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Recovery of ß-galactosidase from the yeast *Kluyveromyces lactis* by cell permeabilization with sarkosyl

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ABSTRACT

We present here a novel method for recovering ß-galactosidase (ß-gal) from the yeast *Kluyveromyces lactis* (NRRL 1118) by means of sarkosyl permeabilization. The yeast was permeabilized with 0.2% (w/v) sarkosyl in 0.1 M potassium phosphate buffer for 30 min at 25 °C. After centrifuging and washing, the permeabilized cells were incubated in buffer at pH 7.2, 35 °C for 6–7 h resulting in a nearly soluble extract containing 80% of the ß-galactosidase and about 40% of the cell protein. Transmission electron microscopy showed that permeabilized cells underwent gross structural changes during enzyme release but did not exhibit significant physical breakage. An extract obtained from a 40 mg mL^{-1} suspension of permeabilized cells was partially purified and concentrated 10-fold through diafiltration and ultrafiltration. The concentrated enzyme solution mixed with 50% (v/v) glycerol proved to be stable for at least 10 months at 5 °C. The kinetic properties of the ß-galactosidase preparation including the hydrolysis of milk, were similar to those exhibited by a commonly used commercial ß-galactosidase derived from the same yeast species. Sarkosyl is a biodegradable detergent with extremely low toxicity, thus making this compound an attractive permeabilizing agent for the downstream -processing of enzymes used in the food industry.

1. Introduction

Kluyveromyces lactis is a yeast that produces one of the ß-galactosidases (ß-gals) most widely used in neutral milk products [1,2]. The enzyme, commonly known as lactase, is produced on an industrial scale by selected yeast strains containing extra copies of the LAC4 gene [3]. Since the ß-gal is located in the cytoplasm of yeasts, cell disruption is necessary to release the enzyme into solution. This step is a critical operation because conditions of disruption may have a profound effect on the later processing and overall efficiency of the commercial process [4,5]. The ideal method of cell disruption would be one that releases the protein selectively, in high yield, and with minimal micronization of cell debris in order to facilitate the processing of the crude extract. Permeabilization of cells under mild physical-chemical conditions provides some of these advantages when compared to the methods of mechanical rupture by homogenization or ball-milling typically used on industrial scale [6]. The objective of permeabilization is to disrupt the cell envelope and the internal membranes in order to release a desired protein [7].

Several permeabilization methods have been used to obtain ß-gal from different *Kluyveromyces* species. In these instances, cells were permeabilized by electrical fields [8], toxic solvents such as toluene or chloroform [9–11], or high ethanol concentrations [12,13]. The characteristics of such systems would likely limit their commercial application [5]. This yeast species have also been permeabilized with organic solvents [14–16] and detergents like Triton X-100 [17], cetyl trimethylammonium bromide [18], and digitonin [19]. Because in those instances the permeabilization was carried out in order to prepare whole-cell biocatalysts for lactose hydrolysis or for *in-situ* determination of the ß-gal activity, the details of the enzyme release were not investigated.

An effective chemical for yeast permeabilization would appear to be the anionic detergent N-lauroylsarcosine (sarkosyl). This detergent is an inexpensive chemical that is widely used for the solubilization of recombinant proteins from inclusion bodies and in many other biological applications [20,21]. This detergent was furthermore highly efficient in permeabilizing *K. lactis* [22] and *Kluyveromyces marxianus* [23] as well as other yeasts such as recombinant *Saccharomyces cerevisiae* and *Saccharomyces pombe* strains expressing lactase [24] and baker 's yeast for measuring catalase activity [25] or to accelerate autolysis for the recovery of intracellular enzymes [26]. Because sarkosyl is biodegradable and exhibits a low toxicity, the compound has been authorized for use in personal-care pharmaceutical products and in food films [27,28]. These characteristics, makes sarkosyl a potential permeabilizing agent for use in the downstream-processing of enzymes used in the food industry [5]. In the present report, we have used sarkosyl

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permeabilization for the release of ß-gal from *K. lactis* cells and developed a simple process for obtaining a partially purified, stable β-gal preparation.

