UNIVERSITAT POLITÈCNICA DE VALÈNCIA

Departamento de Tecnología de Alimentos

Aplicación de ultrasonidos de potencia para la mejora de procesos de inactivación con fluidos supercríticos

TESIS DOCTORAL

Presentada por:
Carmen Ortuño Cases

Dirigida por:
Dr. D. José Javier Benedito Fort

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D. JOSÉ JAVIER BENEDITO FORT, CATEDRÁTICO DE UNIVERSIDAD DEL DEPARTAMENTO DE TECNOLOGÍA DE ALIMENTOS DE LA UNIVERSIDAD POLITÉCNICA DE VALENCIA

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Abstract / Resumen / Resum

ABSTRACT

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-CO_2) inactivation technology represents a promising non-thermal processing method, 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. In order to obtain the required lethality at shorter processing times or with lower treatment intensity, a combination of SC-CO_2 with high power ultrasound (HPU) or with high hydrostatic pressure (HHP) have been developed and used in the present work for microbial/enzyme inactivation purposes.

The main aim of this Thesis was to evaluate non-thermal preservation techniques, based on the combination of SC-CO_2 and HPU, and on the combination of SC-CO_2 and HHP. Regarding the combination of SC-CO_2 with HPU, the influence of the culture growth stage, the process conditions, the nature of the medium and the use or not of HPU on the inactivation kinetics of microorganisms (*Escherichia coli* (*E. coli*) and *Saccharomyces cerevisiae* (*S. cerevisiae*)) or enzymes (pectin-methyl esterase (PME)) was studied. Mathematical models and microscopy techniques have been used in order to describe the inactivation kinetics and to study the inactivation mechanisms involved in the treatments, respectively. In regard to the combination of SC-CO_2 and HHP, the effect of different levels of added carbon dioxide in the package on the efficiency of HHP treatment to inactivate PME, peroxidase (POD) and polyphenol oxidase (PPO) in feijoa puree was evaluated.

The influence of the culture growth stage of *E. coli* and *S. cerevisiae* inoculated in culture medium, LB and YPD Broth, respectively, on their inactivation kinetics using SC-CO_2 (350 bar, 35 ºC) was studied. Cultures of *E. coli* and *S. cerevisiae* were grown to four different growth stages from the early exponential phase to the stationary phase, and then treated with SC-CO_2 at

350 bar and 35 ºC. The combined SC-CO_2+HPU inactivation process was compared to the SC-CO_2 treatment in order to evaluate the effect of HPU on the SC-CO_2 inactivation kinetics of *E. coli* and *S. cerevisiae* in the early stationary phase inoculated in culture medium, and to determine the effect of different temperatures (31-41 ºC, 225 bar) and pressures (100-350 bar, 36 ºC). In order to elucidate the inactivation mechanisms associated to the combined technology (SC-CO_2+HPU) a morphological study was carried out. The differences between untreated, SC-CO_2 (350 bar, 36 ºC, 5 min) and SC-CO_2+HPU (350 bar, 36 ºC, 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-CO_2+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 ºC, 225 bar) and pressures (100-350 bar, 36 ºC). The chosen temperature and pressure ranges were higher than the critical one for CO_2 and lower than lethal for both microorganisms. Both *E. coli* and *S. cerevisiae* have been selected in the present study because they are habitual components of the microbiota involved in food spoilage and are commonly used as indicators of food contamination.

The combination of SC-CO_2 with HHP was assessed to determine the effect of different levels of CO_2 in the package (only HHP (HHP); carbonation and HHP (HHPcarb); carbonation + addition of 8.5 mL CO_2/g puree into the headspace of the package and HHP (HHPcarb+CO_2)) on the efficiency of HHP inactivation treatment on PME, POD and PPO in feijoa puree at different pressures (300, 450 and 600 MPa, for 5 min).

Results showed that the resistance of both microorganisms to the SC-CO_2 inactivation treatment increased progressively as the growth phase advanced, which could be due to the natural protective systems that become activated as cells approach the stationary phase. The inactivation kinetics of *E. coli* and *S. cerevisiae* were fitted to the Weibull Model (R^2 = 0.93; RMSE = 0.59) and to the Gompertz function (R^2 = 0.96; RMSE = 0.53), respectively, which were adapted considering the growth stage as one of the model parameters.

Using only SC-CO_2, the inactivation rate of both microorganisms inoculated in culture media increased progressively as the pressure and temperature rose. The required time to reach the total inactivation of *E. coli* (8 log-cycles) was reduced from 60 to 25 min as pressure increased from 100 to 350 bar (36 ºC), and from 75 to 40 min as temperature increased from 31 to 41 ºC (225 bar). The total inactivation of *S. cerevisiae* (7 log-cycles) was attained only at 350 bar, 36 ºC and after 140 min. In general, higher pressures and temperatures enhance the SC-CO_2 solubilization into the medium and increase the fluidity of the cell membrane, respectively, making the contact and penetration of CO_2 into the cells easier and facilitating the decrease of intracellular pH and the extraction of vital cell constituents. However, when HPU was applied during the SC-CO_2 treatments in growth media, a drastic inactivation effect was observed and a total reduction of about 10^7-10^8 log-cycles was attained after only 1-2 min. Using SC-CO_2+HPU the effect of increasing pressure or temperature did not significantly influenced the inactivation level due to HPU leads a vigorous agitation that would accelerate the SC-CO_2 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-CO_2 and HPU had a greater effect on the microbial inactivation than the addition of their individual effects. For *E. coli*, a reduction of 0.3, 0.9 and 8 log-cycles was attained after 5 min of SC-CO_2, HPU and SC-CO_2+HPU treatments, respectively; for *S. cerevisiae* a reduction of 6.83 log-cycles was achieved after 2 min of SC-CO_2+HPU treatment, while for the same period of time, no inactivation was observed when using only HPU or SC-CO_2.

In all the experiments carried out, *S. cerevisiae* showed higher resistance to the SC-CO_2 treatments than *E. coli*, which could be linked to the fact that *S. cerevisiae* has a thicker and more resistant cell wall than *E. coli*, 124.8 nm, compared to 17.7 nm, respectively. However, combining SC-CO_2 and HPU, the vigorous agitation and cavitation of the medium masked the different resistances showed by both microorganisms in the SC-CO_2 treatments.

LM and TEM images provided evidences that 5 min of SC-CO_2 treatment could generate uneven distribution of the cytoplasm content and slight

modifications in the cell envelope, which was not lethal neither for *E. coli* nor for *S. cerevisiae* cells. 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-CO_2+HPU treatment resulted in the total inactivation of both microorganisms. LM and TEM images revealed greater proportions of empty regions inside of SC-CO_2+HPU-treated cells, indicating clearly a drastic reduction of the cytoplasm content. The cell envelope of *E. coli* cells were totally disrupted, while the cell wall of *S. cerevisiae* cells lost partially their layered structure and some broken walls could be observed. Therefore, the inactivation mechanisms associated to SC-CO_2+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-CO_2+HPU treatment were strong enough to avoid a possible regrowth of cells during post-treatment storage (6 weeks at 4 ºC).

On average, the SC-CO_2+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 CO_2 solubilization. The sugar binds water from the medium, thus the free water where the CO_2 can be dissolved was lower in apple juice (15.6 ºBrix) than in orange juice (11.6 ºBrix); and lower in both juices than in LB (2 ºBrix) or YPD (5 ºBrix) Broth. In addition, the SC-CO_2+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 CO_2 in the SC-CO_2+HPU treatments like in the experiments conducted in culture media, therefore an increase of pressure or temperature could facilitate the solubilization of CO_2.

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-CO_2+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 CO_2 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-CO_2+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-CO_2 treatments can be explained by the lowering of the pH, the inhibitory effect of molecular CO_2 on enzyme activity and the fact that SC-CO_2 causes conformational changes. The enzyme PME was more resistant to SC-CO_2+HPU than *E. coli* or *S. cerevisiae* in orange juice (at 36 ºC, 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.

The Peleg Type A model (R^2 = 0.936; RMSE = 0.561) and the Weibull model (R^2 = 0.923; RMSE = 0.561), were adapted to describe the SC-CO_2+HPU inactivation kinetics of *E. coli* and *S. cerevisiae*, respectively, in apple juice, including pressure and temperature as model parameters. The Biphasic model (R^2 = 0.960; RMSE = 0.391), the Peleg Type B (R^2 = 0.894; RMSE = 0.687) and the fractional model (R^2 = 0.931; RMSE = 0.085), were adapted to describe the SC-CO_2+HPU inactivation kinetics of *E. coli*, *S. cerevisiae* and PME, respectively, in orange juice, including pressure and temperature as model parameters.

Results revealed that the residual activity of PME, PPO and POD decreased as increasing pressure, since pressure causes structural rearrangements that can change the three-dimensional structure of enzymes. The HHPcarb+CO_2 treatment increased the inactivation level of the three enzymes, compared to HHPcarb and HHP treated samples, at any pressure condition selected. This fact could be explained because the amount of dissolved CO_2 should be higher, causing a

larger pH lowering with the consequent wider denaturation of enzymes. Moreover, the CO_2 dissolved into the puree during the HHP treatment, could generate a significant and sudden bubbling during the fast depressurization of the process that could contribute to conformational changes responsible for the inactivation of enzymes.

Finally, it can be concluded that the combination SC-CO_2 with HPU or HHP significantly improved the inactivation mechanisms of microorganisms and enzymes. The application of HPU enhanced the SC-CO_2 inactivation mechanisms accelerating the CO_2 solubilization into the medium, which is the first step in the SC-CO_2 treatments; and generating cavitation which damage the cell wall, facilitating both the penetration of SC-CO_2 into the cells and the extraction of intracellular compounds, which accelerates the death of the microbial cells. Moreover, the combination of SC-CO_2 with HHP demonstrated an improvement of the HHP inactivation of enzymes. Using these combined techniques, reasonable industrial processing times and mild process conditions could be selected, which could result into a cost reduction and lead to the minimization of the impact on the nutritional and organoleptic properties of the treated products.

It is recommended that more research is conducted to elucidate the cell/enzyme inactivation mechanisms using SC-CO_2+HPU and SC-CO_2+HHP. Additional studies on the effect of these non-thermal combined technologies on physico-chemical properties and consumer acceptance of different treated food matrices are also of interest.

RESUMEN

La industria alimentaria, en respuesta a la demanda por parte de los consumidores de alimentos naturales, frescos y libres de conservantes químicos, ha desarrollado tecnologías de conservación no térmicas. El CO_2 supercrítico (SC-CO_2), representa una tecnología no térmica de inactivación prometedora, ya que está encaminada a producir el mínimo impacto sobre las propiedades nutricionales y organolépticas de los alimentos. Sin embargo, en algunos casos se requieren condiciones de presión o temperatura elevadas, así como tratamientos excesivamente largos para garantizar la seguridad y estabilidad de los alimentos. En este sentido, con el objetivo de obtener la letalidad requerida empleando procesos más cortos o de menor intensidad, en el presente trabajo se ha desarrollado una combinación del SC-CO_2 con ultrasonidos de potencia (HPU) y de SC-CO_2 con altas presiones hidrostáticas (HHP), para ser empleadas en procesos de inactivación microbiana y enzimática.

El objetivo principal de la presente Tesis fue evaluar tecnologías no térmicas de conservación basadas en la combinación de SC-CO_2 y HPU, y en la combinación de SC-CO_2 y HHP. Respecto a la combinación de SC-CO_2 con HPU, se estudio la influencia del estado de crecimiento de las células, de las condiciones del proceso, de la naturaleza del medio y del uso o no de HPU, sobre las cinéticas de inactivación de microorganismos (*Escherichia coli* (*E. coli*) y *Saccharomyces cerevisiae* (*S. cerevisiae*)) y enzimas (pectin-metil-esterasa (PME)). Se emplearon modelos matematicos y técnicas de microscopia para describir las cinéticas y los mecanismos de inactivación, respectivamente. En cuanto a la combinación de SC-CO_2 y HHP, se evaluó el efecto de la adición de diferentes niveles de CO_2 en el envase sobre la eficacia del tratamiento con HHP para inactivar PME, peroxidasa (POD) y polifenol oxidasa (PPO) en feijoa pure.

Se estudió la influencia del estado de crecimiento de las células de *E. coli* y *S. cerevisiae* inoculadas en medio de cultivo, LB e YPD Broth, respectivamente, sobre sus cinéticas de inactivación con SC-CO_2 (350 bar, 35 ºC). Cultivos individuales de *E. coli* y *S. cerevisiae* se incubaron hasta que las células

alcanzaron cuatro estados de crecimiento diferentes, desde la fase temprana exponencial hasta la fase estacionaria, para posteriormente ser tratadas con SC-CO_2 a 350 bar y 35 ºC. Se comparó el proceso combinado de SC-CO_2+HPU con el tratamiento de SC-CO_2 para evaluar el efecto de los HPU sobre las cinéticas de inactivación con SC-CO_2 de *E. coli* y *S. cerevisiae* en la fase temprana estacionaria, inoculados ambos microorganismos en medios de cultivo, y se determinó el efecto de diferentes temperaturas (31-41 ºC, 225 bar) y presiones (100-350 bar, 36 ºC). Con el objetivo de conocer los mecanismos de inactivación asociados a esta tecnología combinada (SC-CO_2+HPU) se realizó un estudio morfológico. Se estudiaron las diferencias entre células de *E. coli* y *S. cerevisiae* no tratadas, tratadas con SC-CO_2 (350 bar, 36 ºC, 5 min) y con SC-CO_2+HPU (350 bar, 36 ºC, 5 min, 40 W) usando microscopía óptica (LM) y microscopía electrónica de transmisión (TEM). Se seleccionó el zumo de manzana y de naranja para estudiar la inactivación de ambos microorganismos con SC-CO_2+HPU en matrices reales; además, se estudió la inactivación de la enzima pectin-metil-esterasa (PME) del zumo de naranja. Las experiencias se llevaron a cabo a diferentes temperaturas (31-41 ºC, 225 bar) y presiones (100-350 bar, 36 ºC). Las condiciones de temperatura y presión seleccionadas superan el punto crítico del CO_2 y son menores que las condiones letales para ambos microorganismos. Tanto *E. coli* como *S. cerevisiae* se han seleccionado para el presente trabajo porque son componentes habituales de la flora responsable del deterioro de alimentos y son comúnmente empleados como indicadores de contaminación en alimentos.

Se investigó la combinación de los SC-CO_2 con HHP para determinar el efecto de diferentes niveles de CO_2 (solo HHP (HHP); carbonatación y HHP (HHPcarb); carbonatación + adición de 8.5 ml de CO_2/ g puré en el espacio de cabeza del paquete y HHP (HHPcarb+CO_2)) sobre la eficacia del tratamiento con HHP para inactivar PME, peroxidasa (POD) y polifenol oxidasa (PPO) en puré de feijoa contenido en una bolsa de plástico, a diferentes presiones (300, 450 y 600 MPa, durante 5 min).

Los resultados mostraron que la resistencia de ambos microorganismos a los tratamientos de inactivación con SC-CO_2 aumentó progresivamente conforme la fase de crecimiento avanzó, lo cual podría deberse a la activación de sistemas de

protección naturales que desarrollan los microorganismos conforme se acercan a la fase estacionaria de crecimiento. Las cinéticas de inactivación de *E. coli* y *S. cerevisiae* se ajustaron al modelo de Weibull (R^2 = 0.93; RMSE = 0.59) y al modelo de Gompertz (R^2 = 0.96; RMSE = 0.53), respectivamente, que fueron adaptados para considerar la fase de crecimiento como uno de los parámetros de dichos modelos.

Empleando SC-CO_2, la velocidad de inactivación de ambos microorganismos aumentó progresivamente con la presión y la temperatura. El tiempo necesario para alcanzar una inactivación completa de *E. coli* (8 ciclos-log) se redujo de 60 a 25 min al aumentar la presión de 100 a 350 bar (36 ºC), y de 75 a 40 min al aumentar la temperatura de 31 a 41 ºC (225 bar). La inactivación completa de *S. cerevisiae* (7 ciclos-log) se alcanzó únicamente tras 140 min de proceso a 350 bar y 36 ºC. En general, presiones y temperaturas más elevadas mejoran la solubilización del SC-CO_2 en el medio e incrementan la fluidez de la membrana celular, respectivamente, facilitando el contacto y la penetración del CO_2, lo que favorece el descenso del pH intracelular y la extracción de componentes vitales para la célula. Sin embargo, al aplicar HPU en los tratamientos de SC-CO_2 en medios de cultivo, se observó una drástica inactivación microbiana, alcanzándose una reducción total (10^7-10^8 ciclos-log) tras solo 1-2 min de tratamiento. Aplicando SC-CO_2+HPU no se observó un efecto significativo en el nivel de inactivación al aumentar la presión o la temperatura debido a que los HPU generan una vigorosa agitación que acelera los mecanismos de inactivación asociados a los SC-CO_2 y enmascara el efecto de estas variables del proceso. Además, la cavitación generada por los HPU podría dañar la pared celular de los microorganismos, acelerando su inactivación. El estudio de la existencia de un posible efecto sinérgico entre ambas tecnologías reveló que la combinación de SC-CO_2 y HPU tuvo un mayor efecto en la inactivación que la adición de los efectos individuales de ambas. Para *E. coli*, se alcanzó una reducción de 0.3, 0.9 y 8 ciclos-log tras 5 min de tratamiento con SC-CO_2, HPU y SC-CO_2+HPU, respectivamente; para *S. cerevisiae* se alcanzó una reducción de 6.83 ciclos-log tras 2 min de tratamiento con SC-CO_2+HPU, mientras que tras el mismo periodo de tiempo con sólo SC-CO_2 o HPU no se observó ninguna reducción en el número de microorganismos.

En todos los tratamientos llevados a cabo, la levadura *S. cerevisiae* mostró mayor resistencia a los tratamientos con SC-CO_2 que la bacteria *E. coli*, lo cual podría estar relacionado con el mayor espesor de la pared celular de *S. cerevisiae* comparado con el de *E. coli*, 124.8 nm frente a 17.7 nm, respectivamente. Sin embargo, al combinar el SC-CO_2 y los HPU, la agitación vigorosa y la cavitación del medio enmascaró las diferentes resistencias mostradas por ambos microorganismos en los tratamientos con SC-CO_2.

Las imágenes de LM y TEM mostraron que tras 5 min de tratamiento con SC-CO_2 se produjo una distribución irregular del contenido citoplasmático y aparecieron pequeñas modificaciones en la envoltura celular, no siendo ninguno de estos cambios letales para las células de *E. coli* ni de *S. cerevisiae*. Además, las mayores diferencias entre ambos microorganismos se identificaron en el efecto sobre la envoltura celular: en *S. cerevisiae* se observaron ligeras modificaciones aunque no se apreció rotura de la pared celular, mientras que la pared de las células de *E. coli* aparecieron con un alto grado de disolución, pérdida de cohesividad, protuberancias y algunas áreas desintegradas. Sin embargo, 5 min de tratamiento con SC-CO_2+HPU fueron suficientes para alcanzar una inactivación completa de ambos microorganismos. Las imágenes de LM y TEM revelaron mayor proporción de regiones vacías dentro de las células tratadas con SC-CO_2+HPU, lo que indicó una clara reducción del contenido citoplasmático. La envoltura de las células de *E. coli* se desintegró totalmente, mientras que las paredes de las células de *S. cerevisiae* perdieron parcialmente su estructura laminada y se pudieron observar algunas paredes rotas. Por tanto, los mecanismos de inactivación asociados a los SC-CO_2+HPU podrían estar relacionados con el fenómeno de cavitación generado por los HPU, el cual daña bruscamente la envoltura celular incrementando tanto la ruptura de la membrana celular como la desintegración del contenido intracelular. Los daños generados por el tratamiento de SC-CO_2+HPU fueron tan severos que evitaron una posible recuperación de las células durante un almacenamiento posterior al tratamiento (6 semanas a 4 ºC).

En promedio, la inactivación de ambos microorganismos con SC-CO_2+HPU en zumo de manzana (5.3 min) fue más lenta que en zumo de naranja (4.6 min); y en ambos zumos más lenta que en medios de cultivo (1.5 min). Esto podría

estar relacionado con el contenido de azúcar del medio y la solubilización del CO_2 en el mismo. El azúcar se liga al agua del medio, por tanto, la cantidad de agua disponible donde el CO_2 puede disolverse es menor en zumo de manzana (15.6 ºBrix) que en zumo de naranja (11.6 ºBrix); y menor en ambos zumos que en LB (2 ºBrix) o YPD (5 ºBrix) Broth. Además, empleando SC-CO_2+HPU, la velocidad de inactivación de ambos microorganismos inoculados en zumos aumentó con la presión y la temperatura. Esto podría estar relacionado con la composición de los zumos, los cuales no se saturan rápidamente de CO_2 en los tratamientos con SC-CO_2+HPU como sí ocurre en las experiencias llevadas a cabo sobre medios de cultivo, de manera que un incremento de presión o temperatura puede facilitar la solubilización del CO_2.

Contrariamente a los resultados obtenidos con SC-CO_2+HPU sobre medios de cultivo, donde no se observaron diferencias entre *E. coli* y *S. cerevisiae*, en zumos *E. coli* mostró mayor resistencia que *S. cerevisiae.* En promedio, para alcanzar una completa inactivación de *E. coli* y *S. cerevisiae* se necesitó un tiempo de tratamiento de 6.6 y 3.3 min, respectivamente. En zumos, la vigorosa solubilización del CO_2 generada por los HPU podría estar dificultada por un mayor contenido de azúcar, por tanto los mecanismos de inactivación podrían estar gobernados principalmente por el fenómeno de cavitación y el tamaño de los microorganismos. El tamaño de las células de *S. cerevisiae* es mucho mayor que el de las de *E. coli,* por tanto, la probabilidad de que las burbujas de cavitación afecten a la estructura celular será mayor para *S. cerevisiae* que para *E. coli.*

Por otro lado, la inactivación de la enzima PME mediante SC-CO_2+HPU aumentó con la presión y la temperatura, aunque su inactivación completa no se alcanzó en ninguna de las condiciones estudiadas. La inactivación de enzimas tratadas mediante SC-CO_2 se debe a la bajada de pH, al efecto inhibitorio del CO_2 sobre la actividad enzimática y a los cambios estructurales generados por el SC-CO_2. La enzima PME mostró mayor resistencia a los tratamientos con SC-CO_2+HPU que los microorganismos *E. coli* o *S. cerevisiae* en zumo de naranja (se alcanzó una reducción del 18.9 %, 62.4 % y 88.1 %, a 36 ºC y 225 bar tras 2 min de tratamiento, respectivamente), lo que puede atribuirse a la diferente naturaleza y tamaño de los microorganismos y las enzimas.

El modelo de Peleg Tipo A (R^2 = 0.936; RMSE = 0.561) y el modelo de Weibull (R^2 = 0.923; RMSE = 0.561) se adaptaron para describir las cinéticas de inactivación de *E. coli* y *S. cerevisiae* con SC-CO_2+HPU en zumo de manzana, respectivamente, incluyendo la presión y la temperatura como parámetros de dichos modelos. El modelo Bifásico (R^2 = 0.960; RMSE = 0.391), el modelo de Peleg Tipo B (R^2 = 0.894; RMSE = 0.687) y el modelo fraccional (R^2 = 0.931; RMSE = 0.085), se adaptaron para describir las cinéticas de inactivación de *E. coli*, *S. cerevisiae* y PME con SC-CO_2+HPU en zumo de naranja, respectivamente, incluyendo como parámetros de dichos modelos la presión y la temperatura.

Los resultados revelaron que la actividad residual de las enzimas PME, POD y PPO descendió conforme aumentó la presión, ya que la presión genera un desorden estructural que puede cambiar la estructura tri-dimensional de las enzimas. Las muestras tratadas con HHPcarb+CO_2 mostraron un mayor grado de inactivación de las tres enzimas, comparado con las muestras tratadas con HHPcarb o HHP, en cualquier condición de presión seleccionada. Esto podría deberse a una mayor cantidad de CO_2 disuelto, que provocaría una mayor caída de pH y la consecuente desnaturalización de las enzimas. Además, el CO_2 disuelto en el puré durante el tratamiento de HHP, podría generar un repentino y significativo burbujeo durante la despresurización, que podría contribuir a generar mayores cambios estructurales responsables de la inactivación enzimática.

Finalmente, se puede concluir que la combinación de SC-CO_2 con HPU o HHP mejoró los mecanismos de inactivación de microorganismos y enzimas. La aplicación de HPU agiliza los tratamientos con SC-CO_2, acelerando la solubilización del CO_2 en el medio, que es el primer paso en los tratamientos con SC-CO_2; y generando el fenómeno de cavitación que daña las paredes celulares, facilitando tanto la penetración del SC-CO_2 a las células como la extracción de componentes intracelulares, lo que acelera la muerte de las células microbianas. Además, la combinación de SC-CO_2 con HHP aceleró la inactivación de enzimas en comparación con HHP. Empleando estas tecnologías combinadas, se pueden utilizar tiempos de proceso razonables para la industria alimentaria, así como condiciones de tratamiento suaves, lo que resultaría en una reducción del coste

del proceso y en una minimización del impacto sobre las propiedades nutricionales y organolépticas de los productos tratados.

Se recomienda llevar a cabo mas investigaciones para conocer detalladamente los mecanismos de inactivación de microorganismos y enzimas con SC-CO_2+HPU y SC-CO_2+HHP. Tambien sería interesante conocer el efecto de estas tecnologías no térmicas combinadas sobre las propiedades físico-químicas de los alimentos tratados y sobre la aceptación de los mismos por parte del consumidor.

RESUM

La indústria alimentària, en resposta a la demanda per part dels consumidors d'aliments naturals, frescos i lliures de conservants químics, ha desenrotllat tecnologies de conservació no tèrmiques. El CO_2 supercrític (SC-CO_2), representa una tecnologia no tèrmica d'inactivació prometedora, ja que està encaminada a produir el mínim impacte sobre les propietats nutricionals i organolèptiques dels aliments. No obstant, en alguns casos es requerixen condicions de pressió o temperatura elevades, així com tractaments excessivament llargs per a garantir la seguretat i estabilitat dels aliments. En este sentit, amb l'objectiu d'obtindre la letalidad requerida utilitzant processos més curts o de menor intensitat, en el present treball s'ha desenrotllat una combinació del SC-CO_2 amb ultrasons de potència (HPU) i de SC-CO_2 amb altes pressions hidrostàtiques (HHP), que ha sigut empleada per a processos d'inactivació microbiana i enzimàtica.

L'objetiu principal de la present Tesi va ser avaluar tecnologies no tèrmiques de conservació basades en la combinació de SC-CO_2 i HPU, i en la combinació de SC-CO_2 i HHP. Respecte a la combinació de SC-CO_2 amb HPU, es va estudiar la influència de l'estat de creixement de les cèl·lules, de les condicions del procés, de la naturalesa del midi i de l'ús o no de HPU, sobre les cinètiques d'inactivación de microorganismes (*Escherichia coli* (*E. coli*) i *Saccharomyces cerevisiae* (*S. cerevisiae*)) i enzims (pectin-metil-esterasa (PME)). Es van emprar models matemàtics i tècniques de microscòpia per a descriure les cinètiques i els mecanismes d'inactivación, respectivament. Respecte a la combinació de SC-CO_2 i HHP, es va determinar l'efecte de diferents nivells de CO_2 sobre l'eficàcia del tractament amb HHP per a inactivar PME, peroxidasa (POD) i polifenol oxidasa (PPO).

Es va estudiar la influència de l'estat de creixement de les cèl·lules de *E.coli* i *S. cerevisiae* inoculades en medi de cultiu, LB i YPD Broth, respectivament, sobre les seues cinètiques d'inactivació amb SC-CO_2. Cultius individuals de *E. coli* i *S. cerevisiae* es van incubar fins que les cèl·lules van aconseguir quatre

estats de creixement diferents, des de la fase primerenca exponencial fins a la fase estacionària, per a posteriorment ser tractades amb SC-CO_2 a 350 bar i 35 ºC. Es va comparar el procés combinat de SC-CO_2+HPU amb el tractament de SC-CO_2 per a avaluar l'efecte dels HPU sobre les cinètiques d'inactivació amb SC-CO_2 de *E. coli* i *S. cerevisiae* en la fase primerenca estacionària inoculades en medis de cultiu, i es va determinar l'efecte de diferents temperatures (31-41 ºC, 225 bar) i pressions (100-350 bar, 36 ºC). Amb l'objectiu de conéixer els mecanismes d'inactivació associats a esta tecnologia combinada (SC-CO_2+HPU) es va realitzar un estudi morfològic. Es van estudiar les diferències entre cèl·lules de *E. coli* i *S. cerevisiae* no tractades, tractades amb SC-CO_2 (350 bar, 36 ºC, 5 min) i amb SC-CO_2+HPU (350 bar, 36 ºC, 5 min, 40 W) emprant microscòpia òptica (LM) i microscòpia electrònica de transmissió (TEM). Es va seleccionar el suc de poma i de taronja per a estudiar la inactivació d'ambdós microorganismes en matrius reals; a més, es va estudiar la inactivació de l'enzim pectin-metil-esterasa (PME) del suc de taronja. Les experiències es van dur a terme a diferents temperatures (31-41 ºC, 225 bar) i pressions (100-350 bar, 36 ºC). Les condicions de temperatura i pressió seleccionades superen el punt crític del CO_2 i són menors que les condiones letals per a ambdós microorganismes. Tant *E. coli* com *S. cerevisiae* s'han seleccionat per al present treball perquè són components habituals de la flora responsable del deteriorament d'aliments i són comunament empleats com a indicadors de contaminació en aliments.

Es va investigar la combinació de SC-CO_2 amb HHP per a determinar l'efecte de diferents nivells de CO_2 (només HHP (HHP) ; carbonatació i HHP (HHPcarb) ; carbonatació + addició de 8.5 ml de CO_2/ g puré en l'espai de cap del paquet i HHP (HHPcarb+CO_2)) sobre l'eficàcia del tractament amb HHP per a inactivar PME, peroxidasa (POD) i polifenol oxidasa (PPO) en puré de feijoa contingut en una bossa de plàstic, a diferents pressions (300, 450 i 600 MPa, durant 5 min).

Els resultats van mostrar que la resistència d'ambdós microorganismes als tractaments d'inactivació amb SC-CO_2 va augmentar progressivament conforme la fase de creixement va avançar, la qual cosa podria deure's a l'activació de sistemes de protecció naturals que desenrotllen els microorganismes conforme s'acosten a la fase estacionària de creixement. Les cinètiques d'inactivació de

E. coli i *S. cerevisiae* es van ajustar al model de Weibull (R^2 = 0.93; RMSE = 0.59) i al model de Gompertz (R^2 = 0.96; RMSE = 0.53), respectivament, que van ser adaptats per a considerar la fase de creixement com un dels paràmetres dels citats models.

Emprant SC-CO_2, la velocitat d'inactivació d'ambdós microorganismes va augmentar progressivament amb la pressió i la temperatura. El temps necessari per a aconseguir una inactivació completa de *E. coli* (8 cicles-log) es va reduir de 60 a 25 min a l'augmentar la pressió de 100 a 350 bar (36 ºC), i de 75 a 40 min a l'augmentar la temperatura de 31 a 41 ºC (225 bar). La inactivació completa de *S. cerevisiae* (7 cicles-log) es va aconseguir únicament després de 140 min de procés a 350 bar i 36 ºC. En general, pressions i temperatures més elevades milloren la solubilització del SC-CO_2 en el medi i incrementen la fluïdesa de la membrana cel·lular, respectivament, facilitant el contacte i la penetració del CO_2, la qual cosa afavorix el descens del pH intracel·lular i l'extracció de components vitals per a la cèl·lula. No obstant això, a l'aplicar HPU en els tractaments de SC-CO_2 en medis de cultiu, es va observar una dràstica inactivació microbiana, aconseguint-se una reducció total (10^7-10^8 cicles-log) després de només 1-2 min de tractament. Aplicant SC-CO_2+HPU no es va observar un efecte significatiu en el nivell d'inactivació al augmentar la pressió o la temperatura pel fet que els HPU generen una vigorosa agitació que accelera els mecanismes d'inactivació associats als SC-CO_2 i emmascara l'efecte d'estes variables del procés. A més, la cavitació generada pels HPU podria danyar la paret cel·lular dels microorganismes, accelerant la seua inactivació. L'estudi de l'existència d'un possible efecte sinèrgic entre ambdós tecnologies va revelar que la combinació de SC-CO_2 i HPU va tindre un major efecte en la inactivació que l'addició dels efectes individuals d'ambdós. Per a *E. coli*, es va aconseguir una reducció de 0.3, 0.9 i 8 cicles-log després de 5 min de tractament amb SC-CO_2, HPU i SC-CO_2+HPU, respectivament; per a *S. cerevisiae* es va aconseguir una reducció de 6.83 cicles-log després de 2 min de tractament amb SC-CO_2+HPU, mentres que després del mateix període de temps amb només SC-CO_2 o HPU no es va observar cap reducció en el nombre de microorganismes.

En tots els tractaments duts a terme, el rent *S. cerevisiae* va mostrar major resistència als tractaments amb SC-CO_2 que el bacteri *E. coli*, la qual cosa podria

estar relacionat amb la major grossària de la paret cel·lular de *S. cerevisiae* comparat amb la de *E. coli*, 124.8 nm front a 17.7 nm, respectivament. No obstant això, al combinar el SC-CO_2 i els HPU, l'agitació vigorosa i la cavitació del medi va emmascarar les diferents resistències mostrades per ambdós microorganismes en els tractaments amb SC-CO_2.

Les imatges de LM i TEM van mostrar que després de 5 min de tractament amb SC-CO_2 es va produir una distribució irregular del contingut citoplasmátic i van aparéixer lleugeres modificacions en l'embolcall cel·lular, no sent cap d'estos canvis letals per a les cèl·lules de *E. coli* ni de *S. cerevisiae*. A més, les majors diferències entre ambdós microorganismes es van identificar en l'efecte sobre l'embolcall cel·lular: en *S. cerevisiae* es van observar lleugeres modificacions encara que no es va apreciar ruptura de la paret cel·lular, mentres que la paret de les cèl·lules de *E. coli* van aparéixer amb un alt grau de dissolució, pèrdua de cohesividad, protuberàncies i algunes àrees desintegrades. No obstant això, 5 min de tractament amb SC-CO_2+HPU van ser suficients per a aconseguir una inactivació completa d'ambdós microorganismes. Les imatges de LM i TEM van revelar major proporció de regions buides dins de les cèl·lules tractades amb SC-CO_2+HPU, la qual cosa va indicar una clara reducció del contingut citoplasmátic. L'embolcall de les cèl·lules de *E. coli* es va desintegrar totalment, mentres que les parets de les cèl·lules de *S. cerevisiae* van perdre parcialment la seua estructura laminada i es van poder observar algunes parets trencades. Per tant, els mecanismes d'inactivació associats als SC-CO_2+HPU podrien estar relacionats amb el fenomen de cavitació generat pels HPU, el qual danya bruscament l'embolcall cel·lular incrementant tant la ruptura de la membrana cel·lular com la desintegració del contingut intracel·lular. Els danys generats pel tractament de SC-CO_2+HPU van ser tan severs que van evitar una possible recuperació de les cèl·lules durant un emmagatzemament posterior al tractament (6 setmanes a 4 ºC).

Com a mitjana, la inactivació d'ambdós microorganismes amb SC-CO_2+HPU en suc de poma (5.3 min) va ser més lenta que en suc de taronja (4.6 min); i en ambdós sucs més lenta que en medis de cultiu (1.5 min). Açò podria estar relacionat amb el contingut de sucre del medi i la solubilització del CO_2 en el mateix. El sucre es lliga a l'aigua del medi, per tant, la quantitat d'aigua

disponible on el CO_2 pot dissoldre's és menor en suc de poma (15.6 ºBrix) que en suc de taronja (11.6 ºBrix); i menor en ambdós sucs que en LB (2 ºBrix) o YPD (5 ºBrix) Broth. A més, emprant SC-CO_2+HPU, la velocitat d'inactivació d'ambdós microorganismes inoculats en sucs va augmentar amb la pressió i la temperatura. Açò podria estar relacionat amb la composició dels sucs, els quals no se saturen ràpidament de CO_2 en els tractaments amb SC-CO_2+HPU com sí que va ocórrer en les experiències dutes a terme sobre medis de cultiu, de manera que un increment de pressió o temperatura pot facilitar la solubilització del CO_2.

Contràriament als resultats obtinguts amb SC-CO_2+HPU sobre medis de cultiu, on no es van observar diferències entre *E. coli* i *S. cerevisiae*, en sucs *E. coli* va mostrar major resistència que *S. cerevisiae*. Com a mitjana, per a aconseguir una completa inactivació de *E. coli* i *S. cerevisiae* es va necessitar un temps de tractament de 6.6 i 3.3 min, respectivament. En sucs, la vigorosa solubilització del CO_2 generada pels HPU podria estar dificultada per un major contingut de sucre, per tant els mecanismes d'inactivació podrien estar governats principalment pel fenomen de cavitació i la grandària dels microorganismes. La grandària de les cèl·lules de *S. cerevisiae* és molt major que el de les de *E. coli*, per tant, la probabilitat que les bambolles de cavitació afecten l'estructura cel·lular serà major per a *S. cerevisiae* que per a *E. coli*.

D'altra banda, la inactivació de l'enzim PME per mitjà de SC-CO_2+HPU va augmentar amb la pressió i la temperatura, encara que la seua inactivació completa no es va aconseguir en cap de les condicions estudiades. La inactivació d'enzims tractades per mitjà de SC-CO_2 es deu a la baixada de pH, a l'efecte inhibitori del CO_2 sobre l'activitat enzimàtica i als canvis estructurals generats pel SC-CO_2. L'enzim PME va mostrar major resistència als tractaments amb SC-CO_2+HPU que els microorganismes *E. coli* o *S. cerevisiae* en suc de taronja (es va aconseguir una reducció del 18.9 %, 62.4 % i 88.1 %, a 36 ºC i 225 bar després de 2 min de tractament, respectivament), la qual cosa pot atribuir-se a la diferent naturalesa i grandària dels microorganismes i els enzims.

El model de Peleg Tipus A (R^2 = 0.936; RMSE = 0.561) i el model de Weibull (R^2 = 0.923; RMSE = 0.561) es van adaptar per a descriure les cinètiques d'inactivació de *E. coli* i *S. cerevisiae* amb SC-CO_2+HPU en suc de

poma, respectivament, incloent la pressió i la temperatura com a paràmetres dels models. El model Bifàsic ($R^2 = 0.960$; RMSE = 0.391), el model de Peleg Tipus B ($R^2 = 0.894$; RMSE = 0.687) i el model fraccional ($R^2 = 0.931$; RMSE = 0.085), es van adaptar per a descriure les cinètiques d'inactivació de *E. coli*, *S. cerevisiae* i PME amb SC-CO_2+HPU en suc de taronja, respectivament, incloent com a paràmetres dels models la pressió i la temperatura.

Els resultats van revelar que l'activitat residual dels enzims PME, PPO i POD va descendir conforme va augmentar la pressió, ja que la pressió genera un desordre estructural que pot canviar l'estructura tridimensional dels enzims. Les mostres tractades amb HHPcarb+CO_2 van mostrar un major grau d'inactivació dels tres enzims, comparat amb les mostres tractades amb HHPcarb o HHP, en qualsevol condició de pressió seleccionada. Açò podria deure's a una major quantitat de CO_2 dissolt, que provocaria una major caiguda de pH i la conseqüent desnaturalització dels enzims. A més, el CO_2 dissolt en el puré durant el tractament de HHP, podria generar un sobtat i significatiu bambolleig durant la despressurització, que podria contribuir a generar majors canvis estructurals responsables de la inactivació enzimàtica.

Finalment, es pot concloure que la combinació de SC-CO_2 amb HPU o HHP va millorar els mecanismes d'inactivació de microorganismos i enzims. L’aplicació de HPU agilitza els tractaments amb SC-CO_2, accelerant la solubilització del CO_2 en el medi, que és el primer pas en els tractaments amb SC-CO_2; i generant el fenomen de cavitació que danya les parets cel·lulars, facilitant tant la penetració del SC-CO_2 a les cèl·lules com l'extracció de components intracel·lulars, la qual cosa accelera la mort de les cèl·lules microbianes. A més, la combinació de SC-CO_2 amb HHP va mostrar una millora en la inactivación d'enzims en comparació amb HHP. Emprant estes tecnologies combinades, es poden utilitzar temps de procés raonables per a la indústria alimentària, així com condicions de tractament suaus, la qual cosa resultaria en una reducció del cost del procés i en una minimització de l'impacte sobre les propietats nutricionals i organolèptiques dels productes tractats.

Es recomana dur a terme mes investigacions per a conéixer detalladament els mecanismes d'inactivación de microorganismes i enzims amb SC-CO_2+HPU i SC-CO_2+HHP. També seria interessant conéixer l'efecte d'estes tecnologies

combinades no tèrmiques sobre les propietats fisicoquímiques dels aliments tractades i sobre l'acceptació dels mateixos per part del consumidor.

Introducción

1. INTRODUCCIÓN

1.1. La industria de alimentos

Los alimentos están constituidos por tejidos animales o vegetales formados por agua, hidratos de carbono, proteínas, grasas, sales minerales, vitaminas, enzimas, ácidos orgánicos, pigmentos y aromas. La disposición de los distintos componentes y las fases del alimento, sus proporciones relativas y la naturaleza y características específicas de cada grupo de componentes, determinan las propiedades organolépticas, físicas y nutritivas de los alimentos. Así mismo, los alimentos son productos biológicos complejos que contienen todas las sustancias químicas necesarias para mantener la vida y están sujetos a gran variedad de agentes modificantes siendo susceptibles de cambios o degradaciones.

Las industrias alimentarias abarcan multitud de actividades que pueden englobarse en tres tipos de operaciones principales realizadas sobre los alimentos:

- Extracción de la parte comestible de semillas, frutos, tubérculos y otras materias primas como azúcar, arroz, aceites, etc., conocidas como *industrias extractivas*.
- Transformación de las materias primas originales en productos de características nutritivas y sensoriales diferentes, como es el caso del pan, vino, queso, etc., llamadas *industrias fermentativas*.
- Conservación de los alimentos para facilitar su transporte y almacenamiento como en los congelados, enlatados, etc., denominadas *industrias de conservación*.

En general, la industria alimentaria utiliza materias primas que son sometidas a una serie de tratamientos físicos, químicos o microbiológicos, para dar lugar a productos más estables y adecuados para la alimentación.

1.2. Conservación de alimentos

Desde la antigüedad ha existido una necesidad de conservar los alimentos. El hombre primitivo, en un momento de su evolución observó diferentes fenómenos

que le ocurrían a los alimentos como por ejemplo que la carne y el pescado se resecaban y descomponían, el enranciamiento y pardeamiento de frutas y verduras, la leche se agriaba, etc.

El aumento de la población obligó a utilizar la ganadería y la agricultura como sostén de las sociedades, y surgió la necesidad de almacenar grandes cantidades de alimentos para los tiempos de escasez. Los antropólogos coinciden en que los primeros procesos de conservación de alimentos que dominó el hombre fueron el secado al sol y el salado (Lund, 2002). Algunas aplicaciones fueron el salado de jamón, ahumado de carne, el uso de vinagre para conservar alimentos, secado al sol de ciruelas e higos, etc. (Aleixandre, 1994).

1.2.1. Causas de la alteración de alimentos

Las causas de la alteración de alimentos pueden ser:

- Intrínsecas: producidas por enzimas endógenos naturales. Las principales alteraciones enzimáticas son: pardeamiento enzimático, alteración de las pectinas, alteración por amilasas, destrucción de tiamina (vitamina B_1), destrucción de la vitamina C o degradación de pigmentos, entre otros.
- Extrínsecas: producidas por diferentes fenómenos como: reacciones químicas, ***alteraciones microbiológicas***, lesiones mecánicas (que ponen en contacto las enzimas con sus sustratos, produciendo pardeamientos), insectos, etc.

Las alteraciones microbiológicas tienen una importancia particular ya que representan para una gran parte de los productos alimentarios la alteración más frecuente, y por otro lado, hay numerosos microorganismos que no solo degradan los alimentos, sino que los hacen nocivos para la salud del consumidor.

Los avances en la microbiología de alimentos no se produjeron hasta finales del siglo XIX (1876) a partir de Pasteur, que descubrió que los microorganismos son uno de los principales responsables de la fermentación y descomposición de los alimentos (Mossel y col., 2003).

Los microorganismos utilizan los alimentos como fuente de nutrientes para su propio crecimiento, hecho que puede ocasionar su alteración. Los

microorganismos pueden deteriorar un alimento porque se multiplican en él, porque utilizan sus nutrientes, porque producen modificaciones enzimáticas, y porque comunican sabores desagradables mediante el desdoblamiento de determinadas sustancias o mediante la síntesis de nuevos compuestos. La alteración de los alimentos es consecuencia lógica de la actividad de los microorganismos, ya que, en la naturaleza, una de sus funciones es la reconversión de las formas reducidas de carbono, de nitrógeno y de azufre existentes en las plantas o animales, a otras formas oxidadas que necesitan las plantas, las cuales, a su vez, son consumidas por los animales. Por lo tanto, la naturaleza, tiende a convertir en no aptos para el consumo a los alimentos (Frazier y Westhoff, 1993).

Actualmente, el control microbiológico en la producción de alimentos tiene como finalidad última suministrar productos seguros o inocuos, nutritivos y sabrosos, con una vida comercial adecuada y a un coste razonable para el consumidor (Mossel y col., 2003).

1.2.2. Factores que afectan a la velocidad de degradación

Los alimentos frescos o naturales suelen contener una población microbiana mixta. La velocidad de crecimiento de cada organismo depende de muchos factores, lo que influencia de modo notable el carácter de la población que finalmente predominará en el alimento. Estos factores pueden clasificarse en cuatro grupos principales (Mossel y col., 2003):

- Factores intrínsecos: aquellos que dependen de las características del sustrato de crecimiento, es decir, del propio alimento. Incluyen parámetros químicos, tales como la cantidad de agua disponible, los tipos y niveles de nutrientes disponibles, el pH y la capacidad tampón de los cambios de pH, el potencial redox, y las sustancias antimicrobianas naturales.

- Factores de procesado o tratamiento: aquellos que son consecuencia de los tratamientos aplicados durante la elaboración de los alimentos, tanto a escala industrial como a escala doméstica, que modifican primariamente la composición de la microflora.

- Factores extrínsecos: los impuestos desde el exterior, por ejemplo, la temperatura de almacenamiento, la humedad de la atmósfera y la composición de la fase gaseosa.
- Factores implícitos: estos efectos dependen de la microflora particular dominante que inicialmente se desarrolla en respuesta a los factores intrínsecos, de tratamiento y extrínsecos. Algunos ejemplos pueden ser: que un organismo elimine una sustancia que es inhibidora para otro; que un organismo produzca factores de crecimiento requeridos por otro, etc.

La estabilidad microbiana de los alimentos, lo mismo que su seguridad microbiológica, dependen de la combinación de los efectos ejercidos por los cuatro grupos de factores mencionados. Por ello, estos factores se utilizan a menudo de modo voluntario e intencionado para conservarlos.

1.2.2.1. Factores limitantes

Cuando se tienen en cuenta los efectos de cada uno de los factores que influyen en el crecimiento microbiano, se comprueba que para cada microorganismo, bajo una serie de condiciones de crecimiento, existe un valor máximo y un valor mínimo correspondientes a cada parámetro: medio, temperatura, pH, actividad de agua, etc., que influyen en el crecimiento microbiano. A un factor que ejerce un efecto adverso directo en la velocidad de crecimiento se le conoce con la denominación de factor limitante (Mossel y col., 2003). El conocimiento del efecto de los factores limitantes es empleado en la conservación de los alimentos, siendo los más conocidos y empleados en la conservación de los mismos los siguientes:

- Temperatura: es el factor más importante debido a su gran influencia en todo tipo de reacciones, por ello, gran variedad de procesos incluyen la refrigeración y la congelación de alimentos. Mediante la reducción de la temperatura es posible reducir notablemente la velocidad de deterioro de los alimentos.
- pH: influye en la velocidad de muchas reacciones químicas y enzimáticas. La mayoría de microorganismos crecen entre unos límites de valores del pH y el efecto microbiostático que puede alcanzarse por efecto del pH

depende en gran medida del tipo de microorganismo (Mossel y col., 2003). Son necesarios valores extremos de pH para inhibir el desarrollo microbiano, sin embargo, pequeñas variaciones de pH son capaces de producir drásticas alteraciones en la calidad de algunos alimentos, como por ejemplo la carne.

- Actividad de agua: este parámetro se utiliza como medida de la cantidad de agua disponible en un alimento para el crecimiento microbiano y otras reacciones. Es una de las variables más importantes en el control de la velocidad de las reacciones que se desarrollan en los alimentos, como es el caso de las reacciones enzimáticas o la oxidación de lípidos, entre otras. La adición de solutos o la eliminación física de agua (desecación) reduce la actividad de agua de los alimentos, y por consiguiente su disponibilidad para el crecimiento microbiano.

La aplicación de diferentes metodologías para estabilizar alimentos, aplicadas en paralelo o secuencialmente, ha avanzado utilizando el concepto de "tecnología de barreras". Este concepto está basado en el control de uno o varios de los factores limitantes, que dificultan el crecimiento microbiano y el consiguiente deterioro de alimentos. Cada uno de estos factores actúa de modo diferente, y la combinación entre ellos ha permitido la conservación de alimentos como el requesón o jamón (Leistner y Gorris, 1995). A pesar de ello, las interacciones entre ciertos factores, así como la elevada resistencia de algunos microorganismos, hace necesaria la aplicación de tecnologías más severas para asegurar la estabilidad microbiológica de los alimentos. La más empleada tradicionalmente ha sido el tratamiento térmico.

1.2.3. Tratamientos térmicos de conservación

Los tratamientos térmicos son uno de los métodos más utilizados en la conservación de alimentos y uno de los más empleados en algunos tipos de industrias alimentarias, como por ejemplo en la de conservación de zumos de frutas. El calor permite eliminar numerosos microorganismos e inactivar las enzimas que podrían alterar el producto y hacerlo no apto para el consumo.

La intensidad del tratamiento térmico necesaria para destruir los microorganismos o sus esporas depende de la especie de microorganismo, de su estado fisiológico, y de las condiciones del medio en el momento de efectuar el tratamiento. Según el tratamiento térmico que se emplee, es posible que se destruyan solo algunas células vegetativas, la mayoría de las células o todas las células, parte de las esporas bacterianas o la totalidad de las mismas. El tratamiento térmico elegido dependerá de las especies de microorganismos que sea preciso destruir, de otros procedimientos de conservación que sea preciso emplear, y del efecto que produzca el calor en el alimento.

Los principales métodos térmicos para la conservación de los alimentos son: la pasteurización y la esterilización.

La pasteurización es una operación de estabilización de alimentos que persigue la reducción de la población de microorganismos presentes en éstos, de forma que se prolongue el tiempo de vida útil del alimento (Silva y Gibbs, 2012). Consiste en un tratamiento térmico relativamente suave (temperaturas inferiores a 100 ºC), que puede variar entre un calentamiento durante 30 min a unos 63 ºC, en productos ya envasados (por lotes); o durante 15 segundos a 72 ºC en tratamientos continuos (Mossel y col., 2003). Al ser un tratamiento térmico suave los cambios organolépticos y nutricionales del alimento son poco importantes.

La esterilización es el proceso mediante el cual se alcanza la muerte de todas las formas de vida microbianas, incluyendo bacterias y sus formas esporuladas altamente resistentes, levaduras, hongos y virus (Singh y col., 1997). Se entiende por muerte, la pérdida irreversible de la capacidad reproductiva del microorganismo. Se puede llevar a cabo por calor húmedo (121 ºC-134 ºC, 3-15 min) o por calor seco (121 ºC-180 ºC, 30-360 min). Actualmente, en el caso de alimentos líquidos, este tipo de tratamiento apenas se utiliza y ha sido reemplazado por el proceso de UHT o uperización. En este tipo de tratamiento se alcanzan temperaturas elevadas de hasta 150 ºC, aunque durante periodos muy cortos de tiempo, menos de 5 segundos, seguido de un rápido enfriamiento. Además de alargar la vida útil del producto y garantizar su seguridad, el proceso UHT afecta menos a la calidad sensorial y nutricional.

Sin embargo, a pesar de que los tratamientos térmicos han sido los procesos de conservación más empleados hasta el momento, el uso del calor presenta algunas desventajas asociadas a los cambios que se producen en el producto y que afectan a la calidad y valor nutritivo del mismo, como son la destrucción de vitaminas, la desnaturalización de proteínas, la caramelización de azúcares, la gelificación de almidones, la destrucción de pigmentos, etc. (Li y col., 2012). En cuanto a los cambios organolépticos podemos citar cambios texturales (pérdida de agua, endurecimiento, reblandecimiento), de sabor (rancidez, sabor a cocido o sabores extraños), de color (oscurecimiento, blanqueamiento, desarrollo de colores extraños) o de olor (olor a cocido, olor a rancio, olores extraños).

Estos aspectos negativos asociados a los tratamientos térmicos, unidos al hecho de que el consumidor demanda alimentos cada vez más frescos y naturales, menos procesados pero de rápida preparación y que, además de tener una vida útil prolongada mantengan sus cualidades nutricionales y sensoriales, ha llevado a los investigadores y a las empresas de la industria alimentaria a desarrollar otras tecnologías no térmicas de conservación.

1.3. Conservación de alimentos mediante tecnologías no térmicas

Como tecnologías no térmicas de conservación podemos citar desde las más antiguas como el salado o el secado al sol, hasta las desarrolladas en los últimos años como la irradiación, altas presiones hidrostáticas (HHP), campos de pulsos eléctricos (PEF) o magnéticos, fluidos supercríticos (FSC) o agentes químicos y bioquímicos (Rektor y col., 2004; Monfort y col., 2010; Clariana y col., 2011; Liu y col., 2012).

Tanto la irradiación como las HHP son tecnologías que han recibido especial atención para su aplicación en productos conocidos como "listos para el consumo", los cuales suelen estar envasados en pequeñas porciones donde el tratamiento es aplicado tras el proceso de envasado.

La irradiación ha mostrado ser efectiva en la eliminación de patógenos como *Listeria monocytogenes* (*L. monocytogenes*), *Salmonella* spp., *Yersinia enterocolitica*, *Escherichia coli* O157:H7 (*E. coli*), entre otros (Cabeza y col., 2009; Schilling y col., 2009; Burgess y col., 2010). Cuando un alimento es

irradiado, los átomos/moléculas que lo conforman expulsan electrones y radicales libres. Éstos pueden dañar el ADN de los microorganismos y causar defectos en sus secuencias genéticas que desembocan en su inactivación (Brewer, 2009). La principal limitación de dicha tecnología son los cambios en el aroma, color y olor de los productos irradiados (Brewer, 2009; Rababah y col., 2010), que podrían afectar negativamente a la aceptación de los mismos por parte del consumidor.

La tecnología de HHP ha demostrado una gran efectividad en la eliminación de parásitos, levaduras y mohos, y un amplio rango de bacterias (Renduales y col., 2011). Su aplicación como tecnología en la conservación de alimentos ha recibido especial atención como alternativa, tanto económica como tecnológica, a los procesos térmicos, y está siendo aplicada en alimentos ya comercializados. Una de las principales ventajas de las HHP es la homogeneidad del tratamiento en todo el alimento, que permite la extensión de la vida útil del mismo gracias a la eficaz inactivación de los microorganismos. Dicha inactivación se debe a la modificación de la estructura celular de los mismos por el efecto de la presión, que les hace perder su viabilidad (Renduales y col., 2011), de ahí que las HHP presenten mayor efectividad en microorganismos con un elevado grado de organización celular. Su principal desventaja reside en los elevados costes del equipamiento empleado.

Los PEF han demostrado ser eficaces en la inactivación de microorganismos como la *Salmonella serovars*, *E. coli*, *Bacillus cereus*, *Staphylococcus aureus* (*S. aureus*) y la *Listeria innocua* (*L. innocua*) (Monfort y col., 2010). Los mecanismos asociados a los PEF no están claramente identificados, aunque la mayoría de estudios han aceptado que están relacionados con la permeabilización y rotura de las membranas celulares que desemboca en la desintegración celular (Monfort y col., 2010). El principal inconveniente de los PEF reside en las altas temperaturas requeridas para lograr inactivar ciertos microorganismos, que podrían dañar las propiedades sensoriales de los alimentos (Álvarez y col., 2006).

El uso de los FSC como una alternativa no térmica a la pasteurización de alimentos, fue estudiado por primera vez por Fraser (1951) y Foster y col. (1962). Aunque a partir de esos trabajos se realizaron diversos estudios en este

campo, no fue hasta el estudio llevado a cabo por Kamihira y col. (1987) cuando se consideró seriamente la aplicación de los FSC como una tecnología válida para procesos de inactivación. Desde entonces, se ha publicado un gran número de trabajos en los que se ha investigado el efecto inhibitorio del CO_2 supercrítico sobre diversos microorganismos como virus, bacterias, protozoos, levaduras, hongos y algas, tanto presentes en la flora natural de los alimentos como inoculados para su posterior inactivación (Corwin y Shellhammer, 2002; Gasperi y col., 2009; Fabroni y col., 2010; Liu y col., 2012).

Todos estos estudios han demostrado que la inactivación mediante FSC puede considerarse como una tecnología de pasteurización fría, que inactiva microorganismos y enzimas sin exposición de los alimentos a los efectos adversos asociados a las altas temperaturas de los tratamiento térmicos, y manteniendo sus propiedades físicas, nutritivas y sensoriales (Damar y Balaban, 2006).

1.4. Introducción a los fluidos supercríticos

1.4.1. Definición de fluido supercrítico. CO_2 supercrítico

La Fig. 1.1 muestra un diagrama de fases presión-temperatura para un sistema de un componente, en particular del CO_2. A una temperatura y presión por encima de la temperatura y presión críticas, una sustancia se convierte en un fluido supercrítico (Velasco y col., 2007). Por encima de la temperatura crítica, el gas ya no puede ser licuado independientemente del aumento de la presión. Si se observa un componente al alcanzar el punto crítico, el menisco de la interfase líquido-gas desaparece lo que significa que ya no hay distinción entre las dos fases.

El CO_2 es el fluido supercrítico más utilizado debido a que no es tóxico, ni inflamable, ni corrosivo, es incoloro, de bajo coste, se elimina fácilmente, no deja residuos, sus condiciones críticas son relativamente fáciles de alcanzar y se consigue con diferentes grados de pureza. El CO_2 alcanza el estado supercrítico por encima de 31 ºC y 72.8 atm (Velasco y col., 2007).

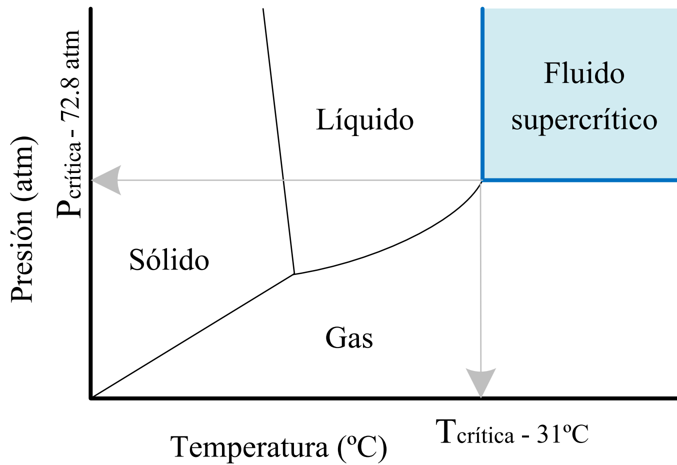


Fig. 1.1. Diagrama de fases presión-temperatura para el CO_2.

Los FSC, y en concreto el CO_2 supercrítico (SC-CO_2) tiene buena capacidad de disolución al presentar una densidad similar a la de los líquidos, pero también exhibe buenas propiedades de transporte al tener una viscosidad y capacidad de penetración parecidas a las de un gas (García-González y col., 2007). Además, la densidad de un fluido supercrítico aumenta al aumentar la presión y, por lo tanto al aumentar la densidad, la capacidad de disolución sobre una matriz líquida o sólida, aumenta de manera notable. Por lo tanto, los FSC y en particular el SC-CO_2 tienen propiedades de transporte muy favorables que los hacen adecuados para procesos de extracción, proporcionando numerosas ventajas tanto tecnológicas como medioambientales.

En la literatura es habitual encontrar el término "dióxido de carbono en fase densa (DPCD). DPCD incluye el estado supercrítico del CO_2, pero además abarca el CO_2 en estado subcrítico, es decir, en estado gas o líquido pero sometido a presiones y temperaturas muy cercanas al estado supercrítico.

1.4.2. Aplicaciones generales de los FSC

Las principales aplicaciones industriales de los FSC en el sector agroalimentario han sido la extracción y el fraccionamiento.

La extracción mediante fluidos supercríticos utiliza las propiedades de los gases por encima de su punto crítico para extraer componentes solubles de

interés de una materia prima. El CO_2 es el solvente ideal para la extracción de componentes naturales ya que no es tóxico, no es explosivo y se elimina fácilmente de los productos. Además, las bajas temperaturas empleadas evitan el deterioro de componentes termolábiles en el extracto (Riera y col., 2010).

La extracción supercrítica seguida por un control riguroso de la densidad del CO_2 hace posible el fraccionamiento de los compuestos extraídos mediante una despresurización en distintas etapas. De este modo, es posible precipitar compuestos de elevada pureza de forma selectiva (Revenchon y De Marco, 2006). En este sentido, Señoráns y col. (2001) aislaron componentes antioxidantes de extractos de naranja obtenidos a contracorriente en un proceso de extracción supercrítica. Por otro lado, Simó y col. (2002) estudiaron la composición en compuestos antioxidantes de extractos de naranja mediante fraccionamiento supercrítico.

En la actualidad, además de para operaciones de extracción y fraccionamiento, los FSC se emplean para un gran número de operaciones debido a su inocuidad y a la creciente presión legislativa relacionada con el empleo de agentes químicos nocivos y de generación de residuos, así como las demandas de los consumidores de productos más naturales y saludables. En este sentido, junto con las aplicaciones implantadas actualmente en el campo alimentario, como la extracción y el fraccionamiento, entre las principales aplicaciones de los FSC pueden citarse las siguientes:

- reducción de actividad biológica: desinfección, desinsectación, inactivación enzimática (Tsuji y col., 2005; Xu y col., 2011).
- procesos de purificación (Sesti y col., 2004)
- eliminación de disolventes (Poletto y col., 2000)
- generación y diseño de partículas en procesos de cristalización, micronización y micro-encapsulación (Aro y col., 2013)
- tratamiento de materiales: modificación de las propiedades de nano-arcillas y polímeros, impregnación de matrices, etc. (Yang y col., 2013).

1.5. Inactivación de microorganismos y enzimas mediante FSC

En los últimos años se han publicado numerosos trabajos relacionados con el uso de los FSC para inactivar bacterias, virus, levaduras, etc. (Perrut, 2012). Este uso se ha extendido gracias a la eficacia mostrada en la reducción de microorganismos en matrices líquidas, como zumos de frutas o medios de cultivo (García-González y col., 2009; Oulé y col., 2006).

1.5.1. Gases usados en procesos de inactivación

Aunque el CO_2 ha sido el compuesto más empleado en los procesos de inactivación en condiciones supercríticas, hay investigadores que han ensayado la capacidad de inactivación de otros gases. Entre éstos se encuentran el nitrógeno (N_2), óxido nitroso (N_2O), etileno, argón (Ar) y tetrafluoretano (TFE). El N_2, Ar y TFE tienen un poder de inactivación significativamente menor, incluso a mayores presiones, que el N_2O y el CO_2 (Fraser, 1951; Enomoto y col., 1997). En este sentido, Wei y col. (1991) inactivaron totalmente una población de *Salmonella typhimurium* (*S. typhimurium*) en yema de huevo y de *L. monocytogenes* en yema de huevo y en zumo de naranja, con CO_2 a 35 ºC y 136 bar, tras 2 h de tratamiento, mientras que el N_2 en las mimas condiciones no mostró ningún efecto.

La temperatura y presión crítica del N_2O (T_C = 36.42 ºC, P_C = 71.5 atm) son similares a las del CO_2, y es también efectivo en procesos de inactivación gracias a su pequeño momento dipolar y a su elevada solubilidad en agua. En el caso del N_2 (T_C = -147.0 ºC, P_C = 33.4 atm) o el Ar (T_C = -122.3 ºC, P_C = 48.4 atm), a pesar de que sus puntos críticos son fáciles de alcanzar, su baja solubilidad en agua (a 1 atm y 25ºC, 0.018 y 0.043 g/L, respectivamente), hace que no sean efectivos en procesos de inactivación, donde una elevada solubilidad de los gases y el contacto entre éstos y los microorganismos es fundamental. El TFE tiene un punto crítico (T_C = 54.8 ºC, P_C = 40.1 atm) más parecido al del CO_2, comparado con el punto crítico del N_2 o el Ar, pero tiene diferentes propiedades químicas, como su momento dipolar (0 y 2.1 para el CO_2 y el TFE, respectivamente) (Erkmen, 2012). En general, el CO_2 es el compuesto elegido mayoritariamente para procesos de inactivación supercríticos debido a que no es tóxico ni inflamable, no requiere de ninguna ventilación especial, es químicamente inerte

(no reacciona con polímeros) y presenta una tensión superficial muy baja, entre otras razones (Erkmen, 2012).

