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

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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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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).

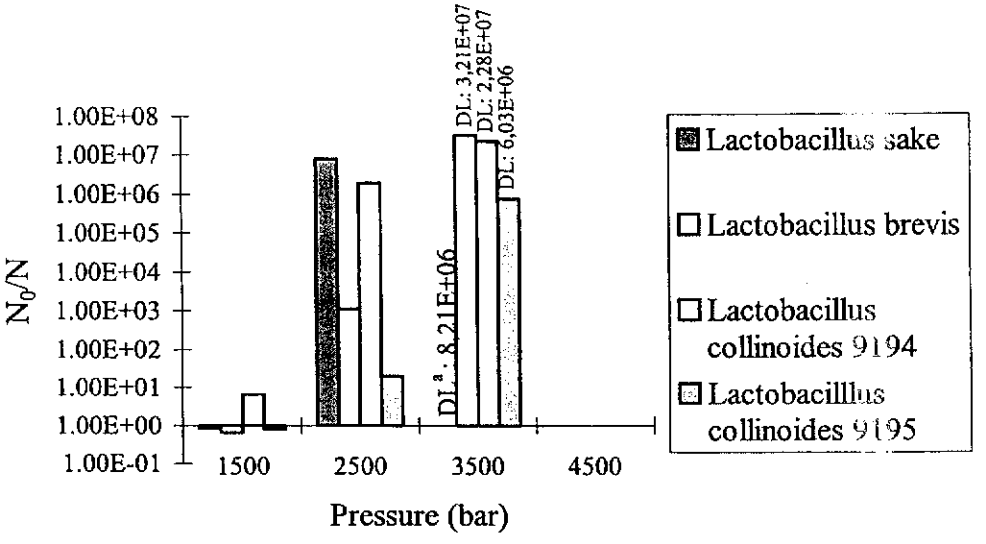

Figure 1: Screening of high pressure sensitivity of Lactobacillus strains at 20°C
Remark: *L. mali* and *L. suebicus* are not shown, but they did not survive 3500 bar.
[a]DL: Detection Limit

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

order kinetics followed by a "tail" corresponding to a small fraction of cells (10^{-2} to 10^{-4}%) that were inactivated at a much slower rate. Other studies with the yeasts *S. cerevisiae* and *Z. rouxii* (Chen and Tseng, 1997) as well as with bacteria like *Escherichia coli* (Patterson *et al.*, 1995) and *Salmonella* spp. (Metrick *et al.*, 1989), have also reported tailing, and it has been suggested that a small portion of the cell population has a higher resistance for pressure than the cells destructed early in the pressurization (Metrick *et al.*, 1989). However, more direct genetic or physiological indications of the nature of this population heterogeneity are missing. In the case of sporulating yeasts, such as *Z. bailii*, a possible cause of tailing could be the presence of ascospores with a higher pressure resistance in the culture. In any case, a consequence of the existence of survivor tails is that the D- and z-value concept should be applied with caution. In concreto, inactivation models based on this concept, such as the model developed in this work, should not be used to extrapolate to levels of inactivation where the first order kinetic is no longer valid.

A remarkable feature is the elliptical shape of the iso-D curves in the P,T diagram (Fig. 3), meaning that at constant pressure, the inactivation rate shows a maximum in function of temperature. Similar behaviour has been observed in *S. cerevisiae* (Hashizume *et al.*, 1995) and in several bacteria (Carlez *et al.*, 1993, Ludwig *et al.*, 1992, Ludwig *et al.*, 1994, Gervilla *et al.*, 1997), suggesting that this is a general phenomenon that must be the consequence of a very fundamental property common to these microbial cells. Since proteins generally also have ellipsoidal stability curves in a P,T diagram (Ludikhuyze *et al.*, 1997, Mozhaev *et al.*, 1994), it is possible that the iso-D curves of the microorganisms reflect the underlying inactivation of a vital protein. Alternatively, it has been proposed that the increase of the inactivation rate at reduced or elevated temperature may be related to compromised membrane integrity. This hypothesis is supported by the observation that pressure treatment causes leakage of intracellular solutes (Shimada *et al.*, 1993, Hauben *et al.*, 1996). At low temperature, this may primarily result from a crystallization of membrane lipids and a destabilization of the interaction with membrane-spanning domains of integral membrane proteins (Cheftel, 1992).

The z_p-values of *Z. bailii* representing the pressure increment needed to reduce the D-value by a factor of 10 were found to be quasi independent of temperature between –1°

and 40°C. In addition, the z_p-value of 66,4 MPa we found for *Z. bailii* at 20°C and pH 6.5 in the pressure range 180-320 MPa corresponds well to the z_p-value of 68 MPa calculated under similar conditions for *S. cerevisiae* by Hashizume *et al.* (1995), and even to the z_p-values of 85,8 MPa for *Z. bailii* inactivation in Sabouraud Dextrose broth at pH3.5 (Palou *et al.*, 1997). A more extensive comparison of z_p-values of different microorganisms is given in Table 7. A general observation seems to be the higher sensitivity to changes in pressure of the inactivation rate of yeasts compared to bacteria. For thermal inactivation (at ambient pressure), no significant difference in z_t-values has been reported between yeasts and vegetative bacteria.

The temperature dependence of the *Z. bailii* D-values at constant pressure is given by two different values: one that is valid for $T \leq 20°C$ and one for $T \geq 20°C$. In each temperature zone, the z_t-value was found to be quasi pressure pressure independent (table 5). Again, a similar conclusion can be made for *S. cerevisiae* using the data of Hashizume *et al.* (1995). However, the z_t-values obtained in this way are smaller in absolute value than those obtained for *Z. bailii*: $z_{t1} = -10°C$ ($T_1 \leq 20°C$), and $z_{t2} = 10°C$ ($T_2 \geq 20$). It can be concluded that the *S. cerevisiae* HP D-values are more temperature sensitive than those of *Z. bailii*.

Finally, it was found that inactivation of *Z. bailii* was not substantially different at low pH compared to neutral pH, except perhaps at 45°C. Detailed observation of the results reported by others for *Z. bailii* (Pandya *et al.*, 1995) and for *S. cerevisiae* (Gola *et al.*, 1994) also reveals a weak dependence of HP inactivation on medium pH. This contrasts to the situation in bacteria where inactivation increases dramatically at pH<4.5 (Garcia-Graells *et al.*, 1998). It seems likely that this difference in HP sensitivity at low pH is related to the ability of yeasts to grow at low pH, as opposed to neutrophilic bacteria. The unexpectedly high inactivation of *Z. bailii* in fruit juices compared to buffers at the same pH, illustrates that other factors in addition to pH must be considered for predicting microbial inactivation in real products. In this case, certain organic acids may be responsible for enhanced inactivation.

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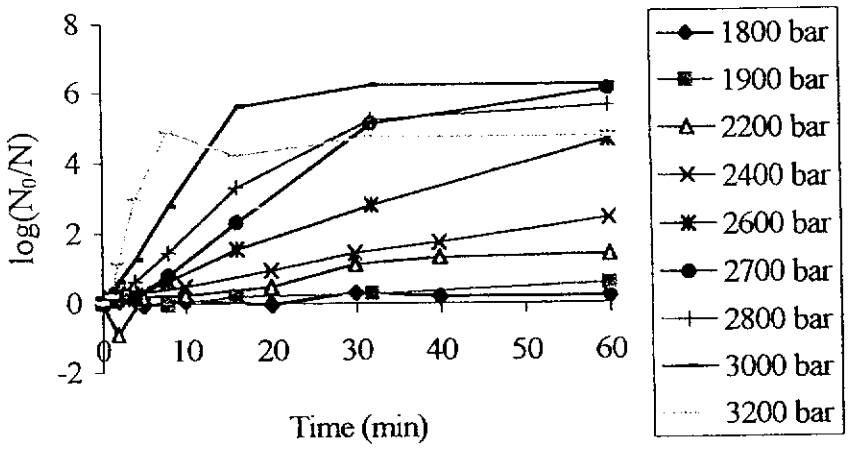


Figure 1: Inactivation kinetics of *Z. bailii* in 40 mM Tris-HCl-buffer at pH 6.5 and 20°C.

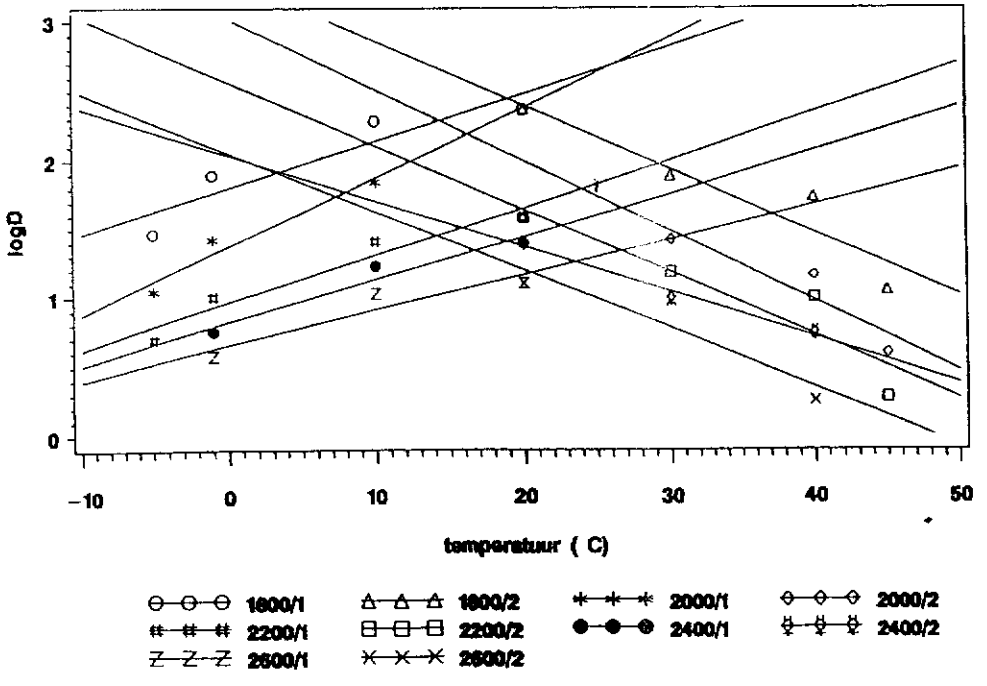


Figure 2: Effect of temperature on the D-values of *Z. bailii* in 40mM Tris-HCl-buffer at pH6.5.

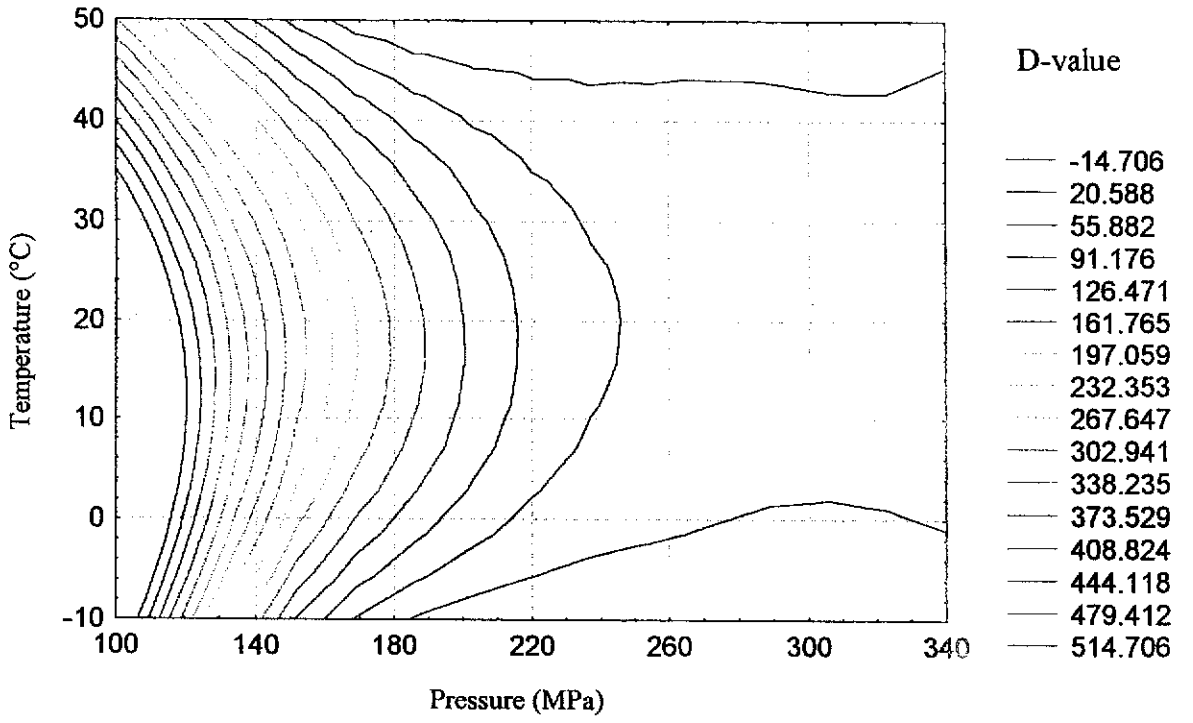


Figure 3: Pressure-temperature diagram or iso-D contour-plot of *Z. bailii*

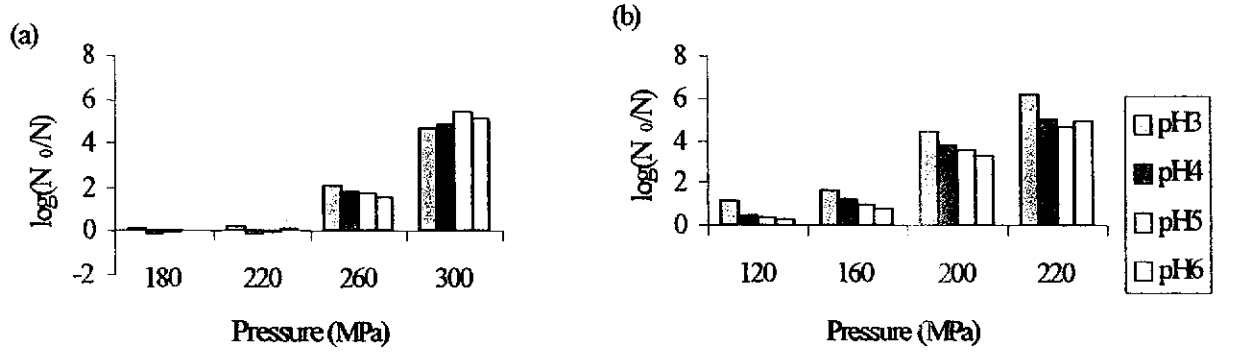


Figure 4: Influence of pH on the HP inactivation of *Z. bailii* at 20°C (a) and 45°C (b).

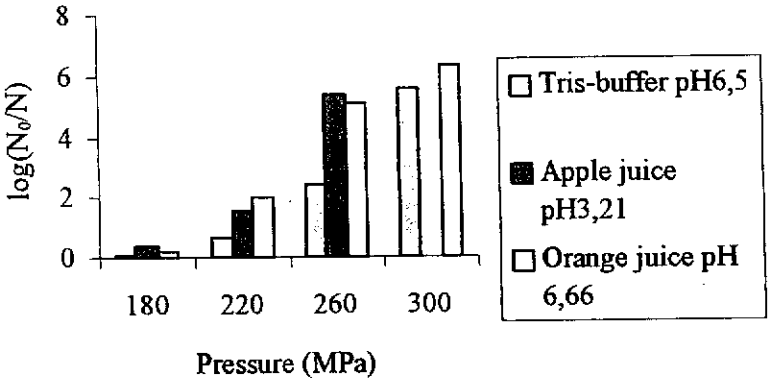


Figure 5: Inactivation of *Z. bailii* in 40 mM Tris-HCl-buffer pH 6.5, apple and orange juice at 20°C.

Table 1: Screening of yeast strains for high pressure resistance

Yeast strain and pressure		Inactivation at[a]:	
		20°C	35°C
Pichia membranifaciens	160 MPa	0	1.0
	200 MPa	3.2	6.5
Zygosaccharomyces rouxii	160 MPa	1.8	4.3
	200 MPa	3.7	4.1
Zygosaccharomyces bailii	160 MPa	0.1	0.1
	200 MPa	0.1	0.3
Zygoascus hellenicus	160 MPa	0.2	0.3
	200 MPa	0.3	1.2
Rhodotorula rubra	170 MPa	3.8	ND[b]
	210 MPa	total inactivation	ND
Schizosaccharomyces pombe	135 MPa	0.5	ND
	185 MPa	3.6	ND
Candida lypolitica	217 MPa	2.8	ND
Torulopsis magni	225 MPa	3.6	ND

[a]Inactivation performed for 15 min and is expressed as $\log(N_0/N)$ being the counts for the untreated control and the pressure-treated sample, respectively.
[b]ND, not determined.

Table 2: Decimal reduction values of some yeasts at 1900 bar/20°C

Species	D-value (min)
Z. rouxii	6
P. membranifaciens	11
Z. hellenicus	180
Z. bailii	180

Table 3: Decimal reduction values (min)[a] for isobaric-isothermal inactivationof *Z. bailii* in 40 mM Tris-HCl-buffer at pH 6,5

Pressure	-5°C	-1°C	10°C	20°C	30°C	40°C	45°C
1200	476.19 (0.330)						232.56 (0.529)
1400	166.67 (0.695)						37.45 (0.936)
1600	75.19 (0.893)	113.64 (0.968)				138.89 (0.827)	16.81 (0.870)
1800	29.41 (0.974)	79.37 (0.932)	192.31 (0.803)	238.10 (0.538)	78.74 (0.895)	55.56 (0.972)	8.80 (0.961)
1900				119.05 (0.906)		26.18 (0.824)	
2000	11.14 (0.968)	26.74 (0.900)	70.92 (0.892)		26.74 (0.942)	14.58 (0.942)	3.95 (0.977)
2200	4.95 (0.984)	8.05 (0.995)	25.91 (0.956)	38.91 (0.906)	12.60 (0.951)	10.06 (0.930)	1.88 (0.953)
2400		4.15 (0.962)	17.01 (0.981)	25.19 (0.993)	9.90 (0.939)	5.46 (0.956)	
2600		3.73 (0.919)	7.73 (0.990)	12.82 (0.992)	9.31 (0.922)	3.11 (0.797)	
2700				6.12 (0.989)			
2800			3.63 (0.988)	5.88 (0.982)	3.63 (0.883)		
3000			2.30 (0.988)	2.88 (0.999)			
3200			1.44 (0.876)	1.54 (0.972)			

[a]Linear regression coefficient R^2 between parentheses.

Table 4: z_p-values for the pressure inactivation of *Z. bailii* at different temperatures

T (°C)	z_p (MPa)
-5	50,66 ± 7,70
--1	62,04 ± 4,83
10	66,89 ± 3,54
20	66,45 ± 1,84
30	84,89 ± 10,94
40	56,15 ± 4,05
45	51,57 ± 5,37

Table 5: z_t-values for the thermal inactivation of *Z. bailii* at different pressures

P (MPa)	z_{t1} (°C) T≤20°C	z_{t2} (°C) T≥20°C
180	-29,46±8,70	21,69±5,76
200	-19,85±4,69	19,25±8,22
220	-28,85±5,19	21,72±6,22
240	-31,81±7,85	30,12±3,85
260	-38,77±14,36	23,37±9,11

Table 6: Kinetic parameters of the fitted model

Kinetic parameter	value
α	-3,102484337±0,08670380327
β	0,003780834±0,00011888609
γ	-0,000000542±0,00000015304
δ	0,055365814±0,00351579415
ε	0,003780749±0,00023140431

Table 7:Comparison of z_p values of some microorganisms

Species	strain	medium	z_p-value (MPa)[a]	Temperature (°C)	P-range	Reference
Z. bailii	Syrup isolate	Sabouraud Dextrose broth, pH3,5, a_w=0.98	85.8[a]	21	241-345	Palou *et al.*, 1997
S. cerevisiae	IFO0234	0,85% NaCl	68[a]	-20 to 40	120-270	Hashizume *et al.*, 1995
Listeria monocytogenes	NCTC11994	Phosphate-buffered saline pH7.0	200[a]	22-24	350-450	Simpson and Gilmour, 1997
L. monocytogenes	Scott A	Phosphate-buffered saline pH7.0	200[a]	22-24	350-450	Simpson and Gilmour, 1997
L. monocytogenes	#2344	Phosphate-buffered saline pH7.0	130[a]	22-24	300-450	Simpson and Gilmour, 1997
Salmonella typhimurium	ATCC7136	63 mM phosphate buffer pH7.0	155[a]	23	240-340	Metrick *et al.*, 1989
S. seftenberg	775WW	63 mM phosphate buffer pH7.0	134[a]	23	240-340	Metrick *et al.*, 1989
E. coli	JMC1649	10 mM Hepes pH5.3	75[a]	20	150-300	Sonoike *et al.*, 1992
Lactobacilus casei	YIT9018	10 mM Hepes pH5.3	100[a]	201	200-400	Sonoike *et al.*, 1992

[a] z_p-values calculated by us from data in reference.

ANNEX 1.3: INACTIVATION OF ASCOSPORES OF *TALAROMYCES MACROSPORUS* BY HIGH HYDROSTATIC PRESSURE

1. Introduction

The potential of novel non-thermal processes such as high hydrostatic pressure (HP) processes to inactivate vegetative cells of bacteria and yeasts and to increase the shelf-life and safety of foods and pharmaceuticals has been demonstrated. At present, HP is mainly applied on acid foods, e.g. fruit-based products. A problem for these products is the high resistance of some fungal ascospores (Butz *et al.*, 1996). In the present study, we describe the effects of high pressure treatment (0-7000 bar) on the heat resistant ascospores from *Talaromyces macrosporus*.

2. Material and methods

After 35 days of sporulation on Malt Extract Agar (Oxoid) at 25°C, ascospores were collected in distilled water. The suspension was filtered through sterile glass wool to remove large mycelial fragments. Before pressurization, ascospore suspensions were given a heat treatment at 65°C/15min to inactivate remaining vegetative cells. This treatment did not influence the behaviour of the ascospores upon a subsequent heat or pressure treatment. Treatments at 80°C/30min were applied to activate dormant ascospores, and/or to assess heat sensitization of ascospores after pressure treatment. Pressure treatments were carried out at 20°C in distilled water. Non dormant viable ascospore counts were determined by surface plating on Rose Bengal Agar (Lab M).

