MODIFICATION OF THE SECONDARY BINDING SITE OF XYLANASES ILLUSTRATES THE IMPACT OF SUBSTRATE SELECTIVITY ON BREAD MAKING

*Sofie Leys, Anneleen Pauly, Jan A. Delcour and Christophe M. Courtin**

Laboratory of Food Chemistry and Biochemistry and Leuven Food Science and Nutrition Research Centre (LFoRCe), KU Leuven, Kasteelpark Arenberg 20, B-3001 Leuven, Belgium

*** Corresponding author:**

Phone: +32 (0) 16 32 19 17

Fax: +32 (0) 16 32 19 97

E-mail address: christophe.courtin@biw.kuleuven.be

22 **ABSTRACT**

23 To investigate the importance of substrate selectivity for xylanase functionality in bread
24 making, the secondary binding site (SBS) of xylanases from *Bacillus subtilis* (XBS) and
25 *Pseudoalteromonas haloplanktis* was modified. This resulted in two xylanases with increased
26 relative activity towards water-unextractable wheat arabinoxylan (WU-AX) compared to water-
27 extractable wheat arabinoxylan, *i.e.* an increased substrate selectivity, without changing other
28 biochemical properties. Addition of both modified xylanases in bread making resulted in
29 increased loaf volumes compared to the wild-types when using weak flour. Moreover, maximal
30 volume increase was reached at a lower dosage of the mutant compared to wild-type XBS. The
31 modified xylanases were able to solubilize more WU-AX and decreased the average degree of
32 polymerization of soluble arabinoxylan in dough more during fermentation. This possibly
33 allowed for additional water release, which might be responsible for increased loaf volumes.
34 Altered SBS functionality and, as a result, enhanced substrate selectivity most probably caused
35 these differences.

36

37 **KEYWORDS**

38 Xylanase, arabinoxylan, bread making, substrate selectivity, secondary binding site

39 **1. INTRODUCTION**

40 Endo-β-1,4-xylanases (EC 3.2.1.8), also referred to as xylanases, are commonly used in bread
41 making processes to enhance dough manageability and bread quality. Although it is evident
42 that their functionality is the result of their capacity to hydrolyze β-(1,4)-linkages in the
43 backbone of arabinoxylan (AX), their mode of action in bread making is not yet fully
44 understood, despite much progress over the last decades[1-5].

45 It has been hypothesized that water-unextractable wheat AX (WU-AX) negatively affects bread
46 volume due to their strong water-holding capacity, while water-extractable wheat AX (WE-AX)
47 and solubilized wheat AX (S-AX) with a high molecular weight have a positive effect since they
48 form highly viscous aqueous solutions, stabilizing the dough foam structure[6-8]. Consequently,
49 xylanases with a high substrate selectivity, *i.e.* the ratio of the capacity to solubilize WU-AX over
50 the capacity to hydrolyze WE-AX, are desired in bread making. Such endoxylanases
51 preferentially solubilize WU-AX but hydrolyze WE-AX and S-AX only to a minimal extent[9].

52 To confirm this hypothesis, extensive research with xylanases that differed in substrate
53 selectivity was conducted[3,10-12]. Disadvantageous for these studies was the use of xylanases of
54 different microbial origins which differed in more biochemical properties than only their
55 substrate selectivity. The use of such xylanases made it difficult to investigate the influence of
56 substrate selectivity in bread making unambiguously.

57 Xylanase functionality in bread making is also influenced by inhibitors. Three different types of
58 xylanase inhibitors have been identified in wheat flour: *Triticum aestivum* xylanase inhibitor
59 (TAXI), xylanase inhibiting protein (XIP) and thaumatin-like xylanase inhibitor (TL-XI)[13]. Due to
60 the formation of enzyme-inhibitor complexes, inhibition sensitive xylanases are less efficient

61 than inhibition insensitive ones. These enzymes need higher dosages to ensure optimal
62 performance[14]. Finally, the temperature and pH activity profile of xylanases determines their
63 efficiency in bread making as well[15].
64 Recently, the existence of a secondary binding site (SBS) situated on the surface of the
65 structural unit was discovered in several single domain enzymes belonging to glycoside
66 hydrolase families (GH) 8, 10 and 11[16-18]. Several functions have been proposed for these SBSs,
67 including targeting of the enzyme towards its substrate, guiding substrate into the active site
68 groove, substrate disruption, enhancing processivity, allosteric regulation, passing on reaction
69 products and anchoring the enzyme to the cell wall of its parent microorganism. These
70 functions correspond well to the functions which are ascribed to carbohydrate-binding modules
71 (CBMs) in modular enzymes[19]. Possibly, SBSs compensate for the lack of CBMs in single domain
72 enzymes[20]. Site-directed mutagenesis to modify this SBS resulted in an increased activity on
73 water-unextractable substrates compared to water-extractable ones[21-23], hence effectively
74 changing the substrate selectivity of the enzyme.
75 In particular, the GH8 xylanase of *Pseudoalteromonas haloplanktis* (XPH) and GH11 xylanase
76 XynA of *Bacillus subtilis* (XBS) are both single domain enzymes which contain an SBS. XBS has a
77 β-jelly roll fold structure, which is often compared to a partially closed right hand, with an SBS
78 located on the 'knuckles' of the enzyme[24]. XPH is a psychrophilic enzyme with a $(\alpha/\alpha)_6$-barrel
79 structure (Fig. 1)[25]. Since both enzymes show good performance in bread making, they are ideal
80 tools to investigate the influence of substrate selectivity on xylanase functionality in bread
81 making.

82 The aim of this study is to validate the abovementioned hypothesis that substrate selectivity
83 determines, at least in part, the intrinsic quality of xylanases for bread making. This is done by
84 selectively modifying the substrate selectivity of XBS and XPH xylanases through modification of
85 their SBS, and studying the impact thereof on the bread making process. This way, nearly
86 identical xylanases are used, which differ only in substrate selectivity and not in other
87 biochemical properties. Therefore, XBS and XPH were modified in their SBS and added to dough
88 and bread followed by evaluating their impact on the AX population and aqueous extract
89 viscosity of dough and bread samples. These results were compared to those obtained with the
90 wild-type enzymes. We here report on the outcome of this work.
91
92 **2. MATERIALS AND METHODS**

93 **2.1 Materials**

94 *Escherichia coli* cells, transformed with expression plasmid pEXP5-CT-*xyna*, were available for
95 heterologous expression of XBS wild-type (XBS WT) (UniProtKB P18429). A stop codon was
96 introduced in the plasmid after the last nucleotide encoding for the C-terminal amino acid of
97 the native protein (W185)[23]. For expression of XPH, *E. coli* cells transformed with expression
98 plasmid pEXP5-CT-*xph* which contained a *de novo* synthesized XPH wild-type (XPH WT) gene
99 (GenBank AJ427921.1), were also available[22]. A triple mutant plasmid of XBS (G56A-T183A-
100 W185A) and a double mutant plasmid of XPH (W249A-Y315A), hereafter referred to as XBS 3A
101 and XPH 2A, respectively, were constructed using a QuickChange site-directed mutagenesis kit
102 (Stratagene, La Jolla, CA, USA) as described by Cuyvers et al.[23] and were available in
103 transformed *E. coli* cells. The selection of amino acids for modification was based on the crystal

104 structures of XBS and XPH soaked with oligosaccharides[16,17]. Amino acids which were able to
105 interact with the substrate by hydrophobic stacking interactions or hydrogen bonds were
106 replaced by alanine to reduce their role in binding of substrate at the SBS (Fig. 1). Xylazyme AX
107 tablets, liquid Azo-wheat AX and xylooligosaccharides up to xylohexaose (X_6) were obtained
108 from Megazyme (Bray, Ireland). Xylazyme AX tablets and liquid Azo-wheat AX both contained
109 highly purified AX from wheat, while the xylooligosaccharides (purity > 90%) were prepared
110 from birchwood xylan. Two European wheat flours, free from additives, were used in bread
111 making. Crousti flour was a commercial bread wheat flour obtained from Dossche Mills (Deinze,
112 Belgium). Soft wheat cultivar Claire from Limagrain (Rilland, The Netherlands) was conditioned
113 to 16.0% moisture and subsequently milled with a Bühler MLU-202 laboratory mill (Bühler AG,
114 Uzwil, Switzerland), yielding three break and three reduction fractions which were combined[26].
115 Protein contents [% dry matter (dm), N x 5.7] were 13.2% and 10.6% for Crousti and Claire
116 flour, respectively, determined using an adaptation of the AOAC Official Method[27] to an
117 automated Dumas protein analysis system (Vario Max Cube, Elementar, Hanau, Germany). Ash
118 contents (% dm) were 0.55% and 0.48%, measured according to Approved Method 08-01.01[28],
119 while the total AX (% dm) and WE-AX contents (% dm) for Crousti and Claire flour were 1.85%
120 and 0.28% and 1.76% and 0.48%, respectively (analyzed as described in Section 2.2.5). Fresh
121 compressed baker's yeast was from AB Mauri (Merelbeke, Belgium). Sodium chloride and
122 sucrose used in the bread making trials were food grade. All chemicals, solvents and reagents
123 were purchased from Sigma-Aldrich (Bornem, Belgium) and were analytical grade, unless
124 specified otherwise.

