New evaluation methods for safety and quality of pressure treated foods

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NEW EVALUATION METHODS FOR SAFETY AND QUALITY OF PRESSURE TREATED FOODS

Laboratory of Food Technology

Laboratory of Food Microbiology

Faculty of Agricultural and Applied Biological Sciences

Katholieke Universiteit Leuven

Prof. dr. ir. M. Hendrickx (promotor), Prof. dr. ir. C. Michiels, dr. ir. L. Ludikhuyze

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I. INTRODUCTION

During the last decade, the growing consumer demand for high-quality, fresh like foods has stimulated research for new preservation methods, minimally affecting quality of foods. In this context, it has become established that high hydrostatic pressure has a potential as a new unit operation in food processing and preservation (Gould, 1995; Barbosa-Canovas *et al.*, 1997). It has been shown that high pressure can inactivate micro-organisms and enzymes while nutritional and sensorial quality aspects are only slightly affected (Cheftel, 1991; Knorr, 1993). Despite these promising results and the small-scale production of some pressure-processed fruit products in Japan, France and the USA (Mertens and Deplace, 1993; Cheftel and Culioli, 1997), large-scale industrial implementation of this new technology is awaiting the development of a generic scientific basis to assess the impact of high pressure processes on safety and quality of food products. From a legislative point of view, industrial application of high pressure technology requires processes to be designed, implemented and carried out according to existing guidelines (HACCP in Europe) to ensure that safe products are delivered. Besides optimal quality of products has to be considered with respect to consumer acceptance (Barbosa-Canovas *et al.*, 1997). In this framework, development of new and improved measuring and control methods are required to define standards that can be applied to preservation of food products by high hydrostatic pressure on a European level.

Especially in Japan, food, pharmaceutical, and biotechnological industries have made considerable research and development efforts in the framework of applying high pressure technology as a unit operation for preservation and processing. Currently also in Europe and the United states, several research projects have been initiated, funded by the European Commission, the governments, national financial institutes and the industry. Indeed several technological questions concerning required capacity, process time, process control, safety, maintenance and cleaning, packaging, and investment costs, still have to be answered.

The general objectives of this project are to develop and to validate methods to quantify the impact of combined high pressure/temperature processes on safety and quality of food products. By analogy with the methods available for traditional thermal processes, two possibilities will be evaluated. In the first method, process conditions (pressure, temperature, time) will be recorded and interpreted in terms of process impact by means of calculation procedures. In the second method, it will be endeavoured to develop and use intrinsic or extrinsic process indicators for process impact determination. Hence, the project will mainly focus on measuring and gathering kinetic data for combined high pressure/temperature inactivation of micro-organisms and enzymes, which could be useful as indicators for safety

and quality of food products. The two main aspects dealt with in this project are product quality involving enzyme inactivation on the one hand and product safety involving microbial inactivation on the other hand and they correspond respectively to the tasks of partner 1 and 2.

II. METHODOLOGY

To reach the specific objectives of this project, the following tasks will be implemented. In a first task, a series of micro-organisms relevant for safety and/or spoilage of acid food products will be investigated with respect to their resistance against combined pressure/temperature processes. This will include moulds with heat resistant ascospores, yeasts and lactic acid bacteria. Initial screening will be carried out in simple buffer media and for a restricted set of pressure-temperature combinations. Selection of one reference organism will be based on a high pressure-temperature stability over the entire pressure-temperature domain studied and on the simplicity of the kinetic model describing adequately the inactivation kinetics. In a final stage, a detailed kinetic study on inactivation op this reference organism under isobaric-isothermal conditions will be accomplished. In a second task, enzyme systems will be evaluated as potential intrinsic and extrinsic indicators for high pressure treatment of fruits and vegetables (α-amylase, peroxidase, lipoxygenase, polyphenoloxidase, myrosinase and pectinmethylesterase). Like for micro-organisms, detailed kinetic studies will be carried out on isobaric-isothermal inactivation of the selected enzymes. Both for micro-organisms and enzymes, simple buffer systems will be used in a first phase, before proceeding to more complex media (different pH values, addition of food compounds such as sugars), simulating real food systems.

All these experiments will be carried out in a multivessel high pressure equipment consisting of eight individual thermostated pressure vessels. This equipment allows to combine pressure between 0.1 and 1000 MPa with temperatures ranging from 0 to 100°C.

Based on the data of isobaric-isothermal inactivation of micro-organisms and enzymes, mathematical models will be developed to describe the course of inactivation as a function of treatment time at constant pressure and temperature. Furthermore it will be endeavoured to formulate a global mathematical model expressing the combined pressure and temperature dependency of the time-dependent parameters (inactivation rate constants). Hereto, existing statistical techniques and software (SAS, 1982) will be used (task 3). Finally in task 4, two test methods to determine the impact of high pressure processes will be evaluated. In the first method, the models mentioned above will be evaluated for variable pressure and temperature conditions. In this case, the impact determined experimentally will be compared with the impact calculated according to the models and concomitant parameters in order to evaluate the validity of this "physical-mathematical" approach. In the second method, some enzymatic

and microbial systems will be selected and evaluated as potential indicators for the impact of pressure processes.

III. RESULTS AND DISCUSSION

Task 1: Microbial inactivation kinetics

In this project, firstly a collection of micro-organisms relevant for spoilage/safety of acid foods were screened for resistance to combined pressure-temperature treatments. In a second phase, more detailed kinetic studies were accomplished for some resistant micro-organisms. The results obtained are briefly discussed below. More detailed results are included in annexes 1.1, 1.2, 1.3 and 1.4.

Screening and selection of relevant micro-organisms (annex 1.1 and 1.2)

A collection of micro-organisms relevant for spoilage/safety of acid foods was screened for resistance to pressure treatments at 20°C. This collection included (i) the moulds *Talaromyces macrosporus*, *Byssochlamys nivea* and *Neosartorya hiratsukae* which can cause spoilage of heat treated fruit juices and are forming heat-resistant ascospores; (ii) the yeasts *Pichia membranifaciens*, *Zygosaccharomyces rouxii*, *Zygosaccharomyces bailii*, *Zygoascus hellenicus*, *Rhodotorula mucilaginosa*, *Schizosaccharomyces pombe*, *Candida lypolitica* and *Torulopsis magni* which are mainly involved in spoilage of acidic (fruit) products; (iii) a number of lactic acid bacteria isolated from acidic products.

