INACTIVATION KINETICS OF VIBRIO PARAHAEMOLYTICUS NATURALLY OCCURRING IN OYSTERS USING SUPERCRITICAL CARBON DIOXIDE TREATMENT

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Abstract. *Vibrio parahaemolyticus* has been involved in a number of food borne outbreaks related to the consumption of raw oysters. As a direct consequence of filter-feeding in often contaminated water, oysters can be loaded with pathogenic microorganisms. The use of supercritical carbon dioxide ($scCO_2$) treatment has increased in importance as an innovative preservation method particularly due to the bactericidal effect of $scCO_2$. This work aims at evaluating the kinetics of $scCO_2$ inactivation of *Vibrio parahaemolyticus* naturally occurring in raw oysters. For this purpose, raw material was exposed to $scCO_2$ (1:0.8 $scCO_2$/product mass ratio) during 0.25, 0.5, 1.0, 1.5, 2.0, 3.0, 4.0, 5.0 and 6.0 hours, at 34°C, submitted to processing pressures from 80 bar up to 200 bar, considering a pressurization and depressurization rate of 100bar/min. All the experiments were done in duplicate. Results showed that an increase in time processing enhanced *Vibrio parahaemolyticus* inactivation in oysters. Treatments at 0.25 and 0.5 hours induced a reduction of approximately 75% of the initial count of *Vibrio parahaemolyticus*. A greater bacteria inactivation level was observed starting from 1.5 hour of treatment. A complete reduction of the initial count of *Vibrio parahaemolyticus* was observed starting at 3.0 hours of treatment. Results obtained in this work confirm the $scCO_2$ capabilities in reducing the *Vibrio parahaemolyticus* load in oysters, which enhanced product safety and has the potential of increasing its shelf life.

Keywords: oyster, carbon dioxide, inactivation, *Vibrio parahaemolyticus*.

1. Introduction

Oysters grown on the coast of Santa Catarina state, in the south region of Brazil, are generally marketed fresh. Its processing is often rudimentary without sanitary control. Besides, oysters are perishable and have a short shelf-life, restricted to only 3 to 5 days (PORTELLA, 2005).

In general, the microbiological characteristics of mollusks vary with the geographical locations of crops, different habitats, method of production, handling practices, environmental conditions, and post-harvest procedures. The microbiological status of oysters after harvesting is directly related to the quality of water, which is also influenced by temperature and salinity, the distance between the place of cultivation and areas polluted with fecal material (close to shore, discharge of sewage, etc.) or the occurrence of bacteria naturally

present in the water (FELDHUSEN, 2000). Therefore, the sanitary quality of water for cultivating mollusks aspect is essential to obtain a safe product.

The potential hazards related to foodborne diseases of marine origin vary according to different factors and include: trematode infections, toxic marine algal blooms, diseases associated with pathogenic bacteria and viruses, waste veterinary pharmaceuticals, pesticide contamination and heavy metals.

Among the pathogenic microorganisms *V. parahaemolyticus, V. cholerae, Salmonella enterica, Aeromonas* spp. *and Plesiomonas* spp. have often been identified in mollusks, and *V. parahaemolyticus* has been indicated as a major cause of disease outbreaks related to the consumption of bivalve mollusks in the world (TEPLITSKI; WRIGHT; LORCA, 2009). *Vibrio parahaemolyticus* occurs naturally in coastal and oceanic waters. In most areas, *V. parahaemolyticus* is most numerous in the environment during the warmer months, so most outbreaks occur during this period.

In this context, the use of nonthermal processes in oysters is an alternative to conventional thermal treatment, to improve the microbiological quality of the product, preserving the sensory characteristics, and promote increased shelf-life, facilitating their marketing, handling and shipping. Treatment with pressurized carbon dioxide has been suggested as a promising option to the use of the pasteurization of food, mainly due to potential to inactivate microorganisms. The aim of this study was to evaluate the kinetics inactivation of naturally occurring on *Vibrio parahaemolyticus* using $scCO_2$ in oysters cultivated in Florianópolis city, in the south region of Brazil.

