J. of Supercritical Fluids 63 (2012) 8–15

Contents lists available at SciVerse ScienceDirect

The Journal of Supercritical Fluids

journal homepage: www.elsevier.com/locate/supflu

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

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ARTICLE INFO

Article history:
Received 18 July 2011
Received in revised form
19 December 2011
Accepted 20 December 2011

Keywords:
Supercritical carbon dioxide
Growth stage
Saccharomyces cerevisiae
Escherichia coli
Inactivation
Modeling

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 ($R^2_{avg.} = 0.96$; $RMSE_{avg.} = 0.48$) 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.

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 [1]; pulsed electric fields [2]; membrane filtration [3]; microwaves and radiofrequency [4] or supercritical fluids [5].

Supercritical carbon dioxide (SC-CO_2) has a liquid-like density, gas-like diffusivity and viscosity, and a zero surface tension [6]. It is, therefore, capable of penetrating into complex structures, which provides it with its ability to inactivate microorganisms [7]. Furthermore, the properties of SC-CO_2 make it an excellent solvent to be used in the food sector, especially in extraction processes [8].

SC-CO_2 showed promising results in the inactivation of microorganisms like *Saccharomyces cerevisiae* (*S. cerevisiae*) in apple juice [9], or naturally occurring microorganisms in liquid whole egg [10].

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 [11,12].

The resistance of a microorganism to different stresses, including high pressure processing, is known to be affected by the growth stage [13]. Hayman et al. [14] found a significant effect of the growth temperature (15 and 43 °C) 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 [15]. Both *E. coli* and *S. cerevisiae* are habitual components of the microbiota involved in food spoilage, with *E. coli* frequently

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doi:10.1016/j.supflu.2011.12.022

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 [16]. 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 [17]. 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 [18] and also the inactivation of *E. coli* by means of new preservation methods, such as high hydrostatic pressure [16]. Moreover, Liao et al. [19] 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: *end*A1 *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) [20], 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 °C, and *S. cerevisiae* was grown in Yeast Peptone Dextrose Broth (YPD Broth, Sigma-Aldrich, USA) at 30 °C, 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 °C 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) [21]. 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 °C. Four subcultures, prepared by inoculating 50 μl from the starter in 50 ml of sterilized medium, were incubated at 37 °C 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 °C overnight. Four subcultures, prepared by inoculating 100 μl from the starter in 50 ml of sterilized YPD Broth were incubated at 30 °C 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), late exponential phase (OD_{600} = 8) and early 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 °C for 48 h and *E. coli* at 37 °C 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 and a N_2-tank, which are kept at room temperature; a chiller reservoir stored at −18 °C; a pump and a thermostatic bath to keep the inactivation vessel 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.

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