Ascospores of *T. macrosporus* were found to be the most pressure-resistant organisms. Among the vegetative organisms, *Zygoascus hellenicus, Zygosaccharomyces bailii* and *Lactobacillus collinoides 95* had the highest pressure resistance. Finally, *Z. bailii* was selected for detailed kinetic studies on pressure-temperature inactivation because (i) it is a more frequent cause of spoilage than *T.* Macrosporus, *Z. hellenicus*, and *L. collinoides* (ii) it can grow in acid (pH 2,5) products, and is resistant to high concentrations of the preservatives benzoate and sorbic acid and (iii) the ascospore suspensions of *T. macrosporus* are highly heterogeneous, displaying highly different susceptibility to HP activation and inactivation. Therefore, it would be very difficult to collect reliable and reproducible HP inactivation data of *T. Macrosporus* ascospores. Nevertheless, the influence of high pressure on these ascospores was studied in a semi-quantitative way to identify the most important parameters affecting HP resistance of the ascospores.

Finally, a kinetic analysis was also carried out on pressure resistant mutants of *Escherichia coli* MG1655. Although they cannot grow at low pH, pathogenic *E. coli* can survive for

several days or even weeks in acidic foods. Due to their low infective dose, these surviving cells can form a health threat.

Zygosaccharomyces bailii (annex 1.2)

Isobaric-isothermal inactivation of *Z. bailii* was investigated in the pressure range of 120 to 320 MPa at temperatures between –5 and 45°C in a buffer of pH 6.5. For all the pressure-temperature combinations, the inactivation could be described by a first order decay down to 10^2-10^3 survivors. At constant temperature, the inactivation rate increases with increasing pressure. On the other hand, at constant pressure, the inactivation occurs more rapidly at temperatures lower and higher than 10-20°C. At -5°C en +45°C, in comparison to moderate temperatures, the z_p-values (pressure dependence of D-value) decreases pointing to an enhanced pressure sensitivity at these extreme temperatures. Z_t-values on the other hand are negative at temperatures below 20°C and positive for temperatures higher than 20°C.
Compared to a buffer system of pH 6,5, the pressure inactivation at 20°C of *Z. bailii* in apple juice (pH 3.21) and in orange juice (pH 3.66) is higher. However in buffer (pH 3-6), there was no significant pH-effect at 20°C, while at low (10°C) and high (45°C) temperature, the inactivation at pH 3 was higher.

Talaromyces macrosporus ascospores (annex 1.3)

Ascospore suspensions were subjected to treatments at 200-700 MPa. Net pressure inactivation occurred only at very high pressures (500–700 MPa). However, the inactivation curves showed a substantial amount of tailing, indicating a large heterogeneity of the ascospore population towards high pressure inactivation. Although direct inactivation by HP at 20°C was very limited, treatment at ≥500 MPa resulted in sensitization of the spores to subsequent heat treatment (80°C/30 min). The level of heat sensitization rapidly reached a plateau (15 sec pressure treatment), and was not substantially increased upon prolonged pressure treatment. Further, it was found that this sensitization is probably the result of ascospore germination, induced by HP treatment. One of the mechanisms leading to germination seems to be loss of the water permeability barrier of the ascospore wall, leading to collapse of the ascospores upon air drying. In conclusion, heat-resistant ascospores are also very HP resistant, but sensitization of the ascospores by HP treatment may create possibilities for more efficient inactivation by combined treatments.

Escherichia coli (annex 1.4)

Unpasteurized acidic foods including fruit juices have been implicated recently in a number of disease outbreaks caused by enteric pathogens such as *E. coli* and *Salmonella,* and have illustrated the potential of these bacteria to survive low pH, and cause disease when ingested in low numbers. In order to provide microbiological safety, HP treatment of fruit juices should therefore cause a sufficient reduction of these bacteria. Therefore, we investigated in this task the HP inactivation in fruit juices of the most HP resistant enteric bacteria known to date, i.e. the HP-resistant *E. coli* strains selected in our laboratory. It was found that treatments up to 500 MPa caused only a limited direct inactivation of the mutants, which was not sufficient to assure safety (5-log reduction). However, the viable cell count after HP treatment declined rapidly upon further storage of the juice, indicating that the surviving cells were injured and had become sensitive to low pH. A 5-log reduction of the bacteria was possible using a 500 MPa pressure treatment at ambient temperature, and a quarantaine storage period of 2 days at 8°C.

Task 2: Enzyme inactivation kinetics

In this project, detailed kinetic studies on pressure-temperature inactivation have been accomplished for the enzymes *Bacillus subtilis* α-amylase (BSAA), peroxidase (POD), lipoxygenase (LOX), polyphenoloxidase (PPO) pectinmethylesterase (PME) and myrosinase (MYR). *Bacillus subtilis* has been chosen as an enzymatic model system, which could possibly serve as an extrinsic indicator. The other enzymes are examples of food quality related enzymes, being potential intrinsic indicators in high pressure processing of fruits and vegetables. Peroxidase is present in most plant materials and is often used as an indicator for impact of thermal processes. Lipoxygenase catalyzes the oxygenation of fatty acids containing methyl interrupted double bonds into the corresponding hydroperoxides. In a secondary reaction strongly volatile compounds are formed, causing rancid off-flavours to be formed. Polyphenoloxidase activity results in enzymatic browning of damaged fruits and vegetables, and thus simultaneously in changes in appearance and organoleptic properties. Pectinmethylesterase is responsible for cloud destabilization of juices, gelation of concentrates and consistency loss of tomato products. Myrosinase is an enzyme that catalyzes the hydrolysis of glucosinolates, a group of sulfur containing glycosides. This hydrolysis results in the formation of sulfate, D-glucose and a series of sulfur- and nitrogen-containing compounds such as isothiocyanates, thiocyanates, nitriles and thiones. Isothiocyanates are responsible for the specific flavor and aroma of several cruciferous vegetables. Below a

summary of the results obtained is given. For more detailed results, we would like to refer to annex 2.

Bacillus subtilis α-amylase

Isobaric-isothermal inactivation of BSAA was investigated in the pressure range 0.1 to 750 MPa at temperatures between 25 and 83°C. Both at atmospheric and elevated pressure, inactivation could be accurately described by a first order kinetic model. In the experimental domain studied, inactivation rate constants systematically increased with increasing pressure and temperature, indicating a synergistic effect of temperature and pressure. Activation energy values (temperature dependence of the inactivation rate constant) at different pressure levels and activation volumes (pressure dependence of the inactivation rate constant) at different temperature levels were determined according to the equation of Arrhenius and Eyring respectively. Besides the influence of glycerol on the inactivation kinetics was studied. In the high pressure area, glycerol seemed to exert a twofold stabilizing effect: the inactivation reaction was retarded and the temperature sensitivity of the inactivation rate constants was reduced. Otherwise, pressure sensitivity of the inactivation rate constants was increased by addition of glycerol.

