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Cell lysis induced by membrane-damaging detergent saponins from *Quillaja saponaria*

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ABSTRACT

This paper presents the results of a study to determine the effect of *Quillaja saponaria* saponins on the lysis of industrial yeast strains. Cell lysis induced by saponin from *Q. saponaria* combined with the plasmolysing effect of 5% NaCl for *Saccharomyces cerevisiae*, *Kluyveromyces marxianus* yeasts biomass was conducted at 50 °C for 24–48 h. Membrane permeability and integrity of the yeast cells were monitored using fluorescent techniques and concentrations of proteins, free amino nitrogen (FAN) and free amino acids in resulting lysates were analyzed. Protein release was significantly higher in the case of yeast cell lysis promoted with 0.008% *Q. saponaria* and 5% NaCl in comparison to plasmolysis triggered by NaCl only.

1. Introduction

The term 'autolysis' means 'self-degradation' and refers to the breakdown of a cell by its own enzymes following the death of the cell [1]. The process of yeast autolysis begins naturally following the death of the cell, with the disintegration of the membranous systems of yeast cells. However, such membrane changes can be also induced chemically. The intracellular yeast proteases, glucanase and nucleases break down cell macromolecules: proteins and mannoproteins, glucans, glycogen, nucleic acids. The conditions must be carefully controlled to ensure cell permeabilisation without inactivating the yeast enzymes [2]. In modified processes, inorganic salts, such as sodium chloride, or organic solvents are often used to accelerate cell lysis [3]. A large number of different substances including proteins, peptides, alcohols or detergents (toluene–ethanol mixture, cethyltrimethyl-ammonium bromide, ethyl ether, digitonin, Triton X-100, hexamethylenediamine, ethanol) may be used to increase the permeability of yeast membranes [4–6]. Among promising natural detergents in this respect are saponins from *Quillaja saponaria*. These are glycoside compounds with chemical structures composed of a fat-soluble triterpenoid aglycone attached to water-soluble sugar glycone through ester linkages. Irradiation, ultraviolet, X-rays or mechanical disintegration, all of which cause profound changes in regulatory and biosynthetical cellular processes (without, however, impairing autolytic capability), may also be used as inductors of yeast cell lysis. At certain concentrations, sodium chloride or sucrose may trigger plasmolysis, by changing the osmotic pressure of the medium. Whether lysis is spontaneous or induced may fundamentally affect the composition of the products obtained.

In our study, two kinds of yeast were tested: conventional fermentative *Saccharomyces cerevisiae* and unconventional *Kluyveromyces marxianus* strains. Crabtree negative yeasts, such as *K. marxianus*, are able to respire even in high glucose concentrations and are more efficient than *S. cerevisiae* at converting available carbon sources into cell biomass [7]. *K. marxianus* strains have been given generally-regarded-as-safe (GRAS) status, as has *S. cerevisiae* [8].

2. Materials and methods

The yeasts *Saccharomyces cerevisiae* Ethanol Red® (Lesaffre) and *Kluyveromyces marxianus* LOCK0026, as well as post-fermentation mixed cultures, were used as pure cultures. Yeast pure cultures were maintained on agar slants (Wort broth; Merck Millipore) at room temperature. Monoculture biomass was grown in Worth Broth (Merck) liquid medium under aerobic conditions at 25 °C for 48 h, while the mixed cultures originated from fermentation processes. Cell suspension density was estimated using a Muse® Cell Analyzer and all results were calculated to 10^9 cells. In order to induce cell lysis, the yeasts were centrifuged (5 min, 11 °C, 3220 × *g*), then washed with sterile distilled water and re-centrifuged (5 min, 11 °C, 3220 × *g*). The yeast cells were then suspended in 5% (w/v) NaCl. The final concentrations of inducers in the samples, following lysis, were: 5% (w/v) NaCl, 5% (v/v) ethanol, 0.008%

