

Sensitivity of *Streptococcus thermophilus* to Chemical Permeabilization

Abstract. *Streptococcus thermophilus* cultures were treated with conjugated and unconjugated bile salts and tested for β -galactosidase activity. Na-deoxycholate and chenodeoxycholate were more efficient permeabilizing agents than cholate, and all three bile salts were superior to their corresponding glyco- and tauro-conjugates. Treatment with sodium dodecyl sulfate resulted in the highest measurable β -galactosidase levels in permeabilized cells, whereas response to Triton X-100 was variable and strain dependent. Na-deoxycholate, chenodeoxycholate, and sodium dodecyl sulfate caused cell injury and arrested culture growth for 4 h or longer. The nongrowing permeabilized biomass of *S. thermophilus* was used to hydrolyze lactose in aqueous solutions and milk.

Streptococcus thermophilus (ST) is a beneficial and economically important microbe that functions as a biocatalyst in the ripening process of many fermented dairy foods (yogurt and cheeses). Since the biomass of ST accumulating during fermentation is consumed together with the product, this harmless microbe has a "food-grade" status.

ST is a well-known producer of β -galactosidase (β gal; β -D-galactoside galactohydrolase, EC 3.2.1.23) [15, 19]. The intracellular enzyme is a 430-kDa tetramer [3] and has been suggested for reducing the lactose content of bovine milk [15, 17, 21]. Low-lactose milk products benefit large population segments around the world that are affected by lactose maldigestion syndrome [10]. The status of ST as "food grade" implies that the β gal of this microbe need not be rigorously purified, unlike the yeast β gal preparations in current commercial use [12]. Recent studies on *S. thermophilus* ST128 have shown that isolation of β gal for lactose hydrolysis is not necessary because treatment of the culture with certain chemicals (solvents, detergents) disrupts cell membranes and results in high-level expression of β gal [22]. Since

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the endogenous β gal is retained by permeabilized cells, the treated biomass may be used directly for hydrolyzing lactose in aqueous solutions and milk [21–24].

We have extended our studies to determine the concentration dependence of free and conjugated forms of bile salts as permeabilizing agents for several high β gal-producing strains of *S. thermophilus*. The synthetic detergents sodium dodecyl sulfate and Triton X-100 were also evaluated.

Materials and Methods

Bacterial strains and media. *Streptococcus thermophilus* strains ST106, ST110, ST119, ST133, ST134, and ST136 were selected from our in-house collection. Selection of strains was based on the level of β gal activity shown by acetone-toluene-treated cells in screening studies with lactose as a substrate [21]. The cultures were maintained in tryptone-yeast extract-lactose (TYL) medium [20] with 3 days between transfers and were incubated at 37°C in a 5% CO₂ atmosphere for 16–18 h. Culture growth was followed by monitoring optical density at 660 nm during incubation at 37°C. Viable cell counts (CFU/ml) were determined in TYL-1.5% agar medium with plates scored after 24-h incubation.

Conditions of cell permeabilization. One ml of each TYL-grown ST culture was inoculated after overnight growth into 200 ml of fresh medium and incubated without agitation for 16 h at 37°C. Cells were harvested at 10,000 g at 4°C in a Sorvall centrifuge and washed once with 50 mM K₂HPO₄/KH₂PO₄-1 mM MgCl₂ buffer (POM), pH 7.4, and resuspended in POM to an A₆₆₀ of 2.0. Samples of 1 ml were dispensed in 1.5-ml Eppendorf tubes and

centrifuged at top speed in a refrigerated microfuge at 4°C for 5 min. Supernatants were removed by aspiration, and pellets were suspended by vortexing in 1 ml detergent solution to induce permeabilization. After holding at ambient temperature (28°C) for 20 min, cells were pelleted as before and tested for β gal activity.

Cell preparations were treated with sodium salts of cholic (C), deoxycholic (D), and chenodeoxycholic (CD) acids, and their respective glyco- (GC, GD, GCD) and tauro- (TC, TD, TCD) conjugates, between 0.1% and 0.8% (wt/vol, in 0.1% increments). Sodium dodecyl sulfate (SDS) and Triton X-100, which were previously shown as effective permeabilizing agents for *S. thermophilus* strain ST128 [23], were respectively used at 0.01%, 0.025%, 0.05% and between 0.1% and 0.5% concentration (in 0.1% increments). Stock solutions of permeabilizing chemicals were prepared in POM. Controls were untreated cells and cells permeabilized with an acetone-toluene mixture (9:1 vol/vol, AT) at 50 μ l/ml cell suspension [19].

