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From Grain By-products to Functional Food through Innovative Processing:

"The influence of xylanase enzyme addition on insoluble and soluble fiber content and water holding capacity of millet bran fraction"

by

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Glossary of Local Terms Used

AACC	American Association of Cereal Chemists
AX	arabinoxylan
CVD	cardiovascular disease
CD	coeliac disease
DF	dietary fiber
SDFP	dietary fiber soluble in water but precipitated in 78% aqueous ethanol
IDF	insoluble dietary fiber
SDFS	dietary fiber soluble in water and not precipitated in 78% aqueous ethanol
NSP	non-starch polysaccharides
SDF	soluble dietary fiber
TDF	total dietary fiber
HMWDF	total high molecular weight dietary fiber
WHC	water holding capacity
WEA	water-extractable arabinoxylan
WUA	water-unextractable arabinoxylan

University of Zagreb

The University of Zagreb is the oldest Croatian university and also the oldest university in South East Europe, established in 1669 by Emperor and King Leopold I Habsburg. It offers education and research in all scientific fields (arts, biomedicine, biotechnology, engineering, humanities, natural sciences and social sciences) and a broad spectrum of courses at all study levels, from undergraduate to postgraduate.

Over the course of more than 300 years, it has become an institution serving society as a whole. It is organized into 29 faculties, three academies of art and the University Centre for Croatian Studies where 72,480 students and 7,900 teachers from all over the world develop knowledge and acquire skills.

Faculty of food technology and biotechnology

The internship was carried out at the heart of the Faculty of food technology and biotechnology, leading institution in terms of science, research and education in the fields of biotechnology, food technology and nutrition science. Specifically in the department of Food Engineering which has around 27 laboratories, including the Laboratory for Cereal Chemistry and Technology where the internship was held under the supervision of PhD. Nikolina Čukelj. According to its name, it focuses on education and different research studies of cereal food products, mainly on gluten and non-gluten bread, with or without sourdough addition.

The laboratory team consists of one full professor, two assistant professors, one senior PhD researcher, and one technician, who work together in the production of fine bakery products with non-traditional raw materials, applying not only innovative technologies in production process for example: vacuum cooling, high pressure, ultrasound and cryomilling, but also modern technologies for the determination of nutritional profile and bioactive compounds content of product, texture measurements, and sensory evaluation.

The internship was completely focused on a new project called “From Grain By-products to Functional Food through Innovative Processing” (IP-2016-06-3) funded by the Croatian Science Foundation, using the millet bran as the main by-product of the cereal industry.

I. Introduction

Cereals are the fruits of cultivated grasses, members of the monocotyledonous family *Gramineae*, one of the largest plant families, comprising about 700 genera and 10,000 species. Nowadays cereals have a considerable interest largely thanks to their highly nutritious edible portion, the "caryopsis", commonly called grain (Kent, 1994; McKevith, 2004).

Many epidemiological studies have shown the positive health effects associated with eating cereals, specifically whole-grains, including protection against the development of chronic diseases such as obesity, the metabolic syndrome, type 2 diabetes, CVD and cancers (Fardet, 2010). Notwithstanding, these beneficial effects are especially credited to the content of dietary fiber in the bran layer of the grains (Slavin *et al*., 1999; Singh *et al.,* 2016).

Unfortunately, the use of bran in food applications is limited due to its technological and sensory challenges and bioavailability of potentially health promoting compounds, (Santala, 2014). Besides that, consumer's interest for this by-product and its usability is relatively low, as is the case of millet bran which is the focus of the present study.

Millet bran, a by-product of millet-based food manufacturing, is an important source of dietary fiber. It approximately consists of 73.18% total dietary fiber (TDF), about 90% of this fiber is insoluble (IDF) while the remaining 10% is soluble (SDF) (Liu *et al.,* 2011). Despite its wide proportion, IDF can negatively influence food texture by its water retention and swelling properties. Furthermore, it is poorly fermented in the colon impeding the absorption of some nutrients. SDF, on the contrary, is easy to incorporate in foods and is characterized by its ability to lower blood cholesterol and regulate blood glucose levels (Fardet, 2010; Santala, 2013).