125

126 **2.2 Methods**

127 ***2.2.1 Recombinant expression and purification***

128 The recombinant expression of XBS, XPH and their mutant variants in *E. coli* BL21(DE3)pLysS
129 cells was according to Van Craeyveld et al. (2010). Subsequently, the xylanases were purified by
130 cation exchange chromatography as described elsewhere[29,30]. The enzyme yields of
131 recombinant XBS and XPH after purification were typically 50-90 mg/L and 100-180 mg/L of
132 culture, respectively. Xylanase purity was verified by SDS-PAGE and silver staining performed on
133 a PhastSystem Unit (GE Healthcare, Uppsala, Sweden) according to GE Healthcare separation
134 technique file 110 and development technique file 210, respectively[31]. Protein concentration of
135 the purified enzymes was estimated by measuring the extinction at 280 nm in triplicate with a
136 Nanodrop-1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) using the
137 molar extinction coefficients calculated with the ProtParam tool
138 (http://expasy.org/tools/protparam.html). These calculations were based on the amino acid
139 sequences of the wild-type and mutant xylanases. Errors in the extinction at 280 nm due to
140 modifications in the amino acid structure were negligible.

141 ***2.2.2 Biochemical characterization of xylanases***

142 <u>2.2.2.1 Specific activities towards different substrates</u>

143 The specific activities of the wild-type and mutant xylanases were determined towards different
144 substrates in order to assess the relative preference for these substrates. All enzyme activities
145 were measured at dough pH, which is 5.5[32], in a 100 mM sodium acetate buffer containing 0.50
146 mg/mL bovine serum albumin (BSA). The pre-incubation and incubation temperature were 30°C
147 in all assays, *i.e.* the dough fermentation temperature.

148 Specific xylanase activities against WU-AX and WE-AX were estimated using the polymeric
149 chromophoric substrates Xylazyme AX and Azo-wheat AX, respectively, as described by Cuyvers
150 et al.[23]. The basis for these measurements were changes in solubility in water or in a water-
151 ethanol mixture, respectively, after hydrolysis of the chromophoric substrate by the xylanase.
152 The color intensity of the soluble fragments was then proportional to the xylanase activity. For
153 the determination of the specific activity on Xylazyme AX, an appropriately diluted enzyme
154 solution (1000 µL) (measurements in the linear area of enzyme concentration *vs.* extinction)
155 was equilibrated for 10 min. Then a Xylazyme AX tablet was added. After 60 min of incubation,
156 the reaction was terminated by addition of 10.0 mL 1.0% (w/v) Tris(hydroxymethyl)-
157 aminomethane, vigorous vortex stirring and immediate filtration through a MN 615 filter
158 (Macherey-Nagel, Düren, Germany). The extinction of the filtrate at 590 nm (E_{590}) [Ultraspec III
159 UV/vis spectrophotometer (Pharmacia Biotech, Uppsala, Sweden)] against a control, prepared
160 by incubating the enzyme solution without substrate. One Xylazyme unit (Xyl-U) corresponds to
161 the enzyme concentration required to obtain an E_{590} of 1.0, under the conditions of the assay.
162 The specific activity on Azo-wheat AX was determined by first pre-incubating an appropriately
163 diluted enzyme solution and the liquid substrate separately for 10 min. After addition of 500 µL
164 of the substrate to 500 µL of the enzyme solution, incubation was extended for 60 min after
165 which the reaction was terminated by addition of 2.5 mL of ice cold ethanol and vigorous vortex
166 stirring. The samples were kept on ice for 10 min. After centrifugation for 10 min at 4000 *g* and
167 4°C in a Sigma 6-16K centrifuge (Sigma Zentrifugen, Osterode am Harz, Germany), the E_{590} of
168 the supernatant was measured against a control, prepared by incubating the enzyme solution
169 without substrate. One Azo-wheat unit (Azo-U) was defined as the enzyme concentration

170 needed to increase E_{590} with 1.0 under the conditions of the assay. The substrate selectivity
171 factor (SSF), defined as the relative preference of xylanases towards WU-AX or WE-AX, was
172 calculated as the ratio of the specific activity toward Xylazyme AX to that toward Azo-wheat AX.
173 Specific xylanase activities against X_6 and reaction products of this hydrolysis were determined
174 by high-performance anion-exchange chromatography followed by pulsed amperometric
175 detection. Sample preparation and quantification of the formed hydrolysis products was done
176 as described by Cuyvers *et al.*[23]. One enzyme unit on X_6 (X_6-U) was then defined as the
177 concentration of enzyme needed for the formation of 1.0 µM xylotriose.

178 _2.2.2.2 Temperature and pH dependency_

179 Temperature dependency of xylanase activity was determined by measuring the activity on
180 Xylazyme AX, under the conditions described above (*cf.* 2.2.2.1), with incubation temperatures
181 ranging from 10 to 80°C with intervals of 10°C. To determine pH dependency of xylanase
182 activity, enzyme dilutions were made in potassium chloride/hydrogen chloride (20 mM, pH 2.0),
183 sodium acetate (100 mM, pH 3.0, 4.0 and 5.0), sodium phosphate (20 mM, pH 6.0, 7.0, 8.0 and
184 9.0), sodium carbonate (50 mM, pH 10.0 and 11.0) and potassium chloride/sodium hydroxide
185 (20 mM, pH 12.0) buffers, all containing 0.50 mg/mL BSA. The enzyme activities of these
186 enzyme solutions on Xylazyme AX were measured at 30°C as described above.

187 _2.2.2.3 Inhibition sensitivity_

188 Wheat flour was suspended in sodium acetate buffer (100 mM) at pH 5.5 (mass ratio 1:10
189 flour:buffer). This suspension was shaken for 30 min at 7°C (Laboshake, VWR International,
190 Leuven, Belgium) and centrifuged (10 min, 4000 g, 7°C), after which the supernatant, containing
191 xylanase inhibitors, was isolated. Xylanase inhibition sensitivity was then measured by

192 incubating an appropriate xylanase dilution (measurements in linear area of enzyme
193 concentration *vs.* extinction) (500 µL; 100 mM sodium acetate buffer pH 5.5) for 30 min at 30°C
194 with the Crousti wheat flour extract (500 µL). Subsequently, the xylanase activity on Xylazyme
195 AX was measured as described above against a control made from wheat flour extract.
196 Inhibition sensitivity was expressed as a reduction (%) of xylanase activity.

