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Permeabilized *Streptococcus thermophilus* in the preparation of low-lactose milk

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Several compounds with detergent activity were used to permeabilize *Streptococcus thermophilus* and allow a high level expression of intracellular β-galactosidase. Treatment with SDS, Triton X-100, sodium deoxycholate and oxgall suppressed cell growth for several hours, which permitted the use of permeabilized *S. thermophilus* biomass in milk without concomitant production of lactic acid. The addition of cell preparations directly or entrapped in an agarose-gel support resulted in up to 90% or higher reduction of lactose content in milk.

Streptococcus thermophilus is a species used extensively in the production of yogurt and cheeses where, in association with other lactic acid bacteria, its main function is the synthesis of lactic acid and precursors of flavour compounds from lactose [1]. The biomass of this microbe accumulates in dairy foods during fermentation, and it is consumed together with the product. Because of its innocuous nature, *S. thermophilus* is an edible and 'generally regarded as safe' (GRAS), food-grade micro-organism.

S. thermophilus has been suggested as a source of β-galactosidase (βgal[2]; β-D-galactoside galactohydrolase; EC 3.2.1.23), suitable for reducing the lactose content of milk [2–5]. Low-lactose dairy foods circumvent the gastrointestinal distress caused by lactose in humans (lactose malabsorption or intolerance) which affects a large segment of the world's population [6]. The *S. thermophilus* enzyme, with its relatively high (50–55 °C) optimum temperature [7], could replace the less-heat-tolerant βgal preparations derived from yeasts [8,9] that are used currently on a commercial scale.

In our laboratory we have studied the characteristics of expression of intracellular βgal activity after treatment of *S. thermophilus* with various permeabilizing agents [10]. We have also recommended the use of permeabilized cells of lactose-transport-system-defective (*lacS*$^-$) strains or detergent-treated cultures of non-growing, wild-type *S. thermophilus* as enzyme microcarriers for lactose hydrolysis in milk in place of purified

βgal from the same or other sources, either as cell suspensions or entrapped in a suitable matrix, such as agarose gels [6,10–12].

The purpose of the present study was to examine the performance of permeabilized non-growing cell suspensions of wild-type *S. thermophilus* and to examine several parameters that influence the hydrolysis of lactose in milk.

Materials and methods[3]

Micro-organism

The culture used in the present study was *S. thermophilus* ST128 from our in-house collection. It was maintained in tryptone/yeast extract/lactose (TYL) broth [13] with 3 days between transfers and incubated at 37 °C for 16 h before storage at 4 °C. Viable-cell counts (colony-forming units/ml) were carried out in TYL/1.5% agar medium and colonies were counted after a 24 h incubation of plates at 37 °C.

Biomass production and permeabilization

16 h broth cultures (200 ml of TYL per 500 ml Erlenmeyer flask) grown at 37 °C without agitation were centrifuged at 10 000 ***g*** at 4 °C, washed once with distilled water and resuspended in 50 mM K_2HPO_4/KH_2PO_4/1 mM $MgCl_2$ (POM) buffer, pH 7.4, to an A_{660} of 0.25 to 5.0, as specified.

Permeabilization of cell suspensions was done with acetone/toluene (AT) mixture (9:1, v/v) at a concentration of 50 μl/ml of cell suspension (A_{660} 2.5), for 20 min at room temperature, in a volume of 5 ml. Other permeabilizing agents used were 0.05% (w/v) SDS (Pierce Chemical Co., Rockford, IL, U.S.A.), 0.5% Triton X-100 (Sigma Chemical Co., St. Louis, MO, U.S.A.), 0.5% sodium deoxycholate (DEO; Sigma) and 0.5% Oxgall (bacteriological grade; Marcor Development Co., Hackensack, NJ, U.S.A.). After treatment, cells were pelleted at 10 000 ***g*** for 5 min, washed once with POM buffer (3 ml) and finally resuspended in 5 ml of ice-cold (4 °C) substrate solution (5% lactose or skim milk). The controls were cell suspensions treated with distilled water.

Measurement of lactose hydrolysis

Permeabilized *S. thermophilus* ST128 cells were mixed with 1 ml of 5% lactose in POM buffer at a final A_{660} between 2.5 and 0.25/ml in increments of 0.5 unit. The reaction mixtures were incubated under standard assay conditions at 50 °C for 10 min, followed by the removal of 50 μl samples, dilution with 0.45 ml of ice-cold POM buffer and centrifugation at 10 000 ***g*** for 3 min. Samples (10 μl) were tested in triplicate for glucose content with the Glucose (HK) Kit (Sigma Diagnostics, St. Louis, MO, U.S.A.), according to the procedure recommended by the manufacturer. Glucose concentrations were calculated by comparing A_{340} values with a standard curve.