2. Materials and methods

2.1. Reagents and buffers

N-Lauroylsarcosine sodium salt (sarkosyl; PubChem CID: 23668817, L5125, ≥94%) and *o*-nitrophenyl-ß-D-galactopyranoside (ONPG) were purchased from Sigma Chemical Company (St. Louis, MO). Other reagents were of analytical grade. PPB contained 0.1 M dipotassium phosphate adjusted to pH 7.0 with HCl 1.0 N. Extraction buffer (EB) contained 50 mM dipotassium phosphate and 0.1 mM Mn^{+2} for ß-gal stabilization, and 0.05% (w/v) methylparabene (PubChem CID: 7456) as a preservative. The pH of EB was adjusted to the desired value with 1.0 N KOH or HCl. The commercial lactase, Maxilact L 2000™, was from DSM (Delft, The Netherlands). Ultrapasteurized whole milk (La Serenisima, Argentina) was purchased from a local market.

2.2. Yeast strain and culture conditions

Kluyveromyces lactis NRRL Y1118 strain (Agricultural Research Service Culture Collection, Peoria, IL, USA) was used throughout. Yeast was grown in lactose-limited aerobic chemostat cultures at a dilution rate of 0.1 h^{-1} in a synthetic medium to induce high levels of β-gal [29]. The culture biomass was recovered from the medium by centrifugation at 5000 × *g* for 15 min and 5 °C, washed twice with distilled water, and frozen in plastic bags at −20 °C. The frozen cells (20–22% dry weight) were used within a period of two months. One mg of cell dry weight contained ~2 × 10^8 cells. The protein content of the biomass was 44%, and the ß-gal and invertase activities measured in a homogenate of mechanically disrupted cells were 6.43 ± 0.16 U mg^{-1} dry cell (~30 U 10^{-9} cells) and 2.81 ± 0.08 U mg^{-1} dry weight, respectively. About 90% of the invertase activity is perisplasmatic [9]. Invertase has been used as a marker enzyme to evaluate cell-wall disruption [7].

2.3. Permeabilization procedure

Frozen cells were thawed at room temperature (usually for 30 min) and then suspended in PPB at a concentration of ~20 mg dry weight mL^{-1}. Twenty to thirty mililiters of the cell suspension was placed in 150-mL screw-capped bottles and gently agitated in a reciprocating shaker bath at 25 °C. Permeabilization was initiated by adding sarkosyl from a concentrated stock solution at different final concentrations (0.05, 0.1, 0.2 and 0.5% [w/v]). A cell suspension without sarkosyl was used as control. Since intact cells are not permeable to ONPG [15], cell permeabilization was assessed by measuring the ß-gal activity (per unit volume) of the total cell suspension hereafter referred to as At. For measuring At, a sample of the cell suspension was diluted 100- to 300-fold in cold PPB in order to stop the permeabilization because to the dilution of the detergent. The diluted sample was kept refrigerated until it was analyzed for enzyme activity. Cell viability during permeabilization was assessed by the methylene-blue test [30] and by plate counting on YPG agar after dilution of the cell suspension in 0.1% (w/v) bactopeptone.

2.4. Enzyme release

Permeabilized cells were centrifuged (5000 × *g* for 15 min), washed once with PPB, and resuspended in EB at a cell concentration of ~20 mg dry weight mL^{-1}. Twenty mililiters of this supension were transferred to a 150-mL screw-capped bottle and incubated in a reciprocating shaker bath (100 r. p. m.). The release of ß-gal from sarkosyl-permeabilized cells was tested at different pHs and temperature values assaying one-factor at-a-time. Accordingly, the ß-gal released was determined in EB at pH 6.6, 7.2, and 7.8 and 30 °C, then at the optimum pH of the EB the release was determined at 35 and 40 °C. The values selected for both parameters were those within the range of maximum stability of the enzyme [1]. The total incubation time was 8 h, and samples were taken every hour for analysis. The time course of β-gal release was followed by measuring both the At and the extracellular activity (Ae) after centrifuging 0.1 mL of the cell suspension in a Thermo Scientific Eppendorf™ microtube centrifuge (10,000 × *g* for 1 min). The suspension and the supernatants were diluted 100-fold with buffer for the enzymatic analysis or 5-fold with water for protein determination. The percent of enzyme released during the incubation time was calculated as Ae/At × 100. Overall changes in the cell structure resulting from the release of cellular components were followed by measuring the decrease in absorbance of the yeast suspension at 620 nm [31] after a 100-fold dilution with EB.

