Journal of Dairy Research

cambridge.org/dar

A comparative study of extraction techniques for maximum recovery of β-galactosidase from the yogurt bacterium *Lactobacillus delbrueckii* ssp. *bulgaricus*

Rabin Gyawali[1], Ayowole Oyeniran[1], Tahl Zimmerman[1], Sulaiman O. Aljaloud[2], Albert Krastanov[3] and Salam A. Ibrahim[1]

[1]Food Microbiology and Biotechnology Laboratory, Food and Nutritional Sciences Program, North Carolina Agricultural and Technical State University, Greensboro, NC, USA; [2]College of Sport Sciences and Physical Activity, King Saud University, P.O. Box 1949, Riyadh 11362, Saudi Arabia and [3]Department of Biotechnology, University of Food Technologies, Plovdiv, Bulgaria

Research Article

Cite this article: Gyawali R, Oyeniran A, Zimmerman T, Aljaloud SO, Krastanov A and Ibrahim SA (2020). A comparative study of extraction techniques for maximum recovery of β-galactosidase from the yogurt bacterium *Lactobacillus delbrueckii* ssp. *bulgaricus*. *Journal of Dairy Research* **87**, 123–126. https://doi.org/10.1017/S0022029919001031

Received: 30 May 2019
Revised: 18 November 2019
Accepted: 2 December 2019
First published online: 3 February 2020

Keywords:
Cell lysis; enzyme extraction; *L. bulgaricus*; lactase; β-galactosidase

Author for correspondence:
Rabin Gyawali, Email: rgyawali@gmail.com

Abstract

The study reported in this research communication evaluates the chemical (solvents) and mechanical (sonication, bead-beater) extraction methods to determine the maximum recovery of β-galactosidase from *L. bulgaricus* spp. Among all extraction techniques, sonication-assisted extraction yielded the highest amounts of enzyme activity (between 1892–2156 Miller Units) in cell-free extract (supernatant). Interestingly, solvent extracted enzyme activities were found to be very low (between 83–153 Miller Units) in supernatant. SDS-polyacrylamide gel electrophoresis and the total protein determination showed that mechanical methods can completely lyse the cells. Our results thus demonstrated that the mechanical extraction method of sonication is the best one for recovering the maximum amount of lactase from *L. bulgaricus* strains.

β-galactosidase (β-gal) (EC 3.2.1.23), also known as lactase, is a commercially important enzyme that is used extensively in food and pharmaceutical industries due to its ability to hydrolyze lactose (Vasiljevic and Jelen, 2001). In addition to hydrolysis, β-gal also catalyzes the transgalactosylation of lactose in order to produce galactooligosaccharides (GOS), which are well known for their prebiotic efficacy (Plou *et al*., 2017). Due to the commercial interest in β-gal, a large number of microorganisms have been assessed as potential sources of this enzyme. Specifically, strains of the yeast *Kluyveromyces* and fungus *Aspergillus* have been widely used for the production of β-gal (Panesar *et al*., 2010). It is estimated that over 70% of the world's adult population have problems digesting lactose due to absent or reduced β-gal activity in the small intestine (Ibrahim and Gyawali, 2013). Therefore, β-gal has found a prominent place in the pharmaceutical industry in treatment of lactose intolerance and in the development of digestive supplements (prebiotics). As a result, there is a considerable market for lactose-free milk and dairy products, which can be obtained by enzymatic hydrolysis using β- gal. To meet this high demand, effective cell extraction techniques are a necessary part of any industrial production line and offer many benefits for the enhancement of product recovery.

Thermophilic lactic acid bacteria (LAB) are in great demand for enzyme production because of their food grade, generally-recognized-as-safe (GRAS) status, and they are stable and active at high temperatures. Among lactic acid bacteria, yogurt bacteria (*Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus thermophilus*) are considered to be the highest β-gal producers (Kreft *et al*., 2001). However, the β-gal from thermophilic LAB such as *L. bulgaricus* is an intracellular enzyme. The efficient recovery of enzymes requires cell disruption, which can be achieved by either mechanical or chemical disruption techniques.

