S. Malfliet, L. De Cooman and G. Aerts

Application of Thermostable Xylanases at Mashing and During Germination

For the evaluation of the intrinsic lautering performance of malt an in-house test, predictive for both lauter tun and mash filter operations, has been used. Commercial thermostable endoxylanases added during mashing differ in their ability to increase the filtration rate. Especially the addition of a family 10 endoxylanase at mashing-in can be very efficient in view of wort filtration. Family 10 endoxylanases degrade barley arabinoxylans in an effective way and are not inhibited by the *Hordeum vulgare* L. xylanase inhibitor (HVXI) purified from barley. Moreover, the addition of this family 10 endoxylanase during germination has a positive effect on the filtration capacity of the kilned malt because the added endoxylanase activity is only partly destroyed during kilning. Furthermore, addition of endoxylanases during germination appears to have no effect on standard malt quality characteristics.

Descriptors: endoxylanase activity, exogenous xylanases, filtration performance

1 Introduction

Mash filtration is considered as the rate-limiting step in wort production and, therefore, represents a factor of increasing economic importance. Until today, several parameters that possibly influence mash filtration behaviour are estimated by conventional malt analyses. Factors causing reduced rate of lautering (*e.g.* viscosity) and thus increased processing time are still poorly characterised [5, 7, 10, 18, 20, 27]. The conventional EBC analysis does not provide sufficient information on malt in terms of mash filtration behaviour [19, 20].

Historically, more research has been done on β-glucans than on arabinoxylans in regard to wort filtration problems. However, nowadays, it has been observed that the impact of arabinoxylans on viscosity and filterability is at least as important as that of β-glucans [3, 9, 14, 21, 25]. In particular higher mashing-in temperatures, relevant to brewing circumstances, increase the amount of arabinoxylans released into the wort, resulting in a higher wort viscosity and low rates of wort separation [17].

During malting, enzymes that hydrolyse arabinoxylans are produced late in the germination process, the maximum of endoxylanase activity occurring only after 72 hours of germination [4, 16, 24]. On the other hand, Kuntz and Bamforth showed the development of xylanases during steeping of barley and early in germination [3, 13]. During malting, some arabinoxylans are solubilised from the cell walls but are not extensively degraded by endogenous xylanases [15]. During the mashing process, because of the restricted temperature stability of endogenous xylanases [15], the addition of heat-stable exogenous xylanases was found to be an effective strategy to degrade arabinoxylans, resulting in an increased filtration rate and a reduced mash viscosity [1, 22].

An optimal cell wall degradation during malting, in combination with the presence of more thermostable xylanases in the kilned malt can increase the filtration rate. Therefore, since incomplete degradation of the endosperm cell walls also reduces the yield of extract [17], thermostable xylanases were added in this study at mashing-in or during germination in order to investigate their effect on the filtration behaviour of the kilned malt.

2 Materials and methods

2.1 Malt samples

Two very different malt samples produced on an industrial scale, were used during this study. Malt A (variety Sebastian, 2009, French harvest) is an evenly well modified 2-row spring barley malt, and malt B (variety Cervoise, 2009, French harvest) is an undermodified 6-row winter barley malt with a relatively high percentage of whole unmodified grains. The analytical data of both malt samples are listed in table 1.

2.2 Malt analysis

The malt samples were characterised according to the analytical methods of Analytica EBC [8] for moisture content (4.2), extract (4.5.1), total protein (4.3.1), soluble protein (4.9.1), friability (4.15), partly unmodified grains (4.15), whole unmodified grains (4.15), wort viscosity (4.8), wort colour (4.18) and wort pH (8.17).

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Tables and figures see Appendix

The in-house filtration test, introduced by a commercial malting plant, was carried out via a modified congress mashing procedure. Fifty grams of malt were milled using a coarse grind (1.5 mm) and two hundred mL of water at 62 °C were added to the grist. The mash was continuously stirred (100 rpm) and the temperature was maintained at 62 °C for 115 min (according to the mashing time of an EBC Congress mash). Whereas in the EBC Congress procedure the mash is filtered at 20 °C, in this test the mash is filtered at 62 °C. The filtration rate of the mashes was determined by transferring the mashed samples into a fluted filter paper (Whatman 597 ½). After recirculation of the initial wort (about 10 mL), the filtrate was collected. The volume of the filtrate was measured every 5 min during the first quarter and then every quarter of an hour.

