Master Thesis

Purification And Characterisation Of Xylooligosaccharides (XOS) From Wheat-Based Dried Distillers Grains With Solubles

Kourosh Hooshmand

Supervised By:

Peter Immerzeel

Henrik Stålbrand

Abbreviations

avDAS: Average degree of arabinose substitution

AX: Arabinoxylan

DDGS: Dried distillers grains with solubles

DDGS-W: Dried distillers grains with solubles originating mostly from wheat

DDGS-W-R: Dried distillers grains with solubles originating exclusively from wheat

DNS: 3, 5-Dinitrosalicylic acid

DP: Degree of polymerization

FOS: Fructo-oligosaccharide

GHF= Glycoside hydrolase families

HPAEC: High-Performance Anion-Exchange Chromatography

LCM: Lignocellulosic materials

MOS: Mannan-oligosaccharides

NaAc: Sodium acetate

NDOs: Non-digestible carbohydrates

PAD: Pulsed amperometric detection

SCFA: Short chain fatty acids

UA: Uronic acid

XOS: Xylo-oligosaccharide

Abstract

The aim of this project is to purify the xylan (the major hemicellulose in wheat bran) from dried distillers grains with solubles (DDGS) as source of NDO's (non-digestible carbohydrate) which is a by-product from the fuel-ethanol industry. The raw material used in the fuel-ethanol industry is wheat grain. The wheat grain is used for the fermentation of starch to produce ethanol in presence of yeast. The ethanol produced in this way is then purified by distillation. The by-product obtained is DDGS which today used as animal feed. In this project we have investigated if it possible to produce a value-added product from DDGS namely prebiotic xylooligosaccharides. The DDGS contains wheat bran rich in xylan and other compounds like starch, proteins, fats and also other forms of hemicellulose. The DDGS was diluted in water and this water extraction step was done to remove the water insoluble impurities like fat and proteins. The major impurity after the water extraction step is starch which is soluble in water. The hemicellulose fraction was purified from starch by using thermostable destarching enzymes, alpha-amylase and amyloglucosidase, followed by ethanol precipitation and dialysis. Xylan can be separated and converted to the novel product (xylooligosaccharides) by treatment of xylan with xylanase. The obtained xylooligosaccharides that can be separated and studied by HPLC (High-performance liquid chromatography) have prebiotic properties. This prebiotic material can be utilised as the source of energy for the bacteria called probiotic bacteria.

Introduction

Plant biomass is material from crops, trees and etc., which is rich in cellulose, hemicelluloses (mainly xylan in cereals), pectin and lignin **(Andersson, 2011)** that can be used for many applications like ethanol fuel production, paper industry and using them as fermentable sugars for growing probiotic bacteria. Hemicelluloses are amorphous heteropolysaccharides and can be hydrolysed relatively easy compare to cellulose because of a lower amount of hydrogen bonds involved in linkage with neighbouring polymers **(Jacobsen et al., 2000).** Probiotics bacteria such as Lactic *acid bacteria (LAB) and Bifidobacteria* groups which can increase the numbers of their cultures by using prebiotic substrates. They are very valuable as they contribute to the health of their host such as humans and animals **(Fuller, 1989)**. They can be used as an additive in dairy products like yoghurt or Japanese Yakult for human consumption to improve their health. In the colon certain probiotic bacteria can utilize prebiotics such as xylo-oligosaccharide (XOS), fructo-oligosaccharide (FOS) and mannan-oligosaccharides (MOS) **(Manning and Gibson, 2004).** Prebiotics are non-digestible carbohydrates (NDOs) that are very stable towards the low pH of the stomach and digestive enzymes on their way to the colon so they can be delivered to the intestine without being hydrolysed **(Okazaki et al., 1990).** Since probiotic bacteria such as *Bifidobacterium* are less capable of hydrolyzing AX (arabinoxylan) to arabinoxylo-oligosaccharides (AXOS) so in the intestine with the help of other bacteria such as *Bacteroides and Roseburia* the hetropolymer of AX can be hydrolyzed to AXOS and xylo oligosaccharides (XOS) followed by entering inside the probiotic bacteria cell in the bowel so the AX can be utilized completely by *Bifidobacterium* **(Hopkins et al., 2003). A** special transport system is required for probiotic bacteria in order to import the prebiotic substrates inside their cells before they are hydrolysed further by *Bacteroides* and *Roseburia* bacteria in the gut. AXOS and more efficiently XOS showed a better growth pattern with *Bifidobacterium* and less efficiently with *Bacteroides, Roseburia and E.coli (***Broekaert et al., 2011)**. After production of AXOS from AX by endoxylanases enzyme it is better that XOS is directly imported inside the cell of *Bifidobacterium* otherwise it can be converted to monomeric sugars by arabinofuranosidases and xylosidases from *Bacteroides and Roseburia* bacteria and further metabolised by competitive bacteria such as *Bacteroides, Roseburia and E.coli* **(Van De Wiele et al., 2007; Broekaert et al., 2011)**. Probiotic bacteria like *Bifidobacterium* have better transport system in their membrane for oligosaccharides than monomeric xylose **(Crittenden et al., 2002).** The anaerobic fermentation of XOS leads to production of short chain fatty acids (SCFA) mainly acetic, propionic and butyric acid. Together the production of SCFA and the growth of probiotic bacteria can result in many health benefits such as the suppression of pathogenic bacteria (because of a lower pH of the environment), colon cancer can be reduced, lipids can be made and it can progress the glucose tolerance and many other health benefits **(Swennen et al., 2006).** Oligosaccharides are more favorable to be used than

monosaccharides as a prebiotic source since monosaccharides can be easily utilized by other competitive bacteria around like *E.coli* but the oligosaccharides can just be utilized by probiotic bacteria such as *Lactic acid bacteria (LAB)* and *Bifidobacteria* **(Maes and Delcour, 2002*).*** Better selectivity of AX in *Bifidobacteria* can be observed in human and rats for AXOS which has DP≤9 **(Håska, 2011).**

Dried distillers grains with soubles (DDGS) is the by-product of the bio ethanol process which is a potential source of dietary fibres. During production of bioethanol from wheat (figure 1) the wheat bran should be first separated from the starch because non-starch polysacchairde can adsorb large amounts of water during the process which influence the amount of water needed. A bigger batch fermenter is needed when wheat bran is not removed **(Misailidis et al., 2009).** So to overcome this problem the wheat should be debranned. Fermentation by yeast will convert starch to ethanol which after distillation can be collected. The remaining liquid will be centrifuged which leads to the production of unfermented by-product DDGS (dried distillers grains with solubles) that contains approximately 85 per cent moisture **(Misailidis et al., 2009)**. DDGS has low amount of starch (because it is already converted to ethanol) but higher amount of fibre, fat, and protein. Two different by-products can be obtained after distillation of ethanol: 1: **DDGS-W** (dried distillers grains with solubles) originating mostly from wheat (contains starch and wheat bran) and up to 25 % can be composed of other species like Rye. 2: **DDGS-W-R** (Dry distiller grains with solubles) originating exclusively from wheat. In the bioethanol process for both DDGS-W and DDGS-W-R materials are ground in order to split the grain into the flour, the germ and bran **(Haskå, 2011).** DDGS-W then will go through the system but DDGS-W-R will be further sieved before fermentation. It means that amount of hemicelluloses in the DDGS-W-R process is lower than DDGS-W process.

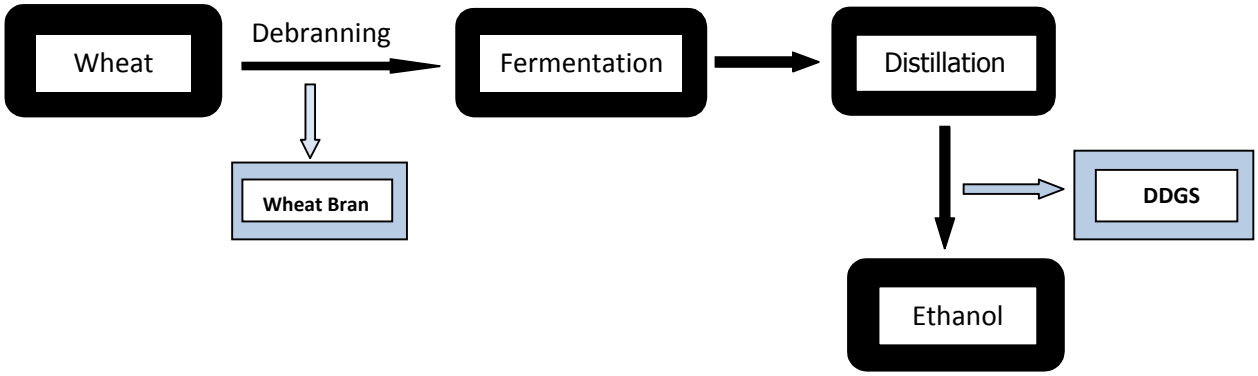

Figure 1: Bioethanol production and production of by-product DDGS

The DDGS's by product is used as a high nutrient feed for animals but nowadays by applying new enzyme technology we aim for producing a novel product that can be candidate as a prebiotics substrate. This novel product can be used in many applications such as prebiotic

sources for probiotic bacteria in food technology, viscosity enhancer **(Misailidis et al., 2009)** or can increase the quality of the dough in bread making **(Courtin and Delcour, 2002).** In cereals such as wheat which is cultivated in most countries all over the world the main and the most abundant hetero polymer which can be found is arabinoxylan (AX). The average degree of arabinose substitution (avDAS) to xylose varies in different parts of wheat kernel. Xylan can strongly bind to polymer of the cell wall which is quite difficult to be extracted **(Andersson, 2007).** Wheat kernels which are the seeds of the wheat plant are composed of three parts: endosperm, germ and wheat bran **(figure 2).** Wheat bran contains most of the AX heteropolymer and it is xylan rich part of each kernel. It is composed of an aleurone layer and pericarp layers which the amount of avDAS is higher in pericarp layers than aleurone layer and endosperm has a higher avDAS than aleurone **(Izydorczyk and Biliaderis, 1995).**

