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AMG	Amyloglucosidase
Ara	Arabinose
AX	Arabinoxylan
AXOS	Arabinoxylan-oligosaccharides, XOS with arabinose side group
BglA	Beta-glucosidase
BSA	Bovine Serum Albumin
CM	Catalytic Module
DH	Degree of Hydrolysis
DTT	Dithiothreitol
DW	Dry weight
FL	Full-length
Gal	Galactose
GI	Gastro Intestinal
Glu	Glucose
GRAS	Generally Recognized As Safe
HPAEC-PAD	High Performance Anion Exchange Chromatography with Pulsed Amperometric Detection
KOH	Potassium Hydroxide
LAB	Lactic Acid Bacteria
MWCO	Molecular Weight Cut-Off
OD	Optic Density

OPA	O-phthaldiadehyde
SDS	Sodium Dodecyl Sulphate
SEC	Size Exclusion Chromatography
SCFA	Short Chain Fatty Acids
UV	Ultraviolet light
XOS	Xylan-oligosaccharides
Xyl	Xylose
Xyl2	Xylobiose
Xyl3	Xylotriose
Xyl4	Xylotetrose
Xyl5	Xylose

RESUMO

O presente trabalho teve como objetivo a melhorar e otimizar o processo de extração, separação e purificação e hidrólise de arabinoxilanos, em ingredientes passíveis de incluir em alimentos funcionais, a partir de material vegetal. Este trabalho foi desenvolvido em conjunto com a Lund University, nomeadamente com o Antidiabetic Food Center que tem como principal objetivo o estudo de doenças metabólicas e o desenvolvimento de novos alimentos que vão contribuir para o combate a essas doenças.

Este trabalho incidiu na melhoria do processo de extração de arabinoxilanos (AX) a partir de farinha de centeio, residual da indústria panificadora e a sua posterior hidrólise em xilo-oligosacarídeos (XOS). Investigaram-se então os diferentes aspetos técnicos da extração de fibras alimentares e caracterizaram-se quimicamente os produtos intermediários ao longo dos processos de extração, separação e purificação. As metodologias utilizadas compreenderam vários passos de hidrólise enzimática usando alfa amilases, proteases e xilanase, bem como solubilização das fibras vegetais usando vapor e purificação de biomoléculas através de precipitação com solventes, diálise e liofilização. Foram empregues diferentes técnicas analíticas para seguir os passos de hidrólise enzimática e de separação e purificação e caracterizar as fibras,

Incluindo o Método OPA, hidrólise ácida em conjunto com cromatografia de troca aniónica HPAEC-PAD e cromatografia de filtração-gel (size-exclusion).

Os oligossacarídeos resultantes do produto final são comprovadamente considerados prebióticos e tem-se, numa perspetiva futura, o objetivo de inclui-los em alimentos funcionais. Há evidências de que XOS contribuem, por exemplo, para uma diminuição da incidência de cancro do cólon, diminuição da absorção de colesterol e uma influência positiva em doenças metabólicas como diabetes de tipo II e obesidade.

Para além de melhorar o processo de extração de AX a partir do material vegetal, pretende-se também que o produto final seja o mais puro possível, nomeadamente, com pequenas quantidades de glucose e pretende-se também obter os oligossacarídeos prebióticos.

De entre os principais resultados deste trabalho destaca-se o facto de que a técnica de extração e purificação de AX foi de facto melhorada dado que se obteve um aumento

na presença de AX na constituição do produto final de 37.57% para 60.45% enquanto a razão AX/glucose também aumentou de 1,84 para um máximo de 10.02. Após várias tentativas e combinações de tempo e concentrações enzimáticas, através do uso de xylanase conseguiram também obter-se os oligossacarídeos pretendidos.

Para além da evidente melhoria que se verifica na composição do produto final, o aspeto físico deste também melhora em relação ao anteriormente obtido, uma vez que resulta num produto liofilizado de cor clara que elimina assim a necessidade de um passo adicional de processamento, no caso de este produto vir a ser utilizado como ingrediente prebiótico na indústria alimentar.

Apesar do objetivo do presente trabalho ter sido atingido com sucesso na medida em que, o produto final obtido tem efetivamente uma composição e características físicas melhoradas, haverá sempre necessidade de adaptar os métodos utilizados consoante a escala a que se faz a extração de arabinoxilanos.

Deste trabalho resultam assim conclusões e metodologias que serão utilizadas pelos colegas da Lund University na continuação deste projeto, otimizando o processo utilizando os oligossacarídeos produzidos como substrato de fermentação para bactérias próbioticas tendo, numa perspetiva futura, o objetivo de os introduzir no mercado como ingredientes em alimentos funcionais com o objetivo de combater doenças metabólicas.

Palavras-chave: Alimentos funcionais, arabinoxilanos, xilanase, prebióticos e probióticos.

ABSTRACT

The purpose of this work is to investigate and improve different technical aspects in the extraction of soluble dietary fibres, mostly AX, from rye bran and to characterize the fibres throughout the process, as well as perform enzymatic hydrolysis of arabinoxylans, producing health promoting oligosaccharides: Xylan-oligosaccharides and arabinoxylan-oligosaccharides. These hydrolysis products may serve as candidate supplements in food and feed industry because of their prebiotic proprieties. They can act as substrates which selectively stimulate the growth of probiotic bacteria that produce metabolic active compounds which can contribute, for instance, to lowering risk of colon cancer incidence, decrease cholesterol absorption and might have a positive influence on type II diabetes and obesity.

The work included several enzymatic steps, using alpha-amylases, proteases and xylanses as well as extraction with steam, separation steps and purification using solvent precipitation, dialysis and freeze drying. Different analytical techniques were used to follow the enzymatic steps and to characterize the carbohydrates, including an OPA-based spectrophotometrical method to study the protein hydrolysis, acid hydrolysis in combination with High Performance Anion Exchange Chromatography with Pulsed Ampherometric Detection (HPAEC-PAD) and size-exclusion chromatography (SEC) to study the carbohydrates.

As main results, regarding the extraction final product, a visible improvement in is physical appearance is achieved, as well as a significant improvement in its content in arabinoxylans which increases from 37.57% (using the previous method) up to 60.45%. Regarding the arabinoxylan / glucose ratio, it also improved from 1,84 up to a maximum of 10.02.

The objective of this work was achieved with success as a significant improvement on the product composition and characteristics was achieved as well as the heath beneficial oligosaccharides were obtained, therefore, this work result on a small but good contribution for the project of my colleagues in Lund University.

Key words: Functional foods, arabinoxylan, xylanase, prebiotics and probiotics.

Today's Western world lifestyle based on an energy-dense diet, low in dietary fibre combined with a lack of physical activity, is believed to play a major role in the development of metabolic disorders. The words of Thomas Alva Edison "The doctor of the future will give no medicine, but will have interest in the care of the human frame, in diet and in the cause and prevention of disease" are relevant in the present scenario where each individual desires to live longer in a healthy manner (Aachary, et al., 2011). There is emerging evidence that functional food ingredients can have an impact on a number of gut-related diseases and dysfunctions associated with changing lifestyle and age. Functional food is a food in which a new ingredient has been added, giving a new function to that food. This function is often related to disease prevention and health-promotion and the ingredients added are known as prebiotics.

This investigation is part of a bigger project, held in Lund University by the Antidiabetic Food Centre with the overall aim to develop novel innovative food concepts and processes which reduces risk for obesity, type II diabetes and other metabolic diseases. In this context, rye bran will be process to extract and recover purified arabinoxylans which, after enzymatic hydrolysis lead to the production of health beneficial oligosaccharides - prebiotics.

RYE BRAN AS RAW MATERIAL

The xylan-type polysaccharides are known to occur in several structural varieties in terrestrial plants and algae and, even in different plant tissues within one plant the structure can differ (Ebringerova, et al., 2000). It is commonly present in high quantities in cereal crops.

Rye (*Secale cereale L.)* bran was chosen as raw material for the extraction of arabinoxylans as rye is one of the cereal grains richest on water extractable arabinoxylans (Ward, et al., 2008) as seen of Figure 1.

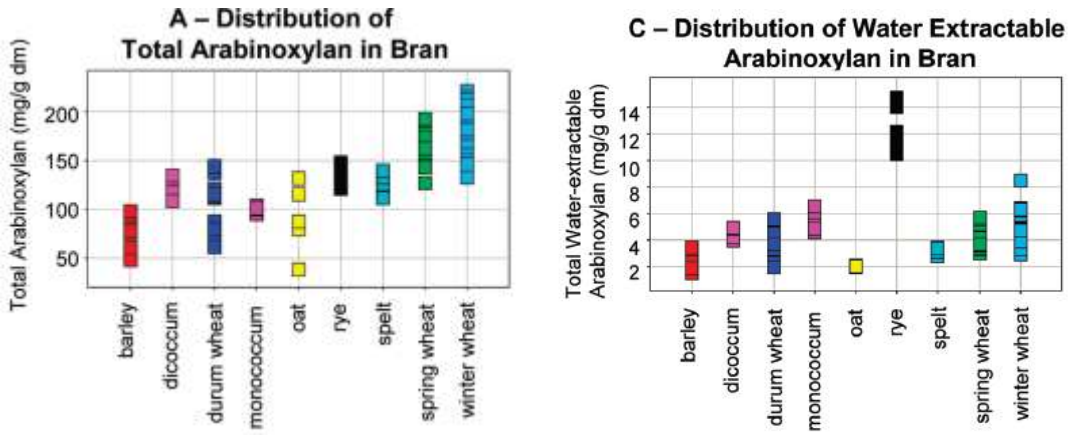

Figure 1 – Distribution of arabinoxylan and water extractable arabinoxylan in different cereal grains bran (Ward, et al., 2008).

In this work, rye bran used as raw material is a side stream from bread making industry.

This raw material was also chosen as it is an especially important cereal crop in the eastern and northern European countries. From an agronomic point of view, rye is the most widely adapted of the cereals because of its extreme winter hardiness and ability to grow on very marginal soils. Typical food applications of rye are the production of bakery products breakfast cereals, and alcoholic beverages. (Nyström, et al., 2008).

The data for 2008 for worldwide production of rye as 17.7 million tonnes (92% produced in Europe, mainly in Nordic countries) with a 20% increase in respect to 2007 (Gullón, et al., 2010). In parallel, huge amounts of low added value by-products (such as straw, bran, hulls or stalks) are generated. These by-products can be used as feedstock for a variety of industrial processes based on the biorefinery approach. Development of biorefineries represents the key for the access to an integrated production of food, feed, chemicals and others, in the future (Gullón, et al., 2010).

In the recent years a discussion arises about the new competition for food markets due to the energetic use or biorefinery of raw material. Besides some ethical concerns, it might be the most comprehensible way from an economic point of view if the focus is set now on the conversion of vegetal waste material that was up to now without any further. In this case, the relative increase in new created value is the highest. As a consequence, crop residues are considered to be one of the most reasonable sources for biorefineries of the second generation (Sedlmeyer, 2011).

AX AND AXOS

Arabinoxylan consist of β-(1,4)-linked D-xylopyranosyl residues to which α-L-arabinofuranose units are linked as side chains (Grootaert, et al., 2007). AX is a type of hemicelullose which is the second most abundant polysaccharide found in nature (Saha, 2003). AX is the main non-starch polysaccharide of cereal grain such as rye, wheat, rice and barley, among others.

Hemicelluloses are non-cellulose and non-starch polysaccharides that exist in both primary and secondary walls of higher plants in association with lining and cellulose (Andersson, 2007) In recent years, bioconversion of hemicelluloses has received much attention because of its practical applications in various agro-industrial processes, such as efficient conversion of hemicellulosic biomass to fuels and chemicals, delignification of paper pulp, digestibility enhancement of animal feedstock, clarification of juices, and improvement in the consistency of beer (Saha, 2003). In this context, the most interesting application is the production of heath promoting oligosaccharides from AX. Besides, AX is present in side-streams from agricultural and forest industry (Andersson, 2007), for instance bread making industry, which make them more appealing from an economical point of view.

XYLAN-OLIGOSACCHARIDES AND ITS PROPRIETIES AS PREBIOTICS

Xylan-oligosaccharides, as an emergent prebiotic have great potential and can be incorporated into many food products. Several studies indicate that XOS seem to exert their nutritional benefits in various animal species, which have an intestinal tract populated by a complex, bacterial intestinal ecosystem.

Oligosaccharides have many beneficial physiological properties, including protection against colon cancer by forming short-chain fatty acids (SCFA) in the large intestine during fermentation and a prebiotic effect promoting the growth of beneficial intestinal bacteria (*Bifidobacterium* and *Lactobacillus*). Arabinoxylan (AX) derived arabinoxylan-oligosaccharides (AXOS) and xylan-oligosaccharides (XOS) are non-digestible oligosaccharides but are fermented in the large intestine by the intestinal flora (Makaravicius, et al., 2012).

In recent years, attention has been paid to the nutritional properties of hydrolytic degradation products of AX, the arabinoxylan-oligosaccharides and the non-substituted xylan-oligosaccharides (Broekaert, et al., 2011) (both can be referred as (A)XOS). The Figure 2 represents a model for the key microbiological and metabolic events in the human colon upon ingestion of either AX or its hydrolysis products.

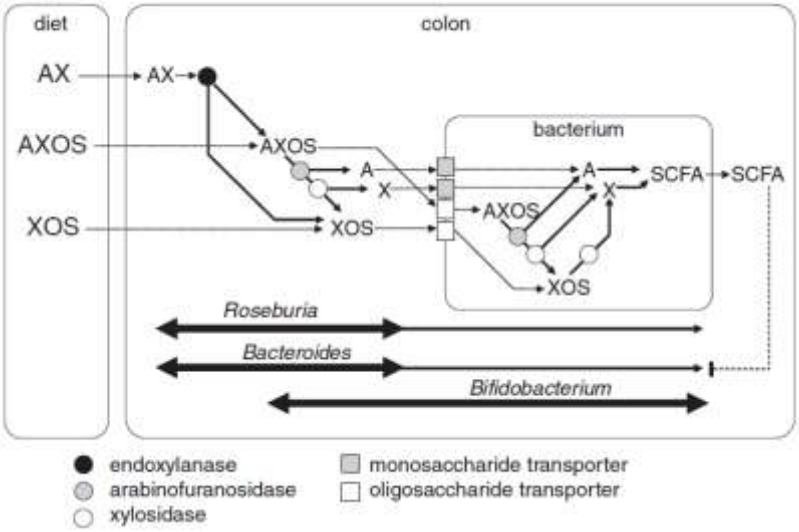

Figure 2 - Microbiological and metabolic events in the human colon upon ingestion of either AX or its hydrolysis products. (Broekaert, et al., 2011)

Prebiotics

Prebiotic was first defined as a "non-digestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon, and thus improves host health". Many food oligosaccharides and polysaccharides (including dietary fiber) have been claimed to have prebiotic activity, but not all dietary carbohydrates are prebiotics(Gibson, et al., 2004). Typically, prebiotics are carbohydrates (such as oligosaccharides), but the definition may include non-carbohydrates. According to the bibliography (Aachary, et al., 2011), such classification requires a scientific demonstration that the ingredient:

1. Resists gastric acidity;
2. Is not hydrolyzed by gastro intestinal (GI) tract enzymes;
3. Is not absorbed in the upper GI tract;
4. Is fermented by intestinal microorganisms;
5. Induces selective stimulation of growth and/or activity of intestinal bacteria, potentially associated with health and well-being.

Prebiotics have the potential to considerably influence the physiology of the whole body and, consequently, health and well-being. Because prebiotics specifically and selectively affect the gut microflora, their importance is likely to become greater and greater.

Probiotics

The microorganisms in gastro intestinal tract that that exerts health-beneficial effects to their host are known as probiotics. It is important that prebiotic stimulate selectively the gut microbiota and therefore, reduce pathogens as *Escherichia coli* and act as a substrate to grow bacteria as lactobacilli and bifidobacteria, well recognized as the representative genera of probiotics.