Because of disadvantages that IDF causes, many approaches have been developed to redistribute the fiber content from insoluble to soluble fractions. Enzymatic treatments have been shown to facilitate the addition of bran to bakery products and the beneficial effects of these processes have been related to the solubilisation of arabinoxylan (AX), the main DF component and responsible behind the adverse effects of insoluble dietary fiber, by using xylanolytic enzymes (Santala, 2011).

Xylanase is the most important xylanolytic enzyme, it depolymerizes and solubilizes AX by cleaving the β-xylosidic bond between two D-xylopyranosyl residues linked in β-(1,4).

Moreover, it has been well documented that the treatment of raw cereal brans with xylanase increase the water holding capacity (WHC) and therefore improve the softness and elasticity of the doughs (Uysal *et al.*, 2007).

Therefore, the purpose of this study was to examine the effect of use xylanase on millet bran fractions by analyzing its content of soluble and insoluble dietary fiber after 24 h enzymatic treatment.

The missions of internship can be determined as:

- To determine both SDF and IDF content of millet bran after xylanase enzyme addition.
- To determine the hydration properties of millet bran after xylanase enzyme addition.

II. Background

2.1 Proso millet

Millets are various grass crops that are extremely important in the semi-arid and sub-humid zones as staples crops for animals and humans. According to Devi *et al.* (2014) millets have resistance to pests and diseases, short growing season, and productivity under drought conditions. Furthermore, nutritional potential of millets in terms of protein, carbohydrate and energy values are comparable to the popular cereals like rice, wheat and barley.

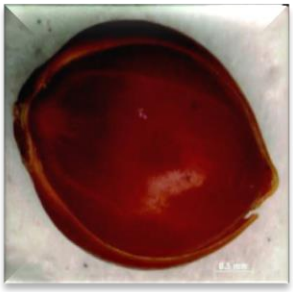

Figure 1. Proso millet (source: Taylor, 2017).

Proso millet (*Panicum miliaceum*) (See Figure 1), also known as common millet, hog millet, broom millet, panic millet, hershey and white millet, is one of the most important varieties of millet. It is considered a self-pollinated crop and, as mentioned above, it is a short season crop (60-90 days) and hence can be grown in different temperature zones and at high altitude (up to 300m) (Saleh *et al.*, 2013; Taylor, 2015).

Common millet does not contain gluten-forming proteins and its gluten-free status renders it suitable for people with coeliac disease (CD). It is a rich source of DF, proteins (14.4%), minerals (1.5 to 4.2%) and phenolic compounds (Kalinová, 2007; Habiyaremye *et al.*, 2017). Nevertheless, proso millet possesses certain phytochemicals with antinutrient effects which may hinder efficient utilization, absorption, or digestion of nutrients. Those antinutritional factors are mostly concentrated in the bran fractions and might be easily removed by decortication (Devisetti *et al.*, 2014).

2.1.1 Millet bran

Millet bran (See Figure 2), which consists of combined aleurone, pericarp and part of germ, is a by-product of the millet processing. It is an important source of DF, proteins and antioxidants, but its use in food application is limited because unprocessed bran is usually detrimental to final product quality, due to its technological and sensory challenges. Fortunately numerous processing methods have been developed to facilitate the use of bran fractions in different types of food products. These methods include enzymatic, chemical, mechanical, hydrothermal and thermo-mechanical treatments (Santala, 2014).

2.2 Dietary Fiber

Dietary fiber, a carbohydrate-based polymer with beneficial physiological properties, is defined by the AACC (2001) as "the edible parts of plants or analogous carbohydrates that are resistant to digestion and absorption in the human small intestine with complete or partial fermentation in the large intestine. It includes polysaccharides, oligosaccharides, lignin and associated plant substances".

DF can be divided into two categories according to their water solubility. Soluble dietary fiber mainly consists of water-extractable arabinoxylan (WEA) and the soluble portion of β-glucan. This fiber form a viscous solution in the intestine and is easily fermented by the colonic organisms, bringing about many desired metabolic effects, as the decrease of serum cholesterol and the stabilization of blood sugar level, thus reducing the chances of diabetes and atherosclerosis (Singh *et al.,* 2016). Regarding bakery products, SDF increases dough extensibility, acts as emulsifiers, has neither bad texture nor bad taste and can significantly increase bread volume and softness. (Devi *et al.,* 2014).