Studies on the recovery of β-gal from *L. bulgaricus* are very limited. Thus, the search for an effective technique that recovers high amounts of β-gal from *L. bulgaricus* spp. remains a significant research area. Therefore, in the present study, the efficacy of different extraction techniques (chemical and mechanical) for the maximum recovery of β-gal from three strains of *L. bulgaricus* was investigated. We measured β-gal not only from supernatant (cell-free extract) but also from cell lysate under different extraction conditions.

Material and methods

Bacterial strains and culture propagation

Three strains of *Lactobacillus delbrueckii* ssp. *bulgaricus* were selected for this study. *L. bulgaricus* strain ATCC 11842 was purchased from the American Type Culture Collection

(Manassas, VA). The *L. bulgaricus* RR strain was provided by Robert F. Roberts (Department of Food Science, Pennsylvania State University). The third strain was isolated from commercial probiotic supplements available in the market. All three strains were maintained in a glycerol stock solution at −80°C. From the glycerol stock, 100 µl of each individual strain was inoculated into 10 ml of deMan Rogosa Sharpe (MRS) broth and incubated for ~16 h at 42°C. The following day, individual strains were sub-cultured in 100 ml of MRS broth and incubated at 42°C until the optical density (OD_{600}) reached ~0.6. Each culture was then spun at 3901 *g* for 20 min at 4°C (Allegra™ X-22R, Beckman, USA). The resulting supernatant was decanted, and the cells were resuspended in 100 ml of Z buffer (0.06 M Na_2HPO_4, 0.04 M $NaH_2PO_4{\cdot}H_2O$, 0.01 M KCl, and 0.001 M $MgSO_4{\cdot}7H_2O$). Each individual culture was then subjected to the different extraction techniques described below.

Extraction of β-galactosidase

Solvents

Cells were permeabilized by adding 10 µl (v/v) chloroform and 50 µl (v/v) toluene-acetone (1:9) into one ml of cell suspension in glass tubes. The samples were then mixed and incubated at 37°C on a shaker with open caps for 30 min. β-gal activity was determined using 0.1 ml of cell lysate, and the remaining portion of cell lysate was spun at 3000 *g* for 20 min at 4°C (Model Z-216 MK, refrigerated microcentrifuge, Wehingen, Germany). The β-gal activity was assayed in the obtained supernatant as well.

Bead-beater

Cells (1 ml) were lysed by bead beating (Bead-Beater, Stratech, London, UK) in the presence of 0.5 g of glass beads (Ø = 0.1–0.11 mm, Sartorius) for 30 s, followed by a 1-min rest on ice and then an additional 30-s beating. The cell lysate and supernatant obtained after lysate centrifugation (3000 ***g*** for 20 min at 4°C) were analyzed for enzyme activity.

Sonication

The cells (80 ml) were lysed using a sonicator (Branson 450 analog sonifier, CT, USA) with a 19-mm probe setting in a beaker with ice throughout the sonication. The cells were then disrupted for 4 min (50% duty cycle, output control 5) with 30 s of sonication pulses followed by 1-min of rest on ice to cool the suspension. The cell lysate and supernatant obtained after centrifugation (3000 ***g*** for 20 min at 4°C) were used for the enzyme activity.

Beta-gal assay

Beta-gal activity was determined by adding 200 µl of β-D-galactopyranoside (ONPG, Sigma Co., St Louis, MO, USA) dissolved in 4 mg/ml of 0.1 M phosphate buffer into each sample tube. Each sample tube contained 0.1 ml of cell lysate, supernatant, and unlysed cells separately added to 0.9 ml of Z buffer (Total vol. 1 ml). The reaction was then stopped by adding 0.5 ml of 1 M Na_2CO_3 after 5 min. Absorbance values were measured at 420 and 550 nm in a microplate reader (Gen 5 2.06, Synergy HT, Biotech, VT, USA), and a unit of β-gal produced was calculated using the following equation (Miller, 1992; Ibrahim and O'Sullivan, 2000):

Miller units (MU): $1000 \times [(OD_{420} - 1.75 \times OD_{550})]/(T \times V \times OD_{600})$, where T = time of the reaction in min, V = volume (ml) of culture used in the assay, OD_{420} measures yellow color present due to ONPG cleavage, OD_{550} corrects for light scattering, and OD_{600} reflects cell density in a Z buffer.