2.5. Transmission electron microscopy (TEM)

Yeast cells before sarkosyl permeabilization (control cells) and sarkosyl-permeabilized cells incubated for 6 h at 35 °C in EB (pH 7.2) were used for TEM observation. The cells were washed with phosphate buffer (pH 7.4) and fixed 2 h at 4 °C in 2% (w/v) glutaraldehyde. The fixed cells were washed three times (30 min) with buffer, osmicated for 1 h at room temperature with 1% OsO4, dehydrated in a graded ethanol series, and embedded in Epon resin. Ultrathin seccions of the Epon blocks were mounted on grids of 3-mm diameter and were stained with 2% (w/v) aqueous uranyl acetate followed by 1% (w/v) lead acetate. The specimens were examinated and photographed with a JEM-1200 EX.II (JEOL) transmission electron microscope at 80–100 kV.

2.6. Comparison of sarkosyl permeabilization and mechanical disruption

Yeast cells were suspended in PPB and permeabilized by exposure to 0.2% (w/v) sarkosyl for 30 min at 25 °C. After treatment with the detergent, the permeabilized cells were separated by centrifugation, washed once with buffer, suspended in EB (pH 7.2) at a cell concentration of ~20 mg dry weight mL^{-1} and incubated for 6 h at 35 °C to release ß-gal. After this time the suspension was sequentially centrifuged at 5000 × *g* for 15 min, 20,000 × *g* for 20 min, and 100,000 × *g* for 1 h (at 5 °C in a Beckman LE-80K ultracentrifuge) to obtain the S5, S20, and S100 supernatant fractions, respectively.

Mechanically disruption of cells was performed by shaking the yeast suspension in the presence of glass beads. Frozen cells were thawed and suspended in PPB at a concentration of ~20–22 mg dry weight mL^{-1}. The suspension (15 mL) was mixed with 15 g of glass beads (diameters 0.45–0.50 µm) and the cells broken in a cell homogenizer (MSK, B Braun, Melsungen, Germany) through eight 15-s shaking cycles under refrigeration. Cell disruption was estimated by microscopical observation. The cell homogenate was then likewise differentially centrifuged to obtain the corresponding S5, S20, and S100 supernatant fractions.

2.7. Preparation of a partially purified ß-gal

To obtain a preparation of partially purified ß-gal, a miniscaled diafiltration plus ultrafiltration was carried out starting with the S5 cell-free extract obtained as described above from sarkosy-permeabilized cells. In this instance the cell concentration during the extraction was ~40 mg dry weight mL^{-1} in order to increase the activity of the final ß-gal preparation. The S5 extract was filtered through a 1-µm glass-fiber microfilter and then diafiltered and concentrated by centrifuging in a 2-mL Centricon™ tube containing an Ultracell YM-100 membrane (nominal-molecular-weight cut-off, 100 kDa). This pore size was selected in order to retain ß-gal, of estimated MW 240–270 kDa [32,33]. Two washing-out steps were carried out for each 2.0-mL batch processed, consisting in a ~10x concentration of the supernatant followed by a dilution with membrane-filtered EB buffer without methylparabene in

order to restore the original volume. The centrifugation for these steps was performed in a GSA Sorvall rotor at 8000 rpm at 5 °C. The final 10x concentrates were pooled, filtered through a 0.45-μm membrane, and mixed with an equal volume of glycerol under aseptic conditions. Methylparabene was added at 0.05% (w/v), and the resulting solution containing partially purified ß-gal was stored at 5 °C for one year to evaluate the stability. The initial activity was ~950 U mL^{-1}. During this storage period, the stability of the preparation was evaluated by measuring the enzyme activity every two months in comparison to the commercial product Maxilact L 2000.

2.8. Analytical methods

ß-gal activity was measured with ONPG as previously described [15]. Fifty microliters of sample was mixed with a 2 mL substrate solution (1.25 mM ONPG in 50 mM dipotassium phosphate buffer, pH 6.6, 0.1 mM Mn^{+2}) and incubated for 5 min at 37 °C. The reaction was stopped with 0.5 ml of 1 M Na_2CO_3 and the absorbance was determined at 420 nm. A blank was made by adding Na_2CO_3 before the addition of the sample. Longer reaction times were used when the enzyme activity was too low. One unit (U) of enzyme activity was defined as the amount of enzyme that hydrolyze one micromol of ONPG per minute under the conditions of the assay, and was calculated by using the molar of *o*-nitrophenol extinction coefficient (4500 M^{-1} cm^{-1}). Invertase were assayed at 37 °C by measuring the glucose released after a 10 min incubation time in a reaction mixture containing 75 mM sucrose in 50 mM acetate buffer, pH 4.8 [15]. Glucose was measured with an enzymatic kit (Glicemia, Wiener). Protein was measured by the Biuret method with bovine-serum albumin as a standard [34]. The protein content of the biomass or the cell homogenate was determined after boiling the sample for 10 min in 1 N NaOH.