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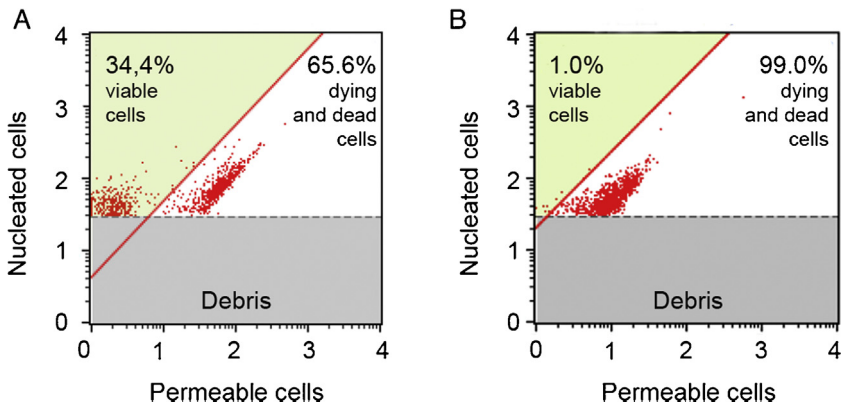

Fig. 1. The distinguish viable and dying/dead cells determined using a Muse® Cell Analyzer; *Saccharomyces cerevisiae* cells incubated with: A – NaCl; B – NaCl + saponin.

(w/v) saponin *Q. saponaria*. Inducers were used separately or in mixed combinations. Lysis was conducted at 55 °C for 48 h. After incubation, the yeast suspensions were centrifuged and the supernatants analyzed.

To observe the integrity of the yeast cells, the samples were stained with the non-specific fluorochrome Calcofluor white, which binds with cellulose and chitin in cell walls. 2 ml of cell suspension after lysis was centrifuged (5 min, 11 °C, 3220 × *g*) and washed twice with sterile distilled water. Microscopic observations were performed at excitation and emission wavelengths of 380 and 475 nm, respectively. With mixed yeast cultures, yeast membrane permeability after 48 h of cell lysis was assessed using a Muse® Count & Viability Assay Kit (Merck-Millipore) and Muse® Cell Analyzer [9]. Infrared spectrometry using the Direct Detect® system was used to quantitatively determine the concentration of proteins, by measuring the number of amide bonds in their chains. The method uses a hydrophilic PTFE membrane engineered to present aqueous samples in a dried format compatible with fast infrared analysis [10]. The Direct Detect® spectrometer had been calibrated to the BSA standard (Sigma).

Free amino nitrogen concentration (FAN) was determined based on the colour reaction of amino acids with ninhydrin and by absorbance measurements at a wavelength of 570 nm [11]. The concentration of ammonia nitrogen was measured using the colorimetric method with Nessler's reagent at a wavelength of 400/425 nm [12]. To assess the concentration of free amino acids in the autolysates, these were first passed through a centrifugal ultrafilter with a cut-off mass of 3 kDa. Samples (50 μl) were then transferred into Eppendorf tubes and evaporated to dryness in a vacuum centrifuge. The free amino acids present in the dried sediment had been converted into a phenylthiocarbamide (PTC) derivative [13]. The derivatives obtained were dissolved in amino acid solvent, added in portions of (200 ml), and 5 μl solutions analyzed using high pressure liquid chromatography (HPLC) on a PicoTag 3.9 × 150 mm column (Waters, USA). Quantitative calibration had been performed using four different concentrations of amino acid standards (Pierce Company, USA).

