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Recovery of cellulase activity after ethanol stripping in a novel pilot-scale unit

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Abstract Recycling of enzymes has a potential interest during cellulosic bioethanol production as purchasing enzymes is one of the largest expenses in the process. By recycling enzymes after distillation, loss of sugars and ethanol are avoided, but depending on the distillation temperature, there is a potential risk of enzyme degradation. Studies of the rate of enzyme denaturation based on estimation of the denaturation constant K_D was performed using a novel distillation setup allowing stripping of ethanol at 50–65 °C. Experiments were performed in a pilot-scale stripper, where the effect of temperature (55–65 °C) and exposure to gas–liquid and liquid–heat transmission interfaces were tested on a mesophilic and thermostable enzyme mixture in fiber beer and buffer. Lab-scale tests were included in addition to the pilot-scale experiments to study the effect of shear, ethanol concentration, and PEG on enzyme stability. When increasing the temperature (up to 65 °C) or ethanol content (up to 7.5 % w/v), the denaturation rate of the enzymes increased. Enzyme denaturation occurred slower when the experiments were performed in fiber beer compared to buffer only, which could be due to PEG or other stabilizing substances in fiber beer. However, at extreme conditions with high temperature (65 °C) and ethanol content (7.5 % w/v), PEG had no enzyme stabilizing effect. The novel distillation setup proved to be useful for maintaining enzyme activity during ethanol extraction.

Keywords Thermostable cellulases · Distillation · Gas–liquid interfaces · Enzyme recycling · Ethanol · Denaturation constant (K_D)

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Introduction

Enzymes for degradation and saccharification of pretreated lignocellulosic biomass are among the most costly factors for cellulosic bioethanol production [9]. Therefore, recycling of enzymes is potentially interesting from an industrial perspective. Enzymes can be recycled after hydrolysis or fermentation, but this requires that either the sugars or ethanol can be efficiently separated from the enzymes and residual solids. Methods to successfully recover enzymes in these steps of the process still need further development [32]. From a process point of view, it could be an advantage to recover enzymes after distillation where no valuable product will be lost. The boiling point of ethanol at atmospheric pressure is 78.5 °C and distillation is consequently performed at a temperature that would rapidly inactivate most of the cellulase preparations commercially available [25]. Thus, recovery of enzymes after distillation either requires decreased temperatures, highly improved thermal stability of the enzymes, or a combination thereof. Thermostable enzymes are, in addition to being stable at elevated temperatures, also known to have higher specific activity and inhibitor tolerance [7, 31]. However, recent studies have shown that the combined effect of ethanol and

elevated temperatures accelerates the inactivation of cellulases [25]. For an economical efficient distillation, the ethanol content should be above 4 % (w/v) [13]. One should therefore be aware of both the ethanol content in fiber beer originated from SSF and the temperature.

A distillation setup consists of two sections; a stripper, where the gaseous phase containing ethanol and water is generated and separated from fiber beer, and a rectifier, where the (evaporated) ethanol-water mixture is concentrated further [19]. Hence, the enzymes in fiber beer will remain in the stripper, while the ethanol will continue to the rectifier. In order to obtain both maximum recovery of enzyme activity and low energy consumption, a novel distillation system has been investigated in this study. It is based on a diabatic horizontal stripping section side by side to a rectifying section separated by a heat transmission wall [12]. In conventional vertical adiabatic stripper columns, the heat needed for evaporation is provided by heating stillage in an external heat exchanger (reboiler) whereby steam will be flashed off when the stillage returns to the bottom of the stripper. The high temperature of and the long contact time between the stillage and the heat transmission wall makes it difficult to avoid enzyme degradation. In the novel stripper, the heat transmission wall is integrated in the stripper, where the average temperature is lower and the contact time between the stillage and the heat transmission wall is shorter than by the conventional stripper. A droplet-generating rotor in the bottom of the stripper is picking up small portions of fiber beer and throwing them against the heat transmission wall, where the absorbed heat will evaporate a mixture of ethanol and water. The enzyme-containing fiber beer will in the novel pilot stripper be exposed to the combined effect of (1) temperature, which is below the boiling point of ethanol, (2) the contact time between the liquid and heat transmission wall, which is short compared to a conventional stripper column, (3) ethanol, which concentration quickly decrease as the fiber beer is passing through the stripper, and (4) the gas–liquid interfaces, which occur when the droplets hit the heat transmission wall.

Lignin is a challenge when recovering enzymes from distillation residue (stillage). It is known to interfere with hydrolytic enzymes via hydrophobic interactions and hence decrease the activity of cellulases by non-productive adsorption during the hydrolysis [22, 30]. In addition, adsorption of cellulases to lignin has also been suggested to induce denaturation of cellulases at elevated temperatures [3]. The issues related to lignin are likely accelerated during distillation. Untreated lignocellulosic biomass consists of ~15–30 % lignin, which increases during bioethanol processing as cellulose and hemicellulose are degraded and/or converted [20]. Different methods to reduce enzyme adsorption to lignin or extract enzymes have been tested such as addition of surfactants or polymers [15, 24]. Research indicates that the surfactant poly(ethylene glycol) (PEG), which is a hydrophobic polymer, can (1) improve the yield of glucan conversion by reducing adsorption of cellulases onto lignin, (2) stabilize the enzyme when exposed to gas–liquid and liquid–liquid interfaces, and (3) form micelles, entrapping the enzymes and hereby avoid denaturation by heat [15, 18, 21, 24].