3. Results

3.1. Activation, inactivation and sensitization of T. macrosporus ascospores

Pressure inactivation of *T. macrosporus* ascospores was studied in function of the treatment time at 20°C for pressures between 2000 and 7000 bar. Neither inactivation nor activation of the ascospores occurred at pressures below 3000 bar. Heat treatment (80°C/30min) of spores treated at <3000 bar resulted in activation of the dormant ascospores in a similar manner as with unpressurized spores. Between 3000 and 5000 bar, pressure activation takes place, but only a fraction of the dormant spores that can be activated by heat, are activated by pressure. It cannot be excluded however, that there is already some ascospore inactivation occurring simultaneously and thereby masking the activation. Net pressure inactivation occurred only at very high pressures (5000–7000 bar). However, the inactivation curves showed a substantial amount of tailing, indicating a large heterogeneity of the ascospore population towards high pressure inactivation (fig.1).

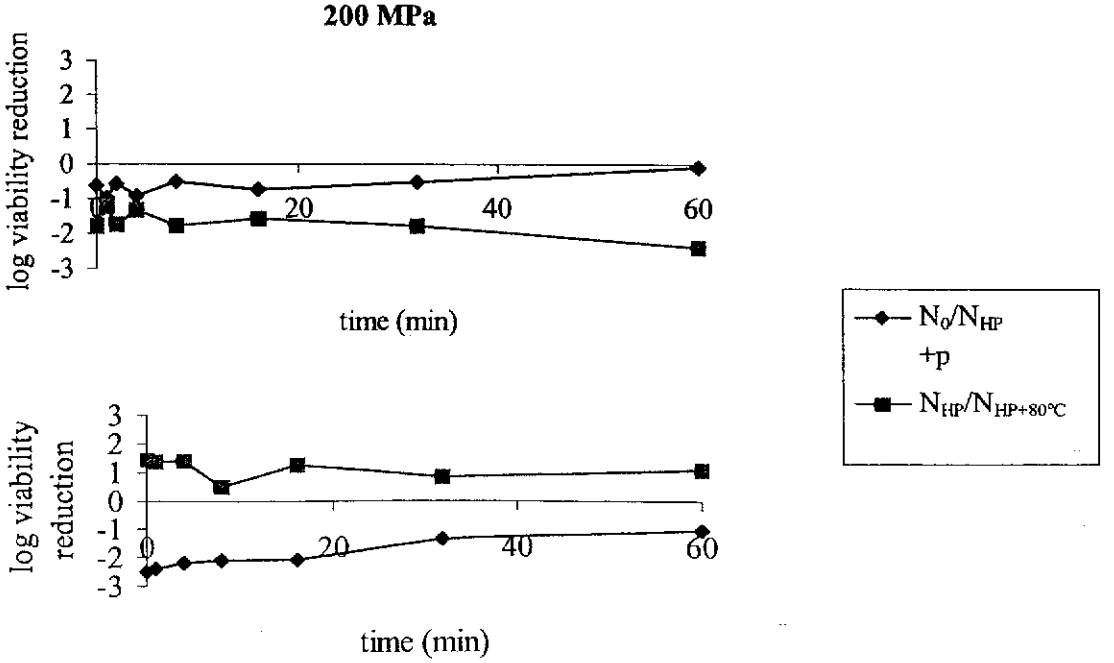


Figure 1: Pressure inactivation kinetics of *T. macrosporus* at different pressures. N_0 and N_{HP} are the non dormant viable ascospore counts before and after high pressure treatment respectively. $N_{HP+80°C}$ is the viable count after the pressure treatment followed by a heat treatment of 80°C.

Although direct inactivation by HP at 20°C was very limited, treatment at ≥5000 bar resulted in sensitization of the spores to subsequent heat treatment (80°C/30 min) (fig.1). The level of heat sensitization rapidly reached a plateau (15 sec pressure treatment), and was not substantially increased upon prolonged pressure treatment.
The effect of five successive short HP treatments (6000 bar/15 sec, 20°C) on the pressure resistance and the heat sensitivity of ascospores was studied in comparison to a single short HP treatment (6000 bar/15 sec, 20°C). Additional pressure cycles did not result in additional inactivation neither additional heat sensitization (fig. 2). This is another indication that the ascospore suspension is heterogeneous and consists of a HP-sensitive and a HP-resistant fraction. Also remarkable is that an important fraction of ascospores that becomes heat sensitive after a pressure treatment, apparently remains pressure resistant, indicating that these phenomena are not necessarily linked.

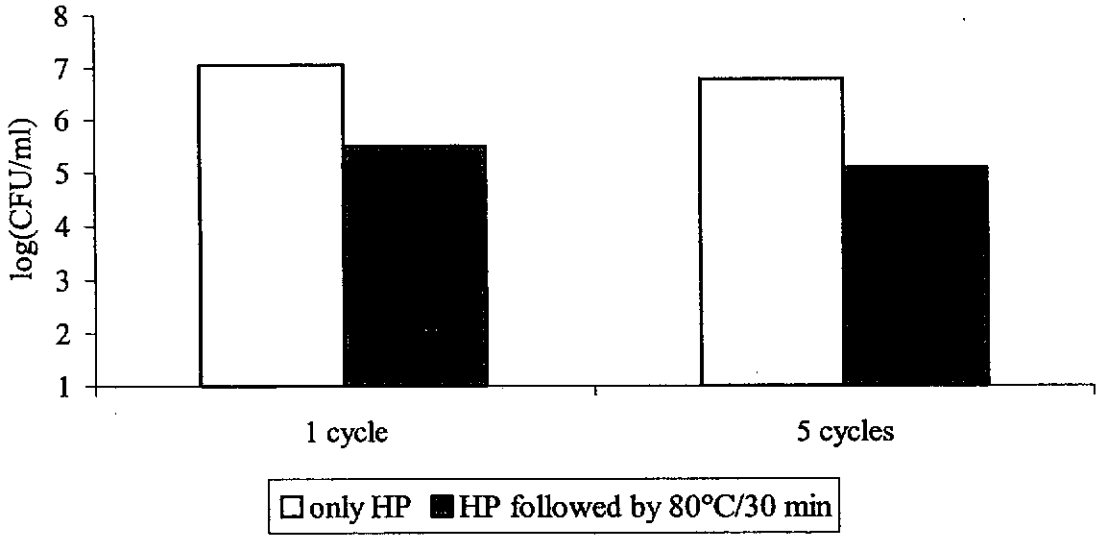
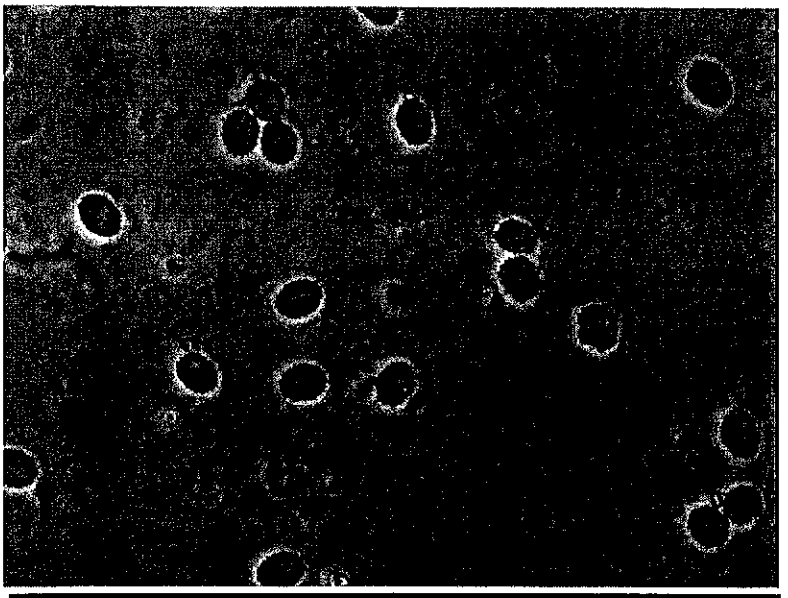


Figure 2: Effect of cycles of successive pressure treatments (HP), each of 6000 bar/15 sec at 20°C, on the high pressure inactivation and heat sensitization of *T. macrosporus* ascospores.

3.2.. HP induces germination of T. macrosporus ascospores

After a short HP treatment (6000 bar/15sec) at 20°C, ascospores suspended in distilled water without any nutrients were incubated for 24 hours at room temperature. After 4-8 hours, the formation of a germination vesicle could be seen under the light microscope (fig. 3) whereas no germinating spores were detected in a non pressurized ascospore suspension. We assume that this germination results in the heat sensitization of the ascospores described above. After 16-24 h, germinated ascospores had developed mycelial growth.

A



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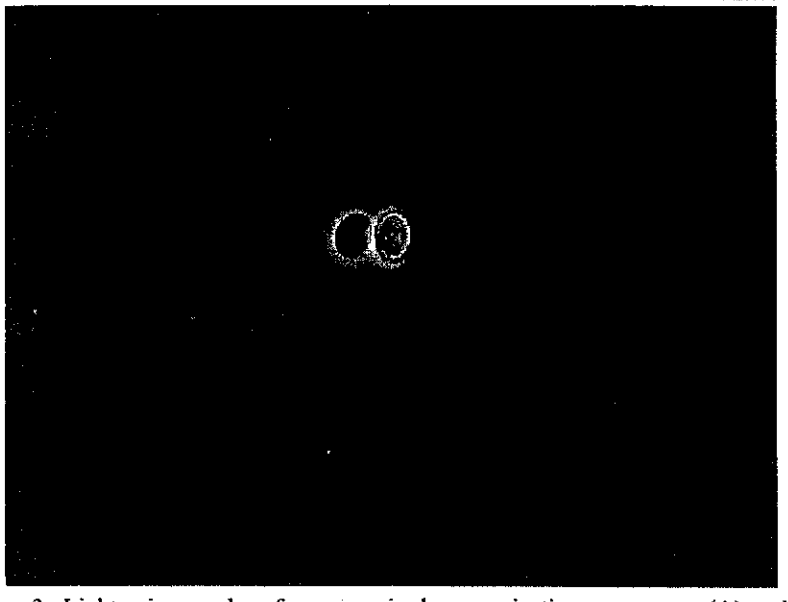
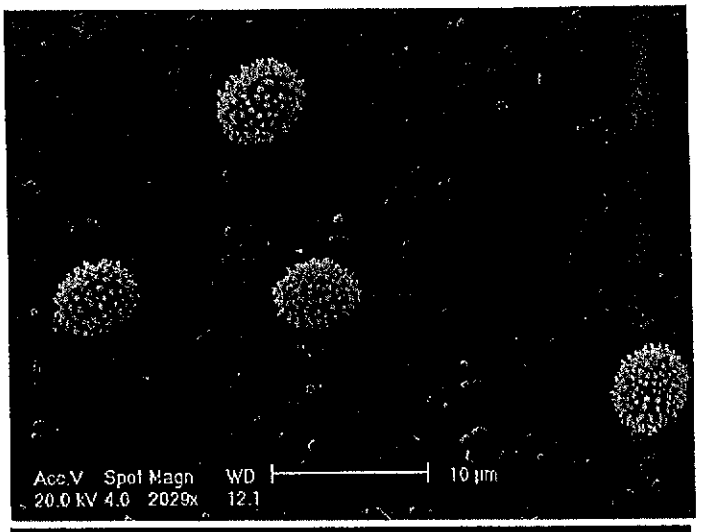


Figure 3: Light micrographs of unpressurized ungerminating ascospores (A) and pressurized (6000 bar/15 sec, 20°C) germinating ascospores (B) of *T. macrosporus* after incubation during 24 hours at room temperature in distilled water.

3.3. HP breaks the hydration barrier of T. macrosporus ascospores

No morphological changes could be seen in wet microscopic mounts of HP-treated ascospore suspensions. However, upon air-drying, the ascospores showed signs of collapse in light microscopy, that could be reversed by wetting. This effect was confirmed by scanning electron microscopy (SEM). Pressure-treated spores collapsed during SEM sample preparation, while untreated spores did not (fig. 4).

A



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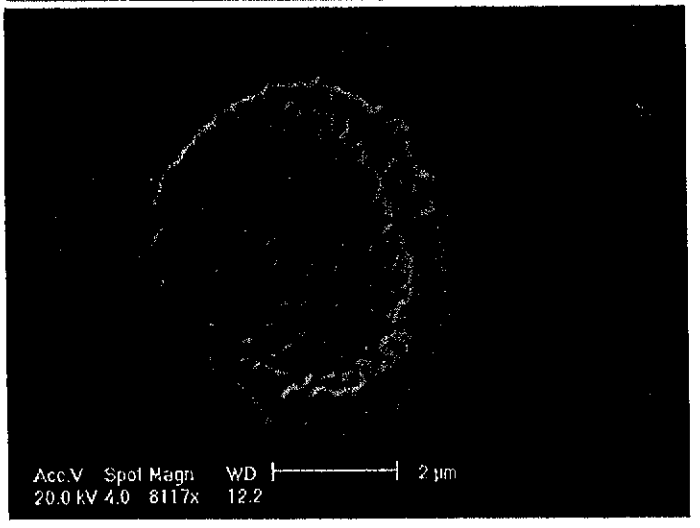

Figure 4: Scanning electron micrographs of non pressurized ascospores (A) and pressurized ascospores (B) of *T. macrosporus* (6000 bar/15 sec, 20°C).

4. Conclusions

1. Heat resistant ascospores of *T. macrosporus* are highly pressure resistant at ambient temperature.
2. At high pressures dormant ascospores can be activated. The permeability and/or rigidity of the spore wall will be affected.
3. High pressure treated ascopores become heat sensitive (80°C).
4. Ascospore populations are highly heterogeneous, displaying highly different susceptibility to HP activation, inactivation and heat sensitization.
5. HP treatment can induce ascospore germination in the absence of nutrients.
6. We suggest that HP treatment leads to hydration of the acospore core by causing irreversible damage to the ascospore wall. This can explain the observed effects, i.e. induction of germination, heat sensitization, and collapse upon drying.

5. Reference

Butz, P., Funtenberger, S., Haberditzl, T. and Tauscher, B. 1996. High pressure inactivation of *Byssochlamys nivea* ascospores and other heat resistant moulds. Lebensm.-Wiss. u.-Technol. 29:404-410.

ANNEX 1.4: HIGH PRESSURE INACTIVATION AND SURVIVAL OF PRESSURE-RESISTANT *ESCHERICHIA COLI* MUTANTS IN FRUIT JUICES

High hydrostatic pressure can be used to inactivate micro-organisms and quality-deteriorating enzymes in foods (6, 7) and, at least in some foods like fruit juices, allows a better retention of the original flavour and taste compared to thermal treatment (9, 10, 11, 12). Pressurized fruit-based foods were first introduced on the Japanese market in 1990, and it is likely that these will also be among the first products to be introduced in Europe and the U.S. Besides good retention of flavour, an important reason for this is that the low pH of fruit products (pH 3-4) does not support growth of pathogenic bacteria which may eventually survive pressurization. We succeeded recently in the isolation of spontaneous extremely pressure-resistant mutants from a pressure-sensitive *E. coli* strain (5). Based on studies in phosphate buffer, we anticipated that these mutants would be able to survive high pressure pasteurizations of food products at very high pressures (800 MPa or more) and mild temperatures (10-40°C). Although it is not yet clear whether pressure resistance occurs naturally in strains of *E. coli* or other bacteria, the possibility can not be excluded that pressure-resistant strains will build up in the selective environment of a high pressure food processing plant.

Although they can not grow at low pH, some strains of *Enterobacteriaceae* like *E. coli, Shigella* and *Salmonella* can survive for several days or even weeks in acidic foods (3, 8, 13). This has been particularly well documented for *E. coli* O157:H7, after this organism had been implicated in a number of recent infectious outbreaks caused by unpasteurized apple juice and cider (1, 2, 4, 14). Clearly, the efficient inactivation of *E. coli* will be a primary and unnegotiable requirement for HP processes for the production of high quality and safe fruit juices. Therefore, the aim of the present work was to study the high pressure inactivation of pressure-resistant *E. coli* mutants in three different fruit juices and in low pH buffers.

MG1655, LMM1010, LMM1020 and LMM1030 are the parental *Escherichia coli* strain and three different pressure-resistant mutants isolated thereof, respectively (5). Cultures in Luria Bertani broth grown to stationary phase at 37°C for 21 h with shaking were harvested by centrifugation (3000×g), and resuspended in juice or in 50mM Hepes buffer. Pressurizations were done for 15 min. at 20°C on small samples (0.5 - 1 mL) sealed in sterile polyethylene bags. The temperature increase due to adiabatic compression never exceeded 10°C. When survival had to be monitored during storage after HP treatment, a sufficient number of replicate bags was prepared and simultaneously treated to allow destructive sampling. All data shown are representative results from three independent experiments. Survivors were enumerated by plate counting on Tryptic Soy Agar (Biokar Diagnostics, Beauvais, France).

As a preliminary experiment, we studied the long-term survival of the strains in the three juices at two different storage temperatures. Survival was found to be similar for all strains, and positively correlated with juice pH, and negatively with temperature (Fig.1). Based on these results, considerable survival levels during several days or weeks can be anticipated also with lower, more realistic, levels of initial contamination, depending on the juice and the storage temperature. Others have also observed that refrigeration enhances acid survival of *E. coli* (3, 4, 14), and this may be due to a reduced membrane permeability for protons and/or a reduced metabolic activity.

GURE 1: Survival of *E.c*
M1030 (O) in fruit juices
10^6 CFU/mL. Detection li

Previously, pre
sphate buffer pH 7.0
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se obtained in Hepes
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most resistant strain

only carried out with this strain. The inactivation of LMM1010 in Hepes buffers pH 3.0-7.0 (Table 1) suggests that the increased pressure sensitivity of the mutants in the juices compared to Hepes pH 7 is at least partly due to the low pH.

TABLE 1. High pressure inactivation[a] (15 min, 20°C) of *E. coli* parental strain and pressure-resistant mutants in Hepes buffer and in apple and orange juice.

	300 MPa				400 MPa			
	MG1655	LMM1010	LMM1020	LMM1030	MG1655	LMM1010	LMM1020	LMM1030
Hepes pH 7.0	4.9	0.4	0.6	0.4	7.0	0.6	2.1	0.8
Hepes pH 4.0	N.D.	0.5	N.D.	N.D.	N.D.	2.6	N.D.	N.D.
Hepes pH 3.5	N.D.	1.2	N.D.	N.D	N.D.	2.3	N.D.	N.D.
Hepes pH 3.0	N.D.	2.9	N.D.	N.D	N.D.	3.3	N.D.	N.D.
apple pH 3.3	>4.4	1.1	2.0	>3.4	>4.4	>4.7	>3.4	N.D
orange pH3.8	3.5	0.8	0.4	1.6	>4.4	1.5	2.4	>3.4

[a] Inactivation was expressed as $\log(N_0/N)$, with N_0 and N the counts for the untreated control and the pressure-treated sample respectively.

In the next set of experiments, we studied the long-term survival of LMM1010 bacteria following a relatively mild pressurization in the three juices and in low pH Hepes buffers. The orange and apple juices were treated at 300 MPa only, while the mango juice was also treated at 400 and 500 MPa, because a lower kill was expected in this juice due to its higher pH. At least 10^6 cfu/mL survivors were counted in each juice immediately after pressure treatment (Fig.2). However, a considerable decrease in numbers of survivors was noticed upon storage of the pressurized juices at 8°C, in several cases resulting in undetectable levels after five days. Comparison of the results obtained with different juices at the same pressure (300 MPa), or in the same juice (mango) at different pressures, revealed that the inactivation rate during storage is inversely correlated with juice pH, and positively correlated with applied pressure. The same conclusion could be drawn from the experiments in Hepes buffers (Fig 3). This secondary inactivation achieved during storage was considerable compared to the primary inactivation caused by the pressurization, even under the mildest conditions. For instance, the low (1.1 log) direct reduction of LMM1010 by a 15 min treatment at 20°C and 300 MPa in apple juice, was followed by an extensive (almost 5 log) further reduction during the first two days of storage (Fig. 2). This phenomenon illustrates that the pressure treatment caused sublethal injury in a large proportion of cells, resulting in a reduced resistance to low pH.

FIGURE 2: Survival of *E.coli* LMM1010 in fruit juices stored at 8°C for up to 15 days after pressure treatment for 15 min. at 20°C. Orange (pH 3.8) 300MPa (◇), apple (pH 3.3) 300 MPa (■) and mango (pH 4.0) 300MPa (▲), 400MPa (●), 500MPa (O). Initial number of cells was 1.0-1.8 x 10^9 CFU/mL. Detection limit was 20 CFU/mL.

FIGURE 3: Survival of *E.coli* LMM1010 in Hepes buffers stored at 8°C for up to 10 days after pressure treatment for 15 min. at 20°C at 300MPa (A), 350 MPa (B) and 400 MPa (C). pH 3.0 (◆), pH 3.5 (■), pH 4.0 (▲). Initial number of cells was 0.35-1.8 x 10^9 CFU/mL. Detection limit was 20 CFU/mL. Inactivation in unpressurized controls was always < 1 log after 10 days of incubation.

From the safety point, a reassuring observation is that even under the mildest conditions applied in this study (mango juice pH 4.0 and Hepes buffer pH 4.0; 300 MPa), a hundredfold further reduction of pressure-resistant *E. coli* takes place during the first three days of refrigerated storage (Fig.1 and 2). Since enterohemorrhagic *E. coli* have a low infectious dose, this reduction may not yet provide the desired level of safety, and a higher pressure would be recommended in this particular case. If we assume that an acceptable safety level requires a 10^5-fold reduction, it can be seen from Fig.2 that this can be achieved, even in a pH 4 juice, within two days of refrigerated storage after a moderate pressure treatment (500 MPa, 20°C, 15 min). In other words, the observation of a two-day quarantaine

period between pressure treatment and consumption may significantly increase the safety of pressure-pasteurized fruit juices.

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ANNEX 2: DETAILED RESULTS ON ENZYME INACTIVATION KINETICS

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1. Enzyme systems studied

The enzyme systems extensively studied during this project are the following:
Bacillus subtilis α-amylase (Fluka, 10069) dissolved at a concentration of 15 mg/mL in 0.01M Tris HCl buffer at pH 8.6
Soybean lipoxygenase (Sigma, L7395) dissolved at a concentration of 0.4 mg/mL in 0.01M Tris HCl buffer at pH 9
Polyphenoloxidase extracted from avocado, dissolved at a concentration of 0.5 mg/mL in 0.1M phosphate buffer at pH 7
Pectinmethylesterase from oranges (Sigma, P5400) dissolved at different concentrations in water.
Pectinmethylesterase from tomatoes (Sigma, P6763) dissolved at a concentration of 0.4 mg/mL in water
Horseradish peroxidase (Sigma), dissolved at different concentrations in phosphate buffer at different pH values.
Myrosinase from broccoli, dissolved at a concentration of 15 mg/mL in 0.1M phosphate buffer at pH 6.55.

