POTENTIALS OF ULTRASOUND AND HIGH PRESSURE CARBON DIOXIDE COMBINED TREATMENT

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ABSTRACT

Innovative non-thermal technologies for the removal of biological contaminants and microorganisms to generate clean or sterile materials are in increasing demand by medical, pharmaceutical as well as food industries. Among the others, pasteurization with high pressure carbon dioxide ($HPCO_2$) has been recognized as one of the most promising method as it assures a total inactivation at low temperature if compared to the thermal treatment, with minimum impact on the properties of the treated matrices. Thanks to its liquid-like density and gas-like diffusivity it allows a faster penetration of CO_2 into microbial membranes compared to an atmospheric environment. However, although $HPCO_2$ inactivation efficiency has been widely demonstrated, harsh process conditions and long treatment times are requested when specific pathogens that often persist in biofilm structures, or fungi commonly associated with nosocomial infections, have to be inactivated.
In this regards, the present work suggests an experimental approach to enhance the inactivation efficiency of $HPCO_2$ by the combination with another preservation technique, namely high power ultrasound (HPU), to obtain additive or synergistic effects and reduce the processing requirements. To this purpose a combined $HPCO_2$ and HPU apparatus was properly designed and tested on *Salmonella enterica*, a highly resistant pathogenic strain. The experimental results showed a drastic decrease of the inactivation time when HPU is applied simultaneously with $HPCO_2$, demonstrating the efficiency of the method and its potentials for future applications in industrial settings.

INTRODUCTION

Conventional thermal methods are the most currently used to inactivate microorganisms in food products. During the last few years innovative preservation technologies have been developed driven by the constant pursuit to reduce the degree of thermal damage to the quality of the processed foods in terms nutritional, sensorial and physical/chemical attributes [1]. The new preservation techniques could pasteurize food products, reducing or eliminating the amount of heat required. These processes are, for the most part, less energy-intensive, therefore more cost-efficient, and environmentally friendly than conventional thermal processing. Among others, a promising alternative to the traditional pasteurization processes is the use of high pressure carbon dioxide ($HPCO_2$) technology.
Nevertheless, long treatment times and temperatures are needed to guarantee the safety and stability of some food products, limiting the efficiency of $HPCO_2$ inactivation processes [2, 3]. That is the reason why there is increasing scientific interest in combining $HPCO_2$ processes with synergistic techniques to enhance the its inactivation mechanisms [4].

Higher-power ultrasound at low frequencies (20 to 100 kHz), which is referred to as ‘‘power ultrasound’’ (HPU), has the potential to be used for the inactivation of bacterial populations; the advantages of ultrasound over heat pasteurization include the minimizing of flavour loss with greater homogeneity and significant energy savings during the process [1, 5]. Unfortunately, very high intensities are needed if ultrasound alone is used for permanent pasteurization. The combination of HPU and $HPCO_2$ and the demonstration of their synergistic effect is quite recent. Ortuño et al. [4, 6] showed that the population of both *S. cerevisiae* and *E. coli* microorganisms inoculated in apple juice was completely inactivated after 5 min (35 MPa, 36 ºC) and 4 min (22.5 MPa, 36 ºC) of treatment, respectively. On the contrary, no microbial reduction was observed if only $HPCO_2$ was applied for the same treatment time and process conditions. It has been also shown that the performance of HPU treatment is affected by several factors including the type, shape or diameter of the microorganisms [7], the growth stage [8] and the medium [6].
However, so far no references have been found in the literature exploring the effect of the combination of HPU and $HPCO_2$ ($HPCO_2$+HPU) on the inactivation of *Salmonella enterica*, a patoghenic bacteria responsible of several human diseases such as gastroenteritis, bacteremia, enteric fever.
In this regards, the objective of this work was to study and compare the effect of $HPCO_2$ alone and $HPCO_2$+HPU combined treatment on two liquid matrices: a simple phosphate buffer solution (PBS) and a coconut water (CW) inoculated with the pathogenic gram-negative *S. enterica* microorganism. The inactivation kinetics were analyzed and compared in terms of the process parameters: pressure, temperature and treatment time of $HPCO_2$. The investigation of the feasibility of such a pasteurization combined technology, may open the door to the exploitation of the technology to different and high values drinks at industrial scale.

