JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

ARTICLE

pubs.acs.org/JAFC

Use of Psychrophilic Xylanases Provides Insight into the Xylanase Functionality in Bread Making

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ABSTRACT: The bread-improving potential of three psychrophilic xylanases from *Pseudoalteromonas haloplanktis* TAH3A (XPH), *Flavobacterium* sp. MSY-2 (rXFH), and unknown bacterial origin (rXyn8) was compared to that of the mesophilic xylanases from *Bacillus subtilis* (XBS) and *Aspergillus aculeatus* (XAA). XPH, rXFH, and rXyn8 increased specific bread volumes up to 28%, 18%, and 18%, respectively, while XBS and XAA gave increases of 23% and 12%, respectively. This could be related to their substrate hydrolysis behavior. Xylanases with a high capacity to solubilize water-unextractable arabinoxylan (WU-AX) during mixing, such as XBS and XPH, increased bread volume more than xylanases that mainly solubilized WU-AX during fermentation, such as rXFH, rXyn8, and XAA. Irrespective of their intrinsic bread-improving potential, the dosages needed to increase bread volume to a similar extent were much lower for psychrophilic than for mesophilic xylanases. The xylanase efficiency mainly depended on the enzyme's temperature activity profile and its inhibition sensitivity.

KEYWORDS: *Triticum aestivum* L., psychrophilic xylanase, arabinoxylan, bread making, substrate selectivity, inhibition sensitivity, temperature optimum

INTRODUCTION

Today, *endo*-β-1,4-D-xylanases (EC 3.2.1.8, xylanases) are often added in bread making to improve dough processability and bread quality. Although xylanase functionality in bread making obviously results from the enzymic modification of the arabinoxylan (AX) population, its functionality is not yet fully understood. Several factors have been reported to play a role.

A first important factor is xylanase substrate selectivity, which is a measure of the relative preference of a xylanase to solubilize water-unextractable AX (WU-AX) or to degrade water-extractable AX (WE-AX) to low molecular mass (MM) fragments.[1,2] WU-AX have a detrimental effect on bread volume, most likely because their insolubility and resulting water immobilization negatively affect gluten development.[1] WE-AX, in contrast, have a positive effect on bread volume, which has been related to their high viscosity in solution and their potential to form a network by hydrogen bonds with itself, starch, and/or gluten. This results in improved dough properties and gas retention during the fermentation and early baking phases.[1] On the basis of research performed over the last 2 decades into xylanase functionality in bread making, it has been put forward that xylanases should solubilize WU-AX, without extensively degrading the formed solubilized AX (S-AX) and native WE-AX.[1−5]

A second important factor that determines xylanase functionality in bread making is its inhibition sensitivity. Wheat (*Triticum aestivum* L.) contains several inhibitors, such as *T. aestivum* xylanase inhibitor (TAXI), xylanase inhibiting protein (XIP), and thaumatin-like xylanase inhibitor (TLXI), which bind to microbial xylanases and inactivate them.[6] It has been demonstrated that the activity of xylanases that are sensitive to these inhibitors is limited to the mixing phase, and that they have to be added in much higher dosages than their uninhibited counterparts.[7]

A third factor that might be important for xylanase functionality in bread making is the xylanase temperature activity profile. Most bread making active xylanases, currently used to improve dough manageability and bread volume, are optimally active at temperatures of 40−60 °C. The functionality of xylanases with higher temperature optima has also been investigated, but contradictory results have been reported. While Dornez et al.[8] found little or no effect of the hyperthermophilic xylanase B from *Thermotoga maritima* on bread volume, Jiang et al.[9] reported an increase in specific bread volume of about 60%. This discrepancy can possibly be explained by differences in enzyme dosage, processing conditions, and bread recipe. The thermophilic *Thermomyces lanuginosus* CAU44 xylanase could improve the specific bread volume by about 30%.[10] Psychrophilic xylanases, displaying optimal activity at lower temperatures, could also have a beneficial effect on bread making.[11−13] Collins et al.[12] found that the psychrophilic xylanase from *Cryptococcus adeliae* TAE85 had no significant effect on bread volume, while the one from *Pseudoalteromonas haloplanktis* TAH3a could increase the bread volume by about 17%. Zheng et al.[13] reported that the psychrophilic xylanase from *Glaciecola mesophila* could increase bread volume by about 26%.

The aim of the present work is to establish whether these psychrophilic xylanases indeed have a selective advantage over mesophilic xylanases in beneficially affecting bread making and hence to investigate the impact of the xylanase temperature activity profile on its bread-making potential. The psychrophilic xylanases tested in this study were the glycoside hydrolase family

Received: May 4, 2011
Accepted: August 1, 2011
Revised: July 29, 2011
Published: August 02, 2011

(GH) 8 xylanase of *P. haloplanktis* TAH3A (XPH), the GH8 xylanase of an unknown bacterium (rXyn8), and the GH10 xylanase of *Flavobacterium* sp. MSY-2 (rXFH). The mesophilic xylanases used in this study were the GH10 xylanase of *Aspergillus aculeatus* (XAA) and the GH11 xylanase of *Bacillus subtilis* (XBS). Because optimum temperature is evidently not the only factor determining xylanase functionality in bread making, a thorough biochemical characterization of each of the enzymes was performed.

MATERIALS AND METHODS

Materials. An enzyme preparation containing the psychrophilic xylanase XPH (Genbank accession no. CAD20872) was supplied by Puratos Group NV (Groot-Bijgaarden, Belgium). Grindamyl H640, an enzyme preparation for the bread making industry, contains XBS (Genbank accession no. P18429) and was obtained from Danisco A/S (Copenhagen, Denmark). Shearzyme 500 L, an enzyme preparation marketed toward the gluten-starch separation process containing XAA (Genbank accession no. AAE69552), was from Novozymes (Bagsvaerd, Denmark). Recombinant plasmids pET22b-*XFH*-His_6 and pET22b-*Xyn8*-His_6, containing the genes coding for rXFH (Genbank accession no. AAY98787) and rXyn8 (Genbank accession no. ABB7189), respectively, were kindly provided by Dr. Charles C. Lee (Western Regional Research Center, Albany, CA). Xylohexaose (X_6), azo-wheat AX, and azurine cross-linked AX (AZCL-AX) were obtained from Megazyme International Ireland Ltd. (Bray, Ireland). *Escherichia coli* strain BL21 (DE3) pLysS was from Stratagene (La Jolla, CA). Manager wheat flour (B-quality class), free of additives, was provided by Prof. M. Lindhauer and Dr. K. Münzing (Max Rubner-Institute, Detmold, Germany). All solvents, chemicals, and reagents were obtained from Sigma-Aldrich (Bornem, Belgium) and were at least of analytical grade, unless stated otherwise.