Lactic acid bacteria (LAB) are defined as bacteria which are able to convert carbohydrates into carboxylic acids, mainly lactic acid. LAB are subdivided into two groups to their carbohydrate catabolism pathways, the subgroup includes homofermentative LAB and heterofermentative LAB. The key characteristic to differentiate between these two groups of LAB are the ability to ferment carbohydrate sources (hexose and pentose sugars) and to produce different end-products. Homofermentative LAB selectively ferments only hexose sugars, mainly glucose, via the Embden-Meyerhof-Parnas (EMP) glycolysis pathway. Heterofermentative LAB are able to utilized hexose and pentose sugar molecules via phosphoketolase (PK) pathway.

Fermentation of prebiotics by colonic bacteria gives rise to production of short chain fatty acids such as acetate, propionate, butyrate, and lactate, which act as electron sinks of respiration in the anaerobic environment of the gut. The presence of short chain fatty acids (SCFA) in the intestines contributes to a lower pH, a better bio-availability of calcium and magnesium, and inhibition of potentially harmful bacteria (Broekaert, et al., 2011). The modulating effects on intestinal microbial populations exerted by prebiotics are, by themselves, not a proof for health beneficial effects, except if such modulations involve a reduction of the number of pathogenic bacteria and the detrimental effects they cause.

Among the biological proprieties of (A)XOS, the most important are:

- Immunomodulatory activity;
- Anti-cancerous activity;
- Anti-microbial activity;
- Effects on stool frequency and stool consistency;
- Effects on lipid metabolism, glucose metabolism, and metabolic disorders;
- Antioxidative activity (conferred by phenolic substituents).
-

Several authors attributed other effects to (A)XOS, including anti-allergy, anti-infection and anti-inflammatory properties and a variety of other properties. These properties are mainly attributed to acidic oligosaccharide containing uronic acid substituents, which can be produced from hardwoods by a combination of enzymatic and/or chemical treatments. Besides biological effects concerning human health, XOS have been employed for phyto-pharmaceutical and feed applications (Aachary, et al., 2011). Figure 3 sums up and represents the potential health benefits of prebiotics.

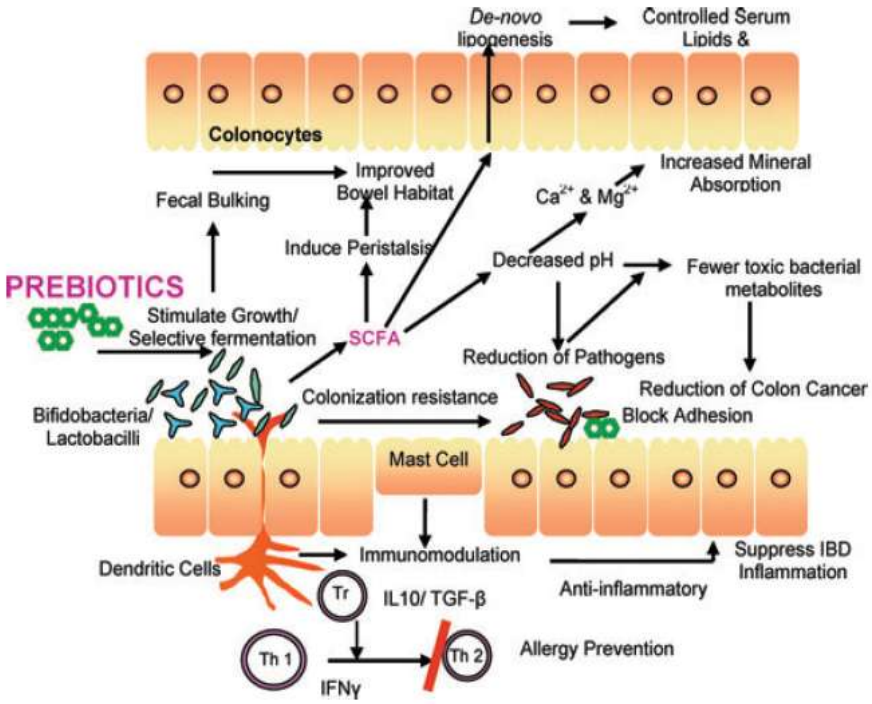

Figure 3 – Potential health benefits of prebiotics (Aachary, et al., 2011).

AX EXTRACTION, PURIFICATION AND HYDROLYSIS INTO OLIGOSACCHARIDES

The extraction and isolation procedures will determine outcome and quality of the final isolated material.

AX Extraction

(A)XOS can be generated in the colon of animals by microbial degradation of AX, or can be present in processed food products, or can be prepared and purified from AX-rich sources and used as a food ingredient (Broekaert, et al., 2011).

Hemicelluloses are non-cellulose and non-starch polysaccharides that exist in both primary and secondary walls of higher plants in association with lining and cellulose. Cellulose it's the most abundant plant component and it's largely used in industry. Hemicelluloses, which are the second most abundant polisschacarides in nature (Saha, 2003), don't have the same degree of utilization. Billions of tonnes of lignocellulosic material are produced every year by plants, offering a tremendous source of raw material. In this context, the development of methods for the isolation and modification of hemicelluloses present in side-streams from agricultural and forest industry, is especially interesting (Andersson, 2007).

In recent years, bioconversion of hemicelluloses has received much attention because of its practical applications in various agro-industrial processes, such as efficient conversion of hemicellulosic biomass to fuels and chemicals, delignification of paper pulp, digestibility enhancement of animal feedstock, clarification of juices, and improvement in the consistency of beer (Saha, 2003). Another application is the production of heath promoting oligosaccharides from arabinoxylans. However, hemicelluloses are tightly connected with the other components of cell wall (cellulose and lignin), and they will have considerable impact on each other during the extraction and isolation. Figure 4 represents a model of a cell wall.

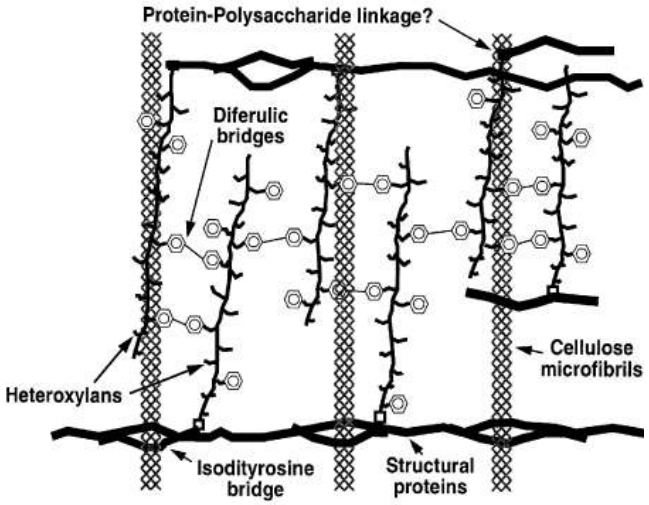

Figure 4- Model of a corn fiber cell wall (Saha, 2003).

Today there are plenty of research groups dedicated to the extraction of AX from plant material with different approaches. So, there are a wide range of methods for the extraction and isolation of AX.

In the extraction step, the major challenges are the possible physical associations and covalent linkages between hemicelluloses, lignin and cellulose.

In the following table there is a resume of the extraction methods used nowadays. Each procedure has advantages and disadvantages.

Table I - Procedures used to extract hemicelluloses from plant material. (Saha, 2003).

METHOD	EXAMPLE
Thermo-mechanical	Grinding, milling, shearing, extruder
Autohydrolysis	Steam pressure, steam explosion, supercritical carbon dioxide explosion
Acid treatment	Dilute acid (H_2SO_4, HCl), concentrated acid (H_2SO_4, HCl)
Alkali treatment	Sodium hydroxide, ammonia, alkaline hydrogen peroxide
Organic solvents treatment	Methanol, ethanol, butanol, phenol

In addition to the examples from Table I there are other procedures such as wet oxidation, microwave irradiation or even application of ultrasound.

Many of the extraction methods such as microwave irradiation (Roos, et al., 2009) and steam extraction are variants of hydrothermolysis. Steam extraction seems to be the

most popular procedure, using an autoclave or a reactor. By aqueous processing of xylan-containing raw materials (autohydrolysis or hydrothermal treatment) the hemicellulosic chains are progressively broken down by the hydrolytic action of hydronium ions (generated from water by autoionization and from *in situ* generated organic acids), yielding soluble products, and leaving both cellulose and lignin in solid phase with little chemical alteration. In the present case, an autoclave was used to extract arabinoxylans by autohydrolysis.

AX Purification Methods

In order to purify and concentrate the extracted hemicelluloses there are several methods that can be applied. The most popular procedures are membrane filtration, size exclusion chromatography (gel filtration), ion exchange chromatography and precipitation with ammonium sulphate or ethanol.

In the present case, several enzymatic steps were followed, using α-amilases, proteases and xylanses as well as separation steps and purification using solvent (ethanol) precipitation, dialysis and freeze drying for instance.

Solvent extraction is useful for removing monosaccharide components of autohydrolysis liquors yielding both a selectively refined aqueous phase and a solvent-soluble fraction mainly made up of phenolics and extractive-derived compounds. The best purification effects were achieved with ethanol, but the process showed limited recovery yields (Aachary, et al., 2011). Dialysis can be used as an alternative to precipitation in order to remove monosaccharides.

AX Hydrolysis into (A)XOS

The health beneficial oligosaccharides are obtained by hydrolysis of AX which almost invariably leads to a mixture of unsubstituted XOS and arabinose substituted AXOS. To produce (A)XOS with chemical and enzymatic methods, AX is generally extracted with alkali, such as KOH or NaOH and extracted AX is converted into (A)XOS by a xylanase enzyme having low exo-xylanase and/or *β*-xylosidase activity.

Xylanases catalyze the hydrolysis of xylan. The use of these enzymes could greatly improve the overall economics of processing lignocellulosic materials for the generation of liquid fuels and chemicals and, in this case ingredients to introduce into

functional foods. Xylanases are found in a cornucopia of organisms and the genes encoding them have been cloned in homologous and heterologous hosts with the objectives of overproducing the enzyme and altering its properties to suit commercial applications. Sequence analyses of xylanases have revealed distinct catalytic and cellulose binding domains, with a separate non-catalytic domain that has been reported to confer enhanced thermostability in some xylanases (Kulkorni, et al., 1999).
In this case, Xylanase Xyn10A from *Rhodothermus marinus* (Eva Nordberg Karlsson, 1997) was used, full-length and the isolated catalytic module, to break down AX into oligosaccharides.

Analysis methods

Different analytical techniques were used to follow the enzymatic steps and to characterize the carbohydrates, including an OPA-based spectrophotometrical method to study the protein hydrolysis, acid hydrolysis in combination with High Performance Anion Exchange Chromatography with Pulsed Amperometric Detection (HPAEC-PAD) and size-exclusion chromatography (SEC) to study the carbohydrates.
Size exclusion chromatography of wood pulp polysaccharides (cellulose and hemicelluloses) has become widely used for the analysis of these polymers including monitoring of transformations occurring under various chemical and biological processes (Eremeeva, 2003) and, in this case, it is used in order to monitoring the hydrolysis of arabinoxylans by the xylanase and, therefore, the production of the health beneficial oligosaccharides.
Other than SEC and HPAEC, NMR and mass spectrometry using MALDI-MS are techniques usually used for this purpose (Aachary, et al., 2011).

(A)XOS APPLICATIONS

The most important applications of XOS in terms of current and potential market demand correspond to ingredients for functional foods (Aachary, et al., 2011). Some examples are in Table II.

Table II - Possible foodstuffs that can be fortified with prebiotics. (Gibson, et al., 2004)

Dairy products
Beverages and health drinks
Spreads
Infant formulae and weaning foods
Cereals
Bakery products
Confectionery, chocolates, chewing gum
Savoury products, soups
Sauces and dressings
Meat products
Dried instant foods
Canned foods
Food supplements

As the main application of (A)XOS is their integration in functional foods, there should be a special concern about the color and odor proprieties of this product.

(A)XOS can also be integrated on animal feeds and pet foods for similar purposes.

Growth regulatory activity of XOS in aquaculture and poultry is another area of application (Aachary, et al., 2011).

INITIAL METHOD AND IMPROVEMENTS

As said before, this experimental work aims to improve the extraction of arabinoxylans from rye bran (Lilla Harrie Valskvarn AB, the Pågen group, Sweden). It is necessary to test the existing method to begin. The procedure described in Appendix I was repeated: extraction of soluble dietary fibres from rye bran by pressure boiling followed by an amylase treatment with α-amylase (from *Bacillus licheniformis* 3000U/mL. *Megazyme*, Wicklow, Ireland), purification by ethanol precipitation and finally, freeze drying. In parallel with this experiment the effect of freezing the soluble extracts after steam treatment, using another amylase (from *Bacillus Amyloliquefaciens*, ≥250U/mL. *Sigma-Aldrich*, St. Louis, Missouri, USA) and the efficiency of ethanol precipitation when compared with dialysis was tested.

This method, as well as all the other is schematically represented in Appendix I.

PRESSURE BOILING

On this first step, the aim is to extract the soluble fibers from the plant material by autohydrolysis under pressure boiling conditions at 121°C inside an autoclave (Thermo Fisher Scientific Inc., Massachusetts, USA).

The first sample was 200mL of 10% DW rye bran in deionized water. The autoclave time was 15h (Falck, 2010).

See Appendix II to further data.

PRIMARY RECOVERY

In order to remove all the solids, the sample was centrifuged at 3000rpm for 30 minutes and then vacuum filtered (Piab, Stockholm, Sweden).

The measured pH of the extract (Mettler Toledo, Ohio, USA) was 5.03, lower than the initial neutral value, before pressure boiling. The pH was adjusted to 6.8, a pH value acceptable for both enzymes in the next step.

Of the 96 mL of extract obtained, 10 aliquots of 8 mL each were divided to use in this experiment.

Two samples were frozen in order to test the amylase treatment after freezing the extract for one the next day.

AMYLASE TREATMENT

In this step, two different enzymes were tested. The initial method used the α-amylase from *Megazyme*, in order to improve the starch hydrolysis, an α-amylase from a different source was also tested, this one supplied by *Sigma-Aldrich*. All samples, except the ones used as *Blank* were amylase treated.

Each sample was treated with 50µL of enzyme (12.5U of *Sigma-Aldrich* α-amylase and 150U of *Megazyme* α-amylase) and incubated at 70°C in a water bath (LAUDA, Germany) for 30 minutes, agitating with a vortex mixer (Scientific Industries, New York, USA) every 5 minutes. The samples were cooled down on the bench before the next steps.

ETHANOL PRECIPITATION

Ethanol precipitation was performed in order to precipitate the polysaccharides and leave the monosugars soluble.

All the samples, except *D-Megazyme* were subjected to this step.

The precipitation condition was 1:4 ethanol 95% (v/v) (Solveco, Kędzierzyn-Koźle, Poland). So, 32mL of ethanol were added to each sample. The precipitate was separate from the supernatant by centrifugation for 30 minutes at 3000 rpm. The supernatant was discarded. Then, the samples were solubilized again in 20mL of deionized water in a hot water bath.

DIALYSIS

Dialysis was used as an alternative to ethanol to separate mono and polysaccharides. In this case, the membrane used had a MWCO of 3500 (SpectumLab, California, USA), so all the monosugars and other relatively small contaminants will pass through and the big polysaccharide chains will remain inside the membrane.

Each of the 8mL sample (*D-Megazyme*) were left overnight in 4L of deionized water. The water was changed two times ($2.5E^5$ times dilution in the end). In the afternoon the samples were transferred into falcon tubes with approximately with deionized

water until obtain a mixture of approximately 20mL. The samples were then kept in the freezer (-20°C).

FREEZE-DRYING

Before this step, samples were frozen at -80°C on dry ice and then freeze-dried (Labconco, Missouri, USA).

The sugar content was then analyzed by HPAEC-PAD (DIONEX, California, USA). The remaining part of the samples were saved in capped flasks with parafilm (SPI Suppliers, New York, USA) at room temperature.

IMPROVED PRODUCT

In this step, the best conditions in combination were used in order to obtain a product with higher amounts of xylan and arabinoxylans and with the least free monosugars possible. The new set of experiments was done following the methods presented above, using *Sigma* α-amylase to perform the amylase treatment and dialysis in order to recover polysaccharides.