Insoluble dietary fiber includes celluloses, pectin, lignin and insoluble hemicellulose, such as water-unextractable arabinoxylan (WUA). IDF have beneficial effects by reducing transit time and increasing fecal bulking, nonetheless is poorly fermented in the colon and it can further negatively influence food texture by its water retention and swelling properties (Fardet, 2010; Rosa-Sibakov *et al.*, 2015).

Therefore, the adverse effects of bran can be related to its insoluble nature. Enzymatic treatments solubilize AX by using endoxylanases, the latter modifies the ratio between soluble and insoluble fibers, raising the level of SDF and releasing health promoting compounds, not to mention the improvement of textural properties of bakery products (Singh *et al.*, 2016).

Figure 2 Proso millet, dehulled (source: Taylor, 2017)

2.2.1 Arabinoxylan

AX is the main non-starch polysaccharides (NPS) of many cereals, it is predominantly found in the outer layers (bran) and starchy endosperm. AX is composed of backbone chains of β-1, 4-linked d-xylopyranosyl residues to which α-L-arabinofuranose units are linked as side

chains in the second and third carbon-positions. AX is categorized into soluble water-extractable and insoluble water-unextractable which differ to their physicochemical properties. WEA has high molecular weight and leads to highly viscous solution when brought in aqueous, whereas WUA is characterized by strong water holding capacity (Lebesi & Tzia, 2012).

There are many techniques available for AX extraction and different extraction methods will give different yields and range of degrees of branching, molecular weight distribution and tertiary conformation (Fader *et al.*, 2018).

2.3 Xylanase

Processing of cereal bran with xylanolytic enzymes has been shown to facilitate the addition of bran to bakery products. They have the ability to increase the water holding capacity and therefore improve the softness and elasticity of the doughs, resulting in a better texture and volume of the final product. The beneficial effects of this enzymatic treatment have been related to the solubilisation of WUA and hence the increase of WEA content (Uysal *et al.*, 2007).

Endo-(1,4)-β-D-xylanase, also called endoxylanase or simply xylanase, is the most important xylanolytic enzyme, it depolymerizes and solubilizes WUA by cleaving the β-xylosidic bond between two D-xylopyranosyl residues linked in β-(1,4) (Juturu &Wu, 2012).

Most xylanases are produced by fungi and bacteria, but they can also be found in plants, insects, snails, etc. The first ones can be obtained from different fungal sources, like *Trichoderma reesei* and *Aspergillus niger*. They are typically stable over a wide pH range (3.0-10.0), whereas their pH optimum is generally (3.5-5.5). On the contrary, bacterial endoxylanases, derived from *Bacillus subtilis* or *Clostridium thermocellum,* usually have higher pH optima (6.0-7.0). Nevertheless, both of them have temperature optima between 40 and 50 °C (Santala, 2014).

III. Materials and methods

3.1 Millet bran samples

The bran was obtained from Proso millet grains harvested in 2017 and provided by Mlinopek d.d., Murska Sobota, a cereal factory located in Slovenia. The grains were decorticated with abrasive disks, subsequently the by-product was collected and size graded using screens of 1000 and 500 µm openings. In previous analysis, the latter demonstrated to have the highest nutritional, it also contained high amount of dietary fiber, of which soluble made 2%. For these reasons, it was chosen for the enzymatic treatment. In order to achieve a particle size similar to flours, it was dry-grinded for 4 min at room temperature by using a Cryogenic Mixer Mill (CryoMill; Retsch GmbH, Hann, Germany) with the following operating elements: 50 mL grinding jar, one 25 mm grinding ball and frequency of 30 Hz. After that, resulting powder was passed through the sieve one more time for 10 min using a screen of 180 µm opening. Finally it was transferred in plastic containers and stored at -18°C before use.

3.2 Enzymatic treatments of millet bran

EL-2018/002879, VERON® RL and VERON® XL are xylanolytic enzymes derived from specific cultures of *Trichoderma reesei*, *Bacillus subtilis* and *Aspergillus niger*, respectively. All this enzymes were used for the enzymatic treatment and were purchased from AB Enzymes GmbH (Darmstadt, Germany).

