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Xylanase and protease increase solubilization of non-starch polysaccharides and nutrient release of corn- and wheat distillers dried grains with solubles

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ABSTRACT

The use of distiller dried grains with solubles (DDGS) as alternative to conventional animal feed for non-ruminants is challenged by the high content of non-starch polysaccharides and varying protein quality. In this study the enzymatic degradation of corn- and wheat DDGS was evaluated, in vitro, by use of four xylanases from two different glycoside hydrolase families, GH10 and GH11, along with protease and phytase. Wheat DDGS showed the highest degree of enzymatic degradation due to a lower degree of cell wall complexity compared with that of corn DDGS. For corn DDGS,the combination of xylanase and protease yielded the highest degree of enzymatic degradation, indicating close association of arabinoxylan and protein within the cell wall matrix. Collectively, the GH10xylanase degraded DDGS more efficiently than the GH11 xylanases, due to the complexity of the DDGS substrate and the substrate affinity of the GH10xylanase. The current in vitro results indicate a high potential of xylanase in combination with protease to efficiently degrade DDGS and promote nutrient release in diets for non-ruminant animals.

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1. Introduction

During the past decade attention on implementing alternatives to conventional feed stuffs (e.g., corn, wheat, and soybean meal) in animal production has become more and more prevalent. The main reason is the increased demand for and consequently increased costs of conventional raw materials together with an increased availability of new low-cost raw materials, many of these being co-products from the grain processing industries. One of these co-products is distillers dried grains with solubles (DDGS), a dried co-product from the production of fuel ethanol [1,2].

As a feed ingredient, DDGS has high potential with a digestible- and metabolizable energy content similar to that of corn, along with a high content of digestible phosphorous [3,4]. In general, DDGS has greater concentrations of protein, fat, vitamins and minerals compared to the parent grain [5,6]. However, disadvantages of using DDGS in the feeding of non-ruminant animals are the 3–3.5 fold higher content of non-starch polysaccharides (NSP), compared to the parent grain [3,7]. Since non-ruminant animals have a limited capacity to utilize NSP, high inclusion levels will inevitably limit feed utilization [8,9,10]. Varying protein quality and the risk of mycotoxin contamination further challenge a high inclusion rate of DDGS in animal diets.

The NSP in DDGS originate from cell walls in the botanical grain fractions; aleurone layer, pericarp, endosperm, germ and tip cap (corn), with arabinoxylan and cellulose as the major components of the NSP fraction [7]. Arabinoxylan consists of D-xylose units joined by β-linkages and substituted with arabinose residues along the chain [11,12], including other substitutes like D-glucuronic acid and acetyl groups [13]. These substitutes together with feruloylated arabinose residues contribute to arabinoxylan cross-linking to form strong intermolecular complexes, affecting the enzymatic degradation and encapsulation of nutrients [14,15]. Xylanases belong to two main families based on their primary sequence and structure

Abbreviations: ADF, acid detergent fiber; AH, acid hydrolysis; A:X, arabinose:xylose ratio; BSA, bovine serum albumin; cDDGS, corn DDGS; DDGS, distillers dried grains with solubles; DF, dietary fiber; DM, dry matter; EE, ether extract; GH, glucoside hydrolase family; NCP, non-cellulosic polysaccharides; NDF, neutral detergent fiber; NIRS, near infrared reflectance spectroscopy; NSP, non-starch polysaccharides; PLS, partial least square; TAXI, Triticumaestivumxylanase inhibitors; UA:X, uronicacid:xylose ratio; wDDGS, wheat DDGS; XIP, xylanasein-hibiting proteins.

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[16], i.e., glycoside hydrolase family 10 and 11 (GH10, GH11). GH10 xylanases have been shown to exhibit greater catalytic versatility or broader substrate specificity than xylanases from the GH11 family. GH11 cleaves primarily unsubstituted regions of the arabinoxylan backbone, whereas GH10 is capable of cleaving in more substituted regions, hence being less hindered by the presence of substitutions [17,18].

Several studies have been conducted to investigate the effects of NSP degrading enzymes on nutrient digestibility of DDGS containing diets. However, the in vivo results are inconclusive [11,19,20], providing evidence for further investigation of the effect of NSP degrading enzymes and other enzymes on DDGS hydrolysis and nutrient release.

The current study was undertaken to investigate the enzymatic degradation of corn DDGS (cDDGS) and wheat DDGS (wDDGS), in vitro, using four different xylanases, alone and in combination with protease and phytase. It is hypothesized that the xylanases comprise different affinities toward c- and wDDGS, thus, affecting DDGS degradation differently. In addition, it is hypothesized that addition of protease and phytase may contribute to an increased degradation by disrupting the feed matrix interactions through hydrolysis of protein and phytate. The effect of enzyme treatment was evaluated by NSP degradation defined as pentosan solubilization, and nutrient release defined as protein solubilization.

2. Materials and methods

2.1. Chemicals

Phloroglucinol (1,3,5-trihydroxybenzene) and D-Xylose ($C_5H_{10}O_5$) were from Merck, and BCA Protein Assay Kit was from Thermo Fischer Scientific. All other chemicals used were of analytical grade.

