EFFECTS OF SUPERCRITICAL PASTEURIZATION OF FOODSTUFF ON MICROORGANISMS VIABILITY EVALUATED BY CELL FLUORESCENT STAINING

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Nowadays high-pressure treatments are receiving an increasing deal of attention as an alternative "mild" technology for pasteurization in foodstuff processing (1). The anti-microbial effect of CO_2 in an experimental set-up was investigated in different operating conditions applied. In order to improve the homogeneity and the mass transfer inside the liquid phase a mechanical stirrer system was exploited. Results confirm that the microorganisms present in the samples are sensitive to the SC-CO_2 treatment at mild temperatures. Furthermore, assessment of high-pressure CO_2 induced damages and death of microorganisms were performed by different advanced techniques. In particular:

- scanning electron microscopy was used to detach the morphological changes in cell structure after the treatment,
- flow cytometry were used for the evaluation of cell integrity (by distinguishing between viable and dead cells) after the staining of cell with fluorescent dyes (2).

Results show that such combined methods provide valuable indication on cell membrane integrity or damages during the pasteurization process applied to foodstuff.

INTRODUCTION

Previous study have demonstrated the feasibility of the treatment with dense gases as an alternative technique for the pasteurization of different substrates and different kind of microbes commonly present in foodstuff, both in their vegetative and latent form.
A review on inactivation of bacteria by dense CO_2 (DCO_2) has been published recently (1). In the last couple of years several new articles have been published (3-5) and a few patents including the one claimed by Praxair Inc. (Bur Ridge, IL, USA), which validates a continuous process as a non-thermal pasteurization technology of fruit juice from lab-scale to commercialization for a feed flow rate up to 120 L/min (Better than Fresh®) (6).
As the mode of operation is concerning, it is evident from the experimental data published so far that the limiting step of the pasteurization batch process is the mass transport of the dense gas into the liquid phase (7). For this reason, in the present work we present an innovative high pressure apparatus set-up with a mechanical agitation system to study the effect of the stirring system on the efficiency of the process. In this regard, the first purpose of this research work is firstly to confirm the effect of CO_2 inactivation as a function of temperature, treating time and mixing rate. The effects of microbial inactivation were investigated on *Saccharomyces cerevisiae* by comparing the conventional methods based on cultivation techniques with the innovative flow cytometric analysis. Flow cytometry allows obtaining single-cell analysis of the microorganisms present in a suspension, on the basis of their

fluorescence, emitted at different wavelengths (eventually induced by specific staining) and their scattering signals. This multiparametric analysis is a powerful tool to study viability and death of microorganisms and the relative dynamics involved in the progressive damage induced by the CO_2 treatment. Flow cytometry provides semiautomatic analysis on a large number of samples in a relatively short time (by counting several thousand of cells in a minute), ensuring high reproducibility and precision with respect to the more traditional cultivation methods.

MATERIALS AND METHODS

Bacterial strains and media

A sourdough yeast strain *S. cerevisiae* was used as test microorganism, suspended in peptonated sterile water (dilution 1:10) and inoculated in a substrate consisting of 70g of glucose, 5g of triptone, 3g of yeast extract, 3 g of malt extract, 4.5 g of KH_2PO_4, 1000 mL of water and HCl to reach a pH of 3.5. The final concentration of the cell was 10^7 CFU/mL.

Counting

Before and after each treatment cell counts of the surviving yeasts were determined by standard plating technique. Every sample was diluted in peptonated water (dilution 1:10), plated in Petri dishes containing a WL nutrient agar (composition: 4% yeast extract, 5% triptone, 50% glucose, 0.55% H_2PO_4 , 0.425% KCl, 0.125% $CaCl_2$, 0.125% $MgSO_4$, 0.0025% $FeCl_3$, 0.0025% $MnSO_4$, 0.022% AGAR, water) and incubated at their optimal condition (48 h at 25°C) before counting. The results are expressed as N/N_0 versus time, where N_0 is the initial number of cells in the control sample and N is the number of cells in the sample after treatment. All the result values are the arithmetic means of N/N_0 of three different runs.