Using polysaccharide assessment, the cell-lytic effect was also investigated. The quantities of β-glucan and mannan were measured in both the extract (supernatant) and the reaction mixture (yeast suspension). Analysis of glucans requires prior partial acid hydrolysis to remove gel-forming properties and covalent links to other polysaccharides (e.g. chitin) or proteins. 1,3:1,6-β-D-Glucan, 1,3-β-D-glucans and α-glucans were extensively hydrolysed by 1.3 N HCl at 100 °C for 2 h. Hydrolysis to D-glucose was completed by incubation with a mixture of highly purified *exo*-1,3-β-glucanase and β-glucosidase. The assay was conducted using a β-Glucan (Yeast & Mushroom) Assay Kit (Megazyme). Mannans were hydrolysed in 2 M hydrochloric acid at 100 °C for 2 h. After this time, a volume of 2 M sodium hydroxide equal to the amount of acid used was added [14,15]. The amounts of mannose and glucose were determined using a D-Mannose/D-Fructose/D-Glucose Assay Kit (Megazyme).

All of the assays were conducted in triplicate and the standard deviation calculated.

3. Results and discussion

Various procedures for cell lysis using *Saccharomyces*, non-*Saccharomyces* or spent brewery yeast strains as starting materials are described in the literature [2,3,16,17]. The most common and simple processes are based on the phenomenon of plasmolysis, and conducted in the presence of sodium chloride and ethanol. A conventional process for producing a yeast extract food for humans with good flavour, palatability, and no bitterness requires adding sodium chloride and ethanol to active yeast and subjecting the resulting mixture to lysis. Sodium chloride and ethanol are added at concentrations of around 2–10% (w/v) and 5–9% (v/v) of the total volume of the lytic reaction system [18]. The higher concentrations of inducers ensure higher microbiological purity. However, the high amount of NaCl may be detrimental to the nutritional quality of the product. We therefore used smaller amounts of NaCl and ethanol, at concentrations of 5% (w/v) and 5% (v/v), respectively. Plasmolysis generally does not cause molecules of high molecular weight (MW), such as enzymes, to be released. The use of membrane permeabilizing agents may therefore be useful [19].

Quillaia is approved as a natural flavouring for use in food and beverages by the United States Food and Drug Administration (US FDA) [20]. In the European Union, the Codex Committee on Food Additives and Contaminants list unpurified extracts from *Quillaia* as suitable for use as foaming agents in 'water-based flavoured drinks' and sets a maximum use level of 500 mg/kg. It has been reported that saponin concentrations of 0.005–0.01% can be used to permeabilize cultured human intestinal epithelial cells [21]. *Quillaia* extracts can contain as little as 20–26% saponins, so in our study a safe additive level of 80 mg/L (0.008% w/v) was used. Saponin as natural surfactant is a relatively unconventional inductor for yeast lysis. However, cells treated with saponin retain sufficient integrity for rapid equilibration [22]. This is the first paper to propose the use of natural saponin from *Q. saponaria* for yeast cell lysis. The proposed method of yeast cell lysis also has the advantages of relatively low cost, lower added salt and well-documented health benefits [23,24].

The inductors NaCl, ethanol and saponin, separately or in mixtures, were tested for both yeast monocultures and mixed spent yeast biomass. For yeast monocultures, saponin treatment was found to lead to increased cell membrane permeability (Fig. 1.) Absolute total cell counts were conducted and a viability assay based on the differential permeabilities of two DNA-binding dyes. The nuclear dye stains only nucleated cells (*y*-axis), while the viability dye brightly stains dying and dead cells (*x*-axis). This proprietary combination of dyes enables viable and dead cells to be distinguished. Moreover, debris is excluded from the results through negative staining by the nuclear dye [25]. Increased dye penetration inside the yeast cells was observed in 65.6% of a population incubated with NaCl solution and in 99% of a population treated with NaCl and *Q. saponaria*.

The effect of saponin on the concentration of total proteins in the yeast lysates was also tested. With both strains, the presence of saponin during lysis resulted in a substantial increase in the concentration of nitrogen compounds, in comparison to tests without this agent. The highest total protein and amino nitrogen concentrations were obtained with *S. cerevisiae* cells incubated in a mixture of NaCl and saponin. Total protein concentrations were found to be 34-times higher in supernatants from *S. cerevisiae*