Table 1 Mixtures for enzymatic treatments

Sample identifier	Enzymes	Dosage of enzyme (g/g bran)	Bran (g)	Distilled water (mL)
XAN	VERON XL	0.0006	24	60
XTR	EL-2018/002879	0.00048	24	60
XBS	VERON RL	0.0012	24	60

The enzymes were weighted according to Table 1, and transferred into a 250 mL glass bottles containing 24 g of millet bran <180 µm. The enzyme powders were mixed with millet bran after the addition of 60 mL of distilled water. The mixtures and one control were incubated in a Stuart shaking water bath (SBS40, UK) at 120 rpm and 30°C for 24 hours. This process was repeated twice. Otherwise, an extra control (C0.0) was prepared by mixing 24 g of millet bran <180 µm and 60 mL of distilled water without enzyme addition, immediately it was frozen at -18°C. After 24 hours, all the samples were removed from the water bath and transferred into new flasks by rinsing remaining particles with 2 mL of distilled water. The pH was measured

using a Jenway pH meter (Jenway 3010; Jenway LTD, Essex, UK) before reaction stopped by freezing the samples at -18°C. Subsequently, the frozen samples were freeze dried and frozen stored before being used for analyses.

3.3 Measurement of xylanase activity

The endoxylanase activity in millet bran samples was analyzed by Xylazyme AX Tablet assay (Megazyme, Ireland) using *A. niger* control xylanase (260 mU/mL at 40°C and pH 4.7) in all of them. Each sample was weighted (0.5 g) in quadruplicate into glass tubes and mixed with 5 mL of 0.1 M acetic acid. In the first pair, 0.2 mL of distilled water were added, whereas for the others two tubes 0.2 mL of control xylanase solution, with vigorous stirring on a vortex mixer (Ika, Staufen, Germany). After 20 min the incubation at room temperature, the tubes were centrifuged (Hettich Rotina 35, Tuttlingen, Germany) at 4000 rpm for 10 min.

Accurately 0.5 mL aliquots of supernatant were transferred in duplicate to glass test-tubes. Subsequently, one Xylazyme AX tablet without stirring was added to each tube and immediately they were placed in a water bath at 50°C for exactly 30 min. The reaction was terminated by adding 5 mL of Trizma base solution (pH ~9) with vigorous stirring on a vortex mixer (Ika, Staufen, Germany). After 5 min at room temperature the tubes were mixed again and the slurry was filtered through a Whatman No.1 filter paper. Finally, the absorbance of the filtrate was measured at 590 nm against a reaction blank prepared according to the assay instructions. Its activity is measured in one unit of activity (U), it is the amount of enzyme required to release one micromole of reducing sugar equivalents from arabinoxylan per minute under standard assay conditions (40°C and pH 4.7).

3.4 Measurement of the content of total dietary fiber

The measurement of the content of total dietary fiber was achieved using a Megazyme Kit called “Integrated total dietary fiber”. It is an assay procedure including resistant starch and non-digestible oligosaccharides. This method combines the key attributes of AOAC Official Methods of Analysis 2009.01 and 2011.15, therefore it is divided in three sections: Enzyme digestion of samples (AOAC Methods 2009.01 and 2011.25), determination of HMWDF (IDF plus SDFP) (AOAC Method 2009.01) and determination of IDF and SDF (AOAC Method 2011.25). A brief summary of each section will be described below (Annex 1).

For enzyme digestion of samples, duplicate test portions (1.000 ±0.005 g) were incubated with pancreatic α-amylase and amyloglucosidase for 16 h at 37°C in sealed 250 mL bottles in a Stuart shaking water bath (SBS40, UK) while mixing with sufficient vigour to maintain continuous suspension. The reaction was terminated by adjustment of the pH to 8.2 with 3.0 mL of 0.75 M Tris buffer solution and incubation without shaking at 95-100°C for 20 min. Proteins in the samples were denatured and digested with 0.1 mL of protease solution, then the samples were incubated at 60 °C in the same shaking water bath for 30 min. The pH of the supernatants were adjusted to 4.3 ± 0.1 with 2 M acetid acid and finally 0.1 mL of D-sorbitol internal standard solution was added to each bottle.

