S. Fischer, W. Ruß, R. Buckow, V. Heinz, H. Ulmer, J. Behr, R. Meyer-Pittroff, D. Knorr and R. F. Vogel

Effects of hydrostatic high pressure on microbiological and technological characteristics of beer

High pressure treatment is well known as a sparing method for the preservation of beverages or food with low pH value, because it has only little effects on color, aromatic compounds and valuable substances like vitamins. Beer has a very sensitive composition and therefore high pressure treatment is an interesting preservation technique.
This study consisted of three parts: The effect of high pressure/temperature combinations was determined on the enzymes β-amylase, amyloglucosidase and β-glucanase. The mathematical modeling of the empirically detected results allowed the calculation of pressure/temperature combinations for varying treatment times which result in different activity, substrate conversion or stability. It was possible to detect an area with maximized substrate conversion for β-amylase extracted from barley malt. Amyloglucosidase shows higher activity at high pressure and high temperature and also β-glucanase reacts faster with increased pressure and temperature. On this basis, the mashing time could be shortened significantly by applying pressure of about 100 to 150 MPa.
Another step during the brewing process which was effected positively by high pressure treatment was the filterability. Independent of prevailing beer filtration problems it was possible to improve the filterability, regardless if the analyses were carried out with cellulose filter pads or kieselguhr. The influenced parameter was the content of β-glucan gel. In beer and in model systems with very high concentrations, a degradation of β-glucan gel was detected. Using different analytical systems it could be shown that β-glucan was formed. The third effect of high pressure was the inactivation of beer spoiling lactobacilli. These microorganisms are tolerant against hop ingredients and low pH values in beer. High pressure treatment at 200 MPa of *Lactobacillus plantarum* and *L. brevis* showed no significant decrease in the surviving cell counts, but after a few hours of storage in presence of hop the damaged *L. plantarum* cells died, whereas highly hop tolerant *L. brevis* cells survived. Therefore, it is necessary to inactivate highly hop tolerant *L. brevis*. A degradation of 5 powers of ten was possible with 40°C at 400 MPa for 30 s.

Descriptors: High pressure, hop resistance, microbiology, enzyme activity, filterability, β-glucan gel

1 Introduction

The preservation of valuable substances in food and beverages is a very important aim for the producing industry because customers ask for higher nutritional and sensory standards. Thermal treatment has an enormous influence on aromatic compounds, antioxidants, texture and color. High pressure treatment has marginal effects on small molecules and therefore the difference between treated and untreated products is very small. Beer is a very sensitive product because of its composition and the consumers demand for freshness of the product. The protein fractions and carbohydrates can be influenced by thermal treatment. For that reason it was very interesting to figure out the effects of high pressure treatment on beer. In a first study the most promising areas for basic and applied research were identified [1].

It is already known that the native conformation of enzymes can be changed by high pressure treatment [3]. Also a change in reaction kinetics is described [2], and the substrate could be influenced, e. g. starch shows gelatinizing at certain pressures [2]. For that reasons the behaviour of β-amylase, amyloglucosidase and β-glucanase in mash in dependence of different pressure/temperature combinations should be detected, especially because of the possible decelerated temperature inactivation at moderate pressures [3].

The influence of high pressure treatment on β-glucanase is important for the filterability of beer, but besides that high pressure treatment of unfiltered beer shows also an improvement in filterability. In this case the content of β-glucan-gel is influenced. Different analyses were made to figure out the reaction of β-glucan gel on high pressure treatment and if the results are transferable from model systems to beer. These results are also very interesting for other areas of the food processing industry because it is the first time that a possible solution of a gel is described.

For the sparing conservation of beer the tolerance of obligatory spoiling microorganisms against the antimicrobial substances (low pH value, ethanol, CO_2, hop ingredients) must be inactivated. The resistance against isohumulon partly relates to the membrane transporter HorA which is located in the cytoplasm. Even at low pressure of 200 MPa for 30 min HorA is completely inactivated, the cells die during storage within a few hours. This inactivation of the hop resistance seems to be adequate for the conservation of beer. In the following it should be figured out if these results, measured in buffer systems will also be valid for beer and if there is also a decrease in alcohol and acid tolerance.

2 Materials and methods

The experimental high pressure tests for the enzymatic reactions were carried out in a multi vessel system (Unipress, Warsaw).

Authors: Steffen Fischer, Winfried Ruß, Roland Meyer-Pittroff, Lehrstuhl für Energie- und Umwelttechnik der Lebensmittelindustrie, Technische Universität München, Weihenstephaner Steig 22, 85354 Freising; Roman Buckow, Volker Heinz, Dietrich Knorr, Institut für Lebensmittelbiotechnologie und -prozesstechnik, Technische Universität Berlin, Königin-Luise-Str. 22, 14195 Berlin; Harald Ulmer, Jürgen Behr, Rudi F. Vogel, Lehrstuhl für Technische Mikrobiologie, Technische Universität München, Weihenstephaner Steig 16, 85354 Freising.

Tables and Figures see Appendix

The five separate vessels are specially adapted for kinetically determinations [4]. The whole system is designed to operate between 20 and 70 °C, the pressure range is up to 700 MPa. It was planned that the final, constant temperature was reached after the adiabatic heating during pressure increase. After high pressure treatment the samples were chilled for five minutes in icy water, than the enzymatic analyses starts. All tests were carried out in a ACES buffer system.