In distillation processes, the efficiency is enhanced when the temperature is increased, as the interaction between warm gas and colder liquid at the gas–liquid interface is increased [29]. Studies have shown that stability of enzymes is weakened in gas–liquid interfaces, but the damage can in some cases be reduced by addition of surfactants [14, 27]. In an aqueous environment, such as in fiber beer, a protein will normally cover its hydrophobic amino acids (AA) in the core of the protein, while the hydrophilic AAs are facing the liquid surroundings. As the protein is exposed to a gas–liquid interface it will start to orient the hydrophobic AAs to the gas interface, while the hydrophilic AAs will orient to the liquid phase. If a rapid expansion of the gas–liquid interface occurs, this contrary orientation of AAs can cause unfolding and degradation of the protein [1]. Addition of surfactants can reduce protein degradation at the gas–liquid interface by (1) diffusing faster to the surface hereby reducing exposure of enzymes to the surface, (2) lowering the surface tension, and (3) strengthening the hydrophobic interaction inside the protein [1, 23].

The purpose of this study was to investigate the potential for recovery of cellulase activity after being exposed to process-relevant conditions in the novel stripper unit. While the enzyme recovery will benefit from the possibility to operate at lower temperatures, the effect of exposure to gas–liquid interfaces is rather unknown. Therefore, the influence of temperature, ethanol, PEG, and gas–liquid interfaces on the activity of different cellulases in a fiber beer was tested. Only a single section of the stripping unit was tested, where ethanol content remained constant. The study was performed at industrial-relevant temperatures and in a fiber beer obtained after hydrolysis and fermentation of hydrothermally pretreated wheat straw. Two enzyme preparations were evaluated, a commercial product and a mixture of thermostable enzymes. The results obtained in the pilot-scale unit were compared to lab-scale experiments that did not expose the cellulases to gas–liquid interfaces and PEG.

Materials and methods

Fiber beer

Fiber beer was the product after pretreatment, hydrolysis, and fermentation of wheat straw at the Inbicon

demonstration plant, located in Kalundborg, Denmark. In short, wheat straw (*Triticum aestivum* L.) was hydrothermally pretreated for 12 min at 190 °C and adjusted to 25 % dry matter (DM). Before hydrolysis, pH was regulated to 5.0 and 0.01 g PEG/g DM was added. Enzymatic hydrolysis was 6 h at 50 °C followed by SSF for 138 h at 33 °C, whereafter fiber beer was sampled [17]. Fiber beer was heated to 80 °C for 10 min to inactivate the enzymes added in the hydrolysis at the demonstration plant, which resulted in a fiber beer containing 2.5 % ethanol (w/v). For lab-scale experiments, the fiber beer was further distilled at 80 °C using a Rotovap to lower the ethanol content. Fiber beer without PEG was made by the same procedure as at Inbicon, but in a small-scale setup. Ethanol and soluble sugar content of fiber beer was measured by HPLC.

Enzymes

Two enzyme mixtures were tested; a commercial and a thermostable mixture. The commercial enzymes were a mixture of Celluclast, which contains enzymes from *Trichoderma reesei*, and Novozym188 (NZ188), which contains β-glucosidase (BG) from *Aspergillus niger*. Celluclast and NZ188 were mixed in the ratio 5:1 (w/w) and is through the paper denoted as CNmix. CNmix was kindly delivered by Novozymes (Bagsværd, Denmark). The thermostable enzymes, denoted Thermomix, were a mixture containing 51 % cellobiohydrolase I (CBHI) from *Acremonium thermophilum*, 17 % cellobiohydrolase II (CBHII) from *Chaetomium thermophilum* and 13 % endoglucanase II (EGII), 14 % endoxylanase (XYL), and 5 % BG from *Thermoascus aurantiacus*. The enzyme ratio was dosed as enzyme protein (EP), i.e., the protein mixture contained 51 mg CBHI/100 mg mixture, 17 mg CBHII/100 mg mixture, etc. [34, 35]. Thermomix was kindly delivered by Roal Oy (Rajamäki, Finland).