1.5.2. Inactivación de bacterias, levaduras, hongos y virus

Las bacterias se clasifican en Gram-positivas y Gram-negativas en función del color que presentan por la tinción de Gram. Esta característica está íntimamente ligada a la estructura de la envoltura celular. La envoltura celular de las bacterias Gram-positivas está constituida por una pared celular compuesta por una gruesa capa de peptidoglicano (90 % de la pared celular), responsable de retener el tinte durante la tinción de Gram. Esta capa rodea la membrana citoplasmática y ambas están unidas mediante moléculas de ácido lipoteicoico. Las bacterias Gram-negativas presentan dos membranas lipídicas entre las que se localiza una fina pared celular de peptidoglicano (10 % de la pared celular). En éstas, al ser la pared más fina, no se retiene el colorante durante la tinción del Gram. Por tanto, la robustez de la pared de las bacterias Gram-positivas confiere mayor resistencia y menor permeabilidad a éstas, comparada con la fina capa de las bacterias Gram-negativas que serán más sensibles a los agentes externos (Erkmen, 2012). Además, se ha demostrado que algunas levaduras como *Saccharomyces cerevisiae* (*S. cerevisiae*) presentan una membrana celular con un espesor similar al de las bacterias Gram-positivas (Villas-Boas y col., 2006), presentando por tanto niveles de resistencia a agentes externos similares a los de éstas.

Múltiples estudios han demostrado la eficacia de los FSC para inactivar bacterias. Dillow y col. (1999) estudiaron el efecto de los FSC (205 bar, 34 ºC, 30 min) sobre dos bacterias Gram-positivas (*L. innocua* y *S. aureus*) y sobre cinco bacterias Gram-negativas (*Salmonella salford*, *Pseudomonas aeruginosa* (*P. aeruginosa*), *E. coli*, *Proteus vulgaris* y *Legionella dunnifii*). En general, los resultados mostraron una mayor resistencia por parte de las bacterias Gram-positivas, comparadas con las Gram-negativas, en todas las condiciones estudiadas. Oulé y col. (2006) redujeron la población de *E. coli* en 6 ciclos log con FSC (250 bar, 40 ºC, 30 min). Soares y col. (2013) estudiaron la inactivación de *L. monocytogenes* inoculada en medio de cultivo mediante FSC. Partiendo de una población de 10^{10} cfu/mL, estos autores lograron reducir la población a 10^{2} cfu/mL tras 120 min de tratamiento (200 bar, 33 ºC).

Por otro lado, el efecto antimicrobiano de los FSC representa una tecnología alternativa y prometedora para la pasteurización de alimentos por la inactivación de hongos y levaduras a temperaturas y presiones moderadas. Spilimbergo y col. (2007) estudiaron la inactivación mediante FSC de *S. cerevisiae* inoculada en zumo de manzana a 100 bar, alcanzando una reducción de 4.6 ciclos log tras 50 min de tratamiento a 36 ºC. Valverde y col. (2010) evaluaron la inactivación de *S. cerevisiae* en pera a distintas presiones, temperaturas y tiempos. En particular, fue necesario un tratamiento de 10 min a 100 bar y 55 ºC para reducir totalmente la población de *S. cerevisiae*.

En cuanto a la inactivación de virus, Chen y col. (2006) investigaron la inactivación de varios tipos de virus (TGEV, PRV, JEV, PRRSV) en biomateriales por contacto con CO_2 a 160 bar y 40-50 ºC durante 45 minutos, demostrando que el CO_2 supercrítico puede inactivarlos de forma efectiva, estando éstos impregnados en un biomaterial sensible al calor sin un descenso significativo en la bioactividad de dicho material.

El grado de inactivación microbiana alcanzado por la aplicación de FSC varía ampliamente entre los tratamientos. Gran parte de esta variación se debe a las diferencias en los parámetros del proceso tales como presión, temperatura, tiempo de tratamiento o medio de tratamiento, entre otros, y principalmente a la naturaleza del microorganismo.

A continuación se describe detalladamente los mecanismos de inactivación del CO_2 supercrítico, así como los factores del tratamiento que afectan a la sensibilidad de los microorganismos.

1.5.3. Mecanismos de inactivación de microorganismos mediante FSC

Los mecanismos asociados al efecto inhibitorio del CO_2 fueron detallados por Daniels y col. (1985). Estos autores describieron la mayoría de teorías acerca de la inactivación de microorganismos con CO_2 supercrítico, y éstas han sido aceptadas por la mayoría de autores para procesos de inactivación mediante FSC (Spilimbergo y Bertucco, 2003; Damar y Balaban, 2006).

Los mecanismos de inactivación de microorganismos mediante SC-CO_2 se resumen en las siguientes etapas (Fig. 1.2): 1, disolución del CO_2 en la fase

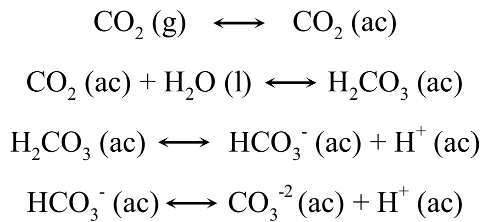
líquida externa donde se encuentran las células suspendidas; 2, modificación de la membrana celular; 3, descenso del pH intracelular; 4, inactivación de enzimas clave; 5, efecto inhibitorio del CO_2 y HCO_3^-; 6, modificación del equilibrio celular; 7, extracción y eliminación de componentes celulares y de la membrana celular; y 8, ruptura celular.

Fig. 1.2. Diagrama de las etapas del mecanismo de inactivación mediante SC-CO_2. A: bicapa fosfolipídica; B: membrana de proteínas; C: membrana; D: componentes intracelulares. Adaptado de: García-González y col., 2007.

Seguidamente se detallan individualmente los mecanismos que tiene lugar en las distintas etapas citadas.

1.5.3.1. Solubilización del CO_2 en la fase liquida

En alimentos líquidos, como zumos, o en medios de cultivo, con un elevado contenido en agua, el CO_2 empleado para presurizar el reactor se disuelve en el agua para formar ácido carbónico (H_2CO_3). Éste se disocia en bicarbonato (HCO_3^-) e iones H^+, que finalmente darán lugar a iones carbonato (CO_3^{-2}) y más iones H^+, de acuerdo al siguiente equilibrio:



$$CO_2\,(g) \longleftrightarrow CO_2\,(ac)$$

$$CO_2\,(ac) + H_2O\,(l) \longleftrightarrow H_2CO_3\,(ac)$$

$$H_2CO_3\,(ac) \longleftrightarrow HCO_3^-\,(ac) + H^+\,(ac)$$

$$HCO_3^-\,(ac) \longleftrightarrow CO_3^{-2}\,(ac) + H^+\,(ac)$$

donde g, l y ac hacen referencia al estado de gas, líquido o disolución acuosa, respectivamente, en el que se encuentra cada compuesto. Este equilibrio se alcanza bajo cualquier condición, viéndose desplazado por el efecto de la presión, de manera que a presiones más elevadas, el contacto entre el CO_2 con el agua es mayor (Erkmen, 2012), facilitándose la disolución del CO_2 en el medio, así como la saturación del mismo.

Esta disolución acidifica la suspensión debido a la liberación de los iones H^+, y esta bajada de pH en el exterior podría reducir la resistencia microbiana a los agentes de inactivación debido a que la mayor parte del consumo energético realizado por los microorganismos sería para mantener el equilibrio quimiostático.

Sin embargo, la reducción del pH externo no es el efecto letal del SC-CO_2, ya que no se ha obtenido un efecto similar en medios acidificados (Kincal y col., 2005). Se ha sugerido por tanto que la bajada de pH contribuye a una modificación de la capa fosfolipídica de la membrana celular, incrementándose la permeabilidad al CO_2 y facilitando la penetración del CO_2 en el interior celular.

1.5.3.2. Modificación de la membrana celular

La membrana celular está constituida por una doble capa de fosfolípidos con capas externas hidrófilas e internas hidrófobas. El CO_2 puede difundir al interior de la membrana celular y acumularse dentro de sus depósitos lipofílicos (en la capa interna), dada la elevada afinidad entre el CO_2 y el plasma de la membrana (Spilimbergo y col., 2002).

Esta acumulación de CO_2 en la fase lipídica de la membrana podría alterar estructural y funcionalmente su orden y romper interacciones lípido-proteicas, además de que el CO_2 podría extraer constituyentes de la membrana, aumentado la fluidez y permeabilidad de la misma (Damar y Balaban, 2006).

Otro posible efecto sobre la membrana podría deberse a la presencia de los iones HCO_3^-, que actuarían sobre los grupos de fosfolípidos y proteínas en la superficie de la membrana, alterando la carga superficial de ésta y su óptimo funcionamiento (García-González y col., 2007).

1.5.3.3. Modificación del pH intracelular

Debido al incremento de la permeabilidad de la membrana detallado anteriormente, el CO_2 podría penetrar fácilmente a través de ésta y acumularse en el interior de las células, donde aumentará la concentración de CO_2 y HCO_3^-, que se disociarán en H^+ y CO_3^{-2}, desestabilizando el equilibrio interno de las células, que es esencial para su correcta viabilidad y actividad celular (Erkmen, 2012).

Muchos microorganismos pueden sobrevivir en ambientes ácidos con pH internos cercanos a la neutralidad. Para compensar el entorno ácido, las células intentan mantener el gradiente de pH entre el interior y el exterior liberando protones desde el citoplasma al exterior, tratando de mantener el gradiente electroquímico a través de la membrana. La suma de ambos gradientes de protones es conocida coma la fuerza motriz de protones (Erkmen, 2012).

Si al citoplasma penetra suficiente cantidad de CO_2 hasta un nivel crítico, el pH se reducirá bruscamente superando y bloqueando la capacidad amortiguadora de las células (García-González y col., 2007).

En este sentido, la inactivación de microorganismos debido al efecto del pH está asociada a una desnaturalización de las enzimas celulares por bajada del pH interno, y a un colapso del sistema celular amortiguador del pH (Erkmen y Bozoglu, 2008a).

1.5.3.4. Inactivación enzimática

Las enzimas son los constituyentes del citoplasma celular que controlan el equilibrio celular, donde el nivel de actividad viene determinado por el pH del medio, presentando cada enzima un pH óptimo. Dada la naturaleza proteica de las enzimas, una acidificación conduce a su desnaturalización, por lo que una bajada del pH en el citoplasma podría causar la inhibición o inactivación de enzimas clave y esenciales para procesos metabólicos y reguladores como la glicólisis, transporte de aminoácidos e iones (García-González y col., 2007; Rezaei y col., 2007).

1.5.3.5. Efecto inhibitorio del CO_2 y HCO_3^-

La concentración intracelular óptima de ciertos sustratos y productos así como un valor adecuado de otros co-factores, como el pH o la temperatura, es esencial para una adecuada actividad celular. La concentración del anión HCO_3^- es importante en la regulación de la actividad enzimática y por tanto en el metabolismo celular, ya que regula la actividad de aniones sensibles presentes en estos enzimas (Damar y Balaban, 2006).

Además, el HCO_3^- y el CO_2 disueltos durante los tratamientos con SC-CO_2, pueden afectar a reacciones de carboxilación y descarboxilación en las células (Damar y Balaban, 2006). La carboxilación es importante para la glucogénesis y la síntesis de aminoácidos y ácidos nucleicos, y el ratio de CO_2-HCO_3^- disuelto determinará la velocidad de estas reacciones. Si las concentraciones de estos compuestos no son las adecuadas, el metabolismo celular se verá alterado y la célula puede ser más susceptible a una posible inactivación (García-González y col., 2007).

Además, a bajo pH interno, la arginina de algunas proteínas podría interactuar con el CO_2 para formar un complejo de bicarbonato, inactivándose y perdiendo su actividad característica (Erkmen, 2012).

1.5.3.6. Modificación del equilibrio celular

La aplicación de SC-CO_2 puede tener efectos letales en el sistema biológico de las células por acumulación en su interior. Al pasar el HCO_3^- a CO_3^{-2}, pueden precipitar electrolitos intracelulares, como Ca^{+2} o Mg^{+2}. Estos iones libres en el citoplasma actúan como reguladores del equilibrio intracelular, de forma que si precipitan, se pierde su actividad reguladora. Estos electrolitos, además de ser importantes reguladores de un gran número de actividades celulares, ayudan en el mantenimiento del equilibrio osmótico entre las células y su entorno, lo que podría afectar al volumen de las mismas (Lin y col., 1993).

Por lo que el efecto letal del SC-CO_2 en las células se incrementa por la precipitación de iones, pérdida del equilibrio celular y desorden del balance osmótico.

1.5.3.7. Extracción de componentes vitales del interior celular y de su membrana

La elevada capacidad de extracción del CO_2, junto con el incremento de la permeabilidad y fluidez de la membrana celular, es suficiente para alterar el equilibrio biológico de las células. El CO_2 penetra en las células y arrastra componentes, como fosfolípidos u otros componentes hidrofóbicos, alterando la estructura de la membrana y el equilibrio biológico celular, por tanto, facilitando la inactivación (García-Gonzalez y col., 2007; Erkmen, 2012). Además, el arrastre de componentes puede estimularse por un descenso repentino de la presión durante la despresurización, permitiendo una rápida salida del material intracelular. En este sentido, Lin y col. (1992) mostraron que repetidos ciclos de presurización-despresurización aceleraban la velocidad de inactivación ya que se aumentaba la transferencia del material intracelular al exterior.

1.5.3.8. Ruptura celular

El efecto directo que el CO_2 ejerce sobre la membrana celular es probablemente determinante para explicar su alta tasa de penetración en las células bacterianas, responsables de modificaciones internas en las células. En este sentido, el estudio del efecto sobre la morfología celular mediante microscopía ha ayudado a entender estos mecanismos de inactivación.

Oulé y col. (2006) observaron mediante microscopía electrónica de barrido (SEM) y microscopía de transmisión electrónica (TEM) que las células de *E. coli* fueron totalmente destruidas tras 30 min de un tratamiento mediante SC-CO_2 (250 bar, 40 ºC). García-González y col. (2010) observaron mediante TEM una distribución irregular y precipitación de agregados del contenido citoplasmático en el interior de células de *E. coli*, *S. cerevisiae* y *L. monocytogenes* tras un tratamiento con SC-CO_2 (210 bar, 45 ºC, 60 min). Mun y col. (2011) detallaron algunas modificaciones en las membranas de *P. aeruginosa* tratada con SC-CO_2 a 100 bar, 37 ºC durante 6 min. Estos autores observaron además de significativos cambios morfológicos en las células y en las membranas citoplasmáticas, fugas del citoplasma y espacios vacíos en el interior de la célula. Li y col. (2012) mostraron mediante SEM células de *S. cerevisiae* dañadas y con arrugas o residuos en la membrana, tras un tratamiento de 120 min con SC-CO_2 (100 bar, 35 ºC). Estos autores mostraron mediante TEM una reducción de la densidad del citoplasma y la ruptura de múltiples células. Sin embargo, otros estudios no han encontrado cambios significativos en la morfología de células de *S. typhimurium* (White y col., 2006) y *L. monocytogenes* (Kim y col., 2008) tras un tratamiento de 60 y 30 min con SC-CO_2 (100 bar, 35 ºC), respectivamente, a pesar de que los ensayos microbiológicos mostraron la inactivación completa de los microorganismos. Las diferencias observadas en los diferentes estudios morfológicos podrían deberse a la variedad de los microorganismos considerados o a la severidad de los tratamientos.

Cabe destacar que los mecanismos descritos en los apartados anteriores (1.5.3.1-1.5.3.8) no ocurren consecutivamente, sino que se llevan a cabo simultáneamente de una forma compleja e interrelacionada.

La velocidad de los diferentes mecanismos descritos depende de múltiples factores referentes tanto a la naturaleza de los microorganismos como a las distintas variables asociadas al proceso.

1.5.4. Factores que afectan a la inactivación de microorganismos mediante FSC

Las tasas de inactivación microbiana por la aplicación de FSC varían notablemente entre los tratamientos descritos en la bibliografía. Gran parte de

estas variaciones se deben a cambios en los factores que pueden ser modificados durante la inactivación supercrítica.

En particular, los factores de mayor importancia son: el tipo de microorganismo, la naturaleza del medio a tratar, la concentración inicial de microorganismos, el estado físico del CO_2, la presión, la temperatura, el tiempo de tratamiento y el tipo de equipamiento.

1.5.4.1. Efecto del tipo de microorganismo

La resistencia que muestran los microorganismos a los FSC varía drásticamente en función de la especie. La comparación entre la resistencia a los FSC mostrada por distintos microorganismos en diferentes estudios es difícil, debido a que los equipos donde se han tratado son distintos, así como la naturaleza del medio, condiciones del proceso, etc., siendo esos factores determinantes en el proceso de inactivación.

En general, las bacterias Gram-positivas presentan mayor resistencia a los FSC que las Gram-negativas, debido a la distinta composición de sus membranas celulares (Sims y Estigarribia, 2003). Las bacterias Gram-negativas tienen una membrana celular más delgada, siendo más fácil que se den la mayoría de los mecanismos de inactivación descritos previamente, como el aumento en la fluidez y permeabilidad de la membrana, la penetración del CO_2 en la célula, etc. (Damar y Balaban, 2006). Las levaduras como por ejemplo *S. cerevisiae*, poseen una pared celular que las hace comparables a las bacterias Gram-positivas (Villas-Boas y col., 2006), presentando por tanto una mayor resistencia a los FSC, comparada con bacterias Gram-negativas como la *E. coli* (Erkmen 2000, 2001a, 2002).

En cuanto a la inactivación de esporas, su resistencia a los FSC es mucho mayor que la de las bacterias o levaduras. Los mecanismos de inactivación no se conocen claramente, aunque parecen ser causados por la ruptura y perforación de la membrana externa de las esporas, especialmente cuando el proceso se lleva a cabo adicionando agentes oxidantes, como el peróxido de hidrógeno, al CO_2 (Perrut, 2012).

La contaminación por virus es una preocupación primordial en bio-productos de origen humano, e incluso en los productos de ADN preparados por la

biotecnología. Como estos productos son muy sensibles a la temperatura, irradiación y agentes químicos, la inactivación de los virus a baja temperatura mediante FSC es de gran interés. Los virus están formados por proteínas, ácidos nucleícos y lípidos. La actividad infecciosa de los virus reside en la combinación y unión entre sus componentes internos, como la agregación de proteínas, que se unen mediante enlaces no covalentes. Las uniones proteína-proteína y proteína-lípido constituyentes de los virus pueden ser fragmentadas o dañadas por el efecto de los FSC, perdiendo total o parcialmente su actividad infecciosa. Bajo condiciones atmosféricas, los virus son químicamente estables, pero bajo condiciones supercríticas, las reacciones químicas que pueden tener lugar, así como la interacción entre el FSC y la estructura de los virus, podrían ser los responsables de su inactivación (Chen y col., 2006; Erkmen, 2012).

Por tanto, los FSC pueden considerarse una nueva técnica para inactivar virus, o una tecnología complementaria a los procesos convencionales de inactivación de virus.

1.5.4.2. Efecto de la concentración inicial de microorganismos

La eficacia de los FSC se ve directamente influenciada por la concentración inicial de microorganismos. Bajo las mismas condiciones, el nivel de inactivación alcanzado es mayor cuanto menor es la concentración inicial de microorganismos (García-González y col., 2007). Una elevada concentración de microorganismos hace que proteínas, lípidos y otros componentes, resultantes de la ruptura de las células microbianas, tengan un efecto protector sobre los microorganismos supervivientes, estando éstos menos expuestos al CO_2 y a los mecanismos de inactivación anteriormente descritos (Tahiri y col., 2006).

1.5.4.3. Efecto de la fase de crecimiento del cultivo

La susceptibilidad de los microorganismos a la mayoría de procesos de inactivación está influenciada por la fase de crecimiento en la que éstos se encuentran.

En general, las células en fases tempranas de crecimiento son más sensibles a los FSC que las células en fase estacionaria. Hong y Pyun (1999) mostraron una mayor velocidad de inactivación con FSC (70 bar, 30 ºC, 100 min) de

Lactobacillus plantarun en la fase logarítmica de crecimiento que en la fase estacionaria.

Los microorganismos al alcanzar la fase estacionaria de crecimiento, sintetizan proteínas y otras biomoléculas que les protegen de la condiciones adversas de su alrededor, como la temperatura o las altas presiones, entre otros factores (Erkmen, 2012).

Hasta la fecha, solo se han observado diferentes velocidades de inactivación entre la fase temprana de crecimiento y la fase estacionaria. Sin embargo, el estudio de las cinéticas de inactivación mediante FSC de un microorganismo seleccionado en las diferentes etapas de su curva de crecimiento no ha sido estudiado en profundidad.

1.5.4.4. Efecto de las propiedades físicas y químicas del medio de suspensión

Las propiedades físicas y químicas del medio donde están suspendidos los microorganismos son determinantes en el efecto de los FSC sobre éstos. La acidez y el contenido en grasa o aceites, agua y proteínas, entre otros componentes, puede influir en la eficacia de inactivación de los FSC. La grasa o los aceites del medio aumentan la resistencia microbiana a los FSC ya que cambian la estructura de las paredes celulares aumentando su resistencia a ser modificadas por el CO_2 (Damar y Balaban, 2006; Erkmen, 2012).

En cuanto al pH del medio donde están los microorganismos suspendidos, en general, la velocidad de inactivación es mayor si el pH inicial es bajo (Hong y Pyun, 1999). Esta acidez podría contribuir a un aumento de la permeabilidad de la membrana, que facilitaría la penetración del CO_2 en las células (Damar y Balaban, 2006; García-González y col., 2007).

En relación al contenido en azúcares, éstos retienen agua haciendo que no esté disponible para que se disuelva el CO_2, es decir, los azúcares reducirían la solubilidad del CO_2 en la fase acuosa, previniendo una bajada de pH (Ferrentino y col., 2010) y por tanto dificultando los mecanismos de inactivación.

La actividad de agua es crítica en los procesos de inactivación con CO_2 supercrítico, ya que es en el agua donde el CO_2 se disuelve y entra en contacto

con las células suspendidas. Además, el agua puede hinchar las células, aumentado la permeabilidad de las mismas al CO_2. Por tanto, microorganismos suspendidos en medios con baja actividad de agua pueden no verse tan afectados por los FSC, como cuando se suspenden en medios con mayor actividad de agua (Erkmen, 2012).

1.5.4.5. Efecto del estado físico del CO_2

Las propiedades físico-químicas del CO_2 dependen de la presión y la temperatura a la que éste se encuentre. El CO_2 a alta presión puede encontrarse en estado subcrítico (líquido o gas) o supercrítico. Se han llevado a cabo numerosos estudios comparando la efectividad de inactivación del mismo en diferentes estados y en general, de dichos estudios se puede concluir que el CO_2 en estado supercrítico es más efectivo que en estado subcrítico (Gunes y col., 2005).

La mayor letalidad del CO_2 supercrítico podría ser atribuida a las particulares propiedades físico-químicas que éste presenta, como una densidad similar a las de los líquidos y una difusividad y viscosidad cercanas a la de los gases. La elevada densidad le proporciona una excelente capacidad de dilución y de extracción de componentes, mientras que la elevada difusividad y baja viscosidad le aporta mayor capacidad de penetración y transferencia de materia. Además, la baja tensión superficial le permite una rápida penetración en las células.

Por lo tanto, el CO_2 supercrítico, es más eficaz tanto en la penetración en las células, como en la extracción de componentes intracelulares, lo que resulta en un aumento de la inactivación, comparado con el CO_2 líquido o gaseoso (Tomasula, 2003; Gunes y col., 2005).

1.5.4.6. Efecto de la presión y la temperatura

La temperatura y la presión definen el estado físico y las propiedades físico-químicas del CO_2, por ello, ambos factores son determinantes en la eficacia de los FSC en los procesos de inactivación microbiana.

En general, la velocidad de inactivación aumenta con la presión, necesitándose tiempos de exposición más cortos a presiones más elevadas (Lin y col., 1993; Hong y Pyun, 1999). La presión controla la solubilidad del CO_2 en el

medio a tratar, por tanto, mayores presiones facilitaran dicha disolución y la acidificación del medio (primer paso de los mecanismos de inactivación), lo que mejorará el contacto entre los microorganismos y el CO_2, y como consecuencia el resto de mecanismos involucrados en el proceso de inactivación con FSC.

La temperatura juega un papel muy importante en los procesos de inactivación con FSC ya que además de facilitar la difusividad del CO_2 en el medio y de aumentar la fluidez de la membrana celular, favorece la desnaturalización de proteínas y el desorden biológico de las células (Erkmen y Bozoglu, 2008b). Sin embargo, los tratamientos con FSC suelen llevarse a cabo a temperaturas cercanas a las del punto crítico del CO_2, por un lado para evitar el deterioro de la calidad de los alimentos debido a temperaturas demasiado altas y por otro debido a que es en esta región donde las propiedades físico-químicas del CO_2 son las más adecuadas para que se den los mecanismos asociados a la inactivación microbiana, como la rápida solubilización del CO_2 en el medio y su difusión al interior celular (García-González y col., 2007).

1.5.4.7. Efecto del tipo de sistema, de la agitación y de la concentración de CO_2

Los tratamientos de inactivación mediante fluidos supercríticos pueden llevarse a cabo en sistemas discontinuos, semi-continuos y continuos, con o sin agitación. El tipo de sistema afecta directamente al contacto entre el CO_2 y el medio a tratar, y por tanto a la concentración del CO_2 en el medio, estando este último factor directamente relacionado con la velocidad de inactivación.

El mayor grado de inactivación se alcanza cuando el medio está saturado de CO_2. La agitación puede mejorar la disolución del CO_2 en el medio a tratar, y por tanto mejorar su contacto con los microorganismos, facilitando todos los mecanismos involucrados en su inactivación, desde la acidificación del medio, hasta la penetración del CO_2 en las células y extracción de componentes intracelulares (García-González y col., 2007). La agitación dentro de un reactor puede llevarse a cabo con un mezclador, una recirculación o un proceso de mezclado previo al tratamiento. Tanto Tsuji y col. (2005) como Oulé y col. (2006) mostraron un aumento en la velocidad de inactivación conforme aumentó la velocidad de agitación en tratamientos de inactivación con FSC.

La agitación también puede verse modificada por el tipo de sistema elegido. En general, para productos sólidos, los sistemas de FSC aplicados han sido en discontinuo (Valverde y col., 2010; Ferrentino y col., 2013) y para productos líquidos existen sistemas discontinuos, semi-continuos y continuos (Spilimbergo y col., 2007; Liu y col. 2012). Los sistemas en discontinuo precisan tiempos de tratamiento más largos que los sistemas semi-continuos, y éstos a su vez son más lentos que los sistemas continuos, para alcanzar niveles de inactivación similares (Ishikawa y col., 1995; Spilimbergo y col., 2003a). El factor crítico es el tiempo en alcanzar la saturación del medio a tratar, siendo más efectivos aquellos sistemas que permiten un mezclado continuo entre el medio y el CO_2. En este sentido, en sistemas continuos, la mayor agitación permite alcanzar la saturación del medio más rápidamente que en sistemas discontinuos. Así pues, el tipo de sistema empleado determinará el nivel de agitación y por lo tanto la velocidad de inactivación y a su vez el tiempo de tratamiento necesario para alcanzar un determinado nivel de inactivación.

1.5.5. Mecanismos y factores involucrados en la inactivación de enzimas mediante FSC

El uso de FSC para la inactivación de enzimas responsables del deterioro de muchos alimentos líquidos ha sido objeto de diversos estudios (Park y col., 2002; Spilimbergo y col., 2007; Xu y col., 2011). La aplicación de FSC a temperaturas moderadas permite la inactivación de algunas enzimas, que un tratamiento térmico a temperaturas similares no sería capaz de inactivar (Balaban y col., 1991).

Entre las enzimas con más incidencia en la industria de alimentos cabe destacar la pectin metil-esterasa (PME) que es responsable de la pérdida de nube de algunos zumos de fruta; la poli-fenol oxidasa (PPO) responsable de la oxidación de frutas, vegetales y zumos; la lipoxigenasa (LOX) que destruye la clorofila y produce olores desagradables en vegetales congelados; y la peroxidasa (PO) que juega un papel importante en la decoloración de alimentos y se utiliza como índice para evaluar la eficacia de un tratamiento térmico en el procesado de frutas y vegetales (Balaban, 2012).

Los mecanismos asociados a la inactivación de enzimas se corresponden con aquellos mecanismos responsables de la desnaturalización de proteínas. Las enzimas están dispuestas en estructuras tridimensionales, determinadas por enlaces covalentes y conexiones intermoleculares (Rezaei y col., 2007). La inactivación de las mismas es causada por la fragmentación o modificación de su estructura y por tanto, todos los fenómenos físicos o químicos que las modifiquen son responsables de su desnaturalización.

Los mecanismos asociados a la inactivación de enzimas por exposición a FSC pueden ser debidos a diferentes efectos como una bajada de pH, el efecto inhibitorio del CO_2 y a cambios estructurales generados por el CO_2 a alta presión (Damar y Balaban, 2006).

Los tratamientos con FSC van acompañados de una bajada de pH debido a la formación de ácido carbónico por disolución del CO_2 en agua, y bajo un ambiente ácido, la arginina de las proteínas interacciona fácilmente con el CO_2 formando complejos carbonatados que precipitan (Rezaei y col., 2007). Adicionalmente, el CO_2 aplicado a alta presión provoca cambios estructurales en las enzimas que les hacen perder sus propiedades características, y por tanto su actividad específica.

A pesar de las citadas teorías, son necesarios más estudios para entender cuáles son los mecanismos de inactivación que tienen lugar en un entorno de CO_2 supercrítico, incluyendo el efecto de la presión, así como el efecto de los iones HCO_3^- y CO_3^{-2} en la estructura de las proteínas y en sus cambios estructurales.

El efecto de los FSC sobre las enzimas depende de la fuente de la que éstas procedan, del contenido de agua, de las condiciones de presión y temperatura, además del tiempo del tratamiento y de las propiedades del medio en el que se encuentren suspendidas. En este sentido, Chen y col. (1992) determinaron que la enzima PPO aislada de patata era más resistente a los FSC que la procedente de langosta y otros mariscos. Zhou y col. (2009) estudiaron la inactivación de PME de zumo de naranja mediante FSC y redujeron la actividad residual de un 83 % a un 9.3 % al incrementar la presión de 80 a 300 bar (55 ºC, 10 min).

La presencia de algunos compuestos en la matriz líquida a tratar, como azúcares, puede tener un efecto protector sobre las enzimas. Tedjo y col. (2000)

obtuvieron una menor inactivación de LOX y PO al estar éstas suspendidas en un medio con una concentración de sacarosa superior al 40 %, comparado con la inactivación en un medio sin sacarosa. Este efecto podría ser debido a que los azúcares retienen CO_2 evitando su disolución en el medio, y por tanto, cuanto menor sea la concentración de CO_2, menor será la capacidad para llevar a cabo los mecanismos de inactivación previamente mencionados.

1.5.6. Efecto de los FSC sobre la calidad y estabilidad microbiológica de los alimentos

La mayoría de trabajos relacionados con el uso de FSC como técnica de conservación están centrados en la inactivación de microorganismos, sin embargo, existe poca información disponible en relación a los efectos sobre las propiedades organolépticas y nutricionales de los alimentos. Es interesante conocer el efecto de los FSC sobre los parámetros de calidad justo tras el tratamiento del alimento, así como la evolución de los mismos durante su almacenamiento. Además, es necesario conocer la evolución de la carga microbiana del producto durante el almacenamiento para, junto con los cambios en las propiedades organolépticas y nutricionales, determinar su tiempo de vida útil.

1.5.6.1. Efecto sobre las propiedades físico-químicas y sensoriales

Los resultados obtenidos en diferentes estudios acerca del efecto de los FSC sobre los alimentos, dependen en gran medida de la naturaleza y características del alimento tratado. En este apartado nos centraremos en el efecto sobre los zumos de frutas al ser el producto empleado en la presente Tesis Doctoral.

En la mayoría de estudios, el pH y ºBrix de zumos tratados con FSC no cambiaron significativamente tras el tratamiento (Spilimbergo y Ciola, 2010; Xu y col., 2011). En general, en cuanto al efecto sobre el color de zumos tratados con FSC, la luminosidad y tonalidades amarillentas aumentaron, mientras que disminuyó la intensidad de los colores iniciales de los productos tratados (Erkemn, 2012). Por otro lado, los FSC pueden mejorar el aspecto físico de algunos alimentos líquidos, como el de los zumos en comparación con zumos no tratados, ya que evitan la clarificación de los mismos gracias a la inactivación

enzimática y la disminución del tamaño de las partículas en suspensión (Xu y col., 2011). Xu y col. (2011) mostraron un aumento significativo de la turbidez del zumo de manzana tras el tratamiento con FSC (220 bar, 60 ºC, 2 min) debido a una disminución del tamaño de partículas, sin embargo, no encontraron diferencias significativas en la viscosidad del zumo tratado.

En cuanto al efecto sobre algunas propiedades sensoriales, Gasperi y col. (2009) compararon zumo de manzana tratado con FSC (100 bar, 36 ºC, 10 min) con zumo no tratado. Las pruebas triangulares revelaron una diferencia significativa ($p<0.001$) entre el olor de los zumos tratados y no tratados y una ligera diferencia en cuanto al sabor. Fabroni y col. (2010) evaluaron las propiedades sensoriales de un zumo de naranja durante 30 días de almacenamiento a 4 ºC, tras un tratamiento con FSC (130 bar, 36 ºC, 15 min). La frescura, acidez, dulzor e intensidad del sabor disminuyeron significativamente tras 20 días de almacenamiento, además el amargor y la presencia de olores extraños aumentaron tras el mismo periodo de tiempo. Spilimbergo y Ciola (2010) compararon zumo de melocotón y kiwi tratado con FSC (35 ºC, 100 bar, 15 min) con zumo no tratado. El zumo de melocotón, tras los FSC presentó un color más claro y con un ligero sabor a avellana. En cuanto al zumo de kiwi, tras el tratamiento con FSC, no se mostraron diferencias significativas respecto al no tratado en cuanto a color, sabor o aroma.

Del Pozo-Insfran y col. (2006) no encontraron diferencias significativas ($p>0.01$) entre el color, aroma y olor del zumo de uva fresco y el tratado mediante FSC (345 bar, 30 ºC, 6.25 min). Sin embargo, sí que se detectaron diferencias significativas ($p<0.01$) entre el zumo no tratado y el pasteurizado (75 ºC, 15 s), identificando la formación de olores a cocido en el zumo pasteurizado. Los panelistas puntuaron en 6.05 y en 4.02 sobre 10 el zumo tratado por FSC y el pasteurizado, respectivamente. Además, Damar y Balaban (2006) concluyeron que los zumos tratados con FSC, en general obtienen peor calificación que los no tratados aunque mejor aceptación que los pasteurizados, en cuanto al sabor, olor y color. Por tanto, aunque se observan mejoras respecto a la pasteurización térmica, es necesario mejorar las tecnologías actuales de inactivación mediante FSC para minimizar el efecto sobre las propiedades

organolépticas del alimento y que éstas sean lo más parecidas a las de los alimentos sin tratar.

1.5.6.2. Efecto sobre las propiedades nutricionales

El efecto sobre las propiedades nutricionales de los alimentos tratados mediante FSC no ha sido analizado en profundidad, aunque algunos trabajos han evaluado su efecto sobre alguna propiedad concreta. Arreola y col. (1991) observaron un aumento significativo de la retención de ácido ascórbico en el zumo de naranja tratado con FSC, en comparación al tratado térmicamente. Del Pozo-Insfran y col. (2006) no observaron cambios en la cantidad total de antocianos, de compuestos fenólicos solubles y en la capacidad antioxidante de zumo de uva tratado mediante FSC (345 bar, 30 ºC, 6.25 min), respecto al fresco, mientras que el zumo tratado térmicamente (75 ºC, 15 s) presentó una reducción del 16, 26 y 10 %, respectivamente, respecto del control. Además, dichas propiedades se retuvieron mejor en el zumo tratado por FSC que en el pasteurizado térmicamente, tras 10 semanas de almacenamiento a 4 ºC.

1.5.6.3. Efecto sobre la estabilidad microbiana

Para asegurar la estabilidad microbiológica de los alimentos tras un tratamiento de inactivación, no solo hay que comprobar la reducción de microorganismos tras el tratamiento, sino que es necesario controlar el crecimiento microbiano durante un tiempo mínimo de comercialización del producto, a temperaturas de refrigeración o ambientales, según el alimento y tratamiento aplicado.

Del Pozo-Insfran y col. (2006) obtuvieron recuentos microbianos comparables entre el zumo de uva tratado mediante FSC (345 bar, 30 ºC, 6.25 min) y el pasteurizado (75 ºC, 15 s) durante las primeras 5 semanas de almacenamiento a 4 ºC. A partir de dicho periodo, la población de levaduras y mohos aumentó en el zumo tratado con FSC, manteniéndose constante en el tratado por calor. Kincal y col. (2005) y Fabroni y col. (2010) evaluaron el crecimiento microbiano en un zumo de naranja durante un almacenamiento a 4 ºC, tras un tratamiento con FSC (1000 bar, 34.5 ºC, 10 min y 130 bar, 36 ºC, 15 min, respectivamente). En ambos trabajos se observó crecimiento de microorganismos durante el almacenamiento,

a pesar de que al inicio del almacenamiento no se detectó su presencia. Estos autores atribuyeron el crecimiento observado con el almacenamiento a una posible recuperación de los microorganismos. Durante el tratamiento con SC-CO_2 los microorganismos podrían sufrir transformaciones y daños en su estructura celular; tras el tratamiento, cuando ya no existe contacto entre el CO_2 y las células, éstas podrían sintetizar nuevas biomoléculas para reparar el daño en las paredes celulares y membranas, continuando con el crecimiento y la división celular (Erkmen y Bozoglu, 2008b).

Por tanto, a pesar de conseguir una reducción de microorganismos aceptable tras los tratamientos con FSC, no siempre se consigue una estabilidad microbiana con el almacenamiento. De manera que, es necesario mejorar la inactivación supercrítica incrementando el efecto de los FSC sobre los microorganismos, de tal forma que se consiga una estabilidad microbiológica durante el almacenamiento a temperaturas de refrigeración, sin alterar las propiedades sensoriales e intentando que éstas sean lo más parecidas a las de los productos sin tratar.

1.5.7. Limitaciones de la inactivación mediante FSC y su combinación con otras tecnologías

Una de las limitaciones de esta tecnología, es que en ciertas aplicaciones es necesario el uso de tiempos largos de tratamiento para alcanzar niveles de inactivación aceptables, lo que podría encarecer el proceso a nivel industrial y afectar a la calidad del producto.

Así pues, para que los FSC puedan reemplazar a las tecnologías tradicionales de pasteurización, éstos deben evitar o minimizar los daños en las propiedades organolépticas y nutricionales de los alimentos, que sí producen los tratamientos térmicos, en un tiempo razonable. Además, deben ampliar la vida útil de los productos tratados, inactivando tanto la flora natural, como posibles patógenos presentes, manteniendo la estabilidad microbiológica durante el almacenamiento.

Para reducir los tiempos de tratamiento en la inactivación con FSC, es necesario incrementar y/o acelerar los efectos que éstos ejercen sobre los microorganismos. Diferentes estudios han evaluado la combinación de FSC con

otras tecnologías no-térmicas para obtener un efecto sinérgico sobre la inactivación microbiana y enzimática y así poder acortar los tiempos de proceso, lo que reduciría el coste del tratamiento y permitiría utilizar temperaturas y presiones más bajas, manteniendo la calidad del producto. En este sentido se ha ensayado cambios en el pH o el uso de PEF y HHP, habiéndose demostrando un efecto aditivo o sinérgico en la inactivación microbiana, lo que permite reducir los tiempos de tratamiento y la intensidad de las condiciones del proceso (Park y col., 2002; Spilimbergo y col., 2003b; Pataro y col., 2010).

Spilimbergo y col. (2003b) investigaron la inactivación de *E. coli*, *S. aureus* y *B. cereus* en agua destilada mediante una aplicación secuencial de PEF (25 kv/cm) y CO_2 supercrítico (80-200 bar, 34 ºC, 10 min). Estos autores mostraron un efecto sinérgico entre ambas tecnologías para los tres microorganismos estudiados. En la misma línea, Pataro y col. (2010) mostraron un efecto sinérgico en la inactivación de *S. cerevisiae* mediante un tratamiento consecutivo de PEF (6, 9 y 12 kV/cm) y FSC (80-140 bar, 25 ºC, 3-30 min) lo que permitió el uso de condiciones menos severas, comparado con el uso solo de FSC.

Varios trabajos han estudiado el efecto sinérgico de la combinación de FSC y HHP sobre la inactivación de enzimas como PPO, LOX y PME. En este sentido, Corwin y Shellhammer (2002) carbonataron zumo de naranja y posteriormente aplicaron HHP (5000 bar y 3 min; 8000 bar, 1 min), mostrando que el CO_2 tuvo un efecto significativo en la inactivación de PME, comparado con el tratamiento solo de HHP. Park y col. (2002) mostraron que una aplicación secuencial de FSC (100-300 bar, 35 ºC, 5 min) y HHP (2000 bar, 25 ºC, 5 min) aumentó la inactivación de PPO, LOX y PME en zumo de zanahoria con una actividad residual de 35 %, 17 % y 45 %, respectivamente, comparado con la actividad residual tras el tratamiento de FSC (40 %, 20 % y 50 %, respectivamente) y tras el proceso de HHP (83 %, 78 % y 80 %, respectivamente).

1.6. Ultrasonidos

El uso de ultrasonidos tanto para procesos de conservación como de extracción, ha suscitado un gran interés en la industria alimentaria. Las técnicas de ultrasonidos se pueden aplicar para mejorar la calidad y seguridad de los alimentos, así como para mejorar otras tecnologías ya existentes.

Los ultrasonidos generan fenómenos físicos y químicos diferentes a los generados por otras tecnologías en el procesado o conservación de alimentos, ofreciendo ventajas en términos de productividad, rendimiento y selectividad, reduciendo el tiempo de procesamiento y mejorando la calidad del producto final, siendo al mismo tiempo respetuosos con el medio ambiente.

1.6.1. Generalidades

Los ultrasonidos se definen como una serie de ondas elásticas que viajan a través del medio conductor produciendo compresión (alta presión) y rarefacción (baja presión) y cuya frecuencia supera a la del sonido audible por el oído humano: 20 kHz.

Al igual que cualquier sonido, los ultrasonidos se transmiten a través de toda sustancia, sólida, líquida o gaseosa, que posea propiedades elásticas. La fuente de producción de ultrasonidos suele ser un cuerpo vibrante. El movimiento de vibración de dicho cuerpo se comunica a las moléculas del medio, cada una de las cuales transmiten, a su vez, el movimiento a las moléculas adjuntas y retornan aproximadamente a su posición original.

Las ondas acústicas, vienen definidas por su frecuencia, velocidad e intensidad o amplitud, siendo clasificadas como longitudinales, transversales y de Rayleigh, en función de si las partículas del medio se mueven en la dirección del desplazamiento de la propagación de la onda, perpendiculares al de la onda, o por la superficie, respectivamente (Corona, 2013).

A su vez, en función de su frecuencia, las ondas acústicas se dividen en cinco grupos: infrasonido (<20 Hz), espectro audible (20 Hz a 18 kHz), ultrasonidos de alta intensidad (18-100 kHz), ultrasonidos de baja intensidad (100 kHz–1 MHz) y los ultrasonidos médicos o de diagnóstico (>1 MHz) (Corona, 2013).

Se puede realizar otra clasificación de los ultrasonidos en función de su aplicación: ultrasonidos de alta potencia o alta intensidad que se caracterizan por tener unas frecuencias que oscilan entre los 20 y los 100 kHz e intensidades superiores a 1 W/cm^2, empleados para mejorar fenómenos de transporte y caracterizados por provocar cambios en los medios en los que se aplican; y ultrasonidos de baja potencia o de señal que se caracterizan por tener frecuencias entre 100 kHz y 20 MHz e intensidades inferiores a 1 W/cm^2, empleados para monitorizar cambios en la composición, textura y otros parámetros de los medios en los que se emplean, sin provocar cambios en ellos (Cárcel, 2003).

En tecnología de alimentos, son importantes tanto las aplicaciones de los ultrasonidos de potencia como los de señal, aunque en el presente trabajo se van a describir los fenómenos y aplicaciones asociados a los ultrasonidos de potencia.

1.6.2. Ultrasonidos de potencia (High Power Ultrasound, HPU)

Para entender el uso de los HPU en las aplicaciones sobre alimentos, es necesaria una descripción de cómo se producen y los fenómenos que generan.

1.6.2.1. Sistemas de generación

En general, la producción de HPU consiste en la conversión de cualquier otro tipo de energía en energía acústica. Los sistemas de ultrasonidos constan de tres partes fundamentales: generador, transductor y emisor. El generador es el encargado de transformar la señal eléctrica de la red a la frecuencia deseada; el transductor convierte la señal eléctrica de alta frecuencia en vibraciones mecánicas; y el emisor irradia la energía acústica generada por el transductor al medio a tratar (Cárcel, 2003).

Los transductores se pueden clasificar en 3 grandes grupos en función de la forma de ser excitados para generar las ondas ultrasónicas: los transductores operados mediante fluidos, los transductores magnetoestrictivos y los transductores piezoeléctricos. El presente trabajo se enmarca en el uso de transductores piezoeléctricos.

El fundamento de los transductores piezoeléctricos se basa en el efecto piezoeléctrico que presentan algunos cristales, que consiste en que al ejercer una

presión sobre el cristal se genera una carga en cada cara del cristal de igual intensidad pero de sentido contrario (Fig. 1.3). El efecto piezoeléctrico inverso ocurre cuando al aplicar una carga igual pero de sentido contrario en cada una de las caras del cristal, éste se contrae o se expande en función de la polaridad de las cargas. Si se aplica una corriente eléctrica alterna de elevada frecuencia se puede provocar una vibración en el material que conlleva la generación de la onda ultrasónica.

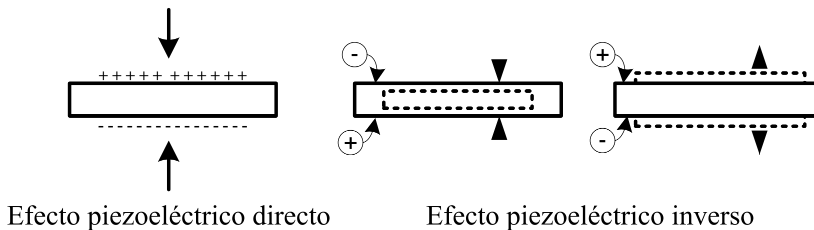


Fig. 1.3. Efecto piezoeléctrico

El efecto piezoeléctrico lo presentan materiales naturales como el cuarzo, la turmalina y la sal de Rochelle. En éstos el efecto piezoeléctrico es muy pequeño, por ello se han desarrollado materiales con propiedades mejoradas, por ejemplo materiales cerámicos ferroeléctricos policristalinos, como el titanato de bario y el zirconato titanato de plomo, conocidos como cerámicas piezoeléctricas (García-Pérez, 2007).

El elemento principal de los transductores son las cerámicas, a las que se le acoplan elementos metálicos de alta calidad, como el titanio, para protegerlas, ya que son frágiles, y para que actúen de sumideros de calor previniendo su sobrecalentamiento. Una de las disposiciones más utilizadas en la aplicación de ultrasonidos de potencia es el sistema “sándwich” (Fig. 1.4). Éste se monta a partir de una pareja de cerámicas, conectadas con las polarizaciones en sentido opuesto, a las que se le acoplan dos masas cilíndricas metálicas que comprimen la estructura con un tornillo colocado en el eje axial. La onda que transmita cada transductor está relacionada con la longitud de los cilindros metálicos. El cilindro frontal será el que aplique la onda ultrasónica generada al medio a tratar.

Los transductores piezoeléctricos son los más utilizados en la actualidad. Sus principales ventajas son la capacidad de producir ultrasonidos a potencias elevadas, cubrir todo el rango de frecuencias y presentar factores de conversión de energía eléctrica muy elevados. Sus principales limitaciones son la despolarización de las cerámicas a elevadas temperaturas, por encima de 100-120 ºC, y el paulatino envejecimiento de las mismas.

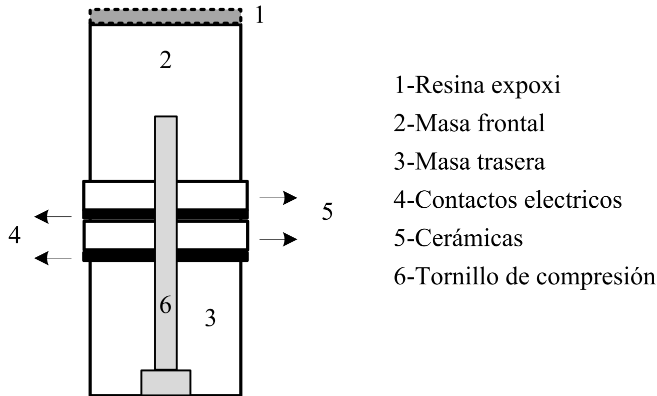


Fig. 1.4. Transductor piezoeléctrico tipo sándwich

1.6.2.2. Sistemas de aplicación

En sistemas de aplicación de ultrasonidos en medios líquidos las dos tipologías más importantes son los baños de ultrasonidos y los sistemas tipo sonda. Éstos últimos son los sistemas empleados en la presente Tesis doctoral.

Los sistemas tipo sonda se basan en la transmisión al medio de la energía ultrasónica que produce el transductor mediante una sonda metálica (Fig. 1.5). El sistema consta de un generador, un transductor y una sonda que transmite la energía acústica al medio a tratar. La intensidad de la onda emitida, es decir, la amplitud de vibración de la punta de la sonda, se puede controlar cambiando la potencia suministrada por el generador. El material utilizado para la fabricación de las sondas debe tener una gran resistencia a la fatiga dinámica, bajas pérdidas acústicas y resistencia a la erosión que produce la cavitación (Cárcel, 2003).

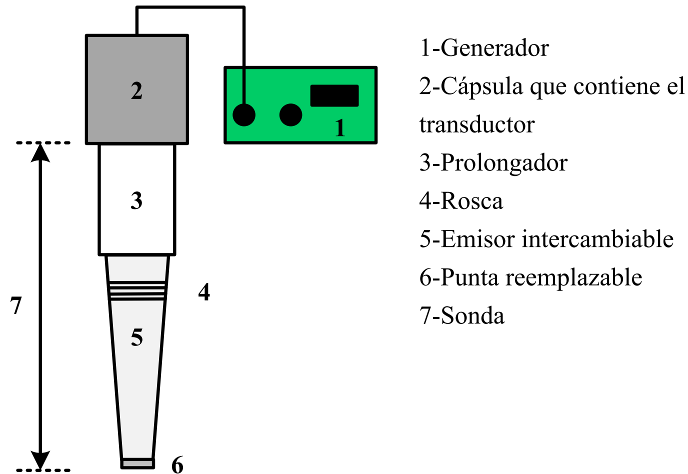


Fig. 1.5. Sistema tipo sonda para la aplicación de ultrasonidos de potencia.

1.6.2.3. Efectos de los ultrasonidos de potencia

Los HPU alteran y modifican el medio en el que son aplicados. Los efectos atribuidos a los ultrasonidos pueden ser muy diferentes, e incluso contrarios, en función del medio físico en el que estos se transmiten. Se emplean en campos tan dispares como la limpieza, el secado, la filtración, la inactivación de microorganismos y enzimas, la extracción, etc. La amplia variedad de efectos asociados a los HPU puede explicarse mediante varios mecanismos asociados.

A continuación se van a exponer los principales efectos y mecanismos asociados a la aplicación de HPU en sistemas sólido-líquido.

- Cavitación: las compresiones y descompresiones que provoca la onda acústica inducen a movimientos moleculares en el medio (Fig. 1.6). Los ciclos de compresión ejercen una presión positiva sobre el líquido y las moléculas tienden a aproximarse. En los ciclos de descompresión el efecto es el contrario, se genera una presión negativa que tiende a separar las moléculas. Si la intensidad de los ultrasonidos es suficientemente elevada, las presiones negativas que se producen durante el ciclo de descompresión pueden vencer las fuerzas atractivas de las moléculas del líquido, principalmente la tensión superficial, y entonces se forma una burbuja de cavitación (García-Pérez, 2007).

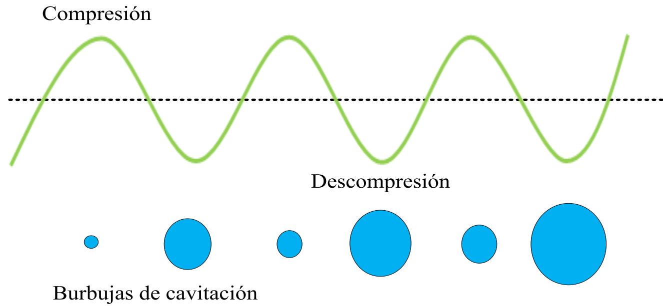


Fig. 1.6. Creación y crecimiento de burbujas de cavitación por difusión rectificada

El colapso de las burbujas libera una cantidad de energía que puede producir diversos efectos químicos y/o mecánicos. En el punto donde se produce la implosión de una burbuja de cavitación, se generan temperaturas de alrededor de 4000 K y presiones que pueden alcanzar los 100 MPa. La vida media de estos "puntos calientes" es de apenas 0,1 µs (Cárcel, 2003). El colapso de las burbujas es muy rápido y potente y produce fuerzas cortantes capaces de romper fuertes enlaces químicos.

Además, pueden presentarse efectos mecánicos significativos que como consecuencia generan corrientes de líquido hacia el interior de la burbuja, atravesándola y pudiendo golpear la superficie de las estructuras sólidas que rodea (Fig. 1.7).

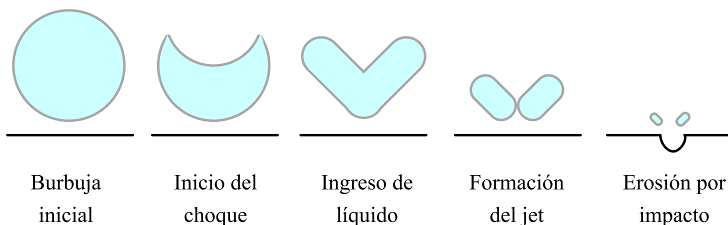


Fig. 1.7. Cavitación cerca de una interfase sólido-líquido.

En general, muchos de los efectos atribuidos a los ultrasonidos ocurren en presencia de cavitación. Algunos de estos efectos son la desgasificación de líquidos, la generación de radicales libres, la aceleración de reacciones, el

incremento de índices de emulsión y de difusión, la mejora en procesos de extracción, la erosión de superficies, etc. (García-Pérez, 2007).

- Calentamiento: como consecuencia de la propagación de las ondas ultrasónicas en un medio, todas las partículas de dicho medio se encuentran vibrando, transformando parte de la energía acústica en calor al oponer resistencia viscosa a esta vibración (Mason y Lorimer, 2002). Por lo tanto, la elevación de temperatura del medio depende de las características viscosas del material atravesado, así como de la frecuencia y la potencia de la onda ultrasónica.
- Microagitación: son turbulencias en las interfases líquido-sólido (García-Pérez, 2007). Esta microagitación se genera por la interacción de la onda acústica, que viaja por el fluido, con el sólido. La importancia de esta agitación estriba en que se forma justo en la interfase, disminuyendo el espesor de la capa límite de difusión e incrementando la transferencia de materia y/o calor.

1.6.2.4. Aplicaciones de los HPU en tecnología de alimentos

Las aplicaciones de los ultrasonidos de alta intensidad en tecnología de alimentos son muy variadas y entre ellas se encuentran el salado de carne (Ozuna y col., 2013) y queso (Sánchez y col., 2000), la deshidratación de vegetales (Ozuna y col., 2011) y también la extracción de productos naturales tanto con disolventes convencionales (Albu y col., 2004; Stavarache y col., 2006; Ahmad-Qasem y col., 2013) como con FSC (Riera y col., 2010). Estas aplicaciones mencionadas están asociadas a procesos de transferencia de materia, pero además, hay otras aplicaciones de los ultrasonidos como la inactivación de microorganismos y enzimas, operaciones de limpieza, mejora de reacciones químicas, tenderización de carne, formación de emulsiones o procesos de corte de alimentos (Mason y col., 1996; Cárcel y col., 1999).

1.6.2.5. Inactivación de microorganismos y enzimas mediante HPU

Los HPU son conocidos por el daño o la ruptura que pueden provocar en células de diferentes microorganismos o sobre la modificación de las estructuras tridimensionales de las enzimas, resultando en la inactivación de las mismas.

Existen numerosos estudios acerca de los efectos de los ultrasonidos sobre los microorganismos, siendo éstos dependientes del tipo de bacteria, del volumen y características del medio donde se aplican, así como de los parámetros ultrasónicos empleados (Piyasena y col., 2003). Desafortunadamente, para conseguir niveles de esterilización aceptables mediante la única aplicación de ultrasonidos, son necesarias intensidades muy elevadas que llevan al sobrecalentamiento del medio donde se aplican. Por ello, se ha probado la combinación de los ultrasonidos con otras tecnologías de inactivación como las HHP, los tratamientos térmicos o el uso de pHs extremos.

La termosonicación (temperatura + HPU) ha sido empleada para la inactivación de microorganismos como *S. typhimurium* o *E. coli* O157:H7 en zumo de mango (Kiang y col., 2013) y enzimas como la PME en zumo de tomate (Raviyan y col., 2005). Para aumentar la intensidad de la cavitación, y por tanto los efectos de los ultrasonidos, se añadió el efecto de la presión a procesos de termosonicación, dando lugar a la manotermosonicación. Ésta también ha sido aplicada para la inactivación de microorganismos en leche (Halpin y col., 2013) y para la inactivación de enzimas como por ejemplo los enzimas pécticos del tomate, lipasas y proteasas (Vercet, 1998; Vercet y col., 2002).

Una de las principales ventajas de la termosonicación frente a la pasteurización, es que las temperaturas empleadas son menores que las asociadas a los tratamientos térmicos, lo que repercute en un ahorro energético, además de que se minimizan las pérdidas de calidad asociadas a las altas temperaturas.

El mecanismo de la inactivación microbiana mediante ultrasonidos de alta intensidad se atribuye fundamentalmente al fenómeno de cavitación, al aumento de temperatura, a la formación de radicales libres, a la turbulencia, al esfuerzo cortante y a la compresión y rarefacción (Knorr y col., 2004). Todos estos fenómenos contribuyen a la destrucción de las células microbianas (Piyasena y col., 2003) y afectan a las estructuras tridimensionales de las enzimas desnaturalizándolas (Raviyan y col., 2005). A pesar de las altas temperaturas, éstas se consideran de poco efecto microbicida por ser muy puntuales, afectando a una región reducida y abarcando por lo tanto a un bajo número de microorganismos. Sin embargo, durante este fenómeno de cavitación, se libera

gran cantidad de energía en forma de presión favoreciendo los procesos de transferencia de materia y diferentes reacciones químicas que también contribuyen a la muerte de los microorganismos.

La extensión de los daños sonoquímicos y físicos varía dependiendo de los parámetros del tratamiento ultrasónico como son la frecuencia y la intensidad. Otros factores que influyen sobre la efectividad microbicida de los ultrasonidos se relacionan con el tipo de microorganismo y su estado fisiológico, el tipo de alimento/medio donde se encuentran y la temperatura del tratamiento (Piyasena y col., 2003).

A pesar de la inactivación microbiana y enzimática lograda mediante la aplicación de HPU, existen limitaciones que hacen necesaria la aplicación combinada de los ultrasonidos con otras tecnologías. Algunas de estas limitaciones están relacionadas con el propio mecanismo de inactivación asociado a los HPU, como la cavitación, responsable de un posible deterioro del alimento por las elevadas temperaturas generadas durante el tratamiento. Asimismo, debido a las elevadas pérdidas de energía conforme aumenta la distancia entre el emisor de ultrasonidos y el medio a tratar, y la elevada cantidad de energía necesaria en el tratamiento de inactivación microbiana, su aplicación queda limitada a tratamientos de contacto o a tratamientos superficiales (Knorr y col., 2004). Además, una limitación importante de los HPU es la dificultad en la comparación entre los trabajos publicados donde se aplica sonicación, termosonicación o manotermosonicación, lo que dificulta la reproducibilidad de los tratamientos. Esta falta de reproducibilidad puede deberse a las múltiples variables involucradas en estos procesos, lo que da lugar a distribuciones muy dispares en el campo acústico y por lo tanto a una elevada heterogeneidad de la intensidad del tratamiento en los diferentes puntos del alimento tratado.

1.7. Combinación de FSC y HPU

La aplicación simultánea de FSC y HPU se ha empleado fundamentalmente en la mejora de los procesos de extracción (Gao y col., 2009; Riera y col., 2010), así como para acelerar reacciones químicas (Trofimov y col., 2001). Sin embargo, la combinación de FSC y HPU en procesos de inactivación microbiana

o enzimática es una tecnología novedosa, de la que no existen referencias bibliográficas.

La nula existencia de trabajos en los que se apliquen simultáneamente ambas tecnologías para la inactivación de microorganismos puede ser debida a la complejidad que conlleva el introducir el sistema de ultrasonidos y sus conexiones eléctricas, en una atmósfera supercrítica, que en presencia de líquidos puede ser altamente conductora y provocar cortocircuitos eléctricos.

En procesos de extracción con FSC asistidos por ultrasonidos, éstos últimos inducen microcorrientes y producen la cavitación de los solventes, con los consiguientes efectos físicos como la ruptura o daño de las paredes y tejidos celulares, el aumento de la difusión del solvente al interior de los poros de la matriz que contiene el soluto, así como la reducción de la resistencia a la transferencia de materia (Gao y col., 2009), favoreciendo estos fenómenos la extracción de los componentes de interés. En este sentido, Riera y col. (2010) aumentaron en un 20 % el porcentaje de aceite de almendra extraído con FSC y ultrasonidos, comparado con la extracción sólo mediante FSC. Por otro lado, Gao y col. (2009) estudiaron la extracción de ésteres de luteína de la caléndula mediante FSC asistidos por ultrasonidos. En este estudio, el coeficiente de transferencia de materia aumentó de $3.1 x 10^{-9}$ a $4.3 x 10^{-9}$ m/s por el efecto de los ultrasonidos, comparado con la extracción supercrítica, y permitió alcanzar grados de extracción similares que con sólo FSC, pero empleando presiones y temperaturas más bajas. Así pues, puede concluirse que la aplicación simultánea de FSC y HPU aumenta la velocidad de los procesos de transferencia de materia.

1.8. Conclusiones

La inactivación de microorganismos y enzimas es fundamental para los procesos de conservación llevados a cabo en la industria alimentaria. En este sentido, los tratamientos con FSC se presentan como una tecnología alternativa prometedora de conservación no térmica.

Los mecanismos de inactivación asociados a esta técnica se basan en la alteración del equilibrio celular como consecuencia de procesos de transferencia de materia, desde el exterior al interior celular, y viceversa.

Factores como la naturaleza de los microorganismos o del medio donde están suspendidos, hacen que, en algunos casos, sea necesario el uso de tiempos largos de tratamiento o condiciones severas de presión y temperatura, que pueden encarecer el proceso y dañar las propiedades sensoriales y nutritivas de los alimentos.

Por otro lado, los HPU son capaces de inactivar microorganismos, pero el uso de los mismos por sí solos como tecnología de inactivación no es factible, por las altas temperaturas alcanzadas en el producto y por los elevados tiempos de tratamiento necesarios. Sin embargo, los HPU son empleados con éxito en numerosos procesos alimentarios para acelerar procesos de transferencia de materia como extracción, emulsión, salado o desalado, deshidratación, etc.

En este contexto, la aplicación de HPU a procesos de inactivación microbiana mediante FSC, donde intervienen mecanismos de trasferencia de materia, tanto del CO_2 al interior celular, como del contenido celular al exterior, podría suponer una mejora de los mismos.

Objetivos

2. OBJETIVOS

La estabilización microbiana y enzimática es un proceso fundamental en la industria alimentaria para garantizar la seguridad microbiológica y mantener las propiedades físico-químicas de los alimentos durante su tiempo de vida útil. Los FSC, tecnología no térmica emergente en los últimos años, han mostrado ser efectivos para inactivar microorganismos y enzimas. Sin embargo, en ciertas ocasiones se requiere de tiempos de tratamiento excesivamente largos o de presiones y temperaturas elevadas, lo que encarece el proceso y podría dañar las propiedades organolépticas y nutricionales de los alimentos.

En este contexto, el objetivo principal de la presente Tesis Doctoral fue estudiar la combinación de los FSC con dos tecnologías no térmicas como son los ultrasonidos de alta intensidad (HPU) y las altas presiones hidrostáticas (HHP), y determinar la influencia de dichas tecnologías sobre los procesos de inactivación llevados a cabo mediante CO_2 supercrítico (SC-CO_2).

Para la consecución de este objetivo general se plantearon diferentes objetivos particulares, entre los que se diferencian los desarrollados con FSC y/o HPU: microbiológicos, llevados a cabo empleando dos tipos de microorganismos, la bacteria *E. coli* DH1 y la levadura *S. cerevisiae* T73, y enzimáticos, llevados a cabo sobre PME; y los desarrollados combinando FSC y HHP: enzimáticos empleando purés de fruta. Estos objetivos particulares pueden resumirse en:

- Estudiar el efecto del estado de crecimiento de los microorganismos sobre su inactivación mediante SC-CO_2. Desarrollar modelos matemáticos que permitan describir las cinéticas de inactivación considerando la fase de crecimiento como una variable del modelo.
- Evaluar el efecto de las condiciones de proceso, tiempo, presión y temperatura, sobre la inactivación microbiana mediante SC-CO_2.
- Desarrollar un dispositivo de HPU acoplado al sistema de SC-CO_2.
- Determinar la influencia de los HPU sobre las cinéticas de inactivación microbiana mediante SC-CO_2.
- Comparar el efecto combinado del uso del SC-CO_2 y los HPU frente al efecto individual de ambas tecnologías.