Horseradish peroxidase

Isothermal inactivation of POD, both at pH 7 and pH 11.5, could be accurately described by a two fraction first order model. The influence of phosphate ions and/or calcium chloride as well as the influence of enzyme concentration on isothermal inactivation kinetics was studied. Isobaric-isothermal inactivation of POD was studied in the pressure range 700-900 MPa at different pH values (4, 7, 9 and 10). Again a two fraction first order model could be used to describe the course of inactivation. It could be concluded that horseradish peroxidase is very pressure resistant. Combinations of high pressure (800-900 MPa) and temperatures in the range of 55 to 70°C are required to induce any significant inactivation. However, the pressure stability could be manipulated by changes in the pH. The highest pressure stability was observed at neutral pH.

Soybean lipoxygenase

Inactivation of soybean lipoxygenase under isobaric-isothermal conditions proceeded in the pressure range 0.1 to 650 MPa at temperatures varying from 10 to 65°C. Again inactivation could be described by a first order kinetic model. At constant temperature, inactivation rate constants consistently increased with increasing pressure. At constant pressure on the other hand, a temperature for maximal pressure stability was observed, generally situated somewhat above room temperature (30-40°C). The activation volume was negative at all temperatures

studied and the absolute value increased with increasing temperature. The activation energy on the other hand was negative at low temperature and positive at high temperature. Besides, it was clearly shown that the inactivation kinetics of LOX are strongly dependent on several intrinsic factors such as enzyme production lot, enzyme concentration, pH and CO_2 concentration. Especially changes in pH and CO_2 concentration revealed promising results with respect to sensitization of LOX to pressure treatment.

Avocado polyphenoloxidase

Isothermal-isobaric inactivation of avocado PPO was studied for temperatures ranging from 25-77.5°C and for pressures ranging from 0.1-900 MPa. The inactivation of the enzyme due to the combined effect of pressure and temperature could, under all conditions studied, adequately be described by a first-order kinetic model. At constant pressure, the first-order inactivation rate constants increased in all cases with increasing temperature. This temperature dependency of the inactivation rate constants could be expressed by the Arrhenius equation. The activation energy appeared to decrease exponentially with increasing pressure. At constant temperature, the pressure dependency of the inactivation rate constants could not be expressed by the Eyring equation, i.e. the activation volume was found to change with pressure. In two pressure domains, namely 0.1-150 MPa and 300-900 MPa, the activation volumes were however rather constant with pressure. The activation volume was positive in the former pressure domain and negative in the latter. The positive activation volume in the pressure domain 0.1-150 MPa refers to an antagonistic effect of pressure and temperature in the high temperature/low pressure domain. It was furthermore shown that the pressure stability of avocado PPO (at room temperature) was dependent on the pH of the chemical environment. The pressure stability of avocado PPO was found to decrease with decreasing pH.

Pectinmethylesterase

Isobaric-isothermal inactivation of pectinmethylesterase was investigated in the pressure range 0.1 to 900 MPa at temperatures between 20 and 63 °C. Two sources of commercially available pectinmethylesterase (PE) were considered, namely tomato and orange PE. The inactivation of tomato PE could be most accurately fitted by a first order model, whereas that of orange PE by a fractional conversion model. For tomato PE, the influence of pH and Ca^{2+}-ions on thermal as well as on pressure-temperature stability was evaluated. Similar intrinsic factors were investigated for orange PE, in addition to the influence of enzyme concentration and sucrose.

Tomato PE is less thermostable than orange PE, but more pressure resistant. At temperatures where tomato PE inactivates at atmospheric pressure, an antagonistic effect of pressure and

temperature was observed, *i.e.* the inactivation was slower at elevated pressure as compared to atmospheric pressure. The antagonistic effect was less pronounced in citric acid buffer pH 3.5, pH 3.8 and 4.5 and in presence of Ca^{2+}-ions than in deionised water. After treatment at 60 °C and 100 MPa a small activation of the enzyme was observed. The activation was more pronounced in presence of Ca^{2+}-ions, and the optimal pressure for activation of tomato PE shifted to higher values.

Orange PE is more pressure and temperature stable in concentrated than in diluted solutions. Moreover, depending on the lot used, a larger percentage residual orange PE was observed after temperature and pressure-temperature treatment in more concentrated solutions. An increased Ca^{2+}-concentration caused sensitization to temperature and increased the residual fraction active orange PE after treatment. A similar behaviour was not found for pressure treatment. Only the lower Ca^{2+}-concentrations increased pressure inactivation and the remaining fraction active orange PE was not influenced by addition of Ca^{2+}-ions. Acidification accelerated thermal as well as pressure-temperature inactivation, whereas in presence of sucrose an increased temperature and pressure stability of orange PE was observed. The remaining fraction was for all additives studied independent on the pressure and temperature level applied, except for the inactivation in an acid medium.

Myrosinase

Thermal and pressure inactivation of myrosinase from broccoli was kinetically investigated. Thermal inactivation proceeded in the temperature range 30-60°C. These results indicate that myrosinase is rather thermolabile, as compared to other food quality related enzymes such as polyphenoloxidase, lipoxygenase, pectinmethylesterase and peroxidase. A consecutive step model was shown efficient in modeling the inactivation curves. Two possible inactivation mechanisms corresponding to this consecutive step model were postulated. Pressure inactivation at 20°C occurred at pressures between 200 and 450 MPa. In addition to its thermal sensitivity, the enzyme likewise is rather pressure sensitive compared to the above mentioned food quality related enzymes. By analogy with thermal inactivation, a consecutive step model could accurately describe pressure inactivation. At 35°C, inactivation was studied in the pressure range between 0.1 and 450 MPa. Application of low pressure (<350 MPa) resulted in retardation of thermal inactivation, indicating an antagonistic or protective effect of low pressure.

Task 3: Quantitative mathematical models to describe inactivation kinetics

The results obtained are briefly summarized below. For more detailed results we would like to refer to annex 3.

Bacillus subtilis α-amylase

Based on the data for isobaric-isothermal inactivation kinetics, a pressure-temperature kinetic diagram was constructed. This is a two-dimensional diagram indicating combinations of pressure and temperature resulting in the same inactivation rate constant. Subsequently, it was endeavoured to fit a mathematical model to the data, describing adequately the combined pressure-temperature inactivation of BSAA. In this case, the Arrhenius equation (1), which appeared to be valid in the entire experimental domain studied, was used as starting point.

$$\ln k = \ln k_{refT} - \left(\frac{E_a}{R} \left(\frac{1}{T_{abs}} - \frac{1}{T_{absref}} \right) \right) \tag{1}$$

In this equation, the inactivation rate constant at reference temperature and the activation energy are pressure-dependent parameters. Hence mathematical models describing the evolution of the latter parameters as a function of pressure were derived and implemented in the general Arrhenius equation. The resulting global model (2) was then verified on its ability to describe pressure-temperature inactivation of BSAA.

