Effect of isothermal and non-isothermal treatments on the viability and stress response of foodborne pathogen and spoilage microorganisms

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Resumen

Los tratamientos térmicos son el proceso de conservación más ampliamente utilizado en la industria alimentaria. Podría decirse que casi todos los alimentos reciben, al menos, un tratamiento térmico durante su manufacturación. Los objetivos de este trabajo fueron determinar el comportamiento de los microorganismos en condiciones isotérmicas y anisotérmicas(tanto en estático como en continuo), determinar el uso correcto de la combinación de calor y antimicrobianos naturales (tecnología de barreras) y encontrar los genes involucrados en la respuesta a los tratamientos térmicos. El uso de la tecnología de barreras se basa en aplicarlas de forma correcta. En este estudio se encontró que aplicar los compuestos antimicrobianos durante el tratamiento térmico no tenía efecto sobre la inactivación del microorganismo, mientras que su aplicación después del mismo, resultó en una reducción significativa del tiempo de tratamiento. El intercambiador de calor usado en esta investigación permite determinar el comportamiento de los microorganismos de forma más real. Los resultados obtenidos muestran que un ligero incremento en la velocidad de calentamiento resulta en una mayor inactivación. La síntesis *de novo* de proteínas y el uso de la cisteína para la estabilización de las proteínas celulares son mecanismos clave en la termorresistencia de *C. sakazakii*.

Palabras clave: Resistencia al calor; Intercambiador de calor; Respuesta genética; Microbiología predictiva.

Abstract

Thermal treatments are the most widely preservation technique used in the food industry. Almost, all food products are heated at least once. The objectives of this study were to determine the behavior of microorganisms under isothermal and non-isothermal heat treatments, to determine the microbial inactivation during a continuous heating process, to find the best balanced application of heat and natural antimicrobials (hurdles), and to determine the genes that could be involved in heat resistance. The basis of the hurdle technology is its correct use in the accurate way. Results show that the application of the natural antimicrobials in the heating medium do not have effect in the inactivation of the microorganism, while its application just before the thermal treatment results in greater inactivation, leading to a reduction of the treatment time. Results on the heat exchanger show that under non-isothermal treatments, a slight increase in the heating rate results in greater inactivation of microorganisms at the end of the process. The *de novo* protein synthesis and cysteine uptake for protein stabilization are key process in the heat resistance of *C. sakazakii*.

Keywords: Heat resistance; heat exchanger; genetic response; predictive microbiology.

1. Introduction

Heat treatment is used to produce safe and shelf stable foods and to eliminate pathogenic microorganisms. It is important to ensure that the food is adequately heat treated and to reduce post-processing contamination. The two most important issues connected with thermal processing are food safety and food quality. There are many conflicts between safety and quality issues. For example, microbial inactivation and food safety is increased by more severe heating conditions, but product quality in general deteriorates [1].

Microbial heat resistance determination performed under isothermal treatments help to set thermal treatments according to the microbial load of the food product being processed. However, industrial thermal treatments involves three distinct stages: heating, holding and cooling, and all three stages may contribute to the microbial inactivation. However, procedures are needed to evaluate the behavior of the microorganisms under a complete (three stages) and during each stage individually [1].

It has been believed that microbial inactivation follows a linear relationship, between the

decimal logarithm of the number of surviving microorganisms and the treatment time at a given temperature, but in many cases the obtained survival curves from thermal treatments show a non-linear relationship. In this case, lineal models are no longer valid, and non-lineal models, such as the one derived from the Weibull distribution, should be used. The advantage of the Weibull model is its simplicity, flexibility and its hardiness, giving the possibility of modeling linear and non-linear survival curves,

Also it is important to determine the response of microorganisms to inactivation treatments. Heat and other lethal agents cause damage to macromolecular cell components; thus the main function of stress proteins is to repair or destroy these damaged components so they do not disrupt cellular metabolism [2]. The heat-shock response is characterized by the induction of a large set of proteins as a result of a rapid increase in the environmental temperature [3].

The demand by consumers for high quality foods having "fresh" or "natural" characteristics has led to the development of foods that are preserved using mild technologies. Since microbial growth may occur at refrigeration temperatures, additional barriers (hurdles) are required to control spoilage and pathogenic microorganisms. The hurdle technology is the use of combined preservation factors (i.e. temperature, water activity, pH) for gentle, but effective, preservation of a variety of foods. To assure the microbiological safety and stability of healthful foods, it is necessary to apply balanced hurdles, achieving a hostile environment to inhibit their growth, shorten their survival or kill them, while not damaging the product's sensory and nutritional properties [4].