The activity of β-amylase from barley (Sigma #A-7130) was analyzed according to the modified method of Bernfeld [5]. Soluble starch by Zulkowski (Merck, Darmstadt, #101257) was used as substrate. Here the change in color of dinitrosalicylic acid was measured spectral photometrical at 490 nm and the activity was determined by using a calibration straight line. The activity of amyloglucosidase was analyzed according to a modified method of Novo [5]. This is also a spectral photometrical method where at 420 nm the difference between sample and blank value is measured. The activity of β-glucanase from *Bacillus subtilis* (Sigma #49106) was indirectly measured by detecting the dissolved maltose monohydrates from azo-barley-glucan after a certain reaction time.

The decreasing activity of the enzymes as function of time is described in the following equations:

$$\left(\frac{dA}{dt}\right) = -k \cdot A^n \qquad (1)$$

After integration at constant temperature it is possible to calculate the velocity constant.

$$\frac{A}{A_0} = \left[1 + A_0^{n-1} \cdot (n-1) \cdot k \cdot t\right]^{\frac{1}{1-n}} \qquad (2)$$

A = activity

A_0 = starting activity

k = velocity constant of the inactivation

t = time

n = order of reactions

The empirical detected results allow to simplify the calculation of k by using the specific velocity constant k`.

$$k` = k \cdot A_0^{n-1} \qquad (3)$$

The examinations of the filterability and the behaviour of β-glucan-gel were carried out with beer and model systems. The beer samples were commercial products with filtration problems. The model systems were produced of isolated β-glucan from barley. It was dissolved in a basic solution (pH 11), afterwards a pH of 6 was adjusted by using HCl. The samples were frozen and thawed 14 times every 24 hours. After this procedure the gel content was the highest. The measurements were carried out with concentrations of 400 and 800 mg β-glucan gel per litre.

The treatment of the samples occurs in different high pressure plants. At the beginning the treatment temperature was 20 °C, and it rose – pressure dependent – up to 30 – 35 °C. After high pressure treatment the beer samples were analyzed with filtration tests according to Sartorius and Raible and a β-glucan analyzer [6; 7]. The model systems were analyzed with a viscosimeter, a β-glucan-analyzer and a NMR-spectrometer.

The online measurements were carried out in an optical cell [8]. The method was the same like the one of the β-glucan analyzer. The difference is that high pressure lowers the fluorescence of Calcofluor. For that reason the changing intensity of the fluorescence of Calcofluor was determined. The achieved results were subtracted from the intensities in fluorescence of the relating samples. Using this method the influence of pressure on the fluorescence of Calcofluor was eliminated.

For the microbiological examinations the beer spoiling organisms *Lactobacillus plantarum* TMW 1.460 (moderately hop tolerant) and *L. brevis* TWM 1.465 (highly hop tolerant) were cultivated. After high pressure treatment the evaluation of the total number of colony forming units was detected with MRS agar and for the calculation of the sublethal damaged cells 4 % NaCl was added. These examinations were also carried out upon storage over various periods in model beers with different pH values and with or without hop addition. The measurement of the intracellular pH value took place according to the diacetate-succimidylester method [9]. This method was adapted to the high pressure process [10]. The ratio of fluorescence at 485 and 410 nm depends on the pH value. Because of the intracellular location of the fluorescence probe and a special preparation of the cell suspensions it was possible to measure the intracellular pH value before and during high pressure treatment [11].

3 Results and discussion

Figure 1 shows examples for inactivation kinetics of β-amylase at 55 and 65 °C. It is obvious that the temperature tolerance increases with rising pressure. The ratio A/A_0 is most negative at atmospheric pressure and above 500 MPa. Between 200 and 250 MPa even at 65 °C, the inactivation rate is not very significant.

The empirical detected graphs fit best with an order of reaction n = 2. For an exact description of the dependence of the velocity constant on pressure and temperature it was necessary to apply regression analysis (Software TableCurve 3D). In Figure 2 the velocity constant in dependence on pressure and temperature is shown in a logarithmic scale. With increasing temperature the graphs show parabolic behaviour, the minimum of this parabola is at 200 MPa. In analogy to Figure 1 this is an obvious hint that the rate of inactivation is lowest at this pressure.

Using equation 2 it is possible to calculate pressure/temperature dependencies of β-amylase in ACES buffer. The elliptical behaviour of the velocity constant in Figure 2 leads to an also elliptical graph for the lines of equilibrium for constant decrease in activity. Figure 3 shows pressure – temperature combinations which effect the same inactivation. In this consideration again at 200 MPa β-amylase is most stable.

Determinations concerning the activity of amyloglucosidase were carried out in the same way. Another way to demonstrate the effect of high pressure is shown in Figure 4. The level curves show the percentage of glucose produced from maltose monohydrate. The optimum is at 370 MPa and 85 °C, it is about 35 % higher in comparison to the optimum at atmospheric pressure and 65 °C.

For a better comparison between the enzymes the pressure/temperature phase diagram is shown in Figure 5. The best temperature tolerance is, differing to the highest transferring rate, at about

700 MPa. To reach an inactivation of 90 %, temperatures of approximately 105 °C are necessary.

The inactivation of β-glucanase tends to show the same behaviour as the already described enzymes. A significant stabilization against thermal influence at 400 MPa and an increase of the transferring rate at 200 MPa and 58 °C of about 40 % was measured. Figure 6 shows the pressure/temperature phase diagram of β-glucanase for different inactivation rates.

All results show that it is possible to increase the transferring rate and the velocity of enzymatic reactions. But it has to be considered that not only the enzymes are influenced by high pressure, also the substrates are effected. Wheat starch for example gelatinizes at defined pressure/temperature combinations (500 MPa / 30 °C or 200 MPa / 60 °C) [2]. The combination of these two effects – better activity and gelatinizing – results in greater transferring rates and for that in higher velocity constants at defined pressure/temperature windows. Another reason for improved reaction kinetics could be the temperature increase due to the pressure induced adiabatic heating. By starting at the optimal temperature it is possible to design the process in that way, that the treatment conditions are in the mentioned optimal pressure – temperature window.