2.2. Materials

The wDDGS was supplied from a European bioethanol plant and cDDGS from a North American bioethanol plant. These two samples were previously analyzed and characterized together with 136 DDGS samples by Pedersen et al. [7].

Purified endo-1,4-β-xylanases (EC 3.2.1.8); XylA (GH10), XylB (GH11), XylC (GH11) and XylD (GH11) from different microbial origins, protease (EC 3.4.21.62) of *Bacillus* origin (*syn.* Multifect P-3000, Danisco Animal Nutrition, Marlborough, UK), and purified microbial phytase (EC 3.1.3.26), were supplied by DuPont Industrial Biosciences, Denmark. The purity of the enzyme solution was confirmed using gel electrophoresis (NU-PAGE Bis/Tris precast gel, Invitrogen, Life Technologies Corp.). Thermostable α-amylase (E-BLAAM 53.7 U/mg) and amyloglucosidase (E-AMGDF 36 U/mg) were obtained from Megazyme International.

2.3. Chemical analysis and calculations

2.3.1. Near infrared reflectance spectroscopy

Ground DDGS (Retsch ZM 200 centrifugal mill fitted with a 0.5 mm sieve) was scanned from 1100 to 2498 nm using near infrared reflectance spectroscopy (NIRS) on a FOSS NIRSystems 5000 (Foss). The spectral data were predicted by Aunir (AB Agri Ltd., UK) for the composition of moisture, fat (ether extract), fat (acid hydrolysis), protein, crude fiber, ash, starch, total sugars, neutral detergent fiber (NDF) and acid detergent fiber (ADF), using the calibration available for DDGS as previously described [7].

2.3.2. Compositional analysis of NSP

Total- and soluble NSP along with their constituent sugars were determined by gas–liquid chromatography for neutral sugars and by a colorimetric method for uronic acids, basically as described earlier [21], except that 2 M of sulfuric acid for 1 h was used for the hydrolysis of the non-cellulosic polysaccharides (NCP) rather than 1 M of sulfuric acid for 2 h. Klason lignin was measured gravimetrically as the residue resistant to hydrolysis by 2 M sulfuric acid after swelling with 12 M sulfuric acid [21].

Percentage solubilization of total pentosan as

$$\frac{0.88 \times \text{measured pentose}(\mu\text{g/ml})}{\text{Total content of anhydrous xylose and arabinose}(\mu\text{g DM/ml})} \times 100$$

Percentage solubilization of total protein as

$$\frac{\text{measured protein}(\mu\text{g/ml})}{\text{Total content of protein}(\mu\text{g DM/ml})} \times 100$$

2.4. Enzyme purification and quantification

The protein content of the purified enzyme solutions were quantified using a NanoDrop 1000 spectrophotometer (Thermo Fischer Scientific Inc.) based on UV absorbance at 280 nm according to Lambert Beer's Law with extension coefficient calculated from sequence. The preparation of protease was performed just prior to incubations, by diluting the stock solution in ice-cooled MQ-water and mixed while kept on ice. One protease unit was defined as the release of 1.0 μg of phenolic compound, expressed as tyrosine equivalents, from a casein substrate per minute.

2.5. Substrate preparation

DDGS was ground and sieved (<212 μm) before mixing with 25 mM citrate buffer pH 6.0–10% w/v suspension followed by final pH-adjustment. Under constant stirring, 175 μl/well of this suspension was dispensed into 96-well plates using Biomek NX (Beckman Coulter, Inc.). Prepared substrate plates were stored at −20 °C, and thawed just prior to incubations.

For the preparation of insoluble substrate (hereon referred to as insoluble DDGS), removal of soluble NSP was performed basically as previously described [21]. Ground DDGS (<212 μm) in sodium acetate/$CaCl_2$-buffer (0.1 M/20 mM, pH 5.0) was mixed with thermostable α-amylase (E-BLAAM 53.7 U/mg) and incubated at 100 °C for 1 h with frequent mixing. Complete degradation of starch was done by incubation with amyloglucosidase (E-AMGDF 36 U/mg) for 2 h at 60 °C. After removal of starch, the soluble NSP was extracted in sodium phosphate buffer (0.2 M, pH 7.0) at 100 °C for 1 h, followed by centrifugation at 3000 rpm. The pellet was then thoroughly washed sequentially with the phosphate buffer, ethanol (85% v/v), and acetone, with centrifugation and discard of supernatant in between these washes. The sample was placed at room temperature until completely dried.

2.6. Enzymatic hydrolysis of DDGS by xylanase, protease and phytase

For each of the four xylanase treatments (0.2 g xylanase/kg feed) a full factorial 3^2 experiment was setup in duplicates with 2 factors in 3 levels; protease (0 U/kg feed, 4.3×10^5 U/kg feed, 8.6×10^5 U/kg feed) and phytase (0 μg/well, 0.1 g/kg feed, 0.2 g/kg feed). A volume of 25 μl of mixed enzyme solution was transferred to the thawed 96-well substrate plates and mixed. Then the substrate plates were sealed and placed in iEMS incubators (Thermo Scientific) at 39 °C, 1100 rpm for 4 h. After incubation, the solution was transferred to a 96-well filter plate (0.22 μm) and centrifuged at 3600 rpm, 5 °C until the retentate was completely dried. Finally, the filtrate was mixed, proper diluted and aliquots taken out for protein- and pentosan quantification.