2.3 Enzyme assays

Endoxylanase activities were determined in two different commercially available enzyme preparations (Enzyme 1 and Enzyme 2) using the colorimetric Xylazyme AX method (Megazyme T-XAX200 10/2008 data booklet), with some adaptations in which xylanase preparations were diluted in maleate buffer (100 mM, pH 5.6) and Xylazyme AX tablets were added after a pre-incubation of 5 min at 40 °C or 62 °C. The suspensions were incubated for exactly 10 min at 40 °C or 62 °C and the reaction was stopped by the addition of 10.0 mL Trizma base solution (2.0 % w/v, pH 9.0) and vigorous vortex-mixing. After centrifugation, $A_{590\,nm}$ of the supernatant was measured against a control, prepared by incubating Xylazyme AX tablets in buffer without enzyme addition. All measurements were performed in triplicate. One xylanase unit is the amount of enzyme required to release one micromole of reducing sugar equivalent (xylose) from arabinoxylan per minute under the conditions of the assay.

Endoxylanase activities were also determined after 1 day, 2 days and 5 days of germination and in kilned malt using the same colorimetric Xylazyme AX method. 10.0 g of (finely milled) barley or malt sample were extracted during 30 min in 25.0 mL maleate buffer (100 mM, pH 5.6) at room temperature. After centrifugation, Xylazyme AX tablets were added to the supernatant after a pre-incubation of 5 min at 40 °C. The suspensions were incubated for 60 min at 40 °C and the reaction was stopped by the addition of 10.0 mL Trizma base solution (2.0 % w/v, pH 9.0) and vigorous vortex-mixing. After centrifugation, $A_{590\,nm}$ of the supernatant was measured against a control, prepared by adding Trizma base solution before the Xylazyme AX tablet.

2.4 Micro malting operations

A 6-row winter barley (variety Cervoise, 2009, French harvest) was malted at a 1 kg scale. Steeping was carried out by immersion (wet stage: 7 h at 18 °C; dry stage: 17 h at 20 °C; wet stage: 1 h at 18 °C). Germination was performed in cylindrical drums at a temperature of 20 °C with continuous moistening of air during the first 2 days and at 18 °C with moistening of air for 1 min every 3 min during the next 3 days. The drums were slowly rotated for 1 min every 7 min. The germinated barley was kilned using three temperature steps (6 h at 55 °C, 9 h at 60 °C and 5 h at 80 °C).

3 Results and discussion

3.1 Evaluation of the malt samples

The malt originating from a 2-row spring barley (malt A) and the malt originating from a 6-row winter barley (malt B) were evaluated for malt quality characteristics according to Analytica EBC [8] (see Table 1). Malt B has a lower friability and higher concentration of partly and whole unmodified grains, indicating a lower degree of modification. Furthermore, malt B gives a lower extract, a higher total and soluble protein content and a higher wort viscosity and wort colour.

The filtration behaviour of malt was measured by the in-house filtration test (Fig. 1). All measurements were performed five times and standard deviations are indicated in the graph. Since mashing-in conditions have changed in industrial brewhouses, insufficient degradation of the non-starch polysaccharides (β-glucans and arabinoxylans) may result in wort filtration problems. With the modified congress mashing procedure of the in-house filtration test, endogenous β-glucanases and xylanases are inactivated because of the higher mashing-in temperature of 62 °C. This mashing procedure is relevant to current brewing practice and reveals differences in the intrinsic lautering performance between both malts tested. Malt A has a significantly higher filtration rate in comparison with the filtration rate of malt B (after 1 h of filtration: Student's t, p-value < 0.0005).

