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Microbial inactivation by ultrasound assisted supercritical fluids

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Abstract

A method combining supercritical carbon dioxide (SC-CO₂) and high power ultrasound (HPU) has been developed and tested for microbial/enzyme inactivation purposes, at different process conditions for both liquid and solid matrices. In culture media, using only SC-CO₂, the inactivation rate of *E. coli* and *S. cerevisiae* increased with pressure and temperature; and the total inactivation (7-8 log-cycles) was attained after 25 and 140 min of SC-CO₂ (350 bar, 36 °C) treatment, respectively. Using SC-CO₂+HPU, the time for the total inactivation of both microorganisms was reduced to only 1-2 min, at any condition selected. The SC-CO₂+HPU inactivation of both microorganisms was slower in juices (avg. 4.9 min) than in culture media (avg. 1.5 min). In solid samples (chicken, turkey ham and dry-cured pork cured ham) treated with SC-CO₂ and SC-CO₂+HPU, the inactivation rate of *E. coli* increased with temperature. The application of HPU to the SC-CO₂ treatments accelerated the inactivation rate of *E. coli* and that effect was more pronounced in treatments with isotonic solution surrounding the solid food samples. The application of HPU enhanced the SC-CO₂ inactivation mechanisms of microorganisms, generating a vigorous agitation that facilitated the CO₂ solubilization and the mass transfer process. The cavitation generated by HPU could damage the cell walls accelerating the extraction of vital constituents and the microbial death. Thus, using the combined technique, reasonable industrial processing times and mild process conditions could be used which could result into a cost reduction and lead to the minimization in the food nutritional and organoleptic changes.

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1. Introduction

Non-thermal food preservation techniques have been developed by the food industry in response to an increasing consumer's demand for natural, fresh and free of chemical preservatives food. Supercritical carbon dioxide (SC-CO₂) inactivation technology represents a promising non-thermal processing method (Liu et al. 2012), as it promotes minimum impact on the nutritional and organoleptic food properties. However, in some cases high pressures or temperatures and too long treatment times are required to guarantee the food's safety and stability (Ortuño et al. 2012). In order to obtain the required lethality at shorter processing times or with lower treatment intensity, a combination of SC-CO₂ with high power ultrasound (HPU) has been developed and used in the present work for microbial/enzyme inactivation purposes. The influence of the process conditions, the nature of the medium and the use or not of HPU on the SC-CO₂ inactivation kinetics was assessed. Additionally microscopy techniques were used in order to elucidate the inactivation mechanisms involved in the combined treatment (SC-CO₂+HPU).

2. Methodology

The combined SC-CO₂+HPU inactivation process was compared to the SC-CO₂ treatment in order to evaluate the effect of HPU on the SC-CO₂ inactivation kinetics of *E. coli* and *S. cerevisiae* inoculated in culture medium, and to determine the effect of different temperatures (31-41 °C, 225 bar) and pressures (100-350 bar, 36 °C). In order to elucidate the inactivation mechanisms associated to the combined technology (SC-CO₂+HPU) a morphological study was carried out. The differences between untreated, SC-CO₂ (350 bar, 36 °C, 5 min) and SC-CO₂+HPU (350 bar, 36 °C, 5 min, 40 W) treated *E. coli* and *S. cerevisiae* cells, were determined using light microscopy (LM) and transmission electron microscopy (TEM).

Apple and orange juice were selected to study the inactivation of these microorganisms with SC-CO₂+HPU in real matrices; additionally, the inactivation of the enzyme pectin-methyl esterase (PME) in orange juice was addressed. Experiments with juices were performed at different temperatures (31-41 °C, 225 bar) and pressures (100-350 bar, 36 °C).

On the other hand, the inactivation of *E. coli* in solid matrices (chicken, turkey ham and dry-cured pork ham) was carried out using SC-CO₂ and SC-CO₂+HPU at different pressures (150-450 bar, 41°C) and temperatures (36-51 °C, 350 bar).