2.7. Enzymatic hydrolysis of insoluble DDGS by combinations of xylanases and protease

The effects of four xylanase treatments (0.2 g xylanase/kg feed) were investigated as mono components and in combination with protease (8.6×10^5 U/kg feed) on the solubilization of pentosan and protein from insouble c- and wDDGS. 88 mg of the prepared insoluble DDGS substrate was weighed into 1.5 ml Eppendorf tubes and mixed with citrate buffer (25 mM, pH 6.0) and enzymes solution to a final reaction volume of 1.0 ml. The incubations were carried out at 39 °C and a stirring speed of 1300 rpm on an Eppendorf ThermoMixer incubator for 4 h. After incubation, the samples were filtered and analyzed for soluble pentosan and protein content. Experiments were performed in duplicates.

2.8. Analysis of hydrolysis products

2.8.1. Protein quantification

Soluble protein was quantified using the BCA Protein Assay Kit with the assay range of 50–2000 μg/ml. In 96-well plates 25 μl sample was mixed with 200 μl premixed assay reagent and incubated at 37 °C and stirring at 1100 rpm for 30 min. The absorbance was measured spectrophotometrically at 562 nm and quantified against a bovine serum albumin standard curve. The measured protein content was corrected for the amounts of added enzymes. Additional experiments were performed to verify that protein quantification was not interfered by the pentosan content present in the samples.

2.8.2. Pentosan quantification

Performed principally as previously described [22]. Soluble pentosan was quantified using a San+ + Continous Flow Analyzer (Skalar Analytical B.V., Breda, Netherlands) equipped with 96-well autosampler unit. The sample was mixed with acetic acid reagent (glacial acetic acid with 2% v/v HCl (37%)) for total hydrolysis and phloroglucinol (1,3,5-trihydroxybenzene). The mixture was heated to 96 °C followed by cooling and absorbance measurement at 510 and 550 nm. Pentosan was quantified against a xylose standard curve (50–500 μg/ml). The pentosan content was determined as mono-sugars. To determine the proportion of solubilized pentosan of total pentosan, the mono-sugars were converted to polysaccharides equivalents (anhydro sugars) by a conversion factor of 0.88, and calculated as described above.

2.9. Experimental design and statistical analysis

The 3^2 full factorial experiments (2 factors in 3 levels) [23] was designed and the response data analyzed using PLS regression analysis with backward elimination of insignificant interactions and outliers, with an overall significance level at $P = 0.05$ (Modde 9.1, Umetrics, Umeå, Sweden). A PLS regression model defines a linear relationship $y = f(X)$ between two blocks of variables: (1) the matrix X contains the 3^2 combinations of different enzymes, and (2) the vector y is the individual responses i.e., pentosan and protein [24]. The PLS models were considered satisfactory when R^2 and Q^2 were above 0.5. Means from incubations with c- and wDDGS were compared based on the 95% confidence intervals generated from the PLS modelling.

For the comparison of means from the incubations with insoluble DDGS, a one-way ANOVA was applied followed by a Tukey's pair wise comparison with overall significance level at $P = 0.05$, using Minitab 16 (Minitab Inc.).

Table 1
Compositional profile of corn- and wheat DDGS g/kg dry matter.

	Corn DDGS	Wheat DDGS
Fat (EE)	79	57
Fat (AH)	102	76
Crude protein	304	366
Crude fiber	95	70
Ash	70	93
Starch	50	7
Total sugar	62	79
NDF	387	330
ADF	118	105
Total NSP	328 (29)	266 (76)
Cellulose	91	62
NCP		
Xylose	94 (5)	81 (22)
Arabinose	70 (7)	55 (17)
Mannose	14 (5)	14 (7)
Galactose	18 (2)	10 (6)
Glucose	22 (5)	33 (21)
Uronic acid	18 (5)	8 (2)
Klason lignin	28	86
A:X-ratio	0.74	0.68
UA:X-ratio	0.19	0.10

EE, ether extract; AH, acid hydrolysis; NDF, neutral detergent fiber; ADF, acid detergent fiber; NSP, non-starch polysaccharides; NSP, non-starch polysaccharides; NCP, non-cellulosic polysaccharides; A:X, arabinose:xylose; UA:X, uronic acid:xylose; Values in brackets are soluble NSP.

3. Results

3.1. Compositional profile of corn and wheat DDGS

The compositional profiles of c- and wDDGS are listed in Table 1. The content of soluble NSP was greater in w- than cDDGS, whereas cDDGS had greater contents of both insoluble and total NSP. The content of arabinoxylan (sum of anhydrous arabinose and xylose) was slightly greater in c- than wDDGS, and cDDGS had greater content of uronic acids than wDDGS. In addition, cDDGS had higher arabinose:xylose ratio (A:X) and uronic acid:xylose ratio (UA:X) than wDDGS.