Recombinant expression of rXyn8 and rXFH in *E. coli*. Electrocompetent *E. coli* BL21 (DE3) pLysS cells were transformed with plasmids pET22b-*Xyn8*-His_6 and pET22b-*XFH*-His_6 and used for recombinant production of rXyn8 and rXFH, respectively. Cultures were grown in Luria−Bertani medium [0.5% w/v yeast extract (LabM, Lancashore, UK), 1.0% w/v tryptone (LabM), and 1.0% w/v sodium chloride] containing ampicillin (100 μg/mL) and chloramphenicol (34 μg/mL) at 37 °C with shaking at 250 rpm until the optical density at 600 nm reached approximately 1.0. At this point, isopropyl-β-D-thiogalactopyranoside (Promega, Leiden, The Netherlands) was added to a final concentration of 1.0 mM to induce the expression of *Xyn8* and *XFH*. Cells were further grown at 18 °C for 18 h and harvested by centrifugation (3400*g*, 20 min, 4 °C). The obtained pellets were resuspended in lysis buffer [50 mM sodium phosphate buffer (pH 7.0) containing 300 mM sodium chloride and 30 mM imidazole]. Cell lysis was accomplished as outlined previously.[14] Cell debris was removed by centrifugation (3400*g*, 20 min, 4 °C), and clear cytoplasmic extracts containing the recombinantly produced xylanases were obtained.

Purification of Xylanases. rXFH and rXyn8 were purified from the cytoplasmic extracts through Ni-Sepharose affinity chromatography using HisTrap HP columns according to manufacturer's instructions (GE Healthcare, Uppsala, Sweden). The enzymes were eluted with an imidazole gradient (from 10 to 500 mM) in a buffer consisting of 50 mM sodium phosphate (pH 8.0) and 300 mM sodium chloride. rXFH was further polished through gel filtration chromatography [Hiprep Sephacryl S-100 (26 × 600 mm), GE Healthcare] using 200 mM sodium phosphate (pH 6.0) as eluent.

XPH, XBS, and XAA were purified from commercial enzyme preparations, essentially as outlined previously. XPH was purified from the enzyme preparation by anion and cation exchange chromatography as described by Pollet et al.[15] XBS was purified from Grindamyl H640 by two-step cation exchange chromatography as described by Pollet et al.[16] and XAA from Shearzyme 500 L by two-step anion exchange chromatography as described by Goesaert et al.[17] All chromatographic procedures were performed with an ÄKTA Purifier system equipped with an UV-detector and controlled by Unicorn software (GE Healthcare).

Protein purification was evaluated using SDS-PAGE and SimplyBlue SafeStain (Invitrogen Corp., Carlsbad, CA). Protein concentrations of purified proteins were quantified by measuring the absorbance at 280 nm using a Nanodrop 1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE) and by using the molar extinction coefficient calculated from the amino acid sequence with the ProtParam tool (http://expasy.org/tools/protparam.html). Low MM markers of GE Healthcare were used to compare band sizes.

Determination of Temperature Dependency of Xylanase Activity and Stability. Temperature dependency of xylanase activity was analyzed by measuring xylanase activity levels at temperatures ranging from 4 to 70 °C using the colorimetric AZCL-AX method (Megazyme International Ireland Ltd.). For rXyn8, rXFH, XPH, and XBS, enzyme activity measurements were performed in a 200 mM sodium phosphate buffer pH 6.0, while for XAA, 200 mM sodium acetate pH 5.0 was used. In each assay, the enzyme was diluted such that the activity measurement could be done in the linear range; i.e., the extinction value never exceeded 1.5. All incubations and subsequent measurements were performed in triplicate and the coefficient of variation was typically around 4%.

Aliquots of enzyme solution (0.5 mL) were preincubated for 10 min at temperatures ranging from 4 to 70 °C. Subsequently, an AZCL-AX tablet was added and incubation was extended for an additional 10 min at the same temperature. The enzymic reaction was stopped by the addition of 5.0 mL Trizma base solution (2.0% w/v), vigorous vortex stirring, and instantaneous filtration. The absorbance at 590 nm was measured against a control, prepared by incubating the AZCL-AX tablet without enzyme. One xylazyme unit (X-U) is the amount of enzyme needed to increase the absorbance at 590 nm with 1.0, under the conditions of the assay.

Temperature stability was monitored by incubating the enzyme solutions for 30 min at temperatures ranging from 4 to 70 °C or to 80 °C for XAA. Subsequently, samples were cooled in an ice bath for 5 min. Residual enzyme activity was assayed in triplicate with AZCL-AX tablets at 40 °C for psychrophilic and 50 °C for mesophilic xylanases.

Determination of pH Dependency of Xylanase Activity and Stability. Dependency of xylanase activity on pH was determined by measuring xylanase activities at pH values from 2.0 to 10.0 using the colorimetric AZCL-AX method as described above. Universal buffers were prepared from a stock solution containing 30 mM citric acid, 30 mM potassium dihydrogen phosphate, 30 mM boric acid, and 30 mM diethyl barbituric acid in deionized water. To 100 mL portions of this stock solution, varying quantities of sodium hydroxide (2.0 M) or hydrochloric acid (2.0 M) were added to reach the desired pH values. For the psychrophilic xylanases rXyn8, rXFH, and XPH, a temperature of 40 °C was used for determination of xylanase activity, while for the mesophilic xylanases XAA and XBS, incubation was at 50 °C.

pH stability was assessed by a similar approach. After 120 min of incubation of the xylanase (20.0 X-U/mL) in universal buffer of pH 2.0−10.0, the enzyme solutions were diluted 10-fold in the appropriate buffer (200 mM sodium phosphate pH 6.0 for rXyn8, rXFH, XPH, and XBS and 200 mM sodium acetate pH 5.0 for XAA). Residual xylanase activity was assayed in triplicate with AZCL-AX tablets as described above.

Determination of Specific Activity of Xylanases against Different Substrates. To allow comparison of the specific activities of the selected xylanases toward diverse substrates, all enzyme activities were measured in comparable conditions, i.e., a 20 mM sodium phosphate buffer pH 6.0 and an incubation temperature of 40 °C.