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and total protein determination

Seven hundred fifty microliters of each group of lysed and unlysed cells, obtained from *L. bulgaricus* strain ATCC 11842 were centrifuged (3000 ***g*** for 20 min at 4°C). The supernatant was removed and precipitated with trichloroacetic acid. The precipitated supernatant samples were then mixed with 20 µl of SDS buffer (315 mM Tris-HCL buffer pH 6.8 containing 50% glycerol, 5% SDS, 100 mM DTT, and 0.25% bromophenol blue) and heated at 95°C for 10 min. The samples were then loaded on 12% SDS-PAGE. Electrophoresis was performed at 200 V for 45 min. The gel was then stained with coomassie brilliant blue and destained with an acetic/methanol solution. The total protein concentration was estimated by the Bradford assay (BioRad, USA) using bovine serum albumin as the standard.

Statistical analysis

Statistical analysis of the data obtained from the β-gal activity was performed by one-way ANOVA using SAS Version 9.4 (Cary, NC, USA) where $P < 0.05$ was considered to be significant.

Results and discussion

In the present study, we investigated the efficacy of various extraction techniques on β-gal production from *L. bulgaricus*. β-gal activity was assayed in the cell lysate and supernatant. The activity of β-gal from the cell lysate of strain ATCC 11842 ranged between 152.57 and 2220 MU, and the activity in the supernatant group ranged from 35.71–2156 MU (Fig. 1a). These results are comparable to that of Vinderola and Reinheimer (2003), who reported that β-gal activity from *L. bulgaricus* strains ranged from 0 to 2053 MU. In the cell lysate, β-gal activity was highest in toluene:acetone (2220 MU) followed by sonication (2053 MU), whereas in the supernatant, sonication produced a significantly higher ($P < 0.05$) level of β-gal activity (2156 MU) compared to other techniques. Between the two solvents, we observed slightly higher β-gal activity in toluene:acetone treated cells compared to chloroform. This result correlates with that from earlier studies that demonstrated the higher permeabilizing efficiency of mixed organic solvents in *Lactobacillus* spp. and yeast cells compared to individual solvents (Kumari *et al.*, 2011; Gobinath and Prapulla, 2015). Similarly, Meira *et al.* (2012) reported a β-gal activity of 47.7 MU for *Lactobacillus casei* and a maximum value of 2503 MU for *Lactobacillus plantarum* when cells were extracted using a mixture of toluene:acetone. Interestingly, in the supernatant, both solvent extracted β-gal activities were found to be very low (82.86–97.71 MU). β-gal obtained from the unlysed cell (control) for cell lysate and supernatant were 152.57, and 35.71 MU, respectively.

The β-gal activity of the other two strains also followed a similar trend. Among all of the extraction techniques, sonication-assisted extraction yielded the highest amount of β-gal activity (1846.66 MU) in the cell lysate and supernatant (1892 MU) for strain RR (Fig. 1b). Similarly, in the case of the commercial strain (Fig. 1c), the β-gal amount was significantly higher ($P < 0.05$) in the cell lysate (1288.80 MU) and supernatant (1241.16 MU) when the cells were disrupted using sonication. However, with this strain, there was no β-gal activity observed

https://doi.org/10.1017/S0022029919001031 Published online by Cambridge University Press