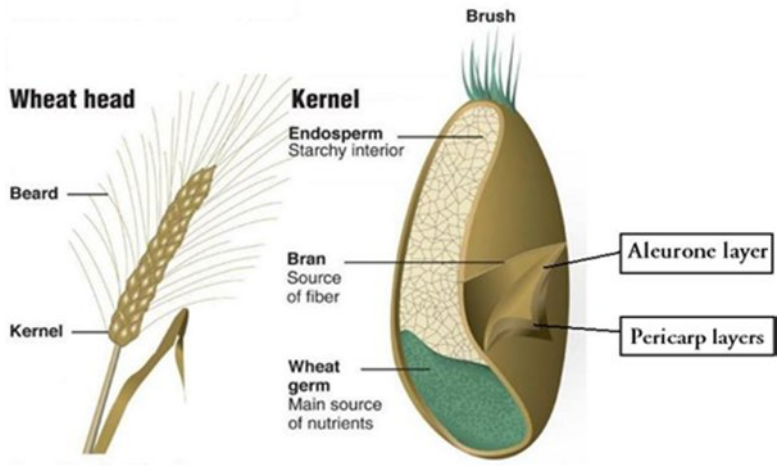

Figure 2: The whole grain of wheat which is composed of many kernels.

The major hemicellulose obtained from cereals is xylan. In wheat bran the polymer which can be found as a major component is arabinoxylan **(Fleming et al., 1983).** The xylan solubility is proportional to the degree of side-chain substitution **(Cacais et al., 2001).** In DDGS materials which mostly comes from wheat bran the xylan is less substituted **(Maes and Delcour, 2002)** which means that it can be dissolved in water which is called water extractable (WE). DDGS xylan is made from β-D-1,4 units of xylopyranosyl units (pyranosyl because it is a five membered ring) which has the α-L-1,4 arabinofuranosyl sugar (furanosyl because it is four membered ring) as a major side-chain substitution which is binds through C-(O)-2 or C-(O)-3 to xylose group which is depicted in figure 3 **(Lequart et al., 1999).**

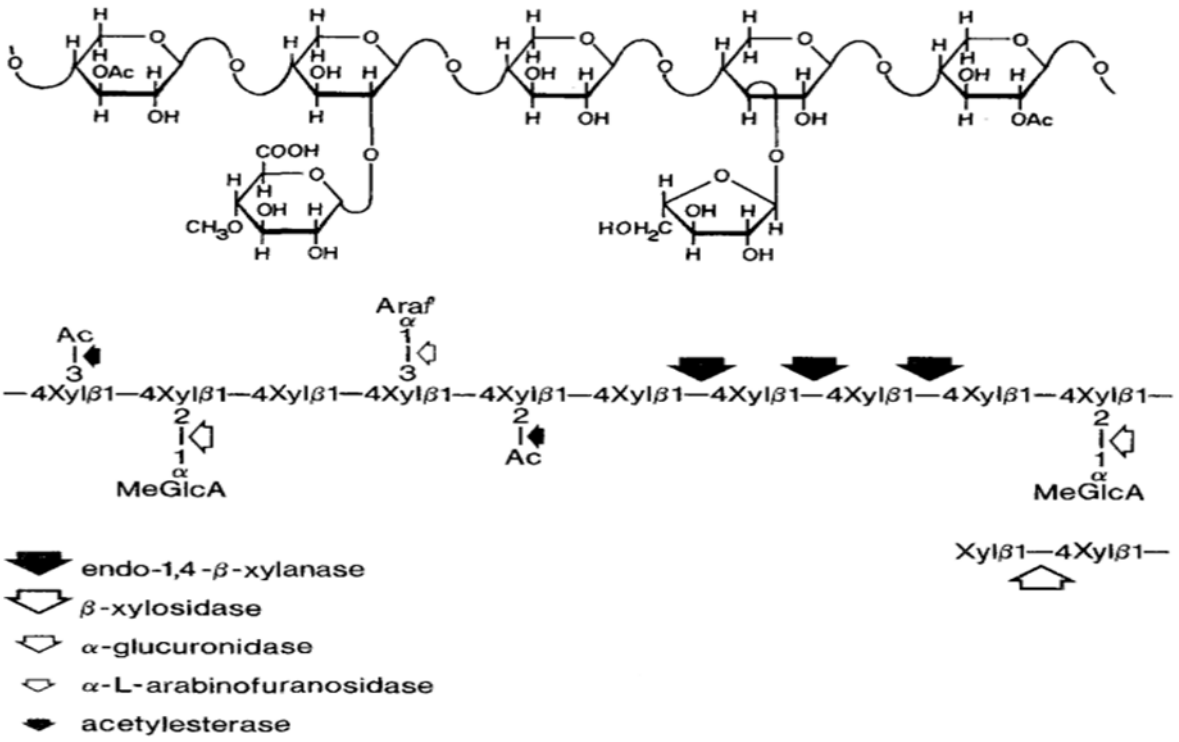

Figure 3: General structure of arabinoxylan and the enzymes which are involved in the hydrolysis (The figure from Biely, P (1985)). **Xyl : xylopyranosyl, Ara: arabinofuranosyl, Ac= acetyl, Glc: glucuronic acid.**

Arabinose substituted in cereals can be mono and disubstituted on D-xylose group **(Andersson and Åman, 2008).** α-D-glucuronic acid can also be substituted to the C-(O)-2 of xylose in the backbone. Acetyl groups can be observed in the structure of AX **(Roberfroid et al., 2010).** Because of the high complexity of heteroxylan cooperation between enzymes is needed for having a proper hydrolysis with low amount of undesirable by products such as furfural that can be produced by acid hydrolysis **(Biely**, **1985).** The hydrolytic enzyme β-D-endoxylanase can cleave the polymeric backbone into the small pieces (oligomers). β-D-Xylosidase can further hydrolyse oligomers to yield the monosaccharide xylose. Two of the main glycoside hydrolase families (GHFs) which contains xylanases are GHF10 and GHF11. The thermostable bacteria *Rhodothermus marinus* (found in Icelandic hot springs) produces endo-1,4-β-xylanase from GHF 10A **(Ramchuran et al., 2005).** Xylanases have many applications like in the paper and pulp industry because hydrolysis of xylan loosen the lignin complex which reduce the consumption of chlorine **(Roberfroid et al., 2010)**. The main aim of this project was purification of xylan, one of the major hemicelluloses from wheat-based dried distillers grains with solubles. Starch as the main contamination was tried to be eliminated from DDGS materials. For this purpose different kinds of destarching enzymes have been used. Xylan was subsequently hydrolysed by using endo-xylanase enzyme. The

potential of xylooligosaccharides as a prebiotic substrate was tested by using *Lactobacillus brevis* probiotic bacteria. By tracing of which XOS have been utilised by *Lactobacillus brevis* bacteria we can judge which size of XOS is optimal to be fermented by probiotic bacteria.

Materials and Methods

Dried Distillers Grains with Solubles (DDGS)

The samples DDGS-W (dried distillers grains with solubles wet) originating mostly from wheat (minimum 75%) and DDGS-W-R (dried distillers grains with solubles wet) originating exclusively from wheat. Moisture content for DDGS-W was 90.4 % and for DDGS-W-R 86.0 % (data from Peter Immerzeel at the center for molecular protein science, Lund University). Samples were stored at cold room -20˚C.

Water Extraction

The DDGS was diluted in MQ-Water. For DDGS-W to 350 gram, 187 gram of MQ-water was added and for DDGS-W-R to 360 gram, 446 gram of MQ-water was added (the final concentration was 62.5 mg/ml for both DDGS-W and DDGS-W-R). It was stirred for about 1 hour followed by centrifugation (10000 rpm, 10 minutes); subsequently the pellet was kept aside. Supernatant was filtered (Ø90 mm, Munktell). Then it was frozen in round bottom flask on dry ice and freeze-dried afterwards. The weight of the obtained supernatant after freeze drying is expressed as a percentage of the total dry mass of the DDGS before extraction.

Destarching

Freeze dried water extracted materials were treated with enzymes in order to remove starch. Samples were placed inside the incubator for about 1 hour with continuous stirring in order to solubilise the materials in citrate buffer, pH=6. Then α-Amylase (Megazyme, Bray, Ireland) (1600 U/ml) and Amyloglucosidase (Megazyme, Bray, Ireland) (3260 U/ml) was applied to the samples and incubated at 40˚C at different times for each sample. The enzyme was denatured by heating at 100˚C for 10 minutes. Three different ethanol concentrations (60, 70 and 80 %) were applied for removing low molecular weight contaminants. Samples were stirred every 5 minutes for 30 minutes. All tubes were placed inside the cold room overnight. The samples were centrifuged at 4000 rpm for 10 minutes. Pellet was saved and sugar composition was determined on a Dionex PA 10 Column. The supernatant which contains most of the starch degradation products was kept aside and analysed for the presence of starch oligosaccharides on a Dionex PA 100 Column.