The examinations concerning the filterability started with the experiments on beer. Unfiltered beer with filtration problems was high pressurized at different pressures. Figure 7 shows the influence of high pressure treatment on the filterability of this beer. The analyses were carried out with a cellulose filter shift (Sartorius test) and kieselguhr (Raible test).

It is obvious that the filterability increased with rising pressure. The optimum is reached between 300 and 500 MPa. With higher pressures an optical remarkable turbidity is given which results in a worse filterability.

The reason for this improvement is the decrease in the β-glucan gel content. Other relevant parameters for the filterability are not influenced. Figure 8 shows the behaviour of β-glucan gel in dependence on the applied high pressure.

To work out if the influence of high pressure only effect a decrease of 10 to 20 mg/l or if a complete degradation is possible, measurements with model systems were carried out. In Figure 9 the lowered content of β-glucan gel in model-systems after high pressure treatment is shown.

For the identification, whether β-glucan gel is degraded into β-glucan or still exists in sol state, viscosity was measured. The influence of the different concentrations of β -glucan gel is shown in the higher viscosity of the 800 mg sample shown in Figure 10. Thermal treatment converts β-glucan gel into sol state which results in a nearly constant viscosity. After high pressure treatment at 500 MPa viscosity was clearly decreased.

The evidence for the degradation of β-glucan gel to β-glucan was supplied. This result was verified by NMR measurements. As shown in Figure 11 the content of free water p_g increases after high pressure treatment. That means water becomes more mobile, which relates to the state of solved molecules. Additionally the content of immobile water p_c decreases, the water which is fixed by gel structures becomes less.

To analyze the influence of time during high pressure treatment online measurements were carried out. These examinations show that the content of β-glucan gel is already reduced during the pressure rise (Fig. 12). This effect stops at 200 MPa. Further degradation does not occur until 300 MPa. After 300 s treatment time no further degradation could be determined. At 400 MPa the content of β-glucan gel is even lower after 300s treatment time.

The detected results show that it is possible to improve the filterability of beer and to reduce the content of β-glucan gel by applying high pressure. Deformation powers cause the destruction of bonding spots during the pressure rise. At pressures above 300 MPa a pressure induced destruction of weak bondings like Van der Waals forces is given. The decrease in viscosity due to high pressure treatment is a very significant evidence for the transfer from the gel state to the single β-glucan molecule.

For the microbiological examinations mainly the influence of the pH value was analyzed. Figure 13 shows the influence of treatment time and pH value on inactivation of *Lactobacillus plantarum*. The top graph shows the whole amount of colony forming units and the bottom graph the stress resistant cells.

It was found that inactivation of *Lactobacillus plantarum* is more effective at lower pH values, the reduction of colony forming units is about five powers of ten. At short pressurization times the number of fully viable (salt stress tolerant) cells is below the number of total survivors. This indicates that sublethal injury occurs at short pressurization times only.

Additionally, the intra cellular pH value of *Lactobacillus plantarum* was measured. Under atmospheric conditions it was 6.3 or even higher, independently of the extra cellular pH value (4.0 – 5.0). This gradient is important to maintain pH homeostasis and for the transport of different substrates into the cells. After high pressure treatment at 300 MPa the intra cellular pH value was equal to the extra cellular one. This process was very fast, occurring during the ramp, and irreversible even at short treatment times.

To work out if the loss of the transmembrane-pH-gradient is responsible for the irreversible inactivation of the acid tolerance high pressure treatment and storage were carried out in different buffer systems (6.5, 5.0, 4.0). Figure 14 shows the colony forming units after high pressure treatment at pH 6.5 and 300 MPa for 60 min and a following storage at pH 6.5, 5.0 and 4.0 for a maximum of 48 h.

High pressure treatment and following storage at a pH value of 6.5 effects only a degradation of 20 % of the content of colony forming units. Storage at pH 5.0 and 4.0 results in a reduction of about 90 to 95 %. If the high pressure treatment is done in buffer systems with lower pH value the effect is even more significant. The simple high pressure treatment effects at pH 5.0 and 4.0 a reduction of three powers of ten. After storage at the same pH value an additional reduction of two powers of ten (pH 4.0) and one power of ten (pH 5.0) is possible (Fig. 15).

The shown reduction of the colony forming units due to high pressure treatment at low pH value relates to the pressure induced loss of the acid tolerance.

High pressure treatment with 200 MPa at neutral pH of *Lactobacillus plantarum* did not effect a significant reduction of the colony forming units. However, in model beer with pH 4.0 the membrane transport system HorA was completely inactivated rendering the cells hop sensitive. Consequently, the cells with lowered hop resistance died in the presences of hops within a few hours. The same behaviour was detected after destroying the transmembrane-pH-gradient due to high pressure treatment. Low pH value during high pressure treatment occurs higher inactivation rates and during storage at low pH value colony forming units become even less (Table 1).

But as demonstrated in Table 1, inactivation of similarly treated highly hop tolerant *L. brevis* was not observed upon storage in hop

containing model beers. This phenomena was observed in beer with low hop content (16,7 mg/l Isohumolone; beer A) and and with high hop content (35,8 mg/l Isohumolone; Bier B). Apparently, this strain possesses additional hop resistance mechanisms and should be completely inactivated by a more intense treatment.

