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Development of an automatable method for the measurement of *endo*-1,4-β-xylanase activity in barley malt and initial investigation into the relationship between *endo*-1,4-β-xylanase activity and wort viscosity

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ABSTRACT

Stages of the brewing process, such as mash separation to produce wort and beer filtration, can in certain cases prove problematic due to the increased viscosity caused by high levels of the non-starch polysaccharides, primarily β-glucan and arabinoxylan. Of these two polysaccharides, β-glucan has been extensively studied, but arabinoxylan has been somewhat overlooked. The concentration of arabinoxylan present during these process stages is principally expected to be inversely related to the malt *endo*-1,4-β-xylansase activity that is available to degrade these polysaccharides. The development of a novel method for the measurement of *endo*-1,4-β-xylansase activity in barley malt extracts is described herein. The method was characterised by two analysts in terms of repeatability (single analyst CVs = 2.2% and 2.3%, n = 8; interanalyst CV = 4.8%, n = 16) and sensitivity (LOD = 10 U/kg, LOQ = 34 U/kg). The assay procedure was then applied to the measurement of xylanase activity in a series of eight standard barley malts and the results obtained were compared with their associated Congress wort viscosities as measured using the conventional EBC Method 4.8, wort viscosity. A highly statistically significant relationship between xylanase activity and wort viscosity was found with a Pearson's correlation coefficient of −0.82 (*p*-value of 0.007).

1. Introduction

In 2014, *Hordeum vulgare* L. (malting barley) was ranked 4th in cereal grains in terms of global production at 144 million tonnes (Food and Agriculture Organization of the United Nations (FAOSTAT), 2014). The majority of this usage is in the brewing industry where malted barley is one of the key ingredients for the production of beer or whiskey. In barley, the cell walls of the aleurone tissue are constituted primarily of arabinoxylan (Bacic and Stone, 1981) while the starchy endosperm cell walls are composed of ~20% arabinoxylan with ~75% of the remainder being made up of mixed linkage (1,3; 1,4)-β-glucan (Fincher, 1975). Traditionally, the brewing fitration issues associated with β-glucan have been more widely studied in brewing science (Narziss, 1993). However, the importance of arabinoxylan has also been demonstrated previously with respect to production issues such as poor mash separation, beer filterability and haze formation (Barrett et al., 1975; Coote and Kirsop, 1976; Li et al., 2015; Lu and Li, 2006). As *endo*-1,4-β-xylanase (xylanase, EC 3.2.1.8) is known to readily degrade the arabinoxylan present in barley (Debyser et al., 1997; Viëtor et al., 1994), it clearly has the potential to significantly effect both wort composition and also the processing efficiency and quality of the finished beer product.

A number of isoforms of barley xylanase have been purified previously and characterised (Benjavongkulchai and Spencer, 1986; Dashek and Chrispeels, 1977; Kanauchi et al., 2013; Slade et al., 1989). Reported molecular weights range from ~29,000–67,000. Isoelectric points have been observed between 4.6 and 6.5, with a pH optima appearing to vary between ~5.0–6.7. However, all previous studies seem to be in agreement that the predominant forms exhibit a broad pH range with > 75% activity maintained from approximately pH 4.5–7. It had been reported that the largest of these enzymes (Mr = 61500) was an inactive form that needed to be processed by endogenous proteolytic machinery to produce active forms with lower molecular weight (Mr = 34,000 and 41,000) (Caspers et al., 2001), but in fact the larger isoform was later shown to be active following recombinant expression by Van Campenhout et al. (2007), a conclusion that has been

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independently confirmed through conventional protein purification techniques from barley by Kanauchi et al. (2013). Xylanase inhibitors of both TAXI (Goesaert et al., 2001) and XIP (Goesaert et al., 2003) type have been reported in barley but these have been shown only to inhibit microbial xylanases, having no measurable effect on the endogenous xylanases. This aspect has been reviewed in depth by Gusakov (2010).