Dialysing

Dialysis membranes tubing with the molecular weight cut-off (MWCO) of 3500 Da (Spectra/Por, USA) was used. The membrane vol/length was 6.4 ml/cm. Dialysis tubing are supplied in rolls and when get wet, will open up into a cylindrical tube that can be tied off at the ends. The Supernatant after destarching from ethanol precipitation (80%) was poured in dialysing tubing. It was immersed in 1L M-Q-Water. The salts, monosaccharides, small oligsaccharides and ethanol went out of tubing and water replaced inside. The conductivity (µS/cm) was measured every 6 and 24 hours. The beakers were kept in the cold room with continuous stirring during the experiment. The water was changed every 24 hours. After 3 days no significant reduction of the conductivity was observed and the dialysis was ended. The dialysing tube was cut subsequently materials inside were freeze dried and sugar composition was determined.

Mono Sugar Analysis

Total sample sugar composition was determined by pre-hydrolysis **(Seaman, 1963)** with a 72 % H_2SO_4 (2700 mg 96 % sulphuric acid to 900 mg MQ-water on ice) for 2 hours at room temperature. Samples were stirred every 30 minutes. MQ-Water was added in order to dilute the acid to 4 % H_2SO_4 and the samples were transferred to reaction tubes with screw cap. The monosugar standard (fucose, arabinose, rhamnose, glactose, glucose, xylose, and mannose) was included. 175 µl of 72 % H_2SO_4 was added to standard (0.5 mg/ml+ UA (uronic acid) 1mg/ml) 4.9 ml in 4 tubes with screw cap to dilute the samples to 4 % concentration of H_2SO_4. All tubes were heated for 3 hours at 100°C. The tubes were cooled down and vortexed. The pH was set to 5-6 by adding $Ba(OH)_2$. The tubes were spun down (3000 rpm for 5 minutes). The supernatant was taken by syringe and filtered with 0.2 µm membrane filters (VWR).

Non-hydrolyzing samples with mono sugars already present in the samples were prepared by following procedure: MQ-Water was added to dry samples then it was stirred about 2 hours afterwards centrifuge was applied (14000 rpm, 5 minutes). The pellet was discarded and the supernatant was taken by syringe which filtered by 0.2 µm membrane filter. Four standards (0.1 mg/ml) were prepared too.

For water extracted materials hydrolysis was done without any pre-hydrolysis treatment by adding H_2SO_4 4 % to the sample. To the standard (0.5 mg/ml+ UA 1mg/ml) (4.9 ml), H_2SO_4 72 % (175 µl), was added in order to get 4 % concentration. The same procedure was done after this stage similar to total sample hydrolysis procedure.

HPAEC-PAD (High Performance Anion Exchange Chromatography with Pulsed Amperometric Detection) has been applied for quantification and qualification of sugars on Dionex IC system. The CarboPac PA10 (Dionex, USA) is an anion-exchange column [Resin Composition

was 10-µm diameter substrate (ethylvinylbenzene 55% crosslinked with divinylbenzene) accumulated with 460-nm micro bead difunctional quaternary ammonium ion (5% crosslinked)]. Operating Pressure was 2600-2700 psi. Eluent bottles were pressurised with nitrogen gas. Flow rate was 1 ml/min and the injection volume was 10 µl. Detection was accomplished with pulsed amperometric detection (PAD) which was equipped with a gold electrode. A small fraction of sugar which has been deprotonated by NaOH subsequently will be oxidized on the surface of gold electrode because of positive potential which is generated. Carbohydrate which has been oxidized can contaminate the surface of electrode. By producing series of potentials the surface of electrode will be cleaned. First after applying the first potential (E1) the gold will be slightly oxidised then after the fourth potential (E4) it would be reduced that causes the carbohydrate washed from the surface and be cleaned. NaOH (1mM) isocratic elution and NaOH (600mM) elution for cleaning the column at pH 14 was used as an eluent. Eluent filtered by using membrane filter cellulose (CHETER), 0.2 µm or Ø 47 mm then they were degassed before applying to the HPLC. Degassing was carried out by stirring the eluents with a magnetic stirrer, and application to a vacuum.

Oligomeric Sugar Analysis

CarboPac PA100 was used to separate oligosaccharides which were coupled with pulsed amperometric detection. Flow rate was 1 ml/min and the injection volume was 10 µl. Operating Pressure was 2600-2700 psi. NaOH (600mM) isocratic elution, NaAc (sodium acetate) (500 mM) gradient elution (it is stronger than NaOH for washing the oligomers which have been bound strongly to the column) and 18-MΩ water were used as an eluent which all were degassed.

Xylanase Activity

To test the activity of the endo-xylanase 10A (from *Rhodothermus marinus*, 12 U/ml, 1 % birch xylan in 0.05 M sodium citrate buffer, pH=6) was used as a substrate for measuring the releasing of reducing sugars inside the solution **(Bailey and Poutanen, 1989)**. Xylose as a standard in different concentrations (0, 2, 2.5, 5, 10 mM) was used to make the standard. Birch xylan was used as a blank. First pre incubation of substrate (360 µl of birch xylan) was done for blank, standards and samples at 50°C for 5 minutes. The enzyme 40 µl (xylanase10A full length in sodium citrate buffer, pH=6) was added only to the samples (triplet) and incubated for 300 seconds at 50°C. DNS (3,5-Dinitrosalicylic acid) 600 µl was added afterwards to stop the reaction and the whole tubes were heated at 100 °C for 10 minutes. After cooling the samples absorbance was measured at 540 nm. Xylose standard (40 µl) in different concentration was added to the substrate tubes (360 µl) and was incubated in water bath at 50 °C for 5 minutes. DNS 600 µl was added and the whole tubes were placed in heating block at 100°C for 10 minutes follow by measuring the absorbance at 540 nm. To

the blank along 360 µl of birch xylan which was already incubated, 40 µl of enzyme was added then immediately DNS 600 µl was added and it was heated at 100°C for 10 minutes subsequently absorbance was measured. The reducing sugar concentration was measured by using the DNS which is reduced. The colour which is produced against the regent blank was measured at 540 nm by uv-visible spectroscopy (SHIMADZU, UV-2401 PC). After concentration curve was made the unknown concentration of reducing sugars after hydrolysis was calculated in nmol. Since the incubation was for 300 second then activity is shown nmol/sec (nkat). After considering volume (0.5 ml) the unit becomes nKat/ml. Since 1U= 16.67 nano katal as a consequence the final unit was defined as U/ml.

Production of XOS (xylose oligosaccharide)

Extracted xylan was converted to XOS by endo-xylanase 10A. Destarched samples were dissolved in sodium citrate buffer (pH~5.3, 0.05 M). Samples were incubated for 1 hour at 50°C. Xylanase (full length, family 10A from *Rhodothermus marinus*, 12 U/ml) added to the samples. Different incubation times have been applied (0, 50, 150 and 220 minutes). The Enzyme was denatured at 100°C. The presence of XOS was determined by analysis of HPAEC-PAD with CarboPac PA100. Arabinose, xylose (X1), xylobiose (X2), xylotriose (X3), xylotetrose (X4) and xylopentose (X5) (Sigma Aldrich, Germany) have been used as a standard.

Growth of the bacteria (anaerobic digestion)

The pre-culture *Lactobacillus brevis* bacteria were prepared in Man Rogosa Sharp (MRS) broth media (pH=6.4) which monomeric glucose was excluded. The pre-culture *L.brevis* was inoculated to new MRS media under anaerobic condition which was incubated at 37°C for 1 day. Xylose and glucose (25 mg/ml) was used as alternative carbon source. MQ-Water was used as control. They were sterilized by using 0.45 µm filters. XOS samples were placed in autoclave at 121°C for 15 minutes to be sterilized. The microplate spectrophotometer reader Expert plus (Biochrom, Cambridge, UK) which contained 96 wells was used for growing the bacteria. Substrates (40 µl), MRS medium (196µl) and *L.brevis* culture (4 µl) was added to the wells. To the remaining wells only 200 µl of MRS medium was added without adding *L.brevis* and carbon sources to be able tracing a contamination by changing the absorbance values. At zero minutes the optical density of plate was measured at 620 nm and samples were collected. The plate was placed in a box which was filled under a constant flow of N_2 gas. Anaerocult C for generating an anaerobic atmosphere and microbiology anaerotest for the detection of an anaerobic atmosphere was placed inside the container too. It was anaerobically incubated at 30°C. After (24, 48 and 72) hours the OD of the samples inside the plate was measured at 620 nm. Sample was collected except at 48 hours. Samples from 24 and 72 hours were pooled. The utilization of XOS during bacteria growth was traced on HPAEC-PAD with CarboPac PA100 by comparing to the standards.