Specific xylanase activities against WU-AX and WE-AX were estimated using AZCL-AX and azo-wheat AX as model substrates, essentially as

Table 1. Genbank Accession Number of Protein Sequence; Glycoside Hydrolase Family (GH); Theoretical Molecular Mass (MM) Assessed by the Protparam Tool (www.expasy.org); Temperature and pH Optimum ($T_{opt.}$ and $pH_{opt.}$) and Stability; Sensitivity Towards Proteinaceous Xylanase Inhibitors; Specific Activities against AZCL-AX, Azo-wheat AX, and Xylohexaose; and the Substrate Selectivity Factor (SSF) and Activity Ratio (AR) of the Psychrophilic rXyn8, rXFH, and XPH and of the Mesophilic XBS and XAA

	rXyn8	rXFH	XPH	XBS	XAA
Genbank accession no.	ABB7189	AAY98787	CAD20872	P18429	AAE69552
GH	8	10	8	11	10
MM (kDa)	46	43	48	23	47
$T_{opt.}$ (°C)	40	40	40	55	60
T stability (°C)	40	40	40	50	60
$pH_{opt.}$	7.0	7.0	8.0	5.0	5.0
pH stability	6.0−8.0	6.0	6.0−8.0	5.0−7.0	5.0−6.0
inhibition sensitivity (TAXI/XIP)	no	no	no	yes (TAXI)	no
specific activity against AZCL-AX (X-U/nmol)[a]	6.0	35.9	60.5	110.7	52.4
specific activity against azo-wheat AX (azo-U/nmol)[b]	0.9	9.9	2.8	3.5	21.2
specific activity against xylohexaose (X_6-U/nmol)[c]	35.0	79.5	1.0	1.5	251.5
SSF	7.0	3.6	21.7	31.4	2.5
AR ($\times 10^{-4}$)	1.7	4.5	620.3	719.5	2.1

[a] One xylazyme unit (X-U) is the amount of enzyme preparation needed to increase the absorbance at 590 nm to 1.0, under the conditions of the assay. [b] One azo-wheat AX unit (azo-U) is the amount of enzyme preparation needed to increase the absorbance at 590 nm to 1.0, under the conditions of the assay. [c] One X_6-unit (X_6-U) is defined as the amount of enzyme preparation needed to release 1.0 μM hydrolysis products from X_6 under the conditions of the assay.

outlined previously by Verjans et al.,[18] with some minor modifications in buffer and temperature as mentioned above. The measurements were performed in triplicate and the coefficients of variation on the AZCL-AX and azo-wheat AX were typically around 4% and 6%, respectively.

Specific xylanase activities against the oligomeric X_6 and primary hydrolysis products thereof were determined by high-performance anion-exchange chromatography followed by integrated pulsed amperometric detection with a Dionex ICS-3000 chromatography system (Dionex Corp., Sunnyvale, CA), as described previously by Verjans et al.,[14] except for the minor modifications in buffer and temperature mentioned above. The measurements were performed in triplicate and the coefficient of variation on the X_6 measurements was typically around 8%.

To assess the relative activity of xylanases toward each of the types of substrates, the substrate selectivity factor (SSF) and activity ratio (AR) were calculated. The SSF is defined as the ratio of the specific xylanase activity toward AZCL-AX (X-U/nmol enzyme) to that toward azo-wheat AX (azo-U/nmol enzyme). The AR is defined as the ratio of the specific xylanase activity toward AZCL-AX (X-U/nmol enzyme) to that toward X_6 (X_6-U/nmol enzyme) and can be regarded as a normalized activity of the enzyme against WU-AX. These ratios can be used to find out whether the inherent potential of xylanases in bread making is determined by their potential to solubilize and degrade AX or by their WU-AX solubilizing potential as such.

Xylanase Inhibition Sensitivity. To measure xylanase inhibition sensitivity, xylanase solutions (0.5 mL, 2.0 X-U/mL 200 mM sodium phosphate pH 6.0 determined at 40 °C) were incubated for 30 min at room temperature with an excess of TAXI (0.5 mL, 70 μg/mL) or XIP (0.5 mL, 20 μg/mL), obtained as described by Gebruers et al.[19] The remainder of the xylanase inhibition assay was identical to the AZCL-AX activity assay (40 °C and pH 6.0) as described above. Inhibition sensitivity was expressed as percentage reduction of xylanase activity. The measurements were performed in triplicate and the coefficient of variation on the inhibition measurements was typically around 5%.

Bread-Making Trials. Breads were made in triplicate using 10.0 g of flour on a 14% moisture base, according to the straight dough method,[20] as outlined previously by Verjans et al.[18] Dough was supplemented with a range of enzyme dosages of rXyn8 (0.03−0.4 ppm), rXFH (0.03−1.0 ppm), XPH (0.01−0.5 ppm), XBS (0.5−2.5 ppm), or XAA (0.25−6.0 ppm). The effect of the enzyme addition on the bread volume, bread height, and bread weight was established as outlined previously by Verjans et al.[18] Bread volumes were analyzed by one-way analysis of variance using Statistical Analysis System software 8.1 (SAS Institute, Cary, NC), with the mean values compared using the Tukey test.

For each combination of flour, enzyme, and enzyme dosage, an overall, subjective appreciation of dough manageability (after mixing and after 52, 77, and 90 min fermentation) was scored from 1 to 5, with a higher score representing better characteristics as outlined previously by Verjans et al.[18]

Analysis of Arabinoxylan Population in Flour, Dough, and Bread. Aqueous extracts of flour, dough, and bread samples were prepared as outlined previously by Verjans et al.[18] AX content of flour and aqueous extracts of flour, dough, and bread samples, as well as AX average degree of polymerization (avDP), were determined by gas chromatography and corrected as outlined previously by Verjans et al.[18] The measurements on flour and bread samples were performed in triplicate, and the coefficient of variation for the determination AX contents was typically around 2%. The measurements on dough samples, in contrast, were only performed in singular.

The MM distribution of the AX populations in the aqueous dough and bread extracts were studied by high-performance size-exclusion chromatography equipped with an evaporative light scattering detector (ELSD) as outlined previously by Verjans et al.[18] MM markers were Shodex (Showa Denko K.K., Kawasaki, Japan) standard P-82 pullulans with MMs of 1600, 788, 404, 212, 112, 47.3, 22.8, 11.8, and 5.9 kDa. The experiments were performed in singular.