- Estudiar el efecto de la presión y la temperatura sobre la inactivación microbiana mediante SC-CO_2 asistido mediante HPU.
- Determinar el efecto de la naturaleza del medio donde están suspendidos los microorganismos, sobre sus cinéticas de inactivación mediante SC-CO_2 con y sin aplicación de HPU.
- Estudiar los cambios morfológicos en las células de los microorganismos tras un tratamiento de SC-CO_2 con y sin aplicación de HPU.
- Determinar el efecto de la presión y la temperatura, sobre la inactivación enzimática mediante SC-CO_2 asistido por HPU en matrices alimentarias.
- Desarrollar modelos matemáticos que describan la inactivación microbiana y enzimática con SC-CO_2 asistido mediante HPU, incluyendo los parámetros del proceso como variables del modelo.
- Estudio de la combinación del SC-CO_2 y las altas presiones hidrostáticas (HHP) para la mejora de los procesos de inactivación enzimática en purés de fruta.

Metodología

3. METODOLOGÍA

3.1. Plan de trabajo

Para alcanzar los objetivos planteados, se desarrolló un plan de trabajo general (Fig. 3.1) y la metodología específica para cada uno de ellos. El desarrollo de estos objetivos ha dado lugar a los seis capítulos que constituyen los Resultados de esta tesis doctoral y que pueden dividirse en cuatro grandes apartados.

El primer apartado está formado por el capítulo 1 de Resultados. En los tratamientos con SC-CO_2, el efecto que éste ejerce sobre los microorganismos depende de diferentes factores como la presión, temperatura o duración del proceso (García-González y col., 2007); además, la resistencia que los microorganismos muestran a los tratamientos de inactivación se ve afectada por la fase de crecimiento en la que éstos se encuentran (Hayman y col., 2007), siendo éste un factor poco estudiado en la bibliografía en el caso del SC-CO_2. Por ello, el capítulo 1 hace referencia a los trabajos realizados para caracterizar la respuesta de dos tipos de microorganismos, una bacteria (*E. coli*) y una levadura (*S. cerevisiae*), a la inactivación con SC-CO_2 en función de su fase de crecimiento. En el mismo, se ha desarrollado un modelo matemático para cada uno de estos microorganismos, incluyendo la fase de crecimiento como una variable del modelo. Este primer trabajo dio lugar a un artículo científico publicado en 'The Journal of Supercritical Fluids'.

En determinadas ocasiones, y sobre todo en sistemas discontinuos de SC-CO_2 como el empleado en esta tesis doctoral, los tiempos de tratamiento necesarios para inactivar completamente una población de microorganismos son excesivamente largos. Con el objetivo de reducir los tiempos de proceso, en el segundo apartado de Resultados se evaluó el efecto de la presión y la temperatura sobre las cinéticas de inactivación mediante SC-CO_2. Además se desarrolló un sistema de HPU acoplado al sistema de SC-CO_2 para intentar acelerar el proceso y se determinó el efecto de los HPU sobre las cinéticas de inactivación a distintas presiones y temperaturas. A su vez, se comparó el efecto de la combinación de las dos tecnologías (SC-CO_2+HPU) frente al efecto individual de ambas. Este segundo apartado se divide en dos capítulos en función del tipo de microorganismo estudiado. El capítulo 2 engloba los resultados de la bacteria

E. coli, y el capítulo 3 abarca los correspondientes a la levadura *S. cerevisiae*. Estos capítulos dieron lugar a la publicación de dos artículos científicos publicados en las revistas ‘Innovative Food Science and Emerging Technologies’ y ‘Food Research International’, respectivamente.

La efectividad de la nueva tecnología desarrollada, basada en la combinación de SC-CO_2 y HPU, puede ser dependiente del medio donde se realice el tratamiento, por ello, el tercer apartado estudia la inactivación de ambos microorganismos (*E. coli y S. cerevisiae)* con SC-CO_2 y HPU inoculados en zumo de manzana y de naranja, a diferentes presiones y temperaturas. Por otro lado, en este apartado se estudió el efecto de la combinación de SC-CO_2 y HPU sobre la inactivación de la enzima PME del zumo de naranja. Se desarrollaron modelos matemáticos para describir las cinéticas de inactivación microbiana y enzimática en zumos, incluyendo en estas ecuaciones las condiciones del proceso, presión y temperatura, como variables. Este tercer apartado se divide en los capítulos 4 y 5, correspondientes a las experiencias con zumo de manzana y de naranja, respectivamente. Además, el capítulo 4 incluye un estudio morfológico de ambos microorganismos sometidos a un tratamiento de SC-CO_2 y de SC-CO_2+HPU. Ambos capítulos (4 y 5) han dado lugar a la elaboración de dos artículos científicos publicados en las revistas ‘Food Research International’ y ‘The Journal of Supercritical Fluids’, respectivamente.

El último apartado abarca el capítulo 6 de esta tesis doctoral y está basado en el trabajo desarrollado durante una estancia en la Universidad de Auckland (Nueva Zelanda), de noviembre de 2012 a marzo de 2013. Este trabajo ha sido publicado en ‘The Journal of Supercritical Fluids’ e incluye la combinación de SC-CO_2 con otra tecnología no térmica, que en este caso fueron las HHP para inactivar enzimas en un puré de fruta. Se estudió el efecto de la presión y el efecto de distintas concentraciones de CO_2 sobre la inactivación de tres enzimas (pectin metil-esterasa, polifenoloxidasa y peroxidasa) en un puré de feijoa, una fruta rica en componentes antioxidantes y característica de Nueva Zelanda.

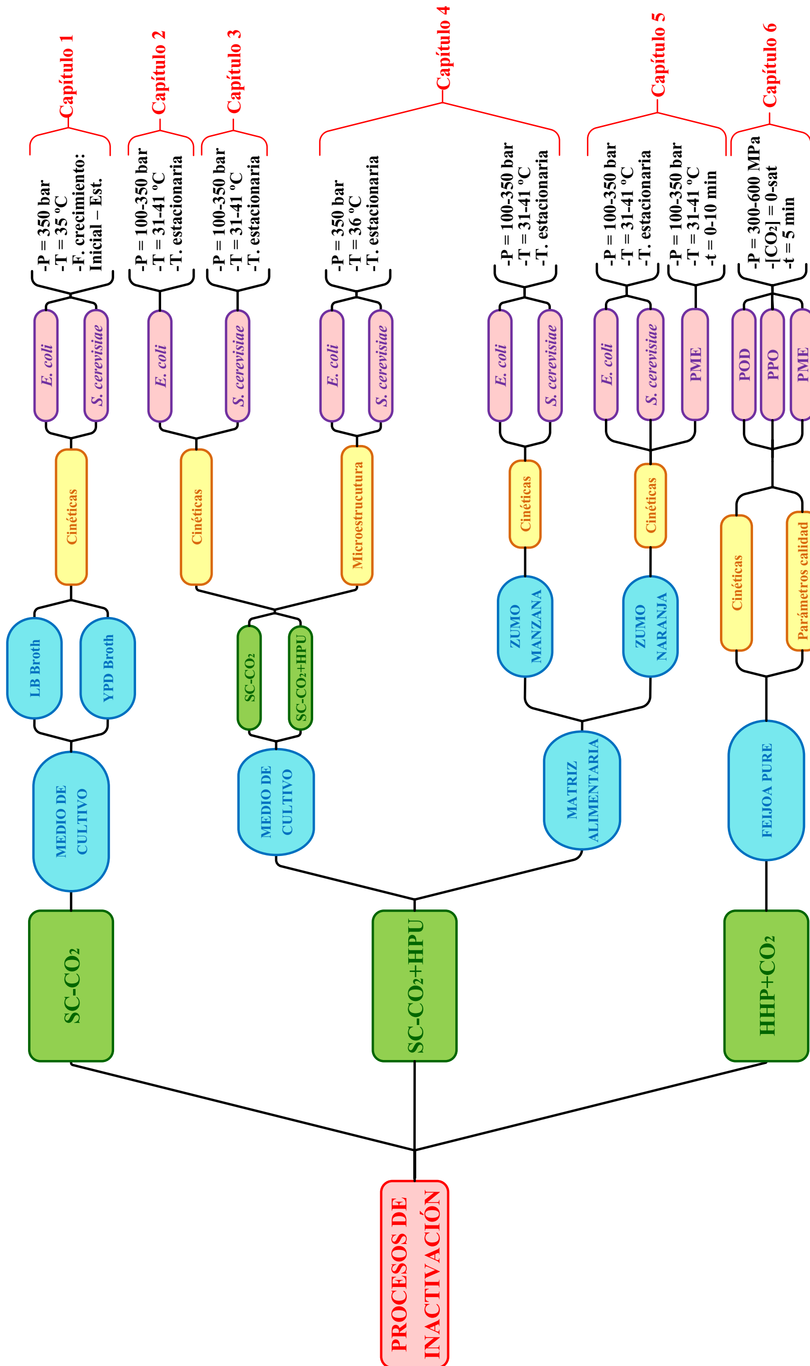


Fig. 3.1. Plan de trabajo

3.2. Microorganismos, medios y condiciones de crecimiento

Los microorganismos empleados en todas las experiencias de inactivación llevadas a cabo en el presente trabajo han sido la bacteria *Escherichia coli* DH1 (*E. coli* - genotipo cromosómico: endA1 gyrA9, thi-1, hsdR179 (rK-, mK+), supE44, relA1) y la levadura *Saccharomyces cerevisiae* Lavin T73 (*S. cerevisiae)*, cepa natural aislada de una fermentación de vino en Alicante (España) (Querol y col., 1992). Estos microorganismos han sido seleccionados porque son componentes habituales de la microflora natural de los alimentos. A menos que se especifique lo contrario, la bacteria *E. coli* creció en Lauira Bertani Broth (LB Broth, Sigma-Aldrich, USA) a 37 ºC y la levadura *S. cerevisiae* creció en Yeast Peptone Dextrose Broth (YPD Broth, Sigma-Aldrich, USA) a 30 ºC, en una cámara incubadora (Fig. 3.2, J.P. SELECTA, Modelo 3000957, Barcelona, España) situada sobre un agitador orbitar a 120 rpm (Fig. 3.2, J.P. SELECTA, Rotabit Modelo 3000974, Barcelona, España).

Los cultivos madre se mantuvieron en Luria Bertani Agar (LB Agar, Sigma-Aldrich, USA) (*E. coli*) y en Yeast Peptone Dextrose Agar (YPD Agar, Sigma-Aldrich, USA) (*S. cerevisiae*), almacenados a 4 ºC y resembrados mensualmente en nuevas placas del agar correspondiente a cada microorganismo.

Fig. 3.2. Cámara de incubación y agitador orbital (J.P. SELECTA)

3.2.1. Estandarización de las curvas de crecimiento

Dado que la fase de crecimiento de los microorganismos afecta a la resistencia que éstos ofrecen a los tratamientos de inactivación, las curvas de crecimiento de *E. coli* y *S. cerevisiae* fueron estandarizadas para conocer el tiempo de incubación necesario hasta alcanzar la fase de crecimiento deseada.

Para ello, una colonia de cada microorganismo fue inoculada en 50 mL de medio de crecimiento estéril y se dejó crecer durante toda la noche a la temperatura adecuada para cada microorganismo. 50 µL del cultivo crecido se inocularon en 50 mL de un nuevo medio estéril, momento inicial de la curva de crecimiento. De dicho cultivo inoculado se determinó la densidad óptica a 600 nm (OD_{600}) mediante un espectrofotómetro (Fig. 3.3, Thermo Electron Corporation, Helios Gamma Model, Unicam, Reino Unido) conforme transcurrió el tiempo de incubación. Para cada medida de OD_{600} se determinó el número de cfu/mL mediante el método de siembra en placa, empleando LB o YPD agar, para *E. coli* y *S. cerevisiae*, respectivamente. De esta forma se obtuvo una curva para cada microorganismo (Fig. 3.4) que reflejó la variación de la OD_{600} con el tiempo de incubación, y que permitió identificar las diferentes fases de crecimiento (Francois y col., 2006).

Fig. 3.3. Espectrofotómetro Thermo Electron Corporation

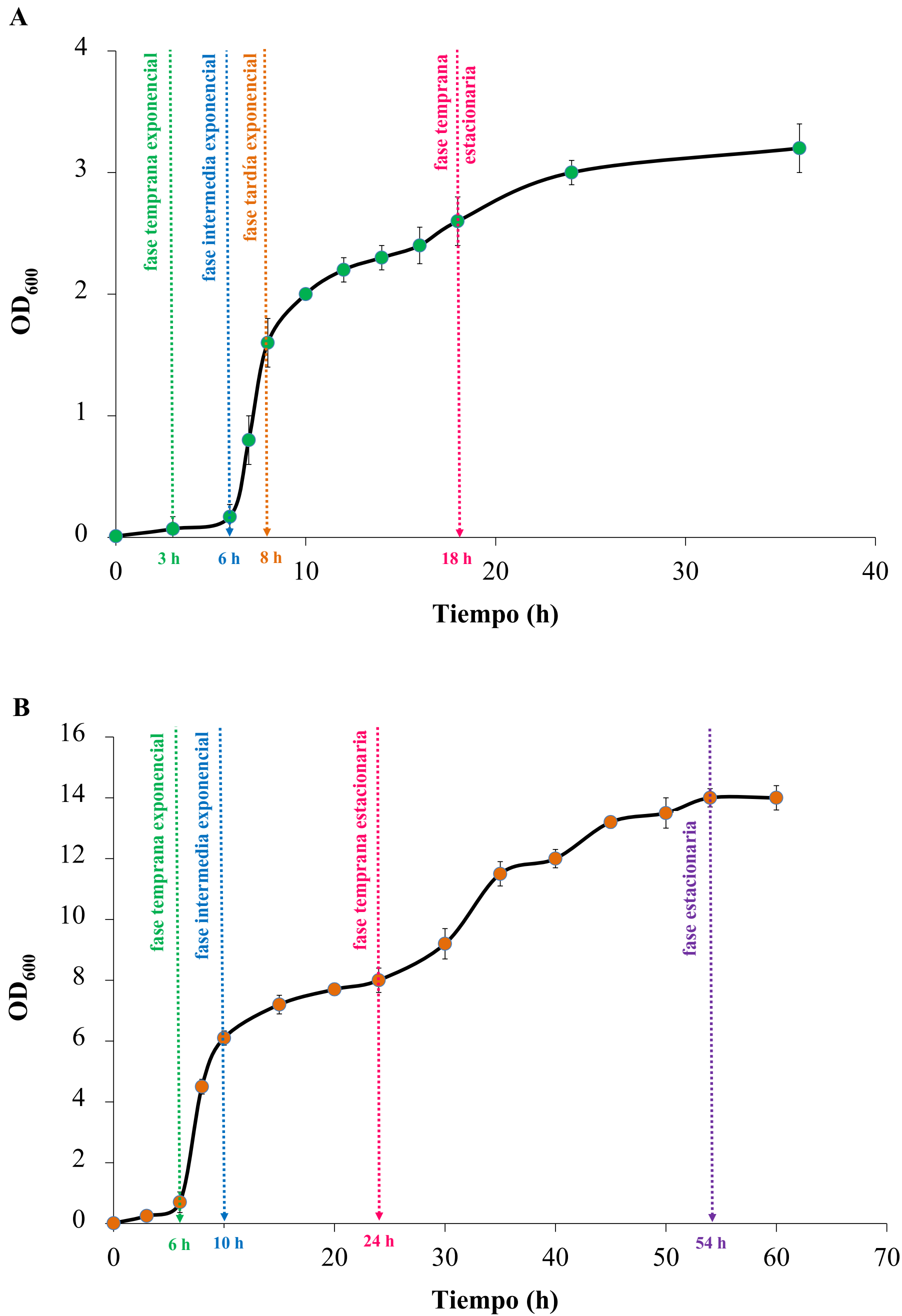


Fig. 3.4. Estandarización de las curvas de crecimiento de *E. coli* (A) y *S. cerevisiae* (B)

3.2.2. Selección de las fases de crecimiento

El primer capítulo de resultados de esta tesis doctoral evaluó la inactivación de los microorganismos seleccionados en diferentes fases de crecimiento, de manera que una vez conocidas las curvas de crecimiento de ambos microorganismos, para trabajar con cada uno de ellos en la fase de crecimiento deseada, la metodología seguida fue la siguiente: una colonia de *E. coli* o *S. cerevisiae* fue inoculada durante una noche en el medio de crecimiento adecuado y a la temperatura idónea para cada microorganismo; 50 µL del cultivo crecido se inocularon en 50 mL de otro medio de cultivo estéril y se dejó creciendo el número de horas adecuado hasta alcanzar la fase de crecimiento seleccionada en cada caso. La bacteria *E. coli* se incubó a 37 ºC durante 3, 6, 8 y 18 h, para obtener las células en fase temprana exponencial ($OD_{600} = 0.07$), intermedia exponencial ($OD_{600} = 0.17$), tardía exponencial ($OD_{600} = 1.6$) y temprana estacionaria ($OD_{600} = 2.6$), respectivamente (Fig. 3.4, A). La levadura *S. cerevisiae* se incubó a 30 ºC durante 6, 10, 24 o 54 h para obtener las células en fase temprana exponencial ($OD_{600} = 0.7$), intermedia exponencial ($OD_{600} = 6$), temprana estacionaria ($OD_{600} = 8$) y estacionaria ($OD_{600} = 14$), respectivamente (Fig. 3.4, B).

En el resto de trabajos llevados a cabo con ambos microorganismos, se seleccionó la fase temprana estacionaria, alcanzada tras 18 h y tras 24 h de crecimiento, para *E. coli* ($OD_{600} = 2.6$) y *S. cerevisiae* ($OD_{600} = 8$), respectivamente.

En cada caso, cuando la fase de crecimiento deseada fue alcanzada, el cultivo crecido se diluyó hasta una concentración de 10^6-10^8 cfu/mL, en el medio donde posteriormente se llevó a cabo el proceso de inactivación.

3.2.3. Medios de tratamiento

En los tres primeros capítulos de Resultados del presente trabajo, las cinéticas de inactivación se llevaron a cabo inoculando los microorganismos, crecidos en sus respectivos medios de cultivo hasta la fase de crecimiento adecuada, en nuevo medio de cultivo estéril, en LB Broth o en YPD Broth, para *E. coli* o *S. cerevisiae*, respectivamente.

En los capítulos 4 y 5, las cinéticas de inactivación se llevaron a cabo en zumo de manzana y zumo de naranja. Para su preparación se partió de fruta fresca. Las manzanas (*Golden delicious*) y naranjas (*Citrus sinensis*) se adquirieron en un mercado local y se mantuvieron a 4 ºC durante dos días hasta su uso. La elaboración de cada zumo se llevó a cabo realizando los siguientes pasos: lavado de la fruta, pelado, troceado y extracción del zumo mediante un extractor de laboratorio (Fig. 3.5, Ultra Juicer, Robot Coupe J80, USA).

El zumo de manzana (pH = 5.4; ºBrix = 15.6) y de naranja (pH = 3.8; ºBrix = 11.6) se repartió en tarros de plástico estériles (60 mL) y se almacenó a -18 ºC hasta su uso.

Fig. 3.5. Extractor de zumo Ultra Juicer

3.3. Equipo de SC-CO_2 asistido por HPU

En el presente trabajo de investigación se ha empleado un equipo de SC-CO_2 diseñado y construido por el grupo ASPA de la UPV, para llevar a cabo los procesos de inactivación microbiana y enzimática. Además, en el marco del presente trabajo, se ha desarrollado un sistema de ultrasonidos acoplado al equipo de SC-CO_2 (Benedito y col., 2011). La Fig. 3.6 muestra el esquema general del equipo de SC-CO_2 asistido por HPU utilizado, que se describirá detalladamente a continuación.

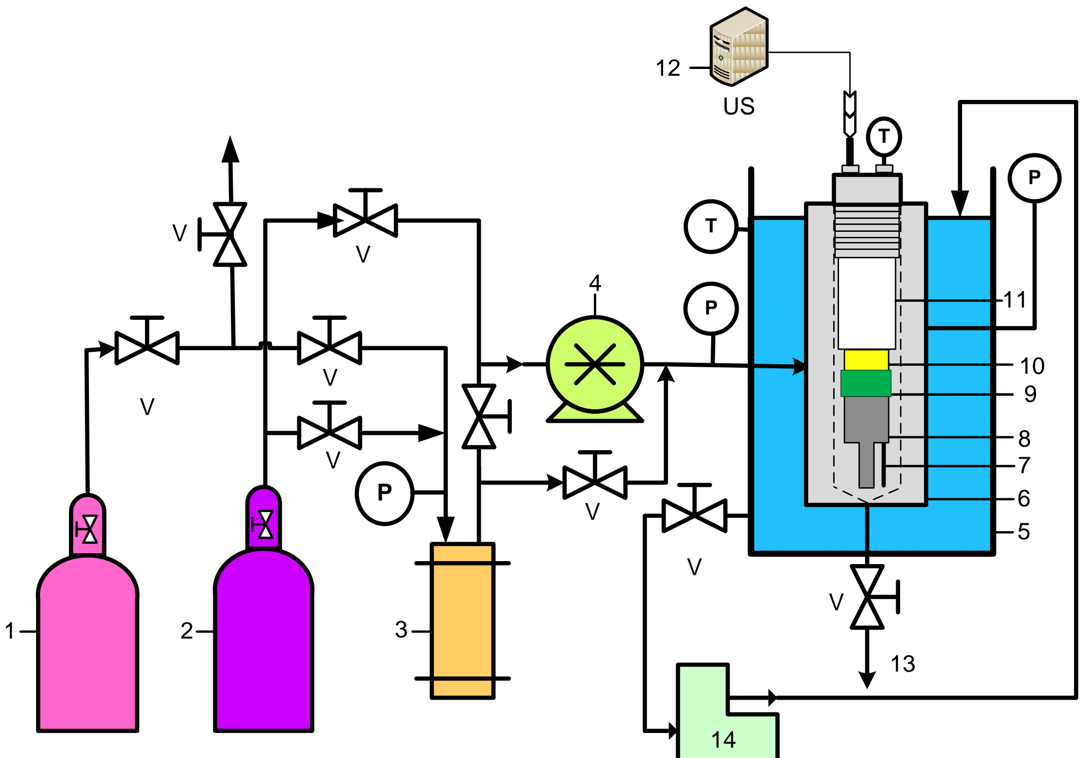


Fig. 3.6. Equipo de fluidos supercríticos asistido por ultrasonidos de potencia. 1-tanque CO_2; 2-tanque de N_2; 3-reserva de CO_2; 4-bomba de CO_2; 5-baño termostático; 6-depósito de tratamiento; 7-sonda de temperatura; 8-transductor; 9-junta de aislamiento; 10-cerámicas; 11-soporte de teflón; 12-generador de ultrasonidos; 13-salida para la toma de muestras; 14-bomba auto-aspirante de recirculación; P: manómetros; T: sensores de temperatura; V: válvulas.

3.3.1. Tanques de CO_2 y N_2

Los tanques de CO_2 (37.5 kg) y N_2 (9.5 m^3) fueron suministrados por la empresa de gases industriales Linde a través de Abelló Linde (Barcelona, España).

Tanto el CO_2 como el N_2 son gases con calidad alimentaria que cumplen las normativas vigentes en cuanto a especificaciones según la Directiva Europea 2008/84/CE y el Real Decreto 1466/2009 en materia de aditivos alimentarios dentro de los países de la UE y en el territorio nacional español.

El tanque de CO_2 se identificó como "dióxido de carbono sonda" y se adquirió a una presión máxima de 60 bar. El tanque de N_2 se adquirió a una presión máxima de 200 bar. Ambos tanques se mantuvieron a temperatura ambiente en un lugar bien ventilado.

3.3.2. Reserva de CO_2

La reserva de CO_2 (3, Fig. 3.6) consistió en un depósito de acero inoxidable tipo 316 de 250 mL situado en el interior de un congelador que se mantiene a -18 ºC ± 3 ºC. Este depósito estaba conectado a la bala de CO_2 y mantuvo en su interior el CO_2 en estado líquido para ser correctamente absorbido por la bomba, y posteriormente inyectado al depósito de tratamiento, donde alcanzó el estado supercrítico.

3.3.3. Bomba

La bomba (Fig. 3.7) consistió en dos bombas de jeringa, de 103 mL cada una, de alta presión y precisión Teledyne ISCO (VERTEX Technics, S.L., Barcelona, España) conectadas a un controlador incorporado al equipo. La bomba de doble jeringa operó en continuo de manera que, mientras un cilindro cargaba CO_2, el otro lo inyectaba y así consecutivamente.

La bomba fue la encargada de absorber el CO_2 desde la reserva de CO_2 a -18 ºC y de inyectarlo al depósito de tratamiento hasta alcanzar la presión requerida. Ésta puede funcionar en modo de presión constante, inyectando el CO_2 al máximo caudal posible hasta alcanzar la presión deseada, y manteniendo ésta constante durante todo el tratamiento; o en modo de flujo constante, inyectando

CO_2 al caudal especificado (1-50 ml/min), teniendo en este caso que controlar manualmente la presión alcanzada en el depósito de tratamiento. La presión máxima de trabajo de la bomba era de 700 bar y las juntas y accesorios de la misma soportaban hasta 200 ºC de temperatura.

Fig. 3.7. Bomba de doble jeringa de alta presión y precisión ISCO

3.3.4. Baño termostático

El baño termostático (1, Fig. 3.8) consistió en un baño de agua en el que estaba sumergido el depósito de tratamiento. Para conseguir la temperatura deseada de proceso, el baño de agua se calentó mediante una resistencia eléctrica sumergible (1500 W-230 V, Ref. 131 CFOR) (2, Fig. 3.8) hasta alcanzar la temperatura deseada en el depósito de tratamiento, que era registrada por un termopar conectado a un controlador digital de temperatura (E5CK, Omron, Hoofddorp, Holanda). Dicho termopar estaba situado en el depósito de tratamiento alcanzando su extremo la parte interna del depósito. La temperatura del tratamiento se cambiaba ajustando el set-point del controlador, manteniéndose constante durante todo el tratamiento.

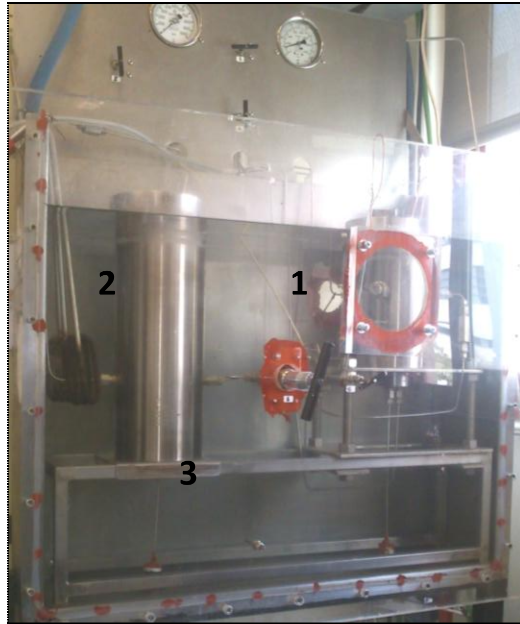


Fig. 3.8. Depósito de tratamiento y elementos auxiliares. 1: Baño termostático; 2-resistencia eléctrica; 3-depósito de tratamiento.

Asimismo, el baño termostático disponía de una bomba auto-aspirante (14, Fig. 3.6) con prefiltro incorporado (Potencia = 1045 W; Modelo SWT75M, Ebara, Madrid, España) que recirculaba continuamente el agua del baño para mantener una temperatura uniforme en el depósito durante todo el proceso. La temperatura del baño también se podía conocer mediante una termorresistencia tipo Pt100 sumergida a una altura intermedia del baño y conectada a otro controlador digital (E5CK, Omron, Hoofddorp, Holanda).

3.3.5. Depósito de tratamiento

El depósito de tratamiento (3, Fig. 3.8) estaba sumergido dentro del baño termostático, y por tanto, su temperatura era regulada por la temperatura del mismo. El depósito estaba fabricado en acero inoxidable tipo 316, tenía un volumen interno de 500 mL y el espesor de su pared era de 3 cm.

Para conocer y mantener la presión del depósito de tratamiento a la presión deseada, se emplearon tres elementos: un manómetro (PTG Pressure Technology, Gesellschaft für Hochdrucktechnik mbH, FModelo EN 837-1, Alemania) de

muelle sumergido en glicerina, con doble escala (psi: 0-14000; bar: 0-1000), un sensor de presión WIKA (Ref. S: 3806415; Ref. P: 8373773) con membrana de acero inoxidable en una escala de 0 a 1000 bar y la bomba inyectora de CO_2, detallada anteriormente.

La presión de trabajo requerida en el proceso se especificó en el set-point del controlador de la bomba inyectora de CO_2, que está a su vez conectado con el sensor de presión del depósito de tratamiento. Desde el controlador se ordenó manualmente que la bomba inyectase CO_2 al depósito, empleando un caudal de 50 mL/min. La bomba se paró automáticamente cuando la presión en el depósito fue la especificada en el set-point, y la mantuvo constante durante todo el tratamiento. El manómetro permitió conocer la presión del depósito en todo momento.

3.3.6. Sistema de ultrasonidos

Una de las limitaciones asociadas a los tratamientos de inactivación con SC-CO_2 en sistemas discontinuos son los largos tiempos de tratamiento. En este contexto, uno de los objetivos de esta tesis fue acelerar el proceso de inactivación supercrítica, para lo cual se desarrolló un sistema de ultrasonidos acoplado al equipo de SC-CO_2. Tras el desarrollo del mismo y en vista de los resultados favorables obtenidos, el proceso combinado de SC-CO_2 y HPU fue patentado junto con el procedimiento de inactivación (Benedito y col., 2011) por la UPV.

El sistema consistió en un transductor insertado en el depósito de tratamiento constituido por dos cerámicas comerciales (4, Fig. 3.9: 35 mm diámetro externo; 12.5 mm diámetro interno; 5 mm de espesor) de 30 kHz de frecuencia y un sonotrodo de aluminio (calidad 7075) (2, Fig. 3.9), especialmente construido para concentrar la mayor cantidad posible de energía acústica en su extremo, y cuyas dimensiones se especifican en la Fig. 3.11 Para controlar la temperatura del producto, el sistema llevaba acoplado una sonda de temperatura (termopar tipo K, 1, Fig. 3.9) sujeto al sistema mediante un soporte de teflón (6, Fig. 3.9), que se conectaba a un controlador de temperatura externo.

El generador de ultrasonidos estaba conectado al transductor mediante una conexión situada en la cabeza del tapón (9, Fig. 3.9). La conexión entre el

generador y el transductor estaba constituida por un cable de cobre recubierto por polietileno (RG178PE) que quedó sujeto al sistema mediante un soporte de teflón (6, Fig 3.9.) para evitar posibles roturas de las conexiones.

El generador de ultrasonidos suministraba una potencia constante al transductor durante el tratamiento de inactivación de 40 W ± 5 W (I = 250 mA ± 10 mA; U = 220 V ± 5 V). La potencia suministrada fue registrada mediante un medidor de potencia digital (Modelo WT210, Yokogawa Electric Corporation, Japón; Fig. 3.10).

Una de las partes críticas del sistema desarrollado es el aislamiento de las conexiones eléctricas. Para ello, las cerámicas se protegieron con una funda de polipropileno (4, Fig. 3.9), y además, se colocó una junta de polipropileno (3, Fig. 3.9) para evitar que el líquido pasara a la zona de las conexiones eléctricas. Este aislamiento protegió al transductor de posibles cortocircuitos que podrían producirse al trabajar en una atmósfera altamente conductora.

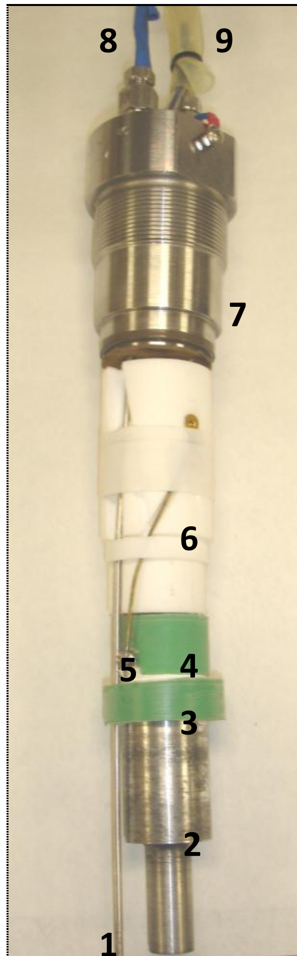


Fig. 3.9. 1: Detalle del sistema de ultrasonidos de potencia. 1-sonda de temperatura; 2-sonotrodo; 3-junta de aislamiento de polipropileno; 4-cerámicas protegidas con polipropileno; 5-conexiones eléctricas; 6-soporte de unión al tapón; 7-tapón del depósito; 8-conexión a un controlador de temperatura; 9-conexión al generador de ultrasonidos.

Fig. 3.10. Medidor de Potencia digital, Yokogawa, Modelo WT210

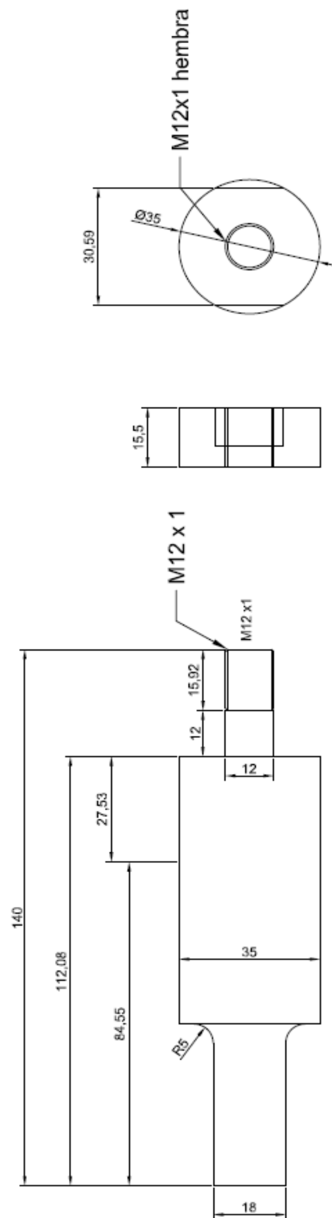


Fig. 3.11. Dimensiones del transductor de ultrasonidos

3.4. Tratamientos de FSC asistidos por HPU

Cada experiencia de inactivación requería de seis pasos: calentamiento del baño termostático, limpieza y desinfección del depósito de tratamiento, carga de las bombas, preparación de la muestra, tratamiento de inactivación y toma de muestras.

En primer lugar, se procedía al calentamiento del baño termostático para lo cual se conectaba la resistencia y la recirculación del baño, y se especificaba la temperatura deseada en el set-point del controlador de temperatura.

El proceso de limpieza se basaba en hacer pasar una disolución (1-4 % v/v) de un desinfectante industrial para superficies externas (Diversey, Dellated, Barcelona) por el depósito de tratamiento durante 5 min a temperatura ambiente. A continuación, se hacía pasar agua destilada por duplicado y por ultimo agua autoclavada.

La carga de las bombas consistía en llenar las mismas de CO_2. Para ello, en primer lugar se despejaba todo el aire contenido en la reserva de CO_2 que estaba a -18 ºC, a continuación se abría el tanque de CO_2 y se llenaba el depósito de reserva. Seguidamente, se abría el tanque de N_2 y se presurizaba el depósito de reserva. Así el CO_2 estaba a -18 ºC y a una presión de 200 bar, para asegurar que se encontraba en estado líquido. Una vez la reserva estaba llena de CO_2 líquido, se procedía al llenado de las bombas, que absorbían el CO_2 procedente de la reserva.

Una vez el baño estaba a la temperatura deseada, el depósito estaba limpio y desinfectado, y las bombas estaban cargadas de CO_2, se procedía a la preparación de la muestra.

La preparación de la muestra a tratar consistía en la disolución de un cultivo concentrado de microorganismos, crecidos en condiciones de temperatura controlada, durante un determinado tiempo, hasta una concentración de 10^6-10^8 cfu/mL. Los microorganismos crecían en un medio de cultivo específico para ello (apartado 3.2), y la disolución podía realizarse en el mismo medio de cultivo estéril o en matrices más complejas como zumos, tal y como se especifica en el apartado 3.2.3. El volumen final de muestra a tratar era de 60 mL.

Una vez preparada la muestra, el tratamiento de la misma consistió en introducirla inmediatamente en el depósito de tratamiento y cerrarlo. En dicho momento, las bombas inyectaban CO_2 hasta que se alcanzaba la presión deseada, tiempo que variaba de 1 a 4 min al operar entre 100 y 350 bar, respectivamente. Para procesos con solo SC-CO_2, el tiempo cero de cada tratamiento se consideraba cuando dentro del depósito se había alcanzado las condiciones de presión y temperatura deseadas. Para tratamientos con SC-CO_2 asistidos por HPU, una vez que se alcanzaba la presión y la temperatura deseada, los HPU eran conectados, momento considerado como tiempo cero de tratamiento. Los HPU suministraban una energía constante de 40 W ± 5 W (I = 250 mA ± 10 mA; U = 220 V ± 5 V) durante todo el tratamiento. Las bombas y el baño eran los encargados de mantener la presión y la temperatura de tratamiento constante durante todo el proceso.

Durante la ejecución del tratamiento, se extraían muestras (13, Fig. 3.6) de 1-3 mL por un tubo (2 mm de diámetro) situado en la parte inferior del depósito de tratamiento, a intervalos de entre 20 s y 5 min, dependiendo de las condiciones del proceso. Entre cada toma de muestra, el tubo se limpiaba y desinfectaba con 3 mL de etanol (96 %v/v).

La viabilidad de los microorganismos tras cada tratamiento era evaluada posteriormente mediante los análisis microbiológicos.

3.5. Viabilidad de los microorganismos

La viabilidad de las células tratadas fue evaluada mediante la siembra y conteo en placa. Durante el tratamiento, se tomaban diferentes muestras del depósito de inactivación a diferentes tiempos, en función de las condiciones del tratamiento. Cada muestra fue diluida en agua autoclavada hasta la dilución adecuada y sembrada en LB Agar o YPD Agar e incubada durante 24 h a 37 ºC o 30 ºC, para *E. coli* o *S. cerevisiae*, respectivamente. El número de células en la muestra inicial no tratada se determinó siguiendo el mismo procedimiento.

Los resultados son el promedio de tres experiencias por condición y cada muestra se replicó en al menos tres placas.

3.6. Modelización

En la mayoría de los capítulos desarrollados en el presente trabajo de investigación se han modelizado matemáticamente las cinéticas de inactivación. El propósito de la modelización matemática de procesos de inactivación fue predecir el efecto de diferentes tratamientos sin llevar a cabo numerosos experimentos así como evaluar matemáticamente el efecto de las variables del proceso.

Tradicionalmente se ha asumido que la inactivación microbiana, tanto térmica como no térmica, podía modelizarse mediante cinéticas de primer orden (Aragao y col., 2007). Sin embargo, hay suficientes evidencias experimentales que demuestran que la inactivación de muchos microorganismos no sigue dicho comportamiento (Peleg, 2006). Estas cinéticas de inactivación que no siguen un comportamiento log-lineal, pueden ser descritas por un amplio número de modelos matemáticos basados en otras suposiciones (Aragao y col., 2007). En este contexto, una curva de supervivencia, por definición, es una forma de expresar una distribución temporal de la mortalidad de microorganismos (Peleg, 2006). Los datos de supervivencia se presentan como la relación N/N_0 frente al tiempo, donde N_0 es el número de microorganismos iniciales en una muestra en el tiempo 0, y N es el número de microorganismos después de un determinado tiempo de tratamiento. Estas curvas de supervivencia han sido ajustadas a un gran número de modelos, algunos de los cuales han sido empleados en el presente trabajo de investigación y se describen a continuación.

- Modelo de Fermi

Este modelo representa una curva de supervivencia en la que existe una fase de latencia seguida por una inactivación lineal (Peleg, 2006). Este tipo de cinéticas de inactivación responde a la Eq. (3.1).

$$\ln\frac{N}{N_0} = -\ln\left(1+e^{k(t-t_c)}\right) \qquad \text{Eq. (3.1)}$$

donde k y t_c son constantes cinéticas; k es la constante de velocidad (min^{-1}) y t_c (min) es el tiempo que transcurre hasta que las células comienzan a verse afectadas por el tratamiento (McKellar y Lu, 2004; Peleg, 2006).

- Modelo de Gompertz

El modelo de Gompertz ha sido empleado para describir curvas de crecimiento sigmoidales, que incluyen una fase de latencia, una fase exponencial y una fase de crecimiento constante relacionada con la fase estacionaria. Este modelo fue modificado por Zwietering y col. (1990) para incluir parámetros con significado biológico, y Kim y col. (2007) lo adaptaron posteriormente para describir cinéticas de inactivación microbiana, obteniendo la Eq. (3.2).

$$\log_{10} \frac{N}{N_0} = A\, e^{-e^{\left(-k_d\, e\, (\lambda - t)/A + 1\right)}} \qquad \text{Eq. (3.2)}$$

donde A es el valor mínimo de la curva, k_d es la velocidad máxima de inactivación, λ es la duración de la fase de latencia y e es la constante 2.718 (Zwietering y col., 1990; Kim y col., 2007).

- Modelo modificado de Gompertz

El modelo de Gompertz fue modificado por Linton y col. (1996) para describir diferentes tipos de curvas de inactivación, expresando dicho modelo con la Eq. (3.3):

$$\log_{10} \frac{N}{N_0} = Ce^{-e^{A+Bt}} - Ce^{-e^{A}} \qquad \text{Eq. (3.3)}$$

donde A, B y C son tres parámetros que representan las diferentes regiones de la curva de supervivencia: la fase de latencia (A), la velocidad máxima de inactivación (B) y la reducción final de supervivientes (C).

- Modelo de Weibull

El modelo de Weibull supone que la inactivación microbiana se puede considerar como la ruptura de partículas bajo una tensión mecánica (Peleg, 2006). Bajo esta suposición, la función que describe este comportamiento en microorganismos viene descrita por la Eq. (3.4), siendo ésta la versión más aceptada del modelo de Weibull (Corradini y Peleg, 2012)

$$\log_{10} \frac{N}{N_0} = -b\, t^{n} \qquad \text{Eq. (3.4)}$$

donde b es un parámetro de velocidad no lineal y n es el parámetro responsable de la forma de la curva. Una curva de supervivencia cóncava hacia arriba estará representada por $n < 1$; una curva cóncava hacia abajo por $n > 1$ y una curva de supervivencia log-lineal es un caso especial del modelo en el que $n = 1$ (Corradini y Peleg, 2012).

- Modelo Bifásico

El modelo bifásico es un modelo de tres parámetros que asume dos comportamientos diferentes en una misma población de microorganismos, una proporción más sensible al tratamiento y otra más resistente, y se define mediante la Eq. (3.5) (Lee y col., 2009).

$$\log_{10}\frac{N}{N_0} = \log_{10}\left[(1-f)\,10^{\frac{-t}{D_{sens}}} + f\,10^{\frac{-t}{D_{res}}}\right] \qquad \text{Eq. (3.5)}$$

donde (1-f) y f son la fracción de células más sensibles y más resistentes al tratamiento, respectivamente. D_{sens} y D_{res} son el tiempo de reducción decimal (min) de la población más sensible y más resistente, respectivamente (Lee y col., 2009).

- Modelo Logístico

El modelo logístico fue propuesto originalmente por Cole y col. (1993) para describir cinéticas microbianas de inactivación térmica. Posteriormente, Chen y Hoover (2003) modificaron la ecuación para reducir el número de parámetros, siendo esta última versión (Eq. (3.6)) la empleada en el presente trabajo.

$$\log_{10}\frac{N}{N_0} = \frac{Q}{1+e^{4\sigma\frac{\tau-\log_{10}t}{Q}}} + \frac{Q}{1+e^{4\sigma\frac{\tau-\log_{10}t_0}{Q}}} \qquad \text{Eq. (3.6)}$$

donde Q es la diferencia entre el valor máximo y el mínimo de los valores de $\log_{10}(N/N_0)$, σ es la velocidad máxima de inactivación y τ es el tiempo para alcanzar la velocidad máxima de inactivación (Lee y col., 2009).

- Curvas sigmoidales y semi-logarítmicas

Los tratamientos de inactivación pueden eliminar rápidamente los microorganismos más débiles de una población, y posteriormente sensibilizar a los más resistentes (Peleg, 2006). Si esto sucede, la curva de supervivencia es cóncava hacia arriba inicialmente, e irá cambiando a cóncava hacia abajo gradualmente a medida que avanza el tratamiento. Este tipo de curva de supervivencia sigmoidal se describe en Peleg (2006) y se ha definido en el presente trabajo como modelo de "Peleg Tipo A" (Eq. (3.7)). Un escenario alternativo podría venir dado por un proceso de inactivación en el que el daño acumulado disminuye la resistencia al tratamiento de los miembros más débiles de la población y, una vez que estos son eliminados, el resto se hacen progresivamente más resistentes. Esto se refleja en una curva de supervivencia semi-logarítmica que es inicialmente cóncava hacia abajo y cambia a cóncava hacia arriba, conforme avanza el tratamiento. Este tipo de curva se ha descrito en Peleg (2006) y se ha definido en este estudio como modelo de "Peleg Tipo B" (Eq. (3.8)).

Peleg Tipo A $$\log_{10}\frac{N}{N_0} = -\frac{a_1\, t^r}{(1+a_2 t)(a_3 - t)} \qquad \text{Eq. (3.7)}$$

Peleg Tipo B $$\log_{10}\frac{N}{N_0} = -\frac{b_1\, t^r}{b_2 + t^r} \qquad \text{Eq. (3.8)}$$

donde a_1, a_2 y a_3 y b_1, b_2 y r, son parámetros del modelo Peleg Tipo A y B, respectivamente.

Además, se modelizaron las cinéticas de inactivación de la enzima PME. Se utilizaron dos modelos, el modelo de Weibull, detallado anteriormente y el Modelo Fraccional, que se describe mediante la Eq. (3.9.).

$$\frac{A - A_f}{A_0 - A_f} = e^{-kt} \qquad \text{Eq. (3.9)}$$

donde A_0 es la actividad enzimática del zumo de naranja sin tratar, A es la actividad enzimática del zumo tras un tiempo de tratamiento t, A_f es la actividad enzimática al final del tratamiento y k es el parámetro de velocidad de inactivación enzimática (Polydera y col., 2004).

Results & Discussion

Chapter 1

Supercritical Carbon Dioxide Inactivation of Escherichia coli and Saccharomyces cerevisiae in Different Growth Stages

C. Ortuño, M.T. Martínez-Pastor, A. Mulet, J. Benedito

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Supercritical carbon dioxide inactivation of *Escherichia coli* and *Saccharomyces cerevisiae* in different growth stages

C. Ortuño, M.T. Martínez-Pastor, A. Mulet, J. Benedito

ASPA Group, Food Technology Department, Universitat Politècnica de València, Camí de Vera s/n, E46022, Valencia, Spain.

ABSTRACT

The aim of this work was to investigate the influence of the culture growth stage on the inactivation kinetics of *Escherichia coli* and *Saccharomyces cerevisiae* using supercritical carbon dioxide (SC-CO_2) and to find models that can describe and predict the inactivation behavior of these microorganisms considering the growth stage as one of the model parameters. Cultures of *E. coli* and *S. cerevisiae* were grown to four different growth stages: early exponential phase, intermediate exponential phase, late exponential phase and early stationary phase and then treated with SC-CO_2 at 350 bar and 35°C. The inactivation kinetics of *S. cerevisiae* and *E. coli* showed that the SC-CO_2 resistance increased progressively as the growth phase advanced. For both microorganisms, the length of the lag phase increased progressively as the growth phase advanced, not appearing at all in the earliest growth stages. For *S. cerevisiae*, an equation based on the Gompertz Function satisfactorily described (R^2_{avg} = 0.93; $RMSE_{avg}$ = 0.59) the inactivation kinetics of this microorganism for the four growth stages selected. Similarly, a single equation that included the dependence on the growth stage was obtained for *E. coli*, based on the Weibull Function (R^2_{avg} = 0.96; $RMSE_{avg}$ = 0.53). The results reveal that the inactivation kinetics using SC-CO_2 are greatly influenced by the growth stage and the application of the developed models could be used to find the optimal process conditions according to the cell's growth stage.

Key words: supercritical carbon dioxide, growth stage, *Saccharomyces cerevisiae*, *Escherichia coli*, inactivation, modeling.

1. INTRODUCTION

Both the increasing consumer demand for natural, fresh food, free of chemical preservatives, and the current trends to avoid processes which may produce a loss of flavor, color and nutrients, such as thermal pasteurization, have led to the food industry developing an interest in non-thermal processing. New preservation technologies are the subject of intense study whose aim is to minimize the effect on the organoleptic and nutritional properties of food. Among these techniques may be cited, high hydrostatic pressure (Erkem & Dogan, 2004); pulsed electric fields (Cortes et al., 2008); membrane filtration (Rektor et al., 2004); microwaves and radiofrequency (Purevdorj et al., 2002) or supercritical fluids (Zhang et al., 2006).

Supercritical carbon dioxide (SC-CO_2) has a liquid-like density, gas-like diffusivity and viscosity, and a zero surface tension (McHugh & Krukonis, 1994). It is, therefore, capable of penetrating into complex structures, which provides it with its ability to inactivate microorganisms (Spilimbergo et al., 2009). Furthermore, the properties of SC-CO_2 make it an excellent solvent to be used in the food sector, especially in extraction processes (Riera et al., 2010).

SC-CO_2 showed promising results in the inactivation of microorganisms like *Saccharomyces cerevisiae* (*S. cerevisiae*) in apple juice (Spilimbergo et al., 2007), or naturally occurring microorganisms in liquid whole egg (Garcia-Gonzalez et al., 2009).

In the SC-CO_2 inactivation treatments, different factors can affect the sensitivity of microorganisms to the treatment, including the species and strain of the microorganism, the composition of the suspending medium, or the pressure, temperature and duration of the process (Garcia-Gonzalez et al., 2007; Spilimbergo & Ciola, 2010).

The resistance of a microorganism to different stresses, including high pressure processing, is known to be affected by the growth stage (Casadei et al., 2002). Hayman et al. (2007) found a significant effect of the growth temperature (15 and 43 ºC) and the growth phase (mid-exponential, late exponential or mid-stationary phases) on high pressure processing. It is, therefore, to be expected

that the growth phase could be a relevant factor in the effectivity of SC-CO_2 treatments.

Numerous microbial strains have been used in order to evaluate their sensitivity to SC-CO_2 treatments. The species that have been investigated ranged from Gram-negative bacteria like *Salmonella typhimurium, Escherichia coli* or *Yersinia enterocolitica,* to Gram-positive or yeasts, like *S. cerevisiae, L. innocua* or *L. monocytogenes.* Several studies have indicated that Gram-negative bacteria are more sensitive to inactivation treatments than Gram-positive bacteria (Ramirez Santos et al., 2005). Both *E. coli* and *S. cerevisiae* are habitual components of the microbiota involved in food spoilage, with *E. coli* frequently being the culprit for the deterioration of fresh meat and meat derivatives. As a natural commensal in human intestines, *E. coli* had for long been considered quite harmless, but in recent years pathogenic strains like *E. coli* O157:H7 have been increasingly involved in food poisoning outbreaks (Buzrul, 2009). On the other hand, several techniques used in food preservation favour the growth of yeast in detriment of bacteria, like a low pH or high sugar levels, and spoil foods such as fruits, juices, vegetables and to a lesser extent, cheese or meat (Loureiro, 2000). Yeast species known to be involved in food spoilage are *Saccharomyces*, *Candida* or *Zygosaccharomyces*.

The purpose of the mathematical modeling of inactivation processes is to assess the effect of different inactivation treatments on microbial population without performing numerous experiments. Moreover, the process parameters can be better understood and different scenarios can be foreseen. Different inactivation models have been described being the Weibull model one of the most used. This model describes the inactivation of *Bacillus* sp. P34 by means of conventional thermal processes (Sant'Anna et al., 2010) and also the inactivation of *E. coli* by means of new preservation methods, such as high hydrostatic pressure (Buzrul, 2009). Moreover, Liao et al. (2010) used the Gompertz Model and a modified Logistic equation to describe the inactivation of *S. typhimurium* using dense phase CO_2 in carrot juice.

Most of the studies covering SC-CO_2 modeled the inactivation kinetics considering the time or the intensity of treatment as the main factors. However,

no study has been found in the literature considering the effect of the microorganism growth stage as a parameter to be included in the SC-CO_2 kinetic inactivation models.

The aim of this work was to study the influence of the culture growth stage of *E. coli* and *S. cerevisiae* on their inactivation kinetics using SC-CO_2 and to find models that can describe and predict the inactivation behavior of these microorganisms considering the growth stage as one of the model parameters.

2. MATERIALS AND METHODS

2.1. Microorganisms, media and growth conditions

The microbial strains used in this study were *E. coli* DH1, (chromosomal genotype: endA1 *gyr*A9, *thi*-1, *hsd*R179 (r_k^-, m_K^+), *sup*E44, ***rel***A1) and *Saccharomyces cerevisiae* T73. *S. cerevisiae* Lalvin T73 (abbreviated as T73) is a natural strain isolated from wine fermentation in Alicante (Spain) (Querol et al., 1992), and is commercialised as Lalvin T73 (Lallemand Inc., Montreal, Canada).

Unless otherwise specified, *E. coli* was grown in Luria Bertani Broth (LB Broth, Sigma-Aldrich, USA) at 37 ºC, and *S. cerevisiae* was grown in Yeast Peptone Dextrose Broth (YPD Broth, Sigma-Aldrich, USA) at 30 ºC, using an incubation chamber (J.P. SELECTA, Model 3000957, Barcelona, Spain) and an orbital shaker at 120 rpm (J.P. SELECTA, Rotabit Model 3000974, Barcelona, Spain). Stock cultures were maintained in Luria Bertani Agar (LB Agar, Sigma-Aldrich, USA) (*E. coli*) and Yeast Peptone Dextrose Agar (YPD Agar, Sigma-Aldrich, USA) (*S. cerevisiae*), stored at 4 ºC and transferred monthly to new plates.

To standardize the growth curves, a single colony of each microorganism was inoculated in 50 ml of sterile medium and grown overnight at the standard temperature. 50 µl or 100 µl of this starter culture were transferred to a new sterilized medium and the growth was determined by both plating and the measurement of optical density at 600 nm (OD_{600}) using a UV-visible spectrophotometer (Thermo Electron Corporation, Helios Gamma Model, Unicam, England (Francois et al., 2006). A linear relationship between OD_{600} and

cell concentration was found for the 0.1-0.7 range (data not shown). Accordingly, as the culture growth advanced, different dilutions of the culture were used in order to read the cell concentration in this range. Consequently, growth phase was determined by OD_{600} measures in all experiments.

2.2. Sample preparation

A single colony of *E. coli* was inoculated on LB Broth and grown overnight at 37 ºC. Four subcultures, prepared by inoculating 50 µl from the starter in 50 ml of sterilized medium, were incubated at 37 ºC for 3, 6, 8 and 18 h to obtain cells in the early exponential phase (OD_{600} = 0.07), intermediate exponential phase (OD_{600} = 0.17), late exponential phase (OD_{600} = 1.6) and early stationary phase (OD_{600} = 2.6), respectively.

A single colony of *S. cerevisiae* was inoculated and grown on YPD Broth at 30 ºC overnight. Four subcultures, prepared by inoculating 100 µl from the starter in 50 ml of sterilized YPD Broth were incubated at 30 ºC for 6, 10, 24 and 54 h to obtain cells in the early exponential phase (OD_{600} = 0.7), intermediate exponential phase (OD_{600} = 6), early stationary phase (OD_{600} = 8) and stationary phase (OD_{600} = 14), respectively.

When the desired growth stage was reached, the culture was diluted to a cell concentration of 10^7 cfu/ml and subjected to the SC-CO_2 treatment.

2.3. Enumeration of viable microorganisms

The viability of *S. cerevisiae* and *E. coli*, was determined by the plate count method. Samples were taken from the inactivation vessel at different times. Each sample was serially diluted with sterilized water, and 100 µl of the appropriate dilution were plated in triplicate in YPD Broth (yeast) or LB Broth (bacteria) plates. Yeasts were incubated at 30 ºC for 48 h and *E. coli* at 37 ºC for 24 h, after which time cells were counted and the arithmetic mean of every three plates calculated. Microbial cells in the initial non-treated sample were counted by the same procedure described for the treated samples. Moreover, each experimental run was carried out in triplicate and the arithmetic mean and the standard deviation of the three experiments was reported as the final result.

2.4. Supercritical fluid plant

SC-CO_2 experiments were carried out in a supercritical fluid pilot-scale plant (Fig. 1). The apparatus was designed in order to withstand up to 1000 bar. The plant includes a CO_2-tank (1) and a N_2-tank (2), which are kept at room temperature; a chiller reservoir stored at -18 ºC (3); a pump (4) and a thermostatic bath (5) to keep the inactivation vessel (6) at the desired temperature.

Liquid carbon dioxide was supplied from the tank to the chiller reservoir in which it was compressed to 200 bar by means of the injection of gaseous N_2. The liquid CO_2 was supplied from the bottom of the chiller reservoir to the pump where it was compressed at the desired pressure prior to injection into the inactivation vessel. The vessel (500 ml internal volume) and the different connections and valves in contact with SC-CO_2 are made of stainless steel type 316. Pressure and temperature gauges are installed in the inactivation vessel to monitor that the supercritical conditions were reached in a short time and subsequently maintained during processing.

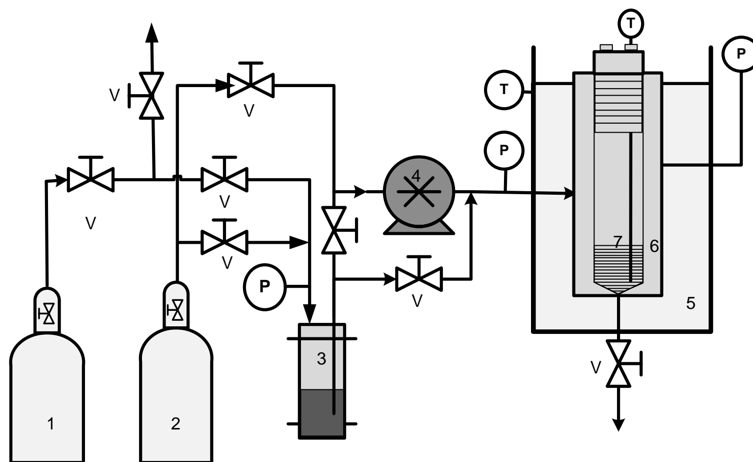

Fig. 1. Supercritical CO_2 treatment system. 1-CO_2 tank; 2-N_2 tank; 3- Chiller reservoir; 4-Pump; 5- Temperature controlled bath; 6- Treatment vessel; 7- Sample; V-Valve; P-Manometer; T-Temperature sensor.

2.5. Supercritical fluid processing

A single experimental run in the plant required four operating steps: plant cleaning, sample preparation, SC-CO_2 treatment and sample extraction. The sample (60 ml) was loaded into the inactivation vessel which had been previously sterilized in the cleaning step, and immediately sealed. The pump filled the vessel with supercritical carbon dioxide reaching the desired pressure in less than 2.5 min. The initial treatment time was considered when the vessel reached the experimental pressure. All the experiments were carried out at 350 bar and 35 ºC and the pump kept the pressure constant during the experiment.

Samples of 3 ml were extracted through a capillary tube located at the bottom of the inactivation vessel at different times until the end of the experiment. In this way, a sudden depressurization of the samples is obtained, changing the pressure from 350 bar to atmospheric pressure. This capillary tube was cleaned and disinfected with 3 ml ethanol (96% v/v) after each extraction.

2.6. Modelling of the inactivation kinetics

A survival curve, by definition, is the cumulative form of a temporal distribution of mortality or destruction events (Peleg, 2006). The survival data are presented as a survival ratio log (N/N_0) vs. time relationship, where N_0 is the number of microorganisms initially contained in the sample at time 0 and N the corresponding number after a determined time of treatment. Certain survival curves exhibit a period of time in which no measurable inactivation takes place (lag-phase) previous to the inactivation period. The mathematical models used in this study to describe the inactivation of the microorganisms were the Fermi Model, the Gompertz Model and the Weibull Function.

2.6.1. Fermi Model

Adhering to the traditional concept of first-order kinetics, the Fermi Model (McKellar & Lu, 2004; Peleg, 2006) is described by Eq. (1).

$$\ln \frac{N}{N_0} = -\ln\left(1 + e^{k(t - t_c)}\right) \quad \text{Eq. (1)}$$

where k and t_c are kinetic constants; k is the rate constant (min^{-1}), t_c (min) is the longest treatment time in which the survival fraction equals 100%, that is, the lag phase, and t is the treatment time.

2.6.2. Gompertz Model

The Gompertz model has been used to describe sigmoidal curves for microbial growth which include lag, growth, and asymptotic phases. This model has been modified by Zwietering et al. (1990), in order to include parameters with biological meaning, and Kim et al. (2007) adapted this model to describe microbial cell inactivation, obtaining Eq. (2).

$$\log_{10}\frac{N}{N_0} = A\, e^{-e^{\left(-k_d\, e\, (\lambda - t)/A + 1\right)}} \qquad \text{Eq. (2)}$$

where A is the lowest asymptote value, k_d is the maximum inactivation rate (min^{-1}), λ is the duration of the lag phase and the constant e = 2.718.

2.6.3. Weibull Function

According to the Weibull Function, an inactivation curve is the cumulative form of the distribution of resistances within the population, measured in terms of the time at which an individual cell is inactivated. The most widely accepted version of the Weibull Function (McKellar & Lu, 2004; Peleg, 2006) is described by Eq. (3):

$$\log_{10}\frac{N}{N_0} = -b\, t^n \qquad \text{Eq. (3)}$$

where the constant b can be considered as a non-linear rate parameter and n is the parameter responsible for the curve shape, a concave upward semilogarithmic survival curve will be represented by n<1; a concave downward curve by n>1 and a log linear survival curve is a special case of the model where n=1.

2.7. Statistical analysis of the inactivation kinetics

The kinetic constants of the models were calculated by minimizing the sum of square differences between experimental and model predicted data using the Solver Microsoft Excel™ tool. The root mean square error (RMSE, Eq. (4)) and the coefficient of determination (R^2, Eq. (5)) were used to evaluate the fitting

goodness of the model and the estimation accuracy. RMSE is a measure of the standard error in the estimation, whereas R^2 is used as a measure of explained variation (Schemper, 2003).

$$RMSE = \sqrt{\frac{\sum_{k=1}^{z}(y_k - y_k^*)^2}{z}} \qquad \text{Eq. (4)}$$

$$R^2 = 1 - \frac{S_{yx}^2}{S_y^2} \qquad \text{Eq. (5)}$$

where y and y* are the experimental data and the estimated value, respectively, calculated as log (N/N_0); z is the number of experimental values and S_y and S_{yx} are the total standard deviation and the standard deviation of the estimation, respectively.

On the other hand, using the Statgraphics 5.1 statistical software, a one-way analysis of variance (ANOVA) with a confidence level of 95 % was carried out to study the effect of the growth stage on the slope of the log-linear section of the inactivation curves.

3. RESULTS AND DISCUSSION

3.1. Effect of the growth stage on SC-CO_2 inactivation

The two microorganisms used in this study (*E. coli* and *S. cerevisiae*) were grown to different stages and then subjected to SC-CO_2 treatment at 350 bar and 35 ºC. The inactivation curves obtained for each species are described separately.

3.1.1. *Saccharomyces cerevisiae*

Fig. 2 shows the inactivation kinetics of *S. cerevisiae* using SC-CO_2 for cells at different growth stages. As can be observed, the growth phase was found to have an effect on the response to SC-CO_2.

Fig. 2 shows that the shape of the curve changes depending on the growth stage. At the earliest stage no lag phase was observed and the kinetics represented a fast-to-slow curve. The viability began to decay immediately, following a first order kinetics, and only after 30 min did it start to slow down.

From the intermediate exponential phase onwards, an initial lag phase appeared when the inactivation treatment was applied - indicated by a shoulder in the curve - followed by a fast inactivation period. The lag time observed in the inactivation curves increased as the growth stage progressed, varying from 30 min in the intermediate exponential phase to 70 min in the early stationary phase. From intermediate growth onwards, a log-linear section of fast inactivation follows once the lag time is exceeded. The inactivation rate in the log-linear section was significantly ($p<0.05$) slower in the early stationary phase than in the intermediate and late exponential phases. These two later phases showed a similar inactivation rate.

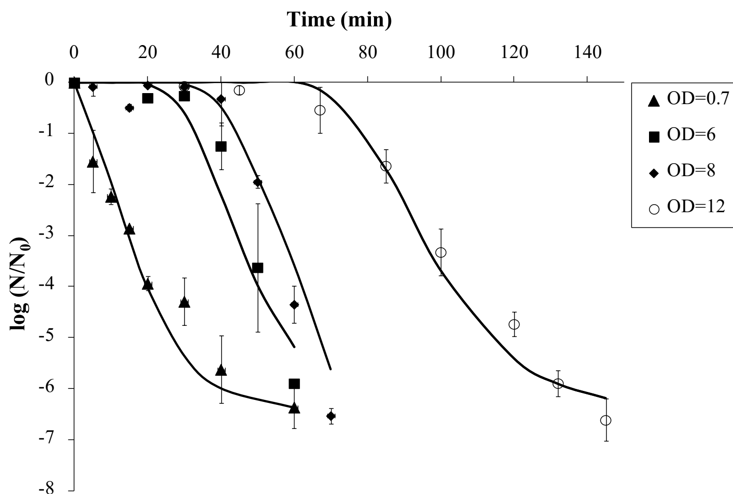

Fig. 2. Experimental data and modeling of the inactivation kinetics of *Saccharomyces cerevisiae* at different growth stages, using SC-CO_2 (350 bar, 35 ºC). Continuous line: Gompertz Function considering the OD_{600} as a model parameter; OD_{600}=0.7-early exponential phase; OD_{600}=6-intermediate exponential phase; OD_{600}=8-early stationary phase; OD_{600}=14-stationary phase.