Lactose hydrolysis in milk was monitored by resuspending permeabilized *S. thermophilus* ST128 in skim milk or whole milk (purchased at a local supermarket) routinely at a cell density eqivalent to an A_{660} of 2.5 or about 10^8 colony-forming units/per ml, unless specified otherwise. Reaction mixtures were kept at 50 °C. At appropriate intervals, samples were withdrawn and worked up as described above.

Immobilization technique

Cell biomass prepared from 16 h-old growth in TYL was treated with DEO (0.5%), concentrated and entrapped in 0.3 cm-thick, 1.5% (w/v) SeaKem ME agarose (FMC Bioproducts, Rockland, ME, U.S.A.) slabs (6.5 cm × 15 cm) prepared on a GelBond film support (FMC Bioproducts) at a final cell density of 36 A_{660} units/ml. The solidified agarose slabs were stored in POM buffer and kept at 4 °C until use. For lactose-hydrolysis experiments, 200 ml of skim milk, whole milk or a 5% lactose solution were heated to 50 °C and stirred magnetically. The agarose slab was separated from the GelBond support and was immersed in a cylindrical configuration until fully covered by substrate solution. Samples (1 ml) were withdrawn during incubation at appropriate time intervals and glucose assays were performed as described above.

Results and discussion

Lactose hydrolysis by permeabilized cells

Permeabilizing agents were used at concentrations that had been reported previously to arrest the growth of *S. thermophilus* ST128 wild-type populations in a lactose medium for 2–3 h or longer [12]. The effect of detergents on growth was similar to that reported for growth suppression by AT (in sucrose medium) of the *lacS*$^-$ mutant *S. thermophilus* NRRL 18818 (previously ST1281), which fails to grow in lactose media [6]. The inability of cultures treated with detergents to resume rapid growth implied that hydrolysis of lactose in either lactose solutions or milk may be more conveniently accomplished by non-growing wild-type *S. thermophilus* cultures permeabilized by a suitable detergent without the need for lactose-transport-negative mutant strains that require special growth conditions.

The extent of lactose hydrolysis established with the 10 min assay method (at 50 °C) showed a direct proportionality to the concentration of permeabilized cells in the reaction mixture (Table 1). Maximum lactose hydrolysis was achieved at a cell density of $A_{660} = 2.5$, regardless of the permeabilizing agent used, while lactose reduction was marginal by control cells treated only with water (Table 1). This value corresponded to a live cell count of about 10^8 colony-forming units/ml, which confirmed previously obtained results [12]. However, differences were observed in the levels of measurable βgal activity following permeabilization.

Table 1 Effect of cell density on lactose hydrolysis[a]

	Glucose (mg/ml)[b] *liberated by cells treated with:*					
A_{660}	*Dist. water*	*AT*	*SDS*	*DEO*	*Triton X-100*	*Oxgall*
0.25	0.42	2.5	7.1	5.0	3.8	5.3
0.50	0.78	4.8	12.8	10.3	6.5	9.2
1.00	1.06	9.5	18.6	13.8	10.6	12.3
1.50	1.28	12.8	20.0	16.7	15.5	15.4
2.0	1.39	16.5	21.2	17.9	17.2	18.4
2.5	1.50	18.0	21.6	19.2	18.8	19.2

[a] The substrate was 5% lactose in POM buffer; conditions of permeabilization and glucose assays are described in the text.
[b] The maximum attainable glucose level was 25 mg/ml.

Table 2 Effect of incubation temperature (*T*) on lactose hydrolysis
ST128 biomass was permeabilized with 0.5% DEO. The cell density in reaction mixtures was equivalent to an A_{660} of 2.5/ml. The length of incubation was 20 min.

		[Glucose] (mg/ml)							
Substrate	*T (°C)...*	*30*	*40*	*45*	*50*	*55*	*60*	*65*	*70*
Skim milk[a]		4.32	6.37	7.80	8.06	8.53	8.92	9.92	1.21
Whole milk[a]		4.37	6.77	8.25	9.27	9.65	10.08	10.80	1.25
5% Lactose[b]		12.41	15.47	16.40	18.20	19.46	19.50	14.32	2.03

[a,b] Maximum attainable glucose values were calculated as 24.2 mg/ml and 25 mg/ml respectively.