The measurement of xylanase for cereal quality analysis was traditionally performed using either viscometric (Preece and MacDougall, 1958) or reducing sugar procedures (Benjavongkulchai and Spencer, 1986) until the advent of commercially available azurine dyed and cross-linked (AZCL) wheat arabinoxylan (McCleary, 1992) in the form of Xylazyme tablets (insoluble substrate) which has since found widespread use, particularly for the analysis of crude cereal extracts (Debyser et al., 1997; Fierens et al., 2008, 2007). Cürten et al. (2018), have recently described an 'on-line' xylanase assay involving the use of HPAEC-PAD where the enzymatic incubations take place in a standard autosampler with multiple injections to monitor the reaction time course. The current authors have previously reported the development of a 'soluble substrate' enzyme coupled assay procedure based on a novel colourimetric hexasaccharide substrate, 4,6-*O*-(3-ketobutylidene)-4-nitrophenyl-β-4^5-*O*-D-glucosyl-xylopentaoside, for the measurement of xylanase (XylX6 assay) (Mangan et al., 2017). The development of a new assay format employing this soluble substrate, that is suitable for the automated analysis of xylanase in barley malt extracts, is described herein. The application of this procedure to the measurement of xylanase in a small sample set of barley malts and demonstration of the apparent correlation between these activities and the viscosity of their resulting worts is also described.

2. Material and methods

2.1. Materials

The XylX6 reagent was obtained from Megazyme (K-XylX6-1 V) and contains lyophilised powder to be dissolved as a 5 mL solution of 4,6-*O*-(3-ketobutylidene)-4-nitrophenyl-β-4^5-*O*-glucosyl-xylopentaoside (3.3 mM) and GH43 β-xylosidase from *Selenomonas ruminantium* (20 U/mL) which is sufficient to perform 50 malt xylanase assays under the procedure described here. The control malt flour (lot number 61002A) from the Malt Beta-Glucanase/Lichenase Assay Kit (K-MBG4) was obtained from Megazyme – hereafter referred to as the Control Malt. Eight malt samples of acceptable quality (201402B, 201403A, 201410B, 201503A, 201505B, 201508B, 201510A, 201514A) sourced from Europe in 2013–2015 were obtained from IFBM (Institut Français des Boissons, de La Brasserie et de la Malterie, Nancy, France). These malts were previously subjected to an interlaboratory analysis on 27 parameters following official EBC methods. The analytical data obtained for the 27 malt descriptors was statistically analysed at IFBM and the complete data set is shown in the Supporting Information. All absorbances were recorded using a Shimadzu UV-1800 spectrophotometer. Graphpad Prism version 6.07 was used for all statistical calculations and to produce all figures. All other reagents were purchased from Sigma Aldrich.

2.2. Enzyme unit definitions

One unit of xylanase activity is defined as the amount of enzyme required to release one μmole of 4-nitrophenol per minute under the XylX6 assay conditions described here (Section 2.3.1.6). One unit of β-xylosidase activity is defined as the amount of enzyme required to release one μmole of 4-nitrophenol per minute from 4-nitrophenyl-β-D-xylopyranoside (5 mM) in a suitable buffer at 40 °C at the enzyme's optimum pH of 5.3.

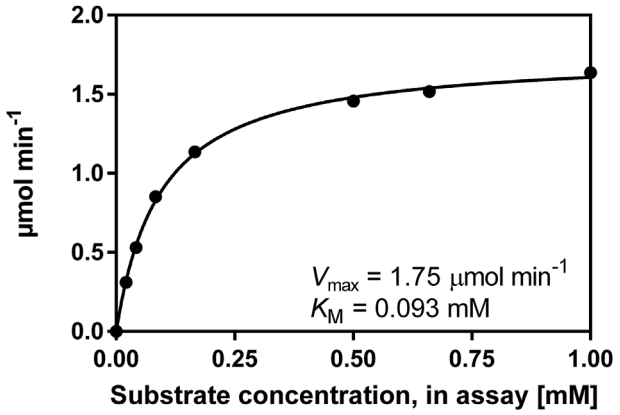

Fig. 1. Kinetic characterisation of a crude xylanase extract obtained from the K-MBG4 control malt flour lot# 61002A using the XylX6 assay format.

2.3. Experimental

2.3.1. Assay development

2.3.1.1. Extraction of barley malt to perform K_M investigation on XylX6 reagent. The Control Malt (5 g) was added to 80 mL of 0.1 M sodium acetate buffer pH 4.6 and the xylanase was allowed to extract at room temperature for 15 min with occasional mixing. The turbid suspension was filtered through glass fiber filter paper to obtain a clear supernatant which was recovered and used immediately.