197 ***2.2.3 Bread making trials***

198 Dough pieces and bread loaves were produced in triplicate at 10 g scale, according to the
199 straight-dough method[33]. Flour (10.0 g, 14% moisture base), 0.53 g fresh yeast, 1.5 g sodium
200 chloride, 6.0 g sucrose, deionized water (4.86 mL for Crousti and 4.20 mL for Claire) and 1.0 mL
201 enzyme solution (dialyzed overnight against 100 mM sodium acetate buffer pH 5.5 (volume
202 ratio 1:160 enzyme solution:buffer)) were mixed in a 10 g pin mixer (National Manufacturing,
203 Lincoln, NE, USA) during 4.0 and 2.5 min for Crousti and Claire flour, respectively. The dough
204 baking absorption and mixing times were based on Farinograph (Brabender, Duisburg,
205 Germany) and Mixograph (National Manufacturing, Lincoln, NE, USA) analyses, respectively[34,35].
206 The xylanase activities of the dialyzed enzyme solutions were measured against Xylazyme AX
207 (as described in Section 2.2.2.1). For XBS and its mutant, dosages between 100 and 700 Xyl-
208 U/kg of flour were used, while for XPH and its mutant, dosages between 5 and 35 Xyl-U/kg of
209 flour were used. For control dough and bread, the enzyme solution was replaced by 1.0 mL of
210 sodium acetate buffer (100 mM, pH 5.5). The dough was fermented for 90 min in a
211 fermentation cabinet (National Manufacturing) at 30°C and 90% relative humidity with
212 intermediate punching at 52 and 77 min. After final punching at 90 min, the dough pieces were
213 molded and proofed for 36 min (30°C and 90% relative humidity). Finally, baking was performed

214 in a rotary oven (National Manufacturing) for 13 min at 232°C and the bread was immediately
215 weighed after baking. Loaf volume was measured 120 min after baking by rapeseed
216 displacement[36]. Dough (immediately after mixing and proofing) and bread samples were frozen
217 in liquid nitrogen. After lyophilization, they were ground with a laboratory mill (model A10, IKA-
218 Werke GmbH and Co. KG, Staufen, Germany) and sieved (Ø = 250 µm) before further analysis.

219 ***2.2.4 Preparation of aqueous extracts from flour, dough and bread samples***

220 Aqueous extracts of flour, (fermented) dough and bread samples were prepared by suspending
221 the samples (1.0 g) in 10.0 mL potassium chloride/hydrogen chloride (20 mM) buffer at pH 3.0.
222 Under these conditions, acid hydrolysis did not occur and enzymatic breakdown of AX was
223 minimized (results not shown). The suspensions were shaken for 30 min at 7°C (Laboshake,
224 VWR International, Leuven, Belgium) and, after centrifugation (10 min, 4000 g, 7°C), the
225 supernatants were analyzed immediately or frozen until further analysis.

226 ***2.2.5 AX levels and compositions***

227 The carbohydrate contents and compositions obtained after hydrolysis of flour or aqueous
228 extracts of flour, dough or bread samples, prepared as described in 2.2.4, were determined by
229 gas chromatography. Hydrolysis of flour (10.0-15.0 mg) was performed in 2.0 M trifluoroacetic
230 acid (TFA) (5.0 mL), while aqueous extracts (2.5 mL) were hydrolyzed in 4.0 M TFA (2.5 mL),
231 both for 60 min at 110°C. Reduction and acetylation were performed with sodium borohydride
232 and acetic anhydride, respectively, according the procedure of Englyst and Cummings[37]. To
233 measure reducing end xylose and arabinose contents, reduction was performed preceding the
234 hydrolysis[38]. A combination of reduction and acetylation, without prior hydrolysis, was
235 performed to determine free xylose and arabinose. The formed alditol acetates (1.0 µl) were

236 separated on a Supelco SP-2380 polar column (30 m x 0.32 mm inner diameter; 0.2 µm film
237 thickness) (Supelco, Bellefonte, PA, USA) in an Agilent chromatograph (Agilent 6890 series,
238 Wilmington, DE, USA) equipped with autosampler, splitter injection port (split ratio 1:20), and
239 flame ionization detector. The carrier gas was helium. Separation was at 225°C while injection
240 and detection were at 270°C. The total AX and WE-AX content of flour and the AX content in
241 dough and bread extracts was 0.88 times the sum of the xylose and arabinose contents. The
242 total arabinose content was corrected for free arabinose as well as arabinose originating from
243 arabinogalactan-peptides (AGP)[39]. The total xylose content was corrected for the presence of
244 free xylose. All contents were expressed on a dm base. The average degree of polymerization
245 (avDP) of the soluble AX was calculated as the sum of the xylose and arabinose content divided
246 by the reducing end xylose content. The arabinose content was corrected for free arabinose
247 and arabinose present in AGP, while the xylose and reducing xylose content was corrected for
248 free xylose.

249 ***2.2.6 Viscosity***

250 The viscosity of extracts of flour, dough and bread samples (500 µL), prepared as described in
251 2.2.4, was determined with a Brookfield DV II+ viscometer (Brookfield Engineering Laboratories
252 Inc., Stoughton, MA, USA) at 30°C with a CP40 cone and a constant shear rate of 60 s^{-1}. The
253 specific viscosity was defined as the relative viscosity, *i.e.* the ratio of the viscosity of the extract
254 and that of a potassium chloride/hydrogen chloride (25 mM; pH 3.0) buffer, minus one[3].

255 ***2.2.7 Statistical analysis***

256 Specific activities towards Xylazyme AX and Azo-wheat AX were determined in fivefold, while all
257 other analyses were done in triplicate. All data were analyzed using statistical software JMP Pro

258 11 (SAS Institute, Cary, NC, USA) to verify whether mean values were significantly different at a
259 difference level (α) of 0.05 using the two-way ANOVA procedure.
260

261 **3. RESULTS**

262 **3.1 Biochemical characterization of xylanases**

263 Biochemical properties of XBS and XPH enzymes modified in their SBS were compared to those
264 of their corresponding wild-type enzymes to investigate whether modifying the SBS of a
265 xylanase influences the SSF without affecting other biochemical properties.

266 ***3.1.1 Specific activity towards different substrates***

267 Since X_6 is too small to reach the active site and SBS simultaneously, it is an ideal substrate to
268 measure differences in inherent activity[23]. For XBS, mutations in the SBS had no effect on the
269 activity on X_6. In contrast, the activity on X_6 increased by 15% by mutating the SBS of XPH WT
270 (Table 1). The hydrolysis pattern of X_6 was identical for all mutant xylanases and their wild-type
271 counterpart (results not shown).

272 On both Xylazyme AX and Azo-wheat AX, significant differences in activity were revealed after
273 mutating the SBS. XBS 3A showed only 34% of the activity of XBS WT for Azo-wheat AX, while
274 for Xylazyme AX, XBS 3A activity was 43% of that of the wild-type enzyme. In contrast to XBS,
275 the activities on both chromophoric substrates increased for XPH 2A compared to its wild-type.
276 Especially the activity on Xylazyme AX (155%) increased, while the activity increase on Azo-
277 wheat AX (128%) was less pronounced. Since the activity towards water-unextractable
278 Xylazyme AX was higher for both mutant xylanases than towards water-extractable Azo-wheat
279 AX, the SSF increased by 26% and 21% for XBS and XPH, respectively (Table 1).

280 ***3.1.2 Temperature and pH dependency of xylanase activity***

281 The wild-type and modified XBS showed maximal xylanase activity at 50°C, while the optimal
282 temperature was 30°C for the psychrophilic wild-type XPH and its mutant (Table 1).
283 Maximal activity was at pH 6.0 to 7.0 for XBS and at pH 9.0 for XPH, for both wild-type enzymes
284 as well as their mutant counterparts (Table 1).

285 ***3.1.3 Inhibition sensitivity***

286 The addition of a wheat flour extract reduced the xylanase activity of the XBS wild-type and
287 mutant with 88% and 90% respectively, compared to the activity measurement in absence of
288 wheat flour extract. Neither XPH wild-type nor mutant were affected by inhibitors.

289 **3.2 Functionality of XBS and XPH in bread making**

290 ***3.2.1 Changes in dough and bread properties***

291 The addition of wild-type and mutant xylanases to the bread making process influenced the
292 manageability of the dough. After mixing, dough supplemented with XPH enzymes felt drier
293 compared to dough supplemented with XBS enzymes. After fermentation, dough
294 supplemented with XPH enzymes became more sticky, while the dough manageability of dough
295 supplemented with XBS enzymes improved. Dough manageability after 90 minutes
296 fermentation limited the enzyme dosages that can be used to 700 Xyl-U/kg flour for XBS
297 enzymes and 35 Xyl-U/kg flour for XPH enzymes but no differences were observed between the
298 wild-type and mutant xylanases.