3.2. Effects of xylanases on DDGS hydrolysis

For cDDGS the four xylanase treatments had varying effects on the solubilization of pentosan and protein compared with control without enzyme. Compared with the control, the greatest increase in pentosan solubilization from cDDGS was observed with XylA treatment (77.3%) followed by XylC (43.6%), XylB (12.2%), and XylD (7.2%), respectively (Fig. 1A). In addition, XylA increased the solubilization of protein from cDDGS by 11.1% followed by XylD (6.1%), XylC (4.1%), and XylB (2.6%), respectively, compared to the control (Fig. 1B).

For wDDGS, the variation in pentosan solubilization was less pronounced between the individual xylanase treatments compared with cDDGS. For wDDGS, the greatest increase in pentosan solubilization compared to the control was observed for XylD treatment (26.7%) followed by XylA (23.3%), XylC (18.3%), and XylB (17.0%), respectively (Fig. 2A). The greatest increase in protein solubilization compared to control was observed for XylD treatment (30.9%) followed by XylC (15.2%), XylB (15.1%), and XylA (14.2%), respectively (Fig. 2B).

Based on modelling, the four individual xylanase treatments solubilized 10.1–16.8% and 80.8–87.5% of the total pentosan content, and 13.0–14.1% and 23.5–27.0% of the total protein content for c- and wDDGS, respectively.

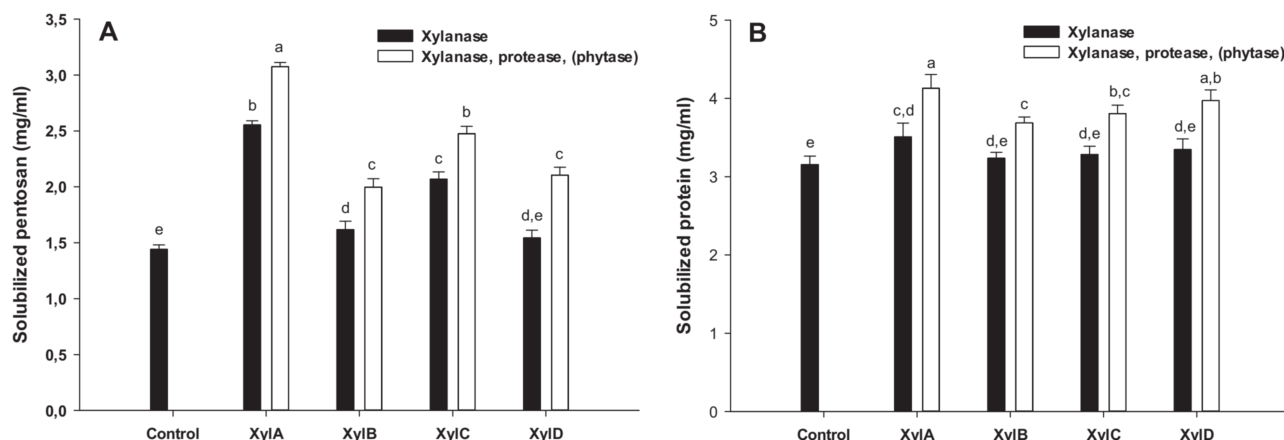

Fig. 1. Solubilization of pentosan (A) and protein (B) from cDDGS with xylanase alone and in combination with protease and phytase (for XylA and XylD) under optimized conditions. Error bars indicate 95% confidence intervals. Bars with different letters are significantly different at $P < 0.05$.

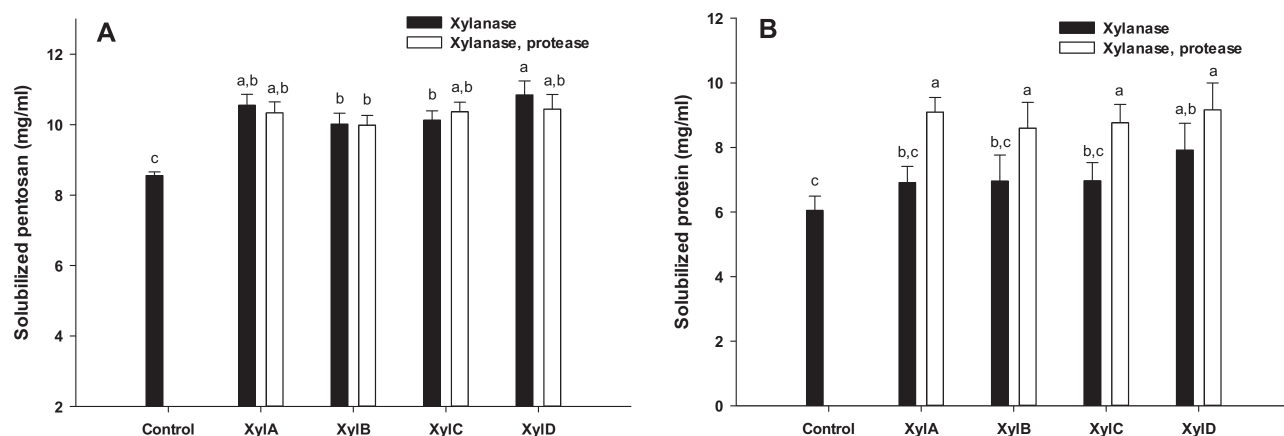

Fig. 2. Solubilization of pentosan (A) and protein (B) from wDDGS with xylanase alone and in combination with protease under optimized conditions. Error bars indicate 95% confidence intervals. Bars with different letters are significantly different at $P < 0.05$.