Tests in pilot stripper (10 l scale)

The influence of temperature, time, and gas–liquid interfaces on cellulases was tested in a pilot stripper without vacuum (Fig. 1). The principle of the distillation system, including stripper and rectifier, is disclosed in US Patent 7972423 B2 [12]. Following Fig. 1, 10 l of fiber beer was added to the stripper via a pipe in the middle (1) and ejected as droplets by a rotor in the bottom rotating at 600 rpm (2). The droplets hit the heat transmission wall (3), where part of the liquid evaporated and the rest returned to the bottom, simulating conditions in an industrial stripper. Samples were taken via a tap in the bottom of the pilot stripper (4). To get a representative sample, 0.5 l of fiber beer was first taken and returned to the stripper before the final sample was taken. The temperature of the heating wall was adjusted

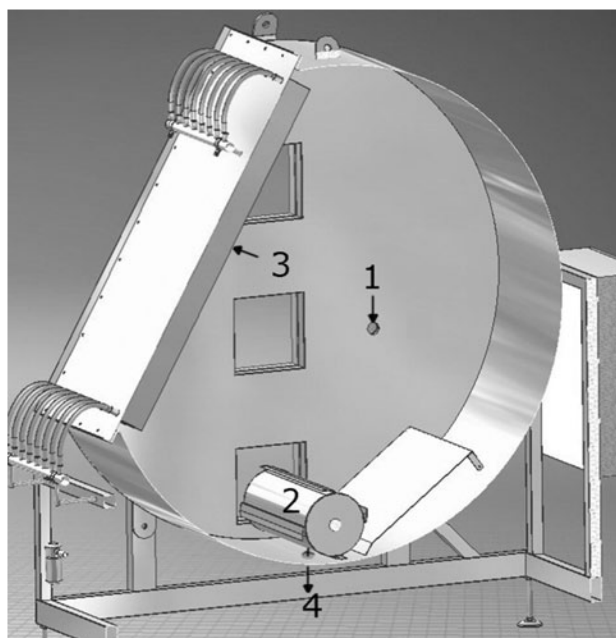

Fig. 1 Drawing of the pilot stripper where fiber beer or buffer (55 to 65 °C) was mixed with enzymes. (*1*) Inlet of slurry and enzyme, (*2*) paddles rotating at 600 rpm, which mixes slurry and enzymes and throw the liquid up on the heat transmission wall (*3*), where droplets transfer to a film of liquid during which a very fast expansion of the gas–liquid interface occur. (*4*) Outlet where samples were taken

to 55–65 °C before enzyme was added. Enzyme dosage was 2 mg EP/g fiber beer, which roughly equals the loading of 8 mg EP/g dry matter (DM) in case of initial 25 % DM. A 50-ml control sample was taken from the distillation plant just before the enzyme was added to control enzyme activity and ethanol content. The enzyme was diluted in 1 l of tap water and directly poured into the equipment. Samples were taken frequently during the 10-min run and stored in freezer until further analysis. The temperature of the fiber beer and heating wall was monitored during the experiment. After each experimental round, the equipment was washed three times with tap water before a new portion of fiber beer was introduced. As a control, the experiments were repeated with 50 mM buffer, pH 4.8, where CNmix was run at 55 °C and Thermomix at 60 °C (temperatures based on [25]).

Temperature and ethanol tolerance in small-scale tests (1 ml scale)

The influence of temperature and ethanol content on enzymes in fiber beer was tested in a small-scale system. Distilled fiber beer was mixed with 96 % ethanol (v/v) to obtain mixtures with 2.5 or 7.5 % ethanol (w/v). In 2-ml Eppendorf tubes, 900 μl of ethanol containing fiber beer was preheated on a heating block (QBD2, Grant, England)

to 55–65 °C for 10 min. To preheated tubes, 100 µl of diluted CNmix or Thermomix was added, mixed well, and returned to the heating block. The enzyme concentration was 2 mg EP/g fiber beer. The following 10 min, the tubes were transferred from the heating block to ice to stop the reaction where after samples were kept frozen until activity measurement. Samples were measured in duplicate.

Enzyme stability in the presence of PEG (1 ml scale)

The effect of PEG was tested in a small-scale system. Distilled fiber beer was mixed with 96 % ethanol (v/v) to gain 7.5 % ethanol (w/v). The fiber beer was divided in two; one containing 0.01 g PEG/g DM (PEG 6000, J. T. Baker, Netherlands) and one without PEG. In a heating block (QBD2, Grant, England), 1.5-ml Eppendorf tubes containing 900 µl of fiber beer was preheated to 65 °C and 100 µl of diluted enzyme was added, mixed well, and returned to the heating block. The enzyme concentration was 2 mg EP/g fiber beer. The following 10 min, the tubes were transferred from the heating block to ice to stop the reaction. Samples were kept frozen until activity measurements.

Protein content and measurement of enzyme activities

The protein content of CNmix and Thermomix was measured following the procedure described by Skovgaard and Jørgensen [25]. For the pure enzyme mixtures, filter paper activity was measured following the procedure described by Ghose [5], CBHI and EGI activities were measured using 4-methylumbelliferyl-β-D-lactopyranoside as substrate [2], while BG activity was measured using *p*-nitrophenyl-β-D-glucopyranoside as substrate [25]. Specific activity of CBHI, EGI, and BG in samples (containing fiber beer or buffer) was measured following [2] and [25]. It was possible to measure CBHI, EGI, and BG activity on fiber beer samples as they had to be diluted at least 150 times to be within the standard curve. The dilution resulted in a less dark fiber beer solution, which made it possible to measure the concentration of the product of enzyme activity, e.g., production of *p*-nitrophenol when measuring BG activity. The enzyme activity in the blank was also measured in fiber beer or buffer. The cellulase activity of enzymes in buffer or fiber beer samples were measured as described in 2.7.