299 Addition of xylanases significantly increased specific loaf volumes, for both flour types used
300 (Fig. 2). Only at 700 Xyl-U/kg Crousti flour, the specific loaf volume obtained with XBS 3A was
301 significantly lower than with XBS WT. In bread making with Claire flour, the maximal increase in

302 specific loaf volume was reached at a lower dosage for XBS 3A (300 Xyl-U/kg flour) compared to
303 XBS WT (500 Xyl-U/kg flour). Moreover, the volume increase at optimal dosages was higher
304 when XBS 3A was used. For Crousti flour, no differences in specific loaf volume were observed
305 when using the wild-type or mutant XPH, regardless of the dosage used (Fig. 2C). Maximal
306 volume increases of 20.3% and 19.1% were obtained with XPH WT and XPH 2A, respectively, at
307 an enzyme dosage of 25 Xyl-U/kg flour. Specific volumes of the Claire loaves supplemented with
308 XPH 2A were higher than those supplemented with XPH WT, for all dosages tested (Fig. 2D).
309 Maximal volume increases of 23.3% and 31.9% were obtained with XPH WT and XPH 2A,
310 respectively.

311 ***3.2.2 AX properties during bread making***

312 To evaluate changes in solubilized WU-AX and avDP, the AX population was monitored during
313 different steps of the bread making process (Fig. 3 and 4). For this, enzyme dosages of 300 Xyl-
314 U/kg flour for XBS wild type and mutant and 25 Xyl-U/kg flour for XPH wild type and mutant
315 were used since these dosages resulted in significant differences in volume of Claire bread
316 between wild-type and mutant xylanases (*cf.* 3.2.1).

317 *3.2.2.1 Solubilization of WU-AX*

318 The level of WU-AX solubilization in dough after mixing and fermentation was significantly
319 higher when xylanases were used (Fig. 3). For Crousti as well as Claire bread making, no
320 differences in solubilized WU-AX content after mixing were observed between incorporation of
321 the wild-type and mutant XBS. During the fermentation stage, XBS 3A solubilized more WU-AX
322 than its wild-type counterpart (Table 2). Immediately after mixing, solubilization of WU-AX in
323 Claire flour had progressed less for XPH 2A compared to the wild-type counterpart. Again, the

324 mutant XPH was able to solubilize more WU-AX during fermentation compared to the wild-
325 type. After baking, the amount of solubilized WU-AX was decreased for all samples tested.

326 *3.2.2.2 avDP of the soluble AX fraction*

327 During mixing and fermentation, the avDP of the soluble AX fraction of xylanase supplemented
328 doughs was significantly lower compared to control dough samples (Fig. 4). When XBS enzymes
329 were incorporated in Crousti bread, no differences between the wild-type and mutant xylanase
330 were observed after mixing. During fermentation, XBS WT was not able to further lower the
331 avDP of the soluble AX fraction and this in contrast to XBS 3A (Table 2). When XBS was added to
332 bread making with Claire flour, the avDP of the soluble AX fraction was already lower after the
333 mixing stage when XBS WT was compared to XBS 3A. During fermentation, the avDP of the
334 soluble AX fraction further decreased. Since this decrease was more outspoken for XBS 3A,
335 there was no significant difference anymore between the wild-type and mutant XBS after
336 fermentation. Furthermore, the avDP of the soluble AX population increased after baking,
337 except when XBS WT was added to Crousti bread. For the control breads, in contrast, avDP
338 decreased after baking.

339 The results obtained with XPH were similar to those of XBS (Fig. 4). After mixing and
340 fermentation in Crousti bread making, no differences were observed between the wild-type
341 and mutant XPH. However, the avDP of the soluble AX decreased more pronouncedly during
342 fermentation when using the mutant XPH (Table 2). After baking, the avDP of the soluble AX
343 fraction was significant lower with XPH 2A than with XPH WT. For Claire bread making, the avDP
344 of the soluble AX fraction after mixing was lower for the wild-type XPH compared to the

345 mutant. During fermentation, the decrease in avDP was more outspoken for XPH 2A. The avDP
346 of the soluble AX population increased again after baking.

347 ***3.2.3 Extract viscosity at different phases of bread making***

348 While XBS enzymes had no impact on extract viscosity after mixing, Crousti dough
349 supplemented with XBS enzymes showed a significantly lower extract viscosity after
350 fermentation than control dough (Fig. 5). No differences were observed between XBS WT and
351 XBS 3A. When XBS enzymes were used in dough and bread samples made with Claire flour, no
352 differences were observed compared to the control.

353 Supplementation of XPH had no impact on extract viscosity after mixing. Dough and bread
354 supplemented with XPH enzymes showed a significant lower extract viscosity after
355 fermentation and baking than control dough and bread. No differences in specific viscosity
356 were noticed between addition of the wild-type and mutant XPH.

357

358 **4. DISCUSSION**

359 Modifying the SBS of XBS and XPH by replacing aromatic amino acids responsible for binding of
360 the substrate by an inert alanine residue resulted in an increased **SSF** and, hence, an increased
361 preference for WU-AX (Table 1). Other biochemical properties were not influenced by these
362 mutations. As shown for XPH 2A, modifying of the SBS did not automatically decrease the
363 activity on polymeric substrates as the activity of the mutant XPH on Xylazyme AX and Azo-
364 wheat AX was increased by 55% and 28%, respectively, compared to XPH WT. With the wild-
365 type XPH, the substrate was probably bound too tightly to the SBS for optimal activity.
366 Modifying this position could therefore allow a better positioning or faster throughput of the

367 substrate with respect to the active site. As mentioned by Cuyvers *et al.*[23], the increased activity
368 of XPH 2A towards X_6 was probably the result of subtle positional change of residues located in
369 the active site after modification of the SBS. Since modifying the SBS provided xylanases that
370 only differed in SSF, they were ideal tools to investigate the specific influence of substrate
371 selectivity in bread making.
372
373 Equivalent units (Xyl-U) of wild-type and mutant xylanases were added to bread making
374 processes based on Crousti and Claire flour. Differences in **dough manageability** were observed
375 after addition of XBS and XPH. Dough supplemented with XPH was dry after mixing but became
376 more sticky during fermentation, while the inverse was observed for XBS. This can be explained
377 by the inhibition sensitivity of the xylanases: XPH is not sensitive to inhibitors and keeps
378 working during the entire process, while the activity of XBS, which is inhibited by TAXI[40], most
379 probably decreases rapidly during bread making. Differences in inhibition sensitivity also
380 explain why XPH was added in lower dosages than XBS. Modifying the SBS had no impact on
381 inhibition sensitivity.
382
383 While little if any significant differences in **specific loaf volume** were observed between wild-
384 type and mutant xylanases in bread making with Crousti (Fig. 2), an additional volume increase
385 of 7.1% and 8.6% was obtained after supplementation of the mutant XBS and mutant XPH,
386 respectively, at their optimal dosages in Claire bread making. The stronger gluten network of
387 dough made with Crousti flour probably masked possible additional improvements of the

388 mutant xylanases. In contrast, when using the weak Claire flour, the effects of incorporation of
389 xylanases with different SSF were more pronounced.
390
391 Since equivalent units (Xyl-U) of wild-type and mutant xylanases were used in bread making, it
392 was expected that also equivalent amounts of WU-AX were solubilized during the different
393 bread making stages. The amount of solubilized WU-AX after **mixing** was indeed similar for
394 wild-type and mutant xylanases (Fig. 3). The increase in solubilized WU-AX was accompanied by
395 a decrease in the avDP of the soluble AX population (Fig. 4). It is possible that the fragments
396 derived from solubilizing WU-AX had a lower avDP than the native WE-AX present in flour.
397 Additionally, native WE-AX and S-AX might have been hydrolyzed into smaller fragments, which
398 would also result in a lower avDP. These fragments would have a minimal chain length of five
399 xylose molecules since XPH and XBS are not able to hydrolyze substrates with a degree of
400 polymerization lower than six.[41] However, since XBS 3A and XPH 2A had a lower activity
401 towards WE-AX and S-AX than the wild-type enzymes (Table 1), we had expected that
402 incorporation of the mutant xylanases in bread making would result in differences in avDP of
403 the soluble AX fraction. This was not the case and, based on this, it can be concluded that the
404 lower avDP of solubilized WU-AX was probably responsible for the decrease in overall avDP.
405
406 During **fermentation**, an additional amount of WU-AX was solubilized (Fig. 3 and Table 2).
407 Especially for Crousti bread making, this was not least caused by the xylanase activity of the
408 flour itself, as shown for the control dough. For xylanase supplemented dough samples, the
409 amount of additionally solubilized WU-AX was lower compared to what was solubilized earlier