2.9. Properties of ß-gal

The Michaelis constant, Km, for lactose hydrolysis was determined at 37 °C by measuring the initial rates of glucose formation in a reaction mixture containing 25 mM K_2HPO_4 (pH 6.6) and 0.1 mM Mn^{+2} at lactose concentrations in the range of 7.0–139 mM. The Km values were estimated by nonlinear regression analyses by means of the Graph-Pad Prism ™ statistical software. Lactose hydrolysis in milk was carried out at 37 °C by incubating 5 mL milk with 50 μL of sterile enzyme solution. At the selected time interval, 0.5 mL of sample was collected, mixed with 1.5 mL of H_2SO_4 0.016 N (final pH of the mixture ~4.7), placed in boiling water for 2 min to inactivate the enzyme and to precipitate the casein, and then kept on ice bath. The precipitate was removed by centrifugation at 5000*g* for 20 min and the clear supernatant used for the determination of glucose. A blank without enzyme and a reference sample containing 2 g L^{-1} glucose were also incubated and treated in the same way.

3. Results and discussion

3.1. Cell permeabilization

In order to study the effect of incubation time and sarkosyl concentration on permeabilization, frozen-and-thawed cells were suspended in buffer at a cell concentration of 20 mg dry weight mL^{-1} and incubated for one hour at 25 °C and sarkosyl concentrations in the range of 0.05–0.5% (w/v). The permeabilization was assessed by measuring the increase in At at different times during the incubation. Fig. 1 shows the time course of permeabilization. The control-cell suspension, to which no sarkosyl was added, exhibited a low degree of permeabilization (~20%)—attributable to damage of the cell membrane produced by freezing and thawing [15,17]. Upon the addition of sarkosyl, a pronounced increase in permeabilization was observed at concentrations higher than 0.05%, with the maximum being achieved at

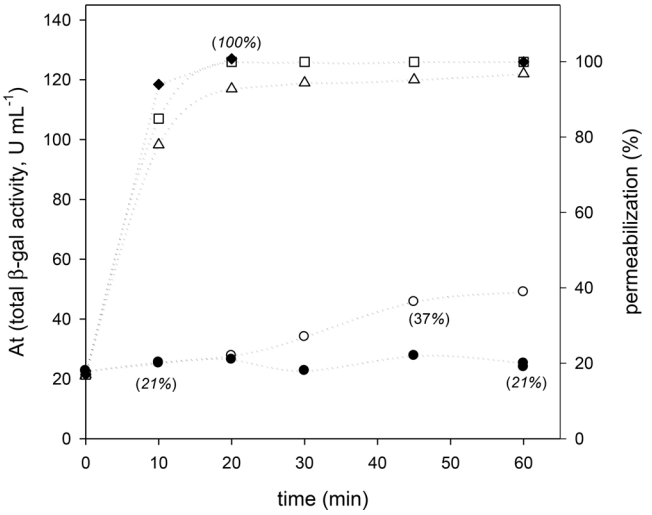

Fig. 1. *Time course of yeast-cell permeabilization by sarkosyl.* Frozen-and-thawed cells were suspended in 0.1 M PPB, pH 7.0 at a cell concentration of ~20 mg dry weight mL^{-1}. Sarkosyl was added from a stock solution to give the final concentrations (w/v) of 0.05% (○), 0.1% (Δ), 0.2% (□), and 0.5% (♦); with control cells receiving no sarkosyl (●). The cell suspensions were gently agitated in a reciprocating shaker bath at 25 °C. The % of methylene-blue–stainable cells is indicated in brackets. Permeabilization was assessed by measuring the ß-gal activity of the cell suspension or total activity (At). Permeabilization (%): $At/At_{max} \times 100$, where At_{max} is the ß-gal activity of the cell suspension after complete permeabilization (~126 U ± 3,8 U mL^{-1}), which value corresponded to 6.3 U per mg^{-1} dry-cell weight. Each data point is the mean value of three replicates of a single experiment. The coefficient of variation of the samples were in the range of 3–10%.

