Inactivation of *Bacillus subtilis* spores in lipid emulsions by supercritical CO_2 combined with high power ultrasound

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Spores are the most resistant form of microorganism, able to survive under extreme environmental conditions. The most extended technique for sterilization is thermal treatment. However, it has some disadvantages in terms of product quality, since high temperatures and long times are required to inactivate spores. Alternative methods such as supercritical carbon dioxide (SC-CO_2) have been studied. However, in many cases long processing times are required to achieve a proper sterilization. High power ultrasound (HPU) can be combined with SC-CO_2 (SC-CO_2 + HPU) to intensify the inactivation effect of carbon dioxide. The aim of this study was to evaluate the effectiveness of the SC-CO_2 + HPU technology to inactivate *B. subtilis* spores in lipid emulsions. For that purpose, the SC-CO_2 + HPU inactivation kinetics were studied at two different temperatures (85 and 95°C) at 350 bar and were compared to SC-CO_2 and thermal treatments at the same temperatures.

B. subtilis vegetative cells were precultivated and inoculated in PCA plates with $CaCl_2$ (100 mg/L) and $MnSO_4$ (40 mg/L) at 30°C for 6 days. Spores were collected from the agar surface and washed by centrifugation. Before being treated, spores were heat-shocked and inoculated in a 20% soybean emulsion to a cell concentration of 10^6-10^7spores/mL. Thermal treatments were performed in a water bath placing 1.5 mL of the inoculated emulsion in glass tubes. SC-CO_2 and SC-CO_2 + HPU treatments were carried out in a supercritical CO_2 plant with an embedded ultrasound system (35 ± 5 W; 30 kHz). Samples were extracted at different times and were analysed in triplicate by the plate count method. The inactivation kinetics were mathematically modelled by the Weibull model.

B. subtilis inactivation levels were higher in the SC-CO_2 treatment compared to the thermal treatments, regardless the treatment temperature. No inactivation was achieved for the thermal treatment at 85°C, while 1.4 log-cycles were reduced, on average for all the processing times, with the SC-CO_2 treatment at the same temperature. At 95°C, *B. subtilis* spores were reduced, on average, 1.6 log-cycles with the thermal treatment and 5.2 log-cycles with the SC-CO_2 processing. The application of HPU had a significant ($p<0.05$) effect on the *B. subtilis* reduction since the average inactivations were 3.9 log-cycles and 6.3 log-cycles, for 85 and 95°C treatments, respectively. The inactivation was significantly ($p<0.05$) higher (2.6 log-cycles on average) when all treatments (thermal, SC-CO_2 and SC-CO_2 + HPU) were performed at the highest temperature (95°C).

Therefore, lower temperatures could be used for the inactivation of spores with SC-CO_2 treatments, compared with conventional thermal ones. In addition, the application of HPU to the SC-CO_2 treatment demonstrated a great potential for the inactivation of spores in lipid emulsions, since lower temperatures or shorter treatment times could be applied.

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Keywords
Bacillus subtilis, spores, supercritical CO_2, ultrasound, lipid emulsion.