2.   Material and Methods

2.1   Raw material

Fresh oysters (*Crassostrea gigas*) cultivated in Florianópolis, Santa Catarina, were obtained at a local market. Samples were placed in an isothermal box containing ice packs and they were transported to the Laboratory of Physical Properties of Food, of the Federal University of Santa Catarina. A total of 24 (twenty four) units of fresh oysters were used for each experiment. Oysters that were not tightly closed shells were discarded.

Samples were washed in running water with a brush to remove dirt adhered in shells. After cleaning, oysters were sanitized with 70% alcohol, and the meat removed manually, using sterile forceps and scissors and the oyster meat part and the liquid were placed in a beaker previously sanitized. Then meat and the liquid were disintegrated in Turrax equipment (TE Turatec-102), for about four minutes using 18,000 rpm. Then, it was weighed a 10 g of the homogenized sample with the aid of a disposable syringe. The sample was kept cool by the time of cell injection in the equipment described in 2.2. The experiments were performed from March of 2012 to February of 2013.

2.2   Experimental apparatus and procedure

Inactivation experiments were conducted employing the static-synthetic method in a high-pressure variable-volume view cell. The experimental apparatus is depicted in Figure 1. Briefly, the experimental set-up consists of a variable-volume view cell, with a maximum internal volume of 27 mL, with two sapphire windows for visual observation, an absolute pressure transducer (±0.3 bar, Smar LD 301), , a data system acquisition of pressure portable programmer (Smar, HT 201) and a syringe pump (ISCO 260D). The cell contains a movable piston, which permits the internal pressure control. Initially, 25 mL of oyster sample was loaded into the cell with help of sterile syringe. The charge of $scCO_2$ was performed with the help of the syringe pump (resulting accuracy of 0.005 g in CO_2 loadings). The mass ratio of $scCO_2$ to oyster sample used in this work was 1:0.8. The temperature control was performed with water using a thermostatic bath coupled at jacket surrounds the cell, so that the cell content was kept at 34°C. The pressure system was increased at pressurization rate of 100 bar/min using $scCO_2$ as pressurizing fluid from its vapor pressure at room (laboratory) temperature (±50 bar) up to 80 bar, which was considered the initial working pressure. At this point the system was hold for a short period (±1 min) to allow system stabilization, and then the pressure was increased until 200 bar and, again the system was hold for certain short period to allow system stabilization. After this procedure, the system pressure was decreased down to 80 bar at the depressurization rate pre-established in the experimental design. This procedure was repeated according to the number of pressure cycles determined by the experimental design.
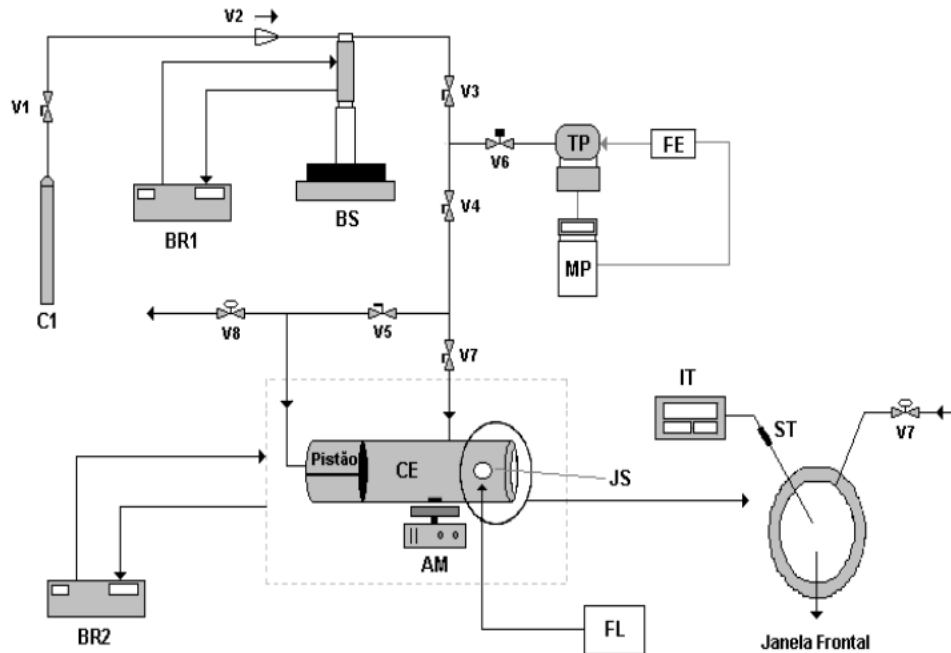
Figure 1. Scheme of the experimental setup.