Bile salts, SDS, and Triton X-100 were purchased from Sigma Chemical Co. (St. Louis, Mo.).

Measurement of β -galactosidase activity. β Gal activity in permeabilized cells was assayed as previously described [21]. Briefly, cell pellets of ST strains treated with chemicals were resuspended by vortexing in 1 ml of 5% lactose prepared in POM. Reaction mixtures were kept in a 37°C water bath for 10 min. A 50- μ l sample of each reaction mixture was diluted with 450 μ l POM buffer and centrifuged at 10,000 g for 5 min. Triplicate 10- μ l samples of each supernatant were tested for glucose content with the Glucose HK Kit (Sigma Diagnostics, St. Louis, Mo.). Glucose concentrations (mg/ml) were calculated by comparing A_{340} values to a standard curve.

β Gal activity in supernatants was tested by mixing 100 μ l of each with 0.9 ml of 5% lactose and incubating as above, followed by triplicate glucose determinations with 10- μ l samples.

A permeabilized cell suspension of ST133 was also entrapped in 1.2% agarose (FMC Bioproducts, Rockland, Me.) at a cell density of $A_{660} = 50$. The 15 \times 25 cm \times 0.25 cm gel slab was placed in a cylindrical configuration in 2 L of 5% lactose solution or skim milk. The substrates were stirred magnetically, and the course of lactose hydrolysis was followed at 55°C by analyzing triplicate samples for glucose content.

Stability of β gal during storage. Pellets of ST cultures were stored at -40°C after treatment with 0.5% D and 0.1% SDS. At monthly intervals, samples were thawed and analyzed for residual β gal activity.

Results

Effect of bile salts on growth. The six strains of ST exposed to different forms of bile salts showed the greatest sensitivity to treatment with 0.6% solutions of D and CD. C was less inhibitory, while the glyco- and tauro-derivatives of all three bile salts had only limited effect on culture growth. At 0.3% concentration, only D remained consistently effective, whereas CD was less so, and most strains were not affected by C. Conjugated forms of bile salts (GD, TD, GCD, TCD, GC, TC) were practically without effect (Table 1).

Treatment with 0.05% SDS stopped the growth

Table 1. Growth of ST strains after treatment with permeabilizing agents^a

Strain	OD ₆₆₀ at 8 h ^b									
	Control	D ^c	GD	TD	CD	GCD	TCD	C	GC	TC
ST106	1.85	0.22	1.70	1.85	1.15	1.52	1.78	1.45	1.75	1.78
ST110	1.72	0.19	1.65	1.77	0.93	1.70	1.69	1.22	1.70	1.74
ST119	1.86	0.24	1.80	1.82	0.80	1.48	1.78	1.76	1.80	1.84
ST133	1.78	0.20	1.62	1.80	0.24	1.67	1.81	1.75	1.78	1.78
ST134	1.95	0.34	1.90	1.90	0.42	1.78	1.89	1.90	1.90	1.92
ST136	1.78	0.27	1.60	1.80	0.34	1.62	1.77	0.56	1.80	1.79

^a Chemicals were used at 0.3% (wt/vol) in POM.

^b Initial OD₆₆₀ values were adjusted to 0.18–0.20.

^c For abbreviations, see Materials and Methods.

of all test cultures for 8 h or longer, and some strains were inhibited by 0.5% Triton X-100. These results were similar to those obtained with strain ST128 [23], although differences in strain-specific response to various chemical treatments were observed.

Cell death induced in the populations of all six test cultures by D and CD was extensive and caused an up to 6-log reduction in viable cell counts (data not shown) in comparison with the controls (10⁸ CFU/ml). However, as in the case of *S. thermophilus* ST128 [23], the cellular injury induced by D and CD was apparently repairable in the surviving cell populations, since all six cultures resumed growth following a 6- to 8-h-long lag period. On the other hand, SDS treatment apparently caused more severe cell injury in still viable cells and prolonged the recovery of culture growth for up to 24 h in all test cultures.

None of the chemicals tested appeared to induce cell lysis in ST cultures. This was supported by the absence of changes in A_{660} values and the lack of detectable β gal activity or A_{260} -absorbing material in the supernatants of cell suspensions after permeabilization.