0.1–0.2%, as had been reported for other yeast species [24]. This concentration was, however, much lower than that obtained at the concentration of 1.5% or 2% reported for *K. marxianus* [23] and baker's yeast [25], respectively. The time required for a complete permeabilization was 20–30 min. Prolonged incubation times even at the highest sarkosyl level (0.5%), did not decrease the maximum ß-gal activity. This data suggest that the enzyme was quite stable in the presence of sarkosyl. Indeed, the maximum At value (126 ± 3.8 U mL^{-1}) which corresponds to ~6.3 U mg^{-1} cell dry weight, was the same as that found in a homogenate of mechanically disrupted cells. An analysis of extracellular protein and enzyme activity after a 30-min incubation with 0.2% sarkosyl indicated that about 12% of the total cell protein was released during permeabilization, but the ß-gal was retained almost completely within the cell.

Permeabilization usually leads to cell death as a result of the leakage of essential compounds from the cell [15]. Although, upon permeabilization with 0.2% (w/v) sarkosyl, all cells were stained; the more sensitive viability assay of plate counting revealed that some yeast cells did remain viable (fewer than 0.001%; ~10,000 colony-forming units mL^{-1}). Yadav et al. [23], also reported a 1% survival of a *K. marxianus* cell suspension (4×10^8 cells mL^{-1}) permeabilized for 20 min with 1.5% sarkosyl at 25 °C. It should be noted here that the relative At value (expressed as a percent of the maximum) correlated well with the fraction of dead cells, as estimated by methylene-blue uptake. Therefore, the relative At activity can be taken as an index of the degree of permeabilization of the cell suspension, as had been previously done with organic solvents [15].

3.2. ß-gal release from sarkosyl-permeabilized cells

Permeabilized cells (30 min incubation at 25 °C with 0.2% [w/v] sarkosyl) were centrifuged, washed once with buffer, and incubated for 8 h in EB to evaluate ß-gal release at different pHs and temperatures by assaying one parameter at a time. Enzyme release was first examined at

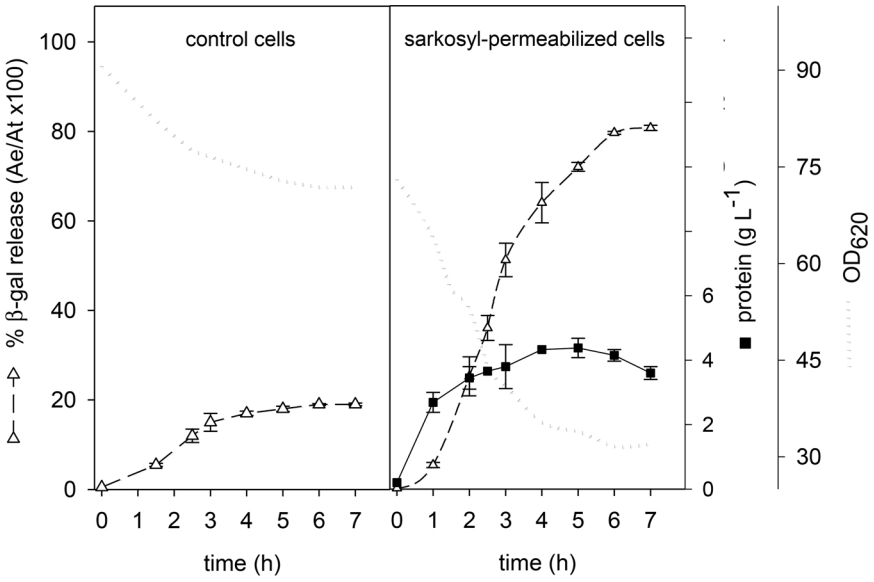

Fig. 2. *Time course of ß-gal release from K. lactis cells.* Sarkosyl-permeabilized and cells were suspended in 0.05 M potassium phosphate buffer (pH 7.2) containing 0.1 mM Mn^{+2} and 0.05% (w/v) methylparabene at a cell concentration of ~20 mg dry weight mL^{-1}. The cell suspensions were gently agitated in a reciprocating shaker bath at 35 °C. OD_{620}: optical density at 620 nm. % ß-gal release: Ae/At × 100 were Ae and At are the extracellular and total ß-gal activity respectively. The initial OD of the control and sarkosyl-permeabilized cell suspensions were 95 and 73 respectively. Each data point is the mean value of three independent experiments and error bars represent the standard deviation.