$$\ln k = (a_2 P^2 + b_2 P + c_2) - \left(\frac{a_1 \exp(-b_1 P)}{R} \left(\frac{1}{T_{abs}} - \frac{1}{T_{absref}} \right) \right) \tag{2}$$

Soybean lipoxygenase

The pressure-temperature kinetic diagram for LOX likewise revealed an antagonistic effect of pressure and temperature in the low temperature area. As opposed to BSAA, the Arrhenius equation was not valid in the experimental domain studied. Therefore, the Eyring equation (3) was used as starting point to develop a mathematical model describing the combined pressure-temperature inactivation of LOX. By implementation of mathematical equations describing the temperature dependence of the inactivation rate constant at reference pressure and the activation volume into the general Eyring equation, a global model was formulated (4).

$$\ln k = \ln k_{refP} - \frac{V_a}{RT_{abs}} (P - P_{ref}) \tag{3}$$

$$\ln k = (a_2 T^2 + b_2 T + c_2) - \left(\frac{a_1 T \exp(-b_1 T)}{R T_{abs}} (P - P_{ref}) \right) \quad (4)$$

Avocado polyphenoloxidase

From the pressure-temperature kinetic diagram for avocado PPO an antagonistic effect of pressure and temperature was apparent in the low pressure/high temperature domain. Since the Arrhenius equation (1) was valid in the entire temperature domain, it was used as starting point to derive a mathematical model describing the inactivation rate constant as a function of both pressure and temperature. The pressure dependent parameters in the Arrhenius equation, namely the activation energy and the inactivation rate constant at reference temperature, were replaced by mathematical expressions reflecting their pressure dependency, hereby yielding equation (5).

$$\ln k = (a_2 P^3 + b_2 P^2 + c_2 P + d_2) - \left(\frac{a_1 \exp(-b_1 P)}{R} \left(\frac{1}{T_{abs}} - \frac{1}{T_{absref}} \right) \right) \quad (2)$$

Zygosaccharomyces bailii

For all the pressure-temperature combinations, the inactivation of *Z. bailii* followed a first order decay down to 10^2-10^3 survivors, the level at which tailing was observed:

$$\log(N) = \log(N_0) - \frac{t}{D} \quad (6)$$

For each temperature, a linear relationship was found between the log(*D*) and pressure:

$$\log\left(\frac{D}{D_{ref}} \right) = -\frac{P - P_{ref}}{z_p} \quad (7)$$

The temperature dependence of the *D*-value could not be expressed by equation (8) over the entire temperature region but could be applied for two separate temperature regions. Z_t-values are negative in the temperature region T ≤ 20°C (z_{t1}) while positive in the region T ≥ 20°C (z_{t2}).

$$\log\left(\frac{D}{D_{ref}} \right) = -\frac{T - T_{ref}}{z_t} \quad (8)$$

Based on the results for isobaric-isothermal inactivation, a pressure-temperature kinetic diagram (iso-*D*-contour-plot) was constructed. This two-dimensional diagram represents the combinations of pressure and temperature resulting in the same *D*-values. The dependence of the *D*-value on pressure and temperature can be formulated by the following global model (9):

$$\ln(2{,}303/D) = \ln(k) = \alpha + \beta(P - P_{ref}) + \gamma(P - P_{ref})^2 + \delta(T - T_{\text{ref}}) + \varepsilon(T - T_{\text{ref}})^2 \qquad (9)$$

where k is the rate of inactivation (cells/min); P, pressure (MPa); P_{ref}, reference pressure (210 MPa); T, temperature (°C) and T_{ref}, reference temperature (25°C).

Task 4: Indicators and procedures to evaluate process impact

The results are briefly summarized below. For more detailed results we would like to refer to annex 4.

4.1. Physical-mathematical test method for impact evaluation

In the context of developing a physical-mathematical method for impact evaluation, the predictive power of the mathematical models formulated to describe inactivation under isobaric-isothermal conditions have to be verified under variable pressure-temperature conditions. Indeed, industrial applications of high pressure are very unlikely to proceed at constant pressure and temperature. Building up of pressure, starting from atmospheric pressure, induces temperature changes as a function of time due to adiabatic heating. Moreover, non-uniform heat transfer throughout the vessel leads to temperature gradients as a function of position, especially when large volume industrial vessels are concerned. In this context, the models for BSAA and LOX were validated under dynamic pressure-temperature conditions, including pulsated pressure treatments.

For BSAA, it was found that the model and its concomitant parameters estimated using isobaric-isothermal inactivation data could likewise predict the extent of inactivation under variable pressure-temperature conditions. On the other hand, when evaluating the kinetic model for LOX and its attendant parameters estimated using isobaric-isothermal inactivation data on its ability to predict activity retention after treatment under variable conditions, the kinetic parameters were noted to be changed. Next to deficiency of the proposed model structure and/or data sets, some other possible reasons were postulated both from mechanistic and experimental point of view. Subsequently, the influence of pulsated pressure treatments was investigated both for BSAA and LOX. In each case, multiple application of high pressure induced an additional inactivation effect. For LOX, this additional inactivation effect became

more pronounced at low temperature and when the number of cycles was increased whereas for BSAA no significant influence of augmenting the number of cycles was observed.

4.2. Indicator-based test method for impact evaluation

Based on the multi-parameter models selected to describe the combined pressure-temperature inactivation of the different micro-organisms and enzymes studied, it has been concluded that the development of indicator-based test methods for impact evaluation will be a very difficult and tedious work. Hence, the physical-mathematical method was focused on in this project.

IV. CONCLUSIONS AND RECOMMENDATIONS

The evaluation of the impact of food preservation/processing methods using high hydrostatic pressure can in general be performed in three ways: by (i) an *in situ* method, (ii) a physical-mathematical method or (iii) using product history indicators. In the *in situ* method, the level of the food safety or quality attribute is evaluated before and after processing. Although this method yields direct and accurate information, its applicability for routine check is limited because it is time-consuming, laborious and expensive. Moreover, the final response is often beyond the detection limit of the available analytical equipment. Therefore, the physical-mathematical method and the indicator-based test method were focused on in this project. In the physical-mathematical method knowledge on the kinetics of the parameter of interest is combined with the actual pressure-temperature profile inside the product. A product history indicator on the other hand is a small device showing a pressure-temperature-time dependent, easily and accurately measurable change that mimics the changes of a target quality or safety parameter undergoing the same pressure-temperature exposure. The major requirement for a system to function as a product history indicator is the kinetic equivalency between the target attribute and the product history indicator. The latter methods both require *a priori* knowledge of the kinetic response of the indicators and parameters of interest. Hence in this context, detailed kinetic studies have been performed regarding the influence of pressure, temperature and some intrinsic factors on the inactivation of some food quality related vegetative micro-organisms and enzymes. Kinetic models have been developed to describe the inactivation of these parameters as a function of pressure and temperature. In all cases, multiple parameter models were required to describe their pressure-temperature dependence. Based on these results, it was concluded that it will be very hard to meet the kinetic requirements necessary for a system to function as a product history indicator. Therefore it is recommended to make appeal to the physical-mathematical method as much as possible.