4 Conclusion

High pressure treatment of enzymes effect a significant influence on activity and substrate transfer. These already known possibilities happen also with high pressure treatment of β-amylase, amyloglucosidase and β-glucanase. At room temperature β-amylase can be inactivated faster by applying high pressure, at 50 °C the inactivation curve becomes parabolic and at higher temperatures the inactivation rate is lowered at 200 MPa. For amyloglucosidase the inactivation curve is similar but the increasing temperature tolerance is located at 700 MPa and for β-glucanase at 400 MPa. But for these results it has to be considered that the swelling of the substrates (starch, maltodextrine) is accelerated under pressure. The best transfer rates are at different pressure – temperature combinations because they are combinations between activity and swelling of the substrate. E.g. for β-glucanase this optimum is located at 210 MPa and 57 °C. It is possible to shorten process time for mashing, the optimum at conventional temperatures is between 100 and 150 MPa.

For the improvement of the filterability of beer high pressure treatment shows the best results between 500 and 600 MPa. These conditions results in a content of β-glucan gel below the detection limit. Other parameters like proteins, polyphenoles or particle size show no changes. But high pressure does not only effect a decrease of β-glucan-gel of about 10 mg/l, in model systems with very high concentrations the content was also lowered under the detection limit. In this process β-glucan gel is dissolved into the single molecule β-glucan, there is not only a transformation into the sol state. The solubilisation happens in two steps, first the pressure independent part between 0.1 and 200 MPa, where deformation powers dissolve bondings and second the pressure related solubilisation above 300 MPa.

For the preservation of beer by using high pressure the most pressure resistant microorganism, *Lactobacillus plantarum*, and highly hop tolerant *L. brevis* were selected. The effect of high pressure treatment depends on the pH value because the acid tolerance of the cells is lowered and storage at pH 4.0 results in a nearly complete inactivation. High pressure treatment at 200 MPa of *Lactobacillus plantarum* and *L. brevis* showed no significant decrease in the surviving cell counts, but after a few hours of storage in presence of hop the damaged *L. plantarum* cells died, whereas highly hop tolerant *L. brevis* cells survived. Therefore, it is necessary to inactivate highly hop tolerant *L. brevis*. A degradation of 5 powers of ten was possible with 40 °C at 400 MPa for 30 s.

At the moment industrial high pressure treatment is a batch process but it is possible to realize pumps which work up to 400 MPa and this is an area which is very interesting for continuous high pressure applications.

5 References

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Appendix

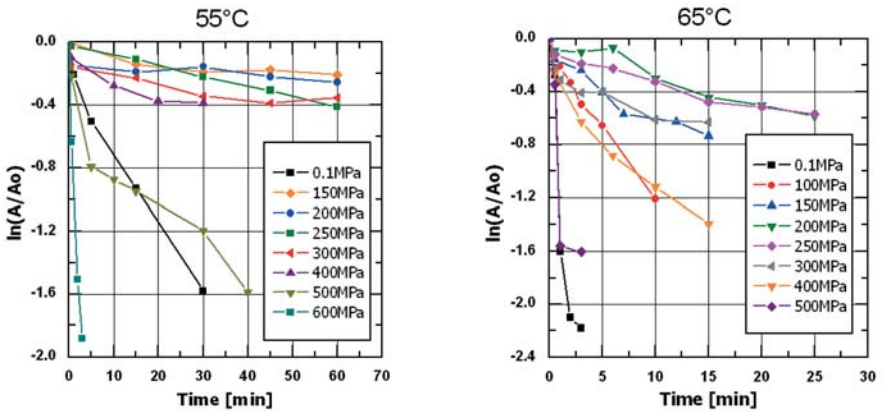

Fig. 1 Inactivation kinetics of β-amylase in ACES buffer at 55 and 65 °C

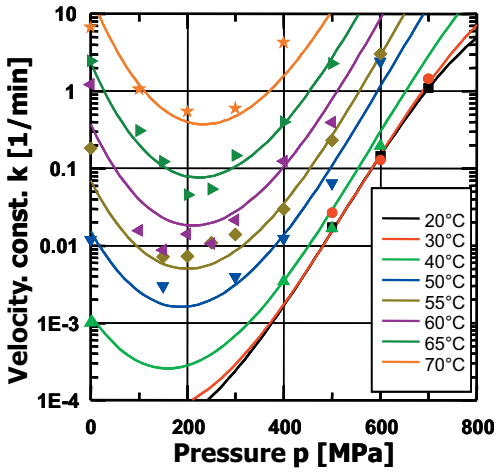

Fig. 2 Pressure/temperature dependence of the specific velocity constant k` of β-amylase in ACES buffer

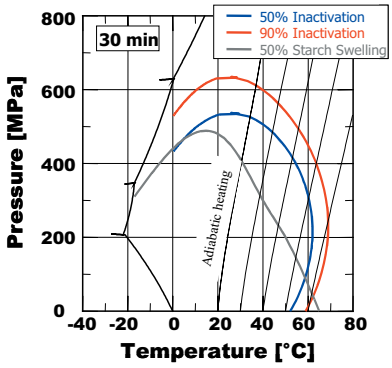

Fig. 3 Pressure/temperature phase diagram of β-amylase in ACES buffer for an inactivation of 50 and 90 % after high pressure treatment for 30 min. For better orientation line of equilibrium for 50 % wheat starch gelatinizing, phase interfaces for water and lines for adiabatic heating of water are given