RESULTS

Recombinant Expression and Purification of Xylanases. The psychrophilic xylanases rXyn8 and rXFH were recombinantly produced in the cytoplasm of *E. coli* cells. As rXyn8 and rXFH both are His_6-tagged proteins, they could easily be purified from the cytoplasmic extract to near homogeneity through Ni-Sepharose affinity chromatography. rXFH was further polished

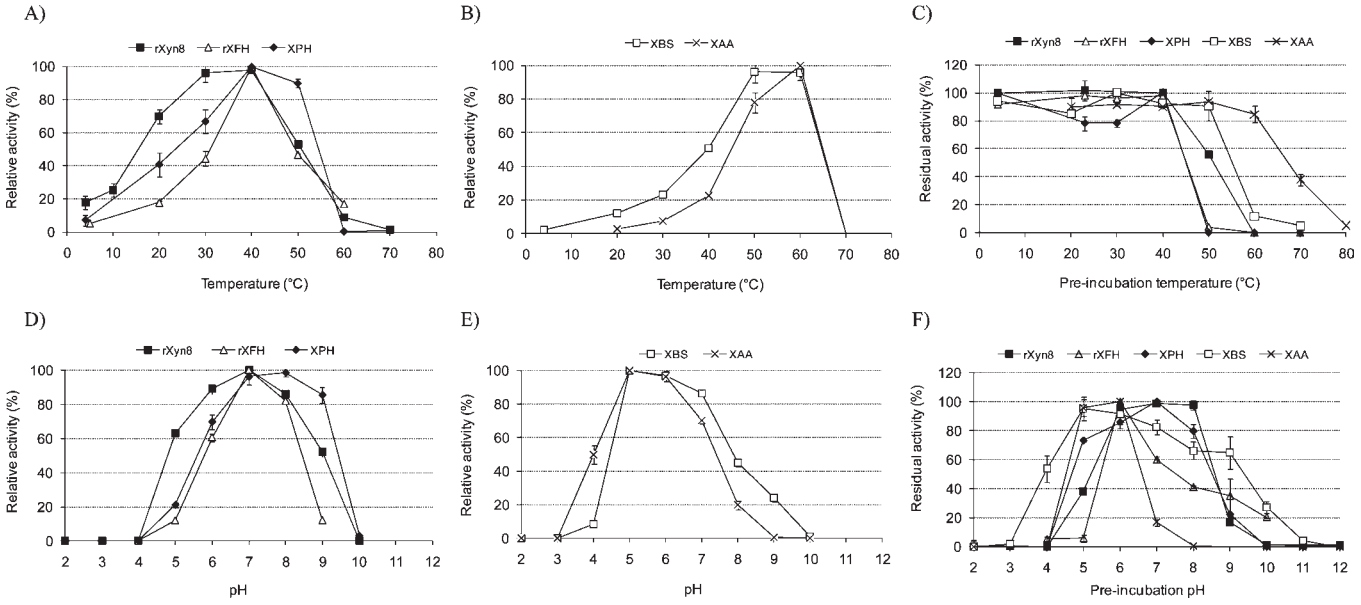

Figure 1. Thermoactivity (A, B) and pH activity (D, E) profiles and thermostability (C) and pH stability (F) profiles of the psychrophilic (rXyn8, rXFH, and XPH) and mesophilic (XAA and XBS) xylanases. For activity measurements, maximal xylanase activities are expressed as 100%, while for stability measurements, initial xylanase activities are expressed as 100%. All values are means of triplicate incubations and subsequent measurements and standard deviations are shown.

by gel filtration chromatography. XPH was purified from the commercially available preparation through ion exchange chromatography by taking advantage of the high p*I* (pH 9.5) of the enzyme. XAA and XBS were purified by anion and cation exchange chromatography, respectively. SDS−PAGE analysis revealed protein bands with MMs corresponding well to the theoretical MMs, which are given in Table 1.

Biochemical Characterization of Xylanases. *Temperature and pH Dependency of Xylanase Activity and Stability.* Under the conditions of the assay used in this study, rXFH, rXyn8, and XPH were not as psychrophilic as previously stated in the literature.[21−23] Instead of being optimally active at 20, 30, and 35 °C, respectively, rXyn8, rXFH, and XPH all showed optimal activity at or near 40 °C (Table 1). The mesophilic XBS and XAA had maximal xylanase activity at approximately 55 and 60 °C, respectively (Table 1). At temperatures below 40 °C, the psychrophilic enzymes (Figure 1A) retained relatively more of their activity compared to the mesophilic xylanases (Figure 1B). rXyn8 was the most psychrotolerant.

Enzyme stability decreased strongly for all xylanases after preincubation at temperatures higher than their temperature optimum (Table 1). Only XBS was not fully stable at its optimal temperature. While XBS retained its activity at 50 °C, its activity was reduced to 13% after 30 min at 60 °C. XAA was the most thermostable xylanase, retaining about 40% of its activity after 30 min incubation at 70 °C.

Maximal xylanase activity was measured at pH 7.0, 7.0, and 8.0, for rXyn8, rXFH, and XPH, respectively (Table 1), which is in line with previous findings.[21−24] XBS and XAA both showed optimal activity at pH 5.0 (Table 1).

rXyn8 and XPH were stable at pH 6.0−8.0, whereas rXFH was only stable at pH 6.0. XBS was fairly stable across a broad pH range (pH 4.0−9.0), while XAA had a very narrow pH stability range (pH 5.0−6.0) (Table 1).

Specific Activity against Different Substrates. Table 1 shows the specific activities of the xylanases against AZCL-AX (X-U/nmol enzyme), azo-wheat AX (azo-X-U/nmol enzyme), and X_6 (X_6-U/nmol enzyme), as well as the ratios of X-U/nmol enzyme over azo-X-U/nmol enzyme (SFF) and over X_6-U/nmol enzyme (AR). The SFF and AR values of GH10 XAA, GH10 rXFH, and GH8 rXyn8 are relatively low, indicating that these xylanases have a comparable substrate hydrolysis behavior (Table 1). They all have a relative preference for degrading WE-AX and X_6 instead of solubilizing WU-AX. XPH, although it is a GH8 xylanase just like rXyn8, resembles GH11 XBS in substrate hydrolysis behavior. Both xylanases display high SFF and AR values, indicating that they have a relative preference for solublilization of WU-AX.

Inhibition Sensitivity of Xylanases. The ability of two classes of xylanases inhibitors, i.e., TAXI and XIP, to inhibit the xylanases was evaluated. Only XBS was sensitive toward one of the proteinaceous xylanase inhibitors, more specifically TAXI, which is in line with earlier observations.[25−27] Under the experimental conditions, TAXI reduced the specific activity of XBS by 80%.[18]

Functionality of Psychrophilic and Mesophilic Xylanases in Bread Making. Psychrophilic and mesophilic xylanases were individually added to the bread making formula. The influence of the xylanases at different enzyme dosages on the dough and bread quality as well as on the AX population is described below. Results for XBS were earlier described by Verjans et al.[18] as they were obtained during an integrated experiment covering several unrelated classes of xylanases, including the psychrophilic xylanases described here, with XBS as the control enzyme.

