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ORIGINAL PAPER

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Efficient mechanical disruption of *Lactobacillus helveticus*, *Lactococcus lactis* and *Propionibacterium freudenreichii* by a new high-pressure homogenizer and recovery of intracellular aminotransferase activity

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Abstract Microbiological studies often involve bacterial cell fractionation, which is known to be difficult for Gram-positive as compared to Gram-negative bacteria. Our purpose was to test the breaking efficiency of a new high-pressure pilot homogenizer for three Gram-positive species involved in dairy technology and to assess the activity of an intracellular aminotransferase. Varied pressures (50, 100 and 200MPa) were applied to concentrated bacterial suspensions (1.2mg dry weight/ml) of *Lactobacillus helveticus*, *Lactococcus lactis* and *Propionibacterium freudenreichii*. Breaking efficiency was estimated by decreases in optical density at 650nm, cellular dry weight and viability. The proteins released were quantified and the residual intracellular aminotransferase activity was estimated using leucine as substrate. One run at 50MPa was sufficient to break 80% of lactobacilli cells whereas 200MPa were required for the same efficiency for *L. lactis* and *P. freudenreichii*. Whatever the pressure, leucine aminotransferase activity was recovered in the supernatant after cell breaking. This new high-pressure pilot homogenizer can allow rapid (20s/run), easy, continuous and highly efficient cell breaking for intracellular enzyme recovery or other purposes. As the species tested were not phylogenetically related, and had different morphologies and cell wall compositions, we conclude that most Gram-positive bacteria may be broken efficiently by this new device.

Keywords Cell disruption · High pressure homogenizer · Aminotransferase · Lactic acid bacteria

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Introduction

Microbiological studies often involve bacterial cell fractionation. This step is required to recover intracellular enzymes or low molecular weight compounds. It is also required in cell wall preparation for subsequent chemical characterization [19] or for studying cell wall-associated enzymes like autolysins [18]. In contrast to Gram-negative bacteria, Gram-positive bacteria are not easily disrupted due to their thick multilayered peptidoglycan cell wall.

Disruption of bacterial cells can be achieved by either mechanical or enzymatic treatments [3]. On a laboratory scale, disruption is carried out by different kinds of presses (French press, X-press), by sonication, by grinding with alumina, or by vortexing with glass beads [6, 9, 16]. For most Gram-positive species, the efficiency of disruption rarely exceeds 50% of the initial cell number after one run. Thus, preparation of large amounts of Gram-positive cell walls in this way can be very tedious and time consuming. Enzymatic treatments exist as a mild alternative involving either lysozyme (muramidase, EC 3.2.1.17), mutanolysin [20, 24] or purified phage endolysins (like lysostaphin), all of which hydrolyze peptidoglycan bonds. Less denaturing compared to physical disruption, these treatments often require optimization for each species and sometimes for each strain [2]. Moreover, peptidoglycan (*O*-or *N*-acetylated) can be resistant to lysozyme and thus require the use of mutanolysin, which is a rather expensive lytic enzyme. Of course, lytic enzymatic treatments are excluded if the purpose is to isolate cell walls and in particular peptidoglycan sacculi.

High-pressure homogenizers appeared in the 1970s. The mechanical principle of cell breaking was the same as for the French press (explosive decompression). However, of all the methods cited, high-pressure homogenization is the only one that works continuously and, because of that, large-scale disruption processes often involve homogenizers rather than other cell

wall disruption methods [5, 12, 17]. Even though the method is widely used industrially, relatively few data exist in the literature. For example, homogenization has been applied to *Escherichia coli*, *Bacillus subtilis* and one yeast in commercial homogenizers at pressures around 55MPa leading to breakage ranging from 12 to 67% in one pass depending on the type of microorganism and other factors [7, 8; for review see 4].

Today, pilot high-pressure homogenizers delivering instantaneous pressure as high as 350MPa and with new valve designs are available. The present work reports experiments showing the disrupting efficiency of this new device toward Gram-positive species of various morphologies: *Lactobacillus helveticus* (rods), *Lactococcus lactis* (cocci) and *Propionibacterium freudenreichii* (complex morphology called ''chinese character''). Those species were chosen (1) because of their morphology, which might resist differently to pressure, and (2) for their wide use and interest in dairy technology. The results showed that whatever the species, a high degree of cell breakage can be achieved in an easy and rapid way using this new homogenizer.