V. APPENDICES

Appendix 1: Literature cited in the final report

Appendix 2: List of publications resulting from the research project

Appendix 3: Detailed results of the different research tasks

Annex 1: Detailed results on microbial inactivation kinetics

Annex 1.1: Detailed results on screening of pressure resistance of moulds and lactic acid bacteria

Annex 1.2: Kinetic analysis of high pressure inactivation of Zygosaccharomyces bailii

Annex 1.3: Inactivation of ascospores of Talaromyces macrosporus by high hydrostatic pressure

Annex 1.4: High pressure inactivation and survival of pressure resistant Eschericia coli mutant in fruit juices

Annex 2: Detailed results on enzyme inactivation kinetics

Annex 3: Detailed results regarding mathematical modelling

Annex 4: Detailed results regarding procedures to evaluate process impact

APPENDIX 1: LITERATURE CITED IN THE FINAL REPORT

Appendix 1: Literature cited in the final report

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APPENDIX 2: LIST OF PUBLICATIONS RESULTING FROM THE RESEARCH PROJECT

Appendix 2: List of publications resulting from the research project

PARTNER 1. LABORATORY OF FOOD TECHNOLOGY

I. Publications in international journals with peer review[1]

Ludikhuyze, L., Van den Broeck, I., Weemaes, C., Herremans, C., Van Impe, J., Hendrickx, M., Tobback, P. 1997. Kinetics for isobaric-isothermal inactivation of *Bacillus subtilis* α-amylase. Biotechnol. Progr. 13: 532-538.

Ludikhuyze, L., Van den Broeck, I., Weemaes, C., Hendrickx, M. 1997. Kinetic parameters for pressure-temperature inactivation of *Bacillus subtilis* α-amylase under dynamic conditions. Biotechnol. Progr. 13: 617-623.

Ludikhuyze, L., Indrawati, Van den Broeck, I., Weemaes, C., Hendrickx, M. 1998. The effect of combined pressure and temperature on soybean lipoxygenase: I. The influence of extrinsic and intrinsic factors on isobaric-isothermal inactivation kinetics. J. Agric. Food Chem. In press.

Ludikhuyze, L., Indrawati, Van den Broeck, I., Weemaes, C., Hendrickx, M. 1998. The effect of combined pressure and temperature on soybean lipoxygenase: II. Modeling inactivation kinetics under static and dynamic conditions. J. Agric. Food Chem. In press.

Ludikhuyze, L., Ooms, V., Weemaes, C., Hendrickx, M. 1998. Kinetic study of the irreversible thermal and pressure inactivation of myrosinase from broccoli (*Brassica Oleracea* L. *Italica*). J. Agric. Food Chem. Submitted.

Weemaes, C., Ludikhuyze, L., Van den Broeck, I., Hendrickx, M. 1998. Kinetics of combined pressure-temperature inactivation of avocado polyphenoloxidase. Biotechnol. Bioeng. In press.

II. Contributions to international congresses and symposia

II.a. Published in a book with ISBN number

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PARTNER 2: LABORATORY OF FOOD MICROBIOLOGY

I. Publications in international journals with peer review[2]

Garcia-Graells, C., Hauben, K.J.A., and Michiels, C.W. (1998). High pressure inactivation and sublethal injury of pressure-resistant *Escherichia coli* mutants in fruit juices. *Applied and Environmental Microbiology* **64**, 1566-1568.

Reyns, K., Soontjens, C., and Michiels, C. Kinetic analysis of high pressure inactivation of *Zygosaccharommyces bailii.* In preparation.

Reyns, K., Veraverbeke, E., Vermeiren, H., and Michiels C. Effects of high hydrostatic pressure treatment on *Talaromyces macrosporus* ascospores. In preparation.

II. Contributions to international congresses and symposia

II.a. Published in a book with ISBN number

García-Graells C., Hauben K., Soontjens C. and Michiels, C.. High pressure inactivation and survival of pressure-resistant *Escherichia coli* mutants in fruit juices. . *In:* "High Pressure Food Science, Bioscience and Chemistry", N. Isaacs (ed.). Proceedings of the 35th Meeting of the European High Pressure Research Group, 7-11 Sept. 1997, Reading (U.K.). The Royal Society of Chemistry, Cambridge, pp. 304-309.

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APPENDIX 3: DETAILED RESULTS OF THE DIFFERENT RESEARCH TASKS

Annex 1: Detailed results on microbial inactivation kinetics
Annex 2: Detailed results on enzyme inactivation kinetics
Annex 3: Detailed results regarding mathematical modelling
Annex 4: Detailed results regarding procedures to evaluate process impact

Appendix 3: Detailed results of the different research tasks

The detailed results of the different research tasks are included in annexes, which are numbered according to the task they refer to.

Annex 1: Detailed results regarding microbial inactivation kinetics

Annex 1.1: Detailed results on screening of pressure resistance of moulds and lactic acid bacteria

Annex 1.2: Kinetic analysis of high pressure inactivation of *Zygosaccharomyces bailii*

Annex 1.3: Inactivation of ascospores of *Tolaromyces macrosporus* by high hydrostatic pressure

Annex 1.4: High pressure inactivation and survival of pressure resistant *Eschericia coli* mutants in fruit juices

Annex2: Detailed results regarding enzyme inactivation kinetics

Annex 3: Detailed results regarding quantitative modelling

Annex 4: Detailed results regarding process impact evaluation procedures

ANNEX 1: DETAILED RESULTS ON MICROBIAL INACTIVATION KINETICS

Annex 1.1: Detailed results on screening of pressure resistance of moulds and lactic acid bacteria
Annex 1.2: Kinetic analysis of high pressure inactivation of Zygosaccharomyces bailii
Annex 1.3: Inactivation of ascospores of Talaromyces macrosporus by high hydrostatic pressure
Annex 1.4: High pressure inactivation and survival of pressure resistant Eschericia coli mutant in fruit juices

ANNEX 1.1: DETAILED RESULTS ON SCREENING OF PRESSURE RESISTANCE OF MOULDS AND LACTIC ACID BACTERIA

1. Microbial strains studied

The moulds and lactic acid bacteria screened in this project are the following (for yeasts see annex 1.2):

species	strain number[a]
Moulds	
Talaromyces macrosporus	CBS 130.89
Neosartorya hiratsukae	CBS 294.93
Neosartorya fischeri	CBS 586.90
Byssochlamys fulva	CBS 132.33
Byssochlamys nivea	CBS 695.95
Penicillium sabulosum	CBS 261.87
Lactic acid bacteria	
Lactobacillus brevis [b]	LMG 11438
Lactobacillus collinoides 9194 [c]	LMG 9194
Lactobacillus collinoides 9195 [d]	LMG 9195
Lactobacillus mali [e]	LMG 6899
Lactobacillus suebicus [f]	LMG 11408
Lactobacillus sake	DSM 6333