GLUCOSE COMPOSITION OF THE IMPROVED PRODUCT

To be able to obtain a more pure arabinoxylan product, it is important to know the composition of the improved final product and find out where the remaining glucose comes from. Two possibilities were explored: β-glucan and resistant starch.

BETA-GLUCAN

In order to access if there was beta-glucan present in the final product, I perform an enzymatic hydrolysis using a β-glucosidase enzyme from glycoside hydrolase family 1 (prepared at Lunds University Biotechnology Department).

Procedure:

1. A stock solution of the substrate was prepared by dissolving 60mg of the freeze-dried improved product in 10mL of Citrate-Phosphate buffer (pH=5) (6mg/mL).

2. In 1ml tubes, were prepared:
    a. *Substrate Blank*: 500µL buffer;
    b. *Enzyme Blank*: 500µL substrate solution;
    c. *Sample 1* and *Sample 2*: 1ml substrate solution.
3. The samples were incubated in a heat block (Grant, Cambridge, England) for 10 minutes at 80°C.
4. To each samples and blank were then added:
    a. *Substrate Blank*: 20µL of β-glucosidase ;
    b. *Enzyme Blank*: 20µL buffer;
    c. *Sample 1* and *Sample 2*: 40µL of β-glucosidase.
5. The samples were maintained at 80°C for 5 more minutes so the reaction could occur and then were frozen.
6. The samples were thawed and analyzed by HPAEC-PAD.

A schematic representation of this procedure can be found in Appendix I.

RESISTANT STARCH

A modified version of the example (c) of *Megazyme* "*Total Starch Assay Procedure (Amyloglucosidade / α-Amylase Method*)" (Megazyme International Ireland Limited, 2009) (Appendix I) was performed in order to assess the total amount of starch and indirectly how much resistant starch that was present in the sample.

Procedure:

1. Preparation of solution and buffer:
    a. Potassium hydroxide solution (2M): 56,22g of potassium hydroxide (Merck, Darmstadt, Germany) were weighed (Sartorius AG, Göttingen, Germany) and added to 450mL of deionized water and dissolved by stirring. The volume was then adjusted to 500mL.
    b. Sodium acetate buffer (1,2M, pH 3,8): 6,69mL of glacial acetic acid (Merck, Darmstadt, Germany) was added to 80mL of deionized water and then the pH was adjusted to 3,8 using 4M sodium hydroxide. The volume was adjusted to 100mL.

2. 20mg of each sample (*Sigma* and *Megazyme*) were accurately weighed in 4 different glass vials.
3. To each vial 40µL of aqueous ethanol (80% v/v) was added to wet the samples and aid dispersion and then mixed in a vortex mixer.
4. Then I add 0,4 mL of 2M KOH to each vial in order to dissolve the resistant starch by continuously stirring using a thermo mixer (HLC Biotech, Pforzheim, Germany) 20 minutes at approximately 4°C (more accurate than the recommended ice/water bath).
5. 1,6 mL of sodium acetate buffer was added to each sample and then, immediately 20 µL of α-amylase and AMG (*Megazyme*, Wicklow, Ireland) were added to the samples (these are the duplicates of the reaction and the remaining two samples of each are the blanks). 40 µL of buffer were added to the *blank* samples to accomplish the same volume.
6. The vials were placed in a water bath at 50°C for 30 minutes with intermittent mixing on a vortex mixer.
7. The samples were then cooled and saved frozen until the next day when they were analyzed by HPAEC-PAD (described next).

A schematic representation of this procedure can be found in Appendix I.

OTHER METHODS

RESISTANT STARCH FORMATION DURING STEAM TREATMENT

To assess if the creamy layer, found when steam treatment extracts were centrifuged, is mainly composed of resistant starch, the following procedure was used.

Procedure:

1. The layer was separated and dissolved in deionized water.
2. The sample was then centrifuged 20 minutes at 3000rpm. In the end the pellet formed was whiter on top and brownish on bottom.
3. The pellet was recovered and dissolved in 10 mL deionized water, frozen and freeze dried.

4. Then I prepared the samples for sugar analysis (monosaccharies and oligosaccharides) by HPAEC-PAD.

ETHANOL PRECIPITATION ASSESSMENT

In order to assess the best ethanol ratio to precipitate the polysaccharides, different ethanol percentages were used and the ethanol precipitation procedure already explained was used.

The starting material was a portion of the previously obtained pressure boiling extract, stored at -20°C. That extract portion was thawed and submitted to *Sigma* α-amylase treatment.

The precipitation was performed with the same amount of extract but, with 5 different amounts of ethanol 95% (v/v): 15, 40, 60, 80 and 95%.

SUGAR ANALYSIS BY HPAEC-PAD

Standards were used for each sugar 1 mg/mL. The standards follow the same treatment as the samples.

It was used an HPAEC-PAD equipment with an ED40 electrochemical detector (Dionex, Sunnyvale, CA, USA), Gradient pump (GP40, Dionex), Autosampler (AS50, Dionex) (injection volume of 10 µL).

The internal method for the chromatography can be found in Appendix I.

FREE MONOSACCHARIDES

Standards

Preparation of standard mixture for analysis:

Standards solutions (1mg/mL): fucose, arabinose, rhamnose, mannose, glucose, arabinose and xylose.

Standard mixture (0.1mg/mL each sugar): were transferred 150µL of each standard solution to one single eppendorf. 450µL of Milli-Q water was added to the mixture totaling 1500µL of solution.

Solutions and Eluents

The solutions prepared for this analysis are:

A= 600mM sodium hydroxide solution;

B= 500mM freshly prepared sodium acetate solution;

C= 1mM sodium hydroxide;

D= Milli-Q water

The amounts of solution depend, obviously, on the amount of samples to analyze.

Sample preparation

To analyze the content in monomeric sugars, approximately 5 mg of each freeze-dried sample was weighed and 1 mL of Milli-Q water was added. The samples were vigorously agitated with a vortex and then continuously stirred for one hour followed by centrifugation for 5 minutes at 10000 rpm. One milliliter of each sample was filtered and 350 µL of filtrate was transferred to vials.

The column used was a *Carbopac PA10* (*Dionex*, Sunnyvale (CA), United States).

Because, most of the times, a whole set of chromatography runs take more than 24 hours, in order to avoid errors, two standards are analyzed in the beginning and other two in the end of the assay.

OLIGOSACCHARIDES

This analysis is very similar to the method to analyze free monosaccharides.

The standards needed are different, as well as the column used to perform the chromatography (column PA100 instead of PA10). Characteristics of the different columns can be found in Appendix IV.

Standards

Preparation of standard mixture for analysis:

Standards solutions (1mg/mL): arabinose, xylose, xylobiose, xylotriose, xylotetrose and xylose.

Standard mixture (0.1mg/mL each sugar): were transferred 50µL of each standard solution to one single eppendorf. 400µL of Milli-Q water was added to the mixture totaling 1000µL of solution.

Sample preparation

Approximately 10 mg of each freeze-dried sample was weighed for this analysis.
In this case, is first necessary to perform Dilute Acid Hydrolysis to hydrolyze polymeric sugars into its monomers. This procedure is a modification of *Seaman Hydrolysis* (Seaman, et al., 1963) as explained below.

Dilute Acid Hydrolysis

1. 5mL of an sulfuric acid solution (4%) was added to each sample;
2. Heat the tubes for 3 hours at 100ºC;
3. Cool down tubes;
4. Neutralize, setting the pH between 5-6 with 0,1M $Ba(OH)_2$ (approximately 4,3ml);
5. Filter with 0,2µm membrane filter;
6. 350µL was then transferred to the analysis tubes.

All samples were analyzed with High Performance Anion Exchange Chromatography - Paired Amperometric Detection.

PRE-TREATMENTS

OPA METHOD

First, rye bran samples were submitted to enzymatic pre-treatments with the aim of following the de-proteinization efficiency of different enzymes. The method used was

the *Novozyme* method: *Determination of the Degree of Hydrolysis (DH) Based on OPA Reaction* (Dennis Petersen, 1995), from now on, simply called OPA Method. The complete description of the method can be found in Appendix I.

Procedure:

1. Reagents preparation
    a. OPA REAGENT
        1. 7,620g di-sodium tetraborate decahydrate (Borax) (Merck, Darmstadt, Germany) and 200mg of sodium dodecyl sulphate (Sigma-Aldrich, Missouri, USA) were dissolved in 150mL of deionized water. It is mandatory to wait until the solution was completely dissolved. This is *solution 1*.
        2. Then, 160 mg of o-phthaldiadehyde 97% (Sigma-Aldrich, Missouri, USA) was dissolved in 4 mL ethanol 95%. This solution was transferred to *solution 1* quantitatively by rinsing with deionized water.
        3. 176mg dithiothreitol 99% (Sigma-Aldrich, Missouri, USA) was added to *solution 1* as well, rinsing with deionized water.
        4. More deionized water was added in order to fill up the flask to 200 mL.
        5. The reagent was kept at 4°C and covered with aluminum foil.
    b. SERINE STANDARD
        1. In 500 mL of deionized water, 50 mg serine (Sigma-Aldrich, Missouri, USA) was dissolved (0,9516mEq/L).
    c. SAMPLE SOLUTION (RYE BRAN)
        1. Dissolve X g rye bran in 100 ml deionized water where X is 0,1-1,0 g for 80-8% protein in sample.

2. General Procedure
    a. First 3 mL of OPA reagent were added to each tube;

        Tubes: 4 standards / 4 blanks / 2 x 2 sample

    b. Blank measuring (2 blanks):

1. 400 µL of deionized water were added to each tube and mixed. Then the mixture stood still for precisely 2 minutes before the absorbance was read at 340 nm in spectrophotometer (Biowave II UV / Visible. *Biochrom Ltd*., Cambridge, England).

c. Standard measuring (all 4 standards):

1. Proceed as for the blanks but adding 400 µL of serine standard to each tube.

d. Sample measuring:

1. Proceed as for the blanks but adding 400 µL of sample to each tube.

e. Blank measuring (remaining 2 blanks).

Typical values: Standard OD ≈ 0,8

Blank OD ≈ 0,07

(The procedure follows the order above.)

3. Calibration Curve

In order to construct a calibration curve, the standard solution was used.

a. The serine standard solution was diluted several times.

b. Each dilution was made in duplicate and each duplicate was analyzed twice, following the general procedure.

4. Scale-Down

As many reagents used in this analysis are environmentally dangerous, a scale down was made. Instead of 3 mL of OPA solution, just 1 mL was used. The samples, blanks and standards were also reduced by a third, approximately 133 µL.

A new calibration curve was then made to see if there is any significant difference by scaling down. If not, all the next experiments can be made in a smaller scale.

5. Sample preparation

Different assays were made to test two different enzymes. To the following experiments using rye bran, the rye bran sample was prepared as explained in 1c.

a. PEPSIN

A pepsin solution (1 mg/mL) from porcine gastric mucosa ≥ 250 U/mg (Sigma-Aldrich, Missouri, USA) pH 2 was prepared and was added in increasing volumes to a mixture of BSA and rye bran, achieving different concentrations. The sample pH must be set to 2 as well. The reaction occurred by incubating one hour at 37°C. Then the samples followed the general analysis procedure.

b. PANCREATIN

The quantity of pancreatin from porcine pancreas (Sigma-Aldrich, Missouri, USA), enough to hydrolyze all the protein content on rye bran samples was calculated as 32,8 mg. The enzyme was added to rye bran sample and BSA solution and incubated at 40°C. Samples were analyzed every ten minutes for an hour.

ENZYMATIC PRE-TREATMENT

Instead of trying to follow the degree of protein hydrolysis using different enzymes, this pre-treatment had a different approach. In order to avoid the Maillard reactions during steam treatment, one solution could be to remove either the proteins or remove starch. In this way, removing the source of amino acids or the main source of reducing sugars during steam treatment would avoid these reactions and thereby, reduce differences in the color and odor on the extracts.

Procedure:

1. Three 50 mL 10% DW rye bran solutions were prepared.
2. To each solution was added:
    - *Control* (*C*): nothing
    - *α-amylase* (*α-A*): 20 µL *Sigma-Aldrich* α-amylase

- *Pancreatin* (*P*): 37 µL *Sigma-Aldrich* pancreatin

3. *Sample α-A* was incubated one hour at 70°C and the *sample P* at 40°C, for one hour.
4. The samples submitted to enzymatic treatment were then centrifuged several times at 3000 rpm followed by washing with deionized water.
5. All three samples were then subjected to steam treatment in autoclave at 121°C for 15 hours.
6. Samples centrifuged for 30 minutes at approximately 3000 rpm.
7. Vacuum filtration.
8. The samples were then prepared for sugar analysis (monosaccharides and oligosaccharides) by HPAEC-PAD (previously described).

A schematic representation of this procedure can be found in Appendix I.

SCALE-UP

A scale up of the procedure was necessary in order to obtain sufficient amount of final product to be able to obtain health beneficial oligosaccharides after xylanase treatment. The scale up was basically the best of all the treatments tested before in order to get a final product as pure and bright as possible as described next.

The starting material was 1500 mL of a rye bran 10% DW mixture in Milli-Q water.

From now on, Milli-Q water (Millipore, Massachusetts, USA) was used instead of deionized water in order to obtain a final product as pure as possible.

Along the procedure small samples (5 to 40mL) were saved for further analysis.

PRE-TREATMENT

The pH was adjusted to 6.8. In order to de-starch the bran, approximately 150 µL (37.5U) of α-amylase (from *Sigma-Aldrich*) was used. The treatment was done at 70°C in a hot water bath for one hour, with continuously stirring using an overhead stirrer (IKA WORKS Inc., North Carolina, USA).

DE-STARCH RYE BRAN RECOVERY

To save time on separation, a square of cotton cloth was used as a filter, the bran was recovered. Otherwise, due to the large volume of the sample, it would be necessary to wait for several centrifuge cycles. Afterwards, the destarched bran was successively washed by pouring 10L of Milli-Q water over the bran.

PRESSURE BOILING

To the previously recovered wet bran, water was added until the weight of this mixture was equal to the starting weight of the material.

The pressure boiling procedure was made in the exact same way as described before (15h at 121°C).

To remove the bran from the extract, a cloth was used again due to the large volume of the sample.

ETHANOL PRECIPITATION

The precipitation was carried out with 1:4 of ethanol in water. The extract was treated in four separated 4 L beakers.

The beakers were left overnight inside a cold room (+4°C).

To recover the pellet, the supernatant on top was poured directly to a different beaker and, afterwards the remaining part was vacuum filtered using a filter which retained gelatinous precipitates (Munktell, Falun, Sweden).

The wet pellet was centrifuged to remove remaining ethanol and then recovered.

In this case, there was no solubilization step before dialysis.

DIALYSIS

The procedure resembled the small scale experiment described above. The gelatinous precipitate fractions obtained by ethanol precipitation were divided in four portions and insert in four dialysis bags. The dialysis was done in a cold room (+4°C) for two days in 4 L Milli-Q water, changing water four times ($3.90E^{6}$ times dilution in the end).

XYLANASE TREATMENT

In order to obtain health promoting oligosaccharides, the recovered arabinoxylan was hydrolyzed by an endo-xylanase. This enzyme is xylanase Xyn10A from *Rhodothermus marinus*, full-length (FL) and the isolated catalytic module (CM), cloned in *E. coli BL21* (Eva Nordberg Karlsson, 1997) were produced in batch cultivations by course students at LTH and purified by Immobilized Metal Affinity Chromatography by another master student at Biotechnology Department (Phositlimpakul, 2011).

The amount of enzyme (36 U/g xylan) used in the experiments was based on the activity of the xylanase on rye bran xylan (see Appendix II).

Activity of the enzymatic solutions:

1210 U/mL (CM)

1293 U/mL (FL)

The substrate used was the final product of the big scale production. The enzymatic hydrolysis of arabinoxylans occurs for 4 or 6 hours.

Procedure:

1. Enzymatic stock solutions and samples
   a) Diluted stock solutions of both Catalytic Module and Full-Length enzyme solutions were prepared (10 U/mL).
   b) Samples were prepared with 22.5mg of arabinoxylan final extraction product in a 4.5mL aqueous solution 5mg/L (total content in xylans about 13.5mg).

2. Test hydrolysis

   In this step, the aim was to screen the stability of the enzymes in stock for long time.