410 during the mixing stage. Both mutant xylanases solubilized WU-AX to a larger extent and
411 decreased the avDP of soluble AX more strongly compared to their respective wild-types. This is
412 remarkable since the same activity on water-unextractable substrate of wild-type and mutant
413 xylanases were added. In the past, several functions have already been described for SBSs[20].
414 Possibly, a number of these functions was slightly enhanced after modification and ensured the
415 xylanase to become more active. This resulted in a more convenient hydrolysis for the mutant
416 xylanases compared to the wild-type.
417 XPH degraded S-AX more severely than XBS as deduced from the avDP of soluble AX after
418 fermentation (Fig. 4). A reason for the lower hydrolyzing capacity of XBS is probably its
419 inhibition sensitivity towards TAXI, as this inhibitor was present in excess in wheat flour. It has
420 already been shown that inhibition-insensitive xylanases show a stronger degradation of S-AX
421 and WE-AX than inhibition-sensitive ones[11,14].
422 WE-AX with a high molecular weight have a high viscosity forming capacity and are able to
423 stabilize the liquid films surrounding the gas cells in bread dough[42]. Despite differences in
424 solubilization degree between wild-type and mutant xylanases, no differences in specific
425 viscosity were observed (Fig. 5). Liquid films in bread dough are indeed more concentrated than
426 the extracts used for measuring specific viscosity. Alternatively, it is possible that differences in
427 bread loaf volume are only determined by WU-AX solubilization degree and accompanying
428 water release and not by the bulk viscosity of aqueous phase. Roels and coworkers (1993)[43]
429 showed that increasing the baking absorption up to 10% above the Farinograph baking
430 absorption results in higher loaf volumes. This could also explain the increased specific loaf
431 volume when mutant xylanases where added to bread making. Since Claire flour is a weak flour

432 type, the impact of additional water release due to degradation of WU-AX might be more
433 pronounced.
434
435 During the early **baking** phase, it was expected that XPH would still hydrolyze AX, what would
436 then result in an additional decrease of the avDP of the soluble AX fraction. A decrease in
437 solubilized WU-AX level was, however, observed (Fig. 3). This indicates that part of the
438 previously solubilized AX turned unextractable again, probably due to crosslinking of AX
439 molecules with other AX molecules or flour components and/or due to physical inclusion in the
440 gelatinized starch matrix since no amylase was added during the extraction[3,44,45]. For Claire
441 bread making, it appears that AX molecules with a low molecular weight preferentially turned
442 unextractable again since the avDP was increased after baking (Fig. 4). On the one hand, smaller
443 AX molecules are more mobile and less sensitive to steric hindrance, what makes that they can
444 cross-link more easily with other constituents. On the other hand, the fact that small molecules
445 are more mobile renders them also easier to extract.
446
447 In **conclusion**, this study shows that increasing the preference of a xylanase for water-
448 unextractable substrates compared to water-extractable ones, *i.e.* increasing its substrate
449 selectivity, can enhance its functionality in bread making. Due to increased WU-AX
450 solubilization during fermentation, these xylanases ensure that more of the WU-AX that is
451 detrimental in bread making is converted into S-AX. This possibly allows for additional water
452 release, responsible for an increase in specific loaf volume and/or a lower optimal dosage when
453 weak flour was used. With the experimental approach followed here, viscosity differences

454 could not be pinpointed as driving mechanism for differences between wild type and mutant
455 enzymes. These conclusions, of course, have to be seen in the right perspective. The
456 functionality of xylanases in bread making is determined by more biochemical properties than
457 only their substrate selectivity.

458

459 **ABBREVIATIONS**

460 α, difference level; AGP, arabinogalactan-peptides; avDP, average degree of polymerization; AX,
461 arabinoxylan; Azo-U, Azo-wheat unit; BSA, bovine serum albumin; CBMs, carbohydrate-binding
462 modules; dm, dry matter; E_{590}, extinction at 590 nm; GH, glycoside hydrolase family; S-AX,
463 solubilized AX; SBS, secondary binding site; SSF, substrate selectivity factor; TAXI, *Triticum*
464 *aestivum* xylanase inhibitor; TFA, trifluoroacetic acid; TL-XI, thaumatin-like xylanase inhibitor;
465 WE-AX, water-extractable AX; WT, wild-type; WU-AX, water-unextractable AX; X_6, xylohexaose;
466 X_6-U, xylohexaose unit; XBS, xylanase XynA of *Bacillus subtilis*; XBS 3A, G56A-T183A-W185A
467 mutant of xylanase XynA of *Bacillus subtilis*; XIP, xylanase inhibiting protein; XPH, xylanase of
468 *Pseudoalteromonas haloplanktis*; XPH 2A, W249A-Y315A mutant of xylanase of
469 *Pseudoalteromonas haloplanktis*; Xyl-U, Xylazyme unit

470

471 **FUNDING SOURCES**

472 Sofie Leys acknowledges the Agency for Innovation through Science and Technology in Flanders
473 (IWT-Vlaanderen, Brussels, Belgium) for financial support. Anneleen Pauly wishes to
474 acknowledge the Research Foundation - Flanders (FWO-Vlaanderen, Brussels, Belgium) for a

475 position as postdoctoral researcher. This research is also part of the Methusalem programme

476 'Food for the future' (2007-2014) at the KU Leuven.

477

478 The authors declare no competing financial interest.

479

480 **ACKNOWLEDGEMENTS**

481 Dr. Emmie Dornez (Mauri Research, the Netherlands) is gratefully thanked for fruitful

482 discussions.

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601

Figure captions

Figure 1. On the left, the overall structures of the xylanases from *Bacillus subtilis* (XBS) (PDB 2QZ3) (A) and *Pseudoalteromonas haloplanktis* (XPH) (PDB 2B4F) (B) in complex with xylooligosaccharides indicate the presence of a secondary binding site (SBS) on the surface of the enzyme. On the right, the amino acids responsible for binding of the oligosaccharides to the SBS are indicated for XBS (A) and XPH (B). All figures were drawn using PyMOL (http://pymol.sourceforge.net/).

Figure 2. Specific loaf volumes of the breads in which XBS (A, B) and XPH (C, D) wild-types (XBS WT and XPH WT) and mutants (XBS 3A and XPH 2A) were incorporated as a function of enzyme dosage. The specific loaf volumes are expressed relative to a control bread without xylanase (fixed at 100%). Enzyme dosages are expressed in Xylazyme units per kg flour. Two types of wheat flour were used: Crousti (A, C) and Claire (B, D). All values are averages of triplicate experiments and error bars show the standard deviations. Values with the same small letter at different enzyme dosages of one xylanase are not significantly different from each other (α = 0.05). Wild-type xylanases which significantly differ from the mutant xylanase at the same dosage are indicated with an asterisk.

Figure 3. Levels of solubilized WU-AX in dough and bread samples supplemented with xylanases of *Bacillus subtilis* [XBS wild-type and XBS mutant (XBS 3A)] (300 Xylazyme units/kg flour) (A, B) and *Pseudoalteromonas haloplanktis* [XPH wild-type and XPH mutant (XPH 2A)] (25 Xylazyme units/kg flour) (C, D) after mixing, fermentation and baking. Two types of flour were used:

Crousti (A, C) and Claire (B, D) flour. No xylanase was incorporated in control dough or bread. According to a Tukey test ($\alpha < 0{,}05$), values with the same small letter at different bread making stages of control bread or with the same xylanase are not significantly different. Values that are not significantly different for control bread or different xylanases at one particular baking stage are indicated with the same capital letter.