2. Materials and Methods

2.1. Isothermal inactivation

Isothermal treatment of the enzyme systems was carried out in a water bath with temperature control. To avoid heating and cooling lags, the samples were contained in capillary tubes. The residence time in the water bath was exactly measured using a stopwatch and after withdrawal from the water bath the samples were cooled either at 25°C or in ice and stored here until determination of the enzyme activity.

2.2. Isobaric-isothermal inactivation

Isobaric-isothermal treatments were performed in a laboratory pilot scale, multivessel high pressure equipment (HPIU-10000, 95/1994, Resato, Roden, Holland). This pressure equipment allows to combine high pressure (up to 1000 MPa) with temperatures between 0 and 100°C. High pressure is generated using a pressure intensifier in the central pressure circuit. The equipment consists of eight individual vessels (volume=8mL, diameter=10mm, length=100mm), surrounded by a thermostated mantle, connected to a cryostat. The vessels are connected to the central pressure circuit using T-joints and valves. The pressure transferring liquid is a glycol-oil mixture (TR15, Resato).
This equipment is ideally suited to perform kinetic inactivation experiments, because the samples in the individual vessels are submitted to inactivation treatments at the same pressure and temperature but for different treatment times. After enclosing the samples in the vessels (filled in 0.25mL microtubes, Elkay), only the central valve is closed and pressure is built up slowly (2-3 minutes) until the desired value is reached. This way, pressurization of the central tubing involves pressurization of the individual vessels. Once the maximum pressure is reached, the valves of the individual vessels are closed, isolating the pressure in each of the vessels and the central valve is opened. As a function of time, pressure can now be released in the individual vessels. In order to ensure isobaric-isothermal treatment of the samples, the activity of the sample from the first vessel, where pressure is released the moment the central valve is opened (after 4 min), is considered as the reference (blanco activity).

3. Data analysis

At first it was tried to define a mathematical model describing the course of thermal or pressure-temperature inactivation as a function of treatment time for each of the enzyme systems studied. For BSAA, LOX, PPO and tomato PE a first order kinetic model (2.1 or 2.2) could describe adequately the course of inactivation. For orange PE a fractional conversion model (2.3) was used while for MYR inactivation could be accurately described by a consecutive two-step model (2.4).

$$A_t = A_0 \exp(-kt) \tag{2.1}$$

$$A_t = A_0 \exp(-t/D) \tag{2.2}$$

$$A_t = A_\infty + (A_0 - A_\infty)\exp(-kt) \tag{2.3}$$

$$A(t) = \left(A_1 - A_2\left(\frac{k_1}{k_1 - k_2}\right)\right)\exp(-k_1 t) + \left(A_2\left(\frac{k_1}{k_1 - k_2}\right)\right)\exp(-k_2 t) \tag{2.4}$$

Secondly, temperature and pressure dependence of the inactivation rate constants was determined according to the Arrhenius (2.5) and Eyring (2.6) equation respectively.

$$\ln k = \ln k_{refT} - \left(\frac{E_a}{R}\left(\frac{1}{T_{abs}} - \frac{1}{T_{absref}}\right)\right) \tag{2.5}$$

$$\ln k = \ln k_{refP} - \left(\frac{V_a}{RT_{abs}}(P - P_{ref})\right) \tag{2.6}$$

Using the thermal death time terminology, temperature and pressure dependence of the D-value can be expressed as the z_T (2.7) and z_P (2.8) value.

$$\log D = \log D_{refT} - \frac{T - T_{ref}}{z_T} \tag{2.7}$$

$$\log D = \log D_{refP} - \frac{P - P_{ref}}{z_P} \tag{2.8}$$

Hence, kinetic parameters (k, D, z_T, z_P, E_a, V_a) were estimated using a two-step regression procedure. Both linear and non-linear regression procedures are performed in the statistical software package SAS (1982).

4. Results and discussion

4.1. Bacillus subtilis α-amylase (BSAA)

Isothermal inactivation

Isothermal inactivation of BSAA proceeded in the temperature range between 70 and 85°C and could be accurately described by a first order kinetic model. Consequently, first order inactivation rate constants could be calculated by plotting on a semilogarithmic scale, the activity retention as a function of time (figure 2.1), the slope being the reverse k-value. Kinetic parameters describing isothermal inactivation of BSAA are presented in table 2.1, together with the standard errors and regression coefficients. The high regression coefficients (0.961-0.997) and the small standard errors (2-8%) indicate the kinetic parameters to be estimated with high accuracy. From this table it could be concluded that BSAA exerts moderate thermal stability, requiring temperatures in the range 70-85°C for inactivation. The inactivation rate constant at 80°C and activation energy were calculated as 11.4×10^{-2} min^{-1} and 214.1 kJ/mol respectively.

Furthermore the influence of glycerol on the inactivation kinetics was investigated. Addition of 15% glycerol clearly retarded thermal inactivation of BSAA in the temperature range 70-85°C, pointing to an enhanced thermostability. The inactivation rate constant at 80°C was reduced to $3.13x10^{-2}$ min^{-1}, while nor reaction order, nor activation energy were affected by addition of glycerol.

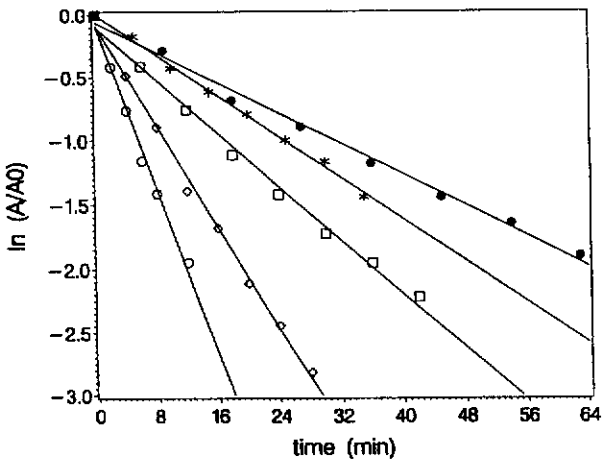

Figure 2.1. Inactivation of BSAA, 15 mg/mL in 0.01M Tris HCl at pH 8.6, at constant temperature: (•) 74°C, (*) 75°C, (□), 76°C, (◊) 79°C and (o) 82°C.

Table 2.1. Kinetic parameters describing isothermal inactivation of BSAA at atmospheric pressure (15 mg/mL in 0.01M Tris HCl buffer at pH 8.6 with or without 15% glycerol)

BSAA in Tris buffer			BSAA in Tris buffer with 15% glycerol		
T(°C)	*k-value (min^{-1})*	r^2	*T(°C)*	*k-value (min^{-1})*	r^2
74	$(2.99\pm0.09)x10^{-2}$	0.994	77	$(1.39\pm0.12)x10^{-2}$	0.961
75	$(4.04\pm0.08)x10^{-2}$	0.997	78.5	$(2.10\pm0.15)x10^{-2}$	0.972
76	$(5.25\pm0.18)x10^{-2}$	0.993	80	$(3.13\pm0.13)x10^{-2}$	0.991
79	$(9.95\pm0.26)x10^{-2}$	0.996	81.5	$(4.22\pm0.15)x10^{-2}$	0.993
82	$(16.2\pm0.85)x10^{-2}$	0.989	83	$(4.63\pm0.36)x10^{-2}$	0.970
E_a=214.1±12.0 kJ/mol r^2=0.991			E_a=214.9±25.2 kJ/mol r^2=0.960		

Isobaric-isothermal inactivation

Pressure-temperature inactivation of BSAA was investigated in the pressure range 100-750 MPa at temperatures varying between 25 and 73°C. The experimental domain of pressure-temperature inactivation had to be demarcated due to limitations in the experimental design. Duration of the experiments was chosen between 0.5 and 3 hours, hereby excluding low-temperature/low-pressure and high-temperature/high-pressure combinations from the data set. At all pressure-temperature combinations studied, the inactivation of BSAA could be accurately described by a first order kinetic model with regression coefficients varying from 0.945 to 0.998. First order inactivation rate constants for 45 combinations of constant pressure and temperature are presented in table 2.2.

Table 2.2. First order rate constants ($x10^{-2}$ min^{-1}) for isobaric-isothermal inactivation of BSAA, 15 mg/mL in 0.01M Tris HCl at pH 8.6. Pressure is given in MPa, temperature in °C.

P/T	25	30	35	40	45	50	55	60	64	67	70	73
100												2.86±0.1
150										0.95±0.0	2.01±0.0	4.55±0.2
200										1.93±0.0	3.82±0.1	
250								0.77±0.0	2.17±0.1	4.69±0.2	7.58±0.3	
300							0.95±0.1	2.08±0.0	5.14±0.2	9.48±0.5		
350						1.21±0.0	2.78±0.2					
400					2.14±0.1	2.74±0.1						
450			1.29±0.0	1.85±0.2	3.13±0.1	5.87±0.3						
500			1.92±0.0	3.43±0.1	4.52±0.1	7.93±0.3						
550		2.11±0.0	3.36±0.1	5.02±0.1	8.28±0.5	11.9±1.3						
600		3.09±0.1	4.66±0.1	9.67±0.3	10.3±0.5							
650	2.39±0.0	3.89±0.2	6.83±0.4	10.7±0.4								
700	2.57±0.0	4.30±0.3	7.17±0.3	10.5±1.0								
750	2.64±0.1	4.96±0.3										

The inactivation rate constants clearly increased with increasing pressure and temperature, i.e. inactivation occurs more readily at higher pressure and temperature. Hence pressure and temperature were found to act synergistically with respect to inactivation of BSAA.

Like for thermal inactivation, the influence of glycerol on pressure-temperature inactivation kinetics was investigated. BSAA in the presence of 15% glycerol was studied in the pressure-temperature range 400-650 MPa and 30-60°C. Again inactivation could be adequately described by a first order kinetic model. Regression coefficients varied between 0.930 and 0.997 and standard deviations ranged from 2 to 8% (table 2.3). The same trend, i.e. enhanced inactivation at higher pressure and temperature was observed in the presence of glycerol. However at corresponding pressure and temperature, *k*-values were significantly reduced, indicating that glycerol exerts not only a protective effect against thermal inactivation but also against pressure-temperature inactivation.

Table 2.3. First order rate constants ($x10^2$ min^{-1}) for isobaric-isothermal inactivation of BSAA, 15 mg/mL in 0.01M Tris HCl at pH 8.6 with 15% glycerol. Pressure is given in MPa, temperature in °C.

P/T	30	35	40	45	50	55	60
400						0.83±0.04	1.65±0.09
450				0.82±0.04	1.14±0.04	2.29±0.12	3.64±0.18
500				2.18±0.15	2.91±0.08	4.36±0.17	7.41±0.58
550			3.12±0.20	3.32±0.09	5.45±0.25	7.11±0.25	
600		3.02±0.09	4.39±0.22	5.77±0.28	6.37±0.52		
650	2.68±0.08	3.97±0.13	5.96±0.20	8.11±0.34			

To determine temperature dependence of the inactivation rate constants, activation energies at different constant pressure levels were derived according to eq. 2..5 from semilogarithmic plots of the inactivation rate constants as a function of reciprocal temperature. Activation energy values together with standard deviations and regression coefficients are summarized in table 2.4, the latter being situated in the range 2-20% and 0.927-0.998 respectively. From these statistical results it could be concluded that the Arrhenius model is valid both at atmospheric and elevated pressure.

Table 2.4. Activation energy (kJ/mol) for isobaric-isothermal inactivation of BSAA, 15 mg/mL in 0.01M Tris HCl buffer at pH 8.6 with or without 15% glycerol, at different constant pressure levels.

	BSAA in Tris buffer		BSAA in Tris buffer with 15% glycerol	
P (MPa)	*E_a (kJ/mol)*	*r^2*	*E_a (kJ/mol)*	*r^2*
0.1	214.1 ± 12.0	0.991	214.9 ± 25.2	0.960
250	220.6 ± 14.6	0.991	/	/
300	179.5 ± 11.7	0.992	/	/
450	83.6 ± 7.5	0.983	90.9 ± 8.8	0.982
500	75.0 ± 6.7	0.983	71.6 ± 7.5	0.979
550	71.1 ± 1.7	0.998	50.5 ± 10.2	0.927
600	69.8 ± 13.8	0.928	41.7 ± 6.9	0.949
650	78.3 ± 2.1	0.997	59.7 ± 2.1	0.998
700	73.5 ± 2.8	0.998	/	/

With respect to the influence of glycerol, two different cases had to be distinguished: at pressure below 500 MPa, activation energy was not affected whereas at higher pressure, there seemed to be a tendency for the activation energy to be reduced. Therefore it could be concluded that in the high pressure area, glycerol exerts a two-fold stabilizing effect: the inactivation reaction is retarded and the temperature sensitivity of the inactivation rate constant is reduced.

Pressure dependence on the other hand was determined according to eq. 2.6 from log-linear plots of the inactivation rate constant as a function of pressure. Activation volumes together with standard deviations and regression coefficients are presented in table 2.5. Standard deviations were situated in the range 3-20% while regression coefficients varied between 0.917 and 0.998, indicating that the Eyring equation could be applied over the entire temperature domain.

Table 2.5. Activation volume (cm^3/mol) for isobaric-isothermal inactivation of BSAA, 15 mg/mL in 0.01M Tris HCl at pH 8.6 with or without 15% glycerol, at different constant temperature levels.

	BSAA in Tris buffer		BSAA in Tris buffer with 15% glycerol	
T (°C)	*V_a (cm^3/mol)*	*r^2*	*V_a (cm^3/mol)*	*r^2*
30	-10.3 ± 1.6	0.932	/	/
35	-18.6 ± 1.9	0.961	/	/
40	-23.6 ± 2.6	0.964	/	/
45	-21.8 ± 1.5	0.985	-29.4 ± 3.5	0.963
50	-30.3 ± 3.6	0.960	-31.1 ± 6.5	0.917
55	/	/	-38.7 ± 4.5	0.973
67	-44.1 ± 1.5	0.998	/	/

The activation volume was negative at all temperatures, indicating that according to the principle of Le Chatelier, inactivation of BSAA is enhanced by an increase in pressure. Furthermore the absolute value of the activation volume seemed to increase with increasing temperature, although in the temperature range 35-45°C, it was rather constant. Upon addition of glycerol, the same trends were observed. Moreover, the absolute value of the activation volume became higher, suggesting that the susceptibility of the inactivation rate constant to pressure may be more pronounced in the presence of glycerol.

4.2. Soybean Lipoxygenase (LOX)

Isothermal inactivation

Thermal stability of soybean LOX at atmospheric pressure was studied in the temperature range 62-68°C. In this temperature range, isothermal inactivation of LOX followed first order kinetics, allowing inactivation rate constants to be determined from a semilogarithmic plot of the activity retention as a function of time (figure 2.2). The kinetic parameters describing isothermal inactivation of soybean LOX, together with standard deviations and regression coefficients are presented in table 2.6.

Table 2.6. Kinetic parameters describing isothermal inactivation of soybean LOX, 0.4 mg/mL in 0.01M Tris HCl at pH 9.

T (°C)	*k-value (min^{-1})*	r^2
62	$(2.02 \pm 0.09)x10^{-2}$	0.987
64	$(4.94 \pm 0.16)x10^{-2}$	0.993
66	$(9.18 \pm 0.32)x10^{-2}$	0.992
68	$(15.5 \pm 0.52)x10^{-2}$	0.992

E_a=319.8 ± 27.3 kJ/mol

r^2=0.986

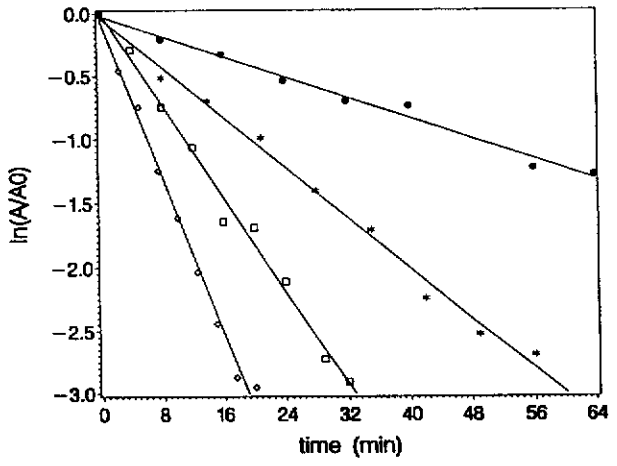

Figure 2.2. Inactivation of LOX, 0.4 mg/mL in 0.01 M Tris HCl at pH 9, at constant temperature: (•) 62°C, (*) 64°C, (□), 66°C and (◊) 68°C.

Isobaric-isothermal inactivation

Isobaric-isothermal inactivation of LOX was studied in the pressure range 50-650 MPa at temperatures between 10 and 64°C. By analogy with isothermal inactivation, first order inactivation kinetics were assumed to analyze isobaric-isothermal inactivation of LOX and verified on semilogarithmic plots of the activity retention as a function of time. Regression coefficients varied between 0.930 and 0.998. First order rate constants for isobaric-isothermal inactivation of LOX together with standard deviations are summarized in table 2.7, showing the latter to be situated between 1 and 10%. This table immediately indicates some antagonistic effects at low temperature (T<40°C) and high pressure (P>475 MPa). In this low temperature region, temperature increase causes the inactivation rate constant to decrease. Minimal inactivation rate constants are observed in the temperature range 30-40°C, showing LOX to exhibit maximal pressure stability at temperatures slightly higher than room temperature. At elevated pressure, both heat and cold denaturation of LOX are feasible.

Table 2.7. First order rate constants ($x10^{-2}$ min^{-1}) for isobaric-isothermal inactivation of LOX, 0.4 mg/mL in 0.01M Tris HCl at pH 9. Pressure is given in MPa, temperature in °C.

P/T	10	15	20	25	30	35	40	45	50	55	62	64
50												5.94±0.2
100											5.47±0.3	7.03±0.8
150											6.55±0.4	8.36±0.8
200											8.17±0.6	8.58±0.6
250											8.52±0.5	9.92±0.6
300										3.01±0.2		11.7±0.1
400										4.18±0.2		
475									1.48±0.0			
500	2.02±0.0	0.90±0.0	0.88±0.0					1.33±0.0	2.18±0.1			
525	3.48±0.2	2.68±0.2	1.81±0.0	1.18±0.0	0.79±0.0	1.19±0.0	1.71±0.0	1.98±0.1	2.74±0.1			
550	6.91±0.4	5.72±0.2	4.18±0.1	2.17±0.1	1.61±0.0		2.50±0.1	3.11±0.2	2.94±0.2	7.46±0.8		
575	14.5±1.4	7.37±0.4	7.24±0.6	7.73±0.3	3.90±0.1	3.83±0.2	3.20±0.1	3.31±0.1	3.67±0.1			
600			13.5±0.6	11.5±0.5	9.84±0.8	8.35±0.4	4.77±0.2	7.95±0.3				
625				18.8±1.1	16.9±1.7	14.8±0.4	11.6±0.9	12.1±0.2				
650				34.5±5.3	17.8±1.1		16.9±0.2					

Pressure dependence of the inactivation rate constant could be analysed using the Eyring eq. 2.6. Activation volumes together with standard deviations and regression coefficients are presented in table 2.8, denoting the Eyring equation to be valid over the entire temperature range. At all temperatures studied, the activation volume was negative as was expected from table 2.7. As opposed to the results obtained for BSAA, the absolute value of the activation volume seemed to be rather constant up to 35°C and to decrease with further increase in temperature, pointing to a reduced pressure sensitivity at high temperature.

Table 2.8. Activation volume (cm^3/mol) for isobaric-isothermal inactivation of LOX, 0.4 mg/mL in 0.01M Tris HCl at pH 9, at different constant temperature levels.

T (°C)	*V_a (cm^3/mol)*	*r^2*
10	-62.6 ± 3.1	0.995
15	-67.7 ± 12.1	0.935
20	-66.5 ± 2.8	0.995
25	-67.2 ± 5.9	0.970
30	-67.9 ± 7.5	0.955
35	-65.3 ± 2.5	0.997
40	-48.9 ± 4.4	0.963
45	-45.6 ± 4.5	0.961
50	-22.7 ± 3.3	0.941
55	-9.9 ± 0.4	0.998
62	-8.6 ± 1.4	0.944
64	-7.1 ± 0.5	0.976

Temperature dependence of the inactivation rate constant on the other hand could not be described by the Arrhenius equation (2.5) over the entire temperature region. Therefore it was investigated whether activation energies could be calculated for distinguished low and high temperature regions. An additional problem arose since these regions could not be demarcated unambiguously as temperature of maximal stability varied with pressure. The results are presented in table 2.9.

Table 2.9. Activation energy (kJ/mol) for isobaric-isothermal inactivation of LOX, 0.4 mg/mL in 0.01M Tris HCl at pH 9, at different constant pressure levels.

	Low temperature area		High temperature area	
P (MPa)	*E_a (kJ/mol)*	*r^2*	*E_a (kJ/mol)*	*r^2*
525	-53.9 ± 3.1	0.990	48.8 ± 3.8	0.982
550	-55.2 ± 6.6	0.956	42.9 ± 12.3	0.802
575	-34.1 ± 5.7	0.879	11.5 ± 3.4	0.916
600	-36.4 ± 7.4	0.889	/	/
625	-24.4 ± 3.7	0.956	/	/

As expected, activation energies are negative in the low temperature area, while positive at high temperature. Qualitatively it could be concluded that both in the low and high temperature area, there seemed to be a tendency for temperature sensitivity to decrease with increasing pressure. The poor statistical results (r^2: 0.800-0.982; standard deviations 7-30%) however indicate that quantitative interpretation of these results could lead to erroneous conclusions.

Influence of intrinsic factors on thermal and pressure inactivation of soybean LOX

As it is well established that kinetics largely depend on extrinsic (e.g. temperature) and intrinsic (e.g. state of the food component, properties of the surrounding medium) factors, a case study was performed with respect to the influence of enzyme production lot, enzyme concentration, pH and flushing with CO_2 on thermal and pressure inactivation kinetics of LOX. Thermal inactivation at atmospheric pressure was performed in the temperature range 54-72°C, depending on the environmental conditions. As the interest in the use of high pressure to inactivate LOX mainly arises from the possibility to avoid detrimental effects caused by heat, pressure inactivation (525 MPa) was performed in the low temperature range (10-25°C). Former results on LOX 0.4 mg/mL in 0.01M Tris HCl at pH 9, were used as reference.

Enzyme production lot

Although the kinetic behaviour was not affected by the enzyme production lot, thermal as well as pressure stability was strongly altered (table 2.10). As compared to the first lot, thermal inactivation of the second was achieved at lower temperature (54-64°C) and at corresponding temperatures *k*-values were increased about eightfold, referring to a reduced thermostability. Also pressure stability was markedly lower. However nor at atmospheric or elevated pressure, temperature sensitivity of the *k*-value was affected by the enzyme production lot. E_a-values for both production lots were not significantly different.