2.3.1.2. Investigation into K_M of malt xylanase extract on XylX6 reagent (Fig. 1). The effect of substrate concentration on the rate of hydrolysis of XylX6 reagent by malt xylanase was determined by addition of 0.1 mL of 4,6-*O*-(3-ketobutylidene)-4-nitrophenyl-β-4,5-*O*-D-glucosyl-xylopentaoside (6 mM–0.126 mM) in the presence of β-xylosidase (1.7 U) to 0.5 mL of malt extract (prepared as per section 2.3.1.1). The mixture was incubated at 30 °C and the reaction was terminated at fixed time intervals by addition of 0.9 mL 2% w/v Tris solution (pH 10.0). The absorbance was measured at 400 nm. K_M and V_{max} values were calculated using Prism Version 7.03.

2.3.1.3. Investigation into incubation time and temperature on malt xylanase assay (Fig. 2). Aliquots (0.5 mL) of extracts of the Control Malt and Malt 201514A (both prepared in triplicate as per section 2.3.1.1) were incubated with XylX6 reagent (0.1 mL) at 30, 35 and 40 °C and reactions were terminated after 0, 10, 15, 20, 30, 40, 50 and 60 min by addition of 0.9 mL of 2% w/v Tris solution (pH 10.0). Absorbance was measured at 400 nm.

2.3.1.4. Investigation into the optimum pH for the extraction and assay of malt xylanase (Fig. S2, supporting information). Extracts of the Control Malt were prepared in triplicate as described in Section 2.3.1.1 but with a series of 7 different buffer solutions; 0.1 M sodium acetate buffer pH 4.0, 4.5, 5.0 and 5.5 and 0.1 M sodium phosphate buffer pH 6.0, 6.5 and 7.0. Aliquots (0.5 mL) were incubated with XylX6 reagent (0.1 mL) at 30 °C and reactions were terminated after 0, 15 and 30 min by addition of 0.9 mL of 2% w/v Tris solution (pH 10.0). Absorbance was measured at 400 nm.

2.3.1.5. Investigation into the effect of BSA content on the extraction and assay of malt xylanase (Fig. 3). Extracts were prepared in triplicate from the Control Malt as described in Section 2.3.1.1 but with BSA added at 0, 0.5 and 1 mg/mL concentration to 0.1 M sodium acetate buffer and extraction time varied from 15 min to 5 h. Aliquots (0.5 mL) of the extract solutions were incubated with XylX6 reagent (0.1 mL) at 30, 35 and 40 °C and reactions were terminated after 0, 10, 20 and 30 min by addition of 0.9 mL of 2% w/v Tris solution (pH 10.0). Absorbance was

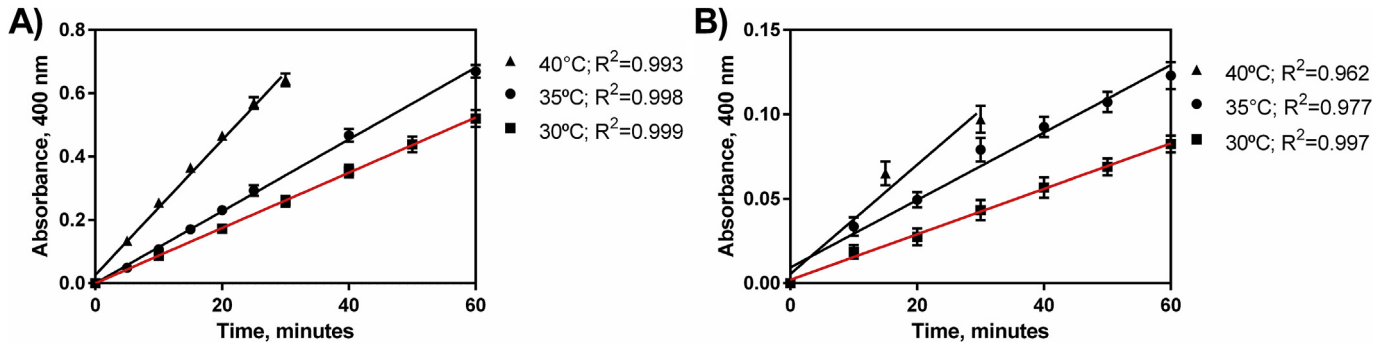

Fig. 2. Investigation into the effect of incubation temperature on the XylX6 assay of crude extracts of A) K-MBG4 control malt flour lot# 61002A and B) IFBM malt 201514A was performed in triplicate as described in Section 2.3.1.3. Error bars show standard deviation.