Figure 4. The degree of polymerization (avDP) of the soluble AX fraction in dough and bread samples enriched with xylanases of *Bacillus subtilis* [XBS wild-type and XBS mutant (3A)] (300 Xylazyme units/kg flour) (A, B) and *Pseudoalteromonas haloplanktis* [XPH wild-type and XPH mutant (2A)] (25 Xylazyme units/kg flour) (C, D) after mixing, fermentation and baking. Two types of flour were used: Crousti (A, C) and Claire (B, D) flour. To the control dough or bread, no xylanase was added. According to a Tukey test ($\alpha < 0{,}05$), values with the same small letter at different bread making stages of control bread or with the same xylanase are not significantly different. Values that are not significantly different for control bread or different xylanases at one particular baking stage are indicated with the same capital letter.

Figure 5. Specific viscosity of aqueous extracts of dough and bread samples relative to a potassium chloride/hydrogen chloride buffer (20 mM; pH 3.0). A wild-type and mutant xylanase of *Bacillus subtilis* [(XBS wild-type and XBS mutant (3A)] (300 Xylazyme units/kg flour) (A, B) and *Pseudoalteromonas haloplanktis* [XPH wild-type and XPH mutant (2A)] (25 Xylazyme units/kg flour) (C, D) were incorporated in dough and bread samples prepared with Crousti (A, C) and Claire (B, D) flour. To the control samples, no xylanase was added. According to a Tukey test (α

< 0,05), values with the same small letter at different bread making stages of control bread or with the same xylanase are not significantly different. Values that are not significantly different for control bread or different xylanases at one particular baking stage are indicated with the same capital letter.

TABLES

Table 1. Biochemical characteristics of wild-type and mutant *Bacillus subtilis* xylanases (XBS WT and XBS 3A) and *Pseudoalteromonas haloplanktis* xylanases (XPH WT and XPH 2A). Values with the same small or capital letter for XBS and XPH respectively, are not significantly different from each other (α = 0.05).

	XBS WT		XBS 3A		XPH WT		XPH 2A	
Genbank accession no.	P18429		-		AJ427921.1		-	
Modified amino acids	-		G56A-T183A-W185A		-		W249A-Y315A	
GH	11		11		8		8	
MM (kDa)	20.4		20.2		46.1		45.9	
Activity on xylohexaose (%) [a]	100 ± 5	a	96 ± 2	a	100 ± 3	A	115 ± 4	B
Activity on Xylazyme AX (%) [a]	100 ± 2	a	43 ± 1	b	100 ± 6	A	155 ± 4	B
Activity on Azo-wheat AX (%) [a]	100 ± 3	a	34 ± 1	b	100 ± 9	A	128 ± 5	B
SSF	8.3 ± 0.5	a	10.5 ± 0.4	b	10.1 ± 1.1	A	12.2 ± 0.3	B
T_{opt} (°C)	50	a	50	a	30	A	30	A
pH_{opt}	6-7	a	7	a	9	A	9	A
Reduction of activity by inhibitors (%)	88 ± 2	a	90 ± 3	a	0 ± 1	A	1 ± 1	A

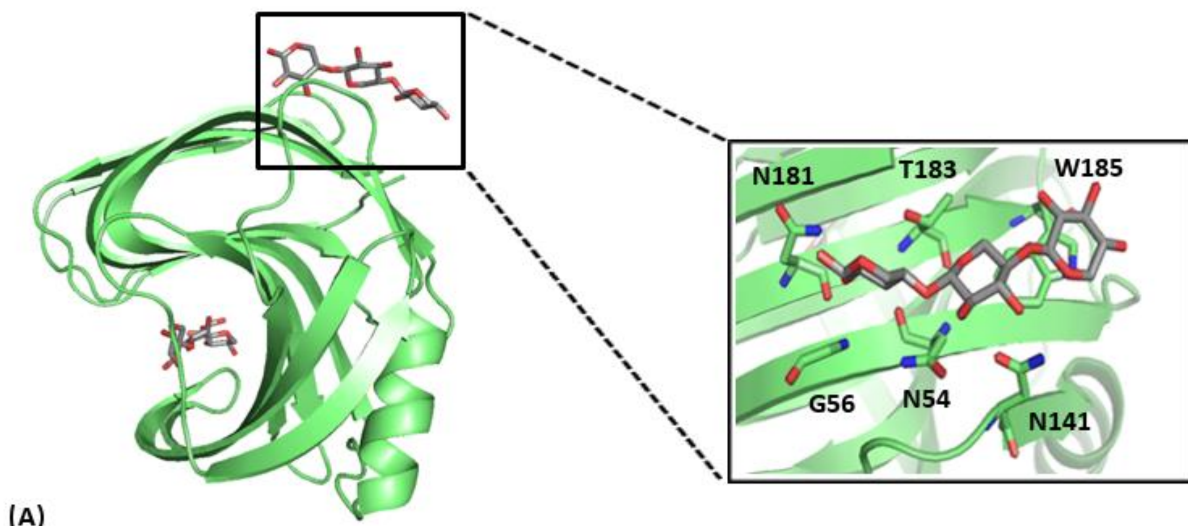
[a] Expressed relative to the activity of the wild-type enzyme (100%). The activities on xylohexaose (X6), Xylazyme AX and Azo-wheat AX were for the wild-type XBS 1 X6-U = 0.17 nM, 1 Xyl-U = 1.03 nM and 1 Azo-U = 8.58 nM, respectively. For the wild-type XPH, 1 X6-U = 4.73 nM, 1 Xyl-U = 2.71 nM and 1 Azo-U = 27.28 nM.

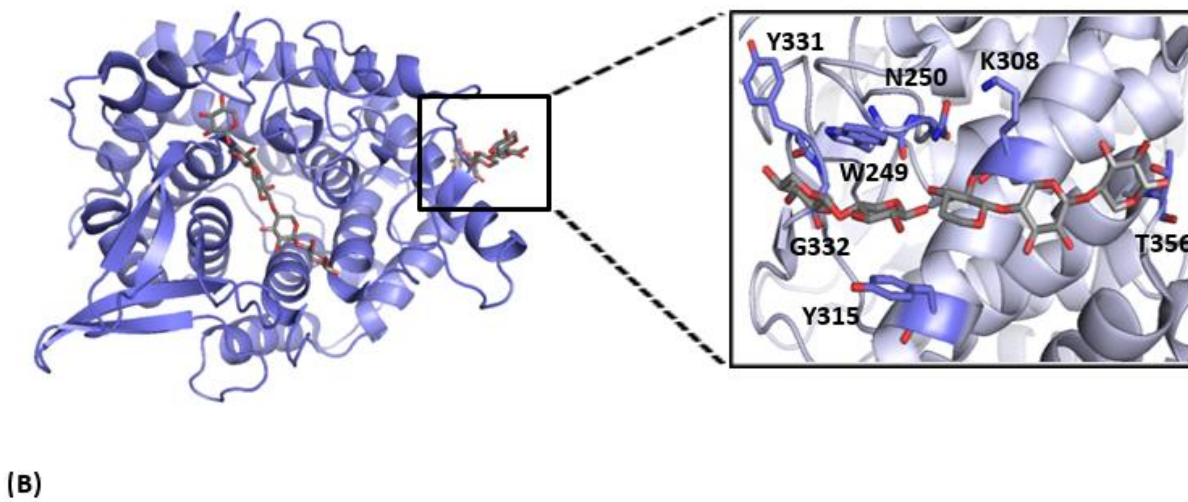
Table 2. Solubilization of water-unextractable arabinoxylan (WU-AX) and decrease of the average degree of polymerization (avDP) during the fermentation stage in Crousti and Claire bread making processes. Wild-types and mutants of the xylanases of *Bacillus subtilis* (XBS) and *Pseudoalteromonas haloplanktis* (XPH) were added to both flour types. A control dough without xylanase addition was also investigated. The results of the statistical analysis are shown per enzyme. Values with the same small letter are not significantly different.