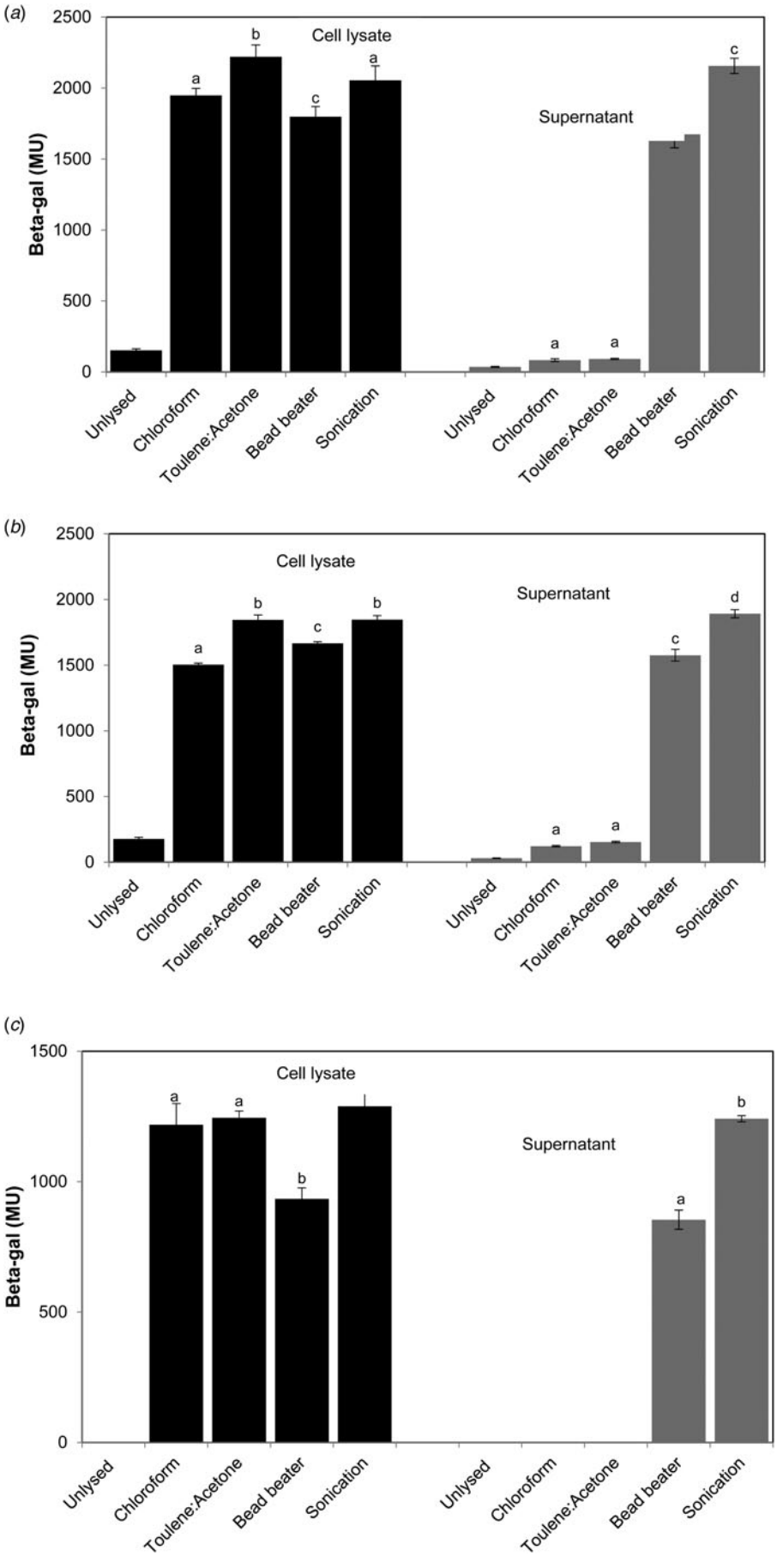

Fig. 1. Beta-galactosidase from *L. bulgaricus* strains using different cell extraction techniques. (a) L.b strain ATCC 11842, (b) L.b strain RR and (c) L.b. strain from probiotic supplement. Data are presented as mean ± standard deviation ($n = 3$), where different letters indicate significant differences within the cell lysate or supernatant categories.

https://doi.org/10.1017/S0022029919001031 Published online by Cambridge University Press

in the cell lysate and supernatant obtained from unlysed cells. Likewise, neither solvent (chloroform and toluene:acetone) produced any amount of β-gal activity in the supernatant.