Both types of SC-CO_2 inactivation curves (fast-to-slow, log-linear kinetics) have been described in the literature for *S. cerevisiae* (Spilimbergo & Mantoan, 2005).

As for the maximum level of inactivation reached, a similar value was obtained for all the growth stages, a 6-log reduction, but differences were found

in the time necessary to achieve this decline, which increased progressively as the growth phase advanced. In the early exponential phase, the 6-log reduction was obtained in 45 min, while in the intermediate exponential phase, 60 min were necessary to reach this level. This time rose to 130 minutes in the early stationary phase.

The inactivation mechanisms of CO_2 are not yet clearly established, but several studies indicate that the main factors are the pressure level and the depressurization rate (Lin et al., 1992), the acidification of the medium (Watanabe et al., 2005), the extraction of intracellular components and the membrane permeability (Oulé et al., 2006; Garcia-Gonzalez et al., 2007).

As far as the pressure factor is concerned, the exponential and stationary phase cells of other microorganisms, like *L. monocytogenes* (Hayman et al., 2007) and *L. plantarum* (Hong & Pyun, 1999), have been reported to offer differing resistance to high pressure treatments, which increases as the growth stage progresses. In the present work, the treated sample is suddenly depressurized from 350 bar to atmospheric pressure. This sudden decrease of pressure can also contribute to the inactivation of cells (Lin et al., 1992; Garcia-Gonzalez et al., 2007).

The initial lag phase has been explained by Lin et al. (1992) as the time needed for CO_2 to penetrate through the cellular envelope which determines when the CO_2 begins to exert its inactivation effect. Thus, as cells in the early exponential phase are the most sensitive, showing no lag phase, it could be deduced that the membrane permeability to CO_2 is maximum at this stage.

On the other hand, the acidification of the medium modifies the properties of the membrane bilayer, allowing CO_2 to penetrate into the cells causing a drop in intracellular pH. The importance of maintaining the intracellular pH during the yeast's response to high pressure treatment was demonstrated by the fact that inactivation was found to be higher in mutant strains lacking either the plasma membrane or vacuole H+-ATPases, enzymes involved in the extraction of H+ from the cytoplasm (Watanabe et al., 2005).

SC-CO_2 has gas-like viscosity and liquid-like density that make easier both the penetration into complex structures and the extraction of solutes. In this regard, SC-CO_2 is considered as an excellent solvent in extraction processes (Riera et al., 2010). Both the extraction of intracellular substances and the inactivation of key metabolic enzymes due to acidification could lethally alter the biological equilibrium; Spilimbergo et al., 2010).

The results of this paper corroborate what other studies have shown as fact; when microbial cells enter or approach the stationary phase they develop greater resistance to different stress conditions, such as heat, ethanol, oxidative stress or lytic enzymes (Werner-Washburne et al., 1996).

It is considered that the resistance to stress increases through the activation of different protective systems, with two main mechanisms involved: one is the production of low molecular weight components such as trehalose, the second is the expression of stress-response genes, such as the heat shock proteins. In yeasts, both mechanisms are included in the so called “General Stress Response”.

This response is mediated through the “general stress” transcription factors, Msn2p and Msn4p, that bind to the STRE sequence (AGGGG or CCCCT) present in the promoter region of a large set of stress-responsive genes. These include both heat shock genes (*SSA3, HSP12, HSP26, HSP104*), and trehalose metabolism genes (Martinez-Pastor et al., 1996).

It has not been documented if these protective routes are activated through SC-CO_2 treatments, but it has been reported that high pressure activates STRE dependent transcription through the Msn2/4 factors, which are also required for cell adaptation to high pressure (Domitrovic et al., 2006). Moreover, Msn2/4 are also involved in the resistance to low pH. Since acidification is one of the mechanisms responsible for the SC-CO_2 microorganism inactivation, it is therefore expected that this pathway could be involved in the response to SC-CO_2 exposure.

3.1.2. *Escherichia coli*

The influence of the growth phase of cells on the *E. coli* inactivation rate is shown in Fig. 3. The treatment conditions were identical to those applied to *S. cerevisiae*. It is known that gram-positive cells are more resistant to pressure than gram-negatives, like *E. coli,* due to their cell wall being thicker (Ramirez Santos et al., 2005). Moreover, it is known that *S. cerevisiae* has a thicker cell wall that makes it similar to gram-positive bacteria. When comparing the inactivation treatment of both microorganisms, it can be observed that the time needed for inactivation was much shorter in *E. coli*. This could be linked to the fact that *S. cerevisiae* has a thicker cell wall than *E. coli*.

As regards the effect of the growth phase on the inactivation kinetics of *E. coli*, the results were qualitatively similar to those for *S. cerevisiae*, that is, a greater SC-CO_2 resistance was observed in the stationary phase compared to the initial growth phases. Cells in the early exponential phase showed the highest sensitivity, decreasing by 5-log after 1 min of treatment and reaching 7-log of reduction after 3 min of SC-CO_2 treatment. Cells in the intermediate exponential phase also decreased after 1 min of SC-CO_2 treatment, but only by 1.5-log; for this growth phase, 8 min were needed to reach 7-log of reduction. In both cases, a biphasic curve (fast-to-slow) was observed. When cells get closer to the stationary phase, a slow-to-fast curve was observed which shows that the resistance to SC-CO_2 treatment increased. Cells in the late exponential phase needed 18 min to reach an inactivation of 8-log. In the early stationary phase, the inactivation rate during the first 14 min is low enough to consider the first period as a lag phase. After this first period, the population decreased progressively until a reduction of 8-log was reached after 25 min of SC-CO_2 treatment.

The factors involved in SC-CO_2 inactivation would be the same as those considered for *S. cerevisiae*, namely: pressure level, acidification, extraction of cell substances and dysfunctions in membrane permeability.

For cells in the exponential growth phase, the effect of SC-CO_2 on the inactivation treatments was noticeable and fast from the beginning, indicating that this is when the cells are at their most sensitive stage. Not having passed through a prior period of stress, the defensive systems have not been previously

activated, and the cells are unable to respond in time to the extreme conditions of SC-CO_2. The rapid inactivation during the first minute in the exponential phase could be related to a fast CO_2 diffusion into the cells, which would exercise its bactericidal effects. As in yeast, it could involve the perforation of the cytoplasmic membrane and the supercritical extraction of intracellular substances, provoking a loss of vital cellular constituents and the fragmentation of the cell envelope (Oulé et al., 2006). In addition to the inactivation mechanism previously mentioned the CO_2 diffusion might cause inhibition and/or inactivation of key enzymes essential form metabolic and regulating processes, such as plycolysis, amino acid and peptide transport, active transport of ions, and proton translocation (Garcia-Gonzalez et al., 2007). When cells get closer to the stationary phase, the inactivation mechanism of CO_2 would be similar but slower, because as *E. coli* cells enter this phase they develop a multiple-stress resistance state, considered to be analogue to sporulation in *B. subtilis* (Ramirez Santos et al., 2005).

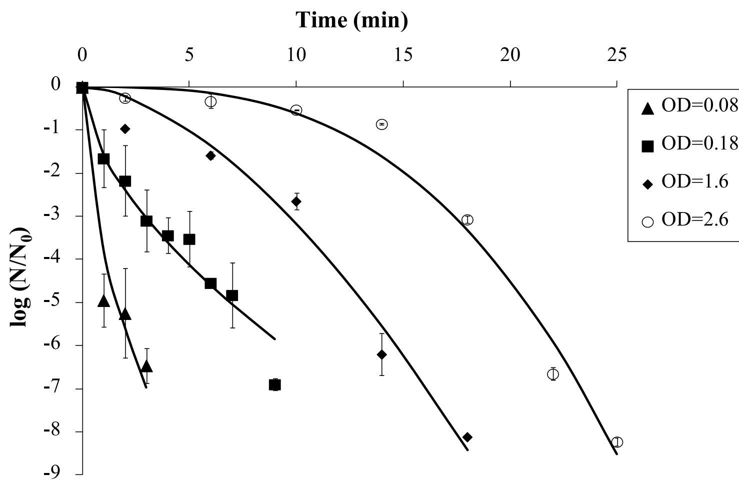

Fig. 3. Experimental data and modeling of the inactivation kinetics of *Escherichia coli* DH1 at different growth stages, using SC-CO_2 (350 bar, 35 ºC). Continuous line: Weibull's Distribution considering the OD_{600} as a model parameter; OD_{600}=0.08-early exponential phase; OD_{600}=0.18-intermediate exponential phase; OD_{600}=1.6-late exponential phase; OD_{600}=2.6-early stationary phase.

The increased pressure resistance in stationary phase cells could be explained by the morphological and physiological changes associated to this phase, like changes in cell size and shape, thickening of the cell wall, or synthesis of osmoprotective compounds, which would make CO_2 penetration difficult and lessen its effects (Ramirez Santos et al., 2005).

As previously mentioned, one of the factors involved in the SC-CO_2 inactivation is the pressurization. In this regard, Casadei et al. (2002) and Hayman et al. (2007) found that the resistance to high pressure treatments was higher in stationary phase cells of *L. monocytogenes* and *E. coli* NCTC 8164, than in exponential phase ones. Similarly to our findings, Isaacs et al. (1995) demonstrated that mid-exponential phase *E. coli* was much more high pressure sensitive than stationary phase cells.

The effects related to the growth phase could be linked to the activation of different protective systems. A combination of three mechanisms could play an important role in the increased resistance of stationary phase cells to SC-CO_2: the variation in cell membrane density, the expression of heat shock proteins (HSPs) and trehalose accumulation.

Previous research attributed the differences in pressure resistance between *E. coli* exponential and stationary phase cells to differences in membrane thickness (Pagan & Mackey, 2000). They found that in exponential growth cells the damage to the membrane was permanent, whereas in the stationary phase the cell membranes become leaky during the treatment, but could reseal to different levels after decompression.

As for the importance of protein management during the exposure to high pressure, Welch et al. (1993) demonstrated that the induction of several heat shock proteins, including several chaperones, occurs during the growth of *E. coli* at pressures of up to 100 MPa and Aertsen et al. (2004) found that the resistance to high hydrostatic pressure in *E. coli* is mediated by HSPs.

It has also been described that the accumulation of carbohydrates, such as trehalose, plays an important role in the response to different stresses. In *E. coli*, the trehalose metabolism genes are controlled by the sigma factor, encoded by

rpoS, the same factor that regulates the general stress response of *E. coli* (Loewen et al., 1998). The trehalose synthesis genes are expressed in the stationary phase, and are known to be involved in the thermotolerance associated to this growth phase (Joseph et al., 2010). Malone et al. (2006) found that the expression of the trehalose synthesis gene OtsA promotes barotolerance (resistance to high hydrostatic pressure) in the *E. coli* pathogenic strain O157:H7, when it is exposed to lethal pressures of 400 MPa. Therefore, it is expected that the accumulation of trehalose which takes place as the cells approach the stationary phase could also play an important role in the increased resistance to SC-CO_2 associated to these cells.

As a conclusion, SC-CO_2 provokes a microbial inactivation and its effect is greatly influenced by the growth phase of the cells, both in *E. coli* and *S. cerevisiae.* The stationary phase cells of *E. coli* and *S. cerevisiae* are more SC-CO_2-resistant than exponential phase cells. The differences are mainly observed in the shape of the curves, the duration of the lag phase and the time needed for inactivation. It may be hypothesized that these differences, involving the activation of the cellular stress responses, might be explained by molecular mechanisms, although further studies would be needed to address this subject.

3.2. Modeling of inactivation kinetics

3.2.1. *Saccharomyces cerevisiae*

In order to analyze the survival curves for *S. cerevisiae* after the SC-CO_2 treatment, the kinetic data was fitted to three mathematical models: the Gompertz Function, Weibull Function and Fermi's Model (Table 1). R^2 and RMSE values indicate that, overall, a good fit was obtained with the three models for the four growth stages considered, with two exceptions: the survival curve in the intermediate exponential phase fitted by Fermi's Model ($R^2 = 0.88$; RMSE = 0.91) and the stationary phase using Weibull Function ($R^2 = 0.91$; RMSE = 0.78). Only the Gompertz Function showed a good fit to all the growth stages studied ($R^2 > 0.95$; RMSE < 0.44).

Table 1. Estimated model parameters for the inactivation kinetics of *Saccharomyces cerevisiae* using SC-CO_2 at P = 350 bar and T = 35 ºC. Influence of the growth stage.

	Growth phase	Parameters		R^2	RMSE
Gompertz Function	Early exponential	A	-6.2 ± 0.3	0.96	0.44
		λ (min)	0.10 ± 0.09		
		k_d (min^{-1})	0.19 ± 0.02		
	Intermediate exponential	A	-9.9 ± 0.4	0.99	0.20
		λ (min)	35.6 ± 1.5		
		k_d (min^{-1})	0.25 ± 0.06		
	Early stationary	A	-8.4 ± 1.7	0.99	0.21
		λ (min)	42.2 ± 1.1		
		k_d (min^{-1})	0.24 ± 0.01		
	Stationary	A	-8.6 ± 1.8	0.99	0.14
		λ (min)	67.2 ± 4.8		
		k_d (min^{-1})	0.09 ± 0.01		
Weibull's Distribution	Early exponential	b (min^{-1})	0.65 ± 0.03	0.98	0.27
		N	0.56 ± 0.05		
	Intermediate exponential	b (min^{-1})	0.012 ± 0.004	0.98	0.31
		N	3.7 ± 0.3		
	Early stationary	b (min^{-1})	0.011 ± 0.003	0.95	0.34
		N	3.7 ± 0.6		
	Stationary	b (min^{-1})	0.009 ± 0.001	0.91	0.78
		N	3.6 ± 0.2		
Fermi's Model	Early exponential	t_C (min)	0.01 ± 0.02	0.99	0.18
		k (min^{-1})	0.12 ± 0.01		
	Intermediate exponential	t_C (min)	35.7 ± 4.7	0.88	0.91
		k (min^{-1})	0.24 ± 0.02		
	Early stationary	t_C (min)	43.2 ± 1.1	0.98	0.21
		k (min^{-1})	0.25 ± 0.01		
	Stationary	t_C (min)	66.1 ± 4.8	0.99	0.19
		k (min^{-1})	0.087 ± 0.006		

According to the definition of the parameters of the Gompertz function, λ values can be correlated with the extent of the lag phase. Therefore, and in agreement with the results shown in the previous section, λ values increased as the growth phase advanced. In the early exponential phase (OD_{600} = 0.7), the lowest λ value (0.1 min) was observed, indicating that the cell population started decreasing immediately after the beginning of the treatment. On the other hand,

this lag time increased by 91% from the intermediate exponential phase (35 min) to the early stationary phase (67 min). Moreover, the maximum inactivation rate, k_d, decreased by 62% from the intermediate exponential phase (0.25 min^{-1}) to the early stationary phase (0.09 min^{-1}). This fact confirmed that the inactivation rate was also slower as the growth stage progressed, due to the increasing resistance of the cells.

3.2.2. *Escherichia coli*

The three previously described mathematical models were fitted to the inactivation data for *E. coli* during the SC-CO_2 inactivation experiments in the four growth stages selected (Table 2). According to the values of the statistical parameters, the three models fitted the experimental data adequately. The Gompertz Function showed the best fit in the early exponential phase ($R^2 = 0.99$; RMSE = 0.30) and the early stationary phase ($R^2 = 0.99$; RMSE = 0.32). Fermi's Model and Weibull Function provided the best fit in the intermediate exponential phase ($R^2 = 0.97$; RMSE = 0.85) and the late exponential phase ($R^2 = 0.97$; RMSE = 0.57), respectively.

Focusing on the values of the model parameters for the Gompertz Function (Table 2), λ values increased as the growth phase advanced, as occurred with *S. cerevisiae*. In the early exponential phase ($OD_{600} = 0.08$) a λ value of 0.18 min was obtained, corresponding with a decay of viable cells at the beginning of the treatment. Also, the obtained k_d value ($k_d = 6.8$ min^{-1}) corresponds to a high inactivation rate, as observed experimentally. In the intermediate exponential phase ($OD_{600} = 0.17$), late exponential phase ($OD_{600} = 1.6$) and the early stationary phase ($OD_{600} = 2.6$), the inactivation rates (k_d) were similar, 0.87, 0.72 and 0.81 min^{-1}, respectively. The greater resistance to the SC-CO_2 treatment of *E. coli* cells in stationary phase compared to those in the early exponential phase, is shown both by a lower inactivation rate, which decreased by 88% (0.81 min^{-1}) and by the longer lag phase (λ = 13.9 min versus 0.18 min).

Table 2. Estimated model parameters for the inactivation kinetics of *Escherichia coli* using SC-CO_2 at P = 350 bar and T = 35 ºC. Influence of the growth stage.

	Growth phase	Parameters		R^2	RMSE
Gompertz Function	Early exponential	A	-6.2 ± 0.9	0.99	0.30
		λ (min)	0.18 ± 0.09		
		k_d (min^{-1})	6.8 ± 1.4		
	Intermediate exponential	A	-6.9 ± 1.1	0.89	0.65
		λ (min)	0.10 ± 0.06		
		k_d (min^{-1})	0.87 ± 0.06		
	Late exponential	A	-8.1 ± 1.5	0.90	1.01
		λ (min)	5.4 ± 1.9		
		k_d (min^{-1})	0.72 ± 0.11		
	Early stationary	A	-11.8 ± 2.4	0.99	0.32
		λ (min)	13.9 ± 0.5		
		k_d (min^{-1})	0.81 ± 0.07		
Weibull's Distribution	Early exponential	b (min^{-1})	5.1 ± 0.4	0.97	0.45
		N	0.14 ± 0.12		
	Intermediate exponential	b (min^{-1})	1.54 ± 0.17	0.96	0.40
		N	0.57 ± 0.07		
	Late exponential	b (min^{-1})	0.26 ± 0.04	0.97	0.57
		N	1.14 ± 0.15		
	Early stationary	b (min^{-1})	0.05 ± 0.004	0.98	0.47
		N	3.1 ± 0.2		
Fermi's Model	Early exponential	t_C (min)	-3.08 ± 0.51	0.96	1.26
		k (min^{-1})	2.36 ± 0.41		
	Intermediate exponential	t_C (min)	-1.36 ± 0.46	0.97	0.85
		k (min^{-1})	1.35 ± 0.05		
	Late exponential	t_C (min)	1.75 ± 0.77	0.93	1.95
		k (min^{-1})	1.09 ± 0.04		
	Early stationary	t_C (min)	13.4 ± 0.5	0.99	0.82
		k (min^{-1})	1.61 ± 0.05		

3.3. Modelling of inactivation kinetics as a function of OD_{600}

Three survival models have been used to describe the inactivation kinetics of *S. cerevisiae* and *E. coli*, in the four growth stages studied. In order to describe the effect of the SC-CO_2 treatment in the different growth stages, a model was proposed for each microorganism that included the OD_{600} as an additional parameter.

3.3.1. *Saccharomyces cerevisiae*

For *S. cerevisiae,* the model adapted to include the effect of the growth stage was the Gompertz Function, since, on average, it was the one that best described the inactivation kinetics for the four stages of growth considered ($R^2_{avg.} = 0.98$; $RMSE_{avg.} = 0.25$). The kinetic parameter A was considered constant, while λ and k_d were related to the OD_{600} values using the following linear equations.

$$\lambda = d\, OD_{600} + f \qquad \text{Eq. (6)}$$

$$k_d = g\, OD_{600} + h \qquad \text{Eq. (7)}$$

By introducing these linear relationships (Eqs. (6) and (7)) in the Gompertz Function (Eq. (2)), a single equation that defined the inactivation kinetics of *S. cerevisiae* was obtained. This equation related the number of surviving cells to the time of treatment (t) and to the growth phase (OD_{600} value) of the initial treated culture.

The model parameters were calculated (Table 3) showing that the proposed model appropriately described the inactivation kinetics for the four stages of growth studied ($R^2 = 0.93$; $RMSE = 0.59$). The close fit obtained between the experimental inactivation data of *S. cerevisiae* and those calculated using this model can be observed in Fig. 2 ($R^2 > 0.90$; $RMSE < 0.71$). The poorest fit was found in the late exponential phase (Fig. 2), for which the model predicts a lower inactivation rate than that depicted by the experimental data for times of over 50 min.

3.3.2. *Escherichia coli*

In order to adapt an inactivation model to include the effect of the growth stage, a similar methodology was followed for *E. coli*. The Weibull Function was

selected because, on average, it was the model that best described the inactivation kinetics for the four stages of growth studied ($R^2_{avg.} = 0.97$; $RMSE_{avg.} = 0.47$). In order to proceed with the model development, the b and n parameters were related to OD_{600} values by means of the following equations.

$$b = i\,OD_{600}{}^{j} - 1 \qquad \text{Eq. (8)}$$

$$n = m\,OD_{600}{}^{2} + p\,OD_{600} + q \qquad \text{Eq. (9)}$$

Introducing these relationships (Eqs. (8) and (9)) in the Weibull Function (Eq. (3)), a single equation that defined the inactivation kinetics of *E. coli* was obtained. This equation related the number of surviving cells to the time of treatment (t) and to the growth phase (OD_{600} value) of the initial treated culture.

Table 3. Estimated model parameters for the inactivation kinetics of *S. cerevisiae* and *E. coli* at different growth stages using the modified Gompertz Function and Weibull's Function, respectively.

Saccharomyces cerevisiae				***Escherichia coli***			
Parameters		**R^2**	**RMSE**	**Parameters**		**R^2**	**RMSE**
A	-6.4 ± 0.2			i	0.28 ± 0.11		
d	4.9 ± 0.3			j	-1.04 ± 0.10		
f	-0.76 ± 0.05	0.93	0.59	l	0.10 ± 0.06	0.96	0.53
g	-0.017 ± 0.003			m	0.19 ± 0.12		
h	0.30 ± 0.03			p	0.41 ± 0.24		
				q	0.51 ± 0.06		

The model parameters were calculated (Table 3) showing that the proposed model appropriately described the inactivation kinetics for the four stages of growth studied (R^2 = 0.96; RMSE = 0.53). The experimental and predicted inactivation kinetics obtained for *E. coli* using this model can be observed in Fig. 3. This model provided good results for late exponential phase (Fig. 3: R^2 = 0.97; RMSE = 0.57) and for early stationary phase (Fig. 3: R^2 = 0.98; RMSE = 0.48). The poorest fit of this model was found in the early exponential phase (Fig. 3: R^2 = 0.89; RMSE = 0.91). As can be observed for Fig. 3, the experimental variability (depicted by the error bars) increases from early exponential to stationary growth stages. This could be due to the differences in

the uniformity of the cells at different growth stages. Both the higher variability and the shorter process times in the exponential phase could be responsible for the poor fit found in Fig. 3 why the model has included the OD_{600} as a new parameter. At times of over 7 min, the fit of the model to the intermediate stationary phase (Fig. 3 $R^2 = 0.94$; RMSE = 0.48) predicted a lower inactivation rate than that of the experimental data.

4. CONCLUSIONS

The supercritical CO_2 inactivation of *E. coli* and *S. cerevisiae* was greatly influenced by the growth stage. The resistance of the microorganisms to the treatment increased progressively as the growth phase advanced, affecting both the lag phase and the inactivation rate. This higher resistance could be linked to the natural protective systems that become activated as cells approach the stationary phase. Models were developed to describe the inactivation kinetics at different growth stages, which could be used to optimize the inactivation process conditions. For future research, the influence of culture mixtures and the use of food matrices, which might contain materials that may interfere with the inactivation, will have to be addressed.

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Chapter 2

An Ultrasound-Enhanced System for Microbial Inactivation using Supercritical Carbon Dioxide

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C. Ortuño, M.T. Martínez-Pastor, A. Mulet, J. Benedito

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An ultrasound-enhanced system for microbial inactivation using supercritical carbon dioxide

C. Ortuño, M.T. Martínez-Pastor, A. Mulet, J. Benedito

Grupo de Análisis y Simulación de Procesos Agroalimentarios, Departamento Tecnología de Alimentos, Universitat Politècnica de València, Camí de Vera s/n, E46022, Valencia, Spain.

ABSTRACT

The effect of ultrasound on the SC-CO_2 inactivation kinetics of *E. coli* at different pressures (100, 225, 290 and 350 bar; 36 ºC), temperatures (31 ºC, 36 ºC and 41 ºC; 225 bar) and varying the composition of the medium (LB Broth, apple and orange juice) was studied. Using only SC-CO_2 the inactivation rate in LB Broth increased progressively as the pressure or temperature rose and the average (for the different process conditions) time needed to achieve a reduction of 8 log-cycles was 50 min. When both SC-CO_2 and ultrasound were used in LB Broth, 95% less time was needed, on average, to achieve the same reduction of 8 log-cycles and the effect of pressure and temperature was minimized; this reduction being achieved after only 2 min at 31 ºC and 225 bar. Using the ultrasonic system led to a shorter process time regardless of the treatment medium, preventing the inhibitory effect that the solutes from the juices had on the SC-CO_2 inactivation. The technology developed permits both a drastic decrease in SC-CO_2 inactivation times and also the use of mild process conditions, which could lead to an increase in the quality of the product treated under this new technique.

Key words: supercritical carbon dioxide, high power ultrasound, *Escherichia co*li, inactivation.

1. INTRODUCTION

Thermal treatment is the most traditional and also the most widespread method used to prevent food microbial spoilage; however, it can cause degradation or changes in flavor, nutrient content, color and texture. For this reason and in addition to consumer demand for fresh-like and natural food products, there has recently been a growing interest in milder, non-thermal food preservation techniques. The aim of the new technologies under development is to ensure food preservation, without the use of preservatives, while maintaining its nutritional value and organoleptic characteristics (texture, color, taste…) unchanged. Among these techniques may be cited high hydrostatic pressure (Clariana et al., 2011), pulsed electric fields (Monfort et al., 2010), membrane filtration (Rektor et al., 2004) or supercritical fluids (Liu et al., 2012).

Supercritical carbon dioxide (SC-CO_2) inactivation technology represents a promising non-thermal processing method, as it promotes minimum impact on the nutritional and organoleptic food properties (Spilimbergo & Bertucco, 2003). SC-CO_2 has been shown to be efficient at inactivating a large variety of microorganisms (*E. coli*, *Enterococcus faecalis, S. cerevisiae*), either in culture media (Debs-Louka et al., 1999; Oulé et al., 2006; Ortuño et al., 2012), or in liquid foods, such as apple juice (Spilimbergo et al., 2007) or liquid whole egg (Garcia-Gonzalez et al., 2009). In this regard, several studies have reported the effect of pressure or temperature on the inactivation kinetics of *E. coli* (Liao et al., 2008), or the effect of the composition of the suspending media (Liao et al., 2006) on the SC-CO_2 inactivation process.

The mechanism of microbial inactivation by SC-CO_2 has not yet been fully elucidated. SC-CO_2 combines the solvent capacity of liquids with the mobility of gases. This is due to the fact that in supercritical state (31.1 ºC; 73.8 bar), the density and viscosity of CO_2 lie midway between a gas and a liquid (Berna et al., 2000). Several theories explaining the inactivation mechanism of SC-CO_2 involve the diffusion of CO_2 into the cells. Due to its properties, SC-CO_2 diffuses easily through the cell membrane causing a cytoplasmic pH decrease, the modification of the cell membrane and the extraction of vital constituents from the cell (Spilimbergo & Bertucco, 2003) to such an extent that the biological

system is altered and disturbed, promoting microbial inactivation (Lin et al., 1992; Garcia-Gonzalez et al., 2007).

However, although the inactivation ability of SC-CO_2 has been widely demonstrated, some cases require high pressures or temperatures and too long a treatment time to guarantee the food's safety and stability (Garcia-Gonzalez et al., 2009; Liu et al., 2012). As an example, Liao et al. (2008) concluded that when using SC-CO_2 at 32 ºC and 200 bar, 75 min were required to reduce the population of *E. coli* by 5 log-cycles, whereas 42 ºC and 300 bar were needed to achieve a reduction of 7 log-cycles in the same time.

In order to obtain the required lethality at shorter processing times or with lower treatment intensity, the possibility of combining SC-CO_2 with another preservation technique and using this as a pretreatment has been studied. Pulsed electric fields (PEF) or high hydrostatic pressure (HHP) (Spilimbergo et al., 2003; Park et al., 2002) have been shown to have additive or synergistic effects on SC-CO_2 microbial inactivation, reducing the SC-CO_2 processing requirements (time, temperature and pressure).

Ultrasound is known to have a significant effect on the velocity of food industry processes involving heat and mass transfer (Chemat et al., 2011). There are many potential applications in food processing, such as extraction, filtration, extrusion, freezing or crystallization (Chemat et al., 2011). The application of ultrasound in food preservation processing is relatively recent. It has been proven that high-intensity ultrasonic waves can cause cell rupture and enzyme denaturalization, although when using only ultrasound, the effects are not sufficiently severe to destroy enough microorganisms (Butz & Tauscher, 2002). An effective microbial inactivation can be achieved by combining ultrasound with either heat (thermosonication), or pressure (manosonication) or with both (manothermosonication). The combination of heat or pressure and ultrasound increases the efficiency with respect to the treatment time and energy consumption, compared to each individual treatment (Chemat et al., 2011).

On the other hand, the simultaneous application of SC-CO_2 and high power ultrasound (HPU) has been shown to improve the extraction processes (Gao et al., 2009; Riera et al., 2010) or to accelerate chemical reactions (Trofimov et al.,

2001). In the ultrasound-assisted supercritical extraction processes, the ultrasound-induced micro-stirring and solvent cavitation had some physical consequences, including cracked or damaged plant cell walls, increased solvent diffusion, interfacial turbulence and a reduction of the external resistance to mass transfer (Gao et al., 2009).

However, no references have been found in the literature covering the simultaneous application of SC-CO_2 and HPU for microbial inactivation purposes.

In this paper, a novel inactivation technique based on High Power Ultrasound embedded in a Supercritical Fluids Plant is presented. This system has been patented in conjunction with the inactivation procedure (Benedito et al., 2011). The aim of the work was to study the effect of ultrasound on the SC-CO_2 inactivation kinetics of *E. coli* at different pressures, temperatures and varying the composition of the medium.

2. MATERIALS AND METHODS

2.1. Microorganisms

The microbial strain used in this study was *Escherichia coli* DH1 (*E. coli*) (chromosomal genotype: endA1 *gyr*A9, *thi*-1, *hsd*R179 (r_k^-, m_K^+), *sup*E44, ***rel***A1). The strain was maintained by freezing (-40 ºC) in Luria Bertani Broth (LB Broth, Sigma-Aldrich, USA) with 15% glycerol added as cryogenic agent.

One loop from each of the deep-frozen cultures was streaked and cultured on Luria Bertani Agar (LB Agar, Sigma-Aldrich, USA) 24 h at 37 ºC. The culture plates were stored at 4 ºC and transferred monthly to generate the stock cultures.

2.2. Sample preparation and growth conditions

A single colony of *E. coli* was inoculated on LB Broth and grown overnight at 37 ºC, using an incubation chamber (J.P. SELECTA, Model 3000957, Barcelona, Spain) and an orbital shaker at 120 rpm (J.P. SELECTA, Rotabit Model 3000974, Barcelona, Spain). For each experiment, a subculture was prepared by inoculating 50 µL from the starter in 50 mL of sterilized medium and incubating at 37 ºC for 18 h to obtain cells in the early stationary phase. Growth curves were

determined in advance by both plating and the measurement of absorbance at 600 nm (data not shown). The culture was diluted in LB Broth, apple juice or orange juice, to a cell concentration of 10^7 cfu/mL and then submitted to the treatment.

2.3. Apparatus

The SC-CO_2 experiments were carried out in a supercritical fluid lab-scale plant specially designed and built in the research group for the application (Fig. 1). The apparatus was designed to withstand up to 1000 bar. The plant includes a CO_2-tank (1, Fig. 1) and a N_2-tank (2, Fig. 1), which are kept at room temperature; a chiller reservoir stored at -18 ºC (3, Fig. 1); a pump (4, Fig. 1) and a thermostatic bath (5, Fig. 1) to keep the inactivation vessel (6, Fig. 1) at the desired temperature. The vessel (500 mL internal volume) as well as the different connections and valves in contact with SC-CO_2 are made of stainless steel type 316. Pressure and temperature gauges are installed in the inactivation vessel to monitor that the supercritical conditions were reached in a short time and subsequently maintained during processing.

Additionally, the system includes the ultrasound equipment (Benedito et al., 2011) which is embedded in the supercritical fluids plant. This system has been patented in conjunction with the inactivation procedure and consists of a high power piezoelectric transducer, an insulation system and a power generator unit. The transducer (>1W/cm^2) is inserted inside the inactivation vessel and includes two commercial ceramics (8, Fig. 1; 35 mm external diameter; 12.5 mm internal diameter; 5 mm thickness; resonance frequency of 30 kHz) and a sonotrode (7, Fig. 1), which was specially constructed to concentrate the highest amount of acoustic energy on the application point. The transducer is powered with constant energy by the power generator unit (9, Fig. 1) during the SC-CO_2 process. One critical part of the equipment is the insulation system for the piezoelectric ceramics. It consists of a polypropylene joint covered with teflon. This insulation protects the transducer from possible electrical short-circuits that can be caused by working in an extreme conductor medium.

2.4. Supercritical fluid processing

Each single experimental run required four operating steps: plant cleaning and disinfection, sample preparation, inactivation treatment and sample extraction. The sample (60 mL) was loaded into the inactivation vessel and immediately sealed. The pump filled the vessel with supercritical carbon dioxide, reaching the desired pressure in less than 2.5 min. Time zero for each treatment was considered to be when the experimental temperature and pressure were reached. For the experiments with HPU, the ultrasound unit was turned on when the desired pressure in the vessel was reached, the applied power during the whole experiment being 40 W ± 5 W (I = 250 mA ± 10 mA; U = 220 V ± 5 V) (Power measured with a Digital Power Meter, Yokogawa, Model WT210). Pressure and temperature were kept constant during the experiment through the pump and the thermostatic bath, respectively.

Samples of 1-3 mL were extracted at different times (time interval ranging from 20 s to 5 min depending on the process conditions) through a small tube (2 mm diameter) located at the bottom of the inactivation vessel until the end of the experiment. This tube was cleaned and disinfected with 3 mL ethanol (96% v/v) after each sampling.

2.5. Enumeration of viable microorganisms

The viability of *E. coli* in the samples periodically taken from the inactivation vessel was determined by the plate count method. Each sample was serially diluted with sterilised distilled water. 100 μL of the appropriate dilution were plated in triplicate on LB Agar plates and incubated for 24 h at 37 ºC before counting. Microbial cells in the initial non-treated sample (control sample) were counted following the same procedure described for the treated samples. The results are expressed as log (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 different times of treatment. The results shown are the arithmetic mean and the standard deviation of log (N/N_0) for at least three plates.

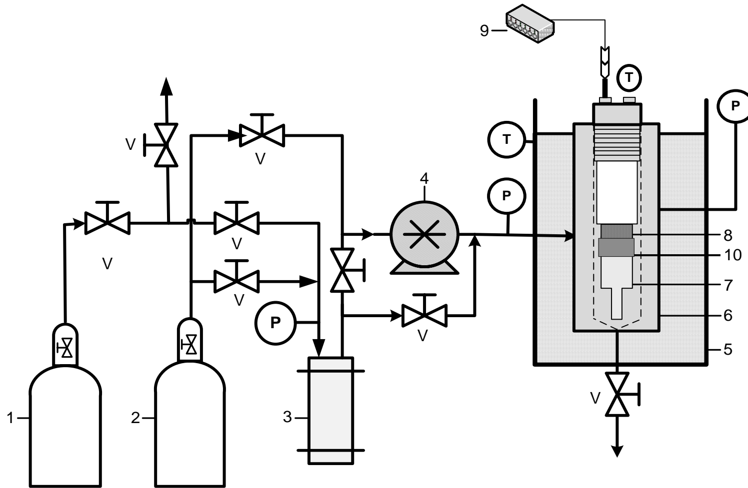

Fig. 1. Supercritical CO_2 treatment system. 1-CO_2 tank; 2-N_2 tank; 3-Chiller reservoir; 4-Pump; 5-Temperature controlled bath; 6-Treatment vessel; 7-Transducer; 8-Ceramics; 9-Power Generation Unit; 10-Insulation joint; V-Valve; P-Manometer; T-Temperature sensor.

2.6. Experimental design

The *E. coli* inoculated into the sterilized LB medium was subjected to the SC-CO_2 treatment under different process conditions. In this regard, to determine the effect of pressure, samples were treated by SC-CO_2 and SC-CO_2+HPU at 36 ºC and 100, 225, 290 and 350 bar. To determine the effect of temperature, samples were exposed to SC-CO_2 and SC-CO_2+HPU at 225 bar and 31 ºC, 36 ºC and 41 ºC (non-lethal temperatures for *E. coli*).

On the other hand, in order to determine the effect of the suspending medium on the resistance of *E. coli* to the SC-CO_2 treatment, apple and orange juice (prepared in the laboratory and stored at -18 ºC until used) were selected to compare with the inactivation kinetics on LB medium. Based on preliminary experiments, 10 mL of *E. coli* LB-incubated subculture was inoculated into 50 mL of juice (apple or orange) and uniformly mixed. Following this procedure, the initial number of *E. coli* in the juices ranged from 10^7 to 10^8 cfu/mL. In this case, the samples were exposed to SC-CO_2 and SC-CO_2+HPU, at 350 bar and 36 ºC.

Finally, to evaluate the possible synergistic effect of SC-CO_2 and HPU, an inactivation experiment using HPU (40 W ± 5 W, 36 ºC) on LB Broth medium was carried out and compared to the SC-CO_2 and SC-CO_2+HPU inactivation treatments at 36 ºC and 350 bar.

2.7. Statistical analysis

All the experiments were run in triplicate, and the data presented are the means of the triplicate experiments. The significant differences between the number of surviving microorganisms and the inactivation rates were assessed with a 5% level of significance ($p<0.05$) by Student's t test.

3. RESULTS

The effect HPU has on the SC-CO_2 microorganism inactivation at different pressures, temperatures and using different culture media are described separately.

3.1. Effect of HPU on the *E. coli* SC-CO_2 inactivation kinetics at different pressures

3.1.1. SC-CO_2 inactivation at different pressures

The cell suspensions of *E. coli* were subjected to four treatment pressures (100, 225, 290 and 350 bar) at a constant temperature of 36 ºC (Fig. 2). The survival curves showed an initial lag phase - indicated by a shoulder in the curve - followed by a log-linear section, known as an exponential inactivation phase. During the lag phase, a slight decrease in the population of microbial cells was observed at every pressure (as the pressure rose this decrease got greater), which could be due to the death of the most sensitive microbial cells. The inactivation kinetics at 100 and 225 bar behaved in a similar way and no significant differences ($p>0.05$) were found for most of the treatment times. However, when increasing the treatment pressure, from 225 to 290 or 350 bar, under constant temperature, both the duration of the lag phase and the time needed to reach a reduction of 8 log-cycles, were substantially reduced. As the pressure increased from 225 to 290 and 350 bar, the lag phase decreased from 30 to 20 and 15 min, respectively. Once the lag phase was exceeded, the number of viable cells decreased exponentially for all pressures. The slopes of the curves were

comprised between -0.67 log-cycles/min for 350 bar and -0.28 log-cycles/min for 100 bar. Using 100 and 225 bar, 55 min and 60 min were required respectively to reach a reduction of 8 log-cycles, while 35 min and 25 min were required to reach a similar inactivation level at 290 and 350 bar, respectively. From these results, it could be thought that it is necessary to exceed a minimum pressure, between 225 and 290 bar, in order to change the *E. coli* inactivation kinetics significantly and, therefore, shorten the treatment times, which is mainly linked to the duration of the lag-phase.

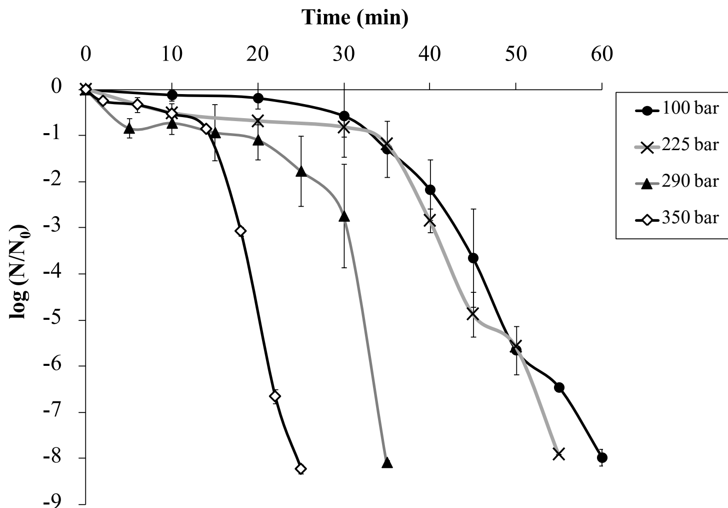

Fig. 2. Inactivation kinetics of *Escherichia coli* DH1 in LB medium at different pressures, using SC-CO_2 at 36 ºC.

Liao et al. (2008) showed that there was not significant ($p>0.05$) differences between the inactivation of *E. coli* exposed to SC-CO_2 at 42 ºC and 100 or 200 bar, while significantly faster inactivation rates were found at 300 bar.

Several studies indicate that the main factors involved in the inactivation mechanisms of CO_2 are the pressure level (Erkmen, 2000), the acidification of the medium (Watanabe et al., 2005), the membrane permeability and the extraction of intracellular components (Liao et al., 2006; Oulé et al., 2006).

In the present study, the shape of the curves showed a shoulder (lag phase). This could indicate that the limiting step in the microbial inactivation is probably

the penetration of CO_2 into the cells, in fact the penetration rate at a constant temperature mainly depends on pressure (Liao et al., 2006). It is known that pressure controls the SC-CO_2 solubilization rate; thus, a higher pressure enhances SC-CO_2 solubilization making the contact and penetration of CO_2 into the cells easier (Liao et al., 2006). Furthermore, CO_2 dissolves in aqueous solutions to form carbonic acid and reduces the pH of the suspending medium. This fact modifies the properties of the membrane bilayer and could also contribute to an increase in cell permeability, allowing more CO_2 to penetrate into the cells and causing a drop in intracellular pH (Watanabe et al., 2005; Liao et al., 2006). Moreover, the penetration of CO_2 into cells permits the extraction of intracellular substances and the inactivation of key metabolic enzymes, due to acidification, which could alter the biological equilibrium of the cell, causing its death. Therefore, factors that ease the entry of CO_2 into the cells could contribute to an enhanced inactivation effect.

3.1.2. Application of HPU to SC-CO_2 inactivation at different pressures

Fig. 3 reports the inactivation curves of *E. coli* cells undergoing a combined SC-CO_2+HPU process at different pressures and at a constant temperature (36 ºC). Remarkable differences in the microbial inactivation rate are evident when comparing the results of the SC-CO_2 treatment (Fig. 2) with those of the SC-CO_2+HPU (Fig 3).

In the inactivation kinetics obtained using SC-CO_2+HPU at different pressures (Fig. 3), no lag phases were observed. The viability began to decrease immediately, following a first order kinetics for all conditions studied. The inactivation curves at 100, 225 and 290 bar showed the same behavior. In these three cases, 1.5 min of treatment were required to obtain a reduction of 8 log-cycles. Using 350 bar, a slower inactivation rate was observed and two phases can be described. For times of under 2 min, a faster inactivation of *E. coli* was observed until a reduction of 6 log-cycles, after which time the inactivation rate decreased for the following 3 min to reach a reduction of 8 log-cycles.

The time required to reach the total inactivation was reduced by 97.5 %, 97.2 %, 95.7 % and 80%, at 100, 225, 290 and 350 bar, respectively, using the patented SC-CO_2+HPU procedure, compared to SC-CO_2 treatments.

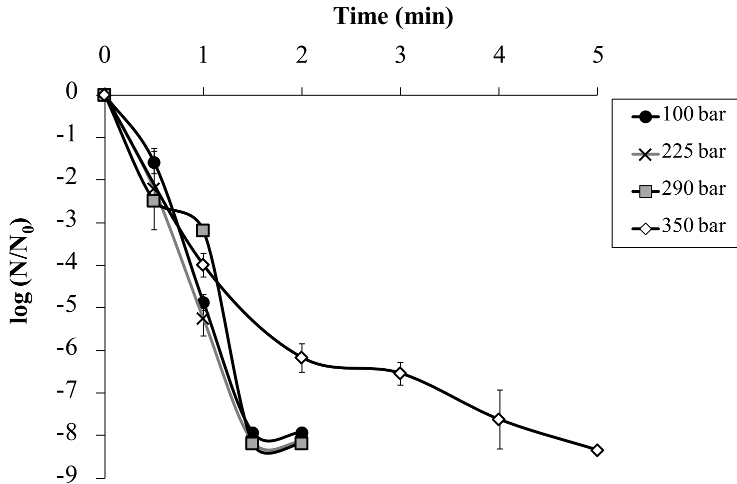

Fig. 3. Inactivation kinetics of *Escherichia coli* DH1 in LB medium at different pressures, using SC-CO_2+HPU at 36 ºC.

The main advantage of using HPU during the SC-CO_2 treatment would include a more effective agitation and consequently a faster mass transfer. Agitation could enhance the solubilization of CO_2, its contact with bacterial cells and the permeation of cell membranes, thus making its penetration into the cells easier and causing a faster drop of intracellular pH (Garcia-Gonzalez et al., 2007). Moreover, HPU improves mass transfer which could also enhance the extraction of vital intracellular components from cells, accelerating its death. In addition, the inactivation effect of ultrasound could also be attributed to the cavitation of the medium. Cavitation refers to the formation, growth, and implosion of tiny gas bubbles in a liquid when ultrasound travels through it, which has been proven to cause the disruption of cell walls (Chemat et al., 2011). The disruption of the microorganisms' walls could contribute to the extraction of intracellular compounds, accelerating the death of microbial cells.

The slower inactivation at 350 bar could be linked to a milder effect of the acoustic field due to the high static pressure, which could affect how the transducer transforms the electrical input into mechanical energy, thus acoustic intensity. Moreover, the transmission of the acoustic field to the medium and the micro-stirring and cavitation produced by the medium

compression/decompression could be affected by the highest pressure, modifying the extraction ability of SC-CO_2+HPU and, therefore, its inactivation capacity.

3.2. Effect of HPU on the SC-CO_2 inactivation kinetics at different temperatures

3.2.1. SC-CO_2 inactivation at different temperatures

Fig. 4 shows the inactivation kinetics of *E. coli* subjected to SC-CO_2 treatments at different treatment temperatures (31 ºC, 36 ºC and 41 ºC) and at a constant pressure of 225 bar. The survival curves under these conditions showed an initial lag phase followed by a log-linear inactivation stage. The length of the lag phase in the inactivation kinetics at 31 ºC and 36 ºC was similar, of approximately 30 min, whereas at 41 ºC it was reduced to 20 min. At the three temperatures studied, a reduction of approximately 0.8 log-cycles was observed during the lag phase, which could be due to the inactivation of the most sensitive cells. Once the lag phase was finished, a log-linear inactivation phase appeared with similar values for the slopes (-0.16, -0.34 and -0.31 log-cycles/min for 31 ºC, 36 ºC and 41 ºC, respectively) and the time required to obtain a complete inactivation of the cells was reduced progressively as the temperature rose and was mainly determined by the duration of the lag-phase. A reduction of 8 log-cycles was achieved in 75 min at 31 ºC, in 55 min at 36 ºC, and in 40 min at 41 ºC.

Erkmen (2001) inactivated a non-pathogenic *E. coli* strain (KUEN 1504), using SC-CO_2 at 75 bar and at different temperatures and obtained a reduction of 8-log cycles in 80 min at 20 ºC, and in 50 min at 40 ºC.

In general, higher temperatures accelerate the microbial inactivation and, consequently, shorter exposure times are needed to reach a desired level of inactivation (Garcia-Gonzalez et al., 2007).

In this regard, higher temperatures enhance the diffusivity of CO_2, and can also increase the fluidity of the cell membrane making penetration easier (Valverde et al., 2010), thus facilitating the decrease of intracellular pH and the extraction of vital cell constituents. However, SC-CO_2 treatments should not be operated at temperatures far above its critical temperature because, within this

region, the density of the solvent and hence its solubilization capacity decreases quite rapidly as the temperature increases (Lin et al., 1994; Hong & Pyun, 1999). Thus, the stimulating effect temperature has on CO_2 penetration can be partly counteracted by its inhibiting effect on CO_2 solubility.

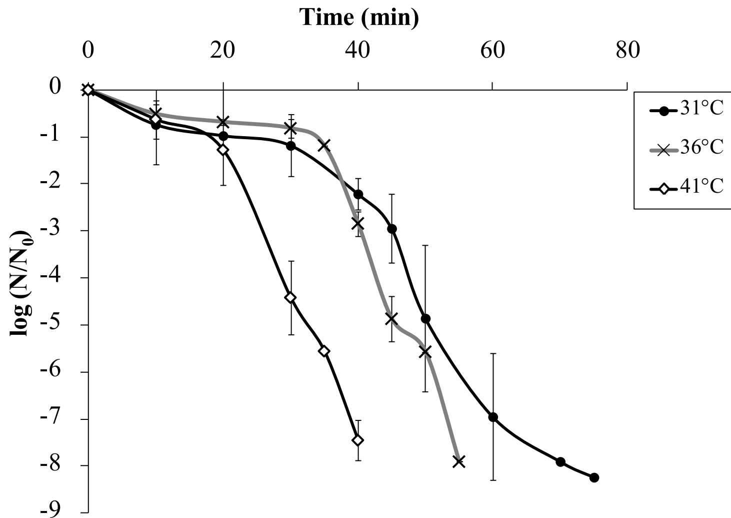

Fig. 4. Inactivation kinetics of *Escherichia coli* DH1 in LB medium at different temperatures, using SC-CO_2 at 225 bar.

3.2.2. Application of HPU to SC-CO_2 inactivation at different temperatures

Fig. 5 shows the inactivation kinetics obtained using the SC-CO_2+HPU patented system at different temperatures and at constant pressure (225 bar). Again, remarkable differences were found compared to when SC-CO_2 is used alone (Fig. 4). The viability began to decrease immediately, following a first order kinetics with no lag phase. No significant ($p>0.05$) effect of temperature was found at the different processing times and the inactivation rate was similar for all the temperatures tested. In every case, only 2 min were needed to reach a complete microbial inactivation (8 log-cycles). Therefore, using SC-CO_2+HPU, 96 % less time is required to obtain this reduction of cells compared to the use of SC-CO_2 alone.

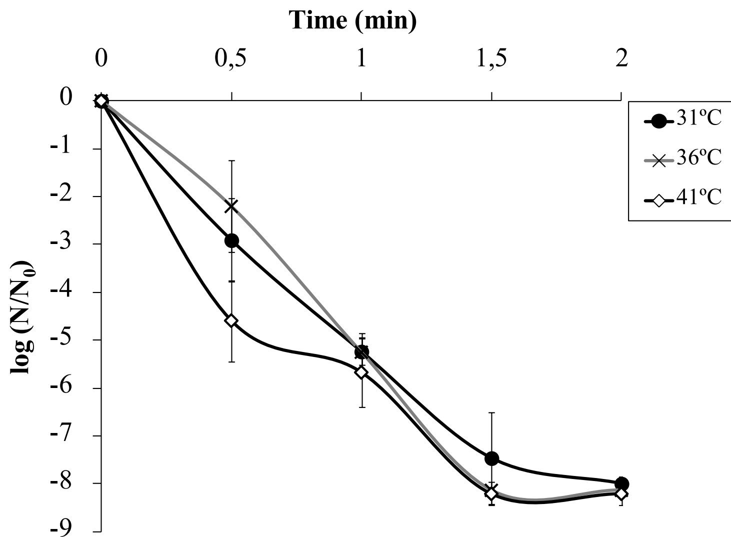

Fig. 5. Inactivation kinetics of *Escherichia coli* DH1 in LB medium at different temperatures, using SC-CO_2+HPU at 225 bar.

As previously explained, the HPU accelerated the inactivation mechanism involved, through the improvement of mass transfer (CO_2 penetration and extraction of cellular components) and the enhancement of CO_2 solubilization. Therefore, the effects caused by HPU would be so intense that the effect of temperature on the inactivation kinetics would be masked. Unlike the case of pressure, the temperatures used had no consequence on either the performance of the transducer or the acoustic field.

3.3. Effect of HPU on the SC-CO_2 inactivation kinetics in different suspending media

3.3.1. SC-CO_2 inactivation in different suspending media

Fig. 6 shows the effect that the nature of the suspending media has on the SC-CO_2 inactivation process at 350 bar, 36 ºC and 25 min of treatment. The results obtained using LB Broth were quite different to those obtained with apple and orange juices. As previously shown, in LB Broth, the survival curve presents an initial lag phase followed by a log-linear section. The microbial reduction during the lag phase was of only 1log-cycle and its length was of approximately 10 min; 8 log-cycles were reached in 22 min.

By contrast, when apple and orange juices were inoculated and treated in the same conditions as LB Broth, only a slight inactivation was observed. The initial population was only reduced by 0.5 and 1 log-cycles in apple and orange juice, respectively, after 25 min of treatment.

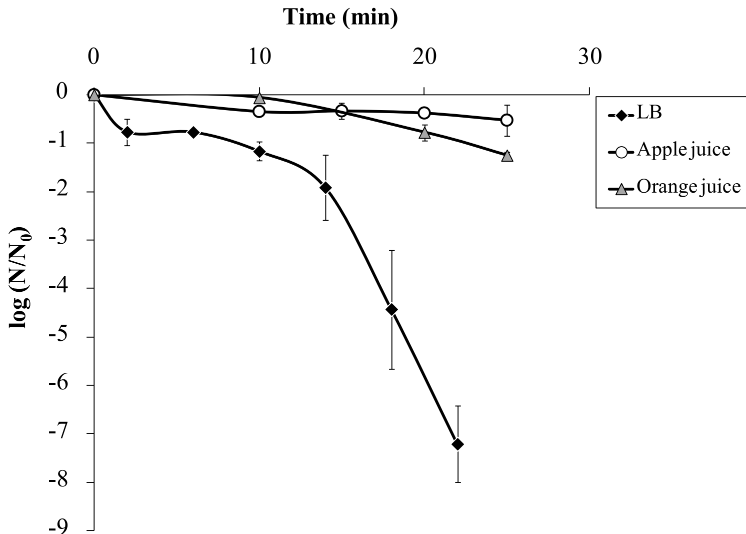

Fig. 6. Inactivation kinetics of *Escherichia coli* DH1using SC-CO_2 at 350 bar and 36 ºC in different media.

The SC-CO_2 microorganisms' inactivation rate is strongly affected by the constituents of the suspending media and/or the nature of the treated foods (Garcia-Gonzalez et al., 2007). Lin et al. (1994) related the increased resistance to SC-CO_2 inactivation of cells suspended in sweetened complex media to the sugar content. They postulated that the presence of sugar in the suspending medium probably led to a decrease in the amount of CO_2 penetrating the cells by changing the structure of cell membranes. Furthermore, the different constituents of the solutions (Hong & Pyun, 1999) give the suspending media a buffering capacity, preventing further pH reduction during SC-CO_2 treatment. Spilimbergo (2002) showed that, while in pure water the pH is greatly affected by the addition of CO_2, the CO_2 effect in orange juice is totally buffered. This buffering effect prevents the acidification of the medium and, consequently, the alteration of the membrane permeability of the cells. This, along with the protective effect of sugar and other components of juice, could make it more difficult for SC-CO_2 to

penetrate the cells. As a consequence, the extraction of vital intracellular components and other physiological changes would not take place, delaying the inactivation process. Under the studied conditions (time, temperature and pressure) the inactivation of *E. coli* in apple or orange juices was too slow to be considered as an effective technique.

3.3.2. Application of HPU to SC-CO_2 inactivation in different suspending media

Fig. 7 shows the inactivation curves of *E. coli* cells undergoing a combined SC-CO_2+HPU process at 350 bar and 36 ºC, LB Broth, apple juice and orange juice being the inoculated and treated media. When comparing these results with those obtained using SC-CO_2 alone (Fig. 6), outstanding differences in the microbial inactivation rate were observed for all the suspending media.

The inactivation kinetics obtained using SC-CO_2+HPU were similar for all the suspending media and were represented by a fast-to-slow curve (Fig. 7). Unexpectedly for times of under 2 min, a significantly ($p<0.05$) faster inactivation of *E. coli* was observed in the juices compared to LB Broth. No lag phase was observed in any media and the viability began to decrease immediately up to 2 min, achieving a reduction of 6 log-cycles and 7 log-cycles using LB Broth or juices, respectively. After this time, the inactivation rate for all the suspending media decreased for the following 3 min, until a reduction of 8 log-cycles was obtained.

Using SC-CO_2+HPU, the influence of the nature of the media is masked by the vigorous agitation of the ultrasonic field. This agitation would disturb the interaction of sugars and other constituents with the cell membrane, preventing the inhibitory effect of these compounds on the cell penetration of SC-CO_2. Moreover, the agitation would be enhancing both the solubilization of CO_2 and the cell-medium mass transfer. This would make the cellular penetration of SC-CO_2 and the extraction of vital intracellular constituents from cells faster, regardless of the suspending media (Garcia-Gonzalez et al., 2007).

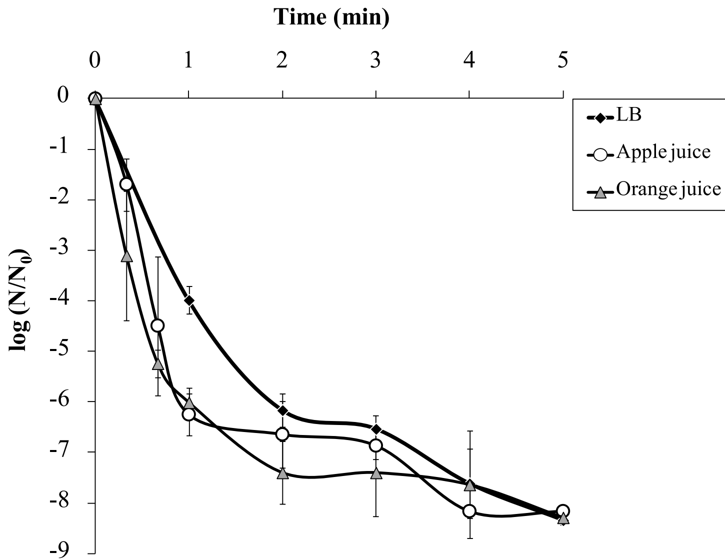

Fig. 7. Inactivation kinetics of *Escherichia coli* DH1using SC-CO_2+HPU at 350 bar and 36 ºC in different media.

3.4. Synergistic effect of SC-CO_2 and HPU.

Fig. 8 shows the inactivation kinetics of *E. coli* DH1 subjected to different treatments: SC-CO_2 (350 bar, 36 ºC), HPU (40 W ± 5 W, 36 ºC) and SC-CO_2+HPU (40 W ± 5 W, 350 bar, 36 ºC). The combination of SC-CO_2 and HPU had a greater effect than the addition of their individual effects. After 5 minutes of treatment with only HPU or SC-CO_2, reductions of 1 and 0.3 log-cycles were observed, respectively. Instead, when both treatments were simultaneously applied, a reduction of 8.5 log-cycles was achieved in 5 min of treatment, which is more than 6 times the inactivation when taking the addition of the individual effects of SC-CO_2 and HPU (1.3 log-cycles) into account. On the other hand, when using only SC-CO_2, 25 minutes were needed to reach total inactivation. For the same time of HPU treatment, only a reduction of 4 log-cycles was achieved. Therefore, the combination of applying SC-CO_2+HPU resulted in a drastic increase of the inactivation rate of *E. coli* cells, which shows a clear synergistic effect of the combination of these techniques.

As far as the shape of the curves is concerned, differences were also found between treatments using the individual techniques (SC-CO_2 and HPU) and the combination of both. In the inactivation kinetics obtained using SC-CO_2+HPU, no lag phases were observed and the kinetics represented a fast-to-slow curve. For times of under 2 min, a fast inactivation of *E. coli* was observed, the inactivation rate being -3.1 log-cycles/min. In the following 3 min, the inactivation rate decreased to -0.66 log-cycles/min up to 8 log-cycles reduction. When only SC-CO_2 is used, a 15 min lag phase appears, which has been previously described as the time needed for the cell penetration of CO_2 and the time during which only the most sensitive cells are inactivated. Once this phase was finished, the log-linear period that followed showed an inactivation rate of -0.5 log-cycles/min. The ultrasound treatment showed two log-linear inactivation stages, the inactivation rate being -0.18 log-cycles/min in the first stage and -0.13 log-cycles/min in the second stage. The absence of a lag-phase could indicate that there was no limiting step in the conditions applied to affect the mechanism of action of HPU. This would also point to the fact that the lag-phase observed for SC-CO_2 at 350 bar may be attributed to a mass transfer limiting step. The application of HPU to the SC-CO_2 treatments involves the elimination of the lag-phase, which is responsible for most of the process time reduction. Moreover, the higher inactivation rate (-3.15 log-cycles/min) during the first stage of the SC-CO_2+HPU compared to the log-linear period of SC-CO_2 treatments (-0.5 log-cycles/min) also contributes to the process time reduction.

In the present study, the synergistic effect of combining SC-CO_2 with HPU has been observed and seems to be linked to mass transfer phenomena at cell wall interfaces. However, the mechanisms under which the synergistic action takes place need to be better understood in order to improve the effectiveness of this phenomenon.

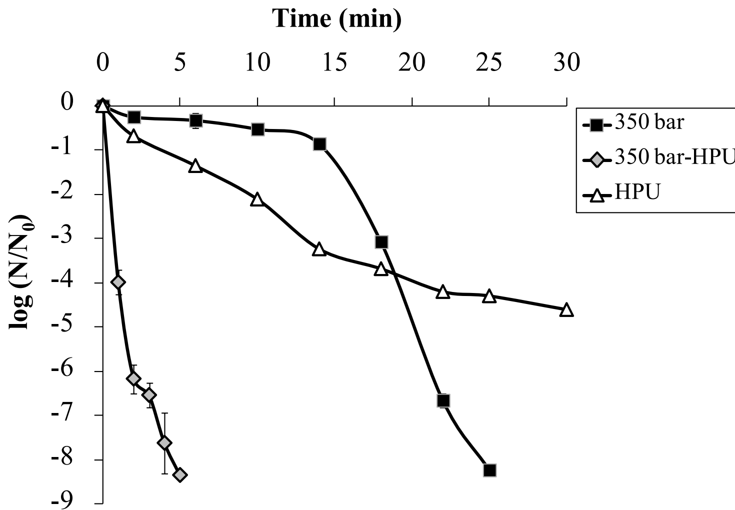

Fig. 8. Synergistic effect. Inactivation kinetics of *Escherichia coli* DH1using HPU (40 ± 5 W, 36 ºC), SC-CO_2 (350 bar, 36 ºC) and SC-CO_2+HPU (350 bar, 36 ºC, 40 ± 5 W).

4. CONCLUSIONS

Experiments have been conducted using a novel inactivation technique based on High Power Ultrasound embedded in a Supercritical Fluids Plant. Using only SC-CO_2, the inactivation rate increased progressively as pressure or temperature were raised, but long treatment times were needed. When SC-CO_2 and HPU were used the time needed to reach a reduction of 8 log-cycles was reduced, on average, by 95 %. The application of HPU could accelerate both the solubilization of CO_2 and the cell-medium mass transfer, causing a drastic drop in intracellular pH and an extraction of vital constituents. The acceleration of these processes even masked the effect of pressure and temperature on the inactivation treatments. Therefore, mild pressure (100 bar) and temperature (31 ºC) conditions could be used with the consequent reduction in cost and increase in the nutritional and organoleptic value of food. Using only SC-CO_2, a protective effect of the different compounds present in juices was found when compared to LB medium. However, when the ultrasound field was applied, similar inactivation times were found regardless of the media. The synergistic effect of SC-CO_2 and HPU was also proved. The new technology developed permits both a drastic decrease in SC-CO_2 inactivation times and also the use of

mild process conditions. Further research should be conducted in order to study the effect of the SC-CO_2+HPU treatment on the organoleptic and nutritional quality of the food processed using this new technology.

Acknowledgments

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Chapter 3

Application of High Power Ultrasound in the Supercritical Carbon Dioxide Inactivation of Saccharomyces cerevisiae

C. Ortuño, M.T. Martínez-Pastor, A. Mulet, J. Benedito

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Application of High Power Ultrasound in the Supercritical Carbon dioxide inactivation of *Saccharomyces cerevisiae*

C. Ortuño, M.T. Martínez-Pastor, A. Mulet, J. Benedito

Grupo de Análisis y Simulación de Procesos Agroalimentarios, Departamento Tecnología de Alimentos, Universitat Politècnica de València, Camí de Vera s/n, E46022, Valencia, Spain.