   Samples (700μL) were taken every hour from the initial time up to four hours and, in the end, analyzed by HPAEC-PAD in duplicated.

   Knowing that, for the total hydrolysis of the xylans present in the samples solutions, it is needed an enzyme amount correspondent to less than 0.50U. The following volumes of stock enzymatic solutions were used:

Table III – Enzyme volumes used in the test xylanase hydrolysis.

SAMPLE	V CATALYTIC MODULE (CM) (µL)	FULL LENGTH (FL) (µL)
1 (0.36U)	36	36
2 (0.50U)	50	50

Those volumes were added to substrate and immediately, the mixture was stirred in the vortex stirrer. A sample was taken (t=0).

a) All the vials were continuously stirred at 900rpm on a continuously stirred heat block at 70°C (HLC Biotech, Pforzheim, Germany). Samples were taken every hour.
b) In order to inactivate the enzyme the samples were boiled for 5 minutes and then centrifuged and filtered (0.2 µm membrane filter).
c) Duplicates were then analyzed by HPEAC-PAD.

3. Enzymatic treatment

From the results of the test hydrolysis, a new procedure was prepared. The hydrolysis time was extended to 6 hours and the amount of each enzyme used was 0.5U. The enzymatic and sample solutions were prepared as in *1*.

a) From each stock enzymatic solution (CM and FL), 50 µL were added to the substrate samples. This procedure was done in triplicate.
b) The same method was followed as above but the reaction time was increased to 6 hours.

The results obtained for the hydrolysis using the xylanase Xyn10A Full Length and the Catalytic Module were than compared.

All the results were analyzed by comparing the chromatograms obtained by anionic exchange chromatography.

SIZE EXCLUSION CHROMATOGRAPHY

In this case, size exclusion chromatography was used as a method to have a visual idea of the degree of hydrolysis of arabinoxylans by xylanase Xyn10A.

This technique was used to see the effect of the hydrolysis in the sample. For that, an hydrolyzed sample was compared with a non hydrolyzed one.

The equipment used in this analysis is a SEC FPLC-system with refractory detector (Pharmacia Biotech, Uppsala, Sweden) set with Columns Hi Load Superdex 75 10/300 and Superdex 200 10/300 (GE Health Care Life Sciences , Uppsala, Sweden).

The standard analysis procedure for the equipment was followed.

Procedure:

1. Non hydrolyzed sample analysis
    a) A 1 mg/mL sample was prepared dissolving the final product obtained in the scale-up extraction process in Milli-Q water.
    b) The sample was then centrifuged for 5 minutes and filtered using a 0.2μm membrane filter.
    c) Although just 500μL of sample was required to the analysis (500μL loop in the equipment), 1mL of sample was injected on the size exclusion chromatography system to make sure that the loop was completely full and then, to have enough material to perform the analysis.

2. After xylanase treatment
    a) A sample CM after 6h hydrolysis was used to compare. It was treated and analyzed in the same way as the first sample.

INITIAL METHOD AND IMPROVEMENTS

The solid content of rye bran was determined as 89,5% by drying the sample at 105°C overnight until constant weight.

The different products obtained (see the procedure scheme on Appendix I) were analyzed by HPAEC-PAD. Relevant images and graphs are shown next. Table IV summarized all relevant results. Analyzing the following results, it should be clarified that *Megazyme* corresponds to the initial method's final product (described in Appendix I).

Table IV – Arabinoxylan, glucose and non sugar composition and yields on the different treatment extracts.

Average results (n=3)

SAMPLE	MG SAMPLE /ML	% GLUCOSE	YIELD GLUCOSE (MG/ML)	% ARABINOXYLANS	YIELD ARABINOXYLANS (MG/ML)	RATIO ARABINOXYLANS /GLUCOSE	% SUGARS
Blank	32.731	47.410	15.518	33.965	11.117	0.716	83.377
Megazyme	28.556	20.422	5.832	37.572	10.729	**1.840**	58.676
Dialysis	22.644	22.959	5.199	42.614	9.649	1.856	66.964
Frozen	23.338	20.916	4.881	40.056	9.348	1.915	61.065
Sigma	21.863	11.534	2.522	41.345	9.039	**3.585**	21.863

Sugars = xylose, arabinose, glucose, fucose and galactose.

Since the objective is to obtain a final product rich in arabinoxylans with low amounts of glucose, the ratio Arabinoxylan / Glucose is a good approach to evaluate the data, thus, *Sigma* sample seems to be the one with the most desirable characteristics.

Keeping these results in mind, the consequences of the different variables introduced on the initial method as well as the physical characteristics of the samples will be analyzed more detailed next.

Further data can be found in Appendix II.

PRESSURE BOILING EFECT ON THE SAMPLE

After pressure boiling, the pH goes from neutral to acidic values (pH=5.03). As the optimum pH value for both α-amylases is around 6.8, the pH was adjusted.

The difference in pH before and after the pressure boiling is due to the autohydrolysis since the acidic groups bound to the hemicelluloses are released at elevated temperatures. These acids, mainly acetic acid, participate in the hydrolysis of the solid lignocellulosic material to soluble oligo and polysaccharides (Roos, et al., 2009). The different color (Figure 5) and smell is due to occurrence of Maillard reactions between carbonyl groups of reducing sugars and free amino groups (Michalska, et al., 2008) during pressure boiling extraction.

Figure 5 - Appearance of the sample before (left) and afler (rigth) pressure boiling.

Since the objective is to produce functional food ingredients, the whiter and odorless the better on each step of the production thus, it would be useful to avoid Maillard reactions.

After centrifuging the steam treatment extract, a white gel-like layer is formed between the extract and the bran below. The composition of such a layer was analysed to determine its composition as shown afterwards.

EFFECT OF FREEZING PRESSURE BOILING EXTRACTS

Keeping the boiling pressure extracts frozen (at -20°C) for a few hours does not affect negatively, in any way, the sugar composition of the final product using the *Megazyme* amylase as seen in Table V and Figure 6.

Table V – Effect of freezing pressure boiling extracts.

Average results (n=3)

SAMPLE	MG SAMPLE /ML	% GLUCOSE	YIELD GLUCOSE (MG/ML)	% ARABINOXYLANS	YIELD ARABINOXYLANS (MG/ML)	RATIO ARABINOXYLANS /GLUCOSE	% SUGARS
***Fresh** Extract*	28.556	20.422	5.832	37.572	10.729	1.840	58.676
***Frozen** Extract*	23.338	20.916	4.881	40.056	9.348	1.915	61.065

Sugars = xylose, arabinose, glucose, fucose and galactose.

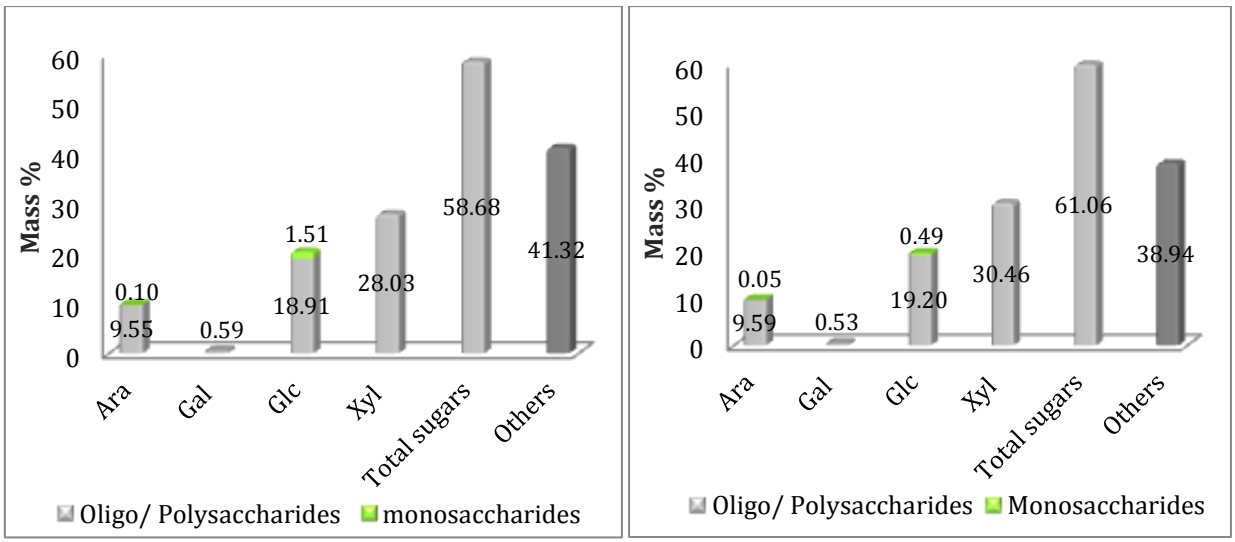

Figure 6 - Comparison of the final products obtained using a fresh sample (right image) and a frozen extract sample (left image). (Y axis: percentage; X axis: components where "others" are the remaining dry content that is not sugars)

Knowing if it is possible to freeze the pressure boiling extracts without disadvantage is very useful, allowing the production of larger batches of extract each time which is, in fact, a time saving benefit.

EFFECT OF AMYLASE TREATMENTS

In order to screen for the best enzyme to use in the amylase treatment, the composition of oligomeric glucose in final products was investigated. Three different final products were analyzed: sample without amylase treatment (*Blank*) and the ones treated with the two different enzymes (Table VI and Figure 7).

Table VI - Comparison of the final products obtained with different enzymatic treatments and without amylase treatment.

Average results (n=3)

SAMPLE	MG SAMPLE /ML	% GLUCOSE	YIELD GLUCOSE (MG/ML)	% ARABINOXYLANS	YIELD ARABINOXYLANS (MG/ML)	RATIO ARABINOXYLANS /GLUCOSE	% SUGARS
Blank	32.731	47.410	15.518	33.965	11.117	0.716	83.377
Megazyme	28.556	20.422	5.832	37.572	10.729	1.840	58.676
Sigma	43.725	11.534	5.043	41.345	18.078	**3.585**	55.783

Sugars = xylose, arabinose, glucose, fucose and galactose.

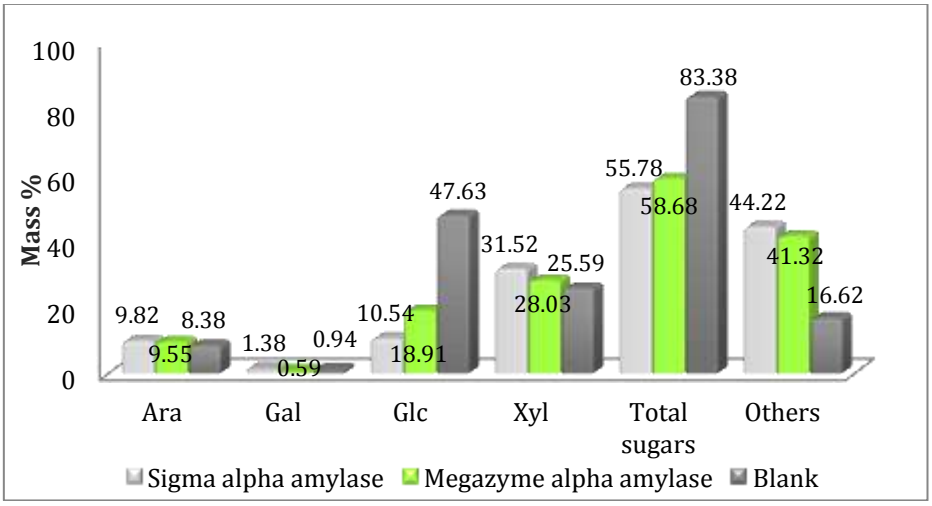

Figure 7 - Comparison of the final products total mass composition obtained with different enzymatic treatments and without amylase treatment. (Y axis: percentage; X axis: components where "others" are the remaining dry content that is not sugars)

The sample without any amylase treatment shows a higher amount of glucose. That is due to all the non-hydrolyzed starch that remains in the final product. Comparing the samples treated with the two different enzymes, this data also shows clearly, that the samples treated with α-amylase from *Megazyme* have a larger amount of glucose than the samples treated with the *Sigma-Aldrich* enzyme, thus, *Sigma-Aldrich* α-amylase is more efficient to remove starch than *Megazyme* α-amylase. To reinforce that, it is good to remember that in this amylase treatment step, the enzyme volume used in both treatments was the same (50µL) but, the activity of *Sigma-Aldrich* α-amylase is about ten times lower that the *Megazyme* enzyme and is still more efficient to hydrolyze starch.

In order to screen the best way to obtain a lower amount of monomeric sugars, it is necessary to compare the samples treated with ethanol precipitation with the one using dialysis. Although it is more common to use ethanol precipitation to isolate AX, dialysis was also reported on the literature (Hartmann, et al., 2005) as an efficient method for this process.

It is very important obtain a product with low (or without) monomeric glucose because the majority of intestinal bacteria assimilate glucose. In this case the goal is to produce a substrate that can be used, in further studies, to assess how the intestinal bacteria will grow on arabinoxylan oligosaccharides. Therefore, it is particularly important to remove free glucose in order to eliminate its interference in the bacterial growth.

Table VII - Comparison of the final products obtained using dialysis and ethanol precipitation.
Average results (n=3)

SAMPLE	MG SAMPLE /ML	% GLUCOSE	YIELD GLUCOSE (MG/ML)	% ARABINOXYLANS	YIELD ARABINOXYLANS (MG/ML)	RATIO ARABINOXYLANS /GLUCOSE	% SUGARS
Precipitation	28.556	20.422	5.832	37.572	10.729	1.840	58.676
Dialysis	22.644	22.959	5.199	42.614	9.649	1.856	66.964

Sugars = xylose, arabinose, glucose, fucose and galactose.

According to the Table VII (an adaptation of Table VI), the samples recovered by ethanol precipitation show a smaller mass percentage on glucose. However, according to the data found in the graphs of Figure 5, Dialysis is clearly a more efficient method to separate the free glucose monomers from the sample.

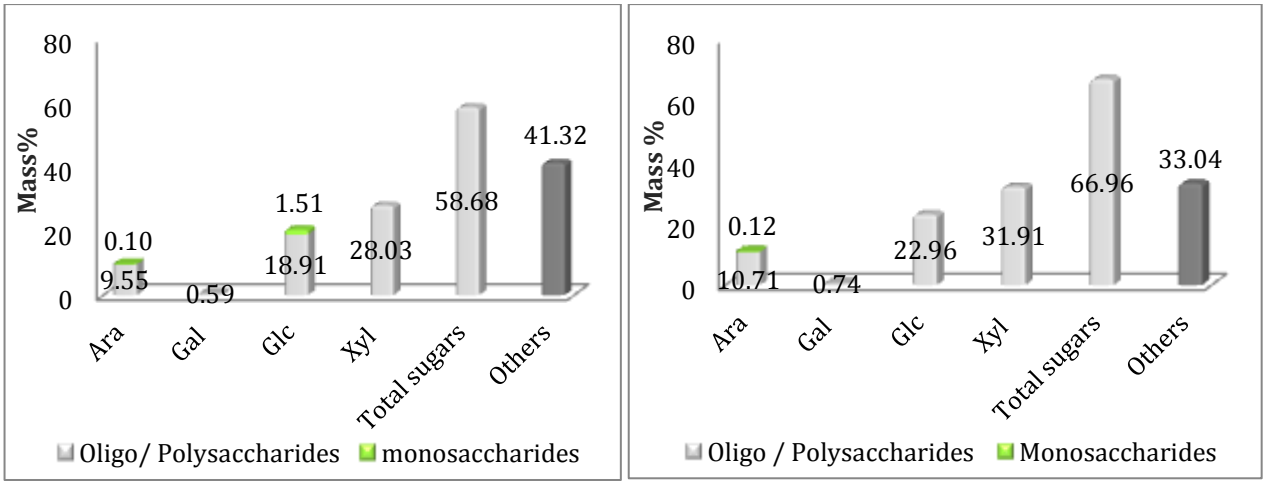

Figure 8 - Comparison of the final products obtained using dialysis (left image) and ethanol precipitation (right image).

(Y axis: percentage; X axis: components where “Others” are all the non sugar components).