3. Results and Discussion

3.1. Effect of HPU on the SC-CO₂ treatments at different process conditions in liquid matrices. Synergistic effect

Using only SC-CO₂, the inactivation rate of *E. coli* and *S. cerevisiae* inoculated in culture media increased progressively as the pressure and temperature rose. Higher pressures and temperatures enhance the SC-CO₂ solubilization into the medium and increase the fluidity of the cell membrane, respectively, making the contact and penetration of CO₂ into the cells easier and facilitating the decrease of intracellular pH and the extraction of vital cell constituents (Erkmen, 2012). However, when HPU was applied during the SC-CO₂ treatments in growth media, a drastic inactivation effect was observed and a total reduction of about 10⁷-10⁸ log-cycles was attained after only 1-2 min at any conditions of pressure and temperature studied. Using SC-CO₂+HPU the effect of HPU leads to a vigorous agitation that would accelerate the SC-CO₂ inactivation mechanisms and mask the effect of these process variables. Moreover, the cavitation generated by HPU could damage the microorganism's cell wall, accelerating its inactivation. The study of a possible synergistic effect revealed that the combination of SC-CO₂ and HPU had a greater effect on the microbial inactivation than the addition of their individual effects.

3.2. Microscopy study

LM and TEM images provided evidences that 5 min of SC-CO₂ treatment could generate uneven distribution of the cytoplasm content and slight modifications in the cell envelope, which was not lethal neither for *E. coli* (Figure 1B) nor for *S. cerevisiae* cells (Figure 1E). Moreover, the greatest differences between both microorganisms

appeared in the cell envelope: minor alterations were observed in *S. cerevisiae* and no disruption of cell wall was appreciated, while the cell envelope of *E. coli* cells was observed with a high degree of dissolution, loss of cohesiveness, protuberances, and some disintegrated areas. On the other hand, 5 min of SC-CO₂+HPU treatment resulted in the total inactivation of both microorganisms. LM and TEM images revealed greater proportions of empty regions inside of SC-CO₂+HPU-treated cells, indicating clearly a drastic reduction of the cytoplasm content. The cell envelope of *E. coli* cells were totally disrupted (Figure 1C), while the cell wall of *S. cerevisiae* cells lost partially their layered structure and some broken walls could be observed (Figure 1F). Therefore, the inactivation mechanisms associated to SC-CO₂+HPU could be related to the cavitation phenomenon, generated by HPU, which drastically damage the cell envelope increasing both the rupture of the cellular membrane and the disintegration of the intracellular content. The damages generated by the SC-CO₂+HPU treatment were strong enough to avoid a possible regrowth of cells during post-treatment storage (6 weeks at 4 °C).

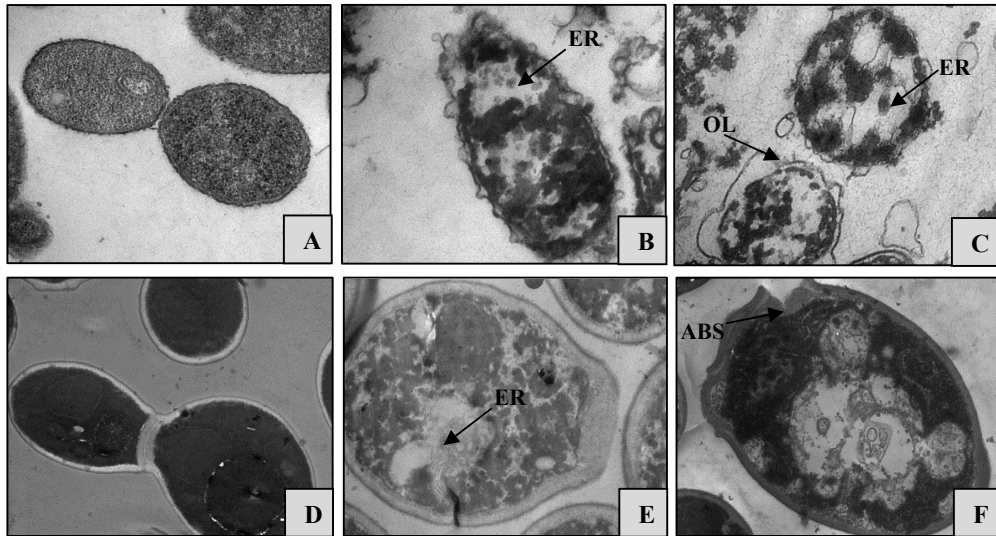


Figure 1. TEM micrographs by ultrathin sectioning of *E. coli* (A-C) and *S. cerevisiae* (D-F). A, D: Untreated; B, E: SC-CO₂-treated cells; C, F: SC-CO₂+HPU-treated. ER: empty regions; OL: cell wall-outer layer; ABS: abnormal bud scars.

