# Preparation and evaluation of arabinoxylan based prebiotics

DIVISION OF BIOTECHNOLOGY | FACULTY OF ENGINEERING | LUND UNIVERSITY
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## Master's Thesis

# Preparation and evaluation of arabinoxylan based prebiotics

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# Preparation and evaluation of arabinoxylan based prebiotics

Biomass characterization, pre-treatments to extract arabinoxylan, production of arabinoxylooligosaccharides (AXOS) by xylanases and utilization of AXOS by potential probiotics

**Master's thesis**

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*Utmost gratitude to all the people who supported me during my research work here at the division of Biotechnology, LTH, Lund University*

# Popular scientific article

Prebiotics, the non-digestible carbohydrate compounds, play an important role to selectively stimulate the growth and activity of health promoting gut microorganisms. Previous research findings have proved that the consumption of prebiotics improves the reduction of blood glucose and cholesterol, enhances mineral absorption from the colon, deactivates pro-carcinogenic enzymes in the gastrointestinal tract as well as their stimulation of the immune system.

By definition, any non-digestible carbohydrates can be considered as prebiotics. Some of the commercially available prebiotics nowadays are inulin, fructooligosaccharide, galactooligosaccharide and guar-gum. Also, products containing xylooligosaccharides (XOS) and arabinoxylooligosaccharides (AXOS) are considered as the potential future prebiotics in the market. This XOS and AXOS are the hydrolysis products of xylan and xylan is the most abundantly available cell wall component of lignocellulosic biomass. Agricultural wastes such as straw, husk, stalk, cob, hull, bagasse and pulp of hard wood have become a major source of xylan. Productions of XOS and AXOS from agricultural residues offer an excellent prospect to the nutraceutical industries owing to the low price and abundant availability of the raw materials.

Brewer's spent grain (BSG) is the production residue from the process of malts (cereal grains) and the separation of wort (fermentation medium to produce beer) during the beer production. BSG is the most abundant by-product generated by brewing industry and accounts for ca. 85% of total by-products. Arabinoxylan, lignin and cellulose are the most abundant compounds in BSG where they weigh roughly 28%, 28% and 17%, respectively. The most common applications of BSG are being used as animal feed and dumping into landfills. This research study was carried out as an attempt to produce AXOS from BSG which is believed to be a source of great potential for making prebiotics. A new technique was developed and presented in this study to extract arabinoxylan from BSG. This technique is compatible to be used in the large scale production of arabinoxylan. Different xylanases were used to hydrolyze the extracted arabinoxylan fractions and to produce short-chain AXOS. Also, *in-vitro* fermentation experiment was carried out to assess the capability of extracted BSG AXOS to be used by health promoting gut bacteria *Lactobacillus*. *In-vitro* fermentation of extracted BSG AXOS fractions using well-known probiotic strain *Lactobacillus* showed the production of short-chain fatty acids.

This study showed that AXOS from BSG have the potential to be utilized by probiotic bacteria and to produce short-chain fatty acids. The technique developed in this study to extract arabinoxylan from BSG can be applied for large scale production of arabinoxylan. Furthermore, the production residues after arabinoxylan extraction can be used as animal feed.

# Abstract

Prebiotics are non-digestible carbohydrates that selectively stimulate the growth and activity of health promoting bacteria in the colon, resulting in homeostasis to the gut environment. It is considered to be the solution of many health-related diseases such as type-2 diabetes, cardiovascular disease and cancer. Arabinoxylooligosaccharides, the hydrolysis product of arabinoxylan from plant cell walls, can be used as potential prebiotics as it is non-digestible to humans and can be fermented by the bacteria in the colon. Brewer's spent grain, the residue from beer production, was used to extract arabinoxylan. The composition of brewer's spent grain was analyzed and arabinoxylan content found to be 14.75% on a dry weight basis. Different pre-treatment methods were used to extract arabinoxylan-rich fractions from brewer's spent grain. The best pre-treatment method was selected based on the purity of extracted arabinoxylan, simplicity of the process and possibility to use in large scale applications. The pre-treatment process simply consists of a heat-treatment step of the de-starched brewer's spent grain and is followed by mild alkaline treatment. Xylanases were used to produce short-chain oligosaccharides from extracted arabinoxylan. Two pure xylanases namely *Rm*Xyn10A from GH family 10 and Pentopan Mono BG from GH family 11 were used to depict their product formation (hydrolysis) pattern. Meanwhile, seven other commercial xylanases were used to compare their efficiency in making short-chain oligosaccharides. Most of the commercial xylanases were found to be efficient in producing mono-saccharides (arabinose and xylose) while two pure xylanases were efficient in making short oligosaccharides. Well-established probiotic strain *Lactobacillus brevis* (DSMZ 1264) was used to perform growth studies by using arabinoxylooligosaccharides as carbon source. Analyzed results of samples after 48 hours of fermentation showed that oligosaccharides were utilized by *Lactobacillus brevis*.

# Introduction

Valorization of underutilized biomass is a topic of wide interest today, due to future needs in the field of food production, energy and chemicals. It is thus of interest to develop technologies that improve use of currently underutilized biomass resources, such as the hemicelluloses.

Lignocellulosic biomass is the most abundant and cheapest biomass having the potential of being used as biofuels and high value carbohydrate products. Lignocellulosic biomass can be treated mechanically (heat treatment), chemically or enzymatically to extract a hemicellulose rich fraction. Hemicellulose is the second most abundant polysaccharide extracted from lignocellulosic biomass which is reported to be utilized for the production of prebiotic xylooligosaccharides (XOS) (Saha, 2003).

The growing demands of novel food products for health and age related issues attached with increasing health care expenses has attracted global attention on prebiotics. Xylan is abundantly available in nature as a major component of hemicellulose, a component of lignocellulosic biomass. Agricultural wastes such as straw, husk, stalk, cob, hull, bagasse and pulp of hard wood represent a major source of xylan. Xylooligosaccharides, the hydrolysis product of xylan is the nutraceutical that can be produced from lignocellulosic biomass.

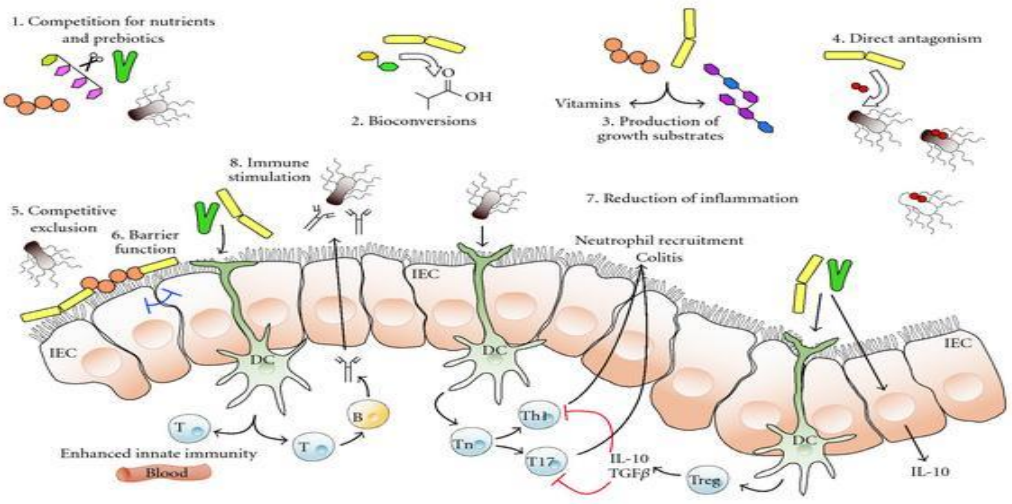
Finding new biomass sources, developing techniques to extract prebiotics, conducting *in-vitro* and *in-vivo* experiments are trending research areas nowadays considering its potential to obtain patent rights, publications, business potentials and prospects to contribute to the prevention of health related diseases.

Production of XOS and arabinoxylooligossaccharides (AXOS) from agricultural residues offers great scope to the nutraceutical industries as the raw material is cheap and abundantly available. The major advantages of XOS consumption are not limited to selective growth stimulation of beneficial gut microflora but also works on the reduction of blood glucose and cholesterol, enhanced mineral absorption from the large intestine, reduced pro-carcinogenic enzymes in the gastrointestinal tract and immune-stimulation. In addition, the sweet taste of XOS enables it to be used as an artificial sweetener. (Samanta et al., 2015)

## Probiotics

Probiotics are defined as live microorganisms which confer health benefits to the host upon ingestion of adequate amounts. Requirements for a microorganism to be considered as probiotic are: safety of the host (the microorganism have to be safe for the host), should be taken as live and should be taken in adequate amounts to show health benefits. Another important point to consider is the survivability of the microorganism through complex human digestion system. The consumed microorganism should survive through digestive system and reach in adequate amounts to the colon to show health benefits. Yoghurts, drinks, capsule and dietary supplements are the most popular food formulations in the functional food market nowadays containing probiotic microorganisms. *Lactobacillus* and *Bifidobacterium* is the genera from where most of the probiotic strains are isolated and the safety of lactobacilli and bifidobacteria is well established since they have been used in fermented foods. (O'Toole and Cooney, 2008)

An overview on how probiotic microorganism works to influence host microbiota can be seen from figure 1. Reported mechanisms are but not limited to: competition for dietary ingredients as growth (nutritional) substrates, production of fermented products by bioconversion of sugars, production of growth substrates (exopolysaccharides or vitamins) for other bacteria, direct antagonism by bacteriocins, competitive exclusion for binding sites, enhanced barrier function, reduction of inflammation by colonization of gut microbiota and stimulation of innate immune response. (O'Toole and Cooney, 2008)




DC: dendritic cells, IEC: epithelial cells and T: T-cells.

*Figure 1: Schematic diagram showing the mechanisms of probiotic impact on microbiota* (O'Toole and Cooney, 2008)

## Prebiotics

Prebiotics are food ingredients that passed through human digestion system (non-digestible) and beneficially affect the host by selectively stimulating the growth and/or activities of health promoting microorganisms. Prebiotic food ingredients are mostly non-digestible carbohydrates as they are not susceptible to gastric acid and gastrointestinal enzymes. Thus, it is important for prebiotic food ingredients to be resistant to digestive system (enzymes) in order to reach to the colon and it should bring a specific change in the gut microbiota composition that will confer positive health benefits to the host. Several studies have proved that non-digestible carbohydrate can reach the large intestine, consumed by gut microbiota and generate short-chain fatty acids. (Figueroa-González et al., 2011)

Inulin, fructooligosaccharide (FOS), galactooligosaccharide (GOS), guar-gum are some of commercially available prebiotics nowadays (Gibson et al., 2004). Also, products containing XOS and AXOS is considered to be the next potential prebiotics and lots of scientific articles mentioning their properties are available nowadays (Rastall, 2010, Broekaert et al., 2011). Faryar et al. reported that probiotic strain *Lactobacillus brevis* (DSM 1269XOS) has grown well in the XOS extracted from wheat straw (Faryar et al., 2015). Broekaert et al. reported that AXOS selectively stimulate the growth and activity of beneficial gut microorganisms and fulfill the criteria to be considered as prebiotics (Broekaert et al., 2011). So, it is quite likely to foresee that products containing AXOS will be the trending prebiotics in few years.

## Structure of plant cell wall and arabinoxylan

The structure of plant cell wall is rather complex and mainly consists of cellulose, hemicellulose (xylans), lignin, starch, protein and glucomannan. A representative graphical view of complex plant cell wall is shown in figure 2. It is important to know the composition of starting material (lignocellulosic biomass) in order to tailor the extraction treatment to obtain maximum yield of the desired compound while reducing the interference of matrix. Apart from matrices, it is also important to know the presence and percentage of other compounds like uronic acids and phenolic acids which offer health promoting effects and makes the biomass demanding when it comes to the topic of commercialization.

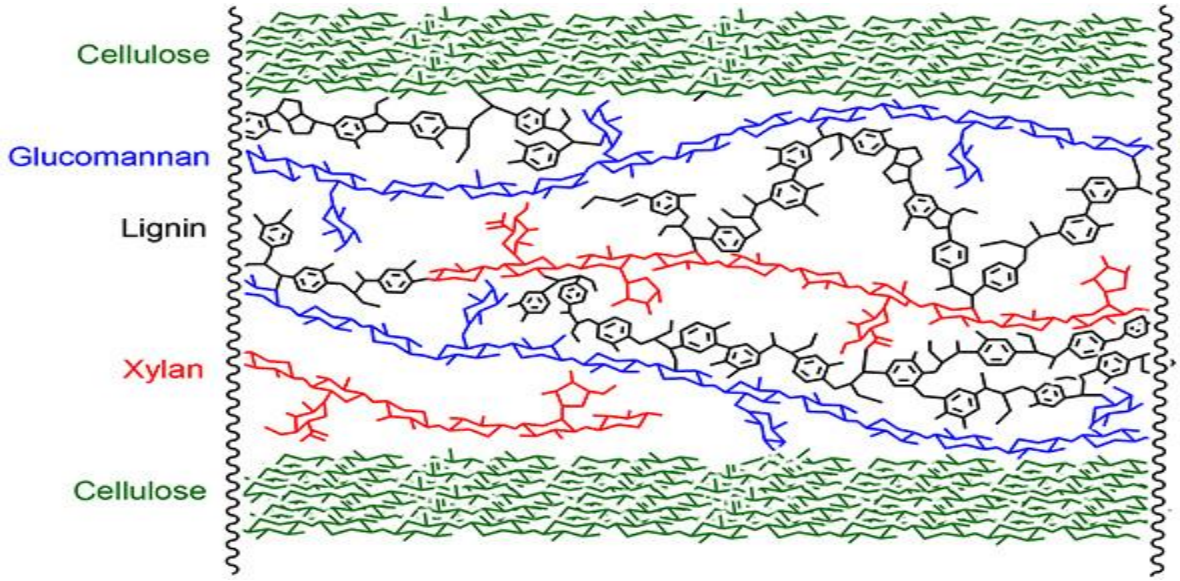



*Figure 2: Typical image of plant cell wall*
(Image reference: http://www.chalmers.se/en/departments/bio/research/industrial-biotechnology/Plant-cell-wall-acting-enzymes/Pages/WWSC.aspx)

Arabinoxylans (xylan chain with arabinose substituents) are non-digestible carbohydrate and mostly found in the outer bran tissues of most cereals (Hopkins et al., 2003). A typical structure of plant cell wall derived arabinoxylan is shown in figure 3. Four possible structures of arabinoxylan has been reported till today, unsubstituted β-(1-4) linked to xylose backbone, α-(1-2) L-arabinofuranosyl linked to the xylose backbone, α-(1-3) arabinofuranosyl linked to the xylose backbone and double α-(1-2) and α-(1-3) arabinofuranosyl linked to the xylose backbone. Uronic acids (galacturonic acid, glucuronic acid and mannuronic acid) can be found on plant cell wall derived AX. Phenolic acids (mainly ferulic acid and p-coumaric acid) can be found in AX which can be ester-linked to the position O-5 on few arabinofuranosyl substituents. Ferulic acid causes oxidative gelation in water by crosslinking of dihydrodiferulic acids and shows anti-oxidative properties upon digestion. (Andersson and Åman, 2008, Saulnier et at., 2007) The structure of xylan, arabinoxylan and degree of substitution varies between plant species. Xylans in annual plants and grasses are typically arabinoxylans. On the other hand, xylans in wood occur as arabino-4-O-methylglucuronoxylan in softwoods and O-acetyl-4-O-methylglucuronoxylan in hardwoods. (Collins et al., 2005)

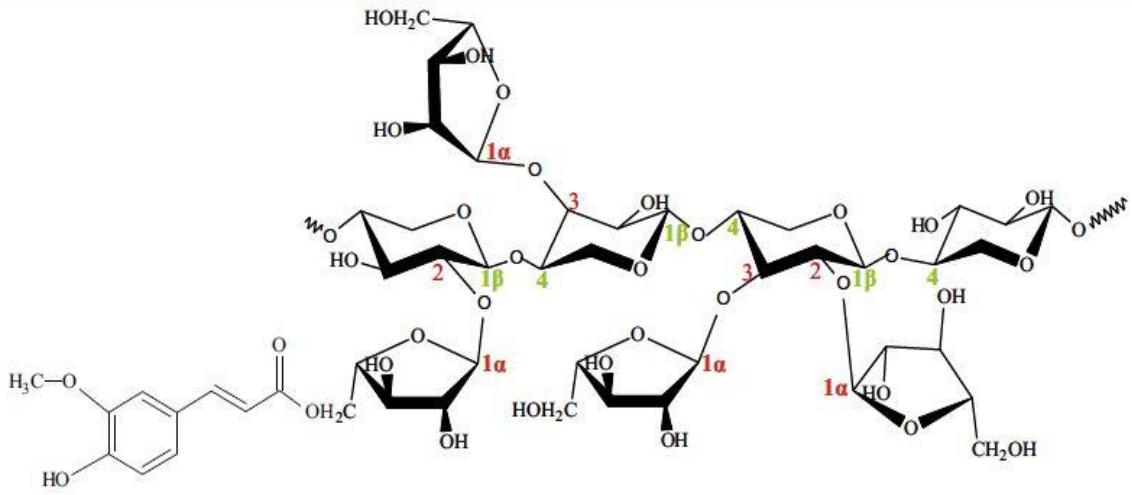


*Figure 3: Structure of arabinoxylan with side groups* (Reis et al., 2015)

Depending on the process of extraction, arabinoxylan is generally classified as water-extractable arabinoxylan (WE-AX) and water-unextractable arabinoxylan (WU-AX). Both these fractions can also be named as water-soluble arabinoxylan (WS-AX) and alkali-soluble arabinoxylan (AS-AX) respectively. The terms of WS-AX and AS-AX is used in this report to represent their respective fraction. The solubility of arabinoxylan in water is limited by covalent, non-covalent or a mixture of both linkages to other cell wall components such as lignin (figure 2), protein and cellulose. (Faulds et al., 2003)

Trogh et al. reported that enzymatic degradability of AS-AX and apparent specific endoxylanase activity decreases with increasing arabinose to xylose (A/X) ratios (Trogh et al., 2005). Also, the extent of fermentation of AX is structure-dependent and decreases with higher arabinose to xylose ratios and higher degree of polymerizations (Annick et al., 2011).

## Brewer's Spent Grain

Brewer's spent grain (BSG) is the residue left after the processing of malts (cereal grains) and separation of wort (fermentation medium to produce beer) during the beer production. Barley is the main cereal grain used worldwide for brewing. Other cereals like wheat, rice, maize, sorghum, millet are also used to some extent to produce beer and research is going on to produce beer from oats. BSG is the most abundant by-product generated by brewing industry accounting to around 85% of total by-products. The most common application of BSG is being used as animal feed or dumping into landfills. (Reis et al., 2015)

Arabinoxylan, lignin and cellulose are the most abundant compounds in BSG where the amounts are roughly 28%, 28% and 17% respectively (Santos et al., 2003, Mussatto et al., 2006). BSG has already gone through hot water extraction process during brewing which makes it difficult to extract compounds using mild treatments like water extraction. Hence, it requires chemical, enzymatical or a combination of both treatments to extract the compound of interest from BSG.

There are only a few studies available till today reporting the prebiotic potential of BSG AX. Reis et al. reported that *in-vitro* fermentation of BSG AX using faecal inocula leads to an increase in bifidobacteria populations and production of propionate. Propionate is one of the short-chain fatty acids (SCFAs) which helps to improve insulin sensitivity and reduce cholesterol synthesis upon absorption in the gut, entering the blood stream and reaching the liver via the portal vein. Thus, it helps to prevent type-two diabetes and cardiovascular diseases. Having the capability to be fermented in the large intestine and produce propionate, BSG AX can be used as an alternative to FOS (fructooligosaccharides). Moreover, the availability and price of BSG is cheaper compared to other commercially available prebiotic sources and it has the potential to draw the interest of investors. (Reis et al., 2014)

## Xylanases

Xylanases are widespread group of hydrolytic enzymes used to cleave the β-1,4 backbone of the xylan chain. In a more specific way, xylanases are glycosidases (O-glycoside hydrolases, EC 3.2.1.x) which catalyze the endo-hydrolysis of 1,4- β -D-xylosidic linkages found in plant cell wall xylan. Xylanases have attracted increased attention during the past decade mostly because of their potential applications in pulp and paper processing, biofuel production, baking and in brewing industries. Recently, the interest has gained to produce prebiotic short-chain oligosaccharides by hydrolyzing lignocellulosic biomass. (Collins et al., 2005)

Xylanases are classified based on their primary structure comparisons of the catalytic domains and grouped in families of related sequences in the carbohydrate-active enzyme (CAZY) database (www.cazy.org). Members in the same family exhibit similar protein folds, same catalytic mechanism and retention or inversion of the anomeric carbon. Research has mainly focused on xylanases belonging to glycoside hydrolase (GH) families 10 and 11 although enzymes from GH family 5, 7, 8 contain catalytic domain with endo-β-1,4 xylanase activity. Depending on their site of attack, GHs can be further divided into endo- or exo-acting where the attack is within the polysaccharide or at the terminal, respectively. (Collins et al., 2005) Xylanase hydrolysis occurs either by one-step direct displacement with water or a two-step mechanism through a glycosyl-enzyme intermediate (McCarter and Withers, 1994).

The complete hydrolysis of xylan requires a large variety of cooperatively acting enzymes because of its complex and heterogeneous structure. Two graphical illustrations showing complex structure of hemicellulose and acting sites of GHs are shown in figure 4 and 5. Endo-xylanase (endo-1,4-β-xylanase) (EC 3.2.1.8) can hydrolyze the xylan backbone by random cleavage. Exo-xylanase (EC 3.2.1.156) works to release xylose and short oligosaccharides from the reducing end of xylo-oligosaccharides. β-xylosidage (EC 3.2.1.37) releases xylose monomers by hydrolyzing short oligosaccharides from the terminal non-reducing end and xylobiose. Side groups in xylan chain can be hydrolyzed by using α-L-arabinofuranosidases (EC 3.2.1.55), acetylxylan esterases (EC 3.1.1.72), α -D-glucuronidases (EC 3.2.1.139) and p-coumaric acid esterases (EC 3.1.1.-). Ferulic acid can be released by feruloyl esterase (EC 3.1.1.73). (Collins et al., 2005) Faulds et al. reported that ferulic acids are probably located in the regions of low substitution on arabinoxylan and use of GH 11 xylanases have synergistic effects with feruloyl esterase for the release of ferulic acids (Faulds et al., 2003).