Result and Discussion

The sugar composition of the dried distiller grains with solubles (DDGS) which came from the industry (DDGS-W and DDGS-W-R) was determined (Table 1). Because there is not a direct way to calculate the polymeric sugar composition, the following method has been used. Mono sugars already present in the sample before hydrolysis have been analysed which are named soluble mono sugars. The value of the calculated polymeric sugar composition is obtained by subtraction the value for the soluble mono sugars from the value of the hydrolysed sample. The carbohydrate composition of the total sample shows a total polymeric glucose content of 16.9% and 24.6 % of dry weight for DDGS-W and DDGS-W-R respectively which can be derived from cellulose or starch (table 1). By comparing the carbohydrate composition of wheat bran (table 2) from the literature study with the starting materials DDGS (table 1) some sugars are present in both substrates: arabinose, glucose, xylose and in less extent: galactose and mannose. Conclusion is that enough xylan is present for isolation. Amount of rhamnose as it is shown in table 1 is zero which we have been expected because pectin is not presented in wheat bran and rhamnose is part of pectin.

Table 1: Total sample carbohydrate composition of starting materials DDGS-W and DDGS-W-R (% dry weight)

Total Sample	Sugar(% Dry Weight)							Total Sugar (%)
	Fuc	Ara	Rha	Gal	Glc	Xyl	Man	
DDGS-W Hydrolysis	0	5.5	0	1.0	17.3	9.6	0.9	34.3
DDGS-W Soluble Mono Sugars	0	1.5	0	0.2	0.4	0.4	0	2.5
DDGS-W Calculated Polymeric Sugar	**0**	**4.0**	**0**	**0.9**	**16.9**	**9.2**	**0.9**	**31.9**
DDGS-W-R Hydrolysing	0	4.5	0	0.6	24.8	7.6	4.0	41.6
DDGS-W-R Soluble Mono Sugars	0	1.3	0	0	0.2	0.1	0	1.6
DDGS-W-R Calculated Polymeric Sugar	**0**	**3.2**	**0**	**0.6**	**24.6**	**7.5**	**4.0**	**39.9**

Fuc: fucose, Ara: arbinose, Rha: rhamnose, Gal: galactose, Glc: glucose, Xyl: xylose, Man: mannose

Hardly any mannan polysaccharide has been found in wheat bran (table 2) but in the DDGS materials especially in DDGS-W-R some mannan has been monitored. Mannan polymers have been derived from the yeast cell wall **(Ballou, 1976).** Less mannan is detected in DDGS-W probably due to the fact that in the batch fermentator a higher amount of non-starch polysaccharides is present as a consequence the ratio yeast to remaining polysaccharides is lower for DDGS-W.

Table 2: Sugar composition of wheat bran from own study and literature (% dry weight)

Wheat Bran	Sugar (% Dry Weight)						
	Fuc	Ara	Rha	Gal	Glc	Xyl	Man
WB analysis Peter I (2010-10-12)	0	7.1	0	0.8	19.8	12.6	0
Kabel, et al (2002)	nD	9	0	1	33(20) starch	16	0
Thender,et al (1986)	nD	7.2	0.1	0.7	34(24) starch	15	0.3

Fuc: fucose, Ara: arbinose, Rha: rhamnose, Gal: galactose, Glc: glucose, Xyl: xylose, Man: mannose nD: not Determined

Work flow (figure 1) describes the procedure set to purify polymeric xylan from by-product of bio ethanol (DDGS) which contains many other polysaccharides. First the starting materials should be water extracted. In order to remove the starch from the extract materials a treatment with α-amylase and amyloglucosidase should be performed. Afterwards by doing ethanol precipitation the carbohydrate composition of destarched DDGS will be determined to judge how much glucose is remained after the destarching to able to judge about the purity of xylan polymer. The dialysis of the supernatant will also be done to see which purification methods give us sufficient amount of xylan polymer with less amount of contamination after destarching by enzymes. Xylanase treatment and producing of xylo oligosaccharide followed by fermentation of XOS by bacteria will be investigated too to judge if XOS derives from DDGS has a positive effect on growth or not.

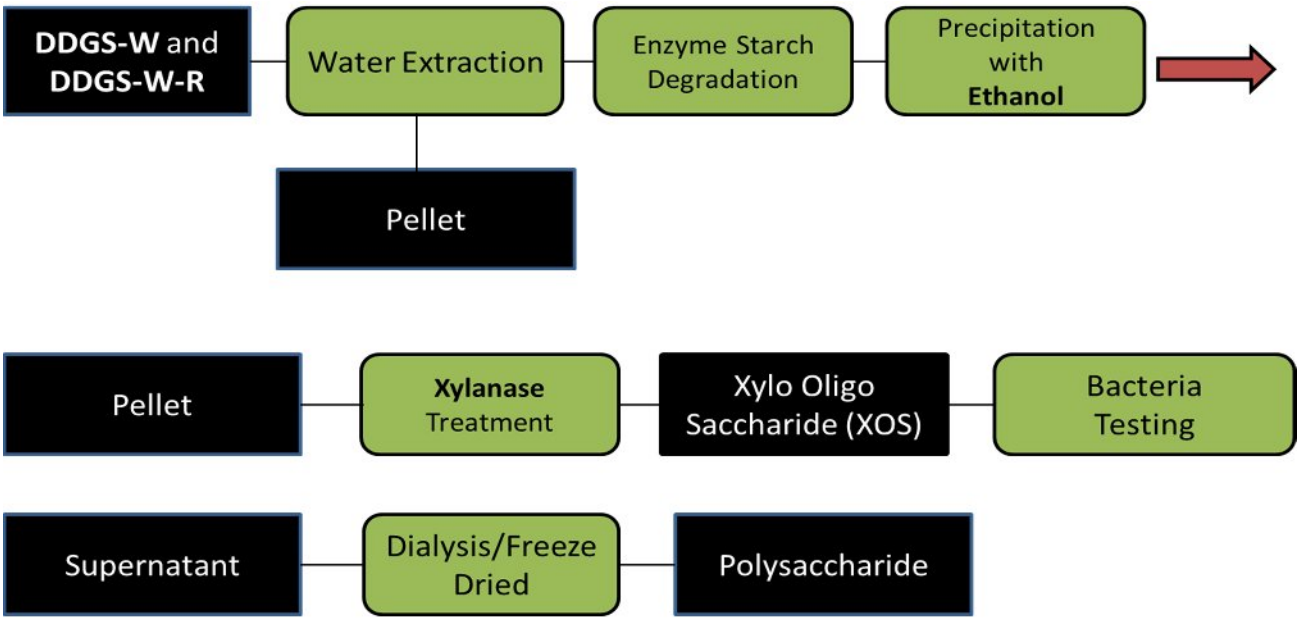

Figure 1: Procedure for the isolation of xylan from DDGS. The purification and the production of xylo oligosaccharide.

Small scale experiment was done (table 3) to make the best decision with the information which has been collected that is it worth the effort to do the large scale for DDGS materials afterwards. Total sample sugar composition was determined with significant quantities of polymeric xylose (table 1). Now the experiment went a step further for water extraction which data are shown (table 3). Significant amount of polymeric xylan was traced that justifies the effort of isolation and purification. Destarching was done following 80 % ethanol precipitation which ended up with 24.1 % and 17.6 % of dry weight yield for DDGS-W and DDGS-W-R water extractable materials respectively. The materials after destarching was further treated with xylanase to get the xylo oligosaccharide. The yield of the xylan recovery was 10.4 % and 12.2 % for DDGS-W and DDGS-W-R respectively.

Table 3: Small sacle carbohydrate composition (DDGS-W and DDGS-W-R) in different stages (% dry weight)

Samples	**Sugar (% Dry Weight)**							**Total Sugar (%)**
Total Sample	**Fuc**	**Ara**	**Rha**	**Gal**	**Glc**	**Xyl**	**Man**	
DDGS-W Calulated polymeric sugars	0	3.3	0	0.9	13.6	6.9	0.9	25.6
DDGS-W-R Calculated polymeric sugar	0	1.4	0	0.7	20.5	4.7	2.4	29.7
Water extraction	**Fuc**	**Ara**	**Rha**	**Gal**	**Glc**	**Xyl**	**Man**	**Total Sugar (%)**
DDGS-W Calulated polymeric sugars	0	5.2	0	1.2	14.1	8.2	0.4	29.1
DDGS-W-R Calculated polymeric sugar	0	2.7	0	0.7	12.5	5	2.4	23.3
After Destarching	**Fuc**	**Ara**	**Rha**	**Gal**	**Glc**	**Xyl**	**Man**	**Total Sugar (%)**
DDGS-W Calulated polymeric sugars	0	9.1	0	2.6	1.59	12.3	0	25.6
DDGS-W-R Calculated polymeric sugar	0	5.8	0	3.3	11.5	8	0	28.6

Fuc: fucose, Ara: arbinose, Rha: rhamnose, Gal: galactose, Glc: glucose, Xyl: xylose, Man: mannose

From the small scale experiment results it was calculated that for obtaining 1 gram XOS product in large scale experiment how much of starting material is required to be used. So for water extraction 33 and 50.1 gram of DDGS-W and DDGS-W-R have been used respectively. As it is shown in (table 4) significantly high yield for DDGS-W (72.5 %) in big scale was observed after water extraction which was 77.7 % for small scale. The value for DDGS-W-R (44.5 %) is lower compared to the small scale extraction which was 64 % yield.

Table 4: The yield of DDGS-W and DDGS-W-R after water extraction from big scale experiment.