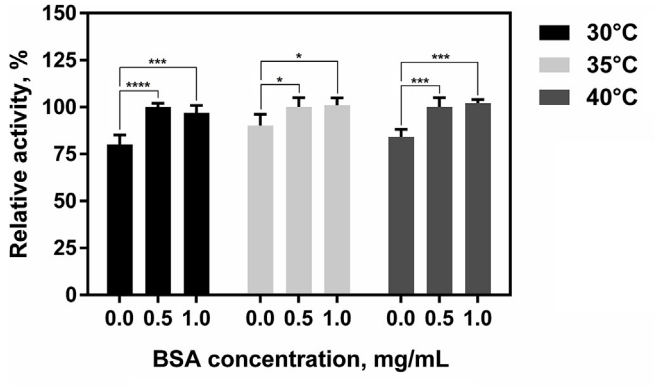

Fig. 3. Investigation into the effect of BSA concentration on the extraction of malt xylanase from the K-MBG4 control malt flour lot# 61002A as measured using the XylX6 assay was performed in triplicate as described in 2.3.1.5. A two-way ANOVA analysis was performed and the statistically significant differences to the mean xylanase activites are shown with $p < 0.05$ = *, $p < 0.01$ = **, $p < 0.001$ = ***, $p < 0.0001$ = ****.

measured at 400 nm.

2.3.1.6. Standard procedure for the extraction and assay of barley malt xylanase. 8 mL of 0.1 M sodium acetate buffer (pH 5.5) containing BSA (0.5 mg/mL) was added to a sample of malt flour (0.5 g, milled to pass a 0.2 mm screen) and the xylanase was allowed to extract at room temperature for 15 min with occasional mixing. The turbid suspension was filtered through glass fiber filter paper to obtain a clear supernatant which was recovered and used immediately. Aliquots (0.5 mL) of the extract solutions were pre-equilibrated to 30 °C for 5 min and then incubated with pre-equilibrated aliquots of XylX6 reagent (0.1 mL) at 30 °C. Reactions were terminated after 0 and 30 min by addition of 0.9 mL of 2% w/v Tris solution (pH 10.0). Absorbance was measured at 400 nm.

3. Results and discussion

In order to develop a procedure for the measurement of xylanase in malt, two separate components required optimisation – the biochemical assay conditions and the malt extraction procedure. To obtain an extract containing sufficient xylanase activity to develop the assay parameters, conditions that were recently employed for the extraction of barley β-glucanase (Mangan et al., 2016) were used without optimisation. The effect of the XylX6 reagent concentration on the assay response was investigated by varying the 'in-assay' concentration from 0.021 to 1.0 mM as described in Section 2.3.1.2 (Fig. 1) and values of 0.093 ± 0.004 mM and 1.75 ± 0.02 μmol/min were obtained for K_M and V_{max} respectively. The standard XylX6 assay format recommends addition of 0.05 mL substrate solution (3.3 mM) to 0.05 mL analyte (xylanase solution) solution, incubated at 40 °C, with the reaction being terminated after 10 min by the addition of 1.5 mL Tris solution at pH 10. Given the low relative abundance of xylanase in malt extracts, it was imperative to maximise the sensitivity of the assay. A new assay format comprising addition of 0.1 mL of substrate solution (3.3 mM) to 0.5 mL malt extract gave rise to an in-assay substrate concentration of 0.55 mM. This concentration is not saturating, but it is ~5 times higher than the K_M for malt xylanase, and the amount of xylanase that is delivered into the assay is increased by a factor of 10. The alkaline "stop solution" was reduced in volume from 1.5 mL to 0.9 mL to minimise dilution of the 4-nitrophenolate released, while still ensuring that the reaction is terminated, and also giving a final assay volume of 1.5 mL which is compatible with all standard spectrophotometers. The XylX6 assay operates on a principle (Fig. S1) involving an ancillary enzyme, β-xylosidase from *Selenomonas ruminantium* (GH43), which must be present at a saturating level. The β-xylosidase content present in the XylX6 reagent was doubled from 20 U/mL to 40 U/mL and this produced no effect under the new modified assay format confirming that no modification to the standard XylX6 reagent was required (data not shown).