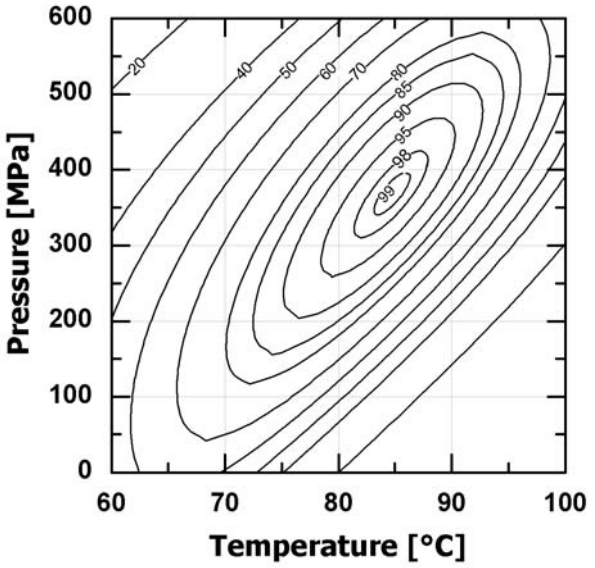

Fig. 4 From maltose monohydrate by amyloglucosidase produced glucose in dependence of pressure and temperature after high pressure treatment for 30 min

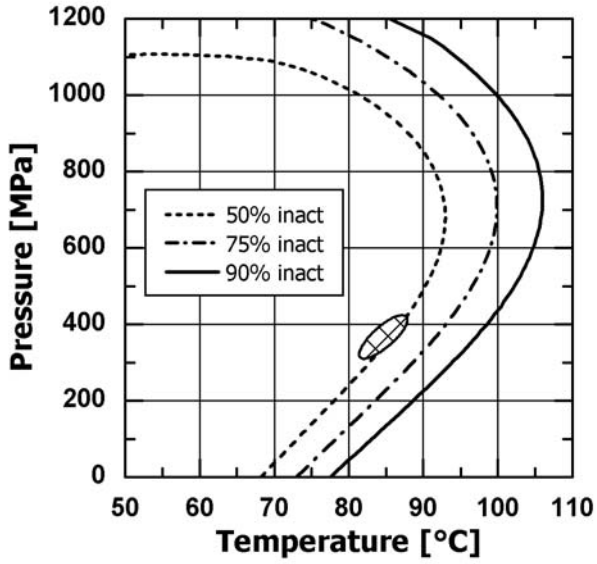

Fig. 5 Pressure/temperature phase diagram of amyloglucosidase in ACES buffer for an inactivation of 50, 75 and 90 % after high pressure treatment for 30 min. The area for the highest transferring rate is marked (Θ)

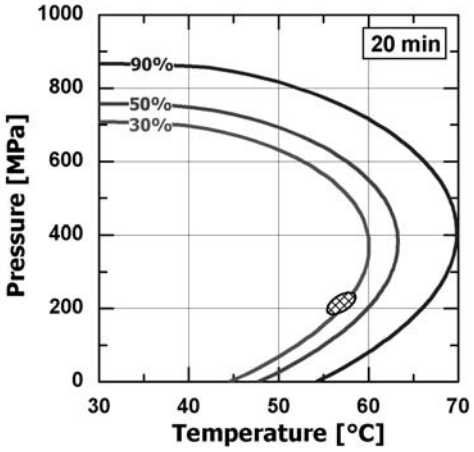

Fig. 6 Pressure/temperature phase diagram of β-glucanase in ACES buffer for an inactivation of 30, 50 and 90 % after high pressure treatment for 30 min. The area for the highest transferring rate is marked (Θ)

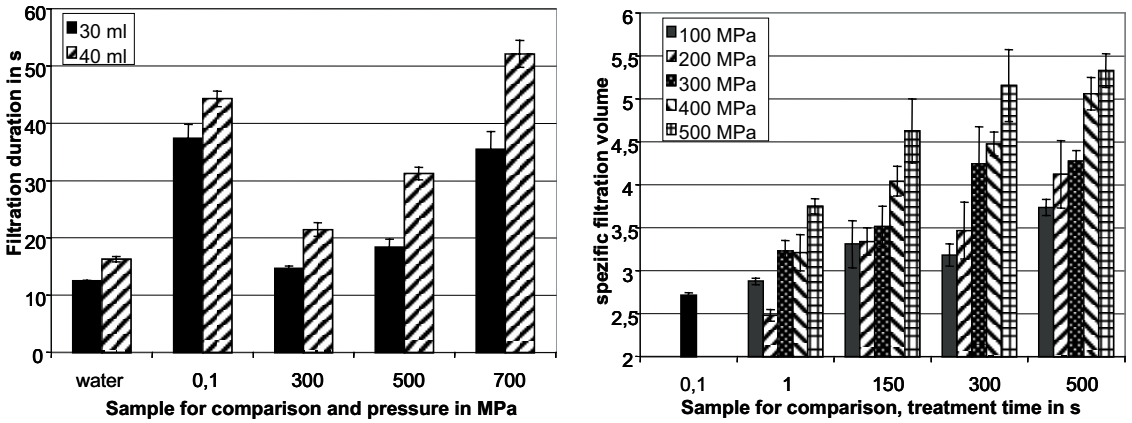

Fig. 7 Filterability of unfiltered beer in dependence of high pressure after a treatment time of 300 s; analyzed with Sartorius test (left) and Raible test (right)