Table 2.10. Influence of enzyme production lot on thermal and pressure-temperature inactivation of LOX, 0.4 mg/mL in 0.01M Tris HCl at pH 9.

Production lot 1		Production lot 2	
P/T area	E_a-value (kJ/mol)	*P/T* area	E_a-value (kJ/mol)
0.1 MPa, 62-68°C	319.8±27.3	0.1 MPa, 54-62°C	277.6±7.1
525 MPa, 10-25°C	-50.9±4.4	525 MPa, 10-25°C	-68.3±15.8

Enzyme concentration

The influence of a reduced enzyme concentration (0.1 mg/mL) on thermal and pressure inactivation kinetics of LOX is summarized in table 2.11. Under all conditions studied, first order kinetics could analyse inactivation of LOX at a concentration of 0.1 mg/mL. Whereas thermal stability of LOX was slightly enhanced at higher enzyme concentration, no such effect was noted with respect to pressure stability, the *k*-values being not significantly different in the latter case. Besides, temperature sensitivity (E_a-value), both at atmospheric and elevated pressure, was not affected by the enzyme concentration.

Table 2.11. Influence of enzyme concentration on thermal and pressure-temperature inactivation LOX in 0.01M Tris HCl at pH 9.

0.1 mg/mL		0.4 mg/mL	
P/T area	E_a-value (kJ/mol)	*P/T* area	E_a-value (kJ/mol)
0.1 MPa, 60-66°C	300.4±35.5	0.1 MPa, 62-68°C	319.8±27.3
525 MPa, 10-25°C	-51.4±7.1	525 MPa, 10-25°C	-50.9±4.4

pH

The influence of pH was investigated the range 4.2-9. Because of restrictions set to buffering capacity of Tris HCl, a McIlvaine buffer was used in the low pH range (4.2-6.6). As solubility problems were encountered at pH 4.2, this pH value was not considered in further experiments. For each of the pH values studied (5.4, 6.6, 8, 9), thermal as well as pressure inactivation could be accurately described by a first order kinetic model. The influence of pH and type of buffer solution is shown in table 2.12.

Table 2.12. Influence of pH and type of buffer on thermal and pressure-temperature inactivation of LOX, 0.4 mg/mL.

Buffer system	*P/T* area	E_a-value (kJ/mol)
0.01M Tris HCl at pH 9	0.1 MPa, 62-68°C	319.8±27.3
0.01M Tris HCl at pH 8	0.1 MPa, 64-70°C	419.5±36.7
0.01M Tris HCl at pH 6.6	0.1 MPa, 66-72°C	428.7±7.7
McIlvaine buffer at pH 6.6	0.1 MPa, 66-725°C	439.8±57.6
McIlvaine buffer at pH 5.4	0.1 MPa, 66-72°C	383.8±77.1
0.01 M Tris HCl (pH 9) flushed with CO_2	0.1 MPa, 66-72°C	486.7±25.7
0.01M Tris HCl at pH 9	525 MPa, 10-25°C	-50.9±4.4
0.01M Tris HCl at pH 8	525 MPa, 10-25°C	-67.8±10.6
0.01M Tris HCl at pH 6.6	525 MPa, 10-25°C	-55.5±5.4
McIlvaine buffer at pH 6.6	400 MPa, 10-25°C	-101.7±11.5
McIlvaine buffer at pH 5.4	400 MPa, 20-25°C	-129.5
0.01 M Tris HCl (pH 9) flushed with CO_2	400 MPa, 10-25°C	-112.4±7.9

Both thermal and pressure stability were largely affected by the type of buffer. LOX seemed to be much more stable in Tris buffer as compared to McIlvaine buffer. Inactivation rate constants for isothermal inactivation at pH 6.6 were quadrupled on switching from Tris to McIlvaine buffer. Activation energy on the other hand was not affected by the type of buffer solution. Furthermore pressure stability in McIlvaine buffer was reduced to such an extent that inactivation could not be studied at the pre-set pressure (525 MPa), because after pressure build-up, the blank activity became too low to investigate sufficiently accurate progressive decrease in activity with time. Therefore, the influence of pH in McIlvaine buffer was investigated at 400 MPa.

For both buffer systems used, thermostability increased with decreasing pH, although this effect became less pronounced at pH 5.4. Thermostability of LOX seems maximal at a pH value corresponding to the isoelectric point. Although temperature sensitivity of the inactivation rate constant seemed lower in Tris buffer at pH 9, it was not possible to detect any significant differences in E_a-values for the enzyme solutions in McIlvaine or Tris buffer at different pH values, based on the 95% confidence interval. As opposed to thermal stability, pressure stability increased with increasing pH. Although the inactivation rate of LOX in Tris buffer at pH 9 and pH 8 was very similar, inactivation could be accelerated by lowering the pH to 6.6. In McIlvaine buffer, this pH effect was even more pronounced. For both buffer systems, no significant influence of pH on the activation energy value could be noted. These results could indicate possible mechanistic differences between thermal and pressure inactivation since at elevated pressure the enzyme is no longer protected against inactivation by pH values near the isoelectric point.

CO_2

To study the influence of CO_2, the enzyme solution (0.4 mg/mL in 0.01M Tris HCl at pH 9) was flushed with CO_2 for 5 minutes at a constant temperature of 25°C. Afterwards the solution was allowed to stand for 10 minutes to avoid supersaturation before the solution was filled in capillaries or microtubes. The kinetic parameters characterising the first order thermal or pressure inactivation of LOX in Tris buffer flushed with CO_2 are presented in table 2.12. Thermostability of LOX was greatly enhanced by flushing with CO_2, as the temperature range for inactivation was shifted to higher values. Next to this stabilisation effect however, sensitivity of the inactivation rate constant to temperature changes became more pronounced. Furthermore it was observed that *k*-values were on the same order of magnitude as those for inactivation of LOX at pH 6.6. Therefore the stabilising effect of CO_2 was completely attributed to a pH effect. The pH of the initial enzyme solution (pH 9) was verified to be

reduced to 6.8 after flushing. Dissolution of CO_2 in water results in the formation of H_2CO_3 which indeed lowers the pH.

Pressure stability on the other hand was largely reduced in the presence of CO_2. After building up pressure to 525 MPa, activity was reduced below the detection limit. Therefore, the experiments were carried out at 400 MPa, in agreement with those in McIlvaine buffer. Even at this lower pressure level, *k*-values were about 2 to 10 times higher. The results did not allow to draw any conclusions with respect to temperature sensitivity as experiments were carried out at different pressure levels and the activation energy for pressure-temperature inactivation of LOX has been shown to be dependent on pressure. Moreover, the activation energy was on the same order of magnitude as those for pressure inactivation in McIlvaine buffer (table 2.12). This may be explained taking into account the distinguished dissociation volumes of the buffers used. A Tris HCl buffer is known to be very insensitive to pressure variations and is characterized by a dissociation volume (ΔV^D) of about 1 cm^3/mol. For McIlvaine buffer and H_2CO_3, ΔV^D values in the range -25 to -30 cm^3/mol were derived. Therefore strong pH changes under pressure must be expected using the latter buffers. This way, extreme sensitisation when using McIlvaine buffer or CO_2 bubbled Tris buffer, as well as the close agreement between the results of pressure inactivation in these respective buffers may be explained.

4.3. Avocado polyphenoloxidase (PPO)

Isothermal inactivation

Thermal inactivation of avocado PPO was apparent at temperatures exceeding about 62.5°C. The thermal inactivation data, recorded at temperatures ranging from 62.5 to 77.5°C, could accurately be described by a first-order kinetic model (figure 2.3). The temperature dependence of the inactivation rate constants (table 2.13) could be expressed by the Arrhenius equation, with an activation energy of 319.28 ± 13.37 kJ/mol (figure 2.4).

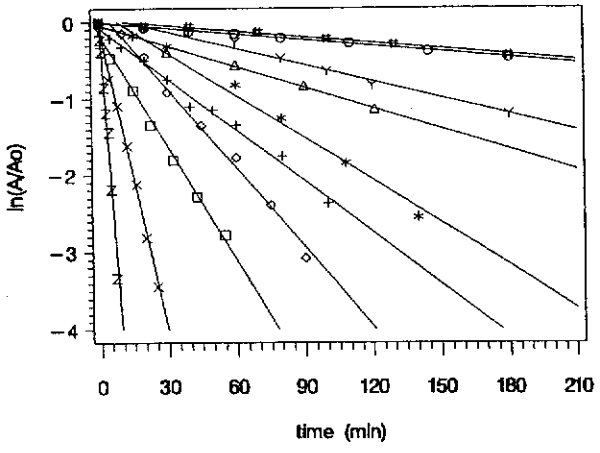

Figure 2.3. Inactivation of avocado PPO 0.5 mg/mL in 0.1M phosphate buffer pH 7, at constant P/T combinations (MPa/°C) : 0.1/65 (+), 0.1/70 (×), 0.1/75 (Z), 850/25 (O), 900/25 (Δ), 150/72.5 (*), 300/62.5 (#), 400/72.5 (◊), 600/70 (□) and 750/40 (Y).

Table 2.13 : k values ($* 10^2$ min^{-1}) for the combined pressure-temperature inactivation of avocado PPO in phosphate buffer pH 7

P (MPa) \ T (°C)	25	30	35	40	45	50	55	60	62.5	65	67.5	70	72.5	75	77.5
0.1									0.67 ± 0.01*	2.23 ± 0.07	4.86 ± 0.08	13.34 ± 0.26	21.61 ± 0.60	44.83 ± 1.68	111.66 ± 2.10
50									0.52 ± 0.02	0.81 ± 0.02	1.84 ± 0.11	4.30 ± 0.32	11.17 ± 0.62		
100									0.31 ± 0.01	0.35 ± 0.03	1.13 ± 0.08	1.84 ± 0.11	3.38 ± 0.10		
150										0.36 ± 0.01	0.66 ± 0.02	1.10 ± 0.05	1.84 ± 0.10		
200										0.26 ± 0.02	0.77 ± 0.05	1.13 ± 0.05	1.63 ± 0.09		
250										0.29 ± 0.02	0.57 ± 0.03	1.06 ± 0.06	1.30 ± 0.06		
300									0.25 ± 0.01	0.43 ± 0.02	0.93 ± 0.06	1.14 ± 0.04	1.75 ± 0.09		
350									0.30 ± 0.01	0.48 ± 0.01	0.83 ± 0.03	1.23 ± 0.07	2.04 ± 0.08		
400									0.37 ± 0.02	0.69 ± 0.14	1.23 ± 0.04	1.90 ± 0.11	2.38 ± 0.11		
450									0.69 ± 0.03	1.04 ± 0.05	1.55 ± 0.08	2.18 ± 0.11	3.44 ± 0.13		
500									0.89 ± 0.01	1.11 ± 0.07	2.30 ± 0.07	3.21 ± 0.06	4.76 ± 0.16		
550								1.10 ± 0.02	1.57 ± 0.03	2.20 ± 0.08	3.24 ± 0.02	3.88 ± 0.13	5.28 ± 0.26		
600							0.72 ± 0.05	1.11 ± 0.28	1.94 ± 0.05	2.91 ± 0.21	3.78 ± 0.23	4.95 ± 0.19	7.36 ± 0.45		
650						0.55 ± 0.11	1.32 ± 0.18	1.92 ± 0.32	2.89 ± 0.15	3.33 ± 0.35	5.64 ± 0.21				
700						1.11 ± 0.11	1.84 ± 0.08	2.44 ± 0.24	4.03 ± 0.19	6.21 ± 0.86					
750	0.09 ± 0.01	0.20 ± 0.01	0.31 ± 0.01	0.71 ± 0.04	0.86 ± 0.03	1.56 ± 0.13	2.18 ± 0.13	2.83 ± 0.05	5.55 ± 0.31						
800	0.15 ± 0.01	0.28 ± 0.01	0.46 ± 0.02	1.07 ± 0.05	1.57 ± 0.07	2.03 ± 0.07	3.31 ± 0.16	5.44 ± 0.27							
850	0.25 ± 0.01	0.37 ± 0.06	0.75 ± 0.04	1.57 ± 0.16	1.91 ± 0.04	2.70 ± 0.15	4.94 ± 0.15								
900	0.90 ± 0.06	0.89 ± 0.06	1.48 ± 0.06	2.92 ± 0.15	3.60 ± 0.16	4.36 ± 0.36									

* standard error

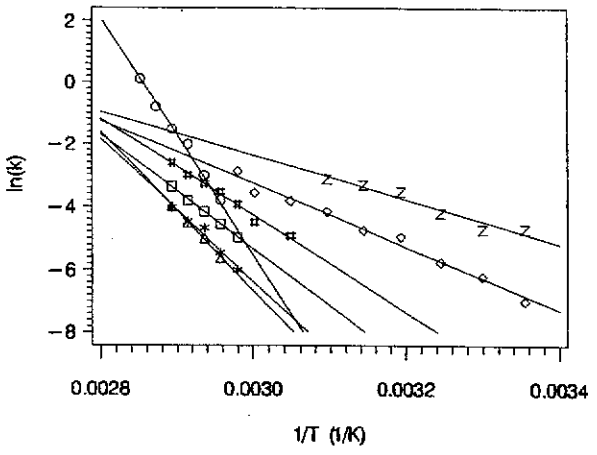

Figure 2.4. Temperature dependence of the inactivation rate constants of avocado PPO at 0.1 (O), 150 (Δ), 300 (*), 450 (□), 600 (#), 750 (◊) and 900 (Z) MPa.

Isobaric-isothermal inactivation

Inactivation of avocado PPO due to the combined effect of pressure and temperature could, under all conditions studied (50-900 MPa; 30-72.5°C), adequately be described by a first-order kinetic model (figure 2.3). Correlation coefficients (r^2) generally ranged between 0.960 and 0.999, however in four cases a value below 0.900 was obtained. The derived inactivation rate constants for these experiments were however in the expected range. The estimated inactivation rate constants are summarised in table 2.13. From this table, it is immediately clear that there is an antagonistic effect of pressure and temperature at high temperature ($T \geq$ 62.5°C) and 'low' pressure ($P \leq 250$ MPa) : in this high temperature/low pressure range, pressure increase resulted in a decrease of the observed inactivation rate constant. From table 2.13, it is furthermore clear that at constant pressure, the inactivation rate constant always increased with increasing temperature. This temperature dependency of the inactivation rate constant could at all temperatures studied adequately be expressed by the Arrhenius equation (figure 2.4). The corresponding activation energies together with their standard errors and correlation coefficients are summarised in table 2.14.

Table 2.14. Estimated activation energies for thermal inactivation of avocado PPO in phosphate buffer (pH 7; 0.1M) at atmospheric and elevated pressures

P (MPa)	E_a (kJ/mol)	r^2	P (MPa)	E_a (kJ/mol)	r^2
0.1	319.28 ± 13.37*	0.991	500	170.28 ± 15.19*	0.977
50	300.29 ± 22.77	0.983	550	119.83 ± 5.13	0.993
100	247.20 ± 30.01	0.958	600	128.30 ± 6.69	0.987
150	210.90 ± 6.64	0.998	650	102.31 ± 7.23	0.980
200	230.98 ± 47.15	0.923	700	89.36 ± 13.24	0.948
250	199.05 ± 30.02	0.957	750	83.56 ± 3.97	0.984
300	189.18 ± 19.50	0.969	800	83.17 ± 3.53	0.989
350	184.36 ± 4.16	0.999	850	80.60 ± 4.77	0.983
400	183.09 ± 17.07	0.975	900	58.45 ± 7.35	0.941
450	151.98 ± 3.54	0.998			

*: standard error

As already mentioned, the inactivation rate constants did not always increase with increasing pressure. Pressure dependence of the inactivation rate constants could therefore not always be

described by the Eyring equation. This means that in some pressure ranges, the activation volume changes with pressure. For pressures exceeding 300 MPa, the activation volumes were about constant with pressure (figure 2.5; table 2.15).

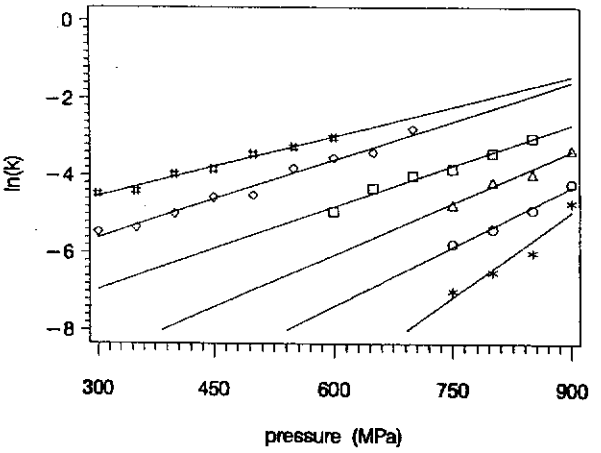

Figure 2.5. Pressure dependence ($P \geq 300$ MPa) of the inactivation rate constants of avocado PPO at 25 (•), 35 (○), 45 (Δ), 55 (□), 65 (◊) and 70 (#) °C.

Table 2.15 : Estimated activation volumes for pressure inactivation ($P \geq 300$ MPa) of avocado PPO in phosphate buffer (pH 7; 0.1M) at room and elevated temperatures

T (°C)	V_a (cm³/mol)	r^2
25	- 36.44 ± 6.59*	0.939
30	- 23.97 ± 4.94	0.922
35	- 26.47 ± 2.41	0.984
40	- 23.98 ± 2.00	0.986
45	- 23.70 ± 3.13	0.966
50	- 18.51 ± 0.91	0.990
55	- 19.48 ± 1.44	0.979
60	- 17.48 ± 2.18	0.942
62.5	- 20.34 ± 0.65	0.985
65	- 18.89 ± 1.04	0.979
67.5	- 15.89 ± 1.16	0.969
70	- 14.75 ± 0.84	0.984
72.5	- 14.17 ± 0.85	0.982

*: standard error

The absolute value of the activation volume ($P \geq 300$ MPa) seemed to decrease with increasing temperature in the temperature domains 25-50°C and 65-72.5°C. In the temperature domain 50-65°C, the activation volume ($P \geq 300$ MPa) was rather constant.
Activation volumes for pressure inactivation at 0.1-150 MPa were likewise about constant with pressure. The estimated values, which are presented in table 2.16 are positive, since pressure inactivation of avocado PPO is retarded in this 'low' pressure domain. The activation volume was found to increase with increasing inactivation temperature, meaning that the counteracting effect of 'low' pressure is more pronounced at high

Table 2.16 : Estimated activation volumes for pressure inactivation ($P \leq 150$ MPa) of avocado PPO in phosphate buffer (pH 7; 0.1M) at elevated temperatures

T (°C)	V_a (cm³/mol)	r^2
62.5	21.52 ± 4.26*	0.962
65	35.59 ± 9.45	0.876
67.5	36.65 ± 4.47	0.971
70	47.67 ± 5.59	0.973
72.5	49.34 ± 4.59	0.983

*: standard error

Influence of pH on the pressure inactivation at room temperature

The minimum pressure needed at room temperature to cause activity loss of avocado PPO in a reasonable time interval was strongly dependent on the pH of the medium. Pressure inactivation in McIlvaine buffer at pH 4, 5 and 6 became apparent at respectively about 450, 650 and 750 MPa. When the enzyme was dissolved in phosphate buffer at pH 6 or 7, pressure induced decay was noticeable upon pressurising at about 750 and 800 MPa, respectively.

Pressure inactivation of enzyme systems with pH ranging from 5 to 8 could satisfactorily be described by a first-order decay model, whereas inactivation of avocado PPO at pH 4 displayed overall non-first-order inactivation behaviour.

Pressure dependence of the first-order inactivation rate constants of avocado PPO solutions with pH values ranging from 5 to 8 could adequately be described by the Eyring equation. The activation volumes for pressure inactivation of avocado PPO at pH 5-8 revealed a decrease of the absolute value of the activation volume with increasing pH (table 2.17).

Table 2.17: Estimated activation volumes for pressure inactivation of avocado PPO at pH 5-8

pH	Buffer	V_a (cm³/mol)
5	McIlvaine buffer	- 33.05 ± 3.01*
6	McIlvaine buffer	- 24.99 ± 4.10
6	phosphate buffer	- 28.41 ± 1.77
7	phosphate buffer	- 22.38 ± 1.44
8	phosphate buffer	- 20.40 ± 2.70

*: standard error

From figure 2.6, it can readily be seen that the resistance of avocado PPO towards pressure decreases in the order pH 8 > 7 > 6 > 5. The pressure stabilities of avocado PPO dissolved in either McIlvaine buffer or phosphate buffer at pH 6 were not significantly different ($p > 0.05$).

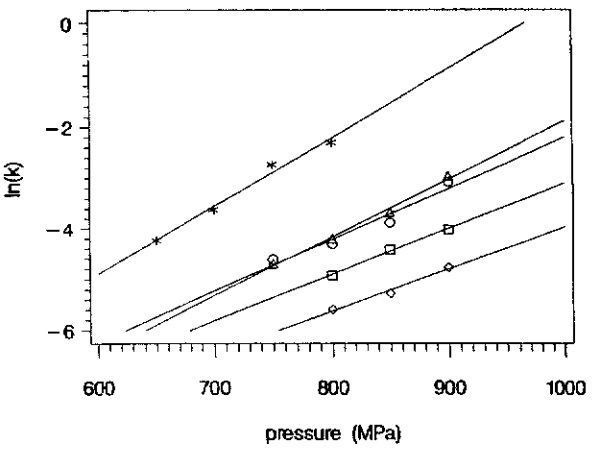

Figure 2.6. Pressure dependence of the inactivation rate constants of avocado PPO at pH 5 (*), 6 [McIlvaine buffer (O), phosphate buffer (Δ)], 7 (□) and 8 (◊).

As already mentioned, pressure treatment at pH 4 resulted in overall non-first-order inactivation behaviour of avocado PPO. In fact, this non-log-linear inactivation behaviour was only observed when treating the enzyme at 550-650 MPa (figure 2.7). At lower (450-500 MPa) and higher (700-900 MPa) pressures, apparent first-order inactivation kinetics were noticed. The inactivation curves recorded at 550-650 MPa were consequently used to discriminate among the different overall non-first-order inactivation models, i.e. general n^{th}-order model, distinct isozyme model, consecutive step model and fractional conversion model. The fractional conversion model appeared to be most suitable for modelling the inactivation data obtained.