ABSTRACT

The objective of the study was to analyse the influence of high power ultrasound (HPU) on the supercritical carbon dioxide (SC-CO_2) inactivation kinetics of *Saccharomyces cerevisiae* and to determine the effect of the temperature (31-41 ºC), pressure (100-350 bar) and composition of the medium (YPD Broth, apple and orange juice) on the process of inactivation. Using a batch-mode SC-CO_2 at 350 bar and 36 ºC, a reduction of 6.7 log-cycles was obtained after 140 min of treatment. However, when HPU (40 W ± 5 W and 30kHz) was applied during the SC-CO_2 treatments, a reduction of 7 log-cycles was achieved after 2 min of treatment for all pressures and temperatures applied. The effect of increasing pressure (from 100 to 350 bar, 36 ºC) or temperature (from 31 to 41 ºC, 225 bar) did not significantly influence this inactivation level. The application of ultrasound leads to a vigorous agitation and cavitation which could accelerate the SC-CO_2 dissolving in the medium. This accelerates the penetration of CO_2 into cells and its inactivation mechanisms. In batch operations the application of HPU increases the speed of reaching saturation solubility of CO_2 in many liquid media and significantly reduces microbial inactivation times.

Key words: supercritical carbon dioxide, high power ultrasound, *Saccharomyces cerevisiae*, inactivation, synergistic effect.

1. INTRODUCTION

Non-thermal processing alternatives have been developed by the food industry in response to an increasing consumer demand for fresh, high quality food products. These preservation technologies aim to preserve the nutrients and functionality of food, extending food shelf-life and minimizing the changes in natural color, taste, flavor and texture (Rawson et al., 2011; Bermúdez-Aguierre & Corradini, 2012), while at the same time being energy-saving, and environmentally safe processes.

The use of supercritical carbon dioxide (SC-CO_2) continues to attract attention as a non-thermal technique for pasteurization processes since the low temperatures used permit the preservation of natural flavors and nutrients of foods (Kincal et al., 2006; Ferrentino et al., 2009; Spilimbergo & Ciola, 2010). SC-CO_2 has been shown to be efficient at inactivating a large variety of microorganisms, such as *Escherichia coli*, *Enterococcus faecalis* or *Saccharomyces cerevisiae* (Garcia-Gonzalez et al., 2007; Ortuño et al., 2012a, 2012b).

SC-CO_2 has a density close to liquid CO_2, while its diffusivity and solubility are similar to those of a gas which results in an improved dissolving capacity (Tomasula, 2003). Theories explaining the inactivating mechanism of SC-CO_2 involve the diffusion and solubility of SC-CO_2 in the culture medium, the decrease in the medium pH, the increase in the membrane fluidity and permeability, the diffusion of CO_2 into the cells, the cell membrane rupture caused by the increase in the internal pressure, and the resultant changes in the cellular environment, such as a decrease in pH, the inactivation of key enzymes, and extraction of critical intracellular materials (Garcia-Gonzalez et al., 2007; Pataro et al., 2010).

Nevertheless, long treatment times and, in some cases, high pressures or temperatures needed to guarantee the food's safety and stability, limit the efficiency of SC-CO_2 inactivation processes (Garcia-Gonzalez et al., 2009; Liu et al., 2012). That is the reason why there is increasing scientific interest in process intensification, which focuses on combining SC-CO_2 processes with synergistic techniques that enhance the SC-CO_2 inactivation mechanisms.

It is known that High Power Ultrasound (HPU) technology accelerates and improves mass transfer processes (Awad et al., 2012). In fact, it has been demonstrated that the application of HPU to the SC-CO_2 extraction process is highly beneficial as a consequence of the mechanical effects produced in the supercritical environment, compared to SC-CO_2 extraction alone (Riera et al., 2010). When ultrasound travels through a medium, it produces effects, such as alternating compressions, cavitation, vibration, streaming and agitation, which enhance mass transfer. Riera et al. (2010) reported that the yield of almond oil was increased by 20% in the presence of ultrasound compared to traditional SC-CO_2 extraction. Ortuño et al. (2012b) showed the advantages of simultaneously applying SC-CO_2 with the HPU treatment when inactivating a Gram-negative bacterium, *E. coli,* compared with the use of SC-CO_2 alone.

The microorganisms investigated by means of SC-CO_2 treatments ranged from Gram-negative bacteria like *Salmonella typhimurium*, *Escherichia coli* or *Yersinia enterocolitica*, to Gram-positive bacteria or yeasts, like *Listeria innocua*, *Listeria monocytogenes* or *S. cerevisiae* (Garcia-Gonzalez et al., 2007; Bermúdez-Aguierre et al., 2012). Most of the studies dealing with inactivation techniques, including SC-CO_2, have indicated that gram-positive cells are more resistant than gram-negative ones due to the fact that their cell wall is thicker (Ramírez Santos et al., 2005). Moreover, it is known that *S. cerevisiae* has a thicker cell wall, which makes it similar to gram-positive bacteria (Villas-Boas et al., 2006). In this regard, Ortuño et al. (2012a) showed that to inactivate *S. cerevisiae* and *E. coli* using SC-CO_2 under 350 bar and 36 ºC, 140 and 25 min are needed to reach a reduction of 7 log, respectively, which would support the connection between wall thickness and inactivation resistance.

A previous study has been performed to analyze the advantages of coupling SC-CO_2 with HPU for the inactivation of a Gram-negative bacterium, *E. coli* DH1 (Ortuño et al., 2012b). This study showed that the combination of both techniques accelerated the death of *E. coli* compared with the use only of SC-CO_2. In this regard, it is of great interest to know how a microorganism with a higher resistance than *E. coli*, such as *S. cerevisiae* (Ortuño et al., 2012a), responds to SC-CO_2+HPU treatment.

The objective of this study was to evaluate the effect of High Power Ultrasound on the SC-CO_2 inactivation kinetics of *Saccharomyces cerevisiae* and to determine the effect of the temperature, pressure and composition of the medium on the inactivation process.

2. MATERIALS AND METHODS

2.1. Microbial preparation

2.1.1. Microorganisms

The microbial strain used in this study was *S. cerevisiae* T73 (*S. cerevisiae*). It is a natural strain isolated from wine fermentation in Alicante (Spain) (Querol et al., 1992), and it is commercialized as Lalvin T73 (Lallemand Inc., Montreal, Canada).

2.1.2. Sample preparation and growth conditions

S. cerevisiae was grown in Yeast Peptone Dextrose Broth (YPD Broth, Sigma-Aldrich, USA) overnight at 30 ºC, using an incubation chamber (J.P. SELECTA, Model 3000957, Barcelona, Spain) and an orbital shaker at 120 rpm (J.P. SELECTA, Rotabit Model 3000974, Barcelona, Spain). For each experiment, a subculture was prepared by inoculating 50 µL from the starter in 50 mL of sterilized medium and incubating at 30 ºC for 24 h to obtain cells in the early stationary phase. Growth curves were determined in advance by both plating and the measurement of absorbance at 600 nm (data not shown).

S. cerevisiae stock cultures were maintained in Yeast Peptone Dextrose Agar (YPD Agar, Sigma- Aldrich, USA), stored at 4 ºC and transferred monthly to new plates.

2.2. Experimental design

To determine the effect of the process conditions (temperature and pressure) on the SC-CO_2 inactivation of *S. cerevisiae*, YPD Broth was selected as a medium. To determine the effect of temperature, samples were exposed to SC-CO_2 at 31 ºC, 36 ºC and 41 ºC at a constant pressure of 225 bar. The temperatures chosen were higher than the critical one for CO_2 and lower than lethal temperatures for *S. cerevisiae*. To determine the effect of pressure, samples

were treated by SC-CO_2 at 100, 225, 290 and 350 bar at a constant temperature of 36 ºC. The pressures chosen were higher than the critical one for CO_2 (73.8 bar) and lower than 350 bar according to a previous study about inactivation of *E. coli* using SC-CO_2 and SC-CO_2+HPU where it was observed that higher pressures of 350 bar was not necessary to reach an acceptable level of inactivation (7 log) using SC-CO_2+HPU (Ortuño et al., 2012b). In the literature, the hydrostatic effect of pressure on microorganisms is negligible below about 200 MPa (Corwin & Shellhammer, 2002), therefore the possible inactivation effect found in the pressure range considered in this study should be attributed to the aforementioned effects of SC-CO_2 on vegetative cells rather than to pressure.

In order to determine whether the combined use of SC-CO_2 and HPU affected the inactivation kinetics, experiments to sonicate the treatment medium (YPD broth) were conducted under the same process conditions of temperature and pressure as the treatments using only SC-CO_2.

In addition, to evaluate the possible combination effect between SC-CO_2 and HPU, an inactivation experiment using HPU (40 W ± 5 W, 36 ºC, 30 kHz) on YPD Broth medium was carried out and compared with the SC-CO_2 and SC-CO_2+HPU inactivation treatments at 36 ºC and 225 bar (intermediate conditions).

In order to evaluate the effectiveness of this novel technique (SC-CO_2+HPU) in food matrices, apple and orange juice were selected to compare them with the inactivation kinetics on YPD Broth. These juices were chosen due to the fact that they are used extensively for microbial inactivation using SC-CO_2 (Kincal et al., 2006; García-Gonzalez et al., 2007; Ferrentino et al., 2009).

Fruit was purchased from a local market and kept at 4 ºC until juice extraction. Apple and orange juices were obtained according to the following steps: washing, peeling and extraction (Ultra Juicer, Robot Coupe J80, USA). The apple juice (pH = 5.4; ºBrix = 15.6) and orange juice (pH = 3.8; ºBrix = 11.6) produced was sealed in plastic containers and stored at -18 ºC and thawed at 4 ºC (overnight) before the treatment. The juice samples were exposed to SC-CO_2 and SC-CO_2+HPU treatments, at 225 bar and 36 ºC (intermediate conditions). All experiments were done in triplicate.

2.3. Supercritical fluid equipment and processing procedure

2.3.1. Apparatus

The SC-CO_2 experiments were carried out in a laboratory scale SC-CO_2 batch system which permits the treatment of liquid foods (Fig. 1). The maximum pressure at which the apparatus may be operated was 1000 bar. The system included a CO_2-tank (1, Fig. 1) and a N_2-tank (2, Fig. 1), which were kept at room temperature; a chiller reservoir stored at -18 ºC (3, Fig. 1); a pump (4, Fig. 1) and a thermostatic bath (5, Fig. 1) to keep the inactivation vessel (6, Fig. 1) at the desired temperature. The inactivation vessel (500 mL), as well as the different connections and valves in contact with SC-CO_2, were made of stainless steel, type 316. The inactivation vessel was 3 cm in thickness, to assure that the process temperature was reached inside the treatment vessel, a temperature probe was installed at the inner vessel surface (7, Fig. 1). This probe was connected to a digital controller (E5CK, Omron, Hoofddorp, Netherlands) which used an electric resistance to heat the bath and keep the temperature in the vessel at the desired value before to start of the treatment. On average, the bath temperature was 5-6 ºC higher than that inside the vessel. A pressure gauge was installed in the inactivation vessel to confirm that only a short time (2.5 min) was needed for the supercritical conditions to be reached and subsequently maintained during processing. In addition, the ultrasound equipment (Benedito et al., 2011) was embedded in the supercritical fluid vessel. The transducer (> 1 W/cm^2) was inserted in the inactivation vessel and included two commercial ceramics (10, Fig. 1; 35 mm external diameter; 12.5 mm internal diameter; 5 mm thickness; resonance frequency of 30 kHz) and a sonotrode (8, Fig. 1), which was specially constructed to concentrate the highest amount of acoustic energy on the application point. The power generator unit (11, Fig. 1) supplied constant energy to the transducer during the SC-CO_2 process. The piezoelectric ceramics were insulated by means of a polypropylene joint (9, Fig. 1) covered with teflon in order to avoid possible electrical short-circuits.

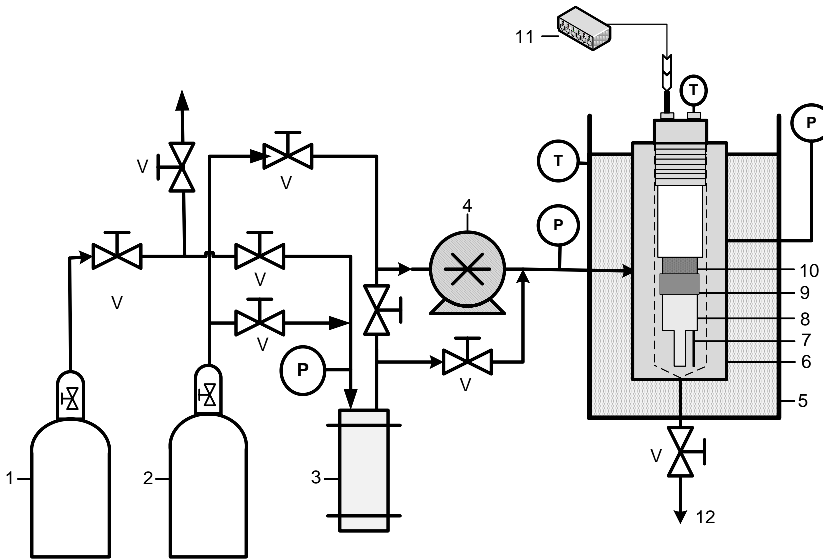

Fig. 1. Supercritical CO_2 treatment system. 1-CO_2 tank; 2-N_2 tank; 3-Chiller reservoir; 4-Pump; 5-Temperature controlled bath; 6-Treatment vessel; 7-Temperature Sensor; 8-Transducer; 9-Insulation joint; 10-Ceramics; 11-Power Generation Unit; 12-Sample extraction; V-Valve; P-Manometer.

2.3.2. Supercritical fluid processing

Prior to each experiment, the inactivation vessel was cleaned and sanitized with disinfectant solution, distilled water and autoclaved water. For each experiment, a subculture was prepared by inoculating 5 mL of cells in the early stationary phase (prepared as described in section 2.1.2) in 50 mL of sterilized YPD Broth, orange or apple juice to a cell concentration of 10^6-10^7 CFU/mL. The inoculated YPD, orange or apple juice (55 mL) was loaded into the inactivation vessel and immediately sealed. The pump filled the vessel with supercritical carbon dioxide, reaching the desired pressure in less than 2.5 min. The ratio (v/v) of SC-CO_2/liquid medium was 3.8. Time zero for each treatment was taken when the experimental temperature and pressure were reached. For the experiments with HPU, the ultrasound unit was turned on when the desired pressure was reached in the vessel, the power applied during the whole experiment being 40 W ± 5 W (W = 0.088 W/cm^{2}mL; I = 250 mA ± 10 mA; U = 220 V ± power applied during 5 V) (Power measured with a Digital Power Meter, Yokogawa,

Model WT210). Pressure and temperature were kept constant during the experiment by means of the pump and the thermostatic bath, respectively.

Samples of 3 mL were extracted periodically through a small tube (2 mm diameter, Fig. 1) located at the bottom of the inactivation vessel until the end of the experiment. This tube was cleaned and disinfected with 3 mL ethanol (96 %v/v) after each sampling. The sample extraction was performed without stopping the treatment. The SC-CO_2 treated samples were collected in individual sterile plastic test tubes.

2.4. Enumeration of viable microorganisms

The cell viability in the samples before and after each treatment was determined by the plate count method. Each sample was serially diluted and 100 µL of the appropriate dilutions were plated in triplicate on YPD Agar. The plates were incubated at 30 ºC for 24 h before counting. The results were expressed as log (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 different treatment times. In the experiments carried out, other microorganisms besides *S. cerevisiae* were not observed before and after the treatment. The experimental results shown were the arithmetic mean and the standard deviation of log (N/N_0) for at least three plates.

2.5. Modelling of the inactivation kinetics and statistical analysis

The microbial mortality during thermal processing has been assumed to follow the first-order kinetics (Corradini & Peleg, 2012) and this assumption has been extended to other microbial inactivation methods, including exposure to a supercritical CO_2 (Corradini & Peleg, 2012). Strong evidence suggests that there is no reason for this assumption for any method of inactivation, be it thermal or non-thermal (Peleg, 2006). Microbial mortality exposed to different inactivation treatment can be considered similar to particulate breakage under mechanical stress. Consequently, the distribution function that could describe this behavior is the Weibull distribution (Peleg, 2006).

The Weibull distribution was used in this study to describe the microbial inactivation kinetics of *S. cerevisiae* obtained with SC-CO_2 and SC-CO_2+HPU.

The most widely accepted version of this model is described by Eq. (1) (Corradini & Peleg, 2012):

$$\log_{10} \frac{N}{N_0} = -b\,(P, T)\, t^{\,n\,(P, T)} \qquad \text{Eq. (1)}$$

where N_0 is the number of microorganisms initially contained in the sample at time 0, N the corresponding number after a determined time of treatment and b (P, T) and n (P, T) are pressure- and temperature-dependent coefficients.

According to the Weibull distribution, the isobaric or isothermal inactivation rate is a function not only of pressure or temperature, respectively, but also of time (Peleg, 2006). Consequently, the pressure- or temperature dependence of the rate parameter b (P, T) was described by the log-logistic model shown in Eq. (2) or Eq. (3), respectively (Peleg, 2006):

$$b(P) = \ln\left(1 + \exp(k_1 (P - P_c))\right) \qquad \text{Eq. (2)}$$

$$b(T) = \ln\left(1 + \exp(k_2 (T - T_c))\right) \qquad \text{Eq. (3)}$$

where k_1 and P_c are two survival parameters of the microorganism at different pressures and isothermal conditions; k_2 and T_c are two survival parameters of the microorganism at different temperatures and isobaric conditions. The application of the log-logistic model does not require that n (P, T) must be pressure or temperature dependent (Corradini & Peleg, 2012) and therefore n was considered constant in the present work.

The kinetic constants of the models were calculated by minimizing the sum of square differences between experimental and model predicted data using the Solver Microsoft Excel^{TM} tool. The root mean square error (RMSE, Eq. (4)) and the coefficient of determination (R^2, Eq. (5)) were used to evaluate the fitting goodness of the model and the estimation accuracy (Schemper, 2003).

$$RMSE = \sqrt{\frac{\sum_{k=1}^{z} (y_k - y_k^*)^2}{z}} \qquad \text{Eq. (4)}$$

$$R^2 = 1 - \frac{S_{yx}^2}{S_y^2} \qquad \text{Eq. (5)}$$

where y and y* are the experimental data and the estimated value, respectively, calculated as $\log_{10}(N/N_0)$; z is the number of experimental data and S_y and S_{yx} are the total standard deviation and the standard deviation of the estimation, respectively.

3. RESULTS AND DISCUSSION

3.1. Inactivation of *S. cerevisiae* using SC-CO_2

3.1.1. Effect of pressure on the SC-CO_2 inactivation kinetics

Fig. 2 shows the inactivation of *S. cerevisiae* in YPD Broth exposed to SC-CO_2 at different treatment pressures (100, 225, 290 and 350 bar) and at constant temperature (36 ºC). Two phases were observed in all the survival curves*:* an initial lag-phase of 80 min, indicated by a shoulder in the curve, followed by an inactivation phase. Once the lag-phase was exceeded, the number of viable cells decreased at every pressure; the higher the treatment pressure, the faster the decrease: 0.018, 0.036, 0.06 and 0.128 log-cycles/min at 100, 225, 290 and 350 bar, respectively. The number of log reductions obtained after 140 min of treatment was significantly different ($P<0.05$): 1.41, 2.33, 3.15, and 6.70 log cycles, at 100, 225, 290 and 350 bar, respectively.

The initial lag-phase has been attributed by Lin et al. (1992) to the time needed for CO_2 to penetrate through the cellular envelope, which determines when the CO_2 begins to exert its inactivation effect. In this case, *S. cerevisiae* T73, required a minimum of 80 min to observe the initial effects of SC-CO_2 and for inactivation mechanisms to take place. The penetration of SC-CO_2 into cells increases with the solubility and saturation of SC-CO_2 into the medium (Kumagai et al., 1997) which is enhanced by a vigorous agitation of sample with CO_2. The inactivation vessel used was not agitated during the experiments with SC-CO_2, so the initial lag-phase (80 min) could be attributed to mass transfer limitations between SC-CO_2 and the cells suspended in the sample.

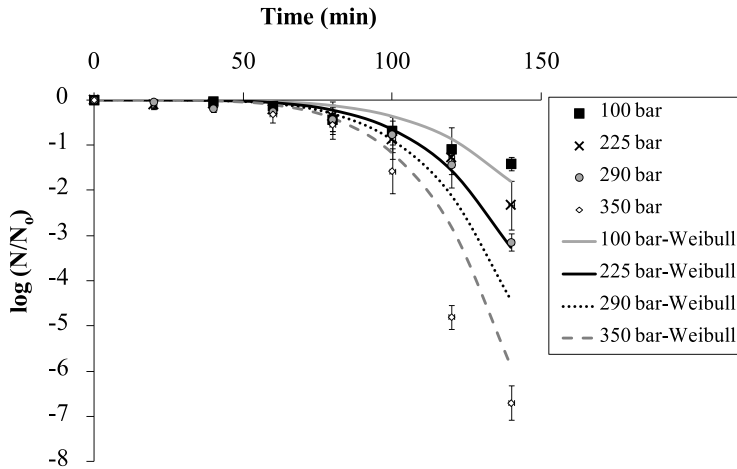

Fig. 2. Experimental data (discrete points) and modeling (curves) of the inactivation kinetics of *Saccharomyces cerevisiae* in YPD medium at different pressures, using SC-CO_2 at 36 ºC.

In a recent study, Ortuño et al. (2012b) reported that the inactivation of *E. coli* DH1 using SC-CO_2 showed lag-phases whose duration was affected by pressure. The similarities between the lag-phases for all the pressures in the SC-CO_2 inactivation of *S. cerevisiae* could be due to the greater resistance shown by the yeast compared to *E. coli*, which is a consequence of its thicker cell wall. This wall thickness could slow down the inactivation mechanisms, such as the chemical modification of the lipid double-layer of the microbial cell membrane, the increase of cell permeability to SC-CO_2 and its penetration into cells (Watanabe et al., 2005), for all studied pressures.

Erkmen (2003) reported that the inactivation rate of *S. cerevisiae* increased with pressure, as a rise from 75 to 100 bar at 30 ºC produced an increase from 0.07 to 0.09 log-cycles/min. This inactivation rate was higher than in the present study, 0.018 log-cycles/min at 100 bar and 36ºC. Both the longer lag-phase and the slower inactivation rate could be attributed to the different nature of the strains used, as it is known that wild strains, like *S. cerevisiae* T73, are usually more resistant to inactivation mechanisms than laboratory strains like *S. cerevisiae* ATCC 9743 (Garcia-Gonzalez et al., 2007). Moreover, these differences could also be linked to the different nature of the treatment medium (Erkmen, 2001; Erkmen & Dogan, 2004).

Once the log-linear inactivation phase was reached, the SC-CO_2 dissolved in the medium would diffuse into cells, interacting with their cytoplasm and permitting the extraction of intracellular substances and the inactivation of key metabolic enzymes. These could compromise the biological balance of the cell (Watanabe et al., 2005; Liao et al., 2006), resulting in its inactivation (Liao et al., 2006; Oulé et al., 2006). Despite the pressure-induced increase in the inactivation rate, it was remarkably slow for *S. cerevisiae* when compared to *E. coli* (Ortuño et al., 2012b), due to its thicker cell wall that would delay the effect of the inactivation mechanisms (such as extraction of cellular components).

Only at 350 bar, 35 ºC and after 140 min a total inactivation level (no growth in enumeration) was reached. This time is too long compared with a continuous SC-CO_2 system, or with systems having agitation. Dagan and Balaban (2006), using a continuous SC-CO_2 system, showed a reduction in yeast population of 7.38 logs at 265 bar and 21 ºC after 4.77 min. In a continuous system the agitation allows a faster saturation of CO_2, and therefore a better contact with cells, compared to batch systems.

3.1.2. Effect of temperature on the SC-CO_2 inactivation kinetics

Fig. 3 shows the inactivation kinetics of *S. cerevisiae* subjected to SC-CO_2 processing in YPD Broth at different temperatures (31 ºC, 36 ºC and 41 ºC) and at constant pressure (225 bar).

All the curves showed a two-phase kinetics, with a shoulder indicating a lag-phase followed by an inactivation phase. During the lag-phase, no inactivation was observed until approximately 80 min at 31 and 36 ºC and 40 min at 41 ºC. Once the exponential phase started, the inactivation rate was quite similar, 0.023, 0.033 and 0.032 log-cycles/min, for 31, 36 and 41 ºC, respectively. Due to the long lag-phase and to the slow and similar inactivation rate, the number of log reductions obtained after 140 min of treatment was not significantly different ($P>0.05$) at any of the three temperatures studied, the average reduction being 2.20 log cycles/min.

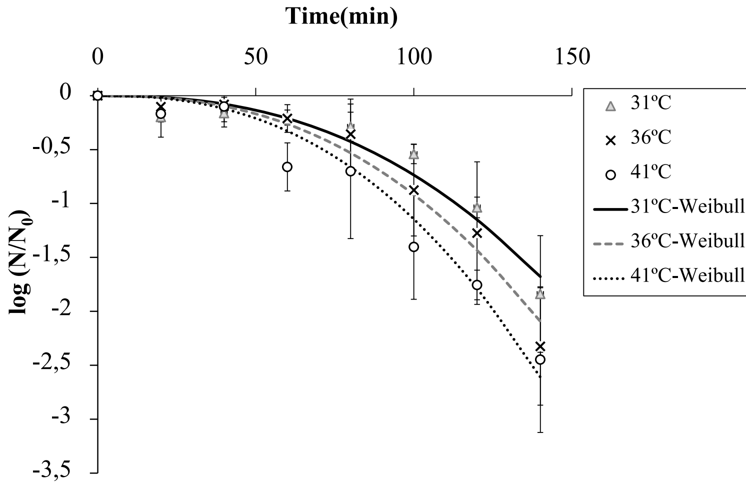

Fig. 3. Experimental data (discrete points) and modeling (curves) of the inactivation kinetics of *Saccharomyces cerevisiae* in YPD medium at different temperatures, using SC-CO_2 at 225 bar.

As temperature increased, the CO_2 viscosity decreased and the cell membrane fluidity augmented, increasing the cell wall permeability. Therefore, it could be thought that a temperature rise would make it easier for CO_2 to penetrate into the cells. Nevertheless, the effect of temperature was not significant since similar inactivation levels were found after 140 min of treatment at the different temperatures used.

Erkmen (2003) showed a shortening of the lag-phase, 50, 30 and 15 min, in the SC-CO_2 inactivation kinetics of *S. cerevisiae* (ATCC 9743) using 30, 40 and 50 ºC, respectively, at 100 bar. Shorter lag-phases and higher inactivation rates were reported for *E. coli* (Ortuño et al., 2012b) than *S. cerevisiae* which, as previously mentioned, could be due to the differences in the cell membrane thickness.

3.2. Inactivation of *S. cerevisiae* using SC-CO_2+HPU

3.2.1. Effect of pressure on the SC-CO_2+HPU inactivation kinetics

Fig. 4 reports the inactivation kinetics of *S. cerevisiae* in YPD Broth using SC-CO_2+HPU at different pressures (100, 225, 290 and 350 bar) and at a constant temperature of 36 ºC. As expected, the inactivation of *S. cerevisiae* was

considerably faster when using SC-CO_2+HPU (Fig. 4) than when using SC-CO_2 alone (Fig. 2), where a minimum time of 80 min was necessary to start inactivation.

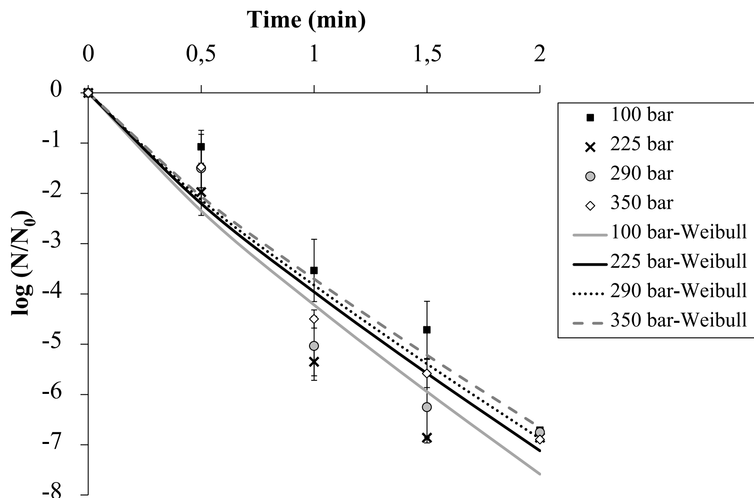

Fig. 4. Experimental data (discrete points) and modeling (curves) of the inactivation kinetics of *Saccharomyces cerevisiae* in YPD medium at different pressures, using SC-CO_2+HPU at 36 ºC.

The kinetics obtained using SC-CO_2+HPU represented a fast-to-slow curve. No lag-phases were observed and the viability began to decay quickly starting to slow down after 1 min. After 0.5 min of treatment, there were significant differences ($P<0.05$) between the inactivation kinetics at 100 and 350 bar, and after 1 and 1.5 min between 100 bar and the other three pressures. After 2 min of treatment, total inactivation (7 log-cycles) was reached for every pressure condition. There were non-significant differences ($P>0.05$) between the inactivation rates in the inactivation phase of the curve at 225, 290 and 350 bar; on average, these were 4.95 log-cycles/min. At 100 bar, a slower inactivation rate of 3.53 log-cycles/min was obtained for the first minute of treatment. Despite this slight difference between 100 bar and the remaining pressures, only 2 min of treatment were required to obtain a complete reduction in all cases. In the SC-CO_2 treatments (Fig. 2), a reduction of 6.7 log-cycles was obtained after

140 min of treatment at 350 bar and 36 ºC, which shows how the application of ultrasound intensifies the inactivation of *S. cerevisiae* using batch mode SC-CO_2.

It is known that high intensity ultrasound generates cavitation in the medium to which it is applied. Cavitation refers to the formation, growth, and implosion of tiny gas bubbles in a liquid when ultrasound travels through it and it has been proven to cause cracked or damaged cell walls, an increase in component diffusion, interfacial turbulence and a reduction in the external resistance to mass transfer (Gao et al., 2009). Due to the fact that *S. cerevisiae* showed a great resistance to the SC-CO_2 treatments and yet the SC-CO_2+HPU treatments bring about a fast inactivation, it could be thought that the acceleration of the solubilization rate of SC-CO_2 into the liquid and the increase in the mass transfer due to the vigorous agitation produced by the ultrasonic field allow to quickly reach the saturation of CO_2 in the medium, which accelerates the inactivation mechanisms. Moreover the damage of the cell walls generated by cavitation could play an important role in both the penetration of SC-CO_2 and the extraction of intracellular compounds, accelerating the death of the microbial cells. Using only SC-CO_2, the inactivation mechanisms were accelerated by increasing treatment pressure since higher pressures increase the solubility of CO_2 in the medium and therefore its contact with cells, moreover higher pressure increases the driving force for mass transfer into cells. Using SC-CO_2+HPU the effects caused by HPU intensify the effects of pressure, so that pressures higher than 100 bar are not necessary to reach an inactivation level of 7 log reductions.

When using SC-CO_2+HPU, no significant differences ($P>0.05$) were found between the inactivation kinetics of *E. coli* DH1 (Ortuño et al., 2012b) and *S. cerevisiae* (Fig. 4). Therefore, the previously mentioned effects caused by HPU would be so intense that, in spite of the different inactivation kinetics observed between *E. coli* and *S. cerevisiae* using only SC-CO_2, when using SC-CO_2+HPU, between 2 and 3 min were necessary to reach 7 log reductions.

3.2.2. Effect of temperature on the SC-CO_2+HPU inactivation kinetics

Fig. 5 shows the inactivation kinetics of *S. cerevisiae* subjected to the SC-CO_2+HPU treatment at different temperatures (31, 36 and 41 ºC), and at a constant pressure of 225 bar. Outstanding differences were found compared with

the use of SC-CO_2 alone (Fig. 3). The viability was observed to decrease after 0.5 min, showing a first order kinetics followed by a slower period that starts after 1.5 min of treatment at 31 ºC and after 1 min at 36 ºC and 41 ºC. The inactivation rate at 31 ºC, 4.2 log-cycles/min, was significantly slower ($P<0.05$) than at 36 or 41 ºC, 5.5 log-cycles/min (average for both temperatures). Despite these differences, total microbial inactivation (7-log cycles) was achieved after a maximum of 2 min in all cases.

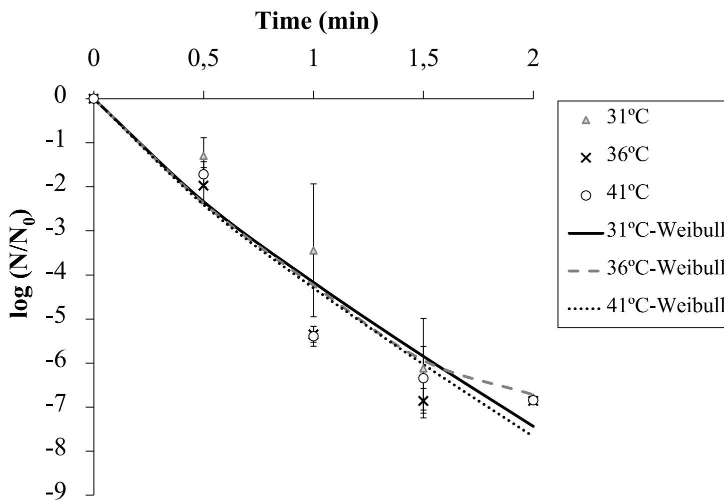

Fig. 5. Experimental data (discrete points) and modeling (curves) of the inactivation kinetics of *Saccharomyces cerevisiae* in YPD medium at different temperatures, using SC-CO_2+HPU at 225 bar.

The short processing times point to the great intensification effect of the combined technique, compared to the use of only SC-CO_2 since, in that case, a maximum reduction of only 2.5 log was attained after 140 min of treatment at 41 ºC and 225 bar.

Therefore, HPU accelerates the inactivation mechanisms involved in the SC-CO_2 treatments, probably through the increase of CO_2 solubilization that allows to reach quickly the saturation of CO_2 in the medium, and enhancing the mass transfer (CO_2 penetration, decreasing internal pH and extraction of cellular components). Similarly to the results obtained from the effect of pressure on SC-CO_2+HPU treatments, the effect of HPU is so intense that the influence of

temperature observed in the SC-CO_2 treatments was not observed, and higher temperatures than (31 ºC) were not necessary.

3.3. Modelling of inactivation kinetics

In order to analyze the survival curves for *S. cerevisiae* after the SC-CO_2 and SC-CO_2+HPU treatments, the kinetic data was fitted to the Weibull distribution (Eq. (1)). The experimental and predicted inactivation kinetics obtained for *S. cerevisiae* using this model can be observed in Fig. 2, 3, 4 and 5.

Table 1 shows the value of the parameters for the different conditions: isothermal (36 ºC) SC-CO_2 and SC-CO_2+HPU treatments at different pressures, and isobaric (225 bar) SC-CO_2 and SC-CO_2+HPU treatments at different temperatures. Therefore, both for the SC-CO_2 experiments with and without HPU, the k_1 and P_c values for the isothermal fitting results can be used to calculate the b value at any pressure between 100 and 350 bars. Similarly, the k_2 and T_c values for the isobaric fitting results can be used to predict the b value at any temperature between 31 and 41 ºC. This model provided good results for SC-CO_2 treatments at different pressures (Fig. 2: $R^2_{avg.}$ = 0.973; $RMSE_{avg.}$ = 0.333) and at different temperatures (Fig. 3: $R^2_{avg.}$ = 0.966; $RMSE_{avg.}$ = 0.141). Using SC-CO_2+HPU, the Weibull distribution model appropriately described the inactivation kinetics at different pressures (Fig. 4: $R^2_{avg.}$ = 0.956; $RMSE_{avg.}$ = 0.75) and temperatures (Fig. 5: $R^2_{avg.}$ = 0.945; $RMSE_{avg.}$ = 0.72).

Table 1. Estimated Weibull distribution parameters for the inactivation kinetics of *Saccharomyces cerevisiae* using SC-CO_2 and SC-CO_2+HPU, at different pressures (at 36 ºC), and temperatures (at 225 bar).

	Parameters		**P (bar)**	**R^2**	**RMSE**	**Parameters**		**T (ºC)**	**R^2**	**RMSE**
SC-CO_2	k_1	0.009	100	0.942	0.400	k_2	0.044	31	0.949	0.131
	P_c	2168.19	225	0.992	0.117	T_c	293.018	36	0.976	0.124
	n	3.646	290	0.981	0.406	n	2.448	41	0.973	0.169
			350	0.977	0.406					
SC-CO_2+HPU	k_1	-0.002	100	0.975	0.935	k_2	-0.012	31	0.963	0.706
	P_c	2097.02	225	0.932	0.858	T_c	393.825	36	0.936	0.747
	n	0.846	290	0.942	0.723	n	0.806	41	0.936	0.706
			350	0.975	0.483					

3.4. Synergistic effect of SC-CO_2 and HPU

The inactivation data of *S. cerevisiae* inoculated in YPD Broth is given in Table 2*,* subjected to different treatments: SC-CO_2 (225 bar, 36 ºC), HPU (40 W, 36 ºC) and SC-CO_2+HPU (40 W, 225 bar, 36 ºC). The combination of SC-CO_2 and HPU had a greater effect than either of the treatments alone, and also greater than the addition of both separate effects. In the first 2 min of treatment, a reduction of 6.83 log-cycles was achieved through the combination of both techniques while, for the same period of time, hardly any inactivation was observed when using only HPU or SC-CO_2. A 99.3 % reduction in the time needed to achieve the same level of inactivation was obtained when SC-CO_2 and HPU were combined, which showed the synergistic effect of the combination.

The mechanisms under which the synergistic action takes place would be due to HPU accelerating the inactivation mechanisms involved in the SC-CO_2 treatments, from the diffusion and solubility of SC-CO_2 in the culture medium, to the diffusion of CO_2 into the cells damaging cellular environment. In addition HPU generates cavitation that damages or cracks cell walls, which increases the diffusion and the interfacial turbulence and also reduces the external resistance to mass transfer (Gao et al., 2009).

Table 2. Log reductions of *Saccharomyces cerevisiae* using HPU (40 W, 36 ºC), SC-CO_2 (225 bar, 36 ºC) and SC-CO_2+HPU (225 bar, 36 ºC, 40 W).

Time (min)	SC-CO_2+HPU	HPU	SC-CO_2
0	0	0	0
0.5	-1.97 ± 0.46	0	0
1.0	-5.35 ± 0.37	0	0
1.5	-6.86 ± 0.00	0	0
20	-	-0.43 ± 0.12	-0.10 ± 0.08
40	-	-0.41 ± 0.11	-0.08 ±0.05
60	-	-1.29 ± 0.33	-0.02 ±0.12
80	-	-0.80 ± 0.14	-0.57 ±0.32
100	-	-2.01 ± 0.54	-0.87 ±0.42
120	-	-2.71 ± 0.67	-1.27 ±0.66
140	-	-3.19 ± 0.61	-2.33 ±0.54

3.5. SC-CO_2+HPU inactivation in orange and apple juice

3.5.1. Effect of the media on the SC-CO_2 inactivation kinetics

The inactivation of *S. cerevisiae* in different media using SC-CO_2 is illustrated in Fig. 6. The inactivation kinetics of YPD Broth showed an initial lag-phase followed by an exponential inactivation phase. The inactivation kinetics obtained using apple and orange juice showed a long lag-phase where little inactivation was observed during the entire treatment.

The duration of the lag-phase using YPD Broth was 80 min, during which time the population of microbial cells did not change significantly. Using YPD Broth, the number of viable cells decreased when the exponential inactivation phase was reached.

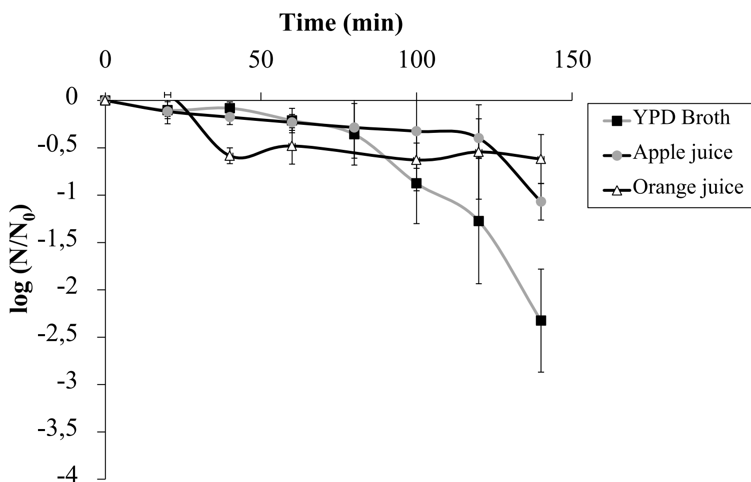

Fig. 6. Inactivation kinetics of *Saccharomyces cerevisiae* using SC-CO_2 at 225 bar and 36 ºC in different media.

No significant differences (P>0.05) were obtained between orange and apple juice in the total log-reduction after 140 min of treatment, which was, on average, 0.83 log-cycles, and this decrease was not significant. However, using YPD Broth, the reduction obtained after 140 min of treatment was significantly different (P<0.05) from that of the juices: 2.33 log reductions. It is known that the inactivation rate is affected by the constituents of the suspending media and

that complex food systems are observed to provide microbial cells with better protection compared with simple solutions (Garcia-Gonzalez et al., 2007; Ortuño et al., 2012b). Ferrentino et al. (2010) studied the inactivation kinetics of *S. cerevisiae*, expressed as a function of CO_2 solubility, and concluded that a decrease in the salt concentration caused an increase in the inactivation level due to the increase of the CO_2 concentration dissolved in the medium. In this study, the sugars of juices bind water from the medium so the amount of free water where CO_2 could dissolve is lower than in YPD Broth. Lin et al. (1992) also attributed the increased resistance to the SC-CO_2 inactivation of microorganisms suspended in complex media either to the existing fat or to the sugar content. These compounds could affect the solubility of CO_2 in the aqueous phase preventing enough pH reduction during the SC-CO_2 treatment (Hong & Pyun, 1999) and, therefore, making the subsequent inactivation mechanisms difficult. Ortuño et al. (2012b) also demonstrated that the inactivation kinetics of *E. coli* DH1 using SC-CO_2 were affected by the constituents of the suspending media, being slower in apple and orange juice, where inactivation were not observed after 25 min of treatment (350 bar, 36 ºC), compared with LB Broth medium, where a reduction of 7 log was obtained after 22 min in the same process conditions.

The slower SC-CO_2 inactivation in apple or orange juices, compared to YPD Broth, could be explained by the effect of the juice constituents. The dissolved sugar in the juice binds water; therefore the solubility of CO_2 is lower than in YPD Broth. The solubility of CO_2 in the medium is the first step in the inactivation mechanisms of SC-CO_2, from which other mechanisms happen (decrease of pH, alteration of membrane cells, penetration into cells or inactivation of key enzymes). Therefore, if the first step was limited by lower free water, the consecutive inactivation mechanisms were delayed and the inactivation kinetics are slower.

3.5.2. Effect of the media on the SC-CO_2+HPU inactivation kinetics

The inactivation kinetics of *S. cerevisiae* inoculated in different media and subjected to a combined SC-CO_2+HPU process at 225 bar, 36 °C and 40 W, is shown in Fig. 7. There are significant differences in the microbial inactivation

rate when comparing the results of the SC-CO_2 treatment (Fig. 6) with those of SC-CO_2+HPU (Fig. 7).

In the inactivation curves obtained with SC-CO_2+HPU (Fig. 7), no lag-phase was observed. The viability decreased in every case, following a first order kinetics for YPD Broth or orange juice and a fast-to-slow curve in the case of apple juice. When using YPD Broth or orange juice, no significant differences (P>0.05) were found at the different processing times involved in the SC-CO_2+HPU treatment, and the inactivation rate was similar in both cases. Only 1.5 min was needed to reach total microbial inactivation with an inactivation rate of 4.66 log-cycles/min.

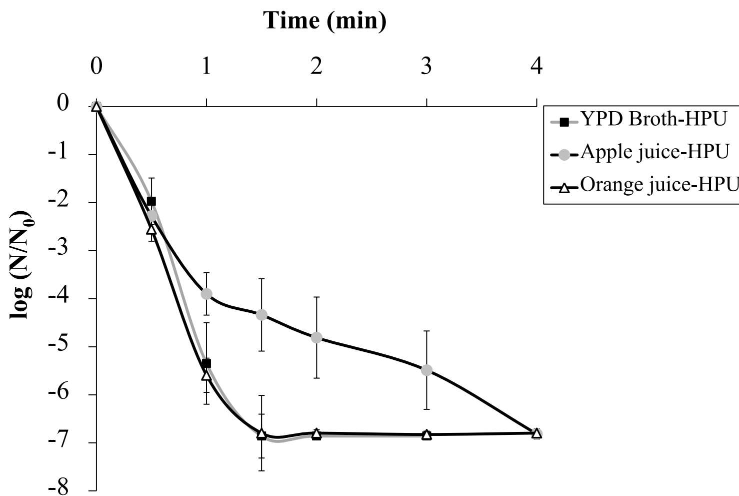

Fig. 7. Inactivation kinetics of *Saccharomyces cerevisiae* using SC-CO_2+HPU at 225 bar and 36 ºC in different media.

However, using apple juice, a slower inactivation rate was obtained and two phases can be observed. For times under 1.5 min, a faster inactivation rate (2.88 log-cycles/min) was observed until a reduction of 4 log-cycles was attained. After this time, the inactivation rate decreased to 0.984 log-cycles/min for the following 3 min, until a reduction of 7 log-cycles was reached. The greater resistance exhibited by apple juice could be related with its higher ºBrix level: apple juice had 15.6 ºBrix compared to 11.6 ºBrix in orange juice. Since the dissolved sugars bind water, and make it less available to dissolve CO_2, the

inactivation mechanisms are delayed. In spite of the differences between apple juice and the other media under SC-CO_2+HPU, 4 min were enough for the total inactivation of the yeast.

The inactivation kinetics for *S. cerevisiae* shown in this paper are very similar to those found for *E. coli* (Ortuño et al., 2012b) after the same treatment media and under identical process conditions.

This noticeable improvement of the inactivation rate when using SC-CO_2+HPU compared to SC-CO_2 alone could be due to effects of a vigorous agitation of the ultrasonic field and the cavitation phenomenon. These effects would accelerate the SC-CO_2 inactivation mechanisms, from the initial solubilization of CO_2 into the medium to the extraction of vital intracellular constituents (Garcia-Gonzalez et al., 2007), regardless of the nature and composition of the medium.

4. CONCLUSIONS

When SC-CO_2 and HPU were simultaneously applied, increasing pressure or temperature does not significantly increase the inactivation kinetics. To reach an inactivation level of 7 log, pressure higher than 100 bar, or temperature higher than 31 ºC was not necessary. On average, total reduction of *S. cerevisiae* was reached after 2 min for every condition, when HPU+SC-CO_2 was applied. The application of ultrasound leads to a vigorous agitation and cavitation that would accelerate the SC-CO_2 inactivation mechanisms, from the initial solubilization and saturation of CO_2 into the medium, to the SC-CO_2 penetration into cells and the extraction of intracellular compounds.

Therefore, using SC-CO_2+HPU, mild process conditions of pressure and temperature and shorter process time could be selected, thus preserving the nutritional and organoleptic properties of food.

It is recommended that more research be conducted to elucidate the mode of cell death with SC-CO_2+HPU and the role of HPU in both the solubility of CO_2 and in the microorganism's inactivation. Additional studies in the area of the effect of SC-CO_2+HPU on physico-chemical properties of juices would also lead to the application of this technology to other food matrices. Further research

should be conducted in order to study the effect of the SC-CO_2+HPU treatment on consumer acceptance of the food processed using this technology.

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Chapter 4

Inactivation Kinetics and Cell Morphology of E. coli and S. cerevisiae treated with Ultrasound-Assisted Supercritical CO_2

C. Ortuño, A. Quiles, J. Benedito

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Inactivation kinetics and cell morphology of *E. coli* and *S. cerevisiae* treated with ultrasound-assisted supercritical CO_2

Carmen Ortuño[1], Amparo Quiles[2], Jose Benedito[1]

[1] Grupo de Análisis y Simulación de Procesos Agroalimentarios, Departamento Tecnología de Alimentos, Universitat Politècnica de València, Camí de Vera s/n, E46022, Valencia, Spain.

[2] Grupo de Microestructura y Química de Alimentos, Departamento Tecnología de Alimentos, Universitat Politècnica de València, Camí de Vera s/n, E46022, Valencia, Spain.

ABSTRACT

The inactivation kinetics of *Escherichia coli* (*E. coli*) and *Saccharomyces cerevisiae* (*S. cerevisiae*) cells in apple juice subjected to supercritical carbon dioxide (SC-CO_2) assisted by high power ultrasound (HPU) at different pressures (100-350 bar, 36 ºC) and temperatures (31-41 ºC, 225 bar) were studied. On average, shorter process times were required to achieve the total inactivation of *S. cerevisiae* (2-6 min) in apple juice than *E. coli* (7 min). The inactivation kinetics of *E. coli* and *S. cerevisiae* were satisfactorily described by the Peleg Type A and the Weibull model, respectively, considering temperature and pressure as model parameters. Transmission electron microscopy (TEM) and light microscopy (LM) techniques were used to study the cellular changes of SC-CO_2 (350 bar, 36 ºC, 5 min) and SC-CO_2+HPU (350 bar, 36 ºC, 5min, 40 W) treated cells. TEM and LM images revealed that 5 min of SC-CO_2 treatment generated minor morphological modifications, although no inactivation of the cells was obtained. However, 5 min of SC-CO_2+HPU treatment totally inactivated the population of both microorganisms. SC-CO_2+HPU produced the degradation of the internal cell content and the disruption of the cell wall and plasmalemma, which prevented the possible regrowth of the cells during refrigerated storage.

Key words: microbial inactivation, apple juice, transmission electron microscopy, cellular morphology, supercritical carbon dioxide, high power ultrasound.

1. INTRODUCTION

The current market share of apple juice is rising, since it is perceived as "healthy" food due to its high content in polyphenols and flavonoids, which contribute to its good antioxidant properties (Kumar et al., 2009). However, apple juice is commonly spoiled by the presence and growth of its natural acid tolerant and osmophilic microflora (Tahiri et al., 2006) and can be a vehicle for external spoilage microorganisms and pathogens.

The preservation technologies developed during the last few years, have been driven by the relentless pursuit to reduce the degree of thermal damage to the quality of thermally processed foods (Rawson et al., 2011). In order to obtain safe products with fresh-like quality attributes, a novel inactivation technique based on High Power Ultrasound (HPU) embedded in a Supercritical Carbon Dioxide (SC-CO_2) System has been developed (Benedito et al., 2011).

The simultaneous application of SC-CO_2 and HPU has been shown to accelerate the death of *Escherichia coli* (*E. coli*) and *Saccharomyces cerevisiae* (*S. cerevisiae*) inoculated in different media. Ortuño et al. (2012a, 2013) showed that the population of both microorganisms inoculated in apple juice, was completely inactivated after 5 min (350 bar, 36 ºC) and 4 min (225 bar, 36 ºC) of treatment, respectively. No microbial reduction was observed with only SC-CO_2 under the same conditions. These authors explored the inactivation in juices using a single combination of pressure and temperature. No references have been found in the literature exploring and modeling the effect of temperature and pressure on the inactivation of microorganisms in real foods, such as apple juice, using SC-CO_2+HPU.

The mechanisms of microbial inactivation by SC-CO_2+HPU have not yet been fully elucidated. Combining SC-CO_2 and HPU the solubilization rate of SC-CO_2 into the liquid and the increase in the mass transfer due to the vigorous agitation produced by the ultrasonic field would permit the rapid saturation of CO_2 in the medium, which might accelerate the inactivation mechanisms (a decrease of the medium pH, an increase in membrane fluidity and permeability, the diffusion of CO_2 into the cells, cell membrane rupture, the alteration of intracellular equilibrium, the inactivation of key enzymes, and the extraction of

critical intracellular materials) of the SC-CO_2 inactivation treatments (Garcia-Gonzalez et al., 2007). The phenomenon of cavitation could damage the cell walls causing the death of the microbial cells (Ortuño et al., 2012a, 2013).

Different authors pointed out that there is a direct relationship between the cellular modification and the inactivation caused by SC-CO_2 (Garcia-Gonzalez et al., 2010; Liao et al., 2010a). Although different studies have been conducted regarding the ultrasound-assisted inactivation of microorganisms using SC-CO_2 (Ortuño et al., 2012a, 2013), no references have been found in literature covering a detailed study of the cellular damage and morphological changes generated by SC-CO_2+HPU treatments in microbial cells.

Therefore, the objective of this work was twofold: to study the effect of HPU-assisted SC-CO_2 treatments on the inactivation kinetics of *E. coli* and *S. cerevisiae* cells inoculated in apple juice and to study the cellular damage caused to microorganisms by this novel technology.

2. MATERIAL AND METHODS

2.1. Apple juice

Apples (*Golden delicious*) were purchased from a local market and kept at 4 ºC for 2 days until juice extraction. The apples were washed, diced and squeezed using a screw juice extractor (Ultra Juicer, Robot Coupe J80, USA) to obtain the juice. ºBrix was measured in triplicate using a digital refractometer (Hand-held Pocket, ATAGO). The apple juice (pH = 5.4; ºBrix = 15.6) produced was sealed in plastic containers and stored at -18 ºC until required.

2.2. Microorganism strains and inoculated media

The microbial strains used in this study were *E. coli* DH1 and *S.cerevisiae* T73. A single colony of *E. coli* or *S. cerevisiae* was grown overnight in Luria Bertani Broth (LB Broth, Sigma-Aldrich, USA) at 37ºC, or in Yeast Peptone Dextrose Broth (YPD Broth, Sigma- Aldrich, USA) at 30ºC, respectively, using an incubation chamber (J.P. SELECTA, Model 3000957, Barcelona, Spain) and an orbital shaker at 120 rpm (J.P. SELECTA, Rotabit Model 3000974, Barcelona, Spain). For each experiment with *E. coli* or *S. cerevisiae*, a subculture

was prepared by inoculating 50 μL from the starter culture into 50 mL sterilized medium and incubating at 37 ºC-24 h or at 30 ºC-24 h, respectively.

For each experiment with juice, a plastic container with 50 mL of apple juice was thawed at 4 ºC for 12 h before processing to evaluate the inactivation kinetics. The inoculated juice was prepared by adding 5 mL of either *E. coli* or *S. cerevisiae* cells to 50 mL of apple juice, to reach a cell concentration of 10^6-10^8 CFU/mL.

To evaluate the individual effect of SC-CO_2 and SC-CO_2+HPU treatments on the cell morphology of *E. coli* and *S. cerevisiae* and the regrowth capacity of these microorganisms (storage test), each sample was prepared by adding 5 mL of either *E. coli* or *S. cerevisiae* cells to 50 mL of LB or YPD Broth culture, respectively. The LB and YPD Broths were selected as the treatment media for the ultrastructural analysis for two reasons: they were the simplest media where SC-CO_2+HPU has been applied to inactivate these microorganisms (Ortuño et al., 2012a, 2013), and also to prevent the suspended solids and sugars present in apple juice to affect the analysis of images.

2.3. Experimental design

In order to evaluate the inactivation kinetics of *S. cerevisiae* and *E. coli*, the inoculated apple juice was subjected to the SC-CO_2+HPU treatment under different pressures (100, 225 and 350 bar, 36 ºC) and temperatures (31, 36 and 41 ºC, 225 bar). The temperatures chosen were higher than the critical one for CO_2 and lower than lethal temperatures for *E. coli* and *S. cerevisiae*. The pressures chosen were higher than the critical one for CO_2 (73.8 bar) and lower than 350 bar according to previous studies where it was observed that pressures higher than 350 bar were not necessary to reach 6-8 log reductions using SC-CO_2+HPU (Ortuño et al., 2012a, 2013).

In order to evaluate the effect of SC-CO_2 and SC-CO_2+HPU treatments on the morphological changes and the regrowth capacity of *E. coli* and *S. cerevisiae* treated cells (storage test), the inoculated culture medium was subjected to SC-CO_2 at 350 bar, 36 ºC for 5 min or to SC-CO_2+HPU treatments at 350 bar, 36 ºC and 40 ± 5 W, for 5 min. These conditions were selected because it has

been previously demonstrated (Ortuño et al., 2012a, 2013) that when using SC-CO_2+HPU, temperatures higher than 36 ºC, pressures higher than 350 bar, or process times longer than 5 min are not necessary to achieve the total inactivation of these microorganism inoculated in the culture medium.

2.4. Supercritical fluid equipment and processing procedure

2.4.1. Apparatus

The experiments were carried out in batch supercritical fluid lab-scale equipment especially designed and built for this application by the research group (Fig. 1). The system includes ultrasound equipment (Benedito et al., 2011) which is embedded in the supercritical fluid vessel. The ultrasound equipment consists of a high power piezoelectric transducer, an insulation system and a power generator unit (40 W ± 5 W).

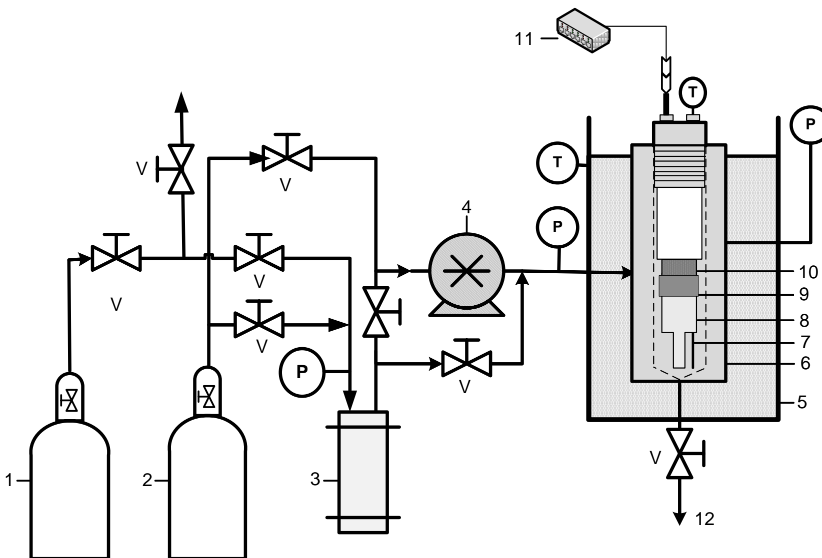

Fig. 1. Supercritical CO_2 treatment system. 1-CO_2 tank; 2-N_2 tank; 3-Chiller reservoir; 4-Pump; 5-Temperature controlled bath; 6-Treatment vessel; 7-Temperature Sensor; 8-Transducer; 9-Insulation joint; 10-Ceramics; 11-Power Generation Unit; 12-Sample extraction; V-Valve; P-Manometer.

The transducer is inserted inside the inactivation vessel and includes two commercial ceramics (35 mm external diameter; 12.5 mm internal diameter; 5 mm thickness; resonance frequency of 30 kHz) and a sonotrode, which was

specially constructed to concentrate the highest amount of acoustic energy on the application point. The equipment is described in detail by Ortuño et al. (2013).

2.4.2. Supercritical fluid processing

Prior to each experiment, the inactivation vessel was cleaned and sanitized with disinfectant solution, distilled water and autoclaved water. For each experiment, a subculture was prepared by inoculating 5 mL of cells in the early stationary phase (prepared as described in section 2.2) in 50 mL of sterilized apple juice or culture medium to a cell concentration of 10^6-10^8 CFU/mL.

The sample (55 mL), inoculated apple juice or culture medium, was loaded into the inactivation vessel and immediately sealed. The pump filled the vessel with supercritical carbon dioxide, reaching the desired pressure in less than 2.5 min. Time zero for each treatment was taken when the experimental temperature and pressure were reached. For the experiments with HPU, 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 being 40 W ± 5 W (I = 181 mA ± 18 mA; U = 220 V ± 5 V) (Power measured using a Digital Power Meter, Yokogawa, Model WT210). Pressure and temperature were kept constant during the experiment through the pump and the thermostatic bath, respectively. All the experiments were run in triplicate.

For the inactivation treatments with inoculated apple juice, samples of 1 mL were extracted periodically through a small tube located at the bottom of the inactivation vessel until the end of the experiment. This tube was cleaned and disinfected with 3 mL of ethanol (96 %v/v) after each sampling. The treated samples were collected in individual sterile plastic test tubes for microbial enumeration.

In the inactivation treatments for the morphological study, all the sample volume was extracted and collected after the treatment.

For the storage test, samples containing the treated cells were collected at the end of each treatment, divided in 7 sterile tubes and placed at refrigerated temperature (4 ºC) for 6 weeks. At time 0 (after the treatment), and weekly from

the 1st to the 6th week of storage, the viability of the microorganisms was evaluated.

2.5. Enumeration of viable microorganisms

The viability of the non-treated and SC-CO_2+HPU treated cells was assessed via a spread plating method on specific selective agars, LB Agar or YPD Agar and incubated for 24 h at 37 ºC or 30 ºC, for *E. coli* or *S. cerevisiae* respectively, before counting. The results were expressed as log (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 the different times of treatment. The data presented for each treatment condition are the means of the triplicate experiments. Moreover, it was shown for each experiment the arithmetic mean and the standard deviation of log (N/N_0) for at least three plates.

2.6. Mathematical models and fitting of data

According to the results of previous studies which have addressed the modeling of microbial inactivation using SC-CO_2, HPU or SC-CO_2+HPU (Peleg, 2006; Lee et al., 2009; Corradini & Peleg, 2012; Ortuño et al., 2013), four different models (Table 1) have been selected in this study to fit the inactivation kinetics of the selected microorganisms treated with SC-CO_2+HPU in apple juice.

2.7. Transmission electron microscopy (TEM) and Light Microscopy (LM)

The cells treated by SC-CO_2 or SC-CO_2+HPU, were collected at the end of the treatment and centrifuged at 2600 rpm and 4 ºC for 5 min. The pellets were collected and fixed with 25 g/L glutaraldehyde solution for 24 h at 4 ºC and post-fixed with 20 g/L osmium tetroxide solution for 1.5 h. The cells were centrifuged and the pellet collected. This procedure was applied after each step of the process.

After this process, cells were stabilized by mixing them with a low gelling temperature agarose solution (3 g/100 mL) at 30 ºC, which facilitates fixation and embedding prior to LM and TEM observation. Hereafter, the cells inserted in the solidified agar were cut into cubes (1 mm^3). These cubes were fixed with

25 g/L glutaraldehyde solution; post-fixed with 20 g/L osmium tetroxide solution; dehydrated with 30 g/L, 50 g/L, 70 g/L ethanol and 100 g/L; contrasted with uranyl acetate solution (20 g/L) and embedded in epoxy resin (Durcupan, Sigma–Aldrich, St. Louis, MO, USA). The blocks obtained were cut using a Reichert-Jung ULTRACUT ultramicrotome (Leica Microsystems, Wetzlar, Germany). Semithin sections (1.5 µ) were stained with 1 g/L toluidine blue and examined in a Nikon Eclipse E800 light microscope (Nikon, Tokyo, Japan). The ultrathin sections obtained (0.1 µm) were collected in copper grids and stained with 20 g/L acetate uranile and 40 g/L lead citrate to be observed in the Philips EM 400 Transmission Electron Microscope (Eindhoven, Holland) at 80 kV.

Table 1. Models used to fit the microbial inactivation kinetics by SC-CO_2+HPU.

Modeling of the microbial inactivation kinetics			
Model	**Equation**	**Parameters**	**Reference**
Weibull	$\log_{10}\frac{N}{N_0} = -b\,t^n$	b, n	Corradini & Peleg, 2012
Biphasic	$\log_{10}\frac{N}{N_0} = \log_{10}\left[(1-f)\,10^{\frac{-t}{D_{sens}}} + f\,10^{\frac{-t}{D_{res}}}\right]$	f, D_{sens}, D_{res}	Lee et al., 2009
Peleg Type A	$\log_{10}\frac{N}{N_0} = -\frac{a_1 t}{(1+a_2 t)(a_3 - t)}$	a_1,a_2 ,a_3	Peleg, 2006
Peleg Type B	$\log_{10}\frac{N}{N_0} = -\frac{b_1 t^r}{b_2 + t^r}$	b_1, b_2, r	Peleg, 2006

N_0: the initial number of microorganisms at time 0; N: the corresponding number after a time t.
b: non-linear rate parameter; n is the shape factor.
(1-f) and f: the fraction of treatment-sensitive and treatment-resistant population, respectively; D_{sens} and D_{res} are the decimal reduction times of the two populations (min)
a_1, a_2, a_3, b_1, b_2, r: model parameters

2.8. Image analysis

The image analysis was carried out using the software ImageJ (Rasband, W.S., ImageJ v. 1.43 s, National Institute of Health, Bethesda, MD, USA). The dimension of cells and the thickness of the cell walls were determined using

TEM images. All the measurements were assessed from at least twelve randomly acquired TEM images.

2.9. Statistical analysis

The statistics package Statgraphics Plus (Statistical Graphics Corp. 5.1, Warrenton, USA) was used to perform a simple ANOVA to determine the effect of the treatments on the dimensions of both microorganisms. Moreover, multifactorial ANOVA, and LSD (Least Significant Differences) were used to evaluate the effect of pressure, temperature and time on the inactivation rate of microorganisms.

The kinetic constants of the models were calculated by minimizing the sum of the square differences between experimental and model-predicted data using the Solver Microsoft Excel™ tool. The root mean square error (RMSE) and the coefficient of determination (R^2) were used to evaluate the model's goodness of fit and the estimation accuracy (Schemper, 2003).