Materials and methods

Origin and growth of the strains

Lactobacillus helveticus CNRZ 243 and *Lactococcus lactis* NCDO 763 were obtained from the CNRZ collection (INRA Jouy en Josas, France). Cultures were stored at −80°C in MRS broth (Difco, Detroit, Mich.) and in M17 broth [21], respectively. *P. freudenreichii* TL 24 (CNRZ 725) was obtained from our laboratory collection (TL, INRA, Rennes, France) and was stored in sodium lactate broth [15]. The media were supplemented with 15% (v/v) glycerol prior to freezing. For propagation of the strains, medium without glycerol was used and growth was monitored at 37°C for *L. helveticus* and at 30°C for the two other species, by measuring optical density at 650 nm (OD_{650}).

Homogenization

When an OD_{650} of approximately 1 was reached (dry weight 0.25–0.35 $mgml^{-1}$ depending on the species), cells from 1l culture were harvested by centrifugation (9,000 *g*, 10 min, 4°C), washed twice with sterile distilled water and suspended in 240ml sterile distilled water to achieve a cell concentration of about 10^9 $cfuml^{-1}$. Cell suspensions were kept on ice until use. Fifteen milliliters of the suspension were kept in the cold as a control (T_o). The remainder of the suspension was divided into 75-ml portions and tested in the pilot homogenizer (Standsted Fluid Power, Stansted, UK) at pressures of 50, 100 and 200 MPa with a cooling flow of water around the disrupting chamber. One hundred milliliters of crude broken suspension was recovered in each case and placed on ice immediately. The diluting effect of homogenization was taken into consideration, i.e., the control was diluted to the same extent (25%) to allow comparisons with treated suspensions. A sample was centrifuged (10,000 *g*, 10 min, 4°C) and the supernatant (cell-free extract) was recovered for analysis. For each species, two independent assays of homogenization were carried out, and the average is as presented in the results.

Enumeration

Cell viability before and after homogenization treatment was determined by plating on the respective medium used for each species containing 1.2% agar. Plates were incubated 2 days at 30°C for *Lactococcus lactis* and *P. freudenreichii* (anaerobiosis, Anaerocult A, Merck) and at 37°C for *Lactobacillus helveticus* (anaerobiosis). The results are expressed as a percentage of the initial viability.

Cellular dry weight

Three milliliters of cell suspensions (control and after homogenization) were centrifuged (10,000 *g*, 10 min, 4°C). The pellet was washed once in sterile cold distilled water and kept at −18°C until dried (Speed Vac; Savant Instruments, Holbrook, N.Y.) and weighed to estimate residual cellular dry weight. The supernatant was kept for protein measurement, SDS-PAGE, and leucine aminotransferase analysis.

Protein measurement

The absorbance of the supernatant at 280 nm was measured. The protein content was also determined by the Bradford [1] procedure using the Bio-Rad Assay Kit (Bio-Rad, Hercules, Calif.), with bovine serum albumin (Sigma, St. Louis, Mo.) as standard.

SDS-PAGE

SDS-PAGE was performed using a 14% acrylamide separating gel (8×8 cm) and a 4% acrylamide stacking gel as described previously [23]. Electrophoresis was performed at 180 V for 1 h. Samples of cell-free extracts (20 μl) were suspended v/v in Laemmli buffer [13] and boiled 3 min before loading. Gels were stained using Coomassie Brilliant Blue R250.

Leucine aminotransferase activity

Leucine aminotransferase activity was assayed according to Yvon et al. [25]. Cell-free extract (20 μl) was added to 150 μl reaction mixture: Tris-HCl (Merck) 70 mM pH 8.0 containing 4 mM leucine (Sigma), 10 mM sodium α-ketoglutarate (Sigma), 50 μM pyridoxal 5′-phosphate (Sigma). Incubation was performed for 2–18 h at 37°C. Glutamic acid was analyzed enzymatically with a Boehringer kit (Diffchamb, Lyon, France). A blank without amino acid was included. Reagents: leucine, α-ketoglutarate, and pyridoxal 5′-phosphate were obtained from Sigma Aldrich (St Quentin, Fallavier, France).