For IDF, SDFP and SDFS determinations the supernatants, obtained in the previous step, were filtered through crucibles using vacuum pump (LVS 105 T - 10 ef, Ilmvac, Ilmenau, Germany). 20 mL of distilled water at 60°C were used to rinse the incubation bottles. The crucibles (IDF) and aqueous filtrates (SDFP) of each sample were collected, the first ones were washed using 78% (v/v) EtOH, 95% (v/v) EtOH and acetone, and dried overnight in 105°C oven (Heratherm, Thermo Fisher, USA), whereas the latters were heated to 60°C and precipitated using 280 mL of 95% (v/v) EtOH at 60°C. To recover SDFP the precipitate were filtered once again through crucibles using vacuum, washed and dried. The aqueous ethanol filtrate from the SDFP fraction is concentrated, desalted and analyzed by HPLC for SDFS. Both the IDF and SDFP residues are corrected for protein, ash and blank values for the final calculation of the IDF and SDFP values.

Calculations for HMWDF, IDF and SDFP:

$$Blank\ determination\ (mg) = \frac{BR_1 + BR_2}{2} - P_B - P_A$$

Where: BR_1 and BR_2 are the residue masses (mg) for duplicate blank determinations respectively. PB and PA are the masses (mg) of protein and ash respectively, determined on first and second blank residues.

$$HMWDF, IDF\ or\ SDFP\ \left(\frac{mg}{100\ g}\right) = \frac{\frac{R_1 + R_2}{2} - P_B - P_A - B}{\frac{M_1 + M_2}{2}} * 100$$

Where: R_1 and R_2 are residue masses 1 and 2 from M_1 and M_2 in mg, respectively. M1 and M2 are the test portion masses 1 and 2 in g, respectively. PA is the ash mass from R1 in mg and PB is protein mass from R2 in mg.

Calculations for SDFS:

$$SDFS\left(\frac{mg}{100\ g}\right) = R_f * (W_t\text{-}IS) * \left(\frac{PA\text{-}SDFS}{PA\text{-}IS}\right) * \frac{100}{M}$$

Where: Rf is the response factor, W_t-IS is weight in mg of internal standard contained in 1 mL of internal standard solution pipetted into sample mixture (100 mg), PA-SDFS is the peak area of the SDFS, PA-IS is the peak area of the internal standard (D-sorbitol) and M is the test portion mass (M1 or M2) in grams of the sample whose filtrate was concentrated and analyzed by LC.

Calculation of integrated TDF:

$$Integrated\ TDF\ (\%) = HMWDF\ (\%) + SDFS\ (\%)$$

3.5 Proximal composition of dietary fiber from millet bran

Protein content was determined by Kjeldhal method, using 6.25 factor for all cases to calculate mg of protein. Ash content was determined by dry ashing using a muffle furnace at 550°C for 5 hours. Both experiments were done in duplicate.

3.6 Moisture content analysis

The moisture content of the freeze dried bran was analyzed by oven (Heratherm, Thermo Fisher, USA), drying a sample of 2 g at 130 °C for 90 minutes. After exactly 90 min the sample was weighted and re-dried at 130°C for 30 min. The analysis was made in duplicate.

3.7 Water Holding Capacity (WHC)

The evaluation of WHC was performed in duplicate according to AACC international method 56-20.01 with minor modification. Two grams of sample were suspended in 50 mL falcon tube containing 40 g of distilled water. The sample was allowed to swell for 10 min at room temperature, inverting three times at end of 5 and 10 min. After being centrifuged (Hettich Rotina 35, Tuttlingen, Germany) for 15 min at 8000 rpm, the supernatant was discarded and the tube was weighted.

$$Hydration\ capacity\ \% = \frac{(weight\ of\ tube + sediment) - (weight\ of\ tube)}{sample\ weight\ (dry\ basis)}$$

3.8 Statistical analysis

All the results were expressed as the means ± standard deviations and carried out in duplicate at least. Data were analyzed by using one-way analysis of variance (ANOVA) by using Tinn-R software version 5.01.02.00 (ASCII and UNICODE, New Zealand).