Changes in Dough and Bread Quality. The addition of psychrophilic or mesophilic xylanases affected dough manageability (Figure 2). In general, dough manageability decreased with increasing enzyme dosages, mainly due to increased stickiness. For the psychrophilic xylanases, dough scores were already slightly lower after mixing, compared to the control. A small increase in dough stickiness was observed with increasing

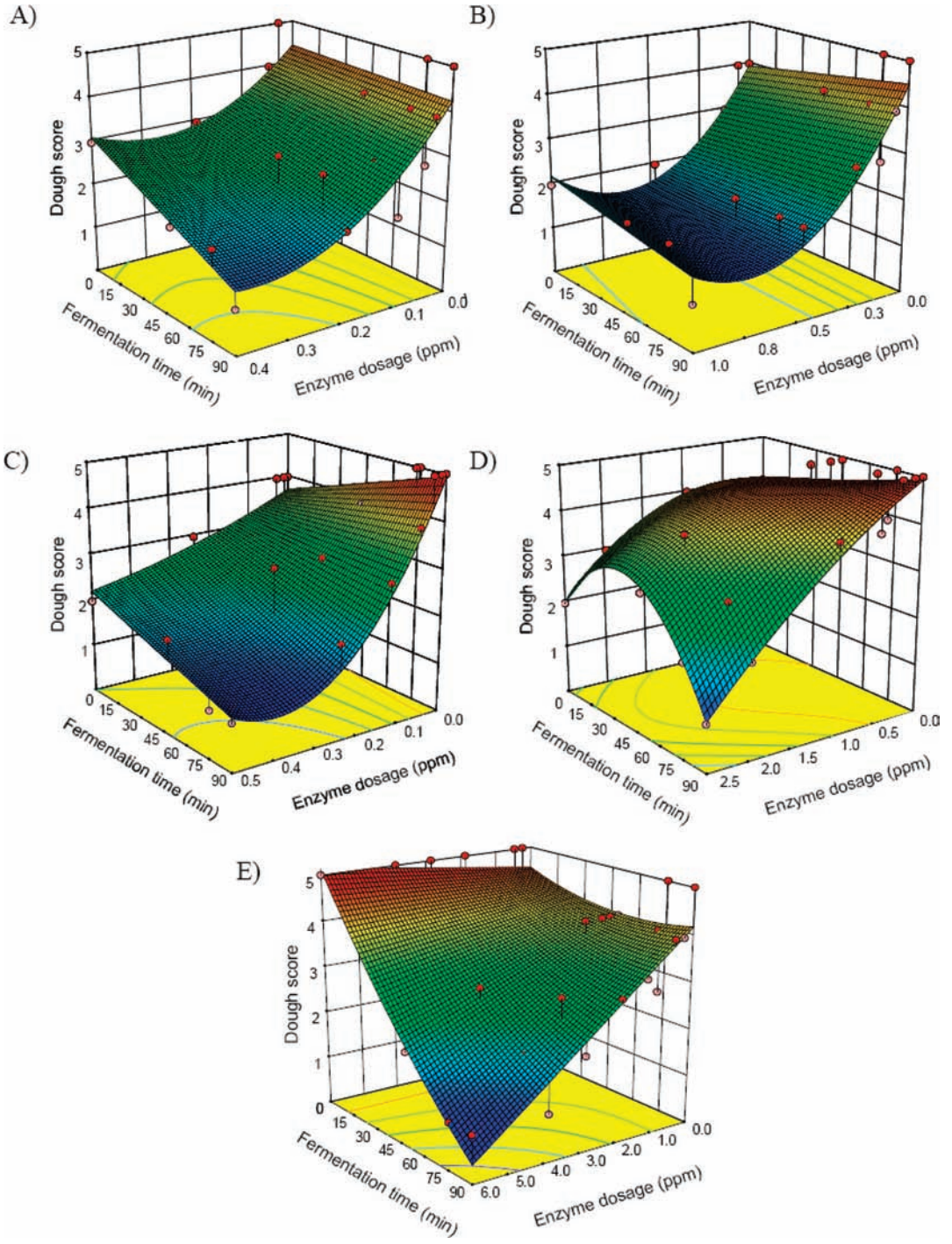

Figure 2. Schematic representation of manageability of dough made with the psychrophilic rXyn8 (A), rXFH (B), or XPH (C) or with the mesophilic XBS (D) or XAA (E) xylanases as a function of enzyme dosage (ppm) and fermentation time (min). Manual dough appreciation was subjectively scored with a score ranging from 1 (blue) to 5 (red), with a higher score representing better characteristics. Dough scores above and below the predicted value are shown.

fermentation time. With XAA, dough scores were somewhat better after mixing than the control, and with XBS, dough manageability increased a bit during fermentation. The limit of dough manageability after 90 min fermentation was reached when 0.4 ppm of rXyn8, 1.0 ppm of rXFH, 0.5 ppm of XPH, 2.5 ppm of XBS, or 6.0 ppm of XAA was added to the dough. Higher dosages caused handling problems during sheeting or molding.

Next to dough manageability, bread volume is considered to be an important quality parameter. Overall, specific bread volumes gradually increased with increasing enzyme dosage (Figure 3). rXyn8, rXFH, and XPH exerted a specific volumetric increase of approximately 18%, 18%, and 28% at the highest enzyme dosages, while XAA and XBS caused an increase of 12 and 23%, compared to the control bread. As noted previously,[2] at