Sample	Initial amount	Water extractable	Yield
DDGS-W	33 (gram)	23.93 (gram)	72.5 %
DDGS-W-R	50.1 (gram)	22.34 (gram)	44.5 %

Data show (table 5) in supernatant of water extraction from DDGS-W-R amount of polymeric xylan is quite low in comparison to the small experiment which was done with similar set up (table 3). In small scale experiment amount of polymeric xylan which was obtained after water extraction was 5% (table 3) but in big scale 3.3% (table 5) which means 1.7% is missing. So sugar composition of pellet after water extraction was determined too (table 5) in order to see the loses went through a pellet or not. Since significant amount of polymeric xylan in DDGS-W and DDGS-W-R samples have been observed in the pellet (table 5) it can be concluded that water extraction method needs to be optimised in future for better purification. It is also shown in table 5 that the massive amount of polymeric glucose exists in the water extraction materials supernatant which should bring into the consideration.

Table 5: Carbohydrate composition of water extraction materials (DDGS-W and DDGS-W-R)

Sample Water extraction **(SUPERNATANT)**	**Sugar (% Dry Weight)**							Total Sugar (%)
	Fuc	Ara	Rha	Gal	Glc	Xyl	Man	
DDGS-W Hydrolysis	0	5.7	0	1.0	14.6	8.6	0.7	30.5
DDGS-W Soluble Mono Sugars	0	1.6	0	0.2	0.4	0.4	0	2.6
DDGS-W Calculated Polymeric Sugar	**0**	**4.1**	**0**	**0.8**	**14.2**	**8.2**	**0.7**	**27.9**
DDGS-W-R Hydrolysing	0	3.6	0	0.6	11.1	4.8	3.1	23.1
DDGS-W-R Soluble Mono Sugars	0	1.2	0	0	3.6	1.4	0	6.3
DDGS-W-R Calculated Polymeric Sugar	**0**	**2.4**	**0**	**0.6**	**7.5**	**3.3**	**3.1**	**16.8**
Sample Water extraction **(PELLET)**	**Sugar (% Dry Weight)**							Total Sugar (%)
	Fuc	Ara	Rha	Gal	Glc	Xyl	Man	
DDGS-W Hydrolysis	0	1.0	0	0	15.0	4.0	0	20.0
DDGS-W Soluble Mono Sugars	0	0.1	0	0	0.1	0.0	0	0.2
DDGS-W Calculated Polymeric Sugar	**0**	**0.9**	**0**	**0**	**14.9**	**4.0**	**0.0**	**19.8**
DDGS-W-R Hydrolysing	0	1.8	0	0	24.3	4.3	0	30.4
DDGS-W-R Soluble Mono Sugars	0	0.1	0	0	0.1	0	0	0.2
DDGS-W-R Calculated Polymeric Sugar	**0**	**1.7**	**0**	**0.0**	**24.2**	**4.3**	**0**	**30.2**

The small scale experiment (pilot study) was set for destarching. Starch hydrolysis enzyme was applied to the water extracted materials in order to get rid of excessive amount of polymeric glucose. Ethanol at different concentrations (60, 70 and 80%) was applied to the samples so that oligomers of sugar will be precipitated but monomer of sugar will be stayed in ethanol. The pellet after ethanol precipitation was freeze dried and the sugar composition was determined (table 6 and 7).

Table 6: Sugar composition of DDGS-W from the pellet after ethanol precipitation with different concentration of ehtanol

Sample (**Ethanol precipitation**)	**Sugar(% Dry Weight)**							Total Sugar (%)
	Fuc	Ara	Rha	Gal	Glc	Xyl	Man	
DDGS-W Hydrolysis (60%)	0	2.1	0	0.6	1.4	3.6	2.0	9.7
DDGS-W Soluble Mono Sugars (60%)	0	0.2	0	0	0.6	0	0	0.8
DDGS-W Calculated Polymeric Sugar (60%)	**0**	**1.9**	**0**	**0.6**	**0.8**	**3.6**	**2.0**	**8.9**
DDGS-W Hydrolysis (70%)	0	3.7	0	1.0	0.9	5.3	0.8	11.6
DDGS-W Soluble Mono Sugars (70%)	0	0.1	0	0	0.3	0	0	0.4
DDGS-W Calculated Polymeric Sugar (70%)	**0**	**3.6**	**0**	**1.0**	**0.6**	**5.3**	**0.8**	**11.3**
DDGS-W Hydrolysis (80%)	0	3.2	0	0.8	1.0	5.1	0.6	10.7
DDGS-W Soluble Mono Sugars (80%)	0	0.1	0	0.0	0.2	0	0	0.2
DDGS-W Calculated Polymeric Sugar (80%)	**0**	**3.1**	**0**	**0.8**	**0.8**	**5.1**	**0.6**	**10.5**

Fuc: fucose, Ara: arbinose, Rha: rhamnose, Gal: galactose, Glc: glucose, Xyl: xylose, Man: mannose

For DDGS-W by increasing the ethanol concentration from 60 to 80 % amount of polymeric xylan which is precipitated was increased (figure 2). The destarching procedure for both DDGS seems to work the best for 80 % concentration of ethanol because the yield which was obtained was higher than other concentrations (table8). The supernatants from ethanol precipitation were collected and tested in order to see whether polymeric xylan is presented

or not. After evaporating the ethanol the supernatant analysed for the presence of oligomeric carbohydrate on HPLC. The chromatogram showed that by increasing the concentration of ethanol from 60 to 80 % less polymeric xylan was present in supernatant. Amount of polymeric form of sugar (starch) has been rapidly reduced after applying starch hydrolytic enzyme. For DDGS-W from 14.2% in water extraction to 0.8 % in destarch materials (table 6). In contrary to DDGS-W-R in comparison with water extraction amount of glucose from 7.5% is reduced to 4.8% which is not a good observation. It means the destarching is not working properly with DDGS-W-R materials.

Table 7: Sugar composition of DDGS-W-R from the pellet after ethanol precipitation with different concentration of ehtanol

Sample (Ethanol precipitation)	Sugar(% Dry Weight)							Total Sugar %
	Fuc	Ara	Rha	Gal	Glc	Xyl	Man	
DDGS-W-R Hydrolysis (60%)	0	2.5	0	0.7	2.9	3.8	2.8	12.7
DDGS-W-R Soluble Mono Sugars (60%)	0	0.1	0	0	0.8	0	0	0.8
DDGS-W-R Calculated Polymeric Sugar (60%)	**0**	**2.4**	**0**	**0.7**	**2.1**	**3.8**	**2.8**	**11.9**
DDGS-W-R Hydrolysis (70%)	0	2.2	0	1.2	6.0	3.3	5.5	18.2
DDGS-W-R Soluble Mono Sugars (70%)	0	0.1	0	0	0.6	0	0	0.7
DDGS-W-R Calculated Polymeric Sugar (70%)	**0**	**2.2**	**0**	**1.2**	**5.4**	**3.3**	**5.5**	**17.5**
DDGS-W-R Hydrolysis (80%)	0	2.6	0	1.1	5.5	3.9	4.4	17.4
DDGS-W-R Soluble Mono Sugars (80%)	0	0	0	0	0.7	0	0	0.8
DDGS-W-R Calculated Polymeric Sugar (80%)	**0.0**	**2.5**	**0.0**	**1.1**	**4.8**	**3.9**	**4.4**	**16.6**

Fuc: fucose, Ara: arbinose, Rha: rhamnose, Gal: galactose, Glc: glucose, Xyl: xylose, Man: mannose

The yield of destarch material is shown in table 8. The numbers are not very high despite the fact that in comparison to the small scale experiment here we have taken 150 mg more starting materials from water extraction materials. But similar yield could be observed in 80 % for both. For very first beginning small scale experiment with 80 % ethanol precipitation 24% yield was obtained for DDGS-W but in second pilot study (table 8) 27.3%. A bit more but it should be considered that amount of starting material is 150 mg more than the first small

scale. For DDGS-W-R for small scale 17.6% yield was obtained after 80% ethanol precipitation but for second pilot study 18.8% which is quite similar to the first pilot study (table 8).

Table 8: Yield of DDGS materials before and after destarching after second times small scale study

Concentration	**DDGS-W Initial amount (mg)**	**DDGS-W Final amount (mg)**	**DDGS-W (Yield)**
Ethanol 60 %	250	20.8	8.32 %
Ethanol 70 %	250	35.1	14.04 %
Ethanol 80 %	250	68.2	27.3 %
Concentration	**DDGS-W-R Initial amount (mg)**	**DDGS-W-R Final amount (mg)**	**DDGS-W-R (Yield)**
Ethanol 60 %	250	28.2	11.28 %
Ethanol 70 %	250	35.2	14.08%
Ethanol 80 %	250	47	18.8 %

For DDGS-W-R as shown in in figure 2 with 80 % ethanol precipitation more amount of polymeric xylan could be purified but in other hand the main problem is that more contamination of polymeric glucose can be obtained. In contrary 60 % ethanol precipitation for DDGS-W-R seems to work better with less contamination of polymeric glucose but it should take into consideration that less amount of polymeric xylan can be gained.