The data found in Figure 8 graphs also shows clearly that the non sugar components in the samples are much lower than in the ethanol precipitated sample. As the membrane used to dialyze the sample has a MWCO of 3500, all molecules with a lower molecular weight will pass trough, therefore, all the lower weight contaminants will be removed, along with the small sugars.

One curious fact is the big difference between color and texture of the different final products. The dialyzed samples show a darker color and a more agglomerate texture than the ethanol precipitated sample as seen on Figure 9. The physical proprieties of AX products may be a very important factor since, in the extrusion processes to incorporate them in food, rheological properties of fibre directly affect its behaviour in the processing system (Madhuvanti, et al.).

Figure 9 - On the left, a final product treated with ethanol precipitation and on the rigth, one of the dialyzed sample.

Based on the observation in Figure 9, as well as the appearance of all the other samples it is safe to suggest that the texture and color of the final product is related to the type of technique used for the polysaccharides recovery. The samples treated with dialysis, result in a final product with a darker color and a more structured and agglomerate texture. With ethanol precipitation, the final product has a whiter color and a more powder-like texture, which are more desirable characteristics plus, it is a quicker method.

From this set of experiments the main conclusions are:

- Using *Sigma-Aldrich* α-amylase it is possible to hydrolyze more starch than using *Megazyme* α-amylase, therefore, more glucose is removed from the sample resulting in ratios Arabinoxylan / Glucose almost three times higher.
- It is necessary to avoid the color and odor that result from Maillard reactions during pressure boiling. This could be achieved by removing proteins (therefore, amino acids) and/or glucose starch (therefore, its reducing sugar: glucose) before steam treatment.
- Freezing the steam treatment extracts seems very handy as it allows a valuable time saving and does not influence negatively the composition and behavior of the sample.
- Dialysis of the samples is more efficient to remove monosaccharides than ethanol precipitation thus, avoiding the use of alcohol, is a good method producing food ingredients. However, the color and texture are not as desirable as when ethanol precipitation is used.

Before proceeding to a larger scale production and therefore, before obtaining health beneficial oligosaccharides, more tests based on the results and observations should be done. So, it is necessary to produce an improved product using all be best conditions determined, before introducing more modifications to the initial method, such as an enzymatic pre-treatment.

IMPROVED PRODUCT

Using *Sigma* α-amylase for the enzymatic treatment and dialysis to recover the polysaccharides, the composition of the final product is shown on Table VIII.

Table VIII - Arabinoxylan, glucose and non sugar composition and yields on the improved product.

Average results (n=3)

SAMPLE	MG SAMPLE /ML	% GLUCOSE	YIELD GLUCOSE (MG/ML)	% ARABINOXYLANS	YIELD ARABINOXYLANS (MG/ML)	RATIO ARABINOXYLANS /GLUCOSE	% SUGARS
Improved product	10.376	5.818	0.604	58.315	6.051	10.022	65.540

Sugars = xylose, arabinose, glucose, fucose and galactose.

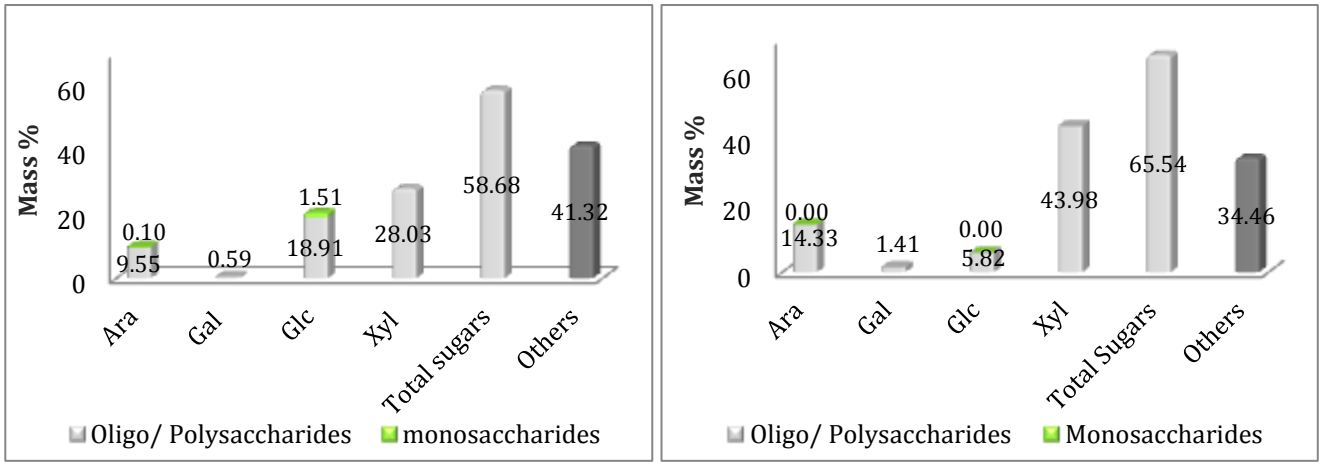

Figure 10 - Comparison of the final products mass composition obtained using the initial method (*Megazyme* α-amylase and ethanol precipitation) on the left image and, on the right image the improved product (*Sigma-Aldrich* α-amylase combined with dialysis).
(Y axis: percentage; X axis: components where "Others" are all the non sugar components).

Looking at Figure 10 and Table VIII, comparing with the results using the initial method (referred as *Megazyme* in Table VI), it is safe to say that, using a different amylase, combined with dialysis, a substantially improved product is achieved.

In the improved product, the ratio Arabinoxylan/ Glucose is more than five times higher than the same ratio observed for the final product using the initial method and the amount of other non-sugar compounds is also 7% (in mass percentage) lower. The yield on glucose is now 4 times lower but, it is still necessary to determine its origin in order to remove it.

From these results, it can be conclude that, using the methods with the best isolated results, actually result on an improved product.

GLUCOSE COMPOSITION OF THE IMPROVED PRODUCT

In order to obtain a more pure arabinoxylan product, it is necessary to remove as much glucose as possible. For that, it is necessary to determine the origin of the non-starch polymeric glucose present on the sample.

Some references (Karppinen, et al., 2001)(Mugaralikrishna, et al., 2007) indicate that rye bran has in its composition beta-glucan and cellulose. Also, severe treatments on cereal grains, such as pressure boiling, lead to resistant starch formation (Kim, et al., 2009).

As cellulose is extremely hard to solubilize, even via pressure boiling thus, it is not likely to be the major source of polymeric glucose.

So, the possibilities explored were: either the remaining glucose is a polymeric form of beta-glucan, resistant starch or other glucose polysaccharide.

BETA-GLUCAN

The sugar composition results, concerning the different samples after enzymatic treatment, are shown next.

According to the literature (Karppinen, et al., 2001)(Mugaralikrishna, et al., 2007) rye bran as 2 to 4% β- Glucan in its composition thus, a small amount of it may be expected.

Finding β- Glucan as the main source of glucose would be very interesting for the purposes of this project since the literature (Gunness, et al., 2010) shown a positive relationship between diets rich in soluble dietary fibers as β- Glucan and reduced serum cholesterol.

Table IX contains the results after sugar analyzed by HPAEC-PAD for the β-Glucan assay.

Table IX - Sugar composition of the different samples used in the β-Glucan assay.
Average results (n=3)

SAMPLE	GLUCOSE (MASS %)
Substrate blank	0.000
Enzyme blank	5.055
Beta-glucan	5.533

(Beta-Glucan: average (n=2) for the enzymatic treatment)

The amount of glucose and therefore, the composition in β-glucan, is very low: around 5 µg/mg sample (≈ 0,5%).

See Appendix II for additional data.

Figure 11 represents the chromatogram obtained by HPAEC-PAD while analyzing a sample treated with BglA and the enzyme blank.

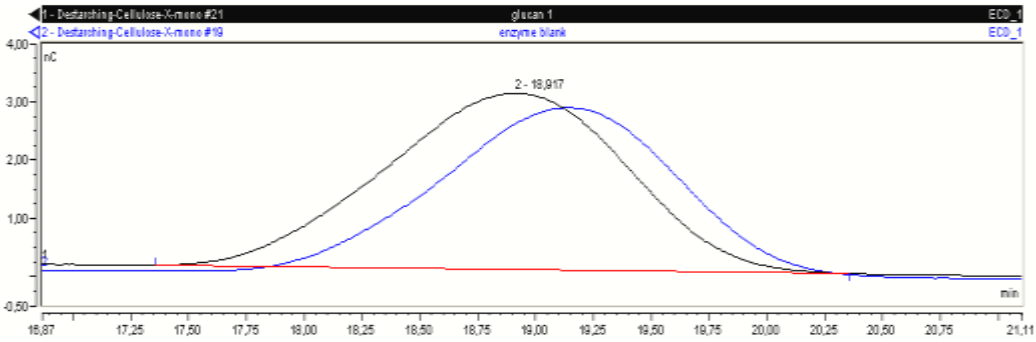

Figure 11- Chromatogram (from HPAEC-PAD chromatography) showing the glucose peak for one sample *Beta-Glucan* (in black) and the *Enzyme Blank* (blue).
(X axis: time in minutes; Y axis: response in nanocoulombs)

The figure above can be used as a visual aid as it clearly shows the similarity on the glucose peak areas on the enzyme treated and blank samples.

Excluding the hypothesis of β-glucan as the main source of glucose, the next step was to investigate the presence of resistant starch.

RESISTANT STARCH

A small amount of resistant starch is detected, higher than the β-glucan composition on the sample, around 4%.

See additional data on Appendix II.

Table X summarizes the glucose and glucose polysaccharide composition and yields of the improved product on.

Table X - Arabinoxylan, glucose and non sugar composition and yields on the improved product.
Average results (n=3)

SAMPLE	MG SAMPLE /ML	% TOTAL GLUCOSE	YIELD GLUCOSE (MG/ML)	% β-GLUCAN	YIELD β-GLUCAN (MG/ML)	% RESISTANT STARCH	YIELD RESISTANT STARCH (MG/ML)
Improved product	10.376	5.818	0.604	0.478	0.050	3.740	0.388

Figure 12 represents the sources of glucose on the mass composition of the improved product.

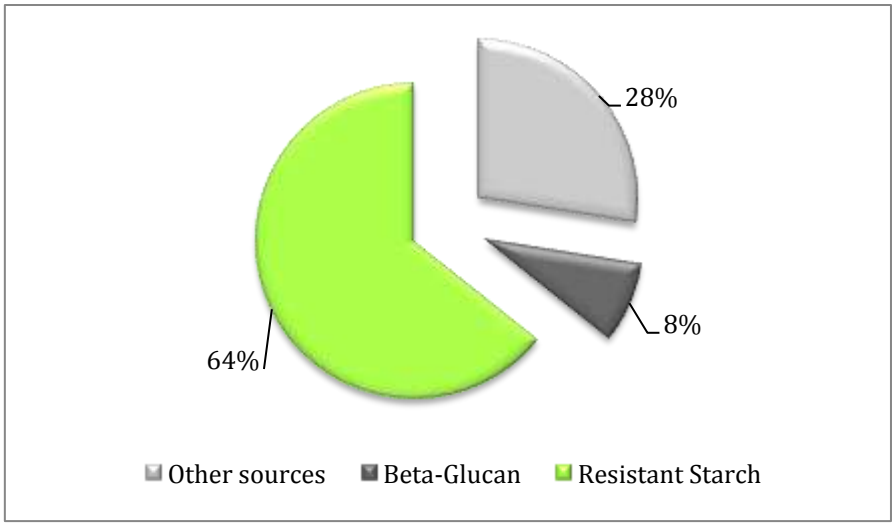

Figure 12 – Sources of glucose in the improved product composition.

Further analyses are needed since the data show that there is still 28% of polymeric glucose from an uncertain origin which could be, for instance, a small portion of cellulose solubilized during pressure boiling.

Knowing the main source of glucose present in the final product, now it is possible to find a strategy to remove it. One solution may be the use of α-amylase pre-treatment, before pressure boiling, hydrolyzing the starch and removing it before the extraction procedure because resistant starch (RS) is formed during hydrothermal treatment of starch-containing food raw materials (Rakha, et al., 2009). This solution would also lead to less free glucose present during pressure boiling so, it would be less reducing sugars available to react with amino acids by Maillard reactions thus, originating less color on the extracts.

In conclusion, avoiding the presence of high amounts of starch in the beginning of pressure boiling will lead to less formation of resistant starch and fewer occurrences of Maillard reactions. These considerations will be assessed next with the pre-treatment results.

RESISTANT STARCH FORMATION

ASPECT OF A CENTRIFUGED PRESSURE BOILING EXTRACT

The aspect of the centrifuged steam treatment extracts can be seen in Figure 13.

Figure 13 - Exemple of a boiling pressure extract aspect after centrifugation.

In Figure 13, it is easy to distinguish three different layers: the extract as supernatant on top, the remaining rye bran on bottom and in the middle there is an intriguing gel-like creamy layer. This as been reported by other coallegues in Lund University before, even when using other cereal as raw material. Its composition was then studied.

Some authores (Rakha, et al., 2009)(Sajilata, et al., 2006)(Juszczak, et al., 2004) stated that under condions as pressure boiling, resistant starch is formed and that its amount increase as the severity conditions increase.

It is knownd now, from the previous results that there is resistant starch present in the sample. Resistant starch is described (Kim, et al., 2009) as amorphous and gel-like and, since 25% of rye bran (dry weigth basis) is starch (Karppinen, et al., 2001) then it is probable that this white layer could be resistant starch formed during pressure boiling.

The goal of assessing the origin of the remaining glucose on the final product is related with the need of eliminate free glucose to prevent its avaliability on later fermentations. However, since resistant starch is considered a dietary fiber (Sajilata, et al., 2006), is presence in the final product could be, in fact positive.

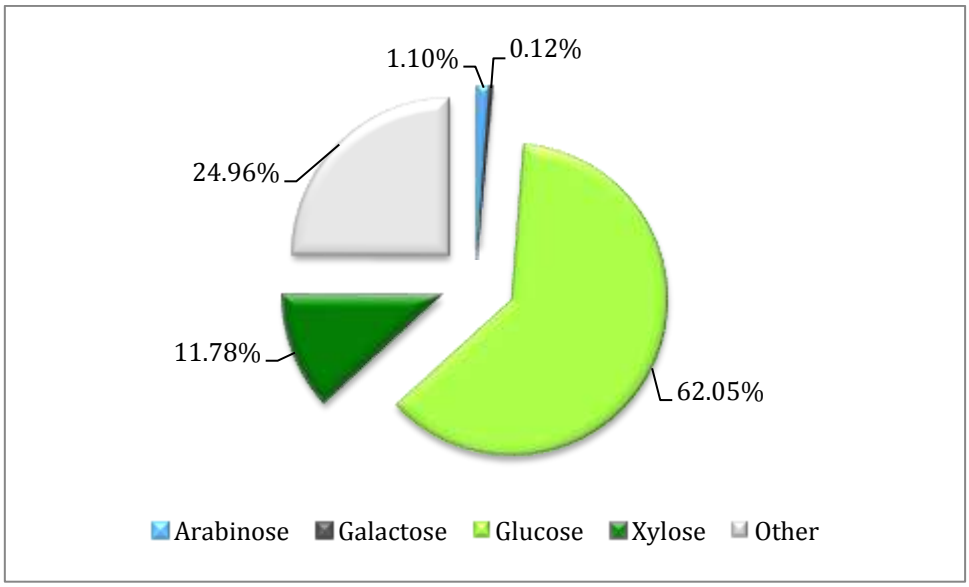

Figure 14 – Composition of the gel-like layer found on the pressure boiling centrifuged product. ("Other" is the remaining dry content that is not sugars).

This gel-like layer is mainly constituted by glucose. Further analyses would have to be done to confirm it is resistant starch but, considering the data found before (see Figure 12), it is very likely.

This gel-like layer, due to its amorphous morphology, is very difficult to completely avoid that a small portion of it would be accidentally included with the soluble extract when it is separated from the rye bran after centrifugation. With more time and higher velocity in the centrifugation step it would be possible to better precipitate this layer and thus, avoid resistant starch in the extract.

