in the chemical and mechanical treatment comparison (Bury *et al.*, 2000; Geciova *et al.*, 2002). Although there are many approaches that work well in the laboratory, only a few are viable for large-scale applications. Thus, this study will be one of the narrow studies which will focus on the comparison of all 3 types

Table 2. Comparison of crude β-galactosidase enzyme activity from selected strain of *Lactobacillus acidophilus* ATCC 4356 using five different methods of cell disruption

Name of the organism	Enzyme activity by different extraction methods				
	Glass Bead (µmol/mL/min)	Toluene (µmol/mL/min)	Sonication (µmol/mL/min)	Lysozyme (µmol/mL/min)	Micro fluidizer (µmol/mL/min)
L. acidophilus 4356	12.08^{a}±0.35	28.16^{b}±1.06	33.29^{c}±0.61	48.27^{d}±0.38	37.41^{e}±0.52

Different alphabets ($^{a, b, c, d, e}$) show significant differences ($p \leq 0.01$) between the samples during the study

of methods, including enzymatic methods. While making a comparison of disruption methods, all other factors like pH, temperature, growth medium, type of cell wall, etc., should also be taken into consideration (Geciova *et al.*, 2002; D'Hondt *et al.*, 2017). The toluene method, being a chemical method, is not suitable for application in milk study as it cannot be added to milk. The lysis by enzyme (lysozyme) method is beneficial because it is accurate and mild, but it is not economical in the long run. Cell lysis by the enzymatic method can be used at small volumes, but it is not advised for large volume production because of the high input cost. Among the other three mechanical procedures, viz. glass beads, micro fluidizer and sonication, the micro fluidizer method yielded the maximum β-galactosidase and also this method enabled the process of the cell suspension 40 times more when compared with glass beads. In conclusion, technology for extraction of crude β-galactosidase extract from *L. acidophilus* ATCC 4356 by using five different extraction methods has been developed successfully.

Conflict of interest: Authors have no conflict of interest in this study.

Author's contribution: DK: Has carried out all the experiments related to this manuscript; SKM: Designed and supervised the study; PKS and NR: Contributed to statistical analysis and writing of the work done.

Data availability statement: All the data supporting the research findings have been presented in this paper. The corresponding author is willing to provide the raw data upon reasonable request.

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