MATERIALS AND METHODS

Sample preparation

Salmonella enterica ATCC 14023 (DSMZ, Braunschweig, Germany) cultures were grown in 10 mL Brain Heart Infusion broth (BHIB) at 37 °C overnight. Then, the culture was transferred to a 200 mL flask of BHIB and grown at 37 °C overnight. Cell growth was carried out in a shaking incubator (220 rpm) and carefully monitored through measurements of the optical density in order to achieve the stationary phase. The microbial suspensions were centrifuged at 6000 rpm for 10 min at 4 °C, the supernatant removed and the pellet resuspended in 100 mL of PBS or CW, reaching a final concentration of about 10^8 - 10^{10} colony forming unit (CFU) per mL. PBS was prepared dissolving a buffer tablet in 500 mL of distilled water reaching a final pH of about 6.8. The solution was sterilized by autoclaving to inoculate *S. enterica*.
Coconut water (CW) was obtained from young green coconuts (*Cocos nucifera, cv Nam Hom*) bought from Thailand and sent to Trento. The coconuts were aseptically opened, the water extracted and accumulated in a 20 liters plastic pail placed in ice. Once the extraction process ended, the coconut water was homogenized, portioned in sterilized glass jars of 200 and 400 ml and immediately frozen at -20° C to prevent any microbial or enzymatic activity.

HPU combined $HPCO_2$ apparatus

The $HPCO_2$ apparatus consisted in a sapphire high pressure visualization cell (Separex S.A.S., France) with an internal volume of 50 ml designed to withstand up to 400 bar and 100°C. The plant includes a CO_2 tank, kept at room temperature, a chiller reservoir, a HPLC pump, and a thermostatic bath to keep the inactivation vessel at the desired temperature. The system was equipped with an ultrasound system (Aktive Arc Sarl, Switzerland) designed on purpose and embedded in the $HPCO_2$ plant. This system consists of a transducer (40 KHz), a buster, a special retainer (M36x1.5), a sonotrode and a power generator unit (Figure 1). Each single experimental run required four operating steps: (1) plant cleaning and disinfection, (2) sample preparation, (3) combined treatment and (4) sample collection. The sample (20 ml) was loaded into the high pressure vessel and immediately sealed. Water was circulated through the jacket of the reactor until the desired temperature was reached. For the experiments with $HPCO_2$, the sample was continuously stirred and pressure and temperature conditions were kept constant at the set-up values during the entire treatment. .

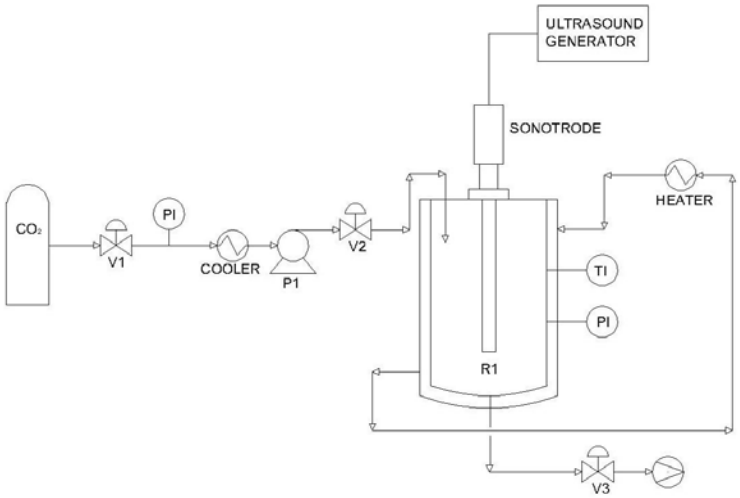

Figure 1 : Combined $HPCO_2$+HPU apparatus.

For the experiments with HPU, after loading the sample in the vessel, the ultrasound unit was turned on for the required treatment time with an applied power of about 10±2 W. To perform the experiments with $HPCO_2$+HPU combined treatment, the ultrasound unit was turned on (time zero) when the desired pressure and temperature were reached in the vessel. The applied power during the whole experiment was 10±2 W. Pressure and temperature were kept constant during the experiment through the pump and the thermostatic bath, respectively. The treated samples were collected in individual sterile tubes for microbial analyses. The vessel was cleaned and disinfected with ethanol (96 %v/v) after each sampling.

Process conditions

Different temperature and time conditions, namely, 25, 30, 35 and 40°C and 1÷15 min, were considered while the pressure was kept constant at 10 MPa for PBS and 12 MPa for CW experiments. Principally, pressure controlled both the solubilization rate and the solubility of CO_2. Its increase is beneficial on microbial inactivation thanks to the dramatic increase in density and solvation power of CO_2 that promotes its contact with the cells inducing the removal of vital constituents from cells or cell membranes. However, this increase is limited

by the saturation solubility of CO_2 in the treatment medium, thus, once the treatment medium is saturated with CO_2, the killing effect of $HPCO_2$ does not change significantly. For instance, the results of the study of Damar et al. [9] performed on CW processed with a continuous $HPCO_2$ system showed that pressure, changing from 13.8 to 34.5 MPa, was not significant in microbial reduction whereas temperature and % CO_2 were significant. The mixing has a main role in the solubilization kinetic of CO_2 inside the liquid phase, more than the pressure, as previously demonstrated [10].