Overall, all three tested *L. bulgaricus* strains exhibited β-gal activity. β-gal is a large enzyme, which makes it necessary to disrupt the microbial cell completely in order to recover high enzyme activity (Ismail *et al.*, 2010). The results of our study showed that sonication technique was the most efficient method to recover intracellular β-gal from *L. bulgaricus* spp. These results corroborate with the previous finding of Degeest and De Vuyst (2000) and Kreft *et al.* (2001) who also found that the sonication method to be the most effective method for releasing protein from *Lactobacillus* spp. Our results thus demonstrated that solvents are not able to lyse the cells as evidenced from the relatively poor release of β-gal in the supernatant from all three strains. Generally, it is likely that the low yield of enzyme observed with solvents is due to the fact that the Gram-positive bacteria, *L. bulgaricus* has high portion of peptidoglycan layer. This provides considerable rigidity to the cell wall and obstructs the release of intracellular enzymes such as β-gal (Geciova *et al.*, 2002). In fact, solvents only permeabilized the cell membrane and facilitated the access of the substrates to intracellular enzymes (Niven and Mulholland, 1998). This result can also be demonstrated through our SDS-PAGE and protein analysis (Supplementary Fig. S1), where no protein bands were detected in unlysed (lane 1), chloroform (lane 2), and toluene:acetone (lane 3) cells. However, in lanes 4 and 5, proteins bands were more distinct suggesting that bead-beater and sonication techniques can completely lyse the cells and release up to 250 kDa of protein into the supernatant. These results also correspond well with the negligible amount of total protein in lane 2 (31.36 µg/ml) and 3 (18.95 µg/ml) when compared to lane 4 (116.42 µg/ml) and 5 (157.34 µg/ml), which is further evidence that the solvents cannot lyse the cells. A schematic diagram of the effect of solvents and mechanical extraction on β-gal is shown in Supplementary Fig. S2.

Mechanical disruption by sonication resulted in the release of the highest amount (2156 MU) of β-gal into solution, making this method the most effective enzyme extraction technique among the three tested. Interestingly, both solvent treatments failed to release β-gal into the solution while at the same time permitting the enzyme reaction to take place. This would indicate that the solvents facilitated the exchange of subtrates and products across the membrane. Further research is warranted both to confirm this model and to determine the mechanism by which this exchange takes place. In some cases, permeabilizing *L. bulgaricus* could be advantageous because β-gal activity could be obtained without the further processing that is required to isolate the β-gal enzyme for downstream applications. β-gal is relatively unstable; consequently, the cost of isolation is high. In addition, it is technically very difficult to recover the active enzyme for reuse when utilized in solution form (Brena *et al.*, 2013; Plou *et al.*, 2017). Presumably, stability could be improved by simply leaving the enzyme untouched inside the cell. In addition, having the enzyme confined within the space of a cell may be preferable for some applications. For instance, enzyme surface mobilization is required for some enzyme-based industrial processes. By pursuing a permeabilization strategy, whole cells could themselves be immobilized for specific enzymatic activities without the prior release or purification of the enzyme in question. Therefore, the ideal treatment method for the exploitation of cellular enzymes -sonication or solvent- depends on the application itself.

In conclusion, we have demonstrated that the mechanical extraction method of sonication is superior to solvent extraction for recovering the maximum amount of lactase from *L. bulgaricus* strains.

Supplementary material. The supplementary material for this article can be found at https://doi.org/10.1017/S0022029919001031.

Acknowledgement. This research was funded by the NIFA through the Agricultural Research Program at North Carolina Agricultural and Technical State University (Evans-Allen Program, project number NC.X-291-5-15-170-1) and in part by the Indiana University.