30 °C and pHs 6.6, 7.2, and 7.8. In all instances the maximum extracellular activity was reached after a 6- to 7-h incubation. During this time, no loss of enzyme activity was observed, as can be infer by the constant values of the total activity; whereas the release of the enzyme was 56%, 63%, and 48% at pHs 6.6, 7.2, and 7.8, respectively. Increasing the temperature to 35 °C at pH 7.2 increased the rate of ß-gal release and the extraction yield. At this temperature, about 80–83% of the total activity was released after a 6-h incubation. Similar results were obtained at 40 °C; but at this temperature about 13–15% of the total activity was lost after a 6-h incubation, thus indicating enzyme inactivation.

Fig. 2 illustrates the time course of the extraction process at pH 7.2 and 35 °C, the conditions finally selected. For the purpose of comparison, control cells were also incubated under the same conditions of pH and temperature. The release of cell constituents during the incubation is clearly indicated by the continuous decrease in the turbidity of the cell suspension. At the time of maximum ß-gal release, the turbidity of the sarcoskyl-permeabilized cell suspension decreased to about 40% of the original value, then remained approximately constant. The initial optical density of this suspension was lower than that measured in the control suspension due to the leaching of compounds during permeabilization. In addition to β-gal, other proteins were released from the cell. Overall protein solubilized during the incubation was around 4.0 g L^{-1} which concentration corresponds to 50% of the permeabilized-cell protein or 40% of the original protein in the biomass. A notable decrease in the Biuret-reactive material occurred beyond this time, probably as a result of proteolysis. Release of ß-gal was also observed in the control-cell suspension, with the turbidity decreasing down to the point where the release of ß-gal became constant. It should be noted here that the total ß-gal activity measured in the control cell suspension did not increase during incubation—*i.e.*, the fraction of permeabilized cells remained unchanged. Therefore, the enzyme liberated presumably originates from cells that were permeabilized by the freeze-thaw cycle. Indeed, the extraction yield was about 18% after a 6-h incubation at 35 °C and corresponded to the degree of cell permeabilization observed after the initial thawing of the cells (~20%). The data suggest that the physical permeabilization is thus also effective for liberating intracellular proteins.

TEM was used to visualize sarkosyl permeabilized cells at the time of maximum ß-gal release with control cells incubated under the same conditions being used for comparison. Most of the control cells exhibited an uniform and compact cell wall and an intact plasma membrane surrounding a very electron-dense cytoplasm (Fig. 3A). After extraction, the sarkosyl-permeabilized cells apparently did not exhibit significant physical breakage, but manifested gross structural changes, with the cytoplasm appearing shrunken and the electron-dense outer layer of the cell wall and the plasma membrane being no longer discernible (Fig. 3B). The chemical treatment had clearly altered the cell envelope, leading to the release of intracellular components. The time elapsed between cell permeabilization and maximum enzyme release may be considered as the initial phase of autolysis since prolonged incubation times will lead to enzyme inactivation and a more complete degradation of the cell structure [35]. That the morphologic characteristics of *K. lactis* cells described here were the same as those previously observed in *Pichia pastoris* after the extraction of recombinant proteins from cells permeabilized with *N*, *N*-dimethyltetradecylamine [36] was most interesting.

3.3. Comparison of cell-free extracts obtained from sarkosyl permeabilization and from mechanical disruption

Table 1 summarizes the data of protein content and ß-gal and invertase activities in the cell-free extracts obtained both from sarkosyl-permeabilized and from mechanically disrupted cells after differential centrifugation. The mechanical disruption was highly effective in releasing ß-gal (> 95%), but also the periplasmatic, cell-wall–associated invertase; which co-release is attributable to the intense shear produced by collision with glass beads [37]. As a result of that extensive cellular fragmentation, the supernatant of the cells ruptured mechanically was difficult to clarify, as demonstrated by the finding that more than 90% of the protein in the homogenate remained in the S5, and about 80% in the S20, turbid supernatants. In contrast, sarkosyl permeabilization produced an almost completely soluble extract, as indicated by the equivalence of the protein concentration ending up in the different supernatant fractions to that of the soluble fraction (S100) of a given cell homogenate after complete disruption. The specific activity of ß-gal in the S5 or S20 supernatants was 1.8-fold higher than in the corresponding supernatants obtained by mechanical disruption. In addition to intracellular proteins, invertase was also released during the incubation. About 20–25% of the total invertase activity was present in the S100 cell-free extract, indicating that this periplasmatic enzyme was completely soluble. The presence of invertase in the soluble extract would indicate that the cell wall had become degraded in some way during the incubation [7], as was suggested by TEM examination of the permeabilized cells after extraction.