Effect of chemicals on β gal activity. The level of β gal expression by treated cells was influenced by the type, nature, and concentration of the detergent used for permeabilization (Figs. 1–3). In addition, strain-dependent fluctuations in β gal values were also observed. In most strains D was slightly more efficient than CD, and both salts required a concentration of 0.3% or higher to induce the maximum level of β gal expression. At higher (up to 0.8%) concentrations of D or CD, enzyme levels remained virtually unchanged. On the other hand, C showed a significantly lower overall efficiency and showed peak activity at ca. 0.6% concentration (Fig. 1).

The conjugated forms of bile salts, GD, GCD, TD, and TCD, were far less efficient in permeabili-

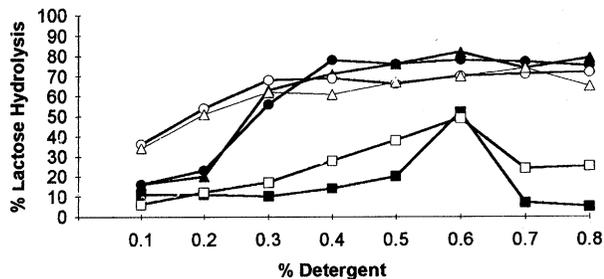


Fig. 1. Effect of deoxycholate (●○), chenodeoxycholate (▲△), and cholate (■□) on β -galactosidase activity in ST106 (●▲■) and ST136 (○△□).

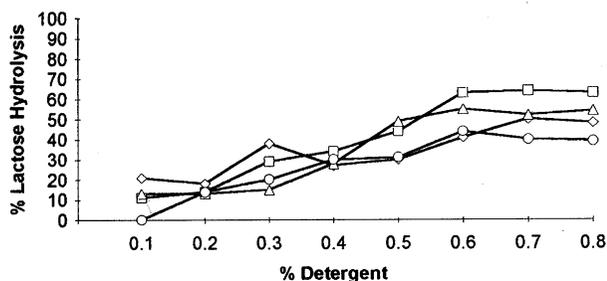


Fig. 2. Effect of glycodeoxycholate (□), taurodeoxycholate (△), glycochenodeoxycholate (◇) and taurochenodeoxycholate (○) on β -galactosidase expression by permeabilized cells of ST106.

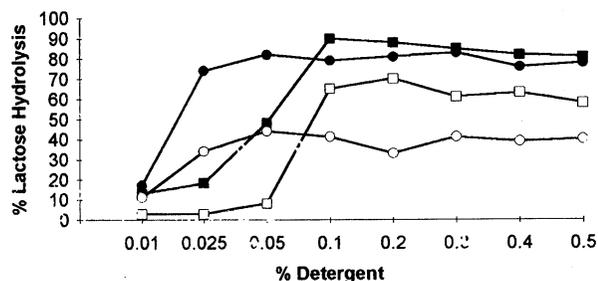


Fig. 3. Effect of SDS (■□) and Triton X-100 (●○) on β -galactosidase activity in ST106 (■●) and ST136 (□○).

zing ST cells than their corresponding unconjugated forms. Furthermore, glyco-conjugates were somewhat more effective than tauro-conjugates of bile salts (Fig. 2). The treatment of cells with glyco- and tauro-conjugates of C, the least effective bile salt, resulted only in a moderate increase in β gal expression.

The synthetic detergent SDS was superior as a permeabilizing agent to both unconjugated and conjugated forms of bile salts, and displayed maximum activity at concentrations three- to five-fold lower than required by D or CD (Fig. 3). The response to treatment with Triton X-100 was less uniform and varied with the strain used.

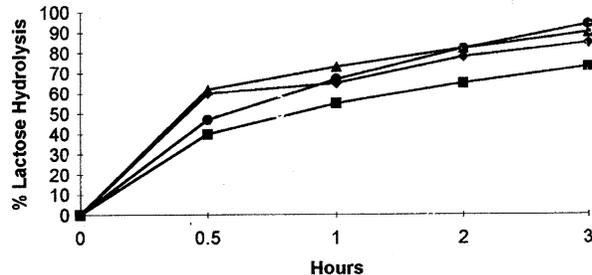


Fig. 4. Time course of lactose hydrolysis in skim milk by permeabilized cells of ST133 at 55°C. Cell density levels (A_{660}): 2.5 (●), 2.0 (▲), 1.5 (◆), 1.0 (■).