When taking into account all observations, i.e. the observed first-order inactivation behaviour at low (450-500 MPa) and high (700-900 MPa) pressure and the fractional conversion behaviour at pressures ranging from 550 to 650 MPa, it could be hypothesised that at pH 4 the two isozymes of avocado PPO highly differed in pressure stability. The most pressure sensitive isozyme already lost its activity upon pressurising at 450 MPa, whereas the more resistant isozyme necessitated 700 MPa for inactivation. The apparent first-order inactivation behaviour at 450-500 MPa could thus be attributed to inactivation of the pressure labile isozyme, while the more resistant isozyme was not affected by the pressure treatment. Because of the duration of these inactivation experiments (2 hours), no 'activity plateau' was reached. The apparent first-order inactivation behaviour of avocado PPO when treated at pressures exceeding 650 MPa could, on the other hand, be ascribed to a *quasi* complete inactivation of the most pressure sensitive isozyme during pressure build-up. This inactivation was excluded from the experiment by taking a blank when reaching the desired pressure and temperature.

The inactivation curves recorded at 450-650 MPa were considered to calculate inactivation rate constants for the most pressure sensitive isozyme. For determination of the inactivation rate constants for the most pressure stable isozyme, the inactivation curves recorded at 700-900 MPa were taken into account. The fractional conversion model was used to estimate the inactivation rate constants for pressure inactivation at or below 650 MPa. The inactivation data obtained at higher pressure were analysed assuming a first-order kinetic model.

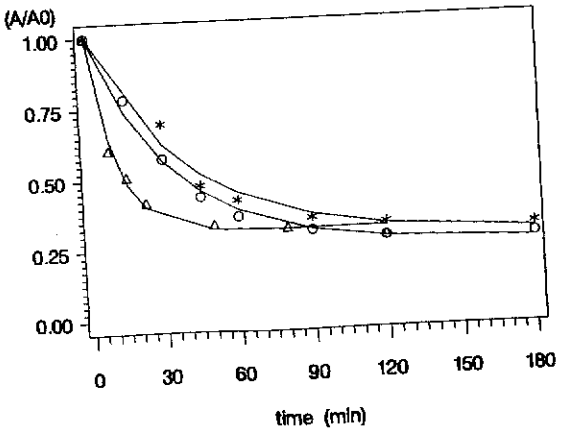

Figure 2.7. Pressure inactivation of avocado PPO in McIlvaine buffer (pH 4) at 550 (*), 600 (O) and 650 (Δ) MPa, modelled using the fractional conversion model.

The pressure dependence of the estimated inactivation rate constants could for both isozymes satisfactorily be described by the Eyring equation (r^2 = 0.964 and 0.976 for the labile and stable isozyme, respectively; figure 2.8). Activation volumes for pressure inactivation of the pressure sensitive and resistant isozymes respectively equalled -34.08 ± 3.81 cm³/mol and -21.05 ± 1.90 cm³/mol.
In the case of pressure inactivation of avocado PPO at pH 5-8, no evidence for the presence of isozymes with different pressure stability was found. In these conditions polyphenoloxidase activity always decreased log-linear in time. It is therefore suggested that the isoforms only display distinct pressure stability below pH 5.

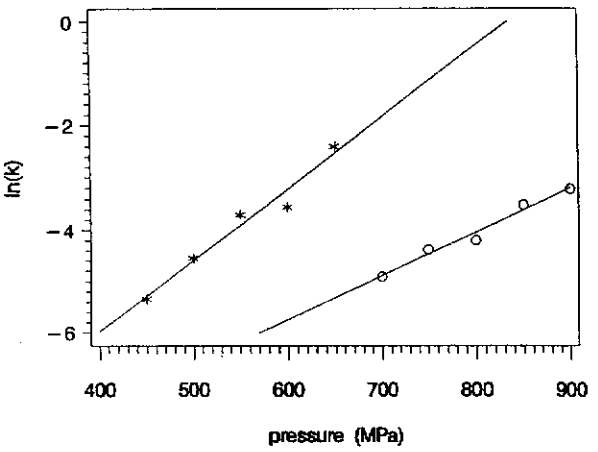

Figure 2.8. Pressure dependence of the inactivation rate constants for avocado PPO at pH 4 : pressure sensitive (•) and resistant (O) isozyme.

4.4. Pectinmethylesterase from tomatoes (tomato PE)

Isothermal inactivation

Model system
Isothermal inactivation of tomato PE in deionised water (0.4 mg/mL) could be accurately described by a first order kinetic model. The temperature range studied varied from 57 to 65 °C. The estimated kinetic parameters for isothermal inactivation of tomato PE are given in Table 2.18. From this table it can be concluded that tomato PE is inactivated by a rather mild treatment. The activity decreased already below detection limit by heating the sample for 3 minutes at 65 °C. The temperature sensitivity of the *D*-value, or the z_t-value, was calculated as 6.5 °C.

Influence of pH
The kinetic parameters for isothermal inactivation of tomato PE at different pH values are presented in table 2.18 and 2.19. For the values reported in table 2.18, the pH was controlled by addition of HCl. From this table, it can be deduced that an acidification of the medium by addition of HCl increases the thermal stability. Moreover, it is clear that the temperature stability increases (larger *D*-value) with decreasing pH. Related to the z_t-value (temperature sensitivity of the *D*-value) no clear influence was noted.

Table 2.18 : *D*- and z_t-values for the thermal inactivation of tomato PE in water, in water acidified with HCl, and in presence of Ca^{2+}-ions.

T (°C)	Water D (min)	pH 4.5 (HCl) D (min)	pH 3.5 (HCl) D (min)	$CaCl_2$ (1M) D (min)
55				46.94 ± 2.12*
57	37.40 ± 1.57*			28.52 ± 0.37
60	15.17 ± 0.42	41.24 ± 4.59*	366.17 ± 55.86*	10.07 ± 0.89
61.5				6.70 ± 0.77
63	4.71 ± 0.12	19.63 ± 1.76		
65	2.20 ± 0.11	9.31 ± 0.94	59.24 ± 5.60	
67		4.16 ± 0.49	23.52 ± 1.86	
70			5.21 ± 0.39	
	z_t-value (°C)	z_t-value (°C)	z_t-value (°C)	z_t-value (°C)
	6.45 ± 0.29*	7.03 ± 0.66*	5.46 ± 0.36*	7.49 ± 0.34*

* : standard error

Next to the use of HCl to acidify the medium also a citric acid buffer was used. The estimated kinetic parameters for the isothermal inactivation of tomato PE in citric acid buffer are presented in table 2.19. By comparing the inactivation in the buffer systems and in deionised water, it can be stated that tomato PE is more thermostable in buffer than in deionised water. In other words, acidification of the medium, or by addition of HCl or by using a citric acid buffer increases the thermal stability. In case of citric acid buffer an increased thermosensitivity of the *D*-value, expressed by the z_t-value, was observed. Moreover, tomato PE is more thermostable in citric acid buffer at higher pH values. This is in contradiction with the results obtained for acidification with HCl. Based on a 95% confidence interval, the z_t-value obtained in a buffer with pH 4.5 is significantly lower than the z_t-value obtained in a buffer with pH 3.5. However the difference in z_t-value between buffer pH 3.8 and 3.5 is not significant. An overall overview of the temperature sensitivity of the *D*-values in buffer systems and in deionised water is given in figure 2.9. In this figure it is visualized that the *D*-value is most thermosensitive for the inactivation in buffer pH 4.5.

Table 2.19 : *D*- and z_t-values for the thermal inactivation of tomato PE in water and in citric acid buffer pH 4.5, 3.8 and 3.5

T (°C)	Water D (min)	pH 4.5 (buffer) D (min)	pH 3.8 (buffer) D (min)	pH 3.5 (buffer) D (min)
57	37.40 ± 1.57*			
60	15.17 ± 0.42			
63	4.71 ± 0.12			46.27 ± 1.03*
65	2.20 ± 0.11	82.81 ± 8.18*	43.54 ± 2.57*	20.87 ± 0.93
67		31.19 ± 3.96	21.62 ± 2.03	9.02 ± 0.42
68.5		16.56 ± 0.74	10.70 ± 0.48	
70		7.05 ± 0.19	5.22 ± 0.21	
	z_t-value (°C)	z_t-value (°C)	z_t-value (°C)	z_t-value (°C)
	6.45 ± 0.29*	4.74 ± 0.18*	5.41 ± 0.32*	5.63 ± 0.09*

* : standard error

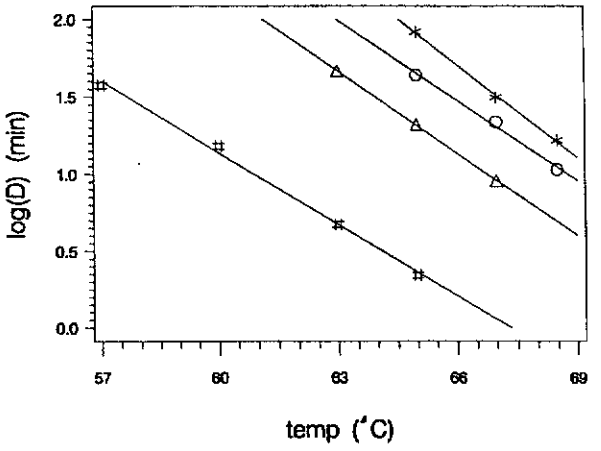

Figure 2.9: Temperature dependence of the *D*-values for the thermal inactivation of tomato PE in citric acid buffer pH 4.5 (*), pH 3.8 (O), pH 3.5 (Δ) and in water (#).

Influence of Ca^{2+}-ions

To investigate the influence of Ca^{2+}-ions on the thermal inactivation of tomato PE, a 1 M $CaCl_2$ solution was used. Again, a first order model was observed, and the estimated kinetic parameters are given in table 2.18. By comparing the estimated kinetic parameters obtained for the inactivation of PE in presence of and without Ca^{2+}-ions, it can be deduced that Ca^{2+}-ions accelerate the thermal inactivation on one hand and reduce the temperature sensitivity of the *D*-value on the other hand. This leads to the possibility that PE will be faster inactivated in natural products in which Ca^{2+}-ions are naturally present.

Pressure-temperature inactivation kinetics

Model systems

Experiments were performed at a temperature of 40 °C and 60 °C, in combination with different levels of pressure ranging from 0.1 MPa to 900 MPa. After pressure release, the residual activity was measured, and it was confirmed that the denatured enzyme did not reactivate after pressure treatment. In case of denaturation, a coagulation of the enzyme was observed after combined pressure-temperature treatment. This is in contrast to thermal inactivation.

At 40 °C, no inactivation of the enzyme was observed for pressures up to 700 MPa. At 900 MPa a D_{40}-value of 48 min was estimated. This points to a synergistic effect of pressure and temperature.

However, at 60 °C, an antagonistic effect of pressure and temperature was observed. This is graphically illustrated in figure 2.10. At atmospheric pressure a D_{60}-value of 15 min was estimated. Increasing the pressure suppressed the inactivation. Even at 900 MPa was the inactivation still slower than at atmospheric pressure ($D_{60\ °C,900\ MPa}$ = 30 min). It can thus be concluded that, although tomato PE is very heat labile it is pressure resistant.

It is also worthwhile mentioning that after a treatment at 60 °C and 100 MPa, a small activation of the enzyme was observed (activity of 126 % after 40 min at 60 °C and 100 MPa compared to the untreated enzyme).

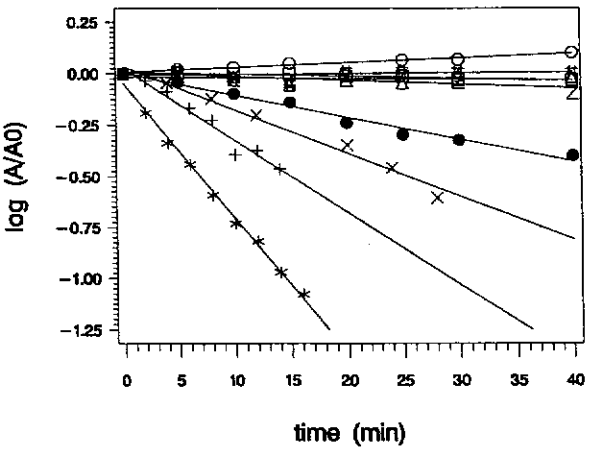

Figure 2.10: Pressure-temperature inactivation of tomato PE in water at 60 °C in combination with pressures of 100 MPa (O), 200 MPa (#), 300 MPa (□), 500 MPa (Δ), 700 MPa (z), 800 MPa (•), 850 MPa (×), 900 MPa (+) and atmospheric pressure (*).

Influence of pH

A study was carried out in order to find out whether pH has an influence on the pressure-temperature inactivation of tomato PE and more specifically on the antagonistic effect of pressure and temperature. As for thermal inactivation a 5 mM citric acid buffer pH 4.5, 3.8 and 3.5 was used. Because of a diminished thermal inactivation of tomato PE in buffer (compared with inactivation in deionised water), a temperature of 63 °C was selected. To facilitate a comparison between the different buffered systems, the temperature was kept constant at 63 °C whereas pressure ranged from 700 MPa to 900 MPa. The estimated kinetic parameters for the inactivation in deionised water as well as in buffer are presented in table 2.20. The decimal reduction times at atmospheric pressure are also given in the table. *D*-values at atmospheric pressure for pH 4.5 and 3.8 are extrapolated values (based on estimated kinetic parameters from table 2.19).

Table 2.20 : *D*- and z_p-values for the combined pressure-temperature inactivation of tomato PE in water and in citric acid buffer pH 4.5, 3.8 and 3.5

P (MPa)	Water D (min) T : 60 °C	pH 4.5 D (min) T : 63 °C	pH 3.8 D (min) T : 63 °C	pH 3.5 D (min) T : 63 °C	$CaCl_2$ (1M) D (min) T : 59 °C
0.1	15.17 ± 0.42*	221	108	46.27 ± 1.03*	
700			396.35±33.74*	190.33±19.54	
800	93.85 ± 5.84	193.91±13.91*	93.19 ± 7.08	70.85 ± 6.51	84.70 ± 3.76*
850	52.25 ± 2.37	74.85 ± 4.89	53.23 ± 3.66	41.49 ± 1.73	36.33 ± 1.24
900	29.60 ± 1.39	45.09 ± 2.43	20.24 ± 3.28		18.08 ± 0.94
	z_p (MPa)	z_p (MPa)	z_p (MPa)	z_p (MPa)	z_p (MPa)
	199.6 ± 1.8*	157.9 ± 33.8*	158.7 ± 9.8*	227.6 ± 4.6*	149.1±8.8*.

* : standard error

Acidification enhanced pressure-temperature inactivation of tomato PE, except at pH 4.5. This is in contrast to thermal inactivation where a diminished inactivation at all pH values was observed. A comparison of the inactivation in the three buffered systems reveals that the inactivation is most enhanced at the lowest pH value.

Although acidification slows down thermal inactivation and accelerates pressure-temperature inactivation, an antagonistic effect of pressure and temperature was still observed, this effect being more pronounced at lower pH values. Indeed, by comparing the different buffered systems mutually, it can be noted that at lower pH values, higher pressures were needed to obtain a similar decimal reduction time as at atmospheric pressure. This can be explained by the fact that a decrease of pH from 4.5 to 3.5 has more influence on thermal inactivation than on pressure-temperature inactivation. But even at the lowest pH value was the antagonistic effect less pronounced than in deionised water.
As related to the z_p-value, the lowest pressure sensitivity of the D-value was observed for the inactivation of tomato PE in buffer pH 3.5.
Concerning the stability of tomato PE in citric acid buffer towards temperature and/or pressure, it can be stated that tomato PE is less stable towards temperature and pressure at lower pH values, while the D-value is more thermosensitive and pressure sensitive at higher pH values.

Influence of Ca^{2+}-ions
To compare the antagonistic effect of pressure and temperature on the inactivation of tomato PE in presence of and without Ca^{2+}-ions, the combined pressure-temperature experiments were performed at a temperature for which a similar thermal decimal reduction time was obtained (*i.e.* D-value = 15 min). In case of thermal inactivation of tomato PE in presence of Ca^{2+}-ions, a temperature of 59 °C was estimated to obtain a D-value of 15 min. So, combined pressure-temperature experiments in presence of Ca^{2+}-ions were performed at a temperature of 59 °C whereas pressures ranged from 100 MPa to 900 MPa. The estimated kinetic parameters are presented in table 2.20 and the results are visualized in figure 2.11.

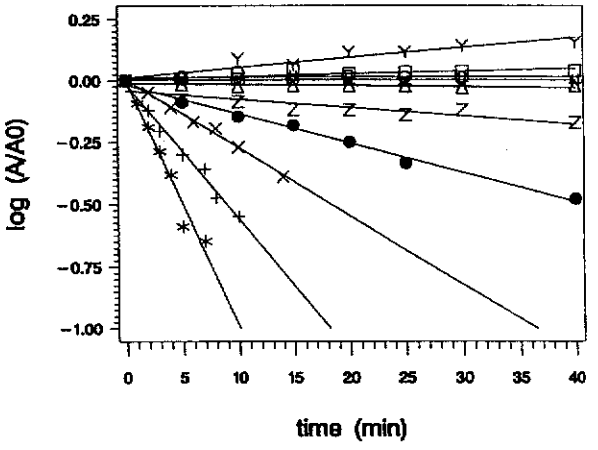

Figure 2.11: Pressure-temperature inactivation of tomato PE in presence of Ca^{2+}-ions at a temperature of 59 °C in combination with pressures of 100 MPa (O), 200 MPa (#), 300 MPa (□), 400 MPa (Y), 500 MPa (Δ), 700 MPa (z), 800 MPa (•), 850 MPa (×), 900 MPa (+) and atmospheric pressure (*).

From table 2.20, it can be derived that pressure-temperature inactivation was enhanced in presence of Ca^{2+}-ions. A similar behaviour was observed for thermal inactivation.
Secondly, an antagonistic effect of pressure and temperature was noted (see figure 2.11). A pressure increase to 700 MPa was necessary to slowly inactivate tomato PE. A further increase in pressure from 700 to 900 MPa accelerated the inactivation process, but even at 900 MPa was the inactivation still slower than at atmospheric pressure. However, the difference between the D_{59}-value at 900 MPa and atmospheric pressure is smaller in presence of Ca^{2+}-ions, which means that the antagonistic effect is less pronounced.
From the D-values estimated at 800, 850 and 900 MPa a z_p-value, which is a measure of the pressure sensitivity of the D-value, was calculated (see table 2.20). This value (149.1 MPa) is

smaller than for the inactivation in deionised water (i.e. the D-value is more pressure sensitive in presence of Ca^{2+}-ions) which is opposed to thermal inactivation where a diminished temperature sensitivity of the D-value was observed in presence of Ca^{2+}-ions.
After low pressure treatment at 59 °C, an increased activity of the enzyme was observed (see figure 2.11). The highest activity was attained at 400 MPa (activity of 143 % after 40 min at 59 °C and 400 MPa compared to the untreated enzyme). By decreasing temperature (40 °C – 50 °C), an increase in activity after low pressure treatment was no longer observed.
Subsequently, pressure-temperature experiments were carried out at 40 °C in combination with higher pressures. Up to 700 MPa no inactivation of the enzyme was observed. By increasing the pressure to 900 MPa, inactivation was attained and a D_{40}-value of 45 min was estimated.

4.5. Pectinmethylesterase from oranges (orange PE)

Thermal inactivation kinetics

Effect of enzyme concentration
Isothermal inactivation of orange PE in deionized water was studied in the temperature range from 57 to 67 °C. Different concentrations of different lots were taken into consideration. The kinetic parameters estimated with aid of the fractional conversion model are presented in table 2.21.
The influence of initial concentration on the inactivation rate constant k, seems to be dependent on the lot. This implies that the same lot should be considered to draw conclusions about the effect of enzyme concentration. By comparing 0.4 mg/mL and 1.0 mg/mL of lot V and 1.0 mg/mL and 1.5 mg/mL of Lot Z, it can be concluded that the enzyme stability against heat is higher in concentrated than in diluted solutions. The temperature dependence of the k-value, expressed by the activation energy E_a, slightly decreases by increasing the enzyme concentration. However, this effect is not pronounced. For lot Z, an increase in the residual fraction was observed by increasing the enzyme concentration. A similar increase was not found for lot V.
Although differences were observed between the different lots, a common characteristic of all the lots used in this study, was a remaining fraction being independent on the temperature level in the temperature domain studied. This is visualized in figure 2.12 for the thermal inactivation of orange PE (lot Z) in a concentration of 1.5 mg/mL.
Lot Z is clearly characterized by a higher heat stable fraction than the other lots. 16 % to 19 % of the original activity, depending on the initial enzyme concentration, remained active after treatment. In previous research, it was demonstrated that the percentage residual PE is dependent on the variety of the oranges used to extract PE. For lot X, Y and V, 5 to 7 % of the original activity was found to be thermostable. This remaining heat stable fraction could be completely inactivated by heating the sample during one minute at a temperature of 85 °C. It was assumed that the residual fraction active PE was a reflection of the heterogeneity of the enzyme. This was confirmed by an electrophoretic study of the enzyme solution. On a silver stained separation pattern of IEF 3-9 gels and 8-25 gradient gels several bands were found. This led to the conclusion that commercially available PE contains multiple enzyme forms differing in isoelectric points and molecular weights and also in heat stability.