Results and discussion

Cell breaking efficiency

For the three species, the indices of cell breakage were all in agreement: the decrease in OD_{650}, the cellular dry weight and cell counts (Fig. 1a–c). The disrupting efficiency increased with the pressure applied, but in three different manners. The breakage curve was linear for *Lactococcus lactis* and logarithmic for *P. freudenreichii*. More than 80% broken cells was obtained in one run at 50 MPa for *Lactobacillus helveticus* whereas a pressure as high as 200MPa was necessary for the same degree of

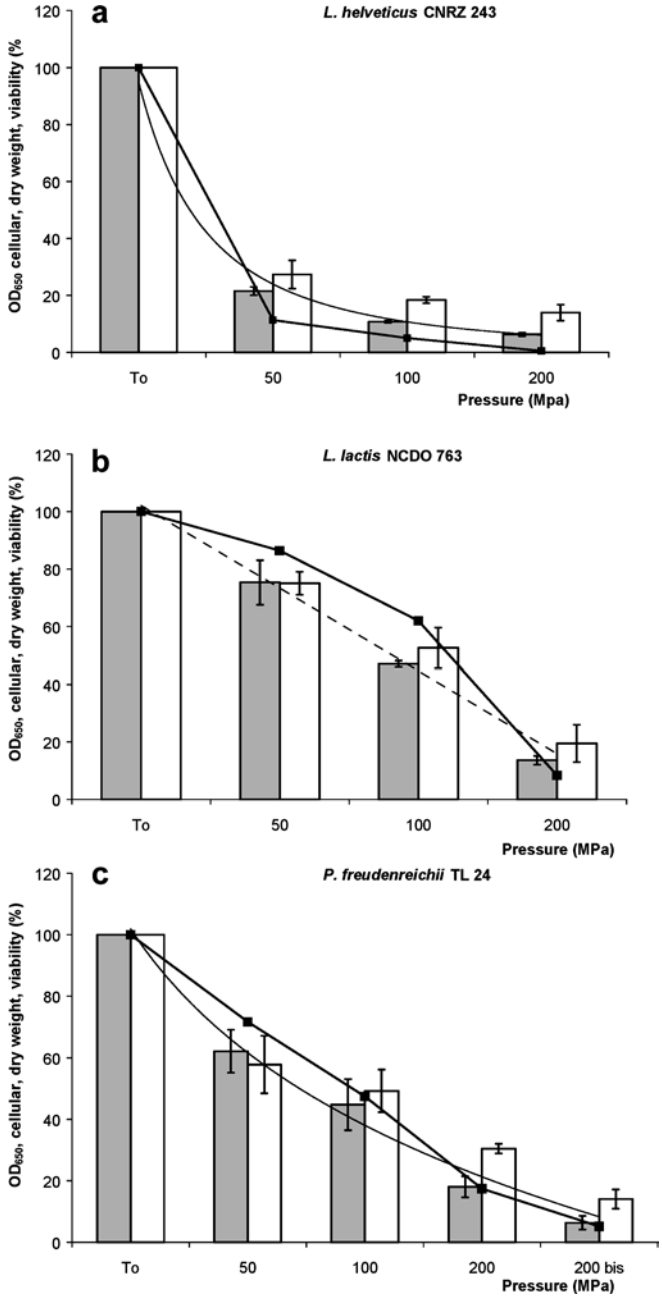

Fig. 1 Optical density at 650 nm (OD_{650}) (*gray bars*), cellular dry weight (*white bars*) and cell viability (*black squares*) of **a** *Lactobacillus helveticus* CNRZ 243, **b** *Lactococcus lactis* subsp. *lactis* NCDO 763 and **c** *Propionibacterium freudenreichii* TL 24 when submitted to homogenization at pressures of 50, 100 and, 200 MPa. - - - Tendency curves