[a]CBS: Centraal Bureau voor schimmelculturen, Baarn, Nederland; LMM: own collection of Laboratorium voor Levensmiddelenmicrobiologie; LMG: LMG culture collection.
[b]Isolated from tomato pulp
[c]Isolated from fermenting apple juice
[d]Isolated from cider and apple juices
[e]Isolated from apple juice from cider press
[f]Isolated from apple mash

2. Material and Methods

2.1. Growth of strains and preparation of cell suspensions

Moulds were grown on Malt Extract Agar (Biokar Diagnostics, Beauvais, France) at 25°C. Samples were examined microscopically for ascospore formation. After 2-4 weeks, when sufficient ascospores were formed the plates were washed with sterile 40 mM Tris-HCl buffer to collect the spores. The suspensions were vigourously shaken, filtered through sterile glass wool and sealed in polyethylene bags for pressurization. Storage of the spore suspensions for up to 5 days at 4°C did not significantly alter their pressure resistance.
Lactobacillus species were grown at 30°C in De Man, Rogosa et Sharpe (M.R.S.) broth to stationary phase, harvested by centrifugation and resuspended in sterile 40mM Tris-HCl buffer pH6,5.
Viable cells were counted before (N_0) and after pressurization (N) by plating dilutions in the same buffer on Rose Bengal Chloramphenicol Agar Base (Lab M, Bury, England) (moulds) or M.R.S. agar (lactic acid bacteria).

2.2. Pressure inactivation

Isothermal-isobaric pressurization was carried out either in a single-vessel thermostated 8 ml vessel driven by a manual spindle pump (Resato, Roden, the Netherlands), or in an eight-

vessel (8 x 8 ml) thermostatted system driven by an automatic pump (Resato, Roden, The Netherlands).

2.3. Heat treatments

Before pressurization, ascospore suspensions were given a heat treatment at 65° or 70°C/15min to inactivate vegetative cells present. Treatments at 80°C/30min were done to activate dormant ascospores, and /or to assess heat sensitization of ascospores by pressure treatment. In both cases, ascospore suspensions in sealed plastic bags were immersed in a thermostatted (±0.1°C) circulating water bath.

3. Results

3.1. Screening of moulds producing heat-resistant ascospores

3.1.a. Formation of ascospores

The six moulds included in the study were grown on four different agar media (OGYE agar base, Malt Extract Agar Base, Nestlé 5 grains Agar, V8 Tomato Juice Agar) and formation of ascospores was followed microscopically during 6 weeks. All strains except *P. sabulosum* formed ascospores on Malt Extract agar in 2-4 weeks, and some strains except *P. sabulosum* also on one or more of the other media. *N. fischeri* and *B. fulva* formed only low numbers of ascospores and were therefore not retained for further work.

3.1.b. Heat activation and inactivation

Ascospores from the three remaining species were treated at 70 and 80°C for various times and colony-forming units were counted (table 1). For all spore suspensions, there was in the first minutes a decrease in counts due to the rapid destruction of the vegetative cells. For *N. hiratsukae* (at 70 and 80°C) and for *T. macrosporus* (only at 80°C), this inactivation was followed by an activation at longer exposure times. The largest extent of activation was seen in *T. macrosporus*, and the spore suspensions from this organism had also the lowest fraction of vegetative counts.

species	70°C				80°C		
	0 min	10 min	30 min	60 min	10 min	30 min	60 min
B. nivea	$1.5x10^6$	$1.8x10^5$	$1x10^5$	$5.0x10^4$	-	-	-
T. macrosporus	$2x10^5$	$3.2x10^4$	$2x10^4$	$2.1x10^4$	$1.3x10^6$	$2.3x10^6$	$1.9x10^6$
N. hiratsukae	$1.5x10^5$	$1.8x10^4$	$3.7x10^4$	$3.3x10^5$	$1.2x10^5$	$3.1x10^5$	$3.6x10^5$

3.1.c. High pressure inactivation

The three remaining species were subjected to high pressure treatments at 20°C and survivors were counted. Up to 5000 bar, no ascospore inactivation was observed for any of the strains (data not shown). Therefore, from the pressure-resistance point of view, all strains can be considered equally important.

3.1.c. Choice of strain for further pressure-heat inactivation studies

T. macrosporus was chosen for further work because (i) its ascospore suspensions contained the highest spore titer (after heat activation at 80°C) and the lowest vegetative cell titer; (ii) its ascospores were highly heat and pressure resistant; (iii) vegetative cells could be inactivated by 65°C/15min treatment without activation of ascospores, which occurred only at 80°C.

3.2. Screening of pressure resistance of Lactobacillus species at 20°C

A number of pressurizations during 15 minutes at 20°C were done and the reduction factor (N_0/N) was calculated for each treatment.
Under the conditions used, *Lactobacillus collinoides 95* was more resistant to high pressure than the other strains (fig. 1).

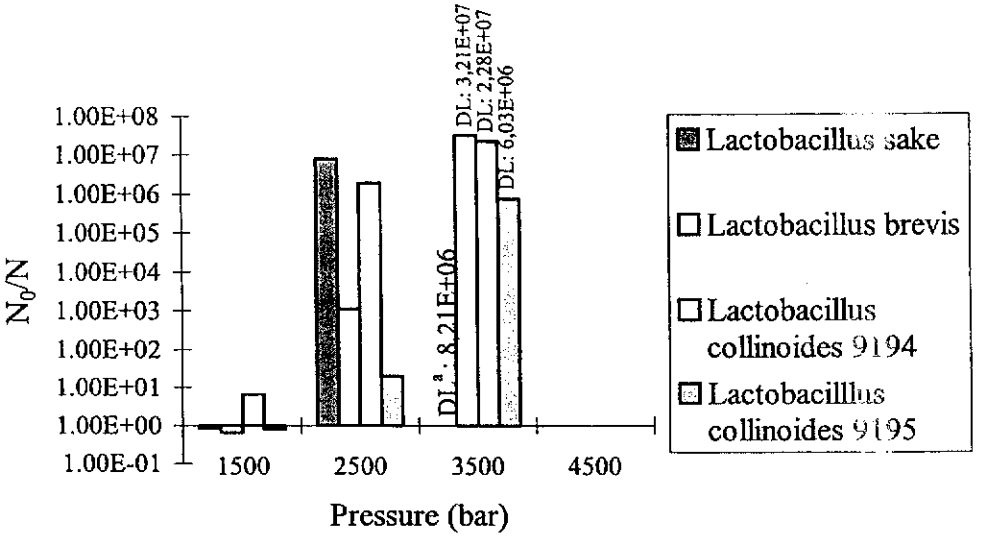

Figure 1: Screening of high pressure sensitivity of Lactobacillus strains at 20°C
Remark: *L. mali* and *L. suebicus* are not shown, but they did not survive 3500 bar.
[a]DL: Detection Limit