Flow cytometry

For the analysis of yeast, the suspensions were stained with two DNA-specific dyes: SYBR-Green I (SG-I, λ_{ex} = 495 nm, λ_{em} = 525 nm; Molecular Probes Inc.) and Propidium Iodide (PI, λ_{ex} = 536 nm, λ_{em} = 617 nm; Molecular Probes Inc.). An amout of 1 mL of yeast suspension was stained with 10 µL of SG-I (after 1:30 v/v dilution of the commercial stock in dimethyl sulfoxide) and 10 µL of PI (concentration of commercial stock equal to 1 mg/mL). Samples were incubated for 15 minutes in the dark and then the flow cytometric analysis was performed. Samples were analysed using a Bryte-HS flow cytometer (Bio-Rad, Hercules, CA), equipped with an arc xenon lamp as light source. Samples were illuminated with an excitation of 470–490 nm. For each cell, the following signals of fluorescence were recorded: (1) green fluorescence, produced by the staining with SG-I, collected at 525±30 nm band-pass filter; (2) red fluorescence, produced by PI, collected with a 600 nm long-pass filter.
SG-I enters into all the cells, while PI is able to enter only into dead cells, that are cells with perrmeabilised membrane. Therefore it is possible to discriminate between viable cells (stained only by SG-I and emitting green fluorescence) and dead cells (stained simultaneously by SG-I and PI and emitting red and green fluorescence). In this context the term “viable” is used as synonymous of “integer” cells, that are cells with an integer membrane. Flow cytometry allows to quantify all the yeast cells (viable, damaged or dead), overcoming some limits of cultivation methods that are able to detect only reproductive cells.

High pressure equipment
The liquid CO_2 (RIVOIRA, carbon dioxide 4.0, purity 99.990%) is fed into a high pressure vessel by a HPLC pump (Gilson, mod 307 drive module) with a maximum flow rate of 25 ml/min. The vessel consists of a 450 ml cylinder with a 5 μm porous metallic filter (frit) which allows the atomization of CO_2 flow in micro-bubbles. The screwed tap is equipped with 2 holes to locate inside: the temperature probe (PtΩ100) and the mechanical stirrer system (Rushton turbine, 1kW, 500-10000rpm). The vessel is thermally controlled and heated by a water bath system (Julabo ME-4). An outlet micrometric valve (Rotarex, mod. 2S-4L-N-SS) which regulates the CO_2 pressure in the autoclave, it is heated by a resistance to prevent freezing during expansion. The operative parameters, temperature and pressure, were continuously recorded by a real time system (NATIONAL INSTRUMENTS, field point FP-1000) and monitored by a specific software (Lab VIEWTM5.0).

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Samples of *S. cerevisiae* subjected to CO_2 treatment have been examined by scanning electron microscope (TMP, 30KV) in order to clarify the inactivation hypothesis.

RESULTS
We believe that the mechanism of sterilization involves the ability of SC-CO_2 to diffuse readily into the cell: as a consequence its regular metabolism and physiological equilibrium are altered irreversibly (8). As a matter of fact, once CO_2 penetrates into the cell it is supposed to react with water and form carbonic acid that leads to a sudden decrease in extracellular and intracellular pH (9). In this respect, the correct experimental set-up is essential for increasing the process efficiency. The time needed for the CO_2 to reach equilibrium in an experimental device, operated in a batch mode, with different mixing rates can vary in a wide range of values. In order to valide our hypothesis, thus determining the effect of mixing on process efficiency, different sets of experiments were carried out at 100 bar, 35°C,different mixing rate (no mixing-500 rpm - 10000 rpm) and different treating time (10-30 min). The sample volume was about 150 mL.