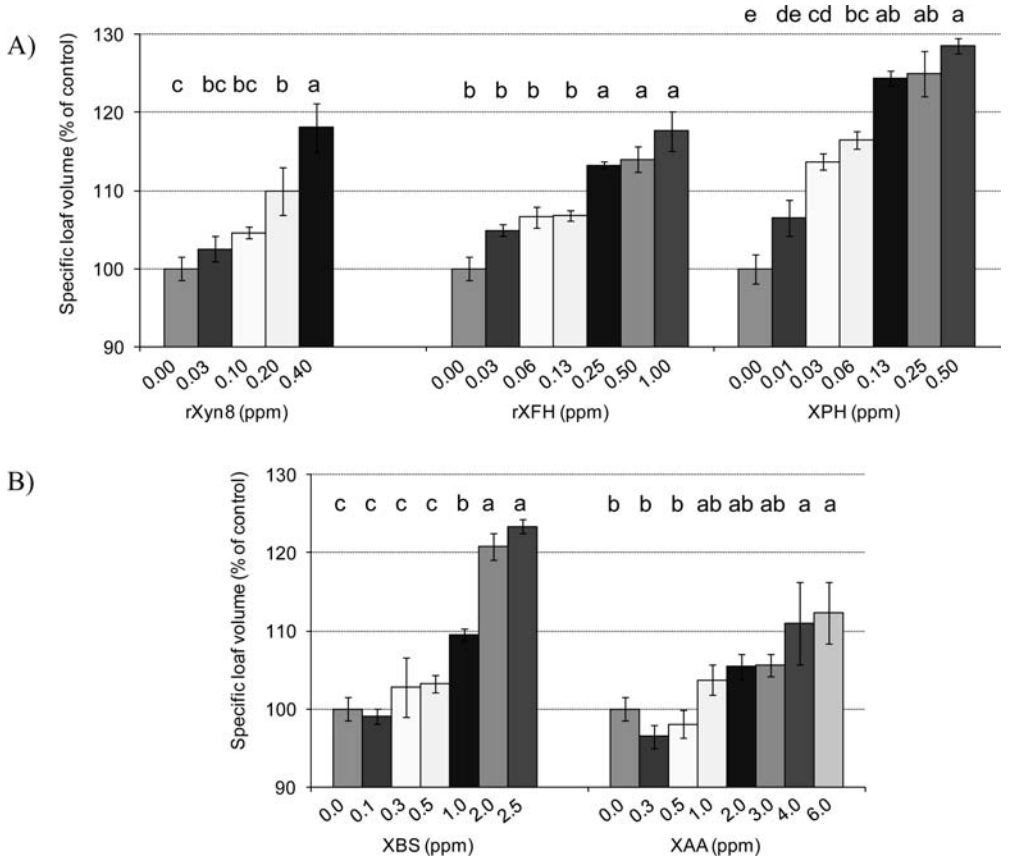

Figure 3. Specific bread volumes made with psychrophilic rXyn8, rXFH, and XPH (A) and mesophilic XBS and XAA (B) as a function of enzyme dosage. Values are the means and standard deviations resulting from analysis of samples from three individual breads. a, ab, b, bc, c, cd, d, de, e: Tukey groups with *P*-value <0.05 for comparison of different enzyme dosages within one enzyme.

the lowest dosages of XAA (0.25 and 0.50 ppm) the specific bread volume decreased somewhat (although not significantly), when compared to the control. Additionally, in contrast to what was observed with XAA and XBS, the maximum specific bread volume was already reached at low enzyme dosages with rXyn8, rXFH, and XPH.

In as far as 10 g breads allow evaluating crumb characteristics, all breads had good and homogeneous crumb structures and colors, irrespective of the xylanase dosage. A slightly increased darkening of the crust was observed as a consequence of xylanase addition. The main reason for this was probably more Maillard reaction together with caramelization, due to the increased amount of reducing sugars in the dough (see below) as a result of enzyme action.

Changes in the Arabinoxylan Population during the Bread Making Process. As determined previously,[18] the total AX content and WE-AX content of the Manager flour were 2.29% and 0.38%, respectively. In the control bread, 12% of WU-AX was solubilized, which resulted in an overall soluble AX level of approximately 0.55 g/100 g of bread. Addition of xylanases increased the S-AX (Figure 4) and thus the soluble AX level in all breads.

XPH and XBS solubilized most WU-AX during mixing, in contrast to what was observed with rXyn8, rXFH, and XAA (Figures 4A,B). In the case of the latter enzymes, most of the WU-AX solubilization took place during the fermentation stage (Figure 4C,D). At this stage, XPH and XBS solubilized an additional amount of WU-AX, especially at the lower enzyme concentrations (Figure 4C,D). In most cases, the amount of solubilized AX obtained after baking (Figure 4E,F) was equal or lower than the level of S-AX after fermentation (Figure 4C,D). Part of the S-AX formed during mixing or fermentation thus became unextractable during baking, probably due to cross-linking or physical entrapment.

The extent to which the AX population is extractable after baking depends on the xylanase used (Figure 4E,F). At the optimal enzyme concentration, i.e., the concentration giving maximal bread volume within the boundaries of dough manageability, rXyn8 increased the S-AX level in the bread to 35%, while 49% and 70% of WU-AX were solubilized in breads supplemented with rXFH and XPH, respectively. In breads treated with XAA and XBS, maximally 59% and 76% of the WU-AX population was extractable after baking. Accordingly, the highest soluble AX levels (1.7 g/100 g of bread) were realized in breads supplemented with the optimal dosage of XPH or XBS, which is consistent with the high SSF and AR values of these enzymes (Table 1).

Analysis of the avDP of the soluble AX in bread indicated that reduction of the MM of AX depended on the type of enzyme and enzyme dosage. At the optimal enzyme dosage, the psychrophilic rXyn8, rXFH and XPH led to an avDP of the AX population of 51, 33, and 34 (Figure 5A), respectively. In breads treated with the optimal dosage of XBS and XAA, the avDP of the soluble AX was 90 and 30, respectively (Figure 5B). Side-by-side comparison