3.3. Effects of protease and phytase in combination with xylanases on DDGS hydrolysis

Coefficients of the PLS regression analyses on the effect of protease and phytase addition to the four xylanase treatments and are presented in Table 2. Protease addition significantly increased the solubilization of protein in both cDDGS ($P < 0.0001$) and wDDGS ($P \leq 0.02$) across all four xylanase treatments. Furthermore, protease significantly increased the solubilization of pentosan in cDDGS across all four xylanase treatments ($P < 0.0001$), whereas no significant effect was observed for protease addition on pentosan solubilization in wDDGS ($P \geq 0.094$). Phytase had no significant effect on the solubilization of pentosan or protein in neither c- nor wDDGS. However, significantly positive interactions between protease and phytase were observed on the solubilization of protein with the XylB treatment in cDDGS ($P = 0.009$).

3.4. Optimizing hydrolysis of DDGS by xylanases in combination with protease and phytase

Modelling of the cDDGS response data predicted the greatest simultaneous solubilization of pentosan and protein at 0.2 g/kg feed phytase and 8.6×10^5 U/kg feed protease for XylA and XylD treatment, and 8.6×10^5 U/kg feed protease for XylB and XylC treatment (Fig. A.1). The greatest increase in pentosan solubilization was predicted for XylA treatment (113.5%) followed by XylC (71.8%), XylD (46.1%), and XylB (38.6%) compared with the control. Furthermore, optimized conditions predicted the greatest increase in protein solubilization for the XylA treatment (30.8%) followed by XylD (25.9%), XylC (20.6%), and XylB (16.9%) compared with the control. When compared with the effects of xylanase treatment by itself, the optimized conditions with protease and phytase further increased the solubilization of pentosan and protein by 19.7–36.3% and 15.1–20.2%, respectively.

Modelling of the wDDGS response data predicted the greatest simultaneous solubilization of pentosan and protein at 8.6×10^5 U/kg feed protease for all xylanase treatments (Fig. A.2). Compared to control, the optimized conditions increased the solubilization of protein by XylD treatment (51.5%) followed by XylA (50.4%), XylC (44.9%), and XylB (42.2%), respectively. The optimized conditions with protease increased the protein solubilization by 15.7–32.9% compared with the four individual xylanase treatments. No effect of the optimized conditions was observed on the solubilization of pentosan.

For cDDGS, the predictability of the PLS models were satisfactory with $R^2 = 0.79$-0.95 and $Q^2 = 0.85$-0.98 for pentosan solubilization, and $R^2 = 0.65$-0.79 and $Q^2 = 0.73$-0.97 for protein solubilization. For wDDGS, the predictability of the PLS models of XylA and XylC were satisfactory for protein solubilization with $R^2 = 0.70$–0.84 and $Q^2 = 0.65$–0.78, whereas, the PLS models for XylB and Xyl D were unsatisfactory. The PLS models for prediction of pentosan solubilization in wDDGS was unsatisfactory with $R^2 = 0.00$–0.41 and $Q^2 = -0.20$ to 0.21, indicating the maximum solubilization of pen-

Table 2
PLS regression models on the release of pentosan and protein by xylanase, protease, and phytase, with parameters and interactions.

	Xylanase A		Xylanase B		Xylanase C		Xylanase D	
	Corn DDGS							
	Pentosan ($N = 17$)		Pentosan ($N = 17$)		Pentosan ($N = 16$)		Pentosan ($N = 18$)	
	Coefficient	*P*-value	Coefficient	*P*-value	Coefficient	*P*-value	Coefficient	*P*-value
Protease	202.2	<0.0001	164.6	<0.0001	165.5	<0.0001	214.4	<0.0001
Phytase	7.3	0.393	−27.2	0.162	0.5	0.974	21.4	0.197
Pro × Pro	−42.8	0.003			−55.0	0.035	−54.3	0.033
Constant	2891.8	<0.0001	1775.6	<0.0001	2360.9	<0.0001	1901.8	<0.0001
R^2	0.95	0.79	0.86	0.85				
Q^2	0.98	0.85	0.92	0.93				
	Protein ($N = 18$)		Protein ($N = 17$)		Protein ($N = 17$)		Protein ($N = 18$)	
Protease	273.1	<0.0001	249.0	<0.0001	215.4	<0.0001	266.9	<0.0001
Phytase	−12.1	0.782	−29.5	0.058	−16.6	0.527	−3.9	0.906
Pro × Pro			−52.3	0.039				
Pro × Phy			46.4	0.009				
Constant	3818.2	<0.0001	3497.7	<0.0001	3542.5	<0.0001	3660.6	<0.0001
R^2		0.65		0.79		0.75		0.77
Q^2		0.73		0.97		0.84		0.82
	Wheat DDGS							
	Pentosan ($N = 18$)		Pentosan ($N = 17$)		Pentosan ($N = 16$)		Pentosan ($N = 17$)	
	Coefficient	*P*-value	Coefficient	*P*-value	Coefficient	*P*-value	Coefficient	*P*-value
Protease	−90.5	0.250	−10.2	0.883	99.6	0.091	−166.3	0.114
Phytase	−91.1	0.247	2.5	0.971	2.0	0.972	−168.5	0.110
Pro × Pro					−159.7	0.049		
Constant	10333.0	<0.0001	10003.7	<0.0001	10486.7	<0.0001	10457.3	<0.0001
R^2	0.16	0.00	0.41	0.29				
Q^2	−0.10	−0.20	0.13	0.21				
	Protein ($N = 16$)		Protein ($N = 18$)		Protein ($N = 18$)		Protein ($N = 18$)	
Protease	934.4	<0.0001	688.1	0.003	756.1	<0.0001	522.8	0.020
Phytase	35.2	0.761	−87.0	0.660	−280.0	0.060	−260.8	0.215
Constant	8113.7	<0.0001	7675.7	<0.0001	7532.2	<0.0001	8228.6	<0.0001
R^2		0.84		0.46		0.70		0.36
Q^2		0.78		0.29		0.65		0.22