3.2 Addition of thermostable xylanases during mashing

Two different commercial xylanase preparations (Enzyme 1 and Enzyme 2), marketed for their ability to improve filterability of brewers' mashes, were investigated in the present study. They differ widely in their endoxylanase activity at 40 °C and 62 °C as demonstrated in table 2 and contain almost no endo-β-glucanase activity (data not shown). Endogenous endoxylanases have the highest activity around 40 °C. The temperature optimum of both xylanase preparations is above 60 °C. The effect of the addition of both enzyme preparations at the start of the mashing process was investigated. Therefore, both xylanases were added at the mashing-in stage of the in-house filtration test. The addition of 50 µL of Enzyme 1 to 50 g malt (corresponding with 170 U at 62 °C) results in a clear increase in the filtration rate in case of the spring barley malt A (after 1 h of filtration: Student's t, p-value < 0.001) as well as in case of the winter barley malt B (after 1 h of filtration: Student's t, p-value < 0.0005) (Fig. 2). Also Enzyme 2 was added at the onset of mashing in different volumes: 10 µL, 25 µL, 50 µL, and 100 µL/50 g malt, respectively (corresponding to 5.6 U, 14 U, 28 U, and 56 U at 62°C respectively). For malt A, the addition of 5.6 U of Enzyme 2 (Fig. 3), already resulted in the same high filtration rate as in case of the addition of 170 U of Enzyme 1 (Fig. 2). This indicates that the lowest addition of Enzyme 2 is already sufficient for a large improvement of the filtration rate of malt A. Through addition of 28 U of Enzyme 2 at mashing-in of malt B (Fig. 4), it is even possible to increase the filtration rate of malt B above the initial filtration rate of malt A without added xylanases (see Fig. 3). Moreover, the addition of only 5.6 U of Enzyme 2 to a mash with malt B also resulted in a high filtration rate (see Fig. 4). The much higher effect of En-

zyme 2 on the filtration behaviour in comparison with Enzyme 1 can be explained by the so-called "family" to which the xylanases belong. Enzyme 1 is a family 11 endoxylanase, while Enzyme 2 is a family 10 endoxylanase. Family 10 endoxylanases produce smaller hydrolysis products from arabinoxylans than family 11 endoxylanases. Family 10 endoxylanases can attack the glycosidic linkage next to a single or double substituted xylose with arabinose [26]. Family 11 endoxylanases show no detectable activity on xylobiose and xylotriose and have only a very low activity on xylotetraose. They preferably cleave in unsubstituted regions of the backbone and require, in contrast to family 10 endoxylanases, three unsubstituted consecutive xylose residues for hydrolysis [6, 26].

Next to the very efficient degradation of arabinoxylans by the family 10 endoxylanases, also the xylanase inhibitors found in barley play a crucial role. *Hordeum vulgare* L. xylanase inhibitor (HVXI) has been purified from barley and is homologous with *Triticum aestivum* L. xylanase inhibitor (TAXI), an endoxylanase inhibitor from wheat flour [11]. TAXI-type inhibitors are strong inhibitors of family 11 endoxylanases but show no inhibition activity towards family 10 endoxylanases [12]. All plant endoxylanases identified so far are family 10 endoxylanases [23], which suggests that TAXI-type inhibitors of vegetable origin do not influence cereal metabolic processes as such, but rather microbial-derived endoxylanase activity [12].

3.3 Addition of thermostable xylanases during germination

Enzyme 1 and Enzyme 2 were sprayed on a 6-row winter barley during moisture adjustment at germination in a micro malting plant of 1 kg. 140 U of Enzyme 1/kg barley were added at the onset of the second or the third day of germination. Next, the changes of endoxylanase activity during the malting process were investigated. Figure 5 shows the endoxylanase activities measured during the 'blank' experiment in which no xylanase was added, as well as during the experiments with addition of Enzyme 1 at day 2 and day 3, respectively. The endogenous endoxylanase activity (see 'blank' experiment) is the highest (10.4 U/kg d.m.) after 5 days of germination, which corresponds to findings in literature that endoxylanases appear relatively late in the germination process [4, 16, 24]. After kilning, the endogenous endoxylanase activity in the finished malt decreased to approx. 2/3 of the highest enzyme activity detected after 5 days of germination, which is also in accordance with literature data [16]. The addition of Enzyme 1 at the start of the second or the third day of germination clearly results in a higher endoxylanase activity during subsequent germination in comparison with the malting experiment without added xylanases ('blank'). Interestingly, even after kilning the added exogenous endoxylanase activity is partly preserved.

Figure 6 displays the endoxylanase activities in the germinating barley and in the kilned malt after addition of 100 U of Enzyme 2/kg barley at the onset of the second or the third day of germination. It appears that similar results are obtained as with the Enzyme 1 treatment and, also after kilning, considerable Enzyme 2 activity is still present. Without milling the barley or malt samples, a high part of the endoxylanase activity can be determined (approx. 80 % of total activity; see Fig. 7). The highest part of the endoxylanase activity appears to be present on the kernel and only a negligible part of the endoxylanase activity probably entered the kernel.