Counting
Table 1 gives a summary of the results obtained in the quantification of cells by using the standard plating technique. Triplicate samples were averaged for each data point.

Table 1: Survival (%) of cells measured by standard plating technique as a function of stirring rate and treatment times, at 100 bar, 36°C

System-operative parameters-	*100 bar - 36°C 10 min*	*100 bar - 36°C 20 min*	*100 bar - 36°C 30 min*
Control (% survival)	100	100	100
No stirring (% survival)	43.3	29.0	15.4
500 rpm (% survival)	5.0	2.0	0.8
10000 rpm (% survival)	3.5	0.2	0

From Table 1 it is evident that an increase of stirring rate assumes a main role in microbial inactivation efficiency. The percentage of survived cells in the sample after a pasteurization treatment at the same operating parameters (treating time of 20 minutes at 100 bar and 36°C) are respectively 30%, 2.8% and 0.3%.

Flow cytometric analysis

The quantification of viable and dead cells in treated yeast suspensions undergone to the different operating conditions was performed by flow cytometry. The interpretation of the flow cytometric analysis is usually performed by means of cytograms. Two examples of cytograms obtained for a treated and an untreated suspension of yeast are shown in Figure 1. In both the cytograms of Figure 1 green fluorescence is plotted vs. red fluorescence. Dead cells can be easily differentiated from the viable ones. In fact viable cells are characterised by a predominant level of green fluorescence due to the staining of SG-I, but without PI emission. On contrary, dead cells exhibit fluorescence both green and red, due to the simultaneous presence of nucleic acid stained by SG-I and PI.

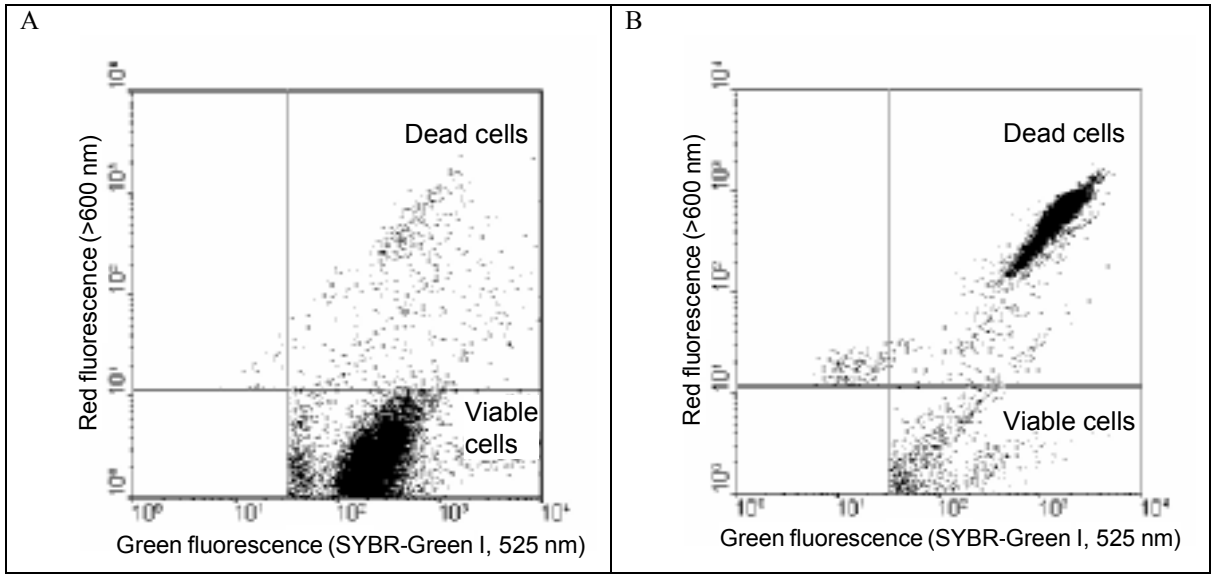


Figure 1. Cytograms of two yeast suspensions: (A) sample without treatment; (B) sample after CO_2 treatment at 36°C, 100 bar and mixing at 10000 rpm for 20 min.