ANNEX 1.2.[illegible]: KINETIC ANALYSIS OF HIGH PRESSURE INACTIVATION OF *ZYGOSACCHAROMYCES BAILII*

Introduction

Over the last ten years, considerable research efforts have been directed towards the development of novel non-thermal processes for food preservation that allow better retention of these quality parameters such as flavour, texture and nutritional value. High hydrostatic pressure (HP) treatment is such an emerging technology. Although the potential of HP to kill microorganisms and to inactivate food enzymes and in this way to extend product shelf-life has been amply demonstrated (Tonello, *et al.*, 1997, Pothakamury *et al.*, 1995), we are still awaiting a commercial breakthrough. One of the major obstructions for the rapid introduction of HP technology is the lack of reliable methods to quantify the impact of actual HP processes on food safety and quality analogous to the concepts of D and z-values that have been developed in thermobacteriology (Bigelow, 1921). However, a quantitative concept will inevitably be more complex and require more experimental work for HP than for thermal treatment, because temperature (T) needs to be considered as a process parameter in addition to pressure (P). Mathematical models describing inactivation over a wide P,T range have been developed for a number of food quality related enzymes (Ludikhuyze *et al.*, 1997, Weemaes *et al.*, 1998), but up to now not for microorganisms. A number of authors have concluded for various bacteria and yeasts from studies under a limited set of conditions that inactivation obeys first order kinetics (Butz and Ludwig, 1986, Carlez *et al.*, 1993, Ponce *et al.*, 1998, Gervilla *et al.*, 1997, Styles *et al.*, 1991, Chen and Tseng, 1997, Hashizume *et al.*, 1995). Others found first order kinetics only for the first part of the inactivation curve, and a slower inactivation rate (tailing) towards the end (Ludwig *et al.*, 1992, Patterson *et al.*, 1995, Simpson and Gilmour, 1997). Although several studies provide D-values for HP inactivation by a number of P,T combinations, information about pressure and/or temperature dependence of these D-values expressed as z-values is scarce. Palou *et al.* (1997) calculated and expressed the pressure dependence of *Z. bailii* inactivation rate as an apparent activation volume or by a z_p-value at 21°C, whereas Chen

and Tseng (1997) described the temperature dependence of the specific death rate constant at different pressures as an activation energy.

The objective of this work was to perform a quantitative study of the inactivation of a yeast strain over a wide pressure and temperature range in order to investigate the interaction of these parameters on the inactivation process, and to demonstrate the feasibility of developing models to describe inactivation as a function of time, pressure and temperature.

2. Material and methods

2.1. Yeast strains

Eight yeast strains causing food spoilage were used: *Pichia membranifaciens* (CBS 5170), *Zygosaccharomyces rouxii*(CBS 733), *Zygosaccharomyces bailii* (CBS 1097), *Zygoascus hellenicus* (CBS 6779), *Rhodotorula mucilaginosa* (MUCL 30592), *Schizosaccharomyces pombe*, *Candida lypolitica* (LMM 02.68) and *Torulopsis magni* (LMM 02.67). Strains were maintained at 4°C on Oxytetracycline Glucose Yeast Extract agar (OGYE) (Lab M, England).

2.2. Growth conditions and preparation of cell suspensions for high pressure treatment

One single colony from an OGYE stockplate (Lab M, England) was transferred to 20 ml YGP containing 1% yeast extract (Biokar Diagnostics, France), 2% D-glucose (Merck, Germany) and 2% bacteriological peptone (Oxoid, England) and incubated on a rotatory shaker (200 rpm) for 48 h at 25°C. The cells were harvested by centrifugation (4000 x g, 5 min) and resuspended in the same volume of sterile 40mM Tris-HCl-buffer pH6.5 in sterile 40 mM Hepes-buffer adjusted to pH 3, 4, 5 or 6, in apple juice (pH 3.21) or in orange juice (pH 3.66), resulting in an initial concentration (N_i) of approximately 10^8CFU/ml.

High pressure treatment

Samples of 0,5 ml were transferred to sterile polyethylene bags. The bags were heat-sealed after careful evacuation of air removed and exposed to HP treatments between 120 and 320MPa at constant temperatures, ranging between -5°C to 45°C. The screening for pressure resistance of yeast strains and the inactivation experiments at different pH and in juices were carried out in a single-vessel thermostatted 8ml equipment driven by a manual spindle pump (Resato, Roden, The Netherlands). Kinetic inactivation experiments were performed in an eight-vessel (8 x 8ml) system driven by an automatic pump (HPIU-10000, 95/1994, Resato, The Netherlands). The samples in the individual vessels were simultaneously exposed to the same pressure and temperature, and then isolated from the high pressure circuit and individually depressurized by a number of valves. A glycol/water mixture (x/y) was used as pressure transmission fluid. Thermostatization was achieved by circulating water around the pressure vessels. Compression speed was 1000 bar/min, but decompression was immediate. The measurement of inactivation kinetics was started (first sample taken at t_0) only one min after the required pressure was reached, to allow dissipation of the adiabatic compression heat. This hold time was experimentally found to be sufficient for equilibration of the temperature inside the pressure vessel to the circulating water temperature (±1°C).

2.3. Viable cell counts and expression of results

Viable yeast counts were determined by surface plating serial dilutions in 40mM Tris-HCl-buffer pH 6.5 on OGYE without oxytetracycline using a Spiral Plater (Spiral Systems Inc., USA). After incubation at 25°C for 2 days, cell counts were expressed as colony forming units per ml (CFU/ml). For each pressure,temperature (P,T) combination, the logarithm of the viability reduction ($\log(N_0/N)$) was plotted against the duration of pressurization to obtain a kinetic inactivation. N_0 is the population at time t_0, i.e. after pressure build-up and temperature equilibration. Decimal reduction (D-) values, i.e. the time needed to

reach a 90% reduction at constant pressure and constant temperature, were calculated as the inverse slope of a straight line fitted through the linear part of the inactivation curves.

