

Permeabilization of *Streptococcus thermophilus* and the expression of beta-galactosidase

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*Studies were carried out to determine the efficacy of several permeabilizing agents in inducing high-level expression of β -galactosidase in *Streptococcus thermophilus*. Sodium dodecyl sulfate, Triton X-100, sodium deoxycholate, and one commercial bile acid preparation were effective as membrane destabilizing agents allowing lactose influx and hydrolysis by cytoplasmic β -galactosidase in treated cells. Cells exposed to Oxgall or Triton X-100 displayed 15 times higher levels of β -galactosidase activity than control cells. Detergent treatment also induced extensive cell death or significant injury to cell populations resulting in long delays before resumption of growth. A permeabilized suspension of *S. thermophilus* corresponding to 10^8 cfu ml⁻¹ released 87% of glucose available in a 5% lactose solution within 10 min at 50°C.*

Keywords: β -Galactosidase; detergent effects; cell permeabilization; *S. thermophilus*

Introduction

Streptococcus thermophilus belongs to a diverse group of industrial microbes known as lactic acid bacteria (LAB) which includes streptococci, lactococci and lactobacilli. In various combinations with one another, LAB are responsible for the development of flavor and physical characteristics of different fermented dairy foods. Recently, interest has been growing in the indirect or direct uses of LAB as sources of enzymes with potential food and nonfood applications. For example, *S. thermophilus*, which has an essential role in the fermentation of yogurt as well as Italian- and Swiss-style cheeses, has been recommended as the source of β -galactosidase (β gal; β -D-galactoside galactohydrolase, EC 3.2.1.23). This enzyme at present is commercially available from yeast^{1,2} for use in low-lactose dairy foods to meet the dietary needs of lactose-intolerant consumers.³

The β gal of *S. thermophilus* has been characterized in detail by several research groups.⁴⁻⁸ Its proposed applications in low-lactose food production include the use of purified enzyme preparations,⁵ enzyme-releasing autolyzing strains,^{8,9} nongrowing lactose transport system defective mutant cultures,¹⁰⁻¹² and *S. thermophilus* cell suspensions dispersed in fluid milk where microbial growth and devel-

opment of sourness are suppressed at refrigeration temperatures.¹³ Since β gal in *S. thermophilus* is a cytoplasmic enzyme, full expression of activity may be achieved only through the perturbation of cell integrity, either by sonic disruption^{7,14} or by chemical treatment such as solvent mixtures.⁴ Sonication of cells results in the release of β gal, whereas permeabilization with organic solvents allows the free passage of lactose to the cell interior where β gal is still fully retained. In the latter case, the permeabilized cells may be viewed as enzyme microcarriers in which β gal remains in a naturally "immobilized" state. Permeabilized cells may be concentrated and used in place of purified β gal in the production of low-lactose foods.

The purpose of this study was to find alternative permeabilizing agents for *S. thermophilus*. The present work shows the effect of a variety of detergents on culture viability and the expression of β gal activity in this important industrial microorganism.

Materials and methods

Microorganisms and cultivation conditions

The test organism *Streptococcus thermophilus* ST128 was taken from our laboratory collection. Cultures were propagated in tryptone-yeast extract-lactose (TYL) broth¹⁵ at 37°C with 3 days between transfers. For preparing stock cell suspensions, overnight cultures (30 ml) were centrifuged at 10,000g at 4°C, washed once with 5 ml of distilled water and finally resuspended to OD₆₆₀ = 2.5 in distilled water (DO), or 50 mM K₂HPO₄/KH₂PO₄-1 mM

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MgCl₂ (POM) buffer, pH 7.4. These stock cell suspensions were used in all subsequent culture viability and enzyme expression experiments.

Cell permeabilization with detergents

Detergents tested for permeabilizing effect included sodium dodecyl sulfate (SDS, Pierce Chemical Co., Rockford, IL), Tween 80, Brij 35 and Triton X-100 (Sigma Chemical Co., St. Louis, MO), sodium deoxycholate and sodium cholate (Calbiochem, San Diego, CA), and two commercial grade bile salt preparations, Oxgall and Bile Salts No. 3 (Marcor Development Co., Hackensack, NJ). Detergent stock solutions were prepared in distilled water at 0.01, 0.025, 0.05, 0.1, 0.25 and 0.5% (w/v) concentration.

Three-milliliter samples of stock cell suspensions were centrifuged at 10,000g for 5 min, and each pellet was dispersed by vigorous vortexing in 3 ml of a detergent stock solution. After exposure for 20 min at room temperature, cells were pelleted as above, washed once with distilled water, and finally resuspended in 3 ml POM buffer. Internal controls included cells treated with a freshly prepared acetone-toluene (AT) mixture (9:1, v/v) at a concentration of 50 µl per milliliter of cell suspension for 20 min with intermittent vortexing,⁴ and cell suspensions exposed to distilled water only.