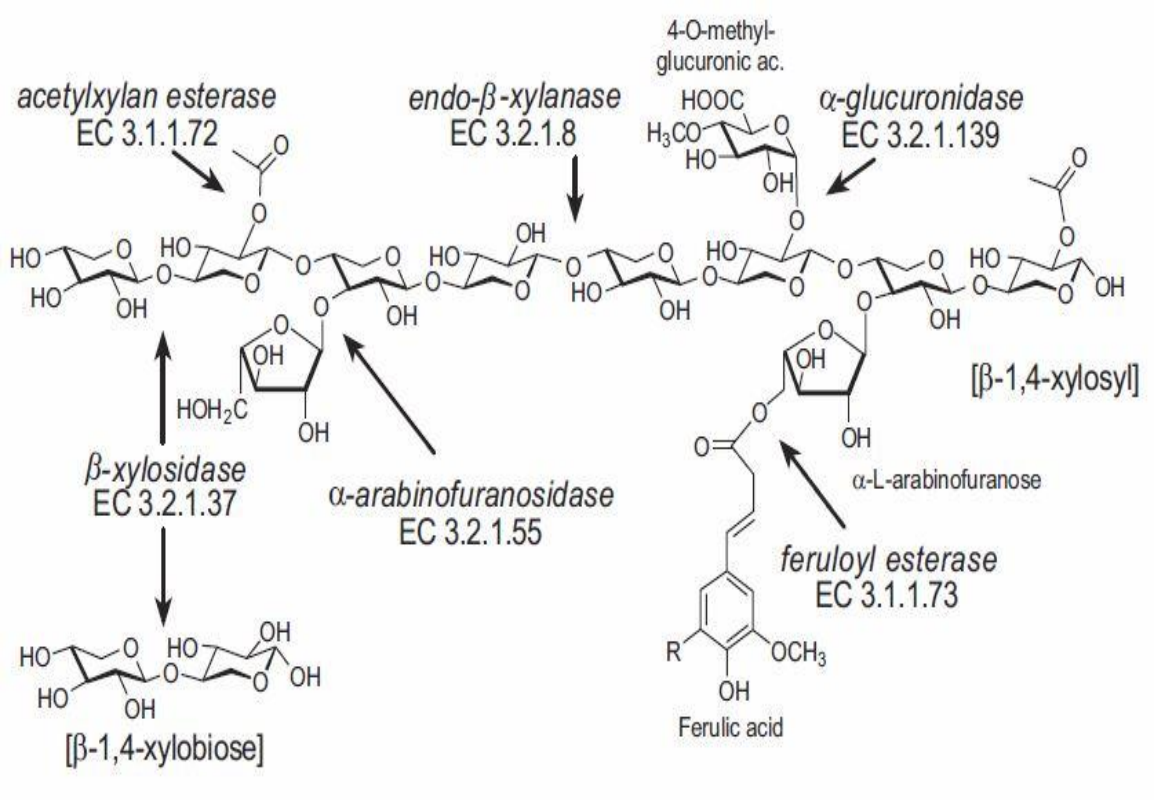



*Figure 4: Glycoside hydrolases acting on hemicellulose* (Linares-Pasten et al., 2014)

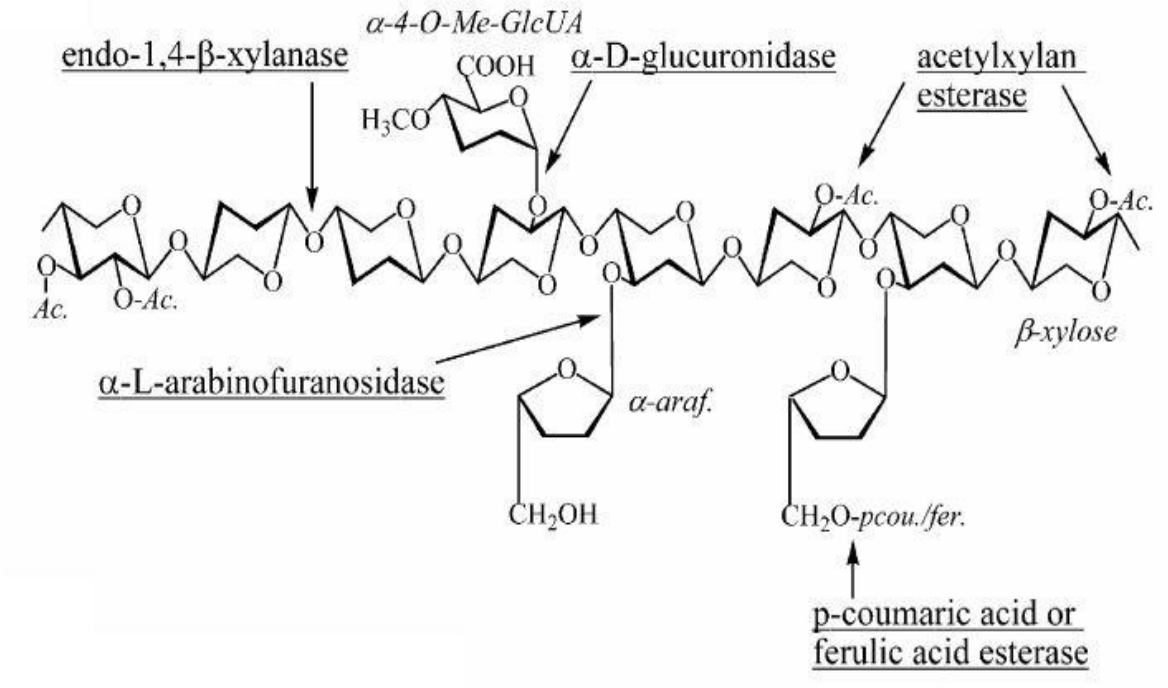



*Figure 5: Xylan backbone and site of attack by xylanolytic enzymes* (Collins et al., 2005)

## Scope of this thesis

The aim of this master's thesis was to find out technique/techniques to isolate arabinoxylan rich fractions from brewer's spent grain and to assess the possibility to use them as prebiotic food ingredients. To achieve this the work plan was divided into:

1. Characterization of the starting materials (Brewer's spent grain). Hydrolysis and quantification of monosaccharides (xylose, arabinose, glucose etc.), protein, moisture, ash, starch and lignin
2. Pre-treatment of the starting material aiming to extract arabinoxylan-rich fractions and analysis of extracted fraction/fractions
3. Treatment of arabinoxylan with different xylanases to produce AXOS
4. Bacterial growth studies on produced AXOS and analysis of fermented products

# Materials and methods

## Raw materials and chemicals

Brewer's Spent Grain was kindly provided by Peter Falck, division of biotechnology. A portion of BSG sample was freeze-dried and meshed through 500 µm sieve. The sample was then stored in closed containers. The rest of the BSG samples were kept frozen at 20ºC and processed when needed. All the chemicals used were of analytical grade unless otherwise stated. Xylanases used were Dyadic, Bio-Cat, Power bake 7430 (Danisco, Denmark), Orbazim HC 2500, GrindAmyl H 460 (Danisco, Denmark), Power bake 900 (Danisco, Denmark), *Rm*Xyn10A, Pentopan mono BG (Novozymes, Denmark) and Pentopan 500 BG (Novozymes, Denmark).

## Characterization of starting material

### Determination of moisture content

Moisture content was analyzed according NREL Laboratory Analytical Procedure (NREL/TP-510-42621, 2008).

Briefly, weighing dishes were pre-dried in a drying oven at 105ºC for a minimum of four hours, cooled in desiccator and weighed to the nearest 0.1 mg. Around 1 g of sample was taken for analysis and thoroughly mixed. The samples are then placed into a convection oven at 105ºC for a minimum of four hours. After completion, the samples were removed from the oven and allowed to cool to room temperature in a desiccator and weighed afterwards. Then the dishes are placed back into the convection oven at 105ºC and dried to constant weight where constant weight is defined as around 0.1 percent change in the weight percent solids upon one hour of re-heating the sample.

### Determination of ash in biomass

Ash content was determined according to NREL Laboratory Analytical Procedure (NREL/TP-510-42622, 2008).

Briefly, empty crucibles were placed in the muffle furnace at 575ºC for a minimum of four hours. Then the crucibles were removed from the furnace to directly into a desiccator and allowed to cool for around one hour and weighed to the nearest 0.1 mg. Then the crucibles were placed back into the muffle furnace at 575ºC and dried to constant weight where constant weight is defined as 0.3 mg change in the weight upon one hour of reheating. Around 2 g of sample is weighed into each crucible and placed into the muffle furnace and heated using furnace ramping program. Upon completion, the crucibles were removed from the furnace directly into a desiccator and cooled for around one hour and weighed afterwards.

## Determination of Total Starch

Total Starch content in the sample was measured using Megazyme Kit and following the instructions specified in the kit.

**Reagents:**

(a) Ethanol (80 % v/v)
(b) 2 M KOH
(c) 1.2 M sodium acetate buffer (pH 3.8)
(d) Thermostable alpha-amylase
(e) AMG - Amyloglucosidase
(f) GOPOD Reagent
(g) D-glucose standard solution

Process-E from the Megazyme Assay Procedure was followed as this is for samples which also contain D-glucose and/or maltodextrins. 100 mg of sample was weighed into a glass centrifuge tube and 5.0 mL of aqueous ethanol (80 percent v/v) was added. The tubes were then incubated at 80 −85ºC for 5 min. The contents were then mixed on a vortex stirrer and another 5 mL of 80 percent v/v aqueous ethanol was added. The tubes were then centrifuged for 10 min at 1,800 g (approx. 3,000 rpm) and the supernatant were discarded. The remaining pellets were re-suspended in 10 mL of 80 percent v/v aqueous ethanol, stirred on a vortex mixer, centrifuged as above and carefully poured off the supernatant. Then the pellets were re-suspended in 2 M KOH by stirring for approximately 20 min in an ice/water bath over a magnetic stirrer. Approximately 8 mL of 1.2M sodium acetate buffer (pH 3.8) was then added to each tube with stirring on the magnetic stirrer. 0.1 mL of thermostable alpha-amylase (bottle -1) and 0.1 mL of AMG (bottle - 2) was added immediately to each tube, mixed well and placed in a water bath at 50ºC. The tubes were then incubated for 30 min with intermittent mixing on a vortex mixer followed by centrifuging at 1,800 g for 10 min.

Duplicate aliquots (0.1 mL) of the solution were then taken into new test tube and 3.0 mL of GOPOD reagent added. D-glucose controls and reagent blanks was also prepared at this stage. D-Glucose controls consist of 0.1 mL of D-glucose standard solution (1 mg/mL) and 3.0 mL of GOPOD Reagent. Reagent Blank solutions consist of 0.1 mL of water and 3.0 mL of GOPOD Reagent. All the samples, D-glucose control and reagent blanks were then incubated at 50ºC for 20 min, cooled to room temperature and the absorbance was measured at 510 nm against reagent blank.

## Determination of Protein content

The protein content was analyzed by dumas method using a FlashEA 1112 Elemental analyzer (Thermo Scientific). 50 and 100 mg of aspartic acid standards were used to obtain the calibration curve. A blank sample was also included to check the precision of the instrument. Around 30 mg of sample was used to perform the analysis. Small crucibles were prepared using aluminum foil and all the samples, standard and blank was weighed in these crucibles which were then used for the analysis. Amount of nitrogen was detected by a thermal conductivity detector after combusting the samples at 900ºC, which was then converted to protein content using a conversion factor of 5.7 (conversion factor used for cereals).

## Determination of Lignin in Biomass

NREL Laboratory Analytical Procedure was followed for this analysis (NREL/TP-510-42618, 2011).

**Reagents:**

(a) Sulfuric acid, 72 % w/w

For around 2 ml, 2700 mg of 96% $H_2SO_4$ was added to 900 mg deionized water on ice

[Density of 96% $H_2SO_4$ is 1.84 g/ml, Density of $H_2O$ is 1.00 g/ml]

(b) Sulfuric acid, 4 % w/w

Prepared by diluting 72% sulfuric acid

**Hydrolysis of the sample:**

300 mg of the sample was weighed and transferred to Schott bottles. 3 ml of 72% $H_2SO_4$ was then added to each bottles and mixed thoroughly using a glass rod. The samples were then placed in a water bath set at 30ºC for 60 minutes and stirred every five to ten minutes. Upon completion of the 60-minute hydrolysis, the samples were removed from the water bath and diluted to 4 % concentration by adding 84 ml of deionized water. The samples were mixed properly by inverting several times to eliminate phase separation between high and low concentration acid layers. Samples were then autoclaved for 1 hour at 121ºC using liquid settings for autoclave. After completion of the autoclave cycle, hydrolysates were allowed to slowly cool to room temperature.

**Determination of Acid Soluble Lignin:**

Acid soluble lignin determination was performed within 6 hours of hydrolysis as specified in the protocol. A small aliquot of autoclaved hydrolysis solution (around 30 mL) was taken and filtered using vacuum filter (0.2 micrometer membrane filter) and captured in a filtering flask. This filtered solution was used for spectrophotometric analysis and quartz cuvette (Quartz SUPRASIL, Hellma Analytics) was

used. 4% sulfuric acid was used as blank (background). On a UV-Visible spectrophotometer (spectrum mode), blanks (4 % sulfuric acid) were used to initialize the baseline. Then the spectrum of the sample was measured in the wavelength range of 190-400 nm to obtain the highest pick near 205 nm region. Both the samples and blanks were diluted accordingly to bring the absorbance below 1.0. Absorbance of the sample was then measured on the same spectrophotometer (photometric mode) using the wavelength that appeared as highest near 205 nm region. 4 % sulfuric acid was used as blank and both the sample and blank was diluted accordingly to bring the absorbance below 1.0.

**Determination of Acid Insoluble Lignin:**

Filtering crucibles were pre-dried to constant weight. Autoclaved hydrolysis solution was filtered using a vacuum filter (0.2 µm membrane filter) and captured in a filtering flask. Deionized water (a minimum of 50 ml) was used to quantitatively transfer all remaining solids out of the pressure tube into the filtering crucible. Then the crucibles containing acid insoluble residue was dried at 105ºC until a constant weight was achieved (around 4 hours). The crucibles were then removed from the oven, cooled in a desiccator and weighed. The crucibles were then placed in the muffle furnace and burned at 575ºC for 3 hours using furnace temperature ramp program. Upon completion, the crucibles were removed and cooled in a desiccator for 01 hour, weighed and re-heated for another 01 hour at 575ºC to constant weight.

## Determination of structural carbohydrates

NREL Laboratory Analytical Procedure was followed to prepare the samples for HPAEC-PAD analysis (NREL/TP-510-42618, 2011).

**Reagents**:

(a) Sulfuric acid, 72% w/w

(b) 0.1 M $Ba(OH)_2$

(c) NaOH

Briefly, pre-hydrolysis of around 15 mg sample with a 72% $H_2SO_4$ for 1 hour at 30ºC followed by dilution of the solution to 4% $H_2SO_4$ concentration. Sugar recovery standards (SRS) were also prepared and $H_2SO_4$ was added to a final concentration of 4% $H_2SO_4$. Then, both the sample and SRS was autoclaved at 121ºC for one hour. Upon completion, the samples were cooled, vortexed and the pH was adjusted to 5-6 by adding $Ba(OH)_2$. The precipitate were spun down (3000 rpm for 5 minutes) and the supernatant was filtered through 0.2 micrometer membrane filters and used for HPAEC-PAD analysis.

Non-hydrolyzing samples with mono sugars already present in the samples were prepared by adding MQ-water to the samples, stirred about 2 hours in the room temperature and centrifuged afterwards (14000 RPM, 5 minutes). The supernatant was filtered through 0.2 micrometer membrane filter and used for HPAEC-PAD analysis. The amount of soluble sugars were subtracted from hydrolyzed amount and presented in the report.

## Characterization of enzymes

### Amylase Activity Assay

**Reagents:**

(a) Buffer solution (20 mM of Sodium Phosphate Buffer with 6.7 mM Sodium Chloride), pH adjusted to 6.9

(b) Starch Solution (soluble), 1.0% (w/v)

(c) DNS solutions (without phenol): prepared by mixing following ingredients step by step and stored in amber bottle at room temperature. This reagent solution is stable for six months at room temperature.

I. 12 ml of deionized water (temperature around 50°C - 70°C)

II. Sodium Potassium Tartrate Solution: 12.0 g of Sodium Potassium Tartrate was added to 8 ml of previously heated (50°C - 70°C) 2 M NaOH

III. 96 mM of 3,5-Dinitrosalicylic Acid Solution

(d) Maltose Standard (0.2%, w/v)

Amylase activity assay was performed according to the protocol mentioned by Sigma (http://www.sigmaaldrich.com/technical-documents/protocols/biology/enzymatic-assay-of-a-amylase.html, Bernfeld, P. (1955) Methods in Enzymology 1, 149-158). Briefly, 5 µl of enzyme solution was added to a mixture of 125 µl of starch solution and 120 µl of buffer solution. The mixed solution was then incubated for exactly 3 minutes at 20°C followed by addition of 125 µl of DNS solution and boiling for exactly 15 minutes. Then this was cooled on ice to room temperature for around 3 minutes and 1125 µl of deionized water was added to each tube to make up the final volume of 1500 µl. Absorbance of the sample was measured at 540nm against reagent blank and expressed after subtracting the absorbance of substrate blank and enzyme blank. Maltose of different concentrations was used to plot the calibration curve and quantify the amount of maltose liberated for the sample.

## Xylanase activity assay (DNS assay)

Xylanase activity assay was performed by the method described by Bailey (Bailey et al., 1992) and DNS reagent (modified) was prepared according to the procedure mentioned by Miller (Miller et al., 1960).

**Reagents:**

(a) DNS solution: following amounts were added the final volume adjusted to 200 ml with deionized water

I. 2 g of 3,5-Dinitrosalicylic Acid
II. 0.4 g of Phenol
III. 0.1 g of Sodium sulfite
IV. 40 g of Rochelle salt (Potassium Sodium Tartarate)
V. 100 ml of 2% (v/v) NaOH

(b) Xylose (Sigma)

(c) Birchwood xylan (Sigma)

(d) Respective buffer solutions for each enzyme

Xylose was used to plot calibration curve. 5, 10, 15 and 20 mM of xylose solutions were prepared by dilutions from a mother stock concentration of 500 mM. 40 µl of each standard solution was added to 360 µl of MQ-water, mixed properly and incubated for 5 min at desired reaction temperature (40°C). 600 µl of DNS reagent was then added to each tube and boiled for 15 mins, cooled down on ice, centrifuged for 5 mins (5000 rpm) and the supernatant was used to measure absorbance at 540nm wavelength.

Working stock for each enzyme was prepared with their respective buffer solutions as mentioned in the table below.

Table 1: Respective buffer solutions

| **Xylanase** | **Buffer solution** | **pH** | **Remarks** |
|---|---|---|---|
| DYADIC | Sodium acetate, 20 mM | 5.0 | Concentration of working solution: 10 g/L |
| BIO-CAT | Sodium acetate, 20 mM | 5.0 | Concentration of working solution: 10 g/L |
| Power bake 7430 | Sodium acetate, 20 mM | 5.0 | Concentration of working solution: 10 g/L |
| ORBAZIM HC 2500 | Sodium acetate, 20 mM | 5.0 | Working stock is 10 times dilution of stock |
| GRIND | Sodium citrate, 20 mM | 4.5 | Concentration of working solution: 10 g/L |
| Power bake 900 | Sodium phosphate, 20mM | 6.0 | Concentration of working solution: 10 g/L |
| *Rm*Xyn10A | Sodium phosphate, 20mM | 7.5 | Working stock is 10 times dilution of stock |
| Pentopan mono BG | Sodium phosphate, 20mM | 5.0 | Concentration of working solution: 10 g/L |
| Pentopan 500 BG | Sodium phosphate, 20mM | 5.0 | Concentration of working solution: 10 g/L |

Birchwood xylan was used as substrate to determine the activity of each xylanase enzyme. Birchwood xylan was prepared to a concentration of 1% (w/v) using the respective buffer for each xylanases and kept at around 60°C until use in the assays (used within 1 hour). For the assay, 360 µl of birchwood substrate was added to a new eppendorf tube and incubated at 40°C for 5 minutes. Then 40 µl of each enzyme solution was added to 360 µl of birchwood substrate and incubated for 5 mins at 40°C. 600 µl of DNS reagent was added to each tube and the mixed solution was boiled for 15 minutes, cooled on ice to room temperature, centrifuged for 5 mins (5000 rpm) and the supernatant was used to measure absorbance at 540nm wavelength. Appropriate dilution of the enzyme solutions was made to bring the absorbance within the range of calibration curve.

## Protein quantification (Bradford assay)

**Reagents**:

(a) Dye reagent (Bio-Rad)

(b) Bovine serum albumin (Sigma)

Bio-Rad dye reagent was used for this analysis and carried out according to the instructions given by Bio-Rad (http://www.bio-rad.com/LifeScience/pdf/Bulletin_9004.pdf). The reagent for experiment was prepared by diluting the original reagent five times using deionized water and kept at room temperature no more than two weeks. Bovine serum albumin (Sigma) was used as calibration standard and prepared in the range of 0.1–1.4 mg/ml. 50 µl of sample solution was taken into tubes and 1.5 mL of diluted reagent added to each tubes. The mixture was mixed properly and incubated for 5 mins at room temperature. The absorbance of the sample was measure afterwards at 595 nm against reagent blank.