Table 2.21: Kinetic parameters for isothermal inactivation of orange PE in water at atmospheric pressure

	Lot V		Lot X	Lot Y	Lot Z	
	0.4 mg/mL	1.0 mg/mL	0.5 mg/mL	0.8 mg/mL	1.0 mg/mL	1.5 mg/mL
k_{57} (min^{-1})	0.059 ± 0.005*	0.041 ± 0.005				
k_{60} (min^{-1})	0.153 ± 0.005*	0.103 ± 0.003	0.124 ± 0.003	0.112 ± 0.008	0.165 ± 0.003	0.113 ± 0.004
k_{63} (min^{-1})	0.507 ± 0.026*	0.266 ± 0.013	0.317 ± 0.006	0.397 ± 0.028	0.469 ± 0.012	0.335 ± 0.014
k_{65} (min^{-1})	0.942 ± 0.026*	0.522 ± 0.029	0.748 ± 0.027	0.680 ± 0.042	0.897± 0.084	0.591 ± 0.033
k_{67} (min^{-1})			1.415 ± 0.024	1.269 ± 0.031	2.359 ± 0.126	1.396 ± 0.055
% residual	6	6	5	7	16	19
Ea (kJ/mol)	326.9 ± 11.7#	301.4 ± 12.3	332.9 ± 15.0	323.6 ± 22.3	350.5 ± 22.8	331.9 ± 16.1

* : asymptotic standard error

: standard error

Table 2.25 : Kinetic parameters for isothermal-isobaric inactivation of orange PE in water at elevated pressure

	Lot V		Lot Y	Lot Z		Lot X	
	0.4 mg/mL	1.0 mg/mL	0.8 mg/mL	1.0 mg/mL	1.5 mg/mL	1.8 mg/mL	1.8 mg/mL
	25°C	25 °C	30 °C	30 °C	30 °C	20 °C	30 °C
k_{600MPA} (min^{-1})			0.029 ± 0.003	0.020 ± 0.002	0.024 ± 0.001		0.018 ± 0.001
k_{700MPA} (min^{-1})			0.046 ± 0.005	0.061 ± 0.003			
k_{750MPA} (min^{-1})							
k_{750MPA} (min^{-1})	0.072 ± 0.002*	0.047 ± 0.003					
k_{800MPA} (min^{-1})	0.119 ± 0.003*	0.073 ± 0.006	0.139 ± 0.024			0.130 ± 0.011	0.196 ± 0.007
k_{850MPA} (min^{-1})	0.241 ± 0.016*	0.117 ± 0.004					
k_{900MPA} (min^{-1})	0.295 ± 0.014*			0.586 ± 0.243			
% residual	5	5	5	14	28	7	7
Va (cm^3/mol)	-24.55 ± 3.44#	-22.48 ± 0.61	-27.52 ± 0.60	-28.23 ± 0.16			

* : asymptotic standard error

: standard error

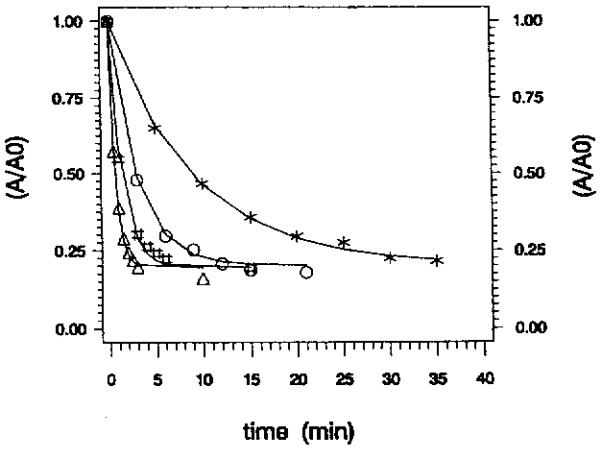

Figure 2.12: Thermal inactivation of orange PE in water at 60 (*), 63 (o), 65 (#) and 67 (Δ) °C.

Influence of pH

The influence of pH on the isothermal inactivation of orange PE was investigated by using a citric acid buffer 5mM. As pH, the pH of commercially available orange juice was selected, i.e. pH 3.7. The inactivation kinetics of two different lots (1 mg/mL of lot Z and 1.7 mg/mL of lot W) were considered. Temperatures ranged from 45 to 60 °C. The estimated kinetic parameters are presented in table 2.22. By analogy with the inactivation in water, is the inactivation behaviour in acid medium clearly dependent on the lot used. Again, lot Z is characterized by a higher percentage residual PE after treatment.

A comparison of the kinetic parameters obtained for lot Z (table 2.21 and table 2.22), reveals that orange PE inactivates faster and at lower temperatures in citric acid buffer than in water, which means that orange PE is less thermostable in an acid medium. It can also be concluded that the inactivation rate constant in citric acid buffer pH 3.7 is significantly less temperature sensitive than the inactivation rate constant in water. The temperature dependence of the *k*-value is visualized in figure 2.13. Regarding the residual fraction active PE, this fraction is obviously more temperature dependent in buffer than in water. A decrease of the residual fraction is observed with increasing temperature; 21 %, 13 % and 12 % of the original activity remained active after treatment at respectively 50 °C, 55 °C and 60 °C. This indicates that the residual fraction active PE after treatment in a similar temperature domain, is smaller in buffer than in water. Orange PE from lot W is even more thermolable and a smaller fraction remains active after treatment.

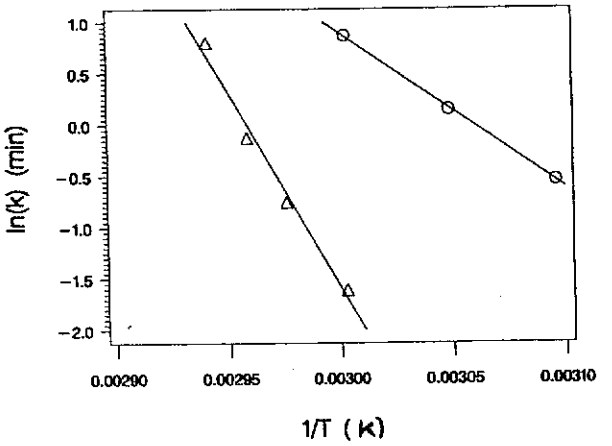

Figure 2.13: Temperature dependence of the *k*-values for the thermal inactivation of orange PE in citric acid buffer pH 3.7 (o) and in water (Δ).

Table 2.22: Kinetic parameters for isothermal inactivation of orange PE in citric acid buffer pH 3.7 at atmospheric pressure

Temp (°C)		Lot Z 1 mg/ml	Lot W 1.7 mg/ml
45	k_{45} (min^{-1})		0.184 ± 0.006
	% residual		8
50	k_{50} (min^{-1})	0.579 ± 0.073*	0.951 ± 0.090
	% residual	21	6
55	k_{55} (min^{-1})	1.158 ± 0.141*	4.530 ± 0.453
	% residual	13	5
57	k_{57}(min^{-1})		6.376 ± 1.184
	% residual		4
60	k_{60}(min^{-1})	2.375 ± 0.267*	
	% residual	12	
	Ea (kJ/mol)	126.2 ± 2.4#	263.0 ± 12.3

* : asymptotic standard error

: standard error

Influence of Ca^{2+}-ions

To investigate the influence of Ca^{2+}-ions on the thermal inactivation of orange PE, the enzyme was dissolved in 0.5 M, 1M and 1.5 M $CaCl_2$-solution. Temperatures from 45 to 58 °C were applied. In all cases, inactivation data could be modelled with the fractional conversion model. The estimated kinetic parameters are presented in table 2.23.

By comparing the inactivation parameters of lot Z from table 2.21 and table 2.23, it can be deduced that Ca^{2+}-ions accelerate the inactivation of PE. Moreover in case of 1.0 M and 1.5 M $CaCl_2$-solution, a decreased temperature sensitivity of the *k*-value was noticed. Although orange PE is less thermostable in presence of Ca^{2+}-ions, the percentage residual PE after treatment is higher. Comparing the different molarities of $CaCl_2$ solution mutually, shows that increasing the salt concentration decreases the thermal stability of orange PE, lowers the inactivation temperature and results in a larger percentage residual PE after treatment (respectively 23 %, 28 % en 36 % for 0.5M, 1M and 1.5M $CaCl_2$ solution). Also a small decreased temperature sensitivity of the *k*-value is observed with increasing salt concentration. To be sure that the significant increase in residual fraction active PE after treatment is not dependent on the lot used, similar experiments were performed with lot V (0.4 mg/mL). Again an increased inactivation, a decreased activation energy and an increased residual fraction active PE after treatment was observed in an 1 M $CaCl_2$ solution. However, it should be stressed that the increase in residual fraction is less pronounced for lot V.

It is also worthwhile mentioning that the initial activity of orange PE in presence of Ca^{2+}-ions decreases as the molarity of the $CaCl_2$ solution increases. This is possibly due to the fact that Ca^{2+}-ions interact with the substrate which makes it less available for the enzyme.

Table 2.23: Kinetic parameters for isothermal inactivation of orange PE in presence of $CaCl_2$ at atmospheric pressure

	Lot Z (1.5 mg/mL)			Lot V (0.4 mg/mL)
	$CaCl_2$ 0.5 M	$CaCl_2$ 1.0 M	$CaCl_2$ 1.5 M	$CaCl_2$ 1.0 M
k_{45} (min^{-1})			0.142 ± 0.004	0.114 ± 0.008
k_{47} (min^{-1})			0.271 ± 0.009	
k_{50} (min^{-1})		0.259 ± 0.003	0.861 ± 0.069	0.716 ± 0.055
k_{52} (min^{-1})				1.260 ± 0.136
k_{53}(min^{-1})	0.224 ± 0.002*	0.789 ± 0.022		
k_{55} (min^{-1})	0.486 ± 0.009*	1.515 ± 0.046	4.315 ± 0.293	
k_{58} (min^{-1})	1.429 ± 0.013*			
% residual	23	28	36	10
Ea (kJ/mol)	331.9 ± 5.4#	312.6 ± 9.9	298.5 ± 6.9	267.6 ± 5.9

* : asymptotic standard error
# : standard error

Influence of sucrose

The influence of sucrose on the thermal inactivation of commercially available orange PE was investigated by heating orange PE (0.4 mg/mL of Lot V) in a 20 % sucrose solution. Temperatures from 60 to 65 °C were applied. The fractional conversion model was the most suitable model to fit the inactivation data. The estimated kinetic parameters are presented in table 2.24.

From a comparison of the inactivation parameters of lot V in absence and presence of sucrose (see table 2.21 and table 2.24), it appears that the inactivation rate decreases in presence of sucrose. However, the decreased stability does not influence the residual fraction: the same fraction remained active as for the inactivation in absence of sucrose. Estimates for the E_a-values indicate that the inactivation rate constant k is slightly more temperature sensitive in presence of sucrose.

Table 2.24: Kinetic parameters for isothermal inactivation of orange PE in presence of 20 % sucrose at atmospheric pressure

	Lot V
	0.4 mg/ml
k_{60} (min^{-1})	0.072 ± 0.007*
$k_{61.5}$ (min^{-1})	0.121 ± 0.009*
k_{63} (min^{-1})	0.239 ± 0.016*
k_{65}(min^{-1})	0.440 ± 0.015*
% residual	6
Ea (kJ/mol)	346.3 ± 18.4#

* : asymptotic standard error
# : standard error

Pressure-temperature inactivation kinetics

Effect of enzyme concentration

Different concentrations of different lots of orange PE were subjected to combined pressure-temperature treatment. Pressure range studied, varied from 600 to 900 MPa, while temperatures of 20, 25 and 30 °C were applied. Also for pressure treatment, a certain fraction of orange PE appears to be pressure stable and hence the fractional conversion model was used to fit the inactivation data. The estimated kinetic parameters are presented in table 2.25. From this table, the synergistic effect of pressure and temperature can be derived; increasing pressure at a fixed temperature increases the inactivation rate. Likewise leads increasing

temperature at a fixed pressure to increased inactivation rates. In table 2.25, the residual fraction is expressed as a percentage of the original activity. Once more, a distinction can be made between lot Z and the other lots, with regard to the remaining active fraction: lot Z is characterized by a higher pressure stable fraction. However, the remaining fraction was of the same order of magnitude for each lot individually, whether orange PE was inactivated by temperature or by pressure.
Similar to thermal treatment was the influence of initial concentration on the inactivation kinetics dependent on the lot used. By considering the same lot (lot V), it can be derived that the enzyme is more pressure stable in more concentrated solutions. But in case of very slow inactivation (see lot Z), enzyme concentration has almost no influence on the inactivation rate constant *k*. Moreover, a small decreased pressure sensitivity of the *k*-value, expressed by the activation volume, was observed by increasing the enzyme concentration. Regarding the residual fraction active PE after pressure treatment, differences were observed depending on the lot. For lot Z, an increase in residual fraction from 14 % to 28 % of the original activity was noticed by increasing the enzyme concentration. However, for lot V, the residual fraction remained the same (i.e. 5 %). A similar behaviour was observed for thermal inactivation.
Because no complete inactivation of the enzyme was attained after pressure treatment, it can be concluded that the PE isoenzymes with high heat resistance may also show pressure resistance. However, to elucidate the relation between the pressure and temperature resistance of this fraction, more experiments are needed.

Influence of pH
The influence of pH on the isothermal-isobaric inactivation of orange PE (1.7 mg/mL of lot W, 0.4 mg/mL of lot V) was investigated in a citric acid buffer 5 mM, pH 3.7. The estimated kinetic parameters are presented in Table 2.26. Figure 2.14 illustrates the pressure inactivation of orange PE (0.4 mg/mL of lot V) in citric acid buffer pH 3.7 at a temperature of 25 °C.

Table 2.26: Kinetic parameters for isothermal-isobaric inactivation of orange PE (lot W : 1.7 mg/ml and lot V : 0.4 mg/ml) in citric acid buffer pH 3.7 at elevated pressure

	Lot W				Lot V
	$k_{18°C}$ (min^{-1}) *% residual*	$k_{23°C}$ (min^{-1}) *% residual*	$k_{28°C}$ (min^{-1}) *% residual*	Ea kJ/mol	$k_{25°C}$ (min^{-1}) *% residual*
400 MPa	0.024 ± 0.003* *7*	0.032 ± 0.002 *7*	0.056 ± 0.001 *7*	62.95±11.11#	
450 MPa					0.027 ± 0.004 *30*
500 MPa	0.107 ± 0.008* *7*	0.138 ± 0.005 *7*	0.208 ± 0.011 *7*	48.42 ± 6.97#	0.035 ± 0.004 *13*
550 MPa			0.323 ± 0.027 *7*		
600 MPa	0.229 ± 0.015* *4*	0.317 ± 0.026 *4*			0.108 ± 0.009 *3*
Va (cm^3/mol)	-27.08 ± 5.18#	-27.76 ± 4.41	-29.29 ± 2.65		-23.43 ± 3.89

* : asymptotic standard error
\# : standard error

By comparing the kinetic parameters of lot W, estimated for the inactivation in an acid medium at atmospheric pressure and elevated pressure (table 2.22 and 2.26), the synergistic effect of pressure and temperature is clear. A similar inactivation rate constant as at 45 °C can be obtained at room temperature if pressure is increased to 500 MPa. The higher the pressure at a fixed temperature and the higher the temperature at a fixed pressure, the faster the

inactivation. A comparison of both tables also reveals that the *k*-value is less temperature sensitive at elevated pressure. Moreover, a small decrease in activation energy is observed with increasing pressure. Regarding the activation volume, this parameter is only slightly influenced by temperature. The remaining fraction is dependent on the pressure level, whereas no differences were observed with respect to the temperature level in the temperature domain studied. The lowest residual fraction was obtained at the highest pressure level.
A comparison of the kinetic parameters of lot V for the inactivation in water and in buffer at elevated pressure (table 2.25 and 2.26) reveals that the enzyme is less pressure stable in an acid medium. Besides this, also a small decrease of the pressure sensitivity of the *k*-value was observed. As for thermal inactivation, the residual fraction is dependent on the pressure applied. The remaining fraction decreased from 30 % to 3 % by increasing the pressure from 450 MPa to 600 MPa. This implies that a smaller residual fraction can be obtained in buffer than in water at a lower pressure level.

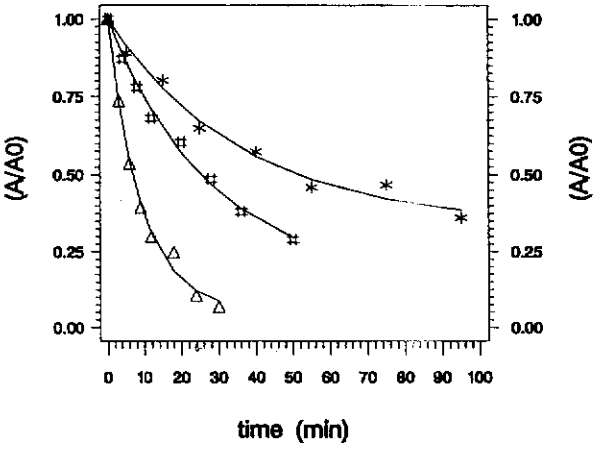

Figure 2.14 : Pressure inactivation of orange PE in citric acid buffer pH 3.7 at a temperature of 25 °C in combination with pressures of 450 MPa (*), 500 MPa (#), 600 MPa (Δ).

Influence of Ca^{2+}-ions
The influence of Ca^{2+}-ions on the pressure inactivation of orange PE (0.4 mg/mL of Lot V) at room temperature, was investigated in an 0.5 M , 1 M and 1.5 M $CaCl_2$ solution. The kinetic parameters, estimated with aid of the fractional conversion model, are presented in table 2.27. A comparison of table 2.25 and 2.27 indicates that an increased pressure inactivation was observed for the lower molarities (0.5 M and 1.0 M), whereas in presence of a 1.5 M $CaCl_2$-solution, a decreased inactivation was noticed. This is contrast to thermal treatment, where a increased inactivation was observed for each of the Ca^{2+}-concentrations studied. Also a slight increase in the pressure sensitivity of the *k*-value was observed. Notwithstanding the altered stability and sensitivity of orange PE, the remaining active fraction remained nearly the same : 4 to 5 % of the original activity was found to be active after pressure treatment in absence as well as in presence of Ca^{2+}-ions.
A comparison of the different molarities mutually, points out that Ca^{2+}-ions have a clearly different influence on pressure and temperature stability of orange PE. Increasing the salt concentration increases the pressure stability whereas it decreases the temperature stability. Moreover, the higher the salt concentration, the higher the residual fraction active PE after thermal treatment. However, no increase or decrease in residual fraction was observed after pressure treatment with respect to different Ca^{2+}-concentrations. For both treatments, a decreased temperature and pressure sensitivity of the *k*-value was observed by increasing the salt concentration.

Table 2.27: Kinetic parameters for isothermal-isobaric inactivation of orange PE in presence of $CaCl_2$ at elevated pressure and 25 °C

	Lot V (0.4 mg/mL)		
	$CaCl_2$ 0.5 M	$CaCl_2$ 1.0 M	$CaCl_2$ 1.5 M
k_{750MPA} (min^{-1})	0.113 ± 0.019*	0.089 ± 0.008	0.053 ± 0.004
k_{800MPA} (min^{-1})	0.148 ± 0.005*	0.146 ± 0.010	0.097 ± 0.012
k_{850MPA} (min^{-1})		0.279 ± 0.014	
k_{860MPA} (min^{-1})	0.455 ± 0.037*		0.172 ± 0.012
% residual	5	4	5
Va (cm^3/mol)	-31.80 ± 9.42#	-28.26 ± 2.34	-26.35 ± 1.64

* : asymptotic standard error
# : standard error

Influence of sucrose
The influence of sucrose on the pressure stability of orange PE (0.4 mg/mL of Lot V) at room temperature was investigated in a 20 % sucrose solution. The kinetic parameters, estimated with aid of the fractional conversion model are presented in table 2.28.
A comparison of table 2.25 and 2.28 reveals that the pressure inactivation decreased in presence of sucrose. A similar behaviour was found for thermal inactivation. Also an increased pressure sensitivity and an increased residual fraction active PE after pressure treatment was observed in presence of sucrose.

Table 2.28: Kinetic parameters for isothermal-isobaric inactivation of orange PE in presence of 20 % sucrose at elevated pressure and 25 °c

	Lot V 0.4 mg/ml
k_{750MPA} (min^{-1})	0.024 ± 0.004*
k_{800MPA} (min^{-1})	0.050 ± 0.003*
k_{850MPA} (min^{-1})	0.071 ± 0.005*
k_{900MPA} (min^{-1})	0.130 ± 0.006*
% residual	8
Va (cm^3/mol)	-26.67 ± 2.40#

* : asymptotic standard error
# : standard error

4.6. Myrosinase from broccoli (MYR)

Thermal inactivation kinetics

Thermal inactivation of myrosinase from broccoli (0.015 g lyophilized powder per mL in 0.1 mol/L phosphate buffer at pH 6.55) was studied at temperatures ranging from 30 to 60 °C. The activity remained rather constant at 30 °C and significant inactivation occurred at 40 °C and higher. A treatment of 3 min at 60 °C was sufficient to reduce enzyme activity by 90%. These results show myrosinase from broccoli to be rather thermolabile.
The course of inactivation could be accurately described by a consecutive two-step model (figure 2.15). This model can be used to describe two different cases of enzyme inactivation: (i) an inactivation process proceeding as a succession of two irreversible reaction steps.

$$N \xrightarrow{k_1} I \xrightarrow{k_2} D$$

In the first step, the native enzyme converts to an intermediate form, the activity of which may be dependent on the inactivation temperature applied. On its turn, this intermediate form is converted to the inactive form. (ii) inactivation of enzymes that exist as two different forms

(e.g. free enzyme and enzyme interacting with impurities or substrates) which are in equilibrium with each other.

$$\begin{array}{ccc} N_1 & \overset{K}{\longleftrightarrow} & N_2 \\ \downarrow k_1 & & \downarrow k_2 \\ D_1 & & D_2 \end{array}$$

The transformation of each of the enzyme forms to the inactive form is characterized by distinct inactivation rate constants. Clearly the position of the equilibrium between the two forms can be dependent on temperature.

The parameters estimated using the consecutive two-step model (2.4) together with standard deviations are presented in table 2.29. This table indicates the activity estimates of the hypothetical intermediate (A_2) to vary significantly with temperature. It can thus be concluded that the activity of the intermediate or the position of the equilibrium between two enzyme forms may depend on the inactivation temperature. The activation energy values of the respective steps were calculated as 113.5±14.1 and 134.1±28.3 kJ/mol respectively, indicating similar temperature sensitivity of the respective inactivation rate constants.

Table 2.29. Consecutive step model: Estimation of the kinetic parameters for thermal inactivation of broccoli myrosinase (0.015 g lyophilized powder per mL in 0.1 mol/L phosphate buffer at pH 6.55).

T (°C)	A_1[a]	k_1 (min^{-1})	A_2[a]	k_2 (min^{-1})
30	NC	NC	NC	NC
35	101.2±1.6[b]	(1.4±0.4[b])x10^{-1}	81.6±3.3[b]	(4.0±0.4[b])x10^{-4}
37.5	99.7±2.7	(3.3±0.9)x10^{-1}	70.2±2.7	(1.8±0.2)x10^{-3}
40	99.9±3.6	(2.4±0.7)x10^{-1}	55.7±4.6	(4.9±0.7)x10^{-3}
42.5	98.6±2.4	(4.2±0.7)x10^{-1}	52.5±2.6	(6.1±1.2)x10^{-3}
45	99.6±3.8	(9.8±0.2)x10^{-1}	49.5±1.3	(1.2±0.2)x10^{-2}
50	99.9±2.5	(9.3±0.1)x10^{-1}	34.1±2.1	(1.5±0.2)x10^{-2}
60	99.9±0.7	(27.9±1.7)x10^{-1}	7.9±0.7	(3.5±0.7)x10^{-2}

[a]: given in % of overall initial activity; [b]: standard error; NC: no convergence

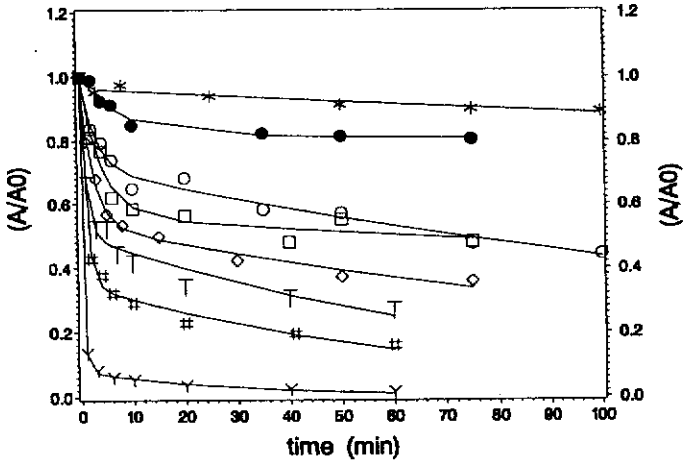

Figure 2.15. Thermal inactivation of myrosinase form broccoli (0.015 g lyophilized powder per mL in 0.1 mol/L phosphate buffer at pH 6.55), modelled using the consecutive step model: (*) 30°C; (•) 35°C; (o) 37.5°C; (□) 40°C; (◊) 42.5°C; (Δ) 45°C; (#) 50°C; (Y) 60°C.