If further analysis confirm that this glucose is in fact resistant starch it would be very interesting because even though several authors described the formation of resistant starch under conditions like pressure boiling, a simple centrifugation as a method to separate resistant starch, taking advantage of its gel-like morphology, was never described before. Unfortunately due to my limited time in Lund University I was not able to perform those analyses myself.

These data reinforce the need of using another enzymatic treatment to remove the glucose, preferentially before steam treatment.

Previous data state that dialisys is a better method to remove monosaccharides than ethanol precipitation (see Table VII and Figure 8) however, this method leads to a product with less desirable physical characteristics as also shown before (Figure 9) and is faster to execute so, it is worth to explore it.

Several authors (Maes, et al., 2002)(Hartmann, et al., 2005) describe that 80% (v/v) is the optimum amount of ethanol to recover polysachharides using different raw materials and it is, in fact, the conditions used in this study to perform precipitation. However, it would be advantageous to use less ethanol, not only because it would be enviromentally friendly and reduce the costs on this step but also because, if this method would be applied on a process to produce food supplements, it is mandatory to reduce all solvents and keep the product as natural as possible.

In order to assess the optimum ethanol percentage to obtain as much polysaccharides as possible and retain less monosaccharides in the pellet different combinations were tested.

It was only possible to recover sufficient amount of samples to analyze when a minimum of 60% ethanol were used.

A first difference between samples can be noticed even before sugar analysis, just by looking to the freeze dried samples on Figure 15.

Figure 15 – Lyophilized pellet from ethanol precipitation. From left to right: 60, 80 and 95% ethanol (95% v/v).

Observing Figure 15, it is easy to see a difference in color as it gets darker with increasing amounts of ethanol.

Also, with increasing amounts of ethanol it is possible to recover larger amounts of pellet (additional data can be found on Appendix II). The reason for that it could be due to the precipitation of other polymers such as proteins present on the extract.

The sugar content of the different recovered pellets were then analysed by HPAEC-PAD and the results are ilustrated on the chromatogram of Figure 16.

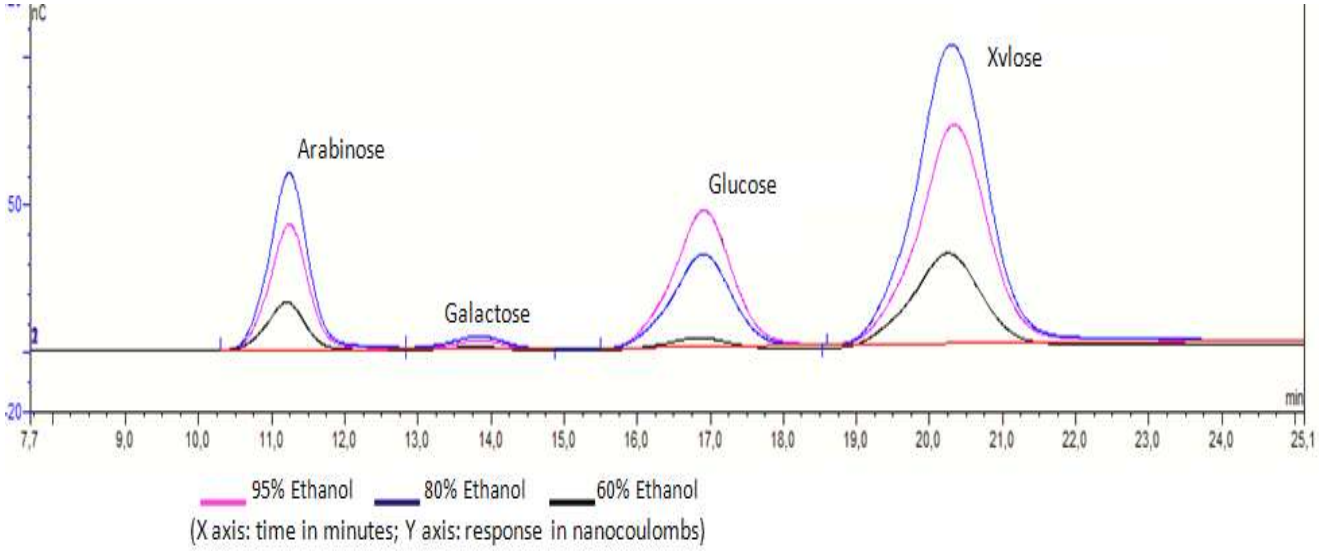

Figure 16 – HPAEC-PAD chromatogram shows the total amount of sugars presented of the differentially precipitated samples.

Figure 16, shows that with increasing amounts of ethanol, increasing amounts of sugars will precipitate, in general. Xylose and arabinose, therefore arabinoxylan seems to precipitate more using a precipitation with 80% ethanol (95% v/v). 80% will also lead to increasing amounts of galactose, a sugar that can be found as a side-chain on arabinoxylans (Mugaralikrishna, et al., 2007).

The A/X-ratio also increases with the increasing ethanol percentage until a maximum of 0.33%, an expected value for similar raw material (Maes, et al., 2002).

Using 95% of ethanol to precipitate arabinoxylans leads to a higher precipitation of glucose, therefore, 80% seem to be the most adequate amount of ethanol to use because it would be the best ratio to use in order to obtain arabinoxylans rather than glucose.

There is still need to infer which ethanol concentration leads to the precipitation of the least amounts of free monosaccharides.

Figure 17 shows the analysis to free monosugars present on the differently precipitated samples.

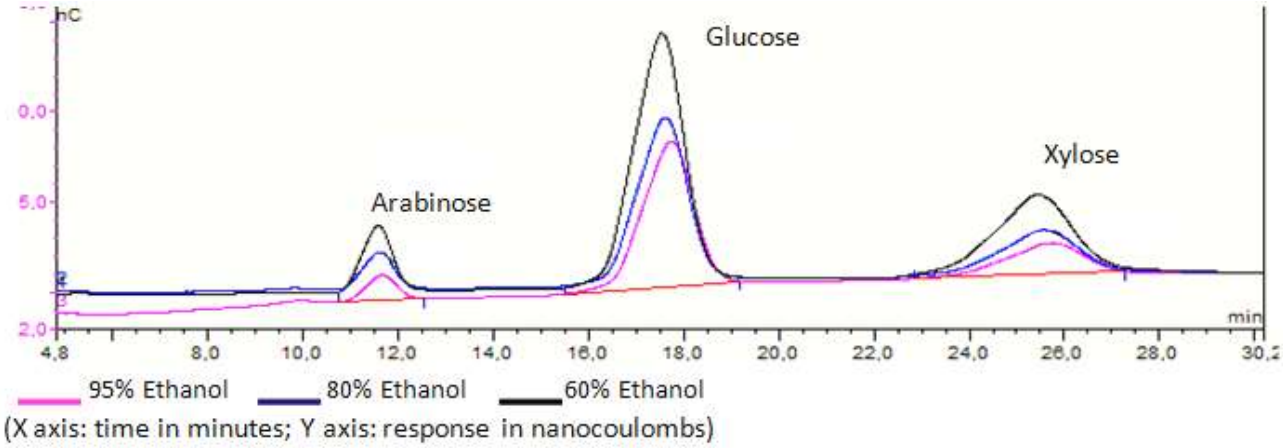

Figure 17 - HPAEC-PAD chromatogram shows the amount of free monosugars presented of the differentially precipitated samples.

The chromatogram presented in Figure 17 show that higher amounts of ethanol will precipitate lower amounts free monosugars in the pellet which is the objective.

If an ethanol concentration should be choosed in order to separate free monosugars from the polysaccharides in the precipitation pellet, it should be 95% (v/v) ethanol. In the other hand, using this concentration it would not be possible to recover the maximum amount of arabynoxylans from the solution.

The data suggest that using 80% ethanol would be the best compromise. In spite of not being the best way to remove monosaccharides, it recovers the maximum amount of arabinoxylan. This data are in concordance with the literature (Hartmann, et al., 2005).

It is good to remind that, the ethanol used for this precipitation is 95% (v/v) so, the real amount of ethanol is a little less than 80%, is 78.3% (see Appendix II for additional data).

This data reinforces how dialysis is a more efficient method to remove small sugars from the sample when compared with ethanol precipitation. Precipitation is a fast method but it will precipitate small molecules along with the polysaccharide chains. Dialysis in the other hand, if enough dilutions were made, it is sure that all molecules smaller than the MWCO of the membrane will be eliminated.

PRE-TREATMENT

The samples were submitted to an enzymatic pre-treatment, in order to avoid the occurrence of Maillard reactions during pressure boiling, and therefore, minimize the changes in the color and odor of the products as well as to minimize the production of resistant starch. As said before, for products which might be used as ingredients in functional foods, its color and odor are very important factors.

OPA METHOD

Several methods of monitoring the Degree of Hydrolysis during protein hydrolysis have been described in the literature, for example, pH-stat, osmometry, soluble nitrogen content, and the trinitro-benzene-sulfonic acid (TNBS) method (Dennis Petersen, 1995). OPA Method was chosen because is the more accurate, easier and faster to carry out that other methods. The aim is to infer the amount of protein that is possible to hydrolyse before steam treatment.

The results shown in this section represent a selection of the best results obtained, among a large number of experiments. This method was selected to understand if it really is advantageous to hydrolyse the proteins present in rye bran, as well as select the best enzyme to use in said hydrolysis, knowing that about 17% (Karppinen, et al., 2001) of rye bran are proteins.

Appendix I contains the standard procedure for this method (Dennis Petersen, 1995).

For additional data please consult Appendix II.

CALIBRATION CURVES

Next, the calibration curves following the general procedure and scaling down the method to save chemicals will be presented in Figure 18.

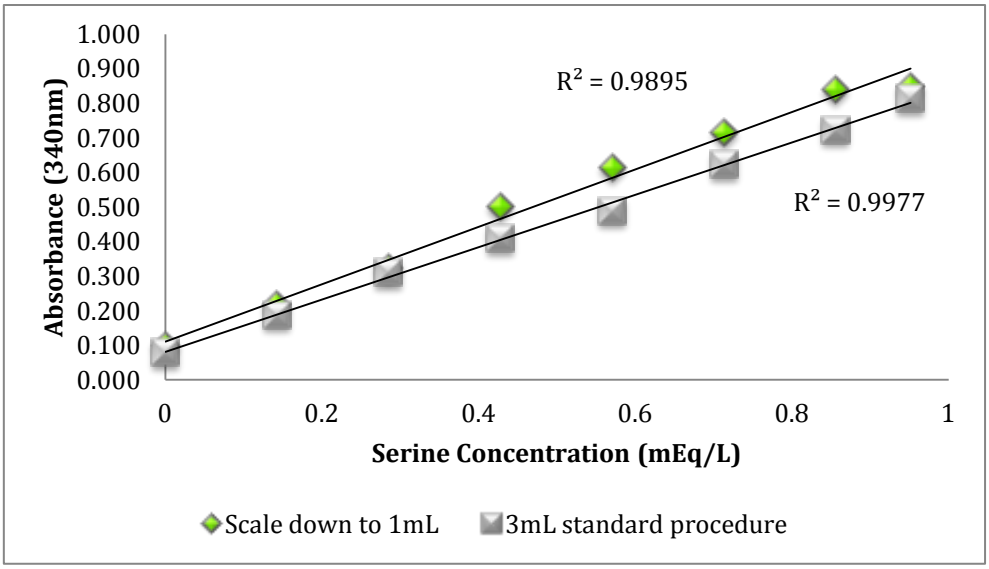

Figure 18 - Calibration curve obtained following the general procedure (3 mL OPA reagent/400µL sample) and the scaled down version (1 mL OPA reagent/133 µL sample).

By repeating the calibration curve in a smaller scale, the similar correlation coefficient values allows to see that scaling down the procedure does not affect negatively the results so, the method will be scaled down.

ENZYMATIC TREATMENT WITH PEPSIN

The first enzyme tested was pepsin ≥ 250 U/mg (Sigma-Aldrich, Missouri, USA). Figure 19 shows the absorbance increase when increasing volumes of 1 mg/mL pepsin solution are added to 3 different samples: rye bran in water, BSA solution and BSA with rye bran until a maximum of 300µL (corresponding to 75U). Specifications about the enzyme can be found in Appendix III.

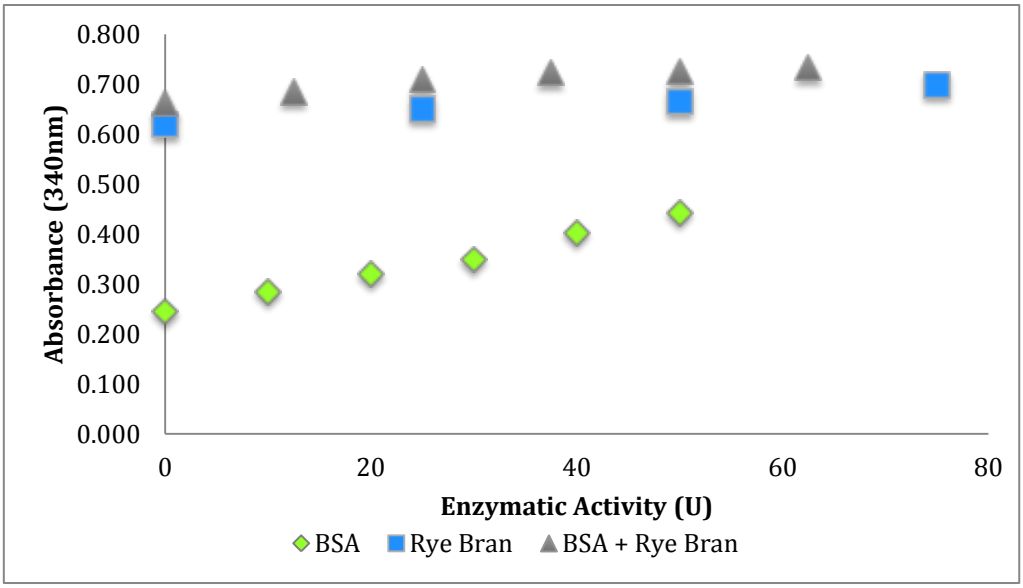

Figure 19 - Optical densities of different samples treated with different concentrations of pepsin. (BSA concentration = 1mg/mL)

When this method was applied just with BSA as a control, it is easy to see an increase in the absorbance with the increase of the enzyme volume.

When the same assay was applied with rye bran, the initial OD values are very high and the increase is not very significant.

To assess if there is something in rye bran that interfere with the absorbance, a new analysis was made but a mixture with rye bran and BSA. From this assay a curve very similar to the last one was seen. So, maybe something in rye bran interferes with the absorbance. It looks like there is a soluble component in rye bran which absorbs in this range of the UV spectrum. The high initial OD values may be due to the low pH necessary for optimum enzyme performance: it can interfere with the method or, it may be contribute to solubilize a rye bran component which can absorb in the UV range.

DEGREE OF HYDROLYSIS

Degree of hydrolysis is defined as the percentage of cleaved peptide bonds.

To calculate the degree of hydrolysis, the following mathematical equations were used:

$$DH = \frac{h}{h_{tot}} \times 100$$

Where:

$$h_{tot} = total\ number\ of\ peptide\ bonds/protein\ \ equivalent$$

$$h = number\ of\ hydrolysed\ bonds$$

H_{tot} is dependent on the amino acid composition of the rye bran. The value for rye bran has not been estimate. Soy values (Table XI) were used.

Table XI – Value of constants α, β and h_{tot} for different protein raw materials (Dennis Petersen, 1995).

PROTEIN	α	β	h_{tot}
Soy	0.970	0.342	7.8
Gluten	1.000	0.400	8.3
Casein	1.039	0.383	8.2
Whey	1.000	0.400	8.8
Gelatin	0.796	0.457	11.1
Meat	1.000	0.400	7.6
Fish	1.000	0.400	8.6

Determination of *h*:

$$h = \frac{(Serine\ NH_2 - \beta)}{\alpha / g\ protein}$$

$$Serine\ NH_2 = \frac{(OD_{sample} - OD_{blank})}{(OD_{standard} - OD_{blank})} \times \frac{0{,}9516 mEq}{L} \times 0{,}1 \times \frac{100}{X \times P}$$

Where:

α and β are shown in Table XI.