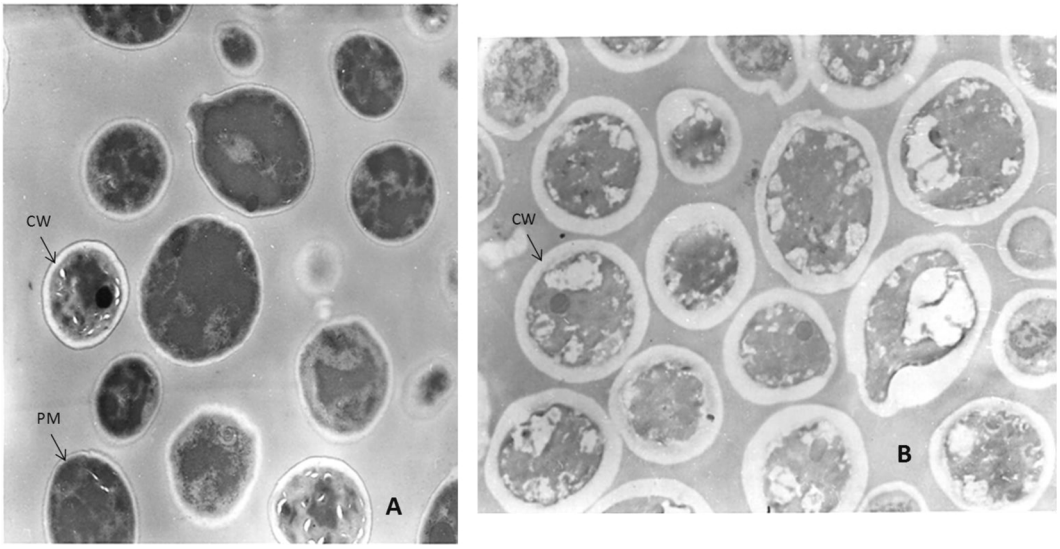

Fig. 3. *Appearance of K. lactis cells as visualized by transmission electron microscopy at x* 6000 *magnification.* A. Control cells B. Sarkosyl-permeabilized yeast cells at the point of maximum enzyme release; 6.0-h incubation in EB (pH 7.2) and 35 °C. CW: cell wall; PM: plasma membrane.

3.4. Preparation of a stable partially purified ß-gal

From a practical point of view, we were interested in evaluating if the released ß-gal could be concentrated and then stabilized through a simple and economical process. For this purpose, an extract from sarkosyl-permeabilized cells was obtained after 6-h, in this instance at a cell concentration of 40 mg mL^{-1} to increase the ß-gal activity in the starting solution. The S5 supernatant containing 230 U mL^{-1} (80% ß-gal release) was microfiltered to remove any insoluble impurities and then concentrated by passage through an Ultracell YM-100 membrane with a 100-kDa cut-off. Two diafiltration or "washing-out" cycles were previously carried out to theoretically reduce by 99% any permeable solute. After these steps, the ß-gal had become concentrated by nearly 10-fold as well as 1.4-fold purified compared to the original extract—*i.e.*, 32.5 U mg^{-1} of protein. Enzyme recovery was 95%. The concentrated extract was then mixed with glycerol at a final concentration of 50%. The ß-gal activity of the final preparation was ~950 U mL^{-1} though invertase was also present (~100 U mL^{-1}). This final mixture was stable for at least 10 months at 5 °C.

3.5. Properties of the ß-gal preparation

Certain properties of the aforementioned ß-gal preparation were compared with those of a partially purified commercial neutral lactase from the same yeast species currently available. Table 2 lists selected data. Both preparations contained invertase as a side activity, had the same Km values for lactose and exhibited similar kinetics of sugar hydrolysis when equal units of enzyme activity were added to the milk. That the analysis of lactose hydrolysis by the enzyme was complicated owing to the concomitant transglycosidase activity of ß-galactosidases is noteworthy [38,39]. An assay of glucose tends to overestimate the remaining lactose since some disaccharide is converted to galacto-oligosaccharides (GOSs) along with the glucose. In the example of the yeast lactase hydrolysing lactose at the latter's normal concentration in milk (~5%), when maximum levels of GOS are achieved (5–7% of the total sugars), the difference between lactose disappearance and degree of hydrolysis estimated by the amount of glucose formed is about 10%. This degree of error occurred when the lactose reduction was in the order of 80–90% [40]. Further research is needed to determine if the production of GOS by both products is equivalent.