Measurement of cellulase activity in samples

Cellulase activity of samples taken during 1-ml and 10-l experiments was measured through sugar release after incubation with filter paper. The fiber beer was very dark due to the concentration of tiny lignin particles, hence colorimetric enzyme activity assays could not be used. The dark color could be reduced by diluting the fiber beer, but the combination of low enzyme dosage and a high degree of dilution would compromise the measurements. To avoid colorimetric measurements, a new method for cellulase activity measurements in microtiter scale was developed inspired by the filter paper assay described in [5] and [33].

Samples (slurry, supernatant, or buffer) were diluted in 50 mM sodium citrate buffer, pH 4.8. A blank was also made, which was a mixture of enzyme and fiber beer or buffer. Diluted samples were mixed and 150 µl was transferred to three wells in a 96-well flat-bottom microtiter plate. The plate was preheated to 50 °C on a heating block (QBD2, Grant, England) where after circular cut filter paper (Whatman no. 1) was quickly added to two of the three wells containing sample (the last well being the blank). Filter papers were 6 mm in diameter and were made with a hole puncher. The plate was covered with PCR film (sealing tape, ref. 95.1994, Sarstedt, Germany) and hydrolyzed for 2 h, 50 °C. After hydrolysis, the heating block was turned up to 95 °C and the plate was heated another 25 min. It took 10 min for the heating block to go from 50 to 95 °C, meaning that the enzymes were inactivated at 95 °C for 15 min. The plate did not lose weight during heating. The plate was afterwards cooled and samples were transferred to a new 96-well filter plate with a 0.45-µm cellulose acetate filter (No. 7,700–1,308, Whatman™, England), placed on top of a 96-well microtiter plate with round bottom, and centrifuged 1 min, 2,000 rpm, 492×*g*. The (round-bottom) plate with the collected supernatant was covered with HPLC foil (adhesive film, ref. 95.1992, Sarstedt, Germany) and sugar content was measured by HPLC.

HPLC

Concentration of soluble sugars was quantified by Ultimate Summit HPLC equipped with a Shodex RI-detector. Separation was performed in a Phenomenex Rezex ROA column at 80 °C with 5 mM H_2SO_4 as eluent at flow rate 0.6 ml/min. Calibration was performed with standard solutions of ethanol, D-glucose, L-arabinose, D-xylose, and D-cellobiose [16].

Calculation of K_D

Enzyme activity and stability is influenced by temperature, substrate, ethanol content, and PEG. Denaturation of an enzyme and thereby loss of residual activity can be modeled as an exponential decay (Fig. 2). The denaturation constant K_D is the rate constant describing the loss of activity. Calculation of K_D is given by Eq. (1), where A is residual activity at time t and A_0 is initial residual activity. Activity was normalized to 100 % initial activity in all experiments, i.e., A_0 is 100 %. In addition, K_D can be used for calculation of $T_{1/2}$ as given by Eq. (2).

$$A = A_0 e^{-t \cdot K_D} \quad (1)$$

$$T_{1/2} = \frac{\ln(2)}{K_D} \quad (2)$$

Results and discussion

Cellulase activity and protein content

During the stability studies, enzymes were dosed based on their protein content, hence enzyme activities in Table 1 are given as activity per mg EP determined at 50 °C. Filter paper activity (FPA) was similar for CNmix and Thermomix even though the specific activities of BG and CBH were remarkably higher for Thermomix. Besides cellulases, the enzyme mixtures (especially CNmix) were expected to contain smaller quantities of other auxiliary enzyme activities, which possibly work synergistically with the cellulases to hydrolyze cellulose in the filter paper assay [11]. In addition, the specific activities were measured at 50 °C, which is likely not the optimum-temperature for the thermostable enzymes.

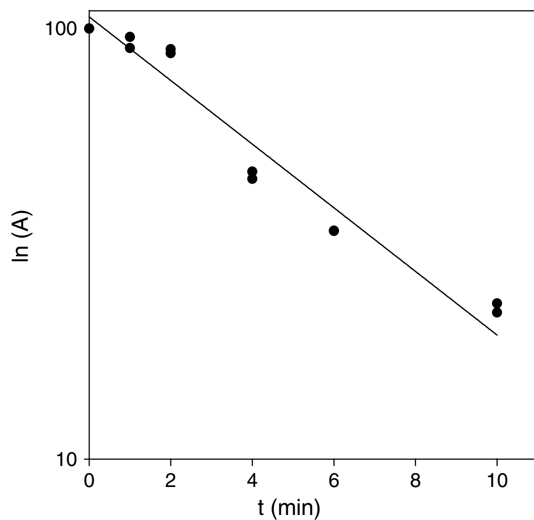

Fig. 2 Residual cellulase activity of CNmix at 65 °C and 2.5 % ethanol (w/v), where $\ln(A)$ is the residual activity at time t (in minutes). The exponential decay is given by $\ln(A) = \ln(A_0) + 0.170 \cdot t$, $R^2 = 0.94$, hence K_D is 0.170