Scaled and centered coefficients of the quadric models fitted with PLS, using Modde 9.1 software. Blanks indicate backward eliminated insignificant interactions at $P > 0.05$. Pro protease; Phy phytase.

tosan in wDDGS was achieved by xylanase treatment, thus, not affected by additional protease or phytase treatment.

Based on modelling, the individual optimized conditions with four xylanases, protease and phytase predicted 13.1–20.2% and 80.6–84.3% solubilization of the total pentosan content, and 14.8–16.6% and 29.3–31.2% solubilization of the total protein content for c- and wDDGS, respectively.

3.5. *Effects of xylanase and protease on solubilization of pentosan and protein from insoluble DDGS fraction*

Similar to the previous observations on DDGS substrate, the four xylanase treatments varied in the solubilization of pentosan and protein for both insoluble c- and wDDGS (Figs. 3 and 4). The greatest solubilization of insoluble cDDGS was observed for XylA with an increased solubilization of 2.2 mg pentosan/ml and 1.8 mg protein/ml. The remaining three xylanase treatments increased the solubilization of pentosan between 0.4 and 1.2 mg/ml and protein between 0.6 and 1.2 mg/ml with XylC > XylB > XylD compared to control with a solubilization of 0.1 mg/ml and 0.2 mg/ml, respectively. Overall, the four xylanase treatments increased the solubilization of pentosan by 338–2052% and protein by 311–1012% compared with the control (Fig. 3).

The greatest solubilization of pentosan from insoluble wDDGS was observed for XylC treatment of 2.4 mg/ml followed by XylA of 2.3 mg/ml. XylD and XylB solubilized more pentosan in w- than cDDGS, with an increase of 1.5 mg/ml and 1.7 mg/ml, respectively (Fig. 4A). The xylanase treatments increased protein solubilization by 0.6–0.8 mg/ml compared to control of 0.2 mg/ml. Overall, the four xylanase treatments increased the solubilization of pentosan by 926–1171% and the solubilization of protein by 421–561% compared with the control.

Protease addition increased the solubilization of pentosan and protein from both insoluble c- and wDDGS, with the most pronounced effect on protein solubilization. Addition of protease increased the solubilization of pentosan by 0.6 mg/ml (572%) and 0.6 mg/ml (298%) compared with control for insoluble cDDGS (Fig. 3A) and wDDGS (Fig. 4A), respectively. Furthermore, protease increased the protein solubilization by 9.4 mg/ml (5263%), and 7.1 mg/ml (4875%) for insoluble cDDGS (Fig. 3B) and wDDGS (Fig. 4B), respectively.

The effect of combining xylanase and protease was most pronounced on the protein solubilization with an increase of 8.5–9.9 mg/ml for insoluble cDDGS, and 6.7–7.1 mg/ml for insoluble wDDGS compared with xylanase treatment alone. Furthermore, the combination of xylanase and protease increased the solubilization of pentosan of 0.5–0.7 mg/ml for insoluble cDDGS, and 0.2–0.4 mg/ml for insoluble wDDGS compared to xylanase treatment alone.

4. Discussion

In the current study supplementation with exogenous enzymes had great effect on solubilizing pentosan and protein from both