$P = protein\ \%\ in\ sample$

$X = g\ sample$

The degree of hydrolysis obtained using 300µl of pepsin 1mg/ml (75U) for one hour is 3.08%. This is a very low value, although a relatively low DH was expected since, in order to obtain higher degrees of hydrolysis, a combination of different enzymes is needed (Dennis Petersen, 1995).

The most probable reason for such a low value of DH is that probably, most proteins are not soluble and therefore, unavailable to be hydrolyzed by the enzyme.

ENZYMATIC TREATMENT WITH PANCREATIN

To avoid such an acidic pH, and due to the previous inconclusive results, the same method was repeated using pancreatin (Sigma-Aldrich, Missouri, USA) instead of

pepsin as proteolytic enzyme. This enzyme has near neutral optimum pH. Specifications about the enzyme can be found in Appendix III.

Figure 20 shows the absorbance changes along the time, when 5.8 mg of pacreatin in solution were added to two different samples.

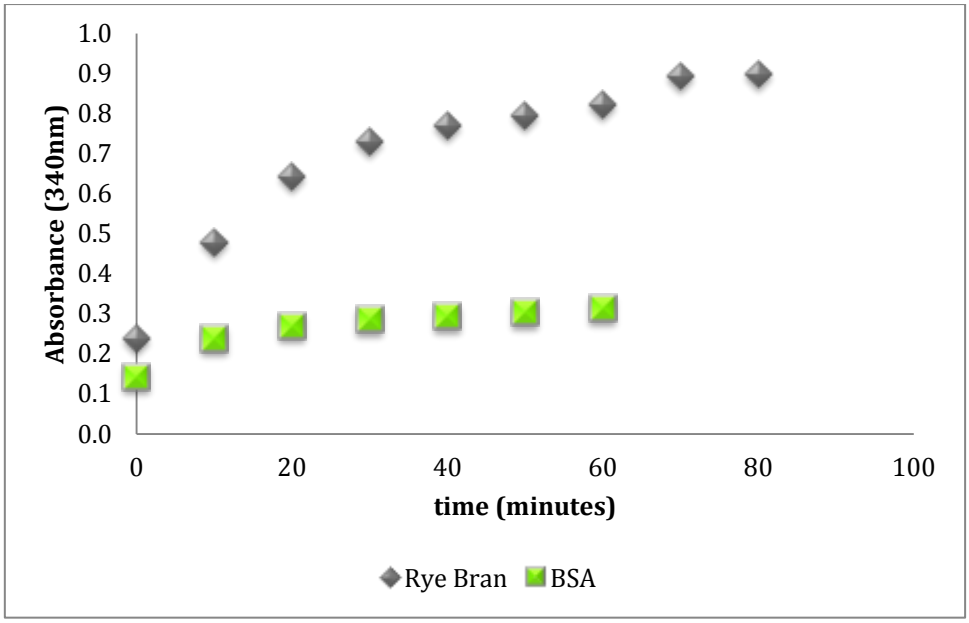

Figure 20- Optical densities along the time for different samples treated with pancreatin. (BSA concentration = 1 mg/ml)

DEGREE OF HYDROLYSIS

After 80 minutes, the degree of hydrolysis obtained using pancreatin in the end of the hydrolysis was 5.34%.

The DH now obtained does not improve significantly in relation to the previous one with pepsin. The optimum pH required for this enzyme is neutral, that is one of the reasons why this enzyme was chosen to be used to remove proteins. The explanations why the DH now obtained is low are the same as the previous assay with pepsin, except in relation to the acidic pH considerations.

OPA method was useful to choose the best enzyme to use on a pre-treatment.

Although the results obtained using this method were not exactly what was expected in the beginning, pancreatin was used on a pre-treatment to remove as much protein as possible before the extraction by steam treatment, in order to avoid Maillard reactions in said process.

ENZYMATIC PRE-TREATMENT

GENERAL ASPECT AFTER PRESSURE BOILING

After pressure boiling, the the physical appearance of the differently treated samples were compared as shown in Figure 21.

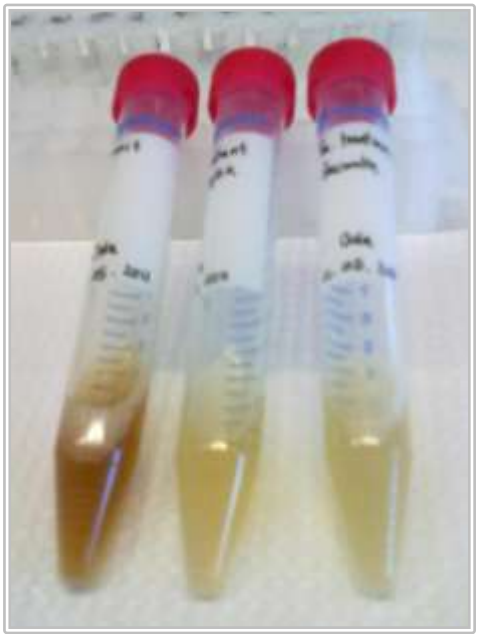

Figure 21 - Comparison between samples after steam treatment (Control/ Alpha-amylase pre treatment /Pancreatin pre treatment, in this order, from left to rigth).

The extract obtained without any pre-treatment is clearly darker than the other samples. Its odor is also stronger. Removing proteins or starch (the main source of reducing sugars) before steam treatment actually reduces the occurrence of Maillard reactions.

YIELDS OF ARABINOXYLAN AND GLUCOSE IN THE PRESSURE BOILING PRE-TREATED EXTRACTS

The yields of arabinoxylan and glucose, on the differently treated samples are analyzed now on table XII and Figure 22.

Table XII - Arabinoxylan and glucose composition and yields on the small scale pre treatment extracts. Average results (n=3)

SAMPLE	MG SAMPLE /ML	% GLUCOSE	YIELD GLUCOSE (MG/ML)	% ARABINOXYLANS	YIELD ARABINOXYLANS (MG/ML)	RATIO ARABINOXYLANS /GLUCOSE
α-amylase	19.000	10.955	2.082	58.860	11.183	5.373
pancreatin	29.367	41.080	12.064	41.980	12.328	1.022

control	54.233	34.811	18.879	25.350	13.748	0.728

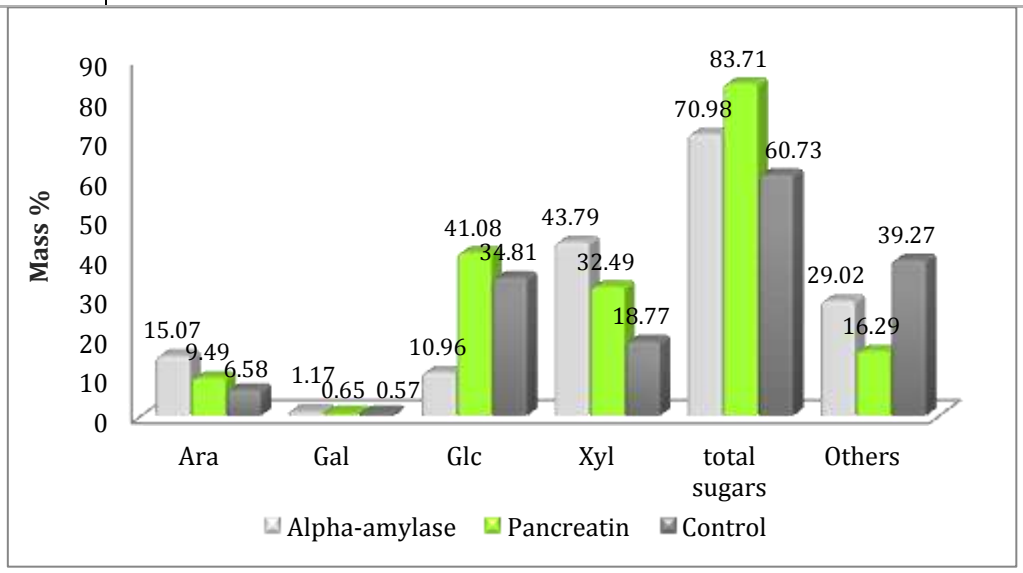

Figure 22 - Comparison of the final products total mass composition obtained with different enzymatic pre-treatments and a control sample. (Y axis: percentage; X axis: components where "others" are the remaining dry content that is not sugars)

Table XII clearly show that the sample treated with α-amylase is the more efficient removing glucose and shows a higher composition on arabinoxylans.

Data on Figure 22 suggest that the sample treated with pancreatin is the one with less protein content since proteins are included in the "others".

Concerning glucose, there is an enormous difference among the samples. The α-amylase pre-treatment is clearly more efficient to de-starch, achieving for times more glucose removal than pancreatin, even though pancreatin is in fact a cocktail of enzymes and, also contains α-amylase. The specifications of both enzymes can be found in Appendix III.

The difference between the yields on mg/mL arabinoxylan of the different pre-treatments is not that significant; however, using α-amylase pre-treatment, it is possible to obtain almost the same amount of arabinoxylans, with higher purity and significantly higher ratio Arabinoxylan/Glucose.

Knowing that with each pressure boiling extraction 28mL of each sample were recovered, on Table XIII, the effects on arabinoxylan content for the three different enzymatic treatments can be observed.

Table XIII - Effects of different enzymatic pretreatments followed by steam treatment. Average results (n=3)

SAMPLE	MASS (G)	MASS YIELD (%)	ARABINOXYLANS (G)	ARABINOXYLANS YIELD (%)	ARABINOXYLANS CONTENT (%)
Rye bran	5.000	100.000	0.942	100.000	18.832
Control extract	1.519	22.304	0.339	35.970	22.304
Amylase extract	0.532	51.795	0.276	29.264	51.795
Pancreatin extract	0.822	36.942	0.304	32.261	36.942

Table XIII clarifies that, for *Control* sample, even though the arabinoxylan content is higher it would just be achieved by sacrificing the purity of the sample and proof, once again, that α-amylase treatment is the best pre-treatment as it allows making a purer product.

In conclusion, the use of a pre-treatment seems to be very efficient, particularly removing glucose. Comparing the results of the sample pre-treated with α-amylase with the *Sigma* sample data on Table IV, the content and yield on glucose for both samples is almost the same. This pre-treatment allows achieving the same results but with the benefit of reducing resistant starching formation as well as Maillard reactions during steam treatment. Therefore, on a next step or on a larger scale production, an α-amylase pre-treatment should be used.

SCALE-UP

The scale up procedure is a sum of all best results obtained so far but, on a bigger scale. Along the process samples were taken and adjustments to the method were needed due to the higher volumes used now. Now those results will be studied. The aim is to follow glucose, arabinose and xylose (arabinoxylan) content and yields along the process.

The starting material was 1500 ml of a rye bran 10% DW mixture in Milli-Q water. The scheme of this experiment, as well as the provenience of each sample can be found in Appendix I.

The following table summarize the principal outcomes of the scaled up process.

Table XIV - Arabinoxylan and glucose composition and yields followed along the big scale process.

Average results (n=3)

SAMPLE	MG SAMPLE /ML	% GLUCOSE	YIELD GLUCOSE (MG/ML)	% ARABINOXYLANS	YIELD ARABINOXYLANS (MG/ML)	RATIO ARABINOXYLANS /GLUCOSE
After α-amylase pre-treatment	50.065	43.795	21.926	5.597	2.802	0.128
After α-amylase pre-treatment and bran wash	0.363	7.948	0.029	5.906	0.021	0.743
Soluble extract after pressure boiling	9.900	9.299	0.921	49.347	4.885	5.307
Final product		10.684	1.377	60.447	7.789	5.658

Observing the data on Table XIV and comparing the final product with the small scale improved product (Table VIII) is that the mass composition on polymeric arabinoxylans is now slightly higher even though the its composition on glucose is also higher and, therefore the ratio Arabinoxylans / Glucose lower. The main reason for that could be related with the adaptations to the method required for larger amounts of sample, for instance, it can be due to the filtration that precedes the steam extraction that is not as efficient as centrifugation on removing glucose (probably resistant starch, see Figures 13 and 14).

To help follow the content on polymeric xylan and glucose along the process, the data were organized in Table XV and Table XVI knowing that, in the rye bran, the content on polymeric arabinoxylans is 18.83% and it is 27% on glucose (see Appendix II).

Table XV – Polymeric arabinoxylans content and mass balance of the scale-up process.

SAMPLE	MASS (G)	MASS YIELD (%)	ARABINOXYLANS (G)	ARABINOXYLANS YIELD (%)	ARABINOXYLANS CONTENT (%)
Rye bran	150.000	100.000	28.248	100.000	18.832
After α-amylase pre-treatment and bran wash	75.446	50.298	24.077	85.233	31.912
Soluble extract after pressure boiling	13.662	9.108	6.742	23.866	49.347
Final product	12.804	8.536	5.160	18.266	60.447

In the end, it was just possible to recover 18.27% of the total arabinoxylans present in the raw material. Analysing Table XV, washing the bran after pre-treatment was one of the steps where more xylans were lost (about 15%) and, even submitting rye bran to pressure boiling, most of arabinoxylans remain unsoluble

In the end, it was just possible to recover 18.27% of the total arabinoxylans present in the raw material. Analysing Table XIV, washing the bran after pre-treatment was one of the steps where more xylans were lost (about 15%) and, even submitting rye bran to pressure boiling, most of arabinoxylans remain unsoluble.

According to Table XV, after the first amylase treatment, almost half of the soluble compounds are glucose as the other half is composed by non-sugars.

Once again, amylase treatment as a pre-treatment before pressure boiling seems to be very appropriate for the means as in this step 81.10% of the glucose present in the raw material is eliminated therefore, a big part of starch will be hydrolyzed into glucose and small glucose oligosaccharides which will be easily removed by simply washing the bran.

Pressure boiling is the central and most important procedure in the process. From the results shown in Table XIV and Table XV, the big majority of arabinoxylans remain unsoluble. The results are in agreement with the literature (Ward, et al., 2008) that states rye bran as the cereal bran with the highest amount of total water extractable arabinoxylan between nine different cereals studied, although the value is rather low.

Table XVI – Total glucose content and mass balance of the scale-up process.

SAMPLE	MASS (G)	MASS YIELD (%)	GLUCOSE (G)	GLUCOSE YIELD (%)	GLUCOSE CONTENT (%)
Rye bran	150.000	100.000	40.500	100.000	27.000
After α-amylase pre-treatment and bran wash	75.446	50.298	7.654	18.899	10.145
Soluble extract after pressure boiling	13.662	9.108	1.270	0.593	9.299
Final product	12.804	8.536	1.368	0.020	10.684

Although the glucose content in the final product is still higher comparing with the improved product obtained on a smaller scale (Table VIII), this process is very efficient removing glucose since it eliminates more than 96% of it.

The graph on figure 23 illustrates the composition of the final product obtained in the scale up process.

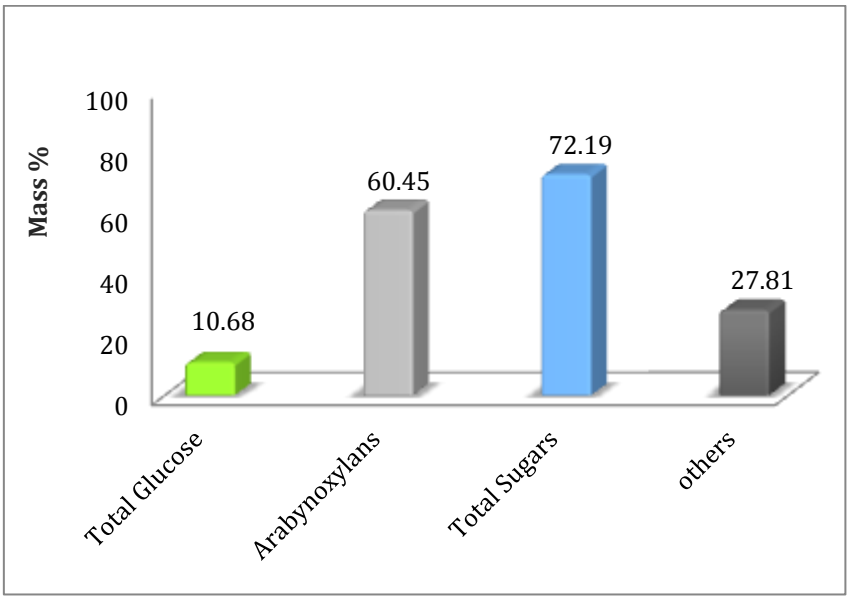

Figure 23 – Final product mass composition obtained by the scale-up process.
(Y axis: percentage; X axis: components where "Others" are all the non sugar components).