Composition of fiber beer

Fiber beer, the residue after hydrolyzing and fermenting wheat straw, was obtained from a demonstration plant running in Denmark. It contained very fine particles, which caused problems when analyzing composition and enzyme activities. Results showed that no residual activity was found in the fiber beer from the production because the fiber beer was heat-treated before a fresh portion of enzyme was added. A detailed analysis of fiber beer from the same plant after distillation, using similar biomass and process conditions as in this study, revealed that the solid fraction consisted of 71 % Klason lignin and 15 % ash [8]. The solid fraction of the fiber beer used in this study was therefore expected to contain mainly Klason lignin and only minor amounts of carbohydrates. Glucose was especially expected to be low, as it was converted to ethanol in the fermentation. The yeast strain applied did not utilize xylose and hence some xylose was present in the fiber beer. The high lignin content could be suspected to reduce enzyme recovery as enzyme adsorption onto lignin is a well-known phenomenon [22]. Therefore, to gain reliable results in this study, the activity of enzyme mixed with fiber beer was used as the blank in the fiber beer studies.

Cellulase stability in a pilot stripper

The stability of CNmix and Thermomix was tested in a pilot stripper working with heat and mass transfer between gas and liquid (droplets) and between liquid and the heating wall under conditions identical to industrial conditions. In addition, the operating temperature was close to the temperature limit of their stability.

For CNmix the effect of temperature was tested at up to 60 °C and for Thermomix up to 65 °C (Table 2), which previously was shown to be their upper limit for stability [25]. To study the effect of fiber beer, control experiments with both CNmix and Thermomix were conducted in buffer only. Fiber beer contained 2.5 % ethanol (w/v) while buffer was without ethanol. The experiments were not run for more than 10 min, since the residence time in an industrial stripper section will be around 5 min for fiber beer with 5 % (w/v) ethanol (pers. comm., B. Holm Christensen, Biosystemer Aps, Denmark).

Table 1 Enzyme activity and protein content of CNmix and Thermomix per gram EP

	FPA (FPA/mg EP)	β-glucosidase (nkat/mg EP)	Endoglucanase (nkat/mg EP)	Cellobiohydrolase (nkat/mg EP)	Protein (mg EP/g)
CNmix	0.66	6.34	94.4	1,432	151 ± 1
Thermomix	0.64	16.2	87.5	2,232	172 ± 2

Data is taken from Skovgaard and Jørgensen [25]

Table 2 Influence of substrate (fiber beer or buffer) and temperature on enzyme activity based on K_D values and $T_{½}$

Substrate	Scale	Temp. (°C)	Ethanol (%)	K_D	$T_{½}$	R^2
CNmix						
Fiber beer	10 l	55	2.5	0.025 ± 0.011	28	0.32
Fiber beer	10 l	60	2.5	0.115 ± 0.032	6	0.64
Buffer	10 l	55	0.0	0.115 ± 0.010	6	0.95
Buffer[a]	1 ml	55	0.0	0.009 ± 0.002	77	0.47
Thermomix						
Fiber beer	10 l	55	2.5	0.015 ± 0.003	46	0.98
Fiber beer	10 l	60	2.5	0.050 ± 0.021	14	0.52
Fiber beer	10 l	65	2.5	0.404 ± 0.109	2	0.72
Buffer	10 l	60	0.0	0.091 ± 0.027	8	0.58
Buffer[a]	1 ml	60	0.0	0.047 ± 0.004	15	0.91

Enzyme dosage was 2 mg EP/ml substrate. CNmix mixed with fiber beer was tested at 55 and 60 °C and Thermomix mixed with fiber beer was tested at 55–65 °C. Ethanol content is given as % (w/v) and $T_{½}$ is in minutes

[a] Data taken from Skovgaard and Jørgensen [25]

In fiber beer, the denaturation constant (K_D) of CNmix at 55 and 60 °C was approximately twice as high as observed for Thermomix at these temperatures, which could also be expected for thermostable enzymes (Table 2). Going from 55 to 60 °C reduced $T_{½}$ nearly fivefold for CNmix, but only threefold for Thermomix. As expected, the experiments in fiber beer indicated that when operating at these temperatures close to the limit of the enzymes, even small deviations in temperature had clear impact on potential recovery of enzyme activity. This imposes that careful control of operation conditions is critical. If the residence time in the stripper section is expected to be 5 min and enzyme recovery should be successful, a temperature of 60 °C would be critical for especially CNmix with a $T_{½}$ of 6 min, whereas it would be more acceptable for Thermomix ($T_{½} = 14$ min). However, if the temperature in the case of Thermomix was increased to 65 °C, recovery would be reduced remarkably as $T_{½}$ at this temperature only was 2 min. Generally, CNmix was less stable than Thermomix, which has also been observed when testing enzyme stability in buffer [25].