The objectives of this study were i) to determine the behavior of microorganisms under isothermal and non-isothermal heat treatments, ii) to determine the microbial inactivation during a continuous heating process, iii) to found the best balanced application of hurdles (heat and natural antimicrobials) and iv) to determine the genes that could be involved in heat resistance.

2. Materials & Methods

2.1 Microorganisms and in batch thermal treatments

Alicyclobacillus acidoterrestris DSM 3922, *Cronobacter sakazakii* DPC 6529, *Staphylococcus aureus* CECT 86T and *Salmonella* Senftenberg CECT 4565 where used in this study. In batch thermal treatments where carried out as described in [5] and [6].

2.2 Thermal treatments on a continuous heating system

Thermal treatments where carried out on a tubular heat exchanger. The description of the equipment and the methodology is described in [7].

2.3 Bacterial response to heat treatments

The methodology used for the determination of the genes involved in the heat resistance is described in [6].

2.4 Data analysis

Data analysis performed to the obtained results is described on [5], [6] and [7].

3. Results and Discussion

The combination of heat treatments and natural antimicrobials has been widely studied, and a synergistic effect on the control of pathogenic and spoilage microorganisms has been shown. The simple addition and combination of preservative factors (hurdles) may not lead to achieve proper food preservation. It is necessary to determine the best order of apply the hurdles, in order to assure food safety and stability. The addition of nisin and citral to the heating and recovery medium in combination with a mild thermal treatment (95ºC) for control of *A. acidoterrestris* has been tested. Fig. 1 shows that the application of a thermal treatment at 95ºC for 2.5 min, followed by the addition of nisin (0.3 mg L^{-1}) and citral (0.34 mM) could inhibit the germination or outgrowth of *A. acidoterrestris* spores, reducing the risk of spoilage by this microorganism. When nisin and citral were added to the heating medium no effect was found.

The right application of the hurdles is not the only parameter to be determined. Accurate calculation of thermal inactivation kinetics is very important to determine the treatment time and temperature to apply. The classic *D* values were used to determine the inactivation kinetics of *A. acidoterrestris* because it followed a linear relationship at all the temperatures tested (Fig. 1). On the other hand the inactivation kinetics of *C. sakazakii* did not show a linear relationship (Fig. 2). This microorganism presented a tailing phenomenon, so the classic *D* value is not accurate to describe its thermal resistance.

Similar results were found for *S. auereus* and *Salmonella* Senftenberg. Calculations of the thermal resistance for these microorganisms were done by applying the Weibull model. The use of this model resulted in a better calculation of the heat resistance under isothermal treatments, which is necessary in order to predict accurately the behavior of microorganisms under non-isothermal treatments.

Nowadays many products are processed in continuous heating systems due to their many advantages [1]. The most common methods used to determine the effect of non-isothermal treatments on the heat inactivation of the microorganisms use batch heating systems (*i.e.* thermoresistometer Mastia, open vials, capillary tubes) to mimic industrial continuous heat treatments. Some authors have used tubular heat exchangers, but these equipments just enable to measure temperature and take samples at the inlet and outlet of the process. These methods do not allow to know the temperature profile and the inactivation kinetics of the microorganisms during the process. Therefore the effect of the heating rates on microorganism inactivation, throughout a process on a continuous heating system, or the behavior of the microbial population inside the system is on a black-box [7]. The heat exchanger used in this investigation provides reliable information about the heating profile during the whole process, permitting to know the heating rate in each section of the equipment, as well as to take samples during the whole process, which enables to plot reliable survival curves. Fig. 3 shows the differences between the inactivation levels reached when using the thermorresistometer Mastia and the heat exchanger, for *S. aureus* and *S.* Senftenberg. For both microorganisms, lower levels of survivors were found at the end of the thermal treatment in the heat exchanger. The treatment temperature in the heat exchanger in some points was slightly higher than in the thermoresistometer, and this could lead to the higher inactivation in the heat exchanger than in the thermoresistometer.

Transposon mutagenesis allowed the identification of some of the molecular mechanisms involved in the response of *C. sakazakii* DPC6529 to heat stress. A transposon mutants library with a total of 2,400 mutants was screened. After a selection step, 28 mutants were found to show a significant decrease in heat resistance as compared to the wild type. These mutants were tested in the thermoresistometer, and only two of them (mutants 7 and 10) showed a significantly higher sensitivity to heat, when compared to the wild type (Fig. 2). Disrupted genes identified for mutant 7 and 10 encoded the ribosome maturation protein RimP and outer membrane porin L (OmpL), respectively. Results suggest that *de novo* protein synthesis, and the uptake of cysteine for the formation of disulfide bonds in proteins for its stabilization, are key processes on heat resistance.