## SDS-PAGE

**Reagents**:

(a) 50 mM sodium acetate buffer at pH 5.0

(1.23045 g of sodium acetate was dissolved in 300 mL distilled water and pH adjusted to 5.0 with Acetic Acid)

(b) Pre-casted gel (Bio-Rad)

(c) Loading buffer (sample buffer), made up of

I. 3.3 ml of TRIS 0.5 M pH 6.8

II. 8 ml of SDS 10% w/v

III. 4 ml of glycerol
IV. 1 ml of bromophenol blue 0.2% w/v
V. 2.62 g of DTT (dithiotreithol)
VI. Volume was adjusted to 20 ml using MQ-water

(d) Electrophoresis (Running) buffer, made up of
I. 7.5 g of Tris
II. 36 g of glycine
III. 2.5 g of SDS
IV. pH was adjusted to 8.3 using HCl and the final volume was made up to 500 ml using MQ-water

This buffer is 5 times concentrated. It was diluted 5 times using deionized water and used in the experiment.

(e) Molecular weight marker

(f) Staining solution
I. 1 g of Coomassie 0.2%
II. 200 ml of MeOH 40%
III. 50 ml of HAc 10%
IV. 260 ml of MQ-water

(g) De-staining solution, made up of
I. 40% methanol
II. 10% acetic acid
III. 50% water

Enzyme solution of 16 µl was added into a new eppendorf tube (mini tube) and 4 microliter of loading buffer was added to each tube and heated at 100ºC for 10 min. Heat will unfold the proteins. It was then centrifuged for 2 mins (10,000 rpm) to remove solids. Pre-casted gel was placed in the SDS-PAGE, power supply and other parts were adjusted accordingly. Running buffer was poured, surrounding the gels to fill the inner part. Then 4 microliter of metabolic weight marker was pipetted into the first well and around 16 µl of enzymes solution to other wells. Then the SDS-PAGE was run using a voltage of 200 for around 45 minutes. Upon completion the gel was taken out carefully, stained and kept in the staining solution overnight. Then the gel was de-stained using de-staining solution and molecular weight of the sample was measured based on the distance covered by the molecular weight marker.

## Pre-treatments of starting material

### Alkali (KOH) treatment

3 g of BSG was treated with 15 ml of 4 M KOH for 2 hours. Two different temperatures (40°C and 80°C) were used to see the influence of temperature on extraction yield. Then the mixture was centrifuged (4500 rpm, 20 min) and the supernatant was transferred to a new tube. The pH of the supernatant was neutralized using saturated solution of citric acid. Ethanol (99.5%) was added to the neutralized solution to a final concentration of 70% v/v and it was kept overnight at 4°C to allow the precipitation of arabinoxylan. The supernatant was discarded after centrifugation (4500 rpm, 20 min) and pellet was washed once with 70% v/v ethanol and centrifuged to remove liquid fraction. The pellet was then solubilized in MQ-water and freeze-dried.

### Sequential extraction

This process was adopted from Vieira et. al. (Vieira et. al., 2014) and the process pathway is attached in the appendix section.

**Reagents:**

(a) KOH 0.1 M
(b) KOH 0.5 M
(c) KOH 4 M
(d) 99.5% ethanol
(e) Citric acid (saturated)
(f) 37% HCl

Around 5 g of BSG was suspended in 50 ml of 0.1 M KOH solution and incubated at 40ºC for 2 hours and centrifuged (3900 x g for 20 min) afterwards. This supernatant fraction was marked as AX-1. The pellet was treated with 0.5 M of KOH at 40ºC for 2 hours, centrifuged and the supernatant fraction was marked as AX-2. The pellet was then treated with 4 M KOH at 40ºC for 2 hours, centrifuged and the supernatant fraction was marked as AX-3. This final pellet was then washed once with MQ-water, centrifuged and the supernatant was freeze-dried and marked as AX-4. All three supernatants (AX-1, AX-2 and AX-3) was then separately acidified to pH 3.0 with a saturated solution of citric acid and allowed to precipitate BSG proteins (mostly hordeins and glutenins), which were separated by centrifugation. All three supernatants were then further acidified to pH below 2.0 using 37% HCl. The arabinoxylan

was then recovered by precipitation with 99.5% ethanol to a final concentration of 80% (v/v) ethanol, centrifuged and the pellets were freeze-dried.

## Ultrasound assisted extraction

This process was adopted from Reis et. al. (Reis et. al., 2015). A flow diagram of this process can be found in the appendix section.

**Reagents:**

(a) KOH 3M

(b) HCl 37%

2 g of BSG sample was suspended in 50 ml of deionized water. The ultrasound probe was submerged up to 25 mm in the sample and performed ultrasound treatment using a probe of 13 mm diameter, 20 kHz frequency and 92% amplitude. Pulse duration was selected as 5 s on and 5 s off. Three different times (treatment duration) was used for this experiment, 7.5 min, 10 min and 15 min respectively. After this treatment, the sample was centrifuged (14,400 rpm for 20 min) and the supernatant was marked as AX-1. The pellet was suspended in 50 ml of MQ-water, autoclaved at 120°C for 30 minutes, centrifuged as above and the supernatant was marked as AX-2. This pellet fraction was then suspended in 3M KOH solution and treated with ultrasound at an amplitude of 75% for 5 minutes. The suspension was then neutralized (pH 6-7) using HCl, centrifuged and the supernatant was marked as AX-3. All three supernatant fractions (AX-1, AX-2 and AX-3) were then precipitated with five volume of ethanol, centrifuged and the pellets were solubilized in MQ-water. Then, these three fractions were dialyzed using dialysis membrane of 6-8 kDa cut off (Spectrapor) and freeze-dried afterwards.

## Auto-hydrolysis

This procedure was adopted from Falck et. al. (Falck et. al., 2014). Process-3 was used to extract arabinoxylan from BSG. A complete process pathway of this process is attached in the appendix section.

**Reagents:**

(a) Sodium Phosphate Buffer (SPB) 100 mM pH 6.9

(b) Termamyl 120 l (Novozyme)

(c) P4860 Protease (Sigma)

(d) Amyloglucosidase (Sigma)

3 g of sample was suspended in sodium phosphate buffer (around 35 ml) and treated with Termamyl 120 l (0.12 U/g) at 90°C for 90 minutes. The mixture was then centrifuged (3900 x g for 20 min) to separate the supernatant. The pellet was washed once with MQ-water and centrifuged as above. This pellet fraction was then suspended in the buffer (around 35 ml) and treated with Protease (0.035 U/g) at 50°C for 4 hours with continuous shaking in a water bath. The mixture was then centrifuged and the pellet was washed once with MQ-water. The pellet was then transferred into 100 ml Schott bottle with MQ-water and autoclaved at 121°C for 15 hours. The sample was then transferred into 50-ml plastic tube, centrifuged and the supernatant was freeze-dried. The freeze-dried supernatant was then solubilized in MQ-water and treated with amyloglucosidase (39.2 U/g) at 50°C for 1 hour. Treated sample was then precipitated with 99.5% ethanol to a final concentration of 80% (v/v), kept overnight at 4°C, centrifuged and the pellet was freeze-dried.

## Pulsed electric field (PEF) treatment

An attempt was made to see the impact of this technology on the extraction of arabinoxylan from brewer's spent grain. Treatment conditions used were: 20 kV/cm, 20 pulse x 2 (performed twice) and 140 Hz resulting a voltage of around 3830 V. A negative control was also used to check the impact of this treatment. The process diagram is given below.

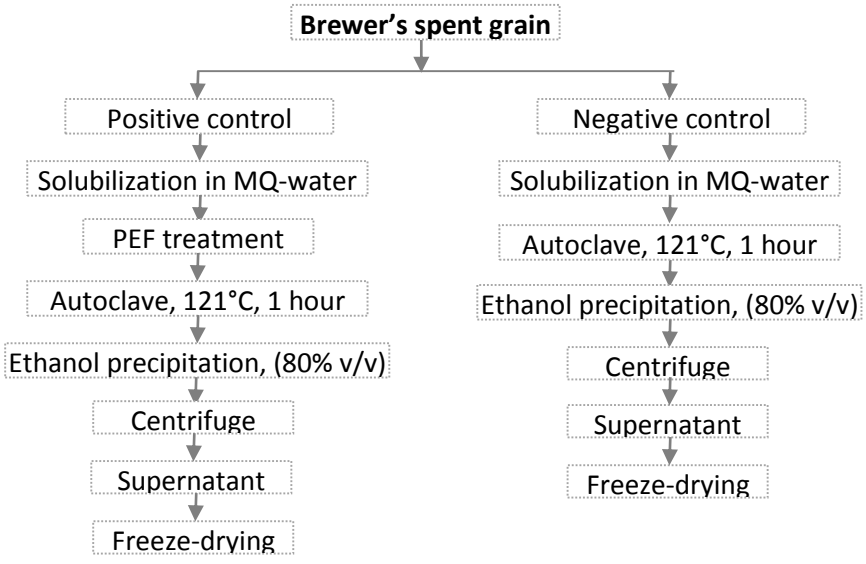



*Figure 6: Process pathways used for PEF treatment*

## Combined extraction method

A combined extraction method was developed based on experience gained from previous extraction methods. The process contains a heat-treatment followed by mild alkali treatment after removing starch from BSG. The process diagram is given below.

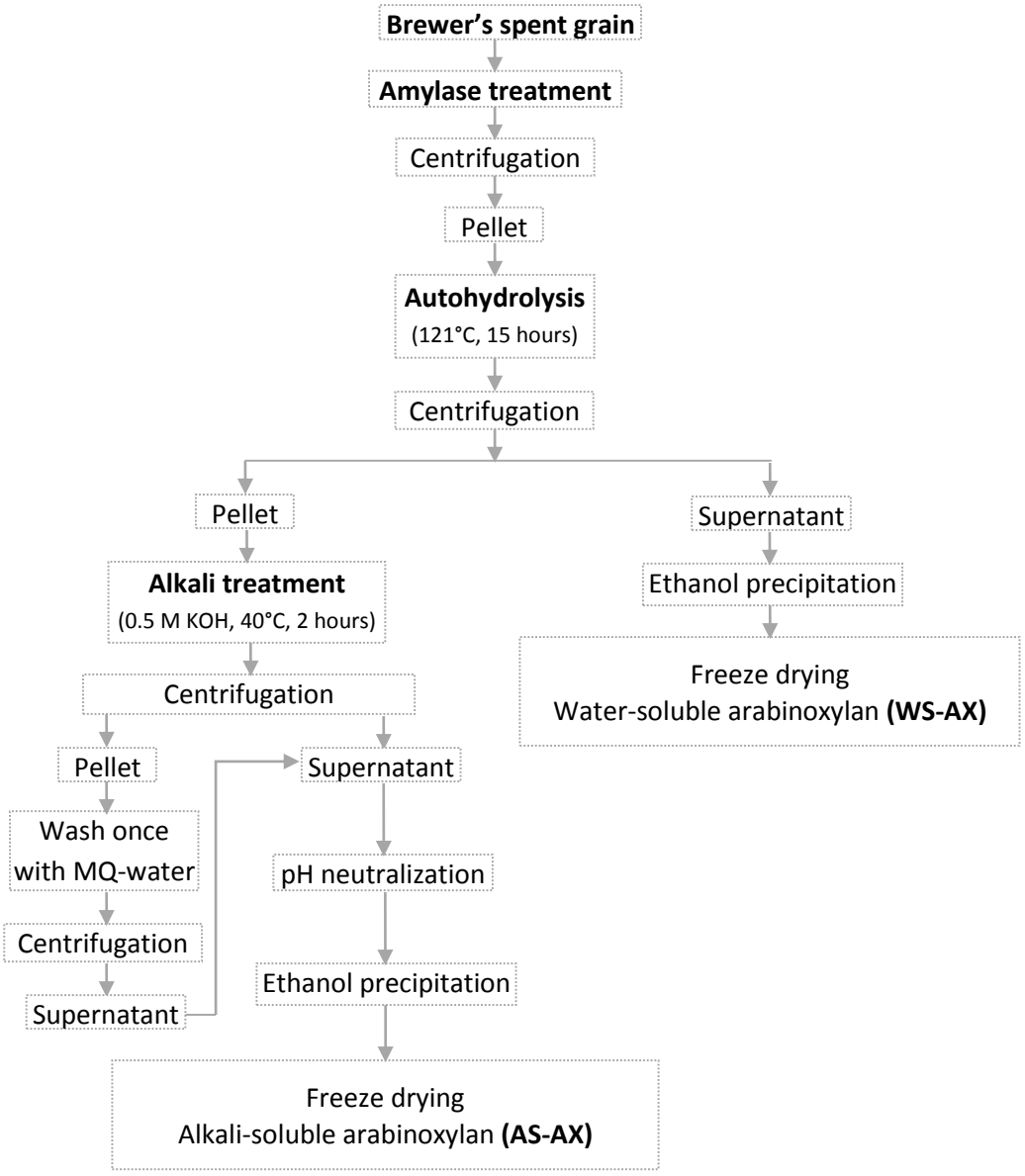



*Figure 7: Combined extraction process*

**Reagents:**

(a) KOH 0.5 M
(b) HCl (37%)
(c) Sodium phosphate buffer, 20 mM, pH 6.9
(d) Termamyl 120 l (Novozyme)
(e) Ethanol 99.5%

20 g of BSG sample was suspended in 500 ml of sodium phosphate buffer and heated until the temperature reached to 90°C. Then termamyl 120 l was added and incubated for 90 minutes at 90°C . Then the suspension was centrifuged (20 min, 4500 rpm) to remove the starch fraction (the supernatant). The pellet was then washed once with 500 ml of deionized-water, centrifuged as above and the pellet fraction was used for further treatment. This pellet was then suspended in 200 ml of MQ-water and autoclaved at 121°C for 15 hours. Upon completion, the suspension was centrifuged and 4 volume of 99.5% ethanol was added to the supernatant (to a final concentration of 80% v/v) and kept at 4°C overnight. This supernatant fraction was marked as WS-AX (water soluble arabinoxylan). The pellet fraction was then treated with 100 ml of 0.5 M KOH for 2 hours at 40°C with continuous shaking. Then the suspension was centrifuged and the pellet fraction was washed once with MQ-water, centrifuged and added to previous supernatant. The pH of the combined supernatant was neutralized using HCl. 4 volumes of 99.5% ethanol was added to this neutralized solution and kept at 4°C overnight. This fraction was marked as AS-AX (alkali soluble arabinoxylan) and this term will be used in this report. Both WS-AX and AS-AX fraction was then centrifuged (20 min, 4500 rpm) and the supernatant was discarded. The pellet fractions were washed once with 80% (v/v) ethanol and centrifuged as above. The pellet fractions were then solubilized in MQ-water and freeze-dried.

## Xylanase hydrolysis

Substrate (Birchwood xylan and BSG arabinoxylan) was prepared to a final concentration of 1% (w/v). The purity of birchwood xylan is more than 99% whereas the purity of BSG arabinoxylan is around 50%. So, in case of BSG arabinoxylan the 1% (w/v) concentration of substrate was prepared considering the purity of arabinoxylan (arabinoxylan content) to make it easier when it comes to comparison. Respective buffer solutions for each xylanase was used to suspend the substrates.

To make this process easier 2% (w/v) concentration of substrates was prepared as mentioned in the table below and amount of xylanses were added based on their activity measured by DNS assay. Then,

the final volume was made up to 10 ml to get the final substrate concentration of 1% (w/v) in the reaction media.

Table 2: Reaction volumes for xylanase hydrolysis

| For 10 ml reaction volume | | | | |
|---|---|---|---|---|
| | 2% AX Solution in respective buffer (µl) | Xylanase* from WS (µl) | Respective Buffer Solution (µl) | Final Reaction Volume (µl) |
| DYADIC | 5000 | 1,21 | 4998,79 | 10000 |
| BIO-CAT | 5000 | 1,79 | 4998,21 | 10000 |
| Power bake 7430 | 5000 | 1107,99 | 3892,01 | 10000 |
| ORBAZIM HC 2500 | 5000 | 0,42 | 4999,58 | 10000 |
| GRIND | 5000 | 48,41 | 4951,59 | 10000 |
| Power bake 900 | 5000 | 693,58 | 4306,42 | 10000 |
| *Rm*Xyn10A | 5000 | 222,22 | 4777,78 | 10000 |
| Pentopan mono BG | 5000 | 6,87 | 4993,13 | 10000 |
| Pentopan 500 BG | 5000 | 54,72 | 4945,28 | 10000 |

*Volumes were calculated to get 1 U xylanase/g of arabinoxylan

1 U xylanase/g of arabinoxylan was used for xylanase hydrolysis. The hydrolysis was performed at 40°C with continuous shaking. Around 1 ml of sample was collected at every 5$^{th}$ hour, boiled at 110°C for 5 minutes, cooled on ice to room temperature and centrifuged (13000 rpm, 2 min) afterwards. The supernatant was transferred into new Eppendorf tube and stored at -20°C until being analyzed in HPAEC-PAD using a column and pre-column for oligosaccharides.

## Bacterial growth studies

**Preparation of inoculum**

MRS broth (Merck) was used to activate the culture of *Lactobacillus brevis* (DSMZ 1264). 10.44 g of MRS media was dissolved in 200 ml deionized water, sterilized (autoclaved for 15 minutes) and stored at 4°C until being used. 5 ml of MRS media was taken into a 15 ml plastic tube and bacterial culture was added to it. The tube was placed in a closed container having Anaerocult (Merck). Anaerocult is a moisturized reaction agent to maintain anaerobic atmosphere. This was incubated for 48 hours at 37°C without shaking. All these works were performed under sterile hood.

**Preparation of carbon source and MRS medium without carbon source**

BSG arabinoxylooligosaccharides (AXOS) was prepared by hydrolyzing BSG AX fractions using Pentopan Mono BG for 5 hours at 40°C and used as carbon source. Carbon source (BSG AXOS) was prepared to a concentration of 25 mg/ml (based on arabinoxylan content) using MQ-water. The suspension was

then boiled in a microwave oven, the headspace was flushed with nitrogen gas, autoclaved at 121°C for 15 minutes and stored at 4°C until being used.

MRS media without carbon source (glucose) was prepared separately. The following components were mixed and the volume was adjusted to 400 ml using deionized water. The pH of the media was adjusted to 6.7, heated for few minutes using a microwave oven, the headspace was flushed with nitrogen. The media was then sterilized and stored at 4°C until being used in the fermentation experiment.

Table 3: Preparation of MRS media without carbon source

| **Components** | **Amount (g)** |
|---|---|
| Casein peptone, tryptic digest | 5 |
| Meat extract | 5 |
| Yeast extract | 2,5 |
| Tween 80 | 0,5 |
| K2HPO4 | 1 |
| Na-acetate | 2,5 |
| (NH4)3 citrate | 1 |
| MgSO4 x 7 H2O | 0,1 |
| MnSO4 x H2O | 0,025 |
| Deionized water | up to 400 ml |

***In-vitro* fermentation**

8 ml of prepared MRS medium without carbon source and 2 ml of carbon source (BSG AXOS) was taken in a 15 ml plastic tube. 200 µl of inoculum (to a final concentration of 2% v/v) was added to the tube, mixed gently and the culture was allowed to grow at 37°C for 48 hours in a closed container having Anaerocult. A negative control containing MQ-water was also used.

Samples were taken at 0, 24 and 48$^{th}$ hour of fermentation, centrifuged (13000 rpm, 2 min) and used for analysis. pH and absorbance at 620nm was analyzed immediately and the sample was stored at -20°C for short-chain fatty acid (SCFA) and HPAEC-PAD analysis. Growth was calculated by subtracting the initial (0 hour) $OD_{620\ nm}$ values from final OD values and reported as percentage of increase in OD values compared to OD values at 0 hour.

## Analytical methods

### Monosugar analysis

Monosugars present in the samples were analyzed according to the process described by Falck et. al. (Falck et. al., 2014). Briefly, HPAEC-PAD (ICS-5000, Dionex, Sunnyvale, CA) system equipped with PA20 column (150 mm x 3 mm, 6,5 µm) and a guard column (30 mm x 3 mm) was used. 0.75mM NaOH was used as eluent concentration with a flow rate of 0.5 ml/min and column temperature of 30ºC. Detection was accomplished with pulsed amperometric detection (PAD) using gold electrode.

### Oligosaccharide analysis

Samples after xylanase hydrolysis and bacterial fermentation were analyzed in HPAEC-PAD (ICS-5000) according to the procedure described by Falck et al. (Falck et. al., 2014). CarboPac PA-200 column (250 mm x 3 mm, 5,5 µm) coupled with pre-column for PA-200 (50 mm x 3 mm) was used for this analysis. A mixture of NaOH and NaOAc was used as mobile phase with a flow rate of 0.5 ml/min for this analysis.

### Molecular weight determination

Molecular weight was determined according to the process described by Sardari (Sardari et al., 2017). Dextran (Sigma) of different molecular weights (5, 12, 50 and 80 kDa) was used as standard. All the samples and standards were passed through a Sephacryl S-200 column (16 mm x 600 mm). The retention time of standards were plotted against the logarithmic values of their respective molecular weights to obtain a calibration curve. Molecular weight of samples were then determined from their retention time.

### Short chain fatty acid (SCFA) analysis

A method described by Patel et al. was used for SCFA analysis (Patel et al., 2013). Samples after fermentation were filtering through 0.20 µm filter. SCFA were analyzed by HPLC (Dionex) using an ion-exclusion column (Aminex 87HPX, Bio-Rad) using a mobile phase of 5 mM $H_2SO_4$ and a flow rate of 0.5 ml $min^{-1}$. The column oven temperature was set at 40°C. Standards of acetic acid (VWR), DL-lactic acid (Fluka), propionic acid (Sigma) and butyric acid (Sigma) were used to identify and quantify the amount of SCFA.

# Results and discussion

## Characterization of starting material

Brewer's spent grain (BSG) is the left over materials after beer brewing process and contains mostly water (around 70%). In case of dry BSG, arabinoxylan content is around 14.75% (w/w) which is second largest amount after glucose. The ratio of arabinose to xylose (ara/xyl) in BSG was found to be 0.44. The composition of BSG on a dry weight basis is presented in table 4. Also, the composition of BSG after brewing process (wet BSG) can be found in appendix 1.