2.3 Preliminary assays

The effects of the mass product to CO_2 ratio, the depressurization rate and the number of pressure cycles were evaluated by means of a Central Composite Design (CCD). The levels of each independent variable of the CCD are presented in Table 1. All results were analyzed using Statistica® 7.0 (Statsoft Inc., Tulsa, OK, USA), considering a significance level of 95% ($p < 0.05$).

Table 1. Level of independent variables investigated in the CCD* to inactivate Aerobic mesophilic, *Vibrio* ssp. and *Vibrio parahaemolyticus* using $scCO_2$

Variables	Levels		
	-1	0	+1
Mass of product to CO_2 ratio (product:CO_2)	1:0.2	1:0.5	1:0.8
Depressurization rate (bar/min)	10	55	100
Pressure cycles	1	3	5

* For the CCD, the treatment time for all runs was always 2 hours.

Before and after each treatment Aerobic mesophilic count (TCB media), total *Vibrio* ssp. count and *V. parahaemolyticus* count (TCBS media) were determined by standard plating techniques according to APHA (2001). Microbiological analyses were performed in duplicate.

2.4 Kinects of inactivation of Vibrio parahaemolyticus naturally occurring in oysters

To evaluate the effect of treatment time on the inactivation of *V. parahaemolyticus*, naturally present in fresh oyster, by use of supercritical CO_2, it was performed an inactivation kinetics with 15, 30, 60, 90, 120, 180, 240, 300 and 360 minutes (process time), with one (1) pressure cycle, product/CO_2 mass ratio of 1:0.8, pressurization and depressurization rates of 100 bar/min (best experimental condition - obtained in 2.3).

Before and after each treatment *V. parahaemolyticus* count (TCBS media) was determined by standard plating techniques. Microbiological analyses were performed in duplicate.

3.   Results and Discussion

Results concerning the number of survivals after scCO$_2$ inactivation of Aerobic mesophilic count, *Vibrio* ssp. and *V. parahaemolyticus* obtained in the CCD are presented at Tables 2, 3 and 4, respectively.

Table 2 presents the results of the effects of pressure, depressurization rate and pressure cycle on inactivation of Aerobic mesophilic. Results presented in this table show that the number of survivors varied greatly after the high-pressure treatment, with observed values of log-reduction of 0.01 (run 3) until almost full inactivation (runs 1 and 5), with 2 log-reduction.

Table 2. Matrix of the experimental results obtained in the CCD to inactivate Aerobic mesophilic count using scCO$_2$

Run	Mass ratio (product:CO$_2$)	Depressurization rate (bar/min)	Pressure cycles	% reduction	Log-reduction
1	-1 (1:0.2)	-1 (10)	-1 (1)	99.41	2.23
2	+1 (1:0.8)	-1 (10)	-1 (1)	6.55	0.02
3	-1 (1:0.2)	+1 (100)	-1 (1)	4.24	0.01
4	+1 (1:0.8)	+1 (100)	-1 (1)	94.13	1.22
5	-1 (1:0.2)	-1 (10)	+1 (5)	99.56	2.35
6	+1 (1:0.8)	-1 (10)	+1 (5)	58.05	0.37
7	-1 (1:0.2)	+1 (100)	+1 (5)	92.80	1.13
8	+1 (1:0.8)	+1 (100)	+1 (5)	50.0	0.30
9	0 (1:0.5)	0 (55)	0 (3)	54.54	0.33
10	0 (1:0.5)	0 (55)	0 (3)	79.61	0.68
11	0 (1:0.5)	0 (55)	0 (3)	62.50	0.42