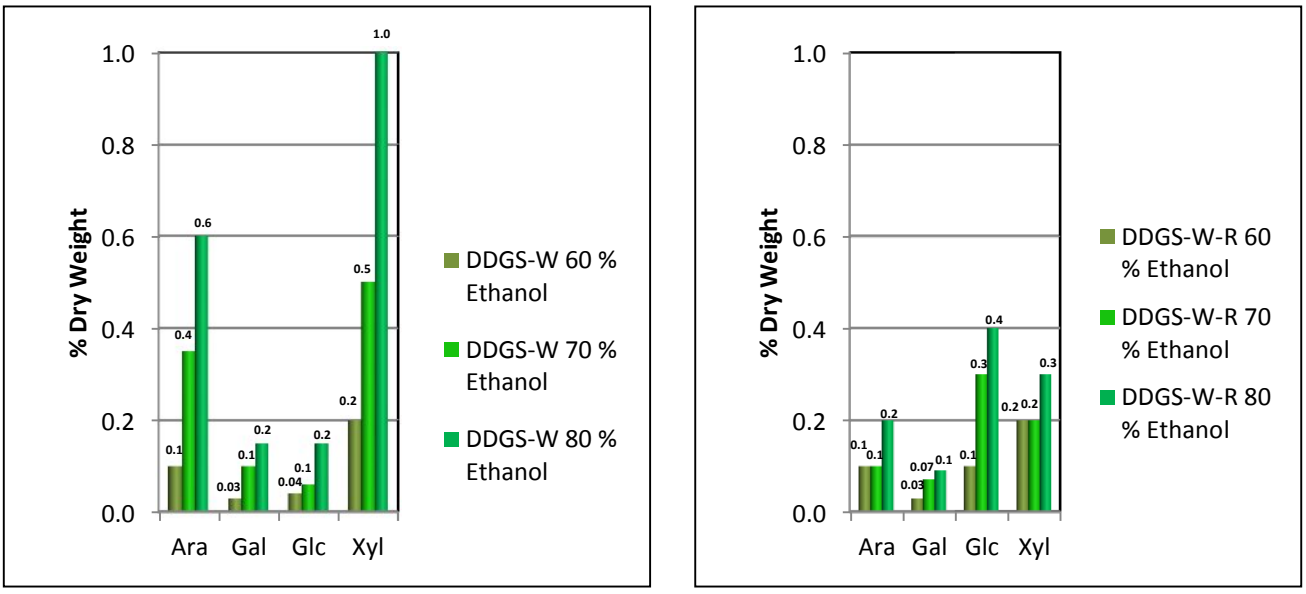

Figure 2: Comparison plot for different concentration of ethanol for DDGS-W left and DDGS-W-R right.

Sugar analysis revealed that a major fraction of the xylan was present in the supernatant. So dialysis of the supernatant was applied in order to recover the xylan. The molecular cut off for dialysis membrane tubing was 3500 Dalton which means that macromolecule are

retained but the molecule with less than 3500 Da pass through the membrane to the water (dialysate). The water was replaced by fresh one every 24 hours. The conductivity which was measured is plotted in figure 3. It shows after 72 hours the equilibrium will be reached with the entire volume of solution.

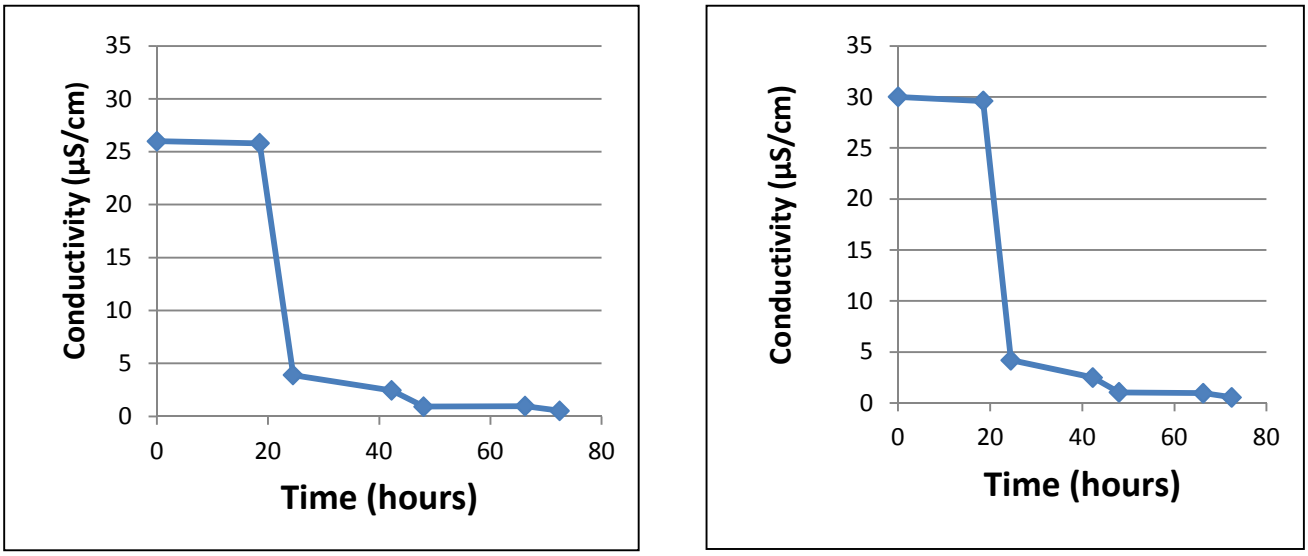

Figure 3: Conductivity graph of DDGS-W and DDGS-W-R during dialysing. The left picture is for DDGS-W and the right one for DDGS-W-R.

Liquid inside the dialysing tubing was freeze dried which sugar composition is shown (table 9). Monomeric sugars was considered zero in the table 9 because it passes through the membrane during the dialysis and could not be remained inside dialysis tubing. High amount of polymeric xylan could be observed (table 9) which confirms that substantial amounts of xylan are not precipitated with ethanol after destarching. As an unwanted side-effect the dialysis has also recovered a high amount of polymeric glucose in both samples. It was deduced from both dialysis and ethanol precipitation that dialysing method cannot be used for this experiment in despite of the fact that higher numbers of xylan can be seen (table 9). But since amount of contamination is higher than ethanol precipitation so ethanol precipitation process should be used to get more pure materials.

Table 9: Carbohydrate composition of supernatant of EtOH 80 % precipitation after dialysing (% dry weight)

Sample	Sugar(% Dry Weight)							Total Sugar %
	Fuc	Ara	Rha	Gal	Glc	Xyl	Man	
DDGS-W Hydrolysis	0	9.2	0	0.3	14.2	22.7	0.8	47.3
DDGS-W Soluble Mono Sugars	0	0	0	0	0	0	0	0
DDGS-W Calculated Polymeric Sugar	**0**	**9.2**	**0**	**0.3**	**14.2**	**22.7**	**0.8**	**47.3**
DDGS-W-R Hydrolysing	0	9.7	0	0.9	26.4	20.2	1.6	58.8
DDGS-W-R Soluble Mono Sugars	0	0	0	0	0	0	0	0
DDGS-W-R Calculated Polymeric Sugar	**0**	**9.7**	**0.0**	**0.9**	**26.4**	**20.2**	**1.6**	**58.8**

Fuc: fucose, Ara: arbinose, Rha: rhamnose, Gal: galactose, Glc: glucose, Xyl: xylose, Man: mannose

After destarching endo-1,4-β-xylanase 10A from *Rhodothermus marinus* was applied to get xylooligosaccarides in small scale (table 10). Xylose monomers cannot be seen on the chromatogram due to the fact that endo-xylanase enzyme has low favour for the substrates of DP=2 such as xylobiose **(Biely et al., 1981).** Different incubation time at 50˚C was done for small scale experiment. Incubation at 150 minutes gives the highest amount of XOS. For second time small scale experiment xylanase treatment (table 10 bold colours) was done at only 150 and 220 minutes. The reason that again the small scale experiment was done because it was not cleared whether this XOS could influence the growth of *L.brevis* bacteria or not. If it is approved that the bacteria can be growing on this substrates then it worthwhile to put effort and work in bigger scale. The sum for the first small scale xylanase treatment is different from the second time small scale (bold colours) in table 10. It could be due to more pre-incubation time for enzyme. For DDGS-W-R due to a lack of AX materials no sample was taken at 150 minuites to be tested by HPLC.

Table 10: Sugar composition of xylo oligosaccharide after treated with xylanase

Sample	Calculated amount of sugar [ug]						
	Ara	X 1	X 2	X 3	X 4	X 5	sum
DDGS-W 0 min	0	0	0	0.08	0.09	0.09	0.26
DDGS-W 50 min	0	0	1.09	1.53	1.13	1.21	4.97
DDGS-W 150 min	0	0	2.49	3.58	2.14	2.06	10.27
DDGS-W-R 0 min	0	0	0	0.09	0.08	0.07	0.24
DDGS-W-R 50 min	0	0	1.38	2.21	1.38	1.51	6.49
DDGS-W-R 150 min	0	0	3.50	4.26	2.16	2.12	12.04
DDGS-W 150 min	**0**	**0**	**9.8**	**19.0**	**13.7**	**13.9**	**56.4**
DDGS-W 220 min	**2.3**	**0**	**10.0**	**18.4**	**14.5**	**10.2**	**53.0**
DDGS-W-R 220 min	**0.8**	**0**	**7.7**	**13.8**	**9.5**	**7.5**	**38.4**

(Arabinose (Ara), xylose (X1), xylobiose (X2), xylotriose (X3), xylotetrose (X4) and xylopentose (X5))

From starting materials which have been water extracted till the xylnase treatment the yield of xylan polymer and xos was calculated which is shown in table 11.