Table 4: Composition of BSG

| **Parameters** | | **Amount (%)** |
|---|---|---|
| Moisture | | 0.68 |
| Protein | | 12.77 |
| Starch | | 13.71 |
| Lignin | Acid-soluble | 3.22 |
| | Acid-insoluble | 11.45 |
| Ash | | 3.28 |
| Monosugars | Arabinose | 4.50 |
| | Galactose | 0.24 |
| | Glucose (including starch) | 37.06 |
| | Xylose | 10.25 |
| | Mannose | 1.18 |
| | Fructose | N.D. |

% w/w; dry weight basis except for moisture (%, w/w), N.D.: Not detected, Values are means, n = 3, SD not more than 0.5%

Figure 8 shows the monosugar chromatogram obtained after acid hydrolysis of BSG. The response for glucose is quite high where it sums the amount of polymeric glucose and starch.

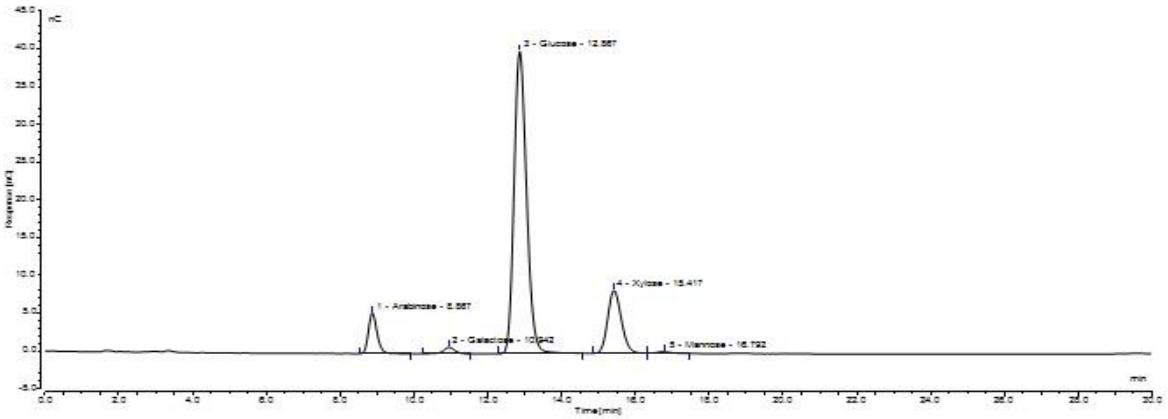


*Figure 8: Chromatogram from monosugar analysis of BSG*

Lignin, the third major compound found in BSG representing around 14.67% (w/w) on a dry weight basis. Lignin is of two types based on their solubility on acid namely acid soluble and acid insoluble.

Acid soluble lignin is determined spectrophotometrically where usually a wavelength of 280nm is used to record the absorbance (Falck et. al., 2014). But, Maekawa et al. reported that a wavelength of around 205nm should be used to determine the acid soluble lignin content in hard woods. While for soft woods the wavelength of 205nm should be corrected as the actual $\lambda_{max}$ in the 196-205 nm region. The author reported that use of 280nm wavelength might give erroneous absorbance as furfural and hydroxymethy furfural (polysaccharide degradation product) give absorbance near 280 nm region. This means that acid hydrolyzed filtrate gives strong absorbance near 280 nm which comes primarily from benzenoid moieties of lignin and partially from furfural and hydroxymethyl furfural. The author suggested to measure the spectra in the range of 190-350nm and use the actual $\lambda_{max}$ in the 196-205 nm region to determine acid soluble lignin content. (Maekawa et al., 1989)

The spectra in the region 190-350 nm was measured for BSG acid hydrolyzed filtrate and the spectra is shown in figure 9. The $\lambda_{max}$ in the region 196-205 nm found was 203 nm and this wavelength was used to measure the absorbance and determine the amount of acid soluble lignin in BSG. Alternatively, acid soluble lignin can be determined by measuring the absorbance at 280 nm wavelength as mentioned by Falck et al. (Falck et al., 2014).

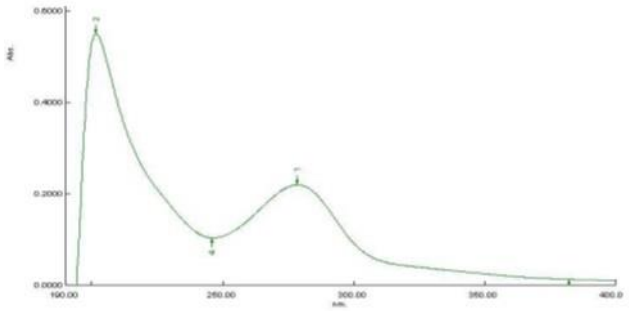



*Figure 9: Spectrum measured to obtain $\lambda_{max}$*

The composition of starting material showed that BSG contains quite reasonable amount of arabinoxylan and it would be feasible to continue into the further process aiming to extract arabinoxylan rich fractions. Also, it showed that removal of glucose (in the form of starch) would give us an enriched portion of arabinoxylan.

## Characterization of xylanases

Nine different xylanases was used in this experiment to hydrolyze arabinoxylan rich fractions extracted from BSG. It is important to characterize the activity and protein content of xylanases in order to get the reproducible results and also to know the exact amount required to hydrolyze the product. Activity of each xylanase enzymes was determined by DNS-assay and presented in table 5. Bio-Cat, one of the commercial enzymes was found to give the highest activity per mg of protein content. Figure 10 shows the purity of studied xylanases determined by SDS-PAGE. A complete band report for figure 10 can be obtained from appendix 2. The only two pure enzymes found was *Rm*Xyn10A and Pentopan Mono BG representing GH family 10 and 11 respectively. Other enzymes gave several bands on SDS-PAGE which made it difficult to determine their molecular weight and GH family.

Table 5: Characterization of xylanases

| Xylanases | Protein Content (mg/ml) | Xylanase Activity (U/mg) | Xylanase Activity (U/ml) |
|---|---|---|---|
| DYADIC | 2.062 | 399.717 | 824.117 |
| BIO-CAT | 0.441 | 1266.604 | 558.786 |
| Power Bake 7430 | 0.037 | 24.457 | 0.903 |
| ORBAZIM HC 2500 | 2.286 | 1037.514 | 2371.901 |
| GRIND Amyl H 460 | 0.059 | 349.181 | 20.656 |
| Power Bake 900 | 0.008 | 187.346 | 1.442 |
| *Rm*Xyn10A | 0.218 | 20.670 | 4.500 |
| Pentopan Mono BG | 0.364 | 400.177 | 145.563 |
| Pentopan 500 BG | 1.369 | 13.349 | 18.276 |

Values are means, n = 3, SD not more than 2.3%,

*****Values are for prepared working stock solutions (concentrations) mentioned in table 1

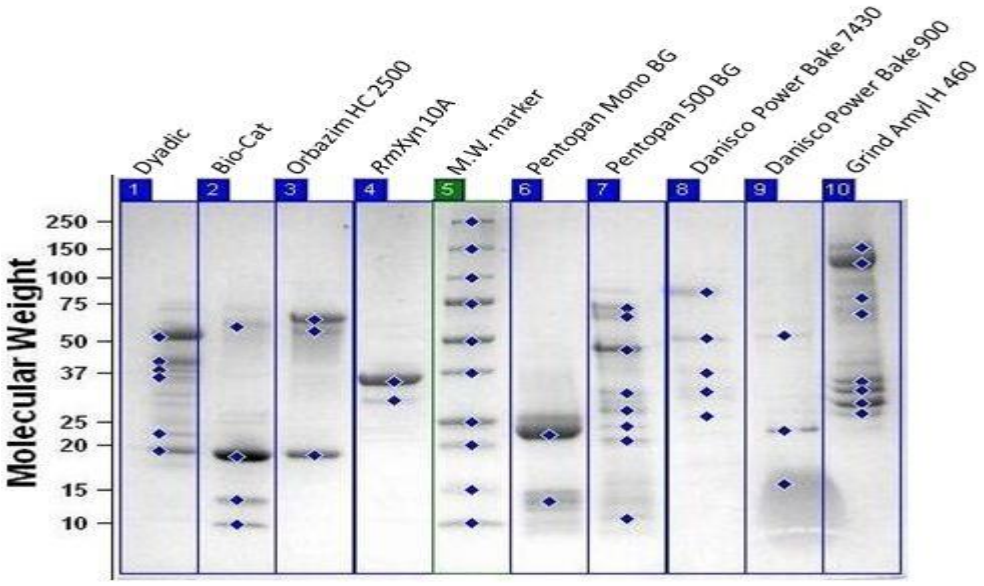



*Figure 10: SDS-PAGE image of studied xylanases*

Beaugrad et al. reported the average molar mass of GH10 and GH11 members where the average molar mass is approximately 40 kDa and 20 kDa respectively (Beaugrad et al., 2004). In this study the molar mass was found to be 33.44 kDa and 22.03 kDa for GH10 and GH11 respectively which is close to the previously reported values. GH11 xylanases are most effective against insoluble polymeric xylans whereas GH10 xylanases are active against soluble substrates and can readily hydrolyze small xylooligosaccharides such as xylotriose. The GH11 family members display a β-jelly roll structure whereas GH10 family members all possess a catalytic domain, which exhibits $(\beta/\alpha)_8$ architecture. GH11 xylanase can better penetrate the cell-wall network which makes it more efficient compared to GH10 xylanase in hydrolyzing xylans from wheat bran. The author also reported that GH10 xylanase tends to create products smaller in size compared to GH11 xylanase which indicates a higher proportion of cleavage sites for the GH10 xylanase. (Beaugrad et al., 2004)

## Pre-treatments of starting material

Several pre-treatments were used to find out the best one in terms of extraction yield, purity of arabinoxylan, simplicity and novelty of the extraction method. Table 6 provides the results mentioning the extraction yield, purity and monosugar contents obtained using different pre-treatments. Purity is expressed as arabinoxylan percentage of the extracted product.

Alkali extraction of two different process conditions (40°C and 80°C) were used which gave extraction yield of 8.15% and 19.46% respectively. Use of alkali treatment at 80°C gave the highest extraction yield and arabinoxylan purity of around 50%. But, this extraction process was not used for further processing as the color of extracted product was black and the product was difficult to dissolve in the water. This could be because of maillard reaction occurred because of amino-acids (protein) and reducing sugars present in the sample.

Auto-hydrolysis provides the benefits of chemical free extraction process. But, the downside of this process is the repeated extraction steps which makes it complicated and the too low final extraction yield. Thus, this method can be a good one in terms of research purpose use but not feasible for industrial scale applications.

Use of ultrasound treatment is a relatively new technique. Ultrasound treatment might help to open up the cell wall structure which makes the later steps easier to extract arabinoxylan. Ultrasound treatment of different time intervals were used and it was found that 10 mins treatment provide the best extraction yield.

Table 6: Extraction yield and purity of different pre-treatments

| Treatments | | Yield (%) | AX (%) | A/X ratio | Monosugar, % (w/w, dry weight) | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Treatment type | Extracted fractions | | | | Ara | Gal | Glu | Xyl | Man |
| 4M KOH, 40°C | | 8.15 | 36.9 | 0.22 | 7.59 | 0.46 | 33.62 | 34.34 | 0.23 |
| 4M KOH, 80°C | | 19.46 | 49.5 | 0.43 | 16.98 | 1.24 | 15.75 | 39.27 | 0.24 |
| Sequential extraction (KOH) | AX-1 | 4.97 | 49.19 | 0.75 | 23.97 | 2.1 | 38.37 | 31.93 | 0.46 |
| | AX-2 | 6.,8 | 61.26 | 0,49 | 22.87 | 1.79 | 14.19 | 46.75 | 0.34 |
| | AX-3 | 13.37 | 41.62 | 0.33 | 11.63 | 1.24 | 23.18 | 35.66 | 0.34 |
| | AX-4 | 1.28 | 27.19 | 0.69 | 12.59 | 1.53 | 26.9 | 18.31 | 19.63 |
| Ultrasound assisted extraction (7.5 min) | AX-1 | 6.46 | 6.84 | 0.85 | 3.56 | 1.0 | 47.53 | 4.21 | 0.33 |
| | AX-2 | 1.42 | 7.25 | 0.76 | 3.56 | 0.87 | 56.76 | 4.68 | 0.47 |
| | AX-3 | 14.61 | 51.,2 | 0.41 | 17.0 | 1.39 | 10.72 | 41.18 | 0.33 |
| Ultrasound assisted extraction (10 min) | AX-1 | 9.26 | 7.33 | 0.86 | 3.85 | 1.01 | 52.22 | 4.48 | 0.45 |
| | AX-2 | 2.59 | 7.89 | 0.76 | 3.88 | 0.92 | 66.75 | 5.09 | 0.58 |
| | AX-3 | 14.48 | 59.1 | 0.43 | 20.33 | 1.54 | 20.03 | 46.82 | 0.49 |
| Ultrasound assisted extraction (15 min) | AX-1 | 10.99 | 5.64 | 0.81 | 2.88 | 0.78 | 48.51 | 3.53 | 0.33 |
| | AX-2 | 1.71 | 5.62 | 0.69 | 2.6 | 0.59 | 47.95 | 3.78 | 0.41 |
| | AX-3 | 13.82 | 53.49 | 0.4 | 17.49 | 1.35 | 16.66 | 43.29 | 0.4 |
| Auto-hydrolysis | | 5.44 | 36.86 | 0.77 | 18.16 | 3.34 | 4.0 | 23.72 | 1.24 |
| Pulsed electric field treatment | Treated | N/A | 7.01 | 0.84 | 3.19 | N.D. | 96.54 | 3.82 | N.D. |
| | Control | N/A | 6.68 | 0.85 | 3.07 | N.D. | 97.05 | 3.61 | N.D. |
| Combined treatment | WS-AX | 5.65 | 44.76 | 0.61 | 16.89 | 0.83 | 9.16 | 27.87 | N.D. |
| | AS-AX | 8.72 | 54.74 | 0.53 | 18.99 | 0.63 | 2.62 | 35.76 | N.D. |

Ara: Arabinose, Gal: Galactose, Glu: Glucose, Xyl: Xylose, Man: Mannose, Values are means, n = 2, SD not more than 2.5%

Combined extraction process was used for further experiments as this pre-treatment gave quite reasonable purity of arabinoxylan and this is a simple process which can be adopted quite easily. Two different fractions (products) was obtained from this pre-treatment and named as WS-AX and AS-AX which represent water-soluble and alkali-soluble arabinoxylan fractions respectively. The plus point of this pre-treatment is the possibility of the process to modify according to the need of extraction yield. WS-AX fraction can be easily obtained without use of any chemical and this provides an environment friendly option. Characteristics of the products obtained from combined extraction process are provided in table 7 and figure 11 and 12 shows the chromatogram obtained from monosugar analysis of each fractions.

Table 7: Characterization of products selected for xylanase hydrolysis

| Fraction | Extraction yield (%) | AX (%) | Ara/xyl ratio | Sugar (%) | | | | Mw (kDa) |
|---|---|---|---|---|---|---|---|---|
| | | | | Arabinose | Galactose | Glucose | Xylose | |
| **WS-AX** | 5.65 | 44.76 | 0.61 | 16.89 | 0.83 | 9.16 | 27.87 | 60.18 |
| **AS-AX** | 8.72 | 54.74 | 0.53 | 18.99 | 0.63 | 2.62 | 35.76 | 59.10 |

Values are means, n = 3, SD not more than 0.5%,

The molecular weight of both WS-AX and AS-AX was determined as 60.18 kDa and 59.10 kDa respectively. In case of AS-AX, the molecular weight is a bit smaller compared to WS-AX which can be explained as loss of arabinoxylan or arabinose substituents alone because of alkali treatment. Also, AS-AX fraction contains less amount of glucose as can be seen from figure-12 which can be because of losing β-glucan due to alkali treatment.

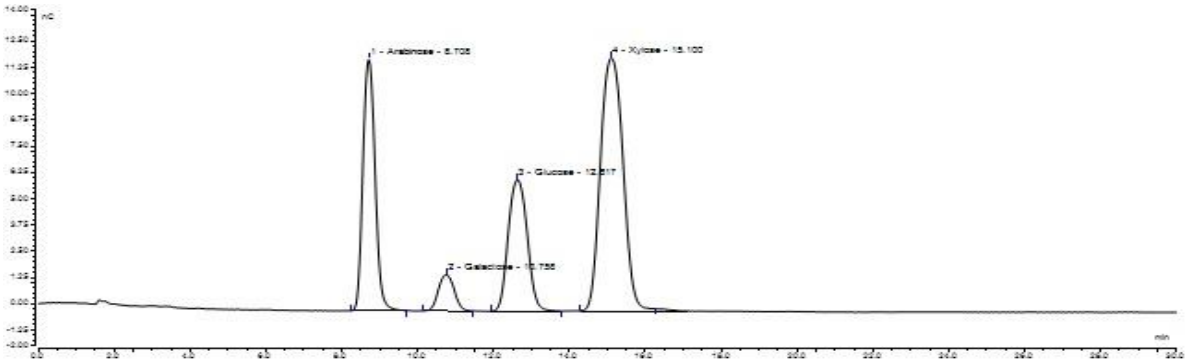


*Figure 11: Chromatogram from monosugar analysis (WS-AX fraction)*

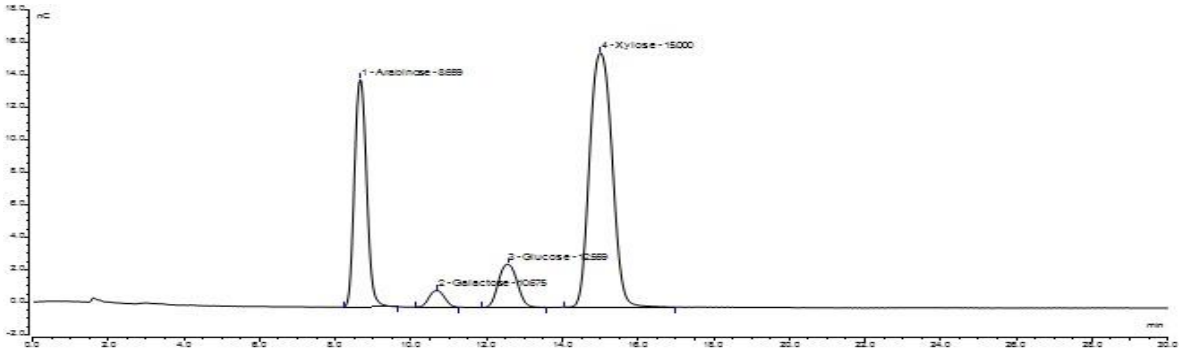


*Figure 12: Chromatogram from monosugar analysis (AS-AX fraction)*

## Xylanase hydrolysis

Hydrolysis efficiency (in terms of formation of short-chain oligosaccharides) of two pure xylanases from GH family 10 and 11 was studied on birchwood xylan (Sigma) and on extracted arabinoxylan rich BSG fractions (BSG WS-AX and BSG AS-AX respectively). Figure 13, 14 and 15 shows product formation pattern of xylanases from GH family 10 (RmXyn 10 A) and GH family 11 (Pentopan Mono BG) respectively. Five hours of hydrolysis time at 40°C was used for this analysis and figure 13, 14 and 15 represent the substrate of birchwood xylan, BSG WS-AX and BSG AS-AX respectively.

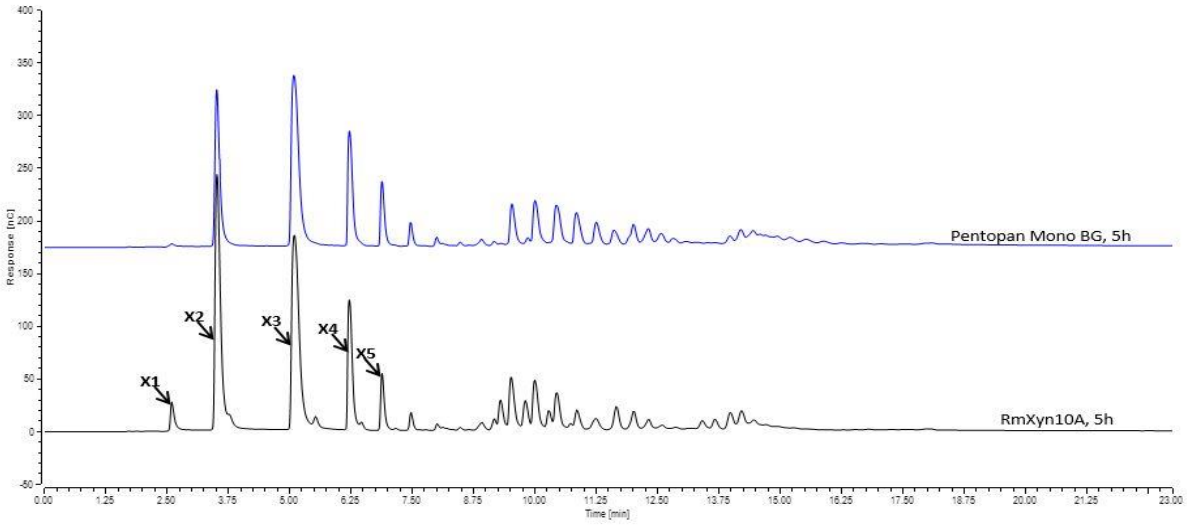



*Figure 13: Birchwood xylan - 5 h hydrolysis - GH 10 (RmXyn 10 A) and GH 11 (Pentopan Mono BG)*

Use of *Rm*Xyn10A on birchwood xylan releases relatively higher amount of xylose sugars compared to Pentopan Mono BG as can be seen in figure 13. This can be explained as *Rm*Xyn10A which is GH10 family xylanase have more cleavage sites compared to GH11 family xylanases and are efficient in making products of smaller size (Beaugrad et al., 2004). The same explanation might works for figure-15 (BSG AS-AX) where use of GH10 xylanase produces more arabinose and xylose compared to GH11 xylanase. The presence of very little amount of arabinose and xylose can be noticed in figure-14 which is unexpected for GH11 xylanase. This could originate from existing monosugars before xylanase treatment. The auto-hydrolysis treatment might hydrolyze a bit of arabinoxylan and this is assumed to be observed in figure-14 in case of GH11 xylanase.