Pressure inactivation kinetics

At first, pressure inactivation of myrosinase from broccoli (0.015 g lyophilized powder per mL in 0.1 mol/L phosphate buffer at pH 6.55) was studied at 20 °C in the pressure range 250-500 MPa. At pressures below 250 MPa, the activity remained constant and no post process activation of the enzyme was noted (data not shown). Significant inactivation was observed between 300 and 500 MPa. These results show myrosinase to be rather pressure sensitive as compared to other food quality related enzymes such as POD, PPO, LOX and PE.

In agreement with thermal inactivation, a consecutive two-step model could analyze pressure-temperature inactivation of myrosinase from broccoli (figure 2.16) The estimated parameters together with standard deviations are shown in table 2.30. Pressure dependence of the distinguished inactivation rate constants were calculated according to the Eyring equation as -34.5±4.4 cm^3/mol and -30.8±1.9 cm^3/mol respectively, pointing out a similar pressure dependence.

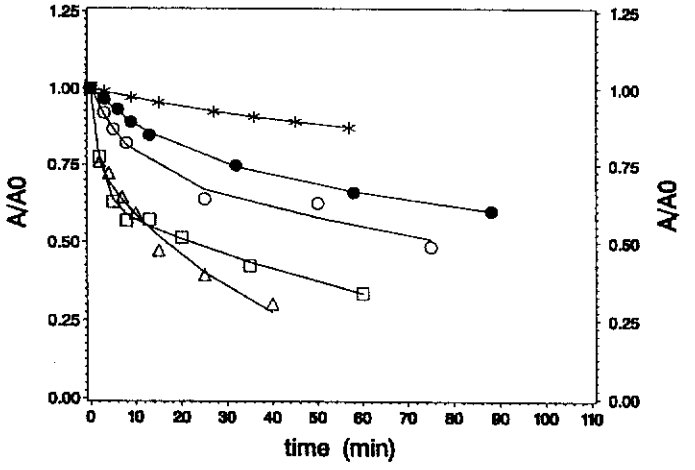

Figure 2.16. Pressure inactivation of myrosinase form broccoli (0.015 g lyophilized powder per mL in 0.1 mol/L phosphate buffer at pH 6.55) at 20°C, modelled using the consecutive step model: (*) 250 MPa; (•) 350 MPa; (o) 400 MPa, (□) 450 MPa; (◊) 500 MPa.

Table 2.30. Consecutive step model: Estimation of the kinetic parameters for pressure inactivation of broccoli myrosinase (0.015 g Lyophilized Powder per mL in 0.1 mol/L phosphate buffer at pH 6.55) at 20 °C.

P (MPa)	A_1[a]	k_1 (min^{-1})	A_2[a]	k_2 (min^{-1})
250	100.0±0.04[b]	(2.6±0.6[b])x10^{-2}	86.3±3.1[b]	(1.0±0.3[b])x10^{-3}
350	100.3±0.6	(5.9±1.2)x10^{-2}	74.1±3.6	(3.1±0.6)x10^{-3}
400	100.4±3.3	(12.3±4.7)x10^{-2}	71.4±9.7	(5.2±2.1)x10^{-3}
450	100.1±1.6	(45.1±6.9)x10^{-2}	61.3±15.3	(10.5±1.1)x10^{-3}
500	99.8±3.1	(76.7±10.1)x10^{-2}	73.1±4.2	(25.3±3.1)x10^{-3}

[a]: given in % of overall initial activity; [b]: standard error

In a second phase, pressure inactivation of broccoli myrosinase was studied at 35 °C, a temperature where thermal inactivation occurs slowly, in order to check whether (low) pressure has an antagonistic (or protective) effect on thermal inactivation. As mentioned

above, a consecutive step model was used to analyze the data of pressure inactivation of myrosinase from broccoli at 35 °C (figure 2.17). The concomitant kinetic parameters are presented in table 2.31. From this table it becomes clear that application of low pressure retards the inactivation of the first myrosinase enzyme form, since inactivation rate constants firstly decreased with pressure increasing upto 350 MPa and then decreased with further increase in pressure. The maximal protective effect was noted at a pressure around 350 MPa. Calculation of the activation volumes in distinct low and high pressure areas was statistically insignificant because of the few data points available for linear regression. This antagonistic effect however, was not observed for the second myrosinase enzyme form. In this case, inactivation rate constants showed a consistent increase with increasing pressure, characterized by an activation volume of -15.7±3.8 cm^3/mol. These results show pressure sensitivity of the inactivation rate of the second enzyme form to be significantly lower at 35 °C than at 20 °C.

Table 2.31. Consecutive step model: Estimation of the kinetic parameters for pressure inactivation of broccoli myrosinase (0.015 g Lyophilized Powder per mL in 0.1 mol/L phosphate buffer at pH 6.55) at 35 °C.

P (MPa)	A_1[a]	k_1 (min^{-1})	A_2[a]	k_2 (min^{-1})
0.1	100.1±1.6	(13.8±4.1)x10^{-2}	81.9±3.3	(4.0±0.4)x10^{-4}
150	100.0±0.2	(12.4±0.6)x10^{-2}	80.6±0.5	(7.3±0.8)x10^{-4}
250	99.9±0.1	(10.4±0.1)x10^{-2}	77.6±0.2	(7.6±0.4)x10^{-4}
350	100.4±0.8	(5.5±0.1)x10^{-2}	69.5±5.2	(16.3±1.6)x10^{-4}
400	100.0±0.2	(10.5±0.1)x10^{-2}	73.9±0.2	(25.5±0.1)x10^{-4}
450	100.1±0.2	(64.0±6.1)x10^{-2}	88.9±0.5	(103.3±0.8)x10^{-4}

[a]: given in % of overall initial activity; [b]: standard error

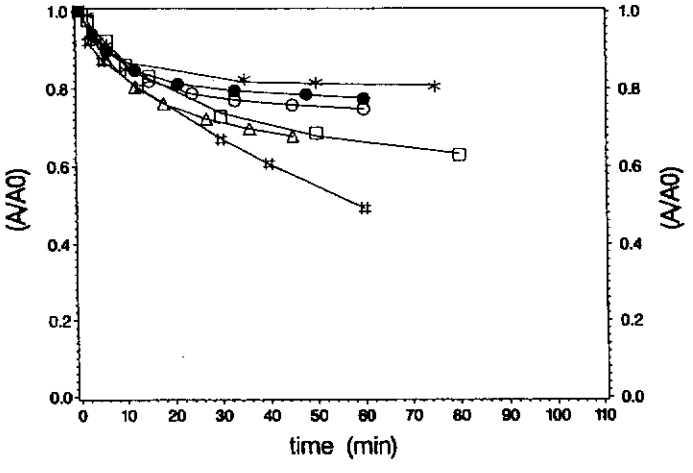

Figure 2.17. Pressure inactivation of myrosinase form broccoli (0.015 g lyophilized powder per mL in 0.1 mol/L phosphate buffer at pH 6.55) at 35 °C, modelled using the consecutive step model: (*) 0.1 MPa; (•) 150 MPa; (o) 250 MPa; (□) 350 MPa; (◊) 400 MPa, (Δ) 450 MPa.

ANNEX 3: DETAILED RESULTS REGARDING MATHEMATICAL MODELLING

ANNEX 3: DETAILED RESULTS REGARDING MATHEMATICAL MODELLING

1. Modelling microbial inactivation kinetics

Based on isobaric-isothermal inactivation data of *Zygosaccharomyces bailii*, a pressure-temperature kinetic diagram was constructed. Subsequently it was tried to develop a mathematical model describing adequately the combined pressure-temperature inactivation of this yeast. The detailed results are included in annex 1.2 (paragraph 3.3).

2. Modelling enzyme inactivation kinetics

2.1. Bacillus subtilis α-amylase

Based on the data for isobaric-isothermal inactivation kinetics, a pressure-temperature kinetic diagram was constructed (Figure 3.1). This is a two-dimensional diagram indicating combinations of constant pressure and temperature resulting in the same inactivation rate constant. From this kinetic diagram it could be derived that pressure and temperature act synergistically with respect to the inactivation of BSAA.

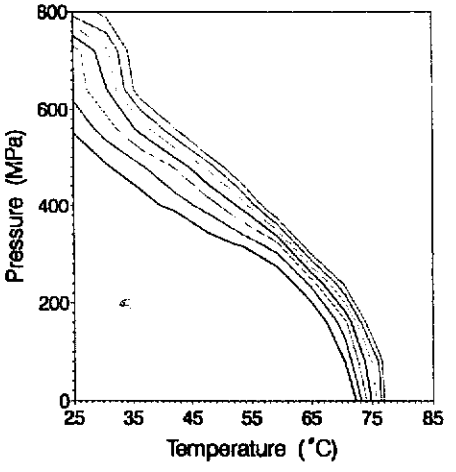

Figure 3.1. Pressure-temperature kinetic diagram for inactivation of BSAA, 15 mg/mL in 0.01M Tris HCl at pH 9. (upper line: k=0.07 min^{-1}; lower line: k=0.01 min^{-1})

Subsequently it was endeavoured to fit a mathematical model to the data describing adequately the combined effect of pressure and temperature. For this purpose, the Arrhenius equation (3.1) was used as starting point, because this equation appeared to be valid over the entire experimental domain. In this equation, the inactivation rate constant at reference temperature (40°C or 313K) and the activation energy are dependent on pressure. Hence mathematical equations describing the evolution of the latter parameters as a function of pressure were developed. The activation energy seemed to decrease exponentially with increasing pressure (3.2). Variation of the inactivation rate constant at reference temperature with pressure could described by a second degree polynomial equation (3.3). Parameter estimates for a_1, b_1, a_2, b_2 and c_2 are presented in table 3.1.

$$\ln k = \ln k_{refT} - \left(\frac{E_a}{R} \left(\frac{1}{T_{abs}} - \frac{1}{T_{absref}} \right) \right) \tag{3.1}$$

$$E_a = a_1 \exp(-b_1 P) \tag{3.2}$$

$$\ln k_{refT} = a_2 P^2 + b_2 P + c_2 \tag{3.3}$$

Substitution of E_a and k_{refT} in the general Arrhenius equation by their respective mathematical expressions (3.2, 3.3) yields a mathematical model, which was investigated on its capability to fit isobaric-isothermal inactivation data of BSAA in the entire pressure-temperature region (3.4). The parameter estimates are summarized in table 3.1 whereas the accuracy of the proposed model and its concomitant parameters is shown in figure 3.2.

$$\ln k = (a_2 P^2 + b_2 P + c_2) - \left(\frac{a_1 \exp(-b_1 P)}{R} \left(\frac{1}{T_{abs}} - \frac{1}{T_{absref}} \right) \right) \tag{3.4}$$

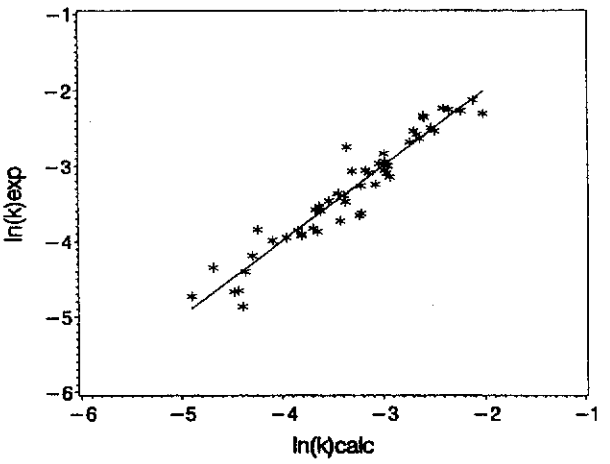

Figure 3.2. Correlation between experimental k-values for isobaric-isothermal inactivation of BSAA and k-values calculated using equation 3.4

Table 3.1. Kinetic parameter estimates for pressure dependence of E_a, pressure dependence of $\ln k_{refT}$ and pressure-temperature dependence of the logarithm of the inactivation rate constant for BSAA, 15 mg/mL in 0.01M Tris HCl at pH 8.6.

Parameter	estimated value (equation 3.2)	estimated value (equation 3.3)	estimated value (equation 3.4)
a_1	435.2 ± 45.5	/	322.1 ± 16.8
b_1	$(3.24 \pm 0.34)\text{x}10^{-3}$	/	2.82 ± 0.08
a_2	/	$(-3.70 \pm 0.34)\text{x}10^{-5}$	$(-2.98 \pm 0.20)\text{x}10^{-5}$
b_2	/	$(5.15 \pm 0.32)\text{x}10^{-2}$	$(4.13 \pm 0.22)\text{x}10^{-2}$
c_2	/	-19.71 ± 0.67	-16.63 ± 0.64

From table 3.1 and figure 3.2, it could be concluded that the proposed model is able to describe isobaric-isothermal inactivation of BSAA in the entire pressure-temperature domain studied.

2.2. Soybean lipoxygenase

As opposed to the results for BSAA, the pressure-temperature kinetic diagram for inactivation of soybean LOX (figure 3.3) clearly showed an antagonistic effect of temperature and pressure in the low temperature/high pressure area. In this experimental domain, inactivation rate constants firstly decreased with increasing temperature, reaching a minimum at temperatures somewhat higher than room temperature and then increased with further increase in temperature. Obviously, the Arrhenius equation is not valid over the entire experimental domain studied. Therefore, the equation of Eyring (3.5) was used as starting point to develop a mathematical model describing pressure-temperature inactivation of

soybean LOX. Mathematical expressions reflecting temperature dependence of the inactivation rate constant at reference pressure (3.6) and of the activation volume (3.7) were fit on the data. In all these equations, T_{abs} is given in K, T in °C and P in MPa. Parameter estimates for a_1, b_1, a_2, b_2 and c_2 are shown in table 3.2.

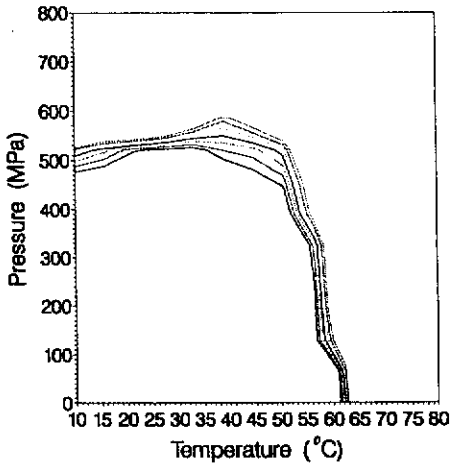

Figure 3.3. Pressure-temperature kinetic diagram for inactivation of soybean LOX, 0.4 mg/mL in 0.01M Tris HCl at pH 9. (upper line: k=0.04 min^{-1}; lower line: k=0.01 min^{-1})

$$\ln k = \ln k_{refP} - \left(\frac{V_a}{RT_{abs}} (P - P_{ref}) \right) \tag{3.5}$$

$$V_a = a_1 T \exp(-b_1 T) \tag{3.6}$$

$$\ln k_{refP} = a_2 T^2 + b_2 T + c_2 \tag{3.7}$$

Implementation of these mathematical expressions into the Eyring equation allows transformation to equation 3.8. Finally it was tried whether the latter equation is able to predict isobaric-isothermal inactivation of LOX, 0.4 mg/mL in 0.01M Tris HCl at pH 9. Parameter estimates are presented in table 3.2 while the correctness of the proposed model and the best fit of parameters is shown in figure 3.4.

$$\ln k = (a_2 T^2 + b_2 T + c_2) - \left(\frac{a_1 T \exp(-b_1 T)}{RT_{abs}} (P - P_{ref}) \right) \tag{3.8}$$

Table 3.2. Kinetic parameter estimates for temperature dependence of V_a, temperature dependence of $\ln k_{refP}$ and pressure-temperature dependence of the logarithm of the inactivation rate constant for LOX, 0.4 mg/mL in 0.01M Tris HCl at pH 9.

Parameter	estimated value (equation 3.6)	estimated value (equation 3.7)	estimated value (equation 3.8)
a_1	-12.2 ± 1.5	/	-15.6 ± 1.4
b_1	(6.11 ± 0.46)x10^{-2}	/	(7.1 ± 0.28)x10^{-2}
a_2	/	(3.09 ± 0.09)x10^{-3}	(2.66 ± 0.27)x10^{-3}
b_2	/	(1.83 ± 0.08)x10^{-1}	(1.39 ± 0.18)x10^{-1}
c_2	/	-2.42 ± 0.18	-3.12 ± 0.28

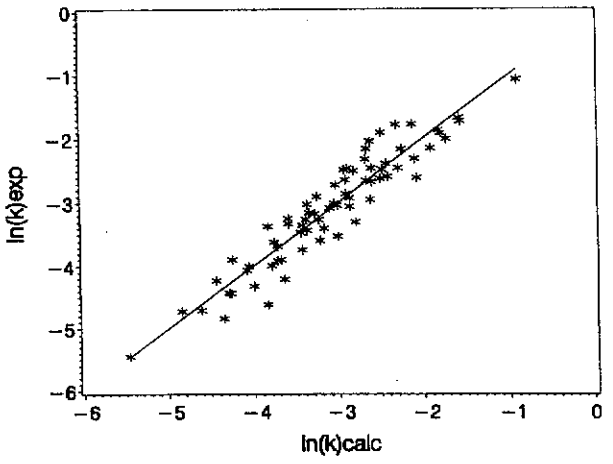

Figure 3.4. Correlation between the experimental *k*-values for isobaric-isothermal inactivation of LOX and the *k*-values calculated according to equation 3.8.

Based on the satisfactory correlation (figure 3.4) and sufficient accuracy of the model and its attendant parameters (table 3.2), it is clear that the proposed model can adequately describe isobaric-isothermal inactivation of LOX under the specified conditions.

2.3. Avocado polyphenoloxidase

The pressure-temperature kinetic diagram for inactivation of avocado PPO (figure 3.5) clearly showed an antagonistic effect of low pressure and high temperature. Application of low pressure at temperatures where thermal inactivation occurs, retarded the inactivation reaction. Low pressure thus protects the enzyme against thermal inactivation.

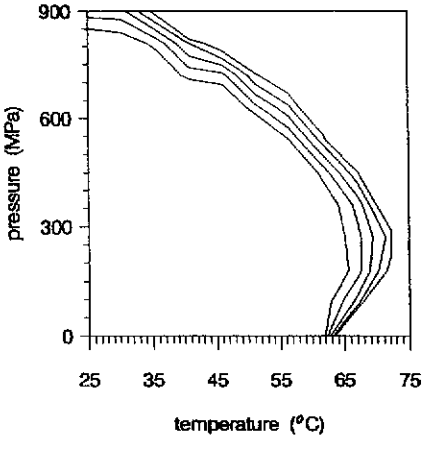

Figure 3.5. Pressure-temperature kinetic diagram for avocado PPO, 0.5 mg/mL in 0.1M phosphate buffer at pH 7. (upper line: k=0.015 min^{-1}; lower line: k=0.005 min^{-1})

In agreement with the results on BSAA, the Arrhenius equation could be applied over the entire experimental domain studied. Hence this equation (3.1) was used as starting point for the development of a mathematical model which can describe the inactivation rate constant as a function of pressure and temperature. Again, the inactivation rate constant at reference temperature and the activation energy were replaced by mathematical equations, expressing their pressure dependency. The activation energy was found to depend in an exponential manner on pressure. Pressure dependency of the inactivation rate constant at reference

temperature could be described by a third degree polynomial equation (3.9). The corresponding parameter estimates for a_1, b_1, a_2, b_2, c_2 and d_2 are presented in table 3.3.

$$\ln k_{refT} = a_2 P^3 + b_2 P^2 + c_2 P + d_2 \tag{3.9}$$

Substitution of E_a and k_{refT} by their respective mathematical equations transforms the Arrhenius equation into equation 3.10.

$$\ln k = (a_2 P^3 + b_2 P^2 + c_2 P + d_2) - \left(\frac{a_1 \exp(-b_1 P)}{R} \left(\frac{1}{T_{abs}} - \frac{1}{T_{absref}} \right) \right) \tag{3.10}$$

It was finally tried whether the proposed model was able to describe the entire inactivation data set. From table 3.3 and figure 3.6, in which the parameter estimates and the accuracy of the corresponding parameter estimation are respectively shown, it is clear that the derived empirical model is able to accurately describe the combined pressure-temperature inactivation of avocado PPO.

Table 3.3. Kinetic parameter estimates for pressure dependence of E_a, pressure dependence of $\ln k_{refT}$ and pressure-temperature dependence of the logarithm of the inactivation rate constant for PPO, 0.5 mg/mL in 0.1M phosphate buffer at pH 7.

Parameter	estimated value (equation 3.2)	estimated value (equation 3.9)	estimated value (equation 3.10)
a_1	309.7 ± 8.58	/	324.3 ± 11.6
b_1	(16.0 ± 0.8)x10^{-4}	/	(16.8 ± 0.6)x10^{-4}
a_2	/	(-56.0 ± 5.8)x10^{-9}	(-23.3 ± 1.3)x10^{-9}
b_2	/	(72.2 ± 5.3)x10^{-6}	(41.1 ± 1.8)x10^{-6}
c_2	/	(-24.8 ± 1.3)x10^{-3}	(-17.2 ± 0.7)x10^{-3}
d_2		-2.08 ± 0.09	-2.42 ± 0.07

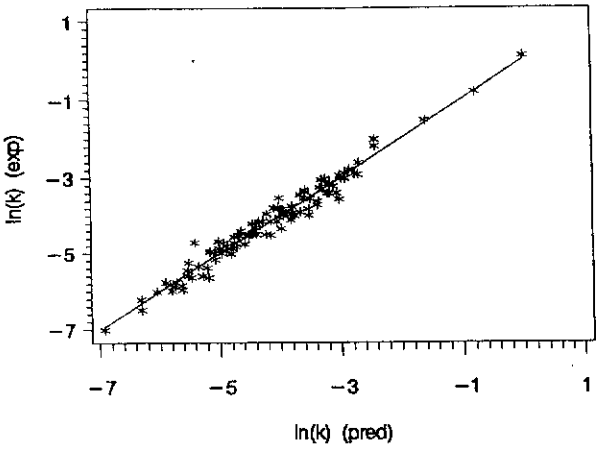

Figure 3.6. Correlation between the experimental k-values for isobaric-isothermal inactivation of PPO and the k-values calculated according to equation 3.10.