Table 1
Characteristics of cell-free extracts obtained from sarkosyl-permeabilized cells and from mechanical disruption.

Disruption method	Extract**	ß-gal U mL^{-1}	Yield %	Protein g L^{-1}	ß-gal U mg protein $^{-1}$	Invertase U mL^{-1}
Sarkosyl	S5	103	82[a]	4.1	25.4	14
Permeabilization*	S20	102		nd	nd	14
	S100	100		4.0	25.6	13
Mechanical (agitation with glass beads)	H	130	95[b]	9.9		59
	S5	126		9.0	14.0	56
	S20	125		7.8	16.0	55
	S100	119		4.0	34.0	54

nd: not determined.
S5, S20, and S100; supernatants obtained by differential centrifugation of the homogenate (H) or the suspension of permeabilized cells (after extraction) at 5000 × *g* for 15 min, 20,000 × *g* for 20 min, and 100,000 × *g* for 60 min, respectively. The protein content of the permeabilized biomass was ~ 38% since 13% of the original content (44%) was released during permeabilization.

* Yeast cells incubated 30 min at 25 °C with 0.2% (w/v) sarkosyl.

** Extract: Sarkosyl-permeabilized cells were centrifuged, washed with buffer, and incubated for 6 h at 35 °C in 50 mM potassium phosphate buffer (pH 7.2) containing 0.1 mM Mn^{+2} and 0.05% (w/v) methylparabene.

[a] Yield % = Ae/At × 100, where Ae = extracellular activity and At = total ß-gal activity of the yeast suspension.

[b] Percent of disrupted cells estimated by phase contrast microscopy. The cell concentration used in both disruption methods and to extract the enzyme from the sarkosyl-permeabilized cells was ~ 20 ± 2 mg mL^{-1}.

Table 2
Characteristics of the soluble lactases.

	Maxilact L 2000TM*	This work
ß-gal activity (U mL^{-1})	2200	950
Km lactose (mM)[a]	28.1 ± 0.23	30.2 ± 1.81
Invertase (U mL^{-1})	360	100
Hydrolysis of ultrapasteurized whole milk (4.5% lactose) at 37 °C. Enzyme concentration: ~540 U mL^{-1}		
Time (h)	Glucose formed g L^{-1} (%)[b]	
1	4.7 (20)	4.7 (20)
2	8.2 (35)	7.5 (32)
4	13.2 (56)	13.4 (57)
10	19.3 (82)	19.0 (80)

* A commercial yeast lactase product.
[a] Km: assay conditions: 20 mM K_2HPO_4 (pH 6.6), 0.1 mM Mn^{+2} at 37 °C, lactose concentration was varied in the range of 7.0–139 mM. The two Km values are statistically indistinguishable ($p > 0.05$).
[b] The percentage of glucose formed is referred to the maximum that would be obtainable upon taking into account the concentration of lactose in milk.

4. Conclusions

The results presented here demonstrate that the permeabilization of cells of *K. lactis* by exposure to sarkosyl is an effective method for releasing ß-gal from the yeast's cytoplasm. Sarkosyl permeabilization, moreover, constitutes an improved method for the release of yeast ß-gal over other permeabilization systems that use toxic solvents or high alcohol concentrations and longer incubation times to release the enzyme [9–13]. The method was more selective than mechanical disruption; furthermore, the lack of significant cell fragmentation facilitated the clarification of the extract by a low-speed centrifugation and the subsequent diafiltration and ultrafiltration used to partially purify and concentrate the crude cell-free extract. A simple formulation obtained by mixing the concentrate with glycerol proved to be stable under refrigeration. The preparation described here may be considered a technical-grade lactase that can be used in different processes—such as GOS production, the elaboration of *dulce de leche* (a sweet, viscous Argentine milk product similar to butterscotch) or other products where the presence of accompanying unrelated activities (*e. g.*, proteases or arylsulfatases) would not constitute a quality problem for a final milk product during long-term storage [41]. The concomitant invertase activity would not be a problem in most applications, but can be a drawback in sugary milk products. Conversely, the presence of invertase may give rise to applications in which the sweetness of the preparation needs to be improved without an excessive increase in the sugar content of the final product [42]. Although this study deals with the ß-galactosidase of *K. lactis*, the procedure could be used as well for the recovery of other intracellular enzymes from various yeast species.

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