IV. Results and discussion

The effect of use xylanase on millet bran was examined by analyzing the content of soluble and insoluble dietary fiber after 24 h treatment. Without addition of exogenous xylanase, proso millet bran contains 34.38% of total dietary fiber, about 95.40% of this fiber is IDF while the remaining 4.60% is SDF (Table 3), compared to the values obtained by Liu *et al.* (2011) normal millet bran has better proportions of both insoluble and soluble fiber, 89.58% and 10.42% respectively. However, proso millet bran looks more promising than rice bran, which 98.09% of its TDF consists by IDF and only 1.95% is SDF (Wen *et al.*, 2017).

This variability in the amounts of soluble and insoluble fiber can be caused by their endogenous xylanase activity, which plays also an important physiological role in cereals. Comparing the endogenous xylanase activity of millet bran with others cereal brans, millet bran value (45.78 U/kg) is lower than wheat bran (3590 U/kg), oat bran (890 U/kg), barley bran (1220 U/kg) and rye bran (290 U/kg). Nevertheless, these fluctuations can be explained by genetic, climatological and agricultural factors (Dornez *et al.*, 2009). This hypothesis is supported by Dornez *et al.* (2008), who reported that endoxylanase activity levels of agami wheat vary strongly over the years. They observed much higher enzyme activity in 2002 crop harvested in a cold and rainy summer (2530 U/kg) than 2004 crop harvested in a dry and warm summer (470 U/kg).

Besides this endogenous xylanase, cereals also contain microbial xylanases from microorganisms populating the outer grain kernels layers which can be removed by debranning. Therefore bran and short milling fractions contain much higher microbial xylanase activity levels than refined flours. In line with the above, the endogenous and microbial xylanase activity influence the percentages of insoluble and soluble fiber present in the bran, which can have a direct impact on processing, yields, and/or end-product quality (Dornez *et al.*, 2009).

Interestingly, after soaking the bran fraction in highly aqueous system (~71.30%) under constant agitation at 30°C for 24 hours and without addition of NSP-degrading enzymes, endogenous xylanase activity of millet bran decreased significantly 21% ($P \leq 0.05$) (Control), whereas, this activity increased significantly 42.11% ($P \leq 0.05$) when the bran was carried out as the same conditions but without incubation time and frozen at -18°C (C0.0). Unfortunately, the latter affected negatively TDF content of millet bran, raising the percentage of IDF 4.63% and decreasing SDF 17.08%. This result indicates that a low content of remaining endoxylanase

activity could mean greater solubilisation of WUAX. This theory is consistent with the report of Santala *et al.* (2011), which indicated that the highest degree of AX solubilisation was found when only 10% of the initial xylanase activity could be detected in the bran extract.

To analyze the residual xylanase activity of the samples treated with exogenous xylanases, their total xylanase activity must be calculated by summing the endogenous xylanase activity of millet bran and each added xylanase activity, results are given in Table 2. After 24 h enzymatic treatment, XTR and XBS remaining xylanase activities, 1.13% and 5.80% respectively, were significantly lower ($P \leq 0.05$). Despite these results, IDF content of XTR increased significantly 5.36% ($P \leq 0.05$), whereas its SDF content increased, not significantly ($P > 0.05$), 0.63% compared to control, as shown in Table 3. On the other hand, the insoluble fiber content of XBS increased significantly 3.07% with respect to control, but the increase in its soluble fiber content was more significant 8.22%. From the results obtained, it can be deduced that high levels of xylanase activity result in extended solubilisation of the soluble AX fragments and their degradation to lower molecular weight fragments. In contrary, the amount of xylanase used in XBS, despite containing a low xylanase activity was enough to break down hemicellulose present in the bran.

This negative correlation has also previously been reported by Li *et al.* (2013), they analyzed two different doses of the same commercial endoxylanase (360 and 600 ppm), concluding that the lowest dose diminished the negative effects of WUA on the bread, due to the conversion of the latter to WEA. In contrast, the addition of 600 ppm of endoxylanase enlarged the adverse effects of the WUA on the bread, causing a further decrease in the volume and a significant increase in the crumb firmness. However, Lebesi & Tzia (2012) demonstrated the opposite effect, they prepared two mixtures of bran and distilled water, 35% and 65%

Table 2 Initial and remaining endoxylanase activity of millet bran after 24 h enzymatic treatment