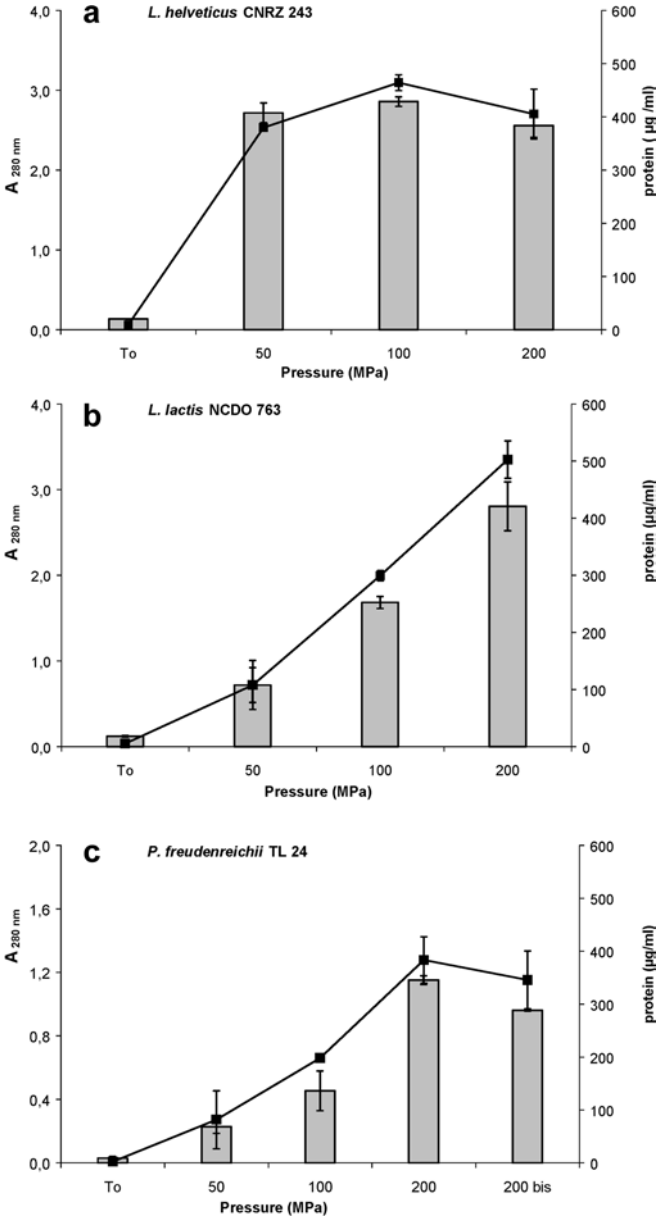

Fig. 2 OD_{280} (*gray bars*), protein (µg/ml) (*black squares*) of **a** *Lactobacillus helveticus* CNRZ 243, **b** *Lactococcus lactis* subsp. *lactis* NCDO 763 and **c** *P. freudenreichii* TL 24 when submitted to homogenization at pressures of 50, 100 and, 200 MPa

breakage for *P. freudenreichii* and *Lactococcus lactis*. Repetition of the treatment at 200 MPa for the propionibacteria led to only a slight increase in cell breakage (Fig. 1c). Lanciotti et al. [14], using applied pressures ranging from 15 to 200 MPa on food spoilage and pathogenic microorganisms, observed a log-linear relationship between surviving cells and pressure. According to Engler [4], the shape and strength of microbial cell walls appear to depend on the structural polymers within the wall and the degree to which they are cross-linked to one another and to other wall components. To disrupt a cell, the major resistance that must be overcome is the covalent bonding of this structural network. To the best of our knowledge, the mechanism of cell disruption by high-pressure homogenization has only recently been investigated more deeply and for only one yeast [11]. In our case, shape seemed to have a significant effect on the breaking efficiency since rods (*Lactobacillus*) were much more fragile toward homogenization than cocci (*Lactococcus*) or pleiomorphic cells like propionibacteria. Interestingly, the percent of residual viability was similar to the percentage of the residual cellular dry

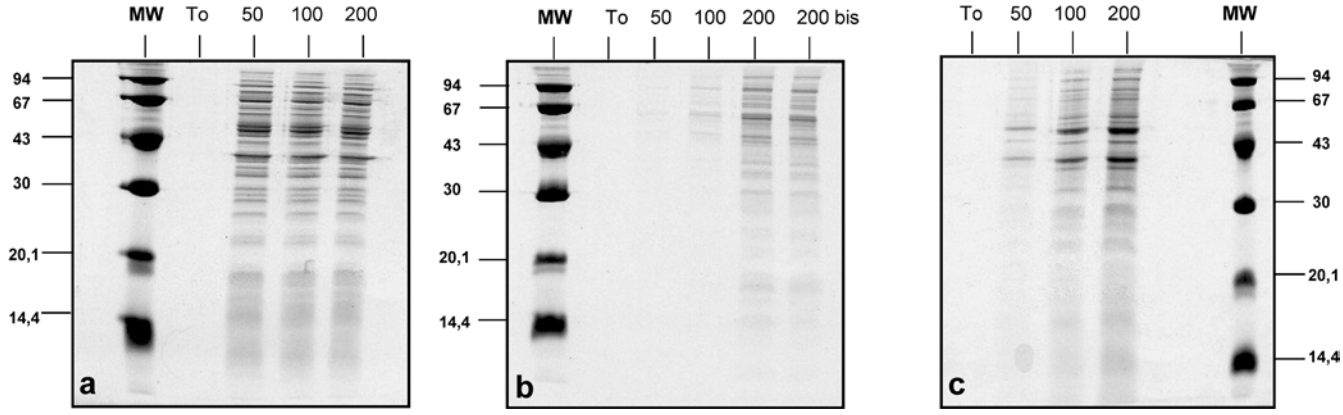

Fig. 3 SDS-PAGE showing the proteins of **a** *Lactobacillus helveticus* CNRZ 243, **b** *Lactococcus lactis* subsp. *lactis* NCDO 763, and **c** *P. freudenreichii* TL 24 released by homogenization depending on the pressure applied (indicated in each line). *MW* Standard molecular weights (kDa)