References

Brena B, González-Pombo P and Batista-Viera F (2013) Immobilization of enzymes: a literature survey. In Guisan JM (ed), *Immobilization of enzymes and cells*. Humana Press, Totowa, NJ, pp. 15–31.

Degeest B and De Vuyst L (2000) Correlation of activities of the enzymes α-phosphoglucomutase, UDP-galactose 4-epimerase, and UDP-glucose pyrophosphorylase with exopolysaccharide biosynthesis by *Streptococcus thermophilus* LY03. *Applied and Environmental Microbiology* **66**, 3519–3527.

Geciova J, Bury D and Jelen P (2002) Methods for disruption of microbial cells for potential use in the dairy industry – a review. *International Dairy Journal* **12**, 541–553.

Gobinath D and Prapulla SG (2015) Transgalactosylating β-galactosidase from probiotic *Lactobacillus plantarum* MCC2156: production and permeabilization for use as whole cell biocatalyst. *Journal of Food Science and Technology* **52**, 6003–6009.

Ibrahim SA and O'Sullivan DJ (2000) Use of chemical mutagenesis for the isolation of food grade β-galactosidase overproducing mutants of bifidobacteria, lactobacilli and *Streptococcus thermophilus*. *Journal of Dairy Science* **83**, 923–930.

Ibrahim SA and Gyawali R (2013) Lactose intolerance. In Young WP and George FW (eds), *Milk and Dairy Products in Human Nutrition: Production, Composition and Health: John Wiley & Sons*. John Wiley & Sons, New Jersey, USA pp. 246–260.

Ismail SA, El-Mohamady Y, Helmy WA, Abou-Romia R and Hashem AM (2010) Cultural condition affecting the growth and production of β- galactosidase by *Lactobacillus acidophilus* NRRL 4495. *Australian Journal of Basic and Applied Sciences* **4**, 5051–5058.

Kreft ME, Roth L and Jelen P (2001) Lactose hydrolysing ability of sonicated cultures of *Lactobacillus delbrueckii* ssp. *bulgaricus* 11842. *Le Lait* **81**, 355–364.

Kumari S, Panesar PS, Bera MB and Singh B (2011) Permeabilization of a yeast cells for β-galactosidase activity using mixture of organic solvents. *Asian Journal of Biotechnology* **3**, 406–414.

Meira SMM, Helfer VE, Velho RV, Lopes FC and Brandelli A (2012) Probiotic potential of *Lactobacillus* spp. Isolated from Brazilian regional ovine cheese. *Journal of Dairy Research* **79**, 119–127.

Miller JH (1992) *A Short Course in Bacterial Genetics. A Labora-Tory Manual and Handbook for Escherichia Coli and Related Bacteria*. Cold Spring Harbor, New York, USA: Cold Spring Harbor Laboratory Press.

Niven GW and Mulholland F (1998) Cell membrane integrity and lysis in *Lactococcus lactis*: the detection of a population of permeable cells in post-logarithmic phase cultures. *Journal of Applied Microbiology* **84**, 90–96.

Panesar PS, Kumari S and Panesar R (2010) Potential applications of immobilized β-galactosidase in food processing industries. *Enzyme Research*. doi: http://dx.doi.org/10.4061/2010/473137

Plou FJ, Polaina JZ, Sanz-Aparicio J and Fernández-Lobato M (2017) β-Galactosidases for lactose hydrolysis and galactooligosaccharide synthesis. In Ray RC and Rosell CM (eds), *Microbial Enzyme Technology in Food Applications*. Boca Raton, FL, USA: CRC Press, pp. 307–317.

Vasiljevic T and Jelen P (2001) Production of β-galactosidase for lactose hydrolysis in milk and dairy products using thermophilic lactic acid bacteria. *Innovative Food Science & Emerging Technologies* **2**, 75–85.

Vinderola C G and Reinheimer JA (2003) Lactic acid starter and probiotic bacteria: a comparative "in vitro" study of probiotic characteristics and biological barrier resistance. *Food Research International* **36**, 895–904.