Enzyme stability was also tested in buffer at similar temperatures, where $T_{½}$ was lower for both enzyme mixtures compared to the $T_{½}$ observed at the same temperature in fiber beer (Table 2). The difference indicates that fiber beer contained one or more components, which functioned to stabilize the enzymes. During tests in the pilot stripper, the enzyme mixtures were exposed to gas–liquid interactions when the stillage hit the heat transmission wall. Earlier studies of the enzyme mixtures in buffer, without being exposed to gas–liquid interactions, showed that when CNmix and Thermomix were heated to 55 and 60 °C, respectively, their activity was close to 100 % even after 10 min at these temperatures [25]. Hence, the mixing and the contact between the heat transmission wall and the fiber affect the enzyme stability in the pilot stripper.

The principle behind this novel pilot stripper is the generation of droplets, which provides a large interface for heat and mass transfer on the surface of the droplets. Furthermore, a rapid evaporation occurs when the liquid and enzymes get into contact with the heat transmission wall where gas bubbles are produced thus exposing the enzymes to large gas–liquid interfaces, which are known to be detrimental to the activity of enzymes [1]. $T_{½}$ for CNmix and Thermomix in buffer without exposure to gas–liquid interfaces was in all cases higher than the values obtained in the stripper (Table 2). $T_{½}$ for CNmix at 55 °C in buffer increased nearly 13 times (from 6 to 77 min) when changing the condition from rapid mixing and exposure to liquid–gas interfaces to conditions with no mixing. The same tendency was observed for Thermomix at 60 °C, where $T_{½}$ went from 8 to 15 min when no mixing occurred. That Thermomix seemed less influenced by gas–liquid interfaces and tough mixing supports the theory that thermostable enzymes generally are more stable when it comes to temperature as well as exposure to external forces.

Recent studies have shown that gas–liquid interfaces and shear forces have a negative impact on the activity of enzymes from *T. reesei* [6, 14]. Activity was reduced when cellulases from *T. reesei* were exposed to increased shear or temperature, where mainly cellobiohydrolase activity decreased, while EG and BG activity remained stable [6, 25]. In this study, a reduction in CBHI and EGI activity was observed for CNmix, which mainly contain cellulases from *T. reesei*, when in buffer, while no changes were observed for the specific enzymes when in fiber beer

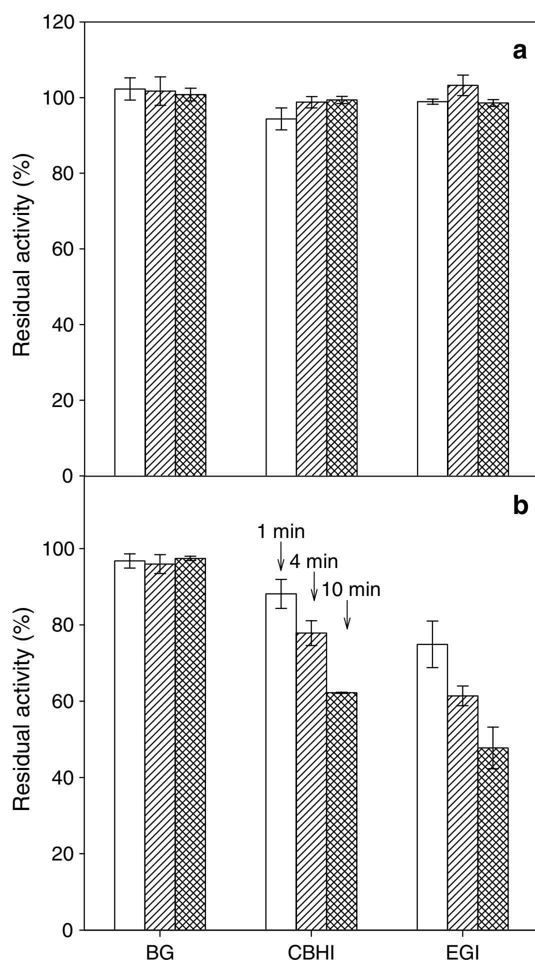

Fig. 3 Residual BG, CBHI, and EGI activity of CNmix in (**a**) fiber beer and (**b**) buffer at 55 °C in the novel pilot stripper. For fiber beer, the whole slurry was used for enzyme activity measurements. Fiber beer contained 2.5 % ethanol and buffer 0 % ethanol. (*white*) 1 min, (*striped*) 4 min, (*cross*) 10 min

(Fig. 3). That activity of EGI was reduced in buffer did not mean that the other endoglucanases (e.g., EGII) in CNmix also dropped, which could explain why other studies have shown that endoglucanases are stable at this temperature (55 °C). Activities in Thermomix were not measured as it only contains EGII and because both Thermomix and CNmix showed good stability of EG but less stability of cellobiohydrolases when tested in buffer with no shear [25]. In fiber beer, the specific enzymes remained stable during the 10 min run, while CBHI and EGI activity decreased in buffer. After 10 min in buffer, the residual activity of CBHI and EGI was 62 ± 0 and 48 ± 6 %, respectively (Fig. 3b). The differences in residual activity in fiber beer and buffer could be due to attachment of enzymes to lignin (or other compounds) or binding to PEG, which probably reduce protein denaturation and degradation in the gas–liquid interface [1].

