

Permeabilization of *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus* with Ethanol

George A. Somkuti, Mary E. Dominiecki,* Dennis H. Steinberg

U.S. Department of Agriculture, Agricultural Research Service, Eastern Regional Research Center,
600 E. Mermaid Lane, Wyndmoor, PA 19038, USA

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Abstract. *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus* cultures were treated with ethanol and tested for viability and β -galactosidase activity. Exposure of the biomass of test cultures to 30%–55% ethanol (vol/vol) caused a 100% loss of viability and up to 15-fold increase in measurable β -galactosidase activity in both streptococci and lactobacilli. Ethanol-treated cell suspensions could be stored for up to 6 months without loss of enzyme activity. The nonviable permeabilized biomass of the more active *S. thermophilus* was used to achieve up to 80% hydrolysis of lactose in aqueous solutions and non-fat milk.

Lactic acid bacteria (LAB) constitute a diverse group of lactococci, streptococci, and lactobacilli with pivotal roles in transforming milk into a variety of fermented dairy foods. The LAB group includes *Streptococcus thermophilus* (ST) and *Lactobacillus delbrueckii* (LDB) subsp. *bulgaricus*, which function synergistically as essential biocatalytic agents in the production of yogurt as well as Italian and Swiss-style cheeses. All known strains of these two species utilize a permease-driven lactose transport system and β -D-galactoside galactohydrolase (EC 3.2.1.23, β gal) to initiate intracellular fermentation of the glucose moiety of lactose [8, 10, 25, 31]. In both organisms, the protein products of the permease and β gal components of the lactose operon (*lacY* and *lacZ*) share 75% and 45% homology respectively, indicating a possibly common origin [17, 27].

In carrying out a variety of biocatalytic functions during dairy fermentations, LAB entrapped in the food matrix become integral components of finished products. Since centuries of human experience with ingesting LAB biomass in fermented dairy foods has not uncovered any harmful effects, LAB are now classified as “food-grade”

organisms. Therefore, interest has grown in LAB as potential sources of enzymes with applicability in food manufacture. The first enzyme of LAB to attract attention was β gal with its potential application in the production of low-lactose milk to benefit a large portion of the world's population troubled by lactose maldigestion [9]. At present, low-lactose milk is produced primarily with β gal isolated from yeast (*Kluyveromyces* sp.), which has only moderate heat resistance [18]. A variety of approaches have been recommended for accessing intracellular β gal in LAB to enhance lactose hydrolysis in milk, including sonication for LDB strains [11, 30], and solvent or detergent treatments for ST cultures [33–36].

The main concern about chemical permeabilization of LAB cultures is solvent or detergent residues remaining associated with treated cells and ending up in finished food products. In this regard, ethanol is an ideal solvent of choice, since it is present in trace amounts in many fermented dairy foods consumed by humans. Ethanol has been used successfully for permeabilizing yeast [4, 7], but has not been evaluated in LAB. In this study, we present data on the effect of ethanol on the survival of ST and LB cultures and its efficacy as a permeabilizing agent for increasing the level of measurable β gal activity in these microorganisms. We also tested the effectiveness of ethanol-treated ST biomass in hydrolyzing lactose in non-fat milk and aqueous solutions.

Mention of brand or firm name does not constitute an endorsement by the U.S. Department of Agriculture over others of a similar nature not mentioned.

*Present address: School of Medicine, New York University, Brooklyn, New York 10012, USA

Correspondence to: G.A. Somkuti

Materials and Methods

Bacterial strains. *Streptococcus thermophilus* (ST) and *Lactobacillus delbrueckii* subsp. *bulgaricus* (LDB) strains were grown at 37°C in tryptone-yeast extract-lactose (TYL) [32] and MRS medium (Difco Laboratories, Detroit, MI), respectively, with culture tubes (each with 6 ml medium) incubated in a 5% CO₂ atmosphere, without agitation. Agar plating media for viable cell counts (colony forming units, CFU/ml) were prepared by the addition of 1.5% (wt/vol) agar to liquid media. Live cell counts of serially diluted samples (CFU/ml) were determined after a 48-h incubation of plates at 37°C.