ANNEX 4: DETAILED RESULTS REGARDING PROCEDURES TO EVALUATE PROCESS IMPACT

ANNEX 4: DETAILED RESULTS REGARDING PROCEDURES TO EVALUATE PROCESS IMPACT

1. Physical-mathematical test method for impact evaluation

1.1. Introduction

Performing isobaric-isothermal treatments is the most straightforward approach to evaluate pressure-temperature inactivation kinetics since it allows to separate the contribution of pressure and temperature to changes in the inactivation rate. Kinetic models describing combined pressure and temperature dependence of the inactivation rate constant have been finalized for BSAA, soybean LOX and avocado PPO. In the context of developing a concept to assess the impact of pressure-temperature processes, the predictive power of the models for BSAA and LOX when applied to inactivation data obtained under variable pressure-temperature conditions, has to be verified. 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.

1.2. Materials and methods

Treatments under variable pressure-temperature conditions were performed in a laboratory pilot scale, warm isostatic press (SO, 5-7442-0, Engineered Pressure Systems International). This equipment is characterized by a vessel volume of 590 mL (l=300 mm, d=50 mm) and filled with a commercial pressure transferring liquid (TR10, Resato). The equipment is connected to a cryostat and enables high pressure (up to 600 MPa) to be combined with temperatures between -30 and 100°C. One external and one internal temperature sensor allow to measure temperature of the thermostated mantle and of the load respectively. Four more thermocouples are provided to measure temperature at different positions inside the vessel, which were calibrated against a mercury in glass thermometer in ice and at maximal process temperature and showed a mutual variation of 0.1°C. They were enclosed in dummy samples, filled with water, in order to take the heat transfer through the microtubes into account and positioned at four levels in the vessel (4, 10, 16 and 22 cm from the bottom of the vessel). Next to these temperature sensors, one pressure sensor served to measure isostatic pressure inside the vessel. After connection of all sensors to a datalogger (Cobra 7-10, Mess und System Technik GmbH), pressure and temperature were recorded at regular time intervals (5 or 10 s) during the entire treatment. The samples were contained in flexible microtubes (0.25 mL, Elkay) and placed in a stainless steel sample holder, fixing them next to the dummy samples at four positions in the vessel.

1.3. Data analysis

It was assumed that the course of enzyme inactivation remained unchanged under variable pressure and temperature, i.e. dynamic, conditions. Hypothesizing first order inactivation kinetics, the integral effect of an inactivation process under dynamic conditions, where the inactivation rate constant is no longer constant as a function of time, can be described by equation 4.1.

$$\ln\left(\frac{A}{A_0}\right) = -\int_0^t k\,dt \qquad (4.1)$$

Implementation of a kinetic model expressing the combined pressure-temperature dependence of the inactivation rate constant (*cfr.* annex 3) under static conditions then yields an equation (4.2) which can be used to check the ability of the proposed kinetic model and its concomitant parameters to predict inactivation under dynamic conditions.

$$\ln\left(\frac{A}{A_0}\right) = -\int_0^t k_{f(P,T)}\,dt \tag{4.2}$$

After treatment, activity retentions were measured spectrophotometrically, whereas the corresponding, recorded pressure-temperature-time profiles were integrated numerically according to Simpson's rule. By applying a non-linear regression procedure to fit equation 4.2 on the data obtained under variable conditions, the proposed kinetic model could be evaluated under dynamic conditions and the kinetic parameters obtained were compared to those from isobaric-isothermal experiments. The problem of finding good initial estimates as starting value for the iterative procedure was solved by performing a grid search in a fixed range of values comprising the estimate obtained from static experiments.

1.4. Inactivation of Bacillus subtilis α-amylase under variable pressure-temperature conditions

Inactivation of BSAA under variable pressure and temperature conditions was thoroughly studied in the pressure range 250-550 MPa at temperatures varying from 25 to 65°C. For BSAA in the presence of glycerol a less extensive data set was gathered in the pressure range 350-550 MPa at temperatures between 30 and 55°C. Subsequently it was checked whether equation 2.4 and its concomitant parameters could be used to evaluate pressure-temperature inactivation of BSAA under dynamic conditions. Hence equation 3.4 was implemented into equation 4.2, hereby formulating an appropriate kinetic model (4.3).

$$\ln\left(\frac{A}{A_0}\right) = -\int_0^t \exp(a_2P^2 + b_2P + c_2)\exp\left(\frac{a_1\exp(-b_1P)}{R}\left(\frac{1}{T_{abs}} - \frac{1}{T_{absref}}\right)\right)dt \tag{4.3}$$

Experimental activity retentions were then equated with activity retentions calculated according to equation 4.3, taking into account the registered pressure-temperature-time profiles. Initially for the calculation of the activity retention after inactivation under variable conditions, parameter estimates obtained from isobaric-isothermal inactivation data (annex 3) were used. When using the latter parameter estimates strong divergences between experimental and calculated activity retentions were established. Moreover the non-linear regression procedure, applied to re-estimate kinetic parameters from dynamic inactivation data, could not converge to a minimal sum of squares and revealed the existence of insignificant parameters. This can be explained from an experimental point of view. The setup for dynamic inactivation experiments does not allow to cover the low-pressure/high-temperature area (P<250 MPa), which is critical in modeling the dependence of the activation energy on pressure (annex 2, table 2.4). Therefore equation 3.4 was simplified, assuming the E_a-value to be constant in the pressure range considered, and implemented into equation 4.2 (4.4).

$$\ln\left(\frac{A}{A_0}\right) = -\int_0^t \exp(a_2P^2 + b_2P + c_2)\exp\left(\frac{-E_{aP}}{R}\left(\frac{1}{T_{abs}} - \frac{1}{T_{absref}}\right)\right)dt \tag{4.4}$$

Kinetic parameters were estimated by fitting this model either on the isobaric-isothermal data set, which was demarcated to pressure levels higher than 200 MPa, or on the data obtained

from experiments under variable pressure and temperature conditions (table 4.1). This model was efficient in predicting activity retention both under static and dynamic conditions. For 90% of the isobaric-isothermal data points and for 75% of the data points for inactivation under dynamic conditions, the percentage error on the predicted value was situated between 0 and 15%. From table 4.1 it could be concluded that kinetic parameters estimated from static experiments can be easily transferred to dynamic experiments and vice versa, since they are not significantly different.

Table 4.1. Kinetic parameters to describe pressure-temperature inactivation of BSAA (15 mg/mL in 0.01M Tris HCl at pH 8.6) at pressure above 200 MPa, according to equation 4.4. The reference temperature was 313K.

	Isobaric-isothermal conditions	Variable pressure and temperature conditions
E_{aP} (kJ/mol)	87.4±6.2	84.7±1.5
a_2	$(7.96\pm1.81)\times10^{-6}$	$(9.25\pm1.0)\times10^{-6}$
b_2	$(1.70\pm0.23)\times10^{-2}$	$(1.69\pm0.09)\times10^{-2}$
c_2	-9.85±0.65	-9.35±0.22
corrected r^2	0.992	0.993

Consequently, experimental activity retentions after inactivation under dynamic conditions largely correlated to activity retentions calculated according to equation 4.4, using parameters estimated from isobaric-isothermal data (figure 4.1). Percentage error on the predicted values was on the same order of magnitude whether static or dynamic parameters were used. Hence it could be concluded that the latter model (4.4) offers the possibility of defining a set of kinetic parameters which is generally applicable whatever the experimental conditions (static or dynamic).

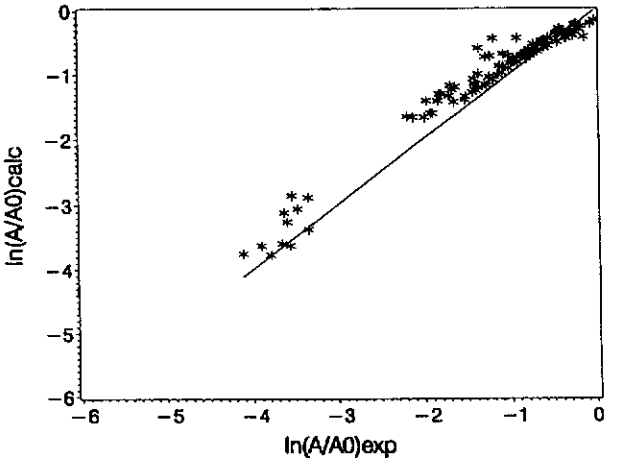

Figure 4.1. Comparison of experimental activity retentions of BSAA (15 mg/mL in 0.01M Tris HCl at pH 8.6), inactivated under variable conditions and those calculated according to equation 4.4 using parameter estimates obtained from isobaric-isothermal data.

Furthermore, the effect of pulsated pressure treatments on inactivation of BSAA was investigated as a special case of dynamic conditions. In view of optimal process design, it may be opportune to investigate the potentials of multi-cycle processes which involve violent pressure and temperature changes during the process. For this purpose, various experiments at the same pressure, temperature and total treatment time, but with different number of cycles were carried out in the pressure range 250-550 MPa and the temperature range 25-55°C. From these experiments it could be seen that multiple application of pressure enhanced the inactivation effect (table 4.2). Activity retention after multi-cycle processes was somewhat lower than that after a single-cycle process at the same pressure and temperature and for the

same total treatment time. Moreover augmenting the number of cycles did not significantly increase the inactivation effect.

Table 4.2. Influence of multi-cycling on the inactivation of BSAA, 15 mg/mL in 0.01M Tris HCl at pH 8.6.

P/T treatment	*Total time (min)*	*cycling time (min)*	*activity retention*
250 MPa, 55°C	18	1x18	0.792
250 MPa, 55°C	18	3x6	0.716
250 MPa, 55°C	18	6x3	0.699
300 MPa, 50°C	20	1x20	0.711
300 MPa, 50°C	20	4x5	0.649
300 MPa, 50°C	20	8x2.5	0.656
350 MPa, 45°C	20	1x20	0.557
350 MPa, 45°C	20	2x10	0.484
350 MPa, 45°C	20	4x5	0.511
350 MPa, 45°C	20	8x2.5	0.413
400 MPa, 40°C	25	1x25	0.384
400 MPa, 40°C	20	4x5	0.164
400 MPa, 35°C	24	6x4	0.263
450 MPa, 35°C	25	1x25	0.297
450 MPa, 35°C	25	2x12.5	0.198
450 MPa, 35°C	16	4x4	0.139
500 MPa, 35°C	20	1x20	0.129
500 MPa, 35°C	20	2x10	0.100

Typical multi-cycle pressure-temperature profiles at four levels in the vessel are presented in figure 4.2. T_1, T_2, T_3 and T_4 represent respectively temperatures at 4, 10, 16 and 22 cm from the bottom of the pressure vessel. The pressure profile was only measured at one position since pressure is isostatic and consequently does not vary as a function of position in the vessel.

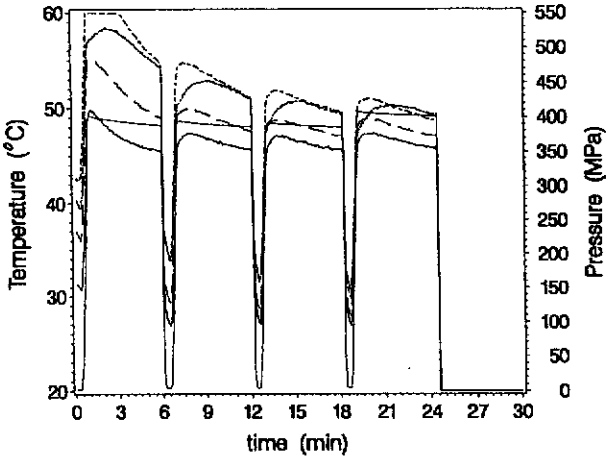

Figure 4.2. Typical multi-cycle pressure-temperature profiles at four levels in the pressure vessel at 350 MPa and 45°C. Each cycle had a duration of 5 minutes (T_1, thin solid line; T_2, ---; T_3, - - - -, T_4, —- - —- ; P, thick solid line).

As can be seen from figure 4.2, each pressure cycle was accompanied by a temperature overshoot due to adiabatic heating, which was obviously most pronounced at the top of the

pressure vessel. At the bottom, where cold pressure transferring liquid is entering the vessel during pressure build-up, temperature overshoot was much smaller. Moreover, it could be seen that, at each level in the vessel, temperature reached its maximum value during the first pressure cycle. Along with depressurization, temperature dropped to a value lower than the starting value, causing the temperature reached when pressure was built up a second time to be lower. In this way, temperature overshoot declined as a function of the number of cycles. This may be the reason why augmenting the number of cycles does not involve additional inactivation effects.

1.5. Inactivation of soybean LOX under variable pressure-temperature conditions

Table 4.3. Influence of multi-cycling on the inactivation of LOX, 0.4 mg/mL in 0.01M Tris HCl at pH 9.

P/T treatment	*Total time (min)*	*cycling time (min)*	*activity retention*
350 MPa, 40°C	40	1x40	0.709
350 MPa, 40°C	40	2x20	0.543
350 MPa, 40°C	40	4x10	0.341
400 MPa, 35°C	40	1x40	0.513
400 MPa, 35°C	40	2x20	0.457
400 MPa, 35°C	40	4x10	0.324
400 MPa, 40°C	40	1x40	0.445
400 MPa, 40°C	40	2x20	0.337
400 MPa, 40°C	40	4x10	0.300
450 MPa, 25°C	60	1x60	0.424
450 MPa, 25°C	60	2x30	0.374
450 MPa, 35°C	40	1x40	0.481
450 MPa, 35°C	40	2x20	0.431
475 MPa, 10°C	40	1x40	0.652
475 MPa, 10°C	40	2x20	0.061
475 MPa, 15°C	60	1x60	0.327
475 MPa, 15°C	60	2x30	0.139
475 MPa, 20°C	60	1x60	0.645
475 MPa, 20°C	60	2x30	0.100
475 MPa, 25°C	60	1x60	0.668
475 MPa, 25°C	60	2x30	0.459
475 MPa, 25°C	60	4x15	0.160
475 MPa, 30°C	60	1x60	0.645
475 MPa, 30°C	60	2x30	0.407
475 MPa, 30°C	60	4x15	0.373
500 MPa, 10°C	15	1x15	0.430
500 MPa, 10°C	15	2x7.5	0.156
500 MPa, 10°C	15	3x5	0.097
500 MPa, 15°C	30	1x30	0.367
500 MPa, 15°C	30	2x15	0.058
500 MPa, 15°C	30	3x10	0.018
525 MPa, 20°C	30	1x30	0.055
525 MPa, 20°C	30	2x15	0.025
525 MPa, 25°C	20	1x20	0.099
525 MPa, 25°C	20	2x10	0.110
525 MPa, 30°C	80	1x80	0.165
525 MPa, 30°c	80	2x40	0.127
525 MPa, 30°C	80	4x20	0.062

At first, the influence of pulsated pressure treatments on the inactivation of soybean LOX was investigated. Several experiments at the same pressure and temperature and for the same total treatment time were performed in the pressure range 350-525 MPa at temperatures between 10 and 40°C, hereby varying the number of cycles (table 4.3). From this table it could be seen that multiple application of pressure exerted an additional inactivation effect on LOX. This additional effect seemed to be most pronounced at low temperature. During low temperature processes, temperature inside the vessel drops below zero upon depressurization, which may be at the base of the enhanced inactivation effect. Besides, pressure inactivation of LOX has yet been shown to be more pronounced at low than at room temperature. As opposed to the results on BSAA, augmenting the number of cycles seemed to further increase the extent of inactivation. This dissimilar behaviour may be explained from the viewpoint of the different temperature sensitivity of both enzyme systems. Since LOX is far more thermosensitive, the declining temperature overshoot as presented in figure 4.2 may still be sufficient to account for the continued decrease in activity retention when cycling becomes more extensive.

Secondly, it was endeavoured to fit the mathematical model expressing the combined pressure and temperature dependence of the inactivation rate constant of LOX under static conditions (3.8), on data obtained under variable pressure-temperature conditions. When using a non-linear regression procedure for this purpose, optimal properties of the parameter estimates can only be assured if the sample size gets very large. The data set on inactivation of LOX under dynamic conditions was not extensive enough to distinguish between single- and multi-cycle processes. Both data had to be merged into one data set (62 individual data points) to meet the requirement of large sample size. As could be expected from the experimental setup, no reliable parameter estimates were obtained using this model. Indeed, dynamic experiments merely covered the temperature range 10-40°C, in which the value of the activation volume seemed to be rather constant (table 2.8). As for BSAA, a model simplification was introduced, assuming the V_a value to be constant, yielding equation 4.5. The latter equation was tried to fit both static and dynamic inactivation data of LOX in the temperature range considered (10-40°C).

$$\ln\left(\frac{A}{A_0}\right) = -\int_0^t \exp(a_2T^2 + b_2T + c_2)\exp\left(\frac{-V_{aP}}{RT_{abs}}(P - P_{ref})\right)dt \qquad (4.5)$$

Firstly the ability of the proposed model structure to predict isobaric-isothermal inactivation in the temperature range 10-40°C was verified. Kinetic parameters together with standard deviations and corrected correlation coefficient are presented in table 4.4. The validity of the model in the temperature range considered was ascertained by the high corrected r^2 and the satisfactory correlation between experimental and calculated activity retentions (figure 4.3). Percentage error on the predicted values varied between 0 and 20%. However, when parameter estimates obtained from static experiments were used to calculate the activity retention after inactivation under variable pressure and temperature conditions, a large and systematic underestimation was notified (figure 4.4). For this reason re-estimation of the kinetic parameters from data obtained under dynamic conditions was evaluated (table 4.4). Where upon re-estimation V_{aT}, a_2 and b_2 remained on the same order of magnitude, the value of c_2 was drastically changed. However, the low value for the corrected r^2 (0.894), as well as the large percentage error on the predicted values (35% of the data points are characterised by an error higher than 50%) may put these results into perspective. Nevertheless, considerable improvement in predicting the activity retentions after inactivation under variable pressure and temperature conditions was observed when using the re-estimated parameters (figure 4.4).

Table 4.4. Kinetic parameters to describe pressure-temperature inactivation of LOX, 0.4 mg/mL in 0.01M Tris HCl at pH 9, in the temperature range 10-40°C, according to equation 6.5. Reference pressure was set 500 MPa.

	Isobaric-isothermal conditions	Variable pressure and temperature conditions
V_{aT} (cm^3/mol)	-56.3±2.7	-83.8±7.1
a_2	(2.14±0.33)x10^{-3}	(3.4±1.1)x10^{-3}
b_2	(-1.38±0.21)x10^{-1}	(-2.41±0.59)x10^{-1}
c_2	-2.69±0.28	(1.04±0.17)x10^{-1}
corrected r^2	0.991	0.894

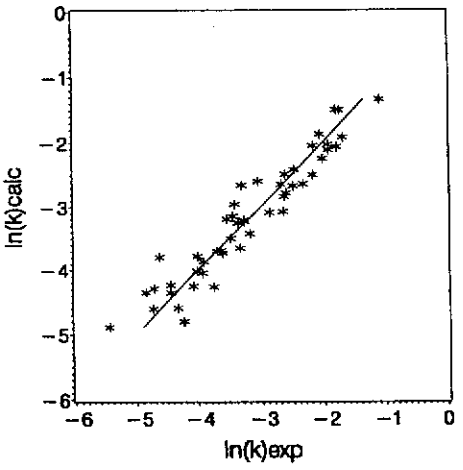

Figure 4.3. Correlation between the experimental activity retentions after isobaric-isothermal inactivation of LOX (10-10°C) and those calculated according to equation 4.5

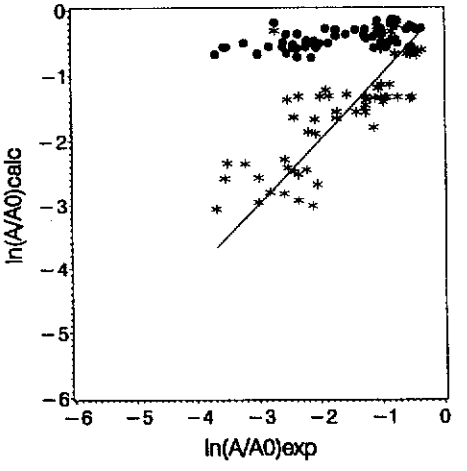

Figure 4.4. Improvement in fitting data for inactivation of LOX under dynamic conditions when using parameters estimated from dynamic (*) instead of static (•) inactivation data.

These results clearly showed the impracticability of estimating a set of kinetic parameters which is generally applicable in expressing the combined pressure-temperature dependence of the inactivation rate constant of LOX. The main reason for this was assumed to be the deficiency of the model and its concomitant parameters. In addition, calibration problems and variations in response time of the temperature measuring devices used in the respective experiments should not be overlooked in this case. LOX is indeed a thermosensitive enzyme so that small deviations on the temperatures considered for calculation purposes may partly account for the divergence in kinetic parameters.

2. Indicator-based test method for impact evaluation

The indicator test method uses the following equation (4.6) to evaluate the impact of combined pressure-temperature processes:

$$F_{HP/T} = \frac{R(X)}{k_{ref}} \tag{4.6}$$

$$R(X) = \ln\left(\frac{X_0}{X_t}\right) \qquad \text{for n} = 1$$

with

$$R(X) = \frac{X_t^{1-n} - X_0^{1-n}}{n-1} \qquad \text{for n} \neq 1$$

The practical and technical requirements to work with indicators is that the indicator response kinetics obey a rate law allowing separation of the variables and that *k*-values of the indicator should be small enough to give detectable changes. The remaining conditions to assure that the process impact determined using the indicator is equal to the actual process impact, depends on whether the high pressure/temperature process is isobaric, isothermal or with variable pressure and temperature. In the latter case, the inactivation rate constant at reference temperature of the indicator should obey the same law concerning pressure- and temperature dependency. Since both activation energy values and activation volumes have shown to be dependent on pressure and temperature respectively and since multi-parameters models are generally required to express the pressure-temperature dependence of the inactivation rate constant, development of pressure-temperature-time indicators will be a very difficult, tedious and time-consuming work. Therefore, it is largely advisable to make an appeal on the physical-mathematical test method for process impact evaluation where possible.