Effect of detergents on culture viability

Culture viability experiments were done two different ways to evaluate growth characteristics and to assess cell death following exposure to permeabilizing agents. In *method A*, 300 µl of each detergent-treated cell and washed cell suspension was mixed with 12 ml of TYL broth. The tubes were incubated at 37°C, and 1-ml samples were withdrawn hourly for a total of 6 h to measure changes in OD₆₆₀ values. In *method B*, detergent-treated cell suspensions were serially diluted with POM buffer (pH 7.0) and 100-µl samples of each dilution were plated out in duplicate on TYL-1.5% agar. After 24 h at 37°C, plates were scored for colony-forming units (CFU), and detergent-induced cell death was estimated by the difference found in CFU ml⁻¹ values between untreated and treated cells.

β-Galactosidase assays

The βgal activity of *S. thermophilus* ST128 and other ST strains was measured by mixing a sample of detergent-treated or -untreated control cell suspensions with 1 ml of 1.25 mM *O*-nitrophenyl-β-D-galactopyranoside (ONPG; Sigma) in POM buffer (pH 7.4), with

the total volume adjusted to 2 ml with POM buffer. Reaction mixtures were incubated for 10 min at 50°C. After stopping the reaction with 4 ml of cold 0.5 M Na₂CO₃, cells were pelleted at 10,000g for 5 min and the OD₄₂₀ values of supernatants were checked against the blank in a Beckman DU-70 spectrophotometer. One enzyme unit (U) was defined as the amount of enzyme catalyzing the release of 1 µmole of *O*-nitrophenol (ONP) per minute under assay conditions. Specific activities were calculated as units of enzyme per milligram of protein. Protein content of cell suspensions was determined by the method of Lowry *et al.*¹⁶

The original supernatants obtained after pelleting cells exposed to permeabilizing agents were also tested for βgal activity as an indicator of possible cell lysis.

Lactose hydrolysis by detergent-treated cells

To determine the extent of lactose hydrolysis by detergent-treated cells, *S. thermophilus* ST128 was exposed to each detergent at the concentration that consistently yielded high expression of βgal activity in earlier experiments. After detergent treatment for 20 min, cell pellets were resuspended in 5% (w/v) lactose solution in POM buffer (pH 7.4) and incubated at 50°C for 10 min. Reactions were terminated by pelleting the cells in an Eppendorf microfuge at top speed for 3 min. The supernatants were collected and 10-µl samples were analyzed in triplicate for glucose content with the Glucose (HK) Kit (Sigma Diagnostics, St. Louis, MO), according to the manufacturer's recommendations. The amount of glucose released was determined by comparing OD₃₄₀ values to a standard curve.

Results

βgal activity of permeabilized cells

In terms of displaying the highest level of βgal activity, the *S. thermophilus* ST128 test culture showed a varying degree of sensitivity to different permeabilizing agents (*Table 1*). Treatment with an organic solvent mixture (AT), a permeabilizing agent used routinely in the pretreatment of streptococci for βgal assays, induced an eightfold increase over untreated cells in the level of βgal activity. Among detergents, four of the eight preparations were also effective in inducing a significant increase in the level of expression of βgal. Sodium deoxycholate and the commercial bile salt preparation Oxgall were most effective at higher (0.5 and 0.25%) concentrations. The results with sodium cholate

Table 1 Effect of detergents on the expression of β-galactosidase activity by *S. thermophilus* ST128

Permeabilizing agent	βgal activity, U mg protein ⁻¹ min ⁻¹ at detergent concentration (%)						
	0.500	0.250	0.100	0.050	0.025	0.010	0.000
dH ₂ O (control)	—	—	—	—	—	—	0.650
AT (control)	—	—	—	—	—	—	5.580
Tween 80	0.510	0.550	0.500	0.510	0.500	0.500	—
Brij 35	0.600	0.600	0.530	0.530	0.520	0.520	—
Triton X-100	5.620	5.580	5.540	5.530	5.440	3.470	—
SDS	5.720	5.530	5.490	5.350	5.230	0.820	—
Deoxycholate-Na	5.260	5.260	1.710	1.000	0.880	0.870	—
Cholate-Na	0.330	0.900	0.550	0.540	NT	NT	—
Oxgall	5.350	4.850	1.280	1.120	0.900	0.940	—
Bile Salts No. 3	2.420	2.380	0.580	0.550	0.550	NT	—

βgal assays were carried out for 10 min at 50°C; NT, not tested

indicated limited permeabilizing effect and also explained the poor performance of the commercial Bile Salts No. 3 preparation, which is a 1:1 ($\pm 10\%$) mixture of cholate and deoxycholate. On the other hand, Triton X-100 and SDS retained high permeabilizing activity even at 0.025% concentration. The increased level of enzyme activity apparently was not the result of cell lysis, since the supernatants obtained from rinsed and pelleted cell suspensions after treatment lacked a measurable level of β gal. Treatment of cells at higher than 0.75% detergent concentration resulted in a loss of enzyme activity, possibly due to protein denaturation. The detergents Brij 35 and Tween 80 were inactive as permeabilizing agents and therefore excluded from further studies.