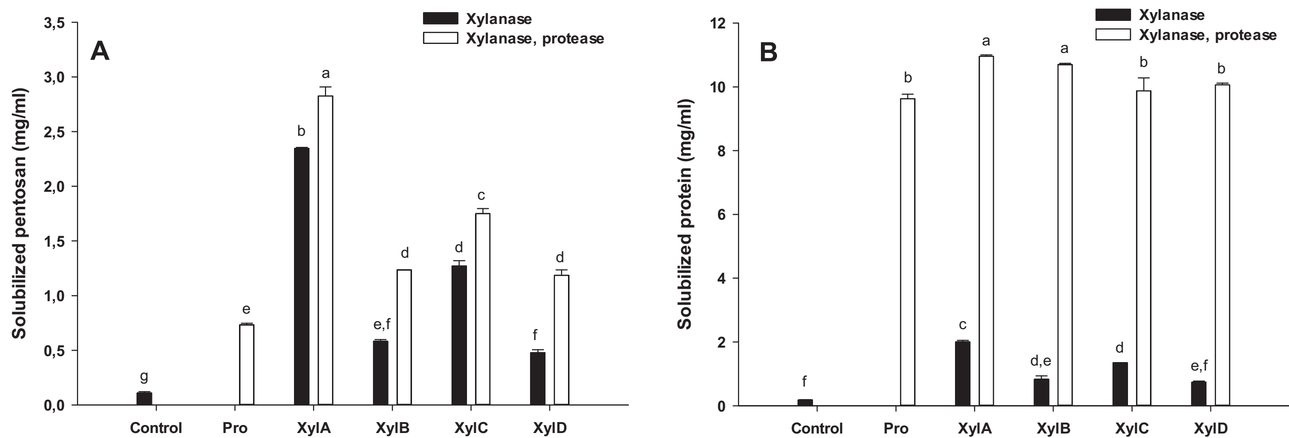

Fig. 3. Solubilization of pentosan (A) and protein (B) from insoluble cDDGS with xylanase alone and in combination with protease. Pro protease. Error bars indicate S.D. Bars with different letters are significantly different at $P < 0.05$.

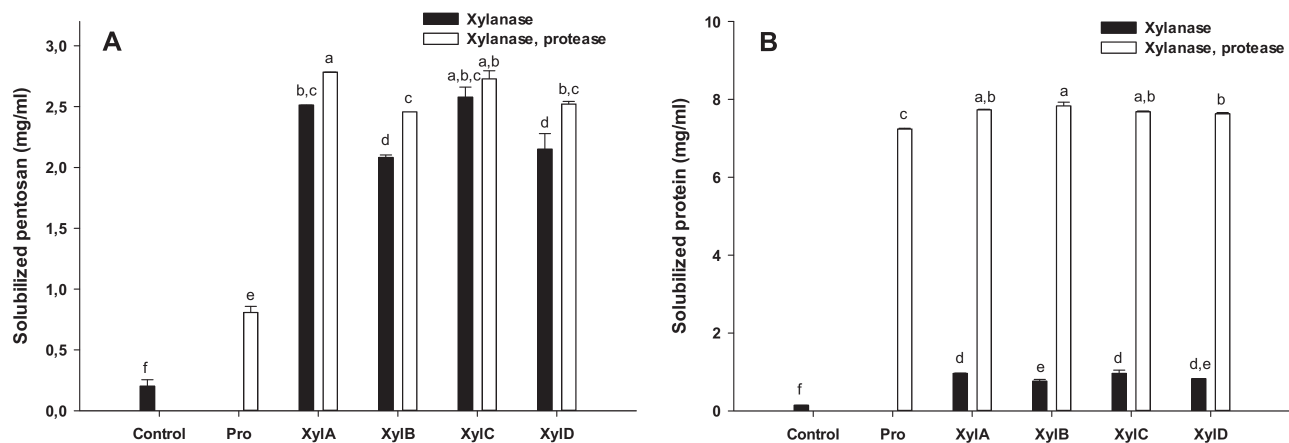

Fig. 4. Solubilization of pentosan (A) and protein (B) from insoluble wDDGS with xylanase alone and in combination with protease. Pro protease. Error bars indicate S.D. Bars with different letters are significantly different at $P < 0.05$.

c- and wDDGS, indicating a high potential of these enzymes to degrade DDGS fiber and release nutrients in vitro. However, large differences was observed in the solubilization of pentosan between the two substrates, illustrated by the highest enzymatic solubilization equals approximately 20% and 85% of the total in c- and wDDGS, respectively. Differences in the compositional complexity between c- and wDDGS are the most likely reason for the markedly different responses to enzymatic treatments between the two substrates. The observed compositional characteristics of the c- and wDDGS used this study were in line with previous reports on DDGS [5,7] and parent grains [21]. Despite similar arabinoxylan content, cDDGS contained a greater fraction of insoluble arabinoxylan and cDDGS had higher A/X–and UA/X ratio, which is indicative for a higher average degree of substitution in c- than wDDGS. Furthermore, a higher ferulic acid dehydrodimers and–dehydrotrimers crosslinking of arabinoxylan [25,26,27] in c- than wDDGS may impair the enzymatic degradation of the plant cell wall [28,29]. It has been found that corn contains approximately 5–7 times more diferulates in the insoluble dietary fiber fraction compared to wheat [30,31]. The combined greater A:X- and UA:X-ratio, along with an expected greater content of ferulic acids in cDDGS than wDDGS implies a more complex structure of the heteroxylans in cDDGS, which is in line with previous reports on the greater branching and complexity of corn arabinoxylan compared to wheat [13,27,32,33]. The observed greater enzymatic effect on degradation of AX in wDDGS than cDDGS is well in line with a previous study describing higher enzymatic degradation of wheat than corn [34]. In addition to the solubilization of pentosan, xylanase addition further increased the release of protein from both c- and wDDGS, indicating close association of pentosan and protein in the cell wall. Fiber degrading enzymes have previously been reported to increase in vitro digestibility of protein in cDDGS, however, with no reported effect on solubilization of NSP [35].