Cell permeabilization. ST and LDB cultures were transferred after 24 h into 200 ml of fresh medium in 500-ml Erlenmeyer flasks (2-ml inoculum per flask) and held at 37°C for 16 h without agitation. Cells were collected under aseptic conditions at 10,000 g for 10 min at 4°C in a Sorvall antifuge and washed once with sterile 50 mM K₂HPO₄/KH₂PO₄-1 mM MgCl₂-pH 7.4 buffer (POM). Cell pellets were resuspended in POM, adjusted to an A₆₆₀ of 2.5, and kept in crushed ice until use.

Cell suspensions were dispensed in sterile tubes (1 ml per tube) and centrifuged in an Eppendorf microfuge at top speed for 5 min at 4°C. After decanting supernatants and draining tubes on sterile paper towels, we resuspended pellets in 1 ml ethanol solution (5–70%, vol/vol) and held them for 20 min at 4°C, 28°C, or 37°C. Cells were collected as before and kept on ice for viability tests and β-galactosidase assays. Controls included cells permeabilized with acetone-toluene (9:1, vol/vol, AT) at 50 μl/ml or 0.1% sodium dodecyl sulfate (SDS) [34].

Cell viability tests. After treatment with ethanol, AT, or SDS, cell pellets equivalent to 10⁹ CFU's were resuspended in and serially diluted with 0.1% peptone water. Triplicate samples (100 μl) from each dilution tube were mixed with 9.9 ml agarized TYL (for ST strains) or MRS medium (for LDB strains). Plates were incubated for 48 h at 37°C before CFUs were counted. Controls included untreated cells and cells exposed to AT. Microbial growth patterns of ST and LDB cultures after ethanol treatment were also followed at 37°C for 24 h in liquid TYL or MRS, respectively, after adjustment of initial cell density to 0.2 at A₆₆₀.

β-Galactosidase assays. Standard conditions used in measuring βgal activity included the rapid mixing of each ethanol-, AT- or SDS-treated pellet from 1 ml cell suspensions (A₆₆₀ = 2.5) with 1 ml of 5% (wt/vol) lactose solution prepared in POM or 1 ml of non-fat milk and incubating reaction mixtures for 10 min at 37°C or 50°C, respectively. To follow lactose hydrolysis over longer time periods, samples (50 μl) from reaction mixtures were withdrawn at predetermined intervals, diluted 10-fold with POM, and centrifuged at 10,000 g at 4°C for 5 min. Glucose levels were measured in triplicate in 10-μl samples of supernatants with the Glucose HK Kit (Sigma Diagnostics, St. Louis, MO), as previously described [34]. Nonpermeabilized cells served as controls.

Results

Viability of permeabilized cell populations. Without exception, the treatment of ST and LDB cell suspensions with AT or SDS for 20 min at a cell density of 10⁹ CFU/ml caused a 100% loss of viability. The magnitude of cell death resulting from ethanol treatment increased with solvent concentration. Survivors could not be detected on TYL or MRS agar plates after a 20-min

Table 1. βGalactosidase activity of LAB cultures permeabilized with 40% ethanol

Strain	βGal (glucose, mg · ml ⁻¹ · 10 min ⁻¹) ^a
<i>S. thermophilus</i>	
ST106	20.3
ST110	18.2
ST119	8.9
ST128	21.0
ST133	20.5
ST136	14.5
<i>L. delbrueckii</i> ssp. <i>bulgaricus</i>	
LDB106	3.2
LDB111	6.2
LDB112	6.0
LDB115	8.5
LDB116	5.1
LDB122	0.3

^a Average values of three replicate assays; maximum attainable glucose concentration: 25 mg · ml⁻¹. Reaction temperature: 37°C.

exposure of cells to 30% or higher aqueous ethanol solutions.

Effect of ethanol on β-galactosidase expression. Notable differences were found in the level of measurable βgal activity between permeabilized ST and LDB cultures surveyed (Table 1). Overall, the βgal activity of permeabilized LDB strains was substantially lower than enzyme levels found in permeabilized ST cells. Even the highest level of βgal found in LDB115 was only about half of that measured in four of the six permeabilized ST strains. Therefore, LDB cultures were excluded from further studies as relatively poor sources of the enzyme.

ST128 was used as a model to test the effect of ethanol concentration on enzyme expression. The level of βgal activity of permeabilized cells increased with alcohol concentration and reached the maximum after treatment with 45–50% ethanol (Fig. 1). On the other hand, permeabilization with 55% or higher ethanol solutions resulted in a decrease in measurable βgal activity, probably caused by protein denaturation. Although the level of maximum βgal activity varied among ST strains (Table 1), they all displayed a similar response to variations in ethanol concentrations.