The effect of fiber beer when exposing enzymes to high temperature and ethanol

The large-scale experiments were conducted at a fixed ethanol concentration of 2.5 % (w/v). However, for the ethanol production process to be economically feasible, the ethanol concentration before distillation should preferably be at least 4 % (w/v) [17]. Previously, the effect of temperature and ethanol on stability of CNmix and Thermomix was investigated in small-scale tests in buffer [25]. To get more information about stability of the cellulases in fiber beer, the effect of temperature and ethanol on CNmix and Thermomix was further tested in a small-scale lab setup (Table 3). The fiber beer batch was the same as used in the pilot studies and therefore contained PEG.

At 55 °C, CNmix generally performed better than Thermomix when the ethanol content was increased to 7.5 % (w/v), indicating that at low temperatures, CNmix was more tolerant to ethanol than Thermomix. At 65 °C and 2.5 % ethanol, $T_{1/2}$ for CNmix was 4 min, which was half of that of Thermomix ($T_{1/2} = 8$ min). Increasing the ethanol content to 7.5 % at 65 °C resulted in similar $T_{1/2}$ (4 min) of the two enzyme mixtures, which could indicate that the stability of the mixtures were about the same at these extreme conditions. However, $T_{1/2}$ for CNmix remained unchanged when going from 2.5 to 7.5 % ethanol, which could be because the denaturation rate at 65 °C mainly was influenced by the high temperature and not the ethanol content. As $T_{1/2}$ is based on the sum of enzyme activities in the mixture, it is not possible to clarify the enzyme(s) contributing to the activity. It could therefore be speculated that one or few enzymes maintain their activity, while the other enzymes denature being exposed to high temperature. For Thermomix, $T_{1/2}$ was halved when increasing the ethanol content, which means that at 65 °C the stability and activity of this mixture is not only influenced by temperature, but also ethanol content. Ethanol with its solvent properties is known to influence the activity of enzymes by being a non-competitive inhibitor, which causes reversible denaturation [10]. Degradation of cellulases from CNmix and Thermomix at even low concentration (<5 % w/v) was recently observed when exposed to ethanol and increasing temperatures in buffer [25, 26]. However, in those studies it was also observed that Thermomix performed better than CNmix when exposed to the combined effect of high temperature and increased ethanol concentration. Considering cellulases for recycling after distillation, one should not only be aware of the temperature, but also the initial concentration of ethanol as both factors influence the efficiency of recovery of active enzymes. If transferring these data to the pilot stripper, increasing the ethanol content from 2.5 % to more than 7.5 % would clearly influence the recovery of enzyme activity. However, in an industrial distillation setup,

Table 3 Influence of ethanol content, PEG, and gas–liquid interfaces (shear) on enzyme activity in fiber beer at 55 and 65 °C

Scale	Ethanol (%)	PEG	Shear	55 °C			65 °C		
				K_D	$T_{½}$	R^2	K_D	$T_{½}$	R^2
CNmix									
10 l	2.5	+	+	0.025 ± 0.011	28	0.32	n.d.	n.d.	n.d.
1 ml	2.5	+	−	0.000 ± 0.003	n.d.	n.d.	0.170 ± 0.018	4	0.94
1 ml	7.5	+	−	0.019 ± 0.002	36	0.87	0.176 ± 0.015	4	0.98
1 ml	7.5	−	−	n.d.	n.d.	n.d.	0.276 ± 0.014	3	0.99
Thermomix									
10 l	2.5	+	+	0.015 ± 0.003	46	0.98	0.404 ± 0.109	2	0.72
1 ml	2.5	+	−	0.000 ± 0.002	n.d.	n.d.	0.090 ± 0.012	8	0.89
1 ml	7.5	+	−	0.038 ± 0.011	18	0.56	0.180 ± 0.019	4	0.95
1 ml	7.5	−	−	n.d.	n.d.	n.d.	0.345 ± 0.065	2	0.92

Calculations are based on K_D values and $T_{½}$. Enzyme dosage was 2 mg EP/ml substrate and PEG dosage was 0.01 g/g DM. The gas–liquid interface was caused by mixing at 600 rpm, which resulted in fiber beer quickly smashing into the heat transmission wall, where the quick gas–liquid interface occurred. For CNmix, no data was available for fiber beer at 65 °C since this enzyme mixture was shown to degrade quickly at this temperature [25]. Ethanol content is given as % (w/v) and $T_{½}$ is in minutes

n.d. Not determined

the stripper is connected to a rectifier where ethanol rapidly is vaporized and separated from the fiber beer. Therefore, the negative effect of high incoming ethanol concentration would be less severe compared to these lab-scale studies where ethanol concentration remained constant during the entire test period. This also means that temperature would be the main limiting factor.