weight, which would indicate that most of the non-broken cells were not damaged but still alive and viable. This point could be easily confirmed by fluorescence microscopy using live/dead staining.

Lastly, ten-fold concentrated suspensions of propionibacteria were submitted to homogenization at 200MPa and equivalent disruption efficiency (70%) was obtained (data not shown). This result is in agreement with the literature, which indicates that performance of homogenizers does not diminish with increasing cell concentration until concentration-related blockages make operation impractical [4].

Analysis of the proteins released

Whatever the method used (A_{280}, protein estimation or SDS-PAGE), no protein was detected in the supernatant of the control. Moreover, in the supernatant of homogenized suspensions, the increase in the quantity of protein released was inversely related to the percentage of cell breakage for each species (Figs. 2, 3). Regardless of the method used, most of the *Lactobacillus helveticus* proteins were already released by a treatment at 50MPa, which is consistent with the already high breakage efficiency at this pressure for that species ($>80\%$). In the case of propionibacterium, 25–30% less protein was released compared to *Lactococcus* for the same breakage efficiency (at 100 and 200MPa). This might reflect differences in the ratio of cell envelope/cytoplasmic proteins since the former were shown to be released more easily and faster by homogenizer treatment [4].

Recovery of leucine aminotransferase activity

Lactobacillus helveticus, *Lactococcus lactis* and *P. freudenreichii* all possess intracellular leucine aminotransferase activity that has a key role in ripening cheese and is for that reason the object of numerous studies [10, 22, 25]. In order to assess the impact of homogenization on this intracellular enzyme, its activity was estimated per milligram of protein released. Whatever the species, and even at the highest pressure, leucine aminotransferase activity was detected in the supernatant (Fig. 4) and no drastic loss of activity was noted for the two independent homogenization assays, even at the highest pressure.

Conclusions

The high-pressure homogenizer pilot used was very efficient in breaking Gram-positive bacteria—with three different morphologies—rapidly (a few seconds), continuously and easily. This can be attributed to the new ceramic decompression valve, which has a sharper cutting edge than the previous design, and also the ability to deliver very high instantaneous pressure (up to 350MPa, maximal values not tested here). For the three species investigated, the activity of an intracellular enzyme, leucine aminotransferase, was recovered in the supernatant after breakage and resisted the highest pressure

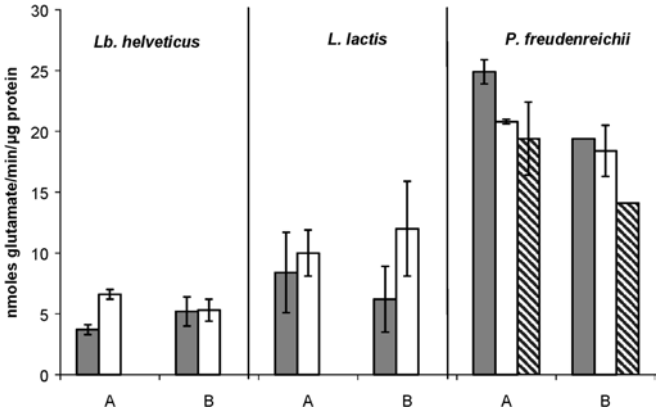

Fig. 4 Leucine aminotransferase activity (nmol $min^{-1}\mu g$ $protein^{-1}$) of cell free extract of *Lactobacillus helveticus* CNRZ 243, *Lactococcus lactis* subsp. *lactis* NCDO 763 and *P. freudenreichii* TL 24, obtained by two different homogenizations (*A* and *B*) at pressures of 100 (*black bars*), 200 (*white bars*) and, 200 MPa twice (*grey bars*)

applied (200MPa). Thus, this new pilot high-pressure continuous homogenizer looks likely to be a valuable device in the preparation of active cytoplasmic enzymes from Gram-positive bacteria.

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