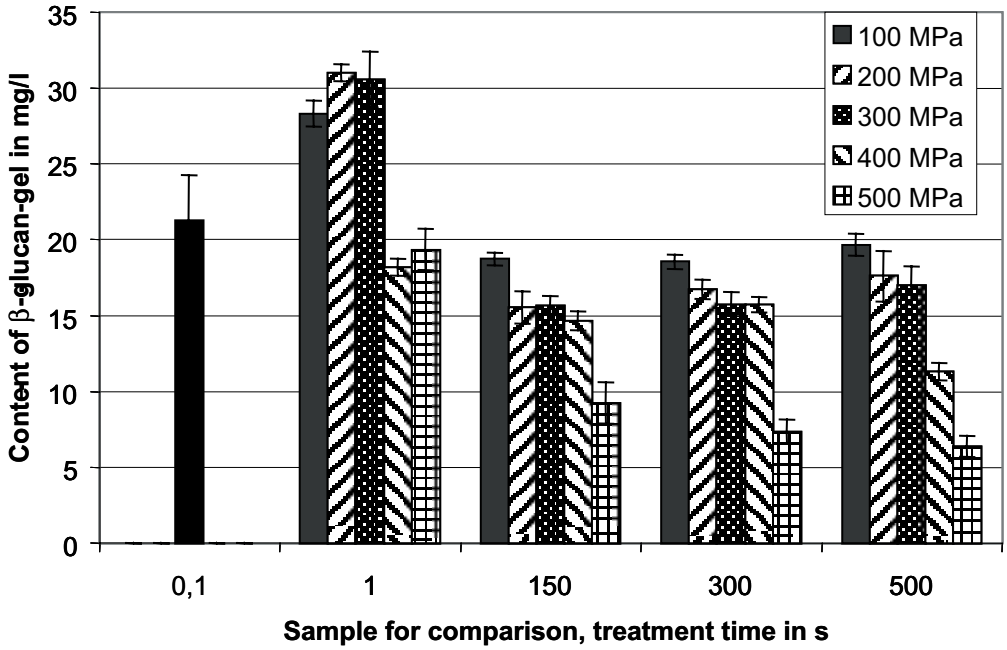

Fig. 8 Content of β-glucan-gel in beer in dependence on pressure and treatment time

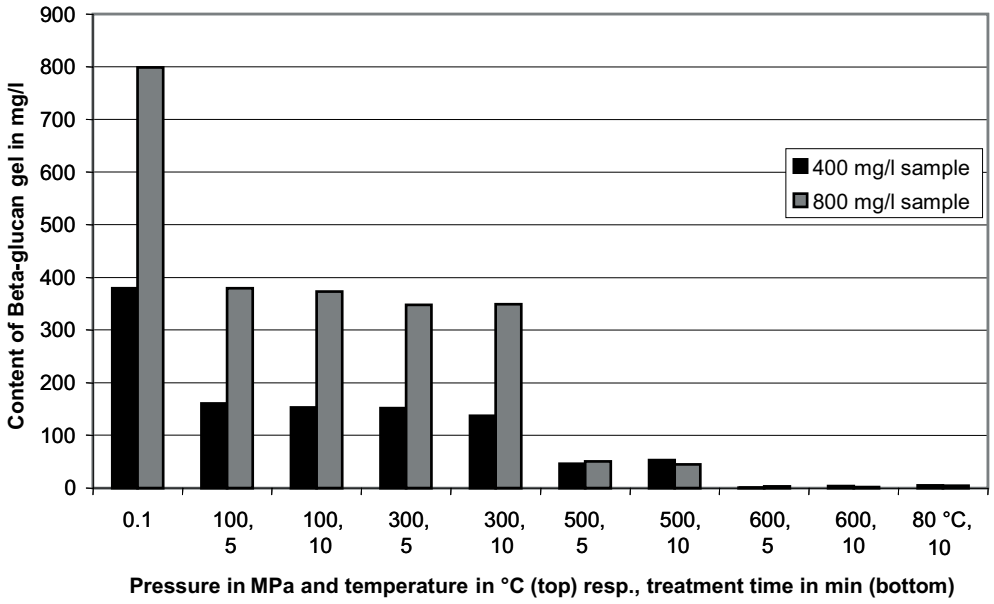

Fig. 9 β-glucan gel content in model-systems after high pressure treatment

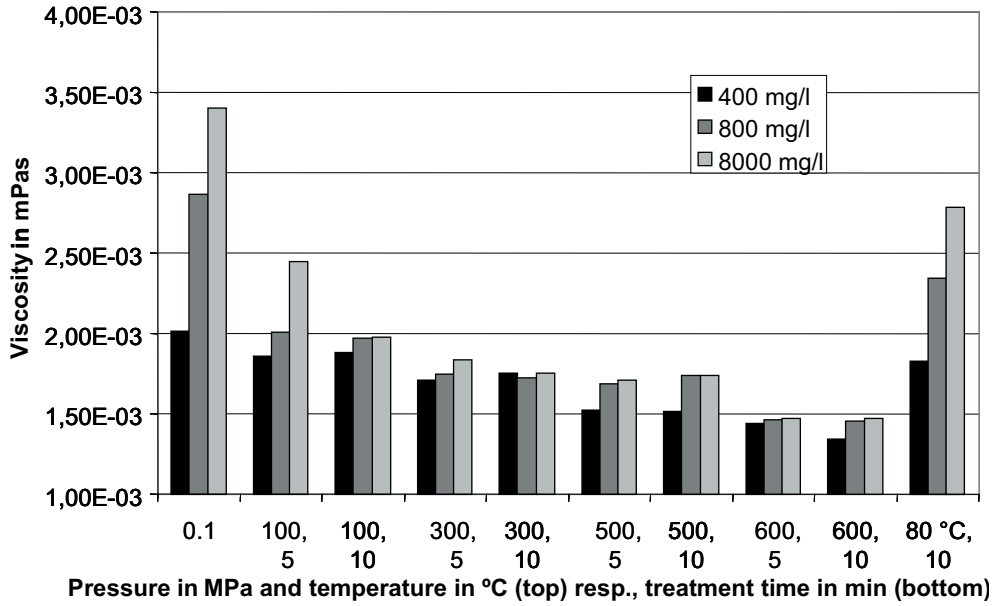

Fig. 10 Viscosity of samples with different β-glucan gel concentrations after high pressure and thermal treatment