Sample identifier	Initial endoxylanase activity (U/kg)	Remaining endoxylanase activity (%)	pH
Control	45.78^{a}	79.00 ± 0.04^{c}	4.25 ± 0.11^{b}
C0.0	45.78^{a}	142.11 ± 0.04^{d}	-
XAN	3445.78^{c}	19.00 ± 0.00^{b}	4.37 ± 0.20^{c}
XTR	4349.11^{d}	1.13 ± 0.04^{a}	4.35 ± 0.08^{c}
XBS	613.78^{b}	5.80 ± 0.02^{a}	4.16 ± 0.08^{a}

- Not determined.
Values followed by different letters in the same column are significantly different ($P \leq 0.05$).

respectively, and treated it with 70 ppm and 700 ppm endoxylanase for 30 min at 40°C. The treatment with the low endoxylanase level resulted in AX fragments of high molecular weight due to solubilisation of WUA. When the high dosage was used similar solubilisation results with the lower dose level were obtained, probably due to the short time of the treatment.

From the results shown in Table 3, the highest content of soluble dietary fiber was obtained using the fungal endoxylanase from *A. niger*, with a significant increase of 24.05% ($P \leq 0.05$), furthermore only an increase of 3.14% in the IDF content was detected, compared to control. Therefore, it can be affirmed that the use of endoxylanases in millet bran can redistribute the content of both soluble and insoluble dietary fiber, attributing this achievement to WUA solubilisation. Lebesi and Tzia (2012) also reported that enzyme treatments by xylanase in bran fractions strongly positively correlate with the increase of water-extractable arabinoxylan and SDF content.

The value of pH for millet bran before enzymatic treatment was 5.86 ± 0.07. After enzyme addition and 24 h incubation time, millet bran showed remarkably low pH values in the range 4.16 ± 0.08–4.37 ± 0.20 (Table 2), which favor the performance of fungal endoxylanases. Napolitano *et al.* (2006) also observed high levels of xylanase activity when fungal strains were used for the treatment of cereal products. On the other hand, moisture content of millet bran without addition of xylanase was 5.68%, after treatment the final moisture content of all samples was statistically different compared to control ($P < 0.05$) (Table 3). This was possibly due to their hydration properties which will be described later.

Table 3 Composition of millet bran dietary fiber (% w/w) treated by different endoxylanases

	Control	C0.0	XAN	XTR	XBS
TDF	34.38 ± 0.02^{a}	35.63 ± 0.08^{bc}	35.78 ± 0.07^{c}	36.15 ± 0.04^{d}	35.53 ± 0.10^{b}
IDF	32.80 ± 0.04^{a}	34.32 ± 0.06^{c}	33.83 ± 0.09^{b}	34.56 ± 0.07^{c}	33.81 ± 0.08^{b}
Ash	5.80 ± 0.06^{e}	4.45 ± 0.07^{a}	5.02 ± 0.04^{b}	5.21 ± 0.03^{c}	5.41 ± 0.05^{d}
Protein	19.10 ± 0.05^{c}	15.75 ± 0.06^{a}	18.30 ± 0.09^{b}	18.31 ± 0.07^{b}	18.07 ± 0.04^{b}
SDF	1.58 ± 0.03^{b}	1.31 ± 0.04^{a}	1.96 ± 0.02^{c}	1.59 ± 0.09^{b}	1.71 ± 0.06^{b}
Ash	40.96 ± 0.07^{b}	56.08 ± 0.09^{d}	32.85 ± 0.06^{a}	47.79 ± 0.04^{c}	39.18 ± 0.07^{b}
Protein	27.36 ± 0.10^{d}	18.61 ± 0.02^{a}	24.12 ± 0.09^{b}	18.46 ± 0.08^{a}	25.75 ± 0.05^{c}
SDFP	1.22 ± 0.05^{bc}	1.00 ± 0.08^{a}	1.55 ± 0.10^{d}	1.14 ± 0.08^{b}	1.26 ± 0.08^{c}
SDFS	0.36 ± 0.02^{ab}	0.31 ± 0.04^{a}	0.41 ± 0.05^{bc}	0.45 ± 0.02^{c}	0.45 ± 0.03^{c}
Moisture	5.68 ± 0.00^{c}	2.10 ± 0.00^{a}	5.93 ± 0.00^{d}	4.11 ± 0.00^{b}	5.88 ± 0.00^{d}

Values followed by different letters in the same raw are significantly different ($P \leq 0.05$). Values expressed on dry basis.