Results presented in Table 2 were used to compute the main effects of the independent variables on the inactivation of the Aerobic mesophilic count, showed by Pareto chart at 95% of confidence level. Fig. 2 shows that the mass ratio and the depressurization rate were the significant variables (p < 0.05) on the microorganism inactivation, while the number of pressure cycle did not present a significant effect on the range evaluated.
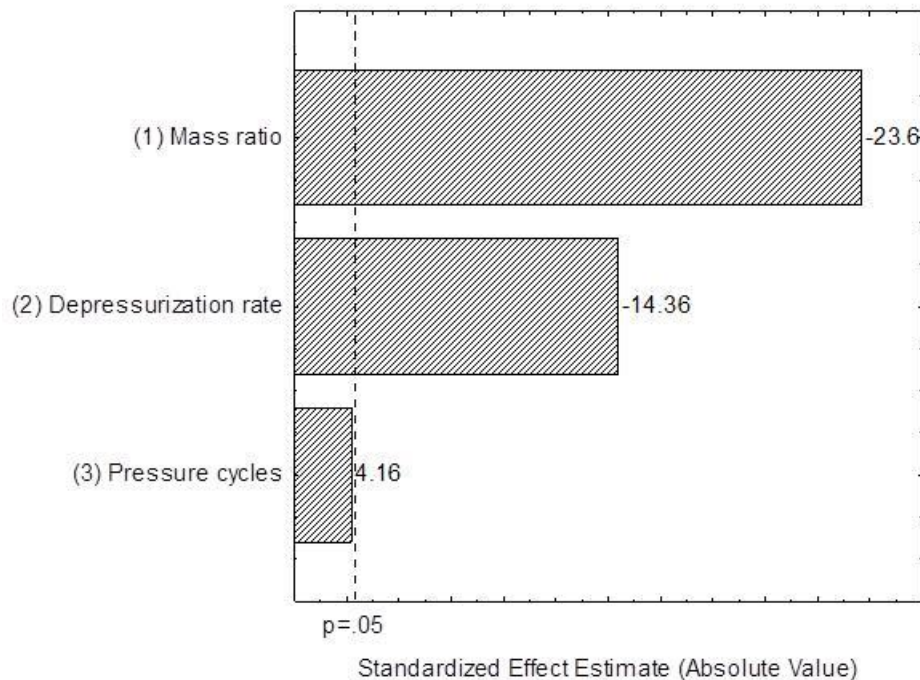

Figure 2. Effects of the independent variables on microbial survivals after scCO$_2$ inactivation of total aerobic count.

Considering the reduction in the total count of *Vibrio* ssp. (Table 3), runs 7, 4 and 3 had the greatest inactivation degree, with a reduction of 2.48, 2.33 and 2.17 log cycles respectively. Inspection the set of runs from 1 to 4, it can be observed that an increase in the depressurization rate resulted in a positive effect in reducing the count of *Vibrio* ssp. naturally present in the samples. On the other hand, considering a larger number of pressure cycles (runs 5 to 8), the increase in the depressurization rate improved the inactivation only when it was used a lower product to CO_2 ratio.

Table 3. Matrix of the experimental results obtained in the CCD to inactivate total *Vibrio* count using $scCO_2$

Run	Mass ratio (product:CO_2)	Depressurization rate (bar/min)	Pressure cycles	% reduction	Log-reduction
1	-1 (1:0.2)	-1 (10)	-1 (1)	43.68	0.25
2	+1 (1:0.8)	-1 (10)	-1 (1)	66.35	0.46
3	-1 (1:0.2)	+1 (100)	-1 (1)	99.31	2.17
4	+1 (1:0.8)	+1 (100)	-1 (1)	99.54	2.33
5	-1 (1:0.2)	-1 (10)	+1 (5)	68.02	0.48
6	+1 (1:0.8)	-1 (10)	+1 (5)	96.03	1.30
7	-1 (1:0.2)	+1 (100)	+1 (5)	99.67	2.48
8	+1 (1:0.8)	+1 (100)	+1 (5)	87.04	0.87
9	0 (1:0.5)	0 (55)	0 (3)	57.94	0.36
10	0 (1:0.5)	0 (55)	0 (3)	62.72	0.41
11	0 (1:0.5)	0 (55)	0 (3)	59.84	0.40