The effect of the addition of exogenous xylanases during germination on the subsequent filtration performance of the kilned malt was further evaluated by the in-house filtration test (Fig. 8). Apparently, in this particular experiment, the addition of Enzyme 1 at the start of the second or third day of germination does not improve the wort filtration rate, although Enzyme 1 activity on the artificial Xylazyme AX substrate was detected via the colorimetric Xylazyme AX method in the kilned malt (see Fig. 5). Probably, in these mashing experiments, the amount of Enzyme 1 introduced via the kilned malt, was too low (approx. only 3 U) to detect any noticeable improvement of wort filtration rate.

Conversely, addition of Enzyme 2 during germination clearly gives a significantly better filtration performance of the kilned malt, independent of the day of germination on which the enzyme preparation was added (after 1 h of filtration: Student's t, p-value < 0.05). As mentioned before, Enzyme 2 belongs to family 10 endoxylanases, known for their very efficient degradation capacity towards arabinoxylans, without possible interference of the xylanase inhibitor (HVXI) present in barley. Differences in standard malt quality characteristics (e.g. extract, protein, degree of modification, etc.) between malt with or without the addition of Enzyme 2 during germination appear to be negligible (see Table 3). Therefore, application of thermostable, family 10, xylanases seems to be very promising in view of accelerated wort filtration, higher productivity, and improved beer flavour quality, related to lower heat load during the mashing process [2].

4 Conclusions

In this study, the in-house filtration test reveals differences in the intrinsic lautering performance of kilned malt.

The addition of exogenous, thermostable xylanases during mashing is highly efficient in view of wort filtration rate, especially when family 10 endoxylanases are applied. Family 10 endoxylanases degrade arabinoxylans very effectively and they are not inhibited by the endogenous *Hordeum vulgare* L. xylanase inhibitor (HVXI).

Besides direct application in mashing, the addition of exogenous family 10 endoxylanase activity during barley germination also results in improved wort filtration performance of the malt. In summary, introducing thermostable xylanase activity in the malting/brewing process shows high potential in respect of more efficient wort production and enhanced beer flavour quality, including flavour stability. Both properties are related to lower heat load. Future research will therefore be focused on heat stable, family 10 xylanase producing microorganisms in malting.

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6 Literature

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Appendix

Table 1 **Malt quality characteristics of the 2-row spring barley malt A and the 6-row winter barley malt B**

	Malt A	Malt B
Moisture (%)	4.3	4.8
Extract (%)	84.1	80.0
Total protein (%)	9.8	10.6
Soluble protein (%)	3.6	4.3
Kolbach index (%)	36.7	40.6
Friability (%)	90.6	80.0
Partly unmodified grains (%)	1.6	5.0
Whole unmodified grains (%)	0.8	3.5
Wort viscosity (mPas)	1.53	1.66
Wort colour (EBC)	3.5	5.0
Wort pH	6.01	6.13

Table 2 **Endoxylanase activity (U/ml) at 40 °C and 62 °C in 2 commercial xylanase preparations**

	40 °C	62 °C
Enzyme 1	1,150	3,400
Enzyme 2	120	560

Table 3 **Malt quality characteristics of the malt without and with the addition of Enzyme 2 during germination**

	blank	addition of Enzyme 2	
		at onset germination day 2	at onset germination day 3
Moisture (%)	3.6	3.5	3.6
Extract (%)	80.3	80.1	80.5
Total protein (%)	10.7	10.7	10.6
Soluble protein (%)	4.1	4.1	4.2
Kolbach index (%)	38.3	38.3	39.6
Friability (%)	89.4	88.5	91.5
Partly unmodified grains (%)	1.6	0.8	0.8
Whole unmodified grains (%)	1.0	0.3	0.3
Wort viscosity (mPas)	1.53	1.65	1.47
Wort colour (EBC)	3.5	3.5	3.5
Wort pH	6.09	6.09	6.07