3. Results

3.1. Screening of yeasts for high pressure resistance

Screening of HP sensitivity of the eight yeasts was done in two steps. First, all strains were subjected to a 15-min treatment at four different pressure,temperature combinations (160 and 200 MP, 20 and 35°C) (Table 1). Two yeasts, *Zygosaccharomyces bailii* and *Zygoascus hellenicus,* were clearly more resistant than the others. For these two, and for *Z. rouxii* and *P. membranifaciens,* the kinetics of pressure inactivation at 1900 bar and 20°C were determined in more detail. The first part of the inactivation curve was linear, and D-values were calculated (Table 2). These values confirm that *Z. bailii* and *Z. hellenicus* are the most pressure resistant strains under these conditions.

Finally *Z. bailii* was selected for further kinetic analysis because this organism is a more frequent cause of spoilage than *Z. hellenicus,* it can grow down to pH 2.5 (Pitt, 1974), and it can develop resistance to high concentrations of the preservatives benzoate and sorbate (Warth, 1985, Neves *et al.*, 1994)).

3.2. Kinetic analysis of Z. bailii inactivation

Inactivation of *Z. bailii* was studied in the multivessel equipment at different combinations of pressure (120-320 MPa) and temperature (-5 and 45°C) in function of the treatment time (0-60 min).

A certain level of inactivation occurred during the come-up time to reach the desired pressure. At he moment when the first sample was taken (N_0), i.e. one minute after the desired ressure was reached, this inactivation was always less than 1.5 log unit, even under the most severe conditions.

Figure 1 shows the inactivation curves for pressurizations at 20°C. Under isobaric and isothermal conditions, the logarithm of the viability reduction (N_0/N) increased with treatment time. For all P,T combinations, the first part, i.e. 4-6 decades, of the semi-logarithmic inactivation curve could be accurately described by a first order relationship:

$$\log\left(\frac{N_0}{N}\right) = \frac{1}{D} * t \qquad (1)$$

Tailing of the curves was observed in the second part of the curve when an inactivation of 4-6 log units was reached and occurred independently of temperature. The D-values for all tested P,T combinations are presented in Table 3. It can be seen from this table that over the entire temperature domain studied, the inactivation rate at a constant temperature increases with pressure. However, at constant pressure, the inactivation rate showed a minimum at 10-20°C, meaning that inactivation proceeds faster at temperatures above and below this temperature region.

By plotting the logarithm of the D-values as a function of pressure at constant temperature, linear relationships were found between log(D) and pressure:

$$\log\left(\frac{D}{D_{ref}}\right) = -\left(\frac{P - P_{ref}}{z_p}\right) \qquad (2)$$

In this equation z_p represents the increase in pressure that causes a tenfold reduction of the D-value at a constant temperature. These values are presented in table 4 and they are not strongly affected by temperature between –5 and 45°C. At –5°C and 45°C, the z_p-values tend to decrease, suggesting an enhanced pressure sensitivity of the inactivation rates at the temperature extremes.

Unlike the pressure dependence, the temperature dependence of log(D) at constant pressure could not be expressed by a first order relation over the entire temperature region. However a first order model could be applied to two separate temperature regions: for temperatures below 20°C and for temperatures between 20°C and 45°C:

$$\log\left(\frac{D}{D_{ref}}\right) = -\left(\frac{T - T_{ref}}{z_t}\right) \quad (3)$$

This results in a set of nearly parallel curves for each temperature region with a slope $-1/z_t$ (Fig. 2). The z_t-values represent the temperature change required to decrease the D-value by a factor ten at a constant pressure, and are given in table 5. z_t-values are negative in the temperature region $T \leq 20°C$ (z_{t1}) while positive in the region $T \geq 20°C$ (z_{t2}). In each temperature region, the z_t-values are not strongly dependent on pressure, and can be considered quasi-constant.

3.3. Mathematical modelling of the inactivation rate

Using the D-values from the isobaric-isothermal inactivation, a P,T kinetic diagram (iso-D contour-plot) was constructed (Fig. 3). Each line represents the combinations of pressure and temperature resulting in the same D-values. The elliptical shape of the curves indicates that around 20°C a higher pressure is needed to achieve the same inactivation rate than at temperatures above 30°C or below 10°C. The P,T inactivation could not be described by the equation of Hawley (1971). Regression analysis of the natural logarithm of the D-value in function of temperature and pressure by the quadratic equation following Hashizume *et al.* (1995) resulted in a bad estimation of the parameter which belongs to the combined temperature-pressure term. Without the combined P,T term, equation 4 was found to describe accurately the dependence of the D-value on pressure and temperature, in the pressure-temperature domain considered.

$$\ln(2{,}303/D) = \alpha + \beta(P - P_{ref}) + \gamma(P - P_{ref})^2 + \delta(T - T_{ref}) + \varepsilon(T - T_{ref})^2 \quad (4)$$

where k is the rate of inactivation (cells/min); P, pressure (MPa); P_{ref}, reference pressure (210 MPa); T, temperature (°C) and T_{ref}, reference temperature (25°C). The values of the kinetic parameters α, β, γ, δ, ε are shown in table 6.

3.4. Validation of pressure inactivation in low pH buffers and juices

All the above kinetic inactivation studies on *Z. bailii* were carried out in a buffer of neutral pH (pH 6.5). However *Z. bailii* mainly causes spoilage problems in acid products. Figure 4 shows the effect of the pH (3-6) on the inactivation of *Z. bailii* at 20°C and different pressures between 220 and 300 MPa. Lowering the pH from 6,0 to 3,0 does not seem to dramatically affect the pressure treatment. For all pH levels, a 1.5- to 2.0-log cycle reduction was observed at 260 MPa after a 20 min exposure, and this corresponds well to the inactivation under the same conditions at pH 6.5. On the other hand, at 45°C the inactivation seems to be enhanced at low pH. For instance, at 200 MPa, a reduction of 3,3 log units was obtained at pH 6 compared to 4.5 log units at pH 3 (Fig. 4).

Finally inactivation in Tris-buffer of pH 6.5 was compared with inactivation in apple juice (pH 3.21) and orange juice (pH 3.66). In this case, even at 20°C, *Z. bailii* was much more sensitive for pressure in the fruit juices than in the buffer at the same pH. For 20-min exposure at 260 MPa, a viability reduction of 2.4 log units was demonstrated in Tris-buffer (pH 6.5) while a viability reduction of 5.4 and 5.1 log units was found in apple and orange juice respectively (Fig. 5).

4. Discussion

Our screening of different yeasts for HP sensitivity revealed *Z. bailii* as one of the most resistant species, and, more generally, also confirmed that yeasts are more sensitive to hydrostatic pressure than most bacteria. Indeed, none of the yeasts survived pressures above 350-400 MPa longer than a few minutes, while some non-sporulating bacteria are known to survive treatments at 500, or even 800 MPa (Patterson *et al.*, 1995, Hauben *et al.*, 1997).

In all our experiments, isobaric isothermal inactivation of *Z. bailii* was described by a biphasic inactivation curve, with a first part covering four to six decades and obeying first