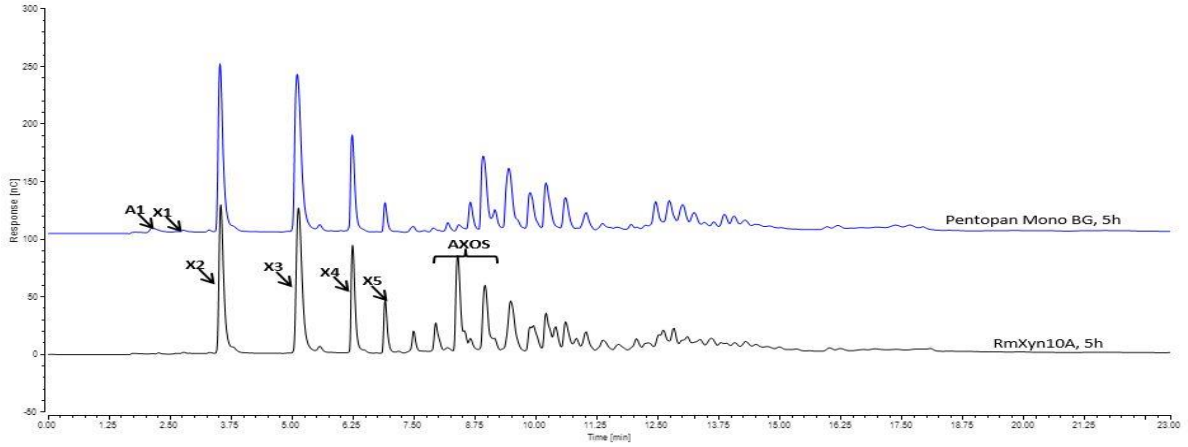



*Figure 14: BSG WS-AX - 5 h hydrolysis - GH 10 (RmXyn 10 A) and GH 11 (Pentopan Mono BG)*

Figure 14 and 15 shows product formation pattern after 5 h hydrolysis of BSG WS-AX and AS-AX fractions using GH10 and GH11 xylanases.

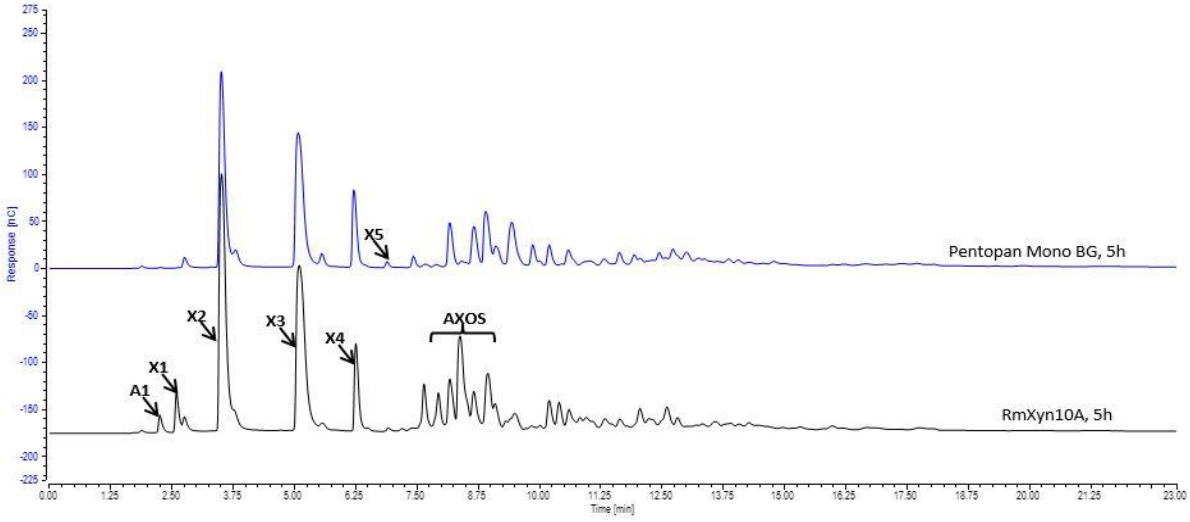



*Figure 15: BSG AS-AX - 5 h hydrolysis - GH 10 (Rm*Xyn *10 A) and GH 11 (Pentopan Mono BG)*

Both xylanases are quite effective on hydrolyzing oligosaccharides into short-oligosaccharides. Thermostable endoxylanase RmXyn10A from *Rhodothermus marinus* has long been used to hydrolyze oligosaccharides and published articles are available (Falck et al., 2013). Pentopan Mono BG was used to hydrolyze products to be used in fermentation experiment.

Figure 16 and 17 shows an overview of products formed from BSG WS-AX and BSG AS-AX after xylanase hydrolysis using all studies xylanases. Hydrolysis was carried out at 40°C for 5 hours. A detailed

hydrolysis profile for each studied xylanases can be obtained from appendix 5 where product formation pattern after every 5$^{th}$ hour of hydrolysis are reported.

Products after pre-treatment were used for xylanase hydrolysis as the starting material was quite complex for the enzymes to work. But it is also possible to use polysaccharide-degrading glycoside hydrolases (cellulases, xylanases, mannanases and pectinases) at the time of pre-treatment which might promote the release of desired compounds like short-chain oligosaccharides.

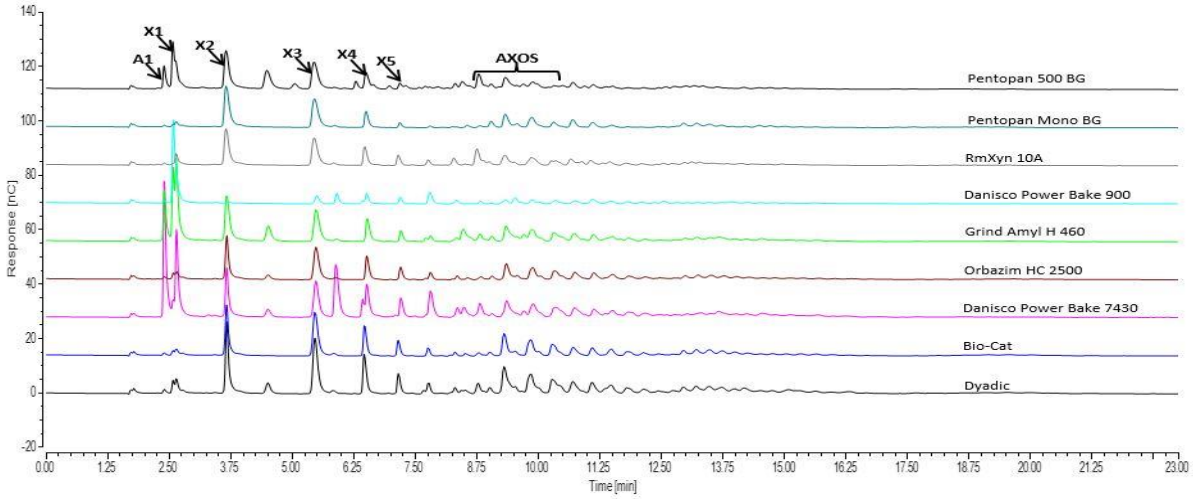



*Figure 16: BSG WS - 5 h hydrolysis - All xylanases*

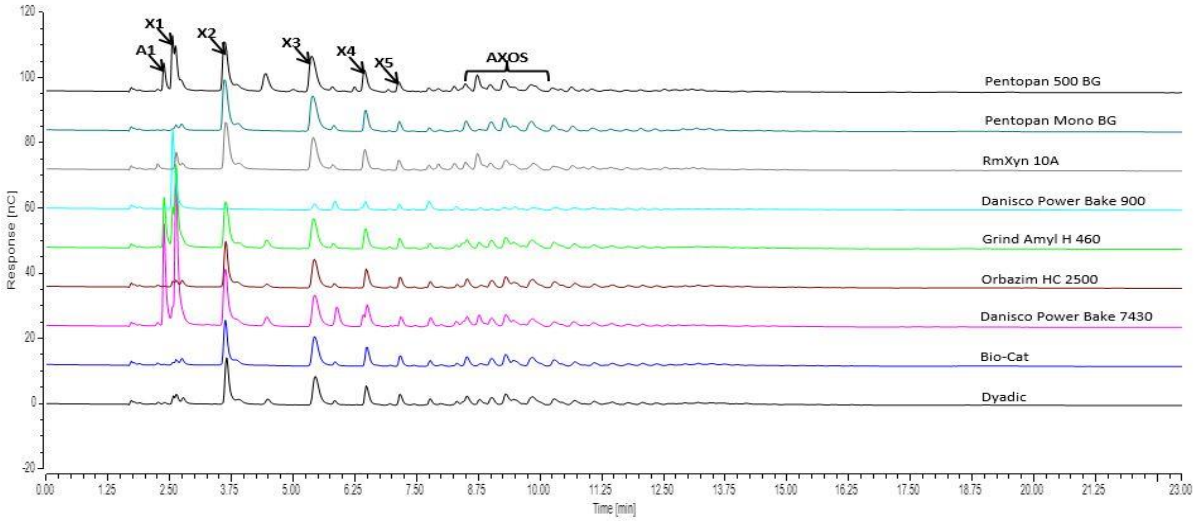



*Figure 17: BSG AS - 5 h hydrolysis - All xylanases*

## Bacterial growth studies on hydrolyzed arabinoxylan fractions

It is important to check the ability of the extracted products to ferment in order to declare it as potential prebiotics. Products extracted using combined extraction method were hydrolyzed for 5 hours at 40°C by Pentopan Mono BG and used as carbon source for fermentation. Well known probiotic

strain of Lactobacillus (*L. brevis* DSMZ 1264) was used for fermentation experiment. Glucose in the media was replaced by AXOS and used as carbon source. Formation of organic acids (SCFAs) after 48 hours of fermentation was determined and reported in table 8. Carbohydrate utilization pattern after 48 hours of fermentation is shown in figure 18 and 19 and represent AXOS from BSG WS and BSG AS fractions respectively.

Table 8: Growth characteristics after 48 h of fermentation

| Substrate | Growth | pH | SCFA (mg/mL) | | | |
|---|---|---|---|---|---|---|
| | | | Lactic acid | Acetic Acid | Propionic Acid | Butyric Acid |
| BSG WS AXOS | ++ | 6.32 | 0.97 | 4.23 | 0.58 | 0.06 |
| BSG AS AXOS | ++ | 6.16 | 1.33 | 4.41 | 0.58 | 0.06 |

Values are means, n = 3, SD not more than 0.5%, growth ($OD_{620\ nm}$): ++ = 26-50%

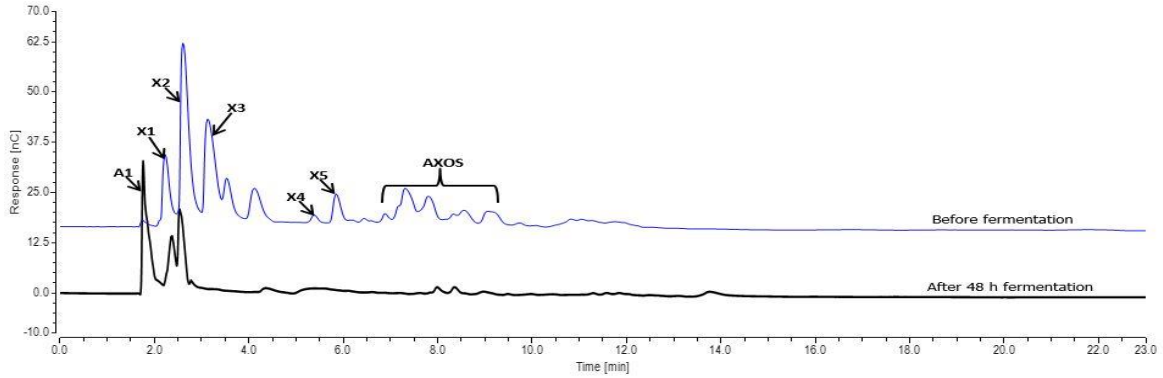



*Figure 18: Carbohydrate utilization pattern of BSG AXOS WS (water-soluble fraction)*

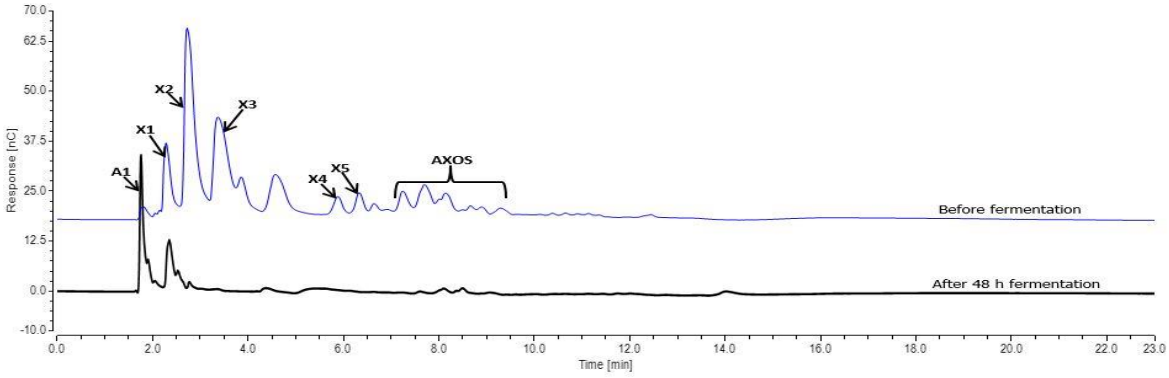



*Figure 19: Carbohydrate utilization pattern of BSG AXOS AS (alkali-soluble fraction)*

# Conclusion

It is well known that the extent of fermentation depends on the structure of carbon source. The simpler the structure of the carbon source the better will be the utilization by the bacteria. Composition of brewer's spent grain suggests the presence of reasonable amount of arabinoxylan which makes it feasible to use for arabinoxylan extraction. Brewer's spent grain has already gone through hot water extraction steps during beer brewing limiting the choice of pre-treatment. Removal of starch before pre-treatment increases the purity of the product.

Auto-hydrolysis of de-starched brewer's spent grain for 15 hours can be used for extraction of arabinoxylan. This pre-treatment step can be used for large scale production of arabinoxylan from brewer's spent grain where no chemicals are needed. Also, the residue after this step can be used for animal feed. Alkaline extraction step can be adopted after auto-hydrolysis to extract further arabinoxylan. Neutralization of pH after extraction is an additional step in case of using alkali extraction. The purity and yield of extraction can be customized by varying the strength of akali, extraction temperature and time. The yield and purity of extracted products can be improved by removing lignin from the biomass before pre-treatment. $H_2O_2$ to a final concentration of less than 3% v/v can be used to remove lignin from the biomass. Risks associated with using H2O2 at elevated temperature should be performed. Alternatively, biomass sample can be treated with some strain(s) of white rot, for example lignin selective *Ceriporiopsis subvermispora* to reduce the content of lignin in it.

Short XOS and AXOS are better compared to long chain arabinoxylan when it comes to fermentability by probiotic bacteria. Xylanases of varying source, function and activity can be used to produce the desired short XOS and AXOS. Pure xylanases were found to be efficient in making short AXOS compared to the commercial ones. Bacterial growth experiment showed the utilization of short oligosaccharides by probiotic strain Lactobacillus brevis and production of short chain fatty acids.

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# Appendices

## Appendix 1: BSG Composition (Sample as received)

| Parameter | | % |
|---|---|---|
| **Moisture** | | 70.98 |
| **Protein** | | 3.76 |
| **Starch** | | 4.01 |
| **Lignin** | Acid-insoluble | 3.35 |
| | Acid-soluble | 0.94 |
| **Ash** | | 0.96 |
| **Monosugars** | Arabinose | 1.31 |
| | Galactose | 0.07 |
| | Glucose | 10.83 |
| | Xylose | 3.00 |
| | Ara/Xyl ratio | 0.44 |
| **Miscellaneous** | | 0.80 |

% w/w, sample as received

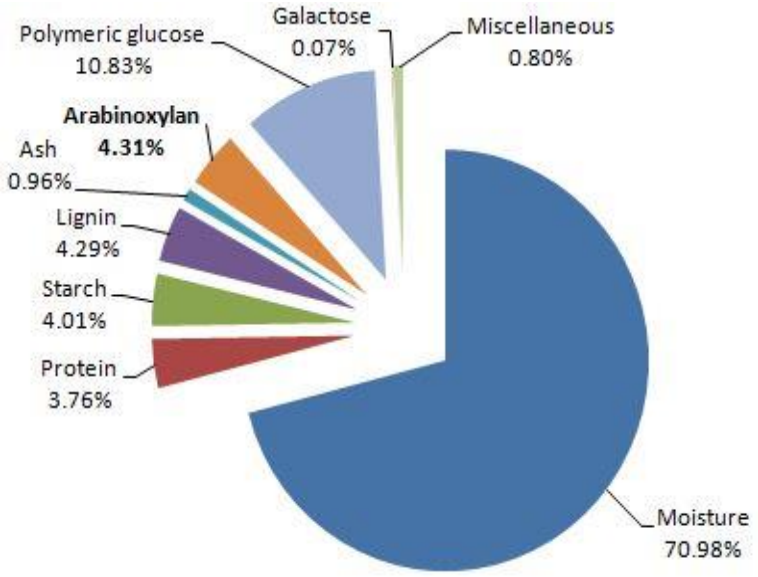


Figure: BSG Composition (% w/w, sample as received)

# Appendix 2: Characterization of xylanases

**Band report:**

| DYADIC | | |
|---|---|---|
| Band | Band % | MW |
| 1 | 42.55 | 52.31 |
| 2 | 18.43 | 41 |
| 3 | 6.924 | 37.93 |
| 4 | 6.791 | 35.68 |
| 5 | 4.633 | 22.35 |
| 6 | 20.68 | 19.07 |

| BIO-CAT | | |
|---|---|---|
| Band | Band % | MW |
| 1 | 13.159 | 58.3 |
| 2 | 65.419 | 18.3 |
| 3 | 12.082 | 13.7 |
| 4 | 9.34 | 9.78 |

| ORBAZIM HC 2500 | | |
|---|---|---|
| Band | Band % | MW |
| 1 | 34.751 | 63.006 |
| 2 | 34.87 | 55.608 |
| 3 | 30.379 | 18.484 |

| *Rm*Xyn10A | | |
|---|---|---|
| Band | Band % | MW |
| 1 | 90.661 | 34.44 |
| 2 | 9.339 | 29.8 |

| Pentopan Mono BG | | |
|---|---|---|
| Band | Band % | MW |
| 1 | 76.16 | 22.03 |
| 2 | 23.84 | 13.48 |

| Pentopan 500 BG | | |
|---|---|---|
| Band | Band % | MW |
| 1 | 11.732 | 71.5 |
| 2 | 10.673 | 65 |
| 3 | 35.527 | 45.8 |
| 4 | 6.763 | 31.5 |
| 5 | 10.329 | 27.6 |
| 6 | 2.584 | 24 |
| 7 | 7.931 | 20.8 |
| 8 | 14.461 | 10.7 |

| DANISCO Power Bake 7430 | | |
|---|---|---|
| Band | Band % | MW |
| 1 | 39.198 | 85.162 |
| 2 | 20.621 | 51.522 |
| 3 | 12.245 | 37 |
| 4 | 23.926 | 31.829 |
| 5 | 4.01 | 26.294 |

| DANISCO Power Bake 900 | | |
|---|---|---|
| Band | Band % | MW |
| 1 | 3.367 | 53.11 |
| 2 | 11.87 | 23.01 |
| 3 | 84.763 | 15.62 |

| GRIND Amyl H 460 | | |
|---|---|---|
| Band | Band % | MW |
| 1 | 7.52 | 154.1 |
| 2 | 29.43 | 119.1 |
| 3 | 1.665 | 79.91 |
| 4 | 5.164 | 67.12 |
| 5 | 10.83 | 34.44 |
| 6 | 15.07 | 32.18 |
| 7 | 23.7 | 29.15 |
| 8 | 6.624 | 26.93 |

| Molecular weight marker | | |
|---|---|---|
| Band | Band % | MW |
| 1 | 10.41 | 250 |
| 2 | 3.044 | 150 |
| 3 | 4.017 | 100 |
| 4 | 17.45 | 75 |
| 5 | 15.16 | 50 |
| 6 | 9.385 | 37 |
| 7 | 17.42 | 25 |
| 8 | 6.39 | 20 |
| 9 | 5.158 | 15 |
| 10 | 11.57 | 10 |