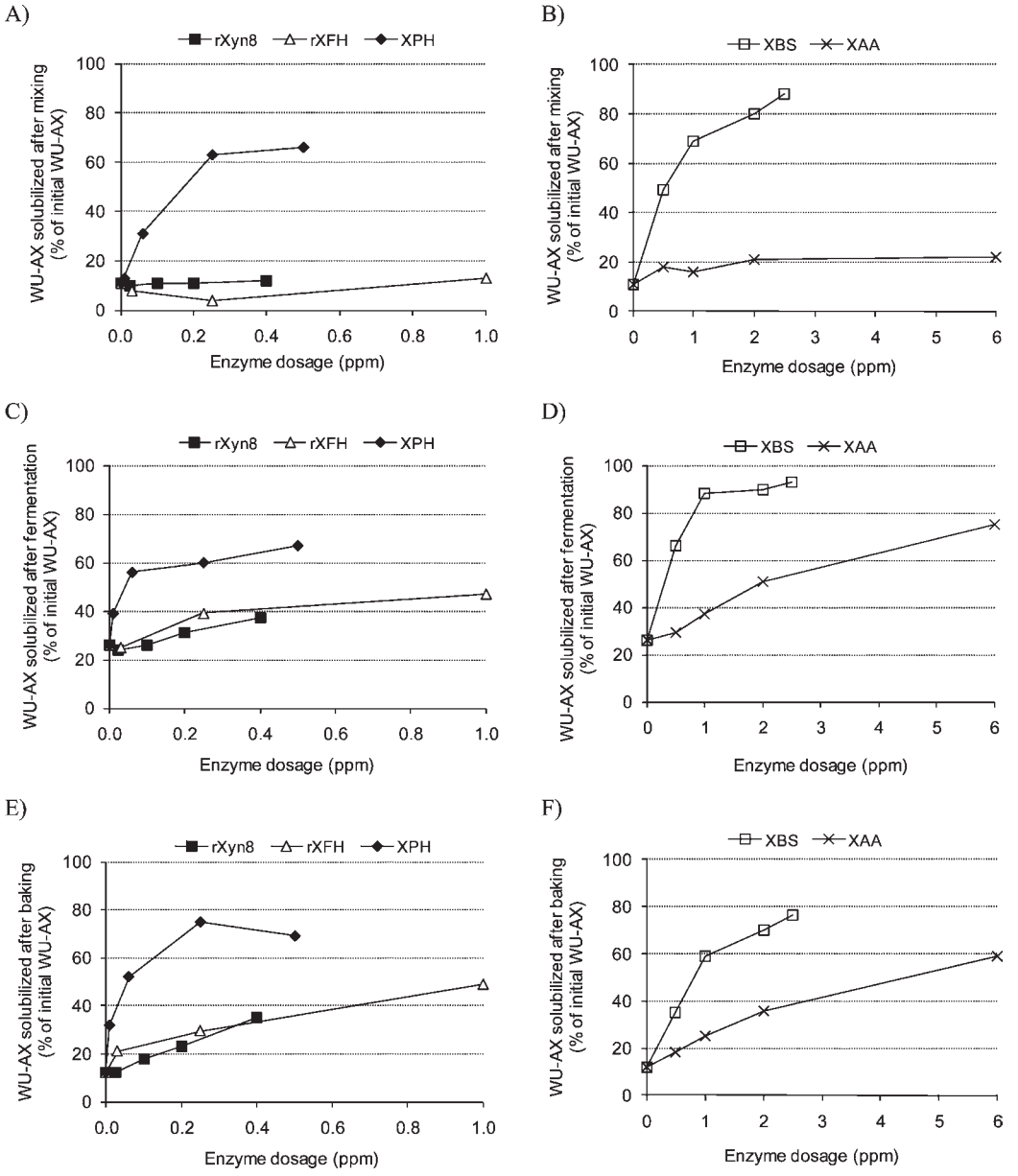

Figure 4. Solubilization of WU-AX in dough or bread samples [after mixing (A, B), fermentation (C, D), and baking (E, F)] made with the psychrophilic rXyn8, rXFH, and XPH (A, C, E) or mesophilic XBS and XAA (B, D, F) as a function of the enzyme dosage.

of the enzyme dosage—avDP response curves revealed that, at low enzyme dosages, GH8 rXyn8 degraded the AX population more intensively than any other xylanase. Comparison of both GH10 xylanases revealed that XAA degraded the AX population faster than rXFH, although both enzymes had the ability to hydrolyze the AX population to a comparable degree at the optimal dosages used.

The ΔELSD curves (results not shown) confirmed the data obtained by gas chromatography analysis. All xylanases were able to solubilize part of the AX population during mixing and more so for XPH and XBS. Due to this solubilization, all xylanases, except XAA, released high MM S-AX fragments larger than 100 kDa. While rXyn8 and rXFH solubilized WU-AX fragments with a MM below 400 kDa, XPH and XBS released S-AX fragments with MM between 400 and >1600 and between 400 and 1600 kDa, respectively, as well. The ΔELSD response in the high MM region decreased after fermentation, indicating that solubilization was followed by degradation of the S-AX. In contrast to what was observed for the other xylanases, XAA addition resulted in degradation of native WE-AX during fermentation, as evidenced by a negative peak in the ΔELSD curves. After baking, the level of high MM S-AX seemed higher in rXyn8-, XPH-, and rXFH-treated breads.

DISCUSSION

When added to the bread formulas, rXyn8, rXFH, and XPH caused maximal specific volume increases of 18%, 18%, and 28%, respectively, while XAA and XBS exerted maximal increases of 12% and 23%, respectively, when compared to the control bread. The volume increases obtained with mesophilic XAA and XBS as control xylanases were in line with previous results.[2] The strong positive effect of XPH on the bread volume also corresponded with previous observations by Collins et al.[12] The results with the

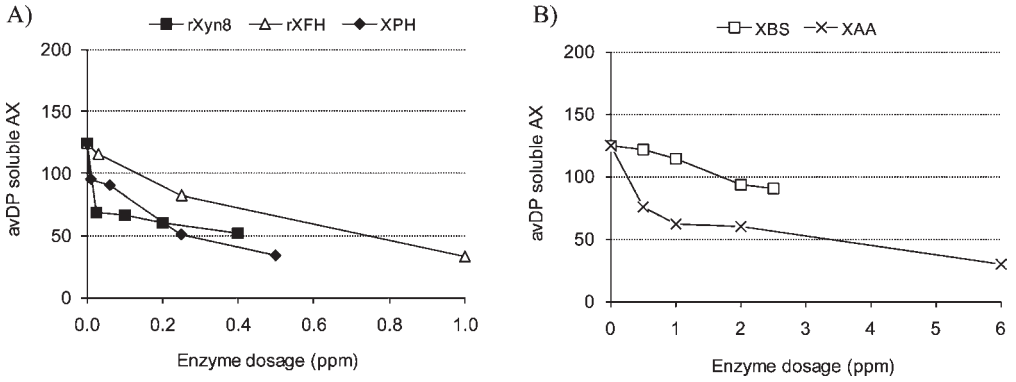

Figure 5. Average degree of polymerization (avDP) of soluble AX in bread made with the psychrophilic rXyn8, rXFH, and XPH (A) or mesophilic XBS and XAA (B) as a function of enzyme dosage.

other two psychrophilic xylanases rXyn8 and rXFH, however, were rather unexpected. Recently, Zheng et al.[13] also found a bread volume increase of about 26% with the psychrophilic xylanase from *G. mesophila*.