3. RESULTS AND DISCUSSION

3.1. SC-CO_2+HPU inactivation of *E. coli* cells. Kinetics and modeling

The inactivation curves of *E. coli* cells in apple juice undergoing a combined SC-CO_2+HPU process at different temperatures (Fig. 2A) and pressures (Fig. 2B) represented a fast-to-slow curve. No shoulders were observed for any temperature and pressure condition studied and the viability began to decrease quickly, starting to slow down after 1 min. Reductions of 4.6, 5.1 and 5.6 log-cycles were obtained after 1 min of treatment at 225 bar and 31, 36 and 41 ºC, respectively; and reductions of 3.3, 5.1 and 4.2 log-cycles were obtained after 1 min of treatment at 36 ºC and 100, 225 and 350 bar, respectively. After the first minute, the population decreased slowly and on average, a reduction of 7.5 log-cycles was obtained after 7 min of treatment under every condition studied. No significant differences ($p>0.05$) were found either between the temperatures or the pressure conditions selected; therefore, the effect of increasing the temperature or pressure did not significantly increase the inactivation level of *E. coli* inoculated in apple juice.

The inactivation of *E. coli* has been explored in previous studies using SC-CO_2. Liao et al. (2006) studied the inactivation of *E. coli* with a batch SC-CO_2 system in cloudy apple juice and found that the inactivation level rose as the temperature and pressure increased: from 5 to 7 log-cycles by increasing the temperature from 32 to 42 ºC (300 bar, 75 min); and from 5.5 to 7.5 log-cycles by increasing the pressure from 100 to 300 bar (42 ºC, 75 min), respectively. Shimoda et al. (1998) studied the antimicrobial effects of pressurized carbon dioxide in a continuous flow system on the population of *E. coli* (10^8-10^9 CFU/mL), inoculated in phosphate buffer. No survivors were found after 15 min of residence time under 35 ºC and 60 bar. This fact could be due to a better agitation in continuous systems that might enhance mass transfer and solubilization rates of pressurized CO_2 in the liquid phase, and increase the contact of CO_2 with microbial cells (Erkmen, 2012). Compared to the results of this work, in the studies where batch systems were used, the inactivation rate increased as the temperature and pressure rose, but much longer process times were needed compared to SC-CO_2+HPU processing. Moreover, the HPU-assisted batch supercritical system of the present study allowed similar inactivation levels to be attained in shorter process times than when using continuous SC-CO_2 systems.

The process time needed in the present study to attain the total inactivation of *E. coli* inoculated in apple juice was 7 min, which was longer compared to the 2-3 min required with LB Broth (Ortuño el at., 2012a). The inactivation rate of the microorganisms treated with SC-CO_2 is seriously affected by the constituents of the suspending media and/or the nature of the treated foods (Garcia-Gonzalez et al., 2007). The sugars of the apple juice (15.6 ºBrix, approximately 93.5 % higher than in LB Broth), bind water from the medium and the amount of free water in which CO_2 could be dissolved is lower than in LB Broth (Ferrentino et al., 2010) despite the intense ultrasound agitation. The effect of higher sugar content of apple juice limited the effect of increasing the pressure or temperature and could not facilitate the solubilization of CO_2 into the medium (Liao et al., 2006) and the subsequent inactivation mechanisms. Therefore, it has been shown that the nature of the medium drastically influences the effect of SC-CO_2+HPU on *E. coli*.

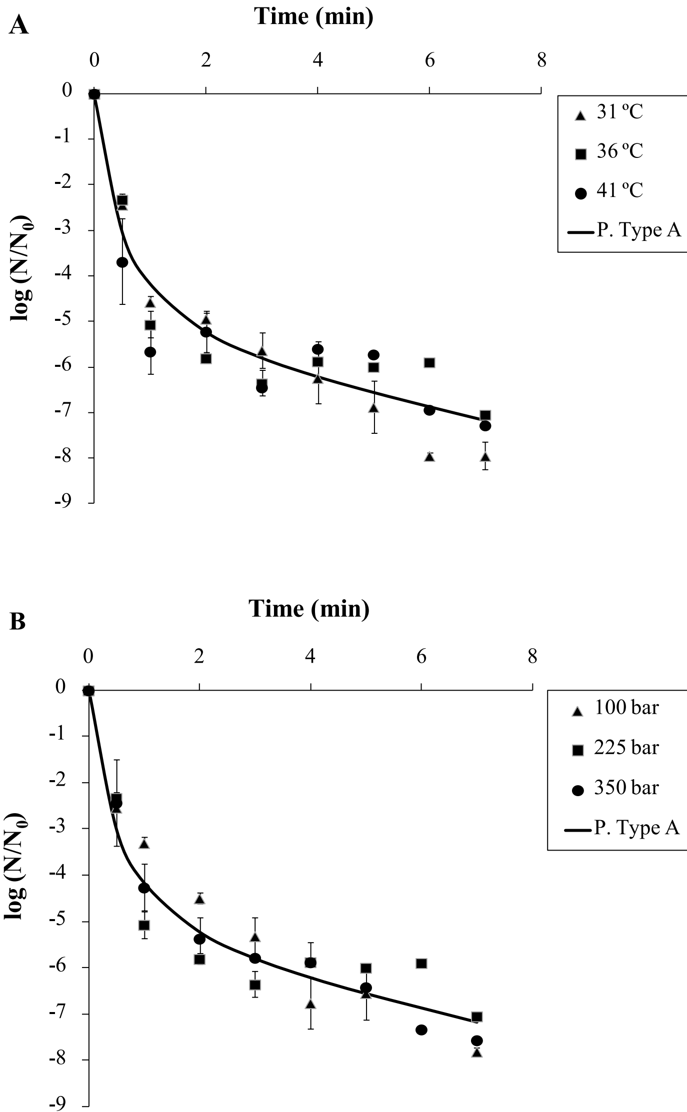

Fig. 2. Experimental data (discrete points) and modeling (continuous line) of the inactivation kinetics of *E. coli* in apple juice treated by SC-CO_2 and HPU at different temperatures (A, 225 bar) and pressures (B, 36 ºC). P. Type A: General Peleg Type A model.

Additionally, the inactivation kinetics of *E. coli* treated with SC-CO_2 and HPU were fitted by using the four models described in Table 1. Table 2 shows the statistical parameters for the fit of the kinetic models to the inactivation data of *E. coli* in apple juice. R^2 and RMSE values (Table 2) indicate that, overall, a good fit was obtained with the four models under the different process conditions considered. $R^2 > 0.94$ were found for most of the conditions studied except using the Biphasic model ($R^2_{avg} = 0.86$; $RMSE_{avg} = 0.591$). On average, the Peleg Type A model ($R^2_{avg} = 0.961$; $RMSE_{avg} = 0.386$) provided the best fit for all the process conditions selected, therefore it was selected in order to predict the inactivation kinetics of *E. coli* at any pressure and temperature in the range of the variables considered.

Since the pressure and temperature were not significant factors ($p>0.01$) in the inactivation of *E. coli*, the inactivation kinetics obtained at different pressures and temperatures were fitted to the same equation using the Peleg Type A model. The parameters, a_1, a_2, and a_3, were calculated by minimizing the sum of square differences between all the experimental data and all the predicted data considered for every pressure and temperature condition studied. The values of the a_1, a_2, and a_3 parameters were 6.38, -0.02 min^{-1} and -0.56 min, respectively. A general expression of the Peleg Type A model was obtained that could be used to predict the inactivation kinetics of *E. coli* in apple juice for any pressure and temperature in the studied range (Fig. 2). The statistical parameters of the general Peleg Type A model exhibited a worse fit of the *E. coli* inactivation kinetics ($R^2_{avg} = 0.936$ and $RMSE_{avg} = 0.561$) compared to the average of the individual fits to each survival curve obtained at each temperature and pressure ($R^2_{avg} = 0.961$; $RMSE_{avg} = 0.386$). However, according to the R^2 and RMSE values, it could be concluded that the proposed general model properly described the SC-CO_2+HPU inactivation kinetics of *E. coli* in apple juice, for any condition of pressure and temperature in the selected range, 100-350 bar and 31-41 ºC, respectively.

Table 2. Statistical parameters for the fit of the kinetic models to the inactivation data of *E. coli* and *S. cerevisiae* in apple juice treated by SC-CO_2 and HPU at three temperatures (31, 36 and 41 ºC, at constant P = 225 bar) and three pressures (100, 225 and 350 bar, at constant T = 36 ºC).

		Escherichia coli				***Saccharomyces cerevisiae***			
Treatment conditions	**Statistics**	**W**	**Bi**	**A**	**B**	**W**	**Bi**	**A**	**B**
225 bar 31 ºC	R^2	0.977	0.993	0.980	0.973	0.977	0.993	0.985	0.973
	RMSE	0.347	0.179	0.303	0.347	0.142	0.071	0.106	0.142
225 bar 36 ºC	R^2	0.887	0.971	0.933	0.868	0.985	0.995	0.973	0.981
	RMSE	0.674	0.313	0.482	0.674	0.232	0.114	0.277	0.230
225 bar 41 ºC	R^2	0.925	0.609	0.926	0.913	0.992	0.992	0.983	0.988
	RMSE	0.530	1.124	0.489	0.529	0.175	0.141	0.211	0.175
100 bar 36 ºC	R^2	0.989	0.866	0.982	0.987	0.985	0.984	0.982	0.981
	RMSE	0.236	0.745	0.270	0.233	0.264	0.239	0.255	0.264
350 bar 36 ºC	R^2	0.976	0.935	0.984	0.977	0.993	0.856	0.977	0.999
	RMSE	0.335	0.507	0.250	0.305	0.177	0.568	0.226	0.001
	$\mathbf{R^2_{avg}}$	0.951	0.860	0.961	0.944	0.986	0.964	0.980	0.985
	$\mathbf{RMSE_{avg}}$	0.425	0.591	0.386	0.446	0.198	0.141	0.212	0.203

W, Bi, A, B: Weibull, Biphasic, Peleg Type A and Peleg Type B model, respectively.

3.2. SC-CO_2+HPU inactivation of *S. cerevisiae* cells. Kinetics and modeling

In the inactivation kinetics of *S. cerevisiae*, no lag phase was observed for any condition studied and the viability began to decrease immediately. Fig. 3A showed a fast-to-slow kinetic for all the temperatures studied. The population reductions obtained after 1 min of treatment were 1.8, 3.9 and 4.8 log-cycle, at 31, 36 and 41 ºC, respectively. Total reduction was reached after 4 and 2 min at 36 and 41 ºC, respectively, but only 3.4 log-cycles reduction was attained after 6 min at 31 ºC. On average, the inactivation rate increased significantly ($p<0.05$) as the temperature rose from 31 ºC to 36 ºC and from 36 ºC to 41 ºC.

Reductions of 2.9 and 3.9 log-cycles were obtained after 1 min of treatment, at 100 and 225 bar, respectively (Figure 3B). The inactivation kinetics at 100 and 225 bar behaved in a similar way and, on average, no significant differences ($p>0.05$) were found between them. However, when the pressure was increased to 350 bar, a significantly ($p<0.05$) faster inactivation was observed than at 100 and 225 bar. At 350 bar, total inactivation was attained after only 1 min of

treatment. Therefore, the effect of increasing temperature or pressure, significantly accelerated the inactivation of *S. cerevisiae* in apple juice, although pressures of over 225 bar were necessary to observe a significant pressure effect.

The inactivation of *S. cerevisiae* with SC-CO_2 has been explored by other authors. Erkmen (2003) reduced the microbial population of *S. cerevisiae* inoculated in potato dextroxe broth with a batch SC-CO_2 system. The time needed to attain total reduction fell from 165 to 50 min and from 125 to 65 min as the temperature increased from 30 to 50 ºC at 75 and 100 bar, respectively. Spilimbergo et al. (2007) used a multi-batch system to study the SC-CO_2 pasteurization of apple juice inoculated with *S. cerevisiae*. The microbial reduction increased from 3.9 to 4.5 logs when the pressure rose from 100 to 200 bar, after 30 min of process. In the aforementioned works, an increase in the inactivation level was also generally observed as the pressure and temperature rose, although they required much longer times than when using SC-CO_2+HPU.

Contrary to the results observed in previous studies using SC-CO_2+HPU to inactivate *S. cerevisiae* in YPD Broth medium (Ortuño et al., 2013), where the effect of increasing the pressure and temperature did not increase the inactivation rate, in the present study with apple juice, the inactivation rate increased with pressure and temperature. As previously explained, the inactivation rate is affected by the composition of the suspending medium (Garcia-Gonzalez et al., 2007; Ortuño et al., 2013). The high sugar content of apple juice could limit the fast CO_2 saturation of the apple juice in spite of the intense ultrasound agitation. As a consequence, an increase in pressure increases the theoretical solubility of CO_2 which raises the level of dissolved CO_2 into the apple juice; and temperature may have a viscosity effect on the dissolved CO_2 by speeding up mass transfer.

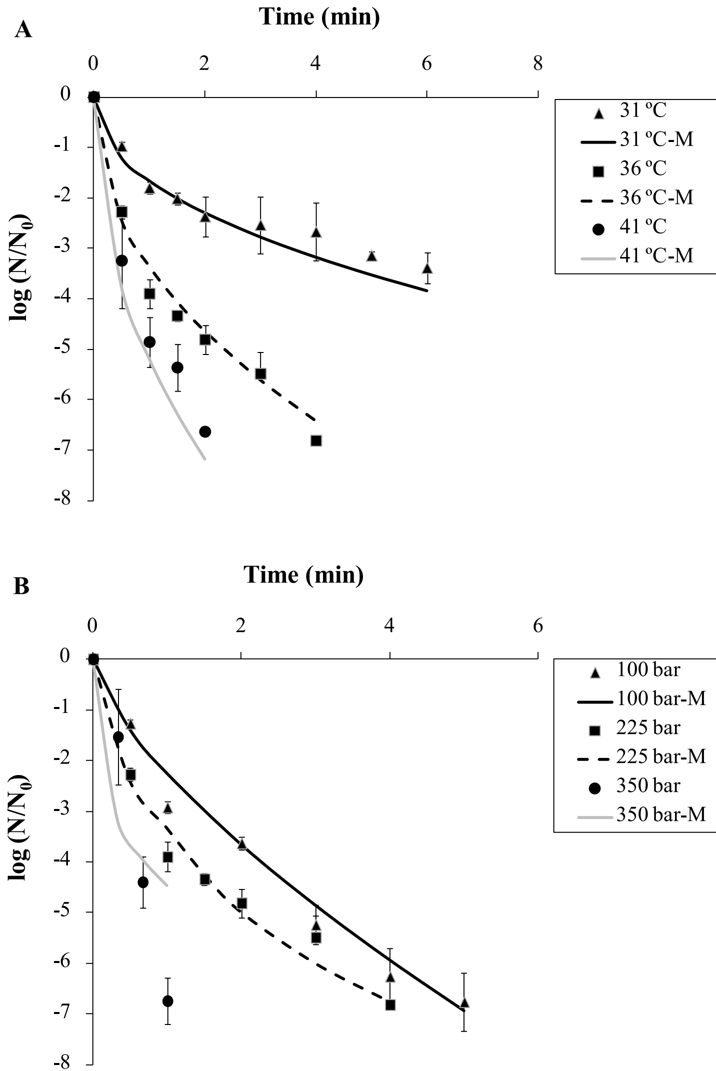

Fig. 3. Experimental data (discrete points) and modeling (continuous line) of the inactivation kinetics of *S. cerevisiae* in apple juice treated by SC-CO_2+HPU at different temperatures (A, 225 bar) and pressures (B, 36 ºC). M: modified Weibull model.

On average, *E. coli* showed more resistance to SC-CO_2+HPU treatments than *S. cerevisiae*, in contrast to the results obtained in previous studies (Ortuño et al., 2012b). In treatments with apple juice, where despite the effect of HPU the high sugar content could limit the fast solubilization of CO_2 into the medium, it could

be thought that the inactivation mechanism would be greatly affected by the cavitation phenomenon and the size of the microorganism. The size of *S. cerevisiae* cells, 8-10 μm (Laun et al., 2001), is much larger than *E. coli* cells, 1.2-2 μm (Nelson & Young, 2000); therefore, the likelihood that the implosion of the cavitation bubbles might reach and affect the cell structure could be higher for *S. cerevisiae* than for *E. coli*. Thus, HPU had a different effect on the SC-CO_2 inactivation of different microorganisms inoculated in apple juice.

Similarly to *E. coli,* the inactivation kinetics of *S. cerevisiae* cells in apple juice subjected to SC-CO_2+HPU were fitted using the models described in Table 1. The statistical parameters obtained from the fit of the experimental data are shown in Table 2. The four models satisfactorily described the inactivation kinetics of *S. cerevisiae* ($R^2 > 0.96$), and on average the Weibull model ($R^2_{avg} = 0.986$; $RMSE_{avg} = 0.198$) provided the best fit for all the process conditions selected. The Weibull model was selected in order to predict the inactivation of *S. cerevisiae* at any pressure and temperature in the range of the variables considered in this study.

From the ANOVA of *S. cerevisiae* inactivation kinetics, both pressure and temperature were found to be significant factors ($p<0.01$); so we assumed that the parameters of the Weibull model, b and n, could be described by a log-logistic model (Peleg, 2006), with simultaneous pressure and temperature dependence (Eqs. (1, 2)).

$$b(T,P) = \ln(1+\exp(x_b(T-T_c)+z_b(P-P_c))) \quad \text{Eq. (1)}$$

$$n(T,P) = \ln(1+\exp(x_n(T-T_c)+z_n(P-P_c))) \quad \text{Eq. (2)}$$

where x_b, z_b, x_n, z_n, T_c and P_c are the characteristic constants of the microorganism. Substituting Eqs. (1, 2) in the Weibull model (Table 1), a general expression of the model was obtained that could be used to predict the inactivation kinetics of *S. cerevisiae* in apple juice for different pressures and temperatures (Fig. 3).

The values of the characteristic constants for this general model were calculated by minimizing the sum of square differences between all the experimental data and all the data predicted by the model considering every

pressure and temperature condition studied. The values of the coefficients x_b, z_b, x_n, z_n, T_c and P_c were 0.373 ºC^{-1}, 0.009 bar^{-1}, -0.001 ºC^{-1}, -0.004 bar^{-1}, 30.195 ºC and 101.896 bar, respectively. As expected, the statistical parameters of the general model exhibited a worse fit (R^2_{avg} = 0.923 and $RMSE_{avg}$ = 0.561) than the average of the individual fits (R^2_{avg} = 0. 986; $RMSE_{avg}$ = 0.198) to each survival curve obtained at each temperature and pressure for *S. cerevisiae*. However, according to the R^2 and RMSE values, the proposed model appropriately described the inactivation kinetics of *S. cerevisiae* with SC-CO_2+HPU as a function of the temperature, pressure and time of treatment, in the practical range of 100-350 bar and 31-41 ºC.

3.3. Morphological changes in *E. coli* cells treated with SC-CO_2 and SC-CO_2+HPU

In Fig. 4A, the typical rod-shaped morphology of untreated *E. coli* cells, uniformly stained with toluidine blue can be observed by LM; these measured 0.67 ± 0.13 µm in width and 1.19 ± 0.16 µm in length. The TEM image revealed (Fig. 4B) that the intracellular organization of untreated *E. coli* cells exhibited an intact cytoplasm with a uniform distribution of the inner material. The cytoplasmic content occupied the whole of the intracellular space that appeared surrounded by an intact cell membrane or plasmalemma and cell wall. In Fig. 4C, the intact plasmalemma and cell wall can be observed in detail, with a well-defined outer membrane, peptidoglycan layer and inner membrane, measuring approximately 4.5 ± 1.6 nm, 8.7 ± 1.5 nm and 4.7 ± 0.7 nm in thickness, respectively.

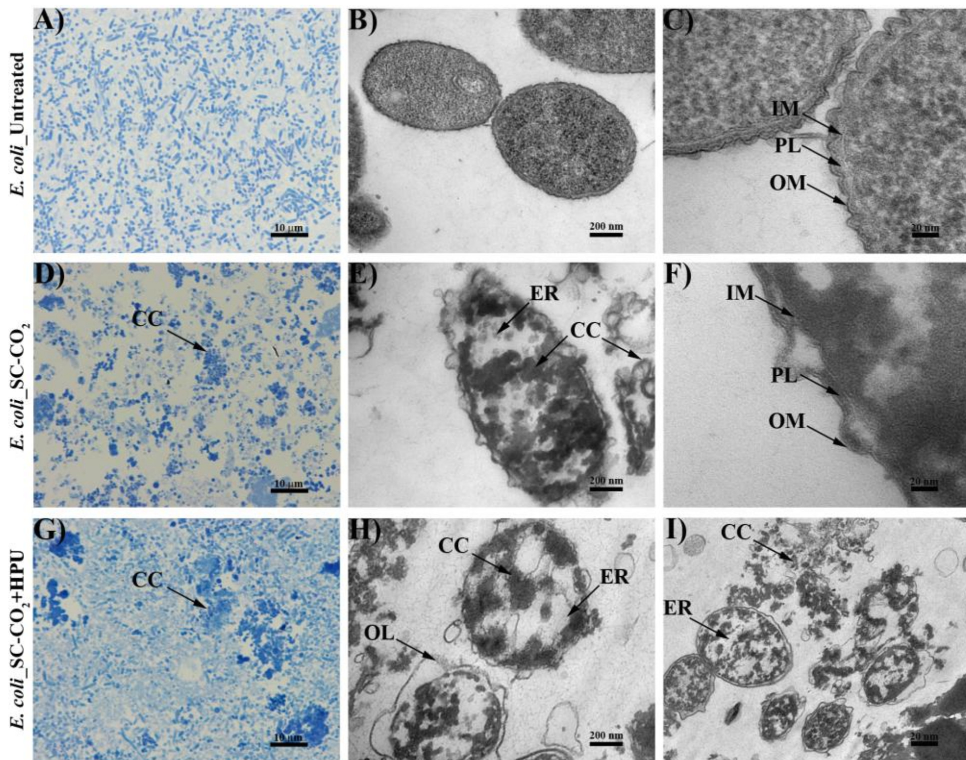

Fig. 4. LM (A, D, G) and TEM (B, C, E, F, H, and I) micrographs by semithin and ultrathin sectioning of *E. coli*. Images A-C represent untreated cells; images D-F show cells treated with SC-CO2 at 350 bar, 36 ºC for 5 min; images G-I show cells treated with SC-CO2+HPU at 350 bar, 36 ºC, 40 W for 5 min. OM: cell wall-outer membrane; PL: cell wall-peptidoglycan layer; IM: cell wall-inner membrane; ER: empty regions; CC: cytoplasmic content; OL: cell wall-outer layer.

The LM image of SC-CO_2-treated *E. coli* cells is shown in Fig. 4D, where little stained cells were observed and the intracellular organization exhibited both an uneven distribution and some aggregation of cytoplasmic content. In addition, a portion of the SC-CO_2-treated *E. coli* cells, which measured 0.76 ± 0.23 µm in width and 1.40 ± 0.78 µm in length, had lost their typical rod-shaped morphology, although no significant differences ($p>0.05$) were found compared to the dimensions of non-treated *E. coli* cells. The cytoplasm content inside the SC-CO_2-treated cells observed by TEM (Fig. 4E and 4F) showed empty regions, which could be due to the aggregation or precipitation of internal cell

components, or to the removal of part of the cytoplasmic content, which could be observed outside cells (Fig. 4D and 4E). The cell wall and the plasmalemma of SC-CO_2-treated *E. coli* cells can be identified in some cells (Fig. 4F), but modifications can be observed, compared to untreated *E. coli* cells. The plasmalemma appeared to be disintegrated in some areas while the thickness of the outer membrane, the peptidoglycan layer and the inner membrane measured 6.2 ± 0.9 nm, 11.8 ± 1.9 nm and 3.8 ± 0.5 nm, respectively, with a significantly ($p<0.05$) thicker outer membrane and peptidoglycan layer than in the untreated *E. coli* cells. This could be due to the fact that the peptidoglycan layer was observed with a higher degree of dissolution and a loss of cohesiveness was observed in the outer membrane, as were protuberances and winding, through which the intracellular content could be extracted. This is a consequence of the amount of CO_2 accumulated in the lipid phase, which structurally and functionally disrupts the cell membrane due to a loss of integrity and order of the lipid chain, which increases the fluidity and, hence, the permeability of the membrane (Giulitti et al., 2011).

Garcia-Gonzalez et al. (2010) compared TEM micrographs of untreated and treated *E. coli* cells, under SC-CO_2 at 210 bar and 45 ºC 60 min, and observed that the cytoplasm of the treated cells bulged through small pores in the cell wall and seemed to have lost its coherence. Liao et al. (2010a) examined the morphology of SC-CO_2-treated *E. coli* (100 bar, 37 ºC, 75 min) by TEM and concluded that the SC-CO_2 treatment provoked morphological changes on the surfaces of treated cells. The process times used in the cited studies (Garcia-Gonzalez et al., 2010; Liao et al., 2010a) were 60 and 75 min and both attained a reduction of 7-9 log-cycles after the treatment. However, in the present study the process time applied was 5 min and only a reduction of 0.3±0.06 log-cycles was achieved. Therefore, 5 min of SC-CO_2 treatment generated the uneven internal cellular distribution; however, although the external morphology was slightly modified, no inactivation of the cells was obtained. It could be due to the fact that these slight alterations inside the cells and in the cell envelope may be reversible. As the contact between cells and CO_2 was broken, the cells probably synthesized new biomolecules to repair damage to the cell walls and membranes so as to continue the cellular division and growth (Erkmen & Bozoglu, 2008).

Spilimbergo et al. (2009) observed that the initial damage of the cellular envelope is not lethal for the cell. These authors observed that SC-CO_2 treatments (100 bar, 36 ºC) of over 10 min were required to induce irreversible damage to the cells, causing their death.

The SC-CO_2+HPU treatment (Fig. 4G-I) generated more morphological changes than the SC-CO_2 treatment (Fig. 4D-F). Significant differences ($p<0.05$) were found in the dimensions of the SC-CO_2+HPU treated *E. coli* cells, 1.20 ± 0.32 µm in width and 2.51 ± 1.15 µm in length, compared to non-treated and SC-CO_2 treated *E. coli* cells. It could be due to the expansion of the cytoplasmic content after depressurization or to the accumulation of CO_2 inside the cells. In the LM image (Fig. 4G), it can be perceived that SC-CO_2+HPU-treated *E. coli* cells appeared less stained than untreated (Fig. 4A) and SC-CO_2 treated (Fig. 4D) ones. Numerous aggregates (intensively blue stained) of cytoplasmic content could be observed surrounding the SC-CO_2+HPU treated cells (Fig 4G). TEM images of SC-CO_2+HPU treated cells (Fig. 4H-I) revealed a higher aggregation and more uneven distribution of the cytoplasmic content compared to SC-CO_2 treated cells. Great proportions of empty regions were observed inside the SC-CO_2+HPU-treated cells, clearly indicating a drastic reduction in the cytoplasmic content. The outer membrane, peptidoglycan layer, inner membrane of the cell wall and plasmalemma appeared to be disintegrated and separated from the inner cell in most of the bacteria (Fig. 4H-I).

In the present study, the SC-CO_2+HPU treatment totally inactivated the population of *E. coli* (8.3 log-cycles) and generated more severe effects on the morphology of cells than the SC-CO_2 treatment. The greatest differences between the effects of both treatments can be found in the integrity of the cell wall and plasmalemma, which were totally disrupted after the SC-CO_2+HPU treatment. This fact could expedite the loss of the *E. coli* cells' integrity, resulting in the microorganism inactivation. The inactivation effect of the SC-CO_2+HPU treatment could be related to the cavitation phenomenon generated by HPU which could damage the cell wall and membranes increasing both the rupture of the cellular envelope and the disintegration and dispersion of the intracellular content. Moreover, the agitation produced by the ultrasonic field could accelerate the solubilization rate of SC-CO_2 into the liquid and increase the mass transfer

rates (Awad et al., 2012), drastically affecting the cell membrane and facilitating the inactivation mechanisms associated with SC-CO_2 treatments.

3.4. Morphological changes in *S. cerevisiae* cells treated with SC-CO_2 and SC-CO_2+HPU

Fig. 5A shows the typical ellipsoidal morphology of untreated *S. cerevisiae* cells observed by LM, which typically measured 3.11 ± 0.40 µm at the large diameter and 2.63 ± 0.23 µm at the small one. The cells appeared homogeneously stained and the budding process could be noted in some of them. TEM images (Fig. 5B, 5C) depict a compact and homogeneous distribution of the cytoplasm, in which the following could be distinguished: a well-defined nucleus, a nuclear membrane, vacuoles, the intact plasmalemma, the cell wall with an electron-transparent internal layer, consisting of β-1, 3-glucan and chitin, the thicknesses of which were about 84.1 ± 16.7 nm and an electron-dense and osmiophilic outer layer, mainly corresponding to glycosylated mannoproteins, of about 40.7 ± 11.3 nm. The plasmalemma or cell membrane also appeared to be well-preserved and close to the cell wall.

The LM image of SC-CO_2-treated *S. cerevisiae* cells allowed less intensely stained cells than untreated yeasts to be observed in Fig. 5D, which indicates a lower intracellular content inside the cells. Moreover, some areas more intensely stained outside the cells, revealing the removal of their cytoplasmic content. In general, no significant differences ($p>0.05$) in size were found between SC-CO_2-treated cells, which measured 3.33 ± 0.50 µm at the large diameter and 2.69 ± 0.48 µm at the small one, and untreated cells. Inside the cells, a loss in cytoplasm integrity could be observed, as could the disappearance of the nucleus, the desegregation of cytoplasmic organelles and the aggregation of some cytoplasmic substances (AC, Fig. 5E). An examination of the treated cells revealed that the cell wall still contained layers (Fig. 5F), an internal layer of about 101.8 ± 19.5 nm in thickness and an outer layer of about 54.7 ± 11.5 nm, both significantly ($p<0.05$) thicker than in the non-treated *S. cerevisiae* cells, which could be due to the accumulation of CO_2 in the cell membrane. The inner layer could be observed as more densely stained and the outer layer thicker than those of untreated cells (Fig. 5F). TEM observations confirmed that in some

cells, the SC-CO_2 treatment provoked the degradation and dissolution of some constituents of cell walls, which could be related to the CO_2 lipophilic solvent characteristics (Giulitti et al., 2011). This fact could increase the permeability and fluidity of cell wall and cell membrane. In many locations, plasmalemma could not be visualized and some cells contained abnormal bud scars (Fig. 5F).

Garcia-Gonzalez et al. (2010) investigated the effect of SC-CO_2 (210 bar, 45 ºC for 60 min) on the morphology of *S. cerevisiae* and concluded that, despite the membrane not being disrupted, its permeabilization could ease the penetration of CO_2 into the cell and the pH drop could induce a denaturation of some key enzymes. Li et al. (2012) explored the differences between untreated and treated *S. cerevisiae* cells at 100 bar and 35 ºC for different process times: 30, 75 and 120 min, by SEM and TEM, and revealed that the intracellular content in the treated cells gradually weakened as the treatment time increased; they also observed the reduction of the cytoplasm density and the extraction of cytoplasmic content, in spite of the fact that the cell walls remained intact. In the present work, after only 5 min of SC-CO_2 treatment, the CO_2 succeeded in penetrating the cells, generating minor irregularities which were not sufficient to observe an important microbial reduction of *S. cerevisiae* (0.2 log-cycles). It is possible that *S. cerevisiae* cells synthesize new biomolecules after 5 min of treatment to repair the damaged cell walls and membranes so as to continue cellular division and growth (Erkmen & Bozoglu, 2008). Thus, longer process times may be required to inactivate enough key enzymes and to affect the cellular envelope, which would allow a significant reduction of surviving *S. cerevisiae* cells to be obtained.

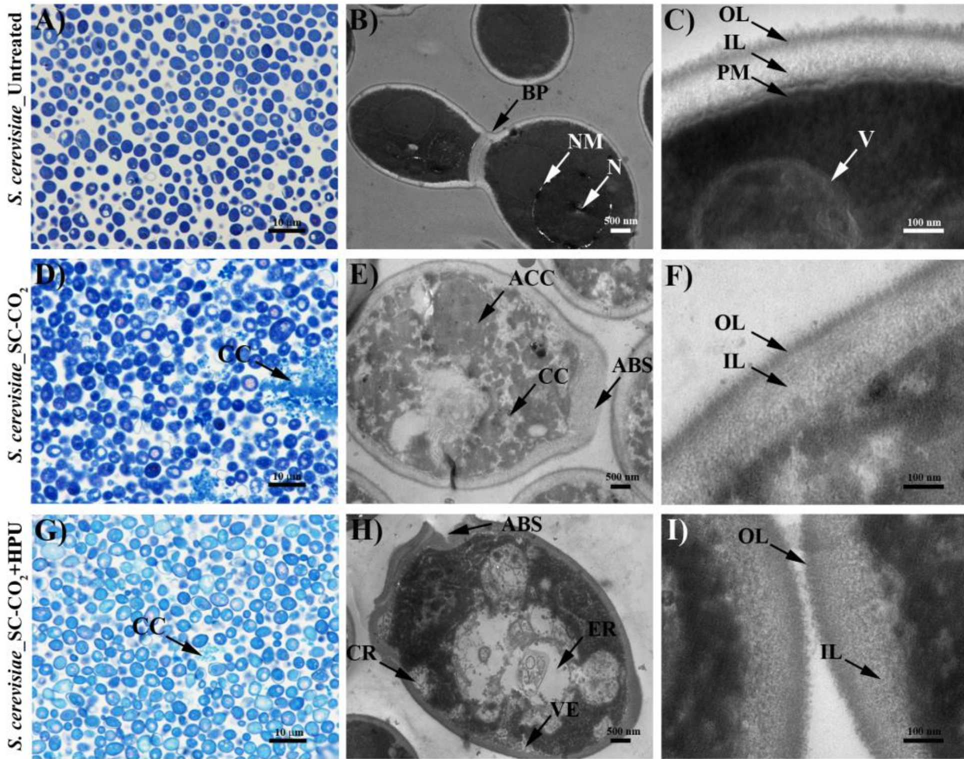

Fig. 5. LM (A, D, G) and TEM (B, C, E, F, H, and I) micrographs by semithin and ultrathin sectioning of *S. cerevisiae*. Images A-C represent untreated cells; images D-F show cells treated with $SC\text{-}CO_2$ at 350 bar, 36 ºC for 5 min; images G-I show cells treated with $SC\text{-}CO_2$+HPU at 350 bar, 36 ºC, 40 W for 5 min. BP: budding process; N: nucleus; NM: nuclear membrane; V: vacuoles; PM: plasmalemma; IL: cell wall-internal layer; OL: cell wall-outer layer; CC: cytoplasmic content; ER: empty regions; ACC: aggregation cytoplasmic content; ABS: abnormal bud scars; CR: cytoplasm retracted; VE: vesicles.

Significant differences ($p<0.05$) were found in the dimensions of the $SC\text{-}CO_2$+HPU- treated *S. cerevisiae* cells, 4.15 ± 0.77 µm in width and 5.76 ± 0.77 µm in length, compared to non-treated and $SC\text{-}CO_2$- treated *S. cerevisiae* cells. The greater size observed in $SC\text{-}CO_2$+HPU-treated cells could be due to the larger expansion of the cytoplasmic content after depressurization or to the accumulation of CO_2 inside the cells. LM images (Fig. 5G) showed that the $SC\text{-}CO_2$+HPU treatment provoked a higher degree of cellular degradation,

compared to the SC-CO_2 treatment. In addition to the fact that the SC-CO_2+HPU-treated cells exhibited a lower degree of staining (Fig. 5G), it was possible to observe a greater amount of intracellular content extracted from the cells. The TEM images revealed lemon-shaped deformed cells (Fig. 5H), with punctured or broken walls, disrupted organelles, cytoplasm retracted from the cell wall and a large proportion of empty regions. Fig. 5I shows that the cell walls had partially lost their layered structure. The inner layer appeared to be more densely stained due to the possible diffusion of intracellular content through it, which hinders a clear differentiation between the outer and the inner layer. The inner and the outer layer measured 165.7 ± 32.3 nm and 96.8 ± 15.7 nm in thickness, respectively, both being significantly ($p<0.05$) thicker than in the non-treated and SC-CO_2 treated *S. cerevisiae* cells. The plasmalemma appeared to be degraded and was not visible. Moreover, it can be noted that the TEM images revealed that the SC-CO_2+HPU treatment caused an important degradation of the content in the majority of cells, with the disappearance of the nucleus, the disruption and degradation of cytoplasmic organelles, and the creation of vesicles on the outer side of the plasmalemma.

The SC-CO_2+HPU treatment produced the total inactivation of *S. cerevisiae* after 5 min of treatment, compared to the 0.2 log-cycles attained after 5 min of the SC-CO_2 treatment. By observing the TEM images of both treatments (Fig. 5E, 5H), it can be observed that the greatest difference appeared to be between the disrupted cell envelope (cell wall) and the plasmalemma of the SC-CO_2+HPU-treated cells and the almost intact ones of the SC-CO_2-treated cells. Therefore, the faster microbial inactivation achieved by the SC-CO_2+HPU treatment compared to that of SC-CO_2, could be due to the cavitation phenomenon generated by HPU which could cause cracked or damaged cell walls. This enhances the penetration of SC-CO_2 inside the cells, changing the cellular equilibrium and facilitating the extraction of intracellular compounds, thus accelerating the death of the microbial cells.

3.5. Stability of treated samples during refrigerated storage

The stability of the samples treated with SC-CO_2+HPU was analyzed while they were stored for 6 weeks at 4 ºC. The regrowth or survival of the

$SC\text{-}CO_2$+HPU- treated *E. coli* and *S. cerevisiae* cells was not observed during the 6-week storage period. These results could suggest that the treated cells were not capable of recovering during their storage on LB or YPD Broth. Therefore, the $SC\text{-}CO_2$+HPU treatment generated irreversible damage to the cells, as observed in the microstructural analysis, preventing a possible synthesis of new biomolecules which would repair the damage to the cell walls and membranes and preventing the cellular division and growth.

Using only $SC\text{-}CO_2$, other studies have observed a growth in the microbial population during a post-treatment storage period (Kincal et al., 2005; Fabroni et al., 2010) although at time 0 (immediately after the treatment) no microorganisms were detected.

Liao et al. (2010b) investigated the inactivation and the possible regrowth of natural microorganisms in apple juice after a $SC\text{-}CO_2$ treatment (200 bar, 52-62 ºC, 30 min). The population of aerobic bacteria in apple juice, subjected to $SC\text{-}CO_2$, exhibited no increase during storage for 35 days at 2 °C; however, the population of yeasts and molds slightly increased after 14 storage days. In the present study, using $SC\text{-}CO_2$+HPU, shorter process times and lower temperatures, no microbial growth was detected during a longer storage period. Therefore, the application of HPU during the $SC\text{-}CO_2$ treatment increased the damage caused to the microorganisms, thus avoiding microbial recovery.

4. CONCLUSIONS

Shorter process times were required to achieve the total inactivation of *S. cerevisiae* than of *E. coli*, despite the fact that the yeast is known to have a greater resistance to $SC\text{-}CO_2$. This could be due to the fact that the *S. cerevisiae* cells are bigger and, as such, the cavitation bubbles associated with HPU have a more marked effect on them.

The microstructural study carried out in the present work revealed that there was a direct relationship between cellular modification/damage and inactivation provoked by the $SC\text{-}CO_2$ and $SC\text{-}CO_2$+HPU treatments on *E. coli* and *S. cerevisiae* cells. Despite the small changes observed in cell morphology after 5 min of the $SC\text{-}CO_2$ treatment, the treatment was not lethal against either *E. coli*

or *S. cerevisiae*. However, 5 min of the SC-CO_2+HPU treatment totally inactivated the population of both microorganisms. After the SC-CO_2+HPU treatment, cell wall and cell membrane were totally disrupted, thus easing the disintegration of the cytoplasm and the inactivation of cells. The damage caused by the SC-CO_2+HPU treatment was serious enough to prevent a possible regrowth of cells during post-treatment storage.

SC-CO_2+HPU is a non-thermal preservation technology that could represent an alternative means to thermal processing to extend the shelf life of foods using mild process conditions.

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Chapter 5

Modelling of the Inactivation Kinetics of Escherichia coli, Saccharomyces cerevisiae and Pectin Methylesterase in Orange Juice Treated with Ultrasonic-Assisted Supercritical Carbon Dioxide

C. Ortuño, M. Balaban, J. Benedito

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Modelling of the inactivation kinetics of *Escherichia coli*, *Saccharomyces cerevisiae* and pectin methylesterase in orange juice treated with ultrasonic-assisted supercritical carbon dioxide

Carmen Ortuño[1], Murat Balaban[2], Jose Benedito[1]

[1] Grupo de Análisis y Simulación de Procesos Agroalimentarios, Departamento Tecnología de Alimentos, Universitat Politècnica de València, Camí de Vera s/n, E46022, Valencia, Spain.

[2] Department of Chemical and Materials Engineering, University of Auckland, Auckland 1142, New Zealand.

ABSTRACT

The combined effect of supercritical carbon dioxide (SC-CO_2) and high power ultrasound (HPU) on the inactivation kinetics of *E. coli*, *S. ceresivisae* and pectin-methyl esterase (PME) in orange juice was studied in order to select models that can predict their inactivation behavior based on process parameters. Experiments were performed at different temperatures (31-41 ºC, 225 bar) and pressures (100-350 bar, 36 ºC). The inactivation rate of *E. coli*, *S. cerevisiae* and PME increased with pressure and temperature during SC-CO_2+HPU treatments. The SC-CO_2+HPU inactivation kinetics of *E. coli*, *S. cerevisiae* and PME were represented by models that included temperature, pressure and treatment time as variables, based on the Biphasic, the Peleg Type B, and the fractional models, respectively. The HPU-assisted SC-CO_2 batch system permits the use of mild process conditions and treatment times that can be even shorter than those of continuous SC-CO_2 systems.

Key words: supercritical inactivation, residual enzyme activity, inactivation kinetics, ultrasound, modeling, synergistic effect.

1. INTRODUCTION

Orange juice is a very popular product due to its high nutritional value, its bioactive components such as phenolics, vitamin C and carotenoids (Abeysinghe et al., 2007) and its well-liked sensory characteristics.

Cloud is a desirable attribute that positively affects turbidity, flavour and the characteristic colour of orange juice. Cloud loss has been primarily attributed to the activity of pectin methyl-esterase (PME), a cell-wall bound pectic enzyme released into the juice during extraction (Balaban et al., 1991). Acid-tolerant bacteria, yeasts, and moulds also play an important role in causing the quality deterioration of citrus products during storage and distribution (Kincal et al., 2005).

In order to prevent cloud loss and to ensure juices with low microbial levels, preservation techniques must be applied. SC-CO_2 has been reported to inactivate different undesirable enzymes (Arreola et al., 1991; Zhou et al., 2009a, 2009b) and many microorganisms (Kincal et al., 2005; Ferrentino et al., 2010; Corradini & Peleg, 2012; Ortuño et al., 2012a) in liquid foods without exposing them to the adverse effects of heat, thereby retaining their fresh-like nutritional and sensory qualities (Damar & Balaban, 2006). Balaban et al. (1991) studied the inactivation of PME in orange juice with a batch SC-CO_2 system. These authors achieved the total inactivation of PME after 145 min at 269 bar and 56 ºC. Fabroni et al. (2010) used a continuous high-pressure carbon dioxide pilot-plant system to reduce the PME activity of blood orange juice. They showed a reduction of 25-35 % in the PME activity after treatments at between 130 and 230 bar at 36 ºC for 15 min.

Kincal et al. (2005) reported that a continuous SC-CO_2 treatment (210 bar, 34.5 ºC, 10 min) caused at least a 5 log-cycle reduction of pathogens (*Escherichia coli* O157:H7, *Salmonella typhimurium*, and *Listeria monocytogenes*) inoculated into orange juice. Ortuño et al. (2012b) reported that by using a batch-mode SC-CO_2 at 350 bar and 36 ºC for 25 min, a reduction of 1 log-cycle of *Escherichia coli* DH1 (*E. coli*) was obtained in orange juice. Batch-mode equipment requires a much longer inactivation time if compared with that of continuous SC-CO_2 systems. In fact, one of the main inconveniences

to the industrial application of batch SC-CO_2 systems is the long treatment time required, a fact which hinders its adoption for use in the food industry (Yuan et al., 2012).

In a continuous system, the agitation caused by the flow of the mixture of treated liquid and SC-CO_2 allows a faster dissolution of CO_2, and therefore its better contact with cells and enzymes, when compared to batch systems (Damar & Balaban, 2006). However, even in continuous systems, the process times needed for the SC-CO_2 inactivation of PME in orange juice are too long to obtain an acceptable enzymatic reduction.

In order to enhance the efficiency of SC-CO_2 microbial and enzyme inactivation processes, a technique based on the combination of SC-CO_2 with high-power ultrasound (HPU) has been developed (Benedito et al., 2011). This simultaneous application has been shown to accelerate the death of *E. coli* and *Saccharomyces cerevisiae* (*S. cerevisiae*) inoculated into a culture medium, compared with the use of only SC-CO_2 (Ortuño et al., 2012b, 2013). These studies have shown that the effect of increasing the treatment pressure or temperature in an SC-CO_2+HPU process conducted on culture media did not significantly enhance the already-rapid inactivation level.

Only two studies have shown that the application of SC-CO_2+HPU in orange juice completely inactivated the population of *E. coli* and *S. cerevisiae* after 5 min (350 bar, 36 ºC) and 1.5 min (225 bar, 36 ºC) of treatment, respectively. No microbial reduction was observed in orange juice under the same process conditions (pressure, temperature and time) when using only SC-CO_2 (Ortuño et al., 2012b, 2013).

The use of mathematical modeling is an important tool that allows the effect of different inactivation treatments and process parameters on microbial loads and enzyme concentrations to be analysed, minimizing the number of experiments to be carried out. To describe microbial inactivation using SC-CO_2, different models have been proposed: the Weibull, Gompertz and Logistic models (Ferrentino et al., 2010; Liao et al., 2010; Corradini & Peleg, 2012; Ortuño et al., 2012a, 2013). Also, PME inactivation was described by first-order kinetics (Balaban et al., 1991), fractional conversion models, and the Weibull

model (Zhou et al., 2009b).

At present, the effect of pressure and temperature on the SC-CO_2+HPU microbial inactivation in juices addressed in the present study has not been evaluated and could differ from that found in culture media reported in the literature (Ortuño et al., 2012b, 2013). Moreover, the effect of this novel combined treatment on the inactivation of enzymes cannot be found elsewhere in the literature.

Therefore, the objective of this work was to study the combined effect of SC-CO_2 and HPU on the inactivation kinetics of *E. coli*, *S. ceresivisae* and PME in orange juice, and to select models that can best describe and predict their inactivation behavior based on the process parameters.

2. MATERIAL AND METHODS

2.1. Orange juice

Valencia Navel oranges (*Citrus sinensis*) were purchased from a local market and kept at 4 ºC for 2 days until juice extraction. Orange juice was obtained by washing, peeling and extracting the fruit (Ultra Juicer, Robot Coupe J80, USA). The orange juice (pH = 3.8; ºBrix = 11.6) was sealed in plastic containers and stored at -18 ºC until required.

2.2. Microorganisms and growth conditions

The microbial strains used in this study were *Escherichia coli* DH1, (chromosomal genotype: *end*A1 *gyr*A9, *thi*-1, *hsd*R179(r_K^-,m_K^+), *sup*E44, *rel*A1), and *Saccharomyces cerevisiae* T73, which is a natural strain isolated from wine fermentation in Alicante (Spain) (Querol et al., 1992) and is commercialised as Lalvin T73 (Lallemand Inc., Montreal, Canada). A single colony of *E. coli* or *S. cerevisiae* was grown overnight in Luria Bertani Broth (LB Broth, Sigma-Aldrich, USA) at 37 ºC, or in Yeast Peptone Dextrose Broth (YPD Broth, Sigma-Aldrich, USA) at 30 ºC, respectively, using an incubation chamber (J.P. SELECTA, Model 3000957, Barcelona, Spain) and an orbital shaker at 120 rpm (J.P. SELECTA, Rotabit Model 3000974, Barcelona, Spain). For each experiment with *E. coli* or *S. cerevisiae*, a subculture was prepared by inoculating 50 μL from the starter culture into 50 mL sterilized medium and incubating at

37 ºC-24 h or at 30 ºC-24 h, respectively, to obtain cells in the early stationary phase. Growth curves were determined in advance by both plating and measuring the absorbance at 625 nm (data not shown).

2.3. Inoculated juice

For each experiment, a container of orange juice was thawed at 4 ºC for 12 h. The juice was inoculated by the addition of 5 mL of either *E. coli* or *S. cerevisiae* cells in the early stationary phase (see section 2.2) to 50 mL of orange juice to reach a cell concentration of 10^7 CFU/mL for *S. cerevisiae* and 10^8 CFU/mL for *E. coli*.

2.4. Supercritical fluid equipment and processing

2.4.1. Apparatus

The supercritical fluid lab-scale batch system was specially designed and built by our research group. It includes a CO_2-tank, a N_2-tank, a chiller reservoir kept at -18 ºC; a pump and a thermostatic bath to keep the treatment vessel at the desired temperature. The system includes ultrasound equipment (Benedito et al., 2011) embedded in the supercritical fluid vessel. The ultrasound equipment consists of a high power piezoelectric transducer, an insulation system and a power generator unit (40 W ± 5 W). The transducer is inserted inside the inactivation vessel and includes two commercial ceramics (35 mm external diameter; 12.5 mm internal diameter; 5 mm thickness; resonance frequency of 30 kHz) and a sonotrode, which was specially constructed to concentrate the highest amount of acoustic energy on the application point.The equipment is described in detail in Ortuño et al. (2013).

2.4.2. Supercritical fluid processing.

Fifty-five mL of inoculated orange juice for microbial inactivation, and 55 mL of non-inoculated orange juice for enzyme inactivation, was subjected to the SC-CO_2+HPU treatment under different process conditions. To determine the effect of pressure, samples were treated by SC-CO_2+HPU at 36 ºC and 100, 225 and 350 bar. To determine the effect of temperature, samples were exposed to SC-CO_2+HPU at 225 bar and 31, 36 and 41 ºC. The temperature and pressure ranges chosen were higher than the critical point for CO_2 and lower than lethal

levels for both microorganisms. According to previous studies of the inactivation of these microorganisms using SC-CO_2+HPU, higher temperatures or pressures were not necessary to reach acceptable levels of inactivation (Ortuño et al., 2012b, 2013). The experimental process has previously been described by Ortuño et al. (2013) in detail. All experiments were run in triplicate.

2.5. Enumeration of viable microorganisms.

The viability of *E. coli* and *S. cerevisiae* in the orange juice samples was determined by the plate count method. Each sample was serially diluted with sterilised distilled water. 100 µL of the appropriate dilution were plated in triplicate on LB Agar or YPD Agar plates and incubated for 24 h at 37 ºC or 30 ºC, for *E. coli* or *S. cerevisiae* respectively, before counting. Microbial cells in the initial non-treated sample (control sample) were counted following the same procedure. The results were expressed as $\log_{10}(N/N_0)$ 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 the different times of treatment. The data presented are the means of triplicate experiments. The results shown are the arithmetic mean and the standard deviation of $\log_{10}(N/N_0)$ for at least three plates.

2.6. PME activity measurements.

The PME activity of orange juice was determined at pH 7 and 25 ºC using the Castaldo et al. (1997) method, with modifications. The reaction mixture consisted of orange juice and a substrate solution that was prepared by dissolving 10 g of pectin powder (Sigma Chemical Co., St. Louis, MO) in 1 L of 0.15 M NaCl. The NaCl solution was heated to 50-55 ºC and added to the blender while pectin powder was sprinkled on the surface and blended. The pectin solution was stored at 4 ºC until required.

The pH of the pectin solution was adjusted to 7 prior to each analysis and 5 mL of orange juice were added to 50 mL of pectin solution. The pH was quickly adjusted to 7 (0.5 M NaOH for gross adjustment, 0.05 M NaOH for fine adjustment). The pH was maintained at 7 by means of the addition of 0.05 M NaOH. The consumption of NaOH was recorded during a period of about 30 min. The dV_{NaOH}/dt slope was determined in the linear part of the titration

curve. The PME activity of the orange juice sample, A, was calculated by Eq. (1) and expressed as microequivalents per min and mL of juice.

$$A = \frac{dV_{NaOH}}{dt} \frac{N_{NaOH}}{V_{sample}} \qquad \text{Eq. (1)}$$

where V_{NaOH} and N_{NaOH} are the volume and molarity of the NaOH solution used for the titration, respectively, and V_{sample} is the volume of the orange juice added to the substrate solution (mL).

Each sample was analyzed in triplicate. The data were normalized to percentage of activity relative to the untreated orange juice and the PME residual activity (RA) was calculated using Eq. (2).

$$\text{PME residual activity} = \frac{\text{Specific activity PME after treatment}}{\text{Specific activity PME control sample}} \text{ x } 100 \qquad \text{Eq. (2)}$$

2.7. Modeling of the microbial and enzyme inactivation kinetics

The modeling of microbial inactivation using SC-CO_2 (Ferrentino et al., 2010; Corradini & Peleg, 2012; Ortuño et al., 2012a) and HPU (Guerrero et al., 2005) processing has been studied for different microorganisms. Six different models which had previously been used in the literature (Linton et al., 1996; Polydera et al., 2004; Peleg, 2006; Lee et al., 2009; Corradini & Peleg, 2012) to fit inactivation kinetics for other non-thermal techniques were selected to describe the inactivation kinetics of microorganisms using SC-CO_2+HPU (Table 1).

Two models, used to fit the residual activity curves of PME treated with non-thermal techniques, have been selected in this study to fit the inactivation curves of PME treated with SC-CO_2+HPU (Table 1).

Table 1. Models used to fit the microbial and enzyme inactivation kinetics.

Modelling of the microbial inactivation kinetics			
Model	**Equation**	**Parameters**	**Reference**
Weibull	$\log_{10}\frac{N}{N_0} = -b\,t^n$	b, n	Corradini & Peleg, 2012
Gompertz	$\log_{10}\frac{N}{N_0} = Ce^{-e^{A+Bt}} - Ce^{-e^{A}}$	A, B, C	Linton et al., 1996
Biphasic	$\log_{10}\frac{N}{N_0} = \log\left((1-f)10^{\frac{-t}{Dsens}} + f10^{\frac{-t}{Dres}}\right)$	f, D_{sens}, D_{res}	Lee at al., 2009
Logistic	$\log_{10}\frac{N}{N_0} = \frac{Q}{1+e^{\frac{4\sigma(\tau-\log t)}{Q}}} + \frac{Q}{1+e^{\frac{4\sigma(\tau-\log t_0)}{Q}}}$	*Q*, σ, τ	Lee et al., 2009
Peleg Type A	$\log_{10}\frac{N}{N_0} = -\frac{a_1 t}{(1+a_2 t)(a_3 - t)}$	a_1,a_2 ,a_3	Peleg, 2006
Peleg Type B	$\log_{10}\frac{N}{N_0} = -\frac{b_1 t^r}{b_2 + t^r}$	b_1, b_2, r,	Peleg, 2006
Modelling of the inactivation kinetics of pectin methyl-esterase			
Model	**Equation**	**Parameters**	**Reference**
Fractional	$\frac{A - A_f}{A_0 - A_f} = e^{-kt}$	k	Polydera et al., 2004
Weibull	$\log_{10}\frac{A}{A_0} = -b\,t^n$	b, n	Corradini & Peleg, 2012

N_0: the initial number of microorganisms at time 0; N: the corresponding number after a time t.

A_0: the PME activity of the untreated orange juice; A: the PME activity of the treated orange juice after time t; A_f: the PME activity at the end of the treatment.

b: non-linear rate parameter; n is the shape factor

A, B and C: different regions of the survival curve: the initial shoulder (A), the maximum death rate (B) and the overall change in the survivor number (C)

(1-f) and f: the fraction of treatment-sensitive and treatment-resistant populations, respectively; D_{sens} and D_{res} are the decimal reduction times of the two populations (min)

Q: the upper asymptote-lower asymptote; σ: the maximum inactivation rate; τ: the log time needed to reach the maximum inactivation rate

a_1, a_2, a_3, b_1, b_2, r: model parameters; k: the inactivation rate parameter

2.8. Statistical analysis of the inactivation kinetics

The Statgraphics Plus (Statistical Graphics Corp. 5.1, Warrenton, USA) statistics package was used to perform multifactorial ANOVA, and LSD (Least Significant Differences) were identified to evaluate the effect of pressure, temperature and time on the inactivation rate of microorganisms and on the residual PME activity of treated orange juice.

The kinetic constants of the models were calculated by minimizing the sum of the square differences between experimental and model-predicted data using the Solver Microsoft Excel™ tool. The root mean square error (RMSE, Eq. 3) and the coefficient of determination (R^2, Eq. 4) were used to evaluate the goodness of fit of the model and the accuracy of estimation. RMSE is a measure of the standard error in the estimation, whereas R^2 is used as a measure of explained variance (Schemper, 2003).

$$RMSE = \sqrt{\frac{\sum_{k=1}^{z}(y_k - y_k^*)^2}{z}} \quad \text{Eq. (3)}$$

$$R^2 = 1 - \frac{S_{yx}^2}{S_y^2} \quad \text{Eq. (4)}$$

where y and y* are the experimental data and the estimated values, respectively, calculated as $\log_{10}$ (N/N_0) or $\log_{10}$ (A/A_0) for microorganisms or enzymes, respectively; z is the number of experimental values and S_y and S_{yx} are the total standard deviation and the standard deviation of the estimation, respectively.

3. RESULTS AND DISCUSSION

3.1. Combined effect of HPU and SC-CO_2 on *E. coli* inactivation.

Figure 1A shows the inactivation curves of *E. coli* in orange juice treated with a combined SC-CO_2+HPU process. The survivor numbers began to decrease immediately and no lag-phase was observed for any temperature or pressure studied. A reduction of 4.12, 4.62 and 6.15 log-cycles was obtained after 1 min of treatment, at 31, 36 and 41 ºC, respectively. There were no significant differences ($p>0.05$) between the inactivation at 31 and 36 ºC; however, when the

temperature was increased to 41 ºC, a significantly ($p<0.05$) faster inactivation was observed. Although the inactivation rate decreased after the first minute in every case, 7 min was needed to attain total inactivation (7-8 log-cycles) at 31 and 36 ºC and only 3 min at 41 ºC.

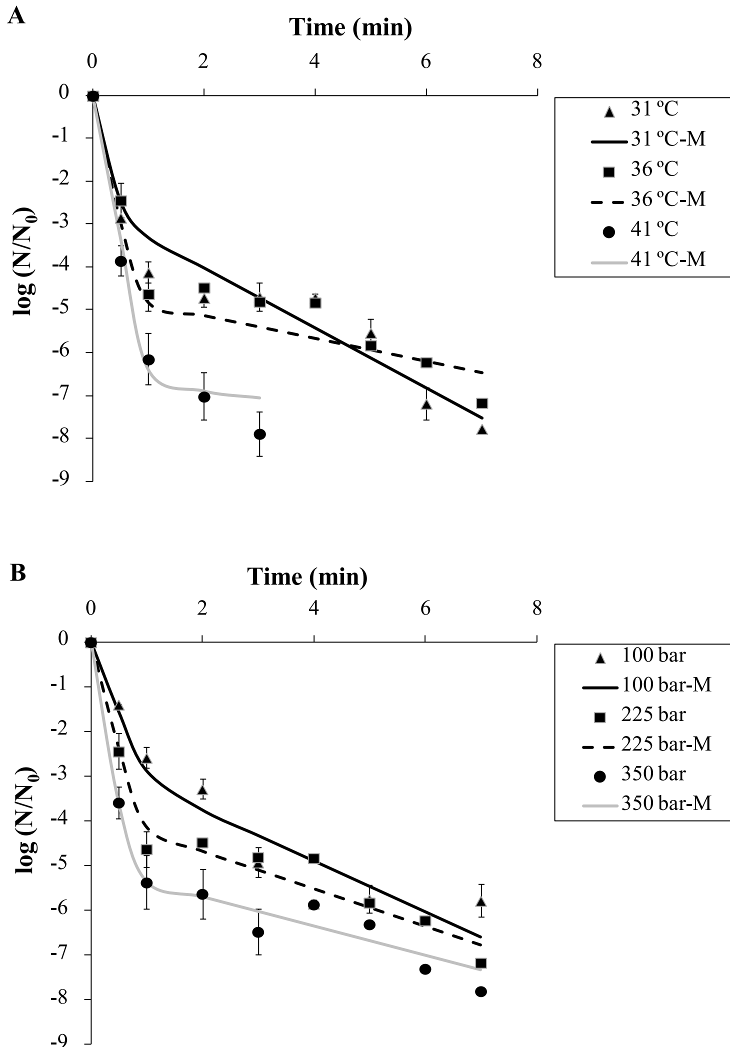

Fig. 1. Experimental data (discrete points) and modeling (M) of the inactivation kinetics of *E. coli* in orange juice treated by SC-CO_2 and HPU at different temperatures (A, 225 bar) and different pressures (B, 36 ºC). M: modified Biphasic model.

Regarding the effect of pressure on the inactivation of *E. coli*, reductions of 2.5, 4.6 and 5.4 log-cycles were reached after 1 min of treatment at 36 ºC and 100, 225 and 350 bar, respectively (Figure 1B). After the first minute, the population decrease was slower and after 7 min of treatment reductions of 5.8, 7.2 and 7.9 log-cycles at 100, 225 and 350 bar, respectively, were reached. On average, the inactivation rate significantly increased ($p<0.05$) as the pressure rose from 100 to 225 bar, and from 225 to 350 bar.

The inactivation of *E. coli* has been explored in previous studies using both techniques (SC-CO_2 and HPU) individually. Liao et al. (2008) studied the inactivation of *E. coli* with a batch SC-CO_2 system in cloudy apple juice at different temperatures and pressures. After 75 min, the microbial reduction increased from 5 to 7 log-cycles as the temperature rose from 32 to 42 ºC (300 bar), respectively; and from 5.5 to 7.5 log-cycles as the pressure increased from 100 to 300 bar (42 ºC). Kincal et al. (2005) tested a continuous high-pressure CO_2 system for the inactivation of *E. coli* inoculated in orange juice. These authors reached a reduction of 4 log-cycles (10^5 CFU/mL initial population) using 34.5 ºC and 380 bar after a residence time of 10 min. Thus, it can be concluded that, in continuous systems, the treatment time is drastically reduced compared to batch systems. However, the HPU-assisted batch supercritical system used in the present study attained similar inactivation levels in shorter process times than in continuous systems. Thus, it can be concluded that, in continuous systems, the treatment time is drastically reduced compared to batch systems due to the agitation of the medium which enhances the solubilization of the SC-CO_2 and the extraction of cellular components. However, the HPU-assisted batch supercritical system used in the present study attained similar inactivation levels in shorter process times than in continuous systems. This fact could be due to the high energy agitation of the ultrasonic waves and to the cavitation phenomenon..

In this regard, using SC-CO_2+HPU, the acceleration of the solubilization rate of SC-CO_2 into the liquid and the increase in the mass transfer due to the vigorous agitation produced by the ultrasonic field would permit the rapid saturation of CO_2 in the medium, which might accelerates the inactivation

mechanisms (a decrease of the medium pH, an increase in membrane fluidity and permeability, the diffusion of CO_2 into the cells, cell membrane rupture, the alteration of intracellular equilibrium, the inactivation of key enzymes, and the extraction of critical intracellular materials) (Garcia-Gonzalez et al., 2007; Pataro et al., 2010). Moreover, the cell wall damage caused by cavitation could play an important role in both the penetration of SC-CO_2 and the extraction of intracellular compounds, accelerating the death of the microbial cells (Ortuño et al., 2013).

Contrary to the results observed in previous studies into the inactivation of *E. coli* in LB Broth medium (Ortuño et al., 2012b), where the effect of increasing pressure and temperature did not enhance the already-rapid inactivation rate, the present study using orange juice showed that increases in both pressure and temperature led to a rise in the inactivation rate. It is known that the inactivation rate is affected by the composition of the suspending medium (Garcia-Gonzalez et al., 2007; Ortuño et al., 2012b). There are approximately 70 % more sugars in the orange juice (11.6 ºBrix) than in LB Broth. They bind water from the medium and there is a smaller amount of free water in which CO_2 could be dissolved than in LB Broth. Despite the intense ultrasound agitation, the orange juice was not as quickly saturated with CO_2 as LB Broth, due to the lower CO_2 solubility as a consequence of the high sugar content. Therefore, increasing pressure or temperature could facilitate the solubilization of CO_2 into the orange juice. This is the first step in the inactivation mechanisms of SC-CO_2+HPU, from which other mechanisms follow. It is also known that the viscosity of the medium directly affects the phenomenon of cavitation. To generate cavitation bubbles, the cohesive forces of the liquid have to be overcome by the negative pressure. The cohesive forces increase as the liquid becomes more viscous; therefore, it is more difficult to obtain cavitation (Carcel, 2003). The orange juice is more viscous than the LB broth. Therefore, cavitation could be less intense and its inactivation mechanisms against microorganisms less severe.

The nature of the medium influenced the effect of HPU and variations between different temperatures and pressures were observed. Therefore, it is important to determine the effect of the combination of treatment medium and

process temperature / pressure on the inactivation of microorganisms, to find optimum SC-CO_2+HPU process conditions. For that purpose, the modeling process is of great importance.