Effect of permeabilization temperature and contact time on β-galactosidase activity.

The effect of incubation temperature was tested with ST128 during 20-min permeabilization with AT, SDS, or 40% ethanol (Table 2). At 4°C, 40% ethanol was less effective than either AT or SDS. However, at ambient temperature or 28°C the discrepancy was largely absent. As expected, permeabilization at 28°C was more effective, but raising the temperature of treatment to 37°C failed to induce an

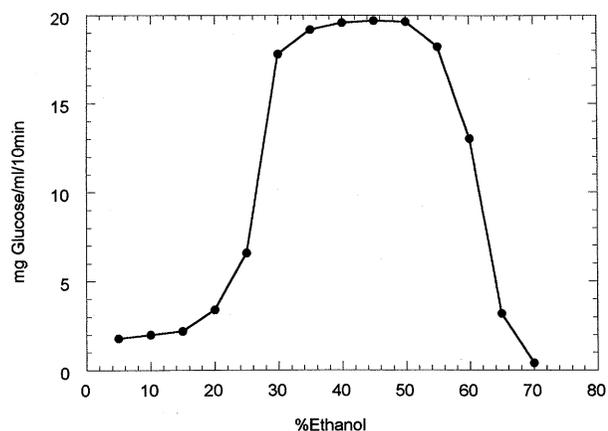


Fig. 1. Effect of ethanol concentration on β galactosidase expression in permeabilized ST128 cells. Standard assay conditions included a cell density of $OD_{660} = 2.5$ and incubation for 10 min at 37°C. Maximum attainable glucose concentration was 25 mg/ml in the 5% aqueous lactose solution used as the substrate. Data points represent average values of three replicate assays.

Table 2. Effect of permeabilization temperature on β gal expression in ST128

Temperature (°C)	β Gal (glucose, mg · ml ⁻¹ · 10 min ⁻¹) ^a		
	AT	40% Ethanol	0.1% SDS
4	11.0	4.5	12.8
28	15.6	16.5	18.0
37	15.9	16.7	18.2

^aAverage values of three replicate assays; maximum attainable glucose concentration: 25 mg · ml⁻¹. Reaction temperature: 37°C.

Table 3. Effect of contact time with permeabilizing agents on β gal activity of ST128

Exposure ^a (min)	β Gal (glucose, mg · ml ⁻¹ · 10 min ⁻¹) ^b		
	AT	40% Ethanol	0.1% SDS
5	7.0	7.3	10.3
10	10.8	11.0	14.2
20	15.8	16.5	18.4
30	15.9	16.8	18.6

^a Cells were treated at ambient (25°C) temperature.

^b Average values of three replicate assays; maximum attainable glucose concentration: 25 mg · ml⁻¹. Reaction temperature: 37°C.

appreciable increase in the β gal activity of permeabilized cells.

The length of contact with ethanol, AT, or SDS also influenced the level of β gal activity (Table 3), and highest values were attained after a 20-min exposure. Contact time longer than 20 min had no further effect on β gal values.

Table 4. Lactose hydrolysis by ST128 in aqueous solutions^a

Time (min)	Glucose (mg · ml ⁻¹) ^b liberated by cells exposed to			
	None (control)	AT	40% Ethanol	0.1% SDS
10	1.3	15.6	16.8	17.8
20	2.4	18.2	19.6	20.3
30	3.2	19.6	20.5	22.3

^a Reaction temperature: 37°C.

^b Average values of three replicate assays; maximum attainable glucose concentration: 25 mg · ml⁻¹.

Table 5. Lactose hydrolysis by ST128 in non-fat milk^a

Time (min)	Glucose (mg · ml ⁻¹) ^b liberated by cells exposed to		
	AT	40% Ethanol	0.1% SDS
10	7.2	8.5	9.6
20	11.2	14.2	14.3
30	15.0	17.0	17.7
40	16.2	18.4	19.2
50	18.8	22.0	21.2
60	19.8	22.2	22.0

^a Reaction temperature: 50°C.

^b Average values of three replicate assays; maximum attainable glucose concentration: 24 mg · ml⁻¹.