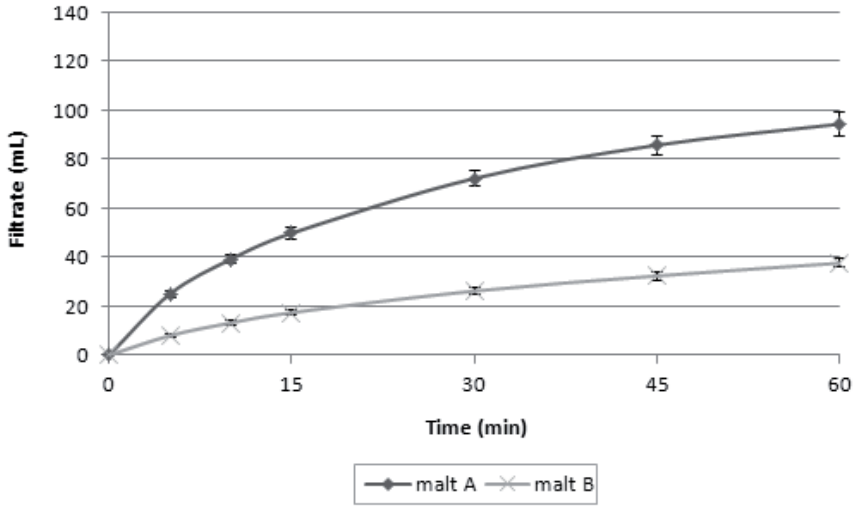

Fig. 1 **Filtration behaviour of malt A and malt B measured by the in-house filtration test (n = 5; error bars = +/– standard deviation (S.D.))**

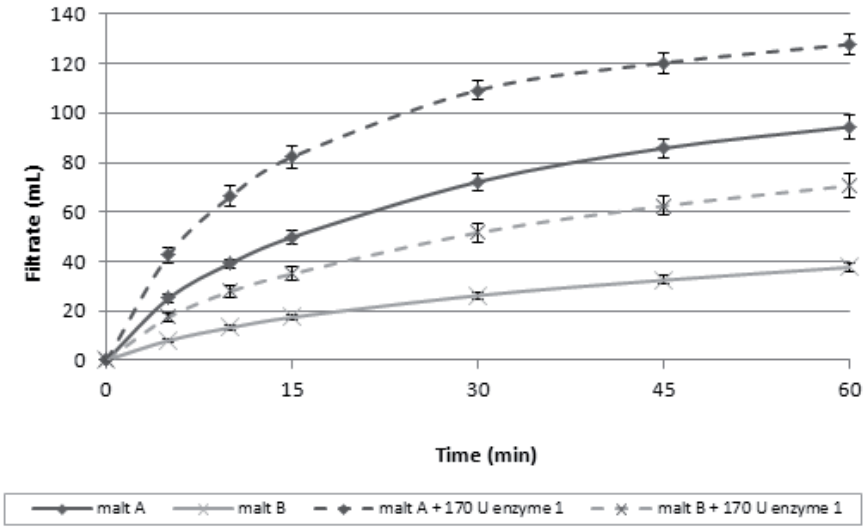

Fig. 2 **Filtration behaviour of malt A and malt B with and without the addition of 170 U of Enzyme 1/50 g malt (n = 5; error bars = +/– S.D.)**

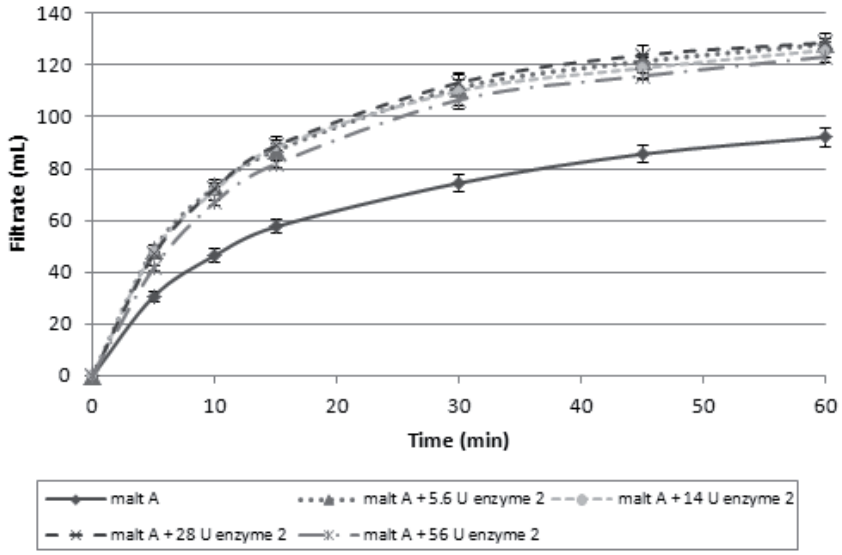

Fig. 3 Filtration behaviour of malt A with and without the addition of 5.6 U, 14 U, 28 U and 56 U of Enzyme 2/50 g malt (n = 3; error bars = +/– S.D.)