Table 11: Comparison table which shows the yields of Xylan polymer and XOS during each step

Sample	Yield	Sample	Yield
DDGS-W xylan polymer **water extraction**	64.5 %	DDGS-W-R xylan polymer **water extraction**	19.32 %
DDGS-W xylan polymer **Destarch (EtOH 80%)**	10.8 %	DDGS-W-R Xylan Polymer **Destarch (EtOH 80%)**	4.43 %
DDGS-W XOS **xylanase (220 min)**	32 %	DDGS-W-R XOS **Xylanase (220 min)**	30.5 %

Different size of XOS which was made after applying the xylanase enzyme was used as a substrate (figure 4) for growing the *lactobacillus brevis* bacteria at 30˚C incubation temperature. The chromatogram (figure 4) shows that different size of XOS from X2 to X5 can be obtained after xylanase treatment. The aim of the experiment was to give this substrate to the bacteria in order to see which size of XOS from X2 to X5 will be utilized by bacteria for their growth. Arabinose also presents in the chromatogram which could be traced to see whether it will be consumed by bacteria for their growing or not. Previous studies have shown that some species of bacteria can be grown with arabinose as carbon source **(Crittenden et al., 2002).** Xylose (X1) was not obtained in the chromatogram due to the fact that β-xylosidase would be needed to produce xylose. Different kind of substrate as a carbon source was used in this experiment to indicate which carbon source promotes growth the most. The growth was measured as cell density by measuring the OD at 620 nm every 24 hours. The growth of bacteria is shown in figure 5. Different growth patterns have been observed in bacterial growth with different substrates (figure 5). For glucose the bacteria had a growth at first day but after 24 hours the growth was reduced then became constant due to using of glucose for caring of the cells **(Perrin et al., 2001)** and no substantial growth could be observed. For xylose substrate at the first day the growth was declined but after 24 hours continues growing could be observed. The graph of water that was used as a control is shown (figure 5) that bacteria can grow at the first day because of using the energy from media but after the second day could not be grown due to the fact that no substrate is present as a carbon source. For DDGS-W and DDGS-W-R they were grown rapidly at the first day but then reached stationary phase after 24 hours. The highest growth rate was observed on xylose. It could be due that *L.brevis* has a better transport system for xylose than glucose **(Palframan et al., 2003).**

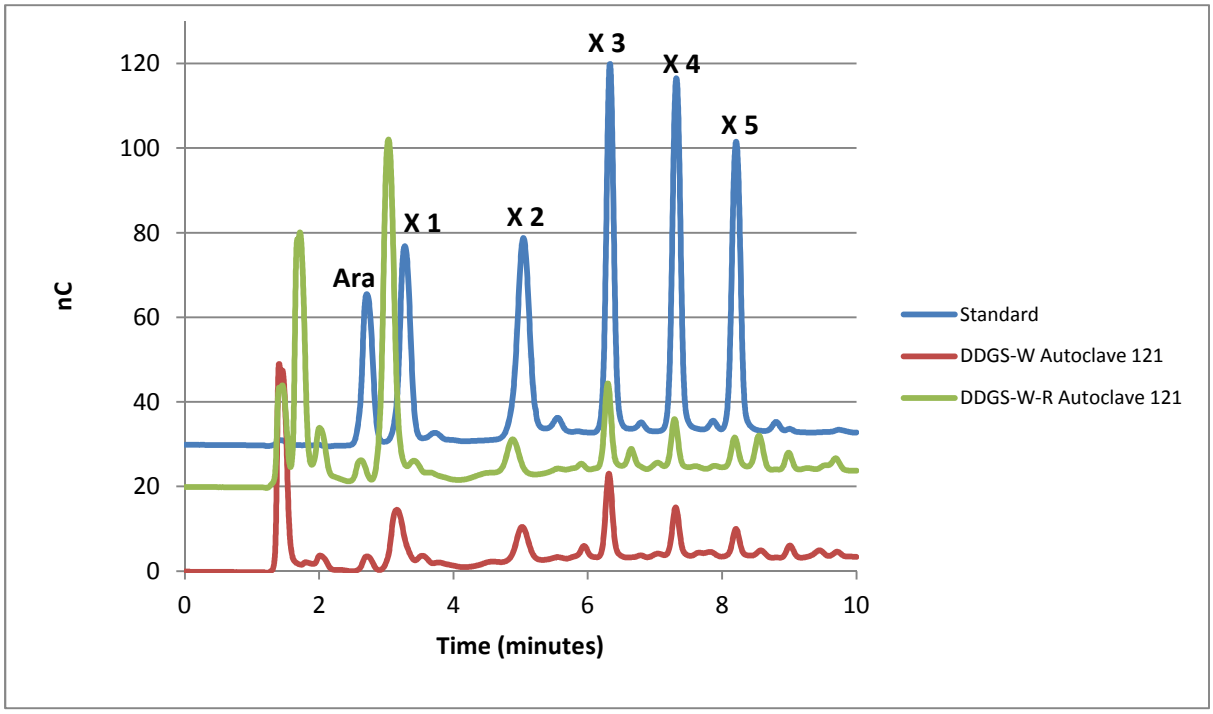

Figure 4: XOS peaks after xylanase treatment with HPLC-PAD PA 100 column and autoclaving at 121°C.
[Arabinose (Ara), xylose (X1), xylobiose (X2), xylotriose (X3), xylotetrose (X4) and xylopentose (X5)]

L.brevis was grown on glucose, xylose or XOS as carbon source (figure 5). To assese the potential uptake of XOS (present in the XOS preparation used as a carbon source, see figure 4) during bacterial growth, samples were withdrawn at (0, 24 and 72) hours and analysed with HPLC (PA 100 column) using X1-X5 as standards (figures 6 and 7). If the the height of the peaks reduced or if the peaks are disappeared in comparison to standard after every 24 hours it means that the bacteria uptake the substrate from the broth and metabolize it that cause the bacteria to grow. However for unexplained reasons the XOS present originally (figure 4) could not be detected even at 0 hour (figures 6 and 7). Control experiment showed that XOS can withstand such sterilization conditions as autoclaving and the peaks corresponding to XOS of DP2-DP5 was present (figure 4). Two explanations can be put forward which is important to consider for the future: First after autoclaving the substrates, the XOS may have been precipitated in the tubes and it was not mixed probably when it was taken by the pipet. Secondly, maybe bacteria have consumed the substrate from the very first beginning since after the zero samples were taken because the tubes were kept 1h at room temperature. The monomeric glucose can also be traced on chromatograms (figures 6 and 7) between arabinose (Ara) and xylose (X1) peak that is coming from after destarching experiment that shows the destarching method did not work properly since the broth media was free of glucose. Glucose was consumed by bacteria for both DDGS-W and DDGS-W-R substrates instead of XOS which was not present in the substrates. The growing of the bacteria in conclusion is due to uptake of the glucose instead of XOS.