Although used at a 10-fold lower concentration than DEO, Oxgall and Triton X-100, SDS was clearly the most effective detergent in inducing the highest level of βgal expression in treated cells. SDS was also superior to treatment with AT.

Incubation temperature and lactose hydrolysis

The effect of temperature on lactose hydrolysis was evaluated with 5% lactose, skim milk and whole milk as substrates (Table 2). In lactose solution, peak activity was detected at 55–60 °C, which was similar to the optimum temperature reported for purified βgal from this organism [7]. On the other hand, in skim milk and whole milk, maximum activity was found at about 60–65 °C. This slight discrepancy was speculated to be the result of a mild protective effect provided by milk proteins. Indeed, other workers have reported the stabilization of *S. thermophilus* βgal by BSA, as well as of milk proteins resulting from the formation of more heat-resistant complexes [14,15].

Preparation of low-lactose milk

The results summarized in Table 3 show that lactose hydrolysis in skim milk progressed at a slower rate than in 5% lactose in POM buffer. First,

Table 3 Lactose hydrolysis in skim milk by cells treated with permeabilizing agents
The permeabilizing agents were used at the concentrations indicated in the Materials and methods section. The cell density in reactions was equivalent to an A_{660} of 2.5/ml.

	[Glucose] (mg/ml)[a]				
Time (min)	*AT*	*SDS*	*DEO*	*Triton X-100*	*Oxgall*
5	2.2	2.9	2.2	2.5	2.4
10	4.4	5.9	5.0	5.6	5.4
20	7.8	9.5	8.1	8.7	8.2
30	9.8	12.6	11.0	11.8	11.2
40	12.0	13.2	12.6	13.4	12.7
60	15.6	16.8	15.8	16.4	16.4
120	23.3	23.6	22.4	24.1	24.2

[a] The maximum attainable glucose value was 24.2 mg/ml.

Table 4 Lactose hydrolysis by immobilized *S. thermophilus* cells

	Lactose hydrolysed[a]	
Time (h)	*Skim milk*	*5% Lactose*
0.5	21.1	44.8
1.0	36.5	67.3
1.5	49.6	78.9
2.0	65.6	91.3
2.5	77.0	95.4
3.0	82.5	99.6

[a] The maximum attainable glucose values were 24.2 mg/ml (skim milk) and 25 mg/ml (lactose solution).

it was assumed that the lower rate of hydrolysis may be due to the lack or unavailability in milk of Mg^{2+}, which is essential for optimum βgal activity in *S. thermophilus* [7]. However, supplementation of skim milk with 5 mM $MgCl_2,6H_2O$ increased the amount of glucose generated by less than 10%.

During incubation the pH value of milk remained at 6.8, indicating the lack of growth and acid production by *S. thermophilus* cells. Up to 30 min of incubation, SDS-treated cells hydrolysed a somewhat higher amount of lactose in milk, similarly to that found with buffered (pH 7.4) lactose solutions. However, after 60 min of incubation, a convergence of glucose values attained with other detergents was apparent. At a cell density of equivalent to an A_{660} of 2.5, more than 90% of the glucose was released within 2 h from lactose present in skim milk. In practice, when only lower cell densities may be attainable, the reduced level of total enzyme available in the permeabilized biomass may be compensated for by extending the length of the incubation period.

Immobilized biomass for lactose hydrolysis in milk

Cells treated with sodium deoxycholate and entrapped in agarose films were also suitable for lactose hydrolysis in either milk or lactose solutions (Table 4). As already observed with the free-floating cells of permeabilized *S. thermophilus*, the amount of glucose released within any given time period was lower in skim milk than in lactose solutions. However, by lengthening the incubation period, the amount of glucose released in milk eventually approximated to that found with lactose solutions. The use of agarose for cell immobilization had a disadvantage: the gel matrix was fragile and tended to tear as a result of handling and repeated uses. Nevertheless, the promising performance in milk of cells immobilized in agarose invites further tests with other support materials that may allow repeated use of the entrapped *S. thermophilus* enzyme.

In summary, the results of this study show the convenience of using the relatively heat-stable βgal of a permeabilized, non-growing *S. thermophilus* biomass for lowering the lactose content of milk. This offers an attractive solution for the production of low-lactose milk products for the benefit of human populations affected by the nuisance disorder of lactose indigestion.

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