The variable number of cycles presented no significant effect ($p < 0.05$) in the inactivation of the total count of *Vibrio* ssp. naturally present in oysters. Also for this case, the stress caused by the possible use of pressurization and depressurization cycles showed no significant effect on reducing these bacteria. The use of cycles of pressurization and depressurization reduces the time in which the sample remains under the effect of the higher process pressure (200 bar), i.e., the condition in principle most adverse. Thus, reduction in the count of *Vibrio* ssp. under high pressure (200 bar) for a longer time, has a greater effect on the inactivation of the microorganism. The other variables showed significant effect in reducing *Vibrio* ssp. sample. That is, the higher the depressurization rate, the more inactivation (consequently, the greater the time that the sample remains in the upper process pressure 200 bar), and the larger the product:CO_2 ratio, the lower the inactivation of *Vibrio* ssp.
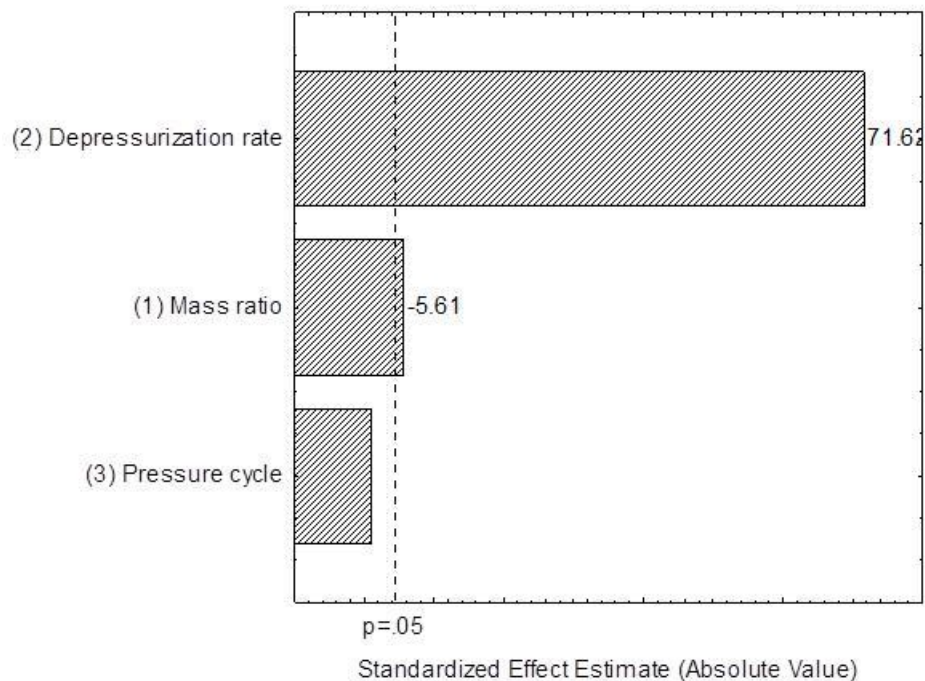

Figure 3. Effects of independent variables on microbial survivals after the $scCO_2$ inactivation of total *Vibrio* ssp. count.

The inactivation of the *V. Parahaemolyticus* is show in the Table 4. Runs 4 and 8 presented levels of reduction very close, of 1.30 and 1.35 logarithmic cycles, representing 95.04% and 95.65% reduction of the initial count presented in the sample.

For *V. parahaemolyticus* inactivation, the increase in the product:CO_2 ratio showed the good results independent of the condition of number cycles pressure and depressurization rate values. For a given mass ratio, increasing the depressurization rate increased the log reduction. Finally, increasing the number of pressure cycles resulted in a slight improvement in all conditions, except for the run 7.

From the Pareto chart (Figure 4), we can see that depressurization rate had significant positive effect on the inactivation of *V. parahaemolyticus* in oysters. That is, an increase in the depressurization rate improves the inactivation degree of the bacteria. This result may be related to the fact that, first, the CO_2 is solubilized within microbial cell and promotes a rapid depressurization abrupt expansion of CO_2, causing destruction of cells and thus inactivation of the microorganism. The number of cycles of pressurization and depressurization also showed significant effect in reducing *V. parahaemolyticus*, indicating that the use of pressurization and depressurization steps can cause stress on the microorganism, and consequently its death. Spilimbergo, Elvassore & Bertucco (2002) and Melo Silva (2013) also found that the number of pressure cycles was beneficial to the inactivation of a pure culture of *Pseudomonas aeruginosa* and *B. subtilis* and *E. coli*, respectively. The variable product:CO_2 ratio also showed significant effect on reducing *V. parahaemolyticus*. Moreover, Soares et al. (2013), when studied the inactivation of *L. monocytogenes*, found no significant positive effect of the variable inoculum:CO_2 ratio.