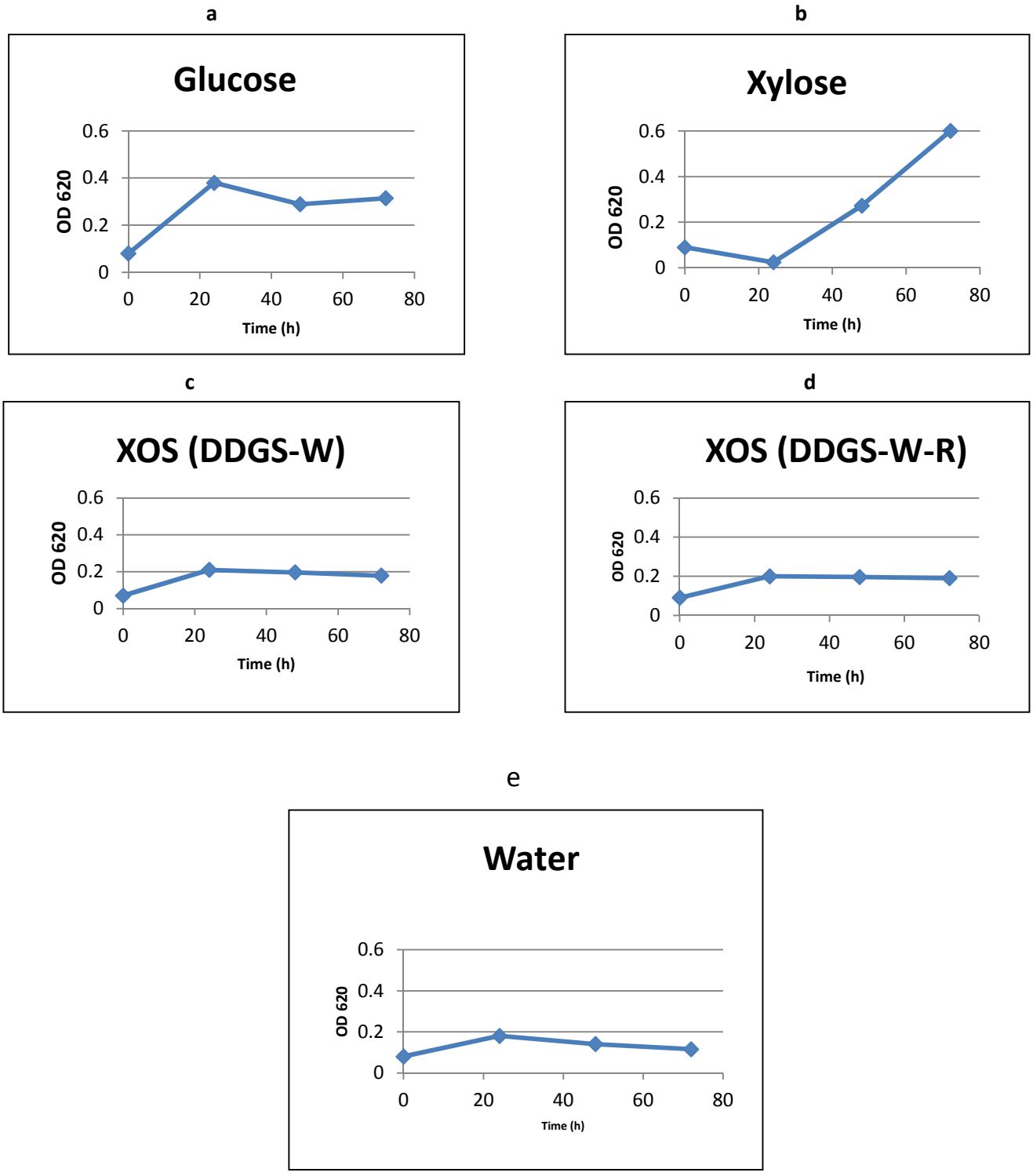

Figure 5: *L.brevis* growth in different substrates and water as a control. Substrates a: glucose b: xylose c: DDGS-W (XOS) d: DDGS-W-R (XOS) e: water respectively.

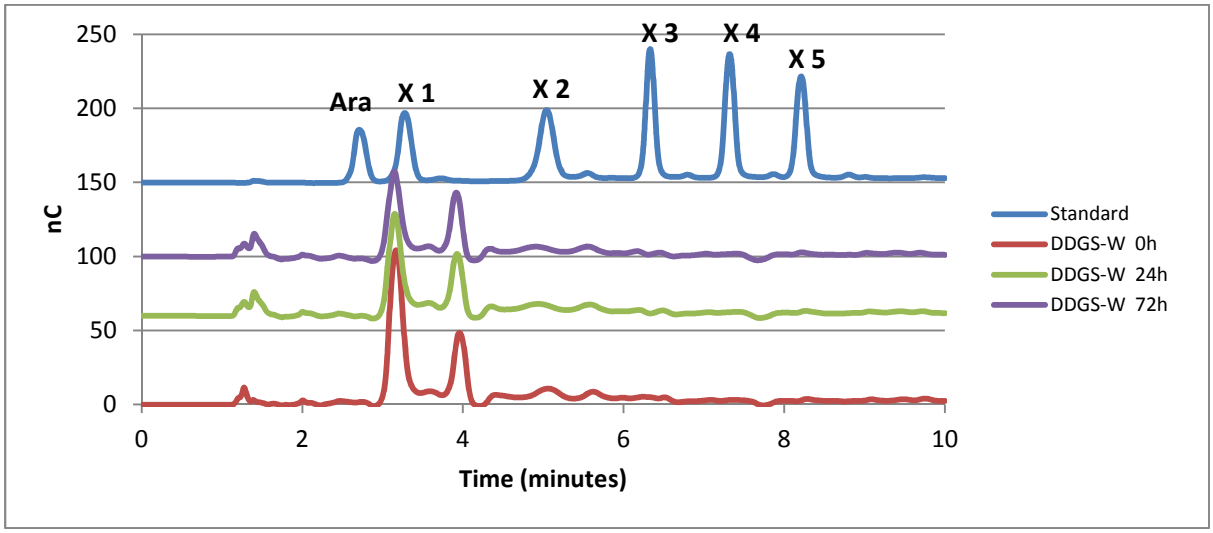

Figure 6: DDGS-W bacteria digest chromatogram with HPLC-PAD PA 100 column. [Arabinose (Ara), xylose (X1), xylobiose (X2), xylotriose (X3), xylotetrose (X4) and xylopentose (X5)]

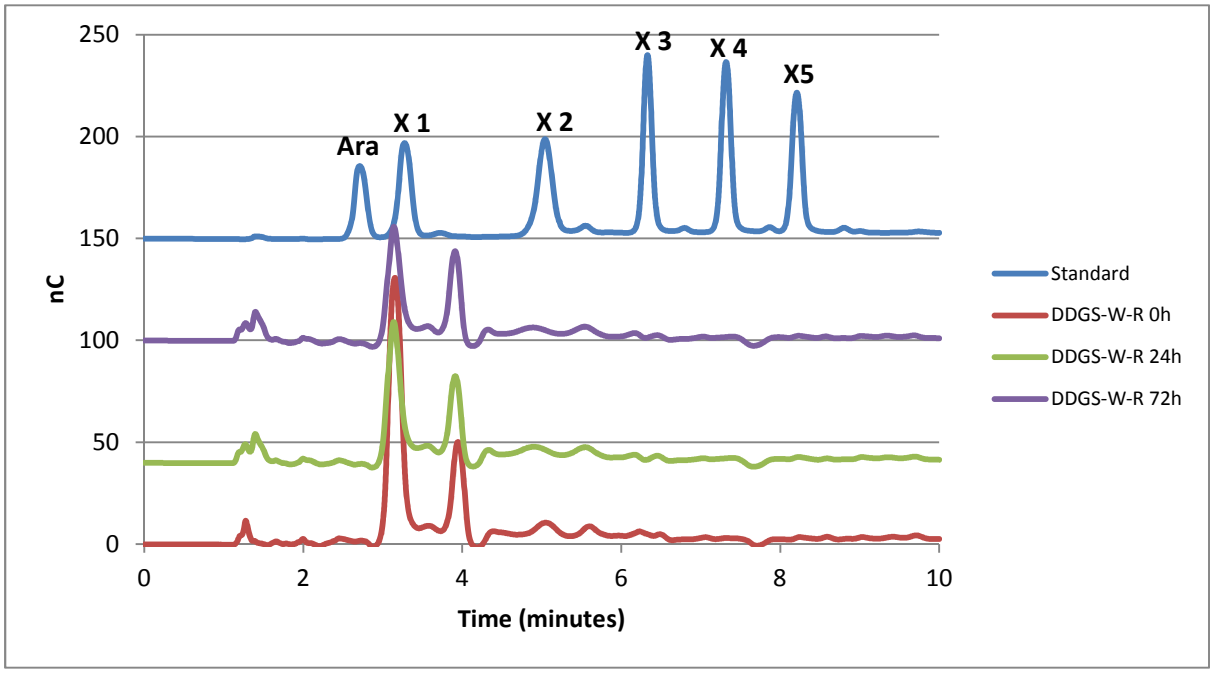

Figure 7: DDGS-W-R bacteria digest chromatogram with HPLC-PAD PA 100 column. [Arabinose (Ara), xylose (X1), xylobiose (X2), xylotriose (X3), xylotetrose (X4) and xylopentose (X5)]

Conclusion and Future works

- The xylan polymer was purified successfully but with low yield (table 11). The yield could be improved by removing polymeric glucose. We do not know exactly which kind of polymeric glucose we are dealing with. Is it starch or cellulose or other sources of polymeric glucose so then the synergism between the enzymes can be used for getting rid of sugar contamination. So destarching method needs to be optimized.

- More xylan is recovered for DDGS-W than DDGS-W-R both with water extraction and destarching (table 5, 6 and 7). It is not cleared what caused this difference because the starting material sources were the same and both were coming from the same wheat source.

- Water extraction leads to different yields of DDGS materials (table 4). It means that this method needs to be optimised to increase the yield for DDGS-W-R.

- Ethanol precipitation is preferred over dialysis because xylan polymer was purified with low contamination by this method. In dialysis more amount of xylan was recovered but higher amount of glucose contaminant was obtained too which makes the method sub-optimal. By using a proper enzyme in future in order to get rid of any kind of polymeric glucose sources we can use dialysis method because amount of polymeric xylan which is recovered is higher than ethanol precipitation.

- Since the experiment was done in small scale no xylan was left after growing the bacteria in DDGS substrates. Enable to analysis better the probiotic effect of DDGS-W and DDGS-W-R substrates in future more amount of xylan should be purified and fermentation in vitro could be done for several strains of bacteria.

- Since after fermentation of probiotic bactria SCFAs are produced by measuring the pH in future it is much simpler to understand whether the prebiotic substrates have been utilized and fermented by probiotic bacteria or not.

- Because standards of monomeric and polymeric sugars are very expensive and we could not use them all the peaks cannot be covered in the chromatogram with our presenting standard. So the structure of unknown peaks can be characterised by using MALDI-TOF (matrix-assisted laser desorption/ionization) and SEC (size-exclusion chromatography).

- Since xylan is very complex heteropolymer and consists of many side chain groups by using different enzymes most of the side chains in polymer can be hydrolysed and

more pure XOS can be gained. But in the case of wheat bran we assumed based on the literature study that the average degree of arabinose substitution (avDAS) is rather lower *(***Broekaert et al., 2011).**

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