3.2. Modeling of *E. coli* inactivation kinetics

Table 2 shows the statistical parameters for the fit of the kinetic models to the inactivation data of *E. coli* in orange juice treated by SC-CO_2 and HPU. R^2 and RMSE values (Table 2) indicate that, overall, a good fit was obtained with the six models for the different process conditions considered, with $R^2 > 0.9$ for most of the conditions studied except for the Gompertz model ($R^2_{avg} = 0.887$; $RMSE_{avg} = 0.549$). The standard deviation of the differences between the values which were actually observed and those estimated by the model was below 0.5 log-cycles. The Biphasic model provided the best fit ($R^2_{avg} = 0.967$) for all the process conditions used, with an accuracy of prediction of 0.286 log-cycles. In this model, to relate f, D_{sens} and D_{res} (see Table 1) to pressure and temperature, we assumed that these parameters were described by a log-logistic model (Peleg, 2006), with simultaneous pressure and temperature dependences (Eqs. (5-7)).

$$f(T,P) = \ln(1 + \exp(a_f(T - Tc) + b_f(P - Pc))) \quad \text{Eq. (5)}$$

$$D_{sens}(T,P) = \ln(1 + \exp(a_{Ds}(T - Tc) + b_{Ds}(P - Pc))) \quad \text{Eq. (6)}$$

$$D_{res}(T,P) = \ln(1 + \exp(a_{Dr}(T - Tc) + b_{Dr}(P - Pc))) \quad \text{Eq. (7)}$$

where a_f, b_f, a_{Ds}, b_{Ds}, a_{Dr}, b_{Dr}, Tc and Pc are the characteristic constants of the microorganism. Substituting Eqs. (5-7) in the Biphasic model (Table 1), a general expression of the Biphasic model is obtained that can be used to predict the inactivation kinetics of *E. coli* in orange juice at different pressures and temperatures.

The characteristic constants of the microorganism were calculated by minimizing the sum of square differences between all the experimental data and all the predicted data obtained from every pressure and temperature condition studied, using the Excel Solver tool. The values of the coefficients a_f, b_f, a_{Ds}, b_{Ds}, a_{Dr}, b_{Dr}, Tc and Pc were: -0.442, -0.021, -0.045, -0.003, 0.057, 0.005, 39.296 and -272.474, respectively. The predicted survival curves of *E. coli* in orange juice, using the described Biphasic general model, can be seen in Figure 1. The

obtained statistics, R^2_{avg} = 0.960 is comparable to that provided by the individual fits to each temperature and pressure combination (Table 1: R^2_{avg} = 0.967). The average prediction error only increased from 0.286 log-cycles to 0.391 log-cycles. Figure 2 shows the comparison between experimental and predicted log reductions with low and randomly distributed prediction errors around the fit of the model.

Table 2. Statistical parameters for the fit of the kinetic models to the inactivation data of *E. coli* in orange juice treated by SC-CO_2 and HPU at three temperatures (31, 36 and 41 ºC, at constant P = 225 bar) and three pressures (100, 225 and 350 bar, at constant T = 36 ºC).

Treatment conditions		Statistics	Weibull	Gompertz	Biphasic	Log-linear	Type A	Type B
225 bar	31 ºC	R^2	0.916	0.752	0.943	0.881	0.961	0.902
		RMSE	0.587	0.932	0.446	0.590	0.367	0.587
225 bar	36 ºC	R^2	0.932	0.818	0.967	0.904	0.947	0.833
		RMSE	0.493	0.743	0.317	0.494	0.402	0.712
225 bar	41 ºC	R^2	0.978	0.987	0.999	0.934	0.989	0.993
		RMSE	0.366	0.226	0.015	0.363	0.214	0.168
100 bar	36 ºC	R^2	0.936	0.972	0.957	0.906	0.973	0.965
		RMSE	0.490	0.296	0.368	0.485	0.291	0.328
350 bar	36 ºC	R^2	0.957	0.906	0.914	0.940	0.963	0.950
		RMSE	0.429	0.586	0.560	0.429	0.370	0.429
		R^2_{avg}	0.944	0.887	0.967	0.906	0.966	0.929
		$RMSE_{avg}$	0.473	0.549	0.286	0.482	0.318	0.449

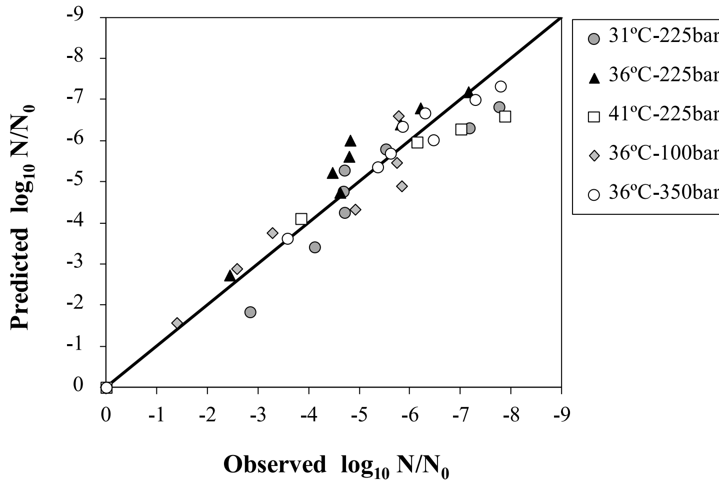

Fig. 2. Predicted (modified Biphasic model) against experimental *E. coli* inactivation data during SC-CO_2+HPU processing at various pressures (100-350 bar) and temperatures (31-41 ºC).

3.3. Combined effect of HPU and SC-CO_2 on *S. cerevisiae* inactivation

At different temperatures and pressures (Figure 3), the viability of *S. cerevisiae* began to decrease immediately and no lag-phase was observed for any condition studied. Figure 3A shows the inactivation for the three temperatures studied. On average, the inactivation rate at 31 ºC was significantly slower ($p<0.05$) than at 36 and 41 ºC, between which no significant ($p>0.05$) differences were observed. After 6 min at 31 ºC, an inactivation of 4 log-cycles was obtained, however for the other two temperatures, the total microbial inactivation (6.5-7 log-cycles) was reached in less than 3 min.

Regarding the inactivation of *S. cerevisiae* at different pressures (Figure 3B), the three survival curves showed a faster inactivation rate for the first minute, then a progressively slower decrease of the population was observed and total inactivation was obtained after 4, 1.5 and 2 min using 100, 225 and 350 bar, respectively. On average, the inactivation levels obtained at 100 bar were significantly lower ($p<0.05$) than those at 225 and 350 bar, between which no significant differences ($p>0.05$) were obtained.

The inactivation levels of *S. cerevisiae* inoculated in orange juice with SC-CO_2+HPU increased with pressure and temperature, although temperatures and pressures higher than 36 ºC and 225 bar, respectively, were not necessary to attain the total inactivation after 1-2 min of treatment.

The inactivation of *S. cerevisiae* by means of SC-CO_2 or HPU alone has previously been studied. Li et al. (2012) reduced the population of *S. cerevisiae* inoculated in bean sprout extract with a batch high pressure CO_2 system. The microbial reduction increased from 2.5 to 4.5 logs as the temperature rose from 25 to 35 ºC (100 bar, 120 min); and from 2.5 to 5 logs as the pressure went up from 100 to 300 bar (25 ºC, 120 min). Shimoda et al. (1998) studied the inactivation of *S. cerevisiae* with a continuous CO_2 system in phosphate buffer with an initial concentration of 10^8-10^9 CFU/mL. After 15 min of residence time at 35 ºC, 60 bar and 20 g CO_2/100 g sample, no survivors were found. Similarly to *E. coli,* the required times for the inactivation of *S. cerevisiae* with SC-CO_2+HPU are much shorter than in batch systems and are comparable or better than in continuous systems.

Different components, such as sugars, etc., lessen the effect of SC-CO_2+HPU during *S. cerevisiae* inactivation treatments in orange juice, compared to that in culture medium reported by Ortuño et al. (2013). In the latter, pressure and temperature increases were not needed for inactivation, since even low process parameters resulted in total inactivation. In the present study, the higher sugar content of orange juice resulted in temperature and pressure having a positive effect on the inactivation levels.

The application of HPU had a different effect against different microorganisms. It is known that Gram-positive cells are more resistant than Gram-negative ones due to their thicker cell wall (Ramirez Santos et al., 2005). It is also known that *S. cerevisiae* has a thicker cell wall, which makes it similar to Gram-positive bacteria (Villas-Boas et al., 2006). Comparing the results of the present study between *E. coli* and *S. cerevisiae*, at 31 ºC and 225 bar, a reduction of 7 and 4 logs was attained respectively, after 6 min of treatment. These results would support the connection between wall thickness and inactivation resistance (Ortuño et al., 2012a).

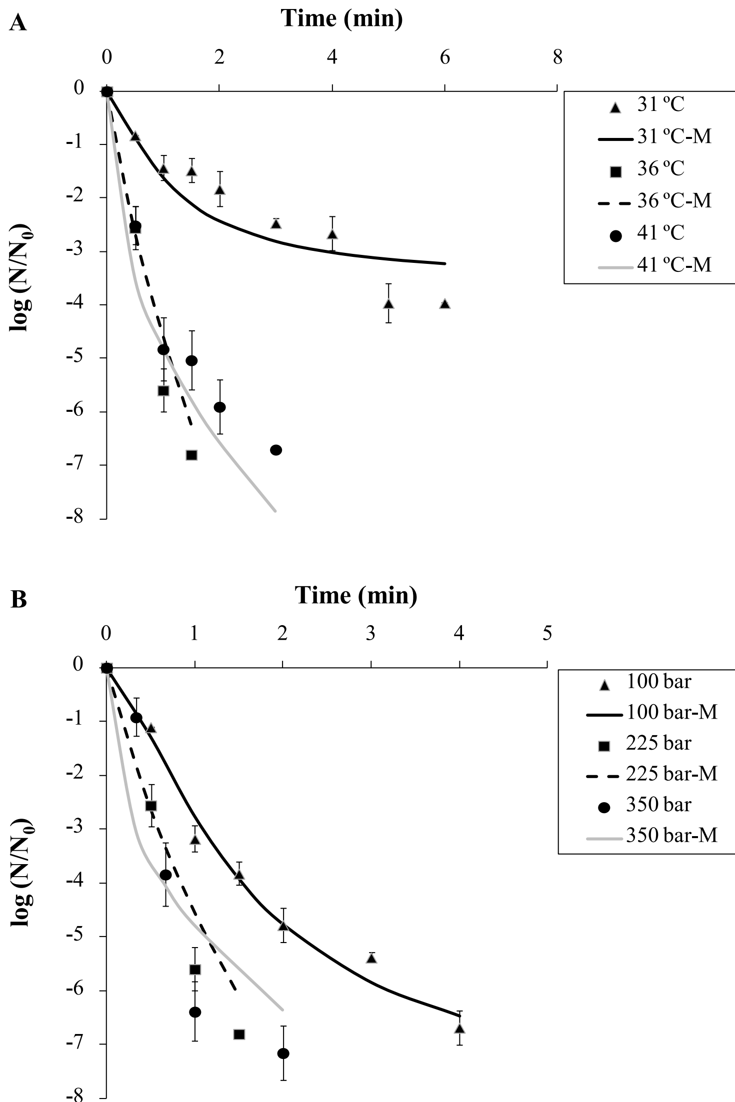

Fig. 3. Experimental data (discrete points) and modeling (M) of the inactivation kinetics of *S. cerevisiae* in orange juice treated by SC-CO_2+HPU at different temperatures (A, 225 bar) and different pressures (B, 36 ºC). M: modified Peleg Type B model.

Therefore, under the same process conditions, a slower *S. cerevisiae* inactivation was obtained. However, *E. coli* showed more resistance to

$SC\text{-}CO_2$+HPU treatments than *S. cerevisiae* for all the other pressure and temperature conditions. This fact could be related to the cavitation phenomenon and the microorganism's size. The *S. cerevisiae* cells, 8-10 µm (Laun et al., 2001) in size, are much bigger than *E. coli* cells, 1.2-2 µm (Nelson & Young, 2000); therefore, there is more likelihood that the cavitation bubbles might affect the cell structure of *S. cerevisiae* than that of *E. coli*.

The nature of the medium influenced the effect of HPU and, in addition to permitting observable effects of increasing temperatures and pressures, it also allowed observable differences between microorganisms.

3.4. Modeling of inactivation kinetics of *S. cerevisiae*

Table 3 shows the statistical parameters for the fit of the kinetic models to the inactivation data of *S. cerevisiae* in orange juice treated by $SC\text{-}CO_2$ and HPU. For all the models $R^2_{avg} > 0.94$ and the standard deviation of the differences between the values which were actually observed and those estimated by the model was below 0.5 log-cycles, with the exception of the Log-linear model ($R^2_{avg} = 0.768$; $RMSE_{avg} = 0.306$). The best fit was obtained by the Peleg Type B model ($R^2_{avg} = 0.983$; $RMSE_{avg} = 0.188$). A general equation was sought to describe the inactivation kinetics of *S. cerevisiae* obtained with $SC\text{-}CO_2$+HPU at any pressure and temperature over the range of these variables considered in the present study. The parameters of the Peleg Type B model, b_1, b_2 and r, were defined using a log-logistic equation that included (Peleg, 2006) a simultaneous pressure and temperature dependence (Eqs. (8-10)).

$$b_1(T,P) = \ln(1 + \exp(a_{b1}(T - Tc) + b_{b1}(P - Pc))) \quad \text{Eq. (8)}$$

$$b_2(T,P) = \ln(1 + \exp(a_{b2}(T - Tc) + b_{b2}(P - Pc))) \quad \text{Eq. (9)}$$

$$r(T,P) = \ln(1 + \exp(a_r(T - Tc) + b_r(P - Pc))) \quad \text{Eq. (10)}$$

where a_{b1}, b_{b1}, a_{b2}, b_{b2}, a_r, b_r, Tc and Pc are the characteristic constants of the microorganism.

Table 3. Statistical parameters for the fit of the kinetic models to the inactivation data of *S. cerevisiae* in orange juice treated by SC-CO_2 and HPU at three temperatures (31, 36 and 41 ºC, P = 225 bar) and three pressures (100, 225 and 350 bar, T = 36 ºC).

Treatment conditions		Statistics	Weibull	Gompertz	Biphasic	Log-linear	Type A	Type B
225 bar	31 ºC	R^2	0.983	0.942	0.969	0.976	0.975	0.980
		RMSE	0.158	0.264	0.193	0.158	0.178	0.158
225 bar	36 ºC	R^2	0.976	0.967	0.993	0.954	0.966	0.999
		RMSE	0.334	0.278	0.124	0.328	0.284	0.002
225 bar	41 ºC	R^2	0.953	0.977	0.994	0.909	0.979	0.982
		RMSE	0.439	0.266	0.140	0.431	0.253	0.234
100 bar	36 ºC	R^2	0.967	0.975	0.985	0.947	0.975	0.959
		RMSE	0.362	0.285	0.218	0.357	0.281	0.361
350 bar	36 ºC	R^2	0.847	0.849	0.925	0.589	0.840	0.993
		RMSE	0.965	0.784	0.550	1.290	0.806	0.172
		$\mathbf{R^2_{avg}}$	0.945	0.942	0.973	0.768	0.947	0.983
		$\mathbf{RMSE_{avg}}$	0.452	0.273	0.168	0.306	0.242	0.188

Substituting Eqs. (8-10) in the Peleg Type B model, a general expression of the model was obtained and used to predict the inactivation kinetics of *S. cerevisiae* in orange juice (Figure 3). The different characteristic constants of the *S. cerevisiae* inactivation model were calculated by minimizing the sum of square differences between all the experimental data and all the predicted data considered for every pressure and temperature condition studied, using the Excel Solver tool. The values of the coefficients, a_{b1}, b_{b1}, a_{b2}, b_{b2}, a_r, b_r, Tc and Pc, were: 9.788, 0.355, -0.157, -0.007, 1.929, 0.070, 3.523 and 973.078, respectively.

The value of R^2 = 0.894, indicate that the Type B model satisfactorily described the survival curves of *S. cerevisiae* (Figure 3). As expected, the statistical parameters of the general model showed a worse fit than the initial individual fits for each survival curve obtained at each temperature and pressure (Table 3: R^2_{avg} = 0.983). The error in the estimation increased from 0.188 log-cycles to 0.687 log-cycles. However, according to the R^2 and RMSE values, the proposed model appropriately described the inactivation kinetics of *S. cerevisiae* under SC-CO_2+HPU treatment as a function of temperature, pressure and time of treatment, over the practical range of 100-350 bar and 31-41 ºC. Figure 4 shows

the correlation between the experimental and predicted log reduction values. For low microbial reductions, between 0 and 3 logs, the modified Type B model predicted higher values. The highest deviation value occurred at 350 bar, 36 ºC and 0.33 min of treatment time and is equal to 2.10 log.

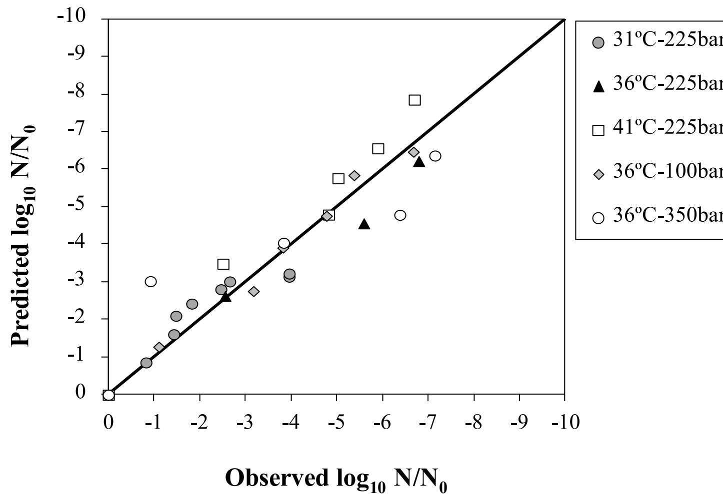

Fig. 4. Predicted (modified Peleg Type B model) against experimental *S. cerevisiae* inactivation data during SC-CO_2+HPU processing at various pressures (100-350 bar) and temperatures (31-41 ºC).

From these results, it could be concluded that the survival models that have previously been used to describe microbial inactivation by means of other non-thermal technologies, such as SC-CO_2 or HPU alone, have appropriately predicted the SC-CO_2+HPU inactivation kinetics of *E. coli* and *S. cerevisiae*.

3.5. Combined effect of HPU and SC-CO_2 on pectin methyl-esterase inactivation.

Figure 5 shows the inactivation of orange juice PME after three SC-CO_2+HPU treatments. The RA of PME decreased as the treatment time increased (Figure 5A), and the higher the temperature, the greater the RA decrease. The effect of temperature was noticeable from the beginning of the process; after 2 min, the RA was 83.63, 81.01 and 50.46 % at 31, 36 and 41 ºC,

respectively. No significant differences ($p>0.05$) were observed in the RA values at 31 and 36 ºC, which decreased until reaching an average 47.5 % after 10 min of treatment. At 41 ºC, however, a significantly faster ($p<0.05$) inactivation was observed when compared to what occurred at 31 and 36 ºC. The lowest value of RA after 10 min of treatment was 10.65 %.

The effect of pressure was not as pronounced as that of temperature (Figure 5B). After 2 min of treatment, no significant differences ($p>0.05$) were found between the pressures studied: on average, 80 % RA was attained. No significant differences ($p>0.05$) were found between 100 and 225 bar: on average, the RA reached 54.2 % after 10 min of treatment. The highest level of pressure studied, 350 bar, produced significantly different ($p<0.05$) results compared to 100 and 225 bar. At 350 bar, after 8 and 10 min of treatment, the % of RA was 32.38 and 15.90 %, respectively.

The inactivation of PME by means of SC-CO_2 or HPU has previously been explored. Balaban et al. (1991) studied the degree of inactivation of PME in orange juice with a batch SC-CO_2 system. Similarly to what occurred in this study, these authors decreased the % RA as the temperature and pressure increased; furthermore, although the inactivation degree reached at 44 ºC and 269 bar after 50 min, 30 %, was similar to the 32% obtained in the present study at 36 ºC and 350 bar after 8 min of treatment, when using the SC-CO_2+HPU system, lower temperatures and much shorter process times were necessary. Fabroni et al. (2010) investigated the inactivation of PME in blood orange juice with a continuous SC-CO_2 system. They obtained an RA of 33.19% and 40.88 %, using 230 and 130 bar (36 ºC, 15 min), respectively. Similar values of RA have been obtained in this study in shorter process times at lower temperatures: an RA of 46 % was attained after 10 min of SC-CO_2+HPU treatment at 225 bar and 31 ºC.

Therefore, it may be concluded that in a batch SC-CO_2+HPU system at lower pressures and temperatures, shorter process times can be used compared to batch and continuous SC-CO_2 systems, which would contribute to the preservation of the nutritional value and desirable sensory characteristics of orange juice.

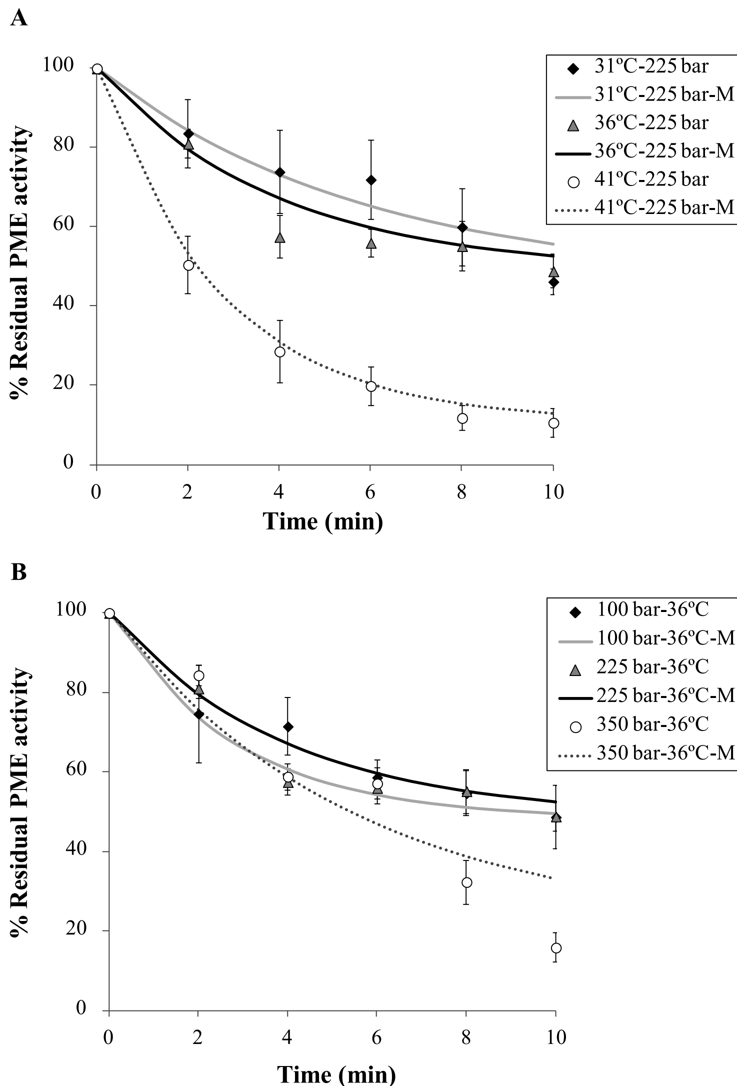

Fig. 5. Experimental data (discrete points) and modeling (M) of the inactivation kinetics of pectin methyl-esterase in orange juice treated by SC-CO_2+HPU at different temperatures (A, 225 bar) and different pressures (B, 36 ºC). M: modified Fractional model.

The mechanisms associated with the inactivation of enzymes are those linked to the denaturation of proteins. Enzymes are folded three-dimensionally, determined by covalent, hydrophobic and ionic intra-molecular forces (Rezaei et al., 2007). The inactivation of enzymes is associated with the fragmentation or

modification of their secondary and tertiary structure; therefore, any mechanism that might affect the structure of enzymes can cause their denaturation.

The inactivation of enzymes exposed to SC-CO_2 treatments can be explained by different mechanisms, such as the lowering of the pH, the inhibitory effect of molecular CO_2 on enzyme activity and the fact that SC-CO_2 causes conformational changes (Balaban, 2012). Treatments with high pressure CO_2 are accompanied by a lowering of the pH because of the formation of carbonic acid from the dissolution of CO_2 in water and under a lower pH environment, protein bound arginine can easily interact with CO_2, forming a bicarbonate complex (Razaei et al., 2007). Therefore, in addition to its pH-lowering effect, CO_2 may directly bind to the enzyme and cause a loss of activity. Moreover, the inactivation of enzymes exposed to SC-CO_2 treatment can be explained by the fact that SC-CO_2 causes conformational changes in the secondary and tertiary structure. Ishikawa et al. (1996) reported that several enzymes, such as lipase, alkaline protease, acid protease and gluco-amylase, were inactivated and their α-helix structures were decomposed after SC-CO_2 treatment.

In the present study, PME was inactivated more quickly in orange juice by applying SC-CO_2 and HPU simultaneously, despite using lower pressures and temperatures and shorter process times than with the single SC-CO_2 or ultrasound treatments reported in other works. The synergistic effect of SC-CO_2+HPU accelerates the solubilization rate of SC-CO_2 into the liquid and the increase in the mass transfer due to the vigorous agitation produced by the ultrasonic field results in the quick saturation of CO_2 in the medium, which accelerates the inactivation mechanisms. The cavitation generated by HPU could contribute to the change in the conformation of the enzyme, accelerating its inactivation.

Comparing the SC-CO_2+HPU inactivation of *E. coli*, *S. cerevisiae* and PME, the enzyme needed longer process times to be inactivated and its total inactivation was not attained in any of the process conditions. This could be attributed to the different nature and size of microorganisms and enzymes.

3.6. Modeling of the pectin methyl-esterase inactivation kinetics

The data obtained for each pressure and temperature condition in the inactivation of PME was fitted to two previously described mathematical models: the fractional conversion model and the Weibull model. Table 4 shows the statistical parameters for the fit of the kinetic models to the inactivation data of PME in orange juice treated by SC-CO_2 and HPU. On average, both models adequately fitted the inactivation kinetics, $R^2_{avg} > 0.9$; $RMSE_{avg} < 0.07$. The best fit was provided by the fractional model ($R^2_{avg} = 0.95$; $RMSE_{avg} = 0.067$).

In order to obtain an estimation of the pectin-methyl esterase inactivation at any pressure and temperature, the equation developed by Polydera et al. (2004) was used to select and modify the fractional model (Eq. 11), including the dependence of parameter k (Fractional model, Table 1) on pressure and temperature.

$$\frac{A - A_f}{A - A_0} = e^{-t\, K_{P,Tref}\, e^{\frac{-E_{aP}}{R} e^{\left(-w\,(P-P_{ref})\right)\left(1/T - 1/T_{ref}\right) - \frac{\left[\left(z\,(T-T_{ref})\right)+V_{aTref}\right]}{R}\frac{\left(P-P_{ref}\right)}{T}}}} \quad \text{Eq. (11)}$$

where w is a kinetic parameter, $k_{P,Tref}$ the inactivation rate at T_{ref} (304 K), E_{aP} is the activation energy at P_{ref} (100 bar), z is a kinetic parameter, T_{ref} is the reference temperature (304 K), V_{aTref} is the activation volume at T_{ref}, R the universal gas constant (8.314 J mol^{-1} K^{-1}). P_{ref} and T_{ref} were selected as the lowest values of each range studied.

The different characteristic constants of the modified model were calculated by minimizing the sum of square differences between all the experimental data and all the predicted data considered for every pressure and temperature condition studied, using the Excel Solver tool. The value of the coefficients were: $w = 2.196 \times 10^{-7}$ bar^{-1}, $k_{P,Tref} = 0.201$ min^{-1}, $E_{aP} = 85.873$ kJ mol^{-1}, $z = 0.704$ mL $min^{-1}K^{-1}$ and $V_{aTref} = 3.124$ mL mol^{-1}. The statistical parameters obtained, $R^2 = 0.931$; $RMSE = 0.085$, were comparable with the individually obtained fit for each pressure and temperature condition studied ($R^2_{avg} = 0.95$; $RMSE_{avg} = 0.067$). Figure 5 shows the modeling of the inactivation kinetics of PME in orange juice by SC-CO_2+HPU.

Table 4. Statistical parameters for the fit of the kinetic models to the inactivation data of pectin methyl-esterase in orange juice treated by SC-CO_2 and HPU at three temperatures (31, 36 and 41 ºC, P = 225 bar) and three pressures (100, 225 and 350 bar, T = 36 ºC).

Treatment conditions		Statistics	Weibull	Fractional
225 bar	31 ºC	R^2	0.942	0.926
		RMSE	0.023	0.085
225 bar	36 ºC	R^2	0.909	0.964
		RMSE	0.030	0.066
225 bar	41 ºC	R^2	0.989	0.998
		RMSE	0.032	0.014
100 bar	36 ºC	R^2	0.979	0.968
		RMSE	0.013	0.059
350 bar	36 ºC	R^2	0.802	0.892
		RMSE	0.107	0.111
		R^2_{Avg}	0.924	0.950
		$RMSE_{Avg}$	0.041	0.067

Figure 6 shows the correlation between the experimental and predicted values obtained by means of Eq. (11). The model properly predicted the experimental RA between 0 and 50 % and for values higher than 80 %; the estimation was slightly poorer from 50 to 80 %. The figure reveals that the highest deviation value occurs at 350 bar, 36 ºC and 8 min of treatment time. All the other treatment conditions fitted using Eq. (11) provided low deviation values. The proposed model provided a satisfactory correlation between experimental and predicted values of % RA in the practical range of 100-350 bar and at 31-41 ºC for SC-CO_2+HPU treatments. Therefore, it has been demonstrated that the fractional model that provided good results for the modeling of PME inactivation with SC-CO_2, also provided good results when HPU is simultaneously applied in an SC-CO_2 treatment.

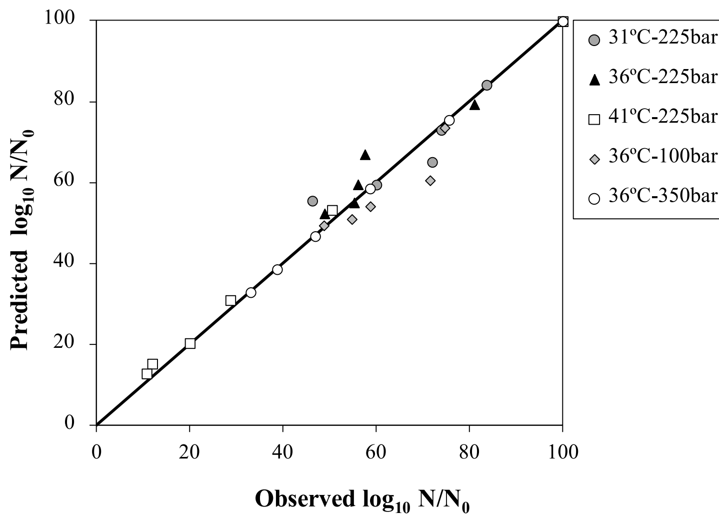

Fig. 6. Predicted (modified Fractional model) against experimental % RA of PME data during SC-CO_2+HPU processing at various pressures (100-350 bar) and temperatures (31-41 ºC).

4. CONCLUSIONS

The application of HPU enhanced the SC-CO_2 inactivation mechanisms and reduced the treatment time needed to achieve a required level of inactivation. HPU leads to a vigorous agitation that would accelerate the SC-CO_2 inactivation mechanisms. The cavitation generated by HPU could damage the microorganism's cell wall and could also change the conformation of the enzymes, accelerating their inactivation.

A rise in pressure or temperature increased the inactivation rate of *E. coli*, *S. cerevisiae* and PME, and the nature of the medium influenced how increasing the pressure and temperature affected the inactivation rate.

HPU had a different effect on the SC-CO_2 inactivation of different microorganisms. The lower resistance showed by *S. cerevisiae* could be related to the fact that they are bigger than *E. coli* cells. The cavitation bubbles might produce a greater effect on the cell structure of *S. cerevisiae* than on that of *E. coli*. The SC-CO_2+HPU inactivation of PME required longer process times

than for microorganisms, and total inactivation was not achieved for any condition.

Models were developed to describe the inactivation kinetics of microorganisms and enzymes at different pressures and temperatures.

It is recommended that more research be conducted to elucidate the effects of the viscosity and water-binding of the treatment media on the SC-CO_2-HPU inactivation treatments as well as to study the effect of applying HPU in a continuous system on the microbial inactivation.

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Chapter 6

Combined High Hydrostatic Pressure and Carbon Dioxide Inactivation of Pectin Methylesterase, Polyphenol Oxidase and Peroxidase in Feijoa Puree

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C. Ortuño, T. Duong, M. Balaban, J. Benedito

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Combined High Hydrostatic Pressure and Carbon Dioxide Inactivation of Pectin Methylesterase, Polyphenol Oxidase and Peroxidase in Feijoa Puree

C. Ortuño[a], T. Duong[b], M. Balaban[b], J. Benedito[a]

[a]Department of Food Technology, Universitat Politècnica de València, Camí de Vera s/n, E 46022, Valencia, Spain.
[b]Department of Chemical and Materials Engineering, University of Auckland, Auckland 1142, New Zealand.

ABSTRACT

A combined treatment of high hydrostatic pressure (HHP) and dense phase carbon dioxide (DPCD) was investigated to inactivate pectin methylesterase (PME), peroxidase (POD) and polyphenol oxidase (PPO) in feijoa (*Acca sellowiana*) puree. The treatments were HHP (HHP); carbonation and HHP (HHPcarb); carbonation + addition of 8.5 mL CO_2/g puree into the headspace of the package and HHP (HHPcarb+CO_2). The different samples were treated at 300, 450 and 600 MPa, for 5 min. The residual POD and PPO activity decreased in the order HHP > HHPcarb > HHPcarb+CO_2 at all pressures used. Treatments with HHP at 300 MPa increased POD activity to 140 %. The residual PME activity of HHPcarb and HHPcarb+CO_2 samples at 600 MPa (45-50 %) was significantly ($p<0.05$) lower than for HHP treatment (65 %). The simultaneous application of HHP and DPCD seems to synergistically enhance the inactivation of the enzymes studied, the CO_2 concentration being a key process factor.

Key words: High hydrostatic pressure, carbon dioxide, enzymes, residual activity, synergistic effect

1. INTRODUCTION

Enzymes and microorganisms in foods cause quality deterioration and spoilage during storage and distribution. In the food industry, non-thermal processing alternatives have been developed in response to an increasing consumer demand for fresh-like and high quality food products. These technologies aim to economically produce safe, nutritious, and tasty foods using less severe processing conditions (Cortes et al., 2008; Oey et al., 2008; Ferrentino et al., 2009).

The application of high hydrostatic pressure (HHP) allows the inactivation of undesirable enzymes (Daryaei et al., 2012) in liquid and solid food systems, without altering their quality to the same extent as thermal treatments and with a comparable preservation effect. Park et al. (2002) reported that by increasing the pressure in HHP treatments (25 ºC-5min) from 200 to 600 MPa, the residual activity of polyphenol oxidase (PPO), lipoxigenase (LOX) and pectin methylesterase (PME) in carrot juice decreased from 83 %, 78 % and 80 % to 10 %, 30 %, and 45 %, respectively. Nevertheless, some undesirable enzymes, such as PPO and some isozymes of PME, are highly pressure resistant (Eisenmenger et al., 2009). In this case, higher temperatures are needed to inactivate these enzymes, thereby negating the non-thermal advantages of HHP process.

Similarly, DPCD has been reported to inactivate different microorganisms in liquid foods (Ferrentino et al., 2009; Corradini & Peleg, 2012; Ortuño et al., 2012, 2013) without exposing them to the adverse effects of heat which allows retain their fresh-like physical, nutritional, and sensory properties (Damar & Balaban, 2006). Similarly to HHP, DPCD has also been proven effective in inactivating many undesirable enzymes, including PPO (Pozo-Insfran et al., 2006; Liu et al., 2008), peroxidase (POD) (Liu et al., 2008), and PME (Arreola et al., 1991; Zhou et al., 2009). However, in some cases the inactivation level was less than satisfactory (Cano et al., 1997; Garcia-Palazon et al., 2004).

Therefore, there is increasing interest in process intensification, with simultaneous application of different non-thermal technologies, seeking for synergistic effects. In this regard, DPCD could be a good candidate to enhance

the effect of HHP processing. It is well known that the effect of HHP is enhanced at lower pH, moreover, it is assumed that CO_2 could dissolve in the hydration layer associated with the enzyme and could decrease the local pH (Tedjo et al., 2000), therefore the presence of CO_2 in sample medium might create an acid environment, and positively interact with pressure to destroy or damage the structure of enzymes. Few studies have shown synergistic effects of combining DPCD and HHP process on inactivation of PPO, LOX and PME enzymes in orange (Corwin & Shellhammer, 2002) and carrot (Park et al., 2002) juice. Corwin & Shellhammer (2002) first carbonated enzyme preparations at atmospheric pressure, then treated them with HHP. They showed that CO_2 had an additional inactivation effect on PME at 500 MPa. Park et al. (2002) reported that a sequential application of DPCD at 4.9 MPa (5 ºC-5 min) and HHP at 200 MPa (25 ºC-5 min) improved the inactivation of the PPO, LOX and PME enzymes in carrot juice with a residual activity of 35 %, 17 % and 45 %, respectively, compared with the residual activity of DPCD (40 %, 20 % and 50 %, respectively) and HHP (83 %, 78 % and 80 %, respectively) treatments.

The extension of atmospheric carbonation could be to add gaseous CO_2 into the headspace of the packaged liquid food before HHP treatment. The CO_2 in the headspace could dissolve into the sample during the HHP treatment and the CO_2 concentration inside the sample could be higher than in carbonated samples. Therefore, the effect associated to CO_2, like the acidification of sample, could be increased, improving the CO_2 effects compared with only carbonated samples. No references have been found in the literature covering simultaneous application of HHP and DPCD techniques involving additional gases in the package for either enzymatic or microbial inactivation purposes.

Feijoa (*Acca sellowiana*), an exotic fruit in New Zealand, has many desirable nutritional characteristics such as good source of vitamin C, low in calories and high in minerals and fiber, and interesting bioactive components such as high antioxidant activity, high phenolics and phytochemicals content (Weston, 2010). Therefore, the preservation of feijoa products by non-thermal technologies is advantageous to retain these desirable characteristics.

The objective of this study was to determine the effect of different levels of added carbon dioxide in a package on the efficiency of HHP treatment to inactivate POD, PPO and PME at different pressures in feijoa puree.

2. MATERIAL AND METHODS

2.1. Raw material

The feijoa (*A. sellowiana*) was supplied by Frans and Tineke de Jong grower, Southern Belle Orchards (Matamata, Waikato), New Zealand. 15 kg of feijoa were stored at room temperature until they started ripening and released a sweet aroma volatile, and then they were put into storage at 4°C for 2-3 days, time necessary to perform the chemical-physical analyses. The fruit that was not used for the chemical-physical analysis was cleaned, peeled and chopped, put in Ziploc bags and stored at -20 ºC until required for the preparation of samples for the inactivation treatments.

2.2. Chemical-physical analysis of feijoa

For the chemical-physical analysis, 30 feijoa pieces were randomly selected. Color, pH and firmness were determined directly on the fruit. Afterwards, a puree was made using the same feijoa fruits, and the moisture, ºBrix and water activity, were determined.

2.2.1. Color determination

Color assessment was conducted at 25 ºC using a CR400-Chroma Meter Colorimeter (Konica Minolta, USA) in CIE L*a*b* color space system after calibration with the reference tile. The fruit color was measured in 9 different sites of the fruit (3 readings around each end of fruit and 3 at the equator) and averaged. 10 fruits from the 30 previously selected were measured and a total of 90 readings were done.

2.2.2. pH

The pH was measured directly inside the feijoa fruit at 25 ºC using a digital pH meter (PerpHec LogR meter, model 320, Orion research Inc., USA) and pH was recorded after stabilization, for 30 selected fruit.

2.2.3. Texture analysis

The firmness of fresh feijoa (Table 1) was measured using a universal texture analyzer (TA.XT Plus Texture Analyser, Stable Micro Systems Ltd., UK) linked to a computer for data acquisition and processing (Exponent software, Stable Micro System Ltd., UK), using a small cylindrical probe (10 mm diameter). The maximum force (firmness, N) was measured and computed with a test speed of 0.03 mm/s and travel distance of 5 mm down on the fruit surface, at the center of its equator and at each side of the fruit (2 punctures per side). 30 pieces of fruit were measured.

2.2.4. Moisture content

The moisture content of fresh feijoa puree was determined using the official method (AOAC, 1997) for a vacuum oven. 5 g of fresh feijoa puree were accurately weighed and placed on a ceramic crucible, dried at 70 ºC and 10 mmHg vacuum for 24 h in a vacuum oven (VT 6205, Haraeus Vacutherm, Germany). The vacuum was released slowly and the dried samples were stored in desiccators at ambient temperature prior to weighing by an analytical balance (ED224S, Sartorius Ag, Germany). The moisture analysis was conducted in triplicate. The moisture content (Table 1) of the feijoa was calculated using the following equation:

$$\text{Moisture content (\%)} = \frac{\text{Total moisture loss after drying (g)}}{\text{Inital weight (g)}} \times 100 \qquad \text{Eq. (1)}$$

2.2.5. ºBrix

The ºBrix of fresh feijoa puree (Table 1) was measured in triplicate at 25 ºC using E-Line ATC range 0-18 ºBrix refractometer (Bellingham + Stanley Ltd., UK).

2.2.6. Water activity

The water activity of fresh feijoa puree was measured in triplicate at 25 ºC using a digital water activity meter (Aqua Lab 4TE, Decagon Devices, USA). The water activity of the fresh feijoa puree was 0.9901±0.0018.

Table 1. Moisture content, ºBrix, pH, firmness, and color of fresh feijoa.

	% Moisture	º Brix	pH	Firmness (N)	Colour		
					L*	a*	b*
Fresh Feijoa	83.33±0.30	11.8±0.8	3.30±0.02	20.67±3.87	54.56±2.56	-8.32±2.07	14.01±3.26

All data shown are means±SD.

2.3. Sample preparation and storage

The frozen fruit was thawed at 4 ºC for 12-14 h before processing. Thawed feijoa were blended (Laboratory blender, Model 38BL40, Waring Commercial, USA), until well mashed and mixed into a puree. 30 g portions of feijoa puree were poured into plastic bags (155 mm x 180 mm x 30 mm, SURT155180, Cas-Pak Products Ltd., New Zealand), vacuum sealed (Vacutherm, VT 6205, Germany) and stored at -20 ºC until required.

2.4. Sample treatment

2.4.1. CO_2 treatment

The frozen feijoa puree was thawed in the bag at 4 ºC for 12-14 h before processing. Three different CO_2 levels were considered in this study. Feijoa puree without CO_2 (HHP); carbonation at 1 atm (HHPcarb); carbonation and addition of 8.5 mL CO_2/g puree into the headspace of the package (HHPcarb+CO_2). The carbonation of samples was carried out by bubbling CO_2 at atmospheric pressure at 1.28 L/min from the bottom of the puree for 5 min at 0-3 ºC by placing the bags of puree in an ice water bath and manually and vigorously agitating to facilitate mass transfer. The bags were immediately sealed without gas loss and were placed on ice until HHP treatment.

2.4.2. High pressure processing

The HPP unit used in this study was Avure 2 L Food Processor (Avure Technologies, Columbus, Ohio, USA). The equipment can operate at a maximum pressure and temperature of 600 MPa and 90 ºC, respectively. The equipment consists of a cylindrical pressure treatment chamber, a pumping system, water circulation and the control system operated through a personal computer with software supplied by the manufacturer. Water was the working fluid in the

pressure chamber where the packaged puree was placed. The temperature history of the water in the chamber was recorded by two thermocouples during processing.

For each pressure run, 3 bags (1 HHP sample, 1 HHPcarb sample and 1 HHPcarb+CO_2 sample) were treated together in the hydrostatic pressure processing unit (HPP). The pressure levels used were 300, 450 and 600 MPa, for 5 min. It is generally agreed that pressures lower than 300 MPa do not have much deactivating effect on enzymes in a process with only HHP (Hendrickx et al., 1998). The process time selected was 5 min in order to reduce the cost of the process and to increase its industrial applicability. Pressure come up times were approximately 0.5 min and 1.5 min to reach 300 MPa and 600 MPa, respectively. Depressurization occurred in less than 2 s. The starting temperature of samples was 25 ºC. The maximum temperature reached at 600 MPa runs was 42 ºC.

Two replicates of each run were carried out for each pressure condition tested. The plastic bags were frozen after treatment at -70 ºC and thawed before enzyme analysis.

2.5. Analysis of treated samples

The treated frozen puree was thawed at 4 ºC for 12-14 h before the analysis. Moreover, feijoa puree without CO_2 and HHP treatments was subjected to the same freezing and thawing processes and it was used as a control sample.

2.5.1. pH

The pH of the puree was measured in triplicate in the control sample and in the treated samples before the enzyme analysis. For the samples with CO_2 (HHPcarb and HHPcarb +CO_2) the puree was decarbonated previously to the pH measurement by agitation under vacuum (10 mmHg, 25 ºC).

2.5.2. Color determination

Color assessment was conducted at 25 ºC in CIE L*a*b* color space system after calibration with the reference tile. The color of control puree was measured in triplicate prior to the enzymes analysis (after the freezing and thawing processes). The color of treated samples was measured in triplicated after the

treatment, just before to the enzymes analysis. Chroma (C*) and hue angle (H°), and total color difference (ΔE) (with respect to control sample after the freezing and thawing processes) were also calculated.

$$\Delta E = [(L^* - L^*_0)^2 + (a^* - a^*_0)^2 + (b^* - b^*_0)^2]^{1/2} \quad \text{Eq. (2)}$$

$$C^* = (a^{*2} + b^{*2})^{1/2} \quad \text{Eq. (3)}$$

$$H^o = \arctan(b^*/a^*) \quad \text{Eq. (4)}$$

where L*: lightness of treated sample at time t; L^*_0: lightness of reference sample; a*: redness of treated sample at time t; a^*_0: redness of reference sample; b*: yellowness of treated sample at time t; and b^*_0: yellowness of reference sample.

2.5.3. PPO and POD assay

The frozen puree was thawed at 4 ºC for 12-14 h before the analysis. 10 g of feijoa puree was homogenized (Laboratory blender, Model 38BL40, Warning Commercial, USA) with 30 mL of 0.05 M potassium phosphate buffer solution, at 13000 rpm, for 2 min. The slurries were centrifuged (SA600 rotor, Sorvall RC28S supraspeed centrifuge, Du Pont Company, USA) at 10000 rpm for 10 min at 4 °C, and the supernatant was filtered through filter paper (Whatman #2) using a suction flask. The pellet was re-extracted and centrifuged. The filtrates of the two extractions were combined and centrifuged at 10000 rpm for 15 min. The supernatant was used to test enzyme activity.

PPO and POD activities were assayed by the method described by Chen et al. (2010) with some modifications. PPO assay medium contained 0.4 mL of the sample and 2.6 mL of substrate solution (1.3 mL 0.05 M sodium phosphate buffer, pH = 6.8, added to 1.3 mL 0.02 M catechol solution); to the blank 0.4 mL of distilled water, instead of sample, was added. POD assay medium contained 0.2 mL of the sample with 3 mL of substrate solution (3 mL of 30 % hydrogen peroxide added to 1.9 mL of liquid guaiacol, made up to 300 mL with 0.2 M sodium phosphate buffer, pH = 6); to the blank 0.2 mL of distilled water, instead of sample, was added.

The increase in absorbance at 420 nm (PPO) or 470 nm (POD) was monitored at intervals of 5 s immediately after the addition of sample to the corresponding substrate solution using an UVmini-1240 spectrophotometer (Shimadzu, Tokyo Japan) at ambient temperature. One unit of specific PPO or POD activity was defined as the change per min and milliliter of sample in the absorbance measured at 420 nm or 470 nm, respectively. The residual activity of each enzyme was obtained using the following equation:

$$\text{PPO (POD) residual activity} = \frac{\text{Specific activity PPO (POD) after treatment}}{\text{Specific activity PPO (POD) control sample}} \text{x } 100 \qquad \text{Eq. (5)}$$

2.5.4. PME activity measurement

Before the PME activity was evaluated, the puree was decarbonated by agitation under vacuum (10 mmHg, 25 ºC). PME activity was determined as described by Castaldo et al. (1997) with some modifications. The substrate solution was prepared by dissolving 10 g of pectin powder (Sigma Chemical Co., St. Louis, MO) in 1 L of 0.15 M NaCl. The NaCl solution was heated to 50-55 ºC and added in the blender while pectin powder was sprinkled on the surface and blended. Pectin solution was stored at 4°C until required.

The pH of pectin solution was adjusted to 7 prior to each analysis and 4 mL of feijoa puree were added into 12 mL of pectin solution. The pH was quickly adjusted to 7 (1 M NaOH for gross adjustment, 0.05 M NaOH for fine adjustment), and PME activity was measured by recording the decrease of pH every 5 s until pH dropped to 6.5. One unit of specific PME activity was defined as the slope of pH vs time in min. The residual activity of PME was calculated using the following equation:

$$\text{PME residual activity} = \frac{\text{Specific activity PME after treatment}}{\text{Specific activity PME control sample}} \text{x } 100 \qquad \text{Eq. (6)}$$

2.6. Statistical analysis

All treatment conditions were duplicated and analyses triplicated. Using the statistical package Statgraphics Plus (Statistical Graphics Corp. 5.1, Warrenton, USA), simple ANOVA and a two-way ANOVA were carried out and LSD (least significant differences) were identified, in order to evaluate the effect of pressure,

CO_2 level and the possible interaction between factors, on the residual PPO, POD and PME activity of treated samples.

A two-way ANOVA was carried out in order to evaluate the effect of pressure and CO_2 level on the pH, and color parameters of the treated samples, compared with the control sample.

3. RESULTS

3.1. POD activity

Fig. 1 shows the effect of 3 types of treatments on residual POD activity. In the HHP treatments, at 300 MPa for 5 min the residual POD activity of feijoa puree increased to 140±5 %. With further increase in pressure, the residual POD activity significantly ($p<0.05$) decreased to 60±9 % and 22±13 % at 450 MPa and 600 MPa, respectively. The addition of CO_2 had a significant effect ($p<0.05$) on the residual POD activity for all the pressures tested. At 300 MPa, the residual POD activities in HHPcarb decreased to 32±7 % compared with 140±5 % of HHP alone; in HHPcarb+CO_2 samples the residual activity drooped to a value of 13±8 %. At 450 MPa and 600 MPa, the residual POD activities in HHPcarb samples were 9±1 % and 10±0.02 %, respectively while in HHPcarb+CO_2 samples were 27±7 % and 6±1 %, respectively. In the samples with CO_2 in the headspace of the package (HHPcarb+CO_2), the residual POD values were by 60 and 45 % lower than in HHPcarb samples at 300 and 600 MPa. Moreover, the addition of gaseous CO_2 in the bag resulted in a residual activity at 300 MPa (13±8 %) that could only be obtained at 600 MPa with high pressure alone (22±13 %).

From the two-way AVOVA it was observed that the residual POD activity obtained at the different pressures significantly decreased ($p<0.05$) in the order HHP (78 $\%_{avg}$) > > HHPcarb (45 $\%_{avg}$) > HHPcarb+CO_2 (25 $\%_{avg}$). On the other hand, for the different CO_2 levels, the residual POD activity was significantly lower ($p<0.05$) as pressure increased: 300 MPa (80 $\%_{avg}$) > 450 MPa (29 $\%_{avg}$) > 600 MPa (13 $\%_{avg}$). These results indicate that, the combined HHP and DPCD processing of feijoa puree had a significant effect on the residual POD activity and this effect was higher with increasing treatment pressure and CO_2 level.

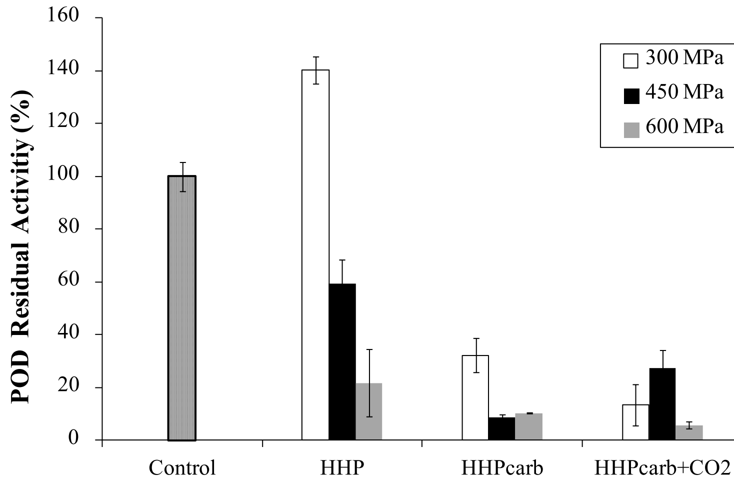

Fig. 1. Residual POD activity in feijoa puree after HHP, HHPcarb and HHPcarb+CO_2 treatments at different pressures (initially at room temperature, 5 min). All data shown are means±SD.

No references have been found in the literature regarding the inactivation of POD in feijoa puree with HHP or HHP+DPCD. Garcia-Palazon et al. (2004) observed residual POD activity in strawberry puree in the range of 11-35 % after 15 min of HHP treatment (600 MPa) at ambient temperature. In another study, no significant inactivation of strawberry POD was observed after 15 min HHP treatment of the puree at pressures ranging from 50 to 400 MPa and temperatures ranging from 20 to 60 °C (1997). DPCD treatment of red beet extract at 37.5 MPa (55 ºC, 60 min) resulted in a reduction of POD activities by approximately 76% (Liu et al., 2008). Other studies suggest that DPCD treatment increases or slightly reduces the POD activity in crude vegetable enzymatic extracts (Fricks et al., 2006; Primo et al., 2007). However, in the present study an increase of POD activity was only observed after 5 min of HHP at 300 MPa, and all HHP+DPCD treatments resulted in a decrease of the POD activity. Based on the results of this study, the addition of CO_2 in the sample allows lower pressures and shorter process times to obtain similar residual POD activities either with HHP or DPCD alone.

3.2. PPO activity

The inactivation of PPO in feijoa puree subjected to HHP, HHPcarb and HHPcarb+CO_2 treatments at different pressures is illustrated in Fig. 2.

The residual PPO activity for HHP, HHPcarb and HHPcarb+CO_2 samples treated at: 300 MPa were 102±8 %, 85±2 % and 56±5 %, respectively; 450 MPa were 47±4 %, 42±6 % and 42±1 %, respectively; 600 MPa were 38±5 %, 44±4 % and 26±3 %, respectively.

On average, the residual PPO activity obtained at different pressures showed a significantly ($p<0.05$) lower value in the HHPcarb+CO_2 samples (52 $\%_{avg}$), compared to HHP (68 $\%_{avg}$) and HHPcarb (62 $\%_{avg}$) between which no significant differences ($p>0.05$) were found. For the different CO_2 levels, on average, the residual PPO activity was significantly lower ($p<0.05$) as pressure increased in the order 300 MPa (81 $\%_{avg}$) > 450 MPa (45 $\%_{avg}$) > 600 MPa (36 $\%_{avg}$). Therefore, similar to the POD, the addition of CO_2 into the headspace of the package allows obtaining higher inactivation levels of PPO when HHP is applied, for all the pressures studied, compared with only HHP or with HHPcarb treated samples.

No treatment combination could fully inactivate PPO. This result was similar to that obtained by Park et al. (2002) using HHP alone, who observed that the residual PPO activity of carrot juice decreased from 83 % to 10 % as pressure increased from 200 to 600 MPa (25 ºC, 5 min). In a sequential application of DPCD (4.9 MPa, 25 ºC, 5 min) and HHP (200 MPa, 5 min) the residual PPO activity in carrot juice decreased to 35 %, compared with 83 % using HHP only (Park et al., 2002). Corwin & Shellhammer (2002) reported that the percent residual PPO activity in carbonated 0.1 M phosphate buffer (pH = 6.5) treated by HHP (500 MPa, 25 ºC, 3 min) was 59.8 %, compared with 98.5 % after HHP alone. Using carbonated 0.1 M phosphate buffer and HHP at 800 MPa, 25ºC for 1 min, the remaining PPO activity was 21.7 % (Corwin & Shellhammer, 2002), similar to residual activity obtained in this study in HHPcarb+CO_2 at 600 MPa for 5 min, 26±3 %.

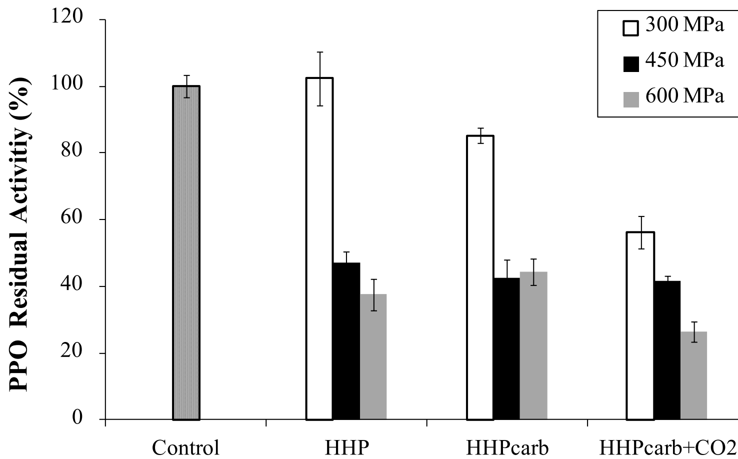

Fig. 2. Residual PPO activity in feijoa puree after HHP, HHPcarb and HHPcarb+CO_2 treatments at different pressures (initially at room temperature, 5 min). All data shown are means±SD.

3.3. PME activity

In the inactivation of PME, pressure showed different effects for the different treatments (Fig. 3). The residual PME activity of HHP samples was not significantly different (p>0.05) with increasing pressure. In the HHPcarb samples, the remaining PME activity at 600 MPa (44±4 %) was significantly lower (p<0.05) than at 300 (83±2 %) and 450 MPa (78±3 %), between which there were no significant differences (p>0.05). For HHPcarb+CO_2 treated samples, only significant differences (p<0.05) were observed between 300 MPa (73±14 %) and 600 MPa (53±3 %). From the two-way ANOVA, it was observed that the residual PME activity of the different treated samples significantly (p<0.05) decreased as pressure increased in the order 300 MPa (78 $\%_{avg}$) > 450 MPa (58 $\%_{avg}$) > 600 MPa (52 $\%_{avg}$). However, no significant differences (p>0.05) were found between the different levels of CO_2 studied. In this case, on average, the addition of CO_2 did not improve the inactivation of PME in a HHP process. The enhancing effect of CO_2 addition to the HHP inactivation process of PME in feijoa puree was only observed at 600 MPa (Fig. 3).

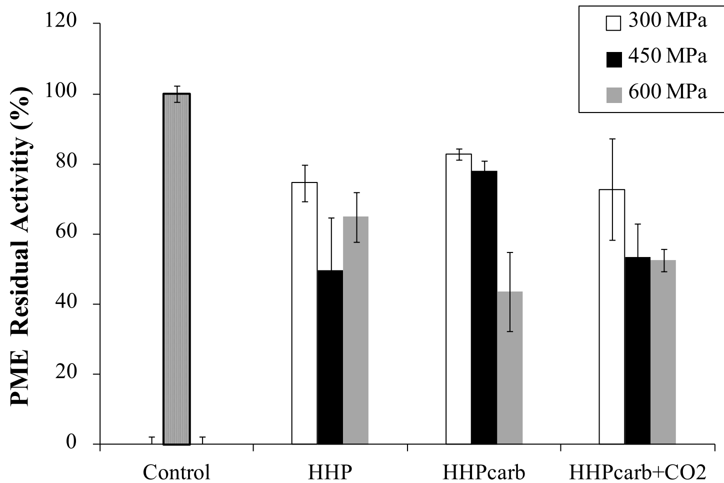

Fig. 3. Residual PME activity in feijoa puree after HHP, HHPcarb and HHPcarb+CO_2 treatments at different pressures (initially at room temperature, 5 min). All data shown are means±SD.

A portion of PME can be inactivated easily by pressure, but an isozyme of PME remains active even after pressurization at 900 MPa (Ishikawa-Nagaia et al., 2009). The lowest remaining PME activity resulting from this study, achieved after HHPcarb treatment (600 MPa, 5 min) was 44±11 %, and no treatment could fully inactivate PME. Similarly, in a sequential application of DPCD (4.9 MPa, 25 ºC, 5 min) and HHP (600 MPa, 5 min) using carrot juice, the lowest residual PME activity was 35 % (Park et al., 2002). Park et al. (2002) observed that the residual PME activity in carrot juice decreased from 80 % to 45 % by increasing pressure from 200 to 600 MPa (25 ºC, 5 min). More significant inactivation of PME was found by many authors using orange juice. Corwin & Shellhammer (2002) reported that the lowest remaining PME activity in carbonated orange juice was 6.8 %, achieved at 25 ºC, 800 MPa for 1 min.

3.4. pH

The value of pH directly measured in feijoa fruit was 3.30 (Table 1) while the pH of the control sample, after the freezing and thawing process was 3.45 (Table 2). The comparison of means shows that the blending, freezing and

thawing processing had a significant ($p<0.05$) effect on the pH of feijoa, before applying CO_2 or HHP.

The pH of the treated samples was compared with the pH of control puree subjected to the same temperature changes (Table 2). The pH values of samples with CO_2 inside the bag were measured after degassing by pulling vacuum. Overall, the pH values of all treated samples at different pressures significantly increased ($p<0.05$) compared to the control sample puree, but no significant ($p>0.05$) effect of pressure on the final pH reached in the puree was found. For the different CO_2 levels, on average, the pH values obtained at different pressures significantly decreased ($p<0.05$) in the order HHP > HHPcarb > HHPcarb+CO_2. This cannot be explained by the possibility of residual CO_2 remaining in the juice, since vacuum was pulled to remove the CO_2 from samples before pH measurement.

Table 2. Values of pH and color of feijoa puree for control and treated samples

	P(MPa)	pH	L*	a*	b*	Chroma	Hue angle
CONTROL		3.45±0.03	55.13±0.86	3.16±0.55	20.76±1.69	21.08±0.76	1.42±0.02
HHP	300	3.63±0.02	55.78±0.53	3.93±0.20	18.6 ±1.07	19.08±0.72	1.36±1.09
	450	3.68±0.01	54.24±0.92	4.43±0.10	18.57±0.17	19.09±0.48	1.34±0.14
	600	3.64±0.01	54.63±0.16	3.97±0.22	17.84±0.65	18.28±0.58	1.35±0.68
HHPcarb	300	3.61±0.02	53.76±0.60	4.30±0.78	18.83±0.82	19.33±0.62	1.35±0.31
	450	3.50±0.01	54.88±0.65	3.61±0.12	21.04±0.09	21.35±0.18	1.40±0.07
	600	3.55±0.03	52.30±0.18	4.58±0.30	17.90±0.16	18.48±0.09	1.32±0.08
HHPcarb+CO_2	300	3.46±0.04	54.84±0.38	2.57±0.80	19.06±0.53	19.25±0.28	1.44±0.42
	450	3.56±0.03	54.58±0.85	3.93±0.20	18.09±0.31	18.52±0.85	1.36±1.85
	600	3.51±0.01	53.68±0.02	4.20±0.09	16.93±0.23	17.44±0.20	1.33±0.25

All data shown are means±SD.

3.5. Color

The ΔE values (taking the control puree color as reference) are shown in Fig. 4 while the L*, a*, b*, Chroma and Hue angle values of the control and treated puree are shown in Table 2.

The ΔE values, on average for the three CO_2 levels studied, were significantly higher ($p<0.05$) in the samples treated at 600 MPa (2.74) compared to samples treated at 300 (2.02) and 450 MPa (2.05). The ΔE values are dependent on L*, a* and b*, and from the two-way ANOVA analysis of these parameters it was observed that pressure also had a significant ($p<0.05$) effect on all of them. The lightness and the yellowness of the samples significantly decreased ($p<0.05$) as pressure increased, while the redness significantly increased ($p<0.05$) as pressure increased. From the two-way ANOVA of Chroma and Hue angle, it was observed that the pressure had a significant ($p<0.05$) effect on them, decreasing their values with increasing pressure.

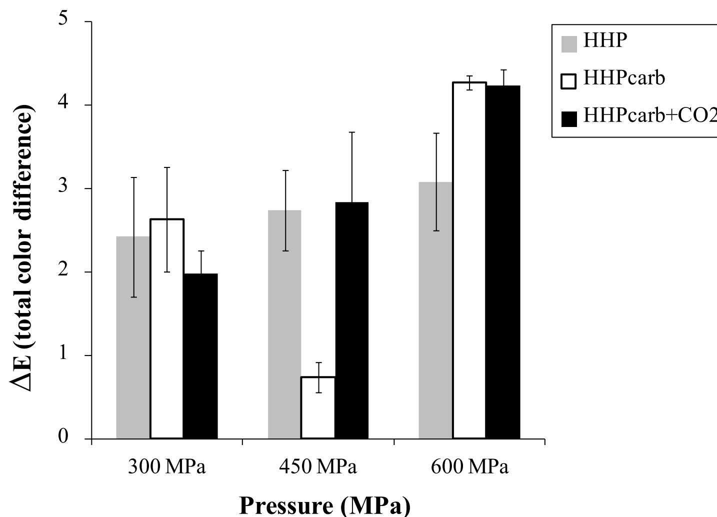

Fig. 4. Total color difference of feijoa puree after HHP, HHPcarb and HHPcarb+CO_2 treatments at different pressures. All data shown are means±SD.

Regarding the different CO_2 levels, on average for the different pressures studied, in the HHPcarb samples the calculated ΔE value was significantly higher than for HHP and HHPcarb+CO_2 samples, between which no significant differences were found. From the two-way ANOVA, the different CO_2 levels had a significant ($p<0.05$) effect on L* and a* values. Therefore the lightness and redness of the samples treated with CO_2 was significantly lower ($p<0.05$) than samples treated only with HHP. However, the yellowness did not change with the addition of CO_2 into the package compared with only HHP. On the other

hand, the CO_2 level had a significant ($p<0.05$) effect on Chroma values, but not on Hue angle values.