	Solubilization of WU-AX during fermentation (% of total WU-AX)		**Decrease in avDP during fermentation**	
	Crousti flour	*Claire flour*	*Crousti flour*	*Claire flour*
Control	14 ± 1 c	3 ± 0 c	6 ± 0 b	6 ± 1 c
XBS WT	19 ± 3 b	7 ± 4 b	4 ± 5 b	12 ± 2 b
XBS 3A	29 ± 3 a	17 ± 4 a	11 ± 2 a	24 ± 2 a
Control	14 ± 1 c	3 ± 0 c	6 ± 0 b	6 ± 1 c
XPH WT	16 ± 3 b	12 ± 3 b	15 ± 4 a	31 ± 1 b
XPH 2A	26 ± 7 a	29 ± 3 a	20 ± 8 a	46 ± 1 a

FIGURES

Figure 1





(B)

Figure 2

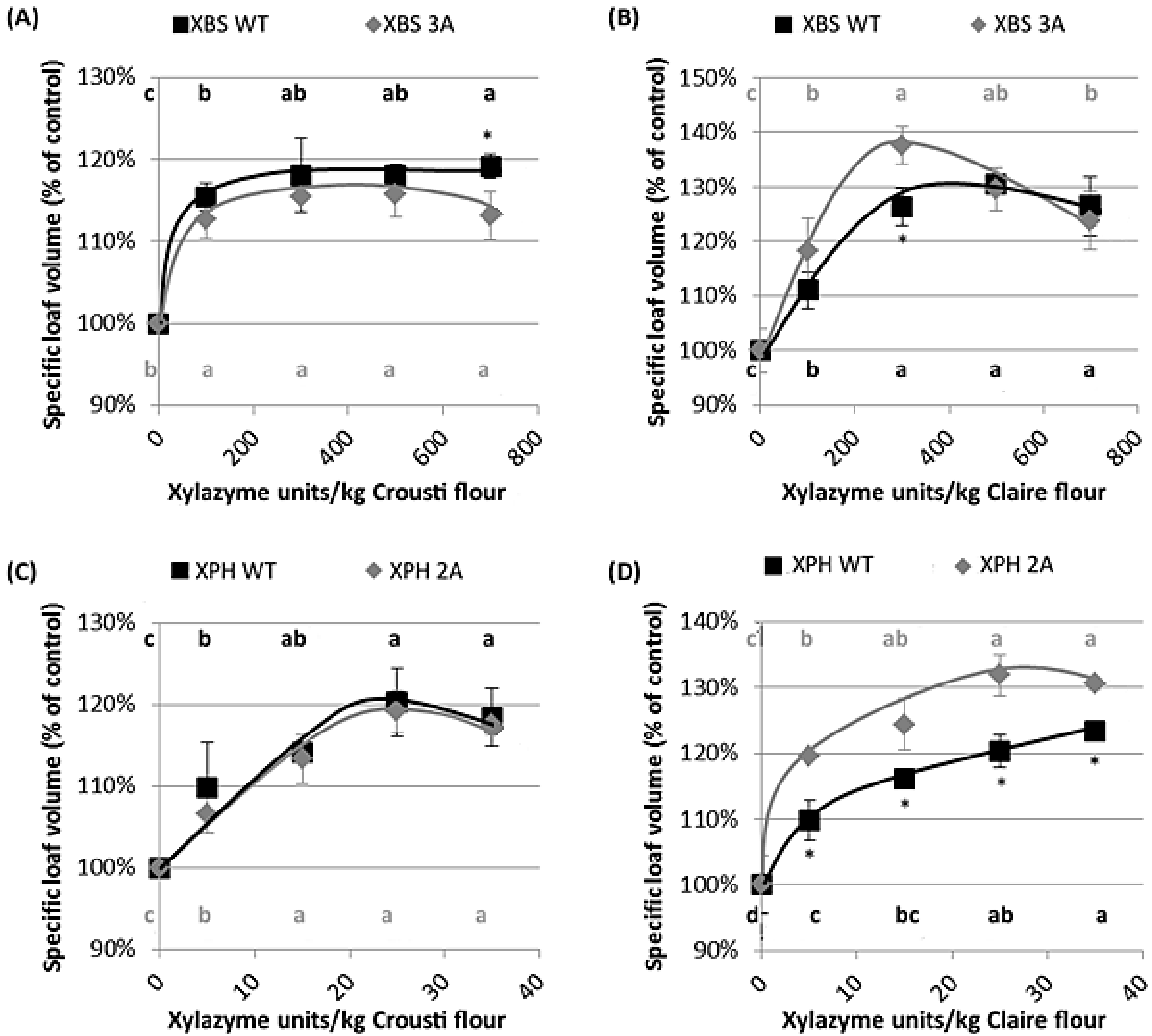
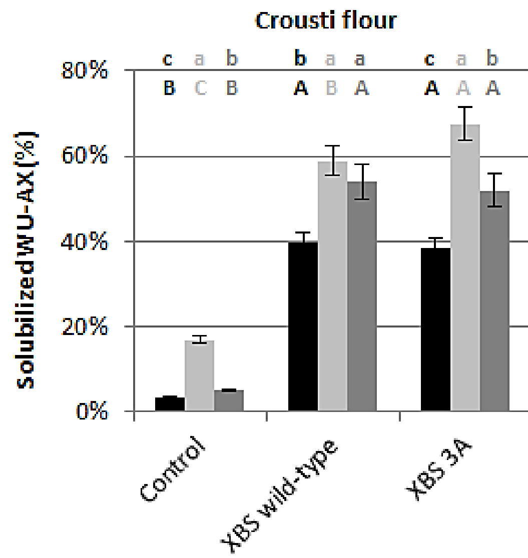
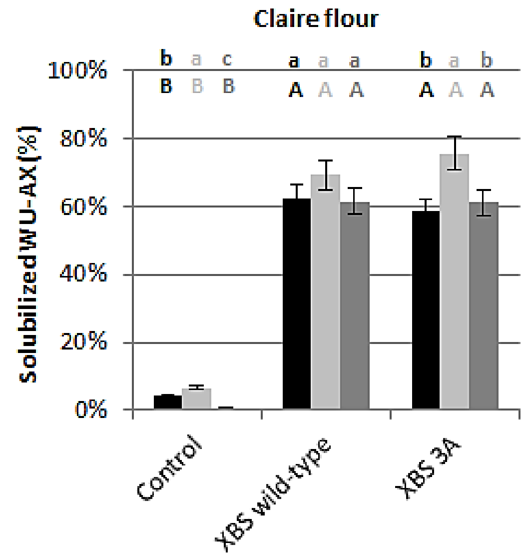


Figure 3

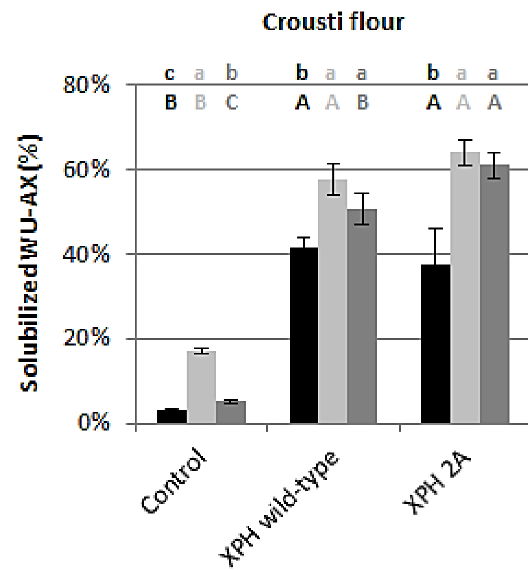
(A)



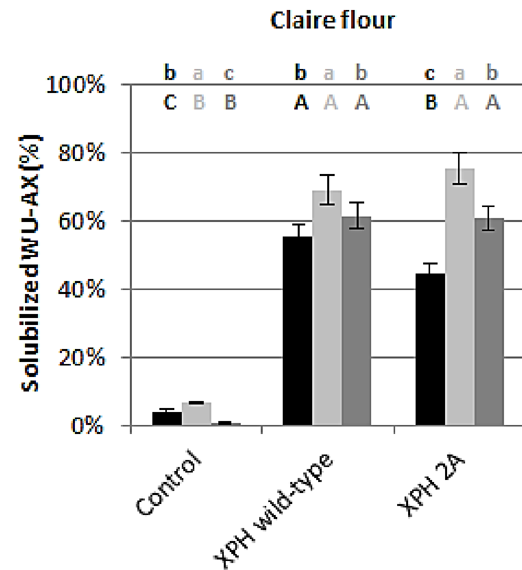
(B)