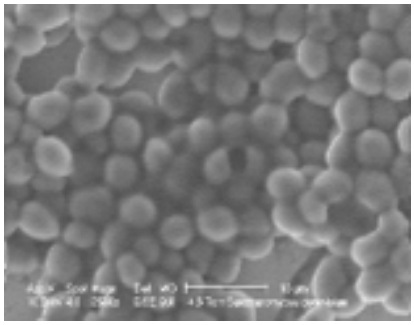
In Figure 1(A) an untreated yeast suspension is indicated. The most part of yeast cells were viable, reaching a percentage of 94.7%, while the other 5.3% is made up of dead cells. Figure 1(B) shows a cytograms of a treated yeast suspensions. The operating conditions imposed during the treatment were the following: temperature of 36°C, pressure of 100 bar and stirring at 10000 rpm for a duration of 20 min. In this case we can observe the significant damage induced in yeast cells, due to the fact that the percentage of dead cells increased up to 95.9%. Only a percentage equal to 4.1% remained as viable cells. In table 2 the synthesis of the results obtained by the flow cytometric analysis are shown.

Table 2: Percentage of viable cells measured by flow cytometry as a function of stirring rate and treatment times, at 100 bar, 36°C

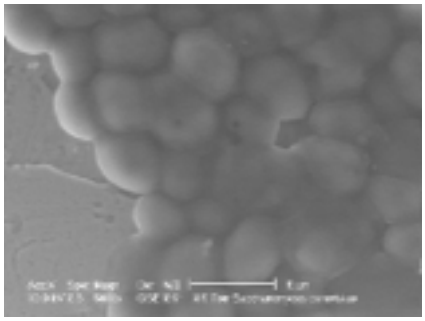
System-operative parameters-	*100 bar - 36°C 10 min*	*100 bar - 36°C 20 min*
Control (% survival)	93.8	94.7
No stirring (% survival)	79.5	27.8
500 rpm (% survival)	65.6	13
10000 rpm (% survival)	12.3	4.1

ESEM analysis

No macroscopic differences can be observed after SC-CO_2 treatment (Figure 1a-1b); anyway the yeasts after the treatment (100 bar, 36°C, 30 min), show a few holes on their surface when compared to the control. This evidence can suggest that the membrane can be damaged under the treatment: CO_2 probably dissolve in the phospholipids of the membrane at such a high extent that deeply modify its characteristics and increasing its fluidity. In these conditions CO_2 would thus easily pass through into the cytoplasm, which would result deeply acidified (8). This hypothesis is confirmed by the quantification of viable and dead cells performed by flow cytometry analysis shown above.



a)



b)

ESEM Images: a) Untreated sample. b) Sample treated at 100 bar, 36°C, 30 min

CONCLUSION

Pasteurization of *S. cerevisiae* in simple solution with supercritical CO_2 was efficiently performed at 36°C, 100 bar on a laboratory apparatus of a total volume of 150 ml at different rate of mixing. The experimental evidences confirm that:

1. the limiting step of the pasteurization is the diffusion of CO_2 inside the liquid phase;
2. the yeast cell membrane is modify under the treatment: CO_2 probably dissolve in the phospholipids at a very high extent;
3. the progressive damage of yeast cells was demonstrated by the flow cytometric analysis that allows to measure directly the increase in the number of dead cells (characterised by a permeabilised membrane) for increasing levels of treatment.

ACKNOWEDGEMENTS

The funding of this research has been provided by the University of Trento (Italy) and the Istituto of S. Michele all'Adige (Italy). The authors wish to thanks the students Nicola Ausserer and Gabriele Tomasi for their helpful participations at the experimental trials and Mr Selvino Marigo and Ugo Pieri for their technical advices.

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