XPH (GH8) and XBS (GH11) hence had the most beneficial effect on bread volume, in line with their substrate hydrolysis profile. Both xylanases displayed high SSF and AR values, indicating that they preferentially solubilize WU-AX. They were indeed the only xylanases able to release a substantial amount of high MM S-AX during mixing. At optimal dosage, XPH generates more S-AX with higher MM during mixing than XBS. During fermentation, both xylanases solubilized an additional amount of WU-AX, but XPH went further in degradation of S-AX than XBS, resulting in stickier and slacker dough. Therefore, the bread volume increase with rXPH (28%) is probably the result of a decrease in the level of WU-AX and an increase in the level of high MM S-AX during mixing, while XBS yields an optimal bread volume increase (23%) through a more extensive solubilization of WU-AX and restricted degradation of high MM S-AX during the entire process. The less pronounced AX degrading potential of XBS during the process can most likely be related to its inhibition sensitivity toward TAXI. As already observed previously,[7,28,29] inhibition-insensitive xylanases induce stronger degradation of S-AX and WE-AX than inhibition-sensitive xylanases.

rXyn8 and rXFH displayed much more limited AX-solubilizing potential. As indicated by their low SSF values, these xylanases preferred WE-AX degradation over WU-AX solubilization. Analyzing aqueous extracts of dough and bread samples supplemented with an optimal dosage of rXyn8 or rXFH revealed that their beneficial effect on bread volume (up to 18% increase) is probably due to their capacity to decrease the WU-AX content during the prebaking phases while leaving the native WE-AX population unaffected during the process. The progressive slackening and softening of the supplemented dough can be ascribed to the reduction of the viscosity-forming properties and the water-holding capacity of the AX.

Although XAA had similar low SSF and AR values as rXFH and rXyn8, it was capable of solubilizing up to 75% of the WU-AX population during fermentation. At this stage low MM S-AX were generated, due to the high specific activity of the enzyme against soluble AX. In contrast to all the other xylanases, XAA also caused a degradation of native WE-AX during fermentation. The extensive degradation of WU-AX and the concomitant reduction in water-binding capacity of AX explains the negative impact of XAA of dough manageability. Conversely, as the WU-AX content further decreased and as the previously AX-bound water was available for film formation and gluten development, XAA exhibited a positive effect on bread volume. The fact that XAA can maximally improve bread volume by 12% can almost certainly be ascribed to the too strong degradation of native WE-AX and S-AX during fermentation, in part counteracting the positive effect of WU-AX solubilization.

From the results above, it is clear that xylanase functionality in bread making cannot be predicted from the GH as such. Xylanases belonging to the same GH can have dissimilar effects in bread making, while xylanases belonging to other GH can act similarly. Indeed, although GH11 xylanases generally have a higher preference for solubilizing WU-AX, and hence a higher SSF value compared to GH10 xylanases,[30−32] for GH8 xylanases, there is no clear trend. While XPH has a very high SSF value, comparable to that of the GH11 XBS, rXyn8 displays a lower value and hence resembles more the GH10 xylanases. The difference between the two GH8 xylanases is even more outspoken for the AR values. Also for substrate specificity, there are large differences between the different GH8 xylanases,[15] while it has generally been accepted that GH10 xylanases can release smaller products compared to GH11 xylanases due to their smaller substrate binding site.[33]

Remarkably, with psychrophilic xylanases, although not optimally active at the pH of dough, much lower enzyme dosages were needed to reach maximal bread volumes compared to XAA and XBS. Zheng et al.[13] and Collins et al.[34] also found that psychrophilic xylanases could be added in much lower dosages compared to the mesophilic ones. This is particularly relevant for industrial application and can probably partially be explained by the fact that psychrophilic xylanases, contrary to the mesophilic ones, are more active during mixing and fermentation. Furthermore, the psychrophilic xylanases tested in this study were insensitive toward the xylanase inhibitors in the wheat flour. This implies that lower dosages are needed compared to those needed for inhibition sensitive xylanases such as XBS.[7]

With regard to the impact of the xylanases on the nutritive value of bread, it can be concluded that addition of xylanases increased the S-AX and thus the soluble AX level in all breads. The highest soluble AX levels in bread (1.7%) were obtained when dough was supplemented with XPH or XBS, consistent with their high SFF and AR values. This implies that, taking the average bread consumption in Europe into account [180 g of fresh bread per person per day, average dm content of 65%[35]], 40% of the recommended daily intake for soluble fiber (i.e., > 5 g/day) is covered by such bread. Simultaneously with the formation of

S-AX, the avDP of soluble AX was gradually reduced during the process. Accordingly, the extent to which bread can be enriched in soluble AX fiber is determined by dough manageability. Within the boundaries of dough manageability, it was not possible to produce arabinoxylan-oligosaccharides with prebiotic potential[36] in situ using psychrophilic or mesophilic xylanases.

In conclusion, this study confirms that the positive effect of xylanases on bread volume is related to their substrate hydrolysis behavior. Both the amount and size of the S-AX produced during mixing and/or fermentation as well as the presence of native high MM WE-AX during the prebaking phases have an effect, as suggested by the previous research on xylanase functionality in bread making. Since the psychrophilic xylanases used are more active during the prebaking phases and insensitive toward xylanase inhibitors in wheat flour, much lower enzyme dosages were needed to reach optimal bread volumes when compared to the mesophilic xylanases. Although this is particularly relevant for industrial bread making, one should take into account that psychrophilic xylanases, due to their higher specific activity, could have a slightly higher deteriorating effect on dough manageability in comparison with the mesophilic ones. Finally, through the use of *P. haloplanktis* xylanase and *B. subtilis* xylanase, bread can substantially be enriched in soluble fiber.

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Funding Sources

This research is part of the Methusalem programme Food for the Future (2007−2014). Financial support from the 'Fonds voor Wetenschappelijk Onderzoek' (F.W.O., Brussels, Belgium) for the postdoctoral fellowship of E.D. and from the European Commission in the Communities 6th framework Programme for the Healthgrain Project (FP6-514008) is gratefully appreciated.

DISCLOSURE

This publication reflects only author's views and the Community is not liable for any use that may be made of the information contained in this publication.

ABBREVIATIONS USED

AR, activity ratio; avDP, average degree of polymerization; AX, arabinoxylan; AZCL-AX, azurine cross-linked arabinoxylan; ELSD, evaporative light scattering detector; GH, glycoside hydrolase family; MM, molecular mass; rXFH, recombinantly produced xylanase of *Flavobacterium* sp. MSY-2; rXyn8, recombinantly produced xylanase of an unknown bacterium; S-AX, solubilized arabinoxylan; SSF, substrate selectivity factor; TAXI, *Triticum aestivum* xylanase inhibitor; TLXI, thaumatin-like xylanase inhibitor; X_6, xylohexaose; XAA, xylanase of *Aspergillus aculeatus*; XBS, xylanase of *Bacillus subtilis*; XIP, xylanase inhibiting protein; XPH, xylanase of *Pseudoalteromonas haloplanktis* TAH3A; WE-AX, water-extractable arabinoxylan; WU-AX, water-unextractable arabinoxylan

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