The use of purified xylanases, deprived of side activities, allowed for a direct comparison of xylanases originating from two different GH families. Considering the difference in catalytic versatility and substrate affinity of GH10 (XylA) and GH11 (XylB, XylC, and XylD) [17,18], the superior effect of XylA on the degradation of cDDGS is likely due to the greater substrate complexity than wDDGS. The three GH11 xylanases showed different effects on especially cDDGS degradation, suggesting that XylC poses greater affinity toward insoluble and more complex substrates than XylB and XylD. Despite GH-relation, xylanases may encompass differences in substrate affinity, and pH and temperature optimum, which may affect the degree of enzymatic degradation [18]. The individual performance of the four xylanases on the insoluble cDDGS was in line with the results on cDDGS. As cDDGS contain lower amounts of soluble NSP than wDDGS, the change in substrate composition caused by removing soluble NSPs is logically less pronounced. However, removal of the soluble NSPs may affect the overall solubilization of pentosan, as the presence of both soluble and insoluble substrate will compete for the xylanase activity, which potentially will

limiting the solubilization from the insoluble NSP fraction. The larger difference in performance between the individual xylanases on pentosan solubilization from insoluble wDDGS than wDDGS may be explained by the different substrate affinities among xylanases toward the insoluble NSP fraction (Fig. 4A).

As expected, addition of protease increased the solubilization of protein from both c- and wDDGS, and from insoluble c- and wDDGS fractions. Compared to the DDGS substrate, the effect of protease addition was more pronounced on the protein solubilization from insoluble DDGS substrate. It can be speculated that removal of the soluble protein increase the protease activity toward degradation of the insoluble protein, consequently increasing the protein solubilization. Addition of protease increased the solubilization of pentosan from cDDGS, indicating a close interaction of protein and arabinoxylan in the cell wall, as previously reported [16]. The latter was further confirmed, as the greatest solubilization of both pentosan and protein was achieved by combining both xylanase and protease, indicating synergistically interactions between the two different hydrolases. Protein has previously been described to be located within the aleurone layer of cereals [36,37], thus, it can be speculated that the addition of xylanase would increase the accessibility of cell wall-encapsulated protein by opening up the cell wall structure through arabinoxylan degradation. Addition of protease could potentially hydrolyze endogenous xylanase inhibitors present in cereals; i.e., *Triticum aestivum* xylanase inhibitors (TAXI) and Xylanase Inhibiting Proteins (XIP) [38,39,40]. Inactivation of these xylanase inhibitors would consequently improve xylanase performance, if inhibited. Addition of protease to wDDGS had no additional effects on pentosan release, likely due to the fact that the solubilization of pentosan was apparently already maximized by the addition of xylanase alone.

Endo-xylanases solubilize arabinoxylan (pentosan) by cleaving the β-1,4-glycosyl linkages within the β-1,4-xylose backbone of insoluble as well as soluble arabinoxylan, thus, partially solubilizing insoluble arabinoxylan and fragmenting soluble arabinoxylan into arabinoxylan-oligosaccharides [17]. The large difference in the proportion of solubilized pentosan out of total pentosan between c- and wDDGS may indicate differences in which botanical grain fractions the pentosan is solubilized from in the two DDGS sources. In corn, endosperm arabinoxylan comprise approximately 20% of the total content of arabinoxylan, whereas endosperm arabinoxylan in wheat comprise approximately 25%, calculated based on data from Bach Knudsen [21] and Watson [41]. As the maximum enzymatic solubilization of pentosan equals approximately 20% the total pentosan in cDDGS, it can be speculated that the majority of the pentosan is solubilized from the endosperm fraction. Corn endosperm arabinoxylan is likely the most readily accessible arabinoxylan for enzymatic degradation, as this arabinoxylan is not cross-linked to the same degree as arabinoxylan in the bran [42,43]. Furthermore, corn bran arabinoxylan has been acknowledged as a recalcitrant substrate regarding enzymatic degradation due to the highly branched structure [44,45]. For wDDGS, the maximum enzymatic solubilization of pentosan equals approximately 85% of the total pentosan in wDDGS, indicating that the pentosan is solubilized from other botanical grain fractions besides the endosperm. To further increase the enzymatic degradation of arabinoxylan in DDGS (especially in cDDGS), other minor enzyme activities may be necessary, such as L-α-arabinofuranosidase, feruloyl esterase, and α-D-glucuronidase.

Overall, phytase had no significant effects on the solubilization of neither pentosan nor protein in both c- and wDDGS. Phytate-bound P in corn is in the range from 61 to 77% of total P [46], whereas values of phytate-bound P in cDDGS have been reported to be 30–35% of total P [20,47]. A decrease in phytate-bound P in fermentation products compared to parent grains has been reported previously [48,49]. Partial degradation of phytate during the fermentation process may be mediated by the presence of endogenous phytase in yeast or exogenous phytase added to increase the starch degradation and ethanol yield [50].

5. Conclusion

The use of xylanases in combination with protease poses a large potential regarding the degradation of arabinoxylan and release of nutrients from both c- and wDDGS. Furthermore, positive effect of protease addition on top of xylanase on cDDGS degradation suggests close protein and fiber interactions in the DDGS matrix. Large differences in xylanase performance were observed between c- and wDDGS, which is related to the different degree of complexity of the arabinoxylan fraction and fiber matrix. The results presented in this study indicate a large potential of using xylanase in combination with protease for efficient degradation of DDGS and nutrient release.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bej.2015.02.036.

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