3.3. Effect of the medium on the SC-CO₂+HPU treatments. Enzyme inactivation

On average, the SC-CO₂+HPU inactivation of both microorganisms was slower in apple juice (5.3 min) than in orange juice (4.6 min); and in both juices slower than in culture media (1.5 min). This fact could be linked to the sugar content and the CO₂ solubilization. The sugar binds water from the medium (Ferrentino et al. 2010), thus the free water where the CO₂ can be dissolved was lower in apple juice (15.6 °Brix) than in orange juice (11.6 °Brix); and lower in both juices than in LB (2 °Brix) or YPD (5 °Brix) Broth. In addition, the SC-CO₂+HPU inactivation of both microorganisms inoculated in juices was accelerated by increasing pressure and temperature. This fact could be related to the composition of juices, which were not so quickly saturated with CO₂ in the SC-CO₂+HPU treatments like using culture media, therefore an increase of pressure or temperature could facilitate the solubilization of CO₂.

Contrarily to the results obtained using culture media, where no difference between *E. coli* and *S. cerevisiae* was found, *E. coli* inoculated in juices showed more resistance to the SC-CO₂+HPU treatments than *S. cerevisiae*. On average, to reach the total inactivation, the treatment time required was 6.6 and 3.3 min for *E. coli* and *S. cerevisiae*, respectively. In juices, the vigorous solubilization of CO₂ generated by HPU could be hindered by the higher sugar content, thus the inactivation mechanisms would be mainly driven by the cavitation phenomenon and the size of the microorganisms. The size of *S. cerevisiae* cells is much bigger than *E. coli* ones, therefore, the probability that the implosion of the cavitation bubbles might affect the cell structure could be larger for *S. cerevisiae* than for *E. coli*.

On the other hand, the SC-CO₂+HPU inactivation of PME increased with pressure and temperature, although its total inactivation was not attained in any of the studied conditions. The inactivation of enzymes exposed to SC-CO₂ treatments can be explained by the lowering of the pH, the inhibitory effect of molecular CO₂ on enzyme activity and the fact that SC-CO₂ causes conformational changes. The enzyme PME was more resistant to SC-CO₂+HPU than *E. coli* or *S. cerevisiae* in orange juice (at 36 °C, 225 bar and after 2 min, a reduction of 18.9 %, 62.4 % and 88.1 % was attained, respectively), which could be attributed to the different nature and size of microorganisms and enzymes.

3.4. SC-CO₂ treatments in solid matrices

Using SC-CO₂, the inactivation rate of *E. coli* in chicken samples did not increase with pressure, however, using turkey ham, the time to reach a reduction of 6.6 log-cycles was reduced from 30 to 20 min as pressure increased from 150 to 350 bar (46 °C). The temperature of SC-CO₂ treatment significantly ($p < 0.05$) increased the inactivation rate of *E. coli* in chicken and turkey ham samples.

On the other hand, on average, the simultaneous application of HPU to the SC-CO₂ treatments did not accelerate the inactivation mechanisms of *E. coli* in solid matrices compared to the SC-CO₂ inactivation treatments. The different effect of HPU on the SC-CO₂ microbial inactivation using liquid or solid matrices could be linked to the free water content of samples. In liquid matrices, the amount of free water where the CO₂ can be dissolved is higher than in solid matrices, which could facilitate the inactivation mechanisms.

In this sense, it was studied the addition of a saline solution to the SC-CO₂ and SC-CO₂+HPU treatments. The presence of saline solution in the SC-CO₂ treatments did not increase the inactivation rate of *E. coli* neither for chicken nor for turkey ham samples, compared to the inactivation using only SC-CO₂. However, the addition of saline solution accelerated the inactivation mechanisms associated to the SC-CO₂+HPU treatments and reduced the time required to reach the total inactivation of *E. coli* in chicken, turkey ham and dry cured ham.

4. Conclusions

The combination of SC-CO₂ with HPU enhanced the microbial/enzyme inactivation process. The application of HPU enhanced the SC-CO₂ inactivation mechanisms in liquid matrices, generating a vigorous agitation that facilitated the CO₂ solubilization and mass transfer, additionally the cavitation damaged the cellular structure accelerating the extraction of vital constituents and reducing the time required to reach a particular inactivation level. Using the ultrasound enhanced SC-CO₂ technique, reasonable industrial processing times and mild process conditions could be used which could lead to the minimization in the food nutritional and organoleptic changes. Therefore, this technology could represent an alternative to thermal processing in order to prevent the deterioration of food and to extend its shelf life.

Acknowledgments

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