Microbial analyses

The standard plate count technique was used to determine the initial microbial concentration and the efficiency of the treatment in reducing the number of S. *enterica*. After the treatments, samples were serially diluted in a phosphate buffer solution (PBS, 0.01 M, pH 7.4) and spread onto *Chromatic Salmonella Agar* colture medium. The plates were incubated at 37 °C for 24 h. The inactivation degree was determined by evaluating the $Log(N/N_0)$, where N_0 (CFU/ml) is the number of colony forming units per ml initially present in the untreated sample, and N (CFU/ml) is the number of survivors after the treatment. Three independent experiments were carried out for each single treatment condition and the results were calculated as the mean value of three replications. Standard deviations, calculated from these replications, were shown by error bars in the figures reported in this study.

RESULTS

Microbial inactivation kinetics of *S. enterica* in PBS

Experiments were performed on an in vitro microbial suspension of *S. enterica* in PBS in order to verify the efficiency and the synergistic effect of $HPCO_2$+HPU combined treatment as previously published by Ortuno et al. [4, 6] for some specific microbial strains. The possible synergistic effect of $HPCO_2$+HPU combined treatment was evaluated at the same process conditions used for $HPCO_2$ treatment by simultaneously applying ultrasound wave with a power of 10 W to the sample.

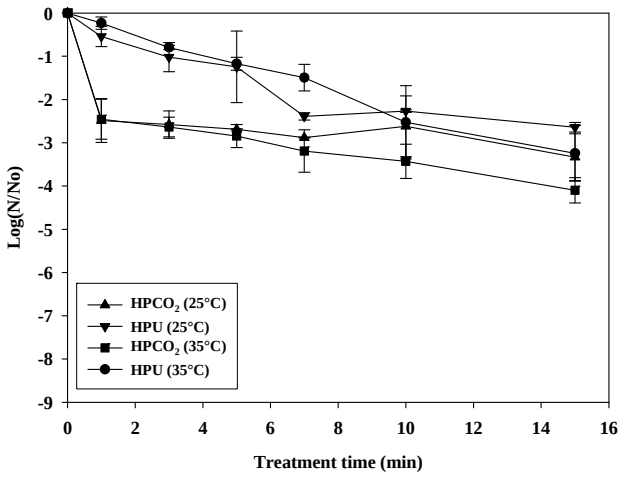

Figure 2 : *S. enterica* inactivation kinetics in PBS at 10 MPa, as function of temperature and time for $HPCO_2$ and HPU treatments.

The $HPCO_2$ and HPU treatments applied separately (Figure 2) induced just 3 and 2.5 Log reductions after 15 min, respectively. The increase of temperature from 25 to 35°C did not result in a substantial increase of microbial inactivation at any treatment times.

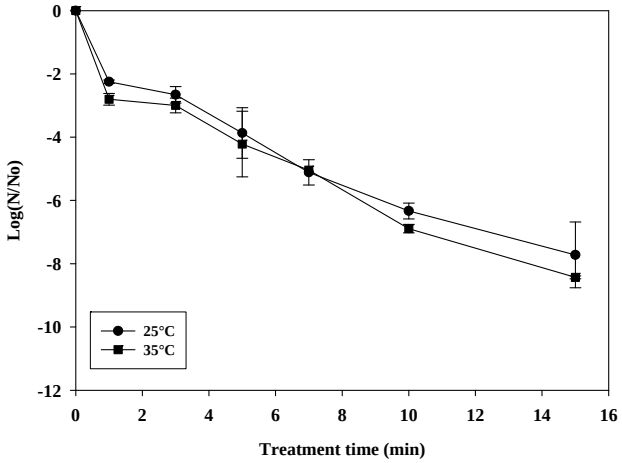

Figure 3 : *S. enterica* inactivation kinetics in PBS at 10 MPa, as function of temperature and time for combined $HPCO_2$+HPU treatment.

The results, shown in Figure 3, clearly indicated the higher efficiency of the $HPCO_2$+HPU combined treatment: inactivation to not detectable levels was obtained after 15 min at both 25 and 35°C. In addition, as shown by Ortuno et al. [4, 6], the effect of increasing the temperature from 25 to 35°C was not significant on the microbial reduction for the combined treatment.

Microbial inactivation kinetics of *S. enterica* in CW

On the basis of the inactivation data obtained for *S. enterica* in PBS, a new set of experiments was carried out considering a complex substrate, such as CW.