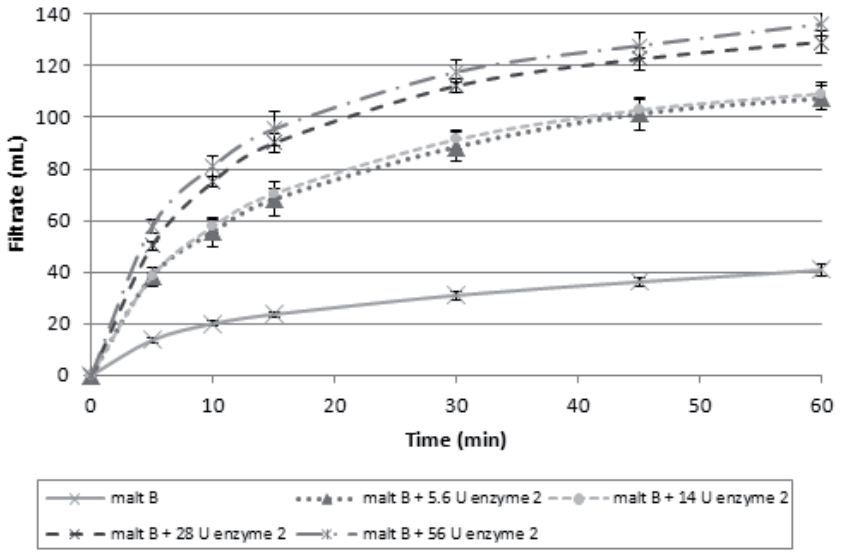

Fig. 4 Filtration behaviour of malt B with and without the addition of 5.6 U, 14 U, 28 U and 56 U of Enzyme 2/50 g malt (n = 3; error bars = +/– S.D.)

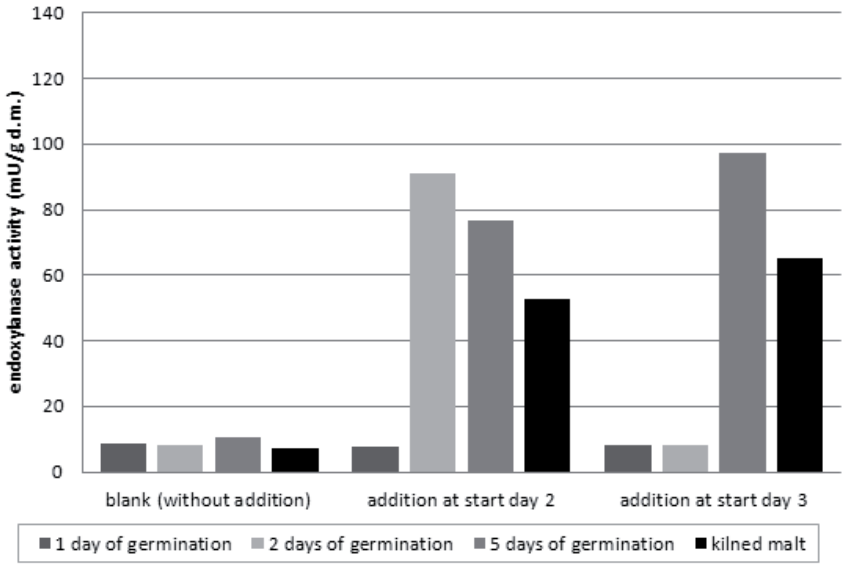

Fig. 5 **Endoxylanase activity during germination and in kilned malt with and without the addition of Enzyme 1 at the onset of the second or the third day of germination**