Insoluble DF such as WUA are known to bind more water than their soluble counterparts, and thus the solubilisation of AX by hydrolytic enzymes can reduce its water holding capacity (Santala, 2014). According to Lebesi and Tzia (2012), WHC means the quantity of water that is absorbed into the fibers without the application of external forces after soaking (except for gravity and atmospheric pressure). It also includes the proportion of water loosely associated to the fiber matrix.

Figure 3 shows WHC values of the bran samples treated by different endoxylanases. Without addition of exogenous xylanase WHC of millet bran was 2.15%, after enzymatic treatment the samples did not significantly differ with each other ($P>0.05$). This situation was unexpected because XAN demonstrated greater amounts of SDF, therefore its WHC should be reduced. In contrary, XTR is the sample with the highest amount of IDF, its WHC should not be the same as the other samples.

An explanation could be that the hydration properties of bran fractions are not only influenced by IDF/SDF ratio, but also by the plant origin of the bran, the fiber porosity, temperature, pH, the fractionation process and histological features and particles mean size distribution of the bran fraction (Soukoulis & Aprea, 2012).

Regarding the particles size, Zhu *et al.* (2010) demonstrated, as particle size decrease, the hydration properties of wheat bran were significantly ($P < 0.05$) decreased, likewise, a redistribution of fiber components from insoluble to soluble fractions was observed, decreasing IDF from 81.13% to 68.65% and increasing SDF from 2.90% to 11.47%. In other words, the solubilisation of IDF is not directly related to the decrease of WHC.

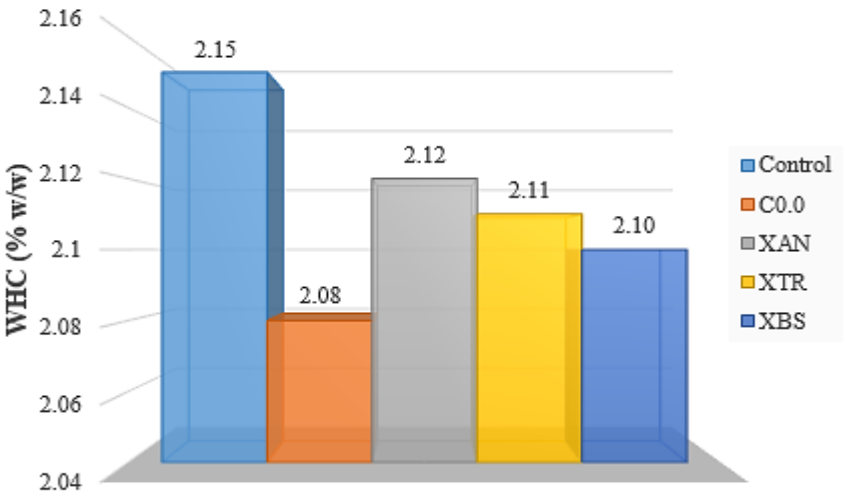

Figure 3 Water holding capacity of millet bran treated by different endoxylanases

V. Conclusions

Proso millet bran fractions contains 34.38% of total dietary fiber, about 95.40% of this fiber is IDF while the remaining 4.60% is SDF. The results of the work showed that application of endoxylanase in millet bran can redistribute the content of both soluble and insoluble dietary fiber, significantly increasing SDF content 24.05%. This achievement is mainly attributed to the solubilisation of WUA.

According to the data obtained, WUA solubilisation depends on different factors such as endogenous, exogenous and microbial xylanase activity, however there are other factors encompassed throughout the enzymatic process that can influence its performance, like water content and particle size. For that reason, it is recommended understand and regulate all these factors to determine the optimal conditions of the enzymatic treatment.

Interestingly, the water holding capacity of millet bran (2.15%) did not significantly change after enzymatic treatment. However, a reduction of the latter was expected due to the increase in the amount of SDF. One explanation to this outcome is that WHC not only depends on the amount of SDF or IDF, but also on the particle size of the bran.

VI. References

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Annex 1 Analytical scheme for the determination of HMWDF (IDF + SDFP) and SDFS