Effect of permeabilization on culture viability

The perturbation of cell integrity by various permeabilizing agents resulted in profound changes in the growth characteristics of *S. thermophilus* ST128. After resuspending cells treated with AT, SDS (0.05%), or sodium deoxycholate (0.5%) in TYL medium (*method A*), the OD₆₆₀ value of cell suspensions failed to change over the entire 6-h incubation period (*Figure 1*), indicating cell death or permanent injury impacting a large segment of the cell population. Treatment with Oxgall (0.5%) depressed the increase in cell density for 3–4 h, indicating that the cell population may have been killed in part or transiently injured, but still capable of eventual recovery. The growth of cell populations exposed to Triton X-100, sodium cholate, or Bile Salts No. 3 at 0.5% concentration, was depressed only slightly when compared to untreated control cells. This indicated a partial kill and a less severe transient injury to the microbial population. The results of *method B* which estimated the number of surviving cells after exposure to permeabilizing agents are shown in *Table 2*. The permeabilizing agents AT, SDS, and Oxgall, at the concentrations indicated, caused the greatest drop in

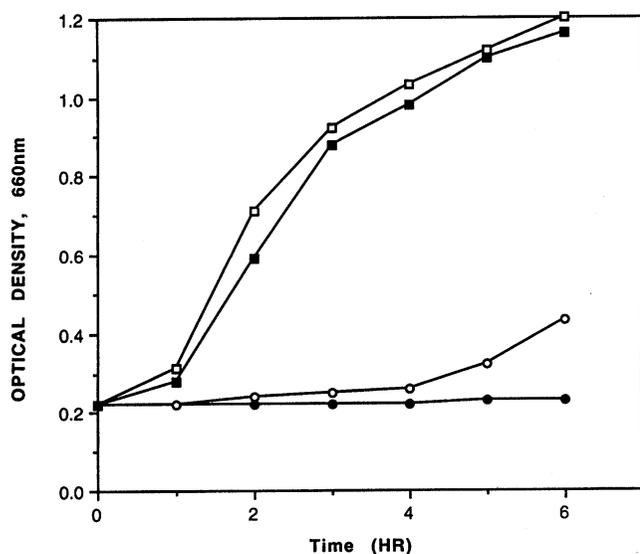


Figure 1 Effect of permeabilizing agents on the growth characteristics of *S. thermophilus* ST128. (□) dH₂O; (■) Triton X-100, sodium cholate, and Bile Salts No. 3; (○) Oxgall; (●) AT, SDS, and sodium deoxycholate. Incubation temperature was 37°C

Table 2 Survival of *S. thermophilus* ST128 after treatment with permeabilizing agents

Treatment ^a (conc.)	Live cell count, CFU ml ⁻¹
None (control)	10 ⁹
AT (50 μl ml ⁻¹)	10 ⁴
SDS (0.05%)	<10 ³
Oxgall (0.5%)	10 ⁴
Bile Salts No. 3 (0.5%)	2 × 10 ⁵
Sodium cholate	2 × 10 ⁵
Sodium deoxycholate (0.5%)	10 ⁵
Triton X-100 (0.1%)	5 × 10 ⁵

^aThe exposure to permeabilizing agents was for 20 min at room temperature

Table 3 Lactose hydrolysis by permeabilized *S. thermophilus* ST128

Treatment (conc.)	Glucose in reaction mixture, mg ml ^{-1a}
None (control)	1.5
AT (50 μl ml ⁻¹)	17.4
SDS (0.05%)	17.6
Oxgall (0.5%)	23.2
Bile Salts No. 3 (0.5%)	4.5
Sodium cholate	2.9
Sodium deoxycholate (0.5%)	17.0
Triton X-100 (0.1%)	23.5

^aTheoretical value of highest possible glucose conc. was 26.3 mg ml⁻¹. Lactose hydrolysis was carried out at 50°C for 10 min

live counts, which amounted to a reduction of 4 to 6 logs in the CFU ml⁻¹ value obtained with untreated cell populations. The CFU ml⁻¹ values with Bile Salts No. 3, sodium deoxycholate, sodium cholate, and Triton X-100 were somewhat higher, amounting only to a 3-log reduction in live cell counts.