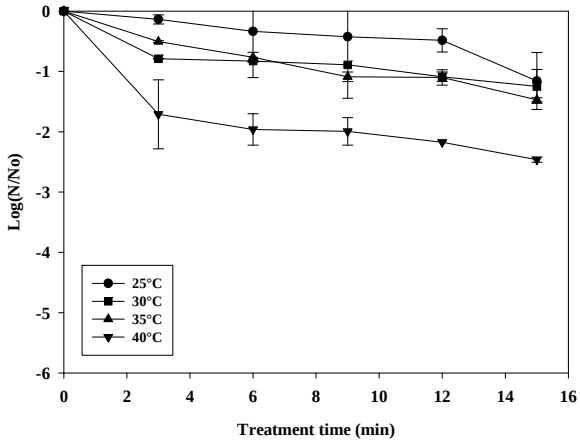

Figure 4 : *S. enterica* inactivation kinetics in CW at 12 MPa, as function of temperature and time for $HPCO_2$ treatment.

The results demonstrated that HPU treatment alone was not beneficial to achieve a faster microbial inactivation (data not shown). In Figure 4 the inactivation kinetics of *S. enterica* after $HPCO_2$ treatments are shown at different temperatures.
When CO_2 alone was used, the inactivation rate increased as the temperature rose: at 12 MPa about 0.5 Log reduction were achieved in 10 min at 25°C, while about 2 Log reduction were achieved at 40°C for the same treatment time (Figure 4).
The same trend was observed for $HPCO_2$+HPU: after 10 minutes of treatment, 3, 4 6 and 9 Log reductions were obtained at 25, 30, 35 and 40°C, respectively but, clearly, a faster reduction of *S. enterica* was reached compared to $HPCO_2$ treatment alone for any temperature considered (Figure 5).

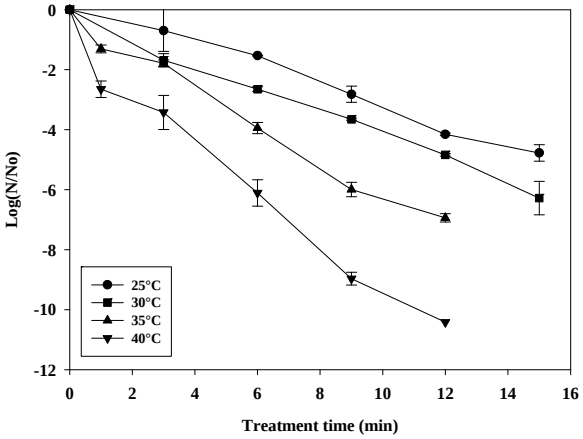

Figure 5 : *S. enterica* inactivation kinetics in CW at 12 MPa, as function of temperature and time for combined $HPCO_2$+HPU treatment.

The results clearly indicated a definite synergistic effect on the inactivation of *S. enterica* when the combination of $HPCO_2$+HPU was used, compared to the single treatments, in agreement with previous works regarding different bacterial strains [6, 8]. Additionally, the results showed that the increase of temperature highly affected *S. enterica* reduction when $HPCO_2$+HPU was exploited in complex solution as CW, while in a simple solution as PBS the effect is negligible.
The different results could be related to the different suspensions where the bacteria were inoculated. It has been previously highlighted that $HPCO_2$+HPU inactivation rate is affected by the type and composition of the suspending medium [4, 11]. The high concentrations of minerals and salts in CW could be able to bind the water molecules, thus to decrease the amount of free water in which CO_2 could dissolve. As a consequence, CO_2 saturation into the liquid phase could be delayed, compared to a simple solution. For this reason an increase in temperature could be beneficial on the inactivation rate increasing the membrane fluidity thus accelerating the inactivation process. Differently, in PBS solution, CO_2 saturation was faster thus the inactivation extremely rapid and the effect of the temperature insignificant.

CONCLUSION

The synergic effect of $HPCO_2$+HPU combined process on the inactivation of *S. enterica* in PBS and CW was demonstrated. The efficiency of the process was influenced by the temperature for CW while no effect was detected for PBS. Different process times were required to achieve the same inactivation for *S. enterica* anyway always shorter than using

$HPCO_2$ or HPU treatments alone. After $HPCO_2$+HPU combined treatment, inactivation to undetectable level (about 8-10 Log reductions) was achieved at 10 MPa, 35°C, 15 min for *S. enterica* in PBS and at 12 MPa, 40°C, 12 min for *S. enterica* in CW.

The technology developed permitted both a drastic decrease of $HPCO_2$ treatment times and the use of milder process conditions, which could lead to an increase of the product quality. It represents a promising alternative to thermal processing for extending the shelf life of thermosensitive and high value foods.

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