Table 4. Matrix of the experimental results obtained in the CCD to inactivate *Vibrio parahaemolyticus* count using scCO_2

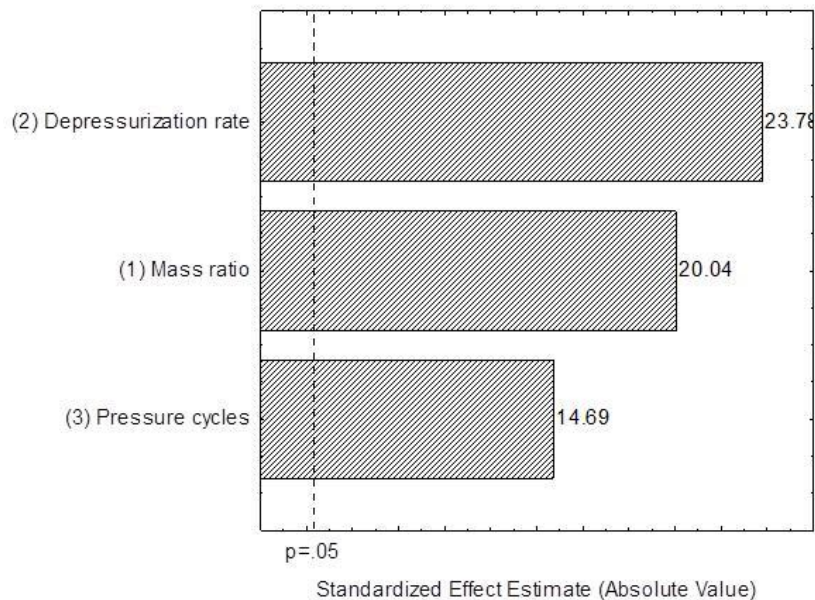| Run	Mass ratio (product:CO_2)	Depressurization rate (bar/min)	Pressure cycles	% reduction	Log-reduction
1	-1 (1:0.2)	-1 (10)	-1 (1)	20.82	0.10
2	+1 (1:0.8)	-1 (10)	-1 (1)	61.94	0.41
3	-1 (1:0.2)	+1 (100)	-1 (1)	82.29	0.74
4	+1 (1:0.8)	+1 (100)	-1 (1)	95.04	1.30
5	-1 (1:0.2)	-1 (10)	+1 (5)	87.92	0.23
6	+1 (1:0.8)	-1 (10)	+1 (5)	84.07	0.79
7	-1 (1:0.2)	+1 (100)	+1 (5)	75.00	0.60
8	+1 (1:0.8)	+1 (100)	+1 (5)	95.65	1.35
9	0 (1:0.5)	0 (55)	0 (3)	47.82	0.27
10	0 (1:0.5)	0 (55)	0 (3)	42.85	0.23
11	0 (1:0.5)	0 (55)	0 (3)	40.91	0.22

Figure 4. Effects of independent variables on microbial survivals after the scCO_2 inactivation of *Vibrio parahaemolyticus*.

The results of reduction of Aerobic mesophilic, *Vibrio* ssp. and *V. parahaemolyticus* were analyzed simultaneously to define the best process condition. The choice was based on the need to eliminate the target microorganism of this product, *V. parahaemolyticus*.

Although the run 8 (product:CO_2 ratio = 1:0.8; depressurization rate = 100 bar / min, 5 cycles of pressurization and depressurization) has provided the greatest reduction in initial count of *V. parahaemolyticus* (1.35 log cycles), this value was very close to the reduction achieved in run 4 (1.30 log cycles). The difference between the runs 4 and 8 is therefore related to the number of pressure cycles. Run 4 used only one cycle of pressurization and depressurization. Moreover, considering greater operational ease and economy of process (initial investment and maintenance) when it is used a single cycle of pressurization and depressurization, run 4 was selected as a potential condition to inactivate *V. parahaemolyticus* in oysters. Thus, recalling the results observed for the reduction of Aerobic mesophilic and *Vibrio* ssp. Only the condition of the run 4 showed reductions logarithmic values relevant to these two counts. Therefore, considering that the run 4 was effective for the reduction of the three counts (Aerobic Mesophilic, *Vibrio* ssp. and *Vibrio parahaemolyticus*), this condition was selected for the experiments of inactivation kinetics.