Hydrolysis of lactose by permeabilized ST cells. ST133 cells permeabilized with 0.6% D were resuspended in skim milk to a cell density equivalent to 10^8 CFU/ml and incubated at 55°C. After 2 h more than 85% of lactose was hydrolyzed (Fig. 4). The time course of lactose hydrolysis followed a similar pattern with permeabilized cells of the other ST cultures. Immobilized cells of ST133 entrapped at a 20-fold higher cell density in 1.2% agarose films hydrolyzed slightly more than 65% of the lactose in 2 L of lactose solution or skim milk after 2 h of incubation.

Stability of β gal in permeabilized cells. Samples were taken on a monthly schedule of predispensed ST cells permeabilized with 0.01% SDA or 0.5% D and checked for β gal activity. No loss of enzyme activity was found after 9 months of storage of the cell preparations at -40°C .

Discussion

The results of this work demonstrate that bile salts, and to a lesser extent their conjugated forms, are suitable for enhancing the level of measurable β gal activity in *S. thermophilus* cells via permeabilization. Although the permeabilizing effect of sodium deoxycholate on group D enterococci and group N dairy lactococci was noted previously and used in β -galactosidase assays [6, 9], this is the first comprehensive evaluation of the effect of pure bile salt preparations on the β -galactosidase of thermotolerant dairy cultures.

The data on the six ST test strains also confirm the consistently superior performance of SDS as a permeabilizing agent and growth inhibitor at very low concentrations [23]. It is noteworthy that, at the concentrations used, SDS does not denature the β -galactosidase in permeabilized ST cells, whereas in the case of group D enterococci, SDS treatment results in the total loss of β -galactosidase activity [9].

The growth inhibition of ST cultures by SDS at very low concentrations is also in sharp contrast with findings on enterobacteria, which continue to grow even in the presence of 5% SDS [1].

On the basis of permeabilizing and growth-inhibitory (cytotoxic) effects, bile salts may be ranked as $D > CD > C$, which is also the order of their ranking based on hydrophobicity. Interestingly, a similar relationship between hydrophobicity and the cytotoxic effects of bile salts was found in liver cells [14]. Our observations support the view that bile salts most likely interfere with the maintenance of integrity of key lipoprotein and protein cell membrane complexes in ST cells, resulting in growth inhibition and a greater accessibility of substrate to the endogenously located β gal enzyme by passive influx.

The intracellular nature of β gal in ST so far has prevented its potential development as an industrial enzyme, although its high temperature optimum (55–60°C) is an advantage over the less heat-tolerant yeast enzymes currently in use [12]. To facilitate the recovery and utilization of this enzyme in food processing, the use of sonically disrupted cells [11, 16] and autolyzing ST strains [2, 17, 18] was explored. In the first approach, scaling up may present a problem, whereas in the second, the parameters of autolysis are difficult to control. On the other hand, studies in our laboratories have confirmed repeatedly the potential of permeabilized ST biomass for direct application for reducing the lactose content of milk, cheese whey, or lactose solutions [22–24]. The advantages of permeabilized ST biomass are several: (a) the enzyme source (ST) is food grade, and the isolation and purification of β gal are unnecessary; (b) the intracellular β gal is retained by permeabilized cells without loss of activity; (c) metabolic activities such as acidulant synthesis that may interfere with applications are arrested by detergent treatment; (d) the solution of the permeabilizing agent may be reused several times without significant loss of activity; and (e) if required, after treatment the ST biomass may be washed free of detergent residues.

The acceptability of specific permeabilizing agents for preparing ST biomass as a source of β -galactosidase for use in dairy foods remains to be determined. Although SDS is allowed as a food additive [4], at present its use in connection with dairy products is not covered. The use of ST biomass permeabilized with deoxycholate and chenodeoxycholate should be acceptable, since both salts are present in human bile where the latter is a major component [8]. Further, deoxycholate is also an ingredient of bovine bile extracts already cleared for use in dairy foods [5].

Traces of deoxycholate or chenodeoxycholate that may be present in the ST biomass after removal of the permeabilizing solution should not be a concern, since both compounds have been used in humans for therapeutic purposes, apparently without deleterious effects. Deoxycholate is useful as a complexing agent for delivering antifungal agents [13], and chenodeoxycholate is an ingredient in formulations for the treatment of gallstones [7]. Therefore, in our view, a strong argument may be presented to support the use of pure bile salts and also SDS in preparing permeabilized ST biomass for lactose hydrolysis in milk on an industrial scale.

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