(C)



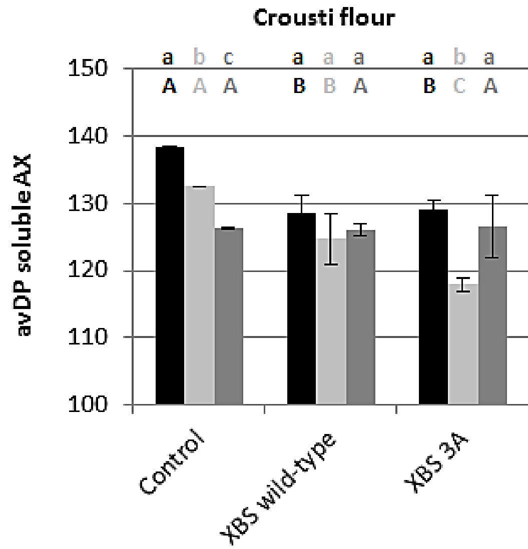
(D)



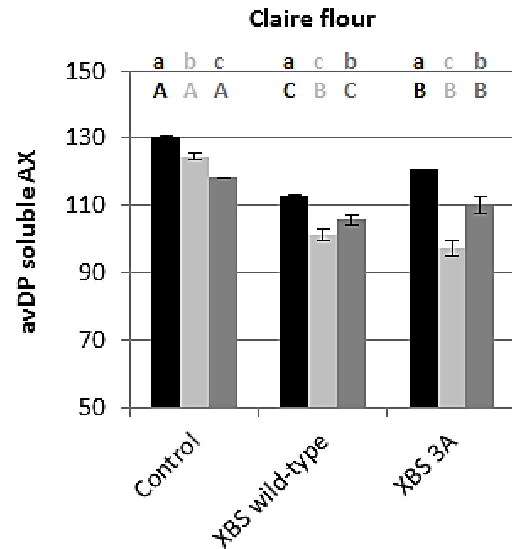
■ After mixing ■ After fermentation ■ After baking

Figure 4

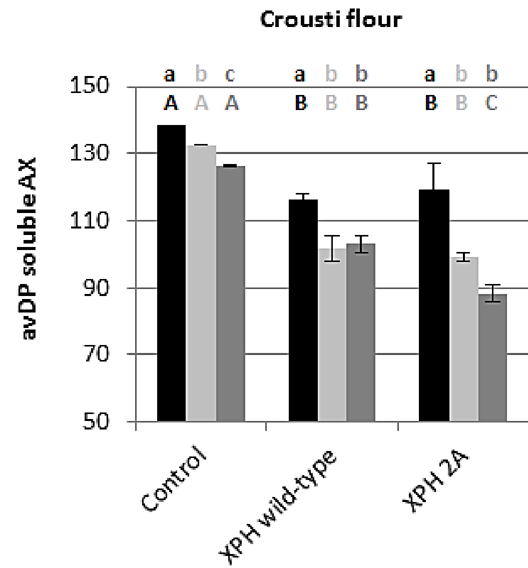
(A)



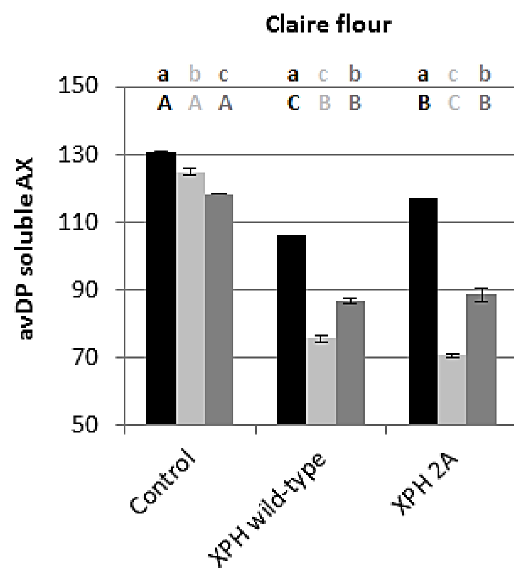
(B)



(C)



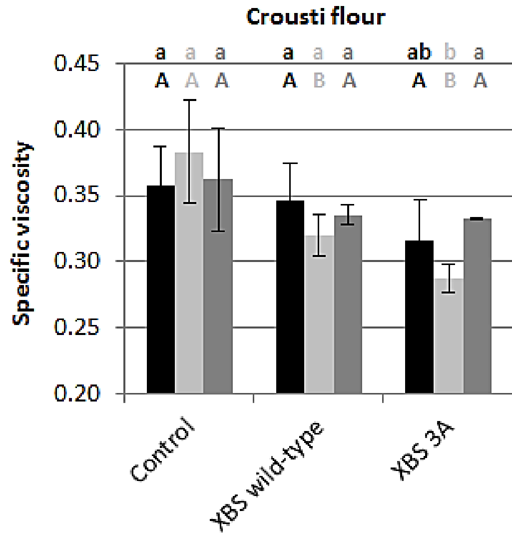
(D)



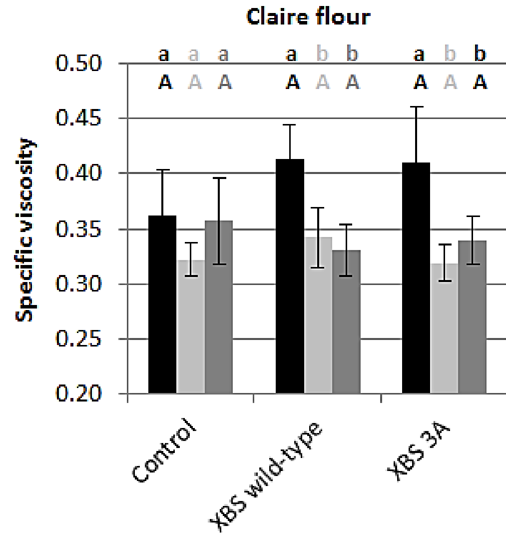
■ After mixing ■ After fermentation ■ After baking

Figure 5

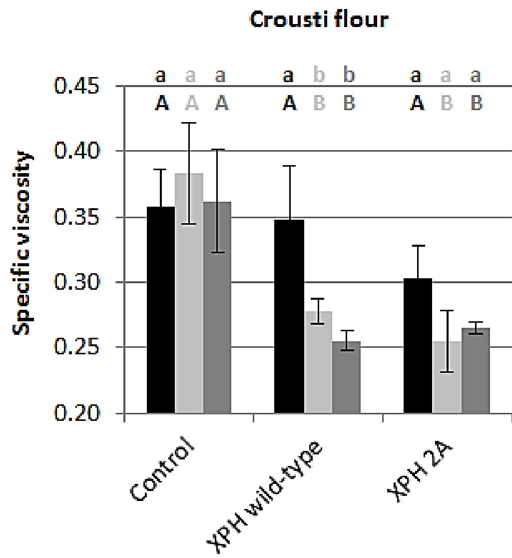
(A)



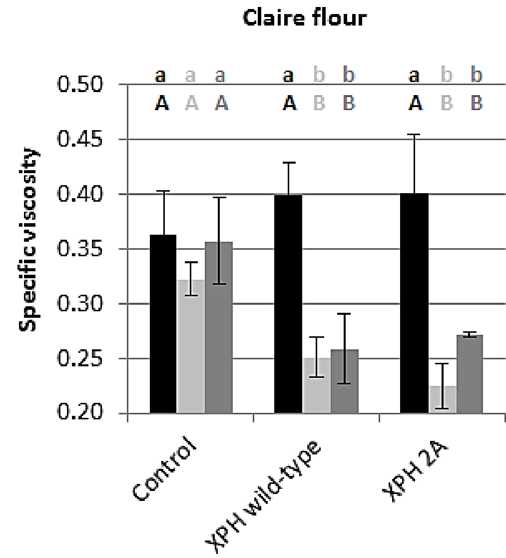
(B)



(C)



(D)



■ After mixing ■ After fermentation ■ After baking

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