## Appendix 3: Pre-treatments of starting material

### Sequential extraction

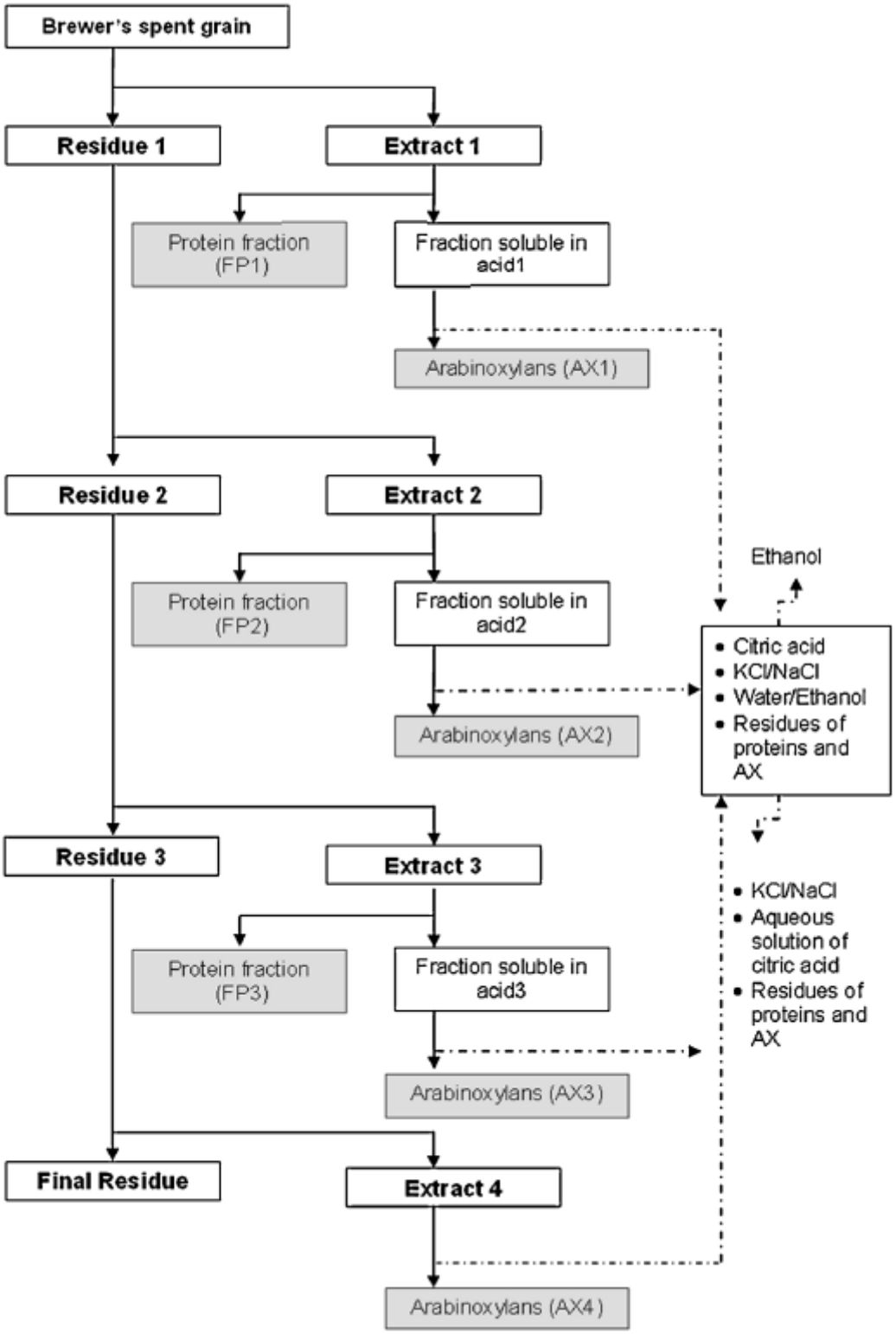


Figure: Process pathways for sequential extraction (Vieira et. al., 2014)

Ultrasound assisted extraction

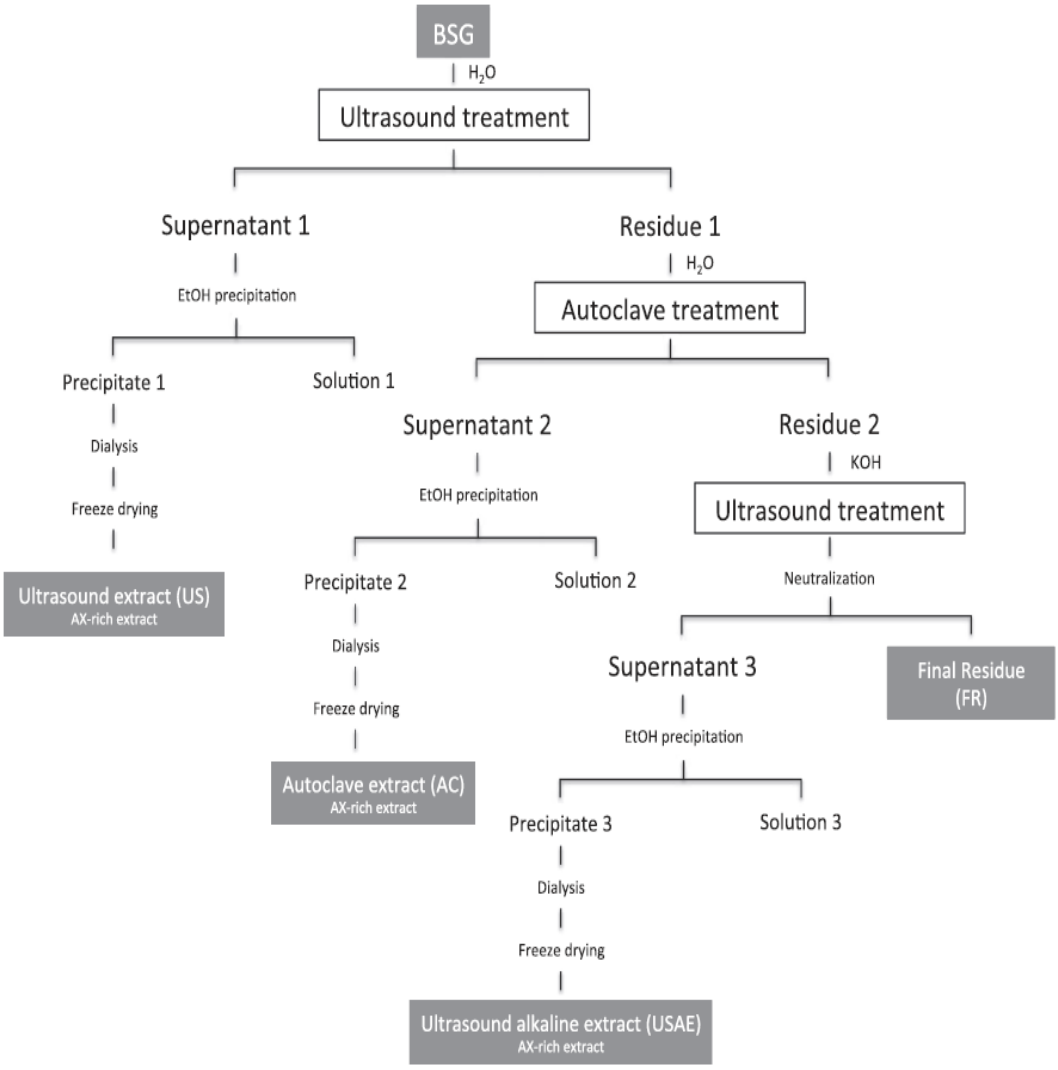


Figure: Process pathways for ultrasound assisted extraction (Reis et. al., 2015)

Autohydrolysis

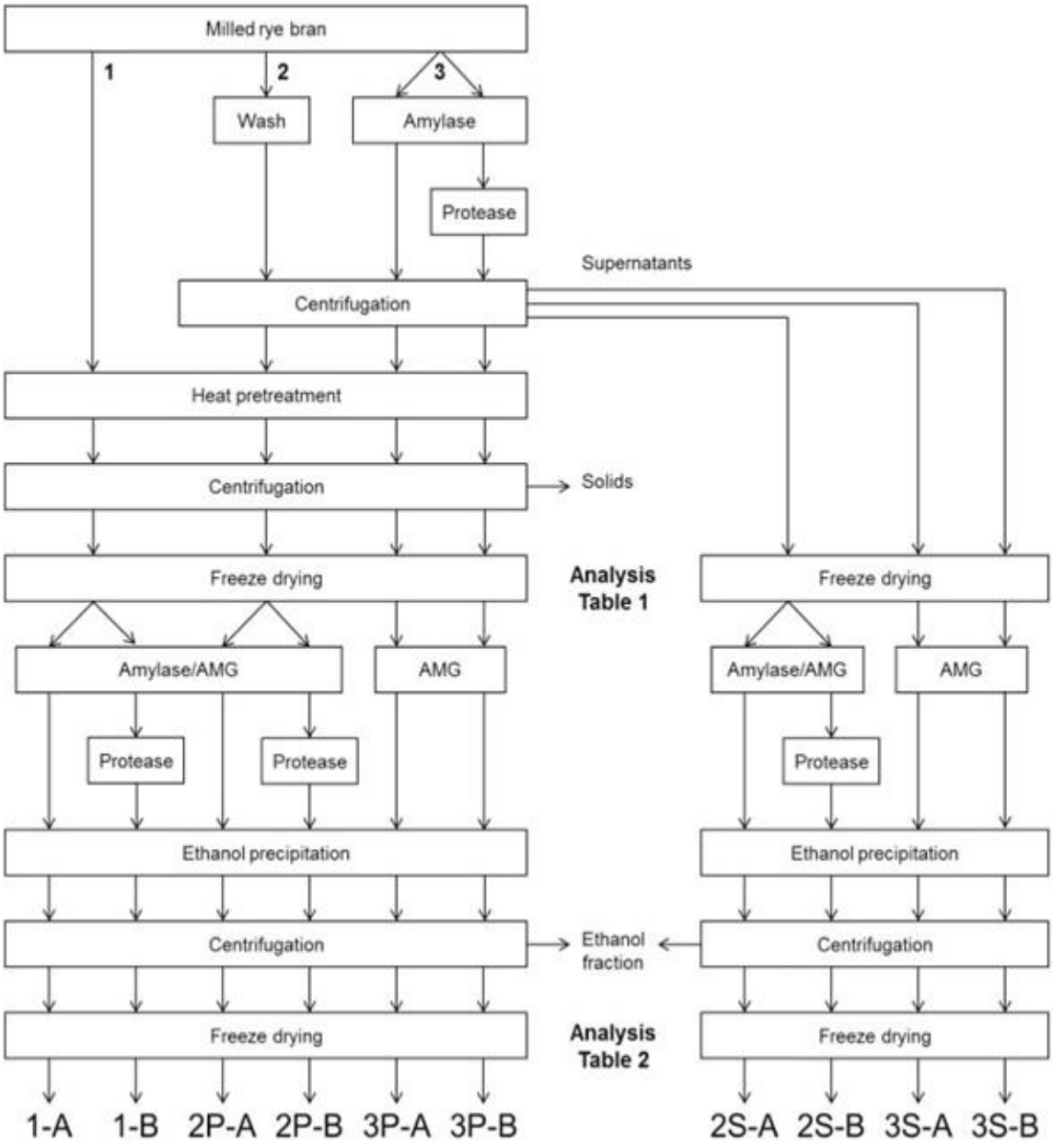


Figure: Process pathway (Falck et. al., 2014)

Appendix 4: Molecular weight of extracted arabinoxylan fractions

| Sample | | Retention Time (min) | Log Mw | Mw (Da) | Mw (kDa) |
|---|---|---|---|---|---|
| BSG | WS | 152,23 | 4,78 | 60178,73 | 60,18 |
| | AS | 153,56 | 4,77 | 59101,16 | 59,10 |

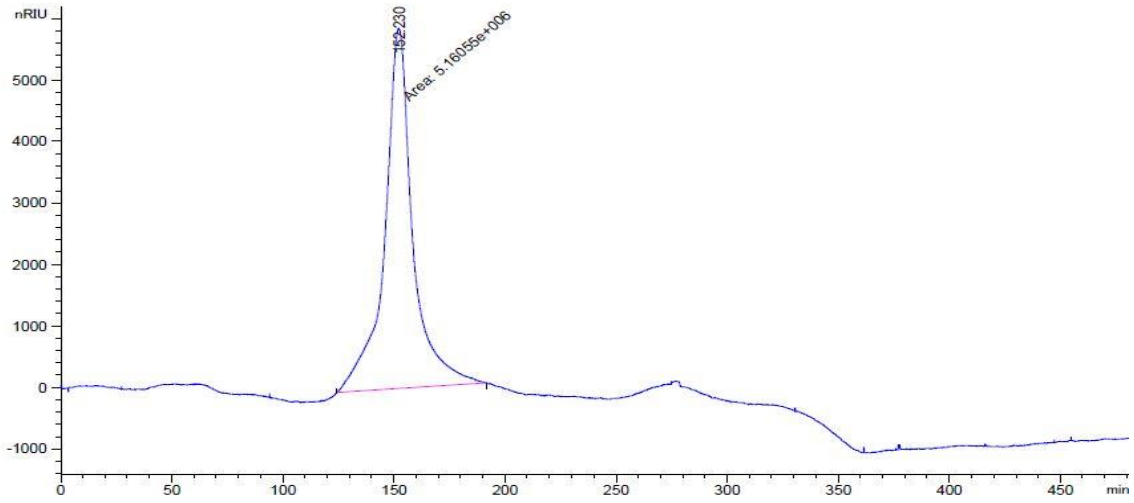


Figure: BSG WS-AX

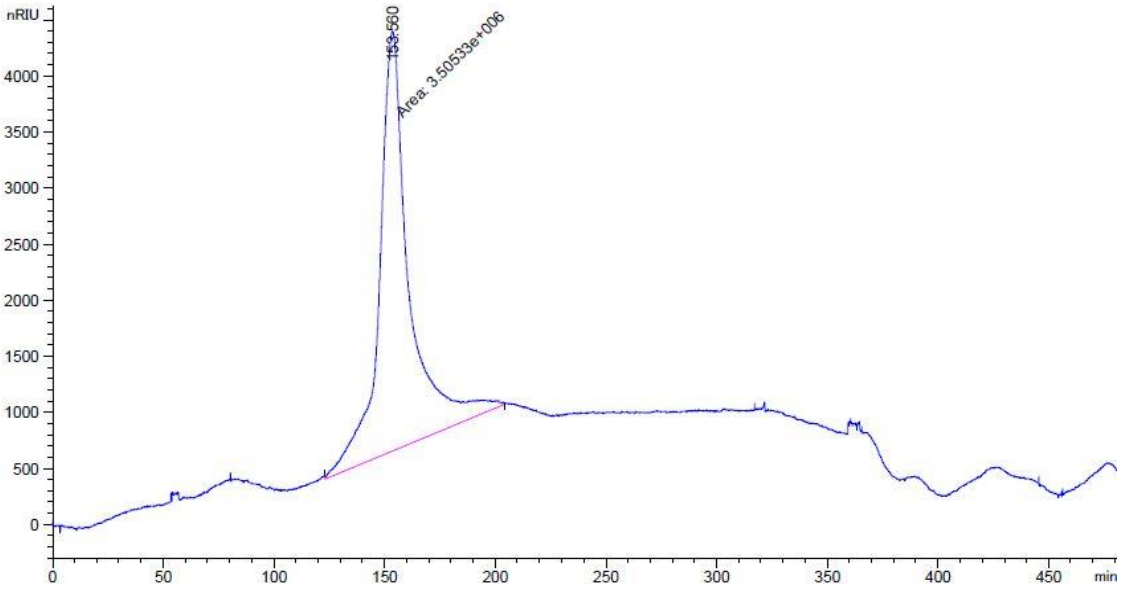


Figure: BSG AS-AX

# Appendix 5: Xylanase hydrolysis

## Xylanase: DYADIC

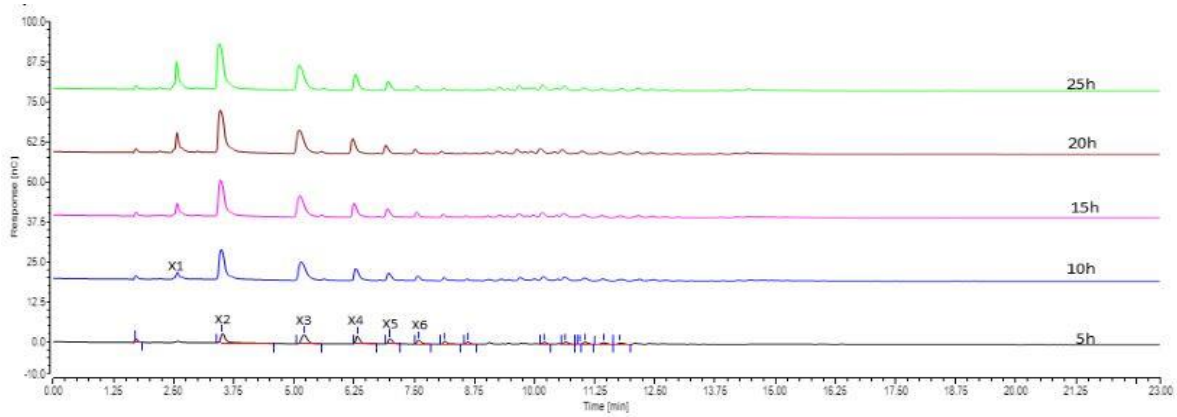


Figure: Hydrolysis of birchwood xylan

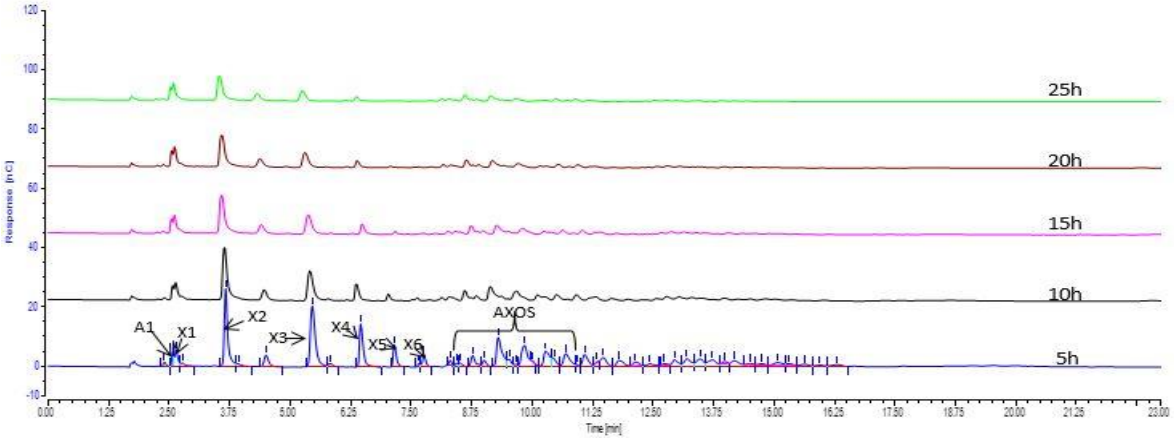


Figure: Hydrolysis of water soluble fraction (BSG WS-AX)

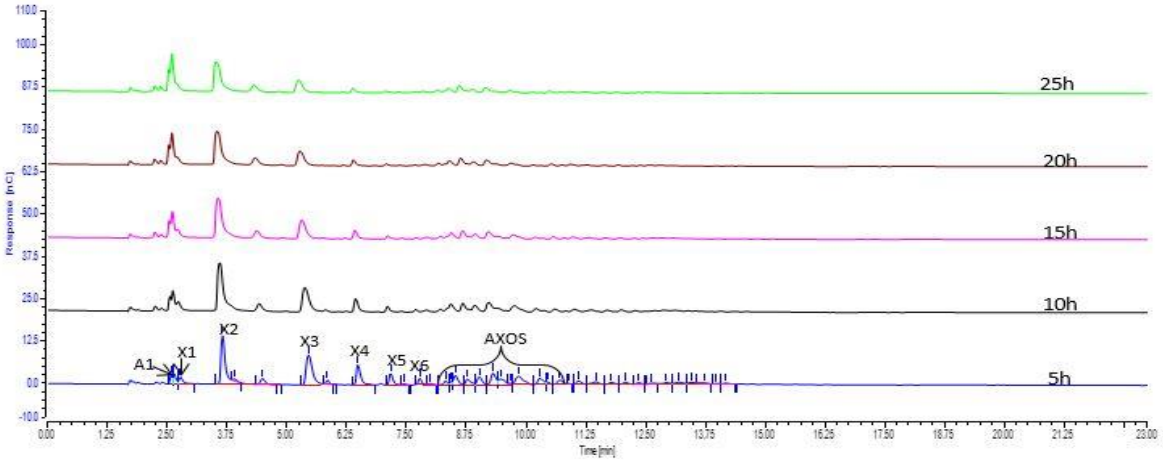


Figure: Hydrolysis of alkali soluble fraction (BSG AS-AX)

Xylanase: BIO-CAT

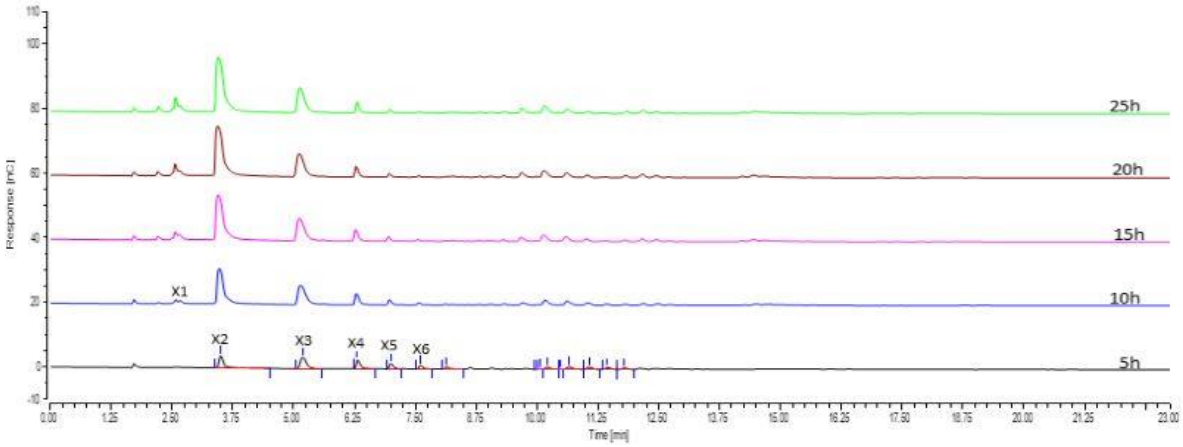


Figure: Hydrolysis of birchwood xylan

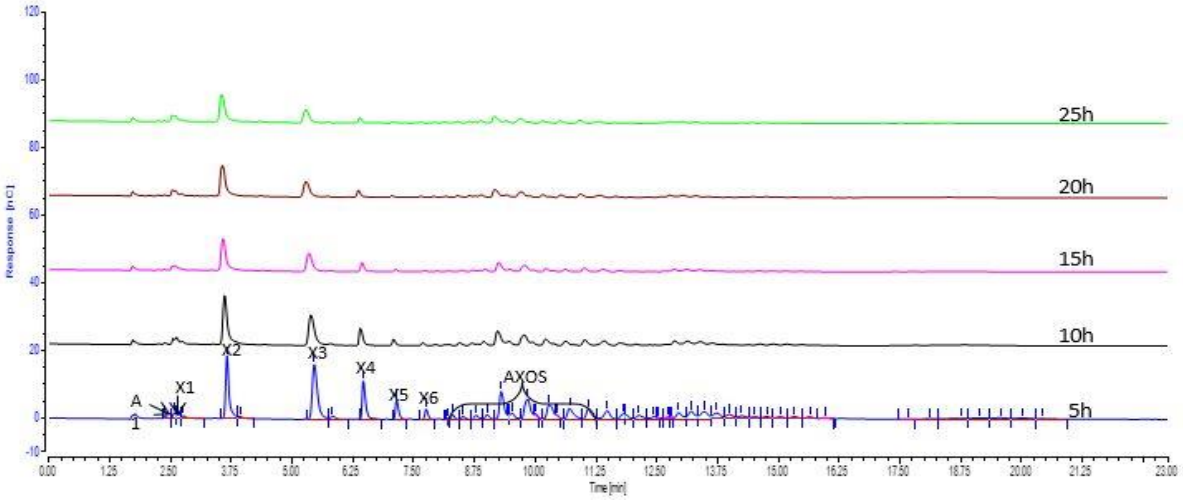


Figure: Hydrolysis of water soluble fraction (BSG WS-AX)