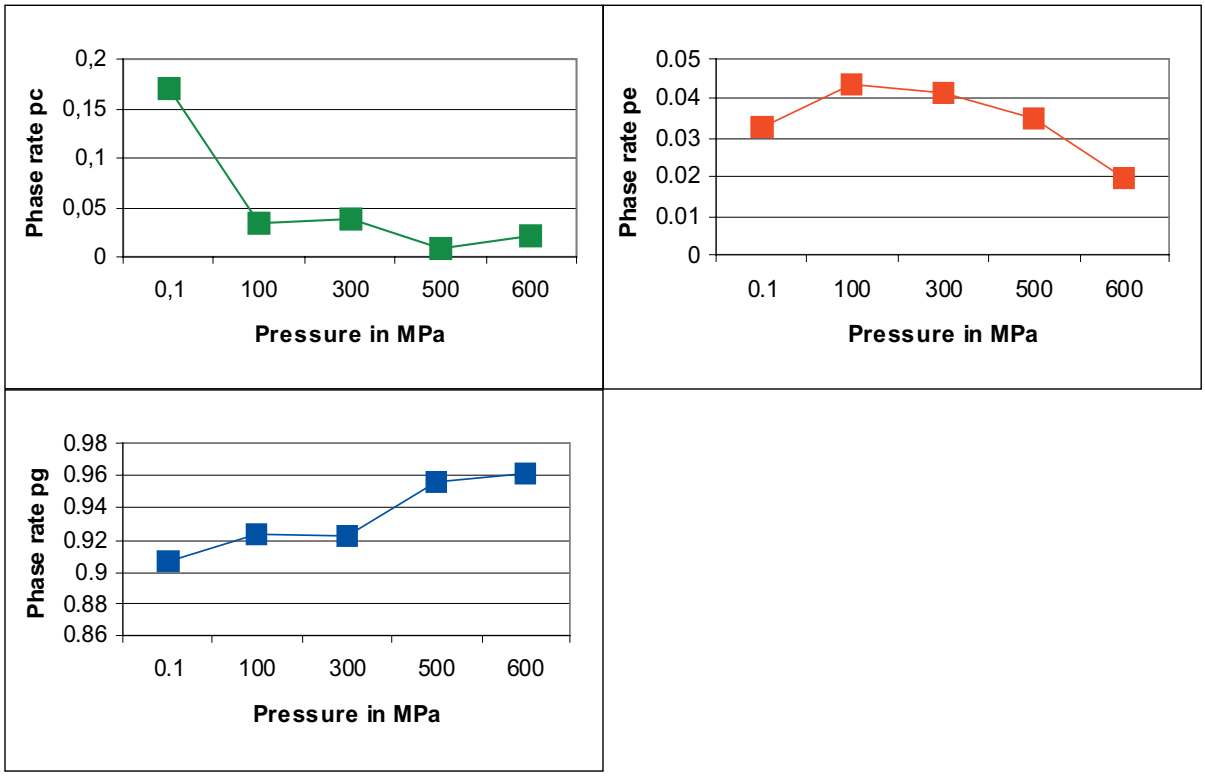

Fig. 11 Phasing rates and relaxation time of immobile water (pc), less mobile water (pe) and mobile water (pg) in dependence of high pressure

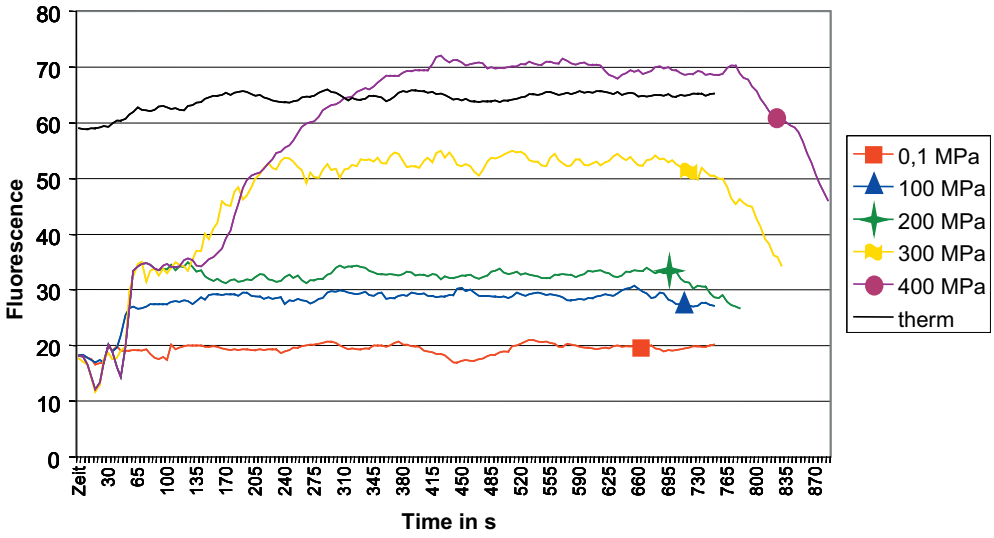

Fig. 12 Online measurements of the β-glucan content at different high pressure treatment conditions