Lactose hydrolysis by permeabilized cells

Table 3 shows the extent of lactose hydrolysis achieved by different treatments of permeabilized ST128 cells under experimental conditions. When the equivalent of 10⁸cfu ml⁻¹ cell suspensions (OD₆₆₀ = 2.5) were treated with sodium deoxycholate, SDS, or AT, the amount of glucose liberated was nearly the same (ca. 64%). Oxgall and Triton X-100 were clearly superior and induced β gal activity levels 15-fold over that measured with untreated cell suspensions. The amount of glucose liberated from lactose by each of these preparations equalled 87% of the theoretically attainable value (26.3 mg ml⁻¹) under assay conditions.

Discussion

Many microbial enzymes with a potential for industrial applications are retained within the cells, which makes recovery and subsequent purification difficult. One example is

the β -galactosidase of *S. thermophilus* which, because of its food-grade classification, has attractive possibilities for application in the production of low-lactose foods.

To achieve maximum recovery of β gal from *S. thermophilus*, the use of sonic disruption¹⁴ and autolysing strains^{8,9} has been proposed. However, the high cost of energy input in sonication and enzyme enrichment steps in the case of pH-induced autolysis remains a drawback to the large-scale application of both methods. Alternatively, *S. thermophilus* may be treated with suitable permeabilizing agents that disrupt membrane structures to allow the passive passage of low-molecular-weight solutes in and out of cells, including lactose and its products of hydrolysis. This approach was already used successfully to induce a high-level expression of β gal activity in *S. thermophilus* permeabilized cells which may be regarded as enzyme microcarriers available for direct food use as concentrated preparations.^{10-12,17}

The results of this study showed that several detergents and the commercial bile salt preparation Oxgall were highly effective in disrupting membrane structures in *S. thermophilus* to allow lactose influx but without inducing enzyme leakage or denaturation. High levels of ONPG hydrolysis were observed with Triton X-100, SDS, deoxycholate, and the commercial bile preparation Oxgall when used at either 0.5 or 0.25% concentration. At a lower than 0.1% concentration, only Triton X-100 and SDS remained effective. However, in terms of actual lactose hydrolysis, treatment with Oxgall (mixture of different bile acids) at 0.5% or Triton X-100 at 0.1% concentration resulted in the highest apparent β gal activity, whereas treatments with sodium cholate or Bile Salts No. 3 was less efficient.

All of the permeabilizing agents used adversely affected culture growth, either by inducing cell death, as evidenced by the precipitously lower live cell counts, or by inducing cell injury, as evidenced by the significant delays in population growth for extended periods of time. This is an especially attractive feature of cell permeabilization, since total cessation or delay of culture growth for 5–6 h would allow the use of *S. thermophilus* biomass for lactose hydrolysis in milk- or lactose-based products in general, without concern about the concomitant production of lactic acid.

Naturally, the concern must be addressed about residual permeabilizing agents that may remain associated with the concentrated cell preparations intended for food applications. However, we found that permeabilized *S. thermophilus* cells may be washed repeatedly with distilled water without the loss of β gal activity. Further, Oxgall or similar bile acid preparations that were previously shown to increase the *in vitro* β gal activity in yogurt,¹⁸ come closest to the definition of a "natural" permeabilizing agent.

The β gal of *S. thermophilus* is believed to contribute to the partial hydrolysis of lactose in dairy foods such as yogurt during product manufacture and again during the passage through the gastrointestinal tract, as the result of permeabilization by bile acids.^{19,20} Therefore, fortification of milk with nongrowing, live cells of *S. thermophilus* has been suggested as a means of increasing the amount of β gal available for *in vivo* lactose hydrolysis following ingestion.¹³ This approach, even if successful, would require special refrigeration conditions to prevent microbial growth and lactic acid synthesis which may cause the souring and coagulation of the product.

Our results reported here on *in vitro* lactose hydrolysis suggest that pretreatment of milk with permeabilized and killed or injured cells of *S. thermophilus* prior to direct consumption or incorporation into milk-based products is a convenient solution to the dilemma of finding uses for this excellent source of β gal. Unlike enzymes from other sources, the β gal of *S. thermophilus* needs no further purification or isolation from the producing microorganism to qualify for food-grade status. Under industrial conditions, lactose hydrolysis in milk could be carried out at 50–55°C for up to 6 h before pasteurization or sterilization by adding the inherently safe and edible biomass of permeabilized and nongrowing *S. thermophilus* at levels corresponding to 10⁸ CFU ml⁻¹. The extent of hydrolysis may be regulated by controlling the length of β gal pretreatment, which may result in up to 90% hydrolysis of lactose present in milk.

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