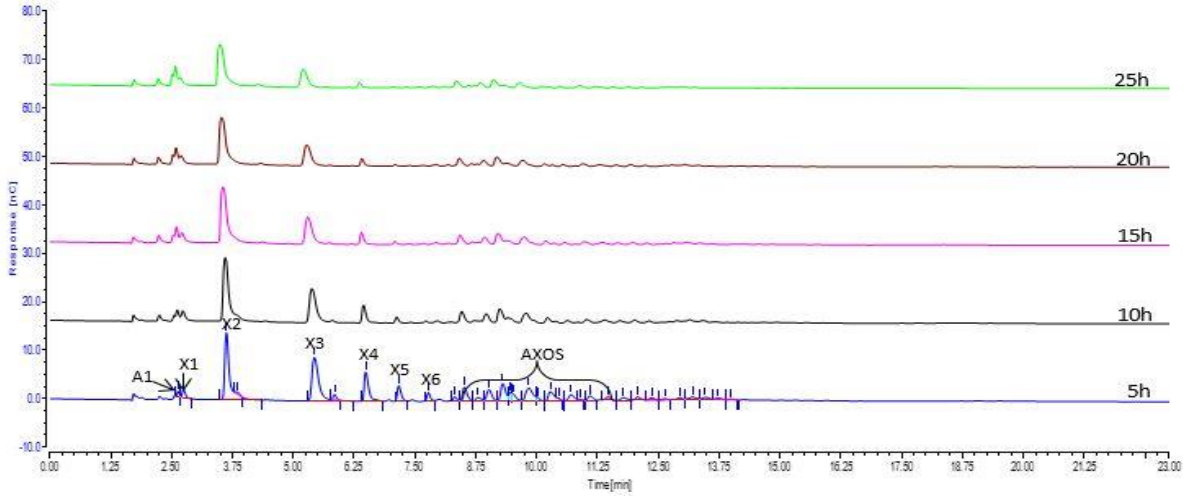


Figure: Hydrolysis of Alkali soluble fraction (BSG AS-AX)

Xylanase: DANISCO Power Bake 7430

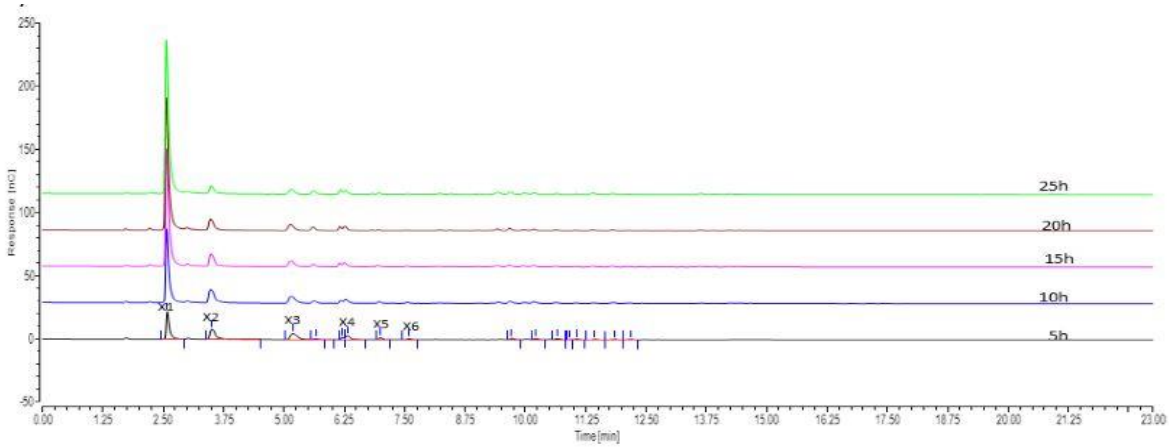


Figure: Hydrolysis of birchwood xylan

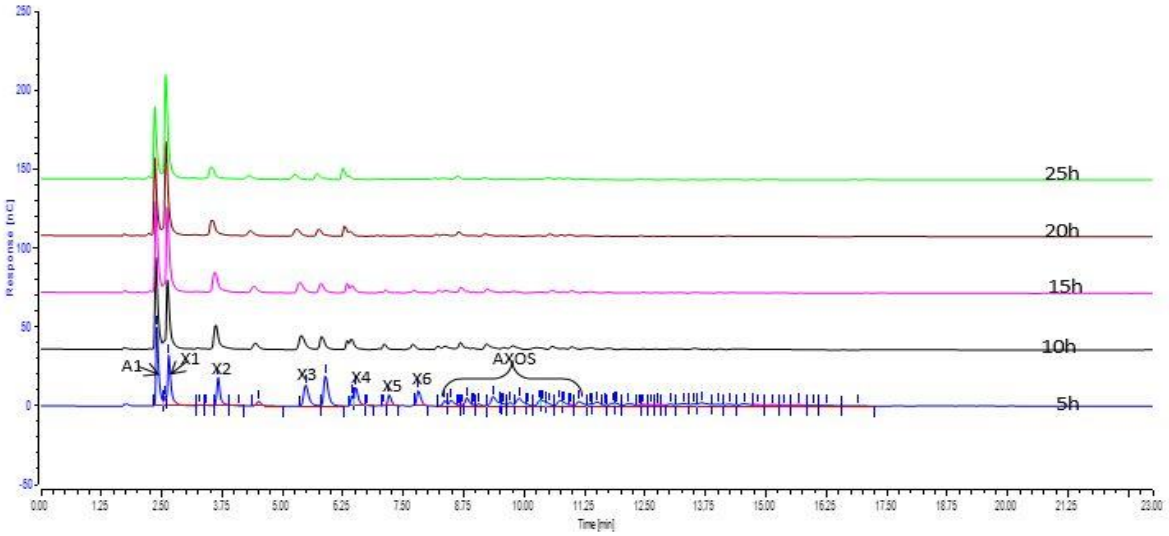


Figure: Hydrolysis of water soluble fraction (BSG WS-AX)

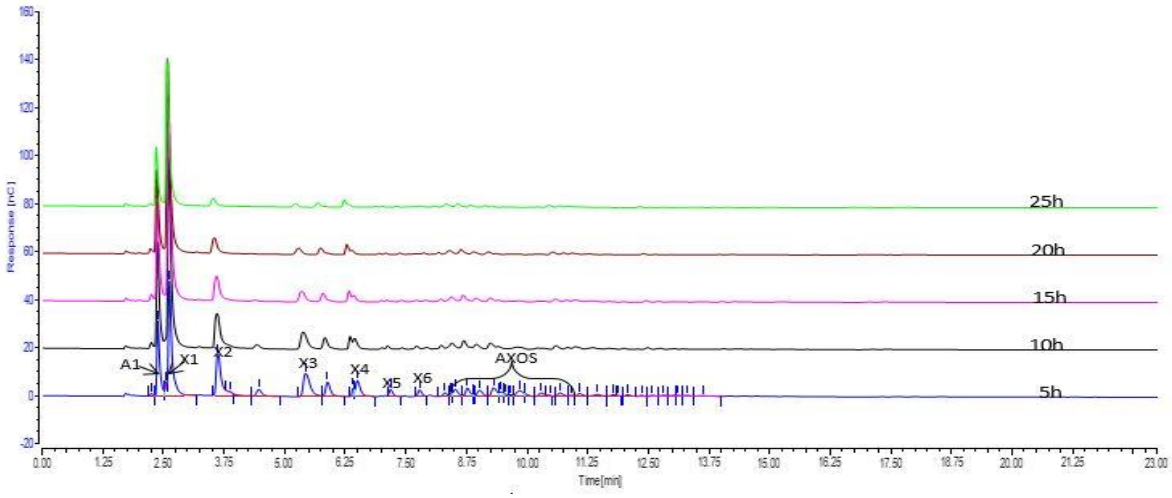


Figure: Hydrolysis of Alkali soluble fraction (BSG AS-AX)

Xylanase: ORBAZIM HC 2500

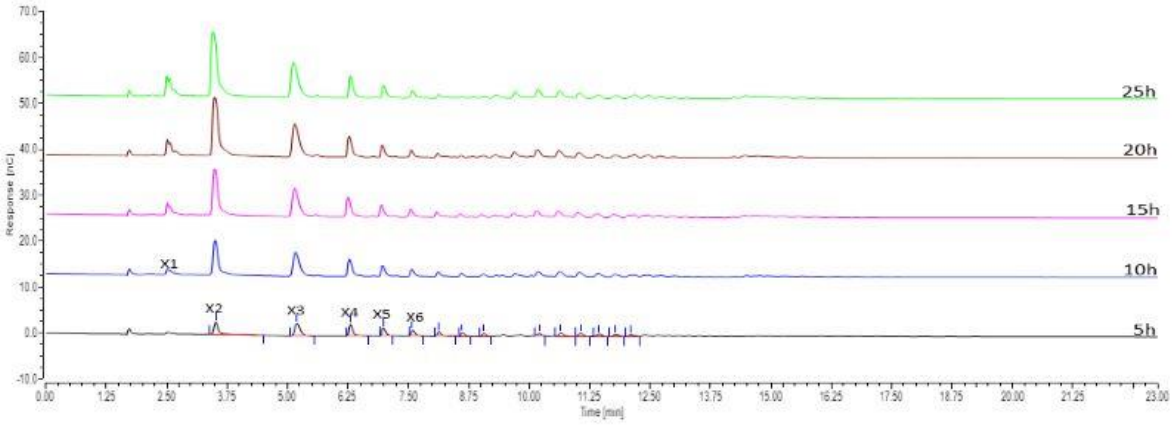


Figure: Hydrolysis of birchwood xylan

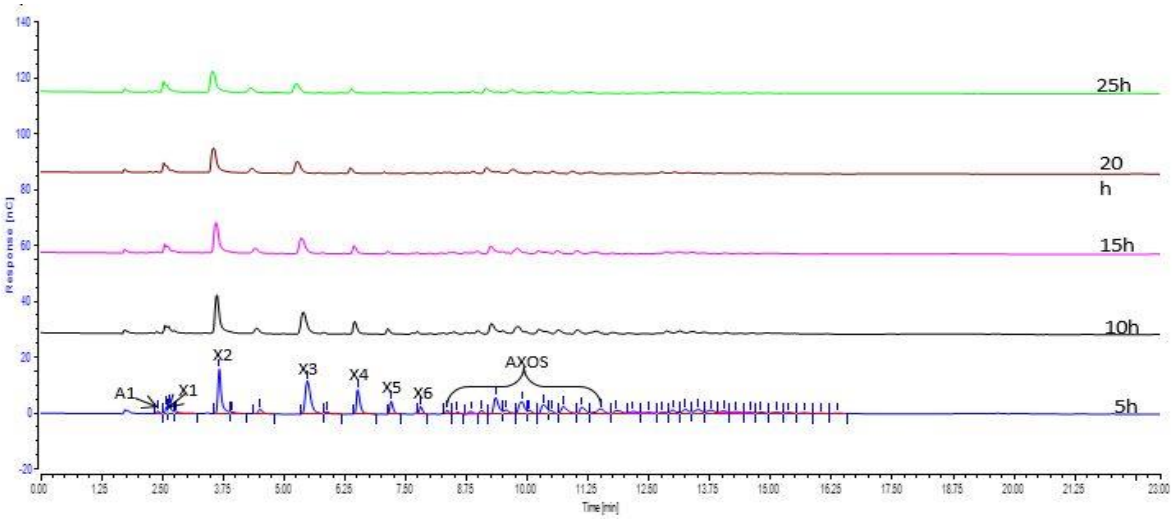


Figure: Hydrolysis of water soluble fraction (BSG WS-AX)

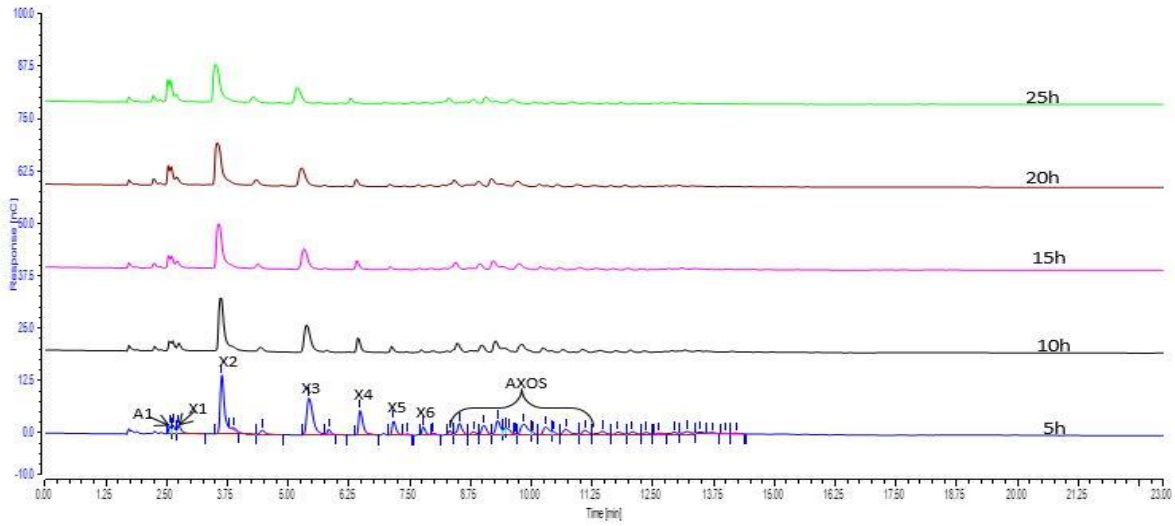


Figure: Hydrolysis of Alkali soluble fraction (BSG AS-AX)

Xylanase: GRIND Amyl H 460

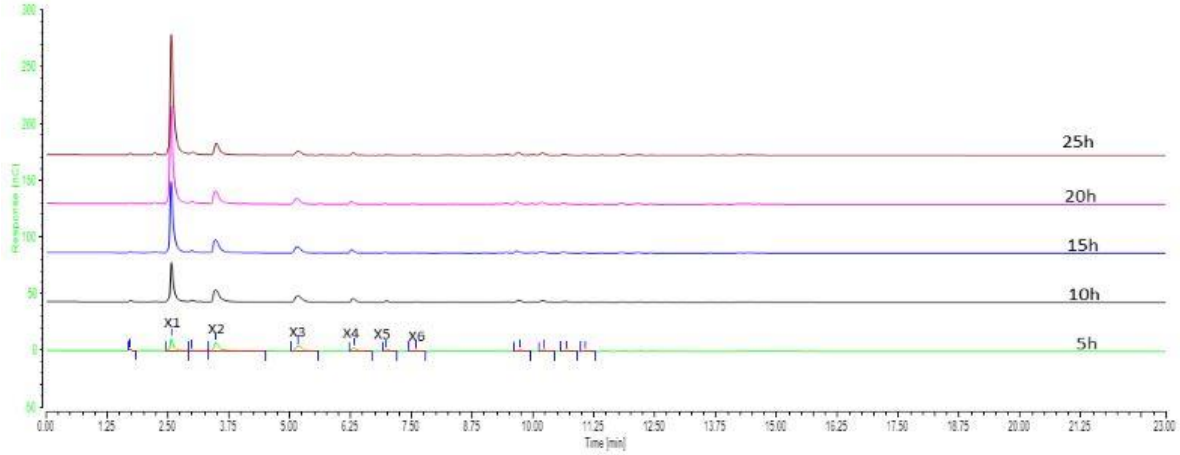


Figure: Hydrolysis of birchwood xylan

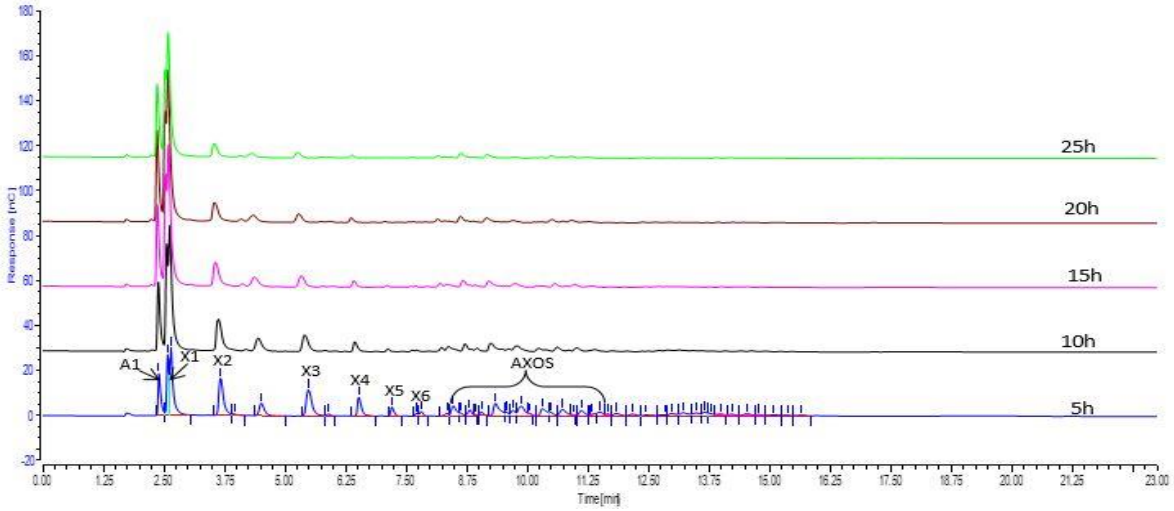


Figure: Hydrolysis of water soluble fraction (BSG WS-AX)

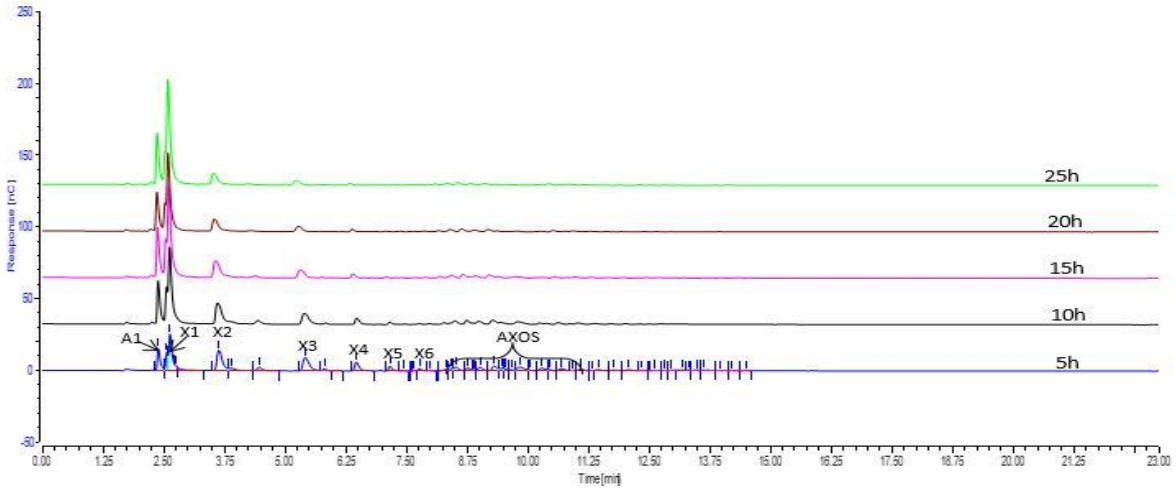


Figure: Hydrolysis of Alkali soluble fraction (BSG AS-AX)