4. Conclusions

The use of the hurdle technology is not just based on the addition of the hurdles, but is based in how to add the hurdles to achieve the best effect on the product safety and preservation. As shown for *A. acidoterrestis,* the addition of nisin and citral after a thermal treatment, led to greater inactivation of the microorganism, enabling to reduce the exposure time to the treatment. It has been shown that the use of adequate models to describe the heat resistance of microorganisms leads to a proper calculation of the thermal treatments intended to be applied for food preservation, avoiding over or under processing of food products. The heat exchanger used in this investigation permit to better understand the inactivation kinetics of microorganisms under continuous heating process, allowing to determine the effect of industrial treatments under a more realistic scenario. The current study also shed a light in the molecular mechanisms involved in the cellular response of *C. sakazakii* to thermal treatments, suggesting that de novo protein synthesis and cysteine uptake for protein stabilization are key process in the heat resistance of this microorganism.

5. Aknowlegments

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Figures

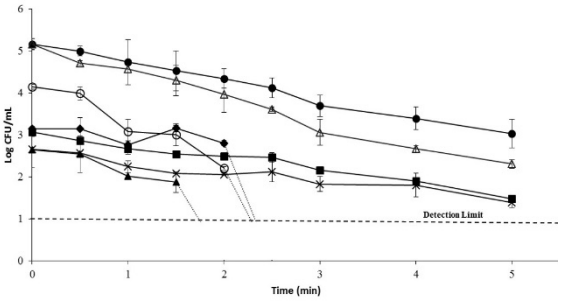

Figure 1. *Survival curves of A. acidoterrestris with the antimicrobials in the recovery medium at 95º C in pH 3.5 McIlvine buffer. Control (●), 0.3 mM nisin (▪),1.5 mM nisin (×), 0.69 mM Citral (Δ), 0.3 mM nisin + 0.34 mM citral (•), 0.3 mM nisin + 0.69 mM citral (○), 1.5 mM nisin + 0.69 mM citral (▲).*

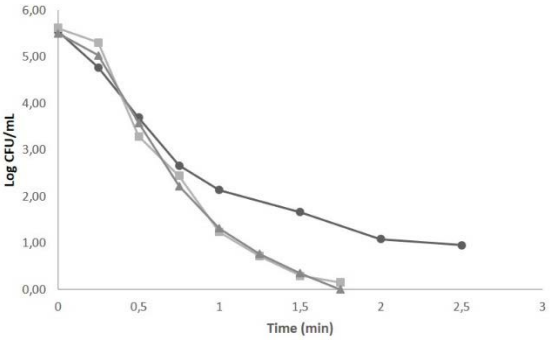

Figure 2. *Thermal resistance of C. sakazakii DPC6529 (●), transposon mutant 7 (■) and transposon mutant 10 (▲) in an isothermal treatment at 58ºC.*

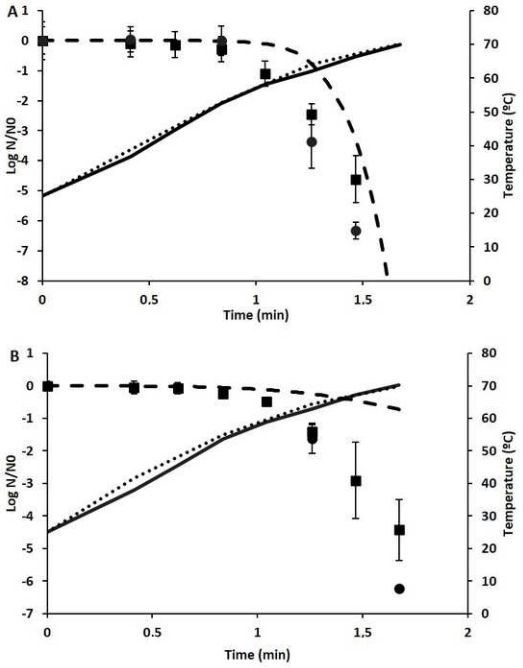

Figure 3. *Survival curves of S. aureus (a) and Salmonella seftenberg (b) in the heat exchanger under a flow of 700 mL/min (●) together with the expected inactivation lines (dashed line) and the corresponding temperature profiles (dotted line) and in the thermoresistometer (▪) mimicking heat exchanger heating profile (continuous line).*