The effect of assay incubation time at 30 °C, 35 °C and 40 °C was investigated as described in Section 2.3.1.3 and the results are outlined in Fig. 2A and B. These data indicated that while the malt flour control sample which contained a relatively high level of xylanase activity appeared to yield an excellent response at 40 °C, xylanase instability (evidenced by lack of linearity in the assay profile) was observed at temperatures greater than 30 °C for a sample from the IFBM barley malts that contained much lower xylanase activity. Given that the IFBM samples are more representative of the standard brewing quality of barley malts currently used in Europe, it was decided that an incubation temperature of 30 °C would be most appropriate.

The optimum pH for the extraction and assay of xylanase was investigated as described in section 2.3.1.4 by varying the pH of the extract buffer from 4.0 to 7.0 using sodium acetate and sodium phosphate to span this range. Bearing in mind that a crude malt extract may contain a number of isoforms of xylanase, and that the relative abundance of each may vary from sample to sample, the pH optimum observed in the region of 5.5–6.0 was in line with previously reported data (Fig. S2, Supporting Information).

Optimisation of the BSA content used in the extraction of malt xylanase indicated that the presence of BSA at 0.5 mg/mL conferred a small but significant positive effect on observed activity but that further increasing the BSA content beyond this level did not result in any further beneficial effect. Malt extracts containing various concentrations of BSA were analysed at 30, 35 and 40 °C in order to assess if BSA content would increase the temperature stability of the enzyme solution for assay. The addition of BSA did not improve the linearity of the assay for samples that had previously exhibited poor linearity at the higher temperatures (linearity data not shown). The results of a two way ANOVA analysis to understand the effect of both BSA concentration and

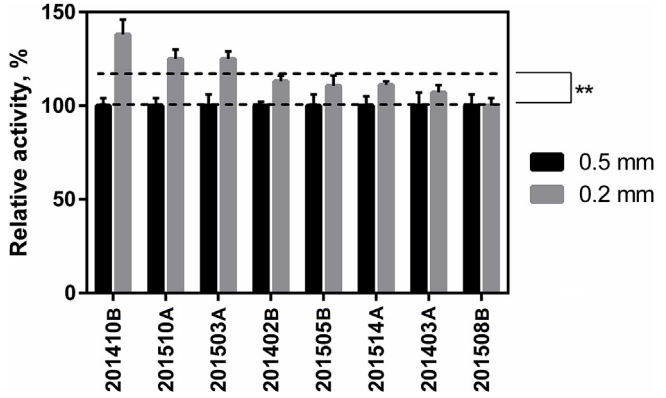

Fig. 4. Investigation into the effect of milling screen size (mm) on the extraction of xylanase from a series of IFBM malt samples. Analysis was performed in triplicate and mean difference in observed xylanase activity of 16.3% was observed (two tailed *t*-test, p = 0.007). Error bars show standard deviation.

temperature revealed that 72% of the variation observed in this dataset could be attributed to the BSA content. All results are summarised in Fig. 3. Investigation into the duration required to fully extract the available xylanase indicated that this was not a critical parameter with no difference observed for incubation times ranging from 15 min to 5 h (data not shown).

Initial investigations revealed the surprising result that the screen size used in the milling of the barley malt grains had a noticeable effect on extractable xylanase levels. This parameter was therefore investigated in more detail by milling a series of 8 IFBM malts from the 2014 and 2015 seasons to 0.2 and 0.5 mm particle size prior to extraction and assay. The results are outlined in Fig. 4 and it emerged that an average increase of 16.3% (p = 0.007, two tailed *t*-test) in measured xylanase activity was observed for those samples which were milled to the 0.2 mm particle size prior to extraction. To the best of our knowledge, this fact has not been reported previously.

By combining the findings of the optimisation of both the biochemical assay procedure and the malt extraction procedure a standard protocol for the measurement of xylanase in barley malt was identified as described in Section 2.3.1.6. Finely milled malt (0.2 mm screen) is extracted and aliquots (0.5 mL) of the filtered extract solutions are pre-equilibrated to 30 °C for 5 min and then incubated with pre-equilibrated aliquots of XylX6 reagent (0.1 mL) at 30 °C. Reactions are terminated after 0 and 30 min by addition of 0.9 mL of 2% w/v tris solution (pH 10.0). Absorbance is measured at 400 nm.