Another interesting observation when comparing small-scale (1 ml) experiments and large-scale (10 l) experiments was $T_{½}$ of CNmix and Thermomix at 55 °C and 2.5 % ethanol (Table 3). In small scale, K_D was close to 0.0 for both enzyme mixtures, i.e., no denaturation occurred. When testing the fiber beer in the pilot stripper under the same condition (55 °C and 2.5 % ethanol), K_D increased resulting in a $T_{½}$ of 28 and 46 min for CNmix and Thermomix, respectively. One likely reason for the changed $T_{½}$ in the pilot setup could be the quick expansion of gas–liquid interfaces during stripping, as discussed in the previous section. Combining gas–liquid interfaces with high temperature enhanced inactivation of the enzymes, and should therefore be taken into consideration when designing a diabetic industrial stripper like the one used in these experiments.

The effect of PEG during distillation

The fiber beer used in the pilot stripper was produced at a demonstration facility where PEG (molar mass 6,000 Da) is routinely added in the beginning of hydrolysis (typically 0.01 g/g DM). The addition of PEG is known to improve the overall enzymatic conversion in the process. In addition, PEG has been shown to reduce protein precipitation when incubating enzymes in buffer without substrate [4]. One hypothesis could therefore be that one of the factors resulting in the higher stability of enzymes in the fiber beer compared to the buffer experiments is the presence of PEG in the fiber beer. A batch of fiber beer with or without PEG was used for comparison of the stability of CNmix and Thermomix when the enzymes were exposed to high temperature (65 °C) and ethanol concentration (7.5 % w/v) (Table 3). At these extreme conditions, PEG had a very small effect on enzyme stability, as $T_{½}$ only was reduced from 4 to 3 min for CNmix and 4–2 min for Thermomix, when it was removed from the fiber beer. The high temperature was therefore likely the reason why $T_{½}$ remained nearly unchanged when PEG was added. Studies with spray drying, where proteins are exposed to large gas–liquid interfaces at high temperatures, have shown a stabilizing effect on proteins when using PEG and Tween80 [21, 23]. It could therefore be speculated that the positive effect of PEG could be more pronounced at lower temperatures when used in this pilot stripper since the stability of enzymes at this point will be less influenced by temperature.

PEG is known to reduce the adsorption of enzyme protein to lignin. Lignin adsorbs enzymes through hydrophobic interactions, which results in less efficient hydrolysis of biomass and enzyme recycling [28]. To recover the enzymes from lignin, which is part of the insoluble solid fraction, either the solid fraction should be reused or the enzymes be desorbed to a liquid fraction, which then can be recycled. Recycling of the insoluble solid fraction at industrial conditions was recently shown to be successful with up to 30 % enzyme dosage reduction [32]. Another technique is to desorb the enzymes from the solid fraction by washing with buffer, which could contain surfactants,

and afterwards filtrating the liquid fraction [9, 32]. However, even though the enzymes can be recovered and desorbed from lignin it does not mean that all the enzymes in the mixture is recovered successfully. This and other studies indicate that especially cellobiohydrolases are sensitive to high temperatures [25]. In addition, β-glucosidases in the newer commercial enzyme mixtures was recently shown to adsorb to the biomass surface instead of being unattached in the liquid fraction, which else has been the general assumption [9].

The differences in stability and activity of the enzyme mixtures indicated that Thermomix generally performed better when exposed to high temperature, ethanol and gas–liquid interfaces (Tables 2, 3). However, the $T_{½}$ values are probably higher than what could be expected in a real ethanol pilot plant. Before the fiber beer was used for these experiments, it was heat treated to inactivate the residual enzyme activity from the bioethanol process, where after fresh enzyme mixture was added. If CNmix or Thermomix were to be used in the full bioethanol process one could speculate that the stability of the mixtures generally were lower when continuing to the distillation. At this point, the enzymes had already been exposed to varying temperature during pre-hydrolysis and SSF, and in addition been mixed and tumbled for several days. Therefore, the residual activity of CNmix and Thermomix would probably be lower in a real process compared to what was tested here.

Conclusions

By testing the stability of CNmix and Thermomix in fiber beer and buffer in a novel pilot stripping section, it was seen that (1) Thermomix was indeed more resistant to high temperatures than CNmix, but more sensitive to an increasing ethanol concentration, (2) fiber beer contained substances that improved the stability of the enzymes when exposed to heat, ethanol, and gas–liquid interfaces, and (3) the formation of gas–liquid interfaces, introduced on the enzymes in the pilot scale unit, had a detrimental effect on stability of the cellulases. Despite the negative effect of the gas–liquid interfaces in the pilot scale unit, the ability to operate at lower temperatures enables high enzyme recovery.

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