Table XVII sum up all major results.

Table XVII - Arabinoxylan, glucose and non sugar composition and yields of the different processes final products.

Average results (n=3)

SAMPLE	MG SAMPLE /ML	% GLUCOSE	YIELD GLUCOSE (MG/ML)	% ARABINOXYLANS	YIELD ARABINOXYLANS (MG/ML)	RATIO ARABINOXYLANS /GLUCOSE	% SUGARS
Previous method	28.556	20.422	5.832	37.572	10.729	1.840	58.676
Using Sigma α-amylase	21.863	11.534	2.522	41.345	9.039	3.585	21.863
Improved product	10.376	5.818	0.604	58.315	6.051	10.022	65.540
Final product	12.885	10.684	1.377	60.447	7.789	5.658	72.189

Sugars = xylose, arabinose, glucose, fucose and galactose.

Observing Table XVII it is clear that, along the modifications on the method, the obtained products composition on arabinoxylans is increasingly improving.

The adaptations in the separation and purification methods to the big scale process, as the elimination of the centrifugation step in the primary recovery step of the extract, lead to a higher composition on glucose and, therefore, to a lower ratio arabinoxylans /glucose on the final product.

In conclusion, even though the big scale procedure lead to a final product with desirable characteristics and composition, there would still be necessary to perform adaptations in the method in order to remove glucose.

The final product obtained in the scale process was then subjected to hydrolysis by xylanase Xyn10A in order to convert arabinoxylan polymers into small oligosaccharides.

The hydrolysis of arabinoxylans into oligosaccharides by xylanase Xyn10A will be analysed next.

ANIONIC EXCHANGE CHROMATOGRAPHY

The HPAEC-PAD chromatogram of Figure 21 illustrates the chromatograms of the two samples used with Xyn10A full length (FL) and catalytic module (CM).

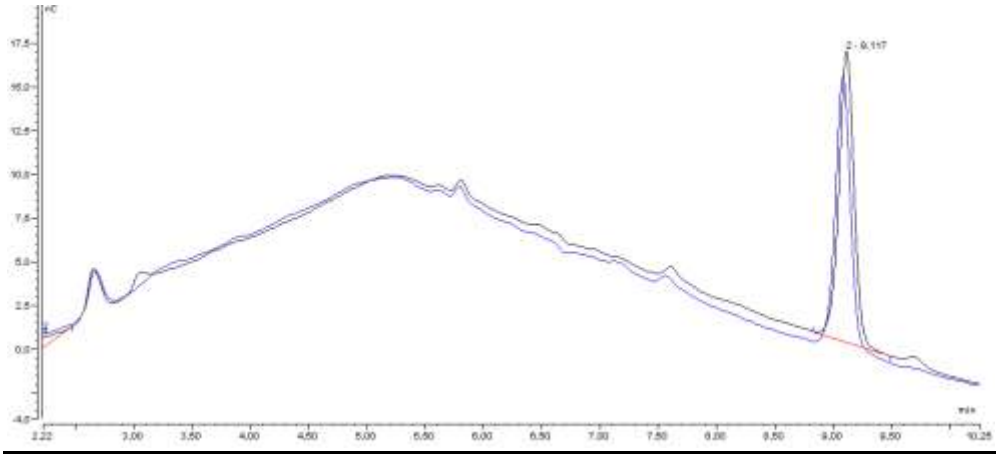

Figure 24 – Chromatogram comparing FL (blue) and CM (black) compositions in the initial time. (X axis: time in minutes; Y axis: response in nanocoulombs)

In the beginning (t=0 hours), the sample chromatograms for both experiments look very similar and that is exactly what was expected since the reactions had not started. The small peaks, before 3 minutes, is most probably some kind of impurities in the sample or chromatographic column.

The small difference between samples is normal as it could be due to a response difference of the equipment between running the two samples.

Figure 22 compares the chromatograms of FL and CM xylanase at 6 hours.

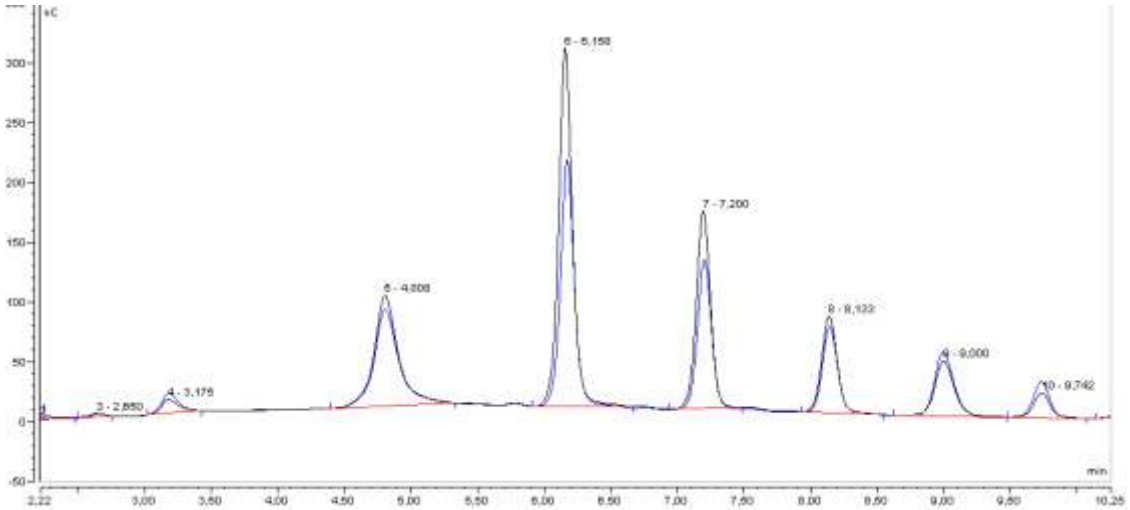

Figure 25 – Comparison between FL (blue) and CM (black) xylanase treatment after 6 hours.
(X axis: time in minutes; Y axis: response in nanocoulombs)

The peaks on the chromatogram correspond to the elution time of the standards and elute in the following order:

Arabinose 2,650 min

Xylose 3,175 min

Xylobiose 4,808 min

Xylotriose 6,158 min

Xylotetrose 7,200 min

Xylopentose 8,133 min

At retention time superior to 9 minutes there are more peaks, most probably continuing with xylohexose and longer xylo-oligosaccharides if compared with the other standard XOS.

The treatment using the xylanase catalytic module seems to be more efficient to produce lower molecular weight oligosaccharides. Therefore, from now on, just the results of CM xylanase treatment will be presented.

Figure 23 allows the peaks to be identified as it compares the chromatograms obtain after 6 hours using xylanase catalytic module with a standard.

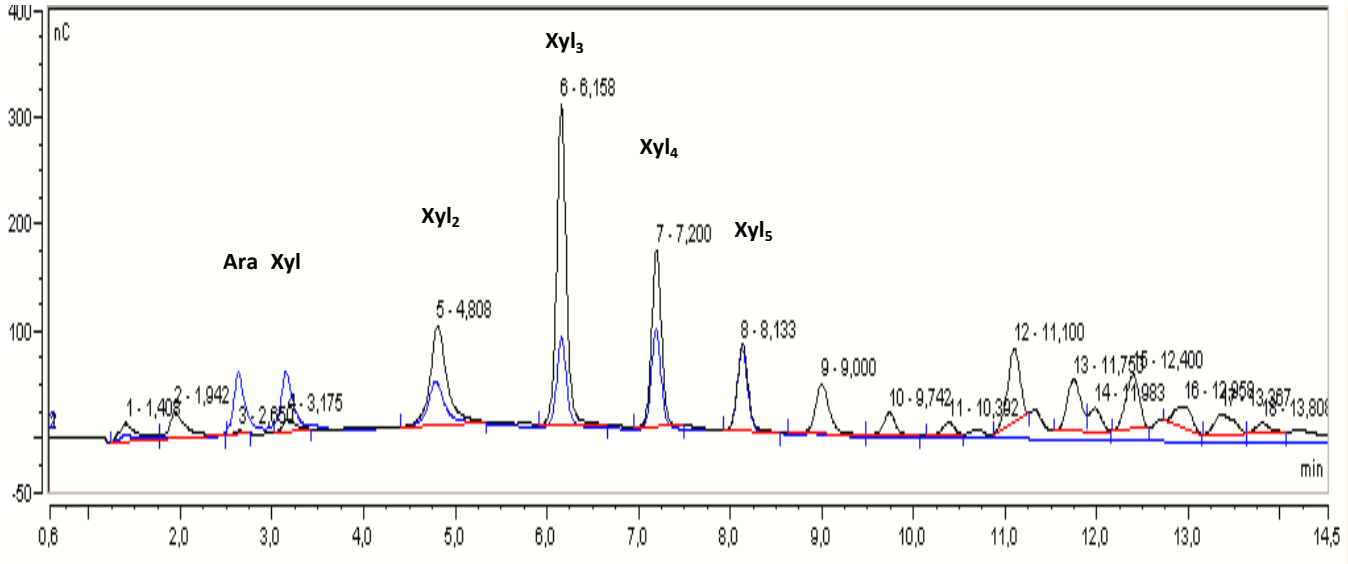

Figure 26 – Sample analysis of Xylanase CM treatment after 6 hours (black) with a standard (blue). (Ara= arabinose, Xyl= xylose, Xyl_2= Xylobiose, Xyl_3= Xylotriose, Xyl_4= Xylotetrose and Xyl_5= Xylopentose) (X axis: time in minutes; Y axis: response in nanocoulombs)

Endo-xylanase family 10A randomly hydrolyzed the glycosidic bonds in the xylan backbone and produced small oligosaccharides with 2-5 degree of polymerization. Between 11 and 13 minutes of retention time, a lot of small peaks are registered, most probably AXOS as reported by other colleagues. It would be very interesting to analyze the fraction of the sample eluted in this range of time. Run the sample with a column which allows collecting and running larger samples would be the next step to take in order to identify these compounds.

In conclusion, this method shows the ability to proof the efficiency of Xyn10A to hydrolyse xylans into oligosaccharides, to identify those smaller sugars and to understand is effect along the hydrolysis reaction.

From the literature (Gullón, et al., 2008) and previous fermentation assays held by colleagues in Lund University (see Appendix II), the utilization of XOS by probiotic bacteria correspond mostly to xylotriose, followed by xylobiose, xylotetrose and xylopentose. Therefore, the results now obtained are highly motivational.

With more time, it would be possible to quantify the different oligosaccharides along the time and to optimize the xylanase treatment in order to obtain larger yields on the oligosaccharides more assimilated for the intestinal flora bacteria.

The aim of this assay is to, once again, clarify qualitatively the effect of xylanase on the xylans present on the product.

Figure 24, compares chromatograms before and after 6h of xylanase treatment with Xylanase Xyn10A catalytic module.

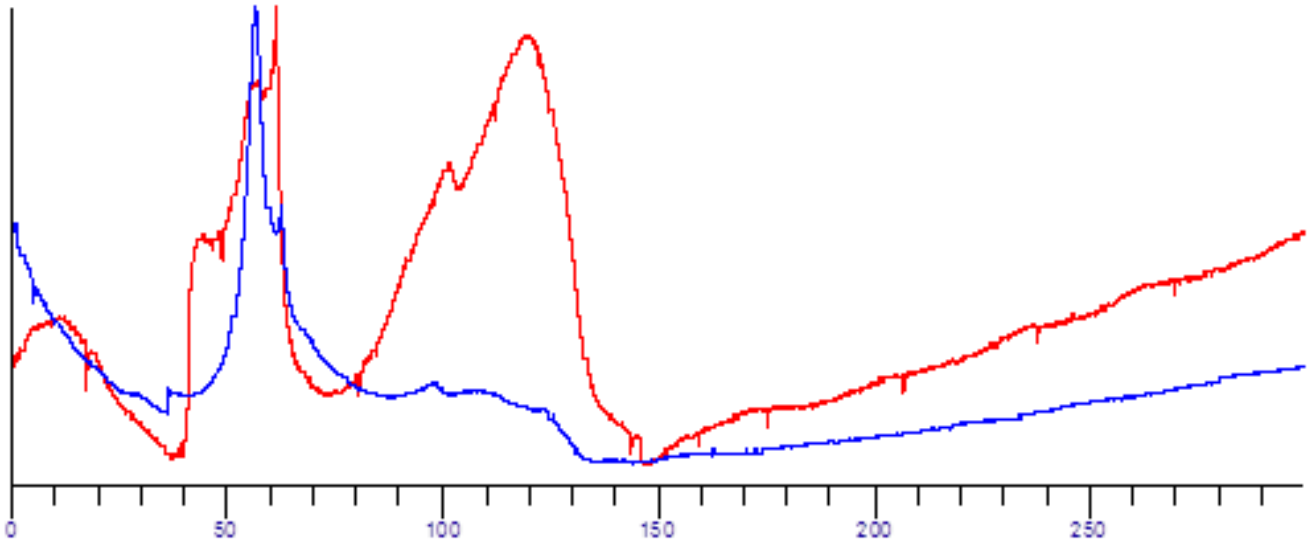

Figure 27 – Adaptation of the obtained Size Exclusion chromatograms before (blue) and after (red) xylanase treatment.
(X axis: time in minutes; Y axis: response in mV).

The complete size exclusion chromatograms as well as more data can be found on Appendix II.

Opposite of what happen in the analysis by anionic chromatography, in this case no standards were used and this analysis its purpose more as a visual.

As no standard was used, this analysis is just to prove once again the degradation of arabinoxylan during the xylanase treatment. On the chromatogram corresponding to the xylanase treated sample, a shift to the right is observed. Knowing the basic principles of size exclusion chromatography (Rasmussen, et al., 2010), molecules with lower molecular weight interact more with the matrix of the chromatographic column therefore, smaller molecules take more time to leave the column and then, to be detected. Hydrolyzing the purified arabinoxylans of rye resulted in a reduction in the molecular weight.

In conclusion, it is worth to compare the sugar composition obtained by the improved method with that of a previous method. The results show the new method as the more efficient and even the physical appearance of the final product seem more attractive for utilizing it as a food ingredient. The composition of AX in the final product increases from 37.57% (using the previous method) up to 60.45%. Regarding the arabinoxylan / glucose ratio, it also improved from 1,84 up to a maximum of 10.02.

In order to get this level of improvement in the final product, modifications were made to the initial method, listed below:

- To de-starch, it is more effective to use α-amylase *from Bacillus amyloliquefaciens* (≥250U/ml) (Sigma-Aldrich*)*, instead of the previously used enzyme from *Bacillus licheniformis* (3000U/ml) (Megazyme).
- The use of pre-treatment is very promising as it allows removing big amounts of starch even before pressure boiling, leading to a whiter and odorless extraction product.
- Dialysis was used to recover polysaccharides and eliminate all the monosugars and small contaminants in combination with ethanol precipitation on the scaled up process, however, dialysis show to be, by itself exceptionally useful successful.
- OPA Method does not seem to be a robust method for this investigation purpose. It was not possible to clearly follow the degree of protein hydrolysis and therefore, it did not shown to be a very useful tool to help making decision about the most adequate protease to use in the pre-treatment.

About the xylanase treatment, the use of the Catalytic Module of xylanase Xyn10A seems to be more efficient to hydrolyze AX than the Full-Length version of the enzyme.

In the end, it was possible to obtain and identify health beneficial oligosaccharides, one of the goals in this project.

In the future it would be interesting to analyze in detail the final product, as well to optimize a variety of steps along the process as the pressure boiling, dialysis and ethanol precipitation or the enzymatic treatments. It would be interesting to look closely on what happened when the extracts from pressure boiling are centrifuged as this step seem to help eliminate high

amounts of glucose as resistant starch, this could be a key step on the purification process. Also, it would be interesting to recover fractions of the oligosaccharides obtained and test its efficiency as a substrate for bacterial growth.

Functional foods develop fast into a market but there is still a lot to be done in utilizing different resources as starting materials. This is a small step forward in achieving this goal.

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