The complete inactivation of the initial load of *V. parahaemolyticus* naturally present in oyster was obtained at three hours of processing, corresponding to a decrease of two log cycles (Figure 5).
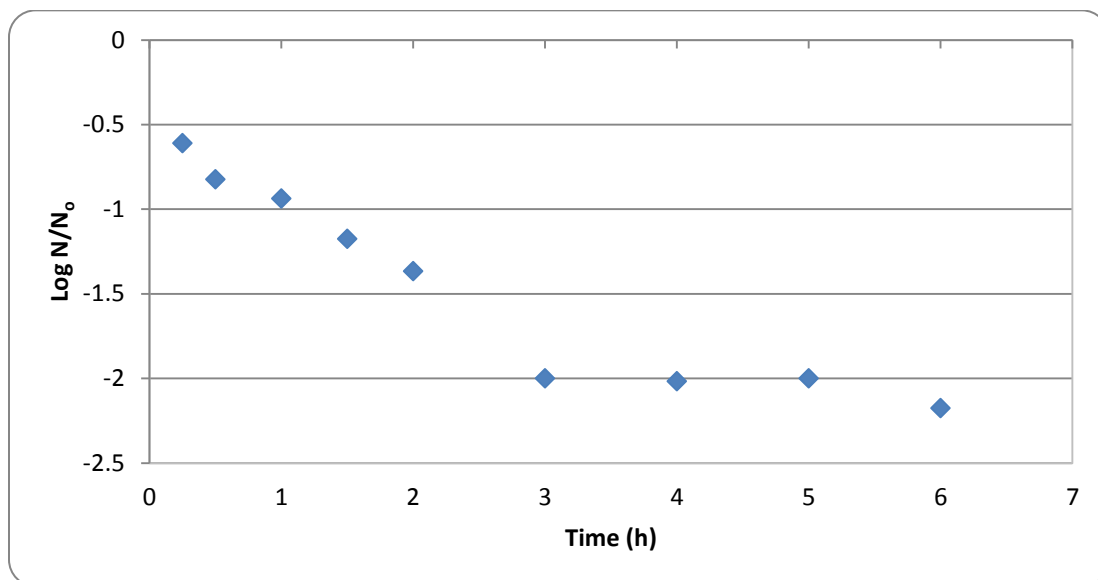

Figure 5. Kinectis inactivation of *V. parahaemolyticus* naturally presented in fresh oysters.

Soares et al. (2013), when studied the inactivation of *L. monocytogenes* (pure culture), observed that the time required for a complete reduction of the initial count of bacteria was approximately 3-hour process. Erkemen and Karaman (2001) studied the inactivation of *S. typhimurium* in two different ways and concluded that the composition of the media influences the inactivation of this microorganism and that a high concentration of solute in the middle has a protective effect for this species of Salmonella.

4. Conclusion

Results presented in this work confirm the $scCO_2$ capabilities in reducing the *Vibrio parahaemolyticus* load in oysters, which enhance the product's safety and it has the potential of increasing shelf life, hence a promising technology for microbial inactivation.

Depressurization rate, product:CO_2 ratio and the pressure cycles number presented positive significant effect on the inactivation of *Vibrio parahaemolyticus*. The best experimental condition to inactivate the microorganisms naturally occurring in oysters was mass of product to CO_2 ratio of 1:0.8, depressurization rate of 100 bar/min, and 1 cycle of pressurization and depressurization, at 34°C for 2 hours of processing. In conclusion, results obtained in this work are valuable to develop a platform for an effective nonthermal

sterilization of food in pilot/industrial scale, and it can be adopted by the shellfish industry as a post-harvest processing to minimize *V. parahaemolyticus* infection associated with raw oyster consumption.

Acknowledgements

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