Xylanase: Power Bake 900

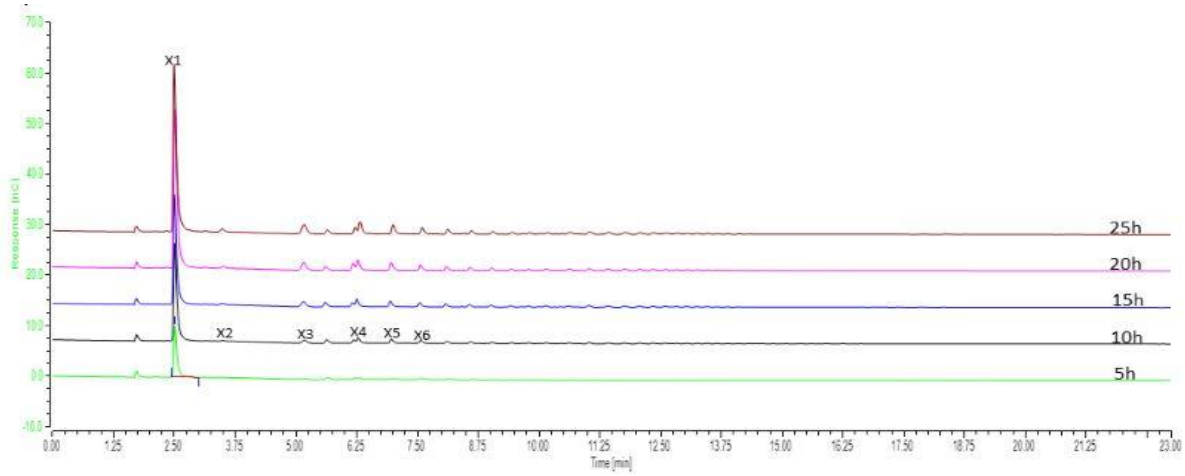


Figure: Hydrolysis of birchwood xylan

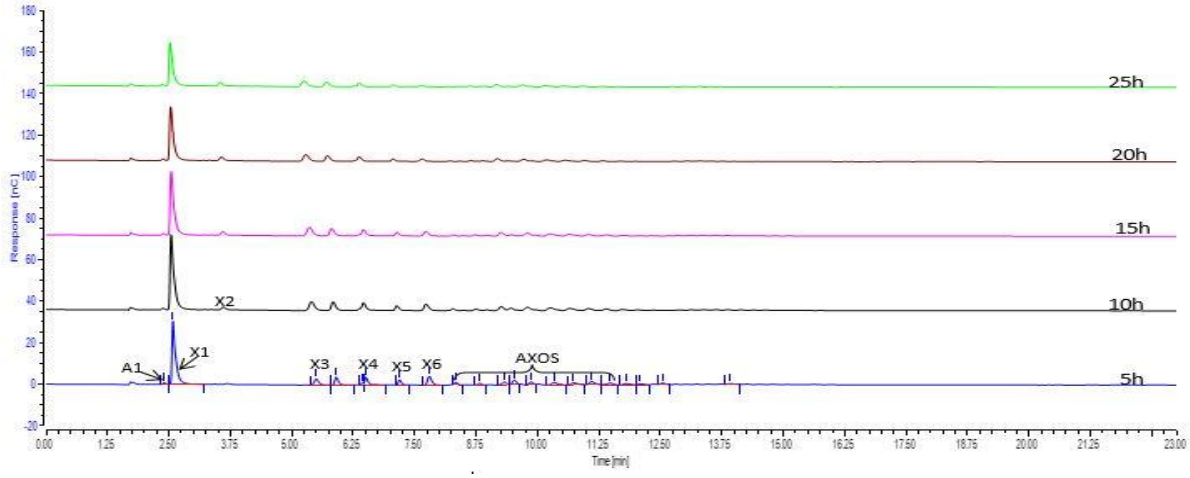


Figure: Hydrolysis of water soluble fraction (BSG WS-AX)

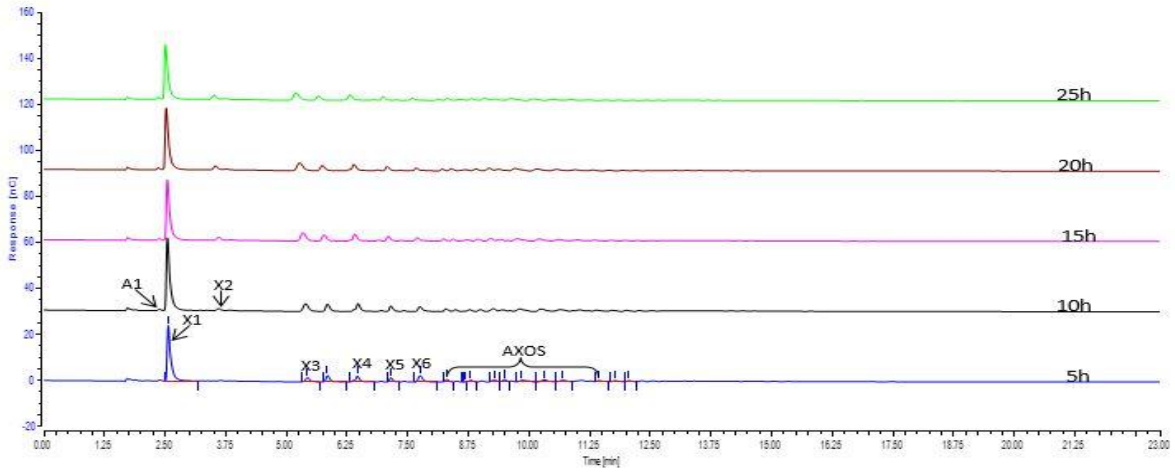


Figure: Hydrolysis of Alkali soluble fraction (BSG AS-AX)

Xylanase: *Rm*Xyn10A

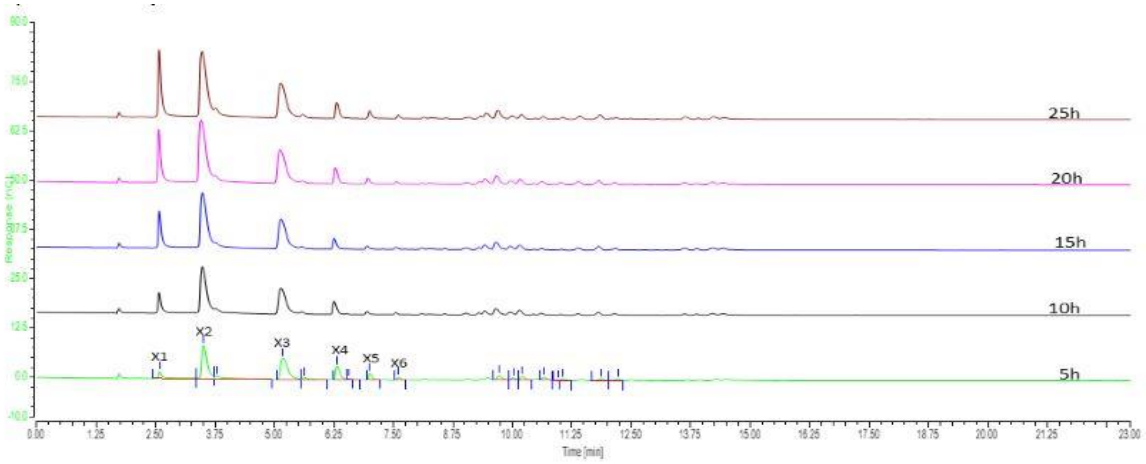


Figure: Hydrolysis of birchwood xylan

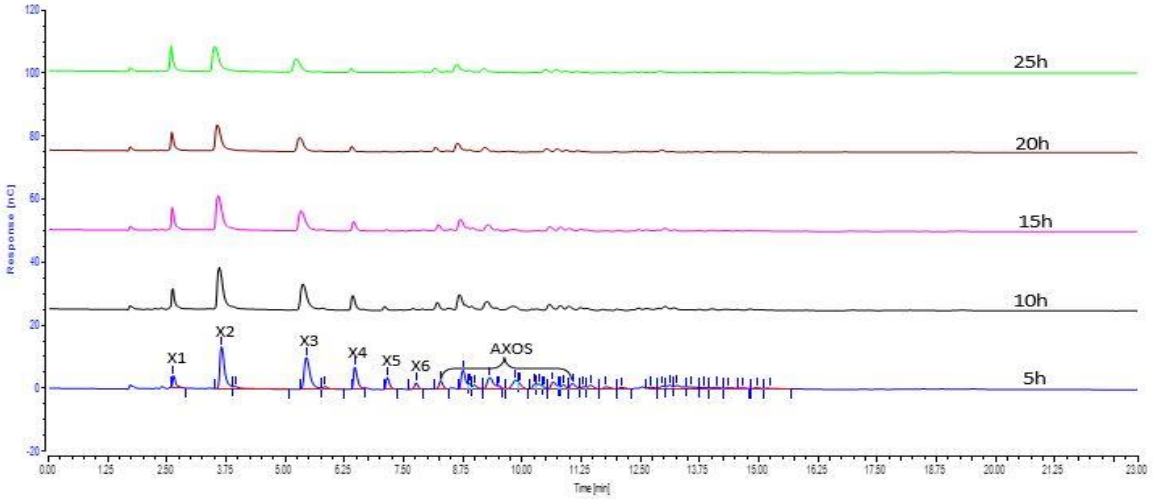


Figure: Hydrolysis of water soluble fraction (BSG WS-AX)

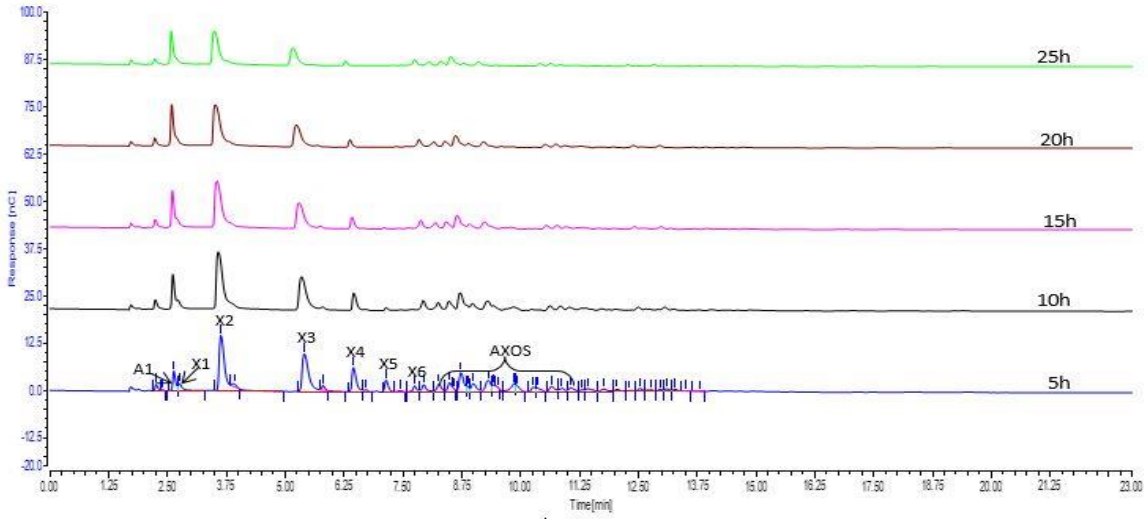


Figure: Hydrolysis of Alkali soluble fraction (BSG AS-AX)

Xylanase: Pentopan Mono BG

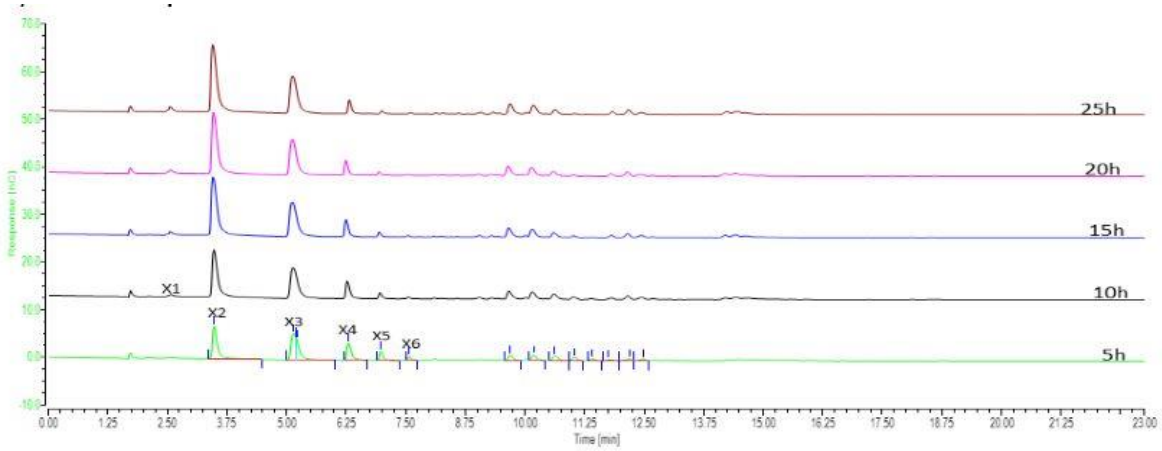


Figure: Hydrolysis of birchwood xylan

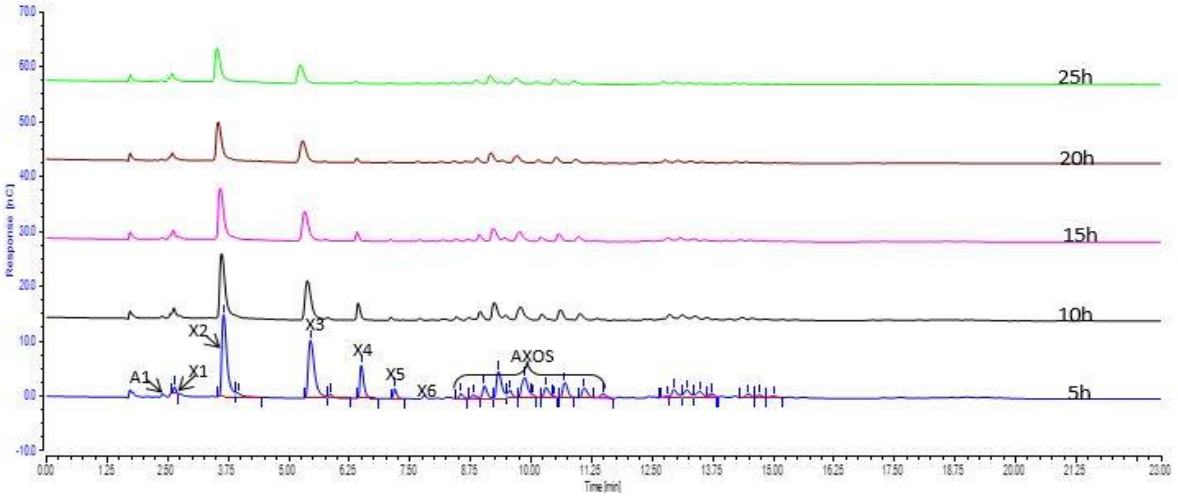


Figure: Hydrolysis of water soluble fraction (BSG WS-AX)

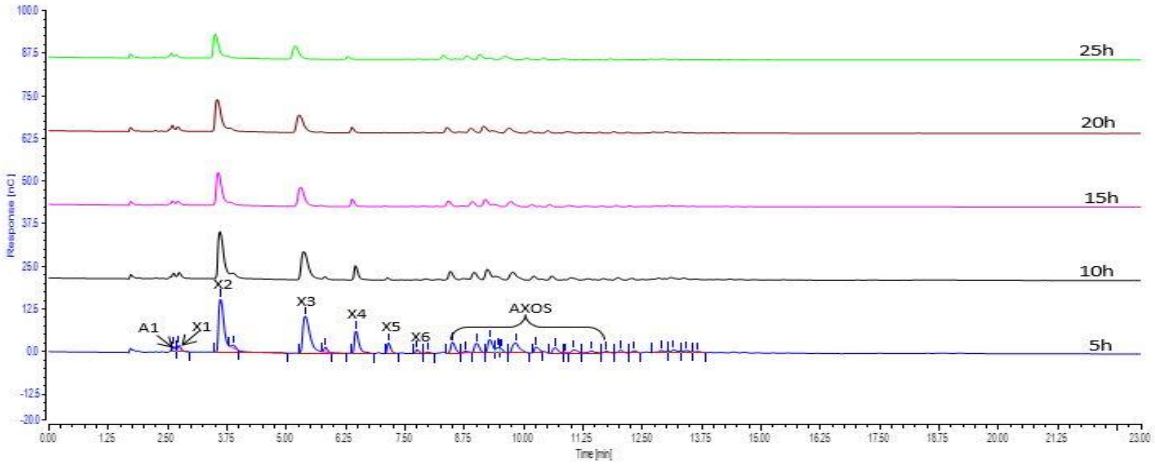


Figure: Hydrolysis of Alkali soluble fraction (BSG AS-AX)

Xylanase: Pentopan 500 BG

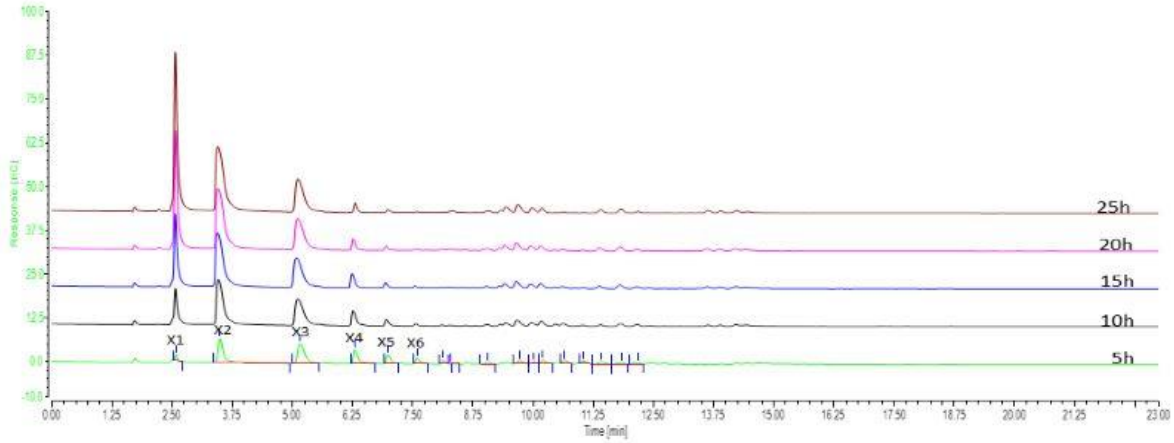


Figure: Hydrolysis of birchwood xylan

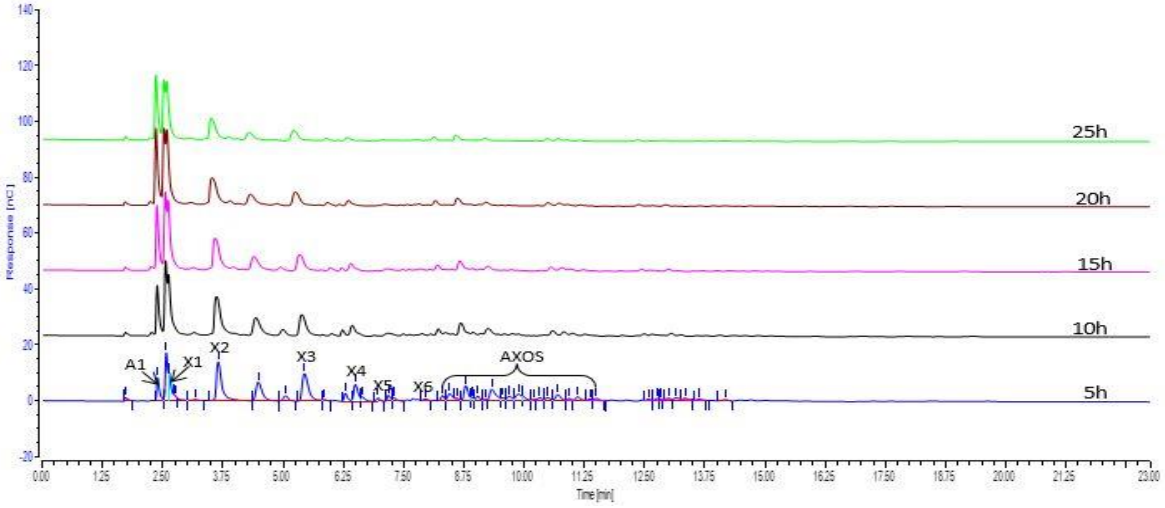


Figure: Hydrolysis of water soluble fraction (BSG WS-AX)

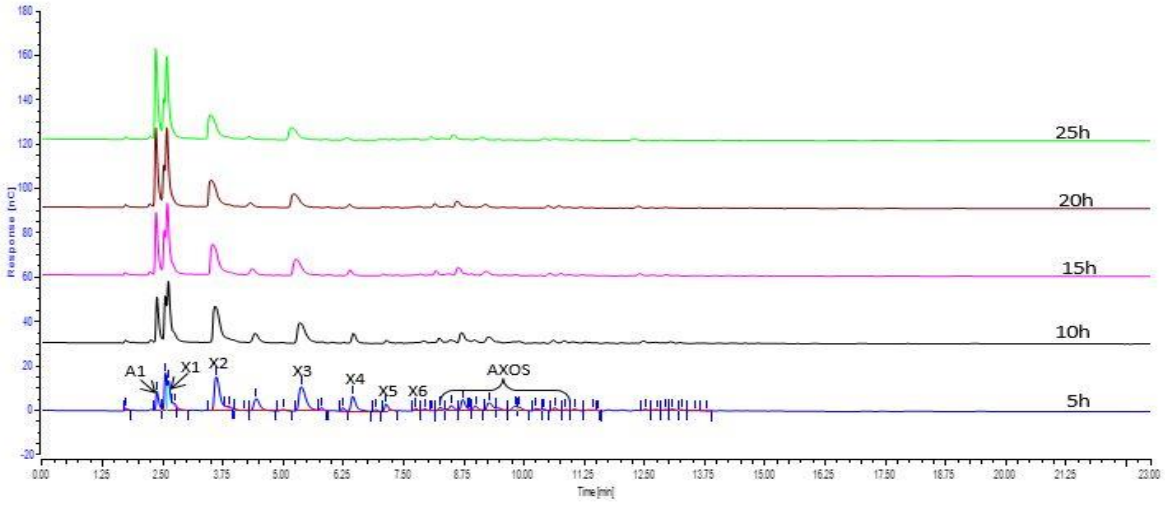


Figure: Hydrolysis of Alkali soluble fraction (BSG AS-AX)