Lactose hydrolysis by ethanol-permeabilized ST128 biomass. Ethanol-treated ST128 cells were similar to AT-treated and SDS-treated cells in efficiency to hydrolyze lactose in a 5% aqueous solution (Table 4). All three treatments resulted in greater than 75% lactose hydrolysis after 30 min of incubation at 37°C. The progression of lactose hydrolysis by ethanol-treated ST128 in non-fat milk at 50°C was comparable to that shown by AT- and SDS-treated cells (Table 5). Regardless of the chemical used for permeabilization, at a cell density of $AD_{660} = 2.5$, more than 75% lactose hydrolysis was achieved after 60 min of incubation.

Storage stability of β -galactosidase. Cell pellets of ethanol-permeabilized ST128 and LDB115 were kept frozen at -40°C. Samples were resuspended on a weekly schedule in 5% lactose in POM at $OD_{660} = 2.5$ and tested for β gal activity. No apparent loss of enzyme activity was detectable after 6 months of storage.

Discussion

Permeabilization destroys the ability of the cellular membranes to control the active transport of solutes. The process is useful in facilitating the extraction of intracellular enzymes or transforming microbial cells into nonviable biocatalysts. The various chemical, biological, and

physical techniques used in cell permeabilization have been extensively reviewed by Schutte and Kula [28].

In yeast, chemical permeabilization has been achieved with organic solvents [1, 2, 4, 6, 7, 29] and surfactants [12–15, 19, 24]. In bacteria, surfactants have been used to measure intracellular β -galactosidase activity [5], to release enzymes [20, 21, 28], and to enhance production of small solutes such as sorbitol and gluconic acid [26] and malic acid [37]. Surfactants are also useful in preparing permeabilized nonviable biocatalysts from LAB cells that are suitable for hydrolyzing lactose in milk [22, 23, 34–36].

Permeabilization of LAB cultures with solvents (e.g., toluene) or solvent mixtures (e.g., acetone–toluene) generally has been restricted to increasing the measurable level of intracellular β -galactosidase activity [31, 33–36]. Similar to other organic solvents, ethanol destroys the structural and functional integrity of cytoplasmic membrane in LAB. This leads to the passive influx and efflux of small solutes including lactose, whose transmembrane transport is normally regulated by complex mechanisms [25]. Although ethanol would most likely be an acceptable solvent for food industrial applications, its efficacy in producing a nonviable but biocatalytically active biomass of LAB as a source of β -galactosidase has not been evaluated.

The results of this study showed that treatment of ST cells with 30–50% ethanol was as effective as the standard permeabilization procedure with AT or treatment with synthetic detergents (SDS, TritonX-100) and bile salt preparations (deoxycholate, Oxgall) previously used for producing nonviable LAB cells with high levels of β gal activity [34–36]. As a rule, *S. thermophilus* strains showed significantly higher β gal activity under assay conditions (i.e., mg glucose released \cdot ml⁻¹ \cdot 10 min⁻¹) than *L. bulgaricus* strains, which makes *S. thermophilus* the species of choice as an enzyme source.

Although ethanol's bactericidal properties were reported to take effect at around 40% [16], in this study the treatment of LAB cultures with 30% ethanol already resulted in total loss of viability. This imparts a significant economic advantage to ethanol-based permeabilization procedures, since in processing LAB cultures the initially 55% aqueous alcohol may be repeatedly used for permeabilization as long as its ethanol concentration, which may be conveniently monitored, stays within the effective 30–55% range.

Unlike sonication [11], chemical permeabilization with solvents or detergents does not result in the release of intracellular β gal in LAB. Thus, similar to LAB cells permeabilized with detergents (e.g., SDS), alcohol-treated ST biomass could be used *in situ* for hydrolyzing lactose in aqueous solutions or non-fat milk. In this study,

application of ST biomass at a density equivalent to ca. 10⁸ CFU/ml live cells resulted in 80% greater hydrolysis of lactose in 5% aqueous solutions at 37°C and in skim milk at 50°C.

So far only one permeabilizing agent with detergent activity (bovine bile extract or Oxgall) has been cleared for use in dairy foods [3]. Although organic solvents such as toluene or acetone–toluene mixtures are excellent permeabilizing agents, their residues would be objectionable in dairy foods. Ethanol, on the other hand, may present an exception, since it is a food grade chemical, and in some fermented milk products (buttermilk, kefir) a small quantity of alcohol is naturally present as a product of starter culture metabolism. Thus, the trace amount of ethanol that may remain in a permeabilized ST biomass used for lactose hydrolysis in milk would be acceptable by consumers and regulatory agencies alike.

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