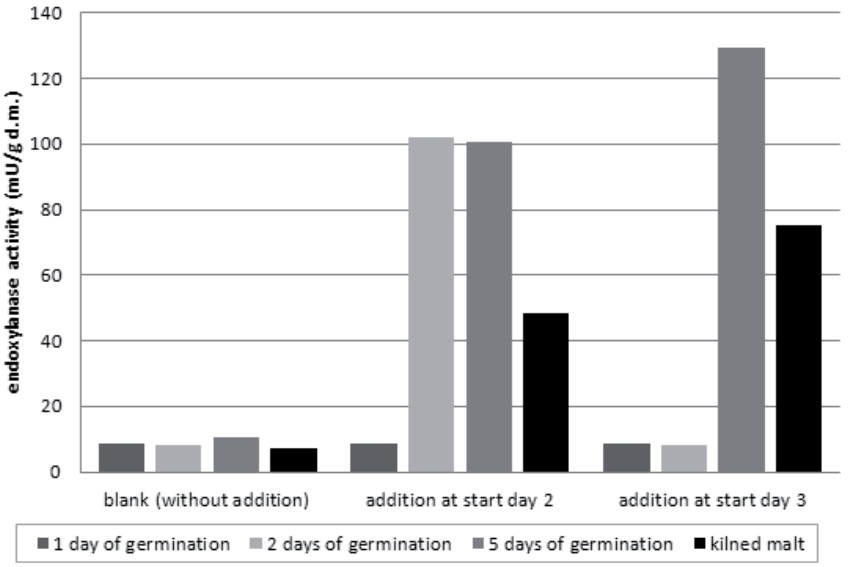

Fig. 6 **Endoxylanase activity during germination and in kilned malt with and without the addition of Enzyme 2 at the onset of the second or the third day of germination**

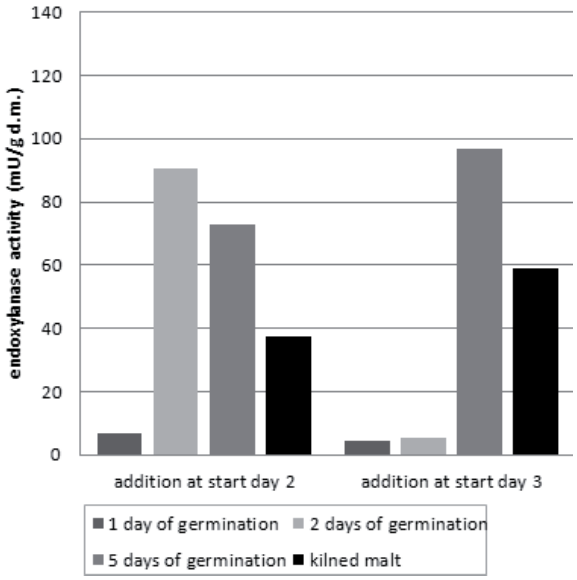

Fig. 7 **Endoxylanase activity during germination and in kilned malt (unmilled samples) with the addition of Enzyme 2 at the onset of the second or the third day of germination**

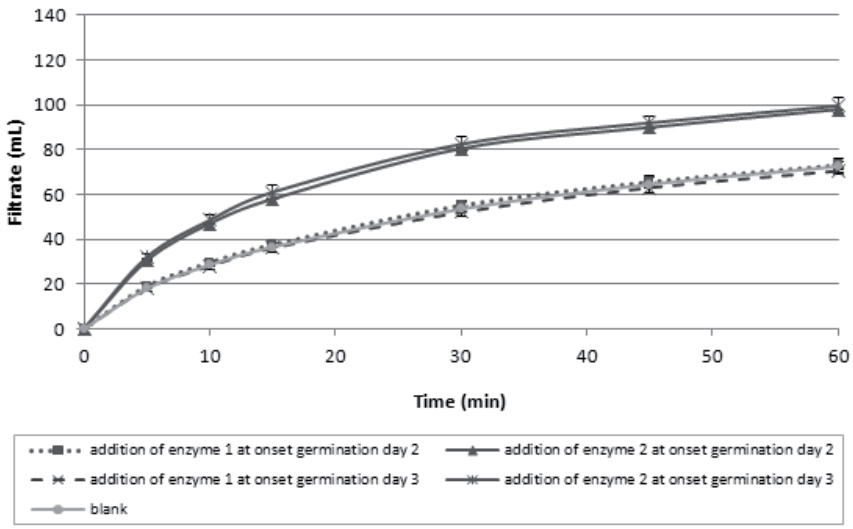

Fig. 8 **Filtration behaviour of a 6-row winter barley malt with and without the addition of Enzyme 1 or Enzyme 2 at the onset of the second or the third day of germination (n = 3; error bars = +/– S.D.)**