As a rule, a ΔE value of 1.6 or less is considered as an imperceptible difference to the human eye (Ishikawa-Nagaia et al., 2009). From Fig. 4 it can be seen that the ΔE values are above this threshold, except for HHPcarb at 450 MPa (0.74), therefore the treatments caused a perceptible color change. The feijoa puree changed from bright yellow tones to shades of brown with lower brightness, after all types of treatments. However, the addition of CO_2 into the headspace of the package did not increase the color change of the samples compared with the samples treated only with HHP.

4. DISCUSSION

The mechanisms associated with the inactivation of enzymes are similar to those associated to the denaturation of proteins because enzymes share the structure and properties of the proteins. Enzymes are folded into a three dimensional state, determined by covalent, hydrophobic and ionic intra-molecular connections (Rezaei et al., 2007). The inactivation of enzymes is caused by the fragmentation or modification of their secondary and tertiary structure; therefore, all the mechanisms that affect the structure of enzymes can be responsible of their denaturation.

The application of HHP causes structural rearrangements in the protein, shifting the system equilibrium toward the state occupying the smallest volume and increasing the degree of ordering of molecules of a given substance (Rezaei et al., 2007). The volume decrease can perturb the balance of intramolecular and solvent-protein interactions and can, therefore, lead to structural changes of the proteins (Hendrickx et al., 1998). A reduction in the pH of suspending media as a result of the pressure-induced transient pH shift leads to a greater enzyme inactivation by HHP, and this has also been reported for food borne vegetative cells (Rezaei et al., 2007).

The inactivation of enzymes exposed to DPCD treatment can be explained by different effects such as pH lowering, the inhibitory effect of molecular CO_2 on enzyme activity and the fact that DPCD causes conformational changes (Damar

& Balaban, 2006). Treatments with high pressure CO_2 are accompanied by a lowering of pH because of the formation of carbonic acid from the dissolution of carbon dioxide in water and under a lower pH environment, protein bound arginine can easily interact with CO_2, forming a bicarbonate complex (Rezaei et al., 2007). Therefore, in addition to its pH-lowering effect, CO_2 may directly bind to the enzyme and cause loss in activity. Moreover, the inactivation of enzymes exposed to DPCD treatment can be explained by the fact that DPCD causes conformational changes in the secondary and tertiary structure.

The present study is the first work where HPP and DPCD have been simultaneously applied in feijoa puree, and where a modified atmosphere of CO_2 has been considered in the treatment of its puree to preserve the nutritional properties of this product.

As a result, the addition of carbon dioxide into the headspace of the package treated with HHP enhanced the inactivation mechanisms of the enzymes POD, PPO and PME, compared with HHP and the HHPcarb samples. This could be explained because pressure increases the CO_2 solubilization, therefore in the HHPcarb+CO_2 samples, the amount of dissolved CO_2 should be higher than in HHPcarb samples, and it is the first step in the inactivation mechanisms of CO_2 from which other mechanisms follow (decrease of pH, alteration of ionic equilibrium and inactivation of enzymes) (Ortuño et al., 2012, 2013).

In addition, the CO_2 dissolved into the puree during the HHP treatment, could generate a significant and sudden bubbling during the fast depressurization of the process (2 s), that could contribute to conformational changes responsible for the inactivation of enzymes. The effect associated to the sudden depressurization would be more intense as pressure drop increases; suggesting that the conformational changes would be higher after treatment at 600 MPa than at 300 MPa. Therefore, various depressurization rates should also be investigated.

The same level of inactivation of POD and PPO was obtained at 600 MPa without CO_2, and at 300 MPa with added CO_2. However, to observe the enhanced HHP inactivation of PME in feijoa puree by the addition of CO_2 it is necessary to use 600 MPa.

The addition of CO_2 significantly improved the inactivation of some enzymes in the HHP process, compared with only HHP. Moreover, CO_2 did not affect the color of the puree, compared with puree treated with only HHP. These results are encouraging to apply this combined technique to other foods systems.

It is recommended that more research be conducted to study the effect of the different CO_2 levels in the bags and to elucidate the mode of enzyme inactivation by the simultaneous HHP and DPCD treatments. Kinetics of inactivation should be measured under this combined method. This typically requires treatments using a series of dwell times. Additional studies regarding the effect of simultaneous HHP+DPCD on physico-chemical properties and consumer acceptance of juices and purees would also bring this method closer to commercial applications.

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General Discussion

5. GENERAL DISCUSSION

As mentioned in the introduction of the present Thesis, the inactivation of microorganisms and enzymes is an essential process in the food industry to ensure the microbiological safety and stability of food. In this regard, there is a need to improve the food preservation techniques avoiding the use of high temperatures characteristic of thermal treatments, which damage the nutritional and organoleptic properties of food. To cover these requirements, the present Thesis was carried out to evaluate the viability of combining Supercritical Fluids (SC-CO_2) with two non-thermal techniques, High Power Ultrasound (HPU) and High Hydrostatic Pressure (HHP).

In the SC-CO_2 inactivation processes, different factors can affect the sensitivity of microorganisms/enzymes to the treatment, including the growth stage of the microorganisms, the pressure and the temperature of the treatment, the composition of the suspending medium or the type of the microorganism/enzyme (Casadei et al., 2002; Garcia-Gonzalez et al., 2007; Spilimbergo & Ciola, 2010). These factors as well as the effect of HPU on the process have been addressed in the present work and will be discussed below.

Effect of the growth stage of microorganisms

The results regarding the effect of the growth stage on the microbial inactivation of *E. coli* and *S. cerevisiae* inoculated in LB or YPD Broth, respectively, showed a greater resistance to the SC-CO_2 treatment (350 bar, 35 ºC) as the growth phase advanced from the early exponential phase to the stationary phase.

Theories explaining the inactivation mechanisms of SC-CO_2 involve different steps: solubilization of SC-CO_2 into the external liquid phase, cell membrane modification, intracellular pH decrease, key enzyme inactivation/cellular metabolism inhibition due to pH lowering, direct inhibitory effect of molecular CO_2 and HCO_3^- on metabolism, disordering of the intracellular electrolyte balance, removal of vital constituents from cells and cell membranes and rupture of cells. It must be noted that most of these steps will not occur consecutively,

but rather take place simultaneously in a very complex and interrelated manner (Garcia-Gonzalez et al., 2007).

The inactivation kinetics of different microorganisms using SC-CO_2 has been characterized by a lag-phase followed by a fast-to-slow curve (Lin et al., 1992). In the present study, both the length of the lag-phase and the time needed to reach a required level of inactivation increased as the growth stage progressed. The initial lag phase has been explained by Lin et al. (1992) as the time needed for CO_2 to penetrate through the cellular envelope which determines when the CO_2 begins to exert its inactivation effect. Thus, it could be thought that the microorganisms become more resistant to the CO_2 penetration as the growth advanced, which could be related to protection systems developed by them that are associated to the different growth stages. Each type of microorganism develops different protection systems.

In yeasts, it is considered that the resistance to stress increases through the activation of different protective systems, with two main mechanisms involved: one is the production of low molecular weight components such as trehalose, the second is the expression of stress-response genes, such as the heat shock proteins. In yeasts, both mechanisms are included in the so called “General Stress Response”. This response is mediated through the “general stress” transcription factors, Msn2p and Msn4p, that bind to the STRE sequence (AGGGG or CCCCT) present in the promoter region of a large set of stress-responsive genes. These include both heat shock genes (SSA3, HSP12, HSP26, HSP104), and trehalose metabolism genes (Martinez-Pastor et al., 1996). Moreover, Msn2/4 is also involved in the resistance to low pH. Since acidification is one of the mechanisms responsible for the SC-CO_2 microorganism inactivation, it is expected that the pH decrease can be involved in the response to SC-CO_2 exposure.

In bacteria, when cells get closer to the stationary phase, they develop a multiple-stress resistance state, considered to be analogue to sporulation in *B. subtilis* (Ramirez Santos et al., 2005). Moreover, in the treatments under pressure, as happens in the SC-CO_2 process, the higher resistance showed in the stationary phase could be explained by the morphological and physiological

changes associated to this phase, like changes in cell size and shape, thickening of the cell wall, or synthesis of osmoprotective compounds (Ramirez Santos et al., 2005), which would make CO_2 penetration difficult and lessen its effects.

Similar results have been obtained in previous studies, when microbial cells enter or approach the stationary phase they develop greater resistance to different stress conditions, such as heat, ethanol, oxidative stress or lytic enzymes (Werner-Washburne et al., 1996).

Effect of pressure and temperature

To determine the effect of the process conditions, namely temperature (31-41 ºC, 225 bar) and pressure (100-350 bar, 36 ºC), on the SC-CO_2 inactivation of *E. coli* and *S. cerevisiae*, both microorganisms were subjected to the treatment inoculated in culture media and selected in one of the most resistant growth stages, the early stationary phase. A similar effect of the process parameters on the microbial inactivation is expected for the remaining growing stages, although shorter times would be necessary to achieve the cells death.

The obtained results showed survival curves with two phases: an initial lag-phase, indicated by a shoulder in the curve, followed by an inactivation phase. When pressure and temperature increased, both the duration of the lag phase and the time needed to reach the same inactivation level, were reduced.

Regarding the effect of pressure, the required time to reach the total inactivation of *E. coli* was reduced from 60 to 25 min as pressure increased from 100 to 350 bar. Similarly, Liao et al. (2008), using a discontinuous SC-CO_2 system, observed a reduction of *E. coli* population of 5 and 6.5 log-cycles as pressure increased from 100 to 300 bar, after 75 min of treatment at 37 ºC and the complete inactivation was not achieved at any of the studied conditions. Only at 350 bar, 36ºC and after 140 min the total inactivation of *S. cerevisiae* (no growth in enumeration) was attained. The number of log reductions obtained after 140 min of treatment was significantly different ($p<0.05$): 1.41, 2.33, 3.15, and 6.70 log cycles, at 100, 225, 290 and 350 bar, respectively. In the same line, Erkmen (2003), using a discontinuous SC-CO_2 system, reported that the inactivation rate of *S. cerevisiae* increased with pressure, reducing the required

time to reach 6 log-cycles from 135 to 60 min, as pressure increased from 50 to 100 bar, at 40 ºC. It is known that pressure controls the solubilization rate; thus, higher pressures enhance SC-CO_2 solubilization, facilitating the contact between CO_2 and cells and increasing the membrane permeability (Giulitti et al., 2011a) therefore making the penetration of CO_2 into the cells easier (Liao et al., 2006), decreasing the length of the lag-phase and the time needed to reach a particular inactivation level.

Regarding the effect of temperature, the required time to reach the total SC-CO_2 inactivation of *E. coli* (8 log-cycles) decreased from 75 to 40 min as temperature increased from 31 to 41 ºC, at 225 bar. Similarly, Erkmen (2001b), using a discontinuous SC-CO_2 equipment, reduced 8 log-cycles the *E. coli* population in 80 and 50 min at 20 °C and 40 °C (75 bar), respectively. In the present study, the total inactivation of *S. cerevisiae* was not achieved at 225 bar due to the long lag-phase and to the slow inactivation rate. The number of log reductions obtained after 140 min of treatment was 2.20 log cycles/min, at any temperature studied. Contrarily, Erkmen (2003) showed a shortening of the lag-phase, 50, 30 and 15 min, in the SC-CO_2 inactivation kinetics of *S. cerevisiae* (ATCC 9743) using 30, 40 and 50 ºC, respectively, at 100 bar. These differences between both studies could be attributed to the different nature of the strains used. In general, higher temperatures enhance the diffusivity of CO_2, and can also increase the fluidity of the cell membrane making CO_2 penetration easier (Valverde et al., 2010), thus facilitating the decrease of intracellular pH and the extraction of vital cell constituents. However, SC-CO_2 treatments should operate around its critical temperature because, beyond this region, the density and solubility of CO_2 decreases quite rapidly as the temperature increases (Lin et al., 1994; Hong & Pyun, 1999).

In addition to the observed increase in the inactivation rate as pressure and temperature augmented, it could also be pointed out that the required time for obtaining pasteurization microbial reductions using the SC-CO_2 batch system of the present study (*E. coli*: 8 log-cycles of reduction after 75 min at 31 ºC and 225 bar; *S. cerevisiae*: 6.7 log-cycles of reduction after 140 min at 36 ºC and 350 bar) were too long compared with continuous or agitated SC-CO_2 systems.

In this regard, Shimoda et al. (1998) reduced 8-9 log-cycles the *S. cerevisiae* population with a continuous CO_2 system after 15 min of residence time at 35 ºC and 60 bar.

This could be due to the fact that in a continuous system the agitation allows a faster saturation of CO_2, and therefore a better contact of the inactivating supercritical fluid with cells, compared to batch systems. In this regard, any mechanical agitation introduced into the inactivation vessel, such as the use of ultrasound, might result into a faster microbial death.

Effect of HPU

In order to overcome the limitations associated to the batch SC-CO_2 system and in the context of the present Thesis, a HPU system was designed and constructed with the aim of enhancing the agitation and solubilization of the CO_2 into the medium where the microbial cells were suspended. This novel inactivation technique, based on High Power Ultrasound embedded in a Supercritical Fluids Plant, was patented in conjunction with the inactivation procedure (Benedito et al., 2011).

The inactivation rate of *E. coli* and *S. cerevisiae* in culture medium using SC-CO_2 assisted with HPU was significantly different ($p<0.05$) compared to the SC-CO_2 treatment. A drastic reduction in the population of both microorganisms was observed, being the viability reduced immediately, following a first order kinetics without any lag phase, at any studied condition. Similarly to SC-CO_2 treatments, the effect of pressure (100-350 bar, 36 ºC) and temperature (31-41 ºC, 225 bar) was studied in the SC-CO_2+HPU treatments. No significant ($p>0.05$) differences were found in the required time to reach the total inactivation of *E. coli* and *S. cerevisiae* at any condition of temperature selected, only 2 min of treatment for both microorganisms. Regarding the effect of pressure, the inactivation of *S. cerevisiae* required on average only 2 min of treatment to obtain a complete inactivation (7 log-cycles), at any condition of pressure selected. For *E. coli*, the time required to reach its total inactivation (8 log-cycles) was 1.5 min at any condition of pressure selected except at 350 bar, where a slower inactivation rate was observed and 5 min were required to attain a similar inactivation level. Thus, using SC-CO_2+HPU the effects

caused by HPU to the microorganisms in culture medium would be so intense that, the effect of increasing pressure and temperature did not significantly influence the inactivation rates.

Both, the process conditions and the treatment time, required in the present study to inactivate microorganisms using a SC-CO_2+HPU batch system, are less severe and shorter, respectively, than those used in studies reported in the literature using continuous SC-CO_2 systems. Kincal et al. (2005), using a continuous high-pressure CO_2 system for the inactivation of *E. coli*, reached a reduction of 4 log-cycles (10^5 CFU/mL initial population) at 380 bar and 34.5 ºC after a residence time of 10 min (in the present work 8 log-cycles of *E. coli* reduction after 5 min at 350 bar and 36 ºC). Shimoda et al. (1998), using a continuous SC-CO_2 system, showed a total reduction of *S. cerevisiae* population (8 log-cycles) at 60 bar and 35 °C after 15 min of residence time (in the present work 7 log-cycles of *S. cerevisiae* reduction after 4 min at 100 bar and 36 ºC). Thus, the HPU-assisted supercritical batch system developed and used in the present study attained higher inactivation levels in shorter process times than in continuous systems, and using milder process conditions. Pressures and temperatures higher than 100 bar and 31 ºC, respectively, were not necessary to reach the total inactivation of *E. coli* and *S. cerevisiae* (7-8 log-cycles).

As it has been previously explained, the agitation could enhance the solubilization of CO_2 into the medium increasing the cell membrane permeability and the penetration of CO_2 into cells, which modify their composition facilitating the inactivation process (Giulitti et al., 2011a). Thus the advantage of using HPU during the SC-CO_2 treatment would include a more effective agitation and consequently a faster mass transfer. Stronger agitation also facilitates the contact between the CO_2 and the bacterial cells and the permeation of cell membranes, thus making CO_2 penetration into the cells easier and causing a faster drop of intracellular pH (Garcia-Gonzalez et al., 2007; Giulitti et al., 2011b). Moreover, HPU mass transfer improvement could also enhance the extraction of vital intracellular components from cells, accelerating its death. In addition, the inactivation effect of ultrasound could also be attributed to the cavitation of the medium. Cavitation refers to the formation, growth, and implosion of tiny gas

bubbles in a liquid when ultrasound travels through it, which has been proven to cause the disruption of cell walls (Chemat et al., 2011). The disruption of the microorganisms' walls could contribute to the extraction of intracellular compounds, accelerating the death of microbial cells.

In order to discern if the good results obtained combining SC-CO_2 and HPU were due to a possible synergistic effect, or just only to the addition of the individual effects of both techniques, individual inactivation treatments in culture medium were carried out and compared to the combined process. The results showed that the combination of SC-CO_2 and HPU had a greater effect on the inactivation of *E. coli* and *S. cerevisiae* than the addition of their individual effects. After 5 min of HPU (40 W), SC-CO_2 (350 bar, 36ºC), and SC-CO_2+HPU (350 bar, 36 ºC, 40 W) treatment, reductions of 1, 0.3 and 8.5 log-cycles were observed in the *E. coli* population, respectively. No microbial reduction of *S. cerevisiae* population was obtained after 1.5 min of HPU or SC-CO_2 treatment, however, a total reduction, 7 log-cycles, was achieved using SC-CO_2+HPU for the same period of time. Therefore, the combination of applying SC-CO_2+HPU resulted in a drastic increase of the inactivation rate of *E. coli* and *S. cerevisiae* cells, showing a clear synergistic effect between these techniques.

With the aim to contribute to improve the knowledge on the microbial inactivation mechanisms associated to the combined technology (SC-CO_2+HPU), a morphological study was carried out to determine the differences between untreated, SC-CO_2 (350 bar, 36 ºC) and SC-CO_2+HPU (350 bar, 36 ºC, 40 W) treated *E. coli* and *S. cerevisiae* cells, using light microscopy (LM) and transmission electron microscopy (TEM). Both types of treatment were applied for 5 min, observing no microbial reduction after the SC-CO_2 treatment and a total inactivation after the SC-CO_2+HPU treatment.

The LM and TEM images of SC-CO_2-treated *E. coli* and *S. cerevisiae* cells showed that the intracellular organization exhibited uneven distribution and some aggregation of cytoplasmic content, compared to untreated cells which exhibited an intact organization of cytoplasm with a uniform distribution of the inner material. The SC-CO_2-treated cells revealed some empty regions, which could be due to the aggregation or precipitation of internal cell components or to the

removal of part of the cytoplasmic content. Minor differences could be appreciated in the thickness of the cell envelope of both microorganisms, due to a possible accumulation of CO_2 into the cell envelope.

In the same line, using SC-CO_2, Garcia-Gonzalez et al. (2010) compared TEM micrographs of untreated and treated *E. coli* cells (totally inactivated), under SC-CO_2 at 210 bar, 45 ºC during 60 min, and observed that the cytoplasm of the treated cells bulged through small pores in the cell wall and seemed to have lost its coherence. Liao et al. (2010a) examined the morphology of SC-CO_2-treated *E. coli* (100 bar, 37 ºC, 75 min) by TEM. These authors indicated that the morphology of cells was significantly damaged, appearing noticeable holes or wrinkles on their surfaces. Moreover, they noticed that the intracellular organization of a large fraction of SC-CO_2 treated *E. coli* cells was significantly disintegrated. Regarding the effect of SC-CO_2 on *S. cerevisiae*, Garcia-Gonzalez et al. (2010) explored its morphology after 60 min of treatment at 210 bar and 45 ºC, and concluded that despite of the membrane was not disrupted, its permeabilization could facilitate the penetration of CO_2 into the cell and the pH drop could induce a denaturation of some key enzymes. Li et al. (2012) explored the inactivation of *S. cerevisiae* using SC-CO_2 at 100 bar, 35 ºC during 120 min, by scanning electron microscopy (SEM) and TEM. They perceived a reduction of the cytoplasm density and the extraction of cytoplasmic content, despite of the cell walls remained intact.

The process times used in the cited studies ranged from 60 to 120 min, after which the total inactivation was attained for all the microorganisms selected. In the present work, it seemed that 5 min of SC-CO_2 treatment allowed the penetration of CO_2 into the cells generating minor irregularities, which were not sufficient to observe an important microbial reduction of both microorganisms. It is possible that after 5 min of treatment, both microorganisms synthesize new biomolecules to repair damage on the cell walls and membranes to continue cellular division and growth (Erkmen & Bozoglu, 2008b). In the same line, Spilimbergo et al. (2009) observed that the initial damaged cellular envelope generated by a short SC-CO_2 treatment (100 bar, 36 ºC) was not lethal for the cells and a minimum of 10 min were required to induce the cells an irreversible

damage that caused their death. Thus, longer SC-CO_2 process times may be required to inactivate enough key enzymes and to affect the cellular envelope, which would allow obtaining a significant reduction of surviving microbial cells.

The SC-CO_2+HPU treatment generated further morphological changes in *E. coli* and *S. cerevisiae* cells, compared to the SC-CO_2 treatment. The dimensions of both microorganisms significantly ($p<0.05$) increased probably due to the expansion of the cytoplasmic content or to the accumulation of CO_2 inside of the cells, which generated a cells swelling. TEM images revealed a higher aggregation and uneven distribution of the cytoplasmic content compared to SC-CO_2 treated cells. Great proportions of empty regions were observed inside of SC-CO_2+HPU-treated cells, indicating clearly a drastic reduction of the cytoplasm content, which could be observed in the surrounding of cells. The cell wall and plasmalemma of *E. coli* cells were totally disrupted and separated of the interior cell content in most of the cells, while the cell wall of *S. cerevisiae* cells lost partially their layered structure and some punctured or broken walls could be observed.

The duration of the SC-CO_2+HPU treatment, 5 min, resulted in the total inactivation of both microorganisms and generated further morphological changes, such as the total disintegration of the intracellular content and the disruption of the cell envelope, comparable to longer SC-CO_2 treatments (Garcia-Gonzalez et al., 2010; Liao et al., 2010a; Li et al., 2012). Therefore, shorter process times could be used applying SC-CO_2+HPU than using SC-CO_2. The faster microbial inactivation of the SC-CO_2+HPU treatment compared to SC-CO_2 could be related to the cavitation phenomenon generated by HPU. The cavitation could cause cracked or damaged cell walls, as observed in the micrographs, enhancing the penetration of SC-CO_2 inside of the cells, changing the cellular equilibrium and facilitating the extraction of intracellular compounds, thus accelerating the death of the microbial cells.

Additionally, the results obtained from the storage study revealed that no growth of SC-CO_2+HPU treated *E. coli* or *S. cerevisiae* cells was detected during 6 weeks of storage at 4 ºC. Therefore, it could be thought that the SC-CO_2+HPU treatment generated irreversible damage on the cells, avoiding a possible cellular

division and growth. Using only SC-CO_2, other studies have observed a growth of microbial population during the storage period (Kincal et al., 2005; Fabroni et al., 2010; Liao et al., 2010b) despite of just after the treatment no viable microorganism was detected. These studies suggested that microbial cells could repair by themselves and revert to their vegetative cultivable state. In the present study, using SC-CO_2+HPU, no microbial growth was detected during a longer storage period and using shorter process times and lower temperatures, which demonstrates the effectiveness of the proposed technique.

Effect of the medium

In order to determine the effect that the nature of the suspending media has on the SC-CO_2 and SC-CO_2+HPU inactivation of *E. coli* and *S. cerevisiae* in the early stationary phase, apple and orange juice were selected to compare them with the inactivation kinetics on LB and YPD Broth.

The obtained results revealed that on average, the SC-CO_2 inactivation of *E. coli* and *S. cerevisiae* in both juices was slower than in culture media. Moreover, on average, the inactivation kinetics were slower in apple juice than in orange juice. This fact could be explained by the effect of the juices' constituents and in particular to the sugar content. The sugar content in the orange and apple juice was 11.6 and 15.6 ºBrix, respectively, compared to 2 and 5 ºBrix for LB and YPD Broth, respectively. The sugar binds water from the medium, thus the free water where the CO_2 can be dissolved was lower in apple juice than in orange juice; and lower in both juices than in LB and YPD Broth. The solubilization of CO_2 into the medium is the first step in the inactivation mechanisms of SC-CO_2, from which other mechanisms happen (decrease of pH, alteration of membrane cells, penetration into cells and inactivation of key enzymes). Therefore, if the first step is limited by a lower content of free water, due to a high sugar content, the consecutive inactivation mechanisms can be delayed and the inactivation kinetics slowed down.

The effect of the constituents of the suspending media on both the CO_2 solubility and the SC-CO_2 microorganisms' inactivation has been explored in previous studies. Calix et al. (2010) demonstrated that the CO_2 solubility in juices was significantly lower than in pure water, since the presence of solutes may

slow down the saturation by increasing viscosity. Lin et al. (1994) related the increased resistance to SC-CO_2 inactivation of cells suspended in sweetened complex media to the sugar content. They postulated that the presence of sugar in the suspending medium probably led to a decrease in the amount of CO_2 penetrating the cells by changing the structure of cell membranes. Spilimbergo (2002) showed that, while in pure water the pH is greatly affected by the addition of CO_2, the CO_2 effect in orange juice is totally buffered. This buffering effect prevents the acidification that the CO_2 could generate into the medium and, consequently, the alteration of the membrane permeability of the cells.

On the other hand, in view of the short process times required to inactivate *E. coli* and *S. cerevisiae* using SC-CO_2+HPU in culture media, where pressures and temperatures higher than 100 bar and 31 ºC were not needed, different process conditions were studied to evaluate the effectiveness of this novel technique (SC-CO_2+HPU) in apple and orange juice, to find the optimal process conditions in real matrices. Contrarily to the results obtained using culture media, the inactivation of both microorganisms with SC-CO_2+HPU in apple and orange juice was accelerated by increasing pressure and temperature. Moreover, on average, longer process times were required to reach the total inactivation of both microorganisms (2-7 min) in juices, compared to the inactivation in culture media (1-2 min). Therefore, despite of the intense ultrasound agitation, the juices were not so quickly saturated with CO_2 as for culture media due to their higher sugar content, and an increase of pressure or temperature could facilitate the solubilization of CO_2. Moreover, it is known that the viscosity of the medium directly affects the cavitation phenomenon and the viscosity of the medium is directly proportional to the concentration of soluble solids (ºBrix) and inversely proportional to the temperature (Carcel, 2003). To generate cavitation bubbles, the cohesive forces of the liquid have to be overcome by the negative pressures (Carcel, 2003). The apple and orange juice viscosity was larger than in culture media, therefore the cavitation in the juices could be less intense and consequently, the mechanical cell damage caused by the cavitation phenomenon, showed in the morphological study, less severe.

The inactivation of *E. coli* and *S. cerevisiae* in juices has been explored in previous studies using batch and continuous SC-CO_2 systems. Liao et al. (2008)

reduced 7 log-cycles the microbial content of cloudy apple juice inoculated with *E. coli*, using a batch SC-CO_2 system at 300 bar, 42 ºC and 75 min of treatment. Kincal et al. (2005), using a continuous high-pressure CO_2 system, reduced 4 log-cycles the population of *E. coli* inoculated in orange juice at 380 bar, 34.5 ºC and 10 min of treatment. Spilimbergo et al. (2007) studied the SC-CO_2 inactivation of apple juice inoculated with *S. cerevisiae* with a multi-batch system. The microbial reduction was of 4.5 log-cycles at 200 bar and 36 ºC, after 30 min of process. Comparing the results of the present work with results reported in the literature using batch systems, much longer process times were needed compared to SC-CO_2+HPU processing (2-7 min) of juices. Additionally, despite in continuous systems the treatment time is drastically reduced compared to batch systems, the batch supercritical system assisted by HPU of the present study allowed attaining similar inactivation levels in shorter process times than in continuous SC-CO_2 systems. Thus, it can be concluded that, with the HPU-assisted supercritical batch system used in the present study, acceptable microbial inactivation levels can be attained in short process times at low pressures and temperatures, which would contribute to the preservation of the nutritional value and desirable sensory characteristics of juices.

Effect of the type of microorganism/enzyme subjected to the SC-CO_2 and SC-CO_2+HPU inactivation treatments

In the inactivation treatments performed to study the effect of the growth stage, pressure, temperature and type of medium, significant differences were found between the microorganisms/enzyme selected.

The required time to attain the total SC-CO_2 (350 bar, 35 ºC) inactivation of *S. cerevisiae* (7 log-cycles) ranged from 60 to 140 min, as the growth stage advanced from the early exponential phase to the stationary phase, compared to 8-25 min required for *E. coli* (8 log-cycles) for the same growth stages. For one of the most resistant phases, the early stationary phase, the total inactivation of *E. coli* using SC-CO_2 was reached after 20-60 min using pressures of 100-350 bar (36 ºC), while the total inactivation of *S. cerevisiae* was attained only at the highest pressure studied, 350 bar (36 ºC), and after 140 min of SC-CO_2 treatment. The SC-CO_2 inactivation at different temperatures, 31, 36 and

41 ºC (225 bar) reduced 8 log-cycles the *E. coli* population after 75, 55 and 40 min, respectively, while *S. cerevisiae* population was reduced on average 2.2 log-cycles at any temperature studied at 225 bar after 140 min of process. Thus, *S. cerevisiae* showed more resistance to the SC-CO_2 treatments than *E. coli*, in all the studies performed in the present work. These results are consistent with previous studies which determined that *S. cerevisiae* has a thicker cell wall, which makes it similar to Gram-positive bacteria (Villas-Boas et al., 2006). It is known that Gram-positive cells are more resistant to different types of stress including SC-CO_2 than Gram-negative ones due to their thicker cell wall (Ramirez Santos et al., 2005). In the present work, the morphological study revealed a cell wall thickness of 17.7 and 124.8 nm, for *E. coli* and *S. cerevisiae*, respectively. Thus, the thicker wall of *S. cerevisiae* could slow down the inactivation mechanisms, such as the chemical modification of the lipid double-layer of the microbial cell membrane, the increase of cell permeability to SC-CO_2 and its penetration into cells (Watanabe et al., 2005). In fact, the morphological study performed to determine the effect of the SC-CO_2 treatment revealed that the greatest differences between both microorganisms appeared in the cell envelope. After the SC-CO_2 treatment, in the *E. coli* cells, the peptidoglycan layer was observed with a high degree of dissolution, the outer membrane showed a loss of cohesiveness, protuberances and winding, and the plasmalemma appeared disintegrated in some areas. However, slight modifications were observed in the examination of *S. cerevisiae* treated cells, in which the inner layer could be observed more densely stained and the outer layer thicker than those of untreated cells, but the cell wall still contained the same layers than untreated cells and no disruption of cell wall was appreciated.

When using SC-CO_2+HPU, no significant differences ($p>0.05$) were found between the inactivation kinetics of *E. coli* and *S. cerevisiae*, in the early stationary phase and inoculated in culture media. The total inactivation of both microorganisms, on average, was attained after 2 min of SC-CO_2+HPU treatment, at any condition of pressure (100-350, 36 ºC) and temperature (31-41 ºC, 225 bar) selected. Therefore, the application of HPU to the SC-CO_2 treatments in culture media, masked the different resistances showed by both microorganisms to the SC-CO_2 treatments. The drastic effects generated by HPU

were observed in the LM and TEM images of *E. coli* and *S. cerevisiae* cells, which have been previously explained. In both microorganisms, the SC-CO_2+HPU (350, 36 ºC, 5 min, 40 W) treatment generated further morphological changes than SC-CO_2 (350, 36 ºC, 5 min) treatment. Therefore, the faster microbial inactivation of the SC-CO_2+HPU treatment compared to SC-CO_2 could be due to the acceleration of the SC-CO_2 inactivation mechanisms and the cavitation phenomenon generated by HPU, which in culture media affected similarly to both microorganisms.

On the other hand, in juices, the SC-CO_2+HPU inactivation showed significant ($p<0.05$) differences between both microorganisms, subjected to the same conditions of pressure (100-350 bar, 36 ºC) and temperature (31-41 ºC, 225 bar). On average, longer process times were required to reach the total inactivation of *E. coli* (7 and 6.3 min in apple and orange juice, respectively), compared to those required for *S. cerevisiae* (3.2 and 2.4 min in apple and orange juice, respectively). Therefore, *E. coli* showed more resistance to SC-CO_2+HPU treatments in juices than *S. cerevisiae*, in contrast to the results obtained with SC-CO_2. It could be thought that in treatments with juices, despite of the effect of HPU, the high sugar content could limit the fast solubilization of CO_2 into the medium, thus the inactivation mechanisms could be greatly affected by the cavitation phenomenon and the microorganism's size. The size of *S. cerevisiae* cells, 3.1 µm and 2.6 µm at the large and the small diameter, respectively, was bigger than *E. coli* cells, 1.2 and 0.7 µm length and width, respectively, therefore, the probability that the implosion of the cavitation bubbles might reach and affect the cell structure could be larger for *S. cerevisiae* than for *E. coli*.

The combined effect of SC-CO_2 and HPU on the inactivation kinetics of pectin-methyl esterase (PME) in orange juice was studied at similar conditions of pressure (100-350 bar, 36 ºC) and temperature (31-41 ºC, 225 bar) than for microorganisms, during 10 min of treatment. The inactivation significantly ($p<0.05$) increased at the highest level of pressure and temperature studied, 350 bar and 41 ºC, respectively, compared to milder process conditions, between which no significant differences ($p>0.05$) were observed. The lowest value of

PME residual activity (RA) obtained, 10.7 %, was attained after 10 min of treatment under 225 bar and 41 ºC.

Similarly to *E. coli* and *S. cerevisiae*, the inactivation of PME by means of SC-CO_2 has previously been described in the literature. Balaban et al. (1991) explored the inactivation of PME in orange juice with a batch SC-CO_2 system. As it was observed in the present study, the RA of PME decreased as the temperature and pressure increased. These authors reached a RA of 30 % at 44 ºC, 269 bar after 50 min, however, in the present work a similar inactivation degree, 32 %, was attained with lower temperatures and shorter process times (36 ºC and 8 min, 350 bar), when using the batch SC-CO_2+HPU system. Fabroni et al. (2010) investigated the inactivation of PME in blood orange juice with a continuous SC-CO_2 system. They obtained a RA of 66.8 % after 15 min of treatment at 230 bar and 36 ºC. This value was similar to the 60 % of the present study attained at 225 bar, 31 ºC after 8 min of SC-CO_2+HPU process. Therefore, using the batch SC-CO_2+HPU system of the present study, shorter process times and lower temperatures can be used to inactivate PME, compared to continuous SC-CO_2 systems, which could avoid the damage of nutritional properties of orange juice.

The inactivation of enzymes is associated to the fragmentation or modification of their secondary and tertiary structure; therefore, any mechanism that might affect the structure of enzymes can cause their denaturation. The inactivation of enzymes exposed to SC-CO_2 treatments can be explained by the lowering of the pH, the inhibitory effect of molecular CO_2 on enzyme activity and the fact that SC-CO_2 causes conformational changes (Balaban, 2012). Additionally, the application of HPU during the supercritical treatment could accelerate the SC-CO_2 inactivation mechanisms and the cavitation phenomenon, generated by HPU, could contribute to the change in the conformation of the enzyme structure, accelerating its inactivation.

The enzyme PME needed longer process times to be inactivated in orange juice with SC-CO_2+HPU, than *E. coli* and *S. cerevisiae*, and its total inactivation was not attained in any of the process conditions considered. These differences could be attributed to the different nature and size of microorganisms and

enzymes, therefore the combined treatment (SC-CO_2+HPU) could affect through cavitation more intensely to the morphology of the microorganisms due to their larger size, while the effect of SC-CO_2+HPU on enzymes could be more intensely related to the chemical effects associated to the solubilization of CO_2, such as the lowering of the pH or the inhibitory effect of the CO_2. Weder et al. (1992) proposed that, under a lower pH environment, protein bound arginine can easily interact with CO_2, forming a bicarbonate complex. Therefore, in addition to the pH-lowering effect, CO_2 may directly bind to the enzyme and cause loss on its activity. Consequently, SC-CO_2+HPU inactivation mechanisms associated to enzymes seem to be more complex and slow than those associated to microorganisms due to the role played by cavitation.

Modelling

In the inactivation kinetics, different behaviours have been observed according to the type of microorganism/enzyme, the different growth stages, the pressure and temperature selected, the media studied, etc. Therefore, it is important to determine the effect of these parameters on the inactivation, to find the optimal SC-CO_2 and SC-CO_2+HPU process conditions. For that purpose, the process modelling is of great importance.

A single equation that included the dependence on the growth stage, related to an OD_{600} value, was obtained for *E. coli* and *S. cerevisiae*, based on the Weibull (Peleg, 2006) and on the Gompertz model (Kim et al., 2007), respectively, in the SC-CO_2 treatments (350 bar, 36 ºC).

In order to analyze the survival curves for *S. cerevisiae* inoculated in culture medium, after the SC-CO_2 and SC-CO_2+HPU treatments, the kinetic data was fitted to the Weibull model (Peleg, 2006) including a pressure-dependence, at isothermal conditions, and including a temperature-dependence at isobaric conditions. This model provided good results for both treatments (SC-CO_2 and SC-CO_2+HPU) at different pressures and at different temperatures, in the range studied, 100-350 bar and 31-41 ºC, respectively.

Using juices as the treatment medium, the SC-CO_2+HPU inactivation kinetics of *E. coli*, *S. cerevisiae* and PME in orange juice were fitted to models that

included temperature, pressure and treatment time as variables, based on the Biphasic, the Peleg Type B, and the fractional model, respectively (Polydera et al., 2004; Peleg, 2006; Lee et al., 2009). These models satisfactorily predicted the experimental values in the practical range of 100-350 bar and 31-41 ºC. In apple juice, a general expression of the Peleg Type A and the Weibull model were used to predict the inactivation kinetics of *E. coli* and *S. cerevisiae*, respectively, for the same range of pressure and temperature that for orange juice.

The inactivation kinetics of microorganisms and enzymes using SC-CO_2 have been modeled in previous studies, although the comparison between works is difficult due to the large variety of microorganisms/enzymes, inoculated media, process conditions studied and the model selected. Ferrentino et al. (2010) demonstrated that the Peleg model was able to correctly fit the experimental supercritical inactivation data of *S. cerevisiae* in distilled water with different sodium phosphate monobasic buffer concentrations, considering pressure, temperature and CO_2 solubility as variables of the model. Polydera et al. (2004) described satisfactorily the PME inactivation rate, using high hydrostatic pressure, as a function of processing conditions by a composite mathematical model, including pressure and temperature as variables of the model. In the present study, similar models were developed considering pressure, temperature and time, with good results of predicted data.

The present study is the first report that covers the modelling of SC-CO_2+HPU inactivation kinetics of different microorganisms and enzymes. In general, the proposed models provided good results for the modelling of microorganism and enzyme inactivation.

Combination of SC-CO2 and high hydrostatic pressure

A combination of SC-CO_2 with other non-thermal technology, HHP, was investigated for the inactivation of PME, polyphenol oxidase (PPO) and peroxidase (POD) in feijoa puree. The preservation of feijoa products by non-thermal technologies is advantageous to retain many desirable characteristics, such as vitamin C. Different pressures (300-600 MPa) and CO_2 levels (0 (HHP), saturation at atmospheric pressure (HHPcarb), and saturation+8.5 ml CO_2/g puree into the headspace (HHPcarb+CO_2)) were studied.

On average, the RA of the three enzymes decreased as increasing pressure. The addition of 8.5 ml CO_2/g puree into the headspace of the package treated with HHP enhanced the inactivation mechanisms of the three enzymes selected, compared to HHP or HHPcarb samples, at all pressures used.

Enzymes are folded into a three dimensional structure, and their inactivation is caused by the fragmentation or modification of this structure. The application of HHP causes structural rearrangements in the enzymes, shifting the system equilibrium toward the state occupying the smallest volume and increasing the degree of ordering of molecules of a given substance (Rezaei et al., 2007). The volume decrease can perturb the balance of intramolecular and solvent-protein interactions and can, therefore, lead to structural changes of the proteins (Hendrickx et al., 1998). A reduction in the pH of the suspending media as a result of the pressure-induced transient pH shift leads to a greater enzyme inactivation by HHP, and this has also been reported for food borne vegetative cells (Rezaei et al., 2007).

Regarding the effect of SC-CO_2 on enzymes, the main inactivation mechanisms involved were pH lowering, the inhibitory effect of molecular CO_2 on enzyme activity and the fact that SC-CO_2 causes enzyme conformational changes (Damar & Balaban, 2006). Treatments with high pressure CO_2 are accompanied by a lowering of pH because of the formation of carbonic acid from the dissolution of carbon dioxide in water and under a lower pH environment, protein bound arginine can easily interact with CO_2, forming a bicarbonate complex (Rezaei et al., 2007). Moreover, the inactivation of enzymes exposed to SC-CO_2 treatment can be explained by the fact that SC-CO_2 causes conformational changes in the secondary and tertiary structure.

The present study was the first work where HPP and SC-CO_2 were simultaneously applied in feijoa puree, and where a modified atmosphere of CO_2 was considered in the treatment of this puree to preserve the nutritional properties of this product. The higher inactivation level obtained in the samples with CO_2 into the headspace of the package could be explained because the amount of dissolved CO_2 should be higher in HHPcarb+CO_2 than in HHPcarb samples. The CO_2 dissolution is the first step in the inactivation mechanisms of

CO_2 from which other mechanisms follow (decrease of pH, alteration of ionic equilibrium and inactivation of enzymes). In addition, the CO_2 dissolved into the puree during the HHP treatment, could generate a significant and sudden bubbling during the fast depressurization of the process that could contribute to conformational changes responsible for the inactivation of enzymes.

The inactivation of PME, PPO and POD has been explored in previous studies but no references have been found in the literature regarding their inactivation in feijoa puree with HHP or HHP+SC-CO_2. Park et al. (2002) explored a sequential application of SC-CO_2 (490 bar, 25 ºC, 5 min) and HHP (600 MPa, 5 min) to inactivate PME and PPO in carrot juice. These author did not attained the total enzyme inactivation at any studied condition, being the lowest residual activity of 35 % and 19 %, for PME and PPO, respectively. Similarly, in the present study the total inactivation of these enzymes was not attained at any condition studied and the lowest remaining PME and PPO activity, 44 % and 26 %, was achieved after the HHPcarb (600 MPa, 5 min) and HHPcarb+CO_2 (600 MPa, 5 min) treatment, respectively. Although the inactivation results of the present work are similar to those of Park et al. (2002), in the case of our treatments the combination of HPP and SC-CO_2 results into a process energy and time saving. Regarding the inactivation of POD, several studies suggest that SC-CO_2 treatment increases or slightly reduces the POD activity in crude vegetable extracts (Fricks et al., 2006; Primo et al., 2007). However, in the present study an increase of POD activity was only observed after 5 min of HHP at 300 MPa, and all HHP+SC-CO_2 treatments resulted into a decrease of the POD activity. Therefore, the addition of CO_2 into the headspace of the package significantly improved the inactivation of some enzymes in the HHP process, compared with only HHP. Additionally, in the present work the addition of CO_2 did not affect the color of the puree, compared with puree treated with only HHP. Thus, these results are encouraging to apply this combined technique to other foods system.

Conclusions

6. CONCLUSIONS

Based on the results obtained in the present Thesis, the main conclusions have been grouped according to the different factors studied in the inactivation treatments using supercritical carbon dioxide.

Effect of the growth stage, pressure and temperature on the SC-CO_2 inactivation treatments

- The growth stage of *E. coli* and *S. cerevisiae* directly affected to their inactivation using SC-CO_2 in growth media.
- The inactivation kinetics showed a lag-phase followed by a fast-to-slow curve. Both the length of the lag-phase and the time needed to reach a required level of inactivation increased as the growth stage progressed.
- The resistance of the microorganisms to the SC-CO_2 treatment increased progressively as the growth phase advanced, which could be linked to the natural protective systems that become activated as cells approach the stationary phase.
- The inactivation kinetics of *E. coli* and *S. cerevisiae* were satisfactorily fitted to the Weibull Model and to the Gompertz function, respectively, which were adapted to include the growth stage as one of the model parameters.
- In the SC-CO_2 treatments, the inactivation rate of both microorganisms, inoculated in growth media, augmented progressively as pressure or temperature rose, shortening both the duration of the lag phase and the time needed to reach a particular inactivation level.
- The Weibull distribution was useful to describe the SC-CO_2 inactivation kinetics of *S. cerevisiae*, inoculated in growth media, at different pressures and temperatures.

Effect of HPU on the SC-CO_2 inactivation treatments

- A novel inactivation technique based on High Power Ultrasound embedded in a Supercritical Fluids Plant has been developed in the context of this Thesis in order to accelerate the SC-CO_2 treatments.
- The application of HPU to the SC-CO_2 treatments resulted in a drastic increase of the inactivation rate of *E. coli* and *S. cerevisiae*. The inactivation kinetics did not show lag phases and the viability began to decrease quickly, following a first order kinetics.
- HPU leads to a vigorous agitation and solubilization of CO_2, and consequently a faster mass transfer that would accelerate the SC-CO_2 inactivation mechanisms causing a drastic drop in intracellular pH and an extraction of vital constituents. Moreover, the cavitation generated by HPU could damage the microorganisms' cell wall, accelerating their inactivation.
- The combination of SC-CO_2 and HPU had a greater effect on the microbial inactivation than the addition of their individual effects, showing a clear synergistic effect between these techniques.
- The simultaneous application of SC-CO_2 and HPU masked the effect of increasing pressure and temperature on the inactivation treatments of *E. coli* and *S. cerevisiae*, inoculated in growth media.
- The Weibull distribution was useful to describe the SC-CO_2+HPU inactivation kinetics of *S. cerevisiae*, inoculated in growth media, at different pressures and temperatures.
- The morphological study revealed that there was a direct relationship between cellular modification/damage and the inactivation provoked by SC-CO_2+HPU treatments on *E. coli* and *S. cerevisiae* cells.
- A short SC-CO_2 treatment (5 min) generated minor morphological modifications such as the uneven distribution of the cytoplasmic content and slight modifications in the cell envelope, however no microbial reduction was obtained.

- TEM and LM images revealed that a short SC-CO_2+HPU treatment (5 min) totally disrupted the cell envelope, facilitating the disintegration of the cytoplasm and the total inactivation of cells. The damage generated by the SC-CO_2+HPU treatment was strong enough to avoid the regrowth of cells during post-treatment storage.

Effect of the medium on the SC-CO_2 and SC-CO_2+HPU inactivation treatments

- The SC-CO_2 inactivation rate of both microorganisms in apple and orange juice was lower than in culture media. Moreover, on average, the inactivation kinetics were slower in apple juice than in orange juice.
- The higher resistance showed by both microorganisms in juices could be due to the sugar content, which binds water from the medium, thus the free water where the CO_2 can be dissolved was lower in apple juice (15.6 ºBrix) than in orange juice (11.6 ºBrix); and lower in both juices than in LB (2 ºBrix) or YPD (5 ºBrix) Broth.
- The effect of HPU on the SC-CO_2 microbial inactivation was affected by the nature of the medium. On average, the SC-CO_2+HPU inactivation rate was lower in apple juice than in orange juice; and in both juices lower than in growth media.
- In SC-CO_2+HPU treatments, the higher sugar content of juices affected the solubilization of CO_2 into the medium and the cavitation phenomenon. The cavitation is directly influenced by the viscosity of the medium, which is proportional to the concentration of soluble solids (ºBrix). In this regard, as apple juice is more viscous than orange juice, and both are more viscous than culture media, the cavitation in juices could be less intense and consequently, the mechanical cell damage less severe.

Effect of the type of microorganism/enzyme subjected to the SC-CO_2 and SC-CO_2+HPU inactivation treatments

- *S. cerevisiae* showed more resistance to the SC-CO_2 treatments than *E. coli* in all the studies performed in the present Thesis due to its thicker cell wall.
- The application of HPU to the SC-CO_2 inactivation treatments in culture media, involved a vigorous agitation and the cavitation of the medium which masked the different resistances showed by both microorganisms in the SC-CO_2 treatments.
- In juices, *E. coli* showed more resistance to the SC-CO_2+HPU treatments than *S. cerevisiae*. In these media, the fast solubilization of CO_2 generated by HPU could be hindered by the higher sugar content, compared to culture media. Therefore, the inactivation mechanisms would be mainly driven by the cavitation phenomenon and the higher likelihood that the implosion of the cavitation bubbles might affect the cell structure of *S. cerevisiae* than of *E. coli* due to its larger size.
- In general, for the SC-CO_2 inactivation treatments assisted by HPU applied in juices, a rise in pressure or temperature increased the inactivation rate of *E. coli* and *S. cerevisiae*.
- The inactivation kinetics of pectin-methyl esterase (PME) in orange juice using SC-CO_2+HPU showed that the inactivation level attained increased as pressure and temperature rose.
- The inactivation of enzymes can be associated with any mechanism that might affect their structure and then cause their denaturation, such as the lowering of the pH, the inhibitory effect of molecular CO_2 on enzyme activity and the fact that SC-CO_2 causes conformational changes. Moreover, the cavitation generated by HPU could contribute to the change in the conformation of the enzyme, accelerating its inactivation.
- The enzyme PME was more resistant to SC-CO_2+HPU than *E. coli* or *S. cerevisiae*, and its total inactivation was not attained in any of the

process conditions tested. This could be attributed to the different nature and size of microorganisms and enzymes.

- The Peleg Type A model and the Weibull model were adapted to satisfactorily describe the SC-CO_2+HPU inactivation kinetics of *E. coli* and *S. cerevisiae*, respectively, in apple juice, including pressure and temperature as model parameters.
- The Biphasic model, the Peleg Type B model and the fractional model were adapted to successfully describe the SC-CO_2+HPU inactivation kinetics of *E. coli*, *S. cerevisiae* and PME, respectively, in orange juice, including pressure and temperature as model parameters.

Combination of SC-CO_2 and high hydrostatic pressure (HHP)

- The combination of SC-CO_2 with high hydrostatic pressure (HHP), resulted in a higher inactivation level of enzymes of feijoa puree (PME, polyphenol oxidase (PPO) and peroxidase (POD)), compared to HHP alone.
- The enzymes inactivation is caused by the fragmentation or modification of their three dimensional structure. On average, the residual activity of PME, PPO and POD, decreased as increasing pressure, since pressure causes structural rearrangements that can perturb the balance of intramolecular and solvent-protein interactions and can, therefore, lead to structural changes of the proteins.
- The effects of SC-CO_2 on enzymes were pH lowering, the inhibitory effect of molecular CO_2 on enzyme activity and the fact that SC-CO_2 causes conformational changes.
- The higher inactivation level obtained as the CO_2 concentration increased, could be explained because the amount of dissolved CO_2 should be higher and the CO_2 dissolution is the first step in the inactivation mechanisms of CO_2 from which other mechanisms follow. In addition, the CO_2 dissolved into the puree during the HHP treatment, could generate a significant and sudden bubbling during the fast depressurization of the process that could

contribute to conformational changes responsible for the inactivation of enzymes.

- Moreover, the addition of CO_2 did not affect the color of the puree, compared with puree treated with only HHP, thus the quality of the treated product can be maintained.

GENERAL CONCLUSION

Finally, it can be concluded that the combination of SC-CO_2 with HPU or HHP enhanced the microbial/enzyme inactivation process. The application of HPU enhanced the SC-CO_2 inactivation mechanisms, generating a vigorous agitation that facilitated the CO_2 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-CO_2 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. Moreover, the simultaneous application of HHP and SC-CO_2 improved the inactivation of some enzymes, compared with only HHP. Therefore, these new technologies developed could represent alternatives to thermal processing in order to prevent the deterioration of food and to extend its shelf life.

Recommendations

7. RECOMMENDATIONS

On the basis of the results obtained in the present Thesis, and in order to improve the knowledge about the new technology developed, it is recommended that more research is conducted addressing the following topics:

Equipment development

- Design, development and adaptation of the batch SC-CO_2 system assisted by HPU to a continuous system that allows continuous process for liquid media.
- Optimize the design of the transducer and treatment vessel in order to maximize the effect of HPU on the SC-CO_2 inactivation.

Media and microorganisms

- To study the effect of SC-CO_2+HPU on the natural microflora of juices.
- To evaluate the effect of SC-CO_2+HPU on pathogen microorganisms, virus, and other strains of spoilage bacteria, yeast and molds, in different media.
- To clarify the inactivation mechanisms involved in the SC-CO_2+HPU treatments exploring the effect of the process on the membrane fluidity and permerability and on the enzymatic activity of the treated cells.
- To study how the composition of the media (ph, ºBrix, proteins, carbohydrates, fats, etc.) affects to the SC-CO_2+HPU inactivation mechanisms.
- To study the microbial inactivation with SC-CO_2+HPU in solid matrices, determining the effect of the HPU, water content, pressure, temperature and process time.
- To determine the effect of the solid matrix structure on the microbial inactivation with SC-CO_2+HPU.

- To determine the effect of HPU, pressure, temperature and residence time on the inactivation of different microorganisms using a continuous SC-CO_2+HPU system in liquid media.

Properties of the final product

- To study the effect of the SC-CO_2 treatment assisted by HPU, on the physico-chemical, organoleptic and nutritional properties of the food processed using this technology.
- To determine the effect of the SC-CO_2 treatment assisted by HPU, on the structure of solid matrices, and the relationship with the quality of the treated food.
- To study the effect of the SC-CO_2+HPU treatment on consumer's acceptance of the food processed using this technology.
- To determine the microbial stability and shelf-life of the food processed using SC-CO_2+HPU.

Combination of SC-CO_2 with HHP

- To study the effect of the different CO_2 levels in the headspace of the package on the microbial inactivation of food treated with HHP.
- To determine the effect of the process time and temperature on the enzyme/microbial inactivation using SC-CO_2+HHP.
- To study the mechanisms of enzyme/microbial inactivation by the simultaneous application of HHP and SC-CO_2.
- To determine the effect of SC-CO_2+HHP treatment on the physico-chemical, organoleptic and nutritional properties of juices and purees treated with this technology.

Energy saving and industrial application

- To determine if the simultaneous application of HPU in the SC-CO_2 treatments, or the addition of CO_2 into the package of food treated with HHP, results in an energy saving, and to study the technical and

economical feasibility of applying these novel technologies in the food industry.

Scientific Contribution

Research papers

Ortuño, C., Pérez-Munuera, I., Puig, A., Riera, E. & García-Pérez, J.V. (2010). Influence of power ultrasound application on mass transport and microstructure of orange peel during hot air drying. Physics Procedia, 3, 153-159.

García-Pérez, J.V., Ozuna, C., Ortuño, C. Cárcel, J.A. & Mulet, A. (2011). Modeling ultrasonically assisted convective drying of eggplant. Drying Technology, 29, 1499-1509.

Garcia-Perez, J.V., Ortuño, C., Puig, A., Carcel, J.A. & Perez-Munuera, I. (2012). Enhancement of Water Transport and Microstructural Changes Induced by High-Intensity Ultrasound Application on Orange Peel Drying. Food and Bioprocess Technology, 5, 2256-2265.

Benedito, J., Cambero, M.I., Ortuño, C., Cabeza, M.C., Ordoñez, J.A. & de la Hoz, L. (2011). Modeling and optimization of sensory changes and shelf-life in vacuum-packaged cooked ham treated by E-beam irradiation. Radiation Physics and Chemistry, 80, 503-513.

Ortuño, C., Martínez Pastor, M.T., Mulet, A. & Benedito, J. (2012). Supercritical carbon dioxide inactivation of *Escherichia coli* and *Saccharomyces cerevisiae* in different growth stages. Journal of Supercritical Fluids, 63, 8-15.

Ortuño, C., Martínez Pastor, M.T., Mulet, A. & Benedito, J. (2012). An ultrasound-enhanced system for microbial inactivation using supercritical carbon dioxide. Innovative Food Science and Emerging Technologies, 15, 31-37.

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Rodríguez, J., Ortuño, C. Benedito, J. & Bon, J. (2013). Optimization of the antioxidant capacity of thyme (*Thymus vulgaris* L.) extracts: Management of the drying process. Industrial Crops and Products, 46, 258-263.

Ortuño, C., Duong, T., Balaban, M. & Benedito, J. (2013). Combined High Hydrostatic Pressure and Carbon Dioxide Inactivation of Pectin Methylesterase, Polyphenol Oxidase and Peroxidase in Feijoa Puree. Journal of Supercritical Fluids, 82, 56-62.

Ortuño, C., Quiles, A. & Benedito, J. (2014). Inactivation kinetics and cell morphology of *E. coli* and *S. cerevisiae* treated with ultrasound-assisted supercritical CO_2. Food Research International. Accepted.

Ortuño, C., Balaban, M. & Benedito, J. (2014). Modeling of the inactivation kinetics of *Escherichia coli*, *Saccharomyces cerevisiae* and pectin methylesterase in orange juice treated with ultrasonic-assisted supercritical carbon dioxide. Journal of Supercritical Fluids, 90, 18-26.

Rodríguez, O., Ortuño, C., Simal, S., Benedito, J., Femenia, A. & Rosselló, C. (2014). Acoustically assisted supercritical CO_2 extraction of cocoa butter: effects on kinetics and quality. Journal of Supercritical Fluids. In Press.

Patents

Benedito, J. J., Martínez-Pastor, M.T., Mulet, A., Ortuño, C. & Peña, R. Procedure of inactivation microorganisms by combination of supercritical fluids and ultrasound. Patent number: P201131099. 2011. Spain.

Book chapters

Authors: Ortuño, C., Martínez, M.T., Mulet, A. & Benedito, J.
Title: Efecto del estado de crecimiento en las cinéticas de inactivación de *Escherichia coli* usando CO_2 supercrítico.
Editorial: University of Extremadura. Publishing Service.

Book (ISBN, ISSN,...): Productos cárnicos para el siglo XXI. Seguros, nutritivos y saludables. ISBN 978-84-7723-949-9.
Year: 2011

Authors: Ortuño, C., Rocha, R., Bon, J., Cárcel J.A. & Benedito, J.
Title: Efecto de la temperatura de secado sobre la capacidad antimicrobiana de extractos de tomillo obtenido mediante fluidos supercríticos.
Editorial: University of Extremadura. Publishing Service.
Book (ISBN, ISSN,...): Productos cánicos para el siglo XXI. Seguros, nutritivos y saludables. ISBN 978-84-7723-949-9
Year: 2011

Authors: Ortuño, C., Bon, J. & Benedito, J.
Title: Obtención de extractos de tomillo (*Thymus zygis*) con capacidad antimicrobiana mediante fluidos supercríticos asistidos por ultrasonidos.
Editorial: University of Extremadura. Publishing Service.
Book (ISBN, ISSN,...): Productos cárnicos para el siglo XXI. Seguros, nutritivos y saludables. ISBN 978-84-7723-949-9
Year: 2011

Authors: Benedito, J., Cambero, M.I., Ortuño, C., Cabeza, M.C., Ordóñez, J.A. & de la Hoz, L.
Title: Modelling Kinetics of sensory changes and shelf-life in e-beam treated cooked ham.
Editorial: University of Extremadura. Publishing Service.
Book (ISBN, ISSN,...): Productos cárnicos para el siglo XXI. Seguros, nutritivos y saludables. ISBN 978-84-7723-949-9
Year: 2011

Congress Contribution

Authors: Ortuño, C., Pérez-Munuera, I., Puig, A., Riera, E. & García-Pérez, J.V.
Title: Influence of power ultrasound application on mass transport and microstructure of orange peel during hot air drying.
Participation: Oral presentation
Congress: International Congress on Ultrasonics (ICU2009)
Publication: ICU Proceedings
Place: Santiago (Chile) **Year**: 2009

Authors: García-Pérez, J.V., Ortuño, C., Cárcel, J.A., Riera, E. & Mulet, A.
Title: Mass transfer improvement by ultrasonic sonication during orange peel drying
Participation: Poster
Congress: IFT Meeting (IFT2009).
Publication: Book of Abstracts IFT2009
Place: Anaheim (EEUU) **Year**: 2009

Authors: Benedito, J., Cambero, M.I., Ortuño, C., Cabeza, M.C., Ordoñez, J.A. & de la Hoz., L.
Title: Modelling kinetics of sensory changes and shelf-life in e-beam treated cooked ham.
Participation: Poster
Congress: Safe Consortium International Congress of Food Safety
Place: Girona, Spain. **Year**: 2009

Authors: Ortuño, C., Martinez-Pastor, M.T., Sanjuán, N., García-Pérez, J.V. & J. Benedito
Title: Effect of the growth stage on the yeast inactivation kinetics using Supercritical Carbon Dioxide.
Participation: Poster
Congress: New Challenges in Food Preservation: Processing – Safety - Sustainability
Place: Budapest, Hungría. **Year**: 2009

Authors: Ortuño, C., Martínez Pastor, M.T., Peña, R., Cárcel, J. & Benedito, J.
Title: Influence of an ultrasound pretreatment and different process variables on the inactivation of wine yeast by Supercritical Carbon Dioxide
Participation: Poster
Congress: New Challenges in Food Preservation: Processing – Safety - Sustainability
Place: Budapest, Hungría. **Year**: 2009

Authors: Ortuño, C., García-Pérez, J.V., Carcel, J., Femenia, A. & Mulet, A.
Title: Modelling of ultrasonically assisted convective drying of eggplant
Participation: Poster
Congress: 17th International Drying Symposium - IDS2010
Place: Magdeburg, Germany. **Year**: 2010

Authors: Bon, J., Ortuño, C., Clemente, G. & Mulet, A.
Title: Effect of the convective drying on antimicrobial activity of thyme: optimization of the operation conditions.
Participation: Poster
Congress: 17th International Drying Symposium - IDS2010
Place: Magdeburg, Germany. **Year**: 2010

Authors: Ortuño, C., Martinez-Pastor, M.T., Irianda, P., García-Pérez, J.V. & Benedito, J.
Title: Antimicrobial activity of oregano and thyme essential oils extracted with supercritical CO_2.
Participation: Poster
Congress: IFT 10 Annual Meeting & Food Expo.
Place: 17-20 July, Chicago. **Year**: 2010

Authors: Hector, C., Ortuño, C., García-Pérez, J.V., Bon, J. & Benedito, J.
Title: Supercritical CO_2 extraction of phenolic compounds from thyme (*Thymus zygis* L.)
Participation: Poster
Congress: IFT 10 Annual Meeting & Food Expo.

Place: 17-20 July, Chicago. **Year**: 2010

Authors: Ortuño, C., Rocha, R.P., Bon, J., Cárcel, J.A. & Benedito, J.
Title: Efecto de la temperatura de secado sobre la capacidad antimicrobiana de extractos de tomillo obtenidos mediante fluidos supercríticos.
Participation: Poster
Congress: XVII Congreso Nacional de Microbiología de los Alimentos.
Place: 19-23 September, Valladolid, Spain. **Year**: 2010
Authors: Ortuño, C., Irianda, P., Rocha, R.P., Bon, J. & Benedito, J.
Title: Extracción Convencional y Supercrítica de compuestos antioxidantes y antimicrobianos del orégano (Origanum vulgare).
Participation: Poster
Congress: VI Congreso Español de Ingeniería de Alimentos.
Place: 6-8 October, Logroño, Spain. **Year**: 2010

Authors: Ortuño, C., Rocha, R.P., Bon, J. & Benedito, J.
Title: Efecto del secado convectivo en la capacidad antioxidante del tomillo (*Thymus vulgaris*): optimización de las condiciones de operación.
Participation: Poster
Congress: VI Congreso Español de Ingeniería de Alimentos.
Place: 6-8 October, Logroño, Spain. **Year**: 2010

Authors: Ortuño, C., Irianda, P., Paniagua, I., Bon, J., Corona, E. & Benedito, J.
Title: Effect of supercritical extraction time on the antimicrobial activity of **thyme** (*Thymus zygis*) extracts.
Participation: Poster
Congress: Food Innova 2010.
Place: 25-29 October, Valencia, Spain. **Year**: 2010

Authors: Paniagua Martínez, I., Rodriguez Jimenes, G.D.C., Ortuño C. Ortuño, García-Pérez, J.V., Benedito, J. & Mulet, A.
Title: Thermal optimization of oregano (*Origanum vulgare*) supercritical fluid extraction.
Participation: Oral presentation

Congress: 4th Internation Congres. Food Science & Food Biotechnology in Developing Countries
Place: Boca de Rio, Veracruz, Mexico. **Year**: 2010

Authors: Ortuño C., Bon, J., Peña, R., Cárcel, J. & J. Benedito
Title: Obtención de extractos de tomillo (*Thymus zygis*) con capacidad antioxidante mediante fluidos supercríticios asistidos por ultrasonidos.
Participation: Oral presentation
Congress: VI Congreso Nacional de Ciencia y Tecnología de los Alimentos.
Place: 8-10 June, Valencia, Spain. **Year**: 2011

Authors: Ortuño, C., Martínez Pastor, M.T., Mulet, A., Peña, R. & Benedito, J.
Title: Ultrasonic enhanced microbial inactivation using supercritical carbon dioxide.
Participation: Oral presentation
Congress: Workshop on food safety: Technologies and innovations applied to food industry.
Place: 5-6 June, Valencia, Spain. **Year**: 2012

Authors: García-Rellán, D., Verdeguer, M., Ortuño, C., Blázquez, M.A. & Boira, H.
Title: Antioxidant activity of five satureja essential oils from the Mediterranean area of Spain.
Participation: Poster
Congress: International Symposium on Essential Oils
Place: 5-8 September 2012, Lisboa, Portugal. **Year**: 2012

Authors: Ortuño, C., Duong, T., Balaban, M. & Benedito, J.
Title: Combined High Hydrostatic Pressure and Carbon Dioxide Inactivation of Pectin Methylesterase, Polyphenol Oxidase and Peroxidase in Feijoa Puree
Participation: Oral presentation
Congress: 6t International Symposium on High Pressure Processes Technology
Place: 8-11 September 2013, Belgrade, Serbia. **Year**: 2013

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