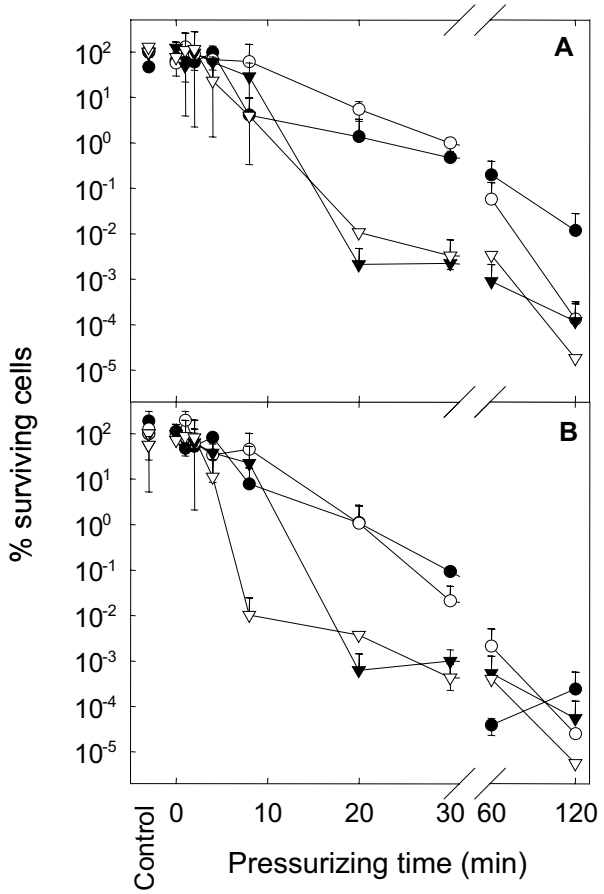

Fig. 13 Inactivation of *Lactobacillus plantarum* due to high pressure treatment at 300 MPa and 20 °C in buffer systems with pH 6.5 (full circles), 6.0 (open circles), 5.0 (full triangles) and 4.0 (open triangles). A: Total amount of colony forming units, B: Salt stress resistant cells

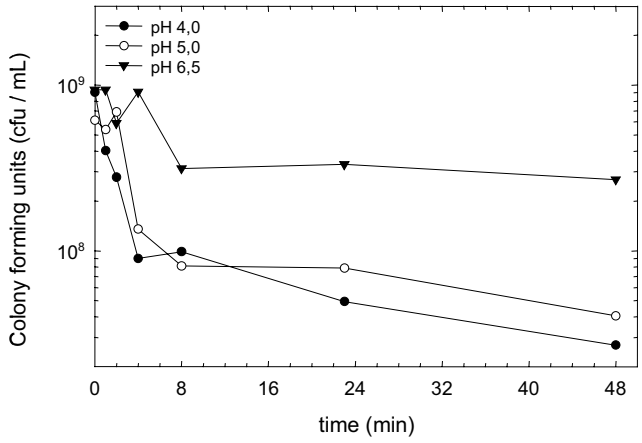

Fig. 14 Inactivation of *Lactobacillus plantarum* due to high pressure treatment at 300 MPa for 60 min in a buffer system with pH 6.5 and following storage at pH 6.5, 5.0 and 4.0

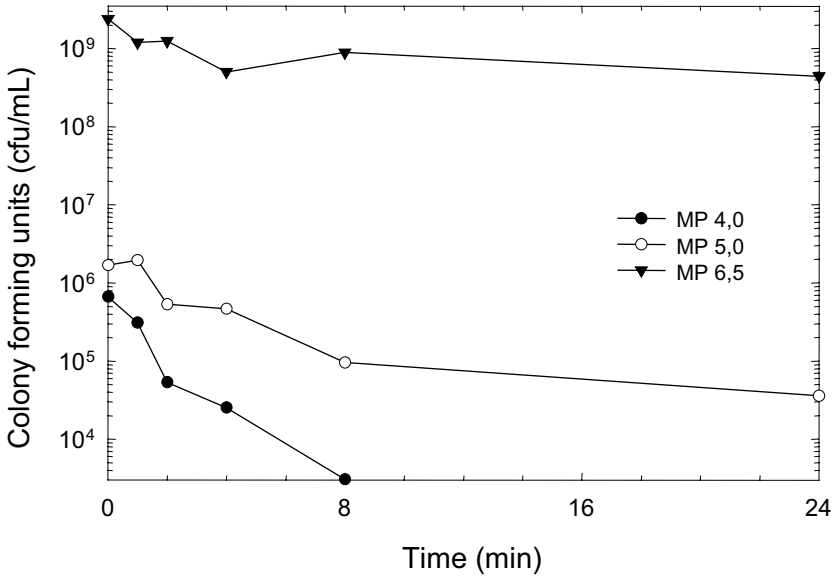

Fig. 15 **Inactivation of *Lactobacillus plantarum* due to high pressure treatment at 300 MPa for 60 min in buffer systems with pH 6.5, 5.0 and 4.0 and following storage at the same pH value**

Table 1 **Comparison of the detection times of *L. plantarum* TMW 1.460, *L. plantarum* TMW 1.332, *L. brevis* TMW 1.465 and *L. brevis* TMW 1.465A in beer A and beer B in dependence of the pressure holding time at 200 MPa. (---) symbolizes no growth within 28 days; no means untreated sample**

Pressure holding time in [min]	Detection time in [h] **TMW 1.460**		Detection time in [h] TMW 1.332		Detection time in [h] TMW 1.465		Detection time in [h] TMW 1.465 ad	
	A	**B**	A	**B**	**A**	B	**A**	**B**
no	**---**	**---**	**---**	**---**	**20**	**---**	**20**	**48**
0	**---**	**---**	**---**	**---**	**20**	**---**	**20**	**48**
4	**---**	**---**	**---**	**---**	**67**	**---**	**20**	**96**
8	**---**	**---**	**---**	**---**	**67**	**---**	**48**	**96**
16	**---**	**---**	**---**	**---**	**67**	**---**	**48**	**96**
24	**---**	**---**	**---**	**---**	**67**	**---**	**96**	**120**
32	**---**	**---**	**---**	**---**	**67**	**---**	**96**	**120**