The repeatability of the assay was investigated by having 2 analysts perform 2 individual extractions of the control malt on 4 consecutive days. Each extract was analysed in duplicate within 30 min of extraction and the results are outlined in Table 1. An acceptable overall CV of 4.8% was obtained (n = 16) although it should be noted that the individual CVs for analyst 1 and 2 were significantly lower (2.3% and 2.2% respectively, n = 8). This was considered a sufficient level of repeatability given the variability that might be expected to arise from the extraction, filtration and pipetting required in the manual assay procedure. The Limit of Detection (LOD) was calculated as 10 U/kg (3 × σ of the 'blank' sample solution absorbance for 10 replicates), which corresponds to an absorbance of 0.023 and the Limit of Quantification (LOQ) was calculated as 33 U/kg (10 × σ of the 'blank' sample solution absorbance for 10 replicates), which corresponds to an absorbance of 0.077.

In order to demonstrate the application of the newly developed method, a series of eight acceptable quality malt samples from the IFBM were analysed for xylanase activity. These activity data were compared with their corresponding values for wort viscosity and as expected, a negative correlation was apparent as shown in Table 2. There is an impressive degree of correlation (−0.82, Pearson's correlation coefficient) between these variables which is substantiated by the low *p*-value obtained (0.007, two tailed *t*-test). This is easily rationalised by the fact that increased xylanase activity should result in depolymerisation of the arabinoxylan. Such an improvement would serve to lower the viscosity of a wort or beer making the mash separation and beer filtration stages of the brewing more efficient and reducing the likelihood of the formation of undesirable hazes (Barrett et al., 1975; Coote and Kirsop, 1976; Li et al., 2015; Lu and Li, 2006). It is tempting to suggest that there is the potential for a predictive test for wort or beer viscosity involving the rapid measurement of xylanase activity in a barley malt using the XylX6 assay. This type of test would obviously be of significant benefit to brewers but such a hypothesis must be tempered by the fact that many other parameters, for example β-glucan content (and also β-glucanase activity), also play a very significant role. A larger study involving an expanded set of 35 IFBM malt samples is now underway in which the correlation between a range of malt enzyme activities and a broad set of brewing parameters will be thoroughly investigated with a view to formulating a multi-component predictive model.

In summary, a method for the measurement of xylanase activity in barley malt extracts has been developed based on the XylX6 reagent. The biochemical assay protocol is suitable for automation using any liquid handling system (Fig. S3, supporting information) while the

Table 1
The repeatability of the modified XylX6 assay for the measurement of xylanase activity in the control malt flour was investigated by 2 analysts across 4 consecutive days.

Day	Analyst	Extraction	Xylanase (U/kg)
1	1	A	26.4
1	1	B	27
1	2	A	25.6
1	2	B	24.8
2	1	A	27.6
2	1	B	28.4
2	2	A	24.6
2	2	B	24.8
3	1	A	27.6
3	1	B	27.4
3	2	A	25.8
3	2	B	25.4
4	1	A	27.2
4	1	B	28.2
4	2	A	26.2
4	2	B	25.2
		Mean	**26.38**
		SDEV	**1.28**
		%CV	**4.83**
		n	**16**

Table 2
The relationship between Congress wort viscosity (EBC mashing protocol) and xylanase activity was examined for a series of 8 barley malts from IFBM.

Barley Malt Sample ID	Variety	Head type/ Growth	Viscosity[a] (MPa.s)	Xylanase[b] (U/ Kg)
201410B	Isocel	6 rows winter	1.47	28.9
201402B	Passerel	6 rows winter	1.49	18.0
201508B	Tipple	2 rows spring	1.50	6.5
201403A	Azurel	6 rows winter	1.52	7.2
201514A	Passerel	6 rows winter	1.52	4.9
201510A	Passerel	6 rows winter	1.53	6.8
201505B	N/A[c]	N/A[c]	1.56	4.6
201503A	Isocel	6 rows winter	1.56	3.6

Note that wort viscosity is negatively correlated with xylanase activity (**R = -0.82, p = 0.007**).

[a] Data provided by IFBM.

[b] Data obtained using XylX6 malt xylanase assay.

[c] Information not provided by IFBM for this sample.

manual extraction procedure is quick and easy to perform. The resulting method was applied to quantify the xylanase activity present in a range of barley malt samples and the values obtained correlated well with the associated Congress wort viscosity data for these samples, a result that has prompted the expansion of our investigations into this area